Determination of ochratoxin A and its occurrence in wines of Slovakian retail

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Summary

The HPLC method using a reverse phase C18 with fluorescence detection and clean up on an immunoaffinity column (IAC) for the determination of ochratoxin A (OTA) in wine and grape juice has been elaborated. The method was validated by assessing the precision (RSD_r were 2.6–5.3% for wines and 3.9–6.9% for grape juice), accuracy (recovery of OTA in spiked grape juice was 94%, in spiked wines the recovery has varied from 72 to 106%), and the extended uncertainty of measurement. The estimated limit of detection (*LOD*) was 0.011 and 0.025 $\mu g.l^{-1}$ for wine and grape juice, respectively; the limit of quantification (*LOQ*) was 0.033 $\mu g.l^{-1}$ for wines and 0.039 $\mu g.l^{-1}$ for grape juice. Thirty nine samples of white and red variety wines from the Slovak wine production of 2005 as well as 16 samples of imported wines have been studied for OTA by this method. OTA was not detected in more than 50% of wine samples. The level of OTA in red wines was a little higher than in white ones (the highest concentration found was 0.463 $\mu g.l^{-1}$). In addition, the distribution of OTA in wines during wine making in the wine season 2006 in the Little Carpathian vineyard region of the Slovakia was investigated.

Keywords

ochratoxin A; HPLC; wine; grape juice; immunoaffinity column

Ochratoxin A (OTA) is mycotoxin produced in food contaminated by fungi, especially by genera *Penicillium* (*P. verrucosum*) and *Aspergillus* (*A. ochraceus, A. carbonarius*) [1, 2]. According to the IARC (International Agency for Research on Cancer), OTA was classified as a carcinogen of the group 2B [3] with a potent nephrotoxic, teratogenic, and immunosuppressive properties [4].

These moulds are capable of growing under different conditions of moisture, pH, temperature on a variety of primary foods, such as cereals (mainly wheat, barley, maize), coffee, and grapes from which they are further transferred into cereal products, dried fruits, as well as into beer and wine [1, 5-7]. Fungi *Penicillium* are probably responsible for the production of OTA in grapes in the southern regions, whilst *Aspergillus* is commonly occurring in the northern regions of Europe [8]. The presence of OTA in wines and musts was described in 1996 by ZIMMERLI and DICK, onward studies confirmed contamination of musts and wines with OTA. This mycotoxin was detected particularly in red and dessert wines [2, 5, 9-11], in grape juice and musts as well [5, 12].

The formation and occurrence of OTA in wines represents a serious economic problem in Europe because of its high share in world vineyard areas, which represent 75% of world-wide wine production. The total intake of OTA due to wine has been provisionally estimated by the Codex Alimentarius Commission to 15% [13]. The estimated OTA daily intakes from food, derived from previous researches, ranged from 0.7 to 4.7 $ng.kg^{-1}$ of body weight. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established the provisional weekly intake of OTA to 120 $ng.kg^{-1}$ of body weight [14]. In accordance with the regulation No. 466/2004 of the European Commission, the wines made in 2005 should comprise maximum concentration of 2 $\mu g.l^{-1}$ of OTA [15].

The possibility of development of moulds producing OTA depends on climatic conditions, though it is more frequent in areas with tropical climate [5]. The favourable factors for the produc-

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tion of mycotoxin comprise suitable temperature, moisture, aeration, and duration of incubation and interaction of fungi [16]. The presence of OTA in wines is the result of the contamination of grape surface, commonly occurring before and after harvest period or during winemaking. The higher level of OTA in red wine in comparison with white wine is due to the different manufacturing concept used in the production of both wine types [8, 17].

According to some studies, the most massive growth of moulds was recorded in the period of grape maturation. Moulds attack and interfuse preferably into damaged berries. The higher the perforation of berries, the higher the feasibility of contamination by moulds. Likewise, grape varieties with thin and fragile skins suffer contamination much easier [18]. For example *A. carbonarius* is a very invasive species which can penetrate berries even without skin damage [19]. This fungi also contributes considerably to the OTA contamination of raisins during drying process - especially sultanas' berries tend to brake after rain during the harvest period [20].

The prevention of grape contamination with OTA in whole reposes by applying the basic sanitary measures and recommended actions. In must which is intended to winemaking it is suggested to monitor the content of OTA, as well as to adhere to appropriate protective programs before and within the fermentation phase [21].

Beside the traditional European vine-growing-countries with high wine production the Slovakia, the Central-European country, has also had a rich vitivinicultural history. According to European territorial segmentation the Slovak vineyards are classified as zone B and represent six vineyard regions with the total of 22,000 hectares of land. The geological and climatic conditions in Slovakia are similar to those in neighbouring Czech Republic, Austria, and Hungary and their diversity creates individual quality characteristics of the Slovak wines.

The objective of this work is to present the results of a random survey of OTA concentration in variety wines processed in the year 2005 in Slovakia, which should comply with the standing regulation of the European Union regarding concentrations of OTA in wine. For comparison, some foreign wines from Europe, Africa, and South America were also analysed too. Quantitative determination of OTA in wines after pre-treatment of wine on immunoaffinity columns was investigated using the HPLC method with fluorescence detection.

MATERIAL AND METHODS

Chemicals and materials

All used chemicals were analytical or HPLC purity grade: sodium chloride, sodium hydrogen carbonate (Lachema, Brno, Czech Republic); glacial acetic acid, toluene, 99% (AFT Bratislava, Slovak Republic); acetonitrile and methanol Chromasolv (Sigma-Aldrich Laborchemikalien, Seelze, Germany); polyethyleneglycol 6000 (Merck, Hohenbrunn, Germany); ochratoxin A, 99% (Sigma-Aldrich Chemie, Steinheim, Germany); immunoaffinity columns Ochraprep (R-Biopharm Rhone, Glasgow, Scotland).

Preparation of standard OTA solution

The stock OTA standard solution was prepared by dissolving 5 mg crystalline OTA in 4 ml of the mixture toluene-acetic acid 99:1 (v/v). This solution was stored at –18 °C. The working standard solutions for calibration and spiking purposes were prepared step by step by vacuum evaporating of necessary stock solution volume, dissolving of the residue in the mobile phase and sequential diluting of this solution. The working standards were stored at 4 °C.

Secondary solutions

The dilution and washing solutions containing sodium chloride, sodium hydrogen carbonate, and polyethylenglycol 6000 were mixed in according to the European Standard prEN 14133 [22].

Samples

The analysis was limited only to wines produced in 2005. The bottled variety wines were purchased in the Slovak retail and wholesale trade. The samples were collected in order to represent each vineyard area in Slovakia. In total 39 samples of variety wines were analyzed: 28 white wines (Rizling rýnsky, Rizling vlašský, Veltlínske zelené, Chardonnay, Müller Thurgau, Muškát Ottonel, Irsai Oliver, Rulandské biele, Furmint, Lipovina Toccata) and 11 red wines (Frankovka modrá, Svätovavrinecké, Cabernet Sauvignon, Cuveé). Some sorts of wines from foreign countries were also analysed for comparison (country of origin - Czech Republic, Hungary, Spain, Italy, Portugal, France, Macedonia, Chile, South Africa, and Australia) – in total 16 samples. The analysed batch was also complemented by a few home-made wines (5 white, 6 red).

In order to monitor the distribution of OTA in wines from the Slovak Little Carpathian region in the wine-campaign 2006, the samples from different technological stages were taken directly in

wine plant and in households. The samples included fresh pressed grape juice, fermented must, wine treated with fining agents (bentonite, gelatin), and a new bottled wine. The wines were sampled from variant batches and comprised both white and red wine sorts.

Wine clean-up on immunoaffinity column (IAC)

Immunoaffinity columns Ochraprep for OTA isolation from wines and grape juices were employed. Immunoaffinity columns commonly stored at 2–8 °C were tempered before use to the ambient temperature. The fill in IAC column was then conditioned with the filling solution present in the IAC column. Ten ml of sample diluted (1:1, v/v) with the dilution solution was then applied on the column and let pass through the column without or with applying a slight vacuum. When analysing a non-clarified grape juice it was necessary to filter the diluted juice through a glass fibre filter (pore size 1.2–1.4 μm). IAC was then washed with 5 ml of washing solution and 5 ml of deionized water to get rid of interfering substances. The column was then dried with air for 10 minutes and the retained OTA was eluted with 2 ml of absolute methanol. The obtained eluate was evaporated on a rotary vacuum evaporator to dryness. The residue was dissolved in 0.25 ml of the mobile phase and quantitatively transferred into 2 ml dark sampler vial.

Apparatus

The HPLC equipment Agilent Technologies 1100 Series (Halbron, Germany) with autosampler and fluorescence detector at excitation wavelength 333 nm and emission wavelength 460 nm was used. The analytical column Zorbax SB-C18, 250 × 4.6 mm with the sorbent particle size of 5 µm together with the precolumn Zorbax SB-C18, 12.5 × 4.6 mm with the same particle size (Agilent Technologies, Halbron, Germany) was applied. The mobile phase mixed of acetonitrile-acidified water (20 ml of acetic acid in 1000 ml of deionized water) 50:50 (v/v) has flown through the system at the rate of 1 ml.min^{-1}. Samples were injected onto analytical column in 100 µl volume. All analyses were carried out at ambient temperature.

Identification of OTA

OTA was identified on the base of retention time, eventually through fluorescence spectra in the range of wavelengths of 365 to 435 nm.

Evaluation of the method

The analytical procedure was internally validated by means of calibration and evaluation of the range of linearity, precision, limit of detection (*LOD*) and quantification (*LOQ*), recovery and expanded uncertainty of measurement for both wine and grape juice. The calibration measurements were carried out with OTA standard solutions. Linear response of fluorescence detector was determined in the range of concentrations 0.032–3.125 μg.l^{-1} which led to the correlation factor $r > 0.999$. The reproducibility expressed as repeatability was checked at two levels of OTA concentration: 0.35 and 2.61 μg.l^{-1} in wines, and the concentration 0.25 and 2.54 μg.l^{-1} in grape juice. *LOD* and *LOQ* were calculated using equations $LOD = X_0 + 3SD$ and $LOQ = X_0 + 5SD$, respectively (where X_0 was the average response of blank samples, *SD* standard deviation for $n = 6$). The recoveries of OTA using IAC columns for sample pretreatment were studied by spiking wines with standard solutions at OTA levels of 0.18 and 2.44 μg.l^{-1} as well as by grape juices at levels of 0.21 and 2.50 μg.l^{-1}. The measurement of uncertainty was evaluated as combined uncertainty (U_c) with the covering factor $k = 2$ (e.g. $2U_c$) and a 95% confidence interval.

Quality control

The stability of calibration curve was checked at two concentration points scoring the slope of the calibration curve. For this purpose the control OTA standard solutions (0.25 µg.l^{-1} and 2.10 µg.l^{-1}) were used for construction of control chart with upper and lower control limit of ± 2*SD* ($n = 9$).

Calculation of results and statistics

The recoveries of OTA from wine and juices were calculated according to formula

$$R[\%] = \frac{c_{spiked\ sample} - c_{nonspiked\ sample}}{c_{spiking\ solution}} \times 100 \qquad (1)$$

For estimation of the final OTA concentration in sample the formula

$$c_{OTA}\ [\mu g.l^{-1}] = c_{OTA\ injected}\ [\mu g.ml^{-1}] \times 1000/20 \qquad (2)$$

was used, where $c_{OTA\ injected}$ was the concentration of OTA in μg.ml^{-1} assigned from the calibration curve, 1000 was a conversion factor to recalculate the OTA concentration to μg.l^{-1}, and 20 was a factor of sample concentrating (from the volume 5 ml to 0.25 ml).

All analyses were done in duplicate from which a standard deviation was calculated. Measured data were processed using chromatographic program Agilent ChemStation (Agilent Technologies).

RESULTS AND DISCUSSION

Method evaluation

The analytical method described was adapted from the European Standard [20] with a few modifications. Wine and grape juices were diluted with polyethylenglycol 6000 to give sufficient pH 8.0–8.5 for appropriate OTA sorption onto IAC filling. Pretreatment on these columns generally offers wine extracts free from complicated interferences as well as lower *LOD*. In addition the chromatograms produced were more lucid and the recorded OTA peak, eluted at 11th minute, more distinct (Fig. 1). The limit of detection and quantification for OTA in wines and grape juices as well as other validation parameters are summarized in Table 1. The data of precision and uncertainty were evaluated for the interval of concentrations 0.35–2.61 $\mu g.l^{-1}$ for wines and 0.25–2.54 $\mu g.l^{-1}$ for grape juices. The recoveries of OTA given in Table 1 were estimated at the fortification level 2.44 $\mu g.l^{-1}$ (white wine - 106%, *RSD* 2.6%), 0.30 $\mu g.l^{-1}$ (red wine - 72%, *RSD* 5.6%), and 2.26 $\mu g.l^{-1}$ (grape juice - 94%, *RSD* 7.2%). Recovery experiments were performed in triplicate. The value of combined uncertainty has involved uncertainty of precision, calibration, recovery, and dilution/concentration of sample volumes.

Tab. 1. Validation parameters of the HPLC method.

Parameter	white/red wine	grape juice
LOD [$\mu g.l^{-1}$]	0.011	0.025
LOQ [$\mu g.l^{-1}$]	0.033	0.039
Precision, RSD_r [%]	2.6–5.3	3.9–6.9
Average recovery [%]	106/72	94
Expanded uncertainty $2U_c$ [%]	55–37	55–39

OTA presence in wines

The goal of the random survey was to find out if wines manufactured in the year 2005 could fulfil the European regulation No. 466/2004 of the European Commission demanding the maximal concentration of OTA in wines and grape juice at the level of 2 $\mu g.l^{-1}$.

Figure 2 provides an overview of vineyard regions in Slovakia. The number of white and red wines tested in monitored regions varied, the most explored areas being the Little Carpathian and Nitra vineyard region (both 12 samples). OTA was not detected in more than 50% of tested wine samples. The remainder of samples comprised OTA concentrations from 0.011 $\mu g.l^{-1}$ up to 0.463 $\mu g.l^{-1}$. Table 2 shows summarized results of OTA incidence in Slovakia as well as in some world countries. In general, levels of OTA were higher in red wines than in white ones, corresponding to comprehensive published findings. In the majority of the Slovak table white wines OTA was not detected (85%), home-made white wines contained not even traces of OTA. Very low levels of OTA, in the range of *LOD–LOQ* concentrations (0.011–0.033 $\mu g.l^{-1}$), were detected in white wines from the Slovak production (7%). In the case of foreign white wines was the number of positive

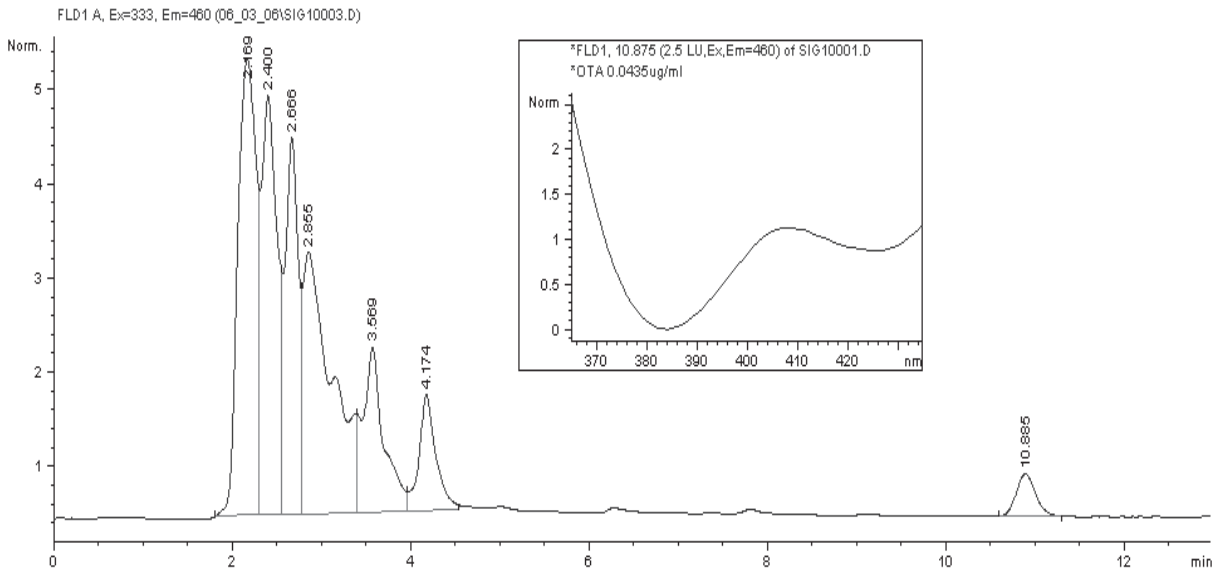

Fig. 1. Chromatogram of a native red wine sample and OTA spectra (retention time of OTA = 10.885 min).

Tab. 2. Occurrence of OTA in wines produced in the year 2005.

Sort of the wine	Total number of samples	Occurrence of OTA in wine [%]			
		N. D.	≈ 0.011 μg.l^{-1}	0.011–0.033 μg.l^{-1}	> 0.033 μg.l^{-1}
Slovak variety white	27	85	8	7	0
Slovak variety red	12	33	8	42	17
Slovak home-made white	5	100	0	0	0
Slovak home-made red	6	33	50	17	0
Foreign white	9	44	34	22	0
Foreign red	7	0	15	71	14

N. D. - not detected.

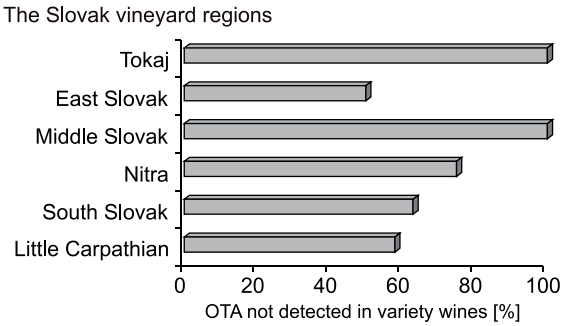

Fig. 2. Fraction of the Slovakian variety wines not contaminated with OTA.

samples higher (22%) in comparison to the Slovak ones.

The presence of toxin has been registered in all tested foreign red wines. The distribution of OTA in red wines was more balanced at toxin levels over 0.033 μg.l^{-1} - there were found two samples of the Slovak (17%) and one sample of foreign red wines (14%) with OTA concentration of 0.036 μg.l^{-1} (SD 0.006), 0.463 μg.l^{-1} (SD 0.030), and 0.122 μg.l^{-1} (SD 0.022), respectively. The highest OTA level was found in the Slovak table red wine - 0.463 μg.l^{-1} originated from the East Slovak vineyard region. The concentration of OTA 0.122 μg.l^{-1} was recognized in sweet red wine from Macedonia. In total, 33% of Slovak table as well as home-made red wines were negative. About 50% of home-made red wines contained OTA level of about 0.011 μg.l^{-1}.

OTA presence in wines during wine making

The presence, or changes of OTA concentration during wine making was examined in the wine campaign 2006 in the Little Carpathian vineyard region. Wines were sampled in according to Table 3. Assayed samples from a private large-scale plant comprised mostly white wines. Samples of red wine were obtained only from the stage of wine racking off, similarly as in wines from private productions. Figure 3 gives a prediction of possible variation of OTA concentration during wine process. OTA concentrations not detected (e.g. under *LOD* level) are marked at level 8 ng.l^{-1} in the Figure 3 for some OTA traces suggestion. OTA concentrations estimated altered in the scope of *LOD–LOQ*. Despite low number of the samples analyzed the depicted graph may indicate some trends.

The process of wine making seemed to be similar for white wines in the stages 1–3 (for both wines from a wine plant and households). Apparently, fermentation has not influenced the OTA level. Concentration of the toxin was nearly identical in the stage 3 (around *LOD* area) for all wines, including red wines. In white wines clarified with bentonite and gelatin OTA was not detected. It can be assumed that these materials could adsorb OTA on their surface, which is then removed by the filtration process. A case in point was the fresh bottled wine, in which no OTA could be detected.

Tab. 3. Technological stage of sampled wines.

Technologic stage	White wine	Red wine
Wine plant		
1. Fresh must	2 sample	3 samples
2. Fermentation	2 samples	3 samples
3. Wine after the first racking off	2 samples	1 sample
4. Wine after clarification (bentonite, gelatin)	2 samples	N. I.
5. Wine after bottling	2 samples	N. I.
Home-made wine		
1. Fresh must	2 samples	N. I.
2. Fermentation	1 sample	N. I.
3. Wine after racking	1 sample	1 sample

N. I. - not investigated.

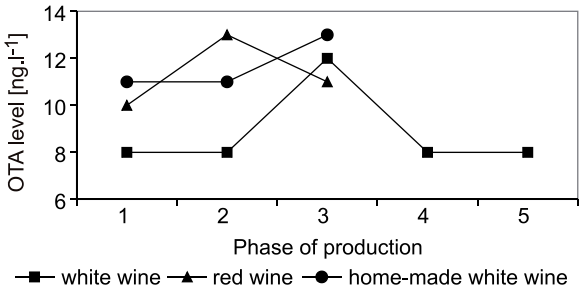

Fig. 3. Changes of OTA concentration during wine making.

Legend: 1 - fresh must (white wine), respectively beginning of fermentation maceration (red wine); from 1 to 2 - period of fermentation (white wine), respectively fermentation maceration (red wine); 3 - after racking off; from 3 to 4 - period of clarification; 5 - fresh bottled wine.

A somewhat different progress was registered in the red wine still in the fermentation period, in which OTA level has marginally increased from 0,010 μg.l^{-1} to 0,013 μg.l^{-1}. The increase might be caused by longer contact of the red grape skin together with pressed juice during maceration fermentation that is typical in red wine production and which commonly takes several days.

CONCLUSION

This limited study is the first information on OTA presence in the Slovak variety wines as well as in processed wine. Moreover, the analytical method presented and used in this survey, has shown sufficient parameters and sensitivity for detecting traceable OTA levels in wine and grape juice. In general, wines produced and sold in Slovakia had lower level of OTA than imported wines and OTA concentrations found were far below the proposed European limit of 2 μg.l^{-1}. Also, minor changes of OTA concentration were recorded during wine making in the Slovak wine season 2006. However it should be emphasized that the presence of OTA in grapes is strongly dependent on climatic conditions during the maturation and harvest of grapes, which are supposed to be moderate in the climate region of the Middle Europe. In spite of that there is the need to monitor OTA levels each year as they can differ from the previous one. On the other hand sanitary requirements have to be adhered to produce wines not contaminated or minimally contaminated with OTA.

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