science and policy
for a healthy future

HBM4EU project

2nd HBM4EU Training School 2018

A08 Mycotoxins and Pesticides biomarker analysis

SESSION 1: GENERAL ASPECTS

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RIKILT, Wageningen, November 22nd 2018, part of
2nd HBM4EU Training School, Nijmegen, November 19th-23rd, 2018

Outline

Trends in instrumental analysis

HBM meets food safety analysis:
What can we learn from mycotoxin/pesticide residue analysis in food or should at least know about....
- Laboratory networks
- Harmonisation of analytical procedures/performance criteria

LOD/LOQ: sense and nonsense

The instrumental tool box

GC-ECD/NPD

LC-UV/DAD
LC-fluorescence

>1970

GC-MS

>1980

>2000

LC-MS/MS
Triple quad/Qtrap

GC-MS/MS

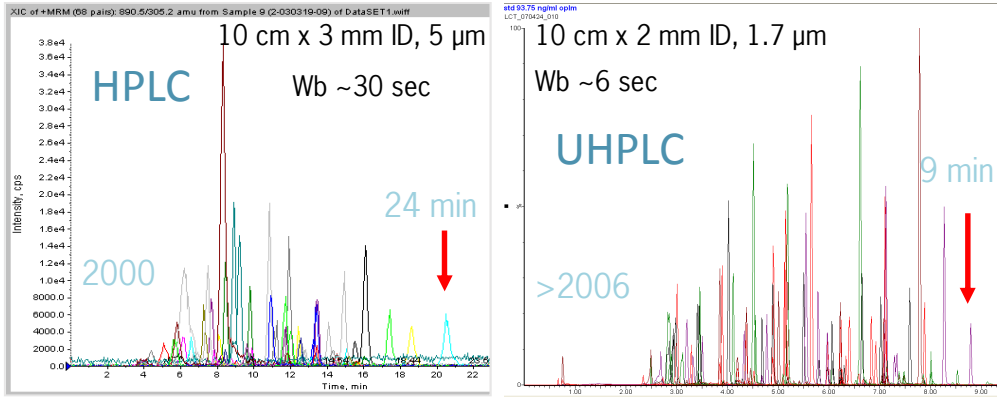
>2005

GC-HRMS
QTOF/Q-Orbitrap

>2010

LC-HRMS
QTOF/Q-Orbitrap

Trends in chromatography-mass spectrometry



Chromatography:

LC: from HPLC to UHPLC
faster *or* better separation
Improved columns for polar analytes

Detection:

MS/MS is king

Faster MS/(MS) measurement:
⇒ more analytes in 1 run *or* shorter runs *or* combined pos/neg, FS, SIM, MS/MS, ...)

Triple quads: ESTABLISHED TECHNOLOGY / CURRENT GOLD STANDARD FOR ROUTINE
sensitivity improvement with each generation
⇒ lower LODs *or* less sample prep (dilute&shoot) *and/or* less matrix into MS

High resolution MS (TOF/Orbitrap): **EMERGING /** MATURING
improvement of sensitivity, resolution (selectivity), dynamic range, scan speed with each generation => becoming a suitable alternative for triple quad quantitative analysis

MS features *Trends*

Sensitivity & selectivity
Scope: highly relevant in pesticide residue analysis in food, in future also for HBM

detector	mass analyzer		acquisition mode	selectivity	sensitivity	scope
MS	single quad	nominal mass	full scan	(-)	+	++++
MS			SIM	(-) /++ [1]	++/+++[1]	++
MS/MS	**triple quad/Qtrap**	**nominal mass**	**SRM/MRM**	**++**	**+++**	**++**
HRMS	TOF/Orbitrap	accurate mass	full scan	+/++	++	++++
MS/HRMS	Q-TOF/Qrbitrap	accurate mass	SRM/MRM; **AIF**	++/+++ ; **+/++**	++	++

[1] GC-NCI-MS for halogenated substances/derivatives
SIM: single ion monitoring
SRM = single reaction monitoring
MRM = multiple reaction monitoring
HRMS = high resolution MS (typically > 25,000 FWHM, mass accuracy < 1 mDa or <5 ppm)
MS/MS = tandem mass spectrometry
MS/HRMS = tandem mass spectrometry with accurate mass detection of product ion

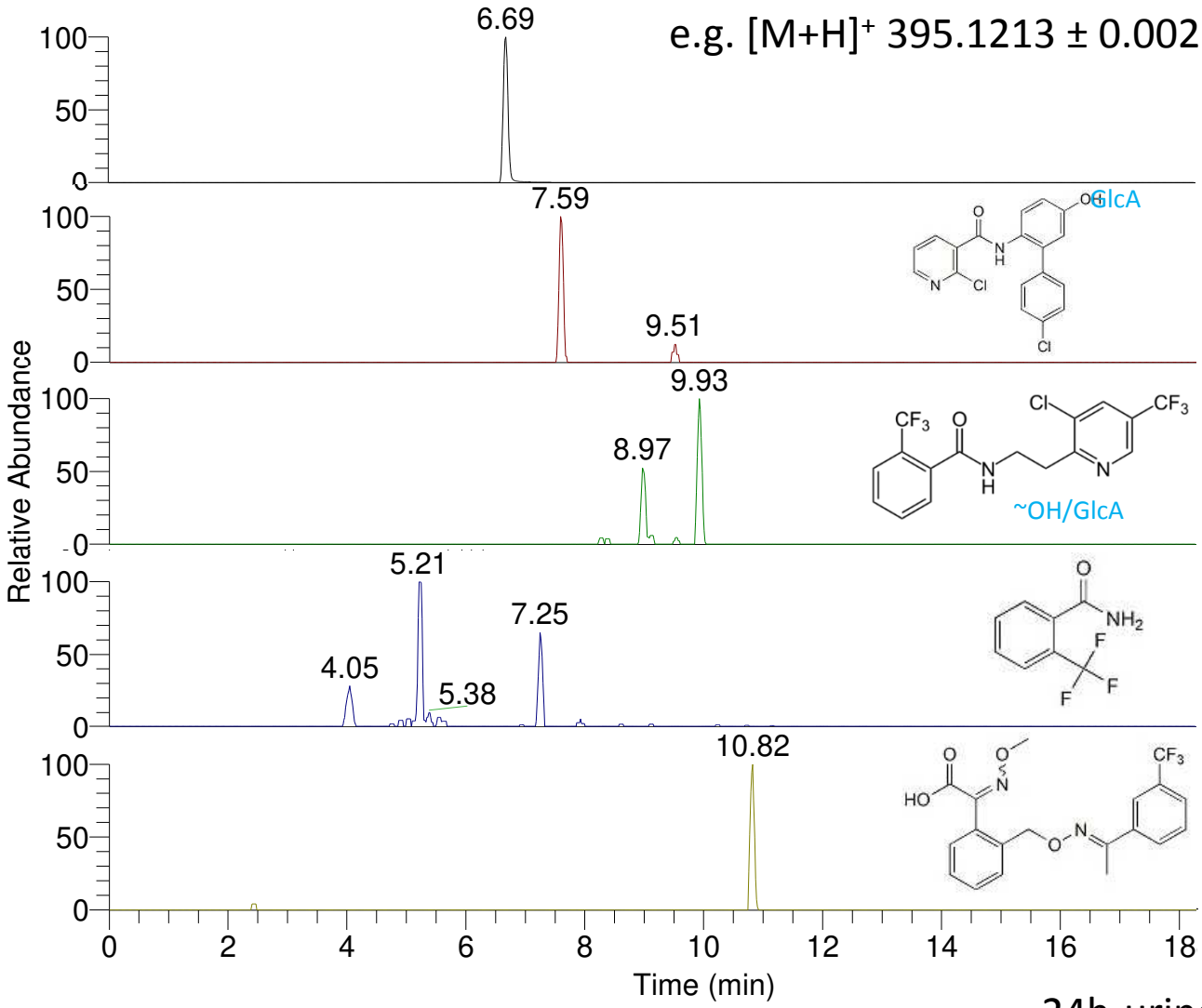
Targeted vs non-targeted measurement *trends*

	Targeted MS/MS	Full scan / untargeted MS
Instrument:	triple quad, Qtrap, QTOF, Q-Orbitrap	High Resolution MS: (Q)TOF, (Q-)Orbitrap
Acquisition:	MS/MS	FS hrMS (+fragm. w/o precursor ion selection)
Scope:	1-300 substances	Anything that enters the source and ionises
Data:	XIC of analyte MS/MS transitions	XIC of analyte exact masses ± x mDa (ppm)
Result:	Quantitative for all subst. incl. in cal.stds/QC samples	Quantitative for all subst. included in cal.stds/QC samples Qualitative/suspect screening
Cons:	- Optimization needed for each analyte - Acquisition in time windows - Only substances in acquisition method can be detected	- Generic acquisition conditions can compromise sensitivity - Very large data files
Pros:	+ Optimized acquisition for each subst. => highest sensitivity/selectivity	+ Straightforward measurement + 'unlimited'# substances + Suspect screening (detection w/o standards + New options for unknowns/profiling + Retrospective evaluation of data

Full scan HRMS: extract biomarkers from raw data

Extract signal of biomarker exact mass ± x Da (ppm)

e.g. $[M+H]^+$ 395.1213 ± 0.002 Da (± 5 ppm)



Caffeine $C_8H_{10}N_4O_2+H^+$
m/z 195.0877 (6.69 min)

Boscalid-OH/GlcA
$C_{24}H_{20}Cl_2N_2O_8+H^+$
m/z 535.0670

Fluopyram-OH/GlcA
$C_{22}H_{19}ClF_6N_2O_8+H^+$
m/z 589.0807

Fluopyram benzamide
$C_8H_6F_3NO+H^+$
190.0474 (7.25 min)

Trifloxystrobin acid
$C_{19}H_{17}F_3N_2O_4+H^+$
395.1213 (10.81 min)

24h-urine sample
Strawberry lover

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- Laboratory networks
- Harmonisation of analytical procedures/performance criteria

LOD/LOQ: sense and nonsense

HBM ⇔ Food safety analysis

Mycotoxins & Pesticides:

General population: food is main route of exposure

⇒ HBM: alternative to traditional exposure assessment
(exposure = dietary intake = food consumption x food concentrations)

Mycotoxins and Pesticides in food (/feed) heavily regulated in EU
Analysis: long established lab networks, guidance documents etc

Laboratory networks

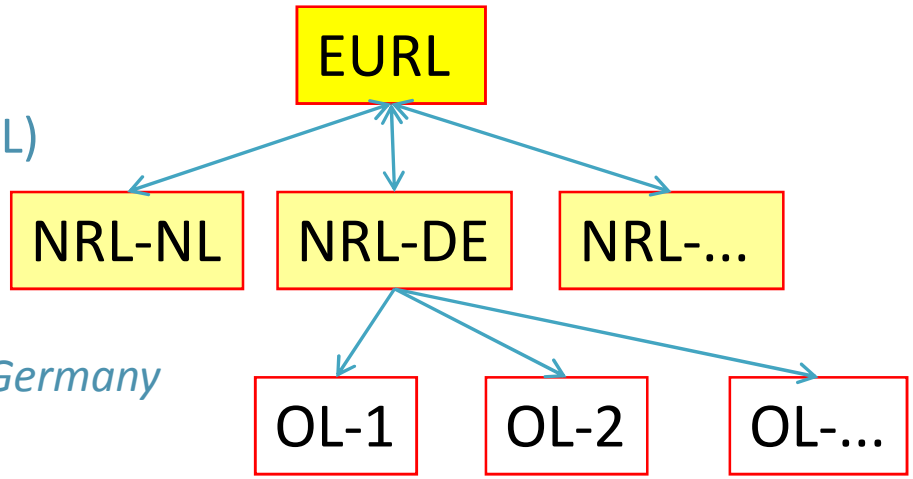
HBM4EU: establishment in progress, multiple labs, assigned by WP9 QAU

Mycotoxins & Pesticides in food/feed:

Regulated at EU level

Assigned by COM (est. 2006)

- EURL mycotoxins/plant toxins (RIKILT, NL)
- EURLs pesticides
  - Fruit&veg, *University Almeria, Spain*
  - Cereals & feed, *DTU, Denmark*
  - Products of animal origin, *CVUA, Freiburg, Germany*
  - 'SRM' pesticides, *CVUA Stuttgart, Germany*



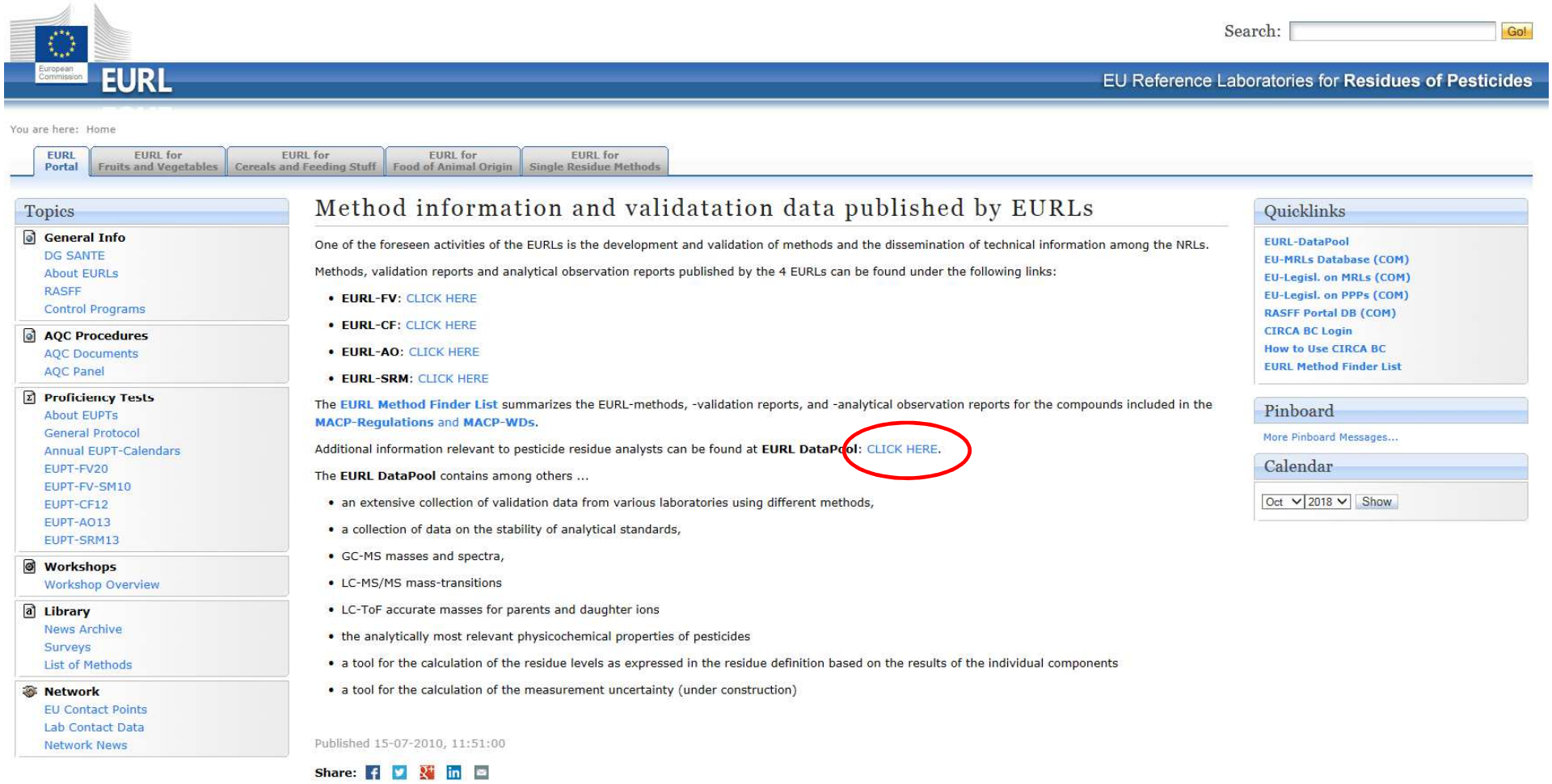
Tasks:

Centre of expertise, method development, guidance documents

Organisation of workshops, organisation of proficiency tests

Advise/respond to questions COM/EFSA

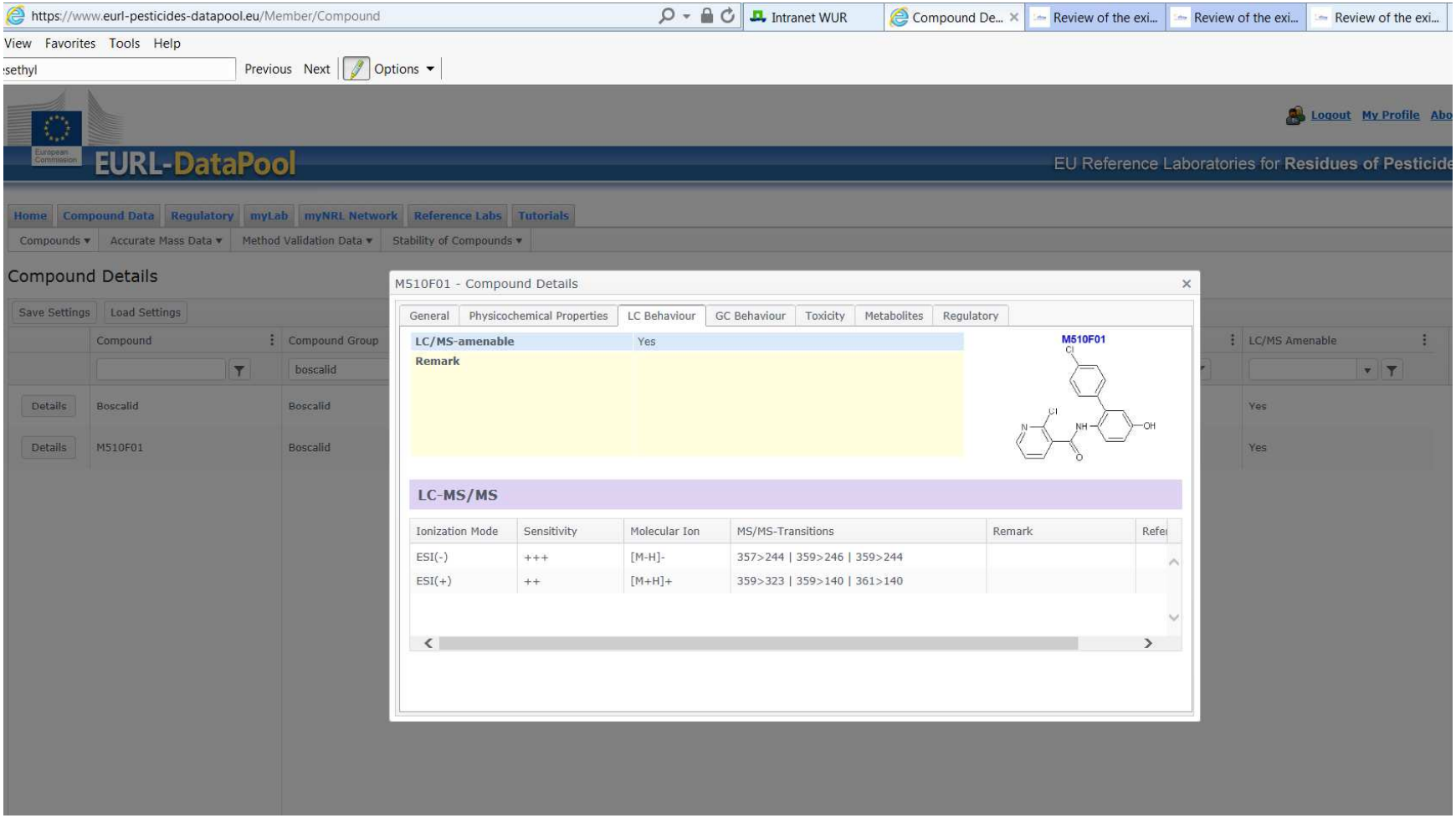
Laboratory networks

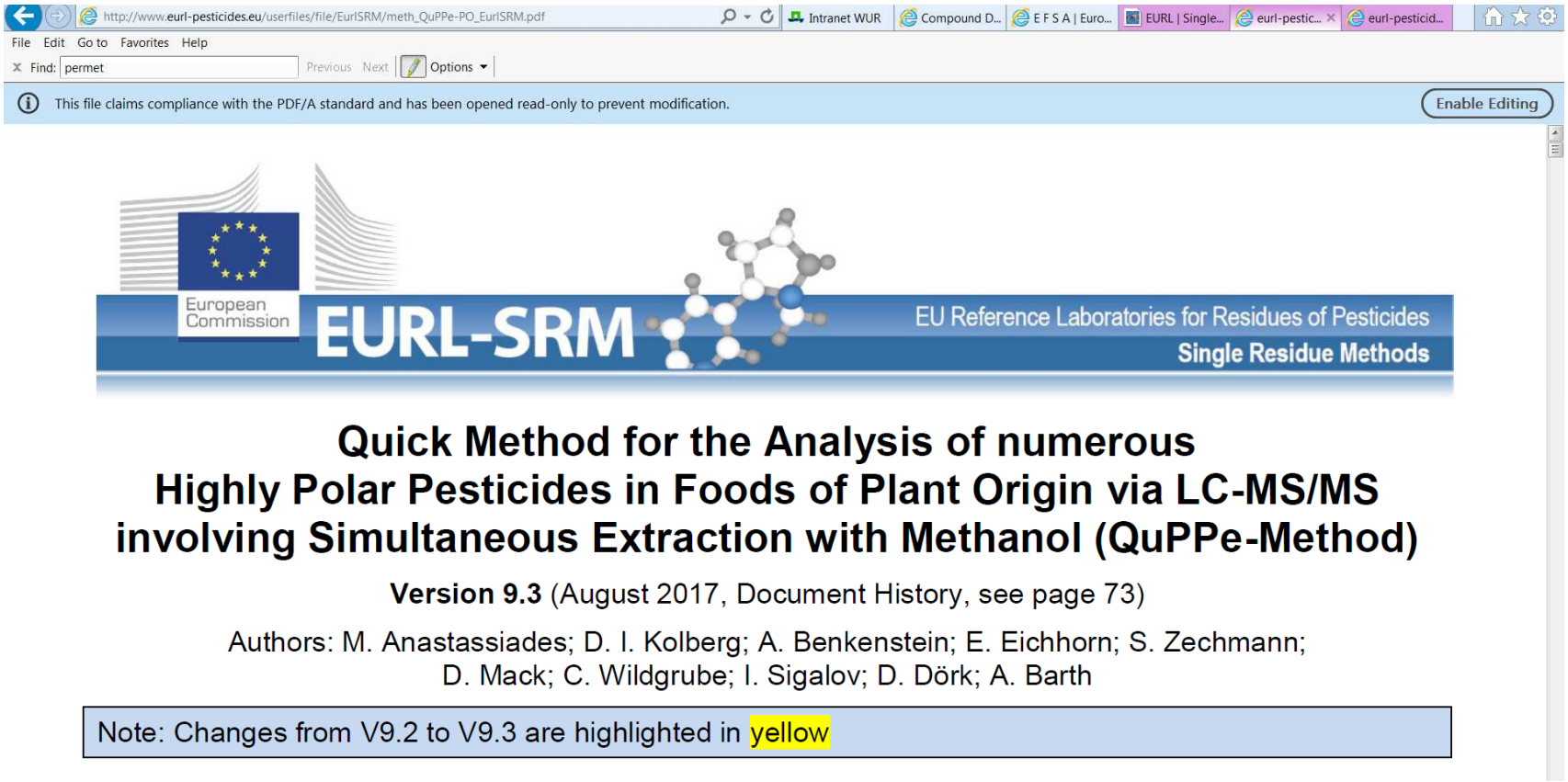


http://www.eurl-pesticides.eu/docs/public/home.asp?LabID=100&Lang=EN

Laboratory networks

HMB meets Food safety





http://www.eurl-pesticides.eu/userfiles/file/EurlSRM/meth_QuPPe-PO_EurlSRM.pdf

QuPPe Method Version 9.3, August 2017

5.7. LC-MS/MS Measurement

Any suitable LC-MS/MS conditions may be used. Some exemplary instrument measurement conditions are given below. An overview of LC-MS/MS conditions proposed within this document is given in Table 3:

Table 3: Overview and scope of the methods proposed within this document for the QuPPe method:

	M 1.1	M 1.2	M 1.3	M 1.4	M 2	M 3	M 4.1	M 4.2	M 5	M 6	M 7	M8	M 9
ESI-mode	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.
Separation princip-le	Anion Exch.	Anion Exch.	Carbon	Carbon	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC	Carbon	HILIC
Column type	AS 11	AS 11-HC	Hyper-carb	Hyper-carb	Obe-lisc-R	Obe-lisc-R	Obe-lisc-R	BEH-Amide	PFP	Obe-lisc-R	Trini-ty P1	Hyper-carb	Trinity P1
NEGATIVE MODE													
Ethephon	✓	✓	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
HEPA	✓	✓	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
Glufosinate	✓	✓	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
N-Acetyl-glufosinate	✓	✓	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
MPPA	✓	✓	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
Glyphosate	✓	✓	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
AMPA	✓	✓	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
Phosphonic acid	(✓)	(✓)	✓	✓	NT	NT	NT	NT	NT	NT	-	NT	NT
N-Acetyl-AMPA	NT	✓	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
Fosetyl-Al	-	✓	✓	NT	✓	NT	NT	NT	NT	NT	✓*	NT	NT
Maleic hydrazide	-	-	✓	NT	✓	NT	NT	NT	NT	NT	✓*	NT	NT
Perchlorate	NT	-	✓	✓	✓	NT	NT	NT	NT	NT	✓*	NT	NT
Chlorate	NT	-	✓	✓	NT	NT	NT	NT	NT	NT	✓*	NT	NT
Bialaphos	NT	NT	✓	NT	NT	NT	NT	NT	NT	NT	-	NT	NT
Cyanuric acid	NT	NT	✓	NT	NT	NT	NT	NT	NT	NT	✓*	NT	NT
Bromide	NT	NT	-	✓	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bromate	NT	NT	(✓)	✓	NT	NT	NT	NT	NT	NT	NT	NT	NT
N-Acetylglyphosate	NT	NT	✓	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Difluoroacetic acid	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	✓
Trifluoroacetic acid	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	✓

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LOD/LOQ: sense and nonsense

Guidance documents / regulations

Analytical procedures/performance criteria

General:

Eurachem 2nd Ed, 2014, The Fitness for Purpose of Analytical Methods, A Laboratory Guide to Method Validation and Related Topics
https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf

Pharma:

EMA Guideline on bioanalytical method validation (EMEA/CHMP/EWP/192217/2009 Rev.1 Corr.2**)
https://www.ema.europa.eu/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf

FDA (US) CDER/CVM Bioanalytical method validation, guidance for industry (May 2018)
https://www.fda.gov/downloads/drugs/guidances/ucm070107.Pdf

Food/Agro:

Animal products (veterinary drug residues) 2002/657/EC

Mycotoxins
Pesticides
} next slides

Which guidance are you using??

EU documents on analysis methods/criteria food

MYCOTOXINS

Regulation 2017/625 'Official Control Regulation' (OCR)
on official controls and other official activities performed to ensure the application of food and feed law

Standardised methods in the EU ('European Norm', EN; 'CEN methods')

Regulation 401/2006
on methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs

Regulation 152/2009
laying down the methods of sampling and analysis for the official control of feed

Guidance Identification criteria SANTE/12089/2016
https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_sampling_guid-doc-ident-mycotoxins.pdf

Guidance LOD/LOQ determination JRC, 2016, DOI: 10.2787/8931
http://publications.jrc.ec.europa.eu/repository/bitstream/JRC102946/eur%2028099%20en_lod%20loq%20guidance%20document.pdf

EU documents on analysis methods/criteria food

PESTICIDES

Regulation 2017/625 'Official Control Regulation' (OCR)
on official controls and other official activities performed to ensure the application of food and feed law

Standardised methods in the EU ('European Norm', EN; 'CEN methods')

SANCO/825/00 rev. 8.1 (2010)
Guidance document on pesticide residue analytical methods
Requirements for methods submitted by Agrochem industry during (re)registration of pesticides, applies to food/feed, water, soil, blood, serum, plasma or urine.
https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_ppp_app-proc_guide_res_post-reg-cont-monitor.pdf

SANTE/11813/2017
Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed
https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2017-11813.pdf

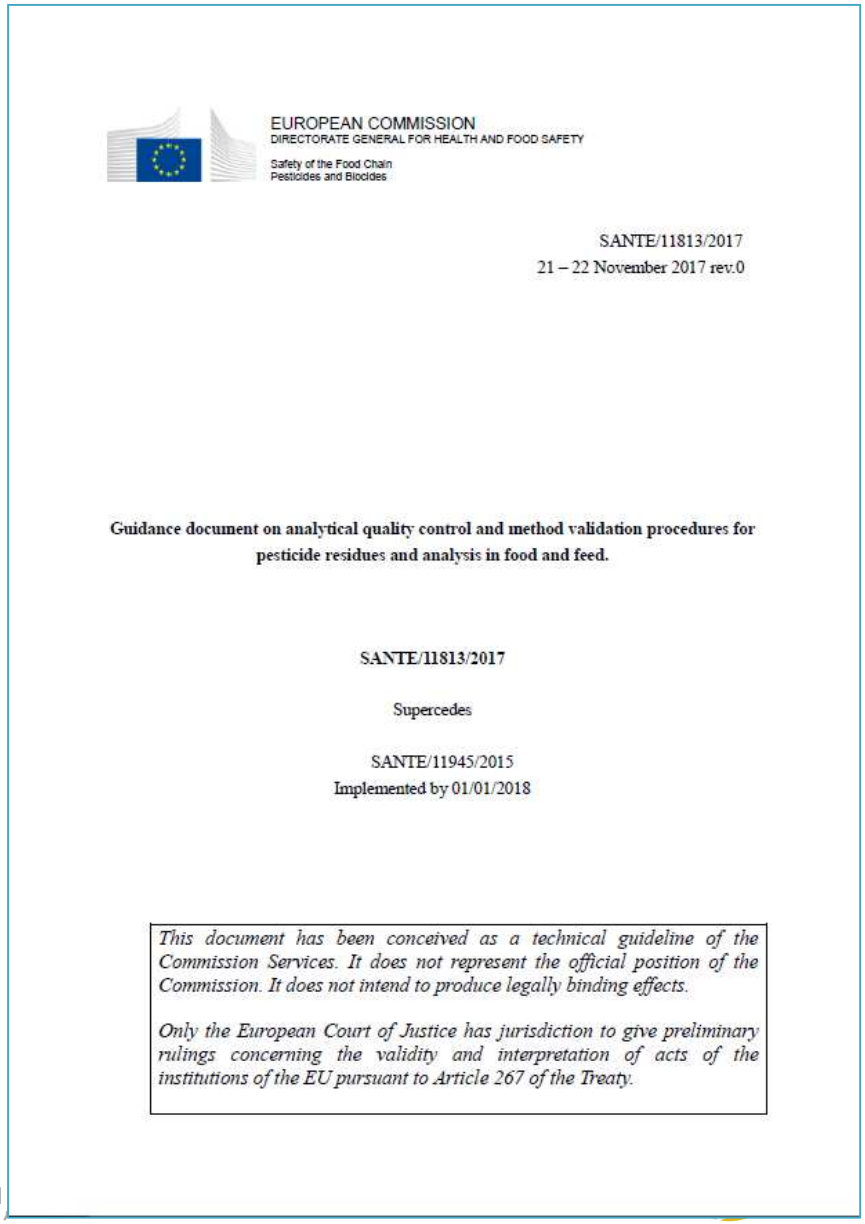
Guidance documents

Pesticide guidance document
AQC, validation, performance criteria

First established 1997 to harmonise Validation and Analytical Quality Control procedures of pesticide residue analysis in food and feed.

Re-evaluated every 2 years
Revised where necessary

Current version SANTE/11813/2017
Next revision: end 2019



EUROPEAN COMMISSION
DIRECTORATE GENERAL FOR HEALTH AND FOOD SAFETY
Safety of the Food Chain
Pesticides and Biocides

SANTE/11813/2017
21 – 22 November 2017 rev.0

Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed.

SANTE/11813/2017

Supercedes

SANTE/11945/2015
Implemented by 01/01/2018

This document has been conceived as a technical guideline of the Commission Services. It does not represent the official position of the Commission. It does not intend to produce legally binding effects.

Only the European Court of Justice has jurisdiction to give preliminary rulings concerning the validity and interpretation of acts of the institutions of the EU pursuant to Article 267 of the Treaty.

Guidance documents

SANTE/11813/2017 Calibration/quantification

Testing/replacing analytical reference standards
Old vs new: ≥5 replicates alternate injections each
Difference of mean old vs new should be ≤10%
Take RSD of mean into account (≤10%)

$$\text{Difference old/new} = \frac{\text{mean } R_{new} - \text{mean } R_{old}}{\text{mean } R_{new}} \times 100\%$$

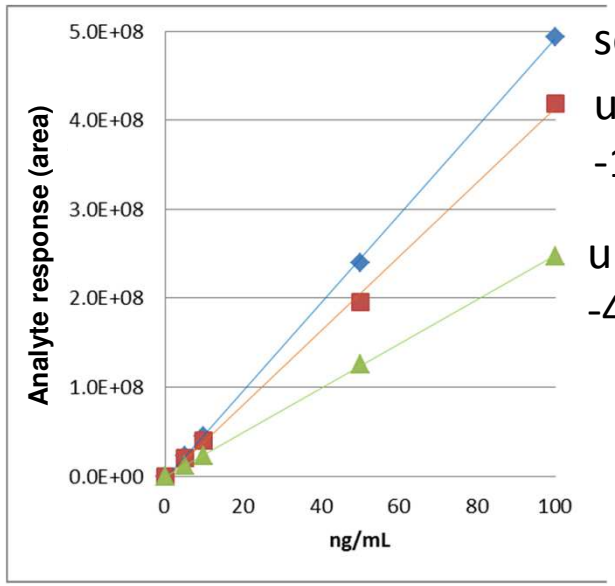
Injection	response	
10 ng/ml old-1	1235	
10 ng/ml new-1	1360	
10 ng/ml old-2	1131	
10 ng/ml new-2	1560	
10 ng/ml old-3	1456	
10 ng/ml new-3	1430	
10 ng/ml old-4	1365	
10 ng/ml new-4	1430	
10 ng/ml old-5	1378	
10 ng/ml new-5	1365	
	average	RSD
old standard	1313	9.8%
new standard	1429	5.6%
difference	8.1%	

SANTE/11813/2017 Calibration/quantification

Matrix effects*

Need to be addressed in calibration when >20%

Difference in response of analyte dissolved in solvent (calibrant) and analyte dissolved in final sample extract => affects quantification



solvent

urine extract A
-16%, 84%

urine extract B
-49%, 51%

Cause:

LC-MS(/MS): ionisation issue
Competition for charge in ESI
Mostly suppression, sometimes enhancement

GC

Active sites in injector (liner)
More pronounced for more polar analytes (-OH, -NH, phosphates, ...)
Mostly enhancement

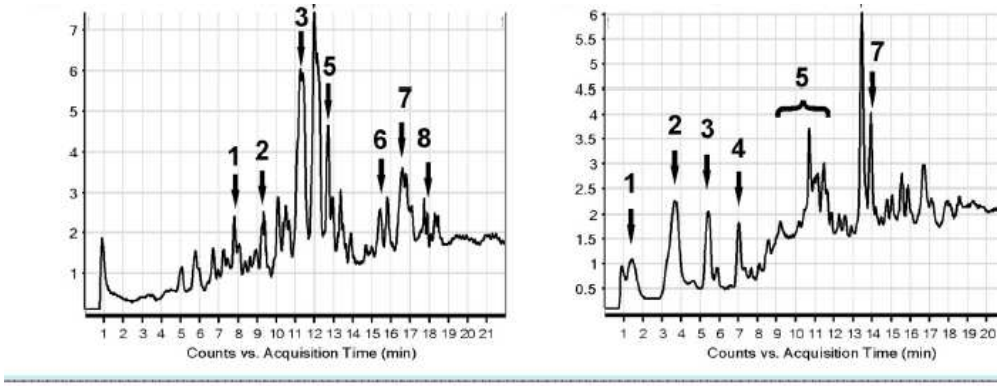
*Reading recommendation: Panuwet et al, (2016) Critical Reviews in Analytical chemistry, 46:2,93-105

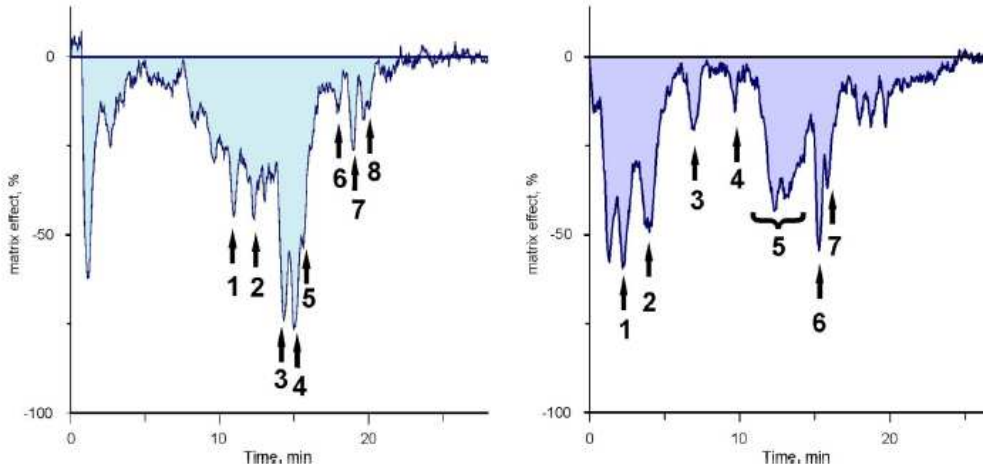
Matrix effects

LC-MS(/MS), a closer look:

Sample matrix A

Sample matrix B





Full scan TIC
LC-TOF-MS

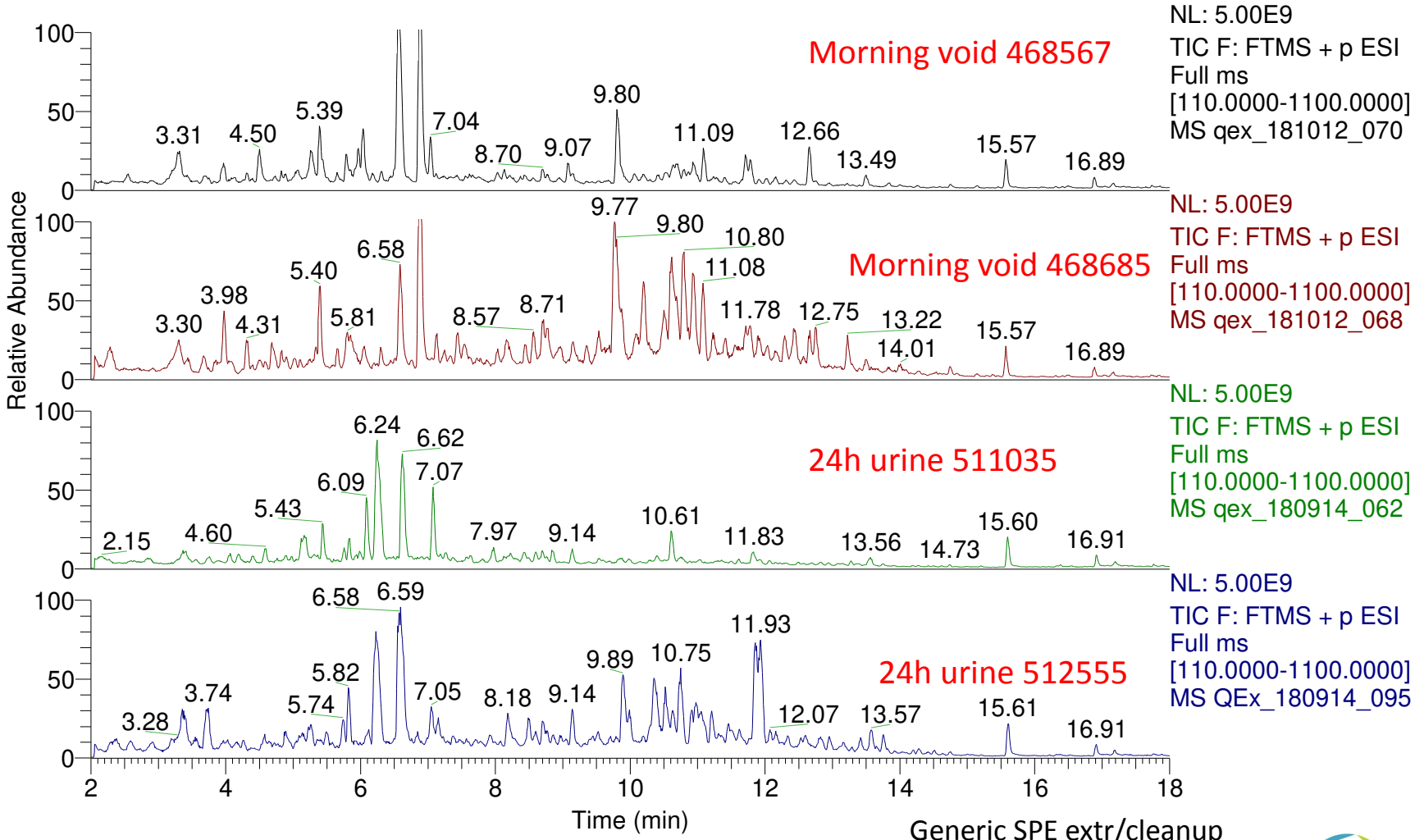
MS/MS response of pesticide
Injection of blank matrix
post-column T-infusion
of the pesticide

Strong suppression typically corresponds to high full scan TIC signals

Courtesy Lutz Alder,
retired from BfR

Matrix effects

LC-MS(/MS), a closer look: every urine is different....



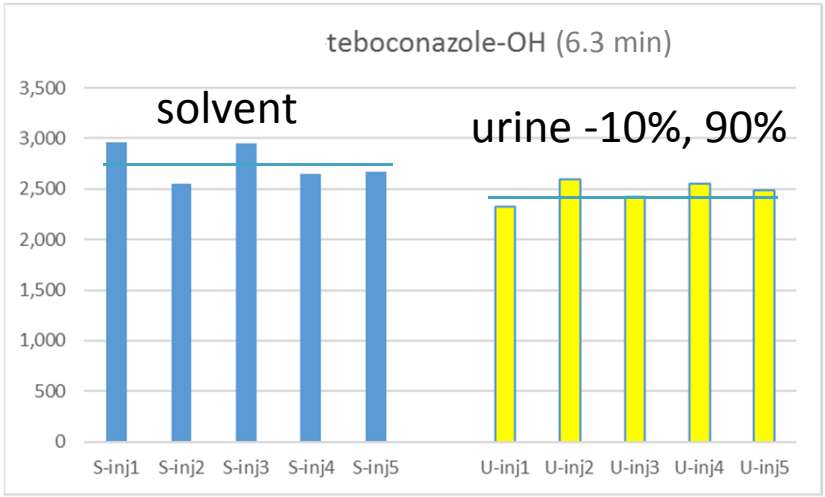
Generic SPE extr/cleanup
Full scan TICs of urine
LC-Q-Orbitrap MS

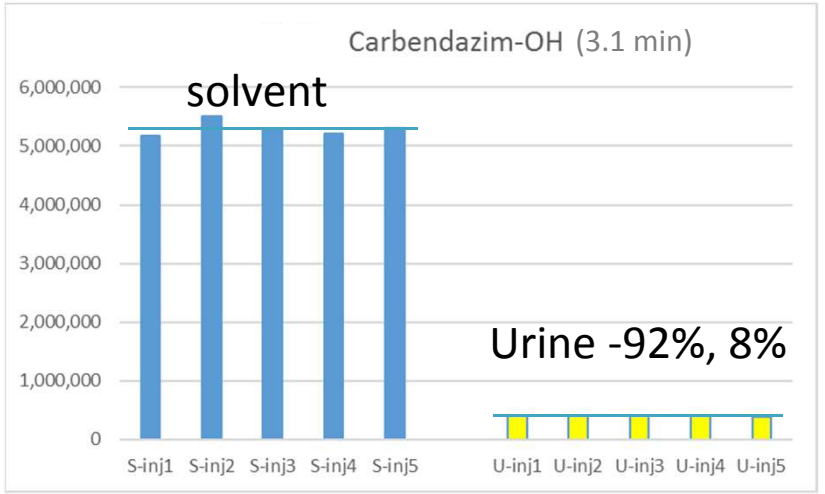
Matrix effects

Intermezzo

LC-MS(/MS), a closer look:

Response of standard prepared in urine extract* vs standard prepared in solvent
5 replicate injections of same concentration





Same urine, hardly any suppression for Teb-OH, very strong suppression for Carb-OH

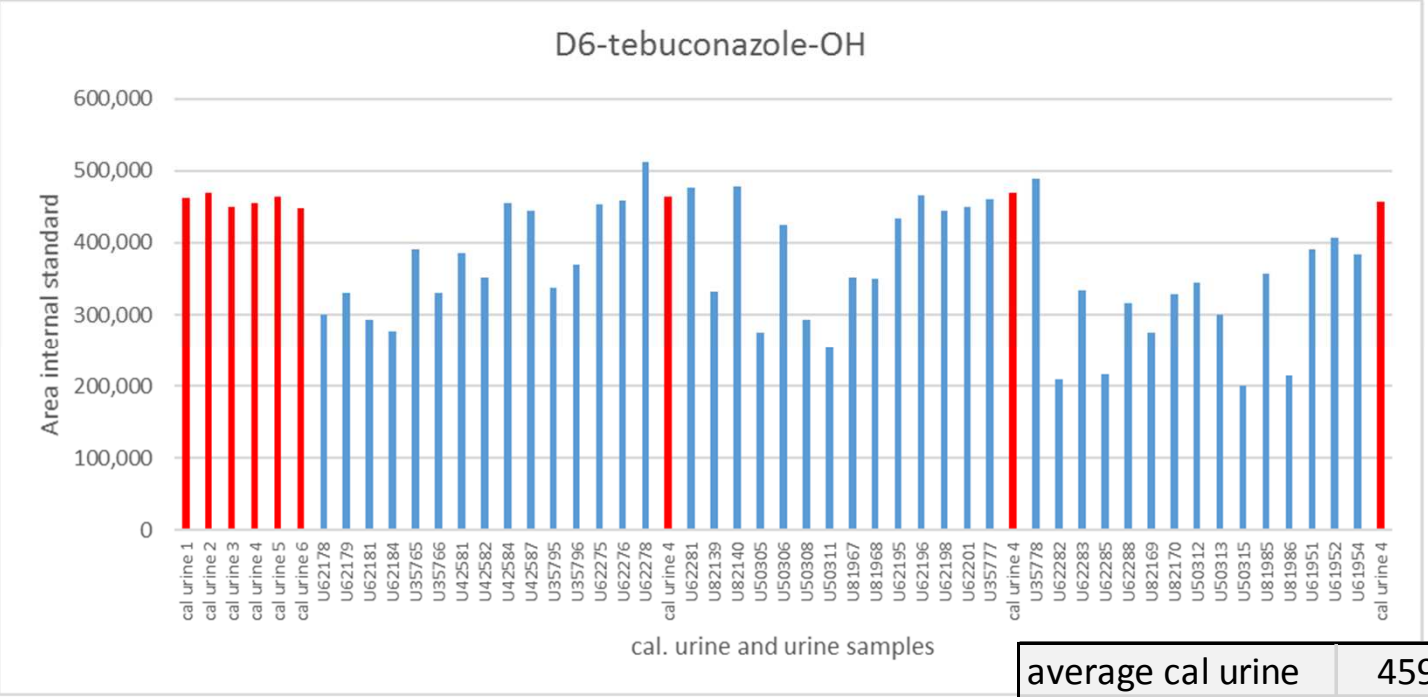
Urine extract*:
Enzymatic deconjugation
"QuEChERS" extraction (ACN partitioning)
Evaporative concentration ACN; reconstitution MeOH/water

Matrix effects

LC-MS(/MS), a closer look:

Different urines, different matrix effects

Variation in absolute response of biomarker in individual urine extracts



average cal urine	459,939
RSD cal urine	2%
min all urines	199,891
max all urines	512,732
average all urines	364,278
RSD all urines	23%

Factor 2.6!

⇒ Same urine consistent response

⇒ Various urines, various degrees of suppression

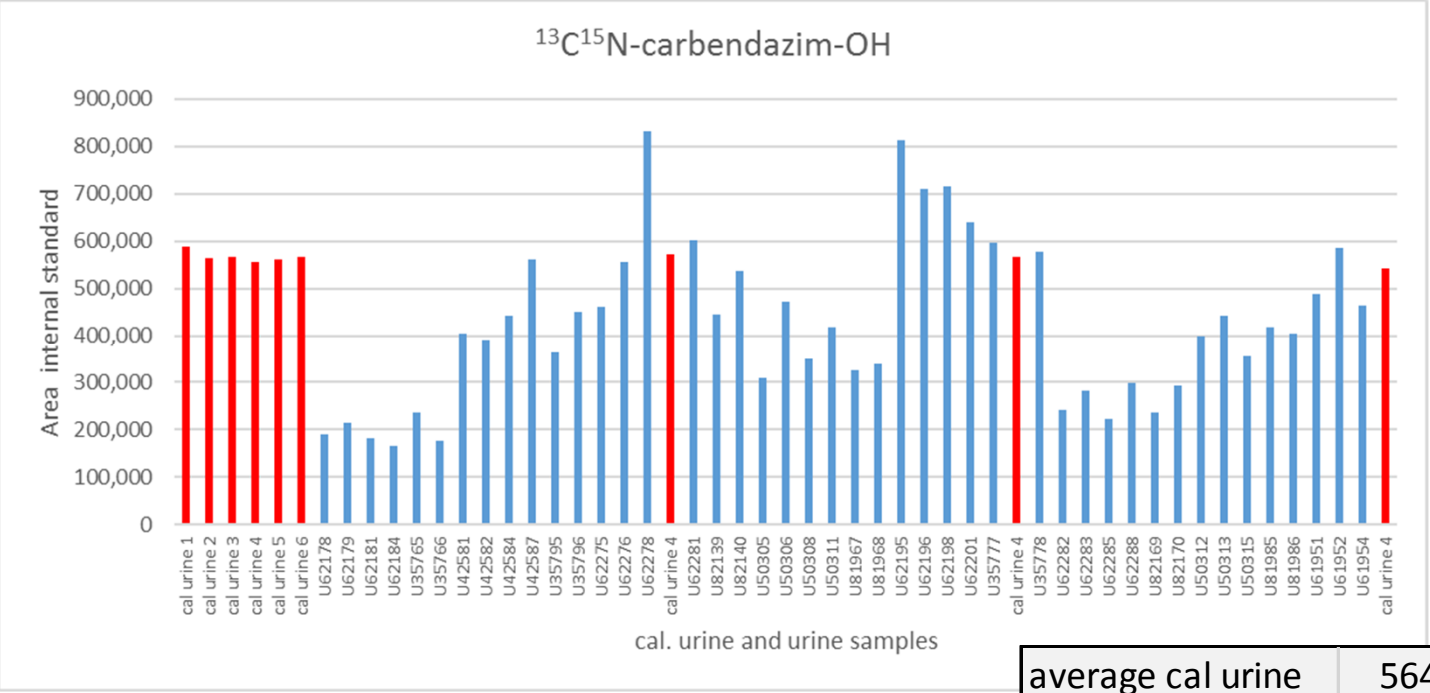
Matrix effects

Intermezzo

LC-MS(/MS), a closer look:

Different urines, different matrix effects

Variation in absolute response of biomarker in individual urine extracts



average cal urine	564,400
RSD cal urine	2%
min all urines	165,187
max all urines	831,044
average all urines	426,329
RSD all urines	40%

Factor 5!

⇒ Same urine consistent response

⇒ Various urines, various degrees of suppression

Matrix effects

Intermezzo

LC-MS(/MS), a closer look:

Parameters affecting matrix effects:

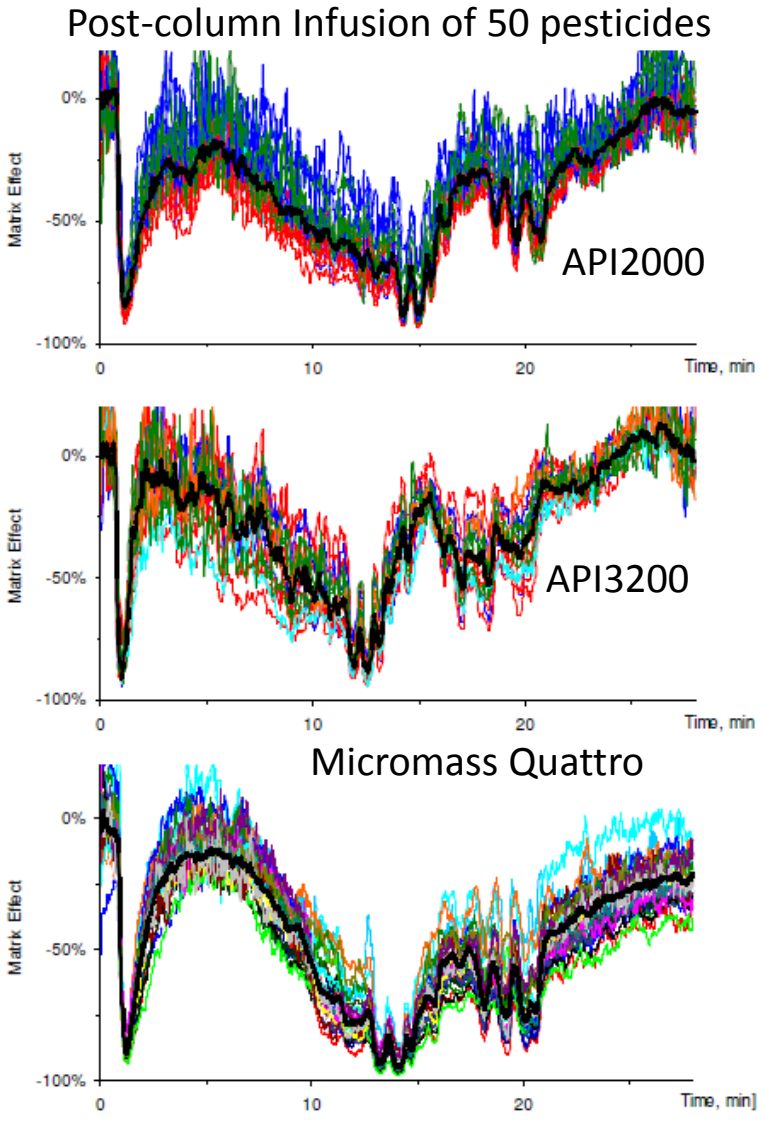
Matrix: **amount of matrix injected into LC-MS**
urine (every urine is different)
sample prep
- dilute&shoot ↔ IAC
- extraction/cleanup
- urine equivalent in extract

injection volume

Chromatographic separation

Analyte

LC-MS Instrument
less for nano/µ-LC?
Source design?
ESI more matrix effects than APCI
ESI pos more than ESI neg



Stahnke et al, Mass. Spectrom. 2012, 47, 875–884
Stahnke et al, Anal Chem. 2012 84(3):1474-82.

Matrix effects

Intermezzo

LC-MS(/MS), a closer look:

How to deal with matrix effects?

1. Reduce matrix effects:

Keep amount of matrix injected low

- inject low urine equivalent
- dilution
  - to go from 80% suppression to <20%, 25-100x dilution needed*

} Issue: LOD

Cleanup

Focus on removal of matrix that co-elutes with the biomarker

(generic C18 cleanup or LLE beneficial for avoiding instrument contamination but may not necessarily be very effective for reducing matrix effects)

⇒ Dedicated cleanup procedures ⇒ con: multiple methods to cover multiple biomarkers

LC separation: separate target biomarker(s) from major matrix peaks

⇒ con: longer chromatographic run time

*Stahnke et al, Anal Chem. 2012 84(3):1474-82.

Matrix effects

LC-MS(/MS), a closer look:

How to deal with matrix effects?

2. Compensate for matrix effects:

Use **isotopically labelled internal standard** (ILIS) of biomarker
Options: add to urine before sample prep or to final extract
Identical phys/chem behaviour, needs to co-elute exactly, requires the isotopically labelled analogue for each biomarker ⇒ requirement: availability of labelled biomarkers

Matrix-matched standards?
Options: add to urine before sample prep or add to final extract
Prepare cal standards in urine/extract ⇒ Which urine?
Differences in matrix effects for different urines

Standard addition
Options: add to urine before sample prep or add to aliquot(s) of final extract
Single level (@2-10x sample concentration) ⇒ requires pre-analysis to estimate conc.
Multi-level ⇒ requires ≥4 measurements/sample

Matrix effects

GC-MS(/MS), a closer look:

How to deal with matrix effects?

1. Reduce matrix effects: low urine equivalent/ml extract, cleanup

2. Compensate for matrix effects:
Use **isotopically labelled internal standard** (ILIS) of biomarker
Typically in blank urine (matrix often improves response/peak shape in GC)

Matrix-matched standards
GC-matrix effects between urines often similar

Use of **'analyte protectants'**
Anastassiades et al, J. Chromatogr. A, 1015 (2003) 163–184.

Standard addition
Only if none of the above works

Guidance documents

Calibration/quantification?

Calibrants in: solvent?
blank urine (what if not available?)
synthetic urine (surine)

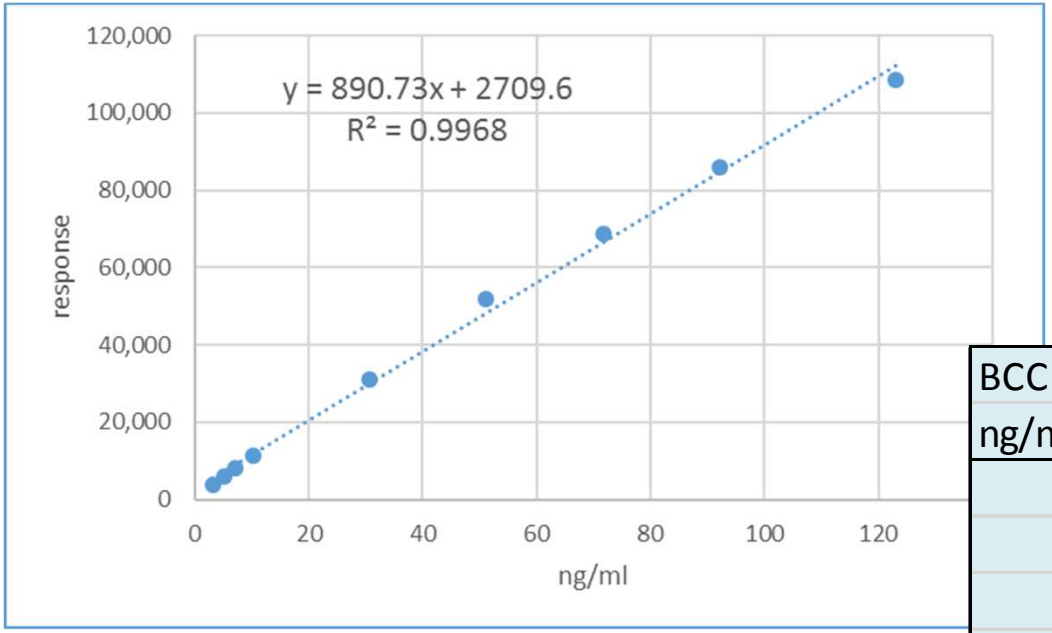
} your procedures/ experiences?

Guidance documents

SANTE/11813/2017 Calibration/quantification

Linearity: Criteria? R^2 (coefficient of determination)?

X	Y
ng/mL	area
3.1	3,805
5.1	5,947
7.2	8,205
10.2	11,502
30.7	31,004
51.2	51,779
71.7	68,903
92.2	86,034
123.1	108,602



BCC	
ng/ml	deviation
1.23	-60%
3.63	-29%
6.17	-14%
9.87	-3%

BCC	weighting 1/x	
ng/ml	deviation	
3.6	15%	4%
5.6	10%	1%
7.8	8%	-3%
10.9	7%	
29.7	-3%	
49.6	-3%	
66.1	-8%	
82.6	-10%	
104.2	-15%	

Do not over-rely on linear regression R^2

Key requirement: back-calculated conc. should not deviate >±20%

Check various options, linear w/wo weighting (1/x), etc

Identification in chromatography – mass spectrometry

your procedures/ experiences?

SANTE/11813/2017 identification

Chromatography + Mass spectrometry required for identification

t_r requirement: ±0.1 min from (average) cal. stds

Table 4. Identification requirements for different MS techniques[2]

MS detector/Characteristics		Acquisition	Requirements for identification	
Resolution	Typical systems (examples)		minimum number of ions	other
Unit mass resolution	Single MS quadrupole, ion trap, TOF	full scan, limited m/z range, SIM	3 ions	S/N ≥ 3[d] Analyte peaks from both product ions in the extracted ion chromatograms must fully overlap. Ion ratio from sample extracts should be within **±30% (relative)** of average of calibration standards from same sequence
	MS/MS triple quadrupole, ion trap, Q-trap, Q-TOF, Q-Orbitrap	selected or multiple reaction monitoring (SRM, MRM), mass resolution for precursor-ion isolation equal to or better than unit mass resolution	2 product ions	
Accurate mass measurement	High resolution MS: (Q-)TOF (Q-)Orbitrap FT-ICR-MS sector MS	full scan, limited m/z range, SIM, fragmentation with or without precursor-ion selection, or combinations thereof	2 ions with mass accuracy ≤ 5 ppm[a, b, c]	S/N ≥ 3[d] Analyte peaks from precursor and/or product ion(s) in the extracted ion chromatograms must fully overlap. Ion ratio: see D12

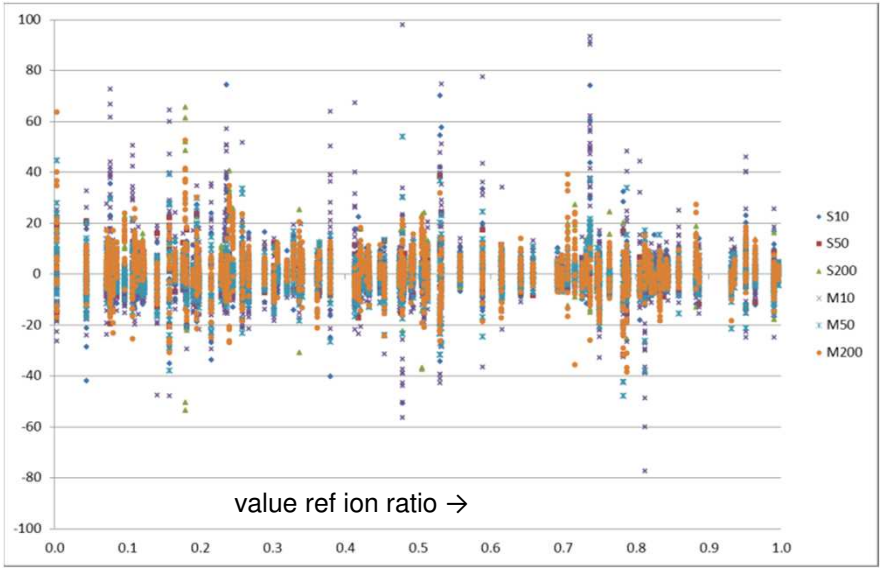
[a] preferably including the molecular ion, (de)protonated molecule or adduct ion
[b] including at least one fragment ion
[c] < 1 mDa for m/z < 200
[d] in case noise is absent, a signal should be present in at least 5 subsequent scans

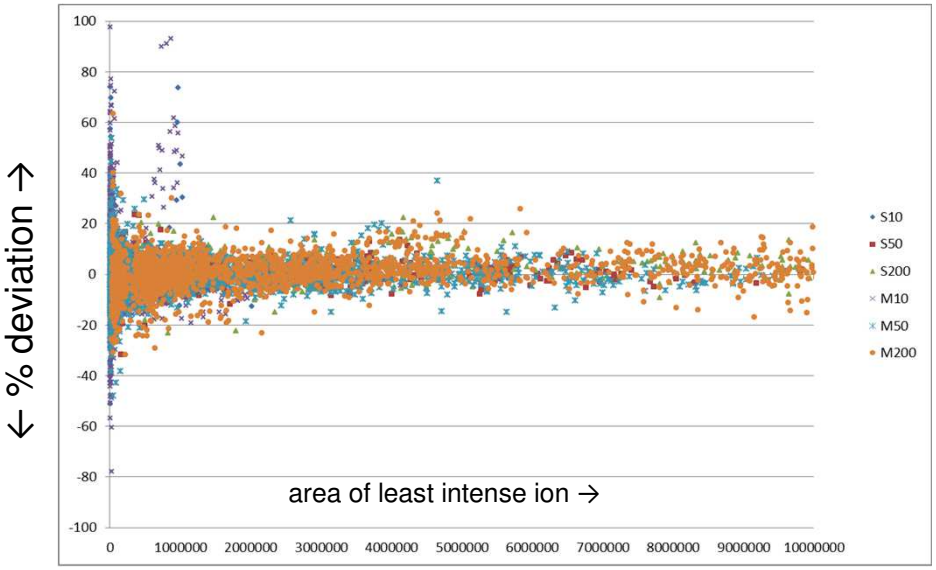
Default criteria, needs to be verified during validation

Guidance documents

SANTE/11813/2017 rationale for the default ±30% ion ratio criterion

LC-MS/MS: deviation of ion ratio in samples vs reference ion ratio in solvent standards for different matrices, different concentrations of >100 pesticides





⇒ Ion ratio is typically within ±30% as long as decent signal is obtained for both ions
Ion ratio is not depending on concentration, ion ratio value, ...

Mol et al, 2015, Analytica Chimica Acta. 873:1–13.
Berendsen et al, 2016, Drug Testing and Analysis. 8:477–490

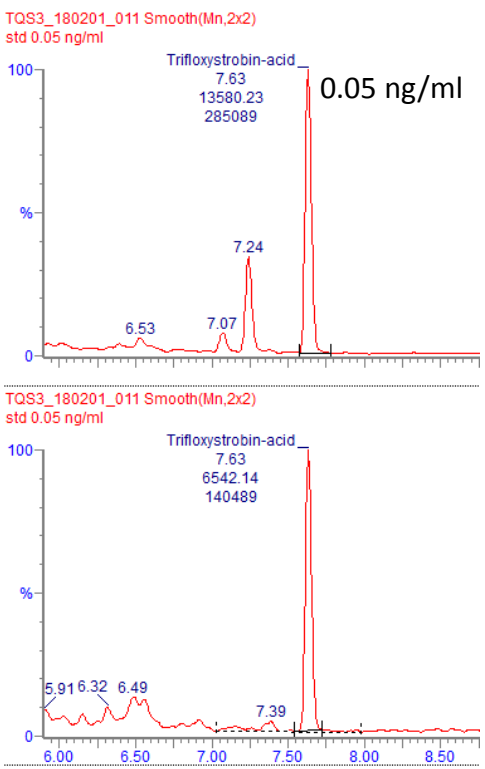
Guidance documents

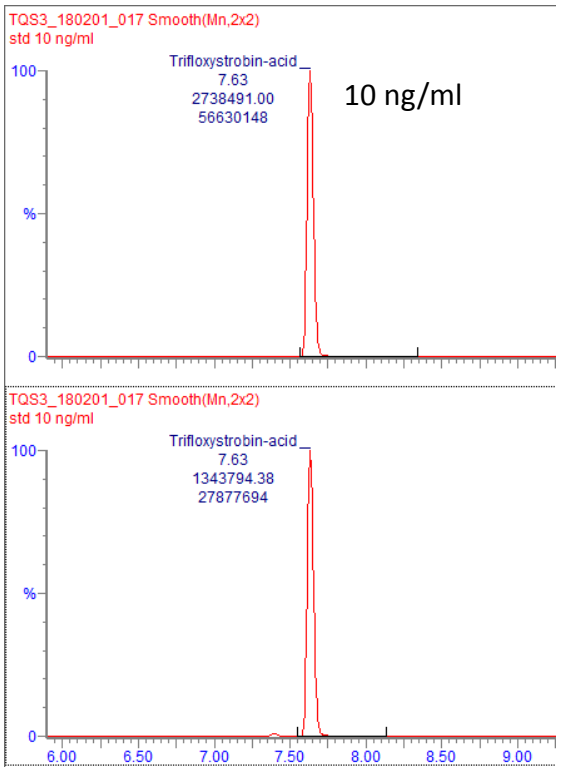
SANTE/11813/2017 identification

Establish reference ion ratio for the sequence

- Based on cal. standards (solvent or urine if interference free)
- Discard responses with poor S/N
- Only use responses within linear range

	Trifloxystrobin-acid			
		quantifier	qualifier	
injection	ng/ml urine	395>186	395>148	ion ratio
1	0	1091	516	
2	0.05	13580	6542	0.482
3	0.1	25494	11912	0.467
4	0.5	118922	59000	0.496
5	1	250027	121722	0.487
6	2	450233	222327	0.494
7	5	1141957	566861	0.496
8	10	2738491	1343794	0.491
22	2	495599	242168	0.489
35	2	516465	255154	0.494
48	2	531695	259670	0.488
61	2	535061	261478	0.489
76	2	545544	267233	0.490
			average	0.489
			RSD	2%
	tolerance window		min -30%	0.342
			max +30%	0.635





Guidance documents

SANTE/11813/2017 validation

Develop or implement method
Check sensitivity/selectivity; matrix effects, choose quantification approach

Two step validation:
1. Basic initial validation (repeatability conditions)
Default: 2 blanks, 5 replicates @ anticipated LOQ, 5 rep's at 10xLOQ or higher if expected

Validation set = 5-6 different* urines
Urine A, B, C, D, E, F; one replicate each, together N=6 for each level

- Analyse as such (n=6)
- Spike at anticipated LOQ (n=6)
- Spike at 2x anticipated LOQ (n=6)
- Spike at 10x LOQ or higher)n=6)

1-2 Procedural blanks

5-6 calibration standards urine / solvent
≤0.5xLOQ to ≥2xhighest spike

→ Check selectivity/interferences
→ Check linearity (matrix effects)
→ Determine average recovery and RSD_r
→ Check compliance identification criteria
→ Check against criteria => pass or fail

*m/f, creatinine, full scan TIC profiles; pre-check for background levels

SANTE/11813/2017 validation

2. On-going validation

With each analysis batch, include QC samples [how many replicates/levels?]

- Samples spiked at LOQ and higher level(s) conjugates usually not available
- Positive sample(s) aliquoted, stored in the freezer for this purpose contains conjugates!

Compile in database or Shewhart chart

Calculate average recovery/trueness and intermediate precision (RSD_{wl})

Other:

Storage stability

Freeze & thaw stability

[Your procedures?]

Guidance documents

SANTE/11813/2017 Validation criteria

Pharma (EMA):
±15%
LLOQ ±20%

≤20% LLOQ

85-115%, LLOQ 80-120%

≤15%, ≤20% LLOQ

Table 5. Validation parameters and criteria

Parameter	What/how	Criterion	Cross reference to AQC document
Sensitivity/linearity	Linearity check from five levels	Deviation of back-calculated concentration from true concentration ≤±20%	C14-C19
Matrix effect	Comparison of response from solvent standards and matrix-matched standards	*	C22-C24
LOQ	Lowest spike level meeting the method performance criteria for trueness and precision	≤ MRL	G6
Specificity	Response in reagent blank and blank control samples	≤30% of RL	C42
Trueness (bias)	Average recovery for each spike level tested	70-120%	G3,G6
Precision (RSD_r)	Repeatability RSD_r for each spike leveltested	≤ 20%	G3, G6
Precision (RSD_{wR})	Within-laboratory reproducibility, derived from on-going method validation / verification	≤ 20%	G3, G6
Robustness	Average recovery and RSD_{wR} derived from on-going method validation / verification	See above	G6, C40-C44
Ion ratio	Check compliance with identification requirements for MS techniques	Table 4	Section D
Retention time		±0.1 min.	D2

* in case of more than 20% signal suppression or enhancement, matrix-effects need to be addressed in calibration (C22-C30)

Outline

Trends in instrumental analysis

HBM meets food safety analysis:
What can we learn from mycotoxin/pesticide residue analysis in food or should at least know about....
- Laboratory networks
- Harmonisation of analytical procedures/performance criteria

LOD/LOQ: sense and nonsense

LOD/LOQ

LOD: limit of detection
LOQ: limit of quantification
LLOQ: lower limit of quantification (pharma)
Decision limit CCα and Detection capability CCβ
LOI: limit of identification
MDL: method detection limit

Various definitions, various ways of determination

[Your procedures?]

Statistical approaches
IUPAC, ISO 11843, DIN 32645, ...

Practical approaches
S/N approach (LOD: S/N = 3; LOQ: S/N=5?, 6?, 10?)
Lowest validated level meeting performance criteria

Eurachem ('3s' approach)

Example LC-MS/MS analysis

What to do
- 10 replicate measurement of test samples with low concentration of analyte
⇒ spike 10 different blank urine samples

Issue
10x 1 urine vs 10 different urines
= close to LOD => range finding needed

- Calibration curve (spike to sample!)
5 points, equidistant, in the range LOD-10xLOD

which urine sample??

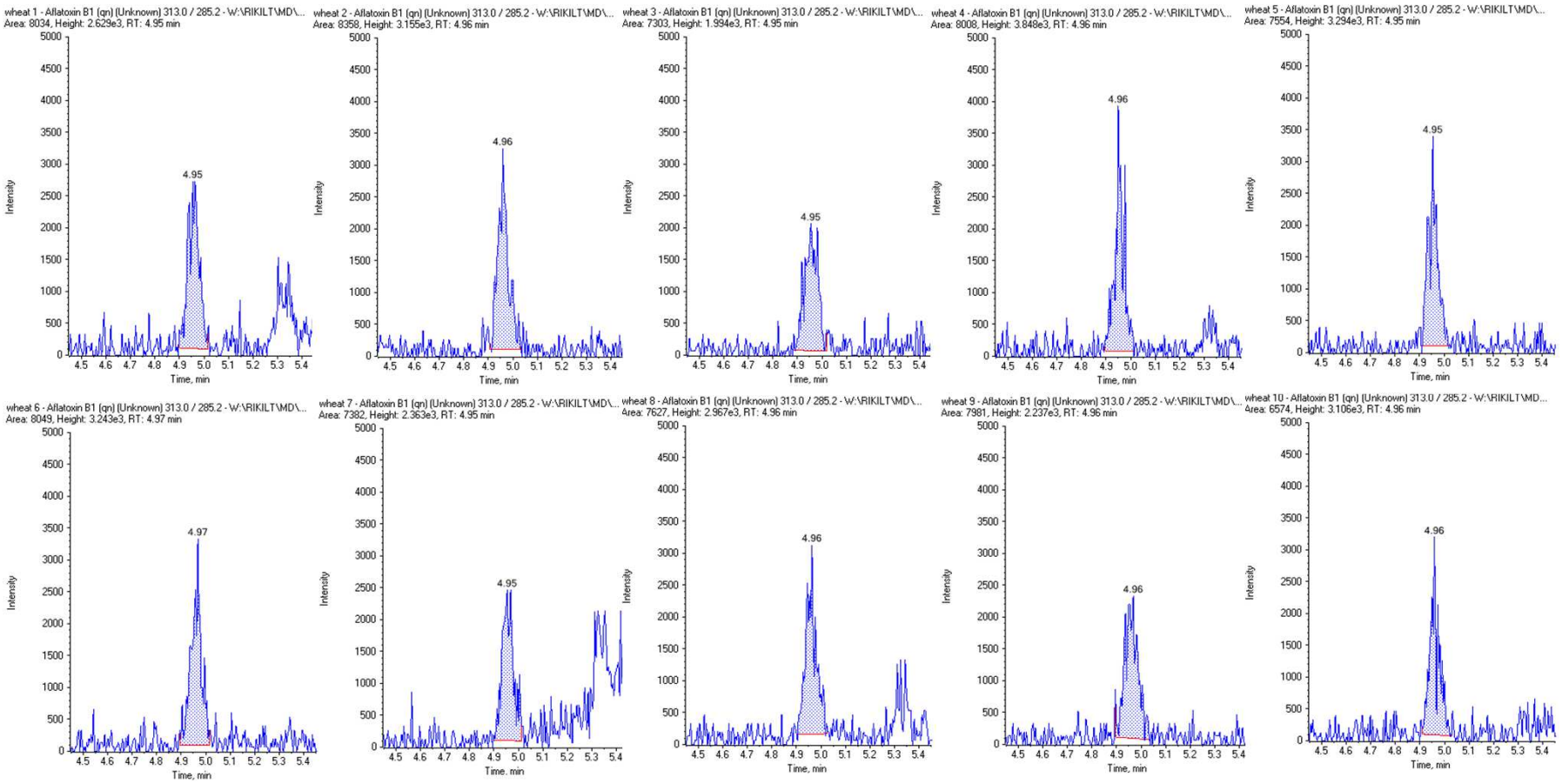
Determine concentration biomarker using the calibration curve
Determine the standard deviation s_0 of the concentrations obtained

SD to be used for LOD determination = $s'_0 = s_0$ in case of single analysis of each sample

LOD = 3 x s'_0
LOQ = 10 x s'_0 (or 6x or 5x?)

Example LOD/LOQ

Aflatoxin B1 spiked @0.26 ng/g (in wheat)



Example LOD/LOQ

Aflatoxin B1 spiked @0.26 ng/g (in wheat)

	ng/g	area
calibrant-1	0.20	5284
calibrant-2	0.40	10355
calibrant-3	0.60	19595
calibrant-4	0.80	20989
calibrant-5	1.00	30883
slope		30916
intercept		-1128
R^2		0.966

Note:
In case of varying matrix effects the LOD may depend on sample used for calibration (slope)

	area	ng/g
sample-1	8034	0.296
sample-2	8358	0.307
sample-3	7303	0.273
sample-4	8008	0.296
sample-5	7554	0.281
sample-6	8049	0.297
sample-7	7382	0.275
sample-8	7627	0.283
sample-9	7981	0.295
sample-10	6574	0.249
average		0.285
SD=S'_0		0.017

LOD = 3*0.017= 0.05 ng/g
LOQ = 10*0.017 = 0.17 ng/g

JRC guidance document

3 methods

‘Blank samples’ (similar to Eurachem)

10 pseudo blank samples (+5 cals)

5 cals equidistant ≤10xLOD

‘Paired observations’

10 pseudo blank samples

Same samples + spike

5 cals equidistant ≤10x LOD

‘Calibration approach’

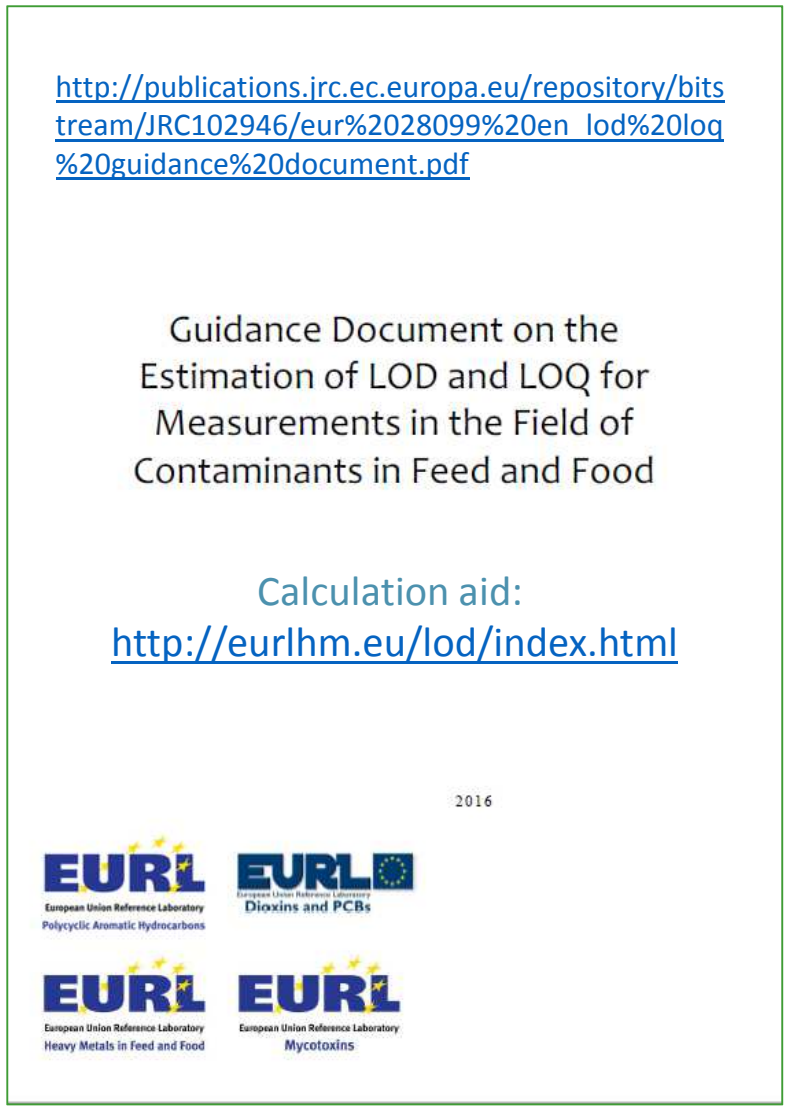
5 cals in duplicate, equidistant, ≤10xLOD

3 methods used, 2 times (one month apart)

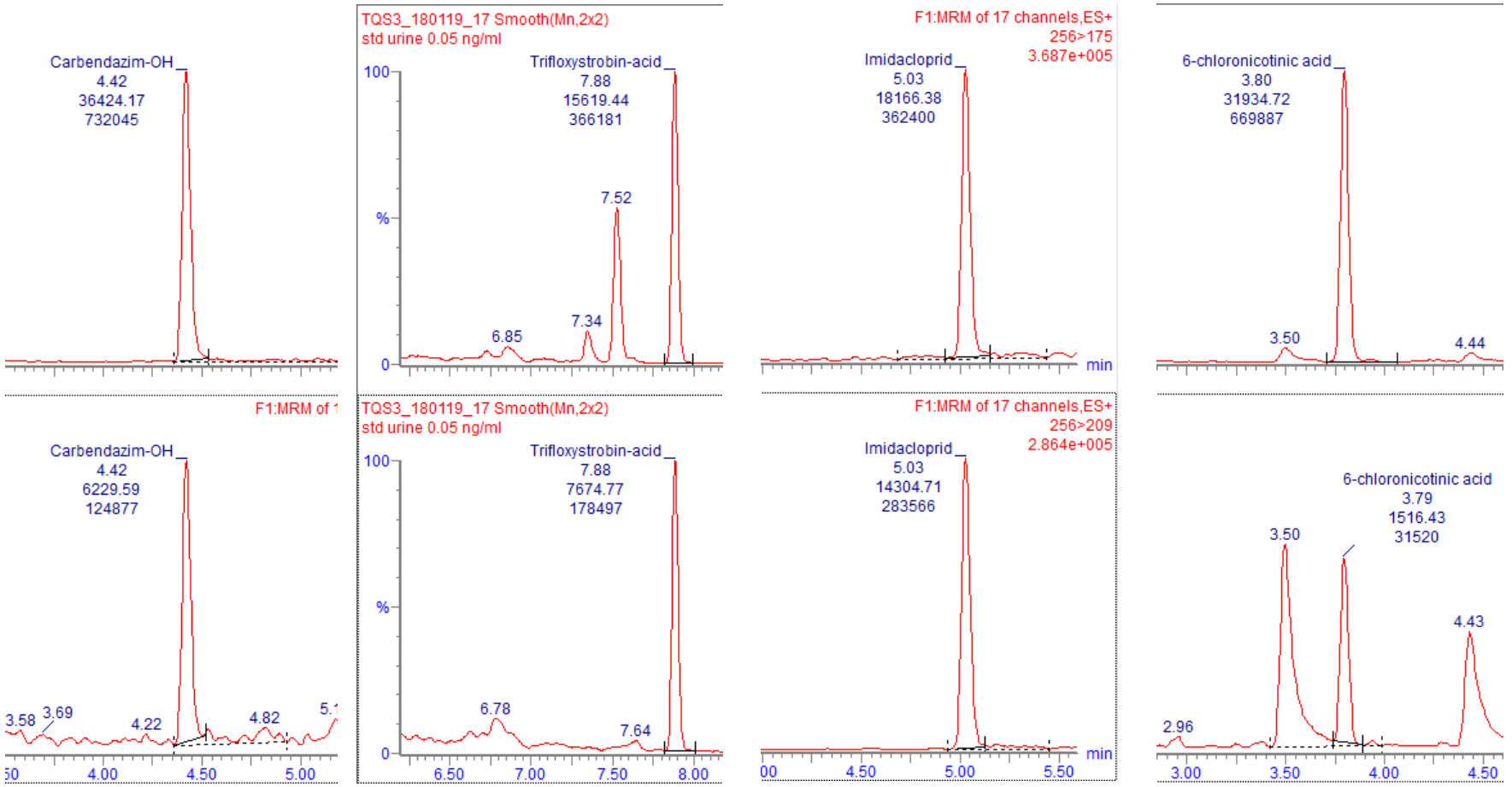
Depending on method and moment

LODs obtained differed by factor 2-6

http://publications.jrc.ec.europa.eu/repository/bitstream/JRC102946/eur%2028099%20en_lod%20loq%20guidance%20document.pdf



Does detection equal identification? *LOD/LOQ*



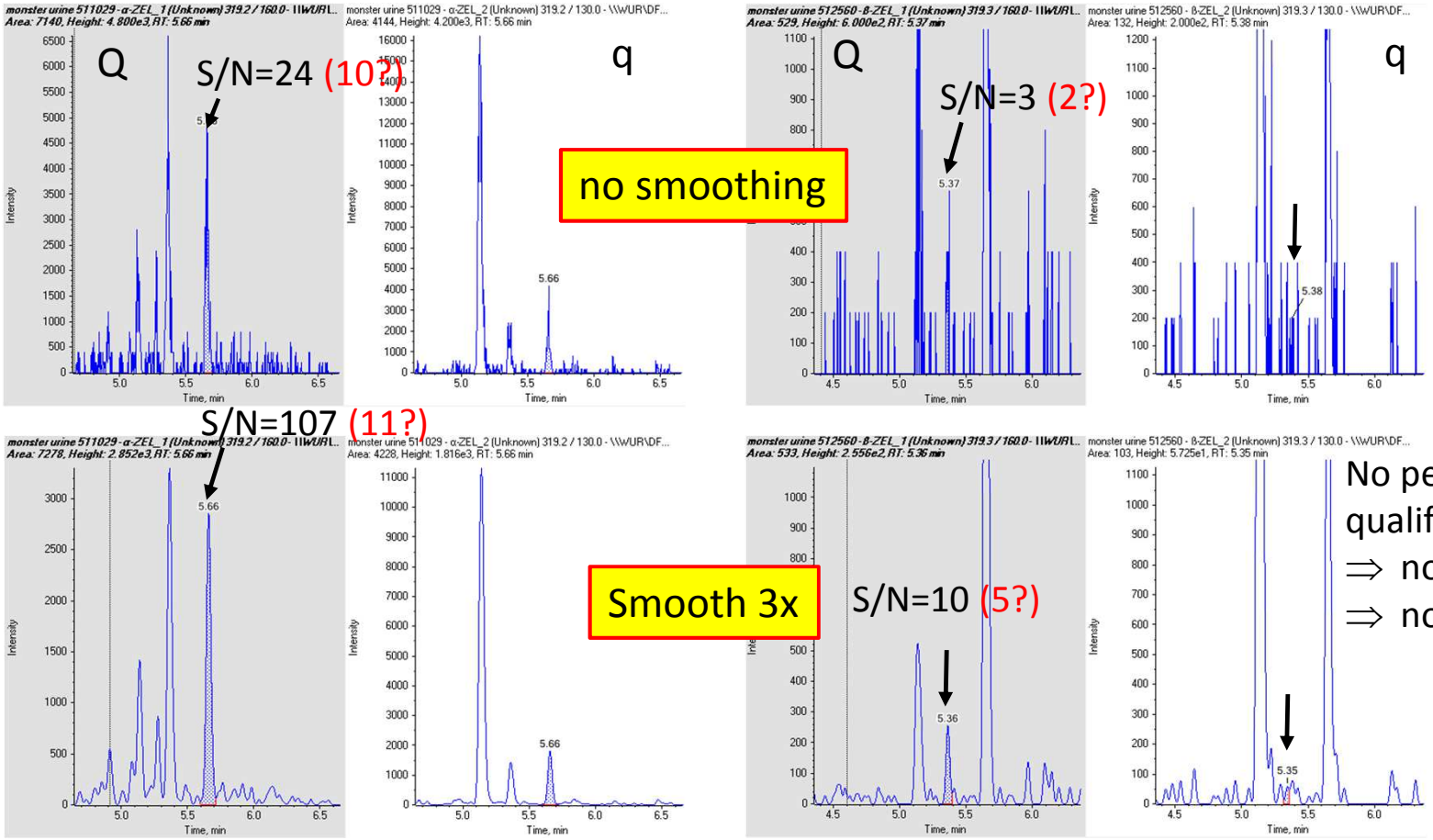
Ion ratio 0.17 | Ion ratio 0.48 | Ion ratio 0.79 | Ion ratio 0.05

S/N approach

Zearalenone biomarkers in urine: S/N software S/N human

α-zearalenol 0.023 ng/ml

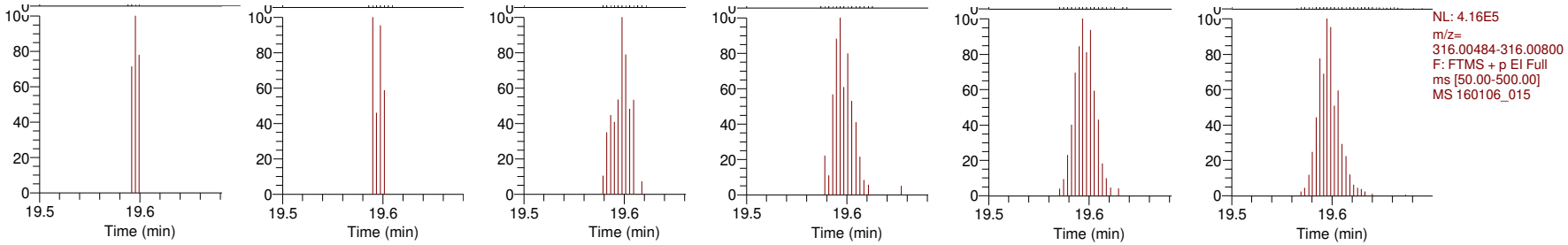
β-zearalenol 0.005 ng/ml



No peak for qualifier ion
⇒ no identification
⇒ not present

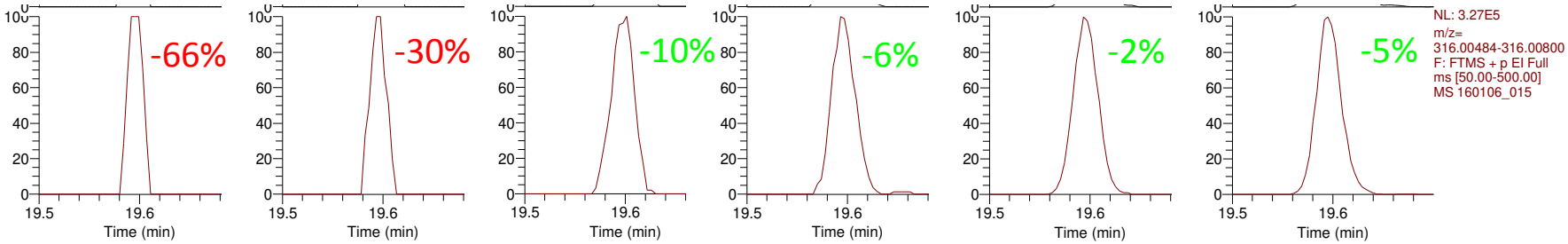
S/N approach

HRMS: no noise.....
Example: Q-Orbitrap:



0.5 pg 1.0 pg 2.5 pg 5.0 pg 10 pg 50 pg on-column

Detection



Quantification

LOD/LOQ

Statistical approaches
Not straightforward
Laborious, iterative
Various options, various outcomes

Practical approaches
S/N approach: affected by software/smoothing, matrix-effects

LOD is not a fixed parameter, it varies with method of determination and in time (with LOQ = n*LOD, same applies for LOQ)

Pragmatic solution:
LOQ = lowest concentration for which it has been demonstrated by (on-going) validation that the criteria for trueness/precision and identification are met.

Use common sense

There are LODs, damned LODs, and LODs from statistics*....
so used common sense like:

Odetokun et al J. Chromatogr B, 878 (2010) 2567–2574
DAPs in urine by LC-MS/MS
"2.6.2. Limits of detection
The LOD was defined as three times the standard deviation of the noise at zero concentration (3S0), where S0 was estimated as the y-intercept of a linear regression analysis of a plot of the standard deviation of the three lowest standards versus the expected concentration from 10 runs [22]. Furthermore, the LOD was compared with the results of the calibration standard samples and low-level spiked samples to ensure that the calculated values agreed with the peak observed and that a minimum signal-to-noise ratio of 3 was present at these low levels."

Schmidt et al, Anal Bioanal Chem (2013) 405:2019–2029
EDCs incl. TCPy in urine by GC-MS/MS)
"Limits of detection (LOD) and limits of quantification (LOQ) were determined by means of a seven equidistant point calibration in pooled urine, according to guideline DIN 32 645. Additionally, the LODs were calculated using a peak-to peak height signal to noise ratio of 3:1, at the lowest calibration concentration of each analyte"

**Rephrased from "There are lies, damned lies, and statistics"*
Quote attributed to Benjamin Disraeli, 19th century British Prime Minister

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Speaker's information

Hans Mol is senior scientist at the department of Natural Toxins and Pesticides, RIKILT, Wageningen, The Netherlands. He is an analytical chemist with more than 20 years of experience in determination of pesticides, mycotoxins, and their metabolites in food, environmental and biological samples.

In HBM4EU he is involved in WP9 (9.4 Quality Assurance, organisation of ICI/EQUAS, 9.1 biomarker/method inventory)

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