Identification and Structural Elucidation by Amino Acid Sequencing of Peptide Impurities by 2D-LC MS/MS

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Background

Peptide impurity profiles require increasingly complex separation methods in order to detect and adequately quantitate peptide impurities. Mobile phase conditions optimal for these separations in most cases consist of non-volatile salt based buffer systems that are not inherently LC/MS compatible. 2D-LC has been demonstrated to overcome this issue by extracting the impurity peaks of interest and transferring them to a mass spectrometry compatible solvent system for further analysis. This case study demonstrates that a 2D-LC system when paired with a Q-TOF mass spectrometer can automate impurity extraction by 2D-LC to facilitate identification by 2D-LC/MS and also further structural elucidation by 2D-LC MS/MS.

Analytical Equipment

Analysis was performed using an Agilent Infinity II 2D-LC system coupled with an Agilent Accurate Mass 6530 Q-TOF system (Figure 1). 2D peak capture was handled using Agilent OpenLab ChemStation and structural elucidation was carried out using Agilent MassHunter software with BioConfirm for amino acid sequencing.

Figure 1: Agilent Infinity II 2D-LC (left) and Agilent Accurate Mass 6530 Q-TOF (right)

2D-LC - A brief overview

A 2D-LC system consists of an Agilent Infinity II Multisampler, 2 Binary Pumps, 2 DAD Detectors and the 2nd dimension valve and loop system. The 1st dimension LC system is used to carry out the sample analysis using the original method unchanged including the MS incompatible buffers. The second dimension LC system runs an MS compatible solvent system with a short LC column to provide the LC separation for any peaks that are captured (heartcut).

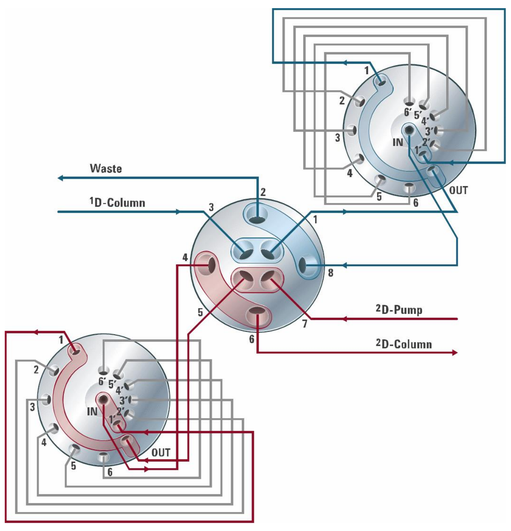

Figure 2: Diagram of Multi-heart cutting valve system

The transfer of a captured peak takes place in the 2D loop and valve region of the system (Figure 2). Twelve 40 µl loops capture and store the eluent of each peak captured by the system allowing the capture of up to 10 analyte peaks in quick succession to be analysed sequentially by the second dimension LC system.

Each loop is filled with a precise volume of the 1st dimension eluent accurately calculated to contain a peak of interest. This peak is then "parked" in the loop and the valves switch to capture the next peak in the next loop. Smart peak management ensures that the throughput of peaks captured and peaks analysed is maximised so that even the most complex of spectra can be managed by the 2D system.

Stage 1: Identify Impurities of Interest in Peptide Separation Method

Peptide samples were analysed using the original LC separations method on the 1st dimension chromatography system. The impurity profile of the sample was reviewed using Agilent ChemStation software and impurities that required MS analysis were flagged within the software for "heartcutting" based on the 1st dimension UV spectrum (Figure 3). ChemStation 2D peak detection was set up using a minimum UV height criteria and a peak slope criteria to prevent incorrect heartcuts.

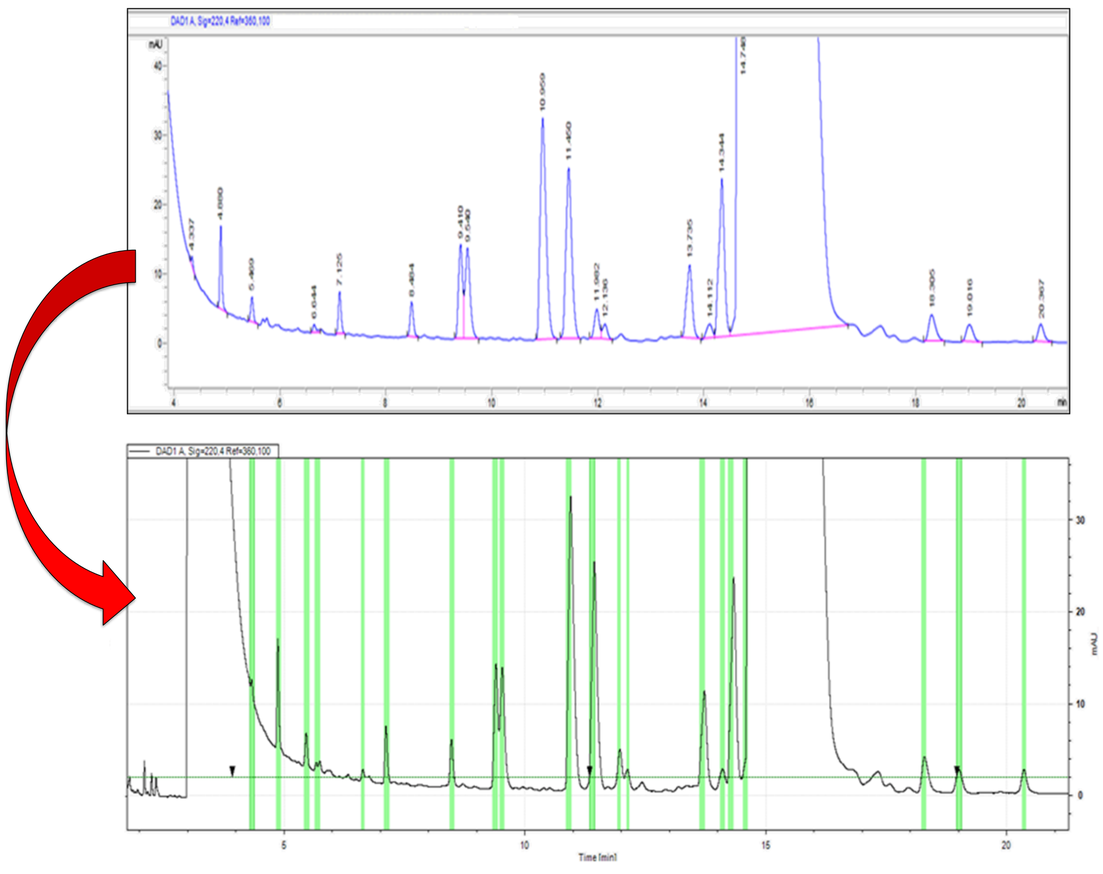

Figure 3: 1st Dimension UV Chromatogram (top) and Heartcut regions assigned by ChemStation for each Impurity (bottom)

Each impurity is collected by the 2D system and then sequentially analysed by the 2nd dimension chromatography system. The 2nd dimension chromatography system uses a fast gradient along with a 50 mm UPLC column to separate the peak of interest from the non-volatile salts and any other background interferences.

Stage 2: Analysis in the 2nd Dimension

Once the heartcut has been separated through the 2nd dimension chromatography system, it is passed to the Q-TOF-MS for accurate mass analysis. Figure 4 presents an example of an impurity Total Ion Chromatogram (TIC) which has been separated by 2D-LC, the first heartcut at approximately 11.2 minutes contains the peptide impurity. The second heartcut at 19 minutes is a blank region for MS subtraction of background. All peptide impurity masses were identified by accurate mass analysis. Specifically a Truncated impurity and three Succinamide Intermediates were identified from the impurity profile and deemed of further interest.

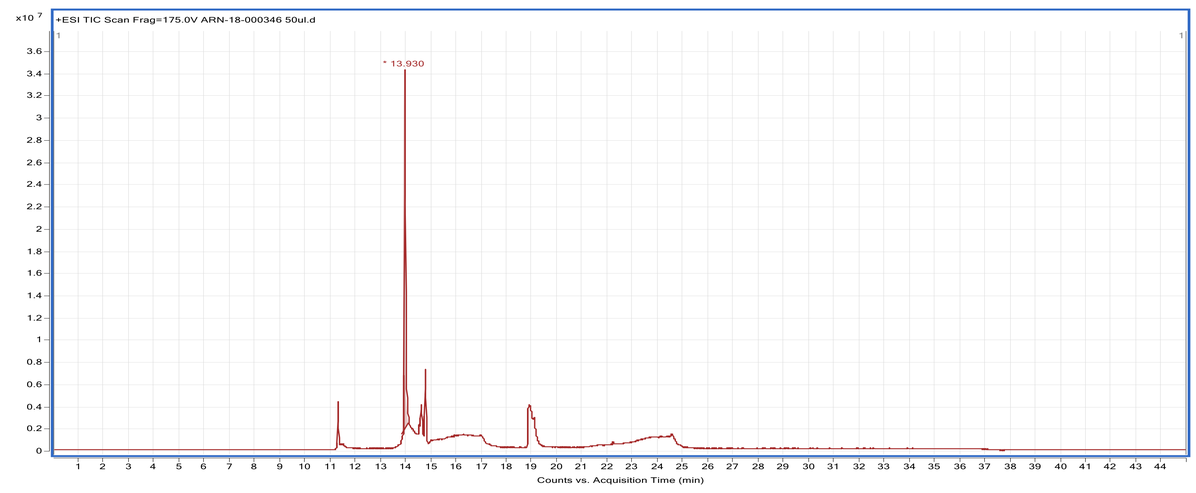

Figure 4: Total Ion Chromatogram of Impurity separated by 2D-LC

Stage 3: Further Elucidation by MS/MS

The peptide impurities identified as being of further interest were reanalysed by MS/MS to elucidate the peptide impurity sequence. Due to the noisy TIC spectra produced from the 2D heartcut, the MS/MS parameters were fine tuned to maximise precursor selection and fragmentation. MS1 scan speed was lowered to reduce the noise and boost the intensity of observed ions, MS2 scan speed was increased to boost detection of low level fragment ions. The injection volume was increased to 100 µL to maximise the intensity of the impurity captured. The Auto MS/MS algorithm was adopted to select expected multiply charged species as a priority, focusing on the $[M+3H]^{3+}$ ion this avoided the selection of singly charged species.

The theoretical sequence of each impurity was input into BioConfirm software and the impurity peaks extracted by the Molecular Feature Extraction (MFE) Algorithm within MassHunter software. The theoretical sequence is compared to the observed collision induced fragments and assigned as b or y ions in the sequence (Figure 5).

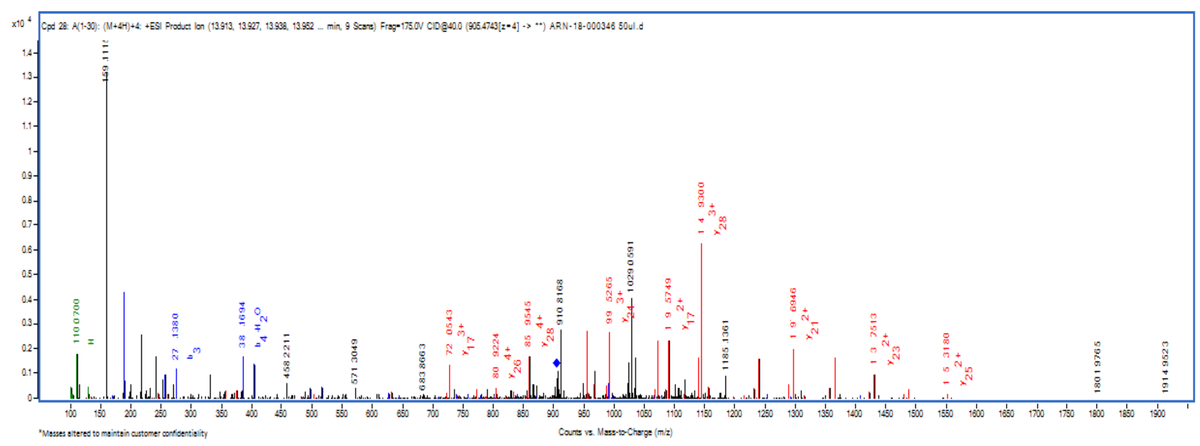

Figure 5: MS/MS Spectra showing assigned b and y ions

BioConfirm Software builds a sequence map from the observed b and y ions by comparison to the theoretical sequence. Lower ppm values indicate better mass accuracy, the observed sequence coverage for the Truncate was >98%.

The Succinamide Intermediates posed more of a problem than the Truncate due to their small size and the fact that the loss of 17 amu could also be attributed to the loss of a terminal peptide bond. The impurities were successfully identified after observing the sequence one amino acid at a time using the BioConfirm software. An observed mass shift in the sequence was attributable to the modified amino acid at three different locations along the peptide.

Ion	Abund	Diff (Bio, ppm)	m/z	m/z (prod.)	Sequence	Z (prod.)
y1	49.2	-9.3	1 4.0435	1 4.0448	D	1
y2	151.2	-12.7	2 2.1	2 2.1034	QD	1
y3	329.7	-6.2	3 5.1851	3 5.1874	QD	1
y4	179	-6.6	5 3.2791	5 3.2824	K QD	1
y5	253	-6.7	6 1.3731	6 1.3774	KK QD	1
y6	81.3	0.7	7 7.479	7 7.4785	KK QD	1
y7	117.8	-80.9	9 0.4897	9 0.5625	L KK QD	1
y8	337.1	-5.4	1 86.636	1 86.6418	WL KK QD	1
y9	107.1	-2.8	1 15.681	1 15.6844	EWL KK QD	1
y10	127.8	-18.2	1 14.7289	1 14.7528	VEWL KK QD	1
y12	99.3	-28.6	8 0.429	8 0.4519	E VEWL KK QD	2
y15	560	2.2	9 6.5117	9 6.5096	NSME VEWL KK QD	2
y17	2355.4	-5.7	1 91.5749	1 91.5811	H NSME VEWL KK QD	2
y17	1334.2	-3	7 8.0543	7 8.0565	H NSME VEWL KK QD	3
y18	454.4	-4.8	1 55.623	1 55.6286	KH NSME VEWL KK QD	2
y19	80.3	-8.1	7 9.7556	7 9.762	GKH NSME VEWL KK QD	3
y20	1565.9	-0.7	1 40.6804	1 40.6814	LGKH NSME VEWL KK QD	2
y21	1984.2	-6.4	1 97.6946	1 97.7028	LGKH NSME VEWL KK QD	2
y22	1659.4	-4.6	1 66.226	1 66.2323	H LGKH NSME VEWL KK QD	2
y23	954.9	-0.8	1 31.7513	1 31.7525	MH LGKH NSME VEWL KK QD	2
y23	2720.9	-0.6	9 4.8369	9 4.8374	MH LGKH NSME VEWL KK QD	3
y24	364.7	-10.8	1 88.2785	1 88.2946	LMH LGKH NSME VEWL KK QD	2
y24	2658.3	-5.7	9 2.5265	9 2.5321	LMH LGKH NSME VEWL KK QD	3
y24	108.7	-18.4	7 4.6372	7 4.6509	LMH LGKH NSME VEWL KK QD	4
y25	139.1	-3.8	1 52.318	1 52.3239	QLMH LGKH NSME VEWL KK QD	2
y26	2325.2	-8.5	1 72.9039	1 72.913	IQLMH LGKH NSME VEWL KK QD	3
y26	426.8	-17.7	8 4.9224	8 4.9366	IQLMH LGKH NSME VEWL KK QD	4
y27	157.6	-13.7	1 15.9119	1 15.9272	IQLMH LGKH NSME VEWL KK QD	3
y28	6226.5	-6.9	1 44.93	1 44.9379	S IQLMH LGKH NSME VEWL KK QD	3
y28	1698.9	-0.8	8 8.9545	8 8.9552	S IQLMH LGKH NSME VEWL KK QD	4

Figure 6: BioConfirm Impurity Sequence Map

Conclusion

2D-Liquid Chromatography has proved to be an excellent choice for sampling peptide impurities using non-mass spectrometry compatible methods. This is extremely valuable when transferring client methods which cannot be used for conventional LC-MS analysis or when trying to separate low-level and chromatographically similar compounds. The extra dimension provides numerous new opportunities to analyse samples which were previously very challenging to analyse using mass spectrometry. In addition, the Agilent 6530 Accurate Mass Q-TOF MS with MassHunter BioConfirm produced accurate and repeatable MS/MS spectra for the robust elucidation of peptide impurity identities.