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Adding Mass Detection to Routine Peptide-Level Biotherapeutic Analyses with the ACQUITY QDa Detector

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APPLICATION BENEFITS

- Add-on to existing Empower® Software-based GMP compliant workflows

- Addition of mass data to routine optical peptide assays for biotherapeutics

- Comparable peptide coverage with either trifluoroacetic acid and formic acid mobile phase additives

- Increased productivity through the use of on-line orthogonal detection techniques

WATERS SOLUTIONS

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Autosampler with FTN

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

ACQUITY UPLC Peptide CSH™ C_{18}, 130Å, 1.7 µm Column, 2.1 x 100 mm

ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm Column, 2.1 x 100 mm

Empower 3 Software

KEY WORDS

Peptides, mass detection

INTRODUCTION

Recently it was shown that the Waters ACQUITY QDa Detector, as a complementary orthogonal detection technique, provides a cost effective means of obtaining mass spectral data for peptides within an existing optically based LC workflow.[1]

This proof-of-principle work, while compelling, was confined to a reference set of seven peptides that ranged in molecular weight from 899 to 2,848 Da.

In contrast, biotherapeutics, such as monoclonal antibodies, when enzymatically treated can produce peptides that span nearly three orders of magnitude in size (150 to 7,000 Da).[2] In addition, ion pairing agents such as trifluoroacetic acid (TFA) are often used for peptide mapping. Given that TFA negatively impacts ionization efficiency, it can be rather challenging to obtain mass spectrometry (MS) based peptide maps.[3,4] As previously established, orthogonal techniques that add value and can be employed with minimal cost and effort are highly desirable in the pharmaceutical industry.[5]

The objective of this application note is to demonstrate that the ACQUITY QDa Detector provides a simple and cost-effective solution for detecting peptides across a wide molecular weight range (one more typical of biotherapeutic peptide maps) and that this mass detection capability is fully compatible with traditional optically based LC peptide monitoring assays that incorporate TFA or formic acid (FA). To this end, we have employed an existing method for the monitoring of peptide maps of trastuzumab, a therapeutic monoclonal antibody (mAb), using the ACQUITY QDa Detector.

Figure 1. An ACQUITY UPLC H-Class System, with the ACQUITY QDa Detector highlighted. The compact footprint of the detector allows for convenient integration into laboratories for improving productivity and strengthening process control and quality assurance in the biotherapeutic production environment.

EXPERIMENTAL

The ACQUITY UPLC CSH 130Å, C_{18} (2.1 x 100 mm, 1.7 µm) and ACQUITY UPLC BEH 300Å, C_{18} (2.1 x 100 mm, 1.7 µm) Columns were conditioned as outlined by the column care and use manual. Chemical reagents were purchased from Sigma Aldrich and used as received. Sequence grade modified trypsin from Promega was used to prepare a digest of trastuzumab (reduced and alkylated) at a concentration of 0.5 mg/mL as outlined by the manufacturers' protocol.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detectors:	ACQUITY UPLC TUV
	ACQUITY QDa
Absorption wavelength:	215 nm
Vials:	Total Recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Columns:	ACQUITY UPLC Peptide CSH 130Å, C_{18}, 1.7 µm, 2.1 x 100 mm (p/n 186006937)
	ACQUITY UPLC BEH 300Å, C_{18}, 1.7 µm, 2.1 x 100 mm (p/n186003686)
Column temp.:	65 °C
Sample temp.:	4 °C
Injection vol.:	8 µL
Mobile phase A:	H_2O, 0.1% TFA
Mobile phase B:	Acetonitrile, 0.1% TFA
Mobile phase C:	H_2O, 0.1 % FA
Mobile phase D:	Acetonitrile, 0.1% FA

Gradient table (BEH column)

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	97	3	0	0
3.00	0.200	97	3	0	0
120.00	0.200	65	35	0	0
127.00	0.200	20	80	0	0
130.00	0.200	20	80	0	0
131.00	0.200	97	3	0	0
140.00	0.200	97	3	0	0

Gradient table (CSH column)

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	0	0	99	1
3.00	0.200	0	0	99	1
120.00	0.200	0	0	67	33
127.00	0.200	0	0	20	80
130.00	0.200	0	0	20	80
131.00	0.200	0	0	99	1
140.00	0.200	0	0	99	1

MS detector settings

Sample rate:	2 points/sec
Mass range:	350 – 1250 Da.
Cone voltage:	10 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C

Informatics for data collection and processing

Empower 3 Software, SR2

RESULTS AND DISCUSSION

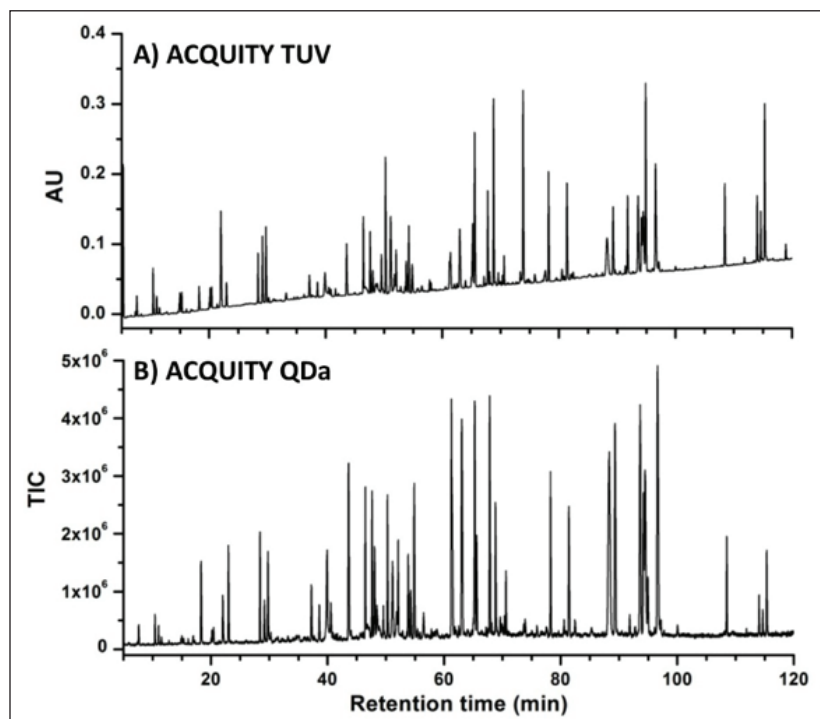*Figure 2. Peptide mapping with the ACQUITY QDa Detector using TFA and an ACQUITY UPLC BEH 300Å, C_{18}, 1.7 μm, 2.1 x 100 mm Column. Mobile phases prepared with 0.1% v/v TFA were used to acquire an A) optical and B) MS based peptide map of trastuzumab simultaneously. A high degree of correlation was observed between both detectors establishing the compatibility of the ACQUITY QDa with legacy methods that incorporate TFA.*

From our initial proof-of-principle work we established that the ACQUITY QDa Detector is well-suited for confirming the identity and purity of peptides across a wide molecular weight range.[1] For that work, the TFA concentration in the mobile phases was kept relatively low, at 0.02%, in order to minimize ion supression.[3,4] However, higher concentrations of TFA are often incorporated in optical based (UV) peptide analyses for improving the performance of conventional C_{18} columns.[6]

To demonstrate that the ACQUITY QDa Detector is compatible with such legacy methods, a peptide map of trastuzumab was acquired using conventional concentrations of TFA. For this experiment, a 0.5 mg/mL solution of trypsin digested, reduced, and alkylated trastuzumab was analyzed using mobile phases prepared with 0.1% TFA v/v. As shown in Figure 2A, a 120 minute gradient (see experimental) was used to generate an optical based peptide map using an ACQUITY UPLC BEH 300Å, C_{18}, 1.7-μm Column. The corresponding mass detector response from the ACQUITY QDa, which was in-line post optical detector, is shown in Figure 2B. A high degree of correlation was observed for the peptides detected using the orthogonal detection configuration. From this data, it is evident the ACQUITY QDa Detector is capable of providing mass spectral data using legacy methods that incorporate ion pairing agents such as TFA.
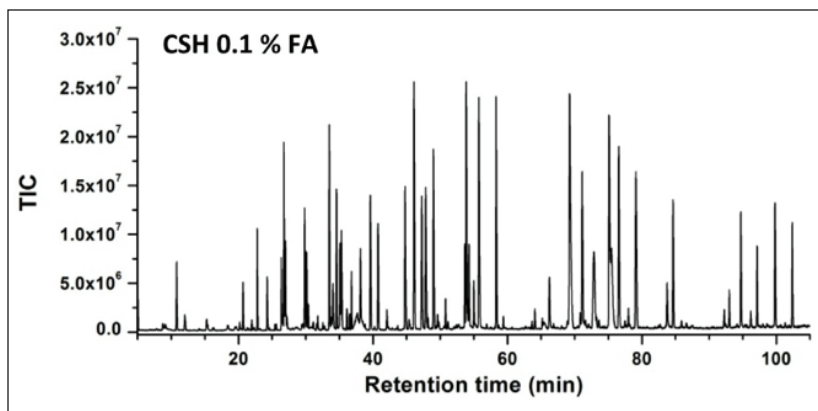
Figure 3. Peptide mapping with an ACQUITY UPLC Peptide CSH C$_{18}$ Column. Mobile phases prepared with 0.1% v/v FA were used to acquire an MS based peptide map of trastuzumab using the ACQUITY QDa Detector. The peptide profile was observed with improved detector response when compared to the one shown in Figure 2B using TFA.

As new columns are introduced to the market with improved performance with FA versus TFA,[7] the question arises if legacy methods can be transferred to new columns more efficiently with the addition of a mass detector.

To test this, an ACQUITY UPLC Peptide CSH C$_{18}$, 130Å, 1.7 µm Column was used to generate a peptide map using the same sample as shown in Figure 2. For this experiment, mobile phases were prepared with FA at a concentration of 0.1% v/v, and a 120-minute gradient was implemented to generate a peptide map.

As shown in Figure 3 and demonstrated in previous work,[8] the unique surface chemistry of charged surface hybrid (CSH) columns facilitates the use of FA, resulting in increased detector response. Given that both columns offer the ability to monitor peptides with the ACQUITY QDa Detector, further interrogation of the data was performed to evaluate the use of different ion-pairing agents.

Charge State

Peptide	Average Mass	[M+1H]$^{+1}$	[M+2H]$^{+2}$	[M+3H]$^{+3}$	[M+4H]$^{+4}$	[M+5H]$^{+5}$	[M+6H]$^{+6}$	[M+7H]$^{+7}$	[M+8H]$^{+8}$	[M+9H]$^{+9}$	[M+10H]$^{+10}$
T39	574.3	575.3	288.2	192.4	144.6	115.9	96.7	83.0	72.8	64.8	58.4
T7	681.3	682.3	341.7	228.1	171.3	137.3	114.6	98.3	86.2	76.7	69.1
T5	830.0	831.0	416.0	277.7	208.5	167.0	139.3	119.6	104.7	93.2	84.0
T21	835.0	836.0	418.5	279.3	209.7	168.0	140.2	120.3	105.4	93.8	84.5
T30	838.0	839.0	420.0	280.3	210.5	168.6					84.8
T9	969.1	970.1	485.5	324.0	243.3	194.8					97.9
T6	1084.2	1085.2	543.1	362.4	272.1	217.8					109.4
T3	1089.2	1090.2	545.6	364.1	273.3	218.8					109.9
T36*	1161.4	1162.4	581.7	388.1	291.3	233.3					117.1
T2*	1167.4	1168.4	584.7	390.1	292.8	234.5					117.7
T8-9	1182.3	1183.3	592.2	395.1	296.6	237.5					119.2
T13	1186.4	1187.4	594.2	396.5	297.6	238.3	198.7	170.5	149.3	132.8	119.6
T10	1310.5	1311.5	656.3	437.8	328.6	263.1	219.4	188.2	164.8	146.6	132.1
T4-5	1311.5	1312.5	656.8	438.2	328.9	263.3	219.6	188.4	164.9	146.7	132.2
T14*	1321.5	1322.5	661.8	441.5	331.4	265.3	221.3	189.8	166.2	147.8	133.2
T11*	1334.4	1335.4	668.2	445.8	334.6	267.9	223.4	191.6	167.8	149.3	134.4
T23	1677.8	1678.8	839.9	560.3	420.5	336.6	280.6	240.7	210.7	187.4	168.8
T33-34	1724.9	1725.9	863.5	576.0	432.2	346.0	288.5	247.4	216.6	192.7	173.5
T26	1808.1	1809.1	905.1	603.7	453.0	362.6	302.4	259.3	227.0	201.9	181.8
T38	1874.1	1875.1	938.0	625.7	469.5	375.8	313.3	268.7	235.3	209.2	188.4
T1	1882.1	1883.1	942.1	628.4	471.5	377.4	314.7	269.9	236.3	210.1	189.2
T22*	2139.4	2140.4	1070.7	714.1	535.8	428.9	357.6	306.6	268.4	238.7	214.9
T26-27	2228.6	2229.6	1115.3	743.9	558.1	446.7	372.4	319.4	279.6	248.6	223.9
T2-3*	2238.6	2239.6	1120.3	747.2	560.6	448.7	374.1	320.8	280.8	249.7	224.9
T37	2544.7	2545.7	1273.3	849.2	637.2	509.9	425.1	364.5	319.1	283.7	255.5
T12	2785.0	2786.0	1393.5	929.3	697.3	558.0	465.2	398.9	349.1	310.4	279.5
T41*	2802.1	2803.1	1402.1	935.0	701.5	561.4	468.0	401.3	351.3	312.3	281.2
T24-25*	3117.1	3118.1	1487.0	1040.4	780.3	624.4	520.5	446.3	390.6	347.3	312.7
T19-20*	3335.9	3336.9	1669.0	1113.0	835.0	668.2	557.0	477.6	418.0	371.7	334.6
T15*	6716.5	6717.5	3359.2	2239.8	1680.1	1344.3	1120.4	960.5	840.6	747.3	672.6
T15-16*	7058.9	7059.9	3530.4	2354.0	1765.7	1412.8	1177.5	1009.4	883.4	785.3	706.9
T15-17*	7187.0	7188.0	3594.5	2396.7	1797.8	1438.4	1198.8	1027.7	899.4	799.6	719.7

Legend:
Trifluoroacetic acid / Formic acid
= not observed

Table 1. Peptide map charge state table. A charge state table constructed from a simulated digest of the heavy chain of trastuzumab was used to identify the observed charge states of peptides detected with the ACQUITY QDa when using TFA or FA as an ion pairing agent. For comparison sake, only peptide fragment assignments that were observed in both the TFA and FA experiments were included in the table.

The volatility and acidic nature of the ion-pairing agent used in the mobile phase can impact the ionization and charge state distribution observed in multiply charged species such as peptides.[3,4] To investigate the effect of TFA versus FA on the charge state distribution of the peptides resulting from the enzymatically treated trastuzumab sample, a charge state table comprised of the heavy chain peptides was constructed from a simulated tryptic digest of trastuzumab (Table 1).

For both the TFA and FA peptide map experiments, the ACQUITY QDa Detector was set to a scan from 350 *m/z* to 1250 *m/z* (maximum) and is highlighted by the thicker line traces inside Table 1. Green and blue highlights were used to indicate if the peptide charge state was observed with TFA or FA, respectively.

Both colors indicate the charge state was observed in both peptide map experiments whereas a grey box represents a charge state within the scan range, but not observed.

From Table 1, it is clear that with only one exception (peptide T12), all peptides were observed to have multiple charge states with either TFA or FA as an ion-pairing agent. In addition, the peptides observed represent 93% of the heavy chain of trastuzumab. Similarly, 92% of the light chain peptide fragments were observed with multiple charge states for the trastuzumab digest when using TFA or FA. Peptides not observed were either not retained or were in a charge state below the 350 *m/z* experimental setting.

From these data, it can be seen that the ACQUITY QDa Detector is compatible with both TFA and FA based methods, affording significant flexibility in method development of monitoring assays.
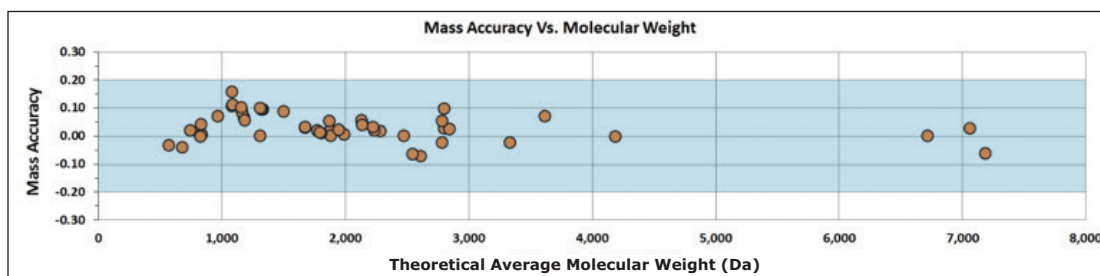

Figure 4. Peptide mass accuracy. A plot of the m/z difference between the theoretical and observed m/z was plotted against the average molecular weight for the peptides detected with the ACQUITY QDa. The mass accuracy of the peptides detected were within the instrument specification of ± 0.2 Dalton.

A natural question that arises from these results is: What is the accuracy with which we are detecting the charge states of the observed peptides?

An assessment of mass accuracy of the ACQUITY QDa was thus performed using the base peak ion (BPI) for the observed peptides. For this assessment, the difference between the observed and theoretical charge state *m/z* value was calculated for the BPI using the average molecular weight of each peptide.

A plot of the mass difference versus the theoretical average molecular weight was constructed as shown in Figure 4. It can be seen from Figure 4 that the BPI derived masses for the observed peptides are within the instrument specification of ± 0.2 Dalton (blue highlight), with a large portion of the peptides falling within ± 0.10 Dalton, demonstrating the ACQUITY QDa Detector is capable of providing accurate mass information for peptides over a broad molecular weight range in assays routinely employed during the analysis of biotherapeutics.

CONCLUSIONS

From previous work, it was shown that the ACQUITY QDa Detector provides a complementary detection technique to optical detection and can thereby improve the productivity of a single workflow. Cost effective techniques that add value and can be implemented into existing workflows with minimal effort are highly desirable in the pharmaceutical industry.

A natural extension of this work was to evaluate the performance of the ACQUITY QDa Detector using a representative biotherapeutic drug. From this work it has been demonstrated that the ACQUITY QDa Detector is compatible with conventional LC mobile phases and is able to detect and accurately report mass information for peptides over a wide molecular weight range typical of biotherapeutic peptide maps.

Collectively, these results establish the ACQUITY QDa Detector as an ideal addition for the biopharmaceutical analyst's lab that will afford increases in productivity and the confidence of data analysis for routine peptide analysis assays.

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