GMO: traceability and coexistence

Yves Bertheau

Background GMO productions

- Main currently concerned productions

New productions and supply chains

Global Adoption Rates (%) for Principal Biotech Crops (Million Hectares, Million Acres), 2011

ISAAA

M Acres

M Hectares	M Acres
445	180
395	160
346	140
296	120
247	100
198	80
148	60
99	40
49	20
0	0

☐ Conventional
☐ Biotech

159
100
30
31

82% Cotton
75% Soybean
32% Maize
26% Canola
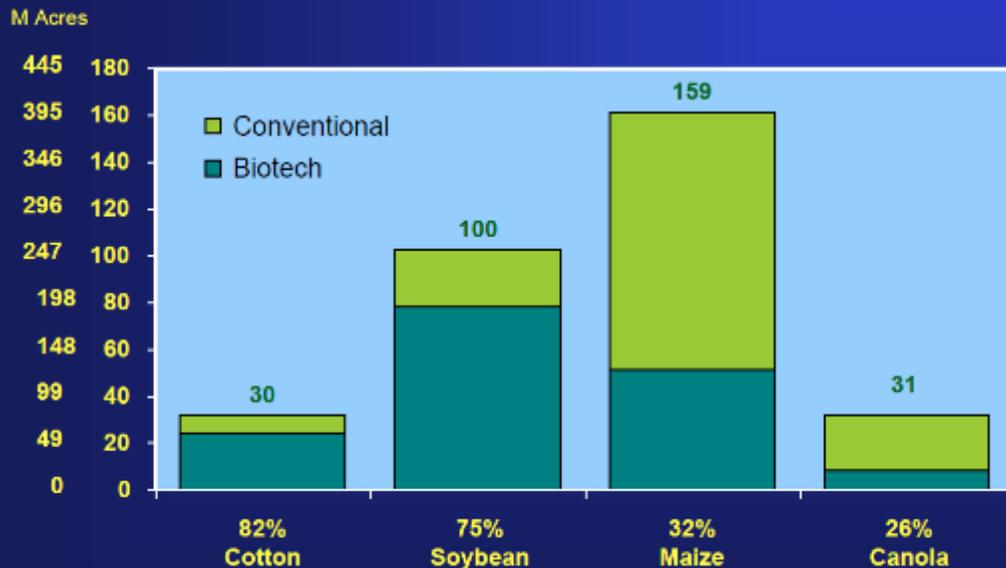
Source: Clive James, 2012

potato rice

tomato squash

papaya sugarbeet

• **Food supply chains contaminations: Starlink, pig vaccine corn, tomato seeds, papaya, Bt10, LLRice601, Bt63…**

Future GMOs for industry, phytoremediation, biofuel, molecular farming… which shall not enter the food supply chains

GLOBAL AREA OF BIOTECH CROPS
Million Hectares (1996-2011)

Legend:
- Total Hectares
- Industrial
- Developing
- 29 Biotech Crop Countries
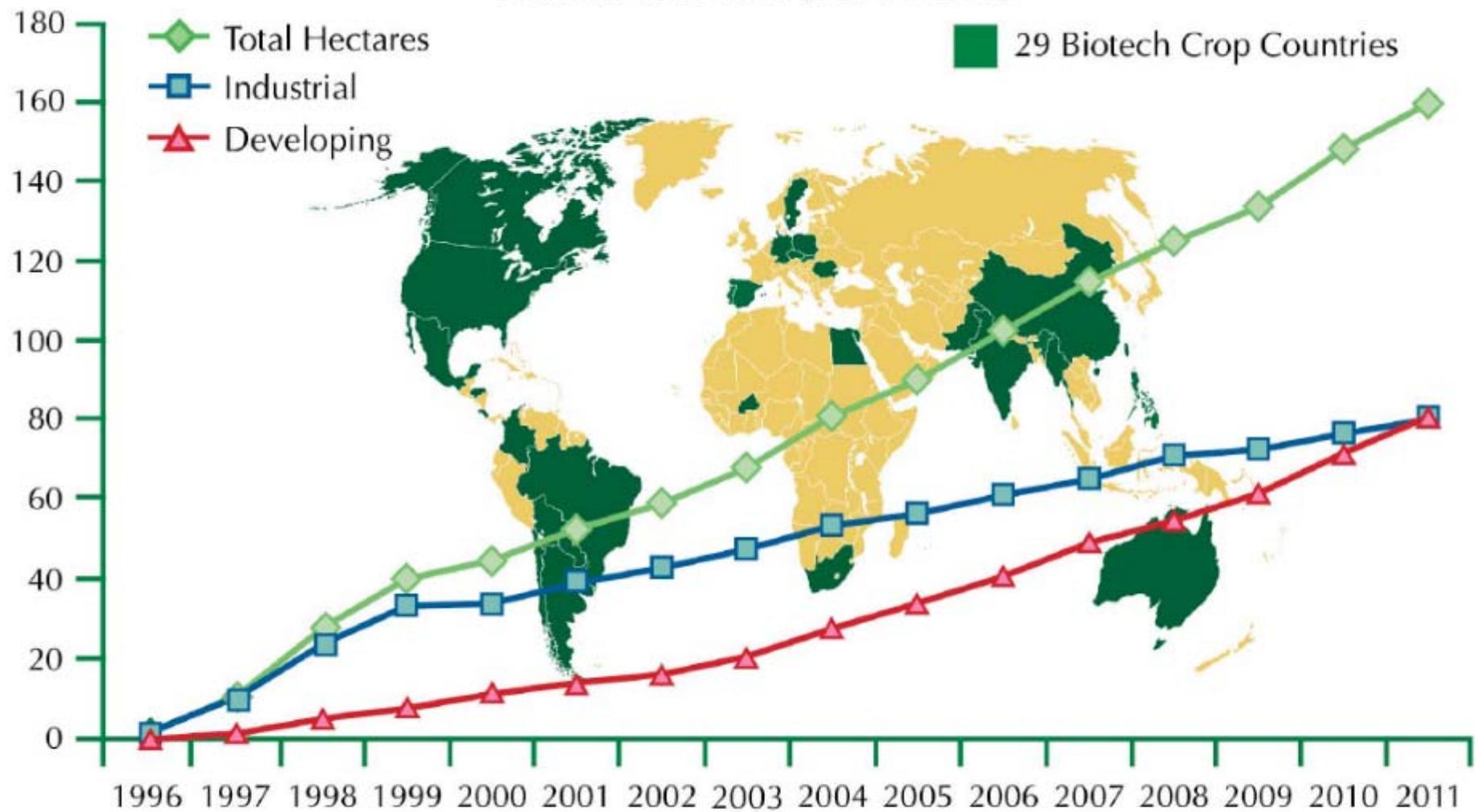
A record 16.7 million farmers, in 29 countries, planted 160 million hectares (395 million acres) in 2011, a sustained increase of 8% or 12 million hectares (30 million acres) over 2010.

Source: Clive James, 2011.

Global Area of Biotech Crops, 1996 to 2011: By Trait (Million Hectares, Million Acres)

ISAAA

M Acres

Legend:
- Herbicide Tolerance
- Insect Resistance (Bt)
- Herb Tolerance/Insect resistance

Y-axis (M Acres / M Hectares): 247/100, 222/90, 198/80, 173/70, 148/60, 124/50, 99/40, 74/30, 49/20, 25/10, 0/0

X-axis: 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011
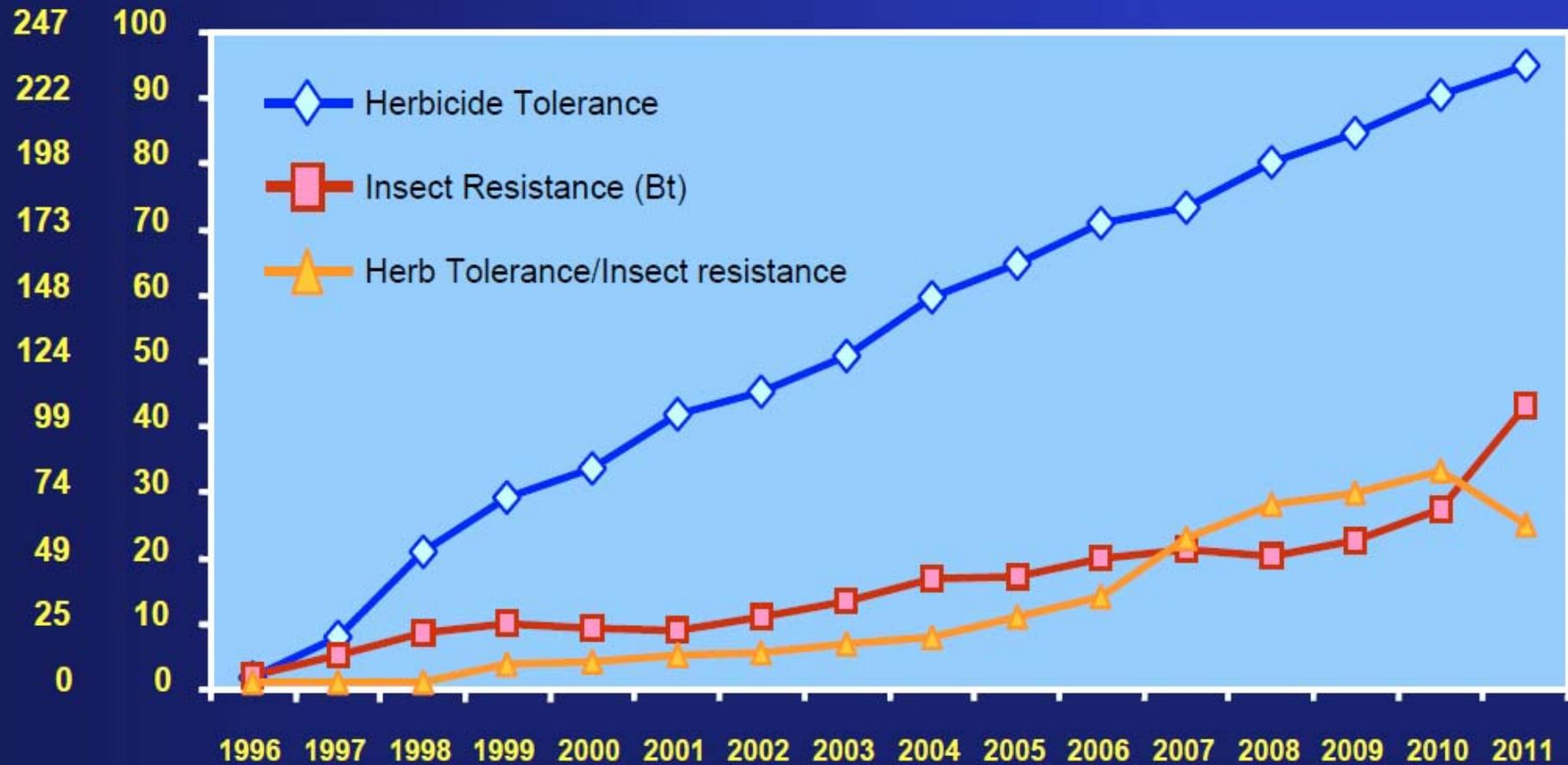
Source: Clive James, 2012

GMO approvals in the EU according to EuropaBio (2011)

FIGURE 1: Number of approved GM products in the EU, US, Brazil and Canada

Note 1:
Canada includes Plant Novel Traits (PNT), some of which are not GM.
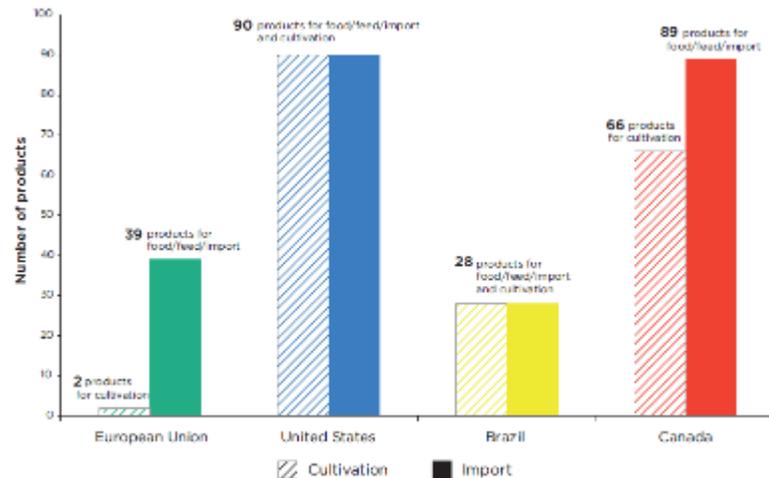
Note that the public perceptions
As well as the regulatory systems are
rather different

Average time required for a GM product approval

Time (in months)
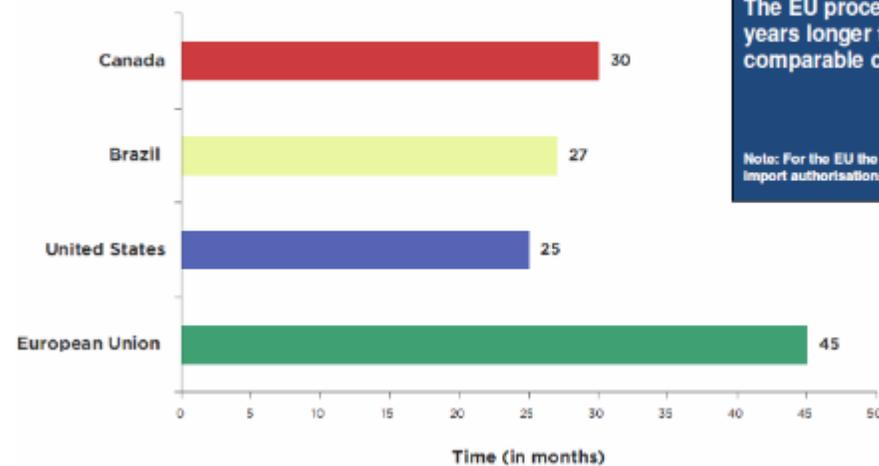
The average time required to achieve authorisations.

The EU process is 1.5 to 2 years longer than in comparable countries.

Note: For the EU the data only applies to import authorisations.

# GMO production

- Currently: random mutagenesis, biolistics, agro-transformation…
- New methods (GMOs? Under discussion since >5 years):
  - ftp://ftp.jrc.es/pub/EURdoc/JRC63971.pdf
  - Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
  - Oligonucleotide directed mutagenesis (ODM)
  - Cisgenesis and intragenesis
  - RNA-dependent DNA methylation (RdDM)
  - Grafting (on GM rootstock, new species?)
  - Reverse breeding
  - Agro-infiltration (agro-infiltration *sensu stricto*, agro-inoculation, floral dip)
  - RNAi / siRNA (effect on the gene regulation of feeding host?)
  - Synthetic genomics
- Risk evaluation procedures:
  - Based on chemicals assessment
  - Still evolving (EFSA, guidelines…)
  - New ways for new products?
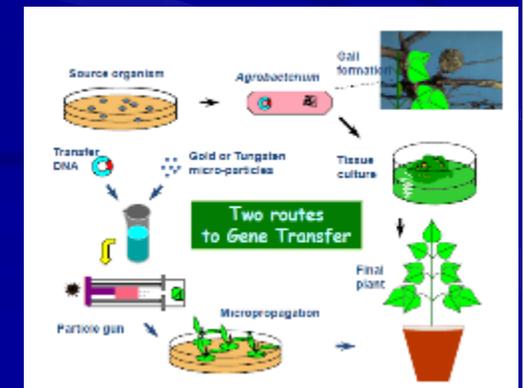
GMO approval process in the EU
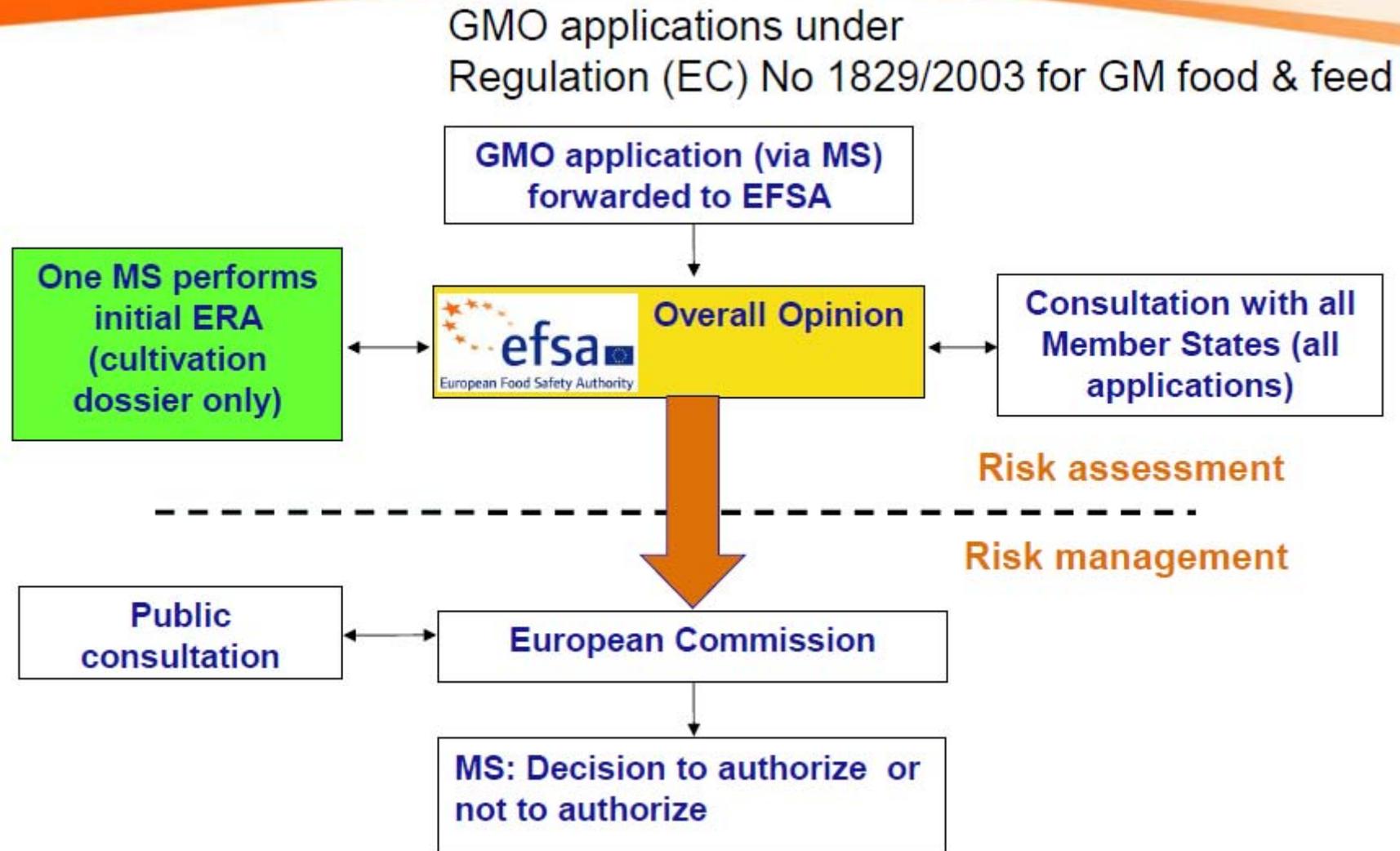
Dossiers and GMO evaluation

- Concepts of familiarity and substantial equivalence

- Molecular characterization

  - Characteristics of the donor and recipient organisms

  - Genetic modification and functional consequences

- Comparative analysis of the GM plant with non GM

  - Agronomic characteristics

  - Compositional and nutritional characteristics

- Food/feed safety in relation to intake

  - Influence of processing on properties of food/feed

  - Potential for changes in dietary intake

  - Potential toxicity and allergenicity of gene products, plant metabolites and the whole GM plant

  - Potential for long-term nutritional impact

Dossiers and GMO evaluation

- Environment impact of GM plant compared to non-GM plant
  - Persistence and invasiveness
  - Selective advantage/disadvantage
  - Potential for gene transfer
  - Interactions between GMP and target organisms
  - Interactions between GMP and non-target organisms
  - Effects on biochemical process
  - Impacts on cultivation, management and harvesting techniques
  - Potential interactions with abiotic environment (e.g. altered sensitivity/tolerance to mineral toxins, salinity…)
- Environmental monitoring plan for the GMP
- Unique Identifier (OECD), sampling and detection methods

Dossiers and GMO evaluation

- A priori and a posteriori risk evaluation and monitoring still evolving:
    - based on chemicals assessment,
    - new requirements for NPBT,
    - RNAi and epigenetics impact on feeding hosts,
    - statistics guidelines for risk evaluation procedures,
    - statistics guidelines for crop production assessment,
    - ERA procedures,
    - guidelines for PMEM procedures / networks / NTO to consider / baseline(s) / statistics and GIS…
- How to consider the appearance of resistant insects and weeds? As for any change of agricultural practices? Despite the introduction of stacked genes involving the reintroduction of harmful pesticides?
- True, independent and reliable cost-benefits analyses still missing

The 2 main European pillars

- 258/97 regulation on novel food and novel ingredients: mandatory labeling (irradiated products, any ingredient new in the EU, GMOs, etc.)

- 178/02 regulation also called "General food Law": mandatory traceability one step up, one step down

178/02/EC ➔ **principle of "one step up" & "one step down"**

ISO 22000
ISO/TC34 WG 8

ISO/CD 22519
ISO/TC34 N 1130
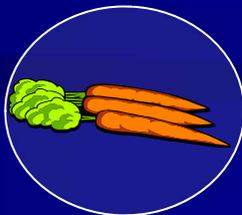
Primary production   **Supplier**   **Producers**   **Transportation**   **Trade**

EU regulations on GMOs

- GMO approval
  - Notifiers under 2001/18 or 1829/03 have to develop and support the costs of validation
- GMO traceability (1830/03/EC, 2001/18/EEC, 1829/03/EC)
  - Obligation: general (178/02/EC) and GMO specific
  - Costs reductions by mostly analyzing raw products to be further traced
  - Labeling of products with or without analytes

178/02/EC → **principle of "one step up" & "one step down"**

ISO 22000
ISO/TC34 WG 8

ISO/CD 22519
ISO/TC34 N 1130
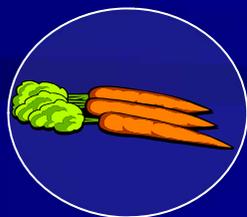
Primary production **Supplier** **Producers** **Transportation** **Trade**

GMO detection organisation

- CA

- National networks (DE, FR, BE, etc.)

- ENGL (28 EU MS, CH, NO) + EC DG observers + third countries observers (China, Black Sea, Maghreb, etc.): methods validation, working groups on e.g. performance criteria, detection of UGM, accreditation, etc. chaired by JRC IHCP

- EURL-GMFF (formerly CRL-GMFF) and IRMM-JRC

Some GMO traceability related FP5 research programs

- Several FP5 programs on food safety and quality, detection methods…

- DNAtrack: N. Marmiroli
- QPCRGMOFOOD: 2000-2003 A. Holst-Jensen
- GMOCHIPS: 2001-2004 J. Remacle & Y. Bertheau
- ENTRANSFOOD Cluster: H. Kuyper

Results
- provided first insights on GMO detection
- Evidenced issues on GMO detection
- Influenced the European regulation: 1829/03, 1830/03

EC FP6 programs on Co-existence and/or Traceability

SIGMEA (FP6, STREP) Sustainable Introduction of GMOs into European Agriculture: 2004-2007
J. Sweet & A. Messéan INRA

Co-Extra (FP6, IP): 2005-2009 Co-existence and traceability in the GM and non-GM supply chains
Y. Bertheau, INRA

PETER (FP6 Specific Support Action) Promoting EC traceability research 2005-2007
M. Debord, CCI Gers

Transcontainer (FP6, STREP) (program on tools for biological containment) 2005-2009
R. De Maagd, Wageningen Univ.

Altogther > 30 M€ spent by the EC on risk assessment, traceability and coexistence issues
http://ec.europa.eu/research/biosociety/pdf/a_decade_of_eu-funded_gmo_research.pdf

FP7 research projects

- GMSAFood biomarker for health post-market monitoring
- GMULTI multiplex detection of unapproved GMOs
- GRACE Risk assessment and communication of Evidence
- AMIGA Assessing and monitoring the impacts of genetically modified plants on agro-ecosystems
- PRICE coexistence issues

In support to: ENGL, EcoB, US AC21…

With several conflict of interests, revolving doors and confirmation bias ("myside bias") issues

In a ordered, logical and technocratic way defined by the EC and MS to which nature and research data shall adapt to…

Numerous sampling plans

- Public: CEN, ISO, etc.
- Private: GAFTA, ISTA, AOSCA, AOAC, etc.
- Mandatory or not (e.g. EC emergency plans)
- For several purposes and products:
  - from seeds to commodities and packaged products,
  - from coexistence research to control plans
  - from biodiversity studies to search for unexpected escape of transgenes in landraces

Detection methods of GMOs

- Phenotype (e.g. herbicide tolerance of seedlings, kernels…)

- Immunoassays

- DNA based methods (PCR, LAMP, SNPlex, NAIMA, micro-arrays, LCR…)

- Different units impacting controls and coexistence issues, see for instance the 2011 HCB advice on coexistence

Model of control plan
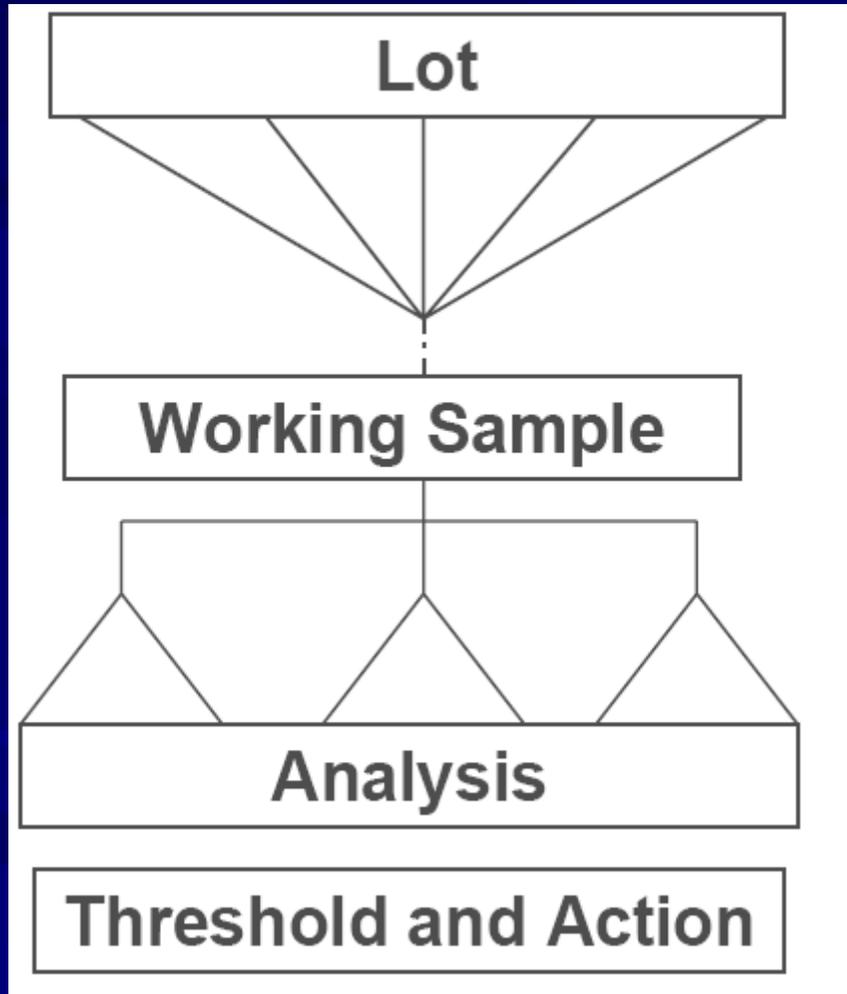
Proportion of GM product Heterogeneity

Number of primary samples: enough primary samples to defend against heterogeneity

Size of working sample (number of kernels): large enough to capture rare events

Particular pattern of replication

%GM DNA, between and within laboratory variation and limit of detection: accurate enough in context of sampling and fitness for purpose

Test portion preparation: an important source of uncertainty
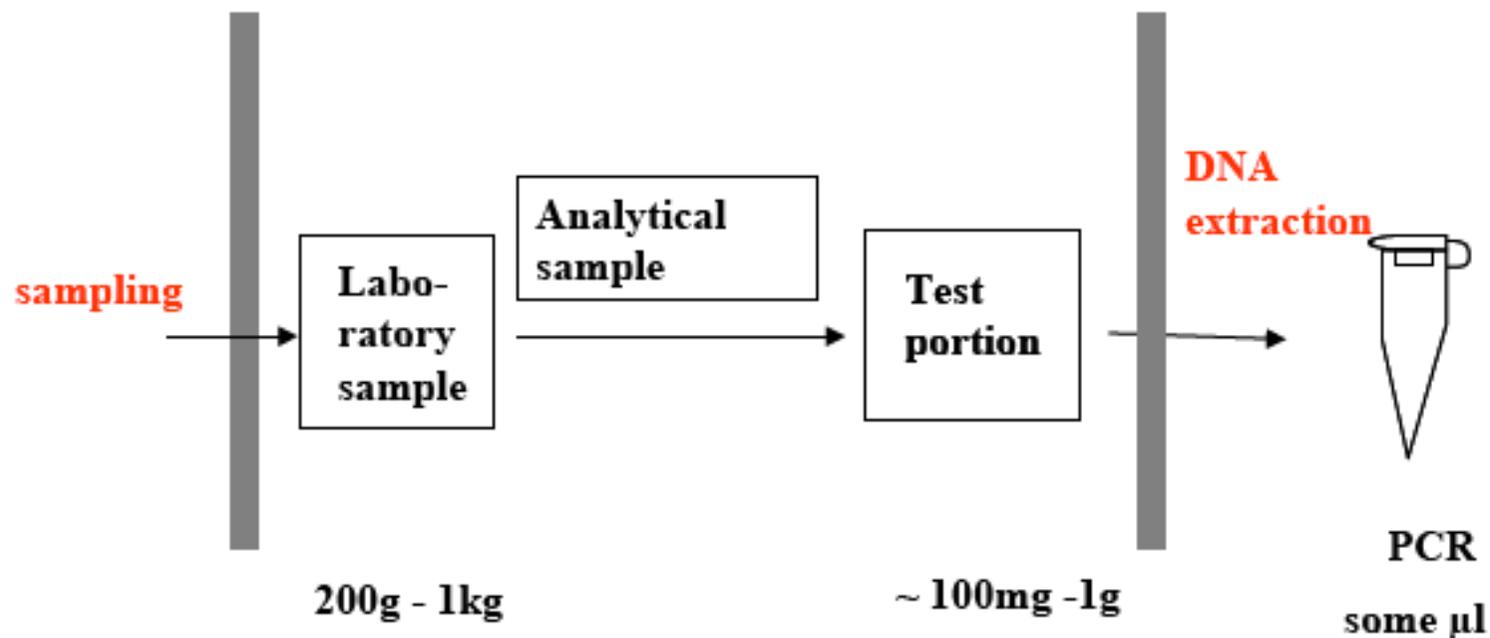
Fitness for purpose

$$RSU_{control} = \sqrt{\frac{S^2 \times z \times \left(100 - \frac{\overline{X}}{z}\right)}{N \times \overline{X}} + \frac{z \times \left(100 - \frac{\overline{X}}{z}\right)}{n \times \overline{X}} + RSU_{analysis}^2 - \frac{m-1}{m} \times RSD_r^2 + \frac{LOD^2}{9 \times \overline{X}^2 \times m}}$$

$$Cost_{control} = F_S + N \times V_S + F_{SS} + n \times V_{SS} + F_a + m \times V_a$$

- Fit for purpose measurement:
  - Cost is constrained by budget
  - RSU is minimised for that cost
- Or:
  - RSU is low enough
  - Cost is minimised for that RSU

Immunoassay Formats

- Each format has advantages and disadvantages
  - Fully automated – clinical analyzers
  - Laboratory kits - ELISA
  - Field tests – "Strip tests"
  - 'Research' methods – western blot
- Choice of method is determined by specific application
  - Performance specifications
  - Ease-of-use (user training)
  - Testing location
  - Cost per test
  - Batch size, testing frequency
  - Turnaround time
  - Equipment costs

Strip Test

(deapstick)

Absorbent Pad

Control Line

Test Line

Au

Protein

Membrane

Gold Pad

Au

Sample

Vial

Filter Pad
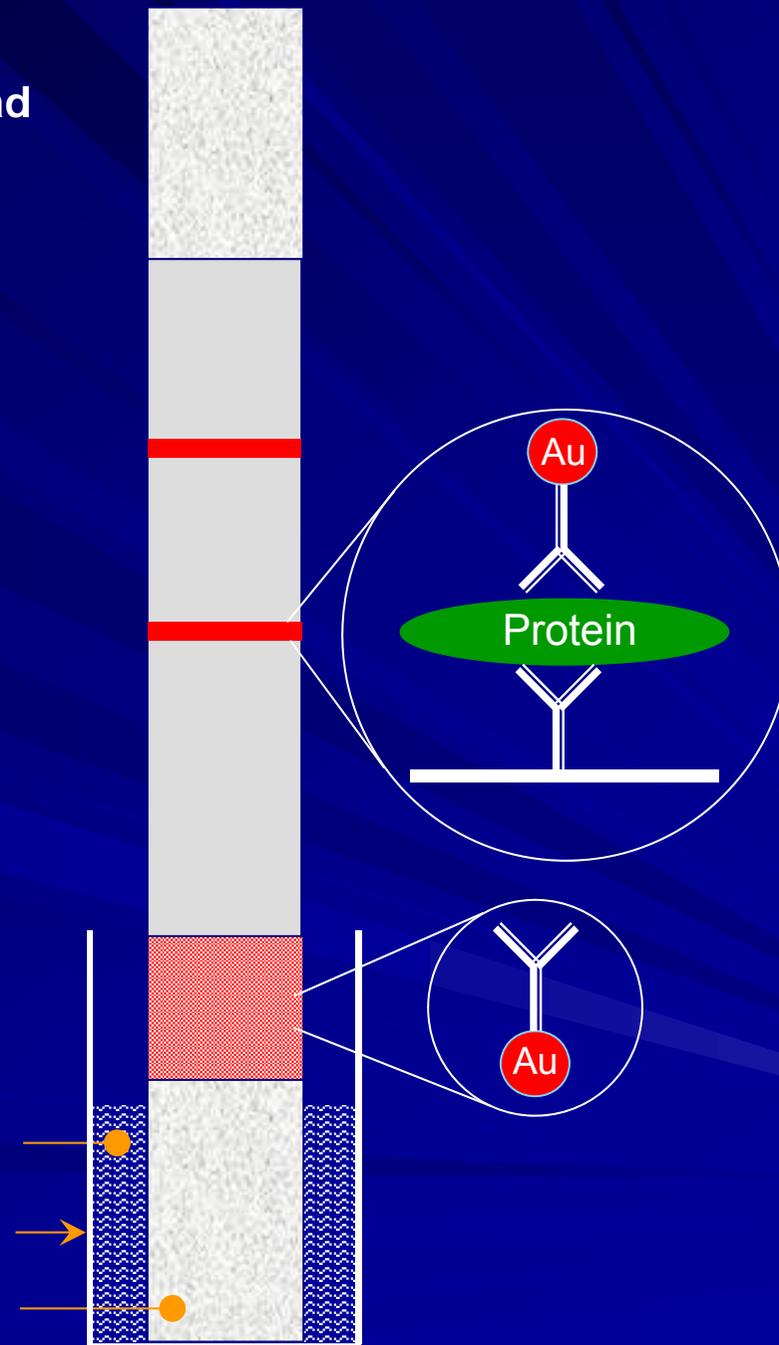
Seed, Leaf & Grain

- Obtain quick, easy & accurate results < 5 minutes
- Analysis in the field
- Significant benefit to the customer
  - Low cost
 - Highly reliable results

Scope extension to some processed products

How that works…

Sampling Station

Sampling Probe
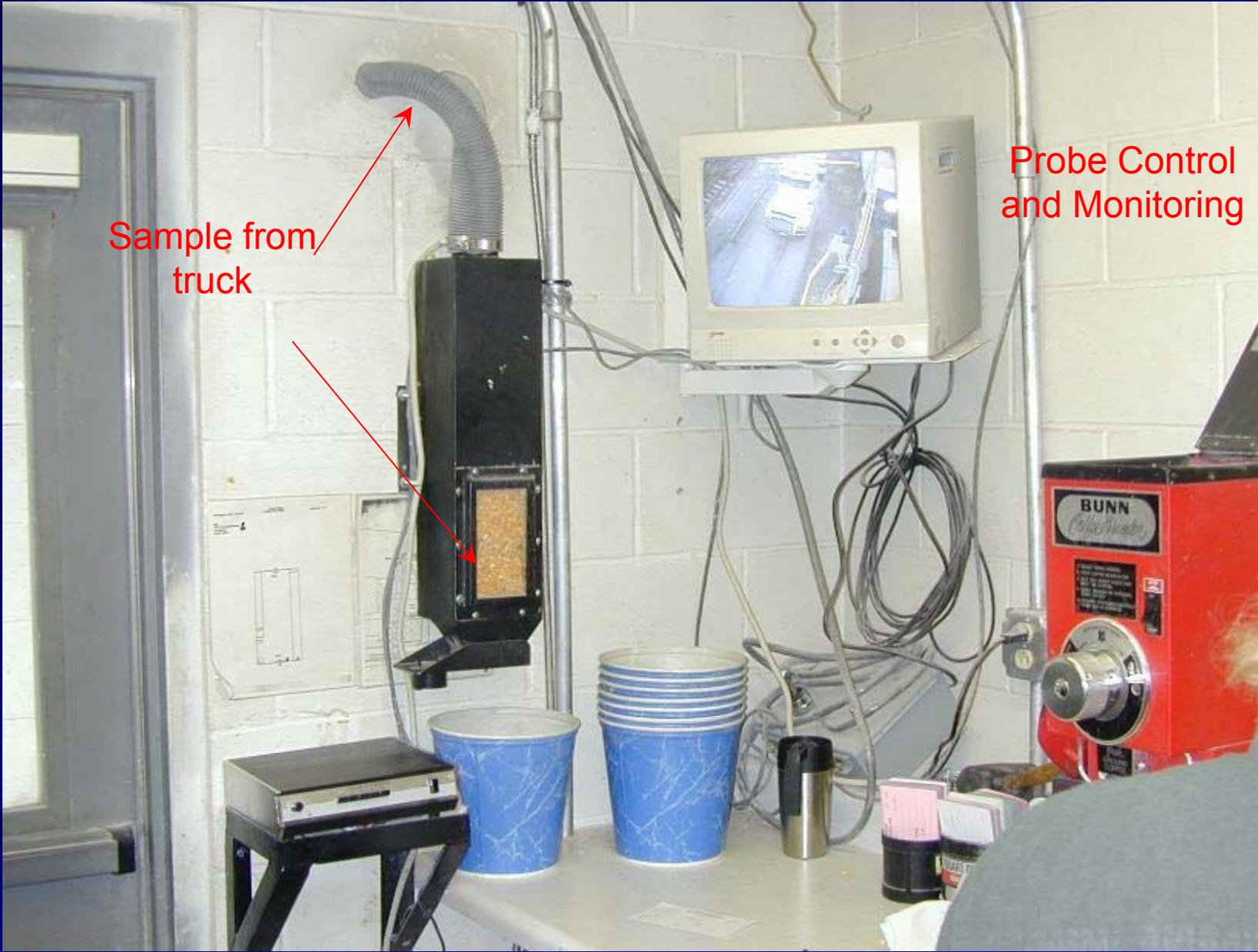
Sample from truck

Probe Control and Monitoring

Detection of common traits by strip test

Negative   Bt11   Bt176   Mon810

Control

Test

ELISA and strip test correlation

Soybean Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
% GMO	-	-	-	<0.1	-	-	1.3	0.2	0.3	-	-	-	-	-	-	0.5
Strip Rating	-	-	-	4	-	-	10	8	8	-	-	-	-	-	-	7

Elements of bulk grain threshold testing

- Estimate probability of detecting 1 positive in a large number (binomial distribution)
- Establish maximum sample size (number of kernels)
    - Only outcome of test is positive or negative
    - 1 positive kernel must always be detected
- Sensitivity (threshold level) determined by:
    - Number of kernels in sample
    - Number of samples
- Reliability of result stated in terms of statistical confidence rather than analytical precision

Advantages of immunoassay Methods

- Directly measure biologically active protein of interest, some resistant peptides

- Validatable/reproducible

- Quantitative analysis

- Qualitative analysis

- High sample throughput

- Easy to perform and transfer to other laboratories

- Widely accepted method by regulatory agencies

- Established use in food industry

- Cost effective

- Timely Analysis

Drawbacks

- Methods are trait specific (generally not GMO specific) and must be validated for each matrix (DNA and Protein-based)

- Limited to use of protein containing processed ingredients and final food products

- Some products may not express a detectable protein in grain

- Antibodies may cross react

No single method will detect all biotechnology-derived products (DNA or protein-based)

Expression of Bt Cry1A(b) in Corn

| Event	Seed (µg/g FW)	Leaf (µg/g FW)
Mon810	0.31	9.35
Bt11	4.76	20.00
Event 176	<0.005	1.00

Source – USDA Petitions
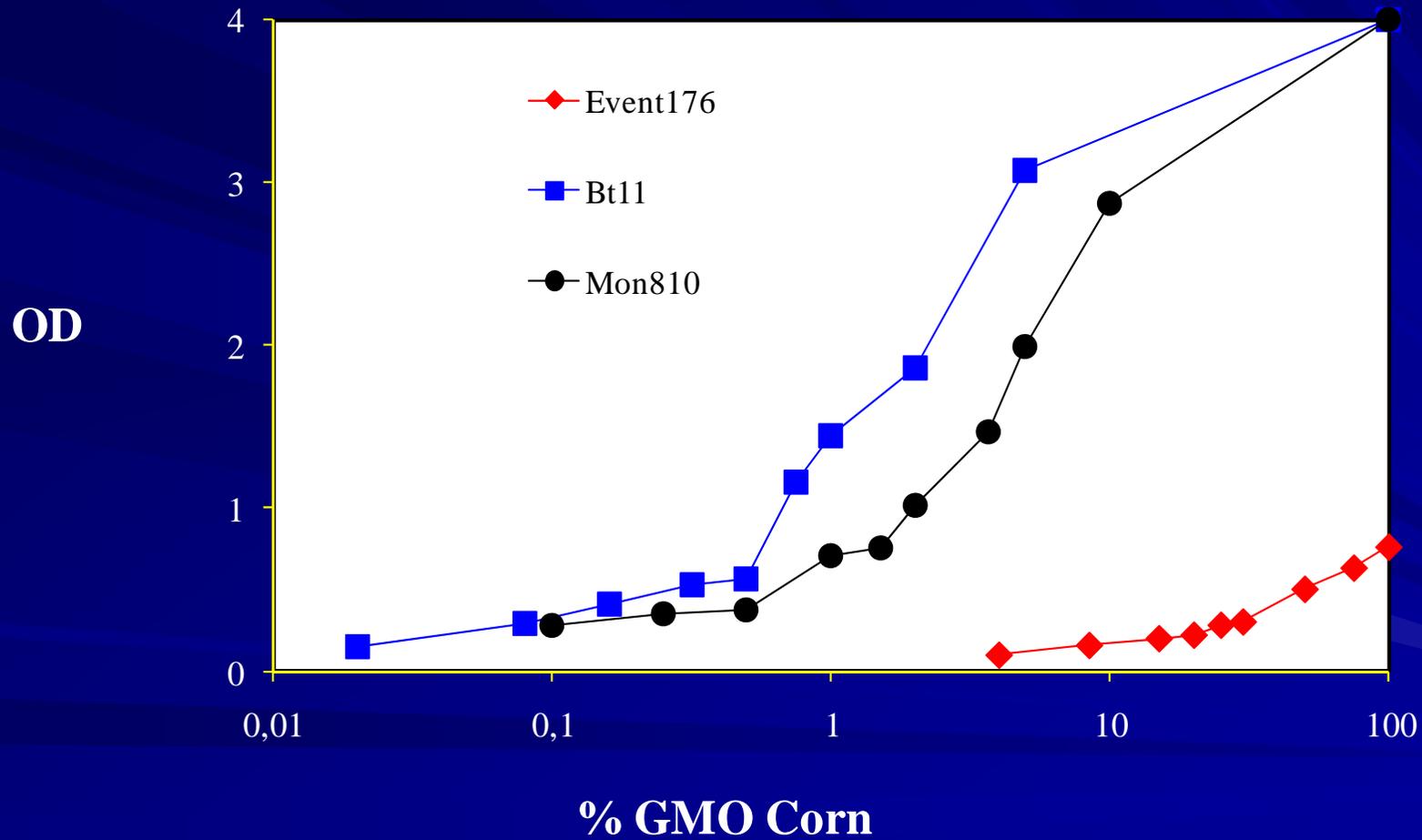
Quantification using Immunoassays

- Variability of protein expression levels
  - **Within an event (crop variety)**
  - **Between events expressing same protein (e.g., Cry1A(b))**

- Varied effects of sample processing on protein conformation and antibody binding

- Quantification of unknown mixed sample is difficult
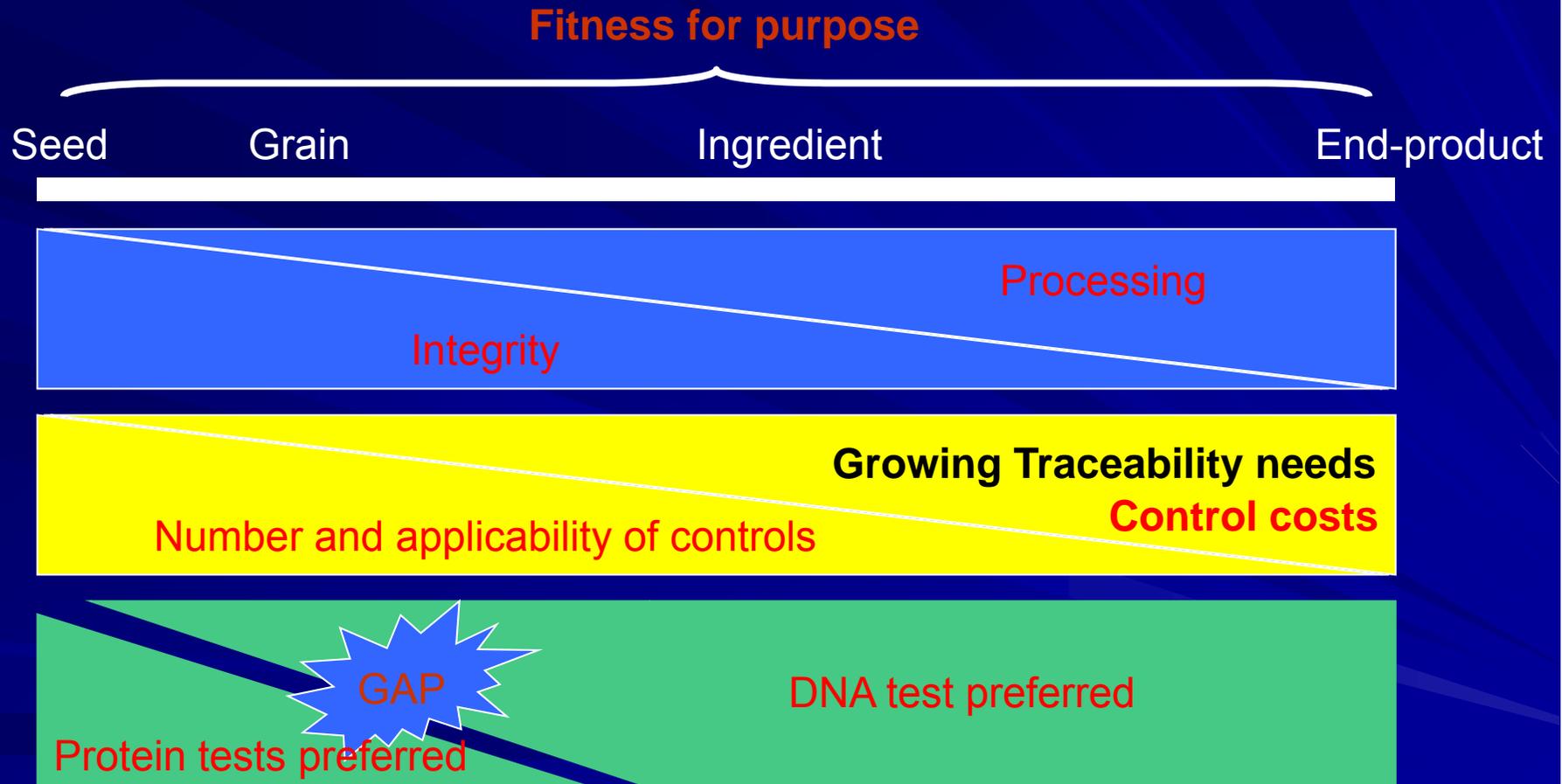
PCR – Potential targets

Plant genome — insert (promoter, gene →, terminator) — Plant genome

3   1   2   1   2   1   3
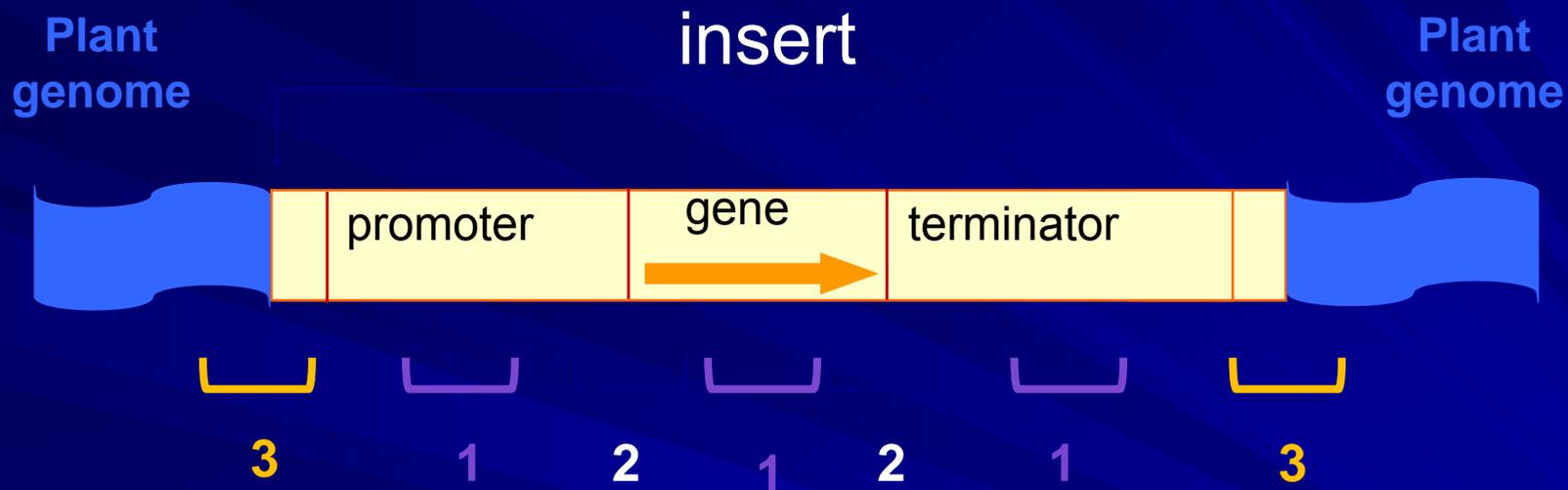
1: Screening (P35S / Tnos / nptII…)
2: Construct-specific test
3: Event-specific test
4: Plant reference genes (from 1 per plant species to one for all)
5. Donor organism when needed (INRA's CaMV test saved tons of seeds)

GMO screening (taxa & controls)

Negative

GMO detection

Positive

Authorized?

GMO identification

Illegal ← No ← → Yes

Assay individual ingredients

GMO quantification

No need for labeling — Less than 0.9%

Labeling required — More than 0.9%

4 kinds of targets: ubiquitous elements (screening), taxa reference genes, construct specific, identification by edge fragments (construct / plan genome)

Genetics of plant seeds – relative parent contributions

monocot (maize):

Chenopodiaceae (beet)

Fabaceae (soya)

seedcoat (2n)

seedcoat (2n)

seedcoat (2n)

endosperm (3n)

embryo (2n)

endosperm (2n)

embryo (2n)

embryo (2n)

	♀	♂
embryo	1	1
endosperm	2	1
seedcoat	2	0

	♀	♂
embryo	1	1
endosperm	2	0
seedcoat	2	0

	♀	♂
embryo	1	1
endosperm	-	-
seedcoat	2	0

Effect of parental contributions

seedcoat (2n)

endosperm (3n)

embryo (2n)

	♀	♂
embryo	1	1
endosperm	2	1
seedcoat	2	0

Maize:
Embryo ≈ 48 % of total DNA?
Endosperm ≈ 49 % of total DNA?
Seedcoat ≈ 3 % of total DNA?

From Trifa & Zhang
J. Agric. Food Chem.
52: 1044-1048 (2004)

GM ♀ X non-GM ♂
GM = 24 + 33 + 3 % = 60 % of total DNA

Non-GM ♀ X GM ♂
GM = 24 + 16 + 0 % = 40 % of total DNA

GM ♀ X GM ♂
GM = 48 + 49 + 3 % = 100 % of total DNA

What does 0.7 % GMO mean in Qn analysis?

Effect of grinding (e.g. beet seeds)

GM male x non-GM female yields non-GM endosperm and GM embryo

100 % GMO

Grinding

10 % GMO
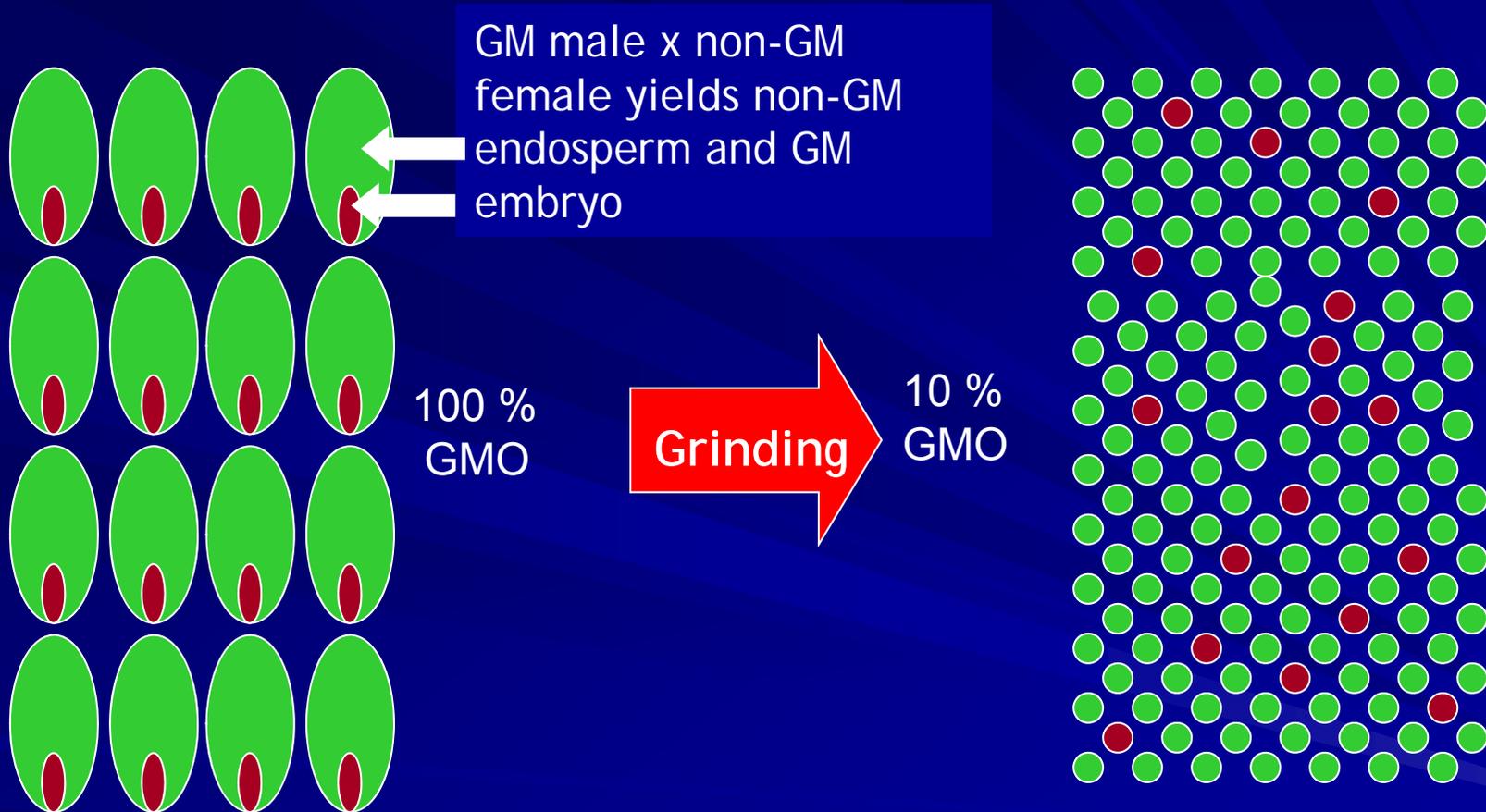
Homogenous (all seeds have GM embryo) → heterogenous sample (embryoderived particles = GM, endosperm derived particles = non-GM)

Taxonomy issue
(e.g. sugarbeet)
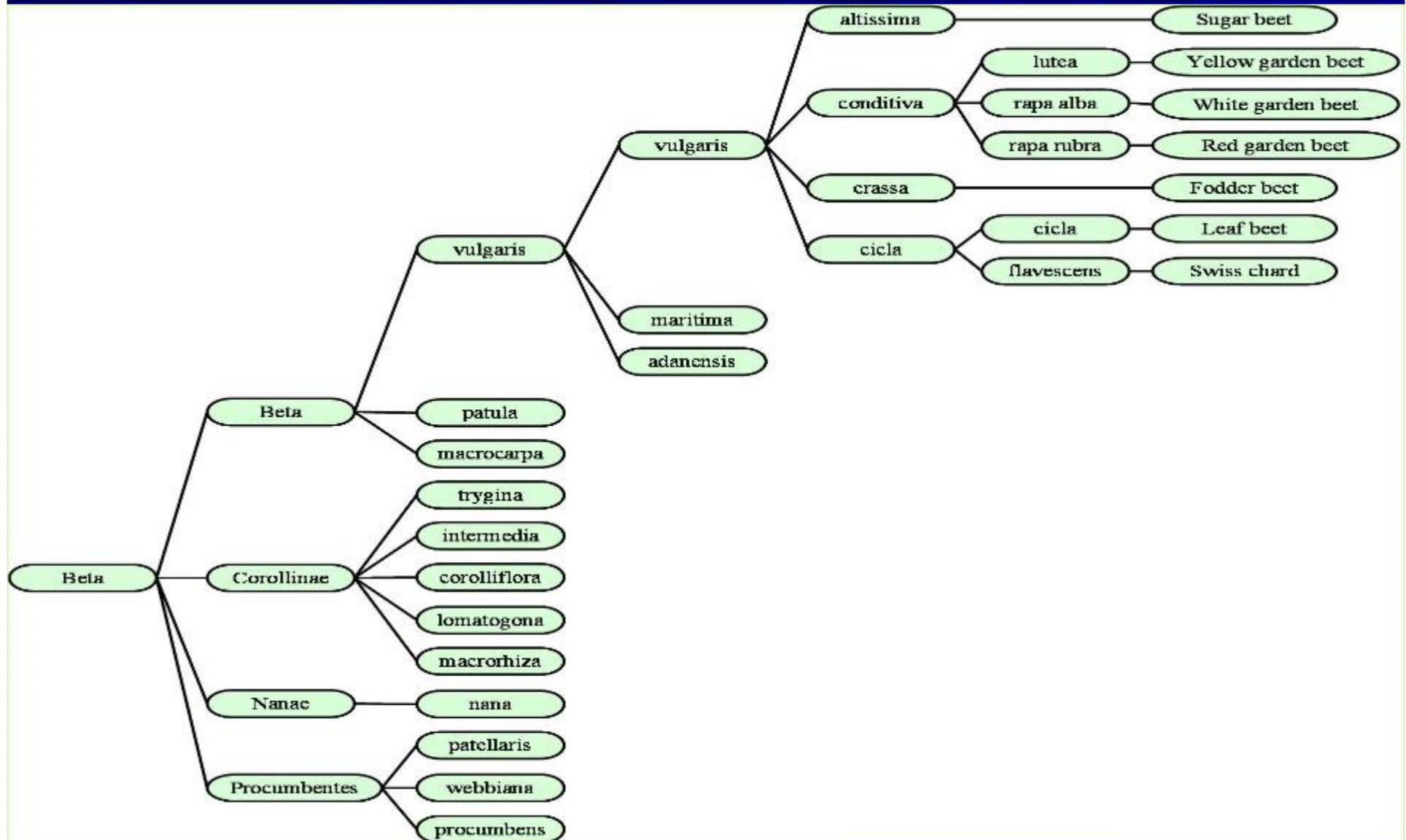
Taxonomy issue (e.g. sugarbeet) — SPS (INRA) — GS2 (notifier test)

| Genus	Species and varieties	B3	L1	L2	Linker	Prom	ST	GS2
Beta vulgaris vulgaris	Sugar beet	+	+	+	+	+	+	+
	Fodder beet	+	+	+	+	+	+	+
	Garden beet	+	+	+	+	+	+	+
	Leaf beet	+	+	+	+	+	+	+
Other species of Beta genus	Beta v. maritima	+	+	+	+	+	+	+
	Beta v. adanensis	+	+	+	+	+	+	+
	Beta patula	+	+	+	+	+	+	+
	Beta macrocarpa	+	+	+	+	+	+	+
	Beta patellaris	-	-	+	+	-	+	+
Other genus of Amaranthaceae family	Atriplex halimus	-	-	-	+	-	-	-
	Atriplex hortensis	+	-	+	+	-	-	-
	Spinacia oleracea	-	-	-	-	+	-	-
	A. glaucum	-	-	-	-	-	-	-
	Suaeda vera	-	+	-	-	-	-	-
Unrelated species	Maize	-	-	-	-	-	-	-
	Rapeseed	-	-	-	+	+	-	-
	Soya	-	-	-	-	-	-	-
	Potato	-	-	+	-	-	-	-

Distribution of GMOs in real lots

Integrating heterogeneous distribution

- JRC's Kelda and Keste project and software

Distribution of GMOs in real lots

Binomial

OK x NO

Distribution of GMOs in real lots

**Other possible scenarios: different GMOs with different distributions**
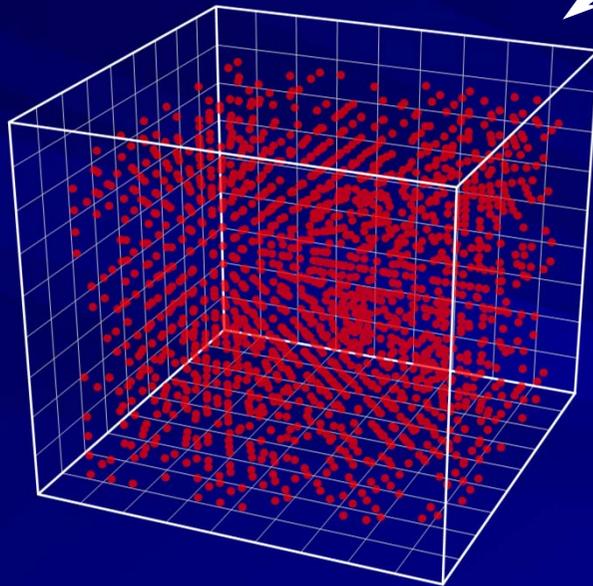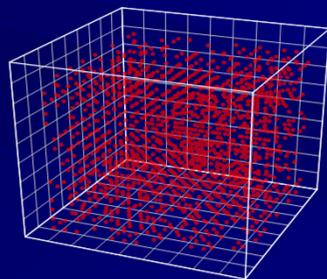
Homogeneous Lot

Heterogeneous Lot

Y Z X

Y Z X
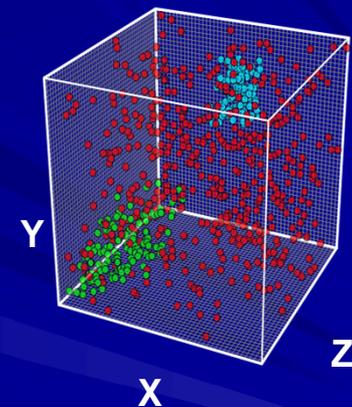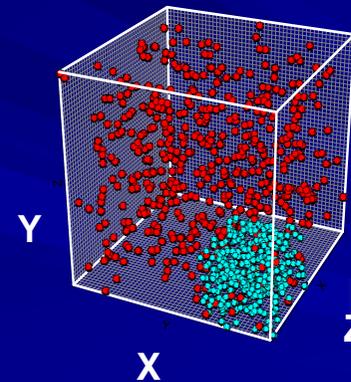
What is the reality?

KeSTE Kernel Sampling Technique Evaluation

development: C.Paoletti, M.Donatelli, S.Kay
programming: M.Donatelli

alpha
v 1.00

JOINT RESEARCH CENTRE Ispra, Italy
Institute for Health and Consumer Protection

ISCI Research Institute for Industrial Crops
Bologna, Italy

The first version of *KeSTE* is now available

2 Lots from the same country
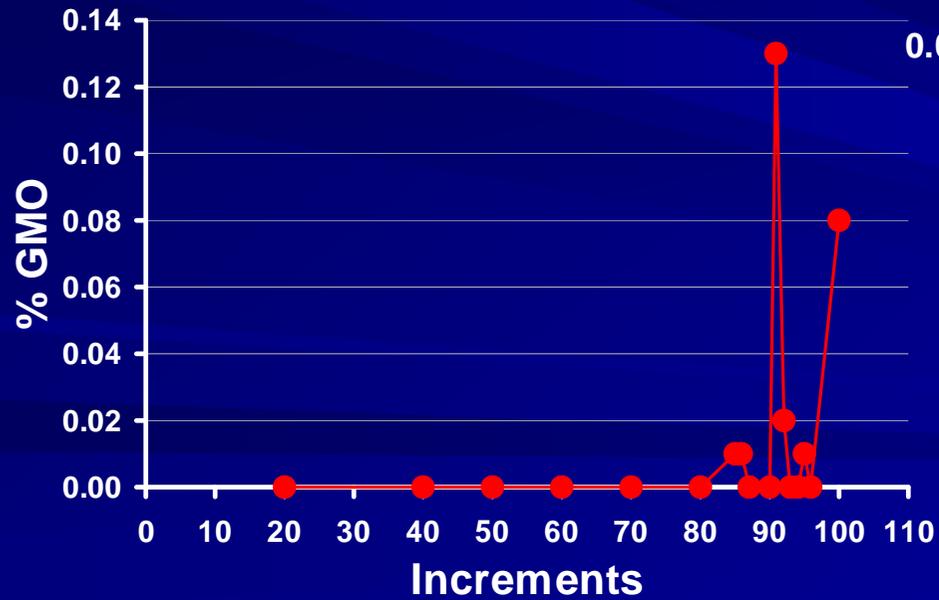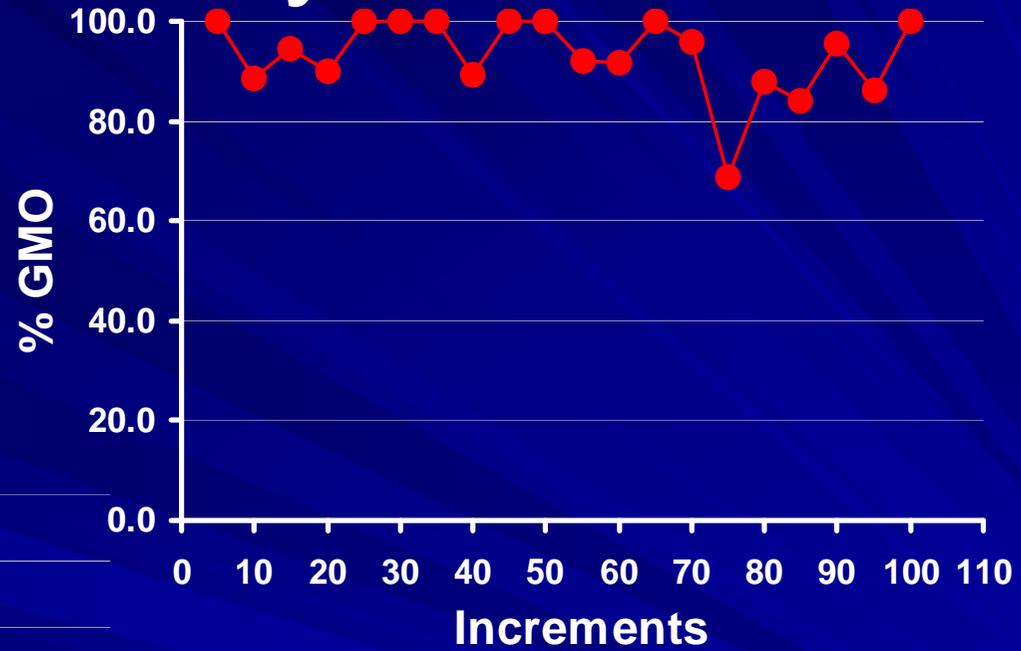
Multiple control plan by attribute
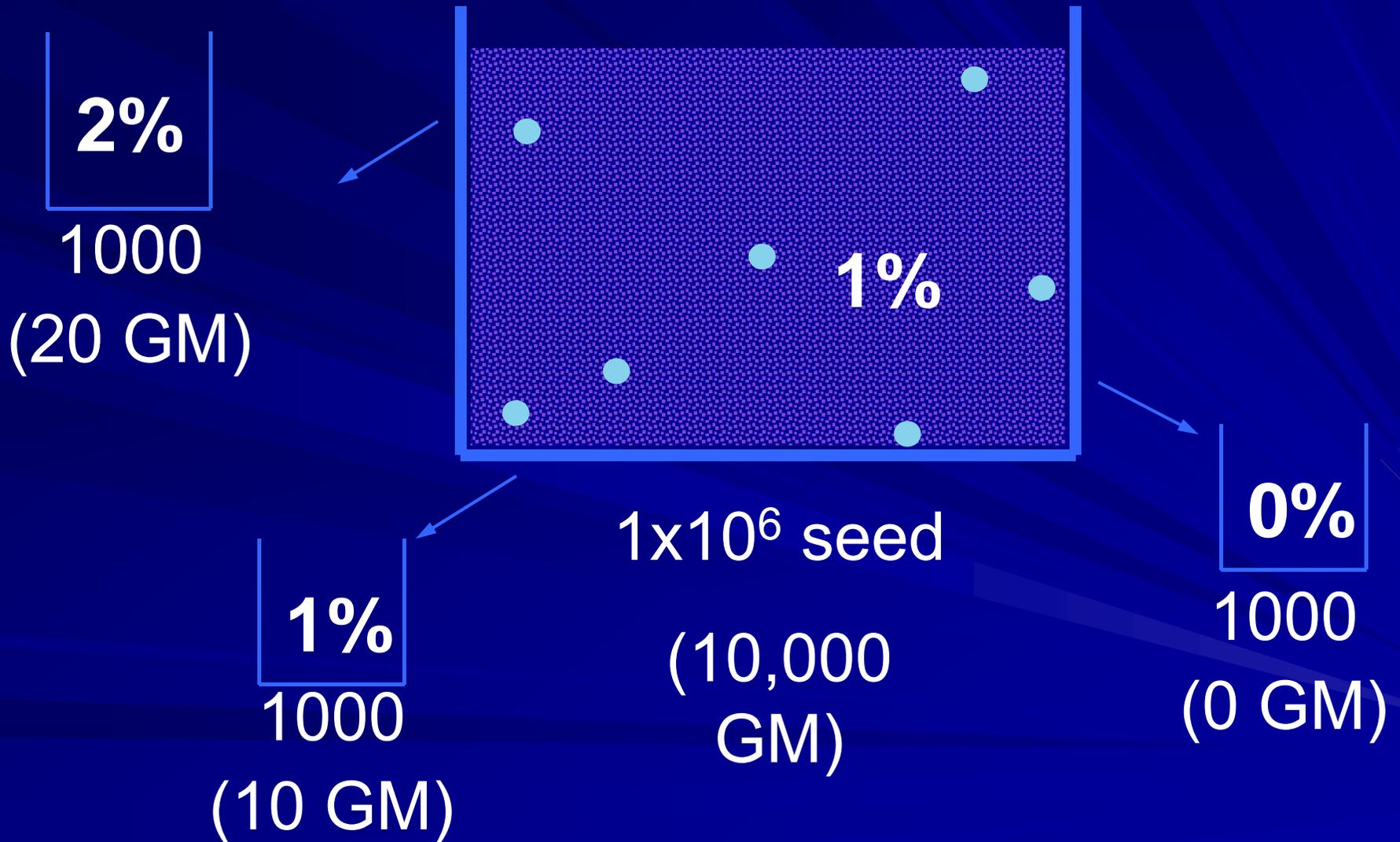
2%

1000

(20 GM)

1%

1x10^6 seed

(10,000 GM)

0%

1000

(0 GM)

1%

1000

(10 GM)

Multiple control plan by attribute
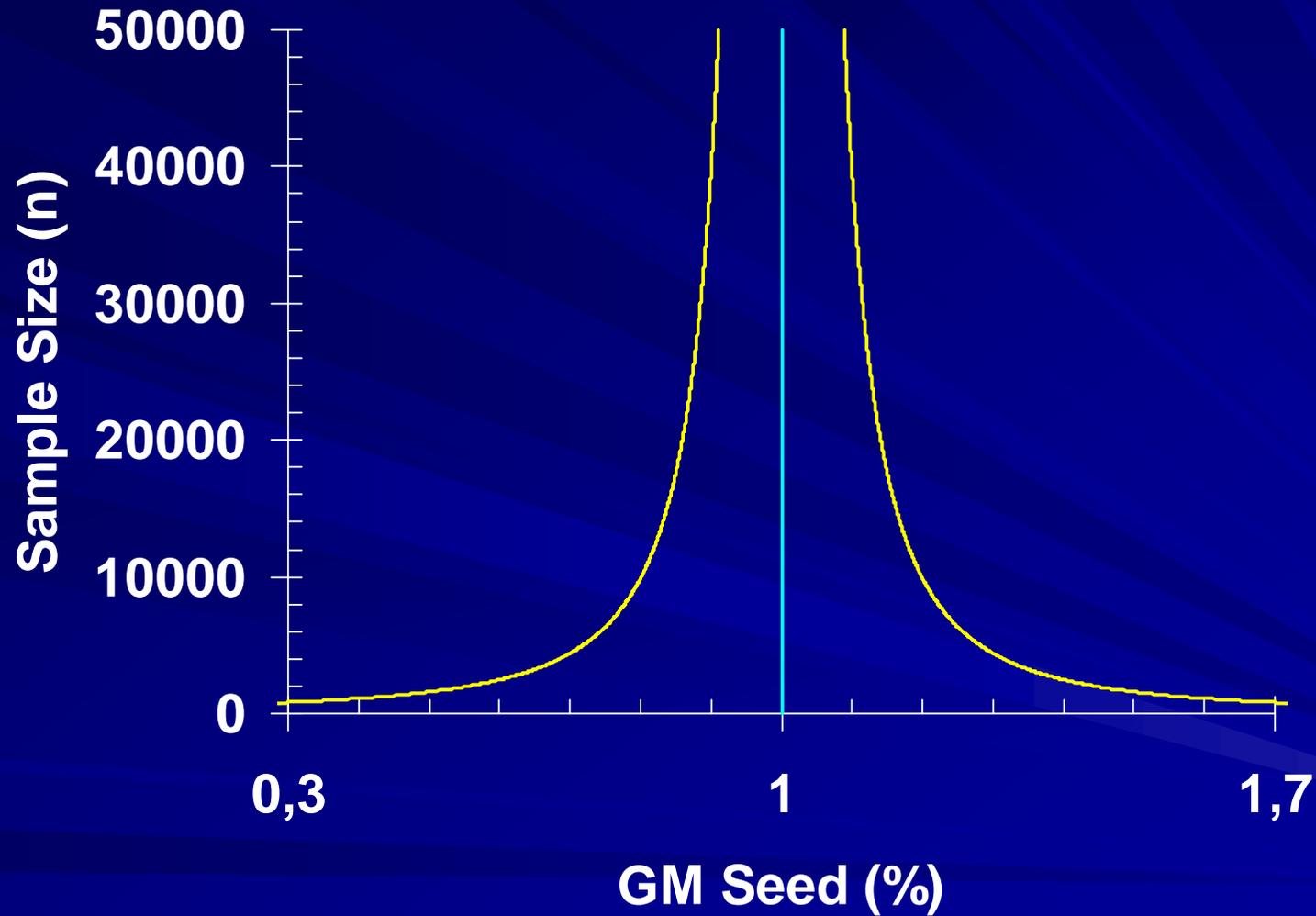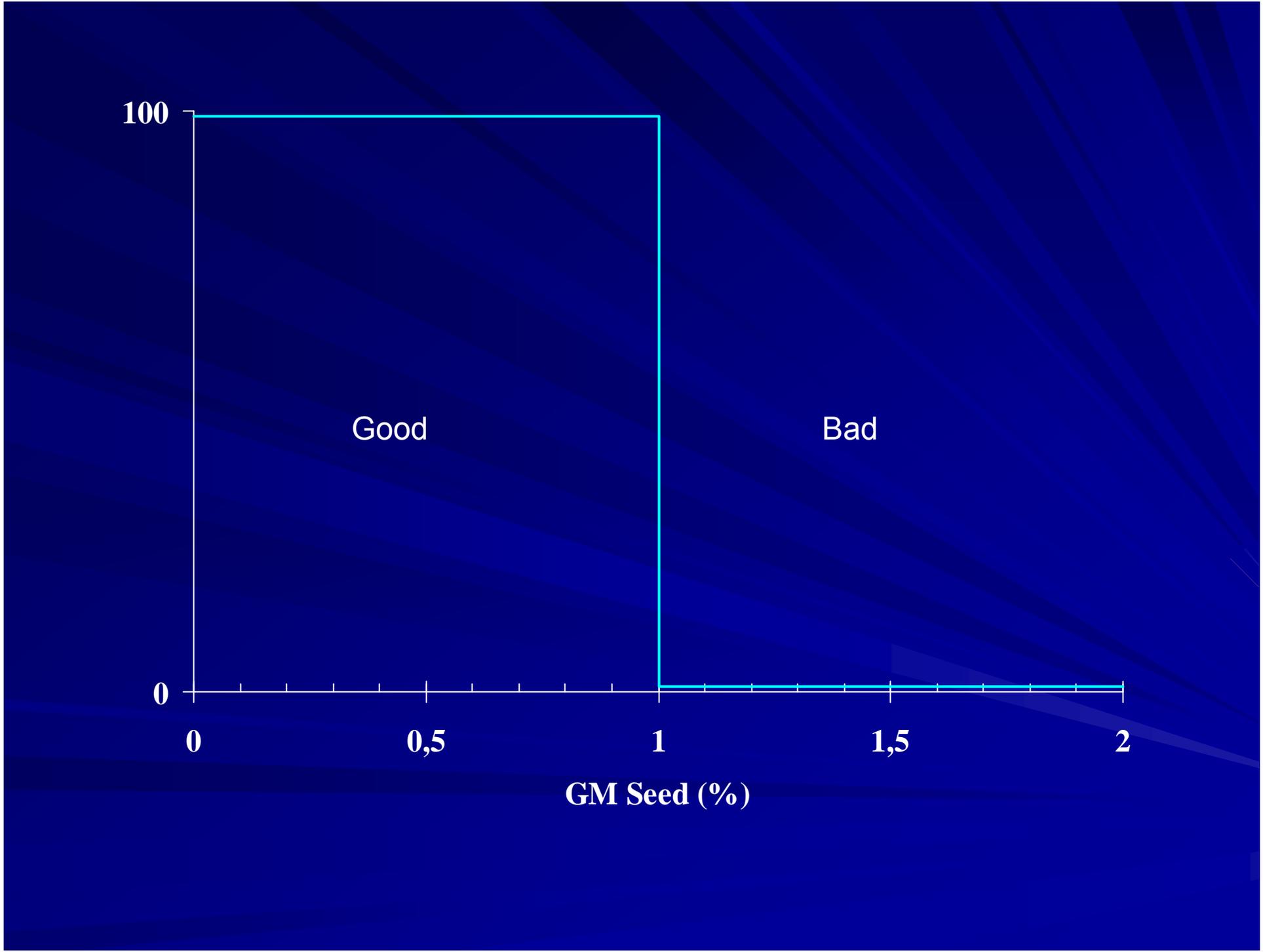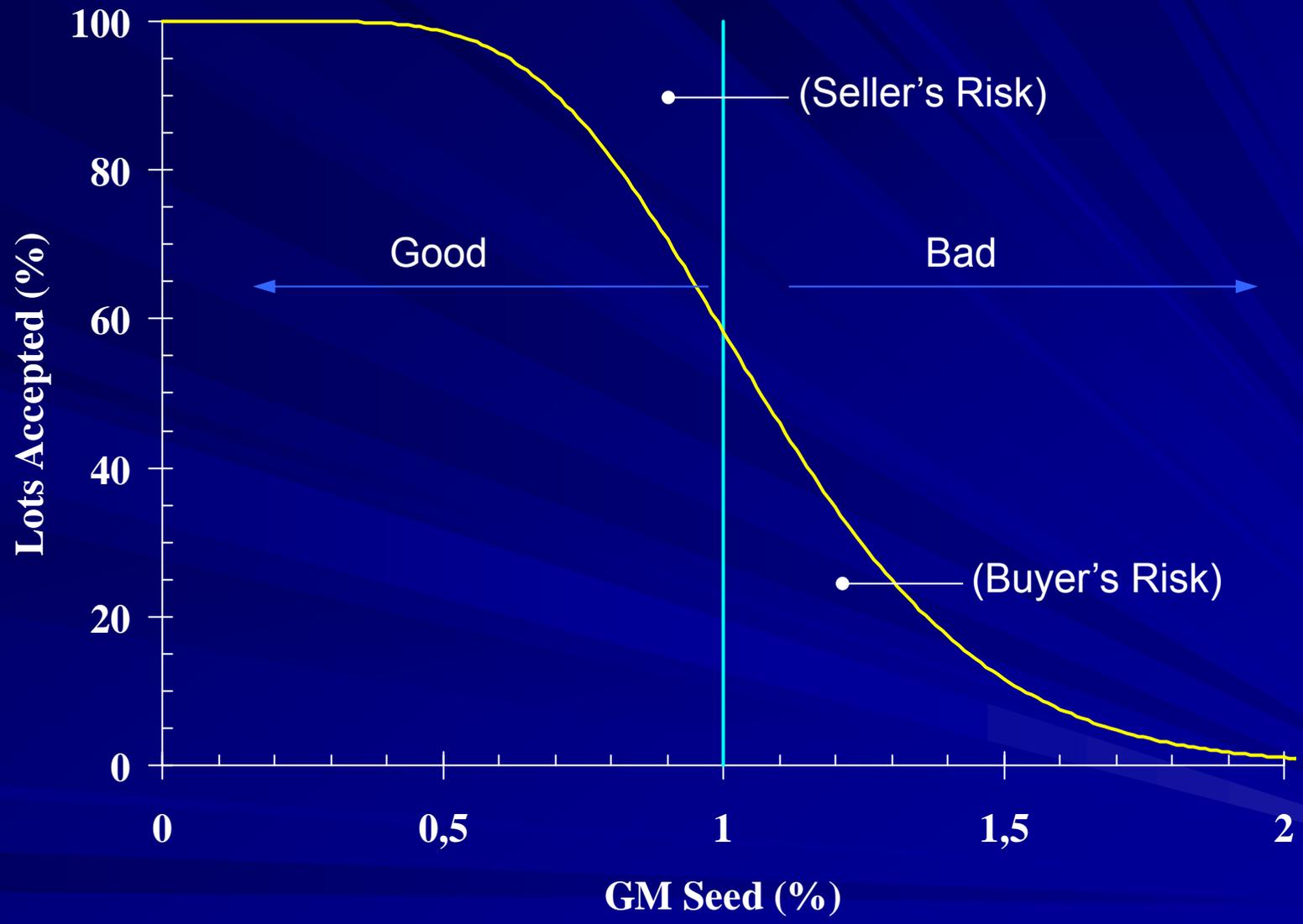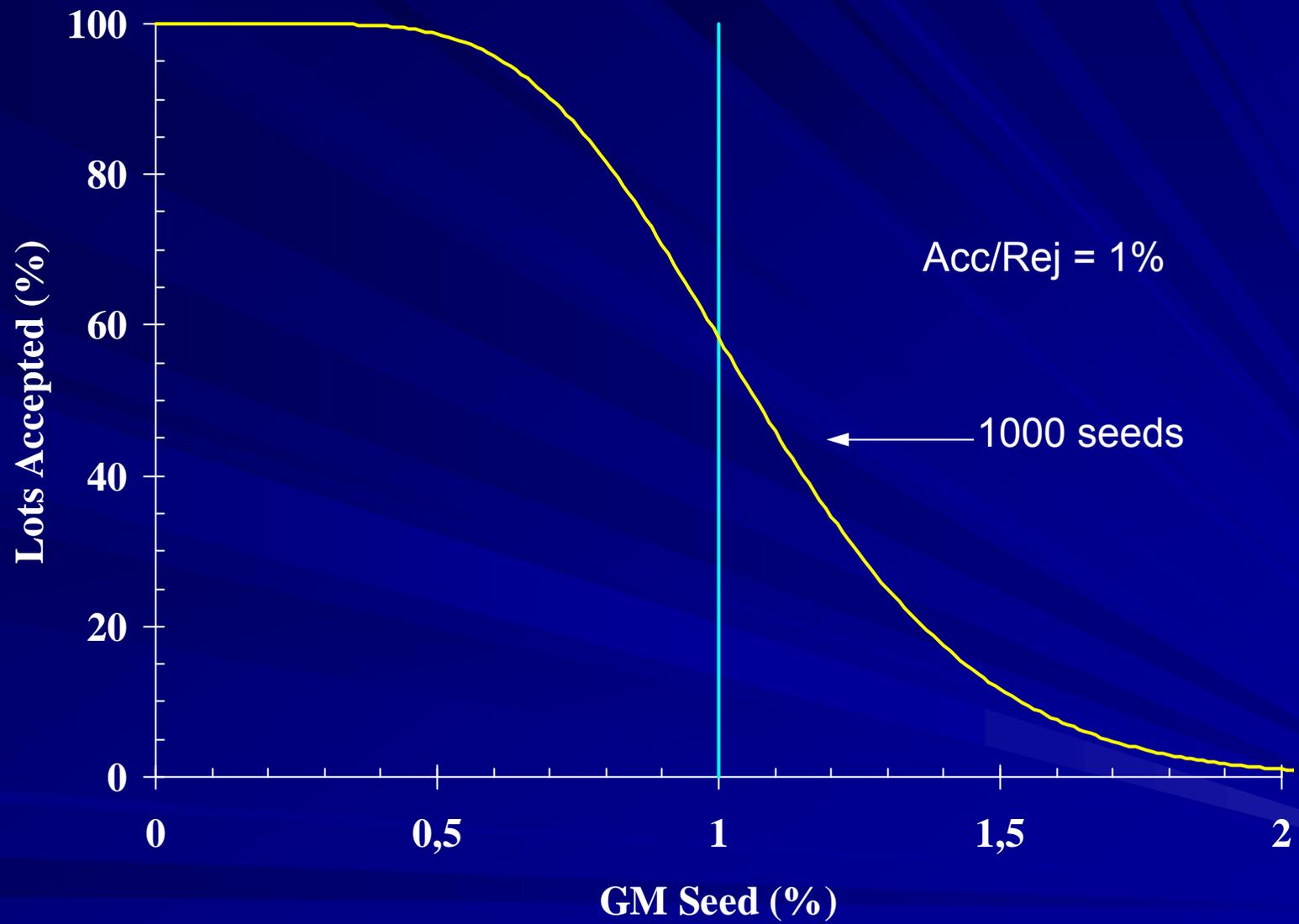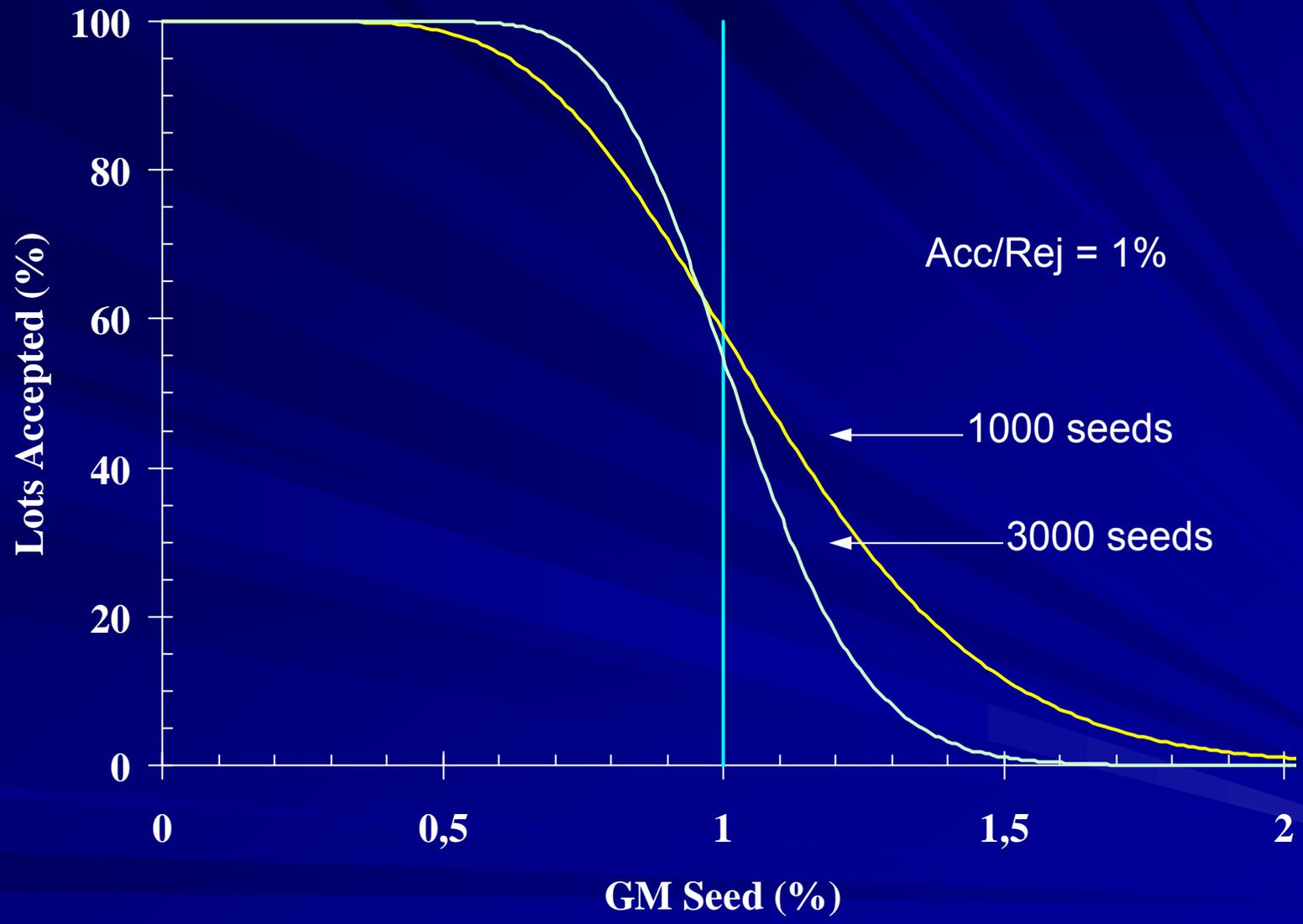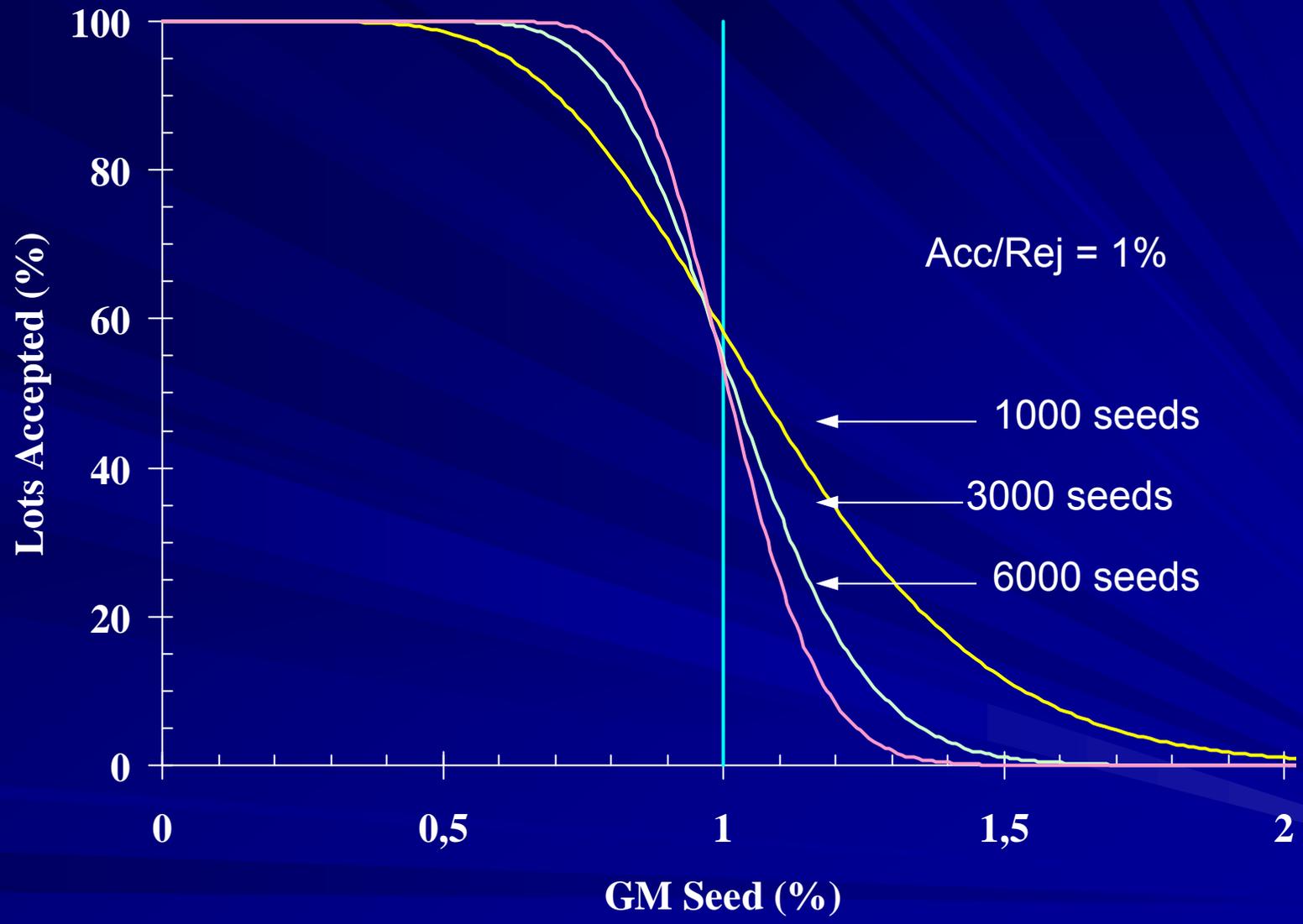
Qualitative analyses with multiple control plans
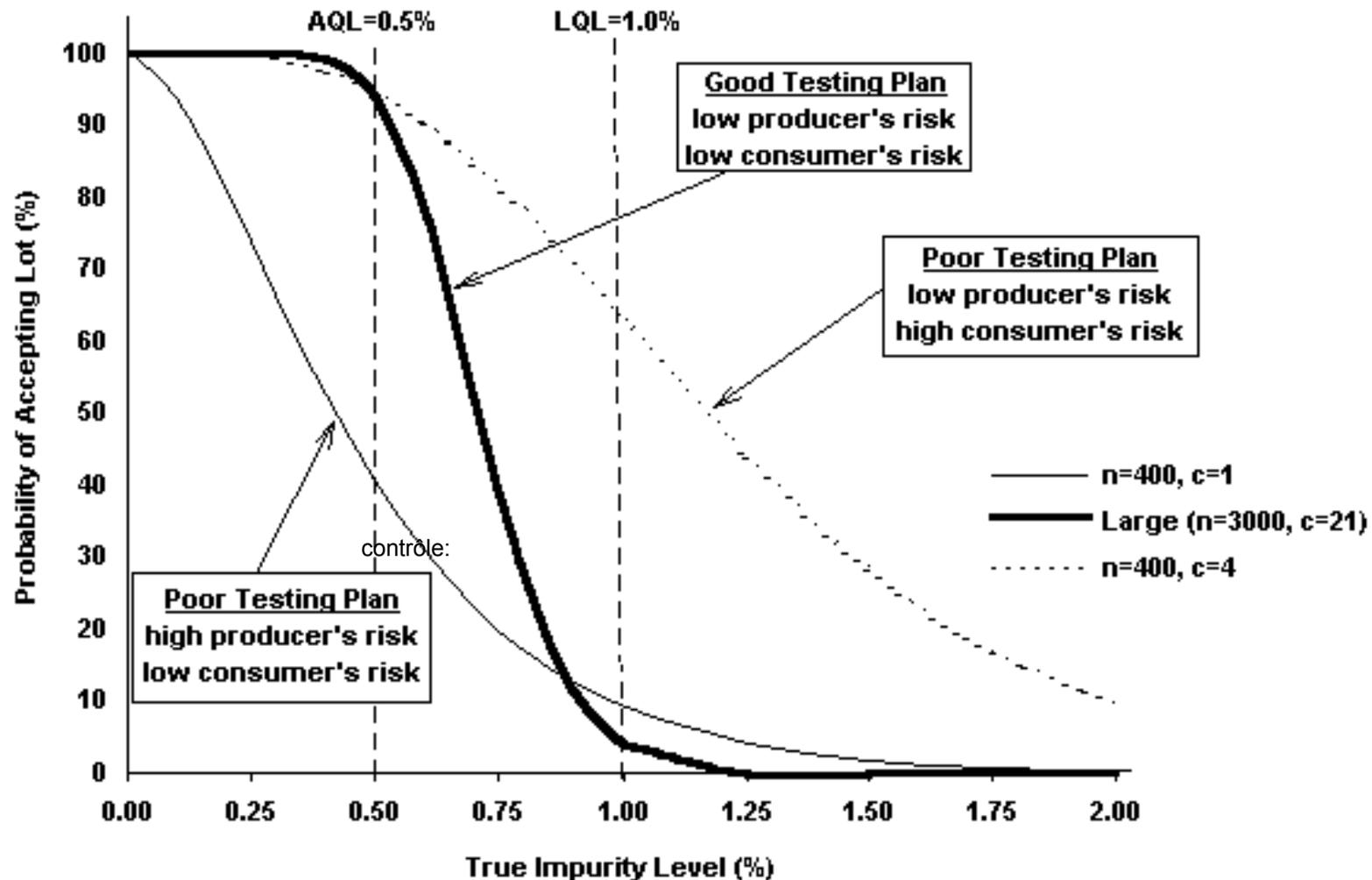
Qualitative analyses with multiple control plans
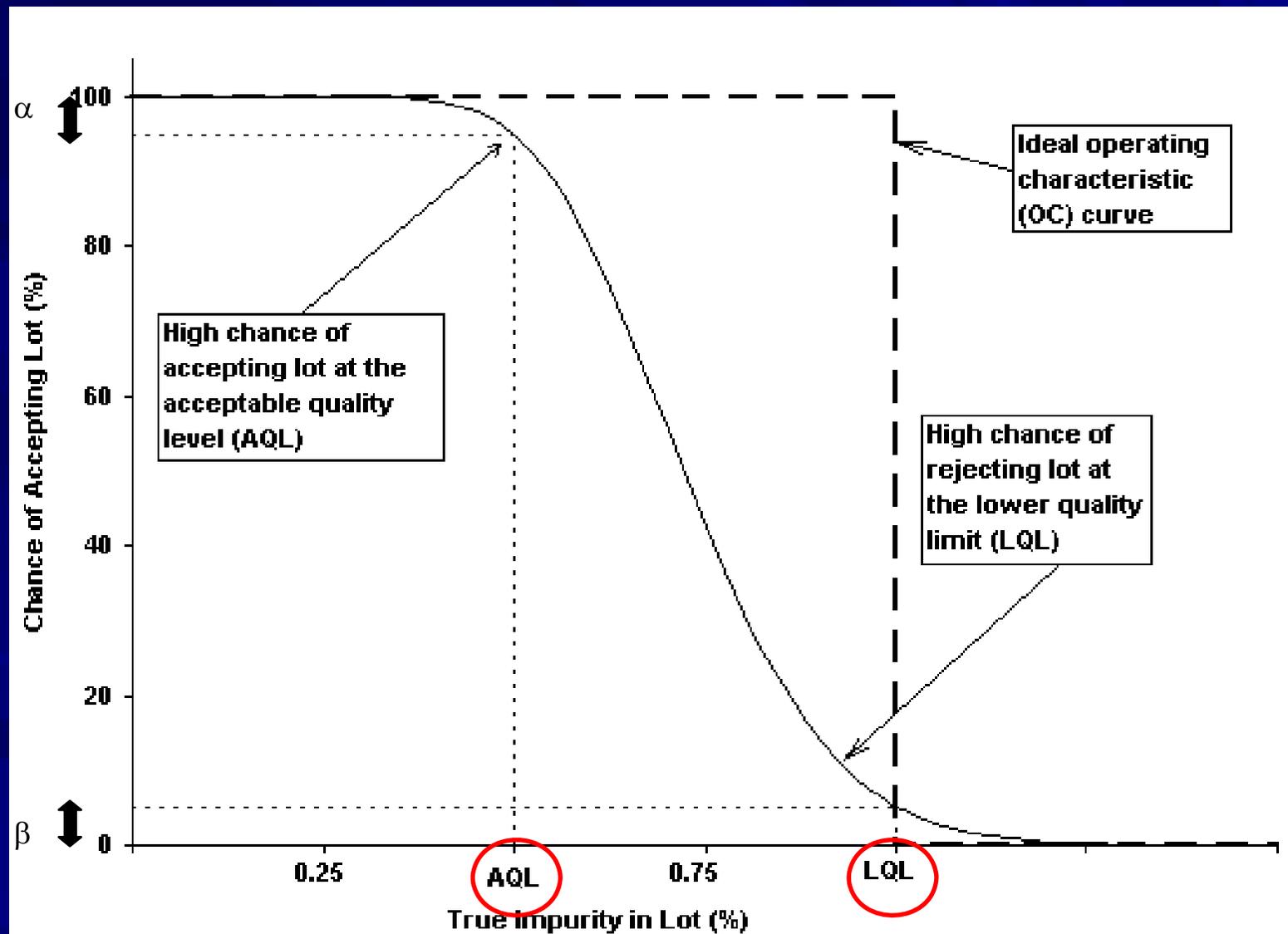
Validation of qualitative methods

A protocol for the validation of qualitative methods of detection†

Roy Macarthur[*a] and Christoph von Holst[b]

This paper presents a draft protocol for analyzing the results of validation studies for qualitative methods of detection which is designed to meet three competing goals: (1) to give correct answers, (2) have a broad scope of application, and (3) be accessible to a wide range of users. The draft protocol can be applied to the validation of methods by collaborative trial or to single-laboratory studies. The protocol produces an estimate of the probability of a positive response with a prediction interval within which 95% of laboratories (or analytical runs) are expected to fall when the method is applied in practice. The interval is calculated from the observed reproducibility (or within-laboratory reproducibility) standard deviation. Then a simple plot of prediction intervals for the probability of detection against the concentration of analyte, where the concentration is known, is used to provide an estimate of the range of limits of detection and false positive probability that we can expect to see when the validated method is applied in practice. The use of the draft protocol is demonstrated using results produced by three collaborative trials. A simulation study showed that a conclusion that a method is fit for purpose that is generated by the draft protocol is likely to be safe.

Decrease of both buyer's and seller's risks

- Increase sample size

- Increase precision of analytical method

European current control plan
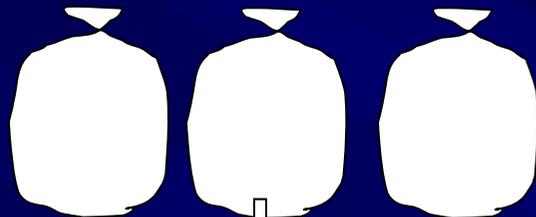
**Bulk sample**

Laboratory sample:
1 attribute

Test portion

Quantitative method e.g. 2 tests portions with duplicate or triplicate on e.g. : precision…

According to the matrix
• Do we need quantitative results or position around a threshold?
• Are multiple control plans by attribute with QL PCR methods and automated extraction as expensive as quantitative PCR methods?

Choosing cost-effective 2 steps control plan by attribute
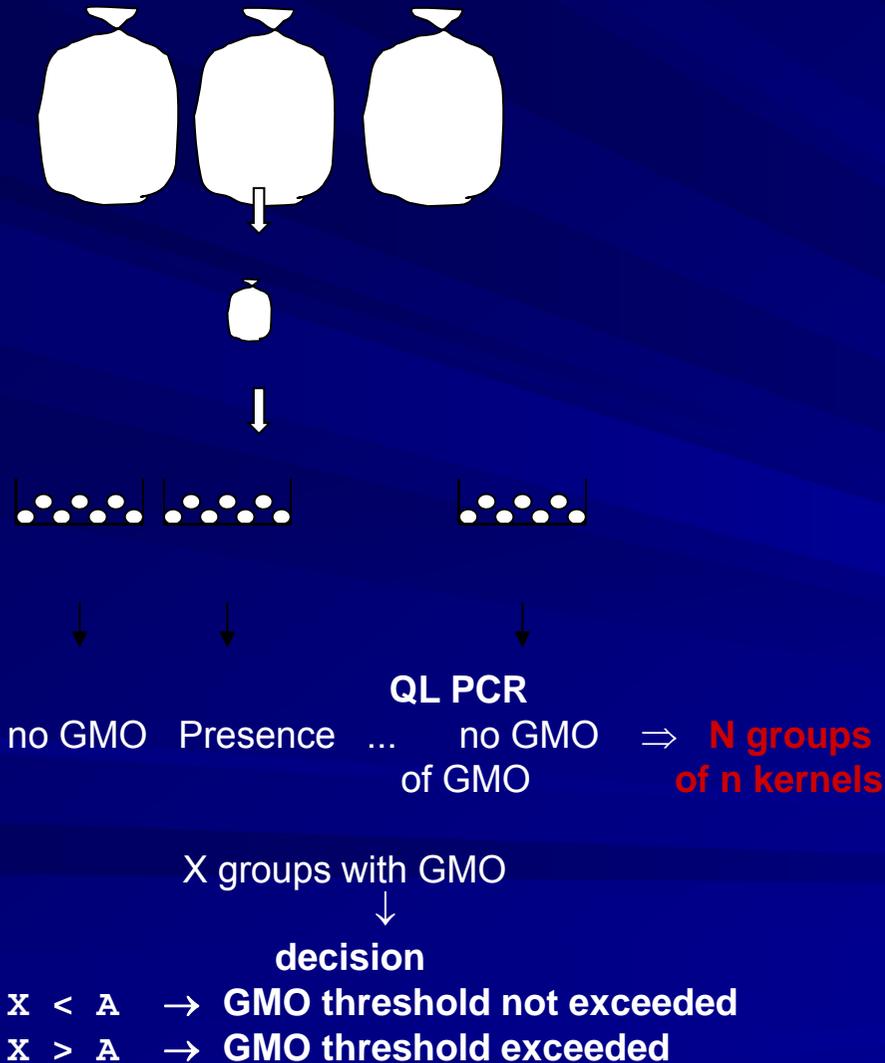
First step: $\mathbf{N_1}$ groups of $\mathbf{n_1}$ grains $\rightarrow$ $\mathbf{X_1}$ GMO-positive

$X_1 \leq A_1$	$\rightarrow$	lot accepted
$R_1 \leq X_1$	$\rightarrow$	lot refused
$A_1 < X_1 < R_1$	$\rightarrow$	**second step**

Second step: $\mathbf{N_2}$ groups of $\mathbf{n_2}$ grains $\rightarrow$ $\mathbf{X_2}$ GMO-positive

$X_2 \leq A_2$	$\rightarrow$	lot accepted
$A_2 < X_2$	$\rightarrow$	lot refused

QL PCR

no GMO   Presence   ...   no GMO $\Rightarrow$ **N groups**
of GMO   **of n kernels**

X groups with GMO
↓
decision

$X < A$ $\rightarrow$ **GMO threshold not exceeded**
$X > A$ $\rightarrow$ **GMO threshold exceeded**

According to a control cost function: calculate the least expensive acceptance sampling plan   Kobilinsky and Bertheau 2005 integrated into ISTA software…

EC DG-JRC & ENGL work

- Lot properties are unknown.

- The optimal sampling technique depends upon the features of the lot.

- Modest heterogeneity strongly affects the definition of the optimal sampling plan.

- *KeLDA* was the first study assessing GMO distribution in real lots.

- *KeSTE* is the first tool to define sampling strategies and to estimate sampling errors as function of lot properties.

- EC recommendation
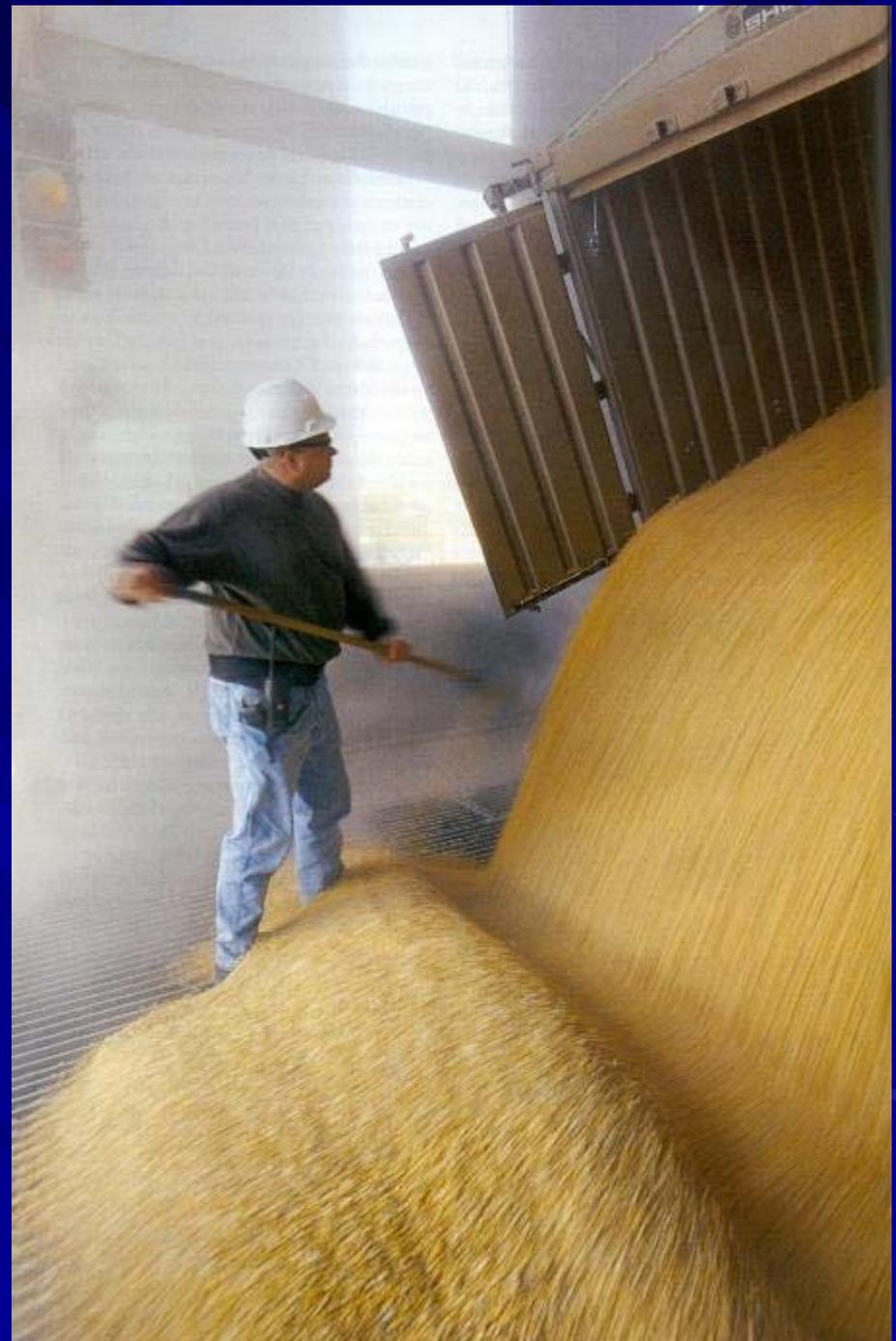
Key features of sampling plans

- The number of incremental samples (locations sampled):

- larger numbers incremental reduce uncertainty caused by heterogeneity.

- The total mass (or number of grains) of analytical sample homogenized for analysis:

- higher masses reduce uncertainty caused by GM product being present at low concentrations in grain lots.

- Larger numbers of incremental samples and higher masses cost more.

And the analytical method used to test the laboratory sample must be considered

Numerous sampling plans

- ISO standards, private standards (e.g. GAFTA), ISTA standards… with numerous possibilities of application,

- Taking into account homogeneous or heterogeneous targets distribution,

- From "light" sampling plans to "drastic" sampling plans for e.g. safety issues such as mycotoxins.

- From field and coexistence related issues to packed processed products

Reducing costs by considering the controls' aims

• Seeds production: field monitoring for research modeling or GM content assessment before harvest, purity certification

• Grain production: production separation in silos

• Environment monitoring, escape of transgene toward landraces

• Food and feed supply chains controls (packaged or not products)

• Detection / quantification of approved vs. unapproved GMOs

• Qualitative testing of 'sub-samples' or quantitative measurement.

- SOME SAMPLING PLANS ARE MORE DRASTIC THAN OTHERS (more attributes or more increments)…

- **QUESTIONS:**

- Does it make sense to use the same sampling plan for different purposes (e.g. use a 'mycotoxin plan' for mycotoxins **and** GMOs)? Zero additional cost for GMOs?

- Are current sampling plans **and** analytical methods right for GMO LLP?

Reducing sampling plan costs

- Using same control plans and laboratory samples for several analytes (mycotoxins, GMO…) would induce labs' re-organization
- Control plans by attributes
  - Simple / multiple
  - Multiple:
    - Single stage,
    - Double stage…
- Quantitative methods *vs.* qualitative methods in relation with automated extractions on smaller test portions and multiple control plans by attributes…

Choosing appropriate sampling/analytical plans

The effect of analytical uncertainty

- We need to know about analytical performance to design the right control plan.
- We express analytical performance as **measurement uncertainty** which tells us how far away the measurement result might be from the true concentration in the sample sent to the laboratory.

- Two major sources of information about analytical performance are
  - proficiency test results
  - method validation studies

Method validation studies

- **Where the validation study includes the whole method (extraction and analysis of extracts)…**
- Variation displayed during method validation

plus

- Uncertainty about the true concentration of GMO in materials used in validation.

gives

- **Prediction of measurement uncertainty when we use the method**

For methods that are 'just good enough…'

- For ENGL validated methods
- "$RSD_R < 35\%$"
- Result display a log-normal distribution.

- Hence, as a general guide, uncertainty is a **factor** of 2
- Result more than 1.8% may demonstrate need for labelling.
- Result less than 0.45% may demonstrate no need for labelling

A method that is 'just good enough'
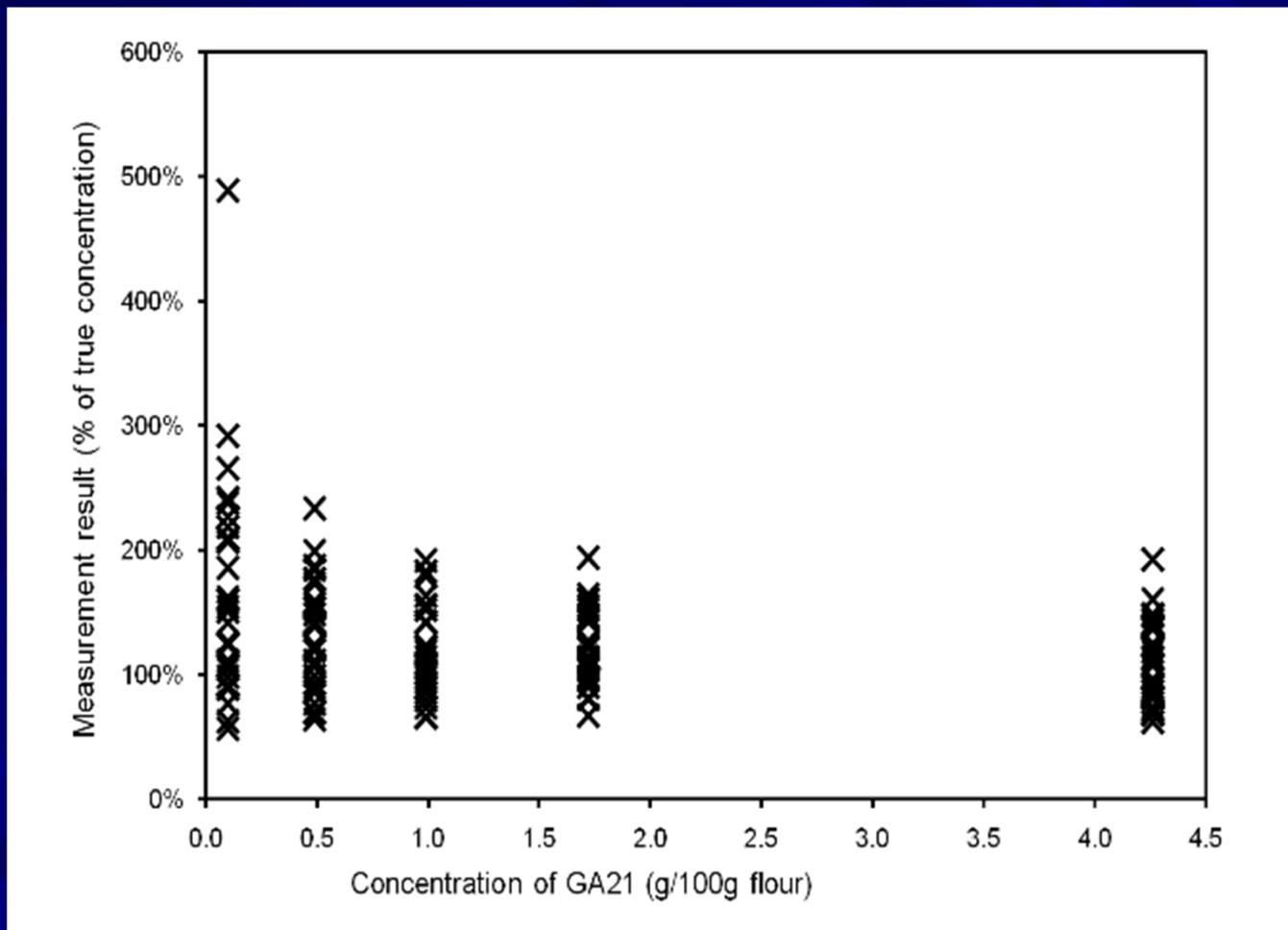
CRL Validation of method for GA21: just meets validation criteria.

A method that is 'just good enough'
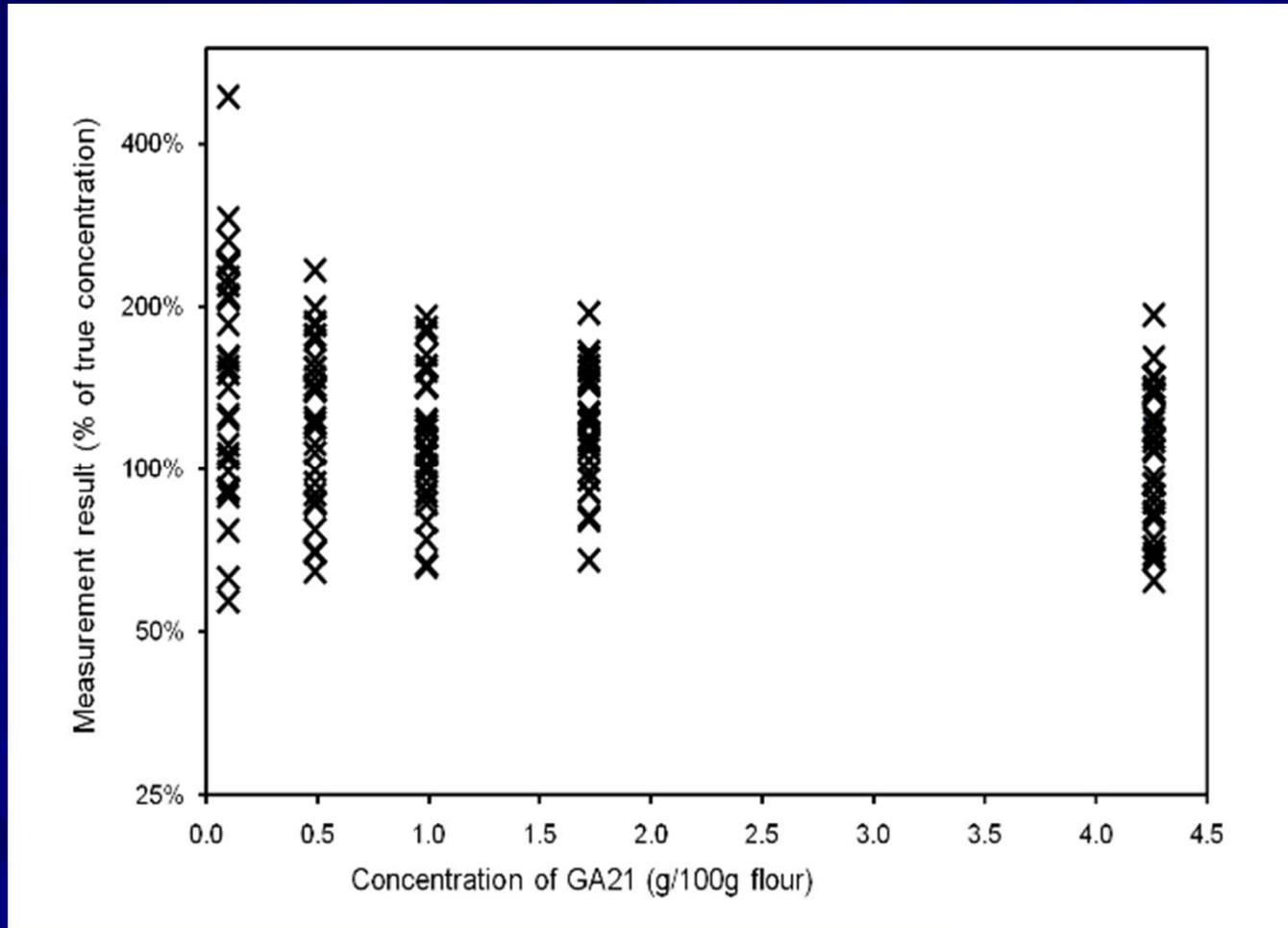
CRL Validation of method for GA21: results are log-normal

A method that is 'just good enough'
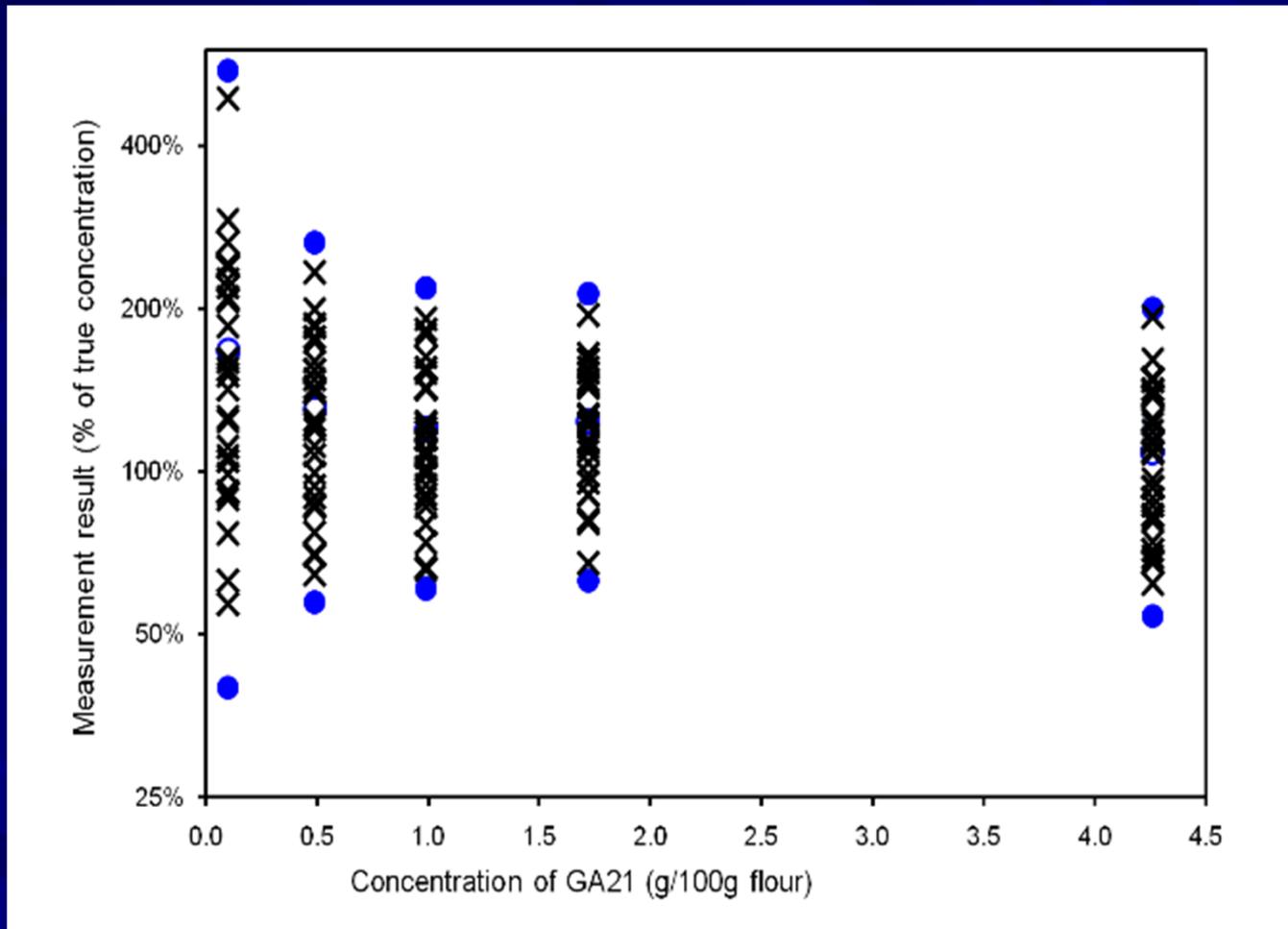
CRL Validation of method for GA21: estimates of measurement uncertainty

A method that is 'just good enough'
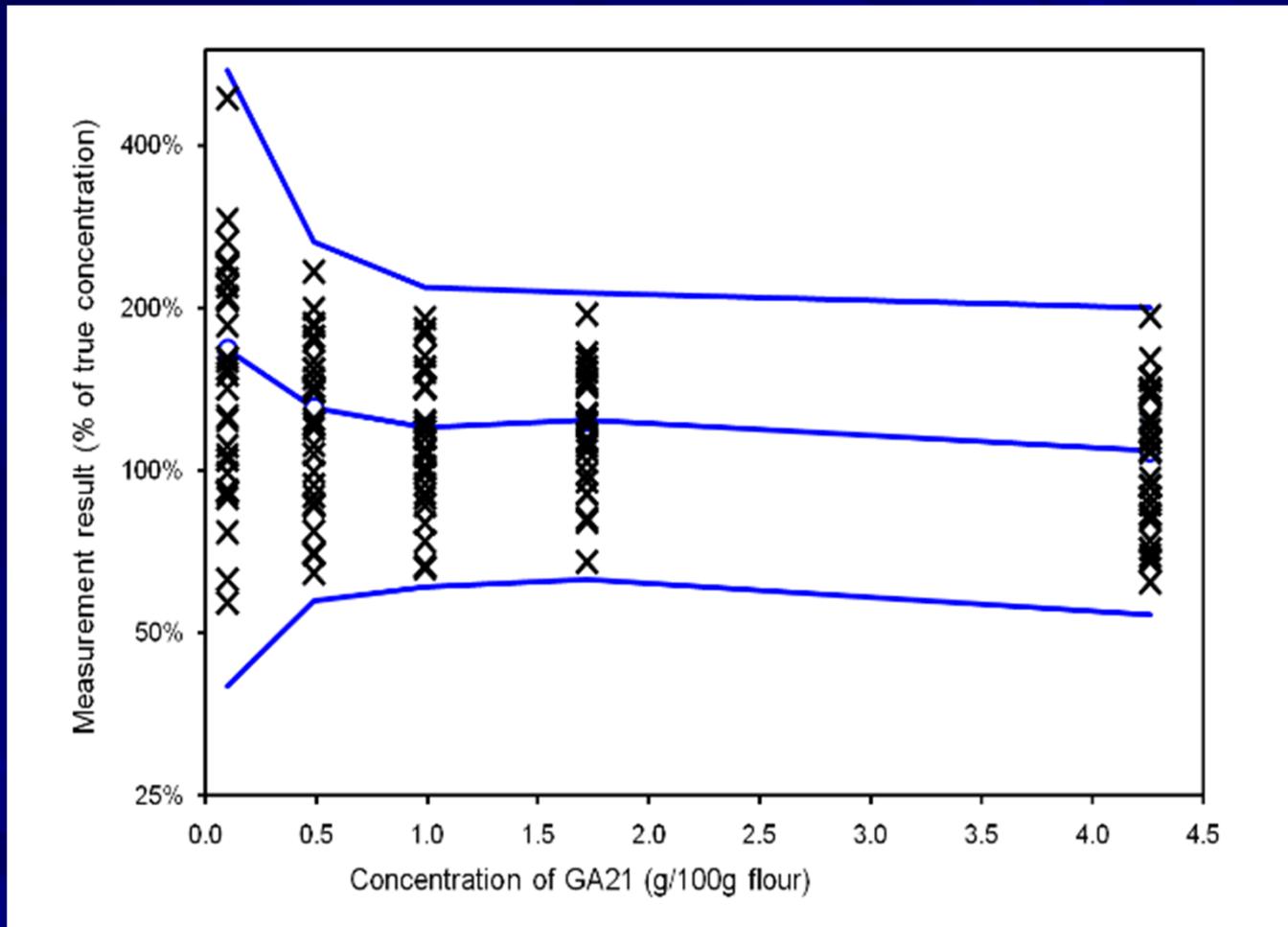
CRL Validation of method for GA21: profile of uncertainty

A method that is 'just good enough'
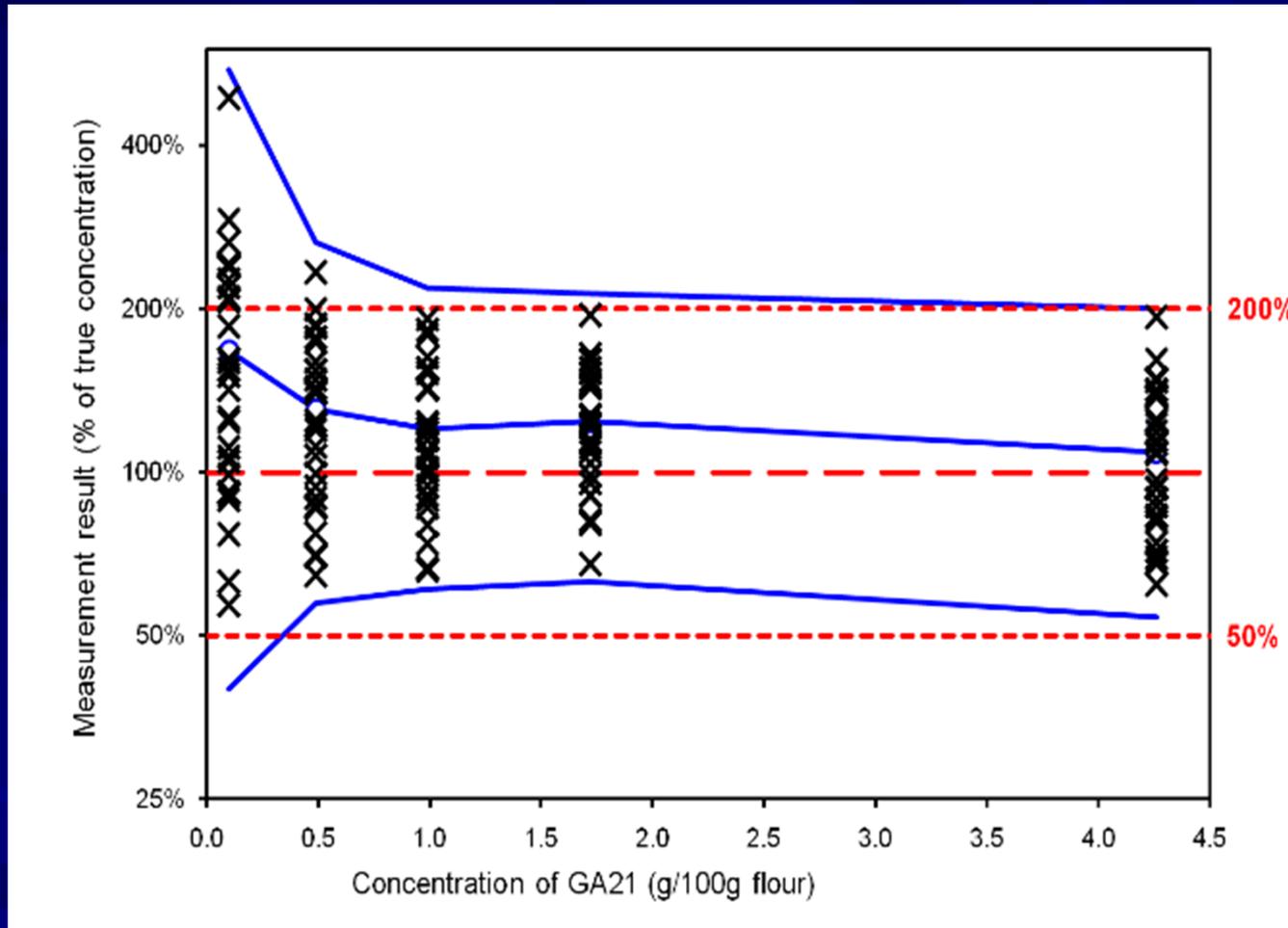
CRL Validation of method for GA21: uncertainty is a factor of approximately 2 for GM product at concentrations greater than 1%

A method that is 'just good enough'
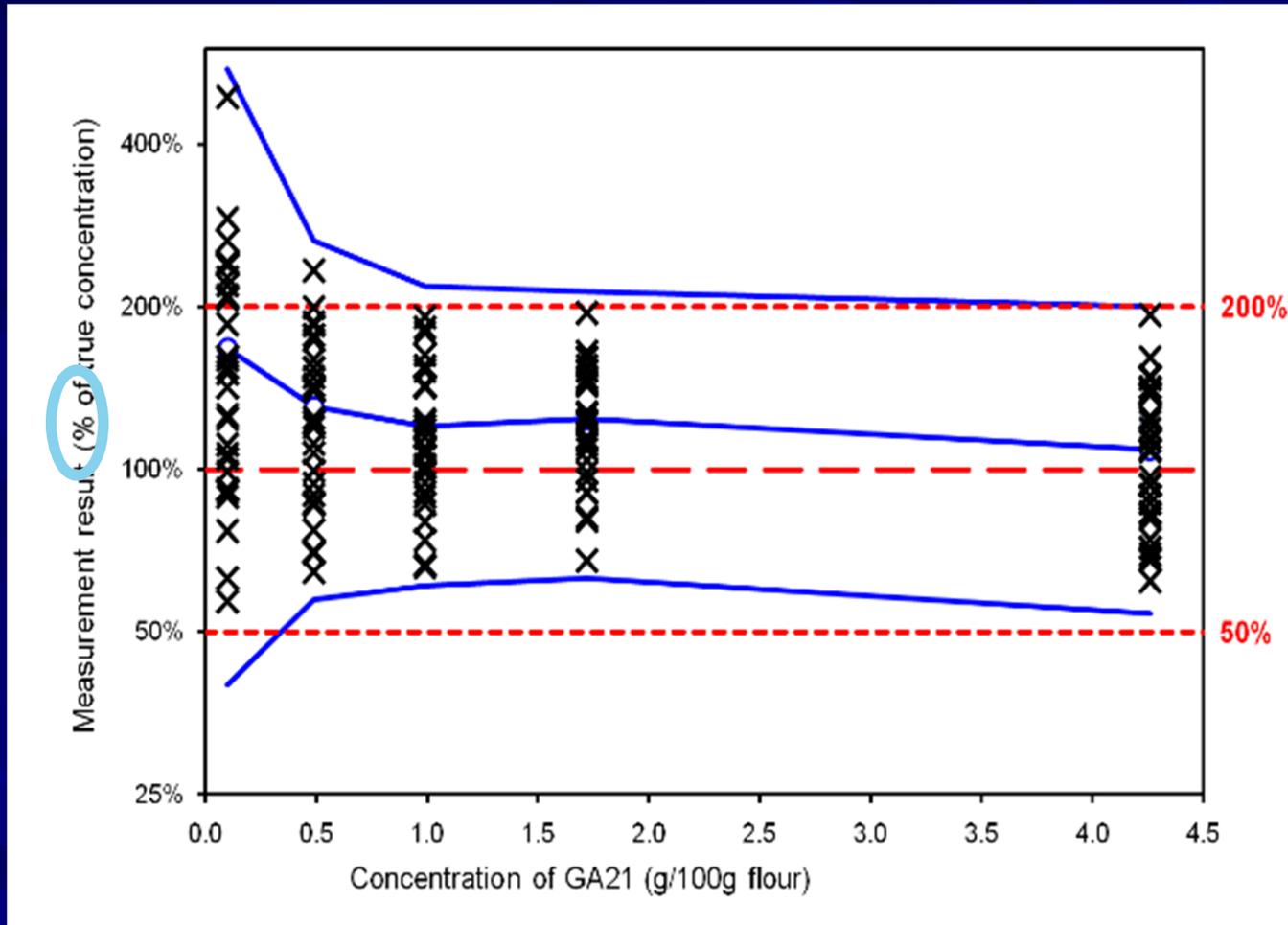
CRL Validation of method for GA21: uncertainty may be larger for lower concentrations

Analytical uncertainty and labelling decisions

• An enforcement authority can be confident that a product requires labelling when **Result – Uncertainty > Limit**

• A producer can be confident that a product does not require labelling when **Result + Uncertainty < Limit**

• Nobody is comfortable with **other results**
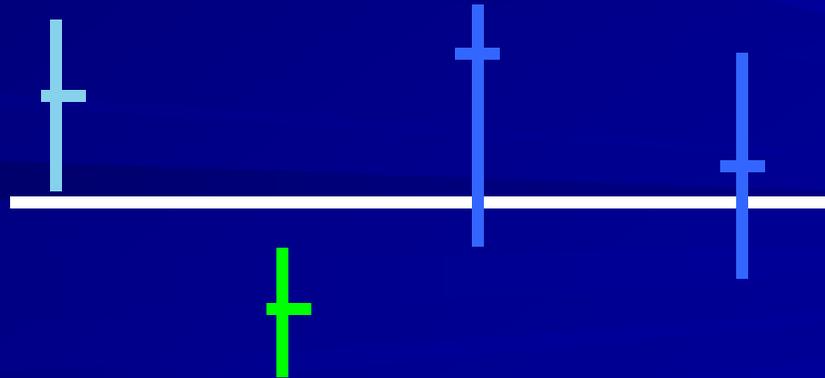
Analytical uncertainty and labelling decisions
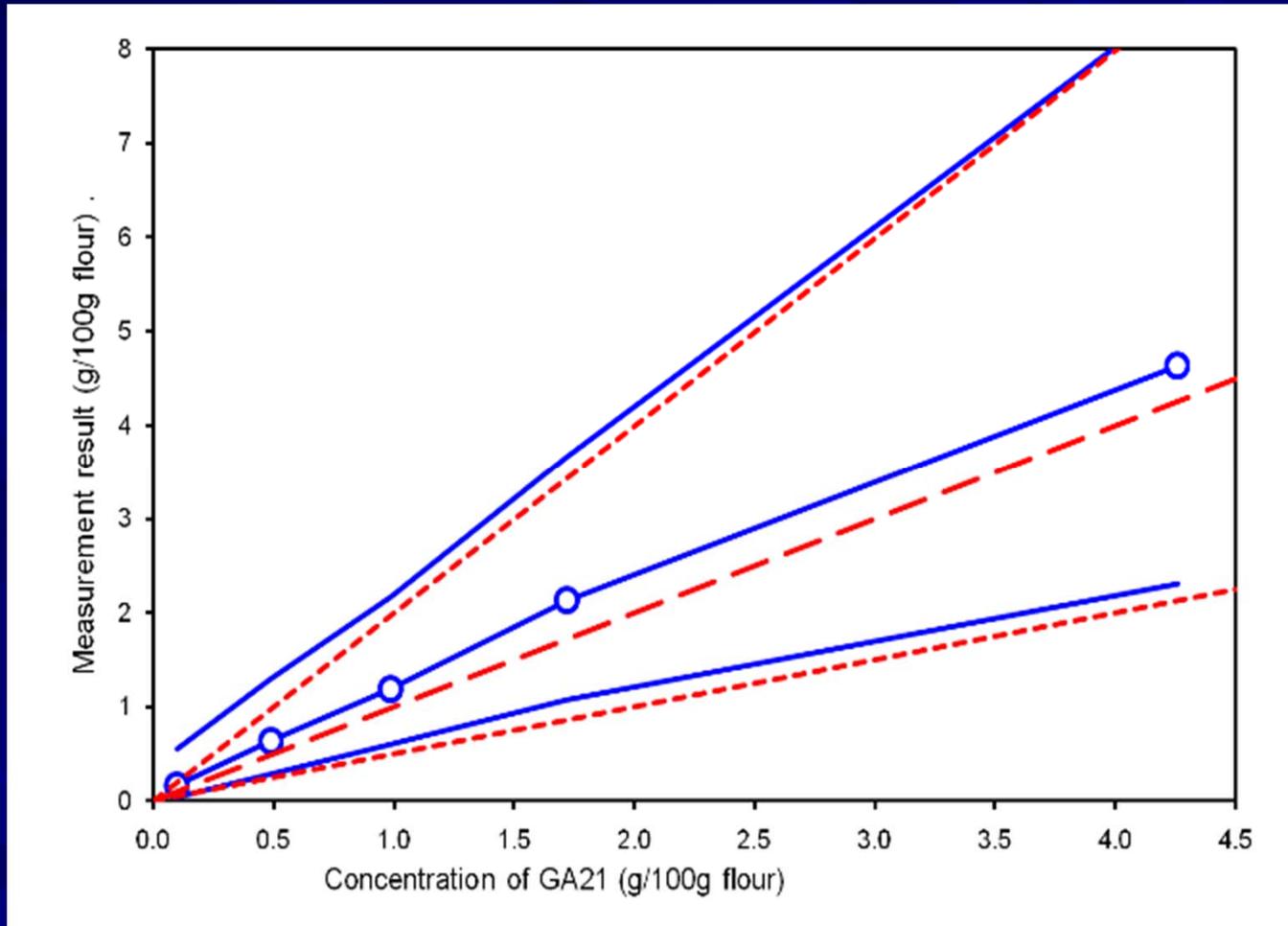
CRL Validation of method for GA21: This shows absolute uncertainty

Analytical uncertainty and labelling decisions
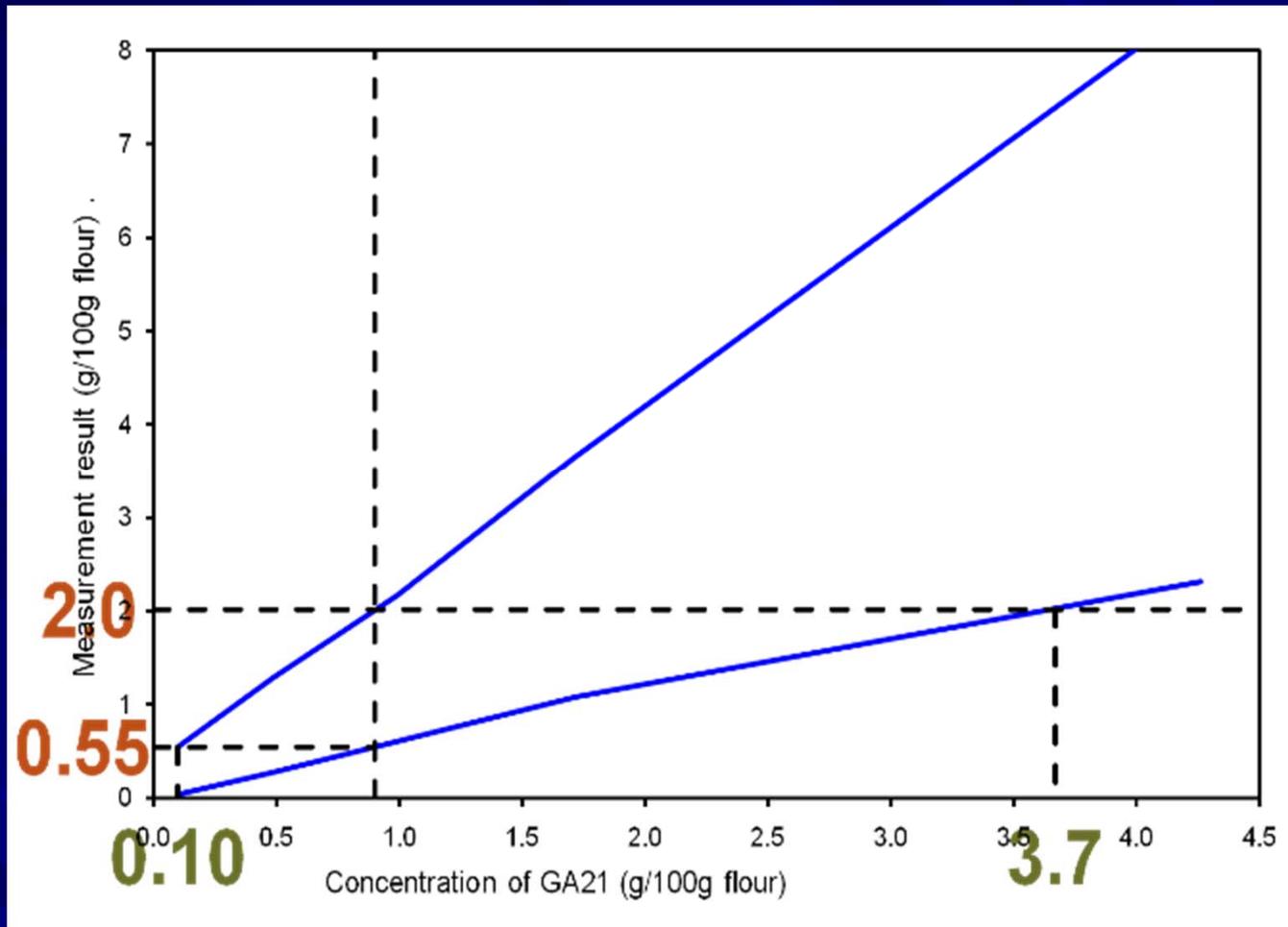
A sample must contain no more than 0.1% GM product to (nearly) always give a result in the green (must not label) zone.

Analytical uncertainty and labelling decisions
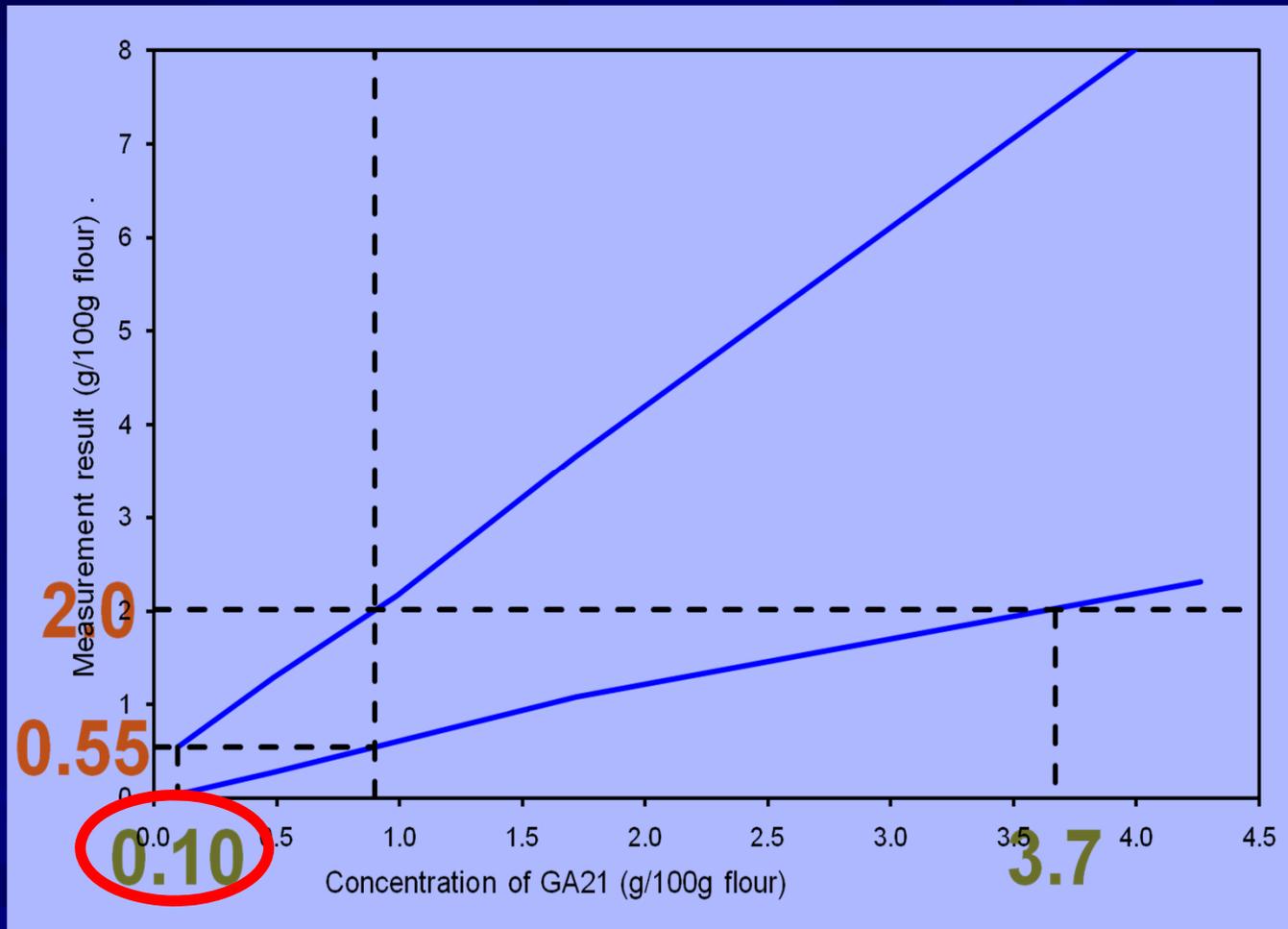
CRL Validation of method for GA21: limit of assurance, the highest concentration of GM product that will (nearly) always give a result that demonstrates no need to label

Analytical uncertainty and labelling decisions

Analyte	Critical result for conc.>0.9%	Limit of control (% GMO)	Critical result for conc.<0.9%	Limit of assurance (%GMO)
NK603	2.45	4.69	0.68	<0.1
GA21	2.02	3.67	0.55	0.1
MON863	1.63	2.60	0.69	0.34
MON810	2.06	>5	0.31	0.1
RRS	1.73	3.03	0.45	<0.5

Even low level presence may have an impact on stakeholders who are subject to demands that measurement results be in the green zone when assessing products against the labelling limit.

Macarthur, Feinberg, and Bertheau. 2010. Construction of measurement uncertainty profiles for quantitative analysis of products derived from genetically modified organisms based on validation data. *JAOAC Int.,* 3: 1046-1056.

Strange use of anal. uncertainty: see 2012 polemics about pesticide content

Proficiency test results

• Samples of known concentration sent to many laboratories.

• Laboratories use any method they like.

•The spread of these results gives us **measurement uncertainty** if we don't know anything about the method used to produce a result.

• Results tell us that if we know nothing about the analytical method, that **measurement uncertainty is a factor of 3**

- – Result more than 2.7% demonstrates need for labelling

- – Result < 0.3% demonstrates no need for labelling

Co-Extra Deliverable 4.5 "The compatibility of control plans".

Thompson, Ellison, Owen, Mathieson, Powell, Key, Wood & Damant. 2006.JAOAC Intl. 89 (1): 232-239

Proficiency testing
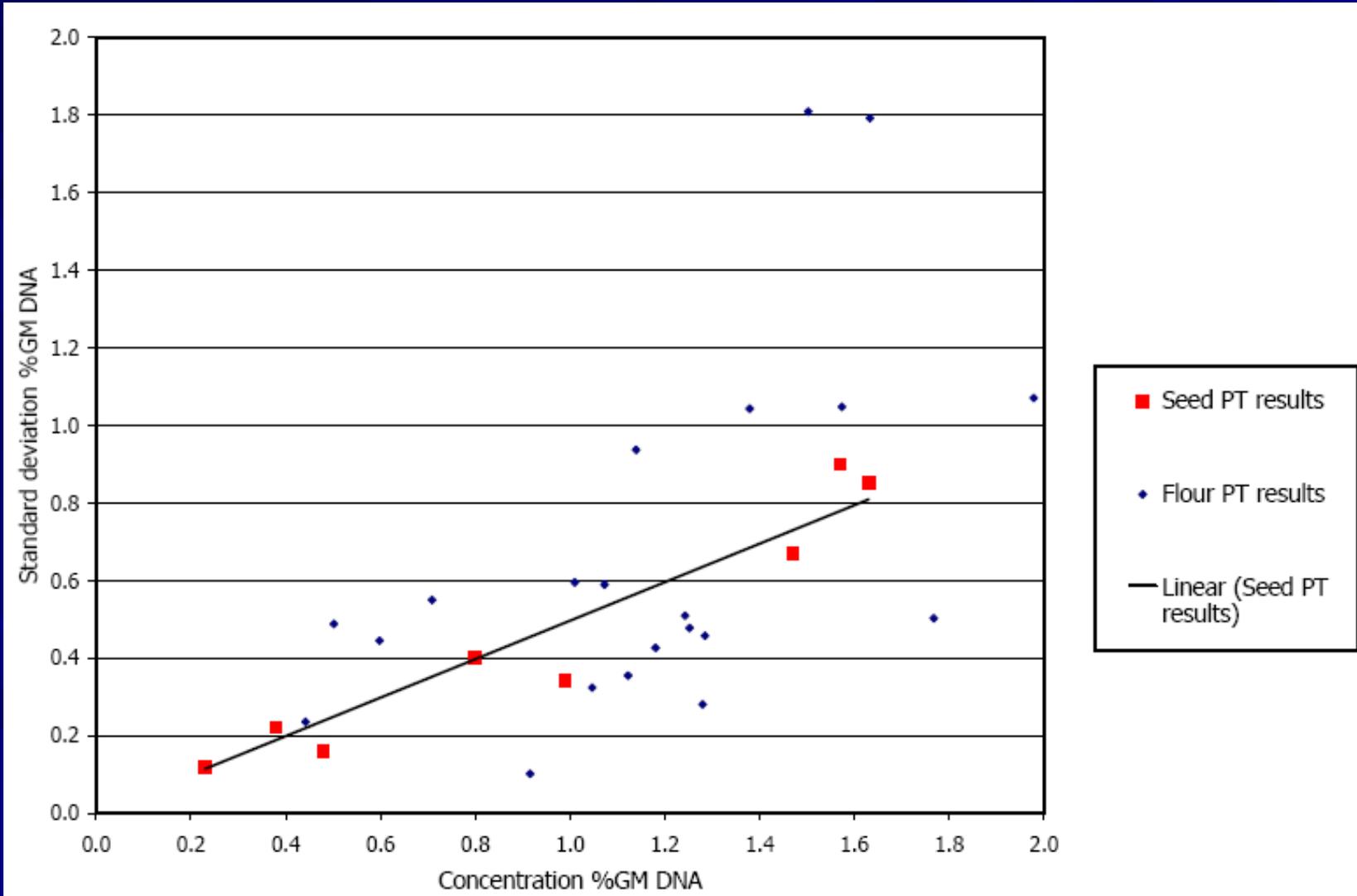
Robust standard deviation of Log$_{10}$ PT results is 0.25
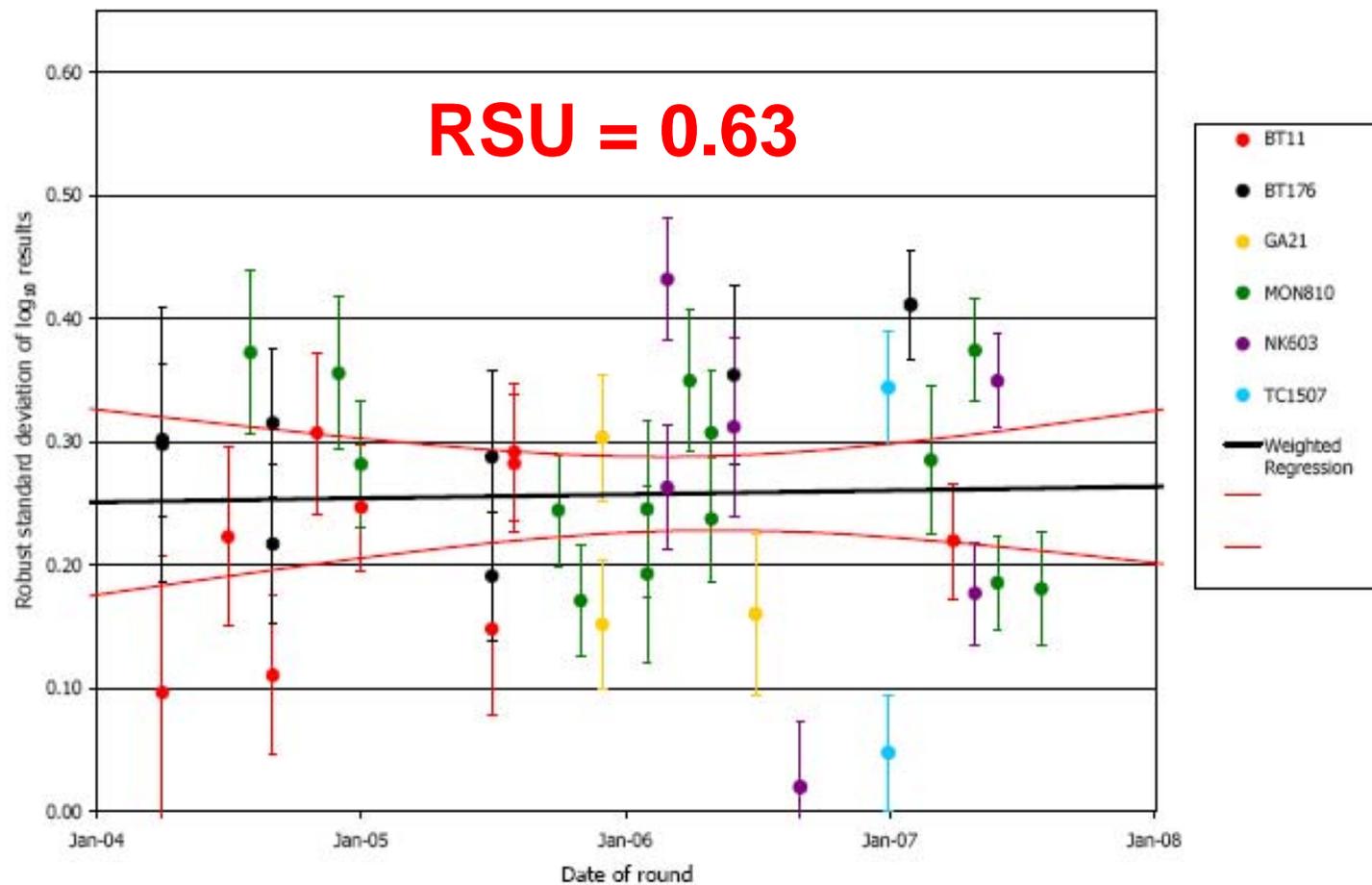
Effect of uncertainty on control plans

$$p_{accept} = \Phi\left(Ln(T), \mu, \sigma\right)$$

$$\sigma = \sqrt{Ln\left(RSU^2_{control} + 1\right)}$$

$$\mu = Ln(\overline{X}) - \frac{1}{2}\sigma^2$$

— RRS

— Other

▲ Result threshold RRS

▲ Result threshold other
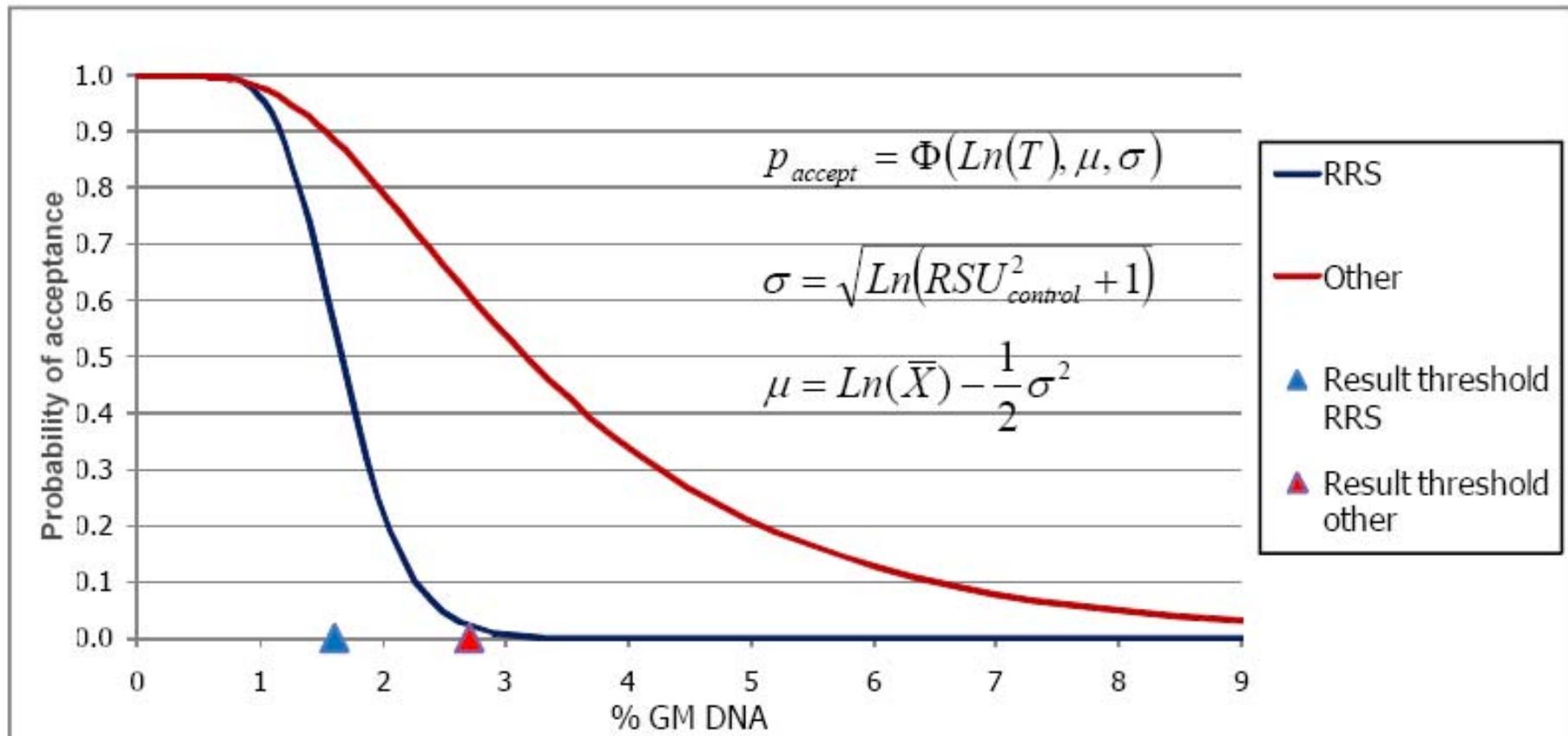
Analytical uncertainty and sampling

- Sampling should be designed with analytical uncertainty in mind. (analysis should be undertaken with sampling uncertainty in mind)
- For example: quantitative DNA based methods have an uncertainty of a factor of approximately 2
  - A result above 1.8% demonstrates a concentration above 0.9%
- Sampling should be good enough to not add any more uncertainty.

BUT

…no more drastic then that required to add 'no uncertainty' from sampling.

Specific guidance on sampling

- **For samples analyzed by quantitative DNA methods...**
- Take samples from of N locations. Where

$$N = 91 \times RSD_L^2$$ and RSD_L is the lot bulk relative standard deviation

- "91" applies when the analytical uncertainty is a factor of 2.
- If analytical uncertainty is higher then heterogeneity uncertainty is effectively where a smaller number of locations are sampled.
- For maize, homogenize an analytical sub sample of **10,000 grains**.
- This plan produces effectively-zero sampling uncertainty and limits based on analytical uncertainty can be used.

Control of Low Level Presence?
(asynchronous approvals issue and withdrawn GMOs)

- We have the analytical/sampling methods capable of detecting LLP.
- LLP is a real issue for stakeholders and CA.

We need a QUALITY INFRASTRUCTURE:

- Formal validation of methods **at low levels.**
- CRMs **at low levels.**
- Proficiency testing **at low levels.**
- **Stakeholders and legal opinion on the required performance.**

Integrating QRT-PCR variability into sampling plans (seeds)

- Using seller's and buyer's risks

- Comparing qualitative and quantitative methods

- Recommendation of 4 attributes with 3 replicates

- Currently limited to assumption of similarity of biological factors between sample and calibrant
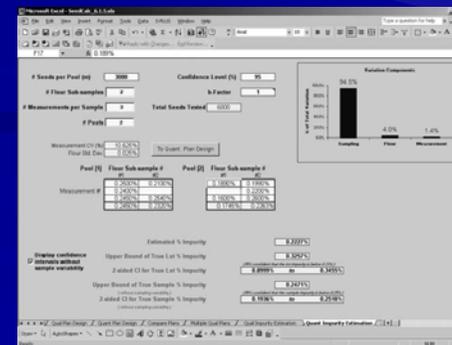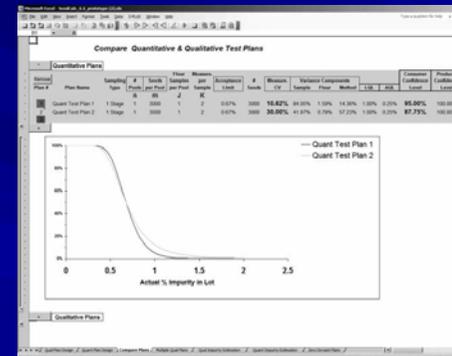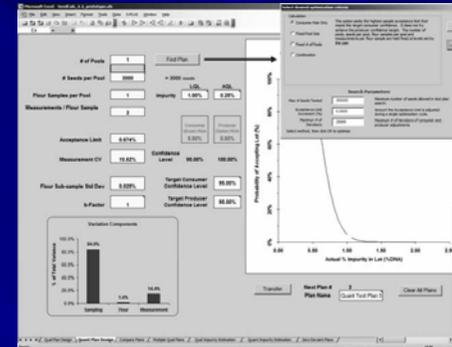
Laffont et al. 2005

Conclusions

- A drastic sampling plan can be used for less drastic control purposes
- Both quantitative and qualitative methods can be used in relation with automated extractions…
- OPACSA could help reduce the number of attributes to be analyzed in a cost-effective and accurate way
- We have a better understanding of analytical uncertainty and how to optimize sampling and analyses
- Low level presence of GMO can be detected but is a real issue for stakeholders and CA

Improving accuracy of detection methods

- **Calibration *vs.* $\Delta\Delta$Ct methods (non need for calibrants): are similar PCR efficiencies a pre-requisite?**
- **Improving precision involve considering several steps**
- **Improved precision should reduce**
  - **Costs (reducing replicates' numbers…)**
  - **Legal disputes**

Guidelines and DSS needed for harmonized ways of interpreting data, reporting and taking decisions

Some insights on technics devoted to GMOs detection which could be helpful in other areas of detection

Global approach
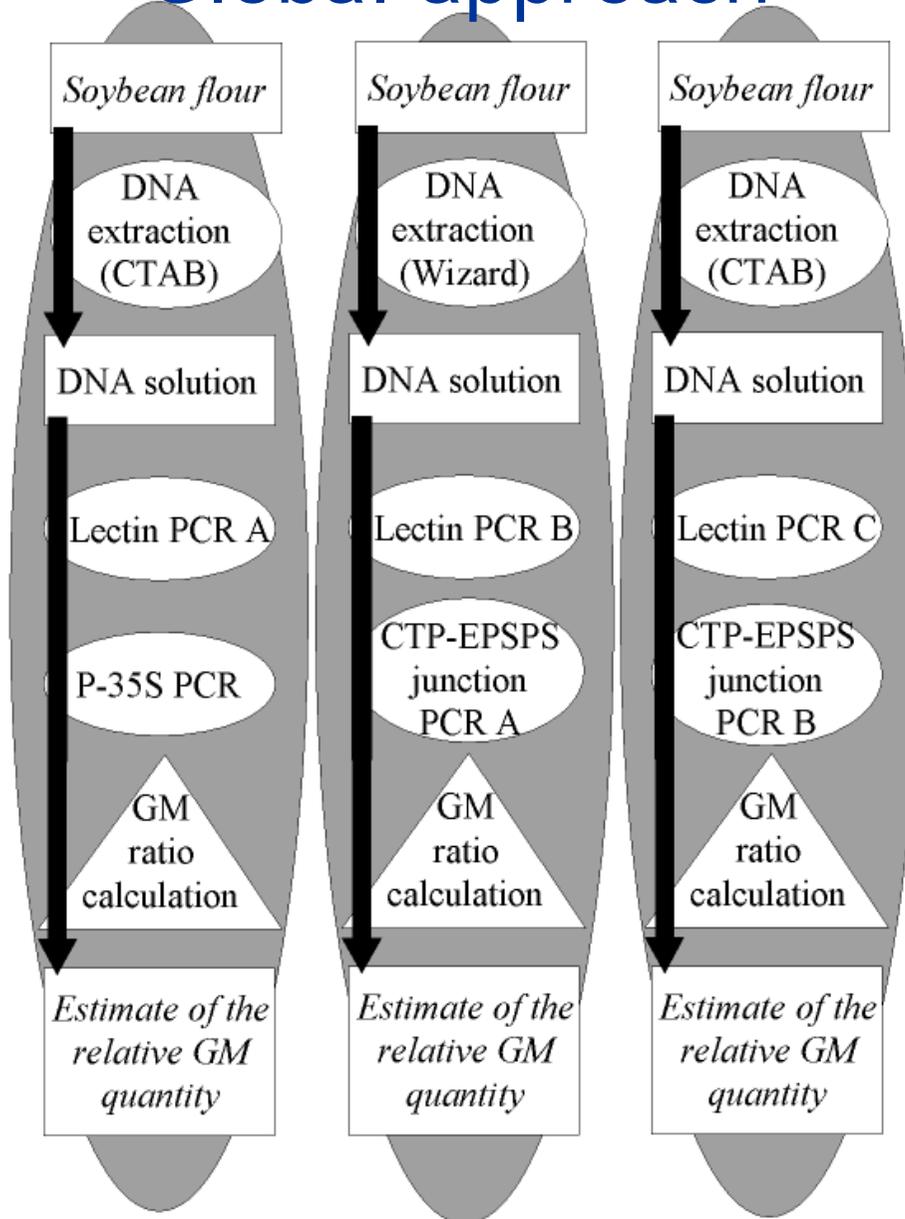
Modular approach
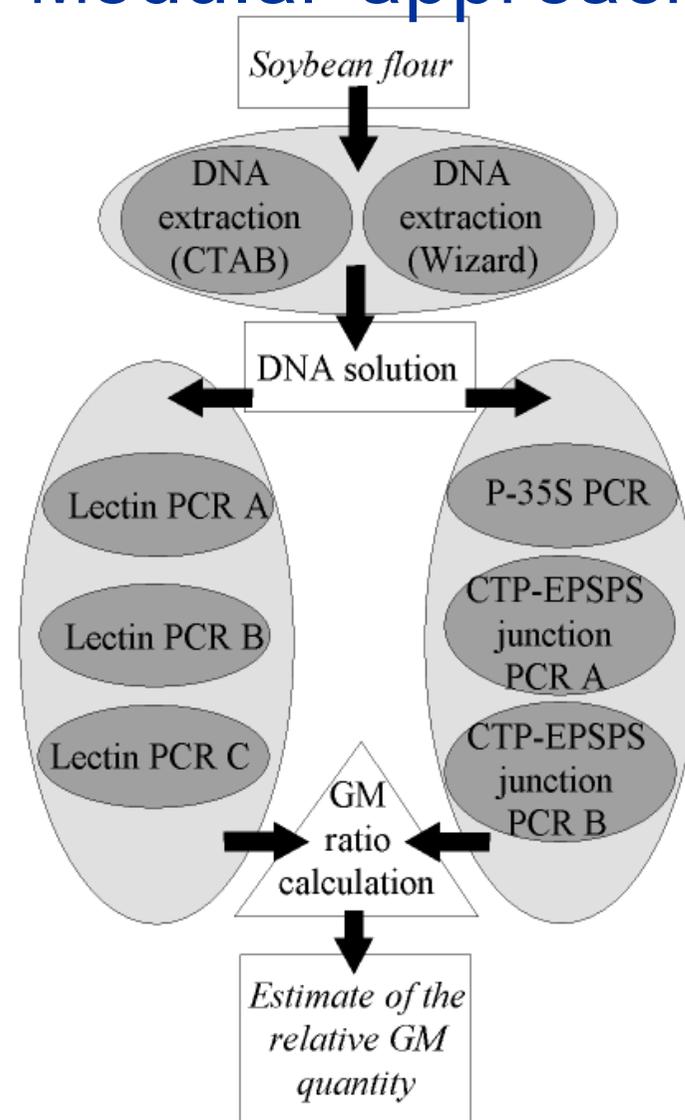
From: Holst-Jensen & Berdal (2004)
J AOAC Int 87(4): 927-936

Approach of high relevance, worldwide used: study of measurements uncertainty

Modular approach

- Highly adaptable as able to integrate new modules (micro-arrays…)
- EURL-GMFF validating only the PCR module
- Residual issues:
  – Multiplication of modules: several modules per taxon and GMO
  – Theoretical commutability of modules (e.g. unique taxon reference system): but shall be internally validated by laboratories (costly)
  – Combination of measurement uncertainties

Search for consensus modules for e.g. each taxon, former proposal of joint Co-Extra Task-force to EuropaBio
Search for unique / universal taxa reference system: QL (plastids) or QN (nuclear)

Certified Reference Material
(not needed in $\Delta\Delta Ct$ methods)

- Current CRM:
  - costly, late release
  - Certified for mass, to be certified for DNA copies
  - Issues about
    - Continuity by withdrawal of GMO e.g. Bt176, Starlink
    - Stability over years, see former Bt176 and Bt11 cases
- Request for DNA based methods of cost-effective, rapidly released CRM

- Data interpretation issues when CRM not available (e.g. UGM as from asynchronous approvals)

Alternative
Certified Reference Material

- Genomic DNA:
  - Lately released as current CRM
  - Stability, continuity issues not resolved
  - Probably commutable with current mass CRM

Issue: disappearance of the current CRM used for seeds and monitoring

- Plasmids
  - Rapidly released
  - Stability and continuity ensured
  - Low cost
  - Commutability to be demonstrated
  - Lack of whole sequence for assessing methods e.g. screening mostly used by stakeholders

JRC IRMM: certification of DNA content based CRM

Fitness of purpose of chemistries and apparatus
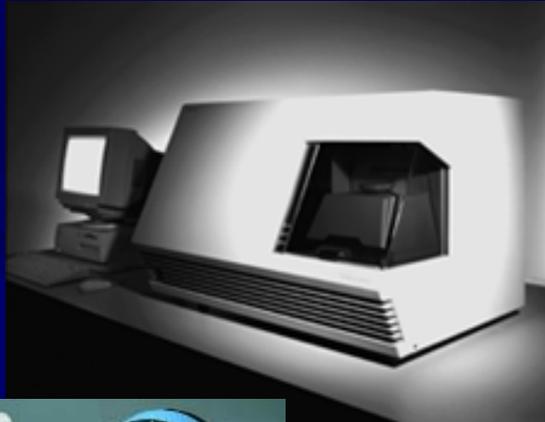
- QC-PCR
- QL Thermocyclers
- Isothermal cyclers
- Lab on a Chip…
- TaqMan chemistry vs. alternatives

Accuracy, comparability of results, cost-effectiveness fitness for purpose of methods and apparatus...
Avoiding monopoly, dictates and fashion effects

Decreasing the number of targets and steps

- **Multiplexing simplex PCR**
- **Decreasing the number of modules:**
  - **The minimum: one standardized reference gene per taxon: former proposal of Co-Extra Task-force to EuropaBio**
  - **An optimum: one reference gene for all plant species (GMOChips and Co-Extra programs)**
- **Identifying several GMOs in a step: Consensus PCR, "Matrix approach" through micro-arrays, SNPlex…**

Limits of the quantitative multiplex strategy

- **Currently: triplex (GeneScan; P35S-Tnos, T35-Pnos, P35S-Tnos-IPC) and tetraplex (INRA; P35S-Tnos-T35S-Pnos)**

- **Interactions between primers and probes to be managed (new softwares)**

- **QRT-PCR:**
    - **limited amount of simultaneous targets because of the limited amount of fluorophores whose spectra can be analyzed simultaneously by the real-time thermocyclers**

- **Need for rapid and user-friendly optimization methods (e.g. by fractionary plans)**

Growing interest for multiplexed QL PCR and other QL methods to be used with control plans by multiple attributes

A High-Throughput Multiplex Method Adapted for GMO Detection

Maher Chaouachi,[†,‡] Gaëlle Chupeau,[†] Aurélie Berard,[†]
Heather McKhann,[†] Marcel Romaniuk,[‡] Sandra Giancola,[†]
Valérie Laval,[‡] Yves Bertheau,[‡,*] and Dominique Brunel[*,†]

CEA/Institut de Génomique/Centre National de Génotypage/INRA UR EPGV, 2 rue Gaston Crémieux,
CP 5724, 91057 Evry cedex, France, and INRA, Laboratoire de Méthodologies de la Détection des
OGM, Unité PMDV, Route de Saint Cyr, RD10, 78026 Versailles cedex, France

A high-throughput multiplex assay for the detection of genetically modified organisms (GMO) was developed on the basis of the existing SNPlex method designed for SNP genotyping. This SNPlex assay allows the simultaneous detection of up to 48 short DNA sequences (~70 bp; 'signature sequences') from taxa endogenous reference genes, from GMO constructions, screening targets, construct-specific, and even specific targets, and finally from donor organisms. This assay avoids certain shortcomings of multiplex PCR-based methods already in widespread use for GMO detection. The assay demonstrated high specificity and sensitivity. The results suggest that this assay is reliable, flexible, and cost and time-effective for high-throughput GMO detection.

KEYWORDS: SNPlex; GMO; detection; multiplex; high-throughput

Consensus PCR and matrix-approach with detection by micro-arrays

Genomic DNA extraction

Sampling grinding

Step I: PCR Amplification

Amplification and labeling

(biotinylated dNTP or primers)

Capture probes consensus and specific

Amplicons hybridization

GMO, plants, control, specific sequences
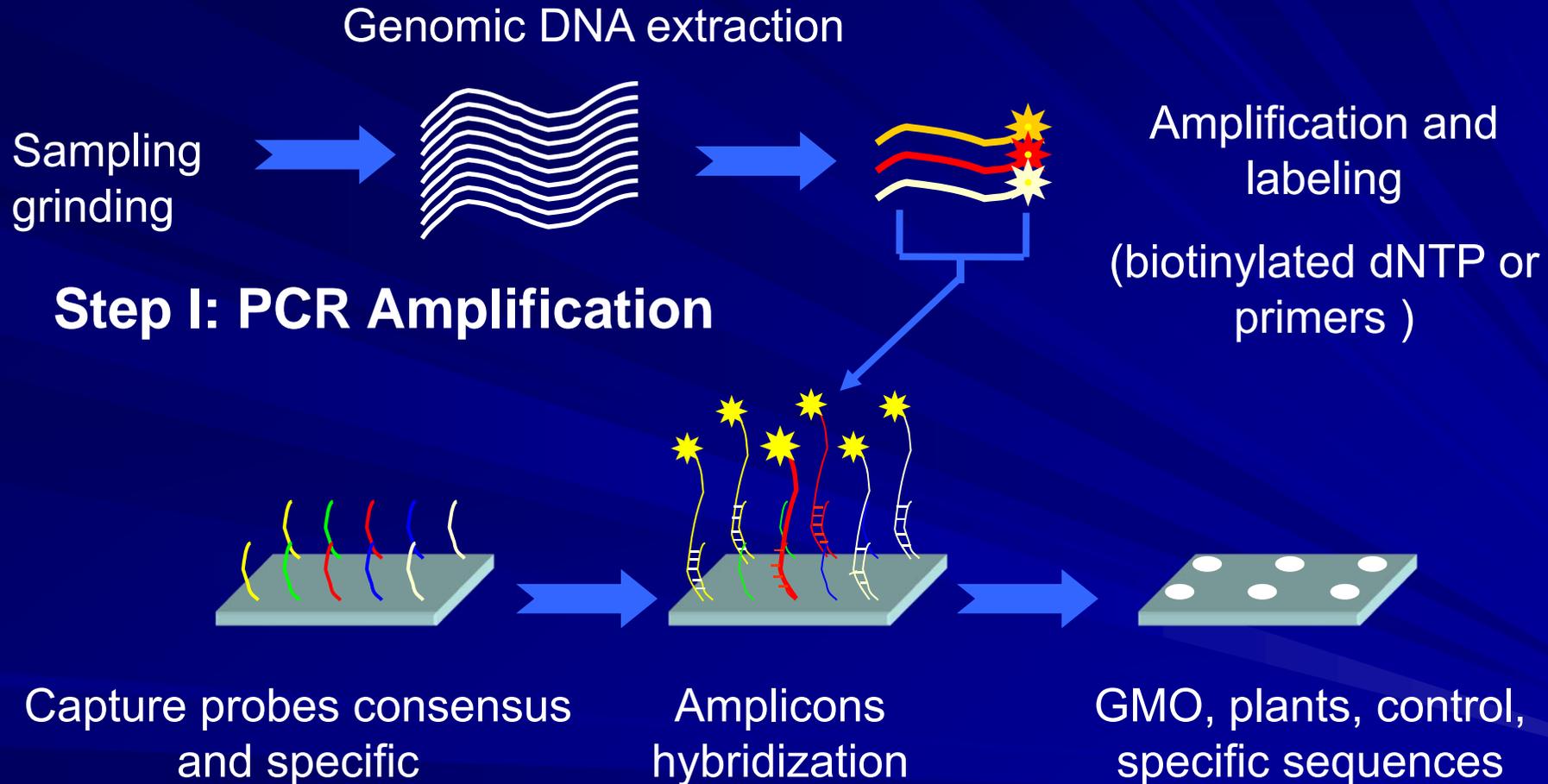
Step II: microarrays hybridization
Detecting several GMO in a step with software
Costs-benefits analysis for acceptability

Unapproved GMOs

Unapproved GMOs

- A residual issue:
  - Asynchronous approvals with e.g. wrong segregation of US approved GMOs: Starlink, US papaya from Brazil and Thailand, etc.
  - Escapes of unapproved GMOs: Bt10, pig vaccine, LLRice601, Bt63 rice, event32, B12 producing microbes…
- A growing concern:
  - New producing countries: China, India, etc.
  - New expected GMOs: molecular farming, phytoremediation, etc. in the same crops

A EU concern shared by USA

United States Government Accountability Office

GAO

Report to the Committee on Agriculture, Nutrition, and Forestry, U.S. Senate

November 2008

GENETICALLY ENGINEERED CROPS

Agencies Are Proposing Changes to Improve Oversight, but Could Take Additional Steps to Enhance Coordination and Monitoring

GAO
Accountability * Integrity * Reliability

GAO-09-60

USDA

U.S. Department of Agriculture

Office of Inspector General
Southwest Region

Audit Report

United States Department of Agriculture
Controls over Importation of Transgenic
Plants and Animals

Report No. 50601-17-Te
December 2008

Sources of UGM

- Third countries and asynchronous approvals (USA, Canada, Brazil, Argentina),
- Illegal uses in some countries of GMOs approved in other countries,
- New producing countries: China, India, Cuba, etc.,
- Biohacking,
- Bioterrorism

UGM: a need of rationalization

- ENGL WG on UGM:
  - 3 to 4 levels of knowledge
  - Survey of existing detection methods and strategies
  - Guidelines for detecting UGM
  - Perspectives and needs

Book's chapter (2013) and EURL-GMFF guidelines:

20
Towards Detection of Unknown GMOs

A. Holst-Jensen, K.G. Berdal, Y. Bertheau, M. Bohanec, J. Bohlin,
M. Chaouachi, K. Gruden, S. Hamels, E.J. Kok, A. Krech, A.B. Kristoffersen,
V. Laval, S. Leimanis, M. Løvoll, D. Morisset, A. Nemeth, N. Papazova,
T.W. Prins, J. Remacle, P. Richl, T. Ruttink, I. Taverniers, T. Tengs,
J.P. van Dijk, D. Wulff, J. Žel, H. Zhang, M. Žnidaršič

Approaches for detecting unapproved GMOs

- **Qualitative differential PCR**
- **Quantitative differential PCR**
- **Matrix approach (micro-arrays, SNPlex)**
- **Fingerprinting (anchored PCR, etc.)**
- **Genome sequencing**
- **Plasmids residues' detection (high density micro-arrays)**

Qualitative differential PCR

- **Sequence detected e.g. P35S**
- **No authorized GMO with such sequence detected**
- **No donor organism for this sequence detected e.g. CaMV**

Suspicion of presence of unknown GMO

Quantitative differential approach (dQ PCR): principle

- **Detect and quantify a consensus element, common to a group of GMOs**

  - set of primers specific of the consensus element (e.g. P35S)

- **Detect and quantify all the approved GMOs presenting this consensus element**

  - sets of event-specific primers for each of these GMO (e.g. edge-fragments)
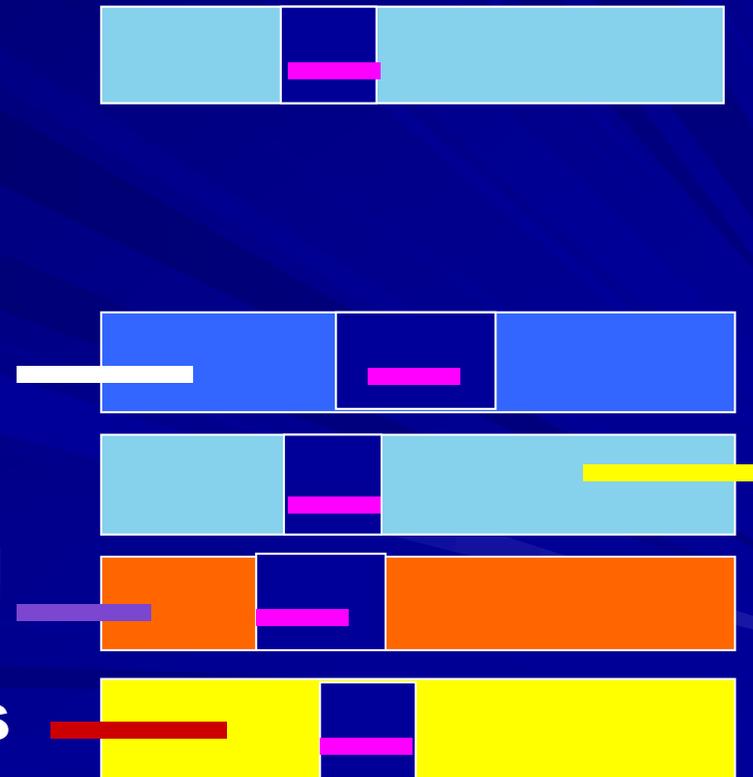
Example of dQ PCR application

- Consensus element : P35S
- Authorized GMO containing P35S: Mon 810, T25, Bt176… + unknown GMO

$$\mu = \text{quant }_{\text{P35S}} - (\text{quant }_{\text{Mon810}} + \text{quant }_{\text{T25}} + \text{quant }_{\text{Bt176}})$$

Testing $\mu = 0$

- Applicable to Tnos, Pnos, T35S, CryIA(b), EPSPS, etc. with appropriate controls (CaMV, *Agrobacterium*, etc.) Cankar *et al.* 2008. *Anal. Biochem.*
- Validation finished.

Detecting unknown GMOs:
the differential quantitative PCR

Contents lists available at ScienceDirect

Analytical Biochemistry
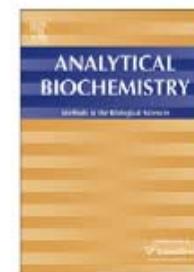
Detection of nonauthorized genetically modified organisms using differential quantitative polymerase chain reaction: application to 35S in maize

Katarina Cankar [a,1,2], Valérie Chauvensy-Ancel [b,1], Marie-Noelle Fortabat [b,1], Kristina Gruden [a], André Kobilinsky [c], Jana Žel [a], Yves Bertheau [d,*]

The first ready to use, accurate, cost-effective and flexible
detection method of unknown GMOs and for confimation of approved GMO data

Results

• Ability to detect unknown GMO containing P35S: between 0 and 5% of UGM for copy numbers between 100, 400 and 1,600 HGE

Results

The power of the test can be calculated according the analysts or CA needs

Results of detection of a nonauthorized GMO for design 4

GMO	DNA quantity (copy number)	Number of samples	Number of samples detected	% Samples detected	Simulation's results of the power of the test (%)
Bt176	100	6	5	83	67.4
	400	9	9	100	95
	1600	7	7	100	100
Mon810	100	6	5	83	61
	400	9	9	100	90.8
	1600	7	7	100	99.8
T25	100	3	1	33	41.6
	400	9	9	100	89.3
	1600	12	12	100	94.2

The accuracy of the test improves with the HGE copy numbers

dQ-PCR validation: Co-Extra / ENGL collaborative trial

- Testing the robustness of the statistical tool
- As far as possible: representative of real-life
- Maize kernels with Bt176, Mon810 and T25... at different levels prepared by BIPEA
- All methods allowed provided they are fully implemented
- No specific SOP
- Work finished: manuscript in preparation

The 'matrix approach'(example)

• **Numerous small sequences common to several GMO ('screening' sequences)**

• **Uses according to their frequency of occurrence**

• **Both primers are located inside those genetic elements such as P35S, T35S, Tnos, Pnos, nptII, CryIA(b), some construct specific…**

• **Used in duplex PCR, EAT DualChip® and SNPLex**

	GMO-1	GMO-2	GMO-3
P35S	x	x	x
Tnos		x	x
T35S	x	x	
cryI(A)b	x	x	x
bar		x	
nptII			

NB: can be extended to other factors: immunological tests, etc.

The Matrix approach

- Ability to both detect unapproved GMOs and identify approved GMOs

- Mostly based on screening sequences

- Needs for reliable and updated data bases of sequences (molecular specificity)

- Need for appropriate CRM (flour or new plasmids)

- Need for specific validation not in the mandate of the EURL-GMFF

ENGL WG working on guidelines

Several DSS currently available

The matrix approach

Research review paper

Detecting un-authorized genetically modified organisms (GMOs) and derived materials

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ARTICLE INFO

ABSTRACT

Genetically modified plants, in the following referred to as genetically modified organisms or GMOs, have been commercially grown for almost two decades. In 2010 approximately 10% of the total global crop acreage was planted with GMOs (James, 2011). More than 30 countries have been growing commercial GMOs, and many more have performed field trials. Although the majority of commercial GMOs both in terms of acreage and specific events belong to the four species: soybean, maize, cotton and rapeseed, there are another 20 + species where GMOs are commercialized or in the pipeline for commercialization. The number of GMOs cultivated in field trials or for commercial production has constantly increased during this time period. So have the number of species, the number of countries involved, the diversity of novel (added) genetic elements and the global trade. All of these factors contribute to the increasing complexity of detecting and correctly identifying GMO derived material. Many jurisdictions, including the European Union (EU), legally distinguish between authorized (and therefore legal) and un-authorized (and therefore illegal) GMOs. Information about the developments, field trials, authorizations, cultivation, trade and observations made in the official GMO control laboratories in different countries around the world is often limited, despite several attempts such as the OECD BioTrack for voluntary dissemination of data. This lack of information inevitably makes it challenging to detect and identify GMOs, especially the un-authorized GMOs. The present paper reviews the state of the art technologies and approaches in light of coverage, practicability, sensitivity and limitations. Emphasis is put on exemplifying practical detection of un-authorized GMOs. Although this paper has a European (EU) bias when examples are given, the contents have global relevance.

DualChip® v2.0 principle

DNA extraction
from food or feed sample

Genomic DNA

Amplification
via 3 different multiplex
PCRs with biotinylated
primers

PCR 1   PCR 2   PCR 3

Hybridization
of the 3 PCR reactions
on 1 DualChip GMO
microarray

Slide with 2 arrays

Detection
with Silverquant®
colorimetric detection
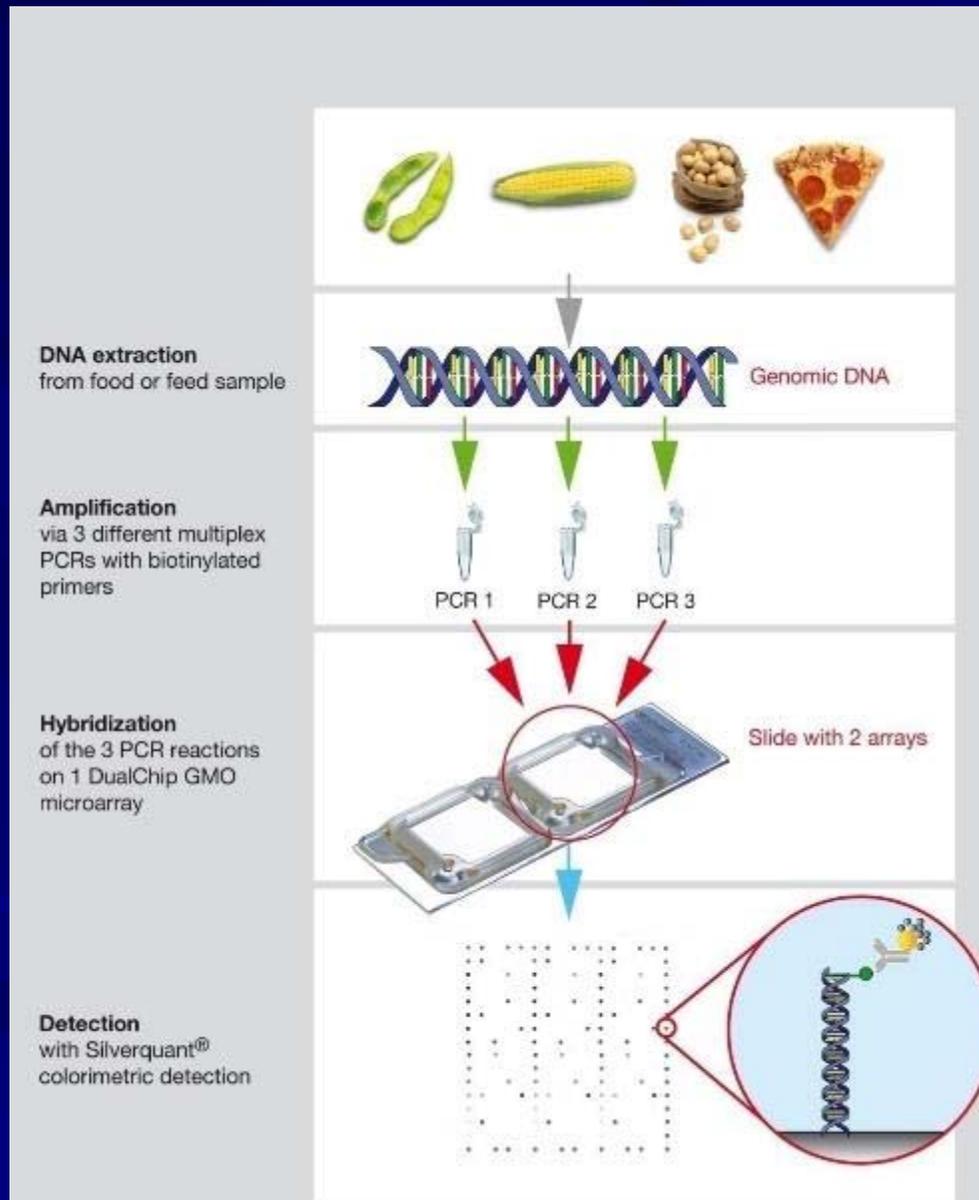
32 genetic elements to be detected

3 multiplex PCR

Collaboration started
With Namur's Univ.
and AAT (spin-off)
then EAT

eppendorf
— *Array Technologies*—

DualChip® v2.0 content

12 Screening targets:
- *P35S*
- *Tnos*
- *pat*
- *bar*
- *Cry1Ab-1*
- *Cry1Ab-2*
- *Cry1Ab-3/Cry1Ac*
- *EPSPS-a*
- *EPSPS-b*
- *EPSPS-c*
- *Pnos-nptII*
- *Cry3Bb1*

1 Contamination control target:
- CaMV

7 Plant species targets:
- Maize
- Rapeseed
- Soybean
- Cotton
- Sugar beet
- Potato
- Rice

11 Event specific insert-to-plant junction targets:
- GA21
- Bt176
- MON810
- Bt11
- MON863
- GTS40-3-2
- T45
- GT73
- MON531
- MON1445
- MON15985

31 targets detected in 1 assay according to ISO standard requests

Report compliance with the ISO 17025

DualChip® GMO Report

Operator name:	John	File name:	File2.txt
Analysis date:	05/06/2008	Experiment date:	26/03/2008
Silverquant detection kit lot:	z654321	Sample number:	1
DualChip GMO Kit Box 1 Lot:	x123456	Sample designation:	XXX
DualChip GMO kit batch number box 2:	y456789	Comments:	none

GM event(s) present

Genetic target elements	
Screening:	
bar	Detected
cry1Ab-1	Not detected
cry1Ab-2	Not detected
cry1Ab-3	
cry3Bb-1	Not detected
EPSPS-A	Not detected
EPSPS-B	Not detected
EPSPS-C	Not detected
P35S	Detected
pat	Not detected
Pnos-nptII	Detected
T-nos	Not detected
Plant species:	
Brassicaceae	Not detected
Cotton	Not detected
Maize	Not detected
Potato	Not detected
Rice	Not detected
Soybean	Detected
Sugar beet	Detected
Event-specific:	
Bt11	Not detected
Bt176	Not detected
GA21	Not detected
GT73	Not detected
GTS 40-3-2	Not detected
MON1445	Not detected
MON15985	Not detected
MON531	Not detected
MON810 a	Not detected
MON810 b	Not detected
MON863	Not detected
T45	Not detected

Contamination control	
CaMV	Detected

Process controls	
Positive hybridization controls:	OK
Negative hybridization controls:	OK
Negative detection controls:	OK
Positive detection controls:	OK
PCR controls:	OK
Array variability	OK

Specified GM events and hybrids based on event-specific target element	
GM event	**Event-specific**
Bt11	Absent
Bt176	Absent
GA21	Absent
GT73	Absent
GTS 40-3-2	Absent
MON1445	Absent
MON15985	Absent
MON531	Absent
MON810	Absent
MON863	Absent
T45	Absent
GM hybrid	**Event-specific**
GA21 x MON810	Absent
MON15985 x MON1445	Absent
MON531 x MON1445	Absent
MON863 x MON810	Absent

Specified GM events and hybrids based on pattern analysis	
GM event	**Pattern analysis**
DAS1507	Absent
DAS59122	Absent
H7-1	Absent
Nk603	Absent
T25	Absent
TOPAS19/2	Absent
GM hybrid	**Pattern analysis**
DAS107 x NK603	Absent
MON863 x NK603	Absent
MS1 x RF1	Absent
MS1 x RF2	Absent
MS8 x RF3	Absent
NK603 x MON810	Absent

Unspecified GM event	Detected screening target element(s)
	bar
	P35S
	Pnos-nptII

Detection of all the currently EU authorized GMO (2007) by:

the event specific elements

Pattern analysis

Detection of " unspecified GM event"

DualChip® Validation report (ISO 5725) in the frame of Co-Extra

JRC Scientific and Technical Reports

Microarray Method for the Screening of EU Approved GMOs by Identification of their Genetic Elements

Report of validation coordinated by the Community Reference Laboratory for GM Food and Feed of the Joint Research Centre

ORIGINAL PAPER

Validation of the performance of a GMO multiplex screening assay based on microarray detection

Serge Leimanis · Sandrine Hamels · Florence Nazé · Guillaume Mbongolo Mbella · Myriam Sneyers · Rupert Hochegger · Hermann Broll · Lillian Roth · Klára Dallmann · Adrienn Micsinai · José Luis La Paz · Maria Pla · Claudia Brünen-Nieweler · Nina Papazova · Isabel Taverniers · Norbert Hess · Britta Kirschneit · Yves Bertheau · Colette Audeon · Valérie Laval · Ulrich Busch · Sven Pecoraro · Katrin Neumann · Sibylle Rösel · Jeroen van Dijk · Esther Kok · Gianni Bellocchi · Nicoletta Foti · Marco Mazzara · William Moens · José Remacle · Guy Van Den Eede

Recent (Co-Extra) applications of the "Matrix approach"

- **Qualitative PCR: up to 9plex**

- **Micro-arrays: (32 targets) DualChip® first inter-laboratories validated chip**

- **SNPlex™: up to 48 targets amplified in a time**

- **Whole genome amplification and micro-arrays detection, including vectors sequences**

ENGL WG guidelines and paper

Quantification of UGM

- dQ-PCR can quantify but does not provide insights on the GMO% (relation sequence / mass)

- The 'Matrix approach' use qualitative methods:
  - Sub-sampling strategy (control plans with multiple attributes) for knowing the content versus a threshold (e.g. when safety reason applies)

Detecting stacked genes

- **Japanese approach: kernel by kernel…**

- **EU: combination of**
  - **qualitative PCR and**
  - **control plans with multiple attributes**
- **Results in a probability of presence of stacked genes**
- **Methodology available (Co-Extra and ISTA)**

- **Generalization with QRT-PCR: mathematical modeling still to be developed…**

How far should the analyses be pursued (costs issues)?

Software which may help: SISSI

- Resampling strategy (see also OPACSA, etc.)

Confalioneri et al. 2007

Taking decisions in uncertain environment

- Matrix data can detect several GMO in a time: interpretation of data
- Matrix approach can detect unknown GMO

Need of DSS for
- Harmonization of data interpretation
- Reporting
- Decision making
- In combination with doc traceability

Conclusion

- While numerous issues on approved GMOs are resolved by 'new' regulations (1829/03 and 1830/03)
- Still pending issues for UGM
  - Issues:
    - Controls for donor organisms (*Agrobacterium*, *Bacillus thuringiensis* when homologous sequences, etc.)
    - A need for keeping two kinds of CRM (grinded seeds and plasmids) for GMOs quantification and "Matrix approach",
  - Validation of such methods (e.g. matrix approach) without reference material?
- EU regulation covering only a part of laboratories' and stakeholders' needs: gaps in regulation and techniques (e.g. CRL validation of screening methods for the 'Matrix Approach' for detecting unknown GMOs): new mandate for EURL?
- Procedures for detecting UGM to be harmonized between EU and third countries such as USA

dQ-PCR: the first cost-effective method directly applicable in day to day analyses of UGM also able to confirm data for approved GMOs (event-specific versus screening methods)

Field sampling

- Coexistence issues (research and in practice)
- diversity centers and landraces (see criollo corn issue in Mexico)

INRA

Coexistence issues

Recipient-Maize

Donor-Maize

Sunflower

Clover/Grass
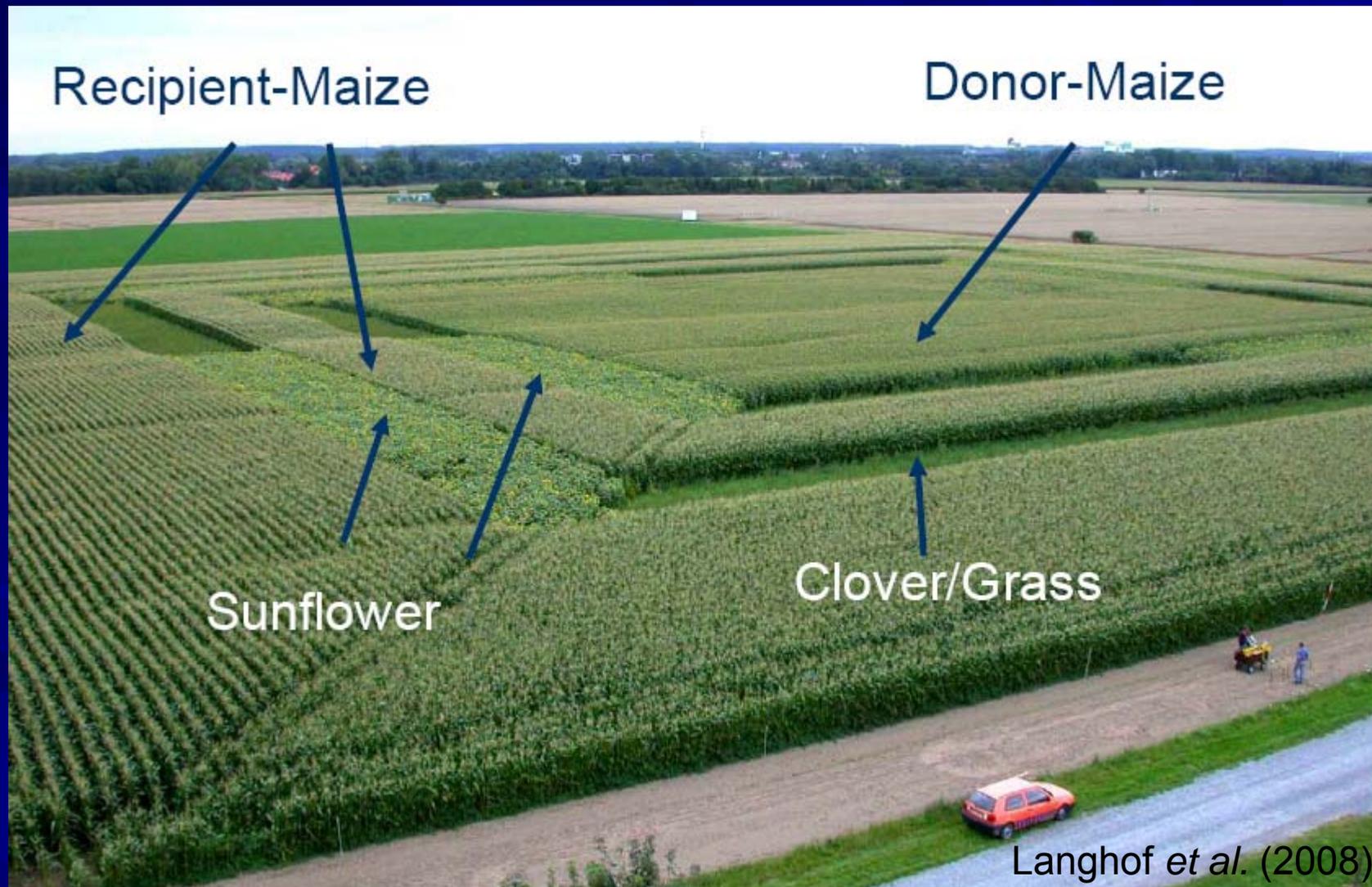
Langhof *et al.* (2008)

Coexistence issues

Langhof *et al.* (2008)
Surfer software 8.0
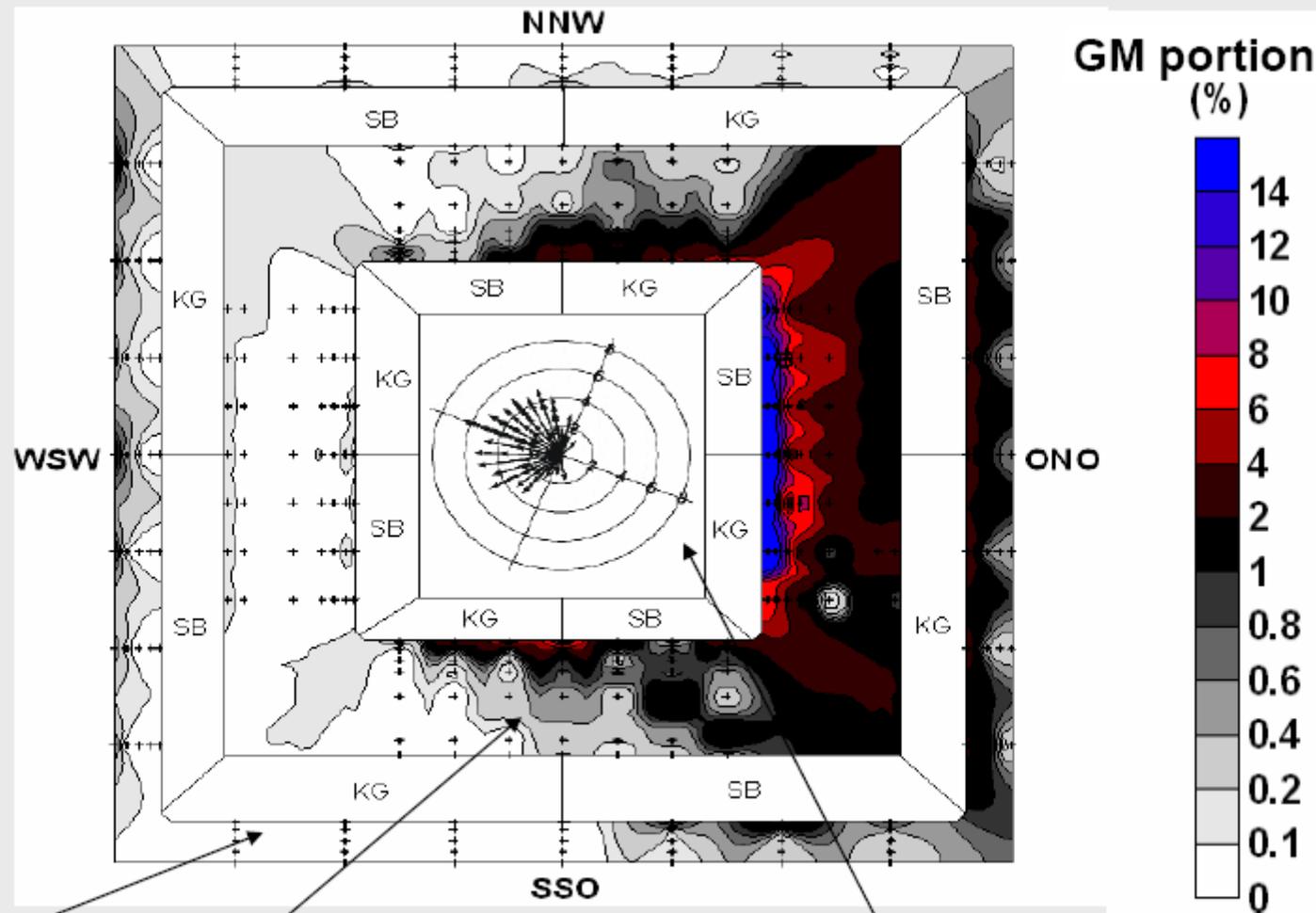
Recipient-Maize

Donor-Maize

Coexistence issues

Donor-Maize
(Mon 810)

Recipient-Maize
(Sandrina)

Buffer crop:
Clovergrass
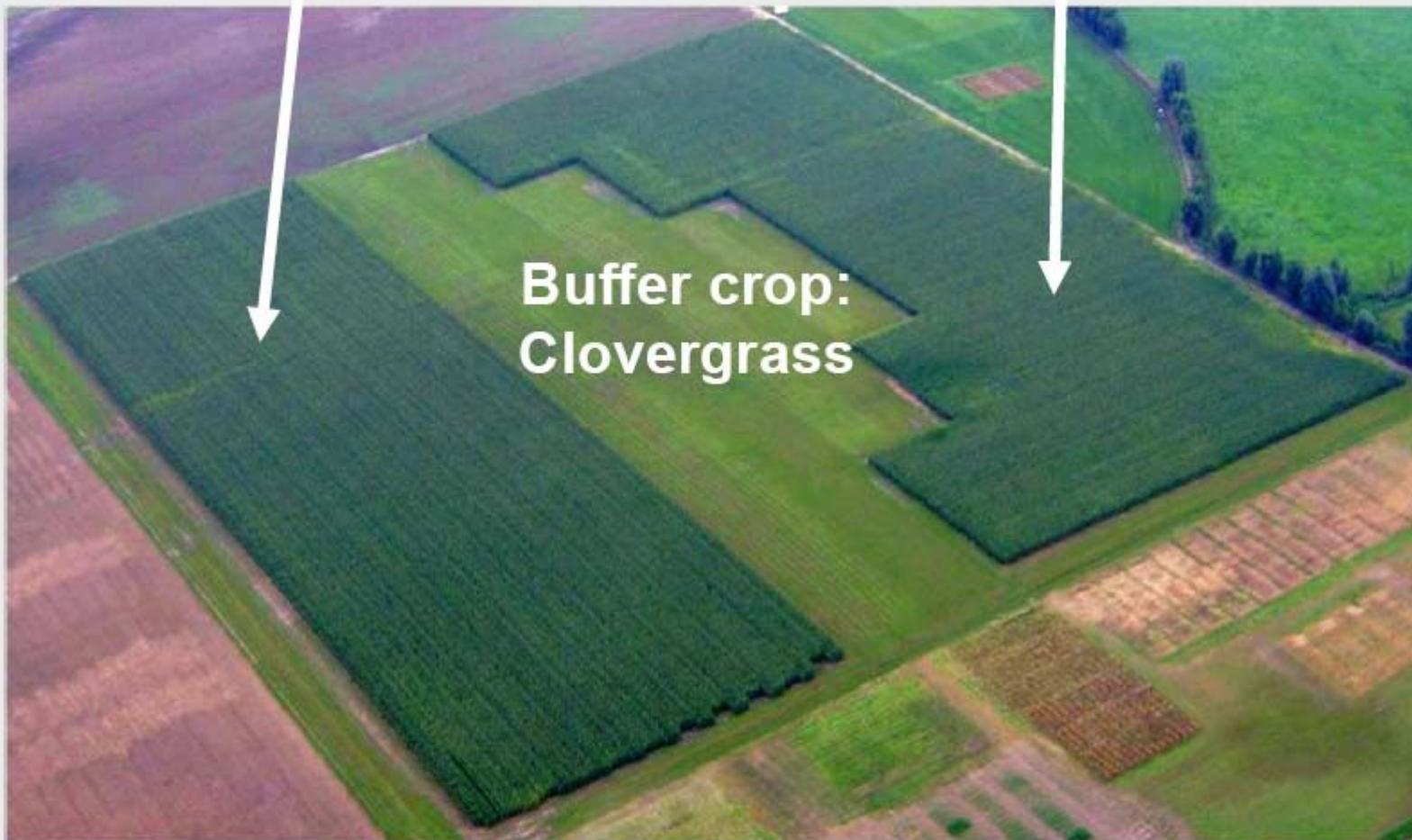
Coexistence issues
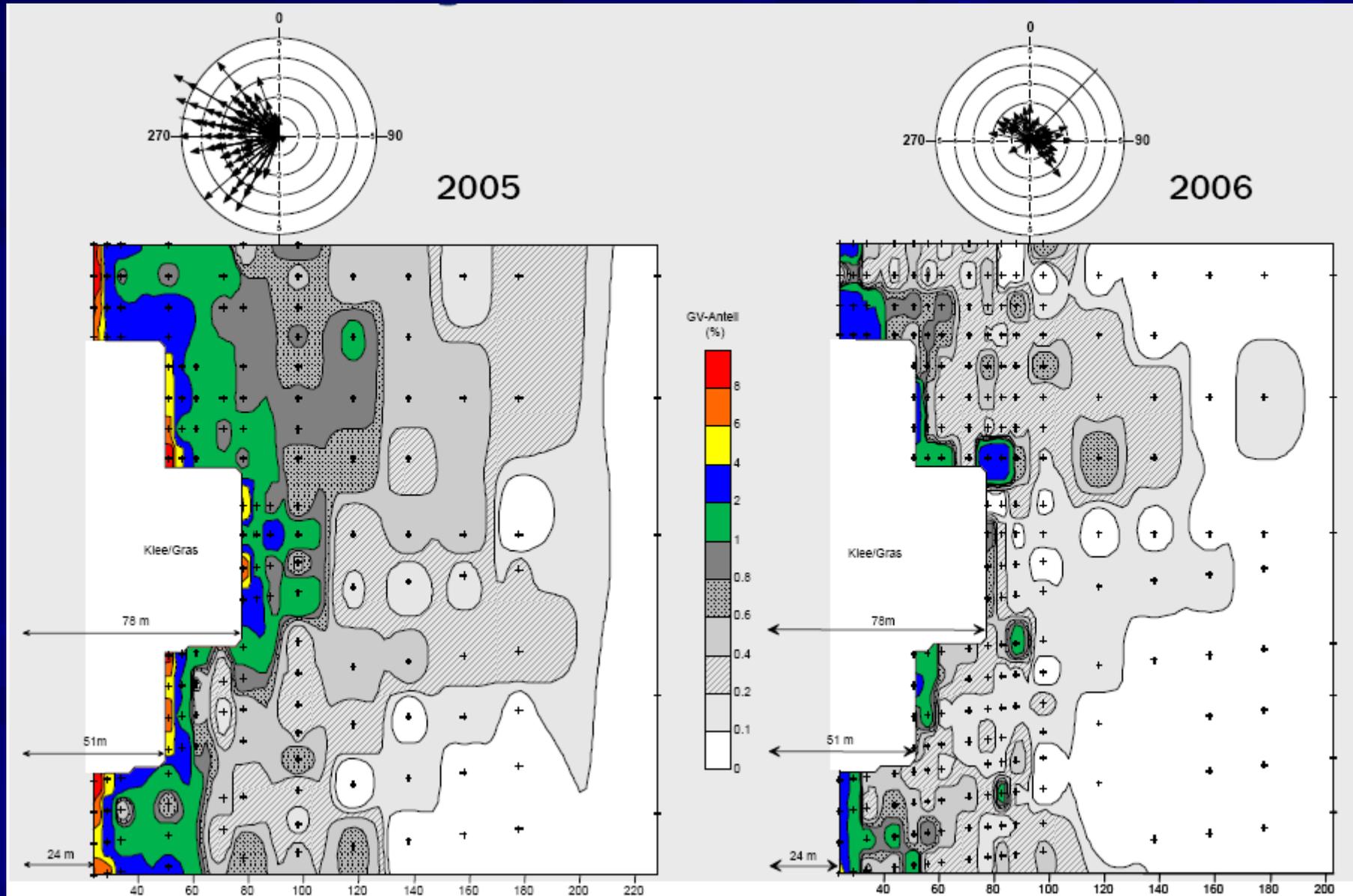
How to estimate the true value for the whole field?

- To calculate the mean rate of cross-pollination over a field area, use of e.g. Inverse Distance Weighting (IDW)

- Zestj
$$\sum \frac{z_i}{(h_{ij} + s)^p} / \sum \frac{1}{(h_{ij} + s)^p}$$

Estimated value for the j point

Z_i analysed value at I point

H_{ij} distance from I to j

S smoothing factor

P power factor


12
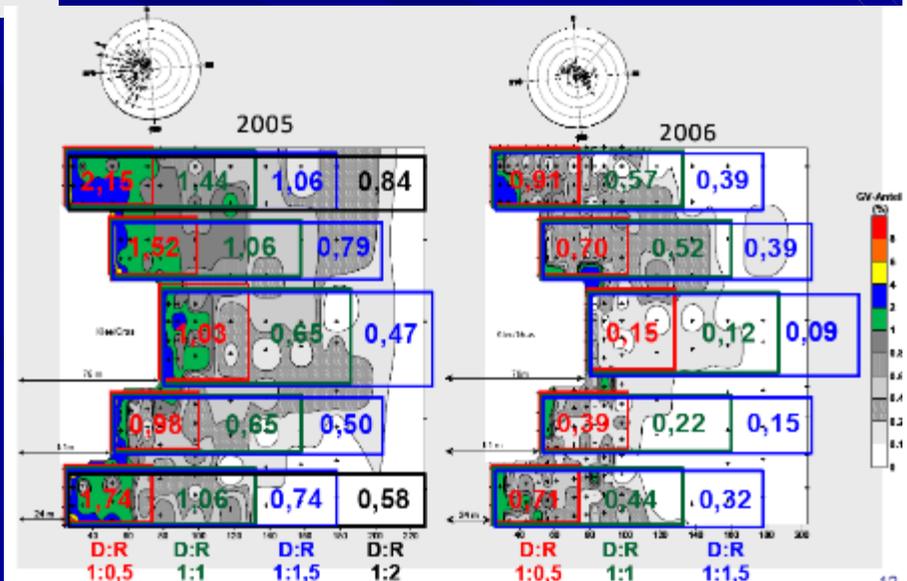
Heavy queue and mix of pollen

2 punctual pollen sources
Same dispersal nucleus
Same pollen quantity
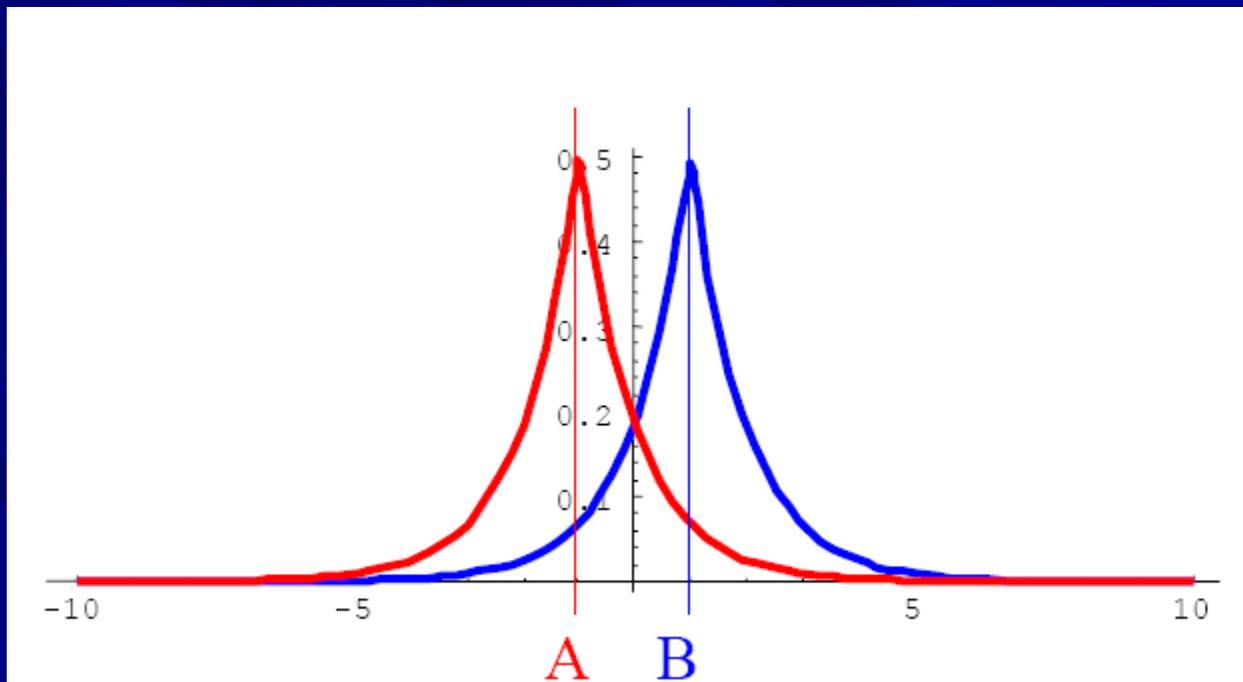
Heavy queue and mix of pollen

Long tailed queue
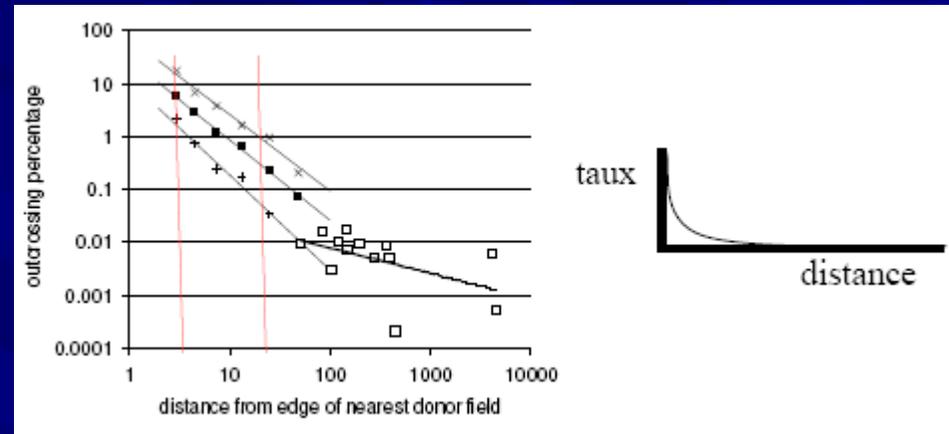
Squire et al., 2007

Pollen dispersal

Allnutt *et al.*, (2008) Sampling and Modelling for Quantification of Adventitious GM Presence in Maize. Agricultural and Food Chemistry 56 (9),
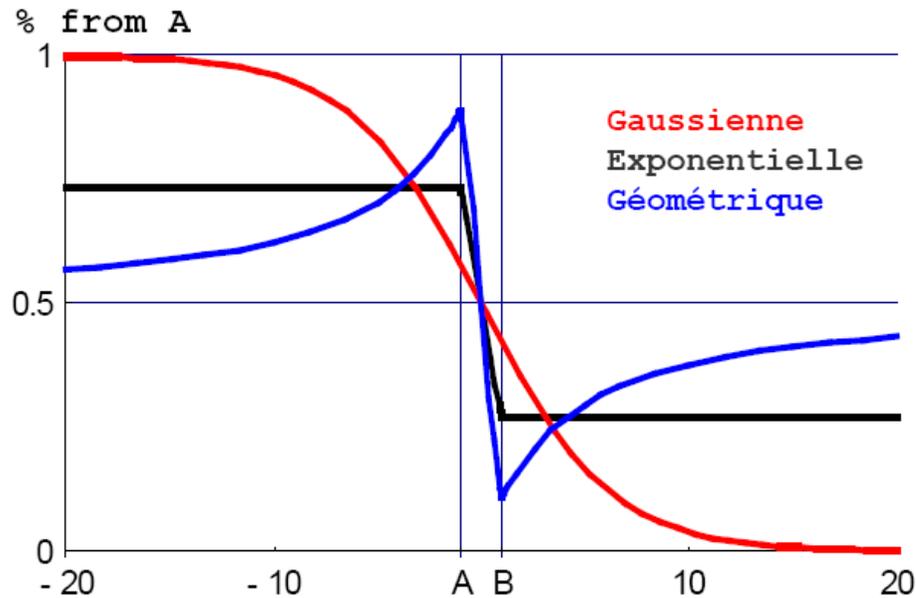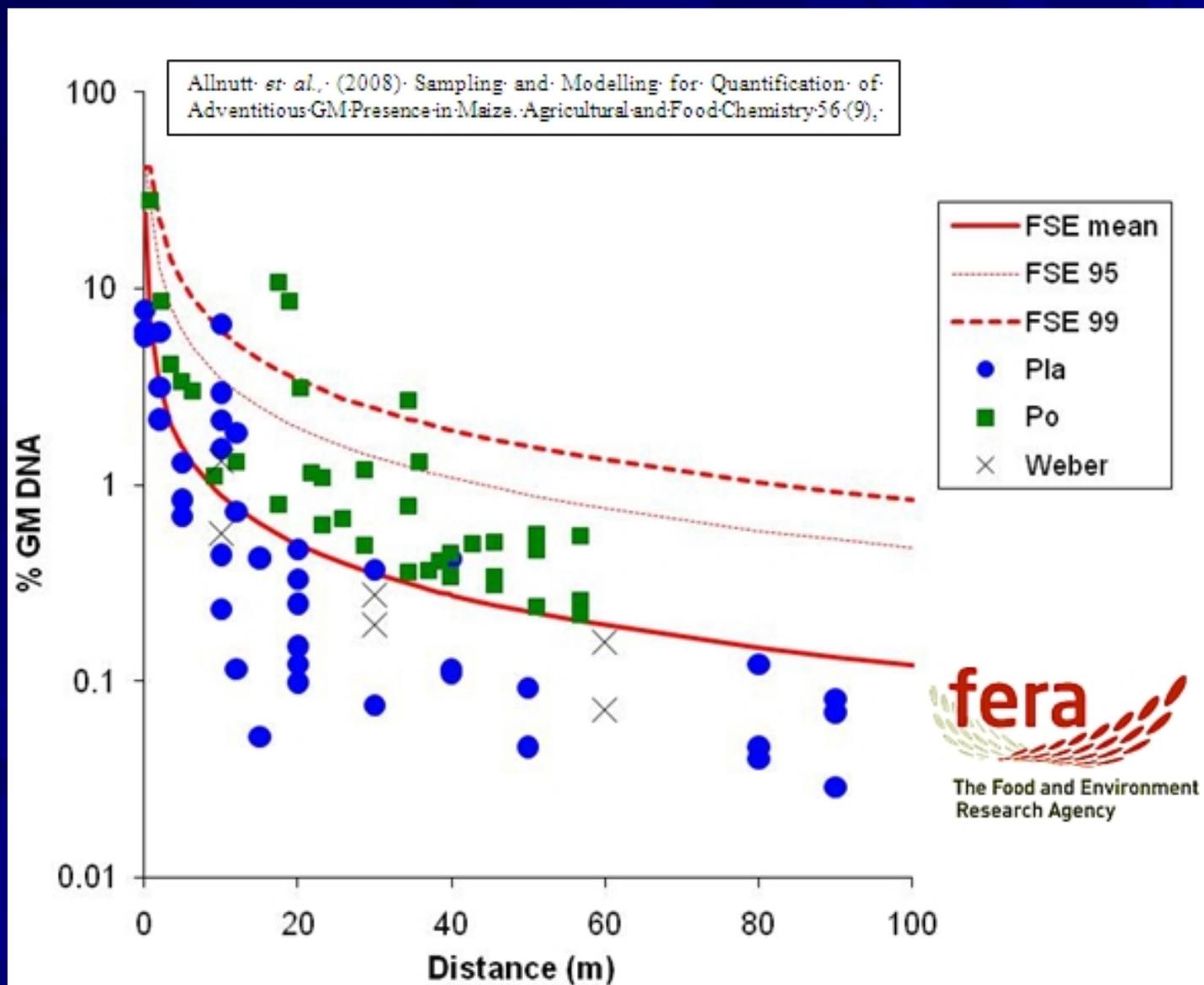
Wheat pollen flow
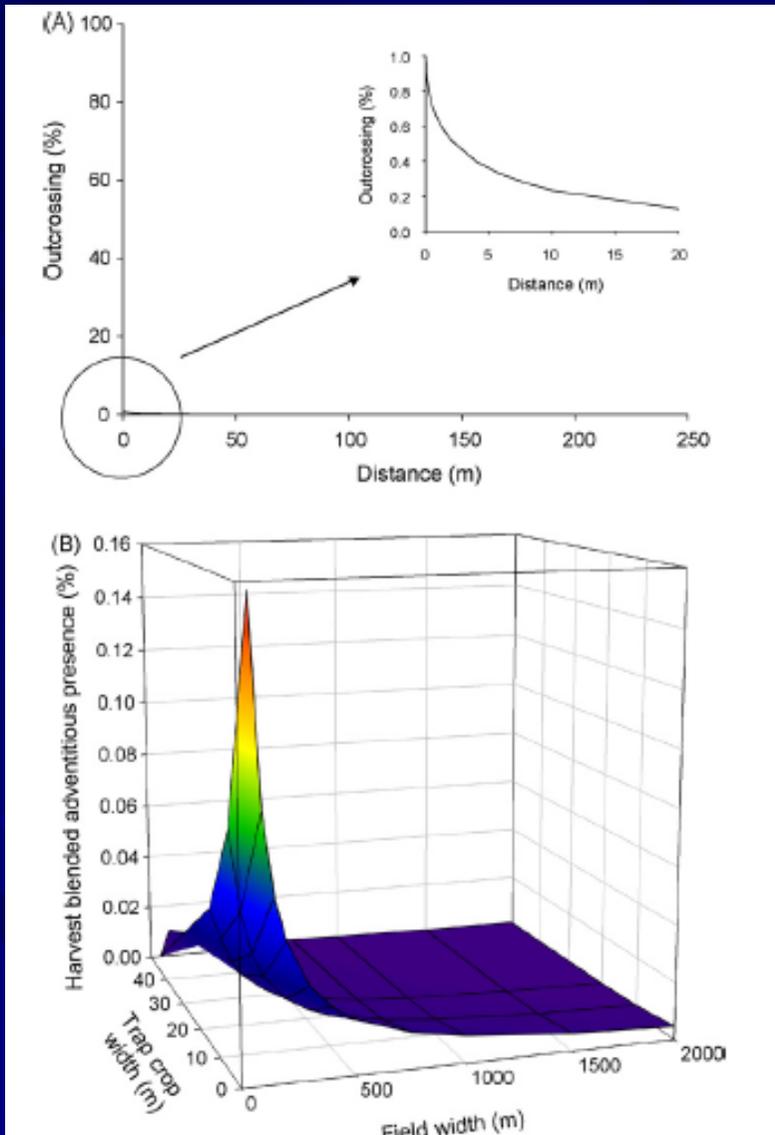
Leptokurtic curve.
AP according to distance:
2 m 0.66%
20 m 0.29%
200 m 0.05%

Rare events of tail contributing little to AP but what about multiple pollen sources and peasants seeds?

(adapted from
Gustafson et al. / Beckie et al.)

Atmospheric Pollen dispersal
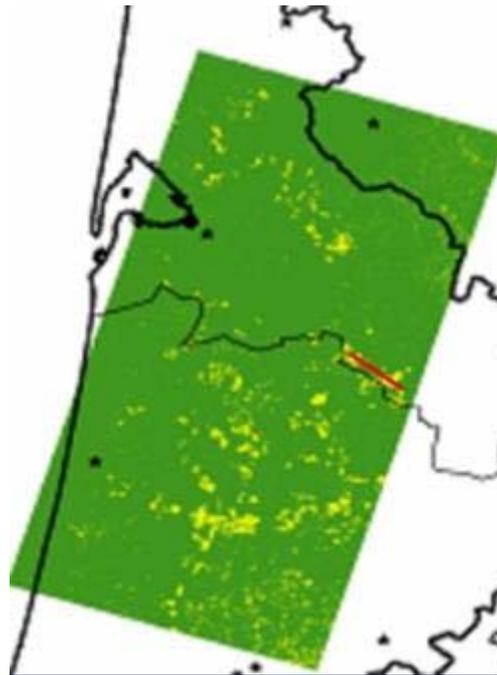
Corn fields
and aircraft transect

Concentration
simulation
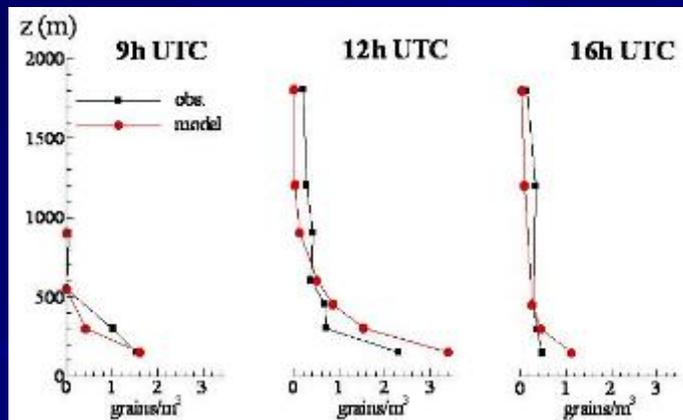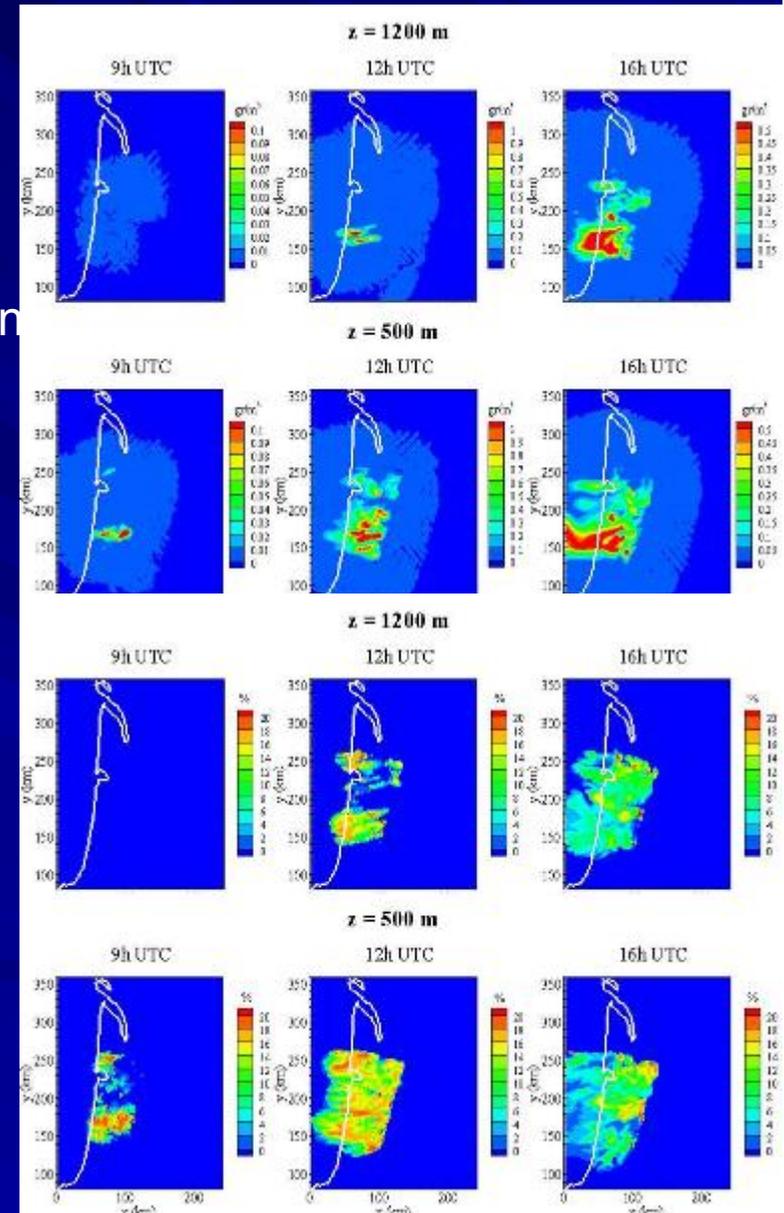
Aircraft observations of pollen
and Meso-NH simulated

Viability
simulation

Issues of tailed queue, atmospheric dispersal and landscape heterogeneity

- Long distance according to pollen characteristics (e.g. *Agrostis stolonifera*: 20 km) and meteorological events
- Sampling and measurement uncertainties practical threshold shall be less than 1/3 of labeling threshold (generally 1/10th)
- GMO-free (2 thresholds in France: 0.9 and 0.1%) and organic farming (EU vs. French definitions)
- Peasants' seeds and participative breeding…
- Importance of landscape composition and heterogeneity, domino effect, multiple sources of pollen flows…

Altogether explain discrepancies between EU MS' coexistence measures such as isolation distance

Land sharing vs. land sparing…

Coexistence and Decision Making Scenarios

Situation	Period	Information	Question	Strategy
Ex-ante	Before sowing	Potential GMO location	Where to allocate GM fields? Which variety/sowing date?	Choice of location/variety/sowing date
Ex-post 1	Between sowing and flowering	Non-GMO and GMO location Variety Sowing date	Which fields to select?	Observation of flowering periods
Ex-post 2	Between flowering and harvesting	+ flowering dynamics and climate	Which fields to select? Where to sample?	Sampling and PCR analysis

Regulation model

- Baseline: 50m isolation distance

Legend

Results

- Conventional maize field within the isolation distance of a GM maize field
- Conventional maize field out of the isolation distance of a GM maize field
- Allowed GM maize field
- Forbidden GM maize field
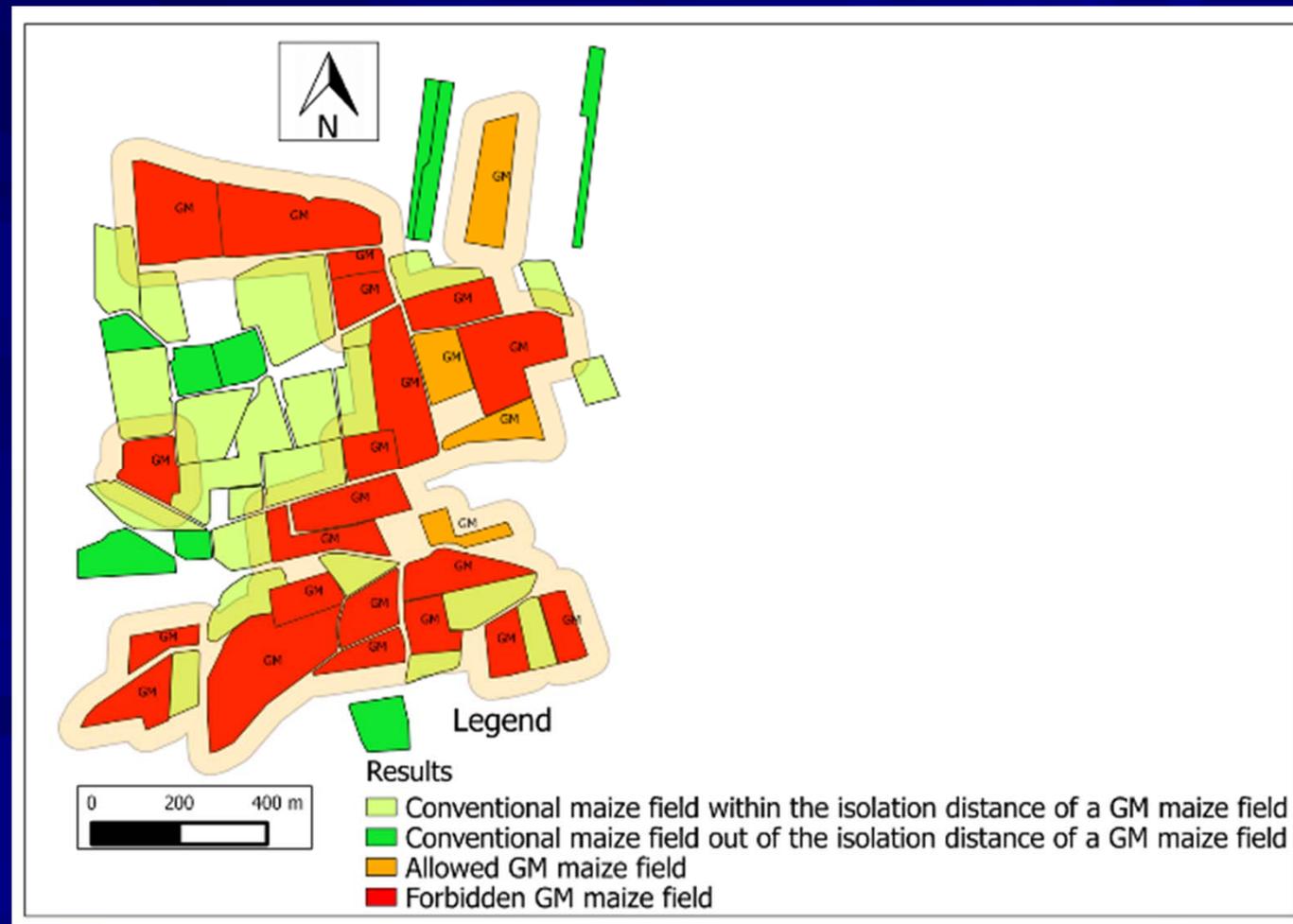
« Model-based » implementation – Evaluation before sowing
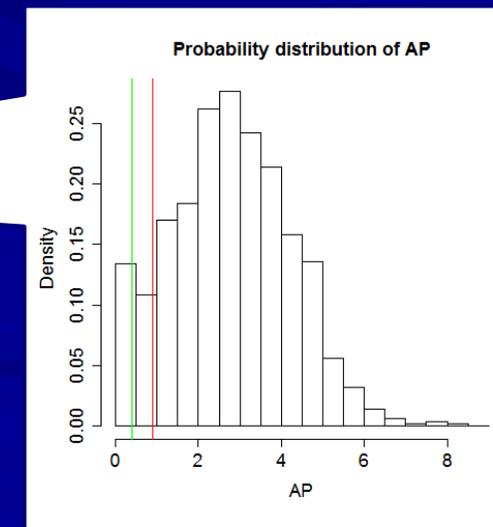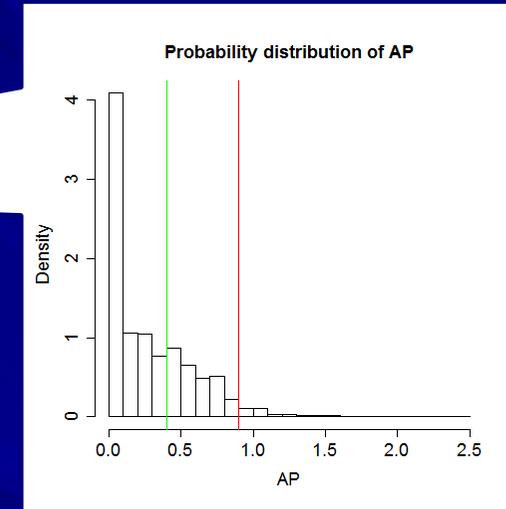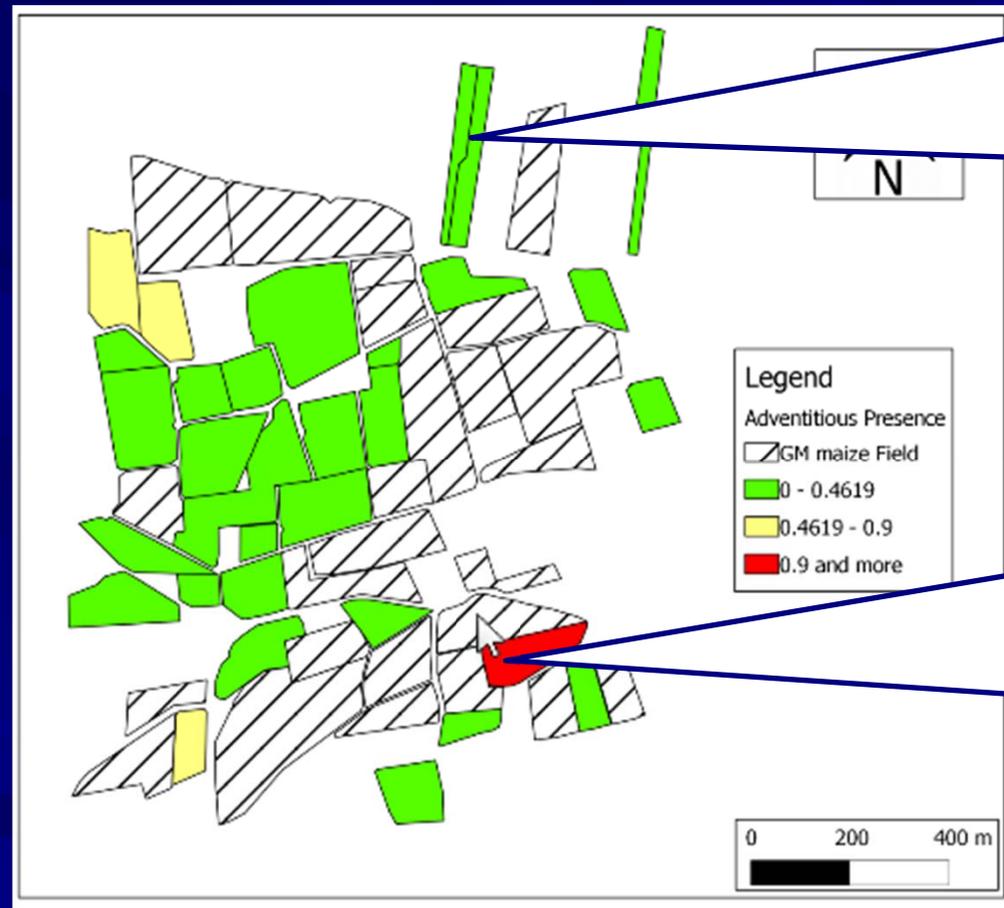
Post-market environmental monitoring plans

- Differentiation between specific and general surveillance plans

- GS:
  - What to observe?
  - How long?
  - Observation spatial scale?
  - Sampling issues…

  See for instance: mirids in China on Bt cotton and fruit trees

EFSA PMEM guidelines moved from "without a priori" (2006) to experimental fields (2011)

Resistance Ratios of Various Bollworm Populations to Bt Proteins
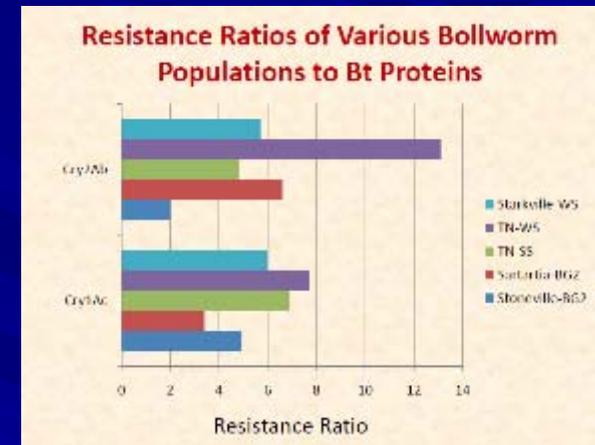
Conclusion

- Truly consensus sampling plans still missing,
- EU regulation covering only a part of laboratories' and stakeholders' needs: gaps in regulation and techniques
- Beside some "ideal" situations (quantification of approved GMOs), there are still numerous decisions to take in uncertain environments

DSS (Decision Support Systems) shall be generalized e.g. for

- Monitoring supply chains
- deciphering data, reporting and taking harmonized relevant decisions (labs and Competent Authorities) after taking into account information of doc traceability

Conclusion

- Numerous sampling issues still to be solved from fields to shelves,
- Costs are a limiting factor of traceability and coexistence
- Sample preparation and measurement uncertainty impact sampling and coexistence issues
- Software and DSS may help operators,
- Stakeholder's pragmatic practical threshold below the labelling threshold: 0.1 to 0.01% (effect on crops' coexistence, seeds' threshold, etc.)
- Genes dispersal – and thus coexistence - is more complex than expected
- Certified (and renewed) seeds *vs.* peasants seeds and participative breeding

Conclusion

- GM animals (see AquaBounty GM salmon) and their dispersal / growth conditions

- Non food / non feed GMOs…

- Expertise crisis: all experts?

Recurrent issues

- Huge difference between supply chains and fields coexistence: mix or not of GM and non-GM products
- Long distance pollen flows, e.g. corn (Hofman et al. 2014; Brunet et al. 2013, Folloni et al. 2012; Bannert & Stamp 2007…), see seeds production with "pollen clouds" and isolated DPA, domino effect of GM vs. non-GM fields… atmospheric and insect mediated…
- Sampling efficiency, related uncertainty *versus* cost-effectiveness and practicability, sampling uncertainty recognized as being largely superior to measurement uncertainty (ca 100 fold?)
- Quantification unit, measurement uncertainty and stacked genes, management of uncertainty whatever the measurement unit (e.g. AQL and LQL for kernels factor >2 for homogeneous distributions, DNA factor >2)
- Stakeholders' *versus* legal *versus* GMO free thresholds: coexistence organization *versus* proportionality… Land sharing *versus* land sparing
- Restricted cultivation areas when wild relatives present (e.g. sugar beet)
- Crop by crop field studies and simulations: currently corn (ca 10 years on this case study), for only small fields within a few landscapes, non standardized methods, need for accumulating new data, missing data on weather… to be integrated for DSS implementation
- Availability of cultivation register and LPIS in the EU, particularly for countries where transparency is rather low
- Alternate sources of gene flows: ferals and volunteers (harbor; transportation infrastructures; climate change), seeds' banks over several years…

Recurrent issues

- Physical and biological containment tools, industrial rights' issues with corn's CMS, yield and economic issues of different sowing dates, development of new cultivars? Autogamy rate, e.g. rice >95% but LL rice issue…
- DSS and simulation's training, calculation and distribution infrastructures (public or private servers and personnel, costs)… Targeted farmers (poor or with connected material)?
- Very complex situations supported by data (missing) and DSS + probabilities: insurance for farmers, liability for predictions, rather simple solution such as land sharing?
- Legal issues for compliance with good practices (see US cases) and controls?
- Representativeness of 4 countries cost/benefit analyses (current EU MS study)?
  - Germany: GMO-free DPA and Spain without coexistence compliance issues except companies' recommendations, Germany without GM potato or UK without oilseed rape or sugar beet
  - legal *versus* stakeholders threshold…
  - HR weeds management and new herbicides
  - Who will bear the costs of coexistence? Compensation claims: funds and insurances (if available)
- LLP and UGM: already well organized corn and soybean supply chains, LLP threshold and stakeholders' threshold (CRM, uncertainty…)
- Surveillance (specific and general):
  - observation networks mobilization and coordination
  - Inherent costs supported by who?
  - what is and how establish a baseline with which bioindicator (see issues in biodiversity studies),
  - How to define "focal species" , particularly for NTO, as GMO traits and growth conditions greatly differ?
  - Data transparency in several MS…
  - Long term and long distance to be studied by ecologists instead of agronomists ?
  - Needs for global biodiversity monitoring without a priori on long term and long distance (cf. China)