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Optimization and validation of a quantitative liquid chromatography–tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices

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ABSTRACT

An LC–MS/MS "dilute and shoot" method for the determination of 295 fungal and bacterial metabolites was optimized and validated according to the guidelines established in the Directorate General for Health and Consumer Affairs of the European Commission (SANCO) document No. 12495/2011. Four different types of food matrices were chosen for validation: apple puree for infants (high water content), hazelnuts (high fat content), maize (high starch and low fat content) and green pepper (difficult or unique matrix). Method accuracy and precision was evaluated using spiked samples in five replicates at two concentration levels. Method trueness was demonstrated through participation in various proficiency tests. Although the method covers a total number of 331 analytes, validation data were acquired only for 295 analytes, either due to the non-availability of analytical standards or due other reasons described in this paper. Concerning the apparent recovery, the percentage of 295 analytes matching the acceptable recovery range of 70–120% lied down by SANCO varied from 21% in green pepper to 74% in apple puree at the highest spiking level. At the levels close to limit of quantification only 20–58% of the analytes fulfilled this criterion. The extent of matrix effects was strongly dependent on the analyte/matrix combination. In general, the lowest matrix effects were observed in apple puree (59% of analytes were not influenced by enhancement/suppression at all at the highest validation level). The highest matrix effects were observed in green pepper, where only 10% of analytes did not suffer from signal suppression/enhancement. The repeatability of the method was acceptable (RSD $\leq$ 20) for 97% of all analytes in apple puree and hazelnuts, for 95% in maize and for 89% in green pepper. Concerning the trueness of the method, *Z*-scores were generally between −2 and 2, despite a broad variety of different matrices. Based on these results it can be concluded that quantitative determination of mycotoxins by LC–MS/MS based on a "dilute and shoot" approach is also feasible in case of complex matrices.

1. Introduction

Mycotoxins are defined as low-molecular-weight natural products produced as secondary metabolites by fungi. By definition, they are toxic to vertebrates and other animal groups in low concentrations, causing acute as well as chronic diseases [1]. Mycotoxins exhibit a great diversity in their chemical structure, which explains that their toxicities and target organs also vary [2]. Over the years, health concerns related to mycotoxins have increased [3] and several regulations have been set into force to control the maximum levels of mycotoxins in food and feed in many countries. For instance, the European Union has laid down maximum levels for certain mycotoxin-matrix combinations in Commission Regulation 1881/2006/EC [4]. Regulations are based on the evaluation of risk assessment (hazard and exposure) but also reflect agriculturally achievable levels in different foodstuffs. As exposure assessment is an important aspect of risk assessment, validated analytical methods and the implementation of analytical quality assurance are necessary to provide a reliable assessment on the toxin intake [5].

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The complexity of food samples together with the low concentrations at which contaminants usually occur require highly sensitive, selective and reliable analytical techniques [6].

During the last decade the coupling of liquid chromatography (HPLC or UHPLC) to tandem mass spectrometry (MS/MS) has enabled the development of highly selective, sensitive and accurate methods for mycotoxin determination. Several methods have been published for the identification and accurate quantification of single or chemically related mycotoxins in several matrices [7]. However, different classes of mycotoxins are often found to co-occur as (i) some fungal species are capable to produce different classes of mycotoxins and (ii) susceptible commodities can be affected by several fungi if the environmental conditions (temperature, water activity) favor their growth. Therefore, different analytical methods are often employed to cover all mycotoxins addressed by the regulations. The techniques used are based on TLC, HPLC-UV, HPLC-fluorescence frequently in combination with time consuming purification step or immunochemical methods such as ELISA [8].

The development of LC–MS/MS based multi-mycotoxin methods tries to overcome the need for sophisticated clean-ups and/or multiple analytical techniques, although the chemical diversity of mycotoxins is a big obstacle to be overcome [2]. For instance, extraction of a wide range of target compounds from a variety of matrices has to be realized. Studies on generic extraction methods for multiple contaminants in different food and feed matrices have demonstrated that mixtures of acidified water with organic solvents (methanol, acetonitrile or acetone) are the most suitable extraction solvents [9,10].

Every clean-up step and even a rather unspecific QuEChERS-like approach [11] limits the number of analytes as some of the target substances might not be amenable to the chosen procedure [12]. On the other hand, reducing the sample clean-up to a minimum (i.e. injection of raw extracts) will result in suppression or enhancement of the analyte response during the ionization process. The influence of these matrix effects is the major challenge in developing reliable quantitative multi-analyte methods [13]. Therefore, considerable efforts to control matrix effects should be carried out to obtain accurate results. The use of stable isotope dilution assays (SIDA) seems to be the best alternative to correct matrix effects. Several methods have been validated using isotopically labeled internal standards [14–16]. However, the limited availability of labeled internal standards for non-regulated toxins and the comparably high costs of isotopically labeled standards are the main drawbacks. Another common approach to deal with matrix effects is the compensation of the signal suppression/enhancement through the usage of matrix matched standards (i.e. blank sample extracts fortified with an appropriate amount of a multi-analyte standard). Here the availability of analyte-free samples (which is especially difficult for certain analyte/matrix combinations such as deoxynivalenol in maize) and repeatable extraction efficiencies as well as matrix effects for all individual samples of a given commodity are the major challenges [17–22].

To assure reliable quantification at a high level of trueness, in-house validation has to be performed, preferably according to international guidelines. The SANCO document for the development of multi-analyte methods in pesticides residue analysis recommends that at least one representative commodity from each commodity group shall be validated and evidence for fitness of purpose shall be provided [22]. This approach has been successfully applied in the field of pesticide analysis [23–25] but has hardly been employed for methods devoted to mycotoxins, for which most methods focus on single commodities (mainly grain-based matrices). However, a few examples can be found for multi-mycotoxin methods which have been validated for a wider range of matrices [9,10,18,26–28].

The aim of this work was to evaluate the performance of a multi-analyte method for mycotoxins and other fungal as well as bacterial metabolites. Furthermore, a validation procedure in accordance to SANCO No. 12495/2011 was developed and applied to four model matrices. The range of analytes finally covered a total of 295 secondary metabolites for which validation data are presented in four different matrices. The model matrices were chosen as representative commodities belonging to the respective commodity groups according to SANCO (each commodity group includes matrices of similar properties). Another aspect of selection was the relevance of the matrix with respect of mycotoxin contamination, i.e. the commodities which are commonly contaminated with mycotoxins. Therefore, apple puree for infants (high water content), hazelnuts (high fat content), maize (high starch or protein content, low fat content) and green pepper (complex matrix) were chosen. In case of the mycotoxins addressed by regulations, the comparability of the method was verified through the participation in proficiency tests.

2. Material and methods

2.1. Chemicals and reagents

LC gradient grade methanol and acetonitrile as well as MS grade ammonium acetate and glacial acetic acid (p.a.) were purchased from Sigma–Aldrich (Vienna, Austria). A Purelab Ultra system (ELGA LabWater, Celle, Germany) was used for further purification of reverse osmosis water.

Standards of fungal and bacterial metabolites were obtained either as gifts from various research groups or from the following commercial sources: Romer Labs®Inc. (Tulln, Austria), Sigma–Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland) and LGC Promochem GmbH (Wesel, Germany). Stock solutions of each analyte were prepared by dissolving the solid substance in acetonitrile (preferably), acetonitrile/water 1:1 (v/v), methanol, methanol/water 1:1 (v/v) or water. Thirty-four combined working solutions were prepared by mixing the stock solutions of the corresponding analytes for easier handling and were stored at −20 °C. The final working solution was freshly prepared prior to spiking experiments by mixing of the combined working solutions.

2.2. Samples

Four samples of different matrix complexity were chosen for the method validation. Apple puree was taken as a high water containing matrix. Matrices with high fat content were represented by hazelnuts, and cereals and high starch matrices by maize. Green pepper was used as a model matrix for the validation of “difficult and unique commodities” [22].

The following proficiency testing samples were used for the verification of the method trueness: (i) FAPAS® testing materials – peanuts (T01044), maize (T2246, T2262), cereals (T1786) and cereal breakfast (T2257) provided by The Food and Environment Research Agency (York, United Kingdom); (ii) Proficiency Testing Scheme samples – peanut cake (04-0231), peanut paste (02-1331, 04-1331), animal feed (02-3031, 03-3031, 04-3031), wheat (05-0631, 03-2331), wheat draff (02-2831), pepper (01-1031, 01-3231), raisins (02-3131), maize (04-0731, 05-0731, 03-0731) milk powder (04-0331), coffee (02-1731), baby food (01-3331, 01-3431), pistachio paste (03-1431), liquorice (01-3531), oat (02-2931) were obtained from Bipea (Gennevilliers, France); (iii) CODA-CERVA proficiency test (oat flour) from 2013 organized by the Belgian National Reference Laboratory for Mycotoxins in Food and Feed.

2.3. Sample preparation

Model samples were ground using an Osterizer blender (Sunbeam Oster Household Products, Fort Lauderdale, Florida, USA). For spiking the model matrices, appropriate amounts of the final working solution were added to 0.5 g of sample. The samples were placed at darkness to avoid analyte degradation and stored overnight at room temperature to allow the evaporation of the solvent and to establish equilibration between analytes and matrix. After this period, 2 mL of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) was added. The samples were extracted for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and subsequently centrifuged for 2 min at 3000 rpm (radius 15 cm) on a GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA). The extracts were transferred into glass vials using Pasteur pipettes, and 350 μL aliquots were diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v). After appropriate mixing, 5 μL of the diluted extract was injected into the LC–MS/MS system without further pre-treatment. It should be noted that the whole procedure was miniaturized only for validation purposes in order to decrease the amount of standards needed for spiking. In routine analysis, 5 g of sample is extracted with 20 mL of extraction solvent.

2.4. Method validation

2.4.1. Spiking experiments and preparation of calibration curves

As outlined above, method validation was performed according to SANCO validation criteria [22]. For the determination of the performance characteristics of the method for all four model matrices, spiking experiments were carried out at four different concentration levels (each in five repetitions) resulting in the relative concentrations of 1:3:10:30 in the final diluted extracts. The concentration ranges of the spiked samples were chosen to cover the respective limits of detection of each toxin, estimated linear range of calibration, legislation limits of regulated toxins, as well as the levels commonly found in naturally contaminated samples. External calibration was prepared by dilution of appropriate amounts of the final working solution with acetonitrile/water/acetic acid (49.5/49.5/1, v/v/v) at levels corresponding to those in spiked samples. Taking into account the matrix induced signal enhancement or suppression of target analytes, two additional calibration points (one above and one below the spiking concentration range) were prepared to ensure that all spiking levels fall into the calibration range. For the assessment of matrix effects and extraction efficiency (recovery), the diluted extracts (blank extracts) of each model matrix prepared according to the Section 2.3 were fortified at the concentration range matching the external calibration.

2.4.2. Data evaluation

The peaks were integrated and linear, $1/x$ weighted, calibration curves were constructed from the data obtained from the analysis of each sample type (spiked sample, neat solvent standard, spiked extract) using MultiQuant™2.0.2 software (AB Sciex, Foster City, California, USA) to evaluate the linearity of the method. Further data evaluation was carried out in Microsoft Excel 2007. All the other performance characteristics of the method (recovery, apparent recovery, repeatability and matrix effects) were evaluated at each spiking level for all model matrices. First of all, the average value from the peak areas of five replicates of spiked samples was calculated. Recovery (R_E) of the extraction step and the apparent recovery (R_A) were obtained by comparing the average area of the spiked samples ($n = 5$) to the average area of two replicates of matrix-matched standard and neat solvent standard, respectively, measured at the beginning and at the end of the set of the respective matrix. Matrix induced enhancement or suppression (SSE) was assessed by comparison of respective matrix-matched standards with the neat solvent standards. All the calculations were performed according to the following equations:

Recoveries (extraction efficiency) were calculated according to the Equation (1).

$$R_E(\%) = \frac{\text{average area (spiked samples)}}{\text{average area (matrix matched standard)}} \times 100 \quad (1)$$

Apparent recoveries (absolute recoveries of the method) were calculated as follows:

$$R_A(\%) = \frac{\text{average area (spiked samples)}}{\text{average area (neat solvent standard)}} \times 100 \quad (2)$$

Matrix effects were expressed as SSE

$$\text{SSE}(\%) = \frac{\text{average area (matrix matched standard)}}{\text{average area (neat solvent standard)}} \times 100 \quad (3)$$

The repeatability of the method was expressed as the relative standard deviation (RSD) calculated from five replicates of the spiked samples. Concerning the limits of quantification (LOQ), they were taken as the lowest validated spiking levels (LL) for which the method performance acceptability criteria were still met, i.e. mean recovery for each representative commodity in the range of 70–120%, with an RSD ≤ 20% [22]. In addition, the more tedious "classic" approach based on the signal to noise ratios (S/N) of 10/1 [29] was applied only to 29 most important analytes including all mycotoxins addressed by EU regulations and several other prevalent fungal metabolites. In this case, the limits of detection (LODs) and the LOQs were estimated with respect to the signal of the less intensive (LOD) and more sensitive (LOQ) selected reaction monitoring (SRM) transition, repectively. S/N ratios were assessed at the lowest reliably visible concentration level of the spiked samples individually for each of the five replicates. LODs and LOQs were calculated from the average of S/N ratios as follows:

$$\text{LOD}\left(\frac{\mu g}{kg}\right) = \frac{\text{spiking concentration}}{\text{average of } S/N} \times 3 \quad (4)$$

$$\text{LOQ}\left(\frac{\mu g}{kg}\right) = \frac{\text{spiking concentration}}{\text{average of } S/N} \times 10 \quad (5)$$

2.5. Instrumental parameters

Detection and quantification was performed with a QTrap 5500 MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboV electrospray ionization (ESI) source and a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C_{18}-column, 150 × 4.6 mm i.d., 5 μm particle size, equipped with a C_{18} security guard cartridge, 4 × 3 mm i.d. (all from Phenomenex, Torrance, CA, US). Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 50% within 3 min. Further linear increase of B to 100% within 9 min was followed by a hold-time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The flow rate was 1000 μL/min. ESI-MS/MS was performed in the scheduled selected reaction monitoring (sSRM) mode both in positive and negative polarities in two separate chromatographic runs. The sSRM detection window of each analyte was set to the respective retention time ±27 s and ±42 s in positive and in negative mode, respectively. The target scan time was set to 1 s. The settings of the ESI source were as follows: source temperature 550 °C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of

nitrogen), ion-spray voltage −4500 V and +5500 V, respectively, collision gas (nitrogen) medium. The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standards (diluted in a 1:1 mixture of eluent A and B) into the MS source using a syringe injection at a flow rate of 10 μL/min, see Appendix A for the corresponding values. The acquisition of two sSRM transitions per analyte (with the exception of moniliformin and 3-nitropropionic acid, that each exhibit only one fragment ion), allowed to confirm the identity of the positive results according to validation guidelines [22,30].

3. Results and discussion

3.1. Method extension and the optimization of the LC–MS/MS parameters

A multi-analyte LC–MS/MS method based on a "dilute and shoot" approach was originally designed for the determination of 39 mycotoxins in cereals in 2006 [17]. Since then the method has continuously been extended to a wide range of additional secondary metabolites of fungi and bacteria. This multi-analyte approach was applied to study newly isolated fungal species, to investigate fungal and bacterial metabolites in indoor environments and to study the spectrum of (toxic) secondary metabolites to which humans and animals are exposed through the food and feed chain [12,27,28]. Although in the meantime UHPLC has become available and has been successfully used in mycotoxin analysis to achieve lower LODs and shorter analysis time [19,21,26] we have refrained from changing from HPLC to UHPLC for two reasons. On the one hand, UHPLC columns are not compatible to turbid samples (as they tend to clog after a few injections) whereas we did not face such problems when using standard HPLC column (we found filtration not to be an option as all tested materials caused losses of certain compounds). In addition on the other hand, the use of UHPLC is related to narrower peaks, which demands a decreased cycle time in LC–MS/MS in order to obtain an appropriate number of data points per peak. This inevitable emphasizes the problem of achieving sufficient dwell times in multi-analyte methods.

One major limitation of multi-analyte analytical methods in repeatable quantification is the time that is available for data acquisition of each SRM transition. In the first place we transferred our previously published method which covered 186 secondary metabolites [28] from a QTrap 4000 to a QTrap 5500 before we extended the method to a greater range of analytes. On the one hand, the QTrap 5500's innovations in the mechanical design (larger orifice entrance, RF-quadrupole QJet, curved collision cell) as well as in the ion path electronics allow higher sensitivity. On the other hand, also an increase in the number of sSRM transitions per chromatographic run (lower dwell time among sSRM transitions) without loss of sensitivity can be achieved due to a higher acquisition speed.

The differences in the mechanical design between the instruments are mainly associated with changes in the ion source parameter settings (curtain gas, ion spray voltage, source temperature, ion source gases). The flow rates of all gases were increased to ensure an efficient evaporation of the mobile phase, and thus avoid passing of liquid through the large orifice entrance. Regarding analyte-dependent MS/MS parameters (declustering potential, collision energy, cell exit potential), we followed the manufacturer's recommendations to increase the declustering potentials (DP) of $[M+H]^+$ and $[M-H]^-$ optimized on the QTrap 4000 by 30 V instead of individual re-optimization of each analyte. Nevertheless, we chose 30 analytes for the individual re-optimization on the QTrap 5500 to check the difference in analyte-dependent parameters values between both instruments. The optimized '5500' DP values of $[M+H]^+$ ions were all 20–30 V higher compared to those on the QTrap 4000. For instance, the DP value of $[M+H]^+$ of ochratoxin A was increased from 61 V to 86 V. The differences for NH_4^+ adducts (T-2 toxin: DP 4000 = 76 V and DP 5500 = 101 V) and Na^+ adducts were lower than the recommended 30 V (HT-2: DP 4000 = 46 V and DP 5500 = 56 V). Similarly, the formation of $[M+CH_3COO]^-$ adducts (deoxynivalenol: DP 4000 = −40 V and DP 5500 = −60 V; 3-acetyldeoxynivalenol: DP 4000 = −45 V and DP 5500 = −55 V)

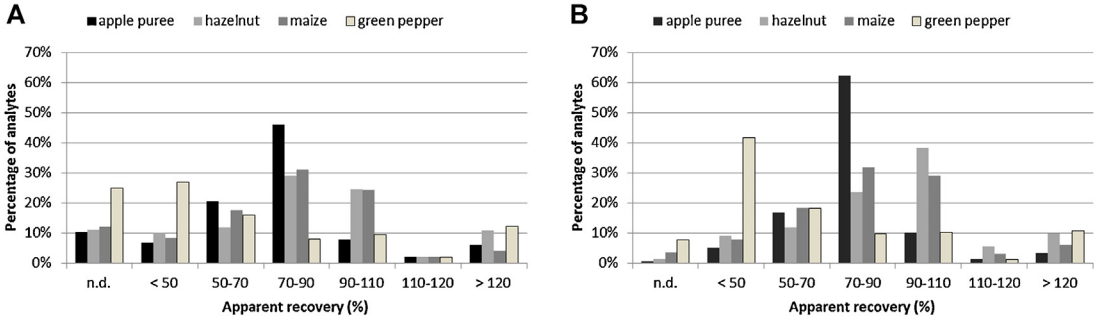

Fig. 1. Distribution of apparent recoveries through the set of 295 analytes (A) at the lowest level, (B) at the highest level.

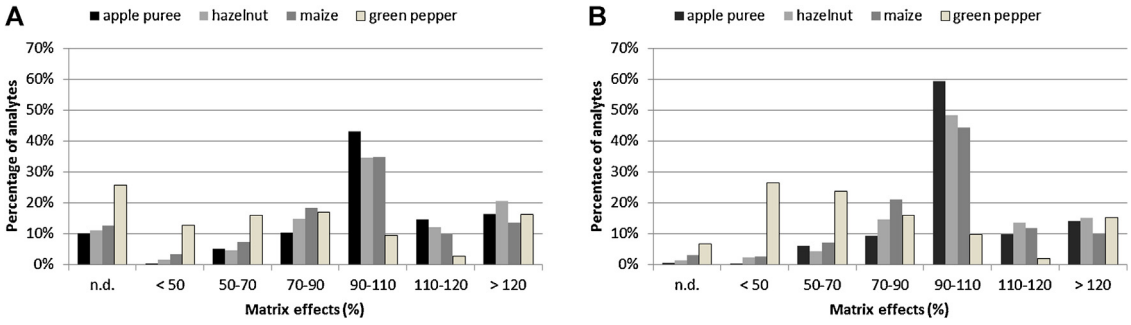

Fig. 2. Distribution of matrix effects through the set of 295 analytes (A) at the lowest level, (B) at the highest level.

Table 1
Performance characteristics of the method for some important analytes in apple puree.

Analyte	RT (min)	LOD (μg/kg)	LOQ (μg/kg)	LL (μg/kg)	R_E (%)	SSE (%)	R_A (%)	RSD (%)	HL (μg/kg)	R_E (%)	SSE (%)	R_A (%)	RSD (%)
3-nitropropionic acid	3.0	0.6	1.9	14.4	80	89	71	7.3	480	78	88	53	8.1
Aflatoxin B_1	8.7	0.6	1.9	1.3	80	103	82	8.0	44	71	107	76	7.3
Aflatoxin B_2	8.4	1.2	4.0	1.3	58	99	57	13.4	44	78	84	65	8.7
Aflatoxin G_1	8.0	2.3	7.6	1.3	58	70	41	18.6	44	70	69	48	7.4
Aflatoxin G_2	7.7	2.6	8.7	1.3	72	141	88	21.6	44	79	92	72	4.5
Aflatoxin M_1	7.2	0.6	2.1	3.4	63	113	71	10.2	114	74	103	76	3.4
Alternariol	11.0	0.5	1.6	2.3	80	107	86	3.3	77	81	108	87	2.1
Alternariol monomethylether	12.8	0.1	0.2	2.3	88	100	88	9.7	77	81	104	84	1.7
Beauvericin	14.4	0.02	0.1	0.1	74	105	78	2.8	4	74	107	79	7.0
Chanoclavine	5.7	0.1	0.3	0.2	81	101	297	2.7	6	77	85	66	3.6
Citrinin	11.8	59.3	197.6	6.9	13	126	14	5.6	231	7	125	9	8.5
Deoxynivalenol	5.6	12.7	42.2	15.1	75	107	80	3.3	629	81	111	90	3.5
Diacetoxyscirpenol	8.5	0.8	2.6	4.7	147	107	129	15.5	155	80	102	81	7.0
Emodin	14.3	0.1	0.4	2.3	73	100	73	3.8	55	77	102	79	3.7
Enniatin B	14.0	0.006	0.021	0.1	91	151	137	13.2	2	82	103	84	2.8
Enniatin B1	14.3	n.e.	n.e.	0.1	105	167	174	8.8	5	80	110	88	4.4
Enniatin A	14.9	n.e.	n.e.	0.1	113	422	475	8.3	0.3	92	142	131	8.8
Enniatin A1	14.6	n.e.	n.e.	0.1	93	378	351	3.3	1.8	87	122	106	5.4
Ergocryptine	8.1	1.5	4.8	2.6	87	71	62	16.9	9	59	126	74	12.9
Ergocryptinine	9.4	0.1	0.4	1.9	66	97	69	5.0	6	74	91	67	7.5
Fumonisin B_1	9.4	2.6	8.6	17.0	72	108	73	3.4	565	76	108	82	3.9
Fumonisin B_2	11.3	2.8	9.2	17.1	69	131	71	6.0	569	78	103	80	2.8
Fumonisin B_3	10.3	2.1	6.9	0.9	72	103	87	20.6	9	77	107	83	5.9
HT-2 toxin	9.7	8.8	29.2	1.6	65	126	81	11.4	155	82	103	84	6.5
Moniliformin	3.2	4.9	16.2	9.2	106	135	143	17.8	306	97	141	137	8.2
Mycophenolic acid	10.7	2.2	7.3	6.5	43	92	58	15	215	80	99	79	4.3
Nivalenol	4.8	2.5	8.3	4.7	89	169	150	6.2	155	67	143	96	5.8
Ochratoxin A	11.9	1.2	3.8	3.9	80	96	76	5.4	130	73	107	78	9.7
Patulin	4.9	35.9	119.7	36.8	77	100	77	5.3	369	82	100	82	4.8
Phomopsin A	7.2	n.e.	n.e.	35.1	n.d.	n.d.	n.d.	n.d.	117	75	96	72	11.8
Phomopsin B*	7.0	n.e.	n.e.	1:30	61	119	73	9.3	1:10	77	103	79	5.5
Sterigmatocystin	12.3	0.2	0.8	2.3	71	111	79	7.7	23	76	104	79	3.4
T-2 toxin	10.7	1.0	3.3	4.6	96	93	90	9.4	154	79	102	80	2.9
Zearalenone	11.9	0.3	1.0	15.5	81	146	118	8.6	155	98	104	101	5.9

Note: RT – retention time; LOD – limit of detection; LOQ – limit of quantification; LL – lowest validation level; HL – highest validation level; R_E – recovery of extraction step; R_A – apparent recovery; SSE – signal suppression/enhancement; n.e. – not evaluated; n.d. – not detected.
* Concentration of the standard not known.

required less than 30 V increase in DP values. However, the increase of all '4000' DPs by 30 V was sufficient, as the optimal range of '5500' DPs of each respective analyte is broader compared to the '4000' instrument. The settings of other analyte-dependent parameters (e.g. collision energy, CE) were the same for both instruments and did not require any further optimization.

Due to the lower QTrap 5500 acquisition mass range of 1250 Da, the analytes of molecular weight higher than 1200 Da had to be excluded from the current method or, if possible, re-optimized as double charged precursor $[M+2H]^{2+}$ ions. For example, actinomycin D (MW 1255.42 Da) does not give a stable $[M+2H]^{2+}$ ion, and could thus not be included in the new method. However, cyclosporine A was successfully re-optimized and transferred from the negative to the positive mode giving an intensive precursor ion of m/z of 601.9. Apart from 186 analytes involved in our previous QTrap 4000 method, further 145 analytes were newly included. The list of all analytes together with the optimized values of ESI-MS/MS parameters is given in Appendix A.

Finally, the developed method accommodated a total number of 331 analytes including 288 fungal and 43 bacterial secondary metabolites and according to the availability of the analytical standards was finally validated for a total of 295 analytes.

As the dwell times in the sSRM mode are automatically generated by the software based on the number of SRM transitions scheduled for a particular point in time and the target cycle time, it is recommended to limit the number of co-eluting compounds (concurrent sSRM transitions). Due to the large number of analytes that are scanned in the positive mode the LC gradient had to be re-optimized to achieve a better distribution of the related sSRM detection windows and thus a more effective utilization of the acquisition time. A steeper gradient elution within 2nd and 5th min in connection with gradient flattening between 5th and 14th min led to a more favorable distribution of sSRM transitions with the exception of the period between 7th and 9th min. For this reason, a few analytes (gibberellic acid, meleagrin, agistatin B and altenuene) eluting in this period were transferred to the negative ionization mode in which an acceptable sensitivity was achieved as well.

3.2. Validation of the method

Currently, no directive or guidance for the validation of analytical methods for the determination of multiple mycotoxins or for multiple analytes in general is established. The only available guideline, the Commission Decision 2002/657/EC [30], provides some requirements and recommendations concerning the performance of analytical methods for official control and the interpretation of results. However, the guidance provided is insufficient for multi-analyte methods for a couple of reasons: a definition of matrix effects and their evaluation is missing, the term recovery is not exactly specified (whether it is extraction efficiency or apparent recovery), and the determination of LOD and LOQ by spiking of 20 replicates at one level for each matrix is not feasible for hundreds of analytes due to the costs of analytical standards. Therefore, we decided to validate the given multi-analyte method according to SANCO protocol No. 12495/2011 [22]. Although the validation criteria have been laid down for pesticide multi-residue determination only, they represent the only "real-life" guidelines available for methods involving hundreds of analytes with a wide range of physico-chemical properties. Since the method is used for

Table 2
Performance characteristics of the method for some important analytes in hazelnut.

Analyte	RT (min)	LOD (μg/kg)	LOQ (μg/kg)	LL (μg/kg)	R_E (%)	SSE (%)	R_A (%)	RSD (%)	HL (μg/kg)	R_E (%)	SSE (%)	R_A (%)	RSD (%)
3-nitropropionic acid	3.0	0.7	2.4	14.4	69	99	68	7.2	480	52	100	53	3.5
Aflatoxin B_1	8.7	0.4	1.9	1.3	94	78	74	2.8	44	97	89	86	9.3
Aflatoxin B_2	8.4	1.0	3.4	1.3	75	80	60	14.3	44	102	79	81	8.8
Aflatoxin G_1	8.0	1.6	5.4	4.4	81	84	68	8.0	44	93	78	73	7.1
Aflatoxin G_2	7.7	0.8	2.8	1.3	107	125	90	14.1	44	104	72	75	9.1
Aflatoxin M_1	7.2	0.6	1.6	3.4	77	94	72	14.2	114	92	90	83	7.0
Alternariol	11.0	0.5	1.8	2.3	89	100	90	9.0	77	88	105	82	4.7
Alternariol monomethylether	12.8	0.1	0.2	2.3	80	106	85	4.8	77	85	97	82	3.6
Beauvericin	14.4	0.01	0.04	0.1	81	125	102	5.9	4	99	106	105	1.9
Chanoclavine	5.7	0.04	0.2	0.6	103	175	306	5.4	6	98	134	131	7.8
Citrinin	11.8	7.8	25.9	6.9	70	143	99	12.6	231	55	174	96	3.6
Deoxynivalenol	5.6	9.6	31.9	4.7	71	82	58	3.1	629	79	79	62	2.8
Diacetoxyscirpenol	8.5	0.6	2.1	4.7	125	66	89	2.2	155	101	94	95	4.7
Emodin	14.3	0.1	0.4	2.3	72	106	76	1.1	55	74	102	75	3.9
Enniatin B	14.0	0.01	0.04	0.1	106	115	122	5.7	2	97	110	107	3.3
Enniatin B1	14.3	n.e.	n.e.	0.1	92	158	145	7.5	5	100	109	108	6.6
Enniatin A	14.9	n.e.	n.e.	0.1	86	184	159	9.4	0.3	85	123	105	5.7
Enniatin A1	14.6	n.e.	n.e.	0.1	111	170	190	11.9	2	101	110	110	5.1
Ergocryptine	8.1	2.9	9.6	2.6	77	71	54	11.9	9	99	68	67	11.9
Ergocryptinine	9.4	0.3	1.2	0.6	68	117	80	6.2	6	106	83	87	8.1
Fumonisin B_1	9.4	5.3	17.8	17.0	44	118	45	5.3	565	60	103	62	6.1
Fumonisin B_2	11.3	4.4	14.7	17.1	56	104	58	2.9	569	75	78	104	3.2
Fumonisin B_3	10.3	7.4	24.6	0.3	61	106	67	10.8	9	73	76	103	7.1
HT-2 toxin	9.7	8.0	26.7	1.6	107	117	96	92.8	155	96	97	93	2.6
Moniliformin	3.2	4.8	16.1	30.6	71	155	110	8.3	307	62	164	102	10.8
Mycophenolic acid	10.7	2.6	8.8	6.5	97	104	77	13.6	215	94	106	100	4.7
Nivalenol	4.8	3.1	10.4	4.7	78	75	59	2.6	155	71	57	80	7.8
Ochratoxin A	11.9	1.3	4.3	3.9	70	110	77	14.0	130	97	103	99	4.7
Patulin	4.9	1.2	4.1	110.6	42	85	35	14.7	369	38	85	33	1.7
Phomopsin A	7.2	n.e.	n.e.	35.1	n.d.	n.d.	n.d.	n.d.	117	105	92	96	3.3
Phomopsin B*	7.0	n.e.	n.e.	1:30	72	161	115	14.4	1:10	81	123	100	9.9
Sterigmatocystin	12.3	0.2	0.7	2.3	85	105	84	10.1	23	91	104	94	5.6
T-2 toxin	10.7	1.3	4.4	4.6	133	104	104	9.0	154	99	104	103	3.9
Zearalenone	11.9	0.1	0.5	46.6	91	93	85	8.6	155	87	94	82	1.8

Note: RT – retention time; LOD – limit of detection; LOQ – limit of quantification; LL – lowest validation level; HL – highest validation level; R_E – recovery of extraction step; R_A – apparent recovery; SSE – signal suppression/enhancement; n.e. – not evaluated; n.d. – not detected.
* Concentration of the standard not known.

the analysis of wide range of matrices, including non-traditional matrices, such as cassava or liquorice, comprehensive validation of each individual matrix for more than 300 analytes would be very expensive and time-consuming. Hence only one representative commodity from each matrix category, as suggested by SANCO, was included in the validation of our multi-analyte method. As mentioned in Section 2.2, we chose four model matrices (apple puree, hazelnuts, maize, green pepper) representing the category groups that are relevant regarding mycotoxin contamination.

The performance characteristics of the method obtained for all 295 analytes are summarized in Appendix B. Despite having 331 compounds included in the current method, not all of them have been successfully validated for several reasons: (i) analytical standard not available (e.g. 4-monoacetoxyscirpenol, decalonectrin, PR-toxin, trypacidin), (ii) instability of an analyte in the final working standard solution (e.g. bacitracin, cephalosporin C, chetoseminudin A, cytromycetin, penicillin G), (iii) low concentration of analytical standard for spiking (15-hydroxyculmoron, chromomycin, lolitrem B, rapamycin, ustiloxin B). The validation for some other analytes (e.g. spyramycin, tylosin, josamycin, fumonisin B6, dinactin) has been done only by spiking with extracts as a substitution of analytical standards which were not available on the market at that time.

3.2.1. Method accuracy

Apparent recovery (R_A) and matrix effects (SSE) strongly vary depending on the analyte/matrix combination. As we use a neat solvent calibration and spiked samples in the routine analysis rather than matrix-matched standards, showing the method accuracy on the apparent recoveries (calculated according Equation (2)), expressing both the extraction efficiency and the matrix effects, is more "real-life" and relevant than showing data on the recovery of extraction step (R_E) (Equation (1)).

3.2.1.1. Apparent recovery. The distribution of R_A for 295 analytes in apple puree, hazelnuts, maize and green pepper is depicted in Fig. 1. The highest validated level (HL) corresponds to 1:10 dilution of the final working solution of the analytical standards. For the lowest validated level (LL), the lowest spiking level reliably detectable at five repetitions (RSD < 20%) through both MRM transitions was taken into account. Concerning the apparent recovery calculated at the HL, 74%, 68%, 64% and 21% of analytes in apple puree, hazelnuts, maize and green pepper, respectively, were in the range of 70–120% as recommended by SANCO [22]. For the analytes out of this range, either high matrix suppression/enhancement (e.g. aflatoxins, alternariol and emodin in pepper) or low extraction efficiency, for instance, 3-nitropropionic acid in hazelnuts was observed (the somewhat lower extraction efficiencies in apple puree are partially due to the water content of the sample (50 rel.%), which accounts for a increase of 12% of the volume of the raw extract.) The lower apparent recovery for some analytes (e.g. chanoclavine and HT-2 toxin in pepper) was caused by a combination of low extraction efficiency and matrix effects which was most pronounced in green pepper (Fig. 2). Therefore, 8% of the analytes (e.g. ergocryptine, α-zearalenol, cerulenin, citrinin) were not detectable in green pepper at all, while for the other matrices less than 4% of the analytes could not be detected. Concerning the LL, the percentage of analytes matching the R_A of 70–120% was lower than at the

Table 3
Performance characteristics of the method for some important analytes in maize.

Analyte	RT (min)	LOD (μg/kg)	LOQ (μg/kg)	LL (μg/kg)	R_E (%)	SSE (%)	R_A (%)	RSD (%)	HL (μg/kg)	R_E (%)	SSE (%)	R_A (%)	RSD (%)
3-nitropropionic acid	3.0	1.2	4.1	14.4	74	90	66	5.3	480	87	87	53	7.2
Aflatoxin B_1	8.7	0.6	1.9	1.3	75	79	59	7.6	44	89	70	62	3.5
Aflatoxin B_2	8.4	0.6	2.0	1.3	105	63	66	9.2	44	91	66	60	3.7
Aflatoxin G_1	8.0	1.2	4.1	1.3	68	75	51	10.5	44	93	69	64	4.0
Aflatoxin G_2	7.7	3.6	12.0	1.3	102	110	62	22.0	44	104	67	69	5.3
Aflatoxin M_1	7.2	0.6	2.1	3.4	77	90	69	7.6	114	87	90	79	4.9
Alternariol	11.0	0.6	2.1	2.3	84	84	71	7.6	77	88	81	72	3.2
Alternariol monomethylether	12.8	0.1	0.3	2.3	86	89	76	6.3	77	95	86	82	2.8
Beauvericin	14.4	0.02	0.06	0.1	105	157	165	9.1	4	108	110	102	4.4
Chanoclavine	5.7	0.4	1.4	0.2	78	44	31	19.4	6	81	32	26	13.6
Citrinin	11.8	12.7	42.4	6.9	20	177	36	10.8	231	18	169	31	5.1
Deoxynivalenol	5.6	5.4	18.2	15.1	95	110	104	3.2	629	93	104	97	7.4
Diacetoxyscirpenol	8.5	1.0	3.4	4.7	101	93	81	13.8	155	99	79	78	3.2
Emodin	14.3	0.2	0.6	2.3	86	100	87	5.5	55	84	101	85	2.2
Enniatin B	14.0	0.01	0.04	0.1	107	114	122	9.8	2	97	102	99	2.1
Enniatin B1	14.3	n.e.	n.e.	0.1	102	105	107	10.2	5	103	102	105	1.6
Enniatin A	14.9	n.e.	n.e.	0.1	87	96	84	13.6	0.3	109	95	103	6.3
Enniatin A1	14.6	n.e.	n.e.	0.2	96	117	112	8.3	2	99	102	101	6.9
Ergocryptine	8.1	2.4	8.0	2.6	91	63	56	12.5	9	96	45	43	10.3
Ergocryptinine	9.4	0.4	1.2	1.9	104	46	48	15.0	6	94	52	49	3.4
Fumonisin B_1	9.4	6.5	21.6	17.0	50	101	51	9.2	565	59	104	62	2.8
Fumonisin B_2	11.3	1.9	6.3	17.1	53	121	61	10.5	569	67	106	72	2.6
Fumonisin B_3	10.3	3.7	12.4	0.9	62	74	74	15.9	9	60	70	116	12.1
HT-2 toxin	9.7	6.6	22.1	1.6	76	58	64	10.1	155	86	83	72	3.6
Moniliformin	3.2	4.8	16.0	9.2	57	151	86	11.1	306	74	112	83	9.1
Mycophenolic acid	10.7	7.2	24.1	21.5	72	97	66	8.9	215	97	96	93	4.4
Nivalenol	4.8	0.8	2.8	4.7	78	110	87	6.4	155	81	110	89	2.1
Ochratoxin A	11.9	0.7	2.2	3.9	70	115	81	7.9	130	88	106	93	2.4
Patulin	4.9	76.1	253.8	36.8	25	98	27	19.0	369	20	100	20	5.5
Phomopsin A	7.2	n.e.	n.e.	35.1	n.d.	n.d.	n.d.	n.d.	117	72	98	71	10.9
Phomopsin B*	7.0	n.e.	n.e.	1:30	40	159	63	16.7	1:10	68	119	81	16.5
Sterigmatocystin	12.3	0.2	0.8	2.3	92	102	97	4.7	23	96	102	98	2.9
T-2 toxin	10.7	1.0	3.4	4.6	110	93	102	10.0	154	99	98	97	3.3
Zearalenone	11.9	0.2	0.7	4.7	109	82	89	3.3	155	87	93	82	2.3

Note: RT – retention time; LOD – limit of detection; LOQ – limit of quantification; LL – lowest validation level; HL – highest validation level; R_E – recovery of extraction step; R_A – apparent recovery; SSE – signal suppression/enhancement; n.e. – not evaluated; n.d. – not detected.
* Concentration of the standard not known.

HL. The R_A for apple puree, hazelnuts and maize was 56%, 56% and 58%, respectively. In green pepper, the R_A of 70–120% was achieved only for 20% of analytes. Altogether 25% of the compounds were not detected in green pepper (for all the other validated matrices only <12% of analytes) at any lower level than the highest validated level.

3.2.1.2. Matrix effects. SSE (Equation (3)) was observed for all matrices. The extent of SSE was strongly dependent on the analyte/matrix combination. As there is no acceptable range concerning the SSE in the SANCO, we have decided that the analytes having the SSE values between 90% and 110% were considered as not to be affected by matrix effects. The distribution of SSE in all validated matrices is displayed in Fig. 2. In general, the lowest matrix effects were observed for apple puree. In this matrix, 59% and 43% of analytes were suppressed/enhanced by only <10% at HL and LL. For instance, signal intensity of patulin, the most common natural toxin found in apples and products thereof, was not affected by matrix in apple puree (Table 1) while it was highly suppressed (SSE of 42%) in pepper (Table 4). Concerning hazelnuts only 48% and 35% of analytes had the SSE in the range of 90–110% at HL and LL, respectively. 3-nitropropionic acid and sterigmatocystin found as the analytes with the highest incidence in hazelnuts in our recent study [31] were not affected by matrix effects at all. Aflatoxins which levels are regulated in nuts by the European Commission (EC) [4] were slightly suppressed in hazelnuts (SSE in the range of 72–89%). However, the R_E values for aflatoxins in hazelnuts close to 100% (Table 2) and the repeatability below 10% still allow to achieve accurate results in routine analysis. In total, only 44% of analytes at the HL and 35% of analytes at the LL were not affected by matrix effects in maize. The mycotoxins with the legislation limits established in maize or cereals by the EC [4], such as deoxynivalenol, zearalenone and fumonisins B_1 and B_2, and ochratoxin A were neither suppressed nor enhanced at the HL (Table 3). As mentioned above, green pepper analysis suffered from the matrix effects the most from all investigated matrices which is obvious from a histogram in Fig. 2. Only 10% of the analytes were not affected by signal suppression/enhancement in green pepper. From the compound on the reduced analyte list (Table 4), only moniliformin was not affected by the matrix effects in green pepper. Some analytes, e.g. paspalic acid and aspyrone, could not be evaluated at all because of huge interferences occurred at the sSRM transitions.

Although mostly matrix-caused signal suppression is being observed in LC–MS [32], here both signal suppression and enhancement occurred in an equal extent in apple puree and hazelnuts independent of the spiking concentration. Interestingly, the same is not true for maize and green pepper. A higher number of analytes was suppressed (34% at HL, 42% at LL) than enhanced in maize (22% HL, 23% LL). Furthermore, the signal suppression was even more pronounced in pepper (Table 4), in which 73% of analytes were suppressed as e.g. HT-2 toxin, T-2 toxin, alternariol, chanoclavine compared to 17% (HL) and 19% (LL) of analytes which were enhanced (e.g. zearalenone, 15-acetyldeoxynivalenol, decarestrictin, giberrellic acid). High enhancement calculated for some compounds such as cyclopiazonic acid, equisetin, CJ 20158 (methylequisetin) and nortryptoquialanine for some or all matrices are probably not caused by matrix effects in the narrower sense i.e. in connection with the electrospray ionization process. In these analyte/matrix combinations co-extracts are likely to work as a protective agent for light- or oxygen-sensitive analytes in the

Table 4
Performance characteristics of the method for some important analytes in green pepper.

Analyte	RT (min)	LOD (μg/kg)	LOQ (μg/kg)	LL (μg/kg)	R_E (%)	SSE (%)	R_A (%)	RSD (%)	HL (μg/kg)	R_E (%)	SSE (%)	R_A (%)	RSD (%)
3-nitropropionic acid	3.0	2.0	6.8	14.4	60	87	52	5.6	480	76	80	63	10.4
Aflatoxin B_1	8.7	8.0	26.5	13.1	75	41	33	12.0	44	78	40	31	4.9
Aflatoxin B_2	8.4	4.3	14.3	1.3	120	49	59	16.5	44	79	40	31	8.8
Aflatoxin G_1	8.0	5.2	17.2	13.2	69	64	44	3.8	44	73	69	50	8.6
Aflatoxin G_2	7.7	13.6	45.5	4.4	n.d.	n.d.	n.d.	n.d.	46	59	53	31	8.0
Aflatoxin M_1	7.2	4.1	13.6	3.4	56	60	33	10.8	114	81	54	43	4.3
Alternariol	11.0	9.4	31.2	7.7	88	46	40	9.4	77	72	40	29	10.9
Alternariol monomethylether	12.8	0.5	1.7	23.3	97	105	102	13.4	77	96	94	90	11.3
Beauvericin	14.4	0.02	0.06	0.1	110	151	167	7.3	4	83	56	46	6.5
Chanoclavine	5.7	1.4	4.8	6.1	50	21	11	22.8	6	50	31	11	22.8
Citrinin	11.8	n.d.	n.d.	69	n.d.	n.d.	n.d.	n.d.	231	n.d.	n.d.	n.d.	n.d.
Deoxynivalenol	5.6	8.6	28.6	15.1	59	86	50	5.2	629	71	66	47	11.7
Diacetoxyscirpenol	8.5	1.3	4.4	4.7	73	80	48	3.4	155	75	69	51	8.8
Emodin	14.3	0.1	0.2	2.3	83	251	207	3.1	55	73	165	121	8.8
Enniatin B	14.0	0.1	0.2	0.1	86	63	54	12.3	2	80	53	42	1.6
Enniatin B1	14.3	n.e.	n.e.	0.5	94	57	54	8.9	5	85	43	37	6.6
Enniatin A	14.9	n.e.	n.e.	0.1	74	61	38	9.2	0.3	74	51	38	9.2
Enniatin A1	14.6	n.e.	n.e.	0.2	81	68	55	6.9	2	82	60	50	5.2
Ergocryptine	8.1	n.d.	n.d.	2.6	n.d.	n.d.	n.d.	n.d.	9	n.d.	n.d.	n.d.	n.d.
Ergocryptinine	9.4	3.6	11.9	6.2	n.d.	n.d.	n.d.	n.d.	6	n.d.	n.d.	n.d.	n.d.
Fumonisin B_1	9.4	5.7	18.8	17.0	74	125	93	10.5	565	71	88	63	6.8
Fumonisin B_2	11.3	5.7	18.9	17.1	71	104	62	6.4	567	76	87	67	6.7
Fumonisin B_3	10.3	2.4	7.9	0.9	71	166	118	15.4	9	95	88	84	7.8
HT-2 toxin	9.7	3.7	12.3	46.6	70	19	16	16.1	155	54	26	14	7.5
Moniliformin	3.2	5.3	17.5	30.6	71	131	93	9.1	306	77	95	73	10.4
Mycophenolic acid	10.7	8.7	28.9	21.5	77	79	60	10.9	215	79	72	57	3.3
Nivalenol	4.8	8.1	27.0	15.5	63	88	48	5.1	155	63	65	41	6.1
Ochratoxin A	11.9	3.9	12.9	3.9	67	69	47	9.7	130	68	55	37	0.5
Patulin	4.9	134.6	448.6	110.6	n.d.	n.d.	n.d.	n.d.	449	50	42	84	4.0
Phomopsin A	7.2	n.e.	n.e.	35.1	n.d.	n.d.	n.d.	n.d.	117.1	83	85	70	19.9
Phomopsin B*	7.0	n.e.	n.e.	1:10	98	71	70	14.6	1:30	69	86	59	6.9
Sterigmatocystin	12.3	3.2	10.6	23.4	78	54	42	5.7	23	75	54	40	2.0
T-2 toxin	10.7	18.5	61.6	46.9	82	57	48	10.0	154	86	50	43	10.2
Zearalenone	11.9	1.2	4.1	15.5	158	134	212	10.4	155	94	136	127	7.6

Note: RT – retention time; LOD – limit of detection; LOQ – limit of quantification; LL – lowest validation level; HL – highest validation level; R_E – recovery of extraction step; R_A – apparent recovery; SSE – signal suppression/enhancement; n.e. – not evaluated; n.d. – not detected.
* Concentration of the standard not known.

matrix-matched standards. The concentration of these analytes in neat solvent standards decreased much faster. For instance, a protective function of ascorbic acid (occurring in many plant matrices) against oxidation of cyclopiazonic acid has already been proven [33]. In addition to that, the matrix might also influence the epimerization rate e.g. of ergot alkaloids.

Besides the SSE evaluation based on one point calibration, also their evaluation by comparing the slopes (matrix-matched standards slope/neat solvent standards slope) was performed. This approach is commonly used for the expression of matrix effects in validation studies. However, we observed that the "slope SSE" do not reflect the real SSE for some analytes despite the linearity of calibration curves. For instance, the "slope SSE" for tryptophol in hazelnut (97%) and maize (97%) did not indicate a matrix effect, but the "one point SSE" calculated at the LL revealed a high signal suppression (139% and 195% for maize and pepper, respectively). Therefore, both approaches should be used for the expression of matrix effects within the validation process. When the "slope SSE" and the "one point calibration SSE" differ, the results should be corrected rather on the "one point calibration SSE" calculated at the closest concentration level to the level found in a sample to avoid an erroneous quantification.

Another difficulty is to estimate the extent of SSE, and also other performance characteristics, for analytes showing epimerization, like ergots alkaloids. A C9=C10 double bond of the ergoline ring is responsible for epimerization with respect to the centre of chirality C8 (Fig. 3). Thus, ergot alkaloids are converted from *–ine* to *–inine* form and back depending on the solvent and pH. The *–ine*/*–inine* ratio can be different in the neat solvent and the matrix-matched environment [34]. For instance, epimers ergotamine and

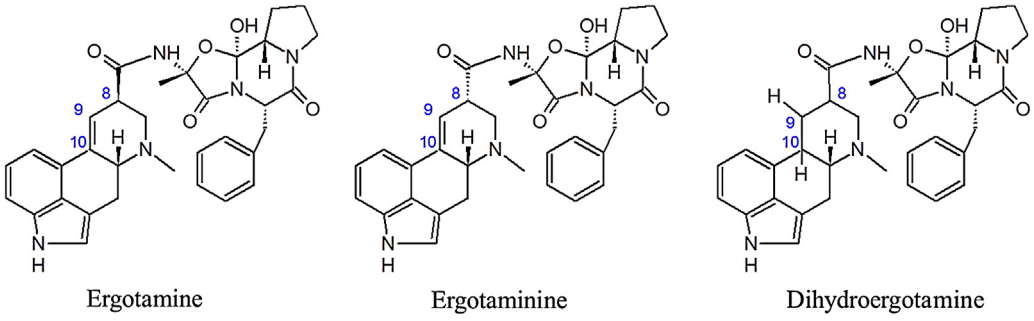

Fig. 3. Chemical structures of ergotamine, ergotaminine and dihydroergotamine.

Table 5
Summary of the performed proficiency tests.

Organizator	Matrix	PT code	Analyte	Reported result (μg/kg)	Assigned value (μg/kg)	Standard deviation (μg/kg)	*Z*-score
FAPAS	Peanut	T01044	Aflatoxin B_1	3.37	3.94	1.74	−0.33
			Aflatoxin B_2	1.62	1.54	0.68	0.12
			Aflatoxin G_1	2.53	2.27	1.00	0.26
	Cereals	T1786	Ochratoxin A	3.01	2.76	1.22	0.20
	Maize	T2246	Fumonisin B_1	1665	1650	110	0.14
			Fumonisin B_2	474	461	32	0.41
	Maize	T2262	Deoxynivalenol	1707	1714	506	−0.01
	Breakfast cereals	T2257	Zearalenone	101.6	69.5	30.6	1.05
BIPEA	Peanut cake		Aflatoxin B_1	452	481	277	−0.10
			Aflatoxin B_2	88.2	77.1	51.1	0.22
			Aflatoxin G_1	58.0	77.1	53.5	−0.36
			Aflatoxin G_2	9.0	7.0	5.1	0.39
	Peanut paste		Aflatoxin B_1	4.9	4.5	1.9	0.21
			Aflatoxin B_2	1.29	0.9	0.3	1.30
	Animal feed		Ochratoxin A	1.29	1.6	1.0	−0.31
			HT-2 Toxin	17.2	15.0	3.0	0.73
			Deoxynivalenol	260	316	98	−0.57
			Zearalenone	30.5	31.0	13.0	−0.04
	Wheat	05-50631	Deoxynivalenol	1844	2223	485	−0.78
			Zearalenone	36.2	20.0	8.0	2.03
	Peanut paste		Aflatoxin B_1	4.2	7.2	3.7	−0.81
			Aflatoxin B_2	0.2	0.7	0.4	−1.25
			Aflatoxin G_1	0.7	2.6	1.5	−1.27
			Aflatoxin G_2	n.d.	0.4	0.2	n.e.
	Wheat draff	02-2831	Ochratoxin A	7.2	5.7	2.6	0.58
			Deoxynivalenol	99	188	96	−0.93
			T-2 toxin	95	84	31	0.35
			HT-2 toxin	105	82	25	0.92
	Pepper	01-1031	Aflatoxin B_1	1.2	2.0	0.8	−1.00
	Animal feed	03-3031	Deoxynivalenol	258	291	82	−0.40
	Raisins	02-3131	Ochratoxin A	2.9	3.7	1.6	−0.53
	Maize	04-0731	Deoxynivalenol	702	563	119	1.17
			Zearalenone	45	53	14	−0.57
			Fumonisin B_1	706	620	289	0.30
			Fumonisin B_2	187	149	68	0.56
	Milk powder	04-0331	Aflatoxin M_1	0.309	0.395	0.173	−0.34
	Maize		Deoxynivalenol	3400	3664	1176	−0.22
			Zearalenone	3478	2891	1836	0.32
			Fumonisin B_1	281	231	120	0.42
	Coffee	02-1731	Ochratoxin A	8.5	8.7	4.2	−0.04
	Wheat		Deoxynivalenol	946	852	230	0.41
			Nivalenol	29.9	n.e.	n.e.	n.e.
			Ochratoxin A	2.4	2.8	0.7	−0.57
	Baby food	01-3331	Aflatoxin B_1	2.3	2.3	0.7	0.00
			Aflatoxin B_2	1.1	1.1	0.2	0.00
			Aflatoxin G_1	2.3	3.5	1.2	−1.01
			Aflatoxin G_2	3.3	1.7	0.6	2.62
			Aflatoxin total	6.4	8.2	3.0	−0.60
			Ochratoxin A	0.85	1.5	0.7	−0.93
	Baby food	01-3431	Ochratoxin A	0.85	1.1	0.4	−0.63
			HT-2 toxin	49	55	30	−0.20
	Type corn		T-2 toxin	54.6	66.0	26.0	−0.44
	Flour		Deoxynivalenol	129	127	55	0.04
			Zearalenone	27.4	32.0	10.0	−0.46
	Pepper	01-3231	Aflatoxin B_1	6.5	12.0	5.9	−0.93
			Aflatoxin B_2	8.7	10.3	4.4	−0.36
			Aflatoxin G_1	6.7	7.7	3.2	−0.31
			Aflatoxin G_2	5.5	5.3	3.2	0.06
			Aflatoxin total	27.3	31.6	15.2	−0.28
			Ochratoxin A	8.9	5.5	3.3	1.03
	Maize	05-0731	Fumonisin B_1	1200	582	208	2.97
			Fumonisin B_2	206	133	52	1.40
			Fumonisin B total	1406	708	256	2.73
			Deoxynivalenol	939	730	178	1.17
			Zearalenone	142	124	32	0.56
			Ochratoxin A	7.0	5.0	1.9	1.05
			Nivalenol	443	453	159	−0.06
			T-2 toxin	111	100	28	0.38
			HT-2 toxin	92	82	20	0.51
			sum T-2/HT-2	203	180	47	0.48
	Pistachio	03-1431	Aflatoxin B_1	17.3	18.3	3.7	−0.27
	Paste		Aflatoxin B_2	10.8	11.7	2.5	−0.36
			Aflatoxin G_1	8.9	9.6	2.7	−0.26
			Aflatoxin G_2	3.0	4.3	1.1	−1.18
			Aflatoxin total	40.0	44.3	9.3	−0.46

Table 5 (*Continued*)

Organizator	Matrix	PT code	Analyte	Reported result (μg/kg)	Assigned value (μg/kg)	Standard deviation (μg/kg)	*Z*-score
			Ochratoxin A	2.3	1.9	1.0	0.35
	Chicken Feed	04-3031	Ochratoxin A	10.2	9.0	2.7	0.44
			Deoxynivalenol	200	243	80	−0.54
			Nivalenol	14.4	n.e.	n.e.	n.e.
			T-2 toxin	68	58	25	0.40
			HT-2 toxin	62	51	15	0.73
			Sum T-2/HT-2	130	111	46	0.41
			Zearalenone	185	145	45	0.89
	Liquorice	01-3531	Aflatoxin B_1	8	21	18	−0.72
			Aflatoxin B_2	0.75	3.4	2.8	−0.95
			Aflatoxin G_1	5.3	15.3	12.7	−0.79
			Aflatoxin G_2	3.5	8.2	6.3	−0.74
			Aflatoxin total	17.6	50.1	39.3	−0.83
			Ochratoxin A	378.0	235.6	168.4	0.85
	Oat	02-2931	Ochratoxin A	2.8	2.8	1	0.00
			Deoxynivalenol	84	128	45	−0.97
			Nivalenol	180	179	75	0.01
			T-2 toxin	37	52	25	−0.60
			HT-2 toxin	120	98	39	0.56
			sum T-2/HT-2	157	146	43	0.26
			Zearalenone	70	79	29	−0.31
CODA-CERVA	Oat flour		Aflatoxin B_1	11.20	12.57	2.77	−0.49
			Aflatoxin B_2	0.53	0.9	0.2	−1.85
			Aflatoxin G_1	5.7	6.0	1.32	−0.25
			Aflatoxin G_2	<0.5	0.48	n.e.	n.e.
			Ochratoxin A	108	79	17	1.67
			Deoxynivalenol	1635	2262	320	−1.96
			Zearalenone	210	191	39	0.48
			HT-2 toxin	72.5	80.5	17.7	−0.45
			T-2 toxin	308	270	53	0.72
			Fumonisin B_1	2310	2313	326	−0.01
			Fumonisin B_2	417	393	72	0.34
			Fumonisin B_3	1280	1530	230	−1.09
			Enniatin A	2.6	2.3	0.5	0.54
			Enniatin A1	30.7	21.5	4.7	1.95
			Enniatin B	944	721	121	1.84
			Enniatin B1	258	194	40	1.63
			Beauvericin	568	459	83	1.32

Note: n.d. – not detected; n.e. – not evaluated.

ergotaminine eluted at 7.51 and 7.67 min showed the ratio shifted more to the latter one (Appendix B). However, as the ratio between these two epimers in the extract is not known, the calculated SSE, R_A and R_E cannot be considered as relevant for evaluation of routine samples. Instead, the data for dihydroergometrine which is eluted between ergotamine and ergotaminine (RT = 7.62 min) could be considered. Dihydroergotamin is hydrogenated at positions C9 and C10, hence it does not exhibit epimerization.

3.2.1.3. Limits of detection and quantification, linearity. The limits of quantification for all analytes were estimated as the lowest spiking level (LL) for which the performance criteria (mean recovery in the range of 70–120% with and RSD lower than 20%) were met (Appendix B). As both parameters, the LOD as well as the LOQ, are strongly dependent on the actual condition of the instrument, i.e. the contamination level of the instrument, the lowest level approach is more feasible than the traditional *S*/*N* one, especially for the multi-analyte methods including more than 100 analytes. In order to compare both approaches to LOQ determination, the calculation of LOD and LOQ according to signal to noise ratio (*S*/*N*) (Equations (4 and 5)) was carried out as well, but only for 29 analytes (including all regulated mycotoxins) listed in Tables 1–4. From Tables 1 to 4 it is obvious that there is not a huge difference between LOQ (estimated from *S*/*N* ratio) and LL. Moreover, the levels of LOQ and LL are strongly dependent on the analyte/matrix combination. The highest difference between LOQ and LL was observed for citrinin in apple puree and for patulin in all matrices. Concerning the matrix influence on the detection capability of the method, the highest levels of LOQ and/or LL were obtained for green pepper.

The linearity of the system for most of the analytes covered two orders of magnitude for all four matrices. For analytes for which a stock solution at high concentration was available and which showed a high sensitivity, e.g. diacetoxyscirpenol and sterigmatocystin, the linear range of three orders of magnitude for all four matrices was obtained.

3.2.2. Method precision

The precision of the method was proven within the laboratory as repeatability of five repetitions at the highest and the lowest spiking level. Most of the analytes fulfilled the criteria of RSD ≤20%. An RSD of ≤20% was achieved for 97% of analytes in apple puree and hazelnuts and for 95% of compounds in maize. The repeatability below 20% of RSD for green pepper was obtained only for 89% of all analytes.

As expected, the method precision at the LL was slightly worse compared to the HL. On average, 85% of the analytes passed the acceptable repeatability in all matrices except for pepper in which only 77% of the compounds fulfilled the recommended RSD at the LL. Ergot alkaloids belong to the analytes with worse repeatabilities (but still below 20%) which is caused by the epimerization between *–ine* and *–inine* form [34]. In general, the highest variation among the five repetitions was observed in green pepper. For instance, for aflatrem the RSD of 94% at the HL was achieved. However, for some analytes the required repeatability was achieved in green pepper but not in any other matrix. Fumiquinazoline A in apple puree with an RSD of 104% and altenusin and geodin in maize with repeatabilities of 69% and 25% are example for this phenomenon (Appendix B).

3.2.3. Method trueness

The trueness of the method has been continuously proven by the participation in various proficiency tests provided by FAPAS®, Bipea and CODA-CERVA. The mycotoxin levels were obtained by means of neat solvent calibration curve and the results were corrected on the apparent recoveries of the respective toxins. Table 5 summarizes the results of the recently performed proficiency tests that our laboratory participated in. The samples cover a wide range of analyte/matrix combinations. Therefore, the method trueness could have been proven also for the matrix types which were not validated, such as animal and chicken feed, coffee, milk powder. An apparent recovery of 100% was assumed for all these matrices. The *z*-scores calculated according to FAPAS®, Bipea and CODA-CERVA proficiency testing protocols for the all of the analyte/matrix combinations lied within the acceptable range of −2 to 2 except aflatoxin G_2 in baby food (*z*-score = 2.62) and fumonisin B_1 in maize (*z*-score = 2.97). Furthermore, it has been proven that the method provides accurate results also for matrices with high sugar content such as raisins. High sugar content matrices were not included into the validation process as they were not amenable to the miniaturization of the sample pretreatment to 0.5 g which is necessary for spiking experiments in order to keep the amount of standards to a minimum.

4. Conclusions

The extension, optimization and validation of the LC–MS/MS method for the simultaneous determination of 295 fungal and bacterial metabolites has successfully been performed. The validation has been carried out for four types of different food matrices – apple puree, hazelnuts, maize and green pepper. Furthermore, the method trueness has been proved by the participation in the official proficiency tests organized by FAPAS®, Bipea and CODA-CERVA. The major outcomes are summarized in the following paragraphs:

- Validation data have been obtained for 295 analytes. In addition, the MS/MS transitions are provided for another 36 metabolites, for which, however, no sound validation could be realized due to non-availability of analytical standards or due to instability of these compounds under the used analytical conditions.
- As compounds comprising a wide range of chemical properties have been included in the method, the extraction and chromatographic conditions had to be compromised. For instance, the acidic conditions essential for the extraction and separation of acidic compounds such as fumonisins, and ochratoxin A are not favorable for basic compounds (e.g. ergot alkaloids). Therefore, the apparent recovery levels varied to a large extent depending on the analyte/matrix combination. In general, green pepper was the most difficult matrix in terms of recovery and matrix effects for the most of the analytes.
- Both signal suppression and enhancement were observed for all four matrices. Their extent was dependent on the analyte/matrix combination and the analyte concentration. The matrix contributing the least to SSE was apple puree, while the highest number of analytes suffering from severe SSE were found in green pepper A.
- Despite some analytes were out of the range of 70–120% apparent recoveries, the repeatability (RSD calculated from five replicates) was below the acceptable level of 20% for the majority (89–97%) of them.
- The LOQs or LLs of the method for the toxins regulated by EC [4] were below the required maximum levels for the respective toxins except of aflatoxins and ochratoxin A in baby food and aflatoxin M_1 in milk.
- *z*-scores < |2| were achieved at all proficiency tests the laboratory participated with the exception of aflatoxin G_2 in babyfood (*z*-score = 2.62) and fumonisin B_1 in maize (*z*-score = 2.97).

In summary this work describes a fully in-house validated LC–MS/MS multi-analyte method covering almost 300 bacterial and fungal metabolites including all relevant mycotoxins in various food matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.08.037.

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