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STUDYING THE CONTAMINATION OF TEA AND HERBAL INFUSIONS WITH MYCOTOXINS (MESSAGE 2)

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The authors performed screening of a wide range of mycotoxins by ultra-high-performance liquid chromatography combined with tandem mass spectrometry (UHPLC-MS/MS) in various tea products distributed on the RF market. Samples were selected in retail outlets and obtained from wholesalers. Seventy-seven tea samples were examined: 54 out of them were Camellia sinensis tea, not packed (semi-finished product) and packed; 23 were mono- and multi-component herbal tea. The analytes were 29 mycotoxins including regulated in food products (aflatoxins, ochratoxin A, deoxynivalenol, fumonisins, T-2 toxin and zearalenone), their derivatives and structural analogues (A and B trichothecenes, structural analogues of zearalenone); emergent mycotoxins (sterigmatocystin, mycophenolic acid, moniliformin, enniatins, beauvericin and Alternaria toxins). C. sinensis tea samples, both green and black, were the least contaminated. In contrast, multi-component herbal tea samples tended to be simultaneously contaminated with several mycotoxins (over five) both regulated in food products and emergent ones. Beauvericin, mycophenolic acid and enniatin B were the most frequently detected. Toxigenic properties of mixed tea microflora were examined in vitro. Model experiments were carried out on a substrate consisting of C. sinensis green tea leaves in the absence of any growth factors. Mixed mycoflora from tea, which contained potentially toxigenic species of mold species proved to be capable to simultaneously produce substantial quantities of several mycotoxins including emergent ones. Mycotoxins accumulation amounted to 290 and 5,600 μg/kg of fumonisins B1 and B2 accordingly; 130 μg/kg of zearalenone; 14 μg/kg of sterigmatocystin; 160 μg/kg of alternariol methyl ester. The present survey indicates there is a potential health risk associated with mycotoxins in teas, especially herbal ones. The systematic study of contamination of tea products distributed in the RF with mycotoxins and their producers has been performed for the first time. Long-term monitoring over variety of mycotoxins in this kind of food products is essential for assessing its safety.

Keywords: *mycotoxins, emergent mycotoxins, C. sinensis tea, herbal tea, UHPLC-MS/MS, mycotoxins producers, mycotoxins occurrence in vitro.*

Mycotoxins (MTs) are secondary metabolites of mold fungi. They are globally recognized food contaminants, which affect its safety for the customers. According to the Food and Agricultural Organization (FAO), approximately 25 % of food and feed worldwide is contaminated with MTs [1]. Toxic fungal metabolites produce a wide range of adverse effects on human health, starting from immune suppression and up to carcinogenesis.

Reduction of risks associated with MTs in food is a vital task of health preservation. To solve it, leading world mycologists and toxicologists adopted the Mycotox Charter (chrter.mycokey.eu) calling for responsibility borne by the present generation for developing and implementing solutions aimed at minimizing MTs exposure worldwide and securing enhanced food safety for future generations [2]. Mold fungi are widely spread and are almost

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inevitable in plant commodities. Toxins can be produced both during vegetation and after harvesting, at any stage in a technological chain (storage, processing and transportation). Contents of the most hazardous MTs (deoxynivalenol (DON), T-2 toxin, zearalenone (ZEA), ochratoxin A (OTA), aflatoxin B1 (AFL B1), fumonisins (FBs)) in food are regulated in most countries. The Technical Regulations of the Customs Union CU TR 021/2011 "On food products safety"[1] and CU TR 015/2011 "On the safety of grains"[2] are the principal regulations in the Russian Federation (RF). There are also about forty state standards and guidelines describing analytical procedures based on up-to-date analytical technologies (ELISA (enzyme-linked immune-sorbent assay), HPLC and HPLC-MS) developed for mycotoxins determination in food.

HPLC-MS/MS provides opportunities for selective and sensitive determination of miltiple contaminants simultaneously. It is widely used for monitoring over broad spectrum of fungal metabolites [3–6]. Their list is being constantly enriched. There are analytical procedures validated for the determination of regulated MTs and their structural derivatives (3- and 15-acetyl deoxynivalenol, nivalenol, fusarenone X – DON group (trichothecenes B); HT-2 toxin, T-2 triol, diacetoxyscirpenol – T-2 toxin group (trichothecenes A); aflatoxins B2, G1, G2, sterigmatocystin (STC) – AFL B1 analogues; zeranol, taleranol, α- and β-zearalenol – ZEA analogues), *Alternaria* MTs (alternairol (AOH), its methyl ester (AME), altenuene, tentoxin (TEN)), as well as 'emergent' MTs (EMTs: enniatins A and B (Enn A and B), beauvericin (BEA)) [7–10]. The number of surveys concerning the occurrence of non-regulated MTs in plant commodities and food products is increasing. Meanwhile, the toxic effects produced by these MTs and their combinations are explored insufficiently. Data on combined chronic dietary exposure to small doses of MTs are insufficient. This determines the relevance of extensive research on plant products that belong to mass consumption segment including tea.

Traditional *C. sinensis* tea is a popular drink all over the world; it is usually associated with beneficial health effects. However, subtropics favorable for tea cultivating, are also suitable for mold fungi development and toxins production. Concentrations of FBs, OTA, AFLs, ZEA, trichothecenes and citrinin detected in tea were reported at dozens and even hundreds of μg/kg. Literature data on analytical methods used for the determination of MTs in tea, their occurrence and legislation were summarized in [11]. At present, only AFL B1 or total AFLs in tea are subject to regulation in some countries. Maximum levels (MLs) for these toxins were set in Argentina, India, Sri-Lanka, Japan and China. MLs vary from 5 to 30 μg/kg. Five μg/kg is set as ML for AFL B1 in dry tea at in the CU countries (Russia, Kazakhstan, Belorussia, Armenia, Kirgizia) (CU TR 021/2011[1]).

Herbal teas are becoming increasingly popular. They often contain medicinal herbs (mint, chamomile, nettle, liquorice, wild dog-rose etc.) and are specified by manufacturers as "health-related products". However, there are reports on the detection of MTs and their producers in foodstuff of herbal origin. AFLs, OTA and *Alternaria* toxins were detected in herbal food supplements of similar composition [12].

In previous studies, the authors obtained data on the levels and frequency of mold contamination of more than 50 tea samples (black and green *C. sinensis* tea, herbal tea) [13]. The maximum levels of contamination in all *Camellia* sp. tea samples were due to fungi of the *Aspergillus* genus, among which the *Aspergillus* species of the *Nigri* section dominated. These species were the major fungi found in herbal teas. Alongside *Penicillium sp., Alternaria sp., Fusarium sp.,* and *Cladospo-*

[1] CU TR 021/2011. On food products safety: The Technical Regulations of the Customs Union / approved by the Decision by the Customs Union Commission on December 9, 2011 No. 880. *KODEKS: the electronic fund for legal and reference documentation*. Available at: http://docs.cntd.ru/document/902320560 (20.11.2019).

[2] CU TR 015/2011.On safety of grains (last edited on September 15, 2017): The Technical Regulations of the Customs Union / approved by the Decision by the Customs Union Commission on December 9, 2011 No. 874. *KODEKS: the electronic fund for legal and reference documentation*. Available at: http://docs.cntd.ru/document/902320395 (20.11.2019).

rium sp. were also detected. Certain species from this group are able to produce toxins. Thus MTs synthesized by those mold fungi can be found in herbal infusions. An issue related to microbiological regulations concerning mold fungi established for tea and the possibility of changing them in order to harmonize national standards with international requirements is being discussed in the RF at present. So far, there has been no full-scale study on the occurrence of wide range of MTs in *C. sinensis* and herbal tea distributed on the RF market. The present research is aimed at filling this gap.

Our research goals were (1) to screen *C. sinensis* and herbal tea for a wide range of MTs: regulated (AFLB1, B2, G1, G2; OTA, DON, FB1, FB2, T-2, ZEA), their derivatives and structural analogues (DAS, T-2 triol, NeoS, HT-2 toxin; 3- and 15-AcDON, NIV, FusX; α–, β–ZEL,α–, β–ZAL), and emergent MTs (STC, MPA, MO, EnnA and B, BEA, AOH и AME, TEN). (2) To reveal possible correlations between MT contents and fungal contamination of studied tea samples (3) we also examined the toxigenic properties of tea microflora *in vitro* under conditions as close to real as it was only possible.

Samples: We examined 77 tea samples. 30 samples were semi-finished (not packed) traditional black and green teas (*C. sinensis)* from six tea-producing regions (Vietnam, India, Indonesia, Kenya, China and Sri-Lanka). They were obtained from wholesalers. 22 samples were traditional packed green and black teas (*C. sinensis*) and teas with additives. Two samples were Pu'er tea. 23 samples were herbal tea: monocomponent ones: Sudan rose *(Hibiscus sabdariffa),* fermented Ivan-tea (*Epilobium angustifolium*), thyme *(Thymus serpyllum),* mint *(Menthae piperita)* and chamomile *(Chamomilae vulgaris)* and multi-component ones. The composition of the latter is presented in Table 1.

Methods: *29 MTs were detected* by UHPLC-MS/MS consisting of UHPLC Vannquish system (equipped with a binary pump, autosampler, and thermostat) combined to triple quadrupole mass-spectrometer with heated electrospray source TSQ Endura conducted by Xcalibur 4.0 QF2 Software (all Thermo Scientific, the USA).

Table 1

Composition of multi-component herbal tea samples

Sample No.	Components
2	Ivan-tea (*E. angustifolium*), currant (*Ríbes sp.*)
8	Hawthorn berries (*Crataegus sp.),* white mistletoe leaves *(Víscum álbum*), melilot (*Melilótus officinális*), motherwort (*Leonurus cardiaca*), valerian root (*Valeriana officinalis*)
9	Echinacea **(***Echinácea purpúrea*), origanum (*Oríganum vulgáre*), brandy mint (*Méntha piperíta*), nettle (*Urtíca sp.*), thyme *(Thymus serpyllum),* chamomile *(Chamomilae vulgaris),* wild rose (*Rōsa sp.*), sage (*Sālvia officinālis*), violet (*Víola sp.*), licorice (*Glycyrrhíza sp.*)
10	Thyme *(Thymus serpyllum),* St. John's wort (*Hypericum perforatum*)
11	Chamomile *(C.vulgaris),* brandy mint (*M.piperíta*), everlasting leaves (*Helichrysi arenarii*), tansy flowers (*Tanacetum sp.*), coriander berries (*Coriándrum sátivum*), holy-thistle (*Sílybum mariánum*), mint (*Méntha sp.),* melilot (*M.officinális*), wild rose berries (*Rōsa sp.*), inula root (*Inula sp.*), hawthorn berries (*Crataegus sp.)*
12	Marigold (*Bídens tripartíta*), sarcarolla (*Caléndula sp.*), everlasting (*Agrimónia eupatória*), chamomile *(C.vulgaris),* St. John's wort (*H.perforatum*), birch leaves (*Betula pendula*)
14	Ivan-tea (*E. angustifolium*), cowberry (*Vaccínium vítis-idaéa*)
19	Ground lemon (*Cymbopogon citratus*), lemon myrtle (*Backhousia citriodora)*, ginger root (*Zīngiber officināle),* licorice root (*G.glábra*), dried lemon peel (*Cítrus límon)*
20	Chamomile *(C.vulgaris),* mint (*Méntha sp.)*
36	Ivan-tea (*E. angustifolium*), sea-buckthorn (*Hippóphaë sp.)*
38	Ivan-tea (*E. angustifolium*), linden (*Tília sp.*)

Analytes were separated on a reversed-phase column Titan C18, 2.1*100 mm, 1.9 μm (Supelco, PA, USA), under gradient elution. The mobile phases were constituted of: (A) methanol-water (10/90 % vol.); (B) methanol-water-acetonitrile (10/10/80 % vol.). Both

phases were modified with 1mM ammonium formate and 0.1 % formic acid. The gradient scheme was as follows: from the start to 1 min. - 0 % B; from 1 to 2 min. - a linear growth to 15 % B; from 2 to 5 min. - to 30 % B; from 5 to 13 min.- up to 70 % B; from 13 to 14 min. - 90 % B; from 14 to 16.5 min. - 95 % B; up to 17 min. - growth to 100 % B and then retention for 3 minutes; from 20 to 20.5 min. – a decrease down to 0 % B; equilibration – until 22 minutes. Mobile phase flow: 0.4 ml/min. The column temperature was set to 30 ºC. Injection volume: 2–4 µl. Run time: 22 minutes. Analytes were detected in the MRM mode. Limit of quantification (LOQ) was determined by 10σ criterion. Recovery was determined for spiked green tea. Summarized method parameters are provided in Table 2.

Neat standards of AFL B1, AFL B2, AFL G1, AFL G2, STC, T-2 and NT-2 toxins, DAS, NIV, DON, 3- and 15-AcDON, FusX, FB1, FB2, ZEA, α- and β-ZEL, α-ZAL, OTA were supplied by Sigma-Aldrich (Russia, Moscow). AOH, AME, BEA, EnnA, EnnB, MPA, MO, NeoS, T-2 triol, TE were obtained from Fermentek (Jerusalem, Israel). Stock standards solutions were prepared in acetonitrile (AFLs, STC, trichothecenes, ZEA and its analogues, OTA, MPA), methanol (*Alternaria* MTs, Enns, BEA, MPA, MO) or water-acetonitrile

Table 2

HPLC-MS/MS method characteristics summary

No.	MT	t_R, min	Parent ion, m/z		RF, V	Daughter ions, m/z (collision energy, V)	LOQ, µg/kg	Recovery, %
1	MO	0.7	$[M-H]^-$	97	54	**41* (12),** 80 (23)	800	76
2	NIV	1.8	$[M+H]^+$	313	100	**125 (10),** 177 (10)	1,000	81
3	DON	2.9	$[M+H]^+$	297	100	**203 (18),** 231 (12), 249 (11)	1,250	63
4	FusX	3.6	$[M+H]^+$	355	103	**175 (20),** 229 (16), 247 (12)	100	80
5	NeoS	3.9	$[M+NH_4]^+$	400	79	**185 (17),** 203 (17), 215 (18), 305 (10)	<10	75
6	AcDON	4.6	$[M+H]^+$	339	97	**203 (17),** 213 (18), 231 (13)	250	94
7	T-2 triol	4.8	$[M+NH_4]^+$	400	76	**215 (10),** 263 (13)	250	75
8	AFLG2	6.2	$[M+H]^+$	331	170	**189 (41),** 245 (30), 313 (24)	4	79
9	AFLG1	6.6	$[M+H]^+$	329	150	**200 (41),** 243 (26), 311 (21)	4	85
10	AFLB2	6.8	$[M+H]^+$	315	170	**243 (39),** 259 (29), 287 (26)	4	85
11	DAS	6.9	$[M+NH_4]^+$	384	89	**247 (14),** 307 (10), 349 (10)	20	95
12	AFLB1	7.3	$[M+H]^+$	313	166	**213 (45),** 241 (37), 285 (22)	4	85
13	AOH	8.0	$[M+H]^+$	259	100	**128 (44),** 184 (30), 213 (27)	1,000	87
14	HT-2	8.0	$[M+NH_4]^+$	442	91	**215 (10),** 263 (10)	500	77
15	FB1	8.0	$[M+H]^+$	772	217	334 (40), **352 (36)**	400	44
16	α-ZAL	8.3	$[M+H]^+$	323	66	**189 (22),** 305 (10)	125	76
17	TE	8.3	$[M+H]^+$	415	130	**302 (13),** 312 (19)	4	78
18	β-ZEL	8.5	$[M+H]^+$	321	88	**189 (20),** 303 (10)	1,000	88
19	MPA	8.9	$[M+H]^+$	321	113	**207 (22),** 275 (16)	50	101
20	α-ZEL	9.5	$[M+H]^+$	321	65	**189 (22),** 303 (11)	1,000	84
21	T-2	9.7	$[M+NH_4]^+$	484	137	**215 (17),** 305 (13)	10	100
22	FB2	9.7	$[M+H]^+$	706	150	**318 (36),** 336 (36)	100	73
23	OTA	10.4	$[M+H]^+$	404	123	**221 (35),** 239 (24), 358 (14)	2.5	78
24	AME	10.5	$[M+H]^+$	273	150	**185 (40),** 199 (40), 258 (30)	750	87
25	ZEA	10.5	$[M+H]^+$	319	90	185 (20), **283 (10),**301 (10)	150	86
26	STC	10.9	$[M+H]^+$	325	152	**253 (44),** 281 (36), 310 (24)	4	78
27	EnnB	15.1	$[M+NH_4]^+$	657	142	196 (30), 214 (31), 527 (27), **640 (17)**	2.5	73
28	BEA	15.5	$[M+NH_4]^+$	801	215	**244 (32),** 262 (30), 784 (17)	2.5	80
29	EnnA	16.2	$[M+NH_4]^+$	699	255	**210 (24),** 228 (24)	6	92

Note: daughter ions selected for quantitative analysis are given **in bold**. Method was verified for *C. sinensis* black tea.

(50/50 % vol.) (FB1 and FB2). Stock individual standard solutions were used for the construction of the multi-analyte standard solution. All the standards solutions were stored at -18 °C.

Dry tea sample preparation. A representative portion (10–20 g) of dried tea sample was ground. 1 g of tea powder was extracted with 10 ml acetonitrile-water-formic acid (80/20/0.5 % vol.) by shaking and ultra sonicating for 30 minutes total; centrifuged for 10 minutes at 10,000 rpm. 1 ml of supernatant was diluted with 1 ml of the mobile phase A. After mixing, the diluted sample was centrifuged. 1.5 ml of supernatant was transferred into a chromatographic vial for analysis.

Screening toxins production in vitro. The nutrient medium constituted of agar with streptomycin (200 mg/l) and 6 % of ground dried tea as an only substrate. We used microbiologically clean green tea (<10 CFU/g of mold fungi and bacteria), tested negative for MTs. The substrate was ground in a mill with sterile disposable grinding chambers and then aseptically added to melted hungry agar at 40±1 ºC. Washouts of dried tea samples (10 g of tea in 90 ml of a sterile phosphate buffer) were used as inoculates. 1 ml of inoculates was placed into Petri dishes and imbedded with a nutrient medium. Cultivation lasted for ten days at 24 ºC in the dark. Next Petri dish contents (substrate mycelium) were thoroughly homogenized. 1 g of substrate mycelium obtained via this procedure was used for the extraction of mycotoxins.

Preparation of substrate mycelium samples for MTs analysis. 1 g of substrate mycelium was thoroughly mixed with 5 ml of water-acetonitrile-formic acid (refer to 'Dry tea sample preparation'). MTs were extracted in an ultrasound bath for 30 minutes and then centrifuged for 10 minutes at 4,000 rpm. After that 1 ml of supernatant was diluted with 1 ml of the mobile phase A, properly mixed and centrifuged for 10 minutes at 10,000 rpm. 1.5 ml of supernatant was transferred into a chromatographic vial for analysis.

Results results. Screening of mycotoxins in tea samples.

Screening of mycotoxins in tea samples. We examined MTs in samples of traditional unpacked loose *C. sinensis* tea from six tea-producing regions: China, India, Indonesia, Sri-Lanka, Vietnam, and Kenya. All but four samples from China were black teas. Eight of 29 analytes were detected, almost all below LOQ: AFLs group (AFL G2 and STC); trichothecenes (FusX, NeoS, T-2); *Fusarium* toxins (EnnB and BEA); MPA - a widely spread plant products contaminant (Table 3). 20 of 30 samples (66.7 %) were MTs positive. BEA was found in 18 samples out of 30 (60 %), occurrence of other MTs was much lower. Black tea samples from Vietnam and Indonesia were the most contaminated with MTs traces; then, in the descending order, tea samples from India, China, Kenya, and Sri-Lanka. Only one green tea sample from China was found to be positive for MTs. We did not detect any samples contaminated with regulated in tea AFL B1.

The results correlated with mycological studies of these samples [13]. Samples from Sri Lanka, Kenya and China characterized by low levels of mold contamination (<10^3 CFU/g) revealed low diversity of detected MTs. On the contrary, the higher diversity of detected MTs was noted for samples from Indonesia and India containing higher levels of mold ((1.5–2.3) 10^3 CFU/g). Samples from Vietnam were the exception: they proved to be microbiologically pure (<10^2 CFU/g), while number of detected MTs was comparable with that found for tea samples from Indonesia. The direct dependence of the content of MT and their producers was more characteristic of fresh samples. As storage proceeds, the viable forms of mold gradually die out. They are not detected within mycological analyses, while the MTs synthesized by them remain in tea.

Screening of MTs in samples of packed (loose and bagged) green and black *C. sinensis* tea and tea with additives revealed the occurrence of 12 MTs. Emergent MTs were detected in 20 of 24 (83 %) examined samples. BEA and MPA were the most frequent ones; they were detected in 13 out of 24 samples (56 %). AcDON and FusX were revealed in six and five samples correspondingly. NeoS, STC, Enn B, AME and TE were sporadic (Table 4). Contamination levels of BEA, MPA and TE in black and Pu'er tea were comparable with quantities occurring in other plant

Table 3

MTs in unpacked loose *C. sinensis* tea samples from six tea-producing regions

Mycotoxin	Vietnam, *n* = 5					Indonesia, *n* = 5					India, *n* = 5					China, *n* = 5					Sri-Lanka, *n* = 5					Kenya, *n* = 5				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2*	3*	4*	5*	1	2	3	4	5	1	2	3	4	5
MTs regulated in other food products																														
T-2	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Structure analogues of regulated MTs and emergent MTs:																														
AFL G2	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
STC	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FusX	–	–	–	+	+	–	–	–	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
NeoS	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MPA	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	200	+	–	–	–	–	–	–	–	–	–	–	–	–	–
EnnB	–	–	+	+	–	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BEA	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	–	–	–
Absolute occur-rence of MTs, cases	12					11					8					5					2					2				

Note: * – green tea samples are marked with;

"+" – MTs concentration is over LOD, but below LOQ;

"–" – MTs concentration is below LOD. The concentration of MPA in sample No1 from China is equal to 200 μg/kg.

Table 4

MTs in packed (loose and bagged) *C. sinensis* tea samples

Mycotoxins	Packed tea samples (*n* – number of samples)					
	Green loose, *n* = 1	Green bagged, |*n* = 3	Black loose, *n* = 10	Black bagged, *n* = 6	Pu'er, *n* = 2	Black with thyme, bagged, |*n* = 2
	Number of positive (>LOD) samples					
Total positive	1	3	7	6	2	1
MTs regulated in food products						
AFL B1	–	–	1	–	–	–
T-2	1	–	–	1	–	–
Structure analogues of regulated MTs						
AFL G2	–	–	–	1	–	
STC	–	–	1	–	–	1 (4.4 μg/kg)*
AcDON	–	2	3	–	1	–
FusX	–	–	2	3	–	–
NeoS	–	–	2	–	–	–
Emergent MTs						
MPA	–	3	4	3 (≤200 μg/kg)	2 (≤3040 μg/kg)	1
BEA	–	2	4 (≤6.0 μg/kg)	5	1	1
EnnB	–	–	–	–	–	1
AME	–	1	–	–	–	–
TE	–	–	–	1 (10)	–	–

Note: "–" – MTs were not detected (below LOD); * – concentration over LOQ is specified in brackets.

commodities [7, 14]. Traces of AFLs were detected in two samples of black tea. STC was detected in two black tea samples (loose tea and bagged black tea with thyme). Its concentration in the latter sample was 4.4 μg/kg. It should be noted, that STC is a biogenic precursor of hazardous AFL B1, and IARC lists it as a potential carcinogen [15].

Analysis of Pu'er tea samples did not reveal the expected variety of MTs; however, we should note that there were not enough analyzed samples to consider the obtained data applicable to the whole group of such products. Traditionally Pu'er is produced via extended fermentation of green tea that can last over 10 years; during this period, an initial microbiome is transformed, and it can create favorable conditions for accumulation of such MTs as patulin [16], AFL, DON [17], and OTA [18]. Hence it is not a coincidence that the first Pu'er brewing is not recommended for consumption. However, a traditional procedure is replaced with pile fermentation at present. It is less time consuming and lasts about 48 days [18]. Within the present study, one, about 3,040 μg/kg of MPA, was detected in one sample of Pu'er tea. MPA is produced by *Penicillium* spp. [19] and can accumulate in a product after harvesting in case storage conditions are improper [20]. Safety requirements for tea subjected to long fermentation, like Pu'er, are not defined clearly to-date. The microbiological standards recommended for teas by Tea & Herbal Infusions Europe (THIE, 2018) do not cover Pu'er [21].

Thus, according to the microbiological survey reported in [13] and screening of MTs, all studied *C. sinensis* tea samples, including those with additives, meet the established MLs concerning fungal and MTs contamination.

Twelve samples of mono-component and 11 samples of and multi-component herbal tea samples were studied. Eight out of 12 (66.6 %) mono-component herbal teas (thyme, mint, Ivan-tea, and hibiscus tea) proved to be positive for ten out of 29 examined MTs (Table 5). Emergent MTs prevailed. The highest occurrence was noted for BEA and MPA; then, in descending order: EnnB and TE, AME, Enn A, DAS, FuzX and STC. As for MTs regulated in food products, two Ivan-tea samples were positive for DON (<LOQ). Thyme and Ivan-tea turned out to be the most contaminated. Thyme samples contained nine MTs that are not subjected to control. It was the highest diversity in mono-component herbal tea samples. Moreover, five of them were in quantities exceeding LOQs. Supposedly, thyme was the source of STC detected in bagged black tea with the addition of this herb (Table 4).

Table 5

MTs in mono-component herbal tea samples

Mycotoxin	Mono-component herbal tea			
	Thyme, *n* = 4	Ivan-tea, *n* = 5	Mint, *n* = 2	Hibiscus, *n* = 1
	Number of MT-positive (>LOD) samples			
Total positive	4	3	1	–
MTs regulated in food products				
DON	–	2	–	–
Structure analogues of regulated MTs				
STC	1 (24 μg/kg) *	–	–	–
FusX	1	–	–	–
DAS	1	–	–	–
Emergent MTs				
MPA	2 (≤100 μg/kg)	2	–	–
BEA	2 (≤4 μg/kg)	1	1	–
EnnA	1	–	–	–
EnnB	2 (≤26 μg/kg)	1	–	–
AME	2	–	1	–
TE	2 (≤13 μg/kg)	–	1	–

Note: "–" – MTs were not detected (below LOD); * – concentration over LOQ is specified in brackets.

Microbiological testing of mono-component herbal tea samples [13] revealed that thyme and mint samples were the most contaminated with mold fungi. Two samples of thyme and two samples of mint herbal tea did not meet existing standards as mold fungi concentration reached 10^5 and 10^6 CFU/g correspondingly. All other samples proved to be safe. Ivan-tea samples provided interesting cases of fungi-bacteria competition. Low fungal contamination (about 50 CFU/g) was coupled with high spore-forming bacteria concentration (up $10^{6–8}$ CFU/g). Some spore-forming bacteria are known to be antagonistic to mold fungi. Their co-occurrence results in mutual competition for the substrate [22]. Thus, MTs found in Ivan-tea samples indicate that fungal contamination preceded bacterial. Therefore, low quantities or even absence of any viable mold fungi detected via mycological analysis do not necessarily mean that samples are MTs-negative. On the other hand, pronounced fungal contamination usually implies MTs occurrence.

The group of multi-component herbal teas included 11 samples (Table 1). They proved to be highly contaminated with MTs: 10 (91 %) out of 11 samples were positive for 18 out of 29 examined analytes (Table 6). Six (54 %) samples contained seven or eight MTs simultaneously. MTs content was considerably higher in these samples than in any other examined in the present study. The most frequently detected emergent MTs were MPA and *Alternaria* metabolite TE. They were found in 7 out of 11 samples; BEA, EnnB and STC were detected in six samples; AME - in five; EnnA - in three; β-ZEL - in two samples. AFL G1, T-2-triol, DAS, ZEA, and FusX were detected sporadically. As opposed to other examined tea products, multi-component herbal tea samples contained a variety of regulated MTs such as AFLB1, OTA (2 μg/kg), DON,

Table 6

MTs in multi-component herbal tea samples

Mycotoxin	Multi-component herbal tea										
	No.14	No.2	No.36	No.38	No.20	No.19	No.8	No.9	No.10	No.11	No.12
MTs regulated in food products											
AFLB1	–	–	–	–	–	+	–	–	–	–	–
OTA	–	–	–	–	–	2.0*	–	–	–	–	–
DON	–	–	–	–	–	+	–	–	–	–	–
T–2	–	–	+	–	–	–	–	–	–	–	9.2
FB2	–	–	–	–	–	–	–	–	100	–	–
ZEA	–	–	–	–	–	–	190	–		–	–
Structure analogues of regulated MTs											
AFLG1	–	–	–	–	–	3.2	–	–	–	–	–
STC	–	–	–	–	–	+	8.0	+	10.0	9.2	9.6
FusX	–	–	–	+	–	–	–	–	–	–	–
T-2 triol	–	–	–	–	–	–	–	–	–	–	+
DAS	–	–	–	–	–	–	–	–	–	+	–
β-ZEL	–	–	–	–	+	–	–	+	–	–	–
Emergent MTs											
MPA	–	–	+	+	+	+	770	690	1760	440	2240
BEA	–	–	–	–	–	20.4	6.0	5.6	5.6	8.0	8.0
EnnA	–	2.8	–	–	–	–	–	+	–	+	–
EnnB	–	–	–	–	–	13.6	22.4	52.0	34.0	55.0	36.0
TE	–	+	–	–	5.6	–	6.0	5.2	5.2	9.2	6.4
AME	–	–	–	–	–	–	+	+	+	+	+

Note: "+" – MTs concentration is over LOD, but below LOQ; "–" – MTs concentration is below LOD. * – concentration exceeding LOQ is in μg/kg.

T-2 (9.2 μg/kg), FB2 (100 μg/kg), ZEA (190 μg/kg). They occurred in quantities close to maximum levels (ML) set by regulations for food products in CU. Thus, five μg/kg of OTA, 200 μg/kg of FBs and 50 μg/kg of T-2 toxin are MLs for these MTs in some cereals for children (CU TR 021/2011). Five μg/kg of OTA is ML for coffee beans set in the EU.

Combined contamination of herbal tea samples with multiple MTs indicates that toxigenic fungi pounced on herbs both during vegetation, processing and storage. "Field fungi", such as *Fusarium* spp. are responsible for DON, T-2, T-2 triol, DAS, ZEN, b-ZEL, EnnA and B, BEA, FBs accumulation. *Alternaria* spp. also invade herbs "in fields" and produce AOH, AME, TE. "Storage" fungi are *Aspergillus* spp. (AFB1, AFG1, STC, OTA) and *Penicillium* spp. (MPA, OTA). This is following the results of the mycological study of these herbal tea samples [13].

Five samples of multi-component herbal teas (No. 8–12) turned out to contain from seven to eight different MTs together with high mold fungi contents (10^4–10^5 CFU/g). Eight MTs were detected in sample No. 19, meanwhile, fungal contamination was low (50 CFU/g). At the same time, the concentration of spore-forming bacteria was high ($8 \cdot 10^5$ CFU/g) in this sample. Similar results were obtained for Ivan-tea samples. They were discussed above in the paragraph, devoted to mono-component herbal tea. Sample No. 19 case also supports the idea that original mold fungal contamination was later suppressed by bacteria development. Therefore, even if a tea sample complies with the microbiological safety requirements fixed in the CU TR 021/2011, this does not mean that they do not contain MTs.

The increase in the number of detected MTs corresponded well with the growing number of components (see Tables 1 and 6). MTs variety in herbal tea was much higher as compared to *C. sinensis* tea samples. This is in accordance with literature data. For example, 12 MTs were discovered in 60 herbal tea samples marketed in Latvia. Co-occurrence of up to eight MTs was noted in 90 % of the samples. EnnB, DON, AFB1, OTA, and ZEA were detected the most frequently [23]. Survey carried out in in Spain revealed 99 % of 84 medical and aroma herbs samples were contaminated. OTA, FBs, AFLs, ZEA, T-2 toxin, DON and citrinin prevailed [24]. A wide variety of MTs was detected in meadow herbs and hay from European Russia regions: 16 MTs were detected including T-2, DAS, DON, ZEA, FBs, AOH, roridine A, AFL B1, STC, cyclopiazonic acid, emodin, OTA, citrinin, MPA, PR-toxin and ergot alkaloids [25].

Table 7 summarizes the occurrence of MTs in the examined *C. sinensis* and herbal tea samples. We detected emergent MTs in all kinds of studied tea. BEA, MPA, EnnB, TE, and FusX were detected the most frequently. We should note that structurally similar BEA and Enns are widely spread and occur in almost all types of plant raw materials and foods. For

Table 7

The occurrence of MTs in *C. sinensis* and herbal tea samples

Tea	MTs-positive samples, %	Quantity of detected MTs	Mycotoxins in decreasing order of occurrence
C. sinensis unpacked (semi-finished)	70	8	BEA>FusX>EnnB> STC>MPA>**T-2**> (AFL G2, NeoS)*
C. sinensis packed	83	12	(BEA, MPA) >AcDON>FusX> (T-2, NEOS) > (EnnB, AME, TE, STC, **AFL B1** and G2)
Mono-component herbal tea	66.7	10	(BEA, MPA) > (EnnB, AME, TE) >**DON**> (EnnA, DAS, FusX, STC)
Multi-component herbal tea	91	18	MPA>TE> (BEA, EnnB, STC) >AME>EnnA> (**T-2**, β-ZEL) >**AFL B1**>AFL G1> (**DON, FB2, OTA**, T-2 triol, DAS, **ZEA,** FusX)

Note: MTs with equal occurrence are put in brackets.

example, BEA was detected in 80 % of tested food, while Enns – in 63 %. A risk assessment carried out by EFSA in 2014 demonstrated that there might be a concern with respect human health effects and chronic dietary exposure to BEA and Enns [9].

Although MTs are detected in low quantities, there is a potential risk that cumulative effects might appear. Toxic impacts that occur due to multiple mycotoxins being consumed simultaneously can become obvious both via additive effects and synergetic ones and in the latter case, overall toxicity can be higher than a simple sum of individual toxicities [26]. Additive effects are described for structurally similar compounds. Results obtained via research on individual and combined toxic effects produced by B-trichothecenes (DON, NIV, 3- and 15-AcDON, DON-3-glucoside and Fus-X) on epithelial cells in a human stomach (GES-1) allowed assuming that their simultaneous occurrence in food products even in low doses can be more or less toxic than a prediction based on data obtained for individual MTs [27]. The same goes for other structural MT analogues from the same species or family when their effects and toxicity profile are similar, for example, FBs or Enns. Synergic effects are described for OTA and AFLs; thus, low mortality due to mycotoxicosis caused by OTA grows considerably when it is combined with AFLs [28]. BEA, DON, and T-2 produce high toxic effects and therefore, their combined exposure can induce certain diseases in people, especially in case exposure is long-term [29]. Overall, in most cases, a combined consumption of MTs results in additive or synergetic effects, and it causes more significant health risks for people and animals [30].

Nominally all the examined tea samples corresponded to hygienic standards. The concentration of AFLB1 did not exceed ML of 5 μg/kg. Still, the co-occurrence of several MTs, especially highly toxic ones, in low doses can cause health risks for people in case of long-term exposure. The obtained data indicate the necessity to assess health risks associated with combined MTs contamination of food, in particular, plant raw materials used in manufacturing specialized food products for babies, dietary products, herbal food supplements, *C. sinensis* teas with additives, herbal tea, spices, etc.

Examination of toxin production by tea microflora *in vitro*. Contamination of traditional *C. sinensis* teas with mold fungi tends to be neglected, by regulatory authorities as well. This is often due to the fact that the risks caused by tea and tea raw materials being contaminated wuth MTs are rather low if production, transportation, and storage conditions correspond to the fixed humidity and temperature [31]. Another opinion is that the lack of growth factors and the content of polyphenolic compounds in tea prevent MTs production even in case there is high mold contamination [32]. Nevertheless, the results of studies of tea samples from various regions confirm that hazardous MTs such as FB, OTA, AFL, T-2, ZEA can be detected in such products in large quantities [11]. For example, 82 % black and green tea samples obtained from retail outlets in Italy were contained OTA, and in 50 % cases its quantity amounted to 7–21 μg/kg (with predominant *A. niger* and *A. tubingensis*). These concentrations exceeded the MLs set for other food products, the consumption volumes of which are comparable to teas, in particular, coffee (5 μg/kg) [33]. Research performed in Switzerland revealed that black mold fungi were one of the most widely-spread ones in 22 samples of herbal teas and isolated strains of *A. niger* and *A. awamori* produced FBs *in vitro* [34]. Several studies have reported the production of toxins *in vitro* on several types of model culture media by certain strains of *Aspergillus* sp. and *Fusarium* sp. which were isolated from plant raw materials including tea made of medicinal herbs [34–36]. The results of these studies show that the types and levels of MTs accumulation by producing fungi in model nutrient media are substrate-specific and do not always reflect toxin production in natural conditions adequately. In nature, different mold fungi compete with each other and exometabolites (MTs) are their weapon used in fighting for a substrate.

We examined a possibility of MTs producing directly in tea substrate *in vitro* under

conditions closest to reality with excess humidity. Green tea was the only substrate, and MTs producers were a consortium of mold fungi that naturally present in certain tea samples. To do that, we selected tea samples with the highest contamination from those previously examined ones; they were multi- and mono-component teas contaminated with mold fungi in quantities equal to 10^3–$7 \cdot 10^4$ CFU/g which was higher than MLs. We made wash-outs out of them (a part of tea and 9 parts of water); wash-outs were used to inoculate an agarized nutrient medium with green *C. sinensis* tea. We used sterile water as an inoculate in a reference sample. Incubation lasted for ten days; then MT were extracted from the substrate and analyzed.

As a result, we revealed that MTs, emergent alongside with regulated ones, which had not been detected in initial dry tea samples, accumulated in extracts from a nutrient medium. Their production was up to: **FB**1 – 294 μg/g; **FB**2 – 4.8–5,694 μg/g; **ZEA** – 128 μg/g; STC – 14.4 μg/g; EnnB – 1.8 μg/g; BEA – 1.36–9.0 μg/g; MPA – 23–303 μg/g; AME – 158 μg/g of a nutrient medium (Table 8).

Obtained results confirmed that toxigenic species of molds from tea samples are capable of accumulating different types of MTs, including emergent ones, simultaneously. It is possible under favourable conditions (humidity - temperature) in a plant substrate with tea leaves as the only nutrient component. This supports the idea that fungal contamination of *C. sinensis* can result in contamination of teas with MTs.

Conclusions. *C. sinensis* and herbal tea samples were screened for 29 MTs. The results revealed that black and green *C.sinensis* tea samples, both bought in retail outlets and obtained from wholesalers, were contaminated with mycotoxins only at low (trace) quantities. A much wider spectrum of MTs, including regulated in other kind of food and emergent,

Table 8

MT production by mold fungi contaminants of selected tea samples *in vitro*

Sample No.	Species of viable mold fungi in initial dry tea samples [13]	Mycotoxins detected in a nutrient medium *in vitro*	
		Content, μg/kg	<LOQ (traces)
2	*Aspergillus sections Nigri, Mucor sp., Fusarium sp., Alternaria sp.*	EnnB-1.8	TE
3	*Aspergillus sp., Penicillium sp., Mucor sp., Fusarium sp., Alternaria sp.*	BEA-9.0	AME
4	*Aspergillus sections Nigri,Mucor sp., Fusarium sp., Alternaria sp.*	**FB1-294**; **FB2-218**; **ZEA-128**	BEA
5	*Aspergillus sections Nigri, Mucor sp., Penicillium sp.,Fusarium sp., Alternaria sp.*	**FB2-952**	STC, **T-2**, BEA, **DAS**
6	*Aspergillus sections Nigri, Mucor sp., Alternaria sp.*	STC-14.4; **FB2**-4.8	β-ZEL, AME
7	*Penicillium sp., Aspergillus sp., Aspergillus sections Nigri, Mucor sp., Epicoccus sp., Fusarium sp., Alternaria sp.*	MPA-23	AFLB1, BEA, **T-2**
8	*Aspergillus sections Nigri, Mucor sp., Fusarium sp., Penicillium sp.,Alternaria sp.*	BEA-1.36	–
9	*Penicillium sp.,Aspergillus sections Nigri, Aspergillus sp., Mucor sp., Fusarium sp. Alternaria sp.*	**FB2-5,624;** MPA-303	EnnA and B, BEA
10	*Penicillium sp.,Aspergillus sections Nigri, Aspergillus sp., Fusarium sp.*	MPA-45	–
11	*Penicillium sp.,Aspergillussp., Aspergillus sections Nigri, Mucor sp.,Fusarium sp. Epicoccussp.,Alternaria sp.*	**AME-158**	BEA
12	*Aspergillus sections Nigri, Aspergillus sp., Penicillium sp.,Mucor sp., Fusarium sp.*	–	**AFLB1**, BEA
Substrate	Not detected	–	–

Note: Substrate: “clean” *C. sinensis* green tea; “–“ – MTs were not detected (<LOD).

was detected in herbal tea samples. Twelve MTs were detected in quantities over LOQs. Neither of 77 samples contained AFLB1 in quantities higher than fixed standards (<5 μg/kg).

Co-occurrence of regulated and emergent MTs in *C. sinensis* and herbal teas can be a potential health hazard under long-term exposure taking into account cumulative effects even at low levels. More representative monitoring and data accumulation is essential for assessing health risks associated with MTs in such products.

Comparison of fungal and mycotoxin contamination in the studied tea samples revealed that low fungal contamination doesn't necessarily mean the absence of mycotoxins. Conversely, an increase in the number and variety of fungal species leads to a greater variety of metabolites.

Toxigenic properties of microflora that occurred in teas were examined *in vitro* in conditions that were as close to real ones as it was only possible; we applied green *C.sinensis* tea leaves as a substrate. Our experiment confirmed the ability of toxigenic mold fungi to accumulate different types of MTs and EMTs simultaneously in significant amounts comparable to MLs established for foods of plant origin (μg/kg): FB1-294 μg/kg; FB2-5,624 μg/kg; ZEN-128 μg/kg; STC-14.4 μg/kg; AME-158 μg/kg.

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Abbreviations: alternariol (AOH); aflatoxins B1, B2, G1 and G2 (AFLB1, B2, G1 and G2); 3- and 15-acetyldeoxynivalenol (3- and 15-AcDON); beauvericin (BEA); deoxynivalenol (DON); diacetoxyscirpenol (DAS); α-zearalanol (zeranol, α-ZAL); β-zearalanol (taleranol, β-ZAL); α- and β-zearalenol (α- and β-ZEL); zearalenone (ZEA); mycophenolic acid (MPA); alternariol methyl ether (AME); moniliformin (MO); neosolaniol (NeoS); nivalenol (NIV); sterigmatocystin (STC); tentoxin (TE); T-2 (T-2 toxin); HT-2 (HT-2 toxin); T-2 triol (T-2 triol); fusarenone X (4-acetyl nivalenol, FusX); fumonisins B1 and B2 (FB1 and FB2); enniatins A and B (Enn A and B).

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