

Lessons learned from the CONffIDENCE

project: Contaminants in food and feed – inexpensive detection for control of exposure

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- Results
 - ✓ Highlights
 - ✓ Inclusion of new requests from DG SANCO and EFSA
 - ✓ Validation and fitness for purpose of screening methods
- Conclusions



CONFIDENCE objectives

- To assure chemical safety and quality in the European food supply; support of EC policies and competitiveness of food and feed industries
 - ✓ Multi-detection: “multiplex”
 - ✓ Inexpensive screening techniques
- To speed-up analysis for factory approval of lots
- To contribute to the assessment of risks of emerging contaminants
 - ✓ e.g. shellfish toxins such as palytoxin and spirolides



CONFIDENCE passport

- FP7 Collaborative Project first call “Food, Agriculture & Fisheries, and Biotechnology”
- Duration: May 2008 – December 2012
- 16 partners from 10 countries, representing universities, research institutes, industry and SMEs
- Budget: 7.5 Mio €
- Coordinator: RIKILT - Institute of Food Safety, part of Wageningen UR (NL)



The commodities

Food

- ✓ Fish/shellfish
- ✓ Cereals
- ✓ Potatoes/vegetables
- ✓ Honey
- ✓ Eggs
- ✓ Meat
- ✓ Dairy products

&

Feed

Fish feed

Cereal-based feed

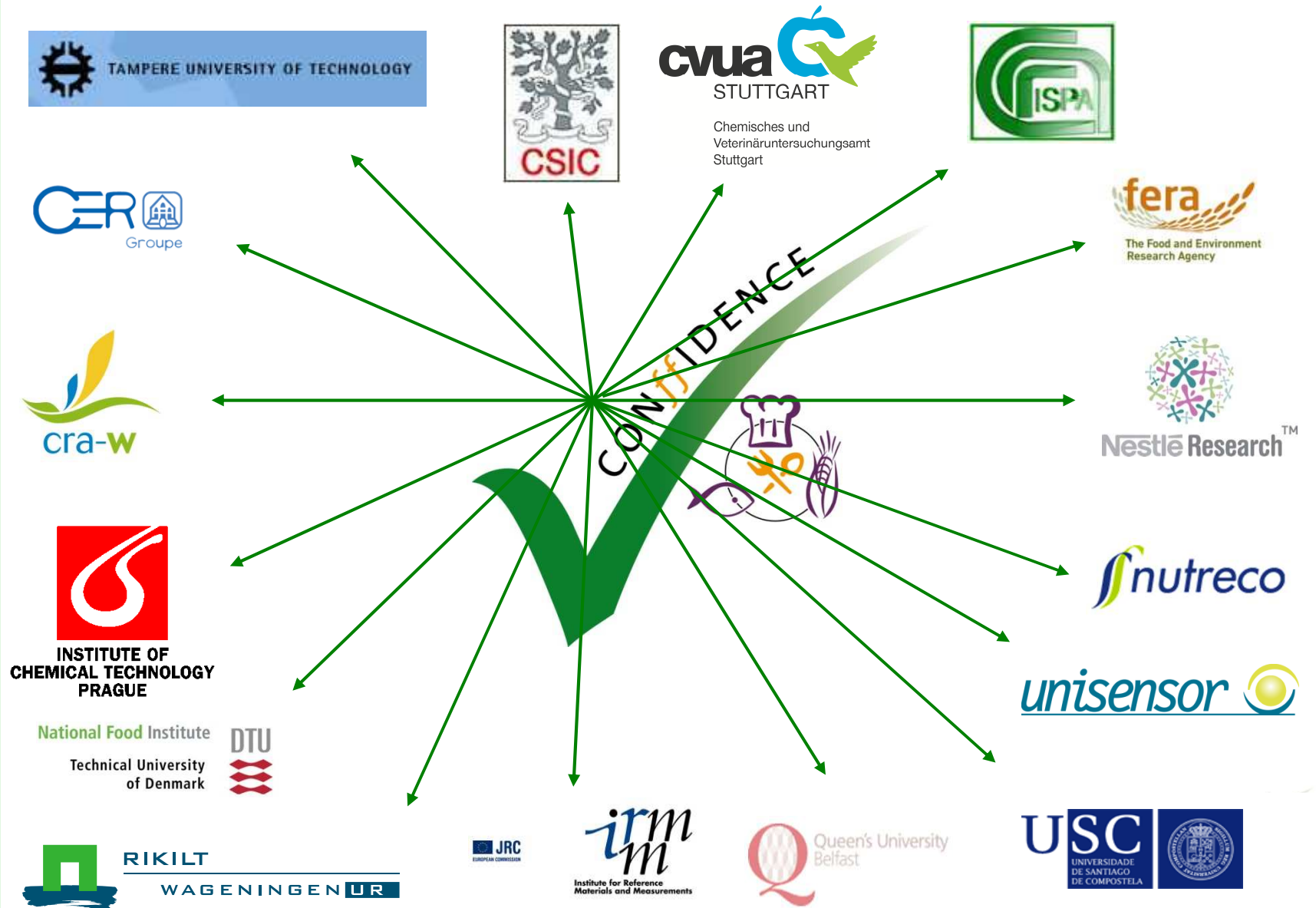


The target contaminants

- Organic pollutants
 - ✓ POPs (Persistent Organic Pollutants) + PAHs
 - ✓ Perfluorinated compounds
 - ✓ Pesticides
- Veterinary drugs
 - ✓ Antibiotics
 - ✓ Coccidiostats
- Heavy metals: speciation of arsenic and mercury
- Biotoxins:
 - ✓ Alkaloids
 - ✓ Marine biotoxins
 - ✓ Mycotoxins



The consortium



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CONFIDENCE Highlights (1)

➤ Biotoxins:

- ✓ Multiplex dipstick for mycotoxins in cereals
- ✓ Multiplex dipstick for ergot alkaloids in cereals and feed
- ✓ NIR Hyperspectral Imaging for ergot sclerotia in cereals
- ✓ Multiplex dipstick for tropane alkaloids in feed
- ✓ Multiplex ELISA for pyrrolizidine alkaloids in honey and feed
- ✓ Multiplex biosensor assay (SPR) for shellfish toxins

➤ Veterinary drugs:

- ✓ Multiplex dipstick for antibiotics in honey
- ✓ Multiplex flow cytometry for coccidiostats in feed and eggs



CONFIDENCE Highlights (2)

- Heavy metal speciation:
 - ✓ Inorganic arsenic in seafood and fish feed: SPE-AAS
 - ✓ Methylmercury in seafood and fish feed: LC-ICPMS
- Organic pollutants:
 - ✓ POPs and PAHs in seafood and fish feed: simplified integrated sample prep + GC-MS/MS or GCxGC-TOFMS
 - ✓ POPs and PAHs in fish: X-map technology
 - ✓ DESI and DART-MS of dithiocarbamates in vegetables
 - ✓ Multiplex electrochemical immunosensor for paraquat and DON in cereals
 - ✓ Perfluorinated compounds in fish and milk: Simplified sample prep and LC-MS/MS
- Cross-cutting surveys, e.g. multiple contaminants in seafood



Mycotoxins: Commodity dedicated **multiplex dipstick tests** for the determination of major *Fusarium* toxins



WHEAT



OAT



WHEAT BASED BREAKFAST CEREALS

Target toxins: **DON, ZEA, T-2** and **HT-2** toxins



MAIZE



MAIZE FEED



MAIZE BASED BREAKFAST CEREALS

Target toxins: **DON, ZEA, FB₁, FB₂, T-2** and **HT-2** toxins



Target levels: EU maximum permitted levels



The assay procedure



Methanol/water extraction

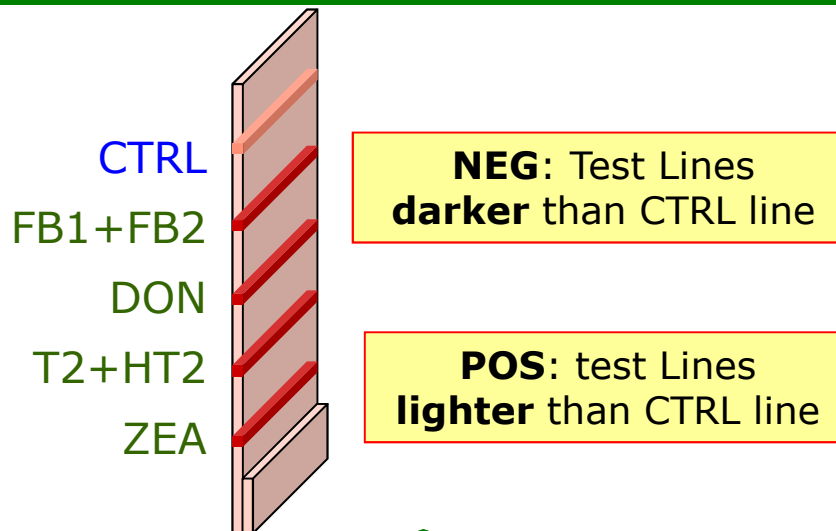


High speed blending

Dilution with buffer



Incubation at 40°C, 10 min
Migration, 10 min



Reading



Total analysis time: 30 min for 6 mycotoxins



The commercial kit



www.unisensor.be

MULTIPLEX: 6 mycotoxin analysed in 1 test

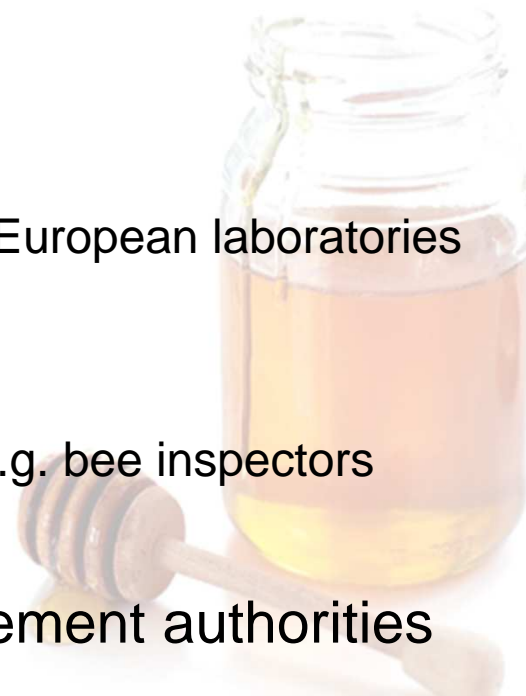
FAST: up to 8 samples in 1 hour (including sample preparation)

SENSITIVE: mycotoxin detection at levels close to EU regulatory limits

USER FRIENDLY: 5 min for sample preparation, easily performed on site

Bee4sensor for honey

- **Multisensor**: Unique **multiplex**, antibody based dipstick **assay**, for the screening of **sulphonamides**, **fluoroquinolones**, **tylosin-A**, and **chloramphenicol** in **honey**
- Laboratory method
 - ✓ Successful Inter-lab validation with 7 European laboratories
- Field-test method
 - ✓ Proof of principle
 - ✓ Global field trial with 16 participants e.g. bee inspectors
- Rapid test for industry and enforcement authorities



Multisensor – bee4sensor for honey

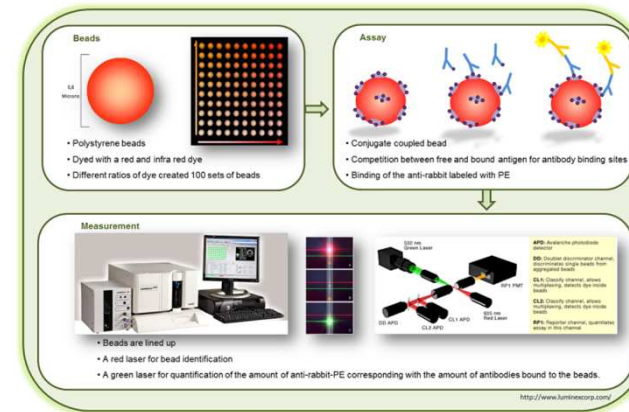
The test kit (bee4sensor) is already marketed by [unisensor](http://www.unisensor.be/en/catalog/antibiotics-28/bee4sensor-45.php)  and will be soon produced, based on customer demand:
<http://www.unisensor.be/en/catalog/antibiotics-28/bee4sensor-45.php>



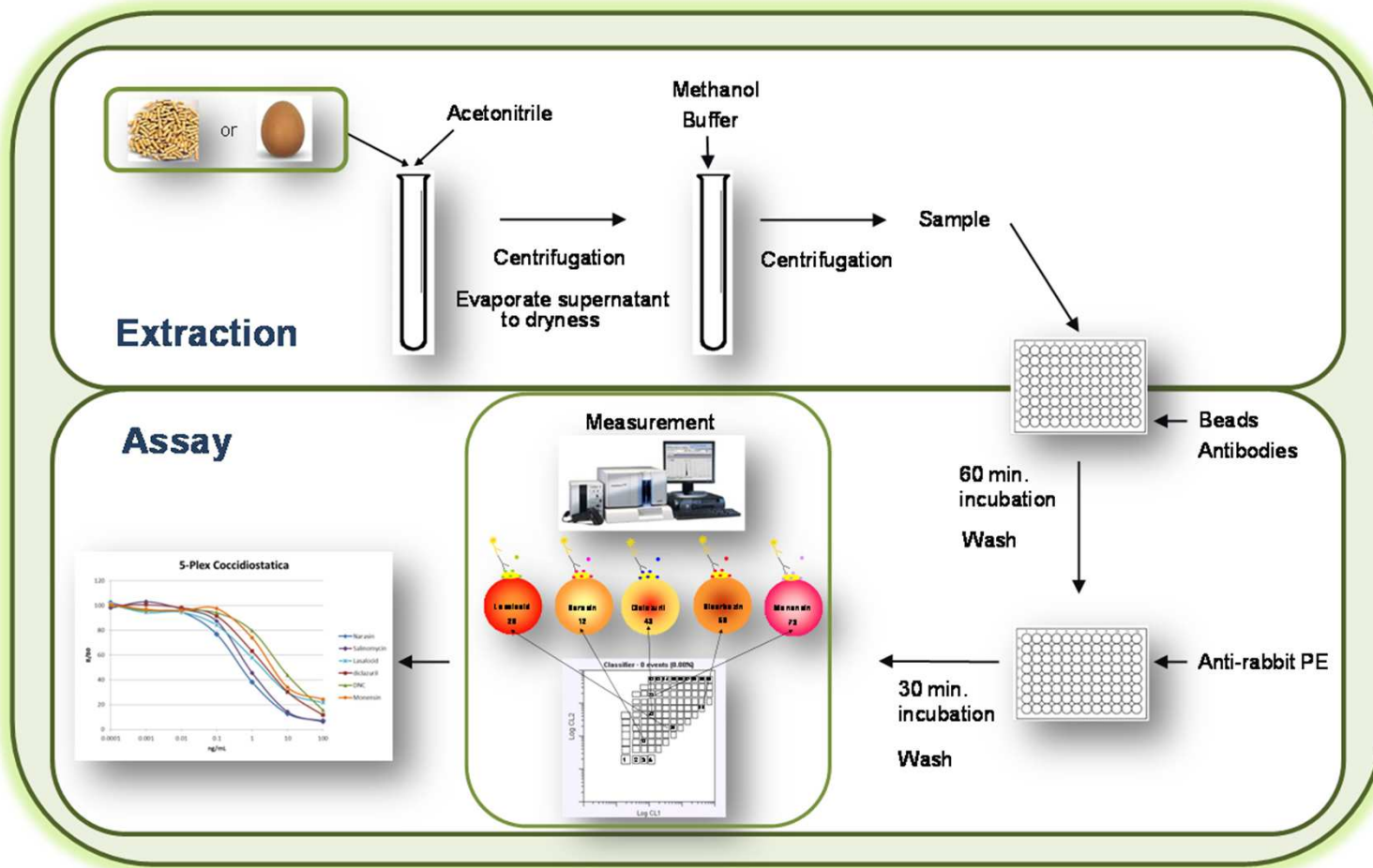
Coccidiostats in feed and eggs

- New **fast** and **inexpensive multiplex** method for the screening of:
 - ✓ Residues of coccidiostats in eggs (Regulation (EU) N° 610/2012)
 - ✓ Coccidiostats at cross-contamination levels in non-target feed (Regulation (EU) N° 574/2011)

- The Technology:
Flow cytometry based
multiplex immunoassay



The assay procedure



Generic extraction; 40 samples (240 analytes) per day in routine



Collaborative study - Overview



	Eggs	Feed
Narasin/Salinomycin	2.89	0.52
Lasalocid	0.17	2.75
Nicarbazin	0.35	10.47
Diclazuril	9.14	93.40
Monensin	2.42	1.65

Rate of false positives in the blank in %

Established at 95% confidence level (maximum rate of false negatives is 5%)



NIR imaging method for ergot sclerotia

- NIR hyperspectral imaging method to detect and quantify ergot bodies in cereals at levels below regulatory limits
- Full conveyer belt system with belt speed of 100 mm/s allows analysis of up to 100 kg grain/hour



Conveyor belt



NIR line scan imaging system

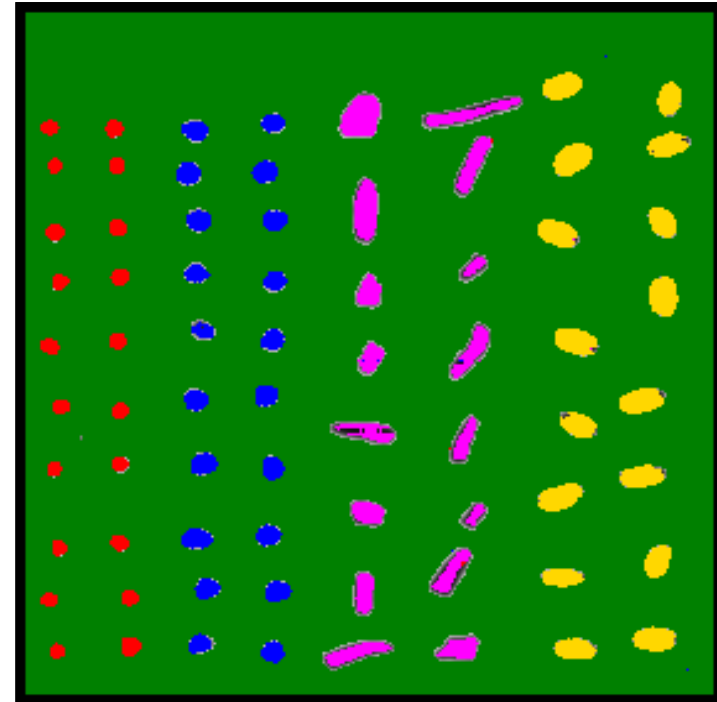


Test system in operation at Nutreco



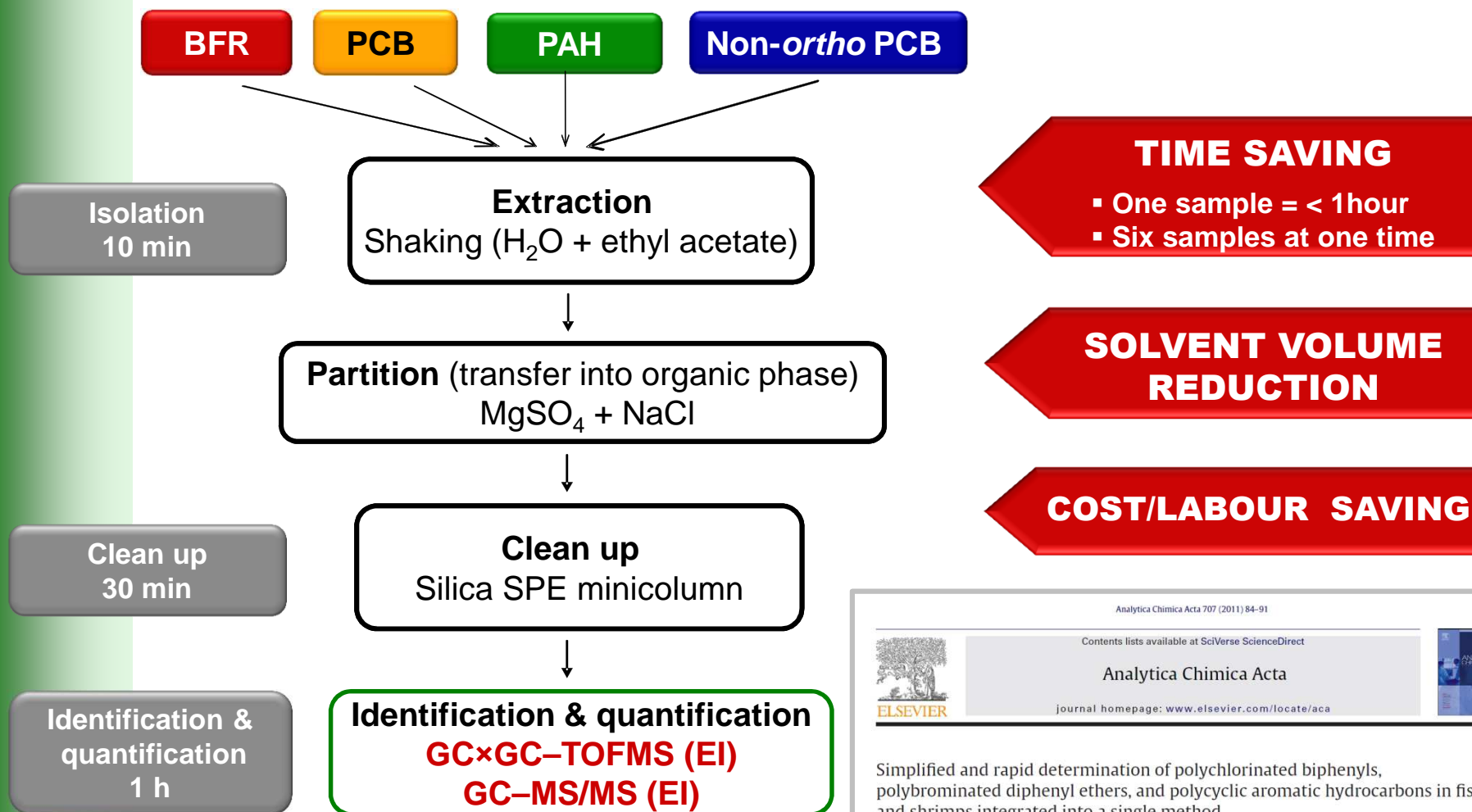
Further developments

- Multicontaminants detection: ergot, Datura, ...

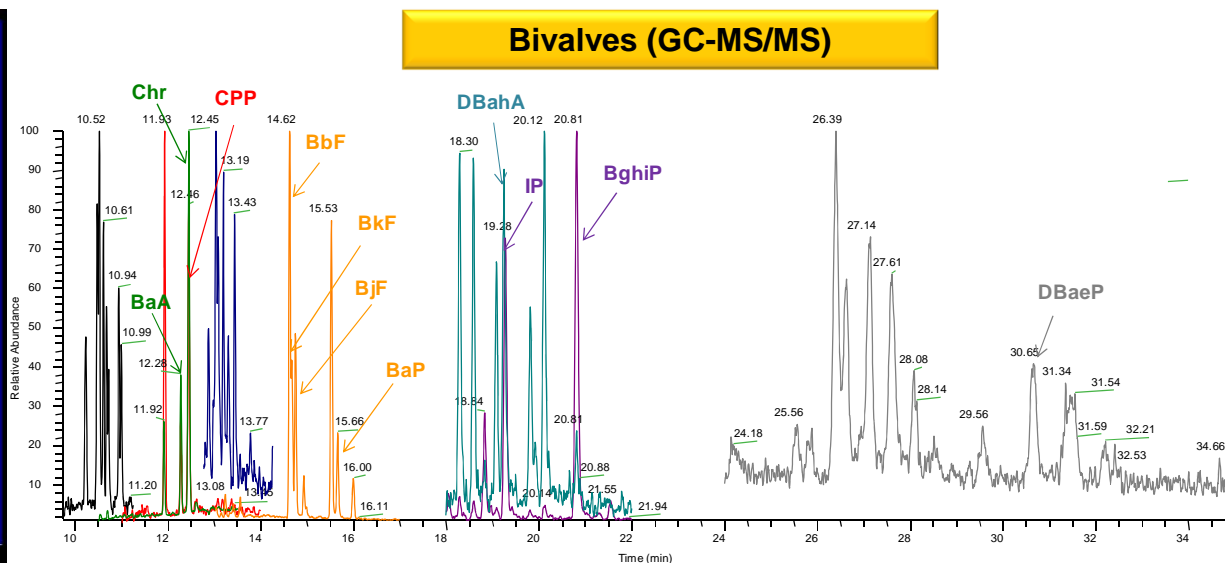
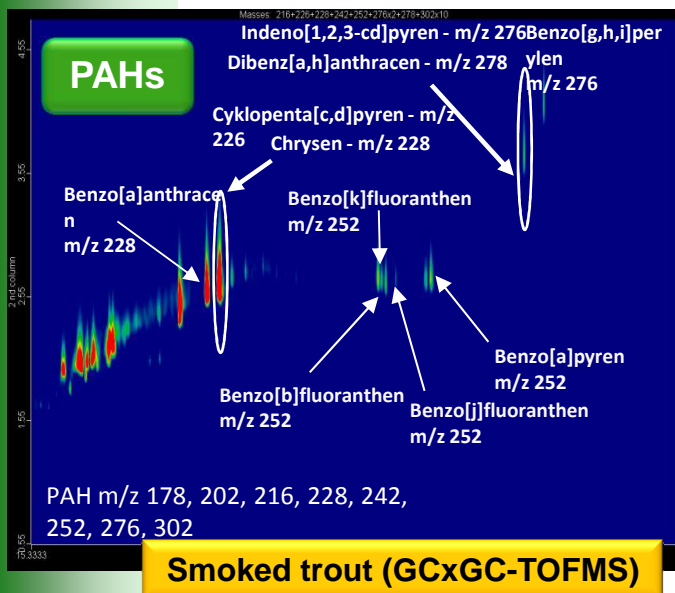


2 lines of rapeseed, Datura seeds, ergot sclerotia and wheat kernels, respectively

Integrated sample prep for POPs/PAHs



GC-MS methods for POPs



Parameter / feature	GCxGC-TOFMS	GC-MS/MS
Chromatographic resolution	+++	+
Selectivity of detection	++ (deconvolution)	++ (products ions)
Detection limits	+	+++
Data handling (time demands)	-	+
Retrospective data mining	+	-
Availability in common control labs	-	++

Anal Bioanal Chem (2012) 403:2813–2824
DOI 10.1007/s00216-012-6095-3

ORIGINAL PAPER

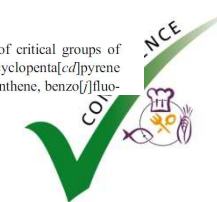
Implementation of comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry for the simultaneous determination of halogenated contaminants and polycyclic aromatic hydrocarbons in fish

Kamila Kalachova · Jana Pulkrabova · Tomas Cajka · Lucie Drabova · Jana Hajslova

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Abstract In the presented study, comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC–TOFMS) was shown to be a

separation of all target analytes even of critical groups of PAHs (group (a): benzo[a]anthracene, cyclopenta[cd]pyrene and chrysen; group (b): benzo[b]fluoranthene, benzo[j]fluoro-





AOAC accepts and validates CONFIDENCE method



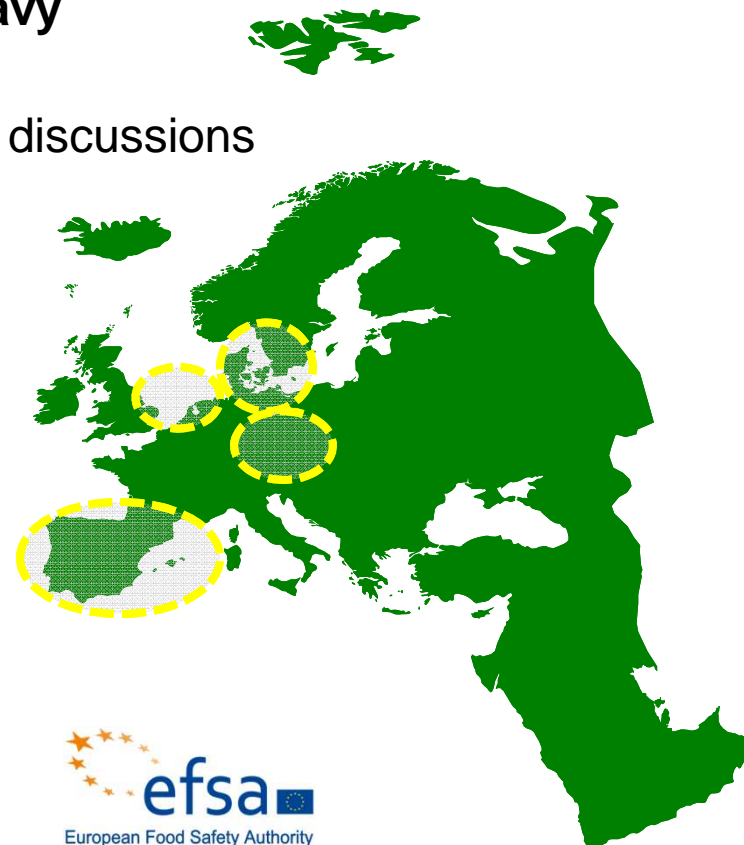
AOAC is reaching out to its Organizational Affiliates (OAs), including technology providers and contract research organizations, in an effort to establish standard method performance requirements (SMPRs) for determining the presence of chemical compounds in seafood resulting from the Gulf oil spill.

AOAC members from state agricultural laboratories are likely to be affected by the oil spill, which started with an oil rig explosion on April 20, 2010, off the coast of Louisiana, and have expressed growing concern that a fully validated analytical method for polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs) in seafood may be required soon. Methods are available from AOAC [Means, J.C. (1998) "Compound-Specific Gas Chromatographic/Mass Spectrometric Analysis of Alkylated and Parent Polycyclic Aromatic Hydrocarbons in Waters, Sediments, and Aquatic Organisms," *J. AOAC Int.* **81**, 657-672] and the National Institute of Standards and Technology (NIST) [NIST 1515, 1516, 1517, 1518, 1519, 1520, 1521, 1522, 1523, 1524, 1525, 1526, 1527, 1528, 1529, 1530, 1531, 1532, 1533, 1534, 1535, 1536, 1537, 1538, 1539, 1540, 1541, 1542, 1543, 1544, 1545, 1546, 1547, 1548, 1549, 1550, 1551, 1552, 1553, 1554, 1555, 1556, 1557, 1558, 1559, 1560, 1561, 1562, 1563, 1564, 1565, 1566, 1567, 1568, 1569, 1570, 1571, 1572, 1573, 1574, 1575, 1576, 1577, 1578, 1579, 1580, 1581, 1582, 1583, 1584, 1585, 1586, 1587, 1588, 1589, 1590, 1591, 1592, 1593, 1594, 1595, 1596, 1597, 1598, 1599, 1600, 1601, 1602, 1603, 1604, 1605, 1606, 1607, 1608, 1609, 1610, 1611, 1612, 1613, 1614, 1615, 1616, 1617, 1618, 1619, 1620, 1621, 1622, 1623, 1624, 1625, 1626, 1627, 1628, 1629, 1630, 1631, 1632, 1633, 1634, 1635, 1636, 1637, 1638, 1639, 1640, 1641, 1642, 1643, 1644, 1645, 1646, 1647, 1648, 1649, 1650, 1651, 1652, 1653, 1654, 1655, 1656, 1657, 1658, 1659, 1660, 1661, 1662, 1663, 1664, 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3491, 3492, 3493, 3494, 3495, 3496, 3497, 3498, 3499, 3500, 3501, 3502, 3503, 35

Cross-cutting survey of seafood (n = 150)

- Co-occurrence of **POPs & PUFA, PFAS, heavy metal speciation**
- Data to be provided to **EFSA** for risk-benefit discussions

Species	Region	Lead for collection of samples
Herring	Baltic sea	DTU
	North sea	RIKILT
	Atlantic ocean	DTU
Cod/whiting/hake	North sea	ICT
	Atlantic ocean	ICT
	Mediterranean sea	CSIC
Trout and salmon	Czech Republic	ICT
	Spain	CSIC
	Scandinavia	DTU
Bivalves	Scandinavia	DTU
	The Netherlands	RIKILT
	Mediterranean Sea	CSIC
Tuna	Canned, in water, preferably from Europe	All
Pangasius	Mostly Vietnam	All



CONFIDENCE Highlights

- Inclusion of new requests from DG SANCO and EFSA, through the *Advisory Board*
 - ✓ Broadening the types of pyrrolizidine alkaloids in honey and feed: inclusion of *Heliotropium* alkaloids
 - ✓ Brominated flame retardants: Hexabromocyclododecane (HBCDD) stereoisomers in fish and fish feed: LC-MS/MS method
 - ✓ Extending the number of ergot alkaloids in cereals
 - ✓ Inorganic arsenic in rice



SPE-HG-AAS – a novel speciation alternative...

μ-wave extraction

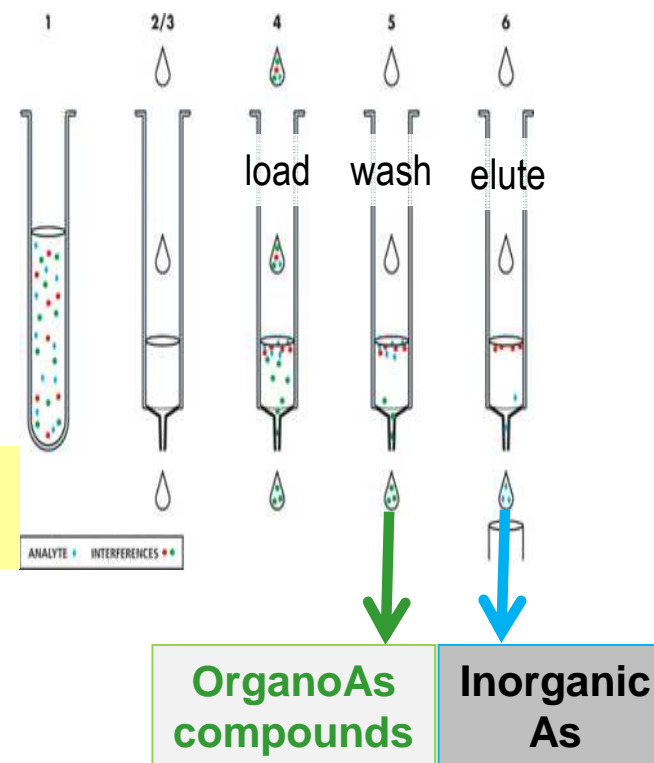
Separation by SPE

Detection by HG-AAS

Inexpensive detection system

Concept: Sequential elution for selective separation of **inorg As** from **organoAs** species by SPE

- Method for inorganic arsenic in marine samples (food and feed)
- Validated through a full-scale collaborative study (10 labs)

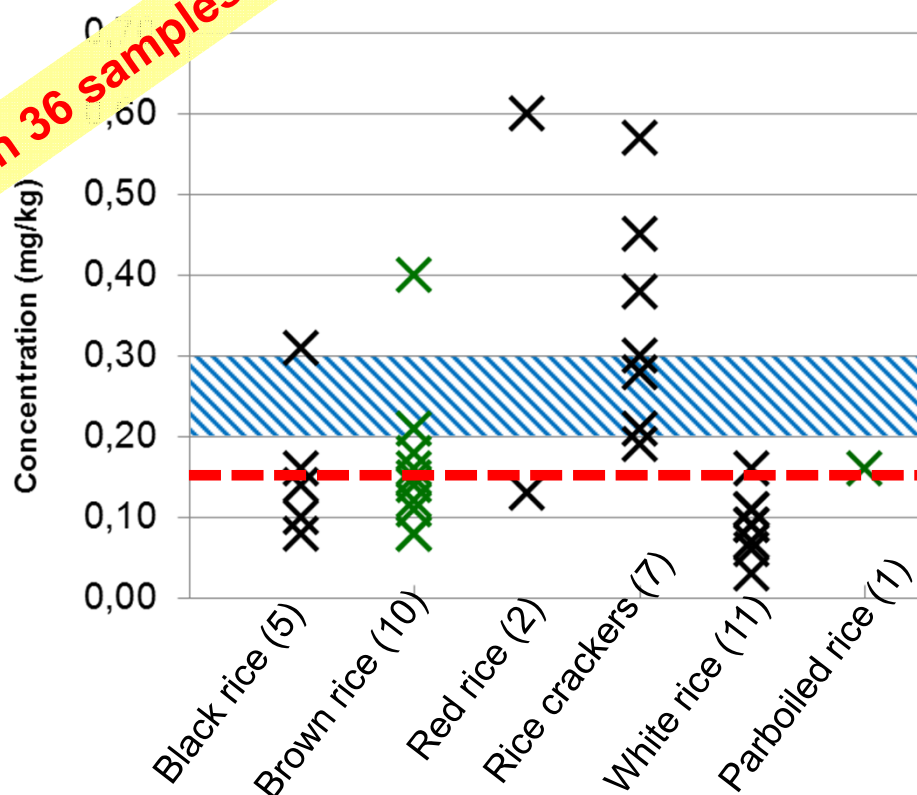


Inorganic arsenic in rice – a hot food safety topic

- Tailoring of SPE HG-AAS method for rice and rice products
- Simplified extraction in waterbath for increased sample throughput (> 50 samples extracted in 1 hour)



Survey on 36 samples



Dataset provides input to:
EFSA (exposure estimation)
EU commission (legislation?)
CODEX (legislation?)

Future EU max level (0.2-0.3 mg/kg)?

Chinese max level (0.15 mg/kg)

National Food Institute
Technical University
of Denmark



Validation of screening methods

- Field of application
 - ✓ Analyzing a high number of samples, of which the majority is contaminated at low levels
- Main feature: Binary result (1 or 0): The sample is above or below a target level (e.g. legal limit)
- Fitness for purpose of the method:
 - ✓ Non-compliant samples should be tested positive (or better: suspect); Criterion is rate of false negative (β -error, safety aspect): should be $\leq 5\%$
 - ✓ Compliant samples should be tested negative; Criterion is rate of false positive (α -error, economical aspect): $\leq 20\%$
- CONFIDENCE: Quantitative methods used in a binary fashion



Principle of quantitative methods used in a binary fashion

- The measurements include a numerical value (e.g. from dipstick reader)
- The numerical value is compared against a predefined cut off value
- Depending on whether the numerical value is below or above the cut off value, the sample is considered as suspect or negative



Steps of validation

- Preparing blank samples, samples with the analyte below and at the target level (TL)
- From the precision experiments of the TL samples we calculate the cut-off value (with β -error = 5% by definition)
- From the precision experiments of the other samples we calculate the α -error
- Example: Mycotoxins



Mycotoxins: the assay procedure



↓ Methanol/water extraction



→ Dilution with buffer

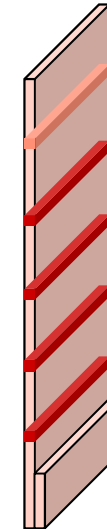


→ Incubation at 40°C, 10 min
Migration, 10 min



→ Reading

CTRL
FB1+FB2
DON
T2+HT2
ZEA



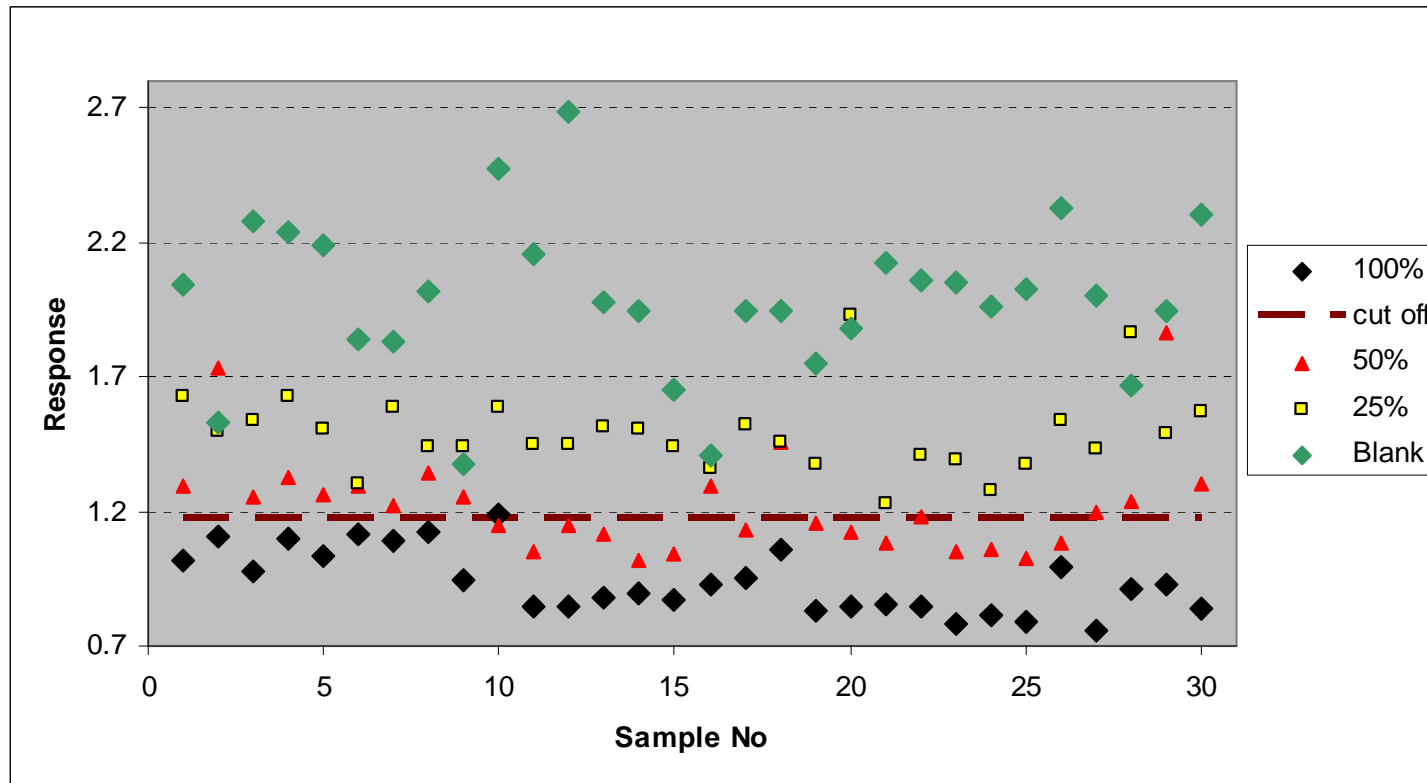
NEG: Test Lines **darker** than CTRL line

POS: test Lines **lighter** than CTRL line

Total analysis time: 30 min for 6 mycotoxins



Example: Zearelenone in maize



- 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 %**



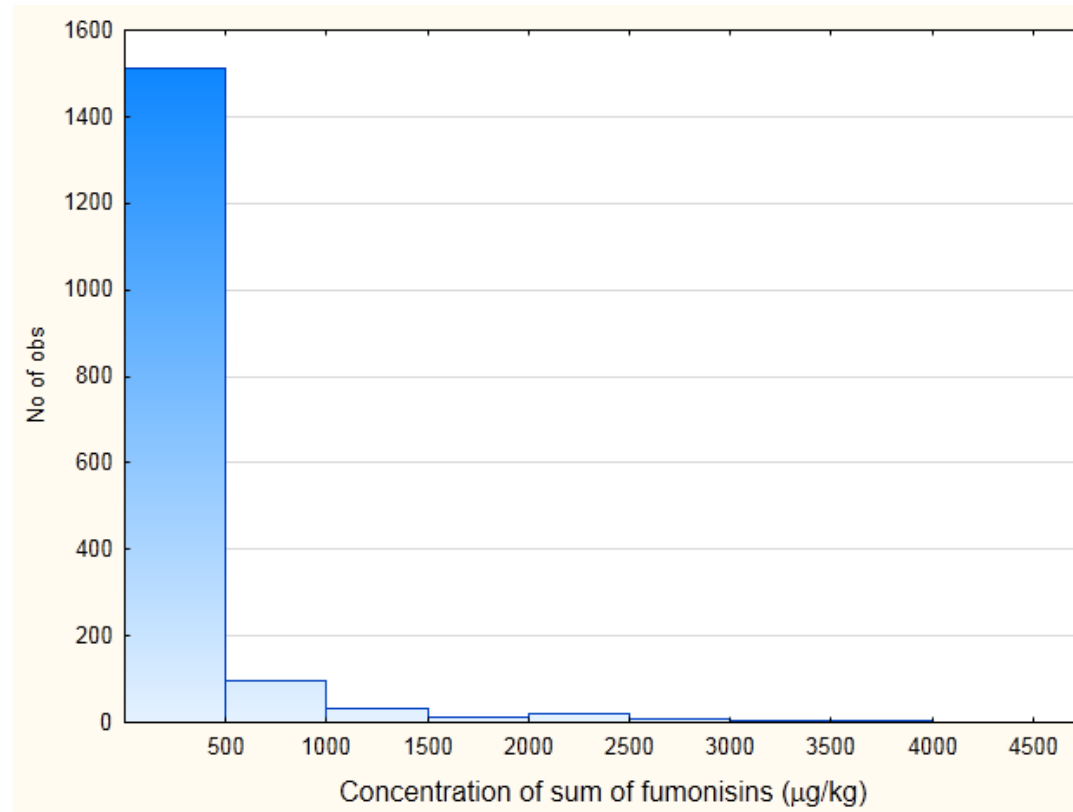
How to know the fitness for purpose?

We need to know three things:

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



Example: Fumonisin concentrations maize – European data. **Step 1**



Most of the samples contain the analyte far below the maximum level (ML) = 4000 µg/kg (food)



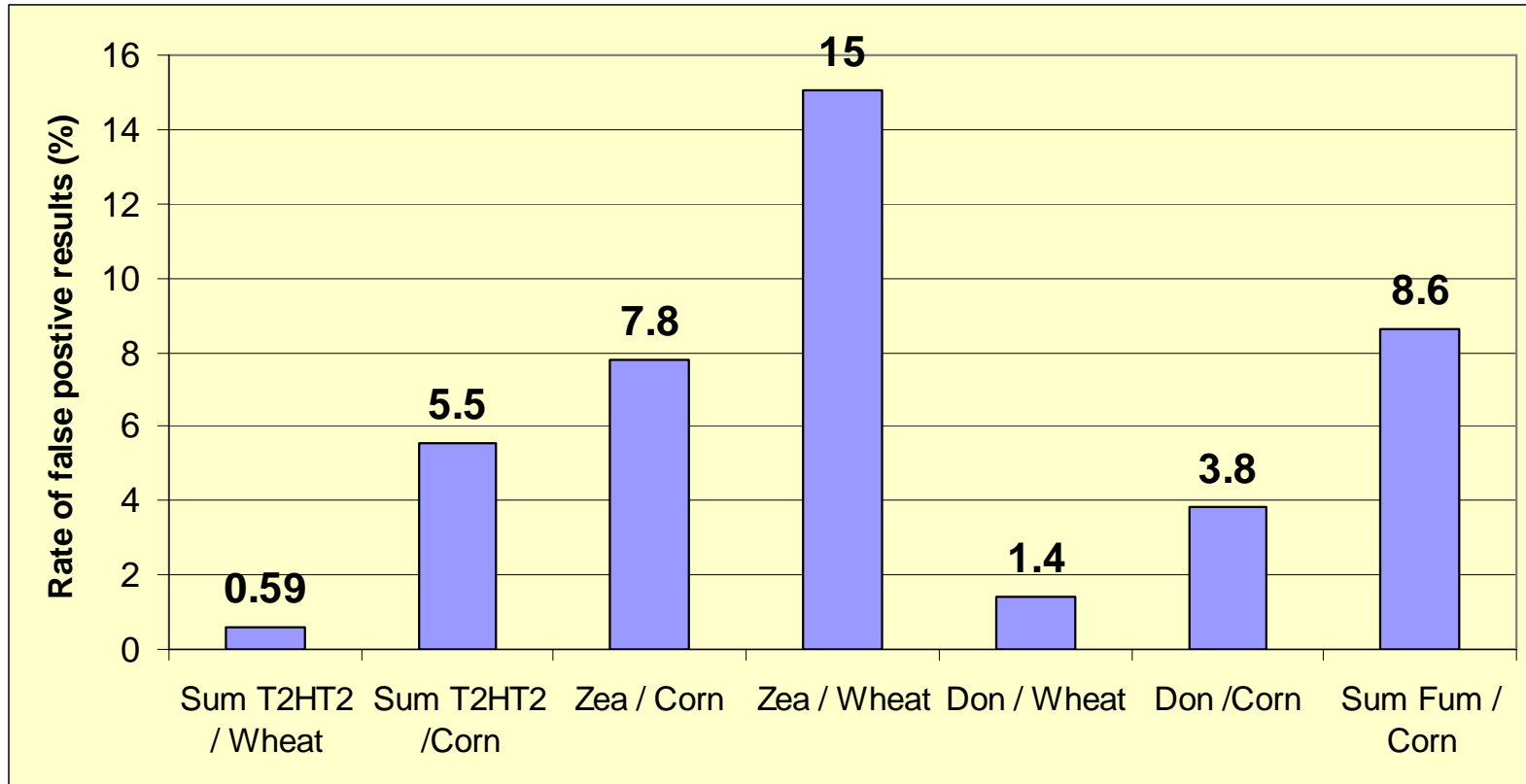
Example: Fumonisin concentrations in maize – European data. **Step 2**

The "rate of false positive results" from the validation is superimposed on the frequency distribution

% of ML with validation data	Range % of ML	Range: $\mu\text{g}/\text{kg}$	No of Samples	Rate of false positive results	Number of false positive
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



Overview for all mycotoxins

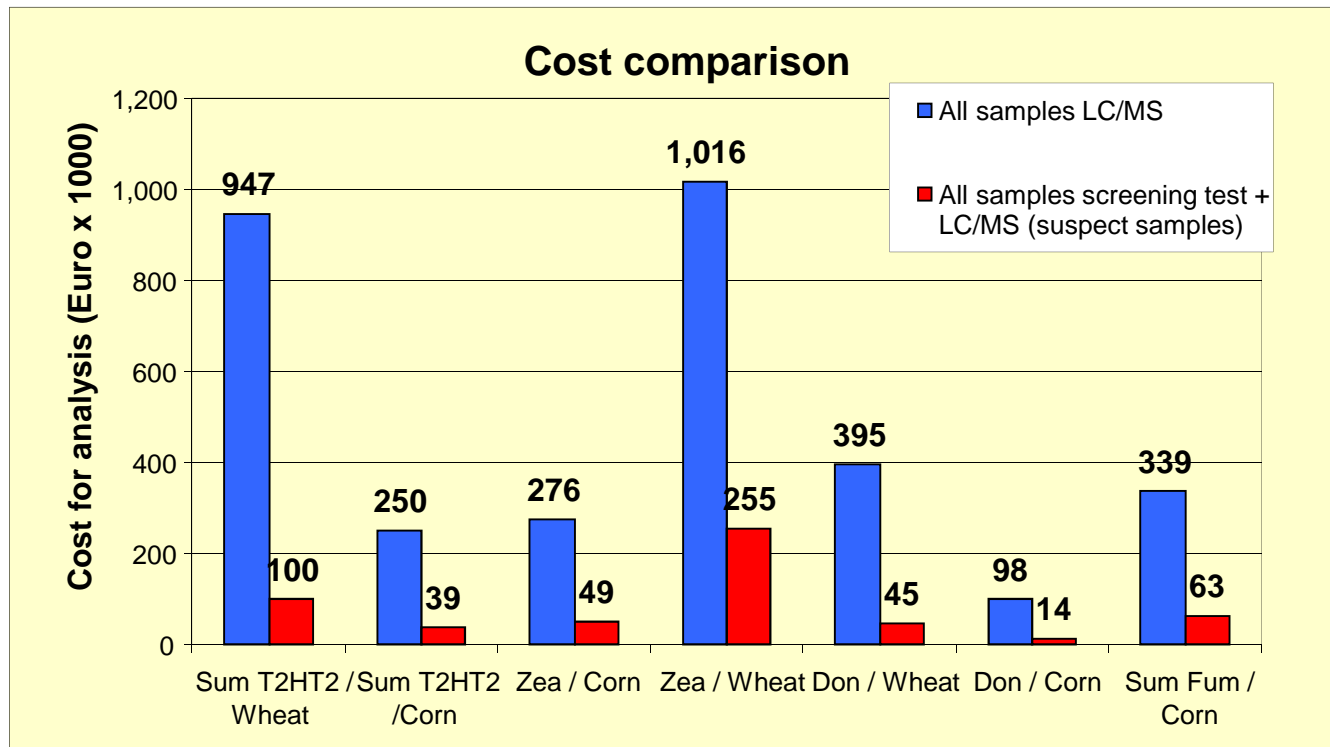


Cost estimation

Estimating total analytical costs for two options:

- Analysing all samples with LC/MS
- Analysing all samples with the screening test and all suspect samples additionally with LC/MS

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



The **test** presented here is considered **fit for purpose**



Summary for validation and fitness for purpose of screening methods

- Safety first: The specific experimental design applied ensured that the rate of false negative results is not above 5 %
- The validation exercise also delivered information about the rate of false positive results
- Final criteria for fitness for purpose of the screening method:
 1. The actual prevalence of contaminated samples in the ground population
 2. Economical consideration of screening versus confirmatory methods



Conclusions (1)

- Some multi-dipsticks are already commercially available



- Multi-dipstick tests can be used in field applications
- Many tests have been validated through small-scale collaborative studies: transferability to other laboratories has been demonstrated



Conclusions (2)

- Some tests have been validated through full collaborative studies, viz. mycotoxins dipstick, inorganic arsenic, perfluorinated compounds
 - ✓ Methods can be adopted by CEN for European standardization
- Major advances in simplified sample prep methods, reducing overall costs and speeding up analysis
- New insights in validation and fitness for purpose of screening tests
- Cross-cutting surveys, viz. fish & seafood: POPs, PFCs, heavy metals and PUFAs
- New topics have been included in the work programme in order to improve the relevance to EU policies

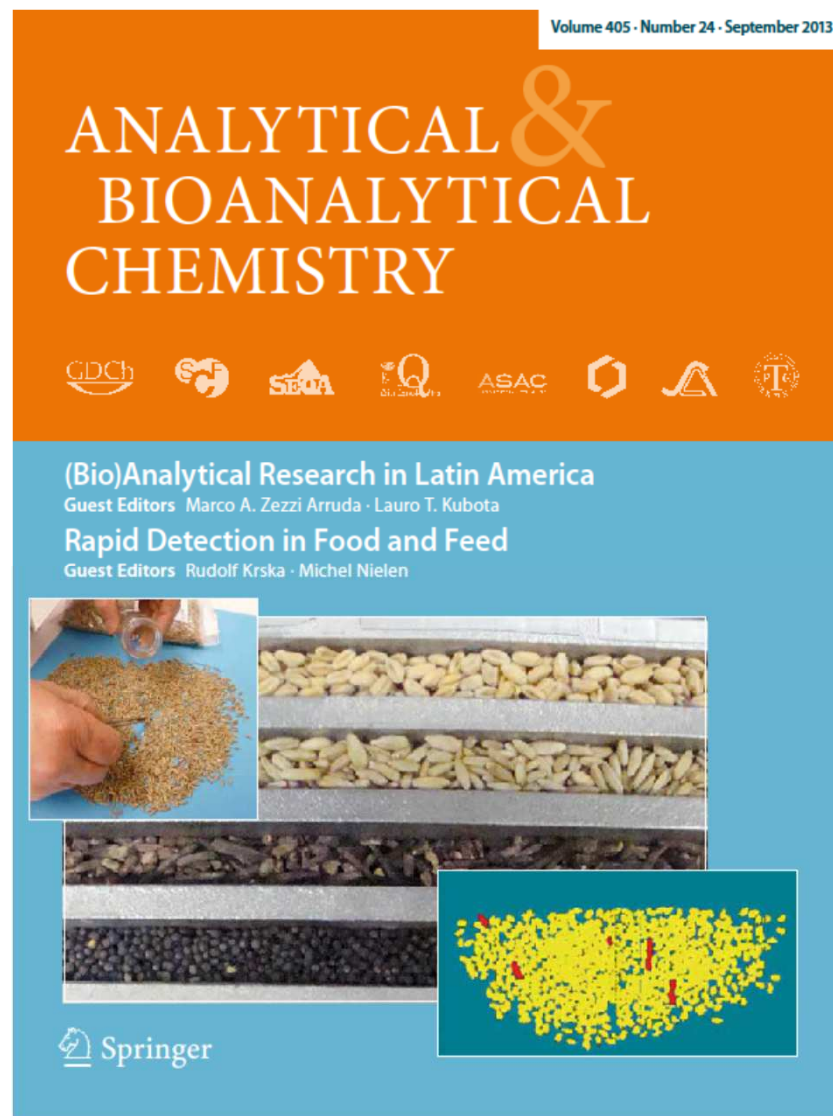


Dissemination

- More than 115 oral presentations and 115 posters at international conferences
- 36 Peer reviewed publications
- Website: www.confidence.eu



Special volume of ABC



Acknowledgements

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Thank you for your attention !

www.confidence.eu

