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# The use of mass defect in modern mass spectrometry

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Mass defect is defined as the difference between a compound's exact mass and its nominal mass. This concept has been increasingly used in mass spectrometry over the years, mainly due to the growing use of high resolution mass spectrometers capable of exact mass measurements in many application areas in analytical and bioanalytical chemistry. This article is meant as an introduction to the different uses of mass defect in applications using modern MS instrumentation. Visualizing complex mass spectra may be simplified with the concept of Kendrick mass by plotting nominal mass as a function of Kendrick mass defect, based on hydrocarbons subunits, as well as slight variations on this theme. Mass defect filtering of complex MS data has been used for selectively detecting compounds of interest, including drugs and their metabolites or endogenous compounds such as peptides and small molecule metabolites. Several strategies have been applied for labeling analytes with reagents containing unique mass defect features, thus shifting molecules into a less noisy area in the mass spectrum, thus increasing their detectability, especially in the area of proteomics. All these concepts will be covered to introduce the interested reader to the plethora of possibilities of mass defect analysis of high resolution mass spectra. Copyright © 2012 John Wiley & Sons, Ltd.

**Keywords:** mass defect; high resolution; labeling; filtering; Kendrick plots; metabolites; proteomics

## INTRODUCTION

The concept of mass defect arises from the fact that each isotope of every element releases a slightly different amount of energy upon the formation and stabilization of its nucleus (nuclear binding energy). By convention, carbon-12 has been defined as the element with zero mass defect and therefore its exact mass is 12.0000 Da. Every other existing isotope has a defined, either positive or negative, mass defect, depending on its relative nuclear binding energy to carbon-12. Each nuclide has a different mass defect and therefore each molecule of different elemental composition will have a unique exact mass, even if the number of neutrons and protons remains equal. The mass defect of a single element or chemical compound is calculated as the difference between the exact mass of the isotope in question and its nominal integer mass which is the simple addition of the number of protons and neutrons in a given formula or elemental isotope. Figure 1 shows a list of exact masses and isotopic abundances for elements of particular interest to this review, as well as a plot comparing mass defect and atomic mass. Uniquely negative mass defect values of some elements displayed in this plot can be used to specifically tag molecules of interest in order to shift them into less noisy regions of complex mass spectra (see section of mass defect labeling).

Modern high resolution mass spectrometry relies on mass defect for measuring accurate masses for the ions formed in the ion source. The fact that each molecule has its own unique exact mass allows us to elucidate chemical formulas from unknown compounds as well as confirm a known compound with great certainty. For instance, nominal mass measurements would not be able to distinguish between the protonated ions of lysine and glutamine at  $m/z$  147.1128 and 147.0764, respectively, however a mass spectrometer with a resolving power of over 10 000 would easily be able to distinguish these two ions. Obviously, the higher the resolution, the more closely spaced ions

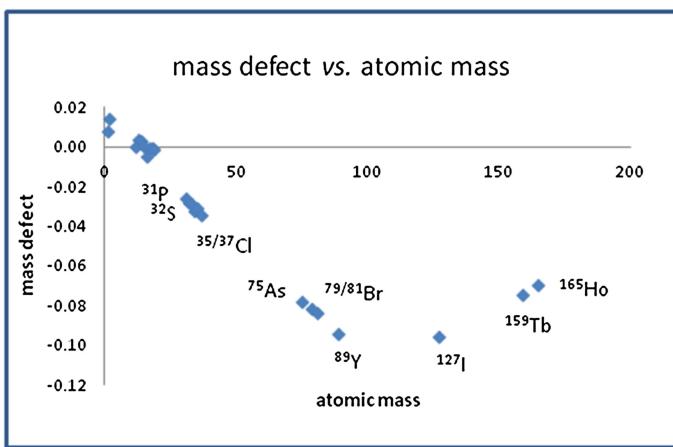
can be easily separated, and with proper calibration of the mass analyser, this high resolution can translate into exact mass measurements. Currently, we have several choices for high resolution mass analysers. Recent progress in time-of-flight (TOF) analyzers has allowed them to easily achieve 20 k resolution and even as high as 60 k for certain TOF machines. Of higher resolution still, orbitrap and Fourier transform ion cyclotron resonance (FT-ICR) analyzers can routinely achieve resolutions of 100 k and even much higher for certain configurations, but at a cost of scan speed as resolving power increases. The orbitrap mass spectrometer has been ameliorated since its inception, in terms of scan speed and sensitivity, to become a more routine instrument, especially in the case of the bench-top version (Orbitrap Exactive<sup>TM</sup>). FT-ICR instruments are extremely powerful and have uniquely high resolving powers, especially when using high field magnets (9.4 or 12 T), however their analysis speed makes them less routine. Nonetheless, there are certain fields of research that necessitate FT-ICR levels of resolution, such as petroleum analysis and highly complex top-down protein analysis.

## KENDRICK MASS SCALES

To represent a complex mass spectrum by separating nominal masses (1 Da apart) on the x-axis and individual mass defects of each measured  $m/z$  on the y-axis, yields a simplified plot for visualizing mass spectral peaks. The mass defect here is the difference between the exact and nominal mass. If you then

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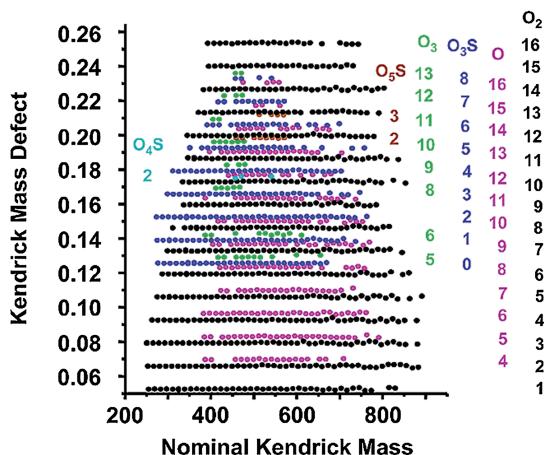


**Figure 1.** A plot of mass defect as a function of atomic mass displaying uniquely negative mass defects of specific elements as well as a table of exact mass, mass defects and natural abundances for elements discussed in this review.

compress the areas of gaps in this plot, important patterns become apparent for extremely complex mass spectra using a relatively simple looking plot. For hydrocarbons systems, we can convert the IUPAC mass to what is known as Kendrick mass by using a simple conversion between the exact mass of a methylene unit ( $\text{CH}_2$ ) to the nominal mass of  $\text{CH}_2$ . All  $m/z$  values are multiplied by 14.0000/14.01565, thus simplifying even further the display and making apparent rectilinear peak patterns for compounds of the same type but with different chain lengths on the same vertical axis. These 'Kendrick' plots are often used for complex petroleum samples since they have the advantage of visualizing thousands of elemental compositions in a single 2D display while identifying compound types and alkylation series. These ultra-complex spectra necessitate, of course, the use of ultra-high resolving power and therefore are most often analyzed by FT-ICR MS. In petroleum analysis, compounds are classified based on their heteroatom content (e.g.,  $\text{SO}_3$ , NS, etc.) and number of double bonds. The repeating mass of methylene is characteristic of hydrocarbons, and therefore we can recognize series of compounds belonging to the same class or type but with different degrees of alkylation by a series of MS peaks differing by 14.01565 Da. If we convert to the Kendrick scale, these peaks will line up horizontally on the plot and thus be more easily recognized<sup>[1]</sup>. Homologous series of peaks will have identical Kendrick mass defect (KMD) which is calculated as the difference between Kendrick nominal mass (KNM) and Kendrick exact mass. Two-dimensional displays of KMD vs. KNM are used as a standard means of visualizing elemental composition patterns in complex organic samples analyzed by high resolution mass spectrometry. An important advantage of these plots is the ease with which high mass members of homologous series are

element	isotope	atomic mass (u)	mass defect	% isotopic composition
hydrogen	$^1\text{H}$	1.00783	0.00783	99.9885
	$^2\text{H}$	2.01410	0.01410	0.0115
carbon	$^{12}\text{C}$	12.00000	0.00000	98.93
	$^{13}\text{C}$	13.00335	0.00335	1.07
nitrogen	$^{14}\text{N}$	14.00307	0.00307	99.632
	$^{15}\text{N}$	15.00011	0.00011	0.368
oxygen	$^{16}\text{O}$	15.99491	-0.00509	99.757
	$^{17}\text{O}$	16.99913	-0.00087	0.038
	$^{18}\text{O}$	17.99916	-0.00084	0.205
fluorine	$^{19}\text{F}$	18.99840	-0.00160	100
phosphorus	$^{31}\text{P}$	30.97377	-0.02623	100
sulfur	$^{32}\text{S}$	31.97207	-0.02793	94.93
	$^{33}\text{S}$	32.97146	-0.02854	0.76
	$^{34}\text{S}$	33.96787	-0.03213	4.29
chlorine	$^{35}\text{Cl}$	34.96885	-0.03115	75.78
	$^{37}\text{Cl}$	36.96590	-0.03410	24.22
arsenic	$^{75}\text{As}$	74.92160	-0.07840	100
bromine	$^{79}\text{Br}$	78.91834	-0.08166	50.69
	$^{81}\text{Br}$	80.91629	-0.08371	49.31
yttrium	$^{89}\text{Y}$	88.90585	-0.09415	100
iodine	$^{127}\text{I}$	126.90447	-0.09553	100
terbium	$^{159}\text{Tb}$	158.92534	-0.07466	100
holmium	$^{165}\text{Ho}$	164.93032	-0.06968	100

recognized. It would be very difficult to ascertain their elemental composition with accurate mass measurement alone, since as molecular weight increases, so does the number of elemental compositions possible within the same mass accuracy window. Kendrick analysis has been applied extensively for petroleum analyses using ultra-high resolution measurements by FT-ICR-MS. Plots of Kendrick MD vs. KNM are used for sorting different compound classes, based on number of N, O and S atoms, number of double bond equivalents, and alkylation ( $\text{CH}_2$ ) distributions. This can extend the upper limit for unique elemental formulae assignments using accurate mass measurements to higher than 900 Da. Kendrick plots and ultra-high resolution MS for this application area are described in a detailed review of 'petroleumics' analysis by Marshall *et al.*<sup>[2]</sup> Figure 2 is reproduced from this article, showing a Kendrick 2D plot of a complex FT-ICR mass spectrum from a Chinese crude oil. This plot illustrates the obvious periodicities of 2 and 14 Da from displaying the data using the Kendrick scale, making it possible to determine molecular class and type over a wide mass range. We can do similar conversion for series of compounds differing in oxidation (O), chlorination (Cl), bromination (Br), etc. by doing analogous calculations (i.e., 16/15.996 for finding compounds differing in oxygen content). A recent example of this type of analogous calculation has been shown for chlorinated and mixed halogenated dioxins and furans<sup>[3]</sup>. Obviously, the more unique mass defect a compound has, the more easily it can be filtered from the chemical background in a complex mixture. Dioxin analysis has always been quite challenging and sensitivity of these compounds by mass spectrometry is still an issue due to the highly toxic potential of these compounds and therefore limits of detection need to be very low in order to ensure public health safety. Taguchi and



**Figure 2.** Kendrick mass defect as a function of nominal Kendrick mass for odd-mass ions from an ESI FT-ICR mass spectrum of a Chinese crude oil. The periodicities of 2 and 14 Da on x-axis (nominal mass), as well as 0.01340 Da on y-axis (associated to the Kendrick mass defect for two hydrogens) on the mass defect axis make it possible to determine molecular 'class' and 'type' over a wide mass range. Figure reproduced with permission from Marshall *et al.* *Acc. Chem. Res.* 2004, 37, 53–59. Copyright 2004 American Chemical Society.

co-workers analyzed samples from vegetation exposed to an industrial fire for halogenated dioxins and furans using GC-FT-ICR MS. The screening of samples was performed using Kendrick plot analysis of ultra-high resolution mass spectra based on units of chlorine substitution for hydrogen, as shown in Figure 3. This example demonstrates the flexibility possible in constructing these types of plots, depending on the compound class of interest. This type of analysis can be used for rapidly determining the extent and severity of contamination of these toxic compounds.

## MASS DEFECT FILTERING

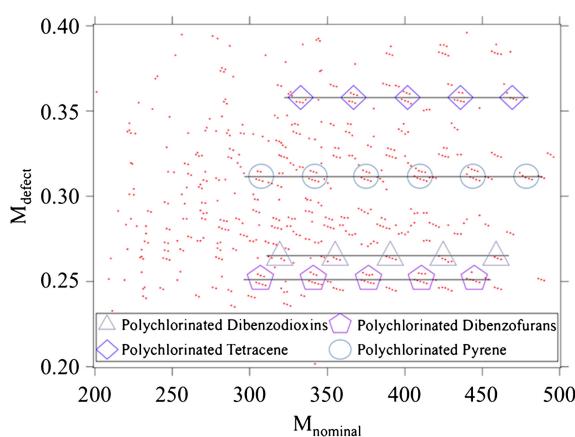
The idea of mass defect filtering (MDF) is to remove all data outside a defined and limited mass defect range from complex high resolution mass spectral data sets. For example, if a compound of interest has a mass defect of 0.135, we can selectively

'filter' out all signals corresponding to other mass defect values through post-acquisition processing of the data with a mass defect filter window centered around 0.135 with a certain user defined width, as needed, based on the resolution and accuracy of mass measurement of the given analysis. What makes the concept of mass defect very useful in mass spectrometry is the fact that elemental formulae are defined based on their accurate mass and, therefore, their mass defects can be used as a specific filtering criteria if a target compound class is known. The more unique the mass defect range of the analytes of interest, the more interesting it becomes to filter complex mass spectra based on mass defect in order to remove any non-interesting 'background' signals which serve only to complicate data analysis when a specific class of molecules is targeted in the analysis. If there are compounds that are of specific interest in a complex mixture, a high resolution mass spectrum can be filtered selectively in order to show signals for specific compounds and reducing the background due to other signals from non-related compounds. Usually, compound classes have similar mass defects or very specific trends with increasing mass, so if we are interested in a given compound or compound class, we can input the chemical formula of that compound of interest, and selectively filter out the related signals. We can also use MDF for discovering compounds related to a given analyte in a complex mixture, such as in the analysis of a target class of endogenous molecules in a complex biological sample or for drug metabolism studies.

### MDF for naturally occurring compounds

Even though drug metabolism represents one of the largest application areas of MDF, which we will see in detail in the next section, we can also apply the same concepts to identifying endogenous compounds in complex biological samples. Here, we will touch on many fields in which we can use mass defect analysis for identifying specific classes of molecules as well as transformation reactions occurring to these compounds in nature.

In a review by Sleighter *et al.*<sup>[4]</sup>, high resolution mass spectrometry and mass defect analysis was shown to be useful for separating different types of naturally occurring compounds in complex mixtures. Natural organic matter, known as NOM, is the complex assemblage of organic molecules present in natural waters and soils or sediments. The analytical challenge is obviously the sample's unique complexity and high polarity; therefore it becomes very difficult to separate species by chromatography prior to mass spectrometry analysis. By infusion experiments, an overview of the sample's constituents can be assessed; however, mass resolving power becomes extremely important. An example highlighted in the review by Sleighter *et al.* exemplifies how classification based on mass defect can be used to distinguish between compound groups. Kramer *et al.*<sup>[5]</sup> used HRMS to compare humic acids from two soils and showed that moving from low to higher MD meant either increasing the number of hydrogens or decreasing the number of oxygens in the compounds present and that hydrogen-rich compounds were found at higher MD values. A snapshot of these two soil samples, analyzed by infusion MS, showed that NOM from various sources have different molecular make-up based only on mass defect analysis. We can also distinguish chemical transformations in natural samples due to increased levels of methylation, hydration, oxidation by looking carefully at mass defect distributions in each sample, especially using Kendrick mass defect analysis. The same



**Figure 3.** Mass defect plotted as a function of nominal mass using mass scale defined by the difference from a substitution of chlorine for hydrogen useful in the identification of unknown polychlorinated compound series in complex samples following FTICR-MS analysis. [Reproduced, with kind permission from Springer Science + Business Media, from Taguchi *et al.* *J. Am. Soc Mass Spectrom.* 2011, 21, 1918–1921.]

group also reported the detection of highly carboxylated polycyclic aromatic compounds, with characteristically low mass defect due to the abundance of oxygen and deficiency of hydrogens in their structures, when analyzing a volcanic ash soil sample by FT-ICR-MS<sup>[6]</sup>.

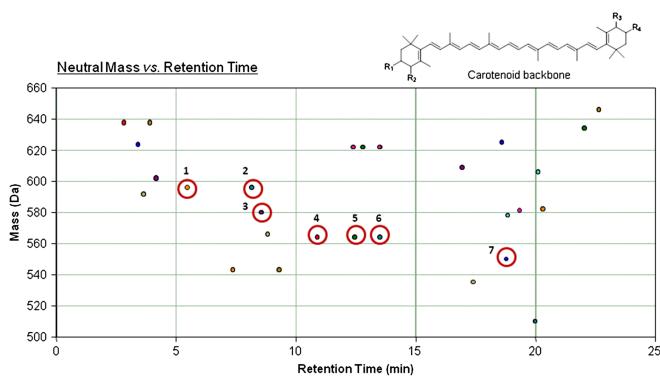
An interesting example of naturally occurring compounds with unique mass defects is seen in the case of carotenoids. These highly conjugated compounds have mass defect values differing considerably from regular aliphatic compounds in a similar *m/z* range. We have recently demonstrated the application of mass defect filtering for carotenoid analysis in complex algal samples using high-resolution TOF-MS<sup>[7]</sup>. In choosing unique windows of mass defects, we were able to filter out all non-carotenoid compounds from a non-polar extract of algal cells in a semi-targeted metabolomics application in order to identify carotenoid compounds up-regulated in algae following high salt treatment. Mass defect filtering based on the MD template of carotenoids is seen to simplify the number of mass spectral peaks which are up-regulated in these samples yielding only signals for carotenoid compounds while removing all other interfering peaks within the same *m/z* range. In Figure 4, we show representative data from the comparison of normal algae and salt-treated algae in the mass region of carotenoids (500–650 Da). The plot of neutral mass vs. retention time based on accurate mass measurements from LC-TOF-MS analysis shows the mass spectral features that have increased at least fourfold in the salt-treated sample. The circled features are those which remain after mass defect and isotope filtering specific to the elemental formulae of carotenoids. The MDF step proved invaluable for identifying differences in carotenoid content between samples<sup>[7]</sup>. Similarly, a new tool based on MDF for screening natural products has been reported using a known elemental formula as a filter template to identify structurally similar compounds. As an example<sup>[8]</sup>, peimine was used as a template compound for screening alkaloids in a crude extract of *Fritallaria thunbergii*. MDF templates can be constructed for any type of natural product and an obvious area of interest would be to adapt it for Traditional Chinese Medicine fingerprinting and

processing. A recent article was published using UPLC-TOF with MDF for profiling constituents and metabolites after administration of *Glechoma longituba* extract<sup>[9]</sup>.

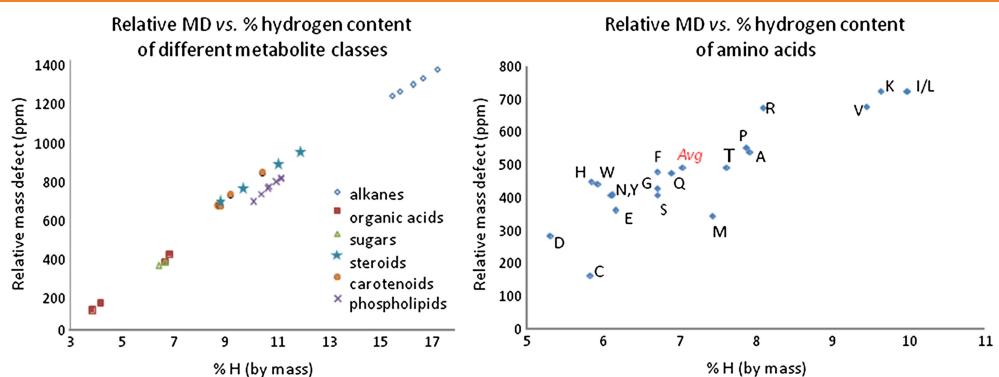
Naturally occurring compounds can be classified based on their relative mass defect, since usually each compound class will have a given trend of mass defect with increasing mass. Most elements have negative mass defects caused by the fact that nuclear binding energy increases with atomic number. Hydrogen has the most notable positive mass defect (7.825 mDa) and therefore has a major contribution to the MD of any naturally occurring compounds. Relative mass defect (RMD) reflects the fractional hydrogen content of compounds and therefore can be used for classifying ions in a mass spectrum. RMD, expressed in ppm, is calculated by dividing the mass defect by the mono-isotopic mass multiplied by 10<sup>6</sup>. Different compound classes will have specific RMD ranges. For instance, alkanes have RMD >1000 ppm, membrane lipids and steroids fall within 600 and 1000 ppm, sugars between 300 and 400 ppm, and organic acids with less than 300 ppm. An example of using RMD for discovering bioactive lipids was shown in combination with bioassay-guided fractionation for the screening an active fraction of FBS<sup>[10]</sup>. In that same paper, a nice correlation between relative mass defect and % hydrogen for various organic compounds was shown. A slightly more comprehensive version of that plot is shown in Figure 5, comparing the relative MD of several naturally occurring compound classes as a function of their % hydrogen content. Relative MDF has also been employed for detecting new selenium-containing volatiles in plants<sup>[11]</sup>. In this example, a simple relative MD-based algorithm was used to simplify high resolution EI spectra from a GC-TOF-MS instrument.

MDF has recently been used for monitoring quaternary ammonium compounds in estuarine sediments<sup>[12]</sup>. Alkylamine ions have uniquely high mass defects and therefore represent a good candidate class of molecules for MDF. For most organic molecules in environmental samples, hydrogen and nitrogen are the only elements possessing a positive MD, so it is relatively easy to discriminate interfering ions from alkylamine and alkylammonium ions. Since quaternary amine compounds containing only carbon, hydrogen and nitrogen, they will end up having the most positive mass defect possible for a given mass range.

Another class of compounds amenable to mass defect filtering is seen when studying protein and peptide phosphorylation, forming species which are important for studying cell signaling and protein function. The analysis of phosphopeptides is extremely challenging not only due to the complexity of samples in proteomic studies but of their low abundance and detectability compared to non-phosphorylated peptides in the sample. Fortunately, phosphopeptides have uniquely negative mass defects and thus can be distinguished in a complex peptide mixture by MDF<sup>[13]</sup>. This leads us into the area of peptide analysis, where MDF has become very important for the analysis of complex proteomic samples. For example, in MS/MS experiments, phosphotyrosine-containing peptides can generate a stable immonium ion with a relatively large mass defect (at *m/z* 216.043), allowing these peptides to be efficiently separated from other non-phosphoryl fragment ions<sup>[14]</sup>. Also, a simple modification of phosphoserine residues with 2-aminobenzylamine (ABA) has been described for the formation of a stable and characteristic fragment ion with unique mass defect due to its phosphorus and oxygen content<sup>[15]</sup>. This cyclophosphoramidate (CyPAA) fragment ion can be used as a mass defect marker since it is easily separated from isobaric ions and thus improves the detection of phosphoserine-containing



**Figure 4.** Analysis of up-regulated carotenoid formation in algal samples treated with high concentrations of NaCl salt. Mass spectral features found to increase at least fourfold in salt-treated samples are shown in this plot of accurate neutral mass vs. retention time. Circled features are those which remain after filtering based on mass defect and isotope abundance of naturally occurring carotenoids (using beta-carotene as template). The filtering parameters used were a mass defect of  $0.4 \pm 0.04$  Da and a  $M+1$  isotope peak abundance of 44% of the monoisotopic peak ( $\pm 20\%$ ). The up-regulated carotenoids were found to be astaxanthin isomers (1–2), adonirubin (3), canthaxanthin isomers (4–6) and echinenone (7).

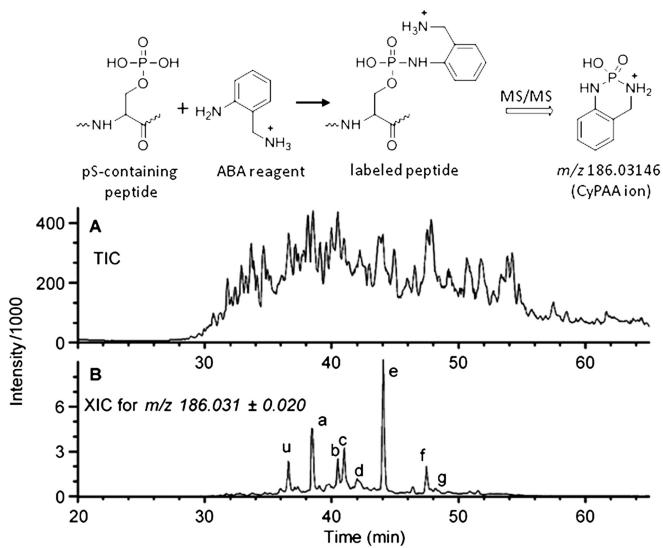


**Figure 5.** Relating relative mass defect values of common naturally occurring compounds classes to their % hydrogen content. Different classes of compounds are represented on the right plot, including alkanes [open diamonds] (including butane, pentane, hexane, octane and decane), organic acids [squares] (including malonic, lactic, citric and adipic acids), sugars [open triangles] (including glucose and sucrose), steroids [stars] (including estradiol, cholesterol, ergosterol, and testosterone), carotenoids [circles] (including beta-carotene, astaxanthin, canthaxanthin and fucoxanthin), and phospholipids [crosses] (including 32:0 and 36:0 versions of PC, PG and PE, as well as lyso PC(16:0)). Amino acids (shown on the left and labeled with their one-letter codes, as well as the theoretical amino acid *averageine* (Avg)) have more diverse values based on their side chains dictating more of their relative MD values than their common structural features.

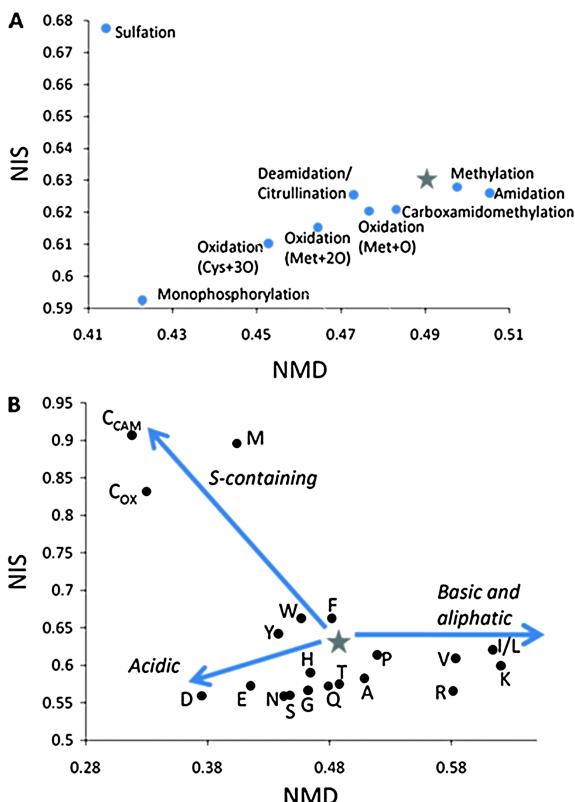
peptides in positive ion mode. The usefulness of this strategy is demonstrated in the filtering of a total ion chromatogram from a 10-protein digest using the characteristic CyPAA fragment ion in order to identify phosphoserine-containing peptides in the complex mixture, reproduced from Shi *et al.* in Figure 6.

A 2D representation of complex data has been proposed for displaying a global overview of MS data from complex peptide samples using relative mono-isotopic mass defect and normalized isotopic shift, which is calculated by the difference between the average and mono-isotopic mass, as distinguishing parameters causing peptides of the same family to cluster together on the 2D plot<sup>[16]</sup>. This method of displaying data was applied to the high throughput analysis of bioactive peptides, but can also be utilized for displaying MS data from any complex mixture. This procedure implies using two mass parameters, including normalized mass defect, having the advantage of grouping

related peptides from the same family. Also, the direction of a change in the 2D plot yields important information on the chemical nature of that change. This procedure could therefore be used to compare different data sets for specific changes occurring based on origin and treatment of samples. The authors found this method particularly helpful in distinguishing differences in peptide content from skin secretions from three different frog species. Figure 7 shows a comparison of



**Figure 6.** Total ion chromatogram from a 10-protein tryptic digest (A) and corresponding extracted ion chromatogram using CyPAA marker ion ( $m/z$  186.031) for identifying phosphoserine-containing peptides. Identified peptides originated from  $\beta$ -casein (a, g, u),  $\alpha$ -casein (b–e) and ovalbumin (f). Figure reprinted with permission from Shi *et al.* *Anal. Chem.* 2008, 80, 7614–7623. Copyright 2008 American Chemical Society.



**Figure 7.** Comparison of normalized isotopic shift and normalized mass defect values for a model amino acid made of nine *averageine* units and the effect of chemical modifications (A) or amino acid substitutions (B). Figure reprinted with permission from Artemenko *et al.* *Anal. Chem.* 2009, 81, 3738–3745. Copyright 2009 American Chemical Society.

normalized isotopic shift and normalized mass defect values for a model 9-amino acid peptide and the effect of chemical modifications or amino acid substitutions. This information can be used when analyzing peptides mixtures or effect of treatments of peptides to better understanding chemical variability of complex samples.

Since different types of peptides can be classified based on their mass defects, there is presumably a use for investigating the region of mass defect important to a given group of peptides. This exercise has recently been performed for improving the detection of human proteins with mass defect filtering<sup>[17]</sup>. An *in silico* tryptic digestion of abundant proteins contained in human serum and seminal fluid yielded a rather restricted range of mass defects which can be used for improving filter selectivity when analyzing human proteins during proteomic data analysis. The same exercise could be explored for a given sample type in order to restrict the detection of signals that would not likely originate from molecules of interest.

### Drug metabolism applications

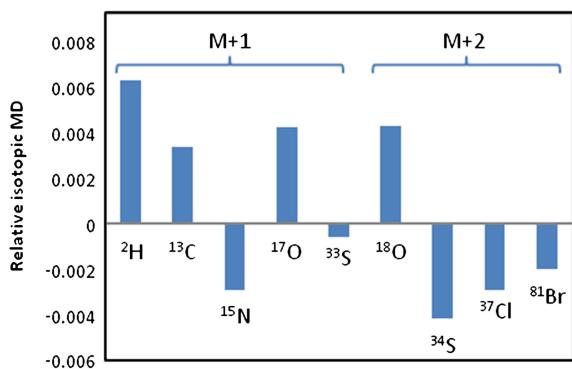
Usually, drug metabolism renders a drug more polar and thus more easily excreted, however, the central structure of the molecule does not change drastically and therefore mass defect usually remains very similar between parent drug and metabolites. There are known biotransformations occurring during drug metabolism that can be informative for filtering out specific drug metabolites. Mass defect filtering is extremely useful for drug metabolism applications since many metabolites can be filtered out of the complex high resolution mass spectral data using a simple mass defect filtering ranges centered on the parent compound with a suitable range allowing for most known metabolic biotransformations, such as oxidation. A recent tutorial<sup>[18]</sup> on the use of MDF in drug metabolite identification efforts serves as an excellent review of how MDF can be employed in such applications and therefore the same information will not be repeated here. However, improvements in MDF methods for drug metabolism studies since this review was published are covered.

Mass defect filtering (MDF) has been used extensively for the identification of reactive drug metabolites by selectively filtering for glutathione (GSH) adducts following trapping of the reactive species formed via drug metabolism. Zhu *et al.*<sup>[19]</sup> showed the applicability of MDF for the screening of reactive metabolites using orbitrap and FT-ICR MS. Using drug metabolite analysis software, mass spectra were filtered specifically for GSH adducts, based on the template of drug + GSH-2H ( $\pm 50$  Da) with a mass defect window of  $\pm 40$  mDa. Dealkylation reactions were also taken into account in this filtering step, since certain drugs can undergo dealkylation of the parent drug which can alter the mass defect of the compound. Therefore, a different template using the substructure of the dealkylated product (instead of the parent drug) was used for searching for dealkylated reactive metabolites trapped with GSH. Using several test compounds, MDF yielded less background peaks during GSH adduct analysis compared to neutral loss scanning, which is often used for this type of analysis, therefore less false positives were seen with the MDF method. For example, when selective MDF was performed for the filter template of clozapine, oxidative metabolites were easily pulled out of the background with very high signal-to-noise. Similarly, when the template for GSH adducts of clozapine was used, non-drug related ions were completely

filtered out and it was possible to easily detect three GSH adducts, including two minor species. The advantage of the MDF technique is that accurate mass LC-MS data can be acquired with a simple generic method regardless of parent drug, followed by specific post-acquisition processing<sup>[20]</sup>. De-alkylation reactions, since these represent more important mass defect shifts than simple oxidation reactions, can also be integrated in MDF processing tools, known as 'chemically intelligent' MDF which balances the need to remove matrix background using a compound specific model for generic de-alkylation. This model includes all one-bond cleavages and simple two-bond C-heteroatom and hetero-hetero atom cleavages, generating a more comprehensive MD target space without having the need for artificially large mass windows to be selected<sup>[21]</sup>. Recently, an approach for reactive metabolite screening by high resolution LC-TOF-MS<sup>E</sup> and data processing using an *in silico* C-heteroatom de-alkylation algorithm for more selective mass defect filtering was shown to yield better results than using a simple linear MDF algorithm<sup>[22]</sup>. Using the heteroatom dealkylation-based specific mass filters ( $\pm 20$  mDa) allowed the detection of specific dealkylation metabolites and therefore much less background from non-drug related ions.

MDF can also be combined with other processing techniques, such as isotope filtering (for chlorinated and brominated drugs, as well as isotopically labeled drugs), neutral loss filtering and comparing control vs. metabolism samples for finding new peaks generated during *in vitro* metabolism incubations<sup>[23]</sup>.

Automated data processing tools for mass defect filtering are now available from most MS vendors and the technique has evolved into using MDF templates for a given parent drug of interest using simulated metabolism with *m/z* ranges and associated mass defect windows to selectively filter out drug metabolite signals<sup>[24,25]</sup>. Certain instruments even have the potential for automatic data-dependent selection of precursor *m/z* values for product ion scan selection, in order to limit the number of data-dependent MS/MS scans to more useful precursor ions for this specific application area<sup>[26]</sup>. Selective filtering algorithms, such as MDF, have caused a recent migration from low resolution MS/MS-based triple quadrupole platforms to accurate mass-based high resolution instruments, such as TOFs and orbitraps, for metabolite identification in the pharmaceutical industry. Recently, the concept of 'relative isotopic MD', the difference between the mono-isotopic mass of a compound and the mass of its *M* + 1 and *M* + 2 isotopic peaks, has been used for unknown identification and could easily be applied to drug metabolism studies. A filtering tool has been described by Thurman *et al.*<sup>[27]</sup>, which employs the relative isotopic MD combined with the intensity of the isotopic cluster for molecular formula assignment from accurate mass measurements. The relative isotopic MD uses the natural abundances of important isotopes of common elements for calculating isotopic cluster intensity, and is plotted for stable isotopes of common elements in Figure 8. Depending on the resolving power of the mass spectrometer used, the *M* + 1 isotopic peak will often consist of several individual isotope peaks together, unless ultra-high resolution is achieved. For example, a resolution of higher than 150 000 is needed to distinguish <sup>13</sup>C and <sup>15</sup>N isotope contributions for a molecule of 500 Da. Therefore, since the *M* + 1 peak is a combination of several 'accurate' mass peaks, each combination of relative intensities for contributing peaks will give a distinct final relative isotopic MD which can then be used to more accurately define elemental formulae. The *M* + 2 peak can also be used, if its signal is abundant enough. For instance, the case where we see a



**Figure 8.** The relative isotopic mass defect for common stable isotopes, calculated as the difference in mass defect between the monoisotopic mass ( $M$ ) of the most abundant isotope and its  $M + 1$  or  $M + 2$  isotopes. [Adapted from Thurman EM, Ferrer I. *Anal Bioanal Chem* 2010, 397, 2807–2816, with kind permission from Springer Science + Business Media.]

positive mass defect for the  $M + 2$  peak indicates no contribution from elements such as S, Cl, or Br. Thurman *et al.* described the application of this tool for the identification of pesticides. For propazine, for example, a positive isotopic mass defect is seen for  $M + 1$  and a negative isotopic mass defect is measured for the  $M + 2$  peak. The concept of isotopic mass defect has also been well described by the same authors in a recent book chapter on mass defect and isotope clusters<sup>[28]</sup>.

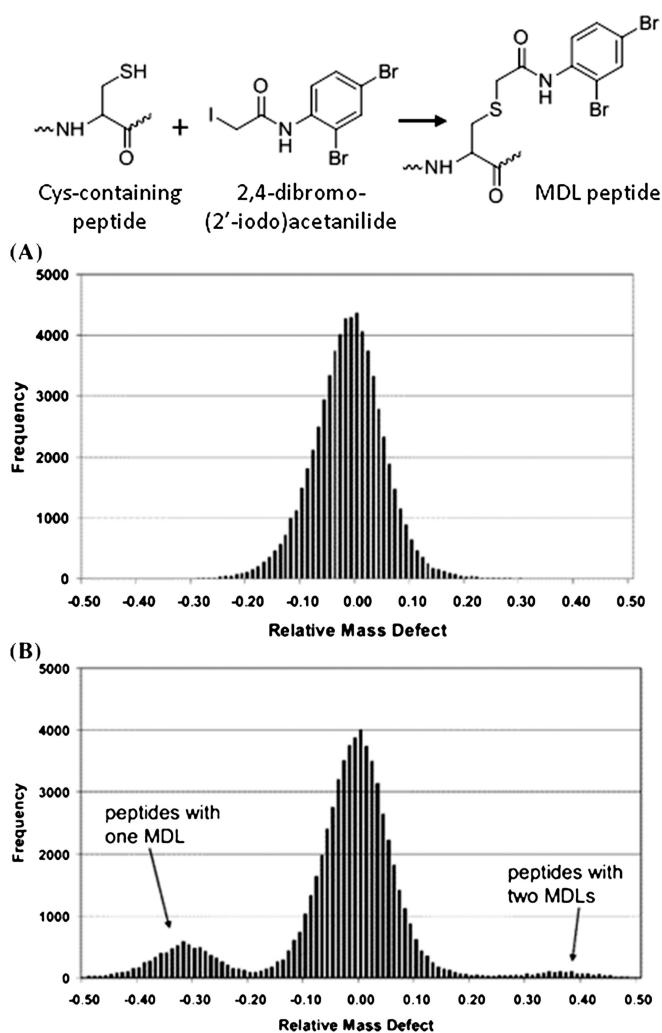
Another improvement for MDF in drug metabolism studies has been reported by using templates based on multiply charged ions instead of only using singly protonated species, especially useful in the case of GSH adducts. This has been shown for the investigation of ticlopidine bioactivation using an LTQ-Orbitrap and an improved MDF technique combining both 1+ and 2+ MDF templates for data processing<sup>[29]</sup>. The usefulness of MDF for drug metabolism applications depends highly on the complexity of the sample matrix. Different biological matrices have been tested for drug metabolite detection using mass defect filtering<sup>[30]</sup>. MDF was shown to exhibit a greater advantage for extracting drug metabolite signals from plasma, bile and feces, however, urine was found to be a more challenging sample type. The authors constructed mass defect profiles by summing mass spectra from most of the chromatographic elution profile presented as a 2D plot of mass defect vs. nominal  $m/z$ , without considering signal intensities. Based on this study, data mining with MDF alone may not be sufficient to filter out all interferences in urine, however, MDF combined with other techniques, including control sample comparison, extracted ion chromatograms analysis and common neutral loss or product ion analysis would improve the selectivity of metabolite identification. A recent *in vivo* drug metabolism study on olaquindox in rat used mass defect filtering to detect drug metabolites in urine and feces matrices in combination with characteristic fragment ions from MS<sup>E</sup> spectra for each metabolite<sup>[31]</sup>. In this example, they were able to characterize 20 metabolites in these complex matrices using MDF. Modern instrumentation allows much faster scanning and therefore what previously needed multiple injections can be done all at once, even in very complex matrices. With sophisticated IDA techniques and fast scanning, the reconstructed full scan accurate mass data can be used for MDF in combination with filtering based on fragmentation patterns to increase the coverage when profiling molecules of interest.

## MASS DEFECT LABELING

### Peptide and protein tagging methodologies

There exist natural gaps in typical mass spectra, usually spaced 1 Dalton apart, where no commonly encountered elemental compositions are seen, even in the most complex spectra. We can take advantage of such areas by tagging our analytes of interest with functional groups having unique or uncommon mass defect values, such as chlorine, bromine, iodine, arsenic, etc. The most common application area which exists for such tagging strategies is proteomics, by either labeling intact proteins or digested peptides of interest in complex samples. If we look at the common elements contained in proteins or peptides, it becomes obvious that important overlap exists resulting in crowded areas in mass spectra of complex proteomics samples. This also creates significant gaps or regions of space between these crowded areas with the potential for shifting specific proteins or peptides to less noisy areas in the mass spectrum by using selective tagging chemistry with chemical groups having unique mass defects. Atoms most commonly present in proteins include  $^{12}\text{C}$  (zero mass defect),  $^{14}\text{N}$  (0.0031) and  $^{16}\text{O}$  (-0.0051) which, for the most part, cancel each other out,  $^1\text{H}$  (0.0078), as well as relatively low abundances of  $^{32}\text{S}$  (-0.0279) and  $^{31}\text{P}$  (-0.0262). Therefore, there is a clear pattern resulting in a slight increase in mass defect with increased molecular weight due to the relatively high proportion of hydrogen atoms in these biomolecules. By calculating the mass defects for all elements of the periodic table, we see that there is a maximum mass defect of about 0.1 Da for elements between Br and Eu (mass numbers 80–150). By high resolution mass spectrometry, masses differing by 0.1 Da can be very easily distinguished even in complex mass spectra especially if they are in less populated areas of the spectrum. Bromine is a particularly good choice of element to be incorporated into a mass defect tag since it is easily incorporated into organic structures as well as having a unique isotopic pattern for  $^{79}\text{Br}$  and  $^{81}\text{Br}$  with near equal abundances which can serve as a secondary filtering criteria in complex spectra for identifying tagged species. Using MD tags containing bromine has been described<sup>[32]</sup> for resolving tagged and untagged species by high resolution mass spectrometry in protein sequencing applications. If intact proteins are labeled on either C or N terminal ends, in-source fragmentation of the labeled protein can yield information on the terminal sequence of amino acids and therefore help identify the protein using top-down mass spectrometry strategies. This method is known as ‘inverted mass ladder sequencing’ or IMLS. In-source fragmentation of intact proteins yields extremely complex spectra with peaks at virtually every  $m/z$  with average spacing of 1.000464 amu. Terminal fragments are easily distinguished by mass defect labeling with a bromine-containing reagent since the mass defect is much larger than that normally found in biomolecules and therefore, tagged peaks stand out in complex spectra and reasonably simple filtering of the mass spectra identifies only those fragments containing mass defect labels and therefore also the terminal amino acid. It was also shown by Hall *et al.* that using multiple mass defect labels allow further discrimination of peaks. For example, one bromine atom in the label allows peaks up to 228 amu to be completely free of interference, while two or three bromines bring this value up to 465 or 686 amu, respectively. Sequencing myoglobin using this tagging strategy was shown as an example by Hall *et al.*

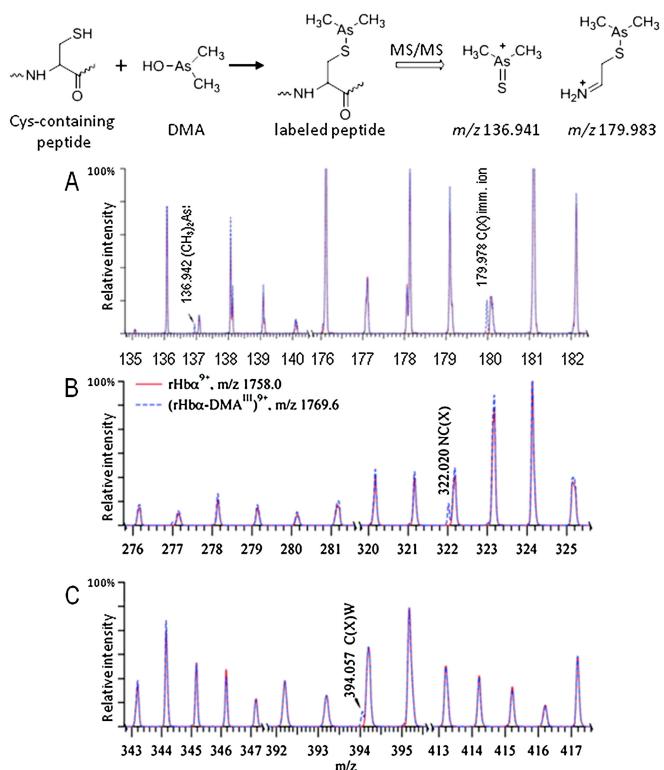
Cysteine residues are often candidates for tagging strategies since their chemistries allow very selective labeling procedures and they are also less common in proteins causing a limited number of peptides to be shifted into less noisy mass spectral regions. A mass defect tag, 2,4-dibromo(2'-iodo)acetanilide has been reported<sup>[33]</sup> as a useful tag for increasing ionization and detectability of cysteine-containing peptides, based on its unique mass defect as well as the ability to filter resulting mass spectra with the selective isotope pattern as a result of the bromine atoms. The effect of this mass defect tagging approach was demonstrated by graphing the distribution of mass defects for all tryptic peptides contained in a complex sample of digested *M. maripaludis* before and after tagging all cysteine-containing peptides with their MD label. This comparison has been reproduced in Figure 9, where modifying peptides with either one or two tags are completely shifted into regions of low chemical complexity. The authors<sup>[33]</sup> characterized an ideal tag as one which exhibits high specificity for low abundance amino acids



**Figure 9.** Distribution of mass defects calculated for all tryptic peptides of *M. maripaludis* between 700 and 3500 Da (A) and effect of tagging cysteine-containing peptides with one or two mass defect labels (B). The horizontal axis is the mass difference (amu) between a peptide's mass defect and the average mass defect for all peptides of the same nominal mass. The central distribution in B corresponds to all peptides that do not contain cysteine. Figure reprinted with permission from Hernandez *et al.* *Anal. Chem.* 2006, 78, 3417–3423. Copyright 2006 American Chemical Society.

(such as cysteine or tryptophan) so as to not shift all peptides, and having a mass defect shift between 0.3 and 0.6 amu<sup>[34]</sup>. The resulting product should be very stable and have no negative effect on solubility or ionization of the peptide. Another unique tag for cysteine-containing peptides has been described using arsenic labeling. Using dimethyl arsinous acid, with an exact mass of 103.9607 g/mol, the resulting labeled peptide (+As<sub>2</sub>H<sub>5</sub>) has a distinct mass defect from other natural peptides in the complex mixture<sup>[35]</sup>. This tag allows arsenic containing ions to be rapidly identified based on specific internal and immonium ions from MS/MS spectra, as shown in Figure 10. The As-S bond is also quite stable under CID conditions, which preserves sequence information. The authors applied their tagging procedure to identify active binding sites on hemoglobin without using enzymatic hydrolysis using a top-down approach by intact protein MS/MS.

Also, most transition and lanthanide series metals have important mass defects which can be used to tag species as chelates. Element-coded metal tags have been reported for peptides using chelates of rare earth metals including yttrium, terbium and holmium, having unique mass defects of approximately 70 mDa each<sup>[36]</sup>. This method has been successfully applied to identify protein oxidation sites using an oxidation-dependent element coded affinity tags coupled to nanospray LC-FTICR-MS, using terbium and holmium as mass defect tags<sup>[37]</sup>. There even exists the possibility for multiplexed experiments with this strategy



**Figure 10.** Comparison of the MS/MS spectra of the parent ions of the rHbα – DMAIII complex ( $m/z = 1769.6$ , 9+) and rHbα ( $m/z = 1758.0$ , 9+) showing two characteristic ions at  $m/z = 136.942$  and  $179.978$  for the arsenic-tagged cysteine specific fragment ions (A). Spectra B and C shows two characteristic internal dipeptide ions with DMAIII tag at  $m/z = 322.020$  and  $394.057$  uniquely present in the MS/MS spectrum of the rHbα – DMAIII complex (in treated rats) but not in that of rHbα itself (in control rats). C(X) denotes that the cysteine residue contains a DMAIII tag [ $(\text{CH}_3)_2\text{As}$ ]. Figure adapted with permission from Meiling *et al.* *J. Proteome Res.* 2008, 7, 3080–3090. Copyright 2008 American Chemical Society.

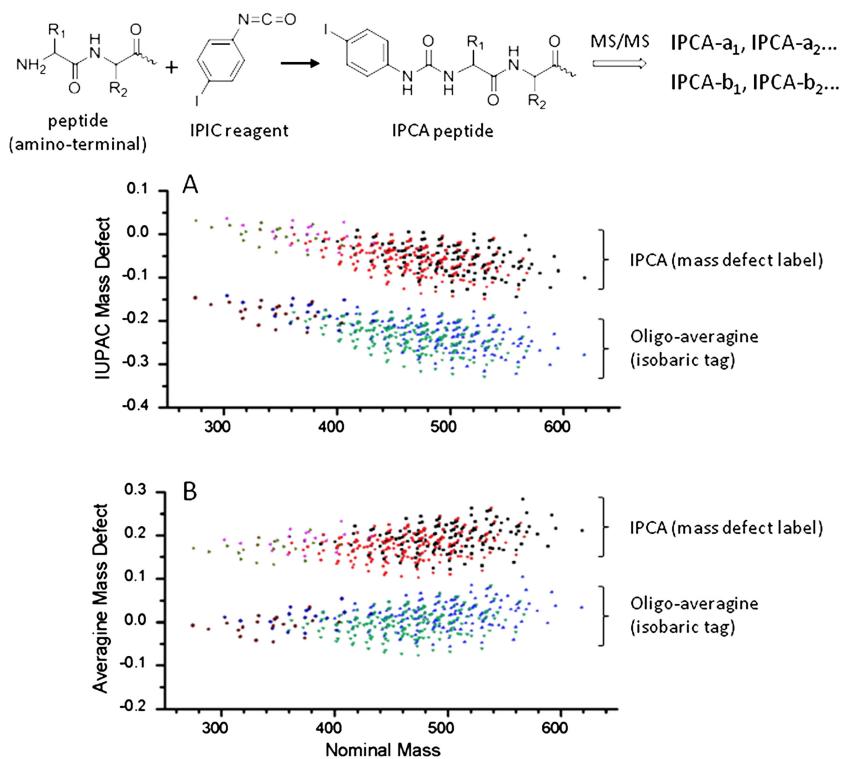
using as many as 7 mono-isotopic rare earth metals<sup>[36]</sup>. This type of experiment would allow the relative quantitation of peptides reactive to the chelated derivatization reagent to be assessed between multiple samples using similar chemistries but with different lanthanides as mass labels. This would allow filtering based on a specific mass defect range for all tagged species to reduce the non-labeled background. The importance is not to have all the tagged species with unique mass defects to each other but to have selective mass shifts based on the lanthanide in each tag as well as unique mass defects compared to the rest of the non-labeled ions.

There has also been recent work in crosslinking labels containing specific mass defect and isotope pattern using a di-brominated cleavable label (dibromo-aniline-dipropionic acid N-succinimidyl ether). The addition of two bromines atoms in the label shifts crosslinked peptides away from simple tryptic fragments. The authors applied this novel strategy on finding interacting regions within a 34 kDa actin protein system<sup>[38]</sup>. A recent review on crosslinking mass spectrometry discusses the use of incorporating mass defect labels into these reagents<sup>[39]</sup>.

Yao and colleagues<sup>[40,41]</sup> have conceived of some interesting mass defect tags for peptides which not only shift the precursor mass into a less noisy region of the mass spectrum, but have specific utility due to their selective fragmentation behavior. Iodine was noted as an ideal element to incorporate into a tag since it has the highest mass defect shift among the halogens and is relatively easily incorporated into reagents for modifying peptides. This effect was demonstrated by comparing mass defect plots for iodine-tagged peptides, using a 4-iodophenylcarbamoyl (IPCA) tag, with those from hypothetical isobaric ions shifted by the same nominal mass using a peptide-like tag, based on *averagine* analysis. This plot display has been reproduced here in

Figure 11<sup>[40]</sup>, demonstrating the effect of displaying MD as a function of IUPAC mass or based on *averagine* mass (using the scaling factor of 111/111.0543052, for the ratio between the nominal and exact mass of the theoretical amino acid *averagine*). *Averagine* is defined as having a representative elemental formula of the average of amino acids found in peptides, and is equal to C<sub>4.9384</sub>H<sub>7.7583</sub>N<sub>1.3577</sub>O<sub>1.4773</sub>S<sub>0.0417</sub><sup>[42]</sup>. The applicability of the different iodine-based labels in terms of induced MS/MS fragmentation and extracted information is described in detail by Shi *et al.*<sup>[31]</sup> They have characterized tags as either *passive* or *active*, depending on the MS/MS spectra produced from the modified peptide for distinct applications in proteome analysis. Passive tags generate multiple *N*-terminal fragment ions yielding good sequence coverage of the peptide. Active tags induce specific fragmentation, either forming the ionized tag itself or the *N*-terminal amino acid still attached to the MD tag, yielding very sensitive ions useful in MRM method development for peptide quantitation applications, for instance.

Alternatively to mass defect tagging, a strategy for removing interfering peptides from the border of the mass defect distribution has been reported for increasing the detectability of phosphorylated peptides<sup>[43]</sup>. Since oxygen is the most common element in peptides having a negative mass defect, peptides with a high number of oxygen atoms lie close to the mass defect border of peptide ion distributions and therefore can interfere with the analysis of phosphorylated peptides. By esterifying carboxylate groups with aliphatic alcohols, oxygen-rich fragment ions are pushed away from the region of phosphorylated fragment ions. Characteristic ions used in the detection of phosphopeptides by MS/MS, such as phosphotyrosine immonium and cyclophosphoramidate ions, are thus more easily detected after treatment with n-butanol<sup>[43]</sup>.



**Figure 11.** Mass defect plots of small *N*-terminal ions up to two amino acid residues (*a*<sub>1-2</sub> and *b*<sub>1-2</sub> ions) on the IUPAC (A) and *averagine* (B) scales with and without IPCA-labeling. Figure adapted with permission from Yao *et al.* *Anal. Chem.* 2008, 80, 7383–7391. Copyright 2008 American Chemical Society.

## Reactive drug metabolites trapped using mass defect label

Most applications of mass defect labeling have involved proteomic analyses, since specific functionalities within proteins and peptides can easily be probed by chemical labels. Recently, we have demonstrated the usefulness of incorporating a mass defect label into a GSH analog for reactive metabolite screening<sup>[44]</sup>. A bromine atom allowed increased selectivity for the detection of GSH adducts following the trapping of reactive drug metabolites in *in vitro* incubations, as well as having a unique isotope pattern for additional filtering of mass spectral data. This method resulted in fewer false positives as well as increased detection of adducts, due to higher sensitivity of the brominated adducts.

## PERSPECTIVES

Mass defect analysis can be applied to virtually all application areas of MS. It is obvious from the increasing amount of MS papers discussing MDF and MD labels in recent years that the prospects are endless. Filtering based on known mass defects allows the selective profiling of specific compound classes. Alternatively, using MD labels for shifting ions into less noisy MS space can increase the detectability of ions of interest. MDF has become so important that automated MD data processing tools are currently incorporated in all high resolution MS platforms.

Certain fields still have numerous opportunities for further applying mass defect analysis for complex sample characterization by HR-MS. For instance, the expanding field of metabolomics could find it useful to label small molecules with MD tags for semi-targeted applications. This concept has been touch upon for the analysis of fatty acids<sup>[45]</sup>, however, many more compound classes could be probed using a MD labeling approach for better filtering of complex data, while potentially incorporating isotope labels for relative quantitation. It would be possible to combine the potential of mass defect filtering with isotope filtering using either isotopically pure bromines (<sup>79</sup>Br vs. <sup>81</sup>Br) or <sup>13</sup>C labels as well as bromine-containing tags, where bromine doublets would be separated by a set number of <sup>13</sup>C labels. Also, it has been previously proposed<sup>[32]</sup> that a similar strategy to sequencing mass defect tags used for protein analysis could be employed for DNA sequencing application using mass defect labels incorporated into non-extendable DNA bases, as well as conceiving tags specific for labeling carboxyl group and phosphorylated residues on proteins<sup>[46]</sup>.

Moreover, many more applications exist for using natural mass defects of endogenous compound classes, such as nucleic acids, fatty acids, sugars and phosphorylated small molecules. Continued imagination with KM plots for displaying specific patterns of interest in complex data sets is also possible, for determining the effect on molecular signatures in complex data sets on specific treatments, for instance during petroleum processing or environmental clean-up strategies. Imaging mass spectrometry could also be combined with mass defect filtering for improved resolution between interfering images from ions of similar *m/z* values, or to evaluate images belonging to a set of compounds of a specific class. This idea has been recently applied for the direct analysis of lipid species by imaging MALDI using MDF for removing background ions not due to lipid species<sup>[47]</sup>, however, many more possibilities exist for this type of filtering of MS images to decipher interesting patterns of biological compounds, either drug and their metabolites, endogenous metabolites and peptides.

## REFERENCES

- [1] C.A. Hughey, C.L. Hendrickson, R.P. Rodgers, A.G. Marshall. Kendrick mass defect spectrum: A compact visual analysis for ultrahigh-resolution broadband mass spectra. *Anal. Chem.* **2001**, *73*, 4676.
- [2] A.G. Marshall, R.P. Rodgers. Petroleomics: The next grand challenge for chemical analysis. *Acc. Chem. Res.* **2004**, *37*, 53.
- [3] V.Y. Taguchi, R.J. Niekarz, R.E. Clement, S. Krolik, R. Williams. Dioxin analysis by gas chromatography-Fourier transform ion cyclotron resonance mass spectrometry (GC-FTICR-MS). *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 1918.
- [4] R. L. Sleighter, P.G. Hatcher. The application of electrospray ionization coupled to ultrahigh resolution mass spectrometry for the molecular characterization of natural organic matter. *J. Mass Spectrom.* **2007**, *42*, 559.
- [5] R.W. Kramer, E.B. Kujawinski, X. Zang, K.B. Green-Church, B. Jones, M.A. Freitas, P.G. Hatcher. Studies of the structure of humic substances by electrospray ionization coupled to a quadrupole-time of flight (QQ-TOF) mass spectrometer. *Spec. Publ. - R. Soc. Chem.* **2001**, *273*, 95.
- [6] R.W. Kramer, E.B. Kujawinski, P.G. Hatcher. Identification of black carbon derived structures in a volcanic ash soil humic acid by Fourier transform ion cyclotron resonance mass spectrometry. *Environ. Sci. Technol.* **2004**, *38*, 3387.
- [7] F. L. Chu, L. Pirastu, R. Popovic, L. Sleno. Carotenogenesis up-regulation in *Scenedesmus* sp. using a targeted metabolomics approach by liquid chromatography – high resolution mass spectrometry. *J. Agric. Food Chem.* **2011**, *59*, 3004.
- [8] L-Q. Wang, Z. Gu. Mass defect filtering: a new tool to expedite screening, dereplication and identification of natural products. 58<sup>th</sup> ASMS Conference on Mass Spectrometry and Allied Topics, Salt Lake City, Utah, May 23–27, **2010**.
- [9] S. Ni, D. Qian, J. Duan, J. Guo, E. Shang, Y. Shu, C. Xue. UPLC-QTOF/MS-based screening and identification of the constituents and their metabolites in rat plasma and urine after oral administration of *Glechoma longituba* extract. *J. Chromatogr. B* **2010**, *878*, 2741.
- [10] M.C. Stagliano, J.G. DeKeyser, C.J. Omiecienski, A.D. Jones. Bioassay-directed fractionation for discovery of bioactive neutral lipids guided by relative mass defect filtering and multiplexed collision-induced dissociation. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3578.
- [11] M. Shah, J. Meija, J.A. Caruso. Relative mass defect filtering of high-resolution mass spectra for exploring minor selenium volatiles in selenium-enriched green onions. *Anal. Chem.* **2007**, *79*, 846.
- [12] X. Li, B.J. Brownawell. Analysis of quaternary ammonium compounds in estuarine sediments by LC-TOF-MS: Very high positive mass defects of alkylammonium ions as powerful diagnostic tools for identification and structural elucidation. *Anal. Chem.* **2009**, *81*, 7926.
- [13] C. Bruce, M.A. Shifman, P. Miller, E.E. Gulcicek. Probabilistic enrichment of phosphopeptides by their mass defect. *Anal. Chem.* **2006**, *78*, 4374.
- [14] H. Steen, B. Küster, M. Mann. Quadrupole time-of-flight versus triple-quadrupole mass spectrometry for the determination of phosphopeptides by precursor ion scanning. *J. Mass Spectrom.* **2001**, *36*, 782.
- [15] Y. Shi, B. Bajrami, M. Morton, X. Yao. Cyclophosphoramidate ion as mass defect marker for efficient detection of protein serine phosphorylation. *Anal. Chem.* **2008**, *80*, 7614.
- [16] K.A. Artemenko, A.R. Zubarev, T.Y. Samgina, A.T. Lebedev, M.M. Savitski, R.A. Zubarev. Two dimensional mass mapping as a general method of data representation in comprehensive analysis of complex molecular mixtures. *Anal. Chem.* **2009**, *81*, 3738.
- [17] M.L. Toumi, H. Desaire. Improving mass defect filters for human proteins. *J. Proteome Res.* **2010**, *9*, 5492.
- [18] H. Zhang, D. Zhang, K. Ray, M. Zhu. Mass defect filter technique and its applications to drug metabolite identification by high-resolution mass spectrometry. *J. Mass Spectrom.* **2009**, *44*, 999.
- [19] M. Zhu, L. Ma, H. Zhang, W.G. Humphreys. Detection and structural characterization of glutathione-trapped reactive metabolites using liquid chromatography – high resolution mass spectrometry and mass defect filtering. *Anal. Chem.* **2007**, *79*, 8333.
- [20] Q. Ruan, S. Peterman, M.A. Szewc, L. Ma, D. Cui, W.G. Humphreys, M. Zhu. An integrated method for metabolite detection and identification using a linear ion trap/Orbitrap mass spectrometer and multiple data processing techniques: application to indinavir metabolite detection. *J. Mass Spectrom.* **2008**, *43*, 251.

- [21] R.J. Mortishire-Smith, J.M. Castro-Perez, K. Yu, J.P. Shockor, J. Goshawk, M.J. Hartshorn, A. Hill. Generic dealkylation: a tool for increasing the hit-rate of metabolite rationalization, and automatic customization of mass defect filters. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 939.
- [22] J.E. Barbara, J.M. Castro-Perez. High-resolution chromatography/time-of-flight MS<sup>E</sup> with *in silico* data mining is an information-rich approach to reactive metabolite screening. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 3029.
- [23] F. Cuyckens, R. Hurkmans, J.M. Castro-Perez, L. Leclercq, R.J. Mortishire-Smith. Extracting metabolite ions out of the matrix background by combined mass defect, neutral loss and isotope filtration. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 327.
- [24] A.C. Li, J. Ding, X. Jiang, J. Denissen. Two-injection workflow for a liquid chromatography/LTQ-Orbitrap system to complete *in vivo* biotransformation characterization: demonstration with buspirone metabolite identification. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 3003.
- [25] J.C.L. Erve, C.E. Beyer, L. Manzino, R.E. Talaat. Metabolite identification in rat brain microdialysates by direct infusion nanoelectrospray ionization after desalting on a ZipTip and LTQ/Orbitrap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 4003.
- [26] N. Bloomfield, Y. LeBlanc. Mass defect triggered information dependent acquisition. International Patent 2007/076606, 12 July **2007**.
- [27] E.M. Thurman, I. Ferrer. The isotopic mass defect: a tool for limiting molecular formulas by accurate mass. *Anal. Bioanal. Chem.* **2010**, *397*, 2807.
- [28] E.M. Thurman, I. Ferrer. In Liquid chromatography Time-of-Flight Mass Spectrometry: Principles, Tools, and applications, (Eds: I. Ferrer, E.M. Thurman). John Wiley & Sons, Inc., **2009**, 17–31.
- [29] Q. Ruan, M. Zhu. Investigation of bioactivation of ticlopidine using linear ion trap/orbitrap mass spectrometry and an improved mass defect filtering technique. *Chem. Res. Toxicol.* **2010**, *23*, 909.
- [30] H. Zhang, M. Zhu, K.L. Ray, L. Ma, D. Zhang. Mass defect profiles of biological matrices and the general applicability of mass defect filtering for metabolite detection. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2082.
- [31] Y. Bi, X. Wang, S. Xu, L. Sun, L. Zhang, F. Zhong, S. Wang, S. Ding, X. Xiao. Metabolism of olaquindox in rat and identification of metabolites in urine and feces using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 889.
- [32] M.P. Hall, S. Ashrafi, I. Obegi, R. Petesch, J.N. Peterson, L.V. Schneider. 'Mass defect' tags for biomolecular mass spectrometry. *J. Mass Spectrom.* **2003**, *38*, 809.
- [33] H. Hernandez, S. Niehauser, S.A. Boltz, V. Gawandi, R.S. Phillips, I.J. Amster. Mass defect labeling of cysteine for improving peptide assignment in shotgun proteomic analyses. *Anal. Chem.* **2006**, *78*, 3417.
- [34] I.J. Amster, R.S. Phillips. Mass defect labelling and methods of use thereof. US Patent 20090061523, 5 March **2009**.
- [35] M. Lu, H. Wang, Z. Wang, X-F Li, X.C. Le. Identification of reactive cysteines in a protein using arsenic labeling and collision-induced dissociation tandem mass spectrometry. *J. Proteome Res.* **2008**, *7*, 3080.
- [36] P.A. Whetstone, N.G. Butlin, T.M. Corneillie, C.F. Meares. Element-coded affinity tags for peptides and proteins. *Bioconjugate Chem.* **2004**, *15*, 3.
- [37] S. Lee, N.L. Young, P.A. Whetstone, S.M. Cheal, W.H. Benner, C.B. Lebrilla, C.F. Meares. Method to site-specifically identify and quantitate carbonyl end products of protein oxidation using oxidation-dependent element coded affinity tags (O-ECAT) and nanoliquid chromatography Fourier transform mass spectrometry. *J. Proteome Res.* **2006**, *5*, 539.
- [38] L.L. Hoffman, P. Griffin, M. Fechheimer, E. Petrotchenko, C. Borchers, I.J. Amster. Development of a mass spectrometry identifiable cross-linker and application to a 34 kDa actin protein system. 57<sup>th</sup> ASMS Conference on Mass Spectrometry and Allied Topics, Philadelphia, Pennsylvania, June 5–9, **2009**.
- [39] E.V. Petrotchenko, C.H. Borchers. Crosslinking combined with mass spectrometry for structural proteomics. *Mass Spectrom. Rev.* **2010**, *29*, 862.
- [40] X. Yao, P. Diego, A.A. Ramos, Y. Shi. Average-scaling analysis and fragment ion mass defect labeling in peptide mass spectrometry. *Anal. Chem.* **2008**, *80*, 7383.
- [41] Y. Shi, B. Bajrami, X. Yao. Passive and active fragment ion mass defect labeling: Distinct proteomics potential of iodine-based reagents. *Anal. Chem.* **2009**, *81*, 6438.
- [42] M.W. Senko, S.C. Beu, F.W. McLafferty. Determination of monoisotopic masses and ion populations for large biomolecules from resolved isotopic distributions. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 229.
- [43] B. Bajrami, Y. Shi, P. Lapierre, X. Yao. Shifting unoccupied spectral space in mass spectrum of peptide fragment ions. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 2124.
- [44] A. LeBlanc, T.C. Shiao, R. Roy, L. Sleno. Improved detection of reactive metabolites with a bromine-containing glutathione analog using mass defect and isotope pattern matching. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1241.
- [45] J. Iglesias, A. Koulman, D.A. Volmer. A differential isotope-coded mass defect label for metabolite discovery and relative quantitation. 58<sup>th</sup> ASMS Conference on Mass Spectrometry and Allied Topics, Salt Lake City, Utah, May 23–27, **2010**.
- [46] M.P. Hall, L.V. Schneider. Isotope-differentiated binding energy shift tags (IDBEST<sup>TM</sup>) for improved targeted biomarker discovery and validation. *Expert Rev. Proteomics* **2004**, *1*, 421.
- [47] R.C. Murphy, J.A. Hankin, R.M. Barkley. Imaging of lipid species by MALDI mass spectrometry. *J. Lipid Res.* **2009**, *50*, S317.