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Two-dimensional liquid chromatography - high resolution mass spectrometry method for simultaneous monitoring of 70 regulated and emerging mycotoxins in Pu-erh tea

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1. Introduction

Mycotoxins are toxic secondary metabolites produced by filamentous fungi under suitable humidity and temperature conditions, contaminating a wide range of agricultural commodities during the planting, harvesting, processing, transportation, as well as storage [1,2]. It was observed that most mycotoxins are stable during processing and are found even in finished products, raising a global concern due to their toxic effects when ingested, inhaled or adsorbed through the skin by humans and animals [3]. Therefore, many mycotoxins have been regulated by the European Union legislation [4] and are monitored in a large number of matrices. Additionally, besides the mycotoxins typically regulated in various crops, there are more than 300 other known mycotoxins, including the emerging *Fusarium* and *Alternaria* mycotoxins, which are of significant interest because of their worldwide occurrence and the insufficient toxicological data. Currently there is a strong interest towards developing multiclass methods for the determination of mycotoxins in various food commodities using LC-MS/MS [5,6]. However, the methods available for quantitative analysis are often inadequate when looking for less-known mycotoxins. The typical difficulties include the detection low-level mycotoxin contamination, applicability to diverse chemical structures of mycotoxins, interference from similar compounds, as well as the complexity of different food matrices in which mycotoxin contamination can be analysed only after complicated sample preparation, extraction, and purification procedures, which become much more challenging upon increasing the number of mycotoxins to be detected.

Tea is one of the most popular and widely consumed beverages in the world (being the leading drink after water in terms of consumption). According to the Intergovernmental Group of tea at the Food and Agriculture Organisation of the United Nations, the average annual increase of tea consumption has been 4.5% over the last decade. Tea has beneficial effects on human health due to the presence of various compounds, such as vitamins, amino acids, polyphenols, caffeine, purine alkaloids etc., which are reported to have antimicrobial, antioxidative, anticarcinogenic activities, enhanced digestion, as well as blood lipid and body mass reduction [7]. Unlike other types of tea, Pu-erh belongs to the best known post-fermented teas, which need a long time of maturation to be considered ripened. It is unique due to the microbial post-fermentation process, which may last from several months to many years, resulting in potential fungal contamination. Other studies have reported about the contamination of tea with heavy metals, pesticides, polycyclic aromatic compounds, microorganisms, plant growth regulators, radionuclides, and mycotoxins as well [8]. Remarkably, there have been very few studies on multi-mycotoxin

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occurrence in Pu-erh teas, generally because of problems with analytical method development for this complex matrix [9,10].

In recent years, two-dimensional liquid chromatography (2D-LC) has gained recognition from researchers in the field of separation science [11]. This methodology is based on combining two solid phases in order to achieve significantly higher separation power and peak capacity, compared to conventional one-dimensional liquid chromatography (1D-LC). This effective separation technique is mostly applied in the case of sophisticated matrix interactions that could be substantial for the determination of selected analytes [12].

2D-LC separations can be carried out either in *offline* or in *online* mode. The *offline* mode is convenient with no time constraint in the second dimension, because of collecting fractions are collected from the first column with later reinjection into a second column. However, in comparison with *online* 2D-LC, this mode in not suitable for automation, as it is time consuming and less reproducible, with possible carry-over issues and sample loss [13]. The two most commonly used modes of *online* 2D-LC separation are known as comprehensive and heart-cutting analysis. Comprehensive 2D-LC (LC × LC) is predominantly used to analyse complex, unknown mixtures, when every peak eluting from the first dimension (^{1}D) chromatographic column is transferred to the second dimension (^{2}D) chromatographic column through a switching valve or another transferring device in order to subordinate the entire ^{1}D effluent to ^{2}D separations [14,15]. Heart-cutting 2D-LC is different from LC × LC involving the transfer of only one or few selected eluent fractions from the ^{1}D column for further separation on the ^{2}D column. The major advantage of the heart-cutting technique versus the comprehensive analysis is that the first and second dimension run times are decoupled and both of them can be operated closer to optimal conditions without any time constraints on the second-dimension separation, providing higher peak resolution [16,17].

While 2D-LC methodology combined with mass spectrometric detection can be very effective for the determination of target compounds in complex matrices, such as spices and herbal medicines [18], there are limited reports of its use for multi-mycotoxin determination where aflatoxin and ochratoxin A were separated using immunoaffinity columns, followed by LC analysis [19,20]. Thus, it would be a relevant challenge in the field of separation technique research to develop a 2D-LC method for simultaneous analysis of hundreds of chemical constituents with different polarities, abundance, as well as structural types in complex matrices, such as Pu-erh tea extract.

Thus, the aim of this study was to develop *online*-heart-cutting-2D-HPLC-TOF-HRMS method as an automated analytical tool for simultaneous, highly selective determination of 70 mycotoxins in Pu-erh tea.

2. Material and methods

2.1. Chemicals, reagents, and materials

The solvents used (acetonitrile, methanol, ethyl acetate) were of pesticide grade and were purchased from Sigma-Aldrich (Steinheim am Albuch, Germany). Dimethylformamide (DMF) of pesticide grade (≥99.8%) and formic acid of analytical grade were purchased from Merck Millipore (Darmstadt, Germany). Deionised water was produced with a Millipore Milli-Q system (Darmstadt, Germany).

The standards of beauvericin (BEA, ≥95%), enniatin A (ENN A, ≥99%), enniatin A_1 (ENN A_1, ≥99%), enniatin B (ENN B, ≥99%), enniatin B_1 (ENN B_1, ≥99%), meleagrin (MEL, ≥98%), cytochalasin A (CCA, ≥98%), cytochalasin B (CCB, ≥98%), cytochalasin C (CCC, ≥99%), cytochalasin D (CCD, ≥95%), cytochalasin E (CCE, ≥98%), cytochalasin J (CCJ, ≥95%), cytochalasin H (CCH, ≥95%), 15-acetyldeoxynivalenol (15-AcDON, ≥99%), 3-acetyldeoxynivalenol (3-AcDON, ≥99%), tentoxin (TNX, ≥99%), citreoviridin (CVD, ≥95%), stachybotrylactam (SBL, ≥95%), alternariol monomethyl ether (AME, ≥98%), dihydrochalasin B (DTC B, ≥98%) and fusaric acid (FA, ≥98%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Deoxynivalenol (DON, 98.3%), aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, ≥99%), HT-2 toxin (HT-2, 99%), T-2 toxin (T-2, 99%), sterigmatocystin (STC, 99.7%), zearalenone (ZEN, 99,66%), citrinin (CIT, 99.6%), ochratoxin A (OTA, 99%), fumonisins B_1 and B_2 (FB_1, 98%; FB_2, 97.5%), fusarenon-X (FUS-X, 99.4%), and deoxynivalenol-3-glucoside (D3G, 96%) were acquired from Romer Labs (Tulln, Austria). Neosolaniol (NEO, 99%), anisomycin (ANC, 98.9%), T-2 toxin tetraol (T-2TET, >99%), apicidin (API, >99%), ansamitocin P3 (AN P3, 99%) altenuene (ALT, 99.3%), alternariol (AOH, >98%), cerulenin (CER, 98%), chaetocin (CTC, 99%), 15-acetoxyscirpenol (15-AcS, >98%), T-2 toxin triol (T-2TRI, 99%), fumonisin B_3 (FB_3, 99%), myriocin (MYR, 99%), brefeldin A (BRF A, 99.9%), 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG, 99.4%), altertoxin I (ATX I, 99%), 17-(allylamino)-17-demetoxygeldanamycin (17-AAG, 99%), aflatoxicol (AFL, 99%), chaetoglobosin A (CHG A, 99%), verruculogen (VCL, >99%), wortmannin (WTM, 99%), helvolic acid (HA, 99%), ochratoxin B (OTB, 99%), destruxin A (DTX A, 99%), destruxin B (DTX B, 99%), paxilline (PXL, 99%), penitrem A (PN A, >99%), gliotoxin (GTX, >99%), curvularin (CVL, 99%), bafilomycin A_1 (BFA_1, >99%) and bafilomycin B_1 (BFB_1, >99%) were purchased from Fermentek (Jerusalem, Israel), while mycophenolic acid (MPA, >99%), penicillic acid (PA, >99%) and roquefortine-C (ROQ-C, >99%) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). (The structural characteristics and origins of the analysed mycotoxins are shown in the Supplementary Material (Table S1)).

Standard stock solutions of all mycotoxins were prepared in acetonitrile, methanol or their mixtures with DMSO, with the exception of BEA and enniatins that were kept in DMF. The spiking solutions and calibration standards were prepared by serial dilution of stock solutions and were stored in UV-protected glassware at 4 °C.

2.2. HRMS conditions

The HRMS instrumentation included an Apollo II electrospray ionisation (ESI) source and a Compact Q-ToF time-of-flight mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) that was controlled by Control 4.0 software (Bruker Daltonik GmbH, Bremen, Germany). The analysis was performed in positive full scan mode for all of the mycotoxins with the *m/z* scanning range from 50 to 1000. The source parameters were the following: capillary cap voltage was set at 4.5 kV, while the spray shield voltage was set at 0.5 kV; desolvation gas temperature was 200 °C; the nitrogen flow rate was 10 L min^{-1}; the nebuliser gas flow pressure was 2 bar. The analyte-dependent parameters are listed in Table 1. External instrument calibration for accurate mass measurements was performed before each batch of samples according to the instrument manufacturer's guidelines by using the sodium formate cluster. Data acquisition was controlled by HyStar 3.2. software (Bruker Daltonik GmbH, Bremen, Germany) and the data analysis was performed with QuantAnalysis 4.3. (Bruker Daltonik GmbH, Bremen, Germany). The analyte-dependent data is summarised in Supplementary Material (Table S2).

2.3. Online-*heart-cutting-2D-HPLC parameters*

Heart-cutting RP-RP 2D-HPLC analysis was performed on an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Waltham,

Table 1
Gradient elution program and valve-switching information.

Time, min	1D-LC separation (Kinetex C_{18} (1.7 µm, 100 Å, 50 mm × 3 mm))			Time, min	2D-LC separation (Luna® Omega Polar C_{18} (3 µm, 100 Å, 100 × 3 mm))			Valve position
	Flow rate (mL/min)	A (%)	B1 (%)		Flow rate (mL/min)	A (%)	B2 (%)	
0	0.35	50	50	0	0.35	45	55	6–1
0.5	0.35	50	50	0.5	0.35	45	55	1–2
1.9	0.35	50	50	1.9	0.35	45	55	
3.0	0.35	50	50	2.7	0.35	45	55	6–1
8.0	0.35	2	98	3.0	0.35	90	10	
11	0.35	2	98	5.0	0.35	90	10	
11.5	0.35	90	10	9.0	0.35	2	98	
13	0.35	90	10	11	0.35	2	98	
13.5	0.35	60	40	11.2	0.35	45	55	
16	0.35	60	40	16	0.35	45	55	

A – deionised water containing 0.1% of formic acid;.
B1 – 0.5 mM ammonium acetate in acetonitrile containing 0.1% of formic acid.
B2 – acetonitrile containing 0.1% of formic acid.

MA, USA) containing a binary pump, degasser, a column oven compartment, an autosampler manager fitted with a 500 μL injection loop, and a programmable Rheodyne® six-port switching valve at the side of the column compartment. A two-position six-port switching valve equipped with a stainless steel 500 μL sampling loop was used to capture the fraction eluted from the ^{1}D analytical column.

The ^{1}D chromatographic separation was performed on a Kinetex C_{18} (1.7 µm, 100 Å, 50 mm × 3 mm; Phenomenex, Torrance, CA, USA) reversed-phase analytical column packed with fully porous silica particles, while the ^{2}D separation was performed on a Luna® Omega Polar C_{18} (3 µm, 100 Å, 100 × 3 mm; Phenomenex, USA) reversed-phase analytical column packed with core-shell silica particles. The ^{1}D and ^{2}D columns shared the column oven and their temperature was set at 40 °C. The elution programs for the ^{1}D and ^{2}D separations, as well as the valve switching program are shown in Table 1 and the generic scheme of the used 2D-HPLC is shown in the Fig. 1. The volume of the injection was set at 15 µL.

2.4. Sample preparation procedure for the raw Pu-erh tea

Two grams of raw and homogenised Pu-erh tea were weighed into a 50 mL PP centrifuge tube. The extraction of mycotoxins was performed using 15 mL of ethyl acetate/formic acid (99:1, v/v) solution. The mixture was shaken (15 min) and then centrifuged at 3000 rpm for 10 min. The sample extract was filtered through a filter paper. The supernatant (3 mL) was collected and evaporated to dryness under a gentle nitrogen flow at 50 °C. The dry residue was dissolved in 1.5 mL of MeOH:H_2O (1:1, v/v) and vortexed for 5 min. This solution is referred to as the first part of the extract. The remaining 12 mL of the extract (referred to as the second part of the extract) were cleaned up by passing through a 500 mg/6 mL NH_2-SPE column which was conditioned with 5 mL of ethyl acetate/formic acid (99:1, v/v). The eluent was collected in a 15 mL PP centrifuge tube, the NH_2-SPE column was kept under a low vacuum for 5 min and the eluate was evaporated under the same conditions as the first part of the extract. Afterwards, the residue was dissolved in 5 mL of MeOH:H_2O (1:1, v/v) and cleaned up by passing together with the first part of the extract through a 500 mg/6 mL C_{18}-SPE column which was conditioned with 5 mL of methanol and 5 mL of milli-Q deionised water. After passing both parts of the extract through the C_{18}-SPE column, the column was dried under low vacuum, then the mycotoxins were eluted with 5 mL of acetonitrile, the eluent was evaporated under a gentle nitrogen flow at 50 °C and redissolved in 150 µL of water/methanol (60:40, v/v) containing 0.1% formic acid.

2.5. Data analysis and the analytical method validation

The developed online heart-cutting-2D-HPLC-TOF-HRMS method was validated according to the performance criteria set in Commission Regulations (EU) No 882/2014, 401/2006, 519/2014, the "Guidance Document on the Estimation of LOD and LOQ for the Measurements in the Field of Contaminants in Feed and Food" [21,22], and ISO 5725:1994. The selectivity, trueness, linearity, repeatability expressed as relative standard deviation (RSD) values and the uncertainty of the method developed during this study were verified by validation of the analytical procedure according to the aforementioned criteria.

The quantification limit of the instrumental method (*i*-LOQ) for which the S/N ratio exceeded 10 was evaluated by analysing a sequence of standard solutions with decreasing concentration. The *m*-LOQ and *m*-LOD values were calculated by taking into account the sample preparation procedures and the matrix effects obtained during the method validation.

3. Results and discussion

3.1. Development and optimisation of the online-*heart-cutting-2D-HPLC-TOF-MS* method

The objective of this work was to develop a 2D-LC method for simultaneous analysis of 70 mycotoxins in Pu-erh teas. First, the mycotoxins and all the other substances from tea samples were separated on the ^{1}D column, then the target fraction of eluent was directed into the sample storage loop, followed by separation on the ^{2}D column. Prior to the analysis of real samples, the two dimensions were optimised independently.

3.1.1. HRMS optimisation

Following the *online*-heart-cutting-2D-HPLC separation, detection and quantification of target compounds was performed by time-of-flight mass analyser. Compared to other mass analysers, especially different quadrupole instruments that are most often used for mycotoxin determination, a TOF system shows higher efficiency operating in single ionisation mode (positive or negative only) within one analysis. This is due to the instrument feature where a much longer time is needed for positive-negative polarity switching, resulting in deterioration of mass accuracy during the analysis [22,23]. In spite of the fact that in several recent studies many mycotoxins were determined using negative ionisation mode all of the 70 mycotoxins that were of interest during our study were analysed using positive ionisation mode, forming protonated molecular ions and/or ammonium/sodium adducts. At the same

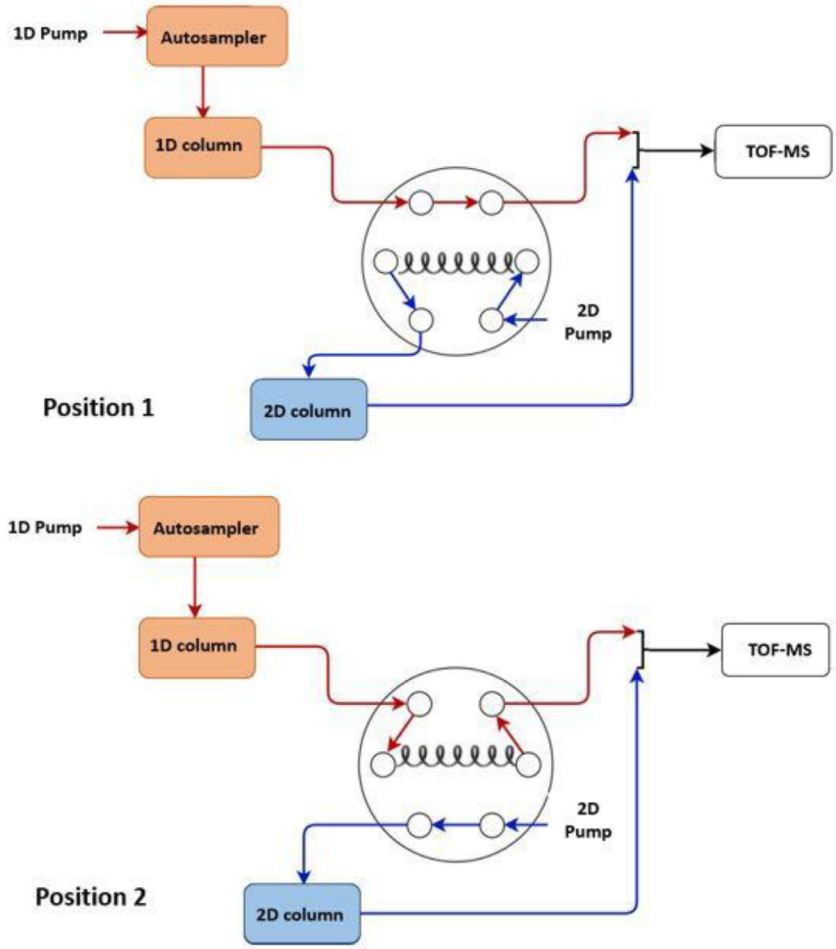

Fig. 1. Schematic representation of the two positions of a 6-port switching valve with a loop-based interface.

time, many of our analysed compounds have not yet been thoroughly described by other researchers. In order to compare both ionisatioin modes and verify that positive ionisation is viable for all of the mycotoxins, a standard solution of each mycotoxin was individually injected directly into the mass analyser. The highest intensities were obtained in the positive mode, so polarity switching or a second run in negative mode could be omitted. Our findings were in accordance with recently published articles specifically directed on optimization and evaluation of HRMS for the mycotoxin analysis [24–27]. The detection was performed in the full scan mode (50–1000 Da), since the molecular weight of mycotoxins ranged from 170.2 Da in a case of penicillic acid to 783.4 Da for the emerging mycotoxin beauvericin.

The elaborated method showed high stability during the experiments. Thus, the retention times of the compounds can also be used as identification criteria of the quantification method. The details of optimised instrumental parameters, as well as the average measured masses of quantification and confirmation ions of all analysed mycotoxins are summarised in Table 1.

The measured mass accuracy compared to the theoretical values was acceptable (from −3.85 to 4.92 ppm), being within the recommended range of ≤5 ppm for $m/z \geq 200$ [28] at a resolution of 10 000 Full Width at Half Maximum (FWHM) values.

3.1.2. Selection of analytical columns

According to other studies, reversed phase (RP) liquid chromatography columns C_{18} are used most frequently for mycotoxin separation [24,29]. Thus, for ^{1}D separation we tested C_{18} columns of two dimensions. To prove the efficiency of the non-polar columns, we compared them to Polar C_{18} analytical RP columns (Table 2). The non-polar Kinetex C_{18} column (1.7 µm, 100 Å, 50 mm × 3 mm) was decided to be used for the ^{1}D separation in order to have shorter analysis time. However, problematic chromatographic separation was observed for polar compounds that are eluted in short retention time (DON and its metabolites, FA, PA, etc.). Previously, those compounds were reported by other authors as being affected by strong matrix interference [30]. Thus, different analytical columns were tested in order to achieve better retention and separation of polar compounds in ^{2}D separation (Table 2).

As the result, a merged RP-LC system in both dimensions (C_{18} - 1.7 µm, 50 mm × 3 mm and Polar C_{18} - 3 µm, 100 × 3 mm)was selected, which is the most widely used 2D-LC approach for different types of analytes [13,31,32]. This system provided better mobile phase mutual compatibility than other modes and the selected stationary phases were not only efficient, but also provided significant orthogonality due to their modifications. Another advantage of RP-LC compared to other stationary phases is that RP-LC analytical columns require short equilibration times, giving high efficiency and hence high peak capacity, which is of prime interest in the context of 2D-LC analysis [33].

3.1.3. Selection of the eluents

The most frequently reported gradient systems for mycotoxin analysis with further MS detection have been acidified methanol/water, acetonitrile/water, or both of these systems con-

Table 2
Comparision of differetnt columns for 2D-LC analysis.

Columns tested	Conclusions
^{1}D separation	
Phenomenex Kinetex C_{18} (1.7 µm, 100 Å, 50 mm × 3 mm and 1.7 µm, 100 Å, 150 mm × 2.1 mm)	The resolution and peak shapes were practically identical.
Luna® Omega Polar C_{18} (3 µm, 100 Å, 100 × 3 mm and 3 µm, 100 Å, 30 × 2.1 mm)	Significantly longer analysis time and tailing peaks of some compounds were obtained.
^{2}D separation	
Luna® Omega Polar C_{18} (3 µm, 100 Å, 100 × 3 mm)	The best retention and separation of analytes was achieved. The obtained analytical signals were eluted in optimal time, not extending the whole 2D analysis.
Luna SCX (5 µm, 100 Å, 50 × 4.6 mm)	Most of the peaks were spread over a wide time range, indicating less time-effective analysis.
Synergi Hydro-RP (4 µm, 80 Å, 150 × 2.0 mm)	Not possible to separate the related compounds of DON from each other.
Luna Phenyl-Hexyl (5 µm, 150 × 4.6 mm)	The first compound was eluted only after the 8th minute, even when using mobile phase composition with high initial organic solvent content (60% ACN).
Synergi Fusion-RP (4 µm, 80 Å, 50 × 2.0 mm)	The satisfactory separation of polar compounds was achieved, but it was necessary to significantly increase the analysis time to reach it.
Synergi MAX-RP (4 µm, 80 Å, 150 × 2.0 mm)	All compounds had nearly identical retention times, preventing the separation of DON from its related compounds.

taining various amounts of ammonium acetate, because of the tendency of many mycotoxins to bind ammonium ions [7,29]. For that reason, all of the mentioned gradient systems were tested for the separation of mycotoxins in both dimensions.

Unlike the previously reported conventional heart-cutting approaches, where two different detectors or one detector in succession for both dimensions have been generally used [11,17,31,34], the method developed during the present study was based on parallel 2D-LC–HRMS detection. In *online* 2D-LC, the compatibility of the mobile phases is very important. Mobile phases with great differences in viscosities should be avoided in 2D-LC systems to prevent problems associated with viscous fingering [35,36]. Our key criteria in choosing the gradient systems were eluent compatibility, maintaining symmetric peak shapes of analytes when both gradient systems blended together before entering the time-of-flight detector. As a result of our experiments, it was concluded that acetonitrile-containing gradient systems were more effective for this application. Methanol/water gradients were less appropriate for some lower mass mycotoxins, showing more pronounced peak expansion, especially after the fraction collected in the storage loop was transferred to the ^{2}D column. Therefore, in order to achieve an effective separation also for small molecules, an acetonitrile/water gradient was used for performing the ^{2}D separation. When choosing the strongest organic phase component for ^{1}D separation, the effects of both methanol and acetonitrile were compared, but, unlike in the ^{2}D separation, the only difference between the chromatograms was the retention time of the compounds, resulting in longer analysis time in the case of methanol. The signal shapes and intensities were comparable, but due to the greater effectiveness of acetonitrile in the ^{2}D separation, as well as the fact that methanol/water solution is more viscous, thus increasing the back pressure in the chromatographic system, it was decided to apply acetonitrile-containing gradient also for the ^{1}D separation.

During the optimisation of HRMS parameters, the strongest signals for many analytes were detected from their $[M+NH_4]^+$ molecular adducts, which were later selected as quantification or confirmation ions. Thus, in order to better characterise the formation of $[M+NH_4]^+$ signals, we also tested systems containing ammonium acetate. In other studies, ammonium acetate solutions with concentrations as high as 10 mM have been used. Therefore, we compared mobile phases containing 0.1 mM, 0.5 mM, 5 mM, and 10 mM of ammonium acetate. Comparing the effects of different ammonium acetate concentrations on the intensity of $[M+NH_4]^+$ signals, the 0.1 mM solution showed practically no improvement, while raising the ammonium acetate concentration to 5 mM enhanced the $[M+NH_4]^+$ ion intensities for many mycotoxins (especially affecting the ionisation of *Alternaria* mycotoxins). The effect of 10 mM ammonium acetate on the chromatographic performance was found to be deleterious, greatly increasing the back pressure on the column (after lasting experiments raising from approximately 350 bar to almost 600 bar), while not improving the ammonium ion formation compared to the 5 mM solution. In order to avoid high back pressure, while still increasing the intensity of $[M+NH_4]^+$ ion signals, 5 mM ammonium acetate in acetonitrile/water was used for ^{1}D separation. The same buffer system was also tested for ^{2}D separation, but there was no marked improvement, thence we decided to avoid complicating the ^{2}D separation step and not to load the column with additional salts in mobile phases. For achieving better ionisation of mycotoxin molecules, all of the solvents of gradient systems were acidified with 0.1% of formic acid.

3.1.4. Optimisation of gradient systems

The aim of the gradient system optimisation was to effectively remove the most problematic matrix components, while collecting all mycotoxins. Thus, ^{1}D gradient system was optimised to achieve the optimal retention of mycotoxins for which ^{1}D separation in Pu-erh tea matrix is feasible. Since the scope of analytes include groups of compounds with very good affinity towards the chromatographic column sorbent, such as the emerging mycotoxins (ENNs and BEA), bafilomycins A_1 and B_1, penitrem A, and other compounds with similar structural characteristics, their effective separation and retention requires longer elution at the absolute acetonitrile composition. Theoretically, the use of a mobile phase with higher initial organic component level can shorten the elution time on a reversed phase column, so ^{1}D chromatography began with 50% of acetonitrile, providing the resolution of 41 analytes in the range from 2.0 to 13.1 min during the ^{1}D separation. Using the same gradient system, a fraction containing 29 analytes was collected in the sample storage loop up to 2.0 min and transferred to the ^{2}D column.

The initial mobile phase composition also turned to be a key factor for developing the ^{2}D gradient system, because of significant tailing of the more polar and acidic compounds eluting at the beginning of ^{2}D separation when starting with a low organic solvent content. In the case of beginning the ^{2}D separation with 10% acetonitrile and gradually increasing its content, significant extension of the chromatography time (up to 25 min) was needed to ensure

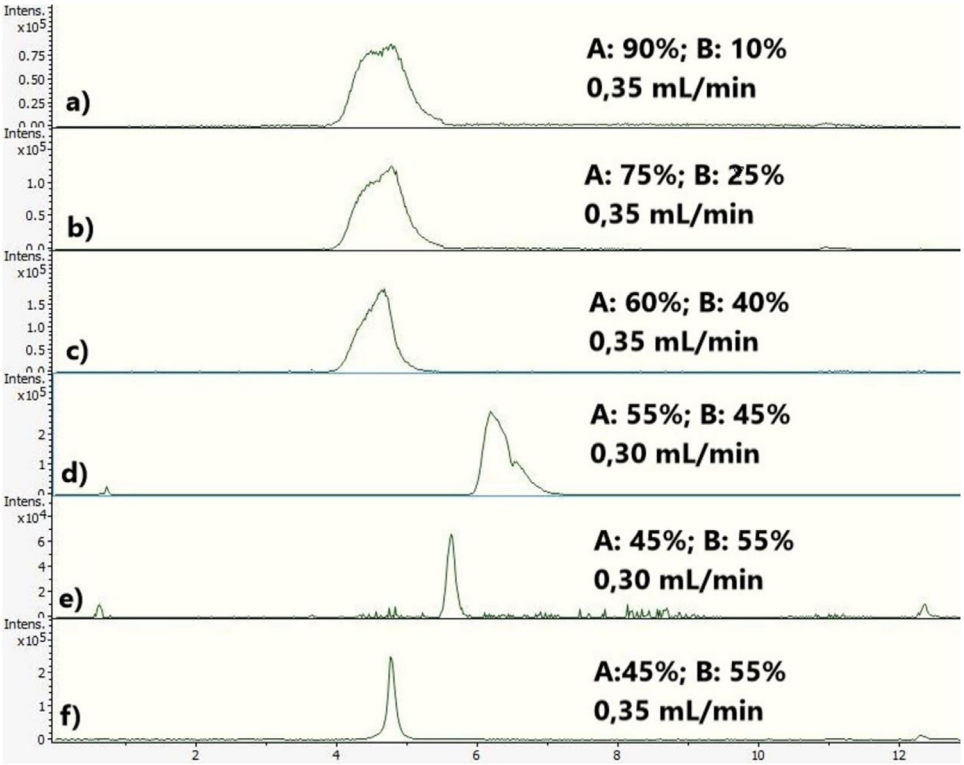

Fig. 2. Changes in the chromatographic signal of DON depending on the initial conditions of ^{2}D separation.

both the elution of all analytes and column re-equilibration under the initial conditions before the next injection. Moreover, the quantification of low molecular and polar compounds under these conditions was impossible.

The determination of DON and its metabolites (15-AcDON and 3-AcDON) in various foodstuffs is a very significant task, because a growing number of publications report their occurrence, in some cases at relatively high concentrations. Fig. 2 shows the optimisation of the initial mobile phase composition for ^{2}D separation (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile) on the chromatographic signal of DON.

It should also be noted that under such conditions it was possible to separate the isomeric analytes 3-AcDON and 15-AcDON, which are usually difficult to simultaneously determine by conventional multi-mycotoxin methods, if their separation from each other is not achieved because of the same molecular mass and the same fragmentation ions in their MS spectrum using MS^2 detection. Some researchers skip this step by describing the concentrations of these compounds as the sum of both isomers [37], but a more complete study on the contamination and objective assessment of possible effects and contributions from both compounds requires their independent quantification. Nevertheless, the retention times of these isomers differed by 0.2 min (5.1 min and 4.9 min), which was sufficient for identification, especially after adding ammonium acetate to the mobile phase, because of the high intensity of ammonium ion adduct for 15-AcDON, which was selected as the quantifying ion.

Since the initial composition of the mobile phase did not significantly influence the separation of high molecular analytes, except for a change in their retention times, it was decided to focus directly on the problematic analytes in an effort to adjust the system parameters to obtain quantifiable analytical signals. Therefore, we started our ^{2}D gradient program with 55% of acidified acetonitrile, ensuring the elution of representative chromatographic peaks. In order to delay the elution of all other compounds from the analytical column in parallel with the compounds analysed in the ^{1}D mode and to overcome peak overlapping, the mobile phase composition after the elution of highly polar compounds was increased to 90% of acidified water and maintained for 2 min. The acetonitrile content was then gradually increased to 98% over 4 min, accelerating the elution of mycotoxins retained on the ^{2}D column. Although the sorption behaviour of these compounds in the analytical column was very similar and the retention times in many cases overlapped, their detection was specific due to the very different masses of quantification ions measured by high resolution mass spectrometry.

The mobile phase flow rate also appeared to be an important factor, which was left equal at 0.35 mL/min in both the ^{1}D and ^{2}D dimensions. The lower flow rate of 0.30 mL/min was tested, but it adversely affected the elution of collected fraction from the ^{1}D on ^{2}D column, broadening the signals of some analytes. The final gradient elution program and valve-switching information for the developed method is shown in Table 1.

3.1.5. Selection of switching time from ^{1}D to ^{2}D separation

During the optimisation of ^{1}D separation, it was obvious that the polar analytes could not be retained on the analytical column with appropriate effectiveness for all of the mycotoxins, and such attempts would prolong the analysis time with no remarkable improvement. Thus, it was decided to collect mycotoxins with short retention times during the ^{1}D separation ($<$2 min) in the sample storage loop for further separation on the ^{2}D column. The collection was performed by switching the valve position in the time interval from 0.5 min until 1.9 min, because all 29 of the highly polar analytes were present in this eluent portion. The generic scheme of switching valve positions is shown in Fig. 1.

The effect of sample loop volume was also studied. Three sample loops of different volume were tested (100 µL, 250 µL, and 500 µL). When testing the 100 µL sample loop, we could collect only a part of the target analytes. Another possibility was using the 250

µL sample loop with the so-called "stop-flow" approach, collecting two eluent portions with interruption of ^{1}D separation in between, but it had the disadvantage of much longer analysis time, as well as the analyte peaks eluted immediately after valve switching during the ^{1}D separation were noticeably broadened. Finally, the 500 µL loop was selected for this study, since the peak widths of target compounds were in the range of 0.6–1.9 min. Corresponding chromatograms of Pu-erh tea samples spiked with DON and its metabolites on different concentration levels obtained during initial experiments by 1D-LC and final outcome of 2D-LC are shown in Supplementary Material (Figs. S1–3).

3.2. Sample preparation and optimisation for the Pu-erh tea matrix

The incidence of mycotoxins has been described for a wide variety of sample matrices, ranging from baby food to spice blends. Pu-erh tea is considered to be a complex matrix, which includes a wide range of different components, depending on the composition of the tea plants and the processing conditions. Thus, one of the most important parameters in the development of this analytical method was to adapt a sample preparation procedure that would be suitable for Pu-erh tea. For this purpose, different extraction solvents and purification techniques were tested.

The most commonly described sample preparation methods (QuEChERS and SPE) for the determination of mycotoxins in samples of plant origin were also approbated and optimised for the purposes of this study.

3.2.1. The application of QuEChERS methodology

According to recent studies devoted to the analysis of mycotoxins in tea matrices, the QuEChERS approach was often used for sample preparation. Therefore, we tested this widely described technique for sample preparation within our study. A QuEChERS method with minimal changes from that reported by Reinholds et al. in 2019 was used [38].

Despite the fact that this extraction and purification procedure showed satisfactory recoveries for emerging mycotoxins and other less polar compounds (such as STC, BFB1, BFA1), the method proved to be unsuitable for the detection of low molecular and polar mycotoxins in Pu-erh tea samples. Quantification was found to be impossible for 49 or approximately 2/3 of the analytes at concentration of 50 µg/kg.

3.2.2. SPE procedure optimisation

Selection of extraction solvent and optimisation of extract clean-up. One of the most important steps during sample preparation, which ensures the overall efficiency of the method, is the choice of an appropriate solvent or solvent system for the extraction of target compounds from the relevant matrix. For mycotoxin extraction, the use of acetonitrile, methanol, ethyl acetate, chloroform, and various mixtures containing these organic solvents has been reported [39,40]. When analysing complex matrices, it should be taken into account that an extra purification step is required for preventing the contamination of mass spectrometer, so different procedures for extractions, as well as different SPE techniques were compared and their appropriateness for direct analysis was evaluated.

Referring to a scientific review on mycotoxin determination in teas [7], the following extraction solvent systems were considered: Acetonitrile/H_2O (84:16, v/v), Acetonitrile/H_2O (50:50, v/v), MeOH/H_2O (50:50, v/v), and EtOAc. When comparing the final extracts, it should be noted that the most cloudy, dark, and intensively coloured extracts were obtained by extraction with acetonitrile mixtures, while the clearest extracts were obtained by EtOAc extraction. As the d-SPE purification according to the QuEChERS approach proved to be unsuitable, it was decided to test sample preparation methods using SPE columns described in earlier articles. The following were selected for comparison purposes: Strata® NH_2, Oasis-MAX, Strata-X, Oasis-HLB, Strata® C_{18}-E, and Discovery® DSC-18 solid phase extraction columns (all column parameters are described in the Materials and Reagents description).

We found that emerging and other less polar mycotoxins can be extracted by acetonitrile NH2systems and EtOAc using all the of the aforementioned SPE columns without preliminary filtration (except for Discovery® DSC-18 and Oasis-MAX), but acetonitrile extracts after the whole sample preparation were still visually opaque. An additional filtration step of the extracts through NH_2-SPE column resulted in chromatographic signal deterioration for ENNs and BEA, as well as the determination of FBs became impossible because of their binding to the sorbent material, which has been also mentioned in an earlier study [41]. On the other hand, this step improved the determination of other compounds by elimination of undesirable matrix components. So, the best solution to improve the determination of the most mycotoxins without significant affecting the ENNs, BEA and FBs determination, is to pass one part of the extract through NH_2-SPE column and leave the rest of extract without additional clean up. During experiments, it was found that the volume of 3 mL is optimal that is needed for filtration.

It should be highlighted that EtOAc extracts became much cleaner after an additional filtration step. Moreover, in agreement with many publications dedicated to mycotoxin determination in different foodstuffs, EtOAc showed comparable extraction efficiency for mycotoxins with a wide range of lgP values [40–45].

Other authors [46] have claimed that the sample preparation procedure based on extraction with EtOAc/FA (99:1, v/v), followed by passing the extract through an NH_2-SPE column with further evaporation, reconstitution with acetonitrile/H_2O (84:16, v /v), and refiltration through C_{18}-SPE was appropriate for the analysis of Pu-erh tea samples. When we performed the same procedure, the expected effectiveness was not achieved, so we replaced the filtration through C18-SPE column by extraction using this SPE. Additionally, Oasis-HLB and Strata-X columns were also tested for this purpose. Every of these three SPE columns was suitable for individual compounds, but the best results were obtained with Strata® C18-E column, giving the best analyte recovery and the largest number of detectable compounds. EtOAc/FA (99:1, v/v) was selected as the extraction solution, resulting in fewer interfering signals in the final chromatograms and giving a clear extract prior to injection, protecting both the LC system and the detector from major contamination.

3.3. Method validation

The elaborated analytical method for the determination of 70 mycotoxins in Pu-erh tea was validated *in-house* with consideration of the criteria and recommendations set in EU legislation and guidelines. The validation was carried out over two days, thus obtaining both inter-day and intra-day data.

3.3.1. Evaluation of matrix effects

Co-extractives originating from the matrix may significantly affect the ionisation of analytes and thereby have an impact on the reliability of the method. The matrix effect was calculated as the percentage (ME,%) of signal enhancement or suppression, according to the equation:

$$ME(\%) = 100 \times \left(\frac{a_m}{a_s} - 1\right) \quad (1)$$

with the slope of the matrix-matched calibration curve denoted as a_m and the slope of the calibration plot with calibration solutions in solvent denoted as a_s.

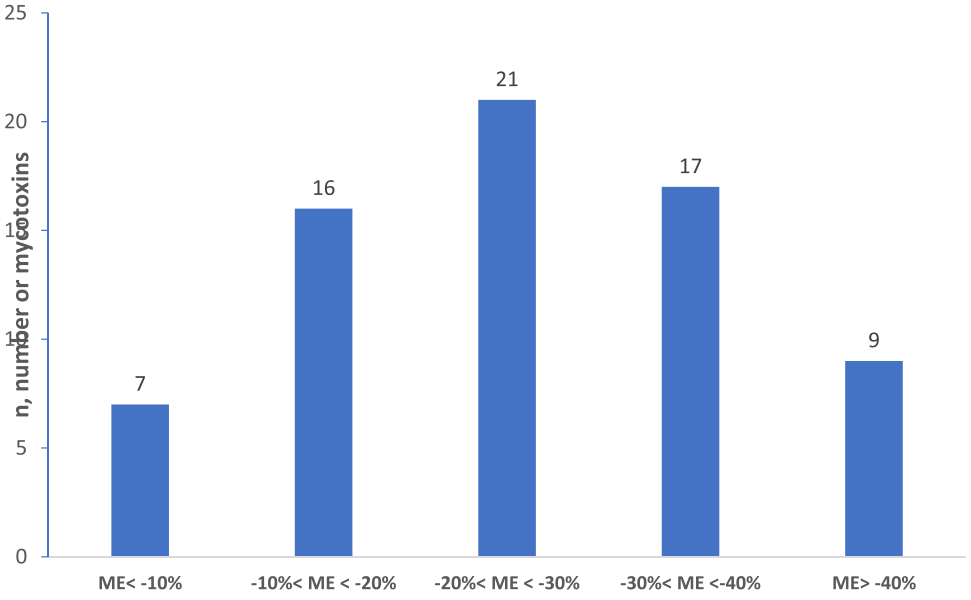

Fig. 3. The matrix effect (ME,%) of Pu-erh tea amongst the analysed mycotoxins.

The calculated matrix effect values were in a good agreement with those observed by other authors. Lower matrix effects were obtained for the mycotoxins that were better retained on the analytical column (ENNs, BEA, BFs, ZEN, etc.), compared to the more polar mycotoxins that were eluted faster (DON, D3G, FA, PA, etc.). None of the analysed mycotoxins showed ion enhancement in Pu-erh tea matrix, while ion suppression was observed for all compounds, being in a range from −4.3% (BFs and emerging mycotoxins) to −62% in the case of MPA. The distribution of matrix effects amongst the analysed mycotoxins is shown in Fig. 3.

Considering the strong adverse matrix effect of Pu-erh tea for most of the analysed mycotoxins, the matrix-matched calibration approach was applied during the further mycotoxin analysis, in order to compensate for the negative effect on the analyte recovery.

3.3.2. Selectivity and linearity

The selectivity of the method was estimated by the analysis of blank matrix samples fortified with mycotoxin standards at LOQ levels. No significant interfering peaks or chromatographic interference effects were observed at the retention times of the targeted compounds. The blank samples of Pu-erh tea are shown along with some examples of Pu-erh tea samples spiked with DON and its related compounds 3-AcDON, D3G, and 15-AcDON that were detected in all of the analysed samples (Supplementary Material, Figure S4).

The linearity of the method was assessed from five-point matrix-matched calibration curves by spiking blank Pu-erh tea samples with standard solutions of mycotoxins. The peak areas of each analyte were plotted against their concentrations and a linear least squares regression model was applied, showing a good linearity ($R^2 > 0.99$). The linear ranges and the respective correlation coefficients are shown in Table 3.

3.3.3. Precision, trueness and uncertainty of the method

The trueness and precision of the method were tested by analysing blank samples spiked with all 70 mycotoxins at low, moderate, and high concentration levels. In particular, 18 spiked samples were processed per day, resulting in a total of 36 spiked samples analysed over two days. The trueness expressed as recovery of mycotoxin determination was calculated from matrix-matched calibrations, indicating that, in turn to the standard solution calibration, it can effectively compensate the matrix effects of Pu-erh tea. The inter-day recovery of mycotoxin determination was in an acceptable range of 83%–116%, as shown in Table 3. However, the individual recovery obtained during the overall validation varied from 79% to 121%.

The precision was expressed as the relative standard deviation (RSD) for both intra-day and inter-day precision experiments. The results of the precision experiments confirmed the good performance of the developed method, and all the values are shown in Supplementary Material (Table S3). The obtained mean RSD values for the between-days validation ranged from 5% to 13%. Compared to the previously reported ^{1}D methods for mycotoxin determination [7,47–49], the parameters obtained in the present study were similar or even superior. A comparison of the validation parameter values to those described in the EU validation guidelines showed that both the trueness and precision met the validation criteria. The uncertainty of the method was estimated by multiplying the intra-day RSD of each mycotoxin by a factor of 2. The uncertainty values for individual mycotoxins ranged from 9% to 28%, which is considered to be an acceptable degree of uncertainty during method evaluation, taking into account the matrix effects and other complications that may affect the final concentration values.

3.3.4. Sensitivity (LODs and LOQs)

The method sensitivity was evaluated by measuring the instrumental limit of quantification (*i*-LOQ) that was determined experimentally as the lowest amount of analyte injected on-column for which the S/N ratio exceeded 10. With regard to the dynamic range of the TOF detector, only signals with intensities above 1×10^4 were used for reliable detection. The *m*-LOQ values were calculated by taking into account the sample preparation procedure and the matrix effects observed during the method validation. The *m*-LOQ and *m*-LOD values were expressed as µg/kg of the raw sample weight, varying from 0.01 to 7.17 µg/kg for *m*-LODs and from 0.04 to 23.9 µg/kg for *m*-LOQs. The obtained *m*-LODs and *m*-LOQs (Table 3) were comparable to those obtained in traditional mycotoxin determination methods based on offline SPE methodology or QuEChERS approach for the analysis of different types of tea, followed by HPLC-MS/MS detection or by other techniques [7,40].

3.4. Analysis of real samples

The applicability of the elaborated *online*-heart-cutting-2D-LC method was assessed through the analysis of 70 mycotoxins in 20

Table 3
Method validation data.

Compound	Linearity range, µg/kg	Linearity, r^2	*i*-LOD, pg	*i*-LOQ, pg	*m*-LOD, µg/kg	*m*-LOQ, µg/kg	*Inter-day* recovery ($n = 36$),%	*Inter-day* RSD ($n = 36$),%	Uncertainty,%
AFB_1	1.0–25	0.998	6.3	21	0.05	0.15	96	9	18
AFB_2	0.25–5	0.9990	3.5	12	0.07	0.24	90	10	19
AFG_1	1.0–25	0.998	7.7	26	0.05	0.18	92	9	17
AFG_2	0.25–5	0.994	10	33	0.08	0.27	93	8	15
AFL	10–250	0.998	250	833	1.44	4.80	100	7	14
AME	10–250	0.994	143	476	1.04	3.45	99	10	20
ANC	1.0–25	0.9991	15	49	0.10	0.33	92	7	13
AN P3	5.0–100	0.9996	77	256	0.47	1.57	93	7	14
ALT	1.0–25	0.9997	46	152	0.30	1.01	92	10	19
AOH	5.0–100	0.996	83	278	0.68	2.25	100	8	16
ATX I	1.0–25	0.9995	19	64	0.13	0.45	95	9	18
API	1.0–25	0.995	21	69	0.11	0.37	100	8	16
BEA	1.0–25	0.9998	9.6	32	0.06	0.19	97	12	24
BFA_1	5.0–100	0.9991	91	303	0.47	1.57	104	9	17
BFB_1	5.0–100	0.9997	143	476	0.77	2.58	99	7	14
BRF A	25–500	0.998	667	2222	3.52	11.7	98	8	16
D3G	25–500	0.9990	250	833	1.90	6.30	96	14	28
DON	25–500	0.996	211	701	1.55	5.15	108	6	11
DTX A	10–250	0.9993	95	317	0.62	2.07	94	6	12
DTX B	10–250	0.997	111	370	0.75	2.52	98	6	11
DTC B	10–250	0.9991	100	333	0.58	1.94	92	7	13
ENN A	1.0–25	0.9993	6.0	20	0.04	0.12	103	8	16
ENN A_1	1.0–25	0.9997	5.0	17	0.03	0.11	103	7	13
ENN B	1.0–25	0.9992	7.8	26	0.05	0.16	102	8	16
ENN B_1	1.0–25	0.9998	14	46	0.08	0.27	97	4	9
CCA	10–250	0.993	154	513	1.00	3.34	104	6	13
CCB	10–250	0.9994	125	417	0.87	2.91	95	7	13
CCC	10–250	0.998	154	513	1.24	4.14	96	8	17
CCD	10–250	0.997	167	556	1.19	3.97	99	7	13
CCE	10–250	0.998	200	667	1.37	4.57	88	6	12
CCH	10–250	0.9991	182	606	1.17	3.92	96	8	15
CCJ	10–250	0.996	133	444	0.97	3.26	94	9	17
CER	10–250	0.9996	154	513	1.16	3.86	94	7	14
CTC	5.0–100	0.9994	83	278	0.50	1.65	91	8	17
CHG A	10–250	0.997	182	606	1.17	3.90	94	7	14
CVD	5.0–100	0.9998	100	333	0.52	1.74	95	6	12
CIT	5.0–100	0.9991	91	303	0.65	2.18	115	7	14
CVL	10–250	0.9990	46	152	0.54	1.81	92	6	12
GT	5.0–100	0.9994	77	256	0.33	1.10	99	5	11
HT-2	5.0–100	0.9996	153	513	1.22	4.07	97	8	16
HA	25–500	0.9998	467	1556	3.03	10.1	96	7	14
FUS-X	5.0–100	0.9992	111	370	0.90	2.30	92	7	13
FA	25–500	0.998	1778	5926	7.17	23.9	88	10	20
FB_1	25–500	0.993	381	1270	2.91	9.69	89	6	13
FB_2	25–500	0.998	471	1569	4.05	13.5	90	7	14
FB_3	25–500	0.9990	333	1111	3.03	10.1	94	5	10
OTA	1.0–25	0.9993	8.3	28	0.07	0.24	93	6	13
OTB	1.0–25	0.9990	7.1	24	0.04	0.13	90	6	13
MEL	5.0–100	0.997	42	139	0.46	1.54	94	7	14
MPA	5.0–100	0.998	25	83	0.33	1.10	96	7	15
MYR	5.0–100	0.9992	46	152	0.31	1.02	97	7	13
NEO	1.0–25	0.992	31	104	0.27	0.90	93	6	13
PA	25–500	0.9997	615	2051	2.69	8.95	96	7	15
PN A	1.0–25	0.996	11	37	0.06	0.21	98	7	14
PXL	25–500	0.9995	250	833	1.44	4.79	102	5	11
ROQ-C	1.0–25	0.997	1.4	4.7	0.01	0.04	96	8	16
SBL	10–250	0.9997	125	417	0.77	2.57	95	9	17
STC	1.0–25	0.9996	13	42	0.08	0.26	104	8	17
T-2	5.0–100	0.9992	167	556	1.22	4.07	94	7	14
T-2TET	5.0–100	0.9992	83	278	0.49	1.63	92	6	11
T-2TRI	10–250	0.997	133	444	0.81	2.69	90	5	10
TNX	5.0–100	0.9990	59	196	0.38	1.27	91	8	16
VCL	5.0–100	0.9998	46	152	0.48	1.61	96	7	15
ZEN	5.0–100	0.9993	100	333	0.69	2.29	96	8	16
15-AcDON	25–500	0.998	381	1270	5.77	19.2	96	7	13
15-AcS	1.0–25	0.9998	23	78	0.14	0.46	103	6	11
17-AAG	5.0–100	0.9990	91	303	0.59	1.97	94	6	13
17-DMAG	5.0–100	0.997	71	238	0.63	2.12	90	7	13
3-AcDON	25–500	0.994	615	2051	4.23	14.2	95	6	13

i-LOD – the instrumental limit of detection, assessed from the analysis of pure standard solutions, expressed as pg per column;.
i-LOQ – the instrumental limit of quantification, assessed from the analysis of pure standard solutions, expressed as pg per column;.
m-LOD – the method limit of detection;.
m-LOQ – the method limit of quantification;.

Table 4
The occurrence and concentrations of mycotoxins in the analysed Pu-erh tea samples.

Mycotoxin	Positive samples	Positive,%	Concentration, µg/kg		
			Average	Minimum	Maximum
15-AcDON	20/20	100	1154	113	3601
3-AcDON	20/20	100	1573	145	4197
AFB1	8/20	40	2.6	1.1	9.2
AFB2	7/20	35	0.87	0.33	2.76
AFG1	8/20	40	2.2	0.99	7.9
ALT	9/20	45	2.7	1.3	18.9
ALX I	10/20	50	15.5	1.4	42
CER	15/20	15	15.3	11.6	19.2
D3G	20/20	100	1007	122	2252
DON	20/20	100	2313	145	8946
ENN A	10/20	50	17.5	0.69	115
ENN A1	5/20	25	23.9	3.8	91.8
ENN B	11/20	55	22.3	1.3	143
ENN B1	11/20	55	22.3	0.5	125
OTA	5/20	25	1.7	0.85	4.1
OTB	12/20	60	11.3	0.76	30.2
STC	8/20	40	21.7	1.2	93.4
T-2	10/20	50	11.5	4.7	18.9
ZEN	7/20	35	23.8	4.7	56.1

Pu-erh tea samples purchased from the Latvian retail market and specialised tea houses. The observed concentrations of mycotoxins in tea samples (Supplementary Material, Table S4) varied from values below the *m*-LOQ, which were not included in the summary, up to 8946 µg/kg for DON, representing a broad range of mycotoxin contamination. A summary of the positive sample percentages, minimum, maximum, as well as the average concentrations of 19 out of 70 analysed mycotoxins found in Pu-erh tea samples are presented in Table 4.

When comparing the concentrations of actually observed mycotoxins in Pu-erh teas, there were significant differences depending on the compound. As shown in Table 4, a total of 19 mycotoxins were detected, including both regulated and less known contaminants. Despite the large number of literature sources reporting the detection of contaminants in Pu-erh tea, a relatively limited number of articles have been focussed specifically on multi-mycotoxin analysis of this matrix.

The results of the present research also include the detection of potentially carcinogenic compounds. The contamination with three aflatoxins (AFB_1, AFB_2, and AFG_1) was detected in 30–40% of the samples, reaching the highest concentration in the case of AFB_1 (9.2 µg/kg).

In the present study, OTA was detected in 25% of the samples, with the highest concentration of 4.1 µg/kg, which is a relatively low value compared to precedents described in the literature [50,51]. Similarly, OTB was also detected during our study, but its incidence in the Pu-erh teas has not yet been reported, since none of the methods discussed in the literature included the determination of OTB.

Three of twenty samples contained cerulenin at the mean concentration of 15.3 µg/kg, but these results are not comparable to literature due to the lack of studies including the determination of this compound, with only its recurrent occurrence in rice and related crop products reported [52]. In our study, 35% of analysed samples were positive for ZEN and 40% were positive for STC. It is important to note that STC is a mycotoxin produced by certain species of *Aspergillus* and is structurally related to the aflatoxins that are also generated by the same fungi. This association can explain the similar frequency of occurrence for both of these toxins in the analysed Pu-erh tea samples. Moreover, the highest concentrations of STC (93.4 µg/kg) were found in the Pu-erh tea sample that was the most contaminated with AFBGs.

The emerging *Fusarium* mycotoxins (ENNs) and two *Alternaria* toxins (ATX I and ALT) were also identified in the analysed samples. The number of studies on these mycotoxins is very limited and do not describe their occurrence in Pu-erh teas. In our study the concentrations of ENNs found in Pu-erh teas varied from 0.5 µg/kg (ENN B_1) to almost 143 µg/kg for ENN B. The samples that were contaminated with ENN B also contained ENN B_1 but in smaller amounts (the maximal value for ENN B_1 was 125 µg/kg). Comparing ENN A and ENN A_1, the occurrence of the second one is only one half as common, as it was found in 25% of the samples.

Both of the identified *Alternaria* mycotoxins occurred in almost half of the samples (45–50%). The only literature source for comparing the occurrence of ALT is a study focussed on the analysis of 91 tea samples, reporting the determination of 27 mycotoxins [46]. No other reported methods included the determination of ALT or ATX I. The observed concentrations were not high and, in line with the absence of regulations, apparently presented no risk to consumers. Nevertheless, the concentrations detected were covered a wide range from 1.3 to 18.9 µg/kg in the case of ALT and from 1.4 to 42 µg/kg in the case of ATX I. The occurrence of *Alternaria* mycotoxins in the previously untested matrices invites further studies for accumulating incidence data and for enabling a more careful assessment of possible risks.

Finally, a widespread contamination of Pu-erh tea with DON and its related compounds (D3G, 15-AcDON, and 3-AcDON) was observed. The concentrations of the aforementioned mycotoxins ranged from 113 µg/kg for 15-AcDON up to almost 8946 µg/kg for DON. Thus, the maximum concentration for these *Fusarium* toxins exceeded 2000 µg/kg, which is comparable to the results for DON obtained by other researchers. [38,51].

4. Conclusions

For the first time, an analytical method for the simultaneous identification and quantification of 70 regulated and emerging mycotoxins has been developed for Pu-erh tea using *online* heart-cutting 2D ultra-high performance liquid chromatography coupled to high-resolution ToF mass spectrometry. The developed method was successfully validated in accordance with the performance criteria set in EU guidelines and applied to the analysis of 20 commercially available Pu-erh tea samples. The occurrence study revealed actual contamination with 19 out of the 70 analysed mycotoxins. The detected concentrations ranged from 0.5 (for ENN B_1) to 8946 µg/kg (DON).

Conflicts of Interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461145.

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