

Short Communication

OCHRATOXIN A IN WINE

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A high-performance liquid chromatography (HPLC) method for determination of ochratoxin A (OTA) in wine was developed and checked on 14 samples of wine produced in the Republic of Croatia. OTA detection in red and white wine was validated checking the linearity of the method ($r^2=0.997$ and 0.999 , respectively), recovery (88 and 95 %, respectively), and precision (RSD=3.1 and 1.4 %, respectively). The detection limit of OTA in both red and white wine was 10 ng L^{-1} . The OTA concentration in all red wines was above the detection limit (mean $22 \pm 11 \text{ ng L}^{-1}$), while only 4 of 7 white wines contained OTA (mean $10 \pm 9 \text{ ng L}^{-1}$). Three OTA-free white wines were from the north of Croatia (inland), while all wines produced in the south (Adriatic coast) were OTA-positive. However, the concentrations were too low to present a risk for a moderate wine consumer.

KEY WORDS: analytical validation, Croatia, HPLC, immunoaffinity columns, mycotoxin

Ochratoxin A (OTA) is a secondary metabolite of moulds that contaminate food and feed. OTA is nephrotoxic, hepatotoxic and immunotoxic in experimental animals, and due to its carcinogenic properties, the International Agency for Research on Cancer (IARC) evaluated OTA as a possible carcinogen in humans (2B group) (1).

Although OTA is found mostly in grains all over the world (2) it was also found in other commodities of plant and animal origin (coffee, spices, herbs, cocoa, grapes, meat, milk, juices, bear and wine) (3). OTA is a frequent contaminant of wine (4) and wine contamination with OTA is considered an important contributor to human exposure to OTA since Zimmerli and Dick proved that higher OTA exposure was related to the consumption of red wine (5). It was estimated that 15 % of daily intake of OTA is due to wine consumption (6). In general, red wines contain more OTA than rosè and white wines due to different winemaking procedures. Some results suggest that wines from the South contain more OTA than those from the North, at least in Europe and North Africa (4, 7). This difference is attributed to climate, grape cultivation and storage conditions. However, it

seems that the presence of OTA-producing moulds is crucial because it was found that grapes from the North, although infected with moulds, did not have a detectable concentration of OTA (8).

The tolerance level for OTA concentrations in wine has not been set in either Croatia or European Union.

This is the first study of OTA contamination of wines produced in Croatia. For the purpose of screening of OTA in commercially available Croatian wines, a HPLC method for OTA determination in wine was developed and tested on 14 samples of wine.

MATERIALS AND METHODS

Chemicals and reagents

OTA (98-percent purity) was obtained from Sigma (St. Louis, MO, USA). Water (Merck, Darmstadt, Germany), and methanol (Kemika, Zagreb, Croatia), used for HPLC mobile phase were of HPLC-grade. All other chemicals were of *pro analysis* grade (Kemika, Zagreb, Croatia). Phosphate-buffered saline (PBS)

contained potassium chloride (0.2 g), potassium dihydrogenphosphate (0.2 g), anhydrous disodium hydrogenphosphate (1.16 g), and sodium chloride (8.0 g) in 1.0 L of distilled water.

Standard preparation

A stock solution of OTA was prepared in methanol (1.0 mg mL^{-1}). The concentration of stock solution was determined spectrophotometrically at 333 nm with $6640 \text{ L mol}^{-1} \text{ cm}^{-1}$ as the extinction coefficient. The working standards were prepared by adding known amounts of diluted stock solution to the HPLC mobile phase to give final concentrations from 0.5 to 10.0 ng mL^{-1} OTA. The working standards were freshly prepared every day.

Spiked wine samples

For the recovery experiment, OTA-free red or white wine samples (10.0 mL) were spiked with the stock solution of OTA in the final concentration of 30 to 500 ng L^{-1} . OTA was extracted and analysed as described for wine samples.

Sample collection

A total of 14 (7 red and 7 white) bottled wines produced in Croatia in 2002 and 2003 were purchased from a local store. Bottles were kept sealed in the refrigerator on $+4 \text{ }^\circ\text{C}$ and analysed within a few days.

Sample preparation

An aliquot of 10.0 mL of wine sample (red or white) was diluted with 10.0 mL of PBS-buffer. After adjusting pH to 7.0-7.5 with 10 mol L^{-1} NaOH, the sample was filtered through a microfibre filter paper. Ten mL of filtrate were applied onto an immunoaffinity column (IAC; OchraTest, Vicam, Watertown, MA, USA). The column was washed with 10.0 mL PBS-buffer and then with 10.0 mL HPLC-grade water. OTA was eluted from the IAC-column using 4.0 mL methanol acidified with acetic acid (98:2) (V/V). Eluate was collected and evaporated to dryness under a stream of nitrogen. Before analysing on HPLC, evaporated samples were dissolved in $200 \text{ } \mu\text{L}$ of mobile phase.

HPLC equipment

The HPLC equipment consisted of an isocratic pump (Gilson 305), manometric module (Gilson

805), injector (Rheodyne 7125) with a $50 \text{ } \mu\text{L}$ loop, fluorescent detector (Thermo Separation Products - Spectra System FL 2000), and recorder (Gilson N1). Guard column and analytical column were reverse-phase (C-18) (LiChrospher, Merck) with $5 \text{ } \mu\text{m}$ particles (4.0×4.0 and $4.0 \times 125.0 \text{ mm}$, respectively).

HPLC conditions

The mobile phase consisting of methanol, water and acetic acid (700:300:20), pH 3.0, was degassed before use by sonication for 15 minutes in an ultrasonic bath. The flow-rate was 0.5 mL min^{-1} . The excitation wavelength of the fluorescence detector was set at 336 nm, and the emission wavelength was set at 464 nm. The injection volume was $50 \text{ } \mu\text{L}$, and the analysis was performed at room temperature. Under these conditions, the retention time of OTA was about 8 minutes.

RESULTS AND DISCUSSION

The analytical procedure, based on IAC clean-up method, simplifies the procedure and enables the detection of very low OTA concentrations. This procedure is timesaving, avoids preliminary liquid-liquid extraction and reduces the use of toxic solvents. Before applying to IAC column, wine samples were diluted with PBS-buffer in order to neutralise wine acids, and the pH was adjusted to 7.0-7.5 to make sample suitable for application on the IAC.

Poor OTA recovery from wine found in our preliminary studies was overcome by controlling pH in the diluted sample as suggested by other authors (6, 9). *Visconti et al.* reported that the pH had to be adjusted at about 7.5 before applying samples to IAC, in order to obtain 97.2 % recovery (6). The mean recovery of the method was calculated as the ratio of found/added OTA in spiked wine samples. It was lower in red than in white wine (88 and 95 %, respectively), which is probably due to the presence of several compounds in red wine that interfere with OTA binding to antibodies on IAC column (6).

Calibration curve for calculating OTA concentration was prepared from OTA standards in mobile phase. The calibration curve was linear in tested range from 0.5 to 10.0 ng mL^{-1} ($r^2=0.9987$). Standard curves prepared from spiked red and white wine were also linear (r^2 were 0.997 and 0.999, respectively) (Table 1).

Table 1 Validation of OTA method

	Red wine	White wine
Recovery / %	88	95
Linearity, r^2	0.997	0.999
Limit of detection / ng L ⁻¹	10	10
Precision, RSD / %	3.1	1.4

The limit of detection for the spiked red and white wine, calculated by taking the average noise signal and adding 3 standard deviations of the noise, was 10 ng L⁻¹.

Precision was calculated from six OTA-spiked red and white wine samples, respectively, carried out independently throughout the procedure. The precision when expressed as relative standard deviation (RSD) for red wine was 3.1 % and for white wine 1.4 %.

Table 2 Ochratoxin A concentration in commercial wines in Croatia

Samples of wine	Positive / total	Mass concentration of OTA/ng L ⁻¹		
		Range*	Median	Mean±SD
Red	7/7	12-47	22	22±11
White	4/7	15-22	15	10±9
Part of Croatia				
South	5/5	15-47	22	25±12
North	6/9	12-22	15	11±8

*Ranges apply to positive samples

It is known that red wine is usually more contaminated with OTA than rosè and white wine, probably due to different winemaking procedures (7). Namely, white grapes are immediately pressed after being picked while red wine grapes are mashed and the skin and juice are put aside for several days (4). In our study all red wines contained detectable concentrations of OTA, and the highest concentration was 47 ng L⁻¹ (Table 2). OTA was found in four of seven white wine samples and the highest concentration was much lower than in red wine (22 ng L⁻¹). The highest concentrations found in our study are lower than in other studies in Europe, which makes Croatian wines more suitable for consumers (4, 6). Low OTA concentrations found in both red and in white wines in our study do not pose a risk to people who drink up to two glasses of red wine (300-400 mL) a day. Namely, an average person weighing 70 kg who drank two glasses of wine containing the highest concentration

of OTA found in our study (47 ng L⁻¹) would have a daily OTA intake of 14.1 to 18.8 ng. Calculated per kg of body weight, this means 0.20 to 0.26 ng kg⁻¹ b. w., which is much lower than the acceptable daily intake recommended by the WHO (16 ng kg⁻¹ b.w.) (1).

Some studies in Europe and Africa have shown that the wines from the South usually contain higher OTA concentrations than from the North (4, 7). Although the number of samples analysed so far is too low for a relevant discussion about regional differences in OTA concentrations in Croatia, it is indicative that all samples from the Adriatic coast (south) contained OTA, while 3 white wines from inland Croatia (north) were OTA-free (Table 2). The low concentrations found in wine collected in Croatia are in agreement with our pilot study on grapes collected in Croatia, when low OTA concentrations were found.

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Sažetak

OKRATOKSIN A U VINU

Uvedena je metoda određivanja okratoksina A (OTA) u vinu uz pomoć tekućinske kromatografije visoke djelotvornosti i testirana na 14 uzoraka vina proizvedenih u Republici Hrvatskoj. Uzorci su pročišćeni s pomoću imunoafinitetnih kolona. Metoda je vrednovana za primjenu u crnim i bijelim vinima određivanjem linearnosti ($r^2=0,997$, odnosno $0,999$), iskorištenja (88, odnosno 95 %) i preciznosti metode (RSD=3,1, odnosno 1,4 %). Detekcijski limit za crna i bijela vina bio je 10 ng OTA L^{-1} . Koncentracija OTA u svim je crnim vinima bila iznad detekcijskog limita ($22 \pm 11 \text{ ng L}^{-1}$; srednja vrijednost \pm standardna devijacija), dok su od 7 analiziranih bijelih vina samo 4 bila kontaminirana ovim mikotoksinom ($10 \pm 9 \text{ ng L}^{-1}$). Tri uzorka bijelih vina koja nisu sadržavala OTA, bila su iz sjevernog dijela Hrvatske, odnosno iz dijela s kontinentalnom klimom, dok su sva vina skupljena iz južnog dijela (Istre, Primorja i Dalmacije) sadržavala OTA. Iako je OTA bio vrlo čest nalaz u vinu, niske koncentracije upućuju na to da OTA u vinu ne predstavlja značajni izvor izloženosti ljudi ovom mikotoksinu u našoj zemlji.

KLJUČNE RIJEČI: *analitičko vrednovanje, HPLC, Hrvatska, imunoafinitetne kolone, mikotoksin*

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