Ochratoxin A in roasted coffee marketed in Czech Republic: determination by liquid chromatography and fluorescence detection

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Summary

This study dealt with determination of ochratoxin A (OTA) in roasted coffee by ultra-performance liquid chromatography and fluorescence detection (UPLC-FLD). Coffee features characteristic sensory properties, contains a wide range of beneficial substances and is among the most sold commodities worldwide. UPLC-FLD method for determination of OTA in ground roasted coffee was optimized and validated. A calibration curve of good linearity ($R^2 > 0.999$) was obtained for the range of 0.3–10 ng·ml^{-1}, and the limit of detection and limit of quantification for OTA were 0.1 μg·kg^{-1} and 0.3 μg·kg^{-1}, respectively. The recovery of certified reference material for OTA ranged between 81.3–112.8 % and the relative standard deviation under repeatibility conditions was 11.7 %. The method was applied to the analysis of OTA in coffee obtained from retail stores in Czech Republic. Sixteen samples (29 %) of roasted coffee were positive, containing OTA in the range from 0.31 μg·kg^{-1} to 1.06 μg·kg^{-1}. None of the samples analysed exceeded the legislative limit according to EU Commission Regulation No. 1881/2006.

Keywords

ochratoxin A; coffee; immunoaffinity column; method validation; ultra-performance liquid chromatography; fluorescence detection

Coffee, one of the most popular beverages worldwide, is consumed by millions of people every day [1]. Coffee drinking has had a long tradition and is a part of various lifestyles. Coffee beans belong to the world's most valuable agricultural export commodities [2]. There are about 80 varieties of the coffee tree (*Coffea* spp.), the most important of them being *Coffea arabica* (Arabica) and *Coffea canephora*, the latter commonly referred to as Robusta [3].

Coffee features characteristic sensory properties and contains a variety of bioactive compounds, including the stimulant caffeine and also health beneficial substances such as phenolic compounds known for their antioxidant effects [4]. Nevertheless, coffee can also contain undesirable compounds, such as contaminants introduced during coffee processing, namely, pesticides, toxinogenic moulds and their secondary metabolites – mycotoxins, including ochratoxins. Ochratoxin A (Tab. 1) is the most widespread and most relevant ochratoxin [5]. Based on the available data, ochratoxin A (OTA) seems to be overall the most toxic of them, followed by ochratoxin C (OTC), ochratoxin B (OTB) and ochratoxin α (Otα) [6].

OTA (*N*-[(3R)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)carbonyl]-L-phenylalanine) is one of the most harmful naturally occurring mycotoxins. It is produced mainly by the fungi *Aspergillus ochraceus* and *Penicillium verrucosum* [6, 7]. International Agency for Research on Cancer (IARC) has classified OTA as a possible human Group 2B carcinogen based on a large number of studies demonstrating carcinogenicity in animals [8]. It is a globally widespread mycotoxin with nephrotoxic, hepatotoxic, immunotoxic, neurotoxic, reprotoxic, teratogenic and carcinogenic effects [9]. It is known to occur in foods

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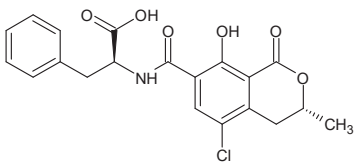
Tab. 1. Ochratoxin A [18].

Trivial name	Ochratoxin A
Summary formula	$C_{20}H_{18}ClNO_6$
Abbreviation	OTA
Molecular weight	403.8 g·mol^{-1}
Partition coefficient	4.7
Structure	

such as cereals, nuts, spices, cocoa, grapes, wine, beer, green coffee, and roasted coffee [10–12]. For these reasons, Commission Regulation (EC) No. 1881/2006 [13] sets the maximum levels for OTA in commodities and finished products. The maximum values are set as follows: for all unprocessed cereals (5.0 μg·kg^{-1}), for all products derived from cereals (3.0 μg·kg^{-1}), for dried vine fruit (10.0 μg·kg^{-1}), for wine-based beverages, grape juices, nectars and musts intended for human consumption (2.0 μg·kg^{-1}), for processed cereal-based foods and baby foods for infants and young children (0.50 μg·kg^{-1}), for dietary foods, roasted coffee beans and ground roasted coffee (5.0 μg·kg^{-1}) and for instant coffee (10.0 μg·kg^{-1}).

Due to the toxicity and occurrence of OTA in coffee and other foods, several analytical methods have been developed and applied to sensitive and effective contamination control. These include thin-layer chromatography, capillary electrophoresis, gas chromatography and liquid chromatography. The most frequently used analytical approach for the intended purpose is liquid chromatography combined with fluorescence detection or mass spectrometric detection [12, 14]. Fluorescence detection can provide lower limit of detection and limit of quantification than mass spectrometric detection for OTA [15, 16]. Due to the complexity of the coffee matrix and the relatively low content of OTA, immunoaffinity column extraction (IAC) or solid-phase extraction (SPE) are usually used for sample preparation [17].

In this context, the aim of this work was to optimize and validate an analytical method based on the use of the OCHRAPREP immunoaffinity column (R-Biopharm, Darmstadt, Germany) for the determination of OTA in ground roasted coffee by ultra-performance liquid chromatography and fluorescence detection (UPLC-FLD). The validated method was used on a set of 55 commercially available samples of roasted coffee.

MATERIAL AND METHODS

Standards and chemicals

The OTA standard solution (10 μg·ml^{-1} in acetonitrile) was purchased from Sigma-Aldrich, (St. Louis, Missouri, USA). A working stock standard solution was prepared at a concentration of 100 ng·ml^{-1} and then was used for the preparation of the calibration curve. Deionized water was prepared using Aqua-Osmotic type 06 system (Aqua-Osmotic, Tišnov, Czech Republic) and the quality of deionized water was characterized by conductivity (0.06 μS·cm^{-1}). Gradient-grade solvents used were acetonitrile (≥ 99.9 %) and methanol (≥ 99.9 %), both obtained from Sigma-Aldrich. Additives and chemicals for pH adjustment were as follows: sodium bicarbonate (≥ 99 %; Lachner, Neratovice, Czech Republic); acetic acid (≥ 99.8 %; Honeywell, Offenbach am Main, Germany); formic acid (≥ 96 %; Sigma-Aldrich); Tween 20 EP (Sigma-Aldrich) and phosphate buffered saline solution (Oxoid, Basingstoke, United Kingdom). For quality assurance and quality control (QA/QC), a certified reference material (CRM) Fapas T17198QC of ground roasted coffee with natural OTA contamination was used, with a mean value of 3.87 μg·kg^{-1} and a range for z-score $|z| \leq 2$ from 2.17 μg·kg^{-1} to 5.57 μg·kg^{-1} ($n = 49$).

Samples

Samples for the determination of OTA ($n = 55$) were obtained from coffee-roasting plants, cafés and retail shops in the Czech Republic as commonly available coffee beans intended for coffee preparation. The types of coffee were Arabica (44 samples) and Robusta (6 samples) or a mixture thereof (5 samples). The composition of the mixtures was taken from the sample package. On a global scale, Arabica and Robusta coffees are produced in a ratio of approximately 3 : 1 [3], but in industry this ratio is close to 1 : 1 [19]. The origin of the samples along with the types of coffee are summarized in Tab. 2.

Sample preparation

Samples were prepared using the commercially available immunoaffinity column (IAC) Ochraprep (R-Biopharm, Darmstadt, Germany). To a weight (10 g) of roasted coffee finely ground by a grinder AR 1105 (Moulinex, Écully, France), 100 ml of 1% $NaHCO_3$ was added, the mixture was shaken for 50 min at 15 000 Hz and then centrifuged for 10 min at 4 000 ×g. A volume of 10 ml of the supernatant were diluted with 10 ml of phosphate buffered saline with 0.1% Tween 20

Tab. 2. Occurrence and content of ochratoxin A in coffee bean samples.

Geographical origin	Number of analysed samples				Positive samples		Content in positive samples [$\mu g \cdot kg^{-1}$]	
	Total	Arabica	Robusta	Mixture*	Number	Rate [%]	Min.	Max.
Africa	15	12	3	0	3	20	0.55	0.86
South America	12	12	0	0	4	33	0.31	0.69
Middle America and Carribean region	10	10	0	0	1	10	0.84	0.84
North America (Mexico)	3	3	0	0	1	33	0.37	0.37
Mixture (Latin America and Asia or Latin America and Africa)	6	1	1	4	2	33	0.76	1.06
Asia (India)	2	2	0	0	2	100	0.52	0.53
Unknown	7	4	2	1	2	29	0.69	0.99
Total	55	44	6	5	16	29	0.31	1.06

* Composition of the mixture was taken from the sample package.

and applied to the immunoaffinity column. Flow rates of 2–3 $ml \cdot min^{-1}$ were achieved using Visiprep SPE vacuum manifold (Supelco, Bellefonte, Pennsylvania, USA). Subsequently, the column was washed with 20 ml of the buffer with Tween and eluted with 2.5 ml of acidified methanol (acetic acid-methanol at 2 : 98, v/v) and then with 1.5 ml of water. During elution, „backflushing“ (reversing the flow direction) was used to ensure complete elution of OTA from the column. The eluate was evaporated to dryness using the digital rotary vacuum evaporator RV 10 (IKA-Werke, Staufen, Germany) at 9 600 Hz and a pressure of 95 kPa. The temperature of the water bath was 50 °C. The residue was dissolved in 1 ml of 50% (v/v) methanol and filtered through a nylon syringe filter (pore size 0.22 μm).

Analysis by UPLC-FLD

Waters Acquity UPLC-FLD equipment controlled by Empower 3 data processing software (Waters, Milford, USA) was used for the analysis. Separation was performed on the Acquity UPLC BEH C18 column (100 μm × 2.1 mm, particle size 1.7 μm; Waters) connected to an Acquity UPLC BEH C18 guard column (50 μm × 2.1 mm, particle size 1.7 μm; Waters). Separation was performed using binary gradient. Mobile phase A consisted of pure acetonitrile (ACN) and mobile phase B was a mixture of water, acetonitrile and formic acid (97.7 : 2 : 0.3, v/v/v). The following programme of gradient elution was used: in 0 min: 40 % A and 60 % B, 0–2 min: 60 % A and 40 % B, 2–2.6 min: 60 % A and 40 % B, 2.6–4 min: 40% A and 60 % B, 4–7 min: 40 % A and 60 % B. The column temperature was 40 °C and the injection volume was 10 μl. The fluorescence detector was set at the excitation wavelength 335 nm, and the emission wavelength 440 nm. The analysis lasted 7 min. Quantification was performed by the external standard method.

Method validation

The analytical method was validated according to International Council for Harmonisation Q2(R1) guideline [20], which provides an overview of the criteria required to fully validate an analytical procedure for selected parameters such as selectivity, linearity, limit of detection (*LOD*), limit of quantification (*LOQ*), precision and accuracy. The calibration curve for OTA was linear for seven calibration points and three replicates at each level in the range between 0.3 $ng \cdot ml^{-1}$ and 10.0 $ng \cdot ml^{-1}$. The determination coefficient (R^2) was 0.9997. The regression equation was

$$y = 13532x - 594 \quad (1)$$

The working range of the method was within the scope from 0.3 $\mu g \cdot kg^{-1}$ (*LOQ*) to 10.0 $\mu g \cdot kg^{-1}$. *LOD* and *LOQ* were determined based on successive dilution steps of the prepared CRM sample (OTA content 3.87 $\mu g \cdot kg^{-1}$) with 50% (v/v) methanol until the signal disappeared. *LOD* was defined as the concentration at which the signal to noise ratio equaled 3. *LOQ* was defined as the concentration at which the signal to noise ratio equaled 10. *LOD* and *LOQ* for OTA in roasted coffee matrix were calculated in this way as 0.1 $\mu g \cdot kg^{-1}$ and 0.3 $\mu g \cdot kg^{-1}$, respectively. Accuracy and precision for OTA were determined using CRM. Recovery of OTA in roasted coffee ranged from 81.3 % to 112.7 % with relative standard deviation (*RSD*) of 11.7 % (n = 6). A system suitability test (SST) was used to determine the quality of the chromatographic system. The factors evaluated were: *RSD* for six injections, symmetry fac-

tor, resolution and number of theoretical plates. The acceptance criterion was that *RSD* must be ≤ 2.0 %. The number of theoretical plates was more than 5 000. The experimental results for SST were: *RSD* for six injections was 0.2 %, symmetry factor was 1.1, resolution $R_{1,2}$ was 14.1, $R_{2,3}$ was 11.6 and the number of theoretical plates was 27 475.

RESULTS AND DISCUSSION

A total of 55 commercially available roasted coffee samples were analysed. In each case, two replicate samples were analysed. The content of OTA in roasted coffee is shown in Tab. 2. Sixteen samples (29 %) were quantified in the OTA content range from 0.31 $\mu g \cdot kg^{-1}$ to 1.06 $\mu g \cdot kg^{-1}$. The median OTA concentration was 0.69 $\mu g \cdot kg^{-1}$ and the arithmetic average was 0.65 $\mu g \cdot kg^{-1}$.

The positivity rate in geographically defined sample groups ranged from 10 % to 33 %, except for two samples originating from India, where the positivity rate was 100 %. However, in these two samples OTA was detected at a low content of 0.52 $\mu g \cdot kg^{-1}$ and 0.53 $\mu g \cdot kg^{-1}$. None of the samples analysed exceeded the legislative limit according to Commission Regulation (EC) No. 1881/2006 [13], which sets the limits for OTA in roasted coffee at 5.0 $\mu g \cdot kg^{-1}$. Regarding the OTA content in roasted coffee, 54 samples (98 %) exhibited content below 1 $\mu g \cdot kg^{-1}$. The relatively low OTA contamination in the samples analysed could be due to higher quality, better storage and also better processing of the coffee beans. From a toxicological point of view, the highest level of OTA found in coffee samples (1.06 $\mu g \cdot kg^{-1}$), together with the annual coffee consumption per capita in the Czech Republic (2.40 kg per person), as reported by the Czech statistical office [21], corresponds to the average daily consumption of 6.57 g of coffee and thus 7.0 ng of OTA per person. This amount is 184 times lower than the tolerable weekly intake (*TWI*) of OTA: 120 ng per kilogram of body weight [22]. In view of this result and together with the results published by Malir et al. [9], it appears that roasted coffee sold in the Czech Republic makes only a small contribution to the daily intake of OTA, because transfer of OTA from roasted coffee to coffee beverage is only partial. Depending on the type of preparation of beverage used, OTA transfer into coffee ranged from 22.3 % to 66.1 %. [9]. These calculations are only of theoretical use since *TWI* of 120 ng per kilogram of body weight, according to the European Food Safety Authority [23], is no longer valid.

Tab. 3. Comparison with current available data on ochratoxin A content in coffee.

Number of analysed samples	Positivity rate [%]	OTA [$\mu g \cdot kg^{-1}$]		Ref.
		Min.	Max.	
24	54	0.11	5.78	[10]
69	71	0.20	2.50	[11]
30	100	< *LOQ*	11.90	[14]
24	100	< *LOQ*	0.84	[17]
50	60	0.48	15.70	[24]
11	27	0.71	10.31	[26]
10	NS	2.00	79.00	[28]
72	49	1.21	4.21	[29]
34	68	0.30	6.50	[30]
71	59	0.10	2.30	[31]
20	85	0.20	2.10	[32]
30	27	< *LOQ*	< 5.00	[33]
490	44	0.15	12.10	[34]
55	29	0.31	1.06	Present study

OTA – ochratoxin A, < *LOQ* – below limit of quantification, NS – not specified.

The natural occurrence of OTA in roasted coffee is a global problem, determination of OTA content in this matrix has been addressed by several research groups and several studies were published (Tab. 3). In comparison to other studies, the level of contamination we measured was lower.

In a recent study, Pakshir et al. [24] published a validated analytical method using immunoaffinity columns and HPLC-FLD for evaluating OTA contamination in 50 coffee samples. Among 50 samples, all of them were contaminated with OTA in the range from 0.48 $\mu g \cdot kg^{-1}$ to 15.7 $\mu g \cdot kg^{-1}$. Armutcu et al. [25] used an advanced automated on-line two-dimensional HPLC to determine OTA in Turkish coffee as an alternative to HPLC coupled to IAC. Tozlovanu and Pfohl-Leszkowicz [14] analysed 30 roasted coffee samples by HPLC-FLD and two extraction methods, namely, IAC and toluene extraction under acidic conditions. The OTA content in samples ranged from < *LOQ* to 11.9 $\mu g \cdot kg^{-1}$. Benites et al. [26] analysed commercially available roasted coffee, Arabica ($n = 6$) and Robusta ($n = 3$) from Portugal. The maximum OTA content determined was 10.31 $\mu g \cdot kg^{-1}$, which exceeded EU regulatory limit according to regulation No. 1881/2006 [13]. A study by Vanesa and Ana [10] described the determination of OTA in 5 green coffee, 24 ground roasted coffee and 22 instant coffee samples. Among the total of 51 samples,

35 samples (69 %) were positive, and 3 samples exceeded the limits set out in the EU Regulation 1881/2006 [13]. The median values obtained from the analysis of the above samples were 2.7 $\mu g \cdot kg^{-1}$ for green coffee, 0.24 $\mu g \cdot kg^{-1}$ for ground roasted coffee, and 0.43 $\mu g \cdot kg^{-1}$ for instant coffee. The authors further evaluated exposure risks similarly to MALIR et al. [9], who studied the transfer of OTA into beverages such as tea and coffee. These authors analysed a total of 48 ground roasted coffee samples with the maximum measured content of 1.37 $\mu g \cdot kg^{-1}$, the arithmetic average was 0.51 $\mu g \cdot kg^{-1}$, and the median was 0.48 $\mu g \cdot kg^{-1}$. JONATOVA et al. [11] used a set of 103 samples in their study (roasted coffee $n = 69$ and instant coffee $n = 34$), collected between 2016 and 2018 from commercial stores in the Czech Republic. The extracts were purified using IAC and analysed with ultra-performance liquid chromatography coupled to tandem mass spectrometry. OTA content in roasted coffee ranged from 0.2 $\mu g \cdot kg^{-1}$ to 2.5 $\mu g \cdot kg^{-1}$, with an average content of 0.6 $\mu g \cdot kg^{-1}$ and 71 % of positive samples. KHANEGHAH et al. [27] focused on estimating the prevalence and content of OTA in different types of coffee and coffee products between 1983 and 2018 with the aid of a systematic review and meta-analysis. From a total of 3 182 samples, the global cumulative OTA content of 3.21 $\mu g \cdot kg^{-1}$ and a prevalence of 53 % were calculated. Among these data, the minimum content of OTA in coffee was 0.35 $\mu g \cdot kg^{-1}$ (Taiwan) and the and maximum content was 79.0 $\mu g \cdot kg^{-1}$ (Turkey). Finally, the study published by YAZDANFAR et al. [28] reported the determined OTA content in roasted coffee from 2.0 $\mu g \cdot kg^{-1}$ to 79.0 $\mu g \cdot kg^{-1}$ with a mean of 26.6 $\mu g \cdot kg^{-1}$, which exceeds the EU limit [13] by more than five times. TOZLOVANU and PFOHL-LESZKOWICZ [14] reported that generally OTA contamination is higher in non-trademark coffees. The above results can be used to develop risk evaluation models to reduce human exposure to this mycotoxin in coffee and coffee products.

CONCLUSION

A fast analytical method using the Ochraprep immunoaffinity column for sample preparation and UPLC-FLD for determination of OTA in roasted coffee was optimized, validated and applied to roasted coffee samples. This method may become a useful tool for control laboratories. None of 55 samples analysed exceeded the maximum limit set out in Commission Regulation (EC) No. 1881/2006 [13]. Such coffee beans are therefore safe for the preparation of the final beverage. However, regular monitoring of OTA and its analogues is necessary to ensure consumer health and safety regarding the high consumption of coffee worldwide.

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