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Multimycotoxin UPLC−MS/MS for Tea, Herbal Infusions and the Derived Drinkable Products

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In recent years the consumption of tea and herbal infusions has increased. These hot drinks are consumed as daily drinks as well as for medicinal purposes. All tea varieties (white, yellow, green, oolong, black and puerh) originate from the leaves of the tea plant, *Camellia sinensis*. All extracts made of plant or herbal materials which do not contain *Camellia sinensis* are referred as herbal infusions or tisanes. During processing and manufacturing fungal contamination of the plant materials is possible, enabling contamination of these products with mycotoxins. In this study a multimycotoxin UPLC−MS/MS method was developed and validated for the analysis of the raw tea and herbal infusion materials as well as for their drinkable products. The samples were analyzed by ultra-high-performance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS), with a mobile phase consisting of variable mixtures of water and methanol with 0.3% formic acid. The limits of detection for the different mycotoxins varied between 2.1 μg/kg and 121 μg/kg for raw materials and between 0.4 μg/L and 46 μg/L for drinkable products. Afterward 91 different tea and herbal infusion samples were analyzed. Only in one sample, Ceylon melange, 76 μg/kg fumonisin B_1 was detected. No mycotoxins were detected in the drinkable products.

KEYWORDS: Multimycotoxin; UPLC−MS/MS; tea; herbal infusion; fumonisin B_1

INTRODUCTION

In recent years the consumption of natural products has increased, probably stimulated by cultural or psychosociological factors. People become aware of possible health benefits of natural products. Tea and herbal infusions are hot drinks which are consumed as daily drinks or for medicinal purposes (*1*). All tea varieties (white, yellow, green, oolong, black and puerh) originate from the leaves of the tea plant *Camellia sinensis* and are subjected to different processes during production. In the first step, leaves are picked and rolled to disrupt cellular compartmentalization followed by steaming or fermentation with or without maturation before the drying process. For green tea the young leaves are steamed to minimize oxidation. For the production of black, puerh, oolong, white and yellow tea the young leaves undergo oxidation by oxidizing enzymes such as polyphenol oxidase and peroxidase in the leaves. This step is referred to as the fermentation step, but it is distinct from the yeast-mediated alcoholic fermentation in which glucose is converted to ethanol and carbon dioxide. White tea undergoes light fermentation and oolong tea is semifermented resulting in a taste and color between those of green and black tea. Yellow tea and puerh tea are respectively lightly fermented and fermented tea followed by maturation for a certain period. The longer the maturation, the better the taste and quality of the tea. All extracts made of plant or herbal materials which do not contain *Camellia sinensis* are referred to as herbal infusions or tisanes (*2*−*4*).

During processing and manufacturing microbiological contamination of the plant materials is possible. Different studies in which medicinal plants, black tea, herbal tea and puerh tea were investigated for fungal contamination and *Aspergillus*, *Penicillium*, *Pacelomyces*, *Cladosporium*, *Alternaria*, *Mucor*, *Fusarium*, *Rhizopus*, *Absidia* and *Trichoderma* species were identified (*1*, *3*, *5*, *6*). *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* species are toxigenic mold species able to produce mycotoxins. Mycotoxins are naturally occurring toxic secondary metabolites which can cause acute toxic effects as well as chronic toxic effects. Therefore the ingestion of contaminated foodstuffs can cause serious health hazards. Over 400 mycotoxins are known, of which aflatoxins, trichothecenes, fumonisins, ochratoxin A, zearalenone and *Alternaria* toxins are the main representatives (*7*). The possible fungal contamination of raw materials for tea and infusions has led to the development of analytical methods for the detection of mycotoxins. The determination of fumonisin B_1 and fumonisin B_2 using a strong anion exchange solid phase extraction (SPE) column for the sample cleanup, *o*-phthaldialdehyde and 2-mercaptoethanol for derivatization and a high-performance liquid chromatography (HPLC) fluorescence detection system was published for the analysis of black tea, herbal tea and medicinal plants (*8*, *9*). A survey of 87 Portuguese black tea and medicinal plant samples resulted in the contamination of 65.5% of the samples with fumonisin B_1 with concentrations ranging from

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20 to 700 μg/kg, while no FB_2 was detected (*8*). A survey of 115 Turkish herbal tea and medicinal plant samples resulted in two contaminated samples with fumonisin B_1 (160−1487 μg/kg) (*9*). No aflatoxins were produced by twenty five *A. flavus* species isolated from tea samples (*5*). A HPLC fluorescence method for the determination of aflatoxins in herb teas and medicinal plants marketed in Italy using immunoaffinity columns for the sample cleanup and electrochemically generated bromine for derivatization was published. No aflatoxins were detected in any of the 48 analyzed samples (*10*).

Because different molds can co-occur and toxigenic fungi are able to produce more than one type of mycotoxin, there is a growing interest in the simultaneous analysis of different mycotoxins. A study was published (*6*) where 73 medicinal plants and herbal teas were examined for mold contamination. In total 12 samples were contaminated with *A. flavus* and subjected to aflatoxin B_1, ochratoxin A and zearalenone analysis using thin-layer chromatography. Traces of ochratoxin A were detected in just one medicinal plant (Flores Tiliae) (*6*). Recently 84 medicinal and/or aromatic herb samples originating from Spain were investigated. Samples were analyzed using enzyme linked immune assay (ELISA) based kits for aflatoxins, zearalenone, deoxynivalenol, T-2 toxin, fumonisins, citrinin and ochratoxin A after sample cleanup using multifunctional columns, polyamide column or no prior cleanup step. Aflatoxins were detected in 96%, zearalenone in 98%, deoxynivalenol in 62%, T-2 toxin in 99%, fumonisins in 13%, citrinin in 61% and ochratoxin A in 63% of the analyzed samples. All the contaminated samples were multicontaminated, and 87% were contaminated with at least four mycotoxins (*11*). Besides the studies of the contamination of raw materials it is important to investigate the drinkable product. Since mycotoxins are heat-resistant within the range of conventional food-processing temperatures (80−121 °C), its degradation or decomposition at the temperature of boiling water during the preparation of the drink is unlikely (*12*). Only the occurrence of mycotoxins in the drinkable products can cause health risks by consumption. Therefore these studies demonstrate the necessity to develop and apply a multimycotoxin method to analyze raw tea and herbal infusion materials as well as the derived drinkable products.

Improvement in chromatographic performance was achieved by the introduction of reversed-phase chromatographic media with 1.7 μm particle size. The decrease in particle size of the stationary phase leads to theoretical advantages in resolution, speed and sensitivity according to the Van Deemter equation. The ultra performance liquid chromatography (UPLC) equipment is adapted to this technological innovation permitting pumping and injection of liquids at high pressure (> 10000 psi). On the other hand it requires a detection system that is fast enough to accept and process data such as the mass spectrometer (MS) (*13*). The application of this technology for mycotoxin analysis led to the development of an UPLC−MS/MS method for the simultaneous analysis of aflatoxins and ochratoxin A in beer (*14*). Multimycotoxin UPLC−MS/MS methods for the analysis of foodstuffs were published (*15*−*18*). Until now no multimycotoxin methods were used for the analysis of tea. Therefore in this study a multimycotoxin UPLC−MS/MS method was developed, validated and applied for the analysis of raw tea and herbal infusion materials as well as for the derived drinkable products.

MATERIALS AND METHODS

Reagents and Chemicals. Methanol and acetonitrile were high-performance liquid chromatography grade (VWR International, Zaventem, Belgium). Ethyl acetate and *N*,*N*-dimethylformamide were purchased from Acros Organics (Geel, Belgium). Water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). Formic acid of Merck (Darmstadt, Germany) was used. Mycotoxin standards nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, neosolaniol, fusarenon-X, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, HT-2 toxin, alternariol, alternariol methyl ether, altenuene, ochratoxin A, zearalenone, fumonisin-B_1, fumonisin-B_2, sterigmatocystin and zearalanone were purchased from Sigma Chemical Co. (Bornem, Belgium). Citrinin and fumigaclavin were purchased from Alexis (Lausen, Switzerland). Mycophenolic acid and paxillin were purchased from Fermentek (Jerusalem, Israel). De-epoxy-deoxynivalenol, diacetoxyscirpenol and T-2 toxin were purchased from Biopure (Tulln, Austria). Fumonisin-B_3 was obtained from Promec unit (Tygerberg, South Africa). Nivalenol (100 μg/mL), neosolaniol (100 μg/mL), diacetoxyscirpenol (101.8 μg/mL) and de-epoxy-deoxynivalenol (50.9 μg/mL) stock solutions were certified solutions in acetonitrile. The fumonisin B_3 standard (1 mg) was dissolved in 1 mL of acetonitrile:water (50:50, v/v). Stock solutions of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon-X, aflatoxin B_1, B_2, G_1, G_2, HT-2 toxin, T-2 toxin, altenuene, ochratoxin A, zearalenone, fumonisin B_1, B_2, sterigmatocystin, zearalanone, citrinin, fumigaclavin, mycophenolic acid and paxillin (1 mg/mL) were prepared in methanol. Alternariol and alternariol methyl ether stock solutions (1 mg/mL) were prepared in methanol:dimethylformamide (60:40, v/v). All stock solutions were stored for 1 year at −18 °C except fumonisin B_3, which was stored at 4 °C. Working standard solutions were made by diluting the stock standard solutions in methanol and were stored at −18 °C for 6 months. From the individual stock standard solutions and working solutions a standard mixture for the analysis of raw materials was prepared with the following concentrations: nivalenol (10 ng/μL); fumonisin B_1 (3.5 ng/μL); fusarenon-X, alternariol methyl ether, fumonisin B_2 and FB_3 (2.5 ng/μL); deoxynivalenol, 3-acetyldeoxynivalenol, alternariol, HT-2 toxin, and zearalenone (1 ng/μL); 15-acetyldeoxynivalenol (0.5 ng/μL); aflatoxin G_2, B_1 and sterigmatocystin (0.4 ng/μL); neosolaniol (0.3 ng/μL); aflatoxin G_1, B_2, altenuene, T-2 toxin and ochratoxin A (0.2 ng/μL); diacetoxyscirpenol (0.1 ng/μL) in methanol and stored at −18 °C for 3 months. A standard mixture for the analysis of drinkable products was prepared with the following concentrations: nivalenol (7.5 ng/μL); alternariol methyl ether and citrinin (5 ng/μL); fusarenon-X and fumonisin B_1 (2.5 ng/μL); deoxynivalenol, 3-acetyldeoxynivalenol, alternariol, HT-2 toxin, zearalenon, paxillin, mycophenolic acid and fumigaclavin (1 ng/μL); 15-acetyldeoxynivalenol (0.5 ng/μL); neosolaniol (0.3 ng/μL); aflatoxin G_2, G_1, B_2, B_1, altenuene and sterigmatocystin (0.2 ng/μL); diacetoxyscirpenol, T-2 toxin and ochratoxin A (0.1 ng/μL) in methanol and stored at −18 °C for 3 months.

Sample Preparation: Analysis of Raw Material. Two grams of raw material, in the case of a tea bag only the tea content was used, was weighed in a 50 mL Teflon centrifugal tube, and the internal standards zearalanone (10 μg/kg) and de-epoxy-deoxynivalenol (25 μg/kg) were added. The mycotoxins were extracted using 10 mL of ethyl acetate/formic acid (99:1, v/v) as extraction solvent during 30 min on an Agitelec overheadshaker (J. Toulemonde & Cie, Paris, France). The sample extract was centrifuged during 15 min (3200*g*). The volume of the extract was adjusted to 10 mL before splitting. Two milliliters was kept apart in a test tube, evaporated under nitrogen and redissolved in 1 mL of acetonitrile:water (84:16, v/v). This part of the extract is referred to as part one of the extract. The remaining part of the extract (referred to as part two of the extract) was cleaned up by passage through a 200 mg/3 mL NH_2-SPE column (Bakerbond aminopropyl, Achrom, Zulte, Belgium) which was conditioned by passage through 3 mL of ethyl acetate:formic acid (99:1, v/v). The eluate was directly collected in a test tube, the NH_2-SPE column was sucked dry for 10 min and the eluate was evaporated under nitrogen. Afterward the residue was dissolved in 2 mL of acetonitrile:water (84:16, v/v) and cleaned up by passage through a 500 mg/6 mL C_{18}-SPE column (Grace octadecyl, Grace Discovery Sciences, Lokeren, Belgium). This C_{18}-SPE column was conditioned by passage through 5 mL of acetonitrile:water (84:16, v/v). After passing through the eluate coming from part two of the extract, part one of the extract was passed through the same C_{18}-SPE column. The eluate of both parts was directly collected and combined in a test tube, the C_{18}-SPE column was sucked dry for 10 min, and the eluate was evaporated under nitrogen. The residue was dissolved in 100 μL of mobile phase for injection containing water:methanol (60:40, v/v) and 0.3% formic acid. Before UPLC−MS/MS analysis the mycotoxins

Table 1. Optimized ESI+ MS/MS Conditions

toxin	precursor ion (*m*/*z*)	molecular ion	cone voltage (V)	product ions (*m*/*z*)	collision energy (eV)	retention time (min)
nivalenol	313.1	$[M + H]^+$	26	125.0[a]	13	2.24
				205.0	12	
deoxynivalenol	297.1	$[M + H]^+$	26	231.2	15	2.76
				249.2[a]	10	
fusarenon-X	355.0	$[M + H]^+$	18	137.0	25	4.18
				174.9[a]	20	
fumigaclavine	299.1	$[M + H]^+$	44	208.3[a]	21	4.21
				239.3	21	
neosolaniol	400.0	$[M + NH_4]^+$	26	185.0	19	5.67
				305.3[a]	12	
3-acetyl-deoxynivalenol	339.0	$[M + H]^+$	28	203.2	12	7.55
				231.2[a]	13	
15-acetyl-deoxynivalenol	339.0	$[M + H]^+$	26	137.1	10	7.88
				321.2[a]	10	
aflatoxin G_2	331.0	$[M + H]^+$	53	313.1[a]	25	9.87
				245.2	30	
aflatoxin G_1	329.0	$[M + H]^+$	44	243.0[a]	25	10.27
				311.2	20	
altenuene	292.9	$[M + H]^+$	21	257.3[a]	14	10.69
				275.3	11	
aflatoxin B_2	315.0	$[M + H]^+$	51	259.2	30	10.72
				287.2[a]	27	
aflatoxin B_1	313.0	$[M + H]^+$	51	241.2	36	11.15
				285.1[a]	24	
diacetoxyscirpenol	384.2	$[M + NH_4]^+$	19	247.0	14	11.64
				307.1[a]	12	
citrinin	250.9	$[M + H]^+$	32	205.4	26	12.37
				233.2[a]	17	
alternariol	258.9	$[M + H]^+$	40	185.1[a]	30	12.41
				213.1	26	
HT-2 toxin	447.1	$[M + Na]^+$	30	285.1	23	13.86
				345.3[a]	21	
mycophenolic acid	321.3	$[M + H]^+$	20	207.2[a]	20	14.30
				303.3	10	
fumonisin-B_1	722.1	$[M + H]^+$	56	352.3[a]	36	14.40
				704.4	29	
T-2 toxin	489.1	$[M + Na]^+$	12	245.1[a]	26	14.95
				327.0	26	
fumonisin-B_3	706.1	$[M + H]^+$	54	336.5[a]	37	15.27
				688.5	31	
zearalenone	319.1	$[M + H]^+$	27	203.0	20	15.27
				283.3[a]	10	
ochratoxin A	403.9	$[M + H]^+$	24	239.0[a]	22	15.93
				358.2	14	
alternariol methyl ether	272.9	$[M + H]^+$	57	199.3	30	15.61
				258.2[a]	26	
fumonisin-B_2	706.1	$[M + H]^+$	61	336.5[a]	40	16.63
				688.5	29	
sterigmatocystin	325.0	$[M + H]^+$	47	281.1	22	15.66
				310.2[a]	25	
paxilline	436.2	$[M + H]^+$	26	130.1[a]	20	18.56
				418.2	10	
de-epoxy-deoxynivalenol (IS)	281.0	$[M + H]+$	26	109.1[a]	19	4.20
				137.1	15	
zearalanone (IS)	321.0	$[M + H]+$	26	189.2	19	15.07
				303.3[a]	13	

[a] Most abundant product ion.

dissolved in the mobile phase were centrifuged in an Ultrafree-MC centrifugal device (Millipore, Bedford, MA, USA) for 15 min at 14000*g*.

Sample Preparation: Analysis of the Drinkable Product. During 2 min 2 g of raw material—in the case of a tea bag only the tea content was used—was extracted in 200 mL of boiling water. To 5 mL of the extract the internal standards zearalanone (10 μg/L) and de-epoxy-deoxynivalenol (25 μg/L) were added, and it was centrifuged during 30 min (3200*g*), and the supernatant was cleaned up by passage through a 500 mg/6 mL C_{18}-SPE column (Grace Discovery Sciences, Lokeren, Belgium) which was conditioned by passage through 5 mL of methanol and 5 mL of milli-Q water. After elution of the mycotoxins with 3 mL of methanol, the eluate was evaporated under nitrogen and redissolved in 100 μL of mobile phase for injection, containing water:methanol (60:40, v/v) and 0.3% formic acid. Before UPLC−MS/MS analysis the mycotoxins dissolved in the mobile phase were centrifuged in a Ultrafree-MC centrifugal device (Millipore, Bedford, MA, USA) for 15 min at 14000*g*.

UPLC−MS/MS Conditions. UPLC−MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Quattro

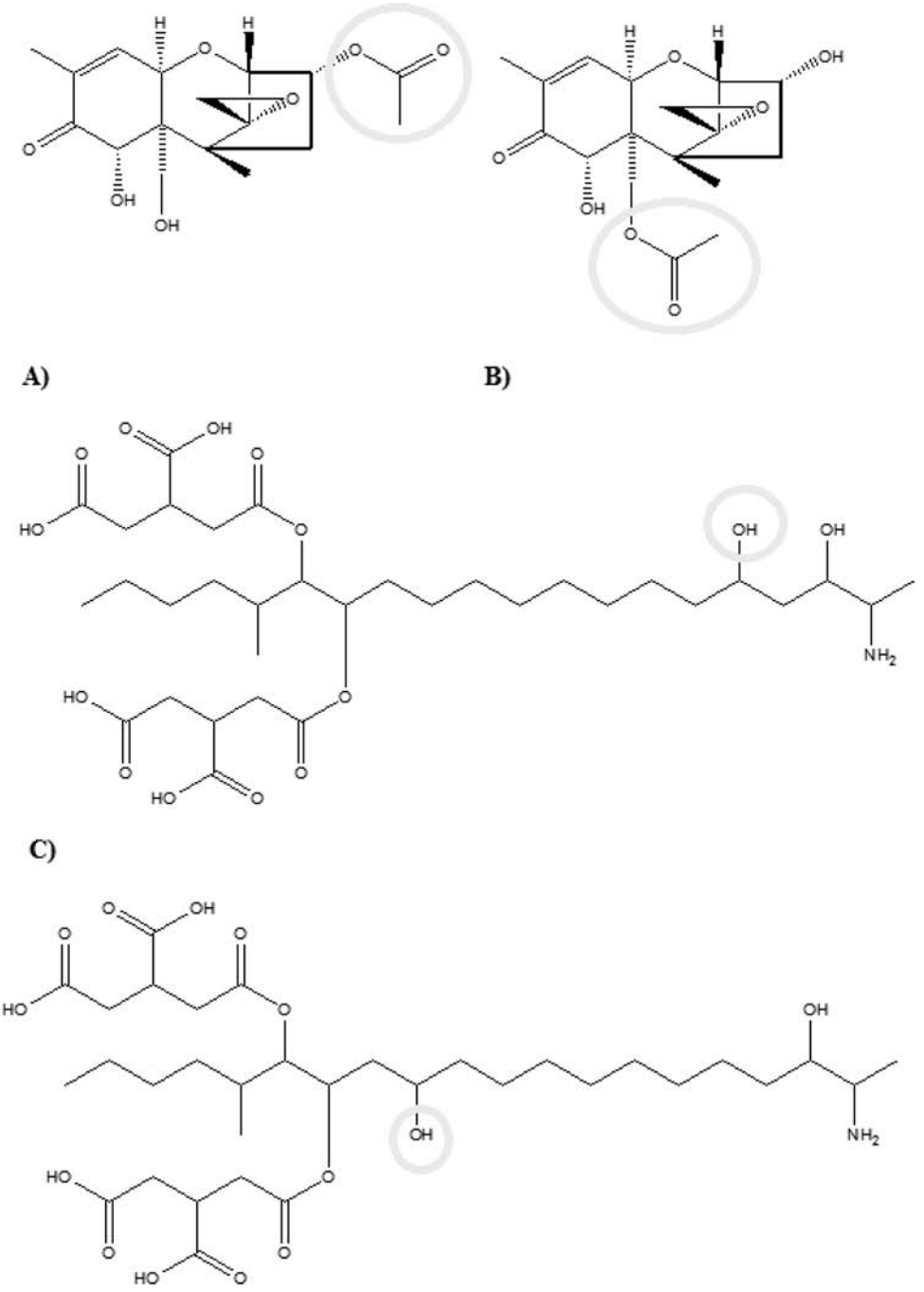

Figure 1. Chemical structures of 3-acetyldeoxynivalenol (**A**), 15-acetyldeoxynivalenol (**B**), fumonisin B_2 (**C**) and fumonisin B_3 (**D**).

Premier XE (Waters, Milford, MA, USA) mass spectrometer equipped with an electrospray interface. The column used was a 100 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC BEH C_{18}, with a 5 mm × 2.1 mm i.d. guard column of the same material (Waters, Milford, MA, USA). The column was kept at 60 °C. A mobile phase consisting of eluents A (water, 0.3% formic acid) and B (methanol, 0.3% formic acid) was used at a flow rate of 0.55 mL/min. A gradient elution was applied as follows: 0−1 min, 0% B; 1−1.5 min 0−15% B; 1.5−8 min 15% B; 8−8.5 min 15−30% B; 8.5−11 min 30−40% B; 11−12 min 40% B; 12−14 min 40−55% B; 14−16 min 55% B; 16−19 min 55−85% B; 19−20 min 85−100% B; 20−22 min 100% B; 22−23 min 100−0% B; 23−25 min 0% B. The injection volume was 10 μL. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. MS parameters for the analysis were as follows: ESI source block and desolvation temperatures, 120 and 400 °C respectively; capillary voltage, 3.2 kV; argon collision gas, 2.01×10^{-4} mbar; cone nitrogen and desolvation gas flows, 50 and 800 L/h, respectively. After selection of the precursor ion for each analyte, product ions were obtained with a combination of cone voltages and collision energies. For increased sensitivity and selectivity, data acquisition was performed working in selected reaction monitoring (SRM). The optimized MS-parameters are presented in **Table 1**. Masslynx and Quanlynx software (Micromass, Manchester, U.K.) was used for data acquisition and processing.

Method Validation. Fifteen black, blank tea samples were spiked in triplicate with a known concentration of mycotoxin mixture at five different concentration levels. Zearalanone and de-epoxy-deoxynivalenol were added as internal standards (IS). This experiment was repeated on three different days. Matrix matched calibration plots were constructed by applying the least-squares method and by plotting the relative peak area (peak area toxin/peak area IS) against the spiked concentration level of the tea sample. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated 3 times and 6 times, respectively, the standard error of the intercept divided by the slope of the calibration curve. For each component the calculated LOD and LOQ were verified by the signal-to-noise

(S/N) ratio, which should be more than 3 and 10. The linearity was tested graphically using a scatter plot, and the linear regression model was tested using a lack of fit test. The specificity was tested by analyzing 20 different blank tea samples. At different concentration levels the apparent recovery was calculated by quantifying the mycotoxins using the matrix-matched calibration plot. For each mycotoxin the observed value was divided by the spiked level. The precision of the method in terms of intraday precision (the analysis of three replicates on the same day at five different concentration levels) and interday precision (the analysis of three replicates at five different concentration levels performed on three different days) was evaluated calculating the relative standard deviation (RSD). The validation parameters were calculated using the relative peak area, in which the peak area was divided by the peak area of the internal standard de-epoxydeoxynivalenol (used for the trichothecenes nivalenol, deoxynivalenol, fusarenon-X, neosolaniol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2 toxin, T-2 toxin and diacetoxyscirpenol) and zearalanone (used for all other described mycotoxins). All calculations were executed in Excel 2007 or SPSS 17.

Sample Collection of the Tea Samples. During 2008−2010 raw tea and infusion materials were collected in China (N = 11) and in Belgium (supermarkets (N = 33), pharmacies (N = 25) and private collections (N = 22)). For each sample the trade name, the type of tea, the batch number and the expiry date, if available, were noticed in a database.

RESULTS AND DISCUSSION

Method Development. *Sample Preparation*. Different recently developed multimycotoxin methods do not require an extensive sample cleanup step. After extraction with a mixture of acetonitrile/water in different proportions with or without the addition of acetic acid, the extract is diluted and/or filtered and injected into the LC−MS/MS system (*19*). In preliminary experiments this fast procedure was tested for raw materials of black tea, resulting in a dark extract and unclear chromatograms, and only traces of fumonisins were detected at the preferred sensitivity. Therefore a sample cleanup was necessary, and it was based on the existing procedure developed for the analysis of sweet pepper (*20*). The new sample cleanup procedure was less time-consuming and did not require a strong anion exchange SPE column. The extract was still divided into two parts to recover all the described mycotoxins, but was cleaned up only using an NH_2-SPE and a C_{18}-SPE column. For the drinkable products, sample preparation was even more simple.

UPLC−MS/MS. The total analysis time was 25 min including 2 min for reconditioning of the column before the next injection. This means that in this method the theoretical advantage of the improved speed for UPLC analysis was not applied. This was in contrast with published UPLC methods where the time of analysis varied between 3.2 and 18 min (*14*−*18*). In this developed UPLC−MS/MS method 28 mycotoxins (including 2 internal standards) were analyzed in one injection in the positive electrospray mode. Special attention was paid to the separation of the isomers 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol and fumonisin B_2, B_3, the structures of which are shown in **Figure 1**. Contrary to the previously developed LC−MS/MS method (*20*) the use of the Acquity UPLC BEH C_{18} column in combination with water and methanol with the addition of 0.3% formic acid as mobile phase made it possible to separate the 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol isomers with a resolution of 2.33, which is shown in **Figure 2**. Although different multimycotoxin LC−MS/MS methods have been described, not all of them are able to separate these isomers. Different strategies are used to circumvent this difficulty, more specifically the development of multimethods with the determination of only one acetyl derivate, the development of multimethods where the separation is ignored, the use of a different electrospray ionization mode for the isomers, and determination of the sum of the acetyl derivates without separation or identification by calculation of the ion ratio of the product ions. Contrary to the numerous existing multimethods, only in a few multimycotoxin LC−MS/MS methods a clear separation of the two isomers was obtained (*15*, *19*, *21*). Multimethods including fumonisin B_1 and B_2 were published, but it is not clear if fumonisin B_2 and B_3 were separated. Multimethods including both isomers achieved a separation of these toxins (*15*, *19*, *21*).

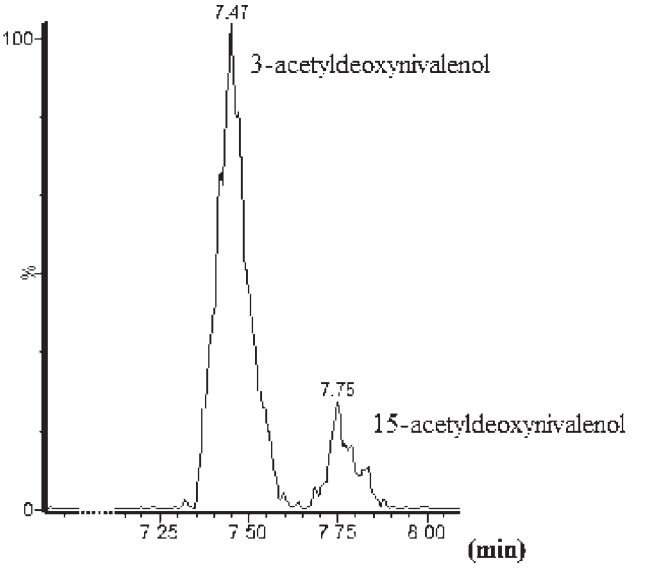

Figure 2. Illustration of the resolution between 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol.

Method Validation. The method for raw materials was validated for nivalenol, deoxynivalenol, neosolaniol, fusarenon-X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, aflatoxin G_2, G_1, B_2, B_1, sterigmatocystin, ochratoxin A, fumonisin B_1, B_2, B_3, alternariol, alternariol methyl ether, altenuene, HT-2 toxin, T-2 toxin, diacetoxyscirpenol and zearalenone. The method for the drinkable product was validated for nivalenol, deoxynivalenol, neosolaniol, fusarenon-X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, aflatoxin G_2, G_1, B_2, B_1, sterigmatocystin, ochratoxin A, fumonisin B_1, alternariol, alternariol methyl ether, altenuene, HT-2 toxin, T-2 toxin, diacetoxyscirpenol, zearalenone, mycophenolic acid, paxilline, fumigaclavin and citrinin. Due to matrix interference present in raw tea materials it was not possible to validate mycophenolic acid, paxillin, fumigaclavin and citrinin in these matrices. The LODs and LOQs of the described toxins are presented in **Table 2**. The LODs for raw material varied between 2.1 μg/kg for altenuene and 122 μg/kg for nivalenol, and the LODs for the drinkable product varied between 0.4 μg/L for ochratoxin A and 46 μg/L for nivalenol. Commission Regulation (EC) No. 1881/2006 is setting maximum levels for certain contaminants in foodstuffs and is amended by Commission Regulation (EC) No. 1126/2007, No. 105/2010 and No. 165/2010 for respectively *Fusarium* toxins, ochratoxin A and aflatoxins (*22*−*25*). Although for the drinkable product of tea and infusions no specific maximum levels are established in these regulations, the obtained sensitivity of the developed method is able to detect the described mycotoxins according to the present European regulations. Commission Regulation (EC) No. 401/2006 is setting criteria for sample preparation and for methods of analysis used for the official control of the levels of mycotoxins in foodstuffs (*26*). In **Table 3** results of the apparent recovery and the precision study including the intraday precision (RSDr) and interday precision (RSDR) are shown. The apparent recovery varied between 91% and 107% for raw materials and between 96% 108% for drinkable products and is for both methods within the range of 70−110%, the strictest range of the regulation.

Table 2. The LODs and LOQs of the Described Multimycotoxin UPLC−MS/MS Method for Raw Tea and Herbal Infusion Materials and Drinkable Products

raw material				drinkable product			
toxin	calibration range (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	toxin	calibration range (μg/l)	LOD (μg/L)	LOQ (μg/L)
nivalenol	100−500	122	243	nivalenol	30−150	46	93
deoxynivalenol	10−50	11	22	deoxynivalenol	4−20	3.6	7.3
fusarenon-X	25−125	28	57	fusarenon-X	10−50	13	26
neosolaniol	3−5	3.2	6.4	neosolaniol	1.2−6	1.6	3.3
3-acetyl-deoxynivalenol	10−50	11	22	3-acetyl-deoxynivalenol	4−20	3.0	6.1
15-acetyl-deoxynivalenol	5−25	5.9	12	15-acetyl-deoxynivalenol	2−10	1.8	3.6
aflatoxin G_2	4−20	3.8	7.5	aflatoxin G_2	0.8−4	1.4	2.8
aflatoxin G_1	2−10	2.5	5.0	aflatoxin G_1	0.8−4	0.8	1.7
aflatoxin B_2	2−10	3.5	7.0	aflatoxin B_2	0.8−4	1.1	2.2
aflatoxin B_1	4−20	3.7	7.4	aflatoxin B_1	0.8−4	1.3	2.6
sterigmatocystin	4−20	4.7	9.5	sterigmatocystin	0.8−4	2.7	5.3
ochratoxin A	2−10	2.6	5.2	ochratoxin A	0.4−2	0.4	0.8
fumonisin-B_1	35−175	37	74	fumonisin-B_1	10−50	25	51
fumonisin-B_2	25−125	36	72	alternariol	4−20	5.8	12
fumonisin-B_3	25−125	25	49	alternariol methyl ether	20−100	30	60
alternariol	10−50	13	26	altenuene	0.8−4	1.3	2.6
alternariol methyl ether	25−125	20	41	HT-2 toxin	4−20	5	10
altenuene	2−10	2.1	4.1	T-2 toxin	0.4−2	0.8	1.5
HT-2 toxin	10−50	12	24	diacetoxyscirpenol	0.4−2	0.7	1.4
T-2 toxin	2−10	3.4	6.8	zearalenone	4−20	8.8	18
diacetoxyscirpenol	5−25	5.1	10	fumigaclavine	4−20	7.9	16
zearalenone	10−50	16	31	paxilline	4−20	5.7	11
				citrinin	20−100	36	72
				mycophenolic acid	4−20	4.4	8.8

Table 3. Method Performance Results for Raw Tea and Herbal Infusion Materials and Drinkable Products

raw material					drinkable product				
toxin	spiked level (μg/kg)	RSDr (%)	RSDR (%)	apparent recovery (%)	toxin	spiked level (μg/L)	RSDr (%)	RSDR (%)	apparent recovery (%)
nivalenol	300	8	9	94	nivalenol	120	14	13	101
deoxynivalenol	30	8	9	102	deoxynivalenol	8	18	16	99
fusarenon-X	75	14	15	100	fusarenon-X	30	13	13	106
neosolaniol	9	11	11	103	neosolaniol	3.6	10	11	105
3-acetyl-deoxynivalenol	30	10	10	102	3-acetyl-deoxynivalenol	8	11	11	100
15-acetyl-deoxynivalenol	15	10	13	93	15-acetyl-deoxynivalenol	4	11	12	99
aflatoxin G_2	8	8	9	99	aflatoxin G_2	3.2	13	11	97
aflatoxin G_1	6	15	15	104	aflatoxin G_1	2.4	13	14	102
aflatoxin B_2	8	11	16	102	aflatoxin B_2	2.4	15	16	108
aflatoxin B_1	8	8	9	103	aflatoxin B_1	3.2	8	14	100
sterigmatocystin	12	9	10	105	sterigmatocystin	4	15	13	99
ochratoxin A	6	9	18	100	ochratoxin A	0.8	18	16	107
fumonisin-B_1	105	7	9	94	fumonisin-B_1	50	10	13	101
fumonisin-B_2	75	13	12	97	alternariol	12	21	16	106
fumonisin-B_3	50	4	8	100	alternariol methyl ether	60	21	14	104
alternariol	30	10	10	106	altenuene	3.2	3	13	101
alternariol methyl ether	50	9	10	107	HT-2 toxin	12	14	13	106
altenuene	6	10	13	97	T-2 toxin	1.6	3	15	97
HT-2 toxin	30	3	8	97	diacetoxyscirpenol	1.6	3	13	101
T-2 toxin	8	16	16	103	zearalenone	20	9	11	98
diacetoxyscirpenol	15	7	10	100	fumigaclavine	16	14	16	105
zearalenone	40	16	21	91	paxilline	12	13	14	106
					citrinin	80	13	16	96
					mycophenolic acid	12	7	11	105

The RSDr and RSDR are in good agreement with the performance criteria. For the toxins not mentioned in the regulation the results were within the limits obtained by the Horwitz equation. No interfering signals in the chromatogram, that can lead to nonconforming results, were detected in the specificity experiments for raw tea materials and the derived products.

Analysis of Collected Tea Samples. To investigate the occurrence of mycotoxins in raw tea and herbal infusion materials, the 91 collected raw materials were analyzed using the described procedure. Only in one sample, Ceylon melange (Orient Sunset, private collection, Belgium), 76 μg/kg fumonisin B_1 was detected. The chromatogram is shown in **Figure 3**. The detection of fumonisin B_1 in raw tea and infusion materials is in agreement with previously obtained results (*8*, *9*, *11*). In our research only 1.1% (1/91) of the investigated samples were contaminated, which is in good agreement with Omurtag and Yazicioglu (*9*),

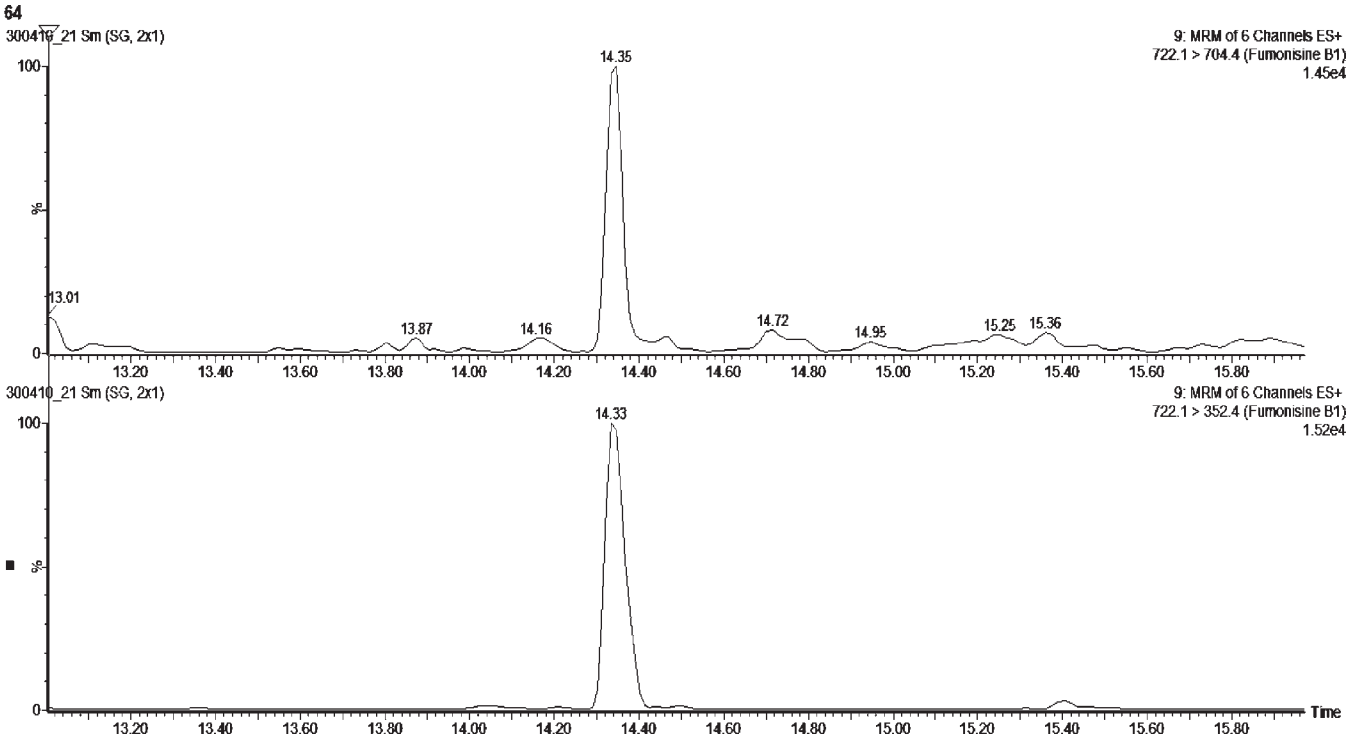

Figure 3. Chromatogram of the fumonisin B_1 contaminated (76 μg/kg) Ceylon melange sample.

who described the fumonisin B_1 contamination of 1.7% (2/115) of the investigated samples. This is in contrast with the results obtained by Santos et al. (*11*) and Martins et al. (*8*), where respectively 13% (11/84) and 63% (55/87) of the investigated samples were contaminated with fumonisin B_1. There was no multimycotoxin contamination observed in the analyzed samples, which is in contrast with the detection of the multicontaminated samples using ELISA screening kits (*11*).

For the analysis of the drinkable products, the fumonisin B_1 contaminated sample was selected and 15 other samples containing traces of mycotoxins, namely, alternariol, alternariol methyl ether, deoxynivalenol, 15-acetyldeoxynivalenol, sterigmatocystin, fumonisin B_1 and T-2 toxin. The identification criteria, as described in Commission Decision (EC) 2002/657 (*27*), were fulfilled. The signal-to-noise ratio for each diagnostic ion was ≥ 3, the relative ion ratio was within the maximum permitted tolerances and the ratio of the relative retention time was within a margin of 2.5%, but the detected concentration was below the LOD. No mycotoxins were detected in the 16 analyzed drinkable products. Despite the fact that fumonisin B_1 is a hydrophilic compound and very soluble in an aqueous environment (*7*), it was not possible to detect it in its drinkable product. This was probably due to the low detected mycotoxin concentration in the tea raw material

The reported multimycotoxin UPLC−MS/MS method for raw materials and the drinkable products fulfilled the performance criteria required by Commission Regulation (EC) No. 401/2006. Special attention was paid to the separation of the isomers 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol and fumonisin B_2, B_3. Two different cleanup methods were developed to analyze raw tea and herbal infusion materials as well as their drinkable products. The application of the developed method for the analysis of 91 samples resulted in the contamination of one sample, Ceylon melange, with 76 μg/kg fumonisin B_1. No mycotoxins were detected in the derived drinkable product. The risk from the occurrence of mycotoxins in the analyzed tea samples is negligible. Based on the obtained results it is hardly likely that the intake of tea and herbal infusion drinks will result in high levels of mycotoxin consumption.

SAFETY

Mycotoxins have been related to carcinogenicity and other toxic effects and should be handled with appropriate caution. Standard safety measures (gloves and laboratory coat) should be used when one is working with standards and samples. Standard and sample preparation was carried out in a fume hood. Extraction and waste disposal should be done according to standard environmental procedures.

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