Utilizing Very High Resolution Fine Isotopic Fragmentation Data to Refine Elemental Composition Determination

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Overview

Purpose: To determine the capability of fine isotopic information to refine the prediction of the elemental composition from very high resolution data.

Methods: Mass spectral information was acquired at very high resolutions (>240,000 FWHM @ m/z 200) for both full scan and fragmentation (MS^2) for known components. The elemental composition was calculated for the full scan observed isotopic pattern using a two different elemental composition sets, a limited "pre-known" set and a more relevant "open" set. The ability of the fine isotopic information to improve composition determination was measured by refining the initial elements and limits in the two sets be the direct observation of elements by their fine isotopic signal in both the full MS and MS^2 data.

Results: The inclusion refined elemental subsets by the direct observation of elemental fine isotopic data in very high resolution data improved the ability to predict an elemental composition by several fold and, in some cases, reduced the complexity by an order of magnitude.

Introduction

The elemental composition of a compound is often an important clue in determining the identification, and subsequently the structure, of an unknown. Accurate mass data can provide a limited number of possible compositions for a given full MS isotopic pattern depending on the observed m/z as well as the elemental subset (the allowed elements and the range of atoms for each element) allowed for the calculation. If the elemental subset is constrained (through prior knowledge of the correct formula) then the total number of possible elemental compositions for an isotopic pattern can be controlled to a reasonable range. In most cases, and especially when performing true unknown detection and identification, the elemental subset cannot be constrained in such an extensive fashion. Instead, other approaches can be used to provide clues to the possible elements present and limits on their range of atoms. One such approach is the direct observation of their presence by utilizing very high resolution. Various isotopes of common elements have small deviations in their mass defect which can be separated at very high resolving powers leading to the direct observation of an element by this fine isotopic fingerprint.

Methods

Sample Preparation

Standard samples of 5 compounds were prepared in a solution of 50:50 water:acetonitrile with 0.1% formic acid.

Liquid Chromatography (or more generically Separations)

Samples were directly infused into the mass spectrometer for analysis by electrospray ionization in the positive mode.

Mass Spectrometry

The analysis was performed on an Orbitrap Fusion Tribrid™ mass spectrometer with the Easy-IC™ calibration option. Easy-IC was turned on during acquisition for both the full MS and MS2. Fragmentation data was acquired using HCD at 25% for tryptophan, 35% for ranitidine, 40% for oxytetracycline, and 60% for guanine and norfloxacin. The fragmentation was acquired using an isolation offset method. The isolation width was set to 4 AMU with an offset of 1 AMU for a net isolation of -1 to +3 AMU. This allowed for fragmentation scans to have isotopes up to A2.

Data Analysis

Determination of possible elemental compositions was performed using the Spectral Distance algorithm in FreeStyle™ data visualization software. For each compound, the 10^{th} full scan profile was taken and used for elemental composition, no averaging of scan data was performed. For fragmentation analysis, the 10^{th} MS^2 spectra was used for analysis, no averaging of scan data was performed.

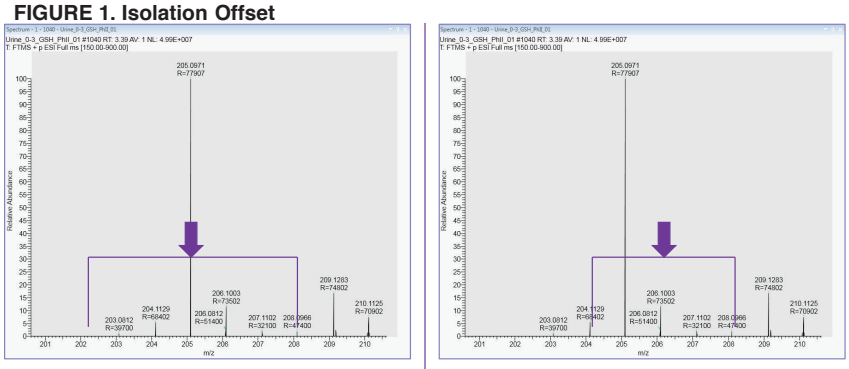
Results

Instrument Considerations – Easy-IC and Isolation Offset

The data was acquired on an instrument with a novel constant source internal calibration (Easy-IC) option. Briefly, a source within the ion optics releases a constant concentration of fluoranthene when on which can be used as an internal calibrant in both positive and negative modes. Fluoranthene ions are generated via Townsend discharge with a stable supply of more than six months continuous. Full MS and MS2 data was calibrated in real time against this constant internal calibrant.

In addition, the mass spectrometer used was capable of performing an offset isolation. This isolation style focuses the isolation of the precursor on the isotopic envelope and allows for the creation of a fragmentation pattern which retains this isotopic pattern without using a significantly wide isolation window reaching significantly below A0. A simple graphical representation of this isolation approach is shown in Figure 1. for ranitidine used in this study.

FIGURE 1. Isolation Offset



Normal Isolation – 6 AMU window centered on tryptophan

Offset Isolation – 4 AMU window offset by +1

The Elemental Composition Subsets – "Pre-Known" and "Open"

Accurate mass alone is insufficient to determine the elemental composition of an unknown. Often, *a priori* knowledge is used to limit the elements in use and constrain the maximum and minimum number of atoms for each element. This can be due to already knowing the answer, or having understanding (real or false) of what a reasonable composition may be. The problem arises when our assumptions are incorrect and we are led to a wrong elemental composition. For this work, we applied two elemental composition subsets. The first was a "Pre-known" set where elements and ranges were applied assuming prior knowledge of the potential "right" answer. The second set, the "open" set, uses the C, H, O, S, N, P elements commonly observed and added elements expected to be possibly observed in compounds found in endogenous samples (containing both endogenous and exogenous compounds) a reasonably wide range for each element. The elemental composition subsets and their ranges are shown in Table 1. It should be noted that inclusion of fluorine greatly increases the possible elemental compositions that can be predicted. For example, inclusion of 0-3 fluorine in the "Open" set increases predictions for Oxytetrycycline from 201 to 324. In order to fully demonstrate the capabilities of this approach, fluorine was kept in the possible elemental set but heavily limited in the "Pre-Known" set.

TABLE 1. The Elements and Ranges used for Composition Determination.

Element	Pre-Known Set		Open Set	
	Min	Max	Min	Max
C	p/2	p+6	1	60
H	p/2	p+12	2	180
O	p-2 or 0	p+3	0	20
N	p-2 or 0	p+2	0	15
S	p-2 or 0	if p>0 then p+1, else 0	0	4
P	p-2 or 0	if p>0 then p+1, else 0	0	3
F	0	p	0	3

p = the number of atoms present in the known parent structure. For the "Pre-known" list, the minimum and maximum values were set as an expansion of the known "right" answer. For the minimum values, 0 was used when the formula provided a minimum value < 0.

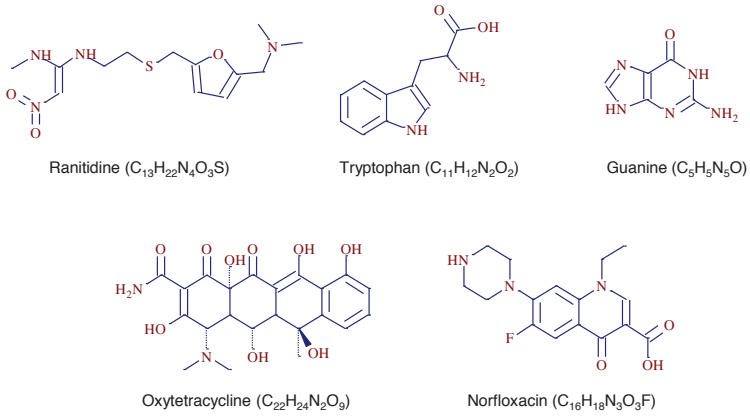
Compounds and Ranges for Elemental Composition Subsets

Five compounds were studied for the utility of fine isotopic information in elemental composition. The compounds had molecule weights that ranged from 150 to 500. The compounds and ranges for the elemental composition subsets for the "Pre-known" values are shown in Table 2 and their structures are shown in Figure 2. The compounds were chosen to cover a range of compound classes and elemental compositions to provide a reasonable test set.

TABLE 2. Compounds and "Pre-known" Composition Subsets.

Element	Ranitidine	Tryptophan	Oxytetracycline	Guanine	Norfloxacin
C	6 – 19	5 – 17	11 – 28	3 – 11	8 – 22
H	11 – 34	6 – 24	12 – 36	3 – 17	9 – 30
O	1 – 6	0 – 5	7 – 12	0 – 4	1 – 6
N	2 – 6	0 – 4	0 – 4	3 – 7	1 – 5
S	1 – 2	0	0	0	0
P	0	0	0	0	0
F	0	0	0	0	0 – 1

FIGURE 2. Structures of Compounds for Composition Determination.



Comparison of Performance – Open vs Pre-Known

For each of the five study compounds, elemental compositions were determined using the pre-known and open sets using the observed fullscan data from a 30,000 resolving power analysis. The results are shown in Table 3 for the two sets. As expected, the more limited range of elements and ranges from the pre-known set significantly reduced the possible compositions indicating it is possible to calculate the right answer when it is already known. However, this may not reflect realistic use cases for unknown analysis. It is also important to note that, even though the mass accuracy was less than 1 ppm for all compounds, it was not possible to predict only a single elemental composition for any compound even using a "pre-known" elemental composition subset. Further limits to the element subset would be required to be able to determine a single composition from only the full scan mass.

TABLE 3. Elemental Composition Determination – No Fine Isotope Refinement.

Compound	Measured Accuracy (ppm)	Total Possible Compositions	
		Pre-Known	Open
Ranitidine	**-0.1**	1	50
Tryptophan	**-0.1**	4	7
Oxytetracycline	**1.0**	2	324
Guanine	**0.8**	1	6
Norfloxacin	**0.5**	2	61

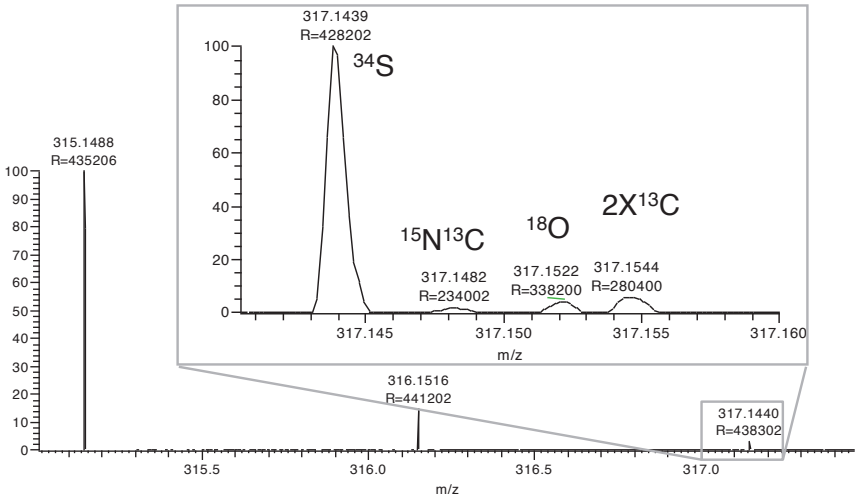
All compositions were determined from a single full MS scan, no scan averaging was performed

Utilizing Fine Isotope Data to Refine Elemental Composition

The determination was repeated using a 480,000 resolving power MS and 240,000 resolving power MS^2 analysis and the fine isotopic information available in the full scan and fragmentation scans was used to refine the elements in use and the minimum number of each. An example of the fine isotopic data available from the fragmentation analysis of ranitidine is shown in Figure 3. The presence of detectable ^{15}N, ^{33}S, ^{34}S, and ^{18}O in several fragments allowed the minimum value for the open set to be raised to 1 for each of these elements.

In addition, the relative maximum number of atoms for sulfur could be estimated by comparing the observed fine isotope ratio between the ^{34}S and $2X^{13}C$ signal in the full MS data. The total number of carbon atoms in a molecule was first estimated by looking at the $^{13}C/^{12}C$ ratio in the A^1 isotope and the $2X^{13}C/^{12}C$ ratio in the A^2 isotope to determine two independent measures of the number of carbon atoms. These estimates were averaged to provide the number of carbon atoms. Subsequently, the ratio of the observed ^{34}S and $2X^{13}C$ isotopes and the known natural abundances of isotopes could be used to provide an estimate of the maximum number of sulfur atoms. Since it was assumed the signal for ^{13}C in A^1 and A^2 could have contained unresolved isotopes (^{15}N, ^{17}O, ^{18}O, etc) this was only used to provide an estimate of the maximum. For example, the determination of a single sulfur atom in the observed data for ranitidine led to a maximum sulfur limit of 3 (allowing a buffer of 1 additional sulfur atom)

FIGURE 3. Full MS Fine Isotopic Pattern - Ranitidine



The fine isotopic pattern observed in full MS and in MS^2 was used t o refine the elements and min/max values for composition determination in the Open set.

Table 4 shows the detected fine isotope refinement possible by direct observation of isotopes in fragmentation data for the compounds studied and the resulting improvement on the possible elemental compositions calculated from the data.

TABLE 4. Refinement to Open Set by Direct Element Observation.

Compound	Refinement from Fine Isotopes			
	Element	Min	Max	Observation
Ranitidine	S	1	2	Full scan $^{33}S/^{34}S$ and $^{13}C/^{34}S$ ratio
	N	1	3	MS^2 ^{15}N and MS $^{13}C/^{15}N$ A_1 ratio
Tryptophan	N	1	3	MS^2 ^{15}N and $^{13}C/^{15}N$ A_1 ratio
	S	0	0	Full Scan Lack of ^{34}S in A_2
Oxytetracyclin	O	3	Unchanged	MS^2 $^{18}O/^{13}C$ ratio
	S	0	0	Full Scan Lack of ^{34}S in A_2
	N	1	Unchanged	MS^2 ^{15}N
Guanine	N	1	Unchanged	MS^2 ^{15}N
	S	0	0	Full Scan Lack of ^{34}S in A_2
Norfloxacin	N	1	Unchanged	MS^2 ^{15}N
	O	1	Unchanged	MS^2 ^{18}O
	S	0	0	Full Scan Lack of ^{34}S in A_2

Comparison of Performance – Using Fine Isotope Refinement

Elemental compositions were again calculated using the fine isotopes directly observed in the MS and MS^2 data to refine the maximum and minimum values. In all cases, the inclusion of the additional limits significantly reduced the number of possible compositions that were determined, for example, the total possible compositions calculated from the HRAM full scan MS data of oxytetracycline was reduced by almost an order of magnitude when taking fine isotopic data into consideration.

Table 5. Comparison of the Three Methods for Elemental Composition.

Compound	Open Set		Pre-Known
	Original	With Fine Isotopes	
Ranitidine	50	5	1
Tryptophan	7	1	4
Oxytetracycline	324	84	2
Guanine	6	1	1
Norfloxacin	61	30	2

Conclusion

The determination of elemental composition from accurate mass alone is insufficient unless the elemental subset is constrained with *a priori* knowledge of the answer. For real world analyses, this prior knowledge doesn't exist and a more open elemental composition set must be used. Here we have demonstrated that inclusion of refinements to the minimum and maximum number of atoms for isotopic elements by direct observation of fine isotope pattern improves our capability to determine the composition.

- Accurate mass, even below 1 ppm, is insufficient for correct elemental composition determination unless *a priori* knowledge is used.
- Very high resolution can give us access to direct observation of fine isotopes.
- Direct observation of the fine patter can refine the determination of elemental composition.
- Fine isotopic refinement of the elemental subset can be applied to real world senarios to improve elemental composition determination..

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