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Journal of Chromatography B, 820 (2005) 183–196
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry

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Received 7 October 2004; accepted 4 March 2005
Available online 26 April 2005

Abstract

Liquid chromatographic/tandem mass spectrometric methods using pneumatically assisted electrospray ionisation (LC–ESI-MS/MS) was developed for determination of 18 mycotoxins and metabolites—ochratoxin A, zearalenone, α-zearalenol, β-zearalenol, α-zearalanol (zeranol), β-zearalanol (taleranol), fumonisin B_1, fumonisin B_2, T-2 toxin, HT-2 toxin, T-2 triol, diacetoxyscirpenol (DAS), 15-monoacetoxyscirpenol (MAS), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), deepoxy-deoxynivalenol (DOM-1) and aflatoxin M_1—in milk. The mycotoxins were extracted and cleaned up simultaneously. Extraction and removal of lipophilic compounds was performed at pH 2 using a two-phase mixture of acetonitrile and hexane. The acetonitrile concentration of the aqueous phase was reduced and the pH was adjusted to 8.5 before clean up by solid phase extraction (SPE) on Oasis HLB. The toxins DON, DOM-1, 3-AcDON, 15-AcDON, ochratoxin A, zearalenone, α-zearalenol, β-zearalenol, α-zearalanol and β-zearalanol were detected in negative ion mode after separation on a Hypersil ENV analytical column, while the toxins T-2 toxin, HT-2 toxin, T-2 triol, DAS, MAS, fumonisin B_1, fumonisin B_2 and aflatoxin M_1 were detected in positive ion mode after separation on a Luna C_{18} column. Two transition products were monitored for each compound. The extraction and SPE conditions were optimised to obtain maximum recovery and minimum signal suppression/enhancement. The detection capabilities related to the transition products of lowest abundance were in the range 0.020–0.15 μg/l. The mean true recoveries were in the range 76–108% at levels of 0.2–10 μg/l.

Keywords: Tandem mass spectrometry; Milk; Mycotoxins; Ochratoxin A; Trichothecenes; Zearalenone; α-Zearalenol; β-Zearalenol; α-Zearalanol; Zeranol; β-Zearalanol; Taleranol; Fumonisin B_1; Fumonisin B_2; T-2 toxin; HT-2 toxin; T-2 triol; Diacetoxyscirpenol; DAS; Monoacetoxyscirpenol; MAS; Deoxynivalenol; DON; Vomitoxin; 3-Acetyldeoxynivalenol; 3-AcDON; 15-Acetyldeoxynivalenol; 15-AcDON; Deepoxy-deoxynivalenol; DOM-1; Aflatoxin M_1

1. Introduction

The mycotoxin of major concern in bovine milk since the late 1960s has been aflatoxin M_1 (AFM_1) because of its toxicity and carcinogenic properties. AFM_1 is a hydroxy derivate of aflatoxin B_1 (AFB_1) and is produced by ruminants by metabolism of AFB_1 [1,2]. Typical sources for ruminants' intake of AFB_1 are cottonseed cake, groundnut meal, maize and feedstuff ingredients based on oilseeds.

However, other mycotoxins may also be present in the feed, especially in cereals and silages. A large family of mycotoxins of general concern is the trichothecenes, which are mainly produced by moulds of the genus *Fusarium*. The trichothecenes are divided into four groups A–D, depending on their molecular structure. Most focus has been on the A and B trichothecenes.

Group A trichothecenes include T-2 toxin, HT-2 toxin and diacetoxyscirpenol (DAS). DAS and T-2 toxin are deacetylated to MAS and HT-2 toxin, respectively in rumen fluid [3]. HT-2 toxin may be further metabolised to T-2 triol [4]. Other metabolic products may also be produced from T-2 toxin [4,5]. Studies where T-2 toxin was administered at relatively high feed concentrations to lactating cows showed that a minor fraction of the T-2 toxin and its metabolites may be transferred to the milk [5,6].

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doi:10.1016/j.jchromb.2005.03.020

The group B trichothecenes, which differ from those in group A in their possession of a keto group at position C-8, include deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON) and 15-acetyldeoxynivalenol (15-AcDON). It has been shown that rumen microorganisms metabolise DON to deepoxy-deoxynivalenol (DOM-1) [7]. Feeding trials have shown that the metabolite DOM-1 may be transferred to milk, but at low excretion rates compared to urine and faeces [8]. Transfer of intact DON to milk was almost negligible or undetectable in feeding trials [8,9].

Fusarium species also produce zearalenone (ZEN), a resorcyclic acid lactone with estrogenic effect mainly found in cereals. ZEN and its metabolites have been reported in milk obtained in some feeding trials [10] but not in others [11]. ZEN is metabolised to α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) in ruminants [3,12]. The estrogenic activity of α-ZEL is higher than for the parent compound [13]. Besides ZEN, α-ZEL and β-ZEL *Fusarium* species may also produce α-zearalanol (zeranol) (α-ZAL) and β-zearalanol (taleranol) (β-ZAL) [14] and the same metabolites may be produced from metabolism of ZEN in ruminants [12,15]. These metabolites have been detected in urine [15–17] and bile [18]. The formation of α-ZAL from ZEN is controversial because α-ZAL is the active component in commercial anabolic growth promoters, which has permitted their use in some countries including the United States, but they are banned in the European Union.

The fumonisins are another group of toxins which are produced by *Fusarium* species. Fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2), found mainly in cereals, have been most thoroughly investigated. Studies show that FB_1, which is considered the most toxic compound of the fumonisin group, is poorly metabolised by rumen microflora [19] and liver microsomes [20]. According to other studies, a low-rate transfer of fumonisins to milk appears possible [21,22].

Other fungal species may also produce mycotoxins of general concern. A common mycotoxin found in, e.g. cereals is ochratoxin A (OTA), produced by *Aspergillus* and *Penicillium* species. OTA is metabolised by rumen fluid microorganisms, producing mainly ochratoxin α [3,23], which has shown no definite toxicity to chicken embryos [24]. Although ochratoxin A is exposed to degradation in the rumen, residues have been reported in cow's milk [25,26].

In general, bovine milk seems not to be a major source of the dietary intake of *Fusarium* toxins in humans. However, it cannot be excluded that a high contamination in feed may result in a significant level in milk when cows are in a state of physiological imbalance, or when the cows are fed solely with the contaminated fodder. The increasing efficiency of milk production and the ongoing striving for higher milk yields may facilitate such situations. For monitoring purposes, broad range analytical methods are needed to reduce analytical costs and allow for a more frequent monitoring of mycotoxins in milk.

Several analytical methods have been published for determination of mycotoxins in cereals, feeds, foods and biological fluids. Methods based on LC–MS or LC–MS/MS have been reported for determination of ZEN in maize and wheat [27], ZEN and ZEL in beer [28], ZEN and its metabolites in urine [29,30] and tissue [30], ZEN and ZEL in fish tissue [31], DON in maize and wheat [32], OTA in food [33], OTA in blood plasma [34], A-trichothecenes in grains [35], B-trichothecenes in urine and maize [36,37], fumonisins in maize [38–40], feed [41] and vegetables [42], and aflatoxins in peanuts [43–46], medicinal herbs [47] and urine [48]. However, except in the case of aflatoxin M_1 where the global concern has resulted in internationally standardised analytical methods, only a few chromatographic methods have been published for the determination of mycotoxins in milk. These include HPLC methods with fluorescence detection for determination of OTA [25,26,49–51], GC with electron capture detection for determination of DON and DOM-1 after derivatisation [52], GC with mass spectrometric (MS) detection for determination of DON [9] and T-2 [53] after derivatisation, HPLC with UV detection for determination of DON [54], and HPLC with fluorescence detection of derivates of FB_1 and FB_2 [21]. To the authors' knowledge, no liquid chromatography tandem mass spectrometric (LC–MS/MS) methods have so far been published for determination of a broad range of mycotoxins in milk.

The object of the present study was to develop a simple and sensitive LC–MS/MS method for the simultaneous determination of a range of the mycotoxins and metabolites in bovine milk which may be present from cows' intake of contaminated fodder.

2. Materials and methods

2.1. Chemicals and reagents

Stock solutions of OTA, FB_1, FB_2, T-2 toxin, HT-2 toxin, DON, 3-AcDON, 15-AcDON, DOM-1, DAS, ZEN, α-ZEL and β-ZEL in acetonitrile were obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria). T-2 triol, MAS, α-ZAL, β-ZAL, AFM_1 and β-glucuronidase type IX from *Escherichia coli* were obtained from Sigma–Aldrich (Schnelldorf, Germany). Chromatography grade acetonitrile and methanol were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Ammonium acetate, sodium hydroxide and sulphuric acid were obtained from Merck (Darmstadt, Germany). Water was purified on a Milli-Q Plus apparatus (Millipore, Bedford, MA).

Stock solutions (1000 μg/ml) of T-2 triol, MAS, α-ZAL, β-ZAL and AFM_1 were prepared in acetonitrile. Combined standard solutions were prepared by diluting stock solutions with 20% methanol. Combined standard solutions containing 1–60 μg/l of the mycotoxins were stable for at least 5 days when stored at 5–7 °C.

A solution of β-glucuronidase containing 100,000 units/ml was prepared in 0.1 M phosphate buffer pH 6.8.

Mobile phases 1A and 1B consisted of 10 and 90% (v/v) methanol, respectively. Mobile phases 2A and 2B consisted of 10 and 90% (v/v) methanol, respectively, acidified with 0.020% acetic acid. Post column reagent contained 0.10 mM ammonium acetate in 80% methanol.

2.2. Equipment

The liquid chromatographic system was an Agilent 1100 series system consisting of binary pump, isocratic pump, solvent degasser, autosampler and column oven (Agilent Technologies, Waldbronn, Germany). The mass spectrometer was a Sciex API 2000 triple quadrupole instrument equipped with a TurboIonSpray ion source (Applied Biosystems, Foster City, CA). Separation was performed on Luna C_{18} 100A (5 μm, 150 mm × 4.6 mm i.d.) (Phenomenex, Torrance, CA) and Hypersil ENV (5 μm, 150 mm × 4.6 mm i.d.) (Thermo Electron Corporation, Cheshire, UK) columns. Oasis HLB cartridges, 60 mg, 3 ml (Waters Corp. Milford, MA) were used for SPE and 17 mm 0.45 μm PTFE filters (Frisenette, Ebeltoft, Denmark) were used for filtration of sample extracts.

Other equipment used was an IKA HS500 horizontal shaker (Staufen, Germany), a Sigma centrifuge model 4K15 (Osterode, Germany), a PHM 93 pH meter (Radiometer, Copenhagen, Denmark), a temperature-controlled heating block with a manifold for nitrogen flow (Mikrolab Aarhus, Aarhus, Denmark), a vacuum manifold for SPE cartridges (Waters Corp.), polypropylene reservoirs for SPE, 50 and 10 ml polypropylene centrifuge tubes with screw cap (Sarstedt, Nümbrecht, Germany), and 600 μl autosample vials in polypropylene (Chromacol, Welwyn Garden City, Great Britain).

2.3. Sample preparation

2.3.1. Non-bound mycotoxins

A volume of 5.0 ml milk was transferred to a 50 ml tube and mixed with 100 μl sulphuric acid 18% (v/v) to obtain a pH of 2.0 ± 0.5. After 10 min standing time, the mixture was shaken horizontally for 10 min (250 strokes/min) with a combination of 10 ml hexane and 16 ml acetonitrile. The mixture was then centrifuged at 3000 × *g* for 10 min. and the hexane phase was removed. A volume of 10 ml of the acetonitrile phase was concentrated to 1 ml by evaporation at 60 °C under a stream of nitrogen. The concentrate was mixed with 10 ml water and the pH was adjusted to 8.5 ± 0.2 with sodium hydroxide. The solution was applied to an Oasis cartridge which had previously been conditioned with 2 ml methanol and 2 ml water. The cartridge was washed with 5 ml water and then vacuum-dried (20 in. Hg) for 15 s. The mycotoxins were eluted with 4 ml methanol and the eluate was evaporated to bare dryness at 60 °C. The residue was redissolved in 500 μl 20% methanol and the solution was filtered through a PTFE filter. Final sample extracts were stable for at least 5 days when stored at 5–7 °C.

2.3.2. Total mycotoxins (non-bound and glucuronides)

A volume of 5.0 ml milk (pH 6.6–7.0) was incubated with 100 μl β-glucuronidase solution at 37 ± 2 °C for 2 h in a water bath. The extraction and clean-up procedure for determination of non-bound mycotoxins was then followed.

2.4. Calibration

External matrix standards were used for calibration. Blank control samples were extracted and cleaned up as per the procedures. The final residues were redissolved in standard solutions to obtain concentrations of 0, 1, 10, 20, 40 and 60 μg/l. The calibration standards were stable for at least 5 days when stored at 5–7 °C.

Calibration curves were created using non-weighted linear regression analysis with lines forced through the origin.

2.5. LC–MS/MS conditions

2.5.1. General conditions

Sample extracts were kept at 5–10 °C until analysis. A volume of 50 μl was injected into the chromatographic system. The column flow rate was 600 μl/min and the column temperature was kept at 25 ± 1 °C. Before ESI the flow was split to obtain a flow rate of 200 μl/min for the interface. The probe temperature was 350 °C and the probe voltage was 4200 V in negative ion mode and 5000 V in positive ion mode. The curtain and nebuliser gas pressures were set at 20 psi, while the heater gas pressure was set at 60 psi. The focusing potential was set at 350 V. The dwell time was 200 ms. Data acquisition and processing were performed using Analyst Software 1.1 (Applied Biosystems).

2.5.2. Negative ion mode: DON, DOM-1, 3-AcDON, 15-AcDON, OTA, ZEN, α-ZEL, β-ZEL, α-ZAL and β-ZAL

The sample extract was injected on to a Hypersil ENV column running with 80% mobile phase 1A and 20% 1B. The gradient was changed to 80% mobile phase 1B over 10 min. After 16 min total time the gradient was returned to 20% 1B over 0.1 min and conditioned for 10 min before the next injection. The eluent was diverted to waste in the intervals 0–5 min and 17–26 min after injection using a post column switch. Selected reaction monitoring (SRM) was applied with conditions shown in Table 1.

2.5.3. Positive ion mode: T-2 toxin, HT-2 toxin, T-2 triol, DAS, MAS, FB_1, FB_2 and AFM_1

The sample extract was injected on to a Luna column running with 30% mobile phase 2A and 70% 2B. After 2.2 min, the gradient was changed to 82% mobile phase 2B over 0.5 min. After 11 min. total time the gradient was returned to 70% 2B over 0.1 min and conditioned for 10 min before the next injection. The eluent was diverted to waste in the intervals 0–3.5 min and 10.8–21 min after injection using a post

Table 1
Transitions and instrument parameter settings used for data collection in negative ion mode

Compound	Transition	DP (V)	EP (V)	CE (V)	Relative abundance
DON	295 → 265	10	5	13	100
	295 → 247	10	5	14	30
3-AcDON	337 → 307	10	5	13	100
	337 → 173	10	5	12	73
15-AcDON	337 → 150	15	10	17	100
	337 → 219	15	10	13	54
DOM-1	279 → 249	8	5	10	100
	279 → 231	8	5	17	33
OTA	402 → 358	20	10	27	100
	402 → 167	20	10	50	90
ZEN	317 → 131	27	10	40	102
	317 → 175	27	10	32	100
α-ZEL	319 → 160	30	10	42	100
	319 → 130	30	10	45	84
β-ZEL	319 → 160	30	10	42	100
	319 → 130	30	10	45	84
α-ZAL	321 → 277	32	10	25	100
	321 → 259	32	10	31	15
β-ZAL	321 → 277	32	10	25	100
	321 → 259	32	10	31	15

DP, declustering potential; EP, entrance potential; CE, collision energy.

column switch. Post column reagent was added in the time intervals 0–5.1 min and 7–9 min with a flow rate of 100 μl/min. Selected reaction monitoring (SRM) was applied with conditions shown in Table 2.

Table 2
Transitions and instrument parameter settings used for data collection in positive ion mode

Compound	Transition	DP (V)	EP (V)	CE (V)	Relative abundance
T-2 toxin	484 → 305	18	5	21	100
	484 → 215	18	5	28	95
HT-2 toxin	442 → 215	10	5	19	100
	442 → 323	10	5	13	45
T-2 triol	383 → 215	8	3	14	100
	383 → 233	8	3	9	29
DAS	384 → 307	12	5	16	100
	384 → 247	12	5	20	58
MAS	342 → 265	15	6	11	100
	342 → 247	15	6	18	15
FB_1	722 → 334	20	11	56	100
	722 → 352	20	11	50	91
FB_2	706 → 336	20	11	50	100
	706 → 318	20	11	57	59
AFM_1	329 → 273	28	9	35	100
	329 → 259	28	9	35	46

DP, declustering potential; EP, entrance potential; CE, collision energy.

2.6. Decision limit and detection capability

The decision limit (CCα, $\alpha = 1\%$) [55] was calculated as three times the signal to noise ratio for the transitions of lowest abundance determined on 20 different blank control samples. The detection capability (CCβ, $\beta = 5\%$) [55] was determined on 20 different blank control samples. The samples were fortified prior to centrifugation and extraction with the individual compounds at the decision limit. The fortified samples were mixed and stored for 1 h at 20–25 °C before extraction. The CCβ was calculated as the CCα plus 1.64 times the standard deviation (S.D.) of the 20 measurements.

2.7. Precision and recovery

The repeatability standard deviation (i.e. the variability of independent analytical results obtained by the same operator, using the same apparatus under the same conditions on the same test sample and in a short interval of time) and the intra-laboratory reproducibility standard deviation (i.e. the variability of independent analytical results obtained on the same test sample in the same laboratory by different operators under different experimental conditions) were determined on blank control samples of pasteurised bulk milk standardised to 3.5% fat and raw milk samples fortified to levels of 0.2, 1 and 10 μg/l with each mycotoxin except for AFM_1, which was added at 10 times smaller concentrations. Two duplicate analyses were performed on different days. The calculation of repeatability was done in accordance with ISO standard 5725-2, 1994 [56]. The intra-laboratory reproducibility was calculated by the same principle used for determination of reproducibility [56].

The true recoveries were determined from results obtained in the precision study.

2.8. Ruggedness

The method was tested for pH-dependence of SPE recovery and matrix-induced effects on signal intensity. Dried extracts from SPE of four different blank control samples redissolved in 500 μl combined standard solution 5 μg/l in 20% methanol, and corresponding blank control samples fortified with 25 μl standard solution 100 μg/l prior to SPE, were analysed in attenuated order together with standard solutions.

The effect of sample pH on recoveries was tested on samples acidified to pH 1.0, 2.0 and 3.0. The effect of standing time before extraction when the sample had been acidified was tested for standing times of 5, 10, 20, 40 and 60 min.

The elution profile of the compounds from the SPE cartridge was investigated using samples fortified to a level of 10 μg/l.

The stability at 5–7 °C of calibration standard solutions and final extracts of six blank control samples fortified with mycotoxins to levels corresponding to original sample levels of 0.20 and 10 μg/l were tested over a period of 8 days.

3. Results and discussion

3.1. Precursor ions and transition products

The dominant precursor ion of OTA obtained from ESI was *m/z* 402 ($[M-H]^-$). When fragmented in the collision cell, several product ions were produced. The most sensitive transition ions were *m/z* 358 and 167. OTA may also be determined in positive ion mode as shown by Becker et al. [33], who used LC–ESI-MS/MS for determination of OTA in food samples, and by Lau et al. [34], who used LC–MS with atmospheric pressure chemical ionisation (APCI) for determination of OTA in plasma samples. However, the negative ion mode was selected in our study because it

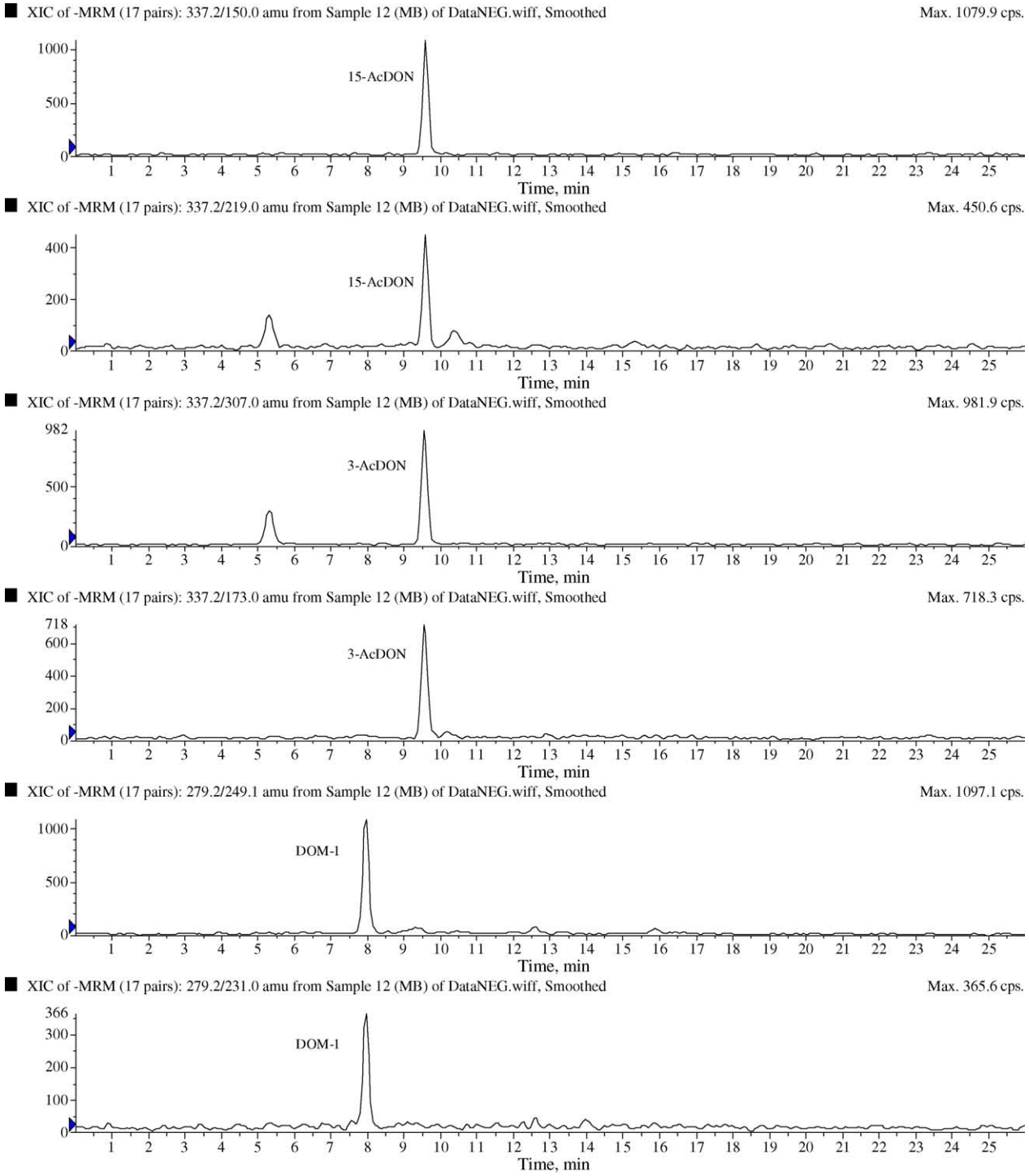

Fig. 1. LC–ESI-MS/MS chromatograms obtained in negative ion mode of blank control sample fortified with 1 μg/l of mycotoxins.

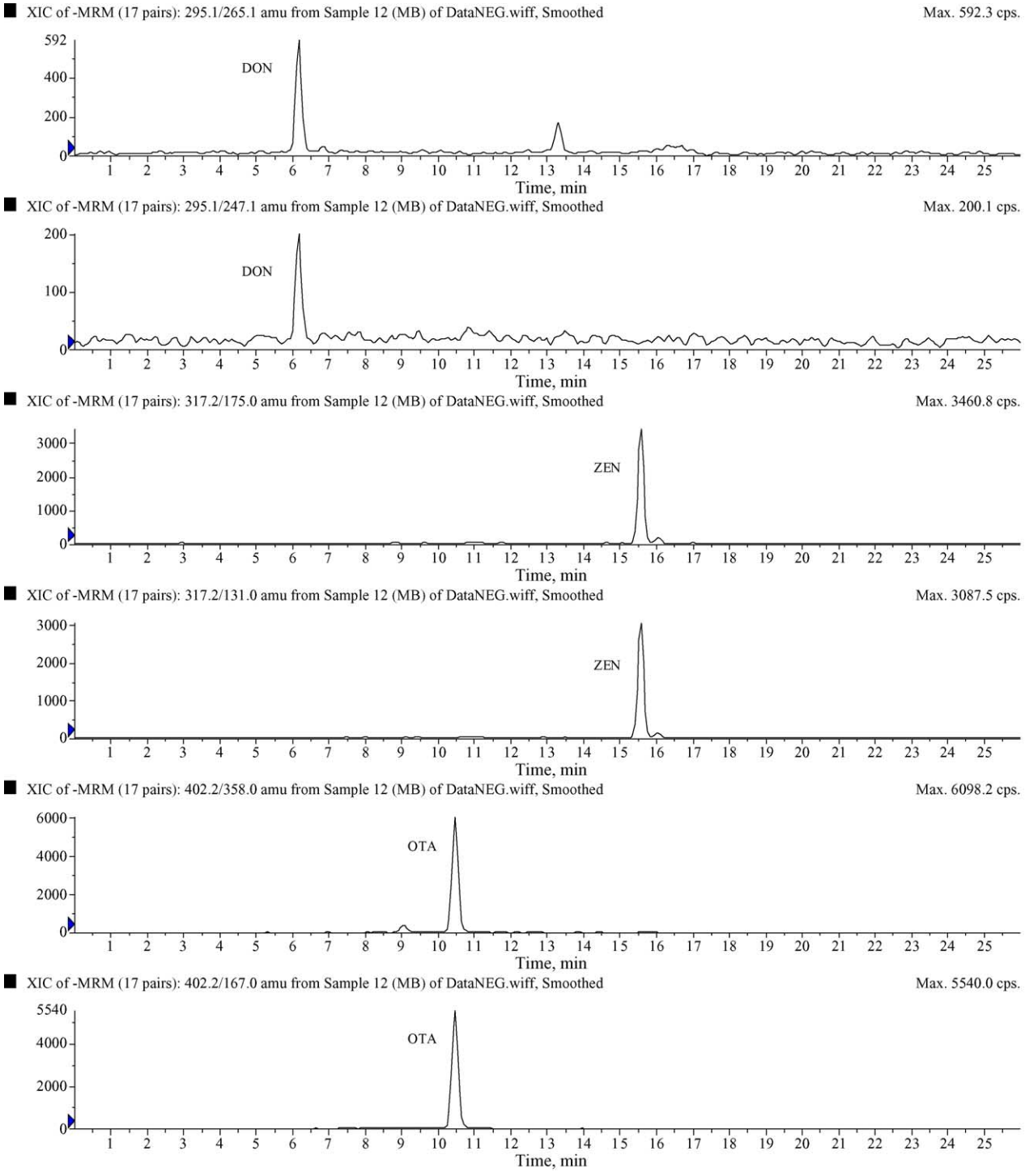

Fig. 1. (*Continued*)

gave considerably higher sensitivity than the positive ion mode.

The dominant precursor ion of ZEN was *m*/*z* 317 $[M-H]^-$, while *m*/*z* 319 $[M-H]^-$ was dominant for the metabolites α-ZEL and β-ZEL. The dominant precusor ion for α-ZAL and β-ZAL was 321 $[M-H]^-$. These ions were also used by van Bennekom et al. [29] in their study on urine using LC–ESI-MS/MS. However, the selected transition products were not identical in all cases. The same transitions could be used for α-ZEL and β-ZEL because they were separated chromatographically. This was also the case for α-ZAL and β-ZAL. The dominant precursor ions of DON, 3-AcDON, 15-AcDON and DOM-1 were the $[M-H]^-$ ions *m*/*z* 295, 337, 337 and 279. These ions were

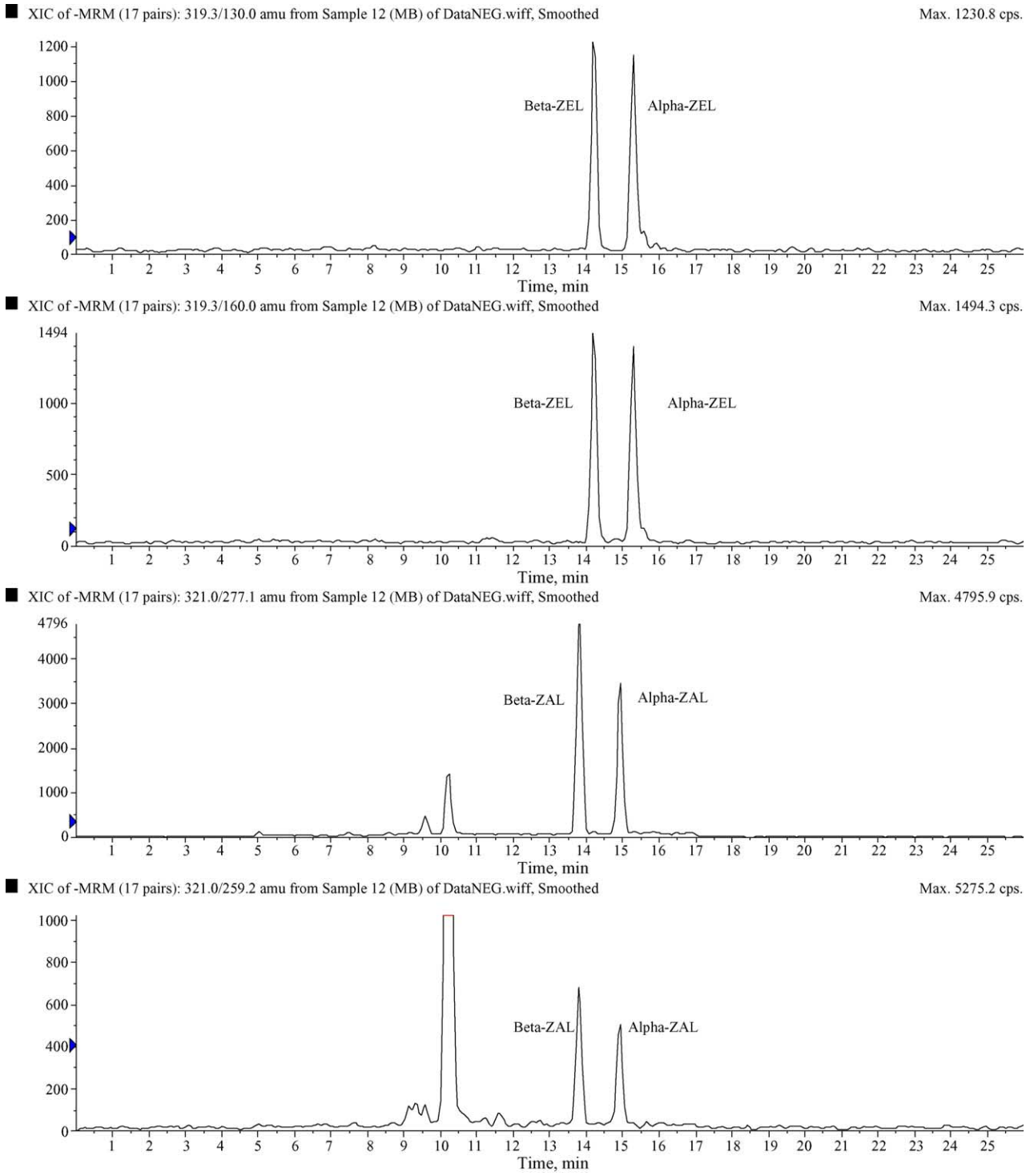

Fig. 1. (*Continued*).

also observed by Razazi-Fazeli et al. [36] in their study using LC–APCI-MS for determination of DON, 3-AcDON and 15-AcDON in pig urine and maize. The most sensitive transitions obtained in negative ion mode without spectral interferences are listed in Table 1. Fig. 1 shows chromatograms of a blank control sample fortified to a level of 1 μg/l.

The dominant precursor ions of T-2 toxin, HT-2 toxin and T-2 triol were *m/z* 484 $[M + NH_4]^+$, 442 $[M + NH_4]^+$ and 383 $[M + H]^+$. The dominant precursor ions for DAS and MAS were *m/z* 384 $[M + NH_4]^+$ and 342 $[M + NH_4]^+$. These ions were also observed in the study reported by Razzazi-Fazeli et al. [35], where an LC–APCI-MS method was developed for cereal analyses. The precursor ions for the FB_1 and FB_2

were 722 $[M+H]^+$ and 706 $[M+H]^+$. The most sensitive transitions obtained in positive ion mode without spectral interferences are listed in Table 2. Fig. 2 shows chromatograms of a blank control sample fortified to a level of 1 μg/l (0.1 μg/l for AFM_1).

The relative abundances of the transitions were constant in the range CCβ—10 μg/l, and the mean difference in relative abundance between pure reference standards and spiked blank control samples was less than 5% for each mycotoxin, which could not be demonstrated to be statistically significant. The use of two transitions gave 4.0 identification points, which corresponds to the EU requirement of 4.0 for confirmation of banned substances in products of animal origin [55]. The most sensi-

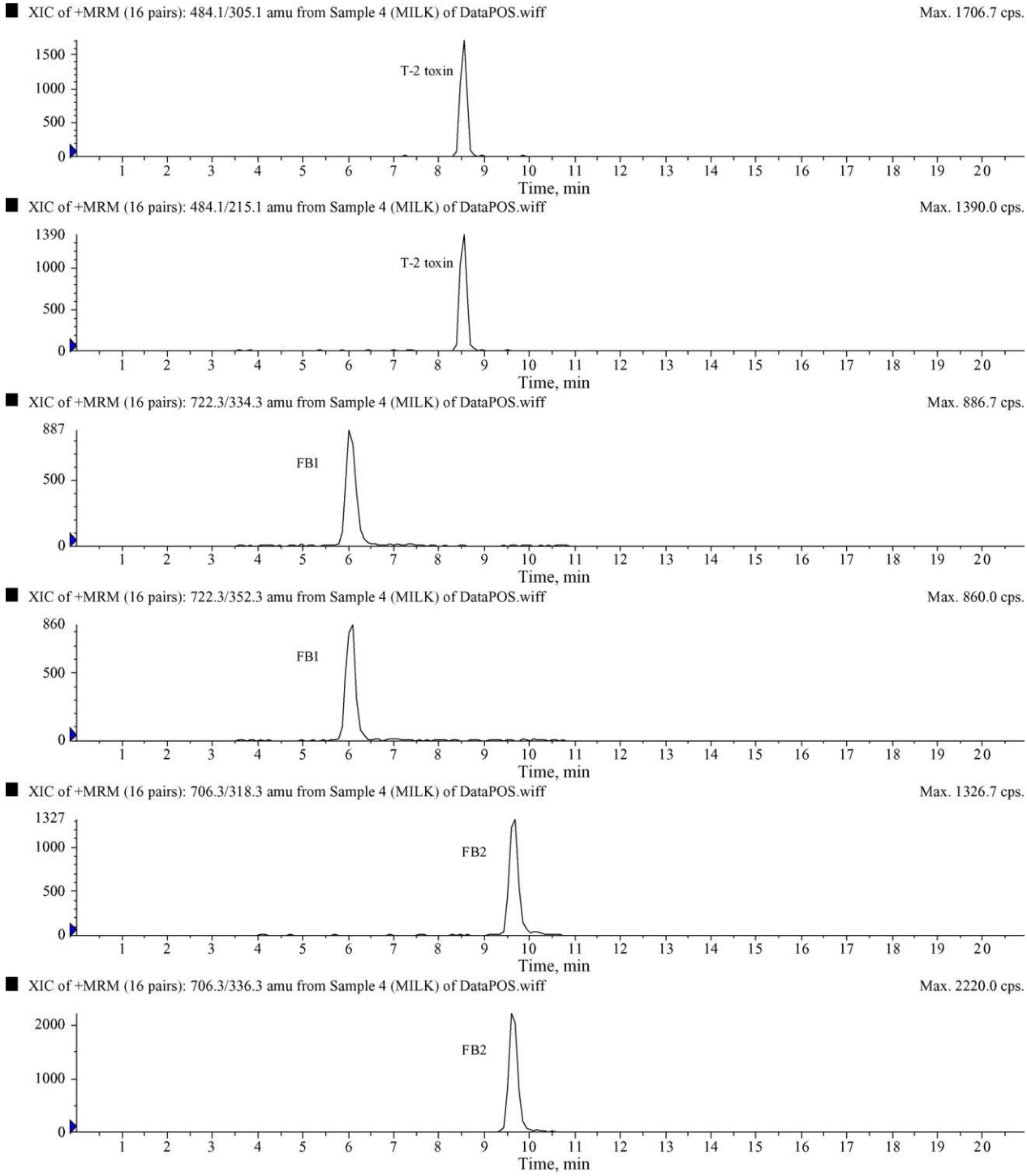

Fig. 2. LC–ESI-MS/MS chromatograms obtained in positive ion mode of blank control sample fortified with 1 μg/l of mycotoxins (0.1 μg/l of AFM_1).

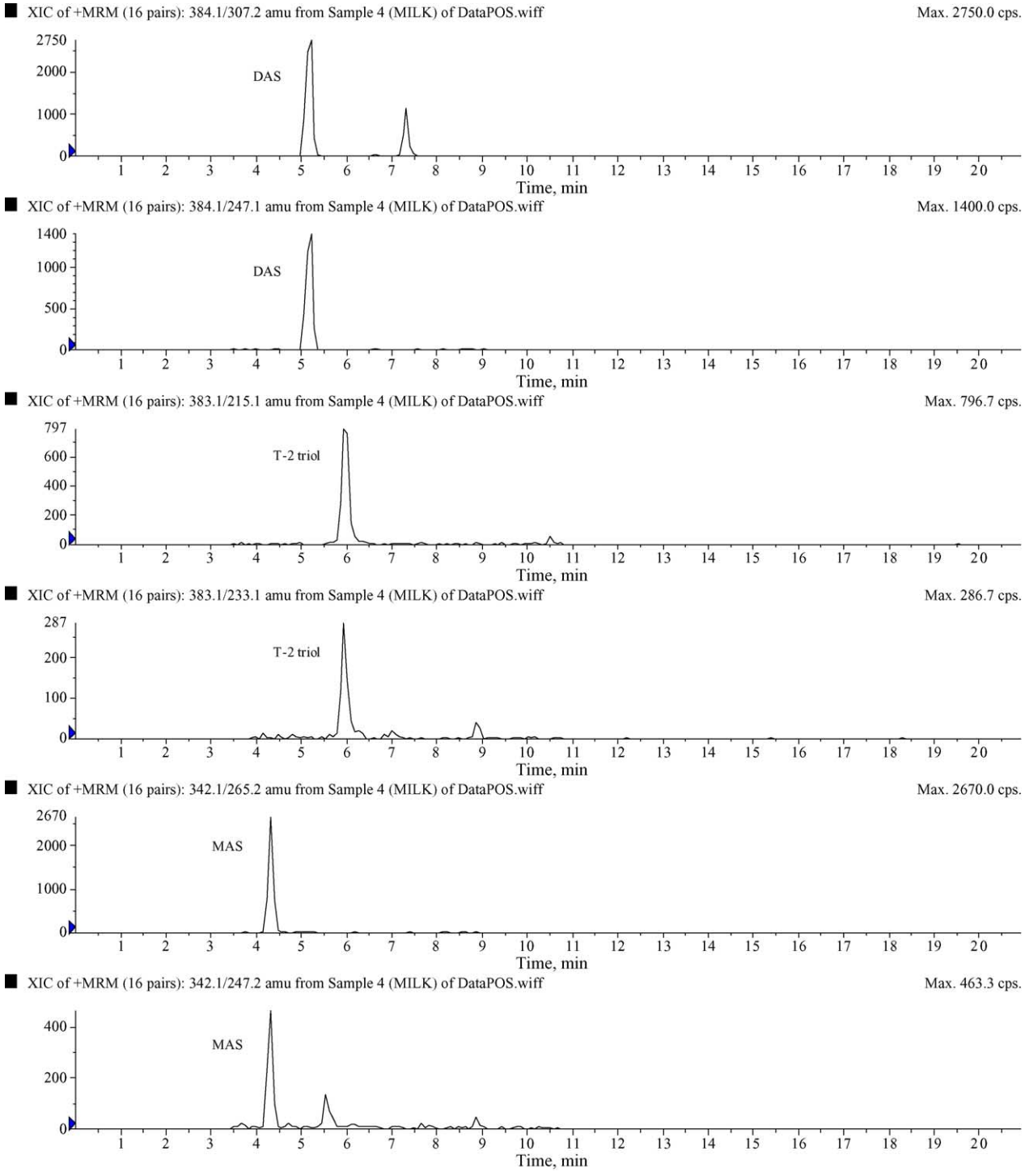

Fig. 2. (*Continued*)

tive transition products were used for quantitative measurements.

3.2. Extraction of mycotoxins

Milk proteins were precipitated by adding acetonitrile to a concentration of ca. 75%. The milk fat was removed by simultaneous extraction with hexane. If fat globules were removed by centrifugation, low recovery of ZEN and its metabolites was obtained.

The samples were acidified before extraction in order to recover the fumonisins. No significant differences in recoveries of the different mycotoxins were observed when the pH was varied in the range 1.0–3.0 and the recoveries were not

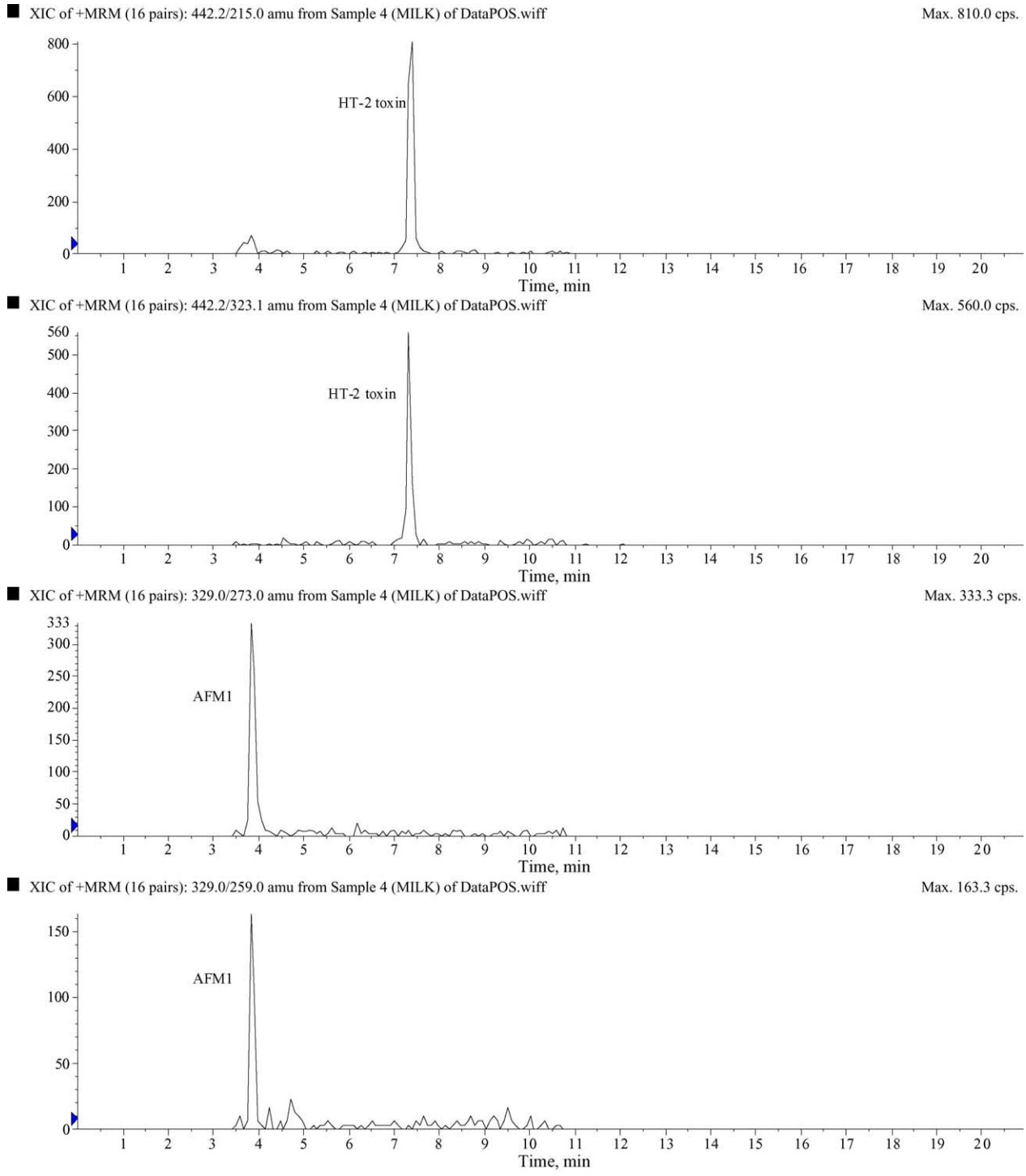

Fig. 2. (*Continued*).

dependent on standing times in the range 5–60 min between sample acidification and extraction.

The extraction was only done once. A second extraction on the pellet from the first extraction recovered less than 3% of the total content of each mycotoxin except DON and DOM-1. In the case of DON and DOM-1, a second extraction recovered 8 and 6%, respectively.

3.3. Clean-up of mycotoxins

The mycotoxins were concentrated and cleaned up by SPE. Before SPE the acetonitrile concentration was reduced to facilitate adsorption to the SPE column.

Except for ZEN and its metabolites, more than 95% of the individual compounds were eluted from the SPE cartridge in

the first 1 ml methanol applied ($n = 4$). Less than 0.5% of ZEN and its metabolites was eluted in the fourth 1 ml methanol.

3.3.1. Dependence of signal suppression on SPE pH

The residual matrix components in the sample extract affected the signal response of most mycotoxins. Generally, the signals were suppressed but the effect decreased with increasing pH used for SPE. At pH 8.5, the signals were 75–110% of corresponding signals of pure standard solutions. To investigate the sample dependence of matrix effects, 20 different sample materials including pasteurised bulk skim milk, pasteurised non-homogenised bulk milk standardised to 3.5% fat, pasteurised and homogenised bulk milk standardised to 1.5% fat and 3.5% fat, respectively and raw milk from Danish Holstein, Red Danish Breed and Jersey breeds were investigated. The samples were extracted and the final extract was spiked to a level corresponding to 1 ppb in the original sample. The analyses were repeated twice under reproducibility conditions. In all cases the mean signal S.D.s from the three sample sets were not statistically significantly different from the mean reproducibility S.D. However, a tendency to a slightly higher signal for zearalenone in low fat milk, i.e. skim milk and standardised milk containing 1.5% fat, was observed. On the basis of these experiments it was decided to use matrix calibration standards for compensation of signal suppression/enhancement effects.

3.3.2. Dependence of recovery on SPE pH

The recoveries of ZEN, α-ZEL, β-ZEL, α-ZAL, β-ZAL, OTA, FB_1 and FB_2 were clearly influenced by the pH of sample extract applied for SPE. The true recoveries of these mycotoxins declined rapidly at pHs below 6.0. It was therefore decided to keep the SPE pH at 8.5 to obtain maximum recovery and minimum matrix effects on signal intensity.

3.4. Filtration of extracts

The final sample extract was filtered through a PTFE filter. Other membranes including nylon and PVDF were tested, but they showed adsorption effects of one or more of the mycotoxins.

3.5. Optimisation of chromatographic and ionisation conditions

The specified gradient for mycotoxins analysed in negative ion mode did not separate 3-AcDON and 15-AcDON. However, the different transitions used for these mycotoxins were quite selective. The abundances of the 337 → 173 and 337 → 307 transitions of 15-AcDON were only 1 and 3% of the same transitions of 3-AcDON at equal analyte concentration. Correspondently, the abundances of the 337 → 150 and 337 → 219 transitions of 3-AcDON were only 1 and 4% of the same transitions of 15-AcDON. A peak resolution of 0.6 could be obtained with a modified gradient: After injection the 20% mobile phase 1B was changed to 40% over 13 min and then to 90% over 5 min before returning to 20% 1B after 23 min.

The mobile phase for mycotoxins analysed in positive ion mode was acidified with acetic acid in order to recover the fumonisins from the analytical column. A concentration of 0.02% was selected to obtain retention times within 4–10 min. Acidified mobile phase could not be used for mycotoxins analysed in negative ion mode because it resulted in poor signal intensities of DON and its metabolites.

Addition of ammonium acetate during ionisation of MAS, DAS, HT-2 toxin and T-2 toxin increased the signal intensity significantly. The precursor ions for these mycotoxins were the NH_4^+ adducts of the molecular ions. In contrast, NH_4^+ reduced the signal of T-2 triol. It was therefore decided to

Table 3
Data from determination of CCα and CCβ

Compound	Transition	CCα (ng/l)	Results on samples fortified to CCα level		CCβ (ng/l)
			Mean (ng/l)	S.D. (ng/l)	
DON	295 → 247	100	99	30	150
3-AcDON	337 → 173	40	35	12	60
15-AcDON	337 → 219	40	44	15	65
DOM-1	279 → 231	80	80	25	120
OTA	402 → 167	10	12	7	20
ZEN	317 → 175	20	16	7	30
α-ZEL	319 → 130	30	26	12	50
β-ZEL	319 → 130	30	25	9	45
α-ZAL	321 → 259	60	58	14	85
β-ZAL	321 → 259	60	61	12	80
T-2 toxin	484 → 215	40	39	11	60
HT-2 toxin	442 → 323	100	83	27	140
T-2 triol	383 → 233	80	78	30	130
DAS	384 → 247	30	35	13	50
MAS	342 → 247	60	68	21	90
FB_1	722 → 352	40	36	7	50
FB_2	706 → 318	20	24	12	40
AFM_1	329 → 259	10	9	4	20

Table 4
Relative repeatability standard deviation (R.S.D.$_r$) and intra-laboratory reproducibility standard deviation (R.S.D.$_{R,intra}$) determined on fortified samples

Compound	Fortification level (μg/l)	R.S.D.$_r$ (%)	R.S.D.$_{R,intra}$ (%)	Recovery mean ± S.D. (%)
DON	10	7.2	13	84 ± 6
	1	5.6	12	86 ± 6
	0.2	10	13	93 ± 10
3-AcDON	10	5.1	9.3	89 ± 7
	1	8.3	8.8	97 ± 6
	0.2	11	11	108 ± 7
15-AcDON	10	5.0	8.4	88 ± 7
	1	8.3	9.8	93 ± 7
	0.2	15	15	108 ± 5
DOM-1	10	3.5	6.0	87 ± 4
	1	8.7	8.7	91 ± 5
	0.2	11	13	97 ± 8
OTA	10	3.8	8.6	102 ± 8
	1	5.5	8.4	104 ± 8
	0.2	5.8	7.8	103 ± 7
	0.05	12	15	104 ± 10
ZEN	10	4.4	8.8	82 ± 4
	1	6.7	10	90 ± 7
	0.2	13	13	92 ± 6
	0.05	11	15	106 ± 10
α-ZEL	10	7.1	15	94 ± 8
	1	7.0	11	92 ± 6
	0.2	13	13	99 ± 8
β-ZEL	10	7.6	9.6	90 ± 7
	1	7.2	11	95 ± 7
	0.2	14	15	97 ± 10
α-ZAL	10	7.0	10	90 ± 7
	1	6.9	9.5	95 ± 7
	0.2	11	14	97 ± 10
β-ZAL	10	6.4	9.2	90 ± 7
	1	6.8	8.7	95 ± 7
	0.2	12	15	97 ± 10
T-2 toxin	10	5.6	7.7	92 ± 6
	1	5.5	9.3	99 ± 5
	0.2	11	14	101 ± 9
HT-2 toxin	10	3.2	7.8	93 ± 7
	1	11	11	89 ± 7
	0.2	15	15	108 ± 11
T-2 triol	10	5.1	8.2	88 ± 7
	1	5.4	10	95 ± 9
	0.2	12	13	101 ± 10
DAS	10	5.7	9.3	94 ± 8
	1	7.3	9.7	100 ± 8
	0.2	10	12	100 ± 9
MAS	10	4.6	6.4	91 ± 5
	1	4.9	8.0	96 ± 7
	0.2	5.4	11	101 ± 11
FB_1	10	4.9	8.8	76 ± 6
	1	5.3	8.9	80 ± 7
	0.2	12	12	83 ± 5
FB_2	10	4.0	8.4	78 ± 6
	1	4.1	7.4	86 ± 6
	0.2	11	12	90 ± 7
AFM_1	1.0	6.2	8.2	80 ± 6
	0.1	5.5	7.2	87 ± 5
	0.02	9.4	12	98 ± 10

apply a segmented post column addition of NH_4-acetate to obtain the highest possible sensitivity for all mycotoxins.

3.6. Method performance parameters

The CCαs and CCβs related to the less sensitive ion transitions were established according to procedures specified for cases where no permitted limit has been established (Table 3). The CCαs and the CCβs ranged from 10 to 100 μg/l and 20 to 150 μg/l, respectively with lowest values for OTA, AFM_1 and ZEN.

The relative repeatability standard deviations ($R.S.D._r$) and relative intra-laboratory reproducibility standard deviations ($R.S.D._{R,intra}$) obtained in a precision study on fortified samples are listed in Table 4. The true recoveries obtained in the same study were in general better than 80% in the concentration range 0.2–10 μg/l (0.02–1 μg/l for AFM_1) (Table 4).

Calibration curves were created using non-weighted linear regression analysis with lines forced through the origin. The coefficients of determination (R^2) for the different transitions were in the range 0.998–1.000.

3.7. Results from practice

The method developed has been used on bulk milk from dairies in Denmark ($n = 42$). In no cases were mycotoxins detected. The method has also been applied to milk samples from selected herds and single cows which showed illness symptoms that could not be diagnosed ($n = 20$). Because mycotoxicoses was suspected as a possible cause milk quality was tested. In five of these samples, DOM-1 was detected at levels up to 0.3 μg/l. The results obtained without β-glucuronidase treatment were 0.18 ± 0.072 μg/l (mean ± S.D.) while the results obtained with treatment were 0.22 ± 0.136 μg/l. The number of samples was not sufficiently high for conclusion of statistical significance of the β-glucuronidase treatment by a paired *t*-test.

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