



Application of a liquid-chromatography tandem mass spectrometric multi-mycotoxin method to different raw cereals and evaluation of matrix effects

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3 1 **Application of a liquid-chromatography – tandem mass**
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6 2 **spectrometric multi-mycotoxin method to different raw cereals**
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10 3 **and evaluation of matrix effects**
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15 5 Michael Sulyok, Rudolf Krska* and Rainer Schuhmacher
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22 8 Christian Doppler Laboratory for Mycotoxin Research, Department IFA-Tulln, University of
23
24 9 Natural Resources and Applied Life Sciences, Vienna, Konrad Lorenzstr. 20, A-3430 Tulln
25
26
27 10
28
29 11

31 12 *corresponding author
32
33

34 13 Phone: +43-2272-66280-401
35

36 14 Fax: +43-2272-66280-403
37
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39 15 Email: rudolf.krska@boku.ac.at
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16 **Abstract**

17 A multi-analyte method for the liquid-chromatography – tandem mass spectrometric
18 determination of mycotoxins in crude grain extracts without any clean-up has been applied to
19 the analysis of spelt, rice and barley. The method performance characteristics were
20 determined after spiking blank samples at multiple levels and were found to be comparable
21 for all investigated matrices as concerns linearity (linear calibration functions were obtained
22 for all analyte/matrix combinations except for moniliformin), precision (coefficient of
23 variations < 6 %) and sensitivity. Matrix induced signal suppression/enhancement was studied
24 in detail and varied significantly between the investigated matrices as well as between
25 individual samples (relative standard deviation was as high as 40% within three rice varieties)
26 and individual toxins. It was therefore concluded that a reliable quantitative analysis using
27 matrix-matched calibration requires a careful consideration of the model matrix as it should
28 match the investigated samples as close as possible.

30 **Keywords:**

31 mycotoxins, multi-target analysis, liquid chromatography, tandem mass spectrometry, matrix
32 effects

34 **Introduction**

35 In the past few years, a trend towards the use of liquid chromatography-tandem mass
36 spectrometry (LC-MS/MS) in mycotoxin analysis has been observed (Sforza et al. 2006,
37 Krska and Molinelli 2007). In contrast to most screening methods for mycotoxins that are
38 based on immunoassays, unambiguous analyte confirmation can be obtained by mass
39 spectrometry (Zöllner and Mayer-Helm 2006). The broad applicability of this analytical
40 approach enables the development of multi-analyte methods and thus the simultaneous
41 screening of different classes of mycotoxins, which is essential to assess the health risks

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3 42 posed by mycotoxin contaminated food and feedingstuffs. Another important aspect is that, in
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5 43 comparison to fluorescence or UV-detection, mass spectrometry drastically enhances
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8 44 selectivity, e.g. minimizes the occurrence of interfering peaks deriving from matrix
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10 45 components. However, this led some researchers to the misconception that the use of LC-
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12 46 MS/MS effectively eliminates matrix effects. In reality, unpredictable increase or decrease of
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15 47 the analytical signal intensities may occur in the analysis of real world samples due to co-
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17 48 eluting matrix components (such as carbohydrates, proteins or fats) that disturb the ionization
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19 49 of the analytes. For details on the mechanism of ion suppression, the interested reader is
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21
22 50 referred to the reviews of Antignac et al. 2005 and of Niessen et al. 2006.
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27 52 Several approaches can be found in the literature to reduce or compensate matrix effects.
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29 53 These include improvements of sample pre-treatment, the use of longer columns or two-
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31 54 dimensional chromatography to improve separation, use of atmospheric pressure chemical
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33 55 ionisation instead of electrospray ionisation, use of mobile phase additives and suitable
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35 56 standardisation. This may lead to success for tailor made clean-up/chromatography for single
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37 57 toxins or toxins with very similar physico-chemical properties. However, the applicability of
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39 58 these approaches must be considered to be problematic as concerns multi-target methods for
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41 59 different classes of analytes exhibiting a broad range of physical and chemical properties. In
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43 60 addition, many of the clean-up methods widely used in mycotoxin analysis are incapable of
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45 61 completely eliminating matrix effects in complex matrices (Cavaliere et al. 2005a, Biselli and
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47 62 Hummert 2005). For example, recoveries significantly higher than 100% have been reported
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49 63 in some studies (Launay et al. 2004, Tanaka et al. 2006), which is a clear indicator that matrix
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51 64 effects were not taken into account (Matuszewski et al. 2003). Therefore, most papers dealing
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53 65 with LC-MS/MS analysis of mycotoxins try to counteract the adverse effect of co-eluting
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55 66 matrix components on the accuracy of the results by applying internal or matrix-matched
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67 standards.

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6 69 Closely related compounds that are not occurring in naturally contaminated samples and show
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8 70 similar chemical structures as the target toxins, such as verrucarol, deepoxy-deoxynivalenol
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10 71 and zearalanone, are frequently applied as internal standards (Zöllner et al. 2000, van
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12 72 Bennekom et al. 2002, Royer et al. 2004, Berthiller et al. 2005, Klötzel et al. 2005, Delmulle
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14
15 73 et al. 2006, Klötzel et al. 2006). However, it has been shown by post-column addition
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17 74 experiments that signal suppression deriving from co-eluting matrix compounds may affect
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19 75 only very small retention time periods (Klötzel et al. 2005). In addition, it is known that the
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21 76 extent of these effects also depends on the properties of the analyte (Rundberget and Wilkins
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23 77 2002, Martinez Vidal et al. 2005, Niessen et al. 2006). Therefore, some authors expressed
24
25 78 doubts about the applicability of a single non-coeluting analogue compound as internal
26
27 79 standard (Royer et al. 2004, Zöllner and Mayer-Helm 2006). Studies investigating ²D- or ¹³C-
28
29 80 labelled internal standards (Lindenmeier et al. 2004, Asam and Rychlik 2006, Bretz et al.
30
31 81 2006, Hartmann et al. 2006, Häubl et al. 2006a and 2006b) have shown that stable isotope
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33 82 labelled mycotoxins are the best choice as internal standards as they completely compensate
34
35 83 matrix effects even in crude extracts due to their identical chemical and chromatographic
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37 84 properties compared to the target toxins. However, this approach covers only a limited
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39 85 number of analytes of a multi-mycotoxin method as the related standards are available only
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41 86 for a few toxins.
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50 88 Another option that is widely used in mycotoxin analysis is the use of matrix-matched
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52 89 calibration, i.e. the fortification of a blank sample extract (representing a typical matrix) with
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54 90 a standard solution containing the analytes. This may be regarded as a simplified version of a
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56 91 standard addition approach in which each individual sample has to be fortified with a series of
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58 92 standard solutions and which is clearly too laborious for routine analysis. Ideally, a single
59
60 93 representative matrix can be used to compensate for matrix effects of both, samples that are

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3 94 allocated to other matrices, as has been investigated for the determination of twenty pesticides
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5 95 in different commodities (Martinez Vidal et al. 2005) as well as of different varieties
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8 96 (different brands, origins, ..) of the same matrix. Differences in the extent of matrix effects
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10 97 between individual samples of the same type of matrix may have a large influence on the
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12 98 precision and the trueness of a method, as has been shown for the analysis of pharmaceutical
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15 99 compounds in different lots of human plasma (Matuszewski et al. 2003).
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19 101 It can be concluded from the data published so far that the existing LC-MS(/MS) methods for
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21 102 mycotoxin analysis still suffer from large matrix effects (in spite of application of clean-up
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23 103 procedures) whereas differences between individual samples of the same matrix seem to be
24
25 104 manageable. In a multi-toxin method developed for milk including protein precipitation and
26
27 105 subsequent SPE on Oasis HLB, matrix effects ranged from 75 to 110%, depending on the
28
29 106 analyte, while differences in matrix effects within 20 investigated milk brands did not
30
31 107 influence the precision of the method, only zearalenone showed slightly higher signal
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33 108 intensities in low-fat milk (Sorensen and Elbaek 2005). Similarly, fluctuations of matrix
34
35 109 effects within 6 corn samples in a multi-*Fusarium* mycotoxin method including a two-step
36
37 110 clean-up occurred only in case of nivalenol (Cavaliere et al 2005a and b). Almost no matrix
38
39 111 effect for deoxynivalenol was observed in grain-based commodities such as wheat flour, oats
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41 112 and rye, whereas strong signal suppression was observed in soybeans and in matrices with a
42
43 113 high sugar content, although the extracts had been cleaned with Mycosep[®] columns (Biselli
44
45 114 and Hummert 2005). Uhlig and Ivanova (2004) analysed enniatins and beauvericin in
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47 115 different types of grain without any clean-up and obtained relative standard deviations of
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49 116 <30% for the recoveries of spiked samples. Signal suppression of trichothecenes and
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51 117 zearalenone in extracts of durum wheat, corn and bread was reduced from >30% to <20% by
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53 118 applying deepoxy-deoxynivalenol as internal standard, and was quite comparable between the
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55 119 three matrices (Klötzel et al. 2005). Zearalanone was used as internal standard for the
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3 120 determination of zearalenone, α - and β -zearalenol in different beer brands including an SPE-
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5 121 based clean-up (Zöllner et al. 2000). The authors concluded that the elimination of the matrix
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8 122 effect is only possible within each beer brand, but not between the different brands.
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12 124 Most of these methods include a clean-up step, as a reduction of sample preparation was
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14 125 found to result in unacceptably large matrix effects and in extreme cases to complete
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16 126 suppression of the detector signal (Biselli and Hummert 2005). In contrast to that, it was
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18 127 shown in our group that the latest generation of MS interfaces tolerates the injection of crude
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20 128 sample extracts of wheat and maize (Sulyok et al. 2006). In spiked wheat extract, significant
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22 129 matrix effects were observed for only few of the 39 analytes, whereas the maize matrix
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24 130 showed a more pronounced influence on the detector signal intensities, especially in case of
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26 131 aflatoxins and ergot alkaloids. The excellent values obtained for the coefficients of variation
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28 132 of the overall process indicated that these matrix effects were stable within the investigated
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30 133 matrices and caused purely proportional errors, which may in principle be compensated by
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32 134 matrix-matched standards. The present work includes the extension of our recently developed
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34 135 method to three further matrices as well as an investigation of the variability of the matrix
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36 136 effects between three individual samples of maize and rice, respectively. Based on these
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38 137 additional data on matrix effects, the applicability of the concept of matrix matched
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40 138 calibration for the developed method is evaluated.
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50 140 **Materials and methods**

51 141 *Chemicals and reagents*

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53 142 Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker
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55 143 (Deventer, The Netherlands), ammonium acetate (MS grade) and glacial acetic acid (p.a.)
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57 144 were obtained from Sigma-Aldrich (Vienna, Austria). Water was purified successively by
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59 145 reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).
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6 147 Mycotoxin standards were dissolved in acetonitrile (ACN) if not stated otherwise. Stock
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8 148 solutions of nivalenol (NIV), deoxynivalenol (DON), fusarenon X (FUSX), 3-
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10 149 acetyldeoxynivalenol (3ADON), deepoxy-deoxynivalenol (DOM), neosolaniol (NEO),
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12 150 diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), T-2 toxin (T-2), zearalenone (ZON), alpha-
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15 151 zearalenol (α -ZOL), beta-zearalenol (β -ZOL), ochratoxins A and B (OTA, OTB), ochratoxin
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17 152 alpha ($OT\alpha$, in ACN/H₂O 1+1, v+v), fumonisins B₁ and B₂ (FB₁, FB₂, in ACN/H₂O 1+1),
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20 153 hydrolysed fumonisin B₁ (HFB₁, in ACN/ H₂O 1+1), and aflatoxins B₁, B₂, G₁ and G₂ (AFB₁,
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22 154 AFB₂, AFG₁, AFG₂) were obtained from Biopure Referenzsubstanzen GmbH (Tulln,
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24 155 Austria). 15-monoacetoxyscirpenol (MAS), verrucarol (VOL), beauvericin (BEA),
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26 156 moniliformin (MON, dissolved in MeOH), ergocornine (ERC, dissolved in MeOH/H₂O 1+1)
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28 157 and ergotamine-D-tartrate (ERA, dissolved in MeOH/H₂O 1+1) were received from Sigma-
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30 158 Aldrich (Vienna, Austria). A stock solution of enniatin A, A₁, B and B₁ (ENN A, ENN A₁,
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32 159 ENN B, ENN B₁) was provided by Dr. Marika Jestoi (EELA Helsinki, Finland). Agroclavine
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34 160 (AGR, dissolved in MeOH) was received from Dr. Miroslav Flieger (Institute of
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36 161 Microbiology, Academy of Sciences of the Czech Republic, Prague).
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38 162 Dihydroergosinmethanesulphonate (DHE, dissolved in MeOH) was purchased from Dr.
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40 163 Danka Pericic (Ruder Boscovik Institute, Zagreb, Croatia). Ergovaline (ERV, dissolved in
41
42 164 MeOH) was purchased from Prof. Forrest Smith (Auburn University, AL, USA).
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44 165 Deoxynivalenol-3-glucoside (D3G) was isolated from wheat treated with DON (Berthiller et
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46 166 al. 2005), zearalenone-4-glucoside (Z4G, dissolved in MeOH) was synthesized according to a
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48 167 modified protocol from Grabley et al. 1992 and zearalenone-4-sulfate (Z4S, dissolved in
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50 168 MeOH/H₂O 1+1) was extracted in our laboratory from rice inoculated with *Fusarium*
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52 169 *graminearum*. Four combined working standard solutions were prepared weekly by dilution
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54 170 of the stock solutions of the analytes in the related solvents, i.e. MeOH (for Z4G, AGR,
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56 171 MON, EV and DHE), MeOH/H₂O 1+1 (for Z4S, ERA and ERC), ACN/H₂O 1+1 (for $OT\alpha$,

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3 172 FB₁, FB₂ and HFB₁) and ACN (for all other analytes), respectively. All solutions were stored
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5 173 at -20°C and were brought to room temperature before use.
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10 175 Maize samples deriving from the harvest 2002 (sample A), 2003 (sample B) and 2004
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12 176 (sample C) were collected from fields in Lower Austria. Spelt, barley, Arborio peeled rice,
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15 177 Arborio brown rice and red rice samples were purchased from a local store in Vienna.
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20 179 *Sample preparation*

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22 180 *Spiking.* 0.5 g of ground sample were spiked by consecutively adding the appropriate amounts
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24 181 of the 4 combined working solutions. The samples were subsequently stored for three days at
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26 182 40°C to allow the evaporation of the solvent and to establish equilibration between the
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28 183 analytes and the matrix.
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34 185 *Extraction.* 2 mL of extraction solvent (CH₃CN/H₂O/HAc 79+20+1) were added to 0.5 g of
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36 186 ground sample. The samples were extracted for 90 min using a GFL 3017 rotary shaker (GFL,
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38 187 Burgwedel, Germany) and subsequently centrifuged for 2 min at 3000 rpm (radius: 15 cm) on
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40 188 a GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA). The extracts were transferred into
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42 189 glass vials using Pasteur pipettes and aliquots of 350 µL were diluted with the same amount
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44 190 of a mixture containing CH₃CN/H₂O/HAc 20+79+1. After appropriate mixing, 5 µL of the
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46 191 diluted extract were injected into the LC-MS/MS system without further pre-treatment.
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53 193 *Instrumental conditions*

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55 194 Parameters of the instrumental set-up have been described in detail in Sulyok et al. 2006.
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57 195 Briefly, a 1100 Series HPLC System (Agilent, Waldbronn, Germany) was coupled to a QTrap
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59 196 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a
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197 TurboIonSpray electrospray ionization (ESI) source. Chromatographic separation was

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3 198 performed at 25°C on a Gemini® C₁₈-column, 150 x 4.6 mm i.d., 5 µm particle size, equipped
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6 199 with a C₁₈ 4 x 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US).
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8 200 Both eluents contained 5 mM ammonium acetate and were composed of
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10 201 methanol/water/acetic acid 10+89+1 (v+v+v; eluent A) or 97+2+1 (eluent B), respectively.
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12 202 ESI-MS/MS was performed in multiple reaction monitoring (MRM) mode in both positive
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14 203 and negative polarity using two separate chromatographic runs per sample.
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20 205 *Performance characteristics*

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22 206 For the determination of the analytical performance of the method in spelt, rice and barley, the
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24 207 whole spiking and extraction procedure was carried out at 8 different concentration levels
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26 208 (each in triplicate) with relative concentrations of 1:4:7:10:40:70:100:400. The concentration
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28 209 ranges of the spiked samples were chosen to cover the respective limits of detection of each
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30 210 toxin as well as the concentration ranges found in naturally contaminated samples. For
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32 211 external calibration, liquid standards were diluted in mobile phase A as well as in blank
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34 212 extracts (diluted 1+1, as described in “Extraction”). The related analyte concentrations were
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36 213 matched on each level to the expected concentrations in the final diluted extract of the spiked
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38 214 samples. Linear, 1/x weighted calibration curves were constructed from the data obtained
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40 215 from the analysis of each sample type (spiked sample, liquid standard, matrix-matched
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42 216 standard) using the Analyst® software version 1.4.1. To differentiate between extraction
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44 217 efficiency and matrix induced signal suppression/enhancement, the slope ratios of the linear
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46 218 calibration functions were calculated to yield the apparent recovery (R_A) and the signal
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48 219 suppression/enhancement (SSE) due to matrix effects. Eventually, the recovery of the
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50 220 extraction step (R_E) was obtained as follows (modified after Matuszewski et al. 2003):
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$$R_A (\%) = 100 * \text{slope}_{\text{spiked sample}} / \text{slope}_{\text{liquid standard}} \quad (1)$$

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$$\text{SSE} (\%) = 100 * \text{slope}_{\text{matrix-matched standard}} / \text{slope}_{\text{liquid standard}} \quad (2)$$

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$$R_E (\%) = 100 * R_A / \text{SSE} \quad (3)$$

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3 224 The coefficients of variation of the end determination (CVs) were calculated from linear, 1/x
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5 225 weighted calibration curves obtained from the analysis of spiked samples using Validata[®], a
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8 226 Microsoft Excel macro developed by Wegscheider et al. (1999).
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12 228 In order to determine the variation of matrix effects between individual samples of the same
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14 229 matrix (rice and maize), blank extracts of three different samples of each matrix were spiked
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16 230 on multiple levels and SSE was calculated according to equation 2.
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21 232 **Results and Discussion**

22 233 *Analysis of rice, spelt and barley*

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24 234 In order to further investigate the limits of the universal applicability of the LC-MS/MS based
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26 235 method developed by our group, the method performance was investigated in 3 additional
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28 236 matrices. Figures 1 and 2 show the chromatograms of the spiked rice samples, Tables I-III
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30 237 summarize the results for peeled rice, barley and spelt, respectively. The number of
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32 238 concentration levels that were evaluated for a given toxin were not identical in all three
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34 239 investigated matrices, as the lowest levels were near the respective limits of detection and the
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36 240 sensitivity of the detector varied depending on the condition of the mass spectrometer. For
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38 241 spelt, data evaluation had to be restricted to the 4 highest concentration levels due to an
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40 242 experimental error.
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51 244 [Insert Figure 1 and 2 approximately here]

52 245 [Insert Table I-III approximately here, preferably on the same double page]

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57 247 The CVs obtained from linear calibration data were < 6.0% for all analytes and matrices with
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59 248 the exception of AFB1 and AFB2 in barley and of Z4S in all matrices. The latter was
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249 probably a result of the tendency of this compound to hydrolyse. The sole analyte exhibiting a

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3 250 non-linear calibration curve was MON that showed a logarithmic response in all three
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5 251 matrices and in the liquid standard. This effect was already observed by Jestoi et al. 2003 in
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7 252 case of the liquid standard (but not in the presence of matrix) and is probably a result of the
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10 253 unfavourable ionisation conditions due to the large water content of the eluent at the retention
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12 254 time of MON. The relative standard deviations of the whole procedure applied to the spiked
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15 255 samples were generally lower than 10% at the highest concentration levels except for AFG2,
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17 256 ERV and DHE in barley and D3G in rice and spelt. The limits of detection have not been
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20 257 calculated for every analyte/matrix combination (as this work focuses on the transferability of
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22 258 the method to other matrices, which is primarily hampered by signal
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24 259 suppression/enhancement effects caused by co-eluting matrix components), but they are
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27 260 comparable to those presented for wheat and maize (Sulyok et al. 2006), as the related
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30 261 concentration ranges during evaluation of method performance characteristics were very
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32 262 similar. The recoveries of the extraction step were comparable between the three matrices for
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34 263 many analytes, slightly larger variations were observed for those analytes showing incomplete
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36 264 extraction (fumonisins and MON and to a lesser extent other polar analytes such as D3G,
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38 265 Z4G, Z4S and NIV) and for several aflatoxins and ergot alkaloids.
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43 267 Signal suppression/enhancement due to co-eluting matrix compounds was pronounced in
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46 268 barley (SSE for 14 analytes outside $100\pm 15\%$) and spelt (9 analytes outside $100\pm 15\%$),
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49 269 whereas in the case of the rice matrix only AFB1 was outside that range. As concerns the
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51 270 prediction of the susceptibility of different analyte classes to signal variations and/or
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53 271 deviations from 100% SSE, the data presented so far indicate that the most critical
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55 272 compounds are aflatoxins, ergot alkaloids and polar analytes (like MON and D3G). The latter
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58 273 are considered to be generally critical due to insufficient chromatographic separation from
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60 274 polar matrix components (Niessen et al. 2006). In this aspect, the different SSE values
275 obtained for the co-eluting DON and D3G seem to be contradictory, but it must be kept in

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3 276 mind that matrix effects are both compound- and matrix-dependent. This might also explain
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5 277 the different SSE values observed for aflatoxins and other analytes such as MAS, DAS and
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8 278 HFB1, that are eluting closely (see Figure 1). Concerning the aflatoxins, the hypothesis of
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10 279 hydrolysis under acidic conditions (Ventura et al. 2006) was tested by analysing spiked
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12 280 neutral maize extracts. In addition, spiked acidic maize extracts were analysed omitting the
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15 281 dilution step with ACN+H₂O+HAc (20+79+1, v+v+v; see “extraction” in the experimental
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17 282 section) to investigate whether aflatoxins adsorb on particulate matter emerging from dilution
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19 283 of the raw extracts (Sulyok 2006). Neither of these two variations led to a decreased signal
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22 284 suppression (data not shown).
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27 286 In general, these results were comparable to those obtained for wheat and maize (Sulyok et al.
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29 287 2006). The main conclusion is that all effects/losses deriving from co-eluting matrix
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31 288 compounds as well as from incomplete extraction were repeatable on all concentration levels
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34 289 for a given analyte/matrix combination: They caused changes of the slope of the linear
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36 290 calibration function of spiked samples in comparison to liquid standards, but did not result in
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38 291 non-linear calibration curves. Moreover, low CV values have been observed, which indicates
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41 292 that the precision of the method is not significantly decreased by those effects. This suggests
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43 293 that the method transfer to other grain matrices is feasible using calibration with pure standard
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45 294 solutions together with correction factors for each analyte/matrix combination (for example
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47 295 0.67 for ZON in barley, see Table II). However, this demands for low variations of matrix-
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49 296 induced signal suppression within a given matrix type.
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55 298 *Variation of matrix effects between individual samples of the same matrix*
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57 299 In order to assess the applicability of the concept of matrix-matched calibration, the variation
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59 300 of SSE between individual samples was investigated for maize and rice by spiking blank
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301 extracts obtained from three different individual samples of the same commodity at multiple

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3 302 concentration levels. Although maize seems to be a more critical matrix compared to rice as
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5 303 concerns the extent of signal suppression/enhancement (especially for aflatoxins and ergot
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7 304 alkaloids), the situation was somewhat different regarding the variation of matrix effects
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10 305 between different varieties (Table IV):
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15 307 [Insert Table IV about here]
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19 309 With the exception of D3G, the relative standard deviation of signal/suppression enhancement
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22 310 was < 15% between the three individual maize samples. In contrast to that, larger differences
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24 311 were observed between the three rice samples (RSD of SSE > 15% for 13 analytes), which
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26 312 was caused by dramatically reduced signal intensities in red rice. It should be pointed out that
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28 313 the variation of the texture of the three rice samples was larger compared to the maize
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30 314 samples: brown and red rice is not peeled and still contains its sprout. In addition, red rice
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32 315 grows on clayey soil, which imparts the colour to the peel. It is highly probable that red rice
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34 316 contains particular matrix components, that are responsible for the pronounced signal
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36 317 suppression. This example shows that the general applicability of standards matched to a
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38 318 single sample for the calibration of all other samples of a given matrix can not be taken as
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40 319 granted.
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48 321 In view of a routine application of the developed method, we recommend that matrix-matched
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50 322 calibration is effectively carried out even if samples allocated to a single, validated matrix are
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52 323 analysed. The use of a standard prepared in pure solvent (if necessary in combination with
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54 324 some sort of empirical correction factor for the matrix effect – e.g. 0.56 for AFG1 in maize,
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56 325 see Table IV) seems to be insufficient. That approach does not take into account that under
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58 326 routine conditions the interface of the mass spectrometer is contaminated with matrix
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60 327 components, which may have an effect on the analytical signal intensities and also on the

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3 328 extent of matrix effects. Indeed, we observed a slight continuous increase in the MS response
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5 329 (similar to Klötzel et al. 2005, who, however cleaned the extract prior to analysis) in the
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8 330 course of some measurement sequences.
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12 332 From the data presented here, it is evident that a thorough compensation of the matrix effects
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15 333 by using standards which are matched to a carefully chosen representative matrix is a
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17 334 prerequisite for a reliable quantitative analysis. Relative biases of as large as 40% must be
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19 335 accepted in a worst-case scenario in the case of the red rice for example, if only a single rice
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21 336 sample is used as typical representative for the matrix rice. This is, however, still acceptable
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24 337 for the purpose of a semi-quantitative screening of a large set of samples followed by target
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26 338 analysis of the positive samples including dedicated clean-up procedures and/or standard
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29 339 addition. Based on the results shown for maize in Table IV, it might be assumed that this error
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31 340 may be significantly reduced if model-samples are available for each individual brand or
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34 341 variety. In view of a routine application, we suggest to carry out standard-addition in case of
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36 342 doubt of positive samples exhibiting mycotoxin concentrations near the levels stated in the
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38 343 related regulations. In addition, an adequate number of random blank samples should be
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41 344 fortified and analysed over a longer period of time in order to obtain statistically meaningful
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43 345 data on the variation of matrix effects within a given type of matrix.
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47 347 **Conclusion**

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50 348 The developed LC-MS/MS multitoxin method may be transferred to other grain matrices
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52 349 without any adaptations concerning the instrumental parameters or sample preparation, as the
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54 350 method performance parameters were found to be comparable in all investigated matrices.
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56
57 351 The main limitation for obtaining accurate quantitative results are differences in the extent of
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59 352 matrix effects between different matrices as well as between variations of a given matrix.
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353 Using a single “model” sample which is used to represent all samples of a chosen matrix, the

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3 354 method is suitable for a semi-quantitative screening of the investigated toxin classes in a large
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5 355 number of grain samples, as there is no clean-up involved. For a quantitative analysis, the
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7
8 356 model matrix used for calibration must match the investigated samples as close as possible.
9

10 357

11 12 358 **Acknowledgements**

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14
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16
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22 362

23 24 363 **References**

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3 508 Figure captions:
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7 510 Figure 1. LC/ESI (+)-MS/MS MRM-chromatograms of spiked Arborio peeled rice; peak
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10 511 heights are given in counts per second (cps); Different time scales are reported for five
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12 512 retention time groups.
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16 514 Figure 2. LC/ESI (-)-MS/MS MRM-chromatograms of spiked Arborio peeled rice; peak
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18 515 heights are given in counts per second (cps); Different time scales are reported for three
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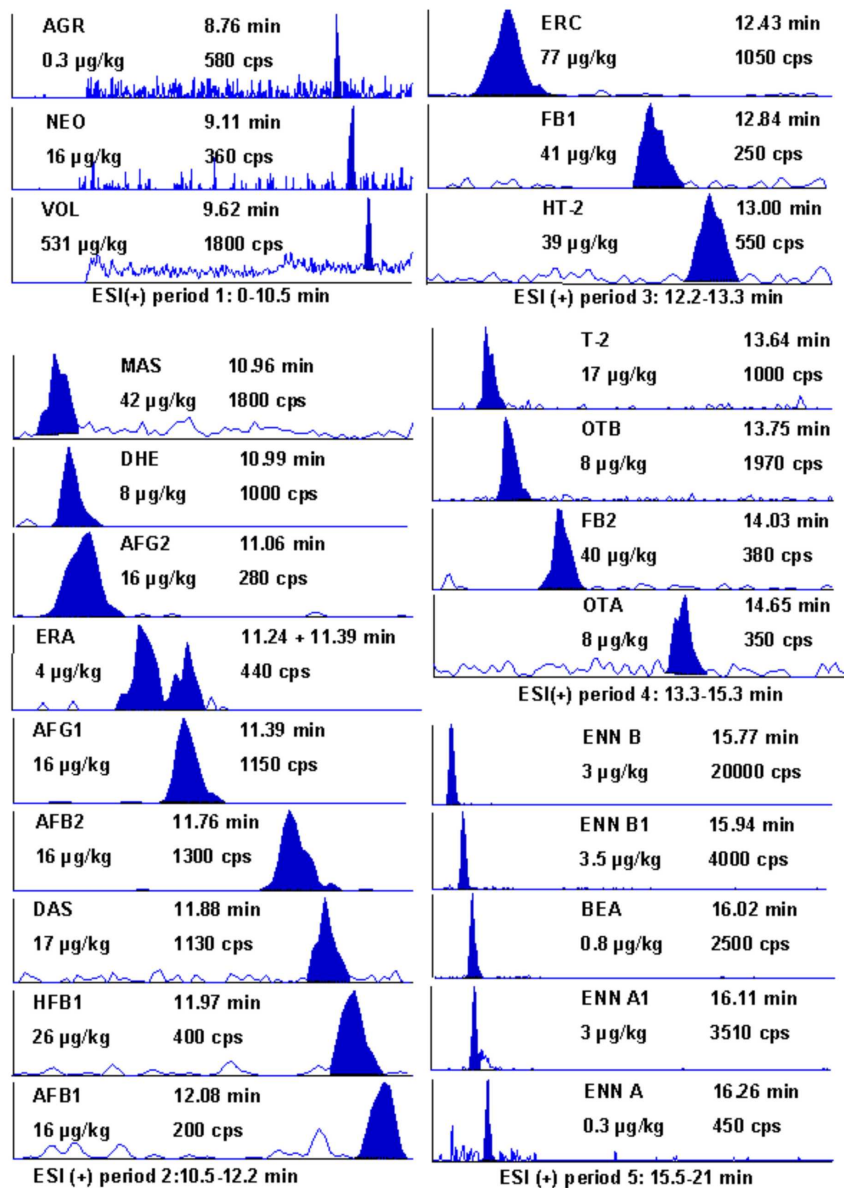


Figure 1: LC/ESI (+)-MS/MS MRM-chromatograms of spiked Arborio peeled rice; peak heights are given in counts per second (cps); different time scales are reported for five retention time groups 158x221mm (600 x 600 DPI)

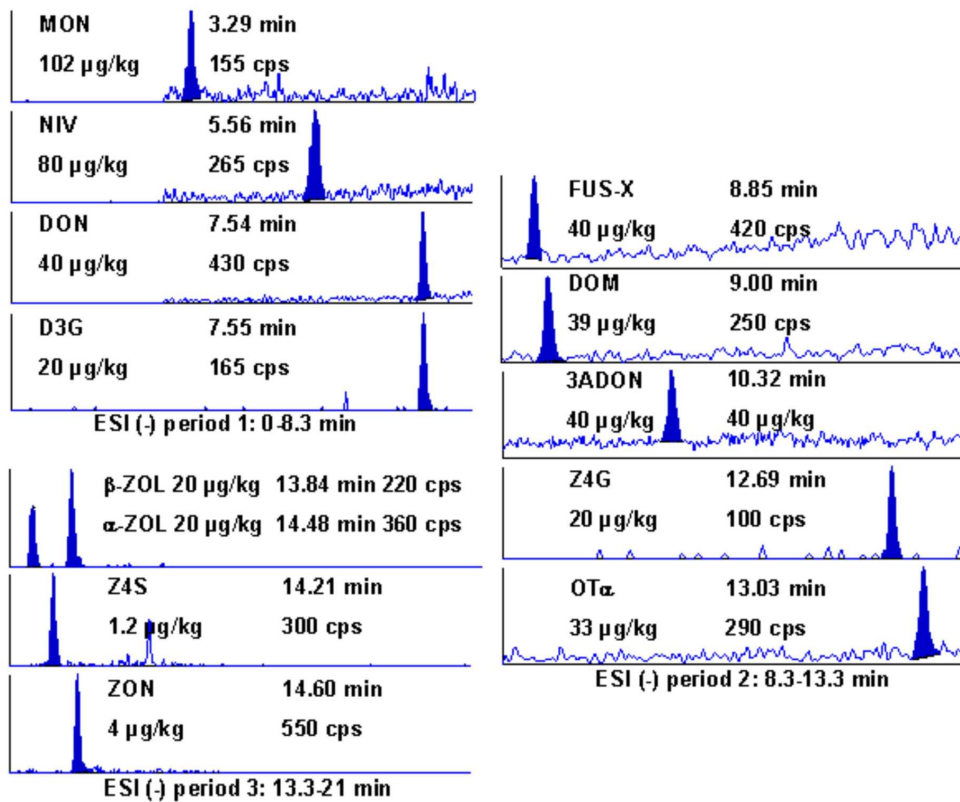


Figure 2: LC/ESI (-)-MS/MS MRM-chromatograms of spiked Arborio peeled rice; peak heights are given in counts per second (cps); different time scales are reported for three retention time groups 160x129mm (600 x 600 DPI)

Table I. Performance characteristics for peeled Arborio rice (n=3): apparent recoveries (R_A), signal suppression/enhancement (SSE), recoveries (R_E), relative standard deviations at the highest concentration levels and coefficients of variation of the overall procedure (CV)

	Conc. range [$\mu\text{g}/\text{kg}$]	evaluable levels	R_A (%)	SSE (%)	R_E (%)	RSD (%)	CV (%)
NEO	16-1620	7	98	99	99	1.7	1.2
VOL	531-5310	4	98	99	99	2.8	0.7
MAS	17-1680	7	105	101	104	10.4	1.6
DAS	4.2-1660	8	105	105	100	2.5	1.0
HT-2	16-1570	7	101	105	96	2.0	1.2
T-2	4.2-1660	8	101	103	97	3.5	1.2
NIV	40-1600	5	88	99	89	6.5	0.9
DON	28-1600	6	96	100	96	4.4	1.5
D3G	10-400	5	93	97	96	26	5.9
FUSX	28-1620	6	105	105	101	2.3	1.2
DOM	27-1560	6	97	106	91	1.8	0.8
3ADON	28-1600	6	102	104	98	2.8	0.7
AFG ₂	4.0-160	5	95	101	93	4.6	3.8
AFG ₁	2.8-160	6	98	93	106	7.0	5.6
AFB ₂	4.0-160	5	94	86	109	8.7	3.7
AFB ₁	16-160	4	96	82	116	5.8	2.7
AGR	0.30-118	8	95	99	96	4.4	3.4
ERV	8.6-86	4	97	95	102	4.0	1.5
DHE	3.4-339	7	93	98	94	6.2	2.1
ERA	4.2-419	7	112	106	106	4.0	1.7
ERC	14-778	6	147	104	142	8.4	2.4
BEA	0.80-320	8	101	100	101	0.3	4.1
ENN B	32-320	4	101	105	97	2.1	2.9
ENN B ₁	6.1-346	6	111	104	107	3.9	2.6
ENN A ₁	3.2-224	6	100	105	96	2.3	1.7
ENN A	0.87-345	8	98	99	99	1.6	1.4
HFB ₁	26-262	4	88	103	85	2.0	1.4
FB ₁	41-1630	5	74	100	74	1.7	0.7
FB ₂	40-1620	5	86	108	80	3.2	0.9
OT α	8.2-330	5	71	114	62	5.5	1.9
OTB	2.0-800	8	98	103	95	3.4	0.9
OTA	3.3-330	7	96	99	97	5.7	2.2
Z4G	20-800	5	89	100	88	3.1	1.3
β -ZOL	2.0-800	8	102	103	99	2.4	1.1
Z4S	0.30-12	5	90	108	83	3.0	8.9
α -ZOL	2.0-800	8	104	99	105	0.5	0.7
ZON	4.0-1620	8	110	109	102	0.8	0.7
MON	71 -4060	6	58	113	51	5.4	-

Table II. Performance characteristics for barley (n=3): apparent recoveries (R_A), signal suppression/enhancement (SSE), recoveries (R_E), relative standard deviations at highest concentration levels and coefficients of variation of the overall procedure (CV); (enniatins and OTA are not included due to contamination of the blank barley)

	conc. range [$\mu\text{g/Kg}$]	evaluatable levels	R_A (%)	SSE (%)	R_E (%)	RSD (%)	CV (%)
NEO	4.1-1620	8	96	96	101	4.4	1.5
VOL	531-5310	4	94	95	99	9.4	0.6
MAS	17-1680	7	93	96	97	8.0	1.2
DAS	4.2-1660	8	99	94	106	4.3	1.8
HT-2	4.0-1570	8	105	105	100	5.8	3.2
T-2	4.2-1660	8	96	97	99	1.5	2.5
NIV	40-1600	5	97	113	85	2.7	0.9
DON	40-1600	5	100	100	100	4.0	0.9
D3G	10-400	5	156	196	80	9.9	5.5
FUSX	162-1620	4	103	101	102	5.8	0.8
DOM	27-1560	6	101	100	101	3.0	0.9
3ADON	28-1600	6	87	82	106	4.6	1.6
AFG ₂	16-160	4	88	75	117	13.1	4.5
AFG ₁	4.0-160	5	76	66	115	4.3	3.7
AFB ₂	2.8-160	6	56	54	104	5.0	7.2
AFB ₁	16-160	4	49	47	105	8.9	7.6
AGR	0.30-118	8	77	89	87	1.7	4.9
ERV	8.6-86	4	79	70	114	11.2	4.8
DHE	3.4-339	7	76	73	104	10.3	4.7
ERA	4.2-419	7	78	89	87	1.7	4.9
ERC	19-778	5	61	66	93	4.3	3.1
BEA	8.0-320	5	94	105	90	2.9	4.9
HFB ₁	26-262	4	98	105	93	2.5	2.8
FB ₁	41-1630	5	69	108	64	3.7	1.5
FB ₂	40-1620	5	83	113	74	2.3	1.9
OT α	58-330	3	87	95	91	5.7	1.7
OTB	2.0-800	8	92	103	89	3.6	1.9
Z4G	20-800	5	104	101	103	6.7	2.0
β -ZOL	2.0-800	8	53	52	103	1.9	1.4
Z4S	0.3-12	5	82	93	88	5.9	12.6
α -ZOL	2.0-800	8	70	69	101	5.2	1.3
ZON	4.0-1620	8	68	67	101	3.7	1.0
MON	71 -4060	7	115	195	59	5.8	-

Table III. Performance characteristics for spelt (n=3): apparent recoveries (R_A), signal suppression/enhancement (SSE), recoveries (R_E), relative standard deviations at the highest concentration levels and coefficients of variation of the overall procedure (CV); (enniatins are not included due to contamination of the blank spelt)

	Conc. range [$\mu\text{g}/\text{kg}$]	evaluabe levels	R_A (%)	SSE (%)	R_E (%)	RSD (%)	CV (%)
NEO	162-1620	4	100	101	99	3.3	0.9
VOL	531-5310	4	102	107	96	2.6	0.1
MAS	168-1680	4	107	101	106	6.8	0.7
DAS	166-1660	4	96	98	99	4.1	0.7
HT-2	157-1570	4	103	100	103	8.4	1.0
T-2	166-1660	4	98	102	96	5.6	0.9
NIV	160-1600	4	91	87	104	6.7	0.2
DON	160-1600	4	99	100	99	4.3	1.0
D3G	40-400	4	78	99	79	15.5	2.8
FUSX	162-1620	4	111	105	105	7.4	0.8
DOM	156-1560	4	115	108	106	1.8	0.6
3ADON	160-1600	4	105	93	113	2.5	0.4
AFG ₂	16-160	4	70	91	77	4.4	4.7
AFG ₁	16-160	4	69	75	92	0.9	5.8
AFB ₂	16-160	4	83	86	97	6.8	3.0
AFB ₁	16-160	4	84	86	98	3.5	4.6
AGR	12-29	3	102	99	104	1.5	5.4
ERV	8.6-86	4	74	77	96	5.2	4.3
DHE	34-342	4	75	70	106	6.3	4.3
ERA	42-419	4	93	78	118	3.0	1.7
ERC	78-778	4	108	81	132	2.2	1.5
BEA	32-80	3	84	87	96	3.2	4.5
HFB ₁	26-262	4	75	105	72	4.7	3.6
FB ₁	163-1630	4	54	102	53	4.0	0.5
FB ₂	162-1620	4	61	102	59	6.4	0.6
OT α	33-330	4	110	127	87	5.9	2.2
OTB	80-800	4	94	101	93	7.0	1.4
OTA	33-330	4	91	102	89	4.0	1.6
Z4G	80-800	4	73	126	58	9.5	2.4
β -ZOL	80-800	4	104	101	103	4.1	1.1
Z4S	1.2-12	4	114	114	100	3.9	11.8
α -ZOL	80-800	4	106	112	95	4.5	1.3
ZON	162-1620	4	130	106	123	1.4	0.6
MON	406-4060	4	91	130	70	4.2	-

Table IV: Variation of signal suppression/enhancement (in %) within spiked blank extracts of individual samples of maize and rice. Values for maize C are taken from Sulyok et al. (2006).

	Maize A	Maize B	Maize C	RSD (%)	Arborio peeled rice	Arborio brown rice	Red rice	RSD (%)
NEO	103	102	90	7.4	110	101	57	31.7
VOL	68	73	78	6.8	118	117	79	21.2
MAS	100	105	91	7.2	106	107	92	8.2
DAS	96	89	89	4.4	122	110	103	8.6
HT-2	86	95	74	12.4	110	106	106	2.2
T-2	99	97	92	3.8	116	110	109	3.4
NIV	100	108	92	8.0	118	119	125	3.1
DON	103	110	108	3.4	121	126	98	13.0
D3G	96	108	157	26.9	140	135	36	56.6
FUSX	100	113	110	6.3	129	134	78	27.3
DOM	99	107	108	4.7	128	135	89	21.1
3ADON	98	102	107	4.4	130	109	83	21.9
AFG ₂	71	76	62	10.2	92	82	65	17.1
AFG ₁	55	57	56	1.8	106	87	66	23.2
AFB ₂	56	52	48	7.7	102	98	56	29.9
AFB ₁	20	24	18	14.8	99	90	92	5.0
AGR	102	103	93	5.5	112	101	87	12.5
ERV	49	62	62	13.0	95	85	40	40.0
DHE	40	44	50	11.3	90	64	34	44.7
ERA	38	43	41	6.2	96	83	61	22.1
ERC	32	34	27	11.6	108	80	45	40.6
BEA	100	108	109	4.7	107	104	111	3.3
ENN B	101	109	101	4.5	112	112	105	3.7
ENN B ₁	87	92	101	7.6	109	106	109	1.6
ENN A ₁	101	107	103	2.9	107	106	101	3.1
ENN A	92	97	104	6.2	107	97	103	4.9
HFB ₁	65	74	63	8.7	115	106	112	4.1
FB ₁	108	104	101	3.4	107	98	96	5.8
FB ₂	113	109	104	4.1	112	116	107	4.0
OT α	84	99	83	10.1	110	104	113	4.2
OTB	107	102	96	5.4	110	109	106	1.9
OTA	102	104	100	2.0	120	101	112	8.6
Z4G	126	125	141	6.9	124	123	128	2.1
β -ZOL	77	77	81	2.9	120	115	112	3.5
Z4S	78	91	78	9.1	110	109	111	0.9
α -ZOL	90	87	95	4.5	128	123	140	6.7
ZON	93	91	108	9.5	128	121	119	3.9
MON	142	147	124	8.8	135	153	119	12.5