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

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12 **Abstract**

13 An LC-MS/MS “dilute and shoot” method for the determination of 295 fungal and bacterial
14 metabolites was optimized and validated according to the guidelines established in the
15 Directorate General for Health and Consumer Affairs of the European Commission (SANCO)
16 document No. 12495/2011. Four different types of food matrices were chosen for validation:
17 apple puree for infants (high water content), hazelnuts (high fat content), maize (high starch and
18 low fat content) and green pepper (difficult or unique matrix). Method accuracy and precision
19 was evaluated using spiked samples in five replicates at two concentration levels. Method
20 trueness was demonstrated through participation in various proficiency tests. Although the
21 method covers a total number of 331 analytes, validation data were acquired only for 295
22 analytes, either due to the non-availability of analytical standards or due other reasons described
23 in this paper. Concerning the apparent recovery, the percentage of 295 analytes matching the
24 acceptable recovery range of 70-120% laid down by SANCO varied from 21% in green pepper
25 to 74% in apple puree at the highest spiking level. At the levels close to limit of quantification
26 only 20-58% of the analytes fulfilled this criterion. The extent of matrix effects was strongly
27 dependent on the analyte/matrix combination. In general, the lowest matrix effects were
28 observed in apple puree (59% of analytes were not influenced by enhancement/suppression at all
29 at the highest validation level). The highest matrix effects were observed in green pepper, where
30 only 10% of analytes did not suffer from signal suppression/enhancement. The repeatability of
31 the method was acceptable ($RSD \leq 20$) for 97% of all analytes in apple puree and hazelnuts, for

32 95% in maize and for 89% in green pepper. Concerning the trueness of the method, Z-scores
33 were generally between -2 and 2, despite a broad variety of different. Based on these results it
34 can be concluded that quantitative determination of mycotoxins by LC-MS/MS based on a
35 “dilute and shoot” approach is also feasible in case of complex matrices.

36 **Highlights**

- 37 • An LC-MS/MS method for 295 bacterial and fungal toxins was developed
- 38 • The method was validated for four very different food matrices
- 39 • The trueness of the method is proven through proficiency test materials
- 40 • The majority of the analytes fulfill the method accuracy criteria
- 41 • It is feasible to apply the method also for complex matrices without prior clean-up

42 **Keywords**

43 apple puree, hazelnuts, maize, green pepper, food contaminants, mycotoxins

44 **1. Introduction**

45 Mycotoxins are defined as low-molecular-weight natural products produced as secondary
46 metabolites by fungi. By definition, they are toxic to vertebrates and other animal groups in low
47 concentrations, causing acute as well as chronic diseases [1]. Mycotoxins exhibit a great
48 diversity in their chemical structure, which explains that their toxicities and target organs also
49 vary [2]. Over the years, health concerns related to mycotoxins have increased [3] and several
50 regulations have been set into force to control the maximum levels of mycotoxins in food and
51 feed in many countries. For instance, the European Union has laid down maximum levels for
52 certain mycotoxin-matrix combinations in Commission Regulation 1881/2006/EC [4].
53 Regulations are based on the evaluation of risk assessment (hazard and exposure) but also reflect
54 agriculturally achievable levels in different foodstuffs. As exposure assessment is an important
55 aspect of risk assessment, validated analytical methods and the implementation of analytical
56 quality assurance are necessary to provide a reliable assessment on the toxin intake [5]. The
57 complexity of food samples together with the low concentrations at which contaminants usually
58 occur require highly sensitive, selective and reliable analytical techniques [6].

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

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

77 Every clean-up step and even a rather unspecific QuEChERS-like approach [11] limit the
78 number of analytes as some of the target substances might not be amenable to the chosen
79 procedure [12]. On the other hand, reducing the sample clean-up to a minimum (i.e. injection of
80 raw extracts) will result in suppression or enhancement of the analyte response during the
81 ionization process. The influence of these matrix effects is the major challenge in developing
82 reliable quantitative multi-analyte methods [13]. Therefore, considerable efforts to control matrix
83 effects should be carried out to obtain accurate results. The use of stable isotope dilution assays
84 (SIDA) seems to be the best alternative to correct matrix effects. Several methods have been
85 validated using isotopically labelled internal standards [14-16]. However, the limited availability
86 of labelled internal standards for non-regulated toxins and the comparably high costs of
87 isotopically labelled standards are the main drawbacks. Another common approach to deal with
88 matrix effects is the compensation of the signal suppression/enhancement through the usage of

89 matrix matched standards (i.e. blank sample extracts fortified with an appropriate amount of a
90 multi-analyte standard). Here the availability of analyte-free samples (which is especially
91 difficult for certain analyte/matrix combinations such as deoxynivalenol in maize) and repeatable
92 extraction efficiencies as well as matrix effects for all individual samples of a given commodity
93 are the major challenges [17-22].

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

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

115 **2. Material and methods**

116 **2.1 Chemicals and reagents**

117 LC gradient grade methanol and acetonitrile as well as MS grade ammonium acetate and glacial
118 acetic acid (p.a.) were purchased from Sigma Aldrich (Vienna, Austria). A Purelab Ultra system
119 (ELGA LabWater, Celle, Germany) was used for further purification of reverse osmosis water.
120 Standards of fungal and bacterial metabolites were obtained either as gifts from various research
121 groups or from the following commercial sources: Romer Labs[®] Inc. (Tulln, Austria), Sigma-
122 Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe
123 (Lausanne, Switzerland) and LGC Promochem GmbH (Wesel, Germany). Stock solutions of
124 each analyte were prepared by dissolving the solid substance in acetonitrile (preferably),
125 acetonitrile/water 1:1 (v/v), methanol, methanol/water 1:1 (v/v) or water. Thirty-four combined
126 working solutions were prepared by mixing the stock solutions of the corresponding analytes for
127 easier handling and were stored at -20°C. The final working solution was freshly prepared prior
128 to spiking experiments by mixing of the combined working solutions.

129 **2.2 Samples**

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

134 The following proficiency testing samples were used for the verification of the method trueness:
135 (i) FAPAS[®] testing materials – peanuts (T01044), maize (T2246, T2262), cereals (T1786) and
136 cereal breakfast (T2257) provided by The Food and Environment Research Agency (York,
137 United Kingdom); (ii) Proficiency Testing Scheme samples – peanut cake (04-0231), peanut
138 paste (02-1331, 04-1331), animal feed (02-3031, 03-3031, 04-3031), wheat (05-0631, 03-2331),
139 wheat draff (02-2831), pepper (01-1031, 01-3231), raisins (02-3131), maize (04-0731, 05-0731,
140 03-0731) milk powder (04-0331), coffee (02-1731), baby food (01-3331, 01-3431), pistachio
141 paste (03-1431), liquorice (01-3531), oat (02-2931) were obtained from Bipea (Gennevilliers,
142 France); (iii) CODA-CERVA proficiency test (oat flour) from 2013 organized by Belgian
143 National Reference Laboratory for Mycotoxins in Food and Feed.

144 **2.3 Sample preparation**

145 Model samples were ground using an Osterizer blender (Sunbeam Oster Household Products,
146 Fort Lauderdale, Florida, USA). For spiking the model matrices, appropriate amounts of the final
147 working solution were added to 0.5 g of sample. The samples were placed at darkness to avoid
148 analyte degradation and stored overnight at room temperature to allow the evaporation of the
149 solvent and to establish equilibration between analytes and matrix. After this period, 2 mL of
150 extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) was added. The samples were
151 extracted for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and
152 subsequently centrifuged for 2 min at 3,000 rpm (radius 15 cm) on a GS-6 centrifuge (Beckman
153 Coulter Inc., Fullerton, CA). The extracts were transferred into glass vials using Pasteur pipettes,
154 and 350 µL aliquots were diluted with the same volume of dilution solvent
155 (acetonitrile/water/acetic acid 20:79:1, v/v/v). After appropriate mixing, 5 µL of the diluted
156 extract was injected into the LC-MS/MS system without further pre-treatment. It should be noted
157 that the whole procedure was miniaturized only for validation purposes in order to decrease an
158 amount of standards needed for spiking. In routine analysis, 5 g of sample is extracted with 20
159 mL of extraction solvent.

160 **2.4 Method validation**

161 **2.4.1 Spiking experiments and the preparation of calibration curves**

162 As outlined above, method validation was performed according to SANCO validation criteria
163 [22]. For the determination of the performance characteristics of the method for all four model
164 matrices, spiking experiments were carried out at four different concentration levels (each in five
165 repetitions) resulting in the relative concentrations of 1:3:10:30 in the final diluted extracts. The
166 concentration ranges of the spiked samples were chosen to cover the respective limits of
167 detection of each toxin, estimated linear range of calibration, legislation limits of regulated
168 toxins, as well as the levels commonly found in naturally contaminated samples. External
169 calibration was prepared by dilution of appropriate amounts of the final working solution with
170 acetonitrile/water/acetic acid (49.5/49.5/1, v/v/v) at levels corresponding to those in spiked
171 samples. Taking into account the matrix induced signal enhancement or suppression of target
172 analytes, two additional calibration points (one above and one below the spiking concentration
173 range) were prepared to ensure that all spiking levels fall into the calibration range. For the

174 assessment of matrix effects and extraction efficiency (recovery), the diluted extracts (blank
175 extracts) of each model matrix prepared according to the Section 2.3 were fortified at the
176 concentration range matching the external calibration.

177 **2.4.2 Data evaluation**

178 The peaks were integrated and linear, 1/x weighted, calibration curves were constructed from the
179 data obtained from the analysis of each sample type (spiked sample, neat solvent standard,
180 spiked extract) using MultiQuant™2.0.2 software (AB Sciex, Foster City, California, USA) to
181 evaluate the linearity of the method. Further data evaluation was carried out in Microsoft Excel
182 2007. All the other performance characteristics of the method (recovery, apparent recovery,
183 repeatability and matrix effects) were evaluated at each spiking level for all model matrices. First
184 of all, the average value from the peak areas of five replicates of spiked samples was calculated.
185 Recovery (R_E) of the extraction step and the apparent recovery (R_A) were obtained by comparing
186 the average area of the spiked samples (n=5) to the average area of two replicates of matrix-
187 matched standard and neat solvent standard, respectively, measured at the beginning and at the
188 end of the set of the respective matrix. Matrix induced enhancement or suppression (SSE) was
189 assessed by comparison of respective matrix-matched standards with the neat solvent standards.
190 All the calculations were performed according to the following equations:

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

192

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

193

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

195

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

196

197 Matrix effects were expressed as SSE

198

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

199
200 The repeatability of the method was expressed as the relative standard deviation (RSD)
201 calculated from five replicates of the spiked samples. Concerning the limits of quantification
202 (LOQ), they were taken as the lowest validated spiking levels (LL) for which the method
203 performance acceptability criteria were still met, i.e. mean recovery for each representative
204 commodity in the range of 70-120%, with an $RSD \leq 20\%$ [22]. In addition, the more tedious
205 “classic” approach based on the signal to noise ratios (S/N) of 10/1 [29] was applied only to 29
206 most important analytes including all mycotoxins addressed by EU regulations and several other
207 prevalent fungal metabolites. In this case, the limits of detection (LODs) and the LOQs were
208 estimated with respect to the signal of the less intensive (LOD) and more sensitive (LOQ)
209 selected reaction monitoring (SRM) transition. S/N ratios were assessed at the lowest reliably
210 visible concentration level of the spiked samples individually for each of the five replicates.
211 LODs and LOQs were calculated from the average of S/N ratios as follows:

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

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

215 **2.5 Instrumental parameters**

216 Detection and quantification was performed with a QTrap 5500 MS/MS system (Applied
217 Biosystems, Foster City, CA) equipped with a TurboV electrospray ionization (ESI) source and a
218 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic
219 separation was performed at 25°C on a Gemini[®] C₁₈-column, 150×4.6 mm i.d., 5 μm particle
220 size, equipped with a C₁₈ security guard cartridge, 4×3 mm i.d. (all from Phenomenex, Torrance,
221 CA, US). Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM
222 ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A)
223 and 97:2:1 (v/v/v; eluent B), respectively. After an initial time of 2 min at 100% A, the
224 proportion of B was increased linearly to 50% within 3 min. Further linear increase of B to 100%

225 within 9 min was followed by a hold-time of 4 min at 100% B and 2.5 min column re-
226 equilibration at 100% A. The flow rate was 1000 $\mu\text{L min}^{-1}$. ESI-MS/MS was performed in the
227 scheduled selected reaction monitoring (sSRM) mode both in positive and negative polarities in
228 two separate chromatographic runs. The sSRM detection window of each analyte was set to the
229 respective retention time ± 27 s and ± 42 s in positive and in negative mode, respectively. The
230 target scan time was set to 1 s. The settings of the ESI source were as follows: source
231 temperature 550°C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1
232 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of
233 nitrogen), ion-spray voltage $-4,500$ V and $+5,500$ V, respectively, collision gas (nitrogen)
234 medium. The optimization of the analyte-dependent MS/MS parameters was performed via
235 direct infusion of standards (diluted in a 1:1 mixture of eluent A and B) into the MS source using
236 a syringe injection at a flow rate of 10 $\mu\text{L/min}$, see Appendix A for the corresponding values.
237 The acquisition of 2 sSRM transitions per analyte (with the exception of moniliformin and 3-
238 nitropropionic acid, that each exhibit only one fragment ion), allowed to confirm the identity of
239 the positive results according to validation guidelines [22, 30].

240 **3. Results and discussion**

241 **3.1 Method extension and the optimization of the LC-MS/MS parameters**

242 A multi-analyte LC-MS/MS method based on a “dilute and shoot” approach was originally
243 designed for the determination of 39 mycotoxins in cereals in 2006 [17]. Since then the method
244 has continuously been extended to a wide range of additional secondary metabolites of fungi and
245 bacteria. This multi-analyte approach was applied to study newly isolated fungal species, to
246 investigate fungal and bacterial metabolites in indoor environments and to study the spectrum of
247 (toxic) secondary metabolites to which humans and animals are exposed through the food and
248 feed chain [12, 27, 28]. Although in the meantime UHPLC has become available and has been
249 successfully used in mycotoxin analysis to achieve lower LODs and shorter analysis time [19,
250 21, 26] we have refrained from changing from HPLC to UHPLC for two reasons. On the one
251 hand, UHPLC columns are not compatible to turbid samples (as they tend to clog after a few
252 injections) whereas we did not face such problems when using standard HPLC column (we
253 found filtration not to be an option as all tested materials caused losses of certain compounds). In

254 addition On the other hand, the use of UHPLC is related to narrower peaks, which demands a
255 decreased cycle time in LC-MS/MS in order to obtain an appropriate number of data points per
256 peak. This inevitable emphasizes the problem of achieving sufficient dwell times in multi-
257 analyte methods.

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

267 The differences in the mechanical design between the instruments are mainly associated with
268 changes in the ion source parameter settings (curtain gas, ion spray voltage, source temperature,
269 ion source gases). The flow rates of all gases were increased to ensure an efficient evaporation of
270 the mobile phase, and thus avoid passing of liquid through the large orifice entrance. Regarding
271 analyte-dependent MS/MS parameters (declustering potential, collision energy, cell exit
272 potential), we followed the manufacturer's recommendations to increase the declustering
273 potentials (DP) of $[M+H]^+$ and $[M-H]^-$ optimized on the QTrap 4000 by 30 V instead of
274 individual re-optimization of each analyte. Nevertheless, we chose 30 analytes for the individual
275 re-optimization on the QTrap 5500 to check the difference in analyte-dependent parameters
276 values between both instruments. The optimized '5500' DP values of $[M+H]^+$ ions were all 20-
277 30 V higher compared to those on the QTrap 4000. For instance, the DP value of $[M+H]^+$ of
278 ochratoxin A was increased from 61 V to 86 V. The differences for NH_4^+ adducts (T-2 toxin :DP
279 4000 = 76 V and DP 5500 = 101 V) and Na^+ adducts were lower than the recommended 30 V
280 (HT-2:DP 4000 = 46 V and DP 5500 = 56 V). Similarly, the formation of $[M+CH_3COO]^-$
281 adducts (deoxynivalenol: DP 4000 = -40 V and DP 5500 = -60 V; 3-acetyldeoxynivalenol: DP
282 4000 = -45 V and DP 5500 = -55 V) required less than 30 V increase in DP values. However, the
283 increase of all '4000' DPs by 30 V was sufficient, as the optimal range of '5500' DPs of each
284 respective analyte is broader compared to the '4000' instrument. The settings of other analyte-

285 dependent parameters (e.g. collision energy, CE) were the same for both instruments and did not
286 require any further optimization.

287 Due to the lower QTrap 5500 acquisition mass range of 1250 Da, the analytes of molecular
288 weight higher than 1200 Da had to be excluded from the current method or, if possible, re-
289 optimized as double charged precursor $[M+2H]^{2+}$ ions. For example, actinomycin D (MW
290 1255.42 Da) does not give a stable $[M+2H]^{2+}$ ion, and could thus not be included in the new
291 method. However, cyclosporine A was successfully re-optimized and transfer from the negative
292 to the positive mode giving an intensive precursor ion of m/z of 601.9. Apart from 186 analytes
293 involved in our previous QTrap 4000 method, further 145 analytes were newly included. The list
294 of all analytes together with the optimized values of ESI-MS/MS parameters is given in
295 Appendix A.

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

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

310 **3.2 Validation of the method**

311 Currently, no directive or guidance for the validation of analytical methods for the determination
312 of multiple mycotoxins or for multiple analytes in general is established. The only available
313 guideline, the Commission Decision 2002/657/EC [30], provides some requirements and
314 recommendations concerning the performance of analytical methods for official control and the

315 interpretation of results. However, the guidance provided is insufficient for multi-analyte
316 methods for a couple of reasons: A definition of matrix effects and their evaluation is missing,
317 the term recovery is not exactly specified (whether it is extraction efficiency or apparent
318 recovery), and the determination of LOD and LOQ by spiking of 20 replicates at one level for
319 each matrix is not feasible for hundreds of analytes due to the costs of analytical standards.
320 Therefore, we decided to validate the given multi-analyte method according to SANCO protocol
321 No. 12495/2011 [22]. Although the validation criteria have been laid down for pesticide multi-
322 residue determination only, they represent the only “real-life” guidelines available for methods
323 involving hundreds of analytes with a wide range of physico-chemical properties. Since the
324 method is used for the analysis of wide range of matrices, including non-traditional matrices,
325 such as cassava or liquorice, comprehensive validation of each individual matrix for more than
326 three hundred of analytes would be very expensive and time-consuming. Hence only one
327 representative commodity from each matrix category, as suggested by SANCO, was included in
328 the validation of our multi-analyte method. As mentioned in section 2.2, we chose four model
329 matrices (apple puree, hazelnuts, maize, green pepper) representing the category groups that are
330 relevant regarding mycotoxin contamination.

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

341 **3.2.1 Method accuracy**

342 Apparent recovery (R_A) and matrix effects (SSE) strongly vary depending on the analyte/matrix
343 combination. As we use a neat solvent calibration and spiked samples in the routine analysis
344 rather than matrix-matched standards, showing the method accuracy on the apparent recoveries

345 (calculated according Equation 2), expressing both the extraction efficiency and the matrix
346 effects, is more “real-life” and relevant than showing data on the recovery of extraction step (R_E)
347 (Equation 1).

348 **3.2.1.1 Apparent recovery**

349 The distribution of R_A for 295 analytes in apple puree, hazelnuts, maize and green pepper is
350 depicted in Figure 1. The highest validated level (HL) corresponds to 1:10 dilution of the final
351 working solution of the analytical standards. For the lowest validated level (LL), the lowest
352 spiking level reliably detectable at five repetitions ($RSD < 20\%$) through both MRM transitions
353 was taken into account. Concerning the apparent recovery calculated at the HL, 74%, 68%, 64%
354 and 21% of analytes in apple puree, hazelnuts, maize and green pepper, respectively, were in the
355 range of 70-120% as recommended by SANCO [22]. For the analytes out of this range, either
356 high matrix suppression/enhancement (e.g. aflatoxins, alternariol and emodin in pepper) or low
357 extraction efficiency, for instance, 3-nitropropionic acid in hazelnuts was observed (the
358 somewhat lower extraction efficiencies in apple puree are partially due to the water content of
359 the sample (50 rel. %), which accounts for a increase of 12% of the volume of the raw extract.)
360 The lower apparent recovery for some analytes (e.g. chanoclavine and HT-2 toxin in pepper) was
361 caused by a combination of low extraction efficiency and matrix effects which was most
362 pronounced in green pepper (Figure 2). Therefore, 8% of the analytes (e.g. ergocryptine, α -
363 zearalenol, cerulenin, citrinin) were not detectable in green pepper at all, while for the other
364 matrices less than 4% of the analytes could not be detected. Concerning the LL, the percentage
365 of analytes matching the R_A of 70-120% was lower than at the HL. The R_A for apple puree,
366 hazelnuts and maize was 56%, 56% and 58%, respectively. In green pepper, the R_A of 70-120%
367 was achieved only for 20% of analytes. Altogether 25% of the compounds were not detected in
368 green pepper (for all the other validated matrices only <12% of analytes) at any lower level than
369 the highest validated level.

370 **3.2.1.2 Matrix effects**

371 SSE (Equation 3) was observed for all matrices. The extent of SSE was strongly dependent on
372 the analyte/matrix combination. As there is no acceptable range concerning the SSE in the
373 SANCO, we have decided that the analytes having the SSE values between 90% and 110% were
374 considered as not to be affected by matrix effects. The distribution of SSE in all validated
375 matrices is displayed in Figure 2. In general, the lowest matrix effects were observed for apple

376 puree. In this matrix, 59% and 43% of analytes were suppressed/enhanced by only <10% at HL
377 and LL. For instance, signal intensity of patulin, the most common natural toxin found in apples
378 and products thereof, was not affected by matrix in apple puree (Table 1) while it was highly
379 suppressed (SSE of 42%) in pepper (Table 4). Concerning hazelnuts only 48% and 35% of
380 analytes had the SSE in the range of 90-110% at HL and LL, respectively. 3-nitropropionic acid
381 and sterigmatocystin found as the analytes with the highest incidence in hazelnuts in our recent
382 study [31] were not affected by matrix effects at all. Aflatoxins which levels are regulated in nuts
383 by the European Commission (EC) [4] were slightly suppressed in hazelnuts (SSE in the range of
384 72-89%). However, the R_E values for aflatoxins in hazelnuts close to 100% (Table 2) and the
385 repeatability below 10% still allow to achieve accurate results in routine analysis. In total, only
386 44% of analytes at the HL and 35% of analytes at the LL were not affected by matrix effects in
387 maize. The mycotoxins with the legislation limits established in maize or cereals by the EC [4],
388 such as deoxynivalenol, zearalenone and fumonisins B₁ and B₂, and ochratoxin A were neither
389 suppressed nor enhanced at the HL (Table 3). As mentioned above, green pepper analysis
390 suffered from the matrix effects the most from all investigated matrices which is obvious from a
391 histogram in Figure 2. Only 10% of the analytes were not affected by signal
392 suppression/enhancement in green pepper. From the compound on the reduced analyte list (Table
393 4), only moniliformin was not affected by the matrix effects in green pepper. Some analytes, e.g.
394 paspalic acid and aspyrone, could not be evaluated at all because of huge interferences occurred
395 at the MRM transitions.

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

407 these analyte/matrix combinations co-extracts are likely to work as a protective agent for light-
408 or oxygen-sensitive analytes in the matrix-matched standards. The concentration of these
409 analytes in neat solvent standards decreased much faster. For instance, a protective function of
410 ascorbic acid (occurring in many plant matrices) against oxidation of cyclopiazonic acid has
411 already been proven [33]. In addition to that, the matrix might also influence the epimerization
412 rate e.g. of ergot alkaloids.

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

424 Another difficulty is to estimate the extent of SSE, and also other performance characteristics,
425 for analytes showing epimerization, like ergots alkaloids. A C₉=C₁₀ double bond of the ergoline
426 ring is responsible for epimerization with respect to the centre of chirality C₈ (Figure 3). Thus,
427 ergot alkaloids are converted from *-ine* to *-inine* form and back depending on the solvent and
428 pH. The *-ine/-inine* ratio can be different in the neat solvent and the matrix-matched
429 environment [34]. For instance, epimers ergotamine and ergotaminine eluted at 7.51 and 7.67
430 min showed the ratio shifted more to the latter one (Appendix B). However, as the ratio between
431 these two epimers in the extract is not known, the calculated SSE, R_A and R_E cannot be
432 considered as relevant for evaluation of routine samples. Instead, the data for dihydroergometrine
433 which is eluted between ergotamine and ergotaminine (RT=7.62 min) could be considered.
434 Dihydroergotamin is hydrogenated at positions C₉ and C₁₀, hence it does not exhibit
435 epimerization.

436 **3.2.1.3 Limits of detection and quantification, linearity**

437 The limits of quantification for all analytes were estimated as the lowest spiking level (LL)
438 detectable at both MRM transitions at all five repetitions (Appendix B). As both parameters, the
439 LOD and the LOQ, are strongly dependent on the actual condition of the instrument, i.e. the
440 contamination level of the instrument, the lowest level approach is more feasible than the
441 traditional S/N one, especially for the multi-analyte methods including more than one hundred
442 analytes. In order to compare both approaches to LOQ determination, the calculation of LOD
443 and LOQ according to signal to noise ratio (S/N) (Equations 4 and 5) was carried out as well, but
444 only for 29 analytes listed in Tables 1-4. From Table 1-4 it is obvious that there is not a huge
445 difference between LOQ (estimated from S/N ratio) and LL. Moreover, the levels of LOQ and
446 LL are strongly dependent on the analyte/matrix combination. The highest difference between
447 LOQ and LL was observed for citrinin in apple puree and for patulin in all matrices. Concerning
448 the matrix influence on the detection capability of the method, the highest levels of LOQ and/or
449 LL were obtained for green pepper.

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

454 **3.2.2 Method precision**

455 The precision of the method was proven within the laboratory as repeatability of 5 repetitions at
456 the highest and the lowest spiking level. Most of the analytes fulfilled the criteria of RSD $\leq 20\%$.
457 An RSD of $\leq 20\%$ was achieved for 97% of analytes in apple puree and hazelnuts and for 95% of
458 compounds in maize. The repeatability below 20% of RSD for green pepper was obtained only
459 for 89% of all analytes.

460 As expected, the method precision at the LL was slightly worse compared to the HL. On
461 average, 85% of the analytes passed the acceptable repeatability in all matrices except for pepper
462 in which only 77% of the compounds fulfilled the recommended RSD at the LL. Ergot alkaloids
463 belong to the analytes with higher repeatabilities (but still below 20%) which is caused by the
464 epimerization between *-ine* and *-inine* form [34]. In general, the highest variation among the
465 five repetitions was observed in green pepper. For instance, for aflatoxin the RSD of 94% at the

466 HL was achieved. However, for some analytes the required repeatability was achieved in green
467 pepper but not in any other matrix. Fumiquinazoline A in apple puree with an RSD of 104% and
468 altenusin and geodin in maize with repeatabilities of 69% and 25% are example for this
469 phenomenon (Appendix B).

470 **3.2.3 Method trueness**

471 The trueness of the method has been continuously proven by the participation in various
472 proficiency tests provided by FAPAS[®], Bipea and CODA-CERVA. The mycotoxin levels were
473 obtained by means of neat solvent calibration curve and the results were corrected on the
474 apparent recoveries of the respective toxins. Table 5 summarises the results of the recently
475 performed proficiency tests that our laboratory participated in. The samples cover a wide range
476 of analyte/matrix combinations. Therefore, the method trueness could have been proven also for
477 the matrix types which were not validated, such as animal and chicken feed, cassava, milk
478 powder. An apparent recovery of 100% was assumed for all these matrices. The *z*-scores
479 calculated according to FAPAS[®], Bipea and CODA-CERVA proficiency testing protocols for
480 the all of the analyte/matrix combinations lied within the acceptable range of -2 to 2 except
481 aflatoxin G₂ in baby food (*z*-score = 2.62) and fumonisin B₁ in maize (*z*-score = 2.97).
482 Furthermore, it has been proven that the method provides accurate results also for matrices with
483 high sugar content such as raisins. High sugar content matrices were not included into the
484 validation process as they were not amenable to the miniaturization of the sample pretreatment to
485 0.5 g which is necessary for spiking experiments in order to keep the amount of standards to a
486 minimum.

487 **4. Conclusions**

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

- 494 • Validation data have been obtained for 295 analytes. In addition, the MS/MS transitions
495 are provided for another 36 metabolites, for which, however, no sound validation could
496 be realized due to non-availability of analytical standards or due to instability of these
497 compounds under the used analytical conditions.
- 498 • As compounds comprising a wide range of chemical properties have been included in the
499 method, the extraction and chromatographic conditions had to be compromised. For
500 instance, the acidic conditions essential for the extraction and separation of acidic
501 compounds such as fumonisins, and ochratoxin A are not favourable for basic
502 compounds (e.g. ergot alkaloids, enniatins). Therefore, the apparent recovery levels
503 varied to a large extent depending on the analyte/matrix combination. In general, green
504 pepper was the most difficult matrix in terms of recovery and matrix effects for the most
505 of the analytes.
- 506 • Both signal suppression and enhancement were observed for all four matrices. Their
507 extent was dependent on the analyte/matrix combination and the analyte concentration.
508 The matrix contributing the least to SSE was apple puree, while the highest number of
509 analytes suffering from severe SSE were found in green pepper a.
- 510 • Despite some analytes were out of the range of 70-120% apparent recoveries, the
511 repeatability (RSD calculated from 5 replicates) was below the acceptable level of 20%
512 for most of them.
- 513 • The LOQs or LLs of the method for the toxins regulated by EC [3] were below the
514 required maximum levels for the respective toxins except of aflatoxins and ochratoxin A
515 in baby food and aflatoxin M₁ in milk.
- 516 • z -scores $< |2|$ were achieved at all proficiency tests the laboratory participated with the
517 exception of aflatoxin G₂ in babyfood (z -score = 2.62) and fumonisin B₁ in maize (z -
518 score = 2.97).

519

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

523

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Figure Captions

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

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

Figure 3 Chemical structures of ergotamine, ergotaminine and dihydroergotamine

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

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

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

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

Table 5 Summary of the performed proficiency tests

Appendix A Parameters of HPLC-MS/MS method

Appendix B Validation data obtained on spiked apple puree, hazelnut, maize and green pepper

Analyte	RT (min)	LOD ($\mu\text{g}/\text{kg}$)	LOQ ($\mu\text{g}/\text{kg}$)	LL ($\mu\text{g}/\text{kg}$)	R _E (%)	SSE (%)	R _A (%)	RSD (%)	HL ($\mu\text{g}/\text{kg}$)	R _E (%)	SSE (%)	R _A (%)	RSD (%)
3-nitropropionic acid	3	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	44	71	107	76	7.3
Aflatoxin B ₂	8.4	1.2	4	1.3	58	99	57	13.4	44	78	84	65	8.7
Aflatoxin G ₁	8	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	0.5	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
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
Emodin	14.3	0.1	0.4	2.3	73	100	73	3.8	55	77	102	79	3.7
Enniatin B	14	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	6	74	91	67	7.5
Fumonisin B ₁	9.4	2.6	8.6	17	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	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
Phomopsis A	7.2	n.e.	n.e.	35.1	n.d.	n.d.	n.d.	n.d.	117	75	96	72	11.8
Phomopsis 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	3.3	4.6	96	93	90	9.4	154	79	102	80	2.9
Zearalenone	11.9	0.3	1	15.5	81	146	118	8.6	155	98	104	101	5.9

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

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	0.6	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	44	71	107	76	7.3
Aflatoxin B ₂	8.4	1.2	4	1.3	58	99	57	13.4	44	78	84	65	8.7
Aflatoxin G ₁	8	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	0.5	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
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
Emodin	14.3	0.1	0.4	2.3	73	100	73	3.8	55	77	102	79	3.7
Enniatin B	14	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	6	74	91	67	7.5
Fumonisin B ₁	9.4	2.6	8.6	17	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	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	3.3	4.6	96	93	90	9.4	154	79	102	80	2.9
Zearalenone	11.9	0.3	1	15.5	81	146	118	8.6	155	98	104	101	5.9

671 Note: RT-retention time; LOD-limit of detection; LOQ-limit of quantification; LL-lowest validation level; HL-

672 highest validation level; R_E-recovery of extraction step; R_A-apparent recovery; SSE-signal

673 suppression/enhancement; n.e.-not evaluated; n.d.-not detected; * concentration of the standard not known

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	0.6	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	44	71	107	76	7.3
Aflatoxin B ₂	8.4	1.2	4	1.3	58	99	57	13.4	44	78	84	65	8.7
Aflatoxin G ₁	8	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	0.5	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
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
Emodin	14.3	0.1	0.4	2.3	73	100	73	3.8	55	77	102	79	3.7
Enniatin B	14	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	6	74	91	67	7.5
Fumonisin B ₁	9.4	2.6	8.6	17	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	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
Phomopsis A	7.2	n.e.	n.e.	35.1	n.d.	n.d.	n.d.	n.d.	117	75	96	72	11.8
Phomopsis 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	3.3	4.6	96	93	90	9.4	154	79	102	80	2.9
Zearalenone	11.9	0.3	1	15.5	81	146	118	8.6	155	98	104	101	5.9

676 Note: RT-retention time; LOD-limit of detection; LOQ-limit of quantification; LL-lowest validation level; HL-

677 highest validation level; R_E-recovery of extraction step; R_A-apparent recovery; SSE-signal

678 suppression/enhancement; n.e.-not evaluated; n.d.-not detected; * concentration of the standard not known

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.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	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.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	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
Phomopsis A	7.2	n.e.	n.e.	n.d.	n.d.	n.d.	n.d.	n.d.	117.1	83	85	70	19.9
Phomopsis 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

681 Note: RT-retention time; LOD-limit of detection; LOQ-limit of quantification; LL-lowest validation level; HL-

682 highest validation level; R_E-recovery of extraction step; R_A-apparent recovery; SSE-signal

683 suppression/enhancement; n.e.-not evaluated; n.d.-not detected; * concentration of the standard not known

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
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 paste	03-1431	Aflatoxin B ₁	17.3	18.3	3.7	-0.27
		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
		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		

686 Note: n.d.-not detected; n.e.-not evaluated

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