

OCHRATOXIN A DETERMINATION IN BEER BY IMMUNOAFFINITY COLUMN CLEAN-UP AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY¹

Guilherme PRADO^{2*}, Marize Silva OLIVEIRA², Eliana Pinheiro CARVALHO³, Luiz Carlos OLIVEIRA LIMA³, Thais VELOSO², Leandro Augusto Ferreira SOUZA⁴, Ana Cristina Ferreira CARDOSO⁴

SUMMARY

Analyses of ochratoxin A (OTA) in domestic and imported beers were performed by immunoaffinity column and high – performance liquid chromatography (HPLC) using a fluorescence detector. Recoveries of OTA from beer samples spiked at levels from 8.0 to 800pg/mL ranged from 81.2% to 95.0%, with coefficient of variation between 0% e 11.0%. Detection limit and quantification limit were 2.0pg/mL and 8.0pg/mL, respectively. Of the total of 26 samples produced in Brazil only 6 (23%), contained trace amounts of OTA. Of the 4 imported beers, in 2, Ireland and Germany, were detected OTA at levels of 25pg/mL and 82pg/mL, respectively.

Keywords: ochratoxin A; beer; immunoaffinity; HPLC.

RESUMO

DETERMINAÇÃO DE OCRATOXINA A EM CERVEJA POR COLUNA DE IMUNOAFFINIDADE E CROMATOGRAFIA LÍQUIDA. Análises de ocratoxina A em cervejas nacionais e importadas foram executadas por coluna de imunoafinidade e cromatografia líquida de alta eficiência (CLAE) com detetor de fluorescência. Recuperações de ocratoxina A em amostras de cervejas contaminadas em níveis de 8.0 a 800pg/mL, foram de 81,2% a 95%, com coeficiente de variação entre 0% e 11%. O limite de detecção e o de quantificação foram 2.0pg/mL e 8.0pg/mL, respectivamente. De um total de 26 amostras fabricadas no Brasil, somente 6 (23%) apresentavam traços (pg/mL) de ocratoxina A. Das 4 amostras de cerveja importadas analisadas, em duas, Irlanda e Alemanha, foram detectadas ocratoxina A em níveis de 25pg/mL e 82pg/mL, respectivamente.

Palavras-chave: ocratoxina A; cerveja; imunoafinidade; CLAE.

1 – INTRODUCTION

Ochratoxin A (OTA), a phenylalaninyl derivative of a substituted isocoumarin, is a secondary metabolite produced by *Penicillium verrucosum* in temperate climates and by a number of species of *Aspergillus* in warmer and tropical parts of the world. The best known species of *Aspergillus* producing ochratoxin A is *A. ochraceus* [16].

OTA is nephrotoxic to all animal species studied so far and most likely to humans, who show the longest half – life time for elimination of this toxin amongst all species examined. OTA is teratogenic, immunotoxic, genotoxic, mutagenic and carcinogenic [3, 5].

The IARC (International Agency for Research on Cancer) has classified OTA as a possible carcinogen to humans (Group 2B) [8]. OTA is suspected to be involved in the Balkan Endemic Nephropathy (BEN), a fatal kidney disease occurring in some areas of south – eastern Europe and to be associated with urinary tract tumors [1, 21].

OTA has been found in human blood serum [28], in human milk [9, 14, 15] and in a wide range of commodities, including cereals, coffee, pork and poultry meat, pulses, beer, wine and grape-juice [10, 11, 17, 19, 20, 22, 26, 27, 30, 33].

Several countries have specific regulations for OTA with maximum permitted levels ranging from 1 to 50µg/kg for foods [7]. Within the Europe, tolerance levels for ochratoxin A have been suggested at 1µg/kg for infant foods and at 5µg/kg for cereals [6, 29]. In Brazil, no OTA levels have been officially set for foods.

There is a possibility of transmission of OTA from contaminated grains into beer. OTA does not survive the malting process and adjuncts would be expected to be the source of any OTA in commercial beer [23, 24].

Provisional estimates of Codex Alimentarius Commission, based on limited European data, suggest that beer is the fourth major source of human exposure to OTA following cereals, red wine and coffee [32]. Recently, the Italian Ministry of Health has issued a directive setting guidelines for OTA in several products, including beer, for which a maximum level of 0.2µg/L has been fixed [31].

Surveys have been carried out in various countries to verify the occurrence of OTA in beer but in Brazil no data has been published. The methods currently used are often based on reversed – phase high – performance liquid chromatography with fluorescence detector [4, 12, 13, 18, 23, 31].

The use of immunoaffinity chromatography in the clean-up step has given a strong impulse to the improvement of mycotoxin analysis, providing a number of advantages: (1) clean extracts; (2) precision and accuracy; (3) rapidity; (4) reduction of the use of dangerous solvents [25, 30]. The main advantage of these columns seemed to be that OTA is bound specifically to the antibody and the matrix interference can be removed nearly completely [2].

The purpose of this work was investigate the occurrence of OTA in Brazilian and imported beers sold

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² Fundação Ezequiel Dias – Núcleo de Micologia e Micotoxinas. Rua Conde Pereira Carneiro, 80. Gameleira – Belo Horizonte – MG – CEP - 30510-010. E-mail: gui@funed.mg.gov.br

³ Universidade Federal de Lavras – MG. Pós-Graduação em Ciência dos Alimentos – Caixa Postal 37 – CEP - 37200-000- Lavras / MG.

⁴ Bolsistas do CNPq.

* A quem a correspondência deve ser enviada.

in food stores and supermarkets of some cities of Minas Gerais/Brazil.

2 – MATERIALS AND METHODS

2.1 – Samples

A total of 20 beer brands, 16 domestic and 4 imported beers, were analysed. Different bottles were collected corresponding to 30 samples. The samples were collected by the Inspection Service of Minas Gerais/Brazil, between March and July/2001. The Brazilian beers corresponding to at least a five large different companies.

Sample preparation consists in the preliminary degassing step, by sonicating beer samples for 1h, previously opened and cooled at + 4°C for 12h.

2.2 – Immunoaffinity column clean-up

The method used was described by VISCONTI, APSCALE & CENTONZE [31]. A 10 mL of degassed beer were diluted with 10mL of a water solution containing 1% polyethylene glycol 8000 and 5% sodium hydrogencarbonate and filtered through Whatman GF/B glass microfibre filter. A 10mL of diluted extract were cleaned up through an ochratoxin immunoaffinity column (Vicam L. P., USA) at a flow rate of about 1 drop per second. The column was washed with 5mL of a solution containing NaCl (2.5%) and NaHCO₃ (0.5%) followed by 5mL distilled water at 1–2 drops per second flow rate. OTA was eluted with 2mL methanol and collected in a vial. The extract was evaporated to dryness under a nitrogen stream at ca. 50°C and reconstituted with 250mL of the mobile phase. All analyses were done in duplicate.

2.3 – Determination and confirmation of ochratoxin A

Chromatographic experiments were performed using a Shimadzu liquid chromatography with a fluorescence detector (Shimadzu LC – 10 AD Model), 333nm excitation, 460nm emission, with Shim – Pack CLC – ODS column, 5µm, 4,6 x 250mm, preceded by a guard column Shim – Pack G – ODS, 5µm, 4 x 10mm. The mobile phase (filtered and degassed) was acetonitrile : water : acetic acid (49.5:49.5:1) eluted at a flow rate of 1.0mL/min. One hundred ml of the reconstituted extract were injected into the chromatographic apparatus. The retention time for OTA varied from 13.8 to 15.4min over the period of the study.

Quantification of OTA was performed by measuring peak area at OTA retention time and comparing it with calibration curve, in the range 0.02 – 1.6ng/mL (correlation coefficient: 0.9998)

The accuracy of the method was evaluated by the samples spiked with OTA, in duplicate, at levels of 8.0, 40.0, 100.0, 200.0 and 800pg/mL.

The identity of OTA was confirmed by methyl ester formation after derivatization of the standards and samples extracts with 14% BF₃ in methanol [23].

3 – RESULTS AND DISCUSSION

Results of recovery experiments are showed on *Table 1*. The overall average recovery (mean of means) of OTA was 88.0%, with minimum value at 81.2%. The coefficients of variation (C.V.) were satisfactory (i.e. < 20%). The limit of detection (LOD) of the method was 2.0pg/mL, based on a signal/noise of 3:1. The limit of quantification (LOQ) of the method was 8.0pg/mL.

TABLE 1. Recovery in beer samples spiked with OTA standard

Amount added (pg/mL)	Amount recovered (pg/mL)	Recovery Mean (%)	Coefficient of variation (%)
8.0	7.0 6.0	81.2	11.0
40.0	38.0 38.0	95.0	0.0
100.0	80.0 90.0	85.0	8.0
200.0	180.0 160.0	85.0	8.0
800.0	730.0 770.0	94.0	4.0
Mean of means		88.0	

The chromatogram obtained for one naturally contaminated sample of beer containing 26pg/mL of OTA, is reported in *Figure 1*, showing free of interfering compounds in the same retention time.

A summary of the results of the survey, not corrected for recovery, is shown in *Table 2*. Of the total of 26 samples of beer produced in Brazil, OTA were not found in 20 (77%) beer samples and in 6 (23%) contained trace amounts of OTA. OTA levels found in this study are considerably lower than the provisional tolerable weekly intake (PTWI) established previously as 0.1µg/Kg body mass[21].

Although a limited number of samples of imported beers (four) has been analysed in this study, OTA levels found suggest that beer samples produced in Europe must be assessed. Some surveys using immunoaffinity column clean-up and high-performance liquid chromatography with fluorescence detection on European beers have proved this contamination with OTA. LEGARDA & BURDASPAL [13] verified the presence of OTA in 37 of 38 Spanish beers (97.4%) with mean value of 24pg/mL. DEGELMANN et al. [4] verified in 35 samples of German beers OTA levels in the range of 100 – 200ng/mL in 9 beer samples, 21 samples contained trace amounts of OTA and in 5 samples no OTA was detectable. In 2000, VISCONTI, PASCALE & CENTONZE [31] found OTA in different brands of beer produced in Europe and Italy. The incidence of positive samples of 46% (25/54) was observed with OTA concentrations ranging from 10.0 to 135pg/mL and mean value of 23pg/mL. A survey of OTA in domestic and imported beers in Japan verified that in 93% (43/46) of the samples of Europe levels ranging from 1.7 to 66.2 rg/mL and mean value of 11.4 rg/mL [18].

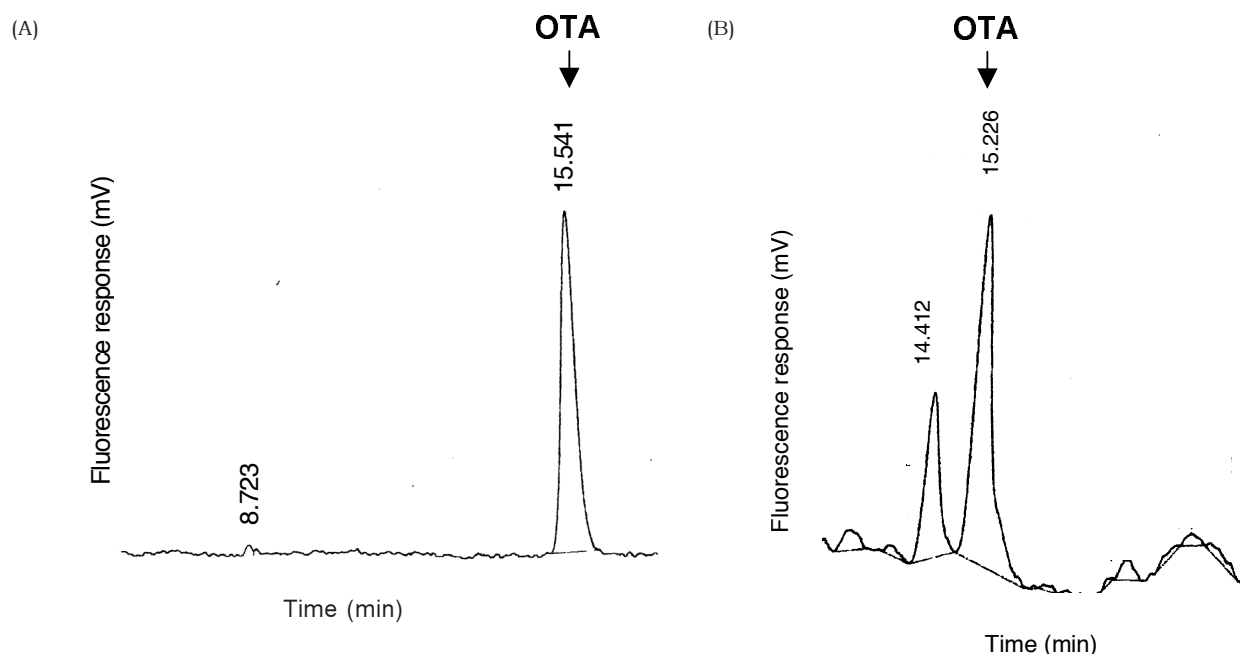


FIGURE 1. Chromatogram showing OTA peak (A) standard of OTA (62.5pg) and (B) beer naturally contaminated (26pg/mL). See Materials and Methods for chromatographic conditions.

TABLE 2. Incidence of OTA in sixteen brands of beer produced in Brazil and four imported beers from different countries

Country	Incidence (Positive / Total)	Mean (pg/mL) ¹
Brazil		
1	0/2	ND ²
2	0/3	ND
3	0/1	ND
4	2/2	> LOD ³ < LOQ ⁴
5	0/1	ND
6	0/1	ND
7	0/1	ND
8	0/1	ND
9	2/2	> LOD < LOQ
10	1/1	> LOD < LOQ
11	0/2	ND
12	0/2	ND
13	0/1	ND
14	0/1	ND
15	1/1	> LOD < LOQ
16	0/4	ND
Total	6/26	- ⁵
Italy		
	0/1	ND
Ireland		
	1/1	26 pg/mL
Mexico		
	0/1	ND
Germany		
	1/1	82 pg/mL

¹ Duplicate Value ² Not Detected ³ Limit of Detection: 2.0 pg/mL

⁴ Limit of Quantification: 8.0 pg/mL ⁵ Not applicable

4 - CONCLUSIONS

The results of this study suggest that beer produced in Brazil presents no risk by human exposure to OTA through its consumption.

Surveys on the incidence of OTA in imported beer are recommended.

5 - REFERENCES

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6 – ACKNOWLEDGEMENTS

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