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Stable isotope dilution assays in mycotoxin analysis

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ABSTRACT

The principle and applications of stable isotope dilution assays (SIDAs) in mycotoxin analysis is critically reviewed. The general section includes historical aspects of SIDAs, the prerequisites and limitations of the use of stable isotopically labelled internal standards along with possible calibrations procedures. In the application section actual SIDAs for the analysis of trichothecenes, zearalenone, fumonisins, patulin and ochratoxin A are presented. The syntheses and availability of labelled mycotoxins for the use as internal standards is reviewed and specific advances in food analysis and toxicology are demonstrated. The review indicates that in particular LC-MS applications require the use of stable isotopically labelled standards to compensate for losses during clean-up and for discrimination due to ion suppression. As the commercial availability of these compounds continues to advance, SIDAs can be expected to find expanding use in mycotoxin analysis.

Key words: LC-MS/MS, Mycotoxins, Ochratoxin A, Patulin, Stable isotope dilution assay; Trichothecenes

INTRODUCTION: PRINCIPLE OF STABLE ISOTOPE DILUTION ASSAYS

General Remarks

The roots of stable isotope dilution assays (SIDAs) date back to the beginning of the 20th century as Soddy [1] discovered the existence of isotopes and George Hevesy used radioactive isotopes to determine the content of lead in rocks and the solubility of lead salts in water [2]. Most elements have a natural distribution of both stable and radioactive isotopes. For example, natural carbon consists of C-12 (98 %), of C-13 (1.1 %), and of radioactive C-14. For organic molecules of known empirical formula the natural isotopic distribution of the entire compound can be calculated from the relative frequency of the individual elements.

Although being very similar in their properties, isotopes can be enriched or depleted due to their different masses leading to an alteration of the natural values. If an element or compound showing a natural isotopic distribution is mixed with such an isotopically different material (fig. 1), the naturally abundant isotopes hence are diluted in the resulting material – which is the origin of the term “dilution” in SIDA. The principle of SIDA is comprehensively sketched in fig. 2. After addition of the labelled standard and its equilibration with the analyte, the ratio of the isotopologues is stable due to their nearly identical chemical and physical properties. Final mass spectrometry allows differentiation between the isotopologues and with the known amount of the internal standard the content of the analyte can be calculated. In contrast to this, a structurally different internal standard may be discriminated against and, therefore, cause systematic errors and imprecision. In other words, losses of the analyte are completely compensated for by identical losses of the isotopologue, whereas the structurally different IS is likely to show different losses and, therefore, is less suited.

Benefits and limitations of using an isotopologic internal standard

As shown above, the ideal compensation for losses renders SIDA a perfect tool for a series of analytical applications. Generally, trace compounds are a typical target for SIDAs. Trace analyses often require tedious clean-up procedure due to matrix interferences, which usually causes losses of the analyte. By using structurally different internal standards, additional recovery and spiking experiments are necessary, which further increase the work-load. But often recoveries are not reproducible, which is an additional cause of imprecision. In all these cases, SIDA offers significant benefits.

Apart from rendering quantitative results accurately by compensation for losses, the use of an isotopologic standard enhances specifity of the determination. In addition to the specific mass spectrometric information on the analyte the coeluting internal standard shows analoguous signals shifted by the introduced mass increment. Therefore, the chromatogram of a SIDA showing the coeluting peaks in the respective mass traces ensures the correct assignment of the analyte (fig 3).

The major prerequisites for analyzing a given mycotoxin by SIDA are the availability of a GC-MS or LC-MS combination and of the labelled internal standard. As the former are basic equipments of a laboratory nowadays, the latter, however, imposes the biggest obstacle for a wider use of SIDA. Of all mycotoxins discovered to date, about 25 have been synthesized as labelled analogues, but only 11 of these are commercially available. The consequence is that the compound aimed at often has to be synthesized. A major psychological hindrance that prevents analysts from synthesizing labelled mycotoxins is the price of labelled educts. However, this is not a convincing argument as can be explained by the following example: one gram of a labelled educt may cost around € 1000 and the yield of the synthesis may be 10 %, which both are realistic figures. Then, the price for 100 mg of the labelled product is € 1000. As for a SIDA only 1 to 10 µg of the labelled standard is

required, 100 mg of the standard enables to perform at least 10000 analyses. Hence the material costs for using a labelled standard would be around € 0.10 per sample, which is negligible compared to the cost of labour and equipment.

Prerequisites for isotopologic standards

As mentioned above, the basic principle of SIDA is to transfer the concentration of the analyte into an isotopologue ratio, which has to be stable during successive analytical steps. Therefore, a stable labelling is the first prerequisite for an internal standard. As carbon-carbon and carbon-nitrogen bonds are very unlikely to be cleaved, labels consisting of [^{13}C] or [^{15}N] are considered to be very stable. On the contrary, losses of [^{18}O] or [^{2}H] can occur, if these labels are at labile positions. On the one hand, [^{18}O] in carboxyl moieties can be exchanged in acid or basic solutions. This was shown by Boni et al. [4], who observed loss of the [^{18}O] label in trypthophan heated in aqueous solutions. On the other hand, deuterium is susceptible to the so-called protium-deuterium exchange if it is activated by adjacent carbonyl groups or aromatic systems. Consequently, mycotoxins containing only labile hydrogens, such as patulin, are not suited as internal standards when carrying deuterium labels.

Another labile labelling position is the trimethylsilyl (TMS) group as demonstrated by Price [5], who added [$^{2}H_9$]-TMS-patulin as an internal standard for patulin to the extract of apple juice. During the derivatization procedure the [$^{2}H_9$]-TMS groups of the internal standard and the [$^{1}H_9$]-TMS groups of the silylating agent exchanged and, therefore, made an exact quantification impossible.

Another difficulty for a stable isotopologue ratio arises from small differences in physical or chemical properties of the isotopologues, commonly referred to as isotope effects (IE). IE are due to different energy contents that are caused by the mass differences of the

isotopes. IE are mainly observable in case of hydrogen, as the mass difference between [^{1}H] and [^{2}H] is proportionally much higher than between [^{13}C] and [^{12}C] or [^{15}N] and [^{14}N]. The well-known and frequently occurring IE influence the chromatographic behaviour and results in different retention time of the isotopolgues in general.

In order to prevent chromatographic separations of the isotopologues during clean-up and thus changes in the isotopologue ratio, isotope effects have to be minimized either by chosing labellings with [^{13}C] or [^{15}N] or by introducing only the necessary number of [^{2}H].

As quantification by means of mass spectrometry is intended, the standard has to be unequivocally distinguishable from the analyte. This requires the presence of signals either of the molecular ion or of fragments showing the mass increment introduced by the labels. Therefore, a loss of the label from the detected signals has to be avoided.

Another problem in isotopologue differentiation arises from spectral overlaps between standard and analyte. In case of the analyte, the natural abundance of isotopes, in particular of [^{13}C], [^{18}O] or [^{34}S] results in isotope clusters of each fragment showing not only the nominal mass m_A, but also to a less extent m_A+1, m_A+2 or even higher masses. In particular [^{13}C] in compounds consisting of a higher number of carbons causes a significant abundance of m+1 and m+2 due to relatively high natural abundance. To avoid overlap of the signals of those natural isotopologues with m_S of the standard, the mass difference, therefore, has to be sufficient. For mycotoxins, which have carbon numbers usually ranging from 10 to 20, a mass increment of at least three units generally meets this requirement. However, the number of labels, especially of deuterium, should not be too high, in order to minimize the mentioned chromatographic isotope effect.

On the contrary, signals of the labelled material falling on the signals of the analyte may also result in spectral overlaps. This occurs, if the labelling is not complete due to low

isotopic purity of labelled educts or due to incomplete labelling in synthetic steps, e.g. during deuterations of aromatic systems.

If a spectral overlap cannot be avoided, calculation procedures have been developed to enable quantification after all. However, these procedures are the more complicated the more pronounced the overlap is.

Another important prerequisite for accurate quantification is the need of equilibration between analyte and standard in the sample to be extracted. As the labelled standard is added to the sample as a solution either in the extracting solvent or directly to the matrix itself, it will likely be recovered to a high extent during the extraction procedure. For the analyte, this might not be true, as it can be trapped in compartments of the matrix and less extractable by the solvent. Therefore, a suitable time has to be chosen, to enable equilibration of standard and analyte in all parts of the sample and to assure that analyte and standard show the same concentration ratio in all compartments as far as possible.

Calibration procedures

The amount of analyte present in the sample appears as the isotopologue ratio, which can be calculated from the intensity ratio of suitable ions measured by mass spectrometry. The relation between isotopologue ratio and intensity ratio has to be established by a series of calibration experiments that consist of analyzing the intensity ratios of defined standard analyte mixtures. If there is no spectral overlap, the calibration function is supposed to be linear. However, usually there are still unlabelled analytes in the labelled material and small intensities of natural isotopologues of the analyte falling at the signals of the standard. Therefore, the calibraton function is linear only in a restricted region, which is outlined in fig. 4. Under the supposition that the standard contains 2 % unlabelled material und natural isotopologues contribute 5 % intensity on the signal of the standard, the calibration function

can only be assumed as a straight line in molar ratios of analyte to standard ranging between 0.2 and 5. In excess of the standard or of the analyte the function is governed by the unlabelled residues in the standard or by the natural isotopologues of the analyte, respectively.

However, in certain cases a spectral overlap cannot be avoided and, therefore, suitable procedures for calculations are required. In general, there are several ways to solve this problem: for example, hyperbolic or polynomial models have been elaborated [6, 7], which approximate the real calibration relation by a mathematic function. As these procedures are rather complicated, several authors proposed linearization methods, which transfer the nonlinear function into a linear one [8]. An isotopologic mycotoxin showing high spectral overlap recently has been introduced by Bretz et al. [9], who demonstrated that the overlap in case of [$^{2}H_{1}$]-deoxynivalenol can be reduced by monitoring a specific MS fragmentation.

APPLICATIONS OF STABLE ISOTOPE DILUTION ASSAYS TO MYCOTOXINS

FUSARIUM TOXINS

The anamorphic genus *Fusarium* is a large group of moulds consisting of about 145 different species [10], which present a hazard to food safety as many of these fungi are plant pathogens and infect crops used for human nutrition. Their capability of producing mycotoxins, especially trichothecenes, zearalenones and fumonisines, was associated with severe food-borne and feed-borne intoxications in the past. In general, *Fusarium* toxins are produced basically on the field during growth of the plant, but wrong storage conditions including high moisture content can also lead to severe mycotoxin accumulation [11].

Trichothecenes

Trichothecenes are a group of tetracyclic sesquiterpenoids that share an epoxide functionality at C-12 and C-13 and a double bond at C-9 and C-10 in general. The great diversity of the trichothecene family is due to both different numbers and different positions of hydroxyl moieties at the basic trichothecene nucleus. As esterification of the hydroxyl groups is also possible with a number of different corresponding acids, up to now over 170 trichothecenes have been isolated from fungal cultures. In order to keep track of this large group of compounds the trichothecenes are classified into four groups A - D [12]. Whereas type C and the macrocyclic type D are of minor regard in food commodities, type A and type B trichothecenes are regularly found in cereal products [13]. The most common members of these two groups are shown in fig. 5.

Type A-Trichothecenes

Already in the late 1980s 3-[$^{2}H_{3}$]-acetyl-T2-toxin and 15-[$^{2}H_{3}$]-HT2-toxin were prepared chemically and used as internal standards for the determination of both toxins in human blood and animal urine samples [14]. Extracts were derivatized with trifluoracetic acid anhydride to enable gaschromatographic separation before tandem mass spectrometric detection. In blood samples the recovery of both components was about 90 % and the limit of detection (LOD) about 0.5 µg/kg. Though the latter authors mentioned that the method using stable isotope labelled standards is superior to that of the external calibration, no further use of isotopically labelled internal standards has been reported for type A-trichothecenes neither in physiological studies nor in food analysis for a long time.

As gas chromatographic analysis of trichothecenes is hampered by the inevitable derivatisation step and severe matrix interferences, alternative methods were needed for trace analysis and, as a consequence, liquid chromatography-mass spectrometry increasingly comes to the fore. One of the first methods for the simultaneous quantification

of type A-trichothecenes with single stage LC-MS [15] used deuterated T2-toxin as internal standard that was commercially available at that time. Recovery for T2-Toxin was only 77.4 %, anyhow, but 90 % for HT2-toxin, 86 % for diacetoxyscirpenol and 95 % for monoacetoxyscirpenol. Limits of quantification (LOQ) were 50 µg/kg for T2-toxin and diacetoxyscirpenol, 60 µg/kg for monoacetoxyscirpenol and 85 µg/kg for HT2-toxin. Recently the recovery values were further improved using carbon-13 labelled standards for T2-toxin, HT2-toxin, diacetoxyscirpenol and monoacetoxyscirpenol and LC with tandem MS detection [16]. With these standards recovery rates of about 112 – 127 % for T2-toxin, 93 – 107 % for HT2-toxin, 90 – 91 % for diacetoxyscirpenol, and 109 – 112 % for monoacetoxyscirpenol could be achieved depending on the spiking level. LODs and LOQs were determined using a statistical approach including additions of the toxins to suitable matrices [17]. These studies resulted in LODs of 3 µg/kg (T2-toxin), 30 µg/kg (HT2-toxin), 1 µg/kg (diacetoxyscirpenol) and 30 µg/kg (monoacetoxyscirpenol) and in LOQs of 8 µg/kg (T2-toxin), 80 µg/kg (HT2-toxin), 4 µg/kg (diacetoxyscirpenol), and 80 µg/kg (monoacetoxyscirpenol).

Type B-trichothecenes

A successful development of a stable isotope dilution assay of the most prevalent type B-trichothecene deoxynivalenol was hindered for a long time by the rather complicated molecular structure of this mycotoxin, which resisted chemical attempts to synthesize isotopically labelled derivates. Only just recently [$^{2}H_{1}$]-deoxynivalenol was synthesized and used in stable isotope dilution assays [9], together with [$^{2}H_{3}$]-3-acetyldeoxynivalenol that was synthesized by the same group before [18]. With this method recovery rates for deoxynivalenol were between 99 and 103 % and between 87 and 96 % for 3-acetyldeoxynivalenol using LC-MS/MS. LOQs and LODs were not determined. But the use of an internal standard, which differs only one mass unit from the analyte leads to spectral

overlap due to natural isotopic pattern. As already stated in the introduction section, special requirements are necessary both in terms of instrumental equipment and mathematic calculations for the suitability of this stable isotope labelled standard.

Broader application found a commercial fully carbon-13 labelled internal standard of deoxynivalenol. Without clean-up the recovery for deoxynivalenol was 95 ± 3 % in wheat and 99 ± 3 in maize with a lower LOQ of 8 µg/L [19]. When a clean-up with Mycosep®225 columns was performed, a recovery rate of 101 ± 2.4 was obtained in maize, whereas external calibration only results in 76 % ± 1.9 [20]. Both studies were performed using LC-MS/MS.

The commercial carbon-13 labelled standard for deoxynivalenol was also used in the first comprehensive stable isotope dilution LC-MS/MS assay for type B-trichothecenes along with carbon-13 labelled 3-acetyldeoxynivalenol, 15-acetyldeoxinvalenol and 4-acetylnivalenol (fusarenon X) that all were synthesized by the authors of this review [21]. Recovery rates were determined at different contamination levels and ranged between 97 and 100 % for deoxynivalenol, between 95 and 105 % for 3-acetyldeoxynivalenol, between 100 and 115 % for 15-acetyldeoxynivalenol and between 94 – 101 % for 4-acetylnivalenol. The LODs were 2 µg/kg for deoxynivalenol, 5 µg/kg for 3-acetyldeoxynivalenol, 4 µg/kg for 15-acetyldeoxynivalenol and 1 µg/kg for 4-acetylnivalenol. The LOQs were 6 µg/kg for deoxynivalenol, 16 µg/kg for 3-acetyldeoxynivalenol, 11 µg/kg for 15-acetyldeoxynivalenol and 4 µg/kg for 4-acetylnivalenol. A summary of performance data of the before mentioned SIDAs is presented in table 1.

Zearalenone

Some Fusarium species such as *F. graminearum* and *F. culmorum* produce a series of macrocyclic resorcylic acid lactones (fig. 6), of which only zearalenone is regularly found in naturally contaminated cereal grains [13]. All members of this group show characteristic

estrogenic effects that lead to severe problems in stock farming, especially infertility of swine when fed over a longer period [22]. The possibility of labelling zearalenone by proton-deuterium exchange is well known, but first was only used for NMR- and conformation studies [23] and for research on the metabolic pathways of zearalenone in animals [24]. Labelled zearalanone synthesized by catalytic deuteration of zearalenone was used as internal standard for the detection of zearalenone metabolites of several feeding trials [25, 26] as well as other labelled commercial available deuterated zearalenone metabolites [27]. The first stable isotope dilution assay (SIDA) for the determination of zearalenon and its metabolites in river water was recently reported [28]. As proton-deuterium exchange is very readily performed with zearalenone the stability of the label under varying and difficult conditions in food extracts may be critical and so far no stable isotope dilution assay for food commodities has been developed.

Instead, a new technique called Alternate Isotope-Coded Derivatization Assay (AIDA) has been reported for the determination of zearalenone as a possible alternative to SIDA, if no labelled standards are available [29]. In this approach both labelled and unlabelled derivatizied internal standards were prepared from stock solutions using a labelled and an unlabelled derivatization reagent, respectively. Samples were split into two parts. One part was spiked with the labelled derivatizied internal standard, extracted and derivatizied with the unlabelled derivatization reagent before LC-MS analysis. The other part was spiked with the unlabelled derivatized internal standard and subsequently derivatized with the labelled derivatization reagent. Thereby, two independent quantitations are performed, in which labelled and unlabelled derivatizied analytes are alternatively used as internal standards. In this way, both quantitations can be used to confirm one another. Using the described AIDA for the quantitation of zearalenone in maize flour the LOD was 5 µg/kg and the LOQ 10 µg/kg. Analysis of certified reference material resulted in an average recovery of 110 %.

Hence, this technique seems to have promise, although it can only be used with analytes that are readily derivatizied. Furthermore losses during sample preparation prior to derivatization are not compensated with these internal standards.

Fumonisins

Few *Fusarium* species, especially *F. verticillioides* (formerly regarded as *F. moniliforme*) and *F. proliferatum*, are able to produce fumonisins, a series of long-chained polyhydroxylamines structurally related to sphingosine (fig. 7). Discovery of fumonisines B_1 and B_2 resulted from intensive research on fungal isolates from moldy corn after severe intoxications of farm animals in South Africa [30]. Concerning human health, fumonisines were reported to disrupt sphingolipid metabolism and to putatively enhance the risk of esophagal cancer [31].

It soon was noticed that feeding liquid cultures of *F. verticillioides* with [$^2H_3$]-methyl L-methionine resulted in highly efficient incorporation of the label into fumonisin B_1. As the label was found to occur only in the methyl groups at C-12 and C-16 a mixture of [$^2H_6$]-FB_1 (90 %) and [$^2H_3$]-FB_1 (9 %) was obtained [32]. In the following SIDAs were developed to determine FB_1 in corn and corn products based on GC-MS and FAB-MS measurement. Using hydrolysis and TFA derivatisation to enable gas-chromatography a limit of detection of 10 µg/kg for FB_1 was achieved. In contrast to this, FAB-MS after strong anion exchange (SAX) clean-up could be applied at contamination levels greater than 100 µg/kg. Recovery was variable and incomplete when no internal standard was used, but 100 % when [$^2H_6$]-FB_1 was applied [40]. The following years saw the development of respective LC-MS and LC-MS/MS methods for the determination of FB_1 and FB_2 in corn and corn products [33, 34], FB_1 and hydrolyzed FB_1 in corn products [35], N-(carboxymethyl)-FB1 in corn products [36] and FB1 in asparagus spears and garlic bulbs [37, 38]. All these methods were based

on the use of [$^{2}H_{6}$]-FB_1. Detailed information about recovery and LODs and LOQs can be found in table 2.

Patulin

Patulin (4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one, PAT) is generated by different species of *Penicillium, Aspergillus* and *Byssochlamys*. The most widespread among these fungi, *Penicillium expansum*, is known to invade fruits, berries, vegetables, bread and meat products. Since PAT is reputed to be acutely toxic, teratogenic [39] and possibly cancerogenic [40] molding of fruits and bread is a serious problem for the whole food chain.

Standard methods in patulin analysis

The most frequently used method to quantify PAT is high performance liquid chromatography (HPLC) coupled to UV-detection [41]. Due to its low specifity, this method is highly susceptible to matrix interferences. Therefore, LC coupled to MS is becoming increasingly popular in PAT analysis. Three LC-MS applications including external calibration have been reported and were applied to quantify PAT in apple products [42, 43, 44]. These methods used detection of PAT in negative mode, either with atmospheric pressure chemical ionization (APCI) or atmospheric pressure photo ionization (APPI). However, the authors admitted that isotopically labelled standards would improve accuracy of their analytical methods.

Gas chromatographic (GC) procedures that determined PAT either as acetate [45], trimethylsilyl(TMS)ether [46] or its heptafluorobutanoic ester [47] did not gain much interest, because of incomplete derivatization and the lack of a suitable internal standard.

Stable isotope dilution analysis

To overcome the aforementioned constraints, [$^{13}C_2$]-PAT as the first isotopologic IS was synthesized in a six-step preparation [48] starting from L-(+)-arabinose. For a SIDA based on GC-MS detection, PATs volatility was increased by trimethylsilylation. The mass spectra

of TMS-[$^{13}C_2$]-PAT and TMS-PAT obtained in the electron impact (EI) mode enabled the differentiation of the isotopomers by monitoring the respective molecular ions [49].

For the SIDA based on LC-MS, extraction and clean-up procedures were similar to those in the GC/MS method without the need for additional clean-up and derivatization. The ratio of PAT and its isotopologue [$^{13}C_2$]-PAT was determined by detecting the deprotonated molecule at *m/z* 153 and *m/z* 155, respectively. These ions were obtained in the negative electrospray ionization mode.

A second SIDA for PAT was developed using [$^{13}C_3$]-PAT and detection by LC/MS in negative ESI mode [50]. However, the synthesis of the standard has not been described. LOD for apple juice was reported to be 1 µg/kg, which was low enough to control PAT contents in fruit products. In contrast to the first SIDA, PAT was extracted from the juice by SPE and desorbed from the solid phase with methanol.

In view of the increasing need for labelled PAT, a new isotopologue of PAT showing an isotopic distribution for [$^{13}C_7$], [$^{13}C_6$], [$^{13}C_5$], and [$^{13}C_0$] of 29%, 3%, 68%, and 0%, respectively, has been generated and commercialized [51]. This IS is particularly in use for LC-MS and reveals increasing acceptance in PAT analysis over LC-MS applications without IS.

Patulin concentration in different food commodities

Application of the GC/MS - stable isotope dilution assay to commercial and home-made fruit products gave results ranging from below 0.02 to 26 µg/kg [52]. The SIDA reported by Ito et al. was applied only to apple juices showing contents ranging from below 1 µg/kg to 45.6 mg/kg [50].

Besides fruits, many other foods are ideal substrates for *Penicillium* moulds, e. g. baked products. These kind of samples require more sophisticated clean-up procedures and, therefore, the use of a labelled IS offers specific advantages. In this respect, [$^{13}C_2$]-PAT

was used to quantify PAT in wheat bread that was, either, spontaneously moulded, or, infected with pure cultures of *Penicillium expansum*. Both types of samples revealed PAT contents of up to 21 mg/kg [49].

The new SIDA was also applied for research into possible PAT contamination in mould-ripened products such as sausages and cheese. Four samples of camembert-like cheese and five samples of mould-ripened sausages were analyzed, but PAT could not be detected.

Application of SIDA in toxicological studies

Although it has been discovered over 60 years ago, the toxicology of PAT is not fully understood yet. In feeding trials its acute and chronic toxicity, teratogenicity, and immunotoxicity has been proven. Moreover, it was shown to be a potent carcinogen after subcutaneous application, whereas orally given PAT produced no tumors [52]. Up to date, a final risk evaluation lacks data on resorption, metabolism and detoxification, which were not available due to missing analytical methods for quantification of PAT in clinical samples. To open new inroads to a better understanding, the newly developed SIDA was used for this purpose.

For studying the gastric absorption of PAT, commercial apple juice enriched with different amounts of the mycotoxin was applied to the model of the perfused rat stomach [53]. The PAT concentrations in juices, perfusate fractions and tissues were quantified by SIDA. After application of PAT, the mycotoxin appeared almost instantly in the vascular fractions and suggested that penetration of PAT occurs by passive diffusion. Moreover, a balance of the applied doses points to the existence of a degrading mechanism, which is very effective in lower doses of the mycotoxin.

In agreement with the ex-vivo study mentioned before, a human physiologic study revealed no detectable PAT in a volunteer’s serum after consumption of an apple juice (1 L) adjusted

to a PAT content of 49.9 µg/L [54]. A possible explanation for this finding is that PAT was degraded when it encountered blood.

To study the degradation's kinetic in whole blood, a small amount of PAT (7 ng) was added to freshly drawn whole blood. But even after a short reaction time of 2 min, no PAT was detectable in the blood by GC/HRMS. Only after adding a much higher amount of PAT (100 µg) the mycotoxin could be detected by GC-MS and revealed a short half-life of 21.2 sec [54].

As PAT bears an α, β, γ, δ-unsaturated carbonyl moiety, it is likely to react with cellular nucleophiles. One of the most abundant nucleophile is the tripeptide glutathione (GSH), which has been shown to form adducts with PAT spontaneously [55].

When reacting the mycotoxin with freshly drawn blood, the respective PAT-GSH adducts were detected by LC-MS and tentatively identified by LC-MS/MS and UV-spectra [56].

OCHRATOXIN A

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

As OTA is a potent hepato- and nephrotoxin [57] and is clearly associated with a kidney disease referred to as Balkan Endemic Nephropathy [58], the European Union has set OTA limits for cereal products, cereals and raisins of 3 µg/kg, 5 µg/kg and 10 µg/kg, respectively [59].

The most frequently used methods to analyze OTA in foods are HPLC with fluorescence detection (LC/FD) and enzyme-linked immunosorbent assays (ELISA) [60]. Whereas the latter are mainly suited for screening purposes, validation studies revealed that LC/FD suffers from several constraints such as widely differing recoveries.

For these reasons, LC-MS/MS is becoming increasingly popular in OTA analysis. The last years have seen a multitude of applications for meat, milk, grapes, wine, beer, spices, feedstuffs, and cereals [61, 62, 63]. However, the uses of labelled standards are scarce. A first study was reported by Jorgensen and Vahl [64], who used OTA [$^{2}H_{3}$]-methyl ester as the standard and included derivatization of OTA to its methyl ester. However, this assay was not a “true” SIDA as the standard was added after methylation, which included the risk of incomplete derivatization.

Application of a SIDA for OTA

A first synthetic approach to stable isotopically labelled OTA was reported by Lindenmeier et al. [65]. The route to [$^{2}H_{5}$]-OTA included hydrolyzing unlabelled OTA and subsequent coupling of the resulting isocoumarin derivative ochratoxin α (OTα) to labelled L-phenylalanine. After hydrolysis of OTA, OTα was activated by addition of thionyl chloride into OTα chloride, which was then reacted with [$^{2}H_{5}$]-phenylalanine methyl ester.

In contrast to this, a fully [^{13}C]-labelled OTA has been made commercially available [66].

LC-MS/MS

Separation of OTA from main interferences in food samples can be achieved on a RP-18 column. The isotopologues could be distinguished in the positive ESI mode by monitoring in tandem MS the ions resulting from the loss of formic acid from the respective protonated molecules [65].

Sample Purification

Generally, immuno affinity clean-up is applied in OTA analysis and the respective extracts are devoid of interferences and display a well shaped and clearly separated OTA peak (fig. 8). However, clean-up on silica cartridges in combination with LC-fluorescence detection without using labelled standards may achieve good results if a correction for recovery is included. This was shown for raisins, wine and nutmeg (table 3, data unpublished).

Validation studies of the SIDA

Addition experiments revealed a LOD of 0.5 and a LOQ of 1.4 µg/kg for OTA. These data proved the SIDAs to be sensitive enough to quantify OTA contents even below the EU limits for foods. SIDA of the reference materials resulted in an OTA content of 8.0 ± 0.3 for CRM 472. Thus, the bias from the certified reference value of CRM 472 was as low as 2.1 %.

Quantification of OTA in food

Quantitation of OTA using SIDA was performed in food samples in which OTA is most likely to occur, such as wheat flour, coffee, liquorice, beer, wine, and spices. 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). Within 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 [65].

DISCUSSION OF PERSPECTIVES

The recent years have seen the increasing use of LC-MS applications in mycotoxin analyses including multimethods that were able to detect as many as 39 different toxins within one run [67]. However, the quantitative value of these assays has to be called in question as for each matrix separate validations including studies on precision, recovery and trueness would have to be performed. Therefore, the use of labelled IS is becoming

increasingly popular. This development is mirrored by the growing supply of commercially available labelled mycotoxins, starting with patulin and followed by trichothecenes and ochratoxin A. However, many of these standards are quite expensive, in particular the [^{13}C]-isotopologues. In case of chemical syntheses of the isotopologues, the effort and expenses are quite manageable as detailed in the introduction section, but the analytical chemist in a routine laboratory often lacks the additional time and personnel for this work. Therefore, another objective of current research is to prepare standards in high yields to enable cheap offers. These mainly include [^{2}H]-labels, which have to be carefully judged as loss of the label and isotope effects may pose additional problems.

As the prices for isotopologic standards still can be expected to remain quite high in the near future, multiple SIDAs will not become widely-used as mycotoxins occur in very different contents and, therefore, would require high standard additions for the most abundant mycotoxins. Thus, selectively combined SIDAs rather than multiple SIDAs will come to the fore. In the course of this development, further aims are not only directed towards combining the detection in one run but also to develop combined extraction and clean-up procedures to enable the respective sensitive quantification of the mycotoxins under study. For selecting the compounds to be quantified, the exact quantification by SIDA has to be preceded by effective screening methods such as already existing multimethods based on LC-MS/MS or immunochemical assays for multiple analytes. Many groups involved in mycotoxin research are concentrating their efforts on these aims and the next years will certainly show significant progress in this respect.

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Table 1. Performance data of stable isotope dilution assays for trichothecenes

Type B-trichothecenes	Analyte	Standard	LOD	LOQ	Recovery
Häubl et al., (2005)	DON	[$^{13}C_{15}$]-DON	n.d.	8 µg/L	95 ± 3 – 99 ± 3 %
Häubl et al., (2006)	DON	[$^{13}C_{15}$]-DON	n.d.	n.d.	101 ± 2.4 %
Bretz et al., (2006)	DON	[$^{2}H_{1}$]-DON	n.d.	n.d.	99 ± 2 – 103 ± 5 %
	3-Ac-DON	[$^{2}H_{3}$]-3-Ac-DON	n.d.	n.d.	87 ± 3 – 96 ± 2 %
Asam et al., (2007)	DON	[$^{13}C_{15}$]-DON	2 µg/kg	6 µg/kg	97 ± 1 – 100 ± 4 %
	3-Ac-DON	[$^{13}C_{2}$]-3-Ac-DON	5 µg/kg	16 µg/kg	95 ± 3 – 105 ± 3 %
	15-Ac-DON	[$^{13}C_{2}$]-15-Ac-DON	4 µg/kg	11 µg/kg	100 ± 4 – 115 ± 7 %
	4-Ac-NIV	[$^{13}C_{2}$]-4-Ac-NIV	1 µg/kg	4 µg/kg	94 ± 2 – 101 ± 2 %
Type A-trichothecenes	**Analyte**	**Standard**	**LOD**	**LOQ**	**Recovery**
Pawlosky et al., (1989)	T2-toxin	[$^{2}H_{3}$]-T2-toxin	0.5 µg/kg	n.d.	70 – 80 %
	HT2-toxin	[$^{2}H_{3}$]-HT2-toxin	0.5 µg/kg	n.d.	90 – 140 %
Razzazi-Fazeli et al., (2002)	T2-toxin	[$^{2}H_{3}$]-T2-toxin	n.d.	50 µg/kg	77 %
Asam et al., (2006)	MAS	[$^{13}C_{2}$]-MAS	30 µg/kg	80 µg/kg	109 ± 3 – 112 ± 1
	DAS	[$^{13}C_{4}$]-DAS	1 µg/kg	4 µg/kg	90 ± 4 – 91 ± 6
	HT2-toxin	[$^{13}C_{2}$]-HT2-toxin	10 µg/kg	80 µg/kg	93 ± 16 – 107 ± 5
	T2-toxin	[$^{13}C_{4}$]-T2-toxin	3 µg/kg	8 µg/kg	112 ± 2 – 127 ± 5

n.d. = not determined, DON = deoxynivalenol, 3-Ac-DON = 3-acetyldeoxynivalenol, 15-Ac-DON = 15-acetyldeoxynivalenol, 4-Ac-NIV = 4-acetylnivalenol, MAS = monoacetoxyscirpenol, DAS = diacetoxyscirpenol

Table 2. Performance data of stable isotope dilution assays for fumonisins

Fumonisins	Analyte	Standard	LOD	LOQ	Recovery
Plattner et al., 1992	FB_1	[$^{2}H_{6}$]-FB_1	10 µg/kg	n.d.	100 %
Lukacs et al., 1996	FB_1	[$^{2}H_{6}$]-FB_1	0.4 µg/kg	0.8 µg/kg	n.d.
	FB_2	[$^{2}H_{6}$]-FB_1	n.d.	n.d.	n.d.
Hartl et al., 1999a	FB_1	[$^{2}H_{6}$]-FB_1	n.d.	20 µg/kg	84 %
Hartl et al., 1999b	FB_1	[$^{2}H_{6}$]-FB_1	5 µg/kg	n.d.	96.4 – 97.4 %
	HFB_1	[$^{2}H_{6}$]-FB_1	8 µg/kg	n.d.	54.6 – 87.6 %
Seefelder et al., 2001	NCM-FB_1	[$^{2}H_{6}$]-FB_1	10 µg/kg	n.d.	50 – 60 %
Seefelder et al., 2002	FB_1	[$^{2}H_{6}$]-FB_1	n.d.	n.d.	79 – 104 %

n.d. = not determined, FB_1 = fumonisin B_1, FB_2 = fumonisin B_2, HFB_1 = hydrolyzed fumonisin B_1, NCM-FB_1 = N-(carboxymethyl)-fumonisin B_1

Table 3. Comparison of OTA concentrations determined in different foods by using a stable isotope dilution assay (SIDA), liquid chromatography-fluorimetric detection (LC-FD) or the ELISA method

Sample	Concentrations (µg/kg) as determined by		
	SIDA	LC-FD [a]	ELISA
CRM (wheat)	8.03	8.26	4.96
Wheat	<0.5[b]	<0.5[b]	1.23
Coffee (instant)	<1.4[c]	<1.4[c]	2550
Liquorice	<1.4	<1.4	2.56
Wine (white)	<0.5	<0.5	0.13
Wine (mulled)	3.30	3.77	4.68
Sultanas	29.80	36.3	30.3
Currants	2.48	n.d.[d]	3.63
Nutmeg	1.79	<1.4	8.56

[a] by application of recovery factors.

[b] Limit of detection.

[c] Limit of quantitation.

[d] not determined

LEGENDS TO THE FIGURES

Figure 1. Stable isotope dilution assays: Addition of a standard with a different isotopic distribution to the analyte – the original isotopic distribution has been “diluted”.

Figure 2. Principle of a stable isotope dilution assay: after addition of an isotopologic standard to the analyte and equilibration the isotopic ratio remains stable until final mass spectral analysis. For a structurally different internal standard, however, the ratio between standard and analyte can alter during sample preparation resulting in systematic errors.

Figure 3. LC-MS/MS run of a maize flour extract containing trichothecenes analyzed with SIDA

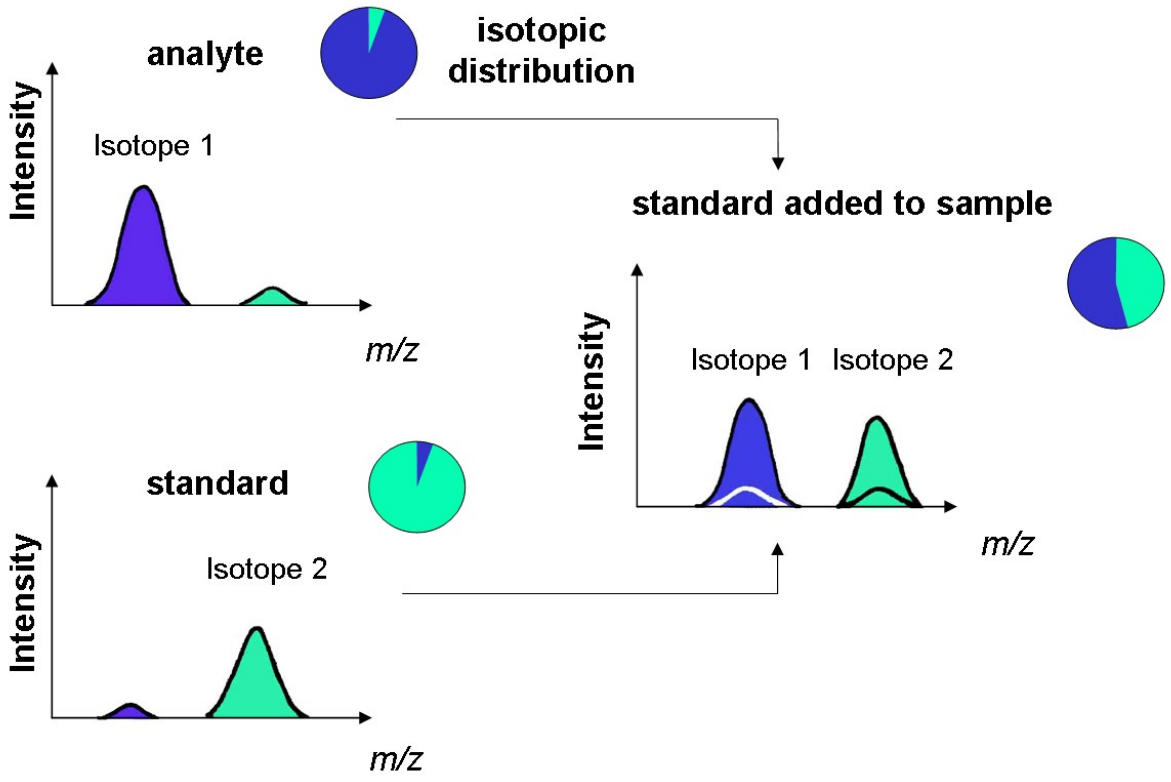
Figure 4. Calibration function (continuous line) for a stable isotope dilution assay under the supposition that the standard contains 2 % unlabelled material und natural isotopologues contributing 5 % intensity on the standard’s signal.

Figure 5. Structure of common type A- and type B-trichothecenes

Figure 6. Structure of resorcylic acid lactones

Figure 7. Structure of fumonisins

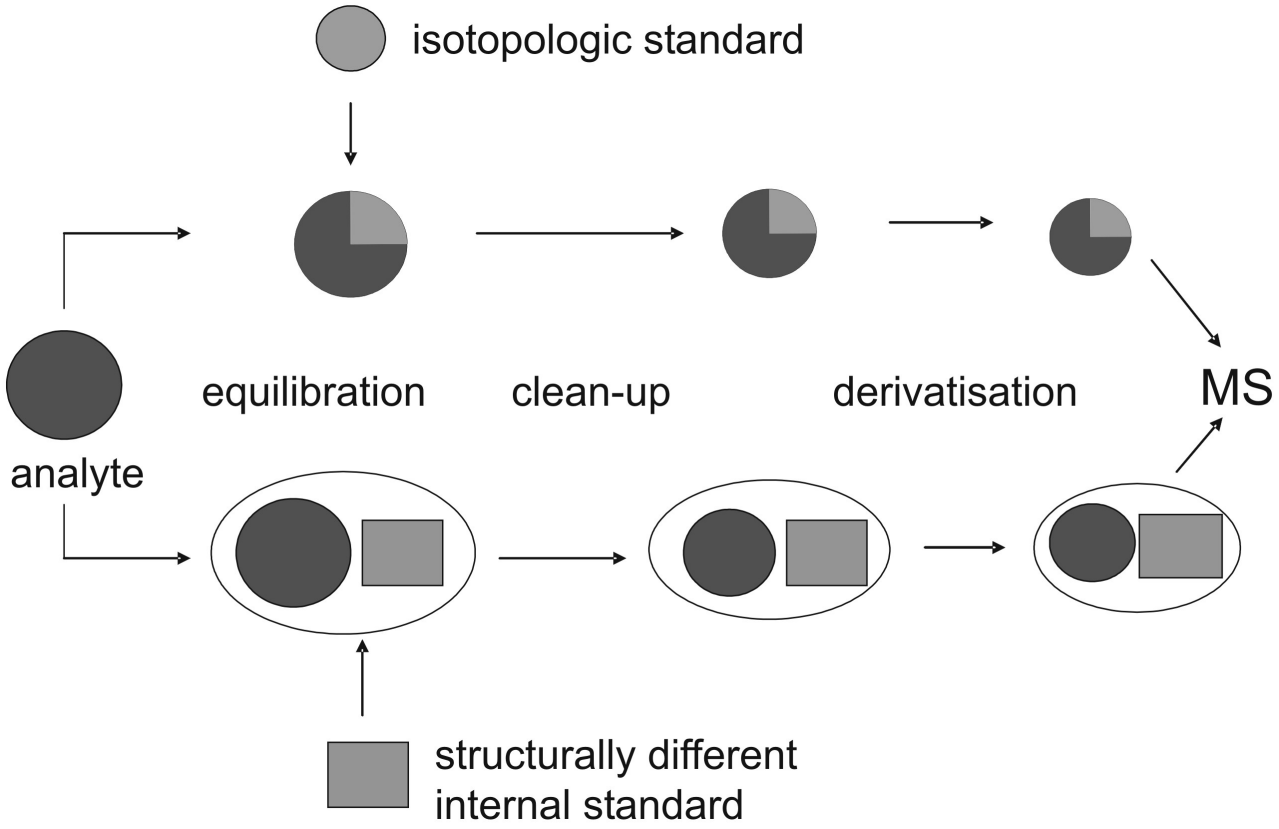
Figure 8. LC-MS/MS run of a blood plasma extract containing ochratoxin A analyzed with SIDA



1

2 **Figure 1.** Stable isotope dilution assays: Addition of a standard with a different isotopic

3 distribution to the analyte – the original isotopic distribution has been “diluted”.



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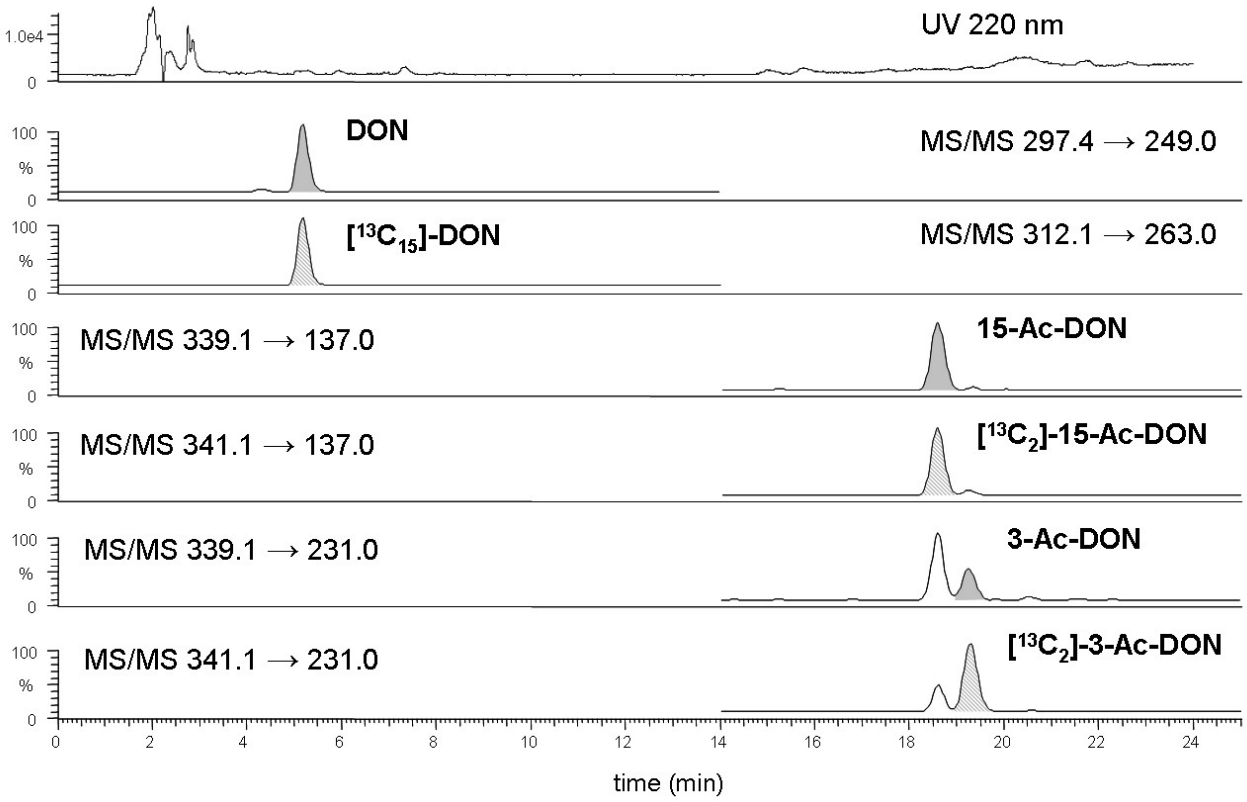


Figure 3. LC-MS/MS run of a maize flour extract containing trichothecenes analyzed with SIDA

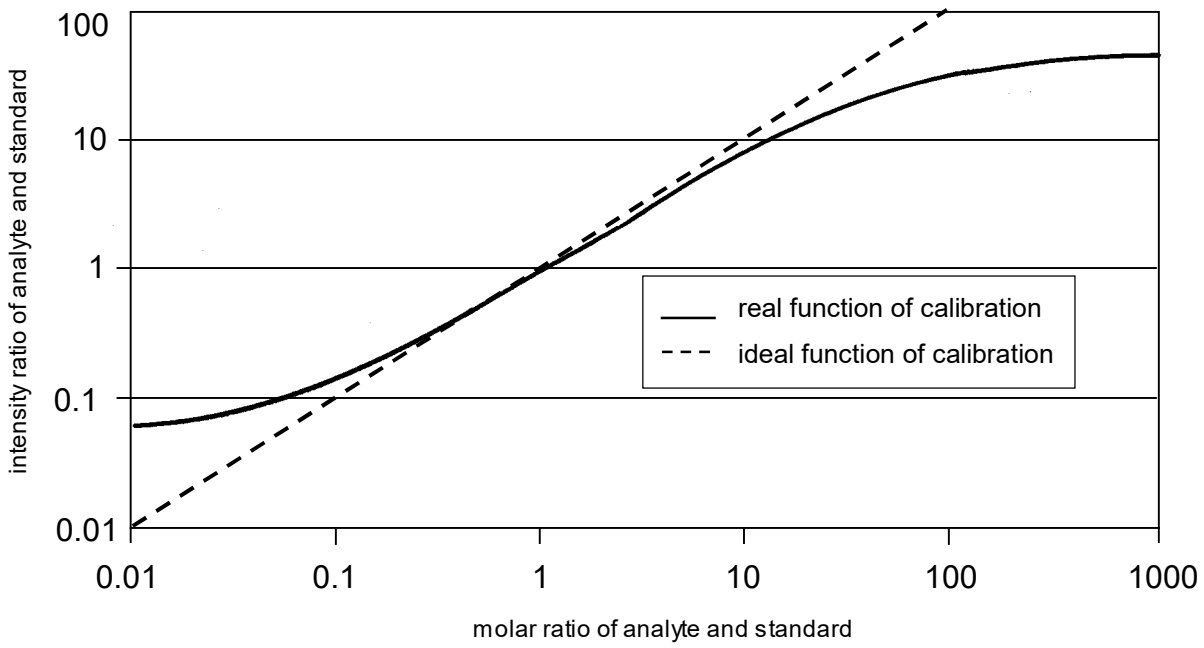
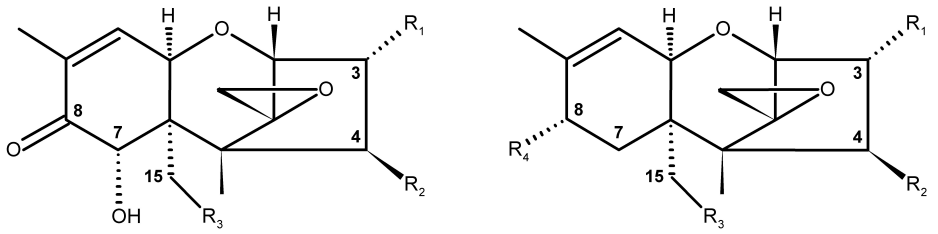


Figure 4. Calibration function (dashed line) for a stable isotope dilution assay under the supposition that the standard contains 2 % unlabelled material und natural isotopologues contributing 5 % intensity on the standard's signal.

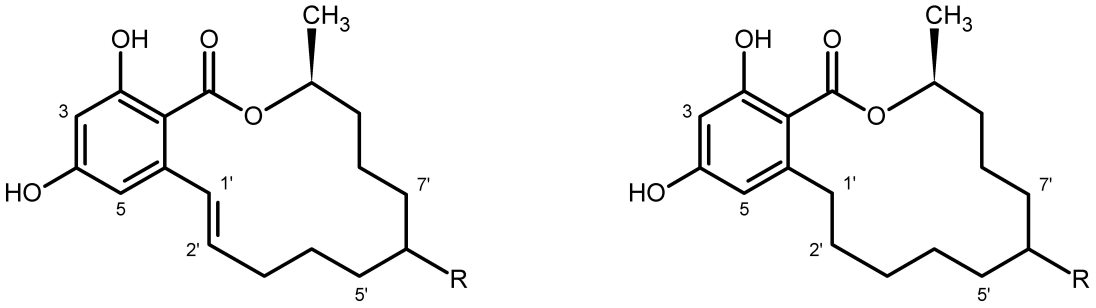
1



Type B	R_1	R_2	R_3	Type A	R_1	R_2	R_3	R_4
deoxynivalenol	OH	H	OH	HT2-toxin	OH	OH	OAc	$OCOCH_2CH(CH_3)_2$
3-acetyldeoxynivalenol	OAc	H	OH	T2-toxin	OH	OAc	OAc	$OCOCH_2CH(CH_3)_2$
15-acetyldeoxynivalenol	OH	H	OAc	monoacetoxyscirpentriol	OH	OH	OAc	H
4-acetylnivalenol	OH	OAc	OH	diacetoxyscirpentriol	OH	OAc	OAc	H

2 **Figure 5.** Structure of common type A- and type B-trichothecenes

3

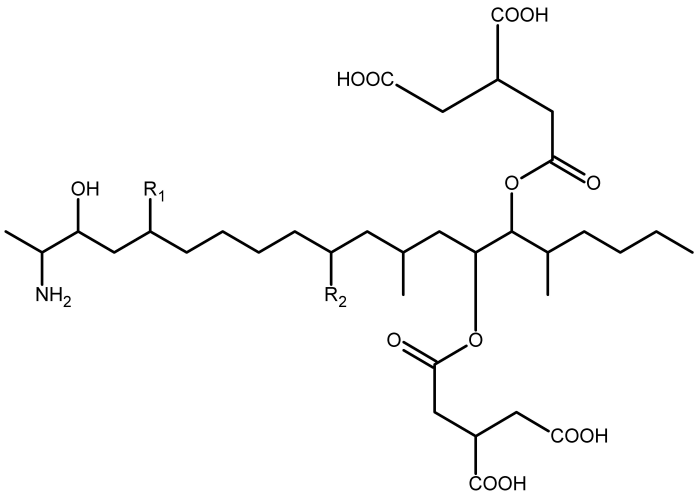


	R		R
zearalenone	=O	zearalanone	=O
α-zearalenol	····OH	α-zearalanol	····OH
β-zearalenol	◄OH	β-zearalanol	◄OH

4 **Figure 6.** Structure of resorcylic acid lactones

5

1



fumonisin	R_1	R_2
B_1	OH	OH
B_2	OH	H
B_3	H	OH

2 **Figure 7.** Structure of fumonisins

3

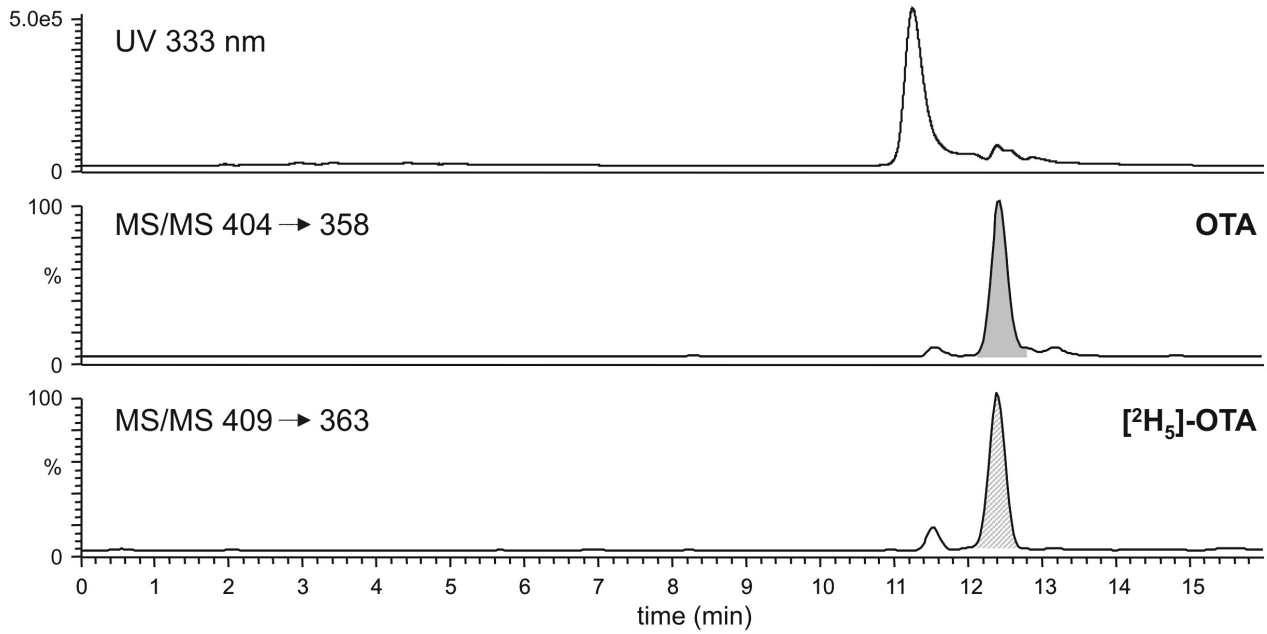


Figure 8. LC-MS/MS run of a blood plasma extract containing ochratoxin A analyzed with SIDA