



# Quantification of 700 mycotoxins and other secondary metabolites of fungi and plants in grain products



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This work reports on the validation of a liquid chromatography–tandem mass spectrometric method for the simultaneous quantification of more than 700 mycotoxins and other secondary fungal metabolites and plant toxins in pasta, biscuits, crackers and musli. The “dilute and shoot” approach was found to be fully applicable to these complex matrices, as only 7–14% of the analytes exhibited significant matrix effects while recoveries of the extraction were outside the target range of 70–120% for only 26 compounds. Data on repeatability (based on 7 brands per matrix) and on intermediate precision was compliant to the related < 20% criterion for 95–98% and 99% of all analytes, respectively. The limits of quantification were much lower than the related regulatory limits set for mycotoxins in cereal products. Application of the method to 157 samples from the European market revealed the presence of enniatins and deoxynivalenol in the majority of the samples. No regulatory limits were exceeded except the sum of ergot alkaloids being higher in a few samples than the 50–150 µg/kg to be implemented as of July 2024.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become the working horse for the quantitative determination of mycotoxins and other low molecular weight contaminants in food samples. The selectivity, robustness and multi-analyte capability of this technique enables developing protocols for sample preparation that include single solvent extraction (sometimes in connection with a QuEChERS-like salting out step) and only minimal or even no clean-up<sup>1</sup>. This facilitates their application to different matrices ranging from raw agricultural commodities like grains or nuts to complex processed products. Although regulatory limits for mycotoxins and plant toxins are laid down (e.g. in Commission Regulation 2023/915) for grain based products (thus requiring reliable methods for compliance testing), there are only a few papers focusing on validation of LC-MS/MS based methods for mycotoxins in products like pasta, biscuits, breakfast cereals or crackers<sup>2–12</sup>.

Individual samples/brands of processed foodstuffs might exhibit significantly larger differences in composition compared to raw grains (e.g. type of grain, whole-grain vs. refined grains, presence of oils, flavours, additives). From an analytical point of view, this might result in significant dispersion of recoveries of the extraction or of matrix effects obtained for different samples belonging to the same matrix (i.e relative matrix effects). Therefore, it might

be questioned if validation data obtained from technical replicates of one “representative” or pooled sample can be considered to be a sufficient proof for the validity of the method for the whole type of grain product. In particular, relative matrix effects have been found to contribute significantly to the uncertainty of the method<sup>13</sup>.

Recent official guidelines on method validation take this issue into consideration to a different extent. Commission Implementing Regulation EC 2021/808 on method performance for pharmacologically active compounds in food producing animals<sup>14</sup> demands to determine the absolute recovery for at least 6 representative lots of a matrix in case neither internal standardization nor matrix matching is applied. In addition, the coefficient of variation shall not be greater than 20% for the matrix factor to be determined in 20 different samples. Instead of giving such a specific criterion Commission Implementing Regulation EC 2023/2782<sup>15</sup> for mycotoxins in food suggests supplementing data on repeatability and within laboratory reproducibility with data from proficiency testing. This seemingly less strict requirement is most probably due to the fact that - in contrast to other contaminant classes such as pesticides or veterinary drugs - stable isotope labelled internal standards are available for most of the mycotoxins addressed by regulatory limits. The use of such standards is considered as the most reliable approach for

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dealing with matrix effects by Official Control Labs<sup>16</sup>, especially as it effectively compensates for relative matrix effects as well.

However, LC-MS/MS based methods with a broader scope and/or targeting contaminants from different classes still require a thorough characterization of relative matrix effects. In a previous study<sup>17</sup> we have shown that for mycotoxins in raw agricultural commodities (grains, nuts and dried fruits) compliance to performance criteria for repeatability can still be obtained if the data derives from different varieties of a given matrix rather than from technical replicates of a single or a pooled sample. The goal of this work was to determine method performance data of an extended version of the method in processed grain matrices (pasta, biscuits, crackers and musli). The robustness of the method was challenged by spiking seven different brands (with potential differences in the type of grains, ingredients, additives, flavours etc.) per matrix. In this way, relative matrix effects as well as potential differences in extraction efficiencies between individual samples were taken into consideration in the calculation of the expanded measurement uncertainty.

## Results and discussion

### Method extension

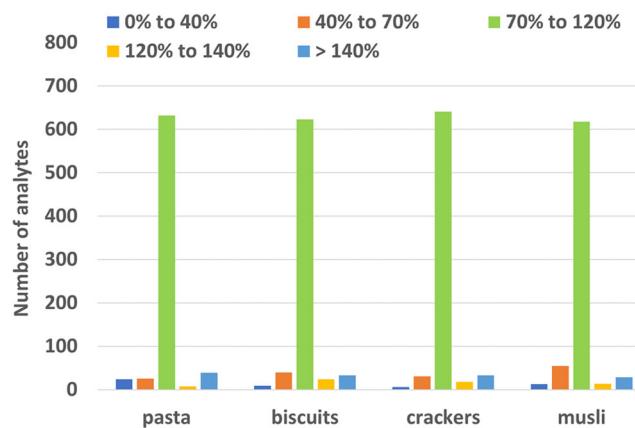
The previously published LC-MS/MS protocol was further extended to cover almost 900 metabolites (170 of them not being amenable to quantification due to lack of a quantitative standard). This required a modification of the general sMRM parameters to achieve acceptable data quality for the 1090 and 736 sMRM transitions acquired in the positive and the negative ionization mode, respectively. As we previously found that increasing the dwell time at the cost of number of data points per peak is advantageous at least on the instrumental platform used in our laboratory<sup>18</sup>, the target cycle time was increased from 1.0 s to 1.4 s, resulting in minimum dwell times of 20 and 25 ms in the positive and the negative ionization mode, respectively. At the same time, at least 10 data points were obtained for each LC peak with a typical width of 15 s.

The newly implemented compounds comprise (besides fungal metabolites) pyrrolizidine and tropane alkaloids, as those compounds may, similar to mycotoxins, contaminate cereals during harvesting and food processing. Similar to the pair of epimers of ergotamine and ergosine, a baseline separation of some individual plant toxins exhibiting the same MS/MS parameters (such as the triplet of indicine, intermedine and lycopsamine as well as that of their N-oxides) cannot be achieved under acidic conditions and an alkaline gradient or a two-dimensional separation<sup>19</sup> would be preferable for a dedicated quantitative analysis of those compounds.

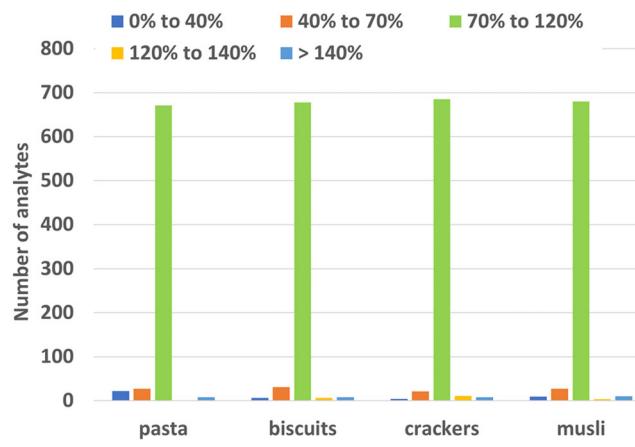
### Apparent recoveries, matrix effects and recoveries of the extraction

The compiled data for 730 metabolites obtained under repeatability conditions in the four matrices (with seven different individual samples for each matrix) is given in Supplementary Table S1. 85–88% of the investigated analytes exhibit  $R_A$  values in the target range of 70–120%, whereas the fractions of  $R_E$  complying this criterion range from 92 to 94% (Figs. 1 and 2). Compared to our investigations in raw commodities of grains, nuts and dried fruits ( $R_A$ : 58–83%  $R_E$ : 84–94%<sup>17</sup>) the fractions of compliant  $R_A$  values have significantly increased while the increase was less pronounced for  $R_E$ . This is due to a decrease of absolute matrix effects as only 7–14% of the analytes were significantly suppressed/enhanced (i.e. a deviation of more than  $\pm 20\%$ ) in the spiked extracts compared to the solvent standards. We consider this to be a counter-intuitive result as we have previously expected that the more complex processed matrices investigated in this study will result in more pronounced matrix effects. We speculate that mechanical and thermal processing of the grains and other ingredients causes a reduction/transformation of low molecular weight matrix constituents that cause matrix effects in the raw commodities.

For 31 compounds, the signal in the spiked extracts was consistently enhanced compared to the solvent standards. For enniatins, abscisic acid, brevianamide F, cordycepin, cyclo (L-Pro-L-Tyr), infectopyron and tryptophol this was due to the non-availability of true blank samples. In contrast, the sample matrix seems to act as a



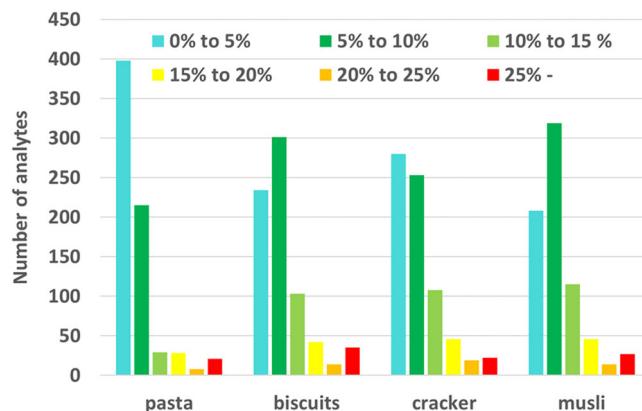
**Fig. 1 | Summary of apparent recoveries obtained under repeatability conditions.** Numerical values for all individual analyte/matrix combinations are given in Supplementary Table S1. The green bars cover the target range of 70–120%.



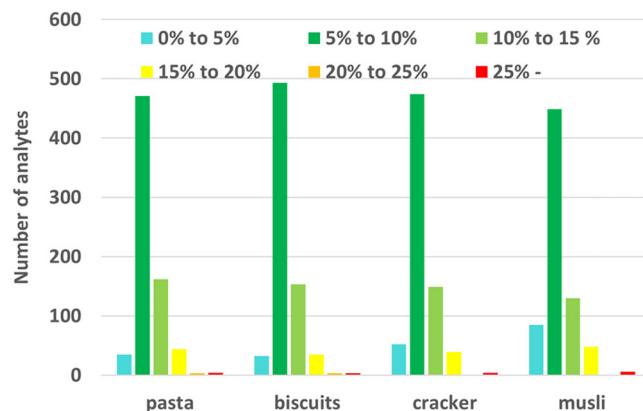
**Fig. 2 | Summary of recoveries of extraction obtained under repeatability conditions.** Numerical values for all individual analyte/matrix combinations are given in Supplementary Table S1. The green bars cover the target range of 70–120%.

protectant against oxygen, light or adsorption in case of some other compounds such as cyclopiazonic acid, altenusin, altersetin, (epi-) equisetin, demethylsulochrin, methylequisetin, rugulosin, sorbicillactone A or violaceol I and II, resulting in a significantly enhanced signal in the spiked samples and spiked extracts compared to the solvent standards. As concerns the latter, matrix effects in the narrow sense (i.e. enhancement of the ionization of the analyte in the ion source due to presence of a particular co-eluting matrix compound) seem to be unlikely to be the cause instead as (I) the increased signals were observed for these analytes in all the matrices investigated in this work and the raw grains/nuts/dried fruits investigated earlier as well and (II) this increase was huge for a few compounds (e.g. a factor of 4 for rugulosin and sorbicillactone A or even a factor of 30–50 for violaceol I and II).

Only half of the 26 analytes not complying to the range of 70–120% for  $R_E$  exhibit very low and/or irreproducible extractions and need therefore to be considered to be incompatible to the sample preparation protocol. The other half (including fumonisin B1) exhibits an  $R_E$  between 50 and 70% with an associated  $RSD_r$  of lower than 20%, which is considered to be still acceptable<sup>15</sup>. Although the use of spiked materials is common practice for the determination of the extraction efficiencies in multi-analyte methods, it might still introduce a bias as spiked samples might be easier to extract in



**Fig. 3 | Summary of repeatabilities RSD<sub>r</sub> obtained for 7 spiked individual samples per matrix.** Numerical values for all individual analyte/matrix combinations are given in Supplementary Table S1.



**Fig. 4 | Summary of intermediate precision RSD<sub>WLR</sub> obtained for spiked technical replicates of one sample.** Numerical values for all individual analyte/matrix combinations are given in Supplementary Table S4.

comparison to naturally contaminated materials. As we did not observe a systematic underestimation of the concentrations in the samples from proficiency testing (see below), we concluded that the extraction efficiencies we have obtained based on the spiked samples are applicable to real-world samples as well.

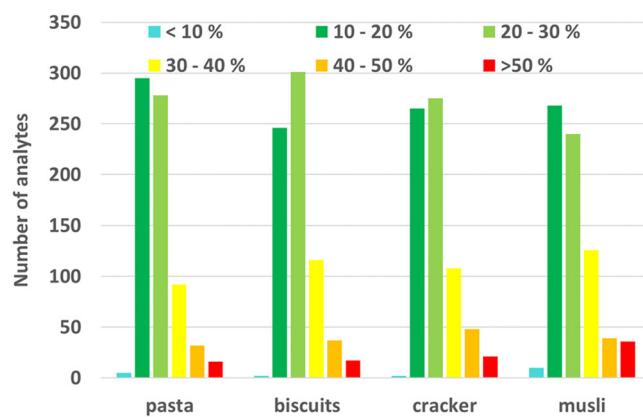
Apparent recoveries obtained on lower concentration levels for biscuits and musli, respectively, did not show any significant differences between the tested levels for the large majority of analytes (Supplementary Tables S2 and S3). This was true as well for analyte/matrix combinations resulting in R<sub>A</sub> values significantly deviating from 100% e.g. 65 ± 8%, 53 ± 7%, 59 ± 5%, 57 ± 10% and 64 ± 7% for citrinin in biscuits or 73 ± 7%, 70 ± 6%, 80 ± 6% and 82 ± 8% for fumonisin B1 in musli. This underlines our previous consideration of spiking experiments on levels near the LOQ not being sufficiently relevant to justify the time effort required in broad multi-methods for a manual cross-check of small peaks.

## Precision

Repeatability data obtained for the seven different individual spiked samples per matrix was better than previously expected with more than 70% of all analyte/matrix combinations exhibiting an RSD<sub>r</sub> < 10% and 95–98% of all analytes complying to the RSD<sub>r</sub> < 20% criterion (Supplementary Table S1 and Fig. 3). Being in line with the observation on reduced absolute matrix effects in the investigated matrices compared to raw commodities (see above), the extent of relative matrix effects was reduced significantly as well. Still, the within-laboratory reproducibility obtained on 7 technical replicates of one sample per matrix (thus cancelling any relative matrix effects) over a period of 7 weeks was still slightly superior (RSD<sub>WLR</sub> < 20% for 98.9 to 99.2% of all analytes depending on the matrix, see Supplementary Table S4 and Fig. 4) to the RSD<sub>r</sub>. The combined measurement uncertainty calculated from RSD<sub>r</sub> and RSD<sub>WLR</sub> was compliant for 95–98% of all investigated analytes depending on the matrix to the U<sub>r,exp</sub> < 50% criterion set in ref. 15 (Supplementary Table S4 and Fig. 5).

## Trueness

The trueness of the method is monitored on a continuous basis by participation in a proficiency testing program. 1118 results were submitted since October 2019 for 30 analytes (5 aflatoxins, ochratoxin A, fumonisins B1 and B2, T-2 and HT-2 toxin, deoxynivalenol and its derivatives, nivalenol, zearalenone, 12 ergot alkaloids, atropine and hyoscine) for grains, nuts, dried fruits, feed, baby food, spices etc. For the sample that was most similar to the matrices investigated in this study (i.e. rusk) all z-scores were between -1 and 1 except 3-acetyldeoxynivalenol with z = 1.15 (Table 1; all other individual results are shown in Supplementary Table S5). Overall, 68.2% and 96.2% of the submitted results exhibited a z-score of -1 < z < 1 and



**Fig. 5 | Summary of the expanded measurement uncertainties U<sub>r,exp</sub> calculated from RSD<sub>r</sub> and RSD<sub>WLR</sub>.** Numerical values for all individual analyte/matrix combinations are given in Supplementary Table S4.

-2 < z < 2, respectively (Fig. 6), indicating the trueness of our dilute and shoot approach is at least comparable to the methods of the other participants. We consider this to be perfectly satisfactory as 1) full validation data is not available for all matrices covered by the proficiency testing scheme and 2) part of the other participants use dedicated methods (e.g. LC with fluorescence detection for aflatoxins) or stable isotope labelled internal standards.

## Sensitivity

LOQs determined both in biscuits and in musli according to EURACHEM<sup>20</sup> are compiled in Supplementary Table S6 and Fig. 7. The numerical values obtained for a given analyte in the two matrices differed by more than a factor of 2 only for 42 compounds. The majority of those exceptions were caused by traces of the respective analytes (e.g. culmorin or chrysogin in musli) or by interferences being present in the samples of one matrix, thus hampering data evaluation of lower levels. This confirms our earlier conclusion drawn from the related data in figs and maize<sup>17</sup> i.e. that it might not be essential to determine LOD/LOQ for each new commodity. Instead, a pragmatic approach for a broad multi-analyte method is to establish a conservative matrix-independent estimation as any lower concentration is irrelevant for most/analyte matrix combinations anyway. Indeed, the obtained LOQs are below the regulatory limits set for mycotoxins in cereal-based products, bread, biscuits, pastries etc. set in Commission Regulation 2023/915<sup>21</sup>, whereas the more stringent limits set for grain-based baby food are in the range or

**Table 1 | Results of the proficiency test in rusk**

Analyte	Determined conc. [µg/kg]	Assigned conc. [µg/kg]	$\sigma_{\text{Proficiency}}$ [µg/kg]	z-score
Aflatoxin B1	3.12	2.65	0.8	0.59
Aflatoxin B2	3.02	2.55	0.77	0.61
Aflatoxin G1	1.74	1.67	0.5	0.14
Aflatoxin G2	1.71	1.35	0.41	0.88
Sum Aflatoxins	9.59	8.13	2.29	0.64
Ochratoxin A	3.66	3.73	1.12	-0.06
Deoxynivalenol	102	127	34	-0.72
15-AcDON	98	112	30	-0.48
3-AcDON	125	95	26	1.15
sum AcDONs	223	197	51	0.50
all DONs	325	324	42	0.02
Nivalenol	92	98	26	-0.23
T-2 toxin	59.9	48	14	0.85
HT-2 toxin	50.3	44	13	0.48
Sum T-2/HT-2	110	91	25	0.77
Zearalenone	63.6	68	19	-0.23

slightly below the respective LOQs for aflatoxins, ergot alkaloids and tropane alkaloids.

### Analysis of real-world samples

Individual data obtained on the 157 investigated samples are compiled in Supplementary Table S7. As considers mycotoxins addressed by regulatory limits, the findings are in fair agreement with published studies<sup>3,6,8–11</sup>. Deoxynivalenol was most frequently identified (in approximately two thirds of the samples) at levels below the EU regulations. This was also true for zearalenone (identified in one out of six samples) as well as for other trichothecenes, and ochratoxin A (which was positively identified in one sample), whereas fumonisins and aflatoxins were not present at detectable levels at all. In contrast to this, the regulatory limit of 50–150 µg/kg (as of 1st July 2024) set in Commission Regulation 2023/915<sup>21</sup> for the sum of ergot alkaloids in milling products has been exceeded in a few samples. Crackers exhibited a significantly higher rate of positive samples (68%) compared to pastries and pasta, which supports the findings of earlier studies on rye-based products<sup>22,23</sup>. Enniatins were detected in the majority of samples, thus confirming earlier studies<sup>3,8,10</sup>, with the median concentrations of the

positive samples ranging from the sub- to the low µg/kg level. Culmorin and 15-hydroxyculmorin were identified in the majority of samples as well at levels similar to deoxynivalenol, which is inline with our findings on naturally contaminated and inoculated wheat<sup>24</sup>. The concentration of alternariol exceeded the indicative level for grain-based baby food of 2 µg/kg set in Commission Recommendation 2022/553<sup>25</sup> in two samples, whereas alternariol monomethyl ether as well as tenuazonic acid were below the related values (2 and 500 µg/kg, respectively) in all samples. Infectopyron was the only metabolite produced by *Alternaria* species that was present at levels exceeding 100 µg/kg, which confirms earlier findings on milling oats intended for human consumption<sup>26</sup>. The remaining fungal metabolites produced by species belonging to *Aspergillus*, *Penicillium* or other genera were found either in only few samples or at low concentrations, with the exception of queuine A that was present in 61 samples with a maximum level of 534 µg/kg.

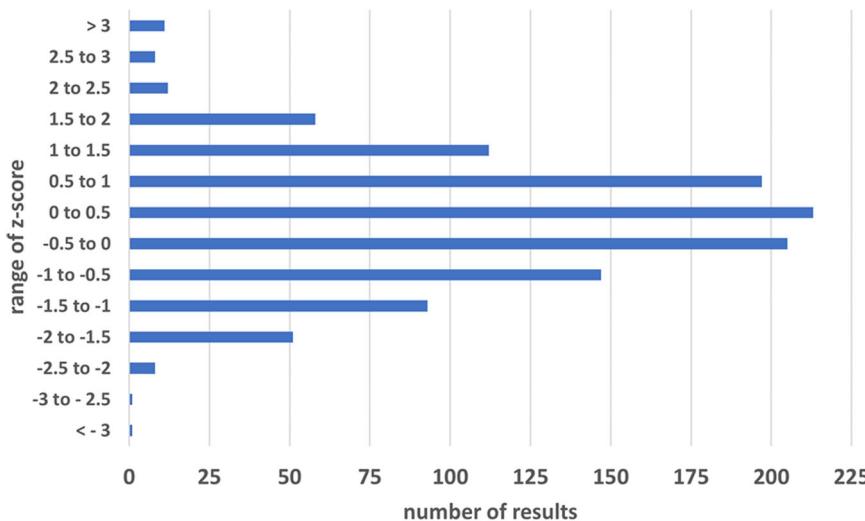
Neither tropane alkaloids nor pyrrolizidine alkaloids addressed by EC 2023/915<sup>21</sup> were identified in any of the samples (with the exception of low levels of erucifolin). In contrast to this, the cyanogenic glucosides linamarin, lotaustralin and prunasin (with the related cyanide equivalent being far below the regulatory limits set for cassava flour), the glycoalkaloids solanine and chaconin (produced by *Solanaceae* like potatoes or tomatoes) as well as xanthotoxin and the phytoestrogen biochanin were present in up to half of the samples with maximum concentrations in the low mg/kg range. Finally, a few of the unspecific metabolites (mostly cyclic dipeptides or anthraquinone derivatives) included in the applied analytical method were present in the majority of samples with levels of up to 40 mg/kg.

The data presented in this work confirm that a quantitative determination of hundreds of contaminants of fungal and plant origin in diluted crude extracts of processed grain matrices is feasible with modern LC-MS/MS instruments. Somewhat surprisingly, the increased complexity of the investigated matrices and the differences in composition between individual samples belonging to the same matrix did not result in an increase of absolute or relative matrix effects compared to the raw grains, nuts and dried fruits investigated in an earlier study<sup>17</sup>. Still, the major conclusions remain the same i.e. 1) relative matrix effects significantly contribute to the uncertainty of the method (as the intermediate precision obtained on 7 technical replicates was compliant to the <20% criterion for an even larger fraction of the analytes compared to the repeatability obtained on 7 different samples) and 2) evaluation of low spiking levels upon transfer of the method to a new matrix is not essential as extraction efficiencies and matrix effects are independent of the analyte concentration and numerical values determined for the LOQ exhibited a reasonable agreement between the investigated matrices.

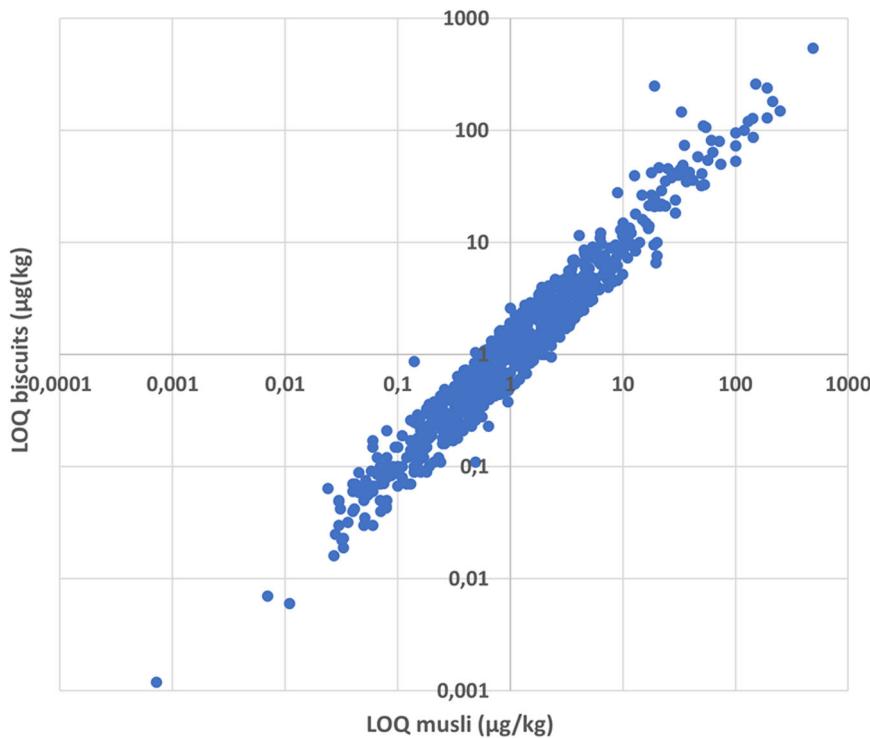
The application of the method to real-world from supermarkets confirmed that the samples are compliant to current EU regulation. However, it is

**Fig. 6 | Compilation of z-scores obtained in proficiency testing (1118 results submitted).**

$z = (\text{measured concentration} - \text{assigned concentration})/\text{standard deviation of the proficiency test}$ ; a z-score between -2 and 2 is considered as a satisfactory result, a z-score between -3 and -2 or between 2 and 3 is considered as a questionable result, a z-score < -3 or > 3 is considered as an unsatisfactory result.



**Fig. 7 | Overview on the LOQs determined according to EURACHEM<sup>20</sup>.** Numerical values for all individual analyte/matrix combinations are given in Supplementary Table S6.



almost impossible to find a sample that is completely free from mycotoxins (most notably free from enniatins) and co-contamination with different compounds is the rule rather than the exception. This underlines the importance of investigations of any potential effects of the chronic uptake of very low levels of (mixtures of) natural toxins.

## Methods

### Chemicals and reagents

LC-MS Chromasolv grade methanol was obtained from Riedel de Haen (Seelze, Germany), LC-MS grade ammonium acetate and glacial acetic acid (p.a.) were purchased from Sigma-Aldrich (Vienna, Austria). Reverse osmosis water was purified by a Purelab Ultra system (ELGA LabWater, Celle, Germany). HiPerSolv Chromanorm HPLC gradient grade acetonitrile was purchased from VWR Chemicals (Vienna, Austria).

Reference standards were either isolated in-house, obtained as gifts or purchased from the following commercial sources: AdipoGen Life Sciences (Liestal, Switzerland), AG Research (Christchurch, New Zealand), AnalytiCon Discovery (Potsdam, Germany), Bio Australis (Smithfield, Australia), BioViotica Naturstoffe GmbH (Dransfeld, Germany), Cfm Oskar Troitzsch GmbH (Marktredwitz, Germany), Chiralex B.V. (Nijmegen, The Netherlands), CSIR Biosciences (Pretoria, South Africa), Enzo Life Sciences (Lausen, Switzerland), Fermentek Ltd. (Jerusalem, Israel), Iris Biotech GmbH (Marktredwitz, Germany), LGC Promochem GmbH (Wesel, Germany), PhytoLab GmbH & Co. KG (Westenbergsgreuth, Germany), Romer Labs<sup>®</sup> Inc. (Tulln, Austria), Santa Cruz Biootechnology Inc. (Dallas, TX, USA), Sigma-Aldrich (Vienna, Austria), Takara Bio Europe (Saint-Germain-En-Laye, France), THP Medical Products (Vienna, Austria) and Toronto Research Chemicals (Toronto, Canada). The related details are given in Supplementary Table S8.

Single-analyte stock solutions were prepared by dissolving the solid substance in acetonitrile, preferably at 250 µg/mL. Due to limited solubility in acetonitrile, a few compounds were dissolved in acetonitrile/water 1:1 (v/v), methanol or water instead. 78 intermediate mixes were prepared by mixing the stock solutions of 10 analytes each for easier handling. The final multi-analyte standard was freshly prepared prior to spiking experiments by mixing of the intermediate mixes. All solutions were stored at -20 °C.

### Samples

For each investigated matrix (pasta, biscuits, crackers and musli) seven different individual samples exhibiting no or very low analyte concentrations were selected for spiking experiments. All pasta samples were made from durum wheat flour, one brand was a whole meal durum flour. As considers biscuits, crackers and muslis, brands based on different cereals with different additives and flavours were selected, details are given in Table 2.

The 157 different real-world commercial samples were provided from the European market. Proficiency test samples were obtained from BIPEA (Gennevilliers, France).

### Sample preparation

Samples were milled and homogenized using an Osterizer blender (Sunbeam Oster Household Products, Fort Lauderdale, FL, USA). 5g-portions were subsequently extracted with 20 mL of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) and shaken for 90 min using a rotary shaker (GFL 3017, GFL; Burgwedel, Germany). 500 µL of the supernatants were transferred into HPLC vials and diluted with 500 µL of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v). After appropriate mixing, 5 µL of the diluted extracts were injected into the LC-MS/MS system without further pre-treatment. For spiked samples a miniaturized approach of 0.25 g sample and 1 mL extraction solvent was applied to economize on standards.

### Instrumental parameters

The method applied in this study is an extension of the version described in detail elsewhere<sup>17</sup>. In brief, a QTrap 5500 MS/MS system (Sciex, Framingham, MA, USA) equipped with a TurboV electrospray ionisation (ESI) source was coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was obtained at 25 °C on a Gemini C<sub>18</sub>-column, 150 × 4.6 mm i.d. with a particle size of 5 µm, which was equipped with a C<sub>18</sub> security guard cartridge, 4 × 3 mm i.d. (both Phenomenex, Torrance, CA, USA). For elution of the analytes a binary methanol/water gradient was applied with a flow rate of 1000 µL/min. 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 hold time of 2 min at 100% A, the

**Table 2 | Composition of the individual samples of biscuits, crackers and musli used for spiking experiments**

Sample	Biscuits	Crackers	Musli
1	Common wheat flour, sugar, sunflower oil, butter, pasteurized milk, butter, cocoa, chocolate, cocoa butter, flavouring, eggs, honey, wheat starch, soy lecithin, natural vanilla	Common wheat flour, extra virgin olive oil, sunflower oil, barley and corn malt extract	Wholemeal flakes (oats, wheat) with 40% dried fruits (figs, sultanas, raisins, apples), sunflower oil
2	Oat flakes, wholemeal common wheat flour, spelt, barley, vegetable oils, glucose syrup, sugar, natural extract of olives,	Wholemeal common wheat flour, vegetable oils, barley and corn malt extract	Wholemeal flakes (oats, wheat, barley) with 22% dried fruits (raisins, apricots, apples), hazelnuts, soy
3	Common wheat flour, sugar, sunflower oil, butter, eggs, pasteurized milk, milk cream, honey, wheat starch, soy lecithin, vanillin aroma	Common wheat flour, vegetable oils, wheat starch, barley and corn malt extract, rosemary extract	Wholemeal flakes (oats, wheat), cornflakes, 40% dried fruits (sultanas, figs, dates, apples, apricots, plums), sun flower seeds
4	Common wheat flour, eggs, sugar, caramelized sugar	Wholemeal rye flour	Wholemeal oat flakes dried apples, cinnamon flavour, sunflower oil
5	Wholemeal common wheat flour, sugar, sunflower oil, cocoa, chocolate, butter, eggs, pasteurized milk, honey	Common wheat, vegetable oils, milk, olives, parsley and chilli, potato starch, modified maize starch	Wholemeal oats flakes, corn flakes, wheat and rice flour, 10% skimmed milk yoghurt, freeze-dried strawberries, caramelized sugar
6	Wholemeal common wheat flour, spelt, sugar, sunflower oil, cane sugar, butter, eggs, pasteurized milk, barley malt extract	Wholemeal rye flour	Wholemeal oats flakes, Cornflakes, 14% nuts (cashews, almonds, hazelnuts), sugar, sunflower oil, coconut flakes
7	Common wheat flour, sugar, chocolate, cocoa butter, sunflower oil, butter, glucose syrup, wheat starch, soy lecithin, vanillin aroma	Wholemeal wheat flour, rape seed oil, poppy seeds (4.7%), sugar	Wholemeal oats flakes, wheat flour, 11% chocolate flakes, 3% hazelnuts, sugar, coconut flakes

proportion of B was increased linearly to 50% between minute 2 and 5. Further linear increase of B to 100% between minute 5 and 14 was followed by a hold time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The injection volume was 5  $\mu$ l.

ESI-MS/MS data was acquired in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarity in two separate chromatographic runs. The detection window width was 40 and 46 s in the positive and negative ionization mode, respectively. The target cycle time was 1400 msec and the MS pause time was 3 msec. Compound dependent LC-MS/MS parameters are listed in Supplementary Table S9. Two sMRM transitions were acquired for each analyte except for 3-nitropionic acid and moniliformin that yield only one product ion. For unambiguous identification of an analyte the ion ratio has to agree with the related values of the standards within 30% as stated in SANTE/12089/2016<sup>27</sup> whereas for the LC retention time a stricter in-house criterion of  $\pm 0.03$  min is applied.

For external calibration based on neat solvent standards, a working solution was prepared by mixing 300  $\mu$ l of the multi-analyte standard (see section 2.1.), 20  $\mu$ l of a certified liquid standard containing fumonisins B1 and B2, 20  $\mu$ l of a certified liquid standard containing fumonisin B3 (the fumonisins were added at this late stage as their concentration is not long-term stable in the multianalyte solution of almost pure acetonitrile) and 260  $\mu$ l of dilution solvent (acetonitrile/water/acetic acid 20/79/1, v/v/v). From this working solution a serial dilution was prepared using acetonitrile/water/acetic acid (49.5/49.5/1, v/v/v) to obtain dilution levels of 1:3, 1:10, 1:30, 1:100, 1:300, 1:1000, 1:3000 and 1:10000, respectively. To check the linearity of the response, linear, 1/x weighted calibration curves were constructed for the neat solvent standards. Peak integration and construction of calibration curves and were performed using MultiQuant™3.0.3 software (Sciex, Framingham, MA, USA).

### Validation

Spiked samples were prepared by adding 100  $\mu$ l of the working solution to 0.25 g of homogenized blank samples, which theoretically results (after extraction and dilution) in a concentration of half of the concentration of the 1:10 dilution level of the external standard. After spiking (and prior to extraction) they were stored overnight in darkness at room temperature to facilitate evaporation of the spiking solvent. For post-extraction spiking, 500  $\mu$ l of raw extracts were mixed with 50  $\mu$ l of the working solution and

450  $\mu$ l of dilution solvent. For determination of LOD/LOQ spiking of biscuit and musli samples was additionally performed on lower levels using 100  $\mu$ l of the respective dilutions of the working solution (1:10–1:1000) as the spike.

Spiked samples and spiked extracts were analysed under repeatability conditions against the neat-solvent standards and the averages of the calculated concentrations were used to determine apparent recoveries ( $R_A$ ), signal suppression/enhancement (SSE) and recoveries of extraction ( $R_E$ ). Data on method repeatability  $RSD_r$  derived from seven different individual spiked samples analysed within one sequence (lasting 2.5 days). For the intermediate precision (within laboratory reproducibility;  $RSD_{WLR}$ ) seven technical replicates of one sample per matrix were spiked and one replicate per matrix was analysed each week within a period of seven weeks. The relative expanded method uncertainty  $U_{r,exp}$  was calculated from data on repeatability and intermediate precision:

$$U_{r,exp} = 2 * \sqrt{RSD_r^2 + RSD_{WLR}^2} \quad (1)$$

LOD and LOQ were calculated from repeatability data on low spiking levels following the EURACHEM guide<sup>20</sup>.

### Data availability

The authors declare that all raw data that support the findings of this study as well as details on LC-MS/MS parameters and on target analytes are available in the supplementary information.

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### References

1. Greer, B., Chevalier, O., Quinn, B., Botana, L. M. & Elliott, C. T. Redefining dilute and shoot: The evolution of the technique and its application in the analysis of foods and biological matrices by liquid chromatography mass spectrometry. *TrAC Trends Anal. Chem.* **141**, 116284 (2021).
2. Frenich, A. G., Martínez Vidal, J. L., Romero-González, R. & del Mar Aguilera-Luiz, M. Simple and high-throughput method for the multimycotoxin analysis in cereals and related foods by ultra-high

performance liquid chromatography/tandem mass spectrometry. *Food Chem.* **117**, 705–712 (2009).

- Blesa, J., Marín, R., Lino, C. M. & Mañes, J. Evaluation of enniatins A, A1, B, B1 and beauvericin in Portuguese cereal-based foods. *Food Addit. Contam. A* **29**, 1727–1735 (2012).
- Beltran, E. et al. Development of sensitive and rapid analytical methodology for food analysis of 18 mycotoxins included in a total diet study. *Anal. Chim. Acta* **783**, 39–48 (2013).
- Capriotti, A. L. et al. Multiclass analysis of mycotoxins in biscuits by high performance liquid chromatography–tandem mass spectrometry. Comparison of different extraction procedures. *J. Chromatogr. A* **1343**, 69–78 (2014).
- De Santis, B. et al. Development of a LC-MS/MS Method for the Multi-Mycotoxin Determination in Composite Cereal-Based Samples. *Toxins* **9**, 169–180 (2017).
- Škrbić, B., Antić, I. & Cvejanov, J. Determination of mycotoxins in biscuits, dried fruits and fruit jams: an assessment of human exposure. *Food Addit. Contam. A* **34**, 1012–1025 (2017).
- Tolosa, J. et al. Multi-Mycotoxin Analysis in Durum Wheat Pasta by Liquid Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry. *Toxins* **2**, 59–70 (2017).
- Zhang, K., Flannery, B. M., Oles, C. J. & Adeuya, A. Mycotoxins in infant/toddler foods and breakfast cereals in the US retail market. *Food Addit. Contam. B* **11**, 183–190 (2017).
- Bouafifssa, Y. et al. Multi-Occurrence of Twenty Mycotoxins in Pasta and a Risk Assessment in the Moroccan Population. *Toxins* **10**, 432–446 (2018).
- Medina, B. G., Sartori, A. V., de Moraes, M. H. P., Cardoso, M. H. W. M. & Jacob, S. D. C. Validation and application of an analytical method for the determination of mycotoxins in crackers by UPLC-MS/MS. *Food Sci. Technol.* **39**, 583–591 (2019).
- Narváez, A. et al. Multi Mycotoxin Method Development Using Ultra-High Liquid Chromatography with Orbitrap High-Resolution Mass Spectrometry Detection in Breakfast Cereals from the Campania Region, Italy. *Toxins* **15**, 148–159 (2023).
- Stadler, D., Sulyok, M., Schuhmacher, R., Berthiller, F. & Krksa, R. The contribution of lot-to-lot variation to the measurement uncertainty of an LC-MS-based multi-mycotoxin assay. *Anal. Bioanal. Chem.* **410**, 4409–4418 (2018).
- Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC. *Off. J. Eur. Union* **L180**, 84–109 (2021).
- Commission Implementing Regulation (EU) 2023/2782 of 14 December 2023 laying down the methods of sampling and analysis for the control of the levels of mycotoxins in food and repealing Regulation (EC) No 401/2006, [http://data.europa.eu/eli/reg\\_impl/2023/2782/oj](http://data.europa.eu/eli/reg_impl/2023/2782/oj) (2023).
- Zhang, K., Wong, J. W., Kryniitsky, A. J. & Trucksess, M. W. Determining mycotoxins in baby foods and animal feeds using stable isotope dilution and liquid chromatography tandem mass spectrometry. *J. Agric. Food Chem.* **62**, 8935–8943 (2014).
- Sulyok, M., Stadler, D., Steiner, D. & Krksa, R. Validation of an LC-MS/MS based dilute-and-shoot approach for the quantification of >500 mycotoxins and other secondary metabolites in food crops: Challenges and solutions. *Anal. Bioanal. Chem.* **412**, 2607–2620 (2020).
- Steiner, D., Sulyok, M., Malachova, A., Mueller, A. & Krksa, R. Realizing the simultaneous liquid-chromatography-tandem mass spectrometry based quantification of >1200 biotoxins, pesticides and veterinary drugs in complex feed. *J. Chromatogr. A* **1629**, 461502 (2020).
- Van de Schans, M. G. M., Blokland, M. H., Zoontjes, P. W., Mulder, P. P. J. & Nielen, M. W. F. Multiple heart-cutting two dimensional liquid chromatography quadrupole time-of-flight mass spectrometry of pyrrolizidine alkaloids. *J. Chromatogr. A* **1503**, 38–48 (2017).
- Magnuson, B. & Örnemark, U. (eds.) *Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics* (Eurachem, 2014).
- Commission Regulation (EU) 2023/915 on maximum levels for certain contaminants in food and repealing regulation (EC) No 1881/2006. *J. Eur. Union* **L119**, 103–157 (2023)..
- Crews, C., Anderson, W. A., Rees, G. & Krksa, R. Ergot alkaloids in some rye-based UK cereal products. *Food Addit. Contam. B* **2**, 79–85 (2009).
- Veršilovskis, A., Mulder, P. P. J., Pereboom-de Fauw, D. P. K. H., de Stoppelaar, J. & de Nijs, M. Simultaneous quantification of ergot and tropane alkaloids in bread in the Netherlands by LC-MS/MS. *Food Addit. Contam. B* **13**, 215–223 (2020).
- Spanic, V. et al. Multiple fungal metabolites including mycotoxins in naturally infected and fusarium-inoculated wheat samples. *Microorganisms* **8**, 578 (2020).
- Commission Recommendation (EU) 2022/553 of 5 April 2022 on monitoring the presence of Alternaria toxins in food. *Off. J. Eur. Union* **L107**, 90–92 (2022).
- Behrens, A. M. et al. Occurrence of Alternaria secondary metabolites in milling oats and its de-hulled fractions from harvest years 2017–2021. *Food Addit. Contam. A* **41**, 188–200 (2024).
- SANTE XXXX/2023. Guidance document on identification of mycotoxins and plant toxins in food and feed. [https://food.ec.europa.eu/system/files/2023-10/cs\\_contaminants\\_sampling\\_guid-doc-ident-mycotoxins.pdf](https://food.ec.europa.eu/system/files/2023-10/cs_contaminants_sampling_guid-doc-ident-mycotoxins.pdf) (2023).

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## Author contributions

Michael Sulyok: Design of experiments, collection of samples, data evaluation, visualization; Writing: Initial draft. Michele Suman: Collection of samples and list of ingredients; Writing: Review and editing. Rudolf Krksa: Acquisition of funding, Design of experiments, Validation; Writing: Review and editing.

## Competing interests

The authors declare no competing interests.

## Additional information

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