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Study on ochratoxin A in cereal-derived products from Spain

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Abstract

A study on ochratoxin A (OTA) in cereal-derived products was carried out. Cereal-based baby foods, breakfast cereals and beers were analyzed for mycotoxin OTA using an in-house developed high-performance liquid-chromatographic method.

OTA was detected in 19 of the 21 samples of breakfast cereals (limit of detection 0.066 μg/kg), in 14 of the 20 samples of cereal-based baby foods (limit of detection 0.035 μg/kg) and in 24 of the 31 samples of beer (limit of detection 0.012 μg/l). The mean concentrations of OTA found were the following: 0.265 μg/kg in breakfast cereals, 0.187 μg/kg in cereal-based baby food and 0.044 μg/l in beer. The influence of different factors, such as the fibre content in breakfast cereals, type of cereals used in cereal-based baby food and alcohol content in beer, on the OTA levels was studied.

Keywords: Mycotoxins; Ochratoxin A; OTA; HPLC; Cereals; Cereal-based baby food; Breakfast cereals; Beer

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by certain toxigenic species of *Aspergillus* and *Penicillium*. It has been shown to be hepatotoxic, nephrotoxic, teratogenic and carcinogenic to animals and has been classified as a possible human carcinogen (category 2B) by the International Agency for Research on Cancer (IARC, 1993). OTA could be a risk factor for Balkan Endemic Nephropathy (Petkova-Bocharova & Castegnaro, 1991).

The natural presence of OTA in food and foodstuffs is widespread, especially in temperate climates (Jørgensen, Rasmussen, & Thorup, 1996), and it is generally associated with a variety of products, such as cereals, coffee beans, cocoa beans, and dried fruit.

OTA is a moderately stable molecule that can survive most food processing operations (Harwig, Kuiper-Goodman, & Scott, 1983; Scott, 1991) and, therefore, it appears in derived products such as cereal products, coffee, wine, beer and grape juice; OTA is also found in products of animal origin.

The main contributors to OTA intake in humans are cereals and cereal-derived products because of the resistance to technological processes by this mycotoxin (Alldrick, 1996). Many countries have regulatory or guideline limits for the presence of OTA in foods and, in the majority of theses countries, OTA content limits have been established for cereals (FAO, 1997). In the EU, regulations list the maximum tolerable levels of OTA in cereals (5 μg/kg), cereal-derived products (3 μg/kg), dried vine fruits (10 μg/kg) (European Commission, 2002a) and in food for infants and young children (0.5 μg/kg) (European Commission, 2004). There is some discussion regarding the limit that should be established for ochratoxin A in some cereal-derived foods. The appropriate level proposed for beer was 0.2 μg/l (Burdaspal, Legarda, & Gilbert, 2001).

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Many studies on the presence and quantity of OTA in cereals have been carried out, worldwide. In spite of the fact that OTA is present in human plasma among the Spanish population (Burdaspal & Legarda, 1998; Jiménez, López de Cerain, González-Peñas, & Bello, 1999; Pérez de Obanos, López de Cerain, Jiménez, González-Peñas, & Bello, 2001), few studies have been carried out in Spain with regard to the presence of OTA in cereals. In a recent study, 115 samples of cereals (wheat, barley and corn) from a northern region of Spain (Navarra) were analyzed for OTA contamination (Araguas, González-Peñas, López de Cerain, & Bello, 2003). OTA was detected in 58 of the 115 samples (limit of detection 0.066 μ g/kg), with a mean concentration of ochratoxin A of 0.219 μg/kg. One sample of corn contained a quantity of this mycotoxin that surpassed the EU legal limit (5 μg/kg). This result, along with the moderate stability of OTA during food processing, adds special interest to the study of OTA in cereal-derived products, especially if they are consumed in large quantities and, as in the case of breakfast cereals and cereal baby food, principally consumed by children.

The technique most often used in the determination of OTA in food is HPLC with fluorescence detection (Valenta, 1998). In the development of new analytical methods for OTA determination there is a tendency toward the use of immunoaffinity columns (IAC), the reduction or elimination of organic solvents in the extraction process (especially chloroform due to its toxicity and environmental impact), and to obtain sensitive methods that permit the detection and quantification of OTA at the levels established by legislation. Moreover, analytical methods for the determination of ochratoxin A must be fully validated if they are to be used for control, monitoring and risk assessment studies.

In this paper, we describe an HPLC method for quantitative analysis of OTA in breakfast cereals, cereal-based baby food and beer, which is versatile, validated and sensitive enough to comply with the regulatory limits; in addition, it is a contribution to the knowledge of OTA contamination in certain cereal-derived products in Spain.

2. Materials and methods

2.1. Samples

Twenty baby food samples, 21 breakfast cereal samples and 31 beer samples of different brands were purchased from supermarkets and pharmacies in Navarra (Spain). The cereal contents in the baby food samples ranged from 56% to 94%. The sample quantities were 600 g for baby food and 375 g for breakfasts cereals. Both, bottled and canned beer were analysed. Ten samples were advertized as being non-alcoholic (<1% of alcohol), whereas twenty-one were advertized as containing alcohol. All of the samples were stored at 4 °C until their analysis.

2.2. Reagents

Acetonitrile and methanol (both of HPLC grade) were purchased from Riedel de Haën (Seelze, Germany). Phosphoric acid, sodium acetate, sodium hydrogen carbonate and hydrochloric acid (37%) were obtained from Merck (Darmstadt, Germany). Water was obtained daily from a Milli-Q system. OTA was supplied by Sigma (Madrid, Spain). Phosphate buffered saline (PBS) was prepared by adding the following to one litre of water: anhydrous dibasic sodium phosphate (2.04 g) and sodium chloride (87.9 g), both from Merck (Darmstadt, Germany), along with sodium dihydrogen phosphate monohydrate (12.62 g) from Panreac (Barcelona, Spain). One hundred millilitres of this solution were diluted to 1 litre and the pH was adjusted to 7.4 with NaOH; 100 μl of Tween 20 from Sigma (Madrid, Spain) were then added.

2.3. Apparatus and chromatographic conditions

The instrument used was an Agilent Technologies 1100 liquid chromatographic system equipped with a fluorescence detector (model G1321A), controlled by the ChemStation 3D software. The chromatographic conditions were those employed in the OTA determination in cereals (Araguas et al., 2003). Briefly, OTA was separated on a 5 μm (25 cm × 0.4 cm) Tracer Extrasil ODS-2 column with a Tracer Extrasil ODS-2 precolumn, both from Teknokroma (Barcelona, Spain). The injection volume was 100 μl and the flow rate was 1.5 ml/min, with a mobile phase of 29:29:42 (v/v/v) methanol–acetonitrile–5 mM sodium acetate acidified to pH 2.2 with phosphoric acid. The aqueous phase was filtered through a 0.45 μm membrane filter (Millipore, Ibérica S. A. Spain). Chromatography was performed at 40 °C and the fluorescence conditions, selected from the OTA excitation spectrum that was obtained when an OTA calibration sample was chromatographied, were as follows: Ex = 225 nm; Em = 461 nm.

2.4. Standard solutions

A standard stock solution of 100 μg/ml was prepared by dissolving 1 mg of OTA in 10 ml of methanol and storing it at −20 °C. The concentration of OTA was determined by UV at 333 nm (Mr: 403.8; ε 5500 M^{-1} cm^{-1}) (Bacha et al., 1988). Standard working solutions and calibration samples were prepared by dilution of the stock solution with methanol.

Qualitative confirmation of positive samples was performed by re-analyzing them after derivatization of

OTA through its methylation with methanol in acidified medium (Li, Marquardt, & Frohlich, 1998).

2.5. Extraction

The method used for OTA extraction from breakfast cereals was based on that described by Trucksess, Giler, Young, White, & Page (1999), with some modifications introduced into the purification step. Three hundred grammes of breakfast cereals were ground in a Restch ZM100 mill, using a sieve size of 0.75 mm; 25 g of ground sample were added to 100 ml of 70:30 (v/v) methanol–1% $NaHCO_3$, and the mixture was blended with a Polytron PT3000 homogenizer with a metal rod PT-DA 3012/2 TS Kinematic AG (Switzerland) at 19,000 rpm for 3 min. The extract was filtered by gravity. Ten millilitres of the filtrate were diluted with PBS and then filtered once more through a glass microfibre filter 934-AH Whatman Inc. (Clifton, USA); 20 ml of this filtrate were passed through an immunoaffinity column Ochratest from Vicam Inc. (Watertown MA, USA) containing inmobilised polyclonal antibodies against OTA. After washing the column with 5 ml of PBS and 5 ml of water, OTA was eluted with 3 ml of methanol (2 drops/s). The eluate was then evaporated to dryness in a water bath at 40 °C under a nitrogen stream and redissolved in mobile phase (200 μl).

For OTA extraction from cereal baby food, some modifications in the aforementioned method have been introduced in order to decrease the quantification limit of the method. After the extraction in the Polytron, the sample was centrifuged for 15 min at 3500 rpm, and 40 ml (instead of 20 ml) were purified through the immunoaffinity column.

In the case of beer, 5 ml of sample were degassed in an ultrasonic bath for 30 min and mixed with 45 ml of PBS before being purified through the immunoaffinity column.

2.6. Statistical methods

When comparing the OTA levels in the different sample groups, the U of Mann–Whitney non-parametric test for non-dependent samples was applied. A probability value of 0.05 has been used in order to determine the statistical significance.

3. Results and discussion

3.1. Method validation

In order to carry out a study on the OTA contamination level in cereal-derived products, we have successfully applied a validated and versatile chromatographic analytical method to the three sample matrices studied and for which all objectives for validation have been matched; moreover, the laboratory has been accredited according to ISO 17025 for the analysis of OTA in the three matrices by ENAC (National Accreditation Body in Spain). Its validation was based on the criteria of linearity, detection and quantification limits, selectivity, recovery, robustness, stability and uncertainty. The method was satisfactory in terms of selectivity. An example of a chromatogram obtained from breakfast cereals, cereal-based baby food and beer is shown in Fig. 1. Under the chromatographic conditions described, OTA had a retention time of 5.2 min. The retention time reported in the reference literature was approximately 10 min (Beretta et al., 2002; Degelmann, Becker, Herderich, & Humpf, 1999; Sharman, MacDonald, & Gilbert, 1992; Trucksess et al., 1999), which is greater than the retention time obtained in this study and, therefore, the analysis of the samples was more rapid and economical. This fact is especially due to the use of a solution of acidified sodium acetate in the mobile phase instead of acidified water.

Two calibration curves, using two different concentration ranges, were generated by plotting OTA peak areas against the corresponding concentrations of calibration samples. Six concentration levels were analyzed in the following ranges: 0.120–0.700 and 0.700–7.00 μg/kg in the case of breakfast cereals, 0.060–0.350 and 0.350–5.00 μg/kg for cereal-based baby food, and 0.020–0.140 and 0.140–1.40 μg/l for beer. All objectives for linearity validation have been matched: $r > 0.999$, relative standard deviation (RSD) of the response factors <5%, the confidence interval of the slope not including the zero value ($\alpha = 0.05$) and the confidence interval of the intercept including the zero value ($\alpha = 0.05$).

The estimated limit of detection (LOD) (signal to noise ratio = 3) and limits of quantification (LOQ) (signal to noise ratio = 6) were 0.066 and 0.132 μg/kg, respectively, for breakfast cereals, 0.035 and 0.070 μg/kg for cereal-based baby food and 0.012 and 0.020 μg/l for beer.

Recoveries were determined from spiked samples of each cereal-derived product at levels of 0.4, 1 and 7 μg/kg for breakfast cereals, of 0.1, 1 and 5 μg/kg for baby food cereals and of 0.02, 0.1 and 0.4 μg/l for beer (three samples for each concentration level). Extraction and analysis of the spiked samples were carried out on three different days. Recovery was calculated by comparing the absolute responses (peak area) of OTA obtained from the samples to the absolute response (peak area) of calibration standards. The recoveries obtained were 89.1% (RSD = 4.3%), 86.2% (RSD = 1.2%) and 101.5% (RSD = 3.5%) for breakfast cereals, cereal-based baby food and beer, respectively; they are good recovery values, corresponding to the levels established by legislation for OTA determination methods (European Commission, 2002b).

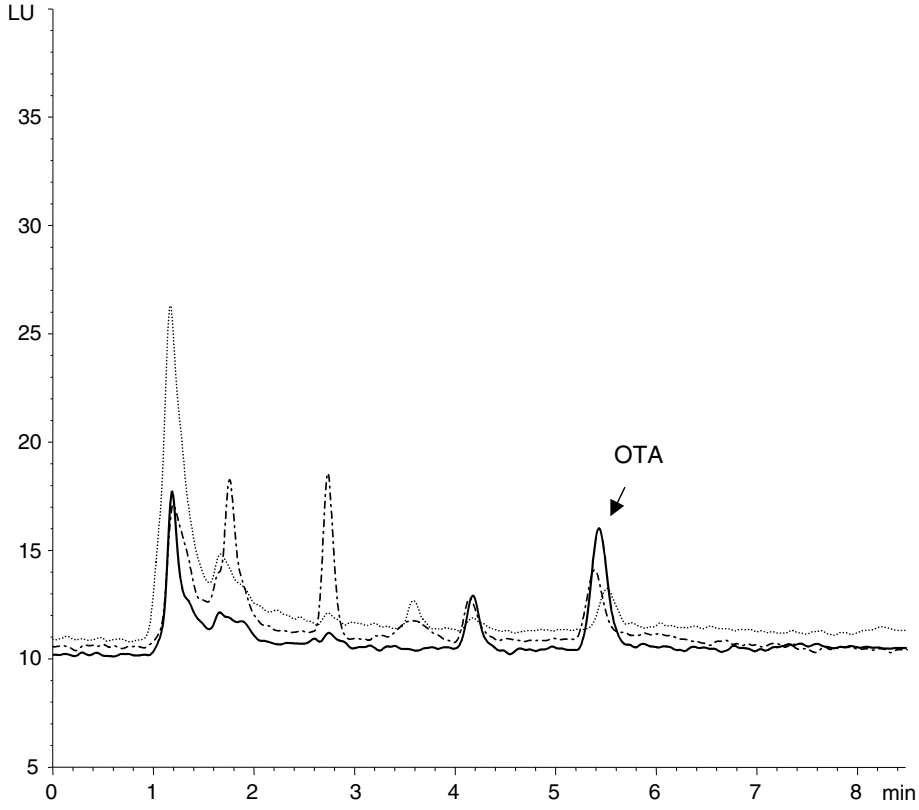

Fig. 1. Chromatograms of positive OTA samples: Cereal-based baby food (—), breakfast cereals and (-·-·-) and beer (· · ·) at 0.40 μg/kg, 0.40 μg/kg and 0.02 μg/l, respectively.

The robustness of a method can be assessed by studying the eventual effects of different sets of conditions placed on the method. In this study, two factors were considered: the change of mobile phase and the chromatographic column batches employed in the HPLC system. The impact of changing the mobile phase was tested by comparing the results obtained from analyzing OTA calibration standards (10, 25, 45, 65, 85, 400 μg/l) using two different mobile phases: (1) 29:29:42 (v/v/v) and (2) 42:29:29 (v/v/v) acetonitrile–methanol–sodium acetate acidified with phosphoric acid. In order to determine the effect produced by changing the batch of the chromatographic columns, the OTA calibration standards (0.4, 0.6, 1, 2.5, 6 and 10 μg/l) were analyzed using three different column batches. The ANOVA test did not show any significant difference among the data obtained in either of the two cases.

Reference literature reports that OTA solutions in methanol are stable for several years at −20 °C (Valenta, 1998), but the stability of other OTA solutions prepared during the analytical process should be assessed. OTA solutions in methanol and in the mobile phase were analyzed during 6 h after being maintained under environmental conditions without light protection. The results showed that OTA was stable under these conditions for at least this period of time.

The uncertainty of the method was estimated, taking into account the following contributions: the uncertainty associated with the balance, the uncertainty associated with the volumetric material, the uncertainty associated with the standard concentration calculation, the uncertainty associated with the reproducibility in the recovery process and the uncertainty associated with the concentration of standards used for spiking in the recovery process. The results obtained were as follows: 18% for breakfast cereals, 8% for cereal-based baby food and 14.3% for beer.

3.2. Ochratoxin a in cereal-derived products

Results obtained from the analysis of OTA levels in the samples are presented in Table 1. OTA was detected in 19 of the 21 samples of breakfast cereals, in 14 of the 20 samples of cereal-based baby food and in 24 of the 31 samples of beer. The mean concentrations of ochratoxin A found were 0.265 μg/kg in breakfast cereals, 0.187 μg/kg in cereal-based baby food and 0.044 μg/l in beer.

No samples of breakfast cereals were above the limit established by legislation (3 μg/kg). According to information on the package, breakfast cereals have been classified into two groups: (1) with "high-fibre content" (11 samples), and (2) with normal-fibre content (10 sam-

Table 1
OTA levels in cereal-based baby food, breakfast cereals and beer

	Samples			OTA (μg/kg) or (μg/l)	
	Total	Positive	Positive (%)	Mean; SD	Maximum
Breakfast cereal					
High fibre content	11	11	100	0.362; 0.256	0.975
Normal fibre content	10	8	80	0.158; 0.107	0.368
Total	21	19	90	0.265; 0.221	0.975
Cereal-based baby food					
Gluten-free	5	0	0	<LOD[a]	<LOD
Multi-cereals	15	14	93	0.245; 0.221	0.740
Total	20	14	70	0.187; 0.216	0.740
Beer					
Non-alcoholic	10	10	100	0.056; 0.052	0.152
Alcoholic	21	14	67	0.038; 0.045	0.205
Total	31	24	77	0.044; 0.047	0.205

[a] LOD, limit of detection.

ples). The statistical study showed significant differences between the two types of samples (U of Mann–Whitney = 23.0; $p < 0.05$). The higher incidence and level of contamination of OTA in high-fibre breakfast could be explained by the fact that OTA accumulates on and directly beneath the epidermis of grain seeds (Osborne et al., 1996; Rafai, Bata, Jakab, & Ványi, 2000).

For cereal-based baby food samples, five "gluten free cereal-based" baby food samples and fifteen "multi-cereal formula" samples were analysed (Table 1). Although most of the samples were below the legislated limit of 0.5 μg/kg, two samples, corresponding to the "multi-cereal formula" group, had an OTA level greater than this value (0.706 and 0.740 μg/kg). The statistical study showed significant differences between the two types of samples (U of Mann–Whitney = 11.00; $p < 0.05$). The highest content of OTA in "multi-cereals", with respect to the "gluten-free" preparations, could be explained by the fact that the types of cereals used for the production process of these two kinds of baby food are different (i. e., principally corn and rye for the former, and wheat, barley, oats and rye for the latter). Consequently, OTA contamination level could also be different. For example, in barley or oats, the OTA content appeared to be higher than in other cereals (Jørgensen et al., 1996; Rafai et al., 2000).

In the case of beer, 10 "non-alcoholic" samples and 21 "alcoholic" samples were analyzed (Table 1). No samples of beer were above the legal limit proposed (0.2 μg/l); only one sample had an OTA level (0.205 μg/l) near this value. The statistical study showed no significant differences between the OTA levels found in the two types of samples: "non-alcoholic/alcoholic" (U of Mann–Whitney = 75.5; $p > 0.05$). Nevertheless, the mean values and the incidence of OTA contamination are higher in the "non-alcoholic" beer samples. This fact could be due to the different production processes used for the two types of beer. If OTA is destroyed in the fermentation process (Scott, Kanhere, Lawrence, Daley, & Farber, 1995), the "non-alcoholic" beer could be more contaminated because, during its production, no fermentation (or controlled fermentation) process is carried out.

Table 2 shows the calculated human OTA intake due to the OTA levels found in the samples analyzed in this study. The relationship between OTA intake and the levels proposed as the daily tolerable intake (DTI) by the Joint FAO/WHO Expert Committee on Food

Table 2
Estimated OTA daily intake (ng/kg body weight) and the percentage that it represents of the proposed daily tolerable intake (DTI) by the Scientific Committee on Food of European Union (SCF) or by the Joint FAO/WHO Expert Committee on Food Additives (JECFA)

	Intake (g or μl/day)	OTA level (μg/kg) or (μg/l)	OTA intake (ng/kg bw/day)	SCF (%)	JECFA (%)
Breakfast cereals	30[a]	0.265	0.13[c]	3	1
Cereal-based baby food	45[a]	0.187	1.05[d]	21	8
Beer	500[b]	0.044	0.36[c]	7	3

[a] Recommended intake indicated on the label of the package.
[b] Recommended intake Saura et al. (2002).
[c] A body weight (bw) of 60 kg is assumed.
[d] A body weight of 8 kg is assumed for a 4-month old child.

Additives (JECFA) (14 ng/kg bw day) and by the Scientific Committee on Food of the European Union (SCF) (5 ng/kg bw/day), has been expressed as a percentage. The values of OTA intake found in this study are below the levels proposed as DTI, and they represent a fraction of DTI which does not exceed 21%. However, it is important to keep in mind that cereals are only one of the many possible sources of OTA for humans and that cereal-derived baby food is the principal source of food for infants. These results reinforce the idea of a vigilant attitude in order to prevent human intake of OTA from food.

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