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Determination of Ochratoxin A in Turkish Wines

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ABSTRACT

A validated, simple and highly sensitive analytical method was described for the determination of Ochratoxin A (OTA) in various wines produced in Turkey. OTA concentrations of 25 wine samples were examined using high performance liquid chromatography coupled with fluorescence detection. OTA and diflunisal (internal standard) were separated using isocratic elution mode with a reversed phase Nucleosil® C_{18} column. The mobile phase consisted of CH_3CN : H_2O : CH_3COOH (50 : 48 : 2, v/v/v) was pumped at a flow rate of 0.4 mL/min. The analytes were detected at 330 nm excitation and 450 nm emission wavelengths within an average time of 13 min. Samples were prepared by simply filtrating the wine samples through a 0.2 µm filter and injected into the system without further extraction or concentration steps. OTA was detected in µg/L levels with adequate chromatographic resolution. It was found that the amount of OTA was higher than the permitted limits (< 2 µg/L) in 14 out of 25 samples, especially in red wines.

Key words: Ochratoxin, Mycotoxins, Liquid chromatography, Wine

INTRODUCTION

Ochratoxin A (OTA, N-[[(3R)-5-Chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl] carbonyl]-L-phenylalanin) is a mycotoxin produced by *Aspergillus ocraceus, Aspergillus carbonarius, Penicillium verrucosum* and other related species, which occur in many cereals (e.g. coffee beans, nuts, wheat), spices, dairy products and beverages. OTA is a potent nefrotoxic, hepatotoxic, immunosuppressive, teratogenic and carcinogenic compound which has been classified as possible carcinogen to humans (Group 2B) by the International Agency for Research on Cancer (IARC)[(1)]. Chemical structure of OTA is shown in Figure 1.

OTA and similar carcinogenic mycotoxins such as trichothecenes, fumonisins and aflatoxins constitute a major group of most analyzed food contaminants worldwide. OTA is found in many commonly consumed food products involving different routes of intake for humans; therefore, studies and improvements on this topic are popular and necessary to determine OTA levels in foods.

Since the beginning of 1980's, frequency and level of OTA occurrence in foods and tissue samples have been studied by many research groups. OTA has been detected in different foods such as dairy products, animal feeds, cereals and beverages, including wines, as well as in tissue samples of urine, blood, kidney, and liver, etc. These findings indicate that many types of foods and animals are subjected to OTA in different stages of production or feeding. Since OTA is produced by fungi of heterogeneous nature, infection of products with these organisms is the source of OTA. Therefore, it is important to trace the sources of vegetable contamination with rapid, selective, sensitive and cost effective assays.

Several methods have been reported for determination of OTA including chromatographic, electrophoretic and ELISA techniques, covering the analysis of

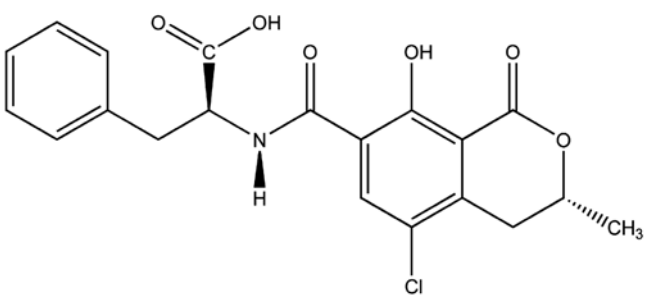

Figure 1. Chemical structure of OTA

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various sample matrices either with or without validation. Many of these studies were compared in terms of instrumentation, application, and determination capabilities and assessed in several reviews[2-4]. In addition, an inter-laboratory study on the determination of OTA in different types of wine samples was carried out by the participation of 24 different laboratories from all continents[5]. However, there are limited studies reported in the literature on OTA in Turkish food products[6-10] .

The methods previously published on OTA generally require intensive extraction and clean-up procedures as well as relatively expensive reagents and instrumentation, which are not economically feasible and preferable for the routine analysis of OTA. The aim of this study was to develop an inexpensive, simple and highly sensitive method for the determination of OTA in wines, based on the procedure described by Aboul-Enein *et al*[6]. Main advantages of the proposed method to the previous ones are low cost, simplicity, high accuracy and precision obtained by utilization of an internal standard (IS). The developed method was validated according to the recommendations of International Conference on Harmonisation (ICH)[12] and United States Pharmacopeia (USP)[13]. Furthermore, this is the first study on OTA analysis of different wines produced in Turkey. In 2005, Turkish wine industry has a market potential about USD 560 million, covering 25% of the Middle East region and 1% globally[11]. Despite this high potential, few studies on the quality control of Turkish wine and grape products are available[6-11]. Therefore, investigation and evaluation of OTA levels in Turkish wines is important to designate the OTA profile of Turkish wine industry.

MATERIALS AND METHODS

I. *Chemicals and Materials*

Standard OTA at ≥ 98% purity level was purchased from Sigma-Aldrich Chemie GmbH (Germany). Diflunisal which was used as internal standard at 99.8% purity level was kindly supplied by Sanovel Pharmaceuticals Inc. (Turkey). Acetonitrile (LiChrosolv® for chromatography) and acetic acid were obtained from Merck KGaA (Germany). Double distilled water was produced in our laboratory. Standard solutions and samples were filtered through PVDF filters (0.20 µm, 13 mm I.D.) from Orange Scientific (Belgium). Wine samples were purchased from local markets and retail stores in Turkey and stored at 4°C prior to analysis. All the information regarding analysed samples such as geographical origin of grapes and production year were collected from the bottle labels.

II. *Apparatus*

Chromatographic analyses were performed using a Shimadzu LC system consisted of an LC-20AT model gradient pump, CBM-20A communication bus module, CTO-10ASvp column oven, RF-10AXL fluorescence detector and DGU-20A5 model degasser (Japan). Samples were injected to the system via 10 µL stainless steel loop which was connected to a Rheodyne 7725i manual injection port (USA). The analytes were resolved in a Teknokroma Nucleosil® C_{18} column (150 × 4.6 mm I.D., particle size 3 µm) (Spain) column. Data were processed by Shimadzu LabSolutions LCsolution v1.11 SP1 data analysis software installed on an IBM-compatible computer.

III. *Chromatographic Conditions*

An isocratic reversed-phase elution was applied throughout the study. The mobile phase consisted of CH_3CN : H_2O : CH_3COOH (50 : 48 : 2, v/v/v) which was degassed and filtered through 0.20 µm filters before analysis. Mobile phase was pumped at a flow rate of 0.4 mL/min. The fluorescence detector was set up at wavelengths of 330 nm for excitation and 450 nm for emission of OTA. A solution consisting of CH_3CN : H_2O : CH_3COOH (80 : 18 : 2, v/v/v) was used to wash the column after each injection for 10 minutes at a flow rate of 0.40 mL/min.

IV. *Standard Solutions*

Standard solutions of OTA were prepared in acetonitrile at the concentration of 0.10 g/L and diluted to the working range using the same solvent. Diflunisal (IS) solutions were prepared in water at the concentration of 22.50 g/L. Since IS is soluble in alkaline media, two drops of 1 M NaOH solution were added to the flask prior to preparation. IS concentration was kept constant at 22.50 µg/L throughout the study. All of the solutions were protected from light and used within 24 h in order to avoid decomposition.

V. *Sample Preparation*

Two mililiters of each wine sample was filtered through a 0.2 µm filter, spiked with IS and directly injected into the chromatographic system. All samples were analyzed in triplicate. No extraction or concentration steps were applied to render the methodology as simple as possible.

RESULTS AND DISCUSSION

I. *Method Development*

An isocratic reversed-phase liquid chromatographic procedure is proposed as a suitable method for the analysis of OTA in wines. A conventional C_{18} column, packed with 3 µm sized Nucleosil® material, was used as stationary phase. Method development began by testing

Journal of Food and Drug Analysis, Vol. 17, No. 6, 2009

different types of mobile phases to provide a fast elution that possesses adequate analytical quality. Several mobile phase compositions were prepared and tested to obtain relatively short retention and good resolution for the analytes of interest. During preliminary experiments it was observed that CH_3CN : H_2O : CH_3COOH (50 : 48 : 2, v/v/v) solution provided the retention time of 11.5 min for OTA. The composition of mobile phase was slightly modified from the one used by Aboul-Enein *et al.*[6] Although CH_3CN : H_2O (50 : 50, v/v) solution was suitable for the resolution, acetic acid was added to to enhance the peak shapes of the analytes. As OTA is a weak acidic compound, the mobile phase was intended to be slightly acidic to avoid tailing and adsorption to the column. In addition, acetonitrile was preferred to methanol as acetonitrile–water mixtures had lower viscosity and better separation efficiency than methanol-water mixtures.

Samples were pumped through the column at a flow rate of 0.4 mL/min, giving a tolerable back pressure of about 90 bars. The retention time was acceptable and suitable for the analysis of wine samples, especially since the matrix signals appeared in the first few minutes of the analysis and did not interfere with the peaks of interest.

Several substances were tested to find a suitable internal standard (IS) for the assay. Detectability and retention properties of several compounds were evaluated. Diflunisal, as it was utilised in a previous study for the analysis of OTA[6], was found acceptable as an internal standard compound. Responses of OTA and IS were evaluated together using the ratio method, i.e. the ratio of a signal was calculated by dividing the area of OTA signal by the area of IS signal. Validation of the method was carried out using this method, employing a constant concentration of IS versus different concentrations of OTA as shown in Figure 2. Signal ratios were chosen instead of area response to minimize the external and internal factors that affect the analysis, such as variation of ambient temperature, mobile phase composition, and injection volume. Fluorescence detector was set up at excitation and emission wavelength of 330 and 450 nm respectively, which were also used at the same values in the previous study[6].

After preliminary studies, system suitability parameters were checked by analysing standard solutions of OTA and IS at the specified conditions. Analyte signals were detected in 11.5 ± 0.1 and 12.6 ± 0.1 min for OTA and IS, respectively. A representative chromatogram is shown in Figure 2 in which OTA and IS peaks are well resolved giving resolution factors higher than 2, with adequate precision. System suitability parameters, which were calculated as recommended by USP, are given in Table 1.

II. *Validation*

The chromatographic procedure was validated

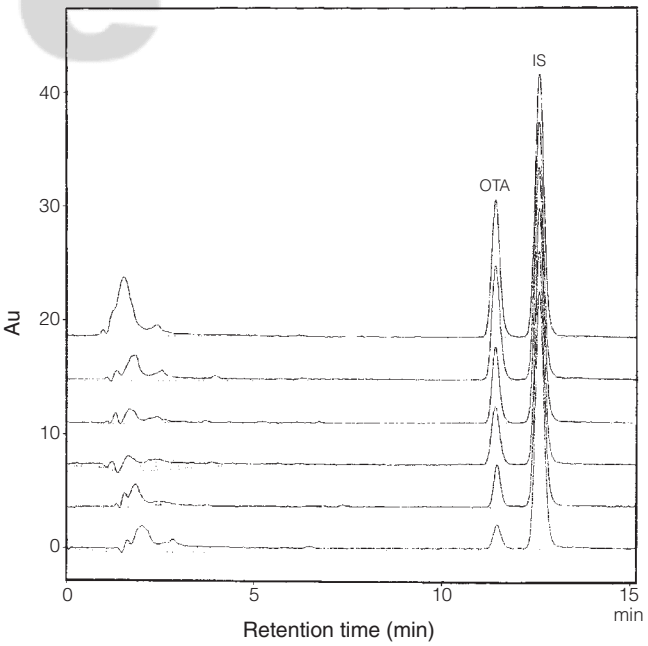

Figure 2. The chromatogram of OTA and IS (C_{OTA} = 2.12 - 11.28 μg/L; C_{IS} = 22.50 μg/L)

Table 1. System suitability data

Parameter *	Assay value	Recommended value
Retention time (min)	11.5 ± 0.1	N/A
Capacity factor (*k'*)	9.9	> 2
Asymmetry factor (A_s)	1.1	0.95 < x < 1.2
Tailing factor (*T*)	1.1	< 2
Selectivity factor (α)	1.2	> 1
Resolution factor (Rs)	2.1	> 2
Theoretical plates (*N*)	8962	> 2000
RSD % of retention time	0.33	< 1

(Column: Teknokroma Nucleosil® C_{18} (150 × 4.6 mm I.D., 3 μm); mobile phase CH_3CN: H_2O: CH_3COOH, 50: 48: 2 v/v/v)

*Capacity factor: $k' = t_r - t_o/t_o$, where t_r is the retention time of the peak and t_o is the dead time of the column. Peak Asymmetry: $A_s = a/b$ where A_s = peak asymmetry, b = distance from the point at peak midpoint to the trailing edge (measured at 5 or 10% of peak height), a = distance from the leading edge of of peak to the midpoint (measured at 5 or 10% of peak height). Tailing factor : $T = (a+b)/2a$, where A_s = peak tailing, b = distance from the point at peak midpoint to the trailing edge (measured at 5 or 10% of peak height), a = distance from the leading edge of of peak to the midpoint (measured at 5 or 10% of peak height). Selectivity factor(α) : $\alpha = k_2/k_1$, where k_2 and k_1 are the capacity factor of the second and first eluted peaks. Resolution factor R_s : $R_s = 2(t_2 - t_1)/wb_2 - wb_1)$, where t_2 and t_1 are the retention time of the second and first eluted peaks, and w_{2b} and w_{b1} are the half peak width of the second and first peaks. Theoretical plate number *N* : $N = 5.55\ t_r^2/w^2_{1/2}$ where t_r is the retention time of the peak and $w_{1/2}$ is the peak width at half hight.

Table 2. Statistical evaluation of test results obtained from linearity studies

	Repeatability*			Reproducibility** (n = 15)
	Set 1 (n = 5)	Set 2 (n = 5)	Set 3 (n = 5)	
Range (μg/L)	2.12 – 11.28			
Slope	$4{,}26 \times 10^{-2}$	$4{,}24 \times 10^{-2}$	$4{,}25 \times 10^{-2}$	$4{,}25 \times 10^{-2}$
Intercept	$1{,}55 \times 10^{-3}$	$2{,}85 \times 10^{-3}$	$1{,}42 \times 10^{-3}$	$1{,}94 \times 10^{-3}$
Σx^2	$3{,}36 \times 10^{1}$	$3{,}49 \times 10^{1}$	$3{,}19 \times 10^{1}$	$3{,}35 \times 10^{1}$
Σy^2	$6{,}10 \times 10^{-2}$	$6{,}26 \times 10^{-2}$	$5{,}78 \times 10^{-2}$	$6{,}05 \times 10^{-2}$
Σxy	$1{,}43 \times 10^{0}$	$1{,}48 \times 10^{0}$	$1{,}36 \times 10^{0}$	$1{,}42 \times 10^{0}$
SD of slope	$1{,}16 \times 10^{-4}$	$1{,}13 \times 10^{-4}$	$1{,}22 \times 10^{-4}$	$1{,}17 \times 10^{-4}$
SD of intercept	$6{,}71 \times 10^{-4}$	$6{,}66 \times 10^{-4}$	$6{,}89 \times 10^{-4}$	$6{,}75 \times 10^{-4}$
R	1,0000	1,0000	1,0000	1,0000
$CL_{0.05}$	$\pm 1{,}10 \times 10^{-4}$	$\pm 1{,}07 \times 10^{-4}$	$\pm 1{,}30 \times 10^{-4}$	$\pm 1{,}16 \times 10^{-4}$

*Repeatability is the variation in measurements taken by a single person or instrument on the same item and under the same conditions. The standard deviation under repeatability conditions is part of precision and accuracy.

**Reproducibility relates to the agreement of test results with different operators, test apparatus, and laboratory locations. It is often reported as a standard deviation.

according to the ICH recommendations in terms of range, linearity, accuracy, precision and analytical limits. The applicable range of the method was derived from the linearity studies, taking the expected OTA concentrations of the samples into consideration. The analytical procedure provided acceptable linearity, accuracy and precision within the range of 2.12-11.28 μg/L. Since the range has comprised OTA concentrations in the analysed samples, it was evaluated as acceptable for the assay.

Linear relationship of concentration versus analyte signal was evaluated across the range of the analysis. Correlation coefficient and regression line was calculated by the method of least squares, along with common statistical data. Linearity studies were repeated for three consecutive days, using standard solutions of OTA and IS at known concentrations. Statistical data are given in Table 2.

Recovery experiments were carried out using standard addition method to study the accuracy and reproducibility of the proposed method. The interference of the co-eluents to the analysis was determined by adding known amounts of standard OTA solution to the pre-analyzed wine samples. The recovery results were calculated via calibration curve using 9 repeated experiments covering 3 replicates of 3 different concentrations of 50%, 100% and 150%. Related data are shown in Table 3.

Precision studies were conducted employing sequential analyses of a standard OTA solution at the concentration of 2.12 μg/L with statistical interpretation of the results. Ten independent determinations for three consecutive days were carried out to determine the repeatability and reproducibility of the method. Relative standard deviation of the test results was found to be precise and was lower than 2.0%. Statistical data are given in Table 4. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as 0.052 and 0.159 μg/L, based on the standard deviation of the response (σ) and the slope of the

Table 3. Results of the accuracy studies assessed using standard addition method

Percentage level	OTA added (μg/L)	OTA found (μg/L)	Average recovery (%)
50%	2.116	2.112	99.82
100%	4.232	4.228	99.91
150%	6.348	6.335	99.79
50%	2.116	2.133	100.82
100%	4.232	4.270	100.91
150%	6.348	6.398	100.79
50%	2.116	2.112	99.81
100%	4.232	4.228	99.90
150%	6.348	6.334	99.78
Mean			100.16
SD			0.505
RSD%			0.504
$CL_{0.05}$			0.330

Journal of Food and Drug Analysis, Vol. 17, No. 6, 2009

Table 4. Statistical evaluation of test results obtained from precision studies

	Repeatability*			Reproducibility** (n = 30)
	Set 1 (n = 10)	Set 2 (n = 10)	Set 3 (n = 10)	
Mean	0.0909	0.0907	0.0908	0.0908
SD	0.0012	0.0013	0.0011	0.0012
RSD%	1.35	1.39	1.30	1.36
$CL_{0.05}$	0.00076	0.00078	0.00073	0.00044

*,**: as noted in Table2.

Table 5. Detection and quantitation limits of the assay

	Set 1 (n = 5)	Set 2 (n = 5)	Set 3 (n = 5)	Average values
Range (μg/L)		2.11-11.28		
SD of slope	1.16×10^{-4}	1.13×10^{-4}	1.22×10^{-4}	1.17×10^{-4}
SD of intercept	6.71×10^{-4}	6.66×10^{-4}	6.89×10^{-4}	6.75×10^{-4}
LOD (μg/L)	0.052	0.052	0.053	0.052
LOQ (μg/L)	0.158	0.157	0.162	0.159

calibration curve (S). S/σ ratio was multiplied by 3 and 10 for calculation of LOD and (LOQ), respectively as shown in Table 5.

III. *Analysis of the Samples*

Direct injection was preferred for the analysis of wine samples, in order to provide a simple and fast assay which requires no sample pre-treatment. It was time effective and economic to just filter, spike IS and inject the sample. This choice was also advantageous for the recovery, which nearly reached 100% due to minimum OTA loss (Table 3).

Twenty-five wine samples, mostly produced in different regions of Turkey, were analysed by the proposed method and the existence and quantity of OTA was investigated. Acceptable sharp peaks with adequate resolution were observed during the analysis of real samples. Since the organic solvent ratio in the mobile phase composition was high, interfering signals were eluted within the first couple of minutes of the analyses, giving clear peaks of analytes. Examples of chromatograms of analysis of OTA in red and white wine samples are shown in Figures 3 and 4.

OTA content of the samples was found variable as a result of variations in production date, geographical region, and variety of grape used for the production of wines. However, construction of a linear relationship between OTA levels and these parameters is difficult. It is known that low level production steps such as ways of cultivation and harvesting are the key points of wine

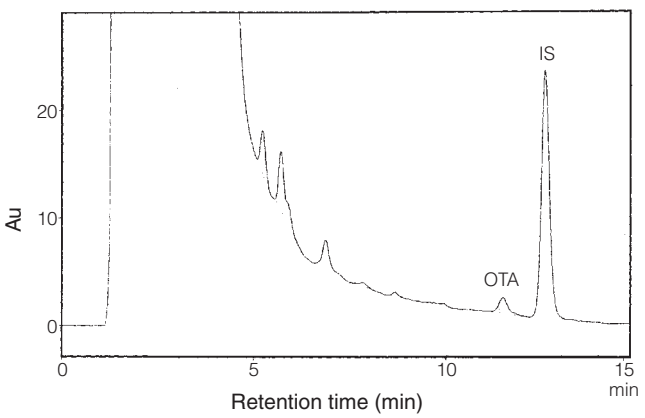

Figure 3. Chromatogram of Do wine sample (Red, 2004, Thrace-Aegean, 1.37 μg/L OTA)

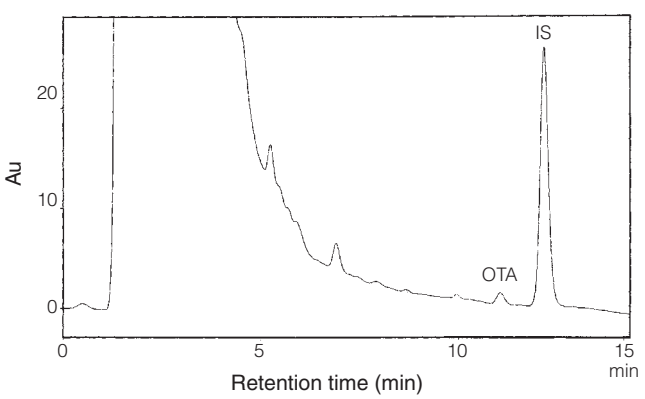

Figure 4. Chromatogram of Trb wine sample (White, 2003, Thrace, 1.07 μg/L OTA)

production that increase or decrease OTA levels. Note that wines produced using Bogazkere and Okuzgozu grapes have shown higher OTA values than other brands. OTA contents of tested wines were found between ranges of 0.25-7.96 μg/L, with an average concentration of 2.55 μg/L. It was observed that OTA levels in red wines were higher than those in the white wines, with average of 2.85 μg/L and 0.87 μg/L, respectively. European Union requires OTA levels to be lower than 2.0 μg/L[14]. OTA concentration of 14 out of samples 25 samples were higher than 2.0 μg/L in this study, indicating more than the half of the samples contained OTA over the permitted limits. Assay results are shown in Table 6.

CONCLUSION

The developed method based on LC with fluorescence detection is relatively fast and simple and has been validated for determination of OTA in wines with good accuracy and precision. The main advantages of the proposed method are of cost effectiveness and simple sample preparation. The overall sensitivity is good.

The results of the analyses performed under specified conditions showed that many wine samples contained OTA higher than the limits set in the EU regulations. The proposed method, which utilises an internal standard for higher reproducibility, could be applied for routine analysis of OTA, considering the advantages of

Table 6. Assay results of wine samples.

Sample ID	Color	Production year	Alcohol content (%, v/v)	Grape varieties	Anatolian region	OTA content (μg/L)
Al	Red	2003	12.0	D, E	Western	5.36
Bb1	Red	2003	12.5	B, M	Central – Eastern	3.20
Bb2	Red	2003	12.5	B, M	Central – Eastern	3.80
Bb3	Red	2003	12.5	B, M	Central – Eastern	2.98
Do	Red	2004	12.0	C, E, I	Thrace – Aegean	1.37
GMk	Red	2000	12.5	E, I, N	Marmara	5.29
Hb1	Red	1999	12.0	F	Thrace	3.20
Hb2	Red	1997	12.0	F	Thrace	1.16
Hb3	Red	1998	12.0	F	Thrace	7.96
Ka	Red	1987	12.0	B, M	Central	2.73
KK1	Red	2003	13.0	G	Central	2.72
KK2	Red	1997	13.5	G	Central	2.91
Ma	Red	2005	12.0	B, D, M	Central	2.90
Me1	Red	1993	13.0	J	Thrace	2.53
Me2	Red	2004	13.0	J	Thrace	0.39
Tbk	Red	2005	11.5	D, I	Aegean	1.20
Trk	Red	2005	11.5	A, E, F	Thrace	2.46
Um	Red	2003	11.0	J	Thrace	1.61
Ve	Red	2005	17.0	K	Central	1.25
Ya	Red	2004	12.0	B, D, M	Eastern	2.01
GMb	White	2000	11.5	O, Q	Thrace	0.25
Trb	White	2003	11.5	O	Thrace	1.07
Ku	White	2003	12.0	O	Thrace	1.80
An	White	2003	11.5	L	Central	0.52
Tbb	White	2004	11.5	O, P	Aegean	0.73

Grape varieties: A: Adakarasi; B: Bogazkere; C: Calkarasi; D: Carignan; E: Cinsault; F: Gamay; G: Kalecik karasi; I: Karasakiz; J: Merlot; K: Misket; L: Narince; M: Okuzgozu; N: Papazkarasi; O: Semillon; P: Sultaniye; Q: Yapincak.

direct injection and high recovery achieved in the analyses of real samples in the described method.

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