Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 843 (2006) 252–261
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of zearalenone and its metabolites in urine, plasma and faeces of horses by HPLC–APCI–MS

P. Songsermsakul [a], G. Sontag [b], M. Cichna-Markl [b], J. Zentek [a], E. Razzazi-Fazeli [c,*]

[a] *Department of Veterinary Public Health, Institute of Nutrition, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria*
[b] *Department of Analytical and Food Chemistry, University of Vienna, Währinger Strasse 38, A-1090 Vienna, Austria*
[c] *vetOmics/Proteomics and Metabolomics centre, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria*

Received 5 December 2005; accepted 8 June 2006
Available online 7 July 2006

Abstract

The paper describes a method for the sensitive and selective determination of zearalenone and its metabolites in urine, plasma and faeces of horses by high performance liquid chromatography and atmospheric pressure chemical ionisation (APCI) mass spectrometry (MS). While only one step sample clean-up by an immunoaffinity column (IAC) was sufficient for plasma samples, urine and faeces samples had to be prepared by a combination of a solid-phase extraction (SPE) and an immunoaffinity column. The method allows the simultaneous determination of zearalenone and all of its metabolites; α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol and zearalanone. Dideuterated zearalanone was used as internal standard for quantification and the study of the matrix effect. Recovery rates between 56 and slightly above 100% were achieved in urine samples, and more than 80% in plasma and faeces samples. The limits of detection ranged from 0.1–0.5 μg/l or μg/kg, the limits of quantification from 0.5–1.0 μg/l or μg/kg. The practical use of the method is demonstrated by the analysis of spiked and naturally contaminated urine, plasma and faeces of horses.

Keywords: Zearalenone; Metabolites; Liquid chromatography–mass spectrometry; Urine; Plasma; Faeces; Horse

1. Introduction

Zearalenone (ZON) [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid lactone] is a secondary fungal metabolite produced by *Fusarium* species, such as *Fusarium culmorum* and *Fusarium graminearum*. *Fusarium* species colonize several grains like maize, barley, oats, wheat and sorghum [1]. Since the chemical conformation of ZON sufficiently resembles 17β-estradiol, it binds to the estrogen receptor and can therefore be classified as an endocrine disruptor. Occurrence of ZON in animal feed causes hyperestrogenism with severe reproductive and infertility problems, especially in swine. Concentration ratios of ZON and its metabolites and the susceptibility to the adverse effects of ZON vary considerably with animal species.

Feeding of naturally contaminated grains with fusarium mycotoxin including ZON has been shown to cause hyperestrogenic and adverse metabolic effect in livestock and poultry [2,3]. In mammals, ZON is mainly metabolized into α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) [4] with α-ZOL being dominant in pigs whereas in cows, β-ZOL is the predominant metabolite [5,6]. α-ZOL is the only ZON derivative which has been found to be generated by *Fusarium* fungi [7]. It has been shown that the reduced forms exhibit a higher estrogenic activity than ZON. Erasmuson et al. demonstrated that α-ZOL and β-ZOL can be reduced further, resulting in significant amounts of α-zearalanol or zeranol (α-ZAL) and β-zearalanol or taleranol (β-ZAL) in mammal urine [8]. Because of its anabolic effect, α-ZAL is used as a growth promoter. Although its application has been banned in the European Union, there is still a need to monitor its abuse. Zearalanone (ZAN) was identified in a few samples of pig and cattle urine [5]. Until now the mechanism of ZAN formation is still unclear (Fig. 1).

There is, however, a lack of information on the effects of fusarium toxins on horses. Raymond et al. described a decrease of feed intake in both unexercised and exercised horses which had been fed a naturally contaminated feed with fusarium

* Corresponding author. Tel.: +43 1 25077 3216; fax: +43 1 25077 3290.
E-mail address: ebrahim.razzazi@vu-wien.ac.at (E. Razzazi-Fazeli).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.06.012

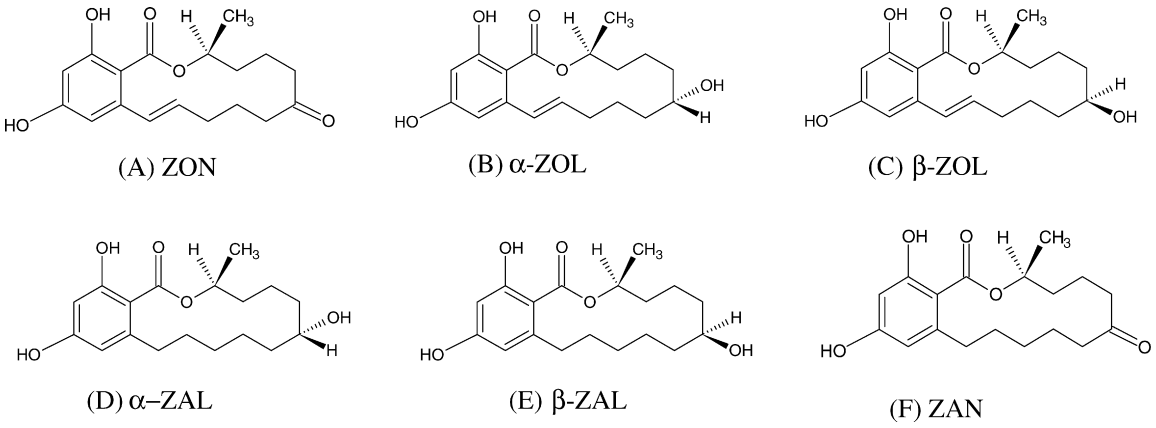

Fig. 1. Structures of ZON and its metabolites.

mycotoxins although one could not observe significant changes of haematology, serum chemistry or athletic performance [9,10].

A number of methods have been developed to determine ZON and its metabolites in various sample materials, frequently complex biological samples containing various interfering matrix components. In order to achieve the required specificity, these methods include sample preparation steps like conventional liquid–liquid extraction, which requires tedious multiple extraction steps, and solid-phase extraction (SPE). In many cases the selectivity of the sample preparation could, however, be improved by antibody-based immunoaffinity chromatography, which has hardly been applied in this field up to now in spite of the additional advantages of being less time consuming and needing less organic solvent.

ZON and α-ZOL analysis can be performed in food and feed by thin layer chromatography (TLC), enzyme linked immunosorbent assays (ELISA), gas chromatography (GC) and high performance liquid chromatography with either fluorescence (HPLC–FLD) or mass spectrometric detection (HPLC–MS) and HPLC–MS–MS [2]. TLC and GC have, however, largely been replaced by HPLC–FLD, HPLC–MS or HPLC–MS–MS since TLC is not sensitive and selective enough for many analysis tasks and GC techniques need a derivatisation step to enhance the volatility of analytes which is time-consuming and error-prone. Compared with HPLC–FLD, HPLC–APCI (atmospheric-pressure chemical ionisation)–MS was found to improve the sensitivity of an analysis method developed for the determination of mycotoxins in food and feed by a factor of about 50 (detection limit is reported to be 2.5 ppb) [11].

The determination of ZON and its metabolites in biological fluids by one of these methods has already been described in some papers. Using HPLC–FLD Dänicke et al. investigated the kinetics and metabolism of ZON in young female pigs [12]. In spite of its inherent disadvantages GC coupled with MS has also been applied in this area [13–15] but the combination of high selectivity and sensitivity make HPLC–MS and HPLC–MS–MS the most suitable techniques for this task. Jodlbauer et al. developed an analysis method to measure ZON, α-ZOL, β-ZOL, α-ZAL and β-ZAL simultaneously in urine and bovine or porcine tissue using HPLC–MS–MS after one step solid-phase extraction clean-up [6]. Recently, Sorensen et al. [16] published a method for the analysis of different mycotoxins in bovine milk by HPLC–MS–MS. The new methods allow the determination of more than one family of co-occurring mycotoxins within a single run. Liquid–liquid extraction, SPE and immunoaffinity chromatography were used in the clean-up of biological samples. A separation scheme combining liquid–liquid extraction and IAC followed by HPLC–FLD was described by Dänicke et al. to determine ZON and its metabolites in broilers [17]. The use of simple detectors such as FLD and single quadrupole mass spectrometer can be compensated by complex sample clean-up. In contrast, the use of high selective MS/MS instrumentation is able to reduce the complexity of sample preparation as reported by Jodlbauer et al. [6]. However, many impurities still remain in one step clean-up which can cause the matrix effect and damage LC column.

So far, atmospheric-pressure chemical ionisation (APCI) and electrospray ionisation (ESI) have been the preferred coupling interfaces for HPLC–MS for determination of ZON and its metabolites in biological samples. The ionisation process in APCI and ESI occurs at atmospheric pressure through ion/molecule reactions. Both of them can be readily coupled with liquid separation techniques. APCI has the advantage over ESI that weakly polar analytes not existing as preformed ions in solution can be readily ionised.

Up to now, few publications dealt with the use of HPLC coupled to MS (single quadrupole) in the determination of ZON and its metabolites in animal samples. Bily et al. developed an analysis method to determine deoxynivalenol, 15-acetyl deoxynivalenol and ZON (but not its metabolites) in fungal liquid cultures, maize grain, insect larvae and pig serum using LC–MS (single quadrupole, positive ion mode) [18].

The present paper aims at providing a versatile analysis method which allows filling the existing gap of data on ZON metabolism in horses. It describes the simultaneous determination of ZON and its metabolites by LC-single quadrupole MS after a new highly selective multi-step sample clean-up procedure including an immunoaffinity step. The method was applied for the determination of ZON and its metabolites in urine, plasma and faeces of horses.

2. Experimental

2.1. Materials

ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL, ZAN standards, phosphate buffer saline (PBS) tablets and sodium hydroxide (analytical reagent grade) were purchased from Sigma (Deisenhofen, Germany). The standards were individually dissolved in methanol to give stock solutions of 1 mg/ml which were stored at −20 °C until use. Standard working solutions were prepared by diluting each stock solution with the mobile phase consisting of water:acetonitrile:methanol (H_2O:ACN:MeOH = 35:30:35, v/v/v). The internal standard (IS), 1′,2′-dideuterated zearalanone (D_2-ZAN), was kindly provided by the laboratory of Prof. Wolfgang Lindner, Department of Analytical and Food Chemistry, University of Vienna. D_2-ZAN was synthesized by catalytic deuteration of ZON. Compound purity (>99.5%), deuterium content (two deuterium atoms per molecule), and structure were established by mass spectrometry and NMR spectroscopy [5]. A methanolic stock solution of D_2-ZAN with a concentration of 80 μg/ml was stored at −20 °C. Calibration curves were set up with the sample matrices spiked with mixed standards and internal standard solutions. All biological samples were taken from the horses at Clinic for Obstetrics, Gynecology and Andrology University of Veterinary Medicine Vienna. ISOLUTE® C18 and ISOLUTE® NH_2 solid-phase extraction columns with 100 mg adsorbent and 3 ml reservoir volume were obtained from IST International Sorbent Technology (Mid Glamorgan, UK). The immunoaffinity columns (IAC) Easi-Extract® Zearalenone were purchased from R-Biopharm (Darmstadt, Germany). Glucuronidase/arylsulfatase from *Helix pomatia* (30/60 U/ml), ammonium acetate, glacial acetic acid (both analytical reagent grade) as well as HPLC-grade methanol and HPLC-grade acetonitrile (ACN) were supplied by Merck (Darmstadt, Germany). Water was purified in a UPW2 system (F&L, Vienna, Austria). All solvents were degassed using a Waters in-line degasser (Milford, MA, USA).

2.2. HPLC–FLD

HPLC system used in preliminary experiment consisted of a L-7100 pump, L-2750 autosampler and L-7480 fluorescence detector (Merck-Hitachi, Darmstadt, Germany). Excitation wavelength 273 nm and emission wavelength 440 nm were set up. The HPLC was operated with a mobile phase system consisting of H_2O:MeOH (45:55, v/v) at a flow rate of 1 ml/min without splitting. Injection volume was 50 μl.

Optimisation of sample clean-up was performed in blank urine with either SPE–SPE combination or SPE–IAC combination. The selection of the washing medium was performed in 5 ml of 75% acetonitrile spiked with 100 μl of ZON (1.5 μg/ml), α-ZOL (1 μg/ml) and β-ZOL (10 μg/ml) and 20 ml of phosphate buffer saline mixture. In order to find the most appropriate washing medium, the influence of the MeOH concentration on the recovery of ZON and its two major metabolites (α-ZOL, β-ZOL) was determined.

2.3. Analytical separation using HPLC–MS

The analytical separation was achieved with a system consisting of a Waters 626-LC pump and Waters 717 plus autosampler (Milford, MA, USA). The HPLC was operated with a mobile phase system consisting of H_2O:ACN:MeOH (35:30:35, v/v/v) at a flow rate of 1 ml/min without splitting. Injection volume was 50 μl. The complex nature of biological samples can cause trapping of matrix components on the LC column. To avoid the trapping phenomena, mobile phase was injected after four sample runs. The results show a satisfactory separation and a reproducible retention time. However, an appropriate solution of this problem would be to increase the organic content to 95–100% of acetonitrile after several injections.

The six analytes (ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL and ZAN) and the internal standard were separated on a Synergi 4 μm Polar-RP 80A (150 mm length × 4.6 mm) and a Synergi 4 μm Hydro-RP 80A column (150 mm length × 4.6 mm) (Phenomenex, Cheshire, UK). The columns were maintained at a temperature of 25 °C (W.O. Electronics, Vienna, Austria).

Mass spectrometric detection was performed with a Platform II mass spectrometer using an APCI interface equipped with a Pepperpot counter electrode (Micromass, Manchester, UK). The optimisation processes were carried out in scan mode (*m/z* 150–500). Pure nitrogen as nebulizing and carrier gas was produced in a Parker Balston generator (Tewksbury, MA, USA). Drying gas flow rate was set at 400 l/h and sheath gas flow rate was held at 200 l/h. The source and APCI vaporizing temperature were maintained at 100 °C and 500 °C, respectively, and the cone voltage was set at 20 V. Quantitative determination of all analytes was applied in the single ion recording (SIR) mode for corresponding deprotonated molecules (α/β-ZOL and ZAN *m/z* 319.1, α/β-ZAL and D_2-ZAN *m/z* 321.1 and ZON *m/z* 317.1) which were analyzed with a dwell time of 0.3 s and a span of 0.2 u.

2.4. Sample preparation

2.4.1. Urine sample

Horse urine (5 ml) was mixed with 25 ml of 0.05 M ammonium acetate buffer pH 4.8 and 25 μl of 1 μg/ml D_2-ZAN. This solution was incubated for 15 h at 37 °C with 100 μl of glucuronidase/arylsulfatase solution. After adjusting the pH with glacial acetic acid to pH 4 the solution was loaded to an ISOLUTE® C18 solid-phase extraction column, which had been pre-conditioned with 5 ml of MeOH, followed by 5 ml of H_2O. After washing the SPE column with 2 ml of 30% MeOH it was dried for 3 min by applying vacuum. The analytes were then eluted with 1.25 ml of MeOH. The eluate was mixed with 20 ml of phosphate buffer saline pH 7.4. This mixture was introduced to an immunoaffinity column Easi-Extract® Zearalenone which had been pre-conditioned with 15 ml of PBS. The immunoaffinity column was washed with 15 ml of water followed by 10 ml of 30% MeOH and dried for 3 min under the vacuum. Elution of the analytes was carried out with 1.5 ml of ACN. The eluate

was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 500 μl of the HPLC mobile phase. A 50 μl amount of this solution was injected into the HPLC–MS system.

2.4.2. Plasma sample

One milliliter of plasma was mixed with 5 ml of 0.05 M ammonium acetate buffer pH 4.8 and 5 μl of 1 μg/ml D_2-ZAN. This solution was incubated for 15 h at 37 °C with 25 μl of glucuronidase/arylsulfatase solution before mixing it with 6 ml of phosphate buffer saline and adjusting pH to 7.4 with 1 M NaOH. After centrifugation at 2500 × *g* with an Eppendorf Centrifuge 5810R (Eppendorf AG, Hamburg, Germany), the clear part was loaded onto an IAC which had been pre-conditioned with 15 ml PBS. The immunoaffinity column was washed with 10 ml water and 3 ml of 30% MeOH and dried for 3 min by forcing vacuum. Elution was performed with 1.5 ml of acetonitrile. The eluate was evaporated to dryness under a stream of nitrogen. The residue was re-dissolved in 250 μl of the HPLC mobile phase. A 50 μl amount of this solution was injected into the HPLC–MS system.

2.4.3. Faeces sample

Faeces were processed in the freeze-drying machine (Gamma 2-20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Two grams of dry matter were extracted with 40 ml of MeOH:H_2O (50:50, v/v). After centrifugation, 20 ml of supernatant were mixed with 40 ml of 0.05 M ammonium acetate buffer pH 4.8 and 20 μl of 1 μg/ml D_2-ZAN. This solution was incubated for 15 h at 37 °C with 80 μl of glucuronidase/arylsulfatase solution. All the following steps were carried out as described above for clean-up of urine samples. The final eluate was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 250 μl of the HPLC mobile phase. A 50 μl amount of this solution was injected into the HPLC–MS system.

3. Results and discussion

3.1. Optimisation of mass spectrometric parameters

Since interface parameters play an important role in APCI–MS measurements, they have to be optimised in order to obtain the highest sensitivity. This was investigated in the flow-injection mode by bypassing the column. All six analytes were injected separately to adjust the parameters for each metabolite. In agreement with the previous studies [6,11,19], the negatively charged deprotonated molecules of ZON were found to be more abundant than the positively charged protonated molecules. This result was also obtained for the other five metabolites. The chemical structures of ZON and its metabolites shows, that acidic phenolic groups enhance a deprotonation of the molecules. The *m/z* 319.1 deprotonated molecule showed higher intensity for α/β-ZOL and ZAN, the *m/z* 321.1 deprotonated molecule for α/β-ZAL and the *m/z* 317.1 fragment for ZON in negative ion mode.

3.2. Chromatographic separation

In the preliminary study, various types of LC columns were used. However, an appropriate baseline separation was not achieved. In order to obtain sufficient chromatographic separation it was necessary to combine two analytical columns: a Polar-RP containing ether-linked phenyl groups with polar end-capping and a Hydro-RP C18 with polar end-capping. Fig. 2 shows the LC–MS chromatogram of six analytes in scan mode. A complete separation of all six metabolites was achieved within 15 min with stable retention times. The total run time was approximately the same as using single analytical column [4–6].

3.3. Sample preparation

3.3.1. Clean-up process (HPLC–FLD)

The complex urine and faeces samples analyzed in the present study made it necessary to develop a highly selective sample

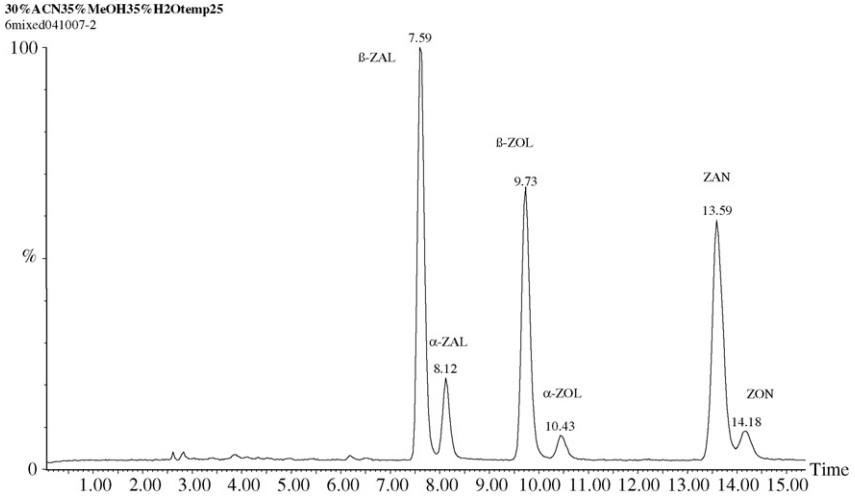

Fig. 2. LC–MS chromatogram in scan mode of ZON and its metabolites standard. ZON 0.075 μg/ml, α-ZOL 0.05 μg/ml, β-ZOL 0.5 μg/ml, ZAN 0.3 μg/ml, α-ZAL 0.25 μg/ml and β-ZAL 0.4 μg/ml (injection volume, 20 μl).

clean-up procedure. The optimisation was performed with combination of SPE–SPE and SPE–IAC. The SPE procedure was modified from a method described by Jodlbauer et al. [6]. Preliminary experiments with blank urine, the eluate from C18-SPE appeared to be turbid and had dark brown colour. However, when the eluate was re-applied onto a second NH_2-SPE column, the final solution still contained many matrix peaks in the HPLC–FLD chromatogram. On the other hand, application of the first eluate from C18-SPE onto IAC resulted in a significantly cleaner solution than from the NH_2-SPE column. The final eluate from the IAC was clear with a slightly yellow colour.

3.3.2. Optimisation of the washing medium for IAC (HPLC–FLD)

The preliminary experiment was processed by injecting a test mixture consisting of 5 ml 75% ACN spiked with 100 μl of ZON (1.5 μg/ml), α-ZOL (1 μg/ml) and β-ZOL (10 μg/ml) and 20 ml PBS. The results did not show significant differences of recovery rate, when washing the column with either water or 10% MeOH or 30% MeOH. However, when increasing the concentration of MeOH to 50%, the recovery rate dramatically dropped more than 50%. Therefore 30% MeOH was chosen as washing medium in further experiments. α-ZAL, β-ZAL and ZAN could not be included into the preliminary study. In order to surpass their high limits of detection in HPLC–FLD, it would have been necessary to overload the IAC.

3.3.3. Urine sample (HPLC–APCI–MS)

While the recovery rates for ZON and α/β-ZOL were slightly above 100% the recovery rates for ZAN and α/β-ZAL were significantly lower. This result is in agreement with Dänicke et al. who used a liquid–liquid extraction–IAC combination for sample clean-up. They reported recovery rates for ZAN and α/β-ZAL of 40–64% in the excreta [17]. Comparing the present method with a one step SPE clean-up method, Jodlbauer et al. obtained a satisfactory rate of recovery of 91–102% in urine and 86–91% in tissue samples [6]. This method was also applied to pig liver with recovery rates ranging from 55–85% and 94–105% in urine. However, a highly sensitive LC/MS–MS system was used, since MRM (multiple reaction monitoring) chromatograms showed interfering peaks [5].

Due to the complexity of urine and faeces samples, the use of SPE prior to IAC was necessary. In the preliminary studies, direct loading of urine or faeces extract into IAC caused obstruction. Moreover, the eluate of IAC contained various interfering peaks. Therefore, single use of IAC with urine or faeces samples is not possible to eliminate matrix associated peaks.

Table 1
Validation data for all analytes of urine, plasma and faeces samples; (A) urine samples, (B) plasma samples, (C) faeces samples

	ZON	α-ZOL	β-ZOL	α-ZAL	β-ZAL	ZAN
(A) Validation data for all analytes determined in urine samples						
Spiked value (μg/l)	3.0	2.0	20.0	10.0	16.0	12.0
Measured value (μg/l)	3.2	2.1	19.0	7.9	14.8	10.7
Deviation (%)	+6.7	+5.0	−5.0	−21.0	−7.5	10.8
Recovery (%)	108.3	104.2	103.8	56.5	69.8	58.3
RSD ($n=6$)	7.5	8.4	8.1	5.3	7.0	3.9
Limit of detection (μg/l)	0.1	0.1	0.1	0.2	0.2	0.2
Limit of quantification (μg/l)	0.5	0.5	0.5	1.0	1.0	1.0
Linear range (μg/l)	0.5–100	0.5–100	0.5–100	1.0–100	1.0–100	1.0–100
	ZON	α-ZOL	β-ZOL	α-ZAL	β-ZAL	ZAN
(B) Validation data for all analytes determined in plasma samples						
Spiked value (μg/l)	0.75	0.5	5.0	2.5	4.0	3.0
Measured value (μg/l)	0.8	0.5	5.2	2.6	4.1	3.2
Deviation (%)	+6.7	0	+4.0	+4.0	2.5	+6.7
Recovery (%)	100.8	84.0	91.9	89.6	88.8	98.3
RSD ($n=6$)	7.8	10.7	6.2	2.0	4.6	6.3
Limit of detection (μg/l)	0.1	0.1	0.2	0.3	0.3	0.3
Limit of quantification (μg/l)	0.5	0.5	0.5	0.6	0.6	0.6
Linear range (μg/l)	0.5–100	0.5–100	0.5–100	0.6–100	0.6–100	0.6–100
	ZON	α-ZOL	β-ZOL	α-ZAL	β-ZAL	ZAN
(C) Validation data for all analytes determined in faeces samples						
Spiked value (μg/kg)	75	50	50	25	40	30
Measured value (μg/kg)	72.8	50.5	45.3	27.1	41.4	28.9
Deviation (%)	−2.9	+1.0	−9.4	+8.4	+3.5	−3.7
Recovery (%)	104.4	104.7	108.2	93.2	104.2	91.9
RSD ($n=6$)	3.0	4.8	3.4	2.1	3.7	2.0
Limit of detection (μg/kg)	0.1	0.1	0.1	0.5	0.5	0.5
Limit of quantification (μg/kg)	0.5	0.5	0.5	1.0	1.0	1.0
Linear range (μg/kg)	0.5–100	0.5–100	0.5–100	1.0–100	1.0–100	1.0–100

Limit of detection (LOD) and limit of quantification (LOQ) in the present method were 0.1–0.2 μg/l and 0.5–1.0 μg/l, respectively. They are comparable to LOD and LOQ of LC/MS–MS method which ranged between 0.1–0.5 μg/l and 0.5–1.0 μg/l, respectively [6]. Therefore the intensive sample preparation can improve the sensitivity of the method. LOD of the present method is significantly lower than the LOD of HPLC–FLD, ranging from 0.5 ng/g to 200 ng/g for all metabolites [16]. Table 1(A) shows the data collected for urine samples.

3.3.4. Plasma sample (HPLC–APCI–MS)

One step IAC clean-up of plasma samples was selective enough to permit a direct injection of the eluate into the LC–MS system after reconstitution without any further modification. The recovery rates for all metabolites were satisfactory, ranging from 84–100%. This one step method is less time consuming and therefore superior to the combination of liquid–liquid extraction and IAC which had been shown to result in recovery rates of 57–99% for broilers plasma using HPLC–FLD [17]. Limit of detection and limit of quantification in the present method are 0.1–0.3 μg/l and 0.5–0.6 μg/l, respectively. The LOD of HPLC–APCI–MS are significantly lower than HPLC–FLD method which has LOD ranging between 5 ng/g and 360 ng/g [17]. Analysis of ZON and its metabolites in human plasma was also performed by GC–MS. The estimated LOD ranged from 0.2 μg/l to 3.0 μg/l and the estimated LOQ ranged from 1.0 μg/l to 10 μg/l [20]. As can been seen in Table 1(B), with the present method a higher sensitivity was obtained than with GC–MS method. There are no validation data available for plasma using LC–MS or LC–MS/MS. The present study set up the recovery rate, LOD and LOQ for ZON and all of its metabolites in LC–MS for the first time. Table 1(B) summarizes the analysis data obtained.

3.3.5. Faeces sample (HPLC–APCI–MS)

SPE–IAC combination was necessary in faeces sample clean-up because of its complex nature. As the high recovery rates given in Table 1(C) demonstrate, the relatively high MeOH concentration in the extracting solution did not interfere with the further clean-up steps. The final eluate from IAC was similar with that one from urine which was clear, containing slightly yellow colour in some samples. The recovery rates of α-ZAL (93.2%), β-ZAL (104.2%) and ZAN (91.9%) were significantly better than those in urine samples. This result may be due to the additional extraction step and also the sample matrix itself. The present method shows higher recovery rates compared to the combined use of liquid–liquid extraction and IAC where the rates of recovery were 108% for ZON, 69% for α-ZOL and 53% for β-ZOL [12]. The validation data of faeces in LC–MS/MS is still missing. However the sample preparation presented in this study should be a versatile method that can be applied in LC–MS/MS as well.

3.4. Matrix effects on the MS response

Co-eluting matrix compounds can be a serious problem in LC–MS and LC–MS/MS. Recent papers have shown, that although highly selective instruments like LC/MS–MS were used, a decrease of accuracy and reproducibility were observed because of the matrix effects in grain samples [19,21,22]. In

Table 2
Determination of all analytes in urine, plasma and faeces samples with and without internal standard (A) urine samples ($n = 6$), (B) Plasma samples ($n = 6$), (C) Faeces samples ($n = 6$)

Analyte	Spiked value (μg/l)	Without internal standard		With internal standard	
		Measured value (μg/l)	Deviation (%)	Measured value (μg/l)	Deviation (%)
(A) Determination of all analytes in urine ($n = 6$) with and without internal standard					
ZON	3.0	3.7	+23.3	3.2	+6.7
α-ZOL	2.0	1.7	−15.0	2.1	+5.0
β-ZOL	20.0	23.0	+15.0	19.0	−5.0
α-ZAL	10.0	6.2	−38.0	7.9	−21.0
β-ZAL	16.0	11.8	−26.2	14.8	−7.5
ZAN	12.0	15.6	+30.0	10.7	−10.8
(B) Determination of all analytes in plasma ($n = 6$) with and without internal standard					
ZON	0.75	1.2	+60.0	0.8	+6.7
α-ZOL	0.5	0.7	+40.0	0.5	0
β-ZOL	5.0	6.0	+20.0	5.2	+4.0
α-ZAL	2.5	3.5	+40.0	2.6	+4.0
β-ZAL	4.0	2.9	−27.5	4.1	+2.5
ZAN	3.0	2.6	−13.3	3.2	+6.7
(C) Determination of all analytes in faeces ($n = 6$) with and without internal standard					
ZON	75.0	66.7	−11.1	72.8	−2.9
α-ZOL	50.0	39.4	−21.2	50.5	+1.0
β-ZOL	50.0	58.0	+16.0	45.3	−9.4
α-ZAL	25.0	31.3	+25.2	27.1	+8.4
β-ZAL	40.0	45.9	+14.8	41.4	+3.5
ZAN	30.0	20.7	−31.0	28.9	−3.7

the present study the influence of matrix effects was investigated for each sample matrix (urine, plasma, faeces) and for each metabolite by comparing the results obtained for spiked sample calculated either with or without internal standard. The results, given in Table 2(A–C) show that without internal standard the accuracy of the data is poor (deviation range −38.0 to +60.0%). A possible explanation is the variation of the detector response, which can be approximately 10% as shown by Zöllner et al. [19]. The use of an appropriate internal standard which does not interfere with the detector response of the analytes allows to correct for part of the matrix effects. In this study D_2-ZAN, which is not found in nature, was used as internal standard. The deviations are obviously reduced in all sample types and for all metabolites (−10.8 to +8.4% deviation).

The method described in the present paper uses both approaches—the use of an internal standard and the use of a very efficient and selective sample preparation. However, as can be seen in the results of urine and faeces samples, even the two steps clean-up with SPE and IAC could not eliminate the matrix effect which consists in either ion suppression or ion enhancement for each metabolite. The results obtained with urine samples indicate the presence of the matrix effect. Even when an internal

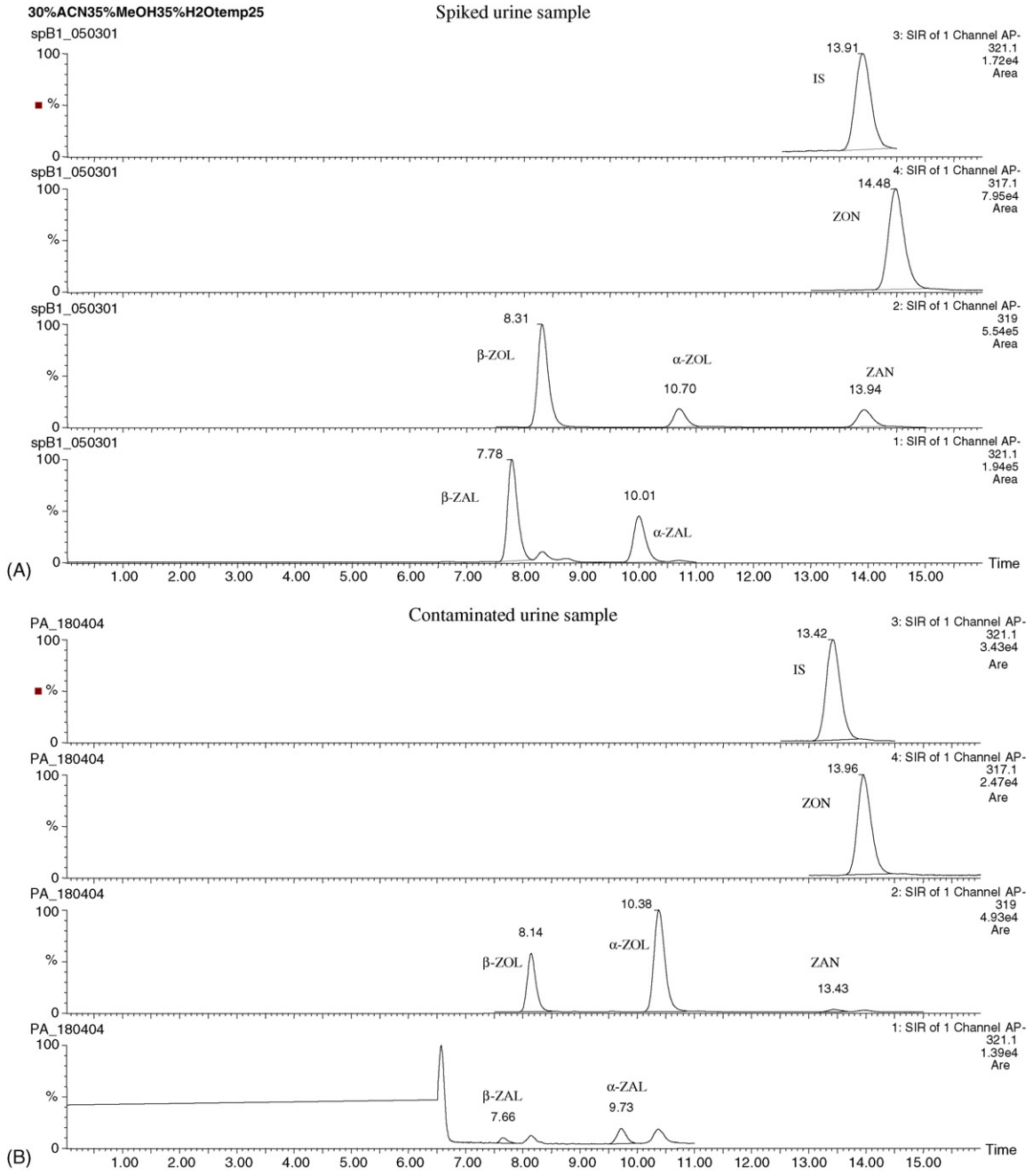

Fig. 3. LC–APCI/MS chromatogram of horses urine: (A) spiked urine sample with ZON 3 μg/l, ZAN 12 μg/l, α-ZOL 2 μg/l, β-ZOL 20 μg/l, α-ZAL 10 μg/l, β-ZAL 16 μg/l and 5 μg/l D_2 ZAN (IS); (B) contaminated urine sample.

standard was used, the deviation was −21%. Ion suppression was found highly in α-ZAL and ZAN and slightly in β-ZOL and β-ZAL.

The deviation rates in plasma samples calculated from results without IS were high (13–60%). Fortunately, the use of IS could reduce the matrix effect. Compared with urine, the matrix effect of faeces samples for α-ZAL was also high (25%). However due to the use of the IS the ion enhancement could be reduced. Ion suppression could be clearly seen in the case of ZAN. The use of IS can reduce the problem. Matrix effect was compensated as can be seen in Table 2. Due to the complex nature of biological samples, it is difficult to predict if ion enhancement or ion suppression can occur.

3.5. Application of the method to spiked and naturally contaminated horse urine, plasma and faeces

The method was applied to determine all six analytes in spiked and contaminated horse urine, plasma and faeces in order to demonstrate its practicality. The LC–MS chromatograms obtained in single ion recording mode (Figs. 3–5) are very clean and show relatively few interferences. α-ZAL and β-ZAL

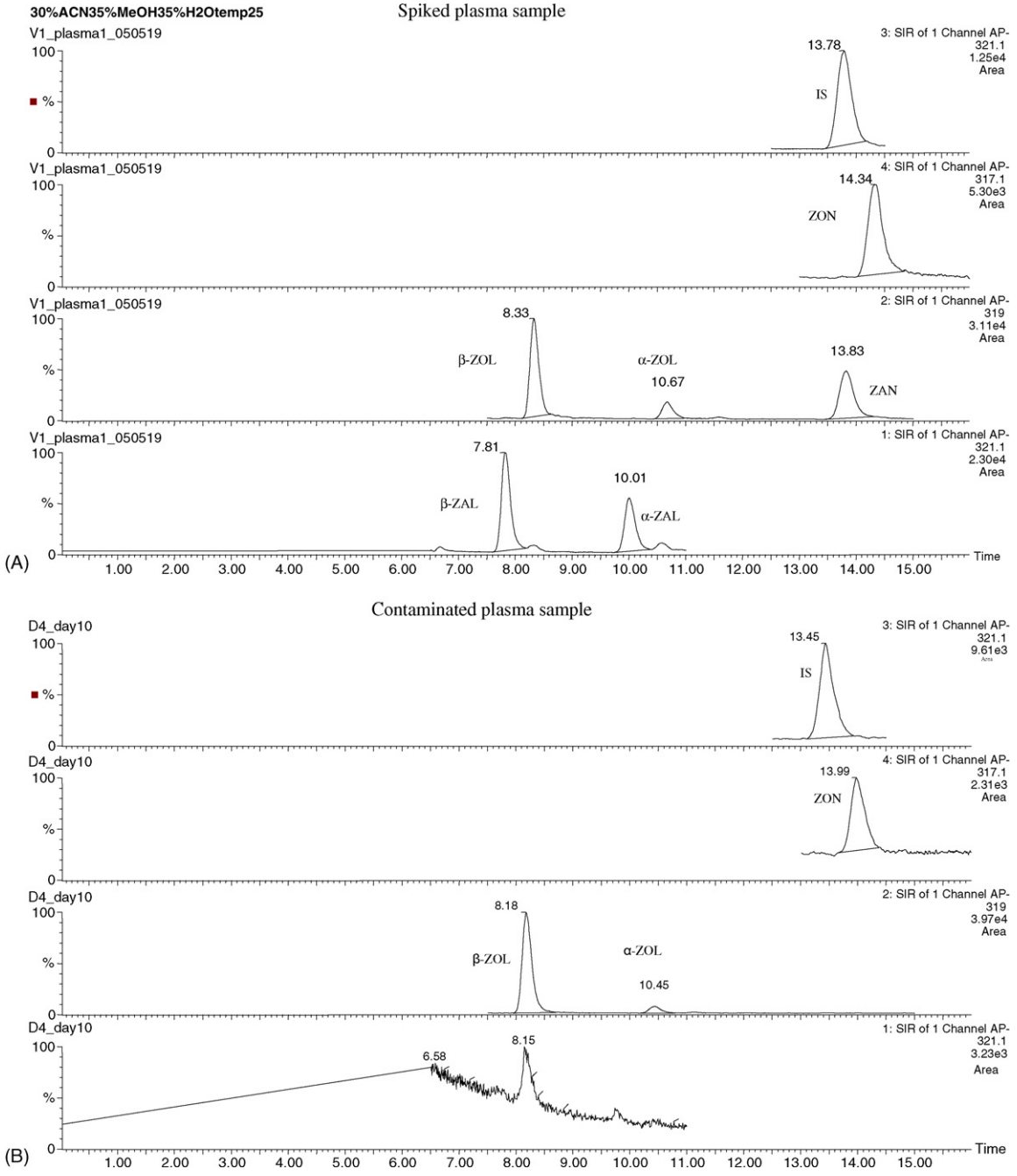

Fig. 4. LC–APCI/MS chromatogram of horses plasma: (A) spiked plasma sample with ZON 0.75 μg/l, ZAN 0.5 μg/l, α-ZOL 5 μg/l, β-ZOL 2.5 μg/l, α-ZAL 4 μg/l, β-ZAL 3 μg/l and 5 μg/l D_2 ZAN (IS); (B) contaminated plasma sample.

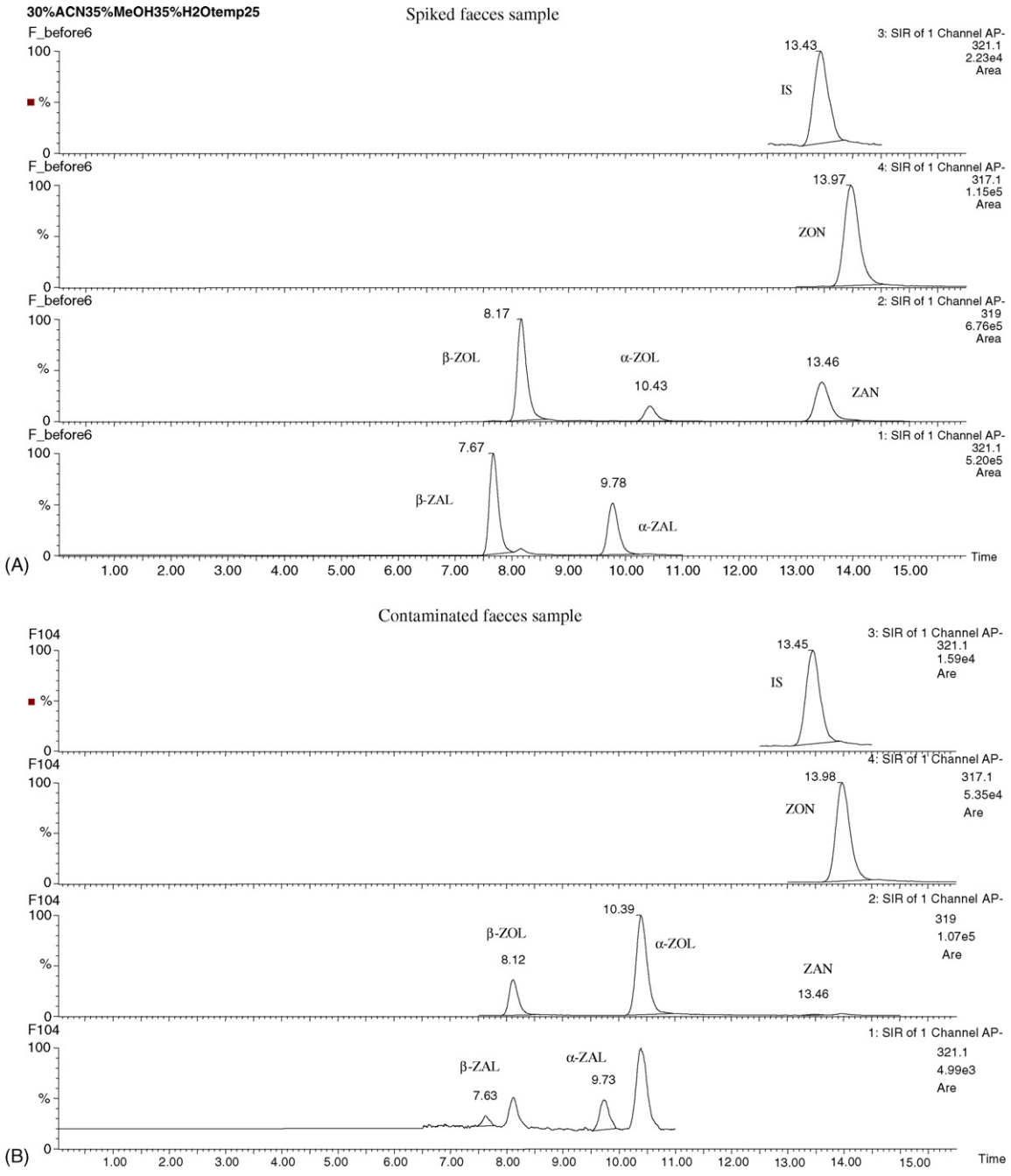

Fig. 5. LC–APCI/MS chromatogram of horses faeces: (A) spiked faeces sample with ZON 75 μg/kg, ZAN 30 μg/kg, α-ZOL 50 μg/kg, β-ZOL 50 μg/kg, α-ZAL 25 μg/kg, β-ZAL 40 μg/kg and 10 μg/l D_2 ZAN (IS); (B) contaminated faeces sample.

were not found in contaminated plasma samples which can be explained by the high method detection limit for these compounds. However, they were possibly not present in the plasma sample since these two metabolites were found only in trace amount even in the urine, the major metabolic pathway of ZON in mammal [4,6]. α-ZAL and β-ZAL were not detected in pig plasma, the only one found metabolite was α-ZOL because the others were absent or below the detection limit of the method [12]. All six metabolites were detected in both contaminated urine and faeces samples.

4. Conclusions

The paper demonstrates that the use of a selective sample clean-up in combination with LC–APCI–MS is a practical method for the sensitive investigation of ZON and its metabolites in biological samples. The combination of SPE and IAC in sample clean-up is presented here for the first time. An effective sample preparation clearly enhances the sensitivity of the method (the superior sample clean-up of this method could even result in higher sensitivity when combined with the powerful

tool like LC–MS/MS). This, however, depends on the nature and complexity of the matrix. Although a one step clean-up by IAC was sufficient for plasma samples its limitations became apparent for other complex biological samples like urine and faeces which could only be analyzed after a clean-up combining SPE prior to IAC. The use of D_2-ZAN as internal standard made it possible to partly compensate for the influence of matrix effects. The chromatographic resolution obtained is good with relatively few interference by remaining background peaks. The six metabolites could be determined in a single run within 15 min.

Acknowledgements

Authors would like to thank Dr. Alexander Leitner and Prof. Dr. Wolfgang Lindner for kindly providing IS, 1′, 2′-dideuterated zearalanone (D_2-ZAN) and Ms. Anita Dockner for her support in the laboratory. This study was financed by Österreichische Nationalbank Jubiläumsfondsprojekt No. 9860. The Austrian Academic Exchange Organisation (ÖAD) is gratefully thanked as well.

References

[1] R. Krska, S. Baumgartner, R. Josephs, Fresenius J. Anal. Chem. 371 (2001) 285.
[2] H.V.L.N. Swamy, T.K. Smith, E.J. Macdonald, H.J. Boermans, E.J. Squires, J. Anim. Sci. 80 (2002) 3257.
[3] H.V.L.N. Swamy, T.K. Smith, T.K. Macdonald, J. Anim. Sci. 82 (2004) 2131.
[4] M. Kleinova, P. Zöllner, H. Kahlbacher, W. Hochsteiner, W. Lindner, J. Agric. Food Chem. 50 (2002) 4769.
[5] P. Zöllner, J. Jodlbauer, M. Kleinova, H. Kahlbacher, T. Kuhn, W. Hochsteiner, W. Lindner, J. Agric. Food Chem. 50 (2002) 2494.
[6] J. Jodlbauer, P. Zöllner, W. Lindner, Chromatographia 51 (2000) 681.
[7] K.E. Richardson, W.M. Hagler Jr., C.J. Mirocha, J. Agric. Food Chem. 33 (1985) 862.
[8] A.F. Erasmuson, B.G. Scahill, D.M. West, J. Agric. Food Chem. 42 (1994) 2721.
[9] S.L. Raymond, T.K. Smith, H.V.L.N. Swamy, J. Anim. Sci. 81 (2003) 2123.
[10] S.L. Raymond, T.K. Smith, H.V.L.N. Swamy, J. Anim. Sci. 83 (2005) 1267.
[11] E. Rosenburg, R. Krska, R. Wissiack, V. Kmetov, R. Josephs, E. Razzazi, M. Grasserbauer, J. Chromatogr. A 819 (1998) 277.
[12] L.K. Sorensen, T.H. Elbak, J. Chromatogr. B 820 (2005) 183.
[13] J. Plasencia, C.J. Mirocha, R.J. Pawlosky, J.F. Smith, J. Assoc. Off. Anal. Chem. 73 (1990) 973.
[14] L.A. van Ginkel, E.H.J.M. Jansen, R.W. Stephany, P.W. Zoontjes, P.L.W.J. Schwillens, H.J. van Rossum, T. Visser, J. Chromatogr. 624 (1992) 389.
[15] M.A.S. Marques, L.A. Lima, C.H.B. Bizarri, F.R.A. Neto, J.N. Cardoso, J. Anal. Toxicol. 22 (1998) 367.
[16] S. Dänicke, E. Swiech, L. Buraczewska, K.H. Ueberschär, J. Amin. Physiol. Anim. Nutr. (Berl.) 89 (7–8) (2005) 268.
[17] S. Dänicke, K.H. Ueberschär, I. Halle, H. Valenta, G. Flachowsky, Arch. Tierernahr. 55 (2001) 299.
[18] A.C. Bily, L.M. Reid, M.E. Savard, R. Reddy, B.A. Blackwell, C.M. Campbell, A. Krantis, T. Durst, B.J.R. Philogene, J.T. Arnason, C. Regnault-Roger, Mycopathologia 157 (2004) 117.
[19] P. Zöllner, J. Jodlbauer, W. Lindner, J. Chromatogr. A. 858 (1999) 167.
[20] M.H. Choi, K.R. Kim, J.K. Hong, S.J. Park, B.C. Chung, Rapid Commun. Mass Spectrom. 16 (2002) 2221.
[21] P. Zöllner, A. Leitner, J. Jodlbauer, B.X. Mayer, W. Lindner, LC-GC Eur. 16 (3) (2003) 163.
[22] P. Zöllner, A. Leitner, D. Berner, M. Kleinova, J. Jodlbauer, B.X. Mayer, W. Lindner, LC-GC Eur. 16 (6) (2003) 354.