Ochratoxin A Occurrence in Romanian Wines

ELISABETA-IRINA GEANA, ANDREEA-MARIA IORDACHE, ROXANA ELENA IONETE
Research and Development Department, National R&D Institute of Cryogenics and Isotopic Technologies – ICSI Rm. Valcea,
4 Uzinei Str., 240050 Rm. Valcea, Romania

Dangerous to health through contamination of food dietary, ochratoxin A (OTA) is a potent nephrotoxic mycotoxin, being produced by Aspergillus and Penicillium species. Certain common foods based on wheat followed by oats, rice and raisins, spices, dried fruits, beer, coffee, grape juice and wine are major sources of human exposure to OTA, mainly in countries with temperate and cold climate. This paper presents results of a study on the Romanian market for OTA in wines. The OTA determination was made by means of commercial immunoaffinity columns for purification followed by HPLC with fluorescence detection for quantification of the toxin, according to EN 14133/2003. The method was applied to 20 dry white wines and 10 sweet wines obtained by various viticultural and oenological practices. The levels of ochratoxin A ranged between less than 0.06 ng/mL and 0.45 ng/mL, sweet wines showing higher levels than dry wines. Most of the wines contained relatively low concentrations of ochratoxin A (0.06 ÷ 0.1ng/mL), which do not represent a serious risk to consumer health.

Keywords: Ochratoxin A, wine, immunoaffinity colunm, HPLC determination

A mycotoxin with carcinogenic, neurotoxic, immunosuppressive and nephrotoxic properties [1, 2]. Ochratoxin A (OTA) is a secondary metabolite of moulds that contaminate food and feed. Classified as potential human carcinogen (group 2B) by the International Agency for Research on Cancer [3] Ochratoxin A (fig. 1) is one of the most common toxins.

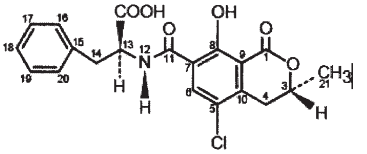

Fig. 1. Ochratoxin A

Found mostly in grains [4] OTA was also found in other commodities of plant (coffee, spices, herbs, cocoa, grapes, juices, bear and wine) and animal origin (meat, milk). Until recently, was thought that OTA contamination of foods, particularly dry foods, grain storage, is produced only by *Aspergillus* ochraceus and *Penicillium* verrucosum. Recent studies have clearly shown that certain species belonging to the black aspergilli, including *Aspergillus* niger aggregate and *Aspergillus* carbonarius, are sources of OTA in foodstuffs such as wine, grapes and dried vine fruit, all over the world [5-15].

OTA is a frequent contaminant of wine [16] the wine contamination with OTA being 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 [17].

There are many methods described for the determination of the ochratoxin A in feed, food, body fluids and animal tissue, wine, beer. Most of them are based on high performance liquid chromatography (HPLC) using reversed phase column followed by fluorescence detection. Solid phase extraction using normal phase or reversed phase adsorbents and more recently immunoaffinity columns have been used for the clean-up of OTA containing extracts [18]. Mass spectrometry has also been coupled with HPLC for the analysis of OTA [19].

The presence of OTA in wine was reported for the first time in 1995. It was detected in commercial Swiss wines as a result of the application of an HPLC method for the determination of this compound in wines. Since then, several studies have been conducted on bottled wines, as well as home-made wines, using various methods. Levels of OTA ranging between 0 ng/mL and 0.533 ng/mL were reported in a study of Hungarian wines [20], and from <0.02 ng/mL to 3.2 ng/mL for Greek wines [21]. An overview on literature revealed that amounts of OTA found in red wines are higher than in rosé wines followed by white wines. In general, OTA mean content in red wines ranged from 0.039 ng/mL [22] to 1.802 ng/mL [23] very similar to that for rosé wines, which ranged from 0.025 ng/mL [22] to 1.348 ng/mL [23]. These average values are slightly higher than the ones for white wine samples, which ranged from 0.011 ng/mL [22] to 0.535 ng/mL [24]. Majerus et al. [25] suggested that the reason for the different levels of OTA concentration of white, rosé and red wines is the different wine-making technique, since longer mash standings in red wine could lead to higher OTA content in wine. The maximum levels of OTA detected were 7.63 ng/mL [24], 2.4 ng/mL [20,25] and 1.2 ng/mL [25] for red, rosé and white wine, respectively.

A maximum permitted level of 2 ng/mL for wine was enforced in the EU. The tolerated maximum concentrations are based on a tolerable daily intake of 5 ng/(kg of body weight day) suggested by the EU Scientific Committee on Food [26].

The objective of this work is to present the results of a random survey of OTA concentration in variety wines processed in Romania, which should comply with the standing regulation of the European Union regarding concentrations of OTA in wine. Quantitative determination of OTA in wines after pre-treatment of wine on immunoaffinity columns was investigated using the HPLC method with fluorescence detection.

---

* *email: irina.geana@icsi.ro; Tel: 0040745752559*

Experimental part

Materials and methods

Chemicals and materials

Ochratoxin A solution in acetonitrile (10 mg/L) was purchased from Supelco. Acetonitrile and methanol were of HPLC grade from Merck. Glacial acetic acid, sodium chloride, sodium hydrogen carbonate and polyethylene glycol (PEG 8000) were purchased from Merck. The diluting solution was prepared by dissolving 10 g of polyethylene glycol and 50 g of sodium hydrogen carbonate in 1000 mL of water, and the washing solution was prepared by dissolving 25 g of sodium chloride and 5 g of sodium hydrogen carbonate in 1000 mL of water.

OTA stock solution was prepared in HPLC mobile phase. Working solution was prepared by appropriate dilution in HPLC mobile phase for the recovery tests and calibration curve. The solutions were stored from -15°C until to -20°C and protected from light. Ochraprep immunoaffinity columns were purchased from R-BIOPHARM RHONE LTD.

Apparatus

Chromatographic analysis was carried out with a Thermo Finnigan Surveyor Plus chromatograph equipped with a fluorescence detector at excitation wavelength 333 nm and emission wavelength 460 nm, Surveyor autosampler, Surveyor LC Pump (Quaternary gradient). Data analysis was done using the Chrome Quest Chromatography Workstation. The analytical column is Hypersil Gold, 100×4.6 mm, with the sorbent particle size of 5 μm. 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. Samples were injected onto analytical column in 25 μL volume. All analyses were carried out at ambient temperature.

Sample preparation and immunoaffinity clean-up (IAC)

Wine were first degassed in an ultrasonic bath for approximately 30 min. Sample preparation of wine sample was achieved according to Visconty method (Visconti 1999) that has been adopted as the official method by the Association of Official Chemists International (AOAC), the European Committee for Standardization (CEN) (EN 14133), and the Organisation Internationale de la Vigne et du Vin (OIV). We used immunoaffinity columns (OCHRAPREP from R-BIOPHARM RHONE LTD). 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. 10 mL of sample diluted (1:1, v/v) with the dilution solution (PEG 1% and $NaHCO_3$ 5%) was then applied on the column and allowed to pass through the column, with or without applying a slight vacuum. When analyzing a non-clarified grape juice it was necessary to filter the diluted juice through a glass fibre filter (pore size 1.2–1.4μm). IAC was then washed with 5 mL of washing solution (NaCl 2.5% and $NaHCO_3$ 0.5 %) and 5 mL of deionized water to get rid of interfering substances. The column was then dried with air for 10 min and the retained OTA was eluted with 2 mL of absolute methanol.

HPLC determination

A 25 μL of reconstituted extract was injected into the chromatographic system by full loop injection system. The mobile phase consisted of the mixture acetonitrile:water: acetic acid (99:99:2) eluted at a flow rate of 1.0 mL/min.

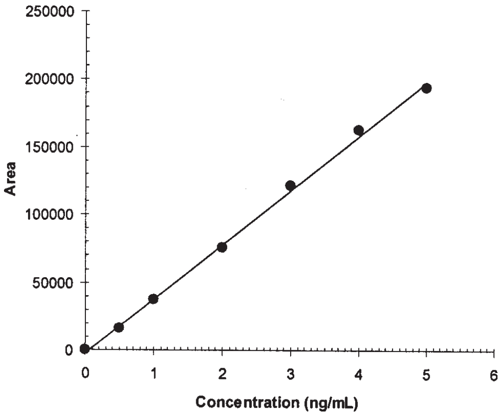

Fig. 2. Calibration curve for OTA

Quantification of OTA was performed by measuring peak areas at OTA retention time and comparing them with the relevant calibration curve.

Wine samples

To measure the concentrations of OTA in Romanian wine, 30 samples of bottled and commercialized wines (5 red, 25 white; 20 dry, 10 sweet) from several Romanian viticultural regions were selected (table 2).

Evaluation of the method

The analytical procedure was internally validated by means of calibration and evaluation of the range of linearity, limit of detection (LOD) and quantification (LOQ) and recovery. The calibration measurements were carried out with OTA standard solutions. Linear response of fluorescence detector was determined in the range of concentrations 0.06 ÷ 5 ng/mL which led to the correlation factor $r > 0.9969$ (fig. 2). The reproducibility expressed as repeatability was checked at two levels of OTA concentration: 0.5 ng/mL (S_r = 0.056 ng/mL) and 2.5 ng/mL (S_r = 0.093 ng/mL) in wines. LOD – 0.059 ng/mL and LOQ – 0.086 ng/mL 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 $n = 10$). The recoveries of OTA using IAC columns for sample pretreatment were studied by spiking wines with standard solutions at OTA levels of 0.1, 1 and 3 ng/mL.

Results and discussions

The analytical procedure, based on IAC cleanup 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.

A typical HPLC Chromatogram with fluorescence detection of contaminated and uncontaminated wine sample is shown in figure 3, respectively figure 4.

For recovery experiments, a wine sample with no detectable amounts of OTA was spiked with various concentration of OTA. Recoveries for spiked wine samples with OTA concentration ranged from 0.1 to 3 ng/mL were between 74 and 96% (table 1). The samples were analyzed in triplicate.

The results of the analysis of 30 wine samples from the Romanian market are summarized in table 1. OTA was founded in 7 samples at concentration ranging from 0.092-0.446 ng/mL. Twenty three sample (75%) contained trace amounts of OTA (<0.06 μg/L).

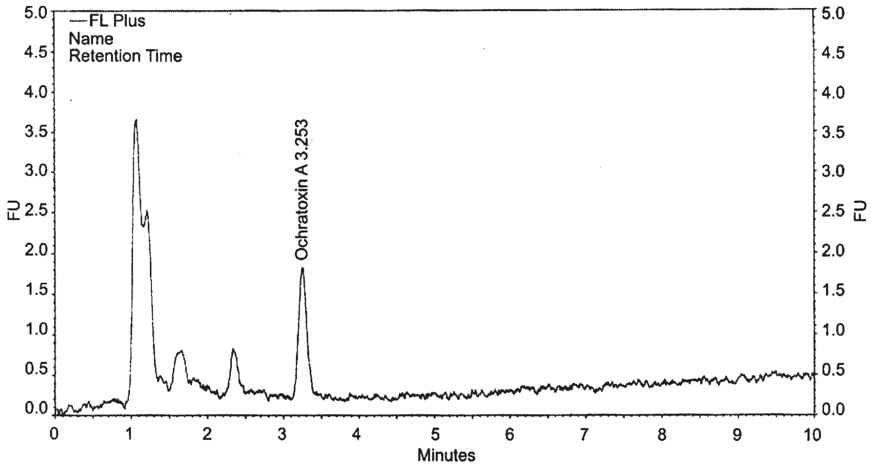

Fig. 3. HPLC chromatogram of contaminated wine sample; excitation 330 nm, emission 460 nm

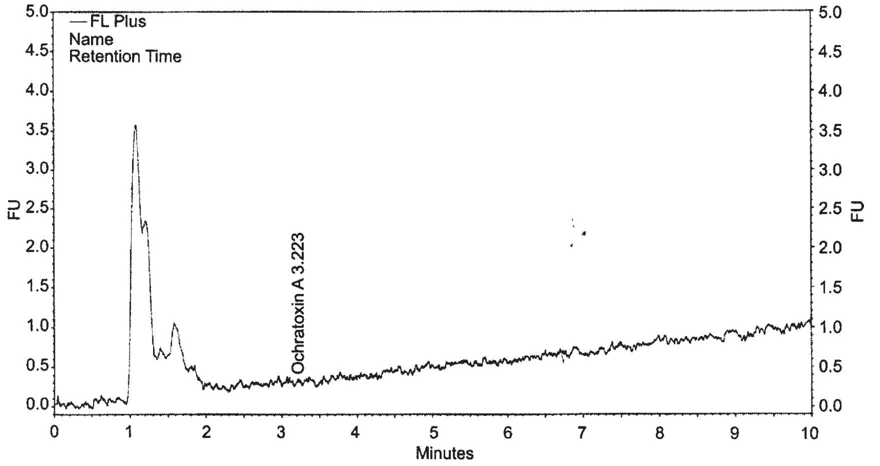

Fig. 4. HPLC Chromatogram of uncontaminated wine sample

Sample	OTA added (μg/L)	Recovery (%)	Number of experiments
1	3.0	96	3
2	1.0	88	3
3	0.1	74	3

Table 1
RECOVERY OF OTA ADDED TO WINE SAMPLES

Sample	Colour	Type	Vintage	OTA content (μg/L)
1	white	semidry	2009	0.092
2	white	semisweet	2009	0.332
3	white	semidry	2010	0.421
4	white	semisweet	2010	0.446
5	white	table wine	2010	0.112
6	red	table wine	2010	0.122
7	red	table wine	2010	0.120

Table 2
OCHRATOXIN A CONTENT OF CONTAMINATED ROMANIAN WINE SAMPLES

Conclusions

This study presented a first picture concerning the presence of OTA in Romanian wines. Moreover, the analytical method presented and used in this survey, has shown sufficient parameters and sensitivity for detecting traceable OTA levels in wine. Generally, wines produced or commercialized on the Romanian market have a lower level of OTA, the concentrations found being far below the proposed European limit (2 ng/mL). 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.

Acknowledgments:L The research was partly supported by the Ministry of Education and Research – NASR under ctr. 19N/2009.

References

1.SAVA, V., REUNOVA, O., VELASQUEZ, A., HARBISON, R., SÁNCHEZ-RAMOS, J., Neuro Toxicology, volume 27, issue 1, 2006, p. 82-92.

2.WALKER, R., LARSEN, J. C., Food Additives and Contaminants, Supplement 1, 2005, p.6–9.

3. *** International Agency for Research on Cancer (IARC), Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 56, Lyon (1993)

4.LIN, L. C., CHEN, P. C., FU, Y. M., YANG-CHIN SHIH, D., Journal of Food and Drug Analysis, Vol. 13, No. 1, 2005, p. 84-92

5.ABARCA, M. L., ACCENSI, F., BRAGULAT, M. R., CASTELLÁ, G., J. Food Prot., 66, 2003, p.504–506.

6.BATTILANI, P., PIETRI, A., BERTUZZI, T., LANGUASCO, L., GIORNI, P., KOZAKIEWICZ, Z., J. Food Prot. 66, 2003, p. 633–636.

7.BAU, M., BRAGULAT, M. R., ABARCA, M. L., MÍNGUEZ, S., CABA ES, F. J., Int. J. Food Microbiol., 98, 2005, p. 125–130

8.BEJAOUI, H., MATHIEU, F., TAILLANDIER, P., LEBRIHI, A., Int. J. Food Microbiol. 111 (Suppl. 1), 2006, p.46–52 .

9.CHULZE, S.N., MAGNOLI, C.E., DALCERO, A.M., Int. J. Food Microbiol. 111, 2006, p.5–9.

10.GUZEV, L., DANSHIN, A., ZIV, S., LICHTER, A., Int. J. Food Microbiol. 111, 2006, p.67–71.

11.LASRAM, S., BELLÍ, N., CHEBIL, S., NAHLA, Z., AHMED, M., SANCHIS, V., GHORBEL, A., Int. J. Food Microbiol., 114, 2007, p.376–379.

12.LEONG, S.L., HOCKING, A.D., SCOTT E, S., J. Appl. Microbiol., 102, 2007, p.124–133.

13.PONSONE, M.L., COMBINA, M., DALCERO, A., CHULZE, S., Int. J. Food Microbiol., 114, 2007, p.131–135.

14.SERRA, R., ABRUNHOSA, L., KOZAKIEWICZ, Z., VENÂNCIO, A., Int. J. Food Microbiol., 88, 2003, p.63–68.

15.TJAMOS, S.E., ANTONIOU, P.P., TJAMOS, E.C., Int. J. Food Microbiol., 111, 2006, p.61–66.

16.DOMIJAN, A.M., PERAICA, M., Arh Hig Rada Toksikol 56, 2005, p.17

17.ZIMMERLI, B., DICK, R., J Chromatogr B; 666, 1995, p.85-99.

18.LINO, C. M., BAETA, M.L., HENRI, M., DINIS, A.M .P., PENA, A.S., SILVEIRA, M.I.N., Food and Chemical Toxicology 46, 2008, p.879-885.

19.DEGELMANN, P., BECKER, M., HERDERICH, M., HUMPF, H.U., Chromatographia 49, no.9/10, 1999, p.543-546.

20.VARGA, J., KISS, R., MATRAI, T., TEREN, J., Acta Alimentaria, Vol. 34 no. 4, 2005, p.381–392.

21.SOUFLEROS, E., TRICARD, C., BOULOUMPASI, E., J Sci Food Agric 83, 2003, p.173–179.

22.ZIMMERLI, B., DICK, R., Food Addit. Contam.13, 1996, p. 655-668.

23.CERUTTI, G., D'AMATO, A., ZUCCHETTI, M., Imbottigliamento 23, 2000, p.39-43.

24.VISCONTI, A., PASCALE, M., CENTONZE, G,, Journal of Chromatography A, 864, 1999, p. 89–101.

25.MAJERUS, P., BRESCH, H., OTTENEDER, H., Arch Lebensmittelhygiene 51, 2000, p. 81-128.

26.*** Commission Regulation EC No 123/2005 of 26 January 2005 amending regulation (EC) No 466/2001 as regards Ochratoxin A. Off. J. Eur. Commun. 2005, L25/3, 28.1.2005

Manuscript received: 6.12.2011