# Somatotropin, overview of research studies

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19-21 May, 2008

# EuroResidue VI

## Conference on Residues of Veterinary Drugs in Food

Hotel Zuiderduin
Egmond aan Zee
The Netherlands

Proceedings

# RESIDUES OF VETERINARY DRUGS IN FOOD

**Edited by:**

**L.A. Van Ginkel**
**A.A. Bergwerff**

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# Volume I

# PREFACE

**Dear friends in residue sciences,**

It is an honour to the EuroResidue Conferences Foundation, the Organising and the Scientific Committees to welcome you at the 6th edition of EuroResidue. Many initiatives for national and international meetings have given opportunity to meet each other very frequently. Despite modern information technology, as web conferencing, video-calling etc, meeting colleagues in 'flesh and blood' remains very necessary. Only face-to-face discussions and time offered at life meetings to spend on discussing varying and numerous subjects can provide optimal inspiration. Inspiration for new residue work, inspiration for old and new friendships …

Two conferences, in particular, the Gent series (the International Symposium on Hormone and Veterinary Drug Residue Analysis) and the EuroResidue series give an international and recognised dedicated platform to discuss all aspects of residues of veterinary drugs in the food production chains. The relevance of such conferences is clearly demonstrated by the start of a new series of similar conferences in Canada, the SaksVal workshops (Saskatoon). There is again a lot to present and to discuss. EuroResidue VI offers you all an attractive and full program with many interesting items. The views of the Scientific Committee are reflected in the specific topics, which are covered in this edition of the conference and which should cover the state-of-the art and future trends in residue science, technologies and policies. They have organised sessions and invited speakers dealing with intriguing subjects as the environment as a source of residues, risk-based monitoring and the progress in residue detection technologies. These advances in technologies are necessary, as there is a growing demand for more and more analysis data-points to be acquired in shorter times at lower prices at a high degree of reliability. It is one of our biggest challenges in our daily work.

Besides the advances, EuroResidue continues to accommodate the presentation of results in 'conventional' residue analysis as well. Many contributions deal with classic ELISA, growth inhibition assays, TLC, HPLC etc etc. Such contributions are highly appreciated and welcomed and EuroResidue encourages participants to remain doing so because such techniques still play an important role in routine residue control programmes. As analytical community, it is important to be open and transparent on how we organise our quality and safety assurance systems in the food chains; for very obvious reasons: fair trade and safe food for every one in the world!

Also during this conference the main program will be supplemented by interesting but specialized workshops dealing with developments in legislation and technology transfer to developing countries. These workshops have increased importance since they contribute to current initiatives to come to further global harmonization of analytical strategies for assuring the world-wide availability of safe and healthy food.

It is the impression of many residue scientists, however, that food contaminants, including residues, starts to settle in the public opinion and that it is fading from the attention of politicians. Attention is more and more attracted to lifestyle and the effect of diet on modern diseases like diabetes. It is our opinion that this development reflects the quality of our work and the outcome of our discussions: outbreaks are increasingly incidents; the control of residues is well-organised and effective. It certainly does not mean that residue analysis is not important (anymore). Does anybody of us keep him/herself to speed-limits while knowing that there is nobody checking speed and/or writing you a fine for speeding? Exactly!

The Organising Committee realised very well that they have to respect the high-standard of this international platform giving all participants an opportunity to discuss residue research and related issues. They have done their outer best to deliver quality to meet this high standard. The venue is perfectly located in a small town close to international traffic services and close to a beautiful environment. Bird lovers should get up early and go into the dunes. The choice for this venue adheres to the philosophy of having accommodation and meeting rooms under a single roof with a pleasant environment closely nearby. It is a growing national tradition to dive into the North Sea at New Years day when the water temperature is only 7-10°C. May we suggest a daily refreshment for the conference …

We wish all participants a very inspiring and a pleasant conference and, although many houses and even a church have disappeared the last 1000 years from the village into the Sea, a relaxing and comfortable stay in Egmond aan Zee.

Leen van Ginkel and Aldert Bergwerff.

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G. Brambilla (Italy)
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# Programme

## Sunday 18th of May 2008

16.00 Registration

17.00 - 20.00 Get-together-party in hotel Zuiderduin

## Monday 19th of May 2008

09.00 - 9.15 Opening of the conference

**First Session** Chairman: C. van Peteghem
Co-chairman: S. Stead

09.15 - 09.45 Opening Lecture
Prof. W. van Dokkum
TNO Quality of Life, Zeist, The Netherlands
*Modern and colourful new methods for the analysis of residues of veterinary drugs in food. Results of a cooperation project between laboratories in Europe.*

09.45 - 10.15 Key Lecture
Prof. Dr. T. Blaha, Dr. D. Meemken
University of Veterinary Medicine, Bakum, Germany
*Risk and epidemiology-based sampling*

10.15 - 10.35 Dr. M.C. Carson
FDA, Washington, USA
*Development of a method to detect anti-viral drug residues in poultry*

**10.35 - 11.15 Coffee Break and poster presentations**

**Second Session** Chairman: L van Leemput
Co-chairman: G. Brambilla

11.15 - 11.35 Dr. A. Cannavan
IAEA, Seibersdorf, Austria
*An investigation into possible natural occurrence of chloramphenicol in poultry litter*

11.35 - 11.55 Dr. W. Reybroeck
ILVO, Melle, Belgium
*Can sulfa-contaminated beeswax lead to residues in honey?*

11.55 - 12.15 Dr. P. Gowik
BVL, Berlin, Germany
*Control of Nitroimidazole residues in food of animal origin; Overview of recent results on suitable matrices, analyte stability and metabolism*

12.15 - 12.35 Dr. R. Clayton
IFAH-Europe, Brussels, Belgium
*Key aspects of the new regulation governing Maximum Residue Limits for pharmacologically active substances*

**12.35 - 13.45 Lunch, posters**

**Third Session** Chairman: L.A. van Ginkel
Co-chairman: M.C. Carson

13.45 - 14.15 Key Lecture
Dr. A. Boxall
CSL, York, UK
*The uptake of residues from plant materials*

14.15 - 14.35 Dr. M. Groot
Rikilt, Wageningen, The Netherlands
*Herbal alternatives for antimicrobial growth promoters*

14.35 - 14.55 Prof. H. De Brabander
Ghent University, Ghent, Belgium
*Analysis of thyreostats: a history of 35 years*

14.55 - 15.15 Dr. G. Brambilla
ISS, Rome, Italy
*Residues of oxytetracyclin in Zea mais: only a matter of food safety?*

**15.15 - 16.00 Tea break and poster presentation**

**Fourth session** Chairman: A.A. Bergwerff
Co-chairman: C.T. Elliot

**Key Lecture**

| | |
|---|---|
| 16.00 - 16.30 | Key Lecture<br>Dr. S. Stead<br>CSL, York, UK<br>*Aptamer technology* |
| 16.30 - 16.50 | Dr. M. Sharman<br>CSL, York, UK<br>*A novel aptamer based procedure for the rapid detection of malachite green in fish tissue* |
| 17.00 - 18.00 | Workshop<br>Associated and third countries and new Member States<br>Convened by Dr. J. McEvoy |
| 18.00 | Dinner at Zuiderduin |
| 19.15 | Departure to the Zuiderzee museum |

## Tuesday 20th of May 2008

**Fifth Session** Chairman: M. Petz
Co-chairman: R. Schilt

9.00 - 9.20 Dr. A.A.M. Stolker
Rikilt, Wageningen, The Netherlands
*Trends in Monitoring the use of veterinary drugs and growth-promoting agents*

9.20 - 9.50 Prof. Dr. P. Fürst
CLNW, Nordrhein Westfalen, Münster, Germany
*Advances in TOF-MS*

9.50 - 10.10 Dr. A. Kaufmann
Official Food Control Authority of the Canton Zürich, Zürich, Switzerland
*Quantitative UPLC-TOF Multiresidue method for 100 different Veterinary Drugs*

**10.10 - 11.30 Coffee break and poster presentation**

11.30 - 11.55 Dr. G. Marchesini
Rikilt, Wageningen, The Netherlands
*SPR Biosensor screening assays coupled to Bio-affinity-directed analytical identification of bioactive substances*

11.55 - 12.25 Prof. Dr. D.G. Kennedy
AFBI, Belfast, NI-UK
*"Break a bone- It's Nandrolone"*

**12.25 - 13.30 Lunch and posters**

**Sixth session** Chairman: D.G. Kennedy
Co-chairman: J.A. van Rhijn

13.30 - 14.00 Prof. Dr. B. Le Bizec
Laberca, Nantes, France
*Naturally occurring hormones in the food chain*

14.00 - 14.20 Dr. G. Pinel
Laberca, Nantes, France
*Somatotropine, overview of research studies*

14.20 - 14.40 Dr. A. Kolkman
RIVM, Bilthoven, The Netherlands
*Development of a proteomic approach for the detection of recombinant somatotropine in bovine milk and serum*

**14.40 - 15.30 Tea break and poster presentation**

**Seventh session** Chairman: B. Le Bizec
Co-chairman: M.W.F. Nielen

15.30 - 16.00 Dr. P. Gowik
BVL, Berlin, Germany
*The state of and update on 2002/657*

16.00 - 16.20 Dr. J. Boison
CFIA, Saskatoon, Canada
*A determinative and confirmatory method for residues of the metabolites of carbadox and olaquindox in porcine tissues. Validation in Canada.*

16.20 - 16.40 Dr. K. Schmidt
BVL, Berlin, Germany
*Validation and multivariate effect analysis of an LC-MS/MS method for the determination of steroids in bovine muscle.*

17.00 - 18.00 Workshop
Update on legislation, a.o. validation of bioassays, measurement uncertainty
Convened by Dr. P. Gowik

19.30 Symposium dinner, beach party
Hotel Lido Egmond aan Zee

## Wednesday 21st of May 2008

**Eighth Session** Chairman: H. De Brabander
Co-chairman: S.S. Sterk

9.00 - 9.30 Prof. Dr. J. van Oostrum
Zeptosens- a division of Bayer (schweiz) AG, Witterswil, Switzerland
*The omics technologies in diagnostics*

9.30 - 9.50 Dr. M.H. Mooney
Queens University, Belfast, NI-UK
*Biomarker-based identification of growth promoter abuse during beef production*

9.50 - 10.10 Dr. J-P Antignac
Laberca, Nantes, France
*Mass spectrometric based untargeted metabolomics: principle and application in the field of food safety*

10.10 - 10.30 Dr. J. de Rijk
Rikilt, Wageningen, The Netherlands
*Microarrays: a screening tool for pro-hormone abuse in bovines*

10.30 - 10.50 Dr. J. van Meeuwen
IRAS, Utrecht, the Netherlands
*Analysis of biomarkers in plasma with a unique multi-assay surface plasmon resonance biosensor to monitor hormone abuse in cattle*

**10.50 - 11.30 Coffee break and poster presentations**

**Ninth Session** Chairman: P. Gowik
Co-chairman: E. Verdon

11.30 - 12.00 Robert Schilt
DUCARES - TNO Company, Zeist, The Netherlands
*LTQ-FT and LTQ-Orbitrap, possibilities of this recently introduced technology in residue analysis*

12.00 - 12.20 Dr. M. Pilar-Marco
AMRg, Barcelona, Spain
*New techniques in residue analysis*

| | |
|---|---|
| 12.20 - 12.40 | Prof. Dr. M.W.F. Nielen<br>Rikilt, Wageningen, The Netherlands<br>*Desorption Electro Spray Ionisation Mass Spectrometry: A rapid screening tool for illegal hormone preparations and forensic samples from hormone crime investigations* |
| 12.40 - 12.50 | Closing of the Euroresidue VI Conference |
| 12.50 | Farewell lunch |
| +/-13.45 | Departure buses to Schiphol airport |

# Key Lectures

Monday 19th of May 2008

## K 1

# OVERVIEW OF APTAMER TECHNOLOGY – AN EMERGING CLASS OF RECOGNITION MOLECULES TO RIVAL ANTIBODIES?

Sara L. Stead
Central Science Laboratory, Sand Hutton, York, Y041 1LZ, UK

**Abstract**
At the present time antibodies are among most popular classes of molecular recognition elements and have been studied and utilised for many decades. As a result, antibodies have made substantial contributions toward the advancement of diagnostic assays and have become an indispensable element in many screening assays in a wide cross-section of applications from the medical, military, environmental, food safety and quality sectors. The development of the Systematic Evolution of Ligands by Exponential enrichment (SELEX) process in the late 1990s (Stoltenburg et al., 2007) made possible the isolation of oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. These oligonucleotide sequences, referred to as "aptamers" are emerging as a class of molecules that could potentially rival antibody performance in a range of diagnostic and therapeutic applications. Aptamers are different from antibodies, yet they mimic their properties. The demand for diagnostic assays to detect the presence of pollutants, contaminants and residues in the analysis of environmental and food samples is increasing and aptamers could potentially fulfill the molecular recognition needs in such assays. Compared with antibody technology, aptamer research is still in its infancy. In order to better harness the unique properties of aptamers, conventional assay formats may require modifications. This paper presents an overview of aptamer technology, the state-of-the-art and the potential for aptamer-based diagnostic products.

**Background and Discussion**
Aptamers, derived from the Latin word aptus meaning "to fit" are specific single stranded oligonucleotides (RNA or DNA) or proteins, which can adopt a vast number of three-dimensional shapes. Due to this property, aptamers can be produced in vitro to bind tightly to a specific molecular target. An extraordinary diversity of molecular shapes exist within the universe of all possible nucleotide sequences thus aptamers may be obtained for a wide array of molecular targets, including most proteins, carbohydrates, lipids, nucleotides, other small molecules and even complex structures such as viruses. Aptamers have the ability to form defined tertiary structures to engage a specific target for binding (Nutiu and Li, 2005). Aptamers were first used as therapeutic agents. For example, the anti-thrombin aptamer was developed with the purpose of application as an anticoagulant (Bock et al., 1992). It is comparatively recently that aptamers have been used as recognition elements in "biosensing".

*Production of aptamers*
Unlike amino acid-based antibodies, aptamers have the advantage of being able to recognise relatively small molecular weight compounds (<1000 Daltons) without prior conjugation to a carrier protein. The identification of rare nucleic acid sequence oligonucleotide libraries was first discovered independently by three research groups in 1990. Tuerk and Gold (Tuerk

and Gold, 1990) patented the process of selection of DNA ligands as a target for T4 RNA polymerase an in vitro evolutionary process called “Systematic Evolution of Ligands by EXponential enrichment” (SELEX) illustrated in Figure 1.
The method is an iterative process based on selection and amplification of the anticipated tight binding aptamer. The SELEX process begins with a random sequence library obtained from combinatorial chemical synthesis of DNA. Each member in a library is a linear oligomer of a unique sequence. The start library for selection of aptamers contains single stranded DNA oligonucleotides with a central region of randomized sequences (up to $10^{15}$ different sequences) which are flanked by constant regions for subsequent transcription, reverse transcription and DNA amplification. The start library is amplified by PCR and transcribed to an RNA start pool by T7 transcription. Target specific RNA is selected from the pool by allowing the pool to interact with the target molecule, only tight binding RNA molecules with high affinity are removed from the reaction cycle, the tight binding RNA molecules are reverse transcribed to cDNA and amplified to double stranded DNA by PCR. These enriched binding sequences are transcribed back to RNA, which is the source for the next selection and amplification cycle. Such selection cycles are usually repeated 5-12 times in order to obtain only sequences with highest binding affinities against the target molecule.

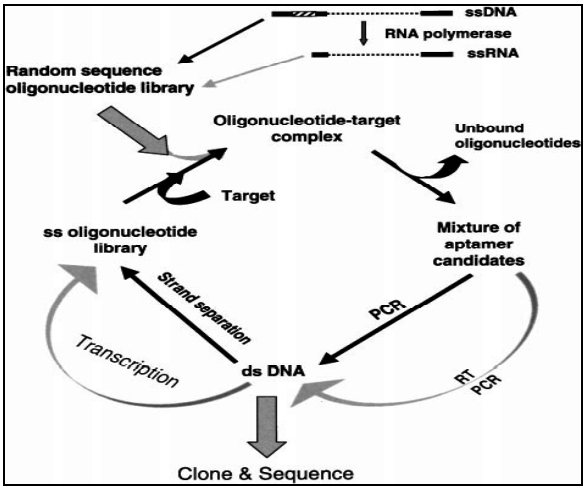


**Figure 1.** Generalised scheme showing the key steps in the SELEX process (Jayasena, 1999)
The general properties aptamers

Aptamers can be generated with remarkable affinity and specificity and there are examples of aptamers that can discriminate targets on the basis of subtle structural differences such as the presence or absence of a methyl or a hydroxyl group and the D vs. L-enantiomer of the target. This property is attributed to the adaptive recognition; aptamers are unstructured in solution and fold upon association with their molecular targets forming molecular architectures in which the ligand becomes an intrinsic part of the nucleic acid structure (Hermann and Patel, 2000). Aptamers have been selected to bind small organic molecules forming complexes that have dissociation constants in the sub-micromolar range and aptamers selected against proteins form complexes that have dissociation constants in the nanomolar range (Luzi, et al., 2003).

This exquisite specificity is achieved practically by a process termed “counter-SELEX”. Oligonucleotide sequences with the ability to bind the targets as well as closely related structural analogs of the target are effectively discarded. In principle, there is no restriction in the type of target for which the aptamer can be selected. To date, aptamers with affinity to various ligands have been synthesized including metal ions, organic dyes, drugs, amino acids, co-factors, antibiotics, toxins and nucleotide base analogs.

Chemically modified oligonucleotide libraries have also been introduced into the SELEX process to generate sequences that are more resistant to nuclease attack. For example, RNA is particularly susceptible to attack by the endonucleases (e.g. RNases) present in biological fluids. Common modifications to the oligonucleotide sequence include modification with a phosphorothiate linkage, backbone modifications and modifications at the 2’ position of the sugar (as shown in Figure 2). The nucleases that are most abundant in biological tissues are pyrimidine-specific endonucleases, substitutions at the 2’ positions of pyrimidine nucleotides alone is sufficient to protect an RNA sequence from degradation. Pyrimidine nucleotides substituted with amino and fluoro functional groups at the 2’ position of the sugar are also substrates for the enzymes used in the SELEX process. Aptamers with enhanced survival times in biological fluids containing pyrimidines modified with 2’$NH_2$ and 2’F functional groups have been reported (Lin, et al., 1994, Davis et al., 1998).

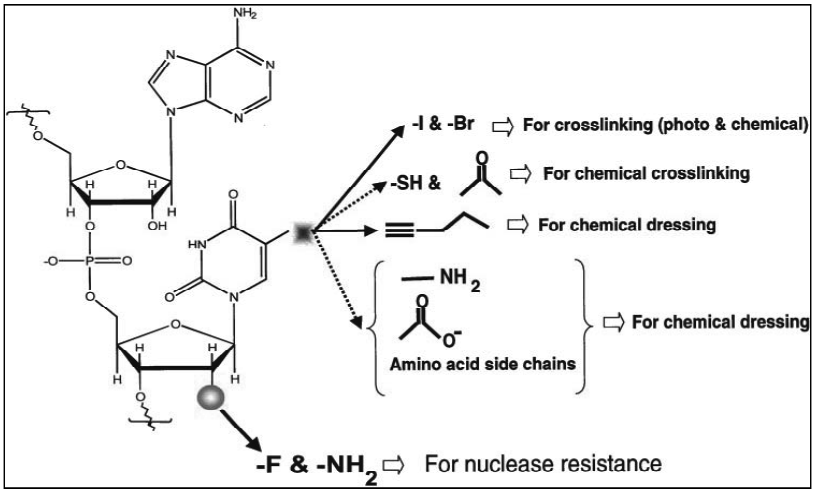


**Figure 2** Sites for possible modifications on an oligonucleotide strand to generate modified oligonucleotide libraries for the SELEX process. Modification at the 2’position of the sugar confers nuclease stability, whereas various modifications at the C-5 position of the pyrimidines could be used either to attract certain classes of targets or to generate covalent cross-links with targets (Jayasena, 1999).

As new aptamers can be identified rapidly via the in vitro SELEX process, the need for the use of animals is removed. Currently, the SELEX is a highly automated procedure and only several weeks are necessary for the development of aptamers for certain ligands. This timescale is much shorter in comparison with the selection of antibodies, where typically

several months are required. By further contrast to antibody production (where it is the animal immune system that selects the sites on the target molecule to which the antibodies bind) within the aptamer-selection the process can be manipulated to obtain aptamers that bind a defined region on the target molecule and also possess specific binding properties under various binding conditions. After the selection process, aptamers are produced by chemical synthesis and purified to a very high degree, which effectively eliminates the batch-to-batch variations, which can occur especially when using polyclonal antibodies.
The recent reports of three-dimensional structural analyses have provided insights into key questions concerning molecular recognition by nucleic acid aptamer complexes, most notably what is the structural basis of highly specific ligand discrimination by aptamers and what are the differences between ligand-binding sites in aptamers versus natural nucleic acids or antibodies? In the example of the aminoglycoside antibiotic aptamers, the ligands are found to be recognised by their aptamers through a combination of electrostatic and shape complementarily along with distinct hydrogen bonds involving the polar groups present on the target molecules (Tereshko et al., 2003).
The variations in ligand recognition between the two different classes of binding molecules, aptamers and antibodies are obviously seated in the different nature of the building blocks of these macromolecules. In the case of antibodies, the diversity of the 20 amino acids allows for a multitude of interactions and precise shape complementarily in open antigen-binding sites. However, for aptamers the structurally more uniform four nucleotides are limited in possible alternative ways to pack around arbitrary ligands. Thus, the ligand fit within the binding sites located in the aptamer fold display a less-than-perfect shape complementarity, which is compensated for through the deep encapsulation of the ligand. The planar nature of the nucleotide bases favours stacking interactions in aptamer complexes whereas intermolecular hydrogen bonds and general acid-base interactions are preferred for substrate binding by proteins (Hermann and Patel, 2000). Single-stranded oligonucleotide sequences have an exceptional ability to assume an array of secondary and tertiary structural motifs with different shapes. The number of possible thermodynamically stable structural variants of an oligonucleotide sequence is much higher than the number of variants of a peptide sequence of the same length due the ability of the nucleotide bases to interact with each other through canonical Watson-Crick as well as unusual base-pairing.

**Conclusions** - *potential applications of aptamers in residue analysis*
The specificity of the molecular recognition, combined with the ease by which target-binding aptamers can be selected, designed and even chemically modified makes these molecules particularly attractive as recognition elements for use within diagnostic, therapeutic and analytical array technology. Aptamers have been used as immobilised ligands in a of separation technologies e.g. affinity probe capillary electrophoresis and capillary electro-chromatography (CEC). Aptamers can be considered as an interesting new class of molecular recognition elements for affinity sensing applications in residue analysis. The development of aptamer-based sensor techniques termed “aptasensors” is being accelerated by the use of existing optical, acoustic and fluorescent methods to analyse biological phenomena in solution in real time or by immobilisation of the aptamer onto a solid support.

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# Oral Presentations

Monday 19th of May 2008

**O 1** **Development of a method to detect anti-viral drug residues in poultry**
Dr. M.C. Carson
FDA, Washington, USA

**O 2** **An investigation into possible natural occurrence of chloramphenicol in poultry litter**
Dr. A. Cannavan
IAEA, Seibersdorf, Austria

**O 3** **Can sulfa-contaminated beeswax lead to residues in honey?**
Dr. W. Reybroeck
ILVO, Melle, Belgium

**O 4** **Control of Nitroimidazole residues in food of animal origin; Overview of recent results on suitable matrices, analyte stability and metabolism**
Dr. P. Gowik
BVL, Berlin, Germany

**O 5** **Herbal alternatives for antimicrobial growth promoters**
Dr. M. Groot
Rikilt, Wageningen, The Netherlands

**O 6** **Analysis of thyreostats: a history of 35 years**
Prof. H. De Brabander
Ghent University, Ghent, Belgium

**O 7** **Residues of oxytetracyclin in Zea mais: only a matter of food safety?**
Dr. G. Brambilla
ISS, Rome, Italy

**O 8** **A novel aptamer based procedure for the rapid detection of malachite green in fish tissue**
Dr. S. Stead
CSL, York, UK

## O 1

# Development of a method to detect antiviral drug residues in poultry

Shani Smith[1], Michelle Smith[1,3], Mary C. Carson[1], Jonathan Tarbin[2], Matthew Sharman[2]

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[3]Current address: Adpen Laboratories Inc, Jacksonville, FL 32224 USA

**Abstract**

Recently, a highly pathogenic and virulent strain of avian influenza emerged in Asia. Scientists speculate that an avian influenza could transform into the next human influenza pandemic. In November 2005, the WHO, FAO and OIE jointly urged their Member States to not use antivirals in animals, in order to preserve their efficacy for human use. By March 2006, the U.S. FDA had published a rule prohibiting the extralabel use of adamantanes and neuraminidase inhibitors in chickens, turkeys, and ducks. We report here a collaborative effort between CVM and CSL on the development of an LC-MS/MS method to enforce these prohibitions. Our method target level is in the low μg $kg^{-1}$ range. The analytes of interest are amantadine, rimantadine, oseltamivir phosphate (Tamiflu®), oseltamivir carboxylate (active metabolite of Tamiflu®), and zanamivir (Relenza®). These compounds have diverse chemistries, with the last two being highly hydrophilic zwitterions. A solution of acidified water and acetonitrile (ACN) is an effective extractant of all five analytes from chicken tissue. Several chromatographic approaches were evaluated. The best results were obtained using the ZIC silica-based HILIC column with an ACN:aqueous gradient. Work is underway to minimize matrix effects by adequate chromatography, sample clean-up, and use of a TurboIonSpray interface on a 4000 Q Trap mass spectrometer.

**Introduction**

In 2005, The Washington Post reported the use of amantadine in poultry in China (Sipress, 2005). This revelation followed a report in the scientific literature that the adamantane class of drugs had lost their efficacy against H5N1 strains of avian influenza isolated from human patients in Asia (Trampuz et al, 2004). International health authorities recommended that antiviral drugs not be used in animals, and FDA/CVM issued an Order of Prohibition on the extra-label use in poultry of anti-influenza drugs in the adamantane class (amantadine and rimantadine, chiefly) and neuraminidase inhibitor class (oseltamivir and zanamivir, chiefly). The purpose of the Order of Prohibition is to prevent the emergence of drug-resistant high pathogenicity avian influenza virus. Virologists speculate that avian influenza could be the source of the next human influenza pandemic, and until specific vaccines are developed, antiviral drugs are the principal defence.

Residue methods are needed to enforce the prohibition of antiviral use in poultry. Ideally, a single method would be used for surveillance of all potential residues, and this method would have low limits of quantitation since these are prohibited compounds. This presents significant challenges to the analytical chemist, as the chemical characteristics of the adamantane class are quite different from the neuraminidase class of antiviral drugs, and none of the drugs possess a strong chromophore. Furthermore, one of the drugs, oseltamivir, is actually a prodrug that is rapidly hydrolyzed in vivo to its active form, so a residue method should be able to monitor both compounds. Most methods for adamantanes involved alkaline extraction with toluene, followed by derivatization and GC analysis (Suckow 2001). Neuraminidase inhibitors, by contrast, are extremely hydrophilic zwitterions, and are analyzed by LC-MS/MS (Allen et al. 1999, Wiltshire et al. 2000). We report here progress toward the development of LC-TOF-MS and LC-MS/MS methods to simultaneously monitor for all five compounds.

## Materials and Methods

*Reagents*

All solvents and ammonium acetate were HPLC grade. Glacial acetic acid was reagent grade. Amantadine HCl and rimantadine HCl were purchased from the U.S. Pharmacopeia (CVM) or Fluka and Aldrich, respectively (CSL); memantadine HCl was purchased from Sigma-Aldrich; oseltamivir and its carboxylate were the generous gift of Roche Pharmaceuticals; zanamivir was obtained as a prescription for Relenza® from a local pharmacy. Each blister pack of Relenza® contained 5 mg zanamivir and 20 mg lactose.

*Instrumentation*

Both CVM and CSL have access to a variety of LC and MS instrumentation from Shimadzu, Waters, Agilent, Thermo, and Sciex. More than one LC-MS system was investigated for its potential in this project by each laboratory. Each laboratory also investigated several analytical columns.

*Tissues*

Control chicken liver and muscle were either purchased at a local market or obtained from untreated birds housed at CVM and CSL facilities. Liver was homogenized in a blender and muscle was homogenized by grinding with dry ice. Both were stored at -80°C until use.

## Results and Discussion

Mass spectrometry. Table 1 lists the antiviral drugs currently included in this study. We also evaluated memantadine, a structural isomer of rimantadine, as a potential internal standard. Each compound could be detected in positive electrospray mode on a ThermoFinnigan TSQ Quantum, Thermo LCQ Deca XP (ion trap), Micromass Quattro micro™ API, Waters Premier™ LCT (TOF), Waters Quattro Premier™ XE, and Sciex 4000 Q Trap. However, the response for zanamivir was weak, and only the Sciex 4000 Q Trap and Micromass Premier™ XE exhibited adequate sensitivity to detect this compound at 10 μg $kg^{-1}$ or lower in tissue. We used triple quadrupole mass spectrometry for most of this study. Each compound produced at least two product ions with good selectivity potential. The product ions for rimantadine and memantadine were identical, though the relative abundances were different.

**Table 1.** Transitions monitored for each compound.

| Compound | Precursor Ion | Product Ions |
|---|---|---|
| Amantadine | 152 | 79, 93 |
| Rimantadine | 180 | 81, 163 |
| Oseltamivir | 313 | 94, 120, 166, 225 |
| Oseltamivir carboxylate | 285 | 120, 180, 197, 198 |
| Zanamivir | 333 | 60, 121 |
| Memantadine | 180 | 107, 163 |

*Chromatography.* Each laboratory had a Waters UPLC and tested it as a potential chromatograph. Oseltamivir, its carboxylate, amantadine, rimantadine, and memantadine could all be chromatographed on a BEH C8, BEH C18, BEH Phenyl, or BEH HILIC. Only the BEH HILIC was able to retain zanamivir; however peak shapes for some of the other compounds were poor with this column. Conventional LC looked more promising. Zanamivir was still not retained by any reversed phase column tested, nor did a CN or C1 column give good results for all five analytes. However, a ZIC-HILIC did give good peak shapes for all compounds (Figure 1). The silica-based ZIC-HILIC performed better than the polymeric ZIC-pHILIC. Regrettably, under these chromatographic conditions, memantadine did not resolve from rimantadine, so it was not included as an internal standard in these analyses.

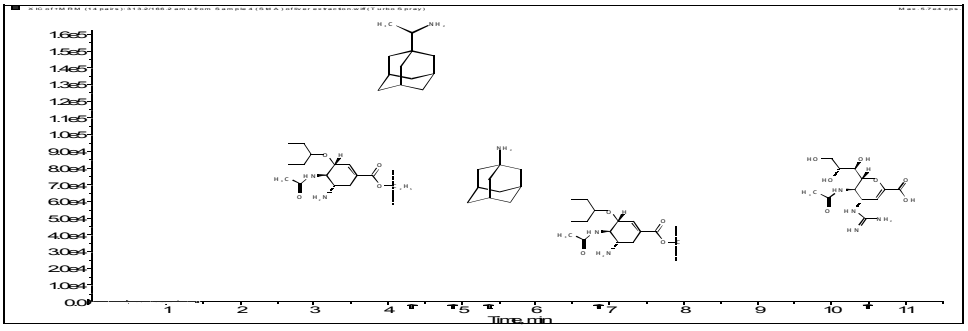


**Figure 1** Total ion chromatogram (14 transition pairs) of five antiviral drugs, 2 µL of 30 ng/mL injected. Separation on a ZIC®-HILIC 150 x 2.1 mm column (Sequant); flow rate 0.3 mL/min with gradient starting at 95 + 5 MeCN + 20m NH4OAc, pH 5.5, with ACN decreasing to 80% at 1-4 min, then to 50% at 10 min. Drugs elute oseltamivir (4.4), rimantadine (5.0), amantadine (5.4), oseltamivir carboxylate (6.9), and zanamivir (10.5). Detection was on a Sciex 4000 Q Trap operated in MRM mode.

*Extraction*

In order to achieve reasonable recoveries for all compounds, we used simple extraction techniques with minimal cleanup. Acidified MeCN (CSL) or acidified $H_2O$-MeCN (CVM) looked the most promising as initial extractants. This was followed with either drying, cleanup on an amino SPE, and evaporation (CSL), or with evaporation, a hexane wash of the

remaining (mostly acetic acid-$H_2O$) phase, and addition of more MeCN (CVM). CVM found that it was critical to not concentrate the acidic $H_2O$-MeCN phase too much, nor to allow the percent $H_2O$ to decrease too much, otherwise zanamivir recovery suffered as it is poorly soluble in MeCN. Conversely, if the aqueous content of the final extract was too high, the quality of the HILIC chromatography was very poor. To compromise between these extremes, we limited injection volumes to 5 µL or less and kept the aqueous content of final extracts between 25-40%.

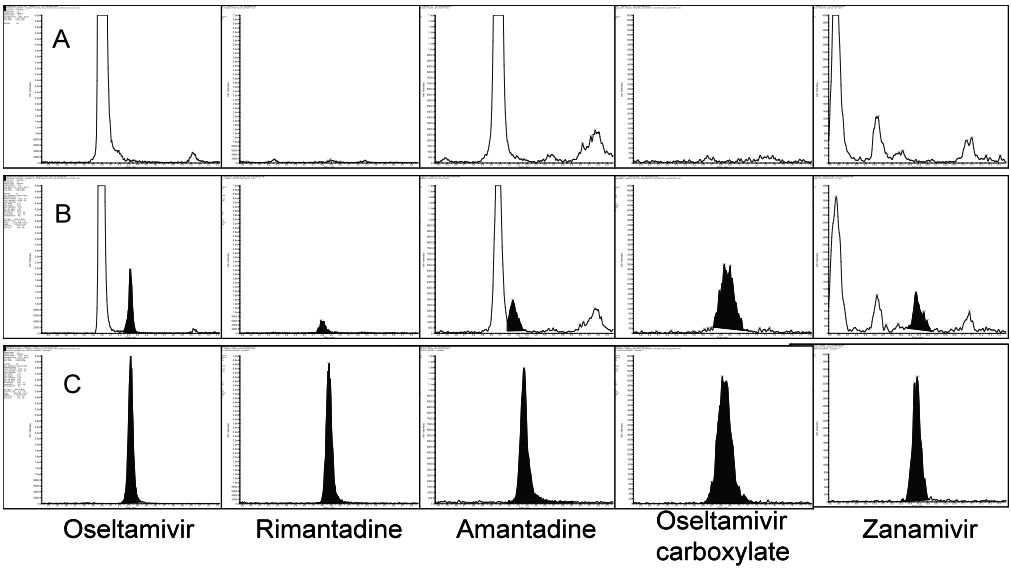


**Figure 2.** Summed extracted ion chromatograms for each compound for three injections. The top set (A) of traces is from control chicken liver extract; set B is from chicken liver fortified with 10 µg $kg^{-1}$; set C is from a 10 ng/mL standard (equivalent to the 10 µg $kg^{-1}$ sample). Chromatography and acquisition conditions as in Fig. 1.

Fig. 2 illustrates results obtained at CVM with chicken liver. Two features are evident. First, there are potential matrix interferences for oseltamivir, amantadine, and zanamivir, though they are resolved in this chromatography. These potential interferences were matrix dependent and different in chicken muscle. Second, the apparent recoveries look low. This is due largely to matrix suppression. Both laboratories observed matrix suppression with HILIC chromatography, as shown in Tables 2 and 3. Matrix suppression was not so severe with the BEH C8 evaluated by CSL, but this chromatography was not suitable for zanamivir analysis. Matrix effects in HILIC could be reduced, or even reversed by injecting a smaller volume, but could not be completely eliminated. These effects were both compound and matrix dependent.

**Table 2.** Matrix effects observed on UPLC-TOF-MS with two different chromatographies. Numbers are the ratio the response of matrix (chicken muscle extracted using MeCN and amino SPE cleanup) standard to the response of a solvent standard. A result near 1 indicates minimal matrix effect.

| Compound | BEH C8 | BEH HILIC |
|---|---|---|
| Amantadine | Near 1 | Near 1 |
| Rimantadine | Near 1 | Near 1 |
| Oseltamivir | Near 1 | Near 1 |
| Oseltamivir carboxylate | 0.85 | 0.11 |
| Zanamivir | Not tested | Not tested |
| Memantadine | Near 1 | Near 1* |

**not resolved from rimantadine in this system.*

**Table 3.** Matrix effects observed on LC-MS/MS (4000 Q Trap) with two different injection volumes on ZIC-HILIC column. Numbers are the response ratio between slopes of standard curves prepared in chicken muscle extract and in solvent.

| Compound | 5 µL inj. | 2 µL inj. |
|---|---|---|
| Amantadine | 0.10 | 0.14 |
| Rimantadine | 0.06 | 0.10 |
| Oseltamivir | 0.09 | 1.71 |
| Oseltamivir carboxylate | 0.34 | 0.41 |
| Zanamivir | 0.86 | 1.41 |
| Memantadine | Not tested | Not tested |

Although there was significant matrix effect on quantitative response, the relative abundance of the product ion ratios in fortified tissue samples generally matched those in both the matrix standards and the solvent standards in the MRM analyses.

## Conclusions

We have made significant progress towards the joint development and validation of a method for antiviral drug residues in poultry tissue. Both laboratories have only had success with ZIC-HILIC column in chromatographing all five analytes. The active versions of the neuraminidase inhibitors, oseltamivir carboxylate and zanamivir, are extremely hydrophilic and gave a relatively weak response in ESI. This, combined with their solubility limitations in MeCN and sample injection volume limitations in HILIC, mean that a mass spectrometer with low detection limits must be used to detect and confirm these residues at 10 μg $kg^{-1}$ or less in tissue. The TOF instrument produced extremely clean high resolution ion chromatographs, but did not detect either neuraminidase inhibitor at the target concentration. Therefore, triple quadrupole mass spectrometry appears better suited for analysis, at least with the instrumentation currently available in our laboratories.

Matrix effects remain an issue, and likely mean that for good quantification, a matched matrix standard curve will be required. Future work will include optimizing the quantification, extending the method to kidney and eggs, and incurring each of the four parent drugs in chicken and turkey (a joint venture between the laboratories).

## Acknowledgments

We are thankful to Roche Pharmaceuticals for the generous gift of oseltamivir and oseltamivir carboxylate.

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## O 2

# AN INVESTIGATION INTO THE POSSIBLE NATURAL OCCURRENCE OF CHLORAMPHENICOL IN POULTRY LITTER

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**Abstract**
In recent years the detection of residues of chloramphenicol (CAP) in foods such as poultry and honey has had a major impact on international trade. Follow-up investigations in Thailand relating to poultry non-compliant findings in Europe have, in some cases, been unable to establish the cause of the residues, since there was no recent history of use of the drug. A possible source of contamination is the biosynthesis of CAP by Streptomyces venezuelae or other actinomycetes. A study was instigated to investigate this possibility in a typical poultry production environment. S. venezuelae in CAP-producing phase was spiked into poultry litter under various conditions and the litter was tested for growth of the organism and for CAP concentration. Results showed that S. venezuelae was not viable after 3-4 weeks and initial levels of CAP from the S. venezuelae/normal saline solution added (maximum 0.6 ppb) decreased rapidly and were below the LOQ of the analytical method (0.04 ppb) by week 3. Litter samples collected from 5 poultry farms with a history of poultry contamination with CAP were tested and found to be negative for both CAP and S. venezuelae. The results suggest that residues of chloramphenicol on the farms tested were extremely unlikely to have been caused by natural biosynthesis of the drug in the production environment.

**Introduction**
Chloramphenicol (CAP) is a broad spectrum antibiotic used in both human and veterinary medicine. Chloramphenicol is biosynthesised by the soil organism Streptomyces venezuelae and several other actinomycetes, but is produced for commercial use by chemical synthesis (Vining and Stuttard, 1994). The drug has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives, most recently at its sixty-second meeting (Wongtavatchai et al, 2005), by the European Committee for Veterinary Medicinal Products (1994) and by a number of other agencies. Because of concerns over its genotoxicity, embryo- and fetotoxicity, carcinogenic potential and the lack of a dose-response relationship for aplastic anaemia caused by treatment with CAP in humans, the drug has been banned for use in food-producing animals in the European Union (EU) and in many other countries. However, because of its efficacy and cost-effectiveness, CAP continues to be used in food animals in some countries, especially in the developing world, as evidenced by sporadic reports in the EU Rapid Alert System for Food and Feed. Because of the detection of CAP and other antimicrobials such as nitrofurans, the European Commission adopted a series of Decisions (2001/699/EC, 2001/705/EC, 2002/251/EC) requiring testing of products for export to the EU

from various countries in East Asia and Latin America to protect the European consumer from imported aquaculture and poultry products containing potentially harmful residues of these substances. A minimum required performance limit (MRPL) of 0.3 μg $kg^{-1}$ was assigned by the European Commission (2003) for analytical methods to detect CAP.
Sporadic non-compliant results continue to arise in products from various countries even after measures have been put in place to control the use of CAP. Various hypotheses have been suggested to explain these results. Residues may be caused by the use of unlicensed and/or counterfeit pharmaceutical products containing undeclared CAP (Cannavan and Elliott, 2004). Other possibilities include persistent environmental contamination resulting from past use of the drug, contamination from topical medicinal preparations containing CAP used by processing workers, and ingestion of naturally occurring CAP from the environment (Wongtavatchai et al, 2005). This last possibility was suggested as an explanation for contaminated products originating from several poultry producers in Thailand where there was no evidence of the recent use of CAP. Any evidence of natural or environmental sources of CAP contamination would have serious implications for the EU's 'zero tolerance' policy within the Union and the application of the MRPL as an action level for imports.
A study was designed and carried out at the Veterinary Public Health Laboratory in Bangkok to test the natural-production hypothesis by introducing S. venezuelae to chicken litter under conditions prevalent in the production facilities of the affected farms and testing for viability of the organism and production of CAP. Litter samples from producers with a history of CAP-contaminated poultry were also tested for the presence of S. venezuelae and CAP.

**Materials and methods**

*Stability of Streptomyces venezuelae in normal saline.* This study was performed to ensure that *S. venezuelae* could survive in normal saline, since a suspension in normal saline would be used in the litter experiments. *S. venezuelae,* strain DSM 40232, was streaked onto GYM Streptomyces agar and incubated at 28°C for 48 hours. Several colonies were harvested and suspended in sterilized normal saline. The suspension was well mixed and centrifuged to obtain a pellet. The procedure was repeated, normal saline added to the combined pellets and the suspension was mixed and stored at room temperature. *S. venezuelae* was enumerated daily for 1 week (trial 1) and weekly for 3 weeks (trial 2).

*Moisture content of litter from poultry producers.* Twenty samples of litter were collected from broiler farms at the broiler ages of 1 day, 1, 2, 3, 4, 5 and 6 weeks and analysed for moisture content using ISO method 1442. Samples were also examined for the presence of *S. venezuelae.*

*Stability of chloramphenicol in litter.* Portions of litter (20 g) were weighed into 11 bags. Chloramphenicol solution (1 ng $ml^{-1}$, 28 ml) was added to 10 of the bags and the contents mixed well. The 10 spiked bags were pooled and again mixed well to homogenize the chloramphenicol distribution. The pooled sample was once more divided into 20 g portions, stored in 10 bags at room temperature along with the control (unspiked) bag and assayed for CAP weekly.

*Capability of Streptomyces venezuelae to produce chloramphenicol in litter.* Portions of litter (20 g) were weighed into 21 bags. *S. venezualae* suspension ($1.0x10^8$ org $ml^{-1}$) was added, 10 ml to bags 1-10 and 20 ml to bags 11-20. The contents were mixed well. The contents of bags 1-5, 6-10, 11-15 and 16-20, respectively, were pooled to produce 4 samples. Each sample was mixed well to ensure homogeneity. Both the suspension and the spiked litter were stored at room temperature and assayed for CAP weekly for 5 weeks. *S. venezuelae* was also enumerated weekly in both the spiked litter samples and the suspension.

*Viability of Streptomyces venezuelae in litter with differing moisture content. S. venezuelae* - normal saline suspension was added to sterile litter with ratios of litter to *S. venezuelae* suspension of 1:1, 1:0.5, and 1:0.25. (g:ml). Each spiked sample was mixed well and analysed for moisture and for presence of *S. venezuelae* weekly for 6 weeks.

*Determination of chloramphenicol.* Chloramphenicol was determined in chicken litter using a LC-MSMS method developed and validated at the VPHL, Bangkok, which will be published elsewhere. Briefly, a portion of well homogenised litter was extracted by sonication with phosphate buffered saline, sodium chloride and acetonitrile. The extract was washed with hexane and extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness under a stream of nitrogen, re-dissolved in water and cleaned up by C18-SPE. The methanol eluate was evaporated to dryness under a stream of nitrogen and re-dissolved in methanol/ water (1:1, v/v) for analysis by LC-MSMS. The method performance characteristics were evaluated and the method was shown to be fit-for-purpose. Quantitation was by the isotope dilution method. The limit of quantitation was approximately 0.04 μg $kg^{-1}$.

*Litter samples from farms with a history of chloramphenicol detection in poultry.* Samples of litter were taken from 5 broiler farms with a recent history of poultry meat containing residues of CAP. The samples were tested for the presence of *S. venezuelae* and assayed for CAP.

**Results and discussion**

The experimental design was guided by preliminary trials in which litter samples were spiked with S. venezuelae in GYM Streptomyces broth. The preliminary results indicated that there may have been some limited production of CAP in the poultry litter. However, it was suspected that the CAP detected was actually contained in the broth used to spike the samples, in which the S. venezuelae had been incubated for 48 hours.

It was also likely that the GYM broth added to the litter was able to support the growth of the organism, whereas the litter alone, without the nutrients contained in the broth, might not support growth. The study was designed, therefore, to use normal saline as the spiking medium. The results of the first experiment in this study showed that S. venezuelae was viable for at least 7 days in normal saline (Figure 1), but the cell count dropped by more than 95% after 2-3 weeks. A suspension in normal saline could therefore be used up to a few days after preparation.

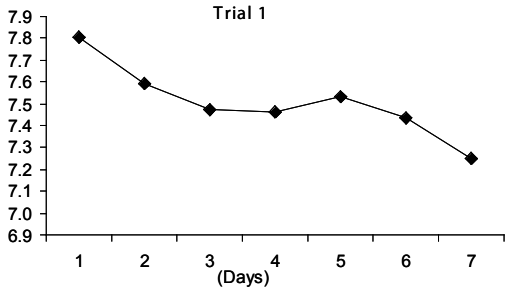


**Figure 1**. Viability of S. venezuelae cells in normal saline

It was further suggested by preliminary studies that CAP, which was present initially at easily measurable concentrations, was unstable in poultry litter, since no CAP was detected after 3 weeks. However, the results of the present study showed that the mean CAP concentration in the 10 litter samples spiked with CAP decreased gradually from 0.60 μg $kg^{-1}$ to 0.37 μg $kg^{-1}$ over 7 weeks (Figure 2).

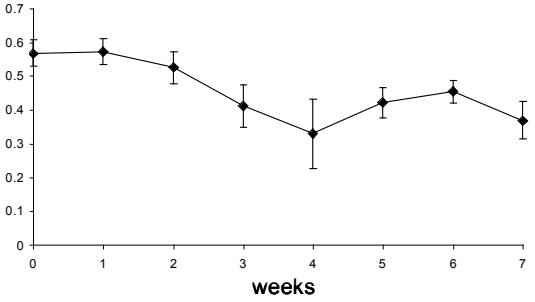


**Figure 2.** Stability of CAP in spiked litter samples (means ± SD, n=10)

The capability of *S. venezuelae* to produce CAP in litter was investigated by spiking litter samples with suspensions of the organism in CAP-producing phase, as described above. Although the CAP concentration in the normal saline suspension remained constant (8.3 ± 0.83 μg $kg^{-1}$, mean ± SD) over the 5 week period of the trial, the concentrations in the 4 litter samples spiked with the *S. venezuelae* suspension decreased from initial measured concentrations (0.26 - 0.6 μg $kg^{-1}$) to less than the detection limit of the method after 3 weeks (Figure 3). In the experiment described above to investigate the stability of CAP in litter spiked with the drug, CAP concentrations, initially in the same range as those in the *S venezuelae* spiked samples, remained easily measurable for at least 7 weeks. The rapid decrease in concentration in litter spiked with *S. venezuelae* may be due to the deactivation and/or degradation of CAP by the organisms. Cultures of *S. venezuelae* not engaged in chloramphenicol synthesis are strongly inhibited if exposed to the antibiotic, but become resistant by inactivating it (Malik and Vining, 1972).

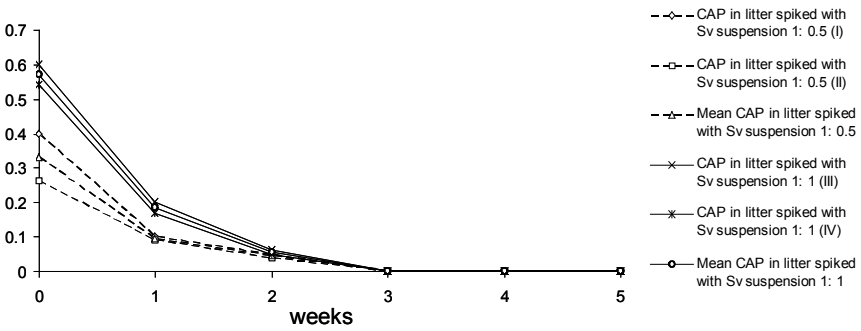


**Figure 3.** Stability of CAP in litter samples spiked with *S. venezuelae* suspension

It was postulated that the moisture content of the litter may be an important factor in its feasibility as a growth medium for S. venezuelae. The experimental protocol affected the moisture content of the litter used in the study, since the S. venezuelae was added as a suspension in normal saline. It is possible that litter with higher moisture content may provide a more suitable medium for the anaerobic growth of the organisms. Many factors affect both the growth of S. venezuelae and the biosynthesis of CAP by the organism. For example, the biosynthesis of CAP is usually uniphasic, linked with vegetative growth of the bacterium, but can also be biphasic and not linked with biomass accumulation, depending on composition and accessibility of available nutrients (Vining and Stuttard, 1994; Bhatnagar, Doull and Vining, 1988). To gain some knowledge of the moisture content, 20 samples of litter from working broiler producers were collected at various time points over the broiler production cycle from day 0 to week 6. The average moisture content almost doubled, from about 20% on day 0 to about 38% after 6 weeks. The investigation into the viability of S. venezuelae in litter with differing moisture content demonstrated that there was no growth of the organism after 3-4 weeks in litter with approximately 30%, 40% or 50% moisture.

The rapid decrease in CAP concentration and the apparent lack of viability of S. venezuelae in the litter may have been at least partly due to inhibition of the growth of S. venezuelae after its addition to the litter by the CAP produced in the starter culture conditions in GYM broth and/or in normal saline, and the simultaneous deactivation of the CAP by the S. venezuelae which was no longer in an environment that facilitated CAP biosynthesis.

The samples of litter collected from 5 farms with a recent history of CAP residues in poultry meat contained no detectable CAP and S. venezuelae was not isolated from any of the samples. This result suggests that the non-compliant results for poultry from these producers arose neither from natural CAP synthesis nor from persisting environmental residues of CAP resulting from historical veterinary uses.

**Conclusions**

The results of this study strongly suggest that chloramphenicol-contaminated poultry tissue samples originating from producers in Thailand were not caused by natural occurrence of CAP in the production environment. This is an important finding in relation to the continuance of the enforcement of the EU MRPL for CAP.

**Acknowledgements**
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# O 3
# CAN SULFA-CONTAMINATED BEESWAX LEAD TO RESIDUES IN HONEY?

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**Abstract**
Despite the prohibition of the use of sulfa drugs in beekeeping, residues of sulfonamides were found in 12 out of 203 tested honey samples in the framework of the quality control of Flemish honey in 2003. In five honey samples, the contamination was limited to concentrations beneath 50 µg/kg. The five producers involved claimed not having used veterinary drugs.
When performing residue control on beeswax intended for the fabrication of wax foundations, also sulfa residues were found.
A migration test was set up to study whether sulfa-containing beeswax can lead to contamination of the honey. Blank beeswax, free from sulfonamides, was spiked with sulfamethazine at three different levels and used for the fabrication of wax foundations. After framing, the wax foundations were placed in different hives to be further constructed by the honeybees to honeycombs and filled with honey. Once the cells were sealed by the bees, the combs were removed and further incubated at 35°C for monthly sampling of the honey for sulfa residue analysis by LC-MS/MS.
The higher the concentration of the sulfonamides in the wax, the more residues were found in the honey. The carry-over of sulfamethazine from beeswax to honey was about 1%.

**Introduction**
In Europe no maximum residue limits (MRLs) are set for sulfonamides in honey (Anonymous, 1990). Some Member States established action limits, taking in account analytical possibilities and available toxicological data. The community reference laboratory proposed 50 µg/kg as the recommended concentration for the screening of sulphonamides in honey (Anonymous, 2007). Despite the prohibition of the use of sulfa drugs in beekeeping, residues of sulfonamides were found in 12 out of 203 tested honey samples in the framework of the quality control of Flemish honey in 2003 (Reybroeck *et al.,* 2004). In most cases it concerned high concentrations of sulfamethazine (till 13 mg/kg), caused by an illegal use of sulfonamides by the beekeeper to prevent or to treat nosemosis. However, in five honey samples the contamination of sulfonamides was limited to concentrations beneath 50 µg/kg. The five producers involved claimed not having used veterinary drugs.
When performing residue control on beeswax intended for the fabrication of wax foundations, sulfa residues were found in imported beeswax as well as in local beeswax from hives treated with sulfonamides (Reybroeck *et al*., 2004). No literature is known if sulfa containing beeswax can lead to contamination of the honey. A migration test was set up to study this possibility.

## Materials and Methods

*Liquid blank* beeswax, free from sulfonamides, was spiked with sulfamethazine at three different levels. From this spiked beeswax, wax foundations were fabricated. A small amount of each wax foundation was sampled for sulfa residue analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). After framing, the three wax foundations were placed in different hives to be further constructed to honeycombs by the honeybees. Afterwards, the combs were placed in the honey supper during full summer blossoming. Once the cells filled with honey and sealed by the bees, the combs were removed and further incubated in the laboratory at 35°C. Monthly honey was sampled for sulfa residue analysis by LC-MS/MS.

For the determination of sulfamethazine in honey and beeswax, a liquid chromatographic-tandem mass spectrometric method was used. For honey the clean-up was based on the method described by Maudens et al. (2004). In short, 1.5 g of honey was dissolved in 1.5 ml of 2 M hydrochloric acid, followed by an extraction with acetonitrile and a SPE clean-up making use of $C_{18}$ columns. For beeswax 5 g of beeswax was dissolved in 30 ml of hexane, followed by the addition of 20 ml of MSU extraction buffer (Charm Sciences Inc., Lawrence, MA). After centrifugation the water phase was transferred to a pre-conditioned $C_{18}$ column. The sulfa residues were eluted using methanol. The methanol was evaporated under $N_2$ at 45°C and the residues were dissolved in the mobile phase.

The extracts were injected into the LC-MS/MS system. A gradient consisting of acetonitrile and water, each containing 0.1 % formic acid, was applied. The abundant precursor ions $[M + H]^+$ produced by positive electrospray ionisation were selected for collisional dissociation with argon. Sulfachoropyridazine was used as internal standard. For sulfamethazine the transition of the precursor ion into a least two product ions was followed in multiple reaction monitoring. For sulfamethazine in honey the decision limit (CC$\alpha$) was 2 µg/kg and the detection capability (CC$\beta$) 2.4 µg/kg. The detection limit in beeswax was 25 µg/kg.

## Results and Discussion

Since doping took place in heated beeswax, parts of the doped wax foundations were analyzed by LC-MS/MS to know the exact concentration of sulfamethazine. The wax foundations A, B and C contained respectively 587, 3761 and 73.098 µg/kg sulfamethazine. The results of the carry-over of sulfamethazine to the honey during storage of the honey in the combs are shown in table 1.

**Table 1.** Concentration of sulfamethazine in the honey stored in the combs made by the bees from the spiked wax foundations; results at different sampling dates.

| Date of sampling | Sulfamethazine concentration (µg/kg) in honey | | |
|---|---|---|---|
| | **Comb A** | **Comb B** | **Comb C** |
| 17/07/2006 | <2 | 10 | 176 |
| 17/08/2006 | 3 | 52 | 628 |
| 18/09/2006 | 2 | 58 | 704 |
| 17/10/2006 | 3 | 70 | 605 |

Comb A, B and C with respectively 587, 3761 and 73.098 µg/kg sulfamethazine in the wax foundation.
The higher the concentration of the sulfonamides in the wax, the more residues were found in the honey. The carry-over from sulfamethazine from the beeswax to the honey was about 1%. From the second month on rather steady values were found in the honey.

**Conclusions**
The results of this study show that sulfonamides are able to migrate from contaminated beeswax to the honey kept in the contaminated combs. Hence the purchase and the use of contaminated wax foundations by the beekeeper can lead to measurable contaminations of the honey. Beekeepers can be advised to recycle their own beeswax for the fabrication of the wax foundations or to ask for a certificate when buying wax foundations from unknown origin. The use of a synthetic foundation wax like Syncera® (Jacobs and Remon, 2001) could also be an effective alternative for contaminated beeswax.

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## O 4

# CONTROL OF NITROIMIDAZOLE RESIDUES IN FOOD OF ANIMAL ORIGIN: OVERVIEW OF RECENT RESULTS ON SUITABLE MATRICES, ANALYTE STABILITY AND METABOLISATION

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**Abstract**
As suspectedly cancerogenic and mutagenic compounds, 5-nitroimidazoles are banned in many countries from use in food-producing animals. In the EU this is explicitly the case for dimetridazole, ronidazole and metronidazole. In order to guarantee an effective control of residues of these drugs, a comprehensive knowledge with respect to metabolisation and the selection of suitable matrices is required. For this purpose pigs were treated with 4 different nitroimidazoles. The animals were slaughtered, muscle, liver, plasma, serum and retina were collected and analysed. Plasma, serum, muscle and retina proved to be suitable matrices for residue control in pigs. The analytes showed comparable residue levels in the first 3 matrices and were stable at least for a few days at +4 °C. It is recommendable to analyse the drugs as well as the hydroximetabolites, since in parts a rapid metabolisation takes place.

**Introduction**
Dimetridazole (DMZ), ronidazole (RNZ) and metronidazole (MNZ) are Annex-IV-substances of Council Regulation 2377/90/EC, and as such are banned from use in food-producing animals in the EU. In addition they have not been authorised as feed additives since 14 November 2001 as per Regulation 2205/2001, so that each positive result discovered in the framework of residue control constitutes a case of misuse.
With respect to an effective residue control of these compounds it is important to have reliable information on their metabolisation, their stability and distribution in different matrices.
The paper summarises the results of recent in-house animal studies with pigs, draws a comparison to results of earlier animal studies and provides recommendations for an effective residue control.

**Animal studies**
In the last few years several animal studies with nitroimidazoles were performed at the BVL. Turkeys, pigs and hens were treated with different nitroimidazoles (DMZ, RNZ, MNZ, ipronidazole, IPZ), the animals were slaughtered and different matrices were collected. The diverse matrices (muscle, plasma, serum, retina, liver, eggs) were tested for analyte stability in matrix and, if applicable, for homogeneity. The ratio of the drugs and their corresponding hydroxy metabolites (DMZ/RNZ -> HMMNI; MNZ -> MNZOH; IPZ -> IPZOH) was calculated.

**Analytical methods**

The analytical procedure is - apart from some minor modifications - similar for all investigated matrices. It includes a pre-treatment with protease, an aqueous extraction at pH 3, a defattening step, a change of pH values and an extraction on diatomaceous earth with ethylacetate/tBME. The extract is evaporated and either derivatised with BSA followed by GC-NCIMS analysis or reconstituted in eluant and analysed by LCMSMS. Due to several advantages LCMSMS has meanwhile become the preferred techniques. Among others the method can easiliy be expanded to additional potentially relevant nitroimidazoles as tinidazole, carnidazole or secnidazole.

Fig 1 depicts a chromatogram of a standard mixture of 9 nitroimidazoles and 3 hydroxy metabolites. The mixture is analysed by UPLC / MSMS ESI+ (Waters Quattro Premier XE) on a 100 * 1 mm BEH $C_{18}$ 1.7 µm UPLC column using a gradient with water/acetonitrile, 0.1 % formic acid. Concentrations of 1 ppb and below can easily be analysed.

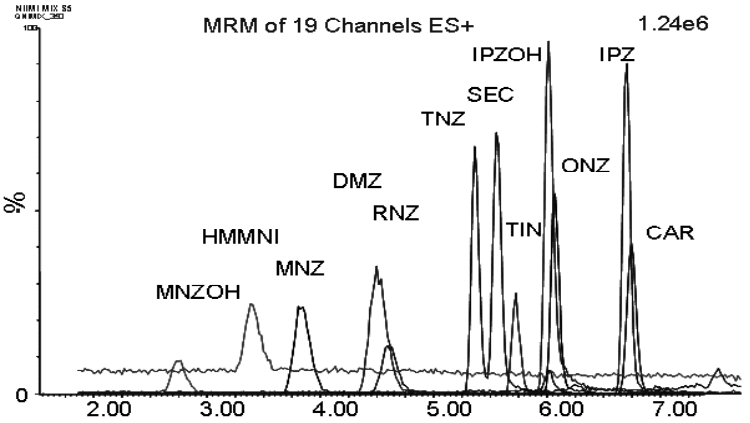


**Figure 1.** Example of an MSMS-chromatogram of nitroimidazoles (standard, 0.01 ng/µl) (in elution order: MNZOH, HMMNI, MNZ, DMZ, RNZ, ternidazole, secnidazole, tinidazole, IPZOH, ornidazole, IPZ, carnidazole; run time 12 min)

**Studies with turkeys**

The analysis of incurred turkey samples was the starting point for this kind of studies. During the preparation of reference material for a proficiency test (incurred muscle with ronidazole) it was discovered that the analyte is unstable in this matrix and not homogenously distributed. Since this had not been reported before, additional animal studies were started in order to obtain more reliable information. The main findings of these studies were that there was an inhomogeneous analyte distribution in muscle samples and a high risk of analyte degradation in muscle and liver samples, at least partly even at +4°C. Hence the target matrices for residue control should be plasma, serum or retina, since higher residue levels can be found in these matrices and analyte stability is given. The drug as well as the hydroxymetabolite should be analysed, as especially IPZ and DMZ are rapidly metabolized. On the other hand the ratio drug/metabolite is not constant throughout the withdrawal period. Details of the results in turkey are described in [1,2].

**Studies with pigs**
4 pigs were treated with one drug each (drugs applied: DMZ [Emtryl™], IPZ [Ipropran™], MNZ [Ursometronid™], RNZ [Ronida Bt™]) in a dosage of 10 mg drug /kg body weight. The animals were treated for 10 days. 18 h before slaughtering the medication was discontinued. The samples were directly collected after slaughtering (plasma: ice-cooled beakers with heparin, centrifugation; serum: stored in beakers overnight at +4°C, centrifugation; muscle: portioned and deep-frozen; liver, eyes, kidney: deep-frozen as a whole).

*Studies on stability and homogeneity in muscle*
For homogeneity testing 5 sub-samples were taken from one piece of muscle. Each of the sub-samples was analysed in triplicate. The results were analysed for the presence of a basic population by applying an analysis of variance. Figure 2a and b give examples for the evaluation of two of the analytes, HMMNI and MNZ.

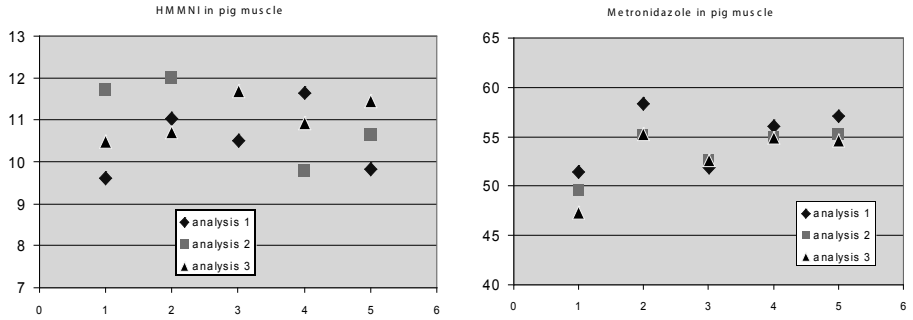


**Figure 2.** Examples (HMMNI and MNZ) of the results of the homogeneity study for nitroimidazoles in pig muscle (5 pieces of meat, each analysed in triplicate)

For HMMNI a basic population was found, for MNZ this was not the case (the difference in the mean values of the samples was greater than it could be expected by chance). The minimum and maximum values of this study for all analytes are included in table 1. Stability studies were performed in a first step with fresh frozen muscle, which was stored under different conditions in an isochronous study design. The reference temperature was -80°C, the additional conditions were – 30 °C, +4°C and + 25 °C with storage durations of 0.5, 1 and two days. The resulting values showed unusual variations and no clear trends, which made an evaluation impossible. A possible explanation was an insufficient homogeneity of the muscle samples. In a second step the study was repeated, but now using minced and homogenized muscle material (a mixture of four different incurred muscle samples). Again aliquots of the mixture were stored at +4°C and + 25 °C (reference temperature: -30°C) for 1 and 2 days. This study produced consistent results, all analytes proved to be stable at least for two days at + 4 °C. After two days of storage at +25°C some of the analytes showed a beginning degradation.

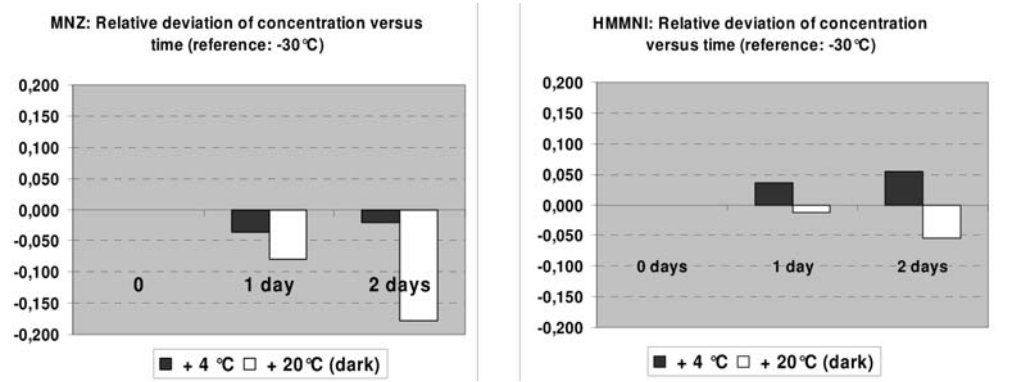


**Figure 3.** Examples of the results of the stability study on nitroimidazoles in pig muscle: MNZ with beginning degradation (at + 20 °C approx -15 % compared to the sample stored at the reference temperature of -30°C) and HMNNI (no significant degradation)

*Studies on stability in plasma and serum*

The stability of the analytes in plasma and serum was tested in a similar way to the muscle samples using an isochronous design. A mixture of incurred plasma was stored for 1 to 5 days at +4°C and + 25 °C with -30°C as reference temperature.

All analytes proved to be stable at least at + 4 °C for up to 5 days (fig. 4). After one day of storage at +25°C a significant degradation can be observed for all analytes. The serum samples showed a comparable behaviour to the plasma samples.

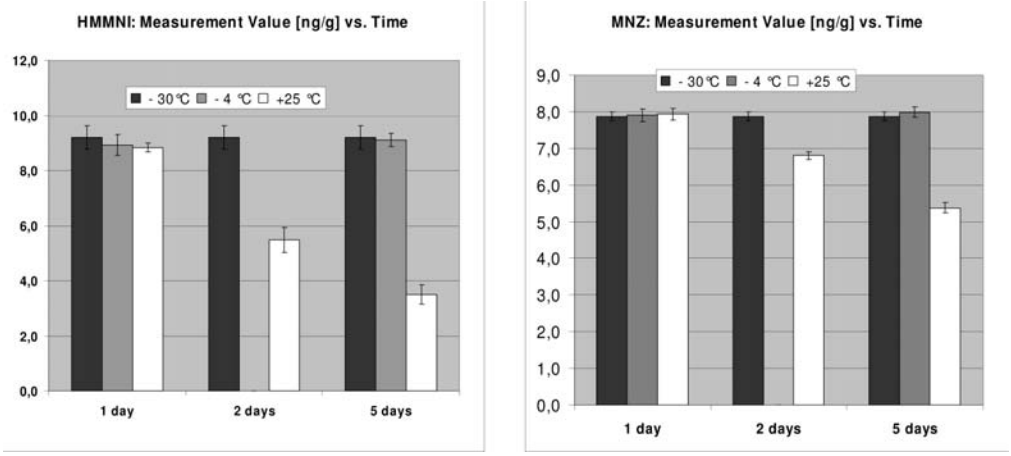


**Figure 4.** Examples of the results of the stability study on nitroimidazoles in pig plasma: stable at +4°C for up to five days, beginning degradation at + 25 °C after 1 day

*Overview of the results of the pig studies*

Table 1 gives an overview of the results of the analysis of different nitroimidazole residues in different matrices. For muscle also the minimum and maximum concentrations determined in the homogeneity study (see above) are indicated.

**Table 1.** Nitroimidazole-medicated pigs: accumulation behaviour of different drugs, drug/ hydroxy metabolite ratios and comparison of the residue levels in different matrices

| Medication: | Muscle ng/g | | Plasma ng/ml | Serum ng/ml | Liver ng/g | Retina ng / g retina |
|---|---|---|---|---|---|---|
| DMZ | nn | (<0,1) | (<0,1) | (<0,1) | nn | |
| HMMNI | 10,7 | | 11,7 | 11,3 | <1 | 123 |
| min/max | 5 | 15,1 | | | | |
| RNZ | 1165 | | 840 | 1100 | nn | 2449 |
| min/max | 1109 | 1210 | | | | |
| HMMNI | 10,8 | | 11 | 14 | nn | |
| min/max | 9,6 | 12 | | | | |
| MNZ | 53,8 | | 61 | 62 | nn | 563 |
| min/max | 47,3 | 58,3 | | | | |
| MNZOH | 107 | | 93* | 97* | 1 | 528 |
| min/max | 100 | 112 | | | | |
| IPZ | nn | (< 0,1) | (< 0,1) | (< 0,1) | nn | |
| IPZOH | 32,1 | | 44 | 46 | nn | 845 |
| min/max | 26,8 | 36,4 | | | | |

The residue levels in plasma, serum and muscle are quite comparable, for the drugs as well as for the metabolites; in liver, no residues at all could be detected. An accumulation takes place in retina (values referring to the weight of the retina prepared out of the eye ball).
The ratio of drug to metabolite is similar for all matrices for one medication; in case of dimetridazole and ipronidazole, the hydroxy metabolite is by far the dominant residue, for ronidazole it is the drug itself. In MNZ-treated pigs drug and metabolite are found, MNZOH being the main residue. Taking into account that all animals received the same drug dosages simultaneously and were slaughtered at the same time, it is clear that RNZ produces the highest residue levels, followed by MNZ and IPZ. The treatment with DMZ yielded the lowest residue levels.

**Summary and conclusions**

Nitroimidazoles showed a different residue behaviour in pigs and turkeys. Whereas the nitroimidazoles were inhomogenously distributed and showed a rapid degradation in turkey muscle, the situation was different for pig muscle: the analytes were stable at least for a few days at +4°C and “more homogenously” distributed. Since the residue levels in plasma, serum and muscle are comparable, all of these matrices (and retina) are suitable for residue control. Liver proved to be unsuitable for pig as well as for turkey.
The ratio of drug to hydroxy metabolite was similar for both species. DMZ and IPZ metabolise rapidly, consequently the hydroxy metabolite is the main residue. For RNZ the drug itself is the main residue. MNZ and its hydroxy metabolite lie in between, i.e. partly the drug, partly the hydroxy metabolite is dominant. Since the ratio of drug to hydroxy metoblite is not constant throughout the withdrawal period, it is always recommendable to control the drug as well as the hydroxy metabolite.

**Acknowledgement:**
The financial support of the European Commission is gratefully acknowledged.

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## O 5

# HERBAL ALTERNATIVES FOR ANTIMICROBIAL GROWTH PROMOTERS

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**Abstract**
Since the use of antibiotics in animal feed is forbidden in the EU, many feed companies have explored the use of botanicals as an alternative. Many herbal feed supplements have been put in the market to improve animal growth and performance. Organic farmers should preferably use natural remedies such as phytotherapy to treat animals, but since few are registered as veterinary medicines, information on the quality and the efficacy of the products on the market is lacking. On request of the Organic farmers organization (Bioconnect) the Dutch Government initiated in 2006 the Fyto-V project, aimed at the rational use of herbal therapies for prevention and treatment of diseases in organic farming. First an inventory was made of the herbal products on the market, their legal status, indication and literature available. With some promising products animal experiments have been performed and quality and biological activity have been investigated. Most herbs are sold as aromatic feed supplements without any health claim. Nevertheless several herbs are known to have pharmacological effects on micro-organisms affecting gut health and performance. Herbal products that are occasionally produced and sold without any involvement of expertise in the field of pharmacognosy and phytotherapy may consist a threat to both animal welfare and human health.

**Introduction**
Fyto-V is the short-name of the project: Development of phytotherapy as a tool for reducing and/or prevention of diseases in farm animals. The project is initiated in 2006 by the Dutch Ministry of Agriculture, Nature and Food Quality on request of the Organic farmers organization (Bioconnect). Organic farmers should preferably use natural remedies such as phytotherapy to treat animals, but since few are registered as veterinary medicines, information on the quality and the efficacy of the products on the market is lacking. Goal of the project is to increase the rational use of herbal therapies for prevention and treatment of diseases in organic farming. The regular animal husbandry is also interested in products that may reduce the need for antibiotics.
In this project the state of the art and the available herbal products were evaluated and recommendations were made for adjustment of legislation to facilitate product development. Another issue that is covered by this project is to improve the acceptation of herbal products

with a proven effect on animal health by regular veterinary medicine.
The project is performed by: RIKILT - Institute of Food Safety of Wageningen UR, Institute for Ethnobotany and Zoopharmacognosy,The Netherlands Association for Phytotherapy, Department of Veterinary Pharmacology and Toxicology, Veterinary Faculty Utrecht University, PhytoGenix, University of Utrecht, Department of Medicinal Chemistry, Faculty of Pharmacy, Animal Science Group (ASG), of Wageningen UR, The Louis Bolk Institute, Driebergen and the HAS Den Bosch

**Material and methods**
The project group used a snowball method to make an inventory of the available herbal products for livestock on the Dutch market. Literature about the efficacy of herbal products in livestock was obtained from the suppliers of these product, as well as through literature search in pubmed, agricola, organic prints and other specialised databases and books. Expert meetings were organised to evaluate the documentation about the products. The evaluation was done both against the available literature and against the need of farmers for the kind of applications the products offered. Pilot animal experiments were performed with eleven products that the experts identified as promising. These experiments (in dairy cows, poultry and pig husbandry) were set out to investigate the claimed action in practice and under experimental conditions and were performed by specialised research institutions.
The project included in vitro research to check the quality and safety of the products. Stability tests on the amount of active compounds using High Performance Liquid Chromatography (HPLC) and measuring of the antioxidant properties of the products (using the ORAC test system) were performed by the Pharmacy department of Utrecht University; Testing of the products with regard to contaminations such as mycotoxins, ionophors and antibiotics as well as bioassays for hormonal action was done by the RIKILT Institute of Food Safety (RIKILT). Due to limits of the budget the number of products taken to test both *in vitro* and *in vivo* was limited to eleven. Five products, that had shown promising results for the control of coccidiosis in broilers, were included in a test for organic laying hens. Three products that documented better growth and health of pigs were tested in field trials. Following the request of the target group the influences of three herbal remedies on the somatic cell count (SCC) of dairy herds were assessed in a pilot study.
An inventory was made of the legal regulations concerning the use of herbal products for farm animals in European countries, The USA, Canada, China and Swiss and this was compared with the regulations that appy for the use of herbal products in humans. The Delphi method was used to include the opinions of several stakeholders and governing institutions in the project.
Several activities were undertaken to share the findings of the project with the target groups. Education in phytotherapy was developed for the professional agricultural universities to increase the scientific acceptance of this method. A website was established, several press announcements were released and about 10 interviews were given.

**Results**

*Many products*
During November and December of 2006 circa 60 Dutch and foreign suppliers were traced,

who had put 255 herbal preparations for pigs, cows or poultry on the market.
This large number of products has been developed partly as a response to the ban on antimicrobial growth promoters in animal feed in the EU, since January 2006 and the new adjustments of the regulations for the use of veterinary medicines which will be implemented shortly. Additional to the product information about these herbals about 30 related (in vitro) studies and 150 clinical studies with herbs were identified that were not confidential, of which only 65 (43%) were published in peer review journals (table 1). Several producers showed us in confidence their documentation on experiences with their products, both performed by independent research institutes as well as results from practice. This indicates that many promising products are available. These products are mostly marketed as aromatic food additives, although many of them are made of herbs that are listed in the European Pharmacopoeia because they are used for medicinal applications in human medicine. The claim is mostly restricted to "increased appetite". In several cases documentation exists of the preventive or damage control effect that these herbs will have in animals, for example in relation to stressful periods as weaning or transport, or during infections with E-coli or clostridia. Dermatologicals and external preparations for (mastitis) is a second group that is well represented in this market.

**Table 1.** Numbers of publications and products* in relation to the indication**

| **Indication** | **A1** | **A2** | **A3** | **A4** | **D** | **G** | **I** | **J** | **M** | **N** | **P** | **R** | **sum** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Publicaties** | 5 | 96 | 7 | 13 | 1 | 22 | 6 | 23 | 0 | 9 | 10 | 0 | 192 |
| **Preparaten** | 13 | 55 | 24 | 19 | 12 | 24 | 2 | 0 | 1 | 5 | 7 | 7 | 168 |

** The nr of publications concerns both clinical studies and in vitro studies. Some of the products are used in more then one animal species for the same indication; that is why the number of products here is lower than the total amount of products mentioned in the text.*

*** Indications: A = Alimentary tract (A1= against diarrhea and constipation; A2 = appetite and growth enhancing products; A3 = other specifics like liver function and special diets; A 4 = prebiotics and dried probiotics) D=dermatological; G= Genito-urinary; I = Immunological; J = antiinfective (systemic); M = musculo-skeletal system; N = nervous system P = antiparasitic products, insecticides and repellents; R = respiratory system.*

**Table 2.** Numbers of products in relation to target species and indication*:

| Indication Animal spp | A1 | A2 | A3 | A4 | D | G | I | J | M | N | P | R | sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cattle** | 6 | 22 | 10 | 8 | 11 | 20 | 0 | 0 | 1 | 0 | 2 | 6 | 86 |
| **Pig** | 6 | 26 | 8 | 9 | 9 | 4 | 0 | 0 | 0 | 3 | 2 | 6 | 74 |
| **Poultry** | 3 | 23 | 9 | 5 | 3 | 0 | 0 | 0 | 0 | 4 | 5 | 2 | 54 |
| **Small ruminants** | 2 | 6 | 6 | 2 | 9 | 10 | 0 | 0 | 1 | 0 | 2 | 3 | 41 |
| **SUM** | 17 | 77 | 33 | 24 | 32 | 34 | 0 | 0 | 2 | 7 | 11 | 17 | 255 |

** Indication legend: see table 1. Only the main indication of each product is mentioned. Some products however have more than one indication.*

*Animal experiments and laboratory tests*
The results of the in vitro and in vivo tests were not yet available at the moment of the deadline for this manuscript.

*Legislation*
For the Fyto-V project a report has been written about the legal use of herbs (Groot et al., 2007). This report describes the legal arrangements for the use of herbs in farm animals. It describes:
- the current legislation in the Netherlands, Europe and in a number of other important countries regarding the use of herbs in animals and in humans
- the bottlenecks in current legislation as noticed by the registration authorities, the industry and the animal production sector
- the recommendations of the project group FYTO-V to solve these bottlenecks.

A distinction is made between the use of herbs as part of animal feed, as animal feed supplemental, as animal feed additive and as veterinary medicinal product. Also, attention has been given to the possibilities of using herbs in organic agriculture and to the legislation abroad. Dutch legislation in the area of animal feed and veterinary medicinal products is based on European legislation and consists of a national implementation of this legislation. Without medical claims herbs can be used in animal feed, provided they are safe and not mentioned on the list of undesirable substances of the European Directive 2002/32/EC, provided they do not contain toxic substances above permitted levels.

*Education*
Education in herbal medicine (phytotherapy) is scarce in The Netherlands. In the regular agricultural courses both on the MBO and HBO level and in the program of the faculty of Veterinary Medicine phytotherapy is not part of the teachings.
For the Fyto-V projects HAS (Agricultural High School) students developed a course in phytotherapy for the higher levels of agricultural education called “Plant, dier en gezondheid”, (applied phytotherapy for production animals [Van Boekel et al. 2007]). In 2008 the first trial of the course will be held at HAS Den Bosch. January 17, 2008 a symposium was organised by HAS Den Bosch named:
“Fyto, diergezondheid in de toekomst” (Phyto, the future for animal health).

On this symposium both farmers, scientists, veterinarians, feed- and herbal industry and growers of herbs were present.

**Discussion and Conclusion**

The introduction of herbal and related natural products in the animal food realm has contributed to maintain the productivity of the livestock after banning the use of antibiotics as growth promoters. To get more benefit of these promising approaches the scientific community should get more involved and support innovative developments in this area. Legislative constrains for this developments should be evaluated and if possible removed. However it is of equal importance that science is involved in monitoring and controlling the risks accompanying these new products. Unfortunately it has been reported that sometimes herbal veterinary products are used as a carrier of illegal pharmacological substances. There may be yet unknown risks involved.

Very little is known about

- The interaction of natural growth promoters with each other and with veterinary medication (be it negative or, under control, positive)
- The residual or other effects these products have on meat quality (again: we must assess risks here but there are also some positive signals, for example the feeding on oregano seems to give more unsaturated fatty acids in meat; the production of milk and eggs as functional food when certain herbs are given to the animals (Gerber, 1997; Wenk, 2005)
- The implementation of the natural products in the specific management situation of each farm. Many general health problems are solved by vaccination and balanced food; the remaining problems are often farm related and require specific additional products (like herbal supplements) to solve them. The choice in natural products is overwhelming and good monitoring of their results in specific situations will be necessary to make a better use of them.

Expertise on these topics should be build up in all involved institutions.

**Acknowledgements**

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M.Y. Noordam and G.A. Kleter (RIKILT staff members) contributed to the legislation report.
M.N. Zijlstra (IEZ) edited the literature studies that were gathered in a spreadsheet; G. de Jong (Plumage) designed the Fyto-V website.
H. van Osch (Professional Agricultural University HAS Den Bosch) and several students of HAS organised the promotional and educational activities in the Fyto-V project.
The project was financed by the Dutch ministry of Agriculture, Nature and Food (LNV) on request of the Bioconnect organisation.

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# O 8

## ANALYSIS OF THYREOSTATS: A HISTORY OF 35 YEARS

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**Abstract**
Thyreostatic drugs (TS) are banned in Europe since 1981, according to the Council Directive 81/602/EC. The abuse of TS results in production of inferior meat quality and represents a potential risk to human health. Therefore, analytical methods need to be developed for the detection of the illegal use of these substances. Various techniques have been described, ranging from investigation of morphological properties of the thyroid gland to chemical detection methods (e.g. colorimetric reaction, thin layer chromatography, gas chromatography, liquid chromatography, GC-MS, LC-MS). This evolution is characterized with a continuous improvement of sensitivity and specificity. The detection methods for monitoring the TS abuse of the last 35 years will be discussed in this review.

**Introduction**
The administration of thyreostatic drugs (TS) to cattle results in a spectacular profit of live weight gain. The weight gain is mainly due to the increased filling of the gastro-intestinal tract as well as the retention of water in edible tissues by inhibiting the thyroid hormone production (Derblom et al. 1963). This results in meat of lower quality and may be considered as an abuse, because water is sold for the price of meat.
Above this, there is the danger of residues: Residues of thyreostatic drugs are teratogenic and carcinogenic (Martinez-Frias et al. 1992; Gissel and Schaal 1974). For these particular reasons, thyreostats are banned within the EU since 1981 (Council Directive 81/602/EC).

**Thyreostats**
Thyreostats are polar and amphoteretic thionamides, which are small molecules derived from thiouracil or mercaptoimidazole. The most important substances in residue analysis within TS are 4(6)-R-2-thiouracil (R= hydrogen, methyl, propyl, phenyl), tapazole and mercaptobenzimidazole (Fig. 1). These TS have a common sequence in their structure: the nitrogen-carbon-sulfur sequence, which is responsible for their thyroid-inhibiting activity. Amongst the thiouracils, especially 2-thiouracil and 6-methyl-2-thiouracil are important.

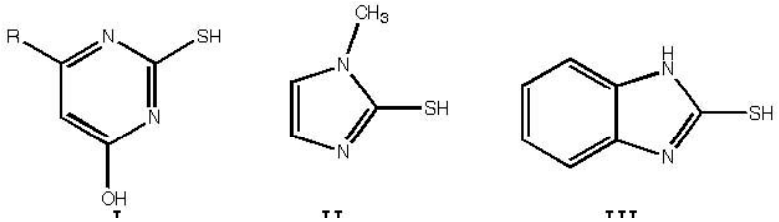


**Fig. 1:** Structural formulae of thyreostats. I: 4(6)-R-2-thiouracil; II: 1-methyl-2-mercaptoimidazole (tapazole); III: 2-mercaptobenzimidazole.

Additionally, it has been described that also a large number of other molecules can act as TS (Heany and Fenwick 1995). For instance inorganic ions as lithium ($Li^+$), perchlorate ($ClO4^-$) and thiocyanate ($SCN^-$), but also some veterinary drugs like sulphonamides.
Natural thyreostats may be formed from precursors (glucosinolates), which are presents in some plant species (a.o. the Cruciferia) (Van Etten and Wolff 1973). This formation is catalyzed by an enzyme, a thioglucosidase (EC 3.2.1.147), which is capable in splitting of the glucose and sulphate moiety. The remainder of the molecule, depending upon the pH, can be transformed in several biological active compounds. At low pH (3-4) sulfur is split off and a nitrile is produced, which can split further forming cyanide ($CN^-$). At pH 5 to 9 isothiocyanates are formed, from which thiocyanates or oxazolidine-2-thiones can be generated. The formation depends entirely upon the structure of the variable side chain.
Of the natural thyreostats, especially goitrine or 5-vinyl-1,3-oxazolidine-2-thione is important (Fenwick et al. 1983; Quinsac et al. 1992).

**Detection of thyreostatic drug abuse**

*Symptoms of hypothyroidism*
Hypothyroidism is a condition characterized by an insufficient production of thyroid hormones, triiodothyronine ($T_3$) and thyroxine ($T_4$), by the thyroid gland (Roberts and Ladenson 2004). The symptom of this disorder is an enlargement of the thyroid gland, also called a goiter. It can be applied for the detection of TS abuse and is furthermore easy to perform at the slaughterhouse.
In fig. 2 three bovine thyroid glands (a lobe) are presented. At the left a lobe of a normal thyroid gland is shown, weighing 17 grams. In the middle, a typical enlargement of the thyroid gland due to the influence of TS, in this case MTU. At the right the largest thyroid gland ever presented in the laboratory, due to the influence of TS (1266g).

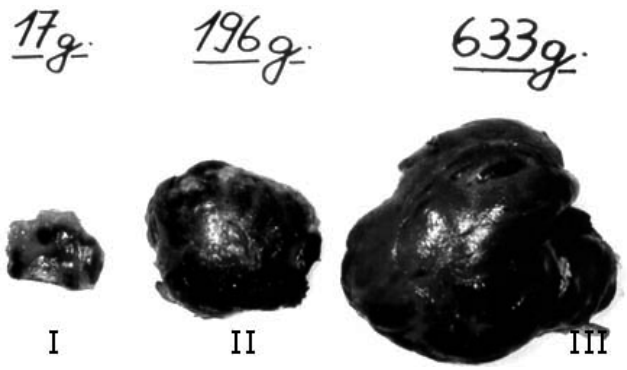


**Fig. 2:** Bovine thyroid glands (a lobe). I: Normal size; II: Enlargement or goiter due to thyreostatic drug; III: Extreme enlargement.

*Chemical detection of thyreostatic drug residues*

In the beginning of 1970's a colorimetric method, the so called "Van Waes" method was applied for TS abuse detection. The reaction was based on the formation of a distinctive yellow color, that resulted from the reaction with 2,6-dichloroquinonimide (Van Waes 1973). In 1973 – 1974, a thin layer chromatographic method (TLC) was reported (Gissel and Schaal 1974). In our laboratory, a TLC method was developed, based on the reaction of TS with NBD–Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazol) (De Brabander and Verbeke 1975). The reaction resulted in a non-fluorescent TS-NBD adducts, which were separated by TLC. For visualization of the TS-NBD adduct, the TLC plate was sprayed with cysteine. In function of time high-performance thin layer chromatography (HPTLC) took over the place of TLC (De Brabander et al. 1988).

Later in the 80's gas chromatography-mass spectrometry (GC-MS) originated (Floberg et al. 1980). In the beginning, the technique was only used for the confirmation of suspected HPTLC spots. In the 90's liquid chromatography-mass spectrometry (LC-MS) was introduced (Blanchflower et al. 1997). Later on, MS technologies evolved to Multiple MS ($MS^n$).

*Animal experiments with MTU*

In order to understand more of the effect of MTU on cattle, animal experiments were set up. Firstly, the MTU concentration was measured in different matrices. Here thyroid, kidney and various muscles were collected from 5 regulatory control animals (bovines). For this purpose, quantitative high performance thin layer chromatography was used. The MTU concentrations (mg $kg^{-1}$) in thyroid were 10 to 100 times larger than those in muscles (De Brabander 1986; Heeremans et al 1998).

Other experiments studied the elimination profile of MTU in bovines. For example, thyroid and meat samples of 13 different animals were tested after various withdrawal periods (Fig. 3). A withdrawal period of approximately 80 days was calculated.

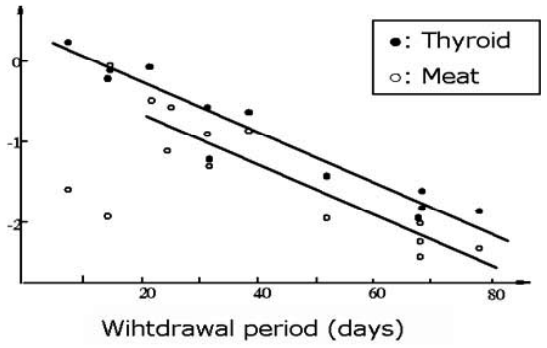


**Fig. 3:** Elimination profile of MTU in bovines in thyroid and meat (n=13).

Meat processing experiments were also conducted, to observe the effect on the MTU levels. 75 % of the residues of MTU remained in the cooked meat and MTU was not destroyed by the heating process.

**Recent developments**

*New methods*
Former detection methods of TS in matrices of animal origin are time consuming. Above this, they demand a lot of organic reagents and are labour intensive. In this section new developments in the framework of TS in urine are highlighted. The new developments are mainly focused on the derivatization step, prior to GC-MS/MS or LC-MS/MS analysis.

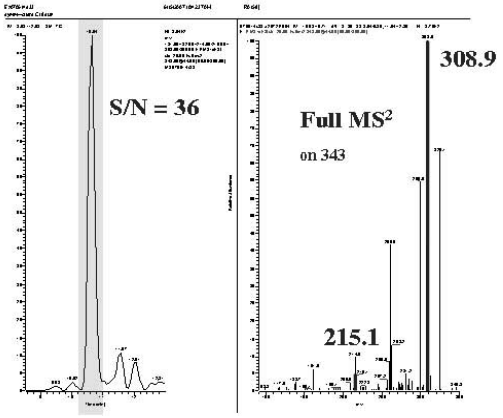


**Fig. 4:** Chromatogram (a) and $MS^2$ spectra (b) of thiouracil in a urine sample at 0.4 µg $L^{-1}$.

The combination of 3-bromobenzylbromide (3-BrBBr) and N-methyl-N-(trimethylsilyl)-trifluoro-acetamide (MSTFA) was found best suited for GC-MS/MS analysis (Le Bizec et al. 2005). For LC-MS/MS analysis, 3-Iodobenzylbromide (3-IBBr) has been selected as the most efficient derivatization reagent (Pinel et al. 2005). Consequently, considerably lower LOD

values of 0.2 to 0.4 µg $L^{-1}$ can be detected, in comparison with 50 to 100 µg $L^{-1}$ before. In fig. 4 a sample of 0.4 µg $L^{-1}$ TU in urine is shown.

*TU… status of semi-endogenous substance?*
An experimental study indicated a correlation between a cruciferous-based diet and the occurrence of thiouracil in urine of cattle (Pinel et al. 2006). This can result in erroneous indications of the possible illegal use of thyreostats in meat production.
The use of an improved detection method has revealed a possible 'natural' presence of TU in urine of non-treated bovine. Further research is needed for the discrimination between abuse and natural presence of TU, and maybe other TS.

**Conclusions**
The detection of thyreostatic drug abuse has evolved immensely during the last 35 years. What started in the beginning of the 1970's with a colorimetric reaction, evolved later on (1973-1974) to thin layer chromatography. TLC was considered as a more specific method, able to reach lower limit of detections, within an acceptable budget. High performance thin layer chromatography succeeded TLC. In the 80's gas chromatography-mass spectrometry was introduced. The early stage of thyreostatic drug abuse detection, using GC-MS existed only as a conformation step for HPTLC. In the 90's liquid chromatography-mass spectrometry followed. Both techniques, GC-MS and LC-MS underwent the evolution from single to multiple MS ($MS^n$). Future developments could involve the application of ultra performance liquid chromatography (UPLC) or ultra-high pressure liquid chromatography (U-HPLC).
The evolution in the detection method ensured continuous improvement of the detection of thyreostatic drugs in various matrices (e.g. urine, meat, plasma, thyroid etc). During the past 35 years, the limit of detection decreased from 5000 µg $kg^{-1}$ to approximately 0.1 µg $kg^{-1}$. Finally, there is a warning for the semi-endogenous status of TU, discovered due to the improved detection techniques. The question is: how will this investigation of thyreostats evolve in the next 35 years?

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## O 7

# RESIDUES OF OXYTETRACYCLINE IN ZEA MAYS: ONLY A MATTER OF FOOD SAFETY?

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**Abstract**
Wastes from intensive animal farming may result in the contamination of the top soil with tetracyclines. To assess the possible risk these residues may recycle in feed materials, an experimental model based on Oxytetracycline (Oxy) and Zea mays has been planned. *On land* and *in pot* plant cultivations were performed. *On land* cultivations were repeated on two subsequent years in a never exposed field, subjected to soil dressing with contaminated slurries (around 10 ppm, on wet basis). Pot cultivation was performed outdoor (Oxy in the range 62.5 – 1,000 ng/g dry soil) and plants harvested after 45 days. Analyses were performed with LC-MS(MS). A single cycle of fertilization with contaminated slurry does not determine the appreciable presence of Oxy in soil (< 1 ng/g). Oxy residues were found in 45 days pot cultivated corn plants in the range of 1- 50 ng/g. Although the residues differences between land and pot cultivations, both batches showed the same biological response: a biphasic growth alteration (hormesis). The analytical and biological results may deserve further attention, not only focused on food safety matters.

**Introduction**
Pig slurry from intensive farming is generally collected for soil top dressing. They may contain drugs, as tetracyclines, macrolides and quinolones, that can become (top) soil pollutants. This implies the possibility that such residues in soil can be actively/passively enter in crop plants. Tetracyclines are widely used in intensive pig farming, mainly in the weaning period, in the range of thousand mg/kg in medicated feed. Taking into consideration their pharmacokynetics and stability when adsorbed to organic matter, this may result in significant residual activity in both animal manure and soil.
This implies that tetracyclines in soil may be absorbed by crop plants and then introduced into the food chains. Previous results on different crop plants and antimicrobials clearly demonstrated these concerns.
To this aim an experimental model based on Oxytetracycline (Oxy) and Zea mays has been planned according to the le Best Available Techniques for soil top dressing. *On land* and *in pot* plant cultivations were performed to give information on: 1) uptake into the different plant compartments, and 2) alteration of normal plant growth.

## Material and Methods

*Experimental design*
*Zea mais* L. plants were cultivated in two different environmental conditions: a) in the field, pig slurry fertilized, and b) in pot, added with known amounts of Oxy. In both cases, plants were cultivated under natural photoperiod and temperature conditions (Rome latitude, April-September. Plants in pot were harvested after 45 days, while open field plants were sampled after 6 months, at the end of their vegetative cycle. Pots were water poured with a medicated solution of Oxy (10 ml/pot), to reach the nominal concentration of 1,000 – 500 – 250 – 125 – 62.5 and 0 (control) ng/g soil (dry weight). Field plants were fertilised with a total 1.8 L of pig slurry containing 10 µg/ mL Oxy and its 4-epimer and also Chlortetracycline and 4-Chlortetracycline-epimer, accounting for the 30% of the Oxy content. A mineral NKP 15-15-15 fertiliser was used in control plants, at concentration in order to comply with a maximum of 300 kg N/ha soil, according to the Dir 91/676/EC on Nitrates from agricultural sources. Slurry was distributed according to Good Agriculture Practices, in a 15 cm depth rows, flanking the plants. The following biometry parameters of plants were recorded: height, number and length of leaves, number of flowers and cobs.

*Chemical analysis*
Oxytetracycline Hydrochloride (OTC) and chlortetracycline Hydrochloride (CTC) were purchased from Sigma Aldrich, their 4-epimers (4epi-OTC, 4-epi-CTC) were from LGC Standards. Doxycycline Hydrochloride (DOXY), used as internal standard, was from Sigma Aldrich. Acetonitrile and methanol ultra gradient HPLC grade, formic acid LC MS reagent, citric acid, copper sulphate, EDTA disodium salt and sodium dihydrogen phosphate were purchased from Baker. Deionized and distilled water was obtained from a MilliQ water purification apparatus. Metal chelating sepharose fast flow was from Amersham Bioscience, SPE box from Supelco, Syncore system evaporator from Buchi, ALC refrigerate centrifuge Analysis were performed on slurries, and on soils, roots, leaves, and cob, for “on field” and on roots and epigeal part on “in pot” plants, after appropriate pooling. Vegetal matrices were brought to dryness at + 35 °C in the dark. Following the procedure previously described by the same authors each considered matrix was finely minced and homogenised, and a test portion of 1 g submitted to liquid-liquid extraction and cleaned up on Metal Chelate Affinity Chromatography.
LC-ESI-MS/MS analysis were performed on a Waters Alliance 2695 LC system cupled to a 4 Premier XE triple quadrupole mass spectrometer. HPLC analysis were run on a Phenomenex Luna C8(2) column (2,0x100 mm,3µm) at 30°C. The mobile phases were constituted with solvent A: water containing 0,1% formic acid(v/v) and solvent B:acetonitrile. The gradient program was: 0,0 min 5% B, 0,0-0,5min 5% B, 0,5-8,0 min 75% B, 8,0-10,0 min 75%B, 10,0-10,1min 100% B, 10,1-11,00 min 5% B, 11,0-20.0 min 5% B; running at a flow rate of 0,3 mL/min. The injection volume was set at 10 µL. Analytes were detected with ESI in positive mode. The source and desolvatation temperatures were 120 e 350 °C respectively, flow rates for desolvation and cone gas were 12 and 4 L/Hr respectively from a $N_2$ generator $N_2$-Mistral-4 (DBS instruments). Capillary voltage was set to 3,5 kV. MS tuning was performed by infusing individual solution of each analyte mixed with a HPLC flow made of solvent A and B (50/50, v/v, 0.3 mL/min) using a Tee connector. MS MS detection was

realized using the Multiple Reaction Monitoring (MRM) acquisition mode (Table 1). Data acquisition and processing were performed using MassLynx 4.1 software.

**Table 1.** LC-MS/MS MRM transition conditions for tetracyclines

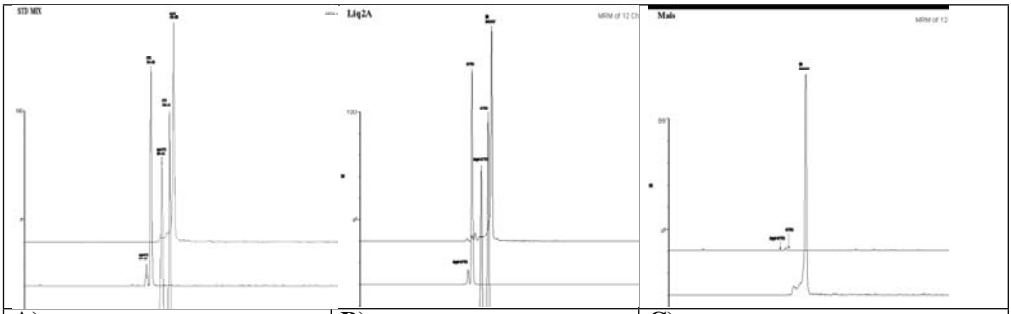
| Analyte | Transition | Collision (eV) | Cone (V) |
|---|---|---|---|
| Oxytetracycline | 461>426 | 20 | 25 |
| 4epi-Oxytetracycline | 461>426 | 20 | 25 |
| Chlortetracycline | 479>444 | 22 | 30 |
| 4epi-Chlortetracycline | 479>444 | 22 | 30 |
| Doxycycline | 445>428 | 20 | 30 |

**Statistics**

Significance ($p < 0.05$) of biometrical data was assessed by Mann-Whitney test, with Bonferroni correction.

**Results**

In the field, corn plants at the end of their vegetative cycle even dressed with 1.8 L slurry containing 15 ppm Oxy (a total antimicrobial amount of about 30 mg/plant) showed no presence of drug residue in the different plant compartments (roots, stalks, leaves, cobs; although the detection limit was been lowered down to 1 ng/g dry weight (Figure 1).




**A) Standards, 100 pg injection** | **B) Contaminated slurry** | **C) Corn**

**Figure 1**. LC-MS/MS chromatograms

On the contrary, in pot cultivation 45 days old corn plants showed Oxy uptake, as reported in Table 2. Uptake is mainly in roots, where a direct relation with the

**Table 2.** Oxy concentration in aerial and radical portion of corn plant cultivated in pot.

| **Soil*** **(ng/g)** | **Aerial portion* (ng/g)** | **Roots*** **(ng/g)** |
|---|---|---|
| Control | Not detected | Not detected |
| 62.5 | 1.84 | 7.5 |
| 125 | 1.69 | 9.7 |
| 250 | 1.01 | 10.29 |
| 500 | 1.88 | 18.23 |
| 1,000 | 2.81 | 54.46 |

** values expressed on dry weight*

amount of administered drug is found; the carry over value is about 5% in roots and 0.5% in the aerial compartment (leaves and internodes).
In pot, corn plants showed a dose response growth trend that can be superimposed to an hormetic curve (Figure 2).


**Figure 2.** Hormetic growth of corn plants

In conclusion, Oxy at concentrations present in pig slurry, affect post-germinative development of *Zea mays* plants: roots, stalk and leaves length is significantly increased and this increase is the highest at intermediate concentration (see Figure 2, T3).
The growth increase in field cultivation can be considered an hormetic response but it is not supported by analytical findings. A low carry-over rate, the low concentrations involved and the huge amount of corn biomass at the end of the vegetative cycle, can reasonably account for this.
These results demonstrated that biological effects can be elicited even at concentrations 2 logs-fold below the regulatory limits stated for residues in animal products and this represents a challenge for the implementation of analytical methods able to find out the triggering amount for hormesis.

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# O 8
# A NOVEL APTAMER-BASED PROCEDURE FOR THE RAPID DETECTION OF MALACHITE GREEN IN FISH TISSUE

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**Abstract**
Malachite green is a triphenylmethane dye that has been used for the control of fungal infections in fish. There are concerns about its safety in human health and there is no authorisation for its use in food-producing animals within the EU. The on-going occurrence of malachite green (MG) and its main metabolite leucomalachite green (LMG) residues in farmed fish samples is still being reported. Laboratory procedures for the detection and quantification of malachite green residues are on the whole complicated and time consuming requiring specialised laboratory equipment.
Aptamers are artificial oligonucleotides, which can show high affinity for a particular molecular target. Unlike antibodies, aptamers can readily recognise relatively low molecular weight compounds, e.g. malachite green. Aptamers can be generated rapidly *in vitro* using a technique called SELEX (Systematic Evolution of Ligands by EXponential enrichment). A ribonucleic acid-based aptamer with an affinity for malachite green has previously been developed elsewhere for use as a probe for the site of incorporation of genes. Within this paper the development and validation of a novel rapid and sensitive aptamer-based method with spectrofluorimeteric detection for malachite green in fish tissue is reported.

**Introduction**
Malachite green (MG) is a triphenylmethane dye that has been used for the control of fungal infections in fish. MG is not listed as a veterinary medicine under Council Regulation 2377/90/EC but is frequently detected as an unauthorised residue in fish. For example, between 2005 and 2007 there were circa 400 EU rapid alerts ("RASFFs") relating to veterinary medicines in foods of animal origin, of which 78 related to the illegal use of MG. An MRPL of 2 μg $kg^{-1}$ (2004/25/EC) has been established as the sum of malachite and leucomalachite green.

Current laboratory procedures for the detection and quantification of MG and its metabolite LMG are relatively slow and are reliant on the use of conventional instrumental methodology e.g. HPLC-with visible detection or HPLC-MS/MS. In most cases, after solvent extraction, sample extracts also require purification via techniques such as liquid-liquid extraction and/or solid phase extraction. Therefore, there is a need to speed up the analytical process and reduce the cost of the analysis by providing an alternative means of screening fish samples for MG and it's metabolite LMG.
Aptamers are single stranded oligonucleotides (DNA or RNA) that have the ability to form defined tertiary structures to engage a specific target for binding (Nutiu and Li,

2005). Unlike amino acid-based antibodies, aptamers have the advantage of being able to recognise relatively small (<1000 Daltons) compounds such as MG and other veterinary drugs. Aptamers can be generated rapidly in vitro, thus removing the need for the use of animals, using a technique called SELEX (Systematic Evolution of Ligands by EXponential enrichment). The mechanism by which aptamers recognise and bind their ligands has been well studied by a combination of multi-dimensional NMR spectroscopy and X-ray crystallography (Sussman et al., 2000). For many aptamers the ligand induces a folding transition from an unstructured, unbound state to a highly ordered bound state. In a different context, a RNA-based aptamer with an affinity for MG has been previously developed elsewhere for use as a probe for the site of incorporation of genes. A side benefit is that, when MG binds with the aptamer, a circa. 2000 times increase in fluorescence signal is observed. Normally MG has an extremely low quantum yield for fluorescence due to easy vibrational de-excitation but, if the molecule's vibrational activity is restricted by binding to the aptamer, fluorescence is greatly enhanced. This facet of the MG–aptamer complex was used to design a rapid screening assay for MG/LMG.
This paper presents the first report of the development of a prototype aptamer based screening assay using spectrophotometric detection for MG and it's major metabolite, LMG.

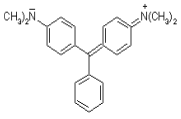
## Material and Methods

*Aptamer Preparation*
Aptamer stock solution was diluted using molecular grade water to give RNA concentration of 5 µg $ml^{-1}$. Reactions were performed in disposable fluorescence curvettes typically containing fortified buffer /or extract and a fixed mass of aptamer material.

*Salmon Extract Preparation*
Fortified salmon (muscle/skin in natural proportions) was extracted using an acidic buffer and purified using a simple liquid-liquid extraction. The organic solvent component was removed, concentrated and then resuspended in buffer prior to measurement.

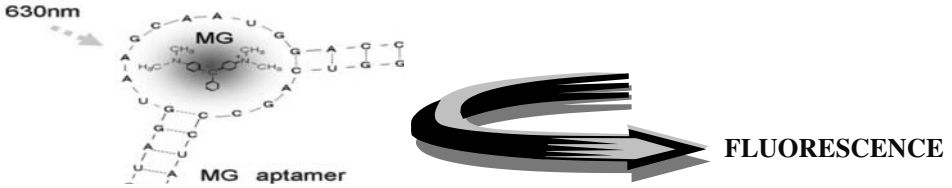
## Results and Discussion

*1. Assay Principle*
The schematic below illustrates the assay principle (steps 1 - 2). The fluorescence signal associated with the target analyte, MG is negligible (the absorption maxima is at 618 nm). The native RNA aptamer (MGA) is also not inherently fluorescent, however, when the aptamer and MG are in the bound conformation (shown in step 2) a strong fluorescence signal can be detected using a conventional spectrofluorimeter. This detection principle was used to determine the initial binding characteristics of the aptamer in the presence of MG and LMG.




**Step 1**. MG in tissue extract solution :– negligible fluorescence signal
Chemical structure of malachite green in solution

**Step 2**. Addition of the aptamer to the extract solution from step 1, followed by a short (5 minute) incubation period.




MG and aptamer in the bound conformation: enhanced fluorescence

*2. Complex Formation -kinetics*

As part of the evaluation experiments, the timescale for the aptamer and ligand binding complex formation was monitored. In order to achieve maximum binding ($B_{max}$) measured as the maximal fluorescence intensity at 645nm, it was found to be necessary to incubate the reaction mixture for a minimum of 5 minutes. Once formed, the MGA-MG complex was found to be stable for circa 18 hours at room temperature.

*3. Fluorescence Spectrum – 3D Scans*

A series of fluorescence scans were performed whereby both the excitation and emission wavelengths were scanned over a fixed range (Ex. 550-650nm) and (Em. 550-750nm) in order to characterize the response. The following control and test samples were included as part of this experiment; MG in buffer, free aptamer (MGA) in buffer and MGA+MG complex. The 3D fluorescence scans are presented in Figures 1a, 1b and 2.

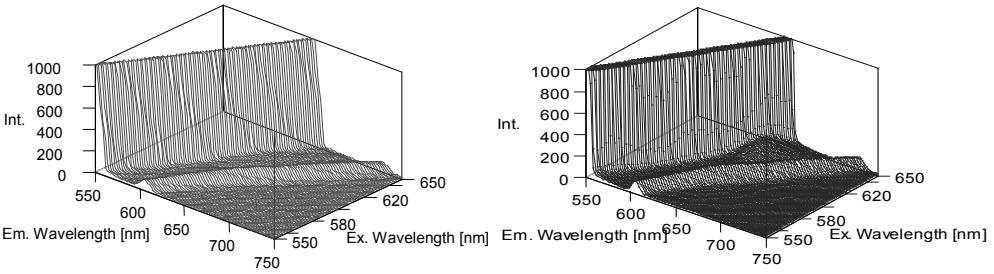



**Figure 1a** (left) and 1b(right) Control MG in folding buffer (0.5ug $ml^{-1}$) and control aptamer (MGA) in folding buffer -3D view

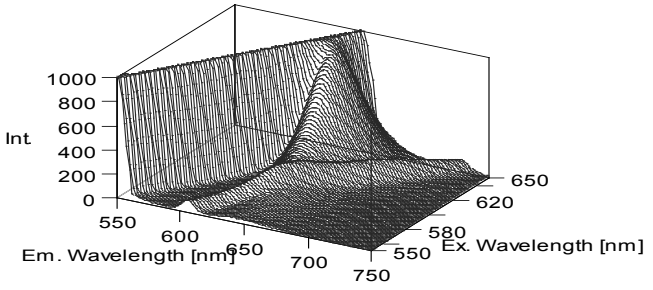



**Figure 3.** MGA-MG complex -3D view

Emission peaks were detected around 645-650nm in the MGA-MG complex (Figure 3), which were not significant in the control samples (Figures 1-2). The intensity of the emission peak was found to be maximal with an excitation wavelength of between 620-630nm.

*4. Linearity of the binding interaction*

In order to determine the linearity of the binding interaction, a fixed mass of aptamer (MGA) was incubated with increasing amounts of MG in the presence of the folding buffer and the fluorescence intensity measured (Ex. 620nm/Em. 650nm). The binding interaction between MG and the MGA (Figure 4) shows that a linear increase in fluorescence intensity is recorded between the range of 0 and 40 ng of MG in solution with an $R^2$ of 0.9975. The linearity of the fluorescence response was seen to plateau between 40 and 50 ng of MG. The signal plateau effect may be related to a fluorescence quenching effect rather than binding domain saturation being reached as the reaction was performed with the aptamer in vast excess of the ligand. The experiment was repeated in the presence of salmon extract fortified with MG and a linear response was also obtained (data not shown).

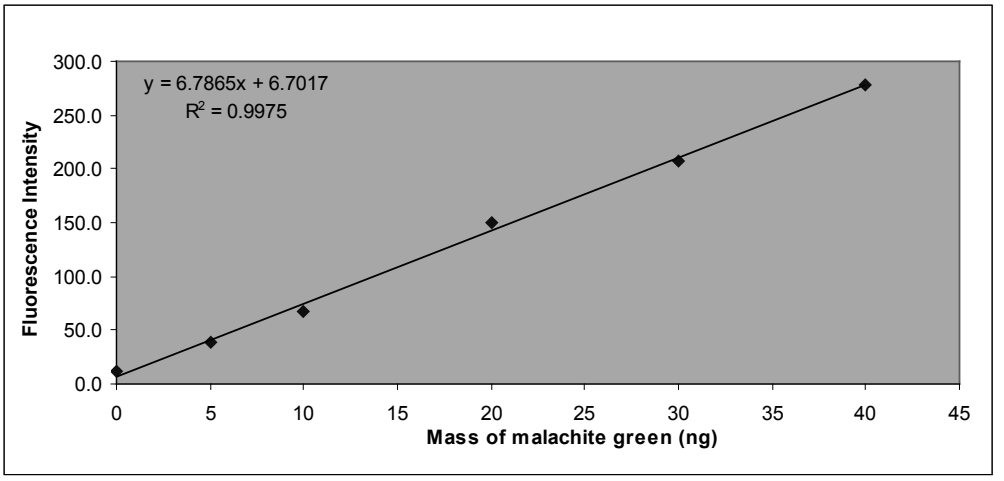



**Figure 4.** Calibration curve showing fluorescence signal recorded following the interaction of MGA and MG in folding buffer

**Conclusions**

An aptamer-based assay for the rapid screening of MG in fish tissue has been developed. The aptamer-based assay employs spectrophotometric detection and shows good detection capability for MG in buffer. The RNA aptamer performs equally well in salmon extract in the presence of a buffer designed to promote complex formation. An oxidation step is required to convert the metabolite (LMG) to the chromagenic form prior to measurement, as the current MG aptamer does not cross-react with LMG. The MGA-MG complex formation is relatively rapid; $B_{max}$ is achieved within 10 minutes. The complex is also found to stable for circa 18 hours. This is first reported application of RNA aptamer as recognition element within a screening assay for residue detection in food.

**Acknowledgements**
The financial support of the Veterinary Medicines Directorate of the UK Department for Environment, Food and Rural Affairs is gratefully acknowledged.

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- Nutiu, R., Li, Y., (2005) Aptamers with fluorescence-signaling properties. Methods 37 16-25.

# Oral Presentations

Tuesday 20th of May 2008

**O 9** **Advances in TOF-MS**
Prof. Dr. P. Fürst
CLNW, Nordrhein Westfalen, Münster, Germany

**O 10** **Trends in Monitoring the use of veterinary drugs and growth-promoting agents**
Dr. A.A.M. Stolker
Rikilt, Wageningen, The Netherlands

**O 11** **Quantitative UPLC-TOF Multiresidue method for 100 different Veterinary Drugs**
Dr. A. Kaufmann
Official Food Control Authority of the Canton Zürich, Zürich, Switzerland

**O 12** **SPR Biosensor screening assays coupled to Bio-affinity-directed analytical identification of bioactive substances**
Dr. G. Marchesini
Rikilt, Wageningen, the Netherlands

**O 13** **The occurrence of α– and β-nortestosterone residues in the urine of injured male cattle.**
Prof. Dr. D.G. Kennedy
AFBI, Belfast, NI-UK

**O 14** **Key aspects of the new regulation governing mrls for pharmacologically active substances**
R. Clayton
IFAH-Europe, Brussels, Belgium

**O 15** **Naturally occurring hormones in the food chain**
Prof. Dr. B. Le Bizec
Laberca, Nantes, France

**O 16** **Somatotropine, overview of research studies**
Dr. G. Pinel
Laberca, Nantes, France

**O 17** **Development of a proteomic approach for the detection of recombinant somatotropine in bovine milk and serum**
Dr. A. Kolkman
RIVM, Bilthoven, The Netherlands

**O 18** **The state of and update on 2002/657**
Dr. P. Gowik
BVL, Berlin, Germany

**O 19** **A determinative and confirmatory method for residues of the metabolites of carbadox and olaquindox in porcine tissues. Validation in Canada.**
Dr. J. Boison
CFIA, Saskatoon, Canada

**O 20** **Validation and multivariate effect analysis of an LC-MS/MS method for the determination of steroids in bovine muscle.**
Dr. K. Schmidt
BVL, Berlin, Germany

## O 10

# TRENDS IN MONITORING THE USE OF VETERINARY DRUGS AND GROWTH-PROMOTING AGENTS

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**Abstract**
At least three major trends are observed in the analysis of veterinary drugs and growth-promoting agents. First, traditionally urine, meat, kidney and liver samples were analysed. Nowadays feed, honey and hair samples are also of interest for monitoring the use of drugs. Second, the techniques used for residue analysis are moving from target orientated methods (LC–QqQ-MS) towards accurate mass full scan MS techniques like time-of-flight (ToF)-MS. Full scan MS makes real multi-residue (>100 compounds in a single run) analyses possible. Third, there is a trend towards the use of more intelligent data evaluation procedures. The systematic study of specific data sets of ToF MS in combination with statistical evaluation makes it possible to mark differences between data sets. These differences, e.g. additional peaks, enhanced or reduced peaks are used to discriminate between a sample obtained from a treated and untreated animal, making non-targeted analysis possible.

**Introduction**
The EU has strictly regulated controls on the use of veterinary drugs including growth-promoting agents, particularly in food animal species, by issuing several Regulations and Directives. In 2005 an extended review was published [Stolker, 2005] describing the analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals. Since then, more than 70 papers on veterinary drug residue analysis have been published. In this paper some trends in monitoring residues will be discussed.
First, the sample selection will be discussed. Traditionally, urine was used as the preferred matrix for the analysis of unauthorized compounds. Urine is relatively easy to collect and the samples can be taken before slaughter. This has the advantage that, when 'non-compliant' (positive) results are obtained, the animals can be destroyed to prevent that they reach the market. In recent years, the analyses of hair and retina have gained popularity because residues are detectable for a long time after treatment. For MRL compounds it is observed that next to bovine meat, liver, kidney and milk samples there is an increasing interest for monitoring drug residues in samples of fish, egg, honey and also animal feed.
Second, the method selection will also be discussed. From recent review articles it is concluded that LC–QqQ-MS is the preferred method for residue analysis of veterinary drugs in complex matrices. However, an emerging trend is recognised from LC–QqQ-MS towards

accurate-mass and full scan alternatives like Time-of-flight (ToF)-MS. The accurate-mass capability allows the reconstruction of highly selective accurate-mass chromatograms of target residues in complex matrices. This is of special interest for confirmation analysis and identification of 'unknowns'. The full scan MS technologies offer also the advantage of retrospective analysis without re-analysis and the possibility to screen for more than a hundred of compounds in one single analysis.
Third, new trends in data evaluation will be discussed. Traditionally samples are analysed for the presence of specific target analytes. Therefore, LC-MS data obtained for sample and (matrix matched) reference standards are compared. Respones of specific MS ions in combination with LC retention times are an indication if a drug or growth promoting agent has been used. Nowadays a more systematic data evaluation approach becomes feasible. Samples (e.g. meat or urine) obtained from a group of animals treated with a specific drug or growth protomoting agent and samples obtained from untreated animals are collected. The samples are analysed by a full scan technique like (LC-)ToF MS. The sets of ToF-MS data obtained are compared and differences between the data sets like additional peaks and/or enhanced or reduced peaks are extracted from these data. The next step is to identify the additional peaks and/or explain the observed differences in intensities of peaks. When an additional peak (or differences in peak responses) is identified as a specific marker for the use of a specific drug, this marker can be used to discriminate a treated animal from a untreated animal. When the ToF-MS data set for a particular population is known, this approach can be used for non-targeted analyses for classes of compounds. Preferably the comparison of data sets is performed automatically and in a statistical acceptable way.

**Practical examples of the trends in monitoring residues are discussed below.**

*First: Sample selection*
The first selection that has to be made when setting up a monitoring programme regards the type of sample material. For monitoring drugs having an MRL animal tissues such as muscle, liver, kidney, fat and milk are most frequently selected. Since the drug concentration in the consumable parts of an animal has to be below the MRL, these matrices are of interest. Analytical methods for the analysis of these matrices are described and discussed frequently [Stolker, 2007].

A second group of matrices which are of interest for monitoring the use of veterinary drugs are eggs, honey and fish. The consumption of these products has increased after the scandals described in the media regarding 'mad cow disease', 'dioxin chickens' and 'MPA crisis'. The consumer is looking for alternatives for meat, and products like eggs and fish are becoming more popular. Furthermore, honey and animal feed are gaining interest in the national monitoring programmes. For the control of the use of unauthorized substances hair and retina are the new sample materials. In these matrices residues of certain growth-promoting agents like beta-agonists and anabolic steroids can be detected even months after treatment. Residues in urine, liver and to some extend also meat will not be detectable anymore at that time.

There are several advantages of using hair as the matrix for detecting veterinary drugs. Hair can be easily collected, transported, stored and extracted. The collection method is non-

invasive and does not cause any damage or pain to the animal. The detection of veterinary drugs in hair offers an enlarged retrospective detection compared with urine, liver, kidney, fat etc. The detection of veterinary drug residues in animal hair started with the detection of the β-agonist clenbuterol in cattle hair. Thereafter stanozolol was the first anabolic steroid detected in farm animal hairs. The presence of anabolic agents like β-agonists and steroidal compounds in hair has been extensively studied in farm animals. Figure 1 presents a LC-QqQ-MS chromatogram of the analysis of steroid-esters in hair.

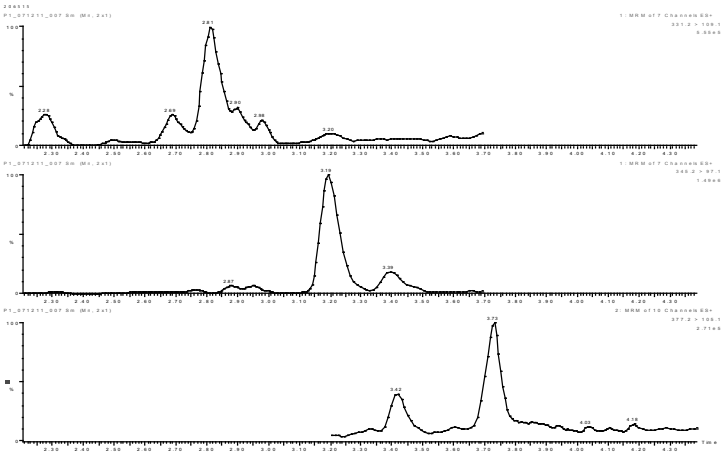

**Figure 1** LC-QqQ-MS chromatogram of the analysis of anabolic-steroids in hair.
The extracted ions (from a suspected sample of bovine hair) are from top to bottom: testosterone acetate Rt=2.81 min, m/z 109.1 (15 µg/kg); testosterone propionate Rt=3.19 min, m/z 97.1 (11 µg/kg); estradiol-3-benzoate Rt=3.73 min m/z 105.1 (24 µg/kg)

A promising field for future investigations could be the identification of specific metabolites or derivatives of active compounds, which are accumulated in the hair structure only as a consequence of a veterinary treatment. As proven in humans, the detection in hair samples of these metabolites could be a useful tool to exclude false positive results due to external contaminations.

*Second: Method selection*
The cost-effectiveness of analytical procedures is becoming an important issue for all laboratories involved in residue analysis. Automation has been introduced to speed up many analytical procedures but instrumentation is expensive. There should be a distinct need to test large numbers of samples to justify such significant capital expenditure. An alternative way to improve cost-effectiveness is to maximise the number of analytes that may be determined by a single procedure or from a single portion of test material. Such an approach is extremely effective when multi-residue techniques, such as LC–QqQ-MS are used. Modern instruments produce high signal to noise ratios, even when relying on short MRM dwell times. This permits the simultaneous monitoring of an increasing number of transitions. However, increasing the number of analyte peaks to be monitored beyond 100-120, requires multiple injections or monitoring specific transitions at a specific retention time window.

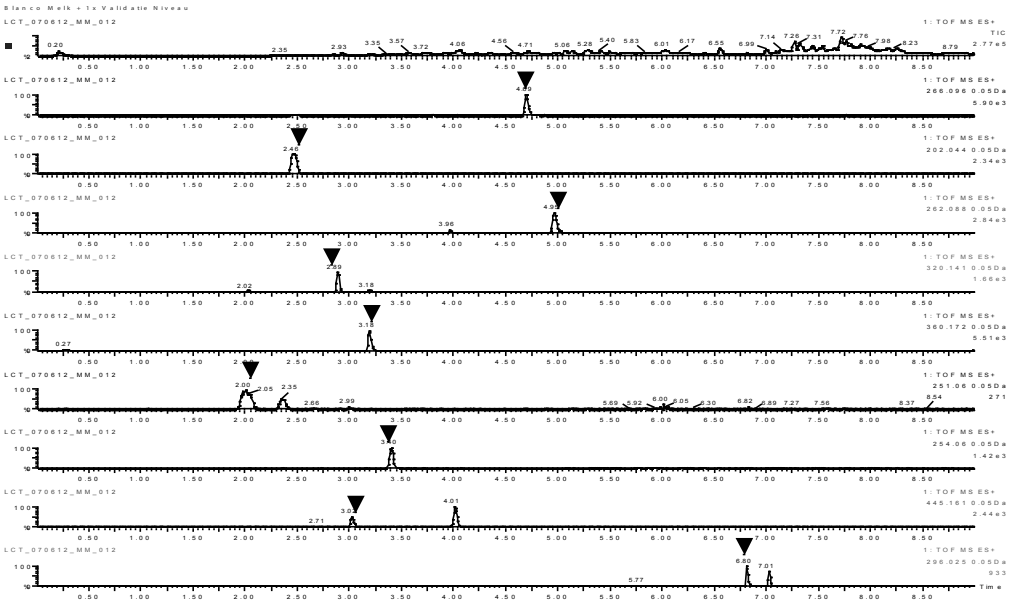

**Figure 2** Typical example of a UPLC–ToF-MS extracted ion chromatogram of blank sample fortified at 10-50 µg/l; The extracted ions, extraction window 0.050 Da, are from top to bottom : albendazole m/z 266.0963; thiabendazole m/z 202.0439; flumequine m/z 262.0879; norfloxacin m/z 320.1410; enrofloxacin m/z 360.1723; sulfadiazine m/z 251.0602; sulfamethoxazole m/z 254.0599; tetracycline m/z 445.1611; diclofenac m/z 296.0250; First track at the top is total ion current (TIC)

An attractive alternative for LC–QqQ-MS is the use of full mass scan MS techniques, for example ToF. The medium to high resolution of 10,000 FWHM of the ToF effects a significant selectivity and therefore sensitivity gain compared to unit-resolution scanning MS instrumentation. A significant advantage of ToF-MS is that no a priori hypothesis about the presence of certain drugs is required; that is, no analyte-specific transitions have to be defined before injecting the sample. The high-resolution, full scan data permits the testing of any a posteriori hypotheses by extracting any desired exact mass chromatogram. Moreover, the accurate mass capability of LC–ToF-MS allows the reconstruction of highly selective accurate mass chromatograms of target residues in complex matrices,
for example the simultaneous determination of different groups of antibiotic compounds in milk (see Figure 2 adapted from Stolker [2008]).

*Third: Data evaluation procedure*
There is a trend towards the use of more intelligent data evaluation procedures. The systematic evaluation of specific sets of ToF MS data in combination with statistical evaluation makes it possible to discriminate between samples obtained from treated and untreated animals. Figure 3 presents an example of such a data evaluation approach. Twenty meat samples fortified with 2 µg/kg of the steroid, melengestrol acetate and twenty blank samples of meat were analysed by using a generic extraction procedure followed by LC-

ToF MS. The full scan data (50-1000 amu) of each sample were stripped from (chemical) noise (see Figure 3) and all detected peaks were identified by using the Agilent Mass Hunter Profiling software. The combined information of all samples was systematically evaluated and the differences - if tested statistical significant - were visualized, plotted. For this process the Agilent Mass Profiler software was used. Figure 4 presents the abundances (log2) of the peaks detected in the blank samples versus the abundances (log2) of the peaks detected in the spiked samples. When a peak is beyond the 4-fold margin (demonstrated by the lines in the data plot) this peak is marked as nearly unique for that group. In this example the peak representing the melengestrol acetate is outside the 4 fold margins and is specific for the group of spiked samples of meat.

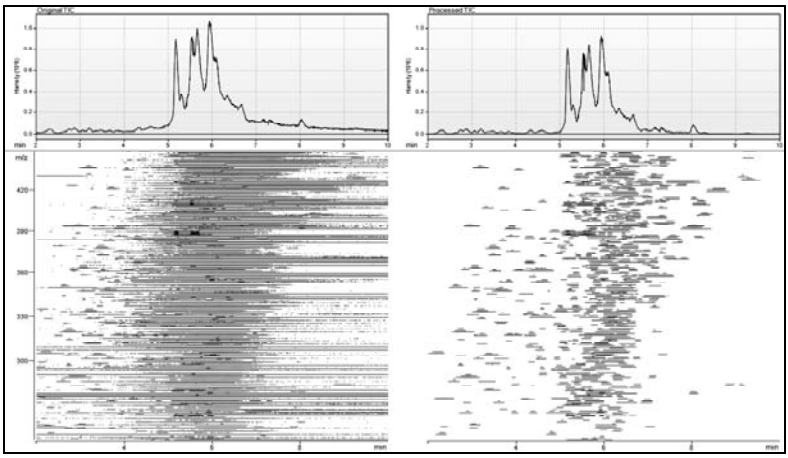

**Figure 3** Contour plots showing the raw data from a meat extract (left) and the stripped data (right)

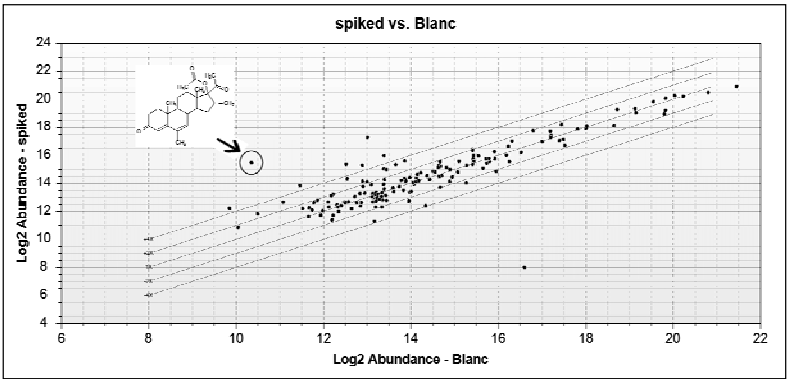


**Figure 4** Melengestrol acetate is uniquely identified in the group of spiked samples

## Conclusions

Three main trends are observed in the analysis of veterinary drugs and growth promoting agents nowadays. First, new matrices like hair, honey and feed are being used for monitoring

purposes. Second, there is a trend towards the use of real multi-residues methods. By using the full mass scan MS techniques like ToF it is possible to detect hundreds of compounds in one single run. Third, there is a trend towards systematic data evaluation of (full scan) MS data. MS data of samples from different group of animals (treated and untreated) are compared. From the – statistical significant –differences, markers (additional peaks, enhanced or reduced peaks) are selected which can be used for monitoring purposes.

**References**


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- Stolker AAM, Rutgers P, Oosterink E, Lasaroms JJP, Peters R, van Rhijn JA, Nielen MWF (2008) Comprehensive screening and quantification of veterinary drugs in milk using UPLC–ToF-MS, *submitted for publication to Anal. Bioanal. Chem.*

## O 11

# QUANTITATIVE UPLC-TOF MULTIRESIDUE METHOD FOR 100 DIFFERENT VETERINARY DRUGS

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**Abstract**
An extraction method followed by solvent exchange and generic SPE clean-up was developed to recover some 100 veterinary drugs. Extraction efficacy for highly polar and apolar drugs was improved by an approach termed bi-polarity extraction. Furthermore, measures were introduced to prevent the loss of analyte caused by precipitating proteins during the solvent exchange steps.
The use of ultra performance liquid chromatography coupled to time of flight mass spectrometry (UPLC-TOF) provided excellent chromatographic resolution and sufficient detection selectivity. Special attention was paid to TOF specific aspects like the dynamic range, and possible isobaric interferences. The proposed method permits the reliable quantification of more than 100 drugs (benzimidazoles, cephalosporines, chinolones, lincosamides, nitroimidazoles, macrolides, penicillines, sulfonamides, tetracyclines and tranquilizers) in one chromatographic run, lasting 14.5 minutes.
The method was validated according to the Commission Decision 2002/657/EC. Minor deviations were required to cope with the widely varying MRL's (1 to 1000 µg/kg) of the different compounds. Aside from a very few exceptions, all analytes could be reliably quantified at their relevant MRL concentrations in the three validated matrices (muscle, kidney and liver).

**Introduction**
The use of multiresidue methods for pesticides in fruit and vegetables is becoming more and more widespread among routine laboratories around the world. This development has not yet reached the field of veterinary drug residue analysis in meat. Most utilized methods cover only one particular veterinary drug family or even only one specific analyte. Multiresidue methods for veterinary drugs are scarce because of some particular difficulties. The lack of volatility and chemical stability essentially prevents the use of gas chromatography coupled to mass spectrometry (GC-MS). The wide polarity range among the various drugs complicates extraction and clean-up. Some drugs are strongly adsorbed or even covalently bound to the matrice, while others have to be quantified at very low levels (e.g. $0.3^{-1}$ µg/kg).
To the best of our knowledge the only quantitative method capable to analyze more than 100 different drugs in meat has been published by Yamada *et. al.*
However, the method does not cover the important class of tetracycline drugs. Furthermore, the provided validation did not follow the Commission Decision 2002/657/EEC and is limited to muscle matrices.

This paper tries to overcome some previous limitations by addressing the following critical points:

- The limited extraction efficacy for highly polar and apolar compounds was improved by a concept termed bi-polarity extraction.
- Poor sensitivity and strong signal suppression effects could be improved by extensive clean-up steps which aimed primarily at removing proteins while marginally affecting analyte recovery.
- Losses of analytes by too selective SPE cleanup were significantly reduced by employing a generic polymeric reversed phase (Oasis HLB) material and careful pH control of the loading solution.
- Adsorption related analyte losses caused by precipitating proteins or by ion exchange effects as occurring on vessel glass walls, were tackled by dedicated rinsing steps and sufficiently high counter ion concentrations.
- Dwell time limitations and the cumbersome establishing and maintaining of time windows, typical for multiresidue methods with LC-MS-MS instruments, were overcome by LC-TOF.
- Selectivity and sensitivity of LC-TOF was improved by the use of long high-resolution sub 2-µm analytical columns and dedicated UPLC equipment.

**Experimental**

*UPLC separation*

The equipment consisted of an Acquity system from Waters (Millford MA). The following linear gradient was used: 0-2 min: 0 %B; 8 min: 30 %B; 12 min 100 %B; 13 min: 100 %B; 13.01 min: 0 %B and 14.6 min: 0 % B. The column flow was adjusted to 0.4 ml/min and the column was maintained at 30 °C. The injection volume was 5 µl. The column was a T3; 2.1mm*100 mm, 1.8 µm particle size (Waters). Mobile phase A: 50 ml of acetonitrile, 3 ml of formic acid and 950 ml water. Mobile phase B: 50 ml of water, 3 ml of formic and 950 ml acetonitrile.

*TOF parameters*

An LCT Premium orthogonal acceleration time of flight mass spectrometer with ESI interface from Waters (Millford, MA) was connected to the UPLC. The ESI capillary was set to +3000 V. The desolvation temperature to 350 °C and the source temperature to 150 °C. The desolvation flow (nitrogen) was 650 l/h and the cone gas flow 50 l/h. The instrument was operated in the "W" mode and tuned to a resolution of 12'000 full width at half maximum (FWHM). Dynamic range enhancement was used to obtain a sufficient dynamic range. Scans (mass range: 100-1000 m/z ) were averaged for 0.2 seconds. Mass windows of 60 ppm were used to extract analyte related exact mass traces.

*Analytes*

The following analytes are quantified:

**Benzimidazoles:** Albendazol, febantel, fenbendazol, fleroxacin, flubendazol, mebendazol, oxibendazol, oxfendazol, parabendazol, thiabendazol.

**Chinolones:** Ciprofloxacin, danofloxacin, difloxacin, enoxacin, enrofloxacin, flumequin,

lomefloxacin, nalidixic acid, norfloxacin, ofloxacin, oxolinic acid, piromidic acid, sarafloxacin, sparfloxacin, virginiamycin.
**Lincomycines:** Clindamycin, iso-pirlimycin, lincomycin, pirlimycin, thiamulin.
**Macrolides:** Azithromycin, eprinomectin, erythromycin, josamycin, oleandomycin, roxithromycin, spiramycin I, spiramycin II, spiramycin III, tilmicosin, tylosin.
**Nitroimidazoles:** Dimetridazol, HMMNI, ipronidazole, ipronidazole-OH, metronidazole, metronidazole-OH, ronidazol, tinidazol.
**Penicillines:** Amoxicillin, ampicillin, cefazolin, cefoperazon, cephalexin, cephapirin, cloxacillin, dicloxacillin, nafcillin, oxacillin, penicilline M, penicillline V.
**Sulfonamides:** Sulfaacetamide, sulfabenzamide, sulfachlorpyrazine, sulfachlorpyridazine, sulfadiazine, sulfadimethoxine, sulfadimidine, sulfadoxine, sulfaguanidine, sulfaisomidine, sulfamerazine, sulfameter, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfamonomethoxine, sulfamoxole, sulfanitran, sulfapyridine, sulfaquinoxaline, sulfasalazine, sulfathiazole, sulfisoxazole.
**Tetrayclines:** Chlortetracycline, demeclocycline, doxycyclin, minocycline, oxytetracycline, tetracycline.
**Tranquilizers:** Acepromacin, azaperol, azaperon, carozolol, chlorpromazin, propionylpromazin, xylazin.
**Various:** Acriflavin, diaveridin, praziquantel, pyrimethamin, rifampicin, rifamixin, trimethoprim.

*Sample preparation*
Sample (muscle, kidney or liver) is extracted with a mixture of McIlvaine buffer and acetonitrile. Phase separation of the two phases is induced and maintained by a high concentration of ammonia sulfate. After centrifugation, the top acetonitrile layer is evaporated under vacuum. The pH of the remaining aqueous phase is adjusted to 6.5 and centrifuged. Evaporation vessels and centrifuged precipitates in the centrifugation tube are rinsed by aqueous dimethysulfoxide. This rinsing solution is sucked through a solid phase cartridge (OASIS HLB) followed by the pH adjusted and centrifuged aqueous extract. SPE elution solvents are acetonitrile and a mixture of McIlvaine buffer with acetonitrile. The combined eluate is evaporated under a stream of air at 50 °C. Evaporation till dryness is prevented by the use of a keeper (dimethylsulfoxide). The concentrated eluate is diluted, centrifuged and injected into the UPLC-TOF.

*Validation*
The method was validated according to the Commission Decision 2002/657/EC for three matrices: muscle, liver and kidney. A dynamic range of two orders of magnitude was covered, which deviates from the commission decision procedure. The spiking levels for forbidden or low MRL substances were 1.0; 3.33; 10; 33.3; 100 µg/kg. Other analytes were fortified at 10; 33.3; 100; 333; 1000 µg/kg. Each level was repeated four times. The whole series was repeated three times on different days, by different operators.

## Results and Discussion

*Extraction and Clean-up*

The use of bi-polarity extraction significantly enlarged the range of extractable analytes. This concept consist of an aqueous buffer, acetonitrile and a high ammonium sulphate concentration which induces phase separation. Hence extraction is performed with an emulsion. Reversed phase polymeric SPE cartridges retain most analytes if loading solutions are free of organic solvents and the solution pH is properly adjusted. Avoiding glass ware as much as possible and the use of a certain ionic strength in all extracts reduces losses of analytes on vessel surfaces and by precipitating proteins. Evaporation till dryness should be avoided under all circumstances. The use of a keeper with high solvation but week elution power like dimethylsulfoxide improves recoveries of non-polar analytes and does not negatively affect peak shape of early eluting polar analytes during chromatography.

*UPLC-TOF measurements*

TOF does not yet produce the same selectivity and sensitivity as triple quadrupole. The combination with UPLC is of very high importance because of the generation of narrow (selective) and tall (sensitivie) chromatographic peaks. A chromatogram showing some 16 selected analyte ion traces (ranging from 33.3 to 333 µg/kg as spiked into a kidney extract) is shown in the figure below.

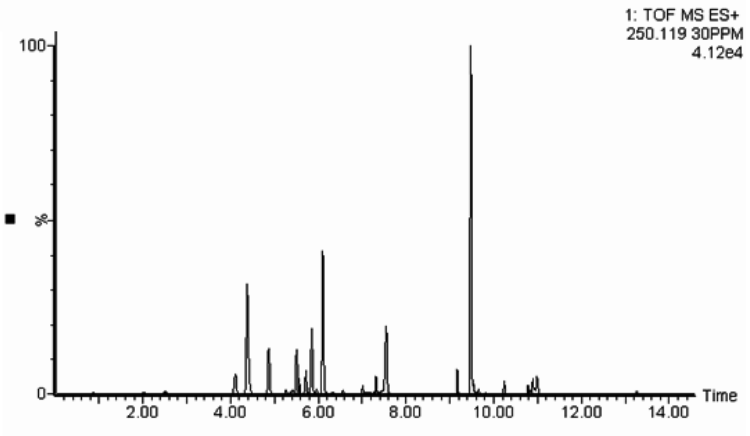


**Figure 1:** Typical UPLC-TOF chromatogram of a fortified kidney extract, showing 16 selected exact mass traces of analytes.

## Validation

A multiresidue method will invariably contain regulated and forbidden compounds. An analyte might have several different MRL's, depending on the matrix and on the animal species. According to the Commission Decision 2002/657/EC, validation levels have to cluster around the decision level (e.g. more blanks have to be analyzed for forbidden than for regulated substances). These different requirements create problems for the validation of multiresidue methods, making required spiking experiments become very difficult. Hence

we validated analytes not for a particular decision level but for a concentration range of two orders of magnitude. The spiking solution contained the high decision level compounds at ten times higher concentrations than the low decision level compounds.
Validation data did not strongly differ for the investigated matrices, which is probably the result of the extensive clean up. Only an average of 11 % of all compounds were recovered at rates of less than 50 %. Around 60 % of all analytes showed rates above 80 %. Poor recovery were observed for very apolar compounds (adsorption losses) and very polar analytes (SPE breakthrough)
Signal suppression is an issue and has to be compensated for. Liver showed the strongest effect where 45 % of all compounds were suppressed by more than 50 %. However, only 3 % of all analytes were suppressed by more than 75 %. The sensitivity, CCα (calculated as if all analytes were forbidden substances) was acceptable. 94 % of all compounds could be detected in muscle at lower concentrations than 5 µg/kg. Sensitivity was only critical for nitroimidazoles. There were some issues of isobaric compounds in liver. They were considered to be not very critical, because of the relatively high MRL in this organ.
50 % of all analytes in muscle and kidney showed coefficients of determination ($r^2$) higher than 0.99. (5 spiking levels covering two orders of magnitude). There is a clear correlation between poor analyte recovery and poor coefficient of determination. This can be explained by the extreme polarity of some compounds and enzymatic or chemical instability of some drugs.

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# SPR BIOSENSOR SCREENING ASSAYS COUPLED TO BIO-AFFINITY-DIRECTED ANALYTICAL IDENTIFICATION OF BIOACTIVE SUBSTANCES

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**Abstract**
Within the EU-project BioCop, we implemented Surface Plasmon Resonance (SPR)-based biosensor immunoassays for the (group) specific detection of fluoroquinolones (FQ) and paralytic shellfish toxins. These assays were used to evaluate two strategies for combining SPR biosensor screening technology with liquid chromatography (LC) and mass spectrometry (MS) for identification. The first strategy was the offline parallel coupling of the technologies. The second, serial strategy, involved using a microfluidic interface between the SPR biosensor and nano ESI- LCMS. The samples suspected non compliant during the SPR immunoassay screening were reinjected onto a recovery chip with similar, but reversed, immunochemistry. The analyte captured in the recovery chip was finally eluted into the injection loop of a nano-ESI LCMS system for identification. The accurate mass determination allowed further element composition calculation and interrogation of chemical substance databases to check whether the bioactive analyte had been ever described in any patent or scientific paper as a known pharmaceutical, or alternatively, must be considered as unknown, an unmarketed drug, or as a synthesis intermediate.

**Introduction**
SPR biosensor immunoassays (BIAs) for the detection of fluoroquinolones (FQ) have been proposed as alternatives to traditional analytical methods on the basis of their shorter analysis time, lower costs and less specialized human resources required for operation. Although, biosensors are indeed useful tools for the biospecific determination of analytes in various matrices, their output provides no clue about the analyte structure or identity. On the contrary, liquid chromatography (LC) separates the analytes based on their physico-chemical properties and unequivocal structural information can be obtained if LC is hyphenated with mass spectrometry (MS). Hence, a synergistic approach can be obtained by combining SPR biosensor technology based on molecular biorecognition events with classical analytical techniques based on physico-chemical properties of the analytes.
SPR biosensors have been combined offline with matrix assisted laser desorption ionization (MALDI) –time of flight (TOF) MS (Krone et al., 1997; Nelson et al., 1997; Sönksen et al., 1998; Nedelkov and Nelson, 2001; Lopez et al., 2003; Borch and Roepstorff, 2004) and micro preparative HPLC (Nice et al., 1994). SPR biosensors were also coupled online as a detector for IgG, using capillary electrophoresis for separation (Whelan and Zare, 2003), in parallel

with LC-diode array detector (DAD)-MS for drug screening (Minunni et al., 2005) and offline and online with MS (Nedelkov and Nelson, 2003).
In the present study, the main challenge was to achieve a coupling between MS and the inhibition BIA (iBIA) format which is most suitable for the detection of small molecules. In this format, during the SPR analysis, the analytes of interest are lost as they flow through to the waste either complexed to the antibodies or as free compounds. When the amount of sample is not limited, one option is to use a split flow strategy for the parallel coupling of the screening and MS. Such a concept was successfully applied with a yeast bioassay for the screening of androgens and estrogens in combination with LC ESI quadrupole (Q) TOF MS (Nielen et al., 2004; Nielen et al., 2006). In the present study, a similar approach was applied with the iBIAs for the screening of FQs instead of the yeast bioassay and only the relevant samples are analyzed with MS. Alternatively, a fast, online system, requiring minimum sample volume and handling, was studied with enrofloxacin (Enro) as model. Here, the SPR-MS interface is a nanoscale immunoaffinity chip that recovered the analyte for subsequent nano-LC-ESI analysis. These two strategies and their advantages/limitations will be compared and discussed.

**Materials and Methods**
Materials, methods, procedures and equipment used during the study are thoroughly described elsewhere (Marchesini et al., 2007; Marchesini et al., 2008).

**Results and Discussion**
The two concepts evaluated for the SPR iBIA coupling with LC-MS, the parallel and the serial, both are depicted in Figure 1. Sample extracts were screened with the SPR iBIA and the suspected non-compliants were pinpointed.

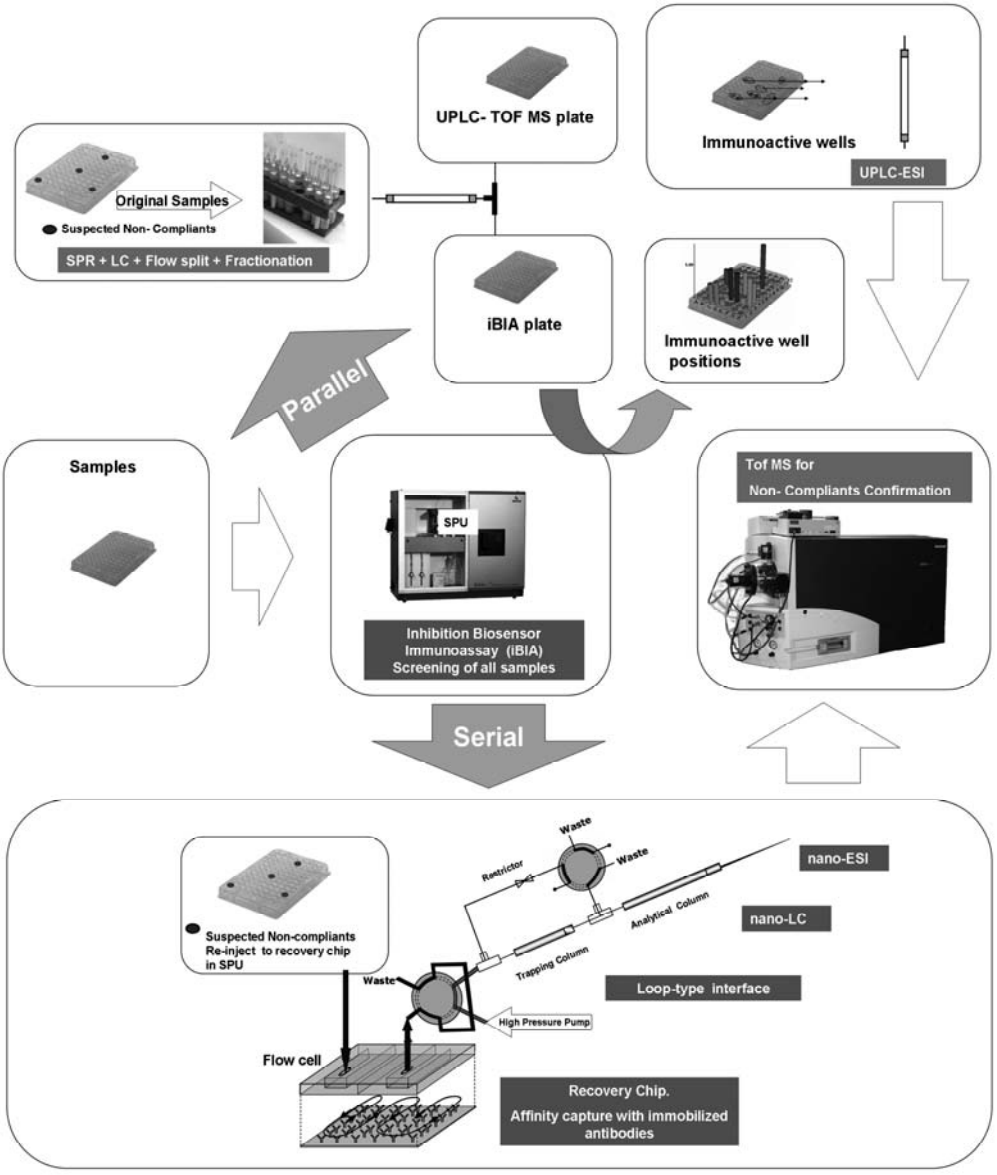


**Figure 1.** Systems setup overview of the two SPR-LC TOF MS interfacing strategies.

In the parallel interfacing, the pinpointed suspected non compliant samples were concentrated using solid phase extraction (SPE), chromatographed, the effluent was splitted and fractionated in two identical 96-wells fraction collectors creating the iBIA plate and the

UPLC-TOF MS plate. In the iBIA plate, the immunoactive LC fractions were screened with the biosensor and only the positive well positions were used in the second 96-well plate for immunoactive oriented identification with UPLC-ESI TOF MS.

This strategy was tested using a dual SPR iBIA in which a specific assay for flumequine was combined with a generic assay for five other FQ (norfloxacin, enrofloxacin, ciprofloxacin difloxacin, sarafloxacin). The presence of the FQs was screened and confirmed in chicken muscle tissue below regulatory-relevant concentrations (Marchesini et al., 2007).

In the serial interfacing strategy (Figure 1), the samples pinpointed as non-compliant during the SPR iBIA screening were reinjected onto a recovery chip (RC) with similar, but reversed immunochemistry (immobilized antibodies). The model analyte was Enro and anti-Enro specific antibodies were used as the biorecognition element during the screening and on the RC. The antibodies in the biosorbent specifically captured the analyte while the sample matrix flowed through. The analyte captured on the RC was eluted into a loop-type interface using an MS-compatible buffer. The loop-type interface solved the pressure incompatibility between the microfluidic system of the biosensor operating at low pressure and the nano LC ESI TOF MS system operating at high pressure. Once in the loop type interface, the analytes enter the nano-LC system and are retained in a trapping column followed by an analytical column connected to the nano-ESI TOF MS for mass analysis. This strategy was applied to the screening and confirmation of identity of Enro in chicken tissue below the MRL level (100 µg/kg). Enro at 0.5 MRL (5 pg/µl, 40 µl injected over the RC) and its metabolite, ciprofloxacin, were successfully detected in chicken muscle extracts. In-depth information of the performance of this coupling strategy can be found elsewhere (Marchesini et al., 2008).

A common problem with SPR biosensor-LCMS hyphenation is the mobile phase incompatibility. To preserve biomolecular interactions, the antibodies in the biosensor usually need a buffer containing salts that might later interfere with the MS analysis. Additionally, nonionic detergents, also incompatible with MS analysis, are generally used in the mobile phase of biosensors to avoid nonspecific adsorption of matrix components or analytes to the fluidic system. In the parallel coupling strategy, the mobile phase incompatibility is not an issue because the mobile phases are separated. On the other hand, in the serial interfacing strategy, the impurities in the recovery chip can be extensively washed but to prevent long lasting impurities, extreme care should be taken in the choice of the SPR buffer. Although the proof of principle of the SPR iBIA screening- MS parallel interface was successful, it had some limitations (Table 1). This strategy requires a relatively large sample volume and a concentration step of the suspected non-compliant samples prior to fractionation. This greatly increases the handling time and the chances of sample contamination and extends the total analysis time up to 24 h.

**Table 1.** Main differences between the two interfacing strategies studied.

| | **Parallel Interfacing** | **Serial Interfacing** |
|---|---|---|
| Sample extract consumption | High (<2 ml) | Low (>0.2 ml) |
| Sample handling of suspected non compliants | High | Minimum |
| Sample extract concentration required | Yes | No |
| Suspected non compliant analysis time | ≈24 hs | ≈25 min |
| iBIA Buffer Limitation | None | High |
| Sample extraction and iBIA analysis time | 20 min | 20 min |

Hence, this makes the parallel strategy unfeasible for screening for bioactive compounds whose half life is short or unknown. An additional limitation is that the fractionation resolution is limited and this might hamper the identification given that complex samples may contain hundreds of peaks separated during the chromatographic step, but pooled back together in the same fraction. Considering these limitations, the serial interface provided has a much faster analysis time, requires a minimum sample volume and handling. The RC interface was successful in the immunoaffinity directed recovery of the analyte and ultimately the resolution of the bioactive peaks was given by the nano-LC ESI TOF MS system.

**Conclusions**

The advantages and limitations of the two strategies for interfacing SPR iBIA with LC-MS were evaluated. The parallel strategy provides the advantage of versatility with respect to the buffers that can be used in the iBIA and with respect to the detection method used because the systems do not need to be physically coupled. This reduced the cost of the system, but at the expenses of extensive sample consumption and handling.

The serial strategy had the advantage of being fast, sensitive and was proven to be capable for a bioaffinity directed capture of the parent compound (Enro) and its metabolite ciprofloxacin. The use of the RC interface enables the acquisition of in depth information about the performance of the biosorbent prior to use and for periodical QA/QC check with SPR.

The SPR system is capable of multianalyte screening, and the RC interface is capable of multianalyte recovery. Hence, current research is focused on the use of the serial SPR-RC-nanoLC TOF MS system for the screening, confirmation and potentially the discovery of paralytic shellfish toxins.

**Acknowledgements**

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# THE OCCURRENCE OF α- AND β-NORTESTOSTERONE RESIDUES IN THE URINE OF INJURED MALE CATTLE.

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**Abstract**
Residues of α- and β-nortestosterone in urine, which heretofore have been considered as proof of nortestosterone abuse in male cattle, have been detected in samples taken from 155 out of 328 injured male animals – leading to the suggestion that these hormones can occur naturally in male, as well as in female cattle.

**Introduction**
Administration of the androgenic anabolic steroid 17ß,-19 nortestosterone (ß-NT), or its esters, to food-producing animals is prohibited (Anon, 1996) within the European Union (EU). The major bovine metabolite of ß-NT is its epimer: 17α-nortestosterone (α-NT). Although it is known that α-NT can occur naturally in the urine of female cattle (Meyer et al., 1992) and that β -NT can occur naturally in the urine of boars (Maghuin-Rogister et al., 1988) and stallions (Houghton et al., 1984), it has been generally accepted that neither compound occurs naturally in the urine of steers (castrated male cattle) or bulls (McEvoy et al., 1999). All EU Member States (EU-MS) are required to monitor for abuse of steroid hormones through their National Residue Control Plans, which are submitted annually to the European Commission for examination and approval. Most testing schemes in the EU include α- and β-NT because they have previously been detected in a number of countries, including Northern Ireland (NI). For example, the presence of β-NT decanoate has been confirmed in preparations or on syringes recovered during enforcement actions carried out by the Department of Agriculture and Rural Development (DARD) in Northern Ireland (NI) in the past three years.

**Materials & Methods**
During the course of the events described below, our testing for hormones in urine was focussed on analysis for nortestosterone. Screening was carried out by extracting nortestosterone from urine using immunoaffinity chromatography, following incubation with E.coli ß-glucuronidase to hydrolyse steroid glucuronide conjugates. Sample extracts were then analysed using an immunobiosensor assay (Biacore Q), with a Detection Capability of < 0.5µg/L. Confirmation was accomplished using high-performance liquid chromatography (HP1100 series binary pump) coupled to tandem mass spectrometry (Micromass Quattro LC), with 20% of the column effluent being directed to the mass spectrometer and 80% to a fraction collector, essentially as described previously (Hewitt et al., 2002). Further confirmation of the presence of α- and β-NT in the samples was achieved by using gas

chromatography coupled to tandem mass spectrometry (GC-MS/MS). In brief, the appropriate fractions from the LC-MS/MS run were dried down and derivatised with heptafluorobutyric acid anhydride. Samples were analysed using a Micromass Quattro micro GC. Two transition products for α- and β-NT (m/z 666.1 > 146.2.2 and 133.2) and one for the internal standard (m/z 669.1 > 136.2) were acquired. A non-compliant result, which will lead to enforcement action, is defined in law (Anon, 2002) as any confirmed concentration in excess of CCα. CCα for α- and β-NT in bovine urine using this procedure are 0.30 and 0.22 µg/L, respectively.

To simulate on-farm emergency slaughter, five healthy Holstein-Freisian steers (approximately 470 kg, aged between 18 and 21 months) were killed by the same method at the laboratory; (stunned using a captive bolt pistol and immediately bled out). Urine was collected directly from the bladder immediately after slaughter, after 3 hours and (if possible) 6 and 24 hours after killing. All samples were split into two aliquots. A portion was frozen at -20°C prior to analysis. The second aliquot was held at 4°C for 72 hours prior to freezing at -20°C to simulate the mean transit time (and storage conditions) of samples from abattoirs to the laboratory. All samples were analysed as described above.

**Results & Discussion**

European law (Anon, 2004) permits the carcases of injured animals, slaughtered on-farm (On Farm Emergency Slaughter – OFES) for welfare reasons, to enter the food chain. Such animals must have had a satisfactory ante mortem veterinary inspection before slaughter. Animals must also be transported within a time limit (3 hours) to abattoirs for processing and post mortem inspection. Injuries may also be detected/reported during ante mortem inspection of live animals considered fit to travel for slaughter (ante mortem findings – AMF). Apart from the acute injury, in both cases the animals should otherwise be healthy to be eligible for human consumption. In the UK, the British Cattle Veterinary Association (BCVA, 2005) has issued a guidance document on emergency slaughter. In NI, OFES and AMF animals are routinely examined for antimicrobial residues, but not for hormones (as is also the case in some other EU-MS).

In March 2006, a Meat Inspector, referred a urine sample taken from a steer to this laboratory for analysis for hormones and ß-agonists. The carcase was detained in the abattoir pending the laboratory result. The presence of α-NT in its urine was confirmed at a concentration of 1.03 µg/L. As this exceeded CCα, the animal was condemned and an investigation was commenced. Over the course of the next 6 weeks, the same Inspection team submitted samples from a further 12 male animals, of which 10 contained either α- or β-NT. It then began to emerge that all of the non-compliant samples were taken from OFES animals. This was initially deemed to be strongly indicative of abuse of β–NT – since OFES animals would not have been subjected to a withdrawal period to minimise the risks of detection. At that point, 100% sampling of all AMF and OFES animals was introduced in NI. All such animals were detained in abattoirs, pending laboratory results, non-compliant animals were excluded from the food chain and a major enforcement effort was initiated. Since then, a total of 155 out of 328 animals tested have been declared non-compliant for α-NT (concentrations ranging from CCα to 17.2 µg/L) and 8 out of 235 animals for β-NT (concentrations ranging from CCα to 1.4 µg/L). The majority of the non-compliant animals were OFES steers (91 %) as summarised in Table 1, below. All of the non-compliant animals were excluded from the food chain.

**Table 1.** Summary of testing for α- and β-NT in injured animals

| Category | Compliant | | | Non-compliant | | |
|---|---|---|---|---|---|---|
| | Bull | Steer | Total | Bull | Steer | Total |
| OFES | 30 | 115 | 145 | 17 | 124 | 141 |
| AMF | 9 | 19 | 28 | 2 | 12 | 14 |
| Total | 39 | 134 | 173 | 19 | 136 | 155 |

An examination of the history available for the 286 OFES animals revealed that fractures accounted for the largest portion of the non-compliant results (44%), with lameness and back injuries accounting for a further 19 and 17% of cases, respectively. There was no strong correlation between a particular condition and the result of the NT analysis (Table 2).

**Table 2.** Clinical histories of the injured male animals (OFES and AMF) involved in this incident.

| History | Compliant | Non-compliant | Total |
|---|---|---|---|
| Fracture | 83 | 70 | 153 |
| Lameness | 37 | 29 | 66 |
| Back injury | 18 | 24 | 42 |
| Down | 5 | 11 | 16 |
| Hindquarter injury | 8t | 6 | 14 |
| Paralysis | 5 | 3 | 8 |
| Other/Unknown | 17 | 12 | 29 |
| **Total** | **173** | **136** | **328** |

It was decided that, in addition to excluding all non-compliant animals from the food chain, all positive incidents would be followed up on farm. Animals deemed (by reason of age, condition, etc) to belong to the same batch were detained on farm and urine collected from at least 10% of all animals in the batch. The owners of the animals represented a broad spectrum of the farming community in NI. It seemed unlikely that such a diverse group of individuals would all be using β-NT or cocktails containing β-NT illegally to promote growth in their cattle. No products, preparations or syringes containing β-NT or its esters were recovered at any of the on-farm follow up visits (in contrast to previous incidents in NI, when such findings have been made). A total of more than 1000 urine samples, collected as follow-ups to the 155 positive results (Table 1) have been tested for nortestosterone. No non-compliant samples have been detected. This suggests that abuse of NT may not have been the cause of the residues. Supporting this is the fact that the prevalence of non-compliant OFES animals did not change significantly throughout this period. Urine samples from approximately 50% of OFES animals tested were shown to contain α-NT and, occasionally, β–NT. This contrasts markedly with the pattern seen in other documented cases of abuse in NI. In the early 1990s, abuse of clenbuterol was widespread. When DARD took action and started to condemn animals, the proportion of non-compliant results detected fell to virtually zero in a few weeks. In this case the prevalence did not change, suggesting that abuse may not be the cause of the residues.

Had NT abuse been widespread in NI, it might have been expected that, in addition to the non-compliant samples taken from injured animals, a significant proportion of uninjured animals would also be non-compliant for NT. Out of 310 samples submitted to this laboratory under the National Residues Control Plan and under other residues tesing schemes, only two contained confirmed concentrations of α-NT. However, neither of these animals could truly have been described as "normal". Both were condemned as unfit for human consumption for other reasons: one because of its emaciation and the other on the grounds that it had clinical tuberculosis. This provides further evidence that there is no underlying problem with NT abuse in NI.

However, proving a negative – that there is no major abuse of NT in NI is impossible. Therefore, we chose to try to demonstrate that this phenomenon was not unique to NI. To do this, we obtained 49 urine samples from three other areas within the EU. The results (Table 3) showed that the presence of α-NT and β-NT could be confirmed in urine taken from AMF and OFES bulls/steers outside of NI. While it is possible that all four areas (including NI) were simultaneously experiencing major misuse of NT in beef production, it is a much more plausible explanation that some heretofore unknown physiological mechanism was producing the residues naturally in injured animals.

**Table 3.** The prevalence of α-NT and β–NT in urine samples from other parts of the EU.

| Region | Compliant | Non-compliant | Total |
|---|---|---|---|
| 1 | 6 | 10 | 16 |
| 2 | 12 | 1 | 13 |
| 3 | 11 | 8 | 19 |

Finally, two steers, belonging to separate farmers, were sampled on their respecive farms (in May and June 2006, respectively) in response to non-compliant results detected in OFES animals sampled at separate abattoirs (in April and May 2006, respectively). The animals tested on-farm were both found to be compliant. However, both steers subsequently suffered injuries and were submitted to abattoirs as OFES animals in June and August 2006, respectively. At that point they were both found to be non-compliant for α-NT. This unusual sequence of events strongly suggested that a physiological event may have been the cause of the residues.

One possibility was that the laboratory had made a procedural error and that we were identifying some other urinary component as α– and β-NT. In order that we might exclude this possibility, however remote, we sent 11 samples to the EU Community Reference Laboratory for anabolic steroids (RIVM, Bilthoven, The Netherlands) and to the French National Reference Laboratory for anabolic steroids (Laberca, Nantes, France) for analysis. We had previously reported some of these samples as compliant and others non-compliant for α-NT and, on occasion, β-NT. No contradictory results were obtained between the 3 laboratories.

One major difference between OFES animals, responsible for the majority of the non-compliant results (Table 1) and our normal sample intake is that the OFES animals were

killed on-farm and transported dead to the slaughterhouse. The maximum permissible time delay between on-farm slaughter and evisceration at an abattoir is 3 hours. We investigated the possibility that this time delay might have led to post mortem carcase formation of α- and β-NT, by killing animals on the laboratory farm and immediately bleeding them out. We took the experiment to extremes – sampling from the bladder not just for 3 hours, but for up to 24 hours post mortem (whenever possible) and by analysing samples frozen either immediately after collection or following a 72 hour incubation period (to simulate transportation time/ conditions that the samples experience). Neither α-NT nor β-NT were present in any of the samples collected. This effectively excludes the possibility of post mortem changes having caused the production of NT as an artefact.

We now believe that the finding of α–NT or β–NT in the urine of male cattle can no longer be ascribed exclusively to abuse of ß-nortestosterone. Work to confirm the natural production of this hormone is continuing at this laboratory. Perhaps this finding should not come altogether as a surprise – since α-NT and β-NT are both known to occur naturally in other species, and in pregnant cattle (which demonstrates the genetic potential for cattle to produce nortestosterone). Nonetheless our findings represent a difficulty for EU-MS who currently rely on measurements α-NT and β-NT to monitor and control the abuse of this perennially popular drug.

The finding of non-compliant residues of unauthorised veterinary drug residues in food-producing animals carries with it the potential for the application of a range of severe penalties for the farmer. However the science underlying a decision to apply enforcement action must be defensible. In the case of NT, there has been no evidence that it, or its metabolites, can occur naturally in male cattle (McEvoy et al., 1999). There is now very considerable evidence that the α- and β-NT detected in these injured animals is not present as a result of abuse of NT.

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## O 14

# KEY ASPECTS OF THE NEW REGULATION GOVERNING MRLS FOR PHARMACOLOGICALLY ACTIVE SUBSTANCES

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**Abstract**
The European Commission's 'Reflection paper on residues in foodstuffs of animal origin' (17/12/2003) discussed the need to review the legislation governing residues in foodstuffs of animal origin to find a better balance between consumer protection and animal health and welfare, and to resolve certain difficulties (e.g. control of imports).
It compiled a list of problems arising from the current legal framework, and it set out the goals of the review. The public consultation was completed with a stakeholder meeting in July 2005. An European Commission proposal for a Regulation to replace Regulation 2377/90 was released in April 2007, thus triggering the start of the co-decision procedure.

This paper reviews the main issues identified with the existing legislation, the main changes proposed by the European Commission, and the main issues that have been raised by the co-legislators (Parliament/Council). Discussion has focussed on reference points for action, extrapolation of MRLs, Codex MRLs and whether biocides should be included in the scope of this Regulation.

**Introduction**
DG SanCo[1] and DG Enterprise[2], of the European Commission (EC), jointly released a 'Reflection paper on residues in foodstuffs of animal origin' for consultation on 18 December 2003. It discussed the need to review the European legislation governing residues in foodstuffs of animal origin. The objectives are to find a better balance between consumer protection and animal health and welfare, to resolve difficulties (e.g. control of imports) and to have a more consistent approach between the different legal texts to control residues of pharmaceutical active substances.
The Reflection paper compiled a list of problems arising from the current legal framework, and it set out the goals of the review. The aim was to bring the relevant legislation in line with the principles of Regulation 178/2002 ('Food Law') and would include modification of the relevant legislative instruments, i.e. Regulation (EEC) No 2377/90, and Directive 96/23/EC. The consultation was completed with a stakeholder meeting in July 2005 to discuss the outcome and the preferred solutions.
An EC proposal for a Regulation to replace Regulation 2377/90 was released in April 2007, thus triggering the start of the co-decision procedure with the European Parliament (EP) and the Council of the EU. The main activities of the committees of EP took place in the last quarter of 2007 and the first half of 2008, when their first reading is expected to be completed.
This paper reviews the main issues identified with the existing legislation, the main changes to the legislation proposed by the EC, and the main issues that have been raised by the EP and

the Council. The key steps in the co-decision procedure are identified and an estimate of the procedural timescale is given.

*1 Directorate General for Health and Consumer Protection*

*2 Directorate General for Enterprise and Industry (Unit F2 Pharmaceuticals and Cosmetics)*

**European Commission's Reflection Paper**

The Reflection Paper (18/12/03) identified the goals of the review to "Determine a new means to balance consumer protection, animal health, welfare and trade; a coordinated approach with related legislation (pesticides, food law); and a more consistent approach for the risk analysis and control of residues in foodstuffs."

The main issues identified in the reflection paper included:
Achieving a correct balance between availability of veterinary medicinal products and consumer protection.
How to set Maximum Residue Levels (MRLs) for control of substances that do not have an MRL.
The timeframe and procedure for submitting national residue control programs to the European Commission.
The content of Annex I of Directive 96/23 (list of product groups that are to be controlled).
Who bears the costs of additional monitoring (to establish actual consumer exposure)?
Imports: equivalence of measures in 3rd countries: timescale and transparency of process.
Distribution of Community Reference Laboratory (CRL) responsibilities, particularly in view of EU enlargement.
Harmonise terminology: non-authorised/illegal use; farm/food-producing animals.
MRL assessment reports: need better distinction between risk assessment and risk management.
Annex II criteria include "other legitimate factors" – these need "addressing".
Reorganisation of, or amendments to, the annexes of Regulation 2377/90 (this should not lead to the need for new MRL applications or data).

The Reflection paper concluded that the legislation needs coordinating, inconsistencies/ contradictions removed, and that certain inadequacies should be rectified, such as: Regulation 2377/90 is too inflexible as it does not cover extrapolation of MRLs and it does not lead to MRLs and analytical methods to control illegal use; the current legislation does not lead to residue limits for substances licensed outside EU, but not licensed in the EU, to control imports; there is no procedure for short-term risk assessment in crisis situations e.g. with imports; alternative funding is required to control illegal use or imports as the pharmaceutical industry is not responsible for these. In addition the Reflection paper recommended that the following aspects should be reconsidered: procedures for national residue surveillance plans; procedures for authorisation of imports; the network of National Reference Laboratories and responsibilities of Community Reference Laboratories; and control and enforcement measures.

*Perspective of European manufacturer's of veterinary medicines*
The current legal framework for MRLs, in force since 1992, has certainly increased consumer safety, but also led to some unintended problems:
While the initial implementation of the Regulation 2377/90 led to the setting of MRLs for each individual food-producing animal species, this turned out to be too expensive for some species. Consequently, the decrease of availability of veterinary medicines for certain species has created negative effects for human health, animal health and welfare. Veterinary medicines are crucial to guarantee animal health and welfare by preventing and curing diseases.
International standards that were assessed scientifically in the framework of the Codex Alimentarius and are supported by the EU cannot at present be included in EU legislation without a new scientific assessment by the European Medicines Agency. This results in duplication and delays.
International trade in animals is a reality of our time, but control services of Member States have no points of reference in particular for substances without MRLs detected in food from third countries.
The current legislation is regarded as difficult to understand and out of date.Consequently IFAH-Europe welcomed and fully supported the Commission's objective to consolidate and improve the legislation.

**European Commission's proposal for a new Regulation**
Addressing these issues, the Commission proposal (17/04/2007) sets out the following measures:
- It formally recognises current scientific knowledge and practice of inter-species extrapolation, whereby under certain conditions the MRLs for a substance for one or more species can be applied to additional species, and creates a legal basis for the Commission to lay down the principles for applying extrapolation.
- It introduces a mechanism allowing the Community to adopt MRLs set by Codex Alimentarius where these have been supported by the EU during the Codex scientific evaluation.
- It creates a specific legal framework to set MRLs for pharmacologically active substances not intended to be authorised as veterinary medicines in the EU, in particular for the control of imported food.
- It creates a specific legal framework to set 'reference points for action' (see below) for substances in imported food that do not have an MRL.
- It simplifies the existing legal texts by deleting expired articles and rearranging their sequence.

*What are Reference Points for Action?*
For residues of pharmacologically active substances which are not subject to an EU MRL, "reference points for action" (RPA) will be set (see Articles 17-19 of COM proposal). These would be based on a harmonised Limit of Detection of the analytical method, i.e. the lowest level detectable by all national laboratories.
RPA would be set by the Community Reference Laboratory (CRL) responsible for that substance, with the consumer safety aspect checked by the European Food Standards Agency (EFSA).

Foodstuffs are subject to controls on residues in accordance with Regulation (EC) No 882/2004. Even if residue limits are not set for such substances pursuant to this Regulation, residues of such substances might occur. Laboratory methods are capable of finding such residues at ever lower levels, but the precise level of detection may vary between laboratories. Such residues have caused different control practices in Member States.
Therefore the EC proposal will establish procedures to set RPAs at concentrations of the residues for which scientific advice indicates that consumer exposure is negligible and laboratory analysis is consistently achievable in all Member States in order to facilitate intra-Community trade and imports.
Control services in Member States will have harmonised points of reference on which to base consistent decisions for certain substances for which there is no European MRL. Food imported from third countries may contain residues of these substances due to the treatment of food-animals with veterinary products not used in the EU, or due to environmental contamination.
The harmonisation of control standards for certain residues in food, with clear reference points, checked by EFSA, will facilitate trade, improve the functioning of the single market, and protect the health of consumers.

**The Co-decision procedure**
the EC Proposal (017/04/07) for a Regulation must be adopted by the co-decision procedure between the European Parliament and the Council.
The first step of the co-decision procedure is the First Reading of the European Parliament. The Committee for the Environment, Public Health and Food Safety ("ENVI") was appointed as the 'lead' committee to prepare a report.
The Committee on Agriculture and Rural Development also elected to prepare its 'opinion'. This was drafted and debated during September to November 2007, and the final AGRI opinion proposed 18 amendments to the EC's draft Regulation. It welcomed the objectives of the draft Regulation, to simplify legislation and improve the MRL procedures, while keeping health protection as the over-riding objective. It supported the extrapolation of MRLs with the objective of making more veterinary medicines available for additional species representing a 'minor' use. However it had objections to the lack of transparency in the CODEX procedure, and insisted that Europe must still be able to take specific measures to maintain consumer protection.
Consequently the majority of the amendments are minor changes aimed at clarifying certain articles, particularly those covering extrapolation of MRLs and provisional MRLs.
The ENVI committee produced a first draft report on 28 November 2007. This was debated in ENVI Committee on 29 January 2008. Other MEPs were given until 20 February to submit additional amendments, prior to a further debate at the 25-27 February ENVI meeting. The vote on the amendments to be retained in the final ENVI report was scheduled for 26 March, and the report would then go forward for a EP plenary session vote during 7-8 May 2008.

The ENVI draft report make reference the EP's resolution on the need for improved availability of veterinary medicinal products, including horses, and the need for good communication and cooperation between EMEA and EFSA. Other amendments seek greater clarity in the text, particularly for the provisions concerning Codex Alimentarius, and the use

of the word ‘risk’ in place of ‘hazard’.
It also proposed that reference points for action should not be “regularly reviewed in the light of technological progress”, as this leads to an endless chase towards zero residues as analytical methodology progresses. If the levels are already safe for the consumer, then this is a purposeless waste of effort. Instead these values should only need reviewing in the light of new toxicological data and advances in the understanding of risks to human health and the food chain.

*First Reading of the Council*
First Reading of the Council does not officially start until it receives the report from the First Reading of the EP. However the Council veterinary experts working group has been examining and discussing the EC’s proposed Regulation since its release in April 2007. This working group met 7 times under the Portuguese presidency in the 2nd half of 2007, and several times under the Slovenian presidency in the 1st half of 2008.
The working group discussions have focussed on 2 questions:
(a) Reference Points for Action (RPA). These are a pragmatic solution to a trade problem. However it raises a matter of principle: should limits be set for residues that should not be present, or should any trace residue lead to rejection of the food commodity? One of the issues is if an MRL exists, should an RPA be set for non target tissues due to, for example, cross contamination in a feed mill. DG Enterprise appears to be opposed to such a scenario.
(b) Inclusion or not of biocides within the scope of the Regulation: the EMEA is responsible for MRLs veterinary medicines: but biocides fall under EFSA’s responsibilities. A biocide could be used in animal housing and end up as a residue in animal produce. Should EFSA set MRLs or RPAs?

*The expected timeline*
The First Reading of the European Parliament is scheduled to be completed in May 2008 (1 year after the Commission proposal was released). The Council 1st reading will then start officially, and will be completed when it adopts a ‘common position’ by a qualified majority. There is no fixed timeline for both these ‘first readings’, however it is likely that an objective of the German presidency during the second half of 2008 will be to successfully negotiate a ‘common position’ and complete the procedure within its presidency.
The 2nd readings in Parliament and in Council have fixed timelines, making the process more predictable. When Parliament receives the Council common position it has 3 months to either approve it, reject it or propose counter amendments. Similarly the 2nd reading in Council has 3 months to adopt the act or the Conciliation Committee is convened within 6 weeks. In order to avoid entering the Conciliation procedure the 2nd readings are characterised by close trilateral discussions between the European Commission, Parliament and Council to agree the final texts and acceptable compromises if necessary.
Consequently it might be anticipated that the co-decision procedure for this draft Regulation could be completed either at the end of 2008 if agreement can be reached after the first readings (not common), or early in the 2nd half of 2009.
The new Regulation will supersede Regulation 2377/90 and will apply on the 20th day following its publication in the Official Journal.

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# O 15
# NATURALLY OCCURING HORMONES IN THE FOOD CHAIN

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**Abstract**
Substances having a hormonal action are prohibited in Europe for use in animals intended for meat production (directive 96/22/EC). EU council Directive 96/23/EC lay down requirements for residue testing in order to ensure compliance with the prohibition. There are no major difficulties as far as the analytical determination is concerned (covering measurement and interpretation) of xenobiotic steroids in most biological matrices collected in cattle; the monitoring of endogenous steroids is significantly made more complicated because of the non existence (very often) of criteria allowing the discrimination between treated and non-treated populations. The most significant problem arises due to the fact that when they are shown to occur naturally within a particular type of animal, a simple qualitative demonstration of their presence does not usually prove abuse. Quantitative decision limit or ratio to another endogenous substance is therefore usually required in order to suspect the abuse of the corresponding compound. Only few techniques are available to definitely confirm the administration of the steroid, and finally few laboratories in Europe are equipped or trained to face these approaches. In this talk, classical approaches applicable to most steroids in animal biological fluids or food from animal origin will be reminded. The importance of steroid metabolism knowledge will be underlined through some examples to show how the efficiency of the control is improved, and detectability of the consecutive residues prolonged in time. Then, major current improvements in technology will be discussed, as well as how new global and high-throughput analytical approaches are developed for mid term introduction in the field**.**

**Introduction**
According to European Union (EU) regulations [1], the use of anabolic steroids as growth promoters is prohibited in food producing livestock. The illegal use of these compounds has raised concerns about human health after consumption, resulting in the introduction of monitoring programs to control their use. Such programs are frequently based on the initial screening of animals for steroid abuse using chemical or immunochemical methods [2–3], followed by the complete chemical confirmation of steroids in suspect samples by mass spectrometry (MS) [4-12].
This strategy does not cause particular problems for strict artificial steroids. Urine and hair samples are normally ad hoc matrices since they are readily available at both slaughterhouse and farm. Until recently, the standard technique for steroid analysis has been gas chromatography (GC)–MS. This required the derivatization of the steroids using silylation, acylation, oxime/ silylation [13] reactions, depending on the individual properties

of the steroid. LC–MS provides a universal detector, since steroids may be analyzed without derivatization. The rules governing screening and confirmation of analytical methods for veterinary drug residues and their validation have been revised by the EU [16]. The rules have been extended to include a number of MS techniques, which have gained in popularity over the last decade, e.g. liquid chromatography (LC)–MS [17-18], and acquisition techniques such high resolution selected ion monitoring (HR-SIM) and selected reaction monitoring (SRM).

The control of natural hormones is more complicated as recently and efficiently shown by James Scarth et al. [19-20]. The occurrence of steroids related to testosterone, nandrolone, boldenone, estradiol, progesterone and cortisol mainly in bovine matrices (but developed in porcine, ovine, caprine, equine, cervine) has been fully reviewed and a suitable subset of the data assessed by statistical analysis, when possible. The measurement of these hormones even at trace levels is nowadays not a challenge as such, but the interpretation of the data (especially when quantitative) is rather trickier. Sporadic action levels have been proposed in the literature, but never clearly mentioned in the European regulation, the few ones being serviceable can be considered as screening thresholds. Recent promising developments to isolate suspicious samples from unequivocal compliant ones are based on target metabolite profiling or full untargeted fingerprinting (metabolomic). The few research projects in the field are still not officially used for official monitoring plans, but first applications are expected soon.

Few confirmatory approaches are currently considered as unambiguous and only a handful of laboratories in Europe are practicing routinely these approaches. The first strategy relies upon the identification of natural hormone esters in hair samples [21-22] with main advantage the long detection window of the corresponding residues in hair, the convenience of sample collection, but with different disadvantages such as its limitation to steroid esters (mis)use (if not, the approach is worthless), the possible contestation of the origin of the detected ester (inter-animals contamination), a lack of knowledge regarding esters incorporation in hair, and a high variability in-between animals, hair colours, distance from injection site… The second strategy is based on the measurement of the $^{13}C/^{12}C$ of steroid metabolite(s) versus the carbon composition of endogenous reference compound(s) [23-25]. This technique has been developed mainly for steroid residues in urine, and in particular to testosterone, estradiol and cortisol, their direct precursors and metabolites. Main advantage is the possibility to demonstrate unambiguously - a couple of weeks after the administration – the exogenous character of the detected metabolite. Further trends include instrumental sensitivity, extension to other biological matrices, and eventually implementation of the technique European official laboratories.

## Material and methods

*Sample preparation*

Apart from untargeted metabolomic applications where sample preparation is kept to strict minimum, analytical protocols dedicated to the extraction and purification of anabolic steroids in biological fluids and tissues are generally based on at least two-stages [11]. Single use solid phase extraction (SPE) columns are generally utilized to extract and purify steroids from biological matrices. C18, C8, $NH_2$ and SiOH are amongst the most popular stationary phase;

SAX (strong anion exchange) columns are used for the isolation of steroid conjugates. Solid samples (faeces, tissues, feeding stuff) are preliminary extracted either by accelerated solvent extraction (ASE), soxhlet or liquid/liquid extraction before any purification step. Conjugates are usually hydrolyzed either by enzymatic (glucuronidase) or chemical approaches (solvolysis or methanolysis). When GC-MS is used, steroids are quite always derivatized, the most popular approach being the silylation, acylation (acetylation, perfluoroacylation) or oxime formation. N-Methyl-N-(trimethylsilyl)-trifluoro-acetamide (MSTFA) is often used as silylating agent in combination with ammonium iodine ($NH_4I$) or trimethyliodosilane (TMIS) as catalyst, and dithiothreitol (DTE) as antioxidant. When LC-MS is used, derivatization is not mandatory but is sometimes used to enhance the signal or improve the specificity.

*Analytical instruments*
GC-MS is routinely used for steroid analysis; mass analyzers are mainly quadrupoles, but ion trap technologies can be found as well. Single MS is progressively replaced by multidimensional approaches, either by triple quadrupoles or ion trap (external sources), for steroid analysis. High resolution mass spectrometer (HRMS) are mainly electromagnetic instruments operated at resolution equal or better than 10,000 (10% valley); they are used in confirmatory processes (control laboratories) or more generally when both sensitivity and specificity are needed. To trace the origin of steroids (endogenous versus exogenous), combustion isotope ratio mass spectrometry (C-IRMS) coupled to gas chromatography is considered today as a serious candidate method for confirmation. In all cases (LRMS, HRMS, C-IRMS) capillary columns are preferred; typical dimensions are 30 m x 0.25-mm id., 0.25 µm film thickness. Stationary phases are non-polar (100% methylpolysiloxane) to medium polar (50% cyano (or –cyano)polysiloxane). Liquid chromatography is either coupled to triple quadrupole analyzer, ion trap detectors (MS/MS or $MS^n$ mode) or high resolution MS on TOF or FT-MS systems for metabolomic approaches. Instruments are generally operated in the positive or negative (for steroid conjugates) electrospray ionization (ESI) mode. Atmospheric pressure chemical ionization (APCI) and to a lower extent atmospheric pressure photon ionization (APPI) are preferred for non-polar steroids. Reversed phase liquid chromatography is generally performed on octadecyl (or octyl) grafted silica stationary phase (e.g. 50 x 2 mm, 5 µm) equipped with a guard column (e.g. 10 x 2 mm, 5 µm). Elution solvents are based on methanol or acetonitrile and 0.5 % (v/v) acetic acid in water. Potentials applied onto the capillary (from 3 to 4 kV), cone (from 15 to 35 V) and collision cell (from 5 to 30 V) are significantly different from one steroid group to another.

## Results and discussion

*LC- or GC-MS/MS*
GC-MS/MS analysis of steroids in hair is one of the only approaches allowing direct detection of natural hormones misuse. The efficiency of this approach is shown on Fig. 1; presence of unchanged residues of 17β-estradiol-3-benzoate after intra-muscular injection of the corresponding anabolic steroid preparation, has been detected in hair up to 3 weeks after injection (EI ionization, SRM acquisition).

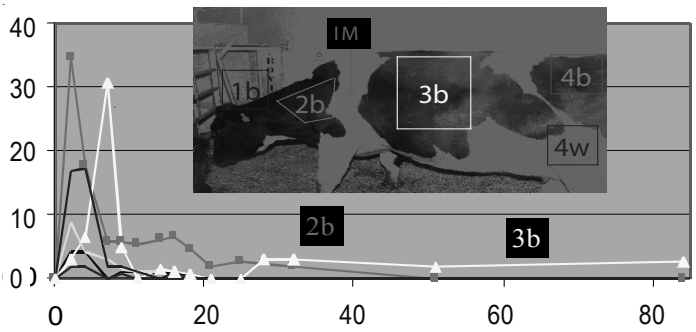


**Figure 1.** Kinetic of fixation/elimination of estradiol benzoate in different hair sample regions collected on a bovine IM injected.

Example of LC-MS/MS applied to 17β-boldenone sulphate or glucuronide in urine sample is given on Fig .2. The simple identification of this steroid phase II metabolite definitely demonstrates the administration of the animal with boldenone, boldenone esters, or boldione.

*GC-C-IRMS*

One of the most challenging tasks for the analyst in the field of chemical residue survey in food is most likely the control of natural hormones (testosterone, estradiol, nandrolone…), and especially the discrimination of endogenous production from exogenous administration. The conventional mass spectrometric approaches permit quantitative assessment of hormones in biological matrices. However, this approach can only be applied for screening purposes because of the intra- and inter-individual physiological variability. Non-ambiguous natural hormone misuse can be demonstrated by measuring the $^{13}C/^{12}C$ of steroid metabolites and precursors.

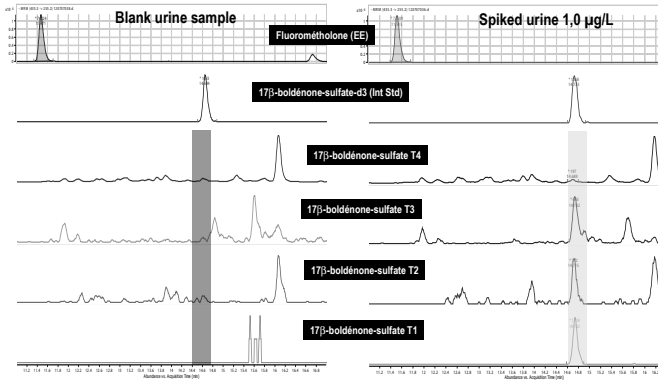


**Figure 2.** Identification of 17β-boldenone sulphate in urine samples by LC-MS/MS (NEG-ESI, QqQ Agilent 6410, SRM). Left: control sample, right: fortified urine sample at 1 $ng.mL^{-1}$.

Indeed, steroid carbon isotopic composition depends strongly on steroid origin. When endogenously produced by the body, testosterone or estradiol derive from cholesterol, i.e., they are directly correlated to the carbon composition of the animal diet. Isotopic deviations ($\delta_{VPDB}$‰) in cattle are in the range -16‰ to -28‰, depending on the feeding, from maize

to hay, respectively. Synthetic steroid sources are characterized by more depleted values contained in-between -28‰ and -34‰. The method using gas chromatography coupled to combustion isotope ratio mass spectrometry (GC-C-IRMS) can measure such differences [23;26]. Whereas 17α-estradiol is the main metabolite allowing to detect 17β-estradiol misuse, etiochalonolone and 5α-androstane-3β,17α-diol (AAdiol) are the main indicators which can prove testosterone administration. In both cases, dehydroepiandrosterone (DHEA) and 5-androstene-3β,17α-diol (AEdiol) are used as endogenous reference compounds (ERC). An illustration is given in figure 3. For grass feeding animals, values of AAdiol vary in-between -26 and -29‰ in blank population and are depleted down to -31‰ in testosterone treated animals.
The ERC isotopic deviation for AEdiol varies traditionally in the -26 to -28‰ range. When fed with maize, δ‰ values of AAdiol vary in-between -20 and -24‰ in non-treated animals, and are depleted down to -31‰ when testosterone is administered, whereas AEdiol can oscillate in the -20 to -23‰ range. Significant difference between ERC and metabolites can be proved during 2-3 weeks after intra-muscular administration of testosterone.

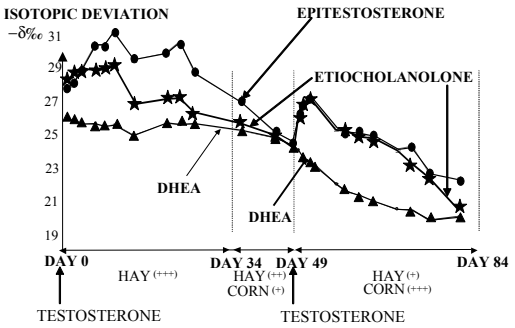


**Figure 3.** $^{13}C/^{12}C$ kinetic of endogenous reference compounds (DHEA) and main testosterone metabolites (17α-testosterone and etiocholanolone) in bovine urine after testosterone enanthate injection. Influence of diet (hay and corn) on $^{13}C/^{12}C$ steroid composition.

*Metabolomic approaches*

Metabolomic approaches either targeted or untargeted, constitute today a great expectation regarding the finding of a (or a set of) biomarkers able to suspect the administration of natural steroid to cattle. LC-HRMS metabolomic fingerprints (LTQ-Orbitrap™, R=30,000, Full Scan [50-800]) was performed on liver samples collected in control versus treated animal with anabolic steroids (1, 2 or 4 implants, Revalor®). As shown on Fig. 3, the different groups of animals can be clearly separated according to their metabolic profiles (around 2000 signals monitored). For this experiment all other sources of potential variability have been minimised (animals of same sex, age and feed). Validation is now necessary on a wide set of samples for testing the robustness of the approach.

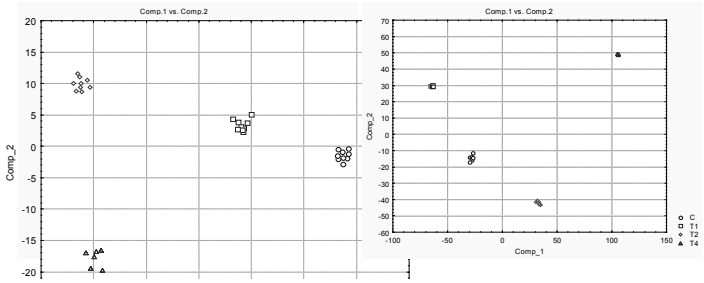


**Figure 4.** Results of linear discriminant analyses (LDA) performed on LC-HRMS Metabolomic fingerprints, liver samples, control animals versus treated animals with 1, 2 or 4 implants of Revalor® and extracted (A) with methanol or (B) with hexane.

## Conclusion

The control of the illegal use of natural steroid hormone in cattle is currently amongst the most challenging ones. Few screening criteria are available; attempt to determine thresholds for the main gonadic steroids did not fully succeed in the past. These proposed limits are mainly concentrations, rarely ratio in-between metabolites or precursors. Few research projects are actually running with main objective to develop metabolomic tools either target (a priori hypothesis) or untargeted (complete metabolite profiling) in order to generate a biomarker or a set of biomarkers able to discriminate compliant population from treated suspected populations. Two main independent confirmatory techniques are today available, the first based on the presence demonstration of steroid esters in hair samples, the second to the accurate isotopic composition of steroids mainly in urine. Even if these approaches are still not widely used by official laboratories, they obviously represent a great hope for the future of natural hormone control in meat producing animals.

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## O 16

# SOMATOTROPIN, OVERVIEW OF RESEARCH STUDIES

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**Abstract**
Growth hormones (GH, somatotropins ST) exhibit numerous biological effects associated with growth functions. Recombinant GH (rGH) are produced in large quantities and widely used outside Europe to stimulate milk production and as a general growth promoter in meat producing animals. The use of this molecule in animal production is strictly regulated by food safety directives in force. The control of its potential misuse constitutes an analytical challenge which many laboratories have attempted to tackle over the last 15 years.
Two strategies can be considered to demonstrate somatotropin treatment to animals, based either on direct or indirect methodologies. Direct methods are focused on the analysis of somatotropin itself, while indirect methods are based on the detection of biological biomarkers with concentrations dependant on ST. Recently, the detection of antibodies produced by the animal after treatment has shown promising results for screening the abuse. In the same time, the challenge of rGH monitoring in biological matrices by means of mass spectrometry has been overcome thanks to the development of efficient purification procedure combined with the use of last MS generation instruments.
This presentation overviews the different strategies for the control of GH misuse, focusing on the more recent and promising ones.

**Introduction**
Growth hormone (GH) also known as somatotropin (ST) is a protein hormone produced by the anterior pituitary gland in mammals. Its biological effects are numerous and associated with growth, development and reproductive functions. Recombinant GH (rGH), differing from the natural form by the addition or substitution of some amino acids at the N-terminal end of the protein, are produced in large quantities and widely used outside Europe to stimulate milk production and as a general growth promoter in meat producing animals (Etherton & Bauman, 1998). The use of this molecule in animal production is however strictly forbidden by European food safety directives (Dir 936/1994/EC, Dir 879/1999/EC) and over the past 15 years, many attempts to set up methods for the detection of its abuse have failed. Two strategies can be considered to demonstrate somatotropin treatment to animals, based either on direct or indirect methodologies. Direct methods are focused

on the analysis of somatotropin itself, while indirect methods are based on the detection of biological biomarkers with concentrations dependant on GH. In the case of indirect methods, most of the researches have focussed on the detection of IGF-I (Popot et al., 2002; Kirsch et al., 2007). Recently, the detection of antibodies produced by the animal after treatment has shown promising results (Bailly-Chouriberry et al., 2008). Unfortunately, and despite a good sensitivity, all these methods are limited to screening and can not be used as confirmatory. Direct strategies, aimed at detecting somatotropin itself, can either be immunoassay or mass spectrometric based methods (Pinel et al., 2004). Despite their excellent sensitivity, immunoassays fail in discriminating recombinant and endogenous forms of the protein (Castigliego et al., 2006). Mass spectrometry on the opposite allows this discrimination and presents furthermore the advantage to be sensitive enough to reach the very low (ppb) and expected levels of detection, providing an efficient purification procedure has been performed preliminary to the MS analysis.
Recent advances in the field concerning both screening and confirmatory strategies will be overviewed in the present lecture.

**Indirect strategies – Screening purposes**
The so called indirect methods are based on the detection of biological biomarkers with concentrations dependant on GH. Several candidate molecules have been investigated, such as haemoglobin alpha chain, pro-collagen type III, IGF binding proteins, but amongst all these potential biomarkers, Insulin-like Growth Factor-I (IGF-I) and antibodies anti-GH have been extensively studied and currently provides the most promising screening tools.

*IGF-I*
The approach is based on the measurement of IGF-I, a protein notably under GH control since produced by liver in response to GH stimulation. As this protein occurs physiologically, detection of GH abuse must rely on detecting levels in excess of those found in an established reference range. This approach has in particular been used to screen for rGH abuse in horseracing with dedicated ELISA tests. Samples are considered as suspicious when an IGF-I concentration above 860 $ng.mL^{-1}$ is calculated. In practice, this approach allows screening during a reduced period of time corresponding to the window of rGH administration (Popot et al., 2002; Bailly-Chouriberry, 2007).

*Antibodies*
The production of specific antibodies upon rGH administration has been documented since the 1960s in humans, and more recently in dogs, horses and cows (Zwickl et al., 1990; Burton et al., 1991, Pinel et al., 2005). Immunoassay based methods such as RIA, ELISA, Western-Blot or Surface Plasmon Resonance (SPR) techniques have been developed in order to detect specific antibodies in plasma or serum samples. Recently, a new high-throughput protocol including a step of antibodies purification through octanoic (caprylic) acid precipitation from serum or plasma samples enabled efficient IgG purification, leading to improved sensitivities of the associated ELISA or SPR methods (Bailly-Chouriberry et al., 2008). The production of specific antibodies upon reGH administrations to 2 thoroughbreds measured in serum samples by ELISA is displayed in Figure 2 (a). The large range of collecting points in the experimental protocol enables to distinguish three steps in the immune response: samples collected

before day 7 corresponds to the period where the animals were not treated and the latency phase where no antibodies could be detected. The second period (D8-D16) is consistent with the exponential production of antibodies while the third one corresponds to antibodies catabolism. Analysis of samples collected during this experiment enabled to detect antibodies in the serum of treated animals over more than 6 months after reGH administrations which significantly increases the window of detection, enabling a long term screening. Results presented on Figure 2b confirm the detection in serum of lactating goats of antibodies produced after 1 single rbGH administration, as soon as 10 days after the administration and for at least 3 weeks.

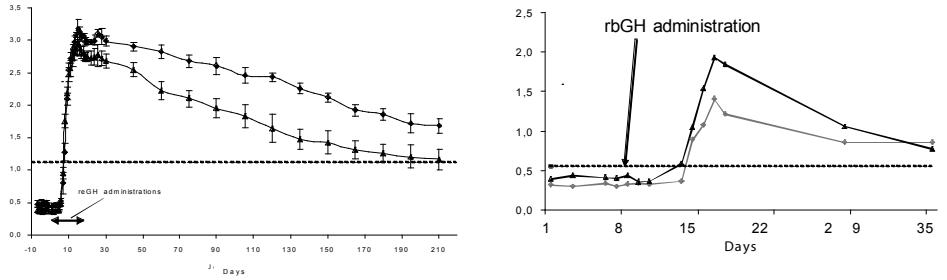


**Figure 2:** (a) Production of antibodies raised against reGH after its administration to 2 thoroughbreds during 2 weeks. (b) Production of antibodies raised against rbGH after one single administration to 2 lactating goats Measurements by ELISA test (DO 405 nm).

**Direct strategies – Confirmatory purposes**

Immunoassay or mass spectrometric based methods have been reported in literature as direct strategies, aimed at detecting somatotropin itself, and thus fitting for confirmatory purposes. However, mass spectrometry is currently the only technique allowing discrimination between the recombinant and the natural forms of growth hormones, thanks to the difference in amino acid sequence at the N-terminal side of the protein (Blokland et al., 2004; Pinel et al., 2004). Mass spectrometry furthermore presents the advantage to be sensitive enough to reach the very low and expected levels of detection ($\mu g.L^{-1}$, $pmol.L^{-1}$) of the target compounds. However and until now, the main issue in the development of a method remained the step of rGH extraction/purification from biological matrices. This analytical challenge has recently been overcome with the development of efficient sample preparation protocol from plasma or serum samples combined with measurements on last generation MS instruments. The sample preparation is based on a precipitation with ammonium sulphate, followed by a clean-up with SPE C4, then a precipitation with cold methanol. A tryptic digestion is performed to generate the specific N-terminal peptides of interest and subsequent MS analyses are performed by LC-ESI(+)-$MS^n$-HRMS on a hybrid instrument composed of a linear ion trap coupled with an orbital trap. N-terminal peptides are identified by selection of their $[M+2H]^{2+}$ in the linear ion trap and monitoring of the resulting fragments in the orbital trap. Typical ion chromatograms of rbGH N-terminal peptide in fortified (10 $\mu g.L^{-1}$) goat plasma are shown in Figure 3.

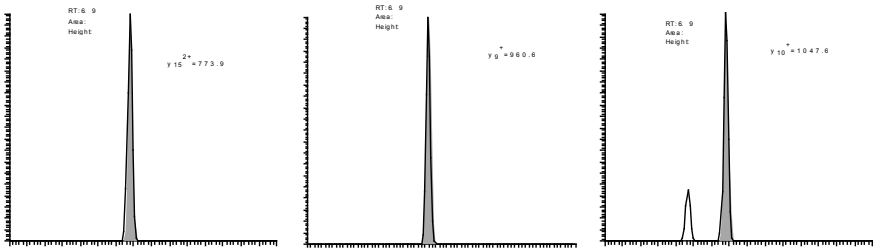


**Figure 3:** Daughter ion chromatogram of a blank plasma from goat spiked at 10 ng.mL$^{-1}$ with rbGH. Analyses performed with a LC-MS$^n$-HRMS instrument in positive mode with resolution R= 30000. Signal corresponding to the tryptic N-terminal rbST. MS2 on 913.3@ CID 18% [260.00-1500.00].

The method was successfully used for the analysis of plasma samples originating from a goat treated with Lactotropin® rbGH and enabled to identify for the very first time rbGH in a sample collected two days after the administration of the hormone (Figure 4). A relative quantification of the signal allowed estimating rbGH concentration in goat plasma around 40 µg.L$^{-1}$.

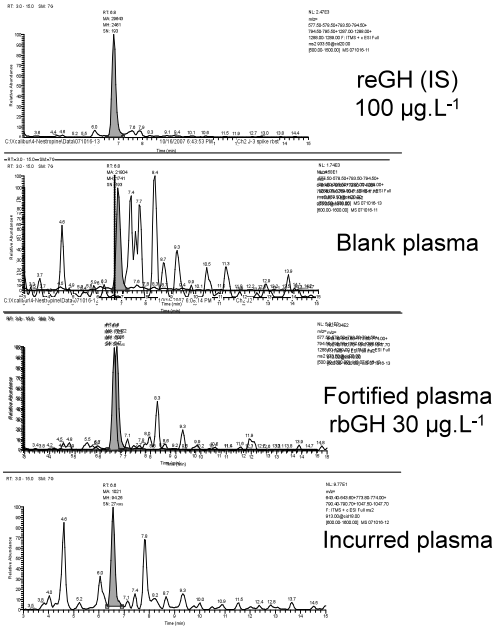


**Figure 4:** Daughter ion chromatograms of goat plasma samples. Signals corresponding to the internal standard (reGH, 100 µg.L$^{-1}$), rbGH in blank plasma, fortified plasma (rbGH, 30 µg.L$^{-1}$), and incurred plasma 2 days after Lactotropin® administration.

## Conclusion

The control of growth hormone potential misuse has constituted an analytical challenge over the last 15 years; however, recent developments in the field have enabled to propose both

screening and confirmatory methods. On the one hand, a strategy focused on the detection of antibodies raised as a consequence of rGH administration to animals enabled to screen animals for rGH abuse from less than 10 days after treatment to several months, on the other hand, a successful confirmatory strategy was set up as the combination of improved sample preparation with the use of last generation MS instruments to identify recombinant growth hormones in plasma or serum samples from treated animals. In parallel of these two approaches, further work is currently on going, focussing on more global strategies aiming at providing untargeted fingerprints of biological matrices as evidence for rGH abuse.

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## O 17

# DEVELOPMENT OF A PROTEOMICS APPROACH FOR THE DETECTION OF RECOMBINANT SOMATOTROPIN IN BOVINE MILK AND SERUM

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**Abstract**
The use of recombinant bovine somatotropin to increase milk production in cows is permitted in a number of countries, e.g. the USA, Brazil and Australia. However, the European Union has prohibited the use of these growth hormones. At the moment, monitoring of the abuse of somatotropin is not possible, because analytical assays are not capable to distinguish between the endogenous and recombinant form. Here we show the development of an analytical method for the detection of recombinant somatotropin in bovine serum and milk. Recombinant- and endogenous somatotropin differ from each other by one amino acid at the N-terminus. Digestion of somatotropin with trypsin in combination with liquid chromatography coupled to mass spectrometry was used to detect the N-terminal peptide. A highly specific MRM assays was developed for specific detection of the N-terminal peptide. It is shown that detection limits were remarkably reduced when switching from conventional liquid chromatography to nanoflow liquid chromatography.

**Introduction**
Somatotropin, also known as growth hormone, is a 22 kDa protein-based hormone produced by the anterior pituitary gland. Biological effects are diverse and include the control of several complex physiologic processes, including growth and metabolism. It is known that administration of somatotropin to cows results in an increased milk production. By recombinant DNA techniques it is possible to produce somatotropin in large quantities. The use of recombinant bovin somatotropin to increase milk production in cows is permitted in a number of countries, e.g. the USA, Brazil and Australia. However, the European Union has prohibited the use of these growth hormones. Therefore an assay to detect somatotropin in biological relevant matrices is necessary to reveal the possible misuse of this compound. Most analytical methods for the detection of somatotropin are immunoassays like radio immuno assays (RIA's) and enzyme linked immuno bound assays (ELISA's ). These assays are not specific enough to distinguish between endogenous- and recombinant somatotropin, and therefore monitoring abuse of recombinant somatotropin is not possible. More recently some methods, which are based on mass spectrometric detection, have been described to discriminate between the endogenous- and recombinant form of somatotropin (Chang et al. 1997; Rochut et al. 2000; Pinel et al. 2004). However, most of these methods use academic solutions. An exception is the method described by Blokland, in which intact recombinant- and endogenous porcine somatotropin at relevant serum concentrations were detected using mass spectrometry (Blokland et al. 2003). In addition, Pinel et al, described a method based on detection of the N–terminal peptide of recombinant somatotropin in milk (Pinel et al.

2005). This research was focused on extending these studies s in order to improve detection limits to detect somatotropin misuse in cattle.
The fact that somatotropin is a protein requires a different analytical approach compared with the "conventional" small molecules in the residue field. The expected concentrations of somatotropin in serum are in the low abundance range according to the Anderson proteome ladder (Anderson et al. 2002) and therefore difficult to detect. Moreover, plasma samples are highly complex samples, which contain large quantities of various proteins and the dynamic range of serum proteins is very large, over 5 orders of magnitude. Sample extraction techniques, such as solid phase extraction (SPE) and liquid-liquid extraction, which are often used to specifically isolate small molecules from serum, can not be applied to extract proteins from serum. Therefore, different techniques need to be applied to enable detection of somatotropin at low serum levels. Here we apply analytical methods from the proteomics research field in order to develop a method to detect somatotropin misuse in cattle.

**Experimental**

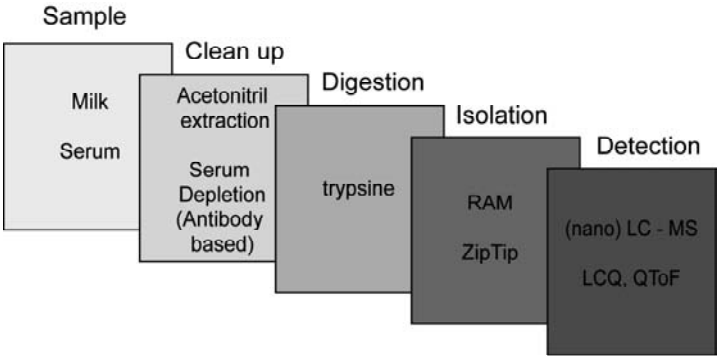


**Figure 1:** experimental strategy used for the detection of recombinant bovine somatotropin in different biological matrices.

Nanoflow liquid chromatography was as described by Meiring at al (Meiring et al. 2002). Briefly, peptides were analyzed by nanoflow liquid chromatography using an Agilent 1100 HPLC system comprising of a solvent degasser, a binary pump, and a thermostated autosampler, coupled online to a LCQ Deca XP (Thermo) or a micrOTOF-Q mass spectrometer (Bruker Daltronic). Aqua C18, 5 µm, (Phenomenex, Torrance, CA) resin was used for the trap column, and ReproSil-Pur C18-AQ, 3 µm, (Dr. Maisch GmbH, Ammerbuch, Germany) resin was used for the analytical column. Peptides were trapped at 5 µl/min in 100% solvent A (0.1 M acetic acid in water) on a 2 cm trap column (100 µm internal diameter, packed in house) and eluted to a 20 cm analytical column (50 µm internal diameter, packed in house) at about 150 nl/min in a gradient from 0 to 60% solvent B (0.1 M acetic acid in 8/2 (v/v) acetonitrile/water). The column eluent was sprayed directly into the ESI source of the mass spectrometer via a butt-connected nano-ESI emitter (New Objectives, Woburn, MA). Mass spectrometry experiments for the specific detection of bovine recombinant somatotropin were performed in MRM mode. The four most abundant ions in the MS2 spectrum of the N-terminal peptide were used.

**Results**

In the work presented here, techniques that are used in the proteomics research field, such as tryptic digestion, nanoflow liquid chromatography and mass spectrometry, were applied to detect the presence of recombinant bovine somatotropin in biological relevant matrices (Fig 1). There are various forms of recombinant somatotropin available on the market. Here, we chose to use recombinant bovine somatotropin from Monsanto (also known as Posilac ®) as a model compound. The detection of recombinant bovine somatotropin was based on the detection of the tryptic N-terminal peptide (MFPAMSLSGLFANAVLR), since this peptide differentiates the endogenous- from the recombinant form of the protein. Recombinant somatotropin was digested with trypsin and the resulting peptides were analyzed using mass spectrometry (Fig 2).

First of all, optimalization of tryptic digestion conditions of recombinant bovin somatotropin in solution was performed. It was observed that the N-terminal peptide was not stable under standard tryptic digestion conditions, e.g. 2M urea in 100 mM ammoniumbicarbonate (Fig 3A). After prolonged digestion times, it was observed that the N-terminal peptide totally disappeared, while other peptides from somatotropin were still present. By using a commercial available buffer that contains a mixture of urea, thiourea, and C7BzO detergent, the digestion efficiency was optimized. Moreover, digestion times were reduced from 12 hours to 1 hour. Within an hour somatotropin was totally digested. In addition, under these conditions the N-terminal peptide was stable for a long period of time (Fig 3B).

Next, a multiple reaction monitoring (MRM) method was developed on a LCQ Deca XP (ion trap) mass spectrometer, to detect recombinant bovine somatotropin. An MS2 spectrum of the doubly charged N-terminal peptide from bovine recombinant somatotropin (m/z = 913.5) is shown in Fig 2C. The amino acid sequence of the peptide can be deduced from this MS2 spectrum. The four highest fragment ions were used for the specific MRM method.

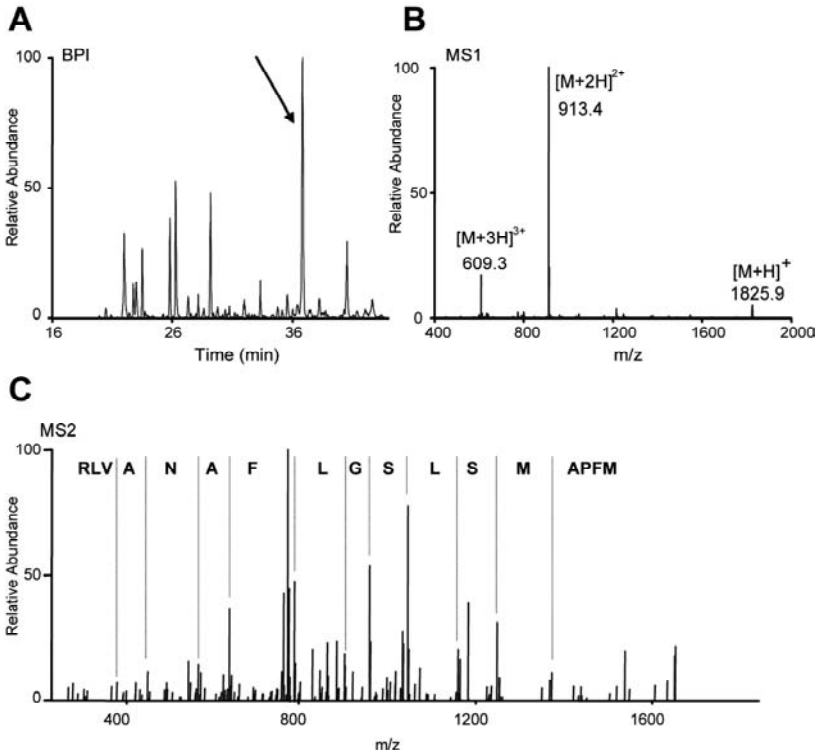


**Figure 2:** Nano LC MS analysis of a tryptic digest of recombinant bovine somatotropin. (A) Base peak chromatogram. The elution of the N-terminal peptide MFPAMSLSGLFANAVLR is indicated with an arrow. In (B) the MS1 spectrum of MFPAMSLSGLFANAVLR is shown, while in (C) the MS2 spectrum of the double charged precursor at m/z 913.5 is shown. The amino acid sequence is indicated in the MS2 spectrum (on the basis of Y" ions).

By using convential LC in combination with this MRM (e.g. 2.1 mm C18 column) a detection limit of 5 ng recombinant bovine somatotropin.on column was achieved. The expected concentration of bovine somatotropin in plasma and milk is 10 ng/ml and 1 ng/ml, respectively. A higher sensitivity is needed to improve detection of somatotropin in biological matrices at these very low levels. Therefore a nanoflow liquid chromatography system was coupled online with the LCQ mass spectrometer. Peptides were separated on a 50 μm ID C18 column at flow rates of about 150 nl/min. This resulted in subpico mole detection limits of recombinant somatotropin in academic solutions, an improvement of a factor of more than 100 (Fig 4).

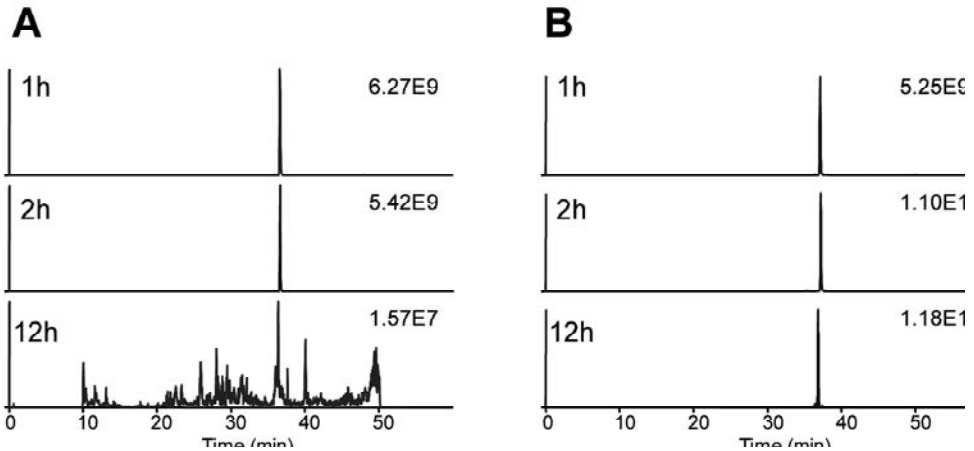


**Figure 3:** Stability of the N-terminal peptide under different digestion conditions. The extracted ion chromatogram of the N-terminal peptide (2+, m/z 913.5) is shown under (A): standard digestion conditions and under (B) optimized digestion conditions, e.g. a mixture of urea, thiourea, and C7BzO detergent. Different digestions times are shown, e.g. t= 1, 2 and 12 hours. Intensity levels of the base peak are shown in the chromatograms.

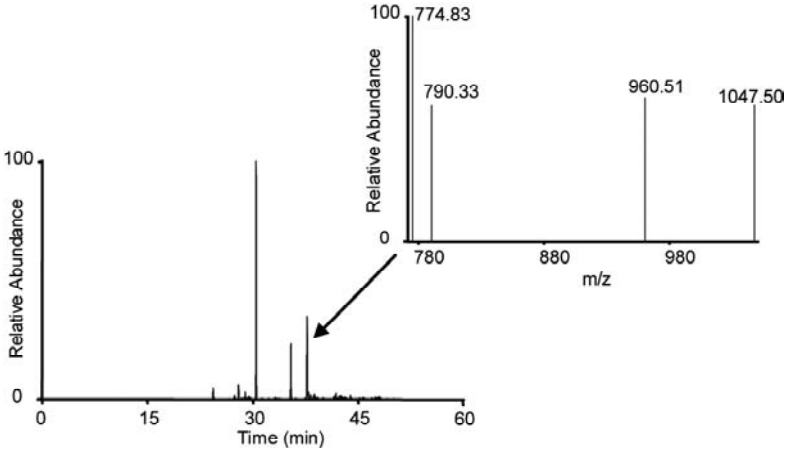


**Figure 4:** Detection of recombinant somatotropin in serum. Chromatogram of serum spiked with recombinant somatotropin which was cleaned up by a general acetonitril extraction method.

The next step was to develop a method for extraction, purification and concentration of somatotropins from complex biological matrices, like serum and milk. Different strategies were tested. First a general method to remove large proteins from serum, e.g. acetonitril extraction was applied. Also a more specific immunodepletion method, to deplete the most abundant proteins using affinity chromatography from serum was tested. In Fig 4 a chromatogram is shown of serum spiked with recombinant somatotropin which was cleaned up using the general acetonitril extraction. The goal of all these methods was to reduce the

sample complexity of serum and to enrich for somatotropin. Moreover, different sample clean up methods, such as ZipTip clean up, restricted access material (RAM) chromatography after tryptic digestion will be tested in the near future.

**Conclusions**

In this study we have developed a method for the detection of recombinant bovine somatotropin. By applying different techniques from the proteomics field, we were able to unambiqiously identify recombinant somatotropin at subpicogram levels. In future, we will continue this work by adding a stable isotope labelled peptide N-terminal for absolute quantification of somatotropin in biological samples.

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## O 18

# THE STATE OF AND UPDATE ON COMMISSION DECISION 2002/657/EC

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**Abstract**
Commission Decision 93/256/EEC laid down its re-examination before 1 January 1996 in order to take account of developments in scientific and technical knowledge (art. 4). The elaboration of the revision was a long process starting in 1996 and ending with the formation of an EC working group in 1998 consisting of 12 members from laboratories of different Member States, mainly from CRL, NRL and other official laboratories. This working group produced a draft version of the revision of CDs 93/256/EC and 93/257/EC within 6 months. The revised version includes completely new ideas on the identification of analytes and the criteria for performance assessment as well as on validation procedures. In the following important aspects of this Decision as well as its development and even a change in meaning and interpretation are described. This contribution is written on behalf of the three CRL for residues involved in the analysis of veterinary drugs.

**Intention of the CD 2002/657/EC**
The goal of this Decision as well as of its predecessors was the establishment of criteria for method assessment in the field of residue control of material from animal origin, which was a very modern approach in contrast to the use of standardised methods. This criteria approach for the first time allowed the continuous application of new methods and analytical techniques and instruments and with this to follow scientific developments more easily. Laboratories were enabled to use new techniques and to apply their own methods. This also led to the introduction of in-house validation, i. e. the verification of the fitness for purpose of individual methods, which in turn needs the fixing of criteria for performance assessment. This approach was first realised with Commission Decision 87/410/EEC and then continuously updated.
CD 93/256/EC still differentiated between routine and reference methods, which was overcome with CD 2002/657/EC.
During the elaboration of the Decision 2002/657/EC several other new aspects were put to discussion, e. g. the identification of the analytes to be determined. In the old legislation several criteria were named to be fulfilled for identification purposes, a. o. the retention time and relative retention time of substances, the use of internal standards and spectra of different analytical techniques (UV, mass spectrometry). Furthermore, when (low resolution) mass spectrometry was applied, it was laid down that at least 4 ions with a particular ratio had to be present when a result was to be claimed positive.
However, no differentiation was made in the application of different techniques for the different substance groups laid down in Council Directive 96/23/EC, Annex I. This was

realised for the first time in CD 2002/657/EC.
Furthermore, as regards quantitative assessment of methods performances, extensive requirements on accuracy (precision and trueness/recovery) existed.
Nevertheless deeper considerations concerning the application of the criteria approach led to the problems of firstly, how to give further advise with regard to positive declarations ("when is a result non-compliant?") and secondly, how to give reasonable advise on how to validate a method, since validation procedures considerably influence the quality of the results of the validation parameters. All members of the EC working group agreed that a precise recipe should be provided to reach a certain harmonisation among the laboratories and a comparability of the individual methods performances. This was a very wise decision, since it led to intensive discussions, misunderstandings which had to be solved, new views and deeper insights into existing problems. All in all it was a fruitful process which is still ongoing, as several additional guidelines and papers show.
In the following some of the most important aspects will be discussed.

**Decision Limit - MRPL**
When is a sample or the positive result of an investigated sample non-compliant? - A question which has preoccupied the residue control for a long time, if not ever since. The question not only comprises the determination of the precision of a measurement result but also the way of how to use this precision information and how to achieve that the precision information is determined in a harmonised manner in all laboratories.
Legislation very often requires the validation at the lowest concentrations possible to achieve. Consequently it happened that laboratories did not know the method's precision at the level of interest (e.g. MRL). However, the knowledge of the precision at the level of interest is the basis for further considerations on how to assess a positive result. Therefore the CD 2002/657/EC requires the validation at the level of interest. But what is the level of interest for methods which are developed to control banned substances (zero-tolerance)? For this reason the term **MRPL** (minimum required performance limit) was established. It was meant as a level to be used for validation purposes. That means each official laboratory in residue control has to be able to validate at this level. The decision limit (**CCalpha**) should be lower than the MRPL. The detection capability (CCbeta) should lower than or equal to the MRPL (SANCO 2726/2005). In the meantime, the MRPL has got a totally different meaning as defined in the Import Decision 2005/34/EC, Art. 2. Here the MRPL mutated to a Reference Point for Action for imported goods. It is the concentration from which on further action is required, i. e. destruction or re-dispatching. There are four substances /substance groups for which MRPL are formally established, namely chloramphenicol, medroxy progesterone acetate and nitrofuran metabolites (Commission Decision 2003/181/EC) as well as malachite green and leuko malachite green (Commission Decision 2004/25/EC). Additionally a so-called "gentleman agreement" exists among the Member States that the import decision should also be applied to the single market. This was agreed upon at the Standing Committee on the Food Chain and Animal Health, Biological Safety, on 21 September 2004. It states "… that any detection of substances whose use is prohibited or not authorised in the Community shall be followed by an investigation into the source of the substance in question and appropriate enforcement measures in particular aimed at the prevention of reoccurrence in the case of documented illegal use."

Notwithstanding, according to CD 2002/657/EC, Art, 6, the decision limit (CCalpha) is still the limit at or above which a result is non-compliant. The term CCalpha denotes a critical concentration concerning the alpha error. It is calculated by taking the level of interest (MRL, or lowest spike concentration in case of banned substances) and adding the 1.64 or 2.33 -fold of the in-house reproducibility standard deviation. With this the measurement uncertainty at the level of interest is taken into consideration and the positive or non-compliant decision is put on a scientifically sound and accepted basis as acknowledged in EURACHEM/CITAC Guide 2007 on "Use of uncertainty information in compliance assessment". So here, with the publishing of CD 2002/657/EC, for the first time an approach was provided allowing the inclusion of a measurement uncertainty for compliance assessment of measurement results which was already determined during the validation procedure.

**Performance Criteria**

For the establishment of performance criteria the methods were divided into screening and confirmatory methods.

For screening methods the only requirement for a technique applied in official residue control was the compliance with the maximum false negative rate of 5%.

Confirmatory methods had to fulfil many more requirements, e. g. the typical parameters like recovery/trueness, precision, specificity and application, the newly introduced parameters CCalpha and CCbeta and the criteria for identification. Furthermore particular techniques were fixed with particular specificities for different usages. This will be explained in more depth in the next chapter.

Concerning the precision of the measurements, in principle the ranges according to Horwitz were to be applied again. However, for concentrations below 10 µg/kg they were not found acceptable any longer. Unfortunately there were no widely accepted other approaches for limiting precision ranges at the time when the Draft was produced. Therefore it was agreed upon to demand that reproducibilities below 100 µg/kg should be as low as possible, but in any case lower than the Horwitz values.

The biggest problem was caused by the introduction of CCalpha and CCbeta. Analysts had to become used to these new limits which are no longer limits laying down the lowest levels for detection and determination, but limits from which on non-compliant decisions can be made having already taken into account the measurement uncertainty at the level of interest. That means that for confirmatory methods for MRL substances, CCalpha and CCbeta are not at the zero concentration level but above the MRL. The limitation of the maximum allowed range between MRL and CCalpha is determined by the limitations applying for in-house reproducibilities.

**Identification Point System**

For the first time it was taken into account that methods for group A substances (banned substances) had to fulfil stricter requirements than methods for group B substances (MRL substances) because of the more severe legal consequences in case of non-compliance. Hence it was decided to introduce an identification point system. Methods for group A substances have to gain 4 points, whereas methods for group B substances only need three points. Furthermore group A substances are to be measured exclusively by mass spectrometric techniques coupled to GC or LC systems. Group B substances may also be detected by means

of other spectrometric techniques like DAD, fluorescence, ECD and others under certain conditions. In table 5 of CD 2002/657/EC different ways of how to earn the respective points are listed. For example, low resolution mass spectrometry (LR-MS) earns 1 point per ion, whereas high resolution mass spectrometry (HR-MS) earns 2 points per ion. Therefore one needs 4 ions when LR-MS is applied and only two when HR-MS is applied. This is only one example and several other combinations are possible.
As regards the group B substances where also other than MS techniques are permitted, the allowed techniques per se earn 3 points, e. g. DAD, fluorescence spectra (cf. table 1 of CD 2002/657/EC), as long as specific requirements are met.
The introduction of the identification point system was a big step towards more clarity for the user/operator. Nevertheless, in the meantime new techniques have been developed which were not accounted for in the Decision, like ion trap techniques or TOF combinations. Here further considerations are required.

**Validation**
The harmonisation of validation procedures was the principle aim of chapter 3 of CD 2002/657/EC.
Two general approaches were described. The first one is the classical validation which requires the analysis of six replicates at three concentrations and three points of time. It should be applied at or around the level of interest. The initial validation usually comprises validation for the primary matrix to be analysed. Other matrices, or identical matrices from other species, can be added to the set of validated analyte-matrix combinations by additional validation ("major change"). Though the applicability of this approach has been demonstrated, a fact is that this approach only allows the validation of one "major change" at any one time, which is considered a drawback. Therefore a second approach is described, the alternative validation. By using statistical experimental plans it allows for the validation of different "major changes" in one go as long as the method is robust enough.
Both approaches have been put to discussion very frequently and many publications have dealt with this topic, so that it does not seem necessary to start the explanations again. It should only be mentioned that there were approx. 12,600 hits in google when "2002/657" was entered (Dec. 2007), 3,000 of which were dealing with "validation" and 140 with "Validierung". The inquiry in the ISI web of knowledge resulted in 147 hits with regard to "2002/657"and "validation". The evaluation of approx. 50 peer-reviewed publications showed very clearly that even such a detailed description of validation procedures as provided in CD 2002/657/EC gives rise to uncountable interpretations. Nevertheless one can say that the original goal, namely the harmonisation of laboratory performances, was advanced since the possible variations in validation were limited to a certain extent.

**Additional Regulations**
The CD 2002/657/EC has never been revised since its publication. Nevertheless some guidelines for additional clearness were published, e. g. SANCO/2004/2726 (topics: recovery correction, use of quality control samples, clarifications concerning validation, parallel extrapolation), SANCO/2004/2726rev. 1 (topics: measurement uncertainty, validation of methods for substances with sum-MRL) and SANCO/2004/2726rev. 2 (topics: guidelines for the implementation of CD 2002/657/EC regarding some contaminants).

Furthermore the definition of the MRPL has changed thanks to the Import Decision 2005/34/EC as mentioned above. Since at present it is not the wish of the Commission to establish further MRPL, but at the same time there are still several banned substances without MRPL which are, according to CD 2002/657/EC, necessary to be able to validate in the correct concentration range, a further term was created: recommended concentrations. Within three years of extensive work especially for each CRL but also for all the NRL and the Commission, a list was produced with recommended concentrations below which the validation of banned substances should start. This list is not an official list of the Commission but a recommendation of the three CRL for residues, and is therefore published on their websites.
Finally it is important to mention that in 2007 the Commission decided with SANCO/2004/2726rev.2 that the requirements of CD 2002/657/EC shall not apply to mycotoxins, dioxins and dioxin-like PCB, to heavy metals like lead, cadmium, mercury and inorganic tin and to 3-MCPD.

**Outlook**

Further support has to be given to NRL and routine laboratories. Therefore at the moment guidelines are being elaborated on how to validate screening methods and how to assess positive results for substances which have sum-MRL.
In connection with the discussed revision of Council Directive 96/23/ the Commission has decided that the requirements and criteria laid down in CD 2002/657/EC should not be applied to pesticides and contaminants (group B3 substances) any longer.
It can be predicted that several other aspects will rise and will have to be clarified. Suggestions are always welcome.

**Acknowledgement**

I wish to thank all colleagues who contributed to the elaboration, development and interpretation of and led discussions on this Decision. They are so numerous that they cannot be named by far.
The European Commission is gratefully acknowledged for financial support.
Last but not least I thank my colleagues from the two other CRL for residues, Leen van Ginkel, CRL Bilthoven, NL, and Pascal Sanders, CRL Fougères, FR, for reading and criticizing my manuscript.

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## O 19

# A DETERMINATIVE AND CONFIRMATORY METHOD FOR RESIDUES OF THE METABOLITES OF CARBADOX AND OLAQUINDOX IN PORCINE TISSUES.

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**Abstract**
Carbadox (CBX) and olaquindox (OLQ) are used in swine feed for growth promotion, to improve feed efficiency, increase the rate of weight gain, control dysentery and bacterial enteritis in young swine. In 1991, JECFA recommended MRLs of 30 and 5 μg/kg in liver and muscle tissues of pigs, based on the concentration of, and expressed as , QCA as marker residue. In 1998, the EC banned the use of CBX and OLQ in food animal production together with 4 other feed additives, following reports that CBX and DCBX are suspect carcinogens and mutagens. In 2001, the sale of CBX was halted in Canada. In 2003, JECFA recommended the withdrawal of the previously recommended ADI and MRLs and concluded that QCA was not the suitable marker residue for CBX, based on new sponsor studies reporting the DCBX, the suspected carcinogen, persisted in animal tissues much longer than had been previously thought. This paper presents a method that was developed by CFIA scientists for the simultaneous determination and confirmation of DCBX at concentrations ≥ 0.050 ppb, and QCA and MQCA at concentrations ≥ 0.50 ppb in bovine muscle, pork liver and muscle tissues.

**Introduction**
Carbadox(CBX) and olaquindox(OLQ) are antimicrobial drugs used in swine feed for growth promotion, improve feed efficiency, increase rate of weight gain and control dysentery and bacterial enteritis in young swine. These drugs have been shown to metabolize rapidly in vivo into the desoxy and quinoxaline-2-carboxylic acid (QCA) products. In 1991, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at its 36$^{th}$ meeting recommended maximum residue limits (MRLs) of 30 and 5 μg/kg in liver and muscle tissues of pigs, respectively, based on the concentration of, and expressed as, QCA. QCA, which is non-carcinogenic, has been used as the marker residue to regulate the use of CBX in swine for many years. In 1998, the European Commission banned their use in food animal production as antimicrobial growth promotants (AGPs) in feed following reports that CBX and its desoxy metabolite, desoxycarbadox (DCBX), are suspect carcinogens and mutagens.
With the ban by the EU, countries like Canada where CBX was still an approved veterinary product, had to demonstrate to the EU that its meat products for export to the EU were free from residues of CBX, OLQ and their metabolites. In a partial response to this requirement, Lau et al. developed an LC-MS/MS method for the determination of CBX and DCBX residues in animal feed and pork liver with detection limits in the low ppb range. The method was, however, unable to extract QCA, the marker residue. Based on new sponsor studies new

reports that DCBX persisted in animal tissues much longer than had been previously thought, Canada requested the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) to review the MRLs set in 1991 for CBX by JECFA. The matter was referred to the 60th JECFA which met in 2003 and recommended the withdrawal of the previously recommended MRLs. This paper describes a very sensitive method developed for the determination of DCBX at concentrations ≥0.050 ng/g of muscle tissue and the two non-carcinogenic metabolites of CBX and OLQ, namely QCA and methylquinoxaline-2-carboxylic acid (MQCA) at concentrations ≥0.50 ng/g in animal tissues.

*Materials and Methods*

All reagents were of analytical grade. Standards for Desoxycarbadox, MQCA, QCA and QCA-d4 were obtained from the sources indicated in Table 1.

**Table 1.** Reference standard sources and information required to prepare standard stock solutions of DCBX, QCA, MQCA and QCA-d4

| **Standard** | **Source** | **Amount to be weighed** | **Concentration in 50 mL of solution** |
|---|---|---|---|
| 1,4 Bis-Desoxycarbadox (DCBX) | RIVM | 2.14 | 42.8* |
| 3-Methylquinoxaline-2-carboxylic acid (MQCA) | Univ of Rennes, France | 5.0 | 100.0 |
| Quinixaline-2-carboxylic acid (QCA) | Aldrich | 5.0 | 100.0 |
| Quinoxaline-2-carboxylic acid deuterated (QCA-d4) | RIVM, The Netherlands | 0.106 | 2.12* |

** Note 1: Concentrations provided by Source.*

*Sample extraction and cleanup*

Weigh six, 5.00 ± 0.05 g each of thawed, drug-free homogenized liver or muscle tissue into 6 labelled 50 mL centrifuge tubes and fortify 5 of the weighed samples with DCBX at 0.05, 0.10, 0.15, 0.25, and 0.50 ng/g. Fortify the same 5 samples with QCA-MQCA standard solutions to provide concentrations at 0.50, 1.0, 2.5, 5.0, and 10.0 ng/g. Do not fortify the 6th sample and leave as negative control. Weigh also, the test samples of interest. Let samples sit for 10 min. The samples are digested with 0.6% formic acid to deactivate naturally-occurring enzymes.

Place samples all samples in water bath at 47 ± 3 °C for 1 hour. After acid digestion, remove from water bath and add 3 mL Tris solution (1.0 M) and vortex mix. Add 1 mL of protease solution and vortex mix. Place in water bath at 47 ± 3 °C for 16 to18 hours. After acid digestion, remove from water bath, and add 20 mL hydrochloric acid (0.3 M) to each sample. Shake at high speed for 5 min. and centrifuge and 3200 x g for 15 min at 20 °C.
After overnight enzymatic hydrolysis with protease, the tissue extract is acidified with 0.3 M HCl, centrifuged and filtered. DCBX, QCA and MQCA are recovered from the tissue extract by using an Oasis MAX solid phase extraction (SPE) cartridge.

Condition an Oasis MAX cartridge (Waters Chromatography) by passing 3 mL methanol followed by 3 mL water. Load the sample extract onto the conditioned column and wash with 30 Ml sodium acetate/methanol (95/5 v/v). Dry the cartridge for at least 15 minutes. DCBX is eluted from the SPE cartridge first (Fraction 1) into a 15 mL glass test tube containing 100 µL of QCA-d4 (0.1 µg/mL) with 4 x 3 mL methylene chloride. The cartridge is washed with different solvents after which QCA and MQCA are eluted (Fraction 2) with 3 mL 2% formic acid in ethyl acetate into a 10 mL test tube containing 100 µL of QCA-d4 (0.1 µg/mL). The two fractions are evaporated to dryness and reconstituted in methanol and water, and filtered through a 0.2 µm PTFE (polytetrafluoroethylene) Acrodisc CR 13mm syringe filter into an HPLC sample vial. The concentrations of DCBX, QCA and MQCA in the two fractions are determined and confirmed by LC-MS/MS.

*LC-MS/MS system*
A Waters Chromatography 2695 Separations Unit interfaced to a Micromass Quattro Micro Triple Quadrupole Mass Spectrometer (Micromass Canada) equipped with an electrospray ionization source (ESI) through a Z-spray interface. Analytical separation was achieved on a Nova-Pak $C_{18}$ 60 Å, 4 µm 2.1 x 150 mm column (Waters WATO23655) preceded by a Metaguard 2.0 mm Inertsil 5 µm $C_8$ guard cartridge (Varian), and controlled by MassLynx 4.0 software.

The Mass spectrometer was operated in the positive ESI mode with a capillary voltage of 2.75 kV, cone voltage of 27 V, source temperature at 100°C with a desolvation temperature and gas flow rates at 150°C and 450 L/h, respectively, and a cone gas flow rate of 50 L/h. The multiplier was set at 989 V and Argon was operated at a pressure of $4.12e^{-3}$ mbar.

The HPLC was operated at a flow rate of 0.2 mL/min in the LC but split at the end of the column (held at 40°C) to permit 80 µL/min flow into the MS.

Table 2 shows the gradient conditions for the analysis of DCBX while Table 3 shows the conditions used for the analysis of QCA and MQCA.

**Table 2.** Gradient conditions for the analysis of DCBX

| **Time (min)** | **Solvent A (%)** | **Solvent C (%)** | **Gradient Profile** |
|---|---|---|---|
| 0.00 | 80 | 20 | 1 |
| 20.00 | 30 | 70 | 6 |
| 23.00 | 30 | 70 | 1 |
| 23.01 | 80 | 20 | 1 |
| Solvent A: 0.04% Formic acid; Solvent C: Methanol | | | |

**Table 3.** Gradient conditions for the analysis of QCA and MQCA

| Time (min) | Solvent B(%) | Solvent C (%) | Solvent D (%) | Gradient Profile |
|---|---|---|---|---|
| 0.00 | 2 | 8 | 90 | 1 |
| 20.00 | 10 | 40 | 50 | 6 |
| 20.01 | 16 | 64 | 20 | 1 |
| 23.01 | 2 | 8 | 90 | 1 |
| Solvent B: Acetonitrile; Solvent C: Methanol; Solvent D: 0.3% formic acid | | | | |

*Quantitative analysis*

The Limit of Quantification (LOQ) was determined as the concentration equivalent to 10 x S/N ratio in the matrix-matched blank. Quantitative analysis was conducted based on a matrix fortified calibration curve in which the relative response ratio of the ion abundance (peak area) of the DCBX peak from its most abundant product ion transition 231 > 143 to that of the external standard QCA-d4, 179 > 133. For quantitative analysis of QCA and MQCA, response ratios based on the most abundant product ion transitions 175 > 129 and 189 > 143, respectively, were measured to that of QCA-d4, 179 > 133.

*Confirmatory analysis*

The Limit of Confirmation (LOC) was determined as the concentration at, and above which the confirmation criteria is met with 95% confidence. Ideally, this concentration should be at or below the LOQ. Confirmation for
the presence of DCBX, QCA and MQCA was considered to have been achieved if the retention time of DCBX, QCA and MQCA peak in an unknown sample matches that of the matrix-matched standard run under the same experimental conditions to within ± 2.5%. In addition, all the

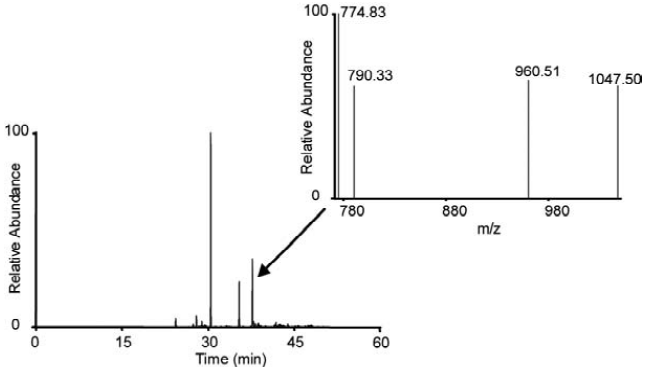


**Figure 1.** Typical MRM chromatogram of a negative control pork liver extract containing 0.20 ppb of the external standard QCA-d4 showing the primary and secondary ion transitions monitored for the determination and confirmation of DCBX.

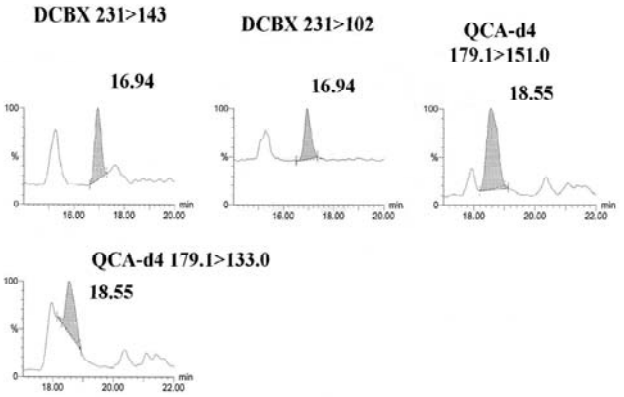


**Figure 2.** Typical MRM chromatogram of a fortified negative control pork liver extract containing 0.075 ppb of DCBX and 0.20 ppb of the external standard QCA-d4 showing the primary and secondary ion transitions monitored for the determination and confirmation of DCBX.

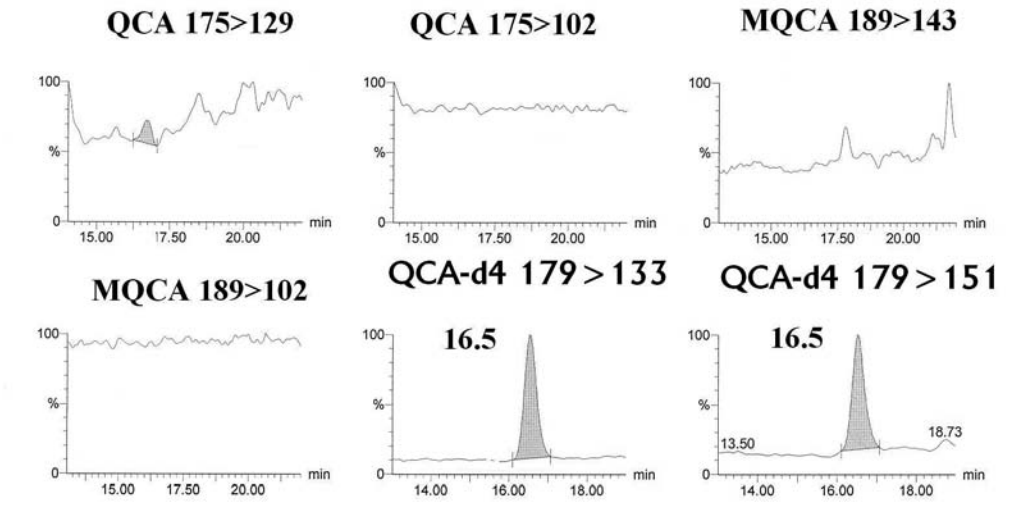


**Figure 3.** Typical MRM chromatogram of a negative control pork liver extract containing 0.20 ppb of the external standard QCA-d4 showing the primary and secondary ion transitions monitored for the determination and confirmation of QCA and MQCA, the metabolites of CBX and OLQ.

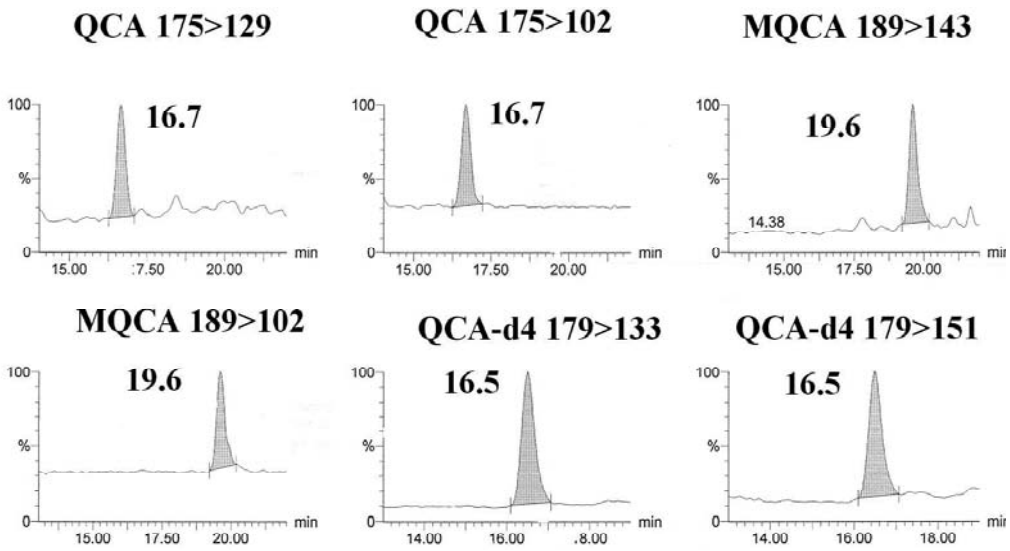


**Figure 4.** Typical MRM chromatogram of a fortified negative control pork liver extract containing 0.75 ppb of QCA and MQCA and 0.20 ppb of the external standard QCA-d4 showing the primary and secondary ion transitions monitored for the determination and confirmation of QCA and MQCA.
product ion transitions including the precursor ion listed in Table 4 are present and the product ion ratios agree within tolerance to what has been specified in the Table 5.

**Table 4a.** Precursor ions and product ion transitions used for the quantitative and confirmatory analysis of DCBX residues.

| Retention time (min) | Precursor ion (m/z) | Ion Transitions | Collision Energy (eV) | Scan Window (min) |
|---|---|---|---|---|
| DCBX<br>16.5 | 231.0 | 231.0>143.0<br>231.0>102.0<br>231.0>171.0 | 20<br>35<br>15 | 15.0 – 19.0 |
| QCA-d4<br>18.9 | 179.1 | 179.1>151.0<br>179.1>133.0 | 15.0<br>20.0 | 14.0 – 17.0 |

**Table 4b.** Precursor and product ion transitions used for the quantitative and confirmatory analysis of QCA and MQCA residues.

| Retention time (min) | Precursor ion (m/z) | Ion Transitions | Collision Energy (eV) | Scan Window (min) |
|---|---|---|---|---|
| QCA<br>16.5 | 175.0 | 175.0>129.0<br>175.0>102.0 | 20<br>30 | 15.0 – 17.0 |
| MQCA<br>19.6 | 189.0 | 189.0>143.0<br>189.0>102.0 | 15<br>35 | 18.0 - 20.0 |
| QCA-d4<br>18.9 | 179.1 | 179.1>151.0<br>179.1>133.0 | 15.0<br>20.0 | 14,0 – 17.0 |

Validation of method and its application in an investigational study

The method was validated (Table 6)and demonstrated to be fit for purpose and was then applied to the analyses of these drugs in a herd of 936 barbecue pigs that had been mistakenly administered carbadox. QCA was detected in a few of the animals that had been sent to slaughter. The herd was, therefore, detained pending further investigation. In May 2004, samples of diaphragm and liver from two randomly selected hogs from each of 3 units (Unit 1 had 155 hogs, Unit 2 had 478 hogs and Unit 3 had 303 hogs) from the suspect herd were delivered to the laboratory. By the time the laboratory received the samples, it was two weeks after the detained hogs had attained final the market weight. All the diaphragm and liver samples were analysed for DCBX, QCA and MQCA residues to determine whether these animals were safe to be released for domestic consumption. Diaphragms from two of the hogs confirmed positive for DCBX at concentrations greater than the LOQ for DCBX; there were, however, no detectable concentrations of DCBX, QCA or MQCA in any of the livers tested. Additionally, no QCA or MQCA residues were detected in any of the samples tested. On June 2nd, the laboratory received another set of 6 pairs of livers and diaphragms obtained from randomly selected pigs in the detained herd. This time, all samples tested negative for all the analytes of interest.

**Table 5.** Product ion transition ratios used for the confirmatory analysis of DCBX, QCA and MQCA residues in tissues and standards.

| Analyte | MRM Product Ion Transition | Chemical Standard | Pork Liver | Pork Muscle | Beef Muscle |
|---|---|---|---|---|---|
| DCBX | 231>143.0<br>231.102.0 | 3.73±0.73 | 3.69±1.30 | 3.36±0.55 | 3.44±0.42 |
| QCA | 175.0>129.0<br>175.0>102.0 | 2.27±0.10 | 2.25±0.26 | 2.22±0.19 | 2.22±0.18 |
| MQCA | 189.0>143.0<br>189.0>102.0 | 4.21±0.39 | 4.21±0.42 | 4.23±0.39 | 4.24±0.29 |

**Table 6.** Characteristic Operational parameters for the determinative and confirmatory method

| Parameter | DCBX | QCA | MQCA |
|---|---|---|---|
| Analytical range (ng/g) | 0.050 – 1.00 | 0.50 – 10.0 | 0.50 – 10.0 |
| LOQ | 0.050 | 0.50 | 0.50 |
| LOC | 0.025 | 0.30 | 0.30 |
| Precision | < 15% | <15% | <15% |
| Selectivity | Yes | Yes | Yes |
| Accuracy | <20% | <20% | <20% |
| Ruggedness | Yes | Yes | Yes |
| Analyte stability in matrix under frozen storage conditions | Stable for at least 63 days at -20°C | Stable for at least 63 days at -20°C | Stable for at least 63 days at -20°C |
| Analyte stability in matrix extract | Stable for at least 48 h under ambient conditions | Stable for at least 48 h under ambient conditions | Stable for at least 48 h under ambient conditions |

**Discussion**

Our findings with respect to the longer persistence of DCBX, the carcinogenic metabolite, in pork tissue from the barbecue hogs would appear to lend further support to the 60th JECFA report that this compound persists in animal tissue longer than had been previously thought. Additionally, the fact that we were able to detect DCBX residues even in the absence of the non-carcinogenic metabolite, QCA, also appears to lend support to the claim that QCA is indeed not the most suitable marker residue for CBX.

**Conclusion**

This paper describes a very sensitive method for the determination and confirmation of residues of the metabolites of CBX and OLX, namely QCA, MQCA and DCBX in food animal tissues by positive electrospray ionization LC-MS/MS. This quantitative and confirmatory method was validated and demonstrated to be specific, selective and suitable for routine analysis for regulating the use/misuse of CBX and OLQ in food animal production. It has been demonstrated that the non-carcinogenic metabolites QCA and MQCA are not suitable markers for the regulation of the use of these AGPs in food animal production. Rather, it is residues of the carcinogenic and mutagenic metabolite, DCBX, that should be monitored for regulatory control. Therefore, this is the method that Canada is using to enforce compliance to its regulation that bans the use of these AGPs in animal feed to ensure the safety of foods of animal origin to the consumer, and to protect international market access for Canadian meat and meat products.

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## O 20

# VALIDATION AND MULTIVARIATE EFFECT ANALYSIS OF AN LC-MS/MS METHOD FOR THE DETERMINATION OF STEROIDS IN BOVINE MUSCLE

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**Abstract**
Anabolic steroids are banned from use in food-producing animals in the EU (Council Directive 96/22/EC). We developed and validated an LC-MS/MS method for the screening and confirmation of most of the relevant natural and synthetic estrogenic and androgenic steroids in bovine muscle which fulfils the requirements of Commission Decision 2002/657/EC. The method allows to confirm and quantify most of the analytes below 1 µg/Kg. The validation was carried out according to a matrix-comprehensive in-house validation concept. Factors supposed to be relevant for the method in routine analysis were systematically varied on two levels. The decision limit CCα, the detection capability CCβ, the recovery, repeatability, within-laboratory-reproducibility and the uncertainty of measurement were calculated. Furthermore, a multivariate effect analysis was carried out to get an estimation of the influence of the individual factors on the measurement results of the individual analytes.

**Introduction**
In the EU hormonally active substances are banned from use as growth promoters in animal production by Council directive 96/22/EEC. The Regulation (EC) No. 882/2004 and Council Directive 96/23/EC mandate residue testing in animal matrices for these substance groups. Furthermore, the EU legislation requires the validation of methods used for such testing [Commission Decision 2002/657/EC)]. Among the requirements are the determination of the critical concentrations CCα (decision limit), CCβ (detection capability) as well as of the repeatability and in-house reproducibility. In addition, it is indispensable to have some information on the robustness of an analytical method to define its scope of applicability and fitness for purpose. In this respect, it is important to know the extent of the influence of noise factors, which cannot be kept fixed in routine analysis (e.g. different cartridge lots, matrix condition, different animals, different storage time of the extracts before LC-MS/MS measurement, etc.). Here we describe an LC-MS/MS method which allows the detection of most of the relevant natural and synthetic estrogenic and androgenic steroids in bovine muscle. The method was validated in accordance with Commission Decision 2002/657/EC. A matrix-comprehensive in-house validation concept was applied using the software “InterVal” [Jülicher et al. 1998, Jülicher et al. 1999]. “InterVal” uses a fractional factorial design which is based on an orthogonal experimental plan. Additionally, a factorial effect analysis was carried out in order to estimate to what extent the selected experimental factors influence the measurement results of the individual analytes. For this purpose, “Optival”, a software for the optimisation of analytical methods, was used.

## Materials and Methods

The samples of homogenised bovine muscle (10 g) were fortified with standards and internal standards (2 µg/Kg). Subsequently, 10 mL of Tris buffer (pH 8) and 100 µL of Protease XIV solution (50 mg/mL, Sigma, Protease Type XIV from Streptomyces griseus) were added. After hydrolysis at 37°C for 16 h the samples were extracted twice with 30 mL of t-butylmethylether. After evaporation of the organic layers to dryness the residue was redissolved in 2 mL of MeOH/water (80/20, v/v) and subsequently washed three times with 2 mL of n-hexane. Oasis HLB cartridges (Waters, 200 mg, 6cc) were preconditioned with 5 mL of methanol and subsequently with 5 mL of water. The extracts were applied and the HLB cartridges were washed with 5 mL of water and 5 mL of methanol/water (40/60, v/v). The analytes were eluted by adding 5 mL of acetone. Subsequently, aminopropylcartridges (Isolute, 500 mg, 3 mL) were preconditioned with 5 mL of methanol and 5 mL of acetone. The eluates of the HLB cartridges were applied to the aminopropyl cartridges. The eluates were collected, evaporated to dryness and then redissolved in 100 µL of acetonitrile/water (1/1, v/v). The HPLC system was a binary solvent delivery system (Agilent 1100 Series, Agilent Technologies). A Luna C18 column (150 mm x 2 mm, 5 µm; Phenomenex) connected to a Phenomenex C18 pre-column (4 mm x 2 mm) was used. The MS/MS measurements were carried out by means of an API 4000 mass spectrometer in the SRM mode using positive atmospheric pressure chemical ionization (APCI) except for stanozolol which was detected in the positive ESI mode. The method was validated according to Commission Decision 2002/657/EC, chapter 3.1.3 “alternative validation”. A matrix-comprehensive in-house validation approach was applied using the software “InterVal Plus” [Jülicher et al. 1998, Jülicher et al. 1999]. The whole validation study consisted of eight individual experiments. Eight muscle samples from eight different calves (both sexes) were used. The fortification levels for the validation curves were 0.3, 0.4, 0.5, 0.6, 0.8, 1.1 and 1.4 µg/Kg. For matrix calibration, an additional calf muscle sample was used. The additional matrix calibration curves for quantification purposes consisted of equidistant measurement points with concentration levels of 0, 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 µg/Kg. One matrix calibration was carried out per series. The whole in-house validation study was performed within five weeks. For the factorial effect analysis the software “OptiVal” (quo Data GmbH, Dresden, Germany) was used.

## Results and Discussion

*Method development*

As a first step, a hydrolysis using Protease XIV was carried out to free potentially protein-bound steroids. This step is considered to be important when analysing incurred material. The further sample preparation consisted of a liquid / liquid extraction with t-butylmethylether, defattening with n-hexane, purification on Oasis HLB and subsequently on aminopropyl columns. Finally, the extracts were analysed by LC-MS/MS.

*Validation*

For the in-house validation study four different validation factors were chosen and each factor was varied on two levels. To simulate real-life conditions mainly “noise factors” were selected which cannot be controlled in routine analysis but which have a potential influence

on the result. Here two different lots of the HLB and the aminopropyl cartridges, two different operators and two different storage durations of the extracts before LC-MS/MS measurement (0 days, 3 days) were selected as validation factors (Table 1). The InterVal validation consisted of eight different bovine muscle samples spiked on at least five concentration levels. The analyte concentrations of the validation curve samples were calculated by means of matrix calibration. On the basis of these results CCα and CCβ, the repeatability, the within-laboratory reproducibility as well as the recoveries corrected by the use of internal standards and matrix calibration curves (Table 2) were calculated. The corrected recoveries of all analytes were between 95.1% and 104.3%, thus meeting the requirements of Commission Decision 2002/657/EC (-50% to + 20%). The within-laboratory reproducibilities $S_{wR}$ at the CCα level were below 14% for most of the analytes. Only for 17β-testosterone, 17β-trenbolone and ethinylestradiol they were slightly higher. The decision limit CCα was calculated according to a worst-case-scenario [Jülicher et al. 1998, Jülicher et al. 1999]. This means that at the lowest spike level an extrapolation is carried out by means of a parallel projection to the y-axis. Although Commission Decision 2002/657/EC allows a linear extrapolation to the y-axis to calculate CCα a parallel projection to the y-axis is preferred as this is supposed to produce more realistic and reliable values. CCβ is calculated from the power curve [Jülicher et al. 1998, Jülicher et al. 1999]. For most of the analytes the CCβ values are below the "recommended concentration that should be screened and confimed" [CRL guidance paper (7 December 2007)] (Table 2). Only for ethinylestradiol the CCβ value exceeds the 1 µg/Kg level. Here the 0.5 µg/Kg level had to be taken as lowest spike level due to the poorer response of this analyte under the measurement conditions. This fact together with a comparably higher within-laboratory reproducibility of 16.4% leads to a higher value for CCα.

**Table 1:** Experimental design

| **Run** | **1**<br>**lot of HLB cartridge** | **2**<br>**operator** | **3**<br>**storage of extracts** | **4**<br>**lot of NH2 cartridge** |
|---|---|---|---|---|
| S01 | B | Z | 3 d | D |
| S02 | B | Z | direct | D |
| S03 | B | G | 3 d | C |
| S04 | B | G | direct | C |
| S05 | A | Z | 3 d | C |
| S06 | A | Z | direct | C |
| S07 | A | G | 3 d | D |
| S08 | A | G | direct | D |

**Table 2:** Critical concentrations and relative coefficient of variation (CV) of the within-laboratory reproducibility $s_{wR}$

| Analyte | CCα (µg/Kg) | CCβ (µg/Kg) | CV $s_{wR}$ at CCα (%) | recomm. conc.* (µg/Kg) |
|---|---|---|---|---|
| 17α-nortestosterone | 0.481 | 0.612 | 13.9 | 1 |
| 17α-testosterone | 0.430 | 0.537 | 12.3 | 1 |
| 17β-boldenone | 0.422 | 0.544 | 13.8 | 1 |
| 17β-nortestosterone | 0.404 | 0.499 | 10.7 | 1 |
| 17β-testosterone | 0.581 | 0.837 | 19.5 | 1 |
| 17β-trenbolone | 0.467 | 0.631 | 15.6 | 1 |
| ethinylestradiol | 0.788 | 1.101 | 16.4 | 1 |
| methylboldenone | 0.148 | 0.193 | 13.9 | 1 |
| methyltestosterone | 0.551 | 0.686 | 12.1 | 1 |
| stanozolol | 0.300 | 0.383 | 13.2 | 1 |

**" recommended concentrations that should be screened and confirmed" [CRL guidance paper (7 December 2007)]]*

*Multivariate effect analysis*

For the application of an analytical method in routine analysis it is indispensable to have some information on its robustness and scope of applicability as well as on its fitness for purpose. In order to obtain more information on the contributions of the different validation factors to the combined measurement uncertainty we carried out a multivariate effect analysis to estimate the influence of each factor/factor level on the result of each individual analyte. To this end the software "OptiVal" was applied. Also being based on factorial experiments, "OptiVal" allows a targeted evaluation of factorial effects with regard to systematic differences not only concerning recovery but also the random error. On the basis of a generalised linear model a model is automatically established from which an estimation of the influence of the factors on the results of the individual analytes at a mean fortification concentration is possible. Figure 1 depicts four diagrams corresponding to the four validation factors examined. Here the factorial effect in percentage of the measurement result at the 0.7 µg/Kg level per analyte is depicted. The mean spike concentration of approximately 0.7 µg/Kg is set to zero in the graphs. The abscissa depicts the relative deviation from the mean value of all measured values in percentages at the mean spike concentration. It can be seen that the analyte 17β-testosterone is the most affected by the factors "lot of HLB cartridge" and "lot of NH2 cartridge". The deviations from the calculated mean concentration amount to ± 7% and ± 5.5%, respectively. Furthermore, the analyte stanozolol seems to be the most influenced by the factor "operator" (± 4%). The factor "storage of extracts" seems to influence the measurement results of ethinylestradiol and 17β-testosterone the most (± 5% and ± 4%, respectively), whereas the effect of this factor on the other analytes is smaller.

**Conclusions**

We have successfully developed and validated an LC-MS/MS method allowing the determination of anabolic steroids in bovine muscle. The validation of this method was

carried out in accordance with Commission Decision 2002/657/EC applying a matrix-comprehensive in-house validation concept using the software InterVal. The critcal concentrations CCα and CCβ were below the "recommended concentrations that should be screened and confirmed" [CRL guidance paper (7 December 2007)]. Furthermore, the ruggedness of the method was sufficiently proven by a systematic variation of the validation factors on two levels. The multivariate effect analysis showed that the influence of these factors on the measurement results was acceptable. Thus, the method is fit-for-purpose and can be applied in routine analysis.

**Acknowledgements**
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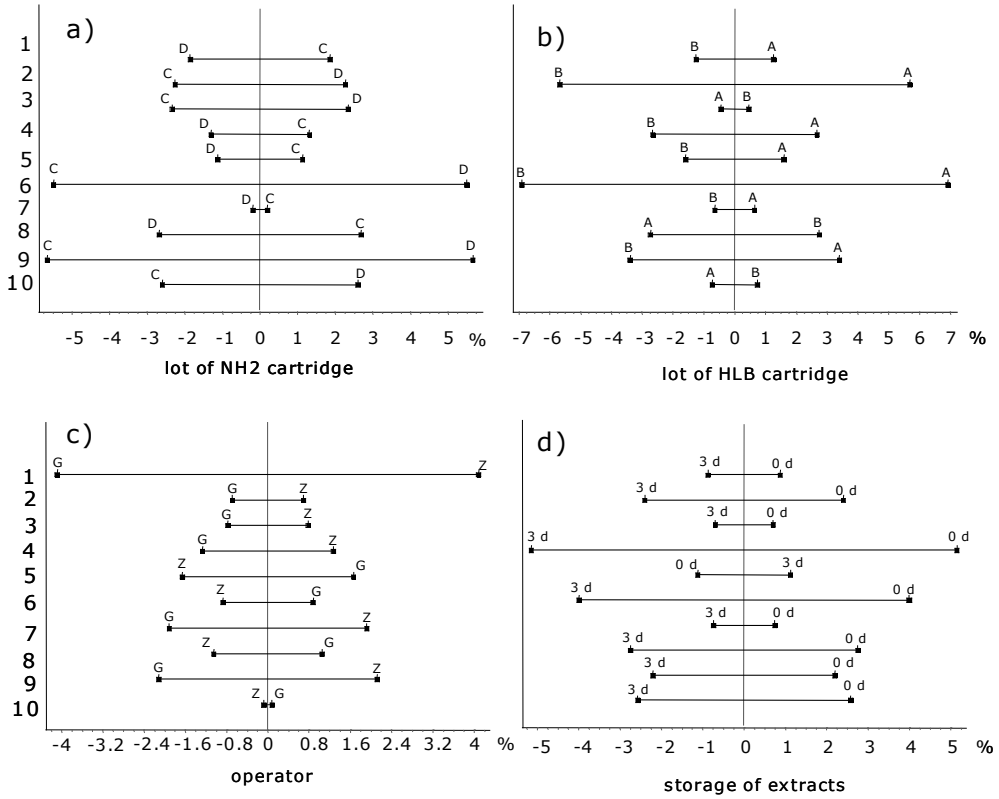


**Figure 1:** Graphical representation of the individual factorial influences per analyte for the factors "lot NH2 cartridge"(a), "lot HLB cartridge" (b), "operator" (c), "storage of extracts" (d), 1: stanozolol, 2: methyltestosterone, 3: methylboldenone, 4: ethinylestradiol, 5: 17β-trenbolone, 6: 17β-testosterone, 7: 17β-nortestosterone, 8: 17β-boldenone, 9: 17α-testosterone, 10: 17α-nortestosterone

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# Oral presentations

**Wednesday 21 th of May 2008**

**O 21** **The omics technologies in diagnostics**
Prof. Dr. J. van Oostrum
Zeptosens- a division of Bayer (schweiz) AG, Witterswil, Switzerland

**O 22** **Biomarker-based identification of growth promoter abuse during beef production**
Dr. M.H. Mooney
Queens University, Belfast, NI-UK

**O 23** **Mass spectrometric based untargeted metabolomics: principle and application in the field of food safety**
Dr. J-P Antignac
Laberca, Nantes, France

**O 24** **Microarrays: a screening tool for pro-hormone abuse in bovines**
Dr. J. de Rijk
Rikilt, Wageningen, The Netherlands

**O 25** **Analysis of biomarkers in plasma with a unique multi-assay surface plasmon resonance biosensor to monitor hormone abuse in cattle**
Dr. J. van Meeuwen
IRAS, Utrecht, the Netherlands

**O 27** **New techniques in residue analysis**
Dr. M. Pilar-Marco
AMRg, Barcelona, Spain

**O 28** **Desi mass spectrometry: a rapid screening tool for illegal hormone preparations and forensic samples from hormone crime and veterinary drug misuse**
M.W.F. Nielen, Wageningen, the Netherlands

## O 21

# ANTIBODY-BASED PROTEOMICS AND THE STUDY OF CELLULAR SIGNALING NETWORKS

Jan van Oostrum,
Zeptosens – a division of Bayer (Schweiz) AG, Switzerland

Systems biology is the study of complex biological processes as systems of many, diverse, interacting components through the integration of experimental, mathematical and computational sciences in an iterative approach.
Experimentally, large scale genomics, proteomics and metabolite measurements are used to monitor gene translation, protein expression, signaling events and metabolite fluxes induced by the systematic perturbation of a biological system by biological, genetic or chemical factors. Furthermore, large scale experimental methods are used to identify the nodes of signaling networks, through the comprehensive identification of interaction partners and protein modifications (e.g. phosphorylations). The comparison of samples from a disease state with those of matched normal donors and the building of animal models of disease (through the knock out of selected genes and/or the introduction of specific mutations and transgenes) represents a well established approach to study a system through the analysis of naturally occurring perturbations.

Understanding the information flow through signaling networks and how these can best be manipulated to halt or redirect the flow of aberrant signaling is a challenging endeavor. A first step would be to describe the full complexity of signaling networks at a molecular level – their systems biology – including activities specific to a particular cell type, dynamic feedback mechanisms, pathway cross-talk, signaling kinetics and of course pathway activation states (see below) in normal and disease situations.

For a “kinase pathway” the information flow or pathway flux, mostly depends on the ratio of phosphorylated and non-phosphorylated protein species, reflecting the activation state of the biological system. If we compare cellular activity over time, at various stages of disease progression or before or after drug treatment, it is likely that a correlation can be found between the activation state on one hand and the biological and disease state, on the other hand. Small molecules that modulate the activity of signaling proteins are useful tools to dissect the functional roles and connections of the individual nodes in a pathway[1]. Using such a ‘systems approach’, one can begin to build a model that will not only provide a contextual understanding of the molecular mechanisms of disease, but also has the potential to facilitate the validation of therapeutic modulation of regulatory and metabolic networks[2]. A direct consequence of such an approach would be the early recognition of “off target” and side effects of drug candidates, as well as the identification of putative biomarkers.

**Phosphoproteomics:**
Different types of tools are available for the analysis of protein phosphorylation, an area of biochemistry often referred to as phosphoproteomics. Mass spectrometry in conjunction with enrichment of phosphopeptides or -proteins has developed into one of the key methods, resulting in studies describing thousands of phosphorylation sites. Protein phosphorylation occurs mostly at three amino acids: at serine (Ser), threonine (Thr) or tyrosine (Tyr). With over 99%, Ser and Thr sites comprise the vast majority of phosphorylations. Nevertheless, there is a strong interest in Tyr-phosphorylation as well, because a significant number of key receptors, such as the EGF receptor family, display Tyr-kinase activity upon ligand binding. Besides, the enrichment of Tyr-phosphopeptides is facilitated by the availability of sequence-independent antibodies, while Ser/Thr-phosphopeptides usually undergo metal-affinity purification. Mapping of phospho-tyrosine residues is also offered as a commercial service termed PhosphoScan by Cell Signaling Technologies[3]. While mass spectrometry is the method of choice for discovery phosphoproteomics, as yet it is not the optimal solution for assaying - known - phosphorylation sites in a large number of samples. Sample preparation is quite laborious and difficult to automate and in most cases relatively large sample amounts (> 1 mg of protein/sample) are required. Novel targeted mass spec methods are currently being developed to address this issue, but screening applications will remain to be dominated by antibody-based approaches for the foreseeable future. The phosphorylation status of signaling pathway components can be measured using sequence-specific anti-phosphoprotein antibodies that specifically recognize the phosphorylated isoforms of such kinase substrates. Thus, the activity status of multiple signaling pathways can be probed through parallel phospho-specific analysis. These antibodies can be utilized in a wide variety of formats, ranging from the ‘good old’ Western Blot to advanced microarray-based platforms.

Because of their minute sample consumption, reverse protein micro-arrays enable truly multiplexed analysis by replicating the same sample many times on separate arrays. This type of array, in which a protein extract is immobilized and queried with antibodies or other reagents that bind to a specific protein in the sample, is often referred to as reverse (phase) protein micro-array (RPA). Opposed to this, forward arrays are those in which the capture reagent (e.g. the antibody) is immobilized[4]. Forward arrays have the inherent advantage of a better sensitivity, owing to the capture step, which enriches the analyte substantially. Reverse arrays require only a single and not a matched pair of antibodies, hence saving significantly on assay development time.

**Reverse protein arrays:**
The simplest, but still commonly used form of a reverse protein arrays is the dot blot, in which small volumes of protein solutions are applied to a membrane, either manually or using a small vacuum manifold. Gradually the technology evolved with the advent of nitrocellulose-coated slides[5] and spotters to what is arguably the most advance reverse protein array setup: the ZeptoMark platform (see below). Much of the work in the reverse array field has been focused on analyzing phosphorylation states in human cancer tissue, to unravel the - more long-term - signaling mechanisms underlying human cancer[5]. However, the scalability and capacity of the latest generation reverse protein array systems (see below) opens the way to profiling pathways almost in ‘real-time’ and hence to provide the necessary basis to model such dynamic systems.

A key requirement for such phospho-specific screening data to feed into pathway models is the quantitative aspect. Among the different proteomics technologies that are suitable for that purpose, we describe here a reverse array platform based on using the planar wave-guide technology for significantly improved sensitivity. Planar wave-guide, reverse protein arrays make it feasible to obtain reproducible and quantitative protein expression information about the dynamic aspects of cell signaling. Samples are titrated in serial dilutions (Figure 1) on the array to ensure that the assay is linear and hence relative or absolute (using an internal standard) quantitation is possible. For this platform, cells or tissue samples are subjected to a one-step extraction using denaturing conditions, under which the potentially labile protein phosphorylations are effectively 'frozen'. Figures 2 and 3 show selected results obtained with our reverse protein micro-arrays.

ZeptoChips using the planar wave-guide technology for improved sensitivity are made of thin film planar waveguides consisting of a 150 nm thin film of a material with high refractive index (e.g. $Ta_2O_5$), which is deposited on a transparent support (glass) with lower refractive index. A laser light beam is coupled into the waveguiding film by a diffractive grating that is etched into the glass. The light propagates within this film and creates a strong evanescent field perpendicular to the direction of propagation into the adjacent medium. The field strength decays exponentially with the distance from the waveguide surface, and its penetration depth is limited to about 100 nm. Upon fluorescence excitation by the evanescent field, excitation and detection of fluorophores is restricted to the sensing surface, while signals from unbound molecules in the bulk solution are not detected. This results in a significant increase in the signal-to-noise ratio compared to conventional optical detection methods.

A typical micro-array has space for 6 arrays each comprising 352 spots. Each array has 4 columns of spots used to calibrate the energy loss when the light travels across the waveguide. Typically 32 samples are spotted in four dilutions (ensuring one remains always within the linear part of the binding curve, see above) and in duplicates. If the concentration of the analytes is known, then the number of dilutions can be reduced. Each array will be probed with one antibody. Each spot will have a volume of around 0.5 nl (ø100  m) and will contain the amount of protein contained in a single cell. Spotting is performed by a non-contact piezo-electric spotter (inkjet technology), with a spotting capacity of about 360 arrays (enabling probing with 360 antibodies) in one overnight spotting run. After printing, chips are blocked with albumin (as done for Western blots) and can be stored in this blocked state for over 1 year at 4°C.

Due to the high sensitivity and high throughput capability of the reverse protein array approach it will be feasible to obtain protein expression profiles and signaling pathway information on a wide variety of cell lines and tissue samples. Interesting applications include i) the comparative analysis of signaling pathway(s) events in normal versus diseased tissue, ii) the comparative analysis of protein expression in various systems, iii) the elucidation of the dynamic aspects of pathway events and iv) the profiling of compounds to reveal signaling and cross-pathway effects of drug candidates. In addition, analysis of healthy versus diseased tissue (including animal models) will provide insights into the pathways' underlying pathologies and provide a platform for molecular diagnostics. In a future approach, the

screening of body fluids with a reverse array approach may enable the investigation a large number of individual body fluid samples for a limited set of proteins contained in them, to establish variations in protein expression levels.

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**Figure Legends:**

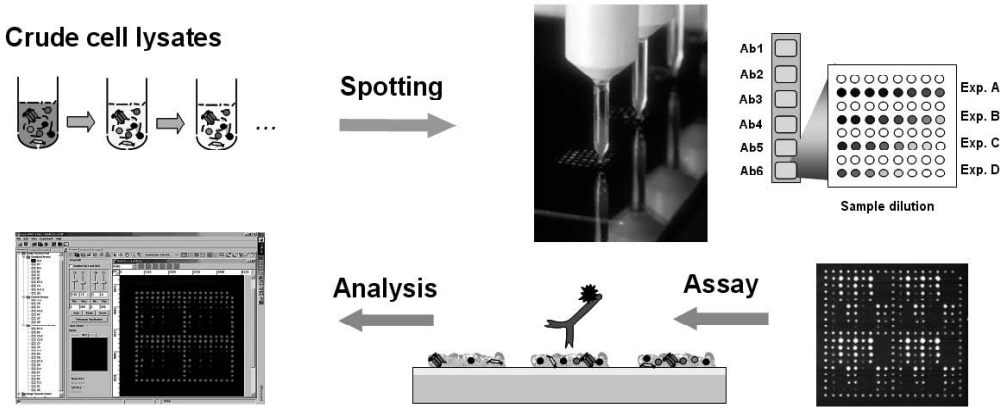


**Figure 1:** Crude cell lysate samples (produced with a denaturing solution) are spotted into arrays onto specially prepared glass chips by non-contact spotting. The samples are probed by e.g. fluorescently labeled antibodies.

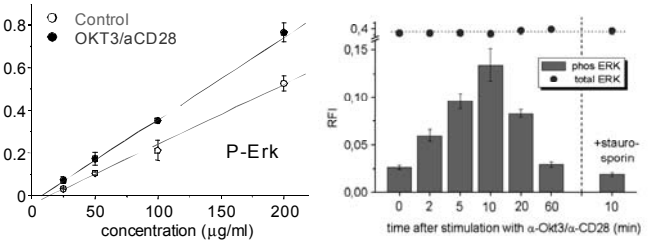


**Figure 2:** Monitoring phosphorylation events using reverse protein array technology: The human T-cell leukemia line Jurkat was either stimulated with OKT3 and anti-CD28 antibodies (blue) or treated with control medium (red). p44/42 MAPK (Erk), a key node

in T-cell signaling, is instantly activated upon stimulation, as shown by the rapid increase in phosphorylation at Thr202/Tyr204 (panels A and B). For every sample (e.g. timepoint) staining with the anti-phospho antibody is plotted against the spotted protein concentration (Panel A). By ensuring linearity of the measurement, the slope of the obtained curve can be used to obtain relative quantification (panel B). This example illustrates how reverse protein arrays can capture highly dynamic signaling events

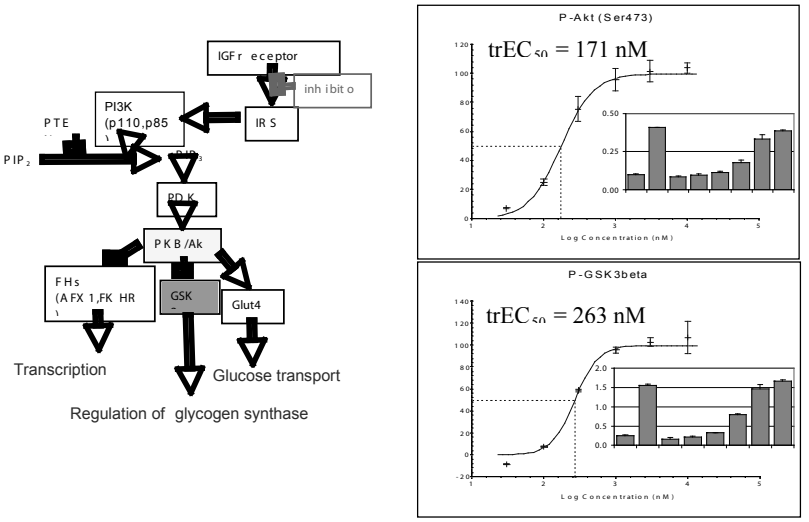


**Figure 3:** Monitoring the downstream effect of cell signaling inhibitors: Starved A431 cells were stimulated with insulin and co-treated with increasing concentrations of an inhibitor of the IGF1-receptor tyrosine kinase. After 30 min of treatment, the cells were lysed and the phosphorylation levels of Akt and GSK3β were monitored with antibodies specific for A) Ser473-Phospho-Akt and B) Ser9-phospho-GSK3β. By plotting the percent inhibition versus inhibitor concentration, one can derive EC50 like data from such experiments, termed trEC50 for signaling transduced downstream EC50 values.

## O 22

# BIOMARKER-BASED IDENTIFICATION OF GROWTH PROMOTER ABUSE DURING BEEF PRODUCTION

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**Abstract**
The effectiveness of analytical techniques currently used for the detection of banned growth promoter administration during meat production is hampered by both the emergent use of endogenous hormones and the limited number of animals which can be routinely tested by such means. Alternative testing strategies with high-throughput capabilities offer the possibility of targeting conventional detection analysis towards suspect animals thereby improving the efficiency of detection programmes. The current study has examined the potential of a biomarker-based strategy centred on the measurement of biochemical and protein-based plasma analytes to identify animals which are growth-promoter treated. During a 42-day study male and female veal calves were randomly allocated to control or treatment groups. Animals within treatment groups were subjected to repeated i.m. doses of either nortestosterone decanoate or 17β-oestradiol benzoate and a single s.c. dose of dexamethasone. Throughout the course of the study blood samples were taken at predefined time points and the concentration profiles of plasma analytes determined. The findings from the analysis of these samples have aided the construction of a panel of biomarkers which cover a spectrum of biological activity. Such a biomarker approach may find use as a tool aiding the identification of animal herds illegally treated with growth promoting agents and help to overcome new challenges which currently circumvent existing testing methodology.

**Introduction**
Whilst the use of growth promoting agents is known to increase the overall efficiency of meat producing processes, the use of these compounds within the European Union is prohibited (Directive 88/146/EEC). The development of sensitive methods for the detection of drug residues has led to a lowering of testing detection limits and as a result abusers have turned to using 'cocktail' administrations consisting of mixtures of multiple growth promoting agents (Courtheyn et al. 2002). The low concentration of individual drug components and the use of naturally occurring hormones within these 'cocktails' has reduced the likelihood of successfully detecting illicit agent use via existing residue testing methodology. However, through detection of perturbations to the circulating concentrations of blood components in response to overall drug administrations, a system of biomarker profiling may possess potential as a tool to aid identification of growth promoter treated animals. Plasma constituents which have their levels elevated or depressed in response to treatments offer

themselves as biomarkers of illicit hormone use. The present study has investigated the variation in the profiles of various plasma components at different stages throughout the period of treatment of calves with a growth-promoting regime typically associated with veal producing processes. Through comparison of the concentration profiles of constituents within the plasma of treated and untreated animals, a series of potential biomarkers of growth promoter treatment have been identified.

## Materials and Methods

*Growth promoter animal treatment study*
24 crossbred (Holstein Friesian x Fries-Hollands) calves underwent treatment in a study as described previously (Eisenberg et al. 2008). Briefly, animals at an average age of 10 weeks and following an acclimatization period of 7 weeks, calves were randomly divided into 2 control and 2 treatment groups (male and female, n=6). The calves were fed milk replacer with free access to water, roughage and concentrate during the whole experiment. Calves within treatment groups received i.m. doses of 17β-oestradiol benzoate (25 mg/animal) and nortestosterone decanoate (150 mg/animal) on Day 0. Treatments were repeated twice at 14-day intervals after which treated animals received a single s.c. dose of dexamethasone (4 mg/animal) at Day 35. Animals within control groups received matching volumes of peanut oil on indicated treatment days. At time-points throughout the 42-day course of the study blood samples were taken from the anterior jugular vein using lithium heparin Vacutainer tubes (Becton Dickinson). After sampling, plasma was obtained by centrifugation at 4ºC (1250 g, 20 min) and stored at -20°C prior to analysis.

*Biomarker identification*
Pooled plasma samples from control and treated male animals at Day -7, 15 and 39 were analysed by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and differentially expressed proteins identified by MALDI-TOF mass spectrometry. Initial comparative analysis was performed on pre- and post-treatment plasma samples from treated animals, and subsequently on plasma from control and treated groups on the same study day - thus accounting for variations in protein levels due to changes in normal physiological activity over time. In order to improve the detection of potential low-abundant biomarker proteins by 2D-DIGE, plasma samples underwent a clean-up procedure prior to analysis by thiophilic depletion of endogenous immunoglobulins and immuno-removal of albumin. In addition, classical assay methodology including ELISA, radioimmunoassay and DHT-binding assays were used to examine plasma samples for levels of proteins reported previously within the published literature to have altered expression following steroid exposure of cells and organisms. Various plasma biochemical constituents were also measured using an Olympus AU600 clinical chemistry analyzer.

## Results
2D-DIGE and MALDI-TOF mass spectrometry analysis
Plasma constituents were considered to be candidate biomarkers if they were found to have their circulating concentrations up- or down-regulated following exposure towards administered androgen, oestrogen and/or glucocorticoid agents. 2D-DIGE and MALDI-TOF

mass spectrometry analysis of plasma from control and treated animals revealed a number of proteins which had altered levels at various time-points in response to treatments. On Day 15, following the second administration of oestradiol/nortestosterone to treatment group animals, 2D-DIGE analysis revealed alpha-2-antiplasmin precursor, serotransferrin precursor and endopin-1 precursor to have reduced levels in treated animals (Table 1). Analysis of plasma samples post-dexamethasone administration at Day 39 found serotransferrin precursor and fetuin-A levels to be reduced in treated animals, whilst L-lactate dehydrogenase B and alpha-1-antitrypsin precursor levels were increased (Table 2).

**Table 1.** Plasma proteins found to be differentially expressed in control and treated male calves at Day 15 following nortestosterone and oestradiol administrations

| Biomarker | Response to treatment | t-test |
|---|---|---|
| Alpha-2-antiplasmin precursor | ↓ | 0.021 |
| Serotransferrin precursor | ↓ | 0.032 |
| Endopin-1 precursor | ↓ | 0.033 |

Using conventional assay techniques, further biomarker candidates (Table 3) were identified by monitoring the concentration profiles of various plasma constituents whose levels have been reported from the literature to be responsive to growth promoter agents. N-terminal propeptide of type III procollagen (PIIINP) levels in female calves were significantly increased (Day 17 and Day 31, $p<0.05$) following exposure to oestradiol and nortestosterone whilst the administration of dexamethasone resulted in a sharp decrease in measured levels. Similar but less significant trends were observed in treated male animals. PIIINP is released during the formation of type III collagen in soft tissue such as muscles, tendons and blood vessels. Osteocalcin, a marker of bone formation, was also observed to have its circulating levels severely depressed in response to dexamethasone administration. The sharp and rapid decrease in PIIINP and osteocalcin levels may be attributed to a glucocorticoid-induced suppression of collagen synthesis and bone formation (Saarela et al. 2003).

**Table 2.** Plasma proteins found to be differentially expressed in control and treated male calve plasma at Day 39 post dexamethasone administration

| Biomarker | Response to treatment | t-test |
|---|---|---|
| Serotransferrin precursor | ↓ | 0.032 |
| Fetuin-A | ↓ | 0.015 |
| L-lactate dehydrogenase B | ↑ | 0.027 |
| Alpha-1-antitrypsin precursor | ↑ | 0.019 |

Over the course of the study significantly lower sex-hormone binding globulin (SHBG) binding capacities were detected within plasma from treated female calves relative to levels within non-treated animals at Day 17 ($p<0.05$), Day 31 ($p<0.001$) and Day 42 ($p<0.001$). In contrast, measured levels within treated males were comparable to those in matching control animals. SHBG is a glycoprotein that binds and transports sex-hormones within the circulation (Petra, 1991) and the administration of anabolic or oestrogenic compounds has

the potential to affect circulating levels of this protein. The finding that changes in female SHBG binding capacities were more sensitive to exogenous oestradiol and nortestosterone administrations may be due to the fact that unlike male animals, females calves are not pre-exposed to high levels of endogenous androgens.
The increased utilization of amino acids for protein deposition and the decrease in protein turnover rates which occurs as a result of elevated anabolic activity due to growth promoter treatment was reflected by the depressed urea levels detected in treated animals of both sexes. By Day 3 post oestradiol and nortestosterone administrations, significantly lower plasma urea levels were found in treated male ($p<0.01$) and female ($p<0.001$) calves compared to control animals. Urea levels remained depressed in treated groups up to the point of dexamethasone treatment at Day 35 when the ensuing elevation in catabolic activity resulted in a transient increase in plasma urea with levels returning to previously depressed levels 2 days post administration. Thyroxine (T4), the primary active hormone synthesised within the thyroid gland which is involved in the regulation of metabolism by controlling the rate of cellular oxidation, was also found to be affected by dexamethasone administration. Total plasma T4 levels remained unaltered over the course of the oestradiol/nortestosterone treatments with measured levels been elevated at Day 37 in treated animals of both sexes in response to dexamethasone administration.

**Table 3.** Potential plasma biomarkers of oestradiol ($E_2$), nortestosterone (NT) and dexamethasone (DEX) administrations identified using conventional assay methodology

| Biomarker | Assay method | Treatment | Response in treated females | Response in treated males |
|---|---|---|---|---|
| N-terminal propeptide of type III procollagen (PIIINP) | RIA | $E_2$/NT<br>DEX | ↓<br>↑ | ↓<br>↑ |
| Osteocalcin | ELISA | DEX | ↓ | ↓ |
| Sex-hormone binding globulin (SHBG) | DHT binding assay | $E_2$/NT | ↓ | — |
| Urea | Clinical analyzer | $E_2$/NT<br>DEX | ↓<br>↑ | ↓<br>↑ |
| Thyroxine (T4) | Clinical analyzer | DEX | ↑ | ↑ |

**Discussion and Conclusions**

The potential of using biomarkers to signal exposure of animals to illegal growth promoting agents has been demonstrated in this study through the identification of a series of plasma components whose circulating levels have been clearly altered in response to exogenous treatments. Whilst the normal concentration range of identified biomarkers in animals of various breed and age under different environmental and husbandry conditions will be required to aid accurate identification of suspect profiles in the general population, this controlled study has provided evidence of the potential of such an approach. The major

advantage of a plasma based biomarker detection method is that the blood samples required to perform such testing can be readily obtained at any point of the animal rearing process. Contrasting biomarker responses observed following oestradiol/nortestosterone and glucocorticoid administrations raise the possibility that distinct biomarker profiles could be used to aid identification of the class of compounds and drug combinations utilised. Biomarkers which may be suitable for this purpose would preferably exhibit minimal day-to-day or diurnal variations under normal conditions and have a low basal concentration spread within the herd population as a whole. If using a single biomarker approach such parameters may be hard to achieve, but the use of a panel of biomarkers reflecting a spectrum of in vivo metabolic activities should offer greater sensitivity to detect growth-promoter abuse. Biomarker profiling could be facilitated by the development of high-throughput plasma screening technologies capable of measuring multiple biomarker levels simultaneously. In conclusion, this study has identified a range of potential biomarkers of growth promoter administrations which could be used to aid the identification of animals suspected of being treated with illegal agents and in doing so improve the effectiveness of existing residue monitoring programs.

Acknowledgements

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## O 23

# THE MASS SPECTROMETRIC-BASED UNTARGETED METABOLOMICS: PRINCIPLE AND APPLICATIONS IN THE FIELD OF FOOD SAFETY

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**Abstract**
For several years, the concept of metabolomics is emerging as new method for characterising biological samples and/or investigating biological processes in an integrated way. This non specific approach is based on the generation of large set of descriptors which correspond to chemical substances (so-called metabolites) present in the studied sample and accessible to the analysis. Although the most widely used technique for this fingerprinting approach was historically nuclear magnetic resonance (NMR), mass spectrometry (MS) is becoming more and more used in this field, due to incomparable advantages in term of sensitivity (giving access to minor but potentially informative metabolites) and specificity (permitting the identification of potential biomarkers of interest). In this general context, the purpose of the present work is first to present the main theoretical and technical principles of such MS-based metabolomics area. A special emphasis will be made on crucial analytical aspects determining the final quality and robustness of the method developed in this field, as well as the interest of the ultra-high resolution achieved on an LTQ-Orbitrap™ instrument. First application examples of this untargeted metabolomics approach in the field of food safety will be then introduced for illustrating these different issues.

**Introduction**
In recent years, the so-called 'omic' techniques (transcriptomics, proteomics, metabolomics) have emerged as powerful tools in the fields of biology and chemistry to profile "life complexity" using unrestricted descriptive methodologies. These approaches are all based on the simultaneous generation of a large set of descriptors that characterise the biological system under study (cell, tissue, organ, or entire organism); these descriptors are then referred to genomic, proteomic or metabolomic entities. In metabolomics, the monitored signals correspond to chemical substances (so-called metabolites) which are produced after the full complexity of absorption, distribution, regulation, and cell metabolism processes. On this point of view, the measurement of chemical species potentially identifiable (and in some extent quantifiable) certainly represents a clear advantage of metabolomics compared to other 'omic' approaches in the scope of establishing an eventual link with an associated physiological function and/or impact on the organism. Basically, the general principle of metabolomics is then to characterise the studied biological system by generating a kind of "fingerprint". When performed on large sample sets, the comparison of such multi-endpoint

measurements facilitates the identification of different sub-groups of samples, and further research ways focusing on relevant metabolites/biomarkers.
In the specific field of chemical food safety, an important conceptual issue linked to metabolomics is that the generated fingerprints are expected to reflect either the eventual presence of chemical residues, contaminants (as well as their eventual degradation and/or biotransformation products), or their potential impact on normal cell metabolism. A particular strength of this approach is that it can potentially identify some physiological effects induced by complex mixtures of chemical metabolites present at trace or ultra-trace concentration levels. Thus, metabolomics may represent a new methodological approach for investigating various issues. One of them is the development of new screening methods for controlling the illegal use of growth promoters in cattle. Basically, the idea is to measure no longer an exhaustive list of specific diagnostic signals of target substances but the global physiological effect associated to the administered drug(s). By this way, the final dream would be to provide statistical models permitting to classify rapidly unknown samples at least in a "compliant" or "suspect" area on the basis of their global MS-fingerprint, even if the administered substance(s) are not known or unambiguously identified. Of course such final expected result would require considerable amount of work before being reality. And experimental, analytical, and validation issues have to be invented for this purpose. But first proofs of feasibility of this approach are now available, which authorise to imagine metabolomics as a next finalised tool for large scale and extended control.
It must be evidenced that metabolomics requires a real synergy between various scientific fields including for instance biology, biochemistry, analytical chemistry, bioinformatics, and statistics. From the analytical point of view, the most widely used technique for this purpose remains nuclear magnetic resonance (NMR), mainly for historical reasons. However, mass spectrometry (MS) is becoming more and more used in this field. Indeed, MS presents some incomparable advantages at least on two crucial points. First, MS present clearly high performance in term of sensitivity, which is useful for measuring species with low abundance (minor but potentially informative metabolites). Secondly, the specificity of MS (through high resolution and/or MSn techniques) permits the chemical structure elucidation of potential metabolites of interest (identification of biomarkers). Besides this technology, many analytical choices have to be made at each step of the metabolomic analysis (sample treatment, mode of sample introduction, ionisation technique, type of mass filter, acquisition mode and parameters, raw signal post-treatment, data processing…) which directly impact the quality of the produced data. The repeatability issue of the fingerprinting process is probably the most crucial point. As a consequence, high level of attention has to be paid to these analytical issues before attempting to generate or interpret metabolomics data.
In this context, the goal of the present work is to present the main basics principles of MS-based metabolomics, as well as to point out some specific analytical issues which appear of primary importance, including the influence of sample preparation procedures, the possible complementarities between various measurement conditions, as well as the advantages and limitations of various software solutions for data processing.

## Materials and Methods

*Reagents and Chemicals*
All solvents used where of analytical (sample preparation) or HPLC (LC-HRMS measurement) grade quality and purchased from SDS (Peypin, France). Reverse phase C18 or normal phase SiOH SPE cartridges (2 g and 1 g of stationary phase, respectively) used for purification of solid samples (tissues) where also provided by SDS. Centrifugal devices (Nanosep 10kD Omega) used for filtration of liquid samples (urine, plasma, serum) where provided by Pall (Pall Life Science Corporation, MI, USA).

*Samples*
Most samples analysed in the present study where collected from twenty Hereford steers. Four groups (n=5 animal per group) were designed as : (1) control, (2) normally treated (intra-muscular injection) using one implant of anabolic steroids (RevalorS®:140 mg trenbolone acetate + 24 mg estradiol), (3) repeatedly treated using two implants at different times, (4) repeatedly treated using four implants received in the same time. Animals have been slaughtered three months after treatment stored in a freezer (at -18°C) until analysis.

*Metablomic LC-HRMS fingerprinting*
LC-HRMS metabolomic fingerprints where acquired on a LTQ-Orbitrap™ instrument (Thermo Fisher) operating in positive electrospray ionisation (ESI+) and high resolution (R=30,000) full scan [50-800] acquisition mode. Liquid chromatographic separation of the extracts was achieved onto a Uptisphere C18 stationary phase (150 x 2.1 mm, 3.5 µm), using a water (A) / acetonitrile (B) elution gradient (0.1% acetic acid in both solvents) from 0:100 to 100:0 (A:B, v/v) in 30 min at 0.2 mL/min flow rate.

*Data processing and analysis*
Various software solutions were used and compared for processing data generated by the LC-HRMS fingerprinting system used. The first one was the proprietary Sieve™ software (Thermo Fisher) directly compatible with the Raw data files produced by the LTQ-Orbitrap™ instrument. The second one was the open source xcms software which requires the previous conversion of the original Raw data files to the generic NetCDF format. Finally, the open source MZMine software was also tested mainly for introducing a normalisation of the Raw data. Processed data were analysed using Statistica® software (v 7.1, Statsoft) through principal component analysis (PCA) and linear discriminant analysis (LDA).

## Results and Discussion

*Measure repeatability*
A good repeatability of the MS fingerprinting process appears as a prerequisite before any metabolomics investigations based on this technology. Indeed, as the main principle of such approach is based on the analysis of the variability revealed by the data processing, each external source of variability (i.e. not related to the biological variability of interest) have to be carefully characterised and kept under control. In this scope, we have tested the potential benefit of a periodic source clean-up performed during a relatively long batch of injected

sera sample extracts. For this experiment, samples were just filtrated (10 kD cut-off) in order to discard macromolecules which can disturb the analysis (mainly during electrospray ionisation). Our results (Figure 1) clearly demonstrated (1) that a consecutive injection of 30 replicates of the same sample without any manual intervention lead to an acceptable absolute repeatability (mean relative standard deviation = 25 %) but (2) that a first level source clean-up (basic sample cone and capillary washing) performed in the middle of the sequence permits to reach even more satisfying value (mean r.s.d. = 15 %). As a conclusion, even if some solutions may be used for signal normalisation (see below), the evidence was confirmed that a preventive periodic source clean-up associated to relatively moderated number of consecutive injections is highly beneficial as first level of action for limiting the analytical variability linked to the MS measurement (source dirtiness and matrix effect).

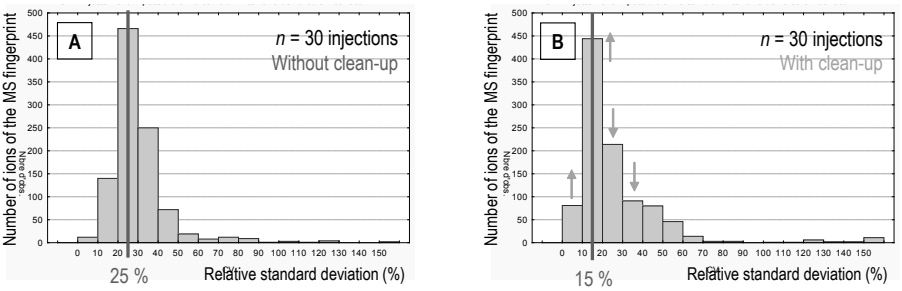


**Figure 1.** Distribution frequency of the relative standard deviation observed for ions constituting the MS fingerprints collected from 30 replicates of the same serum extract injected onto the LTQ-Orbitrap™ instrument in a single batch (A) without any source clean-up or (B)with a middle sequence source clean-up.

*Influence of sample preparation*

One interest of metabolomics is to generate sets of descriptors as large as possible without introducing any selectivity in terms of sample preparation. However, this goal is rarely achievable considering all analytical troubles related to matrix effects especially when MS is used as measurement technique. Moreover, it can be argued that no universal method will be ever provided in this field, as the nature and physico-chemical properties of matrices and compounds of interest are extremely diverse. To illustrate this issue, we have tested various extraction solvents on liver samples collected from control versus treated animals with anabolics. Besides our main objective which was to attempt a discrimination of the two sample types on the basis of the generated LC-HRMS metabolomic fingerprints, we have then compared also the different extracted fractions in terms of information content. Our results (Table 1) confirmed that our main solvents of choice (which have been selected to cover a wide polarity range), led to complementary MS fingerprints. Indeed, the proportions of common signals retrieved in each extracted fraction were poor, excepted for acetone and ethylacetate which present closer similarities. Nevertheless, a very satisfying separation of our 4 groups of animals was obtained whatever the considered sample fraction (Figure 2). In conclusion, the combined used of various sample preparation procedures and/or distinct sample fractions is undoubtedly beneficial for generating complementary and extended metabolomic data sets.

**Table 1.** Proportions (%) of common ions retrieved in the LC-HRMS fingerprints collected from bovine liver samples extracted with 4 different organic solvents.

| Solvent | **Methanol** | **Ethylacetate** | **Acetone** | **Hexane** |
|---|---|---|---|---|
| Methanol | 100 | | | |
| Ethyl-acetate | 7 | 100 | | |
| Acetone | 9 | 62 | 100 | |
| Hexane | 18 | 7 | 11 | 100 |

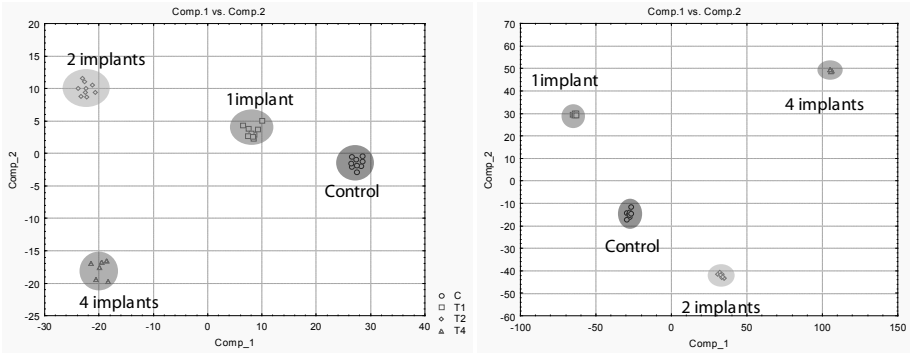


**Figure 2.** Results of linear discriminant analyses (LDA) performed on LC-HRMS metabolomic fingerprints collected from the same bovine liver samples from control versus animals treated with 1, 2 or 4 doses of anabolic steroids and extracted (A) with methanol or (B) with hexane.

**Data processing**

Metabolomic data processing is a current field of extensive development from the bioinformatics community. Various proprietary and open source software solutions are today available for this purpose, which have not equivalent mode of functioning (peak alignment, peak picking, peak sorting). As a consequence, the choice and correct setting of such data processing tool appear as of primer importance and merit to be investigated due to their direct impact on the finally produced processed data table. In this scope we have tested 3 main software solutions on identical data sets; i.e. Sieve™, xcms and MZMine. Each software presents its own advantages and authorises a visualisation of the overlay extracted ion chromatograms corresponding to potential biomarkers of interest (Figure 3). But our results indicates that *xcms* appears as the more powerful in terms of rapidity and peak picking. On the other hand, *MZMine* presents the possibility to normalise the data using single or multiple internal standards.

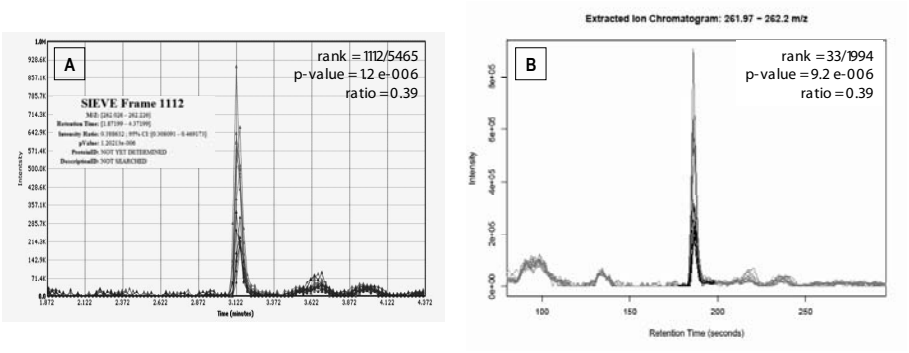


**Figure 3.** Example of extracted ion chromatograms generated by (A) Sieve™ and (B) xcms software for the same compound (m/z 662.1) in the same LC-HRMS metabolomic fingerprints

## Conclusion

Metabolomics is a current field of intense development in various scientific areas. Applied to chemical food safety, it's probably represents a possible future evolution for the control of food products, by authorising a fast and global way to distinguish compliant from suspect samples. However, important analytical issues remain to be deeply considered for each step of the metabolomics process (sample preparation, MS fingerprinting, data processing, and data analysis). The development of efficient tools permitting to link potential ions of interest (m/z) to possible chemical structures is also a current challenge. Validation procedures will be also to invent in this field, especially for applications aiming to propose some finalised diagnostic models. Last but not least, special attention has to be paid to the interpretation of the produced results, the risk of factor confusion being a major trick of such approaches.

# O 24
# MICROARRAYS: A SCREENING TOOL FOR PROHORMONE ABUSE IN BOVINES?

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**Abstract**
In livestock production, illegal use of prohormones is hard to prove since prohormones are strongly metabolized in vivo and metabolites are not significantly different from endogenous levels. In this study we investigated the feasibility of a novel effect-based approach for monitoring abuse of prohormones. Changes in gene expression profiles were studied in livers of bovines treated orally or intramuscular with 1000 mg DHEA, using bovine 44K oligonucleotide microarrays. On the one hand this work demonstrates the strength of a microarray approach for studying the effects of prohormones in bovines and on the other it showed the pitfalls that may be encountered in the identification of robust, transcriptomics-based biomarkers.

**Introduction**
Prohormones are chemical compounds that do not exhibit hormonal action by themselves. However, they are direct precursors of active hormones or they indirectly affect natural hormone levels. Abuse of prohormones in livestock production is hard to prove, because most of these substances are strongly metabolized in vivo and metabolites are either unknown or not significant different from highly fluctuating endogenous levels. Dehydroepiandrosterone (DHEA) is such a prohormone and is the most abundant occurring precursor of both androgens and estrogens in humans (Labrie et al. 1998). It is claimed that DHEA improves muscle strength and lean body mass. Within this context it is anticipated that abuse of prohormones like DHEA is or might become an emerging practice in livestock production.
The application of DNA microarray technology is becoming a common method to describe effects of treatments at the mRNA level and allows screening for changes in expression of multiple genes in one single experiment. Gene expression profiling not only provides detailed mechanistic information on cellular processes and pathways but might also allow the identification of biomarkers for screening (Buterin et al.) and classification of compounds with the same mode of action (gene expression fingerprints) (Labrie et al. 2005).
The rapid progress in veterinary genomics has resulted in an increasing number of commercially available farm animal microarrays, including bovine arrays (Bendixen et al.). In the present work we used bovine 44K oligo microarrays to study the effect of DHEA on gene expression in bovine liver and the potential of using these arrays as a screening tool for

prohormone abuse. We used bovines raised under normal livestock practice but treated with prohormones and compared their gene expression profiles with those of untreated animals. Our study included three small but independently performed experiments.

## Materials and methods

*Animals and treatment*

Frisian cattle were purchased at the local market and housed for 2-3 weeks before the start of the experiment. The treatment with DHEA was repeated in three independent experiments using identical treatment and sample schedules. Each of the three experiment consisted of two animals of which one was orally administrated with capsules containing 1000 mg DHEA (Sigma) and the other was injected intramuscularly with 1000 mg DHEA dissolved in 10 ml Miglyol 812 (Certa). The exposed animals were 8-13 months old and weighed 210-410 kg. Administrations were performed seven times, at 24 hour intervals. Control animals were included in the first (n=3, 6 months old, 153-174 kg) and third (n=4, 13-14 months old, 350-432 kg) experiment. Twenty-four hours after the last treatment, the animals were sacrificed and liver tissue was collected, snap-frozen in liquid nitrogen and stored at -80°C until use.

*Microarray analysis*

Total RNA was extracted from liver tissues by homogenization in Trizol and mixed with chloroform. The lysate was centrifuged for 15 minutes at 12000 g and the aqueous phase was transferred to be mixed with isopropanol which precipitates the total RNA. After washing with 75% ethanol the pellet was resuspended in RNase free water. Upon extraction the RNA was purified using the RNeasy mini kit (Qiagen), RNA quality was determined spectroscopically and by gel electrophoresis.

To generate fluorescently-labelled cRNA the Agilent low RNA input fluorescent linear amplification kit (Agilent technologies) was used. In short, 1 µg of total RNA was reverse transcribed using T7 tagged oligo-dT primer and labelled with Cy3 or Cy5. Liver RNAs of the treated and control animals were individually labelled with Cy5 and RNA of all 7 control animals was pooled and labelled with Cy3. After purification, label efficiency and yield were determined. A mixture of 1 µg of Cy3-labelled and 1 µg of Cy5-labelled cRNA was hybridized onto a 44K bovine oligo microarray using Agilent's gene expression hybridization kit. Hybridization was performed at 65°C for 17 hours in a hybridization oven with rotation function (Agilent technologies). After hybridization, microarrays were washed and fluorescence was measured using an Agilent Technologies G2565B microarray scanner. Fluorescence intensities were quantified using Feature Extraction 8.5 software (Agilent technologies). Data were imported in GeneMaths XT 1.6 (Applied Maths) and signals below two times background were excluded from further analysis. Subsequently, the data were log2 transformed and normalized as described by Pellis et al. Ratio's were calculated between the mean of the DHEA exposed animals (n=6) and the mean of the two different control groups. Analysis of variance was performed by T-test statistics. A difference of at least 1.5 fold and a p-value $<0.05$ was used as a criterion for the selection of differentially expressed genes.

**Results and discussion**

Upon hybridization and data normalization, unsupervised principal component analysis (PCA) was performed based on the three largest principal components (Figure 1). The gene expression profiles of the livers of animals treated with DHEA clustered together but were clearly distinct from those of the controls. Exposed animals (IM 1-3 and O 1-3) and the control animals of the first experiment (C1) are separated along the X-axis, whereas the control bovines of experiment 3 (C3) and the exposed bovines are mainly separated along the Z-axis.

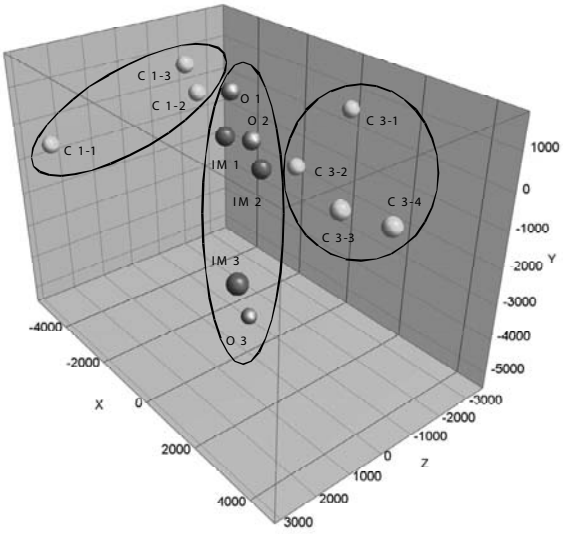


**Figure 1.** Principal component analysis of animals administered DHEA intramuscularly (IM 1-3), orally (O 1-3) and control animals (C1 1-3 and C3 1-4). The projection on the three principal components of greatest variation covering 49.6% of the total variance is shown.

After calculation of the expression ratios between all exposed calves and the controls of respectively experiment 1 (C1) and experiment 2 (C2), t-testing was performed. Of the 579 genes differentially expressed (fold change >1.5 and p-value <0.05) in livers of animals treated with DHEA as compared to the C1 animals, 251 were up-regulated and 328 genes were down-regulated. Comparison of all exposed animals to the C3 control animals revealed 154 differentially expressed genes of which 86 genes are up-regulated and 68 genes are down-regulated. Venn diagram comparison (Figure 2) of the two different gene sets revealed a total of 13 genes (8 up-regulated and 5 down-regulated) that were differentially expressed irrespective of the controls used.

The genes responsible for the separation between groups have the potential to serve as biomarkers for a treatment with the prohormone DHEA. However, the present study also shows that the genes found differentially expressed is determined to a large extent by which control animals are used for comparison and thus demonstrated the pitfalls that may be encountered in the application of microarrays as a screening tool for (pro)hormone abuse in livestock production. When examining calves from practice one is dealing with biological

variation and differences in animal husbandry and age. This variation is likely also reflected in the distance between the expression profiles of the two control groups plotted in the PCA.

**Conclusions**

The present study demonstrates that microarray technology is a powerful tool to analyse the effects of prohormones in male calves. The availability of bovine 44K oligonucleotide arrays offered an opportunity to investigate changes at the level of gene expression and resulted in a set of genes related to the action of DHEA. This eventually may lead to identification of an applicable set of biomarkers, which can be used for screening of DHEA abuse in cattle. However for the correct interpretation of mechanistic data and the identification of robust biomarkers it is important to take into account the biological variation which is inherent to the use of animals of practice. For mechanistic studies, treated animals should be compared with suitable controls. On the other hand, to assure the robustness of a candidate biomarker, its behaviour should be studied in a broad spectrum of daily practice animals reflecting differences in factors such as genetic background, age, climate, environment, nutrition and disease history. A similar approach as described in this research may be followed to study the effects of other (pro)hormones used in livestock production.

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## O 25

# ANALYSIS OF BIOMARKERS IN PLASMA WITH A UNIQUE MULTI-ASSAY SURFACE PLASMON RESONANCE BIOSENSOR TO MONITOR HORMONE ABUSE IN CATTLE

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**Abstract**
Levels of some circulating proteins change specifically upon exposure to exogenic substances and are therefore potential biomarkers of growth-promoter abuse. In BioCop, a surface plasma resonance (SPR) biosensor was developed to analyze multiple protein levels in plasma simultaneously to reveal growth-promotor abuse. Detection was based on indirect affinity assays in which plasma was diluted by adding biomarker-specific antibodies. Samples were run over a 4-channel, 16-spot sensor chip coated with immobilized target proteins reflecting alpha-2-antiplasmin and other immobilized potential biomarkers that were omitted from this manuscript. This biosensor configuration allows up to 16 biomarker assays to be run simultaneously with a throughput of four samples per hour.

**Introduction**
Classical residue analysis methods are in many ways unsatisfactory to detect illegal use of growth-promoting compounds in food-producing animals. In 2005 the BioCop project was funded by the EU 6th Framework to develop approaches to monitor illicit use of growth promoting agents via detection of biological responses to anabolic treatments. As any change in the homeostatic status of an organism will induce specific responses, analysis of these responses may reveal the nature of the change - this concept is generally accepted and exploited in medical and life sciences.
Subjecting animals to anabolics will affect their so-called proteome i.e. the occurrence and levels of protein expression. Through work within Workpackage 2 of the BioCop project, levels of several plasma proteins in cattle were found to be influenced by hormone treatment - these proteins were considered to be biomarkers (BMs). The analysis and measurement of circulating levels of these indicators may thus indicate abuse of anabolics in the sampled animals (Mooney et al. 2008).
This paper describes the development of an assay on a new multi-analyte surface plasmon resonance (SPR) biosensor enabling the detection of a maximum of 16 biomarkers simultaneously on four parallel channels capable of running four different conditions. Here, the first applications on this unique machine are described.

## Materials and Methods

*Instrument & Reagents*
The experimental SPR biosensor instrument and the external immobilization unit were developed by Biacore AB (Uppsala, Sweden) (Fig 1). Carboxymethylated dextran coated chips (Series S Sensor Chip CM5, Biacore AB), buffer (HBS-EP+ buffer containing 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% v/v surfactant P20 at pH7.4), sodium acetate buffers (10 mM sodium acetate at either pH 4.0, pH 4.5 or pH 5.0), amine coupling kit (N-hydroxysuccinimidine (NHS), N-ethyl-N'-(3-diethyl-aminopropyl) carbodiimide (EDC), ethanolamine hydroxychloride (1.0 M, pH 8.5, EtOH-$NH_2$) and normalizing solution (70% glycerol) were obtained from Biacore AB. Water was of Milli-Q quality (Millipore Corp., Billerica, MA, USA).
Fresh plasma was prepared from blood from a single calf (CP) and from an adult animal (AP) collected at a local abattoir (Nijkerk, The Netherlands).

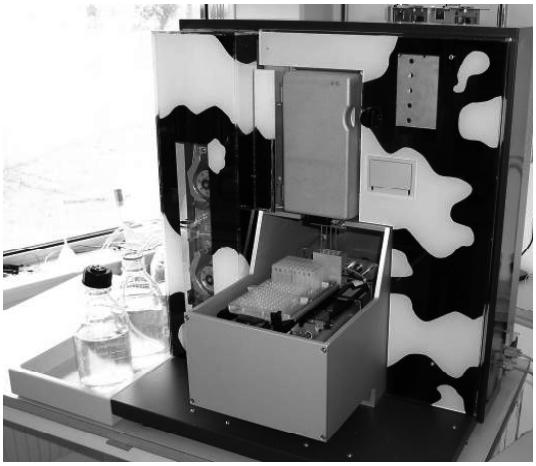

**Figure 1.** Experimental SPR biosensor instrument.

*Proteins and antibody*
Recombinant alpha-2-antiplasmin (further referred to as A2) was supplied by Fusion Antibodies Ltd (Belfast, UK) dissolved in 150 mM imidazole at 100 µg/ml. Anti-A2 antibody was raised in a New Zealand white rabbit (Centre d'Economie Rurale Groupe, Marloie, Belgium). An amount of 0.2 mg A2 emulsion was injected three times, once every two weeks and then twice every four weeks. Sera containing polyclonal antibodies (pAB) were collected from bleeds by centrifugation at 3000 rpm for 10 min. Dilutions of protein stock and pAB were prepared freshly on the day of the experiment, while stocks were stored at a maximum of four weeks at 4°C. Aliquots of frozen stocks were kept at -20°C.

*Preparation of the SPR sensor chip surface*
Amino-coupling of 50 µg/mL A2 on a CM5 chip according to the manufacturer's instructions was performed on an external device that was connected to a computer-controlled pump system. The external device enables the immobilization of a maximum of 16 ligands on

16 corresponding spots with four spots per flow channel configuration. Activation of the surface was achieved by incubation with a mixture of EDC and NHS (1:1, v/v) for 8 min. Per spot, 3 µg A2 dissolved in 60 µL 10 mM sodium acetate (pH 4) containing 0.05% (v/v) P20 surfactant was passed over the sensor surface. After a contact time of 8 min, the surface was rinsed with water and unconjugated active sites were deactivated with EtOH-$NH_2$ for 8 min. The surface was then rinsed with water and dried with $N_2$(g). The immobilization efficiency was reflected by the Rmax, which was determined by allowing diluted pAB to associate with immobilized A2 protein for 15 min at 20 µL/min and then followed by regeneration of the surface.

*SPR biosensor assay*

Each cycle consisted of two sample injections for 180 s at 40 µL/min. The first sample was spiked with pAB (1:100, v/v), whilst the second injection was the same sample minus pAB. The second injection was used to record non-specific binding (NSB) of matrix components. After each sample injection, dissociation was allowed for 30 s before the surface was regenerated with a 30 s pulse of 100 mM NaOH, followed by a 120 s period of buffer flow to enable chip surface stabilization. Baseline levels were defined as the response at 31 s before injection, whereas binding levels were defined as the response at 13 s before the end of the dissociation period (Fig 2).

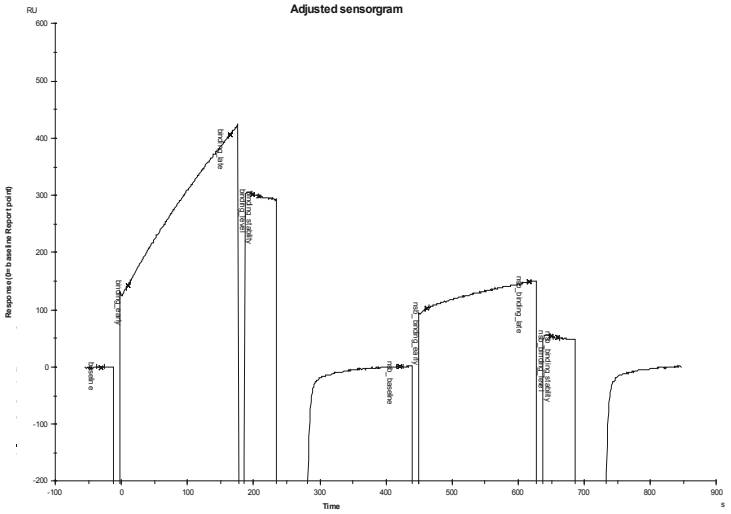


**Figure 2.** Typical sensogram of two repetitive injections of the same sample with and without spiked pAB. Reporter points are depicted.

Specific responses were calculated by subtraction of the recorded NSB in the second run of the sample sequence and were corrected for intra- and inter-experimental differences. Alpha-2-antiplasmin calibration curves were constructed by spiking buffer at 0 to 33 µg/mL. An optimal dilution rate of bovine plasma yielding 50% binding of pAB (EC50) was deduced from a range of dilutions of 1:5 (v/v) to 1:15,625 (v/v) in buffer**.**

## Results

*Calibration curves of A2*

Inspection of the calibration curve revealed 50% inhibition at 1.69 µg/mL for samples of A2 spiked in buffer and 1.62 µg/mL for samples of A2 spiked in buffer following heat treatment through incubation at 65°C for 30 min. This illustrates that heat treatment had no significant effect on immuno-detectable A2 in buffer (Fig 3).

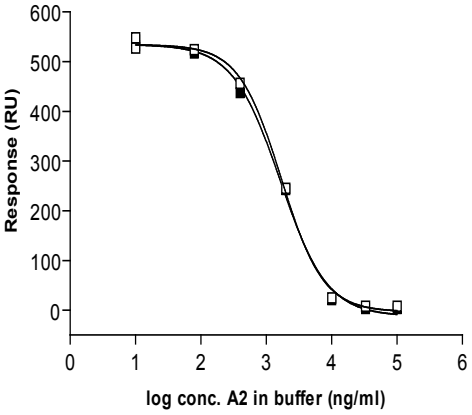


**Figure 3.** Typical calibration curves of A2 spiked in buffer and pAB (1:100)( ) compared to A2 spiked in buffer which was subsequently heat treated at 65 °C for 30 min ( ). Responses are corrected for NSB.

*Pre-treatment of plasma*

Through comparison of effects of pre-treatment of plasma at a range of temperatures, samples incubated at 65°C for 30 min were shown to demonstrate lowest NSB on A2-containing spots. This treatment was found to have no significant effect on immuno-detectable A2 in plasma (Fig 4). Inspection of dilution curves revealed 50% inhibition to occur at 208-fold dilution for non-heat treated adult plasma and at 218-fold dilution for heat-treated adult plasma. For calf plasma these dilution rates were 218-fold and 188-fold respectively. The dilution rate of 210-fold corresponded with an alpha-2-antiplasmin level of 340 µg/ml in adult bovine plasma, while for calve *bovine* plasma this was found to be 324 µg/ml (data not shown). Despite this treatment, residual NSB level was the highest at a 1:5 (v/v) dilution rate and lowest at a 1:15,625 dilution rate.

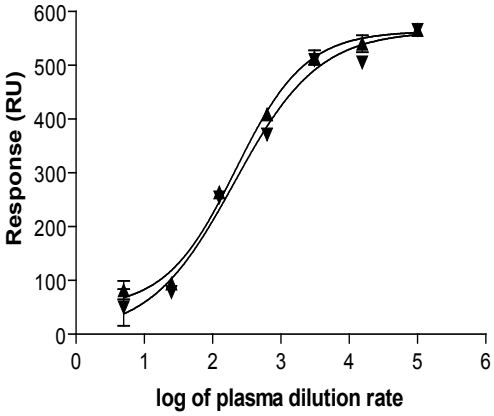


**Figure 4.** Specific A2 responses in diluted adult bovine plasma (AP) ( ) compared to plasma that was treated at 65 °C for 30 min ( ). Responses were corrected for NSB.

*Analysis of A2 in samples from experimental animals*
In calves, the target concentration for A2, estimated from ELISA results, was found to be 20 to 80 µg/mL (results not shown). This concentration drops following steroid treatment. Considering the plasma dilution which induced 50% inhibition and expected levels, plasma was diluted at a rate of 1:100 to 1:300 dilutions to facilitate the determination of A2 using the biosensor.

**Conclusions and Discussion**
This paper describes the first use of a new SPR Biacore biosensor, which can detect 16 analytes simultaneously. Alpha-2-antiplasmin, a potential biomarker of growth promoter abuse, was chosen as a model analyte to illustrate the biosensor's ability to measure multiple protein levels in bovine plasma. Observed plasma NSB effects on this SPR system were found to be dependent on the individual animal from which the sample was acquired, and could be significantly reduced through heat-treatment of plasma samples. Residual NSB was largely compensated by the introduction of an NSB-subtraction strategy involving two sequential sample injections i.e. with and without the presence of reporting anti-A2 pAB. In this way, alpha-2-antiplasmin was successfully detected at levels of 324 µg/ml in calve plasma and at 340 µg/mL in adult plasma. Ultimately, the SPR device will be capable of detecting a maximum of 16 biomarkers per (plasma) sample in 15 min. Compared to the conventional analytical methods used currently, such a high-throughput analytical device will prove beneficial in the detection of illicit hormone abuse hormone abuse through its ability to measure multiple biomarkers representative of a wide range of metabolic activity.

**Acknowledgements**
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## O 27

# NEW TECHNIQUES IN RESIDUE ANALYSIS

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**Abstract**
Different hybrid (bio)materials, nanostructured surfaces and nanoparticles have been the base for the construction of biosensor devices with potential applicability for clinical diagnostics, environmental monitoring, food safety and industrial process control. Antibodies, immunoreagents and bioreceptors to detect different pesticides or veterinary drug residues have been developed, evaluated using microplate-based enzyme-linked immunosorbent assay (ELISA) methods, and immobilized on the top of these materials to construct stable functional surfaces. Examples on novel electrochemical and optical immunosensors for the analysis of residue analysis are described.

**Introduction**
Molecular diagnostics has been evolving rapidly during the last years. Novel molecular diagnostic approaches to refine and extend the limits of detection have emerged as consequence of the combination of nanotechnological and biotechnological advances. Biosensors are between the potential applications of new materials and devices recently and of novel nanobiotechnological approaches. Thus, subtle changes on properties such as the dielectric field or the refractive index produced after biomolecular recognition events can be detected if they are taking place at the surface appropriately developed transducers. Nowadays, the new knowledge on the unique properties of nanostructured materials have opened up the possibility to investigate the influence that biorecognition phenomena produce on the new optical and/or electrical properties of these systems, pointing to the possibility to develop more sensitive and flexible biosensing systems.
Immunosensors using antibodies as biorecognition elements have fascinating features such as the possibility to respond selectively to biological or bioactive substances and the capability to respond in a physiologically manner. The unique properties of certain nanomaterials combined with the excellent features of the antibodies allow envisaging novel exquisitely sensitive chemical and biological sensors. Thus, antibodies are natural molecules with inherent capabilities to specifically react with their counter antigen. Antibodies can be produced, in principle against all kind of substances and their features can be tailored according to the requirements of each application. Their homogeneity regarding chemical structure and properties allow standardization of several procedures. Moreover, antibodies show greater improved stability, if compared to other biomolecules.
Research at the Applied Molecular Receptors Group (AMRg) of the CSIC has been focused for more than ten years on the development of selective natural (i.e. antibodies) and synthetic (i.e. molecular imprinted polymers) receptors, particularly against small organic molecules.

During the last years, this research has been addressed to the incorporation of these receptors on new chemical transducers allowing the development of several electrochemical and optical biosensor devices, in which the functionality of the immunoreagents has been preserved and at the same time, improved analytical features have been accomplished (speed, possibility of automation, etc.). This multidisciplinary research has only been possible thanks to the collaboration established with research groups from the Centro Nacional de Microelectrónica (CNM-CSIC, Bellaterra, Spain), the Centre Suisse d'Electronique et de Microtechnique (CSEM, Newchatel, Switzerland) and the Institut de Ciencies Fotoniques (ICFO, Castelldefels, Spain). In this communication we will show some of the recent achievements regarding detection of interesting bioactive substances by developing biosensing devices based on amperometric (Zacco et al. 2006; Zacco et al. 2007; impedimetric (Ramón et al. in press) and optical (Kreuzer et al. 2006) transducer principles.

## Materials and methods

*Immunoreagents and Bioreagents*
Immunoreagents have been produced after careful hapten design and synthesis using organic chemical synthetic methods. The haptens have been used to prepare bioconjugates using well established chemical conjugation protocols and characterized by matrix-assisted laser-desorbed ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Horseshoe crab hemocyanin (HCH) hapten conjugates have been used to raise antibodies using standard protocols. Bioreceptors have been kindly provided by Benoit Granier (Unisensor SA.

*Materials and Devices*
Gold nanoparticles of 10-20 nm were prepared using well established methods[5]. Nanoparticles of higher size were purchased at BBInternational (Cardiff, UK). The extinction spectra of the gold colloids were measured by conventional dark-field spectroscopy connected to a video camera and a microespectrometer. The wavelength interrogated optical sensor (WIOS) was fabricated at CSEM (Neuchâtel, Switzerland). The interdigitated microelectrodes (IDμE) were fabricated at CNM-CSIC (Barcelona, Spain). The magnetoelectrode used on the amperometric magneto-immunosensor was fabricated in the UAB.

## Results and discussion

*Electrochemical Immunosensor for Antibiotic Detection*
Two electrochemical transducer systems have been investigated. In the first approach, sulfonamide (SA) antibiotics have been detected in milk by using amperometric detection of a SA haptenized enzyme tracer. The system makes use of class-specific anti-sulfonamide antibodies covalently linked to tosyl-activated magnetic beads that are gentle mixed with the milk sample, previously diluted 5 times with PBS. The immunological reaction takes place under competitive conditions using a horseradish-peroxidase enzyme tracer. After about 15 min, a magneto sensor made of graphite–epoxy composite (m-GEC), which is also used as electrochemical transducer captures the modified magnetic beads (see figure 1, A). Detection is achieved through a suitable substrate an electrochemical mediator. A limit of detection of 1.44 g $L^{-1}$ has been achieved when analyzing raw full cream milk samples (Zacco et al. 2007).

On a second approach, antibiotic detection without the use of any label has been intended making use of interdigitated μ-electrodes (IDμE) and recording their impedimetric response. A small-amplitude perturbing sinusoidal voltage applied to the electrochemical cell produces current response that can be measured that is also dependent of the frequency of the voltage. The complex impedance can be presented as the sum of the real, Zre (ω), and imaginary, Zim((ω) components that originate from the resistance and capacitance of the cell. As it can be observed in figure 1B, the antigen is covalently immobilized within the electrodes and exposed to the sample that has been previously mixed and incubated with the specific antibodies. Using this configuration sulfapyridine can be detected with an $IC_{50}$ of 5.6μg $L^{-1}$ in buffer (Ramon et al. in press).

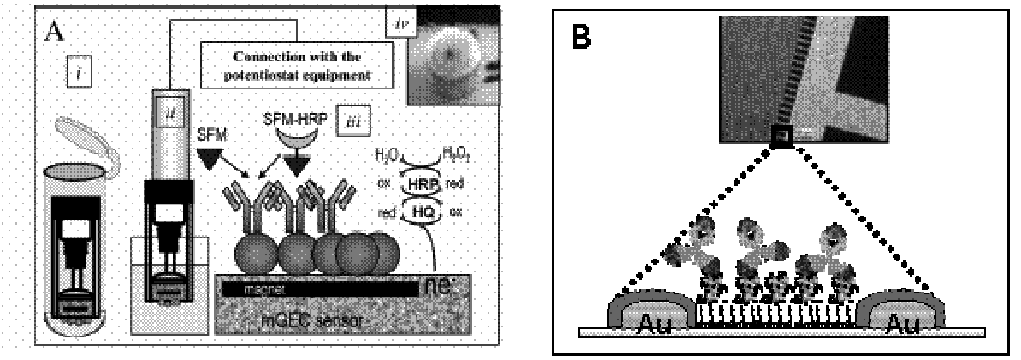


**Figure 1.** Schemes of the two electrochemical approaches investigated for the analysis of antibiotic residues.

*Wavelength Interrogated Optical Sensor (WIOS) for multiple antibiotic detection*
The WIOS system uses the evanescent field of light to probe changes in the refractive index at the surface of a waveguide surface (Cottier et al. 2003) by scanning the resonance condition of the waveguide grating through the wavelength modulation of a laser diode (see figure 2). The second grating couples out the guided light which is collected with optical fibers. Monitoring of the resonance wavelength allows real-time monitoring of the binding of non-labeled molecules on the waveguide grating surface. Since the resonant coupling occurring at the first grating is governed by the a grating equation such as $\lambda_r(t) = \Lambda [n_e(t) - \sin(\theta)]$, in which where, λr(t) is the resonance wavelength at which coupling occurs; $n_e$, the effective refractive index of the waveguide; θ, the incidence angle and Λ, period of the grating, for a given optical configuration (θ, fixed), monitoring λr will give access to effective index variations of the waveguide, and consequently give access to the variations of the refractive index of a sensing layer deposited on the waveguide.

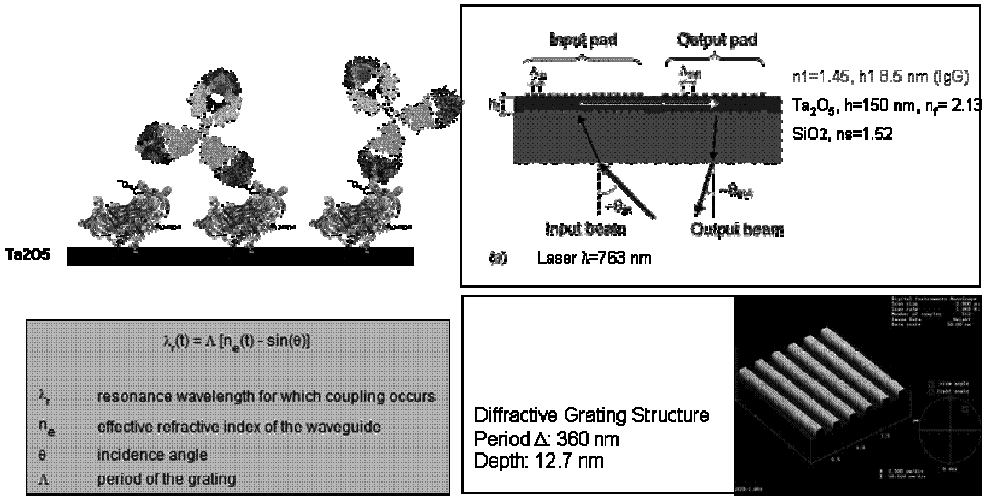


**Figure 2.** Scheme of the WIOS principle. Biorecognition events at the surface of the grating can be recorded due to a variation of the resonance condition.

Residues from the most important antibiotic families have been detected using the WIOS system by immobilizing specific bioreceptors for β-lactames, sulfonamides, fluoroquinolones and tetracyclines on the surface of the chip. Quantitative determination of the antibiotics has been possible reaching detection limits below or very close, depending on the antibiotic family, of the maximum residue limits (MRL) established by the European Community (EC)

*Localized Surface Plasmon (LPR) Immunosensor for Anabolic Androgenic Steroids*

Biosensors based on surfaces plasmon resonance (SPR) have demonstrated a significant commercial success exemplified by devices such as Biacore™. Superficial plasmons are very sensitive to the optical properties of the dielectric media that surrounds the noble metal. Most of the examples found in the literature make use of gold thin layers; however, the potential of using noble metal nanoparticles is still starting. The particle confinement of the surface plasmon gives rise to a spectral resonance in the light scattering that does not occur in thin films. Thus, optical properties of the nanoparticles are not only affected by the dielectric media but also from features such as size, material and shape.

Based on the above mentioned considerations, a label-less immunosensor for the detection of stanozolol (ST), an anabolic androgenic steroid, has been developed. The strategy has consisted on preparing a chip that contains multiple gold nanoparticles covalently immobilized on the surface of a glass slide. The gold nanoparticles are covered by a ST-BSA antigen and exposed to a solution that is a mixture of the sample and the specific antibodies (see figure 3). The characteristic plasmon resonance peak (PRP) of the antigen coated gold nanoparticles (~540 nm) moves about 13 nm to a higher wavelength upon binding of the specific antibody. The presence of the anabolic steroid hormone inhibits this shift on a quantitative manner. At present, about 6 μg $L^{-1}$ ST can be detected in buffer using this configuration.

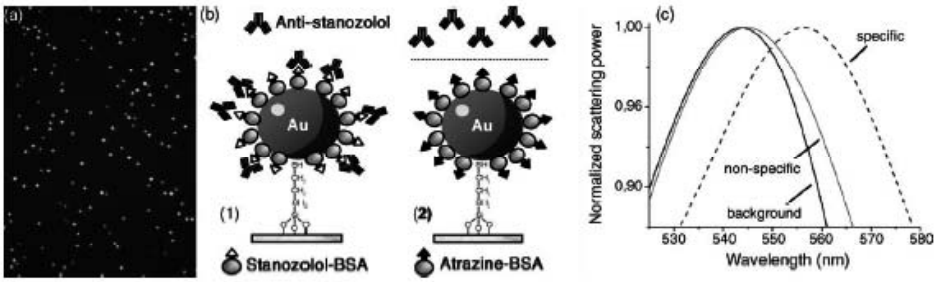


**Figure 3.** (a) Dark-field image of Au particle distribution. (b) Schematic of Au nanoparticles with (1) specific binding of ST-BSA and antiserum and (2) non-specific control using atrazine-BSA. (c) Normalized resonance curves obtained for both conditions described in caption b.

## Conclusions

Research performed at the Applied Molecular Receptors Group of the CSIC (Barcelona, Spain) regarding immunosensors for residue analysis has been presented. Progress in this research area is only possible if research groups from complementary areas (material science, microelectronics, biochemistry organic and analytical chemistry, etc.) work in close collaboration. The optical and electrochemical immunosensors presented here show great potential regarding detectability achieved. However, implication of analytical chemists and researchers willing to work in this interface area is essential to move forward and to really increase the efficiency of analytical laboratories with new technologies. Thus, further work should be addressed to a critical evaluation and validation of the performance of these technologies in real samples.

## Acknowledgements

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## O 28

# DESI MASS SPECTROMETRY: A RAPID SCREENING TOOL FOR ILLEGAL HORMONE PREPARATIONS AND FORENSIC SAMPLES FROM HORMONE CRIME AND VETERINARY DRUG MISUSE

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**Abstract**
Hormone and veterinary drug screening and forensics can benefit from the recent developments in Desorption ElectroSpray Ionisation (DESI) mass spectrometry (MS). Using a linear ion trap or quadrupole time-of-flight MS instrument both full-scan and data-dependent collision induced dissociation MS/MS spectra can be acquired in seconds without any sample preparation. Thus the presence of fake or banned substances can be rapidly disclosed. Data are presented for the rapid screening of food supplements, steroid cocktails and forensic investigations in on-farm medicine mixers. It is envisaged that DESI will achieve a prominent role in hormone and veterinary drug analysis in the near future.

**Introduction**
Desorption mass spectrometry (MS) has been applied for decades towards the characterisation and structure elucidation of involatile chemical substances. Desorption chemical ionisation (DCI), field desorption (FD), fast atom bombardment (FAB), secondary ion mass spectrometry (SIMS), laser desorption ionisation (LDI), matrix-assisted or surface-enhanced laser desorption ionisation (MALDI and SELDI) have been used successfully, but all share a common disadvantage: the probing and ionisation of the sample occurs under vacuum thereby changing the local environment prior to analysis. Although MALDI can be done at atmospheric pressure, the presence of the matrix is significantly altering the sample environment and moreover, interferes with the detection of low molecular mass substances. In 2004 a novel ambient ionisation technology, called Desorption ElectroSpray Ionisation (DESI), was proposed by the group of Cooks at Purdue University (Takáts et al., 2004). For the first time, surfaces could be probed and analysed by mass spectrometry under real native ambient conditions, without any sample preparation or matrix addition. A typical lay-out of a DESI source is shown in Figure 1. In short, a pneumatically assisted electrospray is used to produce charged microdroplets and gas phase solvent ions that are directed onto the sample present on a surface. The ionisation process closely resembles conventional ESI-MS; however, the sample is not present in the solvent nor ionized during the electrospray process, and is therefore less vulnerable to ionisation suppression caused by the presence of salts and other interfering matrix components. Similar to conventional atmospheric pressure ionisation MS, a chemical ionisation version has been developed as well (Chen et al., 2007). In DESI reagents can be added to the electrospray solvent in order to tune the selectivity and the sensitivity of the DESI process, being an advantage over the DART (direct analysis in real time) ionisation alternative (Cody et al., 2005). Depending on the capabilities of the

MS, targeted (selected ion mode), untargeted (full scan mode) or accurate mass analysis is possible. Many successful applications of DESI in forensic and pharmaceutical sciences have been described in

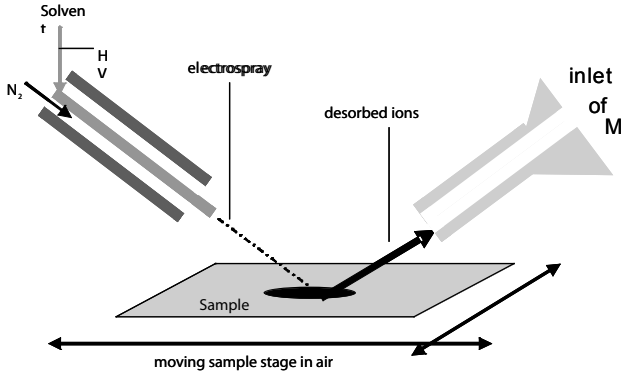



**Figure 1.** Schematic view of a DESI source.

literature (Takáts et al., 2005; Cooks et al., 2006; Rodriguez-Cruz, 2006 ; Williams et al., 2006 ; Kauppila et al, 2007). Very recently, DESI was even successfully applied to the analysis of anabolic steroids in urine, following a simple dipping of an SPME fiber into the raw undiluted urine and in situ derivatisation with hydroxylamine present in the spray (Huang at al., 2007).
In this paper, DESI and its application to the rapid MS and MS/MS screening of hormone preparation findings and forensic samples from related crime investigations will be discussed. Results will be presented for food supplements, steroid cocktails and forensic investigations in on-farm medicine mixers.

**Experimental**
DESI-MS experiments were performed using a Waters (Manchester, U.K.) model QTOFmicro mass spectrometer equipped with a slightly modified Z-source™ and a
pair of tweezers, paper card or wooden spatula to position the sample in between the electrospray and the sample cone (Figure 2a). Alternatively a more dedicated DESI-MS set-up was used consisting of a Prosolia (Indianapolis, IN, U.S.A.) DESI ion source equipped with a rotational and x-y-z positioner, a sample stage with x-y-z positioner, 2 CCD cameras with x60 magnifying optics and associated monitors, and fitted onto a Thermo Fisher (San Jose, CA, U.S.A.) model LXQ linear ion trap (IT) mass spectrometer (Figure 2b). Full scan and data-dependent MS/MS product ion spectra were acquired.

a b

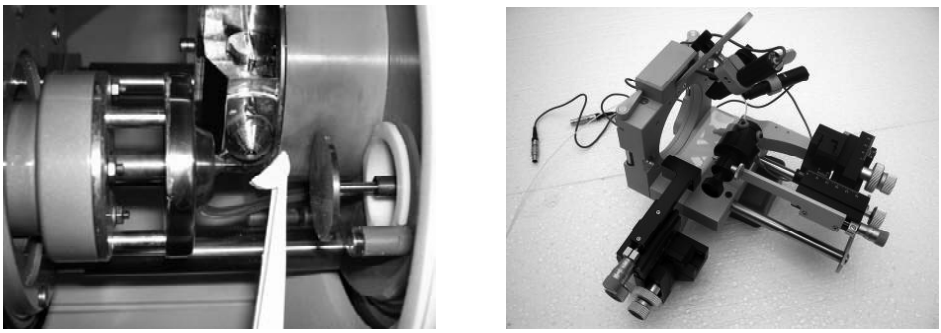


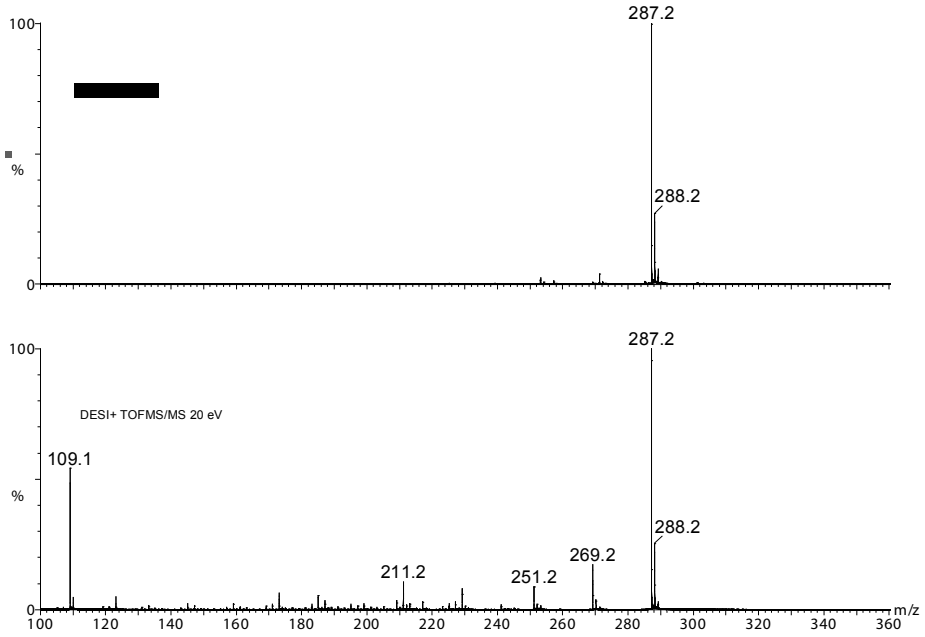
**Figure 2.** DESI ion sources: a, based on a conventional Waters Z-source™ and b, dedicated DESI ion source from Prosolia Inc.

The DESI spray consisted of methanol, water and formic acid (50:50:0.1) and was delivered by the integrated syringe pump at 2.5 µl/min. The desolvation gas was nitrogen (100 psi) and the electrospray needle was at 4.5 kV. Different surfaces for sample deposition were evaluated: PMMA, glass, PTFE, paper. For liquid samples an aliquot of 1.0 µl was pipetted onto the surface. Solid samples were fixed on the surface by using double-sided tape.

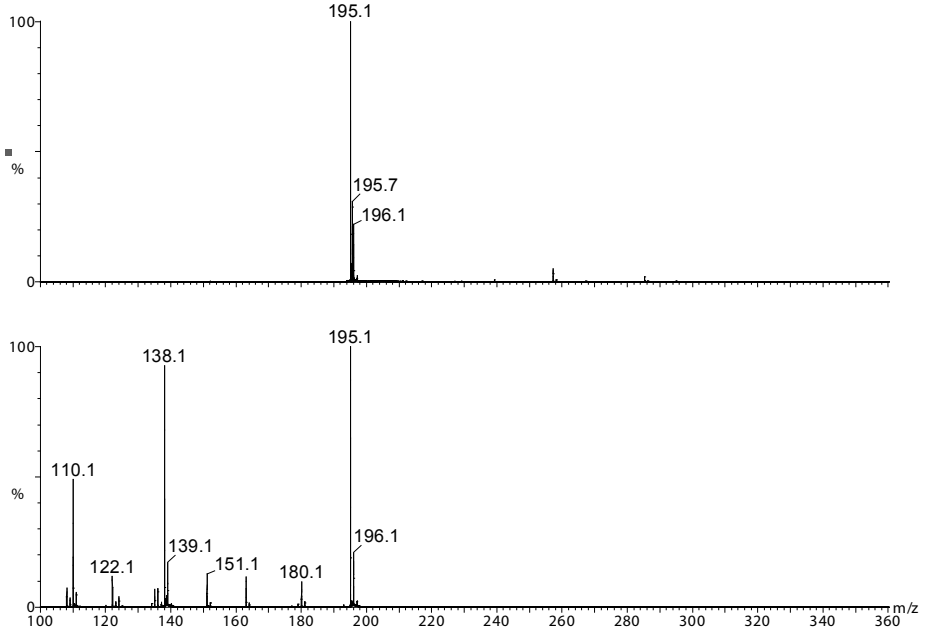
**Results and discussion**

*Screening of food and feed supplements.*
Despite the ban on prohormones in the U.S.A. many preparations can still be ordered through the internet or simply purchased in a pharmacy or a health or organic product shop. Many gaps exist in the European supplement and herb legislation. A specific problem is the lack of quality control: many products do not contain the declared ingredients or even contain banned substances. DESI-MS can provide a rapid screening as illustrated by the examples given in Figure 3a-c. In Fig. 3a the positive ion DESI/QTOF MS and MS/MS spectra are shown for Androcomplex, a supplement. According to the declaration of ingredients each capsule should contain Tribulus terrestris, 4-androstenedione, dehydroepiandrosterone (DHEA), lysine and zinc amine acid chelate. Indeed m/z 287 likely represents the $[M+H]^+$ ion of the prohormone 4-androstene-3,17-dione but the $[M+H]^+$ and $[M+H-H_2O]^+$ ions of DHEA are much less present at m/z 289 and 271 than expected. In Fig. 3b the mass spectra of Vytech 17HD are presented, a supplement which should contain amongst others "17-halo-methyl-dianadrone", beta-ecdysterone and wild yam extract. In reality however the $[M+H]^+$ ion and fragment ions of caffeine are mainly present, indicating a fake anabolic supplement. In Fig. 3c the MS and two MS/MS spectra at 20 and 40 eV collision energy are shown for "Methoxy-TRN", advertised as 17β-methoxytrenbolone and suggested to escape from control. In reality this preparation contains trenbolone itself, being a well-known anabolic steroid banned in the EU.

a


287.2
288.2
DESI+ TOFMS/MS 20 eV
109.1
211.2
251.2
269.2
m/z

b


195.1
195.7
196.1
138.1
110.1
139.1
122.1
151.1
180.1
m/z

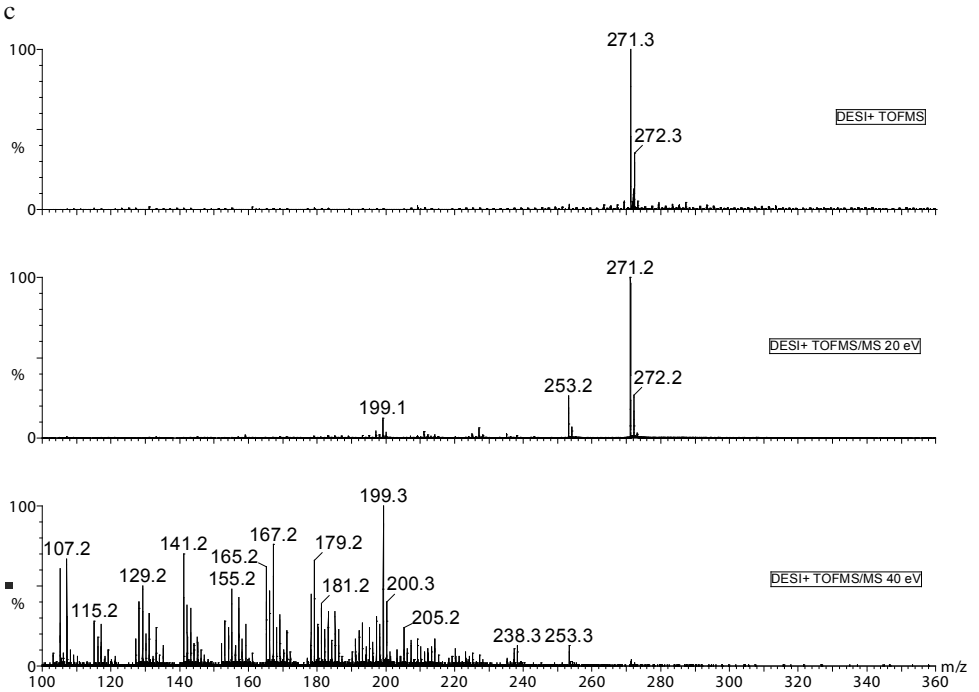



**Figure 3.** Positive ion DESI/QTOF MS and MS/MS spectra of (a) Androcomplex, (b) Vytech 17HD, and (c) Methoxy-TRN

In contrast to direct infusion electrospray ionisation mass spectrometry DESI is less vulnerable for suppression caused by co-introduction of salts, detergents, etc. The secondary ions generated in DESI are the result of the interaction between charged solvent droplets and/or primary ions and the sample molecules in the solid state. Depending on the relative proton affinity some analytes might be more favourably ionised than others, i.e. the DESI mass spectra in Figure 3 do not necessarily reflect the real quantitative composition of the preparations investigated.

*Analysis of steroid cocktails.*

Steroid cocktails are usually analysed by TLC, GC/MS or LC/MS. A much faster screening result can be obtained by DESI MS. In Fig. 4 an example is given of a drop of a steroid cocktail analysed by DESI/QTOFMS. The possible steroid-related explanation of the $[M+H]^+$ ions is given in Table 1. Indeed the main components could be confirmed by H-NMR and according to LC/TOFMS both norethandrolone and methyltestosterone were present. Chlorotestosterone could not be confirmed by H-NMR and LC/TOFMS. On the other hand, estradiol benzoate was confirmed but not observed in the DESI mass spectrum at m/z 377.

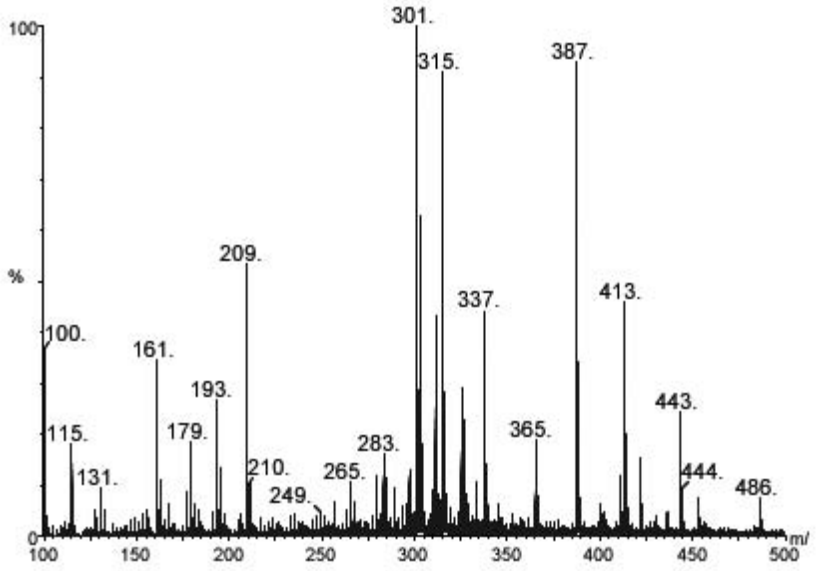



**Figure 4.** Positive ion DESI/QTOF MS spectrum of a steroid cocktail found in a veterinary control programme. For explanation, see table 1.

**Table 1**. Possible steroid-related ions observed in the DESI spectrum of figure 4.

| m/z | can be explanation by the presence of |
|---|---|
| 453 | Boldenone undecenoate |
| 443 | Testosterone decanoate |
| 421 | Testosterone phenylpropionate |
| 413 | Testosterone cypionate |
| 387 | Medroxyprogesterone acetate |
| 365 | Chlorotestosterone acetate |
| 337 | Fluoxymesterone |
| 327 | ? |
| 325 | Chlorotestosterone ? |
| 315 | Progesterone |
| 311 | ? |
| 303 | Methyltestosterone / Norethandrolone |
| 301 | Methylboldenone |

Despite these small discrepancies DESI showed to be a rapid screening tool for complex steroid cocktails and will be the preferred choice when large numbers of unknown preparations have to be screened in a short time.

*Forensic investigations.*
In the past samples for hormone and veterinary drug analysis could be simply classified as preparations (syringes, needles, vials, bottles), feed or biological samples for residue analysis such as urine and hair. Nowadays our laboratory is receiving an increasing number of samples from associated forensic investigations at the farm premises. These include dust samples collected using miniature vacuum cleaners, sweep samples, stirring bars, containers and samples from on-farm medicine mixers. In all cases DESI has the potential for a rapid screening focused on the abuse of steroids and β–agonists, and/or the presence of potentially misused licensed veterinary drugs. In Fig. 5 an example is given of DESI/ITMS featuring an $MS^3$ spectrum from a medicine mixer which was rinsed out with methanol.

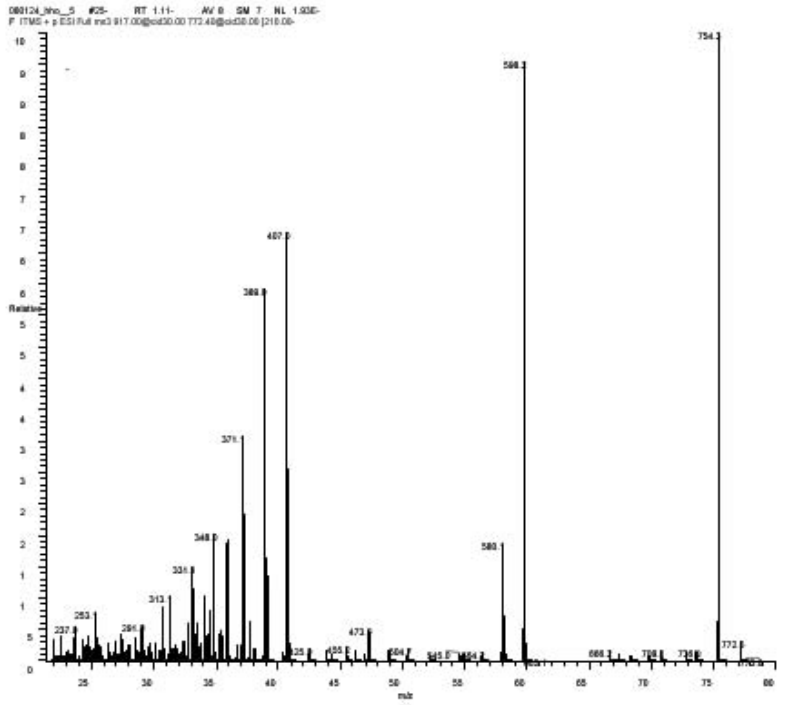



**Figure 5.** Positive ion $MS^3$(916.5->772.5->) spectrum from a methanol rinse of an on-farm medicine mixer.

The full-scan mass spectrum showed the $[M+H]^+$ ion at m/z 916.5 and the MS/MS spectrum m/z 772.5 caused by the loss of a glyco-substructure, followed in $MS^3$ by a loss of water, a second and a third glyco-substructure. The same sample was also analysed by UPLC/TOFMS which confirmed the presence of tylosine. However, tetracyclines were detected as well and missed in the DESI spectrum. Next it was checked whether direct infusion ESI/ITMS of tetracyclines worked out and indeed these antibiotics could be detected without any difficulties. In DESI secondary ionisation must occur from a surface. Different surfaces were evaluated but so far tetracyclines do not ionise in our DESI trials. Even the addition of a complexing agent such as EDTA did not provide a solution to this surface problem.

## Conclusion

DESI mass spectrometry yields answers to real-or-fake questions related to (pro)hormone preparations and supplements in seconds, without any sample preparation. Even more complex samples such as steroid cocktails and unknown samples from forensic investigations can be analysed that fast, but spectrum interpretation and reporting time would definitely benefit from the availability of an $MS^n$ library of known hormones and veterinary drugs. DESI and other ambient mass spectrometry approaches are emerging screening tools in forensic and pharmaceutical sciences. The number of papers in literature is exponentially increasing. It is expected that DESI combined with accurate mass time-of-flight or $MS^n$ ion trap will be increasingly used in hormone and veterinary drug screening, even maybe for residues in urine as demonstrated by Huang et al. (2007).

## Acknowledgements

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# Posterpresentations

P 1 Determination of Dapson in meat and milk by Liquid Chromatography Tandem Mass Spectrometry.
Hadjigeorgiou, et all., Nicosia, Cyprus.

P 2 Total radioactive residue depletion of tritium labeled ivermectin in finfish Shaikh, Badar Laurel, Maryland, USA

P 3 Development and validation of a confirmatory method for the determination of sulfonamides in milk by HPLC with diode array detection.
Dusi, Brescia, Italy

P 4 Determination of tetracyclines in chicken meat by a liquid chromatographic – electrospray tandem mass spectrometric multiresidue method.
De Ruyck, Melle, Belgium.

P 5 Development and validation of a confirmatory method for the determination of resorcylic acid lactones in urine sample by Liquid Chromatography – Tandem mass spectrometry.
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P 6 Histological alterations in veal calves treated with anabolic steroids and a corticosteroid.
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P 8 Demonstration of the treatment of dairy cows with recombinant bovine somatotropin.
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P 9 Confirmation and quantification of 19 ß-agonist compounds in bovine liver by LC-(ESI)MS/MS. Method validated according to CD 2002/657/EC.
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P 10 A confirmatory method for the determination of tetracyclines in muscle with monolithic column HPLC and diode-array detection.
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P 11 About the decision limit for permitted substances.
Galarini, Perugia, Italy

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Crooks, Belfast, UK

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P 16 Validation of an ELISA Test Kit for the determination of fluoroquinolones residues in honey.
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P 18 Determination of Chloramphenicol in Urine Sample Confirmatory GC-MS/NCI Method Using Molecular Imprinted Polymer (MIP) Clean-up.
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P 19 Application of ELISA for the screening analysis of nitrofuran residues in eggs and other edible tissues.
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Niemi, Helsinki, Finland

P 23 Validation of a multiresidue method for UPLC-MS/MS analysis of antibiotics in poultry muscle.
Joaquim, Barcelona, Spain.

P 24 Ion suppression study for tetracyclines in feed.
Joaquim, Barcelona, Spain.

P 25 Tetracyclines in Milk monitoring: the results of a inter-laboratory study.
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P 27 Determination of Lincosamides milk by LC-MS/MS.
Lindfors, Helsinki, Finland

P 28 Validation of an ELISA screening for quinolones in egg and poultry muscle.
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P 29 Depletion of chloramphenicol in trouts after a hypothetic therapeutic treatment.
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P 30 Antibody production: low dose immunogen vs. low incorporation hapten using salmeterol as a model.
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P 31 A replacement for Freund's adjuvant for the production of polyclonal antibodies to veterinary drugs in rabbits.
Fodey, Belfast, UK

P 32 Determination of ten sulphonamides in egg by Liquid Chromatography-tandem mass spectrometry.
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P 33 A Comparison of the FAST, Premi® and KIS™ Tests for Detection of Antibiotics in Beef Kidney Juice and Serum.
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P 34 Degradation of AHD 13 C3 and SEM 15 N2 13 C used as internal standards in nitrofuran residue analysis.
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P 35 Residues of Eprinomectin in sheep milk products.
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P 37 HPLC method for the determination of quinolone residues in various muscle tissues.
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P 39 Flow cytometric immunoassay for sulfonamides in milk, blood serum, meat drip and eggs.
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P 40 Multiplex flow cytometric immunoassay for drug residues using the xMAP technology.
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P 42 Elimination kinetic of 17beta-estradiol 3-benzoate and 17beta-nandrolone laureate ester metabolites in calves urine.
Pinel, Nantes, France

P 43 Exposure assessment of prepubertal children to gonadal steroid hormones.
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P 44 Rapid multi-residue confirmation of antibiotics in muscle by liquid chromatography-tandem mass spectrometry.
Granelli, Uppsala, Sweden

P 45 Control of Cortisol Misuse in Cattle by GC-C-IRMS after a Single Dose Administration.
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P 46 Elimination kinetic of ecdysteroids metabolites and metabolomic fingerprints in calves urine by hyphenated mass spectrometry methods.
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P 47 Determination of a relevant threshold for the control of natural steroid abuse in cattle by GC-C-IRMS.
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P 48 Attempt of discrimination between control versus animals treated with growth hormone using untargeted LC-MS-HRMS metabolomic urinary measurements.
Pinel, Nantes, France

P 49 Rapid multi-residue screening of antibiotics in milk by liquid chromatography-tandem mass spectrometry.
Branzell, Uppsala, Sweden

P 50 Metabolism of boldenone by primary cultures of bovine hepatocytes.
Gallina, Legnaro, Italy

P 52 Validation of a commercial receptor binding assay for sulfonamides in muscle, egg and milk according to the commission decision 2002/657/EC criteria.
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P 53 Uncontrolled oxidative processes of prednisolone in bovine liver extrats: Naturally occurring or method dependent?
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P 54 High- throughput analysis of boldenone and its metabolites calf urine.
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P 56 Detection of Glucocorticoid Bioactivity in Bovine Urine Samples using a Reporter Gene Assay.
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P 57 Dispersive Solid Phase Extraction Cleanup of Tissue Extracts for Veterinary Drug Residue Analysis.
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P 58 Determination of Erythromycin and Associated Degradation Products in Honey.
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P 68 Quantitative analysis of natural hormones in serum and urine from cattle.
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P 70 Analysis of anabolic glucuronide and sulphate conjugates in urine with LC-MS/MS.
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P 71 Confirmation and screening analysis of a large number of steroids in meat using GC-MS/MS.
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P 72 Development of a LC-TOF multi residue method for anabolic compounds in meat.
Sterk, Bilthoven, The Netherlands

P 73 Sample preparation of beta-agonists in urine, comparison of two different techniques; mixed-mode solid phase extraction and molecular imprinted polymers extraction.
Sterk, Bilthoven, The Netherlands

P 74 Validation of a rapid and sensitive routine method for determination of chloramphenicol in honey by LC-MS/MS.
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P 75 Biosensor immunoassay for (fluoro)quinolone antibiotics in fish, egg, and poultry meat.
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P 76 Ringstudy on Salmeterol in Bovine hair.
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P 93 Development of an LC-MS/MS method for the determination of aminoglycosides in animal feed.
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P 94 HAIR! A useful or useless matrix for monitoring growth-promoting agents?
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P 95 Development and validation of a liquid chromatographic-tandem mass spectrometric method for the detection of ten (fluoro)quinolones in milk.
Daeseleire, Melle, Belgium

P 96 Integrated System for the Parallel Detection of Multiple Antibiotics in Milk.
Suarez, Neuchatel, Switzerland

P 97 Confirmatory analysis of malachite green and leucomalachite green in muscle samples of eels, herrings and mackerels applying HPLC-ESI-MS/MS.
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P 98 Synthesis and evaluation of the binding capacities of a molecularly imprinted polymer (MIP) for penicillin G.
Royen, Melle, Belgium

P 99 Multiresidue Determination of Triarylmethane and Phenothiazine Dyes in Fish Tissues.
Tarbin, York, UK

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Carraro, Legnaro, Italy

## P 1

# DETERMINATION OF DAPSON IN MEAT AND MILK BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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**Abstract**
Within the EU the use of Dapson is prohibited in food producing animals; consequently, it's included in the Annex IV of the Directive 90/2377/EC. A quantitative confirmatory method has been developed and validated according to the criteria defined in the Commission Decision 2002/657/EC, for the determination of Dapson in meat and milk. Samples, after homogenization in alkaline conditions and organic solvent extraction, were purified on silica gel solid phase extraction cartridges. The eluate was evaporated and redissolved in mobile phase and was analyzed by Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS) in positive electrospray ionization using deuterium labeled Sulphadimidine-d7 as internal standard. The calculated value for CCα is 0.12 μgkg-1, and the CCβ value is 0.16 μgkg-1.

**Introduction**
Dapson (4,4-diaminodiphenylsulfone) is a synthetic sulfone with bactericidal and bacteriostatic activity [1]. Dapson is used as an antibiotic in animals to prevent and treat diseases. The most prominent side effects of this drug are dose-related hemolysis (which may lead to haemolytic anaemia) and methemoglobinemia [2]. Due to dapson, abnormalities in white blood cell formation, including aplastic anaemia are rare, but cause the majority of deaths [3-5]. For these reasons it has been prohibited from use in food-producing animals according to the Council Directive 90/2377/EC [6]. Therefore analytical methods are needed for monitoring their use through analysis of samples of animal origin such as meat and milk. Such analytical methods must meet the performance criteria set by the European Commission Decision 2002/657/EC [7] to detect and confirm the presence of dapson in food products. Literature on the determination of dapsone in food products is limited, and very few methods have been published. Schuh et al. [8] developed a procedure for the determination of dapson and sulfonamides in muscle, liver and kidney using HPLC-DAD. LC-MS/MS methods for the determination of sulfonamides and dapson in milk have also been implemented with low detection limits [9, 10].
LC-MS/MS is indeed a promising technique for the detection of dapson and therefore this technique has been used in this work. The extraction procedure of the method developed was based on the confirmatory method for the determination of sulfadimidine in samples of animal origin such as muscle, liver, kidney and fat by high performance liquid chromatography with photodiodarray detector [11].

## Materials and Methods

*Chemicals and reagents*

Dapson was purchased from Riedel-de-Haen and the internal standard, sulphadimidine-d7 was purchased from FSL Norwich. Stock solutions of Dapson and sulphadimidine-d7 were prepared in methanol at concentrations of 1000 mgL-1 and 100 mgL-1 respectively. Stock solutions were stored at -18°C. Working standard solutions (10 ngL-1, 1 ngL-1, 0.06 ngL-1, 0,006 ngL-1) were freshly prepared by further dilution with methanol.

Residue grade dichloromethane and acetonitrile were purchased from Labscan and methanol residue grade was purchased from Merck. Analytical grade sodium carbonate and sodium hydrogen carbonate were obtained from Merck and ammonium formate for mass spectroscopy was obtained from Fluka (Sigma Aldrich). Water was deionised by a Milli-Q Plus Water System. Samples cleaned by Solid-phase extraction (SPE) silica gel columns (vac 3cc, 500mg, Waters).

A pH 10 carbonate buffer solution was freshly prepared by mixing 20 ml of 0.2M sodium carbonate solution, 30 ml of 0.2M sodium hydrogen carbonate solution and 130 ml water. The pH was adjusted to 10 with further addition of 0.2M sodium hydrogen carbonate solution and the buffer was diluted to 200 ml with water.

*Equipment*

A MPW-350R centrifuge, an Orbital Selecta platform shaker and a Turbo Vap nitrogen evaporator, obtained from Caliper Life Sciences, were used during the extraction procedure. The liquid chromatography consisted of a Water Alliance 2695 separations Module equipped with a column oven (Waters, Milford, MA, USA). The LC column used was a Phenomenex, Luna 5µ C18(2) 100A column (150mm x 2mm, 5µm) with a precolumn of the same packing (Phenomenex C18, 4mm x 2mm). The column and the precolumn were kept in a column oven at 40$^0$C. A Micromass Quattro Premier XE (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source was operated in positive ion mode. Mass Lynx Software, version 4.1, was used for instrument operation and data handling. The instrument was tuned by infusing dapson and sulphadimidine-d7 standards. The capillary voltage was 1 kV, the source temperature was 120$^0$C and the capillary temperature was 350$^0$C. The cone and desolvation gas were 50 and 900 L/hr respectively. The parent ions, the daughter ions, the cone voltage and the collision energies are shown in table 1.

The final instrumental procedure consisted of a 12 min isocratic LC program of (80:20, v/v) 1mM Ammonium Formate-Acetonitrile at 250 µl.min-1 using 10 µl injections. The divert valve was switched to the MS at 3.5 min and to waste at 9 min.

Data were acquired according to the multiple reaction monitoring (MRM) approach, by selecting the most intense transition reaction from dapson and sulfadimidine-d7.

**Table 1.** Data acquisition method for analysis by LC-MS/MS

| Name (abbreviation) | MRM transition (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|
| Dapson (DAP) | 249.25>156.15 | 0.15 | 14 |
| | 249.25>92.25 | 0.15 | 25 |
| | 249.25>108.25 | 0.15 | 20 |
| Sulfadimidine-d7 | 286.18>193.18 | 0.15 | 22 |

*Sample preparation*
Samples were kept frozen <-18$^0$C, till analysis. Meat samples were cut into pieces and homogenized in a blender obtained from Braun. 2.5g of the homogenized tissues of muscle were weighed in a polypropylene centrifuge tube. Milk samples (2.5ml) were transferred into polypropylene centrifuge tubes.

*Extraction procedure*
Blank samples were fortified with dapson, at the level of CCα and 5 μgKg-1. All the samples, the spiked samples and the blank reagent were spiked with internal standard (sulphadimidine-d7) and were equilibrated for 30 min. In all samples 2 ml of carbonate buffer pH 10 were added to enable the extraction of dapson in the organic solvent. Samples were vortexed for approximately one minute. 15 ml of dichloromethane was added to the samples and extraction was supported on a platform shaker at 230 rpm for 15 min. After centrifugation at 3500 rpm and 4$^o$C for 5 min, the aqueous supernatant liquid was discarded and the samples were placed at -18oC for 30 min.
A silica gel SPE column was conditioned with 6 ml dichloromethane under gravity. The liquid organic phase (sample extract) was passed through a glass wool and applied to the silica gel column via a 20 ml syringe barrel reservoir under gravity (no vacuum). After the entire sample extract was applied, the syringe barrel reservoir walls were washed into the silica gel column with 6 ml dichloromethane. Vacuum (10 psi) was then applied to the column for 10 min. The analytes were eluted with 6 ml of methanol under gravity. This methanol phase was evaporated under a stream of nitrogen at 40 $^o$C. LC-MS/MS standards, were also placed in the Turbo Vap Nitrogen evaporator to evaporate the methanol solvent. Both samples and standards were redissolved in 200 μl mobile phase, placed in the sonicator for 1 min and vortexed for 30 seconds. Centrifuging the samples at 12500 rpm, after vortex, for 5 min produced a cleaner sample. The cleaning step was supported by filtration of the samples through 0.22 mm filters into LC vials.

**Results and discussion**
The method was validated for the determination of dapson in meat according to the procedures described in the Commission Decision 2002/657/EC) [3] covering several performance characteristics, i.e. specificity/selectivity, precision (repeatability, intra laboratory reproducibility), linearity, accuracy, decision limit (CCα) and detection capability (CCβ).
The accuracy, precision, CCα and CCβ were determined by spiking 24 samples at four differed concentration levels ( six replicate at each level). In order to prove the intra laboratory reproducibility of the method a variation in experimental conditions such as

the analyzer and the day of analysis were tested. The results of the validation study are summarized in Table 2.
The repeatability was expressed as the relative standard deviation of the amount within days and the intra-laboratory reproducibility between days. These results were in agreement with the Horwitz accepted limits for each concentration. Lower repeatability and reproducibility were obtained at level of 0.05 μgkg- 1. Also, reproducibility was used to estimate the measurement uncertainty, since reproducibility was the highest contributing factor of error

**Table 2.** Recovery, repeatability, in house reproducibility, CCα and CCb

| Spike level (μgkg- 1) | Recovery (%) | Repeatability (%) | Reproducibility (%) | CCα ($\mu gkg^{-1}$) | CCb ($\mu gkg^{-1}$) |
|---|---|---|---|---|---|
| 0.05 | 138 | 32.8 | 38.6 | 0.12 | 0.16 |
| 0.1 | 118 | 14.6 | 18.5 | | |
| 0.35 | 101 | 12.8 | 16.1 | | |
| 0.60 | 78.9 | 22.9 | 24.5 | | |

Decision limit (CCα) is the limit at and above which it can be concluded with an error probability of α (1%) that a sample is not compliant and is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. Detection capability (CCβ) is the smallest content of the substance that may be detected, identified or quantified in a sample with an error probability of β (=95%) and is the concentration corresponding to CCα + 1.64 times the standard error of the intercept.
Based on the results for CCα it was concluded that analytes at a mass concentration of 0.12μg/kg can be reliably detected. The identification criteria i.e the relative retention time, the relative abundances of the diagnostic ions and the signal to noise were fulfilled. This was proved by comparison with a standard in of the closest concentration in solvent.
Analyte was quantified using matrix-matched calibration standards i.e meat samples spiked with Dapsone and internal standard prior to sample preparation. The linear regression method was used for the evaluation of the correlation factor of X, Y values and the statistical technique of analysing the regression (ANOVA) was used to estimate the fit of the relation to the linear model. The statistical processing of the analytical results proved a satisfactory relation between the concentration and the ratio of the area of the spiked analyte peak to the area of the internal standard peak, $r2 \geq 0.96$. Moreover, the regression factor b proved to be close to zero (t-exp<t-theor) and no significant deviation from the linear model was observed after the application of F-test.
For the study of specificity and selectivity, that is the ability to differentiate between target analytes and interferences, 20 blank meat samples from different animals where analyzed. No significant matrix effects were observed. In addition, 10 different milk samples where analyzed and no interfering peak for the determination of dapsone was observed.
The applicability of the method in milk samples was proven by analysing twelve spiked, six for meat, six for milk samples at a concentration level equal to the CCβ. The F-test showed no statistical difference between the (relative) standard deviations calculated for the meat and milk assays and t-test showed no statistical difference between the two mean values.

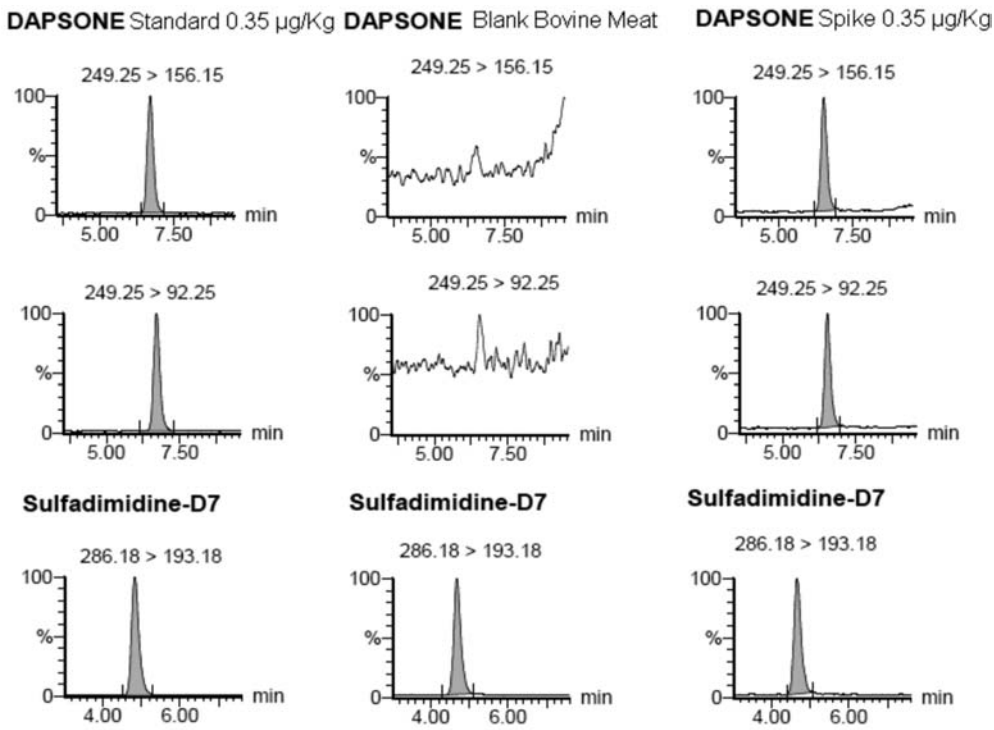


**Figure 1.** Representative MRM chromatograms of a standard, a blank bovine meat sample and the same sample spiked with dapsone and internal standard

## Conclusion

The above method which was developed for the identification of dapson in meat and milk, based on liquid chromatography-positive electrospray tandem mass spectrometry proved to be reliable. The simple sample preparation protocol including solid phase extraction and the addition of internal standard has led to a sensitive and robust method. The achieved confirmatory limits proved the efficiency of this method for the control of trace levels in meat and milk.

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## P 2

# TOTAL RADIOACTIVE RESIDUE DEPLETION OF TRITIUM LABELED IVERMECTIN IN FINFISH

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**Abstract**
Atlantic salmon, tilapia and catfish, were given a single oral dose of 0.1 mg/kg of 3H-ivermectin (9-10 μCi). Six fish of each species were sacrificed and necropsied on post dose days 1, 7, 14, 21 and 28. Filet muscle (with adhering skin, except of catfish) from each fish was removed and homogenized in dry ice. An aliquot of each homogenized muscle tissue was combusted in a sample oxidizer and the resulting solution counted using a liquid scintillation counter for total radioactive residue (TRR) determination.
Of the three fish species studied, the TRR of $^{3}$H-ivermectin depleted fastest from tilapia, a warm water fish and slowest from Atlantic salmon, a cold water fish. The following depletion order was followed by the three fish species: tilapia > catfish > Atlantic salmon. The TRR's of tilapia and catfish were < 10 μg/kg on post dose days 21 and 28, respectively and the TRR of Atlantic salmon was approximately 20 μ/kg on post dose days 28.

**Introduction**
Ivermectin, a broad spectrum antiparasitic drug, is a member of the family of compounds isolated from the fermentation products of the soil microorganism Streptomyces avermitilis, known generically as avermectins (1, 2, and 3). Ivermectin is approved for veterinary use in cattle, sheep, and swine by US Food and Drug Administration (4) but not approved for use in fish. Ivermectin has been shown to be highly efficacious in the treatment of sea lice and other parasitic copepods (5, 6, and 7). Ivermectin is the 22, 23-dihydroderivative of avermectin B1 and consists of two closely related components, containing no less than 80% of the 22, 23-dihydroavermectin B1a ($H_2B_{1a}$) and no more than 20% of the 22, 23-dihydroavermectin $B_{1b}$ ($H_2B_1$b). Figure 1 shows the structure of ivermectin ($H_2B_{1a}$). Ivermectin is highly effective anthelmintic, at extremely low doses, towards a variety of endo- and ectoparasites in many host species, including food and companion animals and humans (8 and 9). The disposition of $^{3}$H-ivermectin in Atlantic salmon smolts was studied by whole-body autoradiography following a single oral dose by gavage (10). Ivermectin was slowly absorbed, the highest concentrations of ivermectin equivalent residues were found in lipid-containing organs and reached maximum in about 4 days. Recently, we reported (11) the slow depletion of total radioactive residue (TRR) of $^{3}$H-ivermectin in bile and muscle tissue of rainbow trout, after a single oral dose. The TRR levels remained high from post dose day 3 to day 7 and thereafter, declined slowly but were still persistent till day 42. The purpose of the current study was to determine the TRR of $^{3}$H-ivermectin in the muscle tissues of other aquacultured fish species, Atlantic salmon, tilapia and channel catfish.

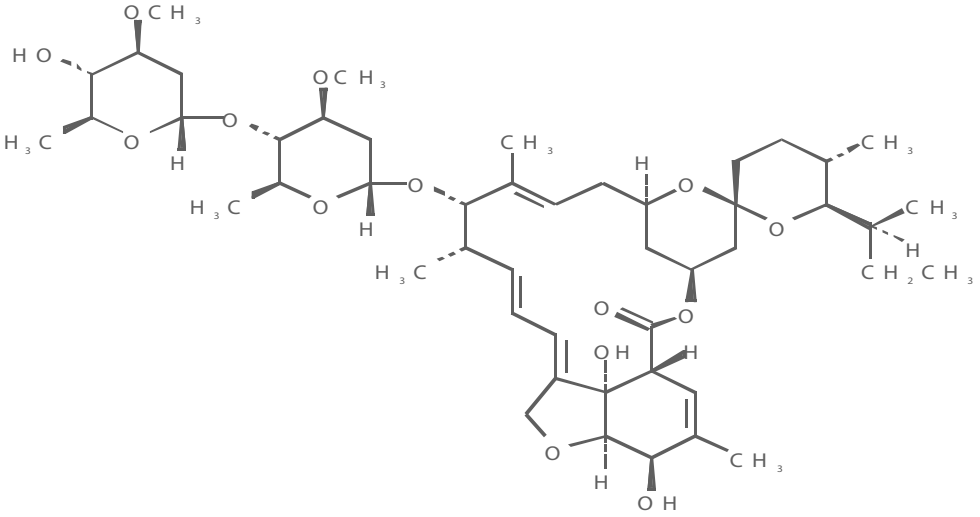

**Figure 1.** Structure of ivermectin $H_2B_{1a}$

## Experimental

*Chemicals*
Tritium labeled ivermectin ($H_2B_{1a}$), labeled at C-22, C23 positions, was synthesized by Perkin-Elmer, Life Sciences Products (Boston, MA, USA) with specific activity of 244.4 mCi/mmol. The drug was re-purified by LC to >98% when needed. The dose was prepared by mixing with unlabeled ivermectin to give a specific activity of 205 dpm/ng (0.081 μCi/μmol) or 212 dpm/ng (0.084 μCi/μmol).

*Apparatus*
A Packard Model 301 Sample Oxidizer (Perkin-Elmer, Life Sciences, Boston, MA, USA) was used to combust the tissue samples. The radioactivity in oxidizer sample vials was enumerated with a Packard Tri-Carb 3100 liquid scintillation counter (Perkin-Elmer Life Sciences, Boston, MA, USA).

*Animal phase*
Atlantic salmon (Salmo salar), tilapia hybrid (Oreochromis nilotica x O. mosambicus), and channel catfish (Ictalurus punctatus) were obtained from commercial or private sources and housed separately in 2000 L re-circulating round fiber glass tanks containing fresh water. Water was maintained at pH of 7.0 ± 0.5 and temperature of 12 ± 2 ºC for Atlantic salmon and 24 ± 2 ºC for both tilapia and channel catfish. The fish were provided ad libitum access to the commercially available diet and were cultured until they reached market weight range. Our laboratory is fitted with four 80-L tanks for dosing with labeled compounds; therefore four fish were weighed at a time and moved to the tanks. The fish were fed drug free gel-food, i.e., gelatin-based fish food (12) daily and allowed to acclimate for at least 7 days and denied food 2 days before dosing. The fish were orally dosed with 0.1 mg/kg of body weight of tritium-labeled ivermectin in size 4 gel-capsules (embedded in gel-food). Tilapia and catfish

were dosed by giving capsules embedded in gel-food nuggets that they had been trained to eat. Salmon and some catfish were given the dose in capsules embedded in gel-food via intragastric tube. Six fish were randomly assigned to each of pre-determined post dose time points of 1, 7, 14, 21 and 28 days. On a sampling day, the tank water was drained to about 20-L and 10-15 g of MS 222 added to the tank water to anesthetize the fish. The fish were removed from the tank and decapitated with a sharp knife. The fish were scaled, muscle fillets with skin removed (except catfish) and stored at 80 ºC.

*Total Radioactive Residue Analysis (TRR)*

Prior to total residue analysis, the muscle fillets were blended to a fine tissue powder as reported previously (11). Triplicate 0.5 g blended samples of muscle were weighed into three combusto-cones containing an absorbent pad and combusted in a continuous flow of oxygen, forming tritiated water using a Packard Sample Oxidizer. Due to high combustion temperatures, water in the form of steam, condensed in an air cooled condenser, and was collected as tritiated water in the tritium counting vial. Uncondensed water was collected in the tritium exchange column and flushed into the tritium counting vial with tritium scintillator (Packard Monophase S). Radioactivity in each vial was enumerated for 2 min with the Packard Tri-Carb- 3100 counter. The recovery of 3H- ivermectin from fortified control muscle tissue was determined throughout sample analyses. Control muscles were fortified with 2504–193429 dpm of 3H-ivermectin and average recovery was 102 % with a coefficient of variation of 5%.

*Results and Discussion*

The mean levels of total radioactive residue (TRR) expressed as nanogram equivalents per gram of muscle tissue of the three species of fish at various time intervals are plotted in figure 2. The TRR peaked on post dose day 1 in catfish, whereas, on post dose day 7 in other two species. The TRR levels in Atlantic salmon were higher than the other two at post dose day 7 and afterwards. The TRR levels at day 7 after the exposure were 41, 22, and 14 µg/kg in Atlantic salmon, catfish, and tilapia, respectively. By day 28, Atlantic salmon and catfish contained 21 and 6 µg/kg, respectively, whereas tilapia had 7 µg/kg by day 21. This indicates that the rate of metabolism and depletion of ivermectin in Atlantic salmon, a cold water fish, was slower than in catfish and tilapia, both warm water fish species. The slower rate of depletion in Atlantic salmon is consistent with our previous study on another cold water fish rainbow trout (11), where the TRR was 40 µg/kg by day 28. HPLC analysis of the muscle tissues of the three species, to determine their marker residue, is in progress and will be reported elsewhere.

In conclusion, the TRR of 3H- ivermectin depleted faster from warm water fish species, tilapia and catfish and slower from cold water species, Atlantic salmon and rainbow trout. The following depletion order was followed by the four fish species: tilapia > catfish > Atlantic salmon > rainbow trout.

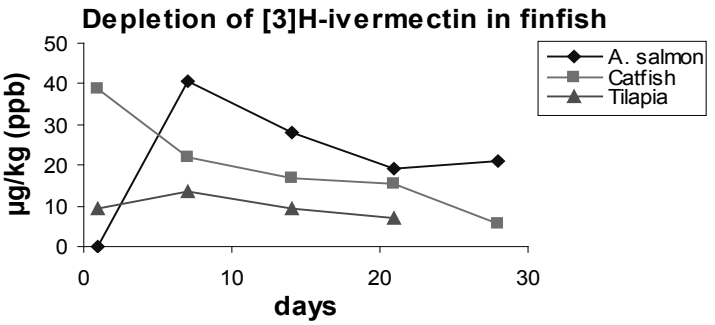


**Figure 2.** Depletion of 3H- ivermectin in finfish

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## P 3

# DEVELOPMENT AND VALIDATION OF A CONFIRMATORY METHOD FOR THE DETERMINATION OF SULPHONAMIDES IN MILK BY HPLC WITH DIODE ARRAY DETECTION

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**Abstract**
A simple multiresidue method for the determination of 7 sulphonamides residues (sulfadiazine, sulfapyridine, sulfamerazine, sulfamethazine, sulfamonomethoxine, sulfadimethoxine and sulfaquinoxaline) in milk samples was developed and validated. The drugs were extracted with a mixture chloroform/acetone and simply cleaned up on a cation exchange solid phase extraction column. The analytes' determination was carried out using HPLC with UV-DAD detection at 270 nm. The procedure was validated as a quantitative confirmatory method according to the EU Decision 2002/657/EC. The developed method shows good linearity, specificity, precision, ruggedness and is able to confirm each sulphonamide residue above 20 µg kg-1. Decision limits around 110 µg kg-1 and recovery above 60% were obtained for all the analytes. The results of the validation process demonstrate that the method is suitable for application, as confirmatory method, in European Union statutory veterinary drug residue surveillance programmes.

**Introduction**
Sulphonamides (SAs) are broad-spectrum antimicrobials used in both humans and animals. They competitively inhibit the bacterial enzyme dihydropholate synthetase. They are commonly used in dairy cattle for treatment or to prevent respiratory or gastro-intestinal tracts infections and mastitis. Beside classic antibiotics, SAs have not lost their interest in veterinary medicine because of their broad-spectrum of activity and low cost. The large scale application of these drugs raises the risk of the occurrence of residues in milk, due to improper observance of withdrawal times. The presence of these drug residues can trigger potential adverse side effects in human, such as allergic reactions in hypersensitive individuals, over long-term health effects, or they can be potential carcinogenic. Prolonged exposure to residue antibiotics can also result in an increase of drug-resistant bacteria. To protect consumers' health, the European Union has adopted for SAs a maximum residue limit (MRL) of 100 µg kg-1 in milk. Following the Decision 98/179/CE, the sanitary controls on the milk destined to the human consumption are very frequent in all the European Community; therefore, monitoring of antibiotic residues is very important in controlling the safety of milk for human consumption. For these reasons, several analytical methods have been developed to determine SAs in milk using high performance liquid chromatography (HPLC) with diode array (DAD) or mass-spectrometry detection. Furthermore, from 2002, the methods

used in official control programmes must be validated according to the validation criteria concerning the performance of analytical methods and the interpretation of results set by EU Decision 2002/657/EC. The present work describes a simple multiresidue method for the determination of 7 SAs residues (Sulfadiazine, Sulfapyridine, Sulfamerazine, Sulfamethazine, Sulfamonometoxine, Sulfadimethoxine and Sulfaquinoxaline) in milk samples, using HPLC with diode array detection. The procedure was validated as a quantitative confirmatory method: specificity, precision, trueness, ruggedness, decision limit (CCα) and detection capability (CCβ) were evaluated for all the seven analytes.

## Materials and Methods

*Chemical and reagents*
All reagents and solvents were of analytical or HPLC grade quality. Acetic acid and Ammonia (30% v/v) were purchased by Carlo Erba (Milan, Italy), all others reagents and solvents were purchased by Sigma (Sigma-Aldrich, Seelze, Germany). Ultrapure water was obtained by Milli-Q system Millipore (Bedford, MA, USA). Aromatic sulfonic acid Speedisk Columns (SPE) (200 mg/6 mL) were supplied by J.T.Baker (Deventer, The Netherlands). Sulfadiazine (SDZ), Sulfapyridine (SPN), Sulfamerazine (SMR), Sulfamethazine (SMZ), Sulfamonomethoxine (SMM), Sulfadimethoxine (SDM), Sulfaquinoxaline (SQX), Sulfamethoxazole (SME), were bought from Riedel-de-Haen (Seelze, Germany). SAs stock solutions at the concentration of 0.1 mg mL−1 in methanol were prepared and stored at 4°C. Suitable working standard solutions were obtained by appropriate dilution of the corresponding stock solution and stored at 4°C.

*HPLC-DAD*
The LC system consisted of a Hewlett-Packard (HP) 1100 series Quaternary Pump, a HP 1100 series DAD detector, a HP 1100 series autosampler all controlled by a computer using HP chemstation software. Separation was carried out on a Supelcosil LC-18 DB (250 x 4.6 mm, 5 µm) column, at room temperature, with a Supelguard LC-18 (20 x 4.6 mm, 5 µm) guard column (both the columns from Supelco, Bellefonte, PA, USA). HPLC eluents were: A) Acetonitrile, B) 2% aqueous acetic acid solution. The gradient initiated with 90% A, continued with a linear increase to 60% A over 16 min and 60% A for 4 min The system returned to 90% A in 1 min and was re-equilibrated for 4 min before the next injection. The flow rate was 1 mL min-1 and the injection volume was 10 µL. The wavelength was set to 270 nm.

*Sample preparation*
Ten grams of milk were weighed and 35 mL of chloroform/acetone solution (65:35 v/v) were added. 100 µL of SME solution at concentration of 10 µg mL−1 was added (100 µg $kg^{-1}$). The mixture was vigorously shaken for 1 min and allowed to stand until the complete separation of the two phases. Organic phase was filtered on a paper filter and collected in a 100 mL flask. The procedure was repeated twice and organic phases were combined and evaporated to dryness in a rotary evaporator. The residue was dissolved in 10 mL of ethyl acetate and transferred in a 50 ml PP-Test tube. The flask was washed twice with 5 mL of ethyl acetate and the organic phases were combined. The organic phase was dewatered with anhydrous

sodium sulphate and loaded on aromatic sulfonic acid speedisk columns (SCX), previously conditioned by 6 mL of n-hexane and 8 mL of ethyl acetate. The SCX cartridge was washed with 2 mL of methanol, then with 5 mL of water and finally with 2 mL of methanol. Analytes elution was done with 5 mL of a mixture methanol-ammonia solution 30% (97:3 v/v). The eluate was evaporated to dryness under a stream of nitrogen. The residue was finally redissolved in 250 µL of a mixture water-acetonitrile (60:40 v/v) and transferred in vials for the HPLC analysis.

*Validation study*

The developed method was fully validated as quantitative confirmatory method according to the EU Decision 2002/657/EC. Parameters taken into account were: specificity, precision, trueness, ruggedness, decision limit (CCα) and detection capability (CCβ). Specificity was tested by analyzing 20 blank milk samples of different origin in order to verify the absence of potential interfering compounds at SAs retention times. Method recovery and precision were evaluated by spiking representative blank milk samples with SAs, resulting in three analytical series, each series with four concentration levels (20 - 50 - 100 - 150 µg kg-1) and six samples per concentration level (6 samples x 4 concentration levels x 3 series → 72 analysis). The same results were used to calculate decision limit (CCα) and detection capability (CCβ). Method ruggedness was estimated for minor changes by means of the Youden robustness test. Seven different factors were chosen in the entire analytical process, because of their possible critical influence. Blank cattle milk samples spiked at 50 µg $kg^{-1}$ were used for all the experiments. Finally SAs instrumental linearity was evaluated by drawing five points calibration curves in solvent, containing a fixed SME amount of 4 µg $mL^{-1}$ with analytes concentrations corresponding to 0.4 – 2 – 4 – 6 - 8 µg $mL^{-1}$.

**Results and Discussion**

The chromatogram of a solvent standard solution in Figure 1 shows very good separation of SAs into a complete run of 25 min. Instrumental linearity was demonstrated for all the analytes in a concentration range from 4 to 80 ng injected using solvent standard solutions in methanol-water (50:50 v/v). Five concentrations, 3 replicates per concentration level, were used to build the regression line using the least square method and to calculate the regression coefficient ($r^2$) and the 95% confidence limits for the y-intercept. Regression coefficients of curves indicated a good fit for all the analytes ($r^2$>0.9996) and confidence limits were satisfactory for every line. Figure 2 shows the chromatogram of a representative blank cattle milk sample. The comparison between the chromatograms reported in Figures 1 and 2 highlighted the specificity of the developed method. The absence of any interfering peaks at the analytes retention times in chromatographic runs demonstrated that the sample clean up procedure was suitable. Trueness was expressed in terms of recovery rate and precision, repeatability (intra-day) and within-laboratory reproducibility (inter-day), as relative standard deviation (CV%). The repeatability and the within-laboratory reproducibility were calculated by using the single factorial analysis of variances (ANOVA). The validation results are reported in Table 1. The data show that the repeatability and the reproducibility for all the analytes were below or equal to 6.4 and 7.7 respectively with recoveries above 60%. Decision limit (CCα) and detection capability (CCβ) were calculated by applying the calibration curve procedure described in EU Decision. Representative blank cattle milk samples were spiked

at 0.5–1–1.5 MRL. As recommended in the Guidelines SANCO/2004/2726 rev.2, we add another spiking level at 20 µg kg-1. This is the lowest experimental concentration at which both qualitative and quantitative criteria are fulfilled. Figure 3 shows the chromatogram of spiked milk at 20 µg $kg^{-1}$.
As regards to the ruggedness, the application of Youden test consists in the introduction of minor simultaneous changes in possible critical factors, chosen in the developed method, according to an established experimental design, with the aim of identifying the critical factors that may have to be controlled in order to obtain accurate assay results. The standard deviation of the differences between two levels of each factor was then calculated. It was demonstrated that all selected factors did not have significantly affect on the analytical performance of the method. Consequently the reported procedure proved to be fairly robust and able to withstand minor fluctuations in the operating factors that may occur in its routine application.

**Conclusion**

A simple method for the simultaneous determination of seven SAs in cattle milk samples was developed and validated according to the EU Decision 2002/657/EC and all parameters were evaluated. The results show that the developed HPLC-DAD method is adequate for the purpose of confirmatory analysis of SAs in cattle milk. In our laboratory it is routinely applied within the Italian Official Residue Control Program.

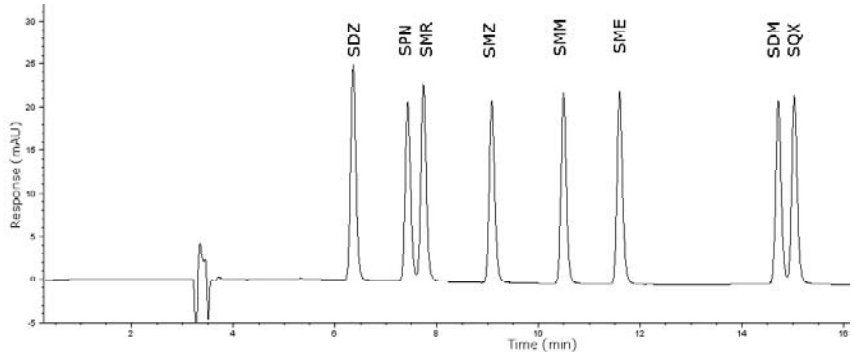


**Figure 1.** HPLC-DAD chromatogram of a solvent standard solution (40 ng inj.)

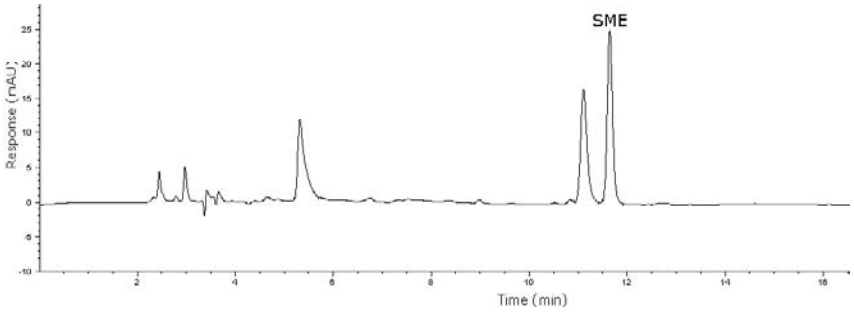


**Figure 2.** HPLC-DAD chromatogram of a representative blank milk sample

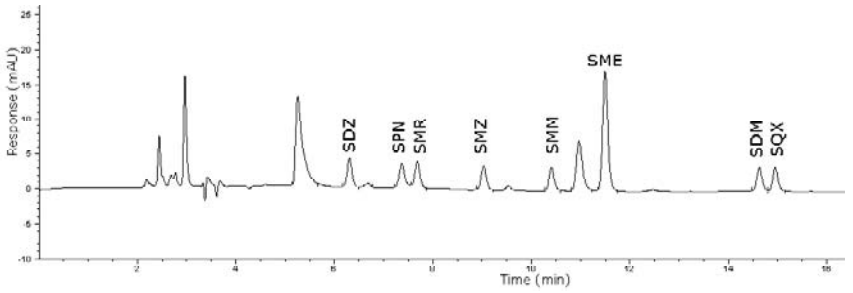


**Figure 3**. HPLC-DAD chromatogram of a representative blank milk sample spiked with all the SAs at 20 μg $kg^{-1}$

**Table 1.** Validation data of SAs

| **Sulphonamides** | **SDZ** | **SPN** | **SMR** | **SMZ** | **SMM** | **SDM** | **SQX** |
|---|---|---|---|---|---|---|---|
| **CCα (μg $kg^{-1}$)** | 112 | 111 | 111 | 109 | 109 | 112 | 113 |
| **CCβ (μg $kg^{-1}$)** | 126 | 124 | 124 | 118 | 118 | 126 | 127 |
| **Repeatability (MRL)[a] (CV%)** | 4.3 | 5.7 | 6.4 | 4.3 | 4.1 | 5.2 | 4.5 |
| **Reproducibility (MRL)[b] (CV%)** | 7.4 | 7.0 | 7.0 | 5.3 | 5.3 | 7.5 | 7.7 |
| **Recovery (MRL)[b] (%)** | 64 | 70 | 72 | 77 | 71 | 75 | 70 |
| **Specificity** | Passed | Passed | Passed | Passed | Passed | Passed | Passed |
| **Ruggedness** | Passed | Passed | Passed | Passed | Passed | Passed | Passed |

*[a]n=6 - [b]n=18*

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## P 4

# DETERMINATION OF TETRACYCLINES IN CHICKEN MEAT BY A LIQUID CHROMATOGRAPHIC - ELECTROSPRAY TANDEM MASS SPECTROMETRIC MULTIRESIDUE METHOD

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**Abstract**
A multiresidue LC-MS/MS method for the simultaneous quantitative determination of the tetracycline antibiotics tetracycline, oxytetracycline, chlortetracycline, their respective 4-epimers and doxycycline in chicken meat has been optimised and validated. The drugs were extracted with a 20% trichloroacetic acid solution. After centrifugation, the supernatant was filtered through a 0.2 µm filter and a thorough sample clean-up with an OASIS HLB SPE column was carried out. The LC separation was performed with an Alliance HPLC system on a reversed-phase Alltech Alltima C18 column (3 µm, 150 x 2.1 mm i.d.) with gradient elution. A mobile phase composed of water and a mixture of methanol/acetonitrile (70/30, V/V), each phase containing 1% formic acid, was used. The analytes were detected and identified with a Micromass tandem quadrupole mass spectrometer by electrospray ionisation in positive mode using multiple reaction monitoring. The method was validated conform the EU requirements. The MRL value is 100 µg/kg for the allowed tetracyclines and all analytes can be detected at residue levels lower than 25 µg/kg. The decision limit values varied between 118 and 128 µg/kg for the various analytes. The detection capabilities ranged from 139 to 154 µg/kg for the envolved components. The mean recovery values varied between 77% for oxytetracycline and 101% for doxycycline.
This analytical method will be used in an integrated system for the confirmation of positive results obtained by screening methods.

**Introduction**
The commonly used tetracycline antibiotics (TCAs) in veterinary medicine are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC). Among the antibiotics, TCAs are frequently used in poultry husbandry because of their broad antibacterial spectra. TCAs have been used to minimise poultry diseases such as chronic respiratory disease, blue comb and infectious sinusitis. Control of infectious synovitus, infectious coryza and fowl typhoid are also described. TCAs will also act against some pathogenic agents unaffected by other antibiotics e.g. rickettsiae and certain large viruses belonging to the psittacosis group in animals. Due to there cost-effectiveness and in spite of the increasing resistance, these drugs are still widely used in the poultry industry for broiler chickens to prevent general infections and, in particular, respiratory diseases. DC is widely used in poultry because of its advantageous pharmacokinetic properties. These include a better bioavailability from the gastrointestinal tract, a stronger lipophilic character and a higher affinity for tissues. However, the higher lipid solubility of DC can result in higher and

more persistent residue amounts in edible tissues.
Following administration of broilers, TCAs are excreted in the tissues. Residue levels in muscle can be present if the recommended withdrawal times are not respected by the poultry farmers. Tetracyclines are susceptible to conformational degradation to their 4-epimers. The tolerable MRL values for TC, OTC and CTC in muscle are 100 µg/kg and as marker residues have to be measured the sum of the parent drugs and their respective 4-epimers. DC is not authorised for broiler chickens.
The objective of this study was to optimise a quantitative LC-MS/MS method that is simple and fast for routine regulatory analysis of residues of the four TCAs OTC, TC, CTC and DC and of the epimers 4-epi-OTC, 4-epi-TC and 4-epi-CTC in chicken muscle.

## Materials and methods

*Reagents and standard solutions*
Analytical standard material of the hydrochlorides of TC, 4-epi-TC, OTC, 4-epi-OTC, CTC and 4-epi-CTC and doxycycline hyclate were dissolved in methanol at a concentration of 0.1 mg/ml. The working standard solutions were made by dilution with water containing 0.1% of formic acid and 25% of methanol.

*Sample preparation*
Five g of homogenised muscle was transferred into a beaker of 100 ml. Spiked samples were conditioned for 30 min. The sample was acidified with 20 ml of 20% (w/v) trichloroacetic acid in water and mixed for 1 min. After 5 min of shaking, the sample was centrifuged (10 min, 5900 g, 5°C). The supernatant was filtered through a 0.2 µm regenerated cellulose filter. A SPE sample clean up using a vacuum manifold with OASIS HLB (hydrophobic-lipophilic-balanced) SPE columns was performed and the TCA residues were eluted with methanol. The extract was evaporated to nearly 2 ml at 40°C under a flow of nitrogen. The remainder was, if necessary, made up to 2 ml with a solution of water containing 0.1% of formic acid and 25% of methanol and transferred into an autosampler vial.

*Liquid chromatography-mass spectrometry*
The liquid chromatographic separation of the tetracycline analytes was performed on an Alliance LC system (Waters) with an Alltima $C_{18}$ reversed-phase column, 3 µm, 150 x 2.1 mm i.d. protected with a guard cartridge of 7.5 x 2.1 mm i.d. The mobile phase A consisted of water containing 1% formic acid to increase the ionisation intensity. The mobile phase B was a mixture of methanol/acetonitrile (70/30, V/V) and was also supplemented with 1% of formic acid. The gradient programme was as follows: 81A:19B (0-5 min), 81A:19B to 40A:60B (5.1-15 min), 40A:60B (15.1-18 min), 40A:60B to 10A:90B (18.1-19 min), 10A:90B (19.1-23 min), 10A:90B to 81A:19B (23.1-24 min), 81A:19B (24.1-39 min). The flow rate was 0.25 ml/min and a volume of 40 µl of the cleaned sample extract was injected into the LC-MS/MS apparatus.
The quattro LCZ tandem quadrupole mass spectrometer (Micromass) was equipped with a z-spray electrospray ion interface and was fully controlled by MASSLYNX 4.0 software. Atmospheric pressure electrospray ionisation in the positive ion mode (ESI+) and nitrogen gas flows of 80 and 600 l/h for respectively nebulising the LC eluent and for drying the

solvents, were used. The source block and solvent desolvation temperatures were set at 130 and 250°C, respectively. Fragmentation of the precursor ions into product ions was obtained in the collision cell by means of argon gas.
The analytes were detected using the Multiple Reaction Monitoring (MRM) mode following two transitions. The specific cone voltage and collision energy of each tetracycline analyte were optimised to maximise the ion current of three induced ions, namely the precursor [M+H]+ ion and two most abundant product ions. Internal calibration by an internal standard procedure based on matrix calibration curves was applied to calculate the residue values.

## Results and discussion

*Liquid chromatographic - mass spectrometric analysis*
A compound derived from chlortetracycline, 4-epi-demethylchlortetracycline (4-epi-DMCTC), was used as internal standard (IS). A summary of the monitored diagnostic product ions and the optimised MS operating parameters obtained for the examined tetracyclines is given in Table 1.

**Table 1.** Summary of the diagnostic ions and the MS operating parameters (positive ionisation mode)

| Analyte | Precursor ion (m/z) | Product ions (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|
| TC and 4-epi-TC | 445.1 | 427.4/ 410.2[a] | 25 | 13/20 |
| OTC and 4-epi-OTC | 461.1 | 444.2/ 426.1[a] | 27 | 18/22 |
| CTC and 4-epi-CTC DC 4-epi-DMCTC (IS) | 479.1 | 462.2/ 444.1[a] | 27 | 20/22 |
| | 445.3 | 410.4/ 428.2[a] | 30 | 30/22 |
| | 465.1 | 430.1/ 448.2[a] | 30 | 22/17 |

[a]*Most abundant product ion*
*TC: Tetracycline OTC: Oxytetracycline CTC: Chlortetracycline DC: Doxycycline*

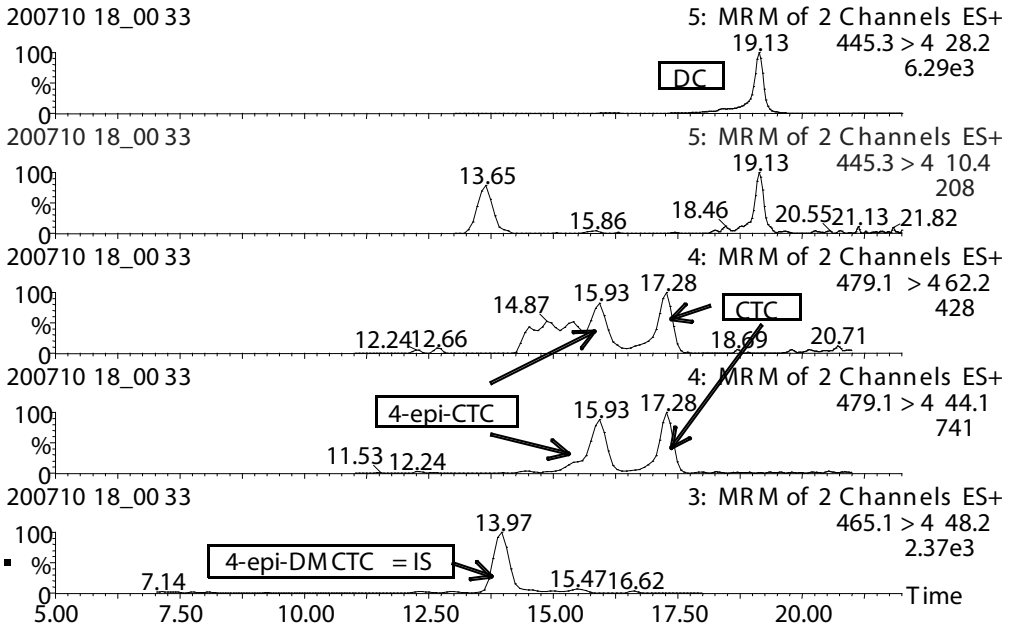
TCAs and their 4-epimers have the same molecular mass and element composition, therefore the TCAs and their 4-epimers had to be separated chromatographically to correctly quantify the residues in egg samples. The previous developed LC-MS/MS analytical method in our laboratory for residues in milk samples was used in this study for residues in muscle samples. Acceptable analyte separations were obtained with phase A consisting of water and phase B composed of a mixture of methanol/acetonitrile (70/30, V/V). Gradient elution was necessary to obtain a good separation of the tetracycline analytes and their respective epimers and to decrease the retention times. Formation of 4-epimers and other conversion derivatives of the TCAs as anhydrotetracyclines, are minimised using a mobile phase with a low pH (< 3) and lowering the temperature and reaction times during the sample preparation.
During the sample preparation a 20% solution of trichloroacetic acid (which was stored at 4°C) was used for the liquid extraction of the analytes. Removal of proteins and other macromolecules was obtained with this agent. The speed of the centrifuge was set at a high value of 5900 g to obtain a clearer supernatant. After filtration with a 0.2 µm regenerated cellulose prefilter, the extracts were further cleaned by the SPE technology using an OASIS HLB column.

Identification and quantitative determination of the seven target compounds and the IS were carried out by MRM. An example of a chromatogram of a blank chicken muscle sample fortified at the respective LOD values is shown in Figure 1. The chromatogram shows two peaks per compound corresponding with the two transitions measured. The TCA compounds eluted within 19 min. A good analyte separation is shown and all compounds are chromatographically separated. The total run time was 39 min which means that 24 cleaned muscle samples can be determinated per night.

*Validation study*

Analytical limits. The LOD values for the authorised TCAs and their 4-epimers and the CCα value for DC were defined as the lowest possible residue concentration spiked in 20 blank muscle samples generating a S/N ratio of at least 3/1 for the lowest abundant ion trace. The obtained concentrations were 15, 15, 15, 10, 10, 15 and 25 µg/kg respectively for TC, 4-epi-TC, OTC, 4-epi-OTC, CTC, 4-epi-CTC and DC.

The LOQ values for the authorised compounds and their 4-epimers and the CCβ value for DC were determined by analysing 20 blank muscle samples fortified at the respective LOD and CCα concentration. The LOD or CCα value plus 1.64 times the corresponding standard deviation equals the LOQ or CCβ value. The respective calculated residues values for TC, 4-epi-TC, OTC, 4-epi-OTC, CTC, 4-epi-CTC and DC were 21, 23, 28, 15, 20, 21 and 41 µg/kg. The CCα values were calculated for the licensed drug substances and their 4-epimers as the sum of the respective MRL value plus the corresponding standard deviation of the residue concentration of 20 blank muscle samples



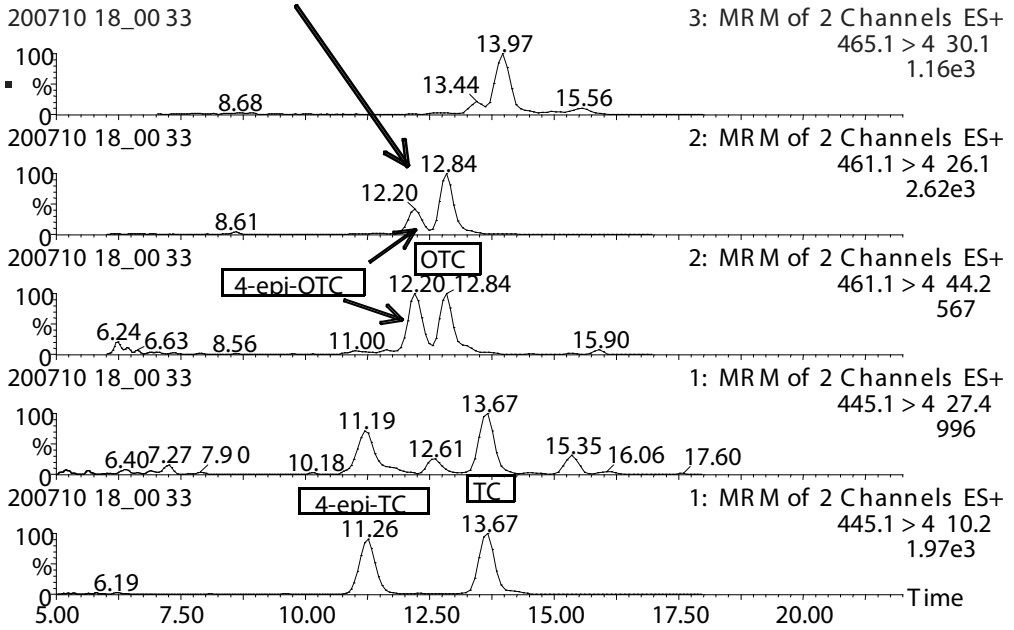



**Figure 1.** Example of a chromatogram of a blank muscle sample fortified at a concentration of 15, 15, 15, 10, 10, 15, 25 and 50 µg/kg respectively for TC, 4-epi-TC, OTC, 4-epi-OTC, CTC, 4-epi-CTC, DC and IS fortified at the MRL concentration of 100 µg/kg.

These limit values for the discussed method are 128, 120, 125, 119, 125 and 119 µg/kg for the respective above mentioned drug compounds.
The CCβ values for the allowed compounds and epimers were calculated as the sum of the CCα value plus 1.64 times the corresponding standard deviation of 20 blank muscle samples fortified at the CCα value. The CCβ values were respectively 155, 139, 145, 154, 155 and 143 µg/kg.
Recovery. The extraction recovery was evaluated at 0.5, 1 and 1.5 MRL concentration of blank muscle samples fortified with the licensed TCAs and epimers. The mean extraction recovery values (n=18, expressed in %) and the variabilities on the individual recoveries (between brackets), expressed as sr values in %, for TC, 4-epi-TC, OTC, 4-epi-OTC, CTC and 4-epi-CTC were respectively 93 (13), 93 (13), 77 (15), 91 (12), 93 (5) and 98 (6). For DC, the recovery was evaluated at 1, 1.5 and 2 CCα (25 µg/kg) concentration. The mean recovery values (n=6, expressed in %) and the variabilities on the individual recoveries (between brackets), expressed as sr values in % were 108 (3), 97 (3) and 98 (5) respectively at concentrations of 25, 37.5 and 50 µg/kg.

**Conclusions**

A relatively simple, fast and specific LC-MS/MS confirmatory multiresidue method has been developed for the simultaneous, quantitative determination of OTC, 4-epi-OTC, TC, 4-epi-TC, CTC, 4-epi-CTC and DC in chicken muscle at residue levels between 10 and 25 µg/

kg. The presented method is also suitable to quantify residues of tetracyclines at MRL level, providing the laboratory with a powerful tool to make decisions for non conform samples for statutory testing purposes. The reliability of the liquid chromatographic-mass spectrometric procedure was proven by fulfilling some validation criteria of the European Commission Decision concerning the performance of analytical methods and the interpretation of results.

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## P 5

# DEVELOPMENT AND VALIDATION OF A CONFIRMATORY METHOD FOR THE DETERMINATION OF RESORCYLIC ACID LACTONES IN URINE SAMPLE BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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**Abstract**
The determination of zeranol, its metabolites taleranol and zearalanone is complicated by the occurrence of other resorcylic acid lactones (zearalenone, α- and β–zearalenol). The aim of this study is a very quick analytical procedure for the determination of all six resorcylic acid lactones. The urine sample was subjected to an enzymatic deconjugation and then simply cleaned up on an immunoaffinity column. The analytes were detected by liquid chromatography-negative ion electrospray tandem mass spectrometry using deuterium-labelled internal standards. The method was validated as a quantitative confirmatory method according to EU Decision 2002/657/EC. The results obtained show good linearity, accuracy and ruggedness. The decision limits obtained were around 0.6 μg L-1 for all the analytes.

**Introduction**
Natural occurrence or illegal treatment of zeranol presence in animal matrices is under debate within the European Union. Zeranol (α-zearalanol, α-ZAL) is one of the non-steroidal estrogenic growth promoters banned by the EU. Zeranol and its primary metabolite taleranol (β-zearalanol, β-ZAL) are resorcylic acid lactones (RALs). RALs also include α-zearalenol (α-ZEL), β- zearalenol (β-ZEL), zearalenone (ZEN) and zearalanone (ZAN). ZEN, known as the Fusarium spp. toxin (F2-toxin), is commonly found in animal feed and recently it was shown that zeranol may also be formed in vivo from it. The main step to distinguish illegal use of zeranol from consumption of food contaminated with Fusarium spp. toxin is the determination of all RALs by quantitative analytical methods. Furthermore the methods must be validated according to the validation criteria concerning the performance of analytical methods and the interpretation of results set by EU Decision 2002/657/EC. Recently published methods suitable for this purpose are based on gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS/MS). All the published methods involve time consuming sample clean up with liquid-liquid extraction and/or two or more solid phase extraction (SPE) columns. In this work we developed an LC-MS/MS method for the determination of RALs in bovine and swine urine samples using α-ZAL-d4 and β-ZAL-d4 as internal standards where the clean up of the sample is done by a simply immunoaffinity (IA) column. The method was validated as a quantitative confirmatory method according to the EU Decision 2002/657/EC: precision, trueness, ruggedness, decision limit (CCα) and detection capability (CCβ) were evaluated for all the analytes.

## Materials and Methods

*Chemical and reagents*
All reagents and solvents were of analytical or HPLC grade quality and supplied by Sigma (Sigma-Aldrich, Milan, Italy). Ultrapure water was obtained by Milli-Q system Millipore (Bedford, MA, USA). Zeranol immunoaffinity chromatography gel (1 mL) was from CER (Marloie, Belgium). Phosphate buffer solution (PBS, 0.05 M) at pH 7.5, prepared by sodium dihydrogen phosphate (0.75 g/L), sodium hydrogen phosphate dihydrate (7.9 g/L) and sodium chloride (9 g/L) were supplied by Carlo Erba (Milan, Italy). α- ZAL, β-ZAL, α-ZEL, β-ZEL, ZAN and ZEN were purchased by Sigma (St. Louis,MO,USA). α- ZAL-d4 and β-ZAL-d4 (ISTDs) were bought from RIVM (Bilthoven, The Netherlands). RALs stock solutions at the concentration of 0.1 mg mL−1 in methanol were prepared and stored at 4°C. Suitable working standard solutions were obtained by appropriate dilution of the corresponding stock solution and stored at 4°C. β-glucuronidase-arylsulphatase from Helix Pomatia was provided by Sigma (St. Louis,MO,USA).

Liquid chromatography-mass spectrometry (LC-MS/MS) system consisted of Finnigan Surveyor™ HPLC system equipped with a Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Electron, San Jose, CA, USA). The mass spectrometer was set in the negative electrospray ionisation (ESI-) mode: capillary voltage was set at 3.0 kV, the ion transfer capillary temperature was set at 300 °C, sheath and auxillary gases (nitrogen for both) were fixed at 30 and 15 respectively. The collision gas was argon at 1.0 mTorr and peak resolution of 0.7 Da FWHM was used on Q1 and Q3. The scan time for each monitored transition was 0.1 s and the scan width was 1 Da. The selected reaction monitoring (SRM) conditions for the analytes is given in Table 1. The analytical column was a Xterra MS C18 Waters (150 x 2.1 mm, 3.5 µm) (Milford, MA, USA) with a guard column Xterra MS C18 Waters (10 x 2.1 mm, 3.5 µm). The LC eluents were: [A] 0.1% (v/v) aqueous acetic acid solution and [B] acetonitrile. The gradient program initiated with 30% [A] for one min., continued with a linear increase to 60 % [A] over 14 min followed by a linear increase to 70 % [A] over 1 min and 70 % [A] for 3 min. In one min [A] returned to 30 % and the re-equilibration time was 5 min before the next injection. The overall run time was 25 min. Column kept in a column oven at 40 °C. The flow rate was 0.2 mL min-1 and the injection volume was 20 µL. Analytes and internal standard MS/MS data points were recorded and elaborated using XcaliburTM version 1.4 SR1 software from Thermo.

**Table 1.** Negative ion MS/MS condition for the SRM acquisition of RALs

| Analyte | Precursor ion (m/z) | Product ion (m/z) | Collision energy (eV) |
|---|---|---|---|
| β-ZAL & α-ZAL | 321 | 259 | 25 (β), 28 (α) |
| | | 277[a] | 24 (β and α) |
| | | 303 | 24 (β), 23 (α) |
| β-ZAL-d4 & α-ZAL-d4 | 325 | 281 | 25 |
| β-ZEL & α-ZEL | 319 | 130 | 40 (β), 42 (α) |
| | | 160 | 35 (β and α) |
| | | 275[a] | 23 (β and α) |
| ZAN | 319 | 161 | 32 |
| | | 205 | 27 |
| | | 275[a] | 23 |
| ZEN | 317 | 131 | 30 |
| | | 175[a] | 28 |
| | | 273 | 24 |

[a] The most abundant product ion used for the quantitative analysis.

*Sample preparation*

Five milliliters of urine sample was spiked at 2 ng mL-1 with 100 µL of ISTDs solution (0.1 ng µL -1). 1 M hydrochloric acid was added to adjust the pH to 5.0. One hundred µL of β-glucuronidase-arylsulphatase was added and enzymatic deconjugation was carried out overnight at 37°C. The sample was cooled to room temperature, and 5 ml of PBS was added. 1 M sodium hydroxide solution was added to adjust to pH 7.5 and then the sample was centrifuged for 10 min at 2000 rpm. All the resulted solution was loaded on a IA column, previously conditioned by 2 x 5 mL of PBS and 2 x 5 mL of water. The IA column was washed with 2 x 5 mL of a mixture methanol-water (10:90 v/v). Analytes elution was done with 5 mL of a mixture methanol-water (80:20 v/v). The IA colum can be used at least 10 times before being discarded. The eluate was evaporated to dryness under a stream of nitrogen at 45 °C. The residue was finally redissolved in 400 µL of a mixture methanol-water (50:50 v/v) and transferred in vials for the LC-MS/MS analysis.

*Validation study*

The developed method was fully validated as quantitative confirmatory method according to the EU Decision 2002/657/EC. Parameters taken into account were: specificity, precision, trueness, ruggedness, decision limit (CCα) and detection capability (CCβ). As regards to the specificity, unfortunately low levels of RALs naturally occur in urines and therefore it was impossible to verify the absence of potential interfering compounds at analytes retention times. As consequence, the validation study was done using representative cattle urines contaminated at very low levels. Method recovery and precision were evaluated by spiking urine samples with the six RALs, resulting in three analytical series, each series with four concentration levels (0.5 – 1.0 - 1.5 - 2.0 ng $mL^{-1}$) and six samples per concentration level (6 samples x 4 concentration levels x 3 series = 72 analysis). The same results were used to calculate decision limit (CCα) and detection capability (CCβ). Method ruggedness was

estimated for both minor and major changes by means of the Youden fractional factorial design. Finally for all the analytes instrumental linearity was evaluated by drawing five points calibration curves in solvent, containing a fixed amount of ISTDs (25 pg $\mu L^{-1}$ each), with analytes concentrations corresponding to 2.5 – 5 – 10 – 20 – 25 pg $\mu L^{-1}$.

**Results and Discussion**

The LC-MS/MS method was developed according to the performance criteria for mass spectrometric detection suggested in EU. Analysis was performed in Selected Reaction Monitoring (SRM) mode, because of its high sensitivity and specificity, and the four ions (one precursor ion and three product ions) monitored are reported in Table 1. This approach allowed to achieve the identification points (IPs) required by the aforesaid document for the identification of banned compounds. Instrumental linearity was demonstrated for all the analytes in a concentration range from 2.5 to 25 pg $\mu L^{-1}$ using solvent standard solutions in methanol-water (50:50 v/v). Five concentrations, 3 replicates per concentration level, were used to build the regression line using the least square method and to calculate the regression coefficient ($r^2$) and the 95% confidence limits for the y-intercept. Regression coefficients of curves indicated a good fit for all the analytes and confidence limits were satisfactory for every line. Figure 1 shows the chromatograms of a urine sample spiked with 0.5 ng $mL^{-1}$ of all the RALs. The precision observed in the bovine urine spiked at 0.5 – 1.0 - 1.5 - 2.0 ng $mL^{-1}$ was calculated applying the one-way analysis of variance (ANOVA). The coefficients of variation (CVs) were lower to 10% and 12% in repeatability or in intra-laboratory reproducibility conditions respectively. The recoveries (no internal standard correction) were good. Decision limit (CCα) and detection capability (CCβ) were calculated by applying the calibration curve procedure described in EU Decision 2002/657/EC as clarified in the document SANCO/2004/2726 rev 2. The obtained values for the six compounds were < 1 ng $mL^{-1}$ (Table 2).

**Table 2.** CCα and CCβ values for studied RALs in cattle urine by LC-MS-MS

| | **β-ZAL** | **β-ZEL** | **α–ZAL** | **α–ZEL** | **ZAN** | **ZEN** |
|---|---|---|---|---|---|---|
| **CCα (ng $mL^{-1}$)** | 0.57 | 0.60 | 0.54 | 0.55 | 0.63 | 0.63 |
| **CCβ (ng $mL^{-1}$)** | 0.61 | 0.67 | 0.56 | 0.60 | 0.76 | 0.75 |

Representative very low contaminated cattle urine samples were spiked at and above the minimum required performance level (0.5 ng $mL^{-1}$). This is the lowest experimental concentration at which both qualitative and quantitative criteria are fulfilled. As regards to method ruggedness, six operational factors were chosen for their possible critical influence (minor changes). Youden approach was also used to verify the method performances when applied to swine urines (major changes). It was demonstrated that all selected factors and the different animal species did not have significantly affect the analytical results.

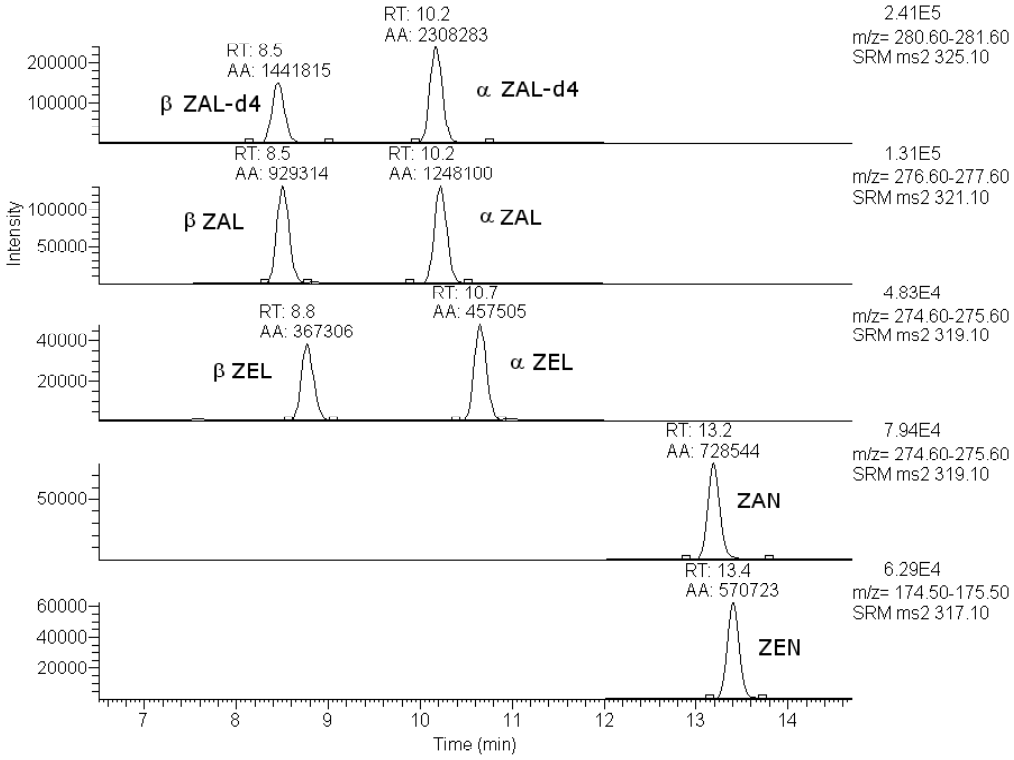


**Figure 1.** Extracted ion chromatogram of a urine sample spiked at 0.5 ng mL-1 with RALs (ISTDs at 2 ng mL-1).

## Conclusion

A LC-MS/MS confirmatory method for rapid and simultaneous determination of six RALs in cattle and swine urine was developed and validated according to the EU Decision 2002/657/EC. In our laboratory it is routinely applied for the confirmation of RALs illegal treatments within the Italian Official Residue Control Program.

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## P 6

# HISTOLOGICAL ALTERATIONS IN VEAL CALVES TREATED WITH ANABOLIC STEROIDS AND A CORTICOSTEROID

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**Abstract**
In the Netherlands and in Italy histological screening for estrogens is performed on the prostate in male calves and the Bartholin's gland in female calves. The thymus is known as target tissue for corticosteroids. In this study 24 veal calves were used. Twelve animals (6 males and 6 females) were 3 times injected with 17 β-estradiol benzoate and 19-nortestosterone laureate ester, with 14 days between the injections. One week after the last injection one dose of dexamethasone was administered. The animals were slaughtered 1-3 weeks after last injection. In all treated male animals both the prostate and the bulbourethral gland showed varying degrees of metaplasia. The glandular tissue was more mucinous in 5 of 6 treated animals and some cystic dilatation was observed. In the treated females most Bartholin's glands showed more ducts than glandular tissue and all showed metaplasia. The thymus of 2 treated males showed slight cortical atrophy and one animal showed marked cortical atrophy. In the thymus of females no changes between the treated and the controls were observed. Anabolic steroids led to distinct changes of histology of prostate and Bartholin's gland, but one dose of dexamethasone only gave alterations in 50 % of the male animals.

**Introduction**
Anabolic steroids are known to influence hormonal target tissues. These alterations can be used as screening method for illegal use of hormonal growth promoters. In the Netherlands and in Italy histological screening for estrogens in veal calves is performed on the prostate in male calves and the Bartholin's gland in female calves. In this study both anabolic steroids and a corticosteroid were used. Corticosteroids can influence the immune system (Cantiello et al., 2007) and lead to cortical atrophy of the adrenal cortex and the thymus (Groot et al., 1998). In this study the histological alterations in both male and female calves are described.

**Material and Methods**
The experimental design of this study was described earlier (Eisenberg et al., 2007). Twelve animals (6 males and 6 females) were 3 times injected with 17 β-estradiol benzoate (5 mg/ml 17β-oestradiol benzoate ester; REG NL 01796; Intervet, Boxmeer, The Netherlands) and 3 ml Decadurabolin (50 mg/ml nandrolon decanoate ester; RVG 00126; Organon; Oss; The Netherlands), with 14 days between the injections. One week after the last injection one dose of 11, 2 ml Dexadreson (2 mg/ml; REG NL 02661; Intervet;) was administered. The animals were slaughtered 1-3 weeks after last injection. The remaining twelve calves were

injected with 2 ml sterile arachide oil subcutaneously at the same time points as the hormone treatment was administered. The animals were euthanized with T61 and dissected. In male animals the following tissues were sampled: prostate and bulbo-urethral gland (frozen), testis, liver, adrenal, kidney, thymus, lymph node, spleen (fixed in formalin).
In female animals the following tissues were sampled: Bartholin's gland and cervix (frozen), ovaries, liver, adrenal, kidney, thymus, lymph node, spleen (fixed in formalin). Frozen and formalin fixed tissues were obtained from the Veterinary Faculty of Utrecht University where the animals had been dissected. Frozen tissues were thawed en fixed in formalin and all tissues were routinely processed for paraffin sections. Sections 4-5 μm were stained with haematoxylin-eosin (HE) according to Mayer (Bancroft and Stevens, 1990) and immunohistochemically stained with cytokeratin 5/6 as described earlier (Groot et al., 2006). The development of testis was evaluated according to Abdel-Raouf (1960).

**Results and discussion**

The organs such as liver, kidney, spleen and thyroid did not show changes due to the hormonal treatment. In the adrenals, lymph nodes and thymus effects of corticosteroid treatment were expected. But as compared with the controls the adrenals showed no marked changes due to the treatment.
The thymus of 2 males showed slight cortical atrophy and one animal showed marked cortical atrophy. In the females no obvious changes between the treated and the control animals were observed. The results are listed in table 1.

**Table 1.** Histological changes in organs, the numbers indicate the number of animals in that groups showing this parameter

| **tissue** | **Group 1 control males (n = 6)** | **Group 2 treated males (n = 6)** | **Group 3 control females (n = 6)** | **Group 4 treated females (n = 6)** |
|---|---|---|---|---|
| Liver | 1 some fibrosis<br>1 much blood<br>2 small infiltrates<br>1 small cells | 4 much blood<br>1 infiltrate<br>1 small infiltrate,<br>2 some fibrosis pp<br>1 some bile duct proliferation | 3 some fibrosis,<br>3 small infiltrates<br>1 hemorrhages<br>1 some bile duct proliferation | 1 much blood, wide vessels<br>3 Some fibrosis<br>3 Some bile duct proliferation<br>1 small cells |
| Adrenal | 5 normal<br>1 thin cortex | 1 Vacuolization zona glomerulosa<br>1 Thin medulla<br>1 broad cortex<br>1Hyperplasia cortex<br>2 thin cortex | 1 Much blood<br>1 Vacuolization zona glomerulosa<br>2 Thin cortex<br>4 normal | 4 Normal<br>1 Thin cortex<br>1 Vacuolization zona glomerulosa |
| Kidney | 3 Normal<br>1slight glomerulo-nephritis<br>1 inflammation | 3 slight glomerulo-nephritis<br>2 normal<br>1 infiltrates | 4 Normal<br>2 infiltrates | 5 normal<br>1 infiltrate |

| | | | | |
|---|---|---|---|---|
| Thymus | 5 normal<br>1 some cortical atrophy and fatty infiltration | 1 absent<br>1 = lymph node<br>2 slight cortical atrophy, some fatty infiltration, some fibrosis<br>1 normal<br>1 severe cortical atrophy | 2 some fatty infiltration<br>1 absent<br>1 moderate fatty infiltration<br>3 many Hasall bodies<br>1 normal | 2 some fatty infiltration<br>1 normal<br>1 some fibrosis<br>1 moderate fatty infiltration<br>1 = lymph node |
| Lymph-node | 6 normal | 2 absent<br>1 normal<br>3 some lympho-depletion | 3 normal<br>1 much blood, active<br>2 vacuoles | 4 normal<br>1 lytic lympho's<br>1 absent |
| spleen | 6 absent | 2 much blood<br>2 absent<br>1 normal<br>1 some follicles | 3 absent<br>3 normal | 4 absent<br>1 normal<br>1 strange |
| thyroid | 4 absent<br>2 normal, inactive | 2 absent<br>1 normal, inactive<br>2 smaller follicles, more active<br>1 more active | 2 varying follicles<br>1 absent<br>1 small follicles<br>2 varying follicles | 1 small follicles<br>1 bigger follicles<br>2 varying follicles<br>2 absent |

The sexual organs of the male animals showed marked effects of the hormonal treatment. In all treated animals both the prostate and the bulbourethral gland showed varying degrees of metaplasia. In the prostate the effects were more pronounced in the peripheral region, in some of the bulbo-urethral glands the whole gland was metaplastic. Also the glandular tissue was more mucinous in 5 of 6 treated animals and in some of the bulbourethral glands cystic dilatation occurred. This is accordance with other experiments using combinations of androgens and estrogens in veal calves (Groot et al. 1990, Groot et al. 1998)
In the testis there was only one animal that showed smaller tubuli seminiferi and one animal had syncytial cells in the tubules. Since the development of the testis is not so far in animals of this age, the effects of treatment are less than in older animals. The histological results of the male sexual tissues are listed in table 2.

**Table 2.** histology of the male sexual tissues

| tissue | Group 1 control males (n = 6) | Group 2 treated males (n = 6) |
|---|---|---|
| Prostate | 2 Thick urethra<br>4 glands with some dilatation and secretion | 2 thick urethra<br>6 peripheral metaplasia<br>5 mucinous gland with dilatation and secretion |
| Bulbo-urethral gland | 3 immature<br>1 absent<br>1 some development<br>1 moderate development | 1 severe metaplasie<br>4 moderate metaplasia<br>1 mild hyperplasia<br>5 mucinous, secretion, hyperplasia<br>3 cystic with secretion |
| testis | 2 Normal stage II<br>3 stage I-II, much stroma<br>1 stage 1 | 1 stage I-II, small tubules<br>4 stage I-II<br>1 stage I-II, some syncytial cells |

The female animals showed normal Bartholin's glands and ovaries with growing and atretic follicles and the cervix had only secretory epithelium in the crypts of the folds.
In the treated animals Bartholin's glands were increased in size and most showed more ducts than glandular tissue. The epithelium of the ducts was in one animal thinned, but 3 animals showed thickened epithelium with vacuoles filled with debris in the epithelium. Two animals showed desquamation of the epithelium of the ducts. In most animals varying degrees of metaplasia was observed in Bartholin's gland.
The ovaries were not sampled in all treated animals. One animal showed a normal ovary in another many atretic follicles were present. The cervix was not always sampled and two times the vagina was sampled.
Two cervices showed apical edema, one was very mucinous with increased secretion and one was very immature. The results of the female tissues are listed in table 3.

**Table 3.** histology of the female sexual tissues

| tissue | Group 3 control females (n = 6) | Group 4 treated females (n = 6) |
|---|---|---|
| Bartholins gland | 6 normal duct-gland ratio<br>1 dilated ducts<br>2 lymph follicles<br>1 inflammation round ducts | 1 much glandular tissue,<br>1 normal ratio<br>5 increased duct-gland ratio<br>1 ducts thin epithelium, dilated<br>3 vacuoles in epithelia ducts<br>2 desquamation epithelium ducts<br>1 dubious metaplasia<br>2 local metaplasia<br>3 moderate metaplasia<br>1 lymph follicles |
| Ovary | 1 normal<br>2 growing follicles, 1 big follicle<br>3 many follicles | 1 growing and atretic follicles<br>4 absent<br>1 many atretic follicles |

| | | |
|---|---|---|
| Cervix | 5 secretory epithelium only in the crypts<br>1 mature secretory epithelium<br>2 some secretion<br>1 moderate secretion | 2 apical edema<br>1 only immature epithelium<br>1 secretory epithelium in the crypts<br>1 very mucinous<br>1 secretion<br>1 autolytic, debris<br>2 some uterus glands<br>2 = vagina |

**Conclusions**

The treatment with hormone cocktails in male and female veal calves did not lead to histological effects in the organs except the prostate and bulbo-urethral gland in the males and in some of the Bartholins gland in the females. Concerning effects of the corticosteroids only 3 male animals showed some cortial atrophy of the thymus, whereas none of the female animals showed effects. This may be due to the fact that the withdrawal time varied between 7 and 25 days after the treatment with dexamethasone.

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## P 7

# ANALYSIS OF THYREOSTATIC DRUGS IN THYROID SAMPLES BY UPLC-MS/MS: COMPARISON OF TWO CLEAN UP STRATEGIES

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**Abstract**
A method based on ultra-performance liquid chromatography electrospray ionisation-tandem mass spectrometry for the determination of six thyreostatic drugs in thyroid tissue was optimised and validated in accordance with European Commission Decision 2002/657/EC. The samples were extracted with methanol. Two clean-up strategies based on either solid-phase extraction (SPE) using silica cartridges or on gel permeation chromatography (GPC) were compared. Recoveries ranged from 40% to 79% for SPE and from 80 to 109% for GPC. Quantification was performed with blank tissue samples spiked with the analytes in the range of 25 to 500 µg Kg-1. 5,6-dimethyl-2-thiouracil was used as internal standard. CCα and CCβ were found at concentrations of 6 to 19 µg Kg-1 and 10 to 23 µg Kg-1, respectively. The accuracy of the method was calculated as percent error, which was less than 10%. Relative standard deviation in reproducibility was between 2.5% and 12.5%.

**Introduction**
Thyreostatic agents, which are derived from 2-thiouracil or 2-mercaptoimidazol, are used in medicine to regulate the activity of the thyroid gland. They have been used to fraudulently increase the weight of cattle before slaughter, leading to the production of lower quality meat. Since residues of thyreostatic drugs in edible animal tissue poses a risk to human health, their use in animal production was banned in the European Union in 1981 [1].
The literature contains very few studies on the analysis of thyreostatic residues. Moreover, most of the methodologies proposed involve complex sample treatments. Analytes are typically extracted with polar solvents, and the extracts are usually cleaned-up with SPE silica cartridges. Most of the proposed methods include a derivatisation step, either for converting the analytes into volatile compounds suitable for GC analysis, or for reducing their polarity and increasing their molecular mass for use in LC separation.
In a previous paper [2], a method for the quantification and confirmation of antithyroid drugs in thyroid samples of bovine, ovine and porcine livestock was presented. The method, based on UPLC separation coupled to a triple quadrupole mass spectrometer, includes an SPE clean up step with silica cartridges and obviates the aforementioned derivatisation step. In this paper, a readily automatable clean up strategy based on gel permeation chromatography (GPC) is presented. The developed method was validated and the two approaches were compared.

## Materials and Methods

*Reagents and solutions*

2-thiouracil (TU), 6-propyl-2-thiouracil (PTU) and 6-phenyl-2-thiouracil (PhTU) were obtained from Sigma (St. Louis, MO, USA); 1-methyl-2-mercaptoimidazole (Tapazole, TAP) and 6-methyl-2-thiouracil (MTU) from Fluka (Buchs, Switzerland) and 5,6-dimethyl-2-thiouracil (DMTU, internal standard) and 2-mercaptobenzimidazol (MBI) from Sigma-Aldrich (Steinheim, Germany). HPLC grade acetonitrile and methanol (Carlo Erba, Rodano, Italy), and analytical reagent grade dichloromethane, cyclohexane, ethyl acetate and formic acid (Merck, Darmstadt, Germany) were used. Doubly-deionised water (Milli-Q, Millipore, Molsheim, France) of 18.2 M $cm^{-1}$ resistivity was used.

Standard stock solutions (1000 mg·$L^{-1}$) were prepared by dissolving the analytes in methanol. An intermediate standard solution containing 20 mg·L-1 of each thyreostat was prepared by dilution of the stock solutions with doubly-deionised water. The 0.4 mg·$L^{-1}$ working solution was prepared dayly by dilution of the intermediate standard solution with water.

*Apparatus*

LC-ESI-MS/MS measurements were carried out with a Waters Acquity UPLC system (Chicago, IL, USA) coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters) using an electrospray source. The column used was an Acquity UPLC BEH C18. (100 x 2.1 mm; 1.7 µm particle diameter) from Waters. The flow rate was 0.3 mL·$min^{-1}$.

GPC was carried out with an Agilent 1100 Series system (Palo Alto, CA, USA) consisting of an isocratic pump, an automatic injector, a UV detector and a G1364C fractions collector. The column used was a Waters Envirogel GPC Cleanup (19 x 300 mm) equipped with a Waters Envirogel GPC Cleanup guard column (19 x 150 mm). A 1:1 mixture of cyclohexane and ethyl acetate was used as mobile phase (flow rate 5 mL $min^{-1}$).

*Procedures*

To a 2 g of minced sample (spiked whit analytes if required), placed in a 50-mL glass tube, 400 µL of a 0.4 mg·L-1 internal standard solution were added. Extraction and clean up using SPE silica cartridges was performed as described previously [2]. When the GPC approach was used, the samples were extracted twice for 10 min in an ultrasonic bath with 5 mL of ethyl acetate, and then centrifuged for 10 min (10 ºC; 3,500 g). The extracts were evaporated to 1 mL under a nitrogen stream, transferred to a 2 mL volumetric flask and diluted to the mark with ethyl acetate. A 1 mL aliquot of this solution was injected into the GPC system, and the fraction eluting between 14 and 21 minutes was collected. The eluents were evaporated to 200 µL under a nitrogen stream, reconstituted with 1000 µL of doubly-deionised water, placed in an ultrasonic bath for 1 min, filtered through 0.45 µm membrane filters (Durapore, Millipore), and then injected (10 µL) into the UPLC system.

The separation was performed at 40 ºC with a binary mobile phase consisting of aqueous 0.1% formic acid solution (phase A) and acetonitrile containing 0.1% of formic acid (phase B). The following gradient program (time in minutes, % phase A) was applied: (0, 100), (2, 100), (8, 50), (9, 50), (9.1, 100). The column was re-equilibrated for 3 min before each run. The electrospray ionisation source was operated in the positive mode under the following

working conditions: capillary voltage, + 3.5 Kv; source block and desolvation temperatures, t 100 and 350 ºC, respectively; desolvation and nebuliser gas (N2) flows, t 450 and 55 L·h-1, respectively; argon pressure in the collision cell, t 4x10-3 mbar. The values of dwell time, cone voltage and collision energy are listen in Table 1. Full scan spectra were obtained over the m/z range of 50 to 250 at a cycle time of 500 ms and an interscan time of 100 ms. Data acquisition for quantification and confirmation was performed in the multiple reaction monitoring (MRM) mode. Although two transitions were followed for the identification, only one of these was used for quantification (shown in bold in Table1).

**Table 1**. Optimal MRM conditions (the fragment ions in bold were used for quantification)

| **Thyreostat** | **$t_R$ (min)** | **Precur-sor ion (m/z)** | **Product ion (m/z)** | **Cone voltage (V)** | **Collision energy (eV)** | **Dwell time (s)** |
|---|---|---|---|---|---|---|
| TU | 1.8 | 129 | 70<br>112 | 25<br>25 | 20<br>15 | 0.5<br>0.5 |
| TAP | 3.0 | 115 | 57<br>88 | 40<br>40 | 18<br>18 | 0.3<br>0.3 |
| MTU | 3.5 | 143 | 84<br>126 | 35<br>35 | 20<br>15 | 0.3<br>0.3 |
| DMTU (IS) | 4.5 | 156 | 98<br>- | 35<br>- | 22<br>- | 0.1<br>- |
| PTU | 5.3 | 171 | 112<br>154 | 40<br>35 | 25<br>20 | 0.2<br>0.2 |
| MBI | 5.5 | 151 | 93<br>118 | 40<br>40 | 23<br>25 | 0.25<br>0.25 |
| PhTU | 6.1 | 205 | 146<br>188 | 40<br>40 | 25<br>20 | 0.4<br>0.4 |

**Results and Discussion**

*Sample treatment*

For the SPE method, the effect of several operational parameters (evaporation to dryness, evaporation temperature, reconstitution solvent prior to and after the SPE, and amount of sorbent in the SPE cartridge) on recovery rates was evaluated and reported in an earlier study [2]. In order to optimise the GPC method, standard solutions of the analytes in either methanol or ethyl acetate were injected into the GPC system, and the absorbance of the column effluent was monitored at 254 nm. As methanolic solutions, the thyreostatic agents eluted within 10 to 18 minutes, whereas when dissolved in ethyl acetate, they eluted within 14 to 21 minutes. Analysis of thyroid gland extracts dissolved in either methanol or ethyl acetate showed that the matrix components elute between 10 and 13 minutes. Since ethyl acetate provided the best separation between the analytes and the matrix components, it was selected for further studies. Recovery rates for the entire procedure were evaluated from the analysis of blank samples spiked with the analytes at four concentration levels (from 25 to 250 µg Kg-1). To ascertain the absolute recovery rates, the analytical responses obtained from samples spiked at the beginning of the sample preparation were compared with those

obtained from extracts spiked after the reconstitution of the SPE and GPC eluents, just before LC injection. Data obtained from a bovine thyroid sample led to absolute recovery rates higher than 85% for all the analytes. These recoveries are clearly superior to those obtained by methanol extraction and subsequent SPE using silica cartridges, which range from 40% to 79 % [2].

*Optimisation of LC-MS/MS parameters*

The samples were separated on a reversed phase column using mixtures of acetonitrile and water containing formic acid (0.1%) as mobile phases. Several gradient programs were assayed and, due to the polar nature of the analytes, a nearly 100% aqueous solution was used as initial mobile phase. The selected gradient, described above in the Procedures section, afforded good resolution for all analytes, with reasonable retention times and good peak shapes. Although complete separation of analytes is not required in MS/MS detection, good resolution prevents ionisation suppression. To achieve maximum sensitivity, the mass spectrometry parameters were optimised by infusion of standard solutions of each compound in 0.1% formic acid. The optimal conditions are given in the Experimental section.

*Methods validation*

The methods were validated according to the criteria specified in European Commission Decision 2002/675/EC for a quantitative confirmation method [3]. Before beginning the validation, a test with thyroid tissues of different animal species was first performed. Bovine, porcine and ovine thyroid samples were spiked with the internal standard at 160 μg $Kg^{-1}$ and analysed. The differences among the measured areas of the chromatographic peaks obtained with the different tissues were lower than 10%. Since a maximal difference of 25% between major and minor areas is acceptable, the assayed species were considered as equivalent for the validation process. Further validation was carried out with porcine thyroid tissue. Validation parameters were determined at concentration levels of 25, 50, 100 and 250 μg $Kg^{-1}$. The parameters measured were specificity, linear range, precision (repeatability and within-laboratory reproducibility), accuracy, decision limit and detection capability.

The specificity was assessed by analysing blank tissue samples. The absence of background peaks with a signal-to-noise ratio greater than 3 at the retention times of the target compounds indicated that the method is free of any endogenous interference.

The calibration curves for each compound were built using blank samples (n = 7) spiked from 25 to 500 μg Kg-1. Linear regression analysis was performed by plotting the peak area ratio of the analyte and internal standard versus the analyte concentrations. Good linearity was observed: correlation coefficients were r = 0.999 for all the analytes, while residuals were below 20% in the low level and below 10% at high concentrations.

Accuracy and precision (repeatability and within-laboratory reproducibility) of the method were determined using independently spiked blank samples at four different levels (16 spiked samples for each level). Samples were analysed on four different days, using standard solutions prepared daily. The results are summarised in Table 2. Values of the within-laboratory coefficient of variation are lower than those calculated by the Horwitz equation.

**Table 2.** Accuracy and precision; data are expressed as a range (min. and max. values of the four concentration levels)

| **Thyreostat** | **TU** | **TAP** | **MTU** | **PTU** | **MBI** | **PhTU** |
|---|---|---|---|---|---|---|
| Methanol-SPE | | | | | | |
| Error (%) | 0.6-4.9 | 3.0-7.2 | 2.4–6.0 | 1.3-6.4 | 0.4-5.4 | 1.6-3.7 |
| Repeatability (%) | 4.8-8.3 | 4.7-7.6 | 3.8-6.2 | 4.3-5.6 | 5.6-10.0 | 5.4-7.7 |
| Reproducibility(%)[a] | 7.9-10.3 | 6.4-8.8 | 5.6-7.8 | 7.3-10.8 | 8.3-11.2 | 6.8-12.1 |
| Ethyl acetate-GPC | | | | | | |
| Error (%) | 0.2-5.8 | 5.5-9.7 | 1.1-5.5 | 0.9-1.9 | 4.4-9.5 | 2.2-9.9 |
| Repeatability (%) | 1.4-4.2 | 1.2-5.1 | 0.9-2.5 | 1.1-2.4 | 1.3-7.4 | 3.0-7.5 |
| Reproducibility(%)[a] | 2.7–10.6 | 3.7-9.9 | 2.9-6.7 | 2.5-8.8 | 7.0-11.5 | 7.2-12.6 |

[a] n =16, on four different days

The decision limit (CCα), calculated as the concentration at the y-intercept plus 2.33 times its standard deviation, ranged from 4 to 16 µg Kg-1 for the SPE method and from 6 to 19 µg Kg-1 for the GPC method. The detection capability (CCβ) ranged from 8 to 27 and from 10 to 23 for SPE and GPC, respectively.

**Conclusions**

The applicability of gel permeation chromatography in the analysis of residues of thyreostatic drugs in thyroid gland samples has been demonstrated in this study. The sample treatment based on extraction with ethyl acetate and clean up by GPC provides recovery rates significantly higher than that based on the use of SPE silica. GPC is a readily automatable thecnique and this allows that a significant part of the sample preparation process can works unattended, leading a saving of operator's time of about 50%. Quality parameters of both, GPC and SPE, methods (accuracy, precision, CC  and CC ) do not differ significantly.

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## P 8

# DEMONSTRATION OF THE TREATMENT OF DAIRY COWS WITH RECOMBINANT BOVINE SOMATOTROPIN

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**Abstract**
Recombinant bovine somatotropin (rbST) is used in dairy cattle to enhance milk production. Despite the ban of this hormone in some countries, especially in Europe, there is so far no method for the direct detection of rbST. An analytical strategy has been developed, including a purification procedure based on a precipitation with ammonium sulphate, followed by a clean-up with SPE C4 and precipitation with cold methanol. The hormone was digested with trypsin and analysed by LC-HRMSn on a hybrid instrument composed of a linear ion trap coupled with an orbital trap. The tryptic N-terminal peptide, specific to the difference between the endogenous and recombinant form of the somatotropin, was fragmented and product ions were analysed at high resolution (R= 30 000 FWHM). This study presents the first results of detection of rbST in plasma from a treated animal. A limit of detection of 10 ng.mL$^{-1}$ of rbST in spiked plasma was reached and the procedure was successfully applied to the analysis of plasma originating from a goat treated with rbST. These results are of a great interest in the field of somatotropin control and are first steps in the development of a method for the detection of rbST not only in bovine plasma but also in other matrices such as milk.

**Introduction**
Growth hormone, also called somatotropin, is a protein hormone produced by the anterior pituitary gland of mammals. Its effects on growth, development and reproductive functions are numerous and still not completely understood. Nevertheless, it is responsible directly or indirectly for growth, improvement of feed efficiency, decrease of fat mass and increase of milk production. The use of recombinant bovine somatotropin (rbST) in order to stimulate milk production in dairy cattle is authorized in some countries, such as the USA, South Africa, Brazil, Mexico… but prohibited within the European Union. However, the close homology between recombinant and pituitary forms, as well as the low level of residue of bST and rbST in biological fluids (between 0 to 5 ng.mL$^{-1}$ in milk and 0 to 30 ng.mL$^{-1}$ in plasma), have prevented until now the development of analytical method able to discriminate rbST treated cows. A few attempts have been described in the literature, but so far no method exists to detect rbST in biological fluids (blood, urine, milk). The objective of the present study was to adapt the method for detection of reST previously described by Bailly-Chouriberry et al. (2008) to the detection of recombinant bovine somatotropin in plasma. This was achieved using the same analytical basis in terms of purification procedure but with a LC-HRMSn measurement. This method was successfully applied for the first time on a sample obtained from a goat treated with rbST.

## Materials and methods

*Reagents and chemicals*
Recombinant bovine somatotropin (rbST) was obtained from the Harbor-UCLA Medical Center, National Hormone and Pituitary Program (Torrance, CA, USA). Recombinant equine growth hormone, reGH (EquiGen-5®) was purchased from Bresagen Limited (Thebarton, Australia). The synthetic peptides with the following amino acid sequence MFPAMSLSGLFANAVLR (N-terminal tryptic rbST) and MFPAMPLSSLFANAVLR (N-terminal tryptic reST) were obtained from Millegen (Labege, France). Pepstatin, EDTA, acetic acid, ammonium bicarbonate and trifluoroacetic acid were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sequence grade modified trypsin (EC 3.4.21.4) was purchased from Promega (Madison, WI, USA). HPLC grade acetonitrile, methanol, ammonium sulphate and formic acid were from SDS (Peypin, France). SPE C4 500mg/6mL were from Interchim (Montluçon, France).

*Sample preparation*
The sample preparation procedure was adapted from a previous method developed for the detection of reST in horse plasma. Briefly, four millilitres of plasma were spiked with 100 ng.mL-1 of reST as internal standard and 18 mL of phosphate buffer 0.1 M pH 6.9 was added. In a first step, proteins were precipitated for 15h at 4°C with 45% ammonium sulphate. The precipitate was re-suspended in 4 mL phosphate buffer 0.1M pH 6 and passed through a SPE C4. The proteins of interest were eluted with 7 mL of a solution of water/acetonitrile 20:80 (v/v) with 0.1% trifluoroacetic acid. After evaporation under nitrogen stream to obtain about 1 mL of the elute, a precipitation with 5 mL of cold methanol (-20°C) for at least 1,5 h eliminated the remaining impurities. A tryptic digestion was performed overnight at 37°C in a buffer composed with ammonium bicarbonate 50mM, EDTA 10mM and pepstatin 1µM at pH 7.9 and with 2 µg of enzyme. The extracts were then concentrated by evaporation, reconstituted in 40 µL of water/acetonitrile 70:30 (v/v) with 0.2% formic acid and analysed by LC-MS/MS.

*LC–MS/MS measurement*
Separation of the peptides was achieved on a Symmetry300 2.1mm×150mm C4 column packed with 3.5 µm beads, 300 Å pore size (Waters, Milford, USA). The HPLC system was a Surveyor from Thermo Finnigan. The solvent flow was set at 300 µL/min. Peptides were separated using a mobile phase composed with acetonitrile + 0.2% formic acid (A) and water + 0.2% formic acid (B). The elution gradient started with 10% A increasing to 50% in 5 min, then decreasing to initial conditions in 5 min and remaining at 10% A for 5 more minutes. A divert valve was used to let the sample pass into the instrument from 4.5 to 9 min. The typical retention time was 6.7 min for the peptide N-terminal rbST and 6.9 min for the peptide N-terminal reST. The API interface was a linear ion trap coupled with an orbital trap allowing high resolution measurements (LTQ-Orbitrap®, Thermo Electron, Bremen, Germany), fitted with an electrospray ion source. The mass spectrometer was operated in positive ion mode. A sample volume of 20 µL was loaded on column using the autosampler. A column heater was used to ensure a stable column temperature of 30°C.
Mass spectrometric analyses were done in the following working conditions: capillary

voltage was set at 42 V, source voltage at 5 kV and capillary temperature at 300°C. Nitrogen was used as sheath, auxiliary and sweep gas at flow rates of 50, 10 and 10 (arbitrary unit) respectively. The linear ion trap mass spectrometer was set to select the ions 933.5 and 913.3 corresponding to [M+2H]2+ of tryptic N-terminal peptides of reST and rbST, respectively. Collision energy of 20% (arbitrary unit) was applied to the ion 933.5 and 18% to the ion 913.3. The analysis of the different fragments was performed in the orbital trap at a resolution of 30000. Acquisition was performed from m/z 500 to 1500. Data were collected and analysed with the Xcalibur software (Thermo Electron, Bremen, Germany).

## Results and discussion

*Selection and fragmentation pathway of the target peptide*

In order to avoid the problem of multi-charged ions yield when electrospray ionization of proteins, the choice was made to digest the protein with an enzyme (trypsin) and focus only on a single peptide representative of this protein. As the difference between the endogenous and recombinant form of the bovine somatotropin is located at the N-terminal of the protein, it was logical to focus on the N-terminal peptide of the molecule, specific of the rbST. Its main form [M+2H]2+ m/z 913.5 was monitored and fragmented. As shown in Figure 1, the fragments obtained were mainly y-type with the most abundant $y_{15}^{2+}$ (m/z 773.9) chosen for quantification purposes. Two other fragments $y_9$ and $y_{10}$ (m/z 960.6 and 1047.6 respectively) were monitored for confirmation. The tryptic N-terminal reST peptide was used as internal standard. The ions monitored were $y_5$, $y_{12}$ and $y_{15}^{2+}$ with the corresponding masses 577.7, 1288.2 and 793.9 respectively obtained with a CID set at 20%.

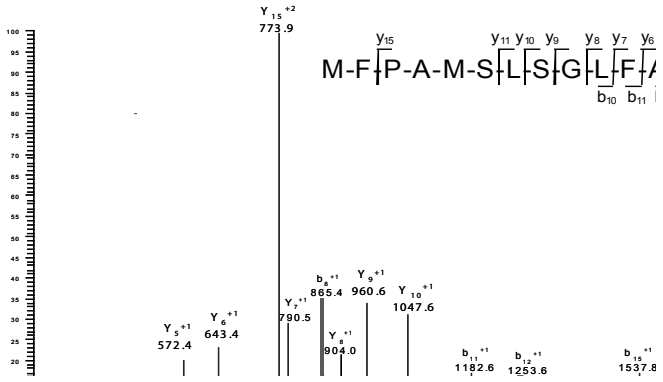


**Figure 1 :** Product ion scan mass spectrum obtained after fragmentation (CID 18%, LC-HRMS) of the tryptic N-terminal peptide of rbST (m/z 913.5). The different fragments obtained experimentally are presented on the peptide amino acid sequence.

*Results on an incurred sample*

The method described in this paper was used for the analysis of plasma spiked at 10 and 30 ng.mL-1 with rbST as well as plasma originating from a goat treated with rbST. The animal was treated twice with Lactatropin and the sample analysed corresponds to blood taken 48h after a second injection of rbST (i.e. 4 days after the beginning of the treatment).

Recombinant bovine somatotropin was successfully detected in the spiked samples as well as in the incurred as shown in Figure 2.

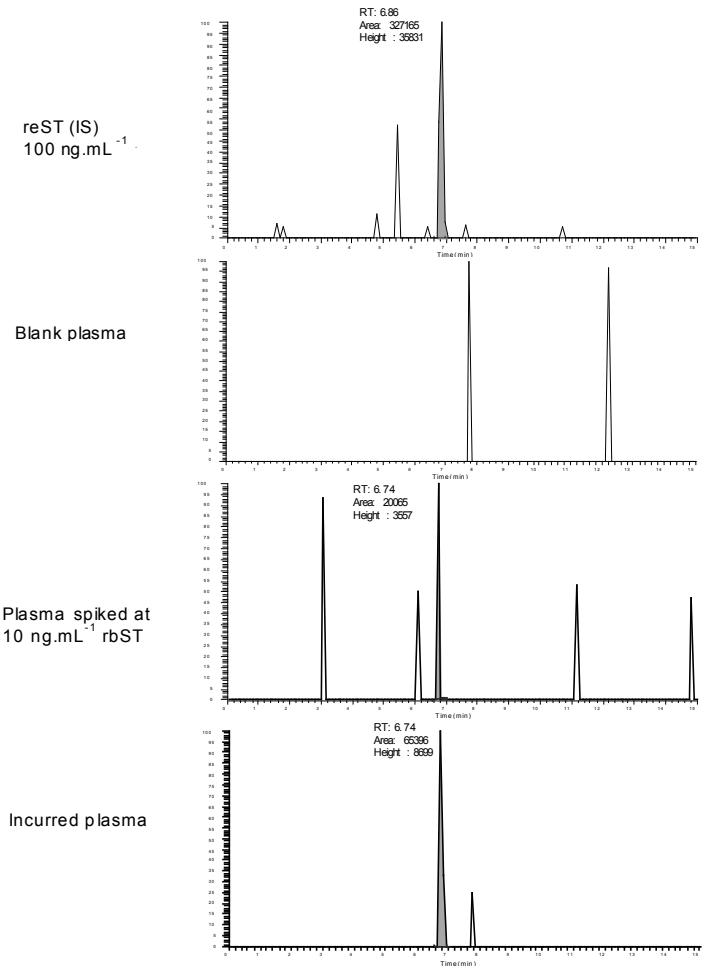


**Figure 2 :** Extracted diagnostic ion chromatograms obtained for goat plasma samples. The different signals correspond to the internal standard (reST 100 ng.mL$^{-1}$), plasma collected before treatment, plasma spiked with 10 ng.mL$^{-1}$ rbST and plasma 48h after a second injection of Lactatropin.

A relative quantification has been performed in comparison with the samples spiked at 10 and 30 ng.mL$^{-1}$ with rbST and gave a result about 41 ng.mL$^{-1}$ of rbST in plasma. This high value can be explained by the fact that the treatment was not adapted to the animal. Indeed, Lactatropin was developed for the treatment of lactating cows and is a prolonged-release formulation.

**Conclusions**

The method described in this study allows for the first time the detection of rbST in plasma from an animal treated with rbST. This approach, especially with the use of LC-HRMSn, a new technology that brings the power of high resolution in addition to the sensitivity of mass spectrometry, coupled with an efficient purification procedure, allowed a limit of detection of 10 ng.mL-1 of rbST in plasma. The objective is now to adapt this method to bovine plasma as well as other biological fluids like milk.

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**P 9**

# CONFIRMATION AND QUANTIFICATION OF 19 β-AGONIST COMPOUNDS IN BOVINE LIVER BY LC-(ESI+)MS/MS. METHOD VALIDATED ACCORDING TO CD 2002/657/EC.

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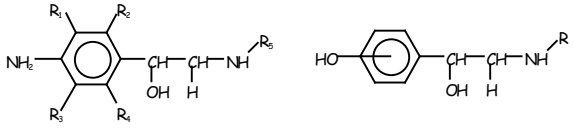
**Abstract**
A sensitive and selective multi-residue analytical method is presented for the simultaneous detection in bovine liver of 19 β-agonist compounds. After an acidify extraction, extracts were adjusted to pH 6 and centrifuged. Samples were cleaned up by solid phase extraction on a CSDAU mixed phase. The method is based on a liquid chromatographic separation using a C8 column and a water (0.02%TFA) – acetonitrile mobile phase with a 35 minutes gradient elution. β-agonist residues were identified and quantified by an LC-tandem quadrupole mass spectrometer using electrospray in the positive ion mode, MRM transitions and with the use of salbutamol-D6 and clenbuterol-D6 as internal standard. Under optimized conditions, detection capabilities below the MRPL were obtained for all β-agonists investigated. The validation of the method was achieved in bovine liver according to the criteria laid down in the Commission Decision 2002/657 EC, by the analysis of several spiked samples. Parameters such as identification criteria, selectivity and specificity, linearity, matrix effect, decision limits CCα and detection capabilities CCβ, accuracy and precision (repeatability and within lab reproducibility) were calculated.

**Introduction**
The use of β–agonists as feed additive for growth promotion in cattle and other farm animals is banned by the European Union since 1996 (ref1). For clenbuterol (CB), MRLs exist in a range of tissues (e.g. 0.5µg/kg in bovine liver). For the majority of β-agonists, a Minimum Required Performance Limit has been suggested by the CRL at 1, 3 and 10 µg/kg respectively for group I, II and III. (ref2). Group I contained clenproperol (CP), clencyclohexerol (CCH), clenpenterol (CPT), mabuterol (MB), mapenterol (MP), brombuterol (B), zilpaterol (Z) and tulobuterol (T). In group II, cimaterol (CM), cimbuterol (CMB), salbutamol (S), salmeterol (SM), isoxsuprine (ISP), ractopamine (R), terbutaline (TB), fenoterol (F) and ritodrine (RI) were involved. Orciprenaline (O) was in group III. Some of them are considered as veterinary drugs and can be use to treat respiratory problems by stimulating β-2adrenergic receptors. Human consumption of meat containing β-agonists may result in increasing heart rate and palpitations (ref3-4).
β–agonists have a chemical structure derived from catecholamine acting on animal growth by modifying meat/fat repartition. These effects lead to the abuse of these drugs in livestock production. This illegal use of clenbuterol (and related compounds) has been reported from a number of countries. Subsequently beta-agonists analyses were included in regulatory control and monitoring programs. While monitoring for clenbuterol has restricted its usage, it has

been demonstrated that unscrupulous producers may switch to other β-agonists for the same purpose (ref5-6). Metabolism action mode implies specific recognition of cellular β-receptors. Belong to the chemical group of substituted phenylethanolamines, β-agonists structure can be aniline type (A), hydroxyl-phenyl type (B) or other type (C). Type A (-NH2) involving CB, MB, MP, CM, CMB, CPT, B, CP and CCH. Type B (-OH) involving TB, R, S, ISP, F, O, SM and RI and type C with tulobuterol and zilpaterol (see fig1).

**Figure 1:** structure of β-agonists: type A=R-NH2; type B=R-OH




A B

The multi-analyte procedure described was developed in 2 stages. The first stage was to improve an old method developed in the laboratory where 11 β-agonists residues were identified in beef liver by modifying the HPLC buffer used. In the second stage, in response to the arrival of new compounds on the market, the method was extended to 8 other β-agonists and CCα and CCβ calculation adjusted with experimental data (decreasing fortified samples) instead of theoretical calculation (intercept of the slope).
The current procedure determined 19 β-agonists in liver using acidification extraction and clean-up on CSDAU (SPE) cartridges, followed by determination of the residues by LC-MS/MS using +ESI. Analyte's identification and validation were achieved according to the criteria laid down in the CD 2002/657/EC (ref7). A semi-quantitative dosage using deuterated internal standards CB D6 and S D6 has evaluated the amount of residues (ref8). The method applicable to liver samples, has been validated in a concentration range of 0.1 to 15 µg/kg depending of the compound.

## Materials and Methods

*Chemicals and Reagents*
All standard compounds were obtained from the Community Reference Laboratory (CRL) in Berlin in charge with β-agonists (ref2). Other chemicals were obtained from local suppliers. All solvents were analytical grade. CSDAU 50 (Clean ScreenDau, C8/benzosulfonic acid, 500mg, 6ml Technicol) SPE column were used for purification.

*Standard solutions*
Individual stock standard solutions at 100mg/L were prepared by dissolution in methanol. Internal standards, deuterated salbutamol and clenbuterol solutions were prepared in a similar way. Individual concentrations of each standard solution were corrected for purity. Adequate dilution in methanol allowed reaching concentrations of 10, 1 and 0.1 mg/L. A concentrated mixed working standard solution (CMWSS) containing 0.1mg/L of clenbuterol, 0.2mg/L of group I compounds, 0.6mg/L of group II compounds and 2 mg/L of orciprenaline was prepared in methanol. CMWSS (at 2MRPL) was diluted to obtain mixed intermediate

working standard solutions (MIWSS) from 0 to 2MRPL. Covering 0,1/5,1/4,1/2,1 and 1.5 times the MRPL values proposed by the CRL.

*Samples*
Bovine liver samples certified to be free of β-agonists were obtained from a local slaughter. Samples were mixed homogeneously and stored at -20°C. Sample treatment procedure was adapted from that described for urine by Montrade et al (ref9). To 10g of minced tissue, 10ng of CB-D6 and 20ng of S-D6 were added as internal standards. After homogenization in a stomacher bag with chlorhydric acid, sample was centrifuged 15 minutes at 9000rpm. The upper layer was removed and adjusted to pH 6.0 with NaOH 1M. Supernatant coming from the second centrifugation was applied to the preconditioned CSDAU cartridges. After washing with acetic acid 1M and methanol, compounds were eluted with ethylacetate/ ammoniac 28% (97/3; v/v) solution. Eluate was evaporated to dryness under a stream of nitrogen (max 70°C) and reconstituted in 150µl of acidified water before injection.
7 portions of blank liver were weighted. One blank was fortified with 100µl of MIWSS to be used as QC sample at the MR(P)L level. Because of matrix effect, the remaining 6 blank portions were extracted and used for constructing the matrix-matched calibration curve. They were fortified after extraction with 100µl of MIWSS and with 100µl and 200µl of respectively CB-D6 and S-D6 to give corresponding matrix tissue standards from 0 to 1.5*MR(P)L levels. Solvent was evaporated to dryness and extracts reconstituted in acidified water.

*Equipment*
A 2690 Alliance HPLC system was used in this study (Waters, Milford, USA). The liquid chromatograph was coupled to a Quattro Micro triple-quadrupole mass spectrometer equipped with a Z-spray source (Waters, Manchester, UK). MS control and spectral processing were carried out using Masslynx software, version 4.0 (Waters). The analytes were separated on two successive C8 Chromspher (Varian-Chrompack), 100*3 mm columns with 5µm particle size, connected to a reversed phase guard column 10*2 mm (Chrompack 28141).
A linear gradient at a flow rate of 0.3ml/min allowed the compounds elution and separation. Mobile phase composition changed from 80/20 to 40/60 (acidified water/acetonitrile) in 10 minutes and hold on during 25 minutes. Autosampler and column temperature were maintained respectively at 4°C and room temperature. The total HPLC effluent was delivered to the MS detector using nitrogen as probe and desolvation gas. Argon was used as collision gas. The block source and desolvation temperature were maintained at 120 and 300°C, and electrospray capillary voltage set at 3.5 kV. Cone voltage and collision energy were tuned for each analyte to optimize the transition of the molecular precursor on the two most abundant product ions. Mass spectrometer was run in +ESI mode using multiple reaction monitoring (MRM).

*Validation experiments*
Validation protocol was performed in accordance with the criteria defined in the Commission Decision 2002/657/EC (ref7). CCα, CCβ, linearity, recovery, matrix effect, accuracy and precision were determined. A representative sample was homogenized and divided into 63 identical sub-samples and fortified at 5 different concentration levels. Twenty-one samples, consisting of 1 blank bovine liver sample, 3 samples spiked at a level of 1/5 MR(P)L, 3 at 1/4 MR(P)L, 3 at 1/2 MR(P)L, 3 at 1 MR(P)L, 3 at 1.5* MR(P)L and 5 samples for the matrix

calibration curve were analyzed on each day for 3 days (3 replicates per level and per run in 3 independent runs). Matrix calibration curves were produced in a concentration range from 0.1 to 0.75 µg/kg for clenbuterol, from 0.2 to 1.5µg/kg for group I compounds, from 0.6 to 4.5 µg/kg for group II compounds and from 2 to 15 µg/kg for orciprenaline.
Matrix effect was studied by comparing the slope of a calibration curve based on solution standards and a calibration curve based on matrix matched standards (n=3 replicates per concentration level). The accuracy of the method was expressed by the overall recovery of β-agonists in the fortified samples while the repeatability and within-lab reproducibility were determined and expressed by the coefficients of variation measured on fortified blank samples. To calculate CCα and CCβ not only quantitative but also qualitative criteria on fortified samples were taken into account (ref10). Identification criteria have to be fulfilled in 50% of the cases to determine CCα and in 95% of the cases to determine CCβ.

**Results and discussion**

*Identification criteria*
According to the EU criteria for the analysis of banned substances listed in Group A5 of Council Regulation 2377/90, a system of identification points (IPs) was used to define the number of ions and their corresponding ratios that should be measured when using confirmatory MS techniques. For the analysis of β-agonists by LC-MSMS, a minimum of 4 IPs was required. The consideration of one precursor ion (1 point) and two transition reactions for each analyte (2*1.5 points) fulfilled this requirement. Additionally the relative abundances of all daughter ions, monitored from the analyte, should match those of the standard analyte in a margin of ± 20; 25; 30 or 50% depending respectively of the relative intensity of the standard (ref7). Furthermore, the relative retention time of the analyte had to equal the retention time of the standard analyte in the respective matrix within a margin of ± 2.5%. For each analyte, these criteria were 100% satisfied at the MR(P)L level. At the half MR(P)L level, those criteria were respected in 89% of cases excepted for CM (78%), SM (67%) and S (100%). Therefore, to confirm the presence of β-agonists in liver, the above mentioned criteria had to be satisfied in comparison with the (QC) fortified sample at the MR(P)L level.

*Linearity and Matrix effect*
Matrix effect was highlighted for 9 compounds : CB, CP, CCH, Z, S, R, TB, F and RI. A matrix-based calibration curve was consequently systematically considered during this validation. The linearity of the matrix calibration curve was verified by the Mandel's fitting test (ref11). Over the concentrations range considered (i.e. from 0 to 15µg/kg), correlation coefficients were always > 0.995.

*Selectivity/specificity*
To improve chromatography and sensitivity, mobile phase was modified from phosphate buffer to acidified water with adaptation of the gradient. In those conditions, no interfering peaks from endogenous compounds were observed at the analyte retention time in blank liver sample. The MRM transitions used for quantification and identification allowed to fulfil all identification criteria at the MR(P)L level.

*Decision Limit CCα and Detection Capability CCα*
The decision limits (CCα) ranged from 0.1 to 2 µg/kg and detection capabilities (CCβ) from 0.25 to 3 µg/kg for all β-agonists studied. In general, for the banned substances, CCα and CCβ were below the suggested MRPL. Acceptable values above the MRL were obtained for the authorized CCB (0.67 and 0.85 µg/kg respectively for CCα and CCβ).
The EU criteria (ISO11843-2) (ref12) proposed several options for the calculation of CCα but a more practical approach based on the respect of the identification criteria (IC) of fortified samples was applied (ref10). For remember, 45 blank samples were fortified at 5 concentration levels (from 1/5 to 1.5*MR(P)L). 9 replicates per concentration level were analyzed as 3 replicates in 3 independent analytical runs. The fortification level where 50% of the IC was fulfilled, determined the CCα limits. While, the fortification level where 95% of the IC was respected was considered as CCβ .

*Accuracy and precision (repeatability and within lab reproducibility)*
As certified reference material was unavailable, the accuracy was expressed as the percentage of residues recovered in the fortified samples. According to the CD 2002/657/EC, the accuracy of a confirmatory method should range from 50 to 120%, 70 to 110% and 80 to 110% respectively for samples fortified at concentration ≤ 1 µg/kg, between 1 and 10µg/kg and above 10µg/kg. Recoveries for B, MP, MB, CPT and T were acceptable at the MRPL concentration and below. The accuracy of the method was at the border and /of outside the permitted range for all other β-agonists. The use of inadequate internal standard and the wide range of chemistries of the 19 compounds currently incorporated in this method could explain those results. In fact, in this validation, recovery was corrected by matrix calibration using only 2 deuterated internal standards, S-D6 for S and O and CB-D6 for all other compounds. To improve quantification, more deuterated analogues should be used for each analyte. Nevertheless, as β-agonists are banned substances, zero tolerance was applied. Therefore, to declare sample non compliant, qualitative identification was sufficient.
Precision repeatability and within-lab reproducibility were also evaluated. The coefficient of variation (CVr) for repeatability and (CVRW) for reproducibility were also determined with fortified samples. According to the legislation, CVr lied below or between ½ and 2/3 of the CV calculated for those concentrations by the Horwitz equation for all β-agonists excepted O, F and SM. At the MRPL level, CVRW did not exceed the level calculated by the Horwitz equation for 11 compounds. It was not the case for MB, MP, B, SM, ISP, R, F and P. For clenbuterol having an established MRL value at 0.5 µg/kg, CVRW was as required below Horwitz CV.

**Conclusion**
A specific and sensitive LC-MS/MS analytical method for the simultaneous determination of 19 β-agonists in beef liver has been developed and validated according to the EU criteria defined in the CD 2002/657/EC. The method was based on MRM transitions and the ratio between the areas of the analyte and its deuterated analogues. Only 2 deuterated standards were available: S-D6 to quantify S and O and CB-D6 for all other compounds. The validated method has been successfully used to quantify CB and S at the respective MRL and MRPL concentrations. For CP, CCH, CM, CMB, TB and Z, high recovery values were calculated. Within-lab reproducibility problems appeared for MP and T while only qualitative determination was achieved for O, SM, R and F. Therefore, the analytical method described

was validated quantitatively for CB and S and qualitatively for the 17 other βagonists studied. The majority of β-agonists being banned substances, it was concluded that this analytical method was applicable for residues control.

Acknowledgements
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## P 10

# A CONFIRMATORY METHOD FOR THE DETERMINATION OF TETRACYCLINES IN MUSCLE WITH MONOLITHIC COLUMN HPLC AND DIODE-ARRAY DETECTION

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**Abstract**
Using high-performance liquid chromatography with diode-array detection (HPLC-DAD) technique, a confirmatory method for determination of trace levels of tetracyclines (oxytetracycline, tetracycline, chlortetracycline and doxicycline) and their 4-epimers (4-epioxytetracycline, 4-epitetracycline and 4-epichlortetracycline) in animal tissues was developed.
The samples are extracted with a mixture of succinic acid 0.1 M (pH 4) and methanol after the addition of metacycline as internal standard. The clean-up is carried out by metal chelate affinity chromatography with a following concentration step on an HLB polymeric reverse phase column. Chromatographic separation of analytes is achieved in ten minutes on a short monolithic column (50 mm x 4.6 mm i.d.) using a gradient elution.
The method was validated in bovine muscle following the Commission Decision 2002/657/EC criteria: samples spiked at three concentration levels (0.5, 1 and 1.5 times the maximum residue limit) were analysed. Method trueness and precision (repeatability and intra-laboratory reproducibility) as well as decision limits (CCα) and detection capabilities (CCβ) are reported.

**Introduction**
Tetracyclines antibiotics (TCs) are globally used as broad spectrum antibiotics in veterinary medicine and aquaculture. Residues in food of animal origin may be found, often because of improper observance of withdrawal times. The European Union laid down maximum residues limits (MRLs) for oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxicycline (DXC): 100 µg kg-1 in muscle and in milk, 200 µg $kg^{-1}$ in egg, 300 µg $kg^{-1}$ in liver and 600 µg $kg^{-1}$ in kidney. These MRLs are expressed as the sum of the parent drug and its 4-epimer, except for DXC for which only the parent compound is included in the MRL.
Several HPLC-DAD methods have been published for the tetracyclines determination in foods, however, none of these present fully satisfactory quantitative validation results, meeting the EU requirements, for parent TCs as well as their 4-epimers. The use of the metal chelate affinity chromatography (MCAC), introduced by Carson (1993), allowed to achieve a very selective cleanup before HPLC analysis. However, this approach needs further concentration of the MCAC eluate to reach low detection limits.
This paper reports a sample cleanup which produces highly concentrated final extracts. Moreover, the use of a monolithic HPLC column allowed the complete chromatographic separation of both parent drugs and epimers in only ten minutes.

## Materials and methods

*Solvents and reagents*

The chemical and chromatographic reagents used were of LC or analytical grade. Disodium hydrogen phosphate dihydrate, methanol, LC grade methanol, succinic acid and sodium chloride were purchased from Carlo Erba (Milano, Italy). Citric acid, EDTA disodium salt dihydrate, oxalic acid, triethylamine were supplied by J.T. Baker (Deventer, The Netherlands). Tetrahydrofuran (THF) and acetonitrile (LC grade) were obtained from Romil (Cambridge, UK) and Carlo Erba (Milano, Italy) respectively. Chelating Sepharose Fast-Flow suspended in ethanol was purchased by GE Healthcare (Uppsala, Sweden) and Oasis HLB Speedisk columns (60 mg/3 mL) by Waters (Milford, MA, USA). Eluents for HPLC and standard solutions were prepared with high-purity water obtained from a Milli-Q system (Millipore, Bedford, MA, USA). LC solvents were filtered with 0.45 μm Durapore membrane (Millipore).

The extraction solution (pH 4.0) was prepared by mixing till dissolution 11.8 g of succinic acid and 7 mL of 5 N sodim hydroxide in 1000 mL deionized water (0.1 M). The McIlvaine-EDTA-NaCl buffer is an aqueous solution of 1% EDTA disodium salt dihydrate and 0.78% NaCl. HPLC oxalic buffer pH 3.0 (0.01 M) mobile phase (B) was prepared dissolving 1.26 g of oxalic acid dihydrate in a 1000 mL volumetric flask with about 700 mL of high-purity water. The pH was adjusted to 3.0 with some drops of triethylamine and then 15 mL of THF was added. The buffer was diluted to volume with Milli-Q water.

*Standards*

CTC, DXC, metacycline (internal standard, IS) and TC were purchased from Riedel-de Haën (Seelze, Germany) and OTC from Sigma (St. Louis, MO, USA). The 4-epitetracycline (4-epiTC), 4-epioxytetracycline (4-epiOTC) and 4-epichlortetracycline (4-epiCTC) were supplied by Acros (Fisher Scientific, Schwerte, Germany). Standard stock solutions were prepared by accurately dissolving approximately 10 mg of each TCs in 10 mL of LC grade methanol and stored at -20 °C. Working standards were prepared daily by appropriate dilution in mobile phase B.

*HPLC–DAD equipment and conditions*

The chromatographic apparatus was a ThermoFinnigan Spectrasystem (Milano, Italy) composed of a P4000 quaternary pump, an AS3000 autosampler equipped with a 7010 Rheodyne valve (Bensheim, Germany) and a diode array (UV6000LP) detector. The chromatographic separation was accomplished in 10 min with the gradient elution reported in Table 1. The mobile phases were methanol (A) and the solution of oxalic acid 0.01 M (pH=3) with 1.5% of THF (B). A RP-18e monolithic column Cromolith® Speed Rod (50 x 4.6 mm i.d.) from Merck (Darmstadt, Germany) was used to separate all of the analytes. TCs and their epimers were detected at 360 nm.

**Table 1.** Gradient timetable

| Time (min) | Mobile phase A (%) | Mobile phase B (%) | Flow (mL min$^{-1}$) |
|---|---|---|---|
| 0.0 | 0 | 100 | 4.0 |
| 2.2 | 0 | 100 | 4.0 |
| 3.0 | 10 | 90 | 4.5 |
| 6.5 | 15 | 85 | 4.5 |
| 8.0 | 0 | 100 | 4.0 |
| 10.0 | 0 | 100 | 4.0 |

*Sample preparation*

The muscle sample was cut into pieces and blended. 3 g of the tissue were accurately weighed in a 50 mL centrifuge tube and, according to the protocol, either spiked with the seven analytes or not (in case of blank samples). The IS was added in each sample at 100 µg kg-1. The extraction was accomplished by shaking the muscle for about 15 min with 20 mL of extraction solution mixed with 20 mL of methanol. The tube was then centrifuged at 4000 rpm for about 15 min and the supernatant transferred in a 100 mL flask. The sample was further extracted with 10 mL of the extraction solution mixed with 10 mL of methanol. The extracts (about 60 mL) were combined and loaded on a pre-prepared MCAC column, previously activated with 6 mL Milli-Q water and 3 mL of 10 mM copper (II) sulphate solution. The column was washed sequentially with 2 mL of the extraction solution, 2 mL of water and 2 mL of methanol. The analytes were eluted with 8 mL of McIlvaine-EDTA-NaCl buffer directly onto the OASIS HLB cartridge, previously conditioned with 3 mL of MeOH, 3 mL of 0.1 N HCl and 3 mL Milli-Q water. The OASIS cartridge was rinced with 3 mL of Milli-Q water and the TCs and 4-epimers were then eluted with 6 mL of methanol. The solvent was removed under nitrogen stream, the residue dissolved in 0.25 mL of mobile phase B and filtrated through 0.45 µm PTFE syringe filter. 50 µL were injected into the HPLC-DAD system.

*Validation study*

The method validation was carried out in following the Commission Decision 2002/657/EC requirements. The specificity, linearity, precision (repeatability and within-laboratory reproducibility), recovery (trueness), decision limit (CCβ, and detection capability (CCβ were evaluated. For validation purposes, blank bovine muscle samples were spiked with the parent drugs and the 4-epimers working solution at concentrations corresponding to 0.5, 1 and 1.5 the MRL.

**Results and discussion**

A typical chromatogram of a standard solution of the TCs and their epimers monitered at 360 nm is shown in Figure 1.

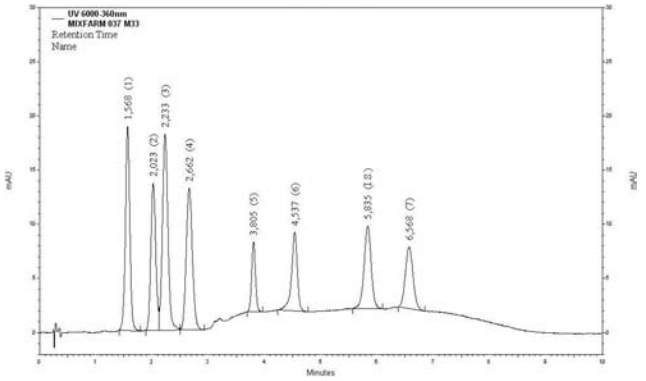
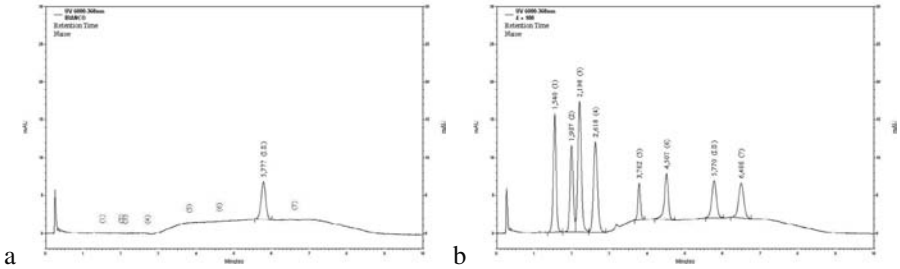



**Figure 1.** LC-DAD chromatogram of a standard solution of 4-epiTC (1), 4-epi-OTC (2), TC (3), OTC (4), 4-epiCTC (5), CTC (6), IS and DXC (7).

The selectivity was assessed studying the absence of any interferences at the eluition times of the analytes. For this purpose twenty muscle samples of different animal species were analysed. The adopted cleanup gives chromatograms free of interferences (Figures 2 and 3).



a b

**Figure 2.** Chromatogram a): blank bovine muscle (IS: 100 µg kg-1); chromatogram b): bovine muscle spiked at the MRL with all analytes (IS: 100 µg kg-1)

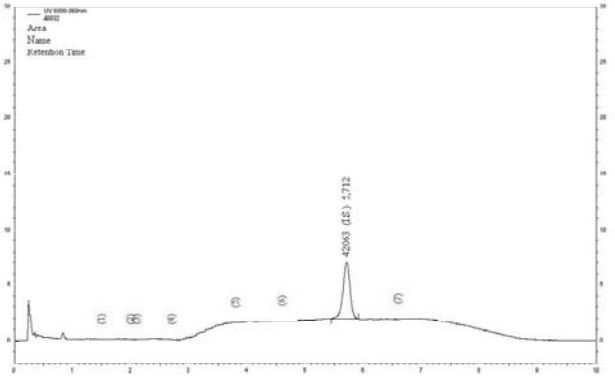



**Figure 3.** Chromatogram of a blank horse muscle (IS: 100 µg kg-1)

The standard calibration curves are linear over the range 0.05-2 μg $mL^{-1}$ (2.5 -100 ng) with determination coefficients greater than 0.9997.
The precision observed in the bovine muscle spiked at 50, 100 and 150 μg $kg^{-1}$ was calculated applying the one-way analysis of variance (ANOVA). The coefficients of variation (CVs) were lower or equal to 8% and 23% in repeatability or in intra-laboratory reproducibility conditions respectively. The apparent recoveries (recoveries obtained after correction with IS) ranged from 91 to 104%.
The decision limits (CCα) and detection capabilities (CCβ) were calculated from the within-laboratory reproducibility (Table 2). The higher CCα (and CCβ) obtained for 4-epiOTC and TC are probably due to the lower chromatographic resolution between these adjacent peaks (Rs=1.1).

**Table 2.** Decision limits and detection capabilities

| | **CCα** (μg $kg^{-1}$) | **CCβ** (μg $kg^{-1}$) |
|---|---|---|
| **4-epiTC** | 117 | 132 |
| **4-epiOTC** | 132 | 166 |
| **TC** | 126 | 152 |
| **OTC** | 113 | 126 |
| **4-epiCTC** | 118 | 135 |
| **CTC** | 109 | 118 |
| **DXC** | 119 | 136 |

**Conclusions**
The reported confirmatory method meets European requirements and will be validated soon in other food matrices (eggs, milk and honey). The use of a suitable internal standard (methacycline) allowed to reach a good trueness which generally represents one of the biggest problem in tetracyclines determination. Finally it is important to point out that the chromatographic analysis is performed with a traditional HPLC-DAD instrument available in almost all the routinary laboratories. The same can not be said of the HPLC-MS-MS systems.

**Acknowledgements**
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## P 11

# ABOUT THE DECISION LIMIT FOR PERMITTED SUBSTANCES

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**Abstract**
This paper describes the problems that came out in the practical application of the decision limit (CCα) in the analysis of substances with an established maximum residue limit (MRL). For confirmatory methods, CCα is a fundamental performance characteristic introduced by EU Decision 2002/657/EC. Since the decision limit takes automatically into account the measurement uncertainty, for permitted compounds, it lies always above the MRL. However, in some cases the permitted limit is fixed as a "sum MRL" (e.g. sulfonamides or tetracyclines) and this implies that, if in a sample two or more of the regulated molecules are found, the analyst faces the lack of criteria for sample judgement (compliance or not).
In these particular circumstances, the uncertainty approach introduced by ISO, taking into account only the intra-lab reproducibility sources of variability, could better help to investigate sample compliance. Moreover the use of uncertainty estimation unifies the criteria for sample judgment with other analytical fields (e.g. environmental contaminants) and avoids the misunderstandings generated by the use of the same term "decision limit" for both banned and permitted substances.

**Introduction**
The EU Decision 2002/657/EC introduced the decision limit and detection capability performances characteristics. Following the definition, "Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant". However this implies, from the analytical viewpoint, the definition of two very different CCα's: one for forbidden substances represents the lowest concentration level at which a method can discriminate with a statistical certainty of 1–α that the particular analyte is present, whereas, for permitted substances, the decision limit is the concentration above which it can be decided with a statistical certainty of 1–α that the permitted limit has been truly exceeded.
Regarding permitted drugs, in some cases the MRLs are fixed for the sum of two or more analytes. In such cases the judgement of compliance with respect to CCα can be difficult. On the other hand, in the field of environmental contaminants, for instance, not the CCα, but the measurement uncertainty approach is used to decide if a specified limit is exceeded. Estimating measurement uncertainty can be done by identifying all possible sources of uncertainty associated with an anaytical procedure, quantifying their magnitude and calculating the total uncertainty by combining the individual uncertainty components following the error propagation rules. Guidelines for this "bottom-up" approach were given in the "Guide to Expression of Uncertainty in Measurement" (GUM, 1993) and were

further interpreted for analytical chemistry by EURACHEM in 2000. According to the ISO/IEC 17025, when assessing compliance, the accredited laboratories must then consider the measurement uncertainty evaluated following preferably the GUM and EURACHEM indications.
This paper describes a simple method to ascertain the compliance of a food sample in which more than one of the regulated substances are found. The reported strategy make use of the uncertainty estimated by the bottom-up method. The aim is to drawn the attention on the problems found by accredited routine laboratories which must follow the ISO/IEC 17025 and 2002/657/EC requirements too.

**Materials and Methods**
In a milk sample 56 µg kg-1 of sulfadiazine and 55 µg $kg^{-1}$ of sulfamerazine (sum = 111 µg kg-1) are found at the same time.
Regarding the MRLs, the Regulation 2377/90 specifies that the total sulfonamides residues in milk should not exceed 100 µg $kg^{-1}$. The results of the validation study done on the applied analytical method (HPLC-DAD) are reported in Table 1. The performances characteristics have been obtained following the 2002/657/EC criteria, testing, in three analytical series, concentrations which are 0.5, 1 and 1.5 times the MRL. Furthermore, as suggested by "Guidelines for the implementation of Decision 2002/657/EC" (SANCO/2004/2726 rev. 2), an additional level at 20 µg $kg^{-1}$ has been investigated (lowest concentration detectable).

**Table 1.** Validation results of the method for the determination of sulfadiazine and sulfamerazine in milk

| **Sulfonamide** | **Nominal concentration** (µg $kg^{-1}$) | **$s_R$[a] (µg $kg^{-1}$)** (n=18) | **CV[b]** (%) | **CCα** (µg $kg^{-1}$) |
|---|---|---|---|---|
| Sulfadiazine | 20 | 1.9 | 9.6 | 112 |
| | 50 | 4.3 | 8.6 | |
| | 100 | 7.4 | 7.4 | |
| | 150 | 5.4 | 3.6 | |
| sulfamerazine | 20 | 2.1 | 11 | 111 |
| | 50 | 3.8 | 7.6 | |
| | 100 | 7.0 | 7.0 | |
| | 150 | 6.1 | 4.1 | |

*[a]The $s_R$ (standard deviation under within-laboratory reproducibility conditions) is calculated via ANOVA; [b]Coefficient of Variation*

**Results and Discussion**
The question is to decide whether the obtained result (111 µg kg-1 as sum of both sulfonamides) implies the compliance or not of the analysed sample, taking into account the decision limits reported in Table 1.
From the validation data, the milk sample appears to be compliant because none of the decision limits is exceeded by the sum of the concentration of the two analytes (111 µg kg-1).

On the other hand, it has been observed from long experience that, over a range of concentrations far from zero, the standard deviation varies proportionally to the level of the analyte (CV almost constant). In this study, in the interval of interest (20-100 µg kg-1), this linear relationship is demonstrated (Figure 1). The variance for the two sulfonamides at the measured levels can, therefore be obtained from experimental data by regressing the standard deviation, sR, versus analyte concentration.

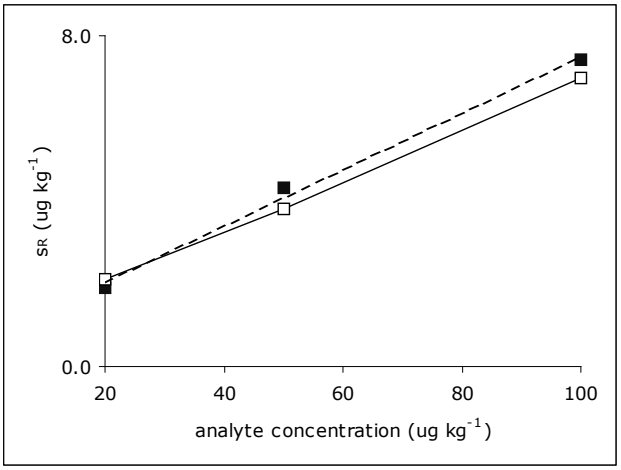


**Figure 1**. Linear regression graph of the standard deviation (sR) vs. the analyte concentration ( ■: sulfadiazine; □: sulfamerazine)

For sulfadiazine at c=56 µg $kg^{-1}$, the interpolated standard deviation, sR, is 4.6 µg $kg^{-1}$, whereas, for sulfamerazine at 55 µg $kg^{-1}$, the sR is equal to 4.3 µg $kg^{-1}$. The error propagation law says that the root square of the added variances gives the standard deviation associated to the sum of two analytes:

$$s_R(\text{sum}) = \sqrt{(4.6)^2 + (4.3)^2} = 6.3 \ \mu g\ kg^{-1}$$

Since the EU Decision 2002/657/EC restricts the sources of uncertainty to be estimated only to method precision (within-laboratory reproducibility), the sR(sum) corresponds, substantially, to a combined standard uncertainty. Applying the safety factor of 1.64, as prescribed by EU Decision for group B of substances, the expanded uncertainty associated to the sum of sulfadiazine and sulfamerazine can be calculated and is 10 µg kg-1. The MRL (100 µg $kg^{-1}$) is, therefore exceeded (111-10 = 101 µg $kg^{-1}$) and the milk sample would be declared non compliant.
It must be observed that the applied safety factor (1.64) derives from a one-tailed z distribution and this constant differs from the coverage factor (k) indicated by ISO in the expanded uncertainty estimation, which is, instead, referred to a two-tailed Student's t distribution.
The reported strategy can be easily generalized because, in residues analysis, frequently it

is possible to define a restricted concentrations range in which a linear relationship between standard deviation and analyte concentration is verified.

**Conclusions**

For banned substances the decision limit and the detection capability are two important performance characteristics which, go beyond the traditional concepts of detection and quantification limits, describing the method performances at low levels as function of alfa (false positive) and beta error (false negative), respectively. However, the same terms are used both for banned and permitted substances generating some misunderstandings because, in the latter case, the CCα is only an action limit. Furthermore, the use of alfa and beta error concepts for substances with MRLs has another drawback: the difficulty in establishing sample compliance in particular analytical cases like the ones illustrated in this paper. The CCα introduction has the great advantage of overcoming the practical problems arising in the estimation of the uncertainty, nevertheless the bottom-up approach, opportunely semplified taking into account only the intra-laboratory reproducibility sources, results surely more flexible.

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# MULTI-RESIDUE SCREENING FOR NITROIMIDAZOLES BY IMMUNOBIOSENSOR

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**Abstract**
An immunobiosensor assay was developed for the multi-residue screening of a range of nitroimidazole compounds in porcine, bovine and ovine kidney. Polyclonal antibodies which bind at least 7 of the major nitroimidazoles and their metabolites were raised in sheep after inoculation with a metronidazole protein conjugate. Sample homogenates were extracted into acetonitrile and subjected to micro-centrifugation prior to biosensor analysis. Validation data obtained from the analysis of 20 fortified samples has shown that the method has a detection capability (CCβ of less than 1 µg kg-1 dimetridazole (DMZ) in each of the species investigated. In addition, cross-reactivity data and the analysis of a smaller number of fortified samples has shown that the method will also detect a range of other major parent nitroimidazoles and their metabolites including ronidazole (RNZ), ipronidazole (IPZ), metronidazole (MNZ), hydroxymetronidazole (MNZOH), hydroxydimetridazole (DMZOH) and hydroxyipronidazole (IPZOH). The cross-reactivity profile and validation data for the detection of these nitroimidazoles is presented.

**Introduction**
Nitroimidazoles are antiprotozoal drugs that have been primarily used in veterinary medicine to treat histomoniasis and trichomoniasis in poultry and haemorragic enteritis in pigs. Growth promotion and improvement of feed efficiency were noted side effects following administration of the drugs. They are now suspected to have genotoxic, carcinogenic and mutagenic properties and their use has been banned in the European Union by Council Regulations 2377/90 and 2205/2001.
In order to monitor for the illegal use of such compounds within Europe, there is a requirement to have robust and reliable screening and confirmatory tests capable of low level detection of nitroimidazole residues. While physicochemical procedures (HPLC and LC-MS/MS) can detect the drugs at concentrations in the low µg kg-1 range, there are significant drawbacks when employing these methods due to time consuming sample extraction procedures and the cost of the analytical equipment required.
In the present study an optical biosensor screening assay was developed for the detection of nitroimidazoles in porcine, bovine and ovine kidney samples, as specified by the United Kingdom National Surveillance Scheme. The procedure was capable of detecting low µg $kg^{-1}$ concentrations of a range of nitroimidazole parent compounds and their metabolites with the additional advantages of high throughput analysis coupled with result generation in real time.

## Materials and methods

*Instrumentation*
An optical biosensor (BIACORE®Q) was obtained from Biacore AB (Uppsala, Sweden). Instrument operation and data handling were performed using BIACORE®Q Control Software (Version 3.0.1).

*Reagents and chemicals*
HBS-EP buffer, CM5 sensor chips (certified grade) and an amine coupling kit containing N-ethyl-N'-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS) and 1 M ethanolamine pH 8.5 were all obtained from Biacore AB (Uppsala, Sweden). Reference standards for DMZ, MNZ, RNZ and IPZ were supplied by Sigma-Aldrich Chemical Company Ltd. (Poole, Dorset, U.K.). The metabolites MNZOH, DMZOH and IPZOH were kindly supplied as gifts by the Community Reference Laboratory for nitroimidazoles (BGVV, Berlin).
All other chemicals were HPLC grade and were supplied by Sigma (Poole, Dorset, U.K.).

*Biosensor assay development*
Immobilisation of MNZ derivative. MNZ was coupled to an amine surface on a CM5 sensor chip via a di-succinimidyl carbonate (DSC) cross-linker. Briefly, the carboxymethyl dextran surface was activated with a 1/1 v/v mixture of 0.2 M EDC/0.05 M NHS for 20 min and the activated surface incubated with 1M ethylenediamine solution for 1 hr. The ethylenediamine solution was removed and the MNZ-DSC derivative was applied to the surface and allowed to incubate overnight at room temperature. After this time any unreacted sites were deactivated by incubating with 1 M ethanolamine pH 8.5 for 20 min. The chip surface was washed with deionised water and stored refrigerated when not in use.
Immunogen and antibody production. An MNZ-DSC-Jeff-BTG immunogen was produced and anti-MNZ antiserum raised in a sheep.
Sample preparation and extraction procedure. Negative and sample kidneys were homogenised and weighed (5 g) into glass universal bottles. Calibrants (0.01, 0.5, 1.0, 2.0, 5.0 and 10.0 μg $kg^{-1}$ DMZ) were prepared by adding working standards (50 μl) to the known negative kidney aliquots (Figure 1). Control and check samples were prepared in the same way using the appropriate working controls. At this point all standards, controls and samples were treated identically. Acetonitrile (10 ml) was added to each universal, vortexed vigorously for 10 s and mixed on a roller mixer for 30 min. All universals were then centrifuged at 3500 rpm for 10 min at 10°C and the supernatants decanted into test tubes. These were then evaporated to dryness using a Turbo-Vap® LV sample concentrator at 60°C under a stream of nitrogen gas. The resultant extracts were reconstituted immediately in HBS-EP buffer (250 μl) by vortexing vigorously for 1 min and transferred to microcentrifuge tubes prior to centrifugation at 13000 rpm for 10 min. Extracts for analysis were transferred (105 μl), in duplicate, to the wells of a microtitre plate.
Biosensor Assay. Kidney extracts were mixed with antibody (70/30 v/v) and injected (80 μl) over the sensor chip surface at a flow rate of 20 μl min-1. Report points were recorded before and after each injection to measure the response generated by antibody binding to MNZ immobilised on the sensor chip surface. The chip surface was regenerated with a 1

min injection of 20% acetonitrile in 0.25 M sodium hydroxide at a flow rate of 20 µl min-1. Including both sample extraction and biosensor analysis time, results for 20 kidney samples could be produced within 15 h.

## Results and discussion

*Antibody characterisation*

The polyclonal antibody produced was assessed for cross-reactivity against a range of nitroimidazoles prepared in assay buffer as well as in negative porcine, bovine and ovine kidney samples. Significant cross-reactivity was found against all of the major parent nitroimidazoles and their metabolites. In addition, extracted calibration curves were produced for all compounds in each of the 3 species and their respective IC 50 values determined (Table 1).

**Table 1.** IC 50 values determined for each parent nitroimidazole and their metabolite in negative kidney extract.

| **Compound IC 50 (µg kg$^{-1}$)** | | | |
|---|---|---|---|
| | **Porcine** | **Bovine** | **Ovine** |
| Dimetridazole | 2.15 | 3.91 | 3.78 |
| Metronidazole | 4.42 | 3.76 | 10.91 |
| Ronidazole | 2.28 | 1.88 | 5.37 |
| Ipronidazole | 0.41 | 0.56 | 5.37 |
| Hydroxymetronidazole | 2.36 | 2.34 | 2.22 |
| Hydroxydimetridazole | 7.70 | 5.80 | >10.0 |
| Hydroxyipronidazole | 7.38 | >10.0 | >10.0 |

*Biosensor assay validation*

The developed immunobiosensor assay was validated in accordance with Commission Decision 2002/657/EC.

Nitroimidazole free porcine, bovine and ovine kidney samples (n = 20) were extracted and analysed as has been previously described. To determine the CCß of DMZ for each of the 3 species, the same blank samples were spiked at 1.0 µg kg$^{-1}$ DMZ. Results obtained showed the method to have a proven CCß for DMZ of <1.0 µg kg$^{-1}$ for each of the 3 species. Furthermore, a smaller number of kidney samples (n = 5) from each species were fortified with the other major parent nitroimidazoles and their metabolites at various levels and the detection capability calculated for each. In all 3 species the detection capabilities for the parent drugs RNZ and IPZ and for the metabolite MNZOH were determined as <1 µg kg$^{-1}$while for MNZ it was determined as <2.0 µg kg$^{-1}$. For both the metabolites DMZOH and IPZOH detection capabilities of <3.0 µg kg-1 were achieved.

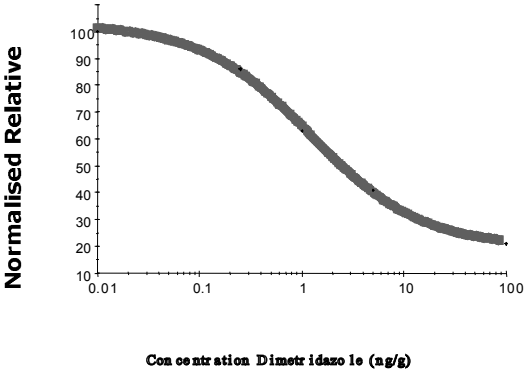


**Figure 1.** Typical example of a DMZ standard curve extracted from porcine kidney extract.

## Conclusions

A simple and reliable screening procedure based on optical biosensor technology has been developed which has been shown to be capable of detecting low concentrations of a range of nitroimidazoles and their metabolites in porcine, bovine and ovine kidney samples. The cross reactivity data obtained has shown that the antibody is suitable for the detection of at least 7 nitroimidazole parent drugs and their metabolites.

The sensitivity of any screening assay employed in laboratories which seek to control the unauthorised use of nitroimidazoles is of great significance due to the fact that they are banned substances within the European Union. While no Minimum Required Performance Limits have yet been set for kidney, in other matrices including muscle they have been provisionally established at 3 μg $kg^{-1}$ for DMZ, MNZ and RNZ and their metabolites. No recommendation has been proposed for IPZ or IPZOH to date. The CCß values obtained in this study have demonstrated that the procedure is suitable for the analysis of these compounds in the low μg $kg^{-1}$ range and is therefore capable of meeting these requirements. Initial studies using this procedure for the detection of a range of nitroimidazoles in other species and matrices (with minor adaptations) have shown promise and full validation of these expanded screening tests is ongoing.

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## P 13

# DETECTION OF NITROIMIDAZOLE RESIDUES IN THE SERUM OF TREATED BROILERS BY IMMUNOBIOSENSOR

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**Abstract**
In this study, groups of chickens (broilers) were treated with the parent drugs dimetridazole (DMZ), metronidazole (MNZ) and ronidazole (RNZ) over a period of 10 days at a target concentration of 10 – 20 mg kg-1 bodyweight per day. Drugs were incorporated into the drinking water and water consumption measured daily to assess drug intake. A serial kill over an extended withdrawal period of 14 days was then undertaken. Screening was performed using a previously developed multi-residue immunobiosensor method which was further adapted and validated for serum analysis. Confirmatory analysis was performed by liquid chromatograpy-mass spectrometry/mass spectrometry (LC-MS/MS). The depletion of each nitroimidazole in serum was then assessed and the suitability of the matrix as a target for residue screening of nitroimidazoles in chickens determined. Results showed that analyte levels remained detectable in serum for an extended period post withdrawal suggesting serum to be an excellent matrix for the control of nitroimidazole abuse in chickens. Full validation and experimental data is presented.

**Introduction**
The nitroimidazoles are a class of veterinary drugs used for the treatment and prevention of histomoniasis and coccidiosis in poultry and haemorraghic enteritis in pigs. Their use in food-producing species is prohibited within the EU by Council Regulations 2377/90 and 2205/2001 as it has been reported that they show mutagenic, carcinogenic and toxic properties. Given this, it is imperative that robust and reliable screening and confirmatory procedures capable of low level detection of these drugs are available to monitor for the illegal use of these compounds.
In a previous study, turkeys and pigs were treated with the nitroimidazoles DMZ, MNZ and RNZ. After slaughtering, various matrices were analysed for their analyte content, the percentage of hydroxy-metabolites present and for homogeneity. In all matrices tested the results showed that for animals treated with DMZ, the hydroxy-metabolite hydroxydimetridazole (DMZOH) is the relevant target analyte, whereas for animals treated with RNZ and MNZ, the parent drug itself is the most relevant analyte. In serum and retina the analytes were found to be present in considerably higher concentrations than in muscle or liver and could be detected for a longer period of time after withdrawal of the medication. Serum and retina were therefore recommended as the target matrices for residue control of nitroimidazoles in turkeys and pigs.
Based upon these findings, the aim of this study was to adapt a previously developed immunobiosensor method to incorporate the analysis of serum samples for the presence of a

range of parent nitroimidazoles and their metabolites. This was followed by a feeding trial to monitor the depletion in serum of both parent and metabolite residues in chickens which had been offered 3 of the major nitroimidazoles in their drinking water for 10 consecutive days over an extended withdrawal period.

## Materials and methods

*Instrumentation*
An optical biosensor (BIACORE®Q) was obtained from Biacore AB (Uppsala, Sweden). Instrument operation and data handling were performed using BIACORE®Q Control Software (Version 3.0.1).

*Reagents and chemicals*
HBS-EP buffer, CM5 sensor chips (certified grade) and an amine coupling kit containing N-ethyl-N'-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS) and 1 M ethanolamine pH 8.5 were all obtained from Biacore AB (Uppsala, Sweden). Reference standards for DMZ, MNZ and RNZ were supplied by Sigma-Aldrich Chemical Company Ltd. (Poole, Dorset, U.K.). The metabolites MNZOH and DMZOH were kindly supplied as gifts by the Community Reference Laboratory for nitroimidazoles (BGVV, Berlin). Carboxymethyl dextran sodium salt (MW 500) was supplied by Ssens (Hengelo, Netherlands). NSB buffer contained 0.1 mM carboxymethyl dextran in HBS-EP buffer. All other chemicals were HPLC grade and were supplied by Sigma (Poole, Dorset, U.K.).

*Biosensor assay development*
Sample preparation and extraction procedure. Negative and sample sera were pipetted (2 ml) into glass universal bottles. Calibrants (0.01, 0.5, 1.0, 2.0, 5.0 and 10.0 µg $L^{-1}$ DMZ) were prepared by adding working standards (50 µl) to the known negative sera aliquots. Control and check samples were prepared in the same way using the appropriate working controls. From this point onwards all standards, controls and samples were treated identically. Acetonitrile (5 ml) was added to each universal and mixed on a roller mixer for 30 min. All universals were centrifuged at 3500 rpm for 10 min at 10°C and the supernatants decanted into test tubes. These were then evaporated to dryness using a Turbo-Vap® LV sample concentrator at 60°C under a stream of nitrogen gas. The resultant extracts were reconstituted immediately in NSB buffer (250 µl) by vortexing vigorously for 1 min and transferred to microcentrifuge tubes prior to centrifugation at 13000 rpm for 10 min. The final extracts (105 µl) were transferred, in duplicate, to the wells of a microtitre plate for analysis.
Biosensor assay. Serum extracts were mixed with antibody (70/30 v/v) and injected (80 µl) over the sensor chip surface at a flow rate of 20 µl $min^{-1}$. Report points were recorded before and after each injection to measure the response generated by antibody binding to MNZ immobilised on the sensor chip surface. The chip surface was regenerated with a 1 min injection of 20% acetonitrile in 0.25 M sodium hydroxide at a flow rate of 20 µl $min^{-1}$.

## Animal study

*Preparation of nitroimidazole incurred material*

*Experimental animals.* One hundred chickens (7 day old broilers) known to be free from any nitroimidazole treatment were purchased from a local supplier. Group A (controls) consisted of 7 birds which received non-medicated water throughout the duration of the trial and were housed in a separate building from the medicated birds. Groups B, C and D consisted of the remaining 93 birds which were randomly divided into 3 groups of 31 each. The 3 medicated groups were housed in separate, concrete-floored, sawdust-bedded pens to ensure no cross contamination was possible. All birds within each group were provided with a constant supply of water from communal galvanised water troughs and fed a diet of protein-reduced and drug free broiler feed (G.E. McLarnon and Sons, Randalstown, Northern Ireland) throughout the course of the trial.

*Medicated water preparation.* Target doses of : DMZ (20 mg $kg^{-1}$), MNZ (20 mg $kg^{-1}$) and RNZ (10 mg $kg^{-1}$ bodyweight) were added to communal drinking water and mixed thoroughly. The medicated water was prepared fresh on a daily basis and offered to the birds on each morning of the medication period.

*Feeding protocol.* All birds used in the trial were offered non-medicated water during a 7 day acclimatisation period prior to commencement of the trial. Nitroimidazoles were then applied via the drinking water for a period of 10 days to each of the 3 groups of 31 birds. Group B received DMZ, group C received MNZ and group D received RNZ medicated water. For the calculation of active substance in the drinking water, a water consumption average of 80ml per day per bird (based on previous studies) was initially assumed and the defined amount of medicated water was supplied to the groups each day ad libitum. On a daily basis (8.30am) the total medicated water consumption was measured and the actual intake of active substance per bird per day determined for each respective group. Using this information the amount of medicated water offered was constantly altered to ensure the correct dose was maintained as the birds matured. Following medication, groups B - D were moved to clean pens and returned to non-medicated water for the duration of the withdrawal period.

*Sampling procedure.* On the morning of days 0, 1, 2, 3, 5, 10 and 14 post withdrawal of nitroimidazoles from the diet, 4 birds from each of groups B - D were removed at random, sacrificed and experimental samples taken. The 7 control animals from group A were sacrificed and samples collected on day 14. All blood samples were centrifuged and the sera collected stored at -20°C until analysed.

## Results and discussion

*Biosensor assay validation*
The biosensor analytical method was validated according to the EC/2002/657 decision requirements.

Significant cross-reactivity was found against all of the major parent nitroimidazoles and their metabolites. In addition, extracted calibration curves were produced for all compounds and their respective IC 50 values determined. These ranged from 2.60 μg $L^{-1}$ for MNZOH to 9.85 μg $L^{-1}$ for DMZOH.

To determine the performance of the biosensor screening assay, nitroimidazole free chicken serum samples (n = 20) were assayed as previously described. The ccβ for the method was determined by spiking the same blank samples at 1.0 μg $L^{-1}$ DMZ and the results obtained provided a proven ccβ of <1.0 μg L-1. Furthermore a smaller number of chicken serum samples (n = 5) were fortified with the other major parent nitroimidazoles and their metabolites at various levels and the detection capability calculated for each. The detection capabilities for the parent drugs MNZ and RNZ and for the metabolite MNZOH were determined as <1.0 μg $L^{-1}$ while for the metabolite DMZOH a detection capability of <3.0 μg $L^{-1}$ was achieved.

*Animal study results*

A summary of all results has been included (Table 1). Concentrations of the 3 parent nitroimidazoles and their metabolites detected using the biosensor screening method were found to be high throughout the entire withdrawal period. Only DMZ concentrations on days 5, 10 and 14 withdrawal fell below 10 μg $L^{-1}$ (the highest calibration curve concentration). Confirmation by LC-MS/MS also showed concentration levels above 10 μg $L^{-1}$ in all medicated groups 14 days after drug withdrawal (the point at which the withdrawal period ended). Results obtained using both technologies confirmed that no nitroimidazole residues were present in the control group.

**Table 1.** Average concentrations (μg $L^{-1}$) per group detected in experimental chicken serum samples by biosensor analysis and LC-MS/MS.

| Withdrawal | Group B | | Group C | | Group D | |
|---|---|---|---|---|---|---|
| **Day** | **Sensor** | **LC-MS/MS** | **Sensor** | **LC-MS/MS** | **Sensor** | **LC-MS/MS** |
| 0 | >10.0 | 1275.6 | >10.0 | 482.2 | >10.0 | 1356.8 |
| 1 | >10.0 | 58.7 | >10.0 | 19.0 | >10.0 | 843.5 |
| 2 | >10.0 | 23.0 | >10.0 | 75.2 | >10.0 | 577.4 |
| 3 | >10.0 | 24.8 | >10.0 | 12.1 | >10.0 | 93.1 |
| 5 | 6.3 | 21.1 | | >10.0 | 33.2 | >10.0 125.5 |
| 10 | 3.6 | 23.1 | >10.0 | 10.3 | >10.0 | 142.6 |
| 14 | 3.5 | 15.1 | >10.0 | 23.9 | >10.0 | 263.4 |

**Conclusions**

A simple and reliable screening procedure based on optical biosensor technology has been developed.

Previously, the analysis of serum has proven difficult by optical biosensor due to non-specific binding associated with the matrix, however, in the current study this was readily overcome by the inclusion of carboxymethylated dextran into the final assay buffer. This resulted in a

simple extraction procedure and method of analysis allowing the processing of 24 samples in a 15 hour period and capable of detecting low concentrations of a range of nitroimidazoles and their metabolites in chicken serum.
Serum has previously been recommended, alongside retina, as the preferred target matrix for residue control of nitroimidazoles in turkeys and pigs with considerably higher concentrations found in this matrix than was detected in either muscle or liver. Concentrations also remained higher for a much longer period post withdrawal of medication.
The results obtained in this study corroborate these findings with nitroimidazole concentrations remaining detectable by both screening and confirmatory technologies throughout the extended withdrawal period of 14 days.
It is therefore recommended that serum is selected as the matrix of choice for analysis in control laboratories because of both the extended withdrawal period during which drug abuse can be detected and also because of the option this sample type offers to authorities in terms of on-farm testing regimes.

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## P 14

# DETECTION OF α-NORTESTOSTERONE IN BOVINE URINE BY IMMUNOBIOSENSOR

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**Abstract**
Administration of 17β-19-nortestosterone (β-NT), an androgenic anabolic steroid, or its esters, to food producing animals is prohibited within the EU. After administration, β-NT is rapidly metabolised to its epimer 17α-19-nortestosterone (α-NT). The detection of α-NT in urine taken from a number of bovine males slaughtered in Northern Ireland (NI) sparked an immediate reaction from regulatory bodies who instructed that all male casualty animals entering NI abattoirs be tested for α-NT. Given the extent of testing required, it became clear that the liquid chromatograpy-mass spectrometry/mass spectrometry (LC-MS/MS) method routinely employed within this laboratory was unsuitable for screening the sample numbers involved. An immunobiosensor (Biacore AB) based screening procedure was therefore established. Method development and validation was completed within 2 weeks. The developed method incorporated an initial deconjugation step followed by immunoaffinity chromatography clean-up and biosensor analysis. Clean up and analysis of a batch of 20 samples could be completed within 15 hours in comparison to the previously employed confirmatory procedure which required 27 hours for the analysis of the same number of samples.

**Introduction**
Council Directive 96/22/EC requires that European Union (EU) Member States prohibit the possession of hormonal growth promoters and the placing on the market for human consumption of animals, or products derived from animals, that have been treated with these compounds. Member States are required to monitor compliance with this legislation through the analysis of samples collected under National Residue Control Plans (NRCP).
One hormone widely included in National Residue Control Plans is β-NT which has been sporadically misused in food animal production and particularly in cattle for many years. β–NT is normally administered as an ester which is rapidly broken down to yield free β-NT. In cattle, the metabolism of β-NT involves the epimerisation of the compound to α-NT which, as sulphate or glucuronide conjugates, are the main urinary metabolites. Urine is the sample matrix of choice for nortestosterone analysis in this laboratory, as is the case in most laboratories within EU Member States, with screening and confirmation for the presence of α-NT and β NT routinely employing LC-MS/MS and GC-MS/MS.
Once thought to be xenobiotic, it is now recognized that β-NT (and its metabolites) can occur naturally in the urine of a number of species, including cattle. α-NT has been detected in pregnant cows in their last trimester and in their calves for up to a week post partum. Until recently however there has been no evidence that either α-NT or β-NT can occur naturally in steers (castrates) or bulls.
During June 2006, a number of urine samples taken from male cattle were screened and

confirmed positive for α-NT and to a lesser degree β-NT during routine analysis in this laboratory. According to EC legislation, the finding of any confirmed concentration (in excess of the CCα of the method employed) requires follow-up action to be taken, including exclusion of non-compliant carcasses from the food chain whenever these are available. The exclusion of carcasses and subsequent intensive follow-up sampling performed by the competent authority dramatically increased the number of samples requiring analysis. It quickly became apparent that the routinely employed analytical method was incapable of handling the incoming sample volume. Rapid development and validation of an alternative screening test was therefore necessary to allow this laboratory to meet its analysis targets and to comply with the demands placed upon it by both the EU and its customers.
This paper describes the development of an optical biosensor based screening procedure which was subsequently employed within this laboratory.

## Materials and methods

*Instrumentation*
An optical biosensor (BIACORE®Q) was obtained from Biacore AB (Uppsala, Sweden). Instrument operation and data handling was performed with BIACORE®Q Control Software (Version 3.0.1).

*Reagents and chemicals*
HBS-EP buffer, CM5 sensor chips (certified grade) and an amine coupling kit containing N-ethyl-N'-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS) and 1 M ethanolamine pH 8.5 were all obtained from Biacore AB (Uppsala, Sweden). α-NT, β-NT and α-nortestosterone 3-0-carboxymethyloxime (α-NT-CMO) were supplied by Steraloids Inc. (Newport, U.S.A.). β-Glucuronidase enzyme (E.coli K12) was obtained from Roche Diagnostics GmbH (Germany). The Ultralink™ biosupport medium and disposable 3 mL polypropylene columns with porous polyethylene 20 µm frits were purchased from Pierce (Rockford, U.S.A.). All other chemicals were HPLC grade and were supplied by Sigma (Poole, Dorset, U.K.).
Polyclonal antisera raised against β-NT (goat) and α-NT (rabbit) had previously been produced within the laboratory.

*Biosensor assay development*

*Immobilisation of α-NT-CMO derivative.* The carboxylmethylated surface of a CM5 biosensor chip was activated with a mixture of EDC (0.2 M) and NHS (0.05 M) to give reactive succinimide esters. These esters were reacted with ethylenediamine (1 M) for 1 h to create an amino-modified surface and then any remaining active ester groups were transformed to amides with ethanolamine (1 M). EDC (5 mg) and NHS (2 mg) were dissolved in 450 µL 10 mM sodium acetate pH 4.5 and mixed with 2 mg α-NT-CMO dissolved in 500 µL dimethylformamide (DMF). This mixture was allowed to react with the amino-modified surface for 2 h at room temperature before being removed. The biosensor chip was then washed with water, dried under a stream of nitrogen gas and stored refrigerated when not in use.

*Preparation of immunoaffinity columns.* Coupling of antibody to the biosupport medium was carried out as described in the manufacturer's instructions. The resultant gel contained 5 mg of purified antibody per mL of swollen gel. Aliquots (equating to 1 mL of coupled gel) of the gel slurry were added to polypropylene columns into which a porous polyethylene frit had been placed, and the contents allowed to settle. A second frit was placed in the column approximately 5 mm above the level of the settled gel and 20% ethanol (3 mL) added. The columns were capped top and bottom and stored refrigerated when not in use.

*Sample preparation.* All urines were centrifuged prior to extraction. Known negative or sample urines (5 mL) were transferred into glass universal bottles and their pH adjusted to between 6 - 6.5 with 1 M acetic acid. Calibrants (0.01 and 0.125 μg L-1 βNT) were prepared by adding working standards (50 μL) to the known negative urine aliquots. Control (0.01 and 0.5 μg L-1 α-NT) and check samples were prepared in the same manner using the appropriate working controls. All fortified urine samples were treated identically to test samples. β-glucuronidase enzyme (25 μL) was added to each universal which were then capped and vortexed before incubation for 2 h (or overnight) in a waterbath at 48°C ± 1°C. After incubation, water (5 mL) was added to each universal prior to the extraction procedure.
Extraction procedure. The immunoaffinity columns (IMAC) were brought to room temperature and placed onto a column holder. The top and bottom caps were removed and the 20% ethanol storage medium was allowed to run through the gel. The columns were then conditioned for use by passing 3 mL of the following solutions through them successively: 0.5 M sodium chloride solution, 0.25 M sodium chloride solution, 80% ethanol and finally water. All solutions were degassed just prior to use and the IMAC procedure run under gravity. Once conditioned, the calibrants, controls, checks and test samples were loaded onto the IMAC in 2 x 5 mL aliquots. The columns were washed by adding the following solutions in sequence: 0.5 M sodium chloride solution (3 mL), 0.25 M sodium chloride solution (3 mL), water (1.5 mL) and 80% ethanol (1 mL). Glass collection tubes were placed below each column and a further aliquot of 80% ethanol (3.2 mL) added. The collected eluant was evaporated to dryness at 70°C under a stream of nitrogen gas and reconstituted immediately in assay buffer (500 μL) by vortexing vigorously for 1 min. Columns were reconditioned for use by adding 80% ethanol (2.5 mL) and water (2.5 mL). If columns were to be stored then 20% ethanol (5 mL) was added and 2 mL allowed to pass through the bed of gel prior to columns being capped and stored refrigerated.
Biosensor assay. Urine extracts (100 μl) were transferred, in duplicate, to the wells of a microtitre plate and mixed with antibody (75/25 v/v) prior to being injected (50 μl) over the sensor chip surface at a flow rate of 25 μl min-1. Report points were recorded before and after each injection and the difference calculated to measure the response generated by antibody binding to the α–NT immobilised on the sensor chip surface. The chip surface was regenerated with a 40 s injection of 20% acetonitrile in 0.25 M sodium hydroxide at a flow rate of 25 μl $min^{-1}$.

## Results and discussion

*Antibody characterisation*

The goat antibody employed in the preparation of the immunoaffinity columns showed cross-reactivity against both α-NT and β-NT (100% and 59% respectively) whereas the rabbit antibody was specific for α-NT. A range of other anabolic steroids and structurally similar compounds were also assessed but no significant cross-reactivity was shown by either antibody.

The antibody prepared in the goat was chosen for use in the immunoaffinity gels as this type of application requires comparatively large volumes of antibody. The plentiful supply obtained during antibody production in goats was therefore ideal.

*Biosensor assay validation*

The biosensor analytical method was validated according to the requirements of Commission Decision 2002/657/EC.

Twenty bovine urine samples known to be free of α-NT were analysed using the extraction procedure described. The same samples were then fortified with α-NT at a concentration of 0.5 ng ml-1 and analysed. The results obtained gave the screening method a proven detection capability (CCβ) of <0.5 ng ml-1 for α-NT in bovine urine compared with the CCα for the LC-MS/MS confirmatory procedure of 0.37 ng ml-1. The efficiency of the deconjugation procedure used was assessed and shown to be 100% efficient between the concentration range of 0 - 0.75 ng ml-1 for the glucuronide metabolite of α-NT in urine when incubated for a minimum of 1 h at 48°C ± 1°C . Inter-assay performance of the assay (n = 10) was determined by calculating the mean and standard deviation of the % inhibition observed by the positive calibration standard and the positive control sample. Values obtained gave a co-efficient of variation (CV) of 7% and 14% respectively.

*Biacore Q vs LC-MS/MS*

Table 1 outlines the time taken to analyse a batch of 20 samples using the sensor based procedure and the previously employed LC-MS/MS method. The introduction of the sensor method allowed the laboratory to reduce the time of analysis for a batch of 20 samples by 46%, which in turn allowed the laboratory to achieve its goal of increased sample throughput.

**Table 1:** Comparison of biosensor and LC-MS/MS analysis times for urine samples.

| | Biacore Q | LC-MS/MS |
|---|---|---|
| Number of samples per batch | 20 | 20 |
| Preparation of extracts (h) | 6 | 18 |
| Switch over time (h) | 0.1 | * |
| Analysis time (h) | 8 | 8 |
| Results calculation (h) | Nil | 1 |
| Time per sample (h) | 0.7 | 1.4 |
| TOTAL TIME (h) | 14.5 | 27 |

*Sample analysis*
In addition to the increased sample throughput achieved by the sensor analysis, the first 4 months after introduction of the new screening procedure resulted in an 80% reduction of the instrumentation time required on the LC-MS/MS system as 20.8% of samples analysed screened non-compliant. This resulted in a 11.3% false non-compliant screening rate. No false negative results were recorded throughout the study.

**Conclusions**
The implementation of the biosensor screening procedure significantly decreased the time taken from receipt of samples into the laboratory to the official reporting of results and issue of the related documentation to the relevant monitoring bodies from 8 days to 3.5 days. This in turn allowed for a decrease in the time during which carcasses were detained pending sample analysis.
This work clearly demonstrates the important role that screening procedures can play during periods of intensive sampling and analysis. Furthermore, the benefits of sensor technology are highlighted in that a suitable procedure can be developed and validated rapidly in response to a crisis.
The biosensor assay was shown to be suitable as a screening assay with a zero rate of false negatives and an 11% false positive rate.
A simple and reliable screening procedure based on optical biosensor technology has been developed which has been shown to be capable of detecting relevant concentrations of α-NT in bovine urine.

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## P 15

# Determination of nitrofurazone metabolite semicarbazide (SEM) residues in eggs by LC-MS/MS: Validation and stability study of SEM in incurred matrix

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**Abstract**
Nitrofurazone is one of the nitrofurans, veterinary drugs prohibited from use in food animals in the EU since 1993 (Annex IV, Regulation (EEC) No 2377/90). The methods for semicarbazide (SEM) determination described in literature were not satisfactorily reproducible in the egg matrix. Using the SPE cleaning step we have modified the method for SEM determination in muscle, originally developed in Netherlands at the Institute of Food Safety (RIKILT), for SEM determination in eggs. This modified procedure was validated with main validation parameters being CCα = 0.20 µg/kg, and CCβ = 0.25 µg/kg. The correlation coefficient for matrix calibration curve was r = 0,9968 in the range of 0 µg/kg - 5 µg/kg. Employing this method we have performed a SEM stability study in incurred egg samples. The time intervals in this study were 1, 2, 4, and 20 weeks, with duplicate samples stored at -20°C and 4°C, in accordance with Commission Decision 2002/657/EC.

**Introduction**
Residue detection of nitrofurans in various matrices remains a timely issue as manifested by the number of available publications on this topic. The metabolite SEM draws special attention, as its parent substance nitrofurazone does not represent the sole source of SEM contamination. Reproducing the previously published methods for SEM detection in eggs we determined unsatisfactory sensitivity with regard to the CD 2002/181 requirements (MRPL 1 µg/kg)(1). Adjusting the separation step of the analytical procedure (4) we acquired a method with desirable sensitivity and satisfactory validation parameters.
The method, as presented in the first part of this paper, was then used to evaluate the stability of SEM in incurred eggs. The data from this study are summarized in the second part of our paper. To our best knowledge, similar data regarding this issue have not been reported in the available literature so far.
A. Validation of LC-MS/MS Method for Quantification of SEM in Eggs.

**Materials and Methods**

*Chemicals and Solvents*
All general reagents were of HPLC grade or gradient grade. The marker of the SEM metabolite, the internal standard ($^{13}C$- $^{15}N_2$)SEM, and the derivatization analyte 2-NP-SEM were produced by VETRANAL by Riedel-de Haen and supplied by Sigma-Aldrich, Czech

Republic. Stock solutions (1 mg ml-1) were prepared in methanol and stored at 2-8°C for a maximum of three months.

*Sample Preparation*
The homogenized egg (1 g) was transferred into a 15ml centrifuge tube. Following addition 40µl of the methanolic solution of internal standard (13C- 15N2-)SEM the contents was vortexed briefly. After a 15 minutes of equilibration, 10 ml of 0.1 M hydrochloric acid plus 250 µl of 0.2 M 2-NBA solution were added and the sample homogenized. The sample was incubated overnight at 37oC with continuous gentle mixing. Following incubation, the sample was cooled to room temperature and neutralized by addition of 500 µl 0.3 M trisodium phosphate. The pH value was adjusted to 7 + 0,5 using 2 M sodium hydroxide.
The tube with the sample was centrifuged for 15 min at 4000 rpm. The supernatant (10ml) was applied to Waters OASIS – MCX cation exchange SPE column conditioned previously with methanol (2ml) and water (2ml). The SPE column was dried for 2 minutes under vacuum and 2-nitrobenzaldehyde derivative of SEM (2-NP-SEM) was eluated with ethyl acetate (3ml) to a vial. The sample eluate was evaporated to dryness at 45°C under a gentle stream of nitrogen and redissolved in 500 µl of water:acetonitrile (1+1, v/v). The final solution was filtered through a 0.45 µm syringe filter to a HPLC vial.

*LC–MS/MS Analysis*
The LC–MS/MS system comprised a LC pump Surveyor and a TSQ Quantum Discovery triple quadrupole mass spectrometer equipped with an APCI ion source (FINNIGAN Termo Electron Corporation, USA). The mobile phase consisted of 0.1% acetic acid in water (A) and acetonitrile (B), using a gradient run at a flow of 0.2 ml min-1 and at temperature of 25°C. The chromatographic separation was performed on a XTerra MS, C18, (3,5µm, 2,1 x 150 mm) column, in combination with a guard column (2,1 x 10mm), all from Waters. The injection volume was 10 µl. The samples were analysed in a positive polarity (APCI +). The mass spectrometer was operated in the selected reaction monitoring mode (SRM) and settings were optimised for maximum sensitivity: spray voltage 4.5 kV, collision gas pressure 0.5 mTorr, scan time 0.30 s, scan width 0.500 m/z, sheath gas flow 30 units, aux gas flow 5 units and capillary temperature 220°C.Data concerning the fragmentation of the semicarbazide of interest are presented in Table 1.

**Table 1.** MS/MS fragmentation conditions for SEM

| **Components** | **Precursor ion (m/z)** | **Product ion (m/z)** | **Collision energy (eV)** |
|---|---|---|---|
| 2NP-SEM | 209 | 166, 192 | 13 |
| 2NP-($^{13}C$-$^{15}N_2$)-SEM | 212 | 168, 195 | 13 |

**Results of the Validation Studies**
Validation of the method for SEM detection in egg homogenate was performed according to the Commission Decision 2002/657/EC (2), the conventional validation approach of which was applied (chapter 3.1.2., Conventional validation procedures). Recovery was not evaluated since the method employs addition of isotopic-marked internal standards.

Repeatability of the method was tested by analysis of three SEM concentrations in 12 spiked egg homogenate samples measured on three different days. Statistical analysis of this data is presented in Table 2.

**Table 2.** Repeatability (coefficient of variation $C_{VSr}$) and within-laboratory reproducibility (coefficient of variation $C_{VSwr}$) of SEM in eggs

| Concentration level (µg/kg) | Date of measurement | n | Test of Normality | $CV_{Sr}$ (%) | $CV_{Swr}$ (%) |
|---|---|---|---|---|---|
| 0.2 | 22.1.2007 | 12 | significant | 11.5 | 11.8 (n = 36) |
| 0.2 | 23.1.2007 | 12 | significant | 11.3 | |
| 0.2 | 24.1.2007 | 12 | significant | 11.9 | |
| 0.5 | 22.1.2007 | 12 | significant | 9.1 | 9.6 (n = 36) |
| 0.5 | 23.1.2007 | 12 | significant | 9.3 | |
| 0.5 | 24.1.2007 | 12 | significant | 9.5 | |
| 1.0 | 22.1.2007 | 12 | significant | 6.5 | 6.3 (n = 36) |
| 1.0 | 23.1.2007 | 12 | significant | 5.8 | |
| 1.0 | 24.1.2007 | 12 | significant | 5.7 | |

Measurements for the matrix calibration curves were performed in model samples over three days using 6 concentration levels of SEM standard (0 - 0.5 - 1.0 - 1.5 - 2.0 - 5.0 µg/kg) and 2.0 µg/kg of the internal standard (IS). Linear regression parameters (Y = a + b*X ) and calibration limits were calculated, as presented in Table 3 and Table 4, respectively.

**Table 3.** Calibration curve parameters for SEM detection in eggs

| Date of measurement | a | b | Sa | r |
|---|---|---|---|---|
| 24.1.2007 | 0.2350 | 1.1213 | 0.0290 | 0.9987 |
| 25.1.2007 | 0.2098 | 1.0989 | 0.0084 | 0.9979 |
| 26.1.2007 | 0.2214 | 1.1256 | 0.0542 | 0.9952 |

**Table 4.** Calibration limits of the validated method

| Method | Yc | Yd | Yq | Xc (µg/kg) | Xd (µg/kg) | Xq (µg/kg) |
|---|---|---|---|---|---|---|
| Method according to ISO11843-2 | 0.4013 | 0.5925 | - | 0.2008 | 0.2461 | - |
| Direct method of analyte | 0.3804 | 0.5475 | 0.7069 | 0.1214 | 0.2302 | 0,3341 |
| Direct method of signal, IUPAC | 0.3805 | 0.5563 | 0.7229 | 0.1214 | 0.2359 | 0.3445 |
| Combined method Ebel, Kamm | 0.3705 | 0.5469 | 0.7063 | 0.1149 | 0.2298 | 0.3337 |
| Method K*Sigma from regression | 0.3804 | 0.5668 | 0.7531 | 0.1214 | 0.2428 | 0.3642 |

*(The index: c = critical, d = detection, q = quantification)*

The calibration curve data complied with all linear regression model requirements. Therefore, the Xc and Xd values, calculated with regard to the ISO 11843-2 norm, were used for the estimation of the decision limit **(CCα)** and detection capability **(CCβ)** parameters. For SEM analysis in eggs, the decision limit (CCα) was 0.2008 µg/kg and the detection capability **(CCβ)** was 0.2461 µg/kg.

**B. Stability Study in Incurred Egg Samples**

**Materials and Methods**

*Production of Incurred Eggs for Stability Study*
The layers were divided into three groups. Eggs were collected in all three groups in two day intervals. The complete egg content was homogenized and stored frozen at -20°C.
The first group (5 layers) was treated with a nitrofurazone dose of 400 mg/kg in the feed (recommended therapeutic dose until the ban of the drug in 1993). The second group (5 layers) received a 30 mg/kg dose of nitrofurazone in the feed. The control group (2 layers) received feed with no nitrofurazone. After 10 days the treatment was terminated and the medicated feed was replaced for the rest of the experiment by identical feed without the drug. Samples of egg homogenate obtained 10 days following nitrofurazone treatment completion were used for the stability study, the results of which are summarized in Table 5.

*Results of the Egg Storage Stability Study*
Initial SEM concentrations in the incurred egg samples selected for the stability study (10 days after treatment completion, labelled T0) were measured using the validated LC-MS/MS method described above (Chapter A). Subsequently, 1 g aliquots of the samples were divided into two groups, stored frozen at -20°C or refrigerated at +4°C to +8°C, respectively. The concentrations of SEM in the sample aliquots were measured after 1, 2, 4, and 8 weeks, and after 6 and 12 months, respectively. The results of these analyses are summarized in Table 5 and graphically processed in Figure 1.

**Table 5.** Stability study of SEM in the egg homogenate performed over 12 months

| **Sample number** | **Storage temp. (°C)** | **SEM Concentration (µg/kg)** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | T0 | T1<br>1<br>week | T2<br>2 weeks | T3<br>4 weeks | T4<br>8 weeks | T5<br>6<br>months | T6<br>12<br>months |
| 1 | 4 - 8 | 17.67 | 17.62 | 17.54 | 17.13 | 17.01 | 16.87 | 16.74 |
| 2 | 4 - 8 | 1.24 | 1.25 | 1.23 | 1.24 | 1.14 | 1.10 | 1.09 |
| 3 | - 20 | 10.63 | 10.71 | 10.62 | 10.63 | 10.32 | 9.95 | 9.79 |
| 4 | - 20 | 0.47 | 0.46 | 0.44 | 0.42 | 0.42 | 0.41 | 0.39 |

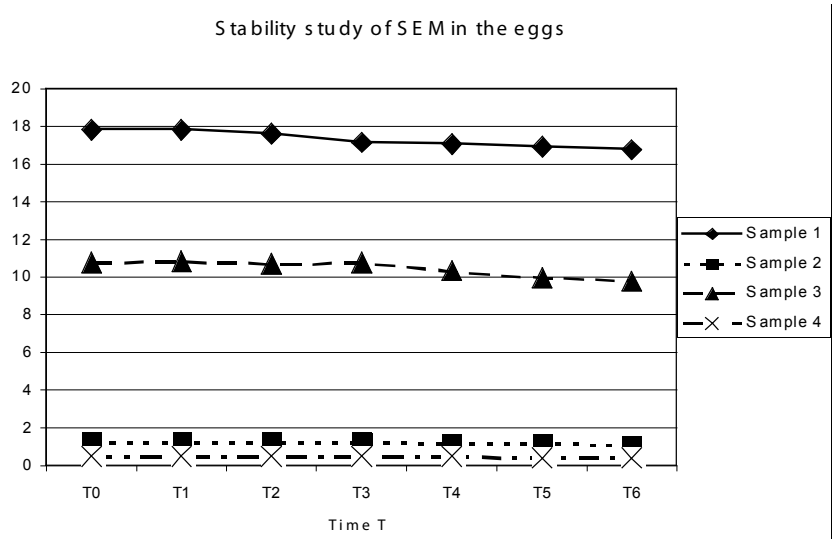


**Figure 1.** Stability study of SEM in egg homogenate

The presented data shows that the residue levels of bound nitrofurazone, evaluated through the SEM metabolite, remained stable for 1 year under the storing conditions described above. This observation was verified via a statistical analysis of the initial SEM concentration at time-point T0, and the SEM concentration measured at time-point T6 (12 months), using the Comparison of Two sampling statistical method. Hypothesis H0: $\mu 1 = \mu 2$ (Means are equal) versus hypothesis H1: $\mu 1 \neq \mu 2$ (Means are not equal) - see Table 6.

**Table 6.** Statistical Comparison of Two-sampling

| Sample n. | μ1 (μg/kg) in time T0 | μ2 (μg/kg) in time T6 | t-stat (exp.) | t-stat (table) | H0 |
|---|---|---|---|---|---|
| 1 | 17.67 | 16.74 | 1.5006 | 2.2281 | accepted |
| 2 | 1.24 | 1.09 | 2.1856 | 2.2281 | accepted |
| 3 | 10.63 | 9.79 | 1.9892 | 2.2281 | accepted |
| 4 | 0.47 | 0.39 | 2.0321 | 2.2281 | accepted |

**Conclusions**

All parameters of the analytical method for detection of semicarbazide (SEM) in eggs using LC-MS/MS proved its validity for testing of this analyte (screening, confirmation) with satisfactory sensitivity.

Statistical evaluation of the stability study results confirmed the stability of the semicarbazide (SEM) marker residue in eggs for a 12 month period at temperatures of -20°C or +4°C to +8°C, respectively.

The findings in our stability study correspond with the results of the semicarbazide stability study in incurred samples of pork muscle as published by Cooper et al. (3).

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## P 16

# VALIDATION OF AN ELISA TEST KIT FOR THE DETERMINATION OF FLUOROQUINOLONE RESIDUES IN HONEY

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**Abstract**
Quinolones are antibiotics widely used in veterinary medicine. Chemical industries, especially in Asia, are producing large quantities of quinolones and fluoroquinolones. In several countries, they have replaced the banned chloramphenicol and nitrofurans. The recent findings of quinolone residues in honey clearly confirmed this trend.
No legislation for quinolones in honey is implemented and hence no residue should be detected. Therefore there is a need to test raw materials for product compliance.
We tested and validated the ELISA test kit for the detection of fluoroquinoles manufactured by EuroDiagnostica. The validation was performed on honey, following the EU Decision 2002/657/EC requirements.
The results obtained with a simple dilution of the honey test sample demonstrated that the kit is reliable and suitable for semi-quantification at 3.13 $\mu g.kg^{-1}$ for norfloxacin, ciprofloxacin and enrofloxacin. A limit of quantification at 1.57 $\mu g.kg^{-1}$ can be reached by adding a lower concentration point in the calibration curve.

**Introduction**
Quinolones are antibiotics widely used in human and veterinary medicine. Until recently they were mainly used as therapeutic drugs due to their relatively high prices. Since the end of the proprietary period of several quinolones, chemical industries, especially in Asia, began to produce large quantities of quinolones for veterinary use. In several countries they have replaced the trouble makers chloramphenicol and nitrofurans.
Many quinolones are used in veterinary medicine on food producing animals, for example, in Switzerland: ciprofloxacin, difloxacin, danofloxacin, enrofloxacin, flumequine, norfloxacin and marbofloxacin are registered.
Quinolones are also sometimes applied on bees to fight the deadly disease American Foulbrood. Unfortunately none of those drugs is authorised to be used on beehive and any residues resulting from the application of quinolone antibiotics can be considered as violative. Following the recent finding of quinolone residues in honey from various countries (European Rapid Alert System for Food and Feed) we looked for a rapid and reliable screening method for the detection of fluoroquinolones residues in honey. We have selected the ELISA from Euro-Diagnostica for the good cross-reactivity with most quinolones detected in honey and because it already proposed an extraction procedure for honey.
However, the test does not detect all fluoroquinolones at the same detection level due to their specific cross-reactivity. The test provides positive or negative result for the fluoroquinolones family. Semi-quantitative results can be estimated. The results obtained demonstrated that the kit is reliable and suitable for the detection at ≥ 3.13 µg.kg-1 of the three fluoroquinolones

we have tested: norfloxacin, ciprofloxacin and enrofloxacin. Using honey contaminated with incurred residues the number of false negative was 0% and the number of false positives was 15%.

## Materials and methods

*Reagents and apparatus*
The Fluoroquinolones enzyme liked immuno assay kits (cat n° 5101FLUQG1p) were purchased from Euro-Diagnostica B.V. (Arnhem, The Netherlands). All reagents used for the validation were provided in the ELISA kits, except for the dilution buffer. This solution was prepared with 0.77 g Na2HPO4, 0.18 g KH2PO4 and 8.94 g NaCl dissolved in 1000 mL of distilled water and adjusted to pH = 7.4 ± 0.1 using NaOH 1M or HCL 1M. The sample dilution buffer is obtained by adding 8% of methanol to the dilution buffer. A microtiter plate spectrophotometer (type µQuant) from Bio-Tek Instruments, Inc. (Winooski, USA) was used to measure the O.D. at 450 nm. A microplate washer (type anthos fluido) from ASYS Hitech GmbH (Eugendorf, Austria) was used to wash the ELISA plates.

*Standards*
Norfloxacin, ciprofloxacin, enrofloxacin, ofloxacin, marbofloxacin, difloxacin, sarafloxacin, danofloxacin, sulfathiazole, chloramphenicol and oxytetracycline were obtained from Riedel-de Haën (Seelze, Germany). Stock solutions at a concentration of 0.1 mg.mL-1 in methanol were prepared and stored at – 20°C. Working solutions at 0.1 µg.mL-1, 0.01 µg.mL-1 and 0.005 µg.mL-1 in sample dilution buffer were prepared in order to spike blank samples. Solutions in the range of 1.25-50 µg.kg-1 were used for recovery tests. Solutions of ofloxacin, marbofloxacin, difloxacin, sarafloxacin, danofloxacin, and sulfathiazole, chloramphenicol and oxytetracycline were used to check the cross reactivity of theses substances on the ELISA plate.

*Sample preparation*
After a 10 times dilution (0.5 g of honey samples in 4.5 mL of sample dilution buffer), the sample solutions were mixed on a shaker for 30 minutes. Honey samples were directly analyzed after centrifugation for 10 minutes at 2000 G.

## Results and discussion

*Performance of the method*
The results obtained with the Euro-Diagnostica ELISA for fluoroquinolones demonstrated that the test is reliable and specific to the determination of the sum of the detectable fluoroquinolones residues in honey, with the following performance: LOD and LOQ at 3.13 $\mu g.kg^{-1}$ (or 1.57 µg.kg-1 by adding one point to the calibration curve).
The calibration curve of this ELISA is linear between 0.157 $ng.mL^{-1}$ to 10 $ng.mL^{-1}$. It corresponds to a concentration of 1.57 µg.kg-1 to 100 $\mu g.kg^{-1}$ in honey.
However, this test is limited for screening only. It does not allow the fluoroquinolone identification in the case of positive sample. A confirmation must be performed in case of positive result.

*Comparison with a confirmatory method*
We analyzed 57 different honey samples with both the ELISA and a LC-MS/MS method. Results of this comparison are displayed in the following table.

**Table 1.** Comparison between ELISA and a confirmatory method

| Response | LC-MS/MS positive (reference method positive) (R+) | LC-MS/MS negative (reference method negative) (R-) | Total (N) |
|---|---|---|---|
| ELISA positive (alternative method positive) (A+) | +/+ positive agreement (PA) 30 (100%) | -/+ positive deviation (PD) 4 (15%) | 34 |
| ELISA negative (alternative method negative) (A-) | +/- negative deviation (ND) 0 (0%) | -/- negative agreement (NA) 23 (85%) | 23 |
| Total (N) | (N+) 30 | (N-) 27 | 57 |

*PD: Positive deviation or false positive results.*
*ND: Negative deviation of false negative results.*
*PA: Positive agreement of positive results with the reference and alternative method.*
*NA: Negative agreement or negative results with the reference and alternative method.*

**Table 2.** Performance indicators

| Matrices | False negative (%) | False positive (%) | Relative accuracy(%) | Relative sensitivity(%) | Relative specificity(%) |
|---|---|---|---|---|---|
| Incurred Honey | 0 | 15 | 93 | 100 | 85 |

*Cross reactivity*
Cross reactivity with different fluoroquinolones was tested and results are displayed in Table 3. The results are similar to those obtained by the manufacturer. However, some variations were noticed between different kits.

**Table 3.** Cross reactivity of fluoroquinolones

| Fluoroquinolones | Supplier results | Nestec results |
|---|---|---|
| Norfloxacin | 100% | 100% |
| Enrofloxacin | 92% | 110% |
| Ciprofloxacin | 124% | 106% |
| Ofloxacin | 18% | 24% |
| Danofloxacin | 89% | 140% |
| Marbofloxacin | 16% | 20% |
| Sarafloxacin | 4% | 16% |
| Difloxacin | 1% | <15% |

## Conclusion

The Fluoroquinolones EIA test kit from Eurodiagnostica can be used for the screening of fluoroquinolones in honey. However, this test is not specific to one molecule, but detects a broad range of compounds from the fluoroquinolone family. The semi-quantitative results depend on the cross reactivity of each fluoroquinolone on the plate and on the recovery rate. Each positive result must be re-tested with a confirmatory method as this is requested by the norms. Confirmation will also tell which fluoroquinolone is present and at which concentration.

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## P 17

# FLOW CYTOMETRIC IMMUNOASSAY FOR FLUORO-QUINOLONES IN CHICKEN SERUM

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**Abstract**
A flow cytometry-based immunoassay (FCI) for fluoroquinolones (FQ) in chicken serum was developed using the Luminex 100 in combination with the MultiAnalyte Profiling (xMAP) technology. In this inhibition FCI, a norfloxacin-NH2 derivative was coupled to carboxylated polystyrene microspheres (5.6 µm). These beads were used in combination with a previously developed multi-FQ polyclonal antiserum and mixed with diluted and ultra-filtrated samples in a 96-wells filter-bottom microplate. After the first incubation (1 h at room temperature in the dark) and filtration by centrifugation, the bound antibodies were detected with fluorescent (phycoerythrin (PE)) labeled second antibodies and the fluorescence intensities on the microspheres were directly measured in the Luminex during 50 s per well (100 microspheres). In buffer, this assay could detect five FQ with 50% inhibition values (IC50) of 0.4, 0.5, 0.6, 2.5 and 2.9 ng/ml for norfloxacin, enrofloxacin, ciprofloxacin, difloxacin and sarafloxacin, respectively. For the detection of the FQ's in chicken sera, an easy ultra-filtration step was selected and all FQ could be detected at or below 100 ng/ml.

**Introduction**
FQ are synthetic antibiotics of broad-spectrum antibacterial activity widely used in The Netherlands for the treatment of infections in farmed fish, turkeys, pigs, calves and poultry. FQ were one group of model compounds in the EU-project "New technologies to screen multiple chemical contaminants in foods" (BioCop; contract FOOD-CT-2004-06988) and antibodies and applications in biosensors were developed. One of the anti-FQ developed was raised in a rabbit against a norfloxacin(Nor)-COOH derivative (Pab CA 65). In a biosensor immunoassay (BIA), with a Nor-NH2 coated biosensor chip, this antiserum showed cross-reactivity towards several FQ and it was used for the detection of FQ in chicken muscle (Marchesini et al., 2007). The same reagents were used in a Dutch research project named "Combined monitoring" to evaluate a, for veterinary drugs, new multiplex screening system which consists of flow cytometry in combination with the MultiAnalyte Profiling (xMAP) technology (Haasnoot et al., 2008). In the present study, the performance of the multi-FQ assay in this new format was compared with the BIA results with chicken serum as the model material. Serum was chosen for its ability to perform serology (anti-pathogens) in combination with residue detection.

## Materials and methods

*Materials and instruments*

Nor, ciprofloxacin (Cipro) and enrofloxacin (Enro) were obtained from Fluka Chemie (Zwijndrecht, The Netherlands). Difloxacin (Diflo) was from Abbott Laboratories (North Chicago, Illinois, USA) and sarafloxacin (Sara) from Dr. Ehrenstorfer (Augsburg, Germany). The amine coupling kit (containing 0.1 M N-hydroxysuccinimide (NHS), 0.4 M 1-ethyl-3-(3-dimethylaminoproplyl)carbodiimide hydrochloride (EDC) and 1M ethanolamine hydrochloride-NaOH (pH 8.5)) was supplied by GE Healthcare (Uppsala, Sweden). Pab CA 65 was obtained from CER (Marloie, Belgium) and the Nor-NH2 derivate from (Health Canada, Ottawa). Goat anti-rabbit IgG (GAR) coupled with R-Phycoerythrin (PE) was obtained from Prozyme (Kempen, Belgium). The 10 kDa cut-off Amicon filters and 96-wells filter-bottom microplates (1.2 µm hydrophil low protein binding) were from Millipore (Billerica, USA) and Protein LoBind Tubes (1.5 ml) from Eppendorf (Hamburg, Germany). The Luminex 100 IS 2.2 system (consisting of the Luminex 100 analyzer, Luminex XY Platform with the StarStation System control sorfware), differents sets of carboxylated microspheres (SeroMAP beads) and sheath fluid were obtained from Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, U.K.). Ethylene diamine (EDA), ovalbumin (OVAL), the Eppendorf centrifuge 5810R and the ultrasonic cleaner were from VWR international (Amsterdam, The Netherlands). The microtiter varishaker was from Dynatech Lab. (Guernsey, UK) and the Snijder test tube rotator from Omnilabo International (Breda, The Netherlands).

*Preparation of the beads*

After acclimatization to room temperature, the stock bead suspension (1.25x107 beads/ml) was resuspended by vortexing for 5 min and sonication for 1 min. Of the suspension, 200 µl (2.5 x 106 beads) were transferred to a 1.5 ml Protein LoBind Tube of Eppendorf in which the beads were pelleted by centrifugation (10.000 rpm for 3 min) and the supernatant was removed. The pelleted beads were resuspended in 100 µl of water by vortex (1 min) followed by sonication (1 min). The suspension was centrifuged as before and after removal of the supernatant, the pellet was resuspended in 80 µl of activation buffer (0.1M NaH2PO4 pH 6.2) by vortex and sonication (both 1 min). Solutions of sulfo-NHS and EDC, both at 50 mg/ml in water, were prepared just before adding 10 µl of each to the 80 µl bead suspension. After mixing by vortex (20 sec), the beads were incubated in the dark at room temperature for 15 min under mixing with the test tube rotator. The activated beads were pelleted by centrifugation and the supernatant was removed. The beads were resuspended in 250 µl of 50 mM MES (pH 5.0) by vortex (20 sec) and pelleted by centrifugation and the supernatant was removed. This wash procedure with 50 mM MES was repeated twice. To the pelleted beads, 0.5 ml of the Nor-NH2 derivative (2.3 mg/ml in borate buffer pH 8.5) and 250 µl DMF were added. After mixing by vortex, the bead suspension was incubated for 2 h in the dark at room temperature. The coupled beads were pelleted by centrifugation, the supernatant was removed and the beads were resuspended in 0.5 ml of blocking buffer (PBS containing 0.1 % BSA, 0.02 % Tween-20 and 0.05 % sodium azide) by vortex ( 20 sec) and incubated for 30 min in the dark. After this blocking, the beads were pelleted by centrifugation and resuspended in 0.5 ml of blocking buffer. This wash procedure was repeated twice. The resuspended beads were stored in the dark at 2-8 ºC.

*Sample preparation*
After homogenising (vortex for 10 sec), 100 µl of the serum sample was diluted with 400 µl of PBS and the mixture was filtered by ultra-filtration (UF) with a 10 kDa Amicon filter by centrifugation (30 min at 14.000 rpm). Spiked samples were prepared in undiluted chicken serum which were incubated for 20 min at room temperature prior to dilution and filtration.

*Assay protocol*
The 96-wells filter bottom plate was prewetted with 200 µl/well of OVAL (0.5%)-containing PBS buffer by centrifugation (800 rpm for 1 min). The Nor-NH2 coated bead suspension was diluted 50 times with the OVAL-containing PBS buffer and 10 µl were added/well and to 100 µl of standard solution or buffer. To this, 10 or 25 µl of the filtered sample and finally 10 µl of the 400 times in OVAL-containing PBS buffer diluted antiserum were added. After incubation for 1 h at room temperature in the dark and under mild shaking, the plate was washed by centrifugation (800 rpm, 1 min) with 100 µl of PBS buffer/well and this wash procedure was repeated twice. Of the 30 times in PBS buffer diluted GAR-PE solution, 5 µl were added/well followed by 95 µl of PBS. This mixture was incubated (30 min at room temperature in the dark and under mild shaking). The beads were directly detected (no wash step) in the Luminex analyzer in which 50 µl/well were measured in 50 s (aiming for around 100 beads/well).

**Results and discussion**
The carboxylated bead surface allows simple chemical coupling of capture reagents such as antibodies, oligonucleotides, proteins, peptides or receptors. For the coating with low molecular weight compounds, carrier proteins are usually applied. However, as shown previously with a sulfonamide derivative (Keizer et al., 2008), direct coupling of the small molecule resulted in higher signals and an improved bead stability. Therefore, the Nor-NH2 derivative was used for the direct coupling to the carboxylated beads using the same chemistry as used for the coupling to a carboxymethylated biosensor chip surface of the previously developed BIA (Marchesini et al., 2007). The antiserum was used in a final dilution (fd) of 5200 or 5800 (dependent of the sample volume applied) which was more diluted than in the BIA (fd of 800 times). The calibration curve of Nor in buffer (Figure 1) showed a 50% inhibition at 0.4 ng/ml which was about five times more sensitive compared with the BIA.

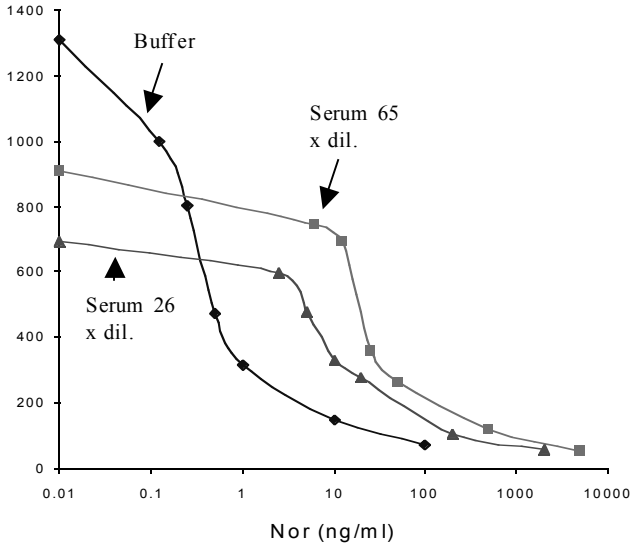


**Figure 1:** Calibration curves of Nor in buffer and chicken serum (after UF and at two different final dilutions).

The sensitivities towards the other FQ in buffer are presented in Table 1 and they were all better compared with the BIA but the calculated cross-reactivities were in a comparable order of magnitude.

**Table 1.** Sensitivities (IC50) and cross-reactivities (%) of the multi-FQ FCI for five FQ in PBS buffer and in chicken serum diluted in PBS (1:65; v/v).

| | **$IC_{50}$ (µg/l)** | | **Cross-reactivity (%)** | |
|---|---|---|---|---|
| FQ | Buffer | Serum | Buffer | Serum |
| Nor | 0.4 | 60 | 100 | 100 |
| Enro | 0.5 | 46 | 80 | 130 |
| Cipro | 0.6 | 77 | 67 | 78 |
| Diflo | 2.5 | 216 | 16 | 28 |
| Sara | 2.9 | 717 | 14 | 8 |

The negative influence of chicken serum on the assay performance was noticed before in the BIA with the same antiserum. An increase in response was observed probably due to non-specific binding of chicken serum proteins to the sensor surface. In the FCI, the presence of chicken serum caused reduced responses, probably due to the same non-specific binding of serum proteins to the bead surface which decreased the accessibility of the immobilised Nor for the antibodies. This effect was reduced after removal of most of the serum proteins by UF prior to the analysis. As shown in Figure 1, the maximum median fluorescence intensity (MFI) in filtered chicken serum was still lower compared to buffer but the detection of Nor at the 100 ng/ml was feasible at both dilutions (26 and 65 times).

**Table 2**: Responses (MFI) obtained with a blank chicken serum sample and the responses and inhibitions (%) obtained with that serum spiked at 100 ng/ml level with different FQ and after two serum dilutions.

| FQ spiked | Level spiked (µg/l) | 65 * diluted serum | | 26 * diluted serum | |
|---|---|---|---|---|---|
| | | MFI found | Inhibition (%) | MFI found | Inhibition (%) |
| | 0 | 909 | 0 | 695 | 0 |
| Nor | 100 | 132 | 85 | 119 | 83 |
| Enro | 100 | 170 | 81 | 121 | 83 |
| Cipro | 100 | 373 | 59 | 250 | 64 |
| Diflo | 100 | 493 | 46 | 321 | 54 |
| Sara | 100 | 628 | 31 | 376 | 46 |

A chicken serum was spiked with the different FQ at 100 µg/l and analysed after the two dilutions. The MFI's (B) were compared with the average MFI's obtained with the blank chicken serum (B0) at the two dilutions. The calculated percentages of inhibition ((BO-B)/ BO * 100 %) are presented in Table 2. The B0 in the 65 times diluted blank chicken serum was 909 ± 79 MFI and in the 26 times diluted serum 695 ± 38 MFI which resulted in action levels (average signal minus 2.33 times the standard deviation (SD)) of 686 and 570 MFI, respectively and all tested FQ could be detected at this level.
In the BIA, the chicken serum sample was diluted 2.5 times and the percentages of inhibition for the different FQ ranged from 53 % for Nor till 46 % for Sara.

**Conclusions**
The previously developed multi-FQ BIA could easily be transferred to the FCI and resulted in a more sensitive assay of which the performance in chicken serum was almost comparable. The major advantage of the new FCI is the possibility to combine it with other assays (multiplex option) and this will be tested in the near future within the Dutch "Combined monitoring" project.

**Ackowledgements**
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## P 18

# DETERMINATION OF CHLORAMPHENICOL IN URINE SAMPLE CONFIRMATORY GC-MS/NCI METHOD USING MOLECULAR IMPRINTED POLYMER (MIP) CLEAN-UP

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**Abstract**
The method for determination of low concentrations of chloramphenicol in bovine and porcine urine was developed. Sample clean-up was performed by Molecular Imprinted Polymer (MIP) columns. The extracts obtained from MIP clean-up procedure were clean enough for chromatografic analyses. Confirmatory analyses were conducted using GC/MS-NCI after derivatisation (silylation). The described method was fully validated according to CD 2002/657/EC, CCa was 0.06 ng/mL, CCb was 0.1 ng/ mL. This method is considerably robust and allows to process even very dirty samples. The described procedure is very simple and little-time-consuming and provides high throughput of examinated samples. This could be used for routine screening and confirmatory analyses as well.

**Introduction**
Chloramphenicol (CAP) is a broad-spectrum antibiotic. Because chloramphenicol has displayed significant toxicological effects on humans its use was banned in food production in the European Community and some other countries. A range of analytical methods was developed for determination of CAP in foods and food raw materials and for monitoring of illegal use of this antibiotic during fattening of food producing animals. All confirmatory methods for CAP determination consist of some clean-up procedure and GC/MS or LC/MS detection.
Determination of trace level of CAP in urine is used for monitoring of illegal use of this compound in live animals during fattening. Because of variation in the composition of urine samples of individual animals, proper clean-up in some cases is difficult. Some extracts from clean-up procedures using conventional SPE columns, in the case of very dirty and concentrated urine samples may be not clean enough for chromatographic analyses. A new type of columns for clean-up of urine samples is available since the last year. These columns are constructed on the base of molecular imprinted polymers (MIP) specific for CAP. We compared clean-up procedure using MIP columns with older procedures used in our laboratory using SPE C18 columns with the same detection by GC-MS/NCI after derivatisation by silylation.

**Materials and Methods**
Chloramphenicol standard was supplied by Sigma-Aldrich, internal standard (deuterated chloramphenicol) CAP-d5 was obtained from Cambridge Isotope Laboratories (USA). Substances were dissolved in methanol and diluted to low concentration working solutions.

MIP columns were obtained from Sigma-Aldrich (SupelMIP SPE – Chloramphenicol, cat.no. Supelco 53210-U). All used solvents were supplied by Merck and they were in SupraSolv quality. Samples of urine were collected from different animal species (pig, cow, goat, sheep) from standard commercial breeding. Internal standard was added at the concentration of 2 ng/mL (20 ng/mL eventually) to each sample and samples were eventually fortified with CAP working solution to achieve different concentration levels for calibration measurement and determination of different validation parameters. Deconjugation was performed by b-Glucuronidase / aryl sulfatase (Merck) overnight at room temperature after pH adjusting to 5.2.
MIP cartridges were conditioned with 1 mL of methanol and 1 mL of water. Then 1 mL of urine sample was applied on the preconditioned cartridge. The columns were subsequently washed with 2 mL of water, 1 mL of 5% acetonitrile in 0.5% aqueous solution of acetic acid, 2 mL of 1% ammonia in water (freshly prepared) and 1 mL of 20% acetonitrile in 1% ammonia water solution. Vacuum was applied to remove residual solvent from cartridge for 5 minutes. The column was washed with 2 mL of dichloromethane and vacuum was applied for 1 minute again. The analyte was eluted from the cartridge with 2 mL of mixture dichloromethane : acetic acid : methanol (89:1:10, v:v:v). Eluate was evaporated at 50°C to dryness using rotary evaporator and transferred by tert-butylmethylether to the derivatisation vial. Solvent was evaporated under the nitrogen stream and derivatisation reagent (0.05 mL BSTFA with 1% TMCS) was added. Sample was treated at 75°C for 45 minutes. The rest of reagent after derivatisation was evaporated under the stream of nitrogen and dry residue was dissolved in 0.05 mL of dried toluene for chromatographic analysis.
Chromatographic analysis was performed using Agilent equipment GC 6890 with cool on-column injector and MSD 5973N in NCI mode (reagent gas methane). Chromatographic column was HP-1MS, length 30m, I.D. 0.25 mm, film thickness 0.25 micrometers, carrier gas He, flow rate 1.2 mL /min. Ions m/z 466,468, 376, 378 for CAP and m/z 471 for internal standard were monitored in SIM mode.

**Results and discussion**

Blank sample of urine and samples fortified at concentration levels 0.2, 0.5, 1.0, 2.0 and 5.0 ng/mL were measured using described method. The concentration of internal standard was 2 ng/mL in this case. The results of these measurements are displayed in fig. 1. The linear regression was applied. The correlation coefficient was higher than 0.999. Blank sample and samples fortified at concentration levels 2, 5, 10, 20, 50 ng/mL were measured to obtain the calibration curve for samples with expected higher concentration level. The results are plotted in fig. 2. The concentration of internal standard was 10 ng/mL in this case.

**Fig. 1** Calibration curve for concentration range 0 – 5 ng/mL

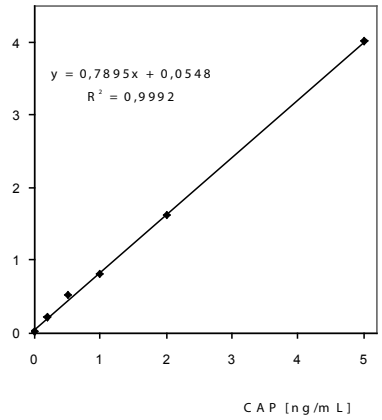



**Fig. 2** Calibration curve for concentration range 0 – 50 ng/mL

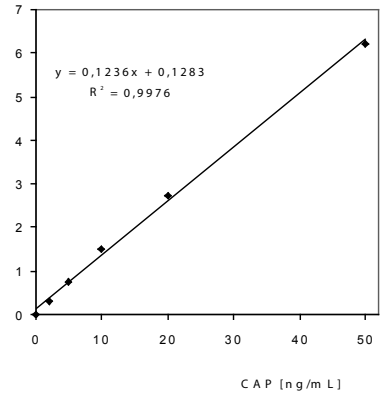
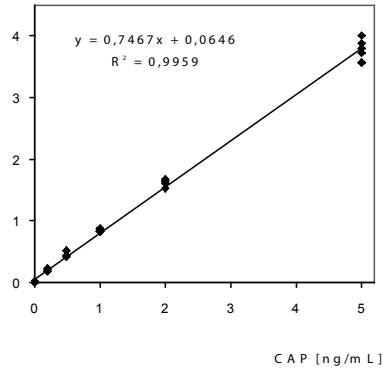



**Fig. 3** Within-laboratory reproducibility



Blank samples and samples fortified at concentrations 0.2, 0.5, 1.0, 2.0 and 5.0 ng/mL were prepared and measured six times in different days for determination of within-laboratory reproducibility. The results are plotted in fig. 3. Correlation coefficient for this set of samples was 0.9959. Decision limit (CCa) and detection capability (CCb) were calculated. CCa = 0.06 ng/mL, CCb = 0.1 ng/mL for used method.

A set of 36 samples fortified at concentration level of 0.1 ng/mL was measured for determination of repeatability at concentration near detection limit and under MRPL for CAP. The results of the measurements for this set of samples are shown in fig. 4. The mean determinated concentration was c = 0.111 ng/mL, standard deviation s = 0.013 and relative standard deviation RSD = 12%.

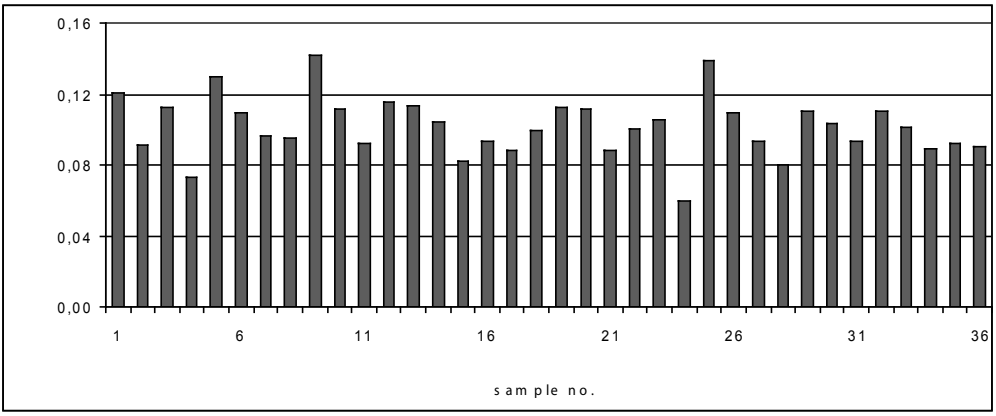



**Fig. 4** The repeatabilits at concentration level 0.1 ng/mL

Described method is useful for the determination of CAP in urine of different species of animals in monitoring of illegal use of this antibiotic during fattening. MIP clean-up procedure, in comparison with SPE C18 one, gives a cleaner extract for GC analyses with less interferences from matrix or from deconjugation with glucuronidase. Sensitivity of determination is sufficient for given purpose. CCa of the method is about five times lower than MRPL for different matrices (see CD 2003/181/EC). Also the robustness of the method is satisfactory. The entire method of sample preparation is very simple and allows high throughput of examinated samples.

**Conclusions**

The described method for the determination of CAP in samples of urine using MIP column clean-up procedure appears to be a simple and robust alternative for common sample preparation. In connection with GC/MS-NCI measurement it allows to achieve low limits of determination (CCa was 0.06 ng/mL, CCb was 0.1 ng/ mL), which offer a possibility to trace the illegal use of CAP for live animals in breeding within the scope of National plan of monitoring. The method is fully validated according to the requirements of CD 2002/657/EC.

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## P 19

# APPLICATION OF ELISA FOR THE SCREENING ANALYSIS OF NITROFURAN RESIDUES IN BABY FOOD, EGGS AND OTHER EDIBLE TISSUES

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**Abstract**
Applicability of ELISA methods for the determination of 3-amino-2-oxazolidone (AOZ) and semicarbazide (SEM) in baby food and other edible tissues is demonstrated. Validated methods enabled the reliable detection of nitrofuran tissue bound metabolites below 1 μg/kg (EU MRPL) in matrices of animal origin. ELISA for SEM was evaluated in different types of baby food. The analytical data obtained by ELISA for both acidic carbohydrate and fatty/protein rich baby food are presented. The LoD and detection capability of the optimised method for SEM was around 1.5 and 3 μg/kg, respectively, with an average recovery of 87.5 to 107.2 %. Variation coefficients of the assay were below 13.5 %.

**Introduction**
Until recently, all tests for banned nitrofuran antibiotics and their metabolites (AOZ, AMOZ, AHD and SEM) employed expensive mass spectrometry methodologies which are unavailable to many laboratories and food producers. The first nitrofuran specific ELISA methods were developed within the EU "FoodBRAND" project several years ago (Cooper et. al., 2004; Diblikova et. al., 2005). The tests were shown to be a viable and effective screening alternative to traditional chemical techniques based mainly on LC-MS methodology.
The use of SEM as a residual marker for the banned drug nitrofurazone has been questioned following the discovery that SEM in food samples may arise from sources other than the use of illegal veterinary antibiotics (Cooper et. al., 2007). Special attention has been devoted to semicarbazide, due to the observation of this contaminant in baby food. The presence of SEM was confirmed in jars of processed baby food, bread and other commodities of non-animal origin. The main source of SEM in baby food was azodicarbonamide, which was used in the manufacture of the plastic gaskets inside the lids of glass jars. It was determined that SEM was a breakdown product of this foaming agent in baby food packed in glass jars. Specific antibodies against derivatised semicarbazide (NPSEM) were produced by Cooper et al. (2007) and Vass et al. (2008a). The antibodies were incorporated into ELISA systems and validated for SEM in chicken meat (Cooper et. al. 2007), porcine muscle (Vass et. al., 2008a) and chicken eggs (Vass et. al., 2008b). This report presents results of baby food sample analysis using introduced ELISA methodology.

## Materials and methods

*Reagents and calibration standards*
All reagents were of analytical grade and are detailed in previous works (Diblikova et. al., 2005, Vass et. al. 2008a, Vass at. al. 2008b). Monoclonal antibody 3B8/2B9 for AOZ and polyclonal (rabbit) antibody MVK31 (IgG) for SEM were produced in the Veterinary Research Institute, Brno and employed in ELISA for the detection of these nitrofuran metabolites in tissues of animal origin and baby food.

*Baby food materials*
Different types of baby food were purchased in retail outlets within the Brno area. All baby foods were typical commercial products packed in glass jars (100 - 200 g) with gasket seals. Samples confirmed free of SEM by analysis using LC-MS/MS were used as blanks for fortification experiments.

*Baby food sample preparation prior to ELISA detection*
The baby food was thoroughly stirred or homogenized upon opening in order to obtain homogeneous materials for testing. One gram sample aliquots or blanks fortified with SEM were weighed in 15 ml plastic disposable tubes and carried through derivatisation and extraction as follows: 0.2 mol/l HCl (4 ml) and 100 µl of 50 mmol/l o-NBA in methanol were added successively to sample tubes and the mixture was vortexed for 10 sec, followed by incubation overnight at 40ºC. Samples were neutralized by the addition of 0.3 ml of 2 mol/l NaOH and 0.1 mol/l PBS (0.5 ml) and vortexed for 20 sec. Ethyl acetate (5 ml) was added to samples, followed by horizontal shaking (10 min) and centrifugation (1600 × g, 15 min, 4ºC). The upper ethyl acetate layer (3.5 ml) was then transferred into glass tubes and evaporated to dryness at 60ºC under nitrogen. The evaporates were re-dissolved in methanol (20 µl) and 0.7 ml PBS (0.7 ml). No hexane washing was used for fruit materials, whereas the preparation of fatty-protein samples (e.g. carrot/beef/potato) required two hexane washes to remove residual fat components, as follows: N-hexane (2 ml) was added to the sample and vortexed thoroughly for 30 sec. Samples were centrifuged (1600 x g, 10 min, 4ºC) and the upper hexane layer discarded. Additional hexane (1 ml) was added, followed by vortexing and centrifugation as describe above. After discarding the upper hexane layer the sample (100 µl) was diluted with PBS (900 µl) and used for ELISA detection.

*ELISA procedure*
Microtitre wells were coated with antibody in coating buffer (200 µl per well) and left to incubate overnight (16 hrs, 4ºC). Plates were washed three times with the washing buffer (0.3 ml per well) and 100 µl of standard or sample, followed by 100 µl of tracer in PBS with 0.5% BSA, was applied to each well. Plates were incubated (1 hr, 4ºC) and the washing step repeated. Substrate solution (200 µl per well) was added and after 15 minutes the enzymatic reaction was stopped by the addition of the stopping reagent (100 µl per well). Absorbance was measured at 450 nm.

**Results**

Table 1 summarizes ELISA methods that were established and validated in this laboratory for the analysis of samples of animal origin. A monoclonal based ELISA coupled with matrix-matched calibration and buffer extraction offers simplified and precise assay performance (Diblikova et.al. 2005) but solvent extraction was easily applicable to a broader scale of matrices and was therefore preferred in routine analysis. All ELISA methods were sensitive enough to meet the performance criteria laid down by Commission Decision 2002/181/EC (2002).

Table 1. In-house validated ELISA methods for the determination of AOZ and SEM in edible tissues

| Assay type | Matrix | Sample extraction | LoD (µg/kg) | $CC_\beta$ (µg/kg) | Citation |
|---|---|---|---|---|---|
| Monoclonal ELISA for AOZ | shrimp | buffer | 0.185 | 0.4 | Diblikova et. al. (2005) |
| | poultry | buffer | 0.134 | 0.4 | Diblikova et. al. (2005) |
| | | solvent | <0.11 | 0.3 | — |
| | beef | buffer | 0.106 | 0.4 | — |
| | | solvent | <0.11 | 0.3 | — |
| | pork | buffer | 0.113 | 0.4 | — |
| | | solvent | <0.11 | 0.3 | — |
| | egg | buffer | 0.162 | 0.6 | Franek et. al. (2006) |
| | | solvent | 0.089 | 0.3 | Franek et. al. (2006) |
| | | solid phase | 0.104 | 0.3 | Franek et. al. (2006) |
| Polyclonal ELISA for SEM | pork | solvent | 0.11 | 0.3 | Vass et. al. (2008a) |
| | egg | solvent | 0.13 | 0.3 | Vass et. al. (2008b) |

Recent efforts have been devoted to the evaluation of ELISA for the detection of SEM in baby food samples. Two types of baby foods were studied: 1) various types of fruit puree and 2) meat/vegetable/side dish meal in different composite combinations. This represented both acidic carbohydrate and fatty/protein-rich products. The analytical data obtained by ELISA performance are presented in Tables 2 and 3.

Fortification of blank samples was carried out at 5, 10 and 20 µg/kg with respect to a potential EU regulatory limit of 10 µg/kg (Ginn et. al., 2006). The ELISA provided intra and inter-assay variation coefficients in the range of 2.4 to 13.5 % for the respective fortified levels. The average recovery of SEM determined from six fruit and six carrot/beef/potato samples was in the range of 93.9 to 107.2 % and 87.5 to 107.2 %, respectively.

Table 2. Precission, recovery and repeatibility of ELISA method for SEM determination in fruit baby food

| Intra-assay variation | | | |
|---|---|---|---|
| Fortified concentration (µg/kg) | Measured concentration (µg/kg) ± SD | CV[a] (%) | Recovery (%) |
| 5 | 5.36 ± 0.45 | 8.4 | 107.1 |
| 10 | 9.79 ± 0.72 | 7.3 | 97.9 |
| 20 | 19.02 ± 1.03 | 5.4 | 95.1 |
| **Inter-assay variation** | | | |
| Fortified concentration (µg/kg) | Measured concentration (µg/kg) ± SD | CV[b] (%) | Recovery (%) |
| 5 | 5.32 ± 0.41 | 7.7 | 93.9 |
| 10 | 9.52 ± 0.55 | 5.8 | 105.0 |
| 20 | 18.66 ± 0.59 | 3.2 | 107.2 |

[a)] Number of different samples analysed = 6
[b)] Number of repeated analysis over 2 weeks = 4

Table 3. Precission, recovery and repeatibility of ELISA method for SEM determination in carrot/beef/potato meal

| Intra-assay variation | | | |
|---|---|---|---|
| Fortified concentration (µg/kg) | Measured concentration (µg/kg) ± SD | CV[a] (%) | Recovery (%) |
| 5 | 4.38 ± 0.31 | 7.1 | 87.5 |
| 10 | 8.88 ± 0.46 | 5.2 | 88.8 |
| 20 | 19.56 ± 0.47 | 2.4 | 97.8 |
| **Inter-assay variation** | | | |
| Fortified concentration (µg/kg) | Measured concentration (µg/kg) ± SD | CV[b] (%) | Recovery (%) |
| 5 | 4.46 ± 0.63 | 13.5 | 93.9 |
| 10 | 8.88 ± 0.46 | 5.2 | 105.0 |
| 20 | 19.7 ± 0.49 | 2.5 | 107.2 |

[a)] Number of different samples analysed = 6
[b)] Number of repeated analysis over 2 weeks = 5

Figure 1 examines ELISA performance by assessment of LoD and detection capability (CCβ) for the testing of commercial baby food products. Twenty selected samples of different composition, batch and origin with regard to producer were employed to determine the CCβ and LoD of the ELISA method (Figure 1a). The CCβ was determined on the basis of the acceptance of 5% false compliant results for a given concentration level according to Commission Decision 2002/657/EC (2002). The LoD was defined as the average level of concentration equivalent at zero SEM concentration plus three times the standard deviation. The same study was carried out using 20 samples of one type of baby meal sample distributed by four producers (Figure 1b). The LoD obtained for fruit or composite samples ranged from 1.40 to 1.66 µg/kg and the respective detection capability values of both sample groups were determined to be 3 µg/Kg.

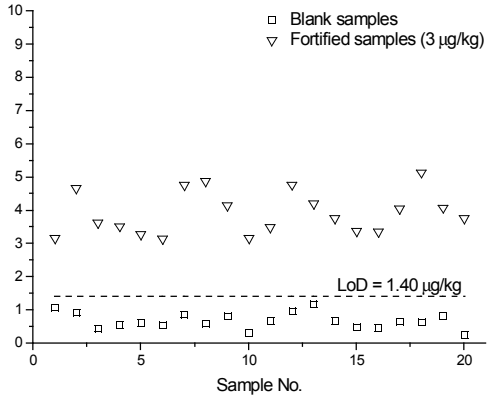


**Figure 1.** Assessment of CCβ for 20 different composite samples

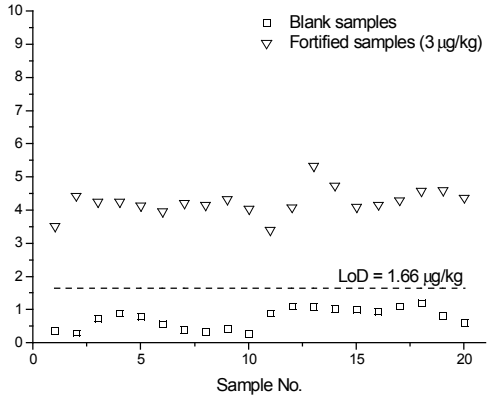


**Figure 2.** Assessment of CCβ for 20 different samples of carrot/beef/potato meal from 4 producers

## Conclusions

Different sample preparation methods linked with ELISA detection were developed and applied for the determination of furazolidone and nitrofurazone metabolites (AOZ and SEM) in different types of food. Whereas application of the ELISA methodology for the analysis of edible tissues of animal origin has been reported in many communications, the determination of SEM in baby foods by ELISA has not been described in available literature so far. The presented results demonstrate that ELISA is a valuable screening tool for the determination AOZ and SEM in edible tissues and baby food.

**Acknowledgements**
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## P 20

# EUROHISTOLOGY: STATE OF THE ART OF HISTOLOGICAL SCREENING FOR HORMONES (2005-2008)

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**Abstract**
In the Netherlands and Italy histological screening for anabolic growth promoters is performed in cattle. In both countries animal experiments have been performed with single sex hormones, hormone cocktails, corticosteroids and prohormones. In Italy the screening for histological lesions in target organs is carried out by the veterinary public health service and, since 2003, by COOP Italia, the largest Italian retailer and V° Valore which certify their meat products with an additional guarantee. Piemonte Region has officially adopted the histological test since 2006, and chemical analysis is performed based on histological results. As a consequence, positive findings for corticosteroid residues in liver has increased from 22 cases in 2005 to 90 in 2006. From 2005 to 2006, the Italian Ministry of Health carried out a pilot project in 8 regions to assess the value of the histological test. The conclusion was that it possesses high sensitivity and allows a good evaluation of the risk for the consumer following the use of growth promoters in bovine production. Additionally it gives information about the nature of employed anabolic molecule. The histological test may be applied in the National Residue Program to strengthen the control of hormone abuse in animal production.

**Introduction**
Histological studies on the effects of growth promoters on target organs of livestock, and their possible application in the monitoring programs, started in the sixties. In the following years several publications have been produced and it is now well known that, besides the better performances induced by these compounds on muscle development and fat deposit reduction, severe changes are produced in organs and tissues which are physiologically susceptible to the hormonal stimulus.
In the Netherlands and Italy a number of animal experiments have been carried out on veal calves and beef experimentally treated with single molecules, growth promoters association, corticosteroids and prohormones (Biolatti et al. 2005, Cannizzo et al. 2007, Groot et al., 1998, Groot et al. 2000,).

In the table 1 the lesions induced by the different drugs are summarized:

**Table 1.** Main changes induced by the administration of growth promoters

| Drug | Target organs | Lesion |
|---|---|---|
| Corticosteroids | Thymus | Atrophy of the cortex and medulla associate with fat replacement |
| Thyreostatics | Thyroid | Hyperplasia of follicular epithelium |
| Estrogens | Prostate<br>Cowper's Gland<br>Bartholin's gland<br>Mammary Gland | Hyperplasia/squamous metaplasia of gland epithelium |
| Androgens | Prostate<br>Cowper's Gland<br>Bartholin's gland | Hypersecretion/hyperplasia |

**The histological test in Italy**

Since the illegal treatment of cattle with growth promoters represents a potential severe risk for the consumer's health, the histological test has been applied in the Italian Public Health Service first by Piedmont Region as screening test in order to have a real evaluation of the prevalence of illegal treatments. More over, from the year 2003, COOP Italia, the largest Italian retail dealer, and V° Valore, a main private provider of quality control systems, certify their bovine meat products with a further guarantee supplied by the histological test.

From 2006 the Public Veterinary Service of Piedmont Region, officially adopted the histological test in the monitoring plans followed by chemical investigation on the samples collected from veal calves or beef in the farms where histological positivity changes were found. As a consequence, the number of positive liver samples to the chemical analysis for corticosteroid residues arose to 90 cases in the year 2006 compared to 22 cases in the year 2005.

The Italian Ministry of Health carried out a pilot study from 2005 to 2006 in order to verify the opportunity to include the test in the Italian Residues Plans (PNR) for the whole country. The project involved 8 Italian Regions, committed to:

- test standardization
- training of veterinarians for samples collection
- training of laboratory technicians
- training of veterinary pathologists
- calculation of the sample of animals to be examined (according to Cannon and Roe, 1982)
- collection and processing of data

Four-hundred and sixty-seven farms were examined from 2005 to 2006, for a total of 1330 animals. Thirty-nine farms (8%) were suspected of illegal treatments. In particular, 143 animals (11%) were suspect at the histological test with the following distribution: 85 animals (6%) for corticosteroid and 63 animals (5%) for steroids.

**Histological test in the Netherlands**

Histological examination of the prostate of male veal calves for tracking the administration of estrogens was developed by the National Institute of Public Health (RIVM) (Ruitenberg et al., 1969). In 1967 the test was introduced in meat inspection practice as a screening method to detect animals suspected of illegal treatment with estrogenic agents. Most veal calves are males, since the females are used for replacement of dairy cows, but since the introduction of the prostate test an increasing number of female veal calves were fattened (Garbis Berkvens et al.,1985). Later Bartholin's glands were used to control the female animals (Kroes et al. 1970). The routine histological control of veal calves is performed by the Food and Consumer Product Safety Authority. Each year 2500 samples are investigated. Based on the outcomes of this test further chemical analysis may be performed on the farms from which the suspect animal originated. Animal experiments are performed by RIKILT-Institute of Food Safety where histological investigation of target tissues is combined with chemical analysis of urine, hair and blood of the animals. Animal experiments are performed with agents that have been found in illegal practice to develop and adapt screening and detection methods accordingly.

Recently a large animal experiment has been performed with not treated animals to update the normal picture of veal calves since during the years feeding and housing systems have been changed. The increased contact between the animals and the changed feeding regimes using more roughage might influence sexual development and so the histology of normal animals. The results of this experiment showed that all male veal calves showed normal histology and only a few female animals showed alterations due to endogenous estrogens (Groot et al., 2006). Moreover histological effects of phyto-oestrogens were investigated, which may be included in milk replacer, to investigate of these products might interfere with the histological test. This appeared not to influence the prostate (Groot, 2006). The Dutch government continues to use the histological screening for veal calves.

**Conclusions**

In Italy the percentages of positive suspect samples to the histological test exceed those positive oft the chemical analyses.

Results of the monitoring programs showed that changes of target organs were mostly the probable consequence of treatments of veal calves and beef with sex hormones and corticosteroids, either alone or associated.

Since the histological test is a screening method having high sensibility, it gives highly reliable information about negative samples, and, when suspectpositive, about the kind of drug categories potentially employed. This allows a good risk evaluation for the consumer health. Preliminary results show the good reliability of histology, also confirmed by the increase of chemical positivity following the application of the results of the histological test to orientate the chemical investigation. Like the increase of chemical positivity for corticosteroids of samples taken from the farms which had previous histological positivity for thymus atrophy. The results strongly suggest that histopathology can be applied by the National Health Service to prevent abuse of growth promoters in cattle, particularly of sex steroids and corticosteroids in veal calves and beef production. The Italian PNR will be integrated and enhanced by the histological test starting from 2008.

In the Netherlands histological test is only used for screening on estrogens and not also for corticosteroids as in Italy. Extension to other tissues might improve the screening results.

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## P 21

# NONPARAMETRIC BOOTSTRAP APPROACH TO ESTIMATE IMPRECISION OF ANALYTICAL RESULTS

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**Abstract**
When calibration is done by weighted least-squares regression the confidence region associated with an analytical result can be rather asymmetric. In such an instance, one may apply nonparametric bootstrapping to the calibration data in order to obtain an adequate confidence interval for the unknown true concentration. The bootstrap is a versatile inferential statistical technique that does not require distributional assumptions about the statistics involved. This contribution presents an illustrative case example from the field of veterinary drug residue analysis.

**Introduction**
Consider a set of $I$ calibration points $(x_i , y_i)_{i=1,...,I}$. Suppose that the relationship between measurement $y_i$ and concentration $x_i$ is given by a model of the form

$$y_i = b_1 + b_2 x_i + \varepsilon_i \tag{1}$$

where $\varepsilon_i$ is a normally distributed random variable with mean zero and heteroscedastic variance $\sigma^2 / w_i$. $\varepsilon_i$ is representing the measurement error. If weights $w_i$ are available, estimates $\hat{b}_1$, $b_2$ and $\hat{\sigma}^2$ can be obtained by performing weighted least-squares regression on the empirical calibration data. An estimator for the expectation value of the response at a certain concentration $x_0$ is

$$\hat{y}_0 = \hat{b}_1 + \hat{b}_2 x_0 \tag{2}$$

The interval in which the mean of $N$ future measurements at a particular $x_0$ is expected to lie with a probability of $1 - \alpha$ (i.e., the prediction interval (Fig. 1 A)) can be approximated as

$$\hat{y}_0 \pm t_{f,1-\alpha/2}\hat{\sigma}\sqrt{\frac{1}{N w_0} + \frac{\sum w x^2 - 2x_0 \sum wx + x_0^2 \sum w}{\sum w \sum wx^2 - (\sum wx)^2}} \tag{3}$$

$t_{f,1-\alpha/2}$ designates the $1 - \alpha$-quantile of the $t$-distribution with $f$ degrees of freedom. Let $y_{0n=1,...,N}$ be $N$ observed response values from a sample with unknown concentration $x_0$, then an estimate of the true concentration, $\hat{x}_0$, can be computed as

$$\hat{x}_0 = \hat{\bar{x}}_{0n=1,...,N} = 1/N \sum_n (y_{0n} - \hat{b}_1)/\hat{b}_2 = (\bar{y}_{0n} - \hat{b}_1)/\hat{b}_2 \tag{4}$$

The $1 - \alpha$ confidence region for the true value $x_0$ covers all those $x$ values at which the prediction interval includes $\bar{y}_{0n}$ (Fig. 1 B). As indicated in Fig. 1 B, the uncertainty interval around $\hat{x}_0$ is asymmetric. Besides obtaining inverse limits through an iterative solution process, a further possible approach to develop an adequate confidence region for $x_0$ is

the nonparametric bootstrap. The so-called bootstrap provides a way to infer measures of the variability in statistical estimates derived from experimental data. It is a computer-based resampling technique usable for determining standard errors and confidence intervals or for conducting hypothesis tests. The bootstrap offers an alternative methodology, especially in situations where classical statistical procedures turn out to be quite complex or are intractable because the appropriate assumptions are not met. A valuable introduction to the theory and implementation of the bootstrap is given by Efron and Tibshirani.

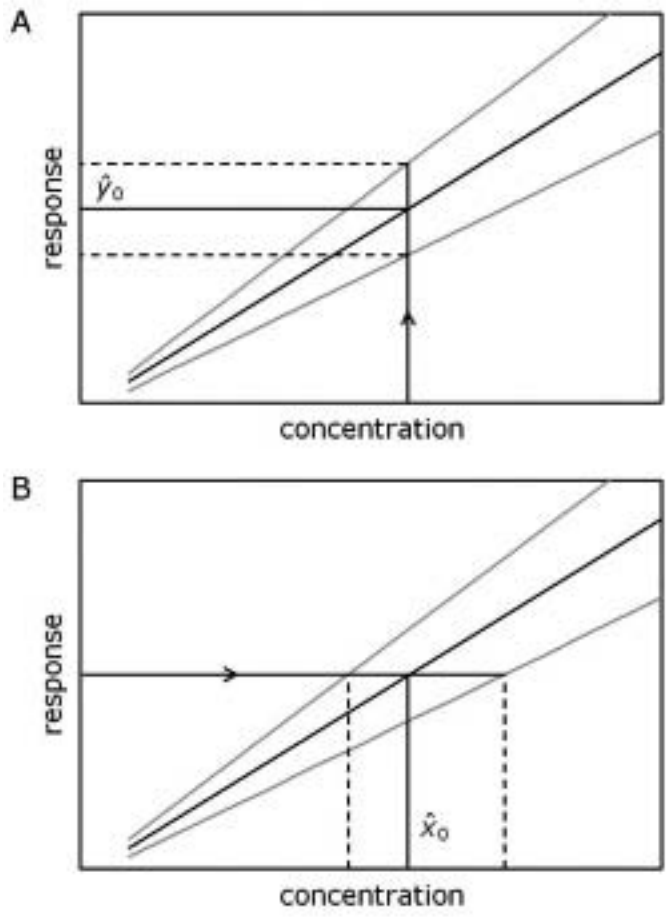


**Figure 1.** A: Prediction interval for $\overline{y}_{0n}$ about $\hat{y}_0$ . B: Confidence region for the true concentration $x_0$ .

**Methods**

Three replicates of a sample (bovine muscle) containing tetracycline in an unknown amount and 15 calibration standards (blank matrices spiked with 25, 25, 25, 75, 75, 75, 125, 125, 125, 250, 250, 250, 500, 500, 500 µg/kg tetracycline) were analysed. Calibration line was established

by weighted least-squares fitting. The weights were set equal to the reciprocal of the response variances and were modelled as a function of concentration. Using Eq. (4), the estimates $\hat{x}_{01}$, $\hat{x}_{02}$, $\hat{x}_{03}$ and their mean value $\hat{x}_0$ were calculated. To construct the confidence interval, Jones et al. approach to the percentile bootstrap was applied. Computations were implemented using Excel 2000 spreadsheets. The algorithm proceeds as follows:

Compute adjusted residuals $R_i$ as

$$R_i = \sqrt{I/(I-2)} \cdot (y_i - \hat{y}_i)\sqrt{w_i} \tag{5}$$

and $R_{0n}$ as

$$R_{0n} = \sqrt{N/(N-1)} \cdot (y_{0n} - \overline{y}_{0n})\sqrt{w(\hat{x}_0)} \tag{6}$$

Pool the adjusted residuals together to form a set of size $k$ $(k = I + N)$.
Sample from the pool of the adjusted residuals $R_{1,\ldots,k}$, randomly with replacement, so obtaining $R^{*1}_{1,\ldots,k} = (R^{*1}_1, \ldots, R^{*1}_I, R^{*1}_{01}, \ldots, R^{*1}_{0N})$.
Compute bootstrap responses $y^{*1}_i$ as

$$y^{*1}_i = \hat{b}_1 + \hat{b}_2 x_i + R^{*1}_i \Big/ \sqrt{w_i} \tag{7}$$

and $y^{*1}_{0n}$ as

$$y^{*1}_{0n} = \overline{y}_{0n} + R^{*1}_{0n} \Big/ \sqrt{w(\hat{x}_0)} \tag{8}$$

Fit a linear model to the data set $(x_i\ ,\ y^{*1}_i)$ via weighted least-squares regression, yielding $\hat{b}^{*1}_1$ and $\hat{b}^{*1}_2$. Calculate bootstrap estimate $\hat{x}^{*1}_0$ as

$$\hat{x}^{*1}_0 = 1/N \sum\nolimits_n (y^{*1}_{0n} - \hat{b}^{*1}_1) / \hat{b}^{*1}_2 \tag{9}$$

Repeat steps 2-4 a large number of times, say $B$. Sort the bootstrap estimates $\hat{x}^{*1}_0, \ldots, \hat{x}^{*B}_0$ into ascending order of magnitude. Chose the $(\acute{a}/2\ B)^{th}$ and the $(1 - \acute{a}/2\ B)^{th}$ of the ordered values as the lower and the upper limit of the $1 - \acute{a}$ confidence interval.

**Results**

Fig. 2 shows the calibration line, Fig. 3 depicts the variance model used for estimating the weights. Raw data and application of the bootstrap procedure are outlined in Table 1. A bootstrap sample consists of $k$ objects drawn with replacement from a sample of size $k$ (for each object, the probability of being drawn is $1/k$). A bootstrap sample comprises members of the original data set, some appearing zero times, some appearing once, some appearing twice, etc. In the concrete case, the resampling procedure was applied to the 18 adjusted residuals ($k = I + N = 18$). 1000 bootstrap data sets were selected. Each data set was then used to generate new responses for the 15 standards and the 3 replicates of the unknown. Calibration procedure was carried out to yield bootstrap estimates $\hat{x}^{*1}_0$, $\hat{x}^{*2}_0$, …, $\hat{x}^{*1000}_0$. The bootstrap estimates were sorted from smallest to largest. The 25$^{th}$ and the 975$^{th}$ of the ordered values were chosen as the limits of the 95 %confidence interval. A histogram of the bootstrap estimates is displayed in Fig. 4.

**Table 1.** Application of the percentile Bootstrap to the data

| Standards | | Adj. residuals | Resampling | |
|---|---|---|---|---|
| $x_i$ [ppb] | $y_i$ | $R_i$ | $R_i^{*1}$ | $y_i^{*1}$ |
| 25 | 8.34744 | -1.39556 | -1.10214 | 8.73517 |
| 25 | 12.92359 | 1.27841 | 1.27841 | 13.0563 |
| 25 | 11.91698 | 0.69022 | -1.39556 | 8.20256 |
| 75 | 39.63756 | -0.40005 | -1.39556 | 34.61400 |
| 75 | 41.63697 | 0.02986 | 1.01084 | 46.48446 |
| 75 | 36.61134 | -1.05074 | -1.39556 | 34.61400 |
| 125 | 66.64413 | -0.75866 | -0.57853 | 67.71779 |
| 125 | 78.67390 | 0.86633 | 0.02986 | 72.49491 |
| 125 | 65.61124 | -0.89818 | 0.00771 | 72.32099 |
| 250 | 157.41806 | 0.59320 | -1.05074 | 133.66328 |
| 250 | 163.22768 | 1.01084 | 0.86633 | 161.94851 |
| 250 | 135.23607 | -1.00142 | 0.02986 | 149.60688 |
| 500 | 303.17960 | 0.00771 | 0.86633 | 326.99639 |
| 500 | 341.21048 | 1.46268 | -0.25096 | 296.01973 |
| 500 | 287.85608 | -0.57853 | -0.89818 | 278.07684 |

**WLS** ($w_i = 1/v_i = (9.41 \cdot 10^{-3} x^{1.82})^{-1}$)

| | | | |
|---|---|---|---|
| $\hat{b}_1$ = | -4.64542 | $\hat{b}_1^{*1}$ = | -5.37314 |
| $\hat{b}_2$ = | 0.61525 | $\hat{b}_2^{*1}$ = | 0.60785 |

| **Unknown** | $y_{0n}$ | $R_{0n}$ | $R_{0n}^{*1}$ | $y_{0n}^{*1}$ |
|---|---|---|---|---|
| | 78.00199 | -1.10214 | 1.35312 | 98.59384 |
| | 96.32443 | 1.35312 | -1.10214 | 76.15352 |
| | 84.35373 | -0.25096 | -1.00142 | 77.07406 |
| | $\hat{x}_0$ = 147.7 | | $\hat{x}_0^{*1}$ = 146.9 | |

**Bootstrap confidence limits** ($B = 1000, 95\,\%$)

$\hat{x}_{lower}$ = 132

$\hat{x}_0$ = 148

$\hat{x}_{upper}$ = 167

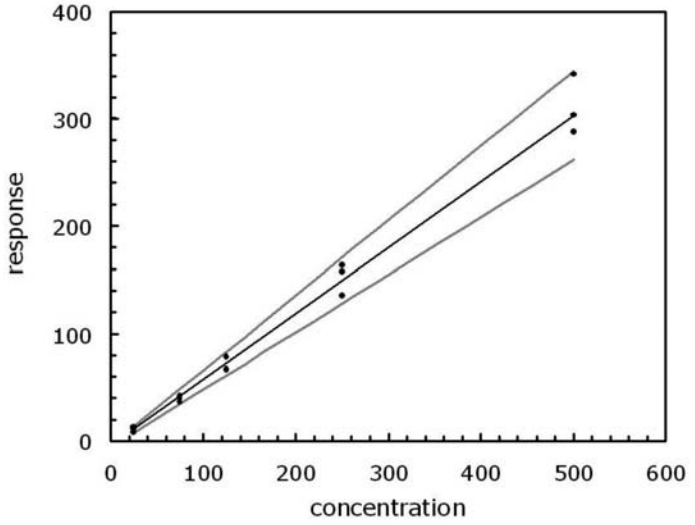


**Figure 2.** Calibration line and 95 % prediction interval for $\bar{y}_{0n}$ ($N = 3$) obtained by weighted least-squares regression.

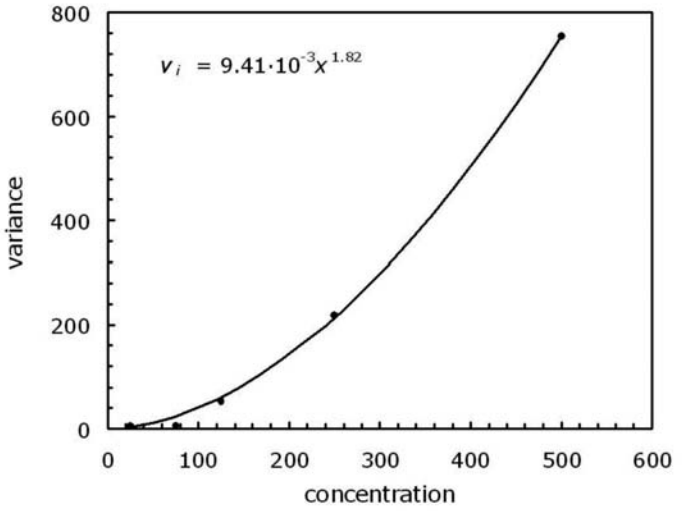


**Figure 3.** Modelling the variance of replicate responses, , as a function of concentration.

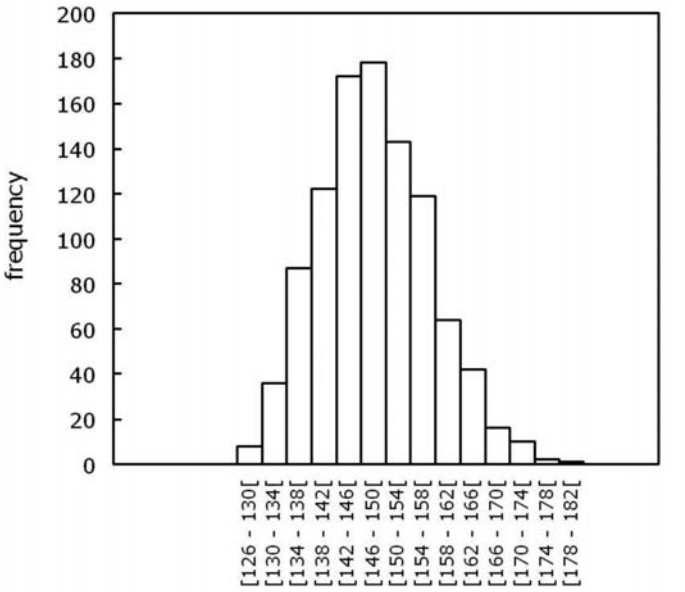


**Figure 4.** Histogram of bootstrap estimates ( ).

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## P 22

# DETERMINATION OF (R)- AND (S)-KETOPROFEN IN PORCINE PLASMA BY USING MIXED-MODE SOLID-PHASE EXTRACTION AND A SUBSEQUENT LC-MS/MS ANALYSIS

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**Abstract**
A simple and sensitive method for analysing ketoprofen (R)-and (S)-enantiomers in porcine plasma has been developed. Plasma samples were purified by using a mixed-mode solid phase cartridge with an anion exchange and reversed-phase resin. The endogenous compounds present in plasma were effectively removed and a smoother baseline in LC-MS/MS analysis was achieved. Ketoprofen enantiomers were separated on a chiral column in combination with MS detection in positive ESI MRM mode. (±)-Ketoprofen-d4 was used as an internal standard for the quantification of (R)- and (S)-enantiomers.

Introduction

Ketoprofen, (R,S)-2(3-benzoylphenyl)propionic acid, belongs to nonsteroidal anti-inflammatory drugs (NSAIDs) and it is used as a racemic mixture. Ketoprofen molecule has one chiral carbon atom, the S-enantiomer being pharmacologically more active than the R-enantiomer, particularly in humans. The enantiomers of the chiral compounds in biological matrices have different pharmacological, pharmacokinetic or toxicological effects. This has raised a great interest in stereospecific pharmacokinetic metabolism and clinical pharmacology data of chiral drug molecules.

The analysis of (R )- and (S)-enantiomers of ketoprofen in plasma requires a very sensitive analytical method. The present study details the development and validation of a method involving mixed-mode solid-phase extraction, the quantification of an internal standard method, chiral LC and electrospray ionization (ESI) MS/MS for the determination of (R)- and (S)-ketoprofen in porcine plasma.

## Materials and methods

*Materials*

Organic solvents were of HPLC grade and other chemicals of analytical quality. Water was purified via a Milli-Q system (Millipore, Bedford, MA, USA). Solid phase extraction cartridges (Oasis® MAX 1cc, 30 mg) were from Waters (Waters, Milford, MA, USA). (R,S)-Ketoprofen was from USP U.S.Pharmacopeia (Rockville, MD, USA) and (±)-ketoprofen-d4 from Qmx Laboratories (Thaxted Essex CM6 2 PY,UK). Optically pure (S)-ketoprofen was obtained from Aldrich (St.Louis, MO, USA).

*Sample preparation*
0.5 ml of plasma was diluted with 0.5 ml of 4 % ammonia followed by OASIS MAX (30 mg) purification. After conditioning with 1 ml of methanol and water, the extract was loaded onto the column. The column was washed with following solvents: 1 ml of 5 % ammonia in water, 1 ml of methanol and 1 ml of 2 % formic acid in methanol/water (45/55, v/v). The compounds were eluted with 1 ml of 2 % formic acid in methanol/water (90/10, v/v). The eluate was evaporated to dryness under a stream of nitrogen at 45 °C and redissolved in 250 µl of the mobile phase. 15 µl of the extract was injected into the LC-MS/MS system.

*LC-MS/MS analysis*
Ketoprofen enantiomers were separated on a chiral column (Chirex 3005, (R)-1-naphtylglycine and 3,5-dinitrobenzoic acid, 250x2.0 mm i.d.) using 20 mM ammonium acetate buffer at pH 3.5 in 95 % methanol and operated on a flow rate of 0.5 ml/min.
The mass spectral analysis was performed on a Micromass Quattro Micro triple quadrupole mass spectrometer, which was operated in a positive ion MS/MS mode. The following parameters were used: capillary voltage 3.52 kV; source temperature 120 °C; desolvation temperature 300 °C; N2 cone gas flow 15 L/h and N2 desolvation gas flow 500 L/h. Argon was used as the collision gas. The Multiple Reaction Monitoring (MRM) mode was used for monitoring ion transitions. The transitions and respective cone voltages and capillary energies are presented in Table 1. The LC-MS/MS system was controlled and the data acquired by the MassLynx 4.0 software (Waters).

**Table 1.** Diagnostic ions and instrumental settings

| **Analyte** | **Precursor ion** | **Product ion** | **Cone voltage(V)** | **Collision energy(V)** |
|---|---|---|---|---|
| (±)-Ketoprofen | 255.1 | 209.3 | 30 | 15 |
| | | 194.2 | 30 | 20 |

Product ion 209.3 is used for the quantificationt

The secondary product ion of (±)-ketoprofen can be used for quantification only in higher concentrations. (±)-Ketoprofen-d4 was used as its protonated molecular form.

*Quantification*
The quantification of (R)- and (S)-enantiomers of ketoprofen was based on the internal standard (I.S.) method. (±)-Ketoprofen-d4 was added in every sample, calibration standard and spiked sample so that the concentration of a racemic internal standard was 50 ng/ml of plasma. The concentration of the enantiomer in plasma was calculated by the ratio of peak area of analyte/(peak area of I.S./added concentration of I.S.) and compared with the external matrix calibration curve.
The matrix standards were prepared by adding (R,S)-ketoprofen to blank plasma samples at the evaporation step of the sample preparation and reconstituted in 250 µl of the mobile phase. The quantification was automatically calculated by the QuanLynx software (MassLynx 4.0, Waters).

*Validation*

The linearity of the method was evaluated for the analytes in the concentration range of 1 – 20 ng/ml of plasma and the correlation coefficients varied between 0.999-0.994.

Six replicates of blank plasma samples fortified at three levels of racemic ketoprofen were analysed on two different days. The precision of the method was expressed as the relative standard deviation (RSD) of replicate measurements. Precision was evaluated as both intra- and inter-day reproducibilities of the analytical method (Tables 2. and 3.)

**Table 2.** Validation results for (R)- and (S)-ketoprofen in porcine plasma (n=6)

| **Analyte** | **Fortification (ng/ml)** | **Mean recovery (%)** | **RSD (%)** |
|---|---|---|---|
| (R)-ketoprofen | 2.5 | 94 | 3.5 |
| | 5.0 | 96 | 9.3 |
| | 10.0 | 94 | 12.7 |
| (S)-ketoprofen | 2.5 | 102 | 4.0 |
| | 5.0 | 96 | 9.5 |
| | 10.0 | 100 | 2.1 |

No interfering peaks were seen in blank and zero plasma samples so the method proved to be specific.

The limit of detection (LOD) was estimated to be 0.5 ng/ml plasma for both enantiomers with a signal-to-noise ratio of 3:1 in MRM mode.

**Table 3.** Average (R)-and (S)-ketoprofen recoveries obtained on different days (n=12)

| **Analyte** | **Fortification (ng/ml)** | **Mean (%)** | **RSD (%)** |
|---|---|---|---|
| (R)-ketoprofen | 2.5 | 92 | 8.6 |
| | 5.0 | 101 | 6.9 |
| | 10.0 | 99 | 11.7 |
| (S)-ketoprofen | 2.5 | 100 | 4.2 |
| | 5.0 | 101 | 9.0 |
| | 10.0 | 103 | 4.4 |

**Results and Discussion**

Special attention has been paid to the purification of plasma samples by multidimensional solid-phase extraction. Phospholipids are identified as a major source of matrix effects in plasma when analyzed by LC/MS. Ion suppression was effectively eliminated by mixed-mode solid-phase extraction used for sample preparation. Samples were clean enough also for

HPLC-UV detection, but lower concentrations were achieved by MS/MS detection.
The Chirex 3005 was chosen for a chiral stationary phase to separate the enantiomers, because it was also possible to run in reversed phase mode using ammonium acetate in methanol that are conducive to ESI. The ion chromatograms can be seen in Figure 1. As can be seen the enantiomers were not completely baseline resolved.
The elution order of (R)- and (S)-ketoprofen was determined with optically pure (S)-ketoprofen. The experiment showed that (R) form elutes first and (S)-ketoprofen elutes second.
Slightly differing mobile phases and negative-ion mode were experimented when optimizing the method. The aim was to yield a more prominent secondary ion for quantification of ketoprofen enantiomers, but this did not come true. LC-MS/MS conditions above gave the most sensitive results.

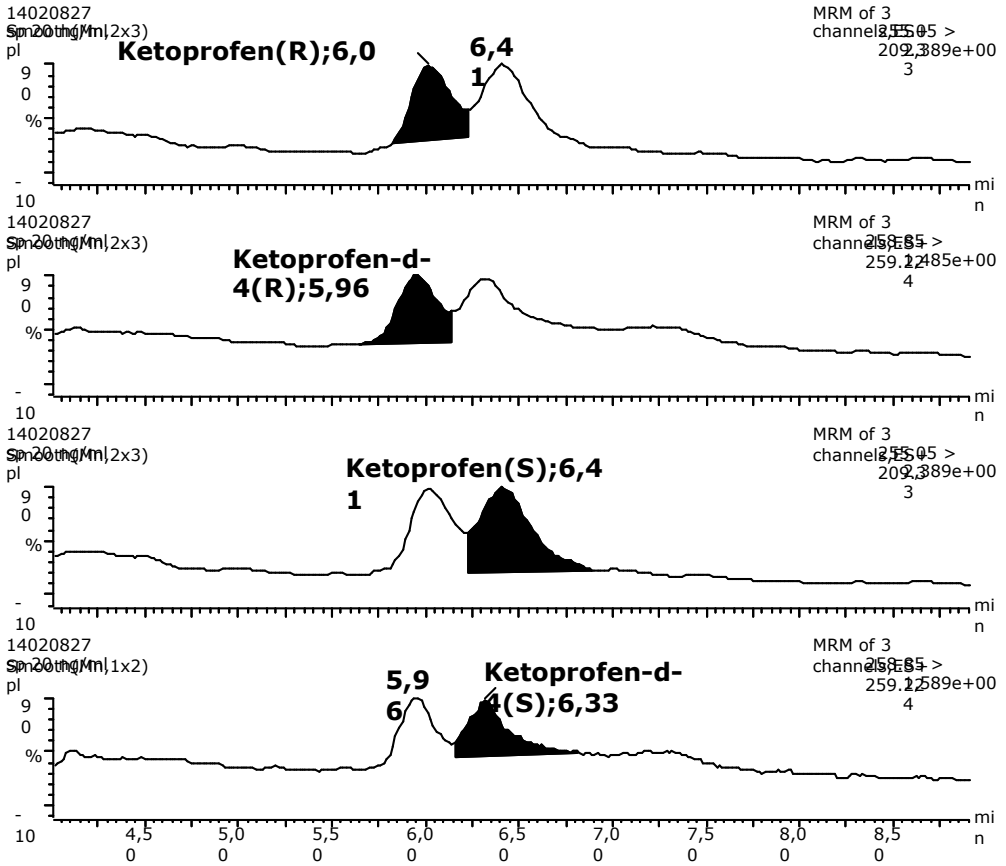


**Figure 1.** Ion chromatogram of a blank plasma sample fortified at 20 ng/ml with (±)-ketoprofen. (±)-Ketoprofen –d4 +was used as an internal standard at 50 ng/ml of plasma.

**Conclusions**

A simple LC-MS/MS method has been developed to determine (R)- and (S)-ketoprofen in porcine plasma. The method can be used in pharmacokinetic study and also in residue control.

The sample preparation is rapid and requires minimum amounts of solvents. Some other chiral columns will be further evaluated to enable baseline resolution.

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## P 23

# VALIDATION OF A MULTIRESIDUE METHOD FOR UPLC-MS/MS ANALYSIS OF ANTIBIOTICS IN POULTRY MUSCLE

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**Abstract**
A simple method for the screening and determination of five families of antibiotics in poultry muscle has been developed. It is specific for 39 analytes: fourteen sulfonamides, four tetracyclines, four macrolides, nine quinolones, seven penicillins and trimethoprim. Sample treatment comprises extraction with methanol-water-EDTA followed by centrifugation, dilution and filtration of the extracts. The analytes and five internal standards elute by UPLC within approximately 13 minutes. Detection of the analytes by tandem mass spectrometry ensures sensitivity and selectivity. The method has been proven to be useful for screening as well as for quantification and confirmation of analytes. It has been validated according to European Union Decision 2002/657/EC.

**Introduction**
A large number of antibiotics are currently used in veterinary practices. Food products from medicated animals can contain drug residues which can be determinental to human health. To protect consumers and ensure food safety, the European Union has set maximum residue limits (MRLs) for several veterinary drugs in different food matrices—including antibiotics—and has established programmes for monitoring veterinary drug residues.
Public health laboratories must analyse numerous food samples of animal origin. Screening methods are often used to initially monitor antibiotics. Among the most common of these are microbiological assays, which are simple and cheap, but non-specific. Thus, when growth inhibition is detected, a more selective screening method is usually applied before proceeding to confirmation analysis, which is usually performed by LC-MS. However, recent advances in LC-MS have enabled researchers to use this technique both for confirmation analysis of suspected non-compliant samples as well as for general screening. UPLC, which allows the separation of numerous compounds in a short time, offers particularly high throughput and can be an excellent tool in residue analysis.
Herein is proposed a method for analysing five families of antibiotics in poultry muscle samples. It combines an effective extraction technique, previously described by Granelli and Branzell, with UPLC-MS/MS, thereby allowing both confirmation and quantification in a single chromatographic run. The multiresidue method has been validated in poultry muscle matrix according to European Union Decision 2002/657/EC.

## Materials and methods

*Stock solutions and samples*
1000 mg L-1 stock standard solutions of each analyte were prepared by dissolving the antibiotics in an appropriate solvent (ACN, MeOH, CH3Cl, H2O or 0.1 M NaOH). The antibiotics included in the study are listed in Table 1. Another 20 mg L-1 intermediate solution was prepared for the five internal standards (IS): sulfamethazine-C13 (sulfonamides and trimethoprim), norfloxacin-d5 (quinolones), roxitromycin (macrolides), piperacillin (penicillins) and demeclocycline (tetracyclines). Working solutions were prepared by aqueous dilution. All solutions were stored at -20 ºC.
Poultry muscle samples for validation studies were obtained from previously analysed compliant samples. They were minced, stored at –20ºC and thawed before analysis.

*Sample treatment*
3 g of minced sample, previously spiked with the appropria
te amount of IS (and analytes, if required), were extracted with 200 µL of 0.1 M EDTA and 10 mL MeOH:H2O (70:30). After vortex shaking for 30 seconds, the samples were extracted for 15 minutes by mechanical shaking and subsequently centrifuged for 5 minutes at 3,800 rpm. Finally, the extract was diluted four times with water, filtered, and injected (10 µL) into the UPLC system.

*LC-MS/MS conditions*
LC-ESI-MS/MS measurements were performed on a Waters Acquity UPLC system (Chicago, IL, USA) coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters) using an electrospray source. The column used was a Waters Acquity UPLC BEH C18 (100 mm x 2.1 mm; 1.7 µm particle diameter).
Separation was performed at 40ºC in gradient elution mode at a flow rate of 0.3 mL min-1. Mobile phase A was an aqueous solution containing 0.2% formic acid and 1 mM oxalic acid. Mobile phase B was 0.1% formic acid in ACN. The following elution program was applied: (time in min, % mobile phase A): (0, 95), (5, 75), (8.5, 10), (9.5, 10) (10, 95). The column was reequilibrated for 3 min before each run.
The electrospray ionisation source was operated in the positive mode. Data acquisition for quantification and confirmation was performed in the multiple reaction monitoring (MRM) mode, splitting TIC acquisition into six windows. Two transitions were followed for each analyte (quantification and confirmation) and one for each IS. Identification was carried out by retention time and confirmation was performed using ion ratio criteria (2002/657/EC). Calibration curves were obtained from spiked blank samples that were processed the same way as the samples. Quantification was performed using the internal standard method, by adding the IS's to all samples (including calibration samples) before extraction. Thus, no correction for extraction recovery was required.

## Results and discussion
Analytes were extracted from poultry muscle samples using a method described by Granelli and Branzell, with slight modifications. It includes simple extraction with methanol-water (70:30) containing EDTA. Absolute recoveries were determined at the MRLs and were satisfactory for

the entire set of analytes: tetracyclines, 60 to 70%; penicillins, 70 to 83%; sulfonamides 73 to 93%; quinolones 74 to 86%; macrolides 87 to 96%; and trimethoprim, 70%.
The MS conditions were adjusted to optimise the signal of the precursor ion (M+H+) and the two most intense product masses for each analyte. The selected transitions are summarised in Table 1.
Owing to the optimised gradient program, all the analytes and IS's eluted within only 13 min. Moreover, monitoring of the two transitions per compound was possible with just one chromatographic run.
Once the whole analytical method was optimised, the method was validated against EU Decision 2002/657/EC.
Linearity was tested in the range from 1/8 MRL to 3/2 MRL (or from 10 μg $Kg^{-1}$ to 75 μg $Kg^{-1}$, for norfloxacine and sarafloxacine) with five points curves. The value of r was higher than 0.99 in all cases, while residuals were lower than 25% for the first calibration point, and lower than 15% for the others.
Accuracy and precision (repeatability and within-laboratory reproducibility) were determined at 1/2 MRL, MRL and 3/2 MRL, except for norfloxacin and sarafloxacin, which have no MRL. These compounds were instead checked at levels of 25, 50 and 75 μg $Kg^{-1}$. For each concentration level 18 muscle samples were processed in three different days. The relative standard deviation (RSD) values obtained were below those calculated by the Horwitz equation. Data corresponding to the MRL level are given in Table 1.
For compounds having an MRL, CCα was calculated according to the following equation: CCα = MRL + 1.64(SD); whereby SD = the standard deviation from 18 samples spiked at MRL level. For those compounds having an MRL for a group of substances (sulfonamides as well as ciprofloxacin and enrofloxacin), the MRL was taken individually for each compound. For sarafloxacin and norfloxacin the calibration curve method was used, and CCα was calculated as the concentration at the y-intercept plus 2.33(SD). CCβ was calculated as CCβ = CCα + 1.64(SD).

The method was then applied to the analysis of quinolones in poultry samples corresponding to a collaborative exercice organised by FAPAS, and the results were satisfactory. Enrofloxacin was found at 61.4 μg $Kg^{-1}$ (mean value of the participants = 72.9 μg $Kg^{-1}$; σ = 16.04) and sarafloxacin was found at 109.1 μg $Kg^{-1}$ (mean value of the participants = 123 μg $Kg^{-1}$; σ = 27.0). Finally the applicability of the method to different matrices was explored. The results obtained in the analysis of muscle samples from other species, or from different tissue samples are promising. For instance, the method was successfully applied to the analysis of a pig kidney sample provided by FAPAS: sulfadiazine was found at 48.9 μg $Kg^{-1}$ (mean value of the participants = 37.9 μg $Kg^{-1}$; σ = 9.51) and sulfamethazine was found at 49.6 μg $Kg^{-1}$ (mean value of the participants = 43.2 μg $Kg^{-1}$; σ = 8.34).

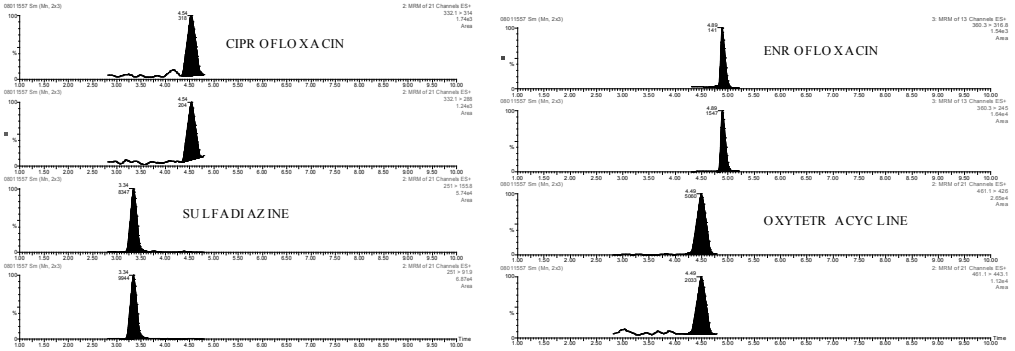


**Figure 1.** Chromatogram of a real poultry sample containing sulfadiazine (325 μg $Kg^{-1}$), oxytetracycline (107 μg $Kg^{-1}$ ), ciprofloxacin (96 μg $Kg^{-1}$) and enrofloxacin (746 μg $Kg^{-1}$ )

**Table 1.** Validation results for the 39 analytes

| **Analyte** | **r.t. min** | **Quantification, confirmation, transition*** | **Precision (MRL) %RSD** | **Accuracy (MRL), %** | **CCα μg $Kg^{-1}$** | **CCβ μg $Kg^{-1}$** |
|---|---|---|---|---|---|---|
| Sulfadimethoxine | 6.95 | 311.0, 155.8, 91.8 | 3.8 | 104.4 | 106.4 | 112.8 |
| Sulfapyridine | 3.97 | 250.0, 155.9. 107.9 | 7.0 | 98.1 | 111.5 | 123.0 |
| Sulfamethoxazole | 6.21 | 254.0, 156.0, 92.1 | 8.5 | 97.7 | 113.6 | 127.2 |
| Sulfaquinoxaline | 6.98 | 301.0, 155.8, 91.8 | 6.9 | 98.7 | 111.5 | 123.0 |
| Sulfathiazole | 3.85 | 255.9, 155.8, 91.8 | 7.5 | 94.4 | 112.6 | 125.2 |
| Sulfamerazine | 4.20 | 265.0, 91.8, 155.8 | 6.7 | 98.7 | 111.5 | 123.0 |
| Sulfadiazine | 3.41 | 251.0, 155.8, 91.8 | 6.1 | 102.1 | 110.1 | 120.3 |
| Sulfamethazine | 4.85 | 279.1, 185.9, 91.9 | 5.6 | 98.4 | 109.1 | 118.3 |
| Sulfisoxazole | 6.52 | 268.0, 155.8, 91.8 | 8.1 | 99.5 | 113.2 | 126.4 |
| Sulfamethizole | 4.98 | 271.0, 156.0, 92.1 | 7.3 | 95.3 | 111.7 | 123.4 |
| Sulfadoxine | 6.18 | 311.0, 155.9, 91.9 | 7.8 | 97.0 | 112.7 | 125.5 |
| Sulfamonometoxine | 5.62 | 281.0, 92.1, 156.0 | 5.3 | 99.8 | 108.7 | 117.4 |
| Sulfamethoxipyridazin | 5.04 | 280.9, 91.9, 155.9 | 5.7 | 99.5 | 109.7 | 119.4 |
| Sulfachloropyridazine | 5.79 | 284.9, 155.8, 91.8 | 6.1 | 99.7 | 110.2 | 120.4 |
| Trimethoprim | 4.30 | 290.9, 230.0, 122.9 | 8.1 | 107.5 | 56.9 | 63.8 |
| Amoxicillin | 2.61 | 366.2, 114.0, 349.1 | 7.0 | 100.8 | 55.8 | 61.6 |
| Ampicillin | 4.32 | 350.2, 106.0, 160.0 | 7.3 | 99.9 | 55.8 | 61.6 |
| Penicillin G | 7.28 | 335.1, 160.0, 176.1 | 7.1 | 97.8 | 55.9 | 61.7 |
| Penicillin V | 7.53 | 351.1, 160.1, 114.0 | 5.8 | 96.6 | 27.3 | 29.6 |
| Oxacillin | 7.66 | 402.1, 160.0, 243.1 | 2.9 | 97.0 | 313.1 | 326.2 |
| Cloxacillin | 7.84 | 436.0, 160.0, 277.1 | 3.8 | 96.6 | 314.0 | 327.9 |
| Dicloxacillin | 7.83 | 470.0, 160.0, 311.0 | 2.7 | 98.1 | 318.6 | 337.2 |
| Ciprofloxacin | 4.62 | 332.1, 288.0, 314.0 | 5.2 | 104.1 | 108.9 | 117.7 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Enrofloxacin | 4.98 | 360.3, 245.0, 316.8 | 5.5 | 102.4 | 109.3 | 118.6 |
| Marbofloxacin | 4.28 | 363.1, 71.9, 129.1 | 4.7 | 99.9 | 161.8 | 173.6 |
| Danofloxacin | 4.85 | 358.0, 96.0, 314.0 | 4.7 | 99.9 | 215.5 | 231.1 |
| Difloxacin | 5.51 | 399.9, 356.0, 299.0 | 2.6 | 107.1 | 316.7 | 333.5 |
| Flumequine | 7.48 | 262.1, 244.0, 201.9 | 5.3 | 102.1 | 217.8 | 235.5 |
| Oxolinic Acid | 6.69 | 262.0, 244.0, 215.9 | 7.5 | 104.3 | 113.0 | 126.1 |
| Sarafloxacin | 5.44 | 386.0, 299.0, 342.0 | 7.3 | 106.5 | 9.9 | 13.4 |
| Norfloxacin | 4.47 | 320.0, 276.1, 233.0 | 6.1 | 103.3 | 4.3 | 6.9 |
| Oxitetracycline | 4.54 | 461.1, 426.0, 443.1 | 5.6 | 106.4 | 109.2 | 118.4 |
| Tetracycline | 4.92 | 445.1, 410.1, 153.9 | 5.6 | 101.8 | 109.2 | 118.4 |
| Doxicycline | 6.08 | 479.0, 444.0, 153.9 | 5.2 | 106.8 | 108.6 | 117.2 |
| Chlortetracycline | 6.42 | 445.1, 428.1, 410.1 | 3.6 | 100.5 | 106.0 | 112.0 |
| Tylosin | 7.02 | 916.5, 174.0, 772.3 | 5.1 | 106.1 | 108.5 | 117.0 |
| Spiramycin | 5.98 | 422.2, 174.1, 101.0 | 6.5 | 103.8 | 220.8 | 241.6 |
| Eritromycin | 6.92 | 734.4, 158.1, 576.3 | 2.7 | 96.9 | 209.0 | 218.0 |
| Josamycin | 7.38 | 828.5, 109.0, 174.1 | 5.8 | 99.7 | 219.5 | 239.0 |

** Transitions are expressed as: precursor ion, product ion 1 (quantification transition), product ion 2 (confirmation transition).*

**Conclusions**

The proposed method has been shown to be amenable to screening as well as to the confirmation and quantification of antiobiotics residues in animal muscle samples, and meets the requirements of 2002/657/EC. It is rapid, as the samples require very little manipulation, and the extracts do not require clean-up prior to UPLC-MS/MS. This helps to shorten the total analysis time per sample, but regular equipment maintenance must be performed. The method has been successfully used for interlaboratory samples and has been established as a routine method in the laboratory of the Barcelona Public Health Agency (Agencia de Salut Pública de Barcelona; ASPB). It is used instead of the five-plates microbiological screening test. It has replaced the five-plates microbiological screening test.

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## P 24

# ION SUPPRESSION STUDY FOR TETRACYCLINES IN FEED

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**Abstract**
LC-MS/MS is widely used for quantitative analysis in the field of residue analysis because of its sensitivity and selectivity. However, ion suppression can be a major drawback of this technique. This paper describes the study of ion suppression in the analysis of tetracyclines in feed. Several parameters have been studied, such as extraction and sample-pretreatment. Two different strategies were used: influence of matrix on a continuous analyte signal and comparison of peak areas between mobile-phase standards and matrix-matched recovery standards. Differences were observed between different feed samples analysed and influence of all parameters was apparent. This is a clear indication of ion suppresion.

**Introduction**
The use of antibiotics in animal husbandry is regulated, as their presence in food coming from these animals can lead to resistance of bacteria to antibiotics. Therefore, the European Union has developed regulation concerning this issue (EU, 2004, EU, 2003). Analysis and control of antibiotics in feedstuffs for animals has become an important issue as only autorised feedstuffs can be medicated under specific conditions as stated in Council Directive 90/167/EEC (EU, 1990). However, the analysis of antibiotics in animal feed has proved to be quite a challenge because the composition of animal feeds is very variable. This considerable variation is introduced by the animal feed industry. Numerous raw materials and additives are added into the feeds, including grains, seeds, beans, rice and soy. These raw materials and additives introduce many interfering components, such as oils, fats, proteins and salts, which can occur at very high levels. This influences the efficiency of sample preparation and causes differences in recovery and ion suppression on LC-MS/MS. Ion suppression can be defined as a change in the efficiency of droplet formation or evaporation in the mass spectrometer source, caused mainly by interfering matrix compounds. The changeable composition of each individual feed leads to variable analysis results for each feed. Presently, feeds are analysed using the standard addition approach. This ensures a correct quantification of each individual feed. Unfortunately this quantification tool is very time consuming and cumbersome, resulting in very few feeds being analysed per day.
More knowledge on the removal of matrix interferences is needed. Tetracyclines were chosen as a model group to perform the experiments. Strategies reported in the literature were used to study the effect of matrix components on actual feed samples (Annesley, 2003, Antignac et al., 2005). This should lead not only to better and more efficient sample preparation, but also to more uniform feed extracts. These feed extracts could then be quantified by external

calibration. This would increase the number of feeds analysed at the same time.
The aim of this work is to investigate some strategies mentioned in the literature to evaluate ion suppression for several different sample treatment procedures for tetracycline analysis in feed and compare them.

## Materials and methods

*Samples*
Five different feed samples were analysed (1x premix sample, 2x dry feed samples and 2x wet feed samples).

**Table 1.** Sample pretreatment methods tested for the clean-up of tetracyclines from animal feeds.

| | Method | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| Extraction | McIlvaine Buffer-EDTA 0,1 M | McIlvaine Buffer-EDTA 0,1 M | McIlvaine Buffer-EDTA 0,1 M | DCM:McIlvaine Buffer-EDTA 0,1 M (1:3) |
| pH | 4 | 2.5 | 10 | 4 |
| SPE cartridge | Oasis® HLB | Oasis® MCX | Oasis® MAX | Oasis® HLB |
| Wash | $H_2O$ | 1. formic acid 2% 2. MeOH | 1. $NH_3$ 2. MeOH | $H_2O$ |
| Elution | MeOH | MeOH:$NH_3$ (95:5) | MeOH:Formic acid (95:5) | MeOH |
| | Evaporation with $N_2$ | Dilution with acetic acid (10%) | Dilution with $H_2O$ | Evaporation with $N_2$ |

*Sample preparation procedures*
The amphoteric properties of tetracyclines (oxytetracycline, OTC, tetracycline, TC, chlortetracycline, CTC, and doxycycline, DC) were used to develop different SPE strategies for sample clean-up. Below their first pKa value (around 3.2) they are positively charged. Between pKa1 and pKa2 they are neutral (zwitterionic state) and above pKa2 (around 7.7) they are negatively charged. Four different sample treatment procedures were tested in order to compare their effect on the ion suppression study (see Table 1). The effect of dichloromethane (DCM) was tested as recent research has suggested that DCM might assist with the removal of some apolar matrix compounds and thus might help to obtain cleaner extracts. An internal standard (demeclocycline) was added to all samples.

*LC-MS/MS Conditions*
The LC system was a Waters 2690 separations module LC system coupled to a triple quadrupole Quattro Ultima mass spectrometer with electrospray ionization source operated in positive mode. Cone voltage and collision energy were optimized for every analyte. The precursor/product ions monitored are listed in Table 2. The system was equiped with a Symmetry C18 (150 mm x 3 mm, 5 µm) operated at 10°C. A binary gradient mobile phase

was used at a flow rate of 0.4 ml min−1 with solvent A (ammonium acetate 1 mM, pH 2.6) and solvent B (ammonium acetate 10 mM:ACN, 10:90). The flow was split 1:1 before entrance into the MS.

**Table 2.** LC-MS/MS precursor/product ion combinations (quantifier underlined) monitored in MRM ESI positive mode.

| **Tetracycline** | **Precursor ion (m/z)** | **Product ions (m/z)** |
|---|---|---|
| TC | 445.2 | 410.1, 154.1 |
| CTC | 479.1 | 444.1, 154.1 |
| DC | 445.2 | 428.1, 154.1 |
| OTC | 461.2 | 337.1, 201.1 |
| DMC | 465.1 | 154.1 |

*Qualitative ion suppression recording*

A blank feed sample extract was injected during continuous post-column infusion of a 5 mg/L tetracyclines solution. The standard solution infusion provided a stable baseline. The signal intensity of the baseline decreased when matrix components causing ion suppression eluted, but when substances eluted that enhanced ionisation the signal increased. Matrix elution profiles were compared to the baseline signal, which was obtained by the injection of mobile phase instead of a sample extract.

*Quantitative ion suppression calculation*

Standard solutions of tetracyclines and matrix matched recovery standards (MMRS) of the same concentration (1 mg/L) were injected on the LC-MS/MS system. Ion suppression or enhancement for each analyte was determined by dividing the peak area in the MMRS with the peak area obtained in a standard solution at the same concentration level. Values lower than 100% were an indicative of ion suppression and values higher than 100% indicated ion enhancement. The ratio between the area of the analyte and the internal standard in MMRS from the five studied samples was used to quantify the variation (RSD %) in ion suppression.

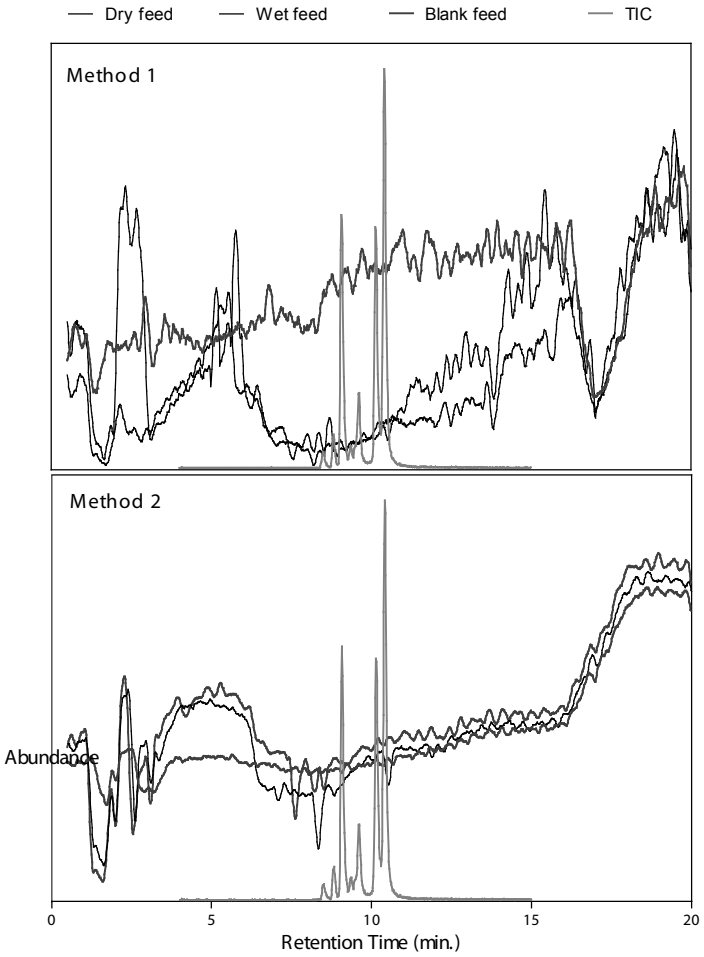


**Figure 1.** Ion suppression profiles, obtained with sample preparation method 1 (upper panel) and method 2 (lower panel), of a blank, a dry feed sample and a wet feed sample for tetracycline.

## Results and discussion

*Ion suppression profiles*

Ion suppression profiles of blank extracts obtained for four sample pre-treatment methods (see Table 1) were recorded. The infusion profiles were studied separately for each analyte and compared. Figure 1 (upper panel) shows the profiles monitored for TC of a blank, a wet feed sample and a dry feed sample cleaned up with method 1. The lower panel of Figure 1 shows similar profiles of feed samples but these were processed with method 2.

Tetracycline eluted at 10.5 minutes. From the infusion profiles (Figure 1) it could be concluded that ion suppression occurred. All analytes showed a similar suppression tendency for extracts obtained with sample treatment method 1 and 4. No differences were observed

between the five feeds. However the infusion profiles of methods 2 and 3 did not differ substantially from the infusion profile obtained with the blank (see Figure 1. No differences were observed between the five feeds. This implies that methods 2 and 3 do a better job at cleaning up samples and removing matrix components. Furthermore the addition of DCM to the extraction solvent proved to be unsuccesful.

*Ion suppression quantification*
Samples were spiked with tetracyclines at 1 mg/kg and extracted according to Table 1. The quantification results overall agreed with the qualitative results (infusion profiles). The results of method 1 showed clear suppression (16-46%), as well as for method 4 (8-51%). Methods 2 and 3 showed less suppression with values 83-124% for method 2 and 92-129% for method 3. Oasis® MCX and MAX cartridges are in general more selective than HLB, which usually leads to cleaner extracts. However, dilution of the final extracts instead of evaporation and reconstitution also plays an important role, as less amount of sample matrix is introduced into the system.

*Variation between samples*
Samples were spiked with tetracyclines at 1 mg/kg and extracted according to Table 1 and the variation between feed samples was determined for every analyte and method. Results are shown in Table 3.

**Table 3.** Variation (RSD, %) between five feed samples processed with four different sample pre-treatment methods.

| | **Method** | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| | **RSD (%)** | **RSD (%)** | **RSD (%)** | **RSD (%)** |
| TC | 12.3 | 11.1 | 2.6 | 19.4 |
| CTC | 17.9 | 12.3 | 21.1 | 25.8 |
| DC | 30.8 | 4.9 | 6.5 | 39.7 |
| OTC | 14.7 | 9.4 | 8.6 | 24.3 |

On a whole, methods 2 and 3 reduced ion suppression effects except for CTC (method 3). Method 3 gave very low SPE recoveries for CTC (around 30%). This indicated that degradation of this analyte occurred, probably due to the high pH required for SPE. The other SPE procedures showed acceptable recoveries for all four analytes.

**Conclusions**
Ion suppression in LC-MS/MS analysis of tetracyclines in feed was studied. Four sample pre-treatment methods were tested with five different feed samples in terms of ion suppression profiles, ion suppression quantification and variation. The method that seemed to provide least ion suppression was method 2, which involved SPE with Oasis® MCX cartridges. Unfortunately, these results are not sufficient to replace the current protocol (method 1) which relies heavily on standard addition. More research should be done, including studies with a larger number of different feed samples.

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## P 25

# TETRACYCLINES IN MILK MONITORING: THE RESULTS OF A INTER-LABORATORY STUDY

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**Abstract**
This proficiency test was focused on screening step in the strategy of control of antibiotic residues in milk. The inter-laboratory study was carried out in cooperation with the Laboratorio Standard Latte of the Italian Breeders Association (AIA-LSL). 53 laboratories took part in this study. The target of the study was to provide the data for a comparative evaluation of KALIDOS TB , a new microbial inhibition assay, developed for the detection of Tetracyclines at MRL (Maximum Residual Limit) according to 2377/90/CE. Six standardized milk samples were sent to all labs as unknown and tested with KALIDOS TB in comparison with the kit currently in use in the laboratory (Delvotest SP-NT in 33 laboratories, Copan Milk Test in 17 labs, and 3 of them not declared). The Six lyophilised milk samples were prepared by the AIA-LSL: Oxytetracycline spiked sample (with ½ MRL) Oxytetracycline spiked sample (with MRL: 100 ng/ml), Oxytetracycline spiked sample (with 2 MRL), negative sample, Penicillin-G spiked sample (with MRL: 4ng/ml) and Sulfadiazine spiked sample (with MRL: 100ng/ml). The results suggest a good correlation between the different kits and demonstrate that KALIDOS TB is an useful tool to detect Tetracyclines according to the 2377/90/CE.

**Introduction**
Antibiotics are widely used in dairy breeding for the treatment of mastitis and other infectious diseases. Any lack in the observance of the withdrawal times designed for such drugs, may cause the presence of same residues in milk. In order to prevent problems for human health of hypersensitivity, allergic reactions and antibiotic resistance development, a serious control is required in accordance with the Directive 96/23/EC for monitoring plans. In order to fulfil the requirements of the regulation EEC 2377/90 laying down maximum residue limits (MRL) of veterinary drugs in foodstuff of animal origin, a suitable analysis flow chart has to be properly set up and validated according to the Commission Decision 2002/657/EC. The non-compliant samples at the screening step, need a confirmation which has to be performed by a specific quantitative method. A reliable screening step is required in milk analysis process; screening methods should have a detection limit in accordance to MRL. Regarding the control of antibiotic residues in milk, the microbial inhibition assays are widely used in the screening analysis due to their capability to detect a wide range of antimicrobial substances including beta-lactams and sulphonamides, although they may not detect some antimicrobials at or below the MRL concentration (e. g. Tetracyclines as described from Le Breton et al. 2007) . There are several commercially available kits, based on growth inhibition of Bacillus stearothermophilus, a thermophilic bacterium sensitive to many antibiotics. The spora of Bacillus stearothermophilus are included in a proper agar medium containing a pH indicator.

In the absence of inhibitory substances, when heated, the spores germinate, grow, produce carbonic acid and the medium colour changes. If the milk sample contain some inhibitory substances, the colour remains unchanged. This paper describes an inter-laboratory study set up to provide the preliminary data of a first evaluation of a new microbial inhibition assay, KALIDOS TB, for the detection of antibiotics and sulphonamides in milk especially designed for the detection of Tetracyclines at MRL according to 2377/90/CE.

**Materials and Methods**

The inter-laboratory study was carry out according to internationally recognised guidelines (Thompson et al. 1993) and in cooperation with the Laboratorio Standard Latte of the Italian Breeders Association (AIA-LSL). 53 AIA laboratories were involved.

*Test Materials*

6 milk samples were prepared by the Laboratorio Standard Latte of AIA. The six samples, supplied to the AIA labs as unknown, were the following: lyophilised Oxytetracycline positive milk sample (spiked with ½ MRL), lyophilised Oxytetracycline positive milk sample (spiked with the MRL: 100ng/ml), lyophilised Oxytetracycline positive milk sample (spiked with 2 fold the MRL), lyophilised negative milk sample (blank), lyophilised Penicillin-G positive milk sample (spiked with the MRL: 4ng/ml) and lyophilised Sulfadiazine positive milk sample (spiked with the MRL: 100ng/ml ). Oxytetracycline, Penicillin-G sodium salt and Sulfadiazine were purchased from Sigma (St. Louis, MO, USA).

*Instructions to the participants*

The 53 AIA laboratories involved in the study, were asked to analyse the samples with Kalidos TB (Euroclone Spa, Milano, Italy) in comparison with the validated analytical methods currently in use: Delvotest SP-NT (DSM Food specialities R&D, Delft, The Netherlands) in 33 laboratories, Copan Milk Test (Copan Italia Spa, Brescia , Italy) in 17 labs, and 3 not declared. All the tests were performed in accordance with the instructions of the producer. In this study only the ampoules were tested not the microplates. The laboratories were asked to give the results as compliant if the colour changes, non-compliant if the colour remains unchanged and suspected to be non-compliant when the colour starts to turn.

**Results**

The results of the study are summarized in the tables below:

**Table1.** Negative samples

| Methods | % of Compliant | % of Suspected of Non-Compliant | % of Non-Compliant |
|---|---|---|---|
| Ref.methods | 98 | 0 | 2 |
| KALIDOS TB | 94 | 0 | 6 |

**Table2.** Penicillin-G with MRL

| Methods | % of Compliant | % of Suspected of Non-Compliant | % of Non-Compliant |
|---|---|---|---|
| Ref.methods | 2 | 0 | 98 |
| KALIDOS TB | 2 | 0 | 98 |

**Table3.** Sulfadiazine with MRL

| Methods | % of Compliant | % of Suspected of Non-Compliant | % of Non-Compliant |
|---|---|---|---|
| Ref.methods | 32 | 2 | 66 |
| KALIDOS TB | 26 | 4 | 70 |

**Table4.** Oxytetracycline with ½ MRL

| Methods | % of Compliant | % of Suspected of Non-Compliant | % of Non-Compliant |
|---|---|---|---|
| Ref.methods | 98 | 0 | 2 |
| KALIDOS TB | 86 | 4 | 10 |

**Table5.** Oxytetracycline with MRL

| Methods | % of Compliant | % of Suspected of Non-Compliant | % of Non-Compliant |
|---|---|---|---|
| Ref.methods | 94 | 0 | 6 |
| KALIDOS TB | 62 | 4 | 34 |

**Table6.** Oxytetracycline with 2 MRL

| Methods | % of Compliant | % of Suspected of Non-Compliant | % of Non-Compliant |
|---|---|---|---|
| Ref.methods | 44 | 10 | 46 |
| KALIDOS TB | 20 | 2 | 78 |

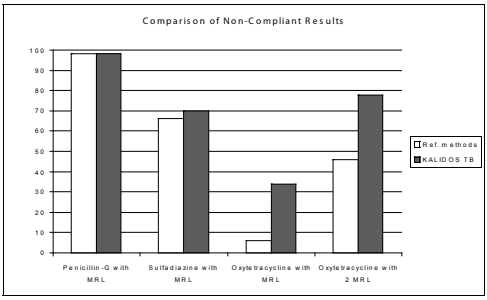


**Figure 1:** a comparison of Non-Compliant results is represented in the graphic above

**Discussion and Conclusions**

The data obtained, suggest a good correlation between methods in use in AIA laboratories and the new KALIDOS TB in detecting Penicillin-G and Sulfadiazine at MRL. KALIDOS TB definitely shows different performances for Tetracyclines detection compared to the other methods. Therefore, it could be considered a more reliable method to reduce the amount of false Compliant results in Tetracyclines milk monitoring screening step.

**Acknowledgements**

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## P 27

# DETERMINAITON OF LINCOSAMIDES IN MILK BY LC-MS/MS

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**Abstract**
A simple LC-MS/MS method has been developed for the indentification and quantification of lincosamide antibiotics lincomycin, clindamycin and pirlimycin in bovine milk. The sample preparation consisted of an acidification with phosphoric acid and a defattening step by centrifugation. The further purification was made on reversed phase cartredges eluted with acetonitrile. The final extracts were analysed by reversed phase liquid chromatography-electrospray tandem mass spectrometry using positive mode iorisation. The method was validated following the European Commission Decision 2002/657/EC.

**Introduction**
Lincomycin, pirlimycin and clindamycin belong to the group of lincosamide antibiotics. Lincomycin is synthetized by actinobacterium Streptomycin lincolnensis. Clindamycin is a semisynthetic derivative of lincomycin and pirlimycin is an analog of clindamycin. They are used for bacterial infections in both humans and animals. Pirlimycin is used to treat mastitis in cows whereas the appearance of lincomycin and clindamycin in milk is more unlikely. Clindamycin is mainly used in therapy for cats and dogs and could thus be considered as an internal standard for residue analysis of the other lincosamides.
There is not a lot of methodology available for the analysis of lincosamide residues, at least not many methods using LC-MS. Hornish et al 1995 and Heller 1996 have determined pirlimycin in milk by LC-MS. Sin et al 2004 analysed lincomycin in animal tissues and milk. A MRL of 150 µg/kg has been established for lincomycin in milk and a MRL of 100 µg/kg for pirlimycin. Clindamycin has no MRL. The development of the method described here had pirlimycin residues in milk as the main target, lincomycin and clindamycin are enclosed as related compounds with the intention to apply the method easily to tissues.

**Materials and Methods**

*Materials*
Organic solvents were of HPLC grade and other chemicals were of analytical grade (J.T. Baker, Mallinkrodt Baker, Deventer Holland). Water was purified via a Milli-Q system (Millipore, Bedford, MA, USA). Solid phase extraction (SPE) cartridges (Oasis® HLB 3cc, 60 mg) were obtained from Waters (Waters, Milford, MA, USA).
Lincomycin hydrochlorid and clindamycin hydrochlorid were from Fluka (Sigma-Aldrich, Steinheim, Germany). Pirlimycin hydrochlorid was obtained from Pfizer. Lincosamide stock solutions were prepared in methanol and diluted in water before analysis.

*Sample preparation*
Blank raw milk samples (5 g) were fortified with lincomycin and pirlimycin at a level of 0.5 MRL, 1 MRL and 1.5 MRL. Clindamycin was fortified at a level of 50, 100 and 150 µg/kg. The samples were mixed with phosphoric acid (5 ml, 4 %) and centrifuged at 3500 g for 10 min at 10 oC. The tubes were placed in -20 oC in an upright position for 10 min and the fat button on the supernatant surface was thereafter immediately removed with a spatula. The supernatant was without delay transferred to a clean tube avoiding the solid material on the tube walls.
The HLB cartredges were conditioned in vacuum with acetonitrile (5ml) and water (5 ml). The supernatant was passed through the cartredges followed by water (5 ml). The cartredges were further dried in vacuum and lincosamides were eluted with 100 % acetonitrile (2 x 0.5 ml). An aliquot of the eluent was diluted with water (1:5), filtered and analysed by LC-MS/MS.

*LC-MS/MS analysis*
Th LC-MS/MS system consisted of a Waters Alliance 2695 Separations Module (Waters, Milford, MA, USA) and a Micro Mass Quattro Micro tandem mass spectrometer with API source in the ESI mode (Waters, Milford, MA, USA).
The chromatographic separation was carried out using an AtlantisTM dC18 column (3 µm, 2.1x150 mm; Waters, Milford, MA, USA). The mobile phase was a mixture of water, acetonitrile and methanol (60:20:20), the flow rate was 0.2 ml/min and the injection volume 10 µl.
The mass spectrometric parameters were: capillary voltage 2.90 kV; source temperature 120 $^{o}$C; desolvation temperature 300 $^{o}$C; N2 cone gas flow 30 L/h, N2 solvation gas flow 700 L/h. Argon was used as collision gas. Ion transitions were monitored using positive mode and Multiple Reaction Monitoring (MRM) mode. The transitions and respective cone voltages and capillary energies are presented in Table 1.
The calibration was based on the external standard method. The calibration standards were prepared in extracts of blank milk handled as the samples ; five concentration points were used for the calibration curve.

*Validation*
The method was validated following the European Commission Decision 2002/657/EC. Six replicates of blank milk samples fortified at three levels around the MRL, including two blank samples, were analysed on three different days.

**Table 1.** Diagnostic ions and instrumental settings

| **Analyte** | **Precursor ion** | **Product ion** | **Cone voltage (V)** | **Collision energy V** |
|---|---|---|---|---|
| Lincomycin | 407 | 126a | 27 | 26 |
| | | 359 | 27 | 18 |
| Clindamycin | 425 | 126a | 27 | 26 |
| | | 377 | 27 | 19 |
| Pirlimycin | 411 | 112a | 25 | 26 |
| | | 363 | 25 | 17 |

[a] *Ion used for the quantification.*

**Results and Discussion**

A quantitative LC-MS/MS method is presented for the determination of lincomycin, clindamycin and pirlimycin in milk. A generic solid phase extraction method was chosen in order to expand the number of analytes in the future to other veterinary medicines. The lincosamides were extracted from SPE column with acetonitrile and diluted before the analysis with water to get a better peak shape and to decrease the amount of matrix impurities getting into the LC-MS. Calibration standards were made in blank milk extracts because the matrix had an enhancing effect on the MS response. The lincosamides are polar in nature and Atlantis dC18, recommended for polar compounds was chosen as the analytical column. Lincomycin had anyhow sometimes the tendency to elute as a double peak which seemed to depend on the way the column had been stabilized before the beginning of the sample set. The ion chromatograms can be seen in Figure 1. The intensity of the secondary product ion was much lower than the intensity of the quantification ion but high enough to be detected in reasonable concentrations.

The validation results are presented in Table 2. The decision limit CCα was determined to be 176 µg/kg for lincomycin and the detection capability CCβ 184 µg/kg. Pirlimycin got a CCα of 126 and a CCβ of 138 µg/kg. Clindamycin has no MRL and the lowest fortification level 50 µg/kg was used as the starting point resulting in a CCα of 71 and CCβ of 86 µg/kg. The recoveries lied for lincomycin between 75-77 %, for clindamycin between 106-114 % and for pirlimycin between 77-82 %. The limit of determination/limit of quantification were estimated to be 15/45µg/kg for lincomycin, 10/30 for clindamycin and 15/45 µg/kg for pirlimycin.

The method proved to be specific. Blank milk samples showed no intefearing peaks at the retention times of the lincosamides or showed peaks with very low intensities. Linear calibration plots were obtained for all analytes and the correlation coefficients were at least 0.99. The method fulfilled the demands presented in the Decision 2002/657/EC neither did The RSD % exceed the ones calculated with the Horwitz equation.

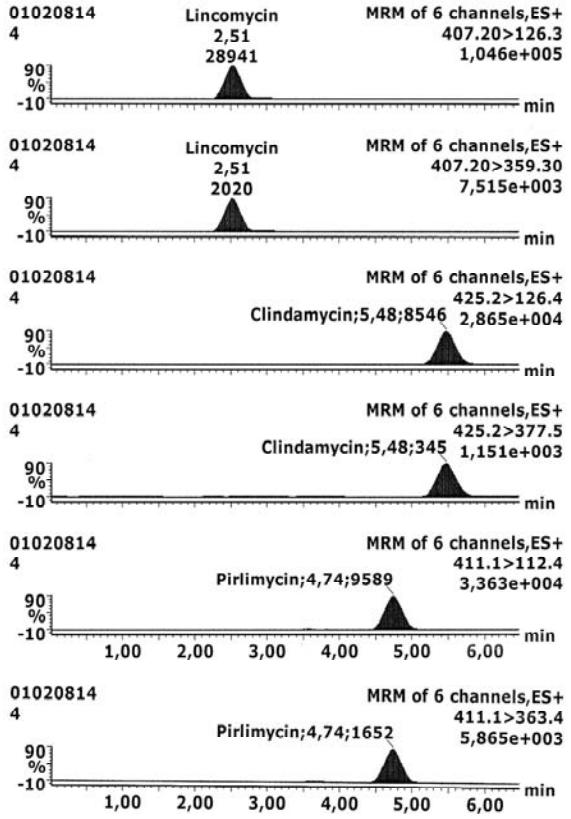


**Figure 1.** Ion chromatogram of a blank milk sample fortified at 50 µg/kg with pirlimycin and clindamycin and at 75 µg/kg with lincomycin.

**Table 2.** Validation results for fortified milk (n = 6)

| Analyte | Fortification µg/kg | Avarage recovery % | SD µg/kg | RSD % | CCα µg/kg | CCβ µg/kg | MRL µg/kg |
|---|---|---|---|---|---|---|---|
| Lincomycin | 75 | 77 | 5.1 | 8.9 | 176 | 184 | 150 |
| | 150 | 76 | 15.7 | 13.8 | | | |
| | 225 | 75 | 21.8 | 12.9 | | | |
| Clindamycin | 50 | 107 | 9.0 | 16.7 | 71 | 86 | - |
| | 100 | 114 | 23.6 | 20.7 | | | |
| | 150 | 106 | 28.2 | 17.7 | | | |
| Pirlimycin | 50 | 82 | 7.1 | 17.2 | 126 | 138 | 100 |
| | 100 | 79 | 15.8 | 20.0 | | | |
| | 150 | 77 | 20.7 | 18.0 | | | |

**Conclusions**

A simple and sensitive method has been developed to quantitate and confirm residues of pirlimycin, lincomycin and clindamycin in milk. The relative easy sample preparation enables a procession of at least 20 samples per day. Further work is going on to add more compounds in the method and to apply it to tissue matrix.

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## P 28

# VALIDATION OF AN ELISA SCREENING FOR QUINOLONES IN EGG AND POULTRY MUSCLE

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**Abstract**
Quinolones (QNs) are a group of chemotherapeutic agents with an excellent efficiency against poultry pathogens. Two commercial ELISA tests have been applied for the qualitative screening analysis of several quinolones (ciprofloxacin, danofloxacin, enrofloxacin, flumequine, norfloxacin and oxolinic acid) in egg and poultry muscle at levels below the European Union maximum residues limits (MRLs). During the validation study, carried out according to the Commission Decision 2002/657/EC criteria, two different sample treatments were compared: the simple and fast procedure suggested by the kit producer and a more complex SPE sample preparation. The results demonstrated that the method based on SPE clean up furnished better characteristic performances, particularly in egg matrix.

**Introduction**
The quinolones are a broad-spectrum antibacterials with an excellent efficiency against poultry pathogens. To ensure human food safety, the European Union (EU) has established maximum residue limits (MRLs) for certain food of animal origin, not including eggs. Thus, the development of sensitive multiresidue screening methods is important in order to easily verify the sample compliance respect to the required levels. A few papers describe relevant immunoassay screening tests and their field of application does not cover the range of analytes and matrices of interest. This work was carried out in order to evaluate the performances of enzyme-linked immuno assay (ELISA) for the determination of several quinolones (flumequine, ciprofloxacin, danofloxacin, enrofloxacin, norfloxacin and oxolinic acid) in eggs and poultry meat in accordance with the EU criteria for qualitative screening methods. Due to the different chemical structure between the flumequine and the 6-fluorinated piperazinyl-derivatives, the ELISA test was performed using two different kits, the first specific for flumequine and the second one generic for the fluoroquinolones. The rapid sample treatment procedure suggested by the ELISA kits producer was compared to a more complex solid phase extraction (SPE) clean up developed with the aim of reducing the matrix effect. In order to evaluate the performances of both sample treatments parallel validation studies were carried out.

## Materials and methods

*Solvents and reagents*
The chemical reagents used were analytical grade. Oasis HLB Speedisk columns (60 mg/3 mL) were supplied by Waters (Milford, MA, USA). Standard solutions were prepared with high-purity water obtained from a Milli-Q system (Millipore, Bedford, MA, USA). FLUOROQUINOLONES EIA (5101FLUQG1p) and FLUMEQUINE EIA (5101FLUM1p) kits were purchased from Euro-Diagnostica B.V. (Arnhem, The Netherlands).

*Standards*
Standard of oxolinic acid (OXO), flumequine (FLU), marbofloxacin (MAR) and difloxacin (DIF) were obtained from Riedel-de Haën (Seelze, Germany). Standard stock solutions were prepared by accurately dissolving approximately 10 mg of each QNs in 100 mL of 0.01 M nitric acid (DIF and MAR) or 0.01 M of aqueous NaOH (FLU and OXO) and stored at 4°C. Working standards at 0.1-1 µg mL-1 were freshly prepared in order to spike the blank samples.

*Sample preparation*
Two different sample preparation procedures (A and B) were carried out. Subsequently an aliquot of each extract (50 µL) was added in duplicate to the microtiter wells of both EIA assay (Figure 1). The EIA determinations were carried out following the manufacturer instructions.

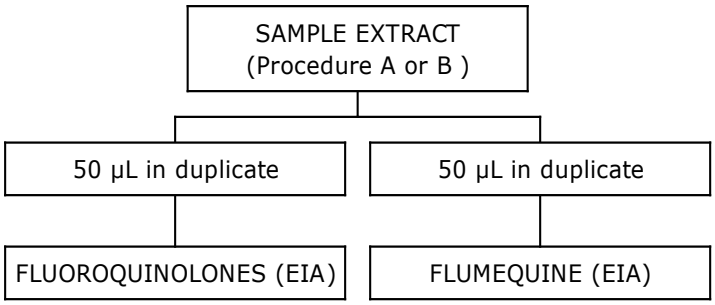


**Figure 1.** Analytical scheme

*A) Manufacturer procedure*
The sample treatment suggested by kit producer (Euro-Diagnostica) is briefly reported.
Egg samples. A 0.5 g of the homogenised whole egg was weighed in a glass vial. The sample was extracted with 1.5 mL of 40% methanol in sample dilution buffer provided by the ELISA kit and mixed for 30 min by shaker. After centrifugation (10 min, 2000 x g), an aliquot of 100 µL of supernatant was diluted with 400 µL of ELISA assay buffer.
Poultry muscle samples. Fat and connective tissues were removed from the muscle. A 1 g of minced muscle was weighed in a propylene tube. The sample was extracted with 3 mL of 80% methanol in sample dilution buffer provided by the ELISA kit and mixed for 15 min by shaker. After centrifugation (10 min, 2000 x g), 2 mL of the supernatant were evaporated at 50 ± 5°C under a nitrogen stream and dissolved in 1 mL of 8% methanol in sample dilution

buffer. After defatting with 1 mL of hexane and centrifugation (15 min, 2000 x g), an aliquot of 50 µl of the aqueous layer was diluted with 250 µL of 8% methanol in sample dilution buffer for Fluoroquinolones EIA; a second aliquot of 50 µL of the aqueous layer was diluted with 950 µL of 8% methanol in ELISA assay buffer.

*B) SPE Procedure*

An accurately weighed 1 g amount of the minced muscle or homogenized whole egg was placed in a 50 mL Falcon® tube. Extraction of QNs was achieved by introducing 4 mL of m-phosphoric acid 0.6% in water/MeOH 60/40 (v/v). The tube was mixed on a vortex mixer for 30 s and then vigorously shaken for about 10 min. After the centrifugation (3000 x g for 15 min), the supernatant was filtered in a 15 mL Falcon® tube and the extraction procedure was repeated with further 4 mL of extraction solution. The extracts (8 mL) were combined and an aliquot of four milliliters was evaporated under a nitrogen stream until the complete evaporation of MeOH (about 1.6 mL). Then the aqueous extract was loaded on the OASIS HLB cartridge previously conditioned with 2 mL of MeOH and 2 mL of Milli-Q water. Subsequently, the cartridge was washed with 5 mL of phosphoric acid solution (0.025 M, pH=3) and with 5 mL of water. The QNs were eluted with 4 mL of MeOH. The solvent was removed under a nitrogen stream and, just before application to the microtiter plates, the residue was dissolved in 4 mL of ELISA assay buffer for muscle and 2 mL for egg.

*Validation study*

The validation study was performed according to the Commission Decision 2002/657/EC criteria established for qualitative screening methods. For both sample preparation procedures (A and B), the extract of twenty (or more) blank and spiked samples (eggs and muscles) were analysed in parallel by the two ELISA kits (fluoroquinolones and flumequine). For eggs CCβ was evaluated analysing twenty blank samples spiked at 10 µg kg-1 of oxolinic acid and flumequine using both procedures. For muscles, instead, the twenty blank samples were spiked at 50 µg kg-1 of oxolinic acid and 200 µg kg-1 of flumequine for procedure A and at 10 µg kg-1 of oxolinic acid and flumequine using procedure B. During spiking experiments the choice of quinolonic compound and the concentrations were based on required levels. Moreover oxolinic acid was selected among the fluoroquinolones according to the cross-reactivity percentages indicated by the ELISA kit producer (Table 1) as it is the substance with the lowest response among those under investigation. The mean signal (B/B0 percent) of 20 blank samples minus 2.33 times the standard deviation (SD) corresponds to the signal (B/B0, %) at the decision limit (CCα). Subsequently this value is used as cut-off to determine the rate of false compliant results in the spiked samples. For B) procedure only, additional experiments were carried out in muscles spiked at ½ MRL with marbofloxacin (75 µg kg-1) and difloxacin (150 µg kg-1).

**Table 1.** Cross-reactivity patterns of antibodies of both ELISA tests

| ELISA test type | Compounds detected | Cross-reactivity (%) |
|---|---|---|
| FLUMEQUINE (5101FLUM1p) | Flumequine | 100 |
| FLUOROQUINOLONES (5101FLUQG1p) | Ciprofloxacin | 124 |
| | Norfloxacin | 100 |
| | Enrofloxacin | 92 |
| | Danofloxacin | 89 |
| | Oxolinic acid | 57 |
| | Marbofloxacin | 16 |
| | Difloxacin | 1 |

**Results and discussion**

In the present work two different sample treatments A) and B) were compared. The first (A) involves a sample extraction with a methanol/buffer mixture followed by defatting with hexane (for muscle only); the latter (B) was based on a sample extraction with a mixture of acidified methanol and a SPE clean up. The summarised validation results are reported in Tables 2 and 3. The specificity was tested analysing twenty different blank samples of each matrix and the obtained data were also used to determine whether the relevant spiked samples were suspect or compliant. No false compliant were obtained neither for eggs nor for muscles prepared using the OASIS clean-up (Table 3), since all the signals of spiked samples were lower than the cut-off. Consequently, the beta error rate was lower than 5% and the detection capabilities (CCβ) were below the fortification levels (Table 4). On the contrary, for flumequine in eggs the procedure A) lead to obtain about 20% rate of false compliant (Table 2) and the method is not suitable for purpose.

**Table 2.** Binding ratios (%) of blank and spiked samples (eggs and muscles) obtained applying the manufacturer procedure (A) followed by EIA tests

| | **Fluoroquinolones EIA** | | **Flumequine EIA** | |
|---|---|---|---|---|
| | **blank eggs** | **OXO 10 µg kg$^{-1}$** | **blank eggs** | **FLU 10 µg kg$^{-1}$** |
| Mean ($B/B_{0,}$ %) | 99 | 71 | 95 | 70 |
| SD ($B/B_{0,}$ %) | 5.1 | 7.6 | 9.6 | 3.1 |
| Signal at CCα ($B/B_{0,}$ %) | 87 | | 73 | |
| | **blank muscles** | **OXO 50 µg kg$^{-1}$** | **blank muscles** | **FLU 200 µg kg$^{-1}$** |
| Mean ($B/B_{0,}$ %) | 95 | 56 | 72 | 37 |
| SD ($B/B_{0,}$ %) | 7.3 | 5.5 | 7.9 | 3.9 |
| Signal at CCα ($B/B_{0,}$ %) | 78 | | 53 | |

**Table 3.** Binding ratios (%) of blank and spiked samples (eggs and muscles) obtained applying the SPE procedure (B) followed by EIA tests

| | Fluoroquinolones EIA | | Flumequine EIA | |
|---|---|---|---|---|
| | **blank eggs** | **OXO 10 µg kg$^{-1}$** | **blank eggs** | **FLU 10 µg kg$^{-1}$** |
| **Mean (B/B$_{0,}$ %)** | 82 | 29 | 58 | 18 |
| **SD (B/B$_{0,}$ %)** | 6.6 | 2.6 | 13 | 2.2 |
| **Signal at CCα (B/B$_{0,}$ %)** | 66 | | 27 | |
| | **blank muscles** | **OXO 10 µg kg$^{-1}$** | **blank muscles** | **FLU 10 µg kg$^{-1}$** |
| **Mean (B/B$_{0,}$ %)** | 90 | 41 | 72 | 25 |
| **SD (B/B$_{0,}$ %)** | 9.4 | 4.4 | 11 | 3.9 |
| **Signal at CCα (B/B$_{0,}$ %)** | 68 | | 48 | |

**Table 4.** Detection capabilities (CCβ) in muscle and egg matrix obtained with SPE procedure (B)

| **CCβ in muscle (µg kg$^{-1}$)** | **CCβ in egg (µg kg$^{-1}$)** | **Compound[a]** |
|---|---|---|
| <10 | <10 | ciprofloxacin[b], norfloxacin[b], enrofloxacin[b], danofloxacin[b], oxolinic acid[b], nadifloxacin, pefloxacin, piromidic acid, enoxacin, flumequine[b]. |
| <150 | Not tested | difloxacin[b] |
| <75 | Not tested | marbofloxacin[b] |

*a Compounds in bold have an established MRL value in muscle (Regulation 2377/90);*
*b Compounds which are determined by confirmatory method (HPLC-FLD)*

Finally, preliminary experiments carried out in poultry blank muscles and spiked with ½ MRL of marbofloxacin and difloxacin gave the results reported in Table 5. Then the EIA fluoroquinolones assay is able to detect at required level also these QNs which have a very low antibody cross-reactivities (16% MAR and 1% DIF).

**Table 5.** Binding ratio (%) of blank and spiked muscles with MAR and DIF obtained applying the B) procedure followed by Fluoroquinolones EIA test (n=3)a

| | Fluoroquinolones EIA | | | |
|---|---|---|---|---|
| | **blank muscles** | **MAR 75 µg kg$^{-1}$** | **blank muscles** | **DIF 150 µg kg$^{-1}$** |
| Mean (B/B$_{0,}$ %) | 82 | 18 | 87 | 17 |
| SD (B/B$_{0,}$ %) | 0.6 | 0.7 | 5.5 | 2.3 |

*a The set MRLs in muscle of various animal species is 150 µg kg-1 for MAR and 300 µg kg-1 for DIF*

**Conclusions**

The results of the present study demonstrate that the method suggested by the kit producer can be used for screening of flumequine in muscle and fluoroquinolones in eggs and muscle. The SPE clean up is necessary for flumequine analysis in eggs and moreover this sample treatment is able to detect lower QNs concentrations. Finally, despite the manufacturer procedure is more cheap and rapid than the preparation process using the OASIS clean up, the latter permits whatever a good sample throughput (about 40 samples per week per operator).

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## P 29

# DEPLETION OF CLORAMPHENICOL IN TROUTS AFTER A HYPOTHETIC THERAPEUTIC TREATMENT

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**Abstract**
The study was intended to evaluate the depletion of chloramphenicol in rainbow trouts (about 300-550 g body weight), after a ten days treatment with fish feedstuff containing chloramphenicol (CAP).
A total of 60 animals were separated in two groups: one was fed with CAP containing feedstuff in order to have a dosage of about 80 mg/kg/day, while a second group of fishes was fed with feedstuff not containing any CAP formulation (negative controls).
The treatment was maintained for 10 days. After this period, groups of 2 to 5 animals were sacrificed at different withdrawal times up to a maximum of 31 days.
Muscle tissues of each group of animals were then analysed for quantitative residual CAP determination both by ELISA and HPLC/MSMS.
The methods applied were in house validated according to the guidelines laid down by the European Decision 657/2002/EC.
Results and considerations are presented.

**Introduction**
Due to its broad antibiotic activity, chloramphenicol (CAP) was widely used in the veterinary therapeutic treatment of several animal species. Unfortunately CAP exhibits a significant toxicity correlated in particular with the risk of producing aplastic anemia [Feder et al; Fraudfelder at al]. Consequently the employment of CAP for veterinary practice has been forbidden within the European Union since 1994.
Nevertheless cases of food commodities containing CAP residues throughout European and extra European countries are still relatively common, as evidenced by EU reports of national monitoring plans even in the last recent years (% of not compliant samples < 0.5 %)
With respect to other species, in cultivated fish and shrimps CAP seems to be more often considered a useful antibiotic, due to its broad spectrum activity, ready availability and low costs. In literature there is not a wide number of publications concerning CAP depletion in fish species (Weifen et al; Zhi-Yong et al.)
For this reason a study to evaluate CAP depletion in rainbow trout is meaningful in the aim of having a better comprehension of the behaviour of this molecule under a probable typical oral administration.

In order to follow CAP elimination from fish tissues, CAP concentration in muscle was estimated by HPLC/MSMS following an internally validated method (based on EU 657/2002 Decision) with a decision limit = 0.1 µg kg-1. Briefly: fish tissues were smashed and extracted in ethyl acetate and ammonia; extracts were purified by C18 SPE, concentrated and analysed by HPLC/MSMS provided with triple quadrupole analyser. Quantification was achieved by means of the internal standard method, deuterated CAP being available.

## Materials and Methods

*Fish treatment*

60 triploid rainbow trout (Oncorhynchus mykiss), individual mean weight 374 g were selected from a larger lot and randomly divided into two groups each consisting of 30 specimens kept under natural day light conditions, in outdoor 0.5 m3 fiberglass tanks supplied with a constant flow (8 l $hr^{-1}$) of well-water at a temperature of 12.2 ± 0.19° C, pH 7.8 ± 0.1, total ammonia nitrogen < 0.05 mg $l^{-1}$. Fish groups were let to adapt to the experimental conditions over 1 week and were fed once a day with a basal diet. After this period one group (30 units) was used as a control and continued to be fed with the basal diet while the second one was given the basal diet supplemented with CAP (8 g $kg^{-1}$) over 10 days. The feeding level during the treatment was set at 1% biomass so as to ensure a virtual CAP intake of 80 mg $kg^{-1}$ $day^{-1}$. At the end of the treatment both groups were weighed in bulk and the actual CAP intake was recalculated to be 73.9 mg $kg^{-1}$ $day^{-1}$ considering the biomass gain occurred in the test group during the treatment.

*Fish sampling*

Treated fishes (2-5) were randomly sampled and sacrificed at days 0 (6.5 hours after the last meal), 2, 3, 4, 9, 16, 24, and 31 after the end of treatment. Control fishes (5 animals) were all sampled 35 days after the end of the treatment. Weight and length were measured and reported (table 2) before external cleaning, followed by organ dissection, fish muscle tissues were weighed again before being frozen at –18° C.

| Time elapsed from last meal | N° of sampled fishes | Mean weight [g] | Daily degrees |
|---|---|---|---|
| 6.5 hours | 5 | 416.0 | 3.3 |
| 2 days | 2 | 371.6 | 24.5 |
| 3 days | 3 | 442.4 | 36.3 |
| 4 days | 3 | 466.6 | 48.7 |
| 9 days | 3 | 438.9 | 110.1 |
| 16 days | 3 | 487.6 | 195.1 |
| 24 days | 3 | 453.8 | 293.3 |
| 31 days | 2 | 579.8 | 379.2 |

**Table 1:** sampling scheme and fish weights

*ELISA analysis*
In order to achieve a fast semi-quantitative estimation of CAP concentration in fish tissues and prepare -if necessary- for sample dilution, an ELISA analysis was performed over a small amount of each sample, following the manufacturer (Diessechem, the Netherland) instruction for sample preparation. Briefly: fish muscle (3 g) were homogenised and extracted with ethyl acetate. After solvent evaporation the residue was re-dissolved with PBS buffer, washed with n-hexane, iso-octane/chloroform, centrifuged and dispensed into plate wells for the assay.
Sample treatment (quantitative standard method)
Fish tissues were thawed. A muscle portion (5 g) -after internal standard fortification (CAP-d5, 0.02 mg $l^{-1}$, 150 ul)-, was homogenised in plastic tube with ethyl acetate and ammonia, 98:2 (20 ml). Extraction was achieved by vortex shaking (10 min) and by ultrasonic bath (15 min). After centrifugation (6000 x g, 10 min), half of organic extracts (10 ml) were transferred into a new tube and dried in Zymark apparatus, at 50° C under a gentle stream of nitrogen. The residue was re-dissolved in acetate buffer (0.01 M, pH 4.6, 15ml) and defatted with n-hexane (7 ml). Aqueous extracts were purified through a SPE (C18 un-endcapped, 500 mg) cartridge, which was previously conditioned with methanol (3 ml), acetate buffer (0.01 M, pH 4.6, 3 ml). The column was washed with water (2 ml), methanol (10% in water, 2 ml) and eluted with methanol (6 ml). The solvent was evaporated at 50° C under stream of nitrogen. Purified extracts were re-dissolved in methanol/water, (50:50, 0.5 ml) for HPLC/MSMS analysis.

*HPLC/MSMS analysis*
HPLC/MSMS analyses were performed on a separation module 2695 (Waters) coupled to a triple quadrupole analyser Micromass Quattro Ultima (Waters). Chromatographic separation was achieved by means of a C18 column (2.1 x 100 mm, 5um) and a mobile phase of methanol and water according to the following gradient elution: 10% methanol for 1 min, from 10% to 80% methanol in 4 min, 80% methanol for 3 min. Flow rate was 0.25 ml min-1, injection volume was 10 µl.
MSMS analysis was performed in Multiple Reaction Monitoring mode. Two transition ions were monitored for CAP, and 1 transition for CAP-d5, as reported in table 2.

| **PARAMETER** | **VALUE** |
|---|---|
| Capillary [kV] | 2.80 |
| Cone [V] | 65 |
| Source Temperature [° C] | 120 |
| Desolvation Temperature [° C] | 325 |
| Desolvation gas flow [l/hr] | 890 |
| Cone gas flow [l/hr] | 50 |
| CAP principal transition (collision energy [V] ) | 321 > 152 (18) |
| CAP secondary transition / (collision energy [V]) | 321 > 257 (12) |
| CAP-d5 principal transition / collision energy [V] | 326 > 262 (12) |

**Results and discussion**

Few papers are available in literature concerning depletion of chloramphenicol (CAP) in animal species: to our knowledge, no depletion study was performed on trout.

Trout were chosen for being a typical fish species frequently adopted for aquaculture: it represents one of the most diffused aquaculture species in fish farms and one of the most widely consumed in the European market.

Cases of fish contamination by CAP were found in the Italian market some years ago. Recently the problem seems to be reduced and more typically focused on fish products from extra-European countries, in which case shrimps represents the species at the highest risk. Nevertheless a deeper knowledge of CAP depletion in the tissues of trout is still of great interest, considering the activity and efficacy of this drug for the treatment of several bacteriological diseases and its consequent possible abuse.

The study was organised in such a way that two separate tanks were prepared to cultivate similar animals (roughly same age, same weights and dimensions) divided into two groups: treated animals (receiving a defined amount of CAP through their diet for a certain period of time) and non treated animals (control).

By verifying average fish weights at the end of the treatment the assumption of CAP dosage (80 mg kg-1 day-1) was corrected taking into account the effective weight increase: recalculated dosage was therefore estimated to be 73.9 mg $k^{g-1}$ $day^{-1}$.

Starting from the last day of treatment (day 0) and at day 2, 3, 4, 9, 16, 24 and 31 after the end of treatment, 2 to 5 fishes were sampled, cleaned and frozen before CAP determination in muscle tissues. All controls were sacrificed at day 35.

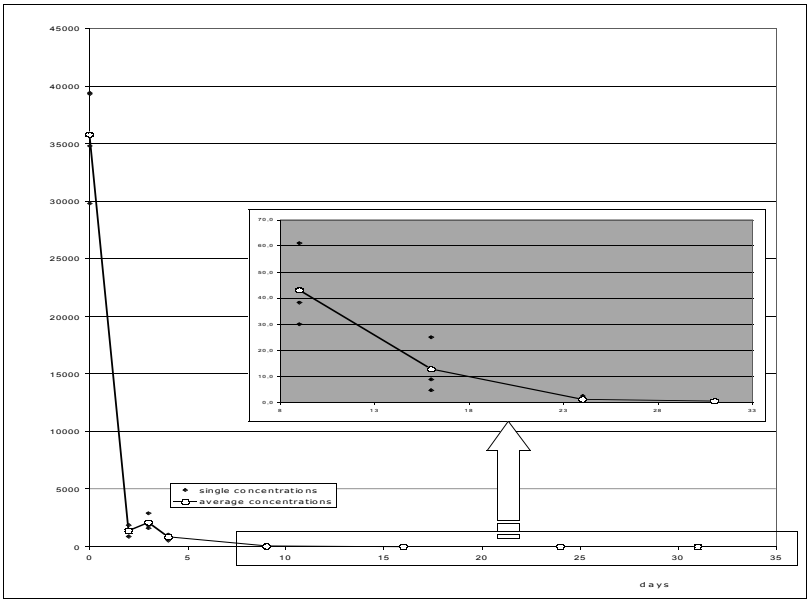
Muscle tissues were prepared for ELISA test to have a rapid estimate of CAP level; then quantitative determination of CAP concentration was achieved by means of an internally validated HPLC/MSMS method with CCα = 0.11 µg kg-1 and CCß = 0.12 µg kg-1, employing CAP-d5 as internal standard: quantification was achieved by means of the internal standard method. During every batch of analyses quality control samples (a blank trout muscle and a blank trout muscle fortified at 0.1 µg kg-1 and at 0.3 µg kg-1) were processed to verify absence of in-lab sample contamination and recovery rates.

In cases of CAP concentration higher than the top of the concentration range for which the method was validated (1 µg $kg^{-1}$) samples were diluted after organic extraction prior to SPE purification and/or before HPLC/MSMS analysis. In some cases –when tissue concentration was far above the analytical range of the method, due to the significant sample dilution, CAP-d5 could not be added to each samples at the beginning of the analytical procedure (as it could not be detected after sample work-up and dilution), therefore it was added to these samples after dilution and before HPLC/MSMS analysis to take into account possible variations in matrix ionisation suppression. In this case CAP concentration was evaluated by means of the external calibration method, taking into account the recovery ratio.

In table 3 for each withdrawal time, concentration range and standard deviations are reported. Graph 1 represents the trend of CAP elimination in trout muscle. Interestingly traces of CAP were still detectable after about one month after the end of the treatment at concentrations around the official MRPL.

| GROUPS OF ANIMALS | ANIMAL | WITHDRAWAL TIME | ANIMAL WEIGHT [g] | SAMPLE CONCENTR, [µg kg$^{-1}$] | AVERAGE CONCENTR, [µg kg-1] | CV gruppo (%) |
|---|---|---|---|---|---|---|
| GROUP 1 | 1 | 0 | 406,3 | 39290 | 35781,00 | 11,0 |
| | 2 | 0 | 430,0 | 34793 | | |
| | 3 | 0 | 432,4 | 39379 | | |
| | 4 | 0 | 421,5 | 35621 | | |
| | 5 | 0 | 389,6 | 29822 | | |
| GROUP 2 | 1 | 2 | 4065,0 | 909 | 1393,26 | 49,2 |
| | 2 | 2 | 3366,0 | 1878 | | |
| GROUP 3 | 1 | 3 | 447,1 | 1614 | 2112,33 | 32,5 |
| | 2 | 3 | 463,3 | 2895 | | |
| | 3 | 3 | 416,9 | 1828 | | |
| GROUP 4 | 1 | 4 | 476,6 | 1088 | 837,00 | 33,2 |
| | 2 | 4 | 444,1 | 539 | | |
| | 3 | 4 | 479,0 | 884 | | |
| GROUP 5 | 1 | 9 | 464,6 | 38,2 | 43,07 | 37,5 |
| | 2 | 9 | 425,0 | 29,9 | | |
| | 3 | 9 | 427,0 | 61,1 | | |
| GROUP 6 | 1 | 16 | 482,5 | 25,0 | 12,73 | 85,1 |
| | 2 | 16 | 555,1 | 8,63 | | |
| | 3 | 16 | 425,3 | 4,54 | | |
| GROUP 7 | 1 | 24 | 511,6 | 0,43 | 1,04 | 110,0 |
| | 2 | 24 | 490,7 | 0,33 | | |
| | 3 | 24 | 359,0 | 2,36 | | |
| GROUP 8 | 1 | 31 | 564,2 | 0,31 | 0,32 | 4,4 |
| | 2 | 31 | 595,4 | 0,33 | | |

**Table 2:** Estimated and average concentration of CAP in trout muscle at different withdrawal time.




**Graph 1:** trend of CAP elimination in trout muscle. Inside graph: expansion of the elimination during the last days of the withdrawal period

**Conclusions**

The study enabled to observe the trend of CAP elimination from trout muscle after a hypothetical oral veterinary treatment with fish feedstuff. High concentrations (slightly above 1 mg/kg) were observed up to 4 days after the end of the treatment. CAP was then significantly eliminated during the following withdrawal days, nevertheless quantifiable traces of this residue were still observable at concentrations levels in the range of the MRPL fixed by the European competent authorities up to 31 days after the end of treatment.

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## P 30

# ANTIBODY PRODUCTION: LOW DOSE IMMUNOGEN VS. LOW INCORPORATION HAPTEN USING SALMETEROL AS A MODEL

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**Abstract**
Haptens are low molecular weight compounds that are non-immunogenic and so must be conjugated to carrier molecules to elicit an immune response.
Doses of 50-1000µg protein conjugate have been suggested for immunisation of rabbits with hapten-protein immunogens. Although larger doses may give a faster response, lower doses may result in higher affinity antibodies. The amount of hapten presented to the host's immune system can be controlled by varying either the quantity coupled to a fixed amount of protein or the amount of immunogen administered. This study investigated which approach was superior for production of antibodies against the β–agonist salmeterol.
A range of salmeterol-HSA conjugates was prepared, with varying molar ratios of hapten: protein (80:1, 30:1, 15:1, 7.5:1), for use as immunogens. The 80:1 immunogen was also administered to different animals at four concentrations (0.5, 0.2, 0.1 and 0.05 mg/dose) while the remaining three immunogens were administered at 0.5 mg/dose. The effects of hapten incorporation and immunogen dose on antibody titre and sensitivity were investigated by comparing the titres of salmeterol specific antibody after each immunisation and by comparing the optimal IC50 obtained from each animal over the immunisation schedule.

**Introduction**
The production of antibodies to a veterinary drug requires conjugation of the low molecular weight compound to a larger molecular weight substance (carrier protein) to enable immune recognition and hence elicit an immune response in the host animal.
The immunogen can be administered to the host at a range of doses (with regard to the amount of carrier protein). Doses of $0.05^{-1}$ mg (Harlow and Lane, 1988) and 0.1-0.5 mg (Liddell, 2001) have been suggested for the immunisation of rabbits with immunogens prepared from hapten-protein conjugates. Although a larger dose may bring about a faster response it has been observed that lower doses and larger intervals between immunisations result in the production of relatively higher affinity antibodies (Cooper et al., 2004). When antigen interacts with cellular antibody the cells bearing it are stimulated to proliferate and differentiate into plasma cells. If antigen is present in critical concentrations it will stimulate those cells which bind it most efficiently. Large doses may stimulate cells bearing antibody of low affinity for long periods of time because of the persistence of a high concentration of antigen (Goidl et al., 1968). The generation of antibodies with increasing affinity with time after immunisation (affinity maturation) is due to a change in the structure of the antibody being synthesised. This change is brought about by somatic mutation of germ-line encoded genes (Griffiths et al., 1984). Furthermore, antigen-selected B cells first proliferate in germinal centres in an unmutated form and then hypermutate and die unless selected

by antigen to differentiate further into plasma cells and memory cells (Liu et al., 1989). Therefore while high-dose immunisation gives rise to a larger accumulation of mutations, selection for high-affinity antibodies is less stringent. Low-dose immunisation promotes selection of a memory cell pool displaying a more focused set of mutations because the genes with the most relevant mutations are selected more efficiently (González-Fernández and Milstein, 1998).

The dose of antigen exposed to the immune system can be controlled through the amount of immunogen administered but also by manipulation of the molar ratio of incorporated hapten to carrier. A high degree of substitution can lead to a stronger primary than secondary response generating antibodies of lower affinity. High titres of antibody with moderate affinity may be produced with intermediate hapten incorporation while a lower incorporation induces a slower response but with the production of higher affinity antibodies (Singh et al., 2004). The aim of this study was to determine the best way of delivering a low dose of low molecular weight hapten to produce sensitive antibodies. Two approaches were compared: an immunogen was prepared for salmeterol with a x 80 molar excess of hapten to carrier protein and administered to rabbits at a dose of 0.5 mg, 0.2 mg, 0.1 mg and 0.05 mg; three further immunogens were prepared with molar excesses of x 30, x 15 and x 7.5 of hapten to carrier protein and administered at a dose of 0.5 mg.

**Experimental**

*Preparation of immunogens.* The immunogens for salmeterol were prepared by coupling the drug to human serum albumin (HSA). Salmeterol was added to the protein at a range of molar excesses (x 80, x 30, x 15, and x 7.5). All the immunogen preparations were purified by dialysis against normal saline.

*Immunisation of rabbits.* The immunogens were mixed with an equal volume of adjuvant (Montanide ISA 50v) to produce emulsions that were administered at a range of doses as shown in table 1. The rabbits were immunised every 4 weeks and the emulsions were introduced subcutaneously into four separate sites. Blood samples were collected 10 days after each immunisation.

**Table 1.** Molar excess of hapten:protein and dose of immunogen administered to each rabbit

| Rabbit | Hapten:protein molar excess | Dose of protein (mg) |
|---|---|---|
| R915 | 80:1 | 0.5 |
| R916 | 80:1 | 0.5 |
| R927 | 80:1 | 0.2 |
| R928 | 80:1 | 0.1 |
| R929 | 80:1 | 0.05 |
| R935 | 30:1 | 0.5 |
| R936 | 15:1 | 0.5 |
| R937 | 7.5:1 | 0.5 |

*Assessment of antibodies.* The antibodies produced were compared for their sensitivity and titre by using a competitive ELISA format employing horseradish peroxidase (HRP) labelled salmeterol. The sensitivity of each antibody was evaluated after the second and subsequent

immunisations by determination of the IC50s (half maximal inhibitory concentration). The titre of each antibody was established at the same time by preparing a range of antibody dilutions and determining which dilution gave an optical density of 0.5 while keeping other assay conditions constant.

**Results and discussion**

Antibody sensitivity. The $IC_{50}$s are shown for each antibody in table 2. The two rabbits receiving 0.5 mg of the 80:1 immunogen (R915 and R916) produced their most sensitive antisera after five and seven immunisations respectively. The three rabbits receiving 0.2 mg (R927), 0.1 mg (R928) and 0.05 mg (R929) of the same immunogen produced their most sensitive antisera after eight, seven and seven immunisations respectively. Two of these antisera (R928 & R929) displayed lower
IC50s than those from the higher dose rabbits (R915 & R916). The rabbits immunised with the 30:1 (R935) and 15:1 (R936) hapten:protein immunogens provided, after eight immunisations, the most sensitive antibodies produced in the study. The final rabbit (R937), administered the 7.5:1 hapten:protein immunogen produced the least sensitive antibody of all which optimised after seven immunisations.

**Table 2.** $IC_{50}$s for each antibody after each immunisation. The antibody with optimum sensitivity for each rabbit is shown in bold text.

| | **IC50s (ng/ml)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **No. of immunisations** | **R915** | **R916** | **R927** | **R928** | **R929** | **R935** | **R936** | **R937** |
| 2 | >200 | 168.0 | >200 | 111.2 | 56.0 | 37.3 | 88.8 | 90.4 |
| 3 | 82.2 | 78.4 | 37.2 | 34.6 | 13.4 | 11.0 | 8.8 | 46.6 |
| 4 | 28.1 | 17.6 | 21.5 | 15.2 | 15.2 | 12.6 | 11.4 | 48.7 |
| 5 | 7.9 | 17.6 | 25.3 | 13.2 | 14.0 | 11.8 | 7.6 | 75.0 |
| 6 | 10.6 | 14.4 | 18.4 | 9.6 | 10.0 | 5.8 | 5.2 | 39.8 |
| 7 | 11.4 | 6.6 | 8.0 | 6.0 | 4.8 | 5.4 | 6.3 | 13.4 |
| 8 | 21.3 | 10.6 | 7.2 | 42.7 | 26.4 | 4.7 | 4.5 | 427 |

*Antibody titre.* The antibody titres obtained from each rabbit over the course of the trial are shown in Figure 1. The highest titres were obtained from the rabbits receiving immunogens with maximal incorporation of hapten, these titres being obtained after 5 (R916), 6 (R915, R927, R928) or 7 (R929) immunisations. As the level of incorporation was reduced in the immunogens (R935 to R937) so the titre of salmeterol specific antibody decreased.

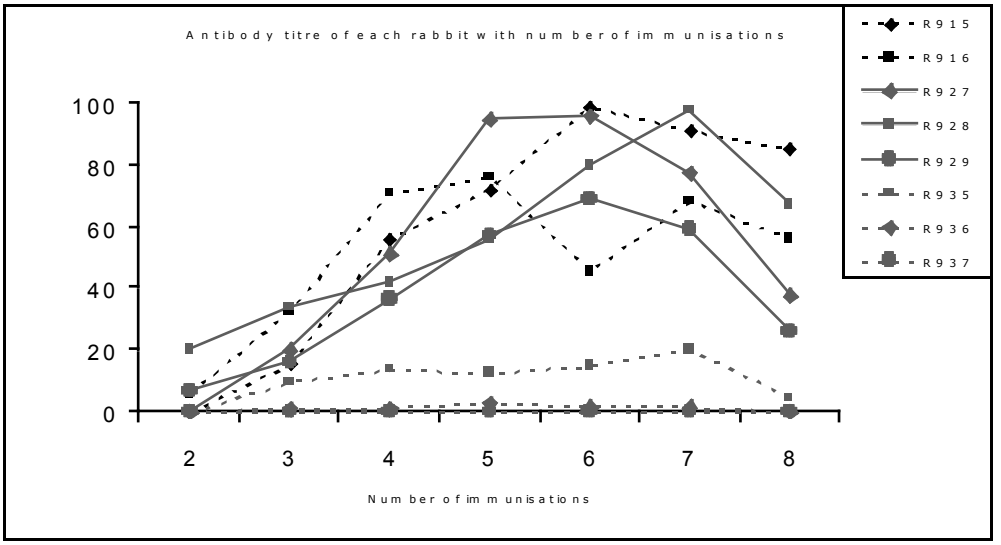


**Figure 1.** Development of antibody titre with number of immunisations for each rabbit Rabbit R929 received the lowest dose of the 80:1 immunogen and although the antibody titres from it were lower than those from the other rabbits receiving the same immunogen it provided the most sensitive antibody. It has been observed before (Fodey et al., 2007) that a relatively highly sensitive antibody will not necessarily possess a relatively high titre. That finding has been supported in this study by the fact that rabbits R935 and R936 produced the most sensitive antibodies overall with relatively lower antibody titres, although adequate for use in an immunoassay. There may be a benefit in selecting the low incorporation approach in that rabbits R935 and R936 produced consistently low $IC_{50}$s from the sixth immunisation onwards whereas over the same time frame the $IC_{50}$s from R928 and R929 were more variable. This trait may make it easier to harvest the preferred pool of antibody with less opportunity to overlook the optimum sensitivity.

**Conclusions**

Reducing the dose of an immunogen, fully incorporated with hapten, may bring about a small reduction in the titre of antibody but improve its sensitivity. However reducing the degree of hapten incorporation in the immunogen (to a limit) can also produce an improvement in sensitivity despite a relatively large reduction in antibody titre. The lower incorporation immunogen seems to produce a more consistent pattern of results regarding antibody sensitivity (after the sixth immunisation in this study) which may make it easier to predict the most suitable time for antibody harvesting.

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## P 31

# A REPLACEMENT FOR FREUND'S ADJUVANT FOR THE PRODUCTION OF POLYCLONAL ANTIBODIES TO VETERINARY DRUGS IN RABBITS

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**Abstract**
Two commercially available adjuvants, Gerbu LQ 3000 and Montanide ISA 50V, were compared with Freund's to assess their ability to produce a similar (or enhanced) immune response in the host animal without the undesirable side effects associated with Freund's. The three adjuvants were used to produce antibodies in rabbits to the veterinary drugs nicarbazin, diclazuril (both coccidiostats) and 3(2 nitrophenylmethylene-amino)-2-oxazolidinone (NPAOZ), a derivatised metabolite of the nitrofuran antibiotic furazolidone. The assessment involved the examination of each injection site and the characterisation of the resulting antibodies with regards to both antibody titre and sensitivity. It was found that the rabbits immunised with Gerbu adjuvant produced some of the most sensitive antibodies. However, titres were relatively low and adverse effects at injection sites were relatively common. Montanide adjuvant produced no adverse effects and the related antibodies were generally more sensitive than those from rabbits immunised with Freund's. It was concluded that Montanide ISA 50V could be considered as a suitable replacement to Freund's for the production of polyclonal antibodies, to low molecular weight compounds in rabbits.

**Introduction**
Antibody production in laboratory animals is a commonly used tool in many fields of biotechnology. Low molecular weight antigens are weakly immunogenic and must be coupled to a large carrier protein in order to elicit an immune response in the host animal.
The immunogenic complex is usually administered in combination with a suitable adjuvant. An adjuvant can operate through one or more of three basic mechanisms. Firstly it can extend the length of time that the immunogen is exposed to the immune system by providing its slow and even release over a long period of time. This "depot" effect allows more time for the immune system to process the antigen as well as conferring an increase in the duration of the antibody response. Secondly the adjuvant can act as a non-specific mediator of immune cell function, either directly or indirectly. Many adjuvants have a degree of surface activity or possess a surface interface, others contain bacterial components that can activate cells of the immune system such as the macrophage. However macrophages can cause excessive inflammation and pain and so the utilisation of bacterial components must be restricted to avoid these adverse reactions (Claassen et al., 1992). Thirdly an adjuvant can act as a vehicle for transporting the immunogen to the lymph nodes. Association with large particulate structures e.g. liposomes is known to increase the delivery of antigen to antigen presenting cells (APCs).

Freund's complete adjuvant (Freund et al., 1937) prepared from a non-metabolizable paraffin oil containing heat killed Mycobacterium tuberculosis, has been commonly used in the laboratory for primary immunisations. Booster immunisations are administered with the incomplete version which does not contain the dead bacterium. The formation of local and systemic lesions in laboratory animals, used for the production of antibodies (Claassen et al., 1992), has prompted investigators to consider using alternative adjuvants, of which there is an extensive list. However a suitable alternative must be capable of producing a satisfactory immune response in the host animal while causing the minimum undesirable side effects. The aim of this study was to find an alternative adjuvant to Freund's for the production of polyclonal antibodies in rabbits immunised with low molecular weight haptens that have been chemically coupled to immunogenic carrier proteins. The antibodies were to be produced against the veterinary drugs nicarbazin, diclazuril (both coccidiostats) and 3(2 nitrophenylmethylene-amino)-2-oxazolidinone (NPAOZ), a metabolite of the nitrofuran antibiotic furazolidone.
Montanide ISA 50, another water-in-oil adjuvant, was chosen for the surfactant activity provided by the mannide oleate mineral oil and its depot effect when emulsified with the immunogen. Gerbu does not provide a depot effect but was chosen for its ability to transport the immunogen to lymphocytes for phagocytosis and to act as an immunomodulator.

**Experimental**

*Preparation of immunogens.* An immunogen for nicarbazin was prepared by coupling N-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide to cationised human serum albumin (HSA) via a carbodiimide reaction (Connolly et al., 2002). A carboxy-diclazuril derivative was coupled to bovine thyroglobulin (BTG) via a carbodiimide reaction (Fodey et al., 2007) to act as an immunogen for diclazuril and the NPAOZ immunogen was produced by conjugating 3(3-carboxyphenyl-methylene-amino)-2-oxazolidinone (CPAOZ) to HSA through an acid anhydride reaction (Cooper et al., 2004).
Preparation of immunogen emulsions. For Freund's and Montanide adjuvants 1ml of immunogen was added to the adjuvant slowly with vortexing and the mixture then thickened. For the Gerbu adjuvant 1ml of immunogen was simply mixed with the pre-emulsified *adjuvant by vortexing.*

*Immunisation of rabbits.* The nicarbazin and diclazuril immunogens were administered at a dose of 0.2 mg while the NPAOZ immunogen was given at a dose of 0.05 mg. The rabbits were immunised every 4 weeks and the emulsions were introduced subcutaneously into four separate sites (left and right front quarters and left and right hindquarters. Blood samples were collected 10 days after each immunisation. Five rabbits were started for each combination of immunogen and adjuvant.

*Assessment of animal health.* When each blood sample was being collected the injection sites were examined for the presence of abscesses, inflammation, ulceration or signs of discomfort to compare the reaction caused by each adjuvant. Adverse reactions were marked on a scale: 0=nothing to report; 1=slight swelling (~1 cm); 2=swelling (1-2 cm); 3=large swelling (>2cm); 4=large swelling with oedema.

Assessment of antibodies. Antibody sensitivities were evaluated at three time intervals (after the 4th, 7th and 10th immunisations) by comparison of individual IC50s (half maximal inhibitory concentration). Antibody titres were established after each immunisation by preparing a range of dilutions and determining which gave the same arbitrary response while keeping other assay conditions constant.

**Results and discussion**

*Animal health.* The degree of adverse effects caused by the three different adjuvants at the injection sites is shown in figure 1.

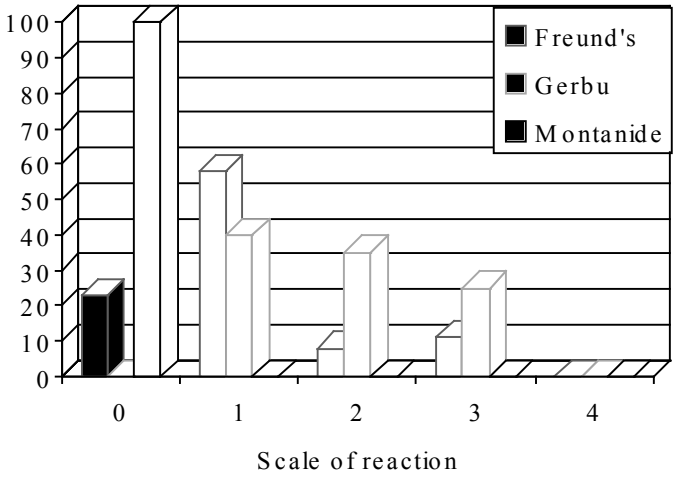


**Figure 1.** Degree of reaction at injection sites. 0=nothing to report; 1=slight swelling ~1 cm; 2=swelling 1-2 cm; 3=large swelling >2cm; 4=large swelling with oedema.

The Montanide adjuvant did not produce local reactions in any of the rabbits. Freund's and Gerbu produced a number of small swellings while the majority of larger swellings were caused by the Gerbu adjuvant. However in a previous study (Ferber et al., 1999) Gerbu adjuvant was found to be less aggressive than Freund's when used in mice. The effects from Freund's were as expected from reports in other studies (Claassen et al., 1992) and from experience in our laboratory.
Antibody sensitivity. The mean IC50s are shown for each group of rabbits (n=5) in table 1. The individually most sensitive antibodies to nicarbazin were produced by rabbits receiving Gerbu and Montanide adjuvants. Four out of the five most sensitive antibodies to diclazuril were obtained from rabbits that were administered the Gerbu adjuvant while three of the four most sensitive antibodies to NPAOZ were acquired using Montanide adjuvant.

**Table 1.** Mean IC50s + S.D. for the antibodies obtained from each group of rabbits after the 4th, 7th and 10 immunisations

| | | $IC_{50}$ (ng/ml) | | |
|---|---|---|---|---|
| **Immunogen** | **Adjuvant** | **4th Immunisation** | **7th Immunisation** | **10th Immunisation** |
| Nicarbazin | Freund's | 17.27 ± 14.71 | 11.19 ± 5.73 | 10.64 ± 5.02 |
| | Gerbu | 6.31 ± 6.22 | 26.72 ± 54.45 | 106.64 ± 220.07 |
| | Montanide | 5.04 ± 2.55 | 2.88 ± 1.71 | 5.61 ± 3.35 |
| Diclazuril | Freund's | 6.47 ± 5.23 | 23.7 ± 15.06 | 10.89 ± 6.48 |
| | Gerbu | 3.64 ± 3.34 | 3.80 ± 6.61 | 10.03 ± 12.89 |
| | Montanide | 4.01 ± 0.69 | 10.97 ± 5.66 | 88.36 ± 131.82 |
| NPAOZ | Freund's | 68.06 ± 15.18 | 43.32 ± 35.73 | 45.51 ± 34.07 |
| | Gerbu | 43.54 ± 50.52 | 83.41 ± 101.76 | 33.51 ± 29.08 |
| | Montanide | 126.52 ± 189.72 | 6.91 ± 5.81 | 3.53 ± 3.20 |

Antibodies produced by the nicarbazin and NPAOZ immunogens were significantly more sensitive when using Montanide adjuvant compared to Freund's after the 7th (P=0.014) and 10th (P=0.05) immunisations respectively. There was no significant difference in sensitivity of the diclazuril antibodies between using Freund's or Monatanide. The antibodies for nicarbazin and diclazuril reached their optimum sensitivity after a lower number of immunisations (4 or 7) when compared to the NPAOZ antibodies (10); probably due to the lower dose of NPAOZ immunogen that was administered. Antibodies produced using the Gerbu adjuvant were generally less sensitive for the NPAOZ immunogen probably due to a combination of the low dose and lack of depot effect provided by this adjuvant. However the fact that the individually most sensitive NPAOZ antibody came from a Gerbu administered rabbit displays the randomness of the immune response.

*Antibody titre.* The average titres obtained for the nicarbazin, diclazuril and NPAOZ antibodies over the course of the trial are shown in Figures 2-4 respectively. For rabbits receiving Freund's and Montanide adjuvants the titre generally increased with time reaching a maximum after 8-10 immunisations. Antibody titres from rabbits receiving the Gerbu adjuvant displayed more fluctuation and were of a lower magnitude. For Montanide it can be seen that greater antibody titres were obtained from the nicarbazin and NPAOZ rabbits compared to the diclazuril rabbits; this is also the case for Freund's though to a lesser degree.

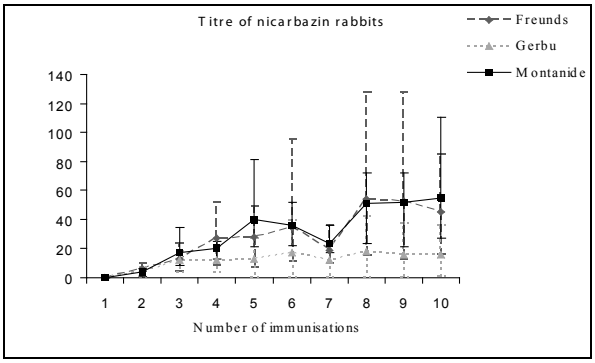


**Figure 2.** Development of antibody titre with number of immunisations for nicarbazin rabbits

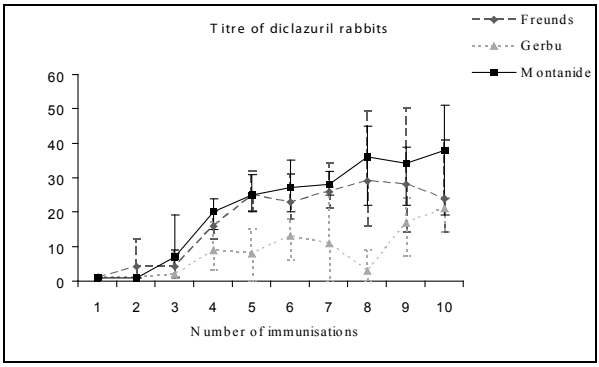


**Figure 3.** Development of antibody titre with number of immunisations for diclazuril rabbits

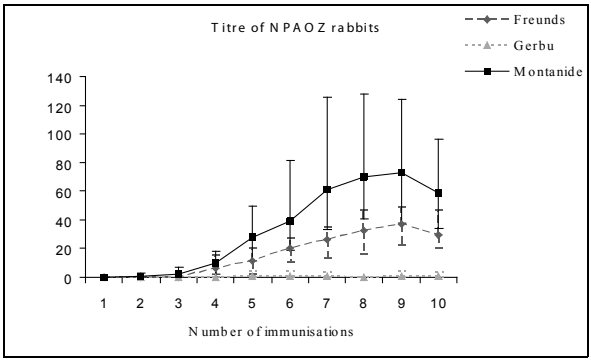


**Figure 4.** Development of antibody titre with number of immunisations for NPAOZ rabbits

The lower titres of Gerbu antibodies can again be explained by the lack of depot effect. With respect to immunogens the higher titres obtained from the nicarbazin and NPAOZ rabbits may be due to the carrier proteins used; HSA as opposed to BTG for diclazuril. In this study it has been demonstrated that higher titre does not necessarily mean greater sensitivity:

six rabbits (1 x nicarbazin, 4 x diclazuril and 1 x NPAOZ) receiving Gerbu presented the most sensitive antibodies but with the lowest titres. Generally Gerbu produced variable responses in individual animals with an elevated number of adverse effects at injection sites. Montanide produced antibodies of adequate sensitivity when compared to Freund's but, more importantly, causing no obvious adverse health effects

**Conclusions**

The surfactant element of the mineral oil and the depot effect of the prepared emulsion make Montanide ISA 50 a suitable replacement for Freund's in the production of polyclonal antibodies to low molecular weight compounds in rabbits, both from the aspect of antibody quality and improved welfare of the animals.

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## P 32

# DETERMINATION OF TEN SULPHONAMIDES IN EGG BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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**Abstract**
A precise and reliable method for the determination of ten sulphonamide antibiotics in egg by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed. Drugs were extracted using a mixture of dichloromethane/acetone (50:50, v/v), acidified with acetic acid and cleaned-up on a cation-exchange solid phase extraction (SPE) cartridge. The chromatographic separation was performed on a C18 column with a mobile phase of methanol-water containing 0.1% formic acid and 5 mM ammonium acetate, then sulphonamides were detected in a triple-quadrupole mass spectrometer operated in positive electrospray ionisation mode (ESI+). The method was validated at 15, 30 and 45 μg $kg^{-1}$, levels much lower than the corresponding maximum residue limit (MRL) of 100 μg $kg^{-1}$ set for sulphonamides in several food matrices but not in eggs, where the occurrence of such residues is not permitted. Results were quantitated against the selected internal standard 13C6-sulphamethazine. The within-laboratory reproducibility, expressed as a relative standard deviation, never exceeded 20.7%. All decision limit (CCα) values lied in the range 16-18 μg $kg^{-1}$ and detection capabilities (CCβ) were between 17 and 21 μg $kg^{-1}$.

**Introduction**
Sulphonamides are a large number of synthetic antibiotics widely used in veterinary therapy for the treatment and prevention of several bacterial and protozoan infections in cattle, swine and poultry. In poultry farming they are commonly employed as both growth promoters and prophylactics of diseases caused by Leukocytozoa and coccidia.
The occurrence of sulphonamide residues in food for human consumption is of concern because of their potential carcinogenic character. Consequently, the European Union (EU) has established a maximum residue limit (MRL) of 100 μg/$kg^{-1}$ (as a total amount) for sulphonamides in tissues from various animal species including poultry. In egg, where no corresponding limits have been set, sulphonamides are "zero tolerance" substances and any residues-incurred samples are violative.
Specific and sensitive analytical techniques are then required for a concrete monitoring of these drugs at residual levels. Moreover, Commission Decision 2002/657/EC prescribes the use of mass spectrometry coupled to an adequate chromatographic separation for the unambiguous identification of banned substances.
In this framework, LC-MS proved to be particularly suitable for the confirmatory analysis of such polar and non-volatile compounds. A variety of LC-MS/MS methods have been developed for the detection of sulphonamides in animal tissues and milk, while only few works describing their qualitative or quantitative analysis in egg are available in literature.

In the present work a sensitive and reliable method for the LC-MS/MS determination of ten sulphonamides is presented, in compliance with the legislation in force.

## Materials and methods

*Solvents and reagents*
All reagents (ammonium acetate, sodium chloride, sodium sulphate, ammonia, acetic acid, formic acid) and solvents (acetone, dichloromethane and methanol) were of analytical or HPLC grade (Carlo Erba, Milan). Ultrapure water was obtained by Milli-Q system Millipore (Bedford, MA, USA). The aromatic sulphonic SPE cartridges (500 mg/3 ml) were purchased from J.T. Baker (Deventer, The Netherlands).

*Standards*
Sulphadiazine, sulphamonomethoxine and sulphadimethoxine standards were obtained from Sigma (Milan, Italy), whereas sulphathiazole, sulphamerazine, sulphamethazine, sulphamethoxypyridazine, sulphachloropyridazine, sulphamethoxazole and sulphaquinoxaline were from Riedel-de Haën (Milan, Italy). Internal standard 13C6-sulphamethazine (13C6-SMZ) was provided by Cambridge Isotope Laboratories (Andover, MA, USA).
Stock solutions of all sulphonamides were prepared at 1 mg ml-1 in methanol and stored at 4°C. A mixed intermediate standard was prepared at 10 µg ml-1 by combining 100 µl of each stock solution and diluting to 10 ml with methanol, then the working solution was obtained by a further ten-fold dilution in methanol (1 µg ml-1). The internal standard stock solution was also prepared at 1 mg ml-1. The corresponding working solution at 1 µg ml-1 was obtained according to the dilution process as above.

*Apparatus*
The LC-MS/MS system consisted of a Series 200 LC micropump and autosampler (Perkin Elmer, Norwalk, CT) coupled to an API 2000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada).
The chromatographic separation was performed on a LUNA ODS(2) C18, 75 x 4.6 mm, 3 µm column (Phenomenex, Torrance, CA), at room temperature. The mobile phase consisted of methanol (A) and water containing 5 mM ammonium acetate and 0.1% formic acid (B). The starting composition of the gradient was 20 (A)/80 (B) v/v, then up to 90 (A)/10 (B) in 10 min, back to 20 (A):80 (B) in 1 min and further 5 min of reequilibration. The flow rate was 0.75 ml min-1, with a split ratio of 1:5. The injection volume was 10 µl.
The mass spectrometer was operated in electrospray positive ion (ESI+) mode with a TurboIon SprayTM source heated at 500°C. The capillary potential was set at 5.5 kV while cone voltage, collision energy and the other transmission parameters were optimised for each molecule. Data acquisition was carried out according to the Multiple Reaction Monitoring (MRM) approach, by selecting the two most intense ion transitions (one parent ion and two fragments) for all sulphonamides (Table 1).

**Table 1.** Selected ion transitions and instrumental parameters for the investigated sulphonamides

| Compound | SMR transitions (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|
| Sulphadiazine | 251®156[a]<br>251®92[b] | 20 | 21 |
| Sulphathiazole | 256®156<br>256®108 | 21 | 21 |
| Sulphamerazine | 265®108<br>265®92 | 16 | 32 |
| Sulphamethazine | 279®124<br>279®186 | 21 | 30 |
| Sulphamethoxypyridazine | 281®156<br>281®108 | 21 | 25 |
| Sulphachloropyridazine | 285®156<br>285®108 | 20 | 25 |
| Sulphamethoxazole | 254®156<br>254®92 | 21 | 27 |
| Sulphamonomethoxine | 281®156<br>281®108 | 20 | 32 |
| Sulphadimethoxine | 311®156<br>311®108 | 16 | 29 |
| Sulphaquinoxaline | 301®156<br>301®108 | 20 | 27 |

[a] More intense ion transition
[b] Less intense ion transition

*Sample preparation*

A 10-g amount of homogenised whole egg was spiked with 30 µg kg-1 of 13C6-SMZ and extracted with 50 ml of a dichloromethane/acetone mixture (50:50, v/v) in Ultra Turrax. After adding 10 g of sodium chloride and anhydrous sodium sulphate the sample was centrifuged for 5 min (1280 x g), then 5 ml supernatant was decanted and 0.25 ml of acetic acid was added.

The aromatic sulphonic SPE column was conditioned with 10 ml of acetone/dichloromethane/acetic acid (47.5:47.5:5, v/v/v). The acidified supernatant was passed through the cartridge and, after washing with 5 ml of water and 5 ml methanol, analytes were eluted with 5 ml methanol/ammonia (97.5:2.5, v/v).

The eluate was evaporated to dryness under a stream of nitrogen at 40-45°C and the remainder was redissolved in 200 µl of the LC-MS/MS mobile phase. The injection volume was 10 µl.

*Method validation*
Method was validated according to the criteria set by Commission Decision 2002/657/EC. The following parameters were taken into account: specificity, response linearity, trueness, precision (repeatability and within-laboratory reproducibility), decision limit (CCα), detection capability (CCβ).
Specificity was tested by analysing 20 egg samples in order to verify the absence of interferences at the retention time of each sulphonamide into account. Response linearity was checked by drawing six points calibration curves in solvent for each sulphonamide, with drug concentrations corresponding to 0, 50, 100, 150, 200 and 250 μg $l^{-1}$ and a fixed amount of 13C6-SMZ (150 μg $l^{-1}$). A triple injection was carried out for any points. A linear regression analysis was performed using the ratio of the standard area to internal standard area against the analyte concentration.
Method trueness and precision were evaluated according to the isotope dilution approach, by analysing 3 batches of egg samples on 3 separate days. Each series consisted of 18 blank egg samples spiked with all sulphonamides at 3 different concentration levels (15-30-45 μg $kg^{-1}$), 6 replicates per fortification level. Trueness was expressed in terms of recovery and precision as relative standard deviation.
Decision limit (CCα) and detection capability (CCβ) were calculated according to the calibration curve procedure reported in Decision 2002/657/EC: CCα was expressed as the concentration corresponding to the signal at the lowest calibration level (15 μg kg-1) plus 2.33-fold the within-laboratory standard deviation at this level. CCβ was calculated as CCα + 1.64-fold the standard deviation of the within-laboratory reproducibility at 15 μg $kg^{-1}$.

**Results and discussion**
Concerning specificity, the choice of a suitable clean-up procedure allowed to eliminate any interference peaks at the retention time of any sulphonamides evaluated in the study.
As these basic drugs present nitrogen-based functional groups that can be protonated, extracts containing such compounds can be easily retained on a cation-exchange SPE cartridge when loaded in 5% acetic acid in dichloromethane/acetone. The following washings with water and methanol are able to remove either polar and non-polar impurities that could affect the subsequent determination. Regarding response linearity, correlation coefficients were above 0.990 for all analytes.
Validation data is summarised in Table 2. Results were all corrected by the internal standard 13C6-sulphamethazine. Calculated recoveries ranged from 37.1 (sulphachloropyridazine) to 105.4% (sulphamethazine) and a clear correlation can be found between these data and the increase of the corresponding pKa values.
For an effective cation-exchange both the analyte and the stationary phase need to be fully ionised. Sulphonamides are weak basic compounds requiring relatively low pH values for protonation. Since sulphonic acid cartridges are ionised over a wide pH range (pka~0.8) they can be used for the extraction of such weak bases capable of carrying a charge at pH ≥ 2.8. In other words, the higher is the sulphonamide pKa value, the more is its retention on the cation-exchange column and, subsequently, the corresponding recovery rate.
Calculated decision limit (CCα) was below 20 μg kg-1 for each sulphonamide. These data are in agreement with the EU legislation, that recommends to reach the highest sensitivity as possible for the detection of those substances, like sulphonamides in egg, which are banned

but not provided with a minimum required performance limit. Such results are mainly due to the good sensitivity achieved in detecting sulphonamides, that allowed to choose a low concentration (15 μg $kg^{-1}$) as a starting point of the validation process.
Moreover, the adoption of the MRM approach permitted to obtain the four identification points (IPs) required by Commission Decision 2002/657/EC for the unequivocal identification of banned compounds.

**Table 2.** Method sensitivity, trueness and precision

| Compound | pKa | CCα[a] | CCβ[a] | Recovery[a] | RSD[a] |
|---|---|---|---|---|---|
| Sulphadiazine | 6.5 | 16.3 | 17.7 | 69.3 | 6.8 |
| Sulphathiazole | 7.2 | 16.4 | 18.0 | 69.5 | 6.1 |
| Sulphamerazine | 7.0 | 17.8 | 20.0 | 102.3 | 6.8 |
| Sulphamethazine | 7.4 | 16.1 | 16.9 | 105.4 | 3.9 |
| Sulphamethoxypyridazine | 7.2 | 17.0 | 18.7 | 93.3 | 7.1 |
| Sulphachloropyridazine | 5.9 | 16.8 | 20.7 | 37.1 | 11.1 |
| Sulphamethoxazole | 5.8 | 17.1 | 20.1 | 59.7 | 20.7 |
| Sulphamonomethoxine | 6.0 | 17.0 | 19.7 | 60.9 | 11.5 |
| Sulphadimethoxine | 6.2 | 16.4 | 18.3 | 58.9 | 11.9 |
| Sulphaquinoxaline | 5.6 | 16.1 | 18.4 | 38.7 | 16.8 |

[a]*Calculated on 54 data (18 replicates, 3 series)*

**Conclusion**

The method described can be used for a sensitive detection and a precise quantitation of sulphonamides in egg. The rapid extraction and the appropriate clean-up procedure make it suitable for the routine control of these drugs in such matrix, in compliance with the legislation in force for the confirmatory analysis of prohibited substances.

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## P 33

# A COMPARISON OF THE FAST, PREMI® AND KISTM TESTS FOR DETECTION OF ANTIBIOTICS IN BEEF KIDNEY JUICE AND SERUM

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**Abstract**
Rapid screening assays play an important role in monitoring the food supply for antibiotic residues and a number of these assays are currently in use around the world. In this study, three different microbial inhibition assays are used to compare results obtained in both beef kidney juice and beef serum. The Fast Antimicrobial Screen Test (FAST) is currently used in the U.S. as a preliminary screen. The Premi®test was developed in Europe as a relatively rapid screening method. The third test, the Kidney Inhibition Swab Test (KISTM) is another relatively rapid screening test which was developed fairly recently. Kidney juice and serum were selected as matrices which could be easily obtained and provide representative samples. Sensitivity levels were determined for each of 8 antibiotics in both matrices using fortified samples and the results compared among the three assays. Results using incurred samples for these rapid assays, as well as LC-MSMS analysis, will also be discussed.

Introduction

Rapid screening assays have been developed as part of a strategy for monitoring antibiotic residues in food. Use of such assays can increase efficiency and decrease the expense involved in residue monitoring. The Premi® test and the KISTM test are two relatively new antibiotic screening kits which have been developed. In this study, we directly compared these two kits with the Fast Antimicrobial Screening Test (FAST), an assay currently used in the U. S. We chose beef kidney juice and beef serum as matrices for this study with the thought that they might provide representative samples, and that serum could have a further benefit as a potential matrix for antemortem testing. The study was carried out using spiked samples, as well as incurred samples in order to best evaluate the utility of the three tests.

## Materials and Methods

*Standards*

Purity corrected stock solutions (1500 µg/mL) were each prepared of penicillin G sodium, tylosin, streptomycin sulfate, neomycin sulfate and spectinomycin.HCl (USP), sulfadimethoxine and oxytetracycline.HCl (Sigma), and danofloxacin (Pfizer) and aliquots were stored at -20°C. All solutions were made using water prepared with an E-pure system (Barnstead), except for sulfadimethoxine, which was prepared in methanol.

*Test Kits*
Materials for the FAST test (agar plates, neomycin standard disks, Bacillus megaterium spore suspension) were supplied by the FSIS Midwestern Laboratory (St. Louis, MO). Premi® Test materials (vials and pre-treatment solution) were obtained from DSM (Geleen, The Netherlands). KISTM materials (vials and feed extraction buffer) were provided by Charm Sciences (Lawrence, MA). All kit supplies were stored at 4°C, although feed extraction buffer aliquots were stored at -20°C.

*Control and Incurred samples*
Control kidneys and blood samples (from steer carcasses) and potentially incurred samples (from inspector-retained carcasses) were obtained at a local processing plant. Blood samples were allowed to clot and then centrifuged (1500 x g, 20 min) and decanted to produce serum, which was then stored, in aliquots, at -20°C. Kidneys were immediately frozen, and then later thawed to produce kidney juice, which was centrifuged (3000 x g, 10 min) and the supernatant stored, in aliquots, at -20°C.

*Screening test kit procedures*
For each day of the spiked study, control kidney juice and serum samples were tested along with a range of spiked concentrations for a given antibiotic. All three tests used the same control and spiked solutions on a given day. For the incurred sample study, a negative control sample was shared among all three tests, and a positive (20 ng/mL penicillin G) control sample was shared between the Premi® and KISTM tests each analysis day.
FAST test: FAST plates were streaked with B. megaterium spores and then 2-3 blank 6 mm paper disks (Becton Dickinson & Co, Sparks, MD) along with a standard neomycin disk (positive control) were placed on each plate. Samples to be analyzed (25 µL) were pipetted onto the blank disks immediately after their placement on the plate. Plates were then incubated 7 hr at 44$^0$C and zones of inhibition were measured with a ruler. Based on variation in zone size surrounding negative control disks, a positive response was determined as a zone size ≥10 mm. Plotting FAST zone sizes vs. spiked antibiotic concentration provided a linear relationship, allowing determination of the concentration corresponding to a 10 mm zone, or the threshold concentration for detectability.
Premi® and KISTM tests: These tests both involved pipetting a sample (100 µL) into a vial and incubation in a 640C heater block until the negative controls turned from purple to yellow in color (2.75 to 3.5 hr, depending on test and lot). The Premi® test required pre-heating of kidney juice vials at 80$^0$C (10 min) to inactivate lysozyme-like materials, prior to the 64$^0$C incubation. Both Premi® and KISTM tests required a pre-treatment of serum samples (100 µL sample and 33 µL pretreatment solution, or 100 µL sample and 100 µL feed extraction buffer, respectively) prior to pipetting the pretreated sample into the vial. In order to determine a minimum detectable concentration for each antibiotic used in the spiked study, Premi® test vials were scanned on a flatbed scanner (Hewlett Packard Scanjet 8200, calibrated daily), using Premi® Scan software. Scanner data (z-values) were plotted against spiked concentration to determine a concentration corresponding to z=0, the positive threshold level. For KISTM test results of the spiked study, the detectability limit was based on concentrations providing a visually positive or negative color result. For incurred sample analysis, both tests used a visual examination to determine a positive (blue/purple color) or negative (yellow/green color) result.

*Extraction of veterinary drugs and LC-MSMS analysis*
The majority of antibiotics were extracted from 1 g samples using either acetonitrile/water (kidney juice) or acetonitrile (serum). Supernatants were treated with C18, evaporated, and taken to 1 mL volume with water. Aminoglycosides were extracted using an established method (http://www.fsis.usda.gov/PDF/CLG_AMG_1_02.pdf), with some modification. Liquid chromatography used a Phenomenex Prodigy ODS-3 column (5 µm, 150 x 3.0 mm), with a C18 guard column, and gradients of either aqueous formic acid and acetonitrile, or aqueous heptafluorobutyric acid and methanol. Screening used a triple quadrupole (majority of antibiotics) or ion trap (aminoglycosides) mass spectrometer. Confirmation and quantitation used the triple quadrupole instrument.

**Results and Discussion**
Our initial goal was to determine the levels at which eight antibiotics would be detectable by the three rapid assay kits in beef kidney juice and serum. The antibiotics, chosen to represent a range of structural classes, included penicillin G, sulfadimethoxine, oxytetracycline, tylosin, danofloxacin, streptomycin, neomycin and spectinomycin. Control beef kidney juice and serum samples were spiked with levels of an antibiotic, and the same spiked samples were used for all three rapid screening tests on each day of an experiment. Each antibiotic was tested as a range of concentrations over 3-6 days. Detectable concentrations were determined for the three assays for each of the 8 antibiotics, and the results are shown in Table 1. As a result of the KIS™ test being interpreted visually, results are reported as a range, with the lower number representing the concentration which consistently provided a negative result, and the higher number, a consistently positive result.

**Table 1.** FAST, Premi® and KIS™ detectability in kidney juice (KJ) and serum (S) and regulatory limitsa

| | | Threshold Concentrations (µg/mL) | | | Tolerance or MRL in kkidney Kidney (µg/g) | | |
|---|---|---|---|---|---|---|---|
| **Analyte[b]** | **Matrix** | **FAST** | **Premi®** | **KIS™** | **U.S.** | **EU** | **Codex** |
| Penicillin G | KJ | 0.4 | 0.005 | 0.005-0.01 | 0.05[c] | 0.05[c] | 0.05 |
| | S | 0.5 | 0.006 | 0.01-0.015 | | | |
| SDMX | KJ | 1.4 | 0.12 | 0.01-0.1 | 0.1[c] | 0.1 | --- |
| | S | 1.7 | 0.12 | 0.2-0.4 | | | |
| OTC | KJ | 1.5 | 2.1 | 0.5-1.5 | 12 | 0.6 | 1.2 |
| | S | 0.9 | 4.1 | 0.1-0.3 | | | |
| Tylosin | KJ | 1.2 | 0.090 | 0.1-0.2 | 0.2 | 0.1 | --- |
| | S | 1.7 | 0.065 | 0.1-0.2 | | | |
| DANO | KJ | 1.7 | 4.5 | 4->6 | 0.2[d] | 0.4 | 0.4 |
| | S | 1.3 | 12 | 3->6 | | | |
| STREPT | KJ | 1.4 | 9.0 | 4-10 | 2.0 | 1.0 | 1.0 |
| | S | 1.6 | 13 | 2-5 | | | |
| Neomycin | KJ | 0.04 | 1.2 | 0.4-0.6 | 7.2 | 5 | 10 |
| | S | 0.04 | 10 | 0.1-0.5 | | | |
| SPECT | KJ | 66 | 3.5 | 5-6 | 4 | 5 | 5 |
| | S | 68 | 1.9 | 1-3 | | | |

*[a]Schneider, MJ, Lehotay, SJ (2008) Anal. Bioanal. Chem., in press [b]SDMX=sulfadimethoxine, OTC=oxytetracycline, DANO=danofloxacin, STREPT=streptomycin, SPECT=spectinomycin; [c]edible tissue; [d]liver*

The results show no clear pattern of detectability, either between the three tests, or the two matrices. In some cases, the FAST displayed a lower threshold concentration, while in other cases, it was higher. In general, the Premi® and KISTM tests had similar thresholds, although there were cases when the Premi® was less responsive than the KISTM in serum.

The ideal screening test would provide a positive threshold concentration at, or very close to the required tolerance or MRL. In comparing the tolerance and MRL values listed above to the assay results, one can see that no one test is clearly the best. Rather, those responsible for residue monitoring will either need to perform multiple tests, or choose the test and matrix which addresses the greatest number of problematic analytes at any given time.

In order to study these tests with potentially incurred samples, kidney juice and serum samples from 235 inspector retained carcasses at a local processing plant were collected and screened. These samples were also analyzed by LC-MSMS to determine which drugs, if any, and their levels were present. In such a way, we determined which antibiotics, of those found, would be detectable by the rapid tests in incurred samples, as well as an approximate concentration. Results from this study are shown in Table 2.

In kidney juice, all three tests successfully detected 2 samples containing high levels of

dihydrostreptomycin. The Premi® and KIS™ tests identified a sample with sulfamethazine above the U.S. tolerance in kidney (both kidney juice and serum), as well, while the FAST did not. LC-MSMS detected three antibiotics having no listed U.S. tolerance in beef. Of these, in serum, the Premi® detected a sample containing <10 ng/mL gentamicin and a trace of kanamycin. None of the tests detected a kidney juice sample containing <5 ng/mL lincomycin.

**Table 2.** Results from incurred samples (KJ=kidney juice; S=serum)

| **Detected, Level Detected, Level False positives[b] > U.S. tolerance[a] < U.S. tolerance[a]** | | | | | | |
|---|---|---|---|---|---|---|
| Test | KJ | S | KJ | S | KJ | S |
| FAST | 2 | 0 | 1 | 0 | 2 | 1 |
| Premi® | 3 | 1 | 5 | 1 | 1 | 30 |
| KIS™ | 3 | 1 | 2 | 1 | 13 | 11 |

[a]U.S. tolerance in kidney; [b]positive test, but negative LC-MSMS result

As for detection of samples containing antibiotics at levels below U.S. tolerance, the FAST detected a sample containing 1 µg/mL dihydrostreptomycin, but no others. The Premi® detected 4 samples with <30 ng/mL pirlimycin (tolerance available only in liver), two of which also had low levels of other drugs, and 2 samples with <10 ng/mL penicillin G or oxytetracycline. The KIS™ detected 2 samples containing <10 ng/mL Penicillin G, one of which had a low level of oxytetracycline, and 1 with pirlimycin (<25 ng/mL) and a very low level of desfuroylceftiofur cysteine disulfide (DCCD).

The FAST provided the fewest false positives (positive test, no antibiotic detected by LC-MSMS). KIS™ and Premi® both had a significant number of false positives, with all but one for the Premi® in serum, while the KIS™ shows them distributed between kidney and serum. The reason for this disparity is unclear, but it suggests kidney juice may be a significantly better matrix for Premi®.

One of the goals of this study was to evaluate kidney juice and serum as potential screening matrices. All 3 tests were easily run in either matrix and, in the spiked study, most of the drugs tested showed no great differences in sensitivity between the 2 matrices. Exceptions included neomycin, streptomycin and oxytetracycline, which were less sensitive in serum in the Premi® test, with the latter two being more sensitive in serum in the KIS™ test. In the incurred study, a potential limitation of serum arose. With the Premi® test, the number of false positives was very high in serum, suggesting kidney juice as a better matrix for this test. Furthermore, the volume of serum to be obtained from a given volume of blood was highly variable, apparently dependent on the hydration state of the animal. Thus serum provides a potentially more variable sampling matrix than had been expected, although it could still provide a good matrix for antemortem screening, given more consistently hydrated animals.

**Conclusion**

In this study, threshold concentrations for positive responses were determined for each of 8 selected antibiotics in both beef kidney juice and serum using the FAST, Premi®, and KIS™ tests. These three tests were also used to analyze kidney juice and serum samples

from 235 potentially incurred carcasses. No one test or matrix was found to be the best for all circumstances, and analysts will need to select the test and matrix to best address their greatest needs for screening.

**Acknowledgements**
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## P 34

# DEGRADATION OF AHD 13 C3 AND SEM 15 N2 13 C USED AS INTERNAL STANDARDS IN NITROFURAN RESIDUE ANALYSIS

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**Abstract**
Monitoring of nitrofuran residues has been focused in the detection of protein-bound nitrofuran metabolites AOZ, AMOZ, AHD and SEM. A multiresidue method for honey has been developed based on an extraction procedure for the isolation and clean-up of the nitrophenyl-derivatised metabolites and further determination by liquid-chromatography-electrospray positive ionization tandem mass spectrometry.
AOZ $^{d4}$, AMOZ $^{d5}$, AHD 13 $^{C3}$ and SEM $^{15}N_2$ $^{13}$C were used as internal standards. A MRM procedure has been applied and the following transitions were monitored: m/z 240>134, 340>296, 252>179 and 212>195 for the four internal standards respectively.
The method was validated in terms of range, linearity, precision, accuracy and recovery (n= 38, two months). Decision limit (Cc$\alpha$) and detection capability (Cc$\beta$) were determined.
In an extended period of time, AHD standard curve slopes increased after 2 months and SEM standard curves slopes after 8 months due to a decrease in the internal standard measures. In both cases the slopes were statistically different than the previous ones.
When recalculating data using AOZ d4 as internal standard, there is no variability in curve slopes.
Degradation of AHD $^{13}C_3$ and SEM $^{15}N_2$ $^{13}$C with time seems to be the explanation.

**Introduction**
Nitrofuran antibacterial agents (4 compounds) have been widely used and their residues have been found in honey, meat and seafood.
Following data which classified furazolidone as a mutagenic and genotoxic drug, legislation was enforced to remove this and similar compounds from the market. Since data concerning the toxicity of furaltadone, nitrofurazone and nitrofurantoine were insufficient, the European Union (EU) prohibited the use of nitrofuran antibiotics by listing them in the Annex IV. A detection of the drug at any concentration should be sufficient to point a violation of the law.
This paper describes the development and validation of a multiresidue analysis for nitrofuran metabolites (AOZ, AMOZ, AHD and SEM) in honey by LC-MS-MS and the problems of degradation of AHD and SEM internal standards with time.

## Materials and Methods

*Materials*

2-nitrobenzaldehyde (NBA) (98%purity) was obtained from Sigma. AOZ, AMOZ, AHD and SEM, their nitrophenyl derivatives and the deuterated internal standards (AOZ-d4, AMOZ-d5, AHD 13C3 and SEM 15 N2 13 C) were obtained from Witega.

Stock solutions of AOZ, AMOZ, AHD and SEM were prepared in methanol HPLC at concentrations of 111,6 µg/ml, 103,8 µg/ml, 110,0 µg/ml and 109,68 µg/ml respectively and stored at 4ºC in the dark. Standard solutions I were prepared mixing from these stock solutions by dilution 1:100 in methanol HPLC to give concentrations of about 1µg/ml of each standard and further standard solutions II were prepared by dilution 1:20 from standard solution I to give concentrations of 52 ng/ml, 52,1 ng/ml 51,31 ng/ml and 51ng/ml for AOZ, AHD, SEM and AMOZ respectively.

Matrix matched standard were prepared by fortification of matrix tissue free from nitrofuran with 10, 20, 40 and 80 µl of standard solutions II that corresponded to approximately 0.5, 1.0, 2.0 and 4.0 ng/g of each compound in the tissue.

*LC-MS/MS.*

All separations were performed in a Symmetry C18 column (5 um, 3 x 150 mm) supplied by Waters equipped with a guard column Symmetry C18 (10x2.1 mm). The mobile phase was a mixture of phase A (0.162 mM ammonium acetate in 0.1% acetic acid in water) and phase B (10 % water in acetonitrile, 0.1% acid acetic) with the following gradient:phase A: phase B 90:10 until min to 55:45 at 14 min. The running was over 22 min from 0.4 ml/min at 0 min to 0.7ml/min at 16 min. The column was at 40ºC and the injection volume was 50 ul. Elution times for the compounds were 7.09 min, 11,93 min, 12,15 min and 13,44 min in average for AMOZ, SEM, AHD and AOZ respectively.

MS detection was performed in electrospray (ESI) positive ion mode using multiple reactions monitoring (MRM). A triple quadrupole mass spectrometer with electrospray ionization (positive mode) was employed (HPLC Waters 2695 Alliance MS-MS Micromass Premier XE). The following parameters were used: capillary voltage: 3.9 Kv, cone voltage: 26 V, source temperature: 120 ºC, desolvatation temperature: 350 ºC, flow rate: 1000 lt/h, cone gas flow: 0 l/h. The carrier gas was argon, p 6xe-3 mbar. Substance identification and quantification were performed selecting one parent ion and two product ions for each analyte, according to the EU guidelines for unambiguous positive identification and quantification of analytes.

*Analytical Procedure*

Samples (1g) of honey were placed in a 15 ml prolypropylene tube and 10, 20, 40 and 80µl of standard solutions II (50 ng/ml) that corresponded to approximately 0.5, 1.0, 2.0 and 4.0 ng/g of each compound in the tissue were added together with 5 ml of HCl 0.2M and 60 µl of 2-nitrobenzaldehyde 100mM, followed by vortex mixing for 2 min before incubated overnight at 37ºC. After cooling, 0.5 ml of Na3PO4 0.3M were added and the mixture was neutralized (pH=7±0.5) with sodium hydroxide (2M) followed by vortex mixing for 30 seg. The samples were extracted with 5 ml ethyl acetate using a shaker (20 min) and centrifuged (2500 rpm, 5 min, 20ºC) before removing ethyl acetate supernatant. The combined ethyl acetate extracts were evaporated under a stream of nitrogen at 55ºC. The residue was dissolved in 500 µl of solvent, vortexed by 30 seg, filtered (13mm, 0.45 µm) and collected in 300 µl HPLC vials.

## Results and Discussion

*Validation*

The method was validated in terms of range, linearity, precision and accuracy. Decision limit (CCα) and detection capability (CCβ) were determined.

Linearity. Calibration curves were performed with four different concentrations levels of metabolites (0.5 ppb, 1.0 ppb, 2.0 ppb and 4.0 ppb) to study the linearity of detector response (y=a.x+b) (n=38, two months)

**Table 1**. Linearity

| | **AOZ** | **SEM** | **AMOZ** | **AHD** |
|---|---|---|---|---|
| a | 0.928 | 0.205 | 0.629 | 0.333 |
| SD | 0.013 | 0.021 | 0.018 | 0.009 |
| b | -0.054 | -0.000 | -0.019 | -0.018 |
| SD | 0.031 | 0.005 | 0.042 | 0.022 |
| R | 0.9855 | 0.9920 | 0.9453 | 0.9461 |

*Accuracy and Precision.* The same four different fortification levels were used for accuracy and recovery determinations (n=3 at each fortification level) studied on different calibration curves in order to establish the precision. The same procedure was repeated on two months involving different operators, reagents and environmental conditions to determine the within laboratory reproducibility (N=38, 2 months)

**Table 2:** Within laboratory Reproducibility and Accuracy.

| | **Nominal Concentration (ppb)** | **S.D.** | **CV %** | **Accuracy %** |
|---|---|---|---|---|
| **AOZ** | 0.52 | 0.081 | 15.62 | 99.82 |
| | 1.04 | 0.118 | 11.37 | 100.22 |
| | 2.08 | 0.213 | 10.26 | 99.91 |
| | 4.16 | 0.371 | 8.93 | 100.01 |
| **SEM** | 0.51 | 0.015 | 2.89 | 98.49 |
| | 1.03 | 0.023 | 2.25 | 100.18 |
| | 2.05 | 0.030 | 1.47 | 100.52 |
| | 4.10 | 0.061 | 1.49 | 99.88 |
| **AMOZ** | 0.51 | 0.066 | 12.90 | 99.60 |
| | 1.02 | 0.136 | 13.33 | 100.50 |
| | 2.04 | 0.245 | 12.01 | 99.80 |
| | 4.08 | 0.541 | 13.33 | 100.03 |
| AHD | 0.52 | 0.044 | 8.62 | 97.91 |
| | 1.04 | 0.061 | 5.88 | 95.88 |
| | 2.08 | 0.141 | 6.78 | 96.72 |
| | 4.17 | 0.240 | 5.75 | 95.02 |

**Table 3:** Decision Limit and Detection Capability (ppb)

| | CCα[a] | CCβ[b] |
|---|---|---|
| AOZ | 0.02 | 0.07 |
| SEM | 0.01 | 0.02 |
| AMOZ | 0.08 | 0.15 |
| AHD | 0.03 | 0.07 |

*a Intercept+2.33xSD intercept b CCα+1.64xSD intercept*

*Variability with operators and equipments.* There were no significant differences in the mean RFs with different pair of operators (n=30) or different LC-MS/MS instruments (n= 120) for each AOZ or SEM concentration (p>0.05, One-Way ANOVA test) (n=30)

**Table 4:** Variability among pair of operators (n=30)

| | RFs AOZ (± SD) | | | | RFs SEM (± SD) | | | |
|---|---|---|---|---|---|---|---|---|
| ppb | 0.52 | 1.04 | 2.08 | 4.16 | 0.513 | 1.026 | 2.052 | 4.105 |
| 1[a] | 0.4300 ±0.0851 | 0.8724 ±0.1149 | 1.7675 ±0.1483 | 3.5797 ±0.5177 | 0.1041 ±0.0204 | 0.2048 ±0.028 | 0.4094 ±0.0526 | 0.8044 ±0.1087 |
| 2[a] | 0.4544 ±0.0548 | 0.9587 ±0.1052 | 0.1957 ±0.1883 | 3.9528 ±0.2501 | 0.1064 ±0.0124 | 0.2177 ±0.0208 | 0.4211 ±0.0317 | 0.8413 ±0.0684 |
| 3[a] | 0.4479 ±0.058 | 0.9086 ±0.0078 | 0.1797 ±0.1291 | 3.8145 ±0.3284 | 0.1134 ±0.0166 | 0.2233 ±0.0313 | 0.4311 ±0.0646 | 0.8834 ±0.096 |

[a] *1,2,3: three different pairs of operators*

**Table 5:** Variability among instruments (n=120)

| | RFs AOZ (± SD) | | | | RFs SEM (± SD) | | | |
|---|---|---|---|---|---|---|---|---|
| ppb | 0.52 | 1.04 | 2.08 | 4.16 | 0.513 | 1.026 | 2.052 | 4.105 |
| A[a] | 0.3684 ±0.0576 | 0.7723 ±0.1149 | 1.5603 ±0.235 | 3.3266 ±0.4539 | 0.1494 ±0.0263 | 0.2926 ±0.0629 | 0.5851 ±0.0715 | 1.174 ±0.1891 |
| B[a] | 0.4366 ±0.0720 | 0.9159 ±0.1083 | 1.8631 ±0.1996 | 3.7804 ±0.3870 | 0.1163 ±0.0178 | 0.2438 ±0.0267 | 0.5268 ±0.4565 | 1.0845 ±0.0879 |
| C[a] | 0.4674 ±0.0552 | 0.9372 ±0.0974 | 1.9302 ±0.1548 | 3.9054 ±0.2976 | 0.1409 ±0.0249 | 0.2822 ±0.0425 | 0.5715 ±0.0856 | 1.145 ±0.169 |

[a] *A,B,C: three different LC-MS/MS instruments*

*Degradation of AHD and SEM internal standards*

In an extended period of time, AHD standard curve slopes increased after the first month due to a decrease in the internal standard measures that leads to higher values of the Rf (calculated as the ratio between the sum of the two daughter ions and the internal standard). There were no significant differences in the slopes during the first 5 months for AOZ (p>0.05), AMOZ (p>0.01) and SEM (p>0.01) analyzed with One-Way ANOVA test.

**Table 6:** AHD standard curve slopes by months (1 to 5)

| Month[a] | Mean | SD |
|---|---|---|
| 1 (n=13) | 0.2251 | 0.0171 |
| 2 (n=21) | 0.3021 | 0.0517 |
| 3 (n=17) | 0.3728 | 0.0715 |
| 4 (n=14) | 0.6376 | 0.0622 |
| 5 (n=15) | 0.7965 | 0.0816 |

*[a] from February to June (1 to 5)*
*For AHD samples, the five means were significantly different from one another (Tuckey test) resulting in significant differences in the slopes along the time ($p<0.01$).*

**Table 7:** SEM standard curve slopes by fortnight (months 5 to 8)

| Fortnight [a] | Mean | SD |
|---|---|---|
| 5- 1st (n=9) | 0.1989 | 0.0173 |
| 5- 2nd (n=8) | 0.1791 | 0.0150 |
| 6- 1st (n=6) | 0.1466 | 0.0365 |
| 6- 2nd (n=9) | 0.1573 | 0.0109 |
| 7- 1st (n=8) | 0.1382 | 0.0349 |
| 7-2nd (n=8) | 0.1736 | 0.0204 |
| 8 -1st (n=6) | 0.2085 | 0.0361 |
| 8- 2nd (n=7) | 0.6018 | 0.1848 |

*[a] from June to September by 1st and 2nd fortnights.*

The SEM slope in the second fortnight of September was significantly different from the others fortnights ($p<0.001$, One-Way ANOVA test, Tuckey test)
If all the data were recalculated using the AOZ (AOZ-d4) internal standard as the reference measure instead of AHD and SEM internal standards (AHD $^{13}C_3$ and SEM $^{15}N_2$ $^{13}C$), there were no significant differences in time in the slopes or Rf measures for both compounds.
The decrease in AHD and SEM internal standard measures are attributed to a degradation of these compounds with time.
Incubation in acid and basic medium and further MS spectra indicate that degradation is not due to ceto-enolic tautomery because MS with and without acid show the same pattern for both compounds. However, the MS pattern is quite different in basic medium where some hydrolysis takes place. Some MS fragments correspond to condensation products of NBA. Other ones have not been identified.
As internal standards are stored in CD3OD, slow hydrolysis by this solvent may happen along the time. This effect would be much slower than with CH3OH because of PIE (primary isotopic effect).
The reasons of the instability and degradation of AHD and SEM internal standards are not clear and require further research.

**Conclusions**

A multiresidue method for nitrofuran metabolites (AOZ, AMOZ, AHD and SEM) in honey by LC-MS-MS has been developed and validated. Degradation of AHD and SEM internal standards with time have been found. This problem can be overcome using the AOZ internal standard as the reference measure. The reasons of degradation of the standards should require further research.

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## P 35

# RESIDUES OF EPRINOMECTIN IN SHEEP MILK PRODUCTS

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**Abstract**
We studied distribution of eprinomectin (EPR) residues from milk to milk products. EPR was administered topically to 30 sheep of dairy crossbreed at a single therapeutic dose of 1.0 mg EPR/kg b.w. 12 collective daily milk samples were collected at selected time intervals (ranging from 1-7 days) from sheep during course of 44 days following drug administration. Samples were processed into following milk products: yoghurt made from raw and pasteurised milk, curd, semi-hard and hard cheese, albumin (whey) cheese and whey with and without albumins and globulins. Samples were cleaned up using SPE, derivatised and analysed using HPLC with fluorescence detection.
Time residue profiles in milk products followed the shape of excretion of EPR into milk, however there were significant differences in residue levels between milk products on the same pooling day. Maximum concentrations were observed on 2nd or 3rd day after the administration of the drug to ewes. The highest level of EPR was found in hard cheese (14.6 µg EPR $B_{1a}$/kg), closely followed by hard cheese on half of ripening period (12.7 µg EPR $B_{1a}$/kg) and curd (11.5 µg EPR $B_{1a}$/kg). Residues fell below limit of detection (0.1 µg EPR $B_{1a}$/kg) after 30th day, except for hard cheese, albumin (whey) cheese and yoghurt made from pasteurised milk. Whey without albumins and globulins had levels of EPR bellow the limit of detection throughout the entire time of experiment.

**Introduction**
Eprinomectin is s macrocyclic lactone (ML) used as antiparasitic drug in many animal species, including lactating dairy animals. There are several papers presenting fate of MLs in milk and milk products, including EPR in sheep milk and some products.
Researchers showed that MLs tend to partition into fat-rich matrices, such as cheese. If MLs are found in water-rich materials like whey, their concentration is very low. In cheese, MLs such as ivermectin can be found even after 20 days of the drug administration. Residence times for other matrices are lower, usually around 15-20 days.
Levels of ML residues in milk products can be significantly higher than those in raw (bulk) milk. Therefore a cheese, produced from milk that had levels of MLs under limit of detection, could contain MLs in a measurable concentration. Since EPR is allowed for use in milk producing animals and is assigned maximum residue limit (MRL) value, concentration levels of EPR residues in milk products could lead to potential risk for consumers.

## Materials and methods

*Animal experiment*

Thirty ewes of milking crossbreed in the highest lactation period (just after weaning), kept indoors, were used in the experiment. They were given Eprinex® (Merial, Lyon, France) topically along the backline in a narrow strip extending from the withers to the tailhead in a single dose of 1.0 mg EPR B1a/kg b.w. The bulk milk obtained from an evening and morning milking of all animals was used for processing.

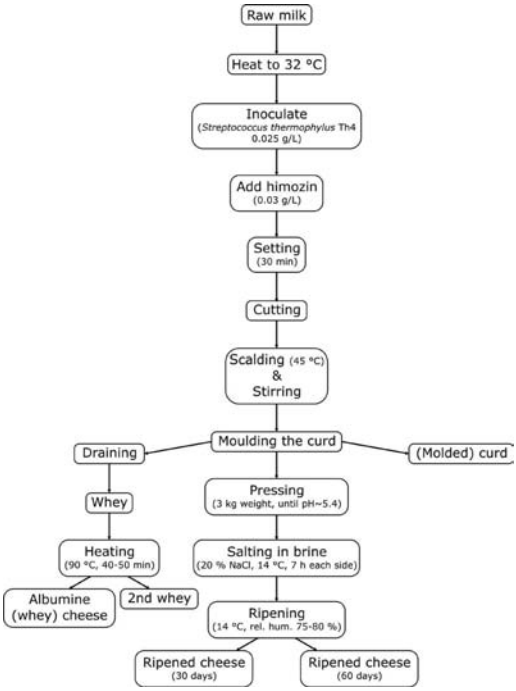


**Figure 1.** Protocol for the manufacturing of cheese samples. "2nd whey" marks whey without albumins and globulins

The first milk sample was taken before EPR administration. After EPR administration, milk was collected on days 1, 2, 3, 4, 7, 10, 14, 18, 23, 30, 37, and 44. In total, 12 daily bulk milk samples were processed during the experiment. An aliquot of each sample was always taken before milk processing and was kept frozen at –20 °C until the analysis.

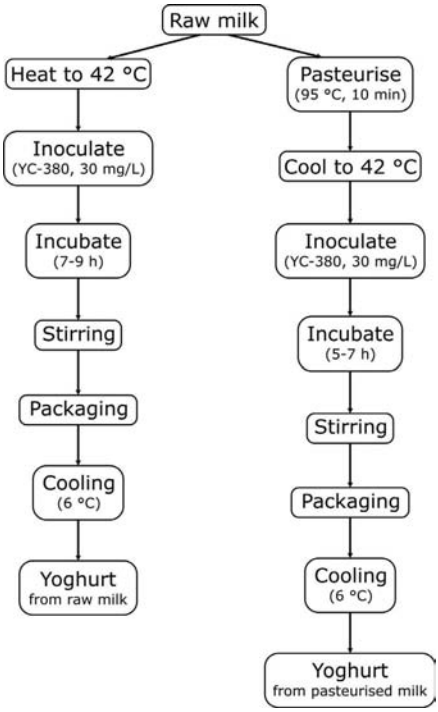


**Figure 2.** Protocol for the manufacturing of yoghurt samples

*Milk products samples*

From daily bulk milk samples collected during the experiment, the following products were made: yoghurt from raw milk, yoghurt from pasteurised milk, curd, hard cheese (samples were also taken at half of ripening time resulting in semi-hard cheese), whey, whey without albumins and globulins and albumin (whey) cheese. Technological protocols are presented in figures 1 & 2. Samples were stored at –20 °C until analysis.

*Analytical determination*

The concentration of EPR in ewes' milk and milk products was determined using a combined HPLC method with fluorescence detection. Sample extraction was performed with acetonitrile followed by SPE clean-up. Cleaned extracts were derivatised to yield compounds appropriate for fluorescence detection. Results were expressed as μg EPR B1a/kg.

Analytical procedure was validated and LOD and LOQ established at 0.1 and 0.2 μg EPR B1a/kg, respectively. Method proved to be linear, robust and repeatable.

**Results**

The fate of EPR residues during ewes' milk processing was studied, including lactic acid fermentation, heating, renneting, salting and ripening (for cheese). Milk products were chosen according to the production in praxis, both industrial and domestic. Because yoghurt from raw (unpasteurised) milk is still common among small private producers, yoghurt made from both raw and pasteurised milk was included in the study.

EPR was excreted into milk of topically treated ewes and residence time of the found residues was long. Time profile of EPR concentrations in the raw (bulk) milk samples of a group of 30 ewes, given a single dose of 1.0 mg EPR B1a/kg b.w. is presented in Table 1 and it can be seen that it essentially determined the subsequent levels in the derived milk products.
EPR residue concentrations in the derived milk products during the experiment are shown in Table 1. A concentration-time profile for milk and its derived products was similar throughout the entire experiment for all matrices, with exception of whey without albumins and globulins (not shown in Table 1) where EPR levels were below the limit of detection from the first day onwards.

**Table 1.**

| Day | Whey | Milk | Yoghurt (raw milk) | Yoghurt (pasteurised milk) | Albumin cheese | Curd | Ripened cheese (30 days) | Ripened cheese (60 days) |
|---|---|---|---|---|---|---|---|---|
| 1. | 0.31 | 2.47 | 2.55 | 2.43 | 5.4 | 10.83 | 7.04 | 11.97 |
| 2. | 0.26 | 3.15 | 3.31 | 2.57 | 5.05 | 11.50 | 12.65 | 14.59 |
| 3. | 0.72 | 3.01 | 3.07 | 3.48 | 9.82 | 11.00 | 9.53 | 9.61 |
| 4. | 0.29 | 2.14 | 2.48 | 2.82 | 4.35 | 6.92 | 7.72 | 9.03 |
| 7. | 0.18[a] | 1.16 | 1.13 | 1.32 | 2.05 | 3.85 | 3.13 | 3.86 |
| 10. | 0.05[a] | 0.58 | 0.59 | 0.63 | 0.92 | 2.42 | 1.74 | 2.60 |
| 14. | < | 0.25 | 0.29 | 0.33 | 0.10[a] | 1.06 | 0.73 | 1.11 |
| 18. | < | 0.13[a] | 0.14[a] | 0.20 | 0.32 | 0.36 | 0.10[a] | 0.44 |
| 23. | < | 0.04[a] | 0.10[a] | 0.12[a] | 0.21 | 0.20 | 0.05[a] | 0.33 |
| 30. | < | < | < | 0.15[a] | 0.25 | 0.15[a] | < | 0.28 |
| 37. | < | < | < | 0.10[a] | 0.16[a] | < | < | 0.14[a] |
| 44. | < | < | < | < | 0.14[a] | < | < | 0.11[a] |

*a … between limit of detection and limit of quantification (semi–quantitative estimate)*
*< … below limit of detection*

The highest EPR residue content was found in 60–day ripened cheese (14.6 µg EPR $B_{1at}$/kg), closely followed by hard cheese on half of ripening period (12.7 µg EPR $B_{1a}$/kg) and curd (11.5 µg EPR B1a/kg). Following the raw (bulk) milk concentration levels, in all matrices investigated, maximum EPR concentrations were found on the second day, with the exceptions of whey, yoghurt made from pasteurised milk and whey proteins (albumin cheese), which had peak concentration on third day after EPR administration. EPR residues fell below the limit of detection (0.2 µg EPR $B_{1a}$/kg) after 23th day following administration of the drug.

**Discussion**

Aim of this project was to determine the effect of technological processes on EPR residue content in milk and derived products. We showed that EPR residues are still present in most dairy products even if raw milk was collected 30 days after EPR administration.
EU Committee for Veterinary Medicinal Products established MRL in bovine milk at 20 µg EPR B1a/kg. This value was derived from calculated allowed daily intake (ADI), which is set

at 5 µg EPR B1a/kg b.w., or 300 µg EPR B1a per person. Even if someone was to consume 20 kg of our most contaminated sample (60-day ripened cheese on 2nd day of experiment), one would still consume less than ADI value.
Important thing to consider here is a concentration factor of EPR between raw milk and its derived products. We showed that levels of EPR residues in milk products can be more that 4.5-times higher that those in milk. Using milk that passes MRL concentration of EPR for further processing could result in milk products with much higher EPR concentrations and would present risk for consumers.

**Acknowledgements**
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## P 36

# DETERMINATION OF MACROCYCLIC LACTONES IN MILK BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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**Abstract**
An analytical method has been developed for the simultaneous determination of the following macrocyclic lactone drugs in milk: ivermectin, abamectin, doramectin, moxidectin, eprinomectin, emamectin and nemadectin. Samples were extracted with acetonitrile, purified with solid-phase extraction on a reversed phase C8, derivatised with N-methylimidazole, trifluoroacetic anhydride and acetic acid to a stable fluorescent derivative, and were further analysed by gradient HPLC on a reversed and deactivated phase Supelcosil LC-8-DB. The method was validated according to the requirements of Commission Decision 2002/657/EC, using fortified raw bovine milk. For the reason of high acceptability regarding the required criteria and applicability also to ovine, caprine and processed milk, giving similar results, this extremely sensitive method has been successfully implemented in the pharmacokinetic research studies as well as statutory residue monitoring in Slovenia.

**Introduction**
Macrocyclic lactones (MLs) are derivatives of the fermentation products of the soil microorganism genus Streptomyces, which are in very low doses extremely effective against -endo and ectoparasites and are probably the anti-parasitic drugs most widely used in food producing animals. Due to their high lipophilicity, MLs are substantially excreted in milk. For the reasons of consumers' safety, their use in animals, from which milk is produced for human consumption, is banned in the European Union, with the exception of eprinomectin and moxidectin, having MRL of 20 µg/kg in bovine and 40 µg/kg in bovine and ovine milk, respectively.
A review over numerous analytical methods for determination of ML residues in biological matrices, including milk, was recently published by Danaher et al. (2006). Liquid chromatography with fluorescence detection of MLs is predominating over MS detection due to high selectivity, sensitivity, and lower cost as well, although a pre-column derivatisation is essential for convertion of parent compounds to fluorescence derivatives.
Using the method described below, residues of eprinomectin (component B1a) (EPR), moxidectin (MOX), emamectin (component B1a) (EMA), nemadectin (NEM), abamectin (component B1a) (ABM), doramectin (DOR) and ivermectin (component H2B1a) (IVM) residues in milk were simultaneously determined. The analytical procedure is a combination of some previously published methods, which were additionally modified regarding the derivatisation and chromatographic separation.

## Materials and methods

*Standard materials*
Reference standards of EPR, EMA, ABM and DOR were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and of IVM from Calbiochem (San Diego, CA, USA). MOX (Cyanamid, Princeton, NJ, USA) and NEM (BASF, Ludwigshafen, Germany) were mediated through CRL (BVL, Berlin, Germany). Standardised solutions were prepared in previously silanised glassware using acetonitrile (MeCN).

*Analytical procedure*
Milk samples (5.0 g) were extracted with 20 mL of MeCN and centrifuged at 3290 g for 10 min. 50 µL of triethylamine was added to 15 mL of MeCN extract, which was further diluted with distilled water to 50 mL and cleaned up using a solid phase extraction (SPE) on Bakerbond C8 cartridges, 500 mg, 6 mL (J.T. Baker, Phillipsburg, NJ, USA). Sample eluates in MeCN were concentrated until dryness at 50 °C under a nitrogen stream and were further derivatised at room temperature with 100 µL of N-methylimidazole solution in MeCN (1:1, v/v), 150 µL of trifluoroacetic anhydride solution in MeCN (1:2, v/v) and 120 µL of glacial acetic acid, and in the meantime diluted to 1.0 mL with MeCN. Samples were further incubated at 65 °C for 50 min and then cooled to room temperature before injection of 20 µL into the HPLC system Agilent Technologies 1100 Series (Palo Alto, CA, USA). Chromatographic separation was performed at 27 °C on a Supelcosil LC-8-DB, 15 cm x 4.6 mm (3 µm particles) analytical column (Supelco, Bellefonte, PA, USA). The mobile phase consisted of two components (A, MeCN : MeOH : H2O = 425 : 425 : 150 (v/v/v) and B, MeCN : MeOH : H2O = 460 : 460 : 80 (v/v/v)), which were mixed at a flow rate of 1.0 mL/min according to a following gradient programme: 0 - 15 min, 100 % A; 15 – 16 min, 100 % A → 100 % B; 16 – 25 min, 100 % B. Fluorescence of the derivatives was detected at excitation and emission wavelengths of 364 and 470 nm, respectively. Calculations were performed according to the external standard method.

*Validation*
Validation was performed with the samples, which did not contain ML (blank material) according to the standard addition procedure. The procedure was validated in accordance with Commission Decision 2002/657/EC, as quantitative confirmation method, considering EPR and MOX as substances with permitted limit in milk, and other substances as not allowed in milk. The following validation parameters were evaluated: selectivity, specificity, linearity, recovery, repeatability, reproducibility, decision limit (CCα), detection capability (CCβ), stability and ruggedness, and in addition limit of detection (LOD) and limit of quantification (LOQ).

## Results and discussion
Modification of the derivatisation step was necessary for a reason of total instability of EPR derivative obtained under conditions suitable for other MLs and was optimised regarding volume of added acetic acid, incubation temperature, and length of incubation. A combination of 120 µL, 65 °C and 50 min, respectively, gave the optimal as well as stable chromatographic peak areas.

At the retention times of MLs there were with exception of MOX, no interfering intrusions, and that is why the method was very selective (Figure 1). In the case of MOX, 10 % negative samples showed background corresponding to approximately 0.1 µg/kg. The specificity between all ML compounds was also clear, and was obtained by programmable mixing of two mobile phase components.

Linearity on a standard level at a wider concentration range, for EPR from 0.0005 – 0.5 µg/mL, for MOX from 0.0005 – 0.75 µg/mL and for EMA, NEM, ABM, DOR and IVM from 0.0005 – 0.25 µg/mL (9 – 11 calibration points) and on a matrix level at two concentration ranges i.e. at a wider concentration range from 0.5 – 60 µg/kg (9 points) and at a lower concentration range from 0.1 – 1 µg/kg (5 points) was evaluated by excellent correlation coefficients (≥0.999).

Recovery and precision of the method were determined on three levels of content that is for EPR 10, 20 and 30 µg/kg (0.5xMRL, MRL and 1.5xMRL), for MOX 20, 40 and 60 µg/kg (0.5xMRL, MRL and 1.5xMRL) and for each EMA, NEM, ABM, DOR and IVM 1, 2 and 3 µg/kg (Table 1). Mean recovery values ranged from 74.9 to 98.8 %, while repeatability and intra-laboratory reproducibility of the measurements, represented by CVr and CVW were from 4.6 to 13.4 % and from 6.6 to 14.5 %, respectively. CVW values were <2/3 of the CVH values after Horwitz equation.

For the CCα of EPR and MOX we determined concentrations of 24.8 and 50.6 µg/kg, respectively. In the retention time windows where other MLs were to be expected there were no disturbing signals and the noise was very low. For this reason we decided to take as CCč value 0.2 µg/kg for EMA and NEM, and 0.1 µg/kg for ABM, DOR and IVM, which were concentrations among the lowest presented in the literature. For the CCčof EPR and MOX we determined concentrations of 27.8 and 55.2 µg/kg, respectively. For the CCčof EMA, NEM, ABM, DOR and IVM concentrations of 0.27, 0.25, 0.14, 0.14 and 0.14 µg/kg were determined, respectively. For the LOD and LOQ of EPR and MOX residue determination we estimated concentrations of 0.2 and 0.3 µg/kg for both substances, respectively. No change of the chromatographic peak area of MLs (as standard solutions or standard additions in blank milk) was observed during 39 hours post derivatisation, so time stability of derivatised final extracts was very good, which significantly contributed to the high flexibility and sample capacity of the method. Moreover, no statistical influence of animal species (bovine, ovine, caprine) or milk processing on method's performance was observed.

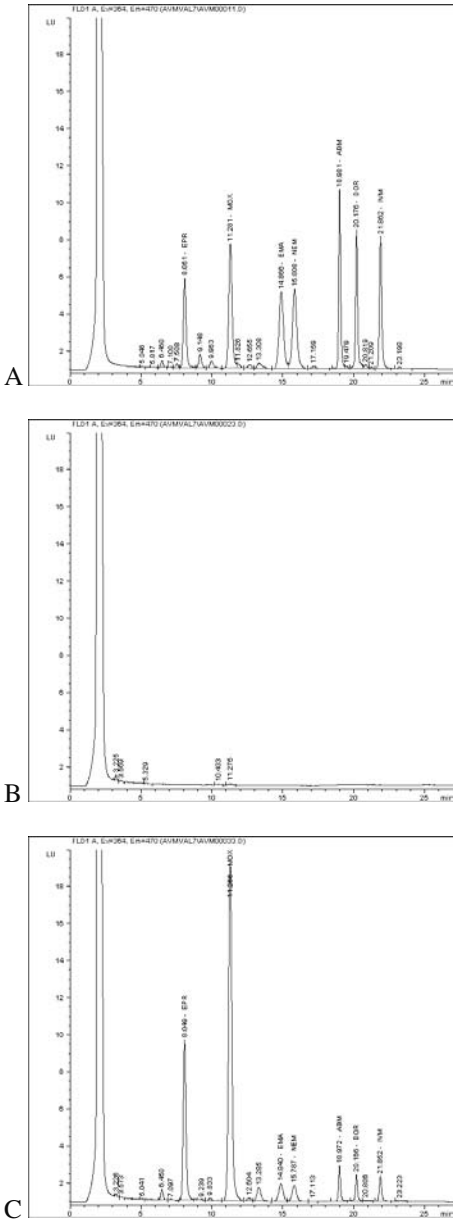

**Figure 1.** Typical chromatograms of ML determination in milk
A standard mixture of individual ML concentration of 0.025 µg/mL
B negative sample
C negative sample with individual ML standard addition of EPR/MOX/EMA/NEM/ABM/DOR/IVM of 20/40/2/2/2/2/2 µg/kg

**Table 1.** Recovery and precision of ML determination in milk

| | Analyte | No of repl. | Level 1 | | Level 2 | | Level 3 | |
|---|---|---|---|---|---|---|---|---|
| | | | Conc. (µg/kg) | | Conc. (µg/kg) | | Conc. (µg/kg) | |
| **RECOVERY** | | | | | | | | |
| Recovery (%) | EPR | 6 | 10 | 94.5 | 20 | 96.3 | 30 | 98.3 |
| | MOX | 6 | 20 | 79.2 | 40 | 79.5 | 60 | 74.9 |
| | EMA | 6 | 1 | 85.7 | 2 | 93.1 | 3 | 97.5 |
| | NEM | 6 | 1 | 91.0 | 2 | 90.4 | 3 | 89.0 |
| | ABM | 6 | 1 | 93.9 | 2 | 97.5 | 3 | 98.8 |
| | DOR | 6 | 1 | 89.7 | 2 | 93.1 | 3 | 93.2 |
| | IVM | 6 | 1 | 93.6 | 2 | 96.8 | 3 | 96.9 |
| **REPEATABILITY** | | | | | | | | |
| Coefficient of variation - $CV_r$ (%) | EPR | 18 | 10 | 10.3 | 20 | 8.9 | 30 | 9.0 |
| | MOX | 18 | 20 | 13.4 | 40 | 12.2 | 60 | 10.8 |
| | EMA | 18 | 1 | 6.7 | 2 | 5.8 | 3 | 5.5 |
| | NEM | 18 | 1 | 8.7 | 2 | 7.4 | 3 | 8.0 |
| | ABM | 18 | 1 | 4.6 | 2 | 6.0 | 3 | 7.2 |
| | DOR | 18 | 1 | 5.4 | 2 | 6.3 | 3 | 7.7 |
| | IVM | 18 | 1 | 7.1 | 2 | 8.5 | 3 | 9.2 |
| REPRODUCIBILITY | | | | | | | | |
| Coefficient of variation (intra-laboratory) – $CV_w$ (%) | EPR | 18 | 10 | 11.0 | 20 | 9.6 | 30 | 13.0 |
| | MOX | 18 | 20 | 13.4 | 40 | 9.0 | 60 | 13.6 |
| | EMA | 18 | 1 | 13.7 | 2 | 14.5 | 3 | 11.4 |
| | NEM | 18 | 1 | 9.9 | 2 | 7.4 | 3 | 9.8 |
| | ABM | 18 | 1 | 11.5 | 2 | 8.4 | 3 | 8.0 |
| | DOR | 18 | 1 | 10.2 | 2 | 8.7 | 3 | 9.4 |
| | IVM | 18 | 1 | 9.5 | 2 | 6.6 | 3 | 7.9 |

With the presented method we successfully participated in Interlaboratory study AVER_07/05 "Avermectins in cow's milk (lyophilised samples)", organised by the EU Reference Laboratory for Residues (CRL) in Berlin. Moreover, the analytical method was successfully accredited in accordance with ISO 17025.

Analytical approach described here also served as a base for the introduction of simultaneous ML residue analysis in other biological matrices, e.g. blood plasma, milk products, fat, muscle tissue and eggs, analysed within the research and monitoring programmes in Slovenia.

**Conclusions**

Presented analytical method for the simultaneous determination of most of MLs in milk has been proved by its working flexibility and sufficient validation parameters according to the required criteria. Moreover, it was substantiated by excellent performance in the inter-laboratory proficiency study, organised by the Community Reference Laboratory, and was further successfully accredited.

**Acknowledgements**

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# HPLC METHOD FOR DETERMINATION OF QUINOLONE RESIDUES IN VARIOUS MUSCLE TISSUES

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**Abstract**
An HPLC analytical method has been developed for the quantitative determination of a wide range of quinolone residues in muscle tissue of various food producing animals. The following substances were simultaneously determined: ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, ofloxacin, orbifloxacin, oxolinic acid and sarafloxacin. Samples were simultaneously extracted with buffer of a pH value of 9.1 and acetonitrile, water phase was then defatted and analysed by gradient HPLC on a polymeric stationary phase and fluorescence detection. According to different chemical structures, quinolones were analysed by three consecutive chromatographic runs, differing in a mobile phase composition, flow rate and detection wavelengths. The method was validated according to the requirements of Commission Decision 2002/657/EC, and has been successfully implemented for confirmatory purposes in the statutory residue monitoring in Slovenia.

**Introduction**
Quinolones and their successors fluoroquinolones are antimicrobial agents, also widely used in veterinary medicine for treatment of pulmonary, digestive and urinary infections as well as treatment of the generalised processes of septicaemia and skin diseases in fish. For the consumers' safety Maximum residue limits (MRLs), for a number of quinolones have been established in the European Union for various animal species and matrices, including meat. The aim of the presented work was development of precise confirmation analysis of all spectrum of the quinolones used in the animal husbandry in line with the Council Regulation (EEC) No 2377/90, as for the screening namely bacteriological approach is used. With the presented method the following twelve quinolones in meat of various food producing animals can be simultaneously determined: ciprofloxacin (CPF), danofloxacin (DAN), difloxacin (DIF), enrofloxacin (EFC), flumequine (FLU), marbofloxacin (MAR), nalidixic acid (NAL), norfloxacin (NOR), ofloxacin (OFC), orbifloxacin (ORB), oxolinic acid (OXO) and sarafloxacin (SAR). The analytical procedure is a combination of previously published methods, which were additionally modified regarding the chromatographic separation.

**Materials and methods**

*Standard materials*
Reference standards of CPF, EFC, FLU, NAL, NOR, OFC, ORB and OXO were purchased from Sigma-Aldrich (Germany), DIF and SAR standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), while DAN and MAR standards were from Pfizer and

Vétoquinol, respectively and were mediated through Community Reference Laboratory (AFSSA, Fougères, France).

*Analytical procedure*
2.5 g samples were extracted by buffer solution with pH 9.1 and acetonitrile (MeCN) using mechanical desintegration and ultrasound. After centrifugation at 3290 g for 10 min, supernatant was evaporated at 50 °C under a stream of nitrogen till the volume of water phase was 2.5 mL, defatted twice by 1.5 mL n-hexane (liquid – liquid extraction), mixed thoroughly, filtrated, and transferred to the vial. HPLC analysis was performed on Agilent Technologies 1100 Series system (Palo Alto, CA, USA), which included polymer analytical column, 150 x 4.6 mm, filled with PLRP-S 100 Å, 3 µm particles connected to the pre-column with the same stationary phase of 5 µm particles (Polymer Laboratories, Shropshire, UK). According to different chemical structures, samples were analysed by three consecutive chromatographic runs, differing in a mobile phase composition, flow rate and detection wavelengths (Table 1).

*Validation*
The method was validated for a quantitative confirmation purpose, according to the requirements of Commission Decision 2002/657/EC, using fortified blank muscle samples. Validation was performed in detail for poultry meat, although some parameters were also evaluated for meat of other food producing animals.

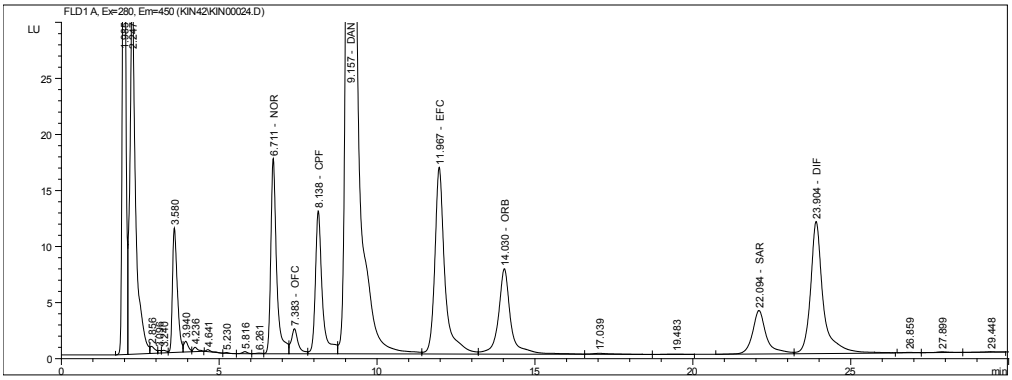
**Results and discussion**
A combination of three HPLC submethods (Table 1) resulted in a very specific and selective determination of all quinolone substances, used in veterinary medicine, as well as some substances used primarily in a human medicine. Optimal residue determination is presented in Figure 1, from which it can be also seen that some quinolones i.e. DAN, ORB, CPF and EFC could be detected by two HPLC working conditions, which gave additional detection options. At the retention times there were with exception of NOR and CPF, no interfering intrusions at all negative tested samples. For NOR nearly for all animal species tested in a certain share of samples (bovine 72 %, porcine 44 %, game 62 %, ovine 33 %, fish 45 %) a background corresponding up to 7.5 µg/kg was observed. For CPF a background corresponding up to 5.7 µg/kg was observed in 8 and 100 % of game and horse samples, respectively.
Linearity on a standard level for two concentration ranges (0.01–1 µg/mL, 0.0001–0.1 µg/mL) was demonstrated by correlation coefficients ≥0.998 and ≥0.988, respectively, while correlation coefficients in poultry, bovine and fish matrices for two concentration ranges i.e. at a wider concentration range from 1–1000 µg/kg and at a lower concentration range from 1–25 µg/kg were 0.987–1.000 and 0.792–1.000, respectively.

**Table 1.** Working conditions on HPLC chromatograph, for the determination of quinolone residues in muscle tissues

| **Parameter** | **Quinolones** | | | | | |
|---|---|---|---|---|---|---|
| | **NOR, OFC, CPF, EFC, SAR, DIF** | | **OXO, NAL, FLU** | | **MAR, DAN, ORB** | |
| Mobile phase composition | Component I (0.02 M $H_3PO_4$:MeCN= 85:15, v/v) and MeCN | | Component II (0.02 M $H_3PO_4$:MeCN:THF= 72:16:12, v/v/v) and MeCN | | Component III (0.02 M $H_3PO_4$:MeCN:THF= 92.5:6.0:1.5, v/v/v) and MeCN | |
| Time programme | 0-16 min | 100 % comp. I | 0-12 min | 100 % komp. II | 0-18 min | 100 % comp. III |
| | 16-28 min (gradient) | 0-10 % MeCN | 12-25 min (gradient) | 0-20 % MeCN | 18-30 min (gradient) | 0-50 % MeCN |
| | 28-29 min (gradient) | 10-0 % MeCN | 25-27 min (gradient) | 20-0 % MeCN | 30-32 min (gradient) | 50-0 % MeCN |
| | 29-31 min | 100 % comp. I | | | | |
| Flow rate (mL/ min) | 0.8 | | 0.7 | | 0.8 | |
| Injection volume (µL) | 30 | | 30 | | 30 | |
| Temperature of pre- and analytical column (°C) | 50 | | 50 | | 50 | |
| Fluorescence wavelengths | $\lambda_{ex}$ = 280 nm $\lambda_{em}$ = 450 nm | | $\lambda_{ex}$ = 326 nm $\lambda_{em}$ = 355 nm | | $\lambda_{ex}$ = 294 nm $\lambda_{em}$ = 514 nm | |

Recovery was determined by using fortified blank poultry matrix on three content levels, that is for quinolones with MRL 0.5 x MRL, MRL and 1.5 x MRL, and for quinolones without MRL LOQ, 2 x LOQ and 3 x LOQ. Each concentration level was analysed within the day in 6 replicates and repeated in two more occasions (n = 18). Mean recovery values ranged from 56.2 to 107.7 % for FLU and ORB, respectively. The second approach was using 20 replicates at MRL value for all animal species (poultry, bovine, porcine, ovine and caprine, rabbits, fish), for the purpose of CCα and CCβ determination (Table 2). Repeatability and reproducibility of the method in poultry was evaluated on the same selected levels as for the recovery determination. CVr values ranged from 3.3 % (SAR) to 28.7 % (FLU) and CVW values ranged from 3.8 (SAR) to 30.6 % (FLU). CVW values were in the majority of the substances less than CVH values after Horwitz equation, while in the cases of DIF and mostly FLU much attention should be taken at a quantitative determination at and above MRL.

A

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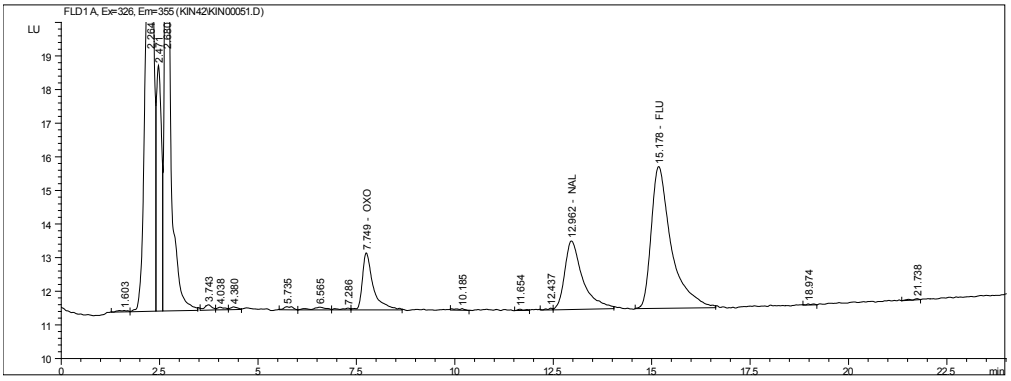


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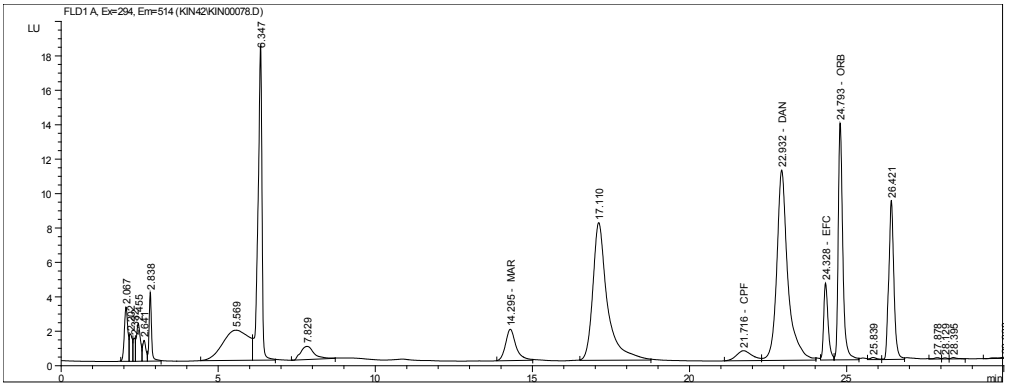


**Figure 1.** Typical chromatograms, representing optimal quinolone residue determination in bovine meat, spiked with individual substance at a concentration of 100 µg/kg (A-determination of NOR, OFC, CPF, EFC, SAR and DIF; B-determination of OXO, NAL and FLU; C-determination of MAR, DAN and ORB)

According to the background interferences, LOQ values ranged from 5 µg/kg (EFC, DIF, DAN) to 30 µg/kg (OFC). For all animal species (poultry, bovine, porcine, ovine and caprine, rabbits, fish), decision limit (CCα) and detection capability (CCβ) values were calculated for the listed substances regarding the particular MRL value and are presented in Table 2. According to unreal low values for the quinolones without MRL in poultry, established by the calibration curve procedure according to ISO 11843, we decided to keep these values, with the exception of NAL, only theoretically, considering LOQ for the real CCα and CCβ values (Table 2).
With the presented method we very successfully participated in Interlaboratory Proficiency Tests 0274 and 0289 "Quinolones and Fluoroquinolones in Chicken Muscle", organised by FAPAS, Central Science Laboratory, Sand Hutton, York, UK.

**Table 2.** Decision limit (CCα), detection capability (CCβ) and LOQ values, together with recovery at MRLs, established at the quinolone residue determination in various muscle tissues

| Substance | Animal Species | MRL | | CCα | CCβ | LOQ |
|---|---|---|---|---|---|---|
| | | MRL value | $Rec_{MRL}$ | | | |
| | | (µg/kg) | (%) | (µg/kg) | (µg/kg) | (µg/kg) |
| NOR | poultry | - | - | 3.05[a] | 5.20[a] | 20 |
| OFC | poultry | - | - | 5.41[a] | 9.21[a] | 30 |
| CPF | poultry | 100 | 77.6 | 104.8 | 109.7 | 20 |
| | bovine | 100 | 72.9 | 104.4 | 108.7 | |
| | porcine | 100 | 73.1 | 105.2 | 110.3 | |
| | ovine and caprine | 100 | 76.4 | 104.2 | 108.4 | |
| | rabbits | 100 | 69.7 | 104.7 | 109.4 | |
| | fish | 100 | 73.3 | 106.2 | 112.3 | |
| EFC | poultry | 100 | 87.2 | 110.5 | 121.0 | 5 |
| | bovine | 100 | 84.5 | 105.7 | 111.5 | |
| | porcine | 100 | 80.8 | 107.3 | 114.6 | |
| | ovine and caprine | 100 | 80.4 | 110.1 | 120.2 | |
| | rabbits | 100 | 77.7 | 105.4 | 110.8 | |
| | fish | 100 | 84.4 | 108.2 | 116.3 | |
| SAR | poultry | - | - | 1.55[a] | 2.64[a] | 10 |
| | fish | 30 | 79.4 | 32.3 | 34.6 | |
| DIF | poultry | 300 | 73.8 | 357.8 | 415.7 | 5 |
| | bovine | 400 | 82.1 | 423.0 | 446.0 | |
| | porcine | 400 | 69.0 | 436.3 | 473.0 | |
| | ovine and caprine | 400 | 68.0 | 462.8 | 525.6 | |
| | rabbits | 300 | 67.5 | 325.8 | 351.5 | |
| | fish | 300 | 78.9 | 326.9 | 353.8 | |
| OXO | poultry | 100 | 86.8 | 112.3 | 124.7 | 20 |
| | bovine | 100 | 52.3 | 104.3 | 108.7 | |
| | porcine | 100 | 42.8 | 104.9 | 109.9 | |

**Table 2, part 2.**

| | | | | | | |
|---|---|---|---|---|---|---|
| OXO | ovine and caprine | 100 | 34.0 | 105.0 | 110.0 | 20 |
| | rabbits | 100 | 52.6 | 104.2 | 108.3 | |
| | fish | 100 | 55.4 | 105.0 | 110.1 | |
| NAL | poultry | - | - | 11.8 | 20.1 | 20 |
| FLU | poultry | 400 | 62.4 | 508.1 | 616.3 | 10 |
| | bovine | 200 | 72.2 | 219.9 | 239.8 | |
| | porcine | 200 | 50.1 | 228.9 | 257.8 | |
| | ovine and caprine | 200 | 68.8 | 240.5 | 280.9 | |
| | rabbits | 200 | 55.5 | 227.3 | 254.6 | |
| | fish | 600 | 78.1 | 686.0 | 772.0 | |
| MAR | poultry | - | - | 3.22[a] | 5.48[a] | 20 |
| | bovine | 150 | 77.7 | 157.6 | 165.3 | |
| | porcine | 150 | 78.4 | 160.1 | 170.3 | |
| DAN | poultry | 200 | 82.6 | 211.8 | 223.5 | 5 |
| | bovine | 200 | 77.8 | 211.5 | 223.0 | |
| | porcine | 100 | 76.4 | 107.1 | 114.1 | |
| | ovine and caprine | 200 | 78.2 | 212.3 | 224.5 | |
| | rabbits | 100 | 75.2 | 107.3 | 114.6 | |
| | fish | 100 | 86.7 | 108.9 | 117.7 | |
| ORB | poultry | - | - | 1.76[a] | 3.00[a] | 10 |

**theoretical value, LOQ is considered as a realistic value*

**Acknowledgements**
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# Comparison of three microbial screening methods for antibiotics using routine monitoring samples

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**Abstract**
Monitoring large numbers of slaughter animals for the presence of antimicrobial residues is preferably done using microbiological screening methods, because of their high cost-effectiveness. An evaluation of the Nouws Antibiotic Test (NAT) was performed on routine monitoring samples and the performance of the method was compared with two other microbial screening methods: Screening Test for Antibiotic Residues (STAR) and Premi®Test. Analysis of 591 samples yielded four MRL violations. Three of them concerned tetracyclines that were only detected with the NAT and the STAR method. The fourth, 172 µg/kg Sulphadiazine, was detected by all three methods. Additionally, 156 µg/kg Tulathromycin was found in porcine meat, while for this residue no MRL in meat has been established.

**Introduction**
Monitoring large numbers of slaughter animals for the presence of antimicrobial residues is often done using microbiological screening methods, because of their high cost-effectiveness. For these methods often only validation data obtained from antibiotic standard solutions are available. It is highly questionable to what extent these data can be considered representative for the performance of a test on incurred tissue. Therefore an evaluation of the Nouws Antibiotic Test (NAT) was performed on samples analysed within the framework of a Dutch national monitoring plan and the performance of the test was compared with two other microbial screening methods: Screening Test for Antibiotic Residues (STAR) and Premi®Test.

**Materials and Methods**
During a period of five months 591 slaughter animals (porcine (423), veal (143) bovine (18), five sheep, one horse and one goat) were analysed with three microbiological screening methods. The NAT is a system comprising three tests: first the NAT-screening is performed using paperdisks impregnated with renal pelvis fluid (Pikkemaat et al. 2008). This test exists of five test plates, specific for either tetracyclines (T), macrolides & beta-lactam antibiotics (B&M), quinolones (Q), sulphonamides & diaminopyrimidines (S) or aminoglycosides (A). When a sample is positive on one of these plates, a post-screening is performed using meat fluid (NAT-meat test) and/or kidney (NAT-kidney test). These post-screening tests are similar to the screening method, but the test plates have been additionally optimized for these specific matrices. Post-screening is only performed on the type of plate that gives a positive NAT-

screening result. Details on these post-screening tests and their validation will be published elsewhere. Results are registered as the diameter of the inhibition zone, including the sample hole diameter of 14 mm.
STAR was performed according to Gaudin et al. (2004). This method also comprises five test plates, each one preferentially sensitive for one or two families of antibiotics: Bacillus subtilis/pH 7.2 (Bs7.2) for detection of aminoglycosides, Kocuria rhizophila/pH 8 (Kv8) for detection of macrolides, Bacillus cereus/pH 6 (Bc6) for tetracyclines, Escherichia coli/ pH 8 (Ec8) for quinolones and Bacillus stearothermophilus/pH 7.4 (Bst) for the detection of sulphonamides and beta-lactams. Meat samples were obtained with a cork borer and were subsequently cut in slices of approximately 2 mm. Results were registered as the diameter of the inhibition zone including the meat disc, which had a diameter of 12 mm. Premi®Test was performed according to the manufacturers instructions.
Samples showing a positive result in any of the three methods were subjected to chemical confirmation. Depending on the test plate giving the positive result, meat was analysed for the presence of tetracyclines, quinolones, macrolides, sulphonamides, penicillins or aminoglycosides. STAR-Bst and Premi®Test positive samples were analysed for penicillin and sulphonamide residues. Kidney was only analysed for the presence of aminoglycosides. Samples were analysed using an LC system coupled to a triple quadrupole mass spectrometer with electrospray ionization source operated in positive mode (sulfonamides, quinolones, aminoglycosides, tetracyclines and macrolides) or negative mode (penicillins). Detection limits were as follows: Sulphonamides 10 µg/kg; Tetracyclines 10 µg/kg; Flumequine 20 µg/ kg, Ciprofloxacin and Enrofloxacin 10 µg/kg; Aivlosin, Tilmicosin and Valnemulin 12.5 µg/ kg, Tylosin, Tiamulin, Pirlimycin and Tulathromycin 25 µg/kg, Erythromycin and Josamycin 50 µg/kg; Amoxicillin, Penicillin and Nafcillin 5 µg/kg, Ampicillin and Dicloxacillin 10 µg/kg, Cloxacillin and Oxacillin 15 µg/kg. Detection limits for the aminoglycosides in meat were: Kanamycin and Gentamicin 25 µg/kg, (DH-)Streptomycin, Spectinomycin, Paramomycin and Neomycin 125 µg/kg, Apramycin 250 µg/kg. For aminoglycosides in kidney they were: Kanamycin 625 µg/kg, Gentamicin and (DH-)Streptomycin 250 µg/kg, Paramomycin 375 µg/kg, Spectinomycin and Neomycin 1250 µg/kg, Apramycin 5000 µg/kg.

**Results and Discussion**

Of the 591 slaughter animals that were tested, 64 gave a positive screening result in one or more of the tests. After chemical confirmation, four MRL violations were established: Tetracycline 214 and 187 µg/kg, Doxycycline 130 µg/kg and Sulphadiazine 172 µg/kg (the MRLs for tetracyclines and sulphonamides in meat are 100 µg/kg). Additionally a porcine sample containing 156 µg/kg Tulathromycin was encountered, but statutorily this is not an MRL violation, because only MRLs in fat (100 µg/kg) and liver and kidney (3000 µg/kg) have been established. Results of the individual tests are shown in Table 1.

**Table 1**. Results of the analysis of 591 slaughter animals

| | NAT-screening | | | | | STAR | | | | | Premi®Test |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 'Suspect' samples | 40 | | | | | 36 | | | | | 6 |
| Plate type | T | B&M | Q | S | A | Bc6 | Kv8 | Ec8 | Bst | Bs7.2 | |
| positive | 29 | 1 | 0 | 1 | 9 | 17 | 5 | 1 | 16 | 3 | |
| MRL violations identified | 4 | | | | | 4 | | | | | 1 |
| Presence of residues confirmed by LC-MS/MS analysis | 23 tetracyclines<br>1 sulphonamide<br>1 macrolide<br>4 aminoglycosides (in kidney) | | | | | 16 tetracyclines<br>1 sulphonamide<br>1 macrolide<br>1 β-lactam | | | | | 1 sulphonamide<br>1 macrolide |
| No residues found with LC-MS/MS analysis | 11 | | | | | 18 | | | | | 4 |

The NAT-screening showed 40 positive results, all of them apparent on only one of the five test plates. STAR showed positive results for 36 animals on one or more test plates. Four samples showed inhibition on two plates, one sample even on three plates. In four out of five cases this could be attributed to the presence of Doxycycline, which appeared on the Bc6 plate, but in some cases also on the Bst and/or Bs7.2 plate as well. Also the Tulathromycin containing sample was detected on both Bc6 and Bst (but not on Kv8). In all cases the inhibition zone on the Bc6 plate was the largest.

Tetracylines were obviously the most important group of residues encountered in this monitoring program. Ultimately, 26 animals were confirmed to contain tetracycline residues in their meat. STAR as well as NAT includes a specific plate for the detection of tetracyclines and this group of residues is detected effectively by both tests. All positives on the STAR Bc6 plate could be confirmed to contain tetracycline residues (except for the inhibition that was caused by the Tulathromycin sample). Although eventually only three out of 16 were actual MRL violations, it could be concluded that any sample showing inhibition on Bc6 should be subjected to chemical confirmation, since the sample containing 214 µg/kg Tetracycline showed a diameter of 17.5 mm (the sample containing 187 µg/kg on the other hand showed a diameter of 22.1 mm) and one of the two samples containing 82 µg/kg Oxytetracycline was not detected (while the other showed 24 mm).

The NAT tetracycline plate showed even more positive samples (29) of which 23 were confirmed by LC-MS/MS. Because of this high number of suspect samples and the absence of any correlation between residue concentration and inhibition, in practice the NAT-screening is followed by a microbiological post-screening. This post-screening is based on the analysis of meat juice or kidney juice. Of the 23 initial positive results for tetracyclines, 11 and 17 were still positive in the NAT-meat and NAT-kidney respectively. Although the post-screening kidney yielded more positive samples, the correlation between the inhibition zones found on the NAT-kidney and the actual concentration in the meat, was much better.

This provided the opportunity to establish a reliable NAT-kidney cut-off inhibition zone, above which the samples require chemical confirmation. Applying this cut-off reduced the number of 'suspect' samples to 9.
A problematic plate in terms of causing false positive results was the STAR Bst plate. This plate showed 16 positive results: two could be attributed to the presence of Doxycycline, and also the Sulphadiazine MRL violation and the Tulathromycin containing sample showed inhibition on this particular plate. The remaining 12 were analysed for the presence of sulphonamides and penicillins, but only for one sample the presence of a low concentration of Cloxacillin could be established. Because of the high incidence of false positives on this particular plate, Fusilier et al. (2000) suggested a cut-off inhibition zone of 4 mm from the edge of the meat disk for this particular plate, which would in this study have reduced the number of false positives to six.
The five samples showing small (up to 19.5 mm) inhibition zones on the STAR Kv8 plate, were analysed for the presence of macrolides, but all turned out negative. The only macrolide that was encountered in this study, Tulathromycin 156 µg/kg, was uniquely identified as a macrolide by the NAT. The only sample that tested positive on the STAR Ec8 plate for quinolones, was chemically analysed for this residue group, but appeared negative.
A major difference between NAT and STAR, primarily caused by the investigated matrix, concerns the detection of aminoglycosides, which were only found by the NAT. Aminoglycosides are specifically bound in the renal cortex and released from this tissue only very slowly, causing detectable residue levels for months after treatment. The NAT-screening showed 9 suspect samples on the aminoglycoside test plate, which were also positive on the post-screening kidney. In four of these, the presence of residues could be confirmed by LC-MS/MS. They contained 2700 and 2300 µg/kg Neomycin, 560 µg/kg DH-streptomycin and 570 µg/kg Gentamicin, so all of them were approximately half (kidney) MRL levels. Interesting, no detectable residue levels in the meat of these animals were found. This observation may have implications for microbiological screening systems based on the analysis of kidney samples and using B. subtilis as a test organism (Nouws et al. 1988). The sensitivity of this microorganism to aminoglycosides may lead to unjust condemnation of meat based on positive screening results for the accompanying kidney.
The Premi®Test gave 6 positive results, of which four were only observed by this specific method. Although two of these four were clearly beta-lactams, since the addition of penicillinase counteracted the inhibition, non of them could be identified by LC-MS/MS. The remaining two were the samples containing 172 µg/kg Sulphadiazine and 156 µg/kg Tulathromycin.

**Conclusions**

NAT-screening and STAR are multi-plate test systems. Compared to a tube-test like the Premi®Test, this type of test is more laborious and requires a longer incubation time. The plate tests do on the other hand track down more residue containing samples. NAT and STAR yielded a comparable number of suspect samples. Both methods effectively detected the four MRL violations encountered in this study, while the Premi®Test was not capable of detecting the tetracycline MRL violations.
An additional advantage of the multi-plate methods is their capability to narrow down the antibiotic family identity of a residue. This significantly reduces confirmatory efforts and

costs. Group identification was unambiguous with NAT, although it must be remarked that the spectrum of residues encountered in this study was still limited. No quinolone residues were found at all, leaving the performance of the tests with respect to this residue group unexplored.

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# FLOW CYTOMETRIC IMMUNOASSAY FOR SULFONAMIDES IN MILK, BLOOD SERUM, MEAT DRIP AND EGGS.

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**Abstract**
A flow cytometry-based inhibition immunoassay (FCI) for sulfonamides was developed using the Luminex 100 flow cytometer in combination with the MultiAnalyte Profiling (xMAP) technology. The FCI included a previously developed biotinylated multi-sulfonamide mutant antibody in combination with fluorescent beads, coated with a sulfathiazole derivative, and streptavidin-phycoerythrin (SAPE) for the detection. In milk and blood serum, this FCI detected at least 11 different sulfonamides which was comparable to the results obtained with a previously developed biosensor immunoassay (BIA) format using the same antibodies. In egg extract, this number reduced to 6 due to extraction efficiencies. Sample preparation procedures for serum, skimmed milk and meat drip were simple (dilution in buffer only). Prior to the analysis of egg extracts, the egg protein avidin had to be removed by ultra-filtration because of its binding to the biotinylated antibodies. Although the FCI procedure included an one hour incubation step, this format is, compared with the BIA, more suitable for multiplex detection (combination of up to 100 assays) and high volume testing. The time necessary for the flow cytometer to measure the 96 wells (10 sec/well) was about 30 min, which is much faster than the BIA (5 min/sample = 8 h per plate).

**Introduction**
Multiplex flow cytometry-based immunoassays (FCI's) are relatively new and, so far, mainly used in clinical diagnostics and for blood analysis. In food analysis, this is a new technology and the Luminex format was previously applied for the development of a multi-sulfonamide FCI in milk (Keizer de et al., 2008). For drug residue measurements, different sample materials are of interest and, in the present study, the influences of some materials (milk, blood serum, meat drip and eggs) on the multi-sulfonamide FCI were investigated and the sensitivities of this FCI towards different sulfonamides were compared with those obtained in a previously described biosensor immunoassay (BIA) using the same antibodies (Bienenmann-Ploum et al., 2005).

## Materials en Methods

*Materials and instrument*

Sulfatroxazole was a gift from Leo Pharmaceutical Products (Weesp, the Netherlands) and the other sulfonamides were obtained from Serva (Heidelberg, Germany) or Sigma-Aldrich (Zwijndrecht, The Netherlands). The HBS-EP buffer was supplied by GE Healthcare (Uppsala, Sweden) and Streptavidin R-Phycoerythrin conjugate (SAPE) by Molecular Probes (Leiden, The Netherlands). Other chemicals were obtained from VWR International (Amsterdam, The Netherlands). Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, UK) delivered the Luminex 100 IS 2.2 system (consisting of a Luminex 100 analyzer, a Luminex SD sheath fluid delivery system and a Luminex XY Platform), the StarStation System control software, the Luminex carboxylated microspheres (beads) no. 015 and sheath fluid. The 96-wells EIA flat-bottom microplates were supplied by Greiner bio-one (Alphen a/d Rijn, The Netherlands). Flat-bottom 96-wells filter-bottom microplates equipped with nonsterile, 1.2 µm hydrophil low protein binding Durapore® Membrane and Microcon YM-30 centrifugal filter devices were obtained from Millipore (Billerica, USA). Previously described were: the preparation of the sulfathiazole derivative (N1-[4-(carboxymethyl)-2-thiazolyl] sulfanilamide (TS)) (Haasnoot et al., 2000) and of the ovalbumin-TS (OV-TS) and TS-coated beads (Keizer de et al., 2008) and the development of the biotinylated mutant multi-sulfonamide antibodies (M.3.4) (Korpimäki et al., 2004), kindly provided by Prof. Timo Lövgren (Department of Biotechnology of the University of Turku (Turku, Finland)).

*Flow cytometric immunoassay*

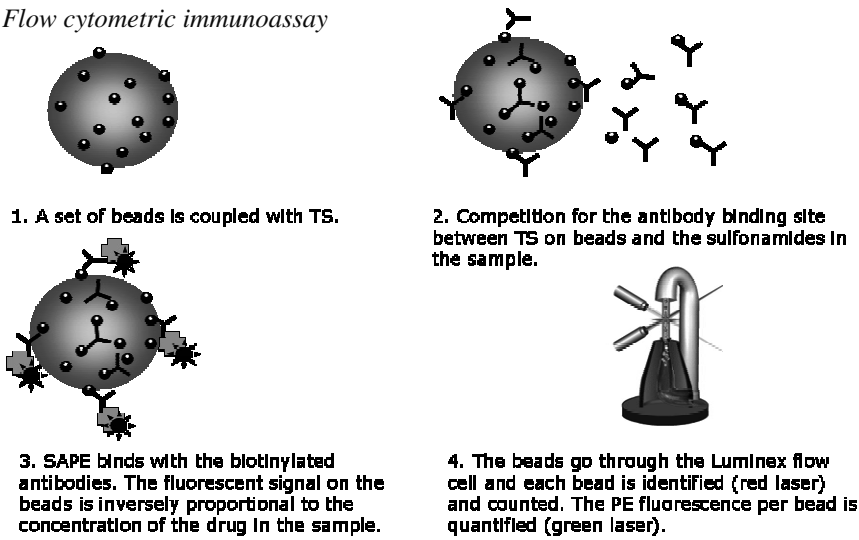


**Figure 1:** Principle of the FCI.

The principle of the Luminex's xMAP technology is described separately (Haasnoot et al., 2008). In this inhibition FCI (Figure 1), the beads were coated with OV-TS or directly with TS (Keizer de et al., 2008) and the coated bead suspension was diluted (about 15 times). In a 96-wells microplate or filter-bottom microplate, 10 µL of this diluted bead suspension,

100 µl of sample (undiluted egg filtrate or 5 times diluted meat drip or 10 times diluted blood serum or milk) and 10 µL of the 200 times diluted antibody solution were added. This mixture was incubated on the microplate shaker in the dark for 1 h which was followed by the addition of 25 µL of a SAPE solution (10 µg/mL). In the case of the use of a filter-bottom microplate, a washing step with HBS-EP was included followed by the addition of 125 µL of SAPE solution (2.5 µg/mL). Subsequently the plate was incubated for 30 minutes prior to the measurements in the Luminex analyzer (about 100 beads/well in 10 s). All dilutions were performed in HBS-EP, unless otherwise stated.

*Sample preparation.*
*Egg.* One gram of homogenized egg was mixed (vortex) with 1 mL of actronitril/HBS-EP (1:1; v/v) and after centrifugation (2500 g for 15 min.), 100 µL of the supernatant was mixed with 400 µL of HBS-EP and transferred to a Microcon YM-30 centrifugal filter device. After centrifugation (5000 g for 15 min.), 100 µL of the egg filtrate was used in the FCI.
*Milk powder.* To 0.5 gram of milk powder, 4.5 mL of demineralised water was added. After 1 hour at the Rock and Roller, this milk was 10 times diluted and 100 µL was used in the FCI.
*Meat drip.* In a 50 mL tube, 10 gram of grounded blank chicken meat was incubated during 20 minutes in a water bath of 60°C. The tube was centrifuged (15,000 g for 10 minutes) and the supernatant (meat drip) was filtered (0.45 µm) and 5 times diluted of which 100 µL was used in the FCI.

**Results**
Sofar, the multiplex flow cytometric technology of Luminex was mainly used for the detection of larger molecules (proteins or DNA/RNA) and coupling protocols to the beads were available for these larger molecules only. Therefore, during the initial assay development, the beads were coated with OV-TS using the standard protein coupling protocol (Luminex, 2008). With these beads, the calibration curves for sulfamethoxazole in milk and meat drip showed 50% inhibition at 81 and 64 ng/mL, respectively (Figure 2). However, these beads were unstable which was noticed by a reduction of the maximum signal during a 6-month storage period. Therefore, a new protocol for the direct coupling of TS to the beads was developed and this resulted in a better stability and sensitivity for the detection of sulfonamides in milk (Keizer de et al., 2008). The assay worked well with skimmed milk after a 10 times dilution. For the application with raw whole milk, a 96-wells microplate-based filtration step was applied to lower the disturbance of the abundantly present milk fat particles (15 x 109/mL) with sizes (3-4 µm) close to the Luminex bead size (5.6 µm).
The assay performance was tested with other sample materials (Figure 2).

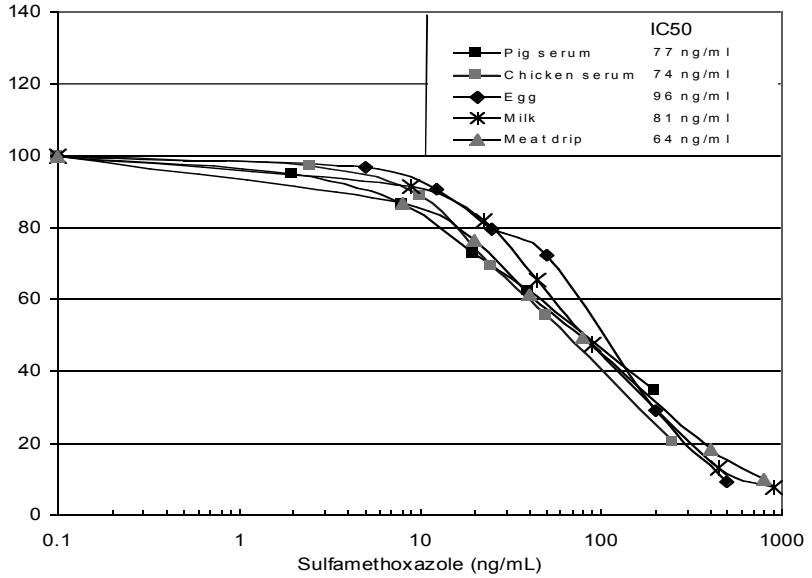


**Figure 2:** Calibration curves of sulfamethoxazole in different sample materials.

The application of 2 times diluted pig and chicken blood serum resulted in significant reductions of the maximum signal compared with HBS-EP buffer (60 %). This was probably due to non-specific binding of serum proteins to the beads which decreased the accessibility of the TS. After a 10 times dilution of the serum in HBS-EP buffer, the problem was sufficiently reduced and the maximum signal was about 2000 MFI. Meat drip showed less non-specific binding and could be used after a 5 times dilution.
Another challenge was the detection of sulfonamides in eggs which contain avidin which interact with the biotinylated antibodies leaving no binding site for the streptavidin-containing fluorescent label (SAPE). A purification step with a Microcon YM-30 centrifugal filter device (cut-off 30 kDa) was sufficient to separate the avidin (67 kD) from the filtered egg extract.
A blank chicken serum was spiked with different sulfonamides at the 100 ng/mL level and analysed, after a 10 times dilution, with a previously developed BIA (Bienenmann-Ploum et al., 2005) and the FCI under comparable conditions. In both assays, 11 sulfonamides showed an inhibition of >50% (Figure 3) whereas the FCI showed about 10 % less inhibition for almost all sulfonamides at this level. The multi-sulfonamide FCI was tested for the sensitivities towards several sulfonamides in different sample materials (Figure 4). In general, the sensitivities in pig serum were, as expected, comparable with those in chicken serum. The sensitivities in milk were tested previously and were comparable with those in serum (Keizer de et al., 2008).

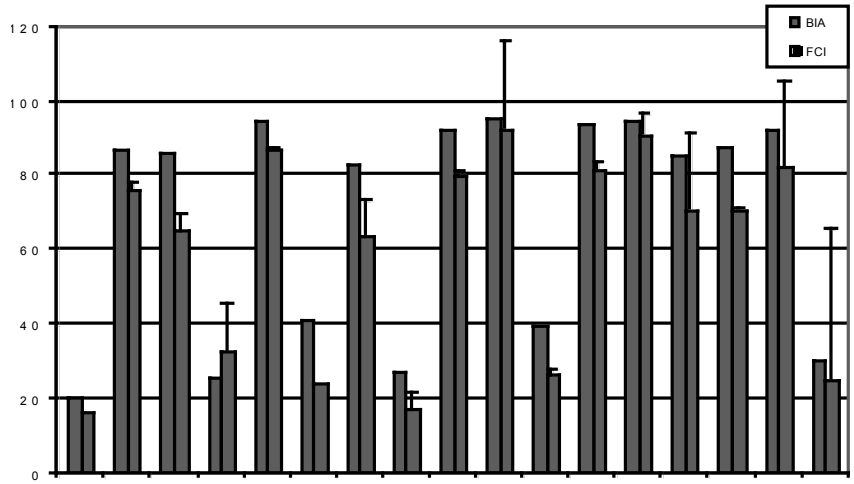


**Figure 3:** Sensitivity of the multi-sulfonamide assays for different sulfonamides (expressed as percentages of inhibition of the maximum signal) measured with BIA and FCI in 10 times diluted chicken serum.

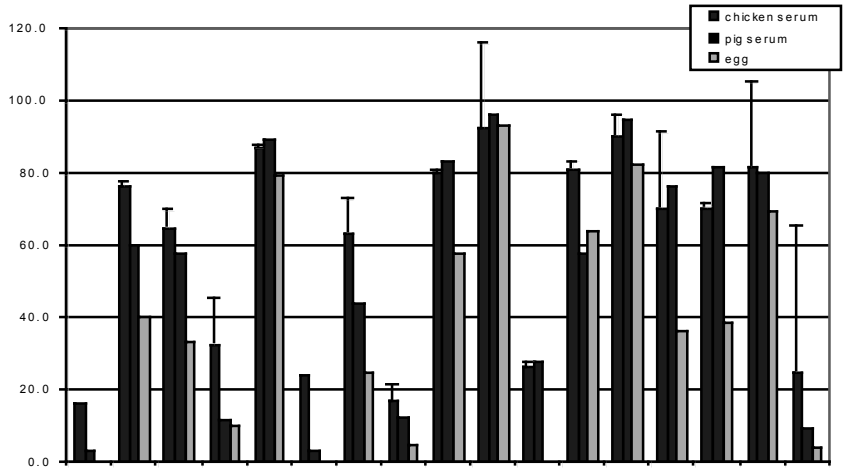


**Figure 4:** Sensitivities of the multi-sulfonamide FCI for different sulfonamides (expressed as percentages of inhibition of the maximum signal) in different sample materials.

Meat drip caused less interference than serum and therefore it was not expected that the sensitivities would differ. Blank egg samples were also spiked with different sulfonamides at the 100 ng/mL. The average inhibition appeared to be 21% less compared with those in chicken serum. This was caused by the extraction procedure which had an average recovery of 62% for sulfamethoxazole.

**Discussion and Conclusions**

The performance of the multi-sulfonamide FCI in blood serum was almost comparable with the previously developed BIA using the same recombinant mutant antibodies (M.3.4). The FCI worked well in pig serum, chicken serum, egg extract, milk and meat drip with IC50 values for sulfamethoxazole of 77, 74, 96, 81 and 64 ng/mL, respectively. In serum, milk and probably meat drip, 11 different sulfonamides could be detected at the 100 ng/mL (MRL) level (>50% inhibition). In egg extract, this number reduced to 6 due to the extraction efficiency. The multi-sulfonamide FCI is insensitive for sulfamethazine which might be solved in the future by the combination with another multi-sulfonamide assay (duplex) which was proven to be very sensitive towards this sulfonamide and others (Bienenmann-Ploum et al., 2005).

**Acknowledgements**

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# MULTIPLEX FLOW CYTOMETRIC IMMUNOASSAY FOR DRUG RESIDUES USING THE xMAP TECHNOLOGY

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**Abstract**
A new screening system for multiplex detection uses the combination of flow cytometry with the MultiAnalyte Profiling (xMAPTM) technology. This technology, developed by Luminex (Austin, Texas, USA), uses polystyrene microspheres (5.6 µm beads) with carboxyl groups on the surface for covalent coupling of ligands. The beads are internally dyed with a red and infrared dye. By varying the ratio of the two fluorophores, up to 100 different bead sets can be distinguished and each bead set can be coupled to a different biological probe. A dual laser instrumentation system allows both the identification of the bead set by its characteristic colour and the quantification of the amount of fluorescent dye corresponding with the amount of reporter molecules (antibodies) bound to the beads. Thus, theoretically, this combination makes it possible to simultaneously measure up to 100 different biomolecular reactions in a single well. Preliminary results obtained with a fourplex flow cytometric immunoassay (FCI), combining three specific aminoglycoside FCI's with the multi-sulfonamide FCI, show that multiplexing in milk and chicken blood serum at relevant levels is feasible.

**Introduction**
A general comment about immunoassays is that they are too specific. For each compound or group of compounds, a different immunoassay has to be performed. Therefore, nowadays, multiplex screening is popular and, as examples, multi-channel biosensor applications have been described for the detection of sulfonamides (Bienenmann-Ploum et al., 2005) and aminoglycosides (Haasnoot et al., 2003) and multiplex biochips are commercially available for the detection of arrays of anti-microbials, growth promotors and synthetic steroids (Randox, UK). However, these multiplex platforms are expensive and/or not flexible for the development of in-house applications.
A new and more flexible open-architecture multiplex format uses a flow cytometer in combination with the MultiAnalyte Profiling (xMAP) technology (Luminex, Austin, Texas, US) and the principle and a model multiplex application for drug residues detection are described in the present paper. Preliminary results obtained with a fourplex flow cytometric immunoassay (FCI) for the combination of three aminoglycoside assays with a separately developed multi-sulfonamide FCI (Bienenmann-Ploum et al., 2008) are presented.
Principle of the Luminex's xMAP technology
The Luminex system is a computer-controlled, bench-top instrument, which contains the complete fluidics and optics system required to measure bead-associated fluorescence (Figure 1). In order to make accurate measurements, the beads must be measured one at a time

and, therefore, must travel single-file through the point of laser interrogation. The method of achieving this ordered stream is known as hydrodynamic focusing (Figure 1A) which is created in a flow cell by injecting a slow-moving sample stream into a faster moving "sheath" fluid (generally PBS) stream.

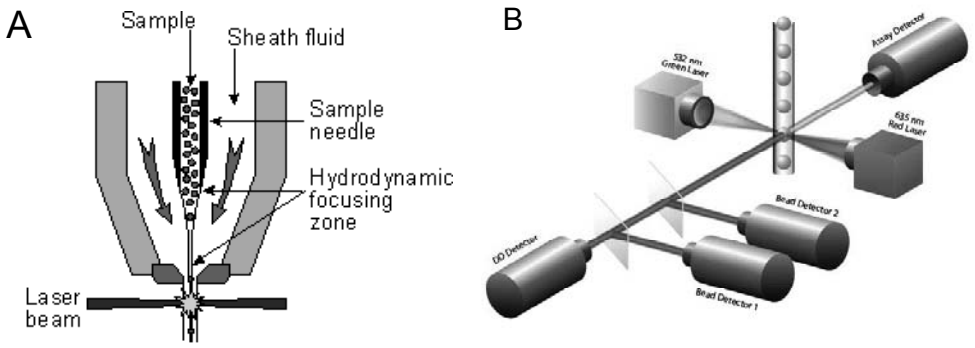


**Figure 1:** Fluidics (A) and optical part (B) of the Luminex flow cytometer.

Careful control of the velocity of the two streams allow for fine control of the width of the centre stream and, therefore, the alignment of the beads within the centre stream.

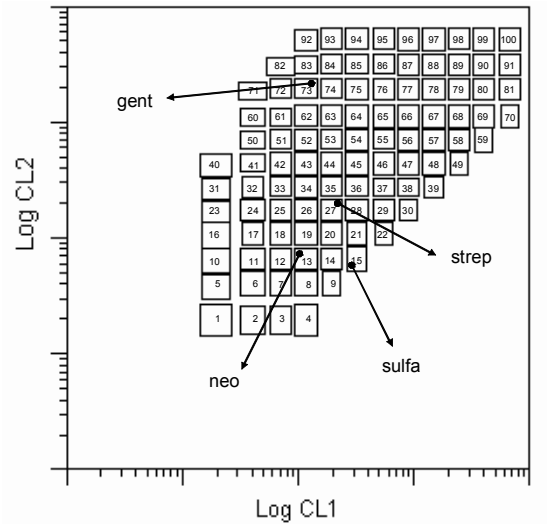


**Figure 2.** Bead map showing the 100 different bead sets and the 4 sets used in this study.

This flow cytometry is combined with the MultiAnalyte Profiling (xMAP) technology of Luminex that uses carboxylated microspheres (5.6 µm) internally dyed with precise ratios of two fluorophores to create 100 different bead sets and each bead set can be distinguished (Figure 2) based on its internal dye ratio using the red laser (635 nm) and two bead detectors (Figure 1B) with wavelengths of 657 and 730 nm. Antibodies or antigens are bound to the

carboxylated bead surface and serve as targets for the green fluorescent (e.g. R-Phycoerythrin (PE))labeled reporter molecules (e.g. antigens or antibodies) to be measured with the green laser (532 nm) and assay detector (580 nm). This combination allows multiplexing of up to 100 unique assays within a single sample.
Since 2007, most of these beads are also available with magnetite encapsulated in the outer coat (MagPlexTM-C Microspheres) enabling easier assay protocols. By introducing a third dye, the three-dimensional multiplexing system (FlexMAP 3D) is built, which will allow for multiplexing of up to 500 tests.

**Materials and methods**
Monoclonal antibodies (Mabs) against neomycin (G 13021M) and gentamicin (G 13032M) were obtained from Biodesign International (Saco, ME, USA). The anti-streptomycin Mab (F62) was previously developed within our organisation and the origin and performance of the recombinant multi-sulfonamide antibody (Rab M.3.4) in a biosensor was described previously as well (Bienenmann-Ploum et al., 2005). Goat anti-mouse (GAM) PE was supplied by Prozyme (Kempen, Belgium) and streptavidin-PE (SAPE) by Molecular Probes (Leiden, The Netherlands). Different bead sets (see Figure 2) were coated, according to a standard Luminex protocol for proteins (Luminex, 2008), with ovalbumin-aminoglycoside conjugates, prepared according to a previously described procedure (Haasnoot et al, 1999), and directly with a sulfonamide (sulfa) derivative (TS) as described previously (Keizer de, 2008). Of these four bead sets, a bead-mixture was prepared which contained about 1500 beads of each set in 40 µl.
In the final fourplex format, to 100 µl of HBS-EP buffer or standard solution or diluted sample (10 times diluted skimmed milk or blood serum), 20 µl of antibody mixture in HBS-EP (250 times diluted MAbs and 50 times diluted Rab) were added followed by 40 µl of the bead-mix. After 1 h incubation, 25 µl of diluted PE (a mixture of GAM-PE and SAPE) were added and after 30 min incubation, the beads were measured in the Luminex (10 µl/well in 10 sec) aiming for 100 beads/set.

**Results and discussion**
Typical results obtained with the fourplex for a skimmed blank milk sample in the Luminex are presented in Figure 3. Figure 3A is the doublet discriminator plot and shows the total amount of events (673) which are detected by the doublet discriminator (DD) detector (Figure 1B). It is used to discriminate primarily upon bead size and to set a gate to ensure that only events caused by single beads are analysed. This gate essentially determines which events are passed to the classifier plot (Figure 3B). In this plot, the number of events in the selected region (singlets) are presented (464), and it identifies which test each bead belongs to by placing them in the four different rectangular bead regions. The individual reporter plots (Figure 3C to F) give the number of events per bead region (from 89 events for Genta to 147 events for Neo) and the amount of fluorescence due to bead surface bound reporter molecules from which the median fluorescence intensity (MFI) per bead set is calculated.

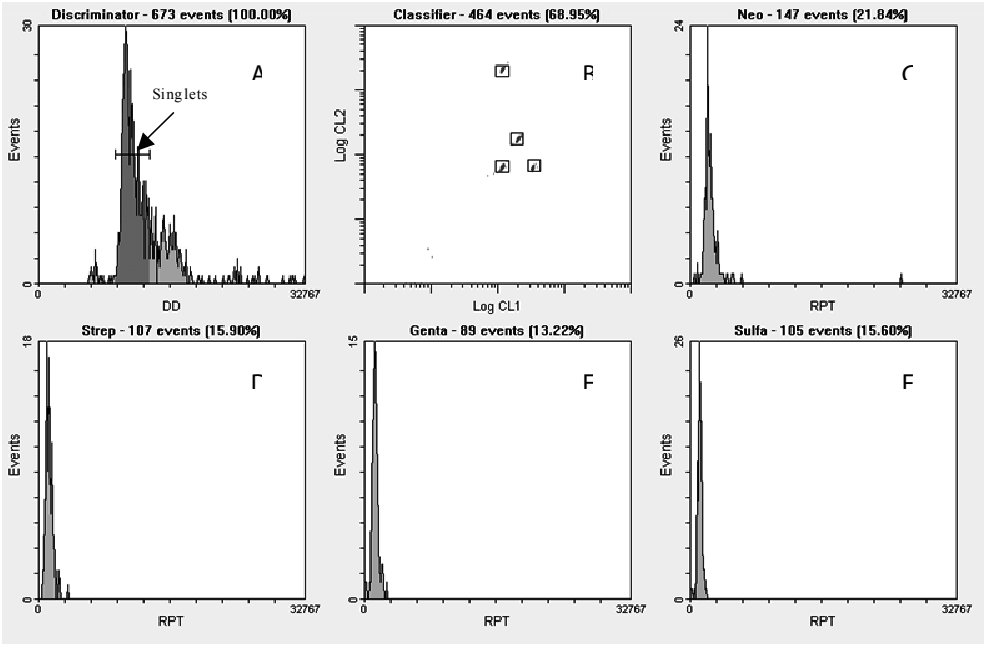


**Figure 3:** Plots of the fourplex showing the discriminator plot (A), the classifier plot (B) and the four reporter plots (C-F).

The multi-sulfonamide FCI was previously applied for the detection of sulfonamides in raw milk (Keizer et al., 2008) in which the many disturbing milk fat particles (15 x 109/ml), with a size (3-4 µm) close to the bead size (5.6 µm), were removed by filtration prior to analysis. Compared with buffer, the presence of filtered milk reduced the maximum response (from 3500 to 1200 MFI). This matrix effect was also observed in the present study in which 10 times diluted skimmed milk (reconstituted from skimmed milk powder) and chicken blood serum were used as model sample materials and, for quantitative analysis, calibration curves had to be made in the matrix. Such curves were prepared in milk for the four drugs and the results are shown in Figure 4. The obtained maximum responses varied per assay from 1100 till 2300 MFI. For a good comparison, the relative responses (% B/B0) were plotted against the concentrations. As shown in Figure 4, the individual assays were very specific, showing no inhibition with the other drugs, and sensitive enough to control on MRL levels (100, 200 and 500 ng/ml for gentamicin, total sulfonamides, streptomycin and neomycin, respectively).

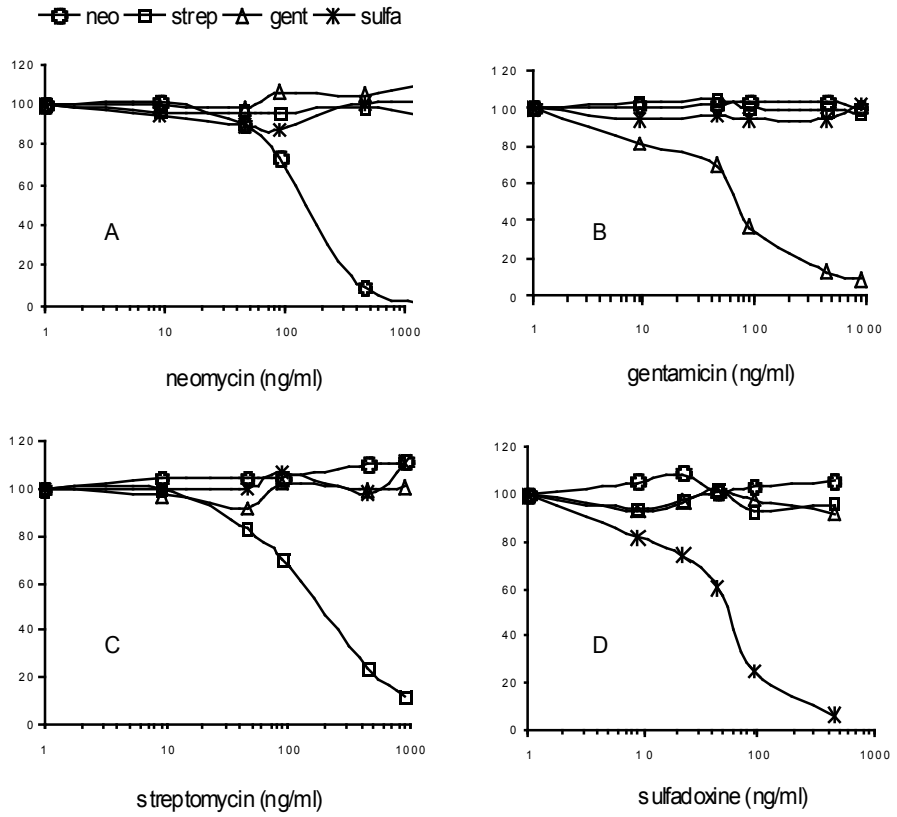


**Figure 4.** Fourplex FCI combining three specific aminoglycoside assays with the multi-sulfonamide assay. The calibration curves in milk for neomycin (A), gentamicin (B), streptomycin (C) and sulfadoxine (D) show the specificity of the individual assays.

## Conclusions

Combined immunoassays could be developed for the detection of three aminoglycosides and several sulfonamides (using the multi-sulfonamide assay) in milk and chicken blood serum at relevant levels. Due to the use of specific Mabs and a Rab, the individual assays were specific and did not influence each other in the combined format. The combination with other drug residue assays (e.g. fluoroquinolones (Gerçek et al., 2008)), as well as the improvement of the assay protocols, will be performed in the near future within a Dutch research project "Combined monitoring" and this promising technology is also included in the new EU-Collaborative Project "CONtaminants in Food and Feed: Inexpensive DEtectioN for Control of Exposure" (CONffIDENCE).

## Ackowledgements

We thank the Dutch Ministry of Agriculture, Nature and Food Quality for their financial support and Prof. Dr. Timo Lövgren (Department of Biotechnology of the University of Turku, Turku, Finland) for the supply of the Rab.

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## P 41

# ELIMINATION KINETIC OF RECOMBINANT EQUINE GROWTH HORMONE (reGH) IN HORSE PLASMA BY LC-MSn (LINEAR TRAP) MEASURMENTS

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**Abstract**
Growth hormones (GH, somatotropins ST) exhibit numerous biological effects associated with growth functions. Recombinant GH (rGH) are produced in large quantities and widely used outside Europe to stimulate milk production and as a general growth promoter in meat producing animals. The use of this molecule in animal production is strictly regulated by food safety directives and many attempts to set up methods for its detection in order to apply the regulation have already failed.
This work describes a successful analytical strategy to detect reGH in plasma samples and discriminate it from the pituitary form: specific extraction-purification steps have been developed and the detection involved tryptic hydrolysis followed by LC-ESI(+)-$MS^n$ (linear trap) detection of specific reGH N-terminal peptide. The method has been validated according to the European Decision 2002/657/EC requirements and applied to plasma samples collected on two thoroughbreds treated with recombinant equine growth hormone during two weeks allowing to establish the first kinetic study of reGH in plasma. This successful strategy leads the way for the control of GH misuse in animal production.

**Introduction**
Growth hormone (GH) also known as somatotropin (ST) is a protein hormone produced by the anterior pituitary gland in mammals. Its biological effects are numerous and associated with growth, development and reproductive functions. Recombinant GH (rGH) are produced in large quantities and widely used outside Europe to stimulate milk production and as a general growth promoter in meat producing animals (Etherton & Bauman, 1998). The use of this molecule in animal production is however strictly forbidden by European food safety directives (Dir 936/1994/EC, Dir 879/1999/EC) and over the past 15 years, many attempts to set up methods for the detection of its abuse have failed. Two strategies can be considered to demonstrate somatotropin treatment to animals, based either on direct or indirect methodologies. Direct methods are focused on the analysis of somatotropin itself, while indirect methods are based on the detection of biological biomarkers with concentrations dependant on ST. In the case of indirect methods, most of the researches have focussed on the detection of IGF-I (Kirsch et al., 2007). Recently, the detection of antibodies produced by the animal after treatment has shown promising results (Bailly-Chouriberry

et al., 2008). Unfortunately, and despite a good sensitivity, all these methods are limited to screening and can not be used for confirmatory purposes. Direct strategies, aimed at detecting somatotropin itself, can either be immunoassay or mass spectrometric based methods (Pinel et al., 2004). Despite their excellent sensitivity, immunoassays fail in discriminating recombinant and endogenous forms of the protein. Mass spectrometry on the opposite allows this discrimination and presents furthermore the advantage to be sensitive enough to reach the very low and expected level of detection (ppb). The first study published on the detection of somatotropin in spiked serum was reported by Blokland in 2003. In the present work, an analytical strategy based on several purification steps of plasma samples, followed by the detection of the specific N-terminal peptide of the hormone by LC coupled to a linear trap mass spectrometer allowed the identification of both the endogenous and the recombinant forms of somatotropin in biological matrices collected on reGH treated horses.

**Experimental**

*Chemicals and solvents*
Recombinant equine growth hormone (EquiGen-5®) was purchased from Bresagen limited (Thebarton, Australia). Recombinant bovine (rbST) and equine (reST) somatotropins were obtained from the Harbor-UCLA Medical Center, National Hormone and Pituitary Program (Torrance, CA, USA). Synthetic peptides with the following amino acid sequences MFPAMPLSSLFANAVLR (N-terminal tryptic reST) and MFPAMSLSGLFANAVLR (N-terminal tryptic rbST) were obtained from Millegen (Labège, France). Sequence grade modified trypsin (EC 3.4.21.4) was purchased from Promega (Madison, WI, USA). HPLC grade acetonitrile, methanol ammonium sulphate and formic acid were from SDS (Peypin, France). SPE C4 were from Interchim (Montluçon, France).

*Biological samples*
The experimental procedure was performed in agreement with animal welfare rules at the administration and sampling center of the Fédération Nationale des Courses Françaises (FNCF). Two 5-year old thoroughbreds identified as H497 (gelding) and H498 (stallion) were daily injected subcutaneously with 18 µg kg-1 of recombinant equine somatotropin (EquiGen 5®) for the first week of the experiment and with 25 µg kg-1 for the second week as described in the protocol supplied by the manufacturer. Two additional injections were given with 30 µg kg-1. 50 mL of blood were daily collected in silicone coated tubes to get 25 mL of serum, and in lithium heparinate tubes to get 25 mL of plasma. Samples were collected everyday from D-7 to D+20 and then every 2 days from D+22 to D+30. All samples were stored at -80°C until their analysis.

*Sample preparation*
Four millilitres of plasma were spiked with 100 ng.mL-1 of rbST. Proteins were precipitated for 15h at 4°C with 45% ammonium sulphate. The precipitate was re-suspended in phosphate buffer 0.1 M pH 6 and applied onto a SPE C4 cartridge previously conditioned with methanol, water and phosphate buffer. Proteins of interest were eluted with a mixture water/acetonitrile 20/80 (v/v) containing 0.1% TFA. After precipitation with cold methanol, a tryptic digestion was performed overnight at 37°C.

*LC-MS/MS conditions*
Peptides were separated on a Surveyor HPLC system (Thermo Finnigan) equipped with a Vydac C4 (2.1 x 150 mm, 300 Å) column (Interchim, Montluçon, France) using a mobile phase composed of acetonitrile and water at a flow rate of 300 µL.min-1. The MS instrument was a linear ion trap (LTQ XL, Thermo Scientific, Bremen, Germany) fitted with an electrospray ion source operated in the positive mode. A collision energy of 20 % was applied in the linear ion trap after selection of the precursor ions m/z 868, 913 and 933 corresponding, respectively to the [M+2H]2+ of the N-terminal eST, rbST and reST. The resulting fragment ions m/z (447, 1287), (643, 774, 790, 960, 1047, 1247) and (578, 794, 1287), corresponding respectively to the fragmentation of the precursor ions m/z 868, 913 and 933, were monitored.

**Results and Discussion**
The different steps of the sample preparation protocol dedicated to growth hormones purification from plasma as well as the determination of the optimal conditions for the fragmentation of their N-terminal tryptic peptides have been optimised. As shown in Figure 1, the fragmentation of the tryptic N-terminal peptide of reST leads to the main fragments y-type especially the y152+ with the mass 794.0.

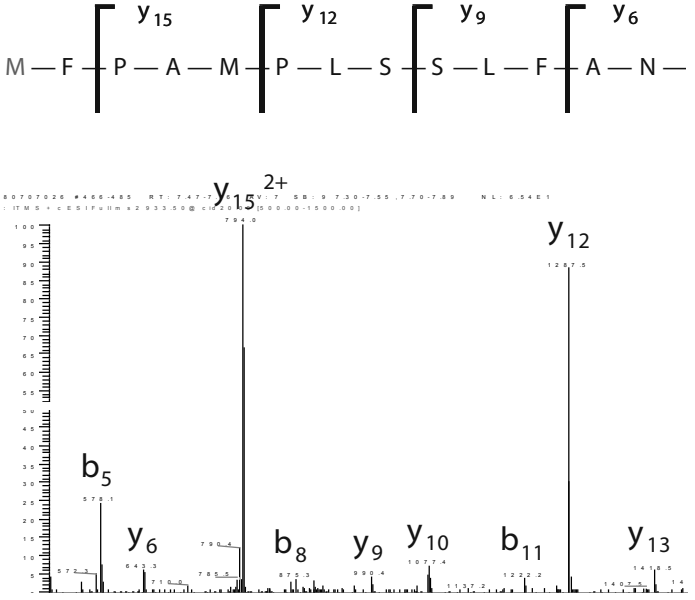


**Figure 1.** reST tryptic N-terminal peptide amino acid sequence. Product ion spectrum (CID 20%) of the peptide [M+2H]2+ acquired on a linear ion trap full scan between 500 < m/z < 1500 (ESI+).

The analysis of a horse plasma sample collected before any administration of reST (Figure 2a) enables the detection of the internal standard (rbST, 100 µg.L-1), but allows furthermore to detect the endogenous circulating form eST. This result is the first ever reported by means of mass spectrometry, it also confirms the performances of the method with the required

sensitivity at trace and physiological level. The analysis of a plasma sample collected 1.5 hour after reST administration to the horse leads to the unambiguous detection of the target molecule (Figure 2b).

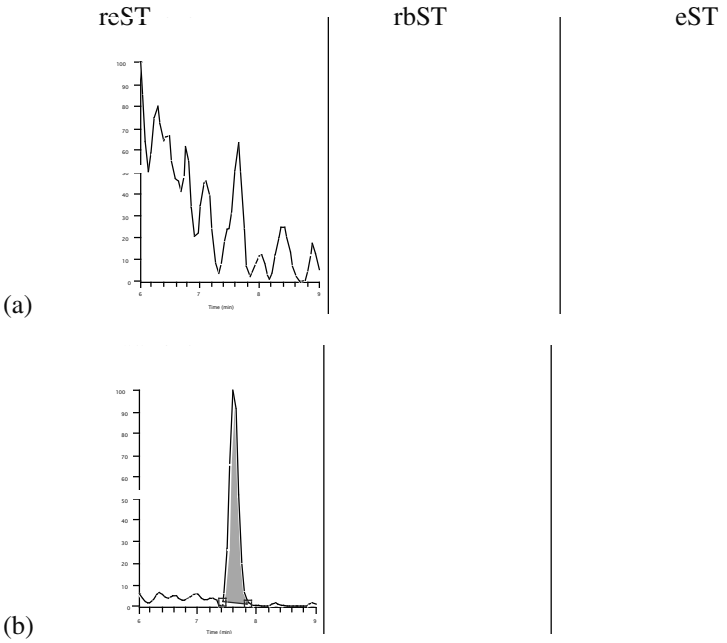


**Figure 2.** Ion chromatograms of reST, rbST and eST N-terminal peptides detected with a linear ion trap (ESI+) in plasma collected (a) before reST administration and (b) 1.5 hour after reST injection.

All plasma samples collected in the frame of the experimental protocol were analysed in the same way, which allowed drawing a kinetic of elimination of the hormone in the blood of the treated horse (Figure 3). It appears that reST can be detected as soon as 35 minutes after its administration. A maximum plasma concentration is reached 4 hours after the injection corresponding to 40 µg.L-1. The hormone is still detectable more than 15 hours after its administration.

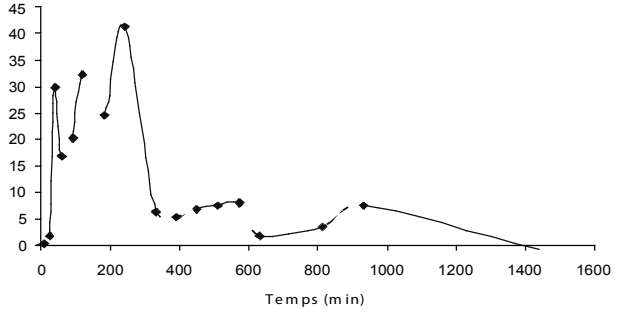


**Figure 3.** Kinetic of elimination of reST in plasma sample after one subcutaneous injection of Equigen-5® to a horse.

**Conclusion**

Growth hormone analysis in biological matrices remained for several years a challenge with, until now and despite the different strategies explored, unsuccessful results. The use of a last generation mass spectrometer (linear ion trap) coupled with an efficient purification procedure allowed to develop a method for the detection of recombinant growth hormone in incurred plasma sample. Sensitivity of the method was evaluated in the ppb range (µg.L-1) which enabled the detection of the administrated molecule in plasma for more than 15 hours after its administration. The detection of the circulating endogenous form was also achieved for the very first time. This successful strategy leads the way for the control of GH misuse in animal production.

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## P 42

# ELIMINATION KINETIC OF 17β-ESTRADIOL 3-BENZOATE AND 17β-NANDROLONE LAUREATE ESTER METABOLITES IN CALVES URINE

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**Abstract**
Efficient control of the illegal use of anabolic steroids must both take into account metabolic patterns with associated kinetics of elimination; in this context, an extensive animal experiment involving 24 calves and consisting of three administrations of 17β-estradiol 3-benzoate and 17β-nandrolone laureate esters was carried out over 50 days. Urine samples were regularly collected during the experiment from all treated and non treated calves. The method used for quantification of target steroids was characterised by decision limits (CCα) below 0.1 µg.L 1. Kinetics of elimination of the administered steroids were established by monitoring 17α-estradiol, 17β-estradiol, estrone and 17α-nandrolone, 17β-nandrolone, 19-noretiocholanolone, 19-norandrostenedione. All animals demonstrated homogeneous patterns of elimination both from a qualitative and quantitative point of view. Most abundant metabolites were 17α-estradiol and 17α-nandrolone (> 20 and 2 mg.L-1, respectively), whereas 17β-estradiol, estrone, 17β-nandrolone, 19-noretiocholanolone and 19-norandrostenedione were found as secondary metabolites at concentration values up to the µg.L-1 level. No significant difference was observed between male and female animals. The effect of several consecutive injections on elimination profiles was studied and revealed a tendency toward a decrease in the biotransformation of administered steroid 17β form.

**Introduction**
Anabolic steroids have been widely used over the last past 50 years in cattle breeding practices with beneficial effects such as animal growth promotion and feed efficiency. They are banned in food producing livestock in Europe (Dir 1996/22/EC). To enforce the prohibition on anabolic steroid abuse, effective monitoring, detection, identification and confirmation methods have been developed. Efficient control of the illegal use of anabolic steroids must both take into account metabolic patterns and associated kinetics of elimination (Le Bizec et al. 2002). This knowledge is mainly available from in vivo experiments and from the scientific literature, most studies have been performed on human subjects (Le Bizec et al., 1999, 2000), but few deal with bovine animals and in particular calves, although this

population has been linked with illegal anabolic treatment in EU member states.
The present study aims at supplying incurred calves' urine samples from a large group of animals (12 treated calves) after administration of a mixture of 17β-estradiol 3-benzoate and 17β-nandrolone laureate esters according to a protocol reflecting likely illegal practices and consisting in three administrations during the 45 days before the animals are slaughtered. Urine samples obtained during the experiment have been used to establish efficiency of the treatment, to study the elimination patterns of administered compounds as well as potential metabolism pathway adjustment upon several administrations and to provide confirmatory data to be compared in a further work to modifications in plasma protein profiles as indicators of steroid administration.

## Materials and methods

*Reagents and chemicals*
Most of the reagents and solvents were of analytical grade quality and provided by Solvants Documentation Synthesis (SDS, Peypin, France). The derivatisation reagents N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), trimethyliodosilane (TMIS) were purchased from Fluka (Buchs, Switzerland) and dithiothreitol (DTE) was from Sigma-Aldrich (St. Quentin Fallavier, France). β–glucuronidase (Helix pomatia) was provided by Sigma-Aldrich (St. Quentin Fallavier, France). The reference steroids were from Interchim (Montluçon, France), Sigma-Aldrich (St. Quentin Fallavier, France), AGAL/NARL (Australia) and RIVM (Bilthoven, The Netherlands). The internal standards used were 17β-nandrolone-d3, 19-norandrosterone-d4, 19-noretiocholanolone-d3, and 17β-estradiol-d3.

*Materials*
96-well SPE filled with 50 mg C18 phase were purchased from Interchim (Montluçon, France). Separation of steroid compounds was performed on non polar capillary column OV-1 (30 m x 0.25 mm i.d., film thickness 0.25 µm) (Ohio Valley, USA). Steroids were analysed on a triple quadrupole mass spectrometer (Quattro II, Micromass, UK) after electron ionisation.

*Animal experiment*
An animal experiment has been conducted within the department of Veterinary Animal Health of the Faculty of Veterinary Medicine of the Utrecht University (The Netherlands). The protocol has been approved by the ethical committee from Utrecht University. Twenty-four calves, 12 male and 12 female animals, were obtained from identified sources at an age of two weeks (10 days minimum and 3 weeks maximum) and subjected to a seven-week acclimatization period. After this period, two groups, each of 6 females and 6 males, were made randomly: treatment group and control group. Over the study period, each animal from the treatment group received three intramuscular doses of a mixture of 5 mL 17β-oestradiol benzoate (Oestradiol Benzoaat®, 5 mg/mL, Intervet, Boxmeer, The Netherlands) and 3 mL 17β nortestosterone laureate (Decadurabolin®, 50 mg/mL, Intervet, Boxmeer, The Netherlands) at day 0, day 14 and day 28. The first dose was administered at the average age of 10 weeks. Urine samples were collected at regular time points starting seven days before administration.

*Methods*
To a quantity of 2 mL urine was added 2 ng of each internal standard, 0.5 mL acetate buffer (2 M, pH 5.2) and 200 µL β-glucuronidase from Helix pomatia. Hydrolysis was performed

over 15 h at 52 °C, before purification on 96-well SPE C18. The reversed-phase wells were conditioned with methanol then water. The extract was applied into the well. The phase was washed with water then cyclohexane. Steroids were eluted with diethylether which was evaporated. Final extracts were derivatised with MSTFA-TMIS-DTE.

**Results and discussion**

*Evaluation of 96-well SPE*
Based on the high number of urine samples which had to be analysed (n > 300), a high sample throughput preparation protocol was developed using 96-well SPE filled with 50 mg C18 reversed-phase. Validation of the developed protocol was performed according to the requirements of the European Decision 2002/657/EC.
Decision limits (CCα) for steroids were below 0.1 µg.L 1, except for 19-norandrosterone (CCα=0.7 µg.L 1) and estrone (CCα=0.3 µg.L 1). Detection capabilities obtained for confirmatory purposes were equal or below the MRPLs (suggested by CRL or LABERCA), which is satisfactory for such a method, especially when taking into account the small urine sample size (2 mL) used.

*Nandrolone and its main metabolites profiles of elimination*
Elimination of 17β-nortestosterone laureate ester after its administration to calves was studied in urine by monitoring 17β-NT and focussing on some relevant metabolites: 17α-NT, NE, NA and norandrostenedione (NAED). For each treated calves and for each compound, except for NA which has never been identified in the samples, kinetics of elimination are shown in Figure 1.

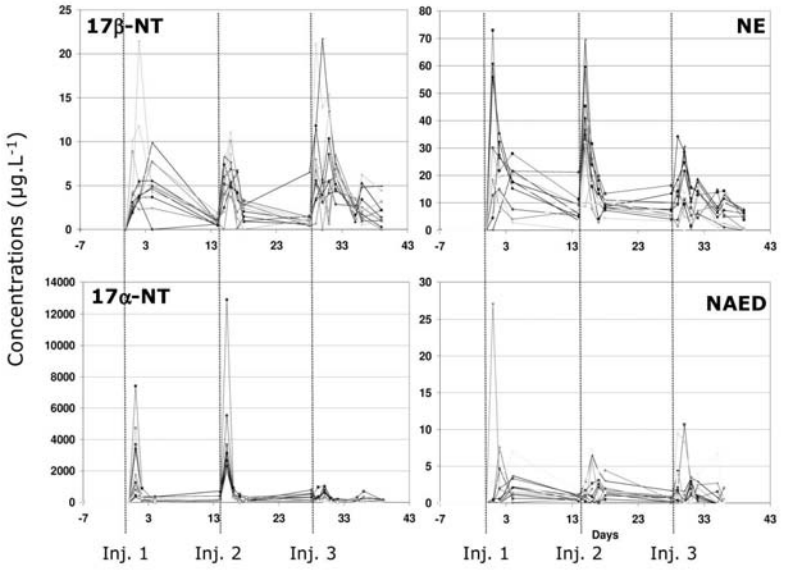


**Figure 1:** Kinetics of elimination over 40 days of 17β–NT, 17α-NT, NE and NAED for the 12 17β-NT laureate / 17β-E2-benzoate treated calves. Concentrations have been calculated from GC-EI(+)-MS/MS analysis of collected urine samples.

When none of the monitored compounds were detected before administration, they could be identified in urine samples of treated animals immediately after the first injection. Intensities of the signals and length of detection post steroid were dependant on the compound of interest. In the case of all compounds and for all animals, profiles of elimination are similar. The excretion rates reached the highest value 24h to 48h after each administration. However, calculated concentrations are different depending on the metabolites, with high concentrations reached for 17α-NT (> 2 mg $L^{-1}$) whereas 17β-NT, NE and NAED mean highest concentration values were at µg L-1 levels. These results are in agreement with the metabolism of 17β-NT in bovine species: C17 epimerization is a major pathway (Benoît et al., 1989; Daeseleire et al., 1993; Calvarese et al., 1994) .

No significant difference could be observed between male and female animals in term of urinary excretions profiles as in term of steroid concentrations detected, excepted for the 17α-NT that was excreted in amounts on average twice as high in female urines than in male samples. A number of trends were observed during the course of the experiment, in particular following the third injection, it was noticed that the concentrations in 17β-NT increased slightly whereas those in 17α-NT and NE relatively decreased or remained stable for NAED. According to the observed concentrations and associated tendencies, a diminution of phase I metabolism seems to be induced following several administrations of nandrolone.

Estradiol and its main metabolites profiles of elimination

Elimination of 17β-estradiol 3-benzoate after the three administrations to calves was studied in urine by monitoring 17β-E2 and its main metabolites, 17α–E2 and estrone (E1). As expected, small quantities of 17α-E2 were detected in calf urine samples before any treatment has been administered, whereas neither 17β-E2 nor E1 were detected at or above the limit of detection. For all treated calves and for each compound, kinetics of elimination are shown in Figure 2.

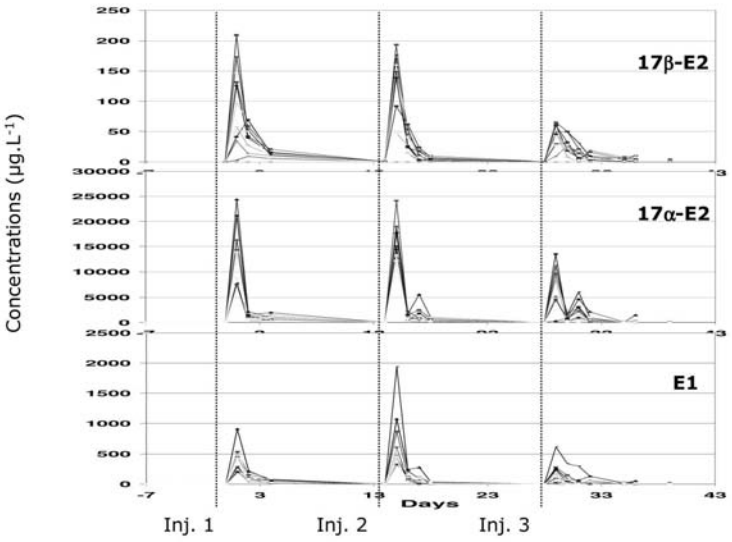


**Figure 2:** Kinetics of elimination over 40 days of 17β-E2, 17α-E2 and E1 for the 12 17β–NT laureate / 17β-E2-benzoate treated calves. Concentrations have been calculated from GC-EI(+)-MS/MS analysis of collected urine samples.

All three estrogenic compounds were detected in urine with intense associated signals immediately after the first injection and were still identified in urine samples collected at the end of the experiment. The time-concentration profiles followed similar elimination kinetics for all estrogenic compounds in all treated animals. Their concentrations peaked 24 h post each administration then declined. Calculated concentrations varied greatly between the measured compounds. Highest concentrations were found in the case of 17α-E2 (> 20 mg $L^{-1}$) as opposed to maximum detected concentrations of E1 and 17β-E2 were around 1 and 0.15 mg $L^{-1}$, respectively.
As previously reported no significant difference could be observed amongst male and female animals in term of residue concentration post steroid administration, with the exception of 17α-E2 that was excreted in concentrations, on average, twice as high in females compared to males. These results confirm that the epimerisation step is the important metabolic pathway for estradiol in bovine (Ivie et al., 1986). Furthermore, a tendency toward a general decrease in 17β-E2, 17α-E2 and E1 concentrations upon three consecutive injections was observed as for nandrolone.

**Conclusion**

The performance of the developed method was established by means of a rigorous validation according to the requirements of the European Decision 2002/657/EC and was in accordance with proposed MRPL values for these compounds.
The results showed efficiency of the treatment since the anticipated compounds were found in urine samples analysed for absorption, transformation and elimination of administered compounds. All animals showed homogeneous elimination profiles which were in strong correlation with the pattern of steroid administrations. The effect of several consecutive injections on elimination profiles was studied and revealed a tendency toward a decrease in phase I metabolism.

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# EXPOSURE ASSESSMENT OF PREPUBERTAL CHILDREN TO GONADAL STEROID HORMONES

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**Abstract**
Global concerns have been raised in recent years over the potential adverse effects that may result from exposure to chemicals owing to their capacity to interfere with the endocrine system. Our main purpose was to investigate to what extent food intake of steroid hormones can represent a risk for prepubertal children. At first, a new analytical strategy for the measurement of estrogens at ultra-trace level in food and complex biological matrices was developed. Subsequently, estrogen measurements in various food products (milk, egg and meat) were performed in order to evaluate the food intake for this population. Then, the quantification of the main estrogens in samples from prepubertal children aged 6-11 years gave access to estradiol plasmatic hormonal rate and consequently to the daily endogenous production of this population. A first interpretation of these data in terms of risk assessment is also provided, in connection with existing JECFA and FDA recommendations regarding the maximal acceptable daily intake for this compound.

**Introduction**
Global concerns have been raised in recent years over the adverse effects that may result from exposure to chemicals that have the capacity to interfere with the endocrine system. Among the different classes of substances that have been pointed out (dioxins, phytosanitary products, phytoestrogens, hormones…), steroid hormones largely remain the most biologically active compounds. A specific concern is related to low-dose effects and long-term exposure consequences, especially for specific populations at critical stage of development (foetus, new born, prepubertal children).
In the field of toxicological substances used in food-producing animals, the US Food and Drug Administration ruled that '… no physiological effect will occur in individuals chronically ingesting animal tissues that contain an increase of endogenous steroid equal to 1% or less of the amount in micrograms produced by daily synthesis in the segment of the population with the lowest daily production' (FDA, 1999). Regarding naturally occurring hormones such as estradiol, daily production rate (PR) and exogenous intake appeared as crucial elements of a risk assessment. However, available data on these topics remain controversial.
Consequently, we decided to re-evaluate these plasmatic hormonal levels as well as concentrations of those compounds in food products using a very sensitive and specific

confirmatory measurement technique (Gas chromatography-tandem mass spectrometry) in order to compare estradiol dietary intake and daily endogenous production of prepubertal children, as a basis of risk assessment.

## Materials and Methods

### *Samples*

*Serum samples.* Serum samples were collected from 26 and 36 healthy prepubertal boys and girls (aged from 6.0 to 11.5 years) recruited from public schools in the Copenhagen area. The experimental protocol was approved by a local ethical committee (approval number: (KF) 11297746) in accordance with the Helsinki declaration, and the informed consent of all participating subjects and their parents was obtained.

*Food samples.* Milk (n=37) and egg samples (n=35) were commercial products randomly collected from a French supermarket in 2006 and 2007. Meat samples (n=90) were collected during two European projects of the 4th and 5th Framework Program (namely EUROESTR and ISOSTER, respectively). Treated animals (n=15), steers were implanted with RevalorS® (1, 2 or 4 implants containing 24 mg estradiol and 120 mg trenbolone) and slaughtered 3 months after treatment.

*Apparatus*
A HP 6890 gas chromatograph coupled to a QuattroMicro GC® (Micromass, Manchester, UK) triple quadrupole mass spectrometer was used equipped with a ZB-MS (30 m x 0.25-mm, 0.25 µm). A HPLC pump (Hewlett Packard HP-1100 system, Palo Alto, CA, USA) with a fraction collector was used for semi-preparative fractionation.

*Chemicals*
All solvents and reagents were of analytical or HPLC grade quality and purchased from Solvent Documentation Synthesis (SDS, Peypin, France). All SPE (ChromP, SiOH) were single use cartridges also provided by SDS. Purified Helix pomatia enzymatic preparation was used for steroid deconjugation (Sigma, St Louis, MO, USA). Derivatisation reagents N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), N,O-Bis (trimethylsilyl)-trifluoroacetamide (BSTFA), and pentafluorobenzylbromide (PFBBr) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTT), while trimethyliodosilane (TMIS) was purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Standard reference steroids were purchased from Sigma (St Louis, MO, USA).

*Sample preparation*
For meat samples, a first liquid/liquid extraction with a methanol/acetate buffer mixture was performed prior enzymatic hydrolysis (with Helix pomatia). For other samples, an extraction of non-polar compounds with diethylether is performed after enzymatic hydrolysis. A first purification step was achieved on a Chrom P SPE cartridge. Then, a liquid/liquid partitioning is performed with n-pentane to separate androgens and estrogens. Estrogen fraction is finally purified on a silica SPE cartridge. A semi-preparative HPLC is used as an ultimate purification step.

*Derivatisation*
Derivatisation was carried out with 20 µL of a solution of PFBBr in acetonitrile (1:20 v/v) and 20 µL of a solution of KOH in anhydrous ethanol (8 mg/mL). The reaction was performed at 60°C for 40 min. The derivatisation reagent was then evaporated to dryness (45°C, N2) and the residue was redissolved in 20 µL BSTFA; this second derivatisation reaction was performed at 60°C for 40 min. For meat samples, derivatisation was performed at 60°C for 40 min in 20 µL of MSTFA/TMIS/DTT.

**Results**

*Occurrence of estrogens in food products*
For all analysed milk and egg samples, the total hormone concentration levels (free plus deconjugated forms) were determined (enzymatic hydrolysis of the glucuronide and sulphate phase II metabolites). Results are summarized in table 1.

**Table 1.** Total estrogen concentrations determined in various food products (in ng.kg-1)

| | **Milk** | **Eggs** | **Meat** | **Meat (from treated animals, 1 implant)** | **Meat (from treated animals, 2 or 4 implants)** |
|---|---|---|---|---|---|
| 17β-estradiol | 14 ± 13 | 360 ±160 | 6 ± 5 | 19 ± 3 | 100 ± 170 |
| 17α-estradiol | 34 ± 10 | 240±190 | 0 ± 0 | 6 ± 1 | 14 ± 9 |
| Estrone | 172 ±84 | 930 ±480 | - | - | - |

In milk, Estrone and 17α-estradiol were measured at global mean concentration levels of 172 ± 84 ng.$L^{-1}$and 34 ± 10 ng.$L^{-1}$, respectively. These two compounds were found to be present mainly as conjugated forms (around 95 %). 17β-estradiol was found to be in lower concentration than the previous compound (mean value: 14 ± 13 ng.$L^{-1}$), with also a lower proportion of conjugated forms (nearly 80%).
Estrone appeared as the major estrogen quantified in egg samples (mean value of 930 ± 480 ng.kg-1), and was found mainly as unconjugated form. 17α-estradiol and 17β-estradiol were also quantified in the same samples, at 240 ± 190 and 360 ± 160 ng.$kg^{-1}$, respectively. In opposition with the previous results obtained for estrone, 17α-estradiol and 17β-estradiol were found mainly conjugated (70-85 %).
Regarding meat samples, 17β-estradiol was found in very weak concentrations in control animals. It can be noticed that after anabolic treatment, residues of estradiol are increased by a factor 3 in muscle from animals treated with 1 implant and about 10 in muscle from animals treated with 2 or 4 implants (in this case, Good Veterinary Practices are not followed).
Plasmatic hormonal rates of 17β-estradiol in prepubertal boys and girls
Estradiol concentration levels (table 2) were confirmed to be extremely low in prepubertal children. Indeed, in boys, it was possible to quantify estradiol in only 6 of 26 samples. When quantified, these levels were in the 1.0 to 3.9 ng.L-1 range (mean value=2.8 ng.L-1). Estradiol levels in prepubertal girls were significantly higher than in prepubertal boys (2.8 ng.L-1 versus <1.0 ng.L-1, respectively, $p<0.0001$); moreover 32 of 36 samples were measurable.

Thus, even before the first signs of puberty, a difference does exist between boy and girl concerning the endogenous production of estrogens. One can thus suspect that even a weak exogenous contribution of estradiol could affect reproductive and development functions.

**Table 2.** Median, Mean, and number of measurements of estrogens in serum samples from boys and girls.

| | | Boys | Girls |
|---|---|---|---|
| **17β-estradiol** | Median ($ng.L^{-1}$) | <1.0 | 2.8 |
| | Mean ($ng.L^{-1}$) | 2.8 | 4.3 |
| | $n_{quantified}$ / $n_{samples}$ | 6/26 | 32/36 |
| **Estrone** | Median ($ng.L^{-1}$) | 10.3 | 23.7 |
| | Mean ($ng.L^{-1}$) | 14.6 | 32.3 |
| | $n_{quantified}$ / $n_{samples}$ | 25/26 | 36/36 |

**Discussion**

On the basis of the previous findings related to the concentration levels of estrogens in various food products, a first interpretation was made in the scope of evaluating the potential risk associated to these endocrine disrupting chemicals for human health, especially for critical population such as prepubertal children. Then, a theoretical food intake, based on the concentration levels determined for 17  estradiol and 17 -testosterone with respect to children consumption data (Table 3) determined in the course of french national investigations (AFSSA, CREDOC and General Directorate for Food), was calculated and compared to:

1)the Acceptable Daily Intake (ADI) established by JECFA for 17 -estradiol (equivalent to 50 ng/kg of body weight per day).

2)the maximum secure daily intake established by FDA for 17 -estradiol (65 $ng.day^{-1}$), this limits corresponding to 1 % of the endogenous levels produced by the segment of the population with the lowest daily production, i.e. prepubertal boys. In this calculation, FDA took data from Angsusingha et al. corresponding to estradiol circulating levels of 4.8 $ng.L^{-1}$.

**Table 3.** Consumption data for children from INCA study (in g / person / day)

| Products | Mean (g) |
|---|---|
| Milk | 219 |
| Egg | 12 |
| Muscle | 53 |

**Table 4.** Estimation of estradiol dietary intake according to chidren consumption of milk, meat and eggs

| | | 17β–estradiol | | |
|---|---|---|---|---|
| | | **Mean (ng/ day)** | **% ADI (1000 ng/day) [a]** | **% maximum secure daily intake (65 ng/day) [b]** |
| Milk | | 3.1 | 0.31 | 4.8 |
| Egg | | 2.2 | 0.22 | 3.4 |
| Muscle | from non treated | 0.5 | 0.05 | 0.7 |
| | from treated (1 implant) | 1.0 | 0.1 | 1.5 |
| | from treated (2 or 4 implants) | 5.8 | 0.58 | 8.9 |
| Total Food intake | If meat is from non-treated | 5.8 | 0.58 | 8.9 |
| | If meat is from treated (1 implant) | 6.3 | 0.63 | 9.7 |
| | If meat is from treated (2 or 4 implants) | 11.1 | 1.11 | 17.7 |

[a] *calculated on the basis of JECFA's ADI for a child weighting 20 kg,*
[b] *maximum secure daily intake of FDA*

It can be observed that the main sources of estradiol are milk and eggs (exogenous intake estimated to 3.1 $ng.day^{-1}$ and 2.2 $ng.day^{-1}$, respectively), and in minor extent meat from untreated animals. Nevertheless, when considering meat from treated animals, and particularly in case Good Veterinary Practices (GVP) is not followed (2 or 4 implants), the resulting intake (i.e. 5.8 $ng.day^{-1}$) reach the same order than the one induced by milk and eggs consumption (i.e. 5.1 $ng.day^{-1}$).
Neither JECFA's ADI nor FDA maximum secure daily intake seem to be reached by consumption of products such as milk, meat and eggs. Nevertheless, our results regarding the determination of the daily production of estradiol in prepubertal boys (estradiol circulating levels < 1 ng.L 1) indicate that the maximum secure daily intake of 65 $ng.day^{-1}$ recommended by FDA should probably be revisited; a first and not definitive estimation should lead to values below 10 ng.day-1. In the case of consumption of meat from treated animals (GVP not followed), the resulting total food intake may over cross this limit.

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