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Increasing Specificity and Sensitivity in Routine Peptide Analyses Using Mass Detection with the ACQUITY QDa Detector

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APPLICATION BENEFITS

- Simple add-on to existing Empower® Software based GMP compliant workflows
- Supplement optical data with mass data for routine peptide assays for biotherapeutics
- Increased assay specificity through the use of on-line orthogonal detection techniques

WATERS SOLUTIONS

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable UV (TUV) Detector

ACQUITY UPLC Peptide CSH™ Column

Empower 3 Software

KEY WORDS

Peptides, specificity, mass detection

INTRODUCTION

Peptide analyses are frequently used during protein-based biotherapeutics development, to assess critical quality attributes (CQAs) of candidate molecules.[1,2] Throughout the development process, many optical based assays, often developed from mass spectrometric based characterization, are used to assess product identity, purity, and monitor CQAs.

While optical detection provides a level of assurance, often there is a need to add an orthogonal detection technique, such as mass detection, to improve specificity, expand detection limits, and increase confidence about peak homogeneity.[3]

The objective of this application note is to demonstrate that the ACQUITY QDa Detector (Figure 1) provides a simple and cost-effective solution for the acquisition of optical and MS-based data, for increased specificity in routine peptide monitoring assays associated with monoclonal antibodies (mAbs), while strengthening confidence in the biotherapeutic production environment.

Figure 1. The ACQUITY QDa Detector. The compact footprint of the ACQUITY QDa Detector allows for convenient integration into laboratories, for improving productivity and strengthening process control and quality assurance in the biotherapeutic production environment.

EXPERIMENTAL

An ACQUITY UPLC Peptide CSH Column was 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
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	215 nm
Vials:	Total recovery vial, 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Column:	ACQUITY UPLC Peptide CSH C_{18}, 130Å, 1.7 µm, 2.1 mm x 100 mm (p/n 186006937)
Column temp.:	65 °C
Sample temp.:	10 °C
Injection volume:	8 µL
Mobile phase A:	H_2O, 0.1% FA
Mobile phase B:	Acetonitrile, 0.1% FA
Mobile phase C:	H_2O
Mobile phase D:	H_2O

Gradient table:

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

ACQUITY TUV Detector settings

Sample rate:	20 Hz
Detector λ:	215 nm
Filter TC:	normal

ACQUITY QDa 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

Data management

Empower 3 SR2 Chromatography Data Software (CDS) with mass analysis

RESULTS AND DISCUSSION

Optical based assays are often used in the biotherapeutic production environment to assess CQAs that impact product efficacy and safety. For biotherapeutics, such as mAbs, monitoring peptides that contain complementary determining region (CDR) sequences are critical in assuring product identity, and to ensure safety standards.[4,5] Optical based techniques that are easily deployed and universally accessible across sites are often used in the development of peptide map profile assays.

As shown in Figure 2A, a chromatographic region containing critical peptides such as the CDR peptide (L3) and its associated deamidated form (L3D) are eluting with non-related neighboring peptides.[5] These factors, combined with baseline noise associated with the use of formic acid, raise the possibility of peak heterogeneity, which can impact the accurate assessment of CQAs. Optimization of such peptide map profiles may require an iterative process where parameters such as gradient, column, temperature, solvent, and ion-pairing agents are systematically changed and evaluated to produce robust separations and ensure peak homogeneity.

With the addition of the ACQUITY QDa Detector into existing workflows, accurate assessment of CQAs can be made with minimal impact on productivity, or the need to modify chromatographic methods.

Using the Empower CDS Software, optical data (Figure 2A) was analyzed to generate an extracted ion chromatogram (XIC) profile (Figure 2B) using mass spectral information acquired with the ACQUITY QDa Detector. From the XIC profile, multiple species were confirmed to be coeluting with the critical pair of interest, demonstrating the ACQUITY QDa Detector's ability to enable rapid assessment of peak homogeneity through the addition of mass detection.

Difficult separations that contain partial or perfectly coeluting peptide species can require extensive optimization and often result in marginal improvements in separation efficiency and subsequent quantification. The ACQUITY QDa Detector features the ability to perform Single-Ion-Recording (SIR) for maximum specificity and sensitivity in routine assays such as peptide mapping profiles.

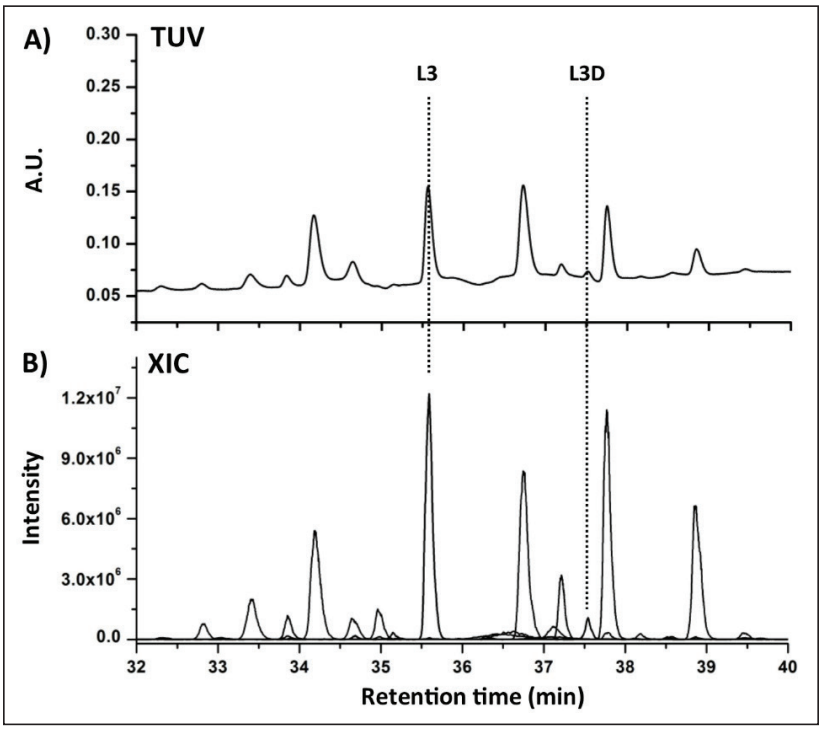


Figure 2. Peptide mapping with the ACQUITY QDa Detector. Using an ACQUITY UPLC Peptide CSH C_{18} Column, 130Å, 1.7 µm, 2.1 mm x 100 mm. A peptide map of trastuzumab was acquired using A) optical and B) mass spectral detectors simultaneously. Unique masses identified from the mass spectra collected for each peak were used to generate an XIC, confirming multiple species were co-eluting in the chromatographic region containing the CDR peptide L3 and its deamidated form L3D.

For peptides with characterized elution times, such as the L3 peptide species containing a critical CDR sequence, timed SIR events can be entered under the advanced option through the instrument method setup screen within the instrument method. As shown in Figure 3A, two timed SIR events were programmed to acquire the +2 and +3 charge state for the non-deamidated (L3), and main deamidated (L3D) form of the L3 peptide species.

Through the use of SIRs, only ions exhibiting the mass of interest are recorded as shown in Figure 3B, resulting in two chromatograms that exhibits high signal to noise ratio (SNR), and are free of co-eluting species (Figure 2) that could otherwise impact the accuracy of quantification, when using optical based techniques.

Determining the relative amount of each peptide form across two data channels is addressed through the use of an inter-channel calculation processing feature of Empower CDS Software, as shown in the workflow of Figure 4.

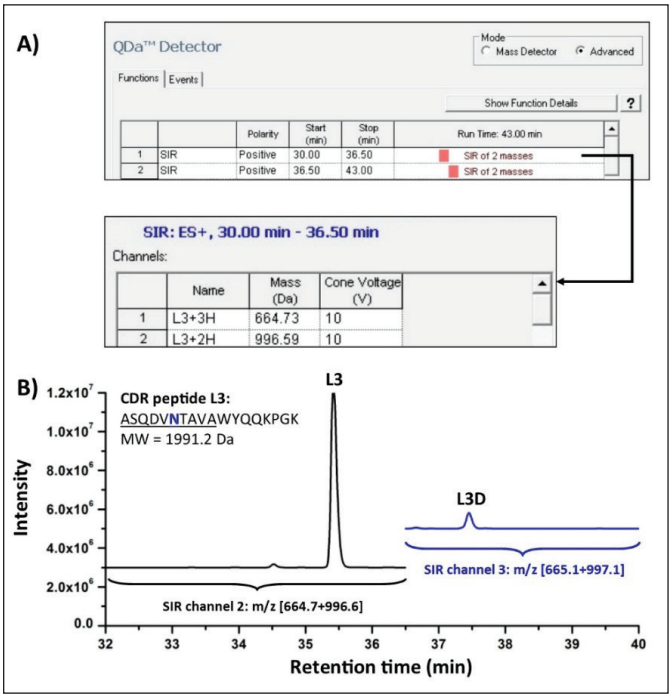


Figure 3. SIR with the ACQUITY QDa Detector. A) Timed SIRs were programmed to acquire non-deamidated (m/z 664.73, m/z 996.59) and deamidated (m/z 665.05, m/z 997.08) masses from 30.00 – 36.50 min and 36.50–43.00 min, respectively for the L3 peptide containing a CDR sequence (underlined). B) Resulting ion chromatograms for each species were summed using a derived channel and plotted with a y-axis offset of 3 x 10^6 (non-deamidated form) and 5 x 10^6 (deamidated form) to contrast the two unique data channels.

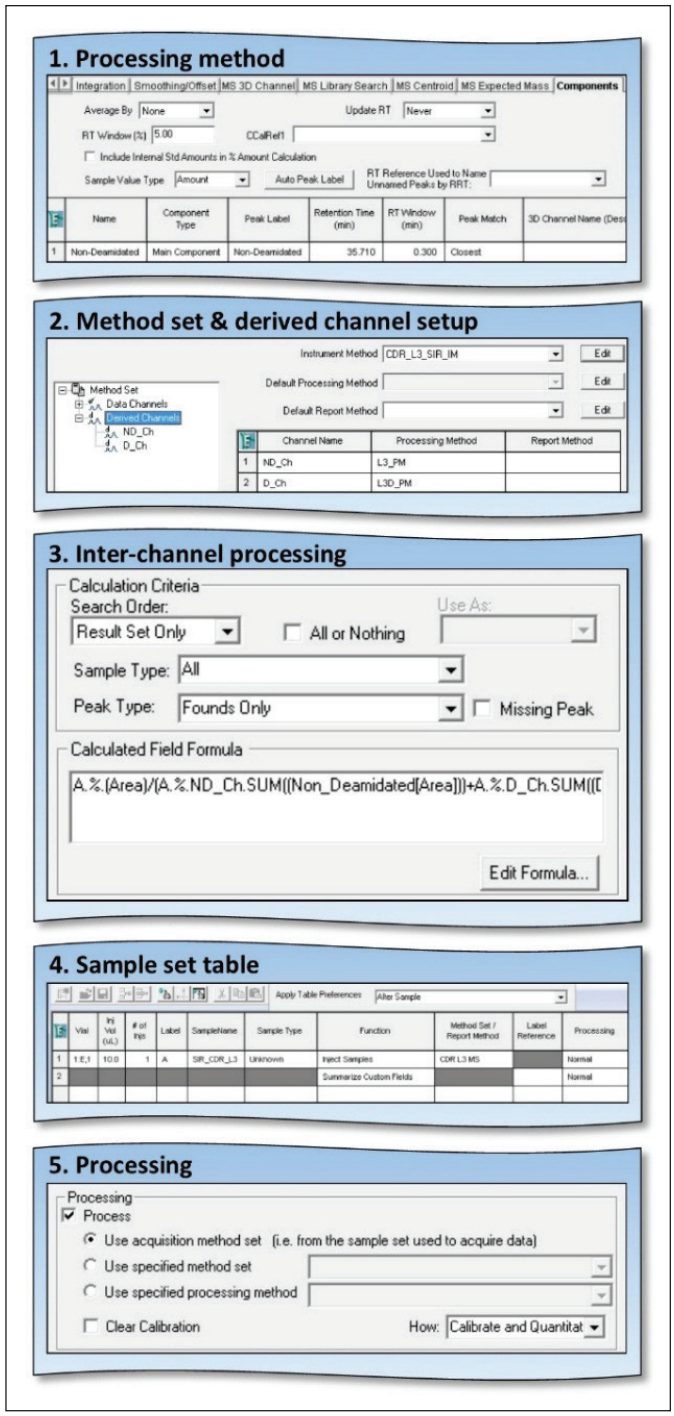


Figure 4. Inter-channel calculation workflow. A generic workflow for determining relative abundance of peaks between two data channels using the inter-channel calculations feature of Empower Software is shown for CDR sequence containing peptide L3.

As an example, the CDR peptide species L3 is used to illustrate the process of inter-channel quantification. As shown in step 1 of Figure 4, a unique processing method for each species form should be created, and include an integration window spanning the peak of interest with the individual component listed in the component table, along with its expected retention time for identification.

In this example the non-deamidated component of L3 is listed in the component table. Once processing methods are defined for each peptide species form, a method is set-up where derived channels are created (Figure 4, step 2), corresponding to the SIR channels from the instrument method. In this example the derived channels ND_Ch and D_Ch correspond to the non-deamidated and deamidated SIR channels, respectively, used for data acquisition in Figure 3A. Upon creation, the individual processing methods defined in step 1 are assigned to the derived channels.

An inter-channel calculation is then set-up using custom fields using the standardized syntax: Label.Injection.Channel.Summary Function(Field)

For this study, relative percent was calculated using the algebraic expression

$$\left(\frac{(\text{Area})}{\text{Area1} + \text{Area2}}\right) \times 100$$

using the following formulas

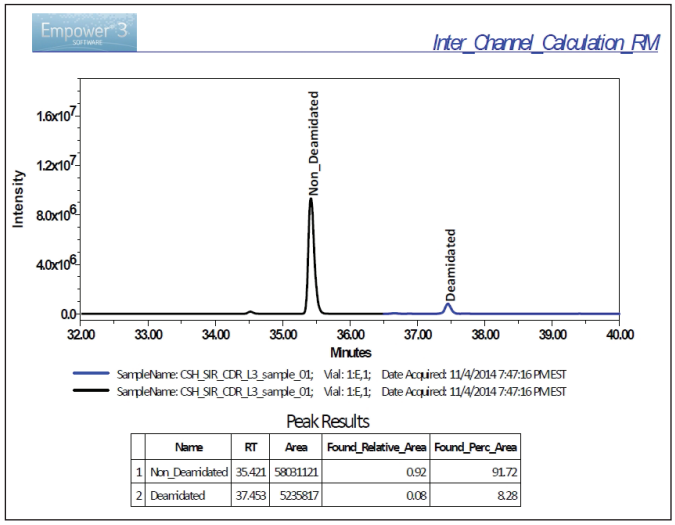
Area: A.%.(Area)

Area1 (non-deamidated area): (A.%.ND_Ch.SUM((Non_Deamidated[Area]))

Area2 (deamidated area): A.%.D_Ch.SUM((Deamidated[Area]))

which were combined in the Calculated Field Formula window as shown in Figure 4, step 3.

Upon entering the inter-channel calculation, a sample set can be created to process the data (Figure 4, step 4). In this example, a sample set was created using the injection named SIR_CDR_L3 and labeled as (A) with the method CDR_L3_MS selected. The summarize custom fields function is selected as shown in Figure 4, step 4, to facilitate the inter-channel quantification. Processing the sample set using the acquisition method set as shown in Figure 4, step 5 will generate a result set containing the two derived channels. Using the preview/publisher option with both derived channels selected allows for the ability to design custom reports for the review of inter-channel processed data, as shown in Figure 5.



	Name	RT	Area	Found_Relative_Area	Found_Perc_Area
1	Non_Deamidated	35.421	58031121	0.92	91.72
2	Deamidated	37.453	5235817	0.08	8.28

Figure 5. Reporting results using inter-channel calculations. Integration, identification, and relative quantification of the non-deamidated (black trace) and deamidated (blue trace) L3 peptide species using SIRs was performed using the inter-channel calculation feature of Empower CDS Software. A report template was created for the review of processed data.

Data acquired using timed SIR channels can be overlayed for easy review, while individual metrics for identified peaks are displayed along with the inter-channel calculations and shown in the last two columns of the associated data tables, as shown in Figure 5.

For this study, the CDR containing peptide L3 was calculated to contain 8.28% deamidation. From this data, it can be seen that the ACQUITY QDa Detector, in combination with Empower 3 CDS Software, affords improved productivity in development of routine peptide monitoring assays in the biotherapeutic environment.

CONCLUSION

Developing efficient methods for the accurate assessment of CQAs that impact product efficacy and safety are highly desirable in the biopharmaceutical production environment. This study has demonstrated orthogonal detection techniques, such as mass detection, can be readily adapted to existing workflows for increased specificity and sensitivity in routine monitoring assays.

The ACQUITY QDa Detector in conjunction with Empower 3 CDS Software affords a means for cost-effective mass detection, with increased productivity, and confidence in the development and analysis of routine monitoring assays.

References

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