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Application of Manual and Automated Systems for Purification of Ochratoxin A and Zearalenone in Cereals with Immunoaffinity Columns

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A manual vacuum manifold and an automated solid phase extraction (ASPEC) system were applied for purification of ochratoxin A and zearalenone in wheat, rye, barley, and oat samples with immunoaffinity columns followed by separation with a high-performance liquid chromatograph and fluorescence detection. The immunoaffinity columns for manual sample purification were purchased from a different manufacturer than were those for the automated system. The limit of detection (LOD) for the method for ochratoxin A with a vacuum manifold and ASPEC was 0.1 μg/kg. For the method for zearalenone, the LODs were 1.5 μg/kg with a vacuum manifold and 3 μg/kg with ASPEC. For the methods for ochratoxin A at spiking levels of 0.6 and 2.5 μg/kg, mean recoveries for different cereals varied from 68 to 106%. For the methods for zearalenone, mean recoveries varied from 78 to 117% at spiking levels of 9 and 25 μg/kg. The relative standard deviations of repeatability with various cereals employing both methods were 2−15 and 2−19% for ochratoxin A and zearalenone, respectively.

KEYWORDS: Ochratoxin A; zearalenone; cereals; immunoaffinity column; ASPEC; HPLC

INTRODUCTION

Ochratoxin A (**1**) and zearalenone (**2**) (Figure 1) are mycotoxins, exposure to which may cause serious health effects in both humans and animals. *Penicillium* and *Aspergillus* molds are both known to produce ochratoxin A, whereas zearalenone is produced by *Fusarium* molds (*1*, *2*). Ochratoxin A has been regarded as a factor in human endemic nephropathy in the Balkan area (*3*) and is considered to be a possible human carcinogen (*4*). Zearalenone may cause reproductive and infertility problems in animals (*5*). The major sources of ochratoxin A as well as of zearalenone in the diet are cereals and cereal products (*2*, *6*). Ochratoxin A has also been detected in several other food products such as wine, coffee, pig kidney, pork, poultry, and cow's milk (*7*−*11*).

High-performance liquid chromatography (HPLC) has often been used for the analysis of ochratoxin A and zearalenone, but thin-layer chromatography and enzyme-linked immunosorbent assay have also been applied (*12*, *13*). For the determination of zearalenone, gas chromatographic techniques have also been used (*13*). Additionally, a liquid chromatographic−mass spectrometric technique has been employed for both mycotoxins (*14*−*18*). To extract ochratoxin A in cereals, a mixture of organic solvent and water, with or without a small amount of acid, has been used (*19*, *20*). Zearalenone has also frequently been extracted with a mixture of organic solvent and water (*21*). Solid phase extraction cartridges have often been applied for the purification of extracts (*19*, *20*, *22*, *23*), and liquid−liquid partition has been used to purify the extract for zearalenone analysis (*24*, *25*). However, immunoaffinity columns have recently been employed successfully for the purification of extracts in several studies (*16*, *26*−*30*).

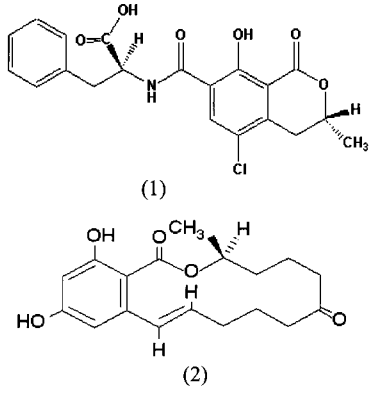

Figure 1. Structures of ochratoxin A (**1**) and zearalenone (**2**).

Ochratoxin A and zearalenone are usually found only in trace amounts in food products, which requires high-performance

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analytical methods. By using immunoaffinity columns even small amounts of mycotoxins can be detected with confidence. Visconti et al. (*31*) stated that the use of immunoaffinity chromatography in the purification step provides a number of advantages over conventional methods, such as clean extracts due to the high specificity of the antibodies for one toxin or a group of related toxins, high precision and accuracy over a wide concentration range of interest, rapidity of the purification step, and reduction in the use of hazardous solvents.

Currently, high efficiency in sample throughput without increased labor is needed in many analytical laboratories. Sample preparation is usually boring, repetitive work and is thus rather susceptible to human errors (*32*); automated systems have therefore been employed. In some studies an automated solid phase extraction (ASPEC) system has been applied to purify food and feed samples by applying immunoaffinity columns for ochratoxin A and aflatoxin analyses (*26*, *33*−*36*). However, to the best of our knowledge, any study of the use of ASPEC for zearalenone analysis employing immunoaffinity columns for sample cleanup has not yet been published.

The primary aim of this study was to introduce the use of immunoaffinity columns for ochratoxin A and zearalenone analysis in wheat, rye, barley, and oats and to validate the two methods. Another aim was to apply the ASPEC system as a stand-alone technique for sample purification by using immunoaffinity columns for ochratoxin A and zearalenone analysis in wheat, rye, barley, and oats and, furthermore, to validate these methods. The two procedures used for the purification steps of ochratoxin A and zearalenone were compared. Additionally, confirmatory methods for both of the toxins were introduced.

MATERIALS AND METHODS

Materials. Ochratoxin A and zearalenone standards were obtained from Sigma, St. Louis, MO. The standard solution for ochratoxin A was prepared in a toluene/acetic acid solution (99:1 v/v) to a concentration of 0.5 μg/mL and the standard solution for zearalenone in acetonitrile to a concentration of 1 μg/mL and monitored spectroscopically. The wavelength and the molar absorption coefficient used were 333 nm and 5440 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ for ochratoxin A, respectively (*37*), and 274 nm and 12623 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ for zearalenone, respectively (R, Krska, personal communication, 2000). Acetonitrile, toluene, acetic acid, and methanol were purchased from J. T. Baker, Deventer, The Netherlands. All solvents were of HPLC grade. Water was purified by a Millipore Milli-Q Plus system. Potassium dihydrogen phosphate, potassium chloride, sodium chloride, and sodium hydrogen carbonate were purchased from Merck, Darmstadt, Germany. Sodium monohydrogen phosphate was from J. T. Baker, and Tween 20 was from Fluka, Buchs, Switzerland. Phosphate-buffered saline (PBS) pellets were obtained from Oxoid Ltd., Basingstoke, Hampshire, U.K. For ochratoxin A and zearalenone analyses, the PBS buffer was prepared according to the Vicam *OchraTest Instruction Manual* (*38*) or by using PBS pellets. The pH of PBS buffer was not adjusted but determined to be ~7.4. For ochratoxin A analysis, washing buffer was prepared as described in the Vicam *OchraTest Instruction Manual* (*38*). A post-column pH shift for ochratoxin A was performed by using 1.1 M ammonium hydroxide solution (pH ~14). Ammonium hydroxide (25%) was purchased from J. T. Baker.

The immunoaffinity columns were purchased from two different producers. For ochratoxin A analysis OchraTest immunoaffinity columns were obtained from Vicam, Watertown, MA, and Ochraprep immunoaffinity columns from Rhône-Diagnostics Technologies, Glasgow, Scotland. For zearalenone analysis ZearalaTest immunoaffinity columns were purchased from Vicam and Easi-Extract zearalenone immunoaffinity columns from Rhône-Diagnostics Technologies. Filter paper 602 $H^1/_2$ was from Schleicher and Schuell, Dassal, Germany, and GHP Acrodisc 13 mm syringe filters with a pore size of 0.2 μm for filtering the samples prior to the HPLC analysis were from Gelman, Ann Arbor, MI.

The certified wheat reference material for ochratoxin A (CRM 472) was obtained from the Community Bureau of Reference-BCR, European Commission, Brussels, Belgium. The certified value with uncertainty for ochratoxin A in wheat (CRM 472) is assigned to be 8.2 ± 1.0 μg/kg (*37*). The wheat test material for ensuring the performance of the zearalenone analysis was obtained from FAPAS, Central Science Laboratory, York, U.K. According to the report of FAPAS (*39*) the measured concentration of zearalenone in the test material varied between 73.8 and 159.5 μg/kg, and the mean concentration was 112.0 μg/kg.

Apparatus. Cereal grains were ground with a laboratory mill model 3100 (Pata-Lab, Helsinki, Finland) with a sieve size of 0.8 mm. A pH meter model PHM95 pH/ion meter was from Radiometer, Copenhagen, Denmark, and a spectrophotometer model Graphicord, UV−visible recording spectrophotometer UV-240 was from Shimadzu, Kyoto, Japan. For manual sample cleanup purposes a Vac Elut SPS 24 vacuum manifold (Analytichem International, Harbor City, CA) was used. To automate the cleanup step by applying immunoaffinity columns, ASPEC was employed. ASPEC was obtained from Gilson, Villiers Le Bel, France, with a sample processor for solid phase extraction, model ASPEC XL, and a 401C dilutor. A Gilson sampler keypad controller version 1.0 controlled the ASPEC system using the Gilson sampler controller software 721 version V2.11 (Gilson Villiers Le Bel, France). The HPLC was a Waters model 2690 Alliance Separation module (Waters, Milford, MA). For peak detection a scanning fluorescence detector, a Waters model 474 was used. Prior to use the HPLC and the detector were validated according to Waters *HPLC Systems Qualification Workbook* (*40*). The analytical column was a Waters C18 reversed-phase Symmetry C18, 150 × 3.9 mm i.d., 5 μm. To confirm the presence of ochratoxin A in the samples by postcolumn pH shift, an additional HPLC pump, a Waters model 501, was used. Millennium[32] software (version 3.05, 1998, Waters) was used to handle and process chromatographic data.

Sample Preparation for Ochratoxin A and Zearalenone Analysis. The sample preparation method for ochratoxin A using an OchraTest immunoaffinity column connected to a vacuum manifold was a modification of the method described in the Vicam *OchraTest Instruction Manual* (*38*). Twenty grams of ground cereal was extracted with 50 mL of an acetonitrile/water (60:40 v/v) mixture for 1 h in a horizontal shaker. The cereal extract was filtered through a paper filter, and 5 mL was diluted with 45 mL of PBS buffer. A 20 mL volume of diluted extract was passed through an immunoaffinity column by using a vacuum manifold (1−2 drops/s). Thereafter, the column was washed with 10 mL of washing buffer and 10 mL of water (1−2 drops/s) followed by elution of ochratoxin A with 1.5 mL of a methanol/acetic acid (98:2 v/v) mixture (1 drop/s). Eluted sample was then evaporated under a nitrogen stream at 40−50 °C, and the dry residue was dissolved in 200 μL of HPLC mobile phase (acetonitrile/water/acetic acid, 99:99:2 v/v/v) for HPLC analysis.

Alternatively, the sample purification was performed automatically using the ASPEC sample preparation system in a sequential mode as described in the *Gilson Guide to SPE Automation* (*41*). The ASPEC system was validated before it was used for sample preparation. The sample cleanup step for ochratoxin A with ASPEC was a modification of the methods described in the instructions for use of the Ochraprep immunoaffinity column (*42*) and in the studies of Sharman and Gilbert (*33*) and Sharman et al. (*26*). To purify the sample by applying ASPEC, Ochraprep immunoaffinity columns were used. Twenty grams of ground cereal was extracted with 100 mL of acetonitrile/water, filtered, and diluted with PBS buffer as described above. Before the sample cleanup step, ASPEC conditioned the immunoaffinity column with 18 mL of PBS buffer with a flow rate of 6 mL/min. Thereafter, ASPEC loaded 40 mL of diluted extract through the immunoaffinity column with a flow rate of 2 mL/min. The column was washed with 19 mL of PBS buffer (5 mL/min), and finally ochratoxin A was eluted with 2 mL of a methanol/acetic acid mixture (98:2 v/v) with a flow rate of 0.3 mL/min. The eluate was evaporated under a nitrogen stream at 40−50 °C followed by reconstitution of the dry residue with 200 μL of mobile phase (acetonitrile/water/acetic acid, 99:99:2 v/v/v).

The method for zearalenone analysis was slightly modified from the method presented earlier by Eskola et al. (*43*). To purify the sample

with a ZearalaTest immunoaffinity column by using a vacuum manifold, 5 mL of extract was diluted with 45 mL of water. A 20 mL volume of the diluted cereal extract was passed through an immunoaffinity column using a vacuum manifold (1−2 drops/s). The column was then washed with 20 mL of water (1−2 drops/s), and finally zearalenone was eluted with 1.5 mL of methanol (1 drop/s). The eluted sample was evaporated under a nitrogen stream at 40−50 °C, and the dry residue was reconstituted with 200 μL of HPLC mobile phase (acetonitrile/methanol/water, 40:52:8 v/v/v) for HPLC analysis.

Alternatively, the cleanup step was performed automatically using ASPEC in a sequential mode as described in the *Gilson Guide to SPE Automation* (*41*). The sample purification step for zearalenone with ASPEC was a modification of the methods described in the instructions for use of the Easi-Extract zearalenone immunoaffinity column (*44*) and in the studies of Sharman and Gilbert (*33*) and Sharman et al. (*26*). When ASPEC was applied, Easi-Extract zearalenone immunoaffinity columns were used. A 5 mL volume of cereal extract was diluted with 45 mL of PBS buffer. Before use, the immunoaffinity column was automatically conditioned with 18 mL of PBS buffer by ASPEC with a flow rate of 6 mL/min. Thereafter, ASPEC loaded 20 mL of diluted cereal extract through the immunoaffinity column with a flow rate of 5 mL/min. The column was then washed with 19 mL of PBS buffer (5 mL/min) followed by the elution step with 2 mL of acetonitrile (0.3 mL/min). The eluate was evaporated under a nitrogen stream at 40−50 °C, and finally the dry residue was dissolved in 200 μL of methanol.

Prior to HPLC analysis all of the samples were filtered through syringe filters into autosampler vials. In ochratoxin A analysis as well as in zearalenone analysis, the injection volume was 40 μL and the flow rate of the mobile phase was 0.9 mL/min. Otherwise, the chromatographic condition for ochratoxin A determination was as presented by Sharman et al. (*26*), with the modification of the emission wavelength that was 450 nm, and for zearalenone determination as presented by Visconti and Pascale (*30*) with the modification of the mobile phase that was acetonitrile/water/methanol (40:52:8, v/v/v). Ochratoxin A and zearalenone were quantified with the external standard method.

Validation of the Ochratoxin A and Zearalenone Methods. Both analytical methods were validated for ochratoxin A and for zearalenone. The methods were validated for wheat, rye, barley, and oats by spiking cereal samples at two concentration levels of 0.6 and 2.5 μg/kg for ochratoxin A and 9 and 25 μg/kg for zearalenone. Standard solution of ochratoxin A (see Materials and Methods) at lower and higher concentration levels, 24 and 100 μL, respectively, were added to a weighed amount of cereal flour. Similarly, 180 and 500 μL of standard solution of zearalenone at lower and higher concentration levels, respectively, were spiked to a cereal sample. Before the validation study wheat, rye, barley, and oats were analyzed to ensure that they were negative for ochratoxin A and zearalenone. Six replicates of each cereal at each concentration level were prepared for purification with a vacuum manifold and with the ASPEC as described above. Recovery and repeatability within day with the same apparatus and operator were calculated from the obtained results. A total of six and four certified reference material replicates (CRM 472) were prepared for ochratoxin A analysis performed with a vacuum manifold and with the ASPEC system, respectively. These samples were prepared on different days with the same apparatus but by a different operator. Six replicates of the test wheat material for zearalenone obtained from FAPAS were analyzed by using ASPEC for sample purification on different days with the same apparatus by a different operator.

The limit of detection (LOD) was calculated on the basis of a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was 2 times the value of LOD. LOD was calculated from the results of the spiked samples at levels of 0.6 μg/kg of ochratoxin A and 9 μg/kg of zearalenone. The highest concentration of ochratoxin A and zearalenone that can be loaded to an immunoaffinity column was not tested because the performance of the method was studied only for low concentrations and saturation of the columns was not presumable. Moreover, the working range for the immunoaffinity columns obtained from Vicam have been reported in the Vicam *OchraTest Instruction Manual* and the Vicam *ZearalaTest Instruction Manual* (*38*, *45*). Information about the highest concentrations possible to load to Ochraprep and Easi-Extract zearalenone immunoaffinity columns was obtained from Rhône-Diagnostics Technologies (personal communication, 2000).

Additionally, the susceptibility of the ochratoxin A and zearalenone methods to variation in pH was tested using the Ochraprep and Easi-Extract zearalenone immunoaffinity columns at pH values of 5, 5.5, 6, 6.5, 7, 7.5, 8, and 9. The spiked sample without cereal matrix was used at concentration levels of 2.5 and 25 μg/kg of ochratoxin A and zearalenone, respectively.

Confirmation. The confirmatory method for ochratoxin A was the method presented by Langseth et al. (*46*), with some modifications in the postcolumn pH shift system. The performance of postcolumn pH shift confirmation for ochratoxin A was studied by analyzing spiked samples prepared with ASPEC for validation studies a second time. To shift the pH of the mobile phase after the analytical column, a 1.1 M ammonium hydroxide solution was pumped with an external HPLC pump at a flow rate of 0.3 mL/min to a mixing T-piece. The pulse in the flow, generated by the external HPLC pump itself, was compensated by pumping the solution via 5 m of a coiled PEEK tubing (0.13 mm i.d.). The eluent from the analytical column was directed to the mixing T-piece, where the ammonium hydroxide solution and the eluent were mixed. Thereafter, the mixture flowed through a reaction coil to the fluorescence detector. The PTFE tubing reaction coil was 1.5 m × 0.5 mm i.d. The analytical column, the mobile phase, the flow rate of the mobile phase, and the injection volume were the same as described above. The excitation wavelength was changed to 375 nm and the emission wavelength to 436 nm as described by Langseth et al. (*46*).

The confirmatory method for zearalenone was the method described in the study of Visconti and Pascale (*30*). The performance of the confirmation method was studied by reanalyzing the spiked samples prepared with ASPEC for validation studies. Confirmation of zearalenone in the samples was performed with the chromatographic procedure described above, using different wavelengths (excitation wavelength of 236 nm and emission wavelength of 440 nm) and comparing the peak response ratios with those of standards and spiked samples.

Use of a Vacuum Manifold and ASPEC. The difficulty in the use of the vacuum manifold was due to the fact that the vacuum was not stable during the sample purification step and created variable flow rates in various immunoaffinity columns. The instability of the vacuum was due to the tap water vacuum system used, which had been installed in the system of water pipes, where the water flow varied with the number of taps used in the laboratory. It is also possible that differences in the columns could be the reason of the variation in flow rates. Unsteady flow rates have been associated with poor recoveries (*26*). In the ASPEC system the flow rate of each sample was similar.

Some cereal extracts diluted with water for zearalenone analysis were cloudy, which caused blockage of the immunoaffinity column during the sample loading step with the vacuum manifold. Some cloudiness was also observed when PBS buffer was used, but for the analysis of ochratoxin A the extracts were clear. The blockage or malfunctioning of the immunoaffinity columns has also been reported by other studies (*28*, *30*). Due to the problems in the functioning of the immunoaffinity columns obtained from Vicam, the columns manufactured by Rhône-Diagnostics were used with ASPEC. When the immunoaffinity columns from Rhône-Diagnostics were applied, blocking was not observed, which was assumed to be due to the wide internal diameter of the column and the constant flow rate produced by ASPEC.

After sample purification with ASPEC for zearalenone analysis, it was observed that the dry sample residue did not completely dissolve in the mobile phase. In the chromatogram, the adjacent interfering peak was not separated from the peak of zearalenone and the baseline was drifting, indicating the presence of insoluble matter in the sample (Figure 2). This was observed in both of the spiked wheat samples and in samples prepared only with the extraction solvent but without cereal matrix. The same was also observed when water and a mixture of PBS buffer/acetonitrile (95:5) were tested for the washing step as recommended by Delaunay et al. (*47*). Additionally, the Vicam immunoaffinity columns were tested in ASPEC instead of the Rhônee-Diagnostics columns, but no improvement was observed in the baseline or peak shapes in the chromatograms. When methanol was used as a solvent agent, peak separation and clean chromatograms were observed.

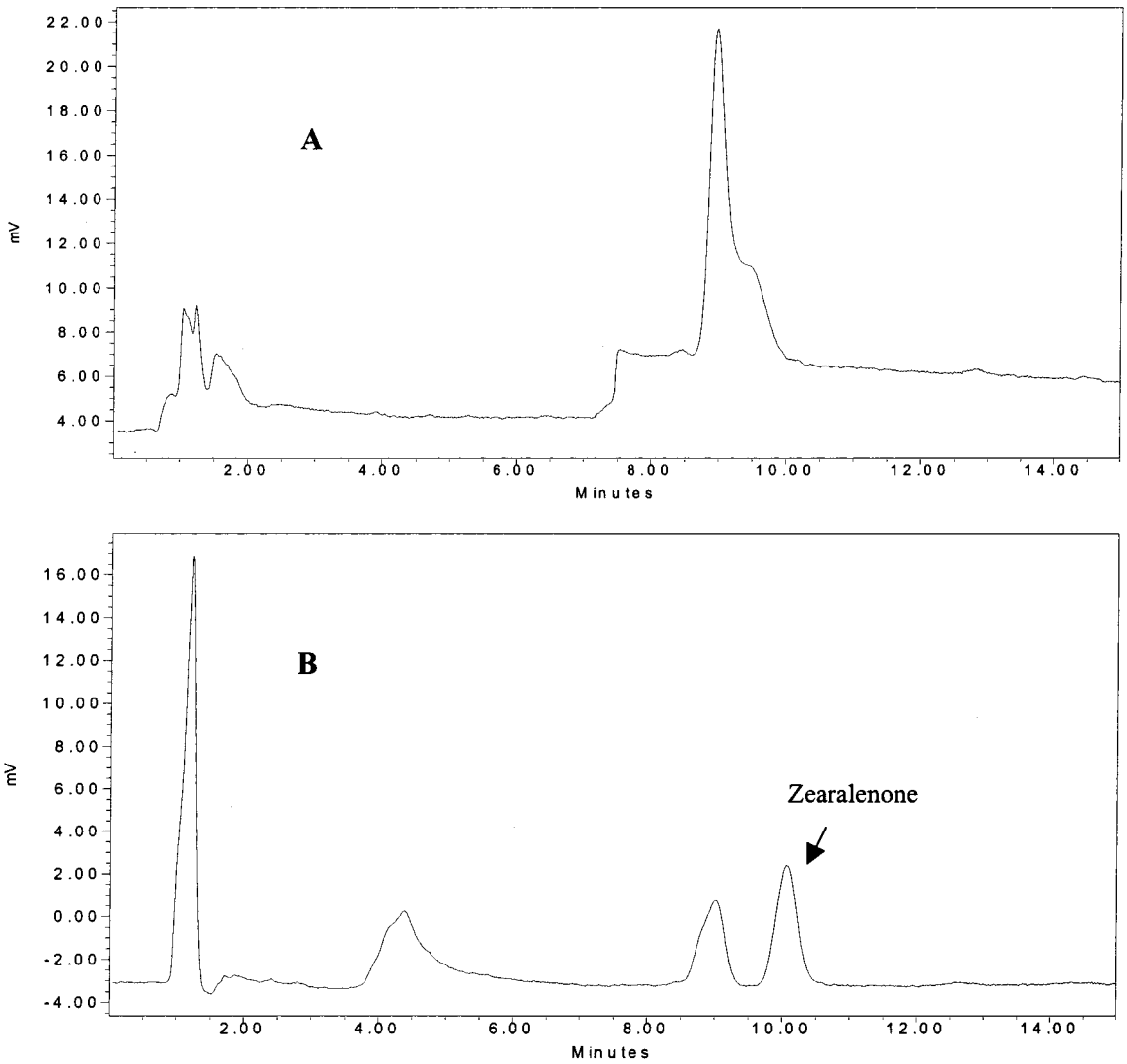

Figure 2. Chromatograms of wheat sample spiked with zearalenone at a concentration level of 25 μg/kg and purified with ASPEC. The dry sample residue was dissolved in the mobile phase in chromatogram A and in methanol in chromatogram B.

However, when a vacuum manifold was applied for sample cleanup, the adjacent interfering peak did separate from the peak of zearalenone when the dry residue was dissolved with the mobile phase. It was assumed that the purification process in ASPEC differed in some way from that of the vacuum manifold, which caused the difference in solubility of the dry residue. The main difference was assumed to be the flow rates of the two different purification methods. When ASPEC was used, the flow rate was usually lower than in a vacuum manifold, but in the latter the steadiness of the flow rate was difficult to control. It is also possible that the origin of the insoluble matter was the immunosorbent matrix of the column itself.

RESULTS AND DISCUSSION

Validation of the Ochratoxin A Method. In the analytical method including the sample purification with the vacuum manifold the LOD was 0.1 μg/kg and LOQ 0.2 μg/kg for all of the various cereal species analyzed. The recoveries of ochratoxin A for different cereals varied from 82 to 101% and from 87 to 95% at the concentration levels of 0.6 and 2.5 μg/kg, respectively (Table 1). The RSDs of the repeatability within day for various cereals were 3−12 and 2−4% at the spiking levels of 0.6 and 2.5 μg/kg, respectively (Table 1). The RSDs for the recoveries were the same as for the repeatability. The mean of the concentration of ochratoxin A in the certified reference material (CRM 472) was 7.3 μg/kg and the RSD 18% ($n = 6$) measured between days.

For the analytical method for ochratoxin A comprising the sample purification step with ASPEC the LOD and LOQ were same as for the method including the sample cleanup with a vacuum manifold. For various cereals at the concentration level of 0.6 μg/kg the recoveries of ochratoxin A varied from 68 to 106% and the RSD for repeatability within day from 4 to 15% (Table 1). At the higher concentration level of 2.5 μg/kg of ochratoxin A, the recoveries of various cereals varied from 85 to 95% and the RSD for repeatability within day from 3 to 9% (Table 1). The RSDs for the recoveries were the same as for the repeatability. The mean of the concentration of ochratoxin A for the four samples of the certified reference material was 8.0 μg/kg and the RSD 8% measured between days.

In both methods used to analyze ochratoxin A the LOD was satisfactorily low and in agreement with the previous studies of Vrabcheva et al. (*48*) and Sharman et al. (*26*). The results obtained from the validation indicate that the recoveries of ochratoxin A were significantly lower at the concentration level of 0.6 μg/kg in the method including ASPEC than in the one including the vacuum manifold, but at a concentration of 2.5 μg/kg no significant difference was observed (two-sample t test, $p < 0.05$, Statistix for Windows, version 2.0). The RSD for the

Table 1. Mean Recovery and Repeatability Expressed as Relative Standard Deviation (RSD) for the Ochratoxin A Analysis in Cereals Performed by Employing the Vacuum Manifold System and the ASPEC System for Sample Purification ($n = 6$ in Each Test)

	analytical method with a vacuum manifold				analytical method with ASPEC			
	spiking level = 0.6 μg/kg		spiking level = 2.5 μg/kg		spiking level = 0.6 μg/kg		spiking level = 2.5 μg/kg	
cereal	recovery (%)	repeatability (RSD%)	recovery (%)	repeatability (RSD%)	recovery (%)	repeatability (RSD%)	recovery (%)	repeatability (RSD%)
wheat	82	10	87	4	68	11	85	3
rye	96	6	95	2	82	15	95	9
barley	85	3	88	3	87	4	85	4
oats	101	12	88	3	106	6	93	6

Table 2. Mean Recovery and Repeatability Expressed as Relative Standard Deviation (RSD) for the Zearalenone Analysis in Cereals Performed by Employing the Vacuum Manifold System and the ASPEC System for Sample Purification ($n = 6$ in Each Test)

	analytical method with a vacuum manifold				analytical method with ASPEC			
	spiking level = 9 μg/kg		spiking level = 25 μg/kg		spiking level = 9 μg/kg		spiking level = 25 μg/kg	
cereal	recovery (%)	repeatability (RSD%)	recovery (%)	repeatability (RSD%)	recovery (%)	repeatability (RSD%)	recovery (%)	repeatability (RSD%)
wheat	95	8	102	5	101	2	104	2
rye	108	4	117	3	88	19	87	5
barley	102	3	112	8	98	7	78	4
oats	96	5	100	5	89	10	87	2

repeatability was slightly higher for rye samples when using ASPEC for purification instead of the vacuum manifold at both spiking levels. Additionally, significant differences between the recoveries of the various cereals at both spiking levels with both purification methods were observed (two-way ANOVA, $p < 0.05$, Statistix for Windows, version 2.0). The recoveries and repeatability of the method including the vacuum manifold were in agreement with previous studies of Vrabcheva et al. (*48*), Bisson et al. (*35*), Scudamore and MacDonald (*28*), and Trucksess et al. (*16*), whereas the validation results of the automated method were in agreement with the study of Sharman et al. (*26*). The certified value with uncertainty for ochratoxin A in wheat (CRM 472) is assigned to be 8.2 ± 1.0 μg/kg (*37*). When the vacuum manifold was applied for sample purification, the mean concentration of ochratoxin A in certified reference material was low, but it was within the range of uncertainty. The RSD was high compared with the repeatability within day, which was due to the measurements performed between days. When the ASPEC was applied, the mean concentration of ochratoxin A in certified reference material was acceptable and the RSD was relatively low.

Validation of the Zearalenone Method. The LOD was 1.5 μg/kg and the LOQ 3 μg/kg for all of the different cereal species when the vacuum manifold was employed for the sample cleanup. The recovery of zearalenone for different cereals varied from 95 to 108% at the concentration level of 9 μg/kg and from 100 to 117% at the concentration level of 25 μg/kg when the vacuum manifold was applied (Table 2). The RSDs for repeatability within day of different cereals varied from 3 to 8% at both concentration levels investigated (Table 2). The RSDs for the recoveries were similar as for the repeatability.

For the method including ASPEC, the LOD was 3 μg/kg and the LOQ 6 μg/kg for various cereals. The recovery of zearalenone for different cereal species varied from 88 to 101% and from 78 to 104% at the concentration levels of 9 and 25 μg/kg, respectively (Table 2). When ASPEC was used, the RSD of the repeatability within day for zearalenone varied from 2 to 19% at the concentration level of 9 μg/kg and from 2 to 5% at the concentration level of 25 μg/kg (Table 2). The RSDs for the recoveries were the same as for the repeatability. When ASPEC was used, the measured concentration of zearalenone in the test material (FAPAS) was 84 μg/kg and the RSD was 18% ($n = 6$) determined between days.

The LOD for the analytical methods of zearalenone including the vacuum manifold as well as the ASPEC was adequately low. However, when the ASPEC system was employed for sample cleanup, the LOD was higher than when the vacuum manifold was used. Nevertheless, the LOD for both methods was in agreement with the earlier investigations of Krska (*29*), Schuhmacher et al. (*49*), and Visconti and Pascale (*30*). The recoveries for zearalenone were significantly lower, especially at the higher spiking level, when ASPEC was employed in comparison with the conventional vacuum manifold (two-sample *t* test, $p < 0.05$, Statistix for Windows, version 2.0). However, no difference was observed between the repeatabilities of the two procedures used for sample cleanup at the higher level. At the lower spiking level of zearalenone the RSD of the repeatability of the method within day was higher when ASPEC was used than when the vacuum manifold was used. No significant difference was observed in the recoveries of various cereals at the lower concentration level when ASPEC was used, but at the higher level and when the vacuum manifold was employed, a significant difference was observed (two-way ANOVA, $p < 0.05$, Statistix for Windows, version 2.0). The recoveries and repeatabilities of the methods were in agreement with earlier studies of Krska (*29*), Schuhmacher et al. (*49*), and Visconti and Pascale (*30*). According to the report of FAPAS (*39*) the measured concentration of zearalenone in the test material ranged between 73.8 and 159.5 μg/kg and the mean concentration was 112.0 μg/kg. The concentration measured in this study was in that range of values, although it was low in comparison with the mean concentration reported by FAPAS (*39*). The RSD for the concentration of zearalenone was higher in comparison with the repeatability within day at the higher spiking level, which was due to the measurements carried out between days.

Both methods of sample purification with the immunoaffinity columns for ochratoxin A and zearalenone were easy to learn and to employ. Using ASPEC saved significant time in manual work, and it was possible to prepare a total of 28 samples per day, including blank, spiked, and parallel samples. The use of ASPEC overnight remarkably enhanced the efficiency of the sample preparation. By using a vacuum manifold 8−10 samples

were prepared in a working day. Furthermore, when ASPEC was applied, it was possible to avoid some of the health risks caused by both the mycotoxins and the solvents used. In several studies, mycotoxins have been analyzed by using ASPEC online with HPLC (*26*, *33*, *34*, *36*), but in the present study this was not done, because it was considered that coupling of HPLC with ASPEC did not allow the effective use of HPLC for other chemical analyses.

Susceptibility of Ochratoxin A and Zearalenone Purification Methods to pH. The susceptibility of the ochratoxin A and zearalenone methods to variation of pH was tested using Ochraprep and Easi-Extract zearalenone immunoaffinity columns. ASPEC was applied for sample cleanup. The height of the peak was measured for every sample. The RSD for the height of the zearalenone peak was 4%, and no effect of pH on the columns was observed. However, for the Ochraprep column clearly lower responses (18% lower) were observed with pH values of 5 and 5.5 than with pH values of 6−9. The RSD for the height of the ochratoxin A peak was 3% in the pH range of 6−9. This study demonstrated that the pH of the sample was a very important factor for ochratoxin A analysis. Stevenson (*50*) stated that the sample should be applied to the immunoaffinity column at a pH close to neutral, typically in the pH range 5−8. Furthermore, Marley et al. (*51*) stated that the presence of acid in the sample extract may be harmful to the antibody in the immunoaffinity column.

Confirmation Methods for Ochratoxin A and Zearalenone. The postcolumn confirmatory method for ochratoxin A performed by shifting the pH in mobile phase caused a 2-fold increase in the response of the ochratoxin A peak in comparison with the response of the ochratoxin A peak in the chromatogram obtained with the normal analytical method. At the same time some of the responses of the other coeluting peaks observed in the chromatogram decreased. This was especially observed with the oat samples confirmed. Langseth et al. (*46*) reported that by using postcolumn pH shift the intensity of the ochratoxin A signal increased 75% and that cleaner chromatograms were obtained. Zimmerli and Dick (*52*) reported a ~6-fold increase of the response for ochratoxin A after the addition of an ammonium hydroxide solution to the eluate, as well as a reduction in coeluting peaks. The confirmation method for zearalenone was also reliable. The ratio of peak response obtained by detecting zearalenone with the excitation wavelengths of 236 and 274 nm was used as described in the study of Visconti and Pascale (*30*). The peak response ratios obtained from the spiked samples were compared with those of standards. The peak response ratio was ~1 (range = 0.9−1.3). Both confirmatory methods were reliable and appropriate to use as qualitative tests.

The present study demonstrated that the analytical methods for ochratoxin A and zearalenone including the immunoaffinity columns for sample purification were adequately reliable, and low detection limits were obtained. The use of ASPEC both facilitated and increased the effectiveness of sample preparation. Furthermore, the confirmatory methods for ochratoxin A and zearalenone were reliable and easy to perform.

SAFETY

Ochratoxin A is a nephrotoxic, teratogenic, and carcinogenic substance, and zearalenone is known to cause reproductive and infertility problems in animals. Therefore, care should be taken in handling samples contaminated with these toxins and associated standards.

ABBREVIATIONS USED

ASPEC, automated solid phase extraction; CRM, certified reference material; FAPAS, food analysis performance assessment; RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantification.

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