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New method for determination of ochratoxin A in beer using zinc acetate and solid-phase extraction silica cartridges

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Abstract

A new method for the determination of ochratoxin A (OTA) in beer has been developed. The new method has been compared with a reference method currently accepted as AOAC official first action. The limits of detection and quantification of the proposed method were 0.0008 and 0.0025 ng/ml, respectively, while they were 0.0025 and 0.0075 ng/ml, respectively, in the AOAC method used as reference. The recovery levels in the 0.025–0.40 ng OTA/ml spiking range for the proposed and the reference methods were 80.6–87.6% and 78.2–83.8%, respectively. The relative standard deviations of recoveries were 2.6–7.5% for the proposed method and 0.7–6.1% for the reference method. Passing and Bablok regression analysis of recovery data obtained by the proposed method versus data obtained by the reference method on an OTA-spiked beer sample showed good correlation ($r^2 = 0.9993$), while the slope and intercept were 1.049 and −0.0013, respectively. The advantage of the proposed method is the low cost of the materials used in sample preparation because expensive immunoaffinity columns are not needed to clean-up samples while it maintains or even increases the good performance of the reference method. The proposed method was applied to 69 beer samples from different geographic origins (national and imported) but purchased in the Spanish market. They were found to be contaminated with OTA in the range from 0.008 to 0.498 ng/ml (average: 0.070 ng/ml). Five samples surpassed the limit recommended by the European Union (0.2 ng OTA/g).
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1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species of the genera *Aspergillus* and *Penicillium* [1–3]. The International Agency for Research on Cancer has classified this toxin as a renal carcinogen to animals and possible carcinogen to humans (group 2B) [4]. OTA is widely found in cereals [5,6], coffee [7,8], grape must and wine [9–11], beer [12–14] and human blood serum after intake of contaminated food [15,16]. An evaluation performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established a provisional tolerable weekly intake of 100 ng/kg of body weight for OTA [17].

The occurrence of OTA in beer has been widely described in refs. [12–14,18,19] and maximum allowable limits for this toxin in fermented beverages such as beer and wine have been laid down in various countries such as the Netherlands (0.3 ng/ml), Finland (0.5 ng/ml) [20] or Italy (0.2 ng/ml) [21]. Although there is no European Union Regulation for OTA in beer, a maximum limit (3 μg/kg) has been set in malt [22]. Therefore, it is necessary to have available analytical methods for the analysis of OTA at very low levels in these matrices. Due to the low concentration levels of OTA usually found in beer and the complexity of the sample, sensitive and selective techniques should be applied.

Immunoaffinity column (IAC) is perhaps the most common clean-up procedure used for OTA analysis due to its specificity. It can be considered the state-of-the art clean-up procedure. In the case of beverages, the sample can be loaded on this type of column directly [21], or after previous processes such as addi-

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tion of $NaHCO_3$ and NaCl to degassed samples [18] or dilution with polyethylene glycol 8000-$NaHCO_3$ solution to eliminate interference materials. This last procedure is applied to beer and wine in the method of Visconti, which has been adopted as official first action by the Association of Official Analytical Chemists (AOAC) [23]. This method is applied worlwide and there is not restriction to its validity with regard to beer variety.

With regard to analysis of OTA in beer, other clean-up procedures such as liquid–liquid extraction and classic solid-phase extraction (SPE) cartridges can be used and have been compared recently [24].

The most widely used technique for analysis of OTA is liquid chromatography with fluorescence detection (LC–FLD) [23]. Alternative detectors, such as photodiode array (LC–DAD) [20,25] or mass spectrometer (LC–MS/MS) [26,27] have also been used. Other techniques for this analysis are TLC, GC–MS of the trimethylsilyl derivative [20], capillary electrophoresis with laser induced fluorescence [28] and enzyme immunoassay (EIA) [29].

Our goal was to develop a new method for the analysis of ochratoxin A in beer having advantages over the existing methods. Emphasis was put on the clean-up step, assaying zinc acetate as a precipitating agent for dyes and other components of beer. Further clean-up was performed using SPE-silica cartridges after liquid–liquid extraction with ethyl acetate. This is the first time that zinc acetate has been used in the analysis of OTA in beer. The proposed method was compared with the official first action of the AOAC [23], also adopted as official by the European Committee of Standardization (CEN) [30], which can be considered the reference method at this time.

2. Materials and methods

2.1. Samples

Sixty-nine beer samples (mainly lager, 4–6° alcohol degree) were purchased in different Spanish retail markets. Thirty-five of them belonged to various brands that had been brewed in Spain but the remaining were imported from The Netherlands (8), Germany (9), Belgium (4), Ireland (1), Australia (1), China (1), USA (4) and Mexico (2). The composition of each sample was not known and depended on the brewing company. The samples were stored in their original bottles or containers in the fridge at 4–5 °C until analysis. Sample bottles or containers were opened the day before analysis to begin degasification and were still kept in the fridge [24].

2.2. Standards and reagents

The ochratoxin A standard was purchased from Sigma (Sigma-Aldrich, Alcobendas, Spain). A stock solution of about 500 mg/l was prepared by solving 1 mg of OTA in 2 ml of toluene–acetic acid (99:1, v/v). A series of working standards was prepared by evaporation of known aliquots of the stock solution and dissolution in filtered LC mobile phase. They were used to calibrate the detector response. The concentration of the stock solution was determined by measuring absorbance at 333 nm of a diluted solution (20–30 mg/l) of OTA in toluene-acetic acid (99:1, v/v) [5]. Acetonitrile, ethyl acetate, and methanol (all LC grade), acetic acid (analytical reagent) and phosphoric acid (85%, analytical reagent) were from J.T. Baker (Deventer, The Netherlands). Pure water was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA) and was used when water was required. Acetic acid was from Merck (Darmstadt, Germany). Sodium hydrogencarbonate was from Panreac (Panreac Química S.A., Montcada i Reixac, Spain). Polyethylene glycol (PEG) 8000, trifluoroacetic acid, ammonium hydroxide (30%, v/v) and zinc acetate dihydrate were from Aldrich (Sigma-Aldrich). Glass microfiber filters (GF/A) were from Whatman (Maidstone, UK). OchraTest immunoaffinity columns were from Vicam Science Technology (Watertown, MA, USA). Sep-Pack Silica cartridges were purchased from Waters (Waters, Barcelona, Spain).

2.3. Sample preparation

About 250 ml of cool beer was thoroughly degassed in ultrasonic bath for 40 min in 500 ml Erlenmeyer flask. Twenty milliliters of degassed beer was transferred to a flask and 200 μl of ammonium hydroxide (30%, v/v) was added to alkalinize the sample and hence to precipitate proteins and other matrix components. The mixture was shaken and let stand for 10 min. Then, 0.6 ml of a 25% aqueous solution of zinc acetate dihydrate was added to eliminate dyes without affecting OTA level. Several trials were needed before finding that this volume of zinc acetate reagent was optimum to perform precipitation without delivering an excessive concentration of Zn^{2+} in the medium. Zn^{2+} excess was controlled with potassium hexacyanoferrate (II) which provided a voluminous precipitate of zinc hexacyanoferrate (II). The mixture was vigorously shaken for 1 min, centrifuged at 4500 rpm for 6 min and the supernatant was collected.

2.4. Extraction

The supernatant was acidified to pH 2.8–3.0 with 20 ml 0.1 M phosphoric acid (1.8–3.8 pH interval indicator strips (Pehanon) were used routinely for test) before performing liquid–liquid extraction with organic solvent. Although chloroform has been described to perform OTA extraction, due to toxicological and environmental problems associated to this solvent, we changed to the less pollutant ethyl acetate, which provided the same results. Thus, the mixture was stirred with 50 ml ethyl acetate in Erlenmeyer flask for 10 min. Forty milliliters of organic phase was collected and passed through a SPE-silica cartridge preconditioned with 5 ml ethyl acetate, according to Domijan et al. [33]. Then, the cartridge was washed with 3 ml of ethyl acetate and the toxin was eluted with 3 ml of ethyl acetate–acetic acid (95:5, v/v) and transferred to a glass vial. The solvent was evaporated at 50 °C under N_2 stream and the residue was solved in 250 μl of mobile phase.

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