Joint Research Centre

the European Commission's in-house science service

Serving society
Stimulating innovation
Supporting legislation

Method of analysis towards feed safety

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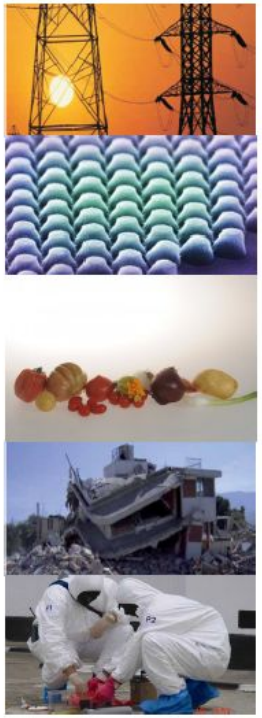
European Commission's Joint Research Centre: Role Facts & Figures

- **In-house** science service of the European Commission
- Independent, evidence-based **scientific and technical support** for many EU policies
- **Established 1957**
- **7 institutes** in 6 locations (Italy, Belgium, The Netherlands, Germany, Spain)
- **Around 3000 staff**, including PhDs and visiting scientists
- **1370 publications** in 2014

JRC's Support to EU policies and societal challenges - examples



- EU's electricity grids; shale gas; biofuels
- Reducing experiments on animals; nanomaterials
- Food and feed safety & quality; GMOs; food security
- Responding to crises; Financial stability
- Nuclear safeguards; Nuclear safety and security

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Activities of JRC in feed safety and quality

- Main activities started about 20 years ago
- Development and validation of methods focusing on
  - Meat and bone meal and processed animal proteins
  - Dioxins
  - Banned antibiotics and growth promotors
- Hosting various European Union Reference Laboratories
  - GMO (Ispra, Italy)
  - Mycotoxins and heavy metals (both Geel, Belgium)
  - *Feed additives (Geel, Belgium)*
- Participation in various European projects

Content of the talk

- Feed safety strategy and analytical methods
- Examples for screening methods and link to confirmatory methods
- Analytical methods within an regulatory environment
- Basic ideas of method validation

Purpose of feed analysis

Feed mill:

- Control of raw materials
- Control of finished products

Official control

- Compliance check with legal limits
- Correct labelling
- Target: Contaminants and key compounds

Example: *Screening methods* for mycotoxins

2015 survey: Mycotoxin threats remain high

11 Mar 2016 3395

Mycotoxin-related threats to livestock production are severe or high in 60% of regions worldwide according to the latest Biomin Mycotoxin Survey.

The survey covered 8271 agricultural commodity samples from 75 countries worldwide in 2015. Over 31,492 analyses have been conducted to identify the presence of six mycotoxins worldwide and their potential risk to livestock animal production. This is more than the **26,200 analyses, done in 2014**. For

home compound feed

EU feed alerts: Trends in 2015

22 Mar 2016 1183

In 2015, a total of 3049 original notifications were transmitted through the RASFF. This is 3.4% less than the 3097 notifications in 2014 but a 14.6% increase in follow-up notifications.

RASFF notifications report on risks identified in food, feed or food contac materials that are placed on the market in the notifying country or detained at an EU point of entry at th border with an EU neighbouring country. In the annual report, the identified risks, the product and its traceability and the measures RASFF has taken are published.

The most notifications in 2014 were done in the hazard categories pathogenic micro-organisms (745), mycotoxins (495), pesticide residues (405) and heavy metals (219). Mycotoxins (mainly **aflatoxins**) were primarily detected in nuts, nut products and seeds coming from China, Iran, Turkey, US, Netherlands, Germany,

Source: http://www.allaboutfeed.net/

Strong need for

- More samples to get analysed. But how to do it in a proper way?
- *Screening methods:* Rapid and simple methods that can be preferably applied on-site
- *Confirmatory methods:* Only suspicious samples measured by more complicated methods

Types of methods: Screening versus confirmatory methods

	Screening	Confirmatory
Example	Dipstick	LC-MS
Costs	Low	High
Required experience of technician	Low	High
Output	High	Low
Specificity	Low	High

LC-MS: Liquid chromatography coupled to mass spectrometry

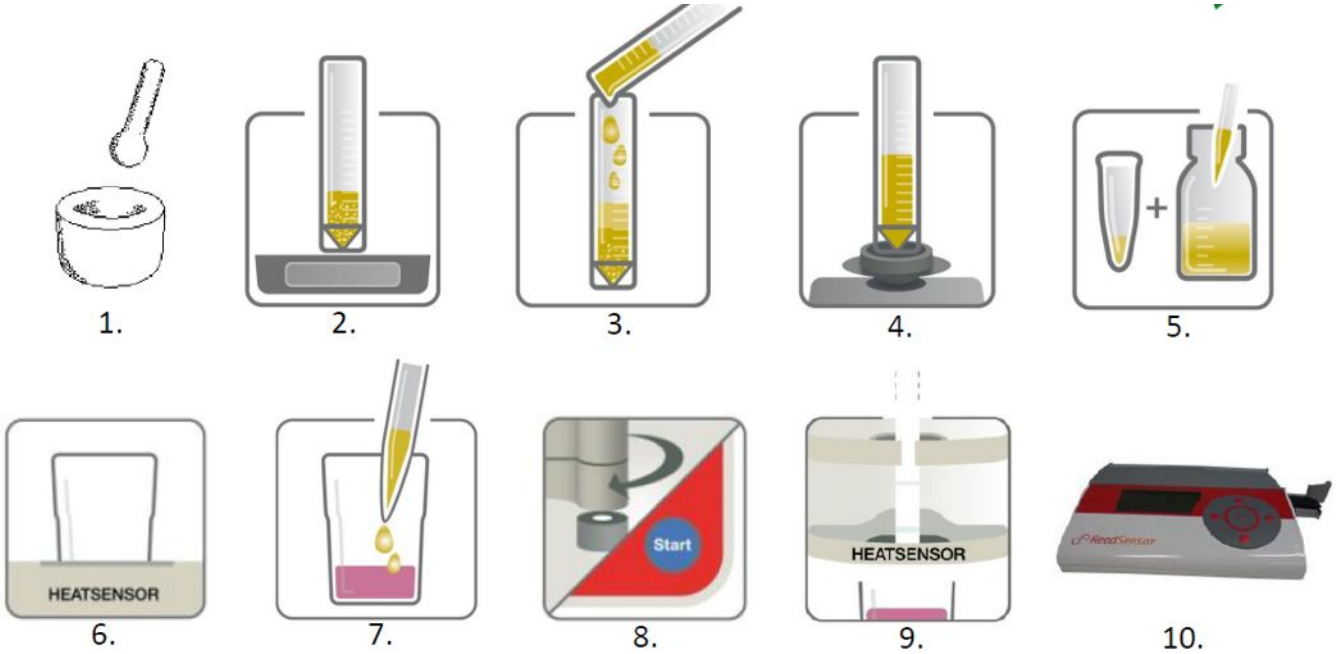
Use of screening method: Embedded in a feed safety strategy

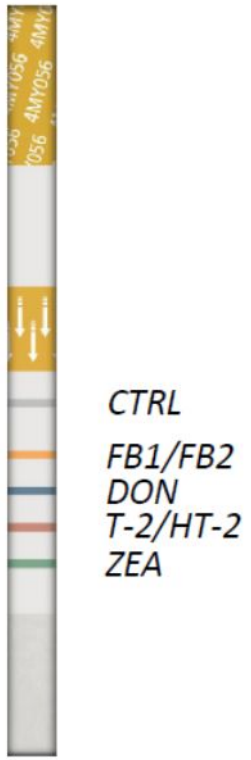
Principle of the strategy:

- Field of application: Many of the samples are compliant, but some of them have contaminants above the legal limit (e.g. 100 out of 1000)
- Many samples are analysed by *screening methods*, but only suspicious samples with the *confirmatory met*hods
- Negative samples measured by the screening method are classified as such and not further analysed.
- The screening method is designed, assuring that majority (e.g 95 %) of samples with the analytes at and above the target level need to be classified as positive. Rate of false negative results is low.
- Positive samples need to be re-analysed using confirmatory methods

Screening methods: Example 1: Dipstick

The 4 mycosensor test





Developed by ©Unisensor within the European FP 7 Project conffidence

Lattanzio V.M. T, von Holst C., Visconti A.: Experimental design for in-house validation of a screening immunoassay kit. The case of a multiplex dipstick for Fusarium mycotoxins in cereals. Anal Bioanal Chem (2013) 405:7773–7782

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How do we ensure that the test does what we expect? First step to validation

- The test of an unknown sample delivers a response from the reader for each sample
- The technician compares the value against a reference value (=cut-off value) established by validation experiments
- Based on this comparison the sample is considered as negative or positive
- Some statistics is required
- At the end we decide, whether the test is fit for purpose
- A simple validation scheme has been included in European Regulation, which is shown here (Regulation 519/2014/EU)

Design of experiments

Experiments with the samples containing the analyte at the target limit to calculate cut-off value

- False negative results fixed at 5% prior to the experiments
- Mean response from the experiments
- Precision data
- t-statistics to set cut-off value

Experiments with samples containing the anlayte below the legal limit are performed to estimate rate of false positive results

- Mean response from the experiments
- Precision data
- t-statistics to calculate rate of false positive results

Target analytes matrices and concentration included in the validation study. In addition blank samples were included

	% of Target level	Spiking level (µg/kg)			
		ZEA	**T-2+HT-2**	**DON**	**FB_1+FB_2**
Wheat	25%	25	125	437	-
	50%	50	250	825	-
	100%	100	500	1750	-
Maize	25%	87	125	437	1000
	50%	175	250	825	2000
	100%	350	500	1750	4000

How to establish the cut-off value?

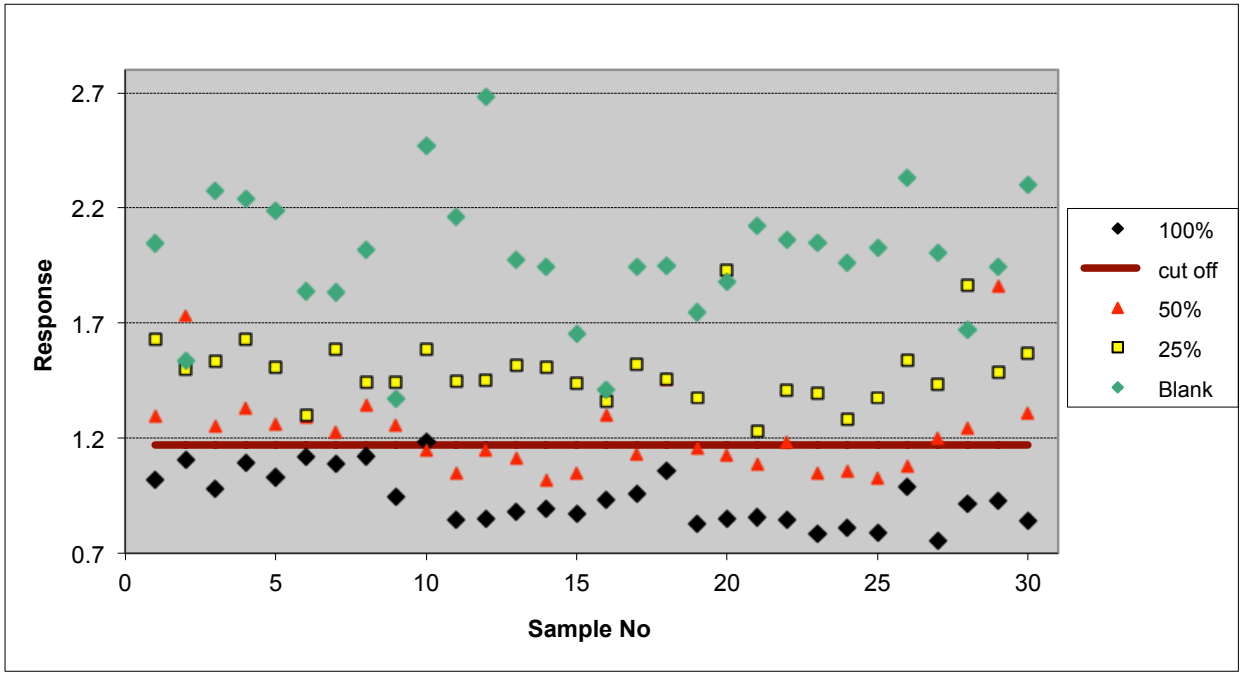
1. To replicate analyses (e.g. 20)
2. Calculate of the mean of the results from the experiments of the samples with the analyte at target level
3. Use total standard deviation from the precision experiments
4. Use one-sided t-value (β = 5 %) from a statistical table
5. Calculate cut-off value as follows:

Cut off value = mean + t-value (β=0.05)*total standard deviation

Example: Zearelenon in maize

Characteristic of this test:

- Low response corresponds to a high concentration
- High response corresponds to a low concentration



- Based on cut-values the rate of false positive samples is estimated (also by t-statistics)
- Rate of false positive:
  - Samples with 50 % of target level: 40 %
  - Samples with 25 % of target level: **2.2 %**
  - Blank samples: **0.6 %**

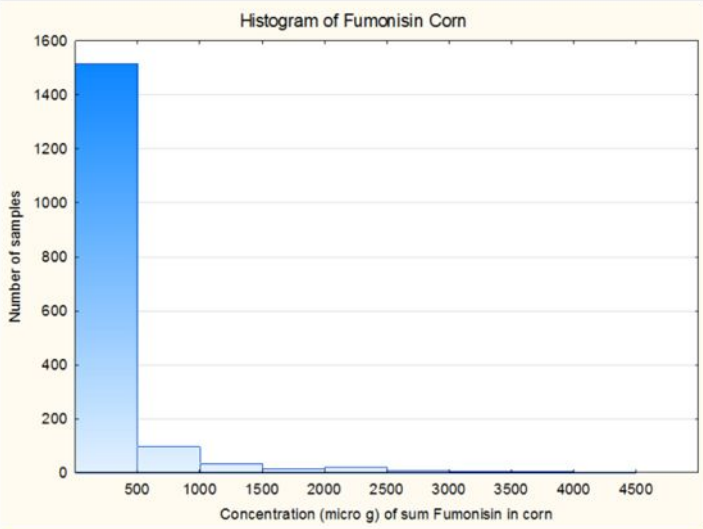
A word in respect to rate of false positive results

- False positive results indicate the application of confirmatory methods of actually compliant samples: Actually superflous!
- They do not impair safety, but increase costs!
- They may look high
- They may not be critical, even if they look high

What do I need to know in order to establish, whether a specific test is fit for purpose?

- A rough idea about frequency distribution of the target analyte in typical samples
- The performance profile including the cut-off value of the test
- The cost per analysis of the screening test compared to the confirmatory method

Mycotoxin concentrations in the target matrices – European data.



Rate of false positive results, based on frequency distribution, *Fumonisins*

% of MRL with validation data	Range % of target level	Range mg/kg	No of Sample	Rate of false posifive	Number false
0	0-12	0-480	1486	0.058	86
25	13-37	481-1480	153	0.12	18
50	38-62	1481-2480	36	0.64	23
100	62-100	2481-4000	19	0.95	18
		Total No of samples:	1694	Total No of false positive results	146
				Total Ratio of false positive results (%)	**8.60**

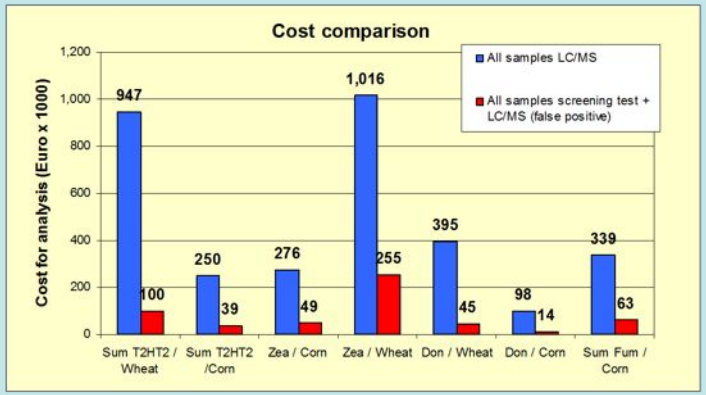
Cost estimation I

- Estimating total analytical costs for two options:
  - Analysing all samples with LC/MS
  - Analysing all samples with the screening test and all false positive additionally with LC/MC

LC MS (Euro) Screening (Euro)		200 20
Total cost (LC/MS)	1694*200	338800
Total (Screening + LC/MS false positive)	(1694 X 20) + (146 X 200)	63008

Cost estimation II

- LC/MS measurement: 200 Euro/sample
- Screening test: 20 Euro/sample



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Example 2: Multianalyte method with high resolution mass spectrometry

LC-Exactive Orbitrap MS (HRMS)


Journal of Chromatography A, 1322 (2013) 38–48

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Multi-residue method for the detection of veterinary drugs in distillers grains by liquid chromatography–Orbitrap high resolution mass spectrometry

George Kaklamanos, Ursula Vincent*, Christoph von Holst

Interesting aspect: The method has potential for screening and confirmatory purposes

Development and single-lab validation:

- **100 Vet. Drugs in distiller grains**
- **Melamine and cyanuric acid in soya bean meal**

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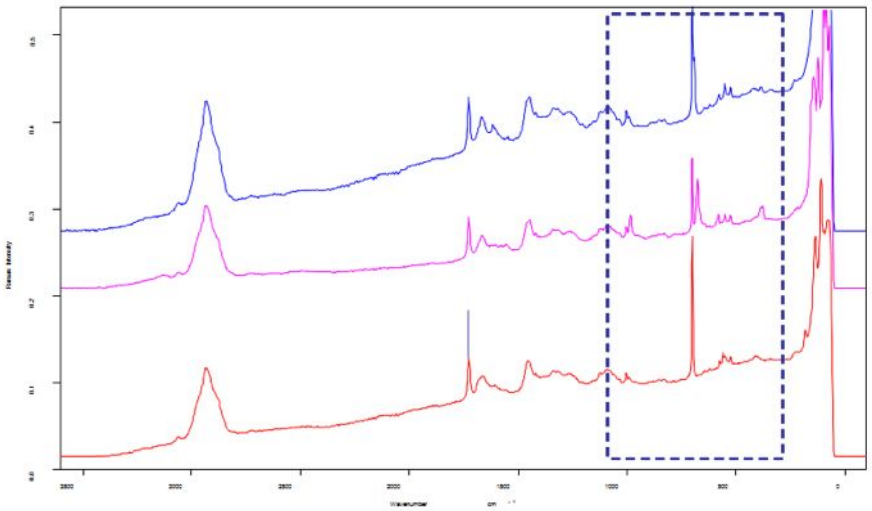
Example 3: Spectroscopy (I)

- Near Infrared (NIR) Spectroscopy, already well established in feed industry, e.g quantification of crude protein
- NIR microsopcy: At the beginning: Identification of bone particles, later contaminants, characterisation of feed additives
- Raman spectroscopy: Recent research show use for detection of melamine or transformer oil adulteration
- Major advantage: Non-destructive methods

Example 3: Spectroscopy (II)

- Application for detection of malamine in soybean
- Possible with Raman and Near Infrared spectroscopy (NIR)



Raman shift of soybean samples containing melamine: Specific peaks showing the presence of melamine

EU FP7 competitive project "Quality and Safety of Feeds and Foods for Europe"

- Also NIR spectra from melamine, soybean and melamine are different
- Application of NIR camera for the detection of melamine*
- Objective identification requires the use of chemometrics
- Decision models are established on spectra of known substances and then applied to new samples

**Fernandez Pierna et al. Line scan hyperspectral imaging spectroscopy for the early detection of melamine and cyanuric acid in feed. J. Near Infrared Spectrosc. 22, 103–112 (2014)*

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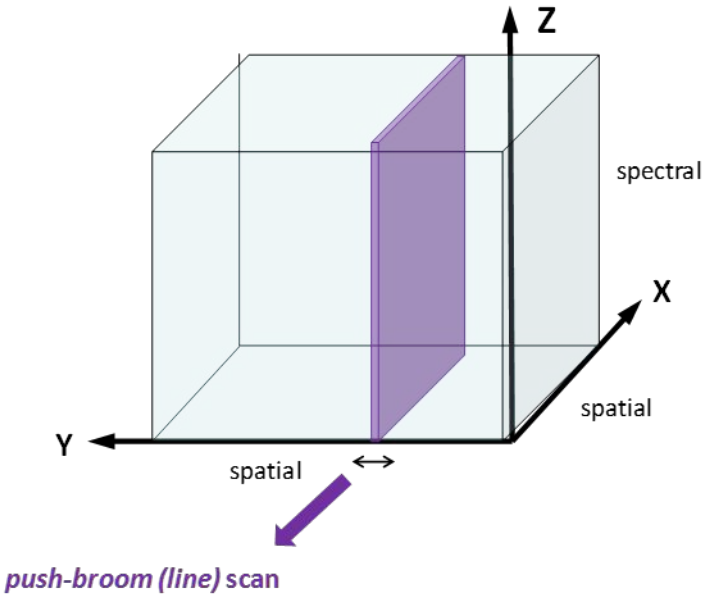
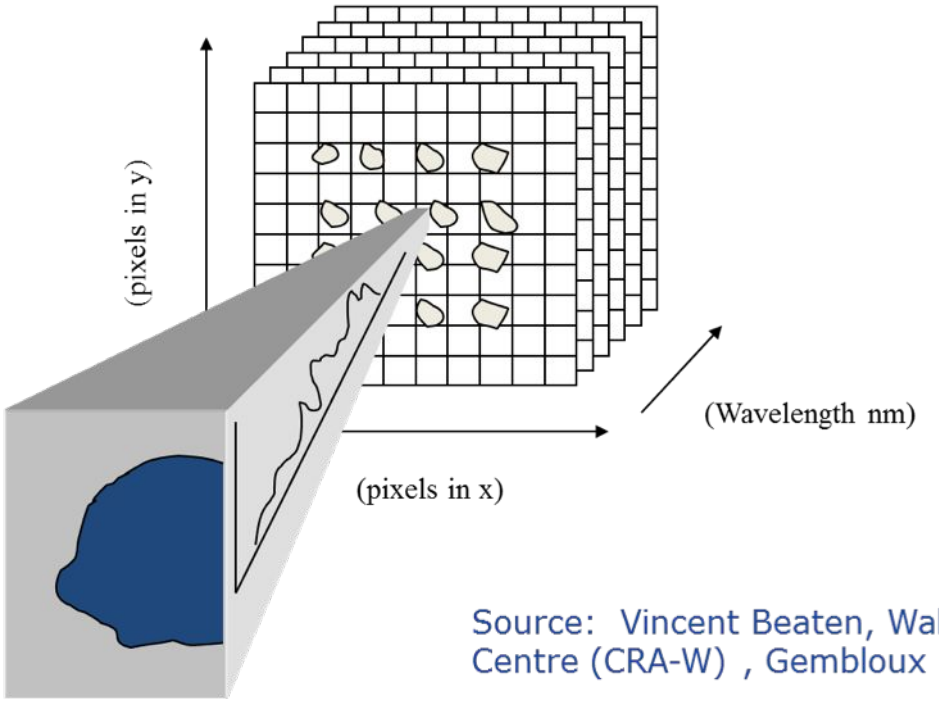
Example 3: Spectroscopy (III), near infrared camera



Source: Vincent Beaten, Walloon Agricultural Research Centre (CRA-W) , Gembloux (Belgium)

Example 3: Spectroscopy (IV), near infrared camera

Datacube (or **Hypercube**) is a matrix of **I x J** pixels and **K** wavelengths



Source: Vincent Beaten, Walloon Agricultural Research Centre (CRA-W) , Gembloux (Belgium)

pixel = spectrum

- Spatial resolution allows the detection of melamine present in the sample at least above 0.5%
- Automatic operation and identification of melamine particles

Confirmatory method: complimentary to screening methods

- Target of screening methods: False negative as low as possible
- Target of confirmatory methods: False positive as possible
- Screening by various spectroscopic methods including NIR and Raman
- High specificity, e.g. via mass spectrometry

Jacob de Jong et al.: Analytical strategies for the early quality and safety assurance in the global feed chain… Trends in Analytical Chemistry 76 (2016) 203–215

Example regulatory frame: Authorisation of feed additives in the European Union and the European Union Reference Laboratory (EURL)

Feed Additives Categories

REG(EC) No 1831/2003

1. Technological

- Preservatives
- Antioxidants
- Emulsifiers
- Stabilisers
- Silage additives

…

- Mycotoxins binders

2. Sensory

- Colourants
- Flavouring compounds

3. Nutritional

- Vitamins
- Trace elements
- Amino acids
- Urea & derivatives

4. Zootechnical

- Digestibility enhancers
- Gut flora stabilisers
- Sustances which favourably effect the environment

5. Coccidiostats

EURL contribution to the authorisation:

Are the applicants' analytical methods suitable to be used for official control ????

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Authorisation regulation: Example

Identification number of the additive	Name of the holder of authorisation	Additive (Trade name)	Composition, chemical formula, description, analytical method	Species or category of animal	Maximum age	Minimum content	Maximum content	Other provisions	End of period of authorisation	Maximum Residue Limits (MRLs) in the relevant foodstuffs of animal origin
						mg of active substance/kg of complete feedingstuff with a moisture content of 12 %				
Coccidiostats and histomonostats										
5 1 771	Janssen Pharmaceutica N.V.	Diclazuril 0,5 g/100 g (Clinacox 0,5 %)	*Additive composition* Diclazuril: 0,50 g/100 g. Protein-poor soybean meal: 99,25 g/100 g Polyvidone K 30: 0,20 g/100 g Sodium hydroxide: 0,05 g/100 g *Characterisation of the active substance* Diclazuril, $C_{17}H_9Cl_3N_4O_2$, (±)-4-chlorophenyl[2,6-dichloro4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl)phenyl]acetonitrile, CAS number: 101831-37-2 Related impurities: Degradation compound (R064318): ≤ 0,1 % Other related impurities (T001434, R066891, R068610, R070156, R070016): ≤ 0,5 % individually Total impurities: ≤ 1,5 % *Analytical method* (1) For determination of diclazuril in feed: reversed-phase high performance liquid chromatography (HPLC) using Ultraviolet detection at 280 nm (Regulation (EC) No 152/2009) For determination of diclazuril in poultry tissues: HPLC coupled to triple quadrupole mass spectrometer (MS/MS) using one precursor ion and two product ions	Guinea fowls	—	1	1	1. The additive shall be incorporated in compound feed in form of a premixture. 2. Diclazuril shall not be mixed with other coccidiostats. 3. For safety: breathing protection, glasses and gloves shall be used during handling. 4. The holder of the authorisation shall carry out a post-market monitoring programme on the resistance to bacteria and *Eimeria* spp.	16 March 2021	1 500 µg diclazuril/kg of wet liver 1 000 µg diclazuril/kg of wet kidney 500 µg diclazuril/kg of wet muscle 500 µg diclazuril/kg of wet skin/fat

(1) Details of the analytical methods are available at the following address of the Community Reference Laboratory: www.irmm.jrc.be/crl-feed-additives

Key data of the EURL regarding methods

- Legal basis: Regulation (EC) No 1831/2003
- Since 2004 the EURL evaluated several hundred dossiers
- EURL reports public domain

European Union Reference Laboratories

EURL feed additives

Authorisation
- Guidance for applicants
- Network laboratories
- Evaluation reports

Official controls
Publications
Contacts

EURL heavy metals

EURL mycotoxins

EURL Polycyclic Aromatic Hydrocarbons

EURL Food Contact Materials

EURL GM Food and Feed*

EURL for alternatives to animal testing*

EURL for feed additives

Evaluation reports

Search help
Use the 'Free text search' field to narrow your seach using 'Product' or 'Active Substances'. You can use the 'FAD Number' field in a search using e.g. syntax FAD-XXX or FAD-XXXX-XXXX. You can also seach by Year 'From' and 'To', or simply use the pagination function.

FAD Number	Free text search	from	to
	xylanase	-Year	-Year

Search | Reset

1-20 out of 36 results — 1 | 2 next › last »

FAD Number	Product	Active Substance(s)	Date of Report
FAD-2010-0213	Feedlyve® AXC	Endo 1,4-β-xylanase	17/12/2015
FAD-2010-0367	Enzymes as silage additives	Alpha-amylase (EC 3.2.1.1), Endo-1,4-beta-glucanase (EC 3.2.1.4), Endo-1,3(4)-beta-glucanase (EC 3.2.1.6), Endo-1,4-beta-xylanase (EC 3.2.1.8)	14/12/2015

https://ec.europa.eu/jrc/en/eurl/feed-additives/authorisation

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Information on methods recommended by the EURL (I)

- Reference to Community methods Regulation (EC) No 152/2009, e.g. Amino acids, trace elements, some coccidiostats
- ISO/CEN methods: E.g. Coccidiostats, Phytase activity
- Pharmacopoeias and food chemical codex: Methods for the characterisation of products
- **Methods proposed by the applicants**

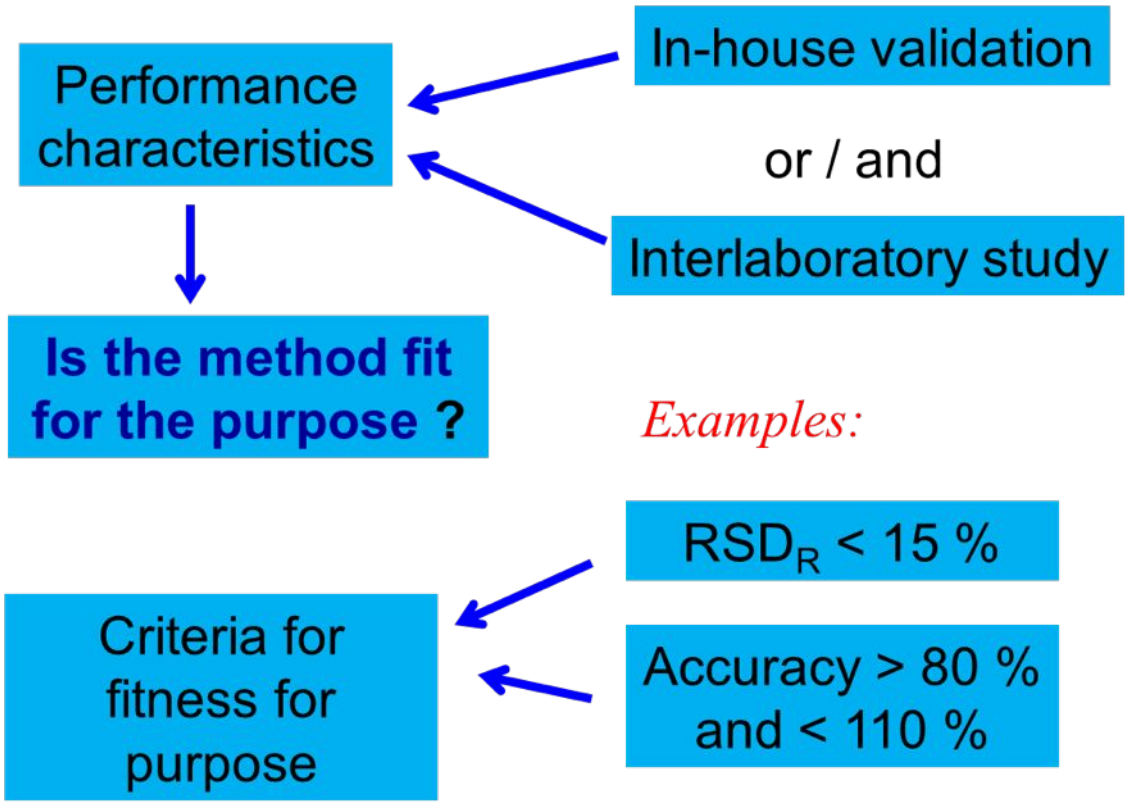
Information on methods recommended by the EURL (II)

- Examples of methods
  - Enzyme activity determination
  - Characterisation of products, e.g. technological feed additives
  - Mycotoxin binder: Protocol for adsorption experiment to check samples against target criterion
- Authorised feed additives
  - Methods recommended by EURL are public domain

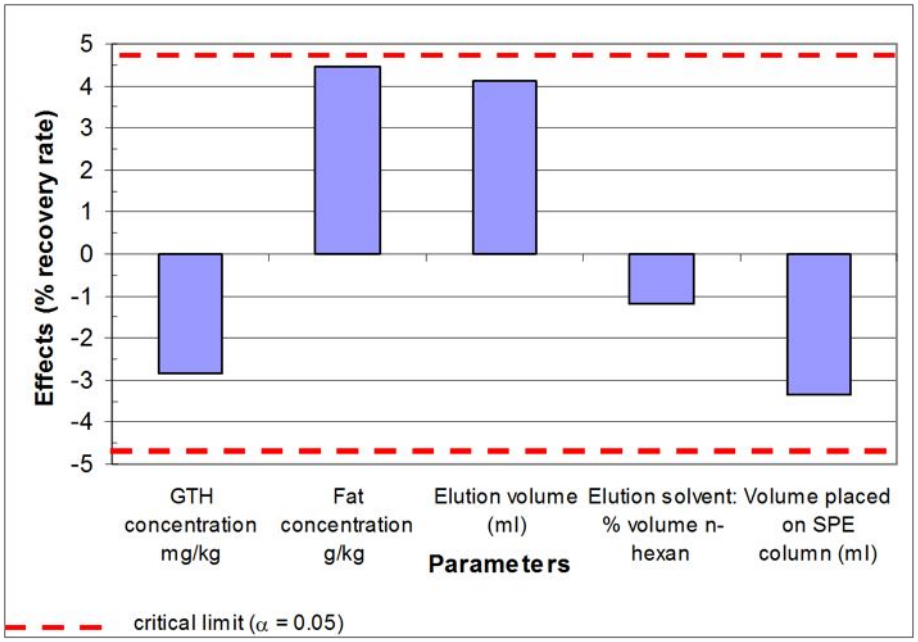
von Holst C., Robouch P., Bellorini S., Gonzálezde la Huebra M.S. & Ezerskis Z. (2016). A review of the work of the EU Reference Laboratory supporting the authorisation process of feed additives in the EU. Food Additives & Contaminants: Part A, 33:1, 66-77

The validation exercise (I):

Is the method is fit for purpose? = Check of method performance *characteristics* against performance *criteria*



The validation exercise II:



- In-house validation: Ruggedness test
- Second laboratory verification
- The gold standard: The interlaboratory study
- A method can only be validated if the purpose of its application has been established beforehand

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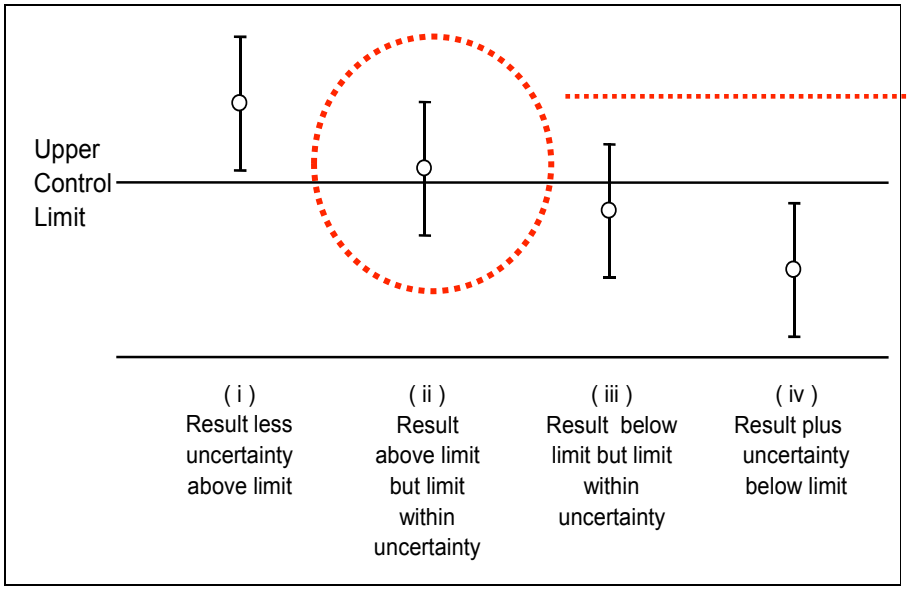
The validation exercise III: In-house validated methods versus standards

	In-house validated	Standard
Validation efforts	Always intermediate	Very high when establishing the standard, but much lower, when used by a lab afterwards
Information from validation	Fitness for purpose just in one lab	Fitness for purpose by group of laboratories
Use of new technology	Immediate	Only with significant delay
Field of application	EU: Residues and contaminants in food of animal origin	Feed analysis
Legal Reference	Commission Decision 657/2002	Regulation (EC) No 152/2009

Legal limits and measurement uncertainty: Beyond reasonable doubt



The analytical result is above the MRL, but the true value could be below this limit

- To take legal actions, the analytical result need to be beyond reasonable doubt above the legal limit
- The MU is subtracted from the analytical result, before establishing non-compliance
- Only case (i) proves non-compliance beyond reasonable doubt
- In the EU currently applied to undesirable substances

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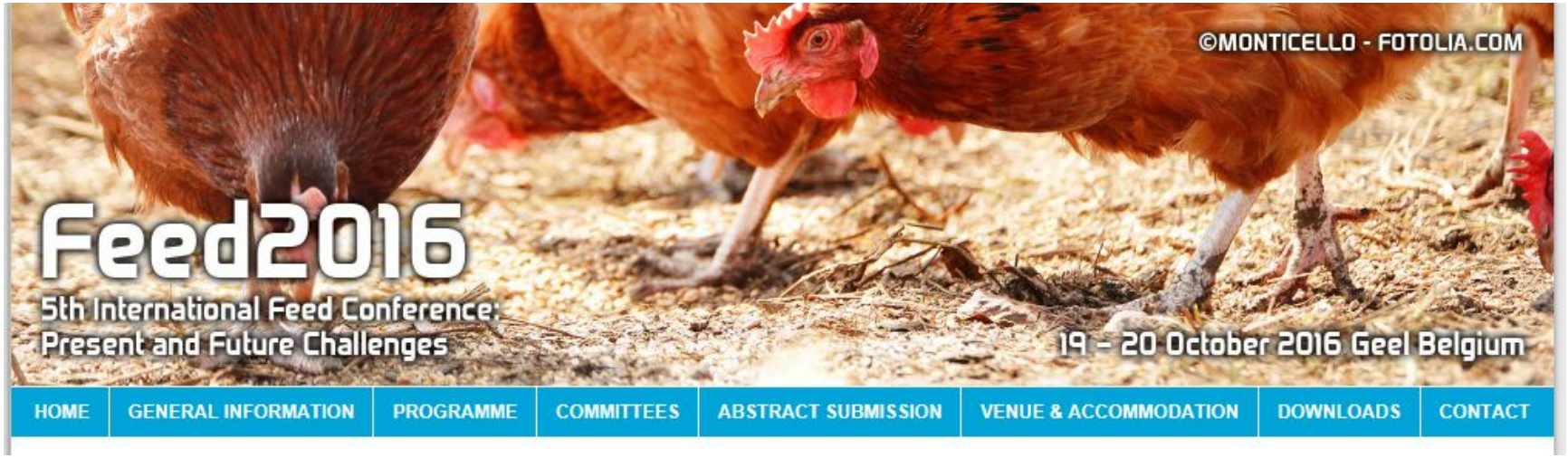
Summary

- The ever increasing number sample/analyte combinations required the smart combination of screening and confirmatory methods
- There is a rapid development of new technology, offering more efficient control of feed materials
- New validation guidelines are available, focusing on the proper use of screening methods
- Methods are getting public domain: for instance from the EURL

Thank you for your attention
…and may be we see us again in our institute
IRMM in Geel (Belgium)

Feed conference: 19th to 20th October 2016
www.feed2016.deu



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