Determination of Ochratoxin A in Beer

Ľubomír DAŠKO, Elena BELAJOVÁ, Drahomíra RAUOVÁ and Milan KOVÁČ

Food Research Institute, Bratislava, Slovak Republic

Abstract

Daško Ľ., Belajová E., Rauová D., Kováč M. (2005): **Determination of ochratoxin A in beer**. Czech J. Food Sci., **23**: 69–73**.**

Ochratoxin A is a very common mycotoxin which can be found rather often, predominantly in various cereal materials and in products from this type of plants. Our aim was to apply an analytical procedure with a suitable detection level of ochratoxin A for its estimation in beer. The detection level of the method suggested was close to 0.001 µg/kg. The analytical procedure is based on HPLC separation with fluorescence detection. The application of this method is demonstrated and analytical results obtained with beer of domestic provenience are reported.

Keywords: beer; ochratoxin A; immunoaffinity cleanup; HPLC

Ochratoxin A (OTA) is produced in contaminated foodstuffs by various toxicogenic fungi of the strain *Aspergillus*, predominantly *Aspergillus ochraceus, A. carbonarius, A. niger* (FAO Food and Nutrition Paper 74 2001). It is also known that there is only one *Penicillium* species producing OTA, namely *Penicillium verrucosum* (FAO Food and Nutrition Paper 74 2001; Chu 1977). Microorganisms differ in the places where they can be found, the plants commodities they are growing in, and the extent of incidence based on the geographical origin. *Penicillium verrucosum* grows only at temperatures below 30°C, the water activity value should be less than 0.8. It is supposed that it is the main source of OTA in cereals in Europe and Canada (FAO Food and Nutrition Paper 74 2001). *Aspergillus* species dominate in mild climate areas where the water activity value is higher than 0.8. These species are the main source of OTA in contaminated coffee (FAO Food and Nutrition Paper 74 2001). OTA is the dominating mycotoxin of five structurally related molecules (Betina 1990). All other similar toxins were reported at very low incidence (Chu 1977; Hayes 1978; Saenz De Rodriguez & Engl 1984; Ueno 1985). Toxicity of OTA was already studied on various sorts of animals (FAO Food and Nutrition Paper 74 2001; Patterson *et al*. 1976; Tsubouchi *et al*. 1984; Pitt 1987; Trucksess *et al.* 1997). LD_{50} values (in mg/kg body weight) are as follows: pig – 1, chicken – 3.3, dog – 0.2 (FAO Food and Nutrition Paper 74 2001). Also long term studies were carried out aimed at the evaluation of the carcinogenic effects of OTA. Higher tumor incidences were observed in the animals tested (Tsubouchi *et al.* 1984). In selected cases also some genotoxic effects were reported (Wiger & Stormer 1990; Wurgler *et al.* 1991; Varga *et al.* 1996; Xiao *et al.* 1996). An excellent review of the published data on the concentration levels of OTA in various commodities can be found in the literature (FAO Food and Nutrition Paper 74 2001). It is evident from this data that the weight average of the content of OTA is the highest in maize – 7.5 µg/kg and the lowest value reported

Supported by State Subprogram of Research "Food – Quality and Safety", No. 2003SP270280E010280E01.

is that in beer, 0.025 µg/kg (FAO Food and Nutrition Paper 74 2001).

Various analytical procedures were suggested for OTA determination. Thin layer chromatography methods are suitable for the detection of 10 µg/kg of OTA in wheat (Larsson & Moeller 1996). Mostly used for OTA determination is high performance liquid chromatography with fluorescence detection. OTA shows very high fluorescence in acidic solutions. The sample extract can be purified either on immunoaffinity columns (Official method 2001.01, 2002; EN 14133 – Determination of ochratoxin A in wine and beer; Visconti *et al*. 2001) or with C18 chemically bonded phases – solid phase extraction (Official method 2000.03, 2002; Official method 991.44, 2002). Immunoaffinity cleanup is suitable for the detection level of 0.1 µg/l in beer. Solid phase extraction is suitable for levels of 2 µg/l (Official method 991.44, 2002).

In our paper, we suggest an analytical method suitable to detect 0.00075 µg/l of OTA. Such very low detection level is suitable for testing the procedures focused on the elimination of OTA from beer.

MATERIALS AND METHODS

Standards and chemicals. All chemicals were of analytical or HPLC purity grade. Sodium chloride, potassium dihydrogen phosphate, potassium chloride, and hydrochloric acid were purchased from Lachema (Brno, Czech Republic); glacial acetic acid from AFT (Bratislava, Slovak Republic); disodium hydrogen phosphate from Merck (Darmstadt, Germany); toluene, min. 99%, from AFT (Bratislava, Slovak Republic); acetonitril Chromasolv from Sigma-Aldrich Laborchemikalien (Seelze, Germany). The standard of ochratoxin A was obtained from Sigma-Aldrich Chemie (Steinheim, Germany), and immunoaffinity columns Ochraprep for cleanup were obtained from R-Biopharm Rhone LTD (Scotland).

Equipment and HPLC analysis conditions. The separation was performed on a liquid chromatograph Agilent Technologies 1100 Series (Halbron, Germany) with fluorescence detector. The wavelengths setting used was 333 nm for excitation and 460 nm for emission in OTA determination. The analytical column was Zorbax SB-C18, 4.6 × 250 mm i.d. with the sorbent particle size of 5 µm, and the precolumn was Zorbax SB-C18, 12.5 × 4.6 mm i.d., with the same particle size, Agilent Technologies (Halbron, Germany). Isocratic separation was carried out with the mobile phase composed of a mixture of acetonitril: acidified water (20 ml glacial acetic acid in 1000 ml of water), 50:50 (v/v). The flow rate of the mobile phase was 1 ml/min. The injection was executed through the autosampler needle of 100 µl volume. The separation proceeded at ambient temperature.

Samples. Beers of Slovak production with different alcohol contents were analysed (pale and dark beers). Several foreign beers were also tested, mainly from the Czech Republic, Ireland, and France. Beers were first degassed in an ultrasonic bath for approximately 30 min and then filtered through a paper filter.

Procedures. The stock standard solution of OTA was prepared at the concentration of 1.25 mg/ml and stored at –18°C in a mixture of toluene:acetic acid, 99:1 (v/v). The working standard was prepared after evaporation of an appropriate volume of the stock solution under a stream of nitrogen, and dissolution of the residue in the mobile phase. The concentration of the working standard prepared was 125 µg/ml. The working standard solution was diluted with the mobile phase to provide five required concentrations in the range of 0.00125–3.05 ng/ml for the calibration curve construction. The standard solutions were stored at 4°C.

Phosphate buffered saline (PBS) solution used in the immunoaffinity cleanup procedure was prepared as follows: 8.0 g of sodium chloride, 1.2 g of disodium hydrogen phosphate, 0.2 g of potassium dihydrogen phosphate and 0.2 g of potassium chloride were dissolved in approximately 990 ml water and the pH value was adjusted to 7.4 with concentrated hydrochloric acid. The solution was made up to the volume of 1 l with deionised water.

Immunoaffinity column cleanup: Ochraprep immunoaffinity columns stored at 2–8°C were equilibrated to the laboratory temperature overnight. Columns were conditioned with 10 ml of PBS and 150 ml of filtered beer was then passed through. Beer passed through the column by the gravity effect. The column was washed with 10 ml of deionised water to eliminate the interfering substances and dried with air. OTA was eluted with 3 ml of absolute methanol. The eluate was partially evaporated on a rotary vacuum evaporator (the temperature of the water bath was 50°C) to the volume of about 0.5 ml, and then brought

to dryness under a gentle stream of nitrogen. The obtained residue dissolved in 500 µl of the mobile phase was ready for injection on HPLC.

RESULTS AND DISCUSSION

The most serious step in OTA determination is the isolation of OTA from beer. At present, it seems not to be any problem because there are many commercially available suitable columns for an effective isolation of OTA from not only beer but also from other food commodities. The Ochraprep immunoaffinity column contains a monoclonal antibody which selectively bonds OTA in such a way that the eluate obtained is free from any interfering compound. Using immunoaffinity column in the cleanup procedure (described above), the beer sample was pre-concentrated 300 times, i.e. its volume was reduced from 150 ml to 0.5 ml. This pre-treatment procedure offered a very sensitive detection level for OTA in beer – in our case the limit of determination was reached that was more than 10 000 times lower than the regulated permitted limit for OTA in beer (10 µg/kg). In general, the routine exploitation of immunoaffinity columns is very convenient but expensive because of their use as disposable items.

The duration of HPLC analysis was shorter than the beer cleanup on immunoaffinity columns. It took about 15 min for the chromatographic run to be recorded in a well-arranged chromatogram (Figures 1 and 2). From this point of view, the iden-

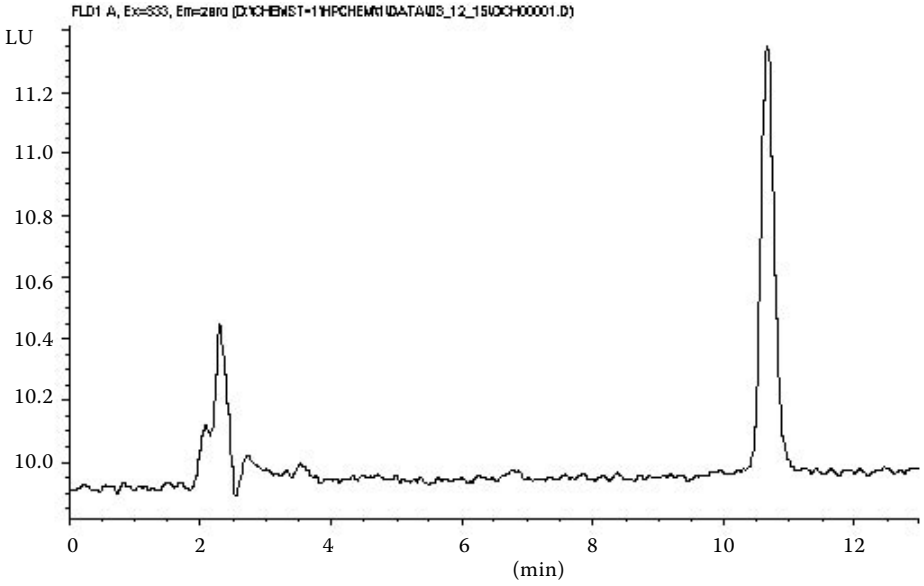

Figure 1. Chromatogram of OTA standard solution (0.0125 µg/ml)

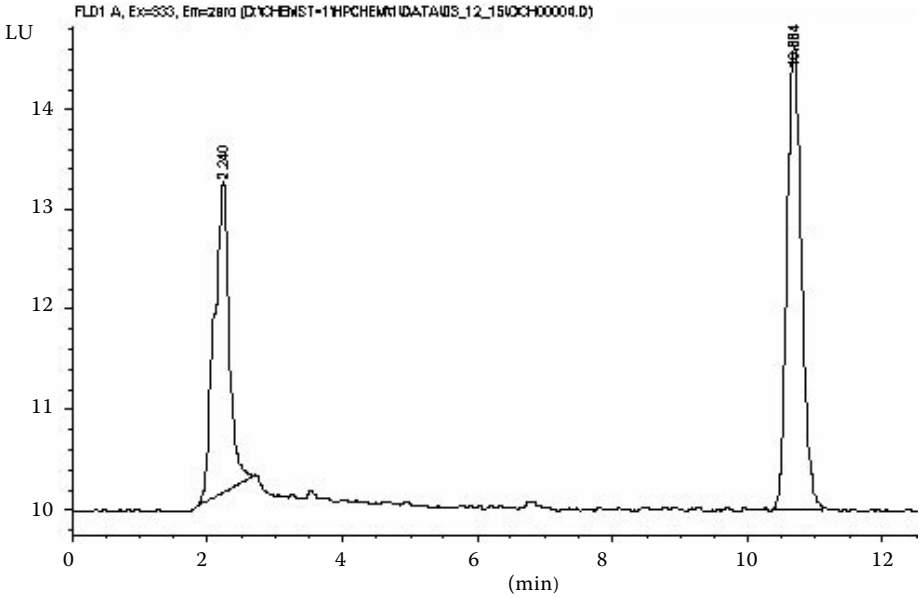

Figure 2. Chromatogram of spiked beer (to OTA concentration level 0.15 µg/l)

Table 1. Validation characteristics measured in spiked beer

Number	Spiked level (µg/l)	Measured level (µg/l)	Spiked level (µg/l)	Measured level (µg/l)
1	0.1974	0.1934	0.004	0.0037
2	0.1974	0.1936	0.004	0.0036
3	0.1974	0.1929	0.004	0.0037
4	0.1974	0.2056	0.004	0.0038
5	0.1974	0.1683	0.004	0.0030
6	0.1974	0.1724	0.004	0.0037
7	0.1974	0.1705	0.004	0.0032
8	0.1974	0.1835	0.004	0.0035
9	0.1974	0.1609	0.004	0.0034
10	0.1974	0.1793	0.004	0.0036
Average (µg/l)		0.1820	–	0.0035
Standard deviation (µg/l)		0.014	–	0.00025
Recovery (%)		92	–	87
Limit of detection (µg/l)		–	–	0.00075
Limit of determination (µg/l)		–	–	0.0025

tification of OTA was very simple – by comparison of OTA retention times of the standard and the sample. Quantification of OTA was done using the calibration curve and the external standard.

Based on the separation conditions, a partial validation of the method was carried out. Some performance characteristics were defined such as the limit of determination, the limit of detection and the statistical results of repeatability, calibration, recovery, and measurement uncertainty. In the repeatability evaluation, naturally contaminated beer was analysed as well as beer spiked with OTA to the level of 0.1974 µg/l (Table 1). In that case, the repeatability standard deviation of 0.014 µg/l was found. In the analyses of not spiked beer, only traces of OTA were observed recorded in the area of the detectable limit. This limit represented the value of 0.00075 µg/l. Calibration data were acquired from five analyses of OTA standards within the range of concentration from 0.00125–3.05 µg/l. The linear range estimated was 0.03–1.25 µg/l with regression coefficient of 0.99999. The recovery at OTA contamination level 0.182 and 0.0035 µg/l was 92% and 87%, respectively.

The measurement of uncertainty was evaluated as the extended combined uncertainty (factor 2) at 95% confidence interval and it resulted in the value of 6.8%. In the calculation of uncertainty were taken into account the standard weighing, standard and sample dissolution in the exact volume, and the beer volume taken for analysis.

The method described was applied in the testing of 20 beer samples from Slovak and 4 from foreign breweries. The Slovak beers were produced from barley grown in 2003. No positive results of OTA presence were found, the mycotoxin level was hardly detectable, being deeply under the quantification limit. The same impact was also observed with foreign beers. Similar screening of domestic beers was done also in the year 2000. In this case, the beer was produced from barley grown in 1999. The method applied was not as sensitive – the detection limit was 0.009 µg/l. 70% of the samples studied were contaminated. The contents found were within the range from 0.02 up to 0.1 µg/l. The difference between the years of study could be due to various climatic conditions.

CONCLUSION

Immunoaffinity cleanup of beer samples for ochratoxin A determination is a crucial step enabling to detect very low concentrations of this mycotoxin. It allows a very effective pre-concen-

tration of ochratoxin A. Therefore, the method suggested has a relatively low detection limit of 0.00075 µg/l. All other performance parameters of the method are also quite interesting. The application of this method is very simple requiring only a larger volume of the sample. Such a sensitive method is very useful as a control tool in the processes destined for the elimination of ochratoxin A from beer. Beer is a very popular drink with a steadily growing consumption.

References

Betina V. (1990): Mykotoxíny: Chémia – biológia – ekológia. Alfa, Bratislava: 112–118.

FAO Food and Nutrition Paper 74 (2001): Safety Evaluation of Certain Mycotoxins in Food. FAO, Rome.

Hayes A.W. (1978): Mycotoxins: a review of biological effects and their role in human diseases. Mycopathologia, **65**: 29–40.

Chu F.S. (1977): Mode of action of mycotoxins and related compounds. Advances in Applied Microbiology, **22**: 83–91.

Larsson K., Moeller T. (1996): Liquid chromatographic determination of ochratoxin A in barley, wheat bran and rye by the AOAC/IUPAC/NMKL method: NMKL collaborative study. Journal of the Association of Official Analytical Chemists International, **79**: 1102–1105.

Official method 2001.01 (2002): Determination of ochratoxin A in wine and beer. AOAC International.

Official method 991.44 (2002): Ochratoxin A in corn and barley. AOAC International.

Official method 2000.03 (2002): Ochratoxin A in barley. AOAC International.

Patterson D.S.P., Roberts B.A., Small B.J. (1976): Metabolism of ochratoxin A and B in the pig during early pregnancy and the accumulation in body tissue of ochratoxin A only. Food and Cosmetics Toxicology, **14**: 439–442.

Pitt J.I. (1987): *Penicillium verrucosum* and production of ochratoxin A. Applied and Environmental Microbiology, **53**: 266–269.

Saenz De Rodriguez C.A., Engl N. (1984): Environmental hormone contamination in Puerto Rico. Journal of Medicine, **310**: 1741–1742.

Trucksess M.W., Giler J., Young K., White K.D., Page S.W. (1997): Determination and survey of ochratoxin A in wheat, barley and coffee. Journal of the Association of Official Analytical Chemists International, **82**: 85–89.

Tsubouchi H., Yamamoto K., Hisada K., Sabake Y. (1984): A survey of occurrence of mycotoxins and toxicogenic fungi in imported green coffee beans. Proceedings of the Japanese Association of Mycotoxicology, **19**: 14–21.

Ueno Y. (1985): Trichotecenes – chemical, biological and toxicological aspects. CRC Critical Reviews in Toxicology, **14**: 99.

Varga J., Kevei F., Rinyu E., Teren J., Kozakiewicz Z. (1996): Ochratoxin production by *Aspergillus* species. Applied and Environmental Microbiology, **60**: 4461–4464.

Visconti A., Pascale M., Centoze G. (2001): Determination of ochratoxin A in wine and beer by immunoaffinity column clean-up and liquid chromatographic analysis with fluorometric detection: Collaborative study. Journal of the Association of Official Analytical Chemists International, **84**: 1818–1827.

Wiger R., Stormer F.C. (1990): Effect of ochratoxin A and B on prechondrogenic mesenchymal cells from chick embryo limb buds. Toxicology Letters, **54**: 129–134.

Wurgler E.F., Friedrich U., Schlatter J. (1991): Lack of mutagenicity of ochratoxin A and B, chinin, patulin and conestine in *Salmonella typhimurin*. Mutation Research, **261**: 209–216.

Xiao H., Madhyastha S., Marquardt R.R., Li S., Vodela J.K., Frohlich A.A., Kemppainen B.W. (1996): Toxicity of ochratoxin A, its opened lactone form and several of its analogs: Structure activity relationships. Toxicology and Applied Pharmacology, **137**: 182–192.

Received for publication October 15, 2004
Accepted after corrections February 9, 2005

Corresponding author:

Ing. Ľubomír Daško, Výskumný ústav potravinársky, Priemyselná 4, 824 75 Bratislava, Slovenská republika
tel.: + 421 2 502 371 51, fax: + 421 2 555 714 17, e-mail: dasko@vup.sk