Determination of Isotopic Purity by Accurate Mass LC/MS

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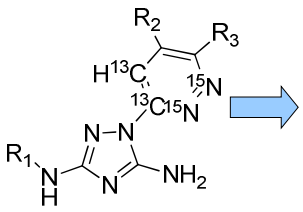
Background

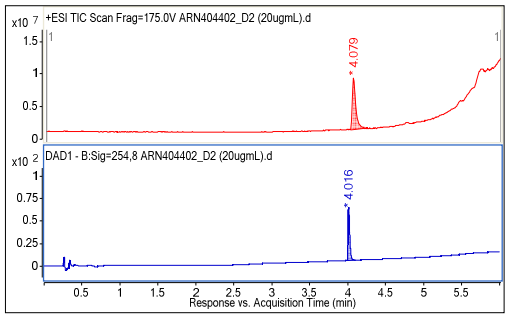
Following the synthesis of isotopic labelled APIs at Almac, or stability testing, it is often necessary to accurately determine the isotopic enrichment of the compounds by mass spectrometry. This process is often complicated if the compound in question contains a variety of labelled atoms, including ^{13}C, ^{15}N and ^{2}H, or where the isotopes are radioactively labelled (e.g. ^{14}C). Advances in time of flight (TOF) mass spectrometry have led to vastly improved resolution between the isotopes of labelled compounds and this has allowed more accurate extraction of these isotopes than ever before. With this high degree of mass accuracy, it is possible to accurately quantify the labelled composition of the compounds in question. A case study for the determination of isotopic purity of a compound manufactured at Almac which contained two ^{13}C atoms and two ^{15}N atoms is presented.

Outline of the impurity isolation and structure elucidation work flow.

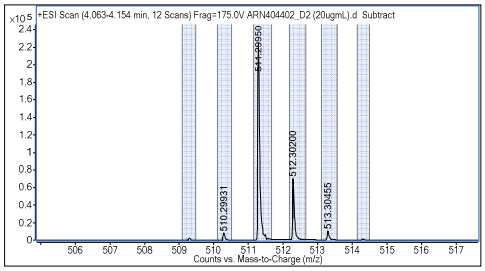
API Structure



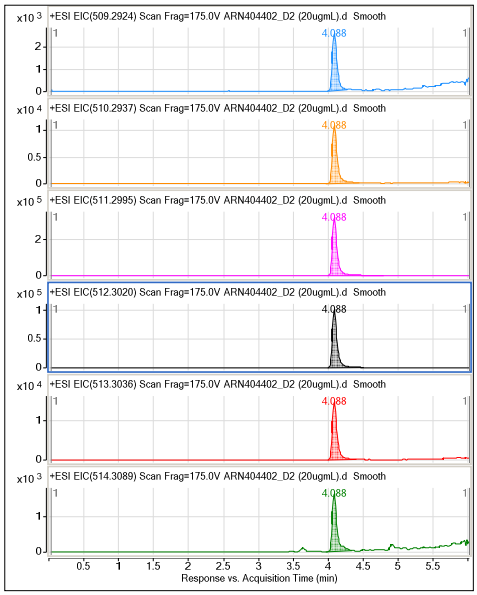
Isolate compound by LC/MS



Extract each of the resolved isotopes



Integrate the EIC for each isotope



The molecular formulas of the various labelled states of the compound are entered into an isotopic distribution calculator to give the predicted isotopic ratios. For compounds containing a number of different labelled atoms (e.g. ^{13}C, ^{15}N, ^{2}H) the average isotopic ratio of the different possible labelled permutations for each mass is calculated.

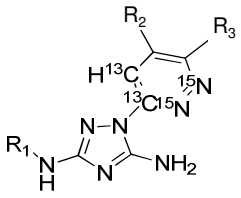
Method

- Sample is dissolved in a suitable solvent, and analysed using an optimised UHPLC/MS method in order to separate the peak of interest from any interfering impurities.
- A background spectrum is subtracted from the sample spectrum to clean the spectra.
- Each of the resolved isotopes related to the compound of interest are extracted to produce an extracted ion chromatogram (EIC).
- The EIC for each isotope is integrated, and the peak areas transferred to a spreadsheet for quantitation.

$[^{13}C_2, ^{15}N_2]$ $C_{28}H_{34}N_6$ Labelled Sample - Extent Of Enrichment

Isotopomer	Total Ion Channel Counts	Natural Isotope Correction	Natural Isotope Corrected Counts
0 x [^{13}C or ^{15}N] 507 m/z	0		0
1 x [^{13}C or ^{15}N] 508 m/z	315	0.00	**315**
2 x [^{13}C or ^{15}N] 509 m/z	12082	113.20	**11969**
3 x [^{13}C or ^{15}N] 510 m/z	53329	4232.10	**49097**
4 x [^{13}C or ^{15}N] 511 m/z	1557482	17636.81	**1539845**
5 x [^{13}C or ^{15}N] 512 m/z	479750	521866.33	**-42116**
6 x [^{13}C or ^{15}N] 513 m/z	69673	70856.18	**-1183**
7 x [^{13}C or ^{15}N] 514 m/z	7673	6235.93	**1437**
Negative Value indicate no more enrichment beyond the 4 x [13C or 15N]			**1559363**

ESI-TOF-MS spectra obtained in postive ion mode from LC-MS (1uL) of samples at 20 ug/mL.
Theoretical abundance of natural isotopes were calculated using Agilent Isotopic distribution calculator
The areas for each of the isotopes are extracted at their maximum intensity in the spectrum



Theoretical Natural Isotopic Distribution for the [M+H]+ ion species of $C_{30}H_{34}N_6$		$C_{30}H_{34}N_6$ Isotopic Distribution	Mono-labelled Average Distribution	Di-labelled Average Distribution	Tri-labelled Average Distribution	$[^{13}C_2, ^{15}N_2]$ $C_{28}H_{34}N_6$ Isotopic Distribution
Isotope	m/z	Relative Abundance	Relative Abundance	Relative Abundance	Relative Abundance	Relative Abundance
M	507	100.0000				
M+1	508	36.6780	100.0000			
M+2	509	6.5353	35.9362	100.0000		
M+3	510	0.7536	6.2701	35.1944	100.0000	
M+4	511	0.0632	0.7076	6.0108	34.4526	100.0000
M+5	512	0.0041	0.0581	0.6636	5.7548	33.7108
M+6	513		0.0036	0.0532	0.6208	5.5047
M+7	514			0.0032	0.0486	0.5800
					0.0029	0.0443
						0.0026

$[^{13}C_2, ^{15}N_2]$ $C_{28}H_{34}N_6$ Labelled Sample

	Total Channel Counts	Natural Isotope Correction	Natural isotope corrected counts	Isotopic Enrichment Relative %		Total Labelled Atoms
0 x [^{13}C or ^{15}N] 507 m/z	0		0	0.00		
1 x [^{13}C or ^{15}N] 508 m/z	315	0.00	315	0.02		0.02
2 x [^{13}C or ^{15}N] 509 m/z	12082	113.20	11969	0.75		1.49
3 x [^{13}C or ^{15}N] 510 m/z	53329	4232.10	49097	3.07		9.20
4 x [^{13}C or ^{15}N] 511 m/z	1557482	17636.81	1539845	96.17		384.67
			1601226	100.00	**Overall Isotopic Purity %**	**98.84**

Data Processing

- In order to remove the natural isotopic contribution of the elements present in the various labelled isotopes from their adjacent isotopes, it is first necessary to determine the level of natural contribution.
- Natural isotopic contributions from preceding peaks (i.e. ^{13}C) are calculated in the adjacent table using data obtained from the theoretical spectra. These are then subtracted from the peak area value of subsequent isotopes, allowing the identification of the natural isotope corrected value, and thus allowing the determination of the true extent of enrichment.

Results

The relative percent of isotopic enrichment is calculated for each isotope species in the compound along with the overall isotopic purity. In the current example, 96.17% of the compound was found to be fully enriched, whilst 3.07% contained 3 labelled atoms (i.e. 2 x ^{15}N and 1 x ^{13}C, or 1 x ^{15}N and 2 x ^{13}C) and less than 1% contained 2 labelled atoms (i.e. 2 x ^{15}N, or 1 x ^{13}C and 1 x ^{15}N, or 2 x ^{13}C). This equated to 98.84% of the potential labelled positions in the compound being occupied by labelled atoms.

Conclusion

Mass spectrometry has been proven to be a very useful technique in accurately quantifying the level of enrichment in labelled compounds. Improvements in baseline resolution between isotopes facilitated by TOF-LC/MS has reduced isotopic overlap between neighbouring isotopes, reducing erroneous results. Furthermore, alignment with LC/MS also makes it possible to remove interferences, perform sample purity and the identification of unknown impurities within the same analysis.