Mycotoxins in herbal tea: transfer into the infusion

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Received: 6 June 2020 / Accepted: 18 January 2021

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OPEN ACCESS

RESEARCH ARTICLE

Abstract

Mycotoxins are natural food contaminants, associated with adverse health effects due to acute intoxication and, what is much more common, chronic exposure. The most hazardous and widespread mycotoxins are subjected to regulation in food in most countries. Alongside with regulated, a wide list of mycotoxins is monitored in various foods. Traditionally mycotoxins in tea are determined in a dry sample, not taking into account their transfer rate into the infusion. This study was aimed at the determination of the transfer rate of several mycotoxins from naturally contaminated herbal tea samples into an infusion. Seven of the most contaminated samples were pre-selected during the monitoring of mycotoxins in *Camellia sinensis* and herbal tea available in the Russian Federation. Ochratoxin A (OTA), sterigmatocystin (STE), mycophenolic acid (MPA), tentoxin (TTX), alternariol (AOH), its methyl ether (AME), zearalenone (ZEN), enniatins A and B (ENN A and B), beauvericin (BEA) were detected in these samples in the range of several µg/kg to several mg/kg. HPLC-MS/MS was used for dry tea samples and their infusion analysis. Mycotoxin polarity and infusion pH (for analytes possessing carboxylic groups) appeared to be factors determining transfer rate. STE transferred into infusion at the average rate of 10%. Average transfer of *Alternaria* toxins varied from 73% (TTX) to 45% (AOH) and about 11% (AME). A third part of ZEN was detected in the infusion. Transfer of ENNs and BEA was low and did not exceed 7%. Infusion pH affected MPA transfer rate; it increased from 23% to 96% in the pH range from 5.5 to 6.3. 83% of OTA was detected in the infusion of a single contaminated sample. Consideration of the mycotoxin transfer rate to herbal tea infusions resulted in the change of the model herbal tea input into mean chronic dietary exposure for most studied mycotoxins.

Keywords: HPLC-MS/MS, *Alternaria* toxins, herbal tea, infusion, transfer rate

1. Introduction

Herbal infusions are traditionally considered safe and are associated with a health-promoting lifestyle. As well as other raw plant commodities, herbs are inevitably contaminated with micromycetes. Prevalence of particular toxinogenic species and their toxin production depend on cultivation, processing and storage conditions. Some fungi infect plants before harvest, others are saprophytic. Tropical and subtropical regions are most at risk for *Aspergillus* spp. (Taniwaki *et al.*, 2018). Dayananda *et al.* (2017) found them dominating in *Camellia sinensis* samples. Herbs cultivated in temperate regions are also contaminated with *Alternaria* and *Fusarium* spp. (Halt, 1998; Rizzo *et al.*, 2004). Most of harmful *Penicillium* infections are post-harvest, fungal

growth and mycotoxin accumulation being induced by inappropriate storage conditions. The predominant mycoflora comprised of *Aspergillus* and *Penicillium* spp. in spices, aroma (Fogele *et al.*, 2018) and medicinal herbs (Chen *et al.*, 2020). A recent survey carried out by Reinholds *et al.* (2020) revealed an almost absolute prevalence of *Aspergillus* spp. in *C. sinensis* and herbal (camomile, peppermint, linden, hibiscus, rosehip fruits) teas, followed by *Penicillium* spp. The fungal species variety in herbal teas also included *Rhizopus*, *Fusarium* and *Alternaria*. Svistova and Paramonov (2011) discovered a 7-10-fold increase in occurrence of toxigenic fungi among soil micromycetes under medical herbs in comparison with black soil under native grasses of the temperate zone of Russia. Health risks for consumers are particularly associated with aflatoxins and

ochratoxin A (OTA) (Kabak and Dobson, 2017; Van Asselt *et al.*, 2018). Aflatoxins are the most potent hazard among fungal metabolites. Most of the regulations concerning tea and herbal tea quality, not taking into account medicinal preparations, set maximum levels (MLs) for aflatoxin B₁ or the sum of aflatoxins. MLs vary from 5 to 30 µg/kg (Sedova *et al.*, 2018). Besides aflatoxins and OTA, surveys report the detection of *Fusarium* (trichothecenes, zearalenone (ZEN), fumonisins (FBs), enniatins (ENN)) and *Alternaria* toxins, cyclopiazonic and mycophenolic (MPA) acids, sterigmatocystin (STE), and citrinin (Bogdanova *et al.*, 2020; Reinholds *et al.*, 2019, 2020; Rocha-Miranda and Venâncio, 2019; Santos *et al.*, 2009; Sedova *et al.*, 2018; Veprikova *et al.*, 2015).

Mycotoxins in tea are usually determined in dry matter. Existing regulations assume complete transfer of contaminants into the infusion. That could be fair for polar water-soluble mycotoxins. Ionogenic groups can also facilitate water solubility. OTA is a good example; its carboxylic group is characterised by pKa about 3.2 (PubChem Database, 2020); at low pH, OTA molecules can be efficiently extracted by water-immiscible organics, while dissociated molecules transfer into the aqueous phase (Dohnal *et al.*, 2010). Malir *et al.* (2014) reported OTA transfer into an infusion at the rate of about 35 and 4% for two naturally contaminated samples of bagged black tea (250 µg/kg of OTA in dry tea; beverage pH 6.7) and fruit tea (104 µg/kg of OTA in dry tea; beverage pH 3.4) within 3 min infusion, respectively. Naturally contaminated coffee samples (0.92 µg/kg of OTA) yielded 22 to 66% transfer depending on the preparation procedure. Later, the authors reported new results concerning OTA in black tea prepared according to Turkish tradition (Toman *et al.*, 2018). Shredded tea leaves were infused for 15 min. OTA transfer into the infusion of the most contaminated sample (about 57 µg/kg) proved to be 41±7%. It was noted, that longer contact of tea with water (15 vs 3 min) could result in higher OTA transfer. Carraturo *et al.* (2018) evaluated average OTA transfer from teabags into the infusion at the level of 54±14% for green tea (2-20 µg/kg of OTA in the dry sample) and 34±4% for black tea (1-22 µg/kg of OTA in the dry sample). A few more projects concerned mycotoxin transfer into infusions. Monbaliu *et al.* (2010) examined 91 *C. sinensis* dry tea samples for over 20 mycotoxins (aflatoxins, STE, OTA, *Fusarium* and *Alternaria* mycotoxins) and found only one contaminated sample, containing 76 µg/kg fumonisin B₁ (FB₁). The authors did not detect any notable transfer of the mycotoxin into the infusion. Sixty samples of medicinal herbal teas were analysed by Reinholds *et al.* (2019) for the presence aflatoxin B₁, STE, OTA, deoxynivalenol (DON), T-2 and HT-2 toxins, ZEN, ENNs and beauvericin (BEA). All of them were detected in dry tea samples. Only DON and ZEN were found in infusions. DON transfer varied in the range 32-100% (7 samples examined, DON concentration

in dry tea ranged from 968 to 5,463 µg/kg), ZEN transferred into infusion completely (1 sample, 90.7 µg/kg in dry tea).

Within the survey of multiclass mycotoxin contamination of *C. sinensis* and herbal tea available in Russia, we have discovered several samples of herbal tea contaminated with *Alternaria* (tentoxin (TTX), alternariol (AOH), its methyl ether (AME)) and *Fusarium* (fumonisin B₂ (FB₂), ZEN, ENNs, BEA) mycotoxins; OTA, STE and MPA (Kiseleva *et al.*, 2020). Mycotoxin concentration reached mg/kg levels. The present study aimed to discover the transfer rate of the above mycotoxins from the raw material into the infusion.

2. Materials and methods

Materials and chemicals

Water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Formic acid (pure, 98+%, Acros Organics, Thermo Fisher Scientific, Geel, Belgium), ammonium formate (for LC-MS, Sigma-Aldrich, Moscow, Russia), HPLC-grade methanol (LiChrosolv, Merck, Darmstadt, Germany) and acetonitrile (Panreac, AppliChem, Darmstadt, Germany) were used for the preparation of standard solutions of mycotoxins and mobile phases. Acetonitrile (pure, Criochem, St. Petersburg, Russia) was used for sample extraction. Anhydrous magnesium sulfate (97%, Acros Organics, Acrus, Moscow, Russia) and sodium chloride (99%, Spektr-Khim, Moscow, Russia) were used as salting out additives during sample preparation.

Three types of water were used to prepare infusions: distilled water (DW) (average pH 5.4 (n=6, CV=2.1%)), bottled deep-well water (D-WW) (average pH 7.9 (n=6, CV=6.4%); total dissolved solids (TDS) = 0.2-0.35 g/l) and bottled still natural mineral water (NMW) (average pH 7.6 (n=5, CV=2.2%); TDS=0.3-0.6 g/l). Bottled water was purchased from the local market.

Standards and standard solution preparation

Individual neat analytical standards of STE (≥98%), ZEN (≥98%), OTA (≥98%) were purchased from Sigma-Aldrich; AOH (99.3%), AME (99.77%), BEA (99.31%), ENN A (99.68%), ENN B (99.62%), MPA (99.59%), TTX (99.84%) were supplied by Fermentek (Jerusalem, Israel). Dry standards were dissolved in an appropriate solvent and diluted to obtain individual stock standard solutions: AOH, AME and TTX – 200 µg/ml in methanol; BEA, ENN A and B – 20 µg/ml in methanol; ZEN – 50 µg/ml in acetonitrile; STE, OTA – 20 µg/ml in methanol; and MPA – 250 µg/ml in acetonitrile. All stock solutions were stored at -18 °C. Stock multi-mycotoxin mixed standard solution was prepared in methanol; mycotoxins concentrations were

as follows: 0.1 µg/ml OTA and STE; 0.4 µg/ml ENNs and BEA; 0.8 µg/ml TTX; 2 µg/ml ZEN, AOH and AME; 5 µg/ml MPA. This solution was used for spiking, its dilutions in the mix of HPLC mobile phases A and B (1/1, v/v) were used to obtain external solvent- and matrix-matched calibrations (L1-L7) and as post-extraction standard additives within quantification of mycotoxins in real samples. The stock multi-mycotoxin standard solution was stored sealed in amber vials and frozen for a month. Calibration solutions were prepared fresh before each analysis.

HPLC-MS/MS

HPLC analysis was performed with a Vanquish UHPLC system consisting of a binary pump, autosampler and column compartment combined with triple quadrupole mass spectrometer with a heated electrospray source TSQ Endura controlled by Xcalibur 4.0 QF2 Software (all Thermo Scientific, Waltham, MA, USA). HPLC column Ascentis Express F5 (100×3.0 mm I.D. pore size 90 Å, particle size 2.7 µm) was supplied by Supelco (Bellefonte, PA, USA). Self-made post-column T-splitter delivered 10% effluent to detector, split ratio 1:9. Column compartment temperature was 25 °C. The autosampler chamber temperature was set to 5 °C.

The mobile phase composition and gradient conditions were optimised to achieve a suitable resolution of chromatographic peaks. The mobile phases were constituted of water/methanol (95:5%, v/v, solvent A) and methanol/water (95:5%, v/v, solvent B), both containing 0.1% (v/v) formic acid and 1 mM ammonium formate. A gradient program was set up as follows: 0–15 min linear gradient from 20 to 95% B; hold at 95% B for 7 min; return to 20% B in 1 min and hold at 20% B for 4 min (total run time 27 min).

Chromatographic separation of mycotoxins was performed on a narrow bore HPLC column filled with pentafluorophenyl silica (PFP). An example of separation is presented in Supplementary Figure S1. This stationary phase offers selectivity different from alkyl bonded phases. Elution order of studied mycotoxins for PFP stationary phase differs from that for traditional octadecyl silica (ODS) (Supplementary Table S1). We observed increased retention of FBs and *Alternaria* toxins AOH and AME. FBs were eluted together with ENNs and BEA, which are usually strongly retained on reversed phase columns. The gradual shift of retention within sequence execution was noted for FB₁ and FB₂. It was +1.6% for both FBs and persisted even after extended column equilibration.

MS/MS detection was performed in positive electrospray ionisation mode. Flow injection analysis set up was used for the optimisation of MS/MS detection of individual mycotoxins. Optimised source parameters differed from the defaults and were set as follows: vaporiser temperature

225 °C, positive spray voltage 4,500 V, ion transfer tube temperature 200 °C, sheath gas – 4 l/min, aux gas – 8 l/min, sweep gas – 2.5 l/min; CID gas (argon) 2 mTorr; dwell time 100 ms, Q1 and Q3 resolution 1.2 and 0.7 FWHM. Detection was performed in selected reaction monitoring mode (Supplementary Table S2).

Samples

The effect of water characteristics on transfer rate of mycotoxins was studied for spiked loose green tea (*C. sinensis*). HPLC-MS/MS analysis showed it could be considered as 'clean' matrix for discussed mycotoxins. Seven the most contaminated samples of tea were selected for the study of mycotoxin transfer into infusion basing on the results of their screening in *C. sinensis* and herbal tea available in the Russian Federation (Kiseleva *et al.*, 2020). All these samples and loose green tea were purchased from a local supermarket (samples No 1 and 7, green tea), and health food store (samples No 2–6) in Moscow. No 1 was bagged tea of a global manufacturer, all others – loose herbs: No 2–7 originated from Krasnodar region of the Russian Federation. Declared sample compositions were the follows:

1. *Cymbopogon citratus* DC. Stapf (lemon grass), *Backhousia citriodora* F. Muell. (lemon myrtle), *Zingiber officinale* Roscoe (ginger root), *Glycyrrhiza glabra* L. (licorice root), *Citrus limon* (L.) Burm.f. (lemon zest);
2. *Crataegus sanguinea* Pall. (hawthorn berries), *Viscum album* L. (mistletoe), *Melilotus officinalis* (L.) Pall. (melilot), *Leonurus cardiaca* L. (motherwort), *Valeriana officinalis* L.s.l. (common valeriana root);
3. *Echinacea purpurea* Moench. (echinacea), *Origanum vulgare* L. (oregano), *Mentha piperita* L. (pepper mint), *Urtica dioica* L. (nettle), *Thymus serpyllum* L. (thyme), *Matricaria recutita* L. (camomile), *Rosa canina* L. (rosehip fruits), *Salvia officinalis* L. (sage), *Viola tricolor* L. (wild pansy), *G. glabra* L. (licorice root);
4. *T. serpyllum* L. (thyme), *Hypericum perforatum* L. (St. John's Wort), *M. piperita* L. (pepper mint), *M. officinalis* (L.) Pall. (melilot), *R. canina* L. (rosehip fruits), *Inula helenium* L. (inula), *C. sanguinea* Pall. (hawthorn berries);
5. *M. recutita* L. (camomile), *M. piperita* L. (pepper mint), *Helichrysum arenarium* L. (yellow everlasting), *Tanacetum vulgare* L. (tansy), *Coriandrum sativum* L. (coriander fruits), *Silybum marianum* (L.) Gaertn. (milk thistle);
6. *Calendula officinalis* L. (marigold), *M. recutita* L. (camomile), *Agrimonia eupatoria* L. (agrimony), *H. arenarium* L. (yellow everlasting), *H. perforatum* L. (St. John's wort), *Betula pendula* Roth. (birch leaves);
7. *T. serpyllum* L. (thyme).

Samples were stored in the original packaging in a dark, cool and dry place.

Sample preparation

Sample preparation for procedure verification

100 µl of stock multi-mycotoxin mixed standard solution per 1.0 g of sample No.3 yielded contamination levels presented in Table 1. The precise volume of mixed standard solution was added into each tested portion of the sample or empty falcon tube or beaker in case of stability test. All recovery experiments were carried out in four replicates.

‘Dilute and shoot’ (DAS) and salts assisted extraction (SAE) approaches were compared for dry tea sample preparation. 1.0 g of milled (IKA Tube Mill, IKA, Staufen, Germany) tested sample was weighted into 50 ml falcon tube, spiked and left for approx. 2 h to let the solvent evaporate. Next, it was ultrasonicated in 10 ml of 1% formic acid for 10 min. After swelling, 10 ml of acetonitrile were added. The mixture was subjected to subsequent agitation in Multi RS-60 orbital shaker (Biosan, Riga, Latvia), ultrasonication and final agitation for 10 min each. An aliquot (0.5 ml) of supernatant was centrifuged at about 25,000×g for 15 min (SL16R, ThermoFisher, Dreieich, Germany) and transferred into the chromatographic vial (DAS extract). 1 g of NaCl and 4 g of anhydrous MgSO₄ (in two portions) were added to the remaining mixture in the falcon tube. Each time the sample was vortexed for about 10 s. Finally, the tube was centrifuged. An aliquot (0.75 ml) of the upper layer was diluted twice with MilliQ water, centrifuged and transferred into the chromatographic vial (SAE extract).

The infusion was prepared as follows: 4.0 g of thoroughly mixed but not milled sample was spiked and left for 2 h to let the solvent evaporate. Next, it was mixed with 200 ml of distilled or drinking water heated to 99.9 °C in thermostat. The infusion was paper filtered after 30 min. 10 ml portion of filtered infusion was mixed with acidified acetonitrile (1% of formic acid) and salts. 5 ml of resulting organic layer was evaporated under a stream of nitrogen (Smart Evaporator, BioChromato, Fujisawa, Japan), reconstituted with 0.5 ml of mobile phase B, followed by 0.5 ml of mobile phase A, mixed and analysed (Infusion_SAE_X5).

Matrix- and solvent-matched calibrations were used for evaluation of matrix effect on detection. Post-extraction standard addition was a method of quantification of mycotoxins in the spiked herbal tea and its infusion extracts. Stability of mycotoxins under brewing conditions was evaluated by brewing bare spike.

The effect of water characteristics (pH and TDS) on mycotoxins transfer rate was studied for artificially contaminated samples of green tea. Spiking levels were 80 µg/kg for OTA, 200 µg/kg for STE and TTX, 1000 µg/kg for ZEN, 2,000 µg/kg for AOH, AME, ENN A, B and BEA, 6,000 µg/kg for MPA. High contamination levels afforded to omit SAE stage in the sample preparation of infusion. Experiments were carried out for each kind of water with pH adjusted to 5 and 6 (using citric acid and/or NaOH solutions) in triplicate. Matrix-matched calibrations were used for quantification.

Table 1. Characteristics of HPLC-MS/MS detection of mycotoxins in the spiked sample and its infusion.¹

Mycotoxin	Found in the sample, µg/kg (CV, %)	Spike, µg/kg	Dry sample				Infusion		
			LOQ, µg/kg	SSE, %	DAS	SAE	LOQ, ng/ml	SSE, %	SAE_X5
					Rec., % (CV,%)	Rec., % (CV,%)			Rec., % (CV,%)
AOH	231 (14)	200	20	-44	89 (20)	90 (18)	1	-25	22 (20)
AME	105 (26)	200	20	-14	76 (14)	82 (20)	1	+7	11 (9)
TTX	10 (27)	80	4	-27	83 (5)	88 (7)	0.1	-13	70 (9)
STE	3.9 (11)	10	0.5	-20	70 (20)	73 (22)	0.02	-4	9 (24)
OTA	<LOD	10	2	-31	75 (9)	91 (12)	0.04	-3	82 (5)
ZEN	<LOD	200	40	-32	80 (6)	90 (8)	1	-10	17 (8)
MPA	663 (12)	500	50	-33	111 (13)	104 (8)	4	-14	69 (27)
ENN B	79 (22)	40	2	+22	66 (20)	110 (16)	0.08	-8	13 (19)
ENN A	4.6 (19)	40	2	+3	60 (18)	82 (15)	0.08	-7	5 (19)
BEA	10.7 (19)	40	1	+12	69 (15)	92 (15)	0.04	+5	<LOQ (<4)

¹ AME = alternariol methyl ether; AOH = alternariol; BEA = beauvericin; CV = coefficient of variation; DAS = dilute and shoot; ENN A = ennatiin A; ENN B = ennatiin B; LOD = limit of detection; LOQ = limit of quantification; MPA = mycophenolic acid; OTA = ochratoxin A; SAE = salt assisted extraction; SSE = signal suppression/enhancement; STE = sterigmatocystin; TTX = tentoxin; ZEN = zearalenone.

Preparation of real samples

For preparation of dry samples, 50 g of dry herbal tea was milled to a powder. 1.0 g of tea powder was weighted into 50 ml falcon tube and ultrasonicated in 10 ml of 1% formic acid for 10 min. After swelling, 10 ml of acetonitrile were added. The mixture was subjected to subsequent agitation, ultrasonication and final agitation for 10 min each. Next, 1 g of NaCl and 4 g of anhydrous MgSO_4 (in two portions) were added to the remaining mixture in the falcon tube. Each time the sample was vortexed for about 10 s. Finally, the tube was centrifuged at $25,000\times g$ for 15 min. An aliquot (0.75 ml) of the upper layer was diluted twice with MilliQ water, centrifuged and transferred into the chromatographic vial.

The infusion was prepared according to the label instruction: 'to prepare one portion of infusion 200 ml of boiling water should be added to 4 g of dry herbal tea; infusion is allowed to stay for 30 min.' Thus, the sample was thoroughly mixed, large particles and berries were distributed equally between four 4.0 g portions (photos of several samples are available in Supplementary Figure S2). Each of them was mixed with 200 ml of distilled water heated in thermostat to 99.9°C and left for 30 min. The infusion was paper filtered. Its 10 ml portion was mixed with 10 ml acetonitrile and acidified with 0.1 ml formic acid. Next, 1 g of sodium chloride and 4 g of anhydrous magnesium sulphate (in two portions) were added. The mixture was vortexed for 10 s each time after salt addition and centrifuged at about $25,000\times g$ for 15 min. 5 ml portion of the acetonitrile fraction was evaporated to dryness and reconstituted with 0.5 ml of mobile phase B, followed by 0.5 ml of mobile phase A. After centrifugation at about $25,000\times g$ for 15 min, the supernatant was transferred into the chromatographic vial.

Sample preparation of dry samples and their infusions was carried out in four replicates. Analytes were quantified by the post-extraction standard addition method. Five-point calibration has been carried out by the addition of L1-L4 standard solutions to dry sample extracts. Four-point calibration (L1-L3) has been used for quantification mycotoxins in infusions. Extracts without spiking were considered as 'zero'. The analyte concentration in the sample was calculated as a ratio of its analytical signal to calibration curve slope.

Method verification

Detection of mycotoxins in the dry sample

DAS and QuEChERS based sample preparation procedures are widely used for extraction of mycotoxins from plant commodities. DAS is a simple one-stage procedure. Water-acetonitrile acidified with acetic or formic acids ($\sim 15\text{-}33/85\text{-}66/1\text{-}2\%$ v/v) provides over 60% recovery

of a wide spectrum of mycotoxins (Da Silva *et al.*, 2019; Juan *et al.*, 2016; Medina *et al.*, 2019; Romera *et al.*, 2018; Sulyok *et al.*, 2007). QuEChERS is a good alternative for DAS, especially for the analysis of matrixes rich in co-extractives and in case analyte enrichment is essential for proper method performance (Perestrelo *et al.*, 2019). The original QuEChERS is a two-step procedure involving analyte extraction and salting-out into acetonitrile phase induced by partitioning salts, followed by its clean-up by dispersive solid-phase extraction. The clean-up stage is difficult to optimise for multi-analyte methods. SAE omits the second step of QuEChERS, tacking benefits of the first one (Bessaire *et al.*, 2019; Juan *et al.*, 2017; Song *et al.*, 2013).

In the present study, the sample preparation was based on the procedures proposed by Veprikova *et al.* (2015) for the determination of mycotoxins in herbal supplements and by Bessaire *et al.* (2019) for mycotoxins in green coffee. In brief, these procedures were as follows: ground samples were treated by acidified water-acetonitrile (1/1, v/v), followed by QuEChERS salts-induced phase separation and acetonitrile layer clean-up by hexane defatting or dispersive or cartridge solid-phase clean-up. Validation revealed high recovery of a wide range of mycotoxins; it exceeded 80% for the majority of analytes considered in the present study. We have carried out SAE of dry herbal tea samples and their infusions without any clean-up steps. Sample preparation effectiveness was verified for sample No 3 and its infusion. It was additionally spiked at the levels close to those of studied real samples.

Recoveries, ESI MS/MS signal suppression/enhancement (SSE, matrix effect) and limits of quantification (LOQs) were evaluated using solvent- and matrix-matched calibrations (Table 1). Matrix effect was calculated as the difference between calibration curves slopes: $\text{SSE, \%} = 100 \times [(\text{the slope of the matrix-matched calibration curve} / \text{the slope of the solvent-matched calibration curve}) - 1]$ (Matuszewski, 2006). LOQ was estimated as the concentration of the analyte, providing a signal to noise ratio (S/N) = 5. Recovery was calculated as a ratio of the difference in mycotoxin concentration in spiked and original sample to the spiking level.

Effectiveness of used partitioning salts was tested for bare spike without matrix and spiked samples. Organic- and water-rich phases separated in accordance with the initial proportion of water and acetonitrile when tested without matrix. 82-107% of spiked mycotoxins were detected in acidified water-acetonitrile (1:1, v/v) solution and 95-108% in the acetonitrile phase after salting-out. Thus, SAE resulted in complete transfer of analytes into the acetonitrile phase. It was difficult to evaluate the phase separation proportion in the presence of the matrix. Assuming it did not change, SAE provided 73-110% recovery for studied mycotoxins from the spiked herbal matrix, while DAS 60-

111%. The most noticeable improvement was for the least polar ENNs and BEA. More hydrophilic analytes, like TTX, AOH and MPA, were transferred from the matrix into solution within DAS and SAE procedures almost equally. SAE has been chosen as a final procedure because for the majority of studied mycotoxins, it proved to be beneficial.

Both sample preparation procedures of herbal tea sample – DAS and SAE – yielded similar signal suppression/enhancement (SSE) values. It exceeded 20% for seven of ten studied analytes, signal suppression prevailed. Diversity of studied herbal tea samples composition made universal matrix-matched calibration impossible. Post-extraction standard addition method allowed reliable quantification.

Detection of mycotoxins in the infusion of spiked model herbal matrix

Stability of mycotoxins within the infusion preparation procedure and their ability to transfer into the water was evaluated for bare spike without matrix. No dramatic loss occurred; only for ENN A and BEA about 20% decline was noticed. So, in the absence of matrix, almost all mycotoxins from the spike readily transferred into water infusion. It should be noted that this is valid only for the examined concentrations of mycotoxins in the spike. Our latter experiments showed that its 50-fold increase resulted in lower recoveries (70-85% for most of mycotoxins, and about 40% for ENN B and BEA).

SAE has been chosen as a sample preparation procedure for the determination of mycotoxins in herbal tea infusions. Prior to HPLC-MS/MS analysis, extracts were concentrated five-fold. Matrix effect decreased, but for AOH SSE remained over 20%. It indicates that competing co-eluting matrix components also transferred into the infusion. Transfer of spiked mycotoxins into infusion differed much. It was up to 70% for TTX and 82% for OTA. About 30% of ZEN and AOH and 9-20% of AME, STC, ENN B and MPA were detected in the infusion. Transfer of ENN A and BEA appeared to be ≤5%.

The effect of water characteristics on mycotoxins transfer

To study the effect of different kinds of water on transfer rate of mycotoxins infusions were prepared from spiked 'clean' matrix using distilled (DW), deep-well (D-WW) and natural mineral water (NMW) with pH adjusted to 5 and 6. Tested water differed in amount of dissolved inorganic salts (ionic strength). Due to organic acids and bases present in tea leaves, pH of resulting infusions changed. It proved to be 5.54 (CV=1.0%); 5.39 (CV=0.3%) and 5.47 (1.3%) for corresponding kind of water adjusted to pH 5 and 5.88 (CV=0.5%); 6.18 (CV=0.6%) and 6.41 (1.2%) for water adjusted to pH6. Studied water parameters did not change transfer rate much (Figure 1).

Excluding ENN B, maximum variation of transfer was noted for AOH – 17%. For TTX, MPA and OTA it did not exceed 11%. It is compatible with repeatability of transfer rate for TTX (12%). Basing on these results and ISO 3103:2019 'Tea — Preparation of liquor for use in sensory tests' further experiments were carried out using distilled water.

3. Results

Transfer of mycotoxins into infusion from naturally contaminated herbal tea

The most contaminated herbal tea samples were chosen for the evaluation of mycotoxins transfer into infusion basing on screening results (Kiseleva *et al.*, 2020). The major mycotoxins found in the selected naturally contaminated herbal tea samples were MPA, STE, ZEN, *Alternaria* toxins and emerging mycotoxins ENNs and BEA (Table 2).

Metabolites of *Penicillium* spp. (MPA: 0.1-1.9 µg/kg), *Alternaria* spp. (AOH: 0.06-0.26 µg/kg, AME: 0.05-0.2 µg/kg and TTX: 4-17 µg/kg), *Fusarium* spp. (ENNs 20-117 µg/kg, BEA: 5-15 µg/kg) and *Aspergillus* spp. (STE: 4-28 µg/kg) were detected in all samples originating from the Krasnodar region (No. 2-7). Bagged herbal tea of the global manufacturer (No 1) contained about 4 µg/kg of *Penicillium* metabolite OTA, 6 µg/kg of STE and about 50 µg/kg of BEA. Microbial contamination of studied herbal teas was examined by Minaeva *et al.* (2019). Content of fungi in loose herbal tea samples exceeded 5×10^3 colony forming units (cfu)/g reaching 10^6 cfu/g. It was much over MLs set for moulds in medicinal herbs in the Russian Federation (10^3 cfu/g; TR CU, 2011). There were identified representatives of several fungal genera: *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Cladosporium* spp. Toxinogenicity tests revealed fumonisins (FBs), ZEN, STE, AME, MPA, ENN B and BEA as major metabolites of extracted fungal strains *in vitro* (Kiseleva *et al.*, 2020). The present survey revealed only trace amounts of FBs in the studied samples. Maximum FB₂ content was detected in sample No. 4 and was about 3.5 µg/kg. The inconsistency of toxinogenicity test results with contamination levels of FBs in the real samples may be due to competition of fungi and spore-forming bacteria. The latter were detected in herbal tea samples, and concentration was up to 2×10^8 cfu/g (Minaeva *et al.*, 2019).

The transfer of mycotoxins into infusion proved to be dependent on infusion pH and mycotoxins' polarity (Figure 2A). Excluding OTA and MPA, studied mycotoxins do not possess ionogenic moieties. Their transfer into infusion was in inverse proportion to their hydrophobicity (logP(o/w)) computed using EPIWEB 4.1 program (EPI Suite; US EPA, Washington, DC, USA). The most polar TTX transferred into infusion at the rate of 59-84%, average transfer 73 (±17%). This matches the result obtained for the spiked

Figure 1. The effect of water characteristics on the transfer rate. AOH = alternariol; BEA = beauvericin; D-WW = deep-well water; DW = distilled water; ENN B = enniatin B; MPA = mycophenolic acid; NMW = natural mineral water; OTA = ochratoxin A; STE = sterigmatocystin; TTX = tentoxin; ZEN = zearalenone. Repeatability of transfer is indicated by vertical bars.

samples (70%, Table 1 and Figure 1). We should note poor repeatability of TTX and STE detection in the sample No 5, though all samples were thoroughly mixed and grounded. The resulting transfer rate was not considered.

AOH transfer varied from 28 to 61%. Strong signal suppression hindered the detection of AOH in infusions: we managed to determine AOH in infusions of only three of six contaminated samples. Its transfer into infusions from spiked samples varied in the range from 12 to 22%. AME signal was less affected by matrix co-eluting compounds in the infusions of studied samples; SSE did not exceed 23%. However, it was not detected in infusions at over LOQ concentrations. Thus, its transfer can be evaluated as less than 26% (calculated from the highest concentration in the studied samples and LOQ value), which is in accordance with 11% transfer from the artificially contaminated sample (Table 1). Transfer rate of STE from naturally contaminated tea varied from 7 to 13%; it was about 10% for spiked samples. Only about 4% of the most hydrophobic BEA and ENN B were found in the infusion. The brewing of bare spike proved that in the absence of matrix, all studied mycotoxins transfer into infusion completely, only ENNs and BEA transferred at the rate of 80%. We can assume that solute-matrix interaction is the main factor affecting the transfer rate.

OTA and MPA are weak acids with pKa about 3.2 (PubChem Database, 2020) and 4.5 (Haynes, 2016) correspondingly; their transfer into infusion is pH dependent. The only sample (No 1), naturally contaminated with OTA, demonstrated 83% transfer of this mycotoxin into the infusion. The same result was obtained for the artificially contaminated herbal tea sample. The corresponding infusions pH were 5.7 and 5.5. This is about two units higher than the pKa of the OTA carboxylic group. Under these conditions it is almost completely dissociated, providing a high transfer rate. However, while testing effect of water characteristics on OTA transfer from spiked green tea matrix transfer rate appeared to be about 60% for infusions with pH from 5.4 to 6.4. MPA was detected in six of seven tested samples at the levels, affording its easy detection in infusions even without preconcentration of infusion extract. Six values of transfer rate were obtained, and they varied in the range from 23 to 96% (Table 2). Correlation of transfer rate with infusion pH was described by quadratic function ($R^2=0.96$).

Health risk evaluation

It was interesting to evaluate the health risk associated with mycotoxins in the studied herbal tea samples. We have compared daily intakes, assuming the complete transfer of mycotoxins into an infusion and taking into account real transfer rates. The following scenario was considered:

Table 2. The concentration of mycotoxins in herbal tea samples and their infusions.¹

Mycotoxin	Sample	1	2	3	4	5	6	7
MPA	Dry sample, µg/kg (CV, %)	<LOQ ²	392 (7)	663 (12)	1,871 (18)	1,623 (16)	1,285 (16)	124 (13)
	Infusion, µg/l (CV, %)		3.9 (34)	4.5 (26)	8.6 (23)	23.8 (19)	22 (24)	3.8 (29)
	Transfer, %		49	34	23	73	86	96
OTA	Dry sample, µg/kg (CV, %)	4.2 (21)	<LOD ²	<LOD	<LOD	<LOD	<LOD	<LOD
	Infusion, µg/l (CV, %)	0.07 (26)						
	Transfer, %	83						
TTX	Dry sample, µg/kg (CV, %)	3.8 (14)	9.2 (20)	10.3 (27)	10.7 (26)	17.1 (43)	7.0 (21)	4.7 (32)
	Infusion, µg/l (CV, %)	<LOQ	0.12 (11)	0.12(24)	0.18 (34)	0.27 (8)	<LOQ	0.08 (19)
	Transfer, %		65	59	84	{78} ³		83
AOH	Dry sample, µg/kg (CV, %)	<LOD	170 (28)	231 (14)	61 (13)	258 (27)	210 (11)	116 (9)
	Infusion, µg/l (CV, %)		2.1 (27)	<LOQ	<LOQ	2.3 (26)	<LOQ	0.7 (13)
	Transfer, %		61	–	–	45	–	28
AME	Dry sample, µg/kg (CV, %)	<LOD	68 (29)	105 (26)	196 (17)	96 (15)	76 (11)	53 (5)
	Infusion, µg/l (CV, %)		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Transfer, %		–	–	<26	–	–	–
STE	Dry sample, µg/kg (CV, %)	6.0 (6)	27.6 (13)	3.9 (11)	15.3 (16)	16.3 (49)	8.4 (11)	16.5 (18)
	Infusion, µg/l (CV, %)	<LOQ	0.06 (34)	<LOQ	0.02 (29)	0.06 (53)	<LOQ	0.04 (32)
	Transfer, %	–	11	–	7	{30}	–	13
ZEN	Dry sample, µg/kg (CV, %)	<LOD	104 (24)	<LOD	127 (17)	<LOD	192 (20)	<LOD
	Infusion, µg/l (CV, %)		<LOQ		<LOQ		0.95 (38)	
	Transfer, %		–		–		25	
ENN B	Dry sample, µg/kg (CV, %)	<LOD	20 (37)	79 (22)	45 (8)	112 (15)	27 (12)	82 (32)
	Infusion, µg/l (CV, %)		<LOQ	0.11 (19)	<LOQ	0.06 (16)	<LOQ	0.07 (24)
	Transfer, %		–	7	–	4	–	4
ENNA	Dry sample, µg/kg (CV, %)	<LOD	<LOD	4.6 (19)	<LOQ	4.7 (29)	1.8 (16)	4.5 (15)
	Infusion, µg/l (CV, %)			<LOQ		<LOQ	<LOQ	<LOQ
	Transfer, %			–		–	–	–
BEA	Dry sample, µg/kg (CV, %)	53.5 (15)	10.1 (17)	10.7 (19)	14.5 (21)	12.6 (34)	10.7 (14)	5.2 (26)
	Infusion, µg/l (CV, %)	0.07 (22)	<LOQ	<LOQ	0.02 (24)	<LOQ	<LOQ	<LOQ
	Transfer, %	6	–	–	6	–	–	–
Infusion pH: average (CV, %)		5.7 (0.9)	5.6 (1.1)	5.5 (1.0)	5.5 (1.3)	5.9 (0.9)	5.9 (0.9)	6.3 (1.2)

¹ AME = alternariol methyl ether; AOH = alternariol; BEA = beauvericin; CV = coefficient of variation; ENNA = ennatin A; ENN B = ennatin B; LOD = limit of detection; LOQ = limit of quantification; MPA = mycophenolic acid; OTA = ochratoxin A; STE = sterigmatocystin; TTX = tentoxin; ZEN = zearalenone.
² LOD and LOQ were estimated for each matrix as 3S/N (signal/noise ratio) and 5S/N, respectively.
³ Transfer calculated from data characterised by poor repeatability was put in braces; it was not used for calculation of average transfer.

the highest detected in the present study concentration of each mycotoxin. Intakes were calculated for an adult person (body weight 60 kg) consuming one 200 ml cup of herbal tea (corresponds to 4 g of herbal tea) per day (Table 3). This volume corresponds well with assessment of chronic consumption of herbal and other non-tea infusions in the EU (Supplementary Table S3): median values for the ‘consumers only’ category vary from 191.6 to 210.8 g/day for adults, elderly and very elderly persons (Comprehensive European Food Consumption Database, EFSA, 2020). Input of herbal teas into dietary exposure to mycotoxins for children is higher. Median consumption by toddlers and infants is approximately half as low, while body weight is 5-12 times lower.

Human exposure to OTA, ZEN, STE, *Alternaria* toxins, ENNs, BEA and MPA through consumption of model herbal tea has been compared with available limits or average dietary exposure: provisional tolerable daily intake (PTDI) set for ZEN and its modified forms; low concern exposure level set in the EU for STE; mean chronic dietary exposure to OTA, *Alternaria* toxins, ENNs and BEA, calculated by EFSA for the EU. In the absence of any other benchmark, baseline daily therapeutic dose value was used for MPA. MPA and its derivatives are used in transplantation as an immune-suppressing agent. Its baseline daily therapeutic dose is 2 g (Doria *et al.*, 2012), that is equal to 33 µg/kg bw/day for 60 kg person.

Figure 2. (A) Correlation of mycotoxins transfer from herbal tea into the infusion with their polarity. (B) The effect of infusion pH on the transfer rate of MPA. AOH = alternariol; BEA = beauvericin; ENN B = enniatin B; MPA = mycophenolic acid; OTA = ochratoxin A; STE = sterigmatocystin; TTX = tentoxin; ZEN = zearalenone.

OTA was found in the only one studied herbal tea. It was bagged tea, containing aromatic herbs, ginger and liquorice root. The latter two components are the most probable OTA sources. There are maximum levels for OTA set by EFSA and European Pharmacopeia in these food categories (liquorice root for herbal infusions – 20 µg/kg, ginger – 15 µg/kg (Altyn and Twaruzek, 2020)). 4.2 µg/kg of OTA in this herbal tea corresponds to an intake of 1.6% of mean chronic dietary exposure. Transfer rate of OTA into infusion is pH dependent. In our study, it was over 80%, the bulk of toxin transferred into the infusion.

Alongside with OTA, MPA transfer rate is also affected by infusion pH. In the pH range from 5.5 to 6.3, it increased from 23 to 96%. In any case, the most contaminated herbal tea infusion could provide only 0.4% of baseline therapeutic dose. It is obvious that the comparison of dietary and therapeutic intakes is not proper. However, the occurrence

of MPA in food at the levels exceeding usual for other mycotoxins and its extensive use in medicine let Gruber-Dorninger *et al.* (2017) assume MPA should not be of any dietary concern.

Unlike OTA and MPA, ZEN and STE average transfer into infusion was about 30 and 10%, respectively. Actual input of infusion into the daily intake was three and ten times lower compared to that calculated from contamination levels of dry tea. It did not exceed 1.0% of PTDI for ZEN and 1.6% of low concern exposure level for STE.

Similar results were obtained for ENNs and BEA. Transfer of these mycotoxins into infusion was low and varied in the range of 2-7%. Contamination levels in studied samples and their infusions were much lower than average daily exposure.

Table 3. Comparison of dry herbal tea and its infusion input into dietary exposure to mycotoxins.¹

Mycotoxin	Dry tea			Infusion		Benchmarks of exposure
	Max contamination, µg/kg	Daily intake ² , ng/kg bw/day	Input ³ , %	Transfer, %	Input ³ , %	
OTA	4.2	0.28	1.6	83	1.3	Mean chronic dietary exposure ⁴ 17.79 ng/kg bw/day (Schrenk <i>et al.</i> , 2020)
ZEN	127	8.4	3.4	30	1.0	PTDI: 250 ng/kg bw/day (Knutsen <i>et al.</i> , 2016)
STE	27.6	1.8	11.5	7-13	0.8-1.6	Low concern exposure: 16 ng/kg bw/day (EFSA, 2013)
TTX	17.1	1.1	8.5	59-84	5-7	Mean chronic dietary exposure ⁴ 13 ng/kg bw/day (Arcella <i>et al.</i> , 2016)
AOH	258	17.2	115	28-61	32-70	15 ng/kg bw/day (Arcella <i>et al.</i> , 2016)
AME	196	13	130	11	14	10 ng/kg bw/day (Arcella <i>et al.</i> , 2016)
ENN A	4.7	0.3	0.4	5	<0.03	1,820 ng/kg bw/day (EFSA, 2014)
ENN B	112	7.5		2-7		
BEA	53.5	3.6	7	4-6	0.3-0.4	50 ng/kg bw/day (EFSA, 2014)
MPA	1,871	125	0.4	23-96	0.1-0.4	Baseline daily therapeutic dose 33 µg/kg bw/day

¹ AME = alternariol methyl ether; AOH = alternariol; BEA = beauvericin; CV = coefficient of variation; ENN A = enniatin A; ENN B = enniatin B; LOD = limit of detection; LOQ = limit of quantification; MPA = mycophenolic acid; OTA = ochratoxin A; PTDI = provisional tolerable daily intake; STE = sterigmatocystin; TTX = tentoxin; ZEN = zearalenone.

² Assuming the complete transfer of mycotoxins into the infusion.

³ The input into tolerable/low concern/average dietary intake or therapeutic dose (for MPA).

⁴ The estimated mean chronic dietary exposure in the adult population across dietary surveys, using an upper bound approach.

Only *Alternaria* toxins found in the studied herbal tea samples provided noticeable input into average dietary exposure. These toxins are associated with fruits and vegetables, cereals and oil crops (Arcella *et al.*, 2016; Crudo *et al.*, 2019; Da Cruz Cabral *et al.*, 2016; EFSA, 2011; Estiarte *et al.*, 2018; Gambacorta *et al.*, 2018; Puntischer *et al.*, 2019). Few surveys are reporting the high occurrence of *Alternaria* toxins in herbs and spices (Gambacorta *et al.*, 2019; Vepríkova *et al.*, 2015). To carry out the present study, we have preliminarily selected the most heavily contaminated herbal tea samples. The concentration of AOH and AME was in the range 50-260 µg/kg; TTX varied from 4 to 17 µg/kg. Contamination levels were of the same order as reported in the literature for herbs. Maximum detected concentrations of *Alternaria* toxins in the dry tea provided 8.5, 115 and 130% of the mean chronic dietary exposure in the adults calculated using upper bound approach level for TTX, AOH and AME, respectively. Considering the transfer of these mycotoxins into the infusion, one cup of herbal tea input decreased about ten-fold for AME; changed to 32-70% for AOH. It changed a little for TTX. AOH and AME proved to be the most noteworthy among the studied fungal metabolites in the pre-selected contaminated herbal tea samples. They provided the highest impact into dietary exposure in comparison with other mycotoxins.

4. Conclusions

Transfer of OTA, STE, MPA, TTX, AOH, AME, ZEN, ENNs and BEA into infusion was studied for seven herbal tea samples. It proved to be dependent on mycotoxins polarity and infusion pH for mycotoxins possessing acidic groups. No difference in transfer rate arose from TDS characteristics of water. STC transferred into infusion at the average rate of 10%. Average transfer of *Alternaria* toxins varied from 73% (TTX) to 45% (AOH) and about 11% (AME). The third part of ZEN was detected in the infusion. Transfer of ENNs and BEA was low and did not exceed 7%. Infusion pH affected MPA transfer rate; it increased from 23% to 96% in the pH range from 5.5 to 6.3. 83% of OTA was detected in the infusion of the single contaminated sample. Accounting for transfer rates of mycotoxins from tea into infusion resulted in decreasing of the input of most mycotoxins into dietary exposure. The decline was minimal for OTA and TTX. *Alternaria* toxins provided the highest impact into dietary exposure in comparison with other mycotoxins detected in selected herbal tea samples.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/wmj2020.2606>.

Figure S1. Separation of mycotoxins in dry herbal tea extract (sample No 5).

Figure S2. Herbal tea samples.

Table S1. Comparison of mycotoxins retention on PFP and ODS stationary phases.

Table S2. Retention and SRMs of studied analytes.

Table S3. Mean consumption of herbal and other non-tea infusions in the EU according to data presented in EFSA Comprehensive European Food Consumption.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to the reviewers for valuable comments and suggestions. This research was funded by Russian Science Foundation grant number 18-16-00077 'Emergent mycotoxins in food of plant origin: development of methods of determination, survey of occurrence, species-specific characterisation of toxigenic moulds, creation of regulatory'.

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