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Identification of the first fumonisin mycotoxins with three acyl groups by ESI-ITMS and ESI-TOFMS following RP-HPLC separation: palmitoyl, linoleoyl and oleoyl EFB1 fumonisin isomers from a solid culture of *Fusarium verticillioides*

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Identification of the first fumonisin mycotoxins with three acyl groups by ESI-ITMS and ESI-TOFMS following RP-HPLC separation: palmitoyl, linoleoyl and oleoyl EFB₁ fumonisin isomers from a solid culture of *Fusarium verticillioides*

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Identification of the first fumonisin mycotoxins with three acyl groups by ESI-ITMS and ESI-TOFMS following RP-HPLC separation: palmitoyl, linoleoyl and oleoyl EFB₁ fumonisin isomers from a solid culture of *Fusarium verticillioides*

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Running head: New fumonisins with three acyl groups

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Abstract

The aim of this study was to apply RP-HPLC/ESI-ITMS and RP-HPLC/ESI-TOFMS to investigate and characterize six new higher molecular weight fumonisins (three pairs of isomers) extracted from a *Fusarium verticillioides*-infected solid rice culture. The ITMS and ITMS² spectra clearly indicated the *m/z* values (960, 984 and 986) of the protonated molecules and the FB₁ toxin-like structures of these compounds, respectively. Moreover, the data evaluation software of the TOFMS equipment unambiguously demonstrated the exact masses of the protonated molecules and the suggested empirical formulae (C₅₀H₈₉NO₁₆, C₅₂H₈₉NO₁₆ and C₅₂H₉₁NO₁₆) of the new fumonisins, with mass accuracy in the range between 0.1 and -1.1 ppm. Subtraction of the empirical formula of FB₁ toxin (C₃₄H₅₉NO₁₅) from these formulae and correction for the mass of water split-off from the fumonisin molecule during ester formation resulted in the empirical formulae of the fumonisin backbone esterifying agents (fatty acids): C₁₆H₃₂O₂ (palmitic acid, PA), C₁₈H₃₂O₂ (linoleic acid, LA) and C₁₈H₃₄O₂ (oleic acid, OA). We denoted the new compounds as esterified FB₁ (EFB₁) toxins, with the suggested names EFB₁ PA, iso-EFB₁ PA, EFB₁ LA, iso-EFB₁ LA, EFB₁ OA and iso-EFB₁ OA. The total amount of these new compounds comprised 0.1% of the FB₁ concentration, which may be rated as significant when it is considered that these new components are significantly more apolar than earlier-described fumonisins, and their uptake into and toxicity elicited in the various tissues of living organisms may therefore also be significantly different from those of other fumonisins.

INTRODUCTION

Fumonisin are structurally related toxic secondary metabolites of fungi, produced primarily by *Fusarium* species (Rheeder et al. 2002), especially *F. verticillioides* and *F.*

1
2 *proliferatum* (Seifert et al. 2003), which are worldwide pathogens of maize (Shephard et
3 al. 1996). However, in recent papers the production of some fumonisin B (FB) analogues
4 by *Aspergillus niger* has also been reported (Frisvad et al. 2007; Noonim et al. 2009;
5 Logrieco et al. 2009; Mogensen et al. 2010). Fumonisin B can cause severe diseases in
6 animals, e.g. leucoencephalomalacia in horses (Kellerman et al. 1990) and pulmonary
7 oedema and hydrothorax in pigs (Colvin et al. 1992; Fazekas et al. 1998; Zomborszky-
8 Kovács et al. 2002), and they can produce toxic effects in chickens (Ledoux et al. 1992)
9 and turkeys (Weibking et al. 1993). Important data have also been published on the
10 nephrotoxicity (Riley et al. 1994), hepatotoxicity and hepatocarcinogenicity (Gelderblom
11 et al. 1991) of FB₁ toxin in laboratory experimental rodents. Although not yet
12 conclusively proved, the consumption of fumonisin-contaminated maize and maize-based
13 foods (at levels of 1-10 mg kg⁻¹) has been linked with the promotion of human primary
14 liver cancer (Ueno et al. 1997) and statistically with human oesophageal cancer in rural
15 areas in different countries (Rheeder et al. 1992; Yoshizawa et al. 1994; Doko et al. 1995;
16 Shephard et al. 2000). Fumonisin B is regarded by the International Agency for Research
17 on Cancer (IARC) as group 2B carcinogens to humans (IARC 2002).

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40 Fumonisin B is characterized by a 19-20 carbon atom long amino-polyhydroxyalkyl
41 chain, which is generally diesterified at the hydroxy groups on C-14 and C-15 with a
42 terminal carboxyl group of propane-1,2,3-tricarboxylic acid (tricarballic acid, TCA)
43 (Rheeder et al. 2002), but in some minor fumonisins the esterification involves other
44 organic acids, such as *cis*-aconitic acid, oxalsuccinic acid and oxalfumaric acid (Bartók et
45 al. 2006, 2008). Partially hydrolysed (e.g. PHFB₁) and fully hydrolysed fumonisins (e.g.
46 HFB₁) have also been described (Rheeder et al. 2002; Bartók et al. 2006, 2008). In
47 partially hydrolysed fumonisins, the backbone is monoesterified, while in fully
48 hydrolysed compounds (usually formed during nixtamalization of maize, baking and
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2 frying of food products or by alkaline hydrolysis in the laboratory) there is no esterifying
3 agent on the backbone. Depending on their chemical structures, the fumonisins can be
4 classified into four main groups (FA, FB, FC and FP); the toxicologically most important
5 ones being the FB analogues (Rheeder et al. 2002), primarily because the FB analogues,
6 and especially FB₁ toxin (Fig. 1), can be found in the highest concentration in maize and
7 maize-based food- and feedstuffs (Stack 1998), and the free primary amine group on C-2
8 contributes significantly to the toxicity of the FB analogues (Bolger et al. 2001; Lemke et
9 al. 2001). The first fumonisins isolated and identified in 1988, were FB₁ and FB₂ toxin
10 (Bezuidenhout et al. 1988). The amine group in the FA analogues is acetylated. The
11 structure of the FC analogues is similar to that of the FB analogues, without the C-1
12 methyl group. The most recently described group of fumonisins comprises the FP
13 analogues, in which a 3-hydroxypyridine moiety can be found on C-2 (Musser et al.
14 1996).

15
16 HPLC/ESI-MS and MS/MS techniques with different types and different
17 combinations of mass analysers have become popular in fumonisin analysis. The most
18 significant advantages of these hyphenated procedures for the analysis of fumonisins are
19 their selectivity and sensitivity, and the fact that, in contrast with UV or fluorescence
20 detection, there is no need for derivatization (pre- or post-column) (Zöllner and Mayer-
21 Helm 2006).

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23 The aim of this study was to apply reversed-phase high-performance liquid
24 chromatography/electrospray ionization – ion trap and time-of-flight mass spectrometry
25 (RP-HPLC/ESI-ITMS and RP-HPLC/ESI-TOFMS) to investigate and characterize six
26 new, higher molecular weight FB analogues of fumonisins (three pairs of isomers with
27 different *m/z* values) extracted from a *Fusarium verticillioides*-infected solid rice culture.
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2 This is the first report on the detection of fumonisins in which, besides two TCA
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4 moieties, there is a third esterifying agent in the molecule.
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8 9 **MATERIALS AND METHODS**

10 11 **Chemicals**

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13 The FB₁ standard and methanol (MeOH, gradient grade) were purchased from Sigma-
14 Aldrich Ltd. (Budapest, Hungary). Acetonitrile (MeCN, hypergrade) was from Merck Ltd
15 (Budapest, Hungary). Purified water with a resistivity of 18 MΩ was delivered with a
16 Nanopure II (Barnstead/Thermolyne Co., Dubuque, IA, USA) water purification device.
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26 **Culturing of *Fusarium verticillioides* and extraction of fumonisins produced**

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28 A *Fusarium verticillioides* isolate acquired from maize stalk (isolate no. Fv 16) was
29 identified via its macro- and micromorphological characteristics and grown on a solid
30 rice culture as described earlier (Bartók et al. 2006, 2010). It was demonstrated in those
31 reports that this isolate has a good fumonisin-producing capability, yielding 18.27 mg g⁻¹
32 FB₁ toxin. The freeze-dried culture material containing fumonisins was extracted with a
33 mixture of MeOH/H₂O (75/25, v/v), using a high-speed homogenizer UltraTurrax T25
34 (IKA, Staufen, Germany) at 9500 rpm for 4 min. After extraction, and centrifugation
35 (10000xg for 10 min), the supernatant was filtered through a PTFE membrane (0.2 μm)
36 into an HPLC autosampler vial and was subsequently analysed with the instruments
37 detailed below.
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51 52 **RP-HPLC/ESI-ITMS and RP-HPLC/ESI-TOFMS analyses of fumonisins**

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54 The instrumental parameters were described in detail recently (Bartók et al. 2010).
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56 Briefly, the fumonisins extracted from the solid rice culture material were analysed by
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RP-HPLC/ESI-ITMS and also RP-HPLC/ESI-TOFMS at two research locations, where different Agilent (Santa Clara, CA, USA) HPLC systems (1090 Series II with DR5 binary pump and 1200 Series with binary pump) were attached to the mass spectrometers. Since the dead volume of the flow path of the 1200 Series HPLC system applied is higher than that of the 1090 Series II HPLC, the pulse damper from the 1200 Series pump was removed in order to decrease the retention time differences for the fumonisins on the two HPLC systems. A YMC-Pack J'sphere ODS H80 (YMC Europe GmbH, Dinslaken, Germany) (250 mm x 2.1 mm, 4 μ m) HPLC column and a flat (24% B to 40% B in 79 min, then increased to 100% B in 15 min) linear binary gradient of H₂O (A) and MeCN (B) (each supplemented with 0.1% (v/v) formic acid) were applied at a flow rate of 0.2 ml min⁻¹ for the separation of the compounds. Mass spectrometric detection was performed with a Varian (Palo Alto, CA, USA) 500MS IT, and on Agilent 6210 TOF mass spectrometers. Both mass spectrometers were equipped with ESI ion sources and operated in positive ion mode. Relative quantitation (% of FB₁) of the new compounds was performed by ESI-ITMS, using an external standard (ESTD) method in a 5 order of magnitude concentration range of an FB₁ calibration standard dissolved in MeCN/H₂O 50/50 (v/v). The quantitative evaluation of the new compounds by using ESTD calibration of FB₁ provided only approximate data, because the polarities of the detected new fumonisins are very different from that of FB₁; naturally, the ionization of the new compounds is assumed, but is not known to be the same as that for FB₁.

RESULTS AND DISCUSSION

Thanks to their outstanding sensitivity in full scan mode, mass spectrometers equipped with IT mass analysers are eminently suitable for the detection and partial identification of new secondary metabolites (e.g. mycotoxins) and their isomers (Josephs 1996; Bartók

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2 et al. 2006, 2008, 2010). As illustrated by the MS² spectrum of FB₁ toxin (Fig. 2), the
3
4 product ion spectra of the molecular ions ([M+H]⁺) of fumonisins are highly
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6 characteristic; in other words, it is easy to decide almost at a glance whether or not a
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8 given product ion spectrum is produced by a fumonisin. In most fumonisins, the
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10 backbone is esterified by two organic acid molecules (most often TCA), and accordingly
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12 the product ion spectra contain three groups of fragment ions (indicated by 1, 2 and 3 in
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14 Fig. 2). The first group of fragment ions consists of the dehydrated molecular ions, the
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16 second is the [M+H-TCA]⁺ group and the third is the [M+H-2TCA]⁺ group. It is
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18 characteristic of the product ion spectra of the fumonisins that, within a given product ion
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20 group, there is a difference of 18 Da between the masses of the neighboring ions,
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22 corresponding to the splitting-off of one H₂O molecule from the larger molecular or
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24 fragment ion. The difference between the *m/z* values of the corresponding ions of the
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26 neighboring product ion groups yields the molar mass of the organic acid esterifying the
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28 backbone (TCA or, in the case of a few minor fumonisins, *cis*-aconitic acid, oxalfumaric
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30 acid or oxalsuccinic acid) (Bartók et al. 2006, 2008). In the course of our earlier
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32 HPLC/ESI-ITMS studies these characteristics indicated that isolates of *Fusarium*
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34 *verticillioides* produce as yet unpublished fumonisins of higher molecular mass. Under the
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36 given experimental conditions, these fumonisins were eluted from the HPLC column at
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38 close to 100% MeCN, demonstrating that these components are considerably more apolar
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40 than other fumonisins so far described. The *m/z* values of the fragment ions in the MS²
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42 spectra, including the hydrocarbon backbone at *m/z* 299, revealed that the structures of
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44 these new fumonisins are highly similar to that of FB₁ toxin, except for the presence of
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46 additional functional groups. We assumed that the new substituents might be carboxylic
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48 acids esterifying the free OH groups of the fumonisin backbone, carboxylic acids that had
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50 not been identified on the fumonisin backbone. We were earlier unable to identify these
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2 fumonisins with our ITMS (lack of exact mass measurement). However, component
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4 identification following RP-HPLC separation was achieved by using an ESI-MS
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6 instrument equipped with a TOF mass analyser suitable for exact mass measurement.
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9 Three research groups had previously mentioned application of the TOFMS technique in
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11 fumonisin research, but neither of them utilized the procedure for the analysis of
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13 fumonisin isomers (Lemke et al. 2001; Senyuva and Gilbert 2008; Mogensen et al. 2010).
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15 We recently verified the usefulness of exact mass measurement by TOFMS during the
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17 identification of new FB₁ isomers (Bartók et al. 2010). Since the HPLC equipment used
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19 in these TOFMS measurements was different from that used in earlier ITMS analyses,
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21 slightly different retention times were obtained for the same components. As shown in
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23 Tables 1 and 2, however, the retention times (relative to FB₁ toxin) of the same
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25 components separated in the two different HPLC systems were highly similar. The
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27 differences between the retention times relative to FB₁ lay in the range 0.085-0.088. After
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29 full scan analysis by RP-HPLC/ESI-TOFMS, the data-processing software (Mass
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31 Hunter) of the equipment was asked to produce both the total ion and the extracted ion
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33 chromatograms (EICs) at *m/z* values of 960, 984 and 986 (Fig. 3). Nevertheless, similarly
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35 to RP-HPLC/ESI-ITMS, the total ion chromatogram obtained by RP-HPLC/ESI-
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37 TOFMS did not display these minor components in addition to the main components
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39 (FB₁, FB₂, FB₃ and FB₄). TOFMS EICs (Fig. 3) and ITMS EICs (chromatogram not
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41 shown), however, unambiguously indicated six new fumonisins in the retention time
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43 range 90.623-91.928 min and 89.324-90.569 min, respectively, i.e. two components at
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45 each of three different *m/z* values. The leading suggestions of the Mass Hunter software
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47 for all six fumonisins were the following empirical formulae (unprotonated) and the
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49 corresponding exact mass values ([M+H]⁺): C₅₀H₈₉NO₁₆ (960.62541); C₅₂H₈₉NO₁₆
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51 (984.62541) and C₅₂H₉₁NO₁₆ (986.64106). Subtraction of the empirical formula of FB₁
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2 toxin ($C_{34}H_{59}NO_{15}$) from these empirical formulae, followed by addition of the H_2O
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4 removed from a OH group of the fumonisin backbone and the organic acid molecules in
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6 the course of esterification, yielded the empirical formulae of the organic (fatty) acids
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8 esterifying the third OH group of the backbone of the FB_1 toxin, i.e. $C_{16}H_{32}O_2$ (palmitic
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10 acid, PA), $C_{18}H_{32}O_2$ (linoleic acid, LA) and $C_{18}H_{34}O_2$ (oleic acid, OA). With regard to the
11
12 currently accepted nomenclature (FB_1 , PHFB₁ (partially hydrolysed FB_1) and HFB₁ (fully
13
14 hydrolysed FB_1)), the new fumonisins were named esterified FB_1 (EFB₁) toxins. The
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16 abbreviation at the end of the name (PA, LA or OA) indicates the organic acid,
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18 esterifying the third OH group of the fumonisin backbone. Thus, the suggested names of
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20 the six new identified fumonisins are EFB₁ PA, iso-EFB₁ PA, EFB₁ LA, iso-EFB₁ LA,
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22 EFB₁ OA and iso-EFB₁ OA. The mass accuracy (based on the measured and the
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24 calculated mass values) of the new EFB₁ toxins was in the range between 0.1 and -1.1
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26 ppm. Data relating to the RP-HPLC/ESI-TOFMS measurements are listed in Table 1.
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33 Our TOFMS measurements confirmed that the signals of the new fragment ion groups
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35 (4, 5 and 6) in the MS^2 spectra obtained by ITMS are due to the presence of the third
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37 organic acid on the fumonisin backbone (Fig. 4). The experimental data obtained by
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39 ITMS allow conclusions that complement the fragmentation pathways published earlier
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41 (Bartók et al. 2006, 2010). Figure 5a-c depicts theoretically possible pathways for the
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43 fragmentation of the newly detected fumonisins. Of these theoretically possible reaction
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45 pathways, dominant routes can be suggested by comparison of the m/z values of the MS^2
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47 spectra shown in Figs 4 and 5a-c. No experiments were performed to address
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49 fragmentations depending on the CID energy, and it is therefore not possible to
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51 distinguish the roles of kinetic and thermodynamic parameters at this point. It is currently
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53 assumed that, the main fragmentation pathways can be characterized by the abundances
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55 of the ions in the product ion spectra. The experimental data mentioned above draw
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1
2 attention to various features regarding the dominant fragmentation pathways. Firstly, it is
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4 immediately apparent that the fragmentation patterns (abundance systems) of the MS²
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6 spectra of the molecular ions of the newly detected fumonisins differ markedly from
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8 those of previously published fumonisins (Fig. 4): for the new fumonisins, there are six
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10 groups of fragment ions instead of the three characteristic of the MS² spectra of the
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12 molecular ions ([M+H]⁺) of most fumonisins (Figs 2 and 4). Secondly, from the six
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14 groups of fragment ions, the ions (*m/z* values) of groups 4, 5 and 6 are also present in the
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16 groups of fragment ions of the mass spectra of the FB₁ toxin and its isomers (1-3 in Fig.
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18 2) (Bartók et al. 2010). The explanation seems obvious: in the MS fragmentation of
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20 EFB₁-type fumonisins, the fatty acid or its ketene form is split off the molecule before
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22 either of the TCAs, and two of the ions characteristic of the MS² spectrum of FB₁ toxin,
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24 i.e. the ions at *m/z* 704 and *m/z* 722, are produced. From this step on, the theoretical MS
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26 fragmentation scheme of the EFB₁ components is identical with that of FB₁ toxin.
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28 Thirdly, it is of diagnostic value that the *m/z* values of the hydrocarbon backbones
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30 detected at the end of the fragmentation in the MS² spectra of the molecular ions of the
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32 newly detected fumonisins (299 Da) are identical with the *m/z* value of the hydrocarbon
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34 backbone in the mass spectra of FB₁ toxin and its isomers. From the predominant
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36 directions of fragmentation, the example of a fragmentation previously unknown among
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38 fumonisins is shown in Fig. 6 for EFB₁ LA (C-10 is the presumed esterification site for
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40 the LA). The fragmentation patterns are similar for the other EFB₁s and for the iso-
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42 EFB₁ components. It follows from the structures of the new esterified fumonisins that groups
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44 modifying the backbone are split off in the course of fragmentation, i.e. C-O and C-N
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46 bond scission leads to the elimination of H₂O, organic acids (TCA, LA, OA or PA) and
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48 NH₃. From the product ions formed as a result of fragmentation, it is clear that TCA can
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50 also be eliminated in a stepwise manner, while H₂O is released with the concomitant
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2 formation of the corresponding anhydride (TCAD 158 Da, Fig. 7). The splitting-off of
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4 TCA and the new fatty acids (LA, OA or PA) esterifying the backbone in the form of
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6 their ketene (TCAK 158 Da, LAK 262 Da, OAK 264 Da and PAK 238 Da, Fig. 7) was
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8 also observed, as can be seen in the theoretical fragmentation pathways (Fig. 5a-c) and
9
10 the MS² spectra (Fig. 4). The MS² spectra permit, the following general observations: (i)
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12 the ions produced by the fragmentation of LA, OA or PA were most abundant; (ii) the
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14 product ions with the lowest abundance were those formed by the splitting-off of 2TCA;
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16 (iii) the sequence of the groups of fragment ions according to abundances is
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18 4>2>5>6≥1>3 for EFB₁ and 4>5>6>1≥2>3 for iso-EFB₁. The characteristic data
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20 (retention time, retention time relative to FB₁, quantity relative to FB₁ and MS² spectral
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22 information on the molecular ions) for the EFB₁ compounds, obtained by RP-HPLC/ESI-
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24 ITMS, are detailed in Table 2. The relative quantities (% of FB₁) of the new fumonisins
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26 were in the range 0.003–0.036%. Their total amount was 0.1% of the FB₁ concentration,
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28 which may be rated as significant when it is considered that these new components are
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30 much more apolar than earlier-described fumonisins, and their uptake into and toxicity
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32 elicited in the various tissues of living organisms may therefore also be significantly
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34 different from those of other fumonisins.

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42 The experimental results presented in this article clearly revealed that the parallel use
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44 of two hyphenated techniques (RP-HPLC/ESI-ITMS and RP-HPLC/ESI-TOFMS) was
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46 essential for identification of new type of fumonisins with three acyl groups, i.e. there are
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48 situations when a single hyphenated technique is not sufficient for the identification of a
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50 new compound. Of course, neither procedure could determine the relative and absolute
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52 configurations of the new fumonisins. In order to elucidate the structures of these toxins,
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54 other spectroscopic techniques, such as NMR, XRD and ORD, must be used. However,
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2 to perform these structural examinations and to examine the toxicity of these compounds,
3
4 the most abundant EFB₁ isomers should first be isolated.
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8 9 **Acknowledgements**

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16 17 18 **References**

19
20
21 Bartók T, Szécsi Á, Szekeres A, Mesterházy Á, Bartók M. 2006. Detection of new
22
23 fumonisin mycotoxins and fumonisin-like compounds by reversed-phase high-
24
25 performance liquid chromatography/electrospray ionization ion trap mass
26
27 spectrometry. *Rapid Commun Mass Spec.* 20:2447-2462.
28

29
30
31 Bartók T, Szekeres A, Szécsi Á, Bartók M, Mesterházy Á. 2008. A new type of
32
33 fumonisin series appeared on the scene of food and feed safety. *Cereal Res Commun*
34
35 *Suppl B.* 36:315-319.
36

37
38
39 Bartók T, Tölgyesi L, Szekeres A, Varga M, Bartha R, Szécsi Á, Bartók M, Mesterházy
40
41 Á. 2010. Detection and characterization of twenty-eight isomers of fumonisin B1
42
43 (FB1) mycotoxin in a solid rice culture infected with *Fusarium verticillioides* by
44
45 reversed-phase high-performance liquid chromatography/electrospray ionization time-
46
47 of-flight and ion trap mass spectrometry. *Rapid Commun Mass Spec.* 24:35-42.
48

49
50
51 Bezuidenhout SC, Gelderblom WCA, Gorst-Allam CP, Horak RM, Marasas WFO,
52
53 Spiteller G, Vleggaar R. 1988. Structure elucidation of the fumonisins, mycotoxins
54
55 from *Fusarium moniliforme*. *J Chem Soc-Chem Commun.* 743-745.

56
57
58 Bolger M, Coker RD, DiNovi M, Gaylor D, Gelderblom WCA, Olsen M. in: Safety
59
60 evaluation of certain mycotoxins in food, Edited by the Fifty-sixth meeting of the Joint

- 1
2 FAO/WHO Expert Committee on Food Additives (JECFA). FAO, Rome. 2001, pp.
3
4 103-280.
5
6
7 Colvin BM, Harrison LR. 1992. Fumonisin-induced pulmonary-edema and hydrothorax
8
9 in swine. *Mycopathologia* 117:79-82.
10
11 Doko MB, Rapior S, Visconti A, Schjoth JE. 1995. Incidence and levels of fumonisin
12 contamination in maize genotypes grown in Europe and Africa. *J Agr Food Chem.*
13 43:429-434.
14
15
16
17
18 Fazekas B, Bajmócy E, Glávits R, Fenyvesi A, Tanyi J. 1998. Fumonisin B-1
19 contamination of maize and experimental acute fumonisin toxicosis in pigs. *J Vet Med*
20 B. 45:171-181.
21
22
23
24
25 Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U. 2007. Fumonisin B-2
26 production by *Aspergillus niger*. *J Agr Food Chem.* 55:9727-9732.
27
28
29
30 Gelderblom WCA, Kriek NPJ, Marasas WFO, Thiel PG. 1991. Toxicity and
31 carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin-B1, in rats.
32 *Carcinogenesis* 12:1247-1251.
33
34
35
36
37 IARC, International Agency for Research on Cancer, Fumonisin B1. IARC Monographs
38 on the Evaluation of Carcinogenic Risks to Humans, Some Traditional Medicines,
39 Some Mycotoxins, Naphtalene and Styrene; IARC: Lyon, France. 2002, 82, pp. 301-
40 366.
41
42
43
44
45 Josephs JL. 1996. Detection and characterization of fumonisin mycotoxins by liquid
46 chromatography electrospray ionization using ion trap and triple quadrupole mass
47 spectrometry. *Rapid Commun Mass Spec.* 10:1333-1344.
48
49
50
51
52
53 Kellerman TS, Marasas WFO, Thiel PG, Gelderblom WCA, Cawood M, Coetzer JAW.
54 1990 Leukoencephalomalacia in 2 horses induced by oral dosing of fumonisin-B1.
55 *Onderstepoort J Vet Res.* 57:269-275.
56
57
58
59
60

- 1
2 Ledoux DR, Brown TP, Weibking TS, Rottighaus GE. 1992. Fumonisin toxicity in
3
4 broiler chicks. *J Vet Diag Invest.* 4:330-333.
5
6
7 Lemke SL, Ottinger SE, Ake CL, Mayura K, Phillips TD. 2001. Deamination of
8
9 fumonisin B-1 and biological assessment of reaction product toxicity. *Chem Res*
10
11 *Toxicol.* 14:11-15.
12
13
14 Logrieco A, Ferracane R, Haidukowsky M, Cozzi G, Visconti A, Ritieni A. 2009.
15
16 Fumonisin B2 production by *Aspergillus niger* from grapes and natural occurrence in
17
18 must. *Food Addit Contam.* 26:1495-15.
19
20
21 Mogensen JM, Frisvad JC, Thrane U, Nielsen KF. 2010. Production of fumonisin B2 and
22
23 B4 by *Aspergillus niger* on grapes and raisins. *J Agr Food Chem.* 58:954-958.
24
25
26 Musser SM, Gay ML, Mazzola EP. 1996. Identification of a new series of fumonisins
27
28 containing 3-hydroxypyridine. *J Nat Prod.* 59: 970-972.
29
30
31 Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA. 2009. Fumonisin
32
33 B2 production by *Aspergillus niger* in Thai coffee beans. *Food Addit Contam.* 26:94-
34
35 100.
36
37
38 Rheeder JP, Marasas WFO, Thiel PG, Sydenham EW, Shephard GS, Van Schalkwyk DJ.
39
40 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal
41
42 cancer in Transkei. *Phytopathology* 82:353-357.
43
44
45 Rheeder JP, Marasas WFO, Vismer HF. 2002. Production of fumonisin analogs by
46
47 *Fusarium* species. *Appl Environ Microbiol.* 68:2101-2105.
48
49
50 Riley RT, Hinton DM, Chamberlain WJ, Bacon CW, Wang E, Merrill AH, Voss KA.
51
52 1994 Dietary fumonisin B-1 induces disruption of sphingolipid metabolism in spargue-
53
54 dawley rats - a new mechanism of nephrotoxicity. *J Nutr.* 124:594-603.
55
56
57 Seifert KA, Aoki T, Baayen RP, Brayford D, Burgess LW, Chulzes S, Gams W, Geiser
58
59 D, deGruyter J, Leslie JF, Logrieco A, Marasas WFO, Nirenberg HI, Odonnell K,
60

- 1
2 Rheeder JP, Samuels GJ, Summerell BA, Thrane U, Waalijk C. 2003. The name
3
4 Fusarium moniliforme should no longer be used. *Mycol Res.* 107:643-644.
5
6 Senyuva HZ, Gilbert J. 2008. Identification of fumonisin B-2, HT-2 toxin, patulin and
7
8 zearalenone in dried figs by liquid chromatography-time-of-flight mass spectrometry
9
10 and liquid chromatography-mass spectrometry. *J Food Protect.* 71:1500-1504.
11
12 Shephard GS, Thiel G, Stockenström S, Sydenham EW. 1996. Worldwide survey of
13
14 fumonisin contamination of corn and corn-based products. *J AOAC Int.* 79:671-687.
15
16 Shephard GS, Marasas WFO, Leggott NL, Yazdanpanah H, Rahimian H, Safavi N. 2000.
17
18 Natural occurrence of fumonisins in corn from Iran. *J Agr Food Chem.* 48:1860-1864.
19
20 Stack ME. 1998. Analysis of fumonisin B-1 and its hydrolysis product in tortillas. *J*
21
22 *AOAC Int.* 81:737-740.
23
24 Ueno Y, Lijima K, Wang SD, Sugiura Y, Sekijima M, Tanaka T, Chen C, Yu SZ. 1997.
25
26 Fumonisin as a possible contributory risk factor for primary liver cancer. A 3-year
27
28 study of corn harvested in Haimen, China, by HPLC and ELISA. *Food Chem Toxicol.*
29
30 35:1143-1150.
31
32 Weibking TS, Ledoux DR, Brown TP, Rottighaus GE. 1993. Fumonisin toxicity in
33
34 turkey poults. *J Vet Diag Invest.* 5:75-83.
35
36 Yoshizawa T, Yamashita A, Lou Y. 1994. Fumonisin occurrence in corn from high-risk
37
38 and low-risk areas for human esophageal cancer in China. *Appl Environ Microbiol.*
39
40 60:1626-1629.
41
42 Zomborszky-Kovács M, Kovács F, Horn P, Vetési F, Repa I, Tornóyos G, Tóth Á. 2002.
43
44 Investigations into the time- and dose-dependent effect of fumonisin B-1 in order to
45
46 determine tolerable limit values in pigs. *Livest Prod Sci.* 76:251-256.
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2 Zöllner P, Mayer-Helm B. 2006. Trace mycotoxin analysis in complex biological and
3
4 food matrices by liquid chromatography–atmospheric pressure ionization mass
5
6 spectrometry. J Chromatogr A. 1136:123-169.
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
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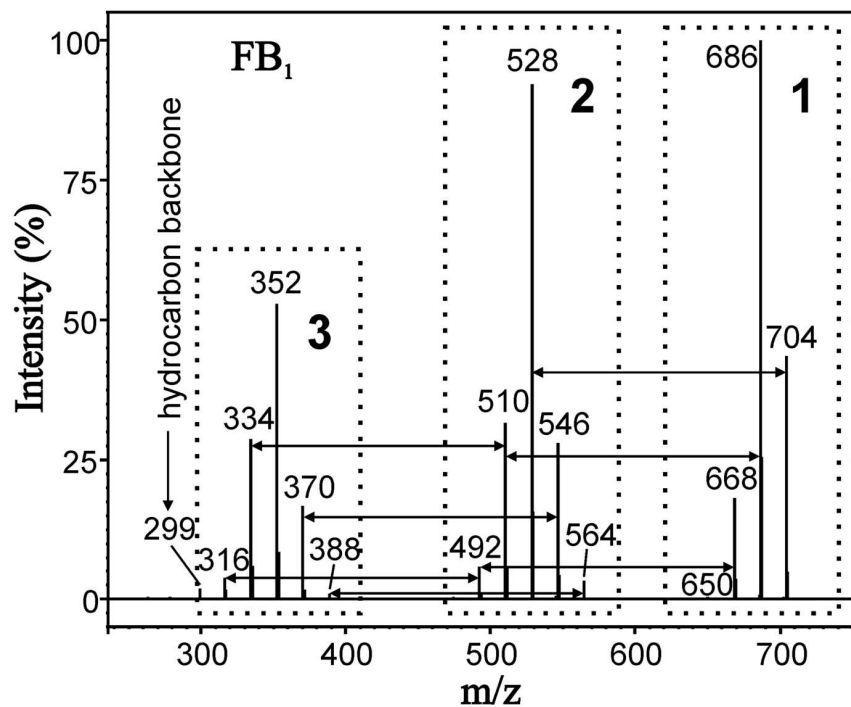


Figure 2. Product ion spectra (CID) of protonated molecules ($[M+H]^+$) of FB_1 with product ion groups (1-3) indicated. The left and right doubled arrows indicate the mass difference of 176 Da (loss of one molecule of TCA) between the corresponding product ions of the neighbouring product ion groups.

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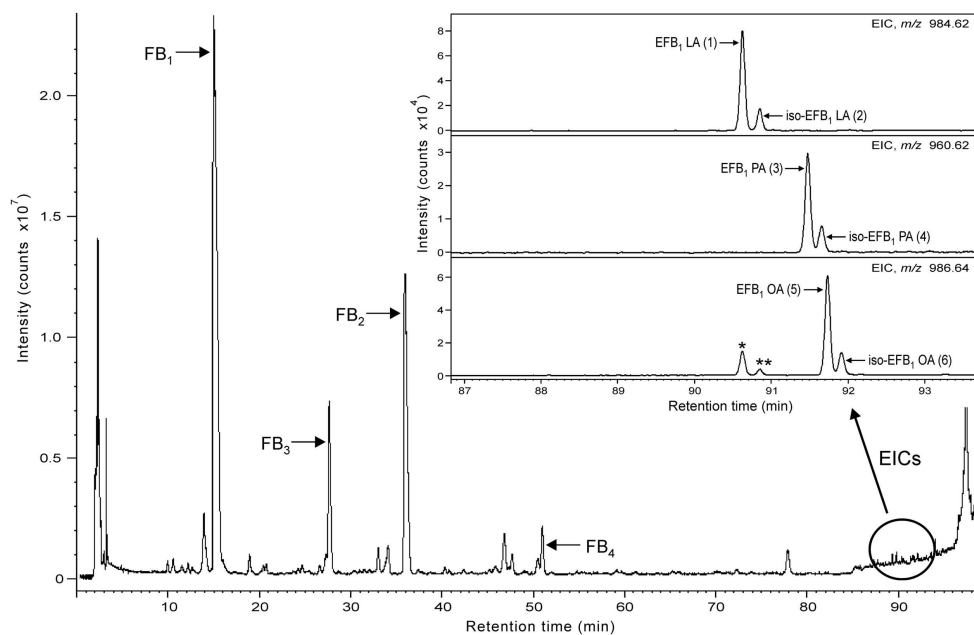


Figure 3. Total ion and extracted ion chromatograms (EICs, enlarged, at m/z 984.62, 960.62 and 986.64) obtained by RP-HPLC/ESI-TOFMS on an extract of a solid rice culture infected with *Fusarium verticillioides*. The asterisks (*) and (**) indicate the isotope peaks of EFB₁ LA (1) and iso-EFB₁ LA (2) at an m/z value of 986.64.
109x74mm (600 x 600 DPI)

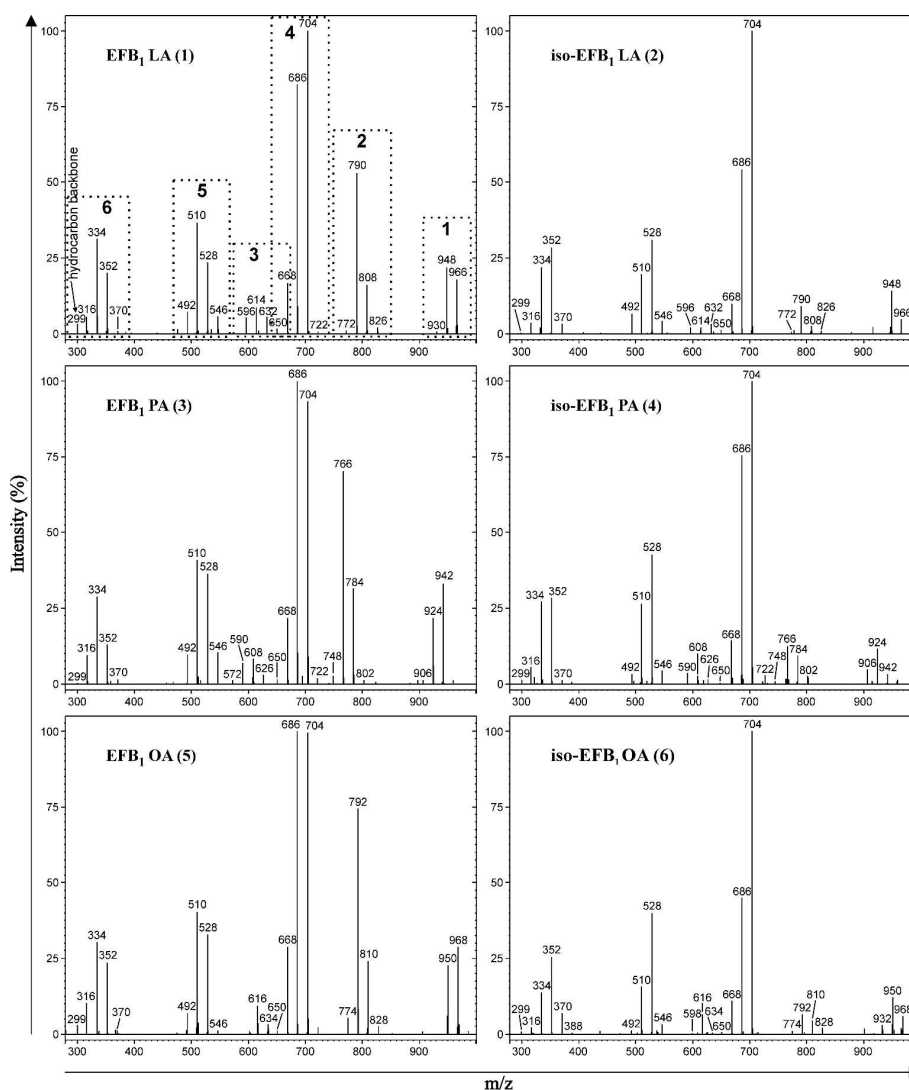
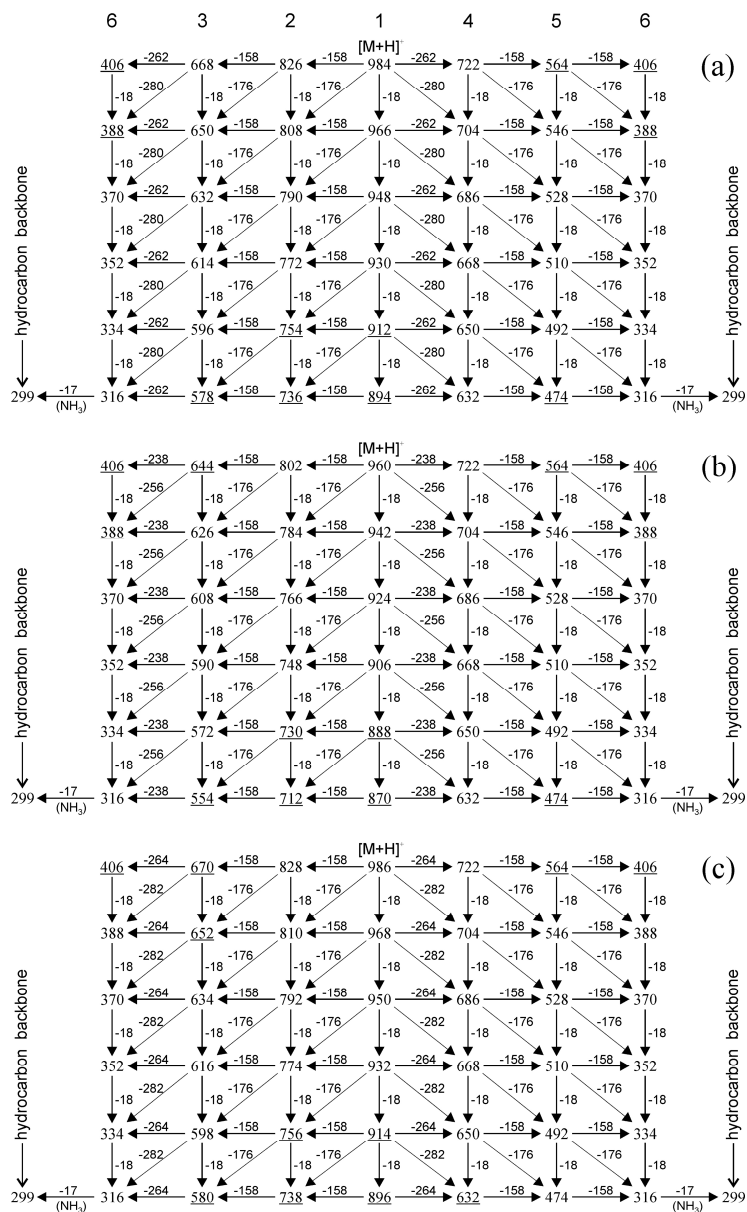


Figure 4. Product ion spectra (CID) of protonated molecules ($[M+H]^+$) of esterified FB₁ (EFB₁) isomers: EFB₁ LA (1), iso-EFB₁ LA (2), EFB₁ PA (3), iso-EFB₁ PA (4), EFB₁ OA (5), iso-EFB₁ OA (6) by ESI-ITMS². The product ion groups (1-6) are indicated in the MS² spectrum of $[M+H]^+$ at m/z 984 of EFB₁ LA (1).
214x243mm (600 x 600 DPI)



132x216mm (600 x 600 DPI)

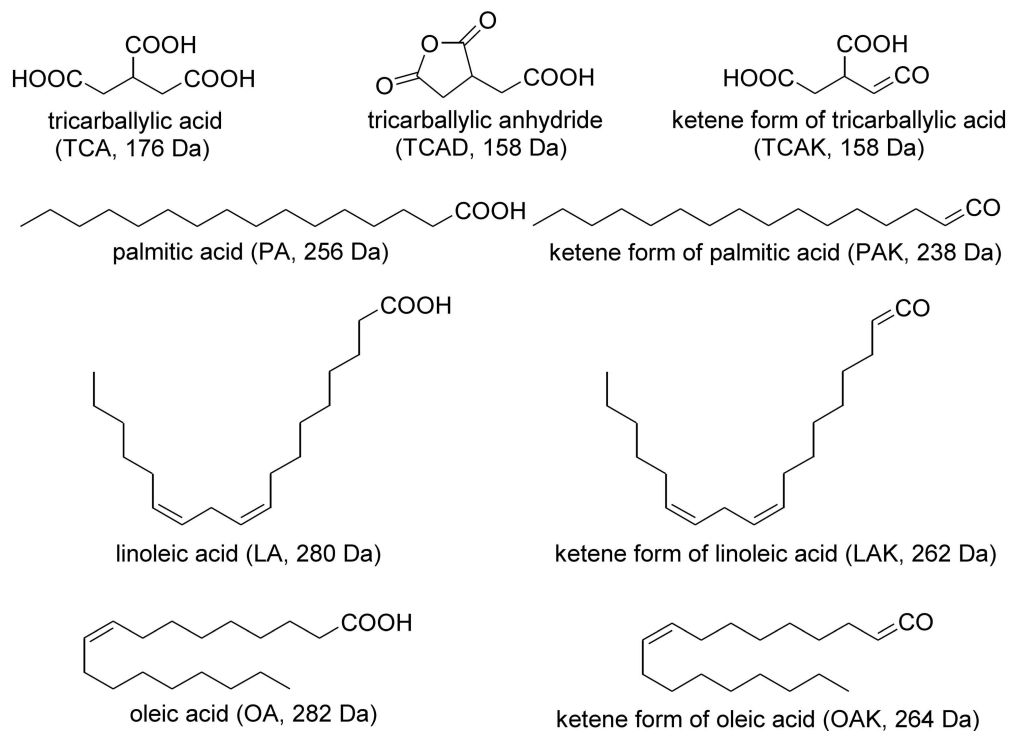


Figure 7. Acids conjugated to the fumonisin backbone and structures of compounds possibly formed in the CID fragmentation processes of protonated molecules of EFB₁ isomers.
128x93mm (600 x 600 DPI)

Table 1. Characteristic data obtained by RP-HPLC/ESI-TOFMS on new esterified FB₁ (EFB₁) isomers.

Compound name	Retention time (min)	Retention time relative to FB ₁	Empirical formula (unprotonated)	Exact mass of [M+H] ⁺ (calculated)	Exact mass of [M+H] ⁺ (measured)	Mass accuracy (ppm)
EFB ₁ LA (1)	90.623	6.809	C ₅₂ H ₈₉ NO ₁₆	984.6254	984.6243	-1.1
iso-EFB ₁ LA (2)	90.852	6.827	C ₅₂ H ₈₉ NO ₁₆	984.6254	984.6248	-0.6
EFB ₁ PA (3)	91.482	6.875	C ₅₀ H ₈₉ NO ₁₆	960.6254	960.6259	0.5
iso-EFB ₁ PA (4)	91.670	6.890	C ₅₀ H ₈₉ NO ₁₆	960.6254	960.6255	0.1
EFB ₁ OA (5)	91.732	6.895	C ₅₂ H ₉₁ NO ₁₆	986.6411	986.6412	0.1
iso-EFB ₁ OA (6)	91.928	6.909	C ₅₂ H ₉₁ NO ₁₆	986.6411	986.6412	0.1

Table 2. Characteristic data obtained by RP-HPLC/ESI-ITMS on new esterified FB₁ (EFB₁) isomers (for abbreviations, see Fig. 7).

Compound name and <i>m/z</i> value (<i>italics</i>) of [M+H] ⁺	Retention time (min)	Retention time relative to FB ₁	Relative quantity (% of FB ₁)	<i>m/z</i> values (<i>italics</i>) and relative abundances (%) of product ions of [M+H] ⁺																								
				[M+H – H ₂ O] ⁺	[M+H – 2H ₂ O] ⁺	[M+H – 3H ₂ O] ⁺	[M+H – TCAK] ⁺	[M+H – TCA] ⁺	[M+H – TCA – H ₂ O] ⁺	[M+H – TCA – 2H ₂ O] ⁺	[M+H – LAK or OAK or PAK] ⁺	[M+H – LA or OA or PA] ⁺	[M+H – LA or OA or PA – H ₂ O] ⁺	[M+H – LA or OA or PA – 2H ₂ O] ⁺	[M+H – LA or OA or PA – 3H ₂ O] ⁺	[M+H – 2TCA] ⁺	[M+H – 2TCA – H ₂ O] ⁺	[M+H – 2TCA – 2H ₂ O] ⁺	[M+H – TCAK – LA or OA or PA] ⁺	[M+H – TCA – LA or OA or PA] ⁺	[M+H – TCA – LA or OA or PA – H ₂ O] ⁺	[M+H – TCA – LA or OA or PA – 2H ₂ O] ⁺	[M+H – TCAK – TCA – LA or OA or PA] ⁺	[M+H – 2TCA – LAK or OAK or PAK] ⁺	[M+H – 2TCA – LA or OA or PA] ⁺	[M+H – 2TCA – LAK or OAK or PAK – H ₂ O] ⁺	[M+H – 2TCA – LA or OA or PA – H ₂ O] ⁺	[M+H – 2TCA – LAK or OAK or PAK – 2H ₂ O] ⁺
EFB ₁ LA (1) <i>984</i>	89.324	6.897	0.036	<i>966</i>	<i>948</i>	<i>930</i>	<i>826</i>	<i>808</i>	<i>790</i>	<i>772</i>	<i>722</i>	<i>704</i>	<i>686</i>	<i>668</i>	<i>650</i>	<i>632</i>	<i>614</i>	<i>596</i>	<i>546</i>	<i>528</i>	<i>510</i>	<i>492</i>	<i>370</i>	<i>352</i>	<i>334</i>	<i>316</i>	<i>299</i>	
iso-EFB ₁ LA (2) <i>984</i>	89.542	6.914	0.010	17.9	21.8	0.7	2.0	16.1	52.9	0.9	0.7	100	82.3	16.6	1.6	5.4	8.6	5.4	5.7	23.5	36.7	7.2	0.8	20.1	31.2	5.4	3.2	
EFB ₁ PA ^a (3) <i>960</i>	90.150	6.962	0.015	<i>966</i>	<i>948</i>	<i>930</i>	<i>826</i>	<i>808</i>	<i>790</i>	<i>772</i>	<i>722</i>	<i>704</i>	<i>686</i>	<i>668</i>	<i>650</i>	<i>632</i>	<i>614</i>	<i>596</i>	<i>546</i>	<i>528</i>	<i>510</i>	<i>492</i>	<i>370</i>	<i>352</i>	<i>334</i>	<i>316</i>	<i>299</i>	
iso-EFB ₁ PA ^b (4) <i>960</i>	90.323	6.976	0.003	4.7	14.1	0	0.8	2.6	9.0	0.6	0	100	54.3	9.8	1.3	3.2	2.0	2.0	4.1	30.9	19.5	6.6	3.2	28.4	21.8	3.6	0	
EFB ₁ OA ^c (5) <i>986</i>	90.395	6.981	0.027	<i>942</i>	<i>924</i>	<i>906</i>	<i>802</i>	<i>784</i>	<i>766</i>	<i>748</i>	<i>722</i>	<i>704</i>	<i>686</i>	<i>668</i>	<i>650</i>	<i>608</i>	<i>590</i>	<i>572</i>	<i>546</i>	<i>528</i>	<i>510</i>	<i>492</i>	<i>370</i>	<i>352</i>	<i>334</i>	<i>316</i>	<i>299</i>	
iso-EFB ₁ OA ^d (6) <i>986</i>	90.569	6.995	0.008	33.0	21.6	1.1	1.1	31.4	70.3	2.5	1.8	93.1	100	21.7	1.6	8.3	7.0	1.2	10.4	36.2	40.8	9.6	1.4	13.0	28.8	9.4	0.2	
				3.2	11.6	4.8	2.0	9.3	12.4	0	0.8	100	75.4	14.4	0	2.8	3.6	0	4.5	42.8	26.3	3.1	1.1	28.4	27.2	5.4	1.3	
				<i>968</i>	<i>950</i>	<i>932</i>	<i>828</i>	<i>810</i>	<i>792</i>	<i>774</i>	<i>722</i>	<i>704</i>	<i>686</i>	<i>668</i>	<i>650</i>	<i>634</i>	<i>616</i>	<i>598</i>	<i>546</i>	<i>528</i>	<i>510</i>	<i>492</i>	<i>370</i>	<i>352</i>	<i>334</i>	<i>316</i>	<i>299</i>	
				28.8	22.8	0	2.6	24.1	74.4	5.3	2.2	99.5	100	28.8	1.1	3.3	9.3	0.2	1.1	32.8	40.4	6.9	1.2	23.5	30.4	10.1	2.8	
				<i>968</i>	<i>950</i>	<i>932</i>	<i>828</i>	<i>810</i>	<i>792</i>	<i>774</i>	<i>722</i>	<i>704</i>	<i>686</i>	<i>668</i>	<i>650</i>	<i>634</i>	<i>616</i>	<i>598</i>	<i>546</i>	<i>528</i>	<i>510</i>	<i>492</i>	<i>370</i>	<i>352</i>	<i>334</i>	<i>316</i>	<i>299</i>	
				5.8	12.2	2.8	2.0	4.5	6.6	0.9	0	100	45.1	10.9	0.7	0.4	6.5	0.2	3.2	39.9	15.6	1.2	7.0	25.6	13.7	2.1	0.7	

^a + *m/z* 626 [M+H – TCA – TCAK]⁺ 3.1%; ^b + *m/z* 626 [M+H – TCA – TCAK]⁺ 1.5%; ^c + *m/z* 474 [M+H – TCA – OA – 3H₂O]⁺ 0.9%; + *m/z* 388 [M+H – 2TCAK – OA]⁺ or [M+H – TCAK – TCA – OAK]⁺ 1.4%; ^d + *m/z* 474 [M+H – TCA – OA – 3H₂O]⁺ 0.2%; + *m/z* 388 [M+H – 2TCAK – OA]⁺ or [M+H – TCAK – TCA – OAK]⁺ 0.4%; characteristic *m/z* values and relative abundances (%), in brackets) of MS² spectrum of FB₁ toxin ([M+H]⁺ at *m/z* 722) according to Bartók et al. 2010: 704 (43.6); 686 (100); 668 (18.1.); 650 (0.4); 564 (3.3); 546 (28); 528 (92.2); 510 (31.5); 492 (5.7); 388 (0.9); 370 (16.7); 352 (52.9); 334 (28.7); 316 (3.9); 299 (1.9)