Chapter

Cunninghamella bertholletiae's Toxins from Decomposing Cassava: Mitigation Strategy for Toxin Reduction Using *Nepenthes mirabilis* 'Monkey Cup' Digestive Fluids

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Abstract

A fermentation technique was utilised to assess a fungus, i.e. *Cunninghamella bertholletiae/polymorpha*, isolated from rotting cassava, ability to produce mycotoxins and resultant oxidation by-products of the mycotoxins using liquid chromatography–mass spectrometry (LC/MS). Thus, the mycotoxins/secondary metabolites, fumonisin B^1 (FB^1) and deoxynivalenol (DON) were produced while, heptadecanone, octadecanamide, octadecenal and 3-keto-deoxynivalenol (DON) were successfully identified as biodegradation by-products in the fermentation broth treated with hydrolysing 'monkey cup' juice from *Nepenthes mirabilis*. Exposure to the mycotoxins and the biodegradation by-products through consumption of contaminated produce including contact due to the cumulative presence in arable agricultural soil can be harmful to humans and animals. Therefore, this work reports on a strategy for the mitigation and reduction of mycotoxins in agricultural soil using natural plant pitcher juices from *N. mirabilis'* 'monkey cup'.

Keywords: biodegradation, carboxylesterases, *Cunninghamella bertholethiae*, LC/MS, mycotoxins, *Nepenthes mirabilis*

1. Introduction

Postharvest storage for cassava is often shortened due to product spoilage caused by bacterial and fungal infestation [1, 2]. Fungal species such as *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. and *Cunninghamella* spp. can produce toxins and/or

secondary metabolites that affect the storage longevity and quality of agricultural product such as cassava [2, 3]. These mycotoxins, which have a negative impact on agricultural products, lead to economic losses due to the contamination of cassava tubers, which makes them inedible. Generally, toxins are biosynthetic compounds produced by numerous microorganisms in a natural or controlled environment.

These microorganisms include the fungus, *Cunninghamella bertholletiae* (also known as *Cunninghamella polymorpha* due to its morphological characteristics and mating/reproductive scheme) [4], is known to be pathogenic to humans and animals [5–7], while its toxins in the environment and on consumable commodities constitute an environmental hazard and a health risk to consumers [8–11]. Some fungi, including their metabolites, are able to contaminate several plant parts as they are endophytes, culminating in infestation of agricultural products such as tomatoes, maize, potatoes, beans, peanuts, yams and wheat, including cassava [1, 5, 12–17] and dairy products such as milk and cheese [1, 18, 19]. Humans' or animals' consumption of contaminated products may lead to foodborne toxin-related intoxication [7, 20] culminating in the degeneration of human internal organs including their functionality and the promotion of diseases such as cancer [8, 15, 21–23]. Some clinical outcomes in animals and humans include liver and oesophageal cancer [21, 23], the destruction of renal and nerve tissues, profound oxidative stress, heart and pulmonary diseases [23].

There are several varieties of mycotoxins, namely aflatoxins (AFB^1, AFB^2, AFG^1 and AFG^2), fumonisins (FB^1, FB^2), deoxynivalenol (DON), ochratoxins (A, B and C), amongst others, which are produced by numerous species, some of which are deleterious to plants/agricultural products, humans and animals [1, 5, 21, 23, 24]. Their production can occur under favourable environmental conditions, such as a high temperature and adequate moisture/humidity, including the availability of nutrients (mostly from the decaying produce) [25]. These concerns have prompted researchers to find cheap, efficient and cost-effective ways to reduce or manage mycotoxin-producing organisms, including mycotoxin contamination, when produced [11, 26] to limit sequential effects including products' contamination.

In a previous study, it was found that *C. bertholletiae/polymorpha*, a common soil organism [7, 23, 26] which was isolated from decomposing cassava, was both cyanide-resistant with the ability to biodegrade free cyanide while being antagonistic towards other soil organisms [15, 27]. Currently, there is minimal literature available on mycotoxins produced by *C. bertholletiae*. Similarly, there is minimal research on a mitigation strategy which could be classified as environmentally benign for combined toxin reduction, via oxidation or hydrolysis. The mitigation method must be implementable *in-situ* in order to minimise deleterious effects observed when other methods are used.

Therefore, the aim of this study was to propose and assess a method for the identification of mycotoxins from the free-cyanide tolerant *C. bertholletiae/polymorpha* isolate; furthermore, to quantitatively assess a mitigation method using oxidative/hydrolysing 'monkey cup' digestive fluids from *N. mirabilis* (green chemistry approach). A *N. mirabilis* is a carnivorous plant which belongs to the genus of *Nepenthes*. This plant is characterised by a pitfall trap commonly known as a 'monkey cup' at the end of the plants' leaf, which contains an acidic and oxidative/hydrolysing fluid. The plants' pitcher juices are known to contain a variety of enzymes useful for prey digestion [28, 29]. As such, these enzymes can oxidise and/or hydrolyse mycotoxins and secondary metabolites via deamination or mechanisms biocatalytically facilitated by esterases for the decoupling of aliphatic chains in mycotoxins or secondary metabolites.

2. Mycotoxin (secondary metabolite) production in food

Several studies discussed about the presence of mycotoxins in food. Thus, during a produce life cycle from harvest, postharvest, selves' life, processing and sometimes distribution, there is a presence of mycotoxins in food worldwide [1]. These toxins occurred during poor storage, handling and processing conditions, sometimes might be the result of the rot/decay foodstuffs [2, 14, 30]. While these mycotoxins constitute a serious threat to food quality and human's health [22, 30].

2.1 Extraction and analysis of mycotoxins (secondary metabolites) and their biodegradation by-products

Literatures abound on the extraction and analysis of mycotoxins, a liquid-phase extraction method seems to be more used. Thus, [31, 32] used liquid-liquid extraction method for their studies in mycotoxins identification, while [33] used a liquid chromatography/tandem mass spectrometry for a combined analysis of aflatoxins, ochratoxin A and *Fusarium* for maize crop. Whereas [34] chose a multiplex approach of Gas chromatography–mass spectrometry (GC-MS), Liquid chromatography-mass spectrometry (LC-MS) and One-dimensional (1D) NMR spectroscopy (1D NMR) techniques for their study on a comparative metabolite profiling and fingerprinting of medicinal licorice roots, to name few.

The samples were analysed using an LC/MS-ToF 6230 (Agilent Technologies Inc., USA) and using mobile-phase parameters as listed in the table below in Supplementary Material, without optimisation as suggested by [31, 34]. The solvent extract phase was steadily evaporated using a blow-down technique to dryness at an ambient temperature for 24 h to minimise mycotoxin evaporation using nitrogen (N2) gas (Afrox, South Africa) [31, 35].

The identification of the mycotoxins from *C. bertholletiae/polymorpha* isolate, including toxin biodegradation by-products, was done through analysis on LC/MS-ToF 6230 (Agilent Technologies Inc., USA) and analytical standard as well as profile data as per [31, 35] using a mycotoxin/biodegradation by-product database, with the assumption that samples were assumed to lose an electron with the H^+ proton being hypothetically the lost ion. Compounds were initially mined based on their molecular features and verified by mining based on their exact formulas. The extracted ion chromatogram (EIC) of matched compounds is presented in **Supplementary Figure 2**.

3. Proposed mitigation strategy

3.1 *N. mirabilis* extracts collection, characterisation and application

The assessment of the physicochemical characteristics of the *N. mirabilis* pitcher juice used was similar to that in [36–38]. Thus, the assessment revealed the following: conductivity: 5.89 S/m, redox potential: 510 mV, specific gravity (SG): 1.02 and a pH of 2.5.

Additionally, a qualitative method for the analysis and enzymes/biochemical tests were done to determine the presence of enzymes in the pitcher juice [36–39]. Furthermore, the VITEK 2 DensiChek™ cards were used (as a supplementary method) to quantitatively determine the enzyme presence in the extracts during the physicochemical analysis of the pitcher juice according to the instrument's/device's user manual instructions [40].

3.2 Enzyme (carboxylesterase) activity: mechanism, specificity and quantification

The quantification of carboxylesterases activity was similar to the method adopted from [41–43] with minor modifications. The overall biocatalysis properties of the *N. mirabilis* pitcher constituents, with a focus on carboxylesterases, are described by [41], who suggested that hydrolysis mechanism associated with carboxylesterases facilitates the biocatalysis of reactions associated with enzymes, including arylesterase, lysophospholipase, acetylesterase, acylglycerol lipase, etc. In the current study, the biodegradation of fumonisin and deoxynivalenol (DON) was achieved using a single enzyme (carboxylesterases).

Furthermore, subsequent reports on the development of a spectrophotometric method used for the determination of carboxylesterase activity for the *N. mirabilis* digestive fluid were used by [29, 42].

3.3 Carboxylesterase activity assay

Previous studies assessed carboxylesterase activity. Thus, the carboxylesterase activity assay was determined spectrophotometrically at an ambient temperature using *p*-nitrophenyl acetate (PNPA) as the substrate as suggested by [36, 43]. While the activity was measured by determining the rate of biocatalysis of PNPA to *p*-nitrophenol (PNP) which was spectrophotometrically monitored at 410 nm. The PNPA exhibits minimal absorbance at 410 nm, whereas the PNP absorbs strongly. The extinction coefficient used for PNP was 17,000 $M^{-1}{\cdot}cm^{-1}$ [36]. Activity was then expressed in U/L, where 1 unit is equivalent to 1 μmol/min (the rate of conversion for PNPA to PNP).

3.4 Spectrophotometer settings: Carboxylesterase activity assay

The JENWAY 6405 UV/Vis spectrophotometer (Agilent Pty, USA) at a kinetics setting was used 410 nm to monitor PNP formation for 2 min at 10 sec intervals, while the cell holder temperature was at 25°C. Eq. (1) Illustrates the mathematical expression used to quantify the activity of carboxylesterases [36].

$$\text{activity}\ (\text{U/L}) = \left[\frac{\frac{dA}{dt} * (\text{dilution factor})}{\text{extinction coefficient}}\right] * 60 * 10^{6} \qquad (1)$$

Where $\frac{dA}{dt}$ is the value of the reaction's initial rate.

4. Mycotoxins identification

Mycotoxins produced by the isolated *C. bertholletiae/polymorpha* were assessed via a fermentation technique in a nutrient broth medium with the liquid-liquid extraction method being done using chloroform, subsequent to a blow-down technique of the samples and reconstitution in absolute methanol. The compounds listed in **Table 1** were identified based on their molecular composition (structural features) and mass-to-charge ratio (m/z), using an LC/MS-ToF.

Toxin identification is important due to observed consequential outcomes of the infested cassava as by-products of bacterial or mycotic infestation which are hazardous to both humans and animals if such agricultural product is consumed. Thus, both fumonisin B^1 and deoxynivalenol were identified as the prevalent compounds associated with the fermentation of the cyanide resistant isolate, *C. bertholletiae*, accession no. KT275316 [15].

FB^1 detection on LC/MS-ToF was done, based on a method developed by [18, 24, 31, 44], for which the analyte produces a signal under a positive MS acquisition mode (**Table 1**).

A, mycotoxins molar mass (g/mol); B, biodegradation by-products molar mass (g/mol); A1, mycotoxins mass (*m*/*z*) to charge ratio-ion form $[M + H]^+$; B1, biodegradation by-products mass (*m*/*z*) to charge ratio-ion form.

For FB^1, mean peak counts of 4×10^3 were observed, while 1.9×10^3 counts were for DON. Similarly, and according to [31], DON detection is easily achieved through HPLC/LC-MS and UV methods. A LC/MS–ToF method, as described above, was used without modification nor optimisation, to also identify the biodegradation by-products for each identified mycotoxins/secondary metabolite as listed in **Table 1**.

Two peaks were observed with a retention time of 23.79 and 35.12 min, with a molecular formula of $C_{34}H_{59}NO_{15}$ and $C_{15}H_{20}O_6$, analogous to FB^1 and DON, respectively. The peaks, A and B, were directly associated with ion *m*/*z* of 722.395 and 297.13, when the ESI was operated in a positive mode [ion form: $M + H^+$]. From the analysis, a combination of the molecular weight, the structure, including *m*/*z* ratio, confirmed the identification of the compounds. It is paramount to indicate that FB^1 was detected in a culture in which CN^- (as KCN) was supplemented; hypothetically, indicating that the FB^1 production was perhaps influenced by strenuous conditions to which the culture was subjected in comparison to DON.

4.1 Biodegradation by-products' identification

To the reported residual samples of the cyanide-resistant *C. bertholletiaee/polymorpha*, in which FB^1 and DON were detected, *N. mirabilis* pitcher juices were added. This was for an assessment of the fungal mycotoxins/toxins' (FB^1 and DON) biodegradation into by-products [36–38], which could be identified using the LC/MS-ToF. Thus, compounds such as heptadecanone, octadecanamide and octadecenal were successfully identified from FB^1 samples with only 3-keto-DON being identified in DON samples, respectively (**Table 1**; **Figure 1**).

Mycotoxins/secondary metabolites	Biodegradation by-products identified	Molar mass (g/mol)		(*m*/*z*) ion form $[M + H]^+$	
		A	B	A1	B1
Fumonisin B^1 ($C_{34}H_{59}NO_{15}$	Heptadecanone $C_{17}H_{34}O$	721	254.45	722.395	256.270
	Octadecanamide $C_{18}H_{37}NO$		283.29		284.282
	Octadecenal $C_{18}H_{34}O$		266.46		267.268
Deoxynivalenol (DON) ($C_{15}H_{20}O_6$)	3-keto-DON $C_{15}H_{18}O_6$	296	294.91	297.13	295.115

Table 1.
C. bertholletiae's *mycotoxins/toxins and mycotoxins biodegradation by-products identified using LC/MS-ToF.*

The findings of this study are similar to those from previous studies which revealed that a biodegradation of FB^1 yielded by-products such as heptadecanone, octadecanamide and octadecenal (**Supplementary Figure 2a–c**) [26, 45]. While a degradation of DON led to an intermediate by-product such as 3-keto-DON [46, 47] (**Supplementary Figure 2d**). By using a similar identification strategy to that used to identify FB^1 and DON, it was clear that *N. mirabilis* had a deleterious effect on both DON and FB^1. The findings of this study are in agreement with those by [38, 48]. From the spectra, the by-product counts indicated octadecenal (1.1×10^2) > octadecanamide (1×10^2) > heptadecanone (0.9×10^2) with molecular ion peaks at *m/z* [M + H^+], 267.268, 284.282 and 256.270, respectively.

Furthermore, for DON residual samples, the by-products observed when subjected to the *N. mirabilis* pitcher juice were indicative of 3-keto-DON; that is, with the ESI spectra showing a molecular ion peak at *m/z* [M + H^+], 295.115 in a positive ion mode which was consistent with the molecular formula ($C_{15}H_{18}O_6$) (see **Supplementary Figure 2d**). Due to the nature of the proposed *in-situ* mitigation strategy, it is prudent to indicate that the applied *N. mirabilis* pitcher juice comprises biocatalytic agents or enzymes [39, 49] known to facilitate the

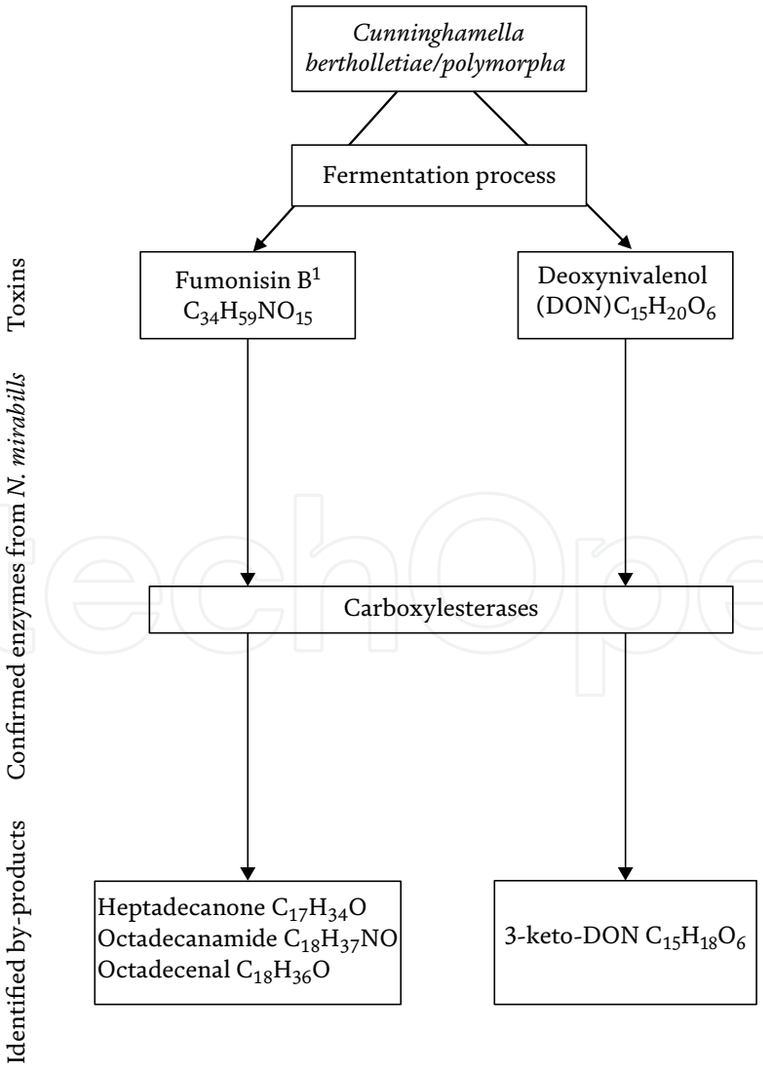

Figure 1.
Summary of a biodegradation process and associated oxidation/hydrolysing enzymes.

biodegradation of mycotoxins, using both qualitative and quantitative techniques. Thus, a degrading ability of the pitcher juice is due to the presence of enzymes such as carboxylesterase, β-glucuronidase, phosphatidyl inositol phospholipase C, xylanases, etc., which are able to biodegrade several organic matters, i.e. agro-waste, hemicellulose, etc., as well as mycotoxins/toxins [36–39, 49–51]. The enzymes found in the *N. mirabilis* pitcher juice originate from decayed multitude of trapped preys/species (insects) and microbial community (fungal and bacterial, etc.) within the plant's fluid [28, 37, 39, 41, 49, 51, 52].

4.2 Enzyme/biochemical activity assays for *N. mirabilis* pitcher juice

The samples' carboxylesterase activity (quantitative) and other biochemical assays (using the VITEK system, qualitative) were also done at room temperatures, whereas the *N. mirabilis* pitcher juice for carboxylesterase, *P*-nitrophenyl acetate (PNPA) were used as a substrate at 75% dilution and 410 nm absorbance which was similar to [36, 37]. For biochemical assays, numerous enzymes (as highlighted in **Table 2**) were positively identified, while the calculation of carboxylesterase activity was found to be 7.8 U/L.

5. Mycotoxin identification from cyanide-resistant *Cunninghamella* spp.

Due to the multitude of methods developed and assessed, a method modified by [44], for toxin extraction from a fermentation of broth, was adopted. It was thus used to produce mycotoxins (FB1 and DON) from the cyanide-resistant *C. bertholletiae*/*polymorpha*, with the extracts being used for LC/MS-ToF analysis due to the method's usability, reproducibility and rapidity, while incurring minimal input/sample-processing costs.

5.1 Biodegradation by-products: outcomes of the mitigation strategy

A digestive fluid of *N. mirabilis* was used as a feasible alternative for the biodegradation of fungal mycotoxins/toxins (Fumonisin and DON) with assays (n = 2) confirming the prevalence of carboxylesterases. However, previous studies mentioned the existence of several enzymes [28, 39, 41, 49, 50] within a *N. mirabilis* digestive fluid/pitcher juice, which counts as a larger enzymatic profile than individual microbial species, as highlighted in **Table 2**.

Furthermore, a few sceptics could express concern about the use of a plant's pitcher juice on mycotoxin-contaminated matrices because of its low pH (2.5), as well as availability, which can be addressed by using appropriate buffers and suitable plant

Enzymes	Activity/outcome	References
Carboxylesterase	7.8 (U/L)	In this study
β-glucosidase	++	[38, 39]
β-glucuronidase	++	[48]
Phosphatidyl inositol phospholipase C	++	[49]

++, positively identified in previous studies.

Table 2.
Carboxylesterase activity and qualitatively identified enzymes.

extracts with similar enzymatic characteristics. Overall, the application of a low pH extract in a matrix such as agricultural soil should not be a major concern because a soil's pH can be amended by an application of lime. A study by [53] revealed that the application of lime on agricultural soil with a low pH increases the soil's pH, improving its respiration capacity, while retaining the soil's microbial community profile at an acceptable level.

6. Conclusions

The identification through LC/MS-ToF of toxins ((fumonisin B^1 and deoxynivalenol (DON)) from a free-cyanide-resistant *Cunninghamella bertholletiae/polymorpha* as well as a mitigation strategy for toxins reduction through a biodegradation/fermentation process using 'monkey cup' juice from *N. mirabilis* (which yielded by-products such as heptadecanone, octadecanamide, octadecenal and 3-keto-DON) is an important step towards ensuring food safety and mitigating humans' health hazards through toxins exposure. As, an exposure or intoxication from these mycotoxins, through consumption of contaminated food or agricultural product, can be hazardous to humans and animals. Therefore, control measures for food and animal feed contamination are needed in order to decrease the levels of these compounds. Additionally, preventative protocols and/or mitigation strategies that would ensure the eradication of these hazardous compounds, using an environmentally benign approach such as *N. mirabilis* digestive fluid/pitcher juices, are paramount. Thus, the application of the digestive fluid to a liquid matrix which culminated in the biodegradation of mycotoxins (fumonisin B1 and DON), with the subsequent formation of the biodegradation by-products such as heptadecanone, octadecanamide, octadecenal for fumonisin B1 and 3-keto-DON for DON, which are easier to biodegrade by other microbial communities, should be encouraged.

However, it is worth noting that at this stage, there is a need to find alternative indigenous plant extracts with similar characteristics to that of the *N. mirabilis*.

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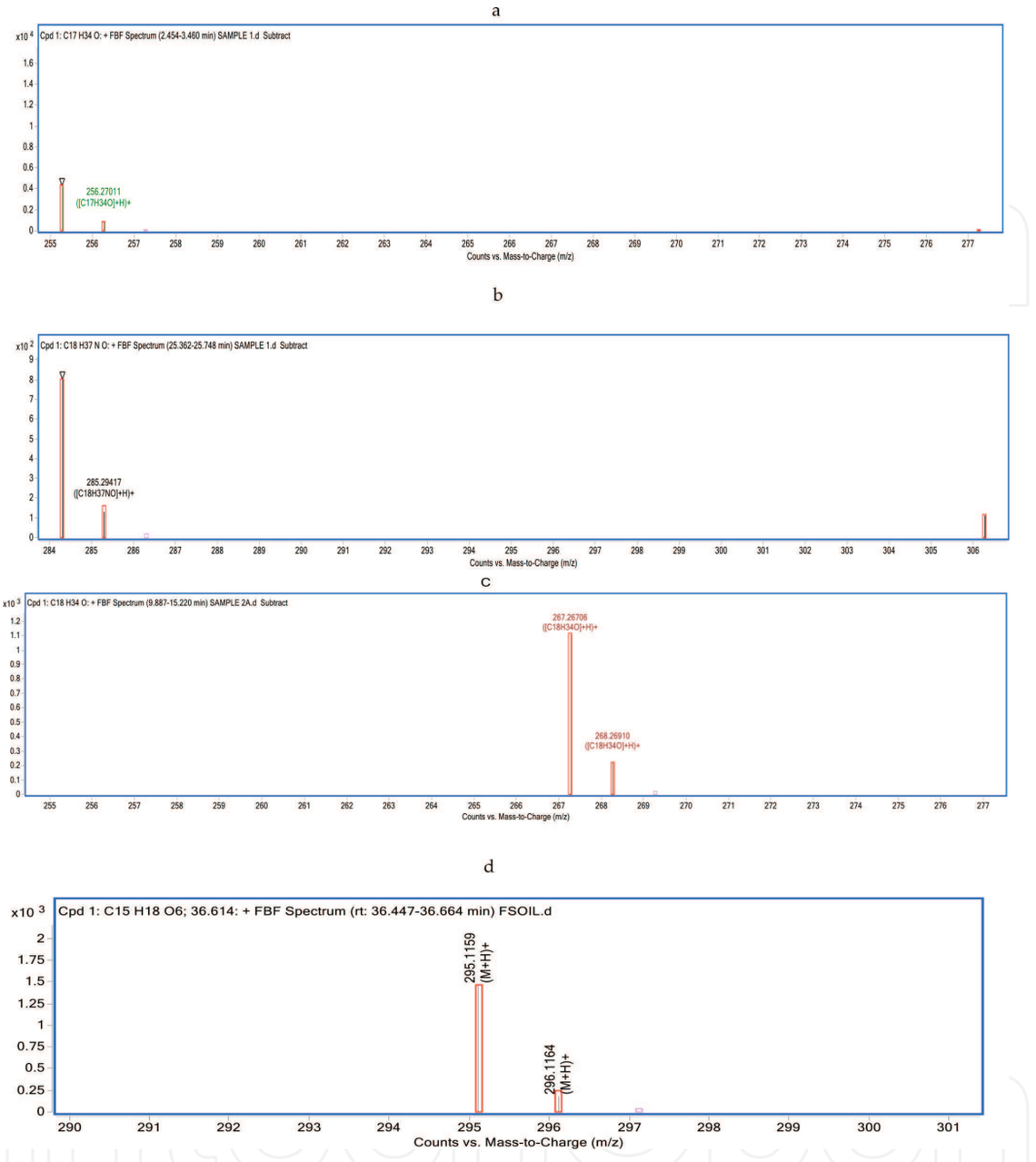
Conflicts of interest

The authors declare no conflicts of interest with respect to the research, authorship and/or publication of this manuscript.

Appendix



Supplementary Figure 2.
Molecular features and the extracted ion chromatograms (EICs)/mass spectrum of mycotoxins/toxins' biodegradation by-products: (a) heptadecanone, (b) octadecanamide, (c) octadecenal and (d) 3-keto-DON.

Gradient (min)	A (H_2O)*	B (MeOH)Y	Flow (mL/min)
0	85	15	0.4
30	0	100	0.4
33	0	100	0.4
45	85	15	0.4
50	85	15	0.4

**, water contained, 0.1% formic acid, pH 3.*
Y, analytical grade methanol.

Supplementary Table S1.
LC/MS-ToF elution and mobile phase parameters.

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