

## 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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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,  $\alpha$ -zearalenol,  $\beta$ -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

### Introduction

Mycotoxins are fungal secondary metabolites toxic for humans and animals and mainly found in food and feed. They also occur in chemically modified forms through acetylation, glucosylation, sulfatation, etc. These conjugated mycotoxins emerge after metabolisation of the native mycotoxins by plants, animals, during food processing or by the fungi themselves. The 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 the analysis of feed and food or biological fluids like blood, serum or urine (Thieu and Petterson 2009; Turner et al. 2009; Köppen et al. 2010; Yunus et al. 2010). 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; Şenyuva 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 one to develop methods with lower limits of detection for the target analytes compared with 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, 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 simply to dilute the sample extracts to minimise 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

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ability to bind also mycotoxin conjugates and metabolites.

Commercially available IACs for zearalenone (ZEN) (Figure 1a) present only cross-reactivity with  $\alpha$ -zearalenol ( $\alpha$ -ZEL),  $\beta$ -zearalenol ( $\beta$ -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; He et al. 2009; Tangni 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 the analysis of mycotoxins, conjugated forms and metabolites in animal serum. A calf's serum sample was tested for the 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

DON (100  $\mu\text{g ml}^{-1}$ ), DON3G (50  $\mu\text{g ml}^{-1}$ ), 3AcDON (100  $\mu\text{g ml}^{-1}$ ), 15AcDON (100  $\mu\text{g ml}^{-1}$ ), DOM-1

(100  $\mu\text{g ml}^{-1}$ ), NIV (50  $\mu\text{g ml}^{-1}$ ), ZEN (100  $\mu\text{g ml}^{-1}$ ),  $\alpha$ -ZEL (100  $\mu\text{g ml}^{-1}$ ) and  $\beta$ -ZEL (100  $\mu\text{g ml}^{-1}$ ) were from Biopure (IFA, Tulln, Austria). Zearalenone diglucoside (ZEN-diG), zearalenone-14-glucoside (ZEN-14-G), zearalenone-16-glucoside (ZEN-16-G), zearalenone-14-glucuronide (ZEN-14-GU) and DON-3-glucuronide (DON3GU) were synthesised in our laboratory on the basis of modified procedures of Zill et al. (1990) (used for the synthesis of ZEN glucosides) and Wu et al. (2007) (used for the synthesis of DON and ZEN glucuronides). The chemical structures of ZEN-14-G and DON3GU were confirmed by LC-MS/MS and  $^1\text{H-NMR}$  (data not shown), while the structure of ZEN-14-GU was identified by fragmentation experiments (MS/MS) in  $\text{ESI}^+$  and  $\text{ESI}^-$ . Furthermore, after incubation with glucuronidase ( $\beta$ -D-glucuronide glucuronosohydrolase, type B-1, from bovine liver), DON and ZEN were detected as reaction products. Fragmentation of ZEN-16-G in MS/MS gave the same fragments as ZEN-14-G fragmentation. Also,  $\beta$ -glucosidase ( $\beta$ -D-glucoside glucosohydrolase from almonds) treatment released ZEN (data not shown). We tentatively identified this molecule as ZEN-16-G. The fragmentation pattern of ZEN-diG clearly showed the presence of two sugars and  $\beta$ -glucosidase treatment resulted in formation of ZEN (data not shown). We tentatively identified this molecule as ZEN-diG, although it is possible that both sugars are on the same position. Stock solutions of DON and its derivatives were prepared in acetonitrile. Stock solutions of ZEN and its derivatives were prepared in methanol. Working standard mixtures containing 0.1, 0.2 and 0.4  $\mu\text{g ml}^{-1}$  of each analyte were prepared in 35% methanol.

### Immunoaffinity columns

Seven different immunoaffinity cartridges for DON or ZEN were tested: DON-prep (R-Biopharm, Rhone, Glasgow, UK) (2000 ng capacity for DON), NeoColumn for DON (DONneo) (Neogen Co., Ayr, UK) (1000 ng capacity for DON), AokinImmunoClean C for DON (DONaok) (Aokin AG, Berlin, Germany) (1500 ng capacity for DON), Easi-Extract Zearalenone

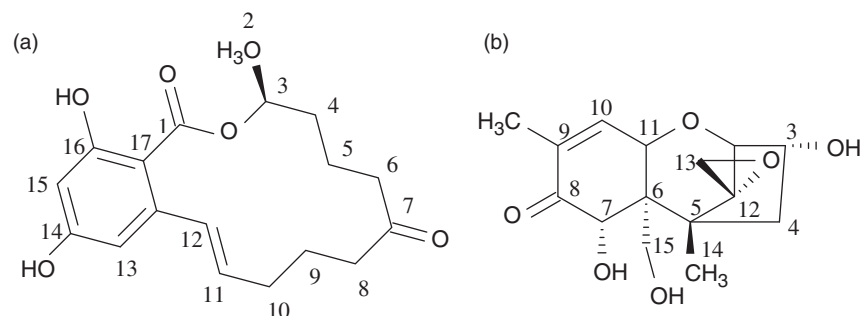


Figure 1. Chemical structures and applied numbering of ZEN (a) and DON (b).

(eeZEN) (R-Biopharm Rhone) (1500 ng capacity for ZEN), NeoColumn for ZEN (ZENneo) (Neogen Co.) (2000 ng capacity for ZEN), AokinImmunoCleanC for ZEN (ZENaok) (Aokin AG) (1500 ng capacity for ZEN), DZT MS-prep (R-Biopharm, Rhone) (capacity not indicated).

### *IAC testing*

Each column was tested by loading a standard mixture containing 40 ng of each analyte (corresponding 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*

A total of 1 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 1 min. A total of 1 ml of MeOH and 2 ml of MeCN were added to the spiked serum to precipitate proteins. The solution was vortexed for 1 min, centrifuged at 14,000 rpm for 10 min. A total of 3 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 the previous section, but after centrifugation, 3 ml of upper layer were evaporated till dryness at 40°C under a nitrogen stream and residue was dissolved in 1 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.).

### *Chromatographic column*

The Acquity UPLC HSS T3 C<sub>18</sub> 100 mm × 2.1 mm i.d., 1.8 µm particle size (Waters Corp.) was used.

### *Chromatographic conditions*

The column temperature was kept constant at 40°C. Flow rate was 0.4 ml min<sup>-1</sup>; a 10 µl partial loop injection was used (loop of 50 µl). Mobile phase A was Milli-Q water with 0.1% of FA and as mobile phase B MeOH containing 0.1% of FA was used. The gradient used was as follows: 0–8 min A – 90%, 8–8.5 min A – 65%, 8.5–17 min A – 15%, 17–18 min A – 0%, 18–18.5 min A – 90%, 18.5–20 min A – 90%.

### *Mass spectrometer parameters*

The main mass spectrometer parameters were as follows: source temperature 140°C, desolvation temperature 450°C, capillary voltage 3 kV, extractor voltage 3 V, RF lens voltage 0.3 V, cone gas (nitrogen) flow 50 l h<sup>-1</sup>, desolvation gas (nitrogen) flow 1000 l h<sup>-1</sup>, and collision gas (argon) flow 0.15 ml min<sup>-1</sup>. The mass spectrometer was operated in electrospray-positive ionisation (ESI<sup>+</sup>) selected reaction-monitoring (SRM) mode. SRM parameters are shown in Table 1. Figure 2 shows the UPLC-MS/MS chromatogram of the multi-mycotoxin standard mixture. All measurements were carried out in duplicate and given as a mean.

### **Results and discussion**

Table 2 shows that DONneo IAC cross-reacts with all DON derivatives tested, DON-prep IAC cross-reacts with all DON conjugated forms and metabolites tested, but not with NIV. DONaok IAC does not bind NIV, DON3GU and DON3G. Surprisingly, DONaok IAC also binds ZEN, β-ZEL and α-ZEL, which can be explained by possible interactions of these analytes with IAC resin or other unknown factors.

Results obtained for DON-prep IAC are in accordance with previous reports on DON-prep IAC ability to bind DOM-1 and acetyl-DONs (Dänicke et al. 2002; Valenta and Dänicke 2005; He et al. 2009; Tangni et al. 2009, 2010; Kostelanska et al. 2011), but do not

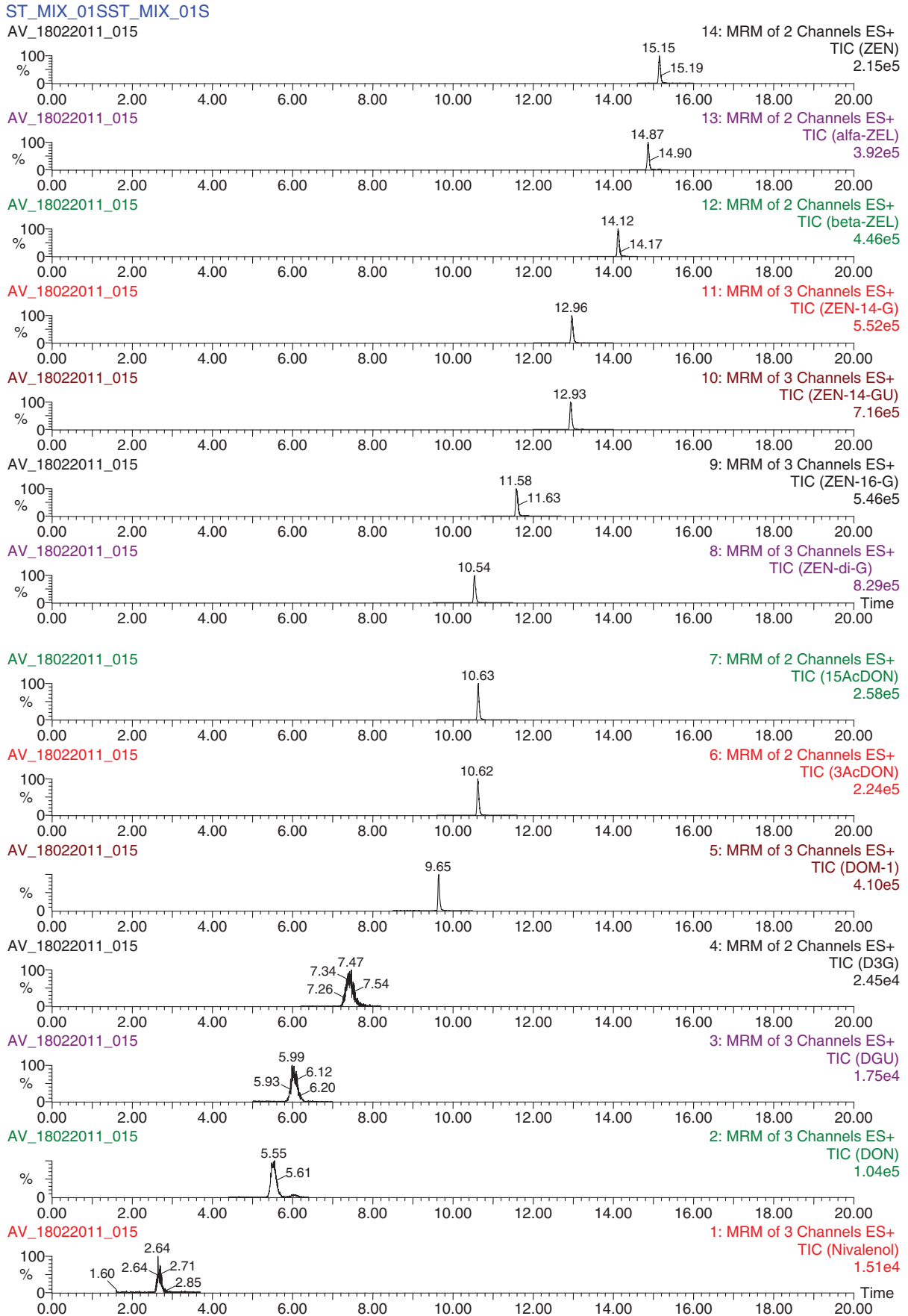


Figure 2. SRM chromatogram of the standard mixture containing 0.1 µg ml<sup>-1</sup> of each analyte.

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

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

Note: <sup>a</sup>Additional qualifier ion.

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

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

comply with Vendl et al. (2009) who reported no cross-reactivity with DON3G.

All ZEN IACs also bind  $\beta$ -ZEL and  $\alpha$ -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  $\beta$ -ZEL and  $\alpha$ -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 bound ZEN,  $\beta$ -ZEL and  $\alpha$ -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

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

Number	Compound	Spike level (ng ml <sup>-1</sup> 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	$\beta$ -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	$\alpha$ -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

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,  $\beta$ -ZEL and  $\alpha$ -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,  $\alpha$ -ZEL,  $\beta$ -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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