Published in:

Analytical and Bioanalytical Chemistry (2015), 407: 7563–7577

The final publication is available at Springer via http://dx.doi.org/ 10.1007/s00216-015-8904-y

Recent developments in stable isotope dilution assays in mycotoxin analysis with special regard to *Alternaria* toxins

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Abstract

Stable isotope dilution assays (SIDAs) are getting more and more common in mycotoxin analysis and the number of synthesized or commercially available isotopically labelled compounds has largely increased in the last seven years following our last review dealing with this topic. Thus, this review is conceived as an update for new applications or improvements of SIDAs for compounds already discussed earlier, but the main focus is laid on newly introduced labelled substances and the development of SIDAs for e.g. fusarin C, moniliformin or the enniatins. Mycotoxin research has concentrated on the emerging group of *Alternaria* toxins in the last years and a series of SIDAs have been developed including tenuazonic acid, alternariol, altertoxins and tentoxin that are discussed in detail in this review. Information about synthetic routes, isotopic purity and mass spectrometric characterisation of labelled compounds are given, as well as about the development and validation of SIDAs and their application to foods, feeds or biological samples. As the number of commercially available labelled standards increases continuously, a general tendency on the use of analytical methods based on liquid chromatography coupled to mass spectroscopy capable of identifying a series of mycotoxins simultaneously ("multi-methods") and using one or more labelled internal standards can be observed. An overview of these applications are given, thus demonstrating that SIDAs increasingly are used in routine analysis.

Key words:

Alternaria toxins; *Fusarium* toxins; LC-MS/MS, Mycotoxins; Stable isotope dilution assay; SIDA

Introduction: State-of-the-art of stable isotope dilution assays

Mycotoxin contamination of food and feedstuffs are regulated in many parts of the world and particularly low maximum limits (MLs) are stipulated in the European Union. Due to severe legal consequences when MLs are exceeded, accurate and reliable analytical methods for these contaminants are essential. Moreover, as MLs for many mycotoxins are at sub ppb levels, e.g. 0.05 µg/kg for Aflatoxin M1, accuracy at these trace amounts poses an additional challenge. In 2008 we detailed in our previous review [1], which particular benefits stable isotope dilution assays (SIDAs) offer, especially for LC-MS methods, and present here an update. The previous review introduced the principle of SIDAs in mycotoxin analysis along with the prerequisites and limitations of the use of stable isotopically labelled internal standards and possible calibration procedures. Moreover the state of SIDA applications at that time for the analysis of trichothecenes, zearalenone, fumonisins, patulin and ochratoxin A was presented. In the subsequent years this methodology has seen tremendous expansion and appreciation. Principles of access to stable isotope-labelled analogues as internal standards (IS), prerequisites and limitations have remained the same, thus we present here new stable isotope labelled analogues and SIDAs that have been developed since then.

Update to Applications of Stable isotope dilution assays to mycotoxins

PART A: *Fusarium* toxins

1. Zearalenone

Zearalenone (ZEN) is a mycoestrogen produced by *Fusarium* species. The synthesis of deuterated ZEN and its use (together with commercially available labeled standards of zearalenols (ZELs) and zearalanols (ZALs)) in a SIDA of environmental samples by has been reported in 2007 [2] and was already included in our preceding review [1]. The

method used for synthesizing labeled ZEN was base-catalyzed protium-deuterium exchange. However, as protons at C-3, C-5, C-5' and C-7' of the ZEN molecule are prone to protium-deuterium exchange, a mixture of different [$^{2}H_{1-9}$]-labeled compounds was obtained, with [$^{2}H_{5}$]-ZEN being the predominant isotopologue (38.3 %). Aware of the discovery that the protons at C-3 and C5 are always almost completely exchanged against deuterium [3], a modified synthetic route was developed, in which the carbonyl moiety at C-6' is protected as a [1,3]-dioxolane prior to base-catalyzed protium-deuterium exchange [4]. Hence, with the partial protium-deuterium exchange at C-5' and C-7' being avoided, double labeled [$^{2}H_{2}$]-ZEN was obtained with high isotopic purity (94.4 %), the rest being singly deuterated (5.4 %) or unlabeled (0.1 %) (Fig. 1). However, the presence of unlabeled compound in the labeled standard and spectral overlap of the double labeled standard with the natural occurring [$^{13}C_{2}$]-isotopologue (2.9 %), required quadratic regression to obtain a linear calibration curve between analyte to standard ratios of 1:10 to 10:1. Recovery rates for ZEN were between 104 ± 3 % and 101 ± 2 % for spiking levels ranging from 5 µg/kg to 300 µg/kg. Precision and limits of detection and quantitation were not determined. The developed SIDA was applied to the analysis of several food commodities from the German retail market not exceeding a content of ZEN higher than 45 µg/kg in a sample of taco shells.

It is generally assumed that *Fusarium* species biosynthesize ZEN with the sidechain double bond only *trans* configured, however the change to *cis* configuration has been reported after artificial UV-radiation [5] or by natural sunlight [3]. Data about estrogenic relevance and occurrence in food and feed of this isomer are limited. However, after synthesis by UV-radiation, purification and characterization of pure *cis*-ZEN as analytical standard [6, 7], it stood to reason to prepare an isotopically labelled standard in the same way. Thus, after UV-radiation of commercially available U-[$^{13}C_{18}$]-ZEN, the isomeric U-[$^{13}C_{18}$]-*cis*-ZEN was obtained with a yield of about 50 % [8]. The developed SIDA showed

low limits of detection (LOD) (0.35 µg/kg for *trans*-ZEN; 0.28 µg/kg for *cis*-ZEN) and limits of quantitation (LOQ) (1.17 µg/kg for *trans*-ZEN; 0.93 µg/kg for *cis*-ZEN) and excellent recovery (104 ± 4 % for *trans*-ZEN; 104 ± 2 % for *cis*-ZEN). However, a survey of 15 edible oils from the German retail market showed low contamination with *trans*-ZEN (2/15) and only one sample exceeding the LOD for cis-ZEN, but not the LOQ [8].

2. Moniliformin

The semisquaric acid or, systematically, 1-hydroxycyclobut-1-ene-3,4-dione, was originally isolated from *Fusarium moniliforme* in 1973 [9], from which its common name "moniliformin" (MON) was deduced, and soon after was structurally characterized [10]. However, many other *Fusarium* species have been found to be able of producing MON, e. g. F. *avenaceum*, F. *tritinctum*, and F. *proliferatum* [11], and contamination of cereal samples with MON (as sodium or potassium salt) has been reported worldwide [12]. Although the principal mode of action is still under discussion, studies with ducklings, mice and rats revealed MON to be an acute cardiotoxin with LD_{50}-values that are comparable with the most toxic *Fusarium* mycotoxins T-2 and HT-2 toxin [13–15]. Recent studies showed rapid urinary excretion of administered MON in rats and no signs of accumulation, but subchronic toxic effects could not be excluded [16]. Synthetic routes to MON have been reported [17, 18], the latter based on a [2+2] cycloaddition that was also used in the synthesis of [$^{13}C_2$]-MON [19]. In the [2+2] cycloaddition 2,3-dihydro-1,4-dioxine was reacted with dichloro-[$^{13}C_2$]-ketene that was generated in situ by reductive dehalogenation of trichloro-[$^{13}C_2$]-acetyl chloride with activated zinc. After hydrolysis and purification [$^{13}C_2$]-MON was isolated with a yield of 15 % and an isotopic purity of 99 % (Fig. 2).

Using this labeled standard a SIDA was developed based on the measurement of the base peaks of the $[M\text{-}H]^-$ ions of MON and [$^{13}C_2$]-MON with high-resolution mass spectrometry (HRMS) after liquid chromatography [20]. It has already been known that the use of triple

quadrupole mass spectrometers is critical for the analysis of MON as only one significant mass transition is formed and selectivity derogated, therefore [21, 22]. The use of HRMS has already been shown to ensure selectivity of the MON analysis by the exact mass measurement [23]. Beside the use of [$^{13}C_2$]-MON as internal standard, selectivity was further improved by monitoring the ratio between MON and its natural occurring [$^{13}C_1$]-isotopologue [20]. Unfortunately, little information was given about the linear range of the calibration and the developed SIDA was principally limited by adding the labeled standard just before the mass spectrometric measurement. For cereal matrices a mean recovery of 75.3 % was calculated, but not used for correcting the results of the samples. The limit of detection (0.7 µg/kg) and limit of quantitation (2.5 µg/kg) of the SIDA determined by the authors showed similar sensitivity to recent conventional methods using LC-MS/MS [24]. The precision of the SIDA (6.5 %) also differs little from published conventional methods [22, 24, 25], although deeper assessment is difficult as method validation was not systematically carried out or reported in most studies, generally.

3. Fusarin C

Fusarin C (Fig. 3) is a mycotoxin produced by several *Fusarium* species including the commonly occurring *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. sporotrichoides*, and many others [26, 27]. It was isolated from corn infected with *F. moniliforme* in the early 1980s and soon found to be mutagenic [28, 29]. Data about contamination of food and feed with this toxin are limited, but corn seems to be affected, especially [30, 31]. High-performance liquid chromatography (HPLC) with ultraviolet detection (UV) has been used for the analysis of this toxin until the recent development of a HPLC method with tandem mass spectrometric detection [32]. Sample preparation was based on dispersive solid phase extraction (“QuEChERs”, Quick, Easy, Cheap, Effective, Rugged, and safe), the limit of detection (LOD) was 2 µg/kg, the limit of determination (LOQ) 7 µg/kg and the

recovery 80 %. Fusarin C was detected in corn-based food and feed with maximum values up to 28 µg/kg in a popcorn sample. However, in this study quantitation had to be done by matrix matched calibration, which is time consuming and expensive. The same group of authors subsequently developed a stable isotope dilution assay (SIDA) for [33]. Using carboxyfusarin C, a biochemical precursor of fusarin C that was enriched in cultures of a knockout strain of *F. fujikuroi*, synthesis of fusarin C was achieved by methylation with deuterated diazomethane. Although the authors took every precaution against deuterium-protium back-exchange, isotopic purity was low, as the synthesis yielded only 62 % [$^{2}H_{3}$]-fusarin C. However, as the synthesized standard contained as little as 0,3 % of the unlabeled compound, it could be used for development of a SIDA based on the earlier LC-MS/MS method. Using SIDA, the LOD and the LOQ decreased (1 and 4 µg/kg, respectively) and the recovery was almost complete (99 %). Unfortunately, no systematic precision studies were performed in both publications. However, based on the given data for e. g. whole corn an improvement of the precision from 55 % (11 ± 6 µg/kg; matrix calibration; [32]) to 3 % (11.5 ± 0.3 µg/kg; SIDA; [33]) can be calculated.

4. Depsipeptides

Some *Fusarium* species, most commonly *F. tricinctum* and *F. avenaceum* are able to produce a series of cyclic depsipeptides [34–36], out of which the enniatins (ENNs) and beauvericin (BEA) are the most frequently analyzed ones. These compounds have been evaluated by the EFSA Panel on Contaminants in the Food Chain (CONTAM) [37], recently. According to this, grain and grain-based products are often contaminated with ENNs and BEA, but the lack of relevant toxicity data prevented a risk assessment. However, as these compounds show distinct biological effects, e. g. acting as ionophores, antimicrobials, or enzyme inhibitors, the panel considered acute and chronic exposure still being a possible concern for human health. The preferred analytical method for the

analysis of ENNs and BEA in food, feed and agricultural goods has been liquid chromatography coupled to mass spectrometry [38–41]. Depending on the matrix, both ion suppression [41] and signal enhancement [38] has been described, thus underlining the need of an isotopically labeled standard for compensation. Although it has been shown that chemical synthesis of ENNs and BEA is possible in principle (e. g. reviewed by [42]), the introduction of labels into the molecules was found to be easily achieved biosynthetically by growing *Fusarium* strains on Czapek-Dox liquid minimal medium with $Na^{15}NO_3$ as the only nitrogen source [43]. Thus, [$^{15}N_3$]-BEA was isolated from a culture of *F. fujikuoi* and [$^{15}N_3$]-ENN A, A1, B and B1, respectively, from a culture of *F. sambucinum*. The developed SIDA revealed values for LODs (1.9 – 4.4 µg/kg) and LOQs (5.8 – 13.1 µg/kg) commonly achievable with modern LC-MS/MS instrumentation. However, both recovery (96 – 110 %) and intraday precision (1.35 – 7.21 %) of the SIDA was much better than for conventional LC-MS/MS methods (e. g. recovery 64 – 121 % and precision 7 – 18 % according to [39]).

PART B: *Alternaria* toxins

The saprophytic moulds of the genus *Alternaria* are ubiquitous plant pathogens invading several agricultural crops, as well as food and feed derived thereof. Mould growth is accompanied with the production of over 70 toxins, out of which the most relevant ones can be classified into the two major groups benzopyrones and perylenequinones, along with the phytotoxin tentoxin (TEN) and the amino acid derivative tenuazonic acid (TA) [44].

1. Benzopyrones

Of all *Alternaria* toxins, the benzopyrone group is the best examined one and was the first to be analysed. This group encompasses the two major toxins alternariol (AOH) and alternariol monomethyl ether (AME) besides altenuene and altenuisol. Both AOH and AME

have been reported to inhibit topoisomerase I and IIa [45] and to be mutagenic in cell culture [46].

As the first labelled isotopologues, deuterated alternariol and alternariol methyl ether were synthesized by palladium catalyzed protium-deuterium exchange from the unlabeled toxins (Fig. 4) [47]. The aromatic protons are most easily exchanged against deuterium, leading to the preferred formation of the [$^{2}H_{4}$]-isotopologue of alternariol (43 % isotopic purity) and alternariol monomethyl ether (59 % isotopic purity). However, as the three benzylic protons are also affected by the exchange reaction a mixture of isotopologues with mass increments of up to M+7 was achieved. But as the internal standards contained no unlabeled material, this isotopic fractionation only resulted in slopes of the response curve deviating from the ideal value 1, whereas the linearity was not affected.

Based on the use of [$^{2}H_{4}$]-AOH and [$^{2}H_{4}$]-AME as internal standards, SIDAs were developed and applied to the determination of AOH and AME in several food commodities using LC-MS/MS [47]. Method validation for fruit juices revealed complete recovery (100.5 ± 3.4 % for AOH and 107.3 ± 1.6 % for AME), low LODs (0.03 µg/kg for AOH and 0.01 µg/kg for AME) and LOQs (0.09 µg/kg for AOH and 0.03 µg/kg for AME) and excellent precision (4.0 % for AOH and 2.3 % for AME). Values for both benzopyrones in fruit juices were low in general (< 1 µg/kg for AOH and < 0.3 µg/kg for AME), but higher values of the toxins were found in wine and vegetable juices amounting to 7.82 µg/kg and 0.79 µg/kg for AOH and AME, respectively.

In another study by the same group of authors, cereal, fruit and vegetable products were analyzed for contamination with both benzopyrones using the developed SIDA [48]. Both toxins were practically not detectable in cereals and cereal products from the German retail market. Higher values were found in cereals intended for animal nutrition (AOH: 13 – 250 µg/kg; AME: 3 – 100 µg/kg). However, AOH and AME were also detected in about 50 % of all analyzed vegetable products in rather high values (2.6 – 25 µg/kg for AOH and 0.1 – 5

μg/kg for AME. Tomato products were affected, especially. The highest content of AOH (25 μg/kg) and AME (5 μg/kg) were found in triple concentrated tomato paste.

Most recently, U-[^{13}C]-labelled AOH and AME have been synthesized by a microbiological procedure using a modified Czapek-Dox medium with low concentrations of [$^{13}C_6$]-glucose and [$^{13}C_2$]-acetate as only carbon sources and ammonium sulfate as only source of nitrogen [49]. Thus, [$^{13}C_{14}$]-AOH and [$^{13}C_{15}$]-AME were isolated in high isotopic purity, albeit in low yield (5 μg absolute each) and applied for developing SIDAs for different food commodities. As by-products [$^{13}C_{15}$]-altenuene and [$^{13}C_{14}$]-altenuisol were also isolated, but not used as internal standards in SIDAs so far.

The alternative labelling of AOH and AME enabled the authors to clarify the fragmentation of both benzopyrones in LC-MS/MS [49]. For AOH, the fragments of the [$^{2}H_4$]- and the [$^{13}C_{14}$]-isotopologue led to the conclusion that the quantifier fragment at *m/z* 147 of AOH must have lost five carbon atoms and one aromatic hydrogen (Fig. 5). For AME, the assignment was much more complicated. From the deuterated isotopologue alone no structural deduction of the fragments was possible [47]. However, the main fragment ions m/z 255 and 256 of AME were accordingly observed with the [$^{13}C_{15}$]-isotopologue (m/z 269 and 270), whereas the [$^{2}H_4$]-isotopologue showed three signals (m/z 258, 259 and 260). Therefore, the loss of the O-methyl radical and of a hydrogen radical is reasonable. The latter must originate mainly from the hydroxyl group and, according to the appearance of a small signal at m/z 258 in the [$^{2}H_4$]-AME spectrum, a minor amount may originate from the aromatic ring (Fig. 6). Using both U-[^{13}C]-labelled benzopyrones a SIDA was developed and validated for cereal matrices. LODs and LOQs of 0.36 μg/kg and 1.1 μg/kg for AOH and 0.09 μg/kg and 0.27 μg/kg for AME were determined. The values for recovery (98 – 103 % for AOH and 109 – 112 % for AME) and precision (4.1 % for AOH and 5.8 % for AME) once more proved the advantages of the SIDA methodology. A survey on a series of food commodities from the German market confirmed the rather low contamination with these

two toxins. However, paprika powder (AOH: 17 – 46 µg/kg; AME: 14-26 µg/kg) and sorghum based animal feed (AOH: 347 – 757 µg/kg; AME: 109 – 215 µg/kg), especially, contained both AOH and AME in amounts that may be critical for human and animal health.

2. Tenuazonic acid

The tetramic acid derivative L-tenuazonic acid [(5*S*,8*S*)-3-acetyl-5-sec-butyl-pyrrolidine-2,4-dione] (TA) is one of the major mycotoxins produced by a series of different *Alternaria* spp. [44] and has attracted increasing attention during the last years. It is regarded as the most acutely toxic metabolite produced by *Alternaria* [50] and is known to occur in high concentrations in commodities infected with this mould [44]. Until now, two different methods leading to isotopologues of TA have been described.

a) [$^{13}C_6$,^{15}N]-TA

The first stable isotope labelled TA was synthesized from [$^{13}C_6$,^{15}N]-methyl isoleucinate by Dieckmann intramolecular cyclization after acetoacetylation with diketene, yielding sevenfold labelled [$^{13}C_6$,^{15}N]-tenuazonic acid (Fig. 7, I) [51]. The obtained labelled standard revealed excellent isotopic purity, however the low yield (about 30 %) of the synthesis was only acceptable because the labeled amino acid is commercial available at a relatively moderate price. Additionally, the conditions employed for the ring closure led to epimerization, the labeled standard being a mixture of the (5*S*,8*S*)- and (5*R*,8*S*)-diastereomers, therefore. However, this reaction is reported to occur with the analyte itself in aqueous solution, but the few analytical methods that are able to separate the diastereomers are not routinely performed [52]. Thus, the labelled standard can be used for general purposes, but if - for example - immunochemical devices become available for sample preparation in the future, this cannot be taken for granted in any case. Using this stable isotope-labelled standard a SIDA was developed and validated. The method was based on derivatization of TA with 2,4-dinitrophenylhydrazine during extraction, solid phase

clean up using C_{18} material and liquid chromatography before mass spectrometric detection. The use of the internal standard during the extraction and derivatization procedure greatly improved the recovery that ranged between 104 ± 2.5 % and 108 ± 1.6 % for tomato products. Without an IS the recovery was 79 ± 11 % for different cereal matrices and 90 ± 22 % for beer [53, 54]. Different tomato products (n = 16) from the German market were analyzed for their content of TA using the developed SIDA. Values were between 15 – 195 µg/kg (tomato ketchup, n = 9), 363 – 909 µg/kg (tomato paste, n = 2) and 8 – 247 µg/kg (pureed tomatoes and comparable products, n = 5).

In a further application of the [$^{13}C_6$,^{15}N]-TA isotopologue, the median contents in different foods were found to be 1.8 µg/kg (fruit juices), 16 µg/kg (cereals) and 500 µg/kg (spices). The amount of positive samples was 86 % (fruit juices), 92 % (cereals) and 87 % (spices) [55], which highlighted the ubiquitous occurrence of TEA.

In another study infant foods and beverages were analyzed by SIDA [56]. The median content of TA in infant tea infusions (n = 12) was 2 µg/L, but values up to 20 µg/L were found in fennel tea infusions. In puree infant food in jars (n = 12), the median content of TA was 7 µg/kg, but higher values were detected in products containing tomato (25 µg/kg), banana and cherry (80 µg/kg) and sorghum (20 µg/kg). Infant cereals on the basis of wheat and/or oats, rice, spelt and barley (n = 4) did not contain TA in values higher than 30 µg/kg, but if sorghum was the major ingredient (n = 12) the mean content of TA was 550 µg/kg and the maximum level was 1,200 µg/kg. This finding raised concerns about the safety of millet infant cereals and the risk was assessed based on the threshold of toxicological concern (TTC) approach [57] by the European Food Safety Authority (EFSA) with a TTC value of 1,500 ng TA/kg body weight per day [58]. The TTC is likely to be exceeded by infants consuming these cereals and, therefore, the Bavarian health and food safety authority stipulated a warning limit for these kinds of products of 500 µg/kg, which led to withdrawal of the highly contaminated products from the Bavarian market.

The [$^{13}C_6,^{15}N$]-labelled TA was also used as internal standard for the quantitation of TA analogues derived from valine (ValTA), leucine (LeuTA), alanine (AlaTA) and phenylalanine (PheTA) that were synthesized from the respective amino acids [59]. Two analytical methods based on high performance liquid chromatography (HPLC) and MS detection were developed, one with derivatization with 2,4-dinitrophenylhydrazine (DNPH) and one without. Beside TA, the analogues LeuTA (about 4 % of TA content) and ValTA (about 10 % of TA content) were found in highly contaminated sorghum infant cereals and sorghum grains. Other analogues were not detected.

Further studies using SIDA were directed towards unravelling the toxic potential of TA. In toxicokinetic studies in humans, the content of TA in human urine was determined by a SIDA extensively re-validated for urine matrix [60]. Interestingly, it was very difficult to obtain blank urines as TA is constantly consumed due to its ubiquitous occurrence. For a blank urine, a diet devoid of spices, cereals, vegetables and fruits was necessary. The method then was applied for two studies dealing with urinary excretion of TA: In the first study, TA was quantified in the 24-hour urine of six volunteers from Germany (3 female, 3 male) having their usual diet in a concentration range of 1.3–17.3 µg/L or 2.3–10.3 ng/mg^{-1} creatinine, respectively. In the second study, two volunteers (1 female, 1 male) ingested 30 µg TA by consumption of naturally contaminated whole meal sorghum infant cereals and tomato juice, respectively. The urinary excretion of the ingested TA was 54–81 % after six hours, depending on matrix and volunteer. After 24 hours 87–93 % of the ingested amount of TA was excreted, but the fate of the remaining about 10 % was not revealed.

In another toxicokinetic study of TA [61] in the plasma of pigs and broiler chickens, each animal received a single dose of 0.05 mg TA/kg body weight orally (p.o.) and intravenously (i.v.) in a two-way cross-over design. The toxicokinetic profile of TA measured by SIDA using [$^{13}C_6,^{15}N$]-TA as IS markedly differed between pigs and broilers. TA was completely absorbed in pigs as well broiler chickens with an absolute oral bioavailability of 147 and 124

%, respectively. However, absorption was slower in broiler chickens with a mean time to maximal plasma concentration of 2.60 h. TA was more rapidly eliminated in pigs compared to broiler chickens, since the mean elimination half-life after i.v. administration was 0.51 h and 2.03 h for pigs and broiler chickens, respectively. This slower elimination is mainly due to the significantly lower total body clearance of TA in broilers, i.e. 448.4 mL/h/kg and 59.3 mL/h/kg after i.v. administration for pigs and broilers, respectively. Possible hypotheses are zero order biotransformation kinetics of TA and/or inhibition of biotransformation enzymes in broiler chickens. Furthermore, the higher area under the plasma concentration-time curve, a measurement of body exposure, in broilers could implicate differences in species susceptibility to the harmful effects of TA, although no clinical signs of intoxication were observed in either species after single bolus administration.

A study from Belgium used [$^{13}C_6$,^{15}N]-TA to analyze a series of derived tomato products using SIDA [62]. The samples had been screened for the mycotoxins AOH, AME, ochratoxin A (OTA) and fumonisin B_1, B_2 and B_3 using liquid chromatography-high resolution mass spectrometry, before. All samples that contained AOH or AME were also contaminated with TA (0.7 – 4.8 mg/kg). A dietary exposure assessment was performed for TA with Belgian consumption data, and the obtained mean value (4230 ng/kg bw/day) was higher than the threshold of toxicological concern (TTC) value of 1500 ng/kg bw/day set by EFSA [58].

A very recent UPLC-ESI$^{+/-}$-MS/MS method for the simultaneous determination of some free (AOH, AME, ALT, TA, TEN, ATX-I) and conjugated (sulphates and glucosides of AOH and AME) *Alternaria* toxins in cereals and cereal products (rice, oat flakes and barley) has been described [63] applying isotopically labelled internal standards ([$^{2}H_4$]-AME and [$^{13}C_6$,^{15}N]-TA) for SIDAs of AME and TA. [$^{13}C_6$,^{15}N]- was also used as internal standard for all other surveyed toxins. Extensive validation of the method has been carried out very thoroghly for all compunds using different matrices and spiking levels. Good values for recovery (> 95 %) and precison (< 10 %) were obtained, in general, but even better validation data were

obtained for TA and AME that were analyzed with SIDA. Finally, 24 commercially available cereal-based foodstuffs were analyzed for all 10 toxins. Beside some trace levels of AOH and TEN in rice samples, TA was the toxin most often detected. A total of 71% (22/31) of the rice samples, and 31% (5/16) of the oat flake samples were contaminated with concentrations in the range of 1.9–113 µg/kg and 2.1–39 µg/kg, respectively.

b) [$^{13}C_2$]-TA

Another synthetic route towards labelled tenuazonic acid was reported recently [64]. [$^{13}C_2$]-tenuazonic acid was elaborated in a three-step procedure starting from unlabelled Boc-protected isoleucine followed by condensation with meldrum's acid, thermal cyclization, decarboxylation and finally introducing the labelled carbons via 3-C-acetylation with [$^{13}C_2$]-acetyl chloride (Fig. 7, II). Apart from the good yield (67 %), the particular benefit of this procedure was the introduction of the label in the last step via a rather cheap reagent. However, epimerization at C-5 also took place during the last step of this approach, so until now there has not been prepared any stereochemically pure labeled standard for TA, yet. Using the [$^{13}C_2$]-TA isotopologue a SIDA was developed for tomato and pepper products based on sample preparation using the QuEChERS procedure. No derivatization was applied and the method was less sensitive (LOD 0.86 µg/kg; LOQ 2.89 µg/kg), therefore. Recovery was determined for different matrices and ranged between 91.0 ± 1.2 % (pepper paste) and 102 ± 4.4 % (tomato products). No data about the precision of the method and the linear range of the calibration were given. The method was applied to 26 tomato samples and 4 bell pepper samples from the German market and TA was found in each sample with levels ranging from 3 to 2330 µg/kg.

3. Perylenequinones

Derivatives of perylenquinone such as altertoxin (ATX) I, II, III, alterperylenol (APOL; syn. "alteichin", ALTCH), and stemphyltoxin (STX) III (Fig. 8) are minor metabolites of *Alternaria*

spp., but are regarded most critical due to their mutagenic properties [65, 66]. Due to the lack of available reference compounds in particular of altertoxins, analytical studies are rare. Therefore, biosynthesis of altertoxins and other perylenes (Fig. 8) by *Alternaria* was studied in a modified Czapek–Dox medium with a low level of glucose as the carbon source and ammonium sulphate as the sole nitrogen source [49]. Under these conditions the perylenequinones were the most abundant metabolites produced by the employed *A. alternata* strain and the required compounds were isolated from the culture broth, purified by preparative HPLC and used as analytical standards in the following. Labeled altertoxins were synthesized in the same way using [$^{13}C_6$]-glucose. However, the obtained [^{13}C]-isotopologues were not completely labelled as unlabeled citric acid in the trace element solution contributed with one $^{12}C_2$ unit into the biosynthetic pathway and caused the emergence of the [$^{13}C_{18}$]-isotopologues, which amounted to about 40% of the [$^{13}C_{20}$]-isotopologues for all compounds. Unfortunately, the mass increment of 18 Da caused an unexpected mass spectral overlap between the internal standards and the unlabelled altertoxins as the hydroxyl group of C-12a (Fig. 9) in the bay corner is easily eliminated during ionization, thus leading to a loss of water equivalent to 18 Da in the ion source. Thus, the fragments (a) were not suitable for quantitation and the fragments (c) were chosen instead. SIDAs were developed for ATX I, ATX II and APOL and validation resulted in good values for precision (3.8–9.9 %) and recovery (96–105 %). Using SIDA, 35 food samples form the German market were analyzed and ATX I and APOL were detected in organic whole grains and paprika powder with maximum values of 4.7 µg/kg (ATX I) and 5.2 µg/kg (APOL). ATX II and III and STX III were not detectable. In addition, if the samples were contaminated with ATX, they were likely to be co-contaminated with other *Alternaria* toxins such as AOH, AME and TEN, but not necessarily vice versa. Much higher values of perylenequinones were found in feed samples based on sorghum. Maximum concentrations

of ATX I and APOL were 43 and 58 µg/kg, respectively, in some cases ATX II was also detected (max. 1.7 µg/kg).

4. Tentoxin

The cyclic tetrapeptide tentoxin (TEN) is produced as secondary metabolite by some *Alternaria* species along with dihydrotentoxin (DHT) and isotentoxin (isoTEN) (Fig. 9) [67, 68]. Their structures differ at the unsaturated bond of the *N*-methyldehydrophenylalanine moiety, which is hydrogenated into a single bond in DHT and "*E"* configured in isoTEN. All three compounds are considered as phytotoxins with TEN being the most potent one, inhibiting the photophosphorylation and inducing chlorosis [69]. However, no toxicological data are available for mammals and the data about occurrence of this toxin in food and feed are limited as well. Triply deuterated TEN was prepared via total synthesis from simple building blocks (Fig. 10) and [$^2H_3$]-DHT and [$^2H_3$]-isoTEN were synthesized starting from [$^2H_3$]-TEN by hydrogenation or isomerization, respectively [70]. The label was introduced with [$^2H_3$]-iodomethane in the last step before cyclization. Although the yield of the whole synthetic procedure was only 0.24 %, it can be calculated to be about 10 % for the steps after introducing the label, which is reasonable and emphasizes the need for thorough planning of the synthetic routes leading to labelled compounds. The method was applied to 103 food samples including bread, cereals, chips, juice, nuts, oil, sauce, seeds and spices. Of these, 85 % were contaminated with tentoxin and 55 % were contaminated with dihydrotentoxin, whereas isotentoxin was not quantifiable. Maximal concentrations of tentoxin and dihydrotentoxin were 52.4 and 36.3 µg/kg, respectively, and were both detected in paprika powder.

PART C: Ochratoxin A

After the first report on the synthesis of a labelled OTA analogue, namely [$^{2}H_{5}$]-OTA [71], two further isotopologues have been made commercially available or have been reported in the literature, respectively. The first one, [$^{13}C_{20}$]-OTA, is commercially available and has been used since its generation in several studies either on the multi-mycotoxin contaminations presented in the table or specifically on OTA, e.g. in coffee [72] or on toxicokinetics in rats [73]. Whereas [$^{13}C_{20}$]-OTA has been prepared microbiologically and can be expected enantiomerically pure in its (3*R*,14*S*) configuration, in chemical syntheses the stereochemistry of the prepared OTA has to be considered. Owing to its two stereo centers at C-3 and C-14, four diastereomers are possible. The first isotopologue reported, [$^{2}H_{5}$]-OTA [71], has not been characterized with regard to its stereochemistry. However, according to its preparation by base catalyzed coupling of ochratoxin alpha (Otα) with [$^{2}H_{5}$]-L-phenylalanine methyl ester, a partly racemization of the phenylalanin moiety can be assumed, thus giving the respective pair of diastereomers. This isomerization also has been observed to occur thermally during roasting of coffee [74] and, for accurate analysis, the respective [$^{2}H_{5}$]-(14*R*)-OTA was prepared by thermal isomerization of enantiomerically pure [$^{2}H_{5}$]-(14*S*)-OTA. This stereo differentiation will enable to evaluate the contribution of heated foods containing OTA to the overall exposure when using blood analyses for assessing the “internal exposure” of humans to the mycotoxin. For OTA, stable isotopologues are increasingly used also in clinical analysis [75]. In the latter study - for the first time in mycotoxin analysis - the quantitation of exact amounts of standards by quantitative NMR was introduced thus providing better accuracy also for SIDAs. During analysis of OTA, stereochemistry is crucial as often immunoaffinity cleanup is applied and the latter may be enantiospecific. This effect has been observed during the use of the (3*S*)-diastereomer as possible internal standard for OTA in a diastereomeric dilution assay (DIDA) as an alternative to SIDA. For this study, both racemic unlabeled and deuterated Otα have been

prepared chemically [76] and coupled to L-phenylalanine. As a result, the respective DIDA was found to show significantly less accuracy than the SIDA used in parallel [77]. In a complementary approach, Gabriele et al. [78] synthesized racemic Otα in a three-step procedure and coupled the latter with protected [$^{2}H_5$]-L-phenylalanine to give a mixture of (3*S*)- and (3*R*)- diastereomersof OTA, which were separated by preparative TLC. In contrast to this, stereoisomeric pure Otα has been prepared in another study to be used as educt for labelled OTA after coupling with labelled phenylalanine. Two new routes for the synthesis of enantiomerically pure (3*R*)-OTα are presented [79]. The key step of both routes is the directed *ortho*-metalation of unprotected and suitably functionalized aromatic carboxylic acids, followed by an alkylation-cyclization reaction with (*R*)-propylene oxide.

Commercially available labelled standards for mycotoxins

The emerging availability of commercial labeled standards led to increased use in mycotoxin analysis. Out of the 30 published LC-MS methods covering the years 2008 – 2013 that have recently been reviewed [80], 10 of them use at least one commercially labeled standard for correction of the analytical results [81–90]. More recent methods follow this trend and perform SIDAs for all or at least some analyzed mycotoxins (table). At present, labelled standards are commercially available for all mycotoxins, the maximum contents of which are internationally regulated in foods and feeds, and are provided as uniformly carbon-13 labelled (“U-[^{13}C]”) isotopologues, in general. The aflatoxins (AF) may serve as example, as for them the labeled internal standards U-[^{13}C]-AFB_1, U-[^{13}C]-AFB_2, U-[^{13}C]-AFG_1, U-[^{13}C]-AFG_2 and U-[^{13}C]-AFM_1 can be purchased from well-known suppliers of chemicals. Further examples are U-[^{13}C]-patulin, U-[^{13}C]-ocharatoxin A, some trichothecenes (U-[^{13}C]-T-2 toxin and U-[^{13}C]-HT-2 toxin) and the fumonisins B_1, B_2 and B_3 that all can be purchased as U-[^{13}C]-isotopologues as well. However, some other labelled mycotoxins not being legally controlled routinely are also commercially available at the

moment, e. g. U-[^{13}C]-nivalenol or U-[^{13}C]-kojic acid and an increasing number of others that may serve the specific purpose of the researcher, but whose long-term availability may be doubted due to lack of general demand. Although the range of labelled compounds does not seem to point to a certain "most needed" lacking compound actually, most likely researchers will not find their desired compound commercially available and have to develop individual strategies towards their synthesis.

Concerning the reliability of the results, a closer look reveals that the majority of the recent methods tend to add the labelled standard to the sample matrix directly at the beginning of the sample preparation (table), which is the best method to counterbalance analyte losses during the analytical procedure. However, in some methods the labelled standard is added just before the LC-MS measurement, which compensates for suppression or enhancement effects in the ion source of the mass spectrometer, but leaves analyte losses during sample preparation aside and, therefore, is less reliable.

Discussion of Perspectives

Since our first review on SIDAs in mycotoxin analysis, the stock of available stable isotopologues has tremendously expanded. All mycotoxins with maximum limits (ML) in foods are commercially available labelled with stable isotopes. When reviewing the synthetic strategies towards labelled mycotoxins, on the one hand, biosynthesis by fungi versus chemical synthesis, on the other hand, can be distinguished, in principal. As a guideline, the more complex the molecule is the more efficient a biotechnologically approach may be. For example, isotopologues for the small molecule TA up to now have only been synthesized chemically. In contrast to this, biosynthesis offers the perspective to fully labelled isotopologues, either with ^{13}C (c.f. altertoxins) or with ^{15}N (c.f. enniatins). Most of the commercially available labelled mycotoxins are biotechnologically produced as

fully carbon-13 labelled isotopologues and - as a matter of fact - are applied more often than the chemically synthesized ones.

Generally, it can be recognized that SIDAs gain a status as reference methods due to several benefits. The surpassing attributes of SIDAs in terms of recovery and precision are essential, if low MLs trace amounts have to be accurately quantitated. Moreover, the versatile and sensitive LC-MS/MS instrumentation has become standard laboratory equipment facilitating the low LODs and LOQs required in MLs supervision, but its susceptibility to matrix interference does not leave many alternatives than using stable isotope labelled isotopologues as internal standard. Finally, more and more analysts realize that the price of the labelled standard is negligible, if calculated for the respective sample and compared to personnel labour costs and general expenses for consumables (solvents, columns for clean-up, etc.) and for acquiring and running LC-MS/MS equipment. However, prices for stable isotope-labelled standards still can be expected to be too high for a breakthrough of multiple SIDAs. This is due to mycotoxins occurring in very different contents in food matrices and, therefore, a multi-SIDA would require high standard additions for the most abundant mycotoxins, at least when added at the initial of a SIDA in its pure philosophy. The addition of the labelled standard just immediately before the LC-MS/MS measurement is sometimes performed, therefore (table), but analyte losses during sample preparation are not compensated for any more in this case, requiring determination of and correction for recovery. Moreover, MLs require highly varying sensitivity and linear ranges of the LC-MS/MS method (up to four orders of magnitude), which is not easy to achieve with standard instruments and calibrations. Thus, specifically combined ones rather than multiple SIDAs will still be used in the medium term.

Further challenges arise from the “emerging mycotoxins” such as *Alternaria* toxins, for which the commercial availability of stable isotopologues is still limited. However, research

groups are increasingly willing to cooperate and exchange labelled isotopologues non-commercially.

Moreover, the issue of “modified mycotoxins” is emerging, which formerly have been known as “masked mykotoxins”, but the latter term is now accepted to be strained [91]. New strategies for identification of modified mycotoxins are required with a promising approach being non targeted metabolomics as exemplified for DON [92]. Risk assessment of modified mycotoxins is limited due to the lack of analytical methods, which in turn restricts studies on toxicology and exposure surveys. First opinions of risk assessment authorities on modified mycotoxins dealt with the best studied compounds, i.e. with DON and its acetates [93] and, more recently, with zearalenone and its metabolites [94].

REFERENCES

1. Rychlik M, Asam S (2008) Stable isotope dilution assays in mycotoxin analysis. Anal. Bioanal. Chem. 390(2):617–628
2. Hartmann N, Erbs M, Wettstein FE, Schwarzenbach RP, Bucheli TD (2007) Quantification of estrogenic mycotoxins at the ng/L level in aqueous environmental samples using deuterated internal standards. J. Chromatogr. A. 1138(1–2):132–140
3. Miles CO, Erasmuson AF, Wilkins AL, Towers NR, Smith BL, Garthwaite I, Scahill BG, Hansen RP (1996) Ovine metabolism of zearalenone to α-zearalanol (zeranol). J. Agric. Food Chem. 44(10):3244–3250
4. Cramer B, Bretz M, Humpf H (2007) Stable isotope dilution analysis of the *Fusarium* mycotoxin zearalenone. J. Agric. Food Chem. 55(21):8353–8358
5. Peters CA (1972) Photochemistry of zearalenone and its derivatives. J. Med. Chem. 15(8):867–868
6. Köppen R, Riedel J, Emmerling F, Koch M (2012) (3*S* ,11*Z*)-14,16-Dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1 H-2-benzoxacyclotetradecine-1,7(8 H)-dione (cis-zearalenone): a redetermination. Acta Crystallogr E Struct Rep Online 68(3):o832
7. Köppen R, Riedel J, Proske M, Drzymala S, Rasenko T, Durmaz V, Weber M, Koch M (2012) Photochemical trans-/cis-isomerization and quantitation of zearalenone in edible oils. J. Agric. Food Chem. 60(47):11733–11740
8. Drzymala S, Riedel J, Köppen R, Garbe L, Koch M (2014) Preparation of ^{13}C-labelled *cis*-zearalenone and its application as internal standard in stable isotope dilution analysis. World Mycotoxin J. 7(1):45–52
9. Cole RJ, Kirksey JW, Cutler HG, Doupnik BL, Peckham JC (1973) Toxin from *Fusarium* moniliforme: Effects on plants and animals. Science 179(4080):1324–1326
10. Springer JP, Clardy J, Cole RJ, Kirksey JW, Hill RK, Carlson RM, Isidor JL (1974) Structure and synthesis of moniliformin, a novel cyclobutane microbial toxin. J. Am. Chem. Soc. 96(7):2267–2268
11. Schütt F, Nirenberg HI, Demi G (1998) Moniliformin production in the genus *Fusarium*. Mycotox. Res. 14(1):35–40
12. Sharman M, Gilbert J, Chelkowski J (1991) A survey of the occurrence of the mycotoxin moniliformin in cereal samples from sources worldwide. Food Addit. Contamin. 8(4):459–466
13. Kriek N, Marasas W, Steyn P, van Rensburg S, Steyn M (1977) Toxicity of a moniliformin-producing strain of *Fusarium moniliforme* var. subglutinans isolated from maize. Food Cosmet. Toxicol. 15(6):579–587
14. Burmeister HR, Ciegler A, Vesonder RF (1979) Moniliformin, a metabolite of *Fusarium moniliforme* NRRL 6322: purification and toxicity. Appl. Environ. Microbiol. 37(1):11–13
15. Jonsson M, Jestoi M, Nathanail AV, Kokkonen U, Anttila M, Koivisto P, Karhunen P, Peltonen K (2013) Application of OECD Guideline 423 in assessing the acute oral toxicity of moniliformin. Food Chem. Toxicol. 53:27–32

16. Jonsson M, Atosuo J, Jestoi M, Nathanail AV, Kokkonen U, Anttila M, Koivisto P, Lilius E, Peltonen K (2015) Repeated dose 28-day oral toxicity study of moniliformin in rats. Toxicol. Lett. 233(1):38–44
17. Bellus D, Fischer H, Greuter H, Martin P (1978) Synthesen von Moniliformin, einem Mycotoxin mit Cyclobutendion-Struktur. Helv. Chim. Acta 61(5):1784–1813
18. Fétizon M, Hanna I (1990) 2,3-Dihydro-1,4-dioxin in Organic Chemistry. Part X. 1 A new synthesis of 3-Hydroxy-3-cyclobutene-1,2-dione (semisquaric acid). Synthesis 1990(07):583–584
19. Lohrey L, Murata T, Uemura D, Humpf H (2011) Synthesis of isotopically labeled *Fusarium* mycotoxin $^{13}C_2$-moniliformin [1-hydroxycyclobut-1-ene-3,4-dione]. Synlett 2011(15):2242–2244
20. Von Bargen KW, Lohrey L, Cramer B, Humpf H (2012) Analysis of the *Fusarium* mycotoxin moniliformin in cereal samples using $^{13}C_2$-moniliformin and high-resolution mass spectrometry. J. Agric. Food Chem. 60(14):3586–3591
21. Jestoi M, Rokka M, Rizzo A, Peltonen KM (2003) Moniliformin in finnish grains: analysis with LC-MS/MS. Aspects Appl. Biol. 68:211–216
22. Jin P, Han Z, Cai Z, Wu Y, Ren Y (2010) Simultaneous determination of 10 mycotoxins in grain by ultra-high-performance liquid chromatography–tandem mass spectrometry using $^{13}C_{15}$-deoxynivalenol as internal standard. Food Addit. Contamin.: Part A 27(12):1701–1713
23. Sørensen JL, Nielsen KF, Thrane U (2007) Analysis of Moniliformin in Maize Plants Using Hydrophilic Interaction Chromatography. J. Agric. Food Chem. 55(24):9764–9768
24. Scarpino V, Blandino M, Negre M, Reyneri A, Vanara F (2013) Moniliformin analysis in maize samples from North-West Italy using multifunctional clean-up columns and the LC-MS/MS detection method. Food Addit. Contamin.: Part A 30(5):876–884
25. Sewram V, Nieuwoudt TW, Marasas, W. F. O, Shephard GS, Ritieni A (1999) Determination of the mycotoxin moniliformin in cultures of *Fusarium subglutinans* and in naturally contaminated maize by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A 848(1–2):185–191
26. Thrane U (1988) Screening for Fusarin C production by European isolates of *Fusarium* species. Mycotox. Res. 4(1):2–10
27. Farber JM, Sanders GW (1986) Production of Fusarin C by *Fusarium* spp. J. Agric. Food Chem. 34(6):963–966
28. Wiebe LA, Bjeldanes LF (1981) Fusarin C, a mutagen from *Fusarium moniliforme* grown on corn. J. Food Sci. 46(5):1424–1426
29. Gelderblom, W. C. A., Thiel PG, van der Merwe, K. J., Marasas, W. F. O., Spies, H. S. C. (1983) A mutagen produced by *Fusarium moniliforme*. Toxicon 21(4):467–473
30. Gelderblom WCA, Thiel PG, Marasas WFO, van der Merwe KJ (1984) Natural occurrence of fusarin C, a mutagen produced by *Fusarium moniliforme*, in corn. J. Agric. Food Chem. 32(5):1064–1067
31. Thiel PG, Gelderblom WCA, Marasas WFO, Nelson PE, Wilson TM (1986) Natural occurrence of moniliformin and fusarin C in corn screenings known to be hepatocarcinogenic in rats. J. Agric. Food Chem. 34(5):773–775

32. Kleigrewe K, Söhnel A, Humpf H (2011) A new high-performance liquid chromatography–tandem mass spectrometry method based on dispersive solid phase extraction for the determination of the mycotoxin fusarin C in corn ears and processed corn samples. J. Agric. Food Chem. 59(19):10470–10476

33. Kleigrewe K, Niehaus E, Wiemann P, Tudzynski B, Humpf H (2012) New approach via gene knockout and single-step chemical reaction for the synthesis of isotopically labeled fusarin C as an internal standard for the analysis of this *Fusarium* mycotoxin in food and feed samples. J. Agric. Food Chem. 60(34):8350–8355

34. Logrieco A, Rizzo A, Ferracane R, Ritieni A (2002) Occurrence of beauvericin and enniatins in wheat affected by *Fusarium avenaceum* head blight. Appl. Environ. Microbiol. 68(1):82–85

35. Morrison E, Kosiak B, Ritieni A, Aastveit AH, Uhlig S, Bernhoft A (2002) Mycotoxin production by *Fusarium avenaceum* strains isolated from Norwegian grain and the cytotoxicity of rice culture extracts to porcine kidney epithelial cells. J. Agric. Food Chem. 50(10):3070–3075

36. Thrane U, Adler A, Clasen P, Galvano F, Langseth W, Lew H, Logrieco A, Nielsen KF, Ritieni A (2004) Diversity in metabolite production by *Fusarium langsethiae*, *Fusarium poae*, and *Fusarium sporotrichioides*. Int. J. Food Microbiol. 95(3):257–266

37. Benford D, Ceccatelli S, Cottrill B, Dinovi M, Dogliotti E, Edler L, Farmer P, Furst P, Hoogenboom L, Knutsen HK, Lundebye A, Metzler M, Nebbia CS, O'Keeffe M, Rietjens I, Schrenk D, Silano V, van Loveren H, Vleminckx C, Wester P (2014) Scientific opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. EFSA J. 12(8):3802/1-3802/174, 174 pp

38. Sørensen JL, Nielsen KF, Rasmussen PH, Thrane U (2008) Development of a LC-MS/MS method for the analysis of enniatins and beauvericin in whole fresh and ensiled maize. J. Agric. Food Chem. 56(21):10439–10443

39. Uhlig S, Ivanova L (2004) Determination of beauvericin and four other enniatins in grain by liquid chromatography–mass spectrometry. J. Chromatogr. A 1050(2):173–178

40. Sewram V, Nieuwoudt TW, Marasas, W. F. O, Shephard GS, Ritieni A (1999) Determination of the *Fusarium* mycotoxins, fusaproliferin and beauvericin by high-performance liquid chromatography–electrospray ionization mass spectrometry. J. Chromatogr. A 858(2):175–185

41. Jestoi M, Rokka M, Järvenpää E, Peltonen K (2009) Determination of *Fusarium* mycotoxins beauvericin and enniatins (A, A1, B, B1) in eggs of laying hens using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Food Chem. 115(3):1120–1127

42. Bräse S, Encinas A, Keck J, Nising CF (2009) Chemistry and Biology of Mycotoxins and Related Fungal Metabolites. Chem. Rev. 109(9):3903–3990

43. Hu L, Rychlik M (2012) Biosynthesis of $^{15}N_3$-labeled enniatins and beauvericin and their application to stable isotope dilution assays. J. Agric. Food Chem. 60(29):7129–7136

44. Bottalico A, Logrieco A (1998) Toxigenic *Alternaria* species of economic importance. In: Kaushal K, Bhatnagar D (eds) Mycotoxins in agriculture and food safety. Marcel Dekker, NY, pp. 65–108

45. Fehr M, Pahlke G, Fritz J, Christensen MO, Boege F, Altemöller M, Podlech J, Marko D (2009) Alternariol acts as a topoisomerase poison, preferentially affecting the IIα isoform. Mol. Nutr. Food Res. 53(4):441–451

46. Brugger E, Wagner J, Schumacher DM, Koch K, Podlech J, Metzler M, Lehmann L (2006) Mutagenicity of the mycotoxin alternariol in cultured mammalian cells. Tox. Lett. 164(3):221–230

47. Asam S, Konitzer K, Schieberle P, Rychlik M (2009) Stable isotope dilution assays of alternariol and alternariol monomethyl ether in beverages. J. Agric. Food Chem. 57(12):5152–5160

48. Asam S, Konitzer K, Rychlik M (2011) Precise determination of the *Alternaria* mycotoxins alternariol and alternariol monomethyl ether in cereal, fruit and vegetable products using stable isotope dilution assays. Mycotox. Res. 27(1):23-28

49. Liu Y, Rychlik M (2015) Biosynthesis of seven carbon-13 labeled *Alternaria* toxins including altertoxins, alternariol, and alternariol methyl ether, and their application to a multiple stable isotope dilution assay. Anal. Bioanal. Chem. 407(5):1357–1369

50. Pero RW, Posner H, Blois M, Harvan D, Spalding JW (1973) Toxicity of metabolites produced by the *Alternaria*. Environ. Health Perspect. (4):87–94

51. Asam S, Liu Y, Konitzer K, Rychlik M (2011) Development of a stable isotope dilution assay for tenuazonic acid. J. Agric. Food Chem. 59(7):2980–2987

52. Siegel D, Merkel S, Bremser W, Koch M, Nehls I (2010) Degradation kinetics of the *Alternaria* mycotoxin tenuazonic acid in aqueous solutions. Anal. Bioanal. Chem. 397(2):453-462

53. Siegel D, Rasenko T, Koch M, Nehls I (2009) Determination of the *Alternaria* mycotoxin tenuazonic acid in cereals by high-performance liquid chromatography-electrospray ionization ion-trap multistage mass spectrometry after derivatization with 2,4-dinitrophenylhydrazine. J. Chromatogr. A. 1216(21):4582–4588

54. Siegel D, Merkel S, Koch M, Nehls I (2010) Quantification of the *Alternaria* mycotoxin tenuazonic acid in beer. Food Chem. 120(3):902–906

55. Asam S, Lichtenegger M, Liu Y, Rychlik M (2012) Content of the *Alternaria* mycotoxin tenuazonic acid in food commodities determined by a stable isotope dilution assay. Mycotoxin Res 28(1):9-15

56. Asam S, Rychlik M (2013) Potential health hazards due to the occurrence of the mycotoxin tenuazonic acid in infant food. Eur. Food Res. Technol. 236(3):491–497

57. Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, Schilter B, Schlatter J, van Schothorst F, Vos JG, Wurtzen G (2004) Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. Food Chem. Toxicol. 42(1):65–83

58. Alexander J, Benford D, Boobis A, Ceccatelli S, Cottrill B, Cravedi J, Di Domenico A, Doerge D, Dogliotti E, Edler L, Farmer P, Filipic M, Fink-Gremmels J, Furst P, Guerin T, Knutsen HK, Machala M, Mutti A, Schlatter J, Rose M, van Leeuwen R, Metzler M, Rauscher-Gabernig E, van Peteghem C, Civera AV, Cioacata G, Curtui V, Eskola M, Heppner C (2011) Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. EFSA J 9(10):2407, 97 pp

59. Asam S, Lichtenegger M, Muzik K, Liu Y, Frank O, Hofmann T, Rychlik M (2013) Development of analytical methods for the determination of tenuazonic acid analogues in food commodities. J. Chromatogr. A. 1289:27–36

60. Asam S, Habler K, Rychlik M (2013) Determination of tenuazonic acid in human urine by means of a stable isotope dilution assay. Anal. Bioanal. Chem. 405(12):4149–4158

61. Fraeyman S, Devreese M, Broekaert N, De Mil T, Antonissen G, De Baere S, De Backer P, Rychlik M, Croubels S In vivo hazard characterization of the *Alternaria* mycotoxin tenuazonic acid in broiler chickens and pigs: Comparative toxicokinetics and absolute oral bioavailability. (submitted)

62. Van de Perre E, Deschuyffeleer N, Jacxsens L, Vekeman F, Van der Hauwaert W, Asam S, Rychlik M, Devlieghere F, De Meulenaer B (2014) Screening of moulds and mycotoxins in tomatoes, bell peppers, onions, soft red fruits and derived tomato products. Food Control 37:165–170

63. Walravens J, Mikula H, Rychlik M, Asam S, Ediage EN, Di Mavungu JD, Van Landschoot A, Vanhaecke L, De Saeger S (2014) Development and validation of an ultra-high-performance liquid chromatography tandem mass spectrometric method for the simultaneous determination of free and conjugated *Alternaria* toxins in cereal-based foodstuffs. J. Chromatogr. A 1372:91–101

64. Lohrey L, Marschik S, Cramer B, Humpf H (2013) Large-scale synthesis of isotopically labeled $^{13}C_2$-tenuazonic acid and development of a rapid HPLC-MS/MS method for the analysis of tenuazonic acid in tomato and pepper products. J. Agric. Food Chem. 61(1):114–120

65. Stack ME, Prival MJ (1986) Mutagenicity of the *Alternaria* metabolites altertoxins I, II, and III. Appl. Environ. Microbiol. 52(4):718–722

66. Fleck SC, Burkhardt B, Pfeiffer E, Metzler M (2012) Alternaria toxins: Altertoxin II is a much stronger mutagen and DNA strand breaking mycotoxin than alternariol and its methyl ether in cultured mammalian cells. Tox. Lett. 214(1):27–32

67. Templeton GE, Grable, C. I., Fulton ND, Bollenbacher K (1967) Factors affecting the amount and pattern of chlorosis caused by a metabolite of *Alternaria tenuis*. Phytopathology 57(5):516–18

68. Kono Y, Gardner JM, Takeuchi S (1986) Nonselective phytotoxins simultaneously produced with host-selective ACTG-toxins by a pathotype of *Alternaria citri* causing brown spot disease of mandarins. Agric. Biol. Chem. 50(9):2401–2403

69. Arntzen CJ (1972) Inhibition of photophosphorylation by tentoxin, a cyclic tetrapeptide. Biochim. Biophys. Acta - Bioenergetics 283(3):539–542

70. Liu Y, Rychlik M (2013) Development of a stable isotope dilution LC–MS/MS method for the *Alternaria* toxins tentoxin, dihydrotentoxin, and isotentoxin. J. Agric. Food Chem. 61(12):2970–2978

71. Lindenmeier M, Schieberle P, Rychlik M (2004) Quantitation of Ochratoxin A in foods by a stable isotope dilution assay using high-performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1023: 57-66

72. Noba S, Uyama A, Mochizuki N (2009) Determination of Ochratoxin A in ready-to-drink coffee by immunoaffinity cleanup and liquid chromatography-tandem mass spectrometry. J. Agric. Food Chem. 57(14):6036–6040

73. Han Z, Zhao Z, Shi, J, Liao Y, Zhao Z, Zhang D, Wu Y, De Saeger S, Wu A (2013) Combinatorial approach of LC–MS/MS and LC–TOF-MS for uncovering in vivo kinetics and biotransformation of ochratoxin A in rat. J. Chromatogr. B 925:46–53

74. Cramer B, Königs M, Humpf HU (2013) Identification and in vitro cytotoxicity of Ochratoxin A degradation products formed during coffee roasting. J. Agric. Food Chem. 56(14):5673–5681

75. Korn M, Frank O, Hofmann T,Rychlik M (2011) Development of stable isotope dilution assays for ochratoxin A in blood samples, Anal. Biochem., 419: 88-94

76. Bouisseau A, Roland A, Reillon F, Schneider R, Cavelier F(2013) First synthesis of a stable isotope of Ochratoxin A metabolite for a reliable detoxification monitoring. Organic Lett. 15(15): 3888-3890

77. Roland A, Bros P, Bouisseau A, Cavelier F, Schneider R (2014) Analysis of ochratoxin A in grapes, musts and wines by LC–MS/MS: First comparison of stable isotope dilution assay and diastereomeric dilution assay methods. Anal. Chim. Acta 818(1): 39-45

78. Gabriele B, Attya M, Fazio A, Di Donna L, Plastina P, Sindona G (2009) A new and expedient total synthesis of Ochratoxin A and d_5-Ochratoxin A. Synthesis 2009(11): 1815-1820

79. Lenz CA, Rychlik M (2013) Efficient synthesis of (*R*)-ochratoxin alpha, the key precursor to the mycotoxin ochratoxin A. Tetrahedron Lett. 54(8):883–886

80. Pereira VL, Fernandes JO, Cunha SC (2014) Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis. Trends Food Sci. Technol. 36(2):96–136

81. Herebian D, Zühlke S, Lamshöft M, Spiteller M (2009) Multi-mycotoxin analysis in complex biological matrices using LC-ESI/MS: Experimental study using triple stage quadrupole and LTQ-Orbitrap. J. Sep. Sci. 32(7):939–948

82. Gottschalk C, Barthel J, Engelhardt G, Bauer J, Meyer K (2009) Simultaneous determination of type A, B and D trichothecenes and their occurrence in cereals and cereal products. Food Addit. Contamin.: Part A 26(9):1273–1289

83. Vaclavik L, Zachariasova M, Hrbek V, Hajslova J (2010) Analysis of multiple mycotoxins in cereals under ambient conditions using direct analysis in real time (DART) ionization coupled to high resolution mass spectrometry. Talanta 82(5):1950–1957

84. Zachariasova M, Lacina O, Malachova A, Kostelanska M, Poustka J, Godula M, Hajslova J (2010) Novel approaches in analysis of *Fusarium* mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. Anal. Chim. Acta 662(1):51–61

85. Tam J, Pantazopoulos P, Scott P, Moisey J, Dabeka R, Richard I (2011) Application of isotope dilution mass spectrometry: determination of ochratoxin A in the Canadian Total Diet Study. Food Addit. Contamin.: Part A 28(6):754–761

86. Pettersson H, Brown C, Hauk J, Hoth S, Meyer J, Wessels D (2011) Survey of T-2 and HT-2 toxins by LC–MS/MS in oats and oat products from European oat mills in 2005–2009. Food Addit. Contamin.: Part B 4(2):110–115

87. Juan C, Ritieni A, Mañes J (2012) Determination of trichothecenes and zearalenones in grain cereal, flour and bread by liquid chromatography tandem mass spectrometry. Food Chem. 134(4):2389–2397

88. Varga E, Glauner T, Koeppen R, Mayer K, Sulyok M, Schuhmacher R, Krska R, Berthiller F (2012) Stable isotope dilution assay for the accurate determination of mycotoxins in maize by UHPLC-MS/MS. Anal. Bioanal. Chem. 402(9):2675–2686

89. Barthel J, Gottschalk C, Rapp M, Berger M, Bauer J, Meyer K (2012) Occurrence of type A, B and D trichothecenes in barley and barley products from the Bavarian market. Mycotox. Res 28(2):97–106

90. Köppen R, Bremser W, Rasenko T, Koch M (2013) Development and certification of a reference material for *Fusarium* mycotoxins in wheat flour. Anal. Bioanal. Chem. 405(14):4755–4763

91. Rychlik M, Humpf H, Marko D, Dänicke S, Mally A, Berthiller F, Klaffke H, Lorenz N (2014) Proposal of a comprehensive definition of modified and other forms of mycotoxins including “masked” mycotoxins. Mycotox. Res. 30(4):197–205

92. Kluger B, Bueschl C, Lemmens M, Berthiller F, Häubl G, Jaunecker G, Adam G, Krska R, Schuhmacher R (2013) Stable isotopic labelling-assisted untargeted metabolic profiling reveals novel conjugates of the mycotoxin deoxynivalenol in wheat. Anal. Bioanal. Chem. 405(15):5031–5036

93. Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2011) Safety evaluation of certain contaminants in food / prepared by the Seventy-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). WHO Food Additives Series: 63

94. European Food Safety Authority (EFSA) (2014) Evaluation of the increase of risk for public health related to a possible temporary derogation from the maximum level of deoxynivalenol, zearalenone and fumonisins for maize and maize products. EFSA J. 12(5):3699–3730

95. Liao C, Wong JW, Zhang K, Yang P, Wittenberg JB, Trucksess MW, Hayward DG, Lee NS, Chang JS (2015) Multi-mycotoxin analysis of finished grain and nut products using ultrahigh-performance liquid chromatography and positive electrospray ionization–quadrupole orbital ion trap high-resolution mass spectrometry. J. Agric. Food Chem. (in print) DOI: 10.1021/jf505049a

96. Mata, A. T., Ferreira, J. P., Oliveira, B. R., Batoreu, M. C., Barreto Crespo, M. T., Pereira, V. J., Bronze, M. R. (2015) Bottled water: Analysis of mycotoxins by LC-MS/MS. Food Chem. 176:455–464

97. Kirincic S, Skrjanc B, Kos N, Kozolc B, Pirnat N, Tavcar-Kalcher G (2015) Mycotoxins in cereals and cereal products in Slovenia - Official control of foods in the years 2008-2012. Food Control 50:157–165

98. Brezina U, Rempe I, Kersten S, Valenta H, Humpf H, Daenicke S (2014) Diagnosis of intoxications of piglets fed with *Fusarium* toxin-contaminated maize by the analysis of mycotoxin residues in serum, liquor and urine with LC-MS/MS. Arch. Anim. Nutr. 68(6):425–447

99. Zhang K, Wong JW, Jia Z, Vaclavikova M, Trucksess MW, Begley TH (2014) Screening multimycotoxins in food-grade gums by stable isotope dilution and liquid chromatography/tandem mass spectrometry. J. AOAC Int. 97(3):889–895

100. Zhang K, Wong JW, Krynitsky AJ, Trucksess MW (2014) Determining mycotoxins in baby foods and animal feeds using stable isotope dilution and liquid chromatography tandem mass spectrometry. J. Agric. Food Chem. 62(36):8935–8943

101. Zhao Z, Rao Q, Song S, Liu N, Han Z, Hou J, Wu A (2014) Simultaneous determination of major type B trichothecenes and deoxynivalenol-3-glucoside in animal feed and raw materials using improved DSPE combined with LC-MS/MS. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 963:75–82

102. Pietruszka K, Sell B, Burek O, Wisniewska-Dmytrow H (2013) Simultaneous determination of multi - component mycotoxin in feeds by liquid chromatography - tandem mass spectrometry. Bull. Vet. Inst. Pulawy 57(4):567–572

103. Han Z, Feng Z, Shi W, Zhao Z, Wu Y, Wu A (2014) A quick, easy, cheap, effective, rugged, and safe sample pretreatment and liquid chromatography with tandem mass spectrometry method for the simultaneous quantification of 33 mycotoxins in *Lentinula edodes*. J. Sep. Sci. 37(15):1957–1966

104. Desmarchelier A, Tessiot S, Bessaire T, Racault L, Fiorese E, Urbani A, Chan W, Cheng P, Mottier P (2014) Combining the quick, easy, cheap, effective, rugged and safe approach and clean-up by immunoaffinity column for the analysis of 15 mycotoxins by isotope dilution liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1337:75–84

105. Blajet-Kosicka A, Twaruzek M, Kosicki R, Sibiorowska E, Grajewski J (2014) Co-occurrence and evaluation of mycotoxins in organic and conventional rye grain and products. Food Control 38:61–66

106. Abia WA, Warth B, Sulyok M, Krska R, Tchana A, Njobeh PB, Turner PC, Kouanfack C, Eyongetah M, Dutton M, Moundipa PF (2013) Bio-monitoring of mycotoxin exposure in Cameroon using a urinary multi-biomarker approach. Food Chem. Toxicol. 62:927–934

107. Gratz SW, Duncan G, Richardson AJ (2013) The human fecal microbiota metabolizes deoxynivalenol and deoxynivalenol-3-glucoside and may be responsible for urinary deepoxy-deoxynivalenol. Appl. Environ. Microbiol. 79(6):1821–1825

108. Osselaere A, Devreese M, Goossens J, Vandenbroucke V, De Baere S, De Backer P, Croubels S (2013) Toxicokinetic study and absolute oral bioavailability of deoxynivalenol, T-2 toxin and zearalenone in broiler chickens. Food Chem. Toxicol. 51:350–355

109. Al-Taher F, Banaszewski K, Jackson L, Zweigenbaum J, Ryu D, Cappozzo J (2013) Rapid method for the determination of multiple mycotoxins in wines and beers by LC-MS/MS using a stable isotope dilution assay. J. Agric. Food Chem. 61(10):2378–2384

110. Zhang K, Wong JW, Hayward DG, Vaclavikova M, Liao C, Trucksess MW (2013) Determination of mycotoxins in milk-based products and infant formula using stable isotope dilution assay and liquid chromatography tandem mass spectrometry. J. Agric. Food Chem. 61(26):6265–6273

111. De Baere S, Goossens J, Osselaere A, Devreese M, Vandenbroucke V, De Backer P, Croubels S (2011) Quantitative determination of T-2 toxin, HT-2 toxin, deoxynivalenol and deepoxy-deoxynivalenol in animal body fluids using LC-MS/MS detection. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 879(24):2403–2415

Tables

Table: Overview of LC-MS/MS methods for the determination of mycotoxins in different matrices using commercially available labeled standards published from 2011 – 2015 and not already been reviewed [72]

Analytes	Standards	Matrices	Time of addition	Reference
AFB1, AFB2, AFG1, AFG2, BEA, DON, DAS, 15-ADON, FB1, FB2, FUSX, HT-2, NEO, OTA, OTB, T-2, ZEN	[$^{13}C_{17}$]-AFB1, [$^{13}C_{20}$]-OTA, [$^{13}C_{18}$]-ZEN, [$^{13}C_{34}$]-FB1, [$^{13}C_{24}$]-T-2	Finished Grain and Nut Products	before MS	[95]
AFB1, AFB2, AFG1, AFG2, FB1, FB2, FB3, NEO, OTA	[$^{13}C_{17}$]-AFB2, [$^{13}C_{17}$]-AFG2, [$^{13}C_{34}$]-FB1, [$^{13}C_{20}$]-OTA,	Bottled water	to the sample	[96]
T-2, HT-2	[$^{13}C_{24}$]-T-2	Cereals and cereal products	before clean-up	[97]
DON, DOM, ZEN, α-ZEL, β-ZEL, ZAN, α-ZAL, β-ZAL,	[$^{13}C_{15}$]-DON, [$^{13}C_{18}$]-ZEN, α-[$^{2}H_{4}$]-ZEL, β-[$^{2}H_{4}$]-ZEL, α,β-[$^{2}H_{4}$]-ZAL	Animal body fluids	to the sample	[98]
AFB1, AFB2, AFG1, AFG2, DON, FB1, FB2, FB3, OTA, T-2, ZEN	[$^{13}C_{17}$]-AFB1, [$^{13}C_{17}$]-AFB2, [$^{13}C_{17}$]-AFG1, [$^{13}C_{17}$]-AFG2, [$^{13}C_{15}$]-DON, [$^{13}C_{34}$]-FB1, [$^{13}C_{34}$]-FB2, [$^{13}C_{34}$]-FB3, [$^{13}C_{20}$]-OTA, [$^{13}C_{24}$]-T-2, [$^{13}C_{18}$]-ZEN	a) food grade gums b) baby food and animal feed	to the sample	[99, 100]
DON, DOM, D3G, 15ADON, 3ADON, NIV, FUSX	[$^{13}C_{15}$]-DON	animal feed	before MS	[101]
DON, ZEN, T-2, HT-2, OTA	[$^{13}C_{15}$]-DON, [$^{13}C_{18}$]-ZEN, [$^{13}C_{24}$]-T-2, [$^{13}C_{22}$]-HT-2, [$^{13}C_{20}$]-OTA	feed	before MS	[102]
33 mycotoxins	[$^{13}C_{17}$]-AFB1, [$^{13}C_{20}$]-OTA, [$^{13}C_{15}$]-DON, [$^{13}C_{24}$]-T-2, [$^{13}C_{18}$]-ZEN	*Lentinula edodes*	to the sample	[103]
AFB1, AFB2, AFG1, AFG2, AFM1, DON, 3-	[$^{13}C_{17}$]-AFB1, [$^{13}C_{17}$]-AFB2, [$^{13}C_{17}$]-AFG1, [$^{13}C_{17}$]-AFG2, [$^{13}C_{17}$]-AFM1, [$^{13}C_{15}$]-DON,	Cocoa, infant formula, green coffee, red	to the sample	[104]

ADON, 15-ADON, NIV FB1, FB2, OTA, T-2, HT-2, ZEN	[$^{13}C_{17}$]-3ADON [$^{13}C_{15}$]-NIV, [$^{13}C_{34}$]-FB1, [$^{13}C_{34}$]-FB2, [$^{13}C_{20}$]-OTA, [$^{13}C_{24}$]-T-2, [$^{13}C_{22}$]-HT-2, [$^{13}C_{18}$]-ZEN	paprika, corn flour, sunflower oil, peanuts		
DON, NIV, 3-ADON, T-2, HT-2, MAS, DAS, ZEN, ZAN	[$^{13}C_{15}$]-DON	organic and conventional rye grain	(not given)	[105]
15 mycotoxins and metabolites	[$^{13}C_{15}$]-DON	Human urine	before MS	[106]
DON, D3G, DOM	[$^{13}C_{15}$]-DON	Human urine	to the sample	[107]
DON, DOM, T-2, HT-2, ZEN; α-ZEL, β-ZEL, ZAN, α-ZAL, β-ZAL	[$^{13}C_{15}$]-DON, [$^{13}C_{24}$]-T-2, [$^{13}C_{18}$]-ZEN	Plasma of broiler chicks	to the sample	[108]
AFB1, AFB2, AFG1, AFG2, DON, FB1, FB2, OTA, T-2, HT-2, ZEN	[$^{13}C_{17}$]-AFB1, [$^{13}C_{17}$]-AFB2, [$^{13}C_{17}$]-AFG1, [$^{13}C_{17}$]-AFG2, [$^{13}C_{15}$]-DON, [$^{13}C_{34}$]-FB1, [$^{13}C_{34}$]-FB2, [$^{13}C_{20}$]-OTA, [$^{13}C_{24}$]-T-2, [$^{13}C_{22}$]-HT-2, [$^{13}C_{18}$]-ZEN	Wines and beers	to the sample	[109]
AFB1, AFB2, AFG1, AFG2, AFM1, DON, FB1, FB2, FB3, OTA, T-2, ZEN	[$^{13}C_{17}$]-AFB1, [$^{13}C_{17}$]-AFB2, [$^{13}C_{17}$]-AFG1, [$^{13}C_{17}$]-AFG2, [$^{13}C_{17}$]-AFM1, [$^{13}C_{15}$]-DON, [$^{13}C_{34}$]-FB1, [$^{13}C_{34}$]-FB2, [$^{13}C_{34}$]-FB3, [$^{13}C_{20}$]-OTA, [$^{13}C_{24}$]-T-2, [$^{13}C_{18}$]-ZEN	Milk based products and infant formula	to the sample	[110]
DON, DOM, T-2, HT2-	[$^{13}C_{15}$]-DON, [$^{13}C_{24}$]-T-2	Animal body fluids	to the sample	[111]

Legends to the Figures

Fig. 1 Synthetic routes to labeled zearalenone: [I] according to [2]; [II] according to [4]

Fig. 2 Synthesis of [$^{13}C_2$]-moniliformin ("■" = ^{13}C) [19]

Fig. 3 Structure of fusarin C (R = CH_3) and its labelled isotopologue (R = $C[^2H]_3$) [33]

Fig. 4 Structures of the [$^2H_4$]-isotopologues of alternariol (R = H) and alternariol methyl ether (R = CH_3) [47]

Fig. 5 Proposed fragmentation of labeled and unlabeled alternariol to the main quantifier ions in LC-MS/MS [49]. In parentheses: the respective *m/z* data of the completely [^{13}C]-labeled isotopologue. In square brackets: the respective *m/z* data of the isotopologue with four [^2H]-labelings in the aromatic rings

Fig. 6 Proposed fragmentation of labeled and unlabeled alternariol monomethyl ether to the main quantifier ions in LC-MS/MS [49]. In parentheses: the respective *m/z* data of the completely [^{13}C]-labeled isotopologue. In square brackets: the respective *m/z* data of the isotopologue with four [^2H]-labelings in the aromatic rings

Fig. 7 Synthetic pathways leading to stable isotope labeled tenuazonic acid ("■" = ^{13}C)
(I) Acetoacylation of [$^{13}C_6$,^{15}N]-methyl isoleucinate with *in-situ* generated diketene followed by Dieckmann intramolecular condensation [47]
(II) Condensation of isoleucine with melldrum's acid, followed by thermal cyclization, decarboxylation and introduction of the label by 3-C-acylation with labeled acetyl chloride [64]

Fig. 8 Structures of perylenequinones produced by *Alternaria* species (ATX = Altertoxin; APLO = alterperylenol; STX = Stemphyltoxin)

Fig. 9 Spectral interferences between ATX I, [$^{13}C_{18}$]-ATX I and [$^{13}C_{20}$]-ATX I in mass spectroscopy [49]. In parentheses: the respective *m/z* data of the ^{13}C-labeled isotopologue (first value [$^{13}C_{18}$], second value [$^{13}C_{20}$]). Arrows indicate the respective spectral overlap

Fig. 10 Structures of cyclic tetrapeptides produced by *Alternaria* species (TEN = tentoxin; DHT = dihydrotentoxin; isoTEN = isotentoxin)

Fig. 11 The last two steps (introduction of the label and ring closure) of the synthesis of [$^{2}H_{3}$]-tentoxin [70] (“D” = [^{2}H]; “HBTU” = O-(benzotriazol-1-yl)-N,N,N’,N’-tetramethyl-uronium hexafluorophosphate; “DMF” = dimethylformamide; “tB” = *tert*-Butyl). The respective origin of the building blocks leading to the intermediate products are given in the light squares (“Leu” = leucine; “MeAla” = *N*-methyl-alanin; “BA” = benzaldehyde; “Gly” = glycine)

Fig. 12 Synthetic routes leading to enantiomerically pure ochratoxin alpha ((3*R*)-OTα) to be used as educt to produce stable OTA isotopologues [71] (“LTMP” = lithium tetramethylpiperidine; “THF” = tetrahydrofurane)

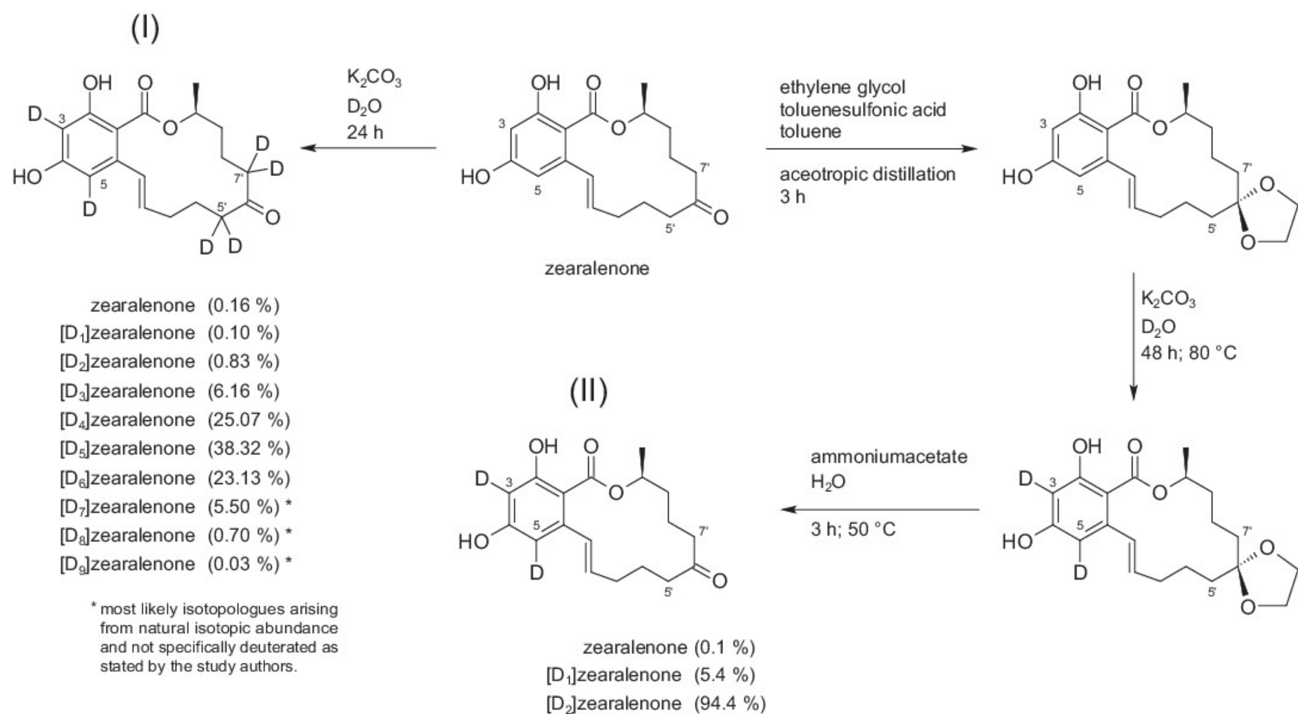

Fig. 1.

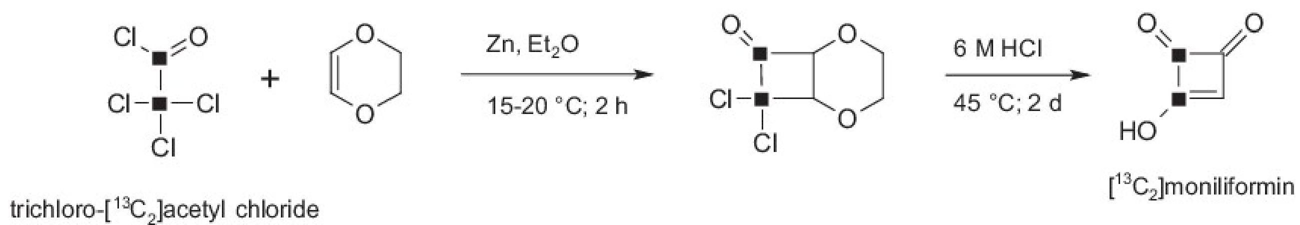

Fig. 2

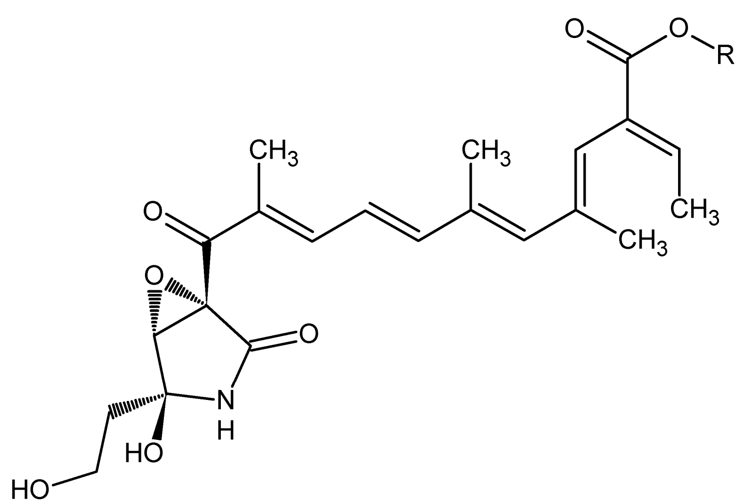

Fig. 3

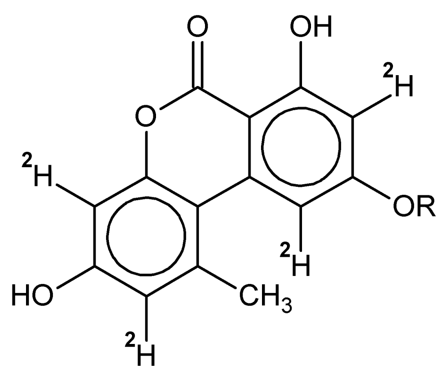

Fig. 4

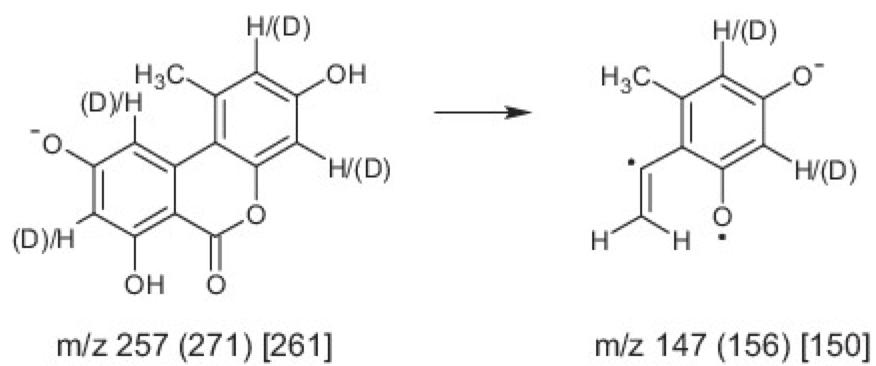

Fig. 5

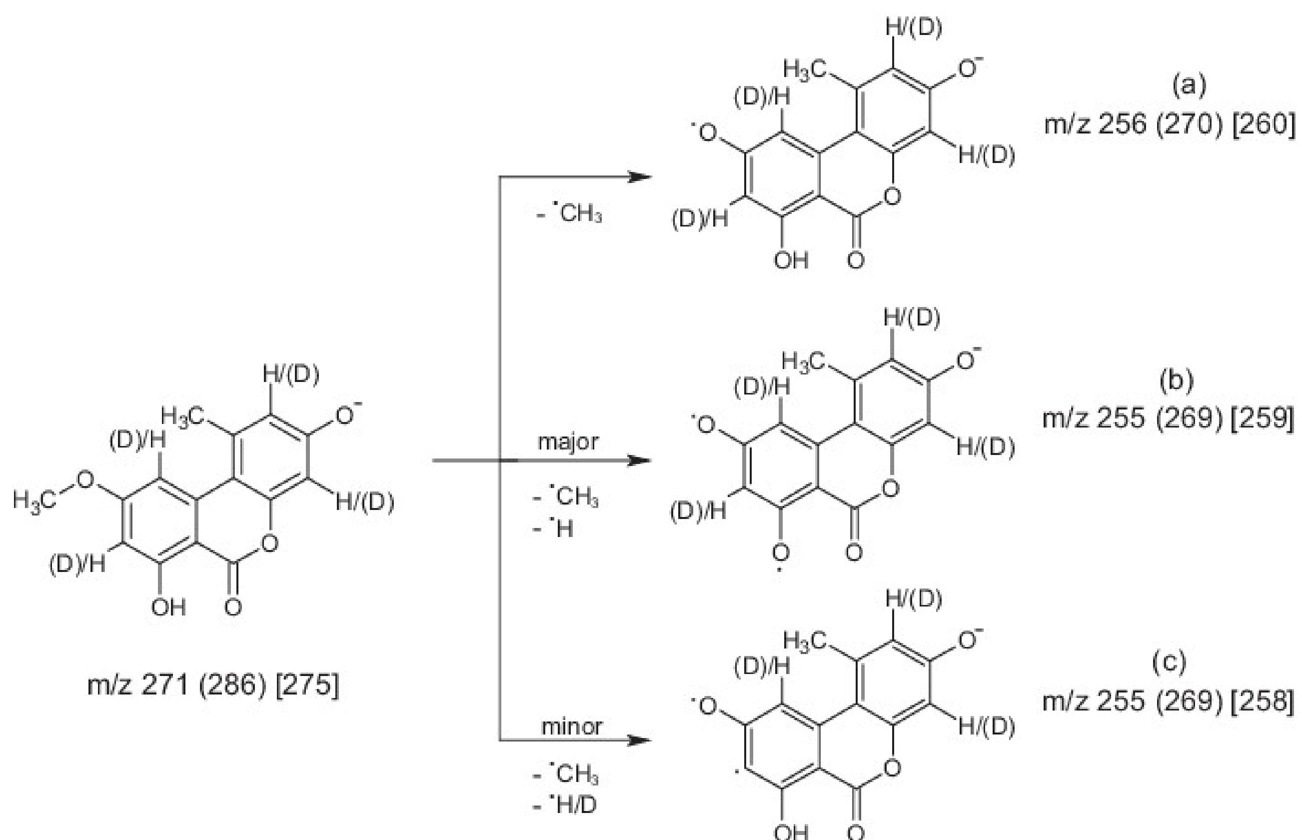

Fig. 6

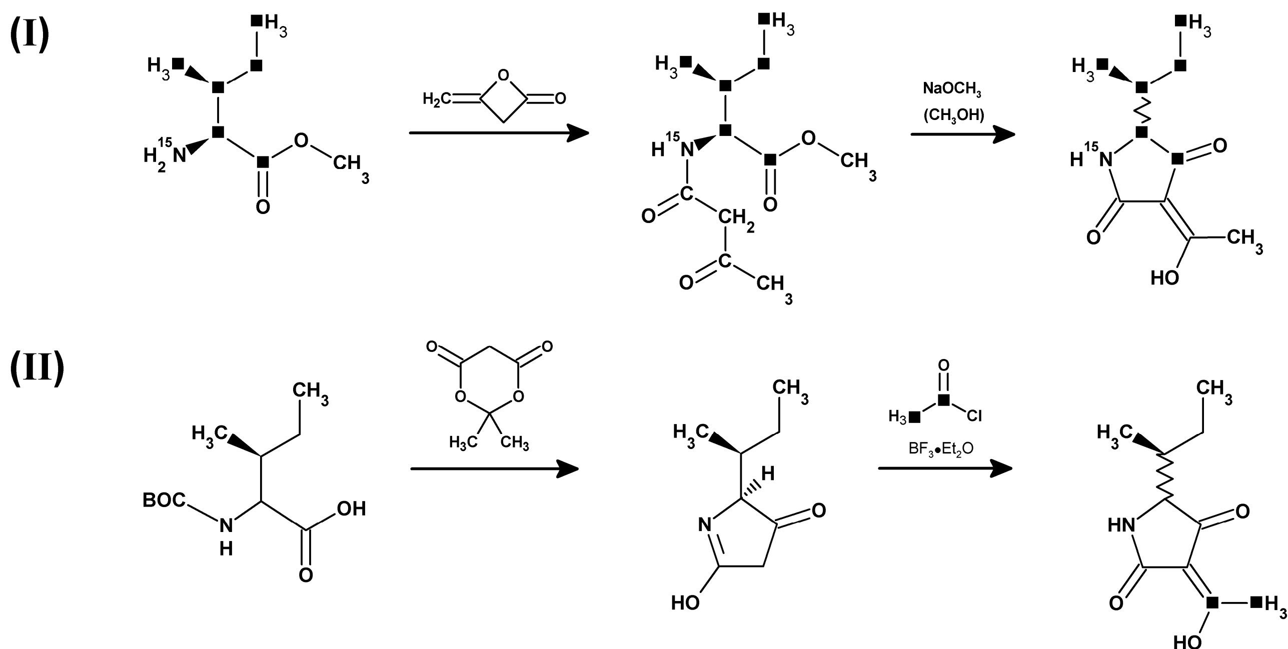

Fig. 7

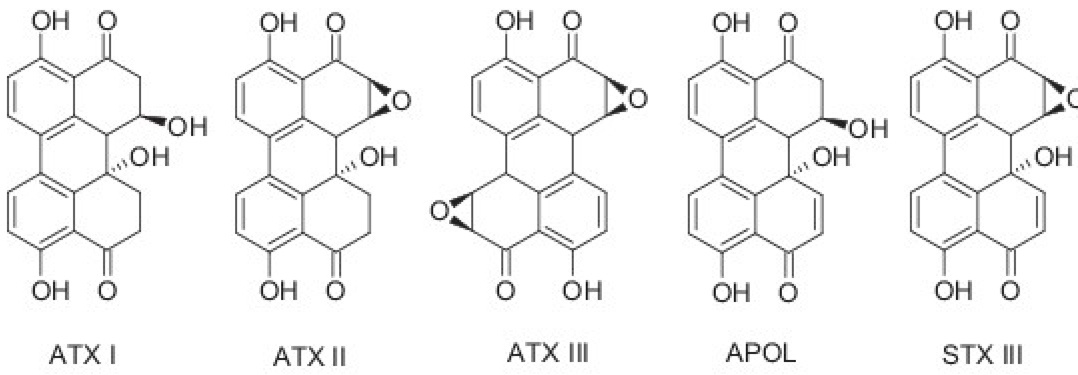

Fig. 8

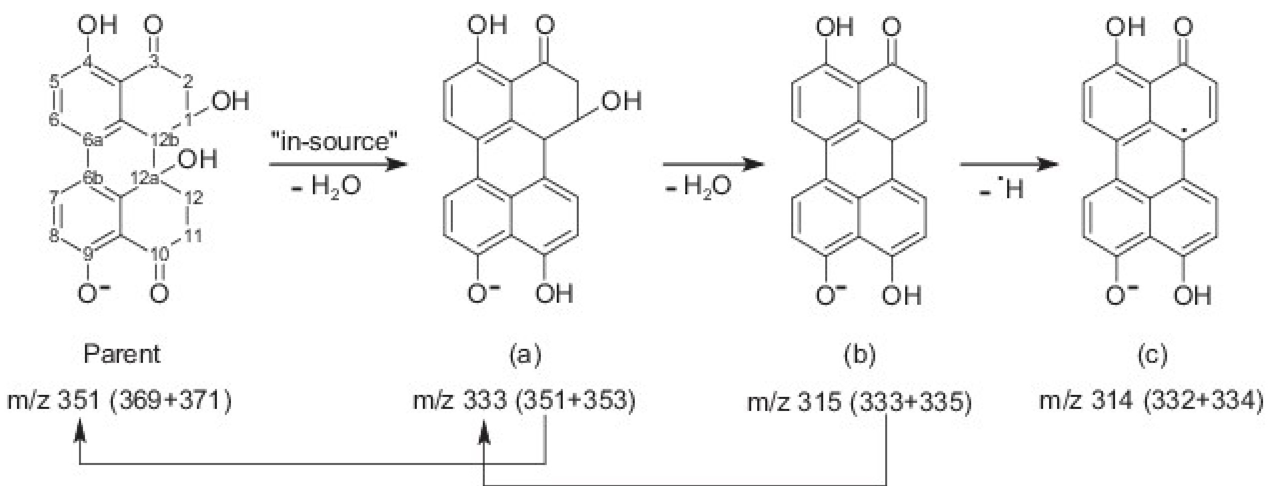

Fig. 9

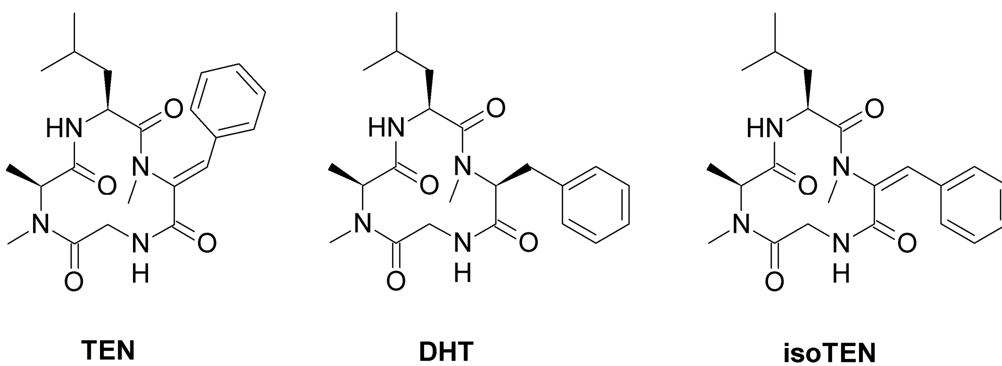

Fig. 10

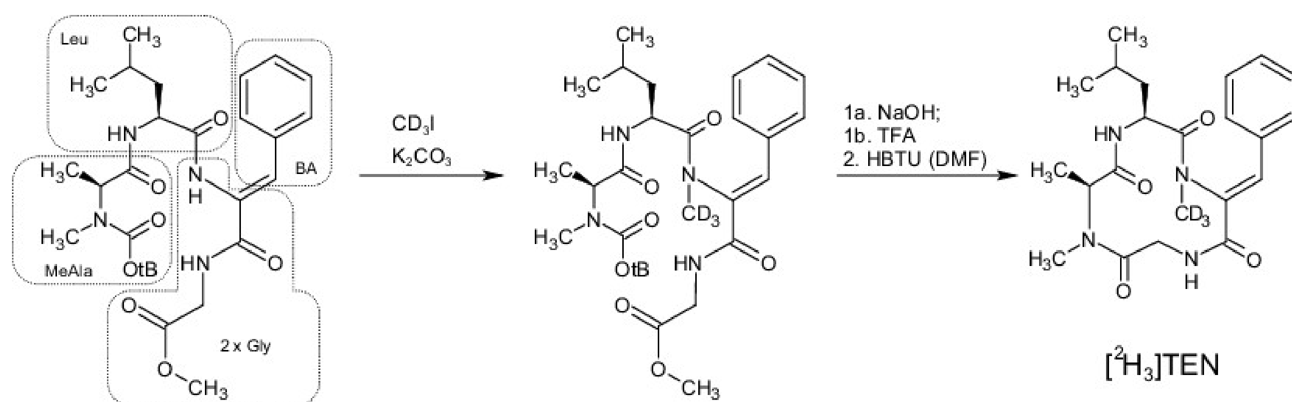

Fig. 11

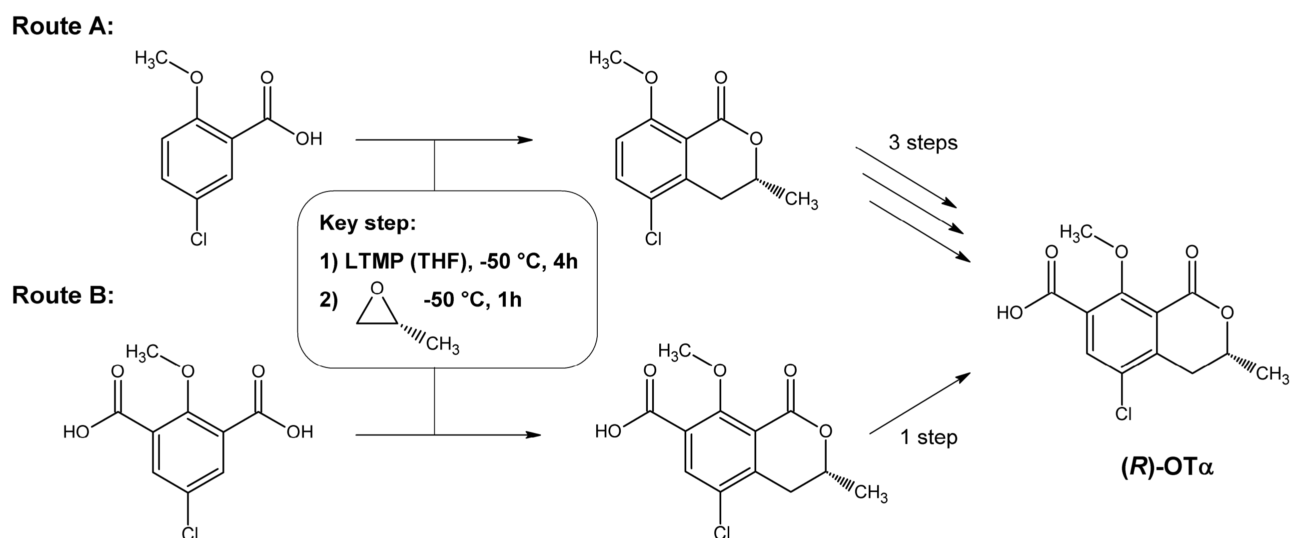

Fig. 12