Analysis of Ochratoxin A in Malt Beverage Samples using Dispersive Liquid–Liquid Microextraction Coupled with Liquid Chromatography-Fluorescence Detection

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Abstract

Maham M., Kiarostami V., Waqif-Husain S., Karami-Osboo R., Mirabolfathy M. (2013): **Analysis of ochratoxin A in malt beverage samples using dispersive liquid–liquid microextraction coupled with liquid chromatography-fluorescence detection**. Czech J. Food Sci., **31**: 520–525.

A simple and economic procedure based on dispersive liquid–liquid microextraction has been applied to extract and pre-concentrate trace levels of ochratoxin A (OTA) in malt beverage prior to analysis using high performance liquid chromatography with fluorescence detection. The method was based on the formation of fine droplets of a water-immiscible extraction solvent in the sample solution using a water-miscible disperser solvent. The influences of various parameters such as the type and volume of extraction and disperser solvents, centrifuging time, sonication time, and salt concentration on the extraction efficiency of ochratoxin A were investigated. Under optimum conditions, the relative standard deviations for five replicates of 2 ng/ml of OTA were 3.4% as within-day and 6.2% as between-day precisions. The detection limit (S/N = 3) was 0.1 ng/ml and the mean recoveries of OTA from malt beverage samples at spiking levels of 0.5, 2, and 4 ng/ml were in the range of 104–108.2%.

Keywords: mycotoxin; beverage; microextraction; HPLC

Mycotoxins are toxic secondary metabolites of several mold species frequently found in a variety of agricultural and food products and beverages. Ochratoxin A (OTA), mainly produced by *Aspergillus carbonarius* and *Penicillium verrucosum*, is one of the most widespread and hazardous mycotoxins (El Khoury & Atoui 2010; Santini *et al.* 2011). OTA is a hepatotoxic, immunosuppressive, nephrotoxic, teratogenic, nephrocarcinogenic mycotoxin and has carcinogenic effects on humans (Group 2B) (IARC 1993; Pfohl-Leszkowicz & Manderville 2007).

Barley, also called malting barley, is the main raw material used in malt production. Steeping, germination and kilning are the three main steps of the malting process. Contamination of malt by OTA can occur or increase in these steps (Gareis 2001). The presence of OTA has been determined in barley, malt and beer samples (Gumus *et al.* 2004; Běláková *et al.* 2011; Mateo *et al.* 2011). One of the important applications of malt is in the manufacture of beverages. Fungal infection of barley (particularly during post-harvest stage) affects the quality of malt used in malt beverage

industry and the amount of OTA in beverages. The European Commission has enacted maximum limits for OTA level as 2 ng/ml for beer (European Commission 2010).

Immunoaffinity column clean up is, due to its selectivity, the most common pretreatment procedure used for the analysis of OTA in different samples (Cicoňová *et al.* 2010; Fabiani *et al.* 2010; Kabak 2012). However, immunoaffinity columns cannot be reused (according to the manufacturer's instructions) and are expensive. Dispersive liquid–liquid microextraction (DLLME), introduced in 2006, is a simple, inexpensive, efficient, and eco-friendly method (Rezaee *et al.* 2006). In DLLME methodology, an appropriate mixture of extraction and disperser solvents is injected into the aqueous sample containing the analytes and a cloudy solution (high turbulence) forms. In this step, the target analytes are rapidly transferred into fine droplets of the extraction solvent as a result of the enhanced surface area between two immiscible phases. The cloudy state is then centrifuged and the enriched droplets of extractant precipitate at the bottom of the conical test tube. The determination of the analytes can be performed by an appropriate analytical technique. DLLME has been widely used for the analysis of organic compounds (Melo *et al.* 2012; Zacharis *et al.* 2012; Karami-Osboo *et al.* 2013; Maham *et al.* 2013) and metal ions (Alexovič *et al.* 2012; Kocot *et al.* 2012).

The aim of this work is the development of a simple, cheap, and fast method based on DLLME for the analysis of OTA in malt beverage samples, which can be used in routine laboratories.

MATERIAL AND METHODS

Reagents and materials. The OTA standard was obtained from Sigma Aldrich Chemical Co. (St. Louis, USA). The stock solution (1 µg/ml) was prepared by dissolving the appropriate amount of OTA in methanol. Deionised water was prepared using a Milli-Q purification system (Millipore, Bedford, USA). Acetonitrile was of HPLC grade. Chloroform, carbon tetrachloride, carbon disulfide, ethanol, methanol, acetone, phosphoric acid, and sodium chloride were of analytical grade and were all purchased from Merck Chemical Co. (Darmstadt, Germany). The malt beverage samples were of commercial type.

Sample preparation. The malt beverage samples were kept in their original bottles or containers in the refrigerator (4°C) throughout the analysis and were used within a few days. The cool sample was thoroughly degassed in an ultrasonic bath for 45 minutes. Five ml of the degassed sample was subjected to DLLME analysis.

Instrumentation. The HPLC system consisted of auto samplers (Waters 717), a binary HPLC pump (Waters 1525), and a Multi λ fluorescence detector (Waters 2487). Excitation λ was set at 330 nm and emission λ at 460 nm. A chromolith RP 18 HPLC column (100 mm × 4.6 mm *i.d.*) from Merck (Darmstadt, Germany) was used for the separation at 30°C. The acetonitrile–water (40:60, v/v) mixture (pH = 3) using phosphoric acid as a modifier was used as the mobile phase at a flow rate of 1 ml/minute.

DLLME procedure. Five ml of spiked sample was transferred to a 15 ml screw cap glass test tube with conic bottom. A mixture of 0.4 ml acetone and 150 µl chloroform (optimum conditions in this study) was added quickly into the sample via a 1 ml syringe. The cloudy state formed in the test tube and the solution was vortexed for a few seconds. After centrifugation at 4500 rpm for 6 min, the dispersed fine droplets of chloroform precipitated along with whitish matrix at the bottom of the test tube. The upper aqueous solution was removed with a syringe and the precipitate was dissolved in 200 µl of acetone and then evaporated to dryness in another test tube. Finally, the residue was reconstituted in 1000 µl mobile phase and filtered through a 0.45 µm membrane before HPLC analysis.

RESULTS AND DISCUSSION

In order to obtain the best experimental conditions for the quantitative extraction of OTA via DLLME, the important parameters were investigated with the malt beverage samples spiked with 2 ng/ml of OTA. The effects of various variables on the extraction process were studied based on the results of the one factor experiment at a time.

Selection of extraction solvent

The first step in optimisation was to select a suitable extraction solvent. The extractant in DLLME should be heavier than water, sparsely

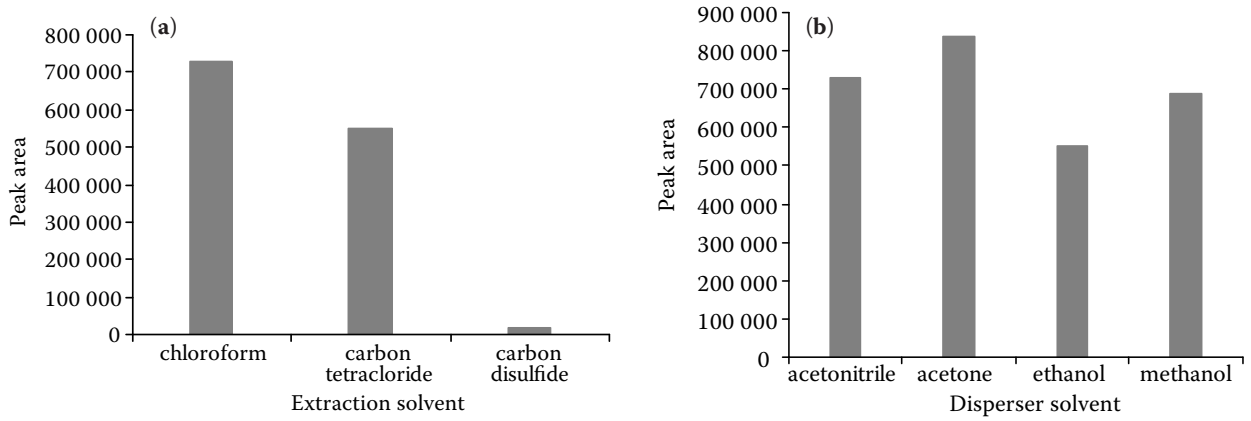

Figure 1. Effect of type of the (**a**) extraction solvent and (**b**) the disperser solvent on extraction efficiency

water soluble and highly capable of the target analyte extraction. The suitability of chloroform, carbon tetrachloride, and carbon disulfide, all of which had these properties, was tested by adding a mixture of 0.8 ml acetonitrile containing 200 µl of each solvent into the sample solution. The results (Figure 1a) showed that the highest extraction efficiency was obtained with chloroform. Thus, chloroform was chosen as the extraction solvent for all subsequent experiments.

Selection of disperser solvent

The disperser solvent must be miscible with both aqueous and organic phases. Therefore, methanol, acetonitrile, ethanol, and acetone were investigated for this purpose. Aqueous samples were extracted using 0.8 ml of each disperser solvent containing 200 µl of chloroform. Based on the results obtained (Figure 1b), the highest fluorescence intensity was obtained with acetone as the disperser solvent. This was attributed to the good miscibility of acetone with chloroform and the tested sample compared with other disperser solvents. Therefore, all further studies were carried out using acetone.

Influence of extraction solvent volume

The influence of the volume of the extraction solvent on the analytical signals was investigated by rapid injections of solutions containing the fixed volume of acetone (0.8 ml) and different volumes of chloroform. As shown in Figure 2a, the extraction efficiency increased by increasing the volume of chloroform to 150 µl and then decreased by further increasing its volume. Therefore, 150 µl of chloroform was used for further optimisation studies.

Influence of disperser solvent volume

The volume of acetone as the disperser solvent should also be optimised. To examine the effect of the acetone volume on the recovery, different volumes of acetone containing 150 µl of chloroform were separately added into 5 ml malt beverage

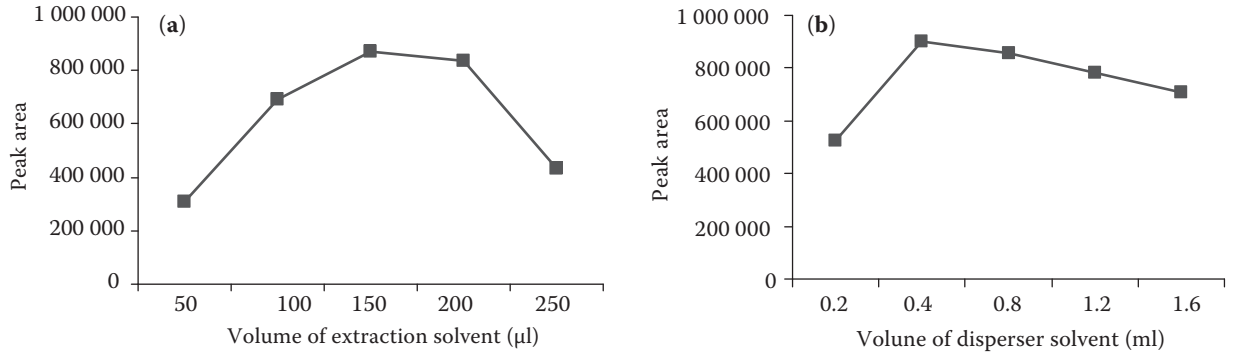

Figure 2. Effect of volume of the (a) extraction solvent and (b) the disperser solvent on on extraction efficiency

samples. The variation of the recovery efficiency versus the disperser solvent (acetone) volume is shown in Figure 2b. The decrease in the performance volumes below 0.4 ml was attributed to the ineffective formation of small droplets of the extraction solvent. On the other hand, at disperser solvent volumes above 0.4 ml, the solubility of the OTA gradually increased in the aqueous sample, which caused lowering the analyte partition with extractant droplets and decreased the extraction efficiency. Based on the obtained results, 0.4 ml acetone was selected as the optimal disperser solvent volume.

Influence of centrifugation time

The centrifugation time is significant in the separation of the organic phase from the aqueous phase. In order to obtain the best separation efficiency, the centrifugation time was evaluated in the range of 1–10 min at 4500 rpm. Based on the obtained results (Figure 3), the extraction efficiency increases by increasing the centrifugation time from 1 min to 6 min and it remains slightly constant by increasing the centrifugation time from 6 to 10 minutes. According to the obtained results, the centrifugation time of 6 min was chosen as the optimum time in subsequent experiments.

Influence of sonication time

Sometimes sonication will make the extraction solvent disperse better into the aqueous solution and thus faster mass transfer can occur between the two immiscible phases. In this study, the influence of sonication time on the extraction efficiency was investigated over the range of 0–7.5 min (0, 2.5, 5, and 7.5 min). The comparison of the obtained fluorescence intensity from the samples extracted (under optimal conditions) with and without ultrasonic radiation did not show any significant difference between them. This was possibly due to the effective dispersion of the extraction solvent by using the disperser solvent in the studied samples. Therefore, in the presence of the disperser solvent, there is no need for ultrasonic radiation, and for ease of the operation, other investigations were performed by using only acetone as the disperser agent.

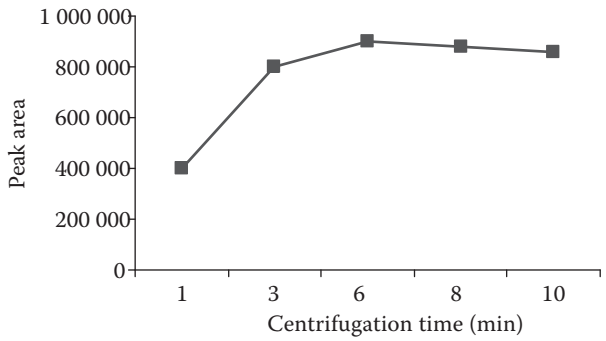

Figure 3. Effect of centrifugation time on extraction efficiency

Influence of salt addition

The influence of salt addition on the extraction efficiency of 5.0 ml spiked malt beverage samples was investigated by adding various amounts of NaCl (0–10%, w/v), while other conditions were kept constant. The obtained results established that the salt addition did not have any considerable influence on the extraction efficiency of OTA. Thus, no salt was added in further experiments.

Quantitative analysis

The analytical characteristics of the DLLME method including the calibration curve, repeatability, limits of detection and quantitation were investigated under the optimised conditions. Calibration curve was prepared for the target analyte after the extraction of a standard series of spiked fresh malt beverage samples with the regression equation being $y = 359473x + 95854$ and the determination coefficient of 0.999. The precision of the proposed method was calculated by five replicated extractions and analysis of spiked sam-

Table 1. Relative recoveries of ochratoxin A (OTA) in beverage samples[a]

Mycotoxin	Concentration (ng/l)			Relative recovery (%)
	initial	added	determined[b]	
OTA	nd	0.5	0.52 ± 0.06	104.0
		2.0	2.13 ± 0.03	106.5
		4.0	4.33 ± 0.04	108.2

[a]extraction conditions: extraction solvent and its volume: 150 µl chloroform, disperser solvent and its volume 0.4 ml acetone, no salt addition; [b]mean ± SD; SD – standard deviation ($n = 3$), nd – not detected

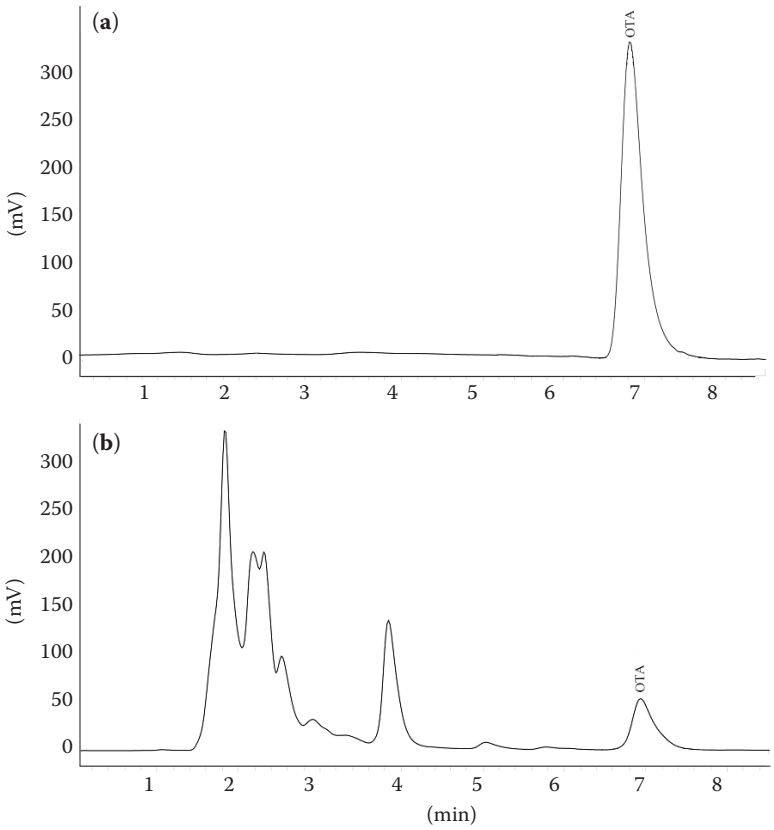

Figure 4. Typical chromatograms of (**a**) a standard solution of OTA in methanol (10 ng/ml) and (**b**) spiked sample (2 ng/ml) after DLLME under optimum conditions

ple (2 ng/ml of OTA), and the relative standard deviations (RSDs) of OTA were 3.4% for within-day precision and 6.2% for between-day precision (n = 5). The limits of detection (LOD, S/N = 3) and quantitation (LOQ, S/N = 10) for the processed spiked fresh malt beverages were 0.1 and 0.3 ng/ml, respectively.

Validity of the method

To assess the applicability of the proposed method, commercial malt beverage samples were obtained and analysed by the proposed DLLME coupled with HPLC-FLD. The results showed that the samples were free of OTA. All samples were spiked with the OTA at levels of 0.5, 2, and 4 ng/ml. The mean recoveries of OTA from malt beverage at spiking the three level concentrations were in the range of 104–108.2% (Table 1). Relative recovery was calculated as follows: RR (%) = $(C_{found}/C_{added}) \times 100$, where C_{found} – concentration of OTA measured in spiked samples after DLLME extraction, and C_{added} – concentration added to the beverage samples. The chromatograms of the standard and spiked samples are shown in Figure 4.

CONCLUSIONS

This study reports the successful analysis of OTA in malt beverage samples based on DLLME. The suggested method offers suitable features of merit such as a low detection limit, good recovery, and precision. Unlike immunoaffinity column clean-up which is time consuming, expensive, and uses much sample, the proposed method has advantages such as simplicity of operation, fastness, low sample consumption (5.0 ml), low cost of the sample preparation step, minimal use of toxic organic solvents (550 µl) and thus minimum waste generation. Therefore, the proposed DLLME method can be considered as an interesting alternative for laboratories performing routine trace analysis of OTA in malt beverage samples.

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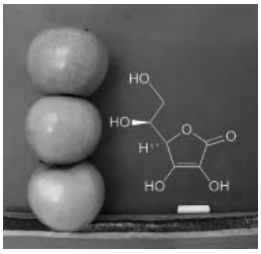

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