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Determination of ochratoxin A in beer by LC–MS/MS ion trap detection

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Abstract

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus ochraceus* and *Penicillium verrucosum*. It was analysed in food and beverages so far. Due to its toxicity, the European community issued directives and some countries own regulations for OTA contents in food, feed, and beverages. This work describes a method for the determination of OTA in beer. It is based on a combined anion exchange/reversed phase clean-up and liquid chromatography with tandem mass spectrometry. This method was compared with a modified standard method and validated on the basis of spiked beer samples. The accuracy was checked with statistical tools (*t*-test). Due to its good reproducibility, repeatability and robustness this method is a promising alternative to LC–FD (fluorescence detection) techniques.

Keywords: Ochratoxin A; Beer; Tandem mass spectrometry; Solid phase/anion exchange extraction; Liquid chromatography

1. Introduction

Ochratoxin A is a naturally occurring toxin mostly produced by *Aspergillus ochraceus* and *Penicillium verrucosum* (van der Merwe, Steyn, Fourie, Scott, & Theron, 1965). Several publications describe the carcinogenic, neurotoxic, and nephrotoxic effects of OTA (Bedele, Carlton, Krogh, & Lillehoj, 1985; Bruinink & Sidler, 1997; Imaida, Hirose, Ogiso, Kurata, & Ito, 1982; Thuvander, Funseth, Breitholtz-Emanuelsson, Hallén Palmiger, & Oskarsson, 1996b). The International Agency for Research on Cancer (IARC) classified OTA as a possible carcinogenic toxin for humans (IARC Monographs on the evaluation of carcinogenic risks to humans, 1993). In the past OTA, was found and analysed in different food and beverages (e.g. beer) (Bauer & Gareis, 1987; Leitner et al., 2002; Nakajima, Tsubouchi, & Miyabe, 1999; Solfrizzo, Avantaggiato, & Visconti, 1998). Due to its toxicity and occurrence in food and beverages, the European community issued directives including maximum levels for OTA in cereals and dried grapes (Commision Regulation, 2002). Furthermore, some countries issued their own regulations with maximum levels for OTA in coffee, dried fruits, figs, and beer (Verordnung, 2004; Visconti, Pascale, & Centonze, 2000). Various methods including reversed phase high performance liquid chromatography with fluorescence (DIN EN14132; DIN EN14133; EN ISO 15141-1) or LC–MS/MS detection (Lau, Scott, Lewis, & Kanhere, 2000; Leitner et al., 2002) have been published for the determination of OTA. The complexity of the samples requires a pre-treatment step such as solvent extraction or immunoaffinity columns, which enables isolation of OTA from the matrix. The use of immunoaffinity columns or reversed phase (RP) columns was reported earlier with good recoveries for beer analysis (Saez, Medina, Gimeno-Adelantado, Mateo, & Jimenez, 2004; citebib13). However, the application of specific stationary phases, especially immunoaffinity columns, is cost intensive. The aim of this work was to establish a reliable

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LC–MS/MS method as an alternative to existent LC methods with fluorescence detection (LC–FD). The proposed method is based on a precipitation step and combined anion exchange/reversed phase clean-up. The envisioned improved clean-up reduces the matrix load on the LC–MS/MS system and avoids the use of expensive immunoaffinity columns. The performance of the new method was compared with a slightly modified standard method and all important parameters in-house validated.

The modifications of the standard method were limited to the elution of OTA from the immunoaffinity column (here: with methanol/acetic acid; 98 + 2 v/v) and the detection technique (here: MS/MS as in the provided method).

2. Experimental

2.1. Chemicals and materials

Crystalline OTA was obtained from Sigma (Deisenhofen, Germany). A first stock solution was prepared gravimetrically by dissolving 1 mg OTA in 100 mL HPLC-grade methanol. The initial weight of OTA was controlled with an ultra microbalance UMT2 from Mettler Toledo (Gießen, Germany). A working solution was prepared gravimetrically by dilution the stock solution with methanol with a resulting concentration of approximately 1.2 μg kg^{-1}.

Working solutions were used over a period of 2 month and stored at 4 °C. The external standard solutions for LC–MS/MS experiments were prepared by further dilution of the s working solution with the mobile phase. All solvents were of HPLC-grade. Methanol, acetone and acetic acid were obtained from J.T. Baker (Griesheim, Germany/Phillipsburg, USA), hydrochloric acid and ammonia by Merck (Darmstadt, Germany). Oasis MAX cartridges were obtained from Waters (Eschborn, Germany). Beer samples were bought in local stores.

2.2. LC–MS/MS

The LC–MS/MS experiments were carried out with an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation was carried out on a personal computer with Data Analysis software (Bruker). For the chromatographic separation a 250 mm × 2 mm i.d. Inertsil ODS 3 (particle size 5 μm) chromatographic column with 10 mm guard column was used. The beer extracts were analysed isocratically with a methanol–water–acetic acid (70/30/1.5) mixture as mobile phase. The flow rate was 0.250 mL min^{-1} and the injection volume 20 μL.

The following parameters were employed throughout all MS experiments: For electrospray ionisation with positive ion polarity the capillary voltage was set to 3.5 kV, the drying temperature to 350 °C, the nebulizer pressure to 30 psi, and the drying gas flow to 10 L min^{-1}. The maximum accumulation time was 250 msec, the scan speed was 27,000 m z^{-1} s^{-1} (ultra scan mode) and the fragmentation time was 40 ms. To determine the product ions of OTA, the protonated molecule ($[M + H]^+$) at *m/z* 404 was isolated, helium gas introduced into the trap to induce collision with analyte molecules and the fragments detected over a scan range of *m/z* 200–500. The most intensive product ion was *m/z* 358 ($[M + H - HCOOH]^+$). In addition, other product ions of lower intensity resulting from loss of water ($[M + H - H_2O]^+$) at *m/z* 386, loss of formic acid and ammonia (($[M + H - HCOOH - NH_3]^+$) at *m/z* 341), loss of phenylalanine ($[M + H - Phenylalanin]^+$) at *m/z* 239, were observed, too (Fig. 1). Throughout all measurements, OTA was detected by multiple reaction monitoring (MRM) of $+M^3$: 404 → 358. For quantification of OTA in beer, external calibration was used. The calibration graph was linear between 3.5 μg kg^{-1} and 38 μg kg^{-1} (Fig. 2). Within the validation study the correlation coefficients varied from 0.990 to 0.998. The retention times for OTA signals were about 8.5 min.

2.3. Clean-up method

To purify about 10 g of beer and enrich OTA, a precipitation step and combined anion exchange/RP cartridges were used. After precipitation of the proteins with 25 mL acetone, the solution was centrifuged. Then, 20 mL water was added to reduce the rate of organic solvent. The resulting solution was used for extraction following this procedure: After activating and conditioning the cartridges by rinsing with 1 mL methanol and 1 mL water about 10 g sample (adjusted to a pH = 6–8 with 1% ammonia solution) were loaded on the cartridges. Then, three washing steps followed: First, 1 mL of 1% ammonia solution was applied through the cartridge to bind OTA and remove ionic compounds. Secondly, 1 mL methanol was utilised to remove neutral and basic interferences. Finally, 1 mL of a methanol, water and hydrochloric acid mixture (40/60/1) was employed to eliminate polar acidic interferences. OTA was then eluted with two portions of 0.5 mL methanol/acetic acid (98/2) and 0.25 mL water was added to conform this solution to mobile phase. Twenty micro liters of this solution were injected into the HPLC.

3. Results and discussion

The combination of anion exchange/RP clean-up and LC–MS/MS yields a reliable and robust method for the determination of OTA in beer. Compared to RP (C18) SPE, which is more an enrichment than clean-up of beer samples, the new washing technique removed several interferences (e.g. polar acidic compounds). It has the following characteristics: In the first wash step with ammonia solution OTA was bound (pK_a of carboxylic group = 4.4 (Uchiyama, Saito, & Uchiyama, 1985)) to the quaternary