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Yann Guitton[1#], Gaud Dervilly-Pinel[1*#], Jandova Renata[2], Sara Stead[2], Zoltan Takats[3], Bruno Le Bizec[1]

1 LUNAM Université, Oniris, Laboratoire d'Étude des Résidus et Contaminants dans les Aliments (LABERCA), UMR INRA 1329, Nantes F-44307, France

2 Waters Corporation, Altrincham Road, SK9 4AX Wilmslow, United Kingdom

3 Faculty of Medicine, Department of Surgery and Cancer, Imperial College London, London SW7 2AZ, United Kingdom

*corresponding author

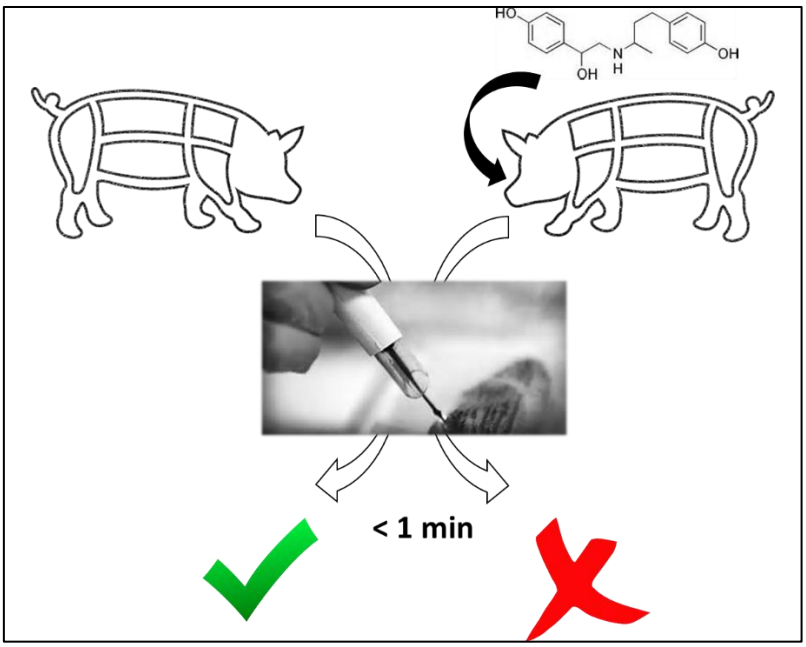
gaud.dervilly@oniris-nantes.fr

laberca@oniris-nantes.fr

# both first-authors, contributed equally

ABSTRACT: In a proof of concept perspective, Rapid evaporative ionization mass spectrometry (REIMS) was explored for the direct analysis of meat samples from β-agonist treated livestock. In this context, the combination of REIMS with untargeted metabolomics was investigated to identify carcasses from treated animals on the basis of a modification of indirect metabolites profile. The REIMS analysis generated specific lipid profiles which enabled the differentiation of meat samples collected from pigs treated with ractopamine *via* their feeding regime. Furthermore, the strategy was found successful when tested on different muscle types (loin, shoulder and thigh), which further expands its applicability. Classification performances were greater than 95% accurate which fully answers requirements of a screening strategy. This research indicates that REIMS implemented in an untargeted-metabolomics workflow can be considered as a high-throughput and accurate strategy for real-time meat classification in relation to ractopamine (and wider β-agonists) treatment in pig production. This approach may subsequently be implemented as a rapid screening test, at the slaughterhouse or at boarder inspection points, to detect such practice.

Key words: β-agonist, pig, muscle, REIMS, iKnife, metabolomics, biomarkers, ractopamine



Graphical Abstract: REIMS for direct detection of meat samples from ractopamine treated livestock

INTRODUCTION

β–agonist drugs are synthetic molecules used for therapeutic purposes in human as well as in veterinary medicine for their anti-asthmatic, bronchodilator, tocolytic and cardiotonic properties. They may also be used for growth promoting purposes in livestock although such practice received extra-attention after outbreak of food poisoning caused by consumption of contaminated bovine tissues in 1990 in Spain (Martinez Navarro 1990): it was the first time that β–agonist residues present in slaughtered cattle were found to have caused acute toxicity in consumers. Later on, similar outbreaks were also reported in France (Pulce et al. 1991), in 1996 in Italy (Brambilla et al. 2000) or 2001 in Portugal (Barbosa et al. 2005). Ractopamine in particular is used in animal feed to improve weight gain, carcass leanness and feed efficiency in more than 20 countries, including the United States, Canada, Japan, South Africa and Mexico. During its 2010 meeting, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established maximum residue limits (MRLs) for ractopamine in muscle (10 $\mu g.kg^{-1}$), fat (10 $\mu g.kg^{-1}$), liver (40 $\mu g.kg^{-1}$) and kidney (90 $\mu g.kg^{-1}$) in pig and cattle (JECFA 2010). On 6 July 2012, the international reference standard *Codex Alimentarius* Commission narrowly approved the adoption of the MRLs although setting any limit was a controversial move. However, recent research confirmed that using ractopamine can affect the heart, thyroid, urethra and prostate (Catalano et al. 2012). Therefore, any proposals to ban ractopamine in food-producing animals is still with controversy. Countries, such as those of the European Union and China, have not approved the usage of any β-agonist, including ractopamine, as growth promoters (Council Directive 96/22/EC 1996; MOA. 2002; EFSA. 2009). However, and despite the ban, its illegal use in food producing animals is regularly suspected in Europe; a particular attention is given at Border Inspection Posts (BIP) to any edible tissues imported from third countries. Consequently, analytical strategies have been developed to comply with the regulation and ensure control of such practices (Le Bizec et al. 2009). Confirmatory strategies targeting β-agonists residues in various relevant biological matrices (urine, serum, tissues, retina, hair) enable long term detection of forbidden practices. However, the extent to which such practice occurs is still difficult to evaluate since screening is an enormous challenge. To meet the requirement for screening illicit practices, “Omics” strategies, based on the observation of physiological perturbations, are promising approaches to tackle drug misuse in breeding animals (Riedmaier et al. 2009; Pinel et al. 2010; Nebbia et al. 2011; Dervilly-Pinel G. et al. 2012; Gallart Ayala et al. 2015). Particularly, metabolomics based on mass spectrometry analytical platforms have proven their efficiency in highlighting candidate biomarkers of anabolic treatments in livestock (Dervilly-Pinel G. et al. 2011; Jacob C et al. 2014; Jacob CC et al. 2014; Jacob C et al. 2015; Kouassi Nzoughet J et al. 2015; Kouassi Nzoughet JJ et al. 2015) and sport animals (Boyard-Kieken et al. 2011; Kieken et al. 2011). During the past few years, several metabolomics studies have been reported for the control of β-agonists in livestock. Courant et al. (Courant et al. 2009) developed an untargeted metabolomics approach based on High

Performance Liquid Chromatography coupled to High-Resolution Mass Spectrometry (HPLC-HRMS) and made it possible to highlight metabolic modifications in calves' urine directly to a clenbuterol administration. Later on, Dervilly-Pinel et al. (Dervilly-Pinel Gaud et al. 2015) proposed a validated LC-HRMS (Orbitrap) metabolomics strategy enabling implementing a robust statistical model to screen for calves which have been treated with β-agonist compounds. At the same time, Wu et al. (Wu et al. 2015) outlined a metabolomics based strategy for detection of the use of "cocktails" composed of mixtures of sub-therapeutic doses of the individual β-agonists (clenbuterol, salbutamol, and ractopamine) *via* pig urine profiling through LC-HRMS (Q-TOF). Until now however, most studies have been focusing on clenbuterol as a model of β-agonists for metabolomics investigation. Peng et al. (Peng et al. 2017) recently reported a number of serum candidate biomarkers specifically associated to ractopamine administration in pig production also based on LC-HRMS metabolomics.

Due to the non-invasive nature of the sample collection for live animals, urine of, e.g. bovine and pig, has primarily been the matrix of choice in these metabolomics studies (Courant et al. 2009; Jacob CC et al. 2013; Dervilly-Pinel Gaud et al. 2015; Wu et al. 2015), while later on, serum or plasma has also been efficiently investigated (Peng et al. 2017). Conversely, sampling the edible tissue (muscle, liver, kidney...) offers the advantage over biofluid analysis of allowing direct control of the food commodity. This is of prime importance for instance in the case of importation at the border. In this context, a single study by Li et al. (2016) reported a successful porcine muscle and fatty tissue metabolomics investigation after clenbuterol treatment using gas chromatography-mass spectrometry (GC-MS). The promising outcome fully justifies considering edible tissues as matrices of interest to highlight β-agonist administration food producing livestock.

In the current international context, developing an efficient and high-throughput metabolomics workflow directly applicable for the sampling of edible meat and enabling immediate prediction of its (β-agonist) status would be considered a breakthrough in the field to reveal biological effects in drug treated animals and efficiently tackle illegal practice.

Rapid Evaporative Ionization Mass Spectrometry (REIMS) is a new emerging technique enabling direct ionization from the sample combined with mass spectrometric analysis (St John et al. 2016). REIMS determines the structural lipid profile of tissues by the on-line analysis of electrocautery smoke and uses this information for the rapid characterization of dissected tissues. The analysis takes only a few minutes and guarantees point-of-control analysis. Originally it was intended for *in vivo* identification of tissues during surgical interventions (Balog et al. 2013); it was recently reported in food applications, in particular, its feasibility was successfully demonstrated for the identification of the species of origin (Balog et al. 2016) or quality (Verplanken et al. 2017) of meat products. A known consequence of the administration of β-agonist compounds to livestock is the development of leaner muscle tissue; therefore, a perturbation in the lipid profiles of tissue from animals treated with β-agonist compounds compared to the control population is hypothesized. In a proof of concept perspective, the present research intended to explore the use of

REIMS to develop a predictive model for the accurate, real-time determination of muscle samples originating from ractopamine treated pigs.

1. EXPERIMENTAL SECTION

1.1 Experimental design

The animal study was approved by the national Ethical Committee (n°6) under agreement 2015092516084715 / APAFIS 1914 (CRIP-2015-054). Ten four months' old female pigs (Axior Porc Charcutier) (Terrena, Ancenis, France) weighing 40 kg were randomly divided into control (n = 5 animals) and treated (n = 5 animals) groups. Animals were allowed a three-day acclimatization period, following which each animal received 1.8 kg feed/day. Pigs from the treatment group received feed containing ractopamine hydrochloride (Sigma Aldrich), at a dose of 10 mg/kg feed, on a daily basis over a 29 day period. The 10 ppm daily dose was selected as corresponding to classical doses used in finishing pigs, where that approval for use exists. Three different muscle type (Shoulder (E), Loin (F) and Thigh (J)) samples were collected from each animal carcass at the end of the experiment, frozen and then stored at -20°C for subsequent REIMS analysis. For ractopamine residue confirmation, muscles were freeze-dried and grounded before analysis.

In addition, three samples of pork muscle were purchased at the local supermarket and used as part of the validation study.

1.2 Methods

REIMS fingerprinting: After thawing, muscles were cut in 1 cm x 4 cm sections and submitted to REIMS analysis as follows. The iKnife hand-held sampling device (Waters, Milford, MA USA) was used to apply a localized high frequency electric current to the surface of each sample, which instantly vaporizes molecules from the latter. It consisted of a monopolar cutting device with a shortened knife blade approximately 6 mm long and was applied in auto-cut mode in combination with a diathermy electrosurgical generator (Erbe VIO 50 C) (Erbe, Tuebingen, Germany) at a power of 25 W. Sampling was carried out for 3 to 5 seconds and for each sample, three technical replicates were analyzed, thus taking into account repeatability of the analysis. Mass spectrometric analysis was performed using a Synapt G2 Si instrument equipped with prototype REIMS source comprising a helical coiled ribbon collision surface heated by a constant current power supply set to 4.5 A (Kanthal D 1.0×0.1 mm) (Waters Corporation, MA USA). All analysis was performed in REIMS TOF MS sensitivity mode with continuum data acquisition. Isopropanol was infused directly into the REIMS source at a constant flow rate of 200 µl min^{-1} to promote the ionization of lipid (fatty acid and phospholipid) species and maintain source cleanliness. The mass resolution was approximately 20 000 FWHM over the mass range of interest. The declustering voltage (labelled cone voltage) is the

DC potential applied between the last electrode of the first stepwave and the differential pumping aperture between the first stepwave vacuum region and the second vacuum region; this was set at difference of 100 V. Mass spectrometric analysis was performed in negative ionization mode over a mass range of 50–1200 *m/z* with an integration (scan) time of 1 s/scan. Prior to use, the instrument was calibrated using sodium formate in isopropanol and was infused via the matrix inlet on the REIMS source. For quality control purposes, the endogenous matrix ion PE (34:1) $[M\text{-}NH4]^-$ $C_{39}H_{76}NO_8P$ with *m/z* 699.497 was used as an internal lock-mass compound to maintain mass accuracy. Furthermore, replicate burns of a QC sample (porcine liver) were collected after every 10 porcine muscle samples. The intensity of the base peak ion at *m/z* 699.497 was recorded and plotted for quality control monitoring. The iKnife blade was cleaned using methanol after every 15 measurements and the three-meter-long transfer tubing and venturi air pump was cleaned in an ultrasonic bath using methanol at the end of each day. A reference sample of porcine liver was analysed after every 10 samples measured. The ion intensity of the base peak at m/z 699.49 obtained from the reference sample spectra was plotted in a control chart so that any deviations in instrument performance would be quickly identified.

An untargeted mass spectrometric analysis was performed to discriminate between muscle samples collected from control and treated animals. The ten loin muscles (MF) were analyzed in triplicate (as a minimum) on each of three separate days and using two different REIMS sources (operated on the same MS instrument), in order to take into account technical variation and intra-laboratory reproducibility. This step thus generated a total of 106 mass spectra (50 spectra for the control and 56 for the treated class), that were used to define the database and consequently train the model. In addition, samples from each of the ten study animals were collected from the shoulder (ME) and thigh (MJ) muscles that were also analyzed in triplicate on the first analysis day and used as an independent validation set to the model created from the loin samples.

Ractopamine analysis: Muscles have been analysed for ractopamine residue according to a validated and ISO17025 accredited method (Antignac et al. 2002). Starting with 20 g of freeze-dried muscle the method briefly consists in a deconjugation step with *Helix pomatia* for 15h at 52 °C, followed by a mixed mode SPE purification step (CSDAU, SDS, Peypin, France). Separation of the compounds was performed on a C18 column (50 x 2,1 mm, 3 µm (Uptisphere 3HDO – Interchim, Montluçon, France)) and MS identification using a triple quadrupole (Waters Xevo T-QS) in the SRM acquisition mode. Criteria of Decision 2002/657/EC (Council Decision 2002/657/EC 2002) were applied. Quantification was performed using isotope dilution principle using $^2H_9$-méthylcimaterol, as the labelled internal standard.

1.3 Chemometric data analysis and recognition

Multi-variate statistical software package LiveID™ (Waters Corporation, Wilmslow, UK) was used as a model builder and recognition tool. In order to generate models from the untargeted profiling REIMS ToF MS data acquired in MassLynx v. 4.1 (Waters Corporation, Wilmslow, UK) the following data treatment steps were performed; lock-mass correction applied using the endogenous matrix ion at *m/z* 699.497; all spectra contained within each "burn event" termed the region of interest (ROI) were combined to form a single continuum spectrum; Adaptive Background Subtraction (ABS) algorithm was applied to reduce the chemical background in the combined spectra; data re-sampling (binning to 0.1 Da) was performed to reduce the data dimensionality; the resulting spectrum was normalised using the TIC For principal component analysis (PCA), the data was centered using the mean value of the entire data set. For linear discriminate analysis (LDA), the data was centered using the mean values of each model class. In either type, the mean for each *m/z* bin is subtracted from the values of that bin. Other than normalisation and centering, no additional manipulation was performed (for example, scaling).

Following data pre-treatment steps, a PCA/LDA model was calculated. Firstly, an unsupervised PCA (Singular Value Decomposition algorithm) transform is applied to the spectral data calculating the scores and loadings; a supervised LDA transform is then applied to the scores calculated by the PCA transform. LDA is a transform that maximizes the inter-class variance, while minimizing the intra-class variance, resulting in a projection where examples from the same class are projected close to each other and, at the same time, the class centres (means) are as far apart as possible. Although it is not a true regularization technique, PCA-LDA is found to reduce the chance of over-fitting that may occur with a pure LDA model.

During the recognition step, the model transforms spectra acquired from test samples into the associated model-space, after which, a classifier decides to which class (if any) the spectra belongs. The model classifier uses a multivariate normal distribution (MVN) for each model class. During the model building phase, these distributions are constructed by transforming the training spectra to generate scores for the *n* principal components/linear discriminants selected for the model. The number of dimensions in the MVNs is also equal to *n*. The MVNs produce a likelihood measure for each class, and Bayes' rule was then applied to derive posterior probabilities.

In silico 5-fold stratified validation was performed to determine the predictive accuracy of the ractopamine screening model. The model-building data set was divided in five partitions (5-fold), each of which contains a representative proportion of each class within it (stratified). Four partitions (80%) of data set are used to build a model under the same conditions as the original model. This model is used to predict the classifications of the one partition (20%) of the training set that was left out. The cycle was repeated iteratively 5 times and each partition was predicted once by a model trained from the other four. The output of the validation details the total number of correct and incorrect

classifications, as well as the number of outliers. Outliers were calculated according to the Mahalanobis distance (Mahalanobis, 1936) to the nearest class center. If this distance is greater than the outlier threshold, the sample is considered an outlier. Following iterations of model optimization, an independent validation step was performed using the ME and MJ sample series not included in the training set.

For independent comparison purposes with LiveID™ outputs, additional and complementary statistical analyses (OPLS-DA, HC) were performed using MetaboAnalyst 3.0 (Xia et al. 2015; Xia and Wishart 2016), Progenesis QI (Non Linear Dynamics, Waters Corporation, Newcastle, UK), EZInfo and SIMCA-P (Umetrics Sartorius Stedim Biotech, Sweden).

The different steps of the workflow are illustrated on the flow diagram presented in Figure S1. Resources and time effort are further indicated.

2. RESULTS AND DISCUSSION

2.1 Ractopamine residue analysis

Muscle samples collected from control pigs were all shown to be free of ractopamine (and associated metabolites), while muscle tissue collected from treated animals was found to be incurred with ractopamine residues. The measured concentrations in the treated population were found to be in the range of 1-2 $\mu g.kg^{-1}$ (ppb), in the three muscle types collected, which confirmed the homogeneous distribution of the drug residue throughout the skeletal muscle as a result of administration of ractopamine *via* feed.

2.2 REIMS - Database training and challenge

The ten loin muscles (coded MF), five from the control and five from the treated populations were analyzed using REIMS in negative ionization mode as a minimum of triplicates on three different days. Within each day, the ten samples were randomized. The mass spectra generated were processed and analyzed with the objective of investigating differences in the metabolic feature profiles between the two populations, control and treated pigs. Data processing enabled feature alignment resulting in 1931 detected deisotoped ions in the range 50-1200 *m/z*. The spectra were binned at 0.1 Da and the region between 600-1000 *m/z* was used to generate a multi-variate model with ten PCA dimensions (for data reduction) followed by one LDA dimension for binary classification. During the model optimization phase, ten PCA dimensions were shown to provide the optimum predictive accuracy as determined *via* the % correctness score generated using *in silico* stratified 5-fold validation following iterative rounds of model training using between 2 and 30 PCA components. The % variance explained by each PCA dimension was

calculated, and greater than 95% of the total variance was shown to be explained with first 10 components. An outlier threshold of three Standard Deviations was applied. As illustrated, in Figure 1 the model enabled discrimination of the mass spectral profiles corresponding to the muscle samples from either control or treated animals. The predictive classification accuracy was calculated as 99.2% *via* stratified 5-fold *in silico* validation which presented only one "false suspicious" sample out of 106 analyses. Clear discrimination between treated and control populations was also observed using both an alternative data analysis algorithm (OPLS-DA) and software (MetaboAnalyst 3.0) (Figure S12) ensuring confident separation of the two classes and confirming the ability of the studied ionization strategy to provide specific fingerprints of muscle samples in relation with growth promoting practice during the breeding period. Some scatter in the data was observed between the different analysis days, however, this did not affect the grouping in the multivariate analysis scores plot.

The model was subsequently challenged using 21 different samples collected from the same animal study (11 control and 10 treated) not included in the model training set originating from different muscle locations (MJ series thigh (n=9), MF series loin (n=2) and ME series shoulder (n=10)) in triplicate using the real-time recognizer functionality (Figure S3). The overall outcome was determined from the mean of the three replicates (Table 1). Individual results having a % confidence matching factor of less than 80% were excluded from the overall outcome. On the basis of the independent validation, all muscle samples except one (an MJ series treated sample) were correctly classified leading to an overall correct assignment rate of ≥95%. Based on these findings a 0% false positive rate and a 10% false negative rate was estimated. Such preliminary performances are very encouraging, especially considering the relatively low number of samples involved and that different muscles types have been considered, in an experimental design corresponding to expected practices of use in finishing animals, where no withdrawal period is applied. Further improvements to the model performance are therefore easily envisaged whereby the model training set is expanded to include representative samples from the other muscle locations due to the observed variation in physiological lipid depositions in different muscle location. However, the preliminary performance shows relevance with the screening requirements as expressed in Dec 2002/657/EC (Council Decision 2002/657/EC 2002) where a maximum of 5% false negative is considered acceptable.

Further, the applicability of the strategy on fresh pork meat samples purchased at local supermarkets was evaluated. Three different meat samples were profiled using the REIMS and corresponding fingerprints were automatically processed for recognition against the established model. The samples were analyzed a minimum of ten times each and were recognized in 100% cases as control pork meat (Figure S4), in accordance with what was expected for meat samples collected in the EU.

2.3 Preliminary pathway analysis

Although the identification of lipid species affected by ractopamine was not the objective of the current work, preliminary hypothesis could be drawn, which will require future appropriate MS/MS investigation. The loading S-plot associated with PCA/LDA (Figure 2a) and OPLS-DA (Figure 2b) shows significant features involved in class separation. Closer investigation shows that members of the diacylglycerophosphoethanolamine (PE), phosphatidylinositol (PI), sphingomyelin (SM) and free fatty acid classes as present in the extremity region are significantly involved in the differentiation of control and ractopamine treated pig populations. Complementary investigation of interesting features was also performed through hierarchical clustering analysis which confirmed relevance of previously highlighted species (Figure 3). The main affected lipids are detailed in Table 2 and Figure S5 (putative identification). The compounds highlighted as lipid species whose profiles would be significantly affected by ractopamine were further selected for pathway analysis using MetabolAnalyst 3.0 software and Lipid Maps (Fahy et al. 2007; Fahy et al. 2009). It could, thus, be hypothesized that glycerophospholipid metabolism was implicated as the most significant involved pathway. REIMS, which specifically allows detection of phospholipids, therefore appears as a technique of choice in this context. Further investigations down to the acyl-composition is now required to conclude on lipids identity and name concrete pathways.

The link between ractopamine and lipid metabolism has been investigated decades ago in farm animals (Dunshea FR. 1993). A reduction in the deposition of adipose tissue in the carcass of pigs fed the diets containing ractopamine has been reported by many authors (Uttaro et al. 1993; See et al. 2004; Mimbs et al. 2005). Such an effect is hypothesised to occur through either or both two metabolic pathways: reduction in lipogenesis and increase in lipolysis. In the 1980s, a number of scientist believed that β-agonists had the property (in various animals such as lamb, swine or steers) of stimulating the lipolysis in adipose tissue, catalysed by the hormone-sensitive lipase and releasing free fatty acids into the blood stream (Baker et al. 1984; Ricks et al. 1984; Jones et al. 1985). These authors, however, who worked with the determination of lipolysis in the pigs through the quantification of serum NEFAs did not find any increase in these elements (Dunshea FR. and King 1994, 1995), thereby indicating that lipolysis might not be the preponderant factor in the reduction in the amount of adipose tissue deposited in the carcass of pigs. The same authors also suggested that ractopamine at doses of 0 and 20 ppm did not stimulate fat mobilization or lipolysis in the pigs or, if it occurred, the effect disappeared after three days. Later (Dunshea FR. et al. 1998) they reported that ractopamine promotes protein deposition with little effect on fat deposition in the pig. In particular, they investigated whether the lack of effect on fat deposition was due to a lack of effect of ractopamine on lipogenesis. They demonstrated that dietary ractopamine increased average daily gain ($P < 0.050$) and glucose turnover by 24%, presumably to support the increased skeletal muscle protein deposition that occurs during dietary ractopamine treatment. They could not demonstrate any effect of dietary ractopamine on the rate of lipogenesis in adipose tissue. More recently and considering the lack of consensus in the literature regarding the mechanisms responsible for the

effects of ractopamine on the metabolism of lipids (da Silva Ferreira et al. 2013) reviewed *in vivo* mechanism of action through which ractopamine reduces the amount of adipose tissue in carcass of animals fed diets supplemented with this drug. The review analysis explained that in the pigs, the use of ractopamine reduced lipid deposition in the carcass due to a greater inhibition of lipogenesis than an increase in lipolysis.

Our results suggest global alteration of lipid profile with mainly higher amounts of some diacylglycerophosphoethanolamine (PE) and phosphatidylinositol (PI) in muscle tissues collected on ractopamine fed pigs. Such observations, however, could not be related to any conclusion on lipogenesis or lipolysis as reported by previous studies above which were performed in serum.

3. CONCLUSION

Recent technological advances using innovative mass spectrometry technologies combined with omics strategies are now well suited to address the issue associated to rapid classification of food samples to guarantee chemical food safety to the consumers (Balog et al. 2016). In this context, the REIMS method has been optimized for real-time analysis of pig muscle tissue, and the results suggest spectral analysis is accurate and rapid for determination of the status of the animals regarding exposure to ractopamine during breeding.

Further work will establish complete scope and robustness of the screening strategy. In particular, the generality of the approach toward different drugs in this action class will be investigated. In parallel, the false negative rate will be established considering samples from populations under different regimes of expected use.

Such emerging strategies are efficient ways to highlight candidate biomarkers of such practices in livestock and are considered by the community as promising tools for ensuring safer food to the consumer in a more efficient way. Furthermore, the LiveID™ software empowers the strategy as it enables real-time classification of unknown samples, through comparison against a trained database. Such workflow can be considered an *in situ* control platform to be implemented at the slaughterhouse or inspection points at the border. A range of food quality applications may also easily be foreseen, whenever high-throughput characterization is a pre-requisite.

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FIGURE CAPTIONS

Figure 1: Image of the 3D PCA score plot generated in LiveID using 10 PCA dimensions generated from the training set of 106 spectra (50 control and 56 treated) across a mass range of 600-1000 m/z with a binning parameter of 0.1 Da.

Figure 2: (a) Loadings plot PCA/LDA model (generated in LiveIDTM) showing the m/z features responsible for the discrimination in the first component in the region between 600-900 m/z. (b) Loadings S-Plot (generated using Progenesis QI and EZInfo 3.0.0.0) showing the significant features (m/z) responsible to the discrimination between treated (upper right quadrant) and control (lower left quandrant) populations. Red circles highlight the 2 example common ions seen in the Heat map representation and compound abundance plots of the data set.

Figure 3: Hierarchal cluster analysis heat map representation (generated using MetaboAnalyst 3.0 software) of the features responsible for the discrimination between the control and treated pig populations created from the top 50 most statistically relevant (ANOVA p-value ≤ 0.05 max fold change ≥ 2 and minimum CV ≥ 30) features (in the region of 600-1200 m/z). The degree of red shading signifies up-regulation whilst blue shading indicates down-regulation at the individual feature level.