Enrichment of Ochratoxin A in Cereals Using Ionic Liquid Based Dispersive Liquid-Liquid Microextraction

Tahıllardaki Okratoksin A'nın İyonik Sıvı Bazlı Dispersif Sıvı-Sıvı Mikroözütleme ile Zenginleştirilmesi

Research Article

Hayriye Mine Antep* and Serap Seyhan Bozkurt
Dokuz Eylül University, Faculty of Science, Chemistry Department, Kaynaklar Campus, İzmir, Turkey.

ABSTRACT

A simple and fast analytical method known as ionic liquid based dispersive liquid-liquid microextraction was proposed for the separation and enrichment of ochratoxin A before high performance liquid chomatography with fluorescence detector. 1-Methyl-3-octylimidazolium hexafluorophosphate and ethanol were used as extraction solvent and dispersive solvent, respectively. The optimal parameters influencing the efficiency of the method such as pH of sample solution, type and volume of extraction solvent, type and volume of dispersive solvent, time of extraction and centrifugation and salting effect were investigated. Under optimum extraction conditions, limits of detection and quantification were 0.05 and 0.13 μg L^{-1}, respectively. The recoveries of ochratoxin A for spiked wheat and corn samples were ranged from 78.55 to 81.05%.

Key Words
Ochratoxin A, dispersive liquid–liquid microextraction, ionic liquid, HPLC.

ÖZET

Okratoksin A'nın floresans dedektörlü yüksek performanslı sıvı kromatografisi ile tayini öncesi, ayrılması ve zenginleştirilmesi için hızlı ve basit bir analitik teknik olarak bilinen iyonik sıvı bazlı dispersif sıvı-sıvı mikroözütleme yöntemi geliştirildi. Özütleme çözücüsü ve dispersif çözücü olarak sırasıyla 1-metil-3-oktilimidazolyum hegzaflorofosfat ve etanol kullanıldı. Örnek çözeltinin pH'ı, özütleme çözücüsünün türü ve hacmi, dispersif çözücünün türü ve hacmi, özütleme ve santrifüj süresi ve tuz etkisi gibi verimi etkileyen optimum parametreler çalışıldı. Optimum özütleme koşulları altında, gözlenebilme ve tayin sınırı sırasıyla 0.05 ve 0.13 μg L^{-1} olarak belirlendi. Mısır ve buğday örnekleri için okratoksin A geri kazanım değerleri % 78.55 -% 81.05 aralığında elde edildi.

Anahtar Kelimeler
Ochratoxin A, dispersif sıvı-sıvı mikroekstraksiyonu, iyonik sıvı, HPLC.

Article History: Received: Jan 17, 2015; Revised: May 26, 2015; Accepted: June 22, 2015; Available Online: Jul 25, 2015.

DOI: 10.15671/HJBC.20154312549

Correspondence to: H.M. Antep, Dokuz Eylül University, Faculty of Science, Chemistry Department, İzmir, Turkey.
Tel: +90 232 301 7909 Fax: +90 232 453 4188 E-Mail: mine.kurtbay@deu.edu.tr

INTRODUCTION

Ochratoxin A (OTA), is a well known carcinogen metabolite that classified by International Agency for Research on Cancer, produced by *Penicillium* and *Aspergillus* [1]. It effects on kidney damage and can damage the liver in higher concentrations [2]. OTA is a mycotoxin commonly occurring in cereals, coffee, spices, dried fruits and in alcoholic beverages [3-5]. The European Union regulations for OTA are 5 μg kg^{-1} for raw cereal grains, 3 μg kg^{-1} for all products derived from cereals and 10 μg kg^{-1} for dried vine fruit [6]. The European Food Safety Authority established a tolerable weekly intake (TWI) of 120 ng kg^{-1} body weight [7]. Therefore, the determination of OTA in food products is very important and various analytical methods have been developed for the encrichment of OTA before analysis.

Dispersive liquid-liquid microextraction (DLLME) is one of the most preferred techniques that are applied for inorganic and organic substances combined with extra vortex assisted ultrasonication and solidification treatments [8-10]. Beside the advantages, using of chlorinated solvents as an extractant is an important disadvantage of this technique. For this, ionic liquids are preferred instead of highly toxic chlorinated solvents. Ionic liquids contain cationic group such as imidazolium, ammonium, phosphonium, and anionic group such as hexafluorophosphate, tetrafluoroborate, bis(trifluoromethanesulfonyl) imide. Ionic liquids have a wide range of applications because of properties such as low toxicity and volatility, high thermal stability, high density [11].

The aim of study is to develop and optimize ionic liquid based dispersive liquid-liquid microextraction (IL-DLLME) for separation and enrichment of OTA before using high performance liquid chromatography (HPLC) with fluorescence detection. The extraction parameters such as pH of sample solution, volume of ionic liquid, type and volume of dispersive solvent, time of extraction and centrifugation, and salt addition were optimized for OTA. The developed method was successfully applied to wheat and corn samples.

MATERIAL AND METHODS

Reagents, standard solutions

OTA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethanol, acetone, acetonitrile and acetic acid were supplied as HPLC grade from Merck (Darmstadt, Germany). The N-methyl-imidazol, 1-bromobutane and 1-bromooctane were purchased from Sigma-Aldrich and ammonium hexafluorophosphate was from Fluka. Ultra pure water was obtained from a Milli-Q apparatus (Milford, USA). A 0.45 μm filter disc (Millipore Millex-HV, Hydrophilic PVDF) was used for filtration of samples. A stock solution of OTA (200 μg mL^{-1}) was prepared in methanol and stored in dark glass vials at -18°C, the working solutions were diluted with appropriate volumes of ultra pure water daily.

Ionic liquids [1-butyl-3-methylimidazolium hexafluorophosphate ($[C_4mim][PF_6]$) and 1-methyl-3-octylimidazolium hexafluorophosphate ($[C_8mim][PF6]$) were prepared according to reported literature [12,13].

Intrumentation

A Thermo Scientific Dionex Ultimate 3000 series LC system equipped with fluorescence detector, pump, autosampler, degasser and column oven was used. The separation was carried out using Thermo hypersil C18 column (150x3 mm, 5 μm) for analysis of OTA at 50°C temperature. The mobile phase was selected as water: acetonitrile: acetic acid (65:34:1, v/v/v) at 1 mL min^{-1} flow rate. Analysis was achieved in isocratic flow in ten minutes. The Chromeleon software was used to control the chromatograms and the process signals. WTW 720 model pH meter and NF 400R model centrifuge device were used for pH adjustments and centrifugation, respectively.

IL-DLLME method

A 5 mL of standard solution containing 0.2 μg mL^{-1} OTA was placed in a 15 mL screw capped test tube with conical bottom. pH of the OTA standard solution was adjusted to 2 with 0.1 M hydrochloric acid. Then, 1 mL of ethanol containing 250 μL of $[C_8MIM][PF_6]$ was injected into the OTA solution, and the mixture was gently shaken for 2 min. After that the cloudy solution formed, the resulting

solution was centrifuged at 4000 rpm for 2 min and the phase of ionic liquid settled at the bottom of the tube. Then, the sedimented phase (200 ± 20 μL) was transferred to a vial and injected to HPLC system after diluted with methanol.

Sample preparation

Wheat and corn samples were obtained from local market in İzmir, Turkey. All samples were purchased with intact packages and were used for analysis OTA as fast as possible. Firstly, the cereal samples were grinded to powder with a blender and then powder samples were extracted with 20 mL of methanol [14,15]. The extracts were filtered from 0.45 μm filter disc and an aliquot of five mL of sample extract was placed in a 15 mL screw capped test tube with conical bottom and applied to IL-DLLME method.

For the recovery experiments, the homogenized cereal samples were directly spiked with OTA standard solution. Then, the samples were stirring using vortex for 60 seconds and IL-DLLME procedure was applied to samples.

RESULT AND DISCUSSION

Selection of ionic liquid

The ionic liquids used as extraction solvent in IL-DLLME should have hydrophobic structure that their insolubilities in water but miscible in dispersive solvent. Also these ionic liquids should ensure good extraction efficiency for the target analytes. At first, the DLLME method was carried out by $[C_4MIM][PF_6]$ and $[C_8MIM][PF_6]$ for OTA under same conditions. It was observed that when $[C_4MIM][PF_6]$ was used, phase separation was not achieved effectively. This may be explained by its higher solubility in water than $[C_8MIM][PF_6]$, relatively. Therefore, $[C_8MIM][PF_6]$ was chosen as extraction solvent for further studies.

Type and volume dispersive solvent

The dispersive solvent in DLLME should be miscible with the extraction solvent and the sample solution. For this reason, ethanol, methanol, acetone and acetonitrile were used as dispersive solvent. The extraction recovery and enrichment factor were calculated for OTA in all dispersive solvent and the highest values were obtained in ethanol. Thus, ethanol was chosen as the dispersive solvent for further studies.

The optimum volume of ethanol was checked to get higher extraction recovery and enrichment factor. The extraction recovery and enrichment factor were almost the same between 50-250 μL of ethanol volumes, but increased slightly after 250 μL of ethanol volume. The best results were obtained at 750 μL of ethanol volume. Thus, the next experiments were carried out with 750 μL of ethanol.

Volume of ionic liquid

The $[C_8MIM][PF_6]$ was used as the extraction solvent in the range of 50-750 μL and optimum volume of the $[C_8MIM][PF_6]$ was determined. The recovery increased up to 250 μL of $[C_8MIM][PF_6]$, and decreased at 500 and 750 μL of $[C_8MIM][PF_6]$. The enrichment factor was same until 250 μL and decreased slightly at 500 and 750 μL of $[C_8MIM][PF_6]$. As a result of this, 250 μL of $[C_8MIM][PF_6]$ was used as the optimum extraction volume for further studies.

Effect of pH

OTA is a weak acid, which has two pK_a values of 4.4 for the carboxylic and 7.3–7.05 for the phenolic groups. OTA presents in the dissociated form under neutral and alkaline conditions [16] and OTA may hydrolysis to phenylalanine at very low pH [15].

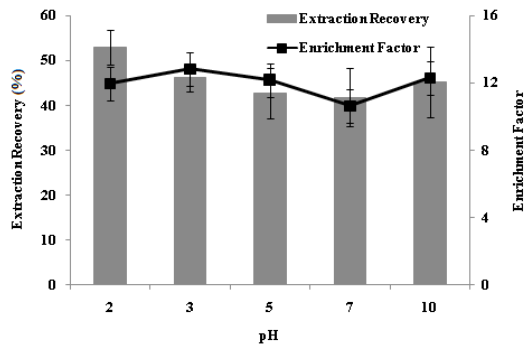

Figure 1. Effect of pH on IL-DLLME. Extraction conditions: volume of synthetic OTA solution: 5 mL; OTA concentration: 0.2 μg mL^{-1}; volume of $[C_8MIM][PF_6]$: 250 μL; volume of ethanol: 0.75 mL.

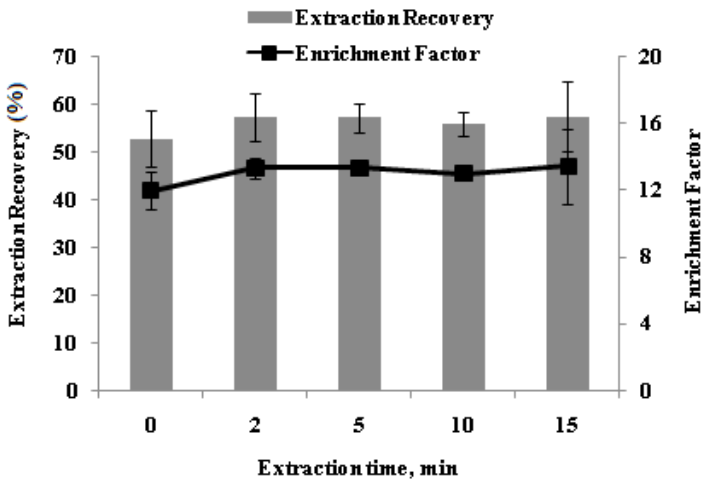

Figure 2. . Effect of extraction time on IL-DLLME. Extraction conditions: volume of synthetic OTA solution: 5 mL; OTA concentration: 0.2 μg mL^{-1}; pH of OTA solution: 2; volume of [C_8MIM][PF_6]: 250 μL; volume of ethanol: 0.75 mL.

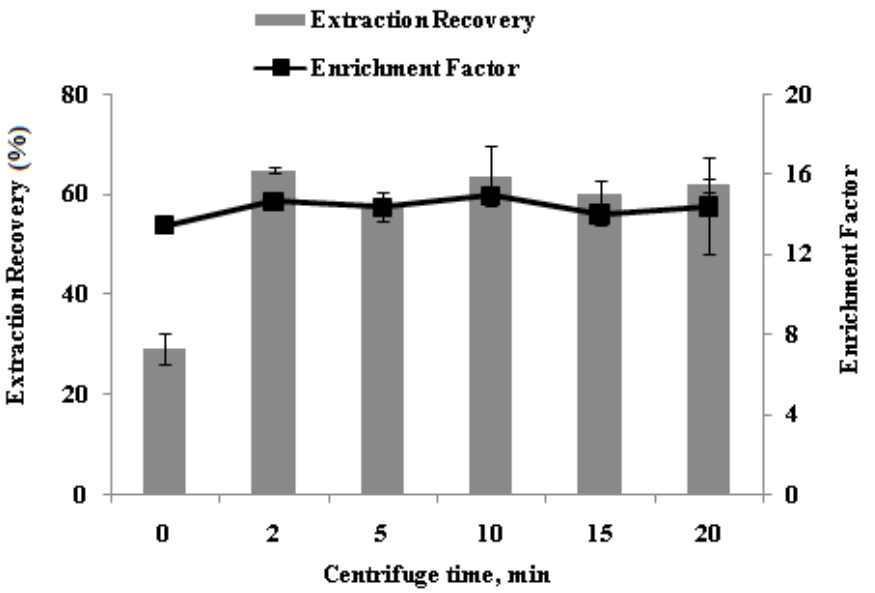

Figure 3. Effect of centrifugation time on IL-DLLME. Extraction conditions: volume of synthetic OTA solution: 5 mL; OTA concentration: 0.2 μg mL^{-1}; pH of OTA solution: 2; volume of [C_8MIM][PF_6]: 250 μL; volume of ethanol: 0.75 mL; extraction time: 2 min.

$$\text{Extraction Recovery (\%)} = \frac{\text{(analyte concentration in the sediment) x (volume of sediment phase)}}{\text{(initial analyte concentration in the aqueous sample) x (volume of aqueous sample)}} \times 100$$

$$\text{Enrichment Factor} = \frac{\text{analyte concentration in the sediment}}{\text{initial analyte concentration in the aqueous sample}}$$

For the optimization of pH on IL-DLLME efficiency, pH was adjusted between 2 and 10 by using 0.1 M hydrochloric acid or 0.1 M sodium hydroxide. As shown in Figure 1, the higher extraction efficiency was obtained at low pH and the increasing of pH from 2 to 10 caused the decreasing of recovery. Thus, pH 2 was selected for further experiments.

Effect of extraction and centrifuge time

In order to optimize the extraction time, IL-DLLME was carried out in the range of 0-15 min. The results showed that extraction time affected slightly the extraction efficiency. The interaction of OTA with [C8MIM][PF6] increased in 2 min (Figure 2). So the extraction time was selected as 2 min for further studies.

Different centrifugation times were studied between 0 and 20 min to evaluate of the centrifuge time. The recovery and enrichment factor increased in 2 min and then were closer to each other (Figure 3). As a result, the optimum centrifugation time was accepted as 2 min and the next experiments were carried out with 2 min centrifugation time.

Salting effect

IL-DLLME experiments were performed by NaCl solution from 0 to 20%, w/v. The extraction recovery and enrichment factor of OTA was not changed so much by increasing salt amount. This could be considered as the partitioning of OTA into organic phase was not effected from the presence of salt. Thus, the NaCl was not added to OTA solution in the following experiments.

Method validation

Under the optimum extraction and chromatography conditions the analytical performance of the proposed method was evaluated in terms of dynamic linearity, limit of detection and quantification, precision, enrichment factor and the extraction recovery. The developed method showed linearity in the range of 1-800 μg L^{-1} concentration with the regression equation y=11983x+15372 (y, peak area and x, μg L^{-1} OTA) and correlation coefficient of 0.9960. Limits of detection and quantification were 0.05 and 0.13 μg L^{-1} for ten blank measurements, respectively (Table 1). The extraction recovery and the enrichment factor were calculated from the following equation [17]:

Table 1. The parameters obtained from the developed extraction method.

Linear working range (μg L^{-1})	1-800
Correlation of coefficient (R^2)	0.9960
Limit of detection (3xS/N) (μg L^{-1})	0.05
Limit of quantification (10xS/N) (μg L^{-1})	0.13
Enrichment factor	14.7
Extraction Recovery (%)	65

Amount of OTA in Corn and Wheat Samples

The applicability of the developed method was demonstrated by wheat and corn samples. The 1 and 5 μg L^{-1} standard OTA solution was spiked to samples and the recoveries were obtained in the range of 78.55 – 81.05%. 2.43 and 1.34 μg L^{-1} OTA were found in the wheat and corn samples, respectively (Table 2).

Table 2. The results obtained from cereal samples (n=3).

Samples	Added (μg L^{-1})	Found (μg L^{-1})	Recovery (%)
Wheat	-	2.43±0.02	
	1	2.78±0.06	81.05
	5	6.22±0.13	79.95
Corn	-	1.34±0.05	
	1	1.89±0.06	80.77
	5	4.98±0.15	78.55

CONCLUSION

Analytical method was developed for separation and enrichment of ochratoxin A before analysis with high performance liquid chromatography with fluorescence detector. Ionic liquid was used as extractant solvent instead of organic solvent. Optimum volume of extraction and dispersive solvent was found to be 250 and 750 μL, respectively. Optimum time of extraction and centrifugation were 2 min. A wide linear range (1-800 μg L^{-1}) was obtained for ochratoxin A. The

enrichment factor for OTA was 14.7 and limits of detection and quantification were 0.05 and 0.13 μg L^{-1}, respectively. The proposed method was successfully applied to cereal samples and the recovery in the range of 78.55-81.05% obtained from samples.

ACKNOWLEDGEMENT

The authors are grateful to the Scientific Research Foundation of Dokuz Eylul University with project numbered "2013 KB FEN 014" for financial support.

References

1. International Agency for Research on Cancer, IARC, Evaluation of carcinogenic risks of chemical to humans. In ''Some naturally-occurring substances: Food Items and Constituents". Heterocyclic Aromatic Amines and Mycotoxins, IARC monographs, Lyon, France, (1993) 3.
2. J.L. Richard, Some major mycotoxins and their mycotoxicoses-An overview, International Journal of Food Microbiology, 119 (2007) 3.
3. K.T.N. Nguyen, D. Ryu, Concentration of ochratoxin A in breakfast cereals and snacks consumed in the United States, Food Control, 40 (2014) 140.
4. O. Galarce-Bustos, M. Alvarado, M. Vega, M. Aranda, Occurrence of ochratoxin A in roasted and instant coffees in Chilean market, Food Control, 46 (2014) 102.
5. A. Roland, P. Bros, A. Bouisseau, F. Cavelier, R. Schneider, Analysis of ochratoxin A in grapes, musts and wines by LC–MS/MS: First comparison of stable isotope dilution assay and diastereomeric dilution assay methods, Analytica Chimica Acta, 818 (2014) 39.
6. FAO, Worldwide regulations for mycotoxins in food and feed in 2003. Food and nutrition paper, 81 (2004) 180 pages.
7. European Commission, Commission Recommendation 2006/576/EC on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding, Official Journal of the European Union, (2006) No L229/7.
8. M. Rezaee, Y. Yamini, M. Faraji, Evolution of dispersive liquid–liquid microextraction method, J. Chromatogr. A, 1217 (2010) 2342.
9. M.I. Leong, S.D. Huang, Dispersive liquid–liquid microextraction method based on solidification of floating organic drop combined with gas chromatography with electron-capture or mass spectrometry detection, J. Chromatogr. A, 1211 (2008) 8.
10. M. Zeeb, M.R. Ganjali, P. Norouzi, Preconcentration and trace determination of chromium using modified ionic liquid cold-induced aggregation dispersive liquid-liquid microextraction: application to different water and food samples, Food Analytical Methods, 6 (2013) 1398.
11. P. Sun, D.W. Armstrong, Ionic liquids in analytical chemistry, Analytica Chimica Acta, 661 (2010) 1.
12. C. Zafer, K. Ocakoglu, C. Ozsoy, S. Icli, Dicationic bis-imidazolium molten salts for efficient dye sensitized solar cells: Synthesis and photovoltaic properties, Electrochim. Acta, 54 (2009) 5709.
13. S. Ozdemir, C. Varlikli, I. Oner, K. Ocakoglu, S. Icli, The synthesis of 1,8- naphthalimide groups containing imidazolium salts/ionic liquids using I-, PF6-, TFSI- anions and their photophysical, electrochemical and thermal properties, Dyes Pigments, 86 (2010) 206.
14. L. Campone, A.L. Piccinelli, R. Celano, L. Rastrelli, pH-controlled DLLME for the analysis of ionisable compounds in complex matrices: Case study of ochratoxin A in cereals, Analytica Chimica Acta, 754 (2012) 61.
15. M.H. Mashhadizadeh, M. Amoli-Diva, K. Pourghazi, Magnetic nanoparticles solid phase extraction for determination ofochratoxin A in cereals using high-performance liquid chromatography with fluorescence detection, Journal of Chromatography A, 1320 (2013) 17-26.
16. T.P. Lee, B. Saad, W.S. Khayoon, B. Salleh, Molecularly imprinted polymer as sorbent in micro-solid phase extraction of ochratoxin A in coffee, grape juice and urine, Talanta, 88 (2012) 129.
17. X. Lai, C. Ruan, R. Liu, C. Liu, Application of ionic liquid-based dispersive liquid–liquid microextraction for the analysis of ochratoxin A in rice wines, Food Chemistry, 161 (2014) 317.