HBM4EU project

science and policy
for a healthy future

2nd HBM4EU Training School 2018

A08 Mycotoxins and Pesticides biomarker analysis

SESSION 2:

MYCOTOXIN BIOMARKER ANALYSIS

Determination of deoxynivalenol urinary biomarker(s)

Hans Mol, Hester van der Top

WAGENINGEN
UNIVERSITY & RESEARCH

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

Outline

Mycotoxins in HBM4EU
Deoxynivalenol
Overview of method options
Example specific method & characteristics

Note: instruments, brand names, methods are for information, not HBM4EU endorsements

Mycotoxins in HBM4EU

Mycotoxins:

100's known
Currently 15 regulated in the EU in food commodities
Emerging issue

Included as substance group in
2nd prioritisation round HBM4EU

Prioritisation within the substance group:

Deoxynivalenol

Fumonisin B1
Aflatoxin B1(?)

Others?

Compound group leaders (CGLs):
Paula Alvito, Susana Viegas, Maria João Silva
INSA / ESTeSL-IPL, Portugal

Currently in progress:
- Inventory of biomarker/matrices & methods
- Drafting of Scoping document

Exposure to mycotoxins

General population: food is the main route of exposure
⇒ EFSA is a main stakeholder

Regulated mycotoxins in food
EU Regulation 1881/2006
recommendation 2013/165/EU

Cereals/-products
Nuts, dried fruits,
various other products

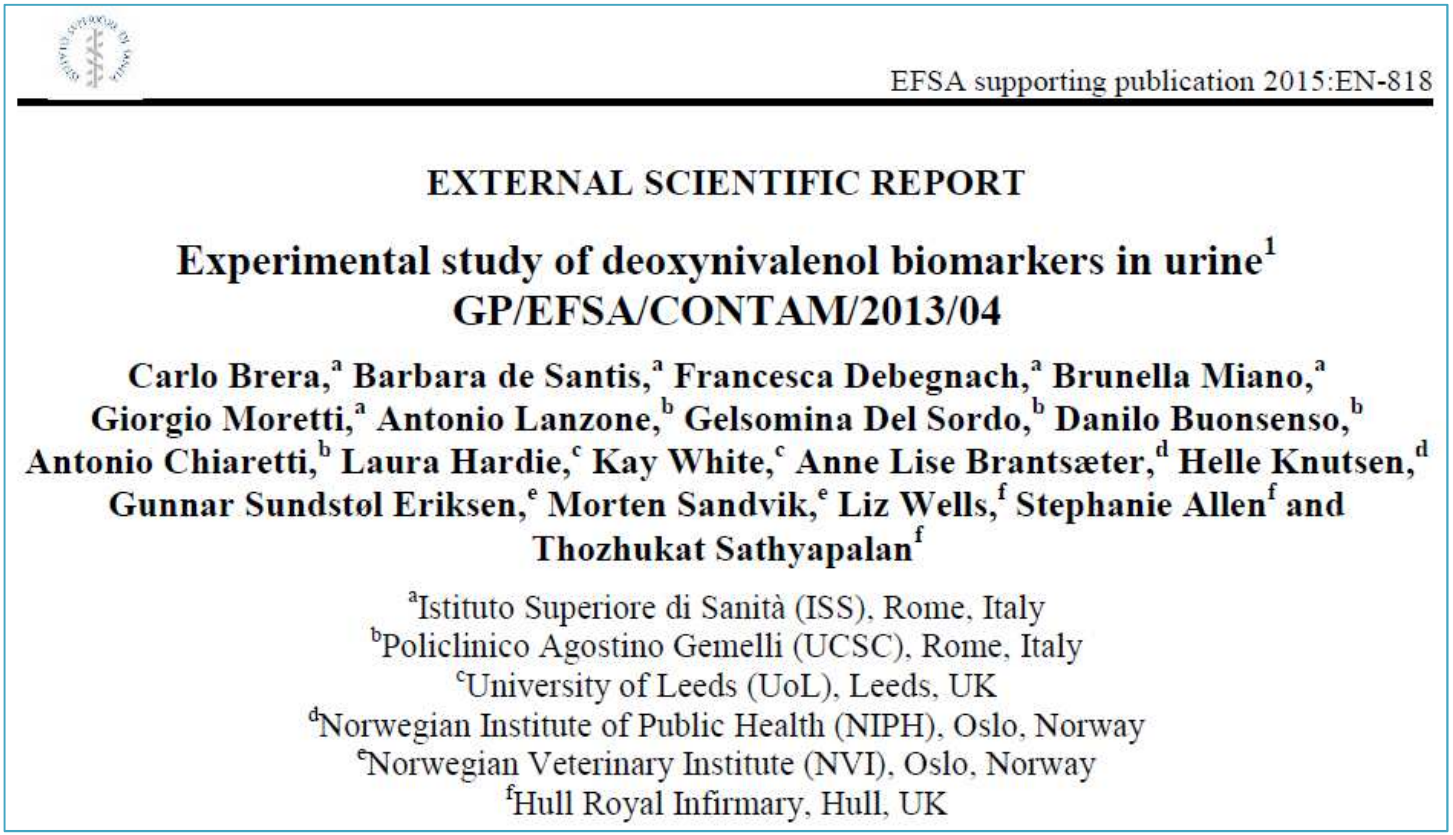
In progress:
Extension other food items
DON derivatives
Ergot alkaloids
Alternaria toxins
.....

Mycotoxins	Max Limit range µg/kg
aflatoxin B1	2-12
aflatoxin B1,B2,G1,G2	4-15
aflatoxin M1	0.05
citrinin	2000 (RYR)
deoxynivalenol	200-1750
ergot sclerotia (kernels)	0.5 g/kg
fumonisin B1	200-4000
fumonisin B2	
ochratoxin A	2-20
patulin	10-50
T-2 toxin	15-1000*
HT-2 toxin	
zearalenone	20-400

*recommendation

HBM of mycotoxins

Relatively low number of studies
Usually NOT included in existing/on-going HBM monitoring programs
Recently increasing interest (modified forms, heterogeneity in food, ...)



EFSA supporting publication 2015:EN-818

EXTERNAL SCIENTIFIC REPORT

Experimental study of deoxynivalenol biomarkers in urine[1]
GP/EFSA/CONTAM/2013/04

Carlo Brera,[a] Barbara de Santis,[a] Francesca Debegnach,[a] Brunella Miano,[a] Giorgio Moretti,[a] Antonio Lanzone,[b] Gelsomina Del Sordo,[b] Danilo Buonsenso,[b] Antonio Chiaretti,[b] Laura Hardie,[c] Kay White,[c] Anne Lise Brantsæter,[d] Helle Knutsen,[d] Gunnar Sundstøl Eriksen,[e] Morten Sandvik,[e] Liz Wells,[f] Stephanie Allen[f] and Thozhukat Sathyapalan[f]

[a]Istituto Superiore di Sanità (ISS), Rome, Italy
[b]Policlinico Agostino Gemelli (UCSC), Rome, Italy
[c]University of Leeds (UoL), Leeds, UK
[d]Norwegian Institute of Public Health (NIPH), Oslo, Norway
[e]Norwegian Veterinary Institute (NVI), Oslo, Norway
[f]Hull Royal Infirmary, Hull, UK

Background information deoxynivalenol (DON)

Main source of exposure:

Cereals and cereal products

Grains, flour

Breakfast cereals

Bread/bakery products

Pasta

Beer

.....

Health based guidance values:

group-TDI of 1 µg/kg bw per day for the sum of the four DON forms

group-ARfD of 8 µg/kg bw per eating occasion

SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 26 January 2017

doi: 10.2903/j.efsa.2017.4718

Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed

EFSA Panel on Contaminants in the Food Chain (CONTAM),
Helle Katrine Knutsen, Jan Alexander, Lars Barregård, Margherita Bignami, Beat Brüschweiler, Sandra Ceccatelli, Bruce Cottrill, Michael Dinovi, Bettina Grasl-Kraupp, Christer Hogstrand, Laurentius (Ron) Hoogenboom, Carlo Stefano Nebbia, Isabelle P Oswald, Annette Petersen, Martin Rose, Alain-Claude Roudot, Tanja Schwerdtle, Christiane Vleminckx, Günter Vollmer, Heather Wallace, Sarah De Saeger, Gunnar Sundstøl Eriksen, Peter Farmer, Jean-Marc Fremy, Yun Yun Gong, Karsten Meyer, Hanspeter Naegeli, Dominique Parent-Massin, Ivonne Rietjens, Hans van Egmond, Andrea Altieri, Mari Eskola, Petra Gergelova, Luisa Ramos Bordajandi, Bistra Benkova, Barbara Dörr, Athanasios Gkrillas, Nicklas Gustavsson, Mathijs van Manen and Lutz Edler

Abstract

Deoxynivalenol (DON) is a mycotoxin primarily produced by *Fusarium* fungi, occurring predominantly in cereal grains. Following the request of the European Commission, the CONTAM Panel assessed the risk to animal and human health related to DON, 3-acetyl-DON (3-Ac-DON), 15-acetyl-DON (15-Ac-DON) and DON-3-glucoside in food and feed. A total of 27,537, 13,892, 7,270 and 2,266 analytical data for DON, 3-Ac-DON, 15-Ac-DON and DON-3-glucoside, respectively, in food, feed and unprocessed grains collected from 2007 to 2014 were used. For human exposure, grains and grain-based products were main sources, whereas in farm and companion animals, cereal grains, cereal by-products and forage maize contributed most. DON is rapidly absorbed, distributed, and excreted. Since 3-Ac-DON and 15-Ac-DON are largely deacetylated and DON-3-glucoside cleaved in the intestines the same toxic effects as DON can be expected. The TDI of 1 µg/kg bw per day, that was established for DON based on reduced body weight gain in mice, was therefore used as a group-TDI for the sum of DON, 3-Ac-DON, 15-Ac-DON and DON-3-glucoside. In order to assess acute human health risk, epidemiological data from mycotoxicoses were assessed and a group-ARfD of 8 µg/kg bw per eating occasion was calculated. Estimates of acute dietary exposures were below this dose and did not raise a health concern in humans. The estimated mean chronic dietary exposure was above the group-TDI in infants, toddlers and other children, and at high exposure also in adolescents and adults, indicating a potential health concern. Based on estimated mean dietary concentrations in ruminants, poultry, rabbits, dogs and cats, most farmed fish species and horses, adverse effects are not expected. At the high dietary concentrations, there is a potential risk for chronic adverse effects in pigs and fish and for acute adverse effects in cats and farmed mink.

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Keywords: Deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, deoxynivalenol-3-glucoside, exposure, toxicity, human and animal risk assessment

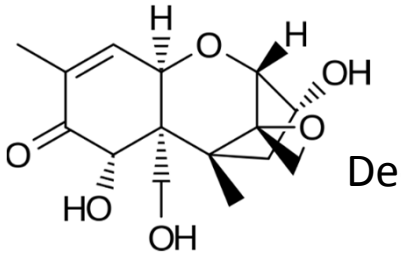
Requestor: European Commission

Question Number: EFSA-Q-2013-00721

Correspondence: contam@efsa.europa.eu

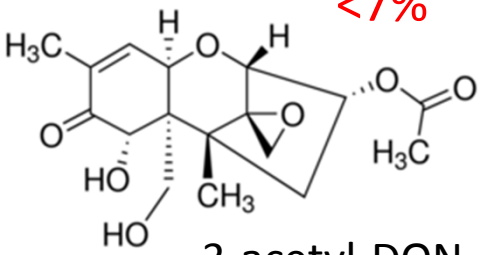
Deoxynivalenol *DON*

Main forms in food:



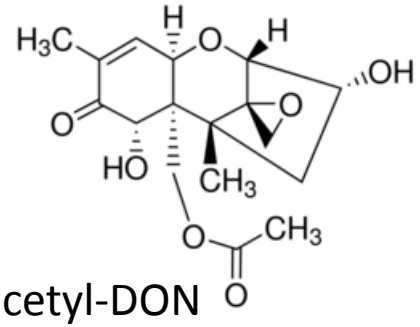
≥70%

Deoxynivalenol (DON)



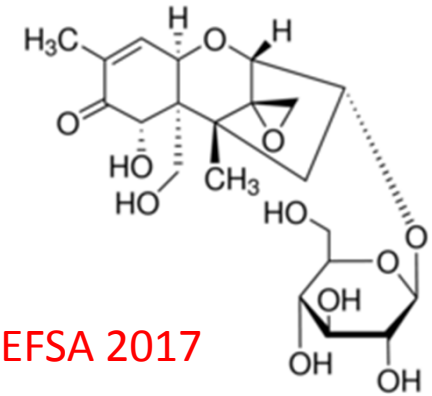
<7%

3-acetyl-DON



<10%

15-acetyl-DON

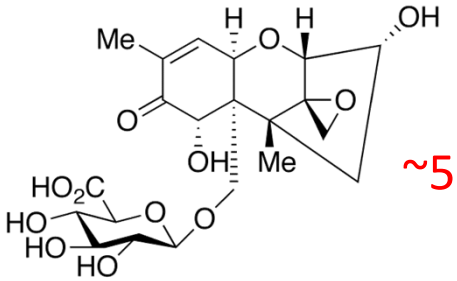


DON-3-glucoside

<20% (higher in beer)

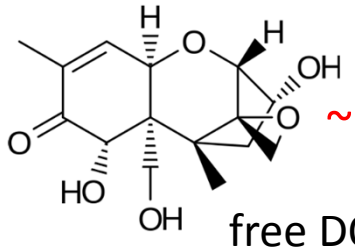
EFSA 2017

Main human biomarkers:



~58%

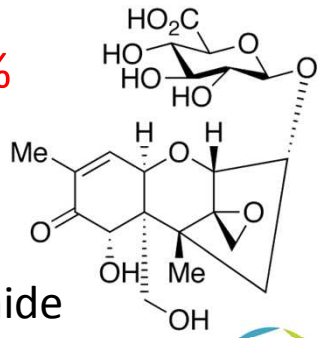
DON-15-glucuronide



~27%

free DON

~14%

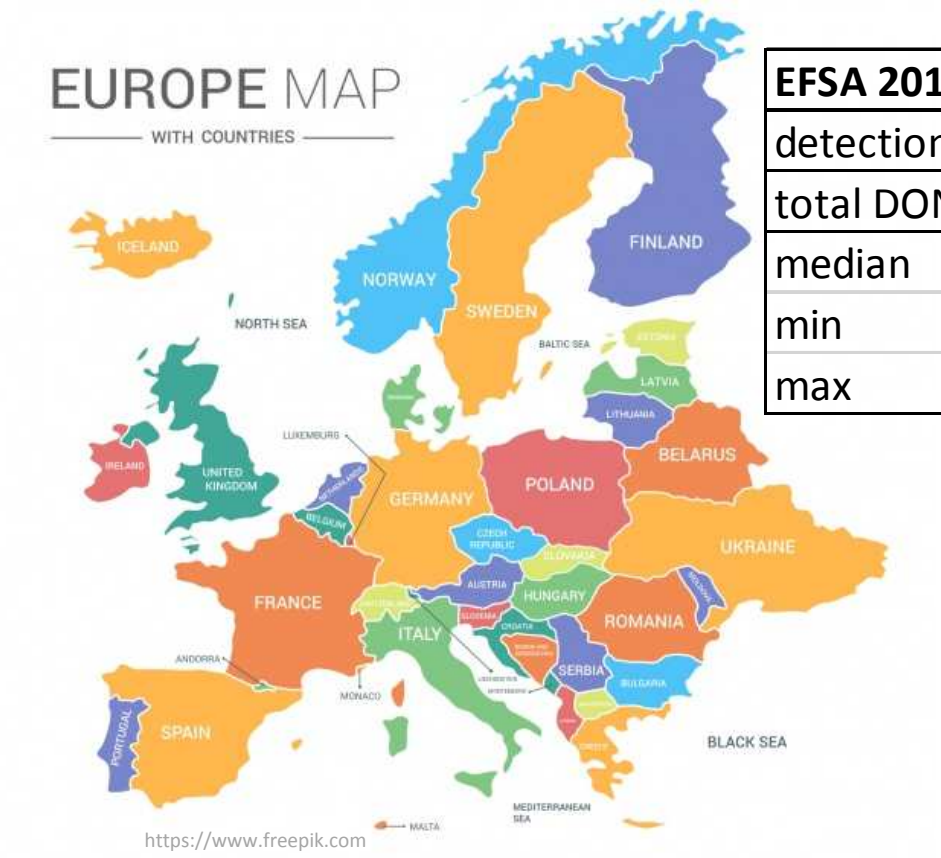


DON-3-glucuronide

Vidal et al, 2018

Deoxynivalenol *DON*

Expected levels DON biomarkers in general population:



https://www.freepik.com

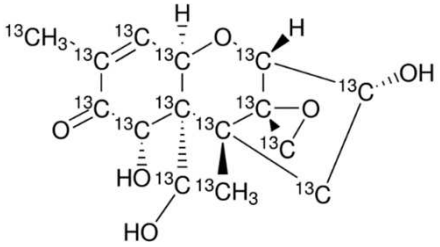
EFSA 2015 supp.pub.	Norway	UK	Italy
detection rate%	99%	93%	76%
total DON	ng/ml morning voids 2 days		
median	3.6-7.3	10.7-12.6	4-13
min	<0.015	0.9-5.1	<0.5
max	19-43	29-59	14-51

⇒ Target LOQ ≤0.5 ng/ml

Deoxynivalenol: target biomarkers

DON

Options for determination of DON biomarkers:



1) Individual three main biomarkers
- Availability of standards:
  - DON and $^{13}C_{15}$-DON readily available
  - DON-15-glucuronide
  - DON-3-glucuronide

} Only custom made $$$$

2) Total DON
free DON + DON-glucuronides after deconjugation glucuronides

Pro: only 1 biomarker, ILIS available
Con: deconjugation step needed

Methods from literature

DON

Without deconjugation:

only free DON or DON-conjugates with custom made standards

[1] Gerding (2015) Mycotoxin Res 31:127–136
[2] Huybrechts (2015), Arch Toxicol 89:1993–2005
[3] Njumbe Ediage (2012) Analytica Chimica Acta 58– 69
[4] Rubert (2011) Food Chem Toxicol 49:2299–2304
[5] Warth (2012) Rapid Com. Mass Spectrom. 26:1533–1540

With deconjugation (total DON, often also including DOM-1)

[6] Cunha (2012) Food Chem Toxicol 50:1019–1026
[7] Turner (2010) J. Agric. Food Chem. 58:5206–5212
[8] Brera (2015) EFSA supporting publication EN-818
[9] Ali (2015) Toxins 7:3845-3857
[10] Solfrizzo (2011) Anal Bioanal Chem 401:2831–2841
[11] Wallin (2013) World Mycotoxin Journal, 6: 439-448
[12] Sarkanj (2018) Analytica Chimica Acta 1019:84-92
[13] Deng (20128) Scientific Reports 8:3901

Methods from literature

DON

Deconjugation

Extraction/cleanup

Instrumental analysis

Deconjugation

DON

Various options

Chemical:	hydrolysis/cleavage under acid or alkaline conditions
Parameters:	type/conc. acid/base, temperature, time
Example:	addition of 37% HCl/70-100°C, 1-3h; 0.1-1 M NaOH
Remark:	degradation of biomarker, degradation of matrix (dirty extracts)
Biological:	**enzymes**, various options differing in activity specificity source: molluscs (*Helix Pomatia*); bacterial (*E. Coli*) most have β-glucuronidase activity, variation in activity/specificity
Parameters:	enzyme, pH, units added, temperature, time, biomarker-conjugate (isomers!)

Helix Pomatia:	optimum pH
β-glucuronidase	pH 4-5
Arylsulfatase	pH ~6.5
Other...(esterase..)	

E. Coli	optimum pH
β-glucuronidase	pH ~6-7

Methods from literature

DON

Deconjugation different enzymes/conditions

Ref	ml	Deconjugation enzyme	pH	time
6	1.0	β-gluc./arylsulf. (Helix P.) (Sigma)	5	18h
7	4	β-gluc (E. coli IX-A)	6.8	18h
8	1.0	β-gluc (E. coli IX-A)	6.8	18h
9	3.0	β-gluc./arylsulf. (Helix P.) (Roche)	5	18h
10	6.0	β-gluc./arylsulf. (Helix P.) (-)	-	overnight
11	1	β-gluc (E. coli IX-A)	7.4	overnight
12	0.5	β-gluc (E. coli IX-A)	7.4	16h
13	1	β-gluc (E. coli IX-A)	6.8	18h

Does it matter?

Deconjugation

DON

[6] Effect of type of enzyme / units (18h, 37°C)

1 mL urine sample (spiked/pos?)

S.C. Cunha, J.O. Fernandes / Food and Chemical Toxicology 50 (2012) 1019–1026

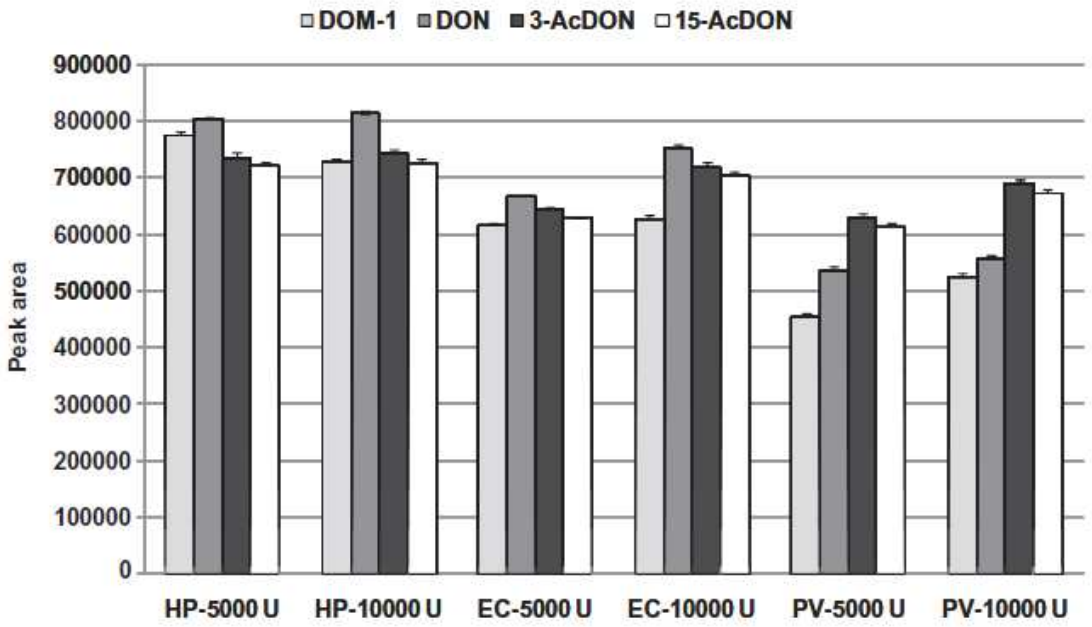


Fig. 2. Average of peak area ($n = 3$) using different types of β-glucuronidase enzymes at 5000 or 10000 U. HP (*H. pomatia*), EC (*E. coli*) and PV (*P. vulgate*).

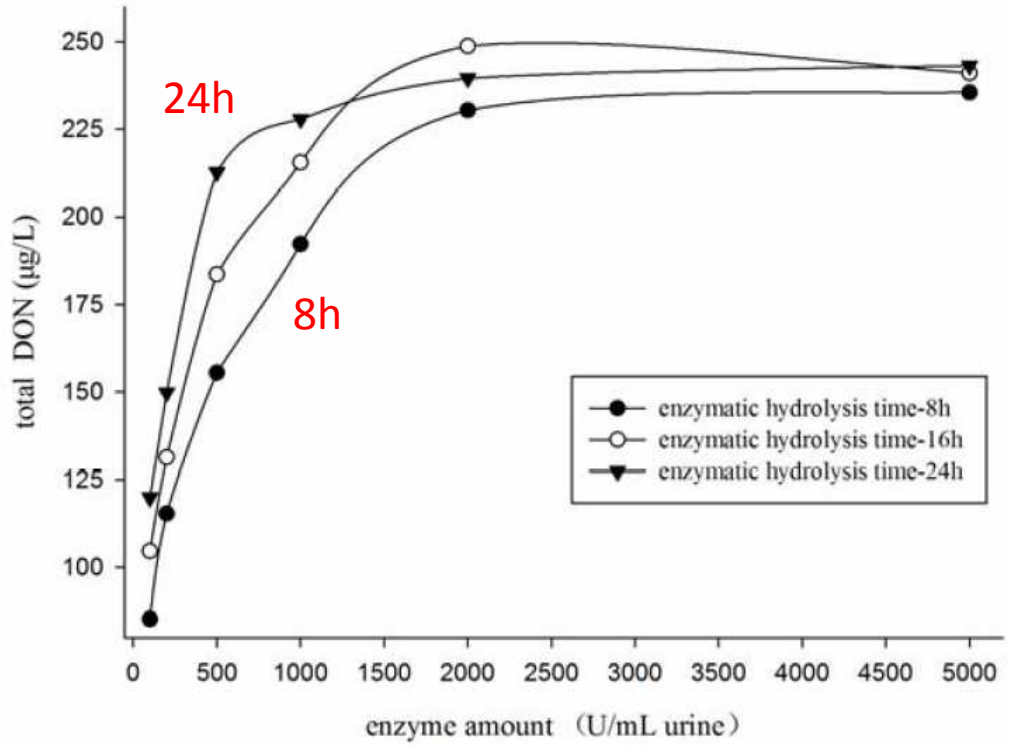
HP = β-Glucuronidase Type 1 from Helix pomatia (Sigma); pH 5
EC = β-Glucuronidase Type IX from Escherichia coli (Sigma); pH 6.8
PV = β-Glucuronidase Type L-II from Patella vulgate (Sigma); pH 5

Deconjugation

DON

[13] Effect of enzyme concentration and time

1 mL positive urine sample + 1.5 ml phosphate buffer (75 mM, pH 6.8) containing X units of β-glucuronidase (Type IX from *E. coli*)



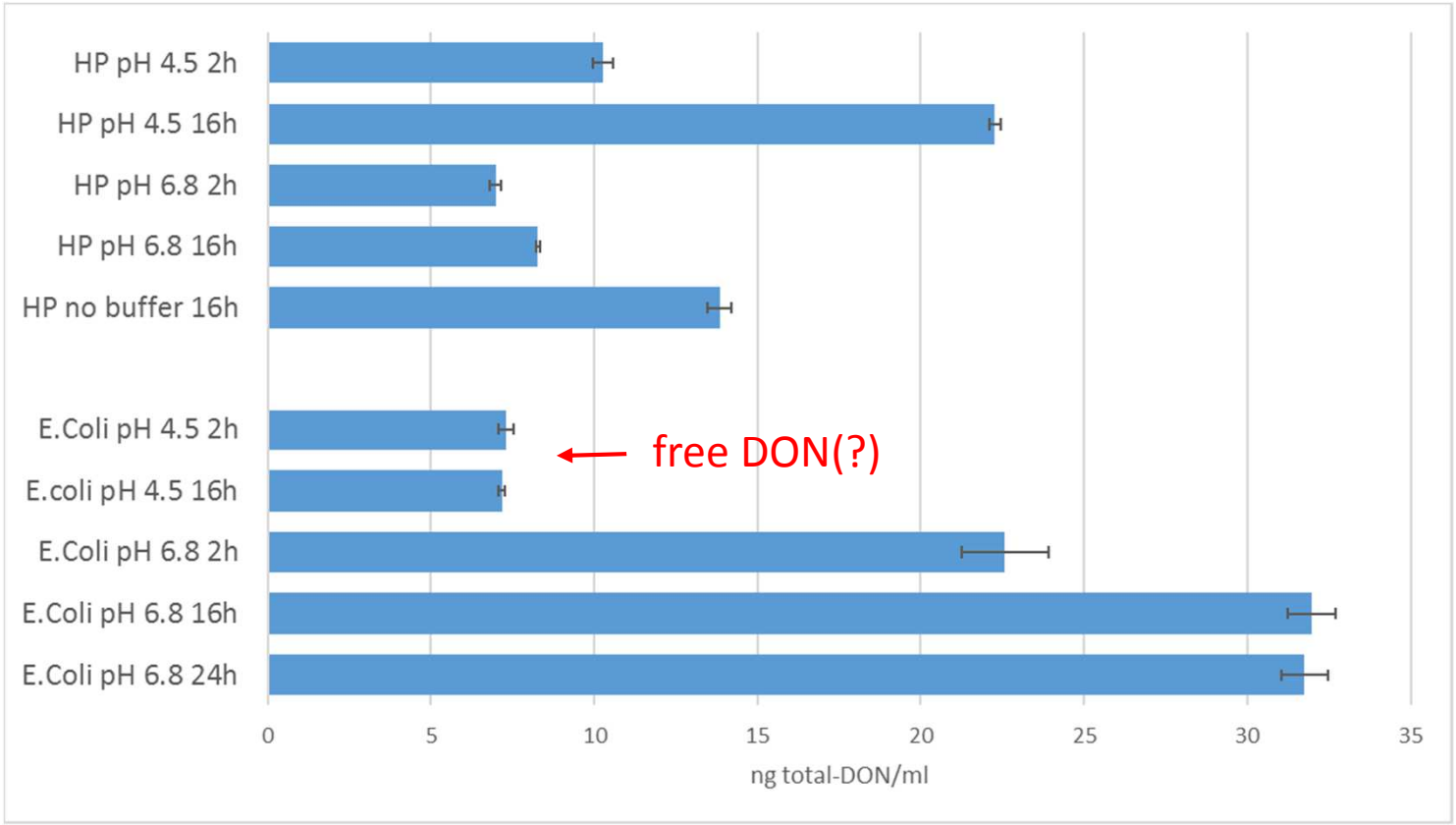
Deng et al (2018) Scientific Reports 8:3901.

Figure S1. Effects of enzyme concentration and incubation time on the extent of deconjugation of DON-glucuronide.

Deconjugation DON-glucuronides

[RIKILT] Effect of enzyme, pH, time

1 mL positive urine sample, experiments in triplicate (+$^{13}C_{15}$-label after deconjugation)



HP= β-Glucuronidase/aryl sulfatase from *Helix Pomatia* (Merck 104114); 1 ml urine + 2 ml 200mM NaAc buffer + 10 µl enzyme
E.Coli = β-Glucuronidase from *Escherichia coli* Type IX-A (Sigma G7396); 1 ml urine + 2 ml 75 mM phosphate buffer + 3000 U enzyme

Extraction/cleanup

DON

Dilute & shoot

+ very straightforward
+ ability to simultaneously detect other mycotoxin biomarkers (and more)
- ion suppression (depending on dilution)
- interferences
- Insufficient sensitivity (depending on instrument)

⇒ LOQs reported for DON 0.5-13 ng/ml

Generic cleanup

± additional step in sample prep
± ability to simultaneously detect multiple other mycotoxins biomarkers
± ion suppression (depending on urine equiv./ml extract)
± Interferences (depending on chemistries of cleanup)
±/+ adequate sensitivity (depending on instrument)

e.g. SPE C18, OASIS (PRiME) HLB, ...

Extraction/cleanup

DON

Specific cleanup

± additional step in sample prep
- only one or few mycotoxin biomarkers in one method
+ minor matrix effects/ion suppression (depending on urine equiv./ml extract)
+ little interferences (depending on chemistries of cleanup)
+ low LOQs (depending on instrument)

Immunoaffinity cleanup columns (IAC)

DONPrep (R-Biopharm Rhone Ltd)
DonStar™ IAC (Romer labs)
DONTest WB (Vicam)
Immunoclean CF DON from Aokin
Neocolumn for DON (Neogen Europe Ltd)
and more.....

Multi IAC cleanup?

[aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, nivalenol, T-2 and HT-2 toxins]
e.g. Myco6in1 [10]

Methods from literature

DON

Deconjugation different enzymes/conditions

Extraction/cleanup: SPE or IAC

Instrumental analysis: LC-MS/MS, GC-MS(/MS)

Ref	ml	Deconjugation enzyme	pH	time	Extr./cleanup
6	1.0	β-gluc./arylsulf. (Helix P.) (Sigma)	5	18h	SPE C18
7	4	β-gluc (E. coli IX-A)	6.8	18h	IAC
8	1.0	β-gluc (E. coli IX-A)	6.8	18h	IAC
9	3.0	β-gluc./arylsulf. (Helix P.) (Roche)	5	18h	IAC
10	6.0	β-gluc./arylsulf. (Helix P.) (-)	-	overnight	multi-IAC+SPE (HLB)
11	1	β-gluc (E. coli IX-A)	7.4	overnight	IAC
12	0.5	β-gluc (E. coli IX-A)	7.4	16h	SPE (HLB)
13	1	β-gluc (E. coli IX-A)	6.8	18h	µSPE HLB (96 well)

GC-MS(/MS): requires derivatisation

[6] dry extract: 100 µL of BSA + TMCS + TMSI (3:2:3); 20 min 80°C

LC-MS/MS:

Table 3. Example MS/MS transitions (not exhaustive).

Mycotoxin	ESI	precursor ion (m/z) [a]		product ion 1 [a]	product ion 2 [a]	product ion 3 [a]
DON	pos	M+H	297	249	231	203
	neg	M+acetate [b]	355	265	295	59 [c]
$^{13}C_{15}$-DON	pos	M+H	312	263	245	216
	neg	M+acetate [b]	370	279	310	59 [c]

[a] the relative abundance or optimum S/N for the transitions depend on the instrument and matrix, and needs to be experimentally optimized/verified.

[b] when formate and/or formic acid is used in the mobile phase, formate adducts instead of acetate adducts can be formed.

[c] measurement of acetate as product ion can be rather non-specific and is therefore not recommended.

https://www.wur.nl/en/Research-Results/Research-Institutes/rikilt/Reference-laboratory/European-Union-Reference-Laboratory-1/Library-EURL-MP.htm#eurl_mp_methods_from_2018

Methods from literature *DON*

Deconjugation different enzymes/conditions
Extraction/cleanup: SPE or IAC
Instrumental analysis: LC-MS/MS, GC-MS(/MS)

Ref	ml	Deconjugation enzyme	pH	time	Extr./cleanup	analysis	scope
6	1.0	β-gluc./arylsulf. (Helix P.) (Sigma)	5	18h	SPE C18	GC-MS*	DONs
7	4	β-gluc (E. coli IX-A)	6.8	18h	IAC	LC-MS	DONs
8	1.0	β-gluc (E. coli IX-A)	6.8	18h	IAC	LC-MS &HRMS	DONs
9	3.0	β-gluc./arylsulf. (Helix P.) (Roche)	5	18h	IAC	LC-MS/MS	DONs
10	6.0	β-gluc./arylsulf. (Helix P.) (-)	-	overnight	multi-IAC+SPE (HLB)	LC-MS/MS	DONs +[a]
11	1	β-gluc (E. coli IX-A)	7.4	overnight	IAC	LC-MS/MS	DONs
12	0.5	β-gluc (E. coli IX-A)	7.4	16h	SPE (HLB)	LC-MS/MS	DONs +[b]
13	1	β-gluc (E. coli IX-A)	6.8	18h	µSPE HLB (96 well)	LC-MS/MS	DONs

*After derivatisation: 100 µL of BSA + TMCS + TMSI (3:2:3); 20 min 80°C
DONs include DOM-1 (acetyl-DONs)
[a] DON, DOM-1, aflatoxin M1, ochratoxin A, fumonisin B1, α-zearalenol, β-zearalenol
[b] DON, DOM-1, aflatoxin M1, alternariol, citrinine/dihydrocitrinine, fumonisin B1, ochratoxin A, α-zearalenol, β-zearalenol, zearalenone

Example specific method

DON

Thaw urine, vortex, take 1.0 ml aliquot

Deconjugation

Add 2 ml 75 mM phosphate buffer pH 6.8

Add 250 µl enzyme solution (~3000 U β-Glucuronidase *E. coli* type IX-A)

37°C overnight (at least 16h)

Cool down, add 2 ml milliQ water

Add $^{13}C_{15}$-DON internal standard

Extraction/cleanup

IAC (Vicam DONTEST)

drain, rinse 2 ml milliQ water

load entire deconjugated sample

rinse 2 ml millQ water

elute 2 ml MeOH (soak & then elute)

Evaporate to dry 55°C/N2

Reconstitute in 200 µl 20% MeOH/water, vortex

Transfer into filter vial/press through

LC-MS/MS analysis

Inject 10-25 µl

Acquity UPLC 100 x 2.1 mm 1.8µm HSS T3; 35°C, 0.40 ml/min

5 mM NH_4Ac, 0.1% acetic acid, MeOH gradient

Sciex Qtrap 6500, ESI positive: m/z 297>249, 297>231; label: 312>263

Quantification: multi-level solvent standards with $^{13}C_{15}$-DON internal standard

Validation

DON

Pre-screen urine analysis for blank samples
6 different urine samples

<u>Validation set:</u>
Procedural blank (water)
Urine
Urine spikes @ 0.5, 1, 5 ng/ml single analysis

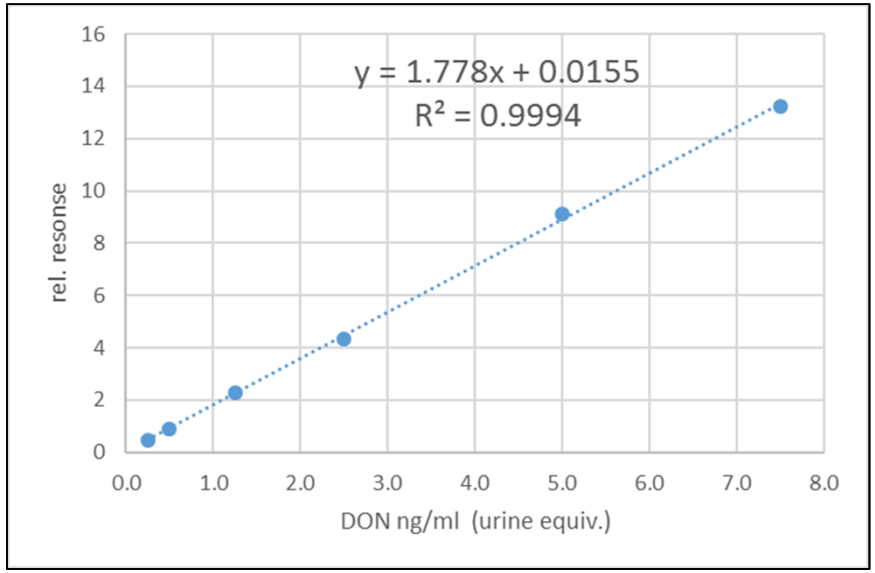
Calibrants in solvent equivalent to 0.25, 0.5, 1.25, 2.5, 5, 7.5 ng/ml urine
Solvent injection <> carry-over

Validation results

DON

Calibration (solvent standards with 13C-DON)

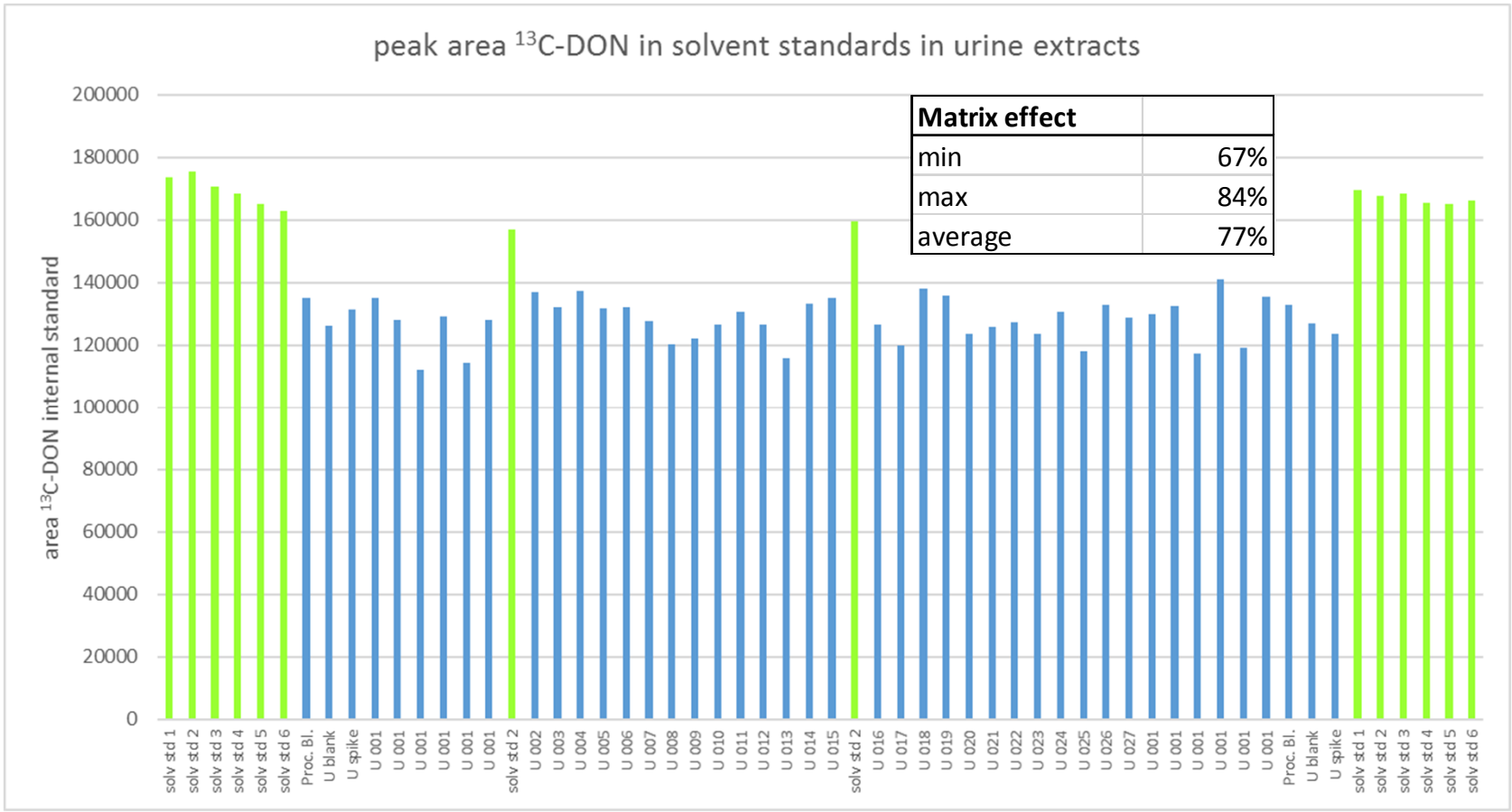
ng/ml (urine equiv.)	rel. response	BCC ng/ml	deviation
0.25	0.467	0.25	2%
0.50	0.901	0.50	0%
1.25	2.255	1.26	1%
2.50	4.320	2.42	-3%
5.00	9.132	5.13	3%
7.50	13.243	7.44	-1%



Validation results

DON

Matrix effects (data from analysis sequence)



Matrix effect	
min	67%
max	84%
average	77%

Solvent standards urine extracts

Validation results

DON

Recovery (accuracy) & precision

Deoxynivalenol in urine (n=6)		
	recovery	RSD
0.5 ng/ml	100%	7%
1 ng/ml	104%	2%
5 ng/ml	106%	3%

Identification*: t_r & ion ratio stability

t_r within sequence within ±0.02 min

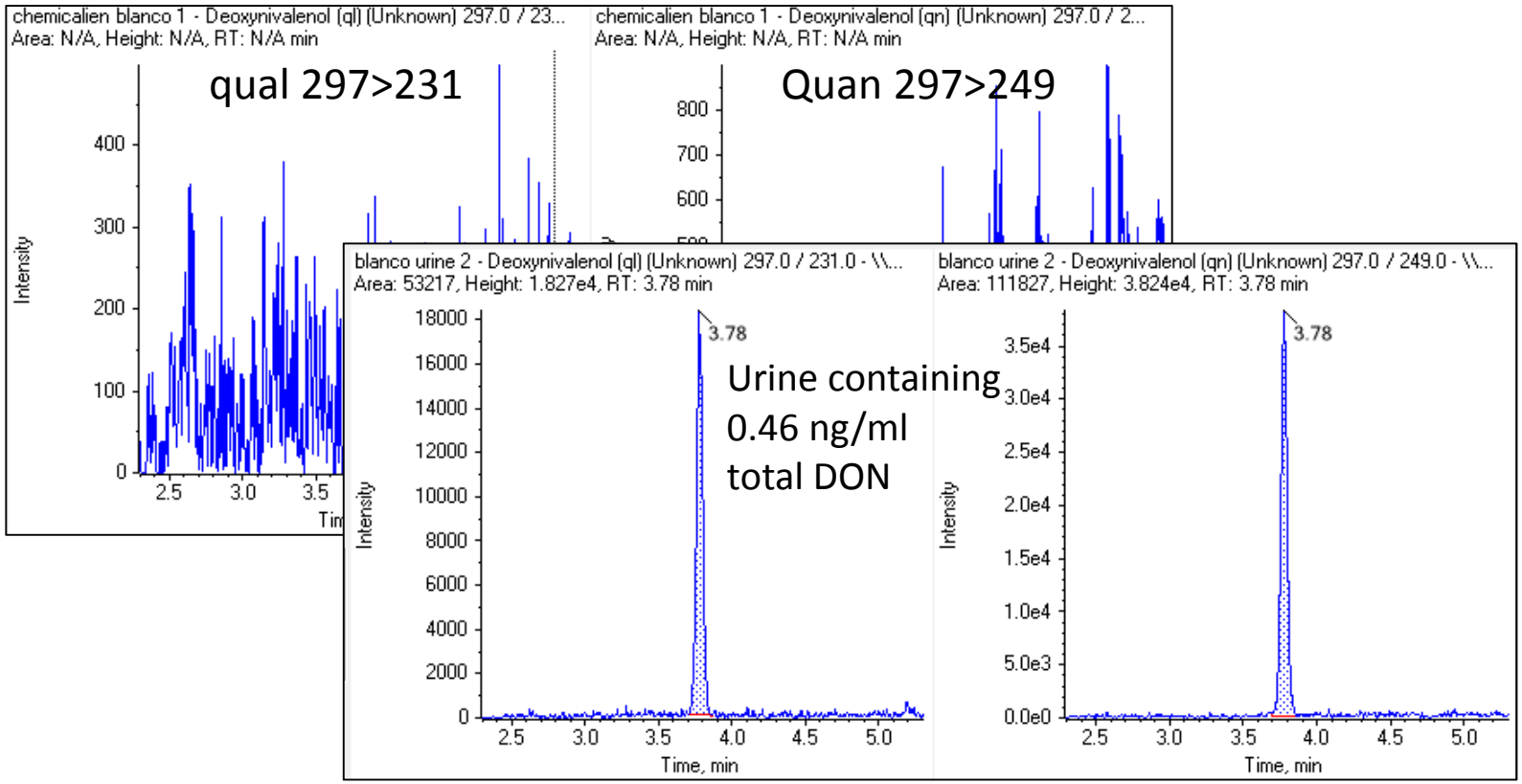
Ion ratio	solvent	urine
average	51%	50%
min	47%	48%
max	52%	51%
tolerance lower	36%	
tolerance upper	66%	

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

Validation

DON

LOQ: lowest validated level meeting quan/qual criteria: 0.5 ng/ml
LOD: not established, S/N of low levels spikes and blanks inspected



Chromatograms indicate lower LOQ is feasible
⇒ If needed: additional validation at lower level

Contacts

Hans Mol hans.mol@wur.nl
RIKILT – Wageningen University & Research

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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