ALMAC

Partnering to Advance Human Health

Whitepaper

Peak Purity of Peptides and Large Molecules by Accurate Mass LC-MS

Author: Peter Smith

Supporting authors: Amanda Bole, Louise Hughes

Introduction

Purity of a chromatographic peak has traditionally been analysed by HPLC analysis using 3D UV to assess compounds spectrally different to the Active Pharmaceutical Ingredient (API) peak. This is a well-established methodology that is routinely and widely used to assess peak purity of small molecules, relying on differences in the 3D UV spectra to capture potential co-eluting impurities.

This method is a robust and widely employed approach in the industry to assess the purity of a peak. However, as molecules increase in size and complexity, there are fundamental limitations to this technique that may render it ineffective for evaluating peak purity in larger molecules such as peptides and proteins. Fundamentally, large molecules and their impurities show no differences in their 3D UV profile, thus, any peptide impurities, even those that are quite abundant, are hidden beneath the principal peak rendering them “invisible” by 3D UV. Additionally smaller impurities, which may be above the reporting threshold for the analytical method, may not be distinct enough at low level to be observed by UV peak purity assessment.

High Resolution Accurate Mass (HRAM) Mass Spectrometry offers an alternative solution to provide peak purity assessment for small and large molecules. This technique offers both an indication of the peak's purity and precise mass data for any observed impurities, enabling the generation of a tentative identity. HRAM also allows an understanding of the level of impurity present underneath the peak, making peak purity a more quantitative assessment than previous techniques.

In this paper, two example case studies are presented for peak purity determination. Firstly, for a typical peptide, determined using Agilent 6230/6530 Accurate Mass TOF/QTOF instrumentation and Agilent MassHunter B.10 software. Secondly, a larger biologics product in the form of a monoclonal antibody determined using a Thermo Q-Exactive Plus BioPharma Orbitrap MS with Chromeleon 7.2.10 Software and BioPharma Finder 4.3 Software.



Peptide Peak Purity

Molecular Feature Extraction (MFE) is a compound mining algorithm from Agilent Technologies MassHunter software suite. MFE analyses mass spectral data and interprets compound "features" These features include ions that are common to a specific mass feature, such as various charged species: salt adducts, clusters, and fragmentation products. By combining all these related features, a chromatographic peak or Extracted Compound Chromatogram (ECC) can be generated pertaining only to those related features.

Extraction takes place for all features that occur in the selected region of the chromatogram, a stacked plot of ECC's and tabulated values for mass, volume, and % volume are generated. Extracted molecular features are measured in "volume" rather than traditional area as the extracted compounds observed are a percentage of the total volume. All features are overlaid separately to create a three-dimensional peak.

Figure 1: Stacked Plot of Extracted Molecular Features

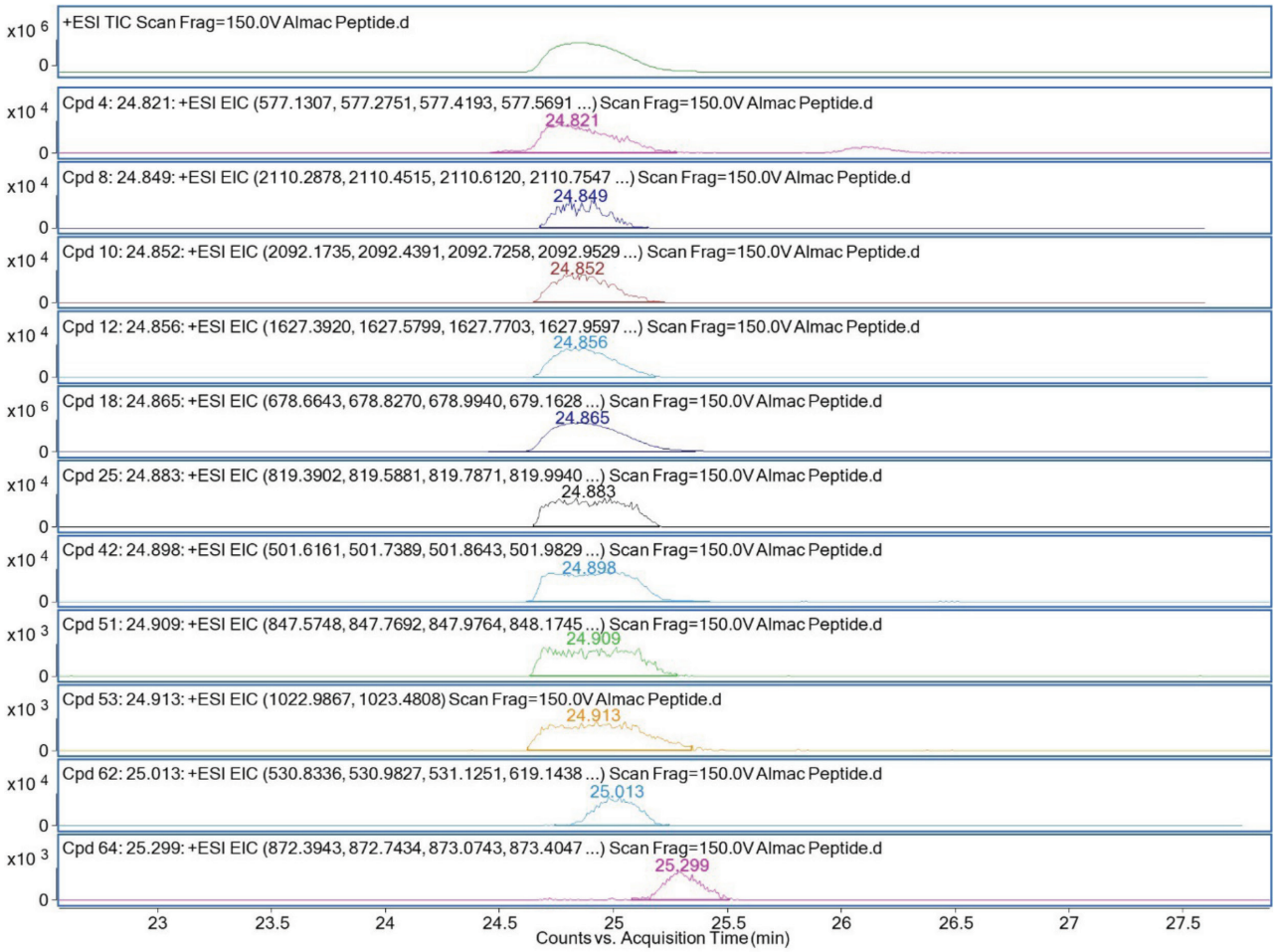


Figure 1 shows the extracted results from MFE performed on an Almac synthetic peptide. The first plot at the top of the stack shows the typical Total Ion Count (TIC) profile for this peptide peak and the plots below this are a sample of the many extracted features beneath the API peak. During interpretation of MFE extracted data many adducts, clusters, and in-source fragmentation products are observed. These features "related" to the main peptide peak share the peptide peaks profile, elution span, and apex.

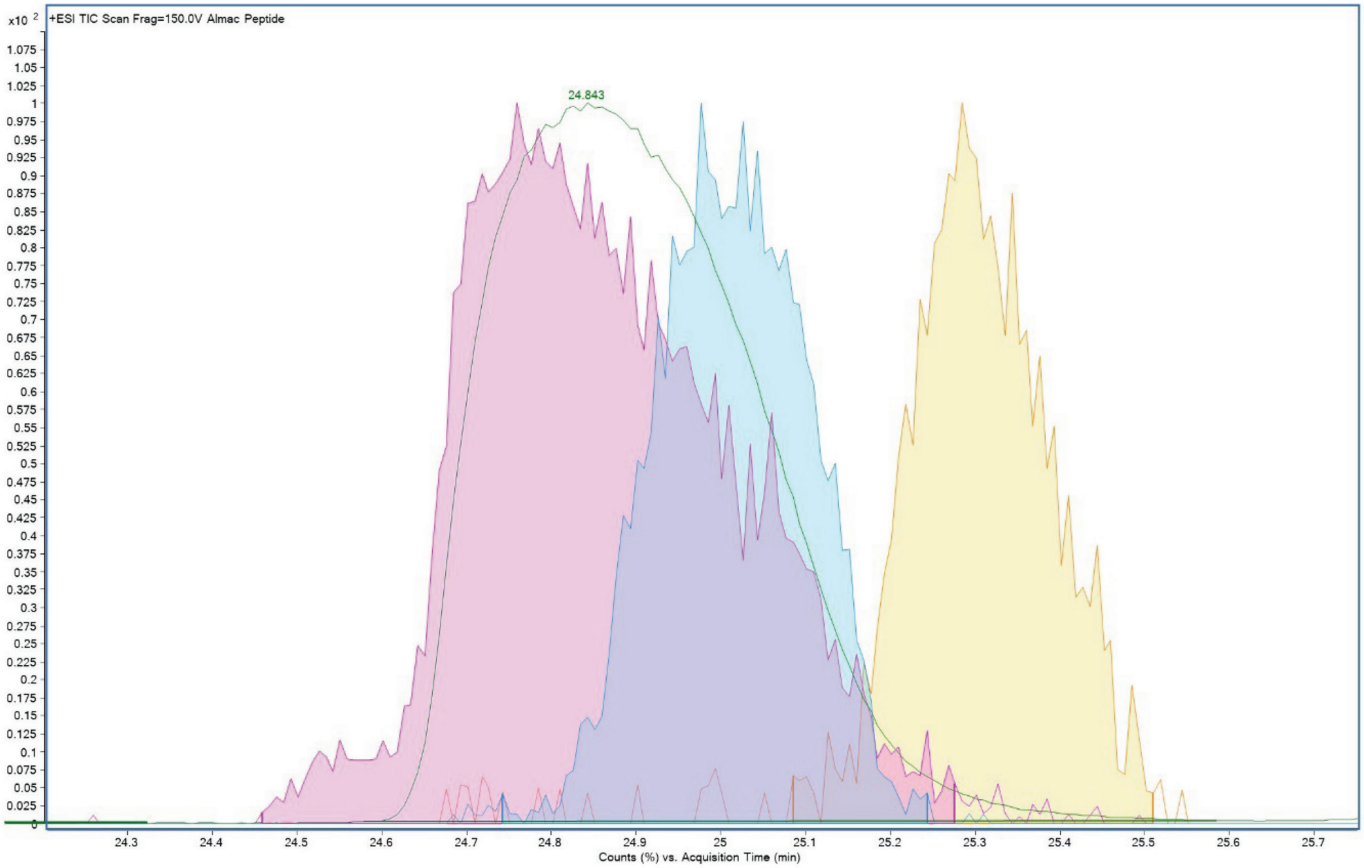
In this instance there are very clear impurities:

- Compound 62, and Compound 64 which when assessed against the profile and span of the peptide peak show clear differences.
- Compound 4 when inspected closely shows a slight peak profile irregularity. When comparing the profile and apex of this molecular feature to the parent TIC profile there is bias towards the front of the peak, indicating a third co-eluting impurity.

Overlay of these features in **Figure 2** makes this clearer. Compound 4 clearly favouring the front of the peak and Compound 62 and Compound 64 present in the tail of the peak.

All classified by peak profile, elution profile and apex as genuine co-eluting impurities.

Figure 2: Overlay of Peptide TIC (Green) with Compounds 4, 62 and 64





Large Molecule Peak Purity (Monoclonal Antibody)

Large molecule peak purity is carried out by Orbitrap LC-MS owing to higher resolution and higher sensitivity for molecules such as proteins or monoclonal antibodies that carry a very large number of charges and produce complex spectra. These molecules render analysis by lower resolution instruments when compared to an Orbitrap insufficient, as there is just not enough spectral fidelity to truly understand all the mass information presented beneath a peak.

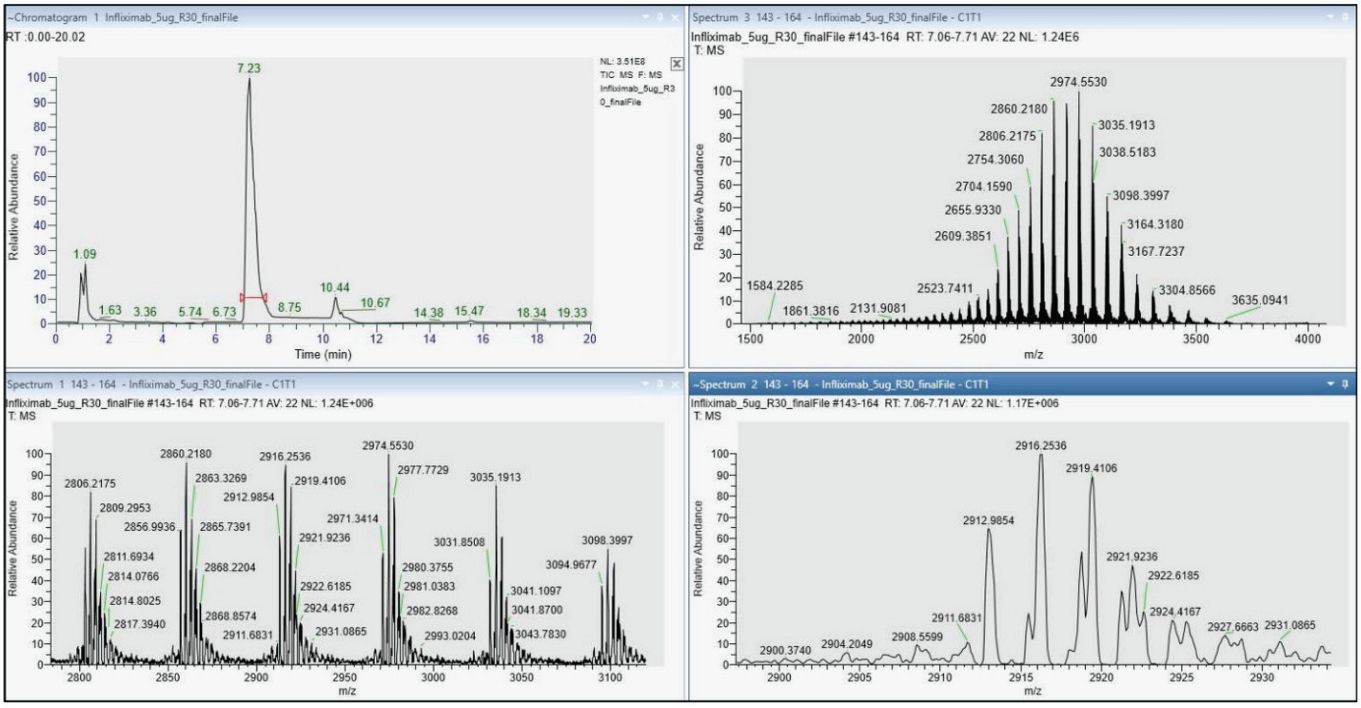
The sequence of the known parent molecule is inserted into the BioPharma Finder software and the peak of interest is extracted using the “Intact Mass” workflow.

The intact mass workflow compares the accurate mass and isotopic distribution to the theoretical based on the input sequence.

The workflow then extracts and annotates any additional features observed within the selected peak region that can be related to the parent sequence.

In this example the monoclonal antibody, Infliximab reference standard, was analysed intact using the Orbitrap’s high resolution mode to produce isotopically resolved data for interpretation. The output spectra from this experiment are presented in **Figure 3.**

Figure 3: Infliximab Intact Mass Spectra



The intact spectra for Infliximab produced a complex map of charged species relating to Infliximab and its many Glycoforms as well as potential impurities. Prior to evaluation of the purity of the peak, the deconvoluted spectra were assigned to map the Glycosylation profile present.

The data was analysed by the “Intact Mass” workflow with the known Glyco-modifications assigned to assess for co-eluting impurity species **(Figure 4 and Figure 5).**

Figure 4: Deconvoluted Spectra showing assigned Glycosylation Profile

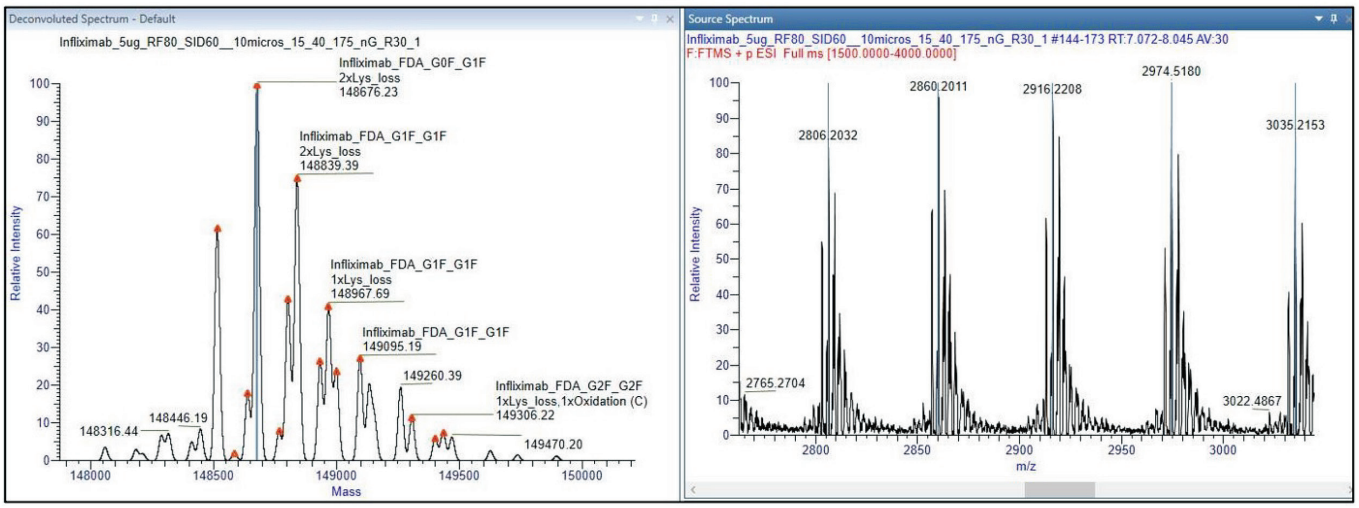


Figure 5: Intact Mass workflow output showing Infliximab Related Features

Protein Name	Modification	Average Mass	Theoretical Mass (Da)	Matched Mass Error (ppm)	Intensity	Delta Mass	Relative Abundance	Fractional Abundance	Number of Charge States
Infliximab_FDA_G0F_G1F	2xLys_loss	148676.23	148674.69	10.4	5.81E+07	0.00	100.00	17.92	18
Infliximab_FDA_G1F_G1F	2xLys_loss	148839.39	148836.83	17.2	4.36E+07	163.16	75.14	13.46	19
Infliximab_FDA_G0F_G0F	2xLys_loss	148514.20	148512.55	11.2	3.60E+07	-162.03	61.92	11.10	18
Infliximab_FDA_G0F_G1F	1xLys_loss	148802.48	148802.86	2.5	2.51E+07	126.25	43.18	7.74	19
Infliximab_FDA_G1F_G1F	1xLys_loss	148967.69	148965.00	18.0	2.39E+07	291.45	41.11	7.37	19
Infliximab_FDA_G1F_G1F		149095.19	149093.17	13.5	1.60E+07	418.95	27.50	4.93	19
Infliximab_FDA_G0F_G1F		148932.97	148931.03	13.0	1.54E+07	256.73	26.59	4.76	19
Infliximab_FDA_G1F_G2F	2xLys_loss	148998.84	148998.97	0.8	1.39E+07	322.61	23.93	4.29	19
		149260.39	0.00	0.0	1.13E+07	584.16	19.51	3.50	19
		149132.27	0.00	0.0	1.13E+07	456.03	19.46	3.49	18
Infliximab_FDA_G0_G0F	1xOxidation (C)	148638.06	148638.75	4.6	1.05E+07	-38.17	18.12	3.25	18
Infliximab_FDA_G2F_G2F	1xLys_loss,1xOx...	149306.22	149305.28	6.3	6.73E+06	629.98	11.58	2.08	19
		149154.27	0.00	0.0	5.98E+06	478.03	10.30	1.85	19
		148446.19	0.00	0.0	4.90E+06	-230.05	8.44	1.51	17
Infliximab_FDA_G0F_G0F	1xH2O loss,1x...	148766.05	148766.88	5.6	4.66E+06	89.81	8.03	1.44	18
Infliximab_FDA_G2F_G2F	1xOxidation (C)	149434.38	149433.46	6.2	4.44E+06	758.14	7.65	1.37	18
		148316.44	0.00	0.0	4.11E+06	-359.80	7.07	1.27	15
		148287.53	0.00	0.0	3.93E+06	-388.70	6.77	1.21	15
		149470.20	0.00	0.0	3.64E+06	793.97	6.26	1.12	16
Infliximab_FDA_G2F_G2F	1xH2O loss	149401.66	149399.44	14.8	3.49E+06	725.42	6.01	1.08	15
		148411.00	0.00	0.0	2.93E+06	-265.23	5.05	0.90	12
		148058.17	0.00	0.0	2.10E+06	-618.06	3.62	0.65	12

Interpretation of the output data showed the presence of several major species consistent with impurities of Infliximab. C-terminal Lysine loss from the Heavy Chain was observed as well as related Oxidation and Dehydration impurities. Lower resolution instruments would have proved challenging to extract and define these impurity features with similar masses to the parent mass.

High resolution instruments returning an average mass at this molecular weight would not have isotopically resolved these impurities. The Q-Exactive Plus BioPharma instrument can return isotopically resolved mass data for monoclonal antibodies, such as Infliximab.

Conclusions

Workflow based analysis of the raw data generated provides robust, repeatable interpretation of data, backed by years of experience in peptide and protein Mass Spectrometry, further challenging the extracted data to provide a broad-spectrum interrogation of all observed species co-eluting with peaks of interest. Almac has the expertise and instrumentation to provide solutions for both small and large molecules. The presented case studies show that co-eluting impurities, which may have been unknown and invisible until intervention by Mass Spectrometry, can now be observed, identified, and potentially quantified with no further development of liquid chromatography separation to elute them.

Peak Purity is currently recommended in ICH Q2. Whilst peak purity is not currently the rule, however with regulatory guidance moving towards more and more stringent interrogation of peaks Almac provides an "ahead of the curve" solution to satisfy the current guidance and beyond for peptides. With guidance for biologics products such as monoclonal antibodies still in their infancy, it is safe to assume that the same level of scrutiny will be required. Almac is equipped and able to apply the same stringent testing on larger molecules to satisfy all future requirements for peak purity.

almacgroup.com

Get in touch

Global HQ
+44 28 3833 2200

US HQ
+1 215 660 8500

almacanalytical@almacgroup.com

© Almac Group Ltd. All rights reserved AM5145