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Determination of Ochratoxin A in Red Wine and Vinegar by Immunoaffinity High-Pressure Liquid Chromatography

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ABSTRACT

A method is described for the determination of ochratoxin A (OTA) in red wine and vinegar using an acidic chloroform extraction, an immmunoaffinity clean-up step, and a high-performance liquid chromatographic determination with fluorescence detection. The detection limit was estimated at 0.002 μg/liter. The mean recovery factors were found at 91.3 and 96.6% for wine and vinegar, respectively. Thirty-one samples of red wine originating from Mediterranean sea countries and 15 samples of vinegar were examined for the presence of OTA. All red wine samples contained OTA. Seventy-two percent of these samples were found to be contaminated over 0.1 μg/liter. Among them, nine samples contained ochratoxin A in the range of 0.5 to 3.4 μg/liter, 12 samples in the range of 0.10 to 0.50 μg/liter (median: 0.176 μg/liter), and 9 samples in the range of 0.010 to 0.100 μg/liter (median: 0.041 μg/liter). All 15 vinegar samples showed the presence of OTA. The most contaminated ones were three balsamic vinegar samples containing 0.156 μg/liter, 0.102 μg/liter, and 0.252 μg/liter of OTA. In the remaining 12 samples, ochratoxin A levels ranged from 0.008 μg/liter to 0.046 μg/liter (median: 0.012 μg/liter). These data are in good agreement with the hypothesis that wine originating from Southern countries might contain significant OTA concentration and showed the possible occurrence of traces of OTA in vinegar.

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Among the various mycotoxins that could occur in agricultural commodities, ochratoxin A (OTA) has received increasing attention because it is suspected to be a nephropathic and carcinogenic molecule *(10).* There is more and more evidence that OTA might be responsible for the Balkan endemic nephropathy and for the high frequency of urinary tract tumors observed in some Balkan areas *(4, 14).* Throughout Western Europe, several authors *(2, 3, 5, 8, 23)* reported that OTA could be found in human sera at the ppb level, betraying consistent exposition of humans to this mycotoxin. Therefore, the question arose of which are the main and/or widespread food vehicles for OTA in the human diet.

OTA has been widely detected in food of plant origin mainly in cereals like barley, wheat, maize, oats, and their by-products, in green coffee, cocoa beans, and also in spices *(9, 11, 17, 21).* Moreover, the mycotoxin could be transferred from feed to edible animal tissues, especially to pork meat *(7).*

In addition, many kinds of beverages made from ripened fruits like raisins (raisin juices, wine) or manufactured from cereals (beer) or seeds (coffee) could contain significant amounts of OTA *(18, 20).* Particularly, the presence of OTA in table wine was recently demonstrated by Zimmerli and Dick *(24).* In addition, the mycotoxin was also detected in dried vine fruits (currants, raisins, and sultanas) from Greece and Turkey *(13).*

The aim of this study was to obtain further quantitative data on the occurrence of OTA in red wine, especially those originating from countries in the Mediterranean basin, and to investigate the possible transfer of OTA in vinegar, especially when made from raisins or red wine.

MATERIALS AND METHODS

Samples. Wine and vinegar samples were purchased from local supermarkets and small shops in France during the summer of 1999. All information on samples was taken from the labels. The prices of the red table wines ranged from about 5 to 60 French Francs per bottle. Samples were stored at 4°C until analysis.

Reagents. All solvents were of high-pressure liquid chromatography (HPLC) grade. Orthophosphoric acid 85%, acetic acid, and boron trifluoride were from Merck (Paris, F). The phosphate-buffered saline (lot 83230-003; potassium dihydrogen phosphate [0.2 g/liter], disodium hydrogen phosphate $12H_2O$ [2.9 g/liter], potassium chloride [0.2 g/liter], sodium chloride [8 g/liter], Thimerosal [0.1 g/liter], pH 7.4) and immunoaffinity columns (OchraPrep) were obtained from Rhone Diagnostic Technologies Ltd. (Glasgow, UK).

Extraction. Ten milliliters of wine or vinegar samples were mixed together with 10 ml of an orthophosphoric acid solution (33.7 ml of orthophosphoric acid 85% and 118 g of sodium chloride per liter, pH 1.6) and stirred for 2 min. The mixture was extracted with 60 ml of chloroform for 3 min. After extraction, the volume of the chloroform was measured and evaporated to dryness by using a rotary evaporator. The residue was dissolved with 2 ml of methanol and mixed thoroughly. The suspended residue was then diluted with 40 ml of phosphate-buffered saline and mixed for 1 min.

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Clean-up. The extract mixture was loaded onto an Ochra-Prep immunoaffinity column operating at a steady flow rate of 2 ml/min. After washing the immunoaffinity column by applying 20 ml of distilled water, the column was dried by pushing air into the column with the help of a syringe.

OTA was carefully eluted by applying 2 ml of a solution of methanol and acetic acid (98 + 2) onto the column. The eluate was then evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 300 to 1,000 μl of mobile phase, and an aliquot was kept at −18°C for confirmation analysis.

HPLC analysis. The HPLC system comprised a pump (Jasco PU-1580, Prolabo, France) and an autosampler from Jasco (AS-1555), a column oven set at 35°C (Hitachi 655A-52, Merck, France), a fluorometric detector (Jasco FP 1520), and a solvent saver (Research model 2907). The chromatographic separation was performed on a C-18 Lichrosphere 100 RP 250 by 4-mm column (Merck). The mobile phase (water + acetonitrile + acetic acid: 500 + 500 + 20) was delivered through the HPLC system at a flow rate of 0.9 ml/min. The fluorometer was set at a wavelength of 335 nm for excitation and 465 nm for emission.

In-house characterization of the analytical protocol for OTA determination. The analytical protocol for OTA determination was in-house characterized regarding the following criteria: linearity, accuracy (by determination of the recovery factor), repeatability, and limits of detection and quantification. With regard to linearity, from a stock solution of 10 μg of OTA/ml in methanol, working solutions (at 1 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml concentrations) were extemporaneously prepared by dilution in the HPLC mobile phase. With regard to accuracy, as no reference material exists for OTA in wine or vinegar, accuracy was indirectly estimated by determining the recovery factor on spiked samples, i.e., 10 wine samples and 3 vinegar samples spiked at 100 ng OTA/liter. With regard to repeatability, this parameter was estimated by analyzing one sample of naturally contaminated red wine 10 times under repetitive conditions. With regard to the limits of determination, calculation for the detection limit was based on a signal-to-noise ratio of 3:1 at the retention time of OTA (i.e., 9.5 min) when analyzing a noncontaminated wine sample.

Confirmation. The confirmation of OTA presence was performed by the methylester technique in producing and identifying the methylester derivative of OTA (METOTA) as described by Lerch and Muller *(12)*. Briefly, an aliquot of the immunoaffinity eluate was dried and dissolved in a methanolic solution of boron trifluoride and heated at 60°C for 15 min. In samples containing OTA, when using the same HPLC chromatographic conditions as for the analysis of OTA, the disappearance of the OTA peak (retention time at 9.5 min) should be observed together with the appearance of the methylester peak (retention time at 23.5 min). METOTA can be calculated from a calibration curve prepared with derivatized OTA standard solutions.

RESULTS

Characterization of the method. The analytical protocol for the OTA determination in wine and vinegar (Fig. 1) was in-house characterized. The calibration curve was established in the range of 1 to 20 ng OTA/ml. Furthermore, the linearity of the method applied in wine was studied by analyzing increasing volumes of one naturally contaminated sample of wine. The final steady test volume was 10 ml, by adding water (Table 1). The regression coefficient r of this curve was found at 0.999, and a Fisher test was applied to confirm the acceptability of the linear regression. The found $F(1.20)$ ratio of 1,811.399 was greater than the critical Fisher value of 8.095 at an alpha risk of 1%.

The mean recovery factors for wine and vinegar were found at 91.3% (coefficient of variation = 8%) and 96.6% (coefficient of variation = 6%), respectively. The coefficient of variation determined under repetitive conditions when analyzing a naturally contaminated sample of wine 10 times (mean 0.178 μg/liter, $n = 10$) was found to be 3.3%. The detection limit was found at 0.002 μg of OTA/liter. The limit of quantification where OTA could be quantified with an acceptable precision ranged from 0.006 to 0.060 μg/liter, depending on the size of the volume (300 to 1,000 μl) in which the immunoaffinity eluate was resuspended and also depending on the injection volume (50 to 100 μl) onto the HPLC column (Table 1).

Occurrence of OTA in wine and vinegar. Table 2 shows the results from the analysis of 31 samples of red wine and 15 samples of vinegar that originated from southern European countries, especially along the Mediterranean Sea. Every sample was found to contain OTA traces at least. The most contaminated samples were one sample from Greece (2.35 μg OTA/liter), four samples from France (2.62 μg/liter, 1.59 μg/liter, 3.21 μg/liter, and 3.40 μg/liter of OTA), and one sample labeled Communauté Europeène (1.54 μg OTA/liter). Furthermore, one sample from Italy and two samples from Morocco were found to be contaminated with OTA at levels of 0.892 μg/liter, 0.551 μg/liter, and 0.554 μg/liter, respectively. The range of contamination for 12 other samples was found to spread from 0.009 to 0.100 μg OTA/liter. Results from the analysis of 15 vinegar samples revealed the presence of OTA traces in all samples. The most contaminated ones were three balsamic vinegar samples containing 0.156 μg/liter, 0.102 μg/liter, and 0.252 μg/liter of OTA. OTA detection in the other 12 samples ranged between 0.008 and 0.046 μg/liter, with a median level of 0.012 μg/liter.

Confirmation. The necessity to confirm the identity of OTA in samples is not so crucial when using the highly specific immunoaffinity columns *(15)*. However, the presence of OTA in the six most contaminated wines (i.e., over 1 μg/liter) was confirmed by the methylester method. The retention time of the methylester derivative METOTA was 23.5 min compared to 9.5 min for OTA, so that no confusion could occur regarding the identification of both peaks. By plotting the results of OTA concentration found in wines against the corresponding METOTA contents, an acceptable linear correlation ($r = 0.967$) was found, with the following equation: $y = 250.49(\pm 303.7) + 0.902(\pm 0.118)x$, where y = ng/liter of METOTA and x = ng/liter of OTA (Fig. 2). When applying a t test at the 95% confidence level, the experimental t value for intercept-zero comparison was 0.824. This was less than the theoretical t value of 2.774, demonstrating that there was no constant systematic error. On the other hand, the t experimental for slope-unity comparison was 0.833, which was less than the t theoretical of 2.774, showing that there was no relative

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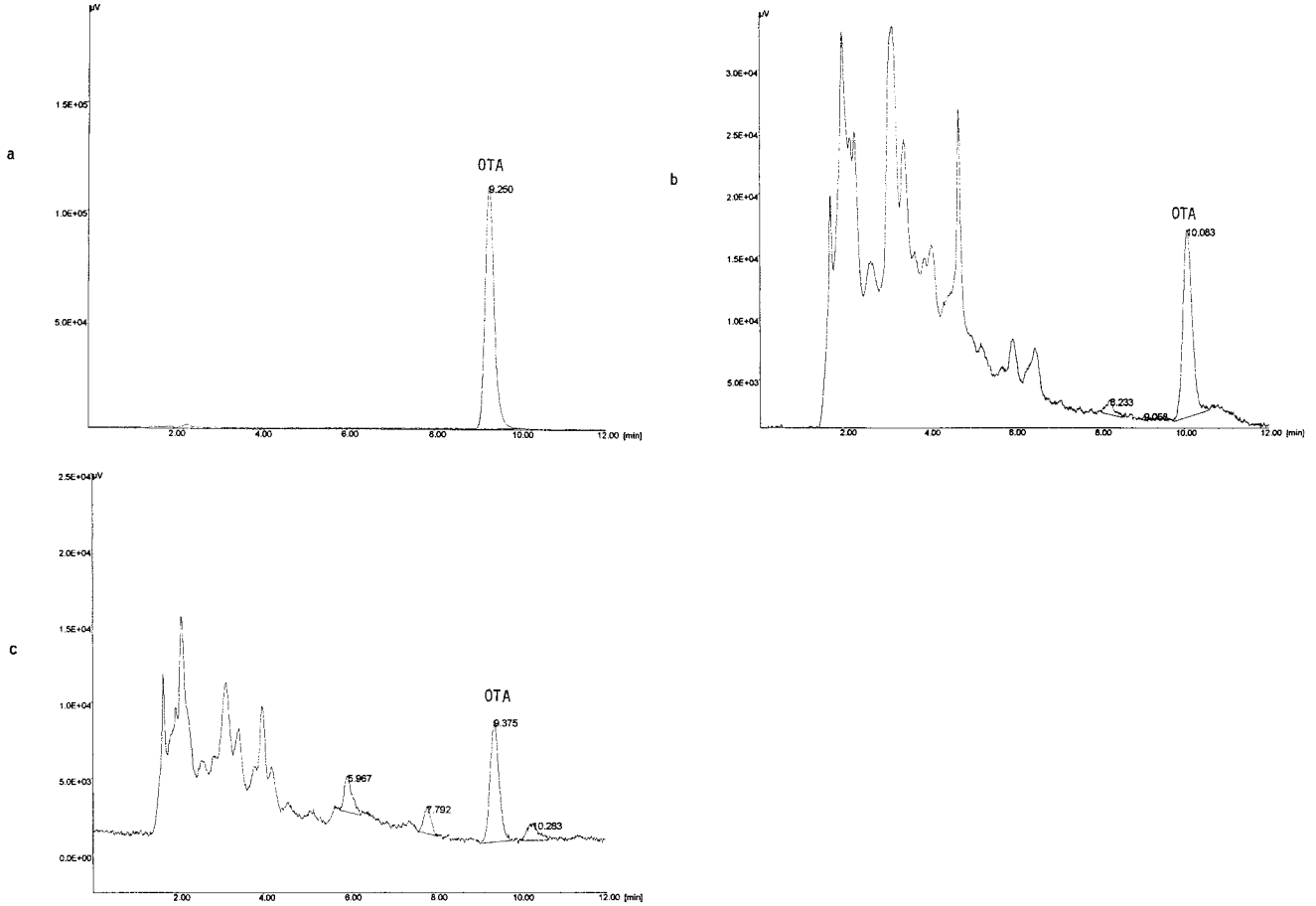

FIGURE 1. *Chromatograms of (a) OTA standard solution, 5 ng/ml; (b) wine naturally contaminated with 0.173 µl/liter of OTA; and (c) vinegar naturally contaminated with 0.046 µg/liter of OTA. The extracts of samples containing OTA were dissolved in the mobile phase of acetic acid + water + acetonitrile (20 + 500 + 500) in the case of b with 1,000 µl and of c with 300 µl. The injection volume was 50 µl.*

systematic error. These results confirmed the identity of the HPLC peak of OTA.

DISCUSSION

The principle of extraction and purification steps for the analysis of OTA from wine by using immunoaffinity clean-up is comparable to other published methods and displays the same characteristics *(16, 22, 24).* The extraction of OTA from red wine by means of organic solvent (chloroform) or polyethylene glycol was demonstrated to be necessary before passing the extract through the immunoaffinity columns. This extraction step makes the separation, as red wine pigments could easily interfere with OTA detection.

The in-house characterization of the immunoaffinity-HPLC method for OTA determination produced very satisfactory performance parameters in terms of repeatability, recovery, and limits of determination. For instance, Zimmerli and Dick *(24)* found a detection limit of 0.003 µg/liter and a mean recovery factor of 84%, while Visconti et al. *(22)* found a detection limit of 0.010 µg/liter and a recovery factor of 88 to 103%.

In this work, all wine samples contained OTA, but most of them had a content below 0.5 µg of OTA/liter. The highest content was found in a French wine at the level of 3.4 µg/liter. As the number of samples was limited, no significant trend could be determined. Visconti et al. *(22)* analyzed 38 samples of red wine for their OTA content, and

TABLE 1. *Determination of the quantification limit for OTA when analyzing increasing volumes of wine test portions*

Levels (p)	Volume of wine (ml)	Volume of water (ml)	Final volume (ml)	Peak height repetitions (n = 5)	Coefficient of variation (%)	Concentration of OTA (ng/liter)
1	10	0	10	9,259.6	1.3	167
2	5	5	10	5,308.5	1.4	101
3	2.5	7.5	10	2,997.0	6	61
4	1.25	8.75	10	1,953.3	26	46
5	0.620	9.38	10	1,177.6	31	30

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TABLE 2. *OTA concentrations in samples of red wine and vinegar*

Origin of samples	*n*	Range (μg of OTA/liter)			
		LOD[a]–0.100	0.10–0.50	0.5–1	>1
Red wine					
Greece	8	4	2	1	1
Maroc	3	1	—	2	—
Spain-Portugal	6	3	3	—	—
France	12	1	7	—	4
Communauté Europeènne	1	—	—	—	1
Italy	1	—	—	1	—
Vinegar					
Balsamic vinegar	3	—	3	—	—

[a] LOD, limit of detection.

these authors found a range of contamination from the detection limit to 7.6 μg/liter. Zimmerli and Dick *(24)* analyzed 79 samples of red wine, and the median OTA level was 0.013 μg/liter. The most contaminated sample, a wine from Tunisia, was found to contain 0.388 μg/liter of OTA. These authors considered the concentration of 0.100 μg of OTA/liter in wine as significantly high. Most likely, the rates of OTA contamination in raisins and consecutively in wine vary from one year to the other depending on the meteorological conditions. Moreover, very little if anything is known about the actual fate of OTA during wine processing.

To our knowledge, no other work on the occurrence of OTA in vinegar has been published yet. It is noteworthy that the most contaminated samples of vinegar were balsamic vinegars, a product that is made from grape musts. The grapevine variety is a specific type of white grapes named Trebiano that are gathered late in autumn to let the grapes completely ripen. These conditions probably make the contamination by molds able to produce some OTA easier. Some traces of OTA (i.e., around 0.010 μg of OTA/L) were detected in samples of vinegar made from cider, plants, or colored alcohol, but this is probably of no significance.

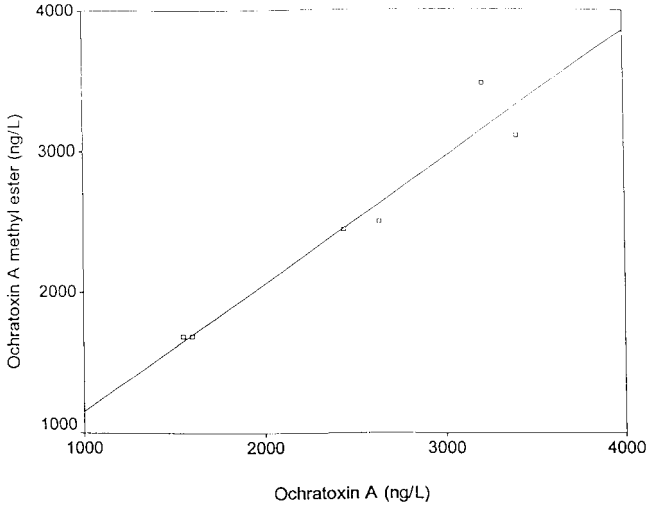

FIGURE 2. *Correlation between OTA content and the production of OTA methylester in the six highly contaminated wines.*

The occurrence of OTA in wine and then in vinegar is linked to the presence of molds on grapes *(6)*. It was suggested that OTA is probably formed prior to the alcoholic fermentation, assuming that OTA is not degraded during wine making and storage. Mold growth in wine itself is inhibited by ethanol and the generally anaerobic conditions in wine *(1)*. OTA found in wine was also supposed to be formed after the harvest of grapes as the climatic conditions during the harvesting period where less humidity occurs are not favorable for mold growth on the field. Zimmerli and Dick *(24)* suggested that failure to remove moldy fruits before further processing or moldy equipment before it comes into contact with grapes or wine would not be in accord with good manufacturing practices, and this could be a good reason for the occurrence of OTA in wine.

The European Union Scientific Committee for Food recommended a tolerable daily intake of 5 ng/kg body weight per day for OTA. After Studer-Rohr *(19)* demonstrated that the terminal half-life of OTA in humans is 10 times longer than in rats, Zimmerli and Dick *(24)* consecutively suggested to decrease the provisional virtually safe dose to the magnitude of 0.5 ng/kg body weight per day and calculated that moderate consumption (300 to 400 ml) of wine containing 0.09 μg of OTA (intake 2 ng/kg body weight per day) would result in a steady-state OTA serum concentration of 0.7 μg/liter. Therefore, consuming wine with OTA levels as reported in this work (1.5 to 3.4 μg/liter) might significantly contribute to the daily OTA intake. Under these conditions, even a moderate drinker would increase his daily OTA intake to about 10-fold. The contribution of vinegar to OTA exposure is certainly minute, but the wide use of this very popular condiment could only increase human exposure to this mycotoxin.

REFERENCES

1. Amerine, M. A., R. W. Kunkee, C. S. Ough, V. L. Singleton, and A. D. Webb. 1980. The technology of wine making, p. 154–616. AVI Publishing Company, Westport, Conn.
2. Breitholz, A., M. Olsen, A. Dahlback, and K. Hult. 1991. Plasma ochratoxin A levels in three Swedish populations surveyed using an ion-pair HPLC technique. Food Addit. Contam. 8:183–192.

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3. Breitholz-Emmanuelsson, A., F. Minervini, K. Hult, and A. Visconti. 1994. Ochratoxin in human serum samples collected in southern Italy from healthy individuals suffering from different kidney disorders. Nat. Toxins 2:366–370.
4. Castegnaro, M., R. Plestina, G. Dirheimer, I. N. Chernozemsky, and H. Bartsch. 1991. Mycotoxins, endemic nephropathy and urinary tract tumours, vol. 115. IARC Scientific Publications, Lyon, France.
5. Creppy, E., M. Castegnaro, Y. Grosse, J. Meriaux, C. Manier, P. Moncharmont, and C. Waller. 1993. Etude de l'ochratoxicose humaine dans trois régions de France: Alsace, Aquitaine, et région Rhône-Alpes, p. 147–158. *In* E. Creppy, M. Castegnaro, and G. Dirheimer (ed.), Human ochratoxicosis and its pathologies, vol. 231. John Libbey Eurotext Ltd., Paris, France.
6. Davis, N. D., and U. L. Diener. 1987. Mycotoxins, p. 517–570. *In* L. R. Beuchat (ed.), Food and beverage mycology. Van Nostrand Reinhold, New York.
7. Gareis, M. 1996. Fate of ochratoxin A on processing of meat products. Food Addit. Contam. 13S:35–37.
8. Hadlock, R. M. 1993. Human ochratoxicosis in Germany: updating 1993, p. 141–145. *In* E. Creppy, M. Castegnaro, and G. Dirheimer (ed.), Human ochratoxicosis and its pathologies, vol. 231. John Libbey Eurotext Ltd., Paris, France.
9. Hubner, M., T. Vrabcheva, and M. Gareis. 1998. Simultaneous detection of ochratoxin A and B in spices and herbs by immunoaffinity column cleanup and HPLC. Rec. Med. Vet. special issue June 98: 507.
10. IARC (International Agency for Research on Cancer). 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins, p. 489–524. *In* IARC monographs on the evaluation of carcinogenic risks to humans, vol. 56. IARC Scientific Publications, Lyon, France.
11. Jorgensen, K., and B. Bilde. 1993. Occurrence and estimated dietary intakes of ochratoxin A in European countries. Results from a SCOOP project. Food Addit. Contam. 13S:15–16.
12. Lerch, C. M., and H. M. Müller. 1990. Analysis of ochratoxins from buffered rumen fluid by reversed-phase HPLC. Chromatographia 30: 424–427.
13. McDonald, S., P. Wilson, K. Barnes, A. Damant, R. Massey, E. Mortby, and M. J. Shepherd. 1999. Ochratoxin A in dried vine fruit: method, development and survey. Food Addit. Contam. 16:253–260.
14. Petkova-Bocharova, T., and M. Castegnaro. 1991. Ochratoxin A in human blood in relation to endemic nephropathy and urinary tract tumours in Bulgaria, p. 135–137. *In* M. Castegnaro, R. Plestina, G. Dirheimer, I. N. Cheznozemsky, and H. Baztch (ed.), Mycotoxins, endemic nephropathy and urinary tract tumours, vol. 115. IARC Scientific Publications, Lyon, France.
15. Scott, P., and M. Trucksess. 1997. Application of immunoaffinity columns to mycotoxin analysis. J. Assoc. Off. Anal. Chem. Int. 80: 943–949.
16. Sharman, M., S. McDonald, and J. Gilbert. 1992. Automated liquid chromatographic determination of ochratoxin A in cereals and animal products using immunoaffinity column clean-up. J. Chromatogr. 603:285–289.
17. Speijers, G. J. A., and H. P. Van Egmond. 1993. World-wide ochratoxin A levels in food and feeds, p. 85–100. *In* E. Creppy, M. Castegnaro, and G. Dirheimer (ed.), Human ochratoxicosis and its pathologies, vol. 231. John Libbey Eurotext Ltd., Paris, France.
18. Stegen, G., U. Jorissen, A. Pittet, M. Saccon, W. Steiner, M. Vincenzi, M. Winkler, J. Zapp, and C. Schlatter. 1997. Screening of European coffee final products for occurrence of ochratoxin A. Food Addit. Contam. 14:211–216.
19. Studer-Rohr, L. 1995. Ochratoxin A in humans: exposure, kinetics and risk assessment. Dissertation No. 11071 of the Swiss Federal Institute of Technology (ETH), Zurich, Switzerland.
20. Thellmann, A., and W. Weber. 1997. Determination of ochratoxin A in cereals, malt and beer after accumulation and separation on immunoaffinity columns and following HPLC chromatograpy with fluorescence detection. Dtsch. Lebensm. Rundsch. 93:1–3.
21. Trucksess, M. W., J. Giler, K. Young, K. D. White, and S. W. Page. 1999. Determination and survey of ochratoxin A in wheat, barley and coffee—1997. J Assoc. Off. Anal. Chem. Int. 82:85–89.
22. Visconti, A., M. Pascale, and G. Centonze. 1999. Determination of ochratoxin A in wine by means of immunoaffinity columns cleanup and HPLC. J Chromatogr. 864:89–101.
23. Zimmerli, B., and R. Dick. 1995. Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by HPLC with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. J. Chromatogr. B:85–99.
24. Zimmerli, B., and R. Dick. 1996. Ochratoxin A in table wine and grape-juice: occurrence and risk assessment. Food Addit. Contam. 13:653–668.

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