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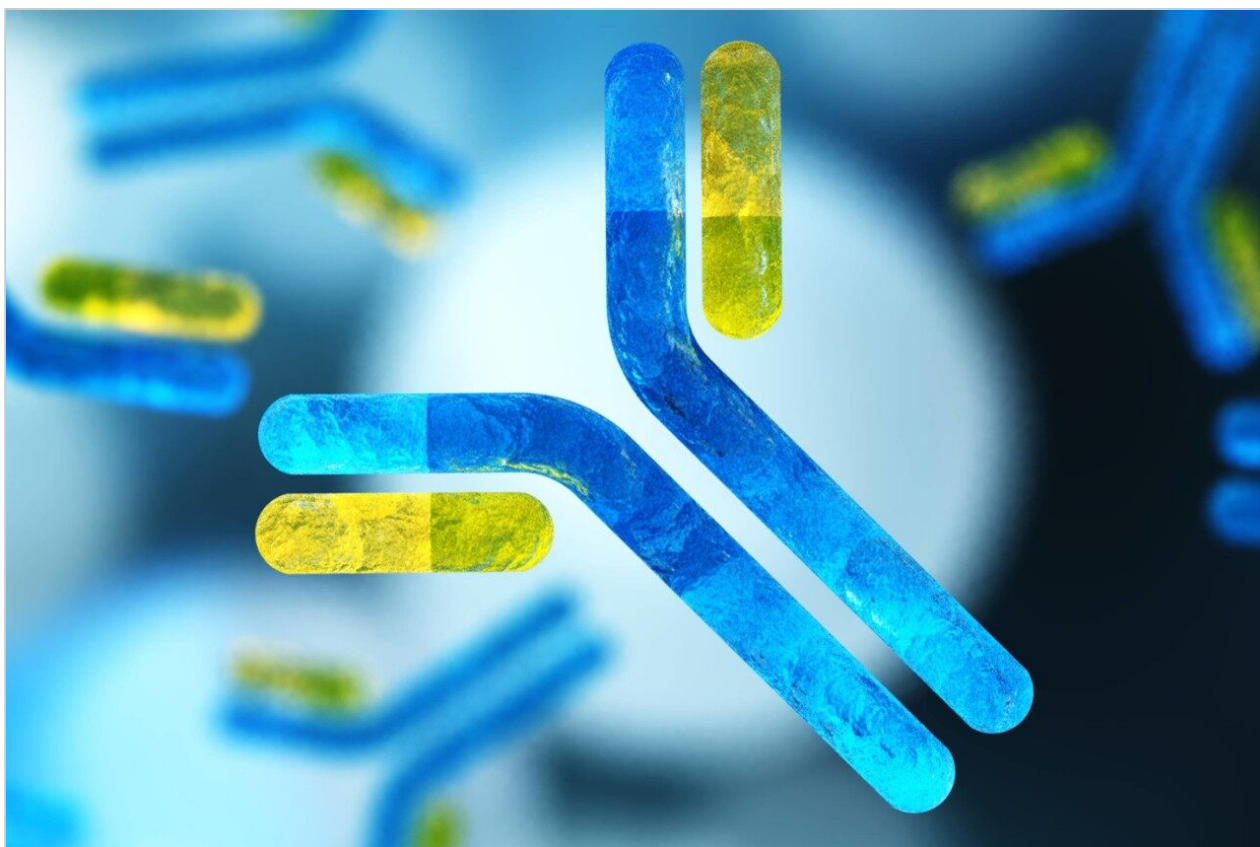
Increasing Specificity and Sensitivity in Routine Peptide Analyses Using Mass Detection with the ACQUITY QDa Detector

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Abstract

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.

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

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}, 130A, 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

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

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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.
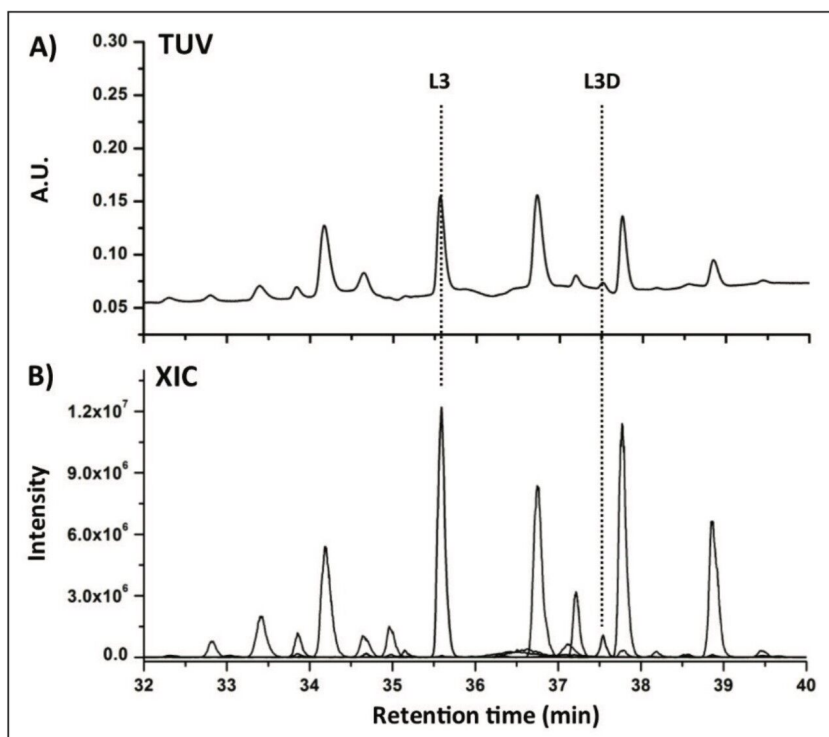
Figure 2. Peptide mapping with the ACQUITY QDa Detector. Using an ACQUITY UPLC Peptide CSH C_{18} Column, 130Å, 1.7 μm, 2.1 mm x 100 mm. A peptide map of trastuzumab was acquired using A) optical and B) mass spectral detectors simultaneously. Unique masses identified from the mass spectra collected for 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.

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.

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.
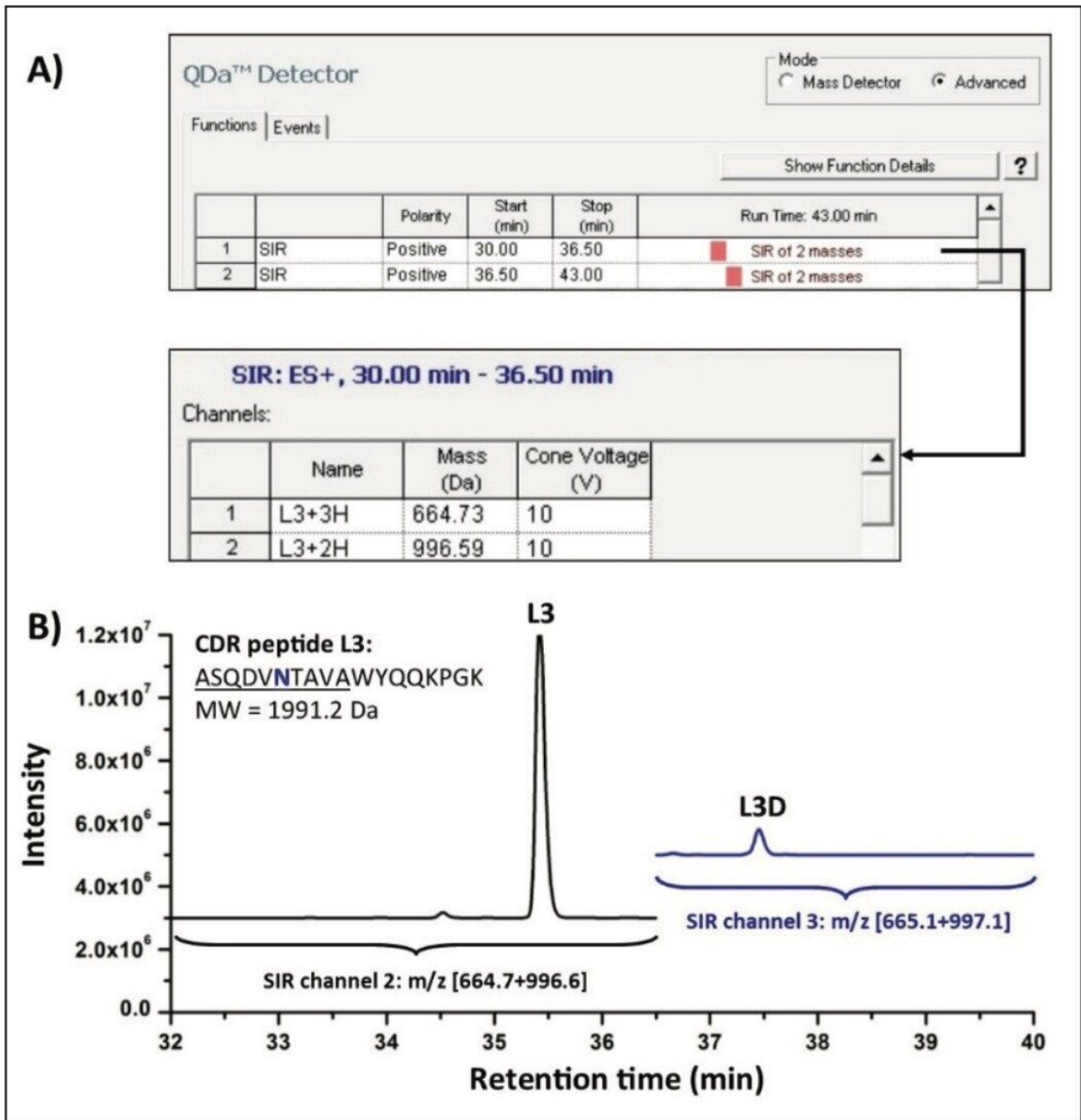
Figure 3. SIR with the ACQUITY QDa Detector. A) Timed SIRs were programmed to acquire non-deamidated (m/z 664.73, m/z 996.59) and deamidated (m/z 665.05, m/z 997.08) masses from 30.00 – 36.50 min and 36.50–43.00 min, respectively for the L3 peptide containing a CDR sequence (underlined). B) Resulting 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.

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.

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

$$((Area)/Area1 + Area2) \times 100$$

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.

1. Processing method

◄ ► | Integration | Smoothing/Offset | MS 3D Channel | MS Library Search | MS Centroid | MS Expected Mass | **Components**

Average By [None ▼]   Update RT [Never ▼]

RT Window (%) [5.00]   CCalRef1 [▼]

☐ Include Internal Std Amounts in % Amount Calculation

Sample Value Type [Amount ▼] [Auto Peak Label]   RT Reference Used to Name Unnamed Peaks by RRT [▼]

	Name	Component Type	Peak Label	Retention Time (min)	RT Window (min)	Peak Match	3D Channel Name (Desc
1	Non-Deamidated	Main Component	Non-Deamidated	35.710	0.300	Closest	

2. Method set & derived channel setup

Instrument Method [CDR_L3_SIR_IM ▼] [Edit]

Default Processing Method [▼] [Edit]

Default Report Method [▼] [Edit]

Method Set
  Data Channels
  Derived Channels
    ND_Ch
    D_Ch

	Channel Name	Processing Method	Report Method
1	ND_Ch	L3_PM	
2	D_Ch	L3D_PM	

3. Inter-channel processing

Calculation Criteria

Search Order: Use As:

[Result Set Only ▼]   ☐ All or Nothing   [▼]

Sample Type: [All ▼]

Peak Type: [Founds Only ▼] ☐ Missing Peak

Calculated Field Formula

A.%.(Area)/(A.%.ND_Ch.SUM([Non_Deamidated[Area]])+A.%.D_Ch.SUM([[

[Edit Formula...]

4. Sample set table

Apply Table Preferences [After Sample ▼]

	Vial	Inj Vol (uL)	# of Inj	Label	SampleName	Sample Type	Function	Method Set / Report Method	Label Reference	Processing
1	1,E,1	10.0	1	A	SIR_CDR_L3	Unknown	Inject Samples	CDR L3 MS		Normal
2							Summarize Custom Fields			Normal

5. Processing

Processing
☑ Process
  ⦿ Use acquisition method set (i.e. from the sample set used to acquire data)
  ○ Use specified method set [▼]
  ○ Use specified processing method [▼]

☐ Clear Calibration   How: [Calibrate and Quantitat ▼]

Figure 4. Inter-channel calculation workflow. A generic workflow for de

Data acquired using timed SIR channels can be overlayed for easy review, while individual metrics for identif

For this study, the CDR containing peptide L3 was calculated to contain 8.28% deamidation. From this data,

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3. Birdsall R, Cosgrave E, McCarthy S. Adding Mass Detection to Routine Peptide-Level Biotherapeutic Analyses with the ACQUITY QDa Detector. Waters Applicaton Note. 2015. p/n 720005266EN.

4. Vlasak J, et al. Identification and characterization of asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody. *Anal Biochem*. 2009 Sep 15;392(2):14–54.

5. Harris R, Kabakoff B, Macchi F, Shen F, Kwong M, Andya J, Shire S, Bjork N, Totpal K, Chen A. Identification of multiple sources of charge heterogeneity in a recombinant antibody. *J Chromatogr B Biomed Sci Appl*. 2001 Mar 10;752(2):233–45.

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720005377, April 2015