

DEVELOPMENT AND VALIDATION OF A SPE-UHPLC-FLUORESCENCE METHOD FOR THE ANALYSIS OF OCHRATOXIN-A IN CERTAIN TURKISH WINES

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A sensitive Ultra-High Performance Liquid Chromatography (UHPLC)-fluorescence method was developed and validated for the determination of ochratoxin-A (OTA) in Turkish wine samples. Naphthalene was used as an internal standard in this study. OTA was separated on a C18 (3.0 mm × 100 mm × 1.8 μm) column and analyses were run under isocratic conditions, with a mobile phase consisting of water/acetonitrile/acetic acid (50:50:1, v/v/v). The flow rate and injection volume were 0.5 ml min⁻¹ and 10 μl, respectively. The excitation and emission wavelengths were 330 nm and 460 nm for OTA, respectively, and 220 nm and 325 nm for internal standard, respectively. A solid-phase extraction (SPE) clean-up procedure on a C18 cartridge was used prior to the analysis of the wine samples by UHPLC. The developed method was validated with respect to linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), stability and robustness. The method presented good RSD (< 4 %) and recovery (102.6–105.2 %) values. The LOD and LOQ values were 0.01 ng ml⁻¹ and 0.05 ng ml⁻¹, respectively. All other parameters were acceptable. OTA amounts were found in the range of 2.72–7.40 μg kg⁻¹ in the Turkish wine samples.

Keywords: UHPLC-FLD; ochratoxin A; Turkish wine; validation; SPE

РАЗРАБОТКА И ВАЛИДАЦИЈА НА SPE-UHPLC-ФЛУОРЕСЦЕНТЕН МЕТОД ЗА АНАЛИЗА НА ОХРАТОКСИН-А ВО НЕКОИ ТУРСКИ ВИНА

Разработен е и валидиран осетлив ултрависокоефикасен течен хроматографски (UHPLC) флуоресцентен метод за определување на охратоксин-А (ОТА) во примероци на турски вина. Како внатрешен стандард во ова истражување беше употребен нафтелен. Раздвојувањето на ОТА беше извршено на колона C18 (3.0 mm × 100 mm × 1.8 μm), а анализите беа извршени во изократски услови, каде мобилна фаза се состоеше од вода/ацетонитрил/оцетна киселина (50:50:1, v/v/v). Протоколот и инјектираниот волумен соодветно изнесуваа 0,5 ml min⁻¹ и 10 μl. Брановите должини за екситација и емисија изнесуваа соодветно 330 nm и 460 nm за ОТА, односно, соодветно 220 nm и 325 nm за интерниот стандард. Пречистувањето беше извршено со цврсто-фазна – SPE постапка на C18 картици пред да се изврши анализата на примероците вино со UHPLC. Разработениот метод беше валидиран во однос на линеарноста, прецизноста, точноста, прагот на детекција (LOD), прагот на квантификација (LOQ), стабилноста и робустноста. Методот покажа добри вредности за релативната стандардна девијација – RSD (< 4%) и аналитичкиот принос (102,6–105,2%). Вредностите за LOD и LOQ соодветно изнесуваа 0,01 ng ml⁻¹ и 0,05 ng ml⁻¹. Сите останати параметри беа задоволувачки. Количеството на ОТА во примероците на турските вина изнесуваа во опсегот од 2,72–7,40 μg kg⁻¹.

Клучни зборови: UHPLC-FLD; охратоксин-А; турски вина; валидација; SPE

1. INTRODUCTION

Mycotoxins are toxic secondary fungous metabolites that have negative effects on humans, plants, animals, and microorganisms. Ochratoxin-A (OTA), the most commonly encountered mycotoxin in food samples, is generated by various genera of *Aspergillus* and *Penicillium* and may exist naturally in several foods, including coffee, cocoa, spices, dried fruits, cereals, cereal products, beans, groundnuts, milk, meat, beer, grape juice and wine. OTA possesses immunotoxic, nephrotoxic, embryotoxic, teratogenic, hepatotoxic, genotoxic, neurotoxic, and carcinogenic effects. Moreover, OTA has a long half-life (about 35 days) in human serum, meaning that it cannot be eliminated quickly from the plasma [1–5]. All of these factors make OTA among the most interesting of mycotoxins.

Humans have been exposed to OTA from an extensive range of foods which show low levels of contamination [6]. Wine is considered one of the most common sources of OTA contamination, especially in European countries, after cereals [7]. Grapes are the origin of OTA contamination in wines, because deteriorated grapes can be readily infected by fungi and generate OTA [3]. The Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) proposed that the tolerable weekly intake of OTA should be 100 ng kg⁻¹ of body weight (b.w.) [8]. The tolerable daily intake of OTA should be less than 5 ng kg⁻¹ of b.w. according to the Scientific Committee for Food (SCF) [9]. European Commission Regulation EC 1881/2006 [10] set the maximum level of OTA to 2.0 µg kg⁻¹ for wines (red, rosé and white). Because of the toxicity of OTA, even in very small amounts, highly selective and sensitive analytical methods are required for the analysis of OTA in food samples [11]. With regard to food, however, there is always a need for the development of new and reliable analytical methods. OTA can be determined in various foods using an array of analytical methods. These include enzyme-linked immunosorbent assay (ELISA) [12, 13], thin layer chromatography (TLC) [14], gas chromatography-mass spectrometry (GC-MS) [15, 16], high performance liquid chromatography (HPLC) [17, 18] and ultra-high performance liquid chromatography (UHPLC) with fluorescence detection (FLD) [19] or mass spectrometry (MS) [20]. Of these methods, HPLC and UHPLC are commonly utilized for the analysis of OTA because of their high separation efficiency and the low limits of quantification of used detectors. In

recent years, UHPLC has replaced HPLC due to its improved instrumentation and column technologies. UHPLC provides high resolution separation, more sensitive analysis and rapid method development due to its lower analysis time and faster column equilibration. Advanced UHPLC detectors are favored for obtaining small peak widths without significant band broadening and also the improved signal-to-noise ratios of detectors allow the detection of analytes at extremely low concentrations. In addition, UHPLC columns packed with sub-2µm particles exhibit enhanced separation efficiency compared to conventional HPLC columns [11, 21–23].

The aim of this study was to develop and validate a new and a sensitive UHPLC-FLD method using an internal standard (IS) for the analysis of OTA in Turkish wines. Numerous studies using liquid chromatographic methods, including HPLC and UHPLC with fluorescence (FLD) [24–26] and mass spectrometry (MS) detectors [27, 28], have reported the presence of OTA in wines originating from various regions. However, there have been few HPLC studies regarding Turkish wines using conventional HPLC columns. Anlı et al. [29] studied the OTA contents in 47 wines from different regions in Turkey using the HPLC-FLD method. They separated OTA in approximately 25 minutes on an SGE Wakosil C18 HPLC column (4.6 mm × 250 mm × 5 µm). They used an extraction method following a Sep-pack cartridge for preparation of the wine samples. However, their sample preparation method was laborious and the HPLC method validation was not exhaustive. Moreover, they did not use an IS during the research. Var and Kabak [7] also studied OTA in different Turkish wines. They separated OTA in 13 minutes using an ACE C18 column (4.6 mm × 250 mm × 5 µm) and they also did not utilize an IS. They used an immunoaffinity column (IAC) to clean-up Turkish wines. Altıokka et al. [30] determined the OTA levels in 25 different Turkish wines by HPLC-FLD with a reversed phase Nucleosil C18 column (4.6 mm × 150 mm × 3 µm). They used diflunisal as an IS and both OTA and IS were separated within 13 minutes. They did not use any extraction or concentration steps for their wine samples and injected the samples directly into the chromatographic system. To the best of my knowledge, there have been no studies of Turkish wines using a UHPLC-FLD method with newly generated small particle UHPLC columns. As a result, it is believed that this study will contribute to the literature associated with OTA determination in Turkish wines. In summary, when the related studies are examined in

the literature, the described method has some advantages such as shorter analysis time, lower costs due to the consumption of less solvent in UHPLC, better performance characteristics and the use of an IS. In addition, a full method validation was performed in this study.

2. EXPERIMENTAL

2.1. Chemicals and materials

The analytical standards of OTA and naphthalene (IS), methanol ($\geq 99.9\%$) and acetonitrile ($\geq 99.9\%$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid (99.5%) was provided by Carlo Erba (Milan, Italy). Solid phase extraction (SPE) was performed using a Supelclean 500 mg C18 SPE cartridge (Supelco, Bellefonte, PA, USA). The Sartorius water purification system (Rotterdam, Netherland) was used to

obtain ultra-pure water. A Sonorex ultrasonic bath (Bandelin, Berlin, Germany) was utilized for the degassing of solutions. A Vortex (2G 560-E, Daigger Scientific Industries, Bohemia, USA) was utilized for the mixing process. All of the standard and sample solutions were filtered from 0.2 μm particle size with 25 mm diameter disposable cellulose acetate syringe filters (Sartorius, Germany) before the UHPLC injections.

2.2. Wine samples

The samples are commercial wines and all the wines used in the experiments were of good quality. Red, rosé and white wine samples were bought from several local markets in Eskisehir (Turkey). In this study, different Turkish wine brands were used from various regions of Turkey; the properties of wine samples are given in Table 1.

Table 1

The properties of commercial Turkish wine samples used in this study

Sample No	Color	Alcohol content (v/v %)	Production year	Region	Grape varieties
1	Red	12	2014	Aegean	Bogazkere&Carignan
2	White	12	2014	Thrace	Sauvignon Blanc&Chardonnay
3	Red	14.5	2013	Denizli-Aegean	Shiraz&Merlot&Kalecik Karası&Cabernet Sauvignon
4	White	11.5	2014	Murefte (Thrace)&Aegean	Sultaniye&Semillion
5	White	12	2014	Cappadocia&Tokat	Anatolian
6	White	11	2014	Aegean	Sultaniye&Chardonnay
7	White	13.5	2014	Denizli-Aegean	Sultaniye
8	Rosé	14.5	2013	Thrace&Aegean	Calkarasi&Cinsault
9	Red	13.8	2014	Izmir&Aegean	Cabernet Sauvignon&Alicante&Merlot&Carignan
10	Red	12.5	2014	Elazığ&Diyarbakır	Okuzgozu&Bogazkere
11	Red	14.5	2013	East-Anatolia&Aegean	Okuzgozu&Shiraz&Alicante
12	Red	13	2014	Tekirdag-Thrace	Shiraz&Merlot
13	Red	15	2013	Denizli-Aegean	Bogazkere&Shiraz
14	Red	13.5	2014	Elazığ&Diyarbakır&Izmir	Okuzgozu&Bogazkere&Alicante&Carignan
15	Red	14.5	2013	Thrace&Aegean	Cinsault&Alicante&Bogazkere

2.3. Instrumentation and UHPLC-FLD Conditions

The analyses were conducted with an Agilent 1290 Infinity UHPLC instrument (Waldbronn, Germany) using a fluorescence detector (FLD). OTA was separated on a C18 Zorbax Eclipse Plus (3.0 mm \times 100 mm \times 1.8 μm) UHPLC column with a mobile phase system composed of water:acetonitrile:acetic acid (50:50:1, v/v/v) under

isocratic conditions. The injection volume and flow rate were 10 μl and 0.5 ml min^{-1} , respectively. Nine μl of OTA standard or wine sample and 1 μl of IS (1.82×10^{-6} M) were mixed by the auto sampler. During the analyses, the UHPLC column and auto sampler were kept at 25 $^{\circ}\text{C}$ and 10 $^{\circ}\text{C}$, respectively. The excitation and emission wavelengths were 330 and 460 nm for OTA, and 220 and 325 nm for IS, respectively.

2.4. Preparation of solutions

One mg of OTA was dissolved in 1 ml of methanol and stored at $-20\text{ }^{\circ}\text{C}$. A working solution of OTA (1000 ng ml^{-1}) was prepared in methanol/water (50:50, v/v) and further OTA standard solutions (0.36, 3.60, 9.00, 18.00, 45.00, 180 ng ml^{-1}) were prepared from this solution. For the preparation of a stock naphthalene (IS) solution, 15.5 mg of naphthalene was weighed and dissolved in 10 ml of methanol. Following this, in order to prepare a working solution of $1.82 \times 10^{-6}\text{ M}$, the appropriate volume of stock IS solution was diluted with methanol/water (50:50, v/v).

2.5. Solid phase extraction (SPE) method

The solid phase extraction (SPE) method was conducted following previous studies by Hernandez et al. [31] and Leitner et al. [28] with slight modifications. A C18 SPE cartridge was preconditioned using 5 ml of methanol and then 5 ml of water without allowing the cartridge to dry. Ten ml of a wine sample was transferred to the cartridge. Subsequently, the cartridge was washed with 2 ml of water and then dried for approximately 30 seconds. OTA was eluted through 2 ml of methanol and the residue was diluted with water. Then, the sample solution was filtered from syringe filter before the UHPLC injection.

2.6. Validation of the UHPLC-FLD method

Validation of the UHPLC-FLD method was conducted via precision, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, stability and robustness, according to ICH [32]. In order to test the precision of the proposed method, repeatability studies were carried out by means of measuring intra-day and inter-day values for three concentration levels of OTA. Accuracy was tested via recovery percentage by spiking with three concentration levels of standard OTA prior to extraction from rosé wine. The spiked samples were extracted twice and injected in triplicate under the previously established optimum UHPLC-FLD conditions. The linearity of the calibration curve was evaluated for six different OTA standard levels in the range of $0.36\text{--}180\text{ ng ml}^{-1}$ under optimum UHPLC-FLD conditions. A fixed concentration of $1.82 \times 10^{-7}\text{ M}$ IS was used in all the experiments. The stability of OTA was evaluated using three different concentrations under various conditions, including short-term stability, long-term stability and freeze-thaw stability. The OTA solutions

were kept for 24 hours at room temperature in order to test short-term stability. For long-term stability, the OTA solutions were left at $-20\text{ }^{\circ}\text{C}$ for two weeks. To test the freeze-thaw stability, the OTA solutions were kept at $-20\text{ }^{\circ}\text{C}$ and then thawed to room temperature three times. The robustness was tested with small variations in the UHPLC-FLD method parameters. In this context, column temperature, flow rate, and the composition of acetonitrile were altered for the robustness study.

In this study, the peak-normalization (PN) method calculating the ratio of peak area (A) values to their retention times (Rt), $\text{PN}=\text{A}/\text{Rt}$, was used. After this, the ratios of PN values for OTA and IS ($R = \text{PN}_{\text{OTA}}/\text{PN}_{\text{IS}}$) were used. All of the statistical calculations were performed at a 95% probability level.

2.7. Statistical analysis

The Graph Pad Prism v6.0 program and Microsoft Excel programs were utilized to carry out the statistical analyses.

3. RESULTS AND DISCUSSION

3.1. Optimization of the UHPLC-FLD method

In general, chromatographic separations of OTA in wine have been carried out using conventional HPLC C18 columns and isocratic elution with acidified acetonitrile or methanol and water in the literature. Sodium acetate-acetic acid and an acetonitrile mobile phase systems were also used in some studies [33]. The proposed UHPLC-FLD method was developed according to the methods used for determination of OTA [26, 34], with slight modifications. In this study, a C18 Zorbax Eclipse Plus ($3.0\text{ mm} \times 100\text{ mm} \times 1.8\text{ }\mu\text{m}$) UHPLC column was utilized during the analyses with similar to Turcotte and Scott [34]. During the analyses, the UHPLC column and auto sampler were kept at $25\text{ }^{\circ}\text{C}$ and $10\text{ }^{\circ}\text{C}$ different from Gentile et al. [26] and Turcotte and Scott [34]. Various flow rates such as 0.4 ml min^{-1} , 0.5 ml min^{-1} and 0.6 ml min^{-1} were applied in order to achieve the best resolution of OTA from other peaks in wine matrices using the mobile phase system of water:acetonitrile:acetic acid (50:50:1, v/v/v). The optimum flow rate was found to be 0.5 ml min^{-1} . In this study, the mobile phase is also similar to that used by Gentile et al. [26] and Turcotte and Scott [34]. The appropriate injection volume was applied as $10\text{ }\mu\text{l}$ when considering the size of the UHPLC column. Different

chemicals including caffeine, diflunisal, methyl, ethyl, propyl and butyl paraben, were tried as an IS and considering the wine matrices, naphthalene was found to be the most suitable IS for this system. Gentile et al. [26] and Turcotte and Scott [34] did not use an IS, unlike this study.

OTA and IS were detected at retention times of 4.2 and 7.9 minutes, respectively, under the previously mentioned optimum UHPLC-FLD chromatographic conditions ('Section 2.3.'). Typical UHPLC-FLD chromatograms of OTA standard (45.0 ng ml⁻¹), white wine and red wine with fixed amounts of IS (1.82 × 10⁻⁷ M) under optimum UHPLC-FLD conditions are shown in Figure 1.

There are very effective HPLC-FLD methods using immunoaffinity (IAC) clean up columns available [2, 26]. Even though IACs allow the highly selective isolation of analyte from a complex matrix, IACs have some drawbacks such as being laborious, their high cost, their limited storage time (usually 12 months), the use of large amounts of solvents and being time consuming [28, 35]. On the other hand, it was reported that IAC columns have shown low recoveries in the analysis of wines [36–38]. Therefore, the SPE clean-up procedure was preferred in this study because it is easier, cheaper and more efficient than IAC.

There have been a number of studies in the literature related to the determination of OTA in wines manufactured in many other countries using UHPLC methods. Table 2 shows a comparison of certain UHPLC methods with this method for the

analysis of OTA in wines. The present study contributes to data on the occurrence of OTA in Turkey by UHPLC-FLD. The described analytical method is different in view of the use of an IS.

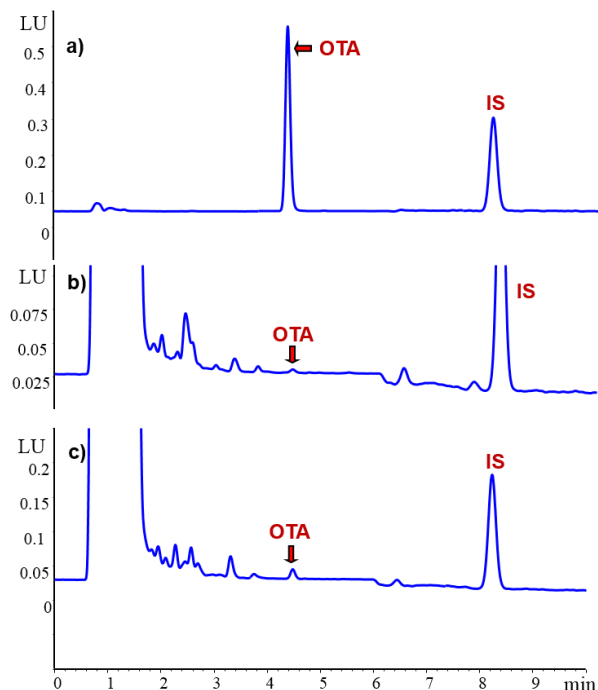


Fig. 1. Typical UHPLC-FLD chromatograms of a) OTA standard (45.0 ng ml⁻¹), b) white wine, c) red wine with fixed amounts of IS (1.82 × 10⁻⁷ M) in the optimum UHPLC-FLD conditions

Table 2

Comparison of analytical methods for analysis of OTA in wines by UHPLC

Method-detector	Rt (min) of OTA	Internal standard (IS)	Clean-up	Mobile phase	Column	LOD (ng ml ⁻¹)	LOQ (ng ml ⁻¹)	Wine origin	Ref.
UPLC-FLD	NA	NA	IAC	Water: acetonitrile: acetic acid (49:49:2, v/v/v) isocratic elution	Waters BEH C18 (2.1 mm × 50 mm × 1.7 μm)	0.01	0.04	Southern Italy (organic farming)	[26]
UHPLC-MS	1.59	NA	SPE	Solvent A: 0.1% formic acid in water Solvent B: 0.1% formic acid in methanol Gradient elution	BEH Shield RPC18 (2.1 mm × 100 mm × 1.7 μm)	0.54	1.81	France, Italy, Australia, Chile	[27]
UHPLC-FLD	4.00	NA	On-line SPE	Solvent A: 80% Acetonitrile Solvent B: 1 % formic acid in water (pH 2.15) Gradient elution	Kinetex XB-C18 100 A° (3.0 mm × 30 mm × 1.7 μm)	0.15	0.51	Romania	[39]
UHPLC-FLD	4.20	Naphthalene	SPE	Water: acetonitrile: acetic acid (50:50:1, v/v/v) isocratic elution	C18 Zorbax Eclipse Plus (3.0 mm × 100 mm × 1.8 μm)	0.01	0.05	Turkey	Present study

NA: Not available

3.2. Validation of the UHPLC-FLD method

3.2.1. Precision

To evaluate the precision of the method, 3.60, 45.0 and 180 ng ml⁻¹ of OTA solutions were injected over three consecutive days, six times each. The data are summarized in Table 3. The relative standard deviation (RSD or RSD_r: within-laboratory RSD) values were found to be less than 4 %. The RSD values were evaluated according to the performance criteria parameters for OTA reported in Regulation (EC) No. 401/2006 [40] and

Regulation (EC) No. 519/2014 [41] and RSD values were within acceptable limits as ≤ 20 (RSD_r % for 1–10 µg kg⁻¹ of OTA level).

3.2.2. Linearity, limit of detection (LOD), limit of quantification (LOQ)

The method was linear in the calibration range of 0.36–180 ng ml⁻¹ for OTA with good r² values being between 0.9982 and 0.9989 (Table 4). LOD and LOQ values were 0.015 ng ml⁻¹ and 0.047 ng ml⁻¹, respectively.

Table 3

Repeatability assay of OTA

3.60 ng ml ⁻¹ OTA	Intra-day results (n = 6 each)			Inter-day results (n = 18)
	First day	Second day	Third day	
\bar{X}	0.16	0.16	0.15	0.16
SD	0.0047	0.0018	0.0056	0.006
RSD%	2.83	1.16	3.60	3.79
45.0 ng ml ⁻¹ OTA	Intra-day results (n = 6 each)			Inter-day results (n = 18)
	First day	Second day	Third day	
\bar{X}	2.31	2.33	2.35	2.33
SD	0.0514	0.0427	0.0443	0.047
RSD%	2.22	1.83	1.88	2.02
180 ng ml ⁻¹ OTA	Intra-day results (n = 6 each)			Inter-day results (n = 18)
	First day	Second day	Third day	
\bar{X}	10.57	10.39	10.24	10.40
SD	0.236	0.246	0.204	0.257
RSD%	2.23	2.36	1.99	2.47

\bar{X} : mean, SD: standard deviation, RSD %: percent of relative standard deviation

Table 4

Linearity assay in the range of 0.36–180 ng ml⁻¹ of OTA

	Intra-day results (n = 6 each)			Inter-day results Pooled (n = 18)
	First day	Second day	Third day	
a ± SD	0.0598 ± 0.0004	0.0708 ± 0.0004	0.0580 ± 0.0003	0.0583 ± 0.0002
b ± SD	-0.1783 ± 0.0337	-0.2030 ± 0.0347	-0.0980 ± 0.0261	-0.0978 ± 0.0178
r²	0.9982	0.9986	0.9989	0.9983

a: slope, b: intercept, r: correlation coefficient

3.2.3. Accuracy

To test accuracy, 1.80, 3.60 and 5.40 ng ml⁻¹ of OTA was added to rosé wine (Table 5). The re-

covery values of OTA were found to be from 102.56 % to 105.16 %, with RSD values below 5 %.

Table 5

Recovery assay for rosé wine ($l = 2; n = 3$)

Spiked OTA concentration (ng ml ⁻¹)	Recovery%	SD	RSD%
1.80	102.56	4.19	4.08
3.60	105.16	1.77	1.68
5.40	104.65	2.94	2.81

l: number of experiments, n: number of injections
 Recovery % = $[(C_{\text{spiked sample}} - C_{\text{unspiked sample}}) / C_{\text{added}}] \times 100$ %

These recovery percentage values were acceptable according to Regulation (EC) No. 401/2006 [40] and Regulation (EC) No. 519/2014 [41] (it is recommended that Recovery % should be 70 to 110 for ≥ 1 $\mu\text{g kg}^{-1}$ of OTA level).

3.2.4. Stability

The short-term stability, long-term stability and freeze-thaw stability were investigated; results are presented in Table 6. The results indicate that OTA is stable under the mentioned conditions.

Table 6

Stability of OTA under various conditions ($n = 3$)

Theoretical concentration (ng ml ⁻¹)	Short-term stability (24 h, room temperature)		Long-term stability (2 weeks, -20 °C)		Freeze-thaw stability (3 cycles)	
	Recovery (%) (mean \pm SD)	RSD (%)	Recovery (%) (mean \pm SD)	RSD (%)	Recovery (%) (mean \pm SD)	RSD (%)
3.6	104.71 \pm 0.33	0.32	96.49 \pm 3.04	3.15	95.89 \pm 2.61	2.72
OTA 45	103.49 \pm 0.03	0.03	94.20 \pm 2.51	2.66	94.87 \pm 2.20	2.32
180	104.80 \pm 0.08	0.08	97.03 \pm 1.98	2.04	94.16 \pm 0.93	0.99

3.2.5. Robustness

The results of the robustness for OTA are introduced in Table 7, and it can be stated that the proposed UHPLC-FLD method is robust with low RSD percentage and standard error of the mean (SE) ($SE = \frac{SD}{\sqrt{n}}$) values.

Table 7

The robustness of OTA (45 ng ml⁻¹)

Parameter	RSD%	SE
Flow rate (ml min ⁻¹)		
0.4	1.84	0.02
0.6	0.05	0.001
Column temperature (°C)		
20	0.08	0.001
30	3.24	0.04
The composition of acetonitrile %		
49	1.39	0.01
51	1.76	0.02

3.3. Application of the UHPLC-FLD to Turkish wine samples

The developed and validated UHPLC-FLD method was performed on fifteen different Turkish

wine samples, including white, rosé and red wines after SPE. The OTA amounts were found to be between 2.72 and 7.40 $\mu\text{g kg}^{-1}$ in the studied Turkish wine samples; the results are shown in Table 8. OTA was not detected in white wine samples 2 and 5 in the proposed method. In all other samples, OTA concentration exceeded the maximum level given in European Commission Regulation EC 1881/2006 (2 $\mu\text{g kg}^{-1}$) [10]. Generally, red wines may contain higher amounts of OTA than rosé and white wines and it is probably due to the different winemaking process [26, 28, 33]. The current study also confirmed this finding and OTA amounts in white wines were found to be lower than those for most of the red and rosé wines. The results indicated that closer OTA amounts were detected in white wine samples 4, 6 and 7, which were produced from similar grape varieties. On the other hand, two red wine samples (sample 10 and sample 14) with the highest OTA contents were from the Eastern and Southeastern Anatolia regions. Wine samples 8 and 15, including similar OTA quantities, were produced in same area (Thrace & Aegean); however, one was rosé wine and the other wine was red wine, and their grape varieties were different from each other, apart from Cinsault. Red wine sample 12, which has a higher OTA content than twelve of the wine samples, was produced only from the grapes of Thrace region,

unlike the other samples. Considering the results for the rest of the studied wines, it can be said that the regions of wines were similar but they were made from different types of grapes. Therefore, their results were not directly comparable. As a result, the lower/higher occurrence of OTA in wine depends on many factors such as wine making techniques, year of production or wine region due to the climatic conditions (temperature and atmospheric relative humidity), grape cultivation, mycoflora composition and storage conditions [26, 28, 31, 33].

Table 8

OTA levels ($\mu\text{g kg}^{-1}$) in various Turkish wine samples ($n = 3$)

Wine sample	\bar{X}	SD	RSD%
1 (Red)	2.72	0.01	0.53
2 (White)	<LOD	—	—
3 (Red)	4.26	0.02	0.54
4 (White)	4.41	0.07	1.68
5 (White)	<LOD	—	—
6 (White)	4.22	0.02	0.51
7 (White)	4.40	0.08	1.92
8 (Rosé)	4.04	0.05	1.22
9 (Red)	4.63	0.03	0.65
10 (Red)	6.68	0.09	1.39
11 (Red)	5.08	0.02	0.47
12 (Red)	6.41	0.04	0.62
13 (Red)	4.90	0.05	1.07
14 (Red)	7.40	0.07	0.94
15 (Red)	4.48	0.04	0.92

4. CONCLUSION

An efficient and a sensitive UHPLC-FLD method has been developed and validated for OTA analysis in different Turkish wine samples. The proposed UHPLC-FLD method is reliable, reproducible and linear for the analysis of red, rosé and white wines. The LOD and LOQ values are quite low at 0.01 ng ml^{-1} and 0.05 ng ml^{-1} , respectively. OTA amounts were found to be in the range of $2.72\text{--}7.40 \mu\text{g kg}^{-1}$ in the Turkish wine samples. OTA contents were found to be below LOD in two of the white wine samples. In the other wine samples, OTA was found to be above the upper permitted limit of $2.0 \mu\text{g kg}^{-1}$ for wines, according to the European Commission Regulation EC 1881/2006 [10]. In conclusion, it is proposed that this

UHPLC-FLD method may be successfully and sensitively apply for the determination of OTA in wines.

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