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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

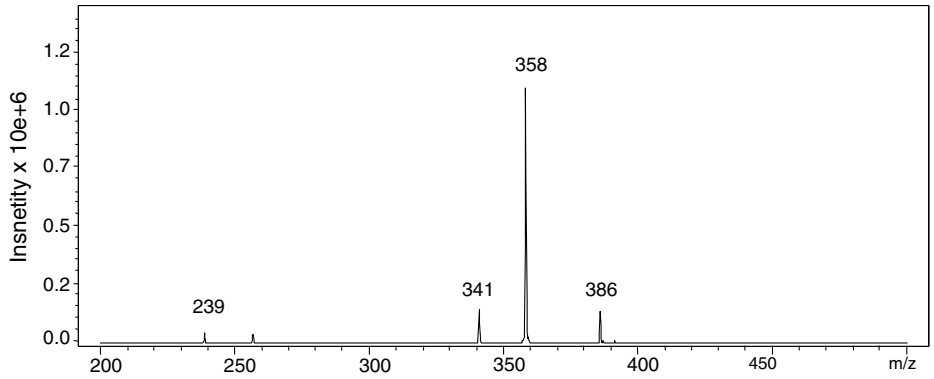

Fig. 1. Product ions spectra obtained after accumulation of $[M + H]^+$ at m/z 404.

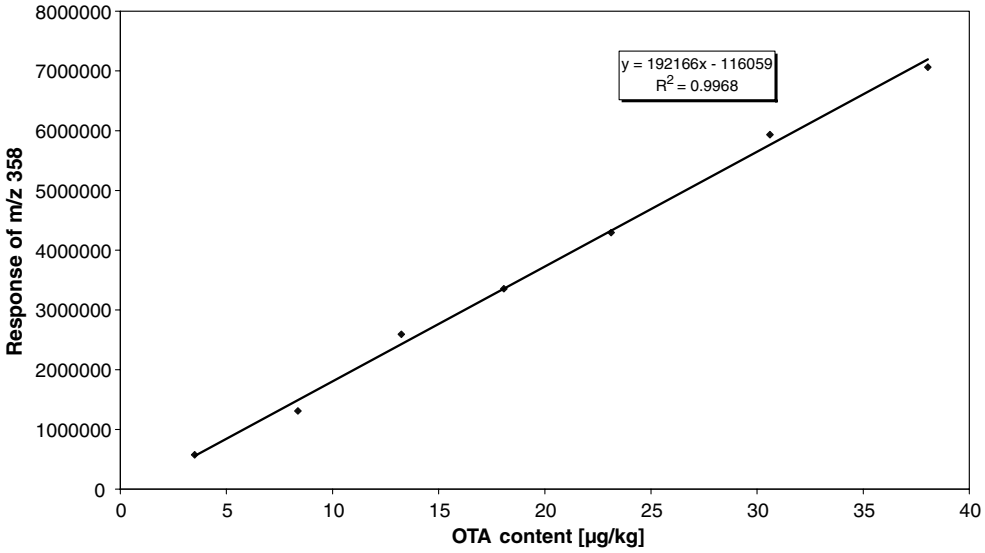

Fig. 2. Linearity of OTA standard solutions analysed by LC–MS/MS.

ammonium groups of the sorbens material and neutral and basic interferences were eluted by flushing the cartridge with methanol. Finally, a mixture of methanol, water and hydrochloric acid was applied to remove polar and acidic interferences. This wash step was optimised by variation of the methanol content, increasing thereby the elution strength. The effluents were collected and analysed subsequently with LC–MS/MS. Fig. 3 displays the intensity of OTA signal as a function of methanol content in the washing solution. The content of methanol in the washing solution should be 40%, respectively, 90% in the eluent. The higher elution ability of this washing solution results in

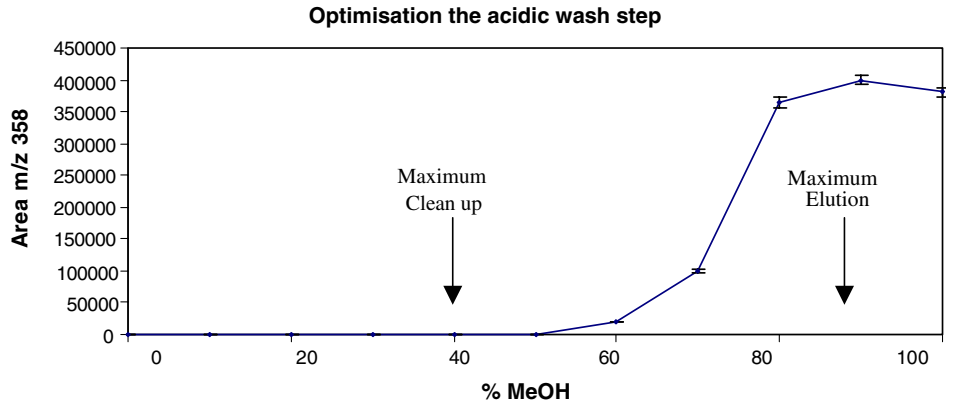

Fig. 3. Optimisation the acidic wash step ($n = 3$).

cleaner samples, less contamination of the mass spectrometer, and better chromatograms with a baseline separation of OTA signal and interfering compounds. Fig. 4 shows LC–MS/MS chromatograms of a beer sample spiked with about 1.5 μg kg^{-1} after four different clean-up procedures. While the proposed clean-up yields in baseline separation of OTA and matrix components with a S/N ratio for OTA of about 8:1, the chromatogram after RP (C18) clean-up shows massive interfering compounds and a lower S/N ratio of about 2:1. The RP (C18) clean-up with a precipitation step, did not show any improvement. The immunoaffinity clean-up displays a better performance compared to the provided method with a S/N ratio of 10:1. However, anion exchange/RP columns are less expensive and easier to handle (e.g. are allowed to run dry). Furthermore, Fig. 4 displays the need of a precipitation step before the extraction of OTA from beer (compare: with and without precipitation).

For checking the linearity and repeatability of our LC–MS/MS system, six calibration levels between 3.5 and 38 μg kg^{-1} were measured. The correlation coefficient was 0.996. The limits of detection (0.4 μg kg^{-1}) and quantifica-

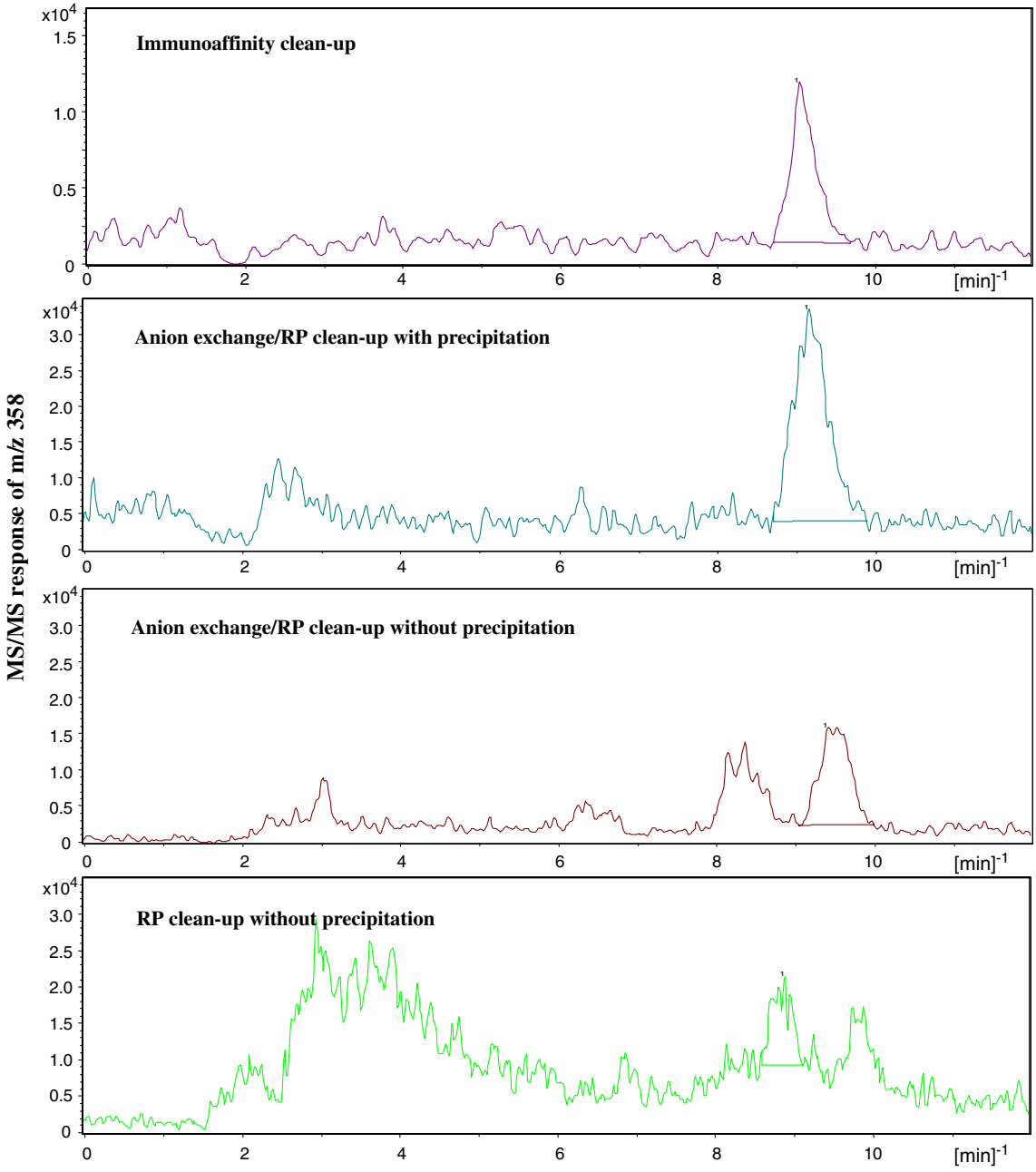

Fig. 4. Chromatograms of beer samples after standard clean-up with immunoaffinity columns, combination of anion exchange and RP clean-up with and without precipitation step and clean-up with C18 only.

Table 1
Validation study results: indices $_{A–C}$: different beers; indices $_{1–6}$: different fortification levels from 1.17 to 5.58 μg kg^{-1}

Sample	Method	Spiking level (μg/kg)	Average (μg/kg)	SD (μg/kg)	RSD (%)	MR (%)
Spiked beer A_1	DIN 14133	**1.44**	**1.44**	0.1	7	99
Spiked beer A_1	LC–MS/MS	1.46	1.50	0.18	11	103
Spiked beer A_2	LC–MS/MS	3.33	2.47	.4	15	86
Spiked beer A_3	LC–MS/MS	3.78	4.5	0.5	11	119
Spiked beer A_4	LC–MS/MS	5.58	5.4	0.3	6	97
Spiked beer A_1 standard addition	LC–MS/MS	1.48	1.30	–	–	88
Spiked beer B_5	LC–MS/MS	1.26	1.46	0.05	3	116
Spiked beer C_6	LC–MS/MS	1.17	1.08	–	–	92

tion (0.8 μg kg^{-1}) for the LC–MS/MS system were calculated by the calibration curve method (DIN 32645) considering an accumulation factor of 10 (ratio of initial weight/measured extract weight).

For testing the accuracy, a method comparison was carried out. First a reference value was determined by six replicates with the modified standard method (DIN EN14133). Then, six replicates with the presented method were measured and the mean values compared (*t*-test). Under the terms of this statistical calculation, the method comparison led to accurate results (Table 1; 1.44 vs. 1.50 μg kg^{-1}). In order to verify the recovery, three different beer samples were spiked with OTA at fortification levels from 1.17 to 5.58 μg kg^{-1}. Furthermore, the standard addition method was applied to prove the new method. All recoveries were related to a reference value determined by the modified standard method or to the fortification levels. The standard addition method was applied by spiking beer sample A_1 with approximately 1.5, 2, and 4 μg kg^{-1} OTA. Table 1 displays the validation characteristics mentioned above. The recovery for beer A_1 with an spiking level of 1.46 μg kg^{-1} is satisfactory (103%) and in the same range compared to RP (C18) (95%; OTA content of 1 μg L^{-1}) and immunoaffinity (75–100%; OTA content of 1 μg L^{-1}) clean-up (Saez et al., 2004; Visconti et al., 2000). The recoveries for higher OTA contents (2.57–5.56 μg kg^{-1}) are satisfactory as well (86–119%). The RSD for the proposed method is between 3% and 15% (mean = 9%), and is in the same range compared to the modified standard method (7%). Since the modification of the standard method is limited to the detection technique, it can be assumed that the contribution to the measurement uncertainty of the provided clean-up is in the same range. Furthermore, an in-house reproducibility test with three chemical technician was carried out. Each technician did three independent replicates of the provided method with beer sample A_1. The mean OTA content was 1.47 μg kg^{-1} (recovery: 101%). The observed reproducibility was 13%, and the repeatability was 10% for each technician (cp. Table 1; RSD = 3–15%). Furthermore, the robustness of our method was checked by increasing the initial weight.

The mean OTA content was 1.23 μg kg^{-1} for sample A_1 (recovery: 84%). Hence it is possible to reduce the LOD by a factor of two. In the validation study not less than three replicates were performed for each sample (except sample C_6: $n = 2$).

4. Conclusion

The presented method is a valuable alternative to the DIN EN 14133 method for the determination of OTA in beer. The combination of anion exchange/RP clean-up and LC–MS/MS yields satisfactory results for OTA contents between 1.17 and 5.56 μg kg^{-1}. The LOD and LOQ (0.4 and 0.8 μg kg^{-1}, respectively) are satisfactory as well and could be improved by a factor of two by increasing the initial weight. Furthermore, this clean-up could be useful for other food, feed, or beverages like spices or cereals.

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DIN 32645 Chemical analysis. Decision limit, detection limit and determination limit. Estimation in case of repeatability, terms, methods, evaluation.

DIN EN14132: Determination of ochratoxin A in barley and roasted coffee-HPLC method with immunoaffinity column clean-up.

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