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Foodomics as a Promising Tool to Investigate the Mycobolome

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Abstract

The huge variety of mycotoxins requires mass spectrometric approaches as they are applied to metabolomics in its targeted and non-targeted analytical forms. For quantitation of mycotoxins in targeted metabolomics, liquid chromatography coupled to triple quadrupole, Orbitrap™ or time-of-flight mass spectrometers provide accurate results. For identification in non-targeted metabolomics, combinations of UPLC and high resolution mass spectrometry (HRMS) are desirable, but mass resolution is compromized by the mass analyzer's scan rates, required for UPLC. In this review, the application of Orbitrap™, time-of-flight and FT-ICR-MS techniques to mycotoxin research is covered and compared to each other. A superior advantage of all HRMS instruments is that they allow a retrospective data treatment thus preserving the information for later reprocessing. Up to date, ultra high resolving FT-ICR-MS is the only method to unequivocally assign elementary sum formulae but cannot be coupled to UPLC without losing resolution power.

Key words

Metabolomics; FT-ICR-MS; Orbitrap; Time of Flight; modified mycotoxins; mycobolome; high resolution mass spectrometry

1. Introduction

Since the advent of the concept “-omics” in “genomics”, “proteomics” and “metabolomics” there has been inflationary use of this term. In case of foods, “foodomics” have been introduced into the scientific literature by Cifuentes [1] and according to a first definition it is a “discipline that studies the Food and Nutrition domains through the application and integration of advanced –omics technologies to improve consumer's well-being, health, and knowledge”. However, this definition could be even broadened to analyze food’s functionality, sensation, nutritional value and safety along with its history, origin, ecologic footprint and/or authenticity. In total, foodomics should provide a holistic and comprehensive understanding of a food’s quality. Moreover, in view ofthe “–omics” terminology the “Foodome” can be seen as the “collection of all compounds present at a given time in an investigated food sample and/or in a biological system interacting with the investigated food” [A. Cifuentes, personal communication].

In the recent years, there has been tremendous progress in high resolution mass spectrometric (HRMS) methods to elucidate the molecular fingerprint of foods particularly in the field of metabolites of low-molecular mass. On the genetic scale (genomics), apart from classical polymerase chain reaction, new developments of isothermal amplifications or next generation sequencing will enable more accurate identification of species. On the protein level (proteomics), specific biomarker peptides are being used. Further methods for profiling include assessing the intensity ratios and positions of stable isotopes in marker molecules (Stable Isotope Ratio Analysis, isotopolomics [2]) or ICP-MS of rare earth elements (metallomics) [3].

Regarding the biochemical pathways in cells or organisms, the proteome has the impact on all metabolites, the set of which is assessed by metabolomics. Among the latter, targeted metabolomics (quantitative analysis of targeted known compounds) can be differentiated from non-targeted metabolomics (profiling of all metabolites in adequation to the analytical limitations; i.e. spectroscopy or spectrometry). As in general many thousands of metabolites have to be expected, the comprehensive non-targeted approach requires ultra high resolution mass spectrometric methods to characterize all of them unequivocally [4].

Apart from authenticity, non-targeted metabolomics may also open new avenues into safety evaluation of foods and food components and, for the latter, in particular of contaminants. In the field of mycotoxins, for those being restricted with maximum limits (MLs) in foods by legislation such as the European Regulation (EC) No. 1881/2006 [5] setting maximum levels contaminants in foodstuffs, targeted metabolomics are the methods of choice. However, in the recent years, „new“ mycotoxins have been sighted and particularly the „emerging mycotoxins“ and the „modified mycotoxins“ require more attention in research. The latter definition can be seen as a more comprehensive extention of the term “masked” mycotoxins. Now the “modified” mycotoxins include all sorts of biological, chemical modifications and matrix associations [6]. This concept differentiates between the originally biosynthesized metabolite by one species and any metabolization or chemical modification by successional processes. In addition, the whole set of fungal metabolites including all modifications may be defined as “*mycobolome*”.

Although a comprehensive definition has been found, a satisfying risk assessment of modified mycotoxins is missing. These modifications have to be considered. For example, the modified toxin deoxynivalenol-3-glucoside has been shown to be cleaved to deoxynivalenol (DON) in the gut and thus contributes to the toxicity of the latter without being regulated yet. Thus, there is an urgent need for suitable analytical methods first to identify as many “new” mycotoxins as possible, and in a second step to obtain reliable exposure data as well as toxicological data.

As mycotoxins are mainly metabolites from fungi along with some further modifications, the foodomics topic will be mainly treated based on metabolomics. Regarding high-resolution mass spectrometric (HRMS) methods in mycotoxin research, there have been two reviews published recently. The first one from Senyuva et al. [7] deals with the Orbitrap™ technology, which is a special instrumentation in LC-HRMS, whereas the second from Righetti et al. [8] covers more LC-HRMS variants and additionally focusses on the application of ion mobility mass spectrometry in mycotoxin analysis. In the review presented here, the principal approach is to differentiate between targeted and non-targeted metabolomics and to review the HRMS methodologies more comprehensively along with their pros and cons.

2. Metabolomics

Commonly, it is accepted that metabolites are rather small molecules in contrast to proteins or nucleic acids. Therefore, they are reasonably analyzed either by gas chromatography coupled to mass spectrometry (GC-MS) or liquid chromatography coupled to mass spectrometry (LC-MS). Each of these methods is applicable

depending on the metabolites' volatility, which is a function of the metabolites' molecular mass and polarity. Volatile metabolites can be best assessed by GC and GC-MS, whereas non-volatiles most likely are accessable by LC and LC-MS, provided that they can be ionized for subsequent detection in mass spectrometry. However, some of the non-volatile metabolites may be detectable by GC-MS after derivatization to volatiles. Apart from the mass spectrometric approaches, another method to comprehensively describe the metabolome is nuclear magnetic resonance (NMR) spectrometry. However, the mycobolome has not been described by NMR yet, most probably due to its lower sensitivity than that of MS. However, there are some applications to assess the metabolism's response to exposure with mycotoxins, such as the study on zearalenone effects on rats [9], which also revealed several new assumptions on the toxicity of this mycotoxin.

The currently known mycotoxins are non-volatile, only few of them can be analyzed by GC-MS such as Patulin [10] or deoxynivalenol (DON) and other trichothecenes [11] after applying trimethylsilylation derivatization techniques. Besides mycotoxins, volatile fungal metabolites have been assessed by GC-MS by Busko et al. [12], but no bioactivity was considered. Although GC has a better resolving power than LC, it is still not sufficient for the many thousand volatile metabolites that have been reported. Therefore, higher chromatographic resolution is obtained with two-dimensional or comprehensive GC, which has been applied by Lima et al. to describe the volatile metabolome of the saprophytic fungus *Memnoniella* sp. [13].

2.1 Targeted Metabolomics for Quantitation

As maximum levels (ML)s for many mycotoxins are at sub ppb levels, e.g. 0.05 µg/kg for Aflatoxin M1, accuracy at these trace amounts poses a particular challenge. To achieve the necessary sensitivity and trueness, targeted metabolomics are the method of choice. In this respect, the development of multi-methods was an important aim for analysts and the availability of recent LC-MS equipments rendered this goal accessible. For being eligible for controlling the MLs, the methods have to meet the respective regulations, which requires a specific minimum of “identification points” (IPs) [14]. Specificity of the methods was achieved either by application of triple quadrupole mass detectors (QqQ) or high-resolution instruments such as time-of-flight (TOF) or Orbitrap™ mass analyzers. For unequivocal identification of a regulated mycotoxin, the European Union requires at least 3 IPs [14], which depend on the applied mass spectrometric method. According to this regulation, 3 IPs are achieved by e.g. monitoring 2 transition products in low resolution LC-MS/MS. LC-HRMS of each ion would yield 2 IPs, which means that in this mode at least 2 ions or one ion and one additional transition would be required. Recent applications of LC-QqQ-MS enabled the detection of almost 300 different toxins in one LC-MS/MS run and could highlight the possible occurrence of much more toxins in foods than ever expected [15]. By using the Orbitrap™ as a high resolution mass analyzer coupled to HPLC, Lehner et al. [16] achieved to identify and quantitate 200 fungal metabolites in foods. Another HRMS method, namely LC-QTOF is also reported in targeted mycotoxin analysis to identify 26 toxins in cereals [17]. When comparing LC-QqQ-MS with LC- Orbitrap™ MS in targeted metabolomics, it has to be mentioned that in a meta-analysis the triple quadrupole coupling revealed a significantly higher sensitivity by at least a factor of 10 for the most important mycotoxins [18].

As targeted metabolomics are mainly used for quantitation, the downside of LC-MS in this respect has to be mentioned. As signal intensity depends on ionization efficiency, matrix interferences are most likely to affect signal intensity [19]. Therefore, accurate quantitation is often limited, particularly if the required sensitivity does not allow for alleviating the matrix effects by simple dilution. A method of choice to circumvent this problem is the use of internal standards labelled by stable isotopes in a so-called stable isotope dilution assay (SIDA), which is decribed in several reviews [19, 20]. Moreover, targeted approaches often involve extensive sample cleanup to reduce matrix interferences and to increase sensitivity and specificity. With regard to superior specificity, immuno affinity cleanup, on the one hand, provides the purest extracts, but is hardly applicable for multi-analyte methods. On the other hand, a rather unspecific, but easy clean-up is available with dispersive solid-phase extraction (DSPE), commonly known as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) [21]. Nevertheless, LC-MS methods without any clean-up (dilute-and-shoot) are popular, but often lack the sensivity for controlling MLs [15].

The use of stable isotopically labelled standards compensate for losses during clean-up and for discrimination due to ion suppression. Moreover, the use of stable isotope-labelled standards allows for additional confirmation as the labelled isotopologue will appear at the same retention time as the analyte in the specified multiple reaction monitoring (MRM) trace. An overview of some currently used labelled standards is presented in Table 1.

One of the most recent applications is shown in Figure1 by introducing a multi stable isotope dilution assays (SIDA) to the quantitation of alternaria toxins [26]. For this method of targeted metabolomis, detection was achieved by a triple quadrupole (QqQ) mass analyzer.

2.2 Non-Targeted Metabolomics for Tentative Identification and Studies on Biosynthesis of Mycotoxins

In non-targeted metabolomics, several thousand features may typically be observed. This requires higher resolving technologies in chromatography, spectrometry or spectroscopy. This capacity can thus be achieved with high resolving electrophoretic or chromatographic methods such as CE, GC or ultra performance liquid chromatography (UPLC) and/or high resolution mass spectrometry (HRMS). High resolving chromatography coupled to HRMS only works, if the scan rate of the mass detector is higher than the typical peak width of the chromatographic method. Among the mass detectors, this is only the case for the Orbitrap^{TM} and the TOF-MS detectors. In the following, this and other features of the different high resolution mass detectors will be detailed and compared to each other.

2.2.1 High resolution mass spectrometers

Using HRMS, accurate masses of each metabolite can be obtained, which enables the analyst to assign an elementary composition for each metabolite. When judging an MS with respect to this capability, the following performance criteria have to be considered: mass resolving power, mass accuracy and sensitivity. These have already been reviewed recently [29], but shall be detailed here again: Mass resolving power is defined as “the observed mass centroid divided by the mass peak width at

50% height for a well isolated single mass spectral peak. This is well known as full width at half maximum (FWHM) of peak height".

Mass accuracy is defined as the ability of any mass analyzer to obtain an experimental mass, which ideally matches (as much as possible) the theoretical mass of a given sum formula. Higher mass accuracy correlates with lower difference between the experimental and theoretical mass of a given sum formula. Sensitivity is the observed change in ion curent per unit mass of sample flow through the ion Source. Sensitivity depends on the limit of detection, which is "the minimal concentration of a compound leading to a peak intensity greater than a specified signal-to-noise ratio (S/N)". Relative sensitivity can be compared on the basis of S/N. The range of mass spectrometers with respect to mass resolution is shown in Figure 2 along with resolving efficiency of different separation methods.

2.2.1.1 Time-of-Flight mass spectrometers

In TOF mass spectrometers the produced ions are accelerated by an electric field to a speed that is dependent on their *m/z* ratio. The different velocities are then measured by an exact quantitation of the time that the ions need to pass a field-free flight tube between the end of the acceleration and the ion detector, provided that all flying ions with different *m/z* ratios have the same initial kinetic energy. Mass resolution generally inceases with the length of the flight path, which can be extended by using reflectors of the ions (ion mirrors or reflectrons). However, the length of the tube is restricted by usual bench lengths or room heights, when the instrument is designed to be used as a bench-top instrument. As time measurements are very accurate, TOFs usually show a scanning rate of 50 Hz, which is sufficient

for on-line monitoring of the effluent from UPLC. Mass resolution of TOF commonly is around 40,000 and mass accuracy around 5 ppm, which means that below masses of 500 Da several dozens of isobars are still possible. As reviewed by Marshall et al. [30], only mass resolutions exceeding 100,000 are able to differentiate between the different isotopic peaks of the elements and to annotate reliably molecular formulae. Therefore, in TOF the assignment of an elementary formula is still tentative and not unequivocal. Further specificity may be achieved with hybrid spectrometers by coupling quadrupoles with TOF detectors. This allows MS^n experiments and reduces ambiguity if the isobars show different fragmentation and is particularly valuable in targeted metabolomics. However, a quadrupole, which is generally used as a mass filter for MS/MS fragmentation studies, has only a unit resolution, so that it has a poor ion isolation within 1 amu isolation window and reduced ion abundance of the isolated signals of interest for running subsequent MS/MS fragmentation experiments.

Due to its lower mass resolution, TOFs have not very often been applied to non-targeted metabolomics of mycotoxins. A recent example of combining non-targeted and targeted metabolomics to elucidate metabolites of cyclopiazonic acid in *Aspergillus flavus* strains by LC-QTOF is reported by Uka et al. [31]. Besides coupling to chromatography and due to their higher mass range, TOF mass spectrometers are often combined with matrix-assisted laser desorption ionization (MALDI). Although MALDI-TOF is mainly used for compounds of higher molecular masses such as proteins, some applications to mycotoxins have been reported [32]. In this study, MALDI-TOF has also been applied to imaging of ochratoxins and fumonisins in mouldy foods.

2.2.1.2 Orbitrap™ MS

The Orbitrap™ detector is based on harmonic axial ion oscillations in electrostatic fields between an inner wire electrode and an exterior cylindrical electrode. The trapping frequency of trapped ions in the Orbitrap™ depends on their *m/z* ratio, and these axial trapping frequencies can be computed by Fourier transformation. Orbitrap™ instruments are capable to exceed a mass resolution of 100,000 at scan times around 2 sec [30]. However, this scan rate would be too slow for HPLC separations and, therefore, Orbitrap™ coupled to HPLC are reasonably run at a mass resolution of 60,000 and a sampling rate of 4 Hz [33]. Although showing a near one order of magnitude lower sampling rate than TOF instruments, Orbitrap™ reach a better mass accuracy below 1 ppm [34]. With respect to dimensions, Orbitrap™ as well as TOF instruments can be used as bench-top equipments.

Several studies for screening and determination of mycotoxins and pesticide residues in several fruits and plants, such as green tea as well as royal jelly supplements have applied an LC-Orbitrap ™ MS system [35]. The auhors could identify aflatoxin B1 (which belongs to the aflatoxin class, one of the major groups of mycotoxins) with a limit of detection < 6μg/kg. LC-Orbitrap™-MS was also utilized for running a quantitative targeted and retrospective data analysis of relevant mycotoxins, pesticides as well as antibiotics in bakery products [36]. In the latter study, the utilized single stage Orbitrap™ mass analyzer was set up to a mass resolving power of 50,000 in order to achieve a good compromise between an adequate chromatographic scan speed and mass spectrometric selectivity. Limit of quantifications between 5 and 100 μg/kg were achieved.

As mycotoxins are formed by the fungi when growing on a substrate or in interaction with a host organism, it is difficult to differentiate the toxin's metabolites from those of the substrate or of the host. Therefore, in mycotoxin research the use of stable isotope labelling (SIL) of precursors for biotransfomation has been introduced by Kluger et al. [37]. The latter authors applied a 1+1 mixture of DON and U-^{13}C-DON to wheat ears and followed the metabolism of DON by LC- Orbitrap^{TM} including MS/MS experiments. Assignment of DON metaboites was achieved by screening for compounds with mass increments between ^{12}C and ^{13}C at the same retention time. Further MS/MS experiments allowed to tentatively identify the metabolites. In this way, the formation of DON-3-glucoside was confirmed along with the identification of new modifications such as DON conjugates with glutathione, cystein and cysteinyl glycine. The workflow of assignment was assisted by new software tools such as MetExtract [38].

Apart from identifying new mycotoxins, LC- Orbitrap^{TM} including MS/MS experiments has also been applied to investigating the biosynthesis of mycotoxins, e.g. of fusarins produced by *Fusarium fujikuroi* [39]. Interestingly, the latter authors combined HRMS with NMR spectroscopic studies and genomics.

2.2.1.3 Fourier Transform Ion Cyclotron Resonance Mass Spectrometers (FT-ICR-MS)

With respect to mass resolution, Fourier Transform Ion Cyclotron Resonance Mass Spectrometers (FT-ICR-MS)26] are the most powerful ones to realize ultra high resolution (uHRMS). Similar to the Orbitrap^{TM} principle, ions are forced to oscillation, but in the ion cyclotron into a circular oscillation by a magnetic field. This circular oscillation occurs at the so-called cyclotron frequency, which is dependent on the

m/z ratio and can be assessed very exactly as it involves a time measurement. In the ion cyclotron, several thousands of ions oscillate with different cyclotron frequencies, which depend on their m/z ratios, and are detected simultaneously, which is the prerequisite for performing metabolomics. In the ICR technique, an ion with *m/z* of 614 amu oscillates at a cyclotron frequency of 300kHz in a 12 Tesla magnet. Given that the ICR cell radius is 23 cm with a cylindrical cell circumference of 144 cm, such an ion can fly for a distance, which exceeds 430 km in only one second. This explains the ultra-high resolution nature of ICR, since the mass resolving power increases with extending the ion flight length. The time domain spectrum detected in the ion cyclotron is transformed into a frequency domain spectrum via Fourier transformation (FT). Thus, ICR represents an FTMS technique. As mass resolution and mass accuracy are dependent on the strength of the magnetic field, superior FT-ICR-MS instruments require strong magnetic fields above 7 Tesla. These are generally produced by a superconducting magnet that requires a room by far exceeding bench-top dimensions. At strong magnetic fields of 9.4, 12 and 15 Tesla, mass resolutions of several hundred thousands are generally achieved, which generate unequivocal elemental compositions of the several thousands of detected ions. A downside of this ultra high resolution is the scanning rate necessary to achieve sufficient resolution and sensitivity. One to several seconds are required to obtain a mass spectrum, which means that this method cannot be reasonably coupled online to high performance liquid chromatography (UPLC). Another restriction of FT-ICR-MS is its limitation in the lower *m/z* ratio. In contrast to TOF instruments that can measure ions at *m/z* values as low as 20 Da, the FT-ICR-MS mass range starts at 125 Da. This is due to electronic limitations of the digitizers, which normally have limited sampling frequency. It should be mentioned that for *m/z*

< 80 amu (for example) very high sampling frequencies should be provided for detection and this represents a real limitation of the working digitizers of the ICR mass analyzers. The higher the magnetic strength, the higher are the generated cyclotron frequencies of the detected ions. This also applies a further limit of functionality of the digitizers, which are coupled to the ICR cells for detection. Given its ultra high resolving power, FT–ICR-MS has been reportedly applied to metabolomics. As coupling to chromatography is not reasonable, either direct infusion of the initial extract or a chromatographic fractionation was applied, prior to direct sample injection of each fraction into the FT-MS instrument (off-line approach). This is due to the fact, that long time domain transient lengths need to be applied in the FT-ICR-MS technique in order to obtain ultra high mass resolving power, especially for relatively high *m/z* ratios above 500 amu. However, UPLC can achieve high resolution separation within only few minutes of a chromatogram, whereas FT-ICR-MS acquisitions can take up to several seconds for only few averaged scans for each chromatographic point plotted in the UPLC chromatogram.Only few studies are reported in the field of fungal metabolites or mycotoxins using direct injection FT-ICR-MS. Therefore, some preliminary results from our lab are presented, which show perspectives and limitations of FT-ICR-MS in metabolomics of mycotoxins. In our study, we aimed at unravelling the option to perform SIDA with FT-ICR-MS after spiking of natural juices with several toxins produced by the fungus *Alternaria alternata*. As a naturally non-contaminated matrix we chose cherry juice and spiked it with the toxins tenuazonic acid (TeA) and alternariol (AOH) successively to contents of 200 µg/L. Unfortunately, sensitivity of FT-ICR-MS was not sufficient for detecting AOH by direct infusion, due to its limited ionization in the negative ionization mode of electrospray given that AOH contains only OH groups that can be deprotonated but

no carboxylic groups. Thus the juice had to be purified and concentrated by solid phase extraction (SPE). Similarly to our SIDAs reported recently, we also added [$^{13}C_6$,^{15}N]-TeA and [$^{2}H]_2$-AOH in different concentrations to the juice. For TeA, the respective signals of the spiked juices in ultra high resolution were clearly visible and linearly increasing with rising concentrations, whereas the blank juice revealed no significant signal for TeA and [$^{13}C_6$,^{15}N]-TeA (Figure 3). For [$^{2}H]_2$-AOH, a similar result was found for the spiked juices. However, in the blank sample, AOH showed a significant signal, which revealed an ion showing the elementary formula of AOH. To further investigate this result, we analyzed the blank juice with our established multi SIDA for alternaria toxins. Surprisingly, no AOH was detected in the latter assay. This pointed to the occurrence of molecules with the same elementary formula as AOH in the juice. A literature review and database search revealed several natural metabolites at this formula, e.g. pannorin or gentisin (Figure 4). This result clearly shows the restriction of FT-ICR-MS and points to the need of orthogonal methods such as MS/MS or ion mobility to clearly separate these isomeric structures, which have the same exact *m/z* ratios but different organic structures.

2.2.2 Data management in HRMS

Generally, non-targeted metabolomics in the field of moulded foods provides thousands of MS signals from the prevalent fungi and from the basic food. If the mass data do not deliver unequivocal elementary formula, the number of conceivable species even exceeds the obtained plethora of signals. Therefore, bioinformatic procedures are required to filter and assign the set of metabolites. Even in the case of FT-ICR-MS, when unequivocal elementary formulae are obtained, several thousands of single molecular formulae have to be considered.

However, recent applications to metabolomics revealed that actually known chemical components in biological samples entered in databases commonly represent less than 10% of its overall chemical diversity revealed by FT-ICR-MS. Therefore, systematic combination with orthogonal analytical methods such as high-resolution QTOF-MS and multidimensional NMR and extensive bioinformatics is required to assign and identify the active components.

In a straightforward workflow, exact masses from FT-ICR-MS and LC-TOF-MS are combined and converted into elementary formulae thus reducing the data set by matching from both HRMS methods. Thereafter, the set is further reduced by using the NetCalc annotation approach based on mass difference and network analysis [40]. The single elementary formulae are checked whether they fit in general metabolic pathways by assuming common transformations equivalent to specific mass differences. In the case of mycotoxins, acetylation, methylation or hydroxylation besides conjugation with glucose, oligosaccacharides, sulfate or glutathione are the most probable ones [8].

Moreover, for conversion of mass spectra from FT-ICR-MS to biologically interpretable data the MassTRIX server (Mass Translator into Pathways, www.masstrix.org) has been developed. MassTRIX [41] is publically accessible and corrects an uploaded mass list corresponding to ionization mode (positive or negative) and possible adducts within a specific mass tolerance window in a given type of organism and compares the corrected masses against possible metabolites

from KEGG, HMDB, Lipidmaps or own uploaded databases. These are mapped in a second step to the respective pathway maps of the chosen organism.

In a later step, the structural characterization of marker compounds then can be realized with combined *in silico* and classical MS/MS analyses, including isolation and structural characterization (HRMS, IR 1D- and 2D-NMR) with candidate structures synthesis. This approach has recently been applied in projects to unravel metabolites associated with lifespan extension [42] or fasting [43]. Extensive experience in foodomic exists for Champagne [44] and wine in general [45].

2.2.3 Approaches for unambiguous identification of metabolites and structural assignment

According to the mass accuracy of the applied MS, elementary formulae can be assigned with a certain validity. Using an ultra high resolving instrument and assuming the most probable bioelements, the calculated formula is trustworthy. Further confirmation of the elementary formula is provided by the distribution of the natural stable isotopologues to be expected in the fine structures. This is achieved by matching the experimental isotopic pattern of a specific metabolite to its theoretical (calculated) isotopic pattern. After identifying and validating a sum formula, identifying the structure of the metabolite comes next. If the formula is entered in a database search, up to dozens of different isomers (organic compounds with exact elemental sum formulae but with different structures) may match it. Therefore, additional complementary methods have to be applied. Structural information can be obtained from MS/MS experiments, but some fragments indicate common moieties that are easily lost. Therefore, in detail MS/MS fragment analysis

might be necessary in some complex cases, when the isomers do not differ significantly from each other. This is the case, when the same organic functional groups exist in several isomers but connected to different positions in the carbon skeleton.

In the case of modified mycotoxins, glucosides can be easily recognized by the loss of the glucose moiety. However, if the glucose may be linked to different hydroxy groups in the molecule, the location of the bond is hardly possible by MS/MS. However, Dall'Asta et al. [46] showed for DON that the loss of glucose from different positions results in significantly different intensities of the fragment. In this way, many new modified mycotoxins have been assigned tentatively including DON oligoglucosides [47]. Besides, MS/MS experiments may allow the differentiation of an α- from a β-glucosidic bond as shown for the case of ascorbic acid 2-O-glucosides [48]. For a more accurate structural assignment the method of choice would be NMR spectroscopy. In case of glucosides or other complex structures two-dimensional NMR spectroscopy will also be essential to clearly elucidate the binding of the single atoms to each other. The last step in unambigous structural assignment then would be the total synthesis of the molecule by using defined chemical procedures.

2.3 Proteomics, Transcriptomics and Genomics

With respect to mycotoxins, the other omics approaches mainly have been applied to elucidate their biosynthesis or regulation of their formation in interaction with other organisms. As these studies were rarely related to food or food quality, they may not be assigned to foodomics. Therefore, only some representative studies are detailed here. In regard to genomics related to biosynthesis of mycotoxins, LC- Orbitrap™

has been applied to unravel the genes responsible for generating aflatoxins [49]. For most of the fungi affecting food almost no efficient strategies for control have been found. Increasing the resistence of plants to fungal infection is one possible option and this issue has been studied ~~for~~ barley by genomics [50]. Further applications of the genomics approach are studies on the adaptation to climate change of *Aspergillus flavus* and its production of aflatoxins [51]. Apart from analyzing the mycotoxins produced by fungi, genomics have been reported either to detect the fungi [52] or to describe the relation of different fungal strains or species within a genus that is not so easy to assign like *Alternaria alternata* [53].

3. Conclusions

Mycotoxins are a particularly complex and huge set of metabolites originating from fungal biological pathways in interaction with other organisms and being susceptible to further biochemical and/or chemical transformations. Given the whole concept of foodomics, metabolomics is a promising tool to identify and quantitate the whole set of mycotoxins to enable accurate risk assessment in the end. For quantitation in *targeted* metabolomics, LC-QqQ-MS, LC- Orbitrap™ MS and LC-QToF-MS provide accurate results, whereas the Orbitrap™ MS coupling was found to be significantly less sensitive than the QqQ-MS for the most important mycotoxins [18]. For identification in *non-targeted* metabolomics, different combinations of UPLC and HRMS are possible. However, the better the peak separation in UPLC is, the higher the scan rate of the employed mass spectrometer has to be. Vice versa, mass spectrometric resolution decreases with higher scan rates, thus compromising the combination applied. The coupling of UPLC to FT-ICR-MS would give the largest set of acurrate elementary formulae, but is not feasible up to date, due to long

acquisition time-domain transients (low MS scan rate compared to quick UPLC separation). Thus a combination of FT-ICR-MS and LC-QToF data currently gives the most comprehensive data set. A very powerful feature of all HRMS instruments is that they allow a retrospective data treatment [54], which renders the assessment of a sample in principle independently from the moment it has been analyzed. Fragmentation of the compounds can be comprehensively applied on-line by data-independent analysis (DIA), which allows to obtaining further structural information in the future, when the signal has to be reprocessed. Thus, the information on the sample is preserved and currently “masked” mycotoxins are already “unmasked” at the time of measurement. Therefore, the newly defined “maskedome” [55] may already be included in the recorded “mycobolome”. This also applies to the differentiation between “expected knowns”, “unexpected knowns”, “expected knowns” and “unexpected unknowns” [8], which all may be preserved in the whole data set recorded. It will only depend on the time when the data are interpreted, whether a signal belongs to the “unexpected unknowns” or the “expected knowns”.

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Conflict of interest

The authors declare that they have no competing interests.

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Tables

Table 1: List of applications of stable isotope dilution assays to mycotoxin analysis

Food	Mycotoxin	Isotopologic standard	Reference
Apple juice	Patulin (PAT)	$[^{13}C]_2$-PAT	[10]
Coffee, Wine, Spices, raisins	Ochratoxin A (OTA)	$[^{2}H]_5$-OTA	[22]
Nuts, corn, spices	Aflatoxin (AF) B_1, B_2, G_1, G_2	$[^{2}H]_2$-AF-B_2; $[^{2}H]_{2\text{-}4}$-AF-G_2	[23]
Tomato products	Alternariol (AOH), alternariol methyl ether (AME)	$[^{2}H]_2$-AOH, $[^{2}H]_2$-AME	[24]
Tomato products	Tenuazonic Acid (TeA)	$[^{13}C_6, ^{15}N]$-TeA	[25]
Div. foods	Altertoxins	u-[^{13}C]-labelled analogues	[26]
Cereals	Div. Fusarium toxins	div. labellings	[27]
Beer	Deoxynivalenol-3-glucoside	deoxynivalenol-3-$[^{13}C]_6$-glucoside	[28]

Legends to the figures

Figure 1. Tageted metabolomics: Multiple stable isotope dilution assay for the quantitation of Alternaria toxins [22].

Figure 2. Characteristic resolutions (peak capacity: total range / half width) of various separation technologies and organic structural spectroscopic methods. The diagram represents a two-dimensional projection of the analytical volumetric pixel space comprising NMR spectroscopy, mass spectrometry and separation technologies that defines our current capacity to depict variance in complex systems with molecular resolution, according to Hertkorn et al. [25].

Figure 3. Fourier Transform Ion Cyclotron Resonance mass spectra of cherry juice spiked with different amounts of tenuazonic acid (TEA) and [$^{13}C_6$,^{15}N]-TeA.

Figure 4. Fourier Transform Ion Cyclotron Resonance mass spectra of cherry juice spiked with different amounts of alternariol (AOH) and [$^{2}H_4$]-AOH. Structures of polyphenol isomers of AOH.

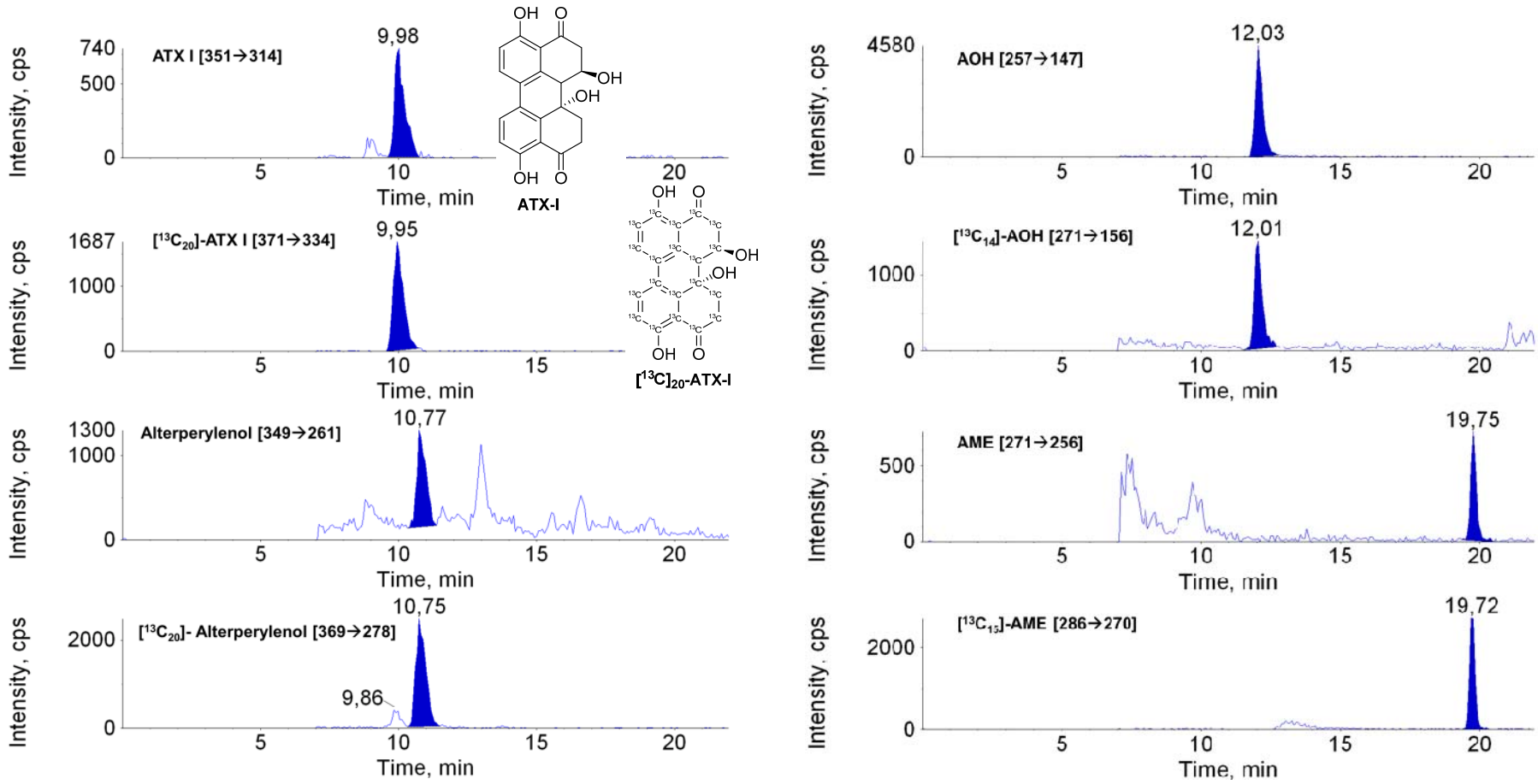
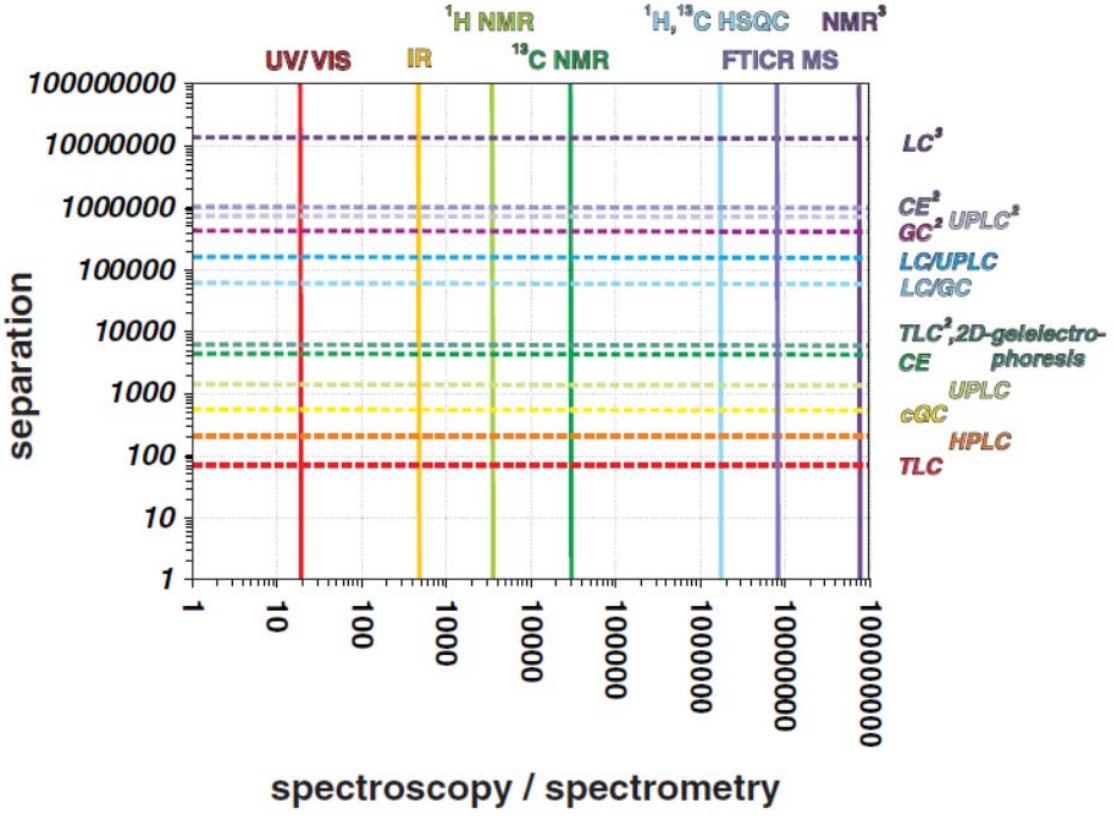

ATX I [351→314]
9,98
ATX-I
[13C20]-ATX I [371→334]
9,95
[13C]20-ATX-I
Alterperylenol [349→261]
10,77
[13C20]- Alterperylenol [369→278]
10,75
9,86
AOH [257→147]
12,03
[13C14]-AOH [271→156]
12,01
AME [271→256]
19,75
[13C15]-AME [286→270]
19,72
Intensity, cps
Time, min

Figure 1


^{1}H NMR
^{1}H,^{13}C HSQC
NMR^{3}
UV/VIS
IR
^{13}C NMR
FTICR MS
100000000
10000000
1000000
100000
10000
1000
100
10
1
separation
LC^{3}
CE^{2}
GC^{2}
UPLC^{2}
LC/UPLC
LC/GC
TLC^{2},2D-gelelectro-
CE
phoresis
UPLC
cGC
HPLC
TLC
1
10
100
1000
10000
100000
1000000
10000000
100000000
spectroscopy / spectrometry

1

2 Figure 2

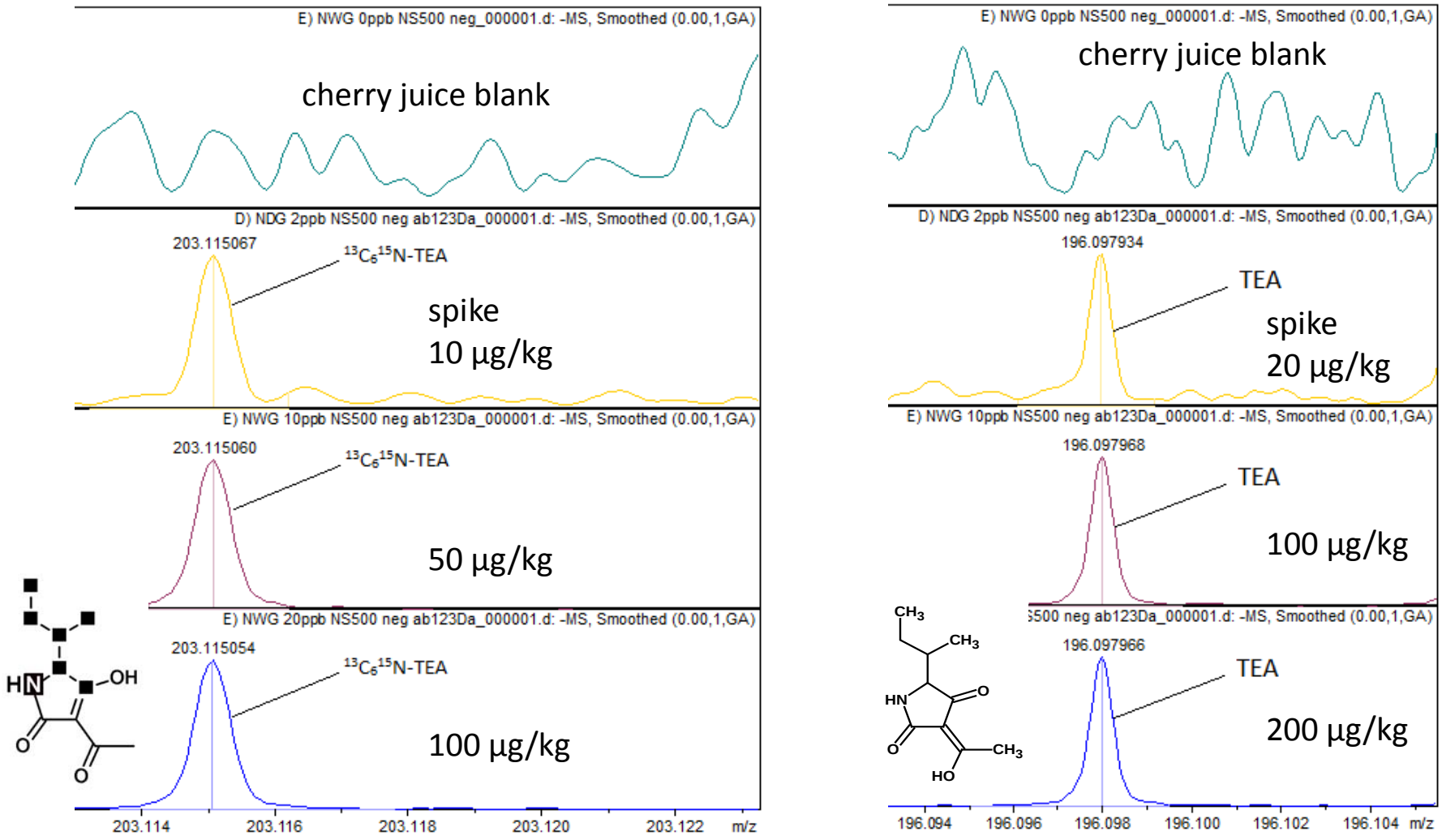

E) NWG 0ppb NS500 neg_000001.d: -MS, Smoothed (0.00,1,GA)
cherry juice blank
D) NDG 2ppb NS500 neg ab123Da_000001.d: -MS, Smoothed (0.00,1,GA)
203.115067
13C6 15N-TEA
spike
10 µg/kg
E) NWG 10ppb NS500 neg ab123Da_000001.d: -MS, Smoothed (0.00,1,GA)
203.115060
13C6 15N-TEA
50 µg/kg
E) NWG 20ppb NS500 neg ab123Da_000001.d: -MS, Smoothed (0.00,1,GA)
203.115054
13C6 15N-TEA
100 µg/kg
203.114
203.116
203.118
203.120
203.122
m/z
E) NWG 0ppb NS500 neg_000001.d: -MS, Smoothed (0.00,1,GA)
cherry juice blank
D) NDG 2ppb NS500 neg ab123Da_000001.d: -MS, Smoothed (0.00,1,GA)
196.097934
TEA
spike
20 µg/kg
E) NWG 10ppb NS500 neg ab123Da_000001.d: -MS, Smoothed (0.00,1,GA)
196.097968
TEA
100 µg/kg
196.097966
TEA
200 µg/kg
196.094
196.096
196.098
196.100
196.102
196.104
m/z

Figure 3

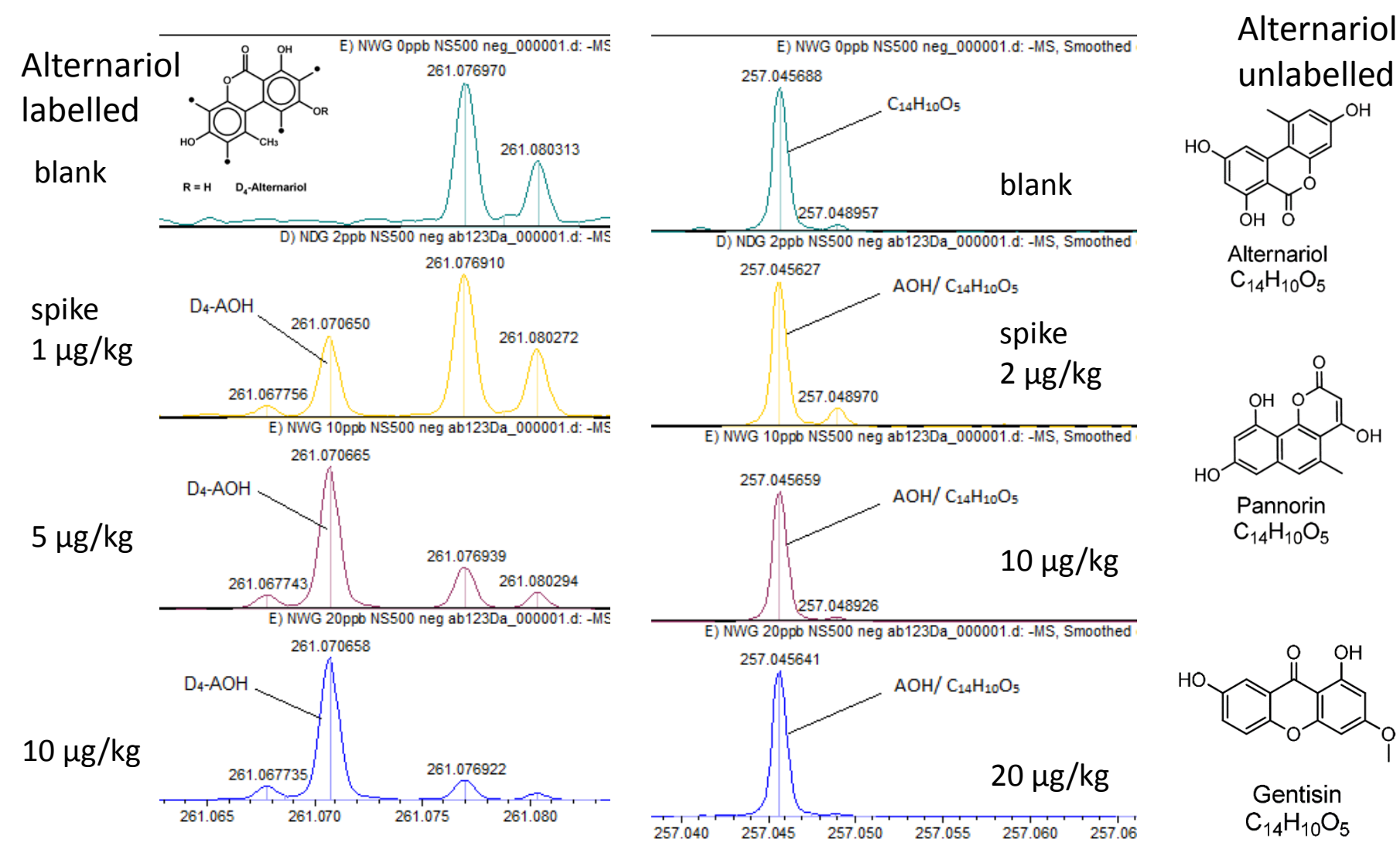

Alternariol
labelled
blank
spike
1 µg/kg
5 µg/kg
10 µg/kg
R = H
D4-Alternariol
E) NWG 0ppb NS500 neg_000001.d: -MS
261.076970
261.080313
D) NDG 2ppb NS500 neg ab123Da_000001.d: -MS
261.076910
D4-AOH
261.070650
261.080272
261.067756
E) NWG 10ppb NS500 neg ab123Da_000001.d: -MS
261.070665
D4-AOH
261.076939
261.067743
261.080294
E) NWG 20ppb NS500 neg ab123Da_000001.d: -MS
261.070658
D4-AOH
261.067735
261.076922
261.065
261.070
261.075
261.080
E) NWG 0ppb NS500 neg_000001.d: -MS, Smoothed
257.045688
C14H10O5
257.048957
blank
D) NDG 2ppb NS500 neg ab123Da_000001.d: -MS, Smoothed
257.045627
AOH/ C14H10O5
257.048970
spike
2 µg/kg
E) NWG 10ppb NS500 neg ab123Da_000001.d: -MS, Smoothed
257.045659
AOH/ C14H10O5
257.048926
10 µg/kg
E) NWG 20ppb NS500 neg ab123Da_000001.d: -MS, Smoothed
257.045641
AOH/ C14H10O5
20 µg/kg
257.040
257.045
257.050
257.055
257.060
257.06
Alternariol
unlabelled
Alternariol
C14H10O5
Pannorin
C14H10O5
Gentisin
C14H10O5

Figure 4

1