Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 993 (2003) 39–45
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of zearalenone from wheat and corn by pressurized liquid extraction and liquid chromatography–electrospray mass spectrometry

Lea Pallaroni, Christoph von Holst*

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Food and Feed Unit, B-2440 Geel, Retieseweg, Belgium

Received 13 December 2002; received in revised form 14 February 2003; accepted 14 February 2003

Abstract

Zearalenone (ZON) was extracted from wheat and corn by using pressurized liquid extraction (PLE) and the PLE extracts were analyzed using liquid-chromatography–mass spectrometry (LC–MS) without further clean-up procedures. A statistical design approach was applied to evaluate the influence of several extraction parameters such as temperature (40 °C; 80 °C; 120 °C), time (5 min; 10 min) and solvent extraction mixture [acetonitrile–water (9:1, v/v); methanol–water (8:2, v/v); methanol–acetonitrile (1:1, v/v)] on fortified cereals. The results showed a strong influence of the solvent composition on recovery of ZON. Quantification of the analytes was performed by LC–MS analysis of the raw extract using matrix-matched standard curves. The method performance was tested in the selected conditions (80 °C; 5 min; two cycles; methanol–acetonitrile) on samples which had been previously used for an international proficiency test. Compared to the assigned value, the recovered ZON was 118% [relative standard deviation (RSD)=5.2%, $n=3$)] and 107% (RSD=2.2%, $n=3$) in wheat and corn, respectively. Therefore, PLE can be used for ZON extraction, achieving good performances and allowing for an automated handling of the sample extraction step. Successively, the influence of temperature and number of cycles was investigated on naturally contaminated corn. From these results it could be concluded that fortified experiments perfectly mimicked naturally contaminated samples.

Keywords: Cereals; Food analysis; Zearalenone

1. Introduction

The extraction step has often proved to be the bottleneck of most analytical procedures, as it is one of the least evolved parts of the whole method. One of the most promising and recent sample preparation techniques is the pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction), which offers the advantages of reducing solvent consumption and allowing for automated sample handling [1]. Although PLE has started to replace conventional extraction in environmental [2,3] and food analysis [4–6], its application in the mycotoxin field has up to now been limited to fumonisin [7,8]. It has been reported that increasing

*Corresponding author. Tel.: +32-14-571-800; fax: +32-14-571-343.
E-mail address: christoph.von-holst@irmm.jrc.be (C. von Holst).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00381-9

the temperature in PLE allows for a higher extraction efficiency of fumonisin from corn products [7].

Zearalenone (ZON) is a Fusarium toxin of great importance due to its negative health effect on animal husbandry, e.g. pigs [9], and on humans [10]. Traditionally, this mycotoxin is extracted by conventional liquid shaking for about 30–60 min [11,12] or by blending for a few minutes [13,14]. Various extraction mixtures have been used to extract ZON from cereals, the most commonly used are acetonitrile–water [13–16], and methanol–water [12,16] mixed at different ratios. Extraction performances of conventional methods for mycotoxins analysis are constantly put under discussion, evaluating the effect of shaking versus blending [17]; additionally, different extraction solvent mixtures [16] as well as single versus multiple extractions are also discussed [18]. Analysis of ZON is commonly performed by applying a clean-up step (liquid–liquid partitioning, solid-phase extraction or immunoaffinity) after the extraction and using HPLC with fluorescence detection [19]. Since the availability of bench-top LC–MS systems equipped with atmospheric pressure interfaces, several LC–MS methods have been published using atmospheric pressure chemical ionisation (APCI) [20–23]. The possibility of injecting raw extract was briefly investigated in one paper, resulting in an overestimation of the ZON level [20], whilst it has been already used for developing a microwave assisted extraction (MAE) method [23].

The aim of this work was to develop a simple method for the determination of ZON in wheat and corn based on PLE and detection in LC–MS equipped with an electrospray ionisation (ESI) interface without performing any clean-up step. This will merge automated and modern sample preparation with sophisticated analysis, resulting in an overall reduction of analysis time, manpower and an increase in throughput. To evaluate the feasibility to use PLE instead of a conventional extraction technique, several extraction parameters such as temperature, static time and solvent composition have been investigated using fortified cereals. Quantification of ZON was carried out by using matrix-matched standard curves to compensate for matrix-related adverse effects, and zearalanone (ZAN) was used as internal standard.

2. Experimental

2.1. Chemicals

ZON and ZAN (Fig. 1) standards were purchased

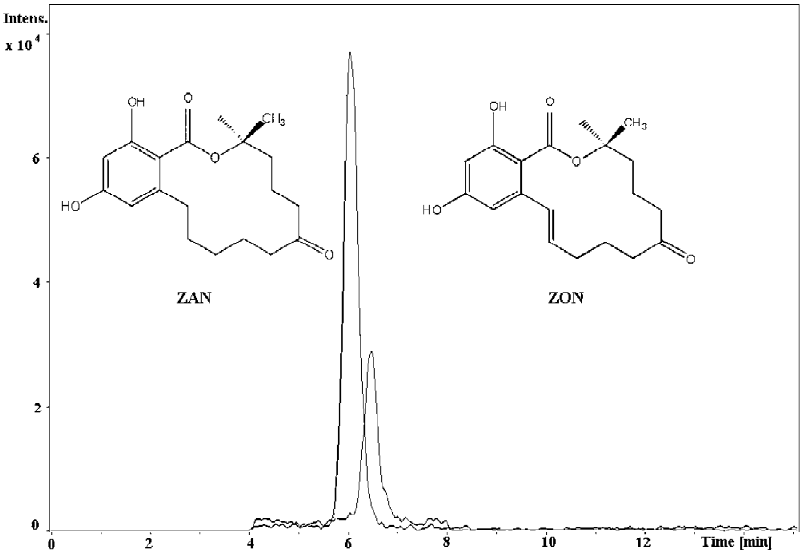

Fig. 1. Extracted ion chromatogram obtained from a corn sample spiked with 80 ng/g of ZON (m/z 317.4) and 200 ng/g of ZAN (m/z 319.4) as internal standard.

from Sigma (Milan, Italy) and diatomaceous earth sorbent Hydromatrix from Varian (Dionex, Milan, Italy). Solvents were of HPLC grade (Aldrich, Milan, Italy). Water was purified in a Super-Q Plus, Millipore, Waters System (Millipore, Milan, Italy).

2.2. Samples

Method development was performed using fortified samples of wheat and corn and a naturally contaminated corn. Samples were ground in a laboratory ultra centrifuge mill ZM100 (Retsch, Haan, Germany) using a 0.5-mm sieve.

The method performance was determined by analysis of wheat [24] and corn [25], which had been previously used in proficiency tests organized by the Food Analysis Performance Assessment Scheme (FAPAS) of the Central Science Laboratory of DEFRA (Department for Environmental Food and Rural Affairs) UK. Characteristics of these materials are described in the respective reports [24,25]. The target ZON concentration (112 ng/g wheat; 285 ng/g corn) and the corresponding uncertainty ($u = \pm 15.8$ ng/g, $\alpha = 0.05$ in wheat; $u = \pm 19.2$ ng/g, $\alpha = 0.05$ in corn) of the test materials derived from the results submitted by the participants of the study.

Naturally contaminated corn sample was provided by a German laboratory. Its ZON target value was established by LC–MS at 5051 ng/g ZON (RSD = 7.4%; $n = 10$) according to a modified Internatioal Standard Organisation (ISO) method [14] as reported in the procedure.

2.3. LC–MS analysis

Analysis of the PLE extracts was performed using an HP 1100 Series HPLC system equipped with a degasser, a binary pump, an autoinjector and thermostat coupled to an ion-trap mass spectrometer equipped with an ESI interface (Hewlett-Packard, Palo Alto, CA, USA). The chromatographic separation was achieved working in isocratic [methanol–water (0.2% acetic acid) 55:45 (v/v)] at a flow-rate of 0.2 ml/min on a Discovery C_8 column (100 × 2.1 mm, 5 μm particle size, 180 Å pore size; Supelco, Milan, Italy) kept at 35 °C. The injection volume was 5 μl.

The ESI interface was used in the negative ionization mode due to its better selectivity. The following ESI parameters were applied: nebulizer 50 p.s.i. (1 p.s.i. = 6894.76 Pa), dry gas 10 l/min, dry temperature 350 °C and high voltage capillary 4000 V. The mass spectra were recorded in full scan mode (200–550 m/z). The quantification was based on extracted ion chromatograms of the deprotonated molecular ion $[M-H]^-$ m/z 317.4 for ZON and $[M-H]^-$ m/z 319.4 for ZAN, which was used as the internal standard for the LC–MS analysis.

2.4. Procedures

All the extractions were performed on an ASE 200 System (Dionex, Sunyvale, CA, USA). An amount of 5 g of sample was weighed in a small beaker and mixed thoroughly with 3 g of Hydromatrix to obtain a porous mixture to enable the extraction solvent to flow through the sample during the extraction. The mixture was poured into a 22 ml thimble, which was packed by adding a layer of Hydromatrix at the base and at the top in order to fill the thimble completely according to the instrument's manufacturer recommendations. The final volume of the solvent after extraction was always close to 35 ml. When the extraction solution reached room temperature, 1.0 ml of internal standard solution (ZAN 2 μg/ml) was added and the volume was filled up to 40 ml. After thoroughly mixing, an aliquot was filtered into vials using a Titan PTFE 0.45-μm filter, which has been tested for not interacting with the target analytes. After filtration, the raw extract was injected directly into the LC–MS system without any further clean-up step. Extracts from low contaminated samples were reduced by a factor of 3 prior to analysis.

Preliminary experiments have been carried out either to evaluate a likely interaction between the Hydromatrix and the target analyte or to guarantee the most appropriate settings of the parameters, which were not further investigated in the study. ZON was spiked directly into the Hydromatrix in the absence of sample matrix and extracted with two sequential static extractions of 5 min each, at two temperatures (40 and 80 °C) applying two different flush volumes of 60 and 75%.

A statistical design approach [26] was applied to evaluate the influence of PLE parameters on extraction efficiency on samples of wheat and corn

fortified at a concentration level of 400 ng/g. This level was chosen in order to work in a range where changes in the response were not given by instrument variation but were imputable to changes in the extraction efficiency. The investigated parameters were temperature (40 °C; 80 °C; 120 °C), static time (5 min; 10 min) and extraction solvent [acetonitrile (ACN)–water (9:1, v/v); methanol (MeOH)–water (8:2, v/v); methanol (MeOH)–acetonitrile (1:1, v/v)]. These three extraction solvent mixtures were selected because ACN–water and MeOH–water have been reported to be the most frequently used for ZON analysis [19], while the third solvent has previously shown satisfactory results by using MAE [23]. The PLE was operated applying the instrument settings reported in Table 1.

Presuming that in a naturally contaminated sample ZON could be bound to the matrix, parameters which did not significantly affect the recovery of ZON from fortified samples could play a role when extracting naturally contaminated sample. In order to evaluate the influence of PLE parameters on naturally contaminated matrices, a naturally contaminated corn was extracted by PLE using MeOH–ACN by applying a 5-min static cycle, varying the temperature (40 °C; 80 °C; 120 °C) and the number of static cycles (2; 3). The target ZON concentration of this naturally contaminated corn sample was determined using a modified ISO [14] method by substituting the suggested solvent mixtures (ACN–water) by MeOH–ACN in order to avoid problems due to phase separation or water absorption by the matrix as reported by Stroka et al. [27]. Finally the chosen extraction conditions (5 min; 80 °C; MeOH–ACN) were tested on the FAPAS wheat and corn samples [24,25].

All experiments were performed in duplicates; the statistical evaluation of the results was performed using STATISTICA™ software (Stat Soft Inc., USA).

3. Results and discussion

3.1. LC–MS analysis

The matrix compounds, which predominantly elute in the first 3.5 min, were directed to the waste by using the divert valve, allowing the target analytes to be separated from the main matrix interferences often leading to under or overestimation of the target analytes. The target analyte peaks were not completely separated by liquid chromatography (Fig. 1); selectivity was achieved by extracted ion chromatogram tool.

Quantification of the extracts was based on a six points matrix match standard curves covering the range of 10–100 ng/ml to achieve better precision. Matrix-matched standard curves were prepared by extracting blank wheat and corn at 40 °C for 5 min using the different solvents investigated in this study, thus assuring a perfect match between samples analysed and standard curves. The need to use matrix-matched standard curves was demonstrated by evaluating calibration curves based on standard diluted into the mobile phase or into matrix extracts. These calibration curves presented good linearity (R^2 value of all curves were >0.995), but different slopes. For example, in the case of ACN–MeOH, a

Table 1
Extraction parameters applied during the PLE extraction

Fixed parameters			
Cell volume	22		
Pre heat time	1 min		
Heat time	5 min (6 min when 120 °C applied)		
Flush volume	75%		
Purge time	100 s		
Cycle	2		
Pressure	1500 p.s.i.		
Investigated parameters			
Temperature	40 °C	80 °C	120 °C
Static time		5 min	10 min
Solvent	ACN–water (9:1)	MeOH–water (8:2)	MeOH–ACN (1:1)

50 ng/g standard diluted in corn or wheat matrix would have resulted in an overestimation of 34 and 23%, respectively, if quantification was based on standard-mobile phase calibration curve (mobile phase $R^2 = 0.9998$; $y = 0.0062x + 0.0194$; corn $R^2 = 0.9999$; $y = 0.009x - 0.0127$; wheat $R^2 = 0.9954$; $y = 0.0071x + 0.0523$). The limits of detection (LODs) [signal-to-noise (S/N) 3:1 and of quantification (LOQs) S/N 10:1] for ZON were determined on the matrices. The method LOD was 5 ng/g in wheat and 4 ng/g in corn, while the LOQ was 15 ng/g (RSD = 8.6%, $n = 5$) and 12 ng/g (RSD = 10.2%, $n = 5$), respectively. Though other methods show lower detection limits compared to the approach presented in this paper, this method has the main advantage of drastically reducing analyte losses during sample handling, time required for the analytical procedure and costs for material and manpower, which are desirable characteristics during method development.

3.2. Pressurized liquid extraction

Preliminary experiments have been carried out to evaluate a likely interaction between the Hydromatrix and the target analyte and to establish the most appropriate settings of the flush volume. All the pre-trials produced recoveries above 92%, ensuring that no losses of the analytes occurred due to adsorption of ZON into the Hydromatrix. A flush volume of 75% was chosen for the following experiments since it provided a statistically significant higher recovery (flush effect of 6.0%).

Subsequently, experiments were devoted to evaluating the influence on extraction efficiency of (1) temperature, (2) time and (3) solvent, with the aim to find the most suitable conditions to be used for ZON extraction. The experimental design and the results of the recovery of ZON in wheat and corn are shown in Table 2.

The effect of each single parameter investigated and their interaction was determined by submitting the presented results to statistical evaluation. The standard deviation (SD) reflecting the analytical error of the method was derived from the duplicate analyses of the factorial design experiments (SD = 7.0% for wheat; SD = 4.0% for corn) and was used to check for the significance of the factor effects calculated from the results of the experimental design. Only the solvent had a significant effect on ZON recovery from wheat and corn. The extraction temperature did not show a significant influence on

Table 2
Recovery of zearalenone (spiking level = 400 ng/g sample) from ground wheat and corn using PLE with different solvent mixtures, times and temperatures

Static time (min)	Temperature (°C)	Solvent	Recovery (%)			
			ZON wheat		ZON Corn	
5	40	ACN–water	100.2	102.5	92.0	94.2
5	80	ACN–water	86.5	105.6	86.6	91.6
5	120	ACN–water	98.4	107.4	97.9	100.9
10	40	ACN–water	86.1	105.3	92.7	90.6
10	80	ACN–water	104.2	96.7	92.9	91.1
10	120	ACN–water	100.0	104.5	86.4	89.1
5	40	MeOH–water	70.7	63.4	80.8	81.4
5	80	MeOH–water	68.8	57.1	83.8	95.5
5	120	MeOH–water	69.0	62.7	83.9	85.9
10	40	MeOH–water	68.8	73.0	66.6	79.9
10	80	MeOH–water	56.4	63.2	94.8	98.8
10	120	MeOH–water	67.9	64.2	84.0	78.5
5	40	MeOH–ACN	93.2	112.7	113.8	112.7
5	80	MeOH–ACN	112.0	116.5	108.4	110.7
5	120	MeOH–ACN	114.1	104.2	104.3	105.0
10	40	MeOH–ACN	115.6	111.3	107.2	107.4
10	80	MeOH–ACN	109.3	104.9	113.5	111.0
10	120	MeOH–ACN	110.8	107.4	105.4	109.2

the recovery of ZON in either matrix irrespective of the extraction solvent used. The results from the experiments in which MeOH–ACN was used showed that the recovery was above 100% at all temperatures, thereby indicating that there was no thermal degradation of ZON at elevated temperatures. On the other hand, the extraction efficiency when using MeOH–water as extraction solvent could not be improved by changing the temperature from 40 to 120 °C since the recovery of ZON was constant at about 65% from wheat and 85% from corn at all applied temperatures. For the subsequent trials, a medium temperature of 80 °C was applied. Since the length of the static cycle did not influence the extraction efficiency, the extraction time was set to 5 min to assure a rapid extraction. The solvent MeOH–water provided low recoveries compared to the other mixtures; ACN–water showed better recoveries for wheat (99.8%) than for corn (92.1%). MeOH–ACN gave significantly better recoveries for both matrices. Therefore, this latter solvent mixture was selected for subsequent extractions.

An increase in colour and in cloudy suspension could be visibly noticed when increasing the temperature (40 °C < 80 °C < 120 °C) and varying the extraction solvent mixture from MeOH–ACN to ACN–water and finally MeOH–water. Extremely dirty extracts were obtained when MeOH–water was used, and also when the extraction was performed at 120 °C. Storing the extracts in the refrigerator overnight facilitated precipitation of the co-extracted matrix component. Consistent results were obtained by analyzing the extract after filtration, after centrifugation and after overnight precipitation.

Presuming that ZON could be bound to the matrix in a naturally contaminated sample, a small statistical design was set up in order to evaluate if parameters which did not significantly affect the recovery of ZON from a fortified sample could play a role when extracting a naturally contaminated sample. This design applied the selected condition, solvent (MeOH–ACN), time (5 min) and varied the temperature (from 40 to 120 °C) and number of static cycles (2; 3). The recoveries of all PLE extractions were between 93 and 103% (RSD = 8.4%), thereby indicating that the experimental conditions established by using fortified material also apply to naturally contaminated corn samples.

3.3. Verification of the final extraction method

Since ZON reference material is still not available on the market, two samples used in an international proficiency study were analysed to evaluate the parameters chosen for the PLE extraction instrument. In this way the extraction method, including the LC–MS analysis, was compared to the most frequently applied methods used by the participants of the proficiency test, which are normally based on purification of the samples using either solid-phase extraction or immunoaffinity clean-up followed by LC–fluorimetry detection. The samples were extracted in triplicate for both matrices. ZON was detected in wheat at a level of 132 ng/g (RSD = 5.2%; $n = 3$), which is 118% of the target value assigned (112 ng/g). In corn the target ZON was at a concentration of 305 ng/g (RSD = 2.2%; $n = 3$), which is equal to a recovery of 107%, the target value being 285 ng/g.

4. Conclusion

Pressurized liquid extraction of ZON in wheat and corn provided results comparable to the most commonly used extraction method. Therefore, PLE could be an optimal choice in the attempt to automate sample handling. In particular, sample extraction could be run overnight. Acceptable recoveries (>90%) were obtained using the solvent mixture ACN–water. However, the alternative extraction mixture MeOH–ACN provided better performances for both matrices (cleaner extracts and higher recoveries). Temperature and time did not play an important role in the extraction efficiency irrespective of whether fortified or naturally contaminated material was used.

The limits of quantification were 15 ng/g in wheat and 12 ng/g in corn, while the RSD values were 8.6% and 10.2%, respectively, showing that this method is suitable for food and feed analysis.

Acknowledgements

We are grateful to U. Jörissen (WEJ GmbH, Hamburg, Germany) for providing us with naturally

contaminated corn and to E. Björklund (Department of Analytical Chemistry, Lund University, Sweden) for the fruitful scientific discussion.

References

[1] E. Björklund, S. Bøwadt, T. Nilsson, Trends Anal. Chem. 19 (2000) 434.
[2] D. Martens, M. Gfrerer, T. Wenzl, A. Zhang, B.M. Gawlik, K.W. Schramm, E. Lankmayr, A. Kettrup, Anal. Bionanal. Chem. 372 (2002) 562.
[3] P. Popp, P. Keil, M. Moeder, A. Paschke, U. Thuss, J. Chromatogr. A 774 (1997) 203.
[4] K. Adou, W.R. Bontoyan, P.J. Sweeney, J. Agric. Food Chem. 49 (2001) 53.
[5] H. Obana, K. Kikuchi, M. Okihashi, S. Hori, Analyst 122 (1997) 217.
[6] E. Korta, A. Bakkali, L.A. Berrueta, B. Gallo, F. Vicente, J. Food Prot. 65 (2002) 161.
[7] J.F. Lawrence, B. Niedzwiadek, P.M. Scott, J. AOAC Int. 83 (2000) 604.
[8] H.S. Klaffke, P. Majerus, E. Märtlbauer, H.J. Stan, R. Tiebach, E. Usleber, R. Weber, Mycotoxin Res. 16B (2000) 79.
[9] V. Betina (Ed.), Bioactive Molecules, Mycotoxins, Vol. 9, Elsevier, Amsterdam, 1989.
[10] P. Szuets, A. Mesterhazy, G.Y. Falkay, T. Bartok, Cer. Res. Commun. 25 (1997) 429.
[11] T. Tanaka, A. Yoneda, S. Inoue, Y. Sugiura, Y. Ueno, J. Chromatogr. A 882 (2000) 23.
[12] B. Fazekas, A. Tar, J. AOAC Int. 84 (2001) 1453.
[13] S.C. Kruger, B. Kohn, C.S. Ramsey, R.V. Prioli, J. AOAC Int. 82 (1999) 1364.
[14] ISO/TC 34/SC 10 N890, Nederlands Normalisatie Instituut, 25 July 2001.
[15] T. Tanaka, A. Hasegawa, Y. Matsuki, U.-S. Lee, Y. Ueno, J. Chromatogr. 328 (1985) 271.
[16] A. Llorens, R. Mateo, J.J. Mateo, M. Jimenéz, Food Addit. Contam. 19 (2002) 272.
[17] A. Visconti, A. Boenke, M. Solfrizzo, M. Pascale, M.B. Doko, Food Addit. Contam. 13 (1996) 909.
[18] M. Solfrizzo, A. De Girolamo, A. Visconti, Food Addit. Contam. 18 (2001) 227.
[19] R. Krska, R. Josephs, Fresenius J. Anal. Chem. 369 (2001) 469.
[20] E. Rosemberg, R. Krska, R. Wissack, V. Kmetov, R. Josephs, E. Razzazi, M. Grasserbauer, J. Chromatogr. A 818 (1998) 277.
[21] P. Zöllner, J. Jodlbauer, W. Linder, J. Chromatogr. A 858 (1999) 167.
[22] P. Zöllner, J. Jodlbauer, W. Linder, J. Chromatogr. A 738 (1999) 233.
[23] L. Pallaroni, C. von Holst, C. Sparr-Eskilsson, E. Björklund, Anal. Bioanal. Chem. 374 (2002) 161.
[24] FAPAS series 22, round 02, Fusarium Toxins, Report 2202, Central Science Laboratory, Sand Hutton, York, (December, 2000).
[25] FAPAS series 22, round 06, Fusarium Toxins, Report 2206, Central Science Laboratory, Sand Hutton, York, (March 2002).
[26] K. Bayne, I.B. Rubin, Practical Experimental Design and Optimisation Methods for Chemists, VHC, Deerfield Beach, 1986.
[27] J. Stroka, M. Petz, U. Joerissen, E. Anklam, Food Addit. Contam. 16 (1999) 331.