

# A fully automated and fast method using direct sample injection combined with fused-core column on-line SPE–HPLC for determination of ochratoxin A and citrinin in lager beers

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**Abstract** A new fast and sensitive method based on on-line solid-phase extraction on a fused-core precolumn coupled to liquid chromatography with fluorescence detection has been developed for ochratoxin A (OTA) and citrinin (CIT) determination in lager beer samples. Direct injection of 100  $\mu\text{L}$  filtered beer samples into an on-line SPE–HPLC system enabled fast and effective sample extraction including separation in less than 6 min. Preconcentration of OTA and CIT from beer samples was performed on an Ascentis Express RP C18 guard column ( $5 \times 4.6$  mm), particle size 2.7  $\mu\text{m}$ , with a mobile phase of methanol/0.5 % aqueous acetic acid pH 2.8 (30:70, v/v) at a flow rate of 2.0  $\text{mL min}^{-1}$ . The flow switch from extraction column to analytical column in back-flush mode was set at 2.0 min and the separation was performed on the fused-core column Ascentis Express Phenyl-Hexyl ( $100 \times 4.6$  mm), particle size 2.7  $\mu\text{m}$ , with a mobile phase acetonitrile/0.5 % aqueous acetic acid pH 2.8 in a gradient elution at a flow rate of 1.0  $\text{mL min}^{-1}$  and temperature of 50 °C. Fluorescence excitation/emission detection wavelengths were set at 335/497 nm. The accuracy of the method, defined as the mean recoveries of OTA and CIT from light and dark beer samples, was in the range 98.3–102.1 %. The method showed high sensitivity owing to on-line preconcentration; LOQ values were found to be 10 and 20  $\text{ng L}^{-1}$  for OTA and CIT, respectively. The found values of OTA and CIT in all tested light, dark and wheat beer samples were significantly

below the maximum tolerable limits (3.0  $\mu\text{g kg}^{-1}$  for OTA and 2000  $\mu\text{g kg}^{-1}$  for CIT) set by the European Union.

**Keywords** On-line SPE–HPLC · Ochratoxin A · Citrinin · Mycotoxins · Beer · Fused-core column

## Introduction

Mycotoxins belong to a wide group of secondary metabolites which are produced by various filamentous micromycetes. Mycotoxins are dangerous chemical substances and they are known as persistent and cumulative toxic contaminants in food [1]. The most frequently contaminated foods are cereals, nuts, coffee, cocoa, spices, oil seeds, dried peas, dried fruits, beans and fruits. Mycotoxins are also often detected in beer and wine where the secondary contamination is caused by the use of primarily contaminated barley, malt, hops, wheat and grapes during their production. Mycotoxins can also carry over to the human food chain via plants, meat or other livestock products such as milk, cheese and eggs [2]. Therefore, the need for protection of public health results in the development of fast, sensitive and selective methods to detect the presence of mycotoxins in various foods at trace concentrations.

The main toxic effects of ochratoxin A (OTA) and citrinin (CIT) and their toxicity in living organisms are well documented and confirmed experimentally in animals. OTA belongs to the secondary metabolites produced by *Aspergillus* species in tropical and subtropical areas, and by *Penicillium* species in colder areas [3, 4]. OTA has been shown to be nephrotoxic, hepatotoxic, immunotoxic, teratogenic and neurotoxic. It acts as a persistent toxic compound, with fast absorption and slow elimination [3, 5]. It is important to keep in mind that contamination of

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brewing materials (such as barley, malt, hops) by OTA or other mycotoxins results in the presence of these mycotoxins in beer [6]. Mycotoxin CIT is a secondary metabolite produced by *Penicillium citrinum* [1] and other species of *Penicillium*, *Aspergillus* and *Monascus*. The main toxic effect of CIT on biological organisms is nephrotoxicity causing the damage of the proximal tubules of the kidney [1, 2]. Food contamination by CIT is usually observed together with contamination by other nephrotoxic mycotoxins, mainly with OTA. These two mycotoxins are considered to be the cause of endemic nephropathy [1, 7, 8]. In comparison with other mycotoxins, CIT contamination of food and food commodities is rather rare. The very low levels of CIT contamination found in processed foods may be because CIT is heat-sensitive and unstable during heating [9]. However, it is reasoned that humans are much more frequently exposed to CIT than generally expected, because CIT is produced by the same fungi species as OTA, which is a common contaminant of human food [10]. The fact that most mycotoxins are toxic in very low concentration levels requires new sensitive, selective and reliable analytical methods for their detection and precise quantification. The wide and varied structures of mycotoxins make it impracticable to use one standard technique for determination of all mycotoxins in one step [2]. The analytical technique of choice for fast and sensitive multi-mycotoxins analysis is LC-MS/MS. The maximum permitted level of OTA in beer marketed in Europe is  $3.0 \mu\text{g kg}^{-1}$  ( $\mu\text{g L}^{-1}$ ). This limit was set in Commission Regulation (EC) No. 1881/2006, as a statement for products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption [11]. Unfortunately, at present, there is only one specific regulation in the European Union (EC No. 212/2014) concerning CIT levels. The limit was set for food supplements based on rice fermented with red yeast *Monascus purpureus*. The maximum permitted level is  $2000 \mu\text{g kg}^{-1}$  [12].

Contamination of CIT or OTA was observed in agricultural commodities, foods, feedstuffs, beverages and plant products across geographically diverse areas [1]. In the case of the beer contamination, various stages of the brewing process show that these mycotoxins (or their metabolites) may be transmitted from contaminated grains, malt, barley and hops into the beer. Because CIT is more heat-sensitive than OTA, short heating during the mashing step can significantly reduce CIT levels in beer. In general, it is believed that CIT is destroyed during the mashing and wort boiling process and is therefore not prevalent in beer [13]; nevertheless, the possibility of trace level contamination of CIT in beer is still possible, because after the short-term beer mashing process trace levels of CIT can persist. It was found that 20 min of heating can

decrease CIT concentration by 50 % [1]. On the other hand, heating CIT with water at  $140 \text{ }^\circ\text{C}$  results in decomposition to more the toxic product CIT H1 [14].

The most widely used analytical techniques available for CIT and OTA determination are high-performance liquid chromatography (HPLC) with fluorescence detection [3, 15, 16], liquid chromatography-mass spectrometry (LC-MS) [17-19], ultrahigh-performance liquid chromatography - mass spectrometry (UHPLC-MS) [20, 21] and other flow or batch methods such as sequential injection analysis (SIA) [22], capillary electrophoresis (CE) [23, 24] and ELISA methods [25, 26]. A wide spectrum of different methodologies has been proposed for mycotoxins extraction, preconcentration and food sample clean-up. The most common approaches are solid-phase extraction (SPE), immunoaffinity sorbents, liquid-liquid extraction (LLE), supercritical fluid extraction (SFE), QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology, dispersive liquid microextraction (DLME) and ELISA methods. Recently published reviews provide a comprehensive overview of the different methods proposed for the determination of mycotoxins in foods, including sample pretreatment techniques [2, 27-31]. Nevertheless, the number of analytical methods dealing with the determination of CIT is rather limited [21, 32-36], presumably because CIT contamination of food and feed is less often observed in comparison with other mycotoxins (e.g. OTA).

HPLC column switching techniques including on-line solid-phase extraction-high-performance liquid chromatography (on-line SPE-HPLC) methods are an efficient and popular tool for increasing peak capacity and improving precision and assay sensitivity [37]. Notably, implementation of an on-line SPE step in addition to HPLC brings advantages for LC-MS and LC-FD analysis. It is an effective method for sample clean-up of complex biological matrices such as the urine, plasma, serum or other food and environmental samples. On-line SPE-HPLC is based on the selective extraction of the analyte on an extraction column and its transfer to an analytical column by using a two-position column switching valve. The clear advantages of this on-line SPE technique are reduced solvent consumption, significant reduction of sample handling and the short time required for all sample preconcentration steps. Nevertheless, optimization and procedure development of on-line SPE methods are not a simple and trouble-free process [38]. In the current literature, the use of short fused-core particle columns for on-line SPE extraction has been described in few cases. The main aim of this work is to present for the first time a new approach for analysis of two mycotoxins (OTA and CIT) in beer samples by using on-line SPE on fused-core particle columns directly in an HPLC system. The

optimized method was validated and the study of OTA and CIT contamination in Czech lager beers (light, dark, wheat) from the whole Czech region was performed.

## Experimental

### Chemicals, materials and samples

Standard of ochratoxin A, citrinin, glacial acetic acid and organic solvents (HPLC grade) methanol and acetonitrile were obtained from Sigma-Aldrich (Chemie GmbH, Germany). The ultra-pure water used for mobile phase preparation was purified through a Milli-Q (Millipore, Bedford, MA, USA). Other chemicals and used materials were of analytical grade. The Czech lager beer samples (light, dark and wheat beers; a total of 49 beer samples) were purchased randomly from local markets across the whole Czech Republic region. All samples were kept in the fridge at 4 °C in the dark before opening the bottle and analysis. The samples with undetectable levels of OTA and CIT were used for spiking, recovery and matrix calibrations studies.

### Equipment and HPLC system

The chromatography equipment was a Shimadzu Prominence (Shimadzu Corporation) system used for method development and validation. A dual pump module LC-20 AD, DGU-AS mobile phase degasser, autosampler SIL-20 AC and fluorescence detector RF-10A XL were also used. The chromatography and extraction columns were thermostatted in a CTO 20 AC column oven and mobile phase flow direction was changed by using an FCV-12AH high pressure column switching valve. The HPLC system was controlled by a

CBM-20A communication module. The data evaluation and acquisition of chromatography analyses were performed by Lab-Solution software (Shimadzu Corporation, Japan).

### Preparation of stock solution and beer samples

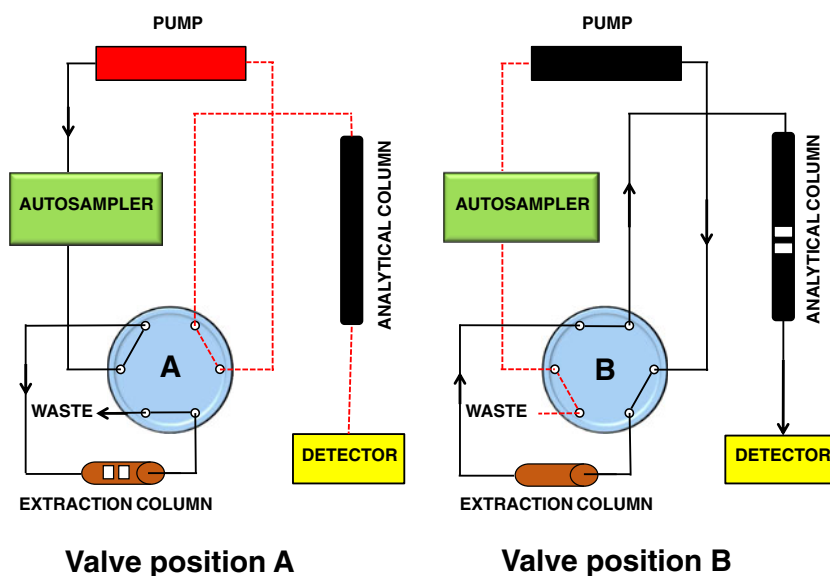
Standard stock solutions of OTA and CIT were prepared by dissolving both substances in methanol in concentration of 5 mg L<sup>-1</sup>. Standard stock solution was stored at 4 °C in the dark. Standard stock solution was further diluted 1000 times with 30 % methanol/water solution to prepare a working standard solution of concentration 5 µg L<sup>-1</sup> for method development. The calibration standard solutions were diluted in the concentration range from 10 ng L<sup>-1</sup> to 5000 ng L<sup>-1</sup> (OTA) and 20 ng L<sup>-1</sup> to 10,000 ng L<sup>-1</sup> (CIT) using ten calibration solutions. The matrix calibration solutions of OTA and CIT in light and dark beer samples were prepared in the same range using ten calibration points.

Untreated and undiluted beer samples were filtered through a 0.45-µm PTFE filter into glass vials. A 100-µL volume of filtered beer solutions was injected directly into the on-line SPE–HPLC system. All beer samples and working standard solutions were prepared fresh daily and injected in triplicate.

### HPLC column switching procedure

The simultaneous beer sample extraction and mycotoxin determinations were performed using the on-line SPE–HPLC system illustrated in Fig. 1. The SPE column used for mycotoxins extraction from beer samples was a fused-core Ascentis Express RP C18 guard column (5 × 4.6 mm), particle size 2.7 µm (Sigma Aldrich, Germany), with a washing mobile phase of methanol/0.5 % aqueous acetic

**Fig. 1** Schematic of on-line SPE–HPLC system for mycotoxins extraction and determination in beer samples. Valve position *A* – extraction procedure and mycotoxins preconcentration; valve position *B* – separation of mycotoxins on analytical column



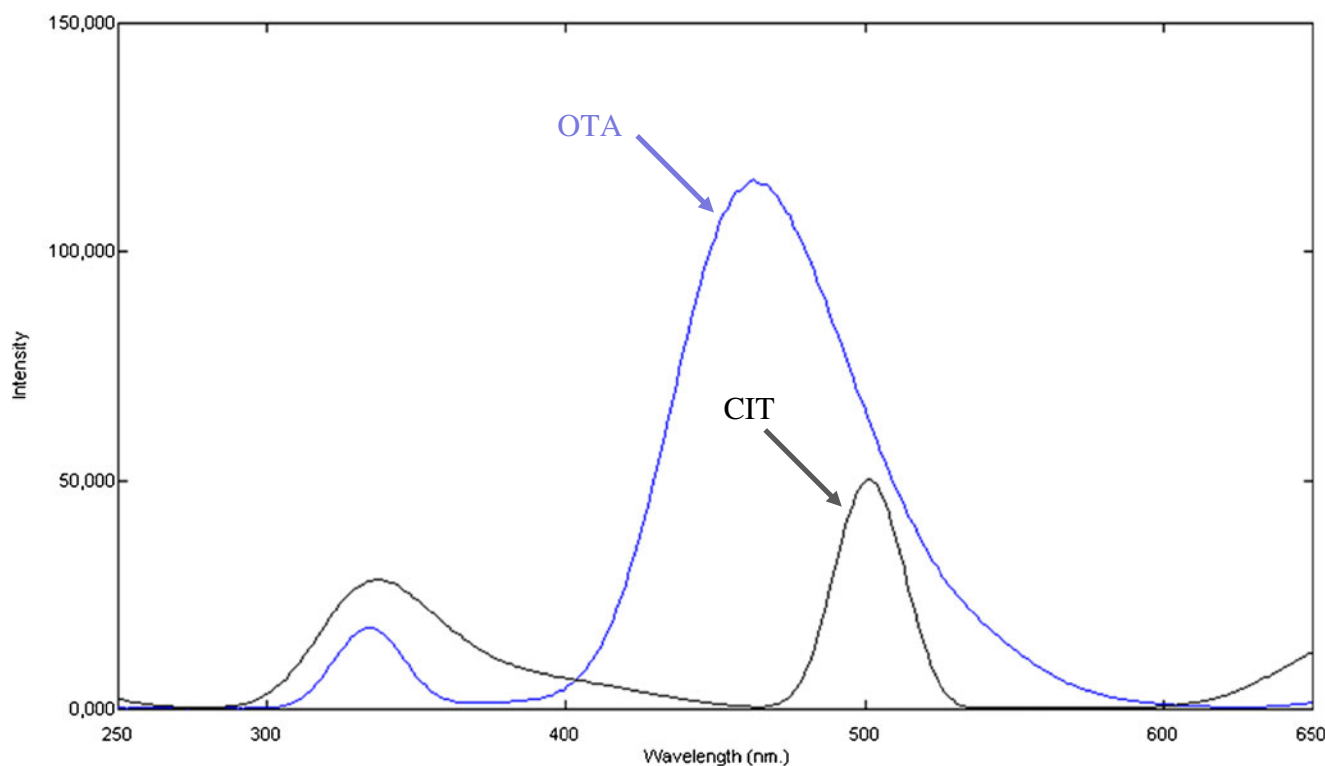
acid pH 2.8 (30:70, v/v) at a flow rate of 2.0 mL min<sup>-1</sup> and temperature of 50 °C. A 100- $\mu$ L volume of the filtered beer sample was directly injected onto the guard column where both mycotoxins were retained while other polar interferences were washed out to the waste. After the valve switch in second minute, the zone of extracted mycotoxins was transferred from the SPE column to the analytical column in the back-flush mode. The separation was performed in gradient elution mode.

The chromatographic separation of OTA and CIT from interfering matrix components of the beer was achieved on the fused-core column Ascentis Express Phenyl-Hexyl (100  $\times$  4.6 mm), particle size 2.7  $\mu$ m (Sigma Aldrich, Germany), using a mixture of acetonitrile (solvent A) and a solution of 0.5 % acetic acid in water, pH 2.8 (solvent B) at a flow rate of 1.0 mL/min and a column temperature of 50 °C. A gradient elution was applied on the analytical column, with the initial conditions (solvent A/B, 45:55, v/v) held for 1 min. Between 1.0 and 3.5 min of the chromatographic run (i.e. between 3.0 and 5.5 min of the total analysis time), the percentage of solvent A was linearly increased to 75 %. Thereafter, the percentage of solvent A was decreased to 45 % over 30 s, while the valve was switched back to the on-line SPE position and the analytical column was equilibrated for 3 min to the initial conditions during the next sample pretreatment step. Fluorescence excitation/emission detection

wavelengths were set at 335/497 nm. The choice of the excitation/emission wavelengths was carried out as a compromise between the fluorescence spectra of OTA and CIT in mobile phase and sensitivity of determination for both mycotoxins (Fig. 2). OTA shows higher fluorescence intensity; therefore the emission wavelength was chosen to ensure the sensitive detection of CIT i.e. the optimal emission wavelength for OTA was not used because of the loss of CIT signal.

## Results and discussion

No method for the simultaneous determination of OTA and CIT in beer samples has been reported in the literature. Beer is a complex matrix which contains water, carbohydrates, proteins, vitamins, phenolic compounds and other antioxidants, mineral salts and alcohol. The ethanol as a fermentation product can partially affect the extraction of polar mycotoxins from the water compartment as a result of early elution during the adsorption step. From these reasons, the procedure for extraction of mycotoxins from beer samples should be optimized. At the same time the composition of the elution solution from the SPE extraction column must be used as a mobile phase for separation on the analytical column. The good compatibility between extraction (SPE) and separation (LC) steps must



**Fig. 2** Fluorescence emission spectra of ochratoxin A (OTA) (blue line) and citrinin (CIT) (black line) at excitation wavelength 335 nm. The spectra were measured in mobile phase comprising acetonitrile/0.5 % aqueous acetic acid (45:65, v/v), pH 2.8

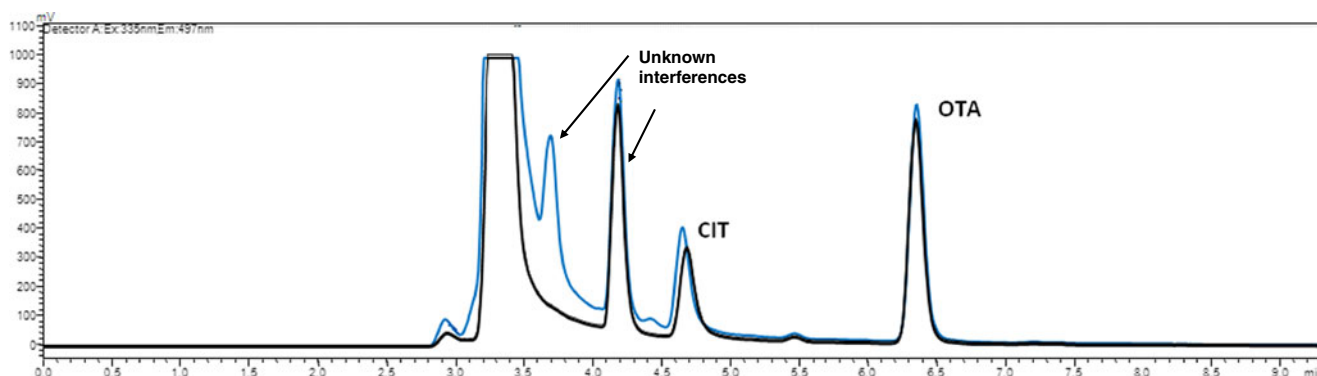
be taken into account during the optimization of each step. Therefore, the parameters of sample pretreatment such as conditions of both mobile phases and stationary phases for extraction and separation were optimized before the method validation and beer sample analysis. The main aim of the work was to show a new, simple and modern analytical tool for the reliable determination of mycotoxins at trace levels in beer.

### Optimization of on-line SPE extraction step

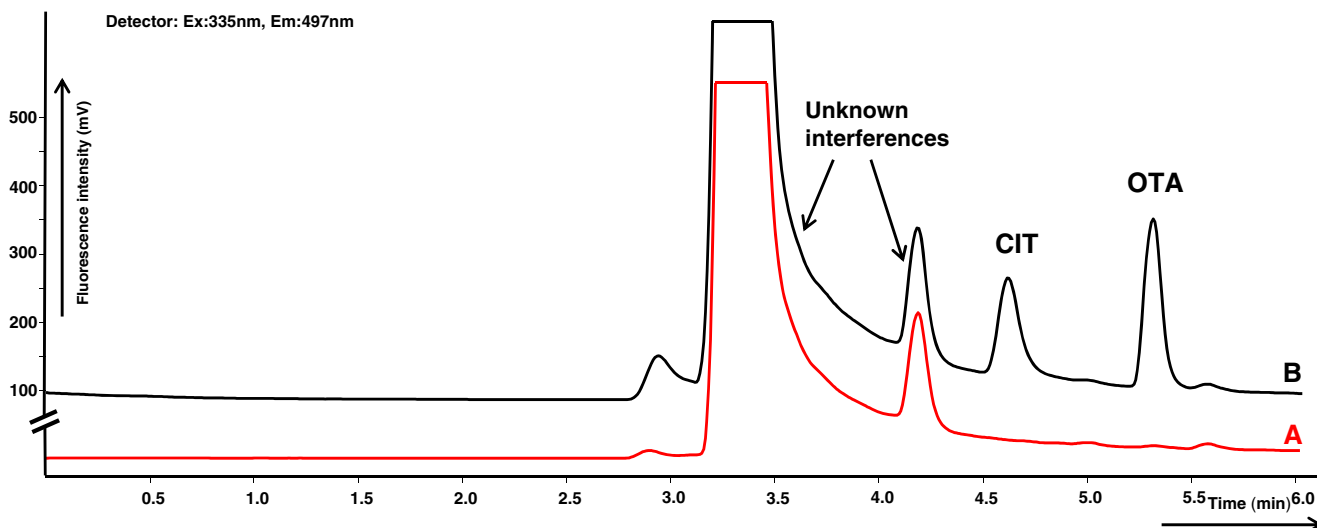
This part was focused on a methodology which would allow fast extraction of mycotoxins from beer matrix with high preconcentration. A commonly available reversed-phase precolumn, an Ascentis Express RP C18 guard column (5 × 4.6 mm), particle size 2.7 μm, was chosen for preconcentration of mycotoxins from beer samples with respect to the physicochemical properties ( $pK_a$ ) and lipophilicity ( $\log P$ ) of the analysed mycotoxins. OTA and CIT are weak organic acids. The  $pK_a$  values of OTA are in the ranges 4.2–4.4 and 7.0–7.3, respectively, for the carboxyl group of the phenylalanine moiety and the phenolic hydroxyl group of the isocoumarin part [39]. CIT is weak organic acid with a  $pK_a$  value of 3.55 and  $\log P$  of 0.81 [40]. The extracted mycotoxins should be in their neutral form to be well retained by hydrophobic interactions. Therefore, for sufficient retention of the mycotoxins of interest on the reversed-phase sorbent, the washing mobile phases were acidified with 0.5 % acetic acid to pH 2.8. A compromise between interference removal and the sufficient recovery of both mycotoxins must be found during washing solution optimization. Therefore, the influence of the washing mobile phase on the SPE procedure was studied to ensure the fast and quantitative transfer of interfering matrix for disposal and simultaneous retention of the target analytes. The key parameters of extraction are the lipophilicity of the analytes, and the chemistry and extraction capacity of the SPE precolumn. The range of washing mobile phase ratios tested was from 5 to 30 % (v/v) of acetonitrile with 0.5 %

aqueous acetic acid pH 2.8. When the percentage of acetonitrile exceeded 10 % (v/v), the more polar CIT was lost during elution of ballast matrix while the huge peak of matrix interferences was not removed. Therefore, the mobile phase with methanol ranging from 5 to 30 % (v/v) was tested. The effect of methanol concentration on clean-up step efficiency is depicted in Fig. 3. Up to percentage 30 % (v/v) the mixtures of methanol with 0.5 % aqueous acetic acid pH 2.8 could be used for the extraction column washing step without the loss of firstly eluted CIT. The breakthrough volume for CIT was not exceeded using 6 mL of washing mobile phase containing 30 % of methanol. It included 3 min washing at a flow rate of 2 mL min<sup>-1</sup>. Since longer washing step times did not improve the clean-up efficiency, the valve switch was performed at the second minute to keep the analyses shorter. These on-line SPE conditions produced a satisfactory elution of interfering compounds to the waste and sufficient resolution of matrix and peak of CIT. The efficiency of the on-line sample pretreatment procedure is depicted on the blank beer chromatogram in Fig. 4A.

Increasing the volume of the injected sample is an effective way to improve the sensitivity of the on-line SPE–HPLC method. Nevertheless, the breakthrough volume of the SPE column resulting in analytes elution should not be exceeded. Therefore, compromise must be found between the washing of the residual peak of the matrix using a larger injected sample volume and efficiency of the clean-up step. Injection volumes larger than 300 μL were not tested in our study because the residual peak of matrix interferences complicated the determination of the CIT peak. Prolonging the washing step time did not lead to a significant elimination of the ballast matrix peak. Therefore, a compromise between the sensitivity and selectivity of the extraction step was found at 100 μL of undiluted beer sample which was injected directly into the extraction column. With 100 μL of injected sample, the chromatographic separation of the huge ballast peak of interferences at the beginning of chromatogram did not negatively influence the evaluation of the CIT peak area.



**Fig. 3** Effect of methanol concentration on clean-up step efficiency during the extraction procedure; 15 % methanol (blue line), 30 % methanol (black line)



**Fig. 4** Efficiency of on-line sample pretreatment procedure: the blank light beer chromatogram (A, red line) and separation of the mycotoxins after on-line SPE step on the spiked beer chromatogram (B, black line). The spiked concentrations of mycotoxins: OTA,  $1 \mu\text{g L}^{-1}$ ; CIT,  $2 \mu\text{g L}^{-1}$

### Chromatographic separation

In order to reflect relatively recent trends in chromatography, columns packed with fused-core particles and monolithic sorbents were tested as the stationary phases for separation of the mycotoxins. Fused-core technology is based on semiporous particles instead of the traditional totally porous ones. In our study, three fused-core particle columns with dimensions of  $100 \times 4.6$  mm and particle size of  $2.7 \mu\text{m}$  with different stationary phases, namely Phenyl-Hexyl, C18, and Pentafluorophenyl F-5 (Sigma-Aldrich), and two reversed-phase monolithic columns, namely Chromolith® FastGradient RP-18e,  $50 \times 2$  mm, and Chromolith® HighResolution RP-18e,  $50 \times 4.6$  mm (Merck), were tested for optimal separation with the aim to achieve narrow and symmetric analyte peaks after on-line SPE extraction. Mobile phases containing mixtures of methanol or acetonitrile with a 0.5 % acetic acid solution in water, pH 2.8 in the range from 35 to 65 % (v/v) were tested in the separation optimization. The elution with methanol showed poor peak symmetry for CIT and higher retention times of both mycotoxins compared with acetonitrile mobile phases. Both monolithic columns showed worse peak symmetry and separation efficiency for CIT and OTA than the tested fused-core particle columns. The optimal peak shapes improving the sensitivity of determination were achieved on Phenyl-Hexyl and C18 fused-core columns with 50 % acetonitrile in the mobile phase for both mycotoxins. The parameter of resolution was almost identical on both columns. However, the back pressure on the Phenyl-Hexyl column was 30 % less than on the C18 column. Thus, the Phenyl-Hexyl column was chosen for further optimization. The column oven temperature was set at  $50 \text{ }^\circ\text{C}$  as a compromise between lowering the back-pressure in the system and stationary phase temperature stability.

In order to optimize the separation conditions in the on-line SPE system, the gradient elution was tuned for better resolution of the CIT peak from the residual peak of interferences. The initial gradient conditions were set with respect to the conditions of the on-line SPE step. The time of the valve switch was set to the second minute. The gradient elution program started at this moment from 45 % of acetonitrile, which was kept for 1 min. The lower content of acetonitrile at the beginning of the separation after the flow switch from the extraction column to the analytical column enabled gradual washing of residual interferences at the beginning of the chromatogram. Thus, the peak of CIT was eluted over a longer time and well separated from the huge peak of ballast matrix. A gradient elution was applied on the analytical column, with the initial conditions (solvent A/B, 45:55, v/v) held for 1 min. Between 1.0 and 3.5 min of the chromatographic run, the percentage of solvent A was linearly increased to 75 %. Thereafter, the percentage of solvent A was decreased to 45 % over 30 s, while the valve was switched back to the on-line SPE position and the analytical column was equilibrated for 3 min to the initial conditions during the next sample SPE step. Separation of mycotoxins after the on-line SPE step is depicted on the spiked beer chromatogram in Fig. 4B. Under the optimal conditions, both mycotoxins were eluted selectively without matrix interferences in less than 6 min including the sample pretreatment step.

### Validation of on-line SPE–HPLC method

The validation and analytical performance of the proposed method was tested in terms of linearity (for standard and matrix solutions), repeatability, limits of detection (LOD), limits of quantification (LOQ), intraday precision and accuracy (recovery). The method validation procedure

**Table 1** On-line SPE–HPLC system suitability parameters (number of replicates,  $n = 6$ )

	$t_R$ (min) <sup>a</sup>	$t_R$ RSD (%) <sup>b</sup>	$k'$ <sup>c</sup>	$k'$ RSD (%) <sup>b</sup>	Peak symmetry	Peak areas repeatability RSD (%) <sup>d</sup>	$R_s$ <sup>e</sup>
CIT	4.63	≥0.1	1.74	≥0.1	1.41	1.0; 0.3; 0.6 <sup>d</sup>	3.75
OTA	5.32	≥0.1	2.46	≥0.1	1.16	0.7; 0.4; 1.1 <sup>d</sup>	

<sup>a</sup> Retention time<sup>b</sup> Number of replicates,  $n = 6$ <sup>c</sup> Capacity factor (reduced – retention times did not include on-line SPE step)<sup>d</sup> RSD was calculated from six injections of standard mixture at concentration levels:  $c_1 = 10 \mu\text{g L}^{-1}$ ,  $c_2 = 2 \mu\text{g L}^{-1}$ ,  $c_3 = 0.1 \mu\text{g L}^{-1}$  (for CIT); and concentrations:  $c_1 = 5 \mu\text{g L}^{-1}$ ,  $c_2 = 1 \mu\text{g L}^{-1}$ ,  $c_3 = 0.05 \mu\text{g L}^{-1}$  (for OTA)<sup>e</sup> Resolution factor for separation CIT and OTA

included the evaluation of the HPLC system suitability test (SST). Standard solutions were injected six times into the chromatographic system. Mean values and standard deviations of retention times, capacity factors, symmetry factors, resolution and repeatability of the analytical run were evaluated. Results of chromatography system suitability parameters are summarized in Table 1. Calibration curves were measured for standard solutions and spiked matrix solutions (light and dark lager beers) at ten concentration levels, depending on the fluorescence intensity of each mycotoxin. The linearity for standard and matrix-

matched calibration between the peak areas and mycotoxins concentration were measured in the range of 0.02–10  $\mu\text{g L}^{-1}$  and 0.01–5  $\mu\text{g L}^{-1}$  for CIT and OTA, respectively. Under the validated conditions, linear relationships with perfect regression coefficients both in standard and matrix calibration curve were found in the range 0.9990–0.9997 for both mycotoxins. Linear regression parameters of CIT and OTA determination for standard and both matrix calibrations are described by the regression equations (slope and intercept) which are mentioned in detail in Table 2. All samples were measured in triplicate.

**Table 2** Analytical characteristics of the validated on-line SPE–HPLC method

	CIT	OTA
Standard linear calibration range ( $\mu\text{g L}^{-1}$ ) <sup>a</sup>	0.02–10	0.01–5
Slope	555,590 ± 8724	1,401,977 ± 11,426
Intercept	4411 ± 32,504	13,386 ± 21,284
Regression coefficient ( $r^2$ )	0.9990	0.9997
Matrix (light beer) linear calibration range ( $\mu\text{g L}^{-1}$ ) <sup>a</sup>	0.02–10	0.01–5
Slope	718,529 ± 6368	1,583,837 ± 14,174
Intercept	52,359 ± 23,723	16,907 ± 26,403
Regression coefficient ( $r^2$ )	0.9997	0.9997
Matrix (dark beer) linear calibration range ( $\mu\text{g L}^{-1}$ ) <sup>a</sup>	0.02–10	0.01–5
Slope	587,867 ± 2831	1,381,239 ± 6107
Intercept	20,251 ± 10,547	9,866 ± 11,375
Regression coefficient ( $r^2$ )	0.99991	0.99992
LOD ( $\mu\text{g L}^{-1}$ )	0.006	0.003
LOQ ( $\mu\text{g L}^{-1}$ )	0.02	0.01
Precision (RSD, %) in light beer <sup>b</sup>	2.8	1.9
Precision (RSD, %) in dark beer <sup>b</sup>	4.8	1.4
Accuracy—spike recovery (%) ± SD in light beer <sup>c</sup>	102.1 ± 2.8	98.5 ± 1.9
Accuracy—spike recovery (%) ± SD in dark beer <sup>c</sup>	101.7 ± 4.8	100.7 ± 1.4

<sup>a</sup> Each concentration level was measured in triplicate<sup>b</sup> Repetitive determination of six spiked light and dark beer samples at one concentration level: 2  $\mu\text{g L}^{-1}$  (CIT) and 1  $\mu\text{g L}^{-1}$  (OTA)<sup>c</sup> Accuracy was determined by a recovery method using spiked beer sample at one concentration level: 2  $\mu\text{g L}^{-1}$  CIT and 1  $\mu\text{g L}^{-1}$  OTA in six samples, each in triplicate (±minimal and maximal standard deviation of recovery determination)

**Table 3** Occurrence of ochratoxin A and citrinin in the Czech lager beer samples

Beer sample	Found amount ( $\mu\text{g L}^{-1}$ )			
	CIT	$\pm\text{SD}$	OTA	$\pm\text{SD}$
<b>Light beers</b>				
Lobkowicz Premium	ND	$\pm 4.E-02$	0.05	$\pm 6.E-04$
Platan 11°	ND		0.04	$\pm 4.E-03$
Rychtář Premium	ND		0.09	$\pm 1.E-04$
Vévoda 11°	ND		0.03	$\pm 2.E-04$
Klášter 12°	ND		0.03	$\pm 1.E-04$
Ježek 11°	ND		0.03	$\pm 2.E-03$
Páter	ND		0.04	$\pm 3.E-04$
Kounic	ND		0.02	$\pm 5.E-03$
Krušovice 12°	0.09		0.03	$\pm 9.E-03$
Břežňák světlý ležák	ND		0.05	$\pm 1.E-03$
Zlatý bažant	ND		0.02	$\pm 2.E-04$
Staropramen Ležák	ND		0.04	$\pm 7.E-04$
Braník Ležák	ND		0.03	$\pm 3.E-03$
Ostravar Premium	ND		0.02	$\pm 2.E-03$
<b>Pilsner Urquell</b>				
Radegast Premium	ND		0.02	$\pm 8.E-04$
Budweiser Budvar	ND		0.06	$\pm 2.E-03$
Pardál Echt	ND		0.04	$\pm 4.E-04$
Holba Šerák	ND		0.02	$\pm 4.E-04$
Bertold Ležák	ND		0.04	$\pm 2.E-03$
Bernard světlý ležák	ND		0.06	$\pm 3.E-03$
Svijanský Máz 11°	ND		0.04	$\pm 1.E-03$
Opat pepper special	ND		1.20	$\pm 2.E-02$
Samson světlý ležák 12°	ND		0.02	$\pm 1.E-03$
Bohemia Regent Premium	ND		0.03	$\pm 8.E-04$
Valdštejn	ND		0.05	$\pm 9.E-04$
Otakar 11°	ND		0.04	$\pm 4.E-03$
Lounský žejdlík 11°	ND		0.02	$\pm 2.E-03$
Chotěboř Premium	0.13		0.03	$\pm 2.E-03$
Poutník světlý ležák 12°	ND		0.02	$\pm 1.E-03$
Bakalář	ND		0.02	$\pm 2.E-03$
Ferdinand	ND		0.02	$\pm 1.E-03$
Chodovar Zlatý ležák	ND		0.03	$\pm 1.E-03$
Vyškovské pivo	ND		0.05	$\pm 1.E-03$
Krakonoš	ND		0.03	$\pm 2.E-03$
Skalák	0.19	$\pm 5.E-03$	0.03	$\pm 2.E-03$
<b>Dark beers</b>				
Master Tmavý 18°	ND		0.03	$\pm 4.E-03$
Kozel Černý	ND		D	
Krušovice Černé	ND		D	
Budvar Tmavý ležák	ND		D	
Pardubický Porter 19°	ND		0.10	$\pm 7.E-03$
Samson tmavý ležák 12°	ND		D	
Bernard Černý ležák	ND		D	
<b>Wheat beers</b>				
Staropramen Nefiltrovaný	ND		0.02	$\pm 8.E-04$

**Table 3** (continued)

Beer sample	Found amount ( $\mu\text{g L}^{-1}$ )			
Krušovice Pšeničné	0.09	$\pm 4.E-03$	0.03	$\pm 6.E-04$
Velen Černá Hora	ND		0.04	$\pm 2.E-03$
Pšeničný ležák Herold	ND		0.05	$\pm 8.E-04$
Primátor Weizenbier	ND		0.03	$\pm 4.E-03$
Fénix	ND		0.05	$\pm 9.E-04$

ND not detected, D detected under the LOQ level

Limits of detection and quantification, calculated as the minimum mycotoxin concentration yielding a signal to noise ratio equal to three and ten times the standard deviation ( $\sigma$ ), respectively, are shown in Table 2. LOQs were established as  $0.02 \mu\text{g L}^{-1}$  and  $0.01 \mu\text{g L}^{-1}$  for CIT and OTA, respectively. These values were experimentally confirmed by multiple dilution of standard solutions and the measurements were included in linearity range evaluation. The obtained LOQ values are low enough to evaluate mycotoxins content, far below the limits set by regulatory authorities in the European Union (approximately 300 times).

The precision of the developed method including on-line sample pretreatment procedure was evaluated with repetitive determination of six spiked light and dark beer samples at one concentration (level  $2 \mu\text{g L}^{-1}$  and  $1 \mu\text{g L}^{-1}$  for CIT and OTA, respectively). Each sample was injected in triplicate. This concentration level corresponds to maximum permitted levels for OTA according to the European Union regulations for wine ( $2 \mu\text{g L}^{-1}$ ) and beer ( $3 \mu\text{g L}^{-1}$ ) [9] marketed in Europe. Relative standard deviations (RSD) for light and dark beer samples were very similar and in both cases did not exceed 5 %. Results are shown in Table 2. Similarly, the accuracy of the proposed method was determined by the recovery studies to evaluate the matrix effects. Peak areas of standard solutions prepared in 30 % methanol in water were compared with peak areas of spiked light and dark beer samples at the same concentrations of  $2 \mu\text{g L}^{-1}$  and  $1 \mu\text{g L}^{-1}$  for CIT and OTA, respectively. The percentage recovery values were calculated as (measured area of spiked placebo beer sample with standard solution/peak area of standard solution at the same concentration)  $\times 100$  %. The beer with no detected mycotoxins was used as the placebo beer sample. The mean recovery values were found to be in the range 98.5–102.1 % for both mycotoxins. Values close to 100 % indicate that there are no interferences or other matrix suppression effects. The on-line SPE–HPLC system was found to be very sensitive, selective and rugged for the analysis of filtered beer samples without additional sample pretreatment. The retention times, peak symmetry, chromatography resolution of mycotoxins and extraction



**Table 4** Statistical evaluation of occurrence of ochratoxin A and citrinin in the Czech lager beer samples

Lager beer	CIT				OTA			
	$n + (\%)^a$	Mean <sub>b</sub>	Median <sub>b</sub>	Range min–max <sub>b</sub>	$n + (\%)^a$	Mean <sub>b</sub>	Median <sub>b</sub>	Range min–max <sub>b</sub>
Light ( $n = 36$ )	8	0.01	<LOD	0–0.19	100	0.07	0.03	0.02–1.20
Dark ( $n = 7$ )	0	<LOD	<LOD	<LOD	100	0.03	0.01	LOD–0.10
Wheat ( $n = 6$ )	17	0.01	<LOD	0–0.09	100	0.04	0.03	0.02–0.05

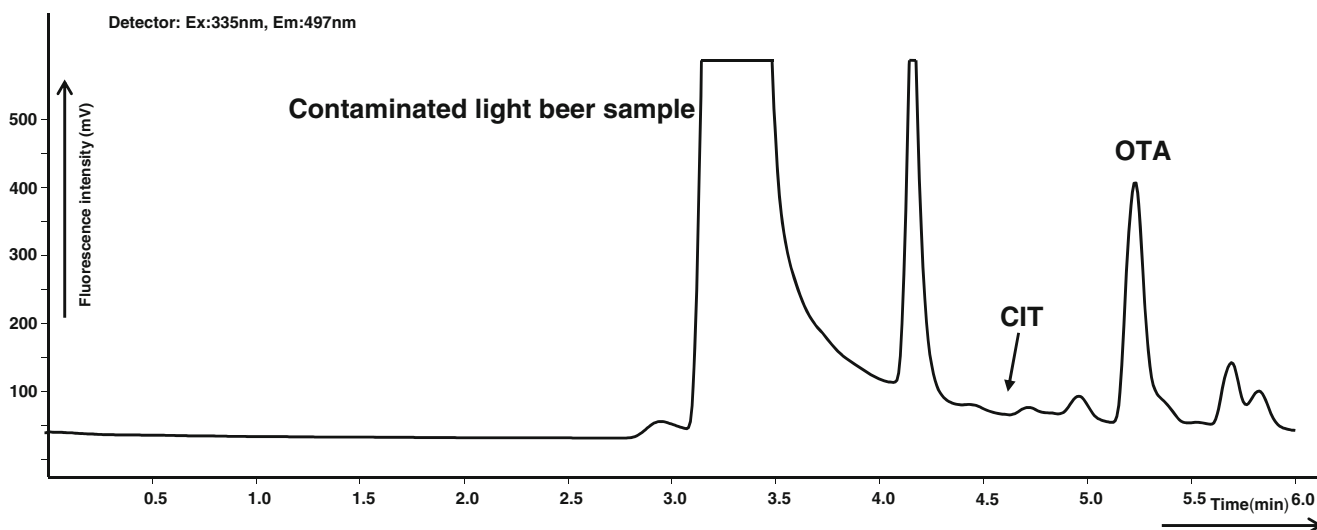
<sup>a</sup> Number of positive samples in %<sup>b</sup> Found concentrations in  $\mu\text{g L}^{-1}$ 

efficiency of the method did not change in the course of all experiments including the method validation and real sample analysis (more than 500 injections). The values of RSD for retention times, peak symmetry and chromatography resolution were not higher than 5 % at the beginning and the end of the study. This was confirmed by using a software integration method. The extraction efficiency showed similar values of RSD ( $\leq 5$  %) in the long-term period (3 months).

#### Analysis of lager beers

In this work, the analysed samples were divided into three categories: light, dark and wheat beer. Forty-nine beer samples from the Czech market were analysed: 36 light, 7 dark and 6 wheat lager beers. The beer samples were randomly purchased in local stores. First of all, OTA levels in the collected samples were found far below the maximum permitted limits of  $3 \mu\text{g L}^{-1}$ . The detected levels of OTA ranged from 0.01 to  $1.20 \mu\text{g L}^{-1}$  (summarized in Table 3). The results confirmed the

presumption that the risk of OTA occurrence in lager beers from the Czech region is very low. Among the samples, 44 beers (90 %) were contaminated by OTA with concentrations lower than  $0.06 \mu\text{g L}^{-1}$ , which is a level 50 times lower than the maximum permitted limit for OTA concentration in beers. The relatively higher concentration of OTA ( $1.20 \mu\text{g L}^{-1}$ ) was found in one sample only. This outlier is almost 40 times higher compared to the median value of the selected samples. The found discrepancy can be explained by the addition of pepper to the beer which was produced as a special pepper beer. Moreover, in the recently published literature [41, 42], concentrations of OTA in pepper were found to be higher than the recently established maximum allowable levels for OTA in spices  $15 \mu\text{g L}^{-1}$  [43]. We can assume that secondary contamination could be caused by contaminated pepper addition during the beer brewing process. The median value of OTA occurrence in all beer samples was  $0.03 \mu\text{g L}^{-1}$ , with an average of  $0.06 \mu\text{g L}^{-1}$ . The detailed statistical values (mean, median, range and number of the positive samples) are presented in Table 4. The found

**Fig. 5** Representative chromatogram of a contaminated beer sample: light lager beer Opat pepper special (found OTA conc.,  $1.2 \mu\text{g L}^{-1}$ )

results confirmed the previous conclusions published by Škarková et al. [44] that the level of OTA contamination in the Czech lager beers is very low, in the range 0.01–0.18  $\mu\text{g L}^{-1}$  (median 0.05  $\mu\text{g L}^{-1}$ , mean 0.06  $\mu\text{g L}^{-1}$ ). Furthermore, the study results showed that the number of detected CIT contaminations of the beers is very low. Among the all tested samples only four beers showed positive occurrence of CIT. For 92 % of beer samples CIT was not detected owing to its low stability and degradation during the beer brewing process. A representative chromatogram of a contaminated light lager beer sample is shown in Fig. 5.

## Conclusion

We have developed a novel approach for sample preparation and separation of two mycotoxins in beer. This new method for on-line solid-phase extraction of ochratoxin A and citrinin from beer samples was optimized and validated. Utilization of on-line SPE sample pretreatment with a relatively large volume sample injection was shown to be the method of choice for fast determination of very low concentrations of mycotoxins in beer samples. The on-line SPE–HPLC technique showed high sample throughput, low solvent consumption and high sample preconcentration of mycotoxins depending on the injected volume. The combination of sensitive fluorescence detection and a highly efficient chromatography process on both fused-core columns together with sample preconcentration resulted in perfect sensitivity of the developed method. The limit of quantification for OTA was 300 times lower than maximum permitted limits for OTA occurrence. The OTA levels found in randomly collected samples confirmed the very low contamination of the Czech lager beers, which is in accordance with previous studies [44]. CIT contamination of beer was found in very few cases which corresponds to the general prediction that CIT is destroyed during the mashing and wort boiling process. Nevertheless, trace level contamination of CIT was found in a few cases in our study showing that negligible levels of CIT remained after the short-term beer brewing process. It corresponds with the fact that the concentration of CIT decreases dramatically after boiling in water; 20 min of heating decreases the CIT concentration by 50 % [1]. Comparing contamination by both mycotoxins, it can be concluded that CIT is more unstable and heat-sensitive than OTA; therefore, significantly lower numbers of contaminated samples were found in our study.

In conclusion, a fast and sensitive on-line SPE extraction with chromatography separation in less than 6 min was developed for determination of OTA and CIT in 49 beer samples. The study showed that the found OTA levels in beers

marketed in the Czech region do not show a serious risk for consumer health.

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## Compliance with ethical standards

**Conflict of interest** The authors report no conflicts of interest in this work.

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