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Short communication

Determination of ochratoxin A in domestic and imported beers in Italy by immunoaffinity clean-up and liquid chromatography

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Abstract

A method first developed to quantify ochratoxin A in wine has been applied to the analysis of domestic and imported beers in Italy. The method uses commercial immunoaffinity columns for clean-up and high-performance liquid chromatography for quantification of the toxin. Beer was degassed, then diluted with a polyethylene glycol–sodium hydrogencarbonate solution and applied to an OchraTest immunoaffinity column. Ochratoxin A was eluted from the immunoaffinity column with methanol and quantified by reversed-phase HPLC with fluorometric detector. Average recoveries of ochratoxin A from blank beer spiked at levels from 0.04 to 1.0 ng/ml ranged from 93.8% to 100.4%, with relative standard deviations between 3.3% and 5.7%. The detection limit was 0.01 ng/ml based on a signal-to-noise ratio of 3:1. The analysis of 61 samples of domestic (10) and imported (51) beers showed ochratoxin A levels ranging from <0.01 to 0.135 ng/ml with an incidence of contamination of 50% and no substantial difference between strong and pale beers.

Keywords: Beer; Food analysis; Ochratoxins; Mycotoxins

1. Introduction

Ochratoxin A (OA), a widely distributed mycotoxin produced mainly by *Aspergillus ochraceus* and *Penicillium verrucosum,* has nephrotoxic, carcinogenic, hepatotoxic, teratogenic and immunotoxic activity towards several animal species, and has been classified by the IARC (International Agency for Research on Cancer) as a possible carcinogen to humans (Group 2B) [1,2]. It occurs in various foodstuffs and beverages including a variety of cereals, beans, groundnuts, spices, dried fruits, pig kidney and blood, coffee, milk, wine and beer [1,3–7]. Provisional estimates of Codex Alimentarius Commission, based on limited European data, suggest that beer is the fourth major source of human exposure to OA following cereals, red wine and coffee [7].

Several countries have specific regulations for ochratoxin A in one or more commodities with maximum permitted levels ranging from 1 to 50 μg/kg for foods and from 5 to 300 μg/kg for animal feeds [8]. Recently, the Italian Ministry of Health has issued a directive setting guidelines for OA in several products, including beer for which a maximum level of 0.2 μg/l has been fixed [9], while no tolerance level has been yet assessed for wine.

The increased awareness of the potential risk for

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the consumer health due to exposure to OA through the consumption of wine and beer prompted the European Committee for Standardisation (CEN) [10] to ask for more accurate analytical methods that can be applied horizontally for quantification of OA in these two beverages largely consumed in Europe.

A rapid and accurate method for the determination of OA in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography (HPLC) has been recently developed in our laboratory [11]. The analytical performances of this method applied to beer are reported in this note together with the results of a survey on the occurrence of OA in domestic and imported beers in Italy.

2. Experimental

2.1. Sample preparation and immunoaffinity clean-up

A total of 61 different beer brands, 10 domestic and 51 imported beers, were purchased in retail grocery stores in Bari, Italy; of these 26 were pale beers and 35 strong beers.

Sample preparation and clean-up procedure were similar to those previously developed for wine [11], with the main difference consisting in the preliminary degassing step. Degassing was performed by sonicating for 1-h beer samples, previously cooled at +4°C for 30 min to prevent fast foam formation that may lead to pouring out of sample. Ten ml of degassed beer were diluted with 10 ml of a water solution containing 1% polyethylene glycol (PEG) 8000 and 5% sodium hydrogencarbonate ($NaHCO_3$) and filtered through Whatman GF/A glass microfibre filter (filtration after dilution was necessary for cloudy solutions or when solid residue was formed). Ten ml of diluted extract (equivalent to 5 ml beer) were cleaned up through an OchraTest immunoaffinity column (Vicam L.P., Watertown, MA, USA) at a flow rate of about 1 drop per second. The column was washed with 5 ml of a solution containing NaCl (2.5%) and $NaHCO_3$ (0.5%) followed by 5 ml distilled water at 1–2 drops per second flow rate. Ochratoxin A was eluted with 2 ml methanol and collected in a clean vial. The eluted extract was evaporated to dryness under a gentle nitrogen stream at ca. 50°C and reconstituted with 250 μl of the HPLC mobile phase (see below). All reagents were ACS grade.

2.2. HPLC determination

One hundred μl of reconstituted extract (equivalent to 2 ml beer) were injected into the chromatographic apparatus by full loop injection system. The HPLC apparatus consisted of a LKB 2150 isocratic pump (LKB, Bromma, Sweden) equipped with a Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, USA), a Perkin-Elmer LC 240 fluorometric detector (λ_{ex}=333 nm, λ_{em}=460 nm) and a Turbochrom 4.0 data system (Perkin-Elmer, Norwalk, CT, USA). The analytical column was a reversed-phase Discovery C_{18} (15 cm×4.6 mm, 5 μm particles) (Supelco, Bellefonte, PA, USA) preceeded by a Rheodyne guard filter (0.5 μm). The mobile phase consisted of a mixture of HPLC grade acetonitrile–water–acetic acid (49.5:49.5:1) eluted at a flow rate of 1.0 ml/min. Quantification of OA was performed by measuring peak area at OA retention time and comparing it with the relevant calibration curve (4 points, in the range 0.02–2.0 ng/ml, *r*-squared=0.99998). Standard solutions for the calibration curve were prepared in the mobile phase from a stock solution containing 1 mg/ml OA (Sigma–Aldrich, Milan, Italy) in toluene–acetic acid (99:1).

Recovery experiments were performed in triplicate on OA-free strong beer (>6% alcohol) samples spiked with OA at levels of 0.04, 0.2, and 1.0 ng/ml.

3. Results and discussion

3.1. Method performances

Analytical methods using immunoaffinity column clean-up and HPLC with fluorometric detection for the quantification of ochratoxin A in beer have been reported by Scott and Kanhere [12], Ueno [13] and Nakajima et al. [14]. With the exception of the one proposed by Ueno [13], using home-made antibodies for the immunoaffinity columns, they have been specifically proposed for the determination of OA in beer. The main objective of the present study was to

Table 1
Recovery data for the proposed method for the analysis of ochratoxin A in beer

Spiking level (ng/ml)	Recovery±SD[a] (%)	RSD[b] (%)
0.04	93.8±5.3	5.7
0.20	95.0±4.5	4.7
1.00	100.4±3.3	3.3
Mean of means	96.4±2.9	3.0

[a] SD=Standard deviation (n=3 replicates).
[b] RSD=relative standard deviation.

fulfil the requirements of CEN for a horizontal method applicable to both wine and beer. The proposed method does not differ substantially from that of Scott and Kanhere [12], the most significant difference consisting in the use of PEG to dilute beer before applying to immunoaffinity column. The presence of PEG in diluting solution was essential to obtain good recoveries of OA from both beers and wine and to reduce the number and intensity of chromatographic peaks unrelated to OA.

Results of recovery experiments of the full analytical procedure proposed herein showed that the overall average recovery (mean of means) in the tested range of concentrations was 96.4±2.9%, with minimum value at 93.8% for the sample spiked at 0.04 ng/ml (Table 1). The limit of detection (LOD) of the method was 0.01 ng/ml (based on a signal/noise of 3:1) obtained by triplicate injections of spiked beer extract. Chromatograms relevant to a blank sample and a naturally contaminated sample containing 0.10 ng/ml ochratoxin A are reported in Fig. 1, showing a very clean chromatographic tracing, free of interfering compounds.

The method proposed herein is easy to apply and time saving, it does not require particular skills and can allow one operator to analyze up to 50 samples per day. It is reliable, accurate and can be applied ''horizontally'' to both beer and wine at levels of OA contamination considerably lower than the possible maximum permitted levels that may come to regulation for these beverages. Therefore, it is suitable for use by beer and wine producers and importers as well as by public laboratories committed to food quality control for the protection of consumer's health. The method fulfils the actual requirements of CEN [15], at least with respect to the within laboratory performances (RSD <20%, recoveries 70–110%), and is actually under evaluation for inter-

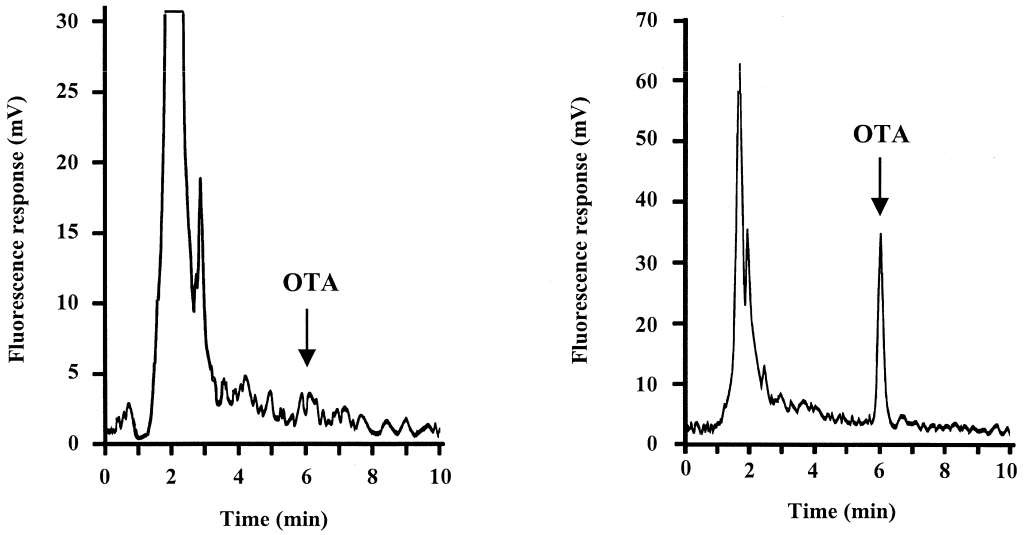

Fig. 1. Chromatograms of a blank beer (*left*) and a naturally contaminated sample of beer containing 0.10 ng/ml OA (*right*) following the extraction and clean-up procedures proposed herein. See Experimental section for chromatographic conditions.

Table 2
Occurence of ochratoxin A in different brands of beer produced in Italy or imported from various countries

Sample origin	Positive/total	Mean of positive (ng/ml)	SD (ng/ml)	Range (ng/ml)
Italy	3/10	0.021	0.001	0.020–0.022
Belgium	6/15	0.049	0.049	0.010–0.135
Denmark	5/7	0.033	0.027	0.010–0.077
Germany	6/9	0.023	0.013	0.010–0.047
UK	2/4	0.052	0.056	0.010–0.091
Ireland	1/2	0.033	–	0.033
Netherlands	1/2	0.021	–	0.021
Austria	0/1	–	–	ND[a]
France	1/3	0.020	–	0.020
Spain	0/1	–	–	ND
USA	2/2	0.067	0.047	0.033–0.100
Canada	1/1	0.011	–	0.010
Argentina	0/1	–	–	ND
Australia	1/1	0.052	–	0.052
Mexico	1/2	0.012	–	0.010
Total	30/61	0.035	0.031	0.010–0.135

[a] ND=not detected, <0.010 ng/ml.

laboratory validation by both the Association of Official Analytical Chemists International (AOACI) and the Office Internationale de la Vigne et du Vin (OIV).

3.2. Survey of OA in beer

The results of the analysis of 61 different brands of beer produced in Italy or imported from several countries worldwide are summarized in Table 2. The same results are reported in Table 3 considering different groups based on alcohol content (above or below 6%), geographical origin (produced in Italy or imported) and starting material (pure malt) of the surveyed products. An overall incidence of positive samples of 50% was observed with OA concentrations ranging from 0.010 to 0.135 ng/ml and mean value of 0.035 ng/ml. There was no substantial difference between strong and pale beers, both showing about 50% incidence of contamination, with mean of positives at 0.040 and 0.031 ng/ml OA, respectively. Pure malt beers showed an incidence of

Table 3
Occurrence of ochratoxin A in different groups of beers commercialized in Italy

Samples	Positive/total (% of positive sample)	Mean of positives (ng/ml)	SD (ng/ml)	Range[a] (ng/ml)
<6% alcohol	17/35 (49%)	0.031	0.027	0.010–0.100
>6% alcohol	13/26 (50%)	0.040	0.036	0.010–0.135
Pure malt beers	8/18 (44%)	0.033	0.028	0.010–0.079
Italian beers	3/10 (30%)	0.021	0.001	0.020–0.022
Imported beers	27/51 (53%)	0.036	0.032	0.010–0.135

[a] Detection limit=0.010 ng/ml.

contamination of about 44%, that was not different from the general trend of the surveyed products. Italian beers seemed to be less contaminated than the imported ones, although the limited number of beers analysed does not allow to make a definitive assessment at this regard.

The hypothesis that, according to previous surveys carried out in Germany [16,17], strong beers (>6% alcohol) are much more contaminated than pale beers (<6% alcohol) is in contrast with the present results that show no difference between these types of beer. Our findings are only apparently different from those of Nakajima et al. [14] reporting for Japanese beers and beers imported in Japan from all over the world very high incidences of OA contamination (higher than 90%). The difference with respect to the present data can be ascribed to the better sensitivity of the Japanese method (detection limit=0.001 ng/ml OA), which uses ion pair chromatography with an alkaline mobile phase to improve OA fluorescence intensity. Based on the mean values reported in the Japanese survey [14], the incidence of contamination at levels above the detection limit of the present method (0.01 ng/ml OA) would be of 46% (54 out of 116 samples), very close to that reported herein.

In summary, with the exception of one report using TLC without confirmatory test [18], our results for occurrence of OA in beer compare well with previous studies carried out in Europe, Japan, USA and Canada [4,12–14,16,17,19–23], which report no contamination or low contamination levels that can only be detected if sensitive analytical methods (detection limit <0.1 ng/ml) are used.

OA levels found in this study were within the Italian recommended value in all the samples analyzed. By assigning the half amount of the detection limit (0.005 ng/ml) to non-detectable toxin levels, the average OA concentrations referred to all the surveyed beers or to Italian beers only would be 0.020 or 0.010 ng/ml, respectively, corresponding to OA daily intakes of 0.167 or 0.083 ng/kg body mass for a 60 kg person consuming daily 0.5 l of beer. These values are considerably lower than the provisional tolerable weekly intake (PTWI=100 ng OA/kg body mass) established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [2], and would lead to an estimate of about 1.2% (for all surveyed beers) or 0.6% (for Italian beers only) for the beer contribution to the level considered at risk for human health due to OA exposure in Italy.

4. Conclusions

Although a limited number of samples has been analysed, the high incidence of contamination indicates a real risk of human exposure to OA through the consumption of beer. Therefore, worldwide surveys on the occurrence of OA in beer are recommended in order to assess the contribution of this beverage to OA human exposure.

Immunoaffinity column clean-up provides several advantages with respect to traditional methods of purification, i.e. limited use of hazardous solvents, provision of clean extracts due to the specificity of the antibody and convenient analysis time-saving.

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