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5 # Quantification of Ochratoxin A in Foods by a Stable Isotope
6 Dilution Assay Using High-Performance Liquid
7 Chromatography-Tandem Mass Spectrometry

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ABSTRACT

A stable isotope dilution assay (SIDA) was developed for quantification of the mycotoxin ochratoxin A (OTA) by using [$^{2}H_{5}$]-OTA as internal standard. The synthesis of labelled OTA was accomplished by acid hydrolysis of unlabelled OTA and subsequent coupling one of the products, ochratoxin α, to [$^{2}H_{5}$]-L-phenylalanine. The mycotoxin was quantified in foods by LC-tandem MS after extraction with buffers containing [$^{2}H_{5}$]-OTA and clean-up by immuno affinity chromatography or by solid phase extraction on silica. The method showed a sufficient sensitivity with a low detection and quantification limit of 0.5 and 1.4 µg/kg, respectively, and good precision in inter-assay studies showing a CV (n=3) of 3.6 %.

The analysis of certified reference materials resulted in a low bias of 2.1 % from the certified values and revealed excellent accuracy of the new method.

To prove the suitability of SIDA, OTA was quantified in a number of food samples and resulted mainly in not detectable OTA contents. However, three samples of raisins exceeded the legal limit of 10 µg/kg and highlighted the need for further controlling the contamination with the mycotoxin.

Key words: Electrospray mass spectrometry, Ochratoxin A; LC/MS/MS, Stable isotope dilution assay

1 **INTRODUCTION**

2 The mycotoxin ochratoxin A (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3*R*-
3 methylisocoumarin-L-β-phenylalanine, OTA) is produced by several species of the fungal
4 genera *Penicillium* (e.g. *veridicatum*) and *Aspergillus* (e.g. *ochraceus*). In particular cereals
5 [1], coffee [2], grape products [3] and liquorice products [4] are frequently contaminated
6 with OTA due to inappropriate conditions during growth, storage and manufacture of the
7 raw material and subsequent invasion by the beforementioned moulds.

8 As OTA is a potent hepato- and nephrotoxin [5] and is clearly associated with a kidney
9 disease referred to as Balkan Endemic Nephropathy [6], there is general consensus that
10 contamination of foods has to be controlled thoroughly. Food intake calculations and
11 surveys of blood plasma concentrations in some European countries revealed that at least
12 one third of the acceptable daily intake (ADI) [7] is covered by the mean intake in countries
13 such as Sweden or Germany [8;9]. Therefore, the European Union has set OTA limits for
14 cereal products, cereals and raisins of 3 µg/kg, 5 µg/kg and 10 µg/kg, respectively [10].

15 The most frequently used methods to analyze OTA in foods are HPLC with fluorescence
16 detection (LC/FD) and enzyme-linked immunosorbent assays (ELISA) [11]. Whereas the
17 latter are mainly suited for screening purposes, validation studies revealed that LC/FD
18 suffers from several constraints. In a collaborative study of OTA quantitation in pig liver
19 recovery values differed widely between 43 and 128 % [12]. Similarly, for barley [13] and
20 wheat bran [14] low recoveries of 56 % and 70 %, respectively, were found.

21 Recently we reported on the excellent accuracy of stable isotope dilution assays (SIDA) for
22 quantification of the mycotoxin patulin [15] or the vitamins of the folate group [16] by
23 employing isotopomers of the analytes as internal standards. This enabled an optimal

1 compensation for losses of the analytes in all analytical steps. The aim of the current study
2 was, therefore, to develop a SIDA for OTA and to verify the accuracy of the new method
3 by analyzing standard reference materials.

4

5 ## MATERIALS AND METHODS

6

7 ### Materials and reagents

8

9 The following compounds were obtained commercially from the sources given in
10 parentheses: acetic acid, acetonitrile, ethyl acetate, formic acid, n-hexane, hydrochloric
11 acid, methanol, L-phenylalanine, toluene, trifluoracetic acid (Merck, Darmstadt; Germany);
12 chloroform, L-phenylalanine methylester, thionyl chloride (Aldrich, Steinheim,Germany);
13 $[^2H_5]$-L-phenylalanine (CDN isotopes, Quebec, Canada); Sep-Pak C18 Cartridges, Sep-
14 Pak Silica Cartridges (Waters, Eschborn, Germany); Mycosep OTA Cartridges; Ochraprep
15 Cartridges (Coring Systems Diagnostik, Gernsheim, Germany). Crystalline Ochratoxin A
16 was purchased from Sigma (Deisenhofen, Germany). Two certified reference materials
17 CRM 471 (wheat flour blank) and CRM 472 (wheat flour contaminated) were obtained from
18 the Community Bureau of Reference of the European Commission (Standard,
19 Measurement & Testing Programme, Brussels, Belgium).

20 All solvents were of gradient quality. The food samples were purchased from local
21 markets.

22 PBS-buffer (pH 7,3) was prepared by dissolving 2.9 g Na_2HPO_4, 0.2 g KH_2PO_4, 8 g NaCl,
23 0.2 KCl in 1 L water and adjusting the solution with HCl to pH 7.3.

1 Synthesis of [$^2H_5$]-OTA

2

3 Deuterated OTA was prepared by hydrolyzing unlabelled OTA and coupling one of the
4 hydrolysis products to [$^2H_5$]-L-phenylalanine using a modified procedure by van der Merwe
5 et al. [17], Steyn and Holzapfel [18] and Rousseau et al. [19].

6 **Ochratoxin α (2).** Ochratoxin A (**1**; 15 mg, 37.1 µmol) was suspended in aqueous
7 hydrochloric acid (18 mol/L, 50 ml) and refluxed for 48 h in an atmosphere of nitrogen. The
8 homogeneous mixture was then cooled to room temperature and extracted with chloroform
9 (3 x 20 mL). After drying the organic phase over anhydrous Na_2SO_4, removal of the
10 solvent gave 5-chloro-3,4-dihydro-8-hydroxy-3-methylisocoumarin-7-carboxylic acid
11 (Ochratoxin α **2**; 9.5 mg, 37.0 µmol).
12 Positive APCI-MS: *m/z (%)*= 257 (100), 239 (80), 259 (40), 241 (35).

13

14 **[$^2H_5$]-L-phenylalanine methyl ester (4)**. [$^2H_5$]-L-phenylalanine (**3**; 100 mg, 588 µmol) was
15 dissolved in a mixture of thionyl chloride in methanol (10%, 100 mL), then cooled to 0 °C
16 for 1h and subsequently kept for another 24 h at room temperature. Rotary evaporation of
17 the solution at room temperature gave the title compound as a white solid (90 mg, 489
18 µmol).
19 Positive APCI-MS: *m/z (%)*= 309 (100), 185 (25), 370 (20)

20

21 **[$^2H_5$]-Ochratoxin A methyl ester (5).** Ochratoxin α (**2**; 5 mg, 19.5 µmol) was dissolved in
22 thionyl chloride (8 mL) and heated under reflux for 2 hr. After evaporating the thionyl
23 chloride under reduced pressure, the resulting Ochratoxin α chloride was taken up in dry
24 pyridine (1 ml) and cooled to 0 °C. [$^2H_5$]-L-phenylalanine methylester (25 mg) in dry

1 pyridine (0.5 mL) was slowly added to the mixture and left at room temperature for 4 h.
2 Subsequently, water (15 mL) was added to the mixture, which was then extracted with
3 chloroform (3 x 10 mL). After washing the organic phase successively with hydrochloric
4 acid (2 mol/L, 2 x 10 mL), aqueous sodium hydrogen carbonate (0.1 mol/L, 2 x 10 mL) and
5 water (2 x 10 mL), the organic layer was dried with $CaCl_2$. Rotary evaporation gave **5** as a
6 solid (1 mg, 2.4 µmol).

7 Positive ESI-MS: *m/z (%)*= 384 (100), 423 (65), 386 (27), 424 (20), 425 (17), 257 (16), 363
8 (14)

9

10 **[$^2H_5$]-Ochratoxin A (6).** [$^2H_5$]-Ochratoxin A methyl ester (**5**; 500 µg, 1.2 µmol) was
11 dissolved in methanol (1.5 ml) and stirred with NaOH (0.8 ml, 1mol/L) at room temperature
12 for 2 h. The mixture was then acidified with aqueous hydrochloric acid (2 mol/L) to pH 3-4
13 and extracted with chloroform (3 x 10 mL). The organic phase was dried over $CaCl_2$,
14 concentrated *in vacuo* to 2 mL and purified by preparative LC/FD.
15 Purification of the [$^2H_5$]-OTA (**6**) was accomplished by injecting 100 µL of the raw solution
16 on a Nucleosil RP18 column (250 x 10 mm i. d., 5 µm, Macherey-Nagel, Düren, Germany)
17 eluted with a mobile phase consisting of variable mixtures of aqueous formic acid (0.1 %,
18 solvent A) and acetonitrile (solvent B). The gradient started at 0 % B and was programmed
19 within 20 min to 70 % B. Then, the content of B was raised to 100 % within 2 min and
20 maintained for further 8 min before being brought back to the initial mixture. The eluting
21 [$^2H_5$]-OTA peak was detected by fluorescence detection (excitation 333 nm, emission 460
22 nm) and pooled from 20 runs. The pooled purified solution was rotary evaporated to
23 dryness and gave [$^2H_5$]-OTA as a white solid (197 µg, 0.488 µmol, 95 % purity by HPLC-
24 UV).

25 Positive ESI-MS: *m/z (%)*= 409 (100), 257 (58), 411 (40), 239 (37)

Preparation and Determination of the Concentration of Standard Solutions

Stock solutions were prepared by dissolving OTA (labelled as well as unlabelled) in methanol. Concentration of OTA was determined by UV spectrometry at 333 nm using the molar extinction coefficient 5550 M^{-1} cm^{-1} reported by Humpf [20]. The UV spectrometer U-2000 (Hitachi, Berks, GB) was calibrated using potassium dichromate [21]. The stock solutions were checked spectrophotometrically revealing stability of OTA at –18 °C over a period of several months.

Sample preparation and clean-up

Solid phase extraction (SPE) on silica

SPE on silica was performed as detailed in the official collection of test methods according to article 35 of the German food law [22]. Briefly, samples (20 g or 20 ml) were mixed with aqueous hydrochloric acid (2 mol/L, 30 mL), aqueous $MgCl_2$ (0.4 mol/L, 50 mL) and toluene (100 mL) containing [$^2H_5$]-OTA (100 ng). After stirring for 1 h at room temperature, the mixture was centrifugated and the supernatant organic phase was subjected to SPE using an 12-port vacuum manifold (Alltech, Bad Segeberg, Germany). The SPE-cartridge (Sep-pak Vac RC Silica, 500 mg, Waters, Milford, MA, USA) was preconditioned with toluene (10 ml), then the sample extract (50 mL) was applied and the cartridge was washed with n-hexane (20 mL), toluene/acetone (95+5, v/v, 20 mL) and toluene (5 mL). Finally, Ochratoxin A was eluted with a mixture of toluene and acetic acid (9+1, v/v, 20 mL).

Extraction and immuno affinity (IA) clean-up

Solid samples (20 g) were suspended in aqueous sodium carbonate (200 ml, 1 %)

containing [$^{2}H_{5}$]-OTA (100 ng) and stirred for 30 min. Subsequently, the mixtures were filtered and the filtrate (60 ml) was passed through the IA column (Ochraprep P13B, Rhone Diagnostics, Glasgow, Scotland). Liquid samples (60 mL) were diluted with PBS-buffer pH 7.3 (60 mL) before application on the IA column. After application of the extract (60 mL), the SPE cartridge was washed with aqueous methanol (20%) and OTA was eluted with methanol/acetic acid (98+2 v+v, 1.5 mL) and water (1.5 mL). The solvent was evaporated *in vacuo* and the residue taken up in methanol (250 µL).

Each sample was analysed in triplicate by LC/MS-MS as described below.

LC/MS/MS

The samples (50 - 100 µL) were analyzed on a spectra series HPLC system (Thermo Separation Products, San Jose, CA, USA) equipped with an Aqua C-18 reversed phase column (250 x 4.6 mm; 5 µm, Phenomenex, Aschaffenburg, Germany) coupled to an UV-Detector and an LCQ ion-trap mass spectrometer (Finnigan MAT, Bremen, Germany).

The mobile phase consisted of variable mixtures of trifluoroacetic acid in water (0.05 %; solvent A) and trifluoroacetic acid in methanol (0.05 %: solvent B), at a flow of 0.8 mL/min. Gradient elution started at 60 % B maintained for 2 min, followed by raising the concentration of B linearly to 100 % within 4 min. After maintaining these conditions for 5 min the concentration of B was brought back within 4 min to the initial mixture and the column equilibrated for 1 min.

To ensure an adequate spray stability, the column effluent was diverted to waste during the first 8 min of the gradient programme. The mass spectrometer was operated in the positive electrospray mode using selected-reaction monitoring (SRM) with the mass transitions (*m/z* precursor ion/ *m/z* product ion) 404/358 for OTA and 409/363 [$^{2}H_{5}$]–OTA,

1 respectively. The spray voltage was set to 5.0 kV, the capillary temperature to 200°C and
2 the capillary voltage to 32.0 V. The maximum ionization time was set to 50 ms and the
3 MS-MS transition was measured using 3 microscans in order to obtain reproducible peak
4 areas. For maximum sensitivity the isolation width of the parent ion was adjusted to 1 Da
5 and the isolation width of the product ion was set to 1 Da in order to detect the product ion
6 most selectively. The sheath and auxiliary gas flow rates were set to 80 % and 20 % of
7 their maximum flow rates, respectively.

8 Flow injection analysis was performed by injecting pure solutions of the compounds to be
9 analyzed into the MS at a flow of 8 µl/min.

10 Atmospheric Pressure ionisation (APCI) was performed by using the APCI interface of the
11 LCQ ion-trap mass spectrometer. The temperature of the vaporizer tube was 450 °C and
12 the corona discharge needle was supplied with a voltage of - 3 kV, the discharge current
13 was 5 µA. The capillary temperature was 150 °C and the capillary voltage - 4 V. The
14 nitrogen flows were 57 % and 43 % of their maximum flow rates for sheath and auxillary
15 gas, respectively.

16

17 Calibration and Quantitation

18 Solutions of unlabelled and labelled OTA were mixed in nine mass ratios ranging from
19 0.11 to 9 to give a total OTA concentration of 0.5 µg / mL. LC/MS-MS analysis of each
20 mixture (20 µL) was performed in triplicate as outlined before. The calibration curve was
21 constructed from these results and revealed a linear response of the peak area ratios to
22 the mass ratios of unlabelled to labelled OTA between the mass ratios 0.2 and 9. The
23 equation for the regression line was $y = 0.960 \bullet x + 0.053$ ($r^2 = 0.9996$), where x is the
24 peak area ratio in the trace MS/MS 404/358 to that in the trace MS/MS 409/363 and y is
25 the mass ratio of unlabelled to labelled OTA.

1 Contents C of OTA in foods were computed using the following equation

2 $$C = (A_{OTA} / A_{d\text{-}OTA} \bullet 0.960 + 0.053) \bullet m_{d\text{-}OTA}$$

3

4 where A_{OTA} is the area of unlabeled OTA in trace MS/MS 404/358; $A_{d\text{-}OTA}$ is the area of
5 labeled OTA in trace MS/MS 409/363; $m_{d\text{-}OTA}$ is the amount of added labeled OTA.

6

7 Detection and Quantitation Limits

8 Detection (DL) and quantitation limits (QL) were determined using a wheat flour devoid of
9 OTA. The following amounts of OTA (unlabelled as well as labelled compounds) were
10 added: 0.5, 1.0, 3.0 and 5 µg/kg. Extraction and SPE sample clean-up was continued and
11 LC/MS-MS analysis was conducted as outlined above. Each addition assay was
12 performed in triplicate and DLs as well as QLs were calculated according to Hädrich and
13 Vogelgesang [23]. In short, a calibration graph of measured versus added OTA amounts
14 was plotted and both the lower and the upper 95 % confidence intervals were included.
15 Considering this graph, DL is the concentration calculated from the maximum height of the
16 95 % confidence interval at the zero addition level. QL is the addition level for which the
17 lower 95 % confidence limit equals the upper 95 % confidence limit of the addition level at
18 the DL [24].

19

20 Stability of Deuterium Labelled Standards to Protium-Deuterium Exchange

21 [$^{2}H_{5}$]-OTA was stirred for 30 min in aqueous sodium carbonate (200 ml, 1 %) and
22 subjected to IA clean-up and LC/MS-MS as detailed above.

1

2 ## RESULTS AND DISCUSSION

3 ### Synthesis of isotopomeric ochratoxin A

4 In the past there have been two attempts to synthesize radioactively labelled OTA to be
5 used in metabolic studies [19; 25]. Both approaches consisted of a metathesis by
6 hydrolyzing OTA and subsequent coupling of the resulting isocoumarin derivative
7 ochratoxin α (OTα) to labelled L-phenylalanine. Following the route reported by Rousseau
8 et al. [19], we transformed ochratoxin α to NHS- OTα and attempted to purify the latter
9 intermediate by HPLC. However, NHS- OTα only appeared in minor amounts, which made
10 the isolation ineffective. Therefore, we chose the acid chloride method to activate OTα. As
11 already described by Steyn and Holzapfel [18], OTα was converted by addition of thionyl
12 chloride into OTα chloride which was then reacted with [$^2H_5$]-phenylalanine methyl ester,
13 hydrolysis of which in sodium hydroxide provided [$^2H_5$]-OTA in a total yield of 4.9 %. A
14 survey of the complete synthetic route is displayed in fig. 1.

15 Flow injection electrospray mass analysis of the synthesized material shown in fig. 2
16 revealed an isotopic purity of 99.7 % and a shift of the molecular mass of 5 dalton
17 corresponding to the introduction of 5 deuteriums by using labelled phenylalanine as
18 reactant. This mass shift was also apparent in collision-induced dissociation (CID)
19 experiments on the respective protonated molecule. As evident from fig. 3, the MS-MS
20 spectrum revealed a conceivable signal corresponding to a loss of formic acid from $[M+1]^+$.

1 **LC/MS/MS**

2 Separation of OTA from main interferences in food samples was achieved on a RP-18
3 column at a gradient consisting of variable mixtures of methanolic trifluoroacetic and
4 aqueous trifluoroacetic acid. LC/MS of standard solutions of mixtures of the isotopomeric
5 OTAs revealed suitable peak shapes and enabled to differentiate unlabelled OTA from its
6 labelled analogue by monitoring the mass traces of $[M+1]^+$at m/z 404 and m/z 409,
7 respectively. Analoguously, the isotopomers could be distinguished in the LC/MS/MS
8 mode by monitoring the ions resulting from the loss of formic acid from the respective
9 protonated molecules.

10 **Calibration**

11 To enable calculation of mass ratios from intensity ratios of OTA isotopomers in their
12 respective mass traces, a calibration function was determined by analyzing mixtures of
13 OTA and $[^2H_5]$-OTA standard solutions the mass ratios of which ranging between 1: 9 and
14 9:1. Plotting the area ratios against the mass ratios revealed a linear calibration function
15 with the respective equation showing a r^2 of 0.9996. This behaviour was expected, as the
16 labelled OTA material is nearly devoid of unlabelled OTA residues and no spectral overlap
17 due to natural isotopomers in unlabelled OTA is likely to occur as the mass shift between
18 the isotopomers is as high as 5 dalton. Linearity and identical response factors were
19 observed in LC/ single stage MS and LC/ tandem MS mode, as well.

20 **Sample Purification**

21 According to the literature, sample clean-up can be achieved by the following methods:
22 solid phase extraction (SPE) either on (a) silica or on (b) reversed-phase cartridges, or (c)

1 on anion exchange columns or (d) by immuno affinity chromatography. In preliminary
2 studies reversed-phase cartridges revealed low recovery and the anion exchange extracts
3 showed significant interferences during LC/MS/MS. Therefore, a direct comparison only
4 between silica cartridges and IAC was carried out using the matrixes wheat, coffee and
5 red wine.

6 In case of wheat and red wine, performance of silica and IA clean-up was quite similar.
7 However, regarding the silica extracts of coffee, the peaks of isotopomeric OTAs were
8 obscured by background compounds as shown in fig 4. In contrast to this, the IA extracts
9 were devoid of interferences and displayed a well shaped and clearly separated [$^{2}H_{5}$]-OTA
10 peak (fig. 5). It can, therefore, be assumed that IA chromatography is the most effective
11 clean-up procedure in OTA analysis.

12 Stability of Deuterium Labelled Standards to Protium-Deuterium 13 Exchange

14 As the labelled OTA contained five deuteriums, a protium-deuterium (H-D) exchange
15 during the course of analysis would result in systematic errors during quantification. In
16 order to exclude H-D-exchange, labelled OTA was stirred in extraction buffer and passed
17 through IAC. The resulting eluate then was analysed by LC/MS/MS and compared with the
18 untreated [$^{2}H_{5}$]-OTA solution, which contained 0.5 % unlabelled material. After sample
19 treatment, the degree of unlabelled material averaged at 0.6 %, which proved that no H-D-
20 exchange did occur.

21 Limits of Detection and Quantification

22 As we detailed in case of SIDA development for the vitamins of the folate goup [16] and
23 pantothenic acid [24] as well as for the mycotoxin patulin [15], the method proposed by

1 Hädrich and Vogelgesang [23] is best suited to consider (i) losses during extraction and
2 clean-up, (ii) background noise due to matrix interferences and (iii) data scattering in low
3 concentration ranges for the determination of detection (DL) and quantitation limits (QL).

4 In analogy, we calculated the DL from the confidence interval of a calibration line prepared
5 by spiking wheat flour devoid of OTA with variable amounts of the analyte.

6 Addition experiments revealed a DL of 0.5 and a QL of 1.4 µg/kg for OTA. These data
7 proved the SIDAs to be sensitive enough to quantify OTA contents even below the EU
8 limits for foods. Moreover, the DL of SIDA was in the same order of magnitude as those of
9 LC/FD methods ranging between 0.04 and 0.9 µg /kg [26]. As sample size for liquids may
10 exceed multiply that of solids, the DL for liquid samples is even lower and can be
11 estimated to 0.1 µg/kg.

12 Accuracy

13 **Trueness.** To check trueness of SIDA, two certified reference materials (CRM) from the
14 Community Bureau of Reference (BCR) of the European Commission were analyzed. The
15 CRMs consisted of wheat flour, one of which was certified to contain 8.2 ± 1.0 (CRM 472)
16 and in the other of which the OTA content was certified to be below the detection limit of
17 0.6 µg/kg (CRM 471). These data had been calculated from the results of nine European
18 laboratories using LC/ fluorescence detection and fulfilling the performance criteria
19 reported by Wood et al. [26].
20 SIDA of the reference materials resulted in an OTA content of 8.0 ± 0.3 for CRM 472, the
21 MS-MS chromatogram of which is displayed in fig. 6. In CRM 471, no OTA was detected
22 above the detection limit of 0.5 µg/kg as mean of triplicate analyses. Thus, the bias from

the certified reference value of CRM 472 was as low as 2.1 % and proved the trueness of the presented method.

Precision and Recovery. Inter-assay precision was evaluated by extracting CRM 472 three times within two weeks and revealed a coefficient of variation of 3.58 %. Recovery was determined by adding unlabeled OTA to wheat flour devoid of the mycotoxin at an addition level of 3 µg/kg in triplicate and quantifying the OTA content by SIDA giving a value of 105.4%. The aforementioned validation data are summarized in table 1.

Quantification of OTA in food

Of those foods in which OTA is most likely to occur, wheat flour, coffee, liquorice, beer, wine, and some spices were quantified. For coffee, cleanup by IA columns was necessary, in the other samples unambiguous identification and quantification was achieved after SPE cleanup on silica.

The majority of foods analyzed did not contain OTA above its DL. However, in soluble coffee, OTA was detectable, but not quantifyable. Higher concentrations were found in mulled wine (n.d.-3.3 µg/kg), nutmeg powder (1.8 µg/kg), and raisins (n.d.-29.8 µg/kg). In the latter products, 8 out of 9 samples contained detectable contents of the mycotoxin, of which 3 samples (CV of each sample less than 3.2%) exceeded the legal limit of 10 µg/kg.

CONCLUSION

The validation data of the SIDA presented here revealed excellent accuracy and sensitivity of the new method for all analyzed samples.

Of all analysed foods, the majority contained OTA below DL. However, three samples of

raisins were found to exceed the legal limit and may, therefore, be a risk for consumers' health. As the survey is not representative due to low sample numbers, a broader survey would be necessary to evaluate the actual hazard due to OTA consumption.
Due to the three-dimensional specifity of LC/MS/MS, SIDA offers the perspective to be suited for clinical matrices, for which the alternative methods show discernable drawbacks. Therefore, a method comparison to the latter methodologies for matrices such as blood plasma or urine is under way.

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Table 1. Performance data of the stable isotope dilution assays (SIDA) for ochratoxin A based on the analyses of certified reference material CRM 472

Performance criterion	
Detection limit	0.5 µg/kg
Quantification limit	1.4 µg/kg
inter-assay coefficient of variation	3.6 %(n=3)
Certified OTA content of CRM 472	8.2 ± 1.0 µg/kg
Quantified OTA content of CRM 472	8.0 ± 0.3 µg/kg
Bias	2.1 %
Recovery (addition level 3 µg/kg) ± standard deviation	105.4 ± 3 %

1

2 ## LEGENDS TO THE FIGURES

3 **Figure 1.** Reaction scheme leading to $[^2H_5]$-Ochratoxin A (**6**)

4 **Figure 2.** LC-ESI(+)-mass spectrum of Ochratoxin A (above) and $[^2H_5]$-Ochratoxin A

5 (below).

6 **Figure 3.** LC-ESI(+)-MS/MS spectrum of Ochratoxin A (above) and $[^2H_5]$-Ochratoxin A

7 (below). Precursor ions were the protonated molecules.

8 **Figure 4.** LC-ESI(+)-MS/MS of a coffee extract not containing OTA after clean-up on a

9 silica SPE cartridge. The internal standard $[^2H_5]$-Ochratoxin A in trace MS/MS

10 409/363 is obscured by matrix interferences, unlabelled Ochratoxin A in trace

11 MS/MS 404/358 cannot be unambiguously confirmed or excluded.

12 **Figure 5.** LC-ESI(+)-MS/MS of a coffee extract not containing OTA after clean-up by

13 immuno affinity chromatography. The internal standard $[^2H_5]$-Ochratoxin A is

14 unambiguously detected in trace MS/MS 409/363, unlabelled Ochratoxin A in

15 trace MS/MS 404/358 is not present.

16 **Figure 6.** LC-ESI(+)-MS/MS of certified reference material CRM 472 wheat flour after

17 clean-up on a immuno affinity cartridge.

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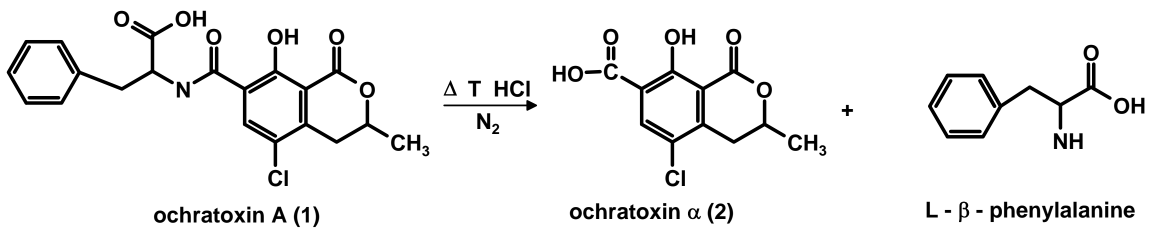

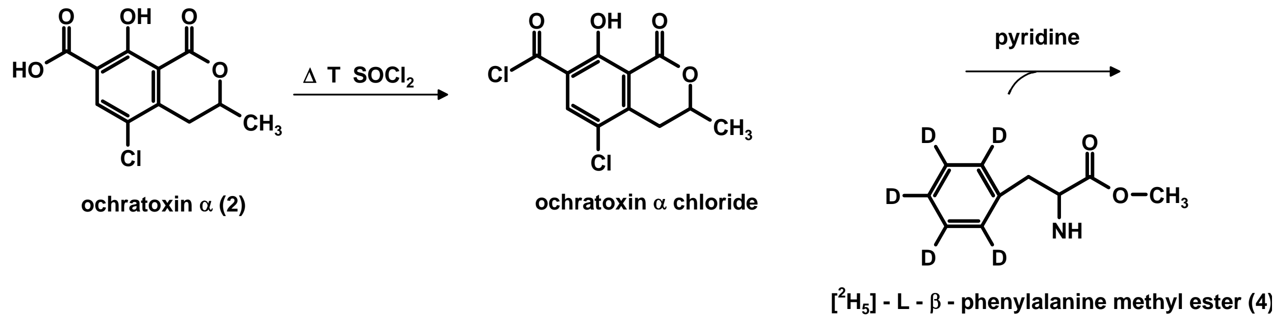

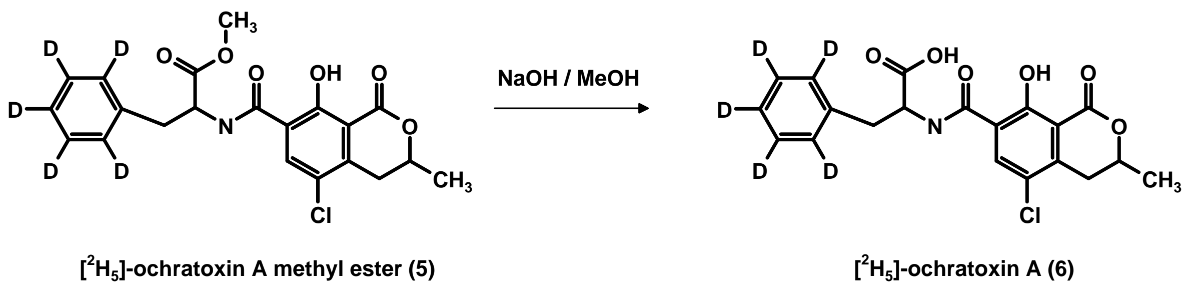

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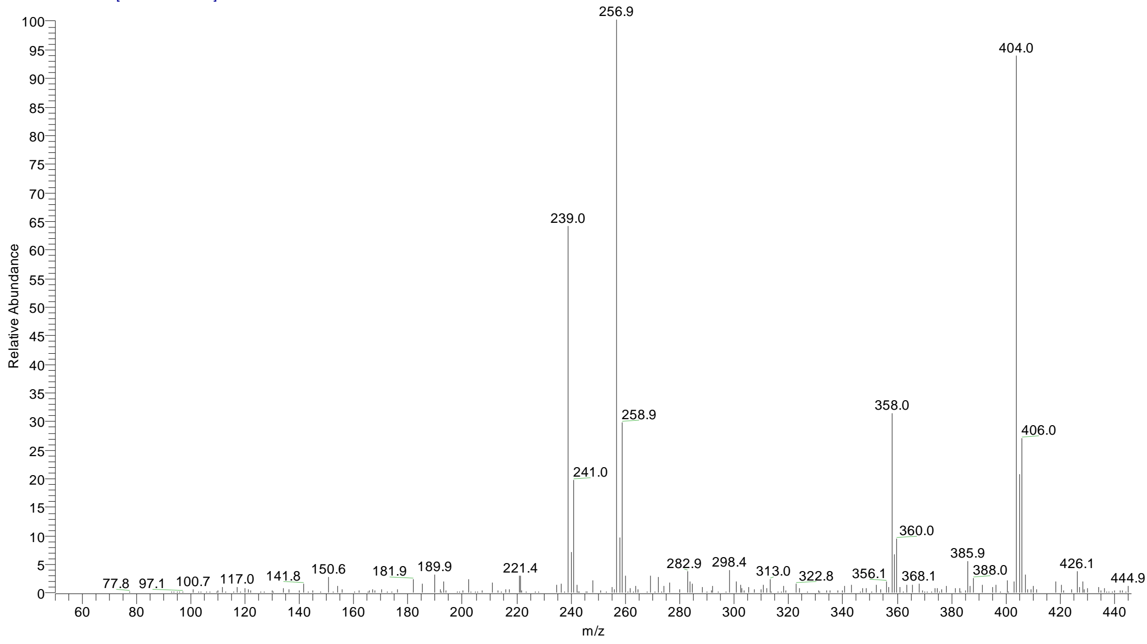
6 Figure 1



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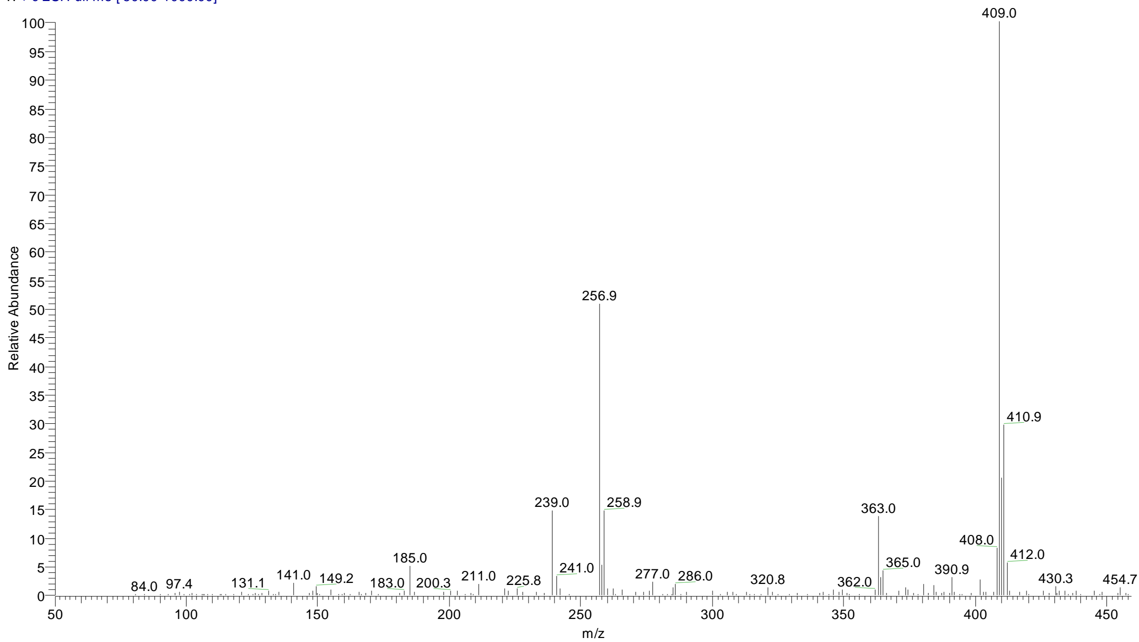

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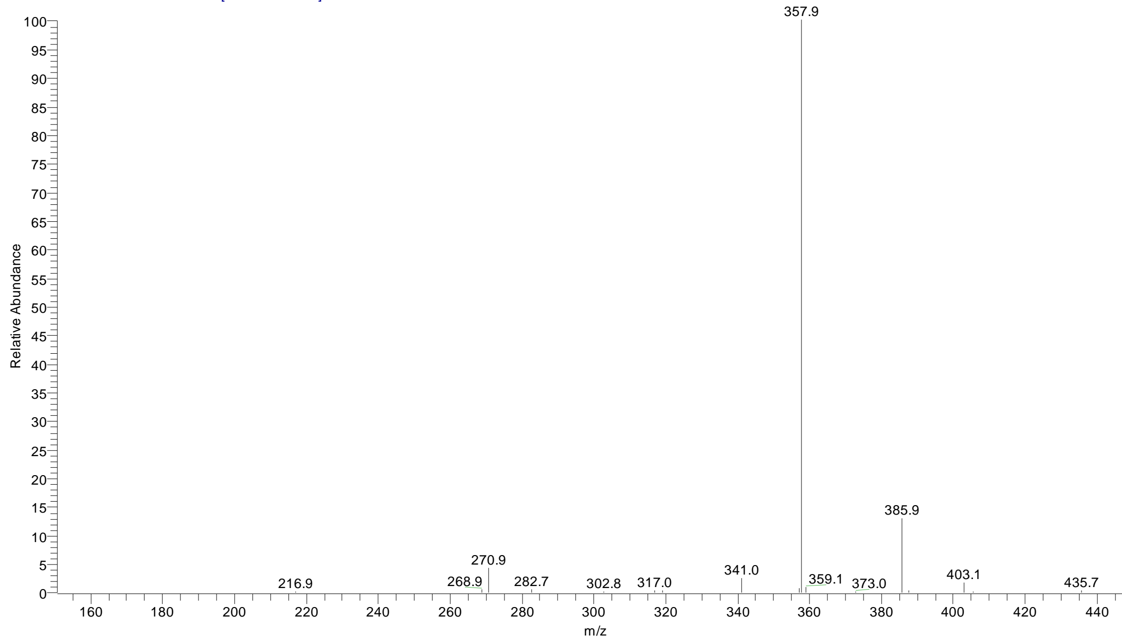

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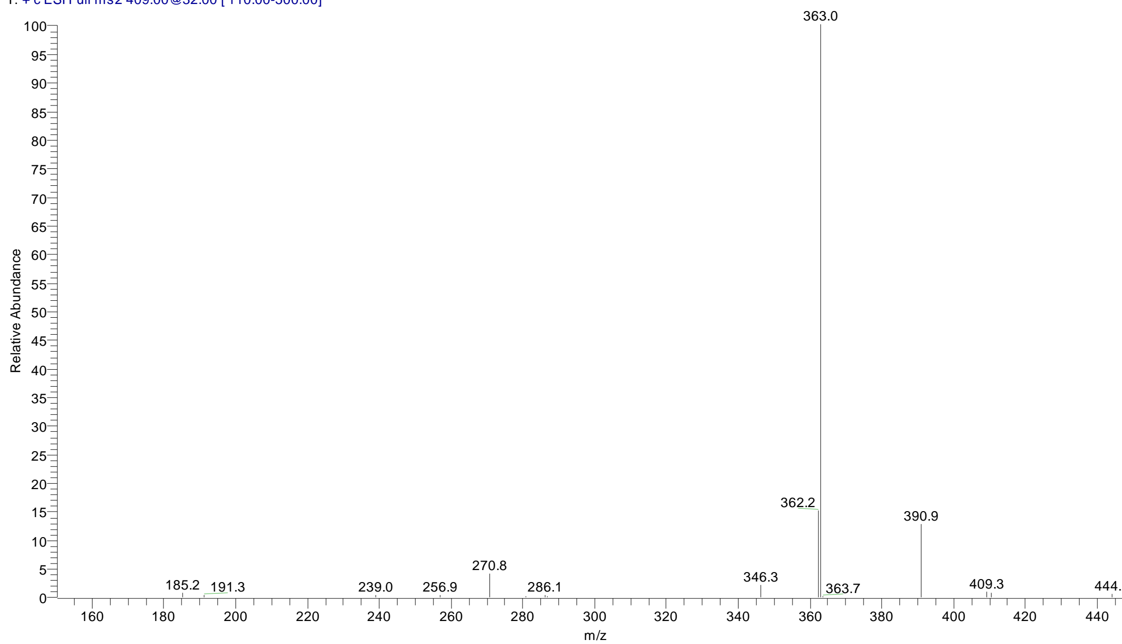

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4 Figure 3

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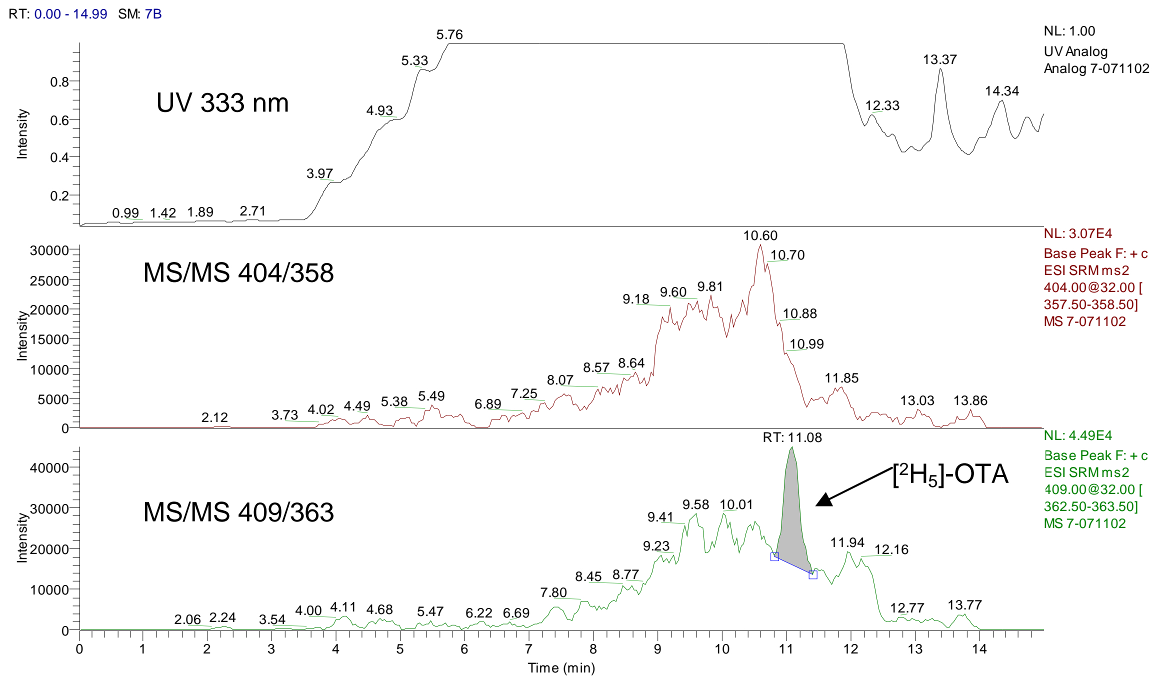

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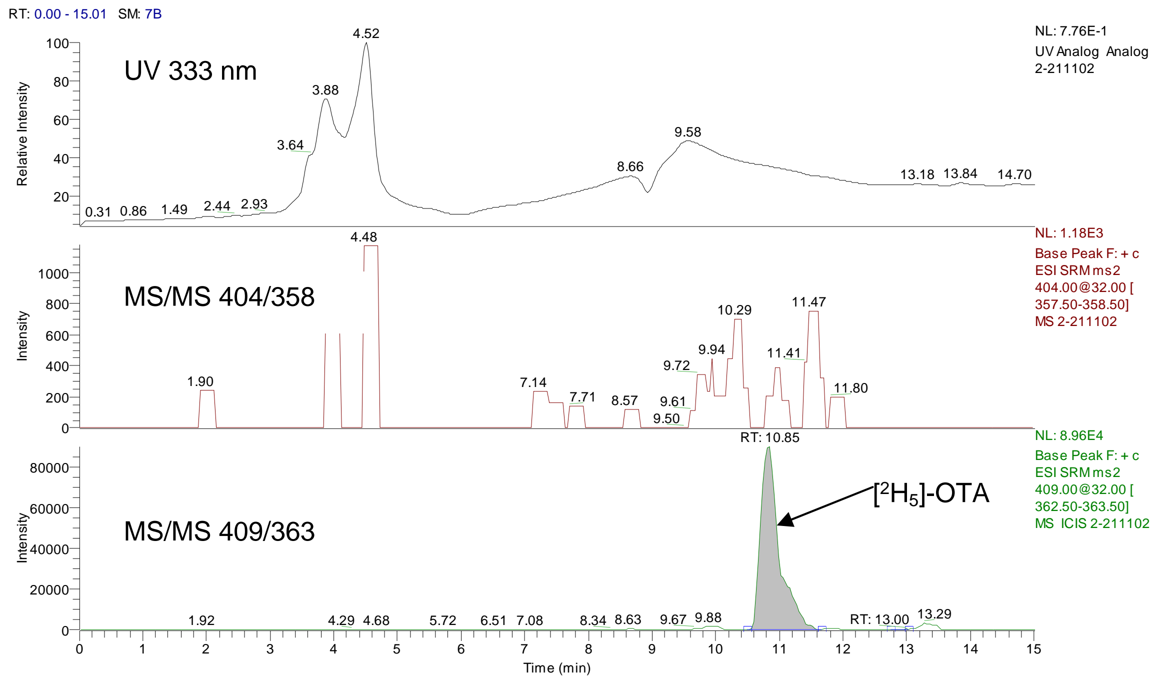
3 Figure 4



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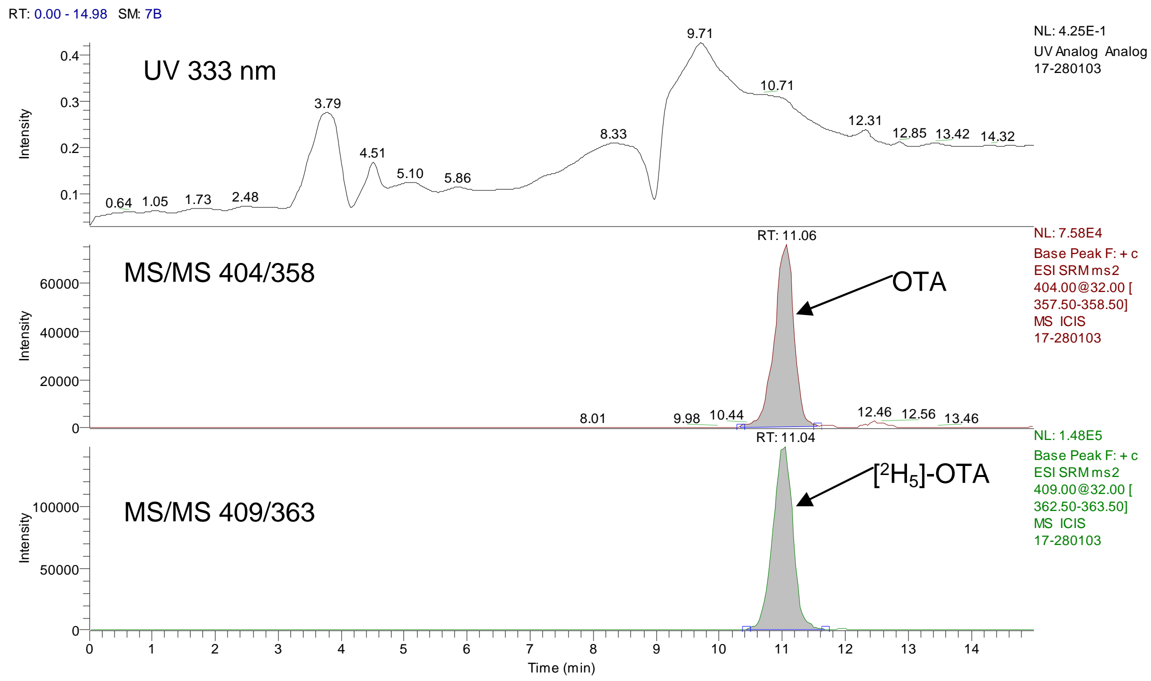
2 Figure 5



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2 Figure 6