A validated dispersive liquid-liquid microextraction method for extraction of ochratoxin A from raisin samples

Rouhollah Karami-Osboo · Ramin Miri · Katayoun Javidnia ·
Farzad Kobarfard · Mohammad Hossein Shojaei AliAbadi ·
Mehdi Maham

Revised: 7 October 2013 / Accepted: 13 November 2013 / Published online: 12 December 2013
© Association of Food Scientists & Technologists (India) 2013

Abstract A method based on dispersive liquid-liquid microextraction (DLLME) was developed for the quantitative extraction of Ochratoxin A (OTA) from raisin samples. The influence of various parameters on the recovery of OTA such as type and volume of DLLME extractant, centrifuging and sonication time, also volume of deionized water was investigated. Recovery values under the optimum conditions were between 68.6 and 85.2 %, the inner and intra-day precision expressed as relative standard deviation (RSD%, $n=3$), were less than 15 % at spiking levels of 2.5–30 $\mu\text{g kg}^{-1}$. Linearity was studied from 0.5 to 30 $\mu\text{g L}^{-1}$, and the limits of detection (LOD) and quantification (LOQ) were 0.7 and 2.0 $\mu\text{g kg}^{-1}$, respectively. Real samples were analyzed by DLLME method and compared with confirmative immunoaffinity Column Chromatography (IAC) clean-up. Low cost, simplicity of operation, speed and minimum consumption of organic solvent were the main advantages of proposed method. The mean contamination of samples was 0.88 $\mu\text{g kg}^{-1}$ that was lower than European Legal Limit.

Keywords Dispersive liquid-liquid microextraction · Ochratoxin A · Raisin · HPLC-FLD

Introduction

The presence of mycotoxins in diet is causing great concern to human in all over the world (Karami-Osboo et al. 2012). Ochratoxin A (OTA), was first isolated from *Aspergillus ochraceus* and is one of the most hazardous mycotoxins (Amézqueta et al. 2012). In moderate climate OTA is produced by *Penicillium* strains (*P. verrucosum*) and in warmer regions is created by several species of *Aspergillus* (*A. ochraceus*, *A. sulphureus*) on different food and feed, especially on dried fruits, wine, grape juice and coffee (Covarelli et al. 2012; Walker 2002). The carcinogenicity possibly of OTA has been recognized by the International Agency for Research on Cancer (IARC) (Walker and Larsen 2005). The European Union recommends legal limits for OTA in dried currants, raisins and sultanas all (10 $\mu\text{g kg}^{-1}$), cereals (5 $\mu\text{g kg}^{-1}$), products derived from cereals (3 $\mu\text{g kg}^{-1}$), wine and grape juice (2 $\mu\text{g kg}^{-1}$), roasted coffee (5 $\mu\text{g kg}^{-1}$) and soluble coffee (10 $\mu\text{g kg}^{-1}$) (Lapera et al. 2008).

The unique phytochemical composition and natural qualities make raisins an appealing source of nutrients, for fresh consumption and also use in baking or confectionery. Just about half of the dried fruits in the international market are raisins. United States, Chile Iran and Turkey are the lead raisin exporter countries. In raisining process, bunch of grapes are dried in a well-ventilated room (air-dried), or placed under sunlight (sun-dried) until the grapes are dried, in this process, moisture may create appropriate condition for fungi growing, and increasing the OTA content (Fang et al. 2010; Hui 2008). Varga et al. analyzed 20 raisin samples, all samples were contaminated with OTA and the heavily contaminated raisin

R. Karami-Osboo · R. Miri · K. Javidnia (✉)
Medicinal and Natural Products Chemistry Research Centre, Shiraz
University of Medical Sciences, Shiraz, Iran
e-mail: kjavidnia@gmail.com

M. H. S. AliAbadi
Farough Life Sciences Research Laboratory No. 96.2,
Tehran 1457844393, Iran

F. Kobarfard
Department of Medicinal Chemistry, School of Pharmacy, Shaheed
Beheshti Medical University, P.O. Box 14155-6153, Tehran, Iran

M. Maham
Department of Chemistry, Aliabad Katoul Branch, Islamic Azad
University, Aliabad Katoul, Iran

sample came from Iran (Varga et al. 2006). Ten percent of 264 sultana sample in Turkey were contaminated over than EU regulatory limits (Meyvacı et al. 2005). Palumbo et al. reported that OTA is a common contaminant of raisin, but average levels for dried fruit are much lower than EU recommends legal limits (Palumbo et al. 2011).

There are numerous methods for extraction and clean-up of OTA in food and feed stuffs. Solid phase extraction (SPE) and immunoaffinity chromatography (IAC) are the most common applied extraction and clean-up methods for extraction of OTA in samples (Sugita-Konishi et al. 2006). These methods are efficient for extraction and clean-up of OTA, but they are time consumer, also use considerable volumes of toxic organic solvents and cause large secondary wastes. SPE has non-selective generic sorbents which easily absorb interferences with similar characteristics to target analytes. IAC is expensive, disposable and has low stability against organic solvents and pH. An interesting alternative to the above mentioned sample preparation methods is the latest developments of liquid phase microextraction, named dispersive liquid–liquid microextraction (DLLME), because of its merits such as simplicity, speed of operation, low cost and eco-friendly, DLLME attracts much attention in mycotoxin analysis for sample pre-treatment (Arroyo-Manzanares et al. 2012; Campone et al. 2011; Karami-Osboo et al. 2013; Maham et al. 2012; Merdivan and Mine 2012; Victor-Ortega et al. 2013). In this method, an appropriate mixture of extraction and disperser solvents quickly is injected into the aqueous sample, and analytes are enriched into the dispersed extraction solvent. Phase separation is accomplished by centrifugation and the enriched analytes in the settled phase are determined by instrumental methods.

Some hyphenated methods were used for quantification of mycotoxins in food and feed (Li et al. 2012, 2013). The aim of this study was to evaluate DLLME as a miniaturized sample pre-treatment technique for preparation of raisin samples contains OTA prior to liquid chromatography with fluorescence detection (HPLC-FLD). In the presented method the solvent used to extract the OTA from samples, was then used as disperser solvent in DLLME procedure. The effect of various parameters on the recovery of OTA such as type and volume of DLLME extractant, centrifuging and sonication time and volume of water was investigated. The performance of the proposed method for the analysis of OTA in samples has also been studied and finally compared with routine clean-up method (IAC) to confirm the results.

Materials and methods

Reagents and materials

HPLC grade acetonitrile and all analytical-grade methanol, chloroform, carbon tetrachloride, carbon disulfide, ethanol,

acetone, phosphoric acid were purchased from Merck (Germany). The OTA standard was achieved from Sigma-Aldrich (USA). A stock solution of OTA ($1 \mu\text{g mL}^{-1}$) was prepared in methanol quantified by measuring its respective absorbance at 330 nm by UV spectrometry and stored in dark vial at -20°C . Deionized water produced by a Milli-Q purification system (Millipore, Bedford, MA). Immunoaffinity columns were Puri-Fast OTA IAC (Libios- France).

Sample preparation

Ten raisin samples were obtained from dried fruit store in Tehran, and were removed from the stems. One hundred gram of samples was homogenized by grinding with dry ice in a Waring blender and stored at -20°C . Twenty gram of ground raisin was blended with 100 mL of methanol: water (80: 20) for 3 min in a Waring blender and filtered through Whatman no. 1 filter paper (Whatman Inc., Piscataway, NJ, USA) (MacDonald et al. 2003), afterward the extract was used in DLLME and IAC process.

Calibration curves and quantification

Working Standard solutions were prepared by dilution of known volumes of Stock standard solution. Seven levels of concentration ($0.5, 1, 2, 2.5, 5, 10$ and $15 \mu\text{g mL}^{-1}$) were used for calibration curve points. The calibration curve was built-in by linear least-squares regression and the value obtained for the determination coefficient (R^2) (0.999) shows the HPLC-FLD method is linear in the range of concentrations studied.

Immunoaffinity column chromatography clean-up procedure

Twelve milliliters of extracted sample was diluted with 100 mL Phosphate Buffer Saline (PBS) and 50 mL of diluted extract was passed through the Puri-Fast OTA column at a about 1 drop/min. The column was washed with 5 mL PBS at the same flow rate. The analyte was eluted with 1.5 mL

Fig. 1 The effect of extraction solvent type on the extraction efficiency

Fig. 2 Volume of CHCl_3 effects on the extraction efficiency

methanol and collected in a 4 mL amber glass vial. The methanol was evaporated at 40 °C, and the residue was reconstituted in 1 mL HPLC grade water: acetonitrile (3:7 v/v; pH=3) (MacDonald et al. 2003). Finally, 100 µL was injected into the HPLC system.

DLLME procedure

Under optimum conditions, a mixture of 0.2 mL chloroform and 0.8 mL methanol 80 % extract (disperser) was quickly injected into a 3.0 mL of deionized water placed in conical tube. Then a cloudy solution that involves of very fine drops of chloroform dispersed into aqueous phase was formed. After centrifugation for 5.0 min at 1,132 g, enriched extraction solvent was settled at the bottom of the test tube. After removing the upper aqueous solution the settled phase was dissolved in 0.2 mL methanol and evaporated to dryness and then the residue was reconstituted in 1 mL mobile phase.

Instrumentation

The HPLC system equipped with a binary HPLC pump (Waters 1525), auto sampler (Waters 717) and a Multi λ fluorescence detector (Waters 2487). Detector set at 330 nm (λ_{em})

Fig. 3 Effect of, the different volume of disperser solvents on the extraction efficiency

Fig. 4 Effect of the, different volume of water on the extraction efficiency

and 460 nm (λ_{ex}). A chromolith HPLC column (15 cm, Merck) was used for separation at 30 °C. The mobile phase was mixture of acetonitrile: water (70:30, v/v) using phosphoric acid as modifier (pH=3) at a flow rate of 1 mL min⁻¹.

Results and discussion

To achieve a satisfactory recovery the effect of various parameters in DLLME were investigated with spiked raisin samples (10 µg kg⁻¹). Spiked raisin samples were prepared by adding different volumes of stock solution of OTA to the blank samples.

Type of extractant

Performance of DLLME is essentially affected by the type of extractant. Extraction solvent in DLLME should have several properties: (1) denser than water; (2) high extraction capability of target analyt; (3) forming a stable cloudy solution (4) minimum solubility in water. In this study, CHCl_3 , CCl_4 and

Fig. 5 Effect of the, centrifuging time on the extraction efficiency

Table 1 Recovery of method for different levels of spiked samples

Intra-day (<i>n</i> =3)			Inner-day (<i>n</i> =3)	
Spiking levels	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
30 µg kg ⁻¹	79.5	4.2	82.6	0.9
15 µg kg ⁻¹	75.4	3.7	74.1	1.4
10 µg kg ⁻¹	85.2	6.1	80.9	3.0
5 µg kg ⁻¹	68.6	5.4	71.3	1.6
2.5 µg kg ⁻¹	74.4	3.9	75.2	2.2

CS₂ were assessed as potential extractants. A series of water (3.0 mL deionized water) was studied by rapidly injection of mixture of different extraction solvent (0.2 mL) and 0.8 mL of extract of sample. According to the obtained results (Fig. 1) CHCl₃ showed the best extraction efficiency.

Volume of extractant

To examine the effect of extraction solvent volume, 0.8 mL methanol 80 % extract containing different volumes of chloroform (50.0, 100.0, 150.0, 200.0, and 250.0 µL) was subjected to the same microextraction procedure and the experimental data are shown in Fig. 2. By increasing the volume of chloroform from 50 to 200 µL, extraction efficiency increased but above that, the recovery was decreased, probably it depends on the change of volume ratio between the disperser solvent and chloroform. Change of this ratio will change the number of droplets available for extraction, also the appropriate volume ratio between extractant and disperser solvent, form a stable cloudy solution that improve the extraction procedure. In this experiment the best volume ratio and performance was achieved at 200 µL of chloroform.

Volume of dispersive

The extraction solvent firstly used to extract OTA from raisin samples and secondly used as a disperser solvent in DLLME process. To study the effect of disperser solvent volume, 200.0 µL chloroform was mixed with different volumes of extract containing (0.4, 0.8, 1.2, 1.6, and 2.0 mL) was subjected to the same microextraction procedure. Figure 3 shows the best performance was obtained by 0.8 mL of disperser solvent volume was.

Table 2 Comparison of two methods for real samples

No of sample	1	2	3	4	5	6	7	8	9	10	Mean
IAC (µg kg ⁻¹) ^a	DL/2 ^a	DL/2	DL/2	DL/2	DL/2	DL/2	0.75	DL/2	DL/2	6.48	0.72
DLLME (µg kg ⁻¹)	0.79	DL/2	DL/2	DL/2	DL/2	DL/2	0.81	DL/2	DL/2	7.21	0.88

^a DL/2: half of the detection limit

Volume of water

For investigation of the required water volume, different volume of water (1 to 5 mL) was exposed to the same experimental conditions (0.8 mL extract containing 200.0 µL of chloroform). According to the obtained results (Fig. 4) recovery was increased to 3.0 mL water and then was decreased by further increasing the volume of water. Thus, 3.0 mL water was chosen for following studies.

Centrifuging time

In DLLME, centrifugation is substantial in order to obtain two separate phases in the extraction tubes. The influence of centrifuging time on the recovery was investigated over the range of 1–9 min (1,132 g). Based on the obtained results, by increasing the centrifuging time from 1 to 5 min, the extraction efficiency was increased. However the further increase didn't have significant effect on extraction performance. So, 5 min was used for subsequent experiments (Fig. 5).

Sonication time

The effect of sonication time on the performance of DLLME was studied by changing its time (0, 2.5, 5 and 7.5 min). Sonication may affect the dispersion of extraction solvent into the aqueous phase, and so may improve the efficiency of extraction. In this study, sonication didn't have considerable effect on recovery. So, for ease of operation the following experiments were done without using sonication.

Analytical figures of merit

The performance of the suggested method was evaluated based on the extraction recovery (ER), determination coefficient (R^2), Intra and inner (between and within)-day precision, limit of detection (LOD) and limit of quantification (LOQ). Standard calibration curve was drawn between 0.5 and 30.0 ng mL⁻¹, the determination coefficient (R^2) was 0.999 at the investigated calibration range. Inner -day precision was determined by analyzing 3 replicates of five levels of spiked sample, and Intra -day precision was determined by analyzing 3 replicates during 3 days consecutives. The precision of the method was calculated from the relative standard deviation (RSD%, *n*=3). Intra day precision was between 3.7

and 6.1 % and inner-day precision of triple measurements of each spiked sample was between 0.9 and 3.0 % (Table 1). LOD based on a signal to noise ratio (S/N) of 3, and LOQ based on S/N of 10, for the IAC and DLLME method were 0.7 and 2.0 ng mL⁻¹, respectively.

Application of the method to real samples

Applicability of the suggested method was investigated by analyzing of raisin samples and results are shown in Table 1. To establish the performance of the method a blank sample was spiked with different volumes of OTA stock standard and then the suggested method was accomplished. The obtained data (Table 1) indicate that the recoveries are in satisfactory agreement (68.6–85.2 %).

The method was applied to analysis of ten real samples with confirmative analyses performed using immunoaffinity Column Chromatography (IAC). Results are shown in Table 2.

Conclusion

Raisins are one of the most important dried fruits, and have great consumption in the world (Fang et al. 2010), and contamination of raisins could be harmful for human. In the present study; we propose a new method for the analysis of OTA in raisin, which was confirmed with IAC. DLLME procedure was used as a clean-up method for extraction of target analyte from solid matrices. The filtrated extraction solvent of extraction procedure was used as a dispersant in DLLME process. DLLME is kind of screening method and results of DLLME will help to find the suspicious samples. In our research only in one sample, result of DLLME and IAC was different, so there was good correlation between DLLME and IAC results. The developed method is a suitable alternative to routine clean-up techniques, such as IAC, because of its several advantages such as simplicity, low consumption of toxic organic solvents, low cost and allows a reduction in the time of analysis. Also, the method offers low detection and quantification limits, good precision and accuracy. Both clean-up methods were used to determine the OTA amount in real samples. In this work, Trace amount of OTA found in three samples, and the mean concentration of OTA was lower than EU legal limit (10 µg kg⁻¹).

References

- Amézqueta S, Schorr-Galindo S, Murillo-Arbizu M, González-Peñas E, López de Cerain A, Guiraud J (2012) OTA-producing fungi in foodstuffs: a review. *Food Control* 26:259–268
- Arroyo-Manzanares N, Gámiz-Gracia L, García-Campaña AM (2012) Determination of ochratoxin A in wines by capillary liquid chromatography with laser induced fluorescence detection using dispersive liquid-liquid microextraction. *Food Chem* 135:368–372
- Campone L, Piccinelli AL, Celano R, Rastrelli L (2011) Application of dispersive liquid-liquid microextraction for the determination of aflatoxins B1, B2, G1 and G2 in cereal products. *J Chromatogr A* 1218:7648
- Covarelli L, Beccari G, Marini A, Tosi L (2012) A review on the occurrence and control of ochratoxigenic fungal species and ochratoxin A in dehydrated grapes, non-fortified dessert wines and dried vine fruit in the Mediterranean area. *Food Control* 26:347–356
- Fang Y, Zhang A, Wang H, Li H, Zhang Z, Chen S, Luan L (2010) Health risk assessment of trace elements in Chinese raisins produced in Xinjiang province. *Food Control* 21:732–739
- Hui YH (2008) Handbook of fruits and fruit processing. Wiley
- Karami-Osboo R, Mirabolfathy M, Kamran R, Shetab-Boushehri M, Sarkari S (2012) Aflatoxin B1 in maize harvested over 3 years in Iran. *Food Control* 23:271–274
- Karami-Osboo R, Maham M, Miri R, AliAbadi MHS, Mirabolfathy M, Javidnia K (2013) Evaluation of dispersive liquid-liquid microextraction-HPLC-UV for Determination of Deoxynivalenol (DON) in wheat flour. *Food Anal Methods* 6:176–180
- Lapera L, Avellone G, Turco VL, Di Bella G, Agozzino P, Dugo G (2008) Influence of roasting and different brewing processes on the ochratoxin A content in coffee determined by high-performance liquid chromatography-fluorescence detection (HPLC-FLD). *Food Addit Contam* 25:1257–1263
- Li P, Zhang Z, Zhang Q, Zhang N, Zhang W, Ding X, Li R (2012) Current development of microfluidic immunosensing approaches for mycotoxin detection via capillary electromigration and lateral flow technology. *Electrophoresis* 33:2253–2265
- Li P, Zhang Z, Hu X, Zhang Q (2013) Advanced hyphenated chromatographic-mass spectrometry in mycotoxin determination: current status and prospects. *Mass Spectrom Rev*. doi:10.1002/mas.21377
- MacDonald SJ, Anderson S, Brereton P, Wood R (2003) Determination of ochratoxin A in currants, raisins, sultanas, mixed dried fruit, and dried figs by immunoaffinity column cleanup with liquid chromatography: interlaboratory study. *J AOAC Int* 86:1164–1171
- Maham M, Karami-Osboo R, Kiarostami V, Waqif-Husain S (2012) Novel Binary Solvents-Dispersive Liquid-Liquid Microextraction (BS-DLLME) method for determination of patulin in apple juice using high-performance liquid chromatography. *Food Anal Methods*:1–6
- Merdivan M, Mine AH (2012) Development of new dispersive liquid-liquid microextraction technique for the identification of zearalenone in beer. *Anal Methods* 4:4129–4134
- Meyvaci K, Altindisli A, Aksoy U, Eltem R, Turgut H, Arasiler Z, Kartal N (2005) Ochratoxin A in sultanas from Turkey I: survey of unprocessed sultanas from vineyards and packing-houses. *Food Addit Contam* 22:1138–1143
- Palumbo J, O'Keeffe T, Vasquez S, Mahoney N (2011) Isolation and identification of ochratoxin A-producing *Aspergillus* section *Nigri* strains from California raisins. *Lett Appl Microbiol* 52:330–336
- Sugita-Konishi Y, Tanaka T, Nakajima M, Fujita K, Norizuki H, Mochizuki N, Takatori K (2006) The comparison of two clean-up procedures, multifunctional column and immunoaffinity column, for HPLC determination of ochratoxin A in cereals, raisins and green coffee beans. *Talanta* 69:650–655
- Varga J, Kocsabé S, Koncz Z, Téren J (2006) Mycobiota and ochratoxin A in raisins purchased in Hungary. *Acta Aliment* 35:289–294
- Victor-Ortega MD, Lara FJ, García-Campaña AM, Olmo-Iruela M (2013) Evaluation of dispersive liquid-liquid microextraction for

- the determination of patulin in apple juices using micellar electrokinetic capillary chromatography. *Food Control* 31:353–358
- Walker R (2002) Risk assessment of ochratoxin: current views of the European Scientific Committee on Food, the JECFA and the Codex Committee on Food Additives and Contaminants. *Mycotoxins Food Saf*:249–255
- Walker R, Larsen JC (2005) Ochratoxin A: previous risk assessments and issues arising. *Food Addit Contam* 22:6–9