Cross-reactivity of some commercially available DON and ZEN immunoaffinity columns to DON and ZEN conjugated forms and metabolites

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Cross-reactivity of some commercially available DON and ZEN immunoaffinity columns to DON and ZEN conjugated forms and metabolites

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Food Types:	
Abstract:	<p>Abstract</p> <p>Seven commercially available deoxynivalenol (DON) and zearalenone (ZEN) immunoaffinity columns (IACs) were tested for cross-reactivity to conjugated forms (3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, DON-3-glucoside, DON-3-glucuronide, ZEN-glucosides, ZEN-glucuronide) and metabolites (de-epoxydeoxynivalenol, α-zearalenol, β-zearalenol) and nivalenol (NIV), using a semi-quantitative multi-mycotoxin ultra performance liquid chromatography – tandem mass spectrometry method. The DON IACs showed cross-reactivity for nearly all DON derivatives tested. The ZEN IACs showed limited cross-reactivity to some of the ZEN derivatives. The</p>

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	IACs were evaluated for their potential use as sample clean-up for mycotoxins in serum.

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Cross-reactivity of some commercially available deoxynivalenol (DON) and zearalenone (ZEN) immunoaffinity columns to DON and ZEN conjugated forms and metabolites

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Abstract

Seven commercially available deoxynivalenol (DON) and zearalenone (ZEN) immunoaffinity columns (IACs) were tested for cross-reactivity to conjugated forms (3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, DON-3-glucoside, DON-3-glucuronide, ZEN-glucosides, ZEN-glucuronide) and metabolites (de-epoxydeoxynivalenol, α -zearalenol, β -zearalenol) and nivalenol (NIV), using a semi-quantitative multi-mycotoxin ultra performance liquid chromatography – tandem mass spectrometry method. The DON IACs showed cross-reactivity for nearly all DON derivatives tested. The ZEN IACs showed limited cross-reactivity to some of the ZEN derivatives. The IACs were evaluated for their potential use as sample clean-up for mycotoxins in serum.

Keywords: deoxynivalenol, zearalenone, immunoaffinity columns, cross-reactivity, multi analyte UPLC-MS/MS, *Fusarium* toxins, conjugated mycotoxins

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Introduction

Mycotoxins are fungal secondary metabolites toxic for humans and animals and mainly found in food and feed. Mycotoxins also occur in chemically modified forms through acetylation, glucosylation, sulfatation etc. These conjugated mycotoxins emerge after metabolization of the native mycotoxins by plants, animals, during food processing or by the fungi themselves. Awareness of such altered forms of mycotoxins is increasing, but reliable analytical methods, reference standards, occurrence and toxicity data are still lacking (Berthiller et al. 2009). Traditional evaluation of human and animal exposure to mycotoxins is based on analysis of feed and food or biological fluids like blood, serum or urine (Turner et al. 2009, Yunus et al. 2010, Köppen et al. 2010, Thieu and Petterson 2009). Due to the complexity of samples, sample clean-up is often performed prior to quantification of target mycotoxins. Sample preparation procedures generally include several steps, such as solvent extraction, defatting with hexane, and solid phase extraction (SPE) (Köppen et al. 2010). The production of specific antibodies for mycotoxins led to the development of immunoaffinity (IA) based columns, which are now widely used. IA column (IAC) chromatography is based upon molecular recognition and can provide an alternative method to isolate, purify and concentrate target analytes from complex sample matrices (Chuang et al. 2007, Senyuva and Gilbert 2010). IACs are easy to use and provide good recoveries, repeatability and reproducibility. The main advantage of IACs is the high selectivity, which allows developing methods with lower limits of detection for the target analytes compared to methods using SPE clean-up. It facilitates the analysis of the most complex matrices starting from cereals-based foods and feeds to biological fluids and animal tissues (Visconti and Pascale 1998a, Visconti and Pascale 1998b). Moreover, IA allows the use of detection methods lacking in separation power such as thin layer chromatography while retaining good method performances (Stroka et al. 2000, Gilbert and Anklam 2002).

Currently, no antibodies specifically developed against conjugated mycotoxins are available. To cope with this gap, numerous multi-analyte liquid chromatography tandem mass spectrometry (LC-MS/MS) methods are used without any clean-up at all. With highly sensitive LC-MS/MS equipment, it has become feasible to simply dilute the sample extracts to minimize possible matrix effects (dilute and shoot approaches) (Vendl et al. 2009). However, mycotoxin concentrations in biological fluids are much lower than in food and feed, so the dilute and shoot

principle is not possible. This is why for biological fluids IACs would be an ideal approach. Existing IACs for mycotoxins can be evaluated with respect to their ability to bind also mycotoxin conjugates and metabolites.

Commercially available IACs for zearalenone (ZEN) (Figure 1a) present only cross-reactivity with α -zearalenol (α -ZEL), β -zearalenol (β -ZEL) and zearalanone (ZAN), also enabling their use for the determination of some growth promoters (Visconti and Pascale 1998b, Sulyok et al. 2006).

Commercially available IACs for deoxynivalenol (DON) (Figure 1b) present cross-reactivity with de-epoxydeoxynivalenol (DOM-1) (Dänicke et al. 2002, Valenta and Dänicke 2005, Tangni et al. 2009, He et al. 2009), 3-acetylDON (3AcDON), 15-acetylDON (15AcDON) (Sulyok et al. 2006, Kostelanska et al. 2011) and only in one case also with DON-3-glucoside (DON3G) (Kostelanska et al. 2011).

The aim of this work was to screen seven commercially available IACs for DON and ZEN for their cross-reactivity, sample clean up ability and as a concentration step for analysis of mycotoxins, conjugated forms and metabolites in animal serum. A calf's serum sample was tested for recovery of selected mycotoxins with and without IAC clean-up. The developed method was applied to calf serum spiked with native and conjugated mycotoxins.

Materials and Methods

Solvents and reagents

Methanol (MeOH) and acetonitrile (MeCN) were ultra-performance liquid chromatography – mass spectrometry (UPLC-MS) grade, purchased from Biosolve (Valkenswaard, the Netherlands). Formic acid (FA) was high performance liquid chromatography (HPLC) grade, purchased from Sigma Chemical Co. (St.-Louis, MO, USA). Water was purified with a Milli-Q system (Millipore SA, Brussels, Belgium).

Standards

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3 93 DON (100 $\mu\text{g.mL}^{-1}$), DON3G (50 $\mu\text{g.mL}^{-1}$), 3AcDON (100 $\mu\text{g.mL}^{-1}$), 15AcDON (100 $\mu\text{g.mL}^{-1}$),
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5 94 DOM-1(100 $\mu\text{g.mL}^{-1}$), NIV (50 $\mu\text{g.mL}^{-1}$), ZEN (100 $\mu\text{g.mL}^{-1}$), α -ZEL (100 $\mu\text{g.mL}^{-1}$) and β -ZEL
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7 95 (100 $\mu\text{g.mL}^{-1}$) were from Biopure (IFA, Tulln, Austria). Zearalenone-diglucoside
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9 96 (ZEN-diG), zearalenone-14-glucoside (ZEN-14-G), zearalenone-16-glucoside (ZEN-16-G),
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11 97 zearalenone-14-glucuronide (ZEN-14-GU) and DON-3-glucuronide (DON3GU) were
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13 98 synthesized in our laboratory on the basis of modified procedures of Zill et al. (1990) (used for
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15 99 synthesis of ZEN glucosides) and Wu et al. (2007) (used for synthesis of DON and ZEN
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17 100 glucuronides). The chemical structures of ZEN-14-G and DON3GU were confirmed by
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19 101 LC-MS/MS and $^1\text{H-NMR}$ (data not shown), while the structure of ZEN-14-GU was identified by
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21 102 fragmentation experiments (MS/MS) in ESI^+ and ESI^- . Furthermore, after incubation with
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23 103 glucuronidase (β -D-Glucuronide glucuronosohydrolase, type B-1, from bovine liver), DON and
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25 104 ZEN were detected as reaction products. Fragmentation of ZEN-16-G in MS/MS gave the same
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27 105 fragments as ZEN-14-G fragmentation. Also, β -glucosidase (β -D-Glucoside glucohydrolase from
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29 106 almonds) treatment released ZEN (data not shown). We tentatively identified this molecule as
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31 107 ZEN-16-G. The fragmentation pattern of ZEN-diG clearly showed the presence of two sugars
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33 108 and β -glucosidase treatment resulted in formation of ZEN (data not shown). We tentatively
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35 109 identified this molecule as ZEN-diG, although it is possible that both sugars are on the same
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37 110 position. Stock solutions of DON and its derivatives were prepared in acetonitrile. Stock
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39 111 solutions of ZEN and its derivatives were prepared in methanol. Working standard mixtures
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41 112 containing 0.1, 0.2 and 0.4 $\mu\text{g.mL}^{-1}$ of each analyte were prepared in 35% methanol.
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44 114 *Immunoaffinity columns*

45 115 Seven different immunoaffinity cartridges for DON or ZEN were tested: DON-prep
46 116 (R-Biopharm, Rhone, Glasgow, UK) (2000 ng capacity for DON), NeoColumn for DON
47 117 (DONneo) (Neogen Co., Ayr, UK) (1000 ng capacity for DON), AokinImmunoClean C for DON
48 118 (DONaok) (Aokin AG, Berlin, Germany) (1500 ng capacity for DON), Easi-Extract Zearalenone
49 119 (eeZEN) (R-Biopharm Rhone, Glasgow, UK) (1500 ng capacity for ZEN), NeoColumn for ZEN
50 120 (ZENneo) (Neogen Co., Ayr, UK) (2000 ng capacity for ZEN), AokinImmunoCleanC for ZEN
51 121 (ZENaok)(Aokin AG, Berlin, Germany) (1500 ng capacity for ZEN), DZT MS-prep
52 122 (R-Biopharm, Rhone, Glasgow, UK) (capacity not indicated).
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58 124 *IAC testing*

Each column was tested by loading a standard mixture containing 40 ng of each analyte (corresponds to 560 ng of total mycotoxins load).

The standard mixture was diluted with 10 mL of PBS buffer in order to obtain a concentration of less than 5% MeOH and allowed to pass the cartridge. Each column was washed twice with 10 mL water and finally eluted with 1.5 mL of 100% MeOH. The solvent was evaporated to dryness under a nitrogen stream at 40°C, the residue was re-dissolved in 200 µL of 35% MeOH and 10 µL was injected in the UPLC-MS/MS system.

Spiked serum sample

One ml of calf serum was spiked with a standard mixture containing 20 ng of each mycotoxin. Two other spiked serum samples were prepared, with 40 and 80 ng of each mycotoxin. The mixtures were vortexed for one minute. One mL of MeOH and two mL of MeCN were added to the spiked serum to precipitate proteins. The solution was vortexed for one minute, centrifuged at 14000 rpm for 10 minutes. Three mL of the upper layer were evaporated till dryness at 40°C under a nitrogen stream. Dry residue was dissolved in 150 µL of 35% MeOH and injected in the UPLC-MS/MS system (injection volume - 10 µL). Recovery was calculated by comparing the signal of the standard in solvent with the signal obtained in a spiked matrix.

Serum sample preparation for testing on selected IACs

DON-prep, DONneo and DZT MS-prep columns were tested with spiked serum. Serum sample preparation was as in previous section, but after centrifugation, 3 mL of upper layer was evaporated till dryness at 40°C under a nitrogen stream and residue was dissolved in one mL of 100% MeOH and then diluted with 20 mL of PBS buffer. The mixture was passed through the IAC column and the column was washed twice with 10 mL of water and finally, the mycotoxins were eluted with 1.5 mL of MeOH. The solvent was evaporated till dryness under a nitrogen stream at 40°C; the dry residue was dissolved in 150 µL of 35% MeOH and injected in the UPLC-MS/MS system (injection volume - 10 µL).

Equipment

UPLC-MS/MS was performed on a Waters Quattro Premier XE (Waters Corp., Milford, MA, USA) connected to an Acquity UPLC system (Waters Corp., Milford, MA, USA).

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3 157 *Chromatographic column*
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5 158 The Acquity UPLC HSS T3 C₁₈ 100 mm x 2.1mm i.d., 1.8 µm particle size (Waters Corp.,
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7 159 Milford, MA. USA) was used.
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10 161 *Chromatographic conditions*
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12 162 The column temperature was kept constant at +40°C. Flow rate was 0.4 mL min⁻¹, and 10 µL
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14 163 partial loop injection was used (loop of 50 µl). Mobile phase A was Milli-Q water with 0.1% of
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16 164 FA and as mobile phase B MeOH containing 0.1% of FA was used. The gradient used was as
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18 165 follows: 0-8 min A – 90%, 8-8.5 min A – 65% , 8.5-17 min A – 15% , 17-18 min A – 0% ,
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20 166 18-18.5 min A – 90% , 18.5-20 min A – 90%.
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23 168 *Mass spectrometer parameters*
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25 169 The main mass spectrometer parameters were as follows: source temperature 140°C,
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27 170 desolvation temperature 450°C, capillary voltage 3 kV, extractor voltage 3 V, RF lens voltage
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29 171 0.3 V, cone gas (nitrogen) flow 50 L.h⁻¹, desolvation gas (nitrogen) flow 1000 L.h⁻¹, collision gas
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31 172 (argon) flow 0.15 mL.min⁻¹. The mass spectrometer was operated in electrospray positive
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33 173 ionization (ESI⁺) selected reaction monitoring (SRM) mode. SRM parameters are shown in
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35 174 Table 1. Figure 2 shows the UPLC-MS/MS chromatogram of the multi-mycotoxin standard
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37 175 mixture. All measurements were carried out in duplicate and given as a mean value.
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44 178 **Results and Discussion**
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48 180 Table 2 shows that DONneo IAC cross-reacts with all DON derivatives tested, DON-prep IAC
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50 181 cross-reacts with all DON conjugated forms and metabolites tested, but not with NIV. DONaok
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52 182 IAC does not bind NIV, DON3GU and DON3G. Surprisingly, DONaok IAC also binds ZEN,
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54 183 β-ZEL and α-ZEL, which can be explained by possible interactions of these analytes with IAC
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56 184 resin or other unknown factors.
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60 186 Results obtained for DON-prep IAC are in accordance with previous reports on DON-prep IAC
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62 187 ability to bind DOM-1 and acetyl-DONs (Dänicke et al. 2002, Valenta and Dänicke 2005, Tangni

et al. 2009, He and al. 2009, Kostelanska et al. 2011, Tangni et al. 2010), but do not comply with Vendl et al. (2009) who reported no cross-reactivity with DON3G.

All ZEN IACs also bind β -ZEL and α -ZEL, but showed no cross-reactivity with the other ZEN derivatives tested. Observed results are in accordance with several studies indicating cross-reactivity of some IACs to β -ZEL and α -ZEL (Visconti and Pascale 1998b, Sulyok et al. 2006), and supports also the study by Vendl et al. (2009) which shows, that eeZEN IAC does not bind any ZEN and ZEL glucosides.

DZT MS-prep column which was designed to bind several mycotoxins at once (DON, ZEN, T-2 toxin and HT-2 toxin) showed cross-reactivity to all DON derivatives tested, except for NIV, and at also binded ZEN, β -ZEL and α -ZEL, but showed no cross-reactivity with the other ZEN derivatives tested.

From these results obtained after application of mycotoxin standards on the IACs, we selected DON-prep, DONneo and DZT MS-prep as clean up step for the DON related mycotoxins in spiked calf's serum sample. Table 3 shows that these three columns are useful as clean-up step for the analysis of DON derivatives. For NIV, only DONneo was useful as clean-up step. For ZEN conjugated mycotoxins only DZT MS-prep was useful as clean-up step, but only for a limited number of ZEN metabolites.

The recovery of DON3GU, DOM-1, 3AcDON, 15AcDON, ZEN, β -ZEL and α -ZEL in serum was improved compared to analysis without sample clean-up. This resulted in lower limits of detection for most of the DON conjugates (results not shown). No ZEN IACs were suitable for the ZEN-diG, ZEN-14-G, ZEN-16-G and ZEN-14-GU clean-up. However the developed UPLC-MS/MS method showed acceptable recovery for those ZEN conjugates without any clean-up.

Conclusions

It is possible to use some commercially available IACs for sample clean-up of conjugated mycotoxins and metabolites in biological fluids such as serum. The IAC clean-up significantly

improved the sensitivity of the developed UPLC-MS/MS method for some conjugated mycotoxins (DON3GU, DON3G, 3AcDON and 15AcDON) and some metabolites (DOM-1, α -ZEL, β -ZEL) in serum. No IACs were found suitable for ZEN conjugates (ZEN-14-G, ZEN-16-G, ZEN-diG and ZEN-14-GU).

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Figure 1. Chemical structures and applied numbering of ZEN (a) and DON (b)

Figure 2. SRM chromatogram of the standard mixture containing 0.1 $\mu\text{g.mL}^{-1}$ of each analyte

Table 1
SRM transitions and analyte retention times of the multi-analyte method

Nr.	Analyte	Precursor Ion (m/z)	Declustering potential (V)	Quantifier ion (collision energy (eV))	Qualifier ion (collision energy (eV))	Retention time (min)
1	NIV	313 [M+H] ⁺	13	175 (20)	125 (20)	2.6
2	DON	297 [M+H] ⁺	20	249 (10)	203 (14)	5.5
3	DON3GU	473 [M+H] ⁺	20	-	297 (18)	6.0
		490 [M+NH ₄] ⁺	15	297 (15)	249 (15)*	
4	DON3G	459 [M+H] ⁺	15	-	297 (10)	7.5
		476 [M+NH ₄] ⁺	15	297 (10)	459 (5)*	
5	DOM-1	281 [M+H] ⁻	17	233 (9)	137 (13)	9.6
				-	109 (19)*	
6	3AcDON	339 [M+H] ⁺	22	231 (13)	189 (18)	10.6
7	15AcDON	339 [M+H] ⁺	15	137 (10)	261 (10)	10.6
8	ZEN-diG	643 [M+H] ⁺	15	-	319 (10)*	10.5
		660 [M+NH ₄] ⁺	15	-	319 (15)	
		665 [M+Na] ⁺	60	323 (40)	-	
9	ZEN-16-G	481 [M+H] ⁺	15	-	319 (10)*	11.6
		498 [M+NH ₄] ⁺	15	-	319 (5)	
		503 [M+Na] ⁺	50	341 (30)	-	
10	ZEN-14-GU	495 [M+H] ⁺	20	319 (20)	283 (20)	12.9
		512 [M+NH ₄] ⁺	15	-	319 (15)*	
11	ZEN-14-G	481 [M+H] ⁺	20	-	319 (10)	12.9
		498 [M+NH ₄] ⁺	15	319 (10)	-	
		503 [M+Na] ⁺	50	-	341 (30)*	
12	β-ZEL	321 [M+H] ⁺	14	175 (29)	285 (9)	14.1
13	α-ZEL	321 [M+H] ⁺	11	285 (11)	175 (27)	14.9
14	ZEN	319 [M+H] ⁺	20	187 (22)	203 (12)	15.1

* additional qualifier ion

Table 2
Recovery (n = 3) of the analytes after application of the standard solutions on the selected IACs

Nr.	Compound name	Recovery ± CV, %						
		DON-prep	DONneo	DONaok	DZT MS-prep	eeZEN	ZENneo	ZENaok
1	NIV	< 1	45 ± 10	< 1	< 1	< 1	< 1	< 1
2	DON	97 ± 12	84 ± 14	102 ± 16	89 ± 15	< 1	< 1	< 1
3	DON3GU	64 ± 19	88 ± 16	<1	31 ± 13	< 1	< 1	< 1
4	DON3G	58 ± 18	48 ± 20	<1	41 ± 16	< 1	< 1	< 1
5	DOM-1	67 ± 15	59 ± 22	51 ± 23	47 ± 12	< 1	< 1	< 1
6	3AcDON	54 ± 8	41 ± 24	5 ± 28	48 ± 18	< 1	< 1	< 1
7	15AcDON	13 ± 12	9 ± 23	24 ± 15	11 ± 20	< 1	< 1	< 1
8	ZEN-diG	< 1	< 1	< 1	< 1	< 1	< 1	< 1
9	ZEN-16-G	< 1	< 1	< 1	< 1	< 1	< 1	< 1
10	ZEN-14-GU	< 1	< 1	< 1	< 1	< 1	< 1	< 1
11	ZEN-14-G	< 1	< 1	< 1	< 1	< 1	< 1	< 1
12	β-ZEL	3 ± 25	2 ± 26	68 ± 18	76 ± 15	92 ± 6	87 ± 9	88 ± 11
13	α-ZEL	7 ± 28	5 ± 22	81 ± 16	90 ± 8	99 ± 5	89 ± 21	95 ± 7
14	ZEN	7 ± 21	4 ± 20	67 ± 22	93 ± 6	101 ± 10	89 ± 16	98 ± 9

Table 3

Recovery (n = 3) of the analytes from the spiked serum sample without clean-up and using selected IACs

Nr.	Compound name	Spike level (ng per mL of serum)	Recovery $\pm$ CV, %			
			Serum + spike (without clean-up)	DONprep IAC	DONneo IAC	DZT MS-prep IAC
1	NIV	20	25 $\pm$ 15	<1	40 $\pm$ 15	<1
		40	21 $\pm$ 13	<1	42 $\pm$ 11	<1
		80	23 $\pm$ 12	<1	44 $\pm$ 12	<1
2	DON	20	115 $\pm$ 18	90 $\pm$ 14	88 $\pm$ 18	89 $\pm$ 23
		40	124 $\pm$ 20	117 $\pm$ 19	92 $\pm$ 14	111 $\pm$ 15
		80	110 $\pm$ 22	115 $\pm$ 17	90 $\pm$ 10	102 $\pm$ 5
3	DON3GU	20	<1	10 $\pm$ 20	6 $\pm$ 20	2 $\pm$ 24
		40	2 $\pm$ 28	13 $\pm$ 16	8 $\pm$ 15	3 $\pm$ 13
		80	8 $\pm$ 25	40 $\pm$ 10	24 $\pm$ 15	3 $\pm$ 15
4	DON3G	20	22 $\pm$ 23	24 $\pm$ 22	21 $\pm$ 24	7 $\pm$ 19
		40	23 $\pm$ 20	26 $\pm$ 20	22 $\pm$ 22	10 $\pm$ 16
		80	16 $\pm$ 18	32 $\pm$ 18	27 $\pm$ 20	14 $\pm$ 12
5	DOM-1	20	39 $\pm$ 18	55 $\pm$ 22	50 $\pm$ 25	47 $\pm$ 26
		40	40 $\pm$ 14	74 $\pm$ 21	64 $\pm$ 23	54 $\pm$ 12
		80	50 $\pm$ 12	85 $\pm$ 18	72 $\pm$ 18	60 $\pm$ 8
6	3AcDON	20	<1	56 $\pm$ 24	44 $\pm$ 26	51 $\pm$ 22
		40	<1	62 $\pm$ 22	58 $\pm$ 24	60 $\pm$ 18
		80	<1	70 $\pm$ 20	65 $\pm$ 21	65 $\pm$ 13
7	15AcDON	20	<1	13 $\pm$ 16	7 $\pm$ 29	11 $\pm$ 24
		40	<1	15 $\pm$ 20	12 $\pm$ 23	15 $\pm$ 20
		80	<1	18 $\pm$ 18	14 $\pm$ 19	18 $\pm$ 14
8	ZEN-diG	20	34 $\pm$ 28	<1	<1	<1
		40	51 $\pm$ 25	<1	<1	<1
		80	56 $\pm$ 20	<1	<1	<1
9	ZEN-16-G	20	20 $\pm$ 34	<1	<1	<1
		40	27 $\pm$ 25	<1	<1	<1
		80	43 $\pm$ 21	<1	<1	<1
10	ZEN-14-GU	20	40 $\pm$ 38	<1	<1	<1
		40	53 $\pm$ 29	<1	<1	<1
		80	67 $\pm$ 23	<1	<1	<1
11	ZEN-14-G	20	41 $\pm$ 23	<1	<1	<1
		40	40 $\pm$ 20	<1	<1	<1
		80	42 $\pm$ 15	<1	<1	<1
12	β -ZEL	20	55 $\pm$ 16	<1	<1	78 $\pm$ 19
		40	54 $\pm$ 12	2 $\pm$ 40	<1	95 $\pm$ 15
		80	52 $\pm$ 11	3 $\pm$ 37	2 $\pm$ 26	98 $\pm$ 12
13	α -ZEL	20	67 $\pm$ 15	<1	<1	90 $\pm$ 11
		40	70 $\pm$ 12	3 $\pm$ 36	<1	105 $\pm$ 8
		80	64 $\pm$ 10	4 $\pm$ 32	3 $\pm$ 23	102 $\pm$ 6
14	ZEN	20	54 $\pm$ 13	<1	<1	93 $\pm$ 10
		40	67 $\pm$ 14	2 $\pm$ 28	<1	98 $\pm$ 8
		80	65 $\pm$ 12	2 $\pm$ 33	2 $\pm$ 20	100 $\pm$ 7