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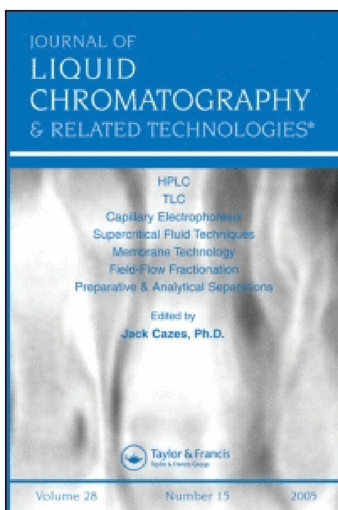

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Comparison of Two Sample Preparation Techniques for the Determination of Ochratoxin A in Grains

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Abstract: Two established, commercially available sample preparation–techniques (automated immunoaffinity purification and push-through solid phase extraction–columns) were tested for the determination of ochratoxin A in grains using high performance liquid chromatography combined with fluorescence detection. The performance of the clean-up techniques was assessed as their impact on the method performance as specified by the validation parameters as well as on their suitability for routine use. Acceptable mean recovery and repeatability assessed with HORRAT-values could be achieved with the two methods and both were able to detect ochratoxin A at the levels needed for legislative purposes. The analyses of the naturally contaminated grain samples and a certified reference material emphasized, that comparable results for most cases could be obtained with these methods. Both techniques were simple and feasible, especially in the case of immunoaffinity-purification which can be automated. A significant matrix effect was observed with both clean-up techniques, and therefore, calibrants prepared with matrix need to be used in the procedures.

Keywords: Ochratoxin A, Grain, Sample preparation, Immunoaffinity, Push-through SPE, Validation

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INTRODUCTION

Ochratoxin A (OTA) is a toxic secondary metabolite, i.e., a mycotoxin, produced by filamentous fungi. The main producers of OTA are *Penicillium verrucosum* in the temperate regions[1] and *Aspergillus* spp. in warm climates.[2] OTA has nephrotoxic, immunosuppressive, teratogenic, and potential carcinogenic properties,[3–5] and therefore, exposure to this mycotoxin may pose serious health risks both to humans and animals. OTA has also been suspected as being an aetiological factor in the endemic nephropathy affecting human populations in the Balkans.[6] The major sources of OTA in diet are cereals and cereal products, coffee beans, beans, pulses, and dried fruits.[7] OTA has also been detected in several other products such as pork and poultry meat, kidney, milk, and wine.[8–11] Due to its toxicity and frequent occurrence, the European Commission has stipulated the maximum permitted levels for the presence of OTA in different food products. The maximum level set for raw cereal grains is 5 μg/kg.[12]

Modern analytical techniques and further method development are still needed in mycotoxin analyses, as many of the existing methods suffer from inherent limitations such as poor recovery of the analytes, variation of the results, or inadequate sensitivity (e.g.[13–16]). Sensitive analytical methods for the determination of the minute concentrations of these agents present in foods and feeds are essential to achieve a meaningful risk assessment of the chronic effects of mycotoxins in humans and animals. In addition, the legislative maximum permitted levels require novel methods or improvements to the existing methods, since false laboratory data may cause food safety problems or have major financial implications due to hindrances to the international food and feed trade.

High performance liquid chromatography (HPLC) connected to a fluorescence detector has been the analytical separation technique most often applied for the analysis of OTA in different food matrices.[13,17–19] That is also the method of choice in the existing official methods for the analysis of OTA in cereals and cereal products.[20,21] However, recently HPLC techniques combined with mass spectrometric detection (LC-MS) have gained popularity.[22–24]

OTA can be found, usually at low concentrations, in a range of matrices with different chemical compositions. This background effect complicates the analytical techniques used for the determination of mycotoxins, and complicated sample preparation is required before the analyte can be injected into the analytical instrument. Sample preparation has shifted from laborious liquid liquid extraction towards more straightforward techniques, such as solid phase extraction (SPE) cartridges. SPE with different, polar or non-polar, silica based sorbents have often been applied for the clean-up of samples in the determination of OTA.[25–27] In addition to "traditional" SPE materials, also columns with immunoaffinity (IA) sorbents have gained popularity.[22,28,29] The main benefit of

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immunoaffinity columns is their high specificity for the target compound.[30] There are supplemental advantages associated with these columns, i.e., suitability for automation and their ability to increase the sample throughput. Quite recently, commercial push-through format SPE columns (Mycosep®229 Ochra) have been introduced as a simple way to purify food samples prior to OTA analysis. Buttinger et al.[31] reported good performance for these columns both in terms of validation results and their suitability for routine use. According to the manufacturer, these columns represent a cost and time efficient alternative to immunoaffinity columns.[32]

In this study, two established, commercially available sample preparation techniques (automated immunoaffinity purification and push-through SPE) were tested for the determination of ochratoxin A in grains in conjunction with high performance liquid chromatography combined with fluorescence detection. The performance of the clean-up techniques was assessed based on their impact on the method performance as specified by the validation parameters, as well as their suitability for the routine method, i.e., the ease of use and cost-efficiency.

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EXPERIMENTAL

Reagents

Acetonitrile (ACN) (J.T Baker, Deventer, Holland) was of HPLC grade, whereas acetic acid and toluene (J.T Baker, Deventer, Holland) were of analytical grade. Deionized water was purified with a Millipore Milli-Q Plus system (Millipore, Espoo, Finland). Phosphate buffered saline (PBS) pellets were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK), and the PBS buffer (pH 7.3) was prepared according to the manufacturer's instructions.

Ochratoxin A (OTA) standard was purchased from Sigma (St. Louis, MO, USA). The stock solution of OTA (1 mg/mL) was prepared in toluene:acetic acid (99:1) and its further dilutions (5 ng/mL, 50 ng/mL and 500 ng/mL, respectively) in ACN. The concentration of the stock solution was determined by ultraviolet light spectrophotometry using a diluted solution (10 μg/mL) and the extinction efficient of 5440 $dm^3\ mol^{-1} \times cm$ (at 333 nm).

Samples

Finnish barley flour purchased from local markets was used as the sample matrix for method validation (selectivity, linearity, repeatability, recovery, limit of detection, and limit of quantification). For further method performance tests, certified reference material CRM472 (wheat) and naturally contaminated barley samples were analysed.

Sample Preparation

Immunoaffinity Columns

The samples for OTA analyses were prepared with immunoaffinity columns (IA) as described in Eskola et al. (2002)[33] with minor modifications. In brief, OTA was extracted from 20.0 g of flour with 100 mL of ACN:water (60:40, v/v) by shaking for 1 h in a horizontal shaker (Edmund Bühler, Bodelshausen, Germany). The extracted samples were filtered through a S&S 602 H1/2 filter paper (Schleicher & Schuell, Dassal, Germany) and 5 mL of the filtered extract was diluted to 50 mL with PBS buffer. Sample purification was automated using ASPEC XL workstation (Gilson Inc., Villier le Bel, France). An IA column (R-Biopharm Rhône Ltd., Glasgow, Scotland) was conditioned with 10 mL PBS buffer before loading the diluted sample extract (40 mL). The column was washed with 14 mL of PBS buffer, and OTA was eluted with 2 mL of ACN:acetic acid (98:2, v/v). The samples collected from ASPEC were evaporated to dryness under a stream of nitrogen at +50°C, followed by reconstitution with 200 μL of HPLC mobile phase (ACN:water:acetic acid, 99:99:2, v/v/v). The dissolved sample was filtered through a 0.2 μm syringe filter (Pall Gelman Sciences, Ann Arbor, MI, USA) into an autosampler vial.

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Push-through SPE Columns

The samples were prepared with push-through SPE-columns (Mycosep™229 Ochra, Romer Labs Inc., MO, USA) according to the instructions of the manufacturer[34] with minor modifications. OTA was extracted from 25.0 g of flour with 100 mL of ACN:water (84:16, v/v) by shaking for 1 h in a horizontal shaker (Edmund Bühler, Bodelshausen, Germany). The extracted samples were filtered through a S&S 602 H1/2 filter paper (Schleicher & Schuell, Dassal, Germany), and 10 mL of the filtered extract was acidified with 100 μL of acetic acid. The acidified extract was pushed through the column; 4 mL of the purified sample extract was transferred to a vial, and evaporated to dryness under a stream of nitrogen at +50°C, followed by reconstitution of the residue with 200 μL of HPLC mobile phase (ACN:water: acetic acid, 99:99:2, v/v/v). The dissolved sample was filtered through a 0.2 μm syringe filter (Pall Gelman Sciences, Ann Arbor, MI, USA) into an autosampler vial.

HPLC Analysis

The high performance liquid chromatography was performed with an Alliance 2960 Separations Module (Waters, Milford, MA, USA) by injecting 40 μL of the sample on a Symmetry C18 column (3.9 × 150 mm, 5 μm) (Waters,

Milford, MA, USA) equipped with a frit (Rep frit A-102X, Upchurch Scientific Inc., Oak Harbor, WA, USA). Separation was achieved using an isocratic elution with ACN:water:acetic acid (99:99:2, v/v/v) with a flow rate of 0.9 mL/min. OTA was detected with a Waters 474 fluorescence detector (Waters, Milford, MA, USA) ($\lambda_{Ex} = 333$ nm, $\lambda_{Em} = 450$ nm). The total run time was 10 minutes.

Preparation of the Calibrants

External calibration was used for the quantification of OTA. Calibrants within the concentration range corresponding to 0–25 μg/kg OTA in the grains were dissolved in mobile phase. Matrix-assisted calibrants were prepared separately for both IA- and push-through SPE column procedures. In brief, blank barley samples were extracted and prepared as described (see Sample Preparation). The purified extracts (2 mL for IA and 4 mL for push-through SPE) were spiked with appropriate amounts of OTA, and evaporated to dryness under a stream of nitrogen, followed by reconstitution with the mobile phase (200 μL). The calibration curves with the matrix were prepared at the same seven levels (0–25 μg/kg) as the calibration curves without the matrix. In both cases, three replicate calibrants were prepared for each of the seven concentrations.

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Method Performance Tests

The method validation included the determination of recovery, repeatability, selectivity, limit of detection (LOD), limit of quantification (LOQ), and linearity. Five replicates of spiked samples at three concentration levels (1, 5, and 10 μg/kg) and calibration curves with and without matrix (see Preparation of Calibrants) were prepared using the two sample preparation procedures described.

In addition to spiked samples, six naturally contaminated barley samples and a certified reference material for OTA (CRM 472, wheat) were analysed with the both techniques.

RESULTS AND DISCUSSION

Validation Parameters

Linearity

The correlation coefficients (R^2) of the calibration curves prepared with or without matrix for both IA and push-through SPE procedures were >0.99

(data not shown). The acceptable linearity of each point of the calibration curves was tested with the method of van Trijp & Roos.[35] A tolerance of $100 \pm 10\%$ was accepted for the separate calibration points as an indication of good linearity (data not shown). On this basis, the calibration curves prepared without matrix can be considered as being linear within the range of 0.25–25 μg/kg OTA in grains. The matrix assisted calibration curves, instead, were linear for both clean-up techniques tested at the range of 1.25–25 μg/kg OTA in grains.

Selectivity and Specificity

Selectivity is the ability of the method to distinguish the response of the analyte from all other responses, i.e., from responses originating from sample matrix compounds. The selectivity of the methods for analysing OTA was tested by comparing the slopes of the calibration curves obtained with and without matrix (see Preparation of the Calibrants).

Statistically significant differences ($p < 0.05$, 2-sided t-test) in the slopes of non-matrix and matrix assisted calibration curves ($n = 3$) were observed with both sample preparation techniques. For the IA procedure, the slopes for calibration curves prepared with matrix were smaller than the slopes for calibration curves prepared without matrix (Figure 1a). This is an indication of a suppressive matrix effect, leading to an underestimation of the sample concentration if calibrants without matrix are used in the quantification procedure. The observed matrix effect was unexpected since the IA columns are claimed to be very selective, and the eluted samples are usually visibly clean and clear. Furthermore, with the push-through SPE procedure, the slopes for the calibration curves prepared with matrix were higher than the slopes for calibration curves prepared without matrix (Figure 1b). Therefore, the matrix effect is of an enhancement type, and the use of calibration curve without matrix would lead to an overestimation of the quantitative result. Although matrix effects leading to decreased selectivity have been frequently described problems when using mass spectrometers as detectors in connection either with liquid chromatography (LC-MS) or gas chromatography (GC-MS), this phenomenon has also been reported to occur when using fluorescence detection.[36]

Specificity is the ability of the method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix under the conditions of the test. The specificity of the methods was tested by observing possible interfering signals at the detection time of OTA. No such peaks were observed in the chromatograms of blank matrices for either of the sample preparation techniques used (Figure 2). Therefore, both methods can be considered specific for the determination of OTA in grains.

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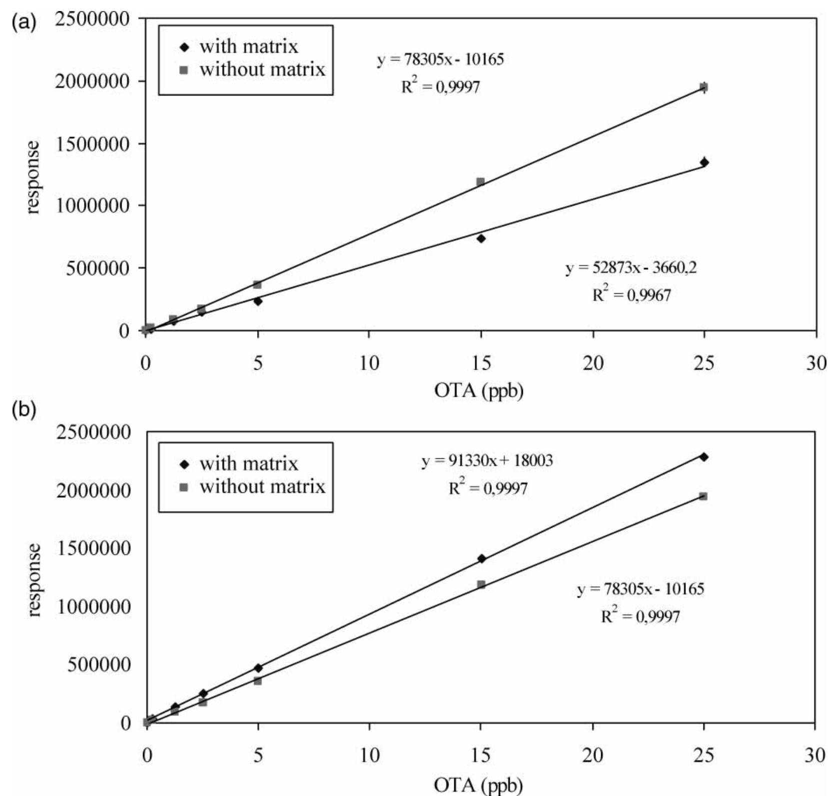

Figure 1. The mean of the responses ($n = 3$) for the calibration curves prepared with and without matrix using the immunoaffinity (a) or the push-through SPE (b) sample preparation technique for ochratoxin A.

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LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) for both of the clean-up -techniques were determined by calculation from the chromatograms of the lowest points of the calibration curves prepared with the matrix ($\mathrm{LOD} = 3 \times S/N$; $\mathrm{LOQ} = 10 \times S/N$). The calculated LOD and LOQ values for the IA method were 0.2 μg/kg and 0.7 μg/kg, respectively. For the push-through SPE method, LOD was 0.8 μg/kg and LOQ 2.7 μg/kg. Thus, the calculated LOD and LOQ values differed between the two methods, i.e., the IA clean-up was able to detect and quantify OTA at nearly four times lower concentrations than the push-through SPE method. More coeluting matrix interferences could be seen in the chromatograms of the push-through SPE columns. In contrast, IA columns showed good

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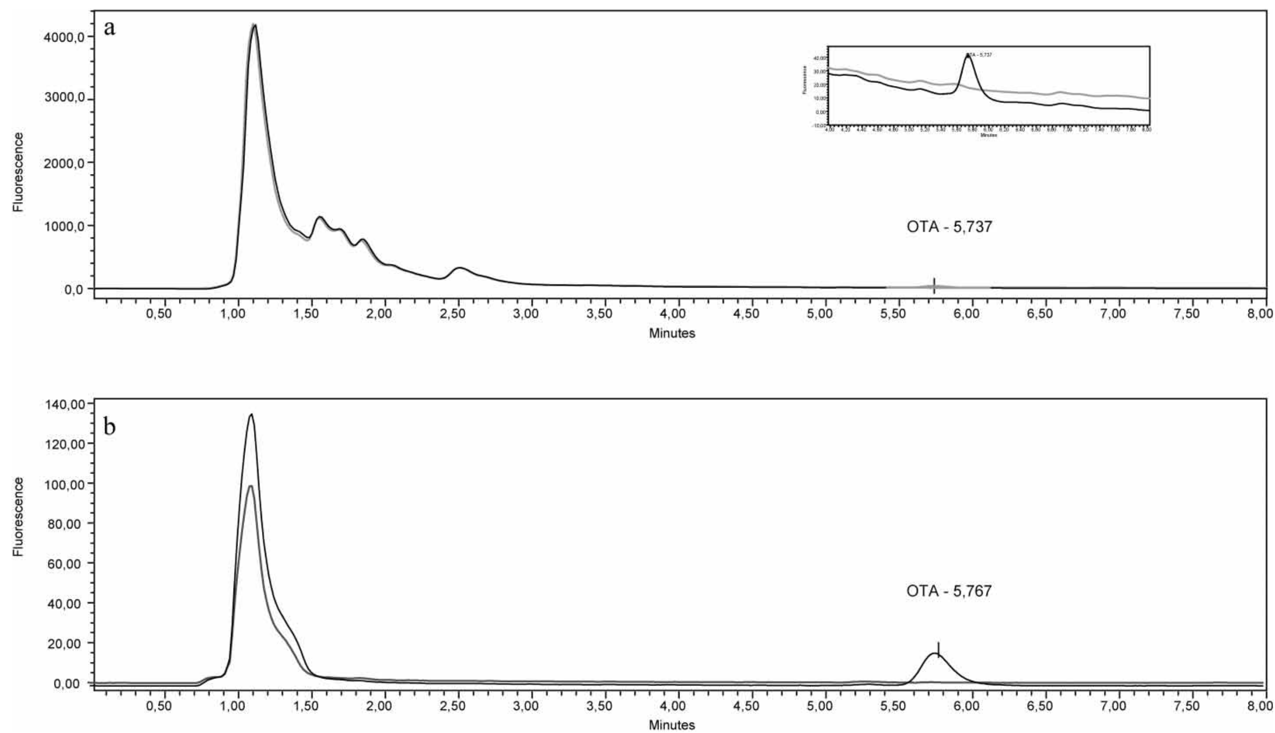

Figure 2. Chromatograms of a blank sample (grey line) and a spiked sample at 5 μg/kg (black line) obtained by the immunoaffinity (a) and the push-through SPE (b) procedures.

clean-up performance, with cleaner chromatograms, which also lead to the lower detection limit. Examples of the chromatograms are shown in Figure 2.

Recovery and Repeatability

The mean recovery and repeatability (RSDs) values of the two sample preparation techniques were determined at three concentration levels, each having five replicates. The results obtained by quantifying with calibrants prepared with and without matrix are presented in Table 1.

In general, the recoveries were acceptable with both techniques. The low relative standard deviation (RSD) values highlighted the good repeatability. With the push-through SPE technique, the RSDs were, however, better than with the IA method, especially at the two higher spiking levels. The matrix effect (see Selectivity and Specificity), i.e., the effect of calibration (with or without matrix) on the quantification can clearly be seen in the recovery results. In other words, for samples purified with IA columns, lower recoveries were obtained when using calibrants prepared without matrix. With the push-through SPE columns, on the contrary, the recoveries were higher when calibrants prepared without matrix were used compared to the results obtained with matrix calibration. However, the calibration had no major effect on the RSDs of the methods.

It is noteworthy that different extraction solvents were used in the two procedures (see Immunoaffinity Columns and Push-through SPE Columns), which may have lead to differences in the initial extraction efficiency, and may account for some of the variations observed in the recoveries. However, we used the extraction procedures recommended by the manufacturers of the purification columns. Therefore, the question whether the solvent had an effect on the recoveries, is not relevant when comparing these two sample preparation methods, as was our aim.

A so called Horwitz Equation ($RSD_R = 2C^{-0.1505}$) is often used to quantify the relationship between the RSD_R (interlaboratory relative

Table 1. The mean recoveries and the corresponding relative standard deviations of ochratoxin A obtained with the two sample preparation techniques using the two different calibration procedures

Spiking level	IA-column (with matrix)[a]	IA-column (without matrix)	Push-through SPE (with matrix)[a]	Push-through SPE (without matrix)
1 μg/kg	99.5 ± 13.0	65.2 ± 11.9	<LOQ	< LOQ
5 μg/kg	118.6 ± 18.1	71.8 ± 17.8	93.4 ± 2.8	115.4 ± 2.7
10 μg/kg	114.4 ± 11.5	68.7 ± 11.5	88.8 ± 2.3	108.7 ± 2.3

[a]According to the validation data, matrix-assisted calibrants should be used for both of the sample preparation methods used.

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standard deviation) and analyte concentration in mycotoxin analysis.[37] Some researchers have suggested that when applying the equation to within laboratory studies, as in our study, the aim value should be 2/3 of the RSD_R predicted from the Horwitz Equation. The Horwitz Equation can be very useful for evaluating analytical methods[38] if one calculates the Horwitz Ratio [$HORRAT = RSD_R(found)/RSD_R(predicted)$].[39] A HORRAT-value <2 indicates that the method is acceptable, precise, and under statistical control.[38] The HORRAT-values (RSD_R (predicted) = 2/3 of the RSD_R obtained from the Horwitz Equation) for ochratoxin A in this study were 0.56–1.06 and 0.61–1.08 for IA purification, as quantified using calibrants prepared without and with matrix, respectively. For the push-through SPE method, the corresponding values were 0.15–0.45 and 0.15–0.50. Thus, the HORRAT values for both methods indicate that they can be considered as being acceptable for analysing OTA in grains.

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Sample Analyses

The results (not corrected for recovery) obtained for the six naturally contaminated barley samples and a certified reference material (CRM 472) are presented in Table 2.

Some variation, depending on the sample preparation technique and the calibration used, was observed between the results. All the sample concentrations determined by the IA method as quantified with calibrants prepared with matrix were higher than the concentrations obtained using calibrants prepared without matrix. For the push-through SPE method, the results were in the opposite direction, supporting the findings of the selectivity studies.

The OTA concentrations determined with the technique using push-through SPE -columns were somewhat higher than the results obtained with

Table 2. The ochratoxin A concentrations (μg/kg, not corrected for recovery) for naturally contaminated barley samples and certified reference material determined with the two methods tested and using the two different calibration procedures

Sample	IA-column (with matrix)	IA-column (without matrix)	Push-through SPE (with matrix)	Push-through SPE (without matrix)
Barley 1	3.5	2.2	7.5	9.2
Barley 2	<0.4	<0.4	<1.6	<1.6
Barley 3	7.5	4.5	8.1	9.9
Barley 4	3.6	2.2	<1.6	<1.6
Barley 5	1.4	0.9	<1.6	<1.6
Barley 6	2.2	1.3	2.7	3.4
CRM472 (wheat)	7.6	4.6	6.4	7.8

IA-columns, except for barley samples 4 and 5. The results obtained with both techniques are, however, in quite good agreement excluding sample "barley 1", especially if the legislative maximum level for OTA (5 μg/kg for raw cereal samples) is considered. In other words, both sample preparation methods would lead, in most cases, to similar conclusions in terms of distinguishing samples below or above the permitted level.

The assigned value for the CRM472 is 8.2 ± 1 μg/kg OTA. The so called z-scores (a measure of the distance in standard deviations of a sample from the mean) calculated for the results obtained in our study were -2.00 and -0.33 for IA purification without and with matrix assisted calibrants, respectively. For push-through SPE purification, the corresponding values were -0.22 and -1.00. As the satisfactory range for z-scores is $|z| \leq 2$, all of the results can be considered as being precise. However, it must be noted that the value obtained for CRM472 (6.4 μg/kg) with push-through SPE purification using matrix assisted calibration as suggested by the selectivity studies, is outside the certified value. For IA purification, the result obtained with non-matrix calibration was also outside of the assigned value, but when matrix assisted calibration was used, the acceptable value was detected. CRM results were also examined as recovery corrected values. When the corresponding correction factors at the level of 10 μg/kg were used, the corrected CRM results for IA purified samples were 6.7 μg/kg and for SPE-purified samples 7.2 μg/kg regardless of the calibration used. The corresponding z scores for these results were -0.83 and -0.55, which are in the satisfactory range. However, on the basis of the recovery corrected results, the results for IA purified samples are outside the acceptable value.

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CONCLUSIONS

Two different sample preparation techniques were tested in the determination of ochratoxin A in grains. According to the method performance parameters, no major differences between immunoaffinity and push-through SPE procedures could be observed, i.e., acceptable mean recoveries and repeatabilities as assessed with HORRAT values could be achieved with both techniques. The procedure utilising immunoaffinity purification was, however, able to detect lower concentrations of the analyte, probably due to the cleaner extracts. Nonetheless, both methods were able to detect ochratoxin A at the levels needed for legislative purposes. The analyses of the naturally contaminated grain samples and a certified reference material showed that despite some differences observed, fairly comparable results could be obtained with both techniques. A significant matrix effect was observed with both of these clean-up methods, and therefore, calibrants prepared with matrix need to be used in the analytical procedure.

Immunoaffinity as well as push-through SPE columns are more expensive than the traditional SPE columns used for sample preparation of OTA.

However, lower detection and quantification levels can be obtained with these newer methods. Push-through SPE columns are possibly the more economic choice of the techniques tested here, but the IA procedure does permit lower detection levels. As both techniques are simple and feasible for routine use, especially if IA purification is automated, the user has to determine which factor (cost or detection limit) is decisive.

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