Research Article

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Transfer of ochratoxin A from raw black tea to tea infusions prepared according to the Turkish tradition

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Abstract

BACKGROUND: Ochratoxin A (OTA) is a natural contaminant of food including tea with multiple toxic effects, which poses a threat to human health. In terms of lifestyle, the Turkish population is a frequent visitor of tearooms, and the traditional Turkish tea preparation is one of the most popular ways of preparing tea infusion.

RESULTS: The aim of this study was to investigate OTA transfer from raw black tea to the tea infusion prepared according to the Turkish tradition. A high-performance liquid chromatography method with a limit of quantification of 0.35 ng g^{-1} was used for OTA determination. The OTA amount in raw black teas from Turkey ranged from $\leq$0.35 ng g^{-1} up to 56.7 ng g^{-1}. An homogenised sample of black tea naturally contaminated with 55.0 ng g^{-1} was used to prepare infusions. The OTA transfer from the black tea to the infusion was found to be 41.5% $\pm$ 7%.

CONCLUSION: These data are important for the realisation of a 'Total Diet study' (TDS). The TDS can be a complementary tool to estimate the population dietary exposure to OTA across the entire diet by analysing main foods prepared 'as consumed' (tea infusions) and not 'as purchased' (raw tea).

Keywords: ochratoxin A; OTA; Black tea; transfer; infusion; HPLC-FLD

INTRODUCTION

Tea, one the most frequently consumed infusion on a global basis,[1] is produced from the leaves of the shrub *Camellia sinensis* (L.) Kuntze.[2] About 18–20 billion cups of tea are consumed daily worldwide.[3] Tea consumption has been attributed various health benefits, including prevention of Parkinson's disease, cardiovascular disease, cancer, immune disorders and decrease of blood cholesterol levels.[4] Tea was used in China as early as 3000 BC or even earlier.[5]

Nevertheless, black tea may contain some contaminants, e.g. pesticides, microorganisms, toxic heavy metals.[6] It can also be contaminated with toxigenic fungi, decreasing its quality.[6–18] Heat-tolerant ascospores and conidia can survive tea pasteurisation.[19] In a study conducted in the Czech Republic, 81 species of microfungi were detected in black, green and herbal teas, with *Aspergillus* Section *Nigri* and *A. ochraceus* being the most frequent fungi.[8] In general, *Aspergillus* Section *Nigri* including *Aspergillus niger*, *A. acidus*, *A. awamori*, *A. tubingensis* and *A. carbonarius* are the most frequent fungi found in black tea,[14,20] notably in teas available on the Swiss market.[21]

The presence of toxigenic microfungi in black tea represents a risk of contamination by mycotoxins, e.g. ochratoxin A (OTA),[22] fumonisin B_1,[23] fumonisin B_2, fumonisin B_4[24] and aflatoxins.[25] It has been shown that 7% of *Aspergillus niger* isolates from herbal teas are able to produce OTA.[21] *Aspergillus niger* and *A. carbonarius* should be highlighted both for their high incidence and for their great capacity to produce OTA.[26]

The present article focuses on OTA, a widespread mycotoxin,[27,28] with several toxic effects,[29–34] which render it one of the most deleterious mycotoxins.

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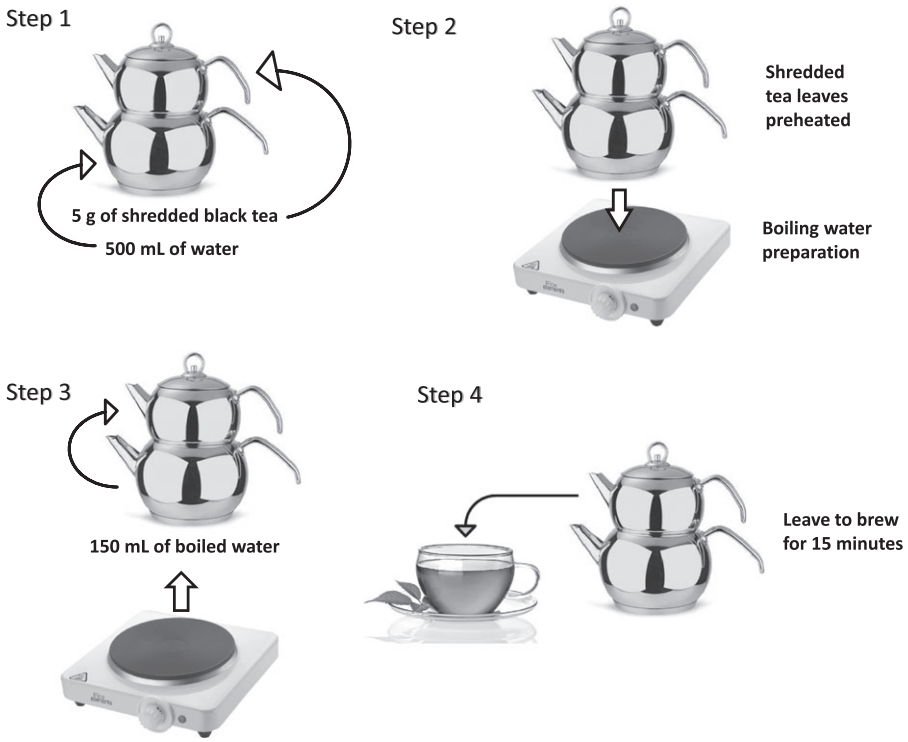

Figure 1. Tea infusion according to traditional Turkish preparation.

In tearooms, tea infusions can be prepared in different ways. There has been a debate within the scientific community on whether OTA could be transferred (and to which extent) from raw black tea to the tea infusion, following the traditional Turkish preparation which is different from processes used in most European countries. Therefore, our study aimed at determining OTA concentrations in some samples of Turkish black teas from the Black Sea region, and to assess OTA transfer from the contaminated teas to their corresponding infusions prepared according to the Turkish tradition.

MATERIALS AND METHODS

Black tea samples

Fifty samples of black tea from the Black Sea region in Turkey were randomly purchased during 2015 from Turkish retail stores (loose tea, $n = 9$; bag tea, $n = 5$) and from different tea-shops and teahouses in the Czech Republic (loose tea, $n = 36$).

Chemicals and materials

A crystalline OTA standard was purchased from Sigma–Aldrich (Prague, Czech Republic). Chemicals used for extraction – sodium hydrogen carbonate, sodium chloride, di-sodium hydrogen phosphate anhydrous, potassium dihydrogen phosphate and potassium chloride (all of AnalaR NORMAPUR purity), and methanol (HiPerSolv CHROMANORM, gradient grade) – were from VWR (Stribrna Skalice, Czech Republic). Sample extracts were evaporated under nitrogen 5.0 SIAD spol. s r.o. (Branany u Mostu, Czech Republic). Paper filters which were used for sample filtration were Whatman No. 4 from Merck (Prague, Czech Republic) and KA 2-M Papirna Pernstejn s r.o. (Pernstejn, Czech Republic). For pre-treatment, Strata Phenyl columns (55 μm, 70A) (500 mg 3 mL^{-1}) (Phenomenex, Torrance, CA, USA) were purchased from AP-servis (Litošice, Czech Republic). OTA immunoaffinity columns OCHRAPREP® (R-Biopharm, Germany) were purchased from Jemo-Trading (Bratislava, Slovakia).

All solutions of chemicals were prepared using Milli Q Plus Millipore (Billerica, MA, USA) ultrapure water. The standard OTA solution and other solutions were prepared as described in Malir *et al.*[35] All calibration solutions were prepared daily from the standard OTA solution.

Black tea homogenised sample preparation

Purchased samples of raw black tea were properly homogenised and successfully tested for their homogeneity by Cochran's test according to ISO 13528:2015, Annex B, 'Homogeneity and stability checks of samples'.[36] The samples were analysed for OTA, and OTA-positive samples were used to prepare an homogeneous sample containing 55.0 ng g^{-1} OTA that would be subsequently used to prepare six infusions.

Black tea sample purification (clean-up)

Ten grams of homogenised raw black tea were purified on a Strata Phenyl column. OTA was recovered by the addition of 10 mL methanol/water (7/93, v/v), to which 30 mL of a phosphate buffer solution (PBS) were added. This mixture was passed through the immunoaffinity column O CHRAPREP® as described in Malir *et al.*[35]

Black tea infusion preparation

The tea infusion was prepared according to the Turkish tradition, and as follows. As described in Fig. 1, a big bowl is filled with 500 mL of drinking water. Five grams of shredded tea are put into the small adapted jar (Step 1). Water is boiled allowing preheating of tea leaves (Step 2). Then, 150 mL of hot water are added to

preheated tea leaves (Step 3). Tea leaves are infused for 15 min. Then, the infusion is ready to drink (Step 4). The cooled tea infusion is used as the matrix to measure the % transfer of OTA.

Black tea infusion purification (clean-up)

Twenty millilitres of the black tea infusion were mixed with 20 mL of a sodium hydrogen carbonate 30 g L^{-1} solution, and 40 mL of the mixture were purified by solid phase extraction on a conditioned Strata phenyl silane column. OTA was recovered by the addition of 10 mL methanol/water (7/93, v/*v*), to which 30 mL of phosphate buffer solution (PBS) were added. This mixture was passed through the immunoaffinity OCHRAPREP® column (R-Biopharm) with a loading speed of around 1 mL min^{-1}. The column was washed with 20 mL PBS. OTA was recovered by slow elution with 4.0 mL methanol. The solution was evaporated to dryness under nitrogen stream and dissolved in 500 μL of methanol.[35]

HPLC-FLD analysis

The validated and accredited[37] method of reversed phase high-performance liquid chromatography (HPLC Spectra System, San Jose, CA, USA) with fluorescence detection (detector 920 FP; Jasco, Tokyo, Japan) was used for OTA detection and quantification in the tea samples. The liquid chromatography equipment consisted of vacuum degasser SCM 400, gradient pump P 2000, autosampler AS 3000 (all from Spectra System) and Solvent saver 2907 (Jour Research, Schenkon, Switzerland). The analytical column Inertsil filled with ODS-3 V, 150 × 4.6 mm and particle size of 5 μm (Hichrom, Reading, UK) coupled with a precolumn Inertsil ODS-3, 10 × 4 mm filled with the same particle size (Hichrom) were used. The calculations and evaluations of the analyses were processed by software CSW 32 (Data Apex, Prague, Czech Republic). The mobile phase contained methanol/acetonitrile/0.005 mol L^{-1} sodium acetate/acetic acid (300/300/400/14, v/v/v/v). The flow rate was 1.5 mL min^{-1}. Parameters of fluorescence detector were: excitation wavelength = 333 nm, and emission wavelength = 465 nm. The injection volume of the sample was 50 μL. Validation of the method was performed according to the protocol approved by the AOAC and to the principles of the ICH Guideline for high-performance liquid chromatography. This method was accredited by the Czech Accreditation Institute. As no reference material (OTA in black tea) was available for method validation, samples of black tea spiked with OTA, and the reference materials T17145QC of roasted coffee (OTA concentration, 4.87 ng g^{-1} naturally contaminated coffee) and T17158QC of roasted coffee (OTA concentration, 3.29 ng g^{-1} naturally contaminated coffee) from JEMO Trading (Bratislava, Slovakia) were used. Repeatability results were obtained by using the same method performed by the same operator using the same equipment on identical testing samples in the same laboratory within short time interval, in accordance with CSN ISO 3534–1.[38] A linear calibration curve was built by measuring the OTA peak areas of six standard solutions of OTA in methanol (concentrations of 0.125, 0.250, 0.500, 1.000, 2.000, and 4.000 ng mL^{-1} OTA), and that of a blank sample (methanol). Each point of the calibration curve was measured in triplicate. The recoveries of the HPLC method ranged from 75% to 85%, and the average relative standard deviation of repeatability (RSD_r) was 9.66%. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.1 and 0.35 ng g^{-1}, respectively.

Measurement of pH

The determination of the pH of drinking water and of the tea infusion was carried out by measuring the potential difference between electrodes immersed in standard and test solutions. The standard solutions were assigned a definite pH value by convention. The pH measurement was done in triplicate on a pH meter WTW (model 340i with combined glass electrode; WTW, Weilheim, Germany).

Statistical evaluation

OTA transfer was assessed by basic calculations (mean, median, 95% percentile, etc.) using MS office Excel.

A difficult step was how to handle data on concentrations reported below the limit of quantification (LOQ) but over the limit of detection (LOD). These data are known as non-detected and the resulting distribution of the occurrence values is left-censored. We preferred, for left-censored data, to use the middle bound approach by replacing the results below LOQ by LOQ divided by 2.[39]

In order to test the homogeneity of raw black tea samples the Cochran's test was used according to ISO 13528:2015, Annex B, 'Homogeneity and stability checks of samples'.[36]

RESULTS AND DISCUSSION

Determination of OTA in raw black teas

In 46 (92%) samples of raw black tea, the concentration of OTA was <0.35 ng g^{-1}. Only four samples (8%) – collected in the Czech Republic and corresponding to loose black tea containing additional ingredients, e.g. citrus pericarp, lemon and orange peel, lemon, flavouring (without further specification) – contained OTA above the LOQ (Table 1); concentrations in the four positive samples were 19.6–56.7 ng g^{-1}.

A HPLC chromatogram of 23.4 ng g^{-1} OTA in black tea is presented in Fig. 2.

In the present study, we note that only 8% of the samples were contaminated, whereas in our previous study (limited by its small sample size, $n = 12$), 25% of the samples contained a significant amount of OTA.[36] Interestingly, in the present study only black tea containing additional ingredients (e.g. citrus pericarp, lemon and orange peel, lemon, flavouring) contained OTA. This either means that OTA came from the ingredients or that the teas had been improperly stored. Since these ingredients were not available individually, we were unable to assess their content in OTA. In any event, and in both cases, the OTA contamination is not negligible.

Determination of OTA in black tea infusion

A black tea sample containing 55 ng g^{-1} OTA was used to prepare six infusions of 150 mL. OTA concentrations in the tea infusions are reported in Table 2.

The OTA transfer from the black tea to the infusions was 41.5% ± 7%.

Determination of the pH of drinking water and black tea infusion

One of the explanations of the transfer of OTA into black tea infusions could be the pH of the prepared infusion. Indeed, the pK_a values of OTA range from 4.2 to 4.4 and 7.0 to 7.3, for the carboxyl group and the phenolic hydroxyl group, respectively.[40] Thus, OTA is expected to be more soluble in an aqueous phase when the pH is above 7.0.[35]

Table 1. OTA contamination in raw black tea samples

Parameter	'Turkish' black tea from Turkey	'Turkish' black tea from the Czech Republic	'Turkish' black tea (all samples)
Number of samples (*n*)	14	36	50
Number of positive samples (*n*)[a]	0	4	4
Mean level (ng g^{-1})[b]	0.175	5.48	3.02
Median level (ng g^{-1})[b]	0.175	0.175	0.175
95% percentile level (ng g^{-1})[b]	0.175	55.26	16.20
Maximum level (ng g^{-1})[b]	0.175	56.70	56.70

[a] Limit of quantification (LOQ): 0.35 ng g^{-1}.
[b] Middle bound approach for left-censored data – when the amount of OTA is <0.35 ng g^{-1}, OTA concentration is considered as being 1/2 LOQ (0.175 ng g^{-1}).

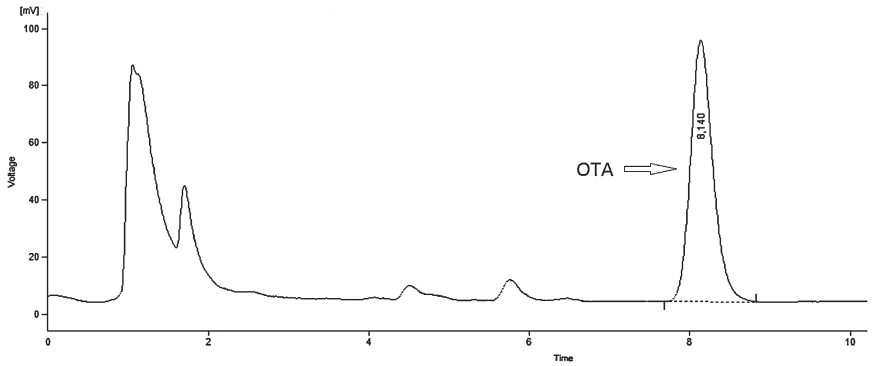

Figure 2. HPLC chromatogram of 23.4 ng g^{-1} OTA in black tea.

Table 2. OTA concentrations and transfer into tea infusions ($n = 6$)

Level	OTA in tea infusions (ng mL^{-1})	Transfer of OTA to tea infusions (%)[a]
Mean level	0.76	41.5
Median level	0.75	41.1
Maximum level	0.89	48.5

[a] Infusions were prepared from 5 g of naturally contaminated black tea containing 55 ng g^{-1} OTA. Thus, a 100% transfer would lead to 275 ng of OTA per 150 mL of infusion (i.e 1.83 ng mL^{-1} infusion).

Table 3. pH of the drinking water and of the tea infusion

Water/tea	pH	Temperature (°C)
Drinking water	7.19	18.4
	7.19	18.7
	7.18	18.8
Tea infusion	7.09	29.0
	7.07	28.6
	7.07	28.4

The pH of the drinking water used to prepare the tea infusion was measured at temperatures between 25.2 and 25.5 °C before boiling. The pH values ranged from 7.18 to 7.19, with an arithmetic mean of 7.19. The pH of tea infusions, at temperatures between 28.4 and 29.0 °C, ranged from 7.07 to 7.09, with an arithmetic mean pH of 7.08 (Table 3).

The above data confirmed that OTA was transferred into the infusion. The transferred amount (41.5% ± 7%) was slightly higher in this study compared with our previous study where 34.8% ± 1.3% of OTA was transferred.[35] This difference could be due to the different processes of tea infusion preparation. Indeed, in our previous study (done in the Czech Republic) tea infusions were prepared from 1.75 g of tea brewed with 250 mL of boiling water for 3 min. This type of preparation (putting a tea bag into boiling water) is very common worldwide. The higher OTA transfer in the present study is probably due to the longer contact of tea with water (15 min).

CONCLUSION

This study confirmed that a significant amount of OTA can be transferred into black tea drinks prepared according to the Turkish tradition (about 41.5%). The longer contact of tea with water, the higher was the amount of OTA in the tea infusion.

As the percentage of OTA passing through the infusion depends on the tea origin, fermentation, pH of drinking water and the process of infusion preparation, the evaluation of the intake should be done using the amount of OTA found in the tea infusion.

These data are very important for the realisation of a 'Total Diet Study' (TDS) and the dietary exposure assessment of OTA. The TDS can be a complementary public health tool to estimate the population dietary exposure to OTA across the entire diet by analysing main foods prepared 'as consumed' (tea infusions) and not 'as purchased' (loose tea or bag tea).[41]

Constant attention must be paid to the transfer of OTA from foodstuffs such as tea or coffee, for better protection of public health.

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