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Optimization and validation of a method for extraction and quantification of ochratoxin A in black pepper

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Abstract

The extraction method for the determination of ochratoxin A (OTA) in black pepper was optimized. The influence of three variables, i.e., type of solvent, solvent-volume-to-sample-size ratio (v/w) and amount of sodium chloride (NaCl) (g), on OTA recovery was evaluated. Analysis of variance was used to compare recovery values obtained from different solvents, and response surface methodology (RSM) was used to determine the optimum amount of NaCl and the solvent-volume-to-sample-size ratio. The concentration of OTA was determined by high-performance liquid chromatography with fluorescence detection. The highest recovery (95.2 %) was obtained when methanol/water (80:20, v/v) was used as the solvent. The RSM results showed that the experimental data could be adequately fitted to a second-order polynomial model with multiple regression coefficients (R^2) of 0.962. The optimum amount of NaCl was determined to be 3 g, whereas the optimum solvent-volume-to-sample-size ratio (v/w) was found to be 4. The proposed method was applied to 20 samples, and the presence of OTA was found in 8 (40%) samples ranging from 0.11 to 3.16 ng g^{-1}.

Introduction

Ochratoxin A (OTA), a phenylalanine derivative of a substituted isocoumarin, is a secondary metabolite mainly produced by some species of *Aspergillus* and *Penicillium*, particularly *A. ochraceus* and *P. verrucosum*, respectively (Sibanda *et al*., 2002). OTA is nephrotoxic, cytotoxic, carcinogenic and teratogenic (Valenta *et al*., 1993) and has been implicated in Balkan nephropathy, a disease characterized by severe kidney damage. It may also induce gene mutation, although the mechanism of genotoxicity is not clear. OTA is listed as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC) (Rahmani *et al.*, 2009; IARC, 1993). There are numerous reports from many countries in the world describing high frequencies of human blood contamination by OTA, although at low levels (Valenta *et al.,* 1993). OTA contamination in humans originates from contaminated foods. Some food products such as cereal, vegetables, spices, fruits and nuts may contain OTA in different levels (Lobeau *et al*., 2005). Spices are a potent source of mold contamination in the food to which they are added. Black pepper is one of the most common spices used for food as a flavoring agent and can be a source of OTA contamination in humans because it is usually consumed raw or added to ready-to-eat food. There are reports on the occurrence of mycotoxins in pepper, owing to climate conditions of high humidity and high temperature in the countries where it is produced and inadequate transport or storage conditions (Cho *et al*., 2008).

The importance of OTA in human health has impelled many countries to set maximum residue levels (MRLs) of OTA in different foods. The European commission (EC, 2002) has established an acceptable level for raw cereal grains (5 ng g^{-1}), cereal products (3 ng g^{-1}) and dried vine fruit (10 ng g^{-1}) and is discussing about maximum limits of OTA in spices, green and roasted coffee and coffee products, wine, beer, grape juice, and cocoa and cocoa products (EC, 2001). In general, OTA can be determined by two different types of methods: immunologic and chromatographic. Immunochemical assays are based on the ELISA (enzyme-linked immuno-sorbent assay) principle, which has been used for the determination of OTA in chili (Thirumala *et al.*, 2000). The chromatographic method includes thin-layer chromatography (TLC) and liquid chromatography (LC), which involves a multiple-step process including extraction, clean-up, separation and detection by a proper detector. High-performance liquid chromatography (HPLC) with a

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fluorescence detector (FD) is more commonly used due to the strong native fluorescence activity of OTA and the high sensitivity of this method. Common extraction methods are based on the solubility of this mycotoxin in organic solvents. A suitable solvent can increase extraction efficiency. The selection of solvent depends on the type of mycotoxin and chemical specification of sample matrix. Pure water (Hancock *et al.*, 2002), methanol/water (Domijan *et al*., 2005), acetonitrile/water (Matos *et al.*, 2006), chloroform/ phosphoric acid (Guillamont *et al*., 2005; Pelegri *et al.*, 1997), dichloromethane (Curtui and Gareis, 2001) and phosphate buffer saline (PBS) (Lino *et al.*, 2005) have been used for OTA extraction from different food samples. However, methanol/water is the most popular system for OTA extraction from different commodities, including cereals and cereal products (Araguas *et al*., 2005), dry beans (Domijan *et al.*, 2005), raisins, green coffee beans (Sugita-Konishi *et al.*, 2006), roasted coffee (Sibanda *et al*., 2002), dried vine fruits (Lombaert *et al*., 2004) and black pepper (Abdulkadar *et al*., 2004; Czerwiecki *et al*., 2005).

However, a solvent system that provides a good recovery with one matrix may not be suitable or is less efficient with another sample matrix. For example, methanol/water (80/20) or a solution of 1% $NaHCO_3$ appeared to be the most useful extraction solvent for the determination of OTA in coriander, ginger, paprika and black pepper depending on the type of matrix. However, in the case of cloves, none of the extraction methods was appropriate (Czerwiecki *et al*., 2005). Apart from the type of solvent, the ratio of the solvent volume to that of the sample is also an important factor in the determination of mycotoxin in food samples. Whitaker (Whitaker *et al*., 1984) reported that the amount of aflatoxin B_1 and B_2 extraction from peanut was a function of both methanol concentration and solvent-to-peanut ratio.

Sodium chloride (NaCl) is usually added to aqueous sample solutions to improve the extraction of mycotoxin and seems to play a role in extraction efficiency. Several researchers have used different types of solvent and dissimilar ranges of solvent-volume-to-sample-size ratios and amounts of NaCl for OTA determination. More studies are needed to investigate the effect of these variables on OTA recovery in different foods or other samples. In such situations where multiple variables may influence the output, response surface methodology (RSM) can be applied. RSM is a particular set of mathematical and statistical methods proper for designing experiments, building models, evaluating the effects of variables and searching optimum conditions of variables to predict targeted responses. The main advantage of RSM is that it allows for the evaluation of the effect of multiple variables and their interactions on the output variables with a reduced number of trials (Sai-Nan *et al*., 2009).

In general, OTA detection in black pepper is somewhat difficult because black pepper contains a large amount of pigments and other natural substances. The objective of the present study was to optimize an accurate method for the determination of OTA in black pepper by HPLC-FD. The study was performed by comparing different solvents and determining the optimum amount of NaCl (g) and the ratio of solvent volume to sample size (v/w) to obtain the highest extraction of OTA from black pepper. The optimized method was then applied to 20 black pepper samples. Despite the solubility of OTA in halogenated solvents, we did not investigate this type of organic compound because the replacement of halogenated solvents has been recommended in view of environmental safety considerations and occupational hazard problems (Monaci *et al*., 2004).

Materials and Method

Chemicals

A standard stock solution (50 μg ml^{-1}) of OTA in benzene/acetic acid (99/1, v/v) was purchased from Supelco (Bellefonte, PA, USA). Making a working standard solution (1.0 μg ml^{-1}) of OTA required a volume of stock solution evaporated under nitrogen steam and dissolved in methanol/water (50/50, v/v). This solution was stored in an amber vial at -20ºC because OTA gradually breaks down under UV light. This working standard was used for spiking and preparing a standard curve. An OTA calibration standard curve for HPLC determination was prepared by diluting a required amount of working standard in the methanol/water (50/50, v/v) to obtain final concentration of 0.05, 0.1, 0.5, 1, 2, 5 or 10 ng ml^{-1}. Methanol and acetonitrile for liquid chromatography, HPLC grade acetic acid, sodium hydrogen carbonate and sodium chloride for analysis were purchased from Merck (Darmstadt, Germany). A glass microfiber filter (11 cm, 934-AH) and fluted filter paper (24 cm) were prepared by Whatman (Maidstone, Kent, UK). PBS and immunoaffinity columns for OTA were purchased from Vicam (Watertown, USA). All of the used glassware was decontaminated with a sodium hypochlorite solution and rinsed with distilled water, followed by pure methanol to remove mycotoxin residues.

Apparatus and chromatographic conditions

A blender (Waring, Torrington, USA), centrifuge (Sartorius AG, Goettingen, Germany) and a water purifier (Elga, Marlow, Buckinghamshire, UK) were used in the study. The HPLC apparatus (Waters 600, Milford, MA, USA) consisted of a Waters 600 controller high-performance liquid chromatographic system equipped with a waters 600E pump, Waters 717 auto-sampler and Waters in-line degasser AF. A multi-wavelength fluorescence detector (Waters 2475) operating at an excitation wavelength of 333 nm and an emission wavelength of 460 nm was applied. The system was controlled by the Empower PDA software from Waters. The chromatographic conditions used were similar to those employed by Solfrizzo *et al*. (1998) in OTA determination in cereals in which the mobile phase was acetonitrile/ water/acetic acid (49.5:49.5:1.0, v/v/v), and the flow rate was 1 ml min^{-1}. OTA was separated on a reversed-phase C18 (5 µm, 25 cm, and 0.46 cm) Purospher star column from Merck (Darmstadt, Germany). The aqueous phase was filtered through a 0.45-µm nylon membrane filter (Whatman, Maidstone, Kent, UK). A 50 µl volume of extract was injected into the HPLC.

Sampling

A total of 30 black pepper seed and powder samples were prepared (from commercially available sizes of 50-100 gr) to obtain about 1.0 kg of each sample. All the samples were purchased from supermarkets located in the city of Kuala Lumpur, Putra Jaya and selected Selangor state of Malaysia and transported to the laboratory under ambient conditions. All of the information about the samples was obtained from the labels. The whole seed samples were ground and used for analysis. Two hundred gram samples were then taken after sampling using quartering techniques and used for the analysis. All the samples were analyzed as soon as possible after purchasing, otherwise kept in polyethylene bag and they were stored at −20◦C until used for analysis.

Method validation

For linearity, six-point (0.1, 0.5, 2, 5, 10, and 20 ng ml^{-1}) calibration curves were separately constructed for OTA. With regard to the accuracy of the method applied, clean black pepper powder samples were spiked with 4 different levels of OTA at concentrations of 0.5, 2, 5 or 10 ng g^{-1}; then, recovery and standard deviation (SD), intraday and inter-day repeatability (RSDr and RSDR respectively) were calculated (n=5).

Experimental design

The effect of using different solvents, i.e., pure water, pure methanol and mixture of methanol/ water, PBS/water and acetonitrile/water (each of the mixtures in three different ratios (40/60, 60/40 and 80/20, v/v)), was investigated on OTA extraction by determining the OTA recovery. In the second step, central-composite experimental design (CCD) was applied to evaluate the effect of two independent variables x1 (solvent volume to sample size ratio (v/w)) and x2 (amount of NaCl (g)) on the response variable Y (OTA recovery). A set of 13 experiments was performed to study the main and combined effect of these independent variables on the response. The two independent variables were set at five levels, the solvent-volume-to-sample-size ratio (v/w) ranged from 1 to 7 and the amount of NaCl ranged from 0 to 5 g. The center point was repeated 5 times. The matrix of CCD, including the values corresponding to the levels of each factor and treatment, is shown in Table 1. The spiking level was set at 10 ng g^{-1} for 25 g of black pepper.

Table1. Central Composite Design of the independent variables along with the values for response variable determined using methanol/ water (80/20, v/v) and HPLC-FD

Standard order	Solvent/ sample ratio	NaCl (g)	Recovery (%)	
			Experimental	Predicted
1	4.0	2.5	101.5	99.6
2	4.0	2.5	99.4	99.6
3	4.0	2.5	96.8	99.6
4	4.0	2.5	105.3	99.6
5	4.0	5.0	95.2	87.2
6	6.1	0.7	63.6	65.7
7	7.0	2.5	56.8	52.9
8	1.0	2.5	30.3	24.2
9	1.9	4.3	47.0	54.9
10	4.0	2.5	94.8	99.6
11	1.9	0.7	40.1	43.8
12	6.1	4.3	67.4	73.7
13	4.0	0.0	75.7	73.7

Statistical analysis

The one-way analysis of variances (ANOVA) and Tukey test were applied to determine statistical comparison between the different solvents used in the study. A value of $p<0.05$ indicated significance differences. Regression analysis and ANOVA were used to determine the regression coefficients, the statistical significance of the model terms and the fitness of the mathematical model to experimental data from central-composite design as well as to determine an overall optimum level of OTA recovery. To achieve the best model, a linear and quadratic polynomial and the interaction of the independent variables were studied. The behavior of the response

surface was investigated for the response function (Y_i) using the regression polynomial equation. The generalized polynomial proposed for predicting the response variable is given as

$$Y_i = a0 + a1x1 + a2\,x\,2 + a11\,x^2 1 + a22\,x^2 2 + a12\,x\,1\,x\,2$$

where, Y_i is the response variable, *a0* is an offset term, *a1* and *a2* are the regression coefficients for the linear effect terms, *a11* and *a22* for quadratic effect terms and *a12* is for interaction effects. The terms *x1* and *x2* are independent variables.

The ANOVA results presented the effect and regression coefficients of individual linear, quadratic and interaction terms that were determined. The significance of the equation parameters for each response variable was also assessed by the F-ratio at a probability (p) of 0.05. The adequacy of the models was determined using model analysis, lack-of-fit test and coefficient of determination (R^2). The statistically non-significant ($p > 0.05$) effects were dropped from the initial models. The experimental design matrix, data analysis and optimization procedure were performed using the Minitab v.14 statistical package (Minitab Inc., PA, USA).

Sample extraction and clean-up

The samples were analyzed for OTA with HPLC–FD. One hundred milliliters of methanol/water (80/20, v/v) was added to 25 g of ground sample containing 2.5 g of sodium chloride. The mixture was blended for 3 min. The extract was filtered by gravity through Whatman fluted filter paper and then centrifuged at 4000 rpm for 10 min at 4ºC. Ten milliliters of the filtrate was diluted with 40 ml of PBS and filtered once more through a glass microfiber filter 934-AH Whatman; 10 ml of this filtrate was passed through an immunoaffinity column OchratestTM containing immobilized monoclonal antibodies against OTA. After washing the column with 10 ml of PBS and 10 ml of water, OTA was eluted with 1.5 ml of methanol (1 drop s^{-1}). The elute was then evaporated to dryness under dry nitrogen stream and dissolved in 500 µl methanol/water (50:50, v/v), in which 50 µl was injected into the HPLC.

OTA confirmation

Confirmation of OTA was carried out using methyl ester formation. The method described by Zimmerli & Dick (1995) was used. Briefly, 2.5 ml methanol and 0.1 ml concentrated HCl were added to 200 µl of the extract. The solution was left standing overnight at room temperature. Then, the methanol was evaporated through a nitrogen stream, and the residue was dissolved in 200 µl methanol/formic acid 0.1 M (70/30, v/v). The HPLC analysis used was the same as that of OTA determination.

Results and Discussion

The linearity in the working standard solutions at three determinations of six concentration levels and two replications was good, as shown by r squared (r^2), which was 0.9996. To optimize the method, different solvents that are common in OTA determination were used to extract the samples. The lowest recovery value, 15.2± 2.3 %, was produced when pure water was used, and the highest recovery value, 95.2± 3.2 %, was produced when methanol/water (80/20, v/v) was used (Table 2).

Table 2. Mean recovery values and standard deviations for solvents used in the Ochratoxin determination by HPLC-FD

Solvent	V / V (%)	Recovery (%)	S.D[e]
Methanol/Water	80:20	95.2[a]	3.2
Methanol /PBS	80:20	94.0[a]	4.3
Acetonitrile/Water	80:20	90.3[a]	3.4
Pure Methanol	-	77.8[b]	6.1
Methanol/Water	60:40	72.8[b]	6.9
Methanol /PBS	60:40	71.6[b]	4.2
Acetonitrile/Water	60:40	56.5[c]	8.4
Methanol /PBS	40:60	53.6[c]	3.8
Methanol/Water	40:60	42.2[c]	4.1
Acetonitrile/Water	40:60	41.3[c]	6.5
Pure Water	-	15.2 [d]	2.3

[a, b, c, d] Significant differences.
[e] Standard deviation

Results obtained from ANOVA testing indicated that there were not significant differences ($p > 0.05$) between recoveries obtained for methanol/water, methanol/PBS and acetonitrile/water at 80/20 (v/v) ratio, which were 95.2 ±3.2%, 94.0±4.3% and 90.3± 4.3%, respectively. This phenomenon may be due to the enhanced solubility of methanol, which can separate OTA from the matrix. Aqueous solvent may penetrate hydrophilic tissues and lead to the most efficient extraction as compared to the non-aqueous solvents (Van Egmond & Paulsch, 1986). Moreover, it has been reported that the solubility of OTA in methanol is high, and methanol is one of the most potent adsorbents; therefore, pure methanol may elute OTA from IAC column (Juan, *et al.*, 2007). However, our results show that the recovery values for pure methanol, methanol/water and methanol/PBS (both at 60:40, v/v) were more than 70%, i.e., 77.8±6.1%, 72.8±6.9% and 71.6±4.2%, respectively; these values are within the limit set in the EC Regulation 401/ 2006 (EC, 2006). Juan *et al* (2005), reported that

the best recoveries (102%) were obtained with PBS/ methanol (50:50) for the determination of OTA in maize bread samples. This difference may be related to the effect of the matrix.

In the second part of this study, to investigate the effect of solvent volume to sample size ratio and amount of NaCl on the extraction efficiency, methanol/water (80:20, v/v) was selected as the solvent. There was no significant difference ($p > 0.05$) between methanol/ water, methanol/PBS and acetonitrile/water at a concentration of 80:20 (v/v). The RSM allows us to study the main effect, interaction and quadratic effect of the solvent-to-sample ratio as well as the amount of NaCl on the recovery of OTA.

Model fitting

By applying multiple regression analysis on the application data, the following second-order polynomial equation was found to explain the recovery of OTA from black pepper in the ranges used in present study.

$Y_i = -52 + 59\ x1 + 17\ x2 - 6.8x1^2 - 2.8\ x2^2$ (Y_i is recovery, *x1* is solvent volume to sample size ratio (v/w) and *x2* is amount of NaCl (g)).

The significance of each coefficient as determined by Student's t-test and the resulting p-value is shown in Table 3.

Table 3. Estimated Regression Coefficients for recovery of OTA using methanol/ water (80/20, v/v) and HPLC-FD

Term	Coef [a]	SE Coef [b]	T- value	p- value
Constant	-55.99	13.5994	-4.117	0.005[c]
x_1	59.53	5.0988	11.676	0.000[c]
x_2	18.81	5.5088	3.415	0.012[c]
x_1^2	-6.78	0.5578	-12.151	0.000[c]
x_2^2	-3.06	0.8032	-3.804	0.007[c]
$x_1 x_2$	-0.21	0.8827	-0.234	0.827[d]

$R^2 = 96.2\%$
[a] Coefficient.
[b] Standard error coefficient.
[c] Significant ($p < 0.05$).
[d] Not significant ($p > 0.05$).
x1, solvent volume to sample size ratio (v/w).
x2, amount of NaCl (g).

The quadratic and linear terms showed that the solvent-volume-to-sample-size ratio ($x1^2$ & $x1$) has the largest effect on OTA recovery. However, the linear and quadratic terms of the amount of NaCl ($x2$, and $x2^2$) show smaller effects. The interaction terms did not have significant influence ($p > 0.05$), which indicates that the interaction between these two factors did not influence the response. Between these two independent variables, the solvent-to-sample ratio played a more dominant role in the extent of OTA recovery in black pepper. Analysis of variance (ANOVA) showed that the resultant quadratic polynomial models adequately represent the experimental data as the coefficient of multiple determinations (R2) for the response of recovery value was 0.962. This result indicates that the fitted model could explain 96.2% of the total variability within the range of values studied. The analysis of the variance (Table 4) showed a high F-value, while the p-value was less than 0.05. The p-value for lack-of-fit testing was 0.082, which implies that the experimental data obtained fit the model well and explained the effect of both the solvent-to-sample ratio and the amount of NaCl on OTA recovery in black pepper.

Table 4. Analysis of Variance (ANOVA) for recovery (%) of OTA using methanol/ water (80/20, v/v) and HPLC-FD by central composite design

source	DF [a]	Seq SS [b]	Adj SS [c]	Adj MS [d]	F- value	p- value
Regression	5	7703.70	7703.70	1540.74	35.16	0.000[e]
Linear	2	1010.91	6006.22	3003.11	68.52	0.009[e]
Square	2	6690.39	6690.39	3345.19	76.33	0.000[e]
interaction	1	2.40	2.40	2.40	0.05	0.822[f]
Residual error	7	206.78	306.78	43.83		
Lack-of-fit	3	239.76	239.76	79.92	4.77	0.083[f]
Pure error	4	67.01	67.01	16.75		
Total	12	8010.48				

[a] Degree of freedom;
[b] sequential sums of squares;
[c] adjusted sums of squares;
[d] adjusted mean squares
[e] Significant ($p < 0.05$).
[f] Not significant ($p > 0.05$).

Optimization of the process

The three-dimensional response surface contour plot, the graphical representation of the regression equation, was drawn to illustrate the effect of the independent variables on the recovery of OTA (Fig. 1).

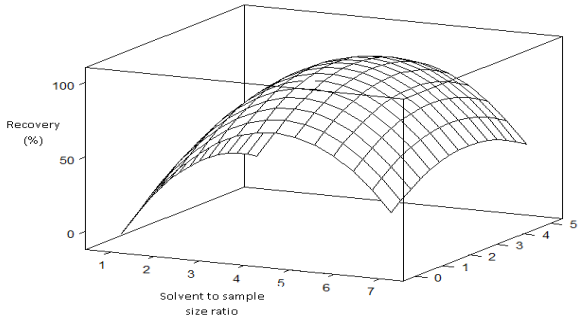

Figure 1. Response surface plot showing the effect of solvent volume to sample size ratio *(x1)* and amount of NaCl *(x2)* on OTA recovery in black pepper

The response surface shows the effect of the solvent-volume-to-sample-size ratio and amount of NaCl on OTA recovery. A quadratic effect for both variables was observed, although the solvent-to-sample ratio was shown to have a greater influence on the response. Increasing the solvent-volume-to-sample-size ratio from 1 to 4 (v/w) resulted in an increase in the OTA recovery; however, by increasing the ratio to more than 4, the OTA recovery started to decrease. Our results were similar to those obtained by Juan *et al.* (2005), which compared OTA recovery by using 50, 75 or 100 ml of solvent for 25 g of sample. They obtained the highest recovery using 100 ml of solvent, which had a solvent-to-sample ratio value of 4. Our study also showed that by increasing the amount of NaCl to 3 g, the recovery increased; however, no change in significance was observed. The results obtained from numerical optimization showed that the solvent-volume-to-sample-size ratio of 4 and 3 g of NaCl provided the highest OTA recovery.

To verify the validity of the final model, the overall optimal condition, including a solvent-volume-to-sample-size ratio (v/w) of 4 and 3 g of NaCl of, leading to the maximum overall OTA recovered, was predicted. Methanol/water (80:20, v/v) was used as extraction solvent. A mean value of 96 ± 2.3 (n = 5) obtained from real experiments demonstrated the validation of the RSM model. The good correlation between these results confirmed that the response model was adequate for reflecting the expected optimization.

Method validation

To assess the method's specificity, three reagents and three blank matrices of black pepper were prepared and injected into the HPLC. No appreciable signal at the retention time of OTA was observed. Figure 2 shows the HPLC chromatograms of the OTA standard and spiked sample. The LOD and LOQ were 0.02 and 0.06 ng g^{-1}, respectively. The accuracy for the optimized method was determined by calculating the mean recovery values for each spike level (Table 5). The recovery values oscillated between 102% and 96% for spike levels of 0.5 and 5 ng g^{-1}, respectively. Our results showed a good recovery as compared to other reports. Fazekas *et al*. (2005) reported OTA recovery of 78% from black pepper at a concentration of 10 mg kg^{-1} in three parallel experiments (Fazekas *et al*., 2005). Abdulkadar *et al*. (2004) have reported that, in research for the determination of OTA in food products including spices, the average recovery value was 80%. In a related study, OTA was determined in various spices: coriander, cloves, ginger, paprika

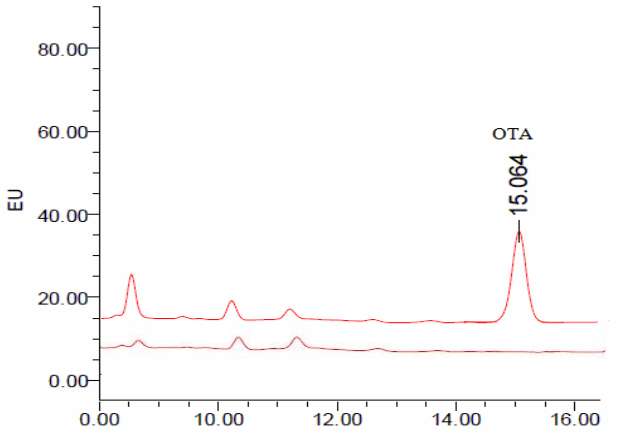

Figure 2. HPLC chromatogram of OTA: (a) spiked sample at 5 ng ml^{-1}; (b) clean matrix of black pepper; Chromatographic conditions: C18 column (5 µm; 250 × 4.6 mm); mobile phase, acetonitrile/water/ acetic acid (49.5: 49.5: 1, v/v/v); flow rate 1ml min^{-1}; fluorescence detector (λex = 333 nm and λem = 460 nm)

Table 5. Accuracy and intra-assay (n = 5) and inter-assay (n = 5) validation results obtained by method extraction consisted of methanol/water (80:20 v/v), solvent to sample size ratio = 4 and 3 g NaCl and by using HPLC-FD

Fortification (ng/ g)	Recovery (%)	RSD_r[a] intraday (%)	RSD_R[b] interday (%)
0.5	102.0	7.5	11.2
2	99.3	5.8	10.7
5	98.6	4.9	8.6
10	96.0	2.4	6.3

[a], intraday relative standard deviation
[b], inter day relative standard deviation

and black pepper. The mean recovery of the method, dependent on the types of sample, was 61-82% (Czerwiecki & Wilczynska, 2005). Ghali *et al.* (2008) obtained recovery between 88 and 91% for food samples including spices; however, there was no report on the recovery in black pepper.

The precision was calculated through the determination of inter-day and intra-day relative standard deviation for repeatability (RSD), (n = 5). The results showed that RSDr was between 7.5% and 2.4%, whereas the RSDR was between 11.2% and 6.3% for 0.5 and 10 ng g^{-1} fortification levels, respectively (Table 5). The values obtained for recovery, RSDr and RSDR, of the optimized method was within the limit set in the European Commission Directive for Method of Analysis of OTA in food stuffs (EC, 2006).

Application to real samples

The optimized method of OTA extraction of black pepper and IAC as a clean-up method, followed by HPLC-FD, was used for the determination of OTA in 20 black pepper samples. A summary of the results

obtained is shown in Table 6. Eight out of 20 samples (40%) were contaminated. The OTA concentration of the positive samples ranged from 0.11 to 3.16 ng g-1. The OTA mean level was 1.5± 0.95 ng g^{-1}. In a related study in Malaysia, the concentration of OTA in 120 commercial peppers was determined. A total of 57 samples (47.5%) were contaminated with OTA ranging from 0.15 to 13.58 ng/g (Jalili *et al*., 2010).

Table 6. A summaries of results obtained from OTA determination in black pepper samples

No. of analysed samples	20
No. of positive samples	8
Frequency (%)	40
Range (ng/ g)	nd[a] – 3.16
Mean ± SD (ng/ g)	1.5 ± 0.95

[a], Not detected

A maximum limit of 10 ng g^{-1} for OTA in spices has been proposed based on data provided (EC, 2001). Malaysia has set a maximum mycotoxin concentration of 5 ng g^{-1} in foods, including spices. All of the samples in the present study were contaminated at a level less than 5 ng g^{-1} (Table 6). There are few reports available for the concentration of OTA in peppers. Some researchers reported different levels of OTA contamination in spices, including peppers, and some of them did not detect OTA in the samples. In Belgium, 43 spice samples were examined for OTA, and the results showed that all of the black pepper samples (n = 6) did not contain OTA (Goryacheva *et al.*, 2006). In another survey of 126 samples obtained from retail shops in India, 14 out of 26 black pepper samples were found to exceed 10 ng g^{-1} of OTA (in the range of 15 - 69 ng g^{-1}), 20 out of 50 coriander samples were in the range of 10 - 51 ng g^{-1}, 2 out of 25 ginger samples were in the range of 23 - 80 ng g^{-1} and 9 out of 25 turmeric samples were in the range of 11-102 ng g^{-1} (Thirumala-Devi *et al*., 2001).

Conclusion

Compared to other solvents, methanol/water (80:20, v/v) showed the highest OTA recovery. The solvent-volume-to-sample-size ratio and the amount of NaCl were successfully optimized using RSM, and a satisfactory prediction equation was obtained. Between these two factors, the sample-to-solvent ratio played a more dominant role in OTA extraction. This optimized analytical methodology provides good results in terms of accuracy, repeatability, intermediate precision and sensitivity, and has shown to be reliable for the determination of OTA in black pepper. The application of the procedure to 20 samples demonstrated that 40% were contaminated, although none of the samples exceeded the set limit.

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