

# Determination of Ochratoxin A in Grape Wines after Dispersive Liquid-Liquid Microextraction Using High Performance Thin Layer and Liquid Chromatography-Fluorescence Detection

## Türk Üzüm Şaraplarındaki Okratoksin A' Nın Dispersif Sıvı-Sıvı Mikroekstraksiyonu Sonrası Yüksek Performanslı İnce Tabaka ve Sıvı Kromatografisi- Floresans Yöntemiyle Tayini

Research Article

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### ABSTRACT

In this study, a method was developed for analyzing ochratoxin A in wines using dispersive liquid-liquid microextraction combined with thin layer and liquid chromatography. Parameters such as type and volume of extraction solvent and dispersive solvent, extraction time and effect of salt were optimized to obtain the best extraction result. The linearity of response was employed in the concentration range of ochratoxin A in wines from 0.03-1.00  $\mu\text{g L}^{-1}$ . Under the optimum conditions, the extraction recovery as 63.9% and the enrichment factor as 34.5 were established.

### Key Words:

Ochratoxin A, wine, dispersive liquid liquid microextraction, TLC and HPLC.

### ÖZET

Bu çalışmada, kırmızı ve beyaz üzüm şaraplarında okratoksin A analizine yönelik dispersif sıvı-sıvı mikroekstraksiyon yöntemi geliştirildi. En iyi ekstraksiyon verimini elde etmek için ekstraksiyon çözücüsü ve dispersif çözücünün türü ve hacmi, ekstraksiyon zamanı, tuz etkisi gibi parametrelerin optimizasyonu gerçekleştirildi. Yöntemin 0.03-1.00  $\mu\text{g L}^{-1}$  derişim aralığında doğrusal olduğu belirlenmiştir. Optimum koşullar altında, ekstraksiyon geri kazanımı % 63.9 ve zenginleştirme faktörü 34.5 olarak belirlendi.

### Anahtar Kelimeler:

Okratoksin A, şarap, dispersif sıvı-sıvı mikroekstraksiyonu, TLC ve HPLC.

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## INTRODUCTION

Ochratoxin A (OTA) is the most common naturally occurring mycotoxin produced by *Aspergillus ochraceus*, *A. carbonarius* and *Penicillium verrucosum* [1]. OTA commonly occurs in sub-tropical and temperate climates [2], and can be found in a number of food products, including cereals, beer, coffee beans, cacao, spices, nuts, dried fruit, grape juice, as well as in human blood and animal-derived products [3].

OTA exerts several toxic effects, mainly involving the kidney and liver [4]. It is a strong carcinogen in rats and mice [5], with immunosuppressive, teratogenic, genotoxic activities, affecting blood coagulation and carbohydrate metabolism [6]. Epidemiological studies evidence a correlation between high OTA levels in blood and the development of Balkan Endemic Nephropathy [7]. The International Agency for Research on Cancer has classified the ochratoxin A as a possible human carcinogen (category 2B) [8]. Controlling limits of OTA in foods have been determined and controlled for their toxic effects by European Commission (EC) in wines and grape juices at 2 µg/kg, however 10 µg/kg for dried wine fruits [9].

The most employed technique for analysis of OTA in wine is high performance liquid chromatography (HPLC) equipped with fluorescence detection on RP-C18 columns [10, 11]. Besides, thin layer chromatography (TLC) is the traditionally method used for mycotoxins analysis for both quantitative and semi-quantitative purposes due to its high throughput of samples, low operating costs, time average and ease of identification of target compounds using new developed densitometers [12-15].

In order to determine OTA trace levels in wine samples, an extraction and a preconcentration step is often required prior to their analysis by chromatographic techniques. There have been uses of immunoaffinity column (IAC) for clean-up [16, 17], silica gel columns for purification using silica gel, octadecylsilane and florisil materials [18,19]. But these methods include some disadvantages like time consuming, general specificity of sorbents,

interferences of similar compounds and high cost due to single use of cartridges.

Dispersive liquid-liquid microextraction (DLLME) is one of the new microextraction technique developed by Assadi and co-workers [20]. This technique consists of two main steps as adding of a binary mixture of a water miscible solvent (disperser), and a high density one with very low water solubility (extractant) to aqueous sample and after centrifugation, forming of a drop of extractant in the bottom of the tube. This is a simple and fast microextraction technique used microliters of chlorinated solvents (chloroform, carbon tetrachloride, chlorobenzene, etc.) as extractant and acetone, methanol and acetonitrile considered as dispersers [21,22]. In this technique fast addition of the extraction mixture to aqueous sample containing the analytes results in a cloudy state consisting of fine droplets of the extractant dispersed in the aqueous matrix. After centrifugation of disperse phase, the analytes are enriched into the fine droplets settled down at the bottom of the conical test tube. The advantage of DLLME is simplicity of operation, rapidity, low cost, high recovery, high enrichment factor and minimal consumption of organic solvents [23, 24]. However, DLLME has been successfully applied to preconcentration of different organic compounds in water samples for the last decade [25, 26].

In this work, unlike classical methods, DLLME has been achieved for extraction and preconcentration of OTA in wine samples using less organic solvents in less analysis time using HPTLC and HPLC.

## MATERIAL AND METHODS

### Reagents, Standards and Samples

Acetonitrile, methanol, 1,2-dichloroethane (C<sub>2</sub>H<sub>4</sub>Cl<sub>2</sub>), tetrachloroethylene (C<sub>2</sub>Cl<sub>4</sub>), trichloroethylene (C<sub>2</sub>HCl<sub>3</sub>), methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>), chlorobenzene (C<sub>6</sub>H<sub>5</sub>Cl), carbon tetrachloride (CCl<sub>4</sub>) and chloroform (CHCl<sub>3</sub>) from Merck (Darmstadt, Germany) were used. All solvents used were HPLC grade. Ultra pure water was obtained from a Milli-Q apparatus (Millipore, Molsheim, France).

OTA was purchased from Sigma-Aldrich (St.

Louis, MO). Stock standard solution of OTA (200 mg L<sup>-1</sup>) was prepared in methanol and stored in a freezer at -20 °C. Working solutions were prepared by dilution of stock standard solution with appropriate volumes of methanol.

Samples of red and white wines were purchased in super markets in Izmir. All sixteen wine samples were stored in their original bottles. Their lids were opened the day before and filtered 0.45 µm filter disk (Millipore Millex-HV, Hydrophilic PVDF).

### Extraction Procedure

A 5 mL of wine sample spiked at level of 0.2 ng µL<sup>-1</sup> OTA was placed in a 15-mL screw capped test tube with conic bottom. A 1.00 mL of acetonitrile (ACN), disperser solvent, containing 100 µL of chloroform, extraction solvent, was rapidly injected into the wine sample, and the mixture was gently shaken for 1 min. After that the cloudy solution formed was centrifuged at 4000 rpm for 5 min and the extraction solvent was sedimented in the bottom of the conical test tube. Then the sedimented phase was transferred to another test tube using a 100 µL syringe and applied to the TLC-densitometer for optimization, to the HPLC-FLD for quantification.

### Instrumentation

Thin layer chromatographic determination was performed using high performance thin layer chromatography equipped with densitometer from Camag. Analysis was performed on 20 cm × 5 cm silica gel 60F<sub>254</sub> HPTLC glass plates previously activated at 110 °C for an hour. Samples and standards were applied to the plates as bands by CAMAG Linomat V semi-automatic sample applicator which operated with 100 µL syringe size, 6 mm band length, 10 cm distance from the side edge and 10 cm distance from the bottom.

Chromatograms were developed in ascending mode, to a distance of 5 cm, at room temperature (22-25°C), with toluene: ethyl acetate: formic acid 6:3:1 (v/v) as mobile phase, in a 20 cm×10 cm CAMAG twin-trough chamber previously equilibrated with mobile phase vapor for 15 min prior to inserting of the plate. After drying the plate at room temperature OTA was detected using fluorescence densitometer

at 333 nm with Hg lamp and K 400 secondary filter. The retardation factor ( $hR_f$ ,  $R_f \times 100$ ) of OTA on the silica gel plate was  $62 \pm 3$ .

Liquid chromatographic determinations were performed by HPLC (Agilent Technologies 1100, Germany) with a fluorescence detection at 333 nm (excitation) and 458 nm (emission), controlled by Chemstation 3D software. The chromatographic conditions were as follows: C18 reverse phase (250 mm × 4 mm, 5 µm) hypersyl gold HPLC analytical column (Thermo), isocratic elution (water/ACN/acetic acid, 48.5:50.5:1, v/v), 1.5 mL/min flow rate at 50 °C. At these chromatographic conditions the retention time of OTA was 4.5 min.

## RESULTS AND DISCUSSION

The type and volume of extractant and disperser solvent, salt effect and extraction time as effective parameters in DLLME procedure were principally optimized. In the optimization procedure, using spiked wine samples, extraction recovery (ER) and enrichment factor (EF) were calculated according to given equations below

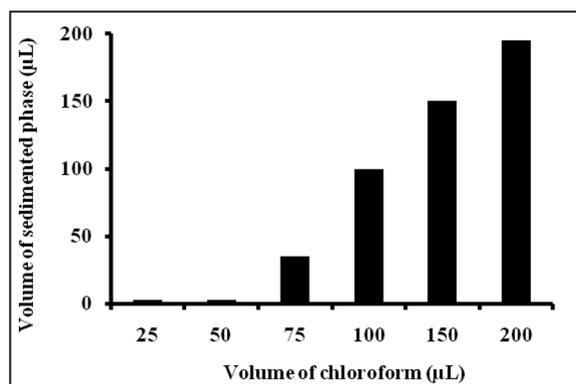
$$EF = (C_{sed}/C_o)$$

$$ER \% = EF \times (V_{sed}/V_{aq}) \times 100$$

where,  $C_{sed}$  concentration of analyte in sedimented phase,  $C_o$ : initial concentration of analyte in aqueous sample,  $V_{sed}$ : volume of sedimented phase and  $V_o$ : volume of aqueous phase. The analyte concentration in the sedimented phase was calculated using the direct calibration curve in the range of 4.0-100.0 ng µL<sup>-1</sup> OTA in methanol.

### Effect of type of extractant and volume

Under DLLME principles, the extraction solvent should have some properties like less solubility in water, high extraction efficiency for analyte compounds, higher density than water and good chromatographic behaviour. Depending on these,  $C_2H_4Cl_2$ ,  $C_2Cl_4$ ,  $C_2HCl_3$ ,  $CH_2Cl_2$ ,  $C_6H_5Cl$ ,  $CCl_4$  and  $CHCl_3$  were performed to determine the effect of solvents on extraction efficiency. Except, chlorobenzene and dichloromethane, phase separation was observed with the others. It could be probably due to higher solubility of these



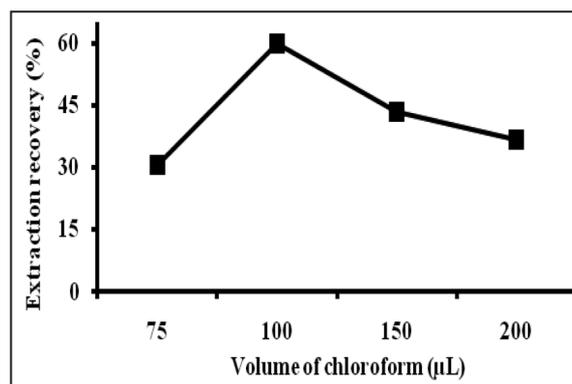
**Figure 1.** Effect of the volume of extraction solvent (chloroform) on the volume of sedimented phase in DLLME. Extraction conditions: wine sample volume, 5.0 mL; disperser solvent: acetone, 1.00 mL; room temperature.

solvents in water. A series of sample solutions were performed by using 1.00 mL of acetone containing 100 μL extractant solvent. The volume of sedimented phase was changed from 40 to 100 μL depending on the extractant solvent used. The highest extraction efficiency was obtained with chloroform under the experimental conditions. The average recovery of triplicate analysis for each extractant and their standard deviations (SD) were given in Table 1. Therefore, chloroform was selected as extractant for further experiments.

**Table 1.** Efficiency of different extractants for extraction

Extractant	ER, %
CHCl <sub>3</sub>	60.0 ± 1.2
C <sub>2</sub> H <sub>4</sub> Cl <sub>2</sub>	36.0 ± 0.0 <sub>4</sub>
C <sub>2</sub> HCl <sub>3</sub>	52.9 ± 5.4
C <sub>2</sub> Cl <sub>4</sub>	18.5 ± 0.4
CCl <sub>4</sub>	38.5 ± 0.9

Extraction conditions: wine sample volume: 5.0 mL; disperser (acetone) volume: 0.90 mL; extractant volumes: 100 μL; sedimented phase volume range: 40-100 μL; room temperature; concentration of OTA 0.5 ng μL<sup>-1</sup>.

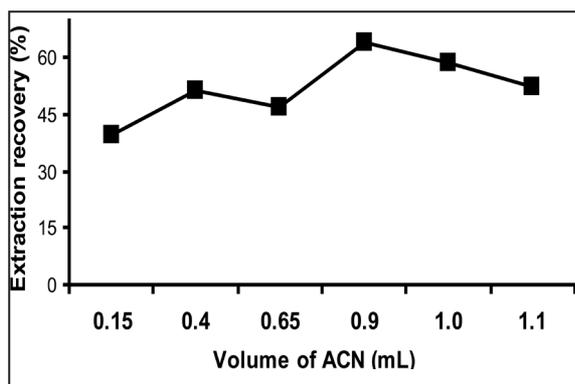


**Figure 2.** Effect of the volume of extraction solvent (chloroform) on the recovery of OTA. Extraction conditions: as with Figure 1; concentration of OTA: 0.5 ng μL<sup>-1</sup>.

In order to evaluate the effect of volume of extractant, 1.00 mL extraction mixture including different volumes of chloroform was used in the same DLLME procedure. The relationship between the volume of sedimented phase and the volume of extraction solvent, CHCl<sub>3</sub> was shown in Figure 1. The volume of sedimented phase increased from 0 to 195 μL by increasing the volume of chloroform from 25 to 200 μL. Concerning the curve of the extraction recovery versus the volume of chloroform in Figure 2, the extraction recovery first increased by increasing the volume of chloroform to 100 μL, then decreased. On the other hand, enrichment factor decreases with increasing the volume of CHCl<sub>3</sub> (45.4 to 9.7). At optimum low volume of chloroform, high recovery (60.0%) and enrichment factor (30.8) were obtained. Later, the volume of chloroform as 100 μL was used.

### Effect of type of dispersive solvent and volume

In DLLME miscibility of disperser solvent with extraction solvent and aqueous phase (sample solution) is the main point for the selection of disperser solvent. In this study, acetone, acetonitrile, 1,4-dioxane, ethylene glycol, dimethyl sulphoxide (DMSO) and tetrahydrofuran were studied using 1.0 mL of dispersive solvent containing 100 μL chloroform. When using THF as a dispersive solvent sedimented phase volume was higher as twice than extractant volume. This may due to lower polarity of THF than others. On the contrary of THF, sedimented phase was less



**Figure 3.** Effect of the volume of ACN on the recovery of OTA obtained from DLLME. Extraction conditions: wine sample volume, 5 mL; sedimented phase volume range: 65-100  $\mu$ L; room temperature; concentration of OTA, 0.2  $\text{ng } \mu\text{L}^{-1}$ .

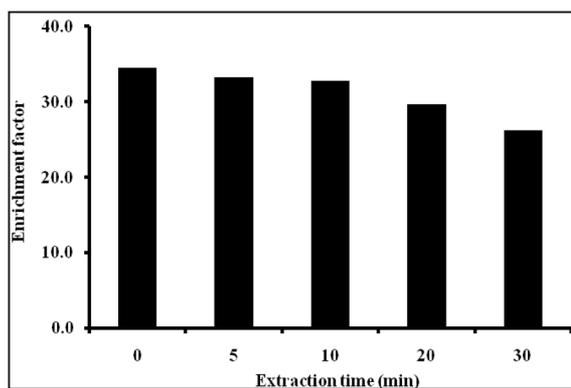
than using ethylene glycol as disperser. White sedimented particules were observed with DMSO. So as shown in Table 2, Recoveries obtained using acetone, 1,4-dioxane and ACN were summarized. Though extraction recoveries with acetone and 1,4-dioxane were almost equal, when used ACN this was a little high. Thus, ACN was chosen of higher recoveries than the others.

**Table 2.** Effect of different dispersive solvents for extraction of OTA by DLLME

Dispersive solvent	ER (%)
Acetone	60.0 $\pm$ 5.5
Acetonitrile	63.9 $\pm$ 2.6
1,4-dioxane	60.0 $\pm$ 0.4

Extraction conditions-wine sample volume: 5.00 mL; extraction solvent (chloroform) volume: 100  $\mu$ L; disperser volume: 0.90 mL; room temperature; concentration of OTA: 0.2  $\text{ng } \mu\text{L}^{-1}$ .

After choosing the ACN as disperser, its volume was optimized by studying different volumes of ACN containing the volume of  $\text{CHCl}_3$  as 100  $\mu$ L. Variation of volume of ACN from 0.15 to 1.10 mL caused change in the volume of sedimented phase from 65 to 100  $\mu$ L. According to the results given in Figure 3, the extraction recovery slightly increased with



**Figure 4.** Effect of extraction time on the enrichment factor of OTA from DLLME.

increasing the volume of disperser up to 0.90 mL. This might caused that ACN could not dispers the extractant sufficiently at low volume. At high volume of ACN, the solubility of OTA in wine increased and the extraction efficiency decreased. Further studies were followed with a 0.90 mL of ACN.

#### Effect of extraction time

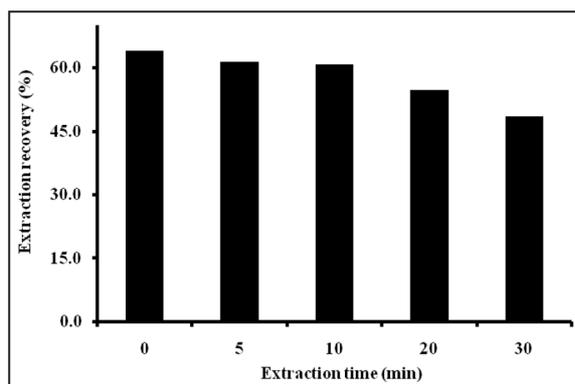
Extraction time is one of the most important parameters in microextraction procedures. In DLLME, extraction time is defined as an interval time between step of injection of extraction mixture and step of centrifugation. For this, different extraction times ranged from 0 to 30 min were studied. Regarding the results (Figures 4 and 5), the variation of ER and EF versus extraction time was not significant. The equilibrium state was achieved quickly.

#### Effect of salt addition

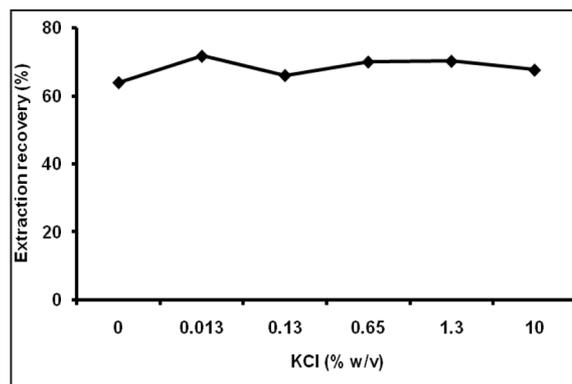
For investigating the influence of ionic strength, various experiments were performed by different concentrations of KCl over the range from 0 to 10%, w/v. The addition of salt had no remarkable change in the volume of the sedimented phase. According to the curve obtained in Figure 6, salt has no remarkable influence on the extraction efficiency.

#### Quantitative analysis Method Validation

Repeatability and linearity were investigated under the optimized experimental conditions. Instrumental calibration curves for OTA standard solutions were linear in the range of 4-100  $\mu$ g



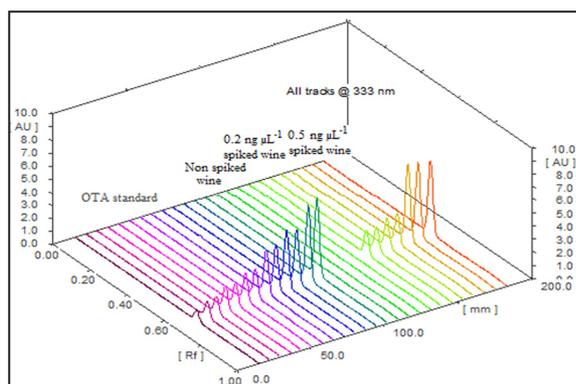
**Figure 5.** Effect of extraction time on the extraction recovery of OTA from DLLME.



**Figure 6.** Effect of salt addition on the extraction recovery obtained from DLLME.

**Table 3.** Results of OTA determination in wine samples (n= 3).

Sample	Added (ng mL <sup>-1</sup> )	Found (ng mL <sup>-1</sup> )	Recovery (R%)	Sample	Added (ng mL <sup>-1</sup> )	Found (ng mL <sup>-1</sup> )	Recovery (R%)
Red Wine 1	-	0.08 ± 0.01	-	Red Wine 9	-	0.04 ± 0.01	-
	0.1	0.16 ± 0.02	86		0.1	0.13 ± 0.02	93
	0.5	0.43 ± 0.02	74		0.5	0.59 ± 0.02	109
Red Wine 2	-	0.10 ± 0.06	-	Red Wine 10	-	0.10 ± 0.01	-
	0.1	0.12 ± 0.00	63		0.1	0.13 ± 0.00 <sub>5</sub>	91
	0.5	0.50 ± 0.03	84		0.5	0.51 ± 0.01	94
Red Wine 3	-	0.05 ± 0.01	-	White wine 1	-	0.07 ± 0.00 <sub>5</sub>	-
	0.1	0.13 ± 0.00	85		0.1	0.16 ± 0.03	94
	0.5	0.64 ± 0.01	103		0.5	0.58 ± 0.00	102
Red Wine 4	-	0.08 ± 0.00	-	White wine 2	-	0.07 ± 0.00	-
	0.1	0.14 ± 0.03	94		0.1	0.12 ± 0.00	73
	0.5	0.58 ± 0.02	100		0.5	0.43 ± 0.03	76
Red Wine 5	-	0.19 ± 0.04	-	White wine 3	-	0.04 ± 0.00 <sub>3</sub>	-
	0.1	0.29 <sub>5</sub> ± 0.01	101		0.1	0.13 ± 0.01	93
	0.5	0.44 ± 0.02	64		0.5	0.52 ± 0.02	96
Red Wine 6	-	0.10 ± 0.01	-	White wine 4	-	0.05 ± 0.00	-
	0.1	0.18 ± 0.01	101		0.1	0.12 ± 0.00	79
	0.5	0.66 ± 0.01	97		0.5	0.51 ± 0.04	91
Red Wine 7	-	0.03 ± 0.00 <sub>5</sub>	-	White Wine5	-	0.04 ± 0.00	-
	0.1	0.11 ± 0.01	84		0.1	0.12 ± 0.01	85
	0.5	0.49 ± 0.00 <sub>5</sub>	87		0.5	0.50 ± 0.00	80
Red Wine 8	-	0.03 ± 0.00 <sub>5</sub>	-	White Wine6	-	0.09 ± 0.03	-
	0.1	0.12 ± 0.05	92		0.1	0.19 ± 0.03	100
	0.5	0.48 ± 0.03 <sub>5</sub>	91		0.5	0.57 ± 0.02	97



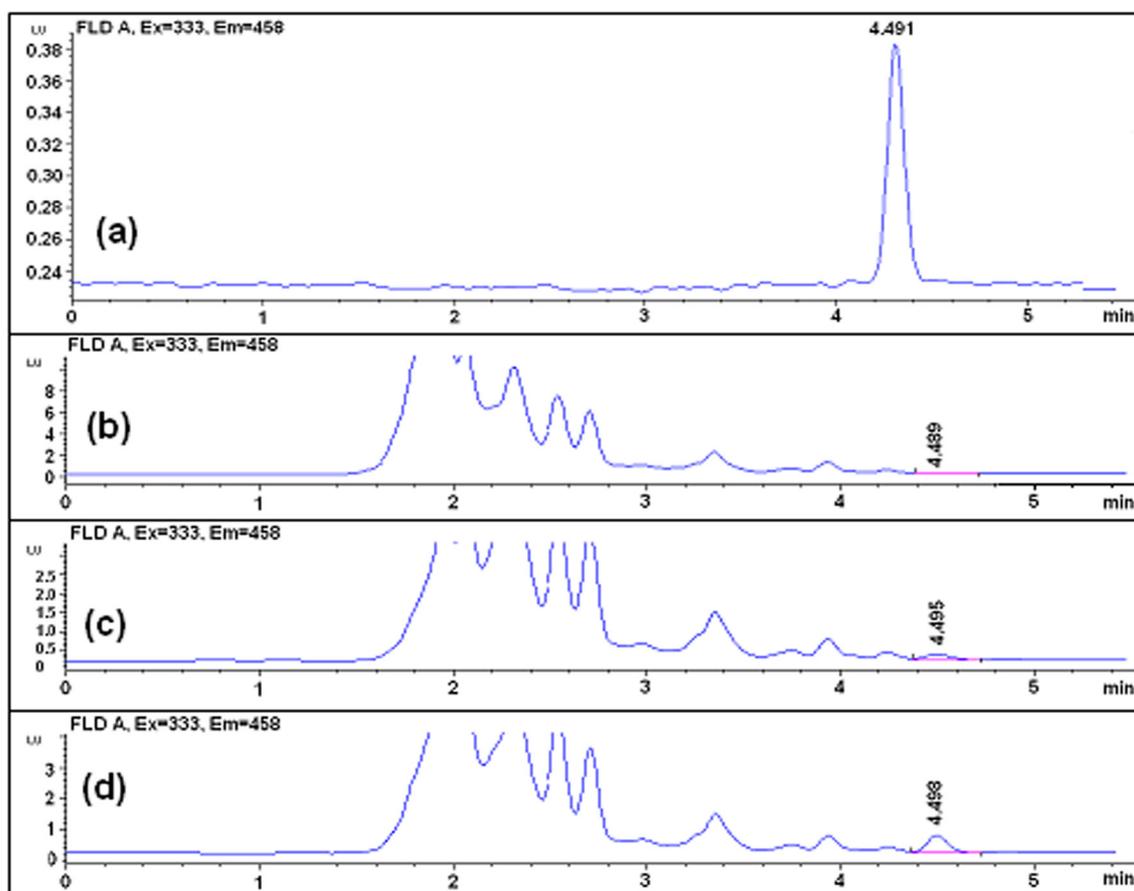
**Figure 7.** Densitograms of OTA standard and OTA spiked and unspiked wine samples on three dimensional spectra.

mL<sup>-1</sup> for HPTLC and 1.0 - 20.0 µg L<sup>-1</sup> for HPLC. The method exhibited linearity for a concentration range from 0.03 to 1.00 µg L<sup>-1</sup> for HPLC and 0.15 to 1.50 µg mL<sup>-1</sup> for HPTLC with correlation coefficients as 0.9974 and 0.9991, respectively. The precision of the proposed DLLME method was

evaluated in terms of repeatability as RSD% < 4.7 and reproducibility as RSD% < 5.3 at 2.5 µg L<sup>-1</sup> OTA solutions for five replicate runs. For each level, five replicate extractions were performed. The limit of detection and the limit of quantification were 0.009 and 0.027 µg L<sup>-1</sup>, based on a signal-to-noise of 3:1 and 10:1, respectively. The enrichment factor and extraction recovery as percentage of the proposed DLLME method were 34.5 and 63.9%, respectively, at 0.2 µg mL<sup>-1</sup> of OTA concentration.

### Real sample analysis

All wine samples were stored in their original bottles and filtered through 0.45 µm filter discs before analysis and analyzed by the proposed DLLME method combined with TLC- fluorescence densitometer. The results showed that all samples were free from OTA contamination (Table 3). For recovery, wine samples were spiked with OTA



**Figure 8.** Typical HPLC chromatogram of a) standard solution containing 2.5 µg L<sup>-1</sup> of OTA, b) a sample of wine unspiked containing approximately 0.03 µg L<sup>-1</sup> of OTA after DLLME method, c) the same wine sample spiked with 0.1 µg L<sup>-1</sup> of OTA after DLLME method, and d) the same wine sample spiked with 0.5 µg L<sup>-1</sup> of OTA after DLLME method.

standards at the concentrations of 0.10 and 0.50  $\mu\text{g L}^{-1}$ . In Figure 7, the HPTLC chromatograms of wine samples before and after spiked with OTA standard and standard solution of OTA in methanol were shown. Also as can be seen in Figure 8, the peak of OTA in wine sample obtained from HPLC chromatogram was free from matrices. Thus, measured concentrations were in reasonable agreement by recoveries from 63 to 103% and 64 to 109 % for spiked OTA concentrations of 0.1  $\mu\text{g L}^{-1}$  and 0.5  $\mu\text{g L}^{-1}$ , respectively, in all wine samples.

## CONCLUSION

With this work the application of DLLME method combined with HPTLC/HPLC-fluorescence detection was achieved for OTA in wine samples. In this technique, OTA was extracted from wine samples with high recovery rates using less amount of organic solvents in a short time. Also, using HPTLC method, paralel analysis of different wine samples and standard of OTA was achieved on the same plate simultaneously.

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