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Determination of zearalenone in grains by high-performance liquid chromatography–tandem mass spectrometry after solid-phase extraction with RP-18 columns or immunoaffinity columns

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Abstract

In this paper a robust, sensitive and selective LC–MS–MS method for the determination of zearalenone (ZON) in several cereals is described. Sample preparation was performed by extraction of the commodities with a mixture of acetonitrile and water followed by solid-phase extraction with RP-18 columns or immunoaffinity columns. The selective determination of ZON was achieved with an atmospheric pressure chemical ionization interface. Using the negative ion mode a detection limit of 0.5 μg/kg and a determination limit of 1 μg/kg grain was achieved, which is by a factor of 100 more sensitive than the positive ion mode. Zearalanone (ZAN), which does not occur in nature, was used as internal standard for quantification. A linear working range from 1.0 μg/kg to 1000 μg/kg could be achieved in grains with a standard deviation of 4% and recovery rates around 100%. All these results were independent from the grain matrices (maize, barley, oats, wheat) when ZAN was used as internal standard. Sample preparation with RP-18 and immunoaffinity materials gave comparable results. In addition, the method was successfully used for the investigation of naturally contaminated maize samples in the course of an interlaboratory comparison test.

Keywords: Solid-phase extraction; Liquid chromatography–mass spectrometry; Mycotoxins Zearalenone; Zearalanone

1. Introduction

Mycotoxins are toxic secondary metabolites produced by filamentous fungi, e.g., *Fusarium*, *Aspergillus* and *Penicillium* species, growing on agricultural commodities in the field or during storage [1,2]. Their occurrence in food has been recognized as potential threat to human health, either caused by direct contamination via grains and grain products or by ''carry over'' of mycotoxins and their metabolites in animal tissues [3], milk and meat after intake of contaminated feedstuffs [3–6]. Three hundred and fifty different mycotoxin species have been discovered so far. Due to their frequent occurence and their severe toxic, estrogenic and carcinogenic properties, guidelines and tolerance levels of diverse mycotoxins have been set in several countries [7].

Zearalenone (ZON, Fig. 1) is produced by *Fusarium* species, which colonize several grains including maize, barley, oats wheat and sorghum [8]. Despite its relatively low acute toxicity [9], ZON exhibits distinct estrogenic and anabolic properties in several animal species. This is due to its argonistic effect on the estrogenic receptor, resulting in severe

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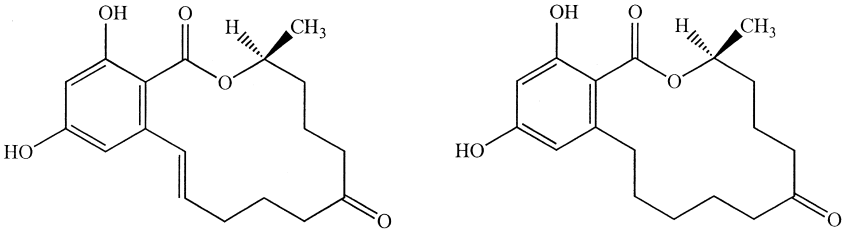

Fig. 1. Structures of ZON and ZAN.

effects on the reproductive system [2]. Tolerance levels in cereals have been set in several countries in the range of 30 to 1000 μg/kg [7]. Data on carcinogenic properties of ZON are not unequivocal and further studies are necessary to evaluate whether ZON should be considered as a potential human carcinogen [10,11].

Many analytical methods have been developed for the determination of ZON in various matrices and advances in this field are annually reviewed [12–15]. Liquid chromatography (LC) has been frequently used for ZON analysis enabling a detection limit of 4 μg/kg in grains with a fluorescence detector [16]. The main advantage of LC-based methods is the reduction of time-consuming and sometimes error-prone sample preparation and derivatization steps. In addition, LC allows the simultaneous investigation of ZON and several other major mycotoxins in cereals in a single chromatographic run [17,18].

Anyway, extensive or selective sample preparation is still required for all analytical methods (a) to remove the major part of matrix compounds that may interfere when using UV and fluorescence detection and (b) to preconcentrate the analyte in order to reach the required low determination limits. The most frequently used method for the clean-up of biological samples is still liquid–liquid partitioning [16], although solid-phase extraction (SPE) is gaining popularity [19]. More recently, analyte selective immunoaffinity columns (IACs) have become popular in mycotoxin analysis as a very selective and time-saving one-step sample clean-up tool facilitating high extraction capacity and an almost complete removal of matrix compounds [15,20,21]. For a variety of mycotoxins, including ZON, such dedicated IACs are commercially available [22]. The highly selective IAC sorbents do not allow multi-toxin analysis, although mycotoxins of the same family occasionally show cross-reactivity; cascades of several different IACs should be used to detect all mycotoxins of interest. This may again be laborious, error prone and costly as the columns are more expensive than SPE materials, such as RP-18.

In addition to selective analyte enrichment, selective and sensitive detection techniques have to be used for mycotoxin analysis in complex biological mixtures. In this respect, mass spectrometry (MS) is a selective, sensitive and universally applicable tool, that is well suited for multi-analyte detection. Single-ion monitoring (SIM) and multi-reaction monitoring (MRM) with tandem MS instruments (MS–MS) enable selective and accurate analysis over a wide linear range. In mycotoxin analysis MS has only been frequently used in combination with gas chromatography (GC) [23,24]. Initial LC–MS investigations were concerned with several mycotoxins [25,26], including ZON [27], mainly using thermospray interfaces. Even more limited is the number of applications for the more recent electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces. These offer the clear advantages of robustness and easy handling, important requirements for a routine technique with high sample throughput. While ESI was used several times for the determination of mycotoxins [28], only two papers have been published using APCI-MS for the detection of sterigmatocystin in bread, maize and cheese [29] and ZON in maize [30]. In the positive ion mode the detection limits were in the low μg/kg range with SIM. Sample preparation was performed with liquid–liquid partitioning or with SPE with a mixture of charcoal, celite, alumina and ion-ex-

change resins or an immunoaffinity material. Very few papers have been published using MS–MS. However, MRM should further enhance the detection performance [28].

This paper describes a robust method to determine ZON in grain at the low μg/kg to ng/kg range, using zearalanone (ZAN, Fig. 1) as internal standard, which does not occur in nature. Sample clean-up with SPE was either performed with selective immunoaffinity material or with nonselective RP-18 material. For the final LC–MS–MS analysis (MRM scan mode) the positive and negative ion mode were compared. To evaluate the broad applicability of the analytical method with regard to different sample matrices and their impact on the ionization capability of the APCI interface, diverse cereals were spiked with ZON to investigate RP-SPE in combination with LC–MS–MS. The LC–MS method was also applied to naturally contaminated maize samples within the course of an interlaboratory comparison test.

2. Experimental

2.1. Materials

ZON and ZAN were purchased from Sigma (Deisenhofen, Germany). Ammonium acetate and potassium chloride (both analytical-reagent grade) as well as HPLC-grade methanol and HPLC-grade acetonitrile were supplied by Merck (Darmstadt, Germany). Grain samples were purchased from local suppliers (Vienna, Austria). Water (conductivity of 18 $M\Omega^{-1}$ cm^{-1}) was purified by an Elgastat water purification system (Bucks., UK). HPLC solvents were filtered before use through GF/A glass microfiber filters (Whatman, Maidstone, UK).

2.2. Instrumental

LC–MS–MS analyses were performed on a PE Sciex API 365 LC–MS–MS system (Perkin-Elmer Sciex, Thornhill, Canada) equipped with a Hewlett-Packard HPLC system, Model 1100 series (Hewlett-Packard, Waldbronn, Germany) and with an APCI interface (Perkin-Elmer Sciex). Chromatographic separation was achieved on a 12.5 cm×3 mm I.D., Superspher 100 RP-18 end-capped column (Merck, Darmstadt, Germany). 15 m*M* ammonium acetate in methanol–water (75:25, v/v, pH 7.5) was used as mobile phase at a flow-rate of 0.5 ml/min. The retention times for ZON and ZAN were 3.36 and 3.23 min (capacity factors, 2.14 and 1.99), respectively. After preliminary experiments, the APCI interface was applied in the negative ion mode at a temperature of 400°C and with a needle current of 4 μA. For MRM the deprotonated molecular species of ZON (m/z 317.15) and ZAN (m/z 319.15) as precursor ions and fragment ions at m/z 131.1 and 175.1 for ZON and m/z 205.1 for ZAN as product ions were selected with a dwell time of 1.1 s for each fragmentation pathway. Product ion spectra of zearalenone and zearalanone were used to select these high abundant fragment ions for MRM experiments. The collisional energy was adjusted by variation of the voltage difference between the high pressure entrance quadrupole (Q0) and the collisional cell quadrupole (RO2) and was found to give higher sensitivity with a value of 30 eV for ZON and ZAN. Nitrogen was used as collisional gas at a setting of 6 characteristic for the Sciex API 365 instrument. For preliminary mass spectrometric experiments and optimization of most of the mass spectrometric parameters an ESI interface was used in combination with a syringe pump (Harvard Apparatus, South Natick, USA) for sample injection at a flow-rate of 5 μl/min stock solution. To obtain a maximum sensitivity and stable performance in the MRM mode, ring and orifice voltages and collisional energy as well as nebulizer gas and interface temperature were finally optimized with the APCI interface.

2.3. Sample preparation

A stock solution of 20.96 μg/ml ZON in methanol was prepared from a solid standard. This solution was used to make standard solutions, with concentrations ranging from 100 ng/ml to 2096 ng/ml. The standards were stored at 4°C under exclusion of light. For calibration and spiking experiments standard solutions were added to the grain sample directly before sample preparation (extraction) was carried out.

Twenty-five grams of finely granulated or milled grain was mixed with 2.5 g of potassium chloride and extracted with 100 ml of acetonitrile–water (75:25, v/v) using an Ultraturrax $T25_{basic}$ apparatus (IKA Labortechnik, Staufen, Germany). After filtration through GF/A glass microfiber filters (Whatman), 10 ml of the extract were diluted with 90 ml of water and adjusted to a pH of 4 with acetic acid. A 10-ml volume of this solution was applied to a SPE column either filled with 100 mg of reversed-phase material (RP-18) on silica gel (Phenomenex, Torrance, CA, USA) or with immunoaffinity material (Vicam, St. Watertown, MA, USA). RP-18 columns were washed and activated with 5 ml of methanol and 5 ml of water. After sample loading, both, RP-18 and immunoaffinity columns, were washed with 2 ml of water. To elute analyte and internal standard from the RP-18 columns, 1.25 ml of 10 m*M* ammonium acetate in methanol–water (70:30, v/v, pH 7.5) was used. In the case of IAC, 5 ml of pure methanol was used. The effluents were evaporated under a stream of nitrogen and the residues redissolved in 250 μl of methanol–water (75:25). A 50-μl volume of this solution was injected into the HPLC–MS–MS system.

2.4. Interlaboratory comparison test

The interlaboratory comparison test (BIOMIN-Intercomparison of Mycotoxin analysis 1998) with 28 participating laboratories from Europe, Singapore and the USA was organized by the Institute of Agrobiotechnology (Tulln, Austria) [32]. Four maize samples were provided, a blank sample (ZON concentration: <5 μg/kg), one spiked sample (ZON concentration: 40 to 300 μg/kg) and two naturally contaminated samples (ZON concentrations: 40 to 300 μg/kg and 100 to 600 μg/kg). Duplicate extractions and analyses were carried out for each sample using the above described method. RP-18 material was used for the SPE step.

3. Results and discussion

3.1. Investigation of mass spectrometric parameters

In agreement with the literature, the negatively charged deprotonated molecular ion of ZON was found to be more abundant than the positively charged protonated molecular ion [30]. This is due to an extensive fragmentation behavior of protonated ZON – not be observed in the negative ion mode (for mass spectra see Ref. [30]). Furthermore, the presence of acidic phenolic groups favors deprotonation of the ZON molecule in the gas phase, leading to an increase of sensitivity by a factor of 100 in the MRM mode. Adjustment of parameters were, therefore, performed in the negative ion mode. A detection limit of 150 pg/ml was obtained for ZON in stock solutions.

3.2. Sample preparation

Extraction of ZON was performed according to a previously described method [16]. Potassium chloride was added to the extraction solvent to enhance cell destruction by osmotic effects [32]. The extraction solution was diluted with water by a factor of 10 resulting in a final content of 7.5% acetonitrile for the 10 ml of sample solution to be applied to the SPE procedure. Under these conditions ZON and ZAN were well retained on the IAC and the RP-18 SPE columns. By fractionation of the effluent during the elution step, the elution of ZON and ZAN was shown to be completed after 1.25 ml of ammonium acetate in methanol–water (75:25, v/v) for the RP-18 SPE column and after 5 ml of methanol for the IAC.

In order to compare the efficiency of the two SPE clean-up procedures, a 25 g maize sample was spiked with ZON (126 μg/kg) and ZAN (480 μg/kg). The filtered extraction solution was split, worked-up separately and analyzed by LC–MS–MS. Results of this comparison are summarized in Table 1.

The overall recovery rates were almost identical for both methods: slightly below 100% and slightly above 100% for the IAC and RP-18 clean up, respectively. This finding was also reflected by the deviation of measured values from mean values. While ZAN was nearly completely recovered with the RP-18 SPE method, it showed approximately 80% recovery with the ZON specific IACs. This value is in good agreement with data given in the literature [20]. Despite this 20% loss of ZAN on an IAC system, standard deviations of both methods

Table 1
Comparison of SPE with RP-18 material and with ZON selective immunoaffinity material ($n=3$)[a]

	RP-18 SPE	Immunoaffinity SPE
Mean value (μg/kg)	126.0	126.0
Obtained value (μg/kg)	129.3	121.7
Deviation (%)	+2.6	−3.4
Recovery of ZON (%)	104.2	96.0
Standard deviation (%)	±4.5	±4.3
Recovery of ZAN (%)	100.0	80.5
Limit of detection (μg/kg)	0.5	0.5
Limit of determination (μg/kg)	1.0	1.0
Linear range (μg/kg)	1–1000	1–1000

[a] Maize sample was spiked with 126 μg/kg of ZON.

were comparable. A limit of detection (LOD) of 0.5 μg/kg maize and limit of quantification (LOQ) of 1 μg/kg maize could be achieved. Calibration curves obtained with both methods had similar linearity within a concentration range of 1 to 1000 μg/kg (RP-18: $r^2=0.9998$; $y=0.0052x-0.0014$; IAC: $r^2=0.9998$; $y=0.0052x-0.0014$). A typical MRM chromatogram of a maize sample is shown in Fig. 2. Neither with the IAC nor with the RP-18 SPE sample clean-up procedures any other peaks than ZON and ZAN could be observed, due to the highly specific detection system. Quantification was easily performed with MRM chromatograms specific for both compounds.

In view of these findings, the combination of a highly selective IAC sample clean-up with a highly selective MS–MS system may be regarded as analytical ''overkill'', since no additional advantages in terms of sensitivity and accuracy could be achieved, as compared to the RP-18 SPE clean-up. On the contrary, the IAC method may have the clear disadvantage based on the incomplete recovery of an internal standard for which the ZON IAC is not specified for. In this light the RP-18 SPE method is more ''universal'', although it is less selective.

3.3. Matrix effects on the MS response

The common perception is that LC–MS–MS practically comes close to the highest degree of selectivity thus termed specificity. Accordingly, sample preparation may be reduced to a minimum or even omitted, due to the mass selectivity of the detection system. Contrary to this common belief, authors have recently reported that even chromatographically co-eluting matrix compounds can severely affect the ion formation process in ESI and APCI interfaces resulting in a decrease of accuracy and reproducibility of LC–MS–MS analyses [33,34]. Ion suppression phenomena by matrix effects have been reported, e.g., for the analysis of indinavir in human

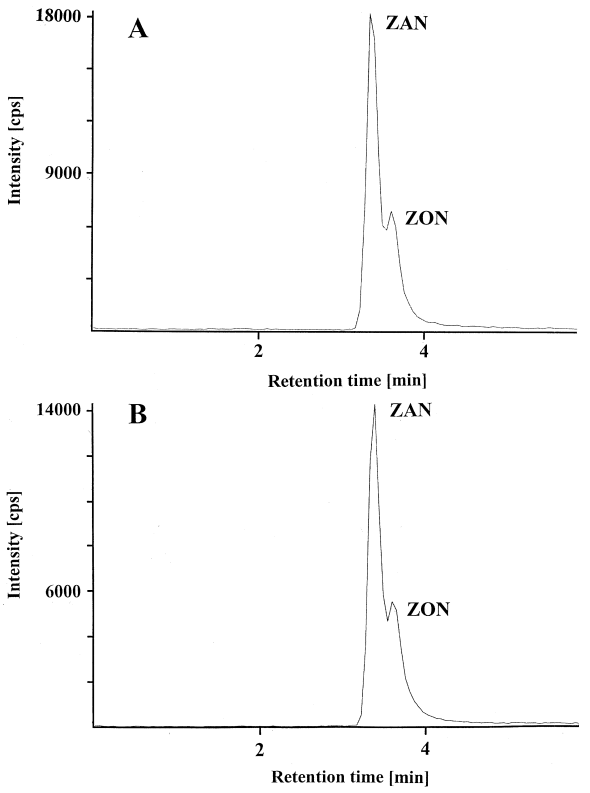

Fig. 2. MRM chromatogram of spiked maize samples with a concentration of 134 μg/kg ZON and 480 μg/kg internal standard ZAN after SPE with (A) RP-18 material, (B) immunoaffinity material.

urine [35] and finasteride in human plasma [36]. To avoid these problems some authors have proved the importance of more efficient sample clean-up and/or improved chromatographic separation to remove such co-eluting and interfering compounds that may disturb the LC–MS–MS analysis [35,36].

In order to estimate if matrix effects can be observed for the detection of ZON by the described LC–MS–MS method, diverse grain samples were spiked with ZON and ZAN, worked-up and analyzed by LC–MS–MS. The ZON concentrations were calculated either with or without internal standard. Results of these experiments are summarized in Table 2.

As indicated in Table 2, the accuracy of data obtained without an internal standard (external calibration) is poor. Deviations ranged from −0.5% to+24%. This finding is, on one hand, due to variations of about 10% of the detector response, as proved by repeated LC–MS–MS analyses of the same sample or standard solution. On the other hand, these deviations are also clearly influenced by matrix effects since repeated sample preparation of the same grain sample resulted in similar scattered data.

There was neither a contribution of ZON to the detector response of ZAN nor a contribution of ZAN to the detector response of ZON as determined at different concentrations of ZON and ZAN. Linearity of each standard curve was confirmed by plotting the peak area ratio of ZON to ZAN versus ZON concentration. The unknown sample concentrations were calculated from the weighted least-square regression analysis of the standard curve.

Using the internal standard method both, the variation of the detector response as well as the matrix effects were almost completely eliminated in all investigated matrices (1.6–3.4% deviation of obtained values from mean values). As a result, for this total analysis method the sample preparation and the final chromatography was sufficient, since ZAN exhibited a similar mass spectrometric behavior to matrix effects as ZON. This is not necessarily always the case, as previously shown for the determination of finasteride in human plasma, when only removal of co-eluting compounds by an improved chromatographic separation or more efficient sample clean-up was suitable to eliminate this problem [35]. To conclude, a deuterated analyte as internal standard seems always superior, but if not available the use of another internal standard seems advantageous to eliminate matrix effects during ion formation.

3.4. Investigation of naturally contaminated maize samples

In order to demonstrate the utility of the described method for the analysis of naturally contaminated samples, the described LC–MS–MS method was finally used in an interlaboratory comparison test of ZON analysis [31]. One spiked and two naturally contaminated maize samples were analyzed. Compared with the average deviation of all laboratories in this intercomparison, data obtained with the LC–MS–MS method are in good agreement with the target and assigned values. Assigned values of natural contaminated samples were calculated from the average results of all participating laboratories. In addition, scattering of analytical data of all participating laboratories can partly explained by the heterogeneity of provided samples. Results of this interlaboratory comparison test are shown in Table 3.

4. Conclusions

A selective LC–MS–MS method in combination

Table 2
Determination of ZON in different cereals (n=5)

Matrix	Mean value (μg/kg)	Value obtained without I.S. (μg/kg)	Deviation without I.S. (%)	Value obtained with I.S. (μg/kg)	Deviation with I.S. (%)	Recovery (%)
Maize	134.0	137.1	+2.3	137.5	+2.6	114
Barley	134.0	145.8	+8.8	136.1	+1.6	120
Oats	134.0	166.2	+24.0	137.6	+2.7	106
Wheat	134.0	133.3	−0.5	138.5	+3.4	112
Wheaten bran	134.0	153.5	+14.6	137.5	+2.6	108

Table 3
Results of the interlaboratory comparison test[a]

Maize sample	Target value (μg/kg)	Assigned value (μg/kg)	Results of the described LC–MS–MS method (μg/kg)	Deviation (%)	Average deviation of all laboratories (%)
ZON blank	–	5.3[b]	6.3	18.9	38.5
ZON spiked	102.4	–	92.7	9.5	27.7
ZON naturally contaminated 1	–	73.0	82.7	13.3	40.5
ZON naturally contaminated 1	–	291.1	375.2	28.9	41.2

[a] Assigned values of the naturally contaminated samples were calculated from the average value of all laboratory results. Each laboratory result was the average of two analysis for each sample.
[b] Only eight out of 28 laboratories were able to determine ZON in this concentration range.

with less selective SPE type sample clean-up is described and proves to be a powerful tool for the sensitive determination of ZON grains. Due to the highly selective MS–MS system, a less selective sample preparation using RP-18 material compared to ZON selective immunoaffinity type adsorption material is more than sufficient. As shown by Rosenberg et al., SPE with immunoaffinity material enables a distinctly higher enrichment of analyte compared to the RP-18 SPE [31]. This compensates the higher MS sensitivity of our method resulting in comparable overall method sensitivities of both methods (LOD of 0.125 μg/kg and 0.5 μg/kg). To combine the superior analyte enrichment of the Rosenberg method and the higher MS sensitivity of the present method should result in an even lower LOD of 0.025 μg/kg. This is, however, distinctly below the concentration range of interest. In view of the lower costs of the RP-18 SPE columns and the fact that the immunoaffinity columns are only limited to the extraction of the dedicated compound(s), the RP-18 SPE method is, therefore, preferable in combination with a HPLC–MS–MS analysis system, in principal allowing multi-mycotoxin analysis.

The use of ZAN as internal standard was found to be ideal to compensate possible matrix effects at the MS detection site. In conclusion, for the development and validation of a robust HPLC–MS method it is of utmost importance to estimate matrix effects and to eliminate them, either by incorporation of an appropriate internal standard or by improving a selective sample clean-up and final chromatography to remove any disturbing co-eluting compounds.

For ZON, having phenolic hydroxy groups, the negative ion mode was found to be distinctly more sensitive than the positive ion mode. This marked improvement of sensitivity seems to be an attractive approach for diverse other mycotoxins and xenobiotics with phenolic hydroxy or carboxylic groups. This could also been shown by preliminary experiments with stilbene anabolics in meat [37].

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