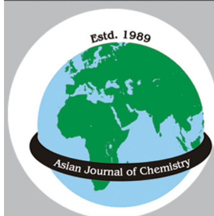

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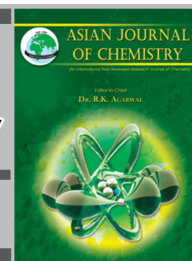

QuEChERS Extraction and HPLC-FLD Determination of Ochratoxin A in Cereals and Cereal Products

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A new method for the determination and analysis of ochratoxin A (OTA) in cereals and cereal products, based on the use of the QuEChERS (quick, easy, cheap, effective, rugged and safe) procedure, enhanced with a high performance liquid chromatography-coupled to a fluorescence detector has been developed. Chromatographic separation was performed within 4 min using a reversed-phase C_{18} 15 cm column at 30 ºC and an isocratic mobile phase composed of 6 % acetic acid in water/acetonitrile 25:75 (%, v/v). The optimal extraction mixture of ochratoxin A compound from cereal extracts was determined by mixture and response surface methodology. Extraction with 20:70:10 (%, v/v) water/acetonitrile/acetic acid offered good validation parameters with a recovery exceeding 85 %, coupled with limits of detection and limits of quantification within the legal limits as set by the European Union.

Key Words: HPLC-FLD, QuEChERS, Mycotoxins, Ochratoxin A, RSM.

INTRODUCTION

Mycotoxins are secondary metabolites produced from molds particularly *Aspergillus*, *Penicillium* and *Fusarium*. There are more than 300 varieties of mycotoxins known today and are classified into different groups such as aflatoxin, ochratoxin and trichothecenes. Plant products such as crops, foods and feeds are widely susceptible to contamination by mycotoxin whenever these foods are kept under adverse conditions of temperature and humidity during drying, transport and storage of foodstuff[1]. The primary source of ochratoxin contamination in food and feedstuff is cereal commodities (maize, oats, barley and wheat) in addition to groundnuts, dried fruits and coffee beans, which have been infected by the *P. verrucosum* and *A. ochraceus*.

The International Agency for Research on Cancer (IARC) has classified ochratoxin A (OTA) as a group 2B carcinogen, as it has been shown to be nephrotoxic, hepatotoxic, immunosuppressive, teratogenic and carcinogenic effect on animal and human[2]. Because of its toxicity and frequent occurrence, several countries have established legal regulations or recommendations to control mycotoxin contamination of various food products[3]. The European Commission has published Commission Regulation (EC) No. 1881/2006 setting maximum limits for ochratoxin A in the unprocessed cereals is 5 μg/kg and, in the case of unprocessed cereal products is 3 μg/kg[4].

However, the analytical determination of ochratoxin A in food matrix is a difficult task, because of the complexity of the matrix and the low concentrations of ochratoxin A are usually present. Therefore, use of appropriate extraction and clean-up procedures in addition to sensitive, reliable and an accurate quantification method for the determination of ochratoxin A in foods are necessary. Various methods have been developed for the determination of ochratoxin A in food and feed commmunity as screening methods including enzyme linked immunosorbent assay (ELISA)[5] and fluorometer or as quantitative methods including high performance liquid chromato-graphy with fluorescence detection (HPLC-FLD)[5,6] and liquid chromatography with tandem mass spectrometry (LC-MS/MS)[7]. To reduce sample handling and toxic waste, consequently to maximize recovery of the analytes and to attain quick sample turnaround time, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure was adopted. This procedure is based on the extraction, liquid-liquid partition and dispersive-solid phase clean-up procedures. QuEChERS has been mainly used for the extraction of different classes of pesticides. To our knowledge, there is no study referring to using QuEChERS in determination of ochratoxin A in foods. The aim of this study was to develop and validate a simple and rapid HPLC-FLD method for the determination of ochratoxin A in cereals at trace levels.

EXPERIMENTAL

Ochratoxin A analytical standard was purchased from Sigma-Aldrich (Malaysia). A working solution was prepared

in acetonitrile and stored at -20 ºC in amber glass vials over a period of 2 months. The external standard solutions used for the calibration curve for the HPLC experiments were prepared by further dilution of the working solution with the mobile phase. HPLC-grade acetonitrile and methanol used for the mobile phase were purchased from Merck (Darmstadt, Germany), whereas analytical grade acetonitrile and methanol used for extraction were purchased from Fischer Scientific (Leicestershire, UK). HPLC-grade glacial acetic acid was purchased from Fischer Scientific (Leicestershire, UK). Water was purified by reverse osmosis followed by an electrodeionization (EDI) system (Maxima Ultra Pure Water, England). The 0.45 µm disposable membrane filters were purchased from Cronus Filter (UK). Anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) were purchased from Agilent Technologies (USA).

HPLC analysis: The HPLC analysis were carried out with a Shimadzu LC-20AT system (Kyoto, Japan) consisting of degasser, tertiary pump, auto sampler, column oven and a fluorescence detector. The chromatographic separation was performed with a C_{18} 150 mm × 4.6 mm Spherisorb 5 ODS-1 (particle size 5 µm) chromatographic column and was purchased from Phenomenex (USA). The cereals extracts were analyzed isocratically using 6 % acetic acid in water and acetonitrile 25:75 (%, v/v) mixture as the mobile phase. The column was kept in a column oven at 30 ºC at a flow rate of 1.0 mL/min. The injection volume was maintained at 10 µL for both the sample and standard solutions.

Food samples: In December 2010, 1-2 kg each of 25 samples of cereals and cereal products (wheat, wheat based noodles, rice, rice based noodles and corn) were randomly obtained from groceries and stores in Kuala Lumpur, Malaysia. The samples were stored in the dark at room temperature (20-25 ºC). The samples were ground and mixed at room temperature for 10 min until a fine and homogeneous powdered material was obtained. The powdered samples were then stored in plastic bags at 4 ºC in a refrigerator prior to analysis.

Sample preparation: Cereal samples were prepared similar to the previous published aflatoxin QuEChERS method with some modification[8].

Step I: A thoroughly homogenized cereal sample (1 g) was weighed in a polypropylene centrifuge tube (15 mL).

Sample recovery was done with (1 g) of the clean wheat, wheat products (wheat-based noodles), rice, rice products (rice-based noodles) and corn samples spiked with 10, 20 and 40 µg/kg of the ochratoxin A standards. The spiked samples were left overnight in the dark at room temperature to allow the solvent to evaporate and for ochratoxin A absorption into the matrix. Subsequently, they were extracted via the following steps (II to IV).

Step II: 3.0 mL of 20:70:10 (%, v/v) water/ acetonitrile/ acetic acid mixture was added and the centrifuge tube was shaken for 1 min to ensure that the solvent has mixed thoroughly with the entire sample, for complete extraction of the analyte.

Step III: 0.8 g of anhydrous $MgSO_4$ and 0.2 g of NaCl were added into the mixture and the shaking procedure was repeated for 1 min to facilitate the extraction and partitioning of the ochratoxin A into the organic layer.

Step IV: The extract was centrifuged for 5 min at 4000 rpm and 0.5 mL of the upper organic layer was filtered through a 0.45 µm nylon syringe filter prior to HPLC analysis.

RESULTS AND DISCUSSION

Optimization of analytical conditions: To give best separation in the chromatograms and to enhance sensitivity, the column type and its length, the mobile phase composition and excitation/emission wavelength were investigated in this study. The heart of a chromatographic system is the column, where the actual separation of the analyte mixture occurs. Column selection depends strongly on prior knowledge of the samples to be analyzed, as well as the matrix. For example, non-polar and moderately polar compounds require conventional C_{18} column while polar compounds requires strong retention power column. Since ochratoxin A is a non-polar compound, thus a conventional C_{18} has been applied in this study. Another criterion that was studied is the column length. Compared to the 25-cm column, the total run time was shorter in the case of the 15-cm column. This reduces the mobile phase consumption and analysis time required for routine work.

Different combinations of water/acetonitrile/acetic acid such as (73:25:2, 48:50:2, 23:75:2 and 13:85:2) were tested as the mobile phase in order to optimize the resolution of the ochratoxin A standard peak and to enhance the sensitivity. Broad peaks and long retention times were produced using the first and second mobile phase systems with high water composition. In contrast, ochratoxin A was not separated from the front peaks by the last mobile phase composition (13:85:2). The third mobile phase offered an adequate separation between the ochratoxin A and front peaks within 4 min retention times. The column temperature effect was also studied by varying the temperature from 20-50 ºC with the objective of increasing the resolution. Furthermore, the ochratoxin A standard that was spiked into the blank wheat sample was applied to ensure that the ochratoxin A mix standards could be distinguished and separated from interfering substances in the sample matrix. The chromatograms demonstrating the selectivity of the procedure are shown in Fig. 1. From the chromatograms of the blank wheat sample that was spiked with 10 µg/kg of ochratoxin A, it is evident that the peak of ochratoxin A is well-separated from interfering substances in the sample matrix with short retention times were also obtained. The selectivity of the isocratic mixture of water/ acetonitrile/acetic acid (23:75:2) at 30 ºC column temperature is considered satisfactory as it enables ochratoxin A quantification in the analyzed food commodities with higher selectivity and sensitivity within a reasonable run time (Fig. 1). The repeatability and reproducibility of the retention times as measured by their relative standard deviation (RSD) range is 0.02 and 0.13 %, respectively.

For the fluorescence detection, a spectrum of ochratoxin A standard solution in the HPLC mobile phase was tested to optimize the detection of ochratoxin A and to obtain the best fluorescence signals in terms of signal-to-noise ratio and sensitivity. Various emission/excitation wavelengths were monitored and compared to obtain the best values in order to enhance the detection for ochratoxin A, as shown in Fig. 2.

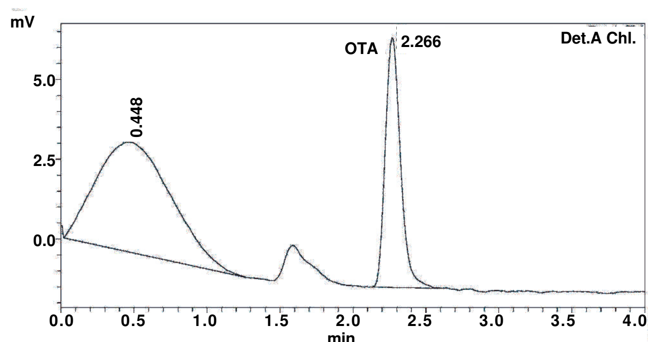

Fig. 1. Representative HPLC chromatogram of blank wheat spiked with ochratoxin A (10 μg/kg each)

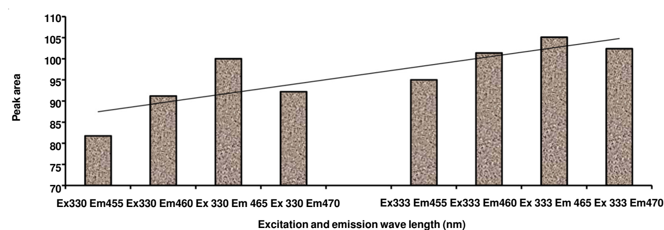

Fig. 2. Effect of excitation and emission wavelength (nm) on the sensitivity of ochratoxin A

When the emission wavelength of ochratoxin A was set at 465 nm, the best fluorescence signals in terms of signal-to-noise ratio and sensitivity were obtained for ochratoxin A. However, by setting the excitation wavelength at 333 nm, strong fluorescence signals were obtained for ochratoxin A. Therefore, the 333 nm excitation/465 nm emission wavelength combination was selected for all the subsequent experiments. The above-mentioned modifications resulted in improved sensitivity.

Sample pretreatment optimization: The sample pretreatment has a crucial impact on the accuracy of the results. The extraction solvent, the type and amount of drying agents, the extraction time and the solvent sample ratio affecting the efficiency of extraction, therefore, they were taken into consideration and optimized. The extraction solvent was found to be the most important factor that heavily affects the extraction efficiency. The optimal extraction mixture of ochratoxin A compound from cereal extracts determined by mixture and response surface methodology (RSM) and analyzed using JMP® 9.0.0 software[9].

From a review of the literature, the use of different volume proportions of water, acetonitrile and acetic acid was frequently employed as the solvent mixture to extract ochratoxin A[6,10,11]. Therefore, the design of experiment (DOE) was applied to find out which proportion of these three solvents (water, acetonitrile and acetic acid) maximizes recovery of the ochratoxin A in the cereal extracts. ABCD design with nine experimental points was performed randomly at all points. Experimental data were fitted to a quadratic polynomial model. The prediction profilers provided in the response surface are shown in Figs. 3 and 4.

Figs. 3 and 4 indicated that high extraction efficiency (100 % recovery) of selected analyte was achieved when using 43.4:65.6 (%, v/v) water/acetonitrile and 20:70:10 (%, v/v) water/acetonitrile/acetic acid. The first one was avoided, since high amounts of water; anhydrous $MgSO_4$ tend to form lumps

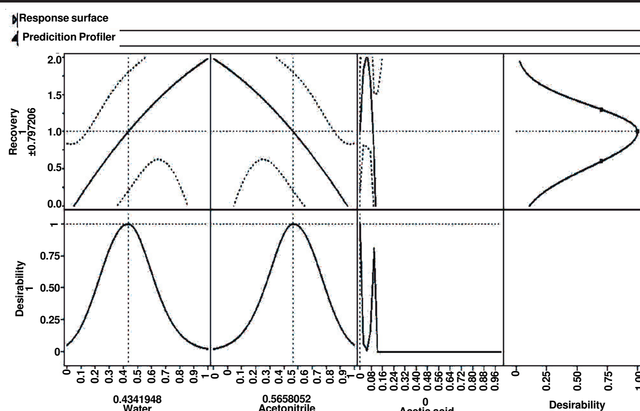

Fig. 3. Profiler in maximum desirability in profiler for mixture analysis, displays optimal settings (rounded) of 0.43 for water, 0.57 for acetonitrile and 0.0 for acetic acid, which give an estimated recovery of 1 (100 %)

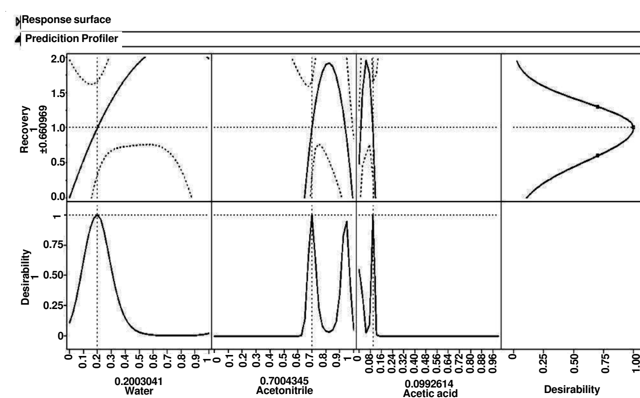

Fig. 4. Profiler in maximum desirability in profiler for mixture analysis, displays optimal settings (rounded) of 0.20 for water, 0.70 for acetonitrile and 0.10 for acetic acid, which give an estimated recovery of 1 (100 %)

that can harden rapidly and reduce the supernatant layer. Therefore, mixtures of 20:70:10 (%, v/v) water/acetonitrile/acetic acid were applied in this study, since it gave recoveries fulfilling the European Union Commission Directive 2006/401/EC for analysis of mycotoxins in food samples.

Method validation: This was an in-house validated method, in terms of linearity, accuracy, intra-day precision, inter-day precision, limit of detection (LOD) and limit of quantification (LOQ). The linearity was tested in triplicate using a standard solution of the ochratoxin A in the concentration range of 3.75 and 120 μg/L. Good linear relationships with correlation coefficients of 0.992 for targeted analyte was obtained. Calibration with standard solutions was used for quantification by the least square method and means comparison was made by ANOVA test ($p < 0.05$).

The accuracy was tested by the determination of the recoveries of the ochratoxin A in clean wheat, wheat products (wheat-based noodles), rice, rice products (rice-based noodles) and corn samples spiked with 20, 40 and 100 μg/kg of the ochratoxin A standards and analyzed in triplicates (Table-1). The recoveries obtained ranged from 85.2 ± 1.2-109.8 ± 2.9 %, with a relative standard deviation (RSD) of less than 12 %. The sensitivity was determined by estimating the limit of detection (LOD) and limit of quantification (LOQ). LODs and LOQs were estimated experimentally as the lowest concen-

TABLE-1
RECOVERY, LOD, LOQ, INTRA- AND INTER-DAY PRECISION OF OCHRATOXIN A

Matrix	Recovery[a]			LOD(µg/kg)	LOQ (µg/kg)	Intra-day precision (n = 5)(10 µg/kg)	Inter-day precision (n = 15)(10 µg/kg)
	20 (µg/kg)	40 (µg/kg)	100 (µg/kg)				
Wheat	99.6±0.4	90.8±5.6	85.2±1.3	0.41	1.37	1.2	2.5
Wheatproducts	109.3±3.3	102.1±3.4	96.5±2.5	0.62	2.07	1.7	2.4
Rice	109.8±2.9	104.1±2.2	98.2±2.4	0.62	2.08	0.8	3.4
Rice products	106.6±2.8	100.6±3.4	86.4±1.7	0.18	0.60	0.4	4.6
Corn	100.0±1.6	98.0±6.0	86.4±1.0	0.47	1.57	1.0	2.1

[a] Recovery ±RSD (%) (n = 5).

tration giving a response of three- and ten-times, respectively, the base-line noise. The LOQ were from 0.60-2.08 µg/kg (Table-1).

Intra-day precision was evaluated by assaying five replicates of the same sample at a spiked level of 10 µg/L ochratoxin A on the same day. For the inter-day precision, five replicates of the same sample at a spiked level of 10 µg/L ochratoxin A were analyzed on three consecutive days. The intra-day precision and inter-day precision were calculated and tabulated in Table-1. The intra-day precision (n = 5) are between 0.8 and 1.7 %, while the inter-day variation (n = 15) values are between 2.4 and 4.6 %. These values determined are much lower than the acceptable maximum, confirming the good reproducibility and repeatability of this technique. Considering the data for method validation, the current HPLC-FLD method and sample preparation procedures employed can be regarded as selective, precise and robust.

Conclusion

The method proposed for ochratoxin A determination in cereal is based on a QuEChERS followed by HPLC-FLD analysis. This study shows that the coupling of simple, rapid and low-cost QuEChERS and HPLC-FLD methodology with a mixture methodology and response surface methodology can result in a powerful tool for solving complex sample matrices and the very low levels of ochratoxin A found in food. Chromatographic separation was within 4 min to allow the rapid determination as well as to reduce the consumption of mobile phase. Sample preparation was carried out in less than 10 min, without employing a prior dSPE clean up. The recovery of the ochratoxin A was over 85 %, limits of detection and limits of quantification are within the legal limits set by the European Union. Finally, the entire QuEChERS HPLC-FLD method appears suitable for application to other types of mycotoxins in different food matrices and, avoids the use of the expensive immunoaffinity columns and the LCMS method.

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