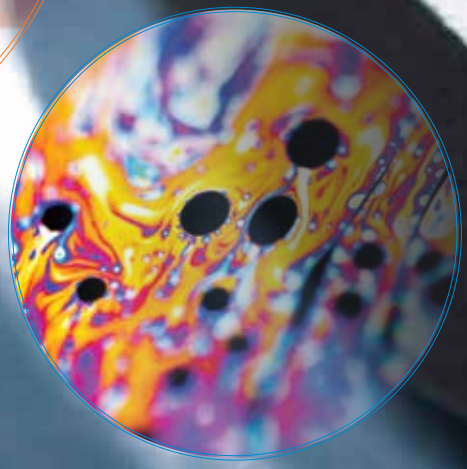
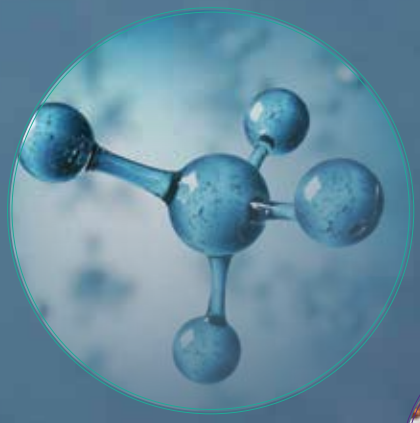
ACQUITY QDa Detector

QC APPLICATIONS COMPENDIUM

EDITION 2





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Dear Colleague

The 2013 introduction of the ACQUITY™ QDa™ Detector was a breakthrough in Chromatography and Mass Spectrometry integration.

It was the fulfilment of a vision 20 years earlier when Waters acquired Micromass, that at some point the blending of MS and LC into a capability that every separation scientist could access, would not only change the way our customers work but would change the industry.

With the combination of ACQUITY, PDA, and QDa, you have everything you need to separate, quantitate and identify your compounds. It adds a whole new dimension to laboratory life; the power of mass data accessible in a way that just fits into the chromatography workflow – and work bench.

The ACQUITY QDa Mass Detector brings an added level of confidence that can help you become more productive and lower costs, at the simple push of a button.

Since its release, the ACQUITY QDa has proved to be our fastest selling MS product. It's received numerous awards for innovation and very positive customer feedback. And it's been adopted across many industries to overcome long-time challenges faced by LC users such as co-eluting peaks, non-chromophoric compounds, and data confidence. The ACQUITY QDa truly redefines separation science.

If you haven't considered the benefits of the ACQUITY QDa Detector until now, we hope that you'll find this compendium informative and inspiring.

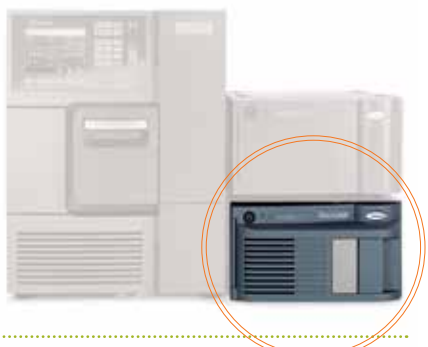
Sincerely,

Ian King

Senior Vice President
Instrument Technology

AUTOPURIFICATION LC PREP

For analytical and preparative chromatography of milligram to multigram quantities, with maximum efficiency on a single, high-throughput HPLC platform.

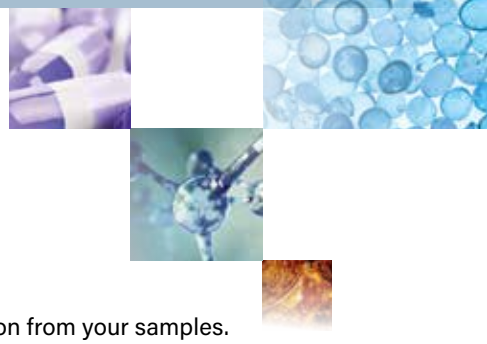


ALLIANCE HPLC

For analytical labs that require versatile and dependable performance, the Alliance HPLC System is the industry gold standard providing exceptional confidence in your analytical results.

ACQUITY UPLC H-CLASS

Discover true UPLC performance with a system that delivers high resolution separations, increases sensitivity, and improves throughput, but also has the flexibility to automate method development and support existing HPLC methods.



SEPARATING BEYOND QUESTION

Bringing the power of MASS DETECTION to you

Mass Detection is widely recognized as a reliable method to derive more meaningful information from your samples. With the ACQUITY QDa Detector, you now have information-rich mass spectral data to compliment your optical detectors, which integrates seamlessly into the same workflow, routinely giving you more complete separation characterization.

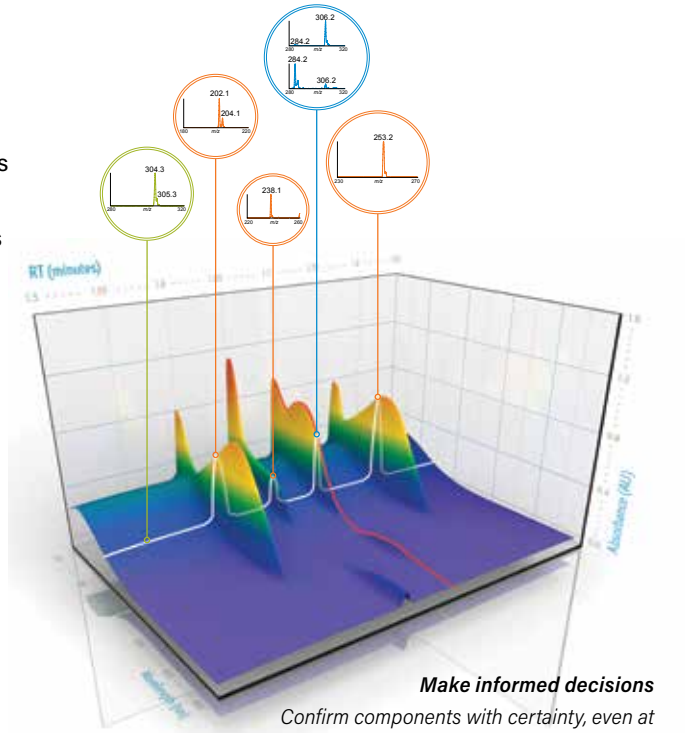
Mass Detection for QC applications

In Quality Control laboratories across all industries, there is an increasing requirement to deliver product to market in an accelerated timeframe. Adherence to dynamic and tightening regulatory standards is obviously also essential in this process. Since the launch of the ACQUITY QDa we've witnessed a growing number of QC laboratories using accessible mass detection to meet these challenges – with many now seeing ACQUITY QDa as a multi-purpose tool enabling QC workflow improvement and improved data confidence.

This application notebook illustrates a selection of that work.

Empower® 3

MassLynx®



Make informed decisions

Confirm components with certainty, even at trace levels (circled orange above), or for components with no UV response (circled green). Minimize the risk of unexpected co-elutions, even for components of similar UV spectral properties (circled blue).

ACQUITY UPC²

Complementary to both LC and GC and providing orthogonal data with greater separation power that you can rely on while also simplifying your laboratory workflow.

ACQUITY UPLC I-CLASS

A binary UPLC System that offers the fastest UPLC separations and maximized peak capacity and minimized carryover, for the most information possible from even the most complex samples.

ACQUITY ARC

Designed for seamless method transfer from any LC, the ACQUITY Arc System is a modern LC that delivers HPLC and UHPLC method compatibility at the flip of a switch.

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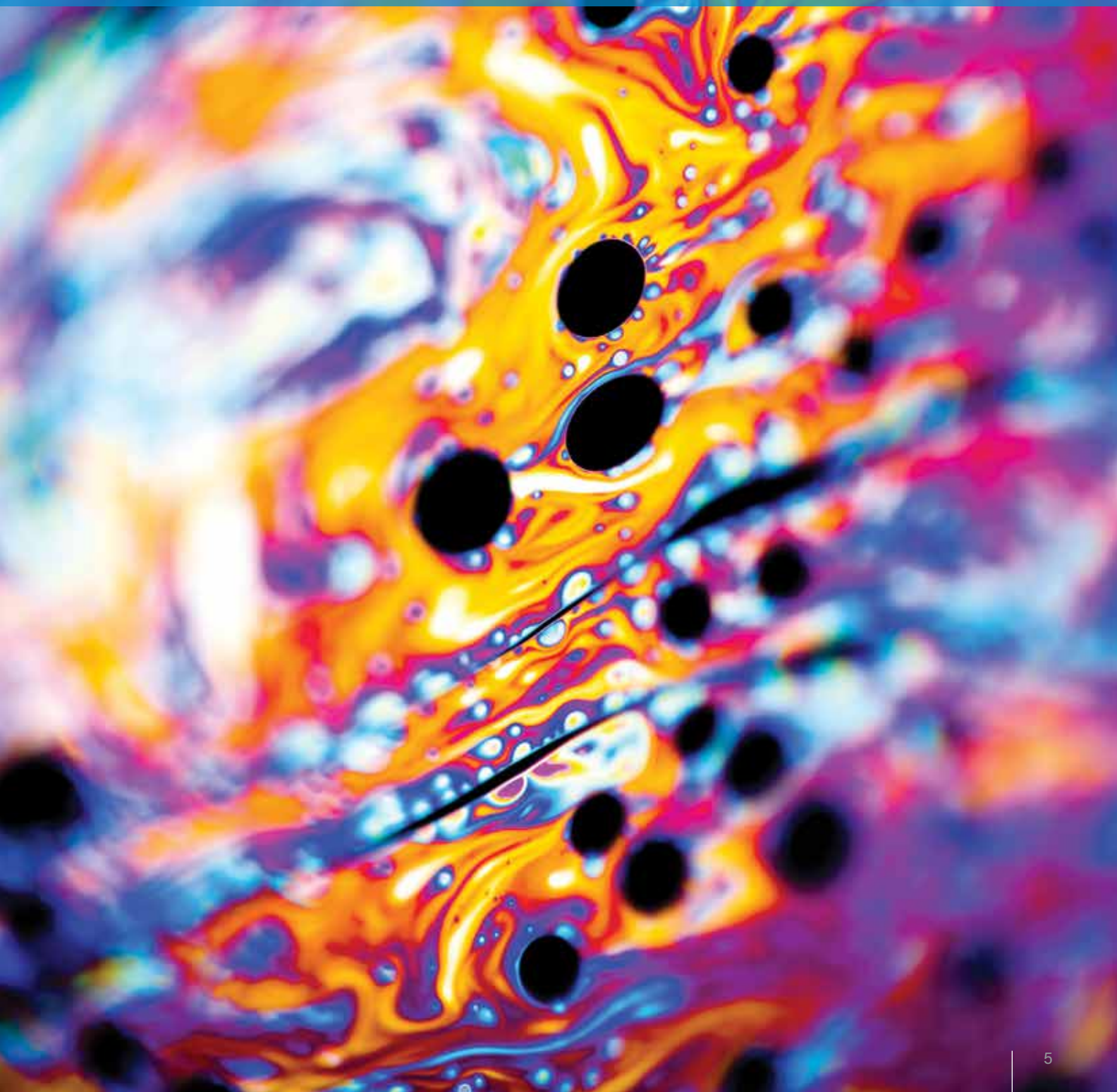
Chemical Materials



Chemical Materials

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Analysis of Disperse Dyes Using the ACQUITY Arc System with PDA and Mass Detection, and Empower Software

Marian Twohig,[1] Michael O'Leary,[1] and Jane Cooper[2]
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APPLICATION BENEFITS

- Enhanced confidence in the profiling of impurities using PDA and mass detection.
- Ease of use with single point control via Empower® 3 Software.
- Dual-flow paths to emulate HPLC and UHPLC separations.

WATERS SOLUTIONS

ACQUITY® Arc™ System

2998 Photodiode Array (PDA) Detector

ACQUITY QDa® Detector

XBridge® C_{18} Column

Empower 3 CDS Software

KEY WORDS

Disperse dyes, consumer products, textile, impurity identification, mass detection

INTRODUCTION

Disperse dyes are low molecular weight synthetic dyes. The structure of the dyes can often contain azo or anthraquinone functional groups.[1] The primary application of disperse dyes is in consumer products such as textiles, paper, toys etc. Several of the dyes have been found to induce an allergic response as a result of prolonged exposure to the skin.[2] The presence of azo groups in the structure of some dyes provides the possibility for them to be converted to potential or known carcinogenic aromatic amines.[2]

The existence of these dyes in consumer products has led to increased awareness of the potential harmful effects to consumer health. Legislation controlling the use of several of these dyes was introduced in Germany in 1996. This led to the development of the DIN 54231 standard procedure which describes a method for the analysis of disperse dyes that employs high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) with either ultraviolet (UV), mass spectrometry (MS), or densitometry detection for the analysis of the dyes.[3-5]

In this application note, we present the analysis of nine disperse dyes (Figure 1) using the standard DIN 54231 procedure with a combination of UV and mass detection, and a dual-flow path liquid chromatography system capable of emulating HPLC or UHPLC separations.[6] The inclusion of the mass detector allowed increased information to be derived from the analysis including confirmation of impurity peaks in specific dye samples. The detection limit when measured using the disperse blue 1 dye standard is specified as 0.7 mg/L in the DIN 54231 method. Using Waters® ACQUITY Arc System and the ACQUITY QDa Detector, the detection limit achieved significantly surpassed the specified detection limit for all compounds evaluated.

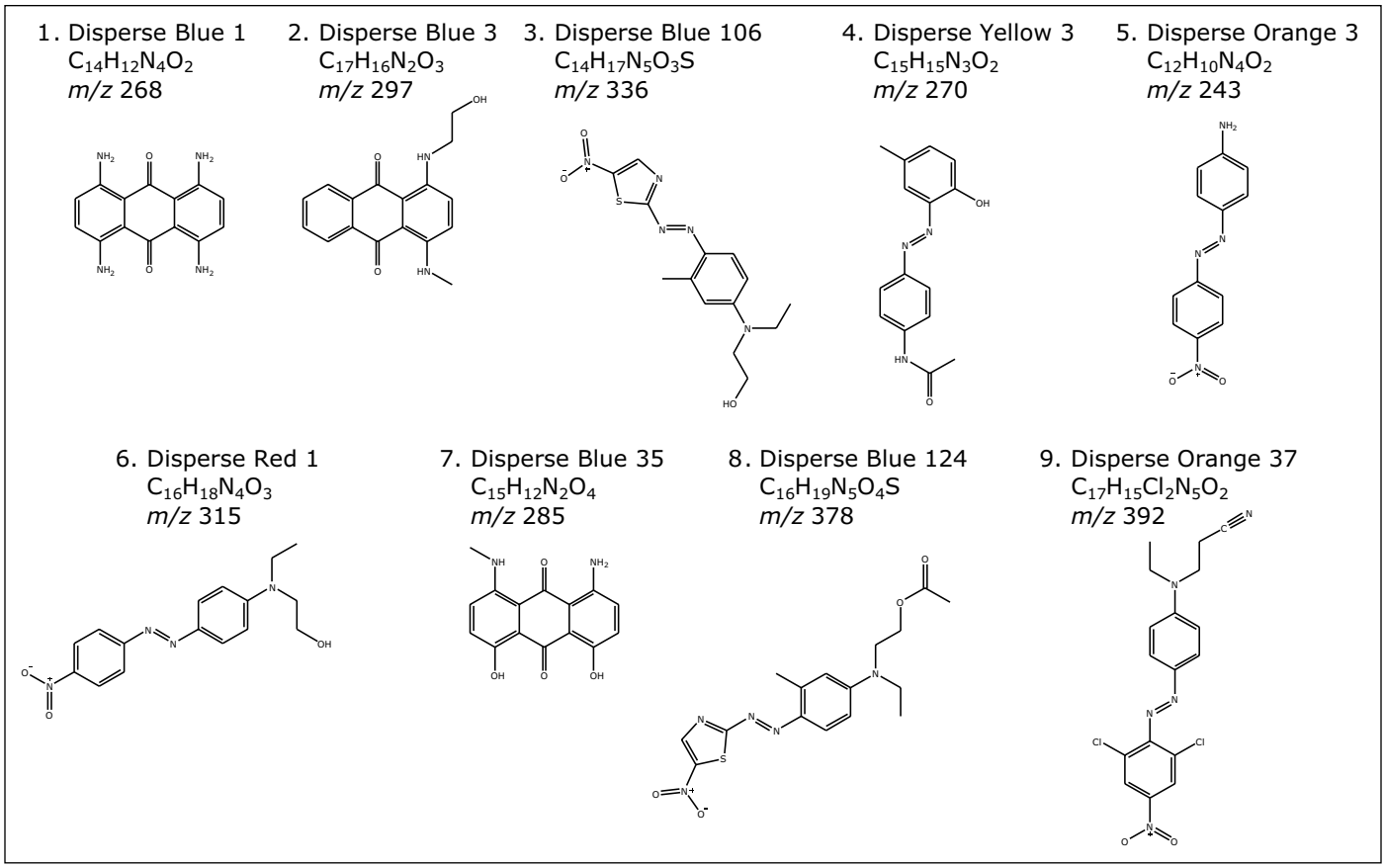

Figure 1. Empirical formulas, structures, and m/z for the disperse dyes used in this study.

EXPERIMENTAL

Instrumentation and software

All separations were performed on the ACQUITY Arc System equipped with a 2998 Photodiode Array (PDA) Detector and positive ion electrospray mass spectrometry (MS) using the ACQUITY QDa Detector. Empower 3 Software was used for data acquisition and processing.

Sample preparation

The dye standards were dissolved in methanol and sequentially diluted in preparation for sample analysis.

LC conditions

HPLC method (DIN 54231)

LC system:	ACQUITY Arc
Separation mode:	Gradient
Column:	XBridge C_{18}, 2.1 x 150 mm, 5 µm
Solvent A:	Ammonium acetate 10 mmol pH 3.6
Solvent B:	Acetonitrile
Flow rate:	0.30 mL/min
PDA detection:	210 to 800 nm
Column temp.:	30 °C
Injection volume:	5 µL
Analysis time:	30 min
Gradient conditions:	0 min 40% B, 7 min 60% B, 17 min 98% B, 24 min 98% B, return to initial conditions.

MS conditions

MS system	ACQUITY QDa
Ionization mode:	ESI +
Capillary voltage:	1.2 kV
Cone voltage:	10 V
Desolvation temp.:	600 °C
Source temp.:	150 °C
MS scan range:	100 to 600 *m/z* and Selected Ion Recording (SIR)
Sampling rate:	5 Hz

Figure 2 shows a PDA chromatogram at 240 nm resulting from the separation of a mixture of nine disperse dye standards (lower trace), and the superimposed SIR channels (top trace) obtained using a 2.1 x 150 mm, 5-µm XBridge C_{18} Column, (Part no. 186003110).

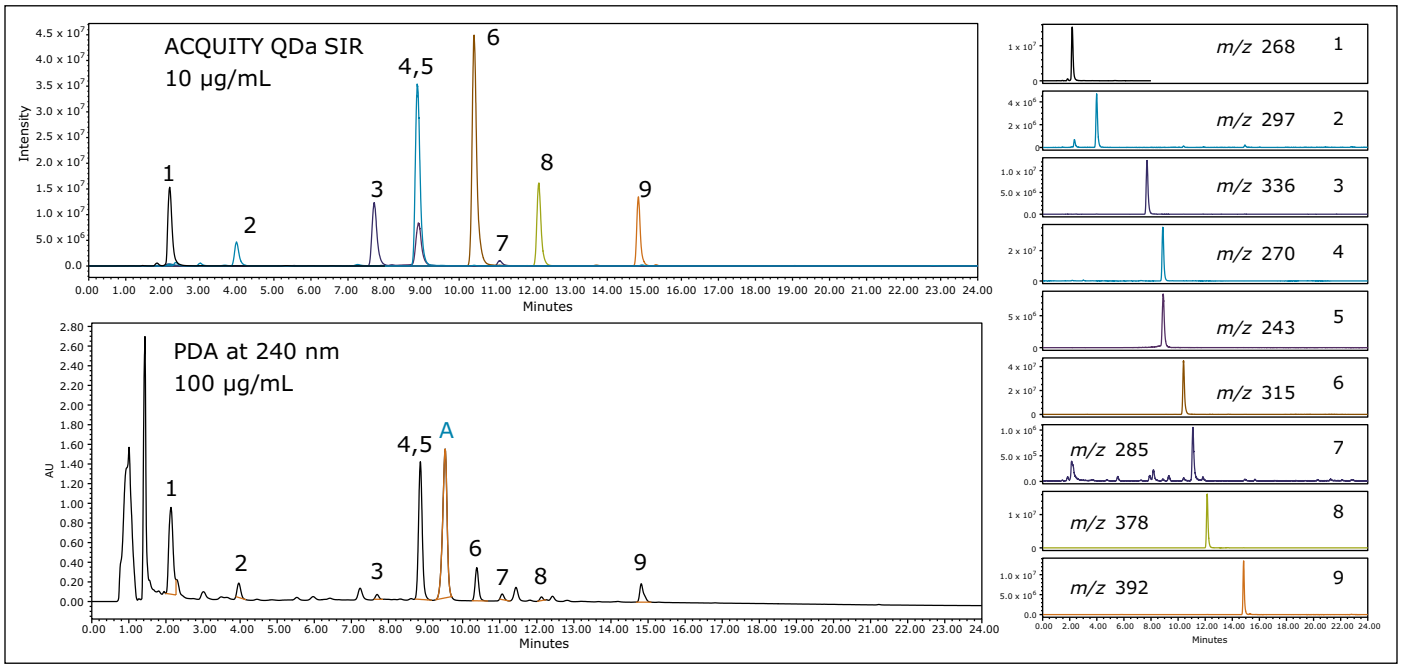

Figure 2. ACQUITY Arc chromatogram from the separation of nine disperse dye standards (100 µg/mL, 5 µL injection) at 240 nm using the DIN 54231 standard method and an XBridge C_{18}, 2.1 x 150 mm, 5.0-µm Column (lower). The superimposed (top) and the individual stacked (right) SIR channel chromatograms (10 µg/mL, 5 µL injection) are also shown.

Note that there is a coelution of the chromatographic peaks resulting from disperse yellow 3 (peak 4), and disperse orange 3 (peak 5) which makes accurate detection by UV alone challenging. Chromatographic separation of the components would be required for accurate detection if UV was to be used which would extend the method development time. The components have different *m/z* ratios, which enabled independent detection using the ACQUITY QDa despite the coelution, as can be seen from the stacked individual SIR chromatograms shown in Figure 2. Detection sensitivity was significantly improved using the mass detector.

Impurity analysis

A prominent peak (peak A) was detected in the PDA data at a retention time (t_R) of 9.5 minutes. This signal was absent from the SIR channels as the specific *m/z* for this unknown component was not monitored in the experimental method. An MS full scan experiment was performed simultaneously with the PDA detector making it possible to determine the mass spectra as well as the UV spectra for all components in the mixture (Figure 3).

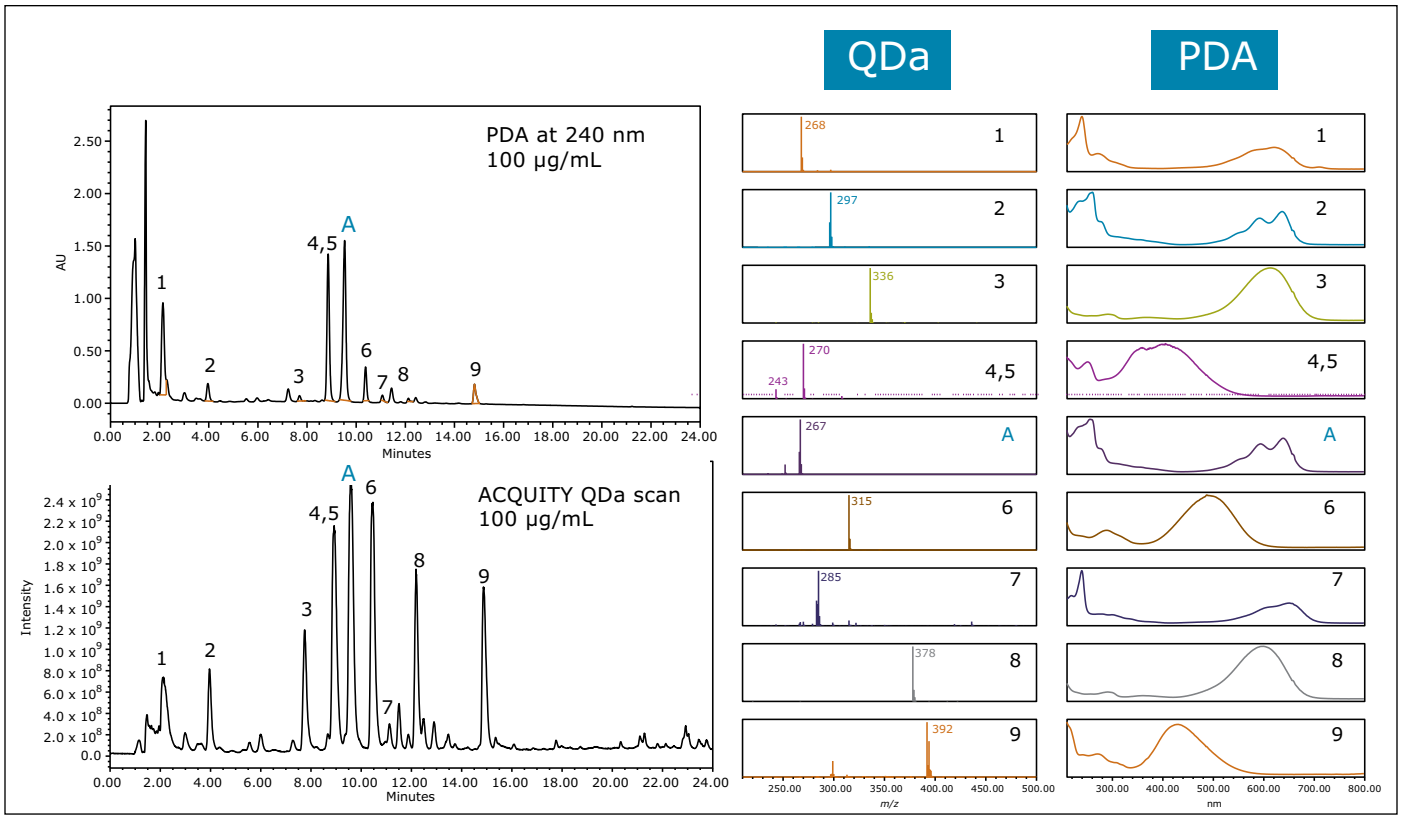

Figure 3. ACQUITY Arc chromatograms from the separation of nine disperse dye standards at 240 nm (top) (100 µg/mL, 5 µL injection) and QDa MS scan (100–600 m/z) (bottom) using the DIN 54231 standard method and an XBridge C_{18}, 2.1 x 150 mm, 5.0-µm Column. The MS and UV spectra are also shown.

The MS spectra for the unknown component A showed a large spectral peak with *m/z* 267. In addition the UV spectra of peak 2 which corresponds to disperse blue 3 and that of unknown peak A had similar features indicating that they may share common structural characteristics. A standard solution containing only disperse blue 3 which had a dye content of 20% was analyzed (Figure 4). The Mass Analysis window from Empower Software allowed rapid confirmation of the identity of disperse blue 3 (*m/z* 297) by displaying both the UV and mass spectra simultaneously. The mass spectrum for unknown peak A indicates that the base peak for this component is *m/z* 267 which matched the previous analysis of the mixture. In addition the t_R and the UV spectra were the same in both analyses. A second unknown component with a t_R of 11.4 minutes, labeled B, with an *m/z* 254 was also detected in the analysis of the disperse blue 3 dye standard. The ACQUITY QDa and PDA data provided complementary information which allowed us to conclude that the impurity A previously detected in the mixture of dyes originated from the disperse blue 3 standard.

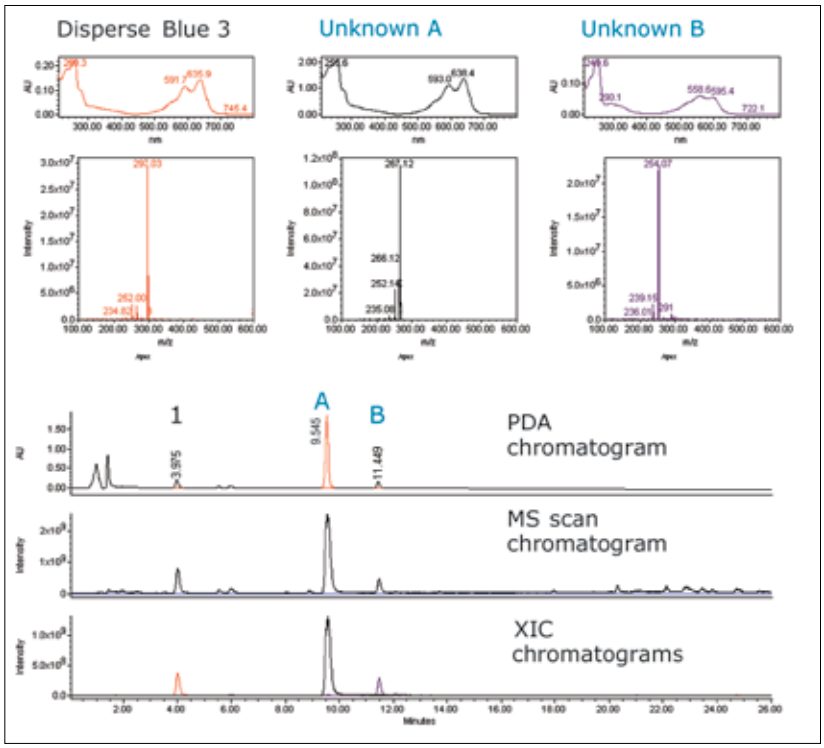

Figure 4. Empower Software's Mass Analysis window showing UV and MS spectra (top). ACQUITY Arc -, PDA, MS scan (100–600 m/z) and superimposed XIC chromatograms of a single standard of disperse blue 3 using the DIN 54231 standard method.

CONCLUSIONS

The addition of mass detection as a complementary analytical detection technique enhances confidence in compound detection and identification. Co-eluting components with different *m/z* ratios can be reliably analyzed using mass detection. The detection limits required for the DIN method can be surpassed for all compounds using the described analytical methodology. The presence of both PDA and mass detection helped confirm that an impurity detected during method development originated in the disperse blue 3 standard. Thus the addition of mass detection acts as a complementary technique for impurity analysis.

The ACQUITY Arc System provides increased flexibility for chromatographic separations and maximizes, productivity by accommodating 3.0 µm to 5 µm particles for HPLC methods, while also supporting rapid and efficient UHPLC separations using 2.5 to -2.7 µm particles.[6]

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UHPLC Analysis of a Pesticide Formulation Using the ACQUITY Arc System with PDA, Mass Detection, and Empower 3 Software

Marian Twohig, Michael O'Leary, and Neil J. Lander
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Enhanced confidence in the profiling of impurities using PDA and mass detection.
- Structural similarities between the active ingredient and unknown components can be identified in a single analysis.
- Ease of use with single point control data analysis and reporting via Empower® 3 Software.
- Dual-flow paths that emulate HPLC and UHPLC separations aid method development and transfer.

WATERS SOLUTIONS

ACQUITY® Arc™ System

2998 Photodiode Array (PDA) Detector

ACQUITY QDa® Detector

CORTECS® C_{18}+ Column

Empower 3 Chromatography Data Software

KEY WORDS

Pesticide, formulations, mass detection, UHPLC, impurity identification, tebuconazole, triazole fungicides, insecticide

INTRODUCTION

Crop protection products provide solutions that decrease crop damage resulting in a food supply that is plentiful and of high quality.[1] In the agricultural chemicals industry the analytical quality control of pesticide products is very important to ensure that a consistent and effective product reaches the customer.[2] Detection, characterization, and quantitation of the active ingredient/s and all other components in the formulation including impurities and degradation products are necessary to support product development, quality control, and product registration. Liquid chromatography (LC) techniques with photo diode array (PDA) detection have been used for the routine analysis of formulation samples.[2-4] The addition of a mass detector in conjunction with UV detection can increase the specificity and selectivity of methods used during analytical testing to provide additional information about a sample in a single analysis.

In this application note, we present the analysis of a commercially available pesticide formulation which contained two active ingredients (AI): an insecticide AI 1, and tebuconazole, a triazole fungicide, AI 2 (Figure 1). The triazole fungicides are a commonly used group of pesticides due to their potent activity against a broad spectrum of crop diseases.[5] The analysis of the formulation employed UV and mass detection and a dual-flow path liquid chromatography system capable of emulating HPLC or UHPLC separations.[6] The ACQUITY Arc System enables existing HPLC methods to be performed, while also allowing the choice of transitioning to a UHPLC method that employs sub-3 µm particles for higher efficiency chromatographic separations.

Empower 3 Software was used for data acquisition and analysis, generating results which were used to flag impurities greater than specified %Area levels. Empower's Custom Fields allowed extra information to be derived from the results as custom calculations which were reported using tailored methods. The combined detection capabilities and data analysis provided the initial structural characterization of the unknown components.

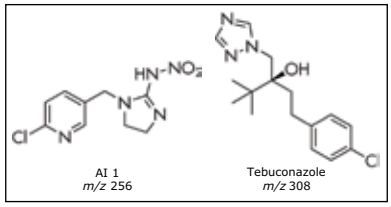


Figure 1. Structures and m/z for AI 1 and tebuconazole.

EXPERIMENTAL

Instrumentation and software

All separations were performed on a Waters® ACQUITY Arc System equipped with a 2998 Photodiode Array (PDA) and an ACQUITY QDa Detector. Empower 3 Software was used for data acquisition and processing.

- Sample preparation: 1 gram of the commercially available pesticide formulation was weighed and 9 mL of 50:50 (v/v) acetonitrile/water was added. The resulting mixtures were sonicated for 20 minutes and syringe filtered into an autosampler vial using a 0.2 µm PVDF filter, in preparation for sample analysis.

LC conditions

LC system:	ACQUITY Arc
Separation mode:	Gradient
Column:	CORTECS C_{18}+ 3.0 x 100 mm, 2.7 µm
Solvent A:	Water with 0.1% formic acid
Solvent B:	Acetonitrile
Flow rate:	0.80 mL/min

UV conditions

UV detector:	2998 Photodiode Array (PDA)
PDA detection:	210 to 400 nm
Column temp.:	50 °C
Injection volume:	5 µL
Gradient conditions:	0 min 20% B, 10 min 80% B, 11 min 90% B, 12 min 90, return to initial conditions.

MS conditions

MS system:	ACQUITY QDa
Ionization mode:	ESI+
Capillary voltage:	0.8 kV
Cone voltage:	10 V
Desolvation temp.:	600 °C
Source temp.:	150 °C
MS scan range:	100 to 1000 *m/z*
Sampling rate:	5 Hz

RESULTS AND DISCUSSION

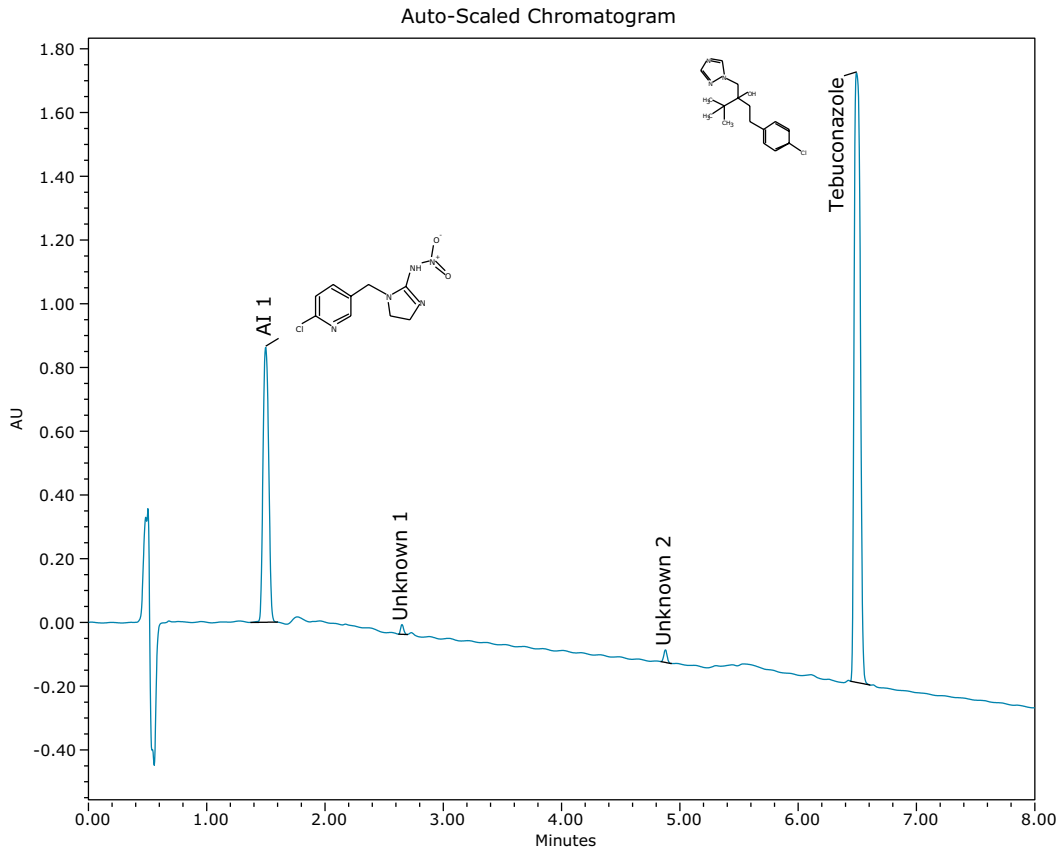
Impurity reporting threshold

The ACQUITY Arc System employs Arc Multi-flow path™ Technology which provides options for selectable dwell volume. This offers increased flexibility for chromatographic separations and maximizes productivity by accommodating 3.0-µm to 5-µm particles for HPLC methods, while also supporting rapid and efficient UHPLC separations using 2.5-µm to 2.7-µm particles.[6] Chromatographic separation of the pesticide formulation sample was performed in UHPLC mode with a CORTECS C_{18}+ Column (3.0 x 100 mm, 2.7-µm solid-core particle technology, part no. 186007402). CORTECS 2.7-µm Columns are compatible on HPLC and UHPLC instrumentation. These columns have high efficiency at HPLC backpressures, resulting in faster analyses with better resolution than current methods using 5 µm, fully porous particles.[7]

Following the analysis of the pesticide formulation and subsequent processing with Empower 3 Software, it was noted that there were two unknown components that exceeded the reporting threshold of 0.1%, which was set in the Empower processing method.[8] The Empower report (Figure 2) shows the ACQUITY Arc UV chromatogram at 220 nm, resulting from the separation of the pesticide formulation sample. The two active ingredients have been identified and the unknown components have been labeled. Beneath the chromatogram, the peak results table displays the component name, area, area%, retention time, impurity response, and the reporting threshold. The UV and MS data suggested that Unknown 1 and Unknown 2 may share common structural features with the tebuconazole AI (Figure 3), therefore an Empower 3 Custom Calculation was employed to calculate the area% of each of the impurities relative to the tebuconazole (Figure 2 table). The tabulated data with the detected impurities highlighted in red makes it easy to interpret which components may require further investigation.

Empower 3

Pesticide Formulation Analysis



Peak Results

	Name	RT	Area	%Area	Impurity response	Impurity	Reporting threshold	Impurity response relative to tebuconazole
1	AI 1	1.499	2875440	29.12		No		
2	*Unknown 1*	2.651	54240	0.55	*0.55*	Yes	0.1	*0.79*
3	*Unknown 2*	4.878	76452	0.77	*0.77*	Yes	0.1	*1.11*
4	Tebuconazole	6.494	6868444	69.56		No		
5	*Total impurities*		130692	1.32	*1.32*	Yes		

Figure 2. Empower Software report showing an ACQUITY Arc UV chromatogram at 220 nm resulting from the separation of the pesticide formulation sample. Peak results are shown beneath and impurities exceeding the threshold are highlighted in red. Structures for the known AI's are also displayed in the report.

Combining chromatographic, UV, and mass detector results in a single place in Empower Software can further ease the burden of data interpretation. The Empower Mass Analysis window (Figure 3) provides a single location to associate chromatographic peaks from all of the detectors used in the analysis with their corresponding spectra. The UV chromatogram and spectra are displayed, along with the total ion chromatogram (TIC) and mass spectra with extracted ion chromatograms (XIC) also shown. The spectra from the detected peaks are time aligned and displayed in a window above the chromatograms facilitating rapid data review.

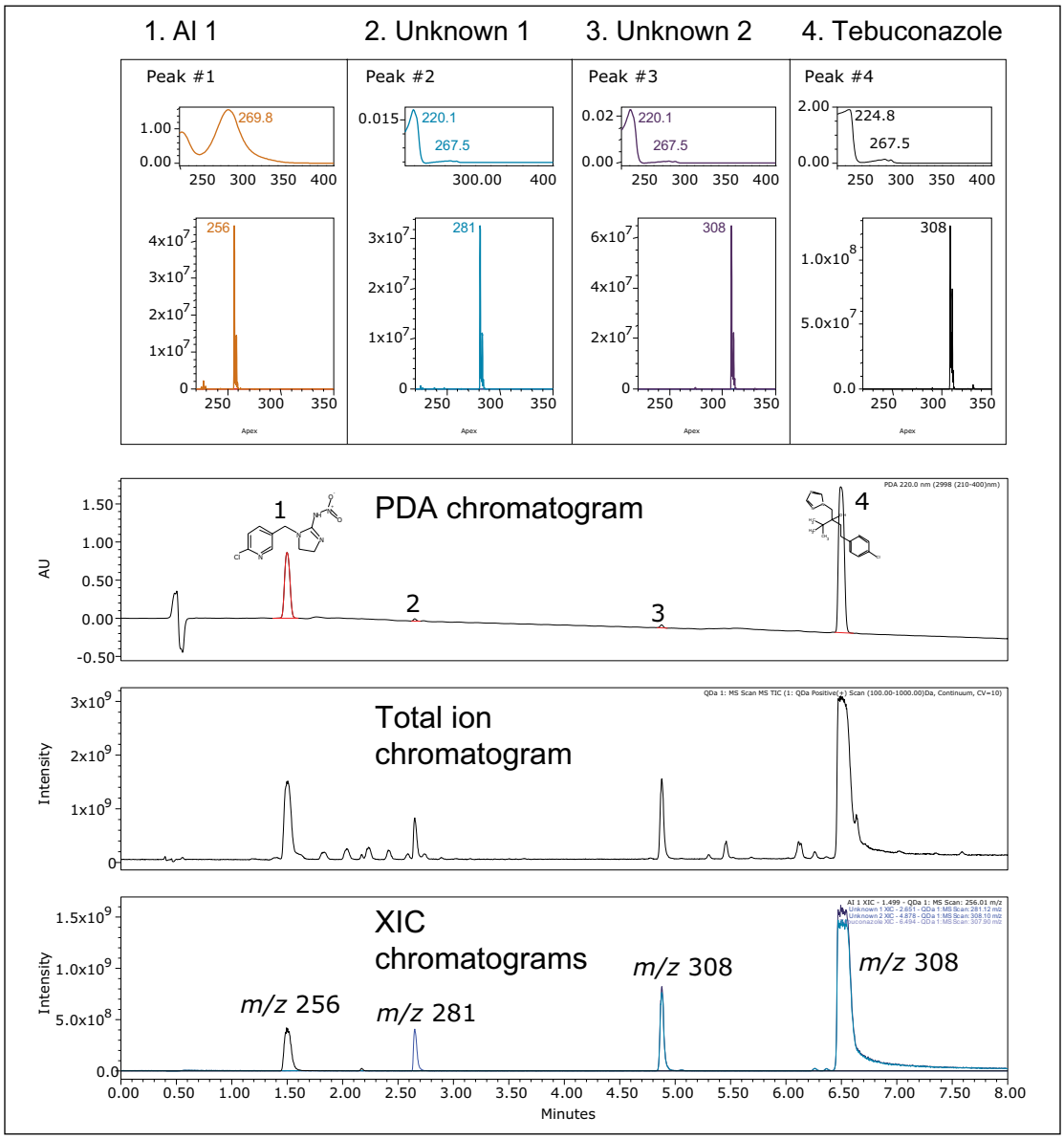


Figure 3. Empower Software Mass Analysis window: UV and MS spectra along with UV and total ion chromatograms (TIC) and extracted ion chromatograms (XIC) can be viewed in a single window.

Interrogation of the data in the Mass Analysis window indicated relationships between Unknown 2 (Peak 3) and the tebuconazole AI (Peak 4). The UV spectra have similar absorption maxima while the mass spectra indicate that tebuconazole, with an $[M+H]^+$ corresponding to *m/z* 308 and Unknown 2 share the same *m/z*. In addition, the isotopic pattern of Unknown 2 is typical of a chlorinated compound (see Figure 4) and is identical to that of the AI. In a single analytical injection, Unknown 2 has been identified as having a similar UV spectrum and the same *m/z* and isotopic pattern as tebuconazole. These results suggest that Unknown 2 is likely an isomer of tebuconazole, and possibly has related structural composition and chemical properties. In addition, the detection sensitivity is greatly improved for the unknown components in the mass chromatogram, especially when the ions corresponding to the peaks of interest have been extracted from the TIC. The increased detection sensitivity provided by the ACQUITY QDa enables improved confidence in the assessment of the data.

The UV maxima of Unknown 1 (Peak 2) showed close similarities when compared with that of Unknown 2 and tebuconazole which may indicate that the impurity is also related to this AI. The *m/z* for Unknown 1 is 281 and the isotopic pattern suggests chlorine in the structure (Figure 4). An Empower Spectrum Index Plot report summarizing the data is shown in Figure 4.

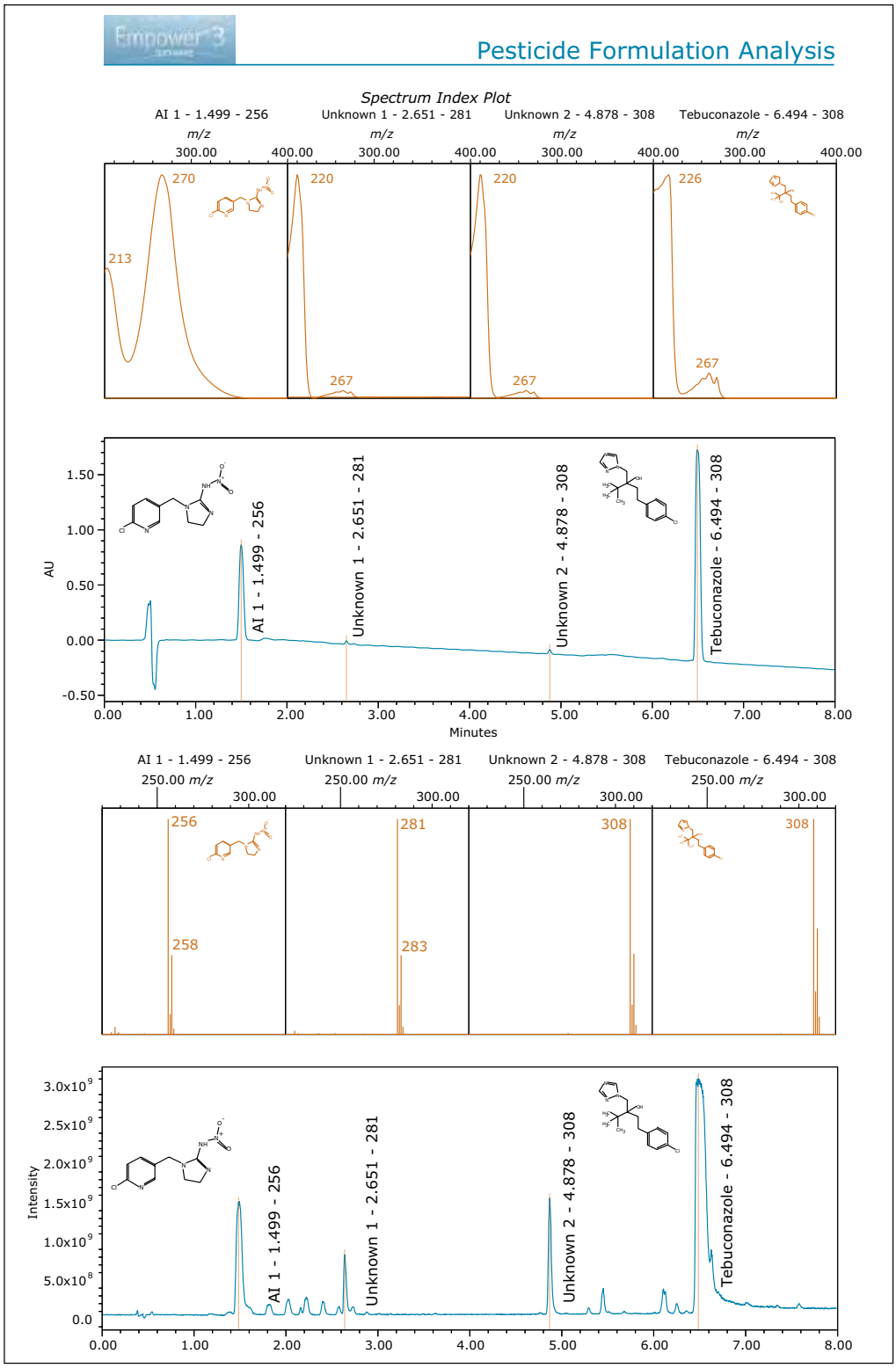


Figure 4. Empower Spectrum Index Plot report showing the ACQUITY Arc UV chromatogram at 220 nm from the analysis of the pesticide formulation, UV spectra of detected peaks are displayed (top), ACQUITY QDa MS scan chromatogram (bottom), and MS spectra for the peaks detected in the UV are also displayed. The structures of the two AI's are shown in the inset.

CONCLUSIONS

- The ACQUITY Arc System provides increased flexibility for chromatographic separations and maximizes productivity by accommodating 3.0-µm to 5.0-µm particles for HPLC methods, while also supporting rapid and efficient UHPLC separations using 2.5-µm to 2.7-µm particles.[6]
- The ACQUITY QDa Detector in combination with the PDA allowed low level components to be detected with increased confidence in the pesticide formulation. The components were identified as having similar optical and structural properties to the triazole fungicide active ingredient present in the formulation sample.
- Empower Software's Mass Analysis window provides a single location to associate the chromatograms and spectra from all of the detectors used in the analysis. The consolidation of this information into one place makes data review and interpretation easy.
- The addition of mass detection as a complementary analytical detection technique enhances confidence in compound detection and identification. With the familiarity of a PDA detector, the ACQUITY QDa Detector provides a cost-effective way to make mass detection routine in laboratories that have previously relied on less selective detectors.
- Empower 3 has numerous features that facilitate the analysis of data. Impurities exceeding a set limit can be automatically flagged. Tailored calculations allow relevant information to be derived quickly which greatly aids in the identification of components that may need further investigation.

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Identification of Skin Lightening Agents in Cosmetics Using UHPLC with PDA, Mass Detection, and Empower 3 Software

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APPLICATION BENEFITS

- UHPLC separations using a CORTECS® T3 Column provided exceptional retention for both polar and non-polar skin lightening agents.
- Enhanced confidence in the detection of skin whitening agents using PDA and mass detection.
- Ease of use with single point control data analysis and reporting via Empower® 3 Chromatography Data Software.
- Dual flow paths to emulate HPLC and UHPLC separations to aid method development and transfer.

WATERS SOLUTIONS

ACQUITY® Arc™ UHPLC System

2998 Photodiode Array (PDA) Detector

ACQUITY QDa® Mass Detector

CORTECS 2.7 µm Columns

Empower 3 Chromatography Data Software (CDS)

KEYWORDS

UHPLC, mass detection, CDS, cosmetics, skin whitening agents, corticosteroids, hydroquinone, tretinoin, nicotinamide, arbutin, salicylic acid, AIs, active ingredients, steroid

INTRODUCTION

Skin lightening/whitening agents in cosmetics are often used to produce a more even skin tone, usually to the face and neck, but sometimes they can be used more extensively over larger areas of skin.[1-3] The products may be marketed to consumers as either a cosmetic to improve appearance, or as a therapy to treat skin conditions (i.e. a drug). The distinction between a cosmetic and a drug is important from a regulatory and safety perspective because, among other things, a cosmetic should not contain pharmaceutical active ingredients (AIs). Otherwise they are classified as a drug according to European Commission's No. 1223/2009 and the US FDA's regulations on Cosmetics.[4,5] In this application note, we investigate misbranded cosmetics products that use pharmaceutical AIs such as hydroquinone, corticosteroids, and tretinoin despite being marketed as cosmetic products. From a safety perspective, the use of steroid ingredients is prohibited in cosmetics due to the potentially undesirable side effects that can occur without the guidance and monitoring by a medically trained professional.[6-8] Hydroquinone is widely used in dermatology to treat hyperpigmentation, however, it is prohibited for use in cosmetics in the EU and several other countries.[4] Hydroquinone is also prohibited at over the counter levels exceeding 2% w/w in the US.[5] Prolonged use of hydroquinone can cause permanent ochronosis which leaves the skin discolored.[9-12] Corticosteroids are highly effective drugs which are used to treat inflammatory skin conditions such as eczema and psoriasis. Topical preparations are usually in the form of creams or gels. Long term use of corticosteroids can cause side effects including pustular psoriasis, permanent skin atrophy and systemic effects such as hypertension, contact dermatitis, and diabetes.[6-8] Tretinoin is retinonic acid in its pharmaceutical form. It is used in the treatment of acne.

These components can still be found in cosmetics marketed as skin lightening products, due to their effectiveness.[1-3] In this study, cosmetic products were obtained from online vendors. The samples were extracted and analyzed using UHPLC with PDA and mass detection on a dual-flow path liquid chromatography system capable of emulating HPLC or UHPLC separations. The ACQUITY Arc System enables existing HPLC methods to be performed while also allowing the choice of transitioning to a UHPLC method employing 2.5 to 2.7 µm particles for higher efficiency chromatographic separations.

The CORTECS Column stationary phase used in the separation contained a 2.7 µm particle designed to give maximum efficiency and exceptional retention for both polar and non-polar analytes. With an optimized pore size, C_{18} ligand density, and endcap, the CORTECS T3 phase is compatible with 100% aqueous mobile phases and provides the perfect balance of polar and non-polar analyte retention.

Several samples tested positive for prohibited skin lightening agents. The packaging labels were often misleading, and in some cases, the skin lightening agent was not listed on the enclosed product information increasing the likelihood of improper long-term use and adverse side effects to consumers.

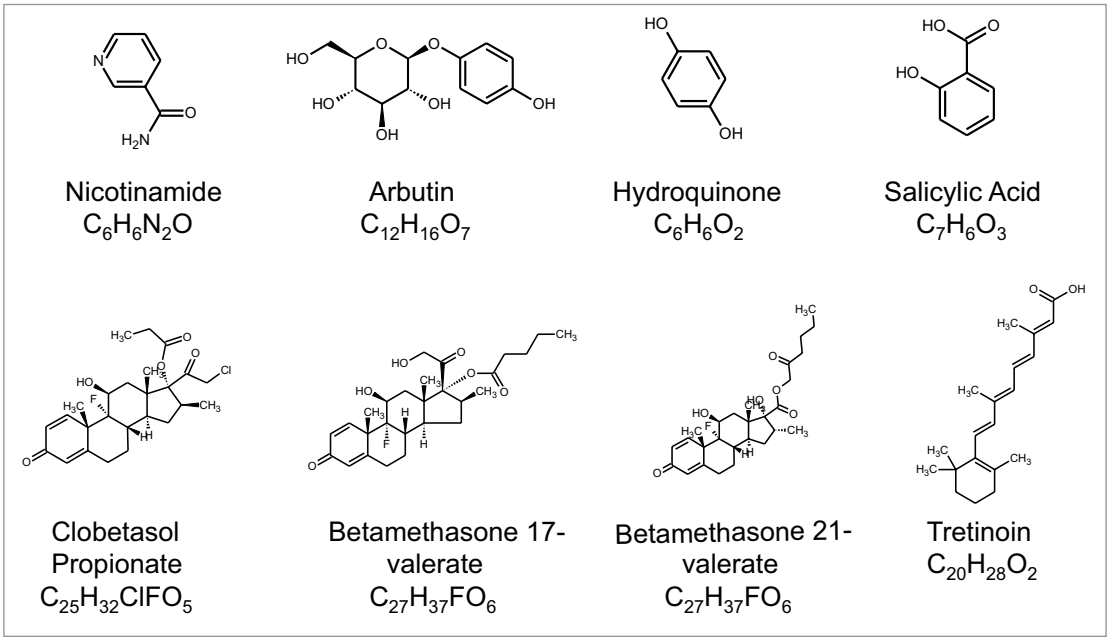

Figure 1. Empirical formulas and structures for the skin lightening agents analyzed in the study.

INSTRUMENTATION AND SOFTWARE

All separations were performed on the ACQUITY Arc System equipped with a 2998 Photodiode Array (PDA) Detector and the ACQUITY QDa Mass Detector. Empower 3 Chromatography Software was used for data acquisition and processing.

SAMPLE PREPARATION

The standard compounds including eight skin lightening agents (Figure 1) and four parabens were dissolved in methanol and sequentially diluted to prepare the spiking solutions. Although parabens are not skin whitening ingredients, they were included in this study because parabens are often used as microbial inhibitors and are commonly found in cosmetic products.[13] Matrix matched curves were prepared using blank cream or gel cosmetics bases obtained from Making Cosmetics Inc. (Snoqualmie, WA, USA). An aliquot (1 g) of the matrices (cream/gel) were weighed into 15 mL centrifuge tubes and spiked with 100 µL of acetonitrile spiking solutions. The mixture was initially vortex mixed and then shaken (Eberbach hand motion shaker 500 rpm) for 10 minutes to equilibrate the spiked analytes. A volume of acetonitrile (4.9 mL) was added and the samples were shaken again for 25 minutes. The samples were then centrifuged at 3000 rpm for 10 minutes. An aliquot of the supernatant was syringe filtered using a 0.2-µm PVDF filter and placed in a vial in preparation for sample analysis. The cosmetics samples (1 g) were extracted in the same way using 5 mL of acetonitrile in the extraction step.

EXPERIMENTAL

Method conditions

UHPLC method

UHPLC system:	ACQUITY Arc
Separation mode:	Gradient
Column:	CORTECS T3 2.7 µm, 3.0 x 100 mm
Solvent A:	0.1% formic acid in water
Solvent B:	Methanol
Flow rate:	0.80 mL/min
Column temp.:	30 °C
Injection volume:	0.5 to 2 µL
Detector:	2998 Photodiode Array (PDA)
PDA detection:	210 to 400 nm

Gradient:

0 min 0% B, 0.5 min 0% B, 2.2 min 2% B, 6.0 min 95% B, 8.0 min 99% B, 9.0 min 99% B, return to initial conditions

MS conditions

MS system:	ACQUITY QDa
Ionization mode:	ESI+ and/or ESI-
Capillary voltage:	1.0 kV (+); 0.8 kV (-)
Cone voltage:	10 V
Desolvation temp.:	600 °C
Source temp.:	150 °C
MS scan range:	100 to 600 *m/z*
Sampling rate:	5 Hz

RESULTS AND DISCUSSION

Figure 2 shows the chromatogram resulting from the separation of a standard mix of 12 compounds encountered during the study using the ACQUITY Arc System with PDA detection. An Empower 3 processing method was developed to identify the analytes using the retention times (t_R) of the standard compounds which were determined experimentally. Four parabens, which are frequently used as preservatives, were detected in many of the cosmetics samples analyzed (Figure 2, peaks 4, 6, 7, 8).[13]

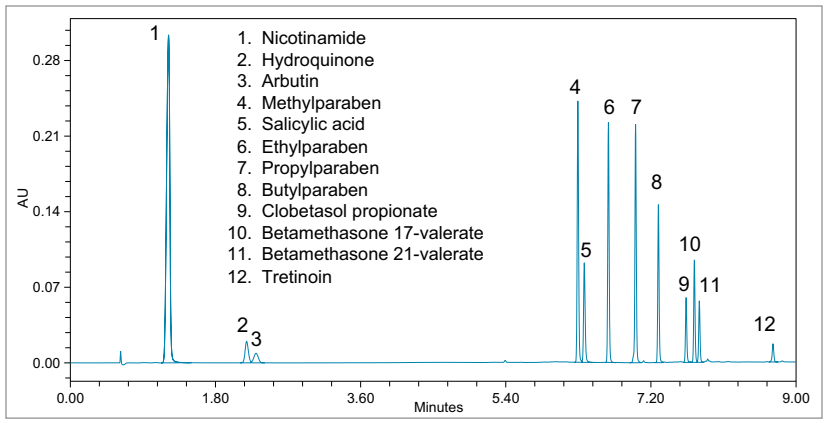

Figure 2. ACQUITY Arc UV chromatogram at 254 nm resulting from the separation of a standard mixture of compounds encountered during the study using a CORTECS T3 Column, 1 µL injection.

CLOBETASOL PROPIONATE IDENTIFIED IN A COSMETIC SAMPLE

Figure 3 (bottom) shows the chromatogram resulting from the separation of the extracted whitening gel sample which was obtained from an internet vendor in the US. The sample was found to contain arbutin, the corticosteroid clobetasol propionate as well as four parabens, which were labeled using the Empower 3 processing method. The t_R of the peaks matched with those in the authentic standard (Figure 3 top).

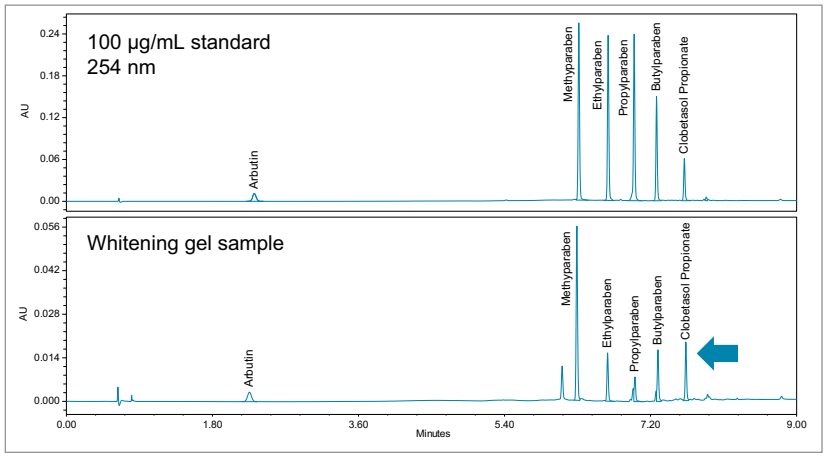

Figure 3. ACQUITY Arc chromatogram resulting from the separation of a skin whitening gel sample (bottom) at 254 nm using a CORTECS T3 Column. Standard compounds for t_R matching are also shown (top), 100 µg/mL, 0.5 µL injection. Clobetasol propionate in the sample is indicated by the arrow.

The inclusion of mass detection in addition to the PDA provided complementary information to the analysis. Figure 4 shows a comparison of both the UV and mass spectrum for clobetasol propionate in the gel sample and in the standard. Clobetasol propionate, with an $[M+H]^+$ corresponding to *m/z* 467, and the component identified as clobetasol propionate by the Empower 3 processing method share the same *m/z*. The isotopic pattern reflects the chlorine present in the chemical structure providing extra confirmation and increased confidence in the identification. The UV and mass spectra were identical in both the standard and the sample. The product label on the cosmetic sample did not declare the presence of the clobetasol.

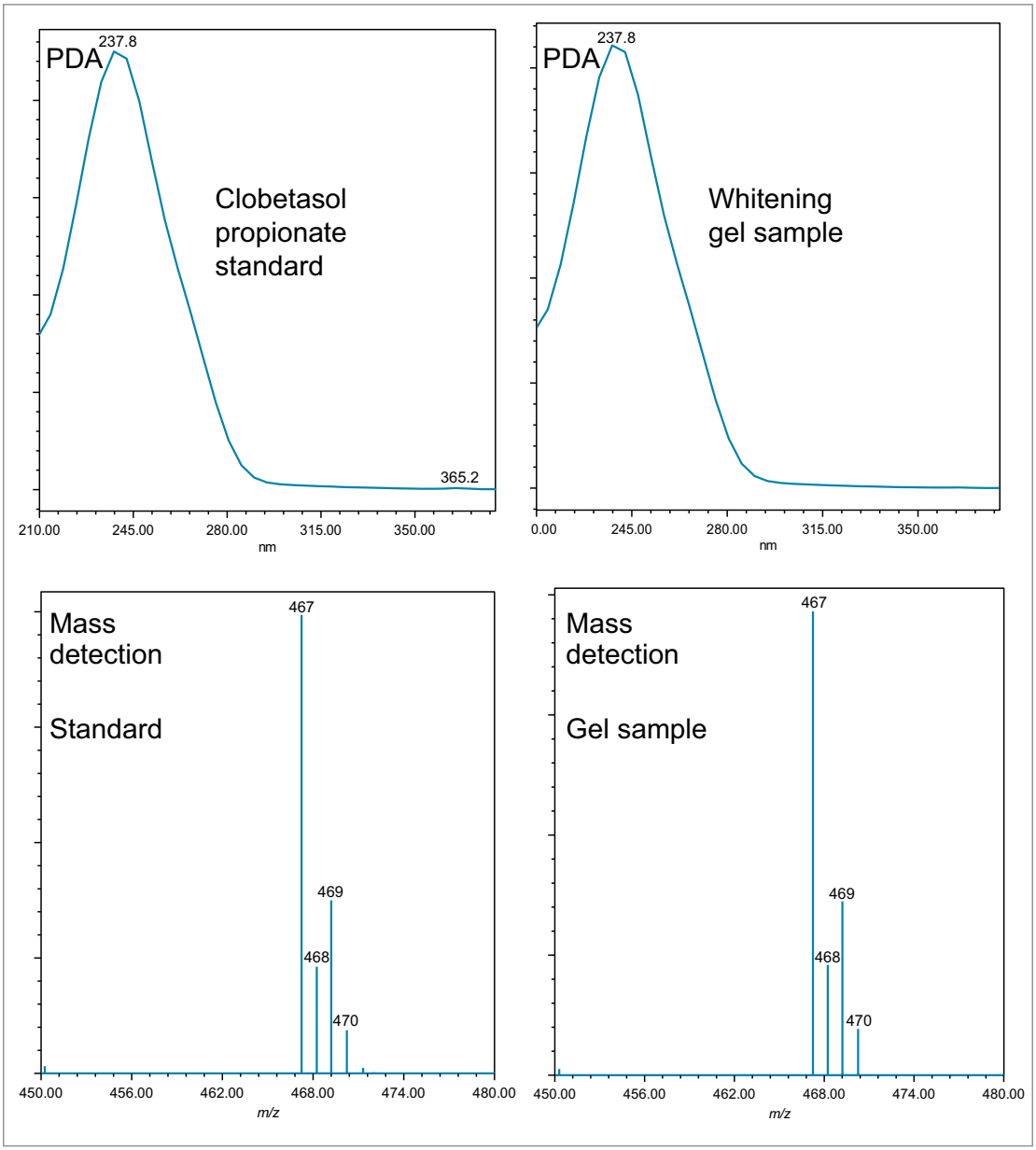

Figure 4. Comparison of the PDA and mass spectra for the clobetasol propionate in the standard and the sample. The same UV spectra, m/z, and isotopic patterns indicating the presence of chlorine were observed.

QUANTITATION

The selected skin lightening agents were pre-spiked into blank cream and/or gel cosmetics bases and extracted using the procedure detailed in the Experimental section in order to create matrix matched quantitative standards. The recoveries of clobetasol propionate and arbutin from the gel matrix were in excess of 93%, and the recoveries of betamethasone 17-valerate, hydroquinine, and tretinoin from the cream matrix were in excess of 98%. The matrix matched calibration curves (R^2 >0.999), were used to quantify the six samples analyzed (Table 1) with the 2D UV channels recorded (Arbutin = 280 nm; Clobetasol propionate, betamethasone 17-valerate and betamethasone 21-valerate = 238 nm; Hydroquinone = 290 nm; Tretinoin = 352 nm. An example of the calibration curve obtained from the cream matrix spiked with hydroquinone is shown in Figure 5.

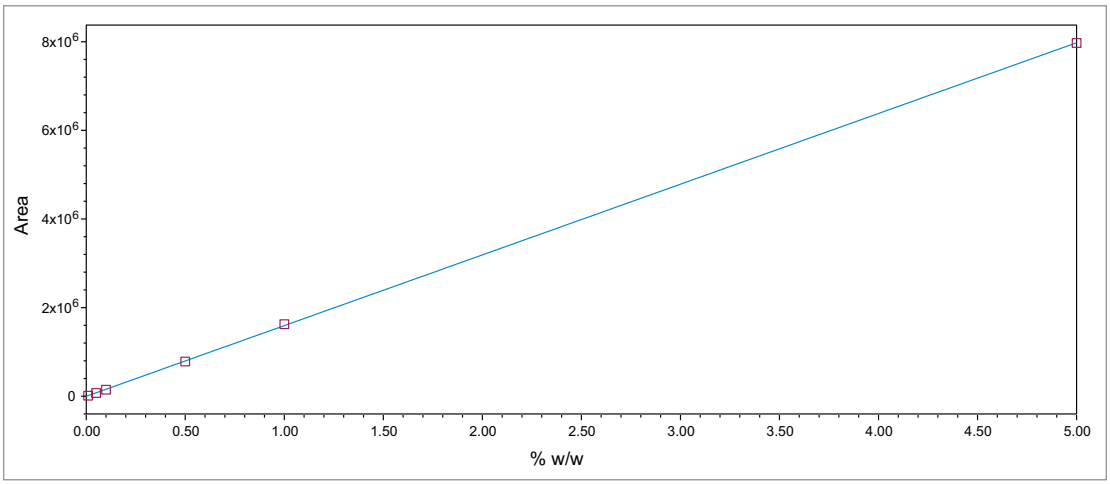

Figure 5. Cosmetic cream matrix-matched calibration curve for hydroquinone 0.01 to 5% w/w.

The results in Table 1 show that the levels of most components detected in the samples were within the typical usage level for the AI.[2] The NS sample was diluted 1:1 to bring the detected hydroquinone into the calibration range of 0.01 to 5%. The results in Table 1 show that clobetasol propionate was detected in a whitening cream sample as well as the whitening gel. The samples MJ-1 and MJ-2 were obtained from an internet shop in the US. This product is marketed as one of a number of skin treatments to be used together in order to provide optimum lightening effects. Hydroquinone, betamethasone 17-valerate, and tretinoin were detected in the MJ-1 sample. The second treatment MJ-2 contained betamethasone 17-valerate and its structural isomer betamethasone 21-valerate as well as tretinoin.

Compounds	Typical usage level % w/w	Whitening gel	Whitening cream	MJ-1	MJ-2	NS	TG
Clobetasol propionate	0.05	0.038	0.060	—	—	—	—
Betamethasone 17-valerate	0.05	—	—	0.035	0.103	—	—
Betamethasone 21-valerate*	N/A	—	—	—	0.038	—	—
Hydroquinone	1 to 5	—	—	3.74	—	7.20	3.00
Tretinoin	0.01 to 0.1	—	—	0.015	<0.01	—	—
Arbutin	4 to 7	0.203	—	—	—	—	—

Table 1. Summary of the quantitation results for selected skin lightening compounds in the samples and the typical usage concentrations.[2]

CONCLUSIONS

This study of cosmetics available for purchase from online vendors in the United States shows that these products contain corticosteroids and tretinoin which are both prohibited for use in cosmetics in the EU and the US. The steroidal components were detected in four of the samples analyzed. Quantified amounts were frequently in the typical usage range or above.

Hydroquinone was detected in three samples at >3% w/w which violated both EU and US regulations.

In some of the samples analyzed, the presence of the active ingredients was not declared on the label or the enclosed product information. Inaccurate or insufficient labeling of the cosmetics products increases the likelihood of adverse side effects, as cosmetics are usually used over long periods of time with no medical supervision.

This study was aided by the use of the ACQUITY Arc System which provides increased flexibility for chromatographic separations, and maximized productivity by accommodating 3.0 µm to 5 µm particles for HPLC methods, while also supporting rapid and efficient UHPLC separations using 2.5 to 2.7 µm particles.

In order to ensure optimal chromatographic performance on the ACQUITY Arc System, the CORTECS T3 Column, which is designed to give maximum efficiency and exceptional retention for both polar and non-polar analytes, was used to facilitate a single analysis for a wide range of the skin lightening agents and parabens.

To ensure data integrity, an Empower 3 processing method, developed with standard compounds was used in the identification and quantitation of the test compounds.

Empower 3 Sofware's UV and MS spectral matching capabilities were used to confirm the identifications of skin lightening ingredients in the samples analyzed. The addition of mass detection as a complementary analytical detection technique enhances confidence in compound detection and identification.

Finally, the described methodology could be used in the routine analysis of cosmetics to screen for skin lightening agents to ensure that they meet regulatory and safety standards.

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Sample Profiling of Pesticide Formulations Using UV and MS Detection for Component Identification

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APPLICATION BENEFITS

- Improved sensitivity over UV for low-level components present in the pesticide formulation
- Increased confidence in peak identification when mass detection is used with PDA detection
- Structural similarities between the active ingredient and the low-level components identified in a single analysis

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY® QDa Detector

ACQUITY UPLC Photodiode Array (PDA) Detector

Empower® 3 CDS Software

KEY WORDS

Chiral pesticides, diastereomer, pesticide formulation, impurity identification, sample profiling, propiconazole, triazole fungicides

INTRODUCTION

Agricultural chemicals decrease crop damage, resulting in a food supply that is both plentiful and of high quality.[1] For the agricultural chemicals industry, the analytical quality control of pesticide products ensures that a consistent and effective product reaches the farm.[2] More specifically, the detection, characterization, and quantitation of the active ingredient(s) as well as all other components in the formulation, including impurities and degradation products, is necessary to support product development, quality control, and product registration. The addition of a mass detector in conjunction with UV detection can increase the specificity and selectivity of methods used during analytical testing to provide additional information about a sample during a single analysis.

Waters® ACQUITY QDa Detector is a novel mass detector that can be integrated into existing liquid chromatography configurations in order to complement the results obtained by UV detectors and increase the detection selectivity. The enhanced selectivity is ideal for the detection and initial identification of low-concentration components present in samples. When MS data is combined with the UV response, it allows the analyst to determine a wider range of analytes in one analytical run with an increased level of confidence.

In this application note, optical and mass detection were combined to provide a thorough profile of a commercially available pesticide formulation concentrate. The formulated product contained the triazole fungicide propiconazole, which contains two chiral centers in its chemical structure, as shown in Figure 1. The triazole fungicides are a commonly used group of pesticides due to their potent activity against a broad spectrum of crop diseases.[3]

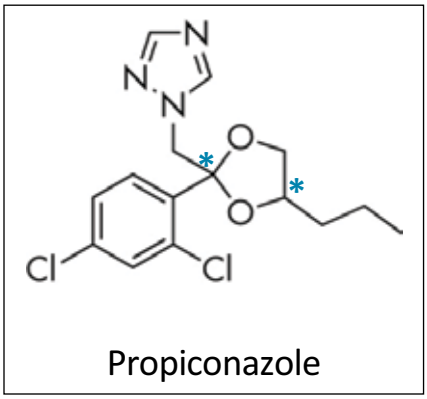

Figure 1. Structure of propiconazole. The asterisks denote the stereogenic centers.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 150 mm, 1.7 µm
Column temp.:	50 °C
Injection volume:	3 µL
Flow rate:	0.60 mL/min
Mobile phase A:	10 mM ammonium formate in water
Mobile phase B:	Acetonitrile

Gradient table:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.60	70	30	6
10.0	0.60	30	70	6
11.0	0.60	10	90	6
12.0	0.60	10	90	6
12.1	0.60	70	30	1

Table 1. UPLC gradient method for analysis of the formulation.

MS conditions

MS system:	ACQUITY QDa Detector
Ionization mode:	ESI+
Capillary voltage:	0.8 kV
Desolvation temp.:	500 °C
Source temp.:	150 °C
Cone voltage:	7 V
Sampling rate:	5 Hz
MS scan range:	100 to 1000 *m/z*

PDA conditions

Detector:	ACQUITY UPLC PDA
Wavelength range:	210 to 400 nm
Sampling rate:	20 Hz

Empower 3 Software Feature Release 2 was used for chromatographic data processing.

Sample preparation

1 gram (g) of the commercially available pesticide formulation was weighed, 9 mL of 50:50 (v/v) acetonitrile/water was added. The resulting mixture was sonicated for 10 minutes, and the sample was syringe filtered into an autosampler vial using a 0.2-µm PVDF filter in preparation for sample analysis. Authentic propiconazole standard was made up in 50:50 (v/v) acetonitrile/water.

RESULTS AND DISCUSSION

A UPLC® UV chromatogram at 220 nm comparing the standard propiconazole and the propiconazole present in the formulation is shown in Figure 2. The ACQUITY UPLC H-Class System's separation of propiconazole resulted in two peaks at retention times (t_R) of 7.45 min (peak 3) and 7.54 min (peak 4). The observed peaks likely originated from the propiconazole diastereomers. The t_R's of the propiconazole standard match those in the formulation. Two minor components (peak 1 and peak 2) at t_R 6.23 min and t_R 6.43 min, respectively, were noted in the UV chromatogram of the formulation. When measured in the UV, the area% contributions from peak 1 and peak 2 were 1.20% and 0.80%, respectively.

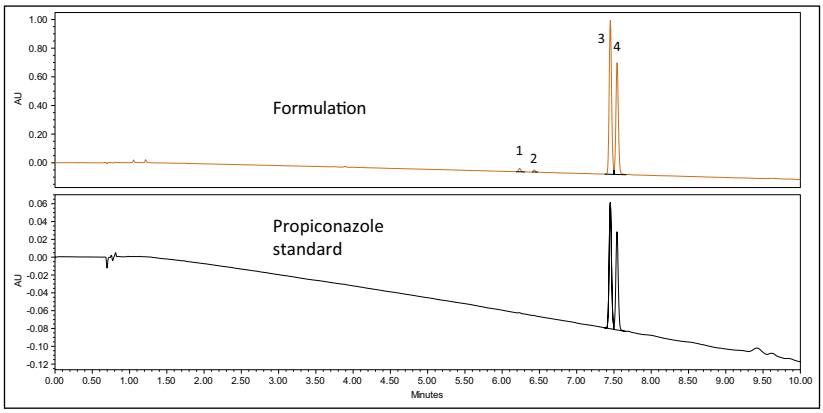

Figure 2. The ACQUITY UPLC PDA Detector's UV chromatogram of the propiconazole present in the formulation and propiconazole standard at 220 nm.

The total ion chromatogram (TIC) from the ACQUITY QDa Detector that was acquired simultaneously with the UV detector is shown in Figure 3. A protonated molecular ion, $[M+H]^+$ that corresponds to a mass-to-charge (*m/z*) ratio of 342 was observed for the propiconazole diastereomers.

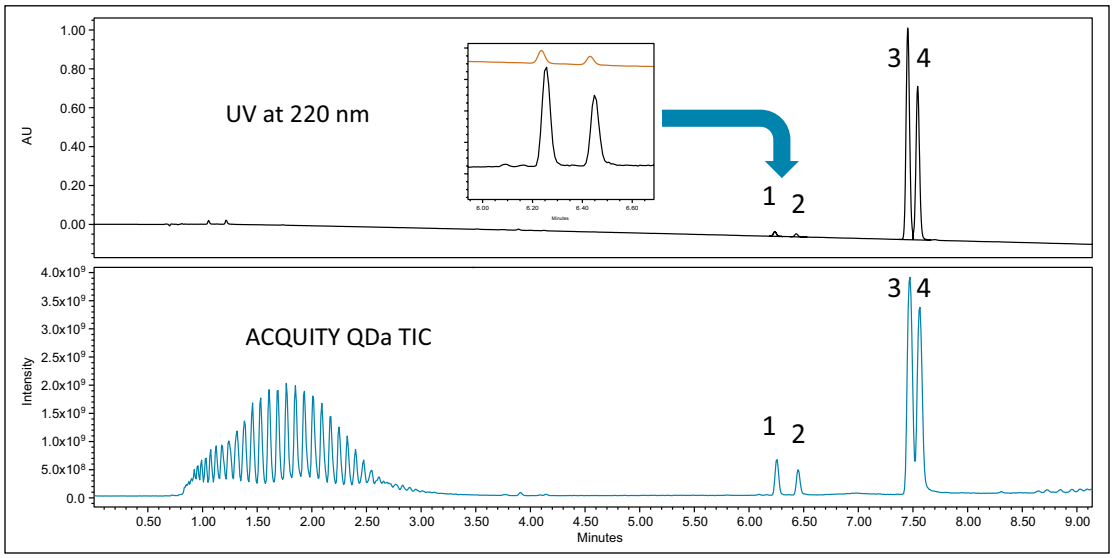

Figure 3. The ACQUITY UPLC PDA Detector's UV chromatogram of the formulation at 220 nm with the ACQUITY QDa Detector's mass chromatogram.

The signal response of all components improved in the mass chromatogram when compared with the UV chromatogram, illustrating the improved likelihood of detecting low-level components using the mass detector (inset Figure 3). The detection limits can be further improved by extracting ions of interest from the TIC to give an extracted ion chromatogram (XIC), shown in Figure 4. This enhances the confidence in compound identification. Other formulation components that could not be seen in the UV were clearly observed in the mass chromatogram, demonstrating that mass spectrometry combined with UV detection can give a more comprehensive sample profile. A series of peaks eluting between 0.80 and 3.0 min, with the *m/z* increasing by 44 amu with respect to the elution order, was observed in the mass chromatogram. The masses are consistent with a polymeric component, which is likely a surfactant present in the formulation that is used to aid in the application of the active ingredient.

Empower 3 Software's mass analysis window provides a single location to associate chromatographic peaks from all detectors used in the analysis with their corresponding spectra. The spectra from the detected peaks are time-aligned and displayed in a window above the chromatograms. The mass analysis window is shown in Figure 4.

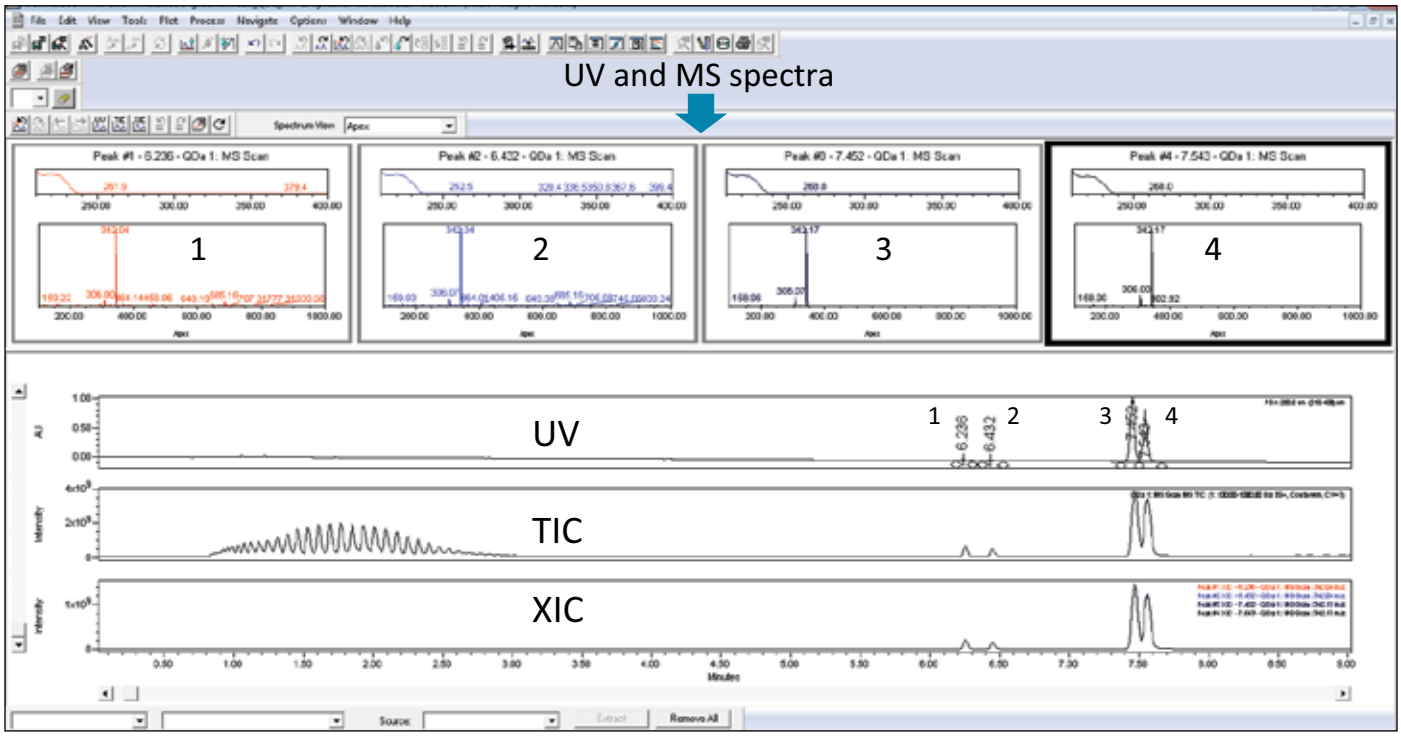

Figure 4. Empower Software's mass analysis window. UV and MS spectra, along with UV and mass chromatograms and extracted ion chromatograms (XIC), can be viewed in a single window.

Interrogation of the data in the mass analysis window indicated relationships between peak 1 and peak 2 with the active ingredient, propiconazole. The UV spectra showed the same maxima at 220 nm with an apparent shift noted in the second absorbance maxima of both peak 1 and peak 2, when compared to the spectra of the propiconazole. The mass spectra show that peak 1 and peak 2 have an *m/z* of 342 which is the same as the active ingredient. In addition, the isotopic pattern is typical of a dichlorinated compound and is identical to that of propiconazole. In a single analytical run, the unknown components were identified as having the same mass and isotopic pattern as the active ingredient. The presence of the mass detector provided additional structural information and increased the confidence in the detection and identification of the compound. The UV and mass spectra for peak 1 and peak 2 and the propiconazole diastereomers are shown in Figure 5.

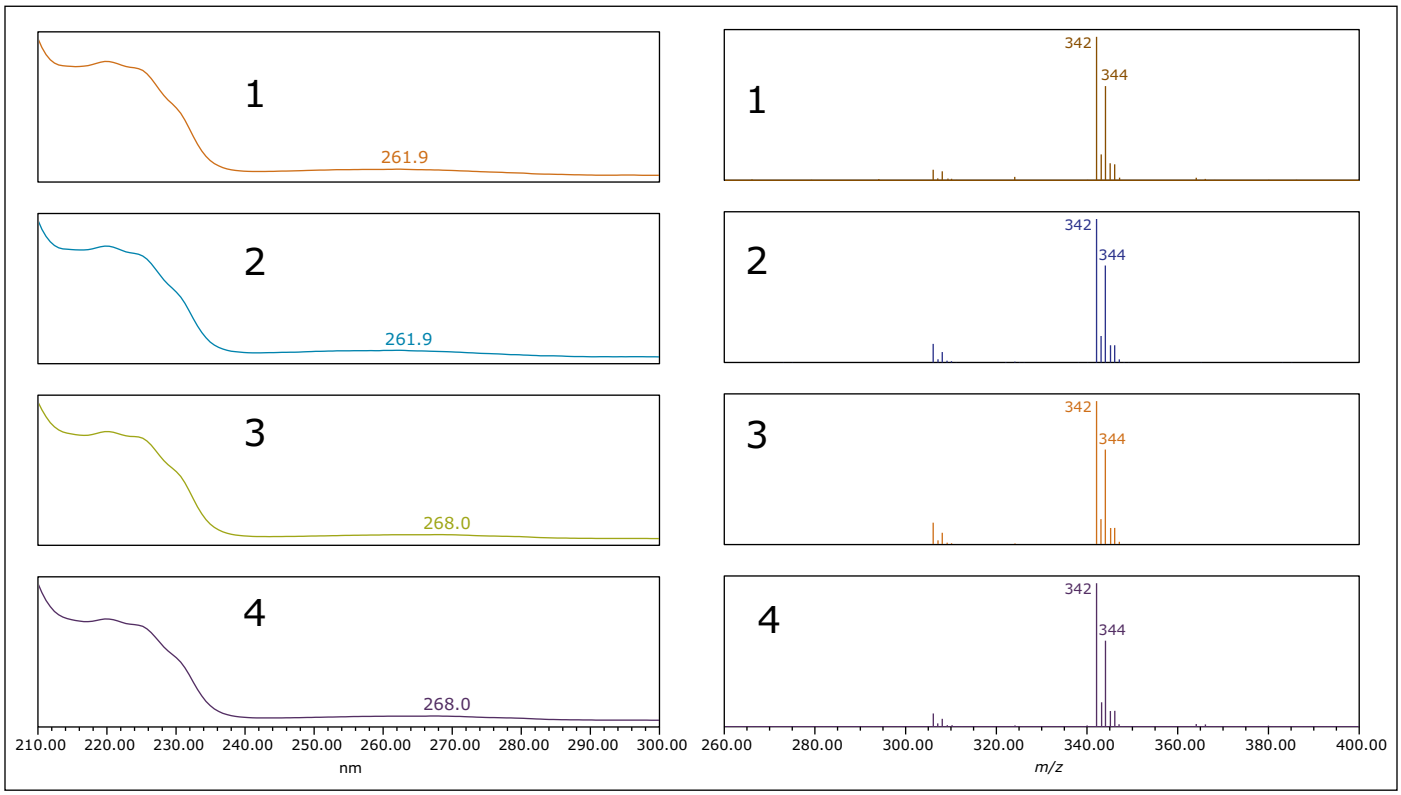

Figure 5. UV and mass spectra for peaks 1 and 2, and the propiconazole diastereomers, peaks 3 and 4, in the formulation.

CONCLUSIONS

The ACQUITY QDa Detector, in combination with PDA detection, allows for low-level components to be detected with increased confidence in pesticide formulations. The components were identified as having similar optical and structural properties to propiconazole, the active ingredient present in the formulation. Inert formulation components not seen in the UV were readily detected by the ACQUITY QDa Detector.

The Empower mass analysis window provides a single place to associate the chromatograms and spectra from all detectors used in the analysis. The consolidation of this information in one place makes data review and interpretation easy.

The addition of mass detection as a complementary analytical detection technique enhances confidence in compound detection and identification. Using the familiarity of a PDA detector, the ACQUITY QDa Detector provides a cost-effective means to make mass detection part of the routine analysis in laboratories that have previously relied on less selective detectors.

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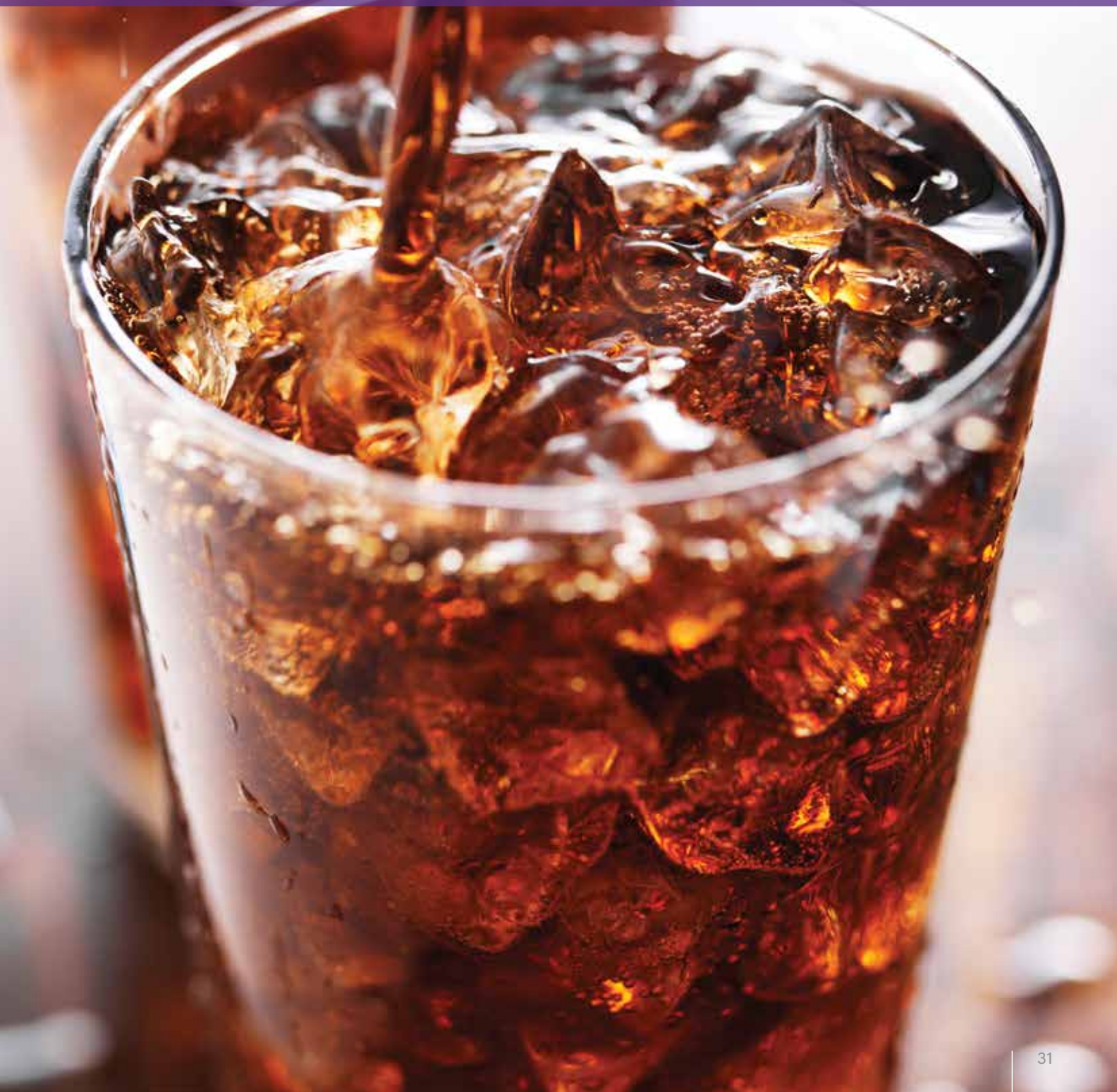

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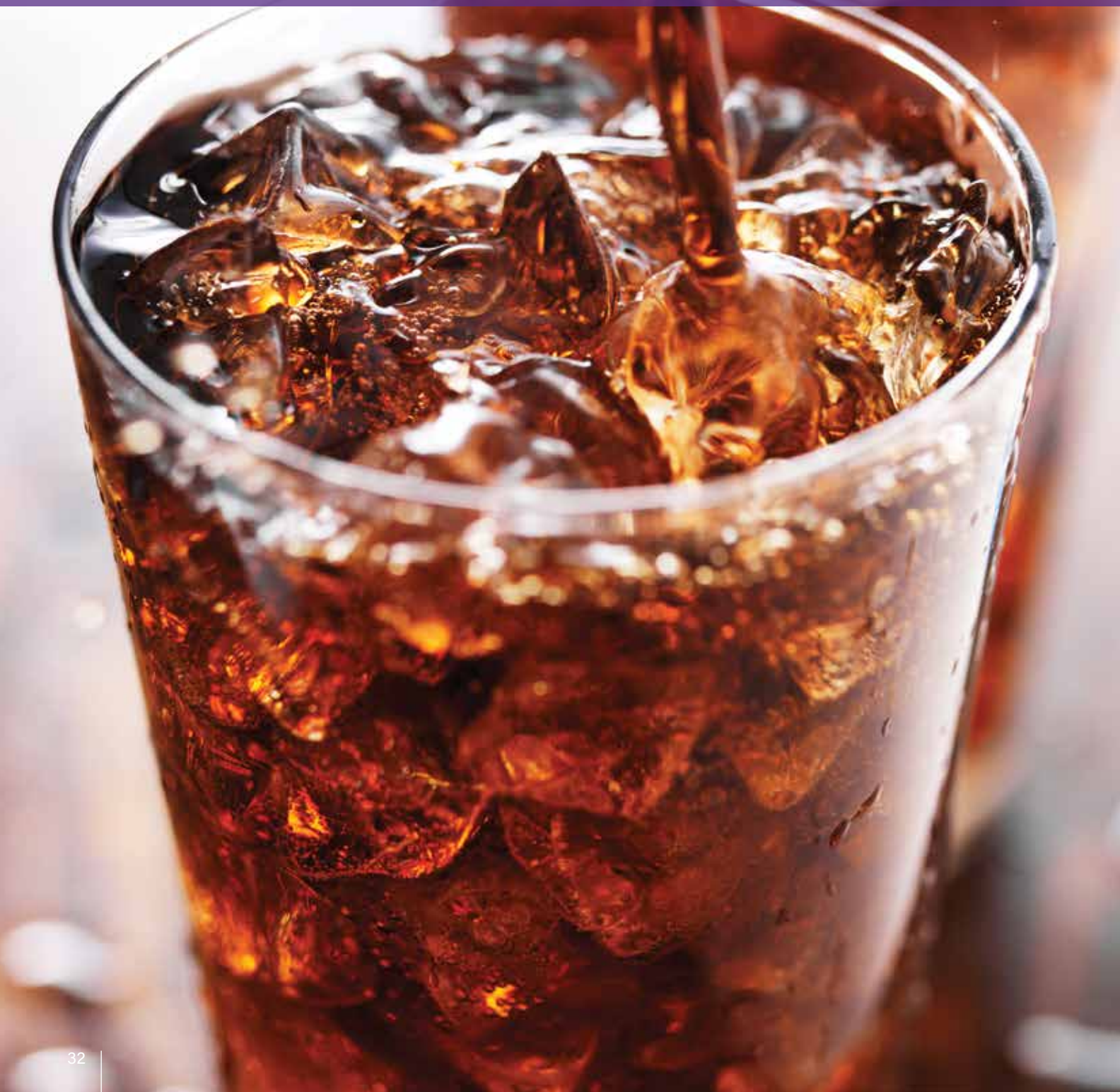

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Soft Drink Analysis on an ACQUITY Arc System

Mark Benvenuti and Jennifer Burgess
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Soft Drink analysis using the ACQUITY® Arc™ System, along with Waters® Beverage Mobile Phase, XBridge® Phenyl Column Chemistry, and a choice of Waters Beverage Standards, or Waters Big-4 Standard concentrate provides multiple benefits such as:

- Ethanol-based, ecologically friendly mobile phase.
- Choice of a single-point calibration suitable for lower levels of analytes common to most soft drinks, or a multi-point calibration especially suited to the higher levels of caffeine found in energy drinks when saccharin and aspartame are not required.
- Dual wavelength monitoring to avoid known co-eluting compounds.
- Use of 2.5 micron particle size columns at system pressures up to 9500 psi.
- Dual flow paths to emulate HPLC or UHPLC separations.

WATERS SOLUTIONS

ACQUITY Arc System

2489 UV/Visible (UV/Vis) Detector

ACQUITY QDa® Detector

XBridge Phenyl XP Column

Beverage Mobile Phase

Beverage Analysis Standard

Big-4 Calibration Stock Standard

KEY WORDS

soft drink, LC, non-nutritive sweetener, acesulfame K, saccharin, caffeine, aspartame, benzoate, sorbate, beverage

INTRODUCTION

The soft drink market is an important revenue source for many major food and beverage producers. Such beverages include traditional carbonated soft drinks, high energy drinks, and recently popular healthy formulations such as vitamin waters and teas.

These products often contain caffeine as a stimulant, benzoate, and sorbate as preservatives; and for diet preparations, non-nutritive sweeteners such as acesulfame K, aspartame, and saccharin.

For quality control purposes the conformance of target concentrations of analytes to specified ranges is critical. In this application note, we show that the Waters ACQUITY Arc System, XBridge Phenyl XP Columns, along with Waters Beverage Mobile Phase, can separate these compounds in under 10 minutes.

Figure 1. ACQUITY Arc System.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY Arc
Runtime:	10.0 min
Column:	XBridge Phenyl ***XP*** 2.5 µm, 4.6 x 50 mm
Column temp.:	35 °C
Mobile phase:	Waters Beverage Mobile Phase
Flow rate:	1.0 mL/min
Flow path:	1 (HPLC emulation)
Injection volume:	5 µL
Detector:	2489 UV/Visible (UV/Vis)
Detection:	214, 247 nm

Standard preparation

A: Beverage analysis standard (single point)

One bottle of Waters Beverage Analysis 5 Standards Solution, p/n 186006008, was poured into one bottle of Waters Beverage Analysis Standards Solid (aspartame), p/n 186006010, and mixed until all of the aspartame was dissolved. This resulted in a standard with a concentration of 150 mg/L acesulfame K, 100 mg/L saccharin, 200 mg/L benzoate, 100 mg/L sorbate, 100 mg/L caffeine, and 500 mg/L aspartame.

B: Standard concentrate (multi-point)

Waters Big-4 Calibration Stock Standard, p/n 186007980, (1000 mg/L each of acesulfame K, benzoate, sorbate, and caffeine) was diluted in water to produce eight separate levels with concentrations listed in Table 1.

Std	Big 4 analytes (mg/L)
1	500
2	400
3	200
4	100
5	50
6	40
7	20
8	10

Table 1. Standard amounts for the Big-4 analytes: acesulfame K, benzoate, sorbate, and caffeine.

Sample preparation

Carbonated beverages were sonicated to remove carbon dioxide. All beverages were filtered through a 0.2-µm PVDF filter, then injected.

RESULTS AND DISCUSSION

Chromatograms resulting from separation of beverage standards using the ACQUITY Arc System with an XBridge Phenyl ***XP*** Column are shown in Figure 2. Depending on the requirements of the analysis, there are two different options for the standards. The first standard, shown in Figure 2A, is the Waters Beverage Standard. This standard contains (in order of elution) acesulfame K, saccharin, benzoate, sorbate, caffeine, and aspartame. Each component has a different UV absorbance spectrum so that the peak height ratios are different as a function of the wavelength. As can be seen from the chromatogram in Figure 2A, two different wavelengths can be programmed and monitored in the same run. In Figure 2A and 2B chromatograms for both 214 nm and 247 nm are shown. Figure 2B shows the chromatograms for the Big-4 Standard, which has the same analytes as the Waters Beverage Standard, with the exception of saccharin and aspartame. These two sweeteners are not required for the analysis of beverages that do not use these ingredients.

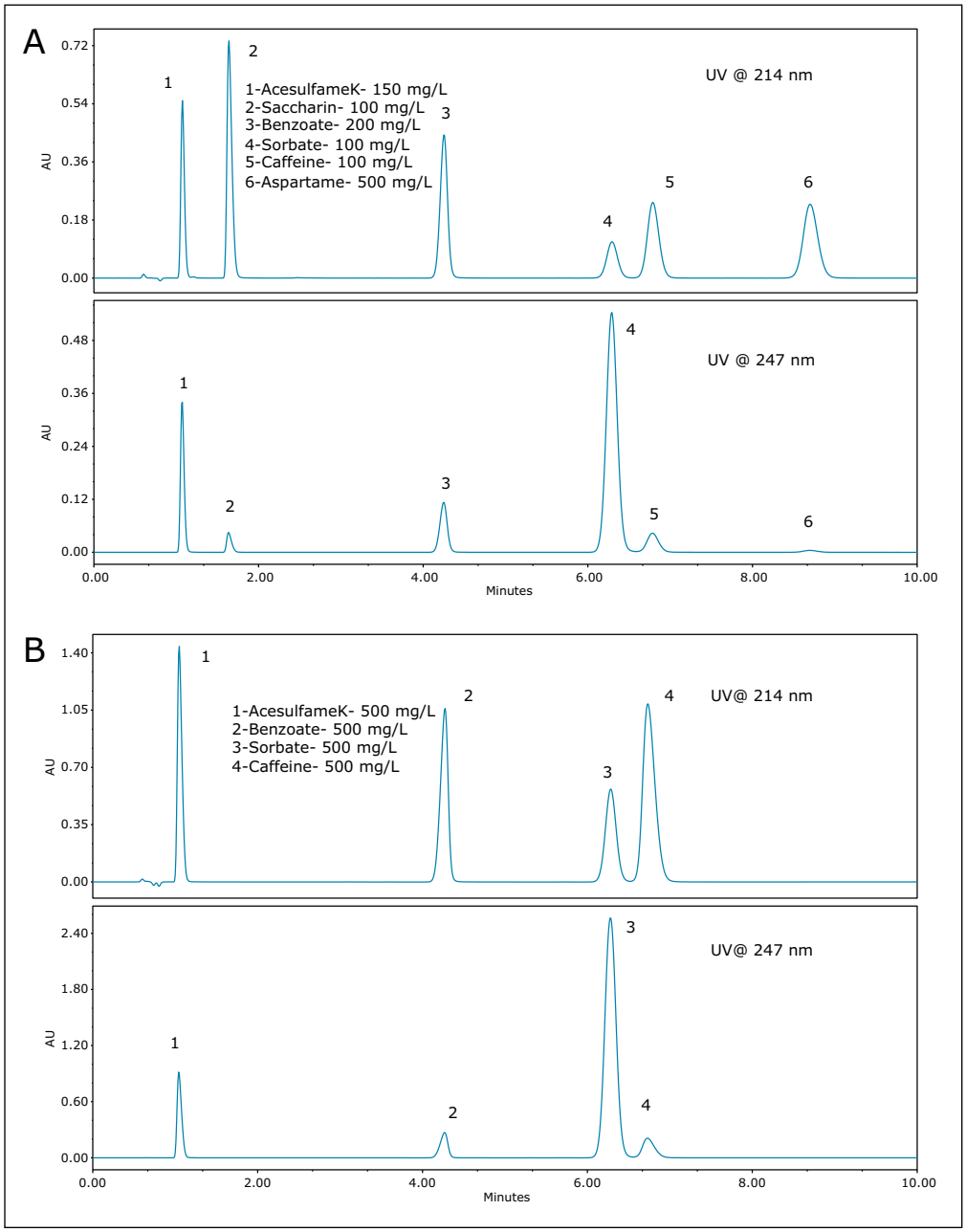

Figure 2A. Beverage Standard.
Figure 2B. Waters Big-4 Beverage Standard.

In order to assess the method with the sample types that are typically encountered, five different beverages were purchased from a local store for analysis. The resulting chromatograms from the analysis of these beverages are shown in Figures 3 to 7. The more traditional-style carbonated beverages are represented by two examples, a diet cola (Figure 3), and a lemon-lime soft drink (Figure 4). For each sample, the amounts of each analyte were calculated using both wavelengths for comparison. In Figures 3 and 4, the calculated amounts using the two different wavelengths agree within 4% for all of the analytes. A single point calibration was used to calculate the amounts shown in Figures 3 to 7.

Newer formulations of soft drinks often include ingredients or additives that have known or perceived health benefits. Examples include essential nutrients, antioxidants, and plant extracts. Figures 5, 6, and 7 show three different examples. Figure 5 displays the chromatograms for a diet vitamin water, and Figure 6 for a diet energy drink. For both of these samples, the quantification at both wavelengths is in agreement (within 2.1%) with the exception of acesulfame K. The diet lemon tea showed excellent agreement at both wavelengths (within 3.4%), even for acesulfame K.

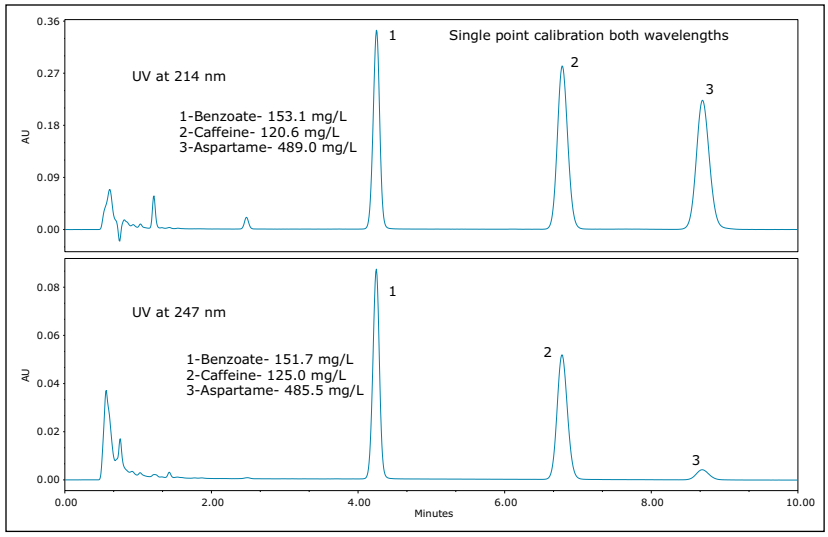

Figure 3. Diet cola chromatograms.

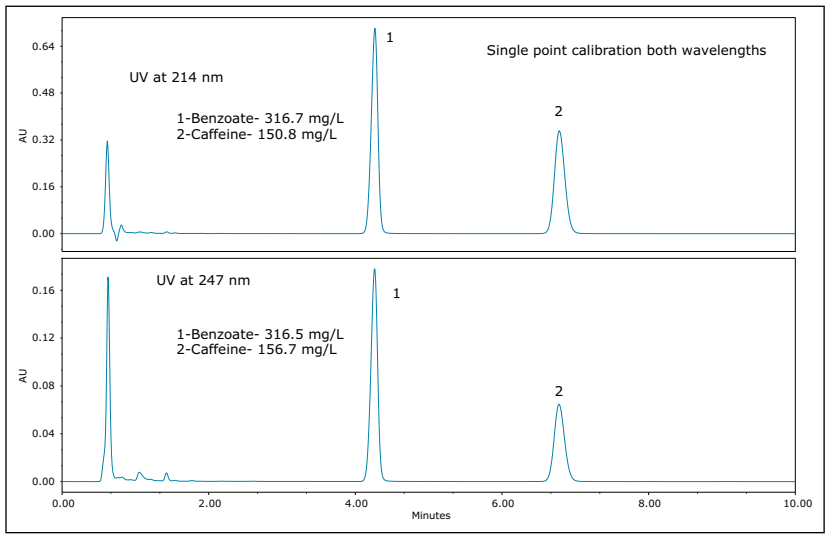

Figure 4. Lemon-lime soft drink chromatograms.

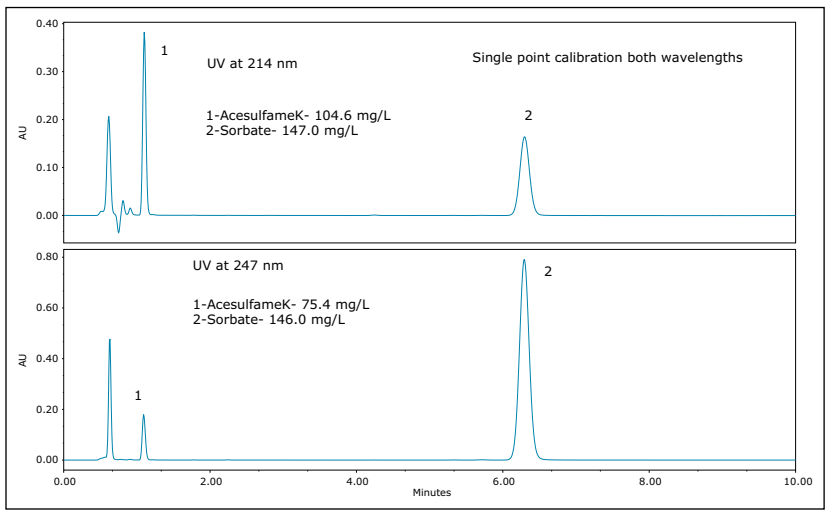

Figure 5. Diet vitamin water chromatograms.

Further investigation of the difference in quantification for acesulfame K in the diet vitamin water and diet energy drink revealed that both of these beverages contain B vitamins, where the other soft drinks did not. Using the ACQUITY QDa Detector, which enables mass analysis of the analytes, it was apparent that the B vitamins closely elute with acesulfame K. Figure 8 shows an overlay of the vitamin water chromatograms of acesulfame K at 214 nm and the individual mass-to-charge ratios of the B vitamins acquired using the ACQUITY QDa Detector. Note that vitamin B6 coelutes with acesulfame K.

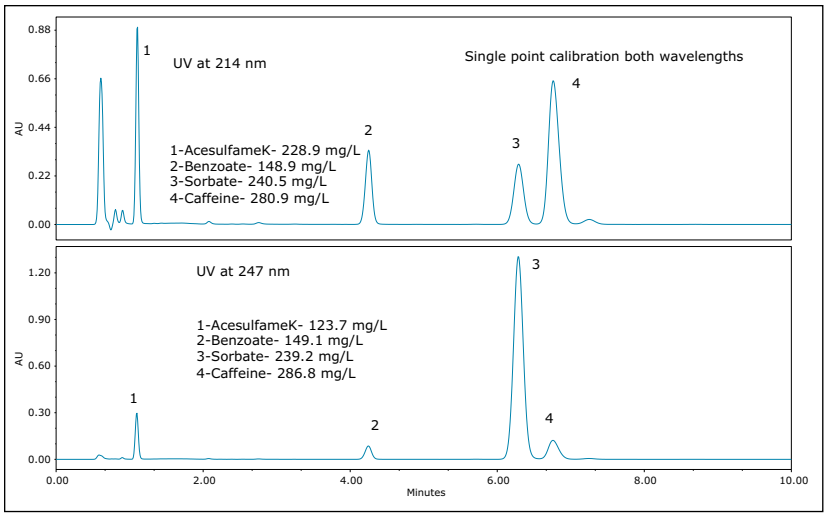

Figure 6. Diet energy drink chromatograms.

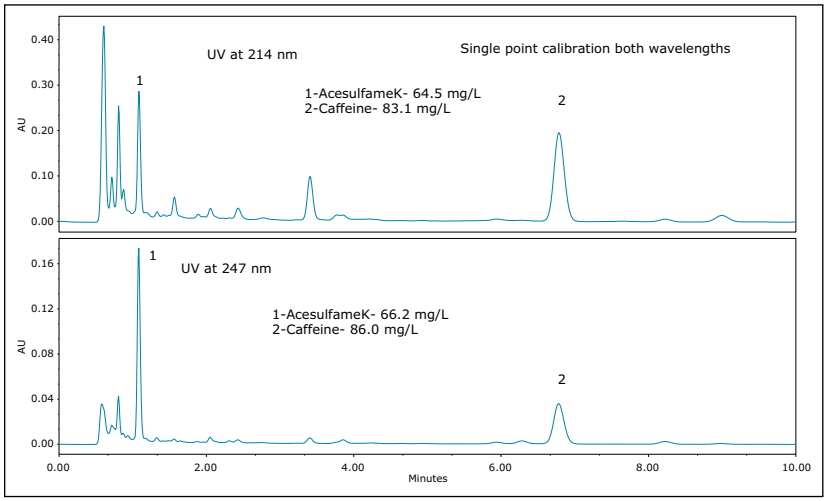

Figure 7. Diet lemon tea chromatograms.

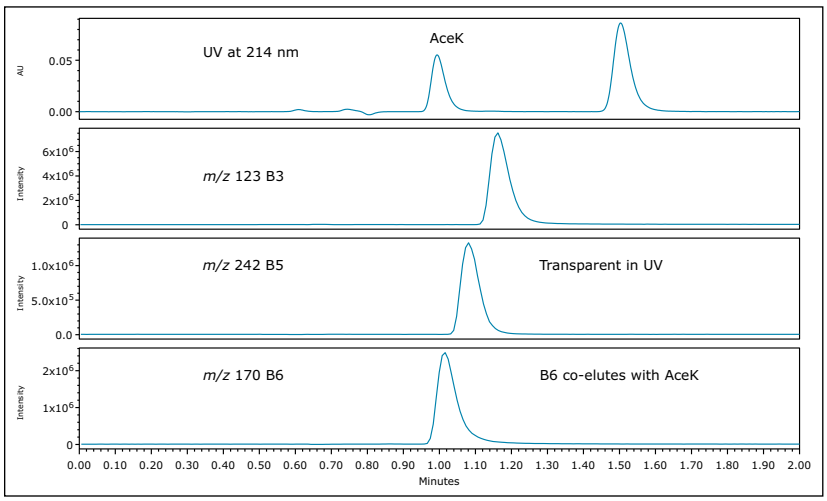

Figure 8. Overlay of UV chromatogram of Acesulfame K with SIR channels of B vitamins.

The UV spectra of vitamin B6 and acesulfame K are shown in Figures 9A and 9B, respectively. At 214 nm, which is non-specific, vitamin B6 has high absorbance and contributes to the overall response at 214 nm, along with acesulfame K. This results in over-estimation of the amount when using 214 nm. As can be seen, vitamin B6 is almost transparent at 247 nm, making this the wavelength of choice for beverages containing acesulfame K and vitamin B6.

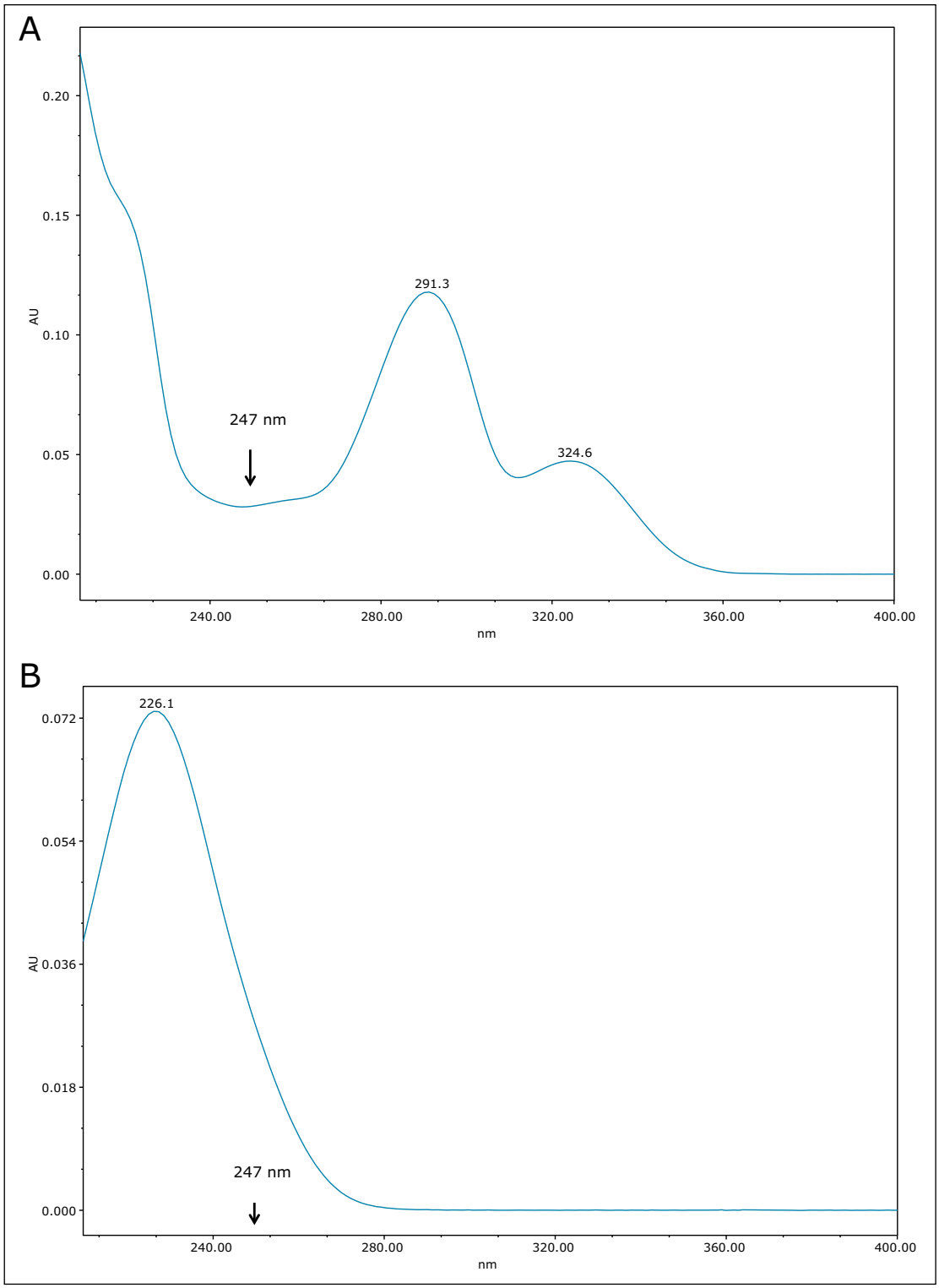

Figure 9A. UV spectrum of vitamin B6. Figure 9B. UV spectrum of acesulfame K.

The calibration curves created using the multi-point calibration from the Waters Big-4 Standard are shown in Figure 10. R^2 values were >0.999 for all compounds. Table 2 lists the retention time reproducibility for six injections of the Waters Beverage Analysis Standard. The %RSD was <0.2% for all analytes. Table 3 lists the reproducibility data for retention time and amounts for seven injections of the lemon-lime soft drink, which contained benzoate and caffeine. Quantification was made using the single-point calibration. Retention time reproducibility was 0.05% or less and the reproducibility for amount was <0.25%. Table 4 compares the quantification of the energy drink using both the single- and multi-point calibration at 247 nm to eliminate B vitamin interference.

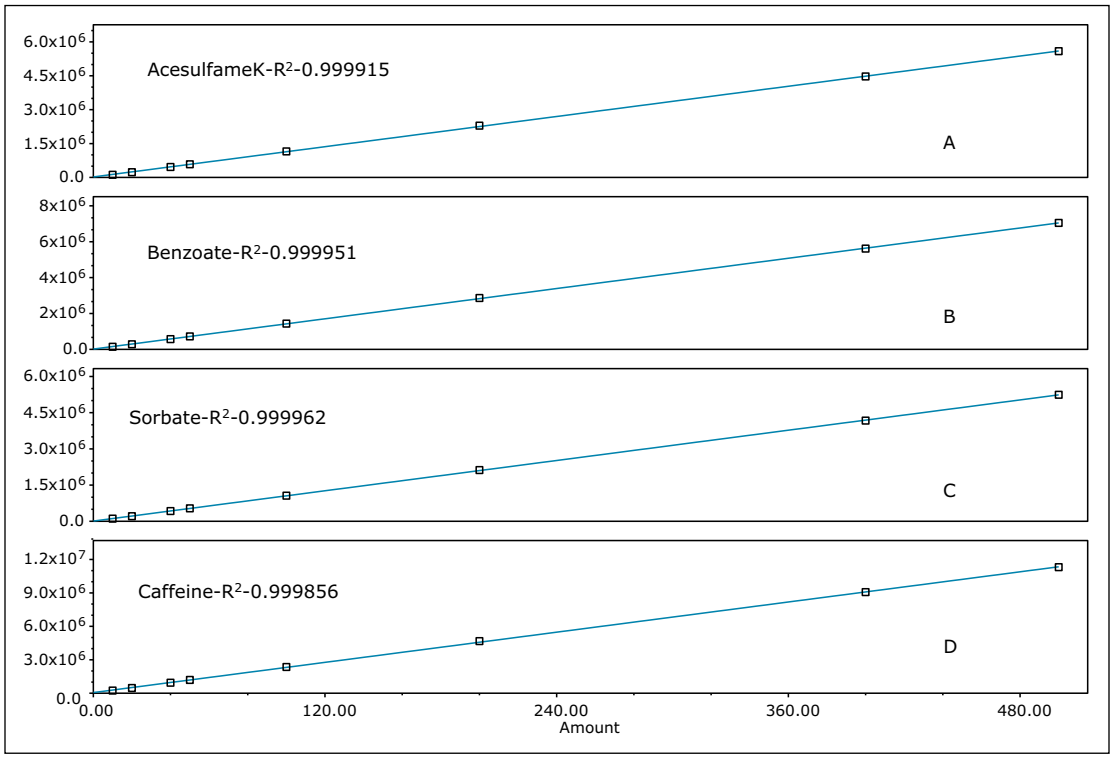

Figure 10. A–D: calibration curves of Big-4 analytes.

Analyte	RT (min)	%RSD
Acesulfame K	1.08	0.16
Saccharin	1.64	0.10
Benzoate	4.25	0.04
Sorbate	6.30	0.03
Caffeine	6.79	0.03
Aspartame	8.70	0.02

Table 2. Retention time reproducibility for six injections of Waters Beverage Standard.

Analyte	RT	%RSD	Amount	%RSD
Benzoate	4.27	0.05	316.64	0.17
Caffeine	6.78	0.04	150.80	0.21

Table 3. Retention time and reproducibility amount for seven injections of the lemon-lime soft drink.

Analyte	Single Point at 247nm	Multi Point at 247 nm	%RSD
AcesulfameK	123.7	123.9	0.24
Benzoate	149.1	148.9	0.13
Sorbate	239.2	245.1	1.24
Caffeine	286.8	288.6	0.33

Table 4. Comparison of the quantitation of the diet energy Drink (mg/L) using single and multi-point calibration at 247 nm.

CONCLUSIONS

Soft drink analysis using the ACQUITY Arc System provides a simple method for the analysis of soft drink additives. Implementation of such a procedure in a manufacturing environment has the capacity to improve overall workplace efficiency as well as:

- The choice of a single- or multi-point calibration, depending on the analytes and analyte levels required.
- Ethanol based mobile phase allows for simple, cost-effective disposal.
- Simple sample preparation requiring only sonication and filtering.
- Choice of UV wavelengths to remove vitamin B6 interference from acesulfame K quantification.

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Profiling and Quantification of Mono and Disaccharides and Selected Alditols in Juice, Beer, Wine, and Whiskey Using the ACQUITY Arc System with Mass Detection

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Detection of sugars at lower levels than with Refractive Index (RI) or Evaporative Light Scattering Detection (ELS).
- Minimum sample preparation required and samples can be diluted to reduce matrix effects.
- Chromatographic separation of the difficult isomer pairs sorbitol and mannitol.

WATERS SOLUTIONS

ACQUITY® Arc™ System

ACQUITY QDa® Detector

XBridge® BEH Amide XP Column

Empower® 3 CDS Software

KEY WORDS

Saccharides, sugars, fructose, glucose, sucrose, maltose, mannitol, sorbitol, whisky, carbohydrate

INTRODUCTION

Sugars and sugar alcohols are classes of carbohydrates that are important in human nutrition and natural constituents of foods. With the increasing incidence of obesity and diabetes across the developed world, interest in monitoring sugar intake has vastly increased in recent years. Consequently, there are now requirements to provide accurate information on product labeling in order to comply with increasingly stringent regulatory demands. Profiling the sugar content of products is also a useful tool in assessing product authenticity and potential adulteration.

The analysis of sugars and sugar alcohols remains a challenging application, owing to the lack of chromophores and the similarity between these molecules. Many of these sugar compounds are isomers of one another, as can be seen in Figure 1, which illustrates the formulae and structures of the compounds analyzed in this study. Due to its separation power, accuracy, and speed of analysis, HPLC has become the method of choice for the analysis of sugars. An alternative to RI and ELS detection is the use of mass detection with electrospray ionization (ESI). Mass detection is complementary to traditional detectors used for LC.

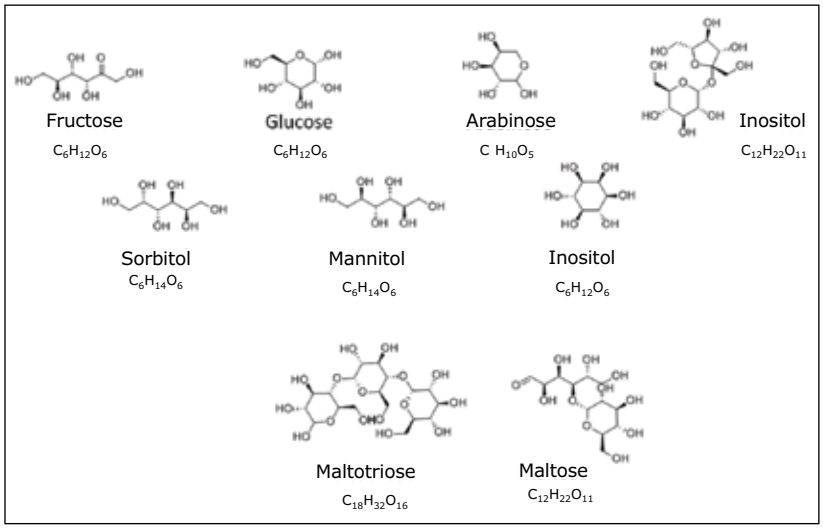

Figure 1. Structures and formulae for the sugar compounds analyzed.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY Arc
Data system:	Empower 3
Runtime:	40.0 min
Column:	XBridge® XP BEH Amide 2.5 µm, 3.0 x 150 mm
Column temp.:	85 °C
Mobile phase A:	90% acetonitrile: 5% IPA:5% water*
Mobile phase B:	80% acetonitrile: 20% water*
Flow rate:	0.8 mL/min
Injection volume:	1 µL

	Time (min)	Flow rate (mL/min)	%A	%B
1.	Initial	0.8	100	0
2.	4.5	0.8	100	0
3.	18.0	0.8	0	100
4.	25.0	0.8	0	100
5.	25.1	0.8	100	0
6.	40.0	0.8	100	0

*Both containing 500 ppb guanidine hydrochloride and 0.05% diethylamine.

MS conditions

MS system:	ACQUITY QDa (Performance mode)
Ionization mode:	ESI-
Capillary voltage:	0.8 V
Cone voltage:	5.0 V
Probe temp.:	600 °C
Acquisition rate:	2 Hz
Full scan:	50 to 800 Hz
Curve fit:	Quadratic, 1/x weighting
Smoothing:	Mean filter, Level 7

SIR channels:

Analyte	Formula	SIR (*m/z*) ($[M+Cl]^-$ ion)
Arabinose	$C_5H_{10}O_5$	185
Fructose	$C_6H_{12}O_6$	215
Glucose	$C_6H_{12}O_6$	215
Inositol	$C_6H_{12}O_6$	215
Sorbitol	$C_6H_{14}O_6$	217
Mannitol	$C_6H_{14}O_6$	217
Sucrose	$C_{12}H_{22}O_{11}$	377
Maltose	$C_{12}H_{22}O_{11}$	377
Maltotriose	$C_{18}H_{32}O_{16}$	539

Standard preparation

A 100 mg/L stock of the nine saccharides listed above was prepared in 1:1 acetonitrile-water. This stock was further diluted to produce nine individual levels (1, 2, 4, 5, 10, 20, 40, 50, and 100 mg/L).

Sample preparation

All samples were purchased locally. The juice samples assessed included orange, apple, pineapple, pomegranate, and grape. The alcoholic beverages assessed included five beers, three lagers (one non-alcoholic), a lemon flavored beer, one hard cider, one sherry, one red wine, and four whiskeys. The beer samples were sonicated to remove carbonation. All of the samples were filtered through a 0.22 µm PVDF syringe filter and diluted in 1:1 acetonitrile-water. The dilution factors are listed in Table 1.

Sample	Dilution factor
Lager 1 and 2	5
Non-Alcoholic	500
Hard Cider	500
Lemon Flavored Beer	50
Sherry Wine	500
Red Wine	500
Whiskey	2
Orange Juice	1000
Apple Juice	1000
Pineapple Juice	1000
Pomegranate Juice	1000
Grape Juice	2500

Table 1. Dilution factors for the "off-the-shelf" samples studied.

It offers the opportunity to decrease detection limits and also to obtain mass spectral information on the components in the sample. The combination of both chromatographic retention time and mass information results in increased selectivity for the analysis of sugars and sugar alcohols. Here we show the application of the Waters® ACQUITY QDa Detector coupled to the ACQUITY Arc System for the profiling and quantification of sugars in juice, wine, beer, and whiskey samples.

RESULTS AND DISCUSSION

Figure 2 shows the ACQUITY Arc System with the ACQUITY QDa Detector and a PDA Detector. The PDA is shown for reference but was not used in this application. Figure 3 shows the SIR chromatograms for a mixed standard at 100 mg/L for each of the analytes listed above. Excellent separation of all of the standards was achieved. Initially, using isocratic conditions the lower mass saccharides were separated, including the difficult pair sorbitol and mannitol. After 4.5 minutes a gradient was started which allowed timely separation of the larger molecular weight saccharides in the mix.

Figure 2. ACQUITY Arc System with the PDA and ACQUITY QDa detectors.

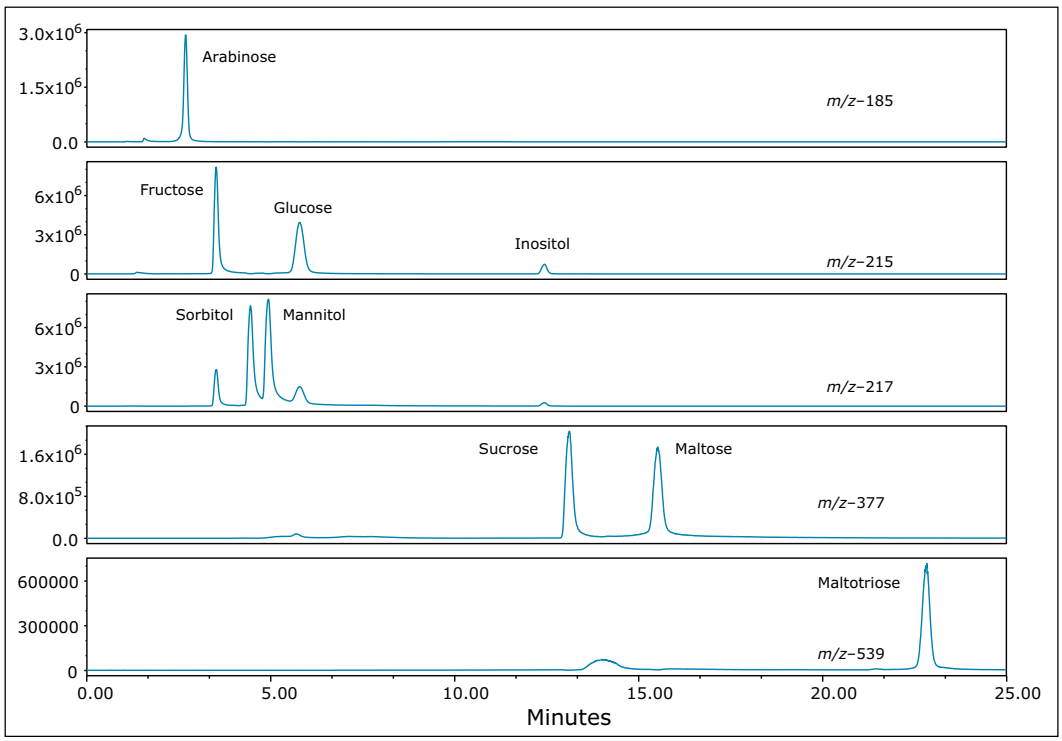

Figure 3. SIR chromatograms of the nine saccharide standards used in the study. The annnotated m/z represents the $[M+Cl]^-$ adducts.

The mass spectra extracted from the SIR of each standard is shown in Figure 4. The use of guanidine chloride in the mobile phase ensured that the compounds were driven to their chloride adduct ($[M+Cl]^-$ ion). The smaller ^{37}Cl adduct response was also present. Figure 5 shows the calibrations curves for the compounds studied. An R^2 value >0.995 was achieved for all of the analytes.

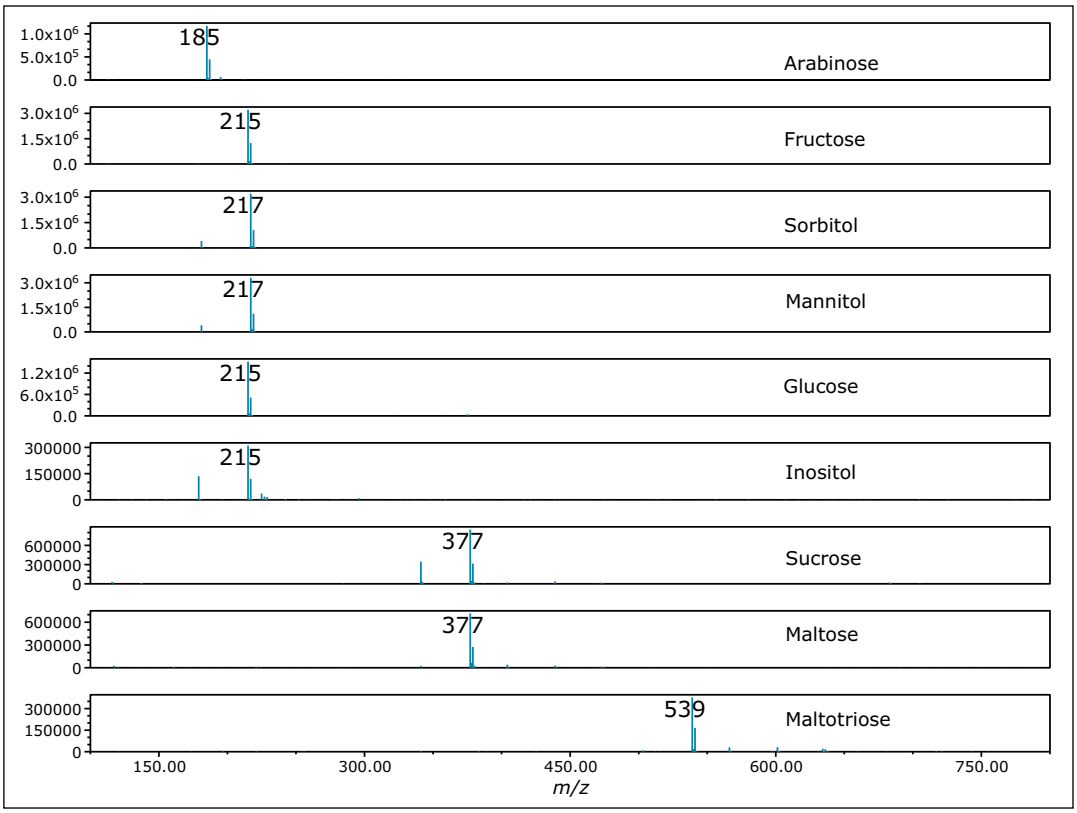

Figure 4. Mass spectral information extracted from SIRs of the nine saccharide standards. The annotated m/z represents the [M+ Cl]- adducts.

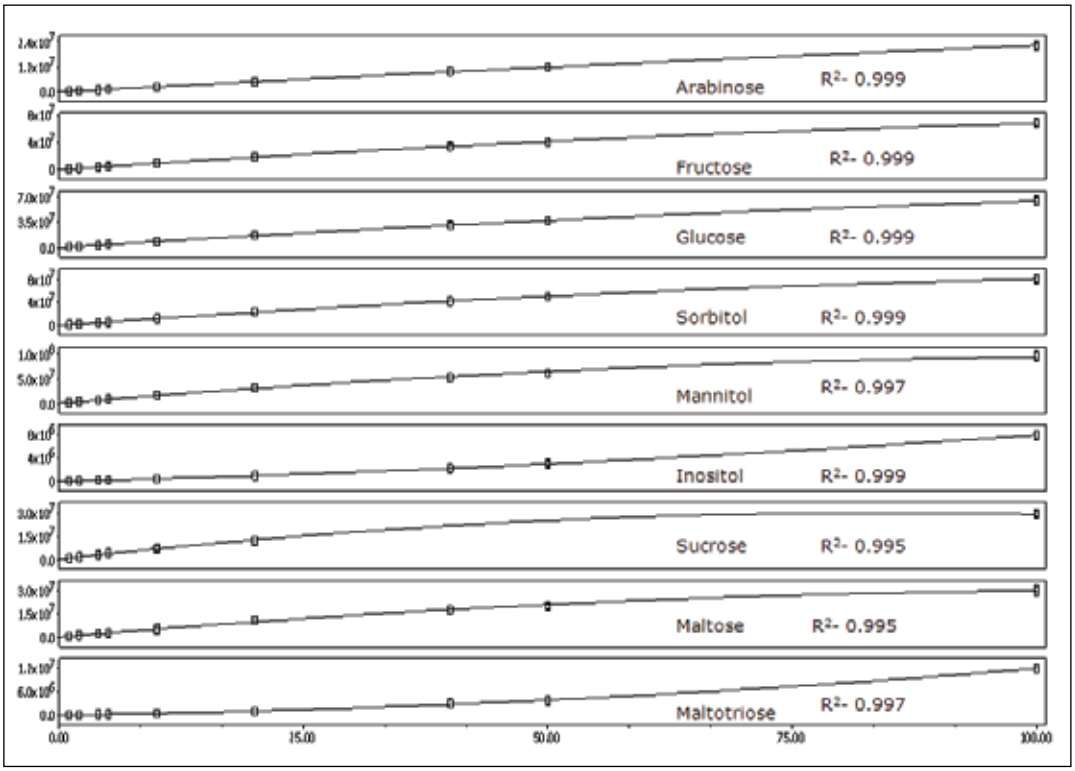

Figure 5. Calibration curves for the nine saccharide standards analyzed showing R^2 values obtained for each analyte.

Figure 6 (A–E) shows the SIR profiles of a lager beer. In Figure 6A (*m/z* 185) arabinose is present. Other peaks are also apparent, suggesting the presence of other pentose saccharides. In Figure 6B (*m/z* 215) traces of fructose and glucose can be seen. The enhanced sensitivity of the ACQUITY QDa allows improved detection of these compounds, as opposed to less sensitive methods such as Refractive Index.[1] In Figure 6C (*m/z* 217) traces of sorbitol and mannitol are present. We also saw small peaks representing the extraction of the Cl^{37} adducts of fructose and glucose, which have the same molecular weight as sorbitol and mannitol. In Figure 5D and 5E (*m/z* 377 and 539 respectively), we observed the DP2 and DP3 compounds maltose and maltotriose, along with isomers of the same mass, which would be expected for a beverage derived from grain.

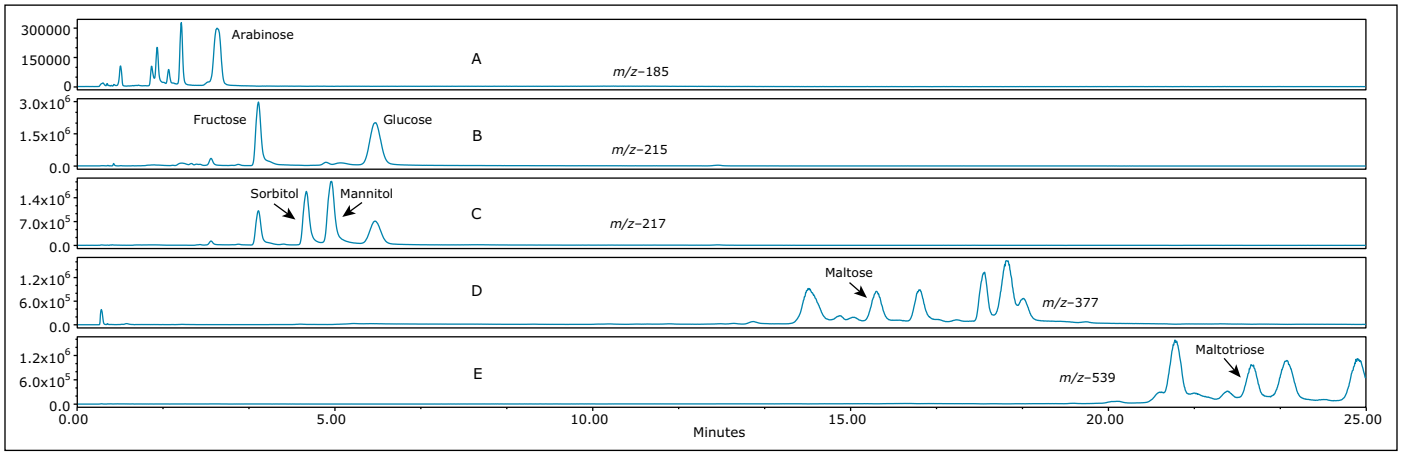

Figure 6. Annotated SIR profile of a lager beer annotated with saccharides found to be present.

A sherry wine profile is shown in Figure 7 (A–E). The main analytes found to be present in sherry are fructose and glucose (Figure 7B). A small amount of arabinose was present (Figure 7A), along with trace levels of sorbitol and mannitol (Figure 7C). Maltose was also apparent (Figure 7D). The DP3 compounds were absent (Figure 7E), as would be expected, since wine is derived from grapes rather than from grains.

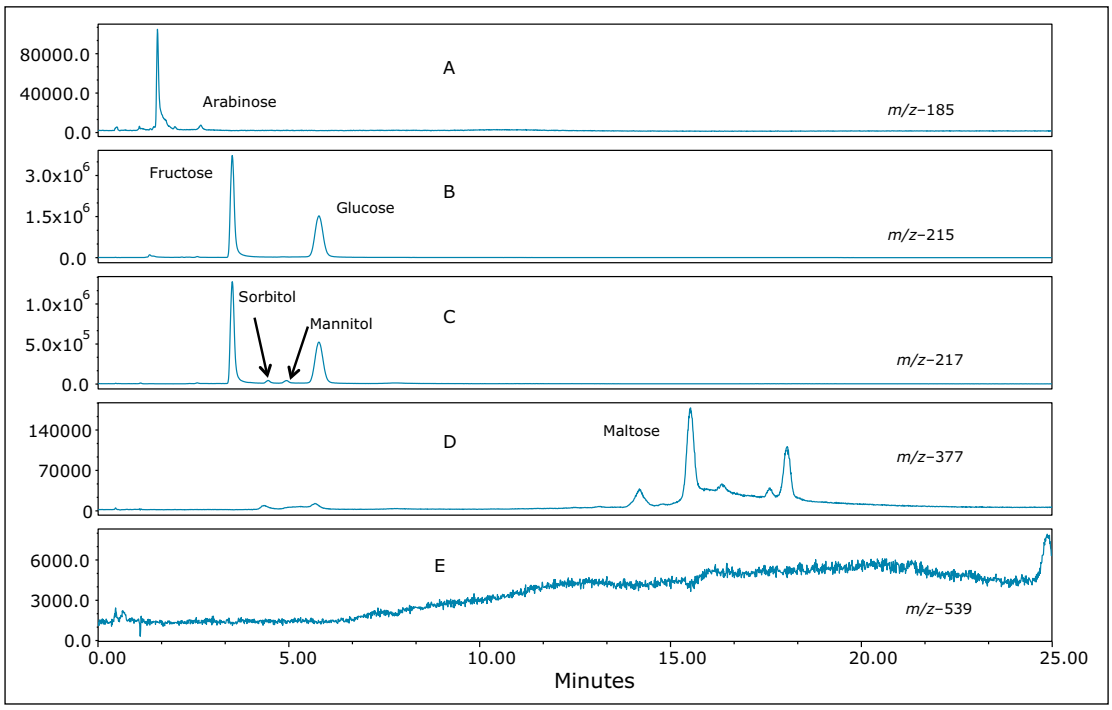

Figure 7. SIR profile of a sherry wine annotated with saccharides found to be present.

Figure 8 (A–E) shows the SIR profile of a whiskey sample. The presence of arabinose (Figure 8A), fructose and glucose (Figure 8B) was evident. Of particular interest was an unknown saccharide apparent in Figures 8B (*m/z* 215) and 8C (*m/z* 217) at retention time 4.85 minutes. Using retention time alone with an RI or ELS detector, this peak would most likely have been misidentified as mannitol. The presence of this peak at both *m/z* 215 and *m/z* 217 indicated that this component has the same mass as a monosaccharide, rather than an alditol. Mannitol does not have an ion at *m/z* 215, as can be seen in Figures 2 and 3.

Finally, the SIR chromatograms from an apple juice sample are shown in Figure 9 (A–E). The presence of arabinose, fructose, glucose, sorbitol, and sucrose are highlighted.

The quantification of various fruit juices is shown in Table 2. Fructose, glucose, and sucrose were present in the orange, apple, and pineapple juices. The amounts and ratios of sugars in these juices are similar to those reported elsewhere.[2,3] Of particular interest was the detection of sorbitol in pomegranate juice. Sorbitol is not usually present in pomegranate juice[4] and its detection could be evidence of adulteration. A second sample tested showed no sorbitol (Figure 10). The grape juice sample showed fructose, glucose, but no sucrose as expected.[2,3]

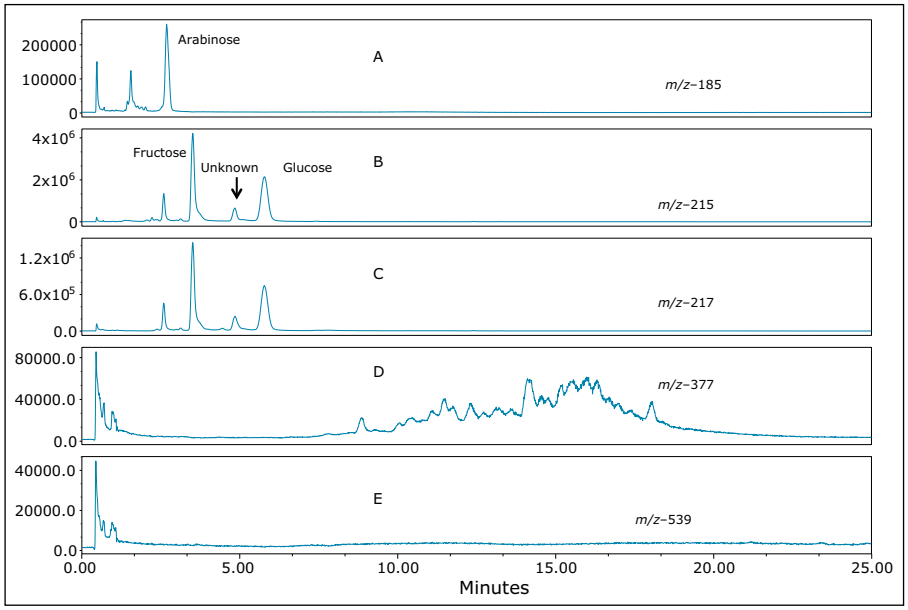

Figure 8. SIR profile of a whiskey annotated with saccharides found, and an unknown saccharide found to be present at m/z 215.

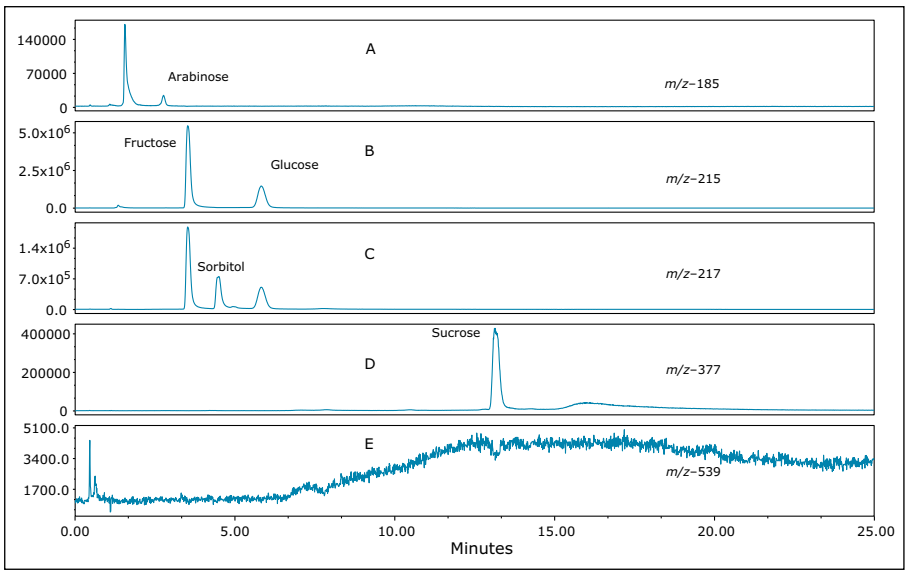

Figure 9. SIR profile of an apple juice sample annotated with saccharides found to be present.

Juice	Fructose	Glucose	Sorbitol	Mannitol	Sucrose
Orange	25.1	23.6	ND	ND	52.6
Apple	70.2	30.2	7.6	ND	10.6
Pineapple	37.4	40.6	ND	ND	41.9
Pomegranate	64.8	71.1	19.7	4.3	0.9
Grape	83.0	82.5	ND	ND	ND

Table 2. Calculated concentrations from the quantification studies of the various fruit juices profiled in this study (g/L).

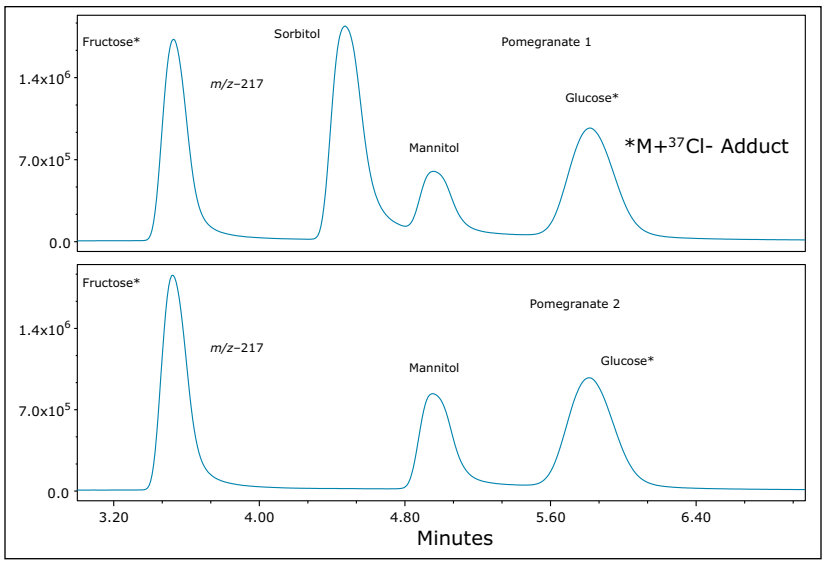

Figure 10. Zoomed-in SIR chromatograms annotated with saccharides found to be present in two pomegranate juice samples. One sample showed the presence of sorbitol, indicating a potential adulteration.

Analyte	RT	% RSD	Amount	%RSD
Fructose	3.54	0.12	25.1	1.33
Glucose	5.84	0.11	23.6	1.82
Sucrose	13.16	0.12	52.6	7.82

Table 3. Reproducibility data for retention time (min) and amount (g/L) based on seven injections of orange juice.

CONCLUSIONS

- The ACQUITY QDa Detector coupled to the ACQUITY Arc System provides improved sensitivity and selectivity to analyze and quantify mono and disaccharide samples in a single injection.
- Mass detection is a viable alternative to Refractive Index (RI) or Evaporative Light Scattering (ELS) methods.
- This enhanced sensitivity allows the analysis of samples at higher dilution levels, which minimizes matrix effects.
- The combination of mass detection and chromatographic separation provides increased selectivity in identifying analytes of interest, while reducing false positives.

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Profiling Mono and Disaccharides in Milk and Infant Formula Using the ACQUITY Arc System and ACQUITY QDa Detector

Mark Benvenuti, Gareth Cleland, and Jennifer Burgess
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- The ACQUITY Arc™ System provides a choice of two flow paths to emulate HPLC or UHPLC separations.
- Chromatographic separation of the difficult isomeric pair, glucose and galactose.
- The ACQUITY® QDa® Detector provides complimentary detection to Refractive Index (RI) or Evaporative Light Scattering (ELS) detectors that are commonly employed for carbohydrate analysis.

WATERS SOLUTIONS

ACQUITY® Arc™ System

ACQUITY QDa®

XBridge® BEH Amide XP Column

KEYWORDS

Mass detection, infant formula, milk, carbohydrate, mono and disaccharide

INTRODUCTION

Sugars and sugar alcohols are classes of carbohydrates that are natural constituents of foods and provide important nutritional benefits. Some sugars are added to processed foods in order to enhance flavor or to mimic fresh food products. With the increasing incidence of obesity and diabetes across the developed world, the need to monitor sugar intake has grown in recent years. Consequently, there are now requirements to provide accurate information about sugar content on food product labels in order to comply with increasingly stringent regulatory demands.

The analysis of these compounds is challenging because they lack chromophores within their compound structures, and because of the close similarity among the various molecules, many of which are simple isomers of one another. Structures and formulae are shown in Figure 1. Due to its separation power, accuracy and speed of analysis, HPLC has become the method of choice for the analysis of sugars.[1] HPLC techniques typically employ RI or ELS detection. RI detection requires careful control of the mobile phase to avoid any changes during the analysis and therefore requires isocratic elution. With RI detection it is also difficult to change the mobile phase composition from one analysis to the next because the RI detector may require several hours to equilibrate when a different mobile phase composition is introduced. ELS detection is more robust for mobile phase composition changes, but ELS often does not meet the sensitivity demands for the detection of sugars in complex matrices.

An alternative gaining traction is the use of mass detection with electrospray ionization (ESI). Waters® ACQUITY QDa Detector offers the opportunity to decrease detection limits as well as the ability to obtain mass spectral information on components in the sample. The combination of chromatographic retention time and mass information can provide improved selectivity for the profiling of sugars and sugar alcohols. The ACQUITY QDa Detector is the only mass detector that has been holistically designed to be incorporated with an LC system. It fits in the LC stack, occupying the same amount of space as a PDA detector. Extensive training is not required, so users already familiar with HPLC can quickly take advantage of the improved selectivity and sensitivity that mass detection affords. In this application note we describe the use of the ACQUITY QDa Detector coupled to the ACQUITY Arc System for the profiling of sugars in milk and infant formula.

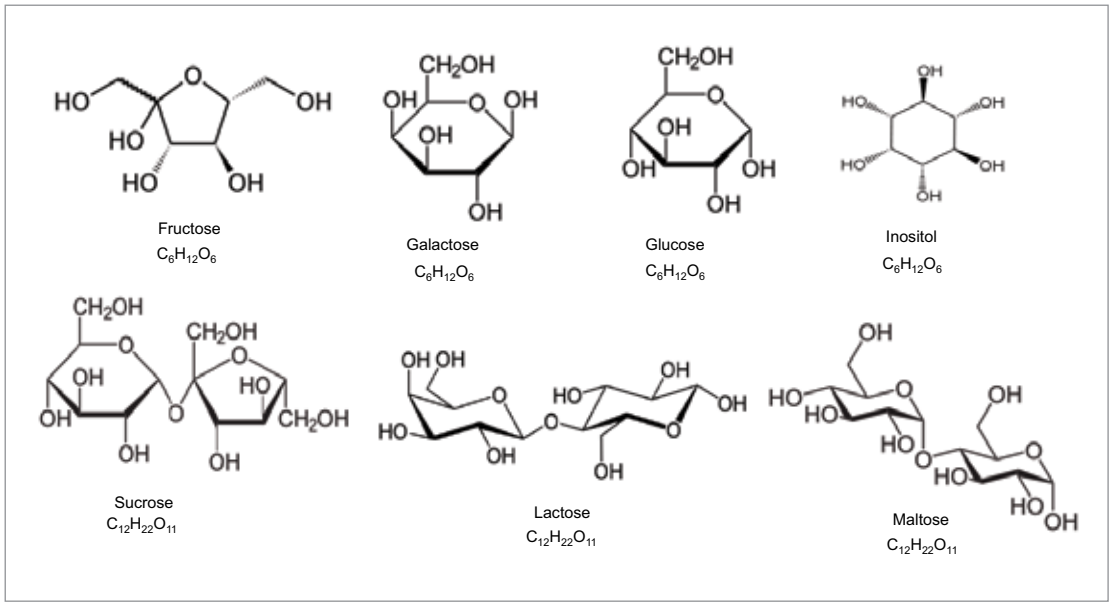

Figure 1. Structures and formulas for the sugar compounds analyzed.

Figure 2. ACQUITY Arc System shown with the PDA and ACQUITY QDa detectors.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY Arc Path 1
Run time:	30.0 min
Column:	XBridge BEH Amide XP 2.5 µm, 3.0 × 150 mm
Column temp.:	85 °C
Mobile phase:	90:5:5 Acetonitrile-water-2-propanol with 0.05% diethylamine and 500 ppb guanidine hydrochloride
Flow rate:	0.8 mL/min
Injection volume:	1 µL

MS conditions

MS system:	ACQUITY QDa (Performance)
Ionization mode:	ESI-
Capillary voltage:	0.8 V
Cone voltage:	5.0 V
Probe temp.:	600 °C
Acquisition rate:	2 Hz
Full scan:	100 to 500 *m/z*
Curve fit:	Quadratic, 1/x weighting
Smoothing:	Mean, Level 7
SIR $\{M+Cl^-\}^-$:	215.0 fructose, glucose, galactose, inositol 377.0 sucrose, lactose, maltose

Standard preparation

A 50 mg/L stock of the seven saccharides listed above was prepared in 1:1 acetonitrile-water. This stock was further diluted to produce nine individual levels (0.5, 1, 2, 2.5, 5, 10, 20, 25, and 50 mg/L).

Sample preparation

Samples of a non-fat dry milk powder, a dairy-based infant formula, a soy based infant formula, and a low fat milk were purchased. These were prepared based on the procedure described by Chavez-Servin et al[2] as follows:

- Add approximately 0.6 g sample to a 25 mL volumetric flask.
- Add 10 mL 1:1 ethanol-water.
- Sonicate in a water bath at 60 °C for 25 min.
- Cool, add 250 µL Carrez 1* reagent, stir for 1 min.
- add 250 µL Carrez 2** reagent, stir for 1 min.
- Add 5 mL acetonitrile, mix.
- Make to 25 mL with 1:1 ethanol-water.
- Mix well and transfer to a 50 mL centrifuge tube, allow to settle.
- Centrifuge at an rcf of 2465 g for 30 min.
- Filter supernatant through a 0.2 µm PVDF filter.
- *Carrez 1 reagent: dissolve 0.36 g $K_4[Fe(CN_6).3H_20$ in 10 mL water.
- **Carrez 2 reagent: dissolve 0.72 g $ZnSO_4.7H_2O$ in 10 mL water.

The supernatant was diluted with 1:1 water:acetonitrile, 1:500 for the analysis of sucrose and lactose, and 1:20 for the analysis of fructose, glucose, galactose and inositol. The initial dilution of 25 was included for the final quantification calculations.

RESULTS AND DISCUSSION

Figure 2 shows the ACQUITY Arc System with the ACQUITY QDa and PDA detectors. Although shown with PDA, UV detection was not used for this analysis. Figure 3 shows the SIR chromatograms of the seven saccharide standards at a 5 ppm level (standard 5) used in the study. The annotated *m/z* (215 and 377) represent the $[m+ Cl]^-$ adducts. Figure 4 shows the mass spectra for the analytes. Addition of guanidine hydrochloride to the mobile phase shifts the equilibrium to the chloride adducts, *m/z* 215 for the monosaccharides and inositol, and 377 *m/z* for the disaccharides. Note the baseline separation of galactose and glucose using this chemistry, and also that lactose eluted before maltose.

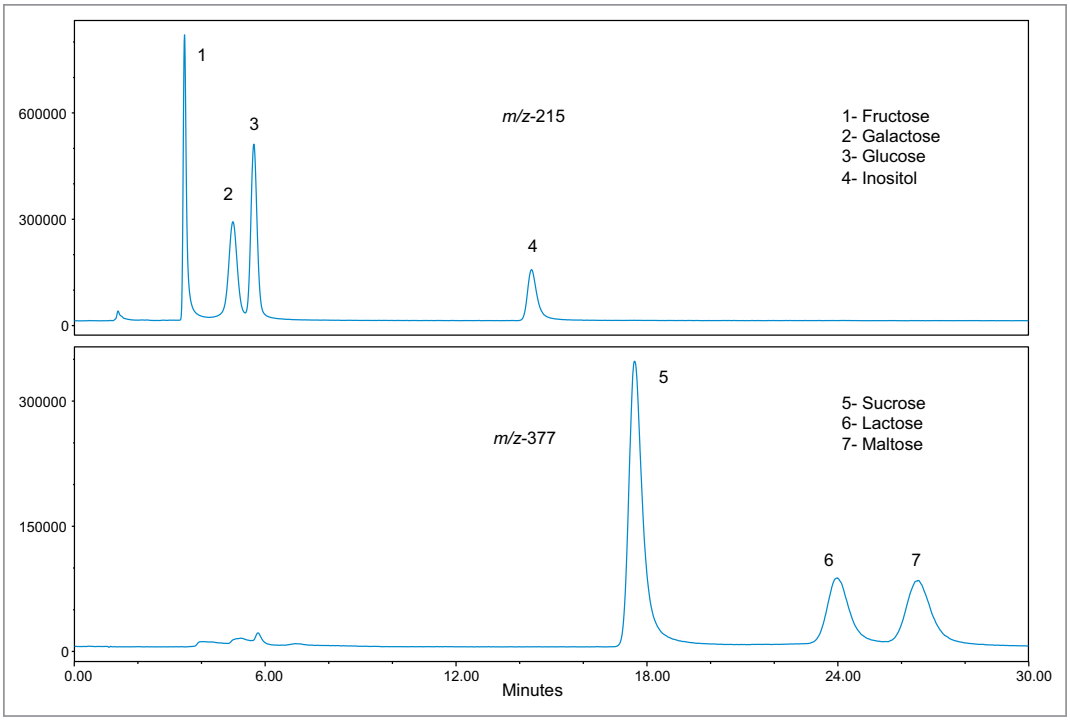

Figure 3. SIR chromatograms of the seven saccharide standards used in the study, the annotated m/z represents the $[m+ Cl]^-$ adducts.

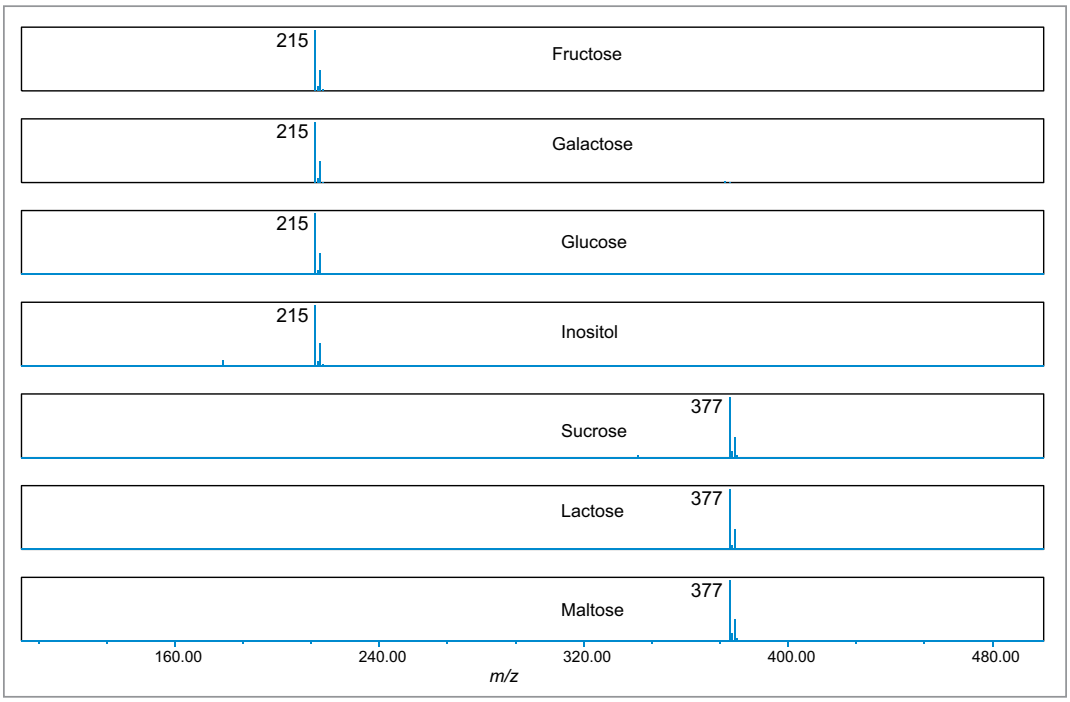

Figure 4. Mass spectral information extracted from SIRs of the seven saccharide standards. The annotated m/z represents the $[m+ Cl]^-$ adducts.

Figure 5 shows the SIR chromatograms of the saccharide standards at 5 ppm (standard 5) along with the dairy and soy based infant formulas at *m/z* 215. Note the absence of galactose in the soy based formula as would be expected. However, inositol is present in both formulations as it is an important nutrient in infant formula.[3] Inositol is highlighted in Figure 6.

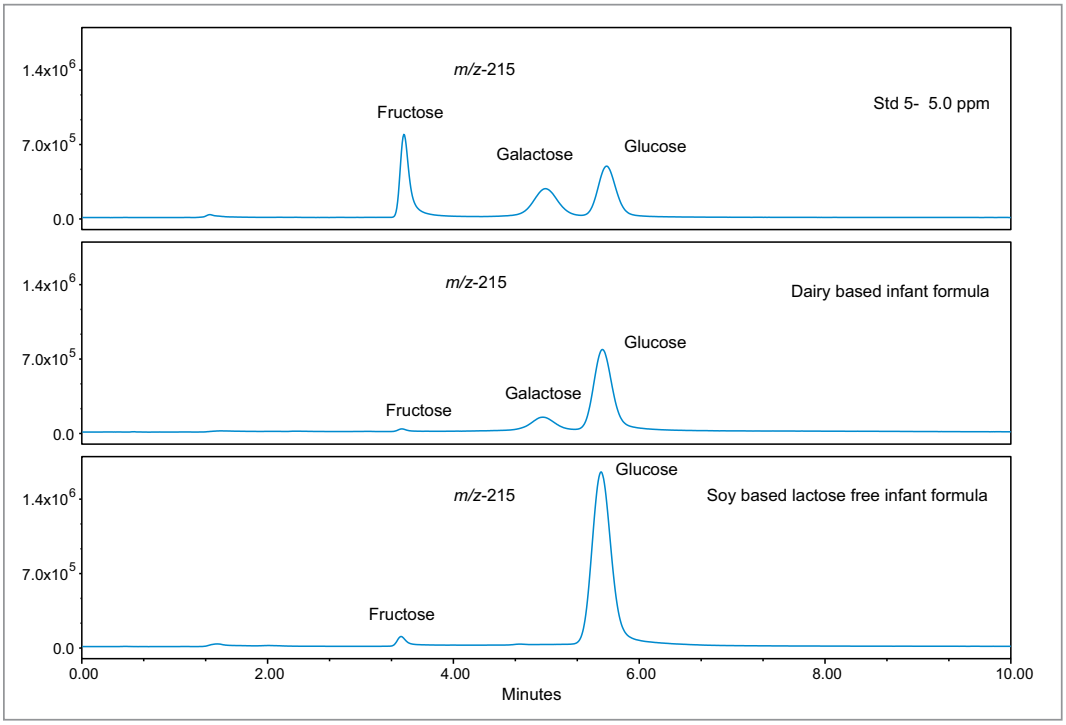

Figure 5. SIR chromatograms of Standard 5 with a dairy and soy based infant formula at m/z 215 for the saccharides fructose, galactose and glucose.

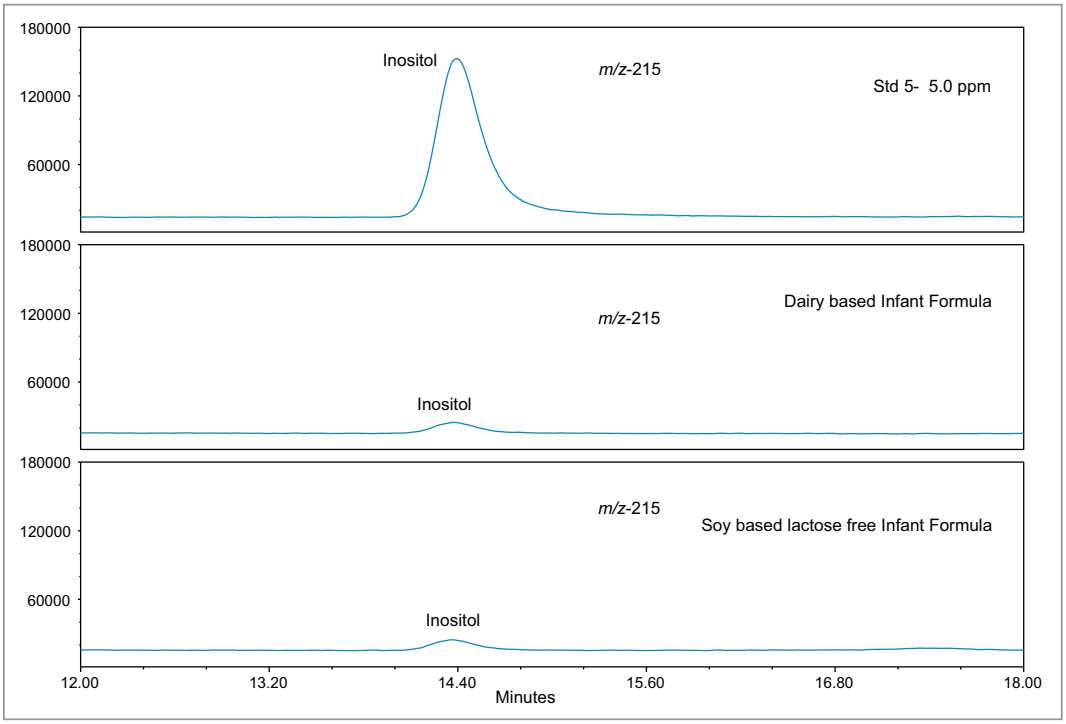

Figure 6. Annotated SIR profile of inositol in standard 5, along with a dairy and soy based infant formula.

Figure 7 shows the SIR chromatograms of the saccharide standards at 5 ppm (standard 5), along with the dairy and soy-based infant formulas at *m/z* 377. Here we see the absence of lactose in the soy formulation. However sucrose and maltose are present, which are absent in the dairy formulation. This is also to be expected as these two sugars are derived from plant based sources and should not be found in a dairy matrix unless added artificially. Figure 8 shows the calibration curves for the analytes. The regression coefficient (R^2) was >0.998 for all analytes.

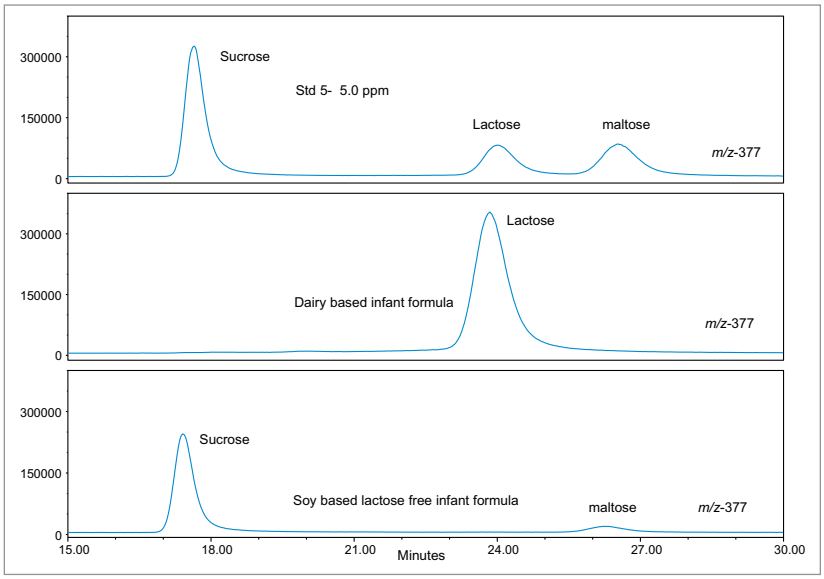

Figure 7. Annotated SIR profile of sucrose, lactose, and maltose in standard 5, along with a dairy and soy based infant formula.

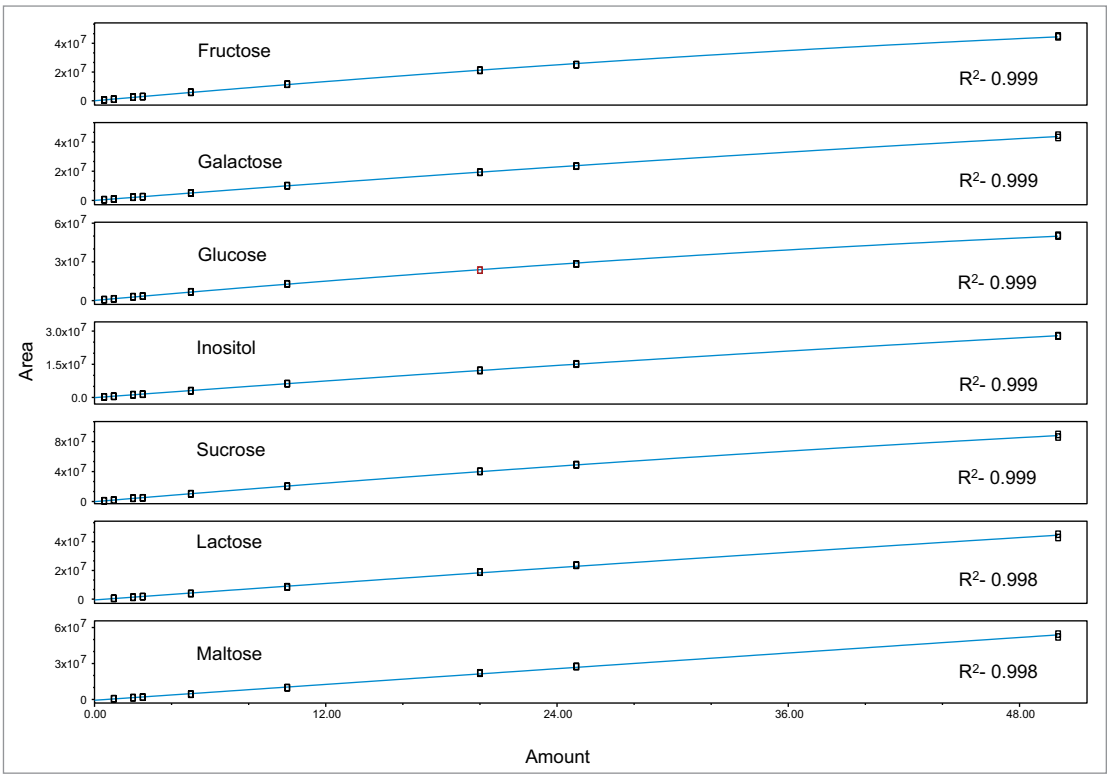

Figure 8. Calibration curves for the seven saccharide standards showing R^2 values, a quadratic fit was used.

Table 1 lists the quantitated values for the saccharides studied. The values for inositol in the dairy and soy based infant formula generally agree with the values reported by Indyk.[4] Likewise, the value for lactose in the dairy infant formula generally agrees with that reported by Ferreira.[5] Table 2 lists reproducibility data for 7 injections of the soy based infant formula. RSD's were <0.25% for retention time and 8.20% for amount.

Sample	Fructose	Galactose	Glucose	Inositol	Sucrose	Lactose	Maltose
Non Fat Dry Milk	0.04	0.837	1.295	0.389	ND	381.647	ND
Dairy Infant Formula	0.113	2.377	7.681	0.339	ND	394.996	ND
Soy Infant Formula	0.481	ND	19.579	0.359	83.705	ND	28.594
Low Fat Milk	ND	0.130	0.125	0.078	ND	42.775	ND

Table 1. Calculated concentrations from the quantification studies of the various dairy and infant formulas profiled in this study (g/kg).

Analyte	RT	% RSD	Amount	%RSD
Fructose	3.44	0.10	0.48	8.17
Glucose	5.59	0.10	19.58	3.71
Inositol	14.34	0.10	0.36	3.14
Sucrose	17.41	0.14	83.71	1.42
Maltose	26.27	0.22	28.59	2.02

Table 2. Reproducibility data for retention time (Min) and amount (g/kg) for seven injections of a soy based infant formula.

CONCLUSIONS

The analysis of carbohydrates in dairy products can be challenging because of the mix of closely related UV transparent compounds. The combination of the ACQUITY Arc System, ACQUITY QDa Detector, and the XBridge BEH Amide Column offers scientists the advanced performance expected of ACQUITY separations, high resolution, sensitivity, and improved throughput, along with a complimentary mass detector to RI and ELS that provides the additional advantages of:

- Improved analytical selectivity by combining both retention time and mass analysis for compound identification.
- Detection of UV transparent molecules using a sensitive and selective detector.
- Chromatographic separation of the difficult isobaric pair, galactose and glucose.

References

1. IMPLVO Ferreira, et al. Determination of Sugars and Some Other Compounds in Infant Formula, Follow Up Milks and Human Milk by HPLC-UV/RI. *Carbohydr Polym.* 37: (3) 225-229, 1998.
2. JL Chavez-Servin et al Analysis of Mono and Di-Saccarides in Milk Based Formulae by High Performance Liquid Chromatography with Refractive Index Detection. *J Chrom A.* 1043: (2) 211-215, 2004.
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4. Ibid p 401.
5. Ferreira, Op. Cit. p 227.

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Selective Quantitative Determination of Water Soluble Vitamins in Various Food Matrices Using the ACQUITY UPLC H-Class System and ACQUITY QDa Detector

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Selectivity of mass detection ensures unambiguous detection of vitamins at low levels, enabling simpler sample preparation protocols with dilution of sample extracts.
- The ACQUITY QDa™ Detector has been designed for integration with UPLC® and HPLC systems to provide robust reliable orthogonal detection to UV spectroscopy and enables new users to quickly take advantage of the most selective ACQUITY detector.
- The ACQUITY QDa Detector can be incorporated into existing liquid chromatography workflows in order to vastly increase selectivity over other LC detectors.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY QDa Detector

KEY WORDS

WSV, vitamins, QDa, mass detection

INTRODUCTION

Many food and beverage products are routinely fortified with vitamins to enhance their nutritional value and to help address any deficiencies in dietary requirements. In order to meet legal requirements, food and beverage manufacturers must label products according to the regulations of the country in which the product is consumed. Examples of these regulations include European Commission (EC) 1925/2006 on the addition of vitamins and minerals, and Title 21 Code of Federal Regulations (C.F.R.), Part 101 on food labeling in the United States.

Rapid, reliable, and cost-effective methods are required by food manufacturers and ingredient suppliers in order to verify product consistency and ensure that label claims are met. This can be a challenging task with the combination of complex matrices and low fortification levels of some vitamins. In addition, many of the methods currently employed stipulate that the vitamins are either analyzed separately, or in small groups. Established techniques include microbiological assays, colorimetric and fluorimetric analysis, titrimetric procedures and HPLC methodologies.[1] LC-MS offers the opportunity to consolidate methods along with the ability to improve detector selectivity and reduce limits of quantification. In order to offer laboratories the opportunity to capture the benefits of mass detection without the challenges associated with the adoption of mass spectrometers, recent advances in technology have focused on improving instrument usability and robustness. These motivations have resulted in the introduction of the ACQUITY QDa Detector.

In this application note, 12 water soluble vitamins (WSVs) were analyzed in dietary supplements and beverage samples using the ACQUITY UPLC H-Class System with the ACQUITY QDa Detector.

EXPERIMENTAL

Table 1 lists the WSVs included in this study along with the observed retention times, single ion recording (SIR) *m/z*, and cone voltage.

Analyte	RT (Min)	SIR *m/z*	Cone voltage (V)
Ascorbic Acid (C)	0.91	177	2
Thiamine (B1)	1.01	265	5
Nicotinic Acid (B3)	1.27	124	15
Pyridoxal (B6)	1.75	168	5
Nicotinamide (B3)	2.48	123	15
Pyridoxine (B6)	2.50	170	10
Ca_Pantothenate (B5)	5.88	242	15
Cyanocobalamin (B12)	7.17	678	2
Folic Acid (B9)	7.22	442	5
B2-5-Phosphate	7.35	457	5
Biotin (B7)	7.50	245	10
Riboflavin (B2)	7.74	377	15

Table 1. Retention times, SIR channels, and cone voltages for the water soluble vitamins.

Standard	Individual B complex vitamins (mg/L)	Vitamin C (mg/L)
1	1.00	50.0
2	0.75	37.5
3	0.50	25.0
4	0.25	12.5
5	0.10	5.0
6	0.075	3.75
7	0.050	2.5
8	0.025	1.25
9	0.010	0.5
10	0.005	0.25
11	0.001	0.05

Table 2. Standards concentrations for B vitamins and vitamin C.

Standard preparation

Individual 1mg/mL WSV stocks were prepared in water. In the case of vitamins B2, B7, and B9, 200 µL of 1 N NaOH were added to effect dissolution. Vitamin C was dissolved in a low pH acetate buffer to enhance stability. From these individual stocks, a mixed stock was prepared by adding 1.25 mL of the vitamin C stock and 0.025 mL of the other stocks and diluting to 25 mL with water. This mixed stock (50 ppm vitamin C, 1 ppm other analytes) was further diluted to provide 11 individual calibration standards, listed in Table 2.

Sample preparation

A packet (8.50 g) of a powdered vitamin beverage was dissolved in 100 mL water and filtered through a 0.2-µm PVDF filter. This sample was then prepared at two additional dilution levels: 1:250 and 1:10. These three dilution levels were injected to cover the different concentrations of vitamins in this sample.

A multi-vitamin supplement tablet was crushed using a mortar and pestle. The powder (1.34 g) was quantitatively transferred to a beaker to which 100 mL water was added. This mixture was sonicated for 15 minutes then stirred and filtered through a 0.2-µm PVDF filter. Three additional dilutions of this sample were prepared in water: 1:1000, 1:100, and 1:20. These dilutions and the initial dissolved tablet solution (undiluted) were analyzed in order to cover the different concentrations of vitamins in this sample.

Two different vitamin water samples were prepared by diluting 1:20 with water and filtering through a 0.2-µm PVDF filter.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC H-Class
Run time:	17.5 min
Column:	ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 100 mm
Column temp.	30 °C
Mobile phase A:	10 mM ammonium formate, 0.1% formic acid in water
Mobile phase B:	10 mM ammonium formate, 0.1% formic acid in methanol
Injection volume:	5 µL

	Time (min)	Flow rate (mL/min)	%A	%B
1.	Initial	0.45	99	1
2.	3.0	0.45	99	1
3.	3.1	0.45	95	5
4.	5.1	0.45	80	20
5.	7.1	0.45	2	98
6.	9.0	0.45	2	98
7.	9.1	0.45	99	1
8	17.5	0.45	99	1

Table 3. UPLC gradient for the separation of water soluble vitamins.

Detector conditions

Detector 1:	ACQUITY UPLC PDA
Wavelength:	Scanning 210 to 400 nm; Analog channel at 270 nm
Scan rate:	10 pts/sec
Detector 2:	ACQUITY QDa
Ionization mode:	ESI+
Run time:	8.0 min
Probe:	600 °C
Capillary voltage:	0.8 kV
Mass range:	*m/z* 50 to 800 (centroid) and select SIRs*
Sampling freq.:	5 Hz
Cone voltage:	Full scan data: 15 V

*See Table 1 for cone voltage of individual SIR channels.

SIR *m/z* were assigned based on previous work.[2]

RESULTS AND DISCUSSION

A chromatogram showing an overlay of all 12 water soluble vitamins used in this study is shown in Figure 1, where all compounds eluted within eight minutes. Using this method, there were two co-eluting pairs (nicotinamide and pyridoxine at ~2.5 minutes and cyanocobalamin and folic acid at ~7.25 minutes). The use of mass detection means that it is no longer necessary to ensure baseline separation of all the analytes. The discrimination offered with mass detection means that these compounds can be accurately measured using their mass-to-charge ratio (*m/z*). This is demonstrated in Figure 2 where the linearity of selected vitamins are shown, including vitamins that co-eluted. Figure 2D and 2F show the calibration curves of folic acid (*m/z* 442) and cyanocobalamin (*m/z* 678), respectively. The selectivity offered with mass detection means that these compounds can be determined quantitatively, even though they co-elute. Figure 2 also shows example calibration curves of vitamins that can be challenging to analyze by UV. For example, biotin (Figure 2A) and calcium pantothenate (Figure 2H) are vitamins that show low responses using UV detection. Those compounds are often analyzed at low wavelengths to obtain a sufficiently sensitive response.[3] At such low wavelengths, the specificity of the analysis may be compromised. Mass detection ensures that the analysis is both specific and sensitive.

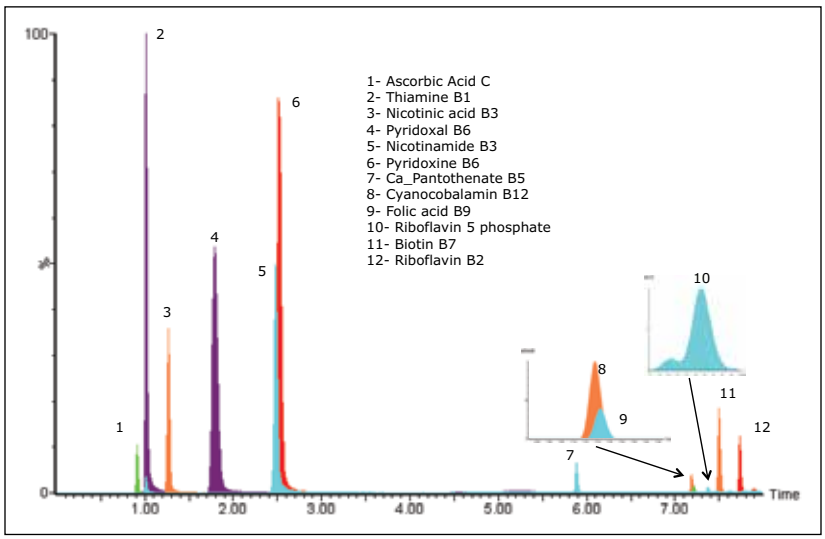

Figure 1. Overlay of SIR chromatograms of 12 water soluble vitamins separated in eight minutes.

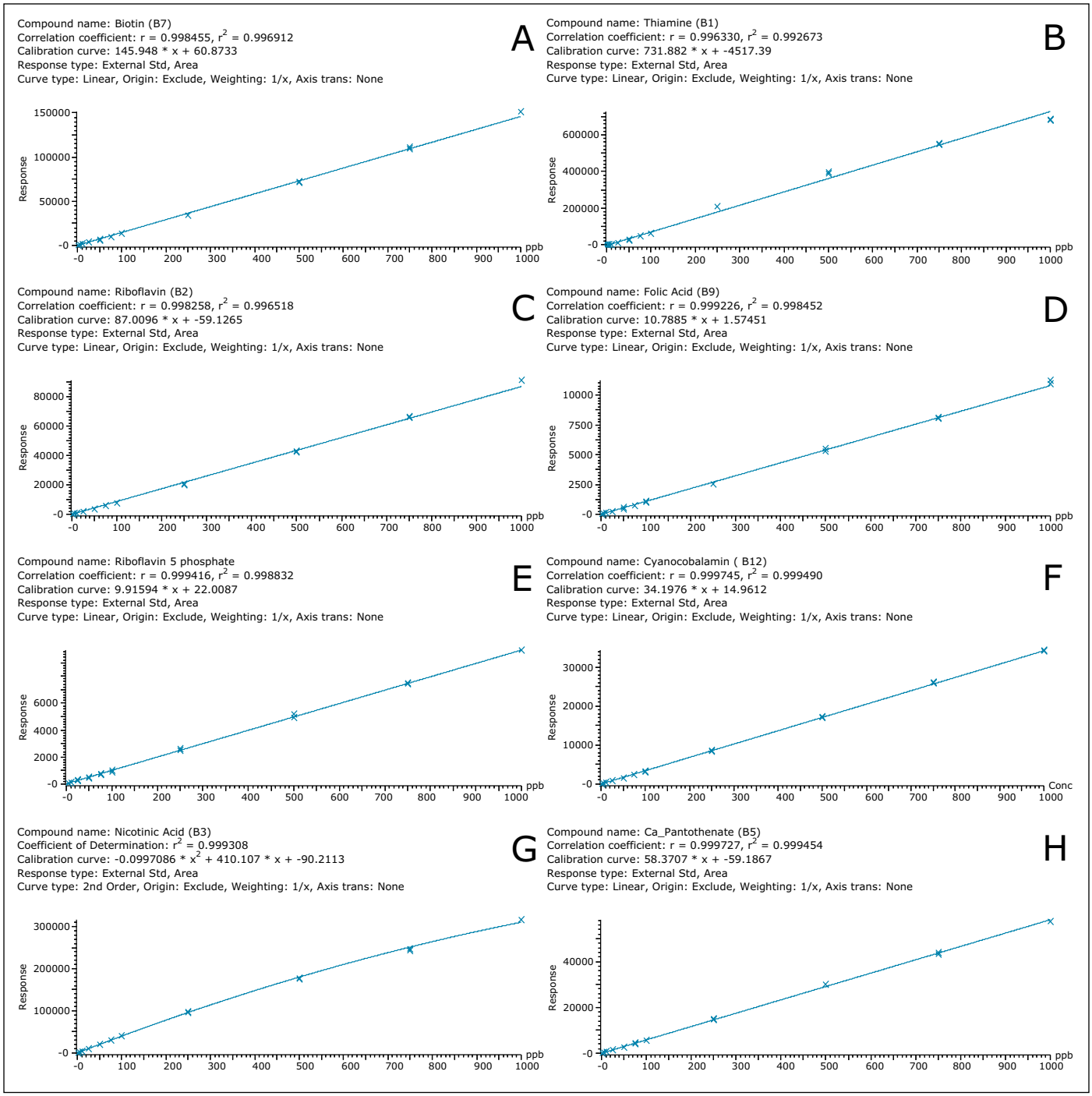

Figure 2. Calibration curves for selected water soluble vitamins.

Mass detection offers the opportunity to detect vitamins at lower levels than can be achieved with UV detection. In Figure 3, the SIR chromatograms of vitamins pyridoxine, pyridoxal, nicotinic acid, and nicotinamide at 5 ppb (5 µg/L) are shown, along with the UV chromatogram (Figure 3A, 270 nm). As shown in Figure 3A, the vitamins could not be detected by UV at this level. The lower limits of quantification that can be achieved with mass detection is important for the quantification of vitamins at low levels. Improved sensitivity also helps to deal with the wide variety of matrices that are encountered by allowing sample extracts to be diluted. In this work, vitamin supplements and drinks were analyzed simply by diluting the sample (in the case of a tablet an initial step to crush the tablet was required).

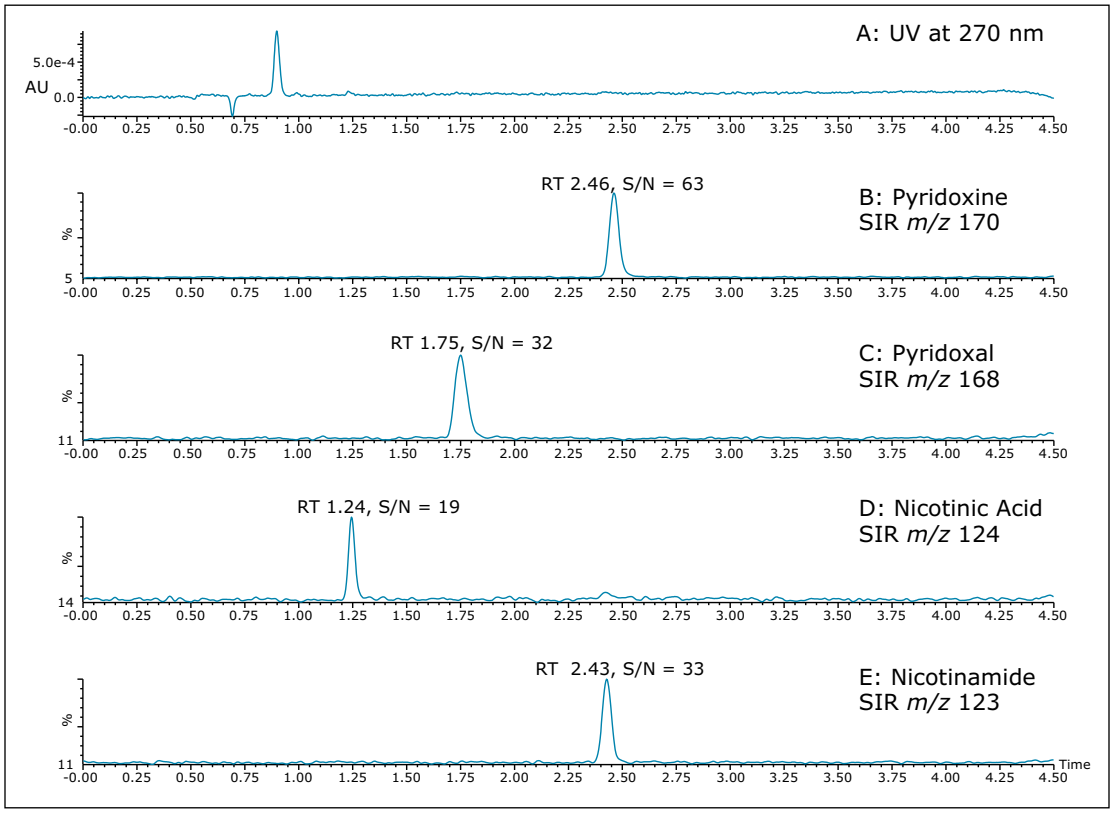

Figure 3. UV chromatogram at 270 nm and SIR chromatograms of a standard mix of vitamins at 5 µg/L. SIR channels for four vitamins are shown. B: pyridoxine, C: pyridoxal; D: nicotinic acid; and E: nicotinamide.

Figure 4 shows the detection of vitamin B5 (calcium pantothenate) in two vitamin water samples. As seen from the UV chromatogram, this vitamin could not be detected by UV without additional sample preparation. Vitamin B1 (thiamine) is another vitamin that is difficult to detect using UV. Figure 5 shows an example of the detection of vitamin B1 and vitamin C (ascorbic acid) in a diluted powdered vitamin beverage. Although vitamin C could be detected in the UV chromatogram, vitamin B1 was not detected. Vitamin B1 however, was clearly detected using SIR with the ACQUITY QDa Detector, as shown in Figure 5A.

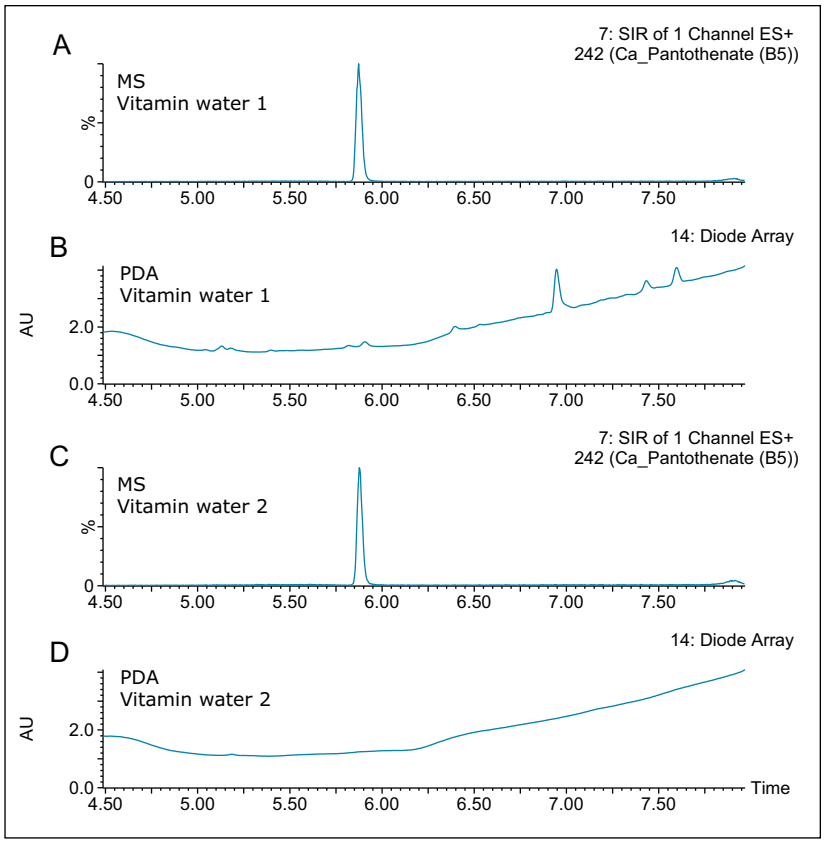

Figure 4. Detection of vitamin B5 in two different vitamin water samples. The peak at 5.9 minutes shows excellent signal-to-noise using mass detection (A and C) but cannot be detected using UV (B and D).

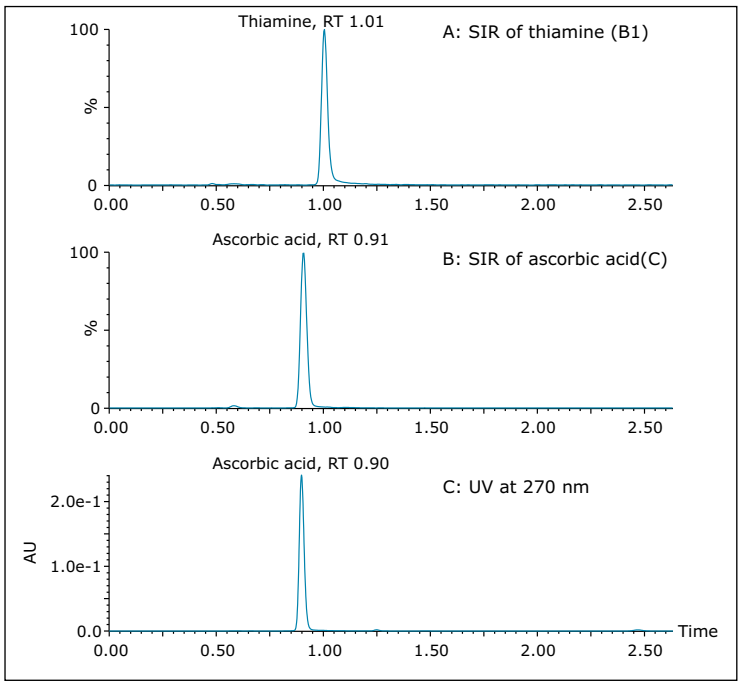

Figure 5. Chromatograms from a 1:250 dilution of a powdered vitamin beverage. A: SIR of vitamin B1 (thiamine), B: SIR of vitamin C (ascorbic acid), C: UV at 270nm; vitamin C was detected in the UV trace while vitamin B1 was not detected by UV.

Cyanocobalamin is a WSV that is fortified at very low levels in supplements and foods and it traditionally requires separate methodologies for its quantification. Two-dimensional chromatography is a routine strategy for the detection of this vitamin.[4] Figure 6 shows an example of cyanocobalamin detected in the multi-vitamin supplement tablet using the UPLC-MS method presented here.

At this level, no peak was apparent in the UV chromatogram (Figure 6B). Mass detection offers the ability to detect vitamin B12 using the same method used to detect vitamins that are fortified at much higher levels. The ACQUITY QDa Detector, which can easily be incorporated into existing LC workflows, offers an easier-to-use method than existing multi-dimensional methods.

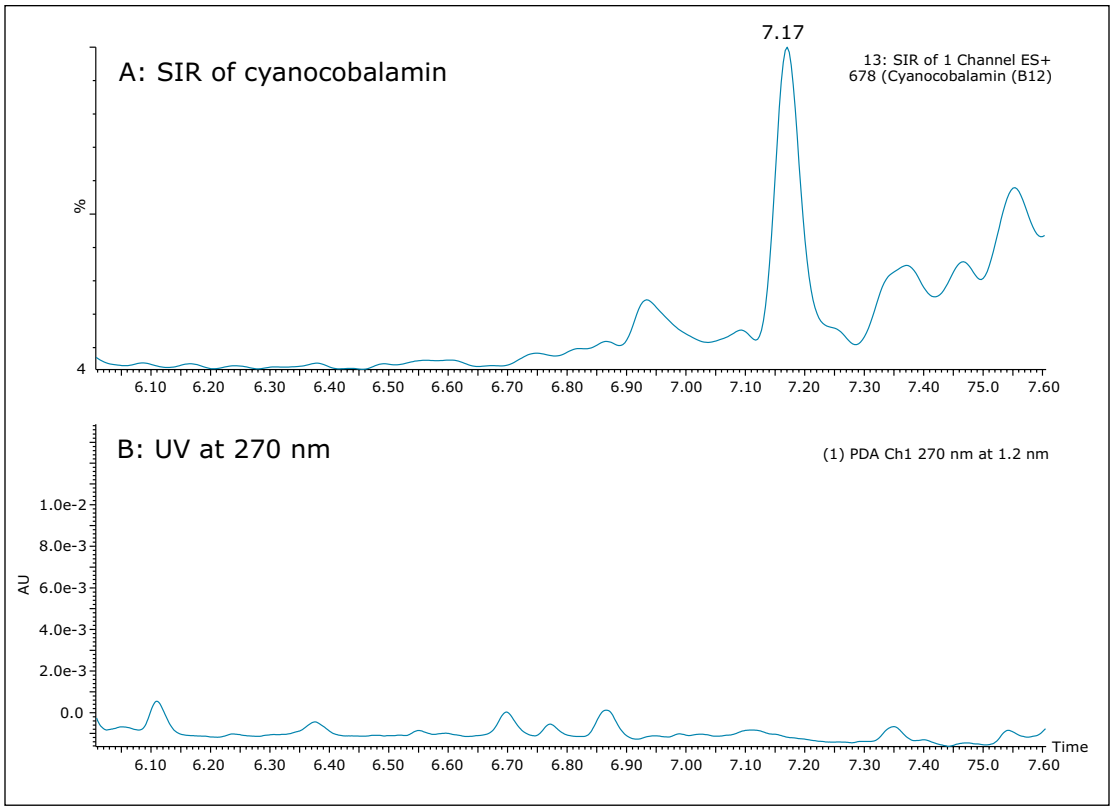

Figure 6. Detection of Vitamin B12 in a vitamin supplement tablet using mass detection (A). The level of the vitamin is below the UV detection limit (B).

One challenge that is encountered in vitamin analysis is the wide range of concentrations at which the vitamins are fortified. For the example of the multi-vitamin supplement in tablet form that was used in this study, the label stated that the B vitamins ranged from 6 µg for vitamin B12 (cyanocobalamin) to 16 mg for B3 (labeled as niacin (nicotinic acid)), with other B vitamins within that range. In this work, the same LC-MS method was used for the analysis of all the vitamins, with different dilution factors of the initial extraction in order to account for the different vitamin levels. Figure 7 shows chromatograms from the analysis of the multi-vitamin tablet. Figures 7A and 7B show the SIR channels of riboflavin (B2) in the 1:100 dilution of the sample and the undiluted sample, respectively. Figure 7C shows the SIR channel of cyanocobalamin (B12) in the undiluted sample. No peak was detected in the diluted sample (data not shown). The UV trace of the undiluted sample at 270 nm is shown in Figure 7D, and the riboflavin peak showed a good response for this sample. The quantified amount for riboflavin and cyanocobalamin were 12.5 ppm and 41 ppb, respectively. These amounts corresponded to 96% and 68% of the label claim of the supplement. Although the label claims were not verified for this work, nor did we undertake a recovery study, this short study demonstrated the feasibility of using the multiple dilution strategy within the calibration range specific in Table 2.

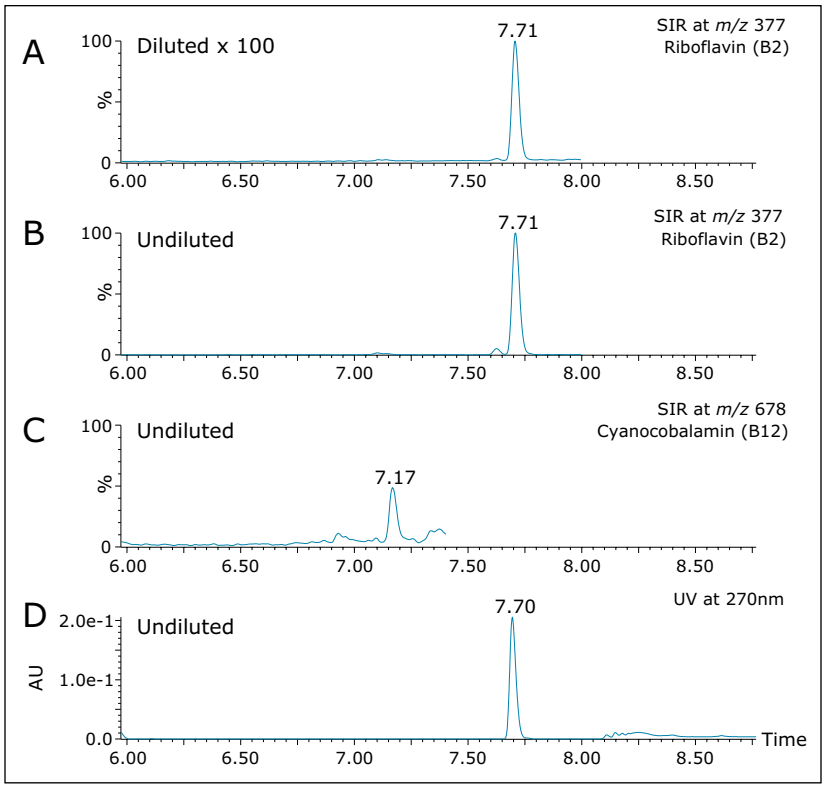

Figure 7. Detection of two B vitamins of very different concentrations in a vitamin supplement tablet. Riboflavin can clearly be detected in a 1:100 dilution of the sample extract (A), whereas B12 is only apparent in the undiluted extract (C). The response of riboflavin in the undiluted extract (B) is outside the calibrated range. At this level it can easily be detected using UV (D).

In order to assess the repeatability of the method for the B vitamins, multiple injections at different vitamin levels were assessed. Results for retention time repeatability, and peak area repeatability are shown in Tables 4 and 5, respectively. In Table 4, 10 injections of two different standards had been combined to give a total of 20 injections. Retention time stability was excellent, even for the early eluting water soluble vitamins, with all RSDs at or below 0.6%. Peak area repeatability was assessed with 10 injections at 0.025 mg/L (Table 5). For the majority of vitamins, %RSDs were well below 10%, with the exception of folic acid and riboflavin 5 phosphate, which were the lower responding analytes mentioned above. Vitamin C was excluded from this study as it is known to degrade over time.

Analyte	%RSD for retention time
Thiamine (B1)	0.6
Nicotinic Acid (B3)	0.19
Pyridoxal (B6)	0.23
Nicotinamide (B3)	0.22
Pyridoxine (B6)	0.26
Ca_Pantothenate (B5)	0.04
Cyanocobalamin (B12)	0.03
Folic Acid (B9)	0.03
Riboflavin 5 phosphate	0.03
Biotin (B7)	0.02
Riboflavin (B2)	0.03

Table 4. Repeatability of retention times for 20 injections using two different standards 0.75 mg/L (10 injections) and 0.025 mg/L (10 injections).

Analyte	%RSD for area
Thiamine (B1)	6.78
Nicotinic Acid (B3)	2.35
Pyridoxal (B6)	2.62
Nicotinamide (B3)	2.24
Pyridoxine (B6)	2.65
Ca_Pantothenate (B5)	4.60
Cyanocobalamin (B12)	7.00
Folic Acid (B9)	11.53
Riboflavin 5 phosphate	14.29
Biotin (B7)	2.77
Riboflavin (B2)	2.28

Table 5. Repeatability of B vitamins peak areas for 10 injections of a standard mix at 0.025 mg/L.

CONCLUSIONS

This work shows the capability of the Waters ACQUITY QDa Detector to accurately quantify water soluble vitamins at levels that cannot be achieved with UV. The acquisition of SIR channels allows for sensitive and selective quantification of analytes, even when co-elution occurs. This helps to remove the burden of ensuring all analytes are baseline separated and enables the detection of lower levels of vitamins.

The ACQUITY QDa Detector allows new users to:

- Quantify analytes which have little or no UV response.
- Selectively quantify compounds that co-elute but have different masses.
- Consolidate water soluble vitamin methods into a single LC-MS method.
- Reduce limits of quantification in order to assess easier sample preparation strategies.
- Easily integrate into existing LC workflows, with the choice of Empower® 3 or MassLynx® software control.
- Quickly take advantage of the ACQUITY QDa's mass detection capabilities – no special mass spectrometry knowledge required.

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Fast Analysis of Isoflavones in Dietary Supplements – USP Method Transfer onto a UHPLC System

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Reduce analysis time of isoflavones from 74 min to 18 min.
- Easily transfer the standard USP isoflavones method to UHPLC with the aid of mass detection.
- Confirm peak identities more confidently using mass detection.

WATERS SOLUTIONS

ACQUITY Arc™ UHPLC System

ACQUITY® QDa® Mass Detector

2998 Photodiode Array (PDA) Detector

CORTECS® C_{18} Column

Empower® 3 CDS Software

KEYWORDS

Isoflavones, dietary supplement, mass detection, soy, *Glycine max*, red clover, *Trifolium pretense*, Kudzu, *Pueraria lobata*, daidzein, glycitein, genistein

INTRODUCTION

Isoflavones are found primarily in plants of soy (*Glycine max*), red clover (*Trifolium pretense*), and Kudzu (*Pueraria lobata*). The 12 major isoflavones found in these plants are daidzein, glycitein, genistein, and their respective glucoside and malonyl- and acetyl- glucoside derivatives. The structures of 12 isoflavones and an internal standard, apigenin, are shown in Figure 1. These hormone-like compounds are often used in remedies to reduce menopausal and post-menopausal symptoms. They are even associated with low breast cancer rate in Asia and the retarded progression of Alzheimer's disease.

Standard methods for isoflavones in dietary supplements have been established by organizations such as USP[1] and AOAC.[2] These methods use reversed-phase LC with C_{18} columns and ultraviolet and visible light (UV-Vis) spectroscopy for separation and quantitation. Because of the close structural similarity of these compounds, the chromatographic run times of these methods are typically over 70 minutes long. It is highly desirable to develop a more rapid isoflavone analysis method.

This application note demonstrates the transfer of the USP method onto Waters® ACQUITY Arc UHPLC System. The analysis time with the ACQUITY Arc System is only 18 minutes, including column wash and equilibration. Waters ACQUITY QDa Mass Detector was used to expedite the method transfer described in this study. The benefits of mass detection in peak identification and method optimization are also highlighted.

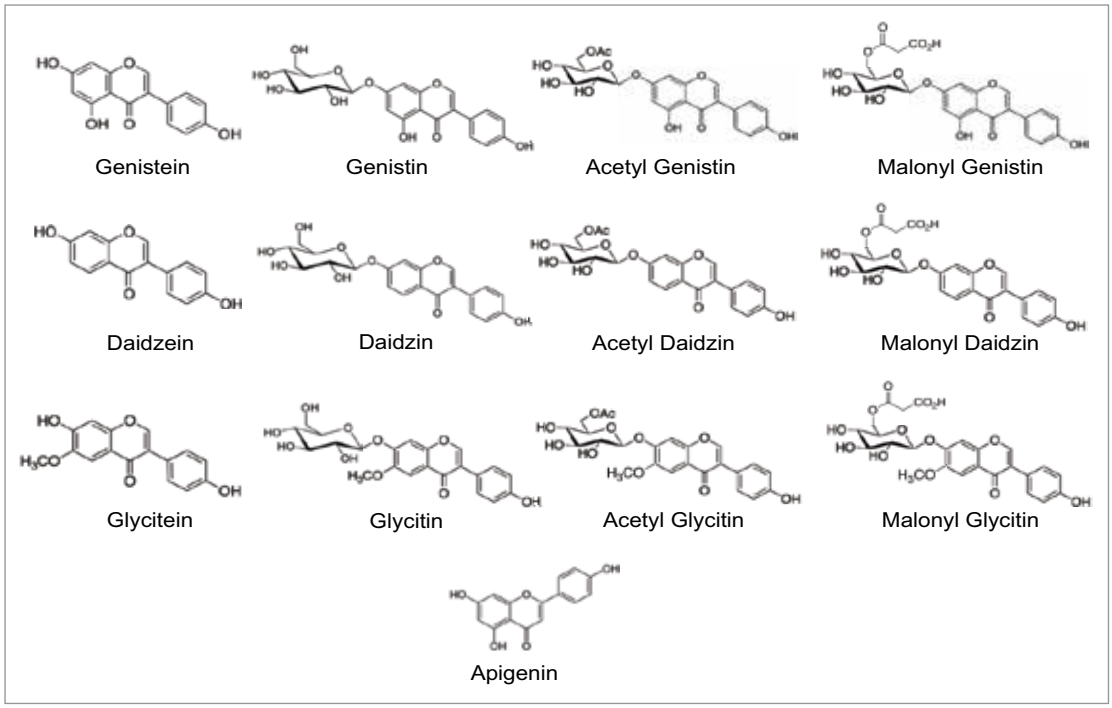

Figure 1. Structures of isoflavones in this study.

EXPERIMENTAL

Sample preparation

The standards, daidzin, glycitin, genistin, daidzein, glycitein, genistein, and apigenin, were purchased from ChromaDex (Irvine, CA) and INDOFINE Chemical (Hillsborough Township, NJ). Defatted powdered Soy RS was purchased from US Pharmacopeia (Rockville, MD). NIST SRM 3238 was purchased from NIST (Gaithersburg, MD). Isoflavone dietary supplement samples from major brands were purchased from online retail stores.

The standard and sample solutions were prepared the same way as in the USP isoflavone method.[1] Sample solutions were further diluted with an acetonitrile water mixture (2/3 by volume) to various levels to fit the calibration range. The concentration of internal standard was always kept constant at 4 ppm.

UHPLC conditions

UHPLC system:	ACQUITY Arc
Detector:	2998 PDA
Software:	Empower 3
Column:	CORTECS C_{18} 2.7 µm, 3.0 x 100 mm
Column temp.:	30 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
Injection volume:	2.0 µL
Flow rate:	1.08 mL/min
Run time:	18.0 min
UV detection:	260 nm
UV resolution:	1.2 nm

Elution gradient

	Time (min)	Flow rate (mL/min)	%A	Curve
1	Initial	1.08	90	6
2	14.40	1.08	70	6
3	14.50	1.08	10	6
4	15.20	1.08	10	6
5	15.40	1.08	90	6
6	18.00	1.08	90	6

MS conditions

MS system:	ACQUITY QDa (Performance)
Ionization mode:	ESI^+
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Probe temp.:	600 °C
SIR masses:	

Table 1. Masses of isoflavone molecular ions.

Analyte	SIR mass (Daltons)
Daidzin	417.1
Glycitin	447.0
Genistin	433.1
Daidzein	254.9
Glycitein	285.0
Genistein	270.9
Apigenin (IS)	270.9
Malonyl Daidzin	503.4
Malonyl Glycitin	533.1
Acetyl Daidzin	459.1
Acetyl Glycitin	489.0
Malonyl Genistin	519.0
Acetyl Genistin	475.1

RESULTS AND DISCUSSION

USP METHOD TRANSFER AND OPTIMIZATION ONTO ACQUITY ARC UHPLC

The USP method (isoflavones powder extract)[1] was transferred to an ACQUITY Arc UHPLC System with a CORTECS C_{18} Column (2.7 µm, 3 x 100 mm), p/n 186007372. The CORTECS Column's 2.7 µm packing material is solid-core particle, which provides higher separation efficiency and lower back pressure than a fully porous particle column of equivalent particle size. The USP method gradient elution program was converted to a new gradient elution program using Waters ACQUITY UPLC Column Calculator.[3] The column parameters in the USP method (5 µm, 3.0 x 250 mm) and the parameters of the CORTECS C_{18} Column (2.7 µm, 3.0 x 100 mm), as well as the USP method's 74 minutes gradient elution program were entered in the column calculator, and a 18 minutes gradient elution program that is equivalent to the USP method was calculated. The mobile phase additive was changed from 0.05% phosphoric acid to 0.1% formic acid, which is a more MS friendly additive.

The factory default ACQUITY QDa Detector instrument parameters were used without any modification. The column temperature of 40 °C was tested, but was later optimized to 30 °C to meet the USP suitability criteria on peak resolution. Figure 2 shows the chromatograms of the USP defatted powdered soy RS, unheated and heated, and their Single Ion Recording (SIR) traces that were obtained using the ACQUITY QDa Mass Detector. Mass detection was used to confirm the peak identities.

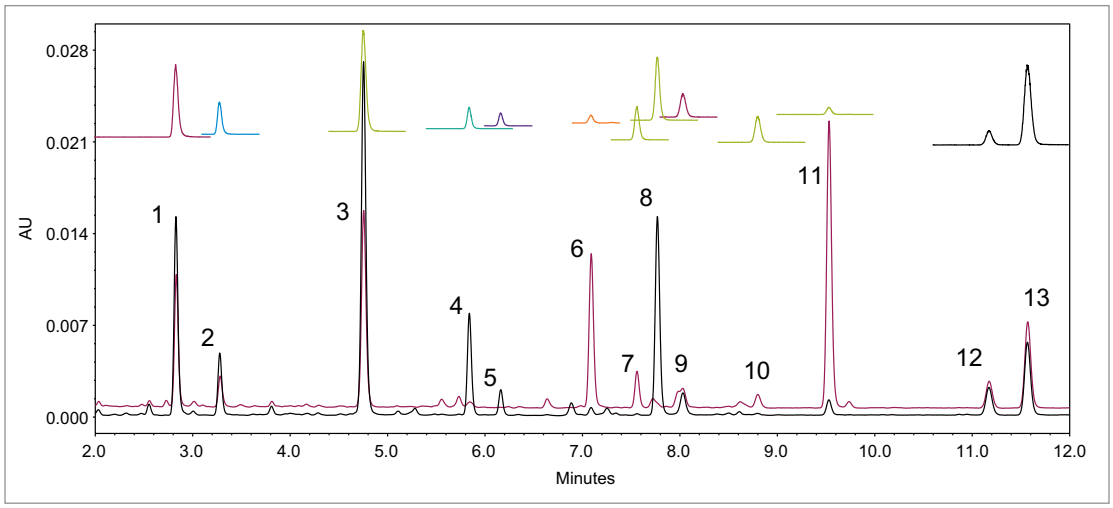

Figure 2. UV chromatograms of unheated defatted soy (black, the bottom trace) and heated defatted soy (red trace next to the bottom), and the corresponding mass detector SIR traces for every compound. Peak ID: 1. Daidzin; 2. Glycitin; 3. Genistin; 4. Malonyl Daidzin; 5. Malonyl Glycitin; 6. Acetyl Daidzin; 7. Acetyl Glycitin; 8. Malonyl Genistin; 9.Daidzein; 10. Glycitein; 11. Acetyl Genistin; 12. Genistein; 13. Apigenin.

METHOD TRANSFER AND OPTIMIZATION USING MASS DETECTION

Since the acetyl and malonyl isoflavone standards were not commercially available, the peak assignment of these compounds were carried out using a reference material and a pattern matching method as described in the USP standard. Heat treatment of defatted soy (DFS) can convert the malonyl forms to the acetyl forms. By comparing the chromatograms of the unheated DFS and the heated DFS and their reference chromatograms, one can assign the peak IDs to those acetyl and malonyl isoflavones. However, this pattern matching approach is not reliable, especially when LC conditions, such as the column, the mobile phase additives, or the LC system are changed. The ACQUITY QDa detects the ions that are formed in electrospray ionization (ESI) at unit mass resolution (0.7 Da). Table 1 lists the molecular ions of these isoflavones. Using mass detection, we were able to selectively detect these compounds and eliminate any possible interference from closely eluting compounds.

Genistein and apigenin have the same mass, but their peaks were well separated by chromatography (see Figure 2). The addition of mass detection (ACQUITY QDa Detector) enabled unambiguous assignment of peak IDs to the correct acetyl and malonyl isoflavones without resorting to individual standards.

The ACQUITY QDa Detector also sped up the method optimization because the selective detection of every compound allowed us to monitor the retention time (RT) changes of all compounds simultaneously with high confidence. This greatly saved the number of injections in method optimization. More details on how the QDa detector benefits the method transfer are discussed in a separate application note.[4]

METHOD PERFORMANCE CHARACTERISTICS

Table 2 shows the UV calibration results of the standards. Apigenin was used as the internal standard for calibration and quantitation of all compounds. The square of the correlation coefficients (R^2) between the relative responses (peak area ratio) and the concentration of standards in solutions (ppm) for all compounds were better than 0.999. The retention time relative standard deviations for all compounds were less than 0.12%.

Table 2. Isoflavones retention times and their relative standard deviation, calibration equations, R^2, and linear ranges.

Analyte	RT		Equation	R^2	Range (ppm)
	Min	RSD (%)			
Daidzin	2.81	0.12	$Y = 2.09x10^{-1} X + 3.09x10^{-3}$	0.9998	0.075–10
Glycitin	3.26	0.09	$Y = 2.42x10^{-1} X + 5.43x10^{-3}$	0.9998	0.075–10
Genistin	4.75	0.05	$Y = 4.53x10^{-1} X + 9.43x10^{-3}$	0.9998	0.05–10
Daidzein	8.03	0.03	$Y = 2.60x10^{-1} X + 4.65x10^{-3}$	0.9998	0.1–10
Glycitein	8.80	0.03	$Y = 4.96x10^{-1} X + 1.07x10^{-3}$	0.9998	0.05–10
Genistein	11.17	0.02	$Y = 4.91x10^{-1} X + 9.73x10^{-3}$	0.9998	0.05–10

Table 3 shows the isoflavone results for the NIST 3238 SRM and the comparison to its certified and reference values. A relative difference of <11% was obtained for the genistin, glycitin, daidzin, genistein, and glycitein. The result of daidzein was 15% higher than the NIST value. A literature search found that a high daidzein value was also reported elsewhere.[5] The accuracy for the daidzein, genistein, and glycitein were also evaluated by a spiking experiment (Table 4). Recoveries of 98% to 101% were obtained for these compounds.

Table 3. Comparison of determined isoflavone values to the certified and reference values of NIST 3238 SRM.

	NIST value (mg/kg)	Determined value (mg/kg)	Relative difference
Genistin	12700±530	11450	-10%
Glycitin	3760±180	3355	-11%
Daidzin	13400±2400	14700	10%
Daidzein	241±5	277	15%
Genistein	108±10	99	-8%
Glycitein	211±5	195	-8%

Table 4. Recovery results from a spiking experiment.

	Original value (mg/kg)	Spiked level (mg/kg)	Determined value (mg/kg)	Recovery (%)
Daidzein	259	499	751	98%
Genistein	41	499	546	101%
Glycitein	68	499	557	98%

ANALYSIS OF ISOFLAVONE DIETARY SUPPLEMENTS

The isoflavone content in four isoflavone dietary supplement samples were measured by the fast 18-minute UHPLC-UV method described above. Sample types included tablets, capsules, and soy powder. The USP calibration and quantitation protocols[1] were followed in the data processing.

The same conversion factors for the acetyl and malonyl derivatives that are specified in the USP HPLC-UV method were used in the analyses. Table 5 shows the determined individual and the total isoflavones, as well as the label claimed total isoflavone contents. For easy comparison, the label claim values were converted to concentration (mg/kg). Two of the three samples (C and D) showed good agreement between the determined values and their label claim values, while one sample (B) contained much less measured total isoflavone content then claimed on its label. The reason for such low total isoflavone content is unknown.

Table 5. Isoflavone contents in dietary supplements and their label claim values.

Sample (mg/kg)	B		C		D		E	
	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
Daidzin	3074	1.8	12540	0.8	442	8.4	0	
Glycitin	693	0.2	4683	0.2	0		0	
Genistin	335	2.0	1786	0.8	730	1.9	303	3.0
Malonyl Daidzin	0		0		0		0	
Acetyl Daidzin	1580	1.5	7222	0.5	0		0	
Acetyl Glycitin	390	2.0	2083	2.6	0		0	
Malonyl Genistin	0		0		354	2.8	143	7.4
Daidzein	6266	0.8	506	0.9	0		0	
Glycitein	0		224	1.8	0		0	
Acetyl Genistin	162	1.9	992	1.7	0		0	
Genistin	0		0		0		159	10.1
Total Isoflavones	12,543		30,006		1,527		605	
Label Claim Value	>31,250		>25,000		867 to 2,600		no claim	

CONCLUSIONS

The USP method for isoflavones was successfully transferred to an ACQUITY Arc UHPLC System with a 2998 PDA Detector. The total analysis time per injection on the UHPLC system was 18 minutes, which was significantly shorter than the 74 minutes for the USP method. This corresponds to a three times increase in the analysis throughput, and a 75% cost savings for solvents used. The ACQUITY QDa Mass Detector provided excellent detection selectivity, which is a great asset in method transfer and development, as well as in the isoflavone analysis of unknowns and challenging samples where potential interference risk is high. Analysis of isoflavones in three dietary supplements showed compliance in the label claims for two samples. Low isoflavone content was found in one of the three tested samples for unknown reason.

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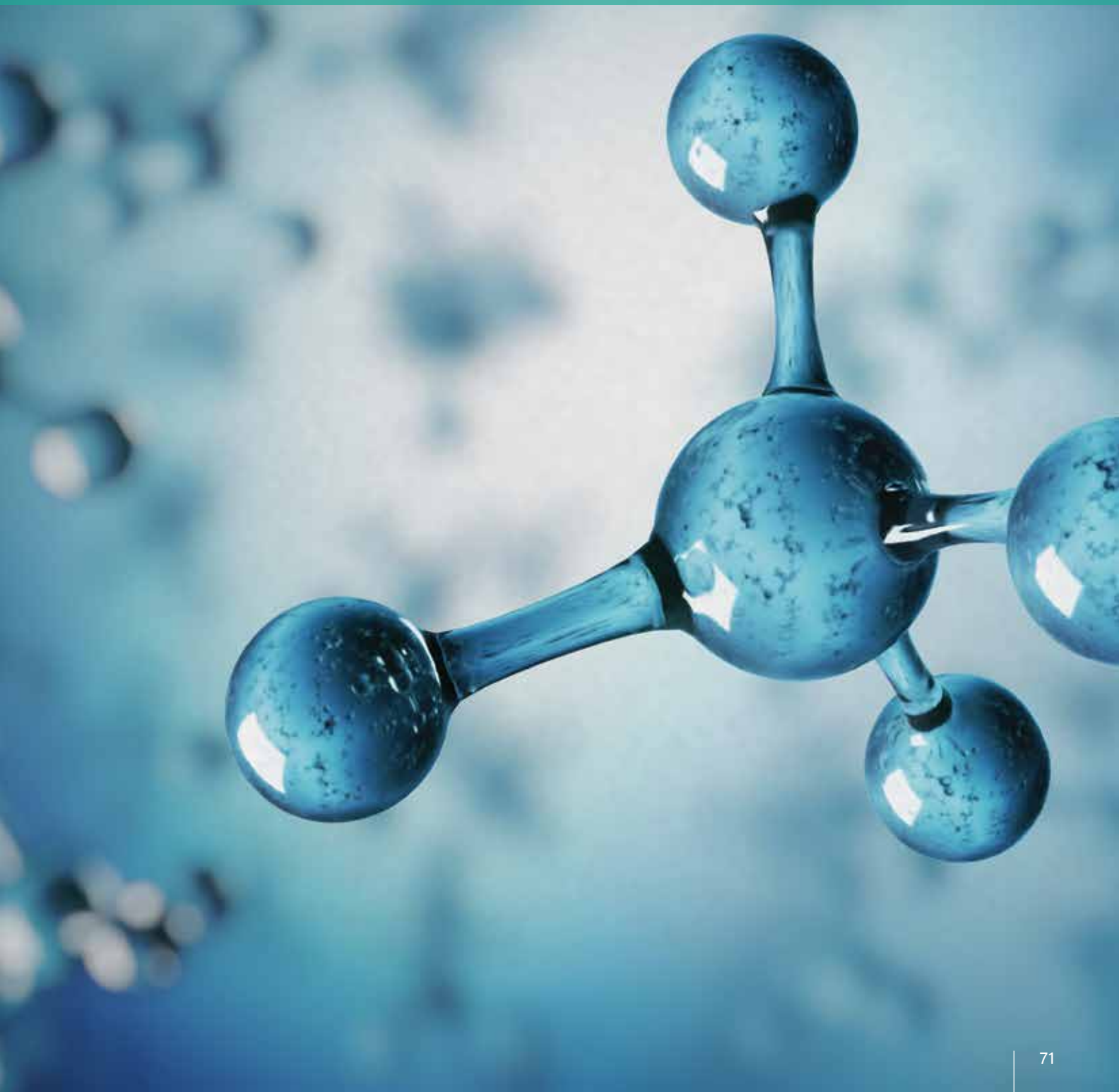

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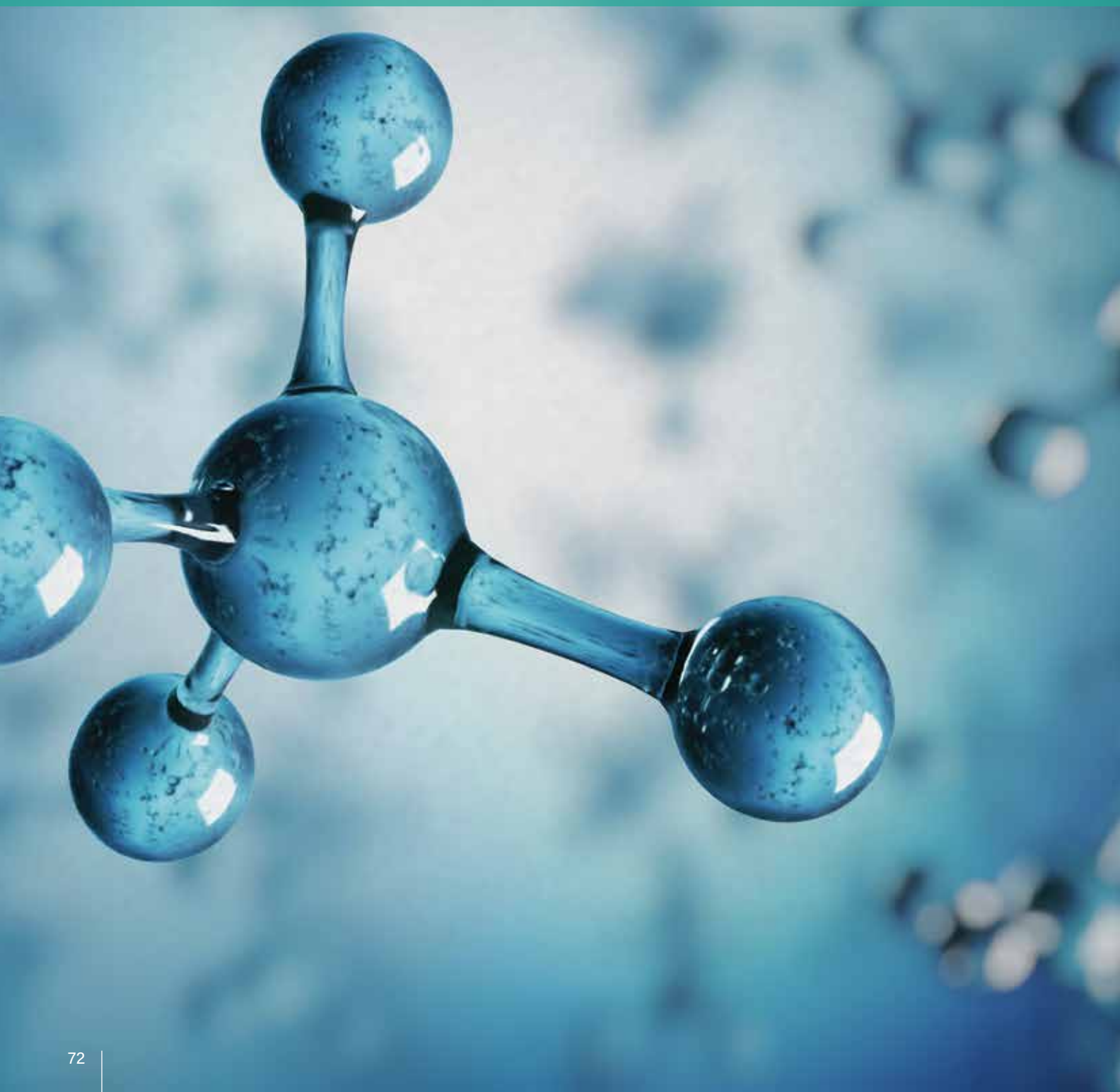

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Exploiting *Rapi*Fluor-MS Labeling to Monitor Diverse N-Glycan Structures via Fluorescence and Mass Detection

Eoin F.J. Cosgrave, Robert Birdsall, and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Reduced sample preparation times for released N-glycan analyses
- Increased confidence in glycan monitoring by obtaining fluorescence and mass detection for every peak

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

ACQUITY® QDa® Mass Detector

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Autosampler with FTN

ACQUITY UPLC Fluorescence Detector (FLR)

ACQUITY UPLC Glycan BEH Amide Column

Empower® 3 FR2 CDS

KEY WORDS

Glycan, mass detection, H-Class, ACQUITY, QDa, *Rapi*Fluor-MS

INTRODUCTION

Glycosylation is one of the most complex post-translational modifications of protein-based biotherapeutics. The efficacy of glycosylated therapeutics is directly related to the glycoprofile. The presence of undesired structures can lead to changes in PK/PD profiles, either positively or negatively, and have been associated with immunogenic responses. For these reasons glycosylation is often designated as a critical quality attribute (CQA). During the development process, the glycoprofile of candidate molecules is extensively studied and characterized. Characteristic profiles are then monitored through process development, commercialization, and post-approval studies to maintain product efficacy and safety.

In this application note, we present a streamlined approach to label released N-glycans with *Rapi*Fluor-MS and analyze the labeled N-glycans with the ACQUITY UPLC H-Class Bio System with fluorescent (FLR) and ACQUITY QDa Mass Detectors. This new monitoring workflow allows researchers to prepare samples from glycoprotein to UPLC-FLR/MS analysis in 30 minutes. In addition to reduced sample preparation times, *Rapi*Fluor-MS yields 14 times greater fluorescence response and 160 times greater MS response when compared to 2-AB. These improvements enable the use of FLR and mass detection with the ACQUITY QDa for routine analysis. In this application note we present the utility of *Rapi*Fluor-MS coupled with UPLC®-FLR-MS for monitoring labeled glycans ranging across a range of properties, masses, and relative abundance.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC FLR and ACQUITY QDa Mass Detector
Column:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	2 µL
Data management:	Empower 3 FR2 CDS

FLR settings

Data rate:	5 points/sec.
Excitation wavelength:	265 nm
Emission wavelength:	425 nm

QDa settings

Sample rate:	5 points/sec
Mass range:	500–1250 Da
Cone voltage:	15 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C
Ionization mode:	ESI+
Mobile phase A:	Acetonitrile (Pierce, LC-MS Grade)
Mobile phase B:	50 mM ammonium formate, pH 4.4, (LC-MS Grade, Waters ammonium formate concentrate)
Mobile phase C:	Acetonitrile (LC-MS grade)
Mobile phase D:	Acetonitrile (LC-MS grade)

Time	Flow rate (mL/min)	% A	% B	%C	% D
Initial	0.400	75	25	0	0
35.0	0.400	54	46	0	0
36.5	0.200	0	100	0	0
39.5	0.200	0	100	0	0
42.5	0.200	75	25	0	0
47.4	0.400	75	25	0	0
55.0	0.400	75	25	0	0

A sample of murine IgG1 mAb N-Glycans was prepared from Waters Intact mAb Mass Check Standard (p/n 186006552), which is included in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003606). N-Glycans were also prepared from RNase B and bovine fetuin (Sigma Aldrich). Released and labeled N-glycan pools were generated using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit following the protocol provided in the Care and Use Manual (715004793). Following release and labeling, samples were dried using a CentriVap™ and reconstituted in 25 µl of a mixture of ACN/Water/DMF at a ratio of 22.5%:55.5%:22%, respectively. In each case the targeted mass load was 30 pmoles of released glycan. The ammonium formate mobile phase was prepared using Waters ammonium formate concentrate (p/n 186007081).

RESULTS AND DISCUSSION

N-glycosylation is a non-template driven process that generates a vast array of glycan structures that vary in size, charge, and extent of branching depending on the protein and expression system. To evaluate the capacity of the ACQUITY QDa to detect glycans both within and beyond its mass range, three glycoproteins (human IgG, RNAse B, and bovine fetuin) were selected to provide typically observed glycans ranging from neutral bi-antennary structures to tetra-sialylated structures. N-glycans from each protein were released using Rapid PNGase F and labeled with *Rapi*Fluor-MS following the provided sample preparation protocol. Labeled glycans were separated via UPLC-HILIC and detected using both an ACQUITY FLR and ACQUITY QDa.

As is evident in Figure 1, each glycan structure is chromatographically resolved using a single gradient method. In addition, each glycan structure observed in fluorescence (top panel) is also observed by the ACQUITY QDa Mass Detector (bottom panel), indicating the ability of the ACQUITY QDa to detect glycans across a range of possible structures and attributes when labeled with *Rapi*Fluor-MS. For traditional labeling technologies this is not possible due to poor ionization efficiency.

While it is useful that glycan structures can be observed by mass detection, it is important to understand the quality of the resulting spectra and the charge states of the glycan ions obtained within. To understand this aspect, we integrated peaks spanning a range of glycan properties and measured the relative abundances of species in each sample using FLR integrated data. The spectra shown in Figure 2 demonstrate the ability of the ACQUITY QDa to generate high quality spectra for glycan structures across a wide range of properties and masses. The data also demonstrate that both high and low abundance glycan structures can be readily detected. Our data indicates that high quality spectra are generated for structures present in the fluorescence profiles at abundances as low as 0.5% highlighting the sensitivity of ACQUITY QDa mass detection combined with the improved ionization efficiency afforded by *Rapi*Fluor-MS. Our data also demonstrate how the improved charging of glycan structures by the use of *Rapi*Fluor-MS allows small structures such as A2, as well as very large structures, such as the tetrasialylated A3G3S4, to be detected with the QDa.

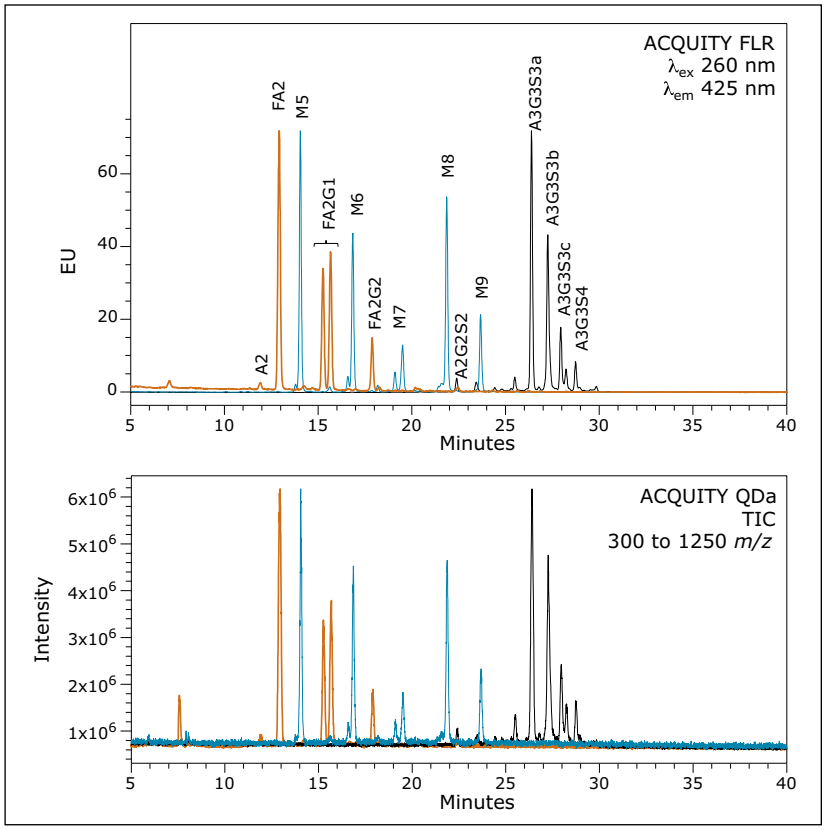

Figure 1. The ACQUITY QDa can detect an array of RapiFluor-MS labeled N-glycans. Glycans from human IgG (red trace), RNAse B (black trace), and bovine fetuin (blue trace) were released with Rapid PNGase F, labeled with RapiFluor-MS reagent. Individual glycan pools were then separated via HILIC and detected with both fluorescence (A) and mass detection (B).

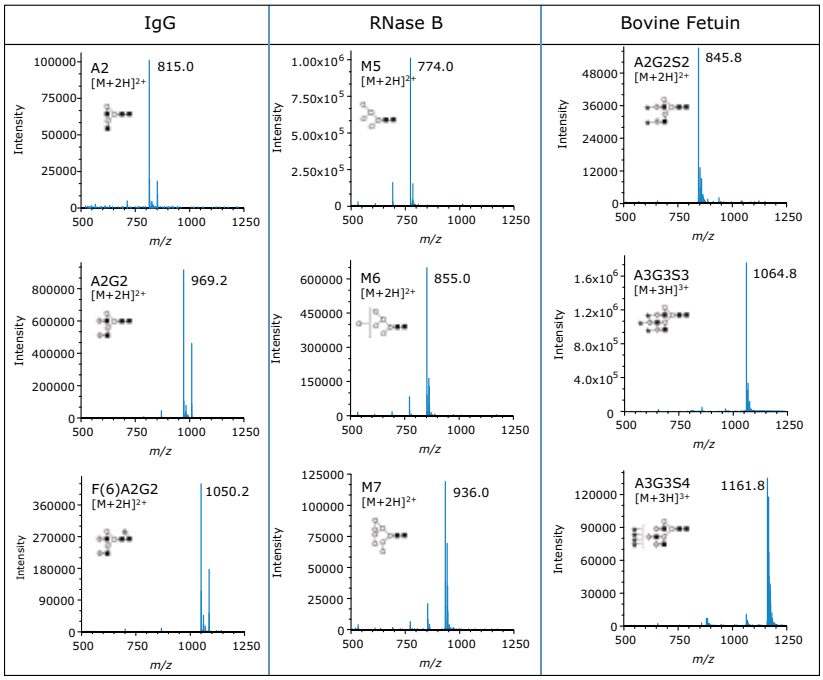

Figure 2. Spectra of selected RapiFluor-MS labeled glycans detected with an ACQUITY QDa Mass Detector. Glycans from murine IgG1 mAb, RNase B, and bovine fetuin were released with Rapid PNGase F and labeled with RapiFluor-MS. Shown are representative spectra for selected glycan structures separated in Figure 1.

CONCLUSIONS

Glycosylation of is a complex and critical aspect of most therapeutic proteins which must be well characterized. Often, the profile of N-glycans is identified as a critical quality attribute and as a result is monitored throughout the lifecycle of products. As discussed in this application note, preparation of samples with a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit can dramatically reduce sample preparation time and complexity. In addition, the use of *Rapi*Fluor-MS yields improved FLR sensitivity and dramatically improved MS sensitivity. Through improving glycan MS sensitivity *Rapi*Fluor-MS labeling permits the use of mass detection with the ACQUITY QDa and thereby affords greater confidence in peak monitoring across the range of structures encountered during biopharmaceutical development. Taken together, *Rapi*Fluor-MS labeling and HILIC-FLR-MS with ACQUITY UPLC H-Class Bio System and the ACQUITY QDa Mass Detector offer an unparalleled solution for monitoring the N-glycan profiles of biotherapeutics.

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LC-UV-MS-based Synthetic Peptide Identification and Impurity Profiling Using the ACQUITY QDa Detector with ProMass Software

Ximo Zhang, Brooke Koshel, Jing Fang, William Alley, Robert Birdsall, and Yinq Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Automated workflow with MassLynx® and ProMass for synthetic peptide target mass confirmation and impurity profiling
- A cost-effective solution for quality control of synthetic peptides using the ACQUITY® QDa® Detector
- Quantitative determination and confirmation of peptide impurities by online UV and MS detection

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY QDa Detector

ACQUITY UPLC Tunable Ultraviolet (TUV) Detector

Waters® CSH column

MassLynx 4.1 Software

KEYWORDS

Synthetic peptide, impurity profiling, batch processing, mass detection, ACQUITY QDa, ProMass

INTRODUCTION

The market for peptide-based drugs is growing due to the broad range of activity and low toxicity of peptides.[1] The use of solid-phase synthesis to produce the majority of peptide drugs often introduces process impurities such as incomplete deprotection of peptides and side reaction products.[2,3] In addition, product-related impurities, such as oxidation and deamidation, add to the complexity of the impurity profile of the drug product.[2,3] To ensure product efficacy and safety, the International Council for Harmonisation (ICH) guidelines require impurities to be identified based on toxicity and dosage levels of a new product.[4] Optical thresholding based on LC-UV detection is commonly used to determine the impurity levels. However, optical detection can only be applied to known or pre-characterized samples, which typically require a laborious process of fractionation and enrichment followed by analysis using MS-based techniques for identification. The continuous growth in peptide-based therapeutics requires efficient workflows that can bring products to market in a timely manner while preserving product quality. Techniques that incorporate orthogonal detection methods, such as mass spectrometry (MS), offer the ability to address the challenges of impurity profiling in synthetic peptide production.

The ACQUITY QDa Detector offers an efficient and cost-effective solution to incorporating MS detection to LC-UV-based workflows.[5] As demonstrated by previous work, the software ProMass (Novatia, LLC) works with MassLynx to provide an automated processing method for MS data generated by the ACQUITY QDa Detector and therefore enables high throughput screening for synthetic peptides.[6]

The purpose of this application note is to demonstrate a cost-effective LC-UV-MS-based workflow using ProMass with MassLynx for mass confirmation and impurity monitoring of synthetic peptides using the ACQUITY QDa Detector as an in-line orthogonal detector. To demonstrate this workflow, a clinically relevant synthetic peptide, eledoisin, is used for analysis.

EXPERIMENTAL

Chemical and reagents

The synthetic peptide eledoisin (pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH2) was purchased from New England Peptide. HPLC grade water, acetonitrile, and formic acid were purchased from Fisher Scientific and used as received. A stock solution of the peptide was prepared at a concentration of 2 mg/mL in water and diluted to the desired concentration. Assay optimization was performed by varying the mass load of the peptide from 0.05 µg to 5.00 µg, while the injection volume was kept constant at 5 µL. A 2 µg sample load was found to be the optimal mass load and was used for all analyses unless otherwise noted.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC TUV Detector, 5 mm flow cell, λ = 215 nm ACQUITY QDa Detector (Performance model)
LC column:	ACQUITY UPLC CSH C_{18}, 1.7 µm, 130 Å, 2.1 mm × 100 mm (P/N 186006937)
Column temp.:	60 °C
Sample vial:	12 mm × 32 mm glass vial, Total recovery (P/N 600000750cv)
Injection volume:	5 µL
Mobile phase A:	H_2O, 0.1% formic acid
Mobile phase B:	Acetonitrile, 0.1% formic acid

Gradient table:

Time (min)	Flow rate (min)	%A	%B
Initial	0.200	82.0	18.0
2.00	0.200	82.0	18.0
2.01	0.200	82.0	18.0
22.01	0.200	72.0	28.0
22.02	0.200	20.0	80.0
24.02	0.200	20.0	80.0
24.03	0.200	82.0	18.0
30.00	0.200	82.0	18.0

ACQUITY QDa Detector settings

Mass range:	350–1,250 *m/z*
Mode:	ESI Positive
Collection mode:	Continuum
Sample rate:	2 points/sec
Cone voltage:	10 V
Probe temp.:	500 °C
Capillary voltage:	1.5 kV

Data management

MassLynx v 4.1

ProMass for MassLynx

RESULTS AND DISCUSSION

METHOD OPTIMIZATION

Synthetic peptide impurity profiling via LC-UV-based techniques requires optimization of peak resolution and detector response. For this study, a 20 minute gradient was found to resolve 11 impurity peaks from the main peak as shown in Figure 1. ICH guidelines recommend impurity identification to be based on total daily intake. For this study, a cut-off of 0.2% relative abundance was used along with S/N ≥ 10 to determine the broadest applicability of the method. Eight of the 11 peaks shown in Figure 1 were identified as peaks meeting this criteria based on automatic peak integration performed by MassLynx and are annotated in blue. Peaks above the optical cut-off of S/N ratio were all observed in the MS spectrum (Figure 1B).

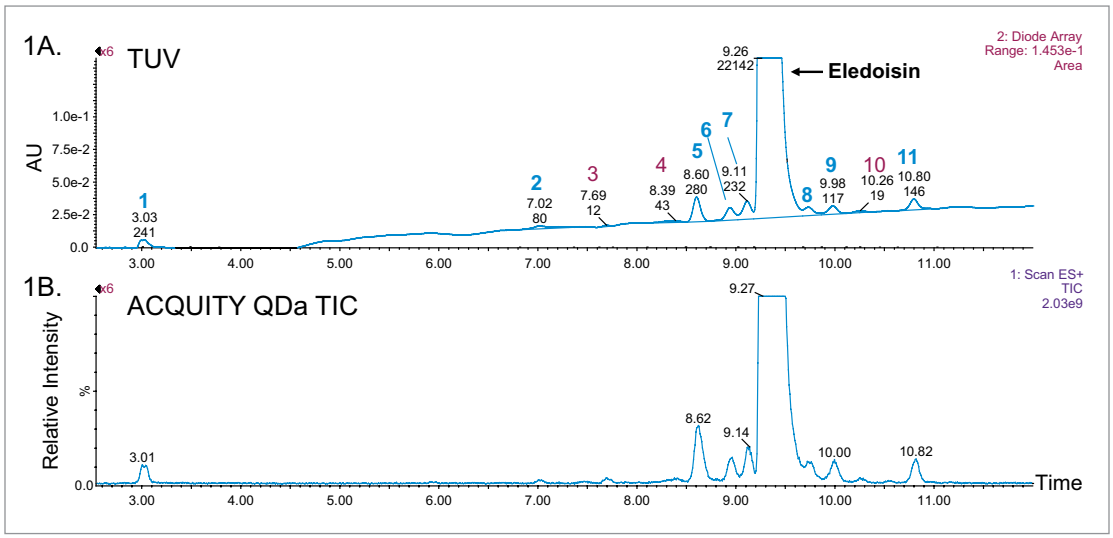

Figure 1. Optimized separation of synthetic peptide eledoisin with orthogonal mass detection. 1A) UV detection at 215 nm. Using the MassLynx integration tool, 11 impurity peaks were identified and annotated with their retention time and area. Peaks above 0.2% area are labeled in blue, otherwise in red. 1B) MS detection by ACQUITY QDa Detector.

Percent purity determined by mass load was used in lieu of a spiking study to determine the working range of the assay. Ten samples were prepared with increasing concentration (from 0.01 mg/mL to 1.0 mg/mL) and evaluated with the optimized gradient. Purity assessment based on total peaks detected in the optical trace was determined to be stable above mass loads of 0.5 µg at 92.74 % ± 0.45%. To evaluate assay precision, three replicate runs were acquired to measure the %RSD of purity. Results showed the %RSD was below 5% at all mass loads. Using the combined data it was determined that a 2 µg mass load offered optimal chromatographic performance while maintaining an S/N ≥10 for eledoisin and its impurities as shown in Figure 2.

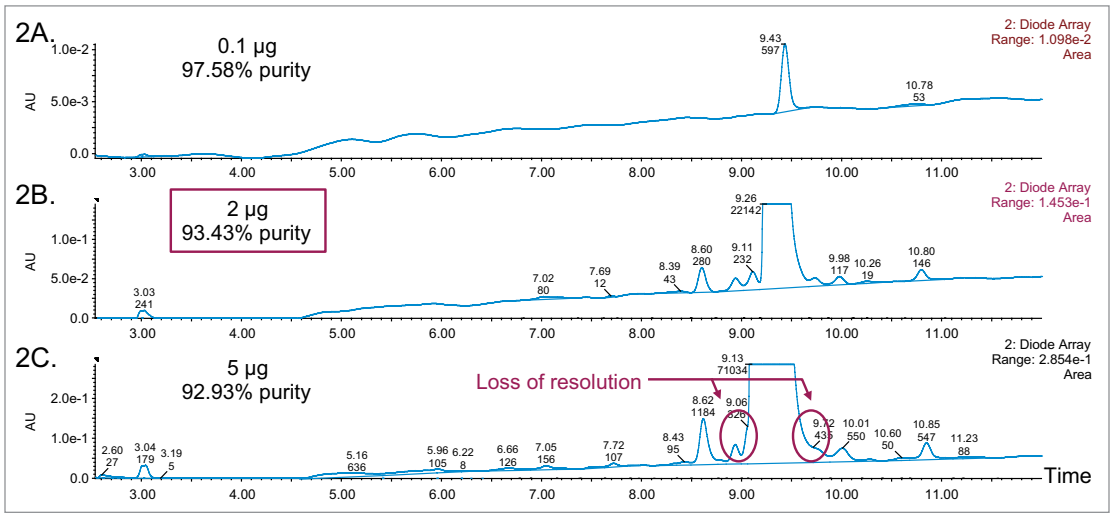

Figure 2. Mass load optimization. Comparison of mass load at 2A) 0.1 µg, 2B) 2 µg, and 2C) 5 µg for synthetic peptide separations are performed using a 20 minute gradient from 18% B to 28% B. Purity was calculated and reported as area percent of target peptide relative to total peak area detected. The optimal mass load was determined to be 2 µg, as higher mass loads resulted in a loss of resolution.

PARAMETERS OF SPECTRUM DECONVOLUTION

With the optimized method determined, the benefit of mass detection can now be fully utilized. The ACQUITY QDa Detector enables the software to determine mass difference between impurities and the target peptide for impurity identification. Automated data acquisition and processing of synthetic peptides can be performed by MassLynx when used in conjunction with ProMass. Figure 3A is an example of the modified Sample List in MassLynx, which contains the instrument methods for data acquisition, as well as information needed for target mass calculation, and parameter files for data deconvolution of synthetic peptides using ProMass. For peptide impurity profiling, the information of the target peptide needs to be entered in the Sequence column as well as the desired Target Info as shown in the zoom-in image of Figure 3A. In this example, 'Ladder = internal' was used to detect all possible fragments with either termini. The target synthetic peptide, eledoisin, has a primary amine group (NH_2) at the C-terminus rather than an OH, thus requiring the termini to be specified as such for correct mass determination. Parameters for MS spectra analysis are defined by the files entered into the ProMassBridge Parameter File column and the Znova Parameter file column. The ProMassBridge Parameter File enables communication between ProMass and MassLynx, and contains user definable parameters such as the retention time window to process, smooth functions and baseline subtraction. As part of the ProMass software, the Znova™ Parameter file provides parameters for spectrum deconvolution, and thresholding for streamlining reporting.

The Znova processing file contains a customizable database for target identification and impurity profiling as shown in Figure 3B. Modified and derivatized amino acids can be entered along with naturally occurring amino acids as part of the "building-blocks" for calculating target masses. In the case of eledoisin, Pyroglutamic Acid (/Pyr/=111.1) was entered to account for the amino acid derivative at the terminus of the synthetic peptide. Similarly, previously characterized impurities can be manually entered as their calculated mass differences from the target peak. As shown in the blue rectangle of Figure 3B, deletion of leucine from the oxidized target peptide (loss of Leu from OxiMet = -97.2), possible peptide fragment (Impurity A = -313.9), and C-terminal substitution (OH terminal group instead of NH_2 = 1) were previously identified by high resolution MS prior to this study. Matching mass differences will be shown in deconvolution reports along with their identities.

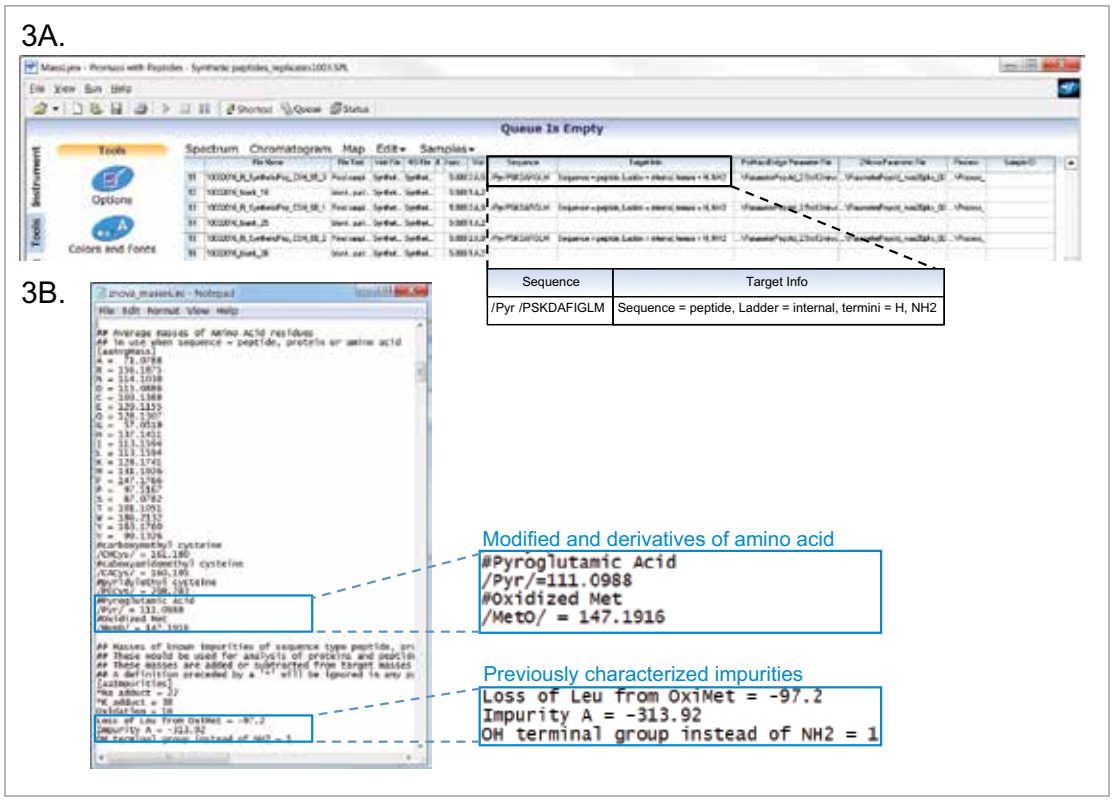

Figure 3. Parameters for data deconvolution. 3A) An example of the modified Sample List in MassLynx. Zoom-in image shows the format for peptide target mass calculation. 3B) The customizable database in Znova Processing File. Naturally occurring amino acids and selective impurities were already included in the database, while entries in the blue rectangle were manually added for target identification and impurity profiling.

RESULTS OF IMPURITY PROFILING

Using the method described above, an analysis of eledoisin was performed for target mass confirmation and impurity screening. Purity of the sample was reported to be 88.87%, which is estimated based on percent abundance of the target mass (including adducts) in the MS spectrum and normalized by chromatographic peak area percent. Percent purity can be found in the Target Mass Summary table, along with other information of the target peptide such as mass error and percent abundance (Figure 4). The sequence Ladder Summary table shows the sequence of identified peptide fragments. The Chromatogram Summary table lists all detected peaks above threshold (0.2%) across the chromatogram. The Spectral Quality, which is based on abundance and the number of charge states detected, was reported "ok" for all impurities with the exception of peaks 4 and 5 which were below the cutoff value (Score ≥4). In this example, the Low Score warning at 8.40 min (peak 4) results from a high degree of spectral noise, and the one at 8.62 min (peak 5) is from the misidentification of the base peak charge due to only one charge state being present in the spectrum. The Spectrum Quality threshold, which can be set by the user, enhances the confidence of impurity identification results by indicating the quality of both input and output spectra for deconvolution.

ProMass results:

Target Mass Summary

RT (min)	Target Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	% Abundance (in Spectrum)	%Purity (Estimate)	Identity	Result Code
9.28	1188.4	1188.2	-0.2 Da (-0.017 %)	2.42E+009	91.67	88.87	Target Mass	

Sequence Ladder Summary

RT (min)	Calculated Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	Sequence
9.28	1188.4	1188.2	-0.2 Da (-0.017 %)	2.42E+009	/Pyr/1-M11
8.40	1058.2	1057.2	-1.0 Da (-0.095 %)	9.47E+005	/Pyr/1-L10
8.95	566.6	566.4	-0.2 Da (-0.035 %)	3.67E+005	S3-F7

Hyperlinks

Chromatogram Summary

Peak	RT (min)	Base Peak Mass (Da)	Intensity	Spectral Quality	LC/MS Peak Area	LC/MS Area Percent
1	3.02	1204.9	1.43E+007	ok	2.58E+006	0.68
2	7.02	874.8	3.78E+006	ok	4.62E+005	0.12
3	7.70	1091.2	4.08E+006	ok	6.98E+005	0.18
4	8.40	1003.6	2.83E+006	low score	7.03E+005	0.19
5	8.62	637.4	2.42E+007	low score	9.02E+006	2.38
6	8.95	1131.1	2.04E+007	ok	2.77E+006	0.73
7	9.12	1116.9	2.79E+007	ok	2.92E+006	0.77
Eledoisin	9.28	1188.2	2.42E+009	ok	3.52E+008	92.80
8	9.76	1259.5	1.70E+007	ok	1.21E+006	0.32
9	9.99	1101.0	1.27E+007	ok	3.02E+006	0.80
10	10.26	1188.5	2.71E+006	ok	5.25E+005	0.14
11	10.82	1189.1	2.17E+007	ok	3.38E+006	0.89

Figure 4. Summary tables of peptide impurity profiling. Target mass and percent purity are shown in the Target Mass Summary table. Peaks above 0.2% with UV detection are labeled in blue numbers in the Chromatogram Summary table. All color coded entries in the tables are hyperlinks that lead to further details of deconvolution.

As part of the ProMass software, additional details can be obtained for each analysis by clicking the hyperlinks in Chromatogram Summary table. An example for the peaks at 9.99 min is shown in Figure 5. The top panel shows a list of peaks with their scores and presumed identities. The bottom panel shows the deconvoluted mass spectrum for all detected peaks at 9.99 min. In this list, it is suggested that the mass 1101.0 Da could be a deletion of Serine (S), from the target peptide. Data processing results obtained from ProMass are summarized in Table 1, including retention time, percent area and its %RSD, mass, mass difference between impurity and the target peptide, and presumed identity of detected impurities. The identification of impurities were highly reproducible for all monitored impurities in three replicate runs demonstrating the reliability of LC-MS based workflows in impurity profiling of synthetic peptides.

Hyperlinked embedded data:

Deconvolution Peak Report

Mass Peak List Sorted by Intensity:

Mass(Da)	+- Std. Dev.	Intensity	Score	Delta Mass	%Relative	%Total	Presumed Identity
1101.0	0.2	1.27E+007	4.62	0.0	100.00	55.48	1188.4 (Minus S)
1154.3	0.5	3.65E+006	4.94	53.3	28.61	15.87	
1241.1	0.4	2.42E+006	3.98	140.1	19.02	10.55	
1242.6	0.7	1.75E+006	3.01	141.6	13.75	7.63	
1123.7	0.9	1.41E+006	3.87	22.7	11.03	6.12	
1189.6	2.0	3.44E+005	3.64	88.6	2.70	1.50	
1170.7	0.8	3.35E+005	3.26	69.7	2.63	1.46	
759.9	1.5	1.77E+005	3.10	-341.1	1.39	0.77	
769.6	0.7	1.42E+005	3.21	-331.4	1.11	0.62	

Mass Peak List Sorted by Mass:

Mass(Da)	+- Std. Dev.	Intensity	Score	Delta Mass	%Relative	%Total	Presumed Identity
759.9	1.5	1.77E+005	3.10	-341.1	1.39	0.77	
769.6	0.7	1.42E+005	3.21	-331.4	1.11	0.62	
1101.0	0.2	1.27E+007	4.62	0.0	100.00	55.48	1188.4 (Minus S)
1123.7	0.9	1.41E+006	3.87	22.7	11.03	6.12	
1154.3	0.5	3.65E+006	4.94	53.3	28.61	15.87	
1170.7	0.8	3.35E+005	3.26	69.7	2.63	1.46	
1189.6	2.0	3.44E+005	3.64	88.6	2.70	1.50	
1241.1	0.4	2.42E+006	3.98	140.1	19.02	10.55	
1242.6	0.7	1.75E+006	3.01	141.6	13.75	7.63	

Deconvoluted Mass Spectrum

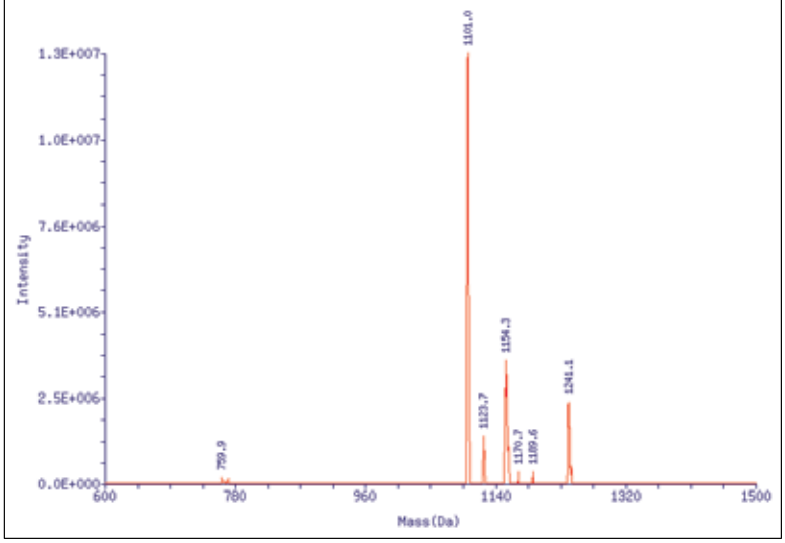

Figure 5. Hyperlinked embedded details of impurity profiling results, such as deconvoluted mass spectra and peak reports, can be accessed through the hyperlinks in the summary tables in Figure 4.

RT (min)	MS %area	%RSD	Mass (Da)	ΔMass (Da)	Score	Identity
3.02	0.68	3.53	1204.9	16.7	4.86	Oxidation
7.02	0.12	5.09	874.8	-313.4	5.01	Impurity A
7.70	0.18	9.35	1091.2	-97	4.09	Minus P or L
8.40	0.19	12.01	1003.6	-184.6	3.77	pE-KDAFIGLM-NH_2
8.62	2.38	2.73	637.4	-550.8	3.04	Not identified
			1188.9	0.7	5.11	pE-PSKDAFIGLM-OH
			1245.0	56.8	5.34	Plus G
8.95	0.73	1.57	1131.1	-57.1	4.95	Minus G
9.12	0.77	2.95	1116.9	-71.3	5.12	Minus A
9.28	92.80	0.08	1188.2	0	6.66	pE-PSKDAFIGLM-NH_2
9.76	0.32	8.17	1259.5	71.3	4.59	Plus A
9.99	0.80	4.56	1099.5	-88.7	4.62	Minus S
10.26	0.14	13.32	1188.4	0.3	4.34	pE-PSKDAFIGLM-NH_2
10.82	0.89	2.90	1189.1	0.9	4.82	pE-PSKDAFIGLM-OH

Table 1. Summary of impurities with their percent peak area, %RSD of percent peak area based on three replicate runs, mass, mass difference between impurity and the target peptide, score, and presumed identity.

CONCLUSION

An automated workflow of synthetic peptide mass confirmation and impurities profiling was developed using the ACQUITY QDa Detector with MassLynx and ProMass. Impurities above 0.2% optical threshold were successfully detected and identified for eledoisin at 2 µg mass load. Retention time, mass, and presumed identity of both the target peptide and impurities were summarized in ProMass deconvolution summary tables and reports. Confidence of deconvolution was represented as a Score for each peak. The addition of the ACQUITY QDa Detector to an optical workflow significantly reduces the time of impurity analysis. With ProMass for MassLynx, the automated workflow allows high throughput screening and therefore improves the productivity of synthetic peptide impurity profiling in development and quality control environments.

References

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Monitoring Multiple Attributes in a Single Assay Using the ACQUITY QDa Detector for Product Confirmation and Process Monitoring of Product Quality Attributes

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Multiple attribute monitoring through a single acquisition to enable both product confirmation and routine screening of post-translational modifications
- Empower® Software enables an automated and compliant workflow for data acquisition, processing, and reporting of multiple product quality attributes

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Tunable Ultraviolet (TUV) Detector

ACQUITY QDa® Detector

ACQUITY UPLC Peptide CSH C_{18} Column

Empower 3 Chromatography Data Software

KEYWORDS

Monoclonal antibody, mAb, multi-attribute monitoring, MAM, product quality attributes, PQA, ACQUITY QDa Detector, peptide map

INTRODUCTION

The concept of using a single LC-MS-based analytical method to monitor multiple product quality attributes (PQAs) is a strategy that has started to gain momentum in the biopharmaceutical industry. The idea behind this concept is that a single LC-MS method can be used to assess a product's important quality attributes simultaneously, as opposed to running a panel of optically-based chromatographic methods, which are unable to assess product attributes at a molecular level. The motivation for extending mass spectrometry (MS) beyond characterization into all stages of development and even quality control, is largely due to the complexity of protein-based therapeutics compared to small molecule drugs.[1] Replacing conventional chromatographic methods with a single, more sophisticated LC-MS method ultimately provides greater product and process understanding, which is required to support Quality by Design (QbD) regulatory submissions. Implementation of QbD is encouraged by regulatory agencies as a way of improving product quality and patient safety by offering a systematic and proactive approach to product development.[2,3]

It has recently been shown that a method for identity testing which monitors the complementarity determining region (CDR) peptides of a monoclonal antibody (mAb) has been developed and validated using the ACQUITY QDa Detector.[4] Using this work as the foundation of our study, we consider the need for a single test to be used for both product confirmation and also for monitoring a number of pre-characterized post-translational modifications (PTMs) using the ACQUITY QDa Detector, which provides a cost-effective solution for incorporating mass data into analysis. To align with the published work, a sample of trastuzumab and Waters Intact mAb Standard can be checked against the CDR peptides of a trastuzumab reference standard to verify method specificity through retention time and mass determination. Because a drug product's critical quality attributes (CQAs) would need to be independently determined, the current study is meant to serve as a proof of concept for providing a strategy for identifying multiple attributes within a single study using the ACQUITY QDa Detector with compliant-ready chromatographic data software such as Empower.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC TUV ACQUITY QDa Detector (Performance Model)
Absorption wavelength:	215 nm
Column:	ACQUITY UPLC Peptide CSH C_{18} 130 Å, 1.7 µm, 2.1 mm x 100 mm (P/N 186006937)
Column temp.:	65 °C
Mobile phase A:	H_2O with 0.1% (v/v) formic acid
Mobile phase B:	Acetonitrile with 0.1% (v/v) formic acid
Sample temp.:	10 °C
Injection volume:	10 µL

Gradient:

Time (min)	Flow rate (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	67	33	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
150.00	0.200	97	3	0	0

Detector settings

Sampling rate:	2 Hz
Mass range:	350–1250 Da
Ionization mode:	ESI+, centroid
Cone voltage:	10 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C

Data management

Empower 3 CDS, SR2

RESULTS AND DISCUSSION

MONITORING MULTIPLE ATTRIBUTES USING EXTRACTED ION CHROMATOGRAMS FOR IDENTITY CONFIRMATION

The variable region of an antibody contains CDR peptides that are unique to that specific antibody, which allows these peptides to be used for identification purposes. To begin the evaluation of the ACQUITY QDa Detector for reporting multiple attributes, we began by collecting a peptide map of trastuzumab. A trypsin digest of reduced and alkylated trastuzumab was prepared and injected at a final concentration of approximately 0.5 mg/mL without further dilution. The peptide mapping method described above was used with the ACQUITY QDa Detector set to collect a full scan so that extracted ion chromatograms (XICs) could be used to identify the attributes of interest. Figure 1 shows a strong correlation between the optical trace and the corresponding mass data. This data suggests that the ACQUITY QDa Detector provides an effective way to incorporate mass measurements into an LC-UV based peptide map assay.

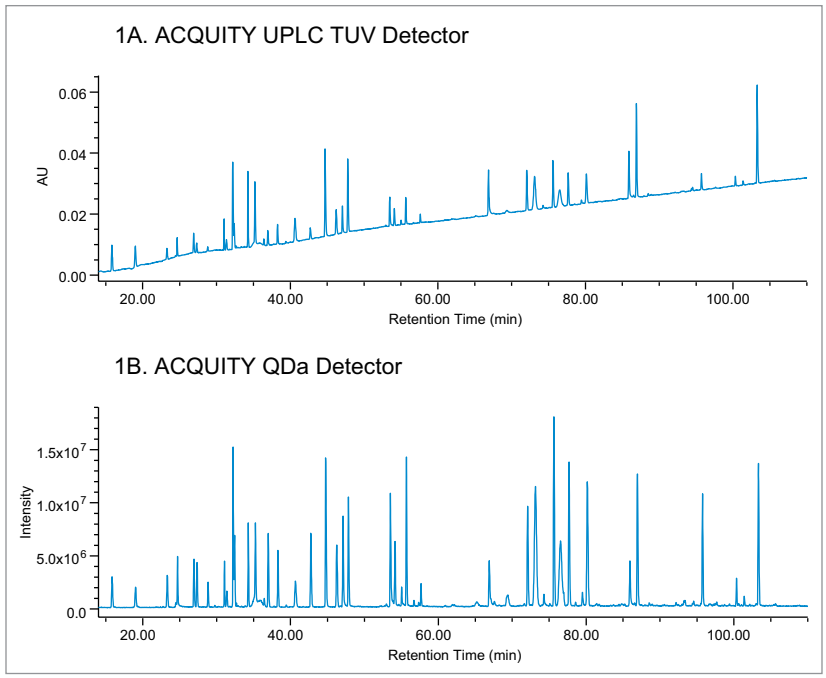


Figure 1. Peptide map detection. 1A) Optical detection of a trypsin digest of trastuzumab. 1B) Corresponding ACQUITY QDa data.

The CDR peptides can be used to confirm the identity of a given antibody by using XICs to extract the *m/z* of each CDR peptide. To avoid manual integration of each of the respective XIC channels, derived channels can be used. The process for creating derived channels has been previously described.[5] In brief, a method set can be created to contain a derived channel containing the *m/z* of each of the CDR peptides. This will essentially extract the *m/z* of each of the desired peptides into a single channel. By linking the derived channel to a processing method with associated retention time and component labels, the result is a single channel containing each of the CDR peptides which are now identified according to mass and retention time. Figure 2A shows a screen capture from Empower that illustrates how a method set can be created to contain derived channels, where a new derived channel can be created for each of the attributes of interest. In the case of the CDR peptides, the mass of six CDR peptides can be entered into the Formula field of the derived channel as shown in Figure 2B. In this example, a single dominant charge state is used to identify each of the CDR peptides, but the user could sum over additional charge states if desired.

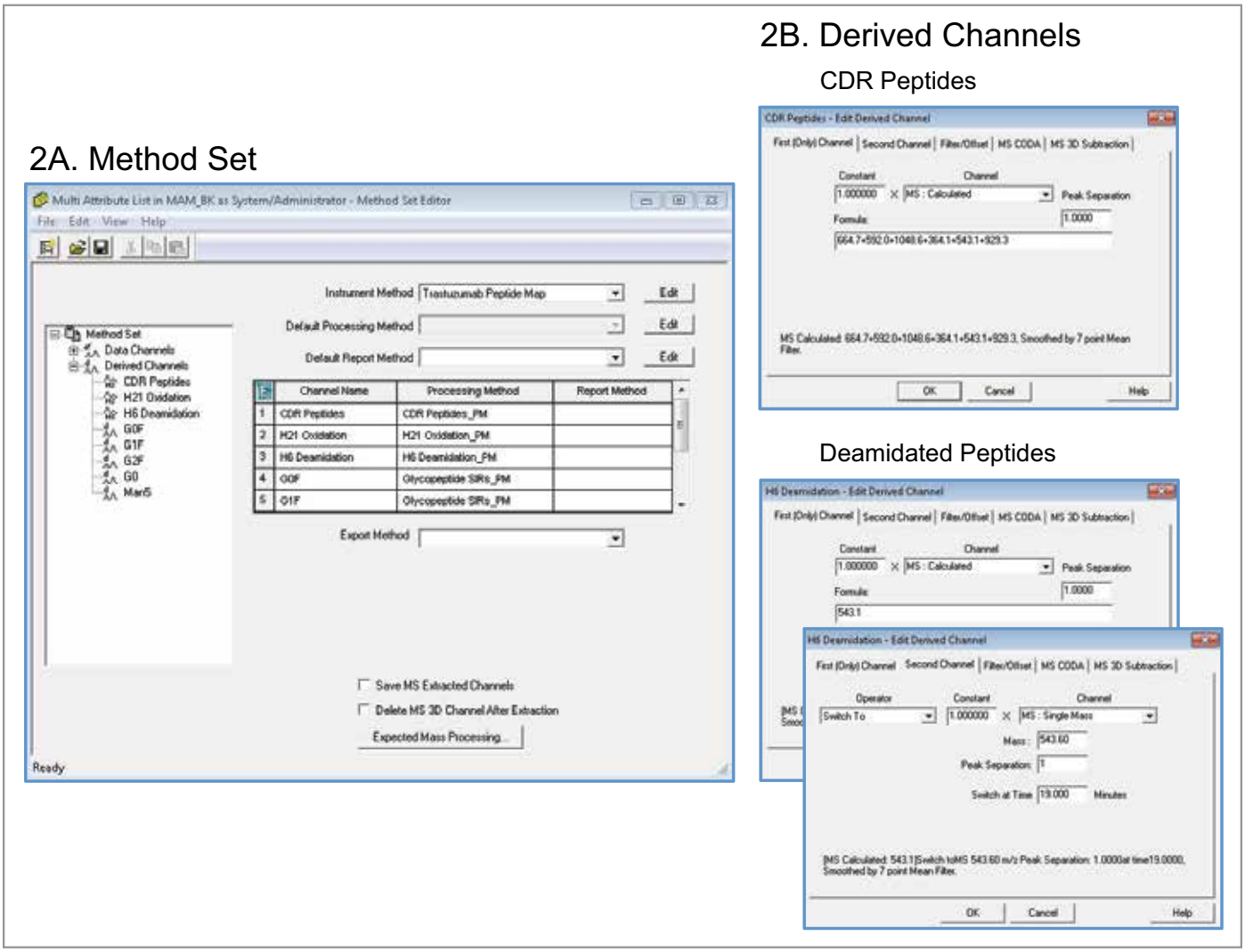


Figure 2. Empower screen captures. 2A) A method set containing derived channels used to determine if previously characterized attributes are present in a sample. The attributes of interest in this case are CDR peptides, an oxidized peptide, a deamidated peptide, and glycopeptides. Each attribute has an independent processing method associated with it. Attributes to be quantitated using XICs can be monitored through a single derived channel for that attribute, while attributes to be quantitated using SIRs must be monitored through individual channels and quantitated through custom calculations. 2B) Examples of derived channels. To create a derived channel for CDR peptides, the m/z of each peptide can be entered into the formula field. Six CDR peptides are monitored in this example. For the deamidated peptide, a time switch can be entered to switch from one m/z to another at a given time. This avoids signal overlap from using XICs having only a small difference in mass between the native and modified peptides.

The average mass for each of the CDR peptides is reported in Table 1 as well as the calculated *m/z* values used for this study. This table also contains mass information for additional attributes to be monitored as discussed below. The chromatogram resulting from the derived channel can be seen in Figure 3A. In this figure, XICs of each of the six CDR peptides are clearly identified, which confirms sample identity.

MONITORING MULTIPLE ATTRIBUTES USING EXTRACTED ION CHROMATOGRAMS FOR PROCESS MONITORING

A similar approach can be used to quantify chemical modifications, such as deamidation or oxidation. Process changes during manufacturing or storage conditions can affect the rate of modification, which could potentially impact antibody activity or antigen binding.[6] In this example, we consider asparagine deamidation and methionine oxidation. Again, we make the assumption that these modifications would have been characterized using high resolution MS and determined to be important quality attributes.

Because the mass difference of the oxidation modification is large, this modification can be tracked in derived channels in a similar manner as the CDR peptides. The resulting XICs can be seen in Figure 3B. The deamidation event, however, shows a much smaller difference in mass between the native and deamidated peptides, so the data must be treated in a different way to reliably determine the relative abundance of each of the peptides. In this case, because the peaks are chromatographically resolved, a derived channel can be used that switches from one calculated mass to another at a time established by the user. This process is more clearly illustrated in Figure 2B. From the Empower screen captures, the mass to be monitored switches from *m/z*=543.1 Da (native peptide, z=2) to 543.6 Da (deamidated peptide, z=2) at 19 minutes. Using a second channel avoids signal overlap from the native and deamidated species. The XICs of the native and deamidated peptide can be seen in Figure 3C.

Peptide	Identification/ Modification	Average mass (Da)	Charge state	Calculated (*m/z*)
L3	CDR	1991.17	$[M+3H]^{+3}$	664.7
L5	CDR	1773.04	$[M+3H]^{+3}$	592.0
L7	CDR	4190.48	$[M+4H]^{+4}$	1048.6
H3	CDR	1089.21	$[M+3H]^{+3}$	364.1
H6	CDR	1084.18	$[M+2H]^{+2}$	543.1
H6	Deamidation	1085.17	$[M+2H]^{+2}$	543.6
H12	CDR	2785.01	$[M+3H]^{+3}$	929.3
H21	Native	834.43	$[M+2H]^{+2}$	418.2
H21	Oxidation	850.42	$[M+2H]^{+2}$	426.2
H25	G0F	2634.53	$[M+3H]^{+3}$	879.2
H25	G1F	2796.67	$[M+3H]^{+3}$	933.2
H25	G2F	2958.81	$[M+3H]^{+3}$	987.3
H25	G0	2488.39	$[M+3H]^{+3}$	830.5
H25	G1	2650.53	$[M+3H]^{+3}$	884.5
H25	Man5	2406.28	$[M+3H]^{+3}$	803.1

Table 1. Peptide information for reported attributes.

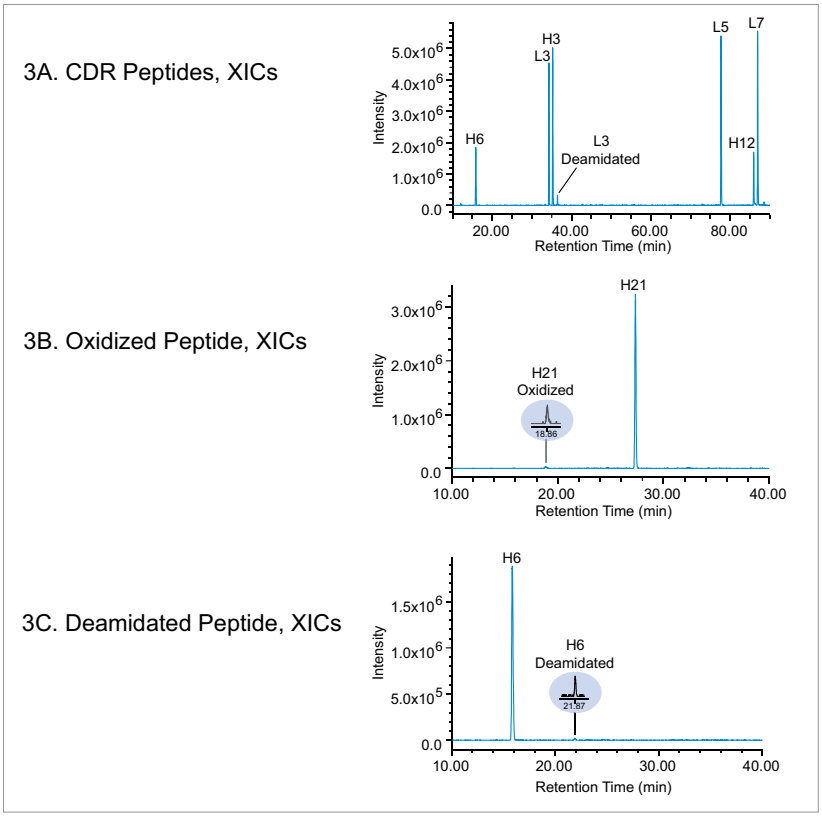


Figure 3. XICs used to identify attributes of interest. Peak labels "H" and "L" refer to heavy chain and light chain peptides, respectively. 3A) The CDR peptides unique to trastuzumab. 3B) A native peptide and its oxidized form. 3C) A native peptide and its deamidated form. Note that the native peptide, H6, is also a CDR peptide. This peptide can function to establish identity but can also be used along with its deamidated form to quantitate the percent modification independent of the CDR channel. Insets in 3B. and 3C. show a 10X zoomed in image of the oxidized and deamidated peptides, respectively. Signal-to-noise is well above minimum requirements to reliably quantitate these low level modifications.

MONITORING MULTIPLE ATTRIBUTES USING SELECTED ION RECORDING FOR PROCESS MONITORING

When additional specificity and sensitivity are needed for peak monitoring, selected ion recording (SIR) can be used. By incorporating SIRs, a single *m/z* is selected and passed through to the detector. To demonstrate the utility of SIRs, the dominant charge state of the five most abundant glycopeptides (G0F, G1F, G2F, G0, and Man5) was previously determined. Figure 4 shows an overlay of the five SIR channels.

For monitoring glycopeptides, the additional sensitivity afforded by the SIRs is required for accurate quantitation. Because each SIR is associated with its own channel, custom calculations can be used to calculate the relative abundance of each glycopeptide.

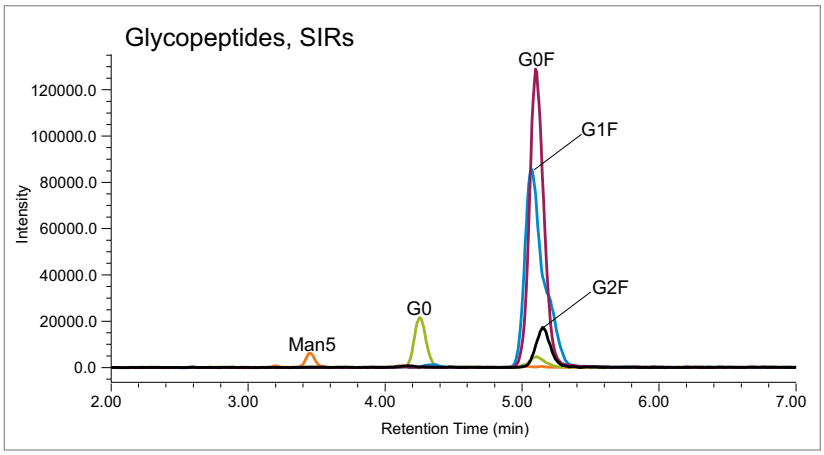
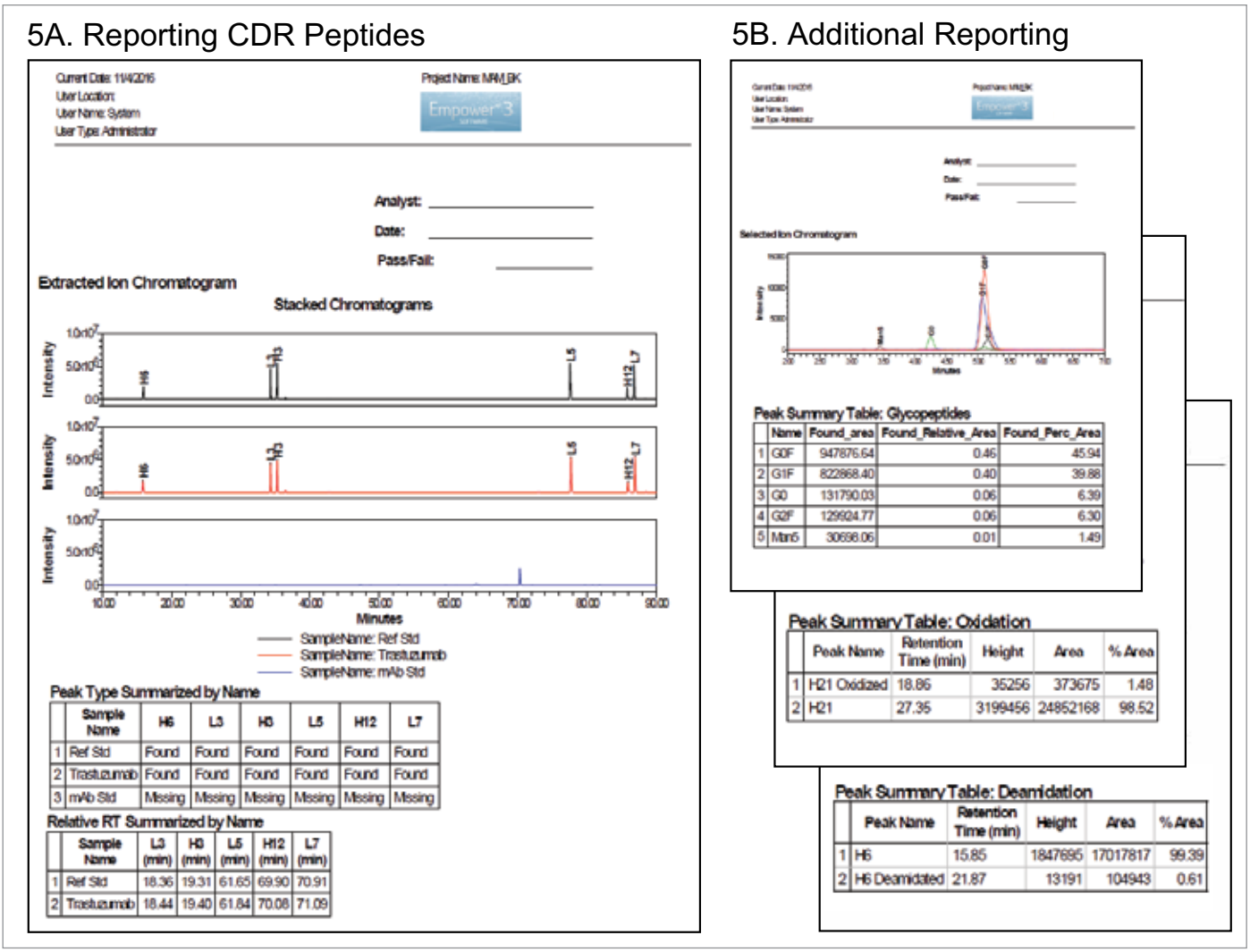


Figure 4. Overlay of five SIR channels used for determining relative abundance of five glycopeptides. Because each SIR is collected in an individual channel, custom calculations must be used to automate the process of determining relative abundance of each glycopeptide.

EMPOWER SOFTWARE ENABLES AUTOMATED REPORTING OF MULTIPLE ATTRIBUTES

We have just shown how a method set can be created to contain derived channels used to monitor attributes of interest, each of which can have an independent processing method associated with it. This same method set can be used to associate independent reporting methods for each of the attributes of interest, which aids to further automate the monitoring process. Should a user wish to import all results into a single report, Empower can accommodate this functionality as well. Figure 5 shows screen captures of Empower reports generated to monitor each of the attributes previously discussed.



	Sample Name	H6	L3	H3	L5	H12	L7
1	Ref Std	Found	Found	Found	Found	Found	Found
2	Trastuzumab	Found	Found	Found	Found	Found	Found
3	mAb Std	Missing	Missing	Missing	Missing	Missing	Missing

	Sample Name	L3 (min)	H3 (min)	L5 (min)	H12 (min)	L7 (min)
1	Ref Std	18.36	19.31	61.65	69.90	70.91
2	Trastuzumab	18.44	19.40	61.84	70.08	71.09

	Name	Found_area	Found_Relative_Area	Found_Perc_Area
1	G0F	947876.64	0.46	45.94
2	G1F	822868.40	0.40	39.88
3	G0	131790.03	0.06	6.39
4	G2F	129924.77	0.06	6.30
5	Man5	30698.06	0.01	1.49

	Peak Name	Retention Time (min)	Height	Area	% Area
1	H21 Oxidized	18.86	35256	373675	1.48
2	H21	27.35	3199456	24852168	98.52

	Peak Name	Retention Time (min)	Height	Area	% Area
1	H6	15.85	1847695	17017817	99.39
2	H6 Deamidated	21.87	13191	104943	0.61

Figure 5. Empower reporting. 5A) Comparing a reference standard, a sample of trastuzumab, and an intact mAb standard (negative control). Peak tables summarize whether the peaks of interest were detected and the relative retention time for each CDR peptide. In lieu of an internal standard, H6 was used to calculate relative retention time. 5B) Additional reporting for a sample of trastuzumab. Each of the items reported is the result of designing a processing method and derived channel for that specific attribute. In reporting relative abundance of the glycopeptides, Found_area, Found_Relative_Area, and Found_Perc_Area are defined by the custom calculations functionality in Empower. These fields are created so that peak area from individual channels can be reported relative to one another.

CONCLUSIONS

The ACQUITY QDa Detector provides an efficient and cost-effective solution for monitoring important product attributes in a development or QC environment when characterization has been previously carried out using a high resolution MS instrument. This application note demonstrates that CDR peptides, oxidized and deamidated peptides, and glycopeptides can be identified, quantitated, and reported from a single acquisition using the derived channel and inter-channel calculation functionalities in the Empower software. The list of attributes to be monitored would need to be determined for each respective antibody, but could be readily expanded to meet the individual user's needs.

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Adding Mass Detection to Synthetic Oligonucleotide Analyses with the ACQUITY QDa Detector

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APPLICATION BENEFITS

- Addition of mass data to synthetic oligonucleotide assays using MassLynx® enabled with MaxEnt™ deconvolution algorithm
- Complementary mass information using traditional IP-RPLC mobile phases
- Increased productivity through the use of on-line orthogonal detection techniques

WATERS SOLUTIONS

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

ACQUITY UPLC BEH Column

MassLynx SCN 9.25 with MaxEnt1

KEY WORDS

Oligonucleotide, mass detection, H-Class, ACQUITY, QDa

INTRODUCTION

The Waters® ACQUITY QDa Detector has been established as a cost-effective means for obtaining mass spectral data within existing optically-based LC workflows in the biopharmaceutical manufacturing environment when used as an orthogonal detection technique.[1-3] Research into therapeutic oligonucleotides has received steadily increasing attention from the pharmaceutical industry due to potential applications using deoxyribonucleic acid (DNA) sense/antisense oligonucleotides and interfering ribonucleic acid- (iRNA) based therapies.[4-5] IP-RPLC has become a prevalent technique in the analysis of synthetic oligonucleotides in part due to the selectivity offered by such techniques as well as its ability to incorporate MS-friendly reagents and buffers as first demonstrated by Apffel and colleagues.[6-7] Mass information afforded by MS detection offers an efficient means of identifying challenging base modifications for improved productivity in synthetic therapeutic oligonucleotide workflows. A natural extension of the ACQUITY QDa portfolio is to evaluate its applicability as an orthogonal detection technique in the analysis of synthetic oligonucleotides.

The objective of this application note is to demonstrate that the ACQUITY QDa Detector provides a simple and cost-effective solution for detecting oligonucleotides across a wide molecular weight range and can be readily integrated into existing UV-based workflows. A set of polyT standards ranging from 15 nt to 35 nt in length were used in this study to evaluate the accuracy and compatibility of the ACQUITY QDa Detector in oligonucleotide analyses using traditional IP-RPLC mobile phases comprised of triethylamine (TEA) and 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP).

EXPERIMENTAL

Triethylamine (99.5% purity) and 1,1,1,3,3,3-Hexafluoro-2-propanol (99.8% purity, LC-MS grade) were purchased from Sigma Aldrich. LC-UV grade solvents (Optima series) were purchased from Fisher Scientific. Mobile phase buffers were newly prepared prior to experiments. PolyT oligonucleotide standards were purchased from Waters (p/n 186004135) and prepared at a concentration of 10 pmol/uL. Mass loads on column were kept constant at 50 pmol or 5 µL injections.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detectors:	ACQUITY UPLC TUV w/Ti flow cell, ACQUITY QDa Detector
Absorption wavelength:	260 nm
Column:	ACQUITY UPLC Oligonucleotide BEH C_{18} Column, 130Å, 1.7 µm, 2.1 mm x 50 mm (p/n 186003949)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Mobile phase A:	15 mM TEA, 400 mM HFIP prepared in H_2O, pH 8.0
Mobile phase B:	15 mM TEA, 400 mM HFIP prepared in MeOH

*mobile phases prepared gravimetrically

Gradient:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	81.0	19.0	0	0
15.00	0.200	73.5	26.5	0	0
16.00	0.200	50.0	50.0	0	0
17.00	0.200	81.0	19.0	0	0
21.00	0.200	81.0	19.0	0	0

QDa Detector settings

Sample rate:	2 points/sec
Mass range:	410–1,250 Da
Mode:	negative
Collection mode:	continuum
Cone voltage:	20 V
Capillary voltage:	0.8 kV
Probe temp.:	600 °C

Data management

MassLynx SCN 9.25 with MaxEnt1

RESULTS AND DISCUSSION

IP-RPLC has become the prevalent separation technique in the analysis of oligonucleotides in part due to the high separation efficiency afforded by such methods when compared to alternative charge-based separations.[5] The use of MS-friendly buffers such as TEA/HFIP – as first demonstrated by Apffel and colleagues – provides an efficient means in the identification of challenging base modifications of oligonucleotides based on orthogonal mass spectrometry data.[6-7] To demonstrate that the ACQUITY QDa is compatible with such IP-RPLC methods, a separation of five polyT standards of increasing length were separated using a TEA/HFIP buffer. As shown in Figure 1, the optical chromatogram (TUV) and response from the mass detector (QDa) – which was in a serial configuration post optical detector – show a high degree of correlation. From this data, it is evident that the ACQUITY QDa Detector is capable of providing mass spectral data using traditional IP-RPLC methods that incorporate ion pairing agents such as TEA buffered in HFIP. As shown in Figure 1, the intensity profile of the ACQUITY QDa Detector is similar but not identical to the UV profile. Interrogation of the raw MS spectrum was performed to gain insight into intensity profile differences.

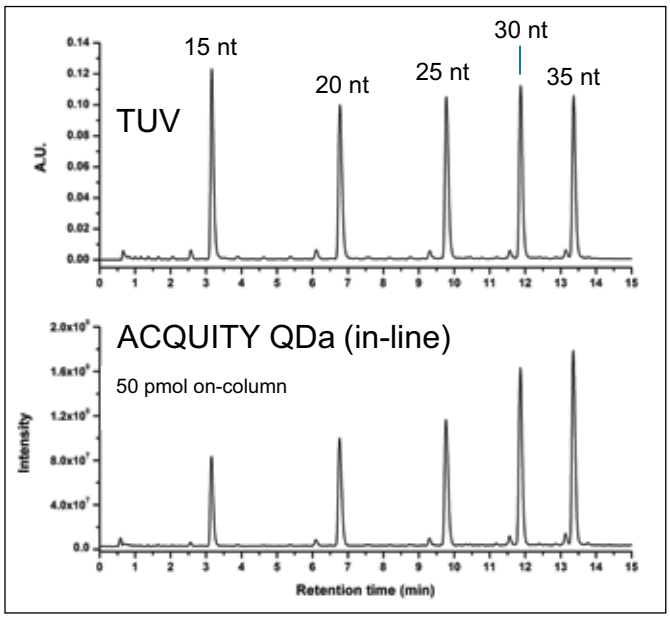

Figure 1. Oligonucleotide analysis with QDa. An IP-RPLC separation of five polyT standards with the ACQUITY QDa in a serial configuration post UV detection.

Table 1 shows that multiple charge states are observed (green highlight) for each of the oligonucleotide standards. Closer inspection of the 35 nt standard indicates at least nine charge states were observed. The shorter sequences, which had greater disparity in intensities, were observed with fewer charge states, indicating potential lower charge states may not be observed as they are outside the detector scan range (>1250 *m/z*). This phenomena does not affect mass data for therapeutic oligonucleotides, as even the shortest standard (nt 15) had five charge states observed.

green = observable charge state

nt	Avg. MW	$[M-4H]^{-4}$	$[M-5H]^{-5}$	$[M-6H]^{-6}$	$[M-7H]^{-7}$	$[M-8H]^{-8}$	$[M-9H]^{-9}$	$[M-10H]^{-10}$	$[M-11H]^{-11}$	$[M-12H]^{-12}$	$[M-13H]^{-13}$	$[M-14H]^{-14}$	$[M-15H]^{-15}$	$[M-16H]^{-16}$	$[M-17H]^{-17}$
15	4,500.9	1124.2	899.2	749.1	642.0	561.6	499.1	449.1	408.2	374.1	345.2	320.5	299.1	280.3	263.8
20	6,021.9	1504.5	1203.4	1002.6	859.3	751.7	668.1	601.2	546.4	500.8	462.2	429.1	400.5	375.4	353.2
25	7,542.9	1884.7	1507.6	1256.1	1076.5	941.9	837.1	753.3	684.7	627.6	579.2	537.8	501.9	470.4	442.7
30	9,063.8	2265.0	1811.8	1509.6	1293.8	1132.0	1006.1	905.4	823.0	754.3	696.2	646.4	603.2	565.5	532.2
35	10,584.8	2645.2	2116.0	1763.1	1511.1	1322.1	1175.1	1057.5	961.2	881.1	813.2	755.0	704.6	660.5	621.6

Table 1. Oligonucleotide m/z table. A charge state table based on average molecular weight of the polyT standards was used to identify the observed charge states detected with the ACQUITY QDa. Green highlight indicates charge states observed for each standard.

As shown in Table 1, the number of charge states observed across the polyT standards gives rise to the question of instrument accuracy across such a diverse range. An assessment of charge state accuracy of the ACQUITY QDa Detector was thus performed using the observed charge states for the oligonucleotide standards. For this assessment, the difference between the observed and theoretical charge state *m/z* value was determined using the average molecular weight of each oligonucleotide. The results for a technical triplicate of the 30 nt standard are listed in Table 2 as a representative sample. It can be seen from Table 2 that the derived masses for the observed charge states are within the instrument specification of ± 0.2 Da with a high degree of method repeatability demonstrated by the low RSD of 0.02% or lower for each charge state. Similar results were obtained for observed charge states for the remaining standards (data not shown) demonstrating the ACQUITY QDa is capable of providing accurate mass information for oligonucleotide analyses.

30 nt, N=3	$[M-8H]^{-8}$	$[M-9H]^{-9}$	$[M-10H]^{-10}$	$[M-11H]^{-11}$	$[M-12H]^{-12}$	$[M-13H]^{-13}$	$[M-14H]^{-14}$	$[M-15H]^{-15}$
Expected	1132.0	1006.1	905.4	823.0	754.3	696.2	646.4	603.2
Observed	1132.1	1006.1	905.4	823.1	754.3	696.2	646.5	603.4
S.D.	0.07	0.12	0.04	0.00	0.04	0.04	0.14	0.08
%R.S.D.	0.01	0.01	0.00	0.00	0.01	0.01	0.02	0.01

Table 2. m/z accuracy evaluation. Expected charge states based on average molecular weight of the 30 nt standard were compared to the observed charge states and were within instrument specification of ± 0.2 Da.

The increased number of charge states observed with oligonucleotides as shown in Figure 2A result in mass spectrums that are not straightforward to interpret. Workflows that can deliver accurate mass information in an efficient manner are highly desirable in the analysis of therapeutic oligonucletoides. To this end, deconvolution algorithms such as MaxEnt1 can be incorporated to provide mass data of oligonucleotide spectra for improved productivity. To evaluate this functionality, a one minute window centered across the peak apex of the 30 nt standard was used to combine the MS spectrum data acquired by the QDa. Deconvolution was performed with a peak width of approximately 0.7 Da and a binning resolution of 0.5 Da. As shown in Figure 2B, the spectrum of the 30 nt standard was deconvoluted to a zero charge state mass of 9,064.5 Da, which was within 0.7 Da of the expected average molecular weight. A minor sodium (Na^+) adduct was also observed with a relative intensity of 6%. Trace salts are routinely encountered in LC-based separations because of their high affinity towards the phosphodiester backbone of oligonucleotides. Similar to the charge state evaluation, an assessment of the mass accuracy of the deconvolution algorithm for the complete set of standards was performed.

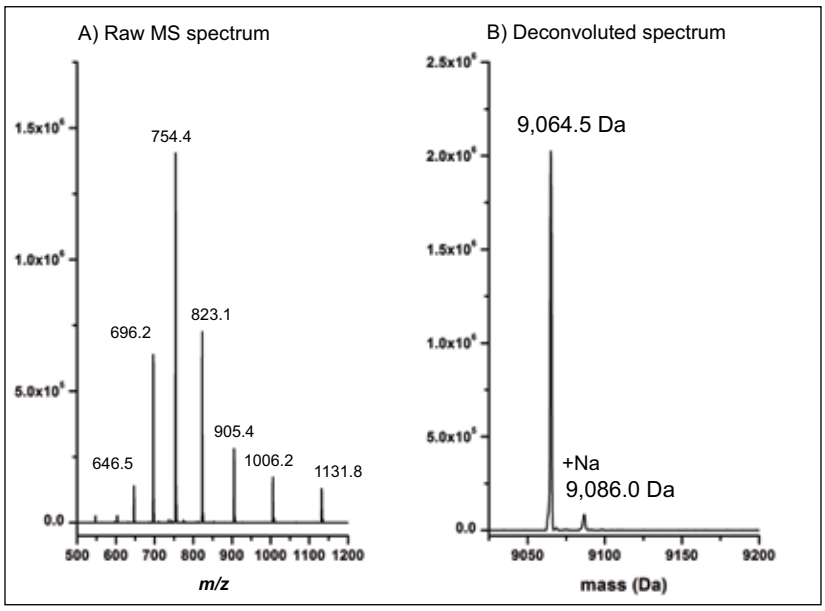

Figure 2. MaxEnt1 Deconvolution of MS spectrum. A) Multiple charge states associated with the raw spectrum of the 30 nt standard when acquiring continuum data using the ACQUITY QDa. B) Deconvolution of the raw MS spectrum of the 30 nt standard resulted in a parent peak mass of 9,064.5 Da (+0.7 Da) and a minor sodium (Na^+) adduct peak with a relative intensity less than 6%.

Using the same methodology as before, the difference between the observed and theoretical deconvoluted mass value was determined using the average molecular weight of each oligonucleotide. An identical number of scans and *m/z* range was used to combine MS spectrum for the polyT standards. Deconvolution parameters were kept constant for the standards with the results listed in Table 3. As shown in Table 3, the deconvolution results were observed to be highly reproducible across a technical triplicate with no deviation observed in the deconvoluted mass. Mass accuracy was observed from +0.0 Da to +0.7 Da across the polyT standards with increasing deviation associated with increasing sequence length. This is not entirely unexpected, as instrument error increases with higher charge states. In light of this, a mass accuracy of ±1.0 Dalton for oligonucleotides ranging from 15–35 nt demonstrates that the ACQUITY QDa is capable of providing adequate mass information in an efficient manner for routine identification and purity assessments in the manufacturing process of synthetic oligonucleotides.

N=3	nt 15	nt 20	nt 25	nt 30	nt 35
Expected	4500.9	6021.9	7542.9	9063.8	10584.8
Trial 1	4500.9	6022.5	7543.5	9064.5	10585.5
Trial 2	4500.9	6022.5	7543.5	9064.5	10585.5
Trial 3	4500.9	6022.5	7543.5	9064.5	10585.5
Average	4500.9	6022.5	7543.5	9064.5	10585.5
Δ Mass (Da)	0.0	0.6	0.6	0.7	0.7

Table 3. Mass accuracy evaluation of MaxEnt1 deconvoluted mass. Expected mass based on average molecular weight of the polyT standards were compared to the average deconvoluted mass. Mass accuracy ranged from +0.0 Da to +0.7 Da across the set of standards.

CONCLUSIONS

Cost-effective techniques that add value and can be implemented into existing workflows with minimal effort are highly desirable in the pharmaceutical industry. The addition of complementary mass information in a single workflow afforded by the ACQUITY QDa Detector provides analysts an efficient means to improve productivity in routine assays. A natural extension of the ACQUITY QDa portfolio is to evaluate its applicability with biopharmaceuticals beyond that of mAbs. From this work it has been demonstrated that the ACQUITY QDa is compatible with IP-RPLC mobile phases and is able to detect and report mass information for oligonucleotides over a wide molecular weight range. Collectively, these results establish the ACQUITY QDa as an ideal addition to an analyst's lab for increased productivity and confidence of data analysis for routine identification and purity assessments in the manufacturing process of synthetic oligonucleotides.

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An Efficient UV-Based Method for the Assessment of Oleic Acid Content in Biotherapeutic Drug Products Containing Polysorbate-80

Robert E. Birdsall and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- A robust and sensitive method in the analysis of PS-80 degradants
- UV-based workflow that enables rapid deployment in regulated environments
- Minimal sample preparation with ability to perform high throughput analysis

WATERS SOLUTIONS

ACQUITY® UPLC® H-Class System

ACQUITY UPLC Autosampler with FTN

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

ACQUITY QDa® Mass Detector (optional)

ACQUITY UPLC Protein BEH C_4 Column

KEYWORDS

Polysorbate 80, fatty acid, Oleic acid, H-Class, UPLC, ACQUITY QDa

INTRODUCTION

Surfactants such as polyoxyethylene sorbitan monooleate (PS-80) are commonly used in formulated biopharmaceuticals to reduce protein denaturing, aggregation, and surface adsorption to vials and syringes.[1] While PS-80 plays a critical role in stabilizing drug products, degradation of surfactants in formulated drugs can decrease overall product efficacy and safety. To this end, characterization, identification, and quantitation of excipients such as PS-80 must be performed to demonstrate the drug product is safe and efficacious in its formulated state. Due to its non-volatile and UV-inactive nature, direct analysis of PS-80 has resulted in methods that incorporate alternative detection techniques such as evaporative light scattering detection (ELSD).[2] Yet, given the ubiquitous nature of UV-based instruments in pharmaceutical labs, methods that can utilize existing equipment would be beneficial. Conveniently, the degradation of PS-80 through radical autoxidation or enzymatic processes results in the cleavage of the hydrophobic ester tail (Figure 1) to produce the UV-active fatty acid, oleic acid.[1,3,4] Various extraction techniques of fatty acids from biological matrices have resulted in assays that range in degree of sample preparation required, sensitivity, and reproducibility.[3-5] UV-based methods that are robust, sensitive, and can be readily deployed in regulated environments are highly valuable in ensuring product quality and safety.

The objective of this study is to demonstrate a robust UV-based method for the determination of oleic acid concentration in drug products that is sensitive and can be performed with minimal sample preparation for straightforward deployment in a laboratory setting.

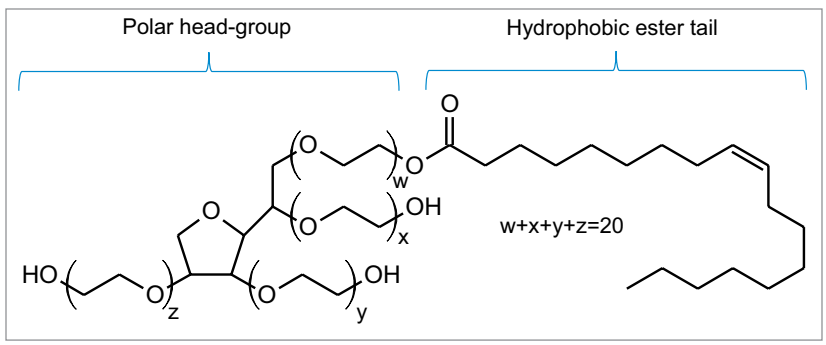

Figure 1. Polyoxyethylene sorbitan monooleate. PS-80 contains a polar head-group and a hydrophobic tail where the head group is comprised of approximately 20 polyoxyethylene (POE) groups.

EXPERIMENTAL

Polysorbate-80, oleic acid standard, cis-10 nonadecenoic acid standard, and mass spectrometry grade solvents (Optima series) were purchased from Sigma Aldrich. Stock solutions of standards were prepared gravimetrically in acetonitrile. Serial dilutions of oleic acid standards and spiked in samples were prepared using the stock 0.1% solutions. Internal standard cis10-nonadecenoic acid stock was prepared gravimetrically in acetonitrile at a concentration of 0.3%. Formulated samples of infliximab and trastuzumab were used without dilution at a concentration of 20 mg/mL.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC TUV w/titanium flow cell
Absorption wavelength:	200 nm
Column:	ACQUITY UPLC Protein BEH C4 300Å, 1.7 µm, 2.1 mm x 100 mm (P/N 186005590)
Column temp.:	30 °C
Vials:	TruView LCMS certified 12 x 32mm total recovery vial (P/N 186005669CV)
Sample temp.:	10 °C
Injection volume:	1 µL
Mobile phase A:	H_2O, 0.1% FA
Mobile phase B:	MeCN, 0.1% FA

Isocratic conditions

Flow:	0.200 mL/min
Mobile phase:	35% B

Extraction protocol

		Recovery		Formulated sample		
		Control	Experimental	Control	Esterfied FA experimental	Free FA
Reagent	Concentration	Experimental	Volume (µL)	Volume (µL)	Volume (µL)	Volume (µL)
IS	0.3% in MeCN	10	10	10	10	10
Sample	~	15	15	0	20	20
MeCN	Neat	275	0	290	0	0
H_2O	Neat	0	15	0	5	35
NaOH	0.5M	0	30	0	30	0
Incubate 65 °C	~	1 hr	1 hr	1 hr	1 hr	0 hr
HCl	1.0M	0	15	0	15	0
NaCl	Saturated	0	50	0	50	50
H_2O	Neat	0	0	0	0	15
LLE						
(Top layer)	~	No	Yes	No	Yes	Yes
MeCN	Neat	0	75	0	90	90
MeCN	Neat	0	100	0	100	100
MeCN	Neat	0	100	0	100	100
Final volume after LLE		300	300	300	300	300

RESULTS AND DISCUSSION

DYNAMIC RANGE

The analysis of fatty acids has been well established in literature over the last few decades using a diverse set of techniques. Legacy methods such as these are quickly becoming outdated as manufacturing processes are modernized as part of a pharmaceutical quality system (ICH Q10). Specifically, HPLC-based separations using wide-bore columns often lack the sensitivity and efficiency needed by today's standards when considering factors such as post-column dispersion and long run-times and their impact on assay performance.[6,7] In light of these factors, some considerations were made in an effort to update the UV-based methodology of the current study. The ACQUITY H-Class Bio System was used as a UPLC platform to minimize system dispersion under isocratic conditions for improved separation performance. In addition, the ACQUITY UPLC Protein BEH C_4 Column (300Å, 1.7 µm, 2.1 mm x 100 mm) was used as a lower retentivity column to reduce carryover and increase analysis throughput.

Evaluation of the system and column performance was carried out using a serial dilution series of an oleic acid standard using concentrations ranging from 1000 ppm (0.1%) to 0.24 ppm (0.000024%). Considering the scope of the current study is to isolate and identify one target species, a five-minute isocratic method was used with 35% organic to increase assay robustness. As shown in Figure 2, the lower retentivity of the C_4 bonded phase offers the ability to separate highly hydrophobic fatty acids such as oleic acid in under five minutes using conditions that are suitable for both UV and MS-based detection. The high throughput separation enables the ability to maintain good peak shape with minimal dispersion under isocratic conditions. A plot of area versus concentration (Figure 2, inset) showed a high degree of linearity over 3-orders of magnitude with a limit of detection (LOD) of 0.24 ppm, demonstrating UPLC platforms are well suited for UV-based assays that are efficient and sensitive in the detection of oleic acid.

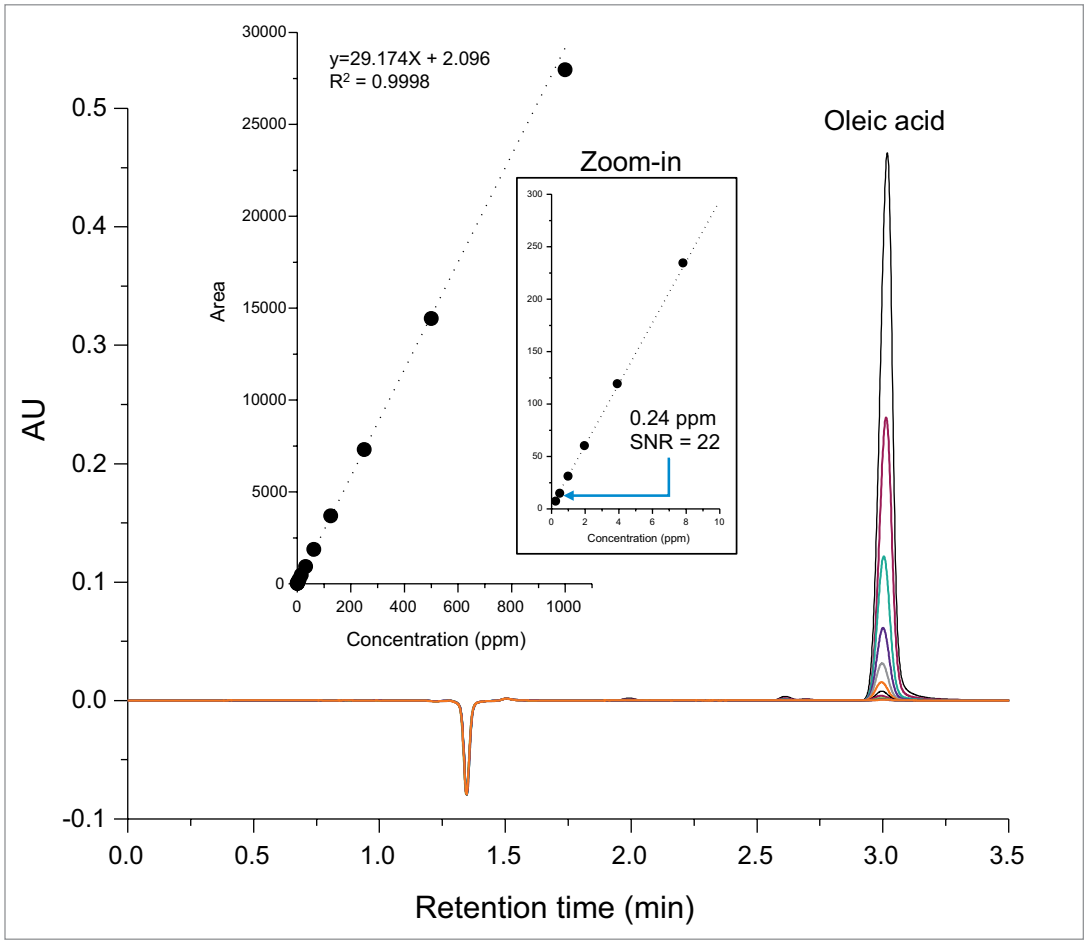

Figure 2. Oleic acid calibration plot. Using an isocratic method, a serial dilution injection series of oleic acid was observed to be highly linear over a range of 0.24 ppm – 1000 ppm using UV-based detection.

RECOVERY

Determination of oleic acid content (free and esterified) has been shown to be used successfully in the assessment and extrapolation of PS-80 degradation and concentration in drug products.[2] More recently oleic acid has been used successfully as a predictor for functionality related characteristics such as critical micelle concentration.[8] Analyses such as these often employ liquid-liquid extraction (LLE) to isolate fatty acids from sample matrices. However, LLE techniques are not without their own challenges such as matrix effects, extraction volume, and solvent type, which can negatively impact recovery efficiencies and impair accurate quantification. Furthermore, organic extraction solvents are often immiscible with aqueous buffers resulting in laborious dry-down and reconstitution steps. These challenges are addressed in the current study with the development of an extraction protocol that can be used for the extraction of free and esterified fatty acids (after hydrolysis) and is amendable for direct injection onto columns with minimal sample preparation.

The extraction protocol was evaluated for stability, recovery efficiency, and column performance prior to drug product analysis using an oleic acid standard. Cis-10-nonadecenoic acid was used as an internal standard to correct for non-specific loss of oleic acid during the extraction process. Two sets of oleic acid samples representing experimental and control were prepared as outlined in the extraction protocol (experimental section). Briefly, stock samples of oleic acid were prepared in MeCN at concentrations 0.8%, 0.4%, 0.2%, 0.1%, 0.05%, 0.025%, and 0.0125%. Experimental samples were prepared by taking 15 µL aliquots of each stock and spiking in 10 µL of the IS. NaOH was added to the experimental set and incubated at 65 °C for 1-hr to assess oleic acids stability at the elevated temperature and high pH required for base hydrolysis of PS-80 and drug product biological matrices. The solution was then acidified with HCl to increase solubility of residual biological material in the aqueous phase as well as minimize the column exposure to elevated pH that could decrease column longevity. After acidification, a saturated solution of NaCl was added to induce a phase separation between the acetonitrile and aqueous phase.[3] Initial extraction of the top-layer was performed with a reduced aliquot of MeCN to account for the MeCN present in the oleic acid sample and internal standard. A control set was prepared in MeCN to determine recovery efficiency and correct for non-specific sample loss. As shown in Figure 3, a direct injection of 1 µL of the extracted spiked in sample using the same isocratic conditions as before is sufficient to separate oleic acid from the IS with baseline resolution. A water blank (blue trace) performed after the injection showed no observable carry-over on the column. Recovery efficiencies up to 98% were observed for samples containing trace amounts of oleic acid as shown in Table 1 with a marginal decrease in recovery efficiency observed at higher concentrations. Sample loss was determined to be non-specific, as similar trends were observed with the IS as well.

Table 1. Recovery results. Liquid-liquid extraction results indicated high recovery efficiencies across a broad concentration range of oleic acid. An internal standard was used in both the control and experimental results to correct for non-specific loss of oleic acid.

		Control		Experimental				
		Oleic acid	Internal standard	Oleic acid	Oleic acid	Internal standard	Internal standard correction factor	Oleic acid corrected area
Sample	Expected OA (ppm)	Area	Area	Area	Recovery (%)	Area		
1	400	10053	2608	8253	82.09	2085	1.29	10322
2	200	5438	2716	4202	77.28	2002	1.34	5701
3	100	2754	2571	2430	88.26	2325	1.16	2688
4	50	1376	2654	1304	94.80	2472	1.09	1400
5	25	708	2619	653	92.23	2440	1.10	701
6	12.5	375	2968	344	91.59	2514	1.07	406
7	6.25	184	2677	180	98.18	2493	1.08	194

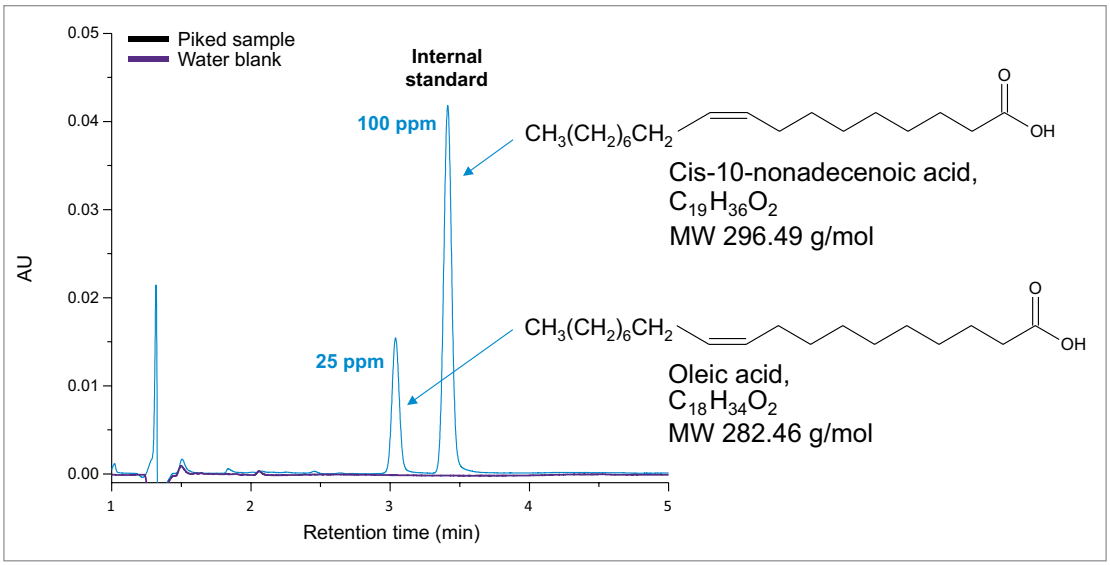

Figure 3. Liquid-liquid extraction chromatogram. Liquid-liquid extraction was used to recover a sample of oleic acid (25 ppm) spiked with an internal standard (100 ppm). Peaks of interest were baseline resolved with no observable carry-over (purple trace).

Non-specific loss was corrected for using the average response of the IS in the control sample. As shown in Figure 4, a comparison of the IS corrected experimental and control data showed a high degree of linearity with good agreement between both data sets over the dynamic range of the assay (slope ratio = 1.03). Collectively, these results demonstrate oleic acid and a corresponding IS can be extracted with a high degree of efficiency over a broad working range with conditions that are compatible with direct injection of samples for efficient and accurate analysis of fatty acids.

DRUG PRODUCT ANALYSIS

Polyoxyethylene sorbitan monooleate (PS-80) and polyoxyethylene sorbitan monolaurate (PS-20) are two commonly used stabilizing surfactants found in biotherapeutic drug products.[1] Representative samples of infliximab (PS-80 containing) and trastuzumab (PS-20 containing) were used to test the applicability and specificity of the proposed method. To determine if free oleic acid was present in the samples as a degradation product, a similar extraction protocol was used as before where the IS was spiked into 400 µg of each sample and the LLE was performed without hydrolysis. Controls were run alongside both samples again to account for non-specific loss.

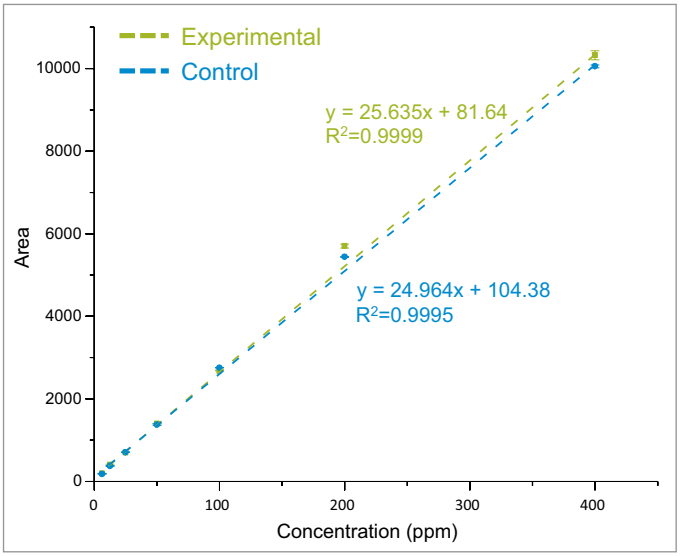

Figure 4. Evaluation of internal standard. A comparison of control data to experimental data corrected for with the internal standard showed a high degree of linearity with a slope ratio of 1.03 between the data sets.

As shown in Figure 5 oleic acid was not detected in either the infliximab or trastuzumab UV chromatograms. To ensure trace levels of oleic acid were not overlooked an ACQUITY QDa mass detector was placed in-line as an orthogonal detectorfor increased sensitivity. Selected ion recording (SIR) at 283.3 *m/z* and 297.2 *m/z* was used to detect oleic acid (OA) and the IS. As shown in the inset of Figure 5 oleic acid was not detected in either sample with the increased sensitivity of the ACQUITY QDa which was previously determined to have a LOD of 60 PPB for oleic acid (data not shown). To determine if esterified oleic acid was present in the form of PS-80, a duplicate sample set with controls was prepared in which the mAb samples underwent base hydrolysis (see Experimental).

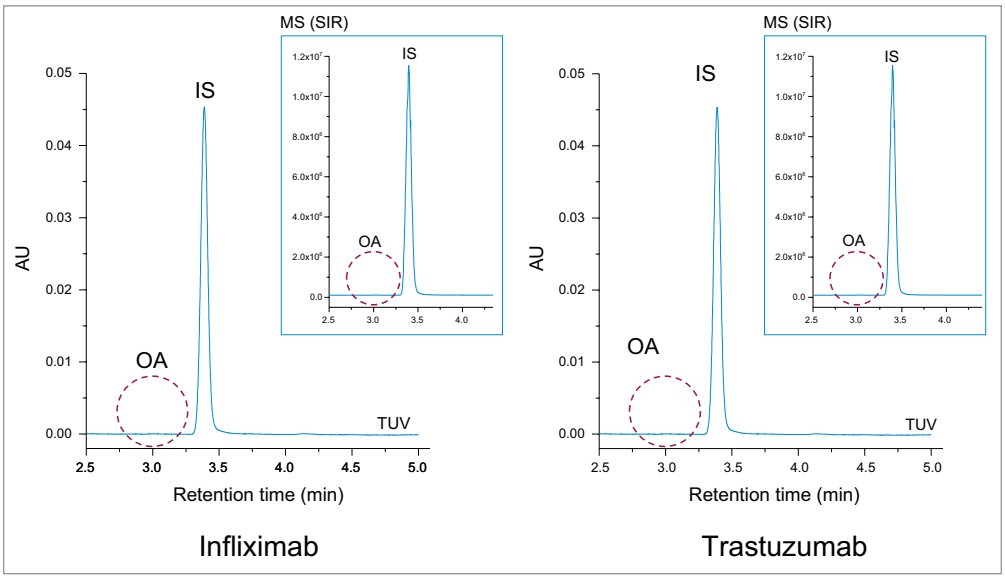

Figure 5. Free oleic acid results. Control samples of infliximab and trastuzumab that were spiked with cis-10-nonadecenoic acid (IS) as an internal standard did not exhibit free oleic acid (OA) in their UV or MS chromatograms (inset) following liquid-liquid extraction in the absence of a base hydrolysis.

As shown in Figure 6, oleic acid was observed to be present in the infliximab sample at less than 1 % relative to the IS (99.08%) peak. The presence of oleic acid in the infliximab sample was confirmed with MS detection (inset). The absence of an oleic acid peak in the hydrolyzed trastuzumab sample highlights the specificity of the assay and confirms that the infliximab sample showed no detectable signs of degradation in the free oleic acid test. These results demonstrate the current method is applicable in the detection of PS-80 degradants in formulated samples.

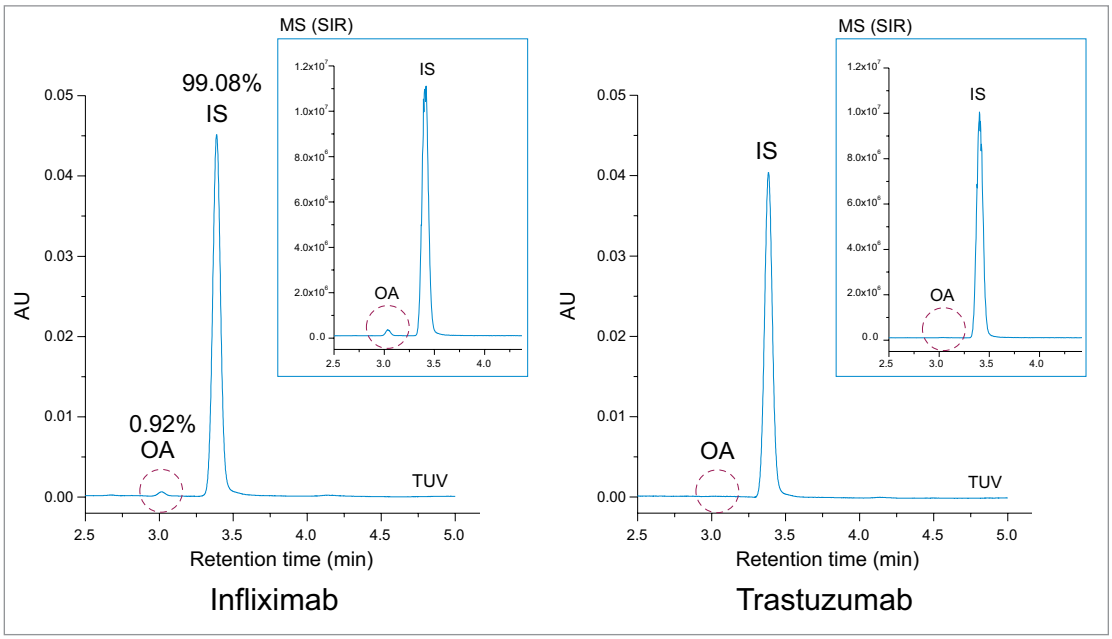

Figure 6. Hydrolyzed mAb oleic acid results. Oleic acid (OA) was detected in the infliximab sample at a concentration below 1% relative to the internal standard (IS) following base hydrolysis. Oleic acid was not detected in the trastuzumab sample. Results were confirmed using MS-based detection (inset).

QUANTIFICATION

Formulation optimization can benefit from high throughput screening assays for determining conditions that ensure product quality and adequate long-term stability when faced with short timelines. To address this challenge, the current study compared absolute quantification of oleic acid content in the infliximab samples using a multi-point and single-point calibration method. Briefly, the corrected area from the infliximab results was used with the multi-point calibration plot from Figure 2 to determine the oleic acid concentration after accounting for the dilution factor (DF = 15) of the hydrolyzed mAb sample. For the single-point calibration method, the relative peak area of oleic acid (Figure 6) was used to calculate the concentration based on the IS peak area (100 ppm) as well as accounting for the dilution factor. As shown in Table 2, both methods determined the oleic acid content in the hydrolyzed mAb sample to be within 1 ppm of each other with comparable CVs. Furthermore, the oleic acid concentration was used to extrapolate the original concentration of PS-80 in the sample which were determined to be on the same order of magnitude as the product monograph (0.05 mg/mL), assuming the original PS-80 was of high purity. These results suggest a single-point calibration method offers an efficient pathway for the accurate determination of oleic acid concentration in a high throughput manner when compared to a more resource dependent method that relies on multiple calibration points.

Table 2. Quantification comparison. Comparison of a multi-point and single-point calibration method showed results that were in agreement with comparable CV's for the determination of absolute oleic acid content in the infliximab sample. Original sample PS-80 concentration was extrapolated and determined to be in the correct order of magnitude based on product monographs.

Method	Mean oleic acid concentration (ppm)	Avg mass PS-80 (mg)	PS-80 sample conc. (vol%)	PS-80 sample conc. (mg/mL)	RSD (%)
Multi-point calibration	12.91	0.00119	0.0051	0.059	4.40
Single-point calibration	13.95	0.00129	0.0055	0.065	4.07

CONCLUSIONS

Stability screening of excipients in drug products plays a critical role in ensuring drug product quality and safety. Methods that enable accurate assessment of excipients and their related impurities in an efficient manner which can be adapted to current instrumentation are highly valuable in industry. The current study successfully demonstrated an efficient UV-based method for the detection and quantitation of oleic acid that is robust as well as sensitive and can be readily deployed in regulated biopharmaceutical environments.

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Using Mass Detection as an Orthogonal Technology to Improve Routine Analysis of Biotherapeutics

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved specificity and confidence for routine analysis of biotherapeutics
- High throughput analysis with enhanced chromatographic resolution and MS sensitivity
- Highly specific platform method that can be applied to identity test of various insulin analogues

WATERS SOLUTIONS

ACQUITY® UPLC® H-Class Bio System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

ACQUITY QDa® Detector

ACQUITY UPLC Peptide CSH™ C_{18} Column

Empower® 3 Software

KEYWORDS

Biotherapeutics, insulin analogue, ACQUITY QDa Mass Detector, ion-pairing reagent, DFA

INTRODUCTION

Biotherapeutics have undergone rapid development with global sales of innovator and off-patent biologics forecasted to reach over $300 billion by 2020.[1] Currently, many analytical methods used in the characterization and monitoring of biotherapeutics are HPLC-UV workflows based on US and EU Pharmacopeia monographs.[2] While sufficient, many of these assays potentially lack the specificity needed to support the diverse portfolio of biotherapeutics coming to market in the near future. Recently, we discussed method modernization as part of a sound pharmaceutical quality system outlined by the International Committee of Harmonization (ICH Q10).[3] New and innovative technologies, such as mass detectors offer the ability to enhance current UV-based techniques in the manufacturing environment for improved product knowledge and product quality. The ACQUITY QDa is a single quadruple mass spectrometer that can be easily integrated into an existing LC-UV-based workflow for the simultaneous collection of both UV and MS data, offering an efficient means to improve selectivity, sensitivity, and confidence in routine assays.[4]

Using insulin as an example, a high throughput LC-UV/MS workflow is used to demonstrate the applicability of the ACQUITY QDa to provide supplemental mass information for deeper product understanding in a manufacturing environment.

EXPERIMENTAL

Chemical and reagents

Insulin human, insulin lispro, and insulin glargine were purchased from USP, while insulin glulisine was from Besse Medical. Endoproteinase Glu-C from S. aureus was purchased from Promega. Digestion procedures were as outlined in the *USP monograph: insulin human.*[3] HPLC grade water, acetonitrile, formic acid, difluoroacetic acid (DFA), and TFA were purchased from Fisher Scientific and used as received.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY TUV Detector 5 mm flow cell, λ = 215 nm
LC column:	ACQUITY UPLC CSH C_{18} 1.7 µm, 130 Å, 2.1 mm × 100 mm (P/N 186006937)
Column temp.:	40 °C for peptide mapping 60 °C for intact analysis
Sample vial:	12 × 32 mm glass vial Total recovery (P/N 600000750cv)
Mobile phases:	Water and acetonitrile
MP additive:	0.1% formic acid, DFA, and TFA
Mass load:	0.86 µg for peptide mapping 0.75 µg for intact analysis

Gradient table for intact insulin separation:

Time (min)	Flow rate (min)	%A	%B
Initial	0.300	95.0	5.0
2.00	0.300	75.0	25.0
22.00	0.300	65.0	35.0
23.00	0.300	20.0	80.0
24.00	0.300	20.0	80.0
24.01	0.300	95.0	5.0
30.00	0.300	95.0	5.0

Gradient table for insulin peptide mapping:

Time (min)	Flow rate (min)	%A	%B
Initial	0.300	90.0	10.0
10.00	0.300	50.0	40.0
11.00	0.300	0.0	100.0
13.00	0.300	0.0	100.0
13.50	0.300	90.0	10.0
20.00	0.300	90.0	10.0

ACQUITY QDa settings

Mass range:	350–1250 *m/z*
Mode:	ESI+
Collection mode:	Continuum
Sample rate:	2 points/sec
Cone voltage:	10 V
Probe temp.:	500 °C
Capillary voltage:	1.5 kV
Informatics:	Empower 3

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

Identity, purity, and stability tests are common assays routinely performed in regulated environments such as QC labs. However, legacy UV-based methods such as these may not be optimized for MS compatibility due to ion-pairing choice (TFA) or buffer additives such as salt, which can impair MS performance.[2] To this end, a degree of method development may be necessary in the evaluation of new technology such as MS. In the case of identity tests for insulin analogues, a high throughput LC-UV/MS method was developed using formic acid as a mobile phase additive for improved MS-performance. As shown in Figure 1A, with a 10 min gradient from 10% to 40% acetonitrile, peptides from a Glu-C digested insulin were well resolved and MS data (Figure 1B) were acquired simultaneously for each peptide fragment (I-IV) using the ACQUITY QDa. However, the above condition was not suitable for insulin glargine, as shown in Figure 2 with the co-elution of peptide fragments III and IV. While the gradient could be extended to resolve the peaks at the cost of analysis time, an alternative approach is to change the selectivity of the separation through choice of mobile phase additive (e.g. ion pairing agent). Previous studies have reported enhanced resolution using difluoroacetic acid (DFA) for a monoclonal antibody separation compared to formic acid.[5]

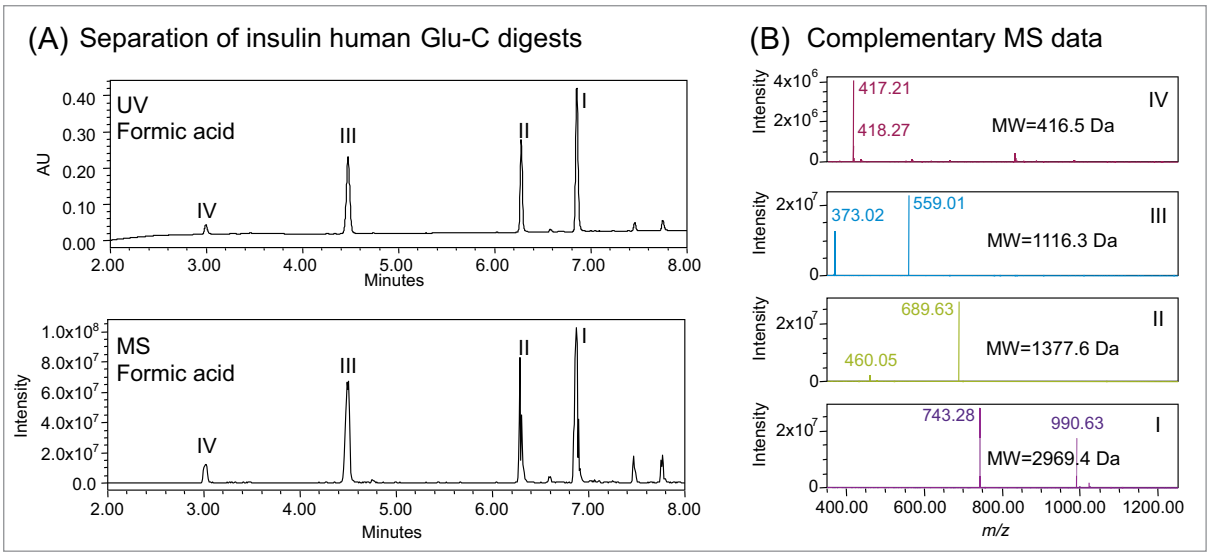

Figure 1. Separation of insulin human digest with orthogonal detection system using formic acid as mobile phase additive. Four peptide peaks were detected using (A) UV detection and MS detection by an ACQUITY QDa Mass Detector, with complementary MS spectra shown in (B).

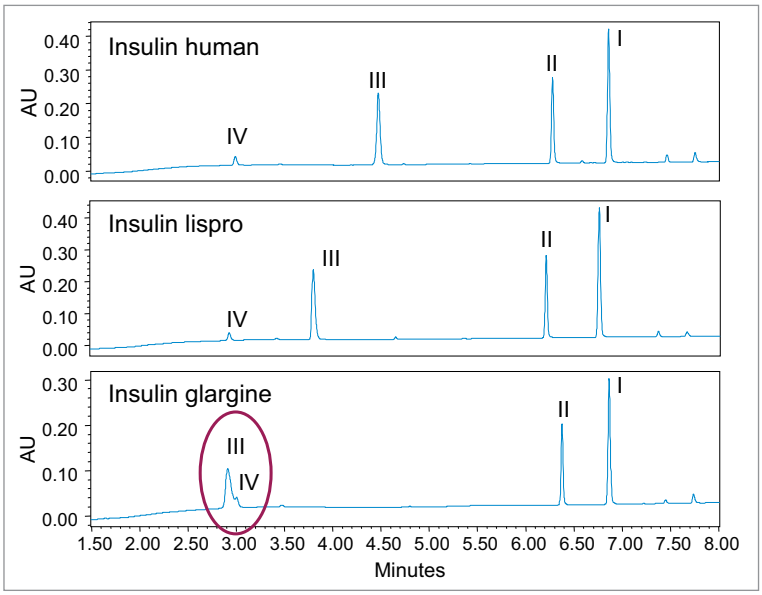

Figure 2. Identity test of insulin human, insulin lispro, and insulin glargine using formic acid as mobile phase additive. Peak III and IV were partially resolved in the identity test of insulin glargine.

Along with TFA and formic acid, the effect of DFA on critical pair resolution (Peak III and IV in glargine digest) was studied while keeping the gradient slope unchanged (Figure 3A). As shown in Figure 3B, the chromatographic results indicated that TFA and DFA provided baseline resolution above the USP criteria of 1.5 (dashed line) in comparison to formic acid. However, the ion pairing reagent can also affect the ionization efficiency of analytes and must be considered in conjunction with the desired resolution. As shown in the bar plot of MS signal to noise ratio (Figure 3C), TFA provides the lowest MS intensity for most peaks where DFA offered acceptable resolution with marginal impact on MS response in comparison to the often preferred formic acid, and therefore DFA was selected as the ion-pairing agent moving forward.

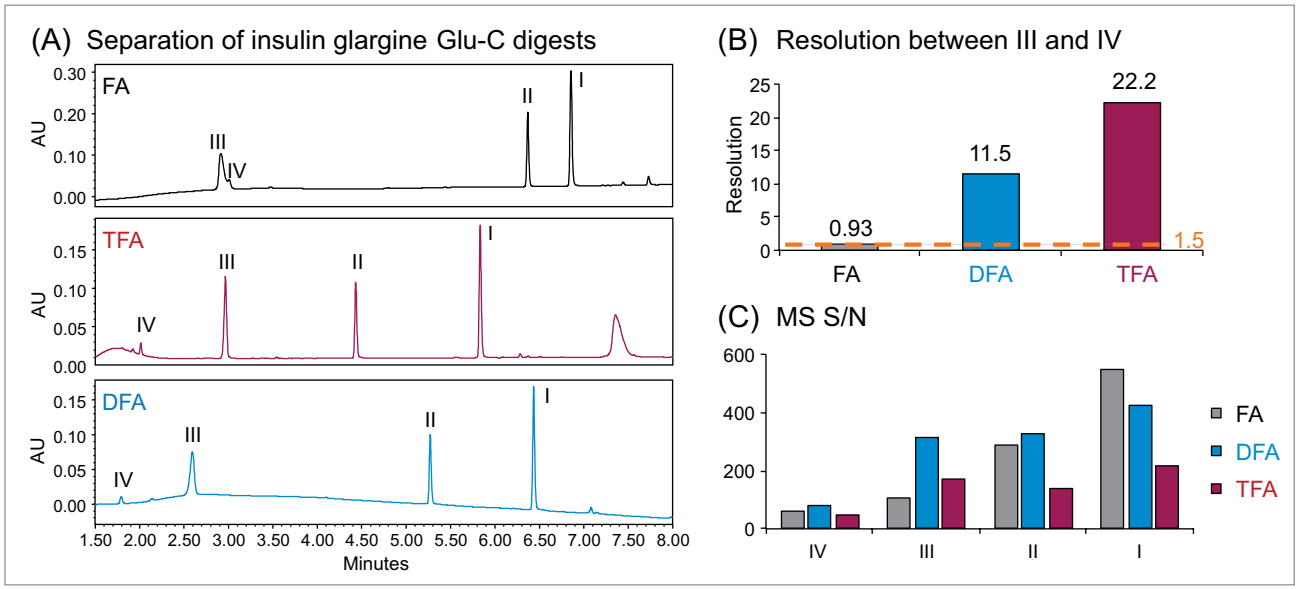

Figure 3. Comparison of different mobile phase additives. (A) Separation of insulin glargine digests using formic acid, TFA and DFA as mobile phase additive while keeping the gradient slope unchanged. (B) Bar plot of chromatographic resolution between the critical pair (Peak III and IV). Resolution using both DFA and TFA are above the 1.5 threshold. (C) MS signal to noise ratio (S/N) comparison for the four peaks.

Application 1: Identity test

One of the benefits of adding MS detection to UV-based identity tests is the complementary mass information provided for investigation of the failed tests. Using the same gradient as the DFA in Figure 3, an LC-UV/MS based platform method was developed with three insulin analogues as reference standards, including insulin human, insulin lispro, and insulin glargine. As shown in Figure 4, all peaks were well resolved while the peptide fragments (Peak I, II, IV) with the same peptide molecular weight were aligned across samples as indicated by the blue dash lines. Using this platform method, an identity test was performed on two insulin drug products: Sample 1 and 2. As shown in Figure 4, the retention times of peptide peaks in sample 1 were aligned well with insulin human, while Peak I and III of Sample 2 shifted significantly. According to the collected MS data, a delta mass difference of 14.7 Da and 100.2 Da was observed for fragment I and III respectively (Figure 4), suggesting the inconsistency in retention times was caused by a single amino acid change in fragment III (KT to E) and I (N to K). Based on the sequence information, Sample 2 was identified as another insulin analogue: insulin glulisine. Collectively, the developed method demonstrates high robustness and specificity as a platform assay for identity test of various insulin analogues.

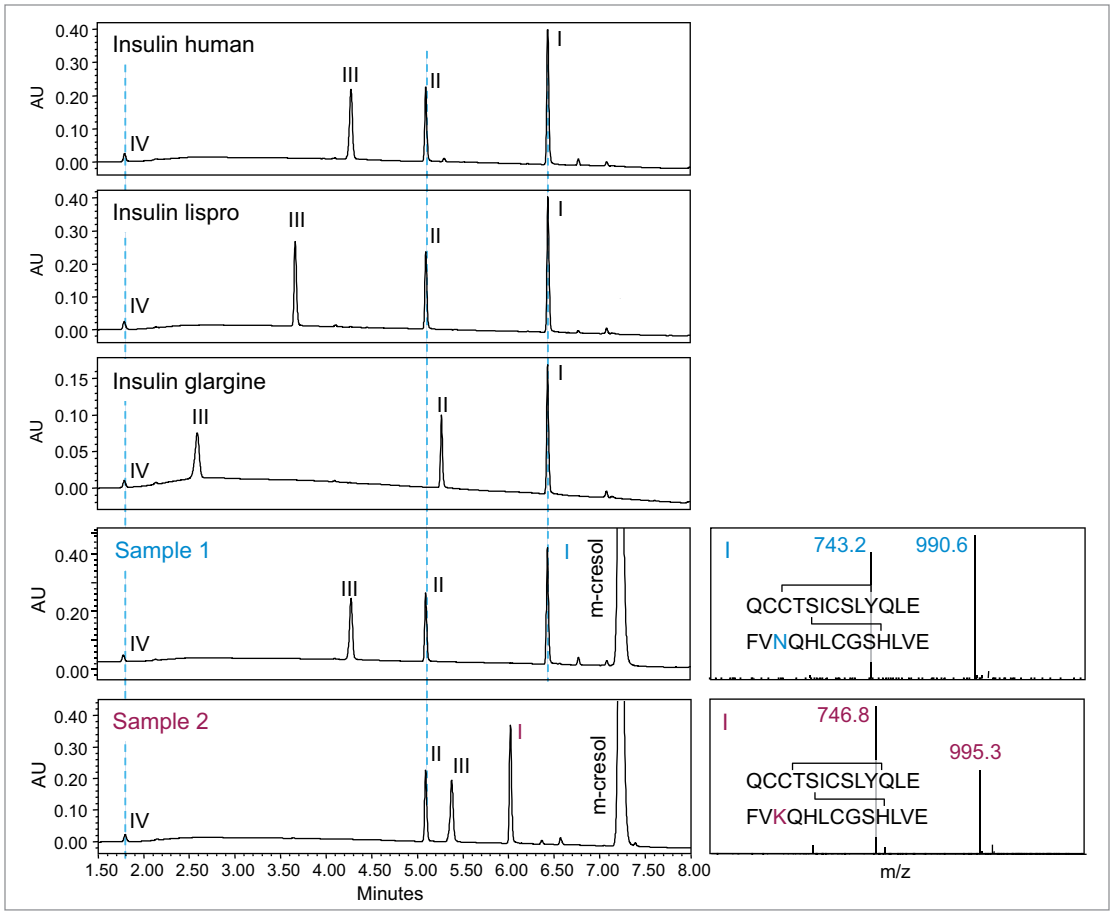

Figure 4. The developed platform method for identity test of insulin analogues using DFA as mobile phase additive. Blue dash lines indicate the same retention time for the peptides with identical peptide molecular weight. Sample 1 showed the same retention times with insulin human. Peak III and I in sample 2 have significantly different retention time compared to the standards, suggesting it could be another insulin analogue or sample degradation. With the delta mass information derived from MS data, this sample was determined to be insulin glulisine.

Application 2: Purity analysis

Another benefit of using orthogonal detection is verifying purity assessment of intact insulin with mass information. UV detection alone provides limited information about peak purity. With added mass information, purity of the target peak can be evaluated via the built-in mass analysis function within Empower CDS. Figure 5A shows the separation of intact insulin human using the gradient conditions optimized for peak-to-peak resolution. With a 20 min gradient from 25% to 35% mobile phase B, three impurity peaks were separated from the main peak. In the Empower mass analysis window (Figure 5B), the MS spectra are displayed for the leading, apex, and trailing edge of the main peak via an automated processing method. In this case, the apex and trailing window showed almost identical MS spectra, suggesting the main peak is pure and free of significant amounts of co-eluting impurities.

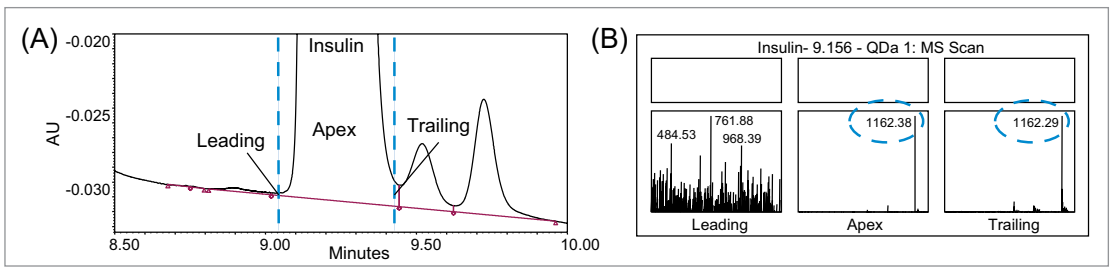

Figure 5. Mass Analysis assisted purity assessment of intact insulin. (A) separation of intact insulin. Blue dash lines indicate the cut-off of leading, apex, and trailing edge for the spectra displayed in (B) the Mass Analysis window in Empower. The apex and trailing window showed almost identical MS spectra, suggesting the main peak is relatively pure.

Application 3: Stability testing

The addition of orthogonal mass detection can also be used to improve product understanding during stability testing. A commonly used approach to monitoring the stability of protein based drug products is to run a peptide map and look for new peaks and/or shifts in peaks over time that may be indicative of degradation occurring. Taking insulin human peptides as an example, the Peak I and II degraded over time at room temperature and yielded Peak I′ and II′, as shown in Figure 6A. According to the MS data (Figure 6B), the Peak II and II′ were observed to have a mass shift of +1 Da, suggesting this degradation could be from deamidation (N to D). Similarly, the +17 Da mass difference between I and I′ suggests the spontaneous transfer from glutamine to pyroglutamic acid on the terminal amino acid. With proper method development, this workflow can be transferred to other biotherapeutics for monitoring degradation and product stability.

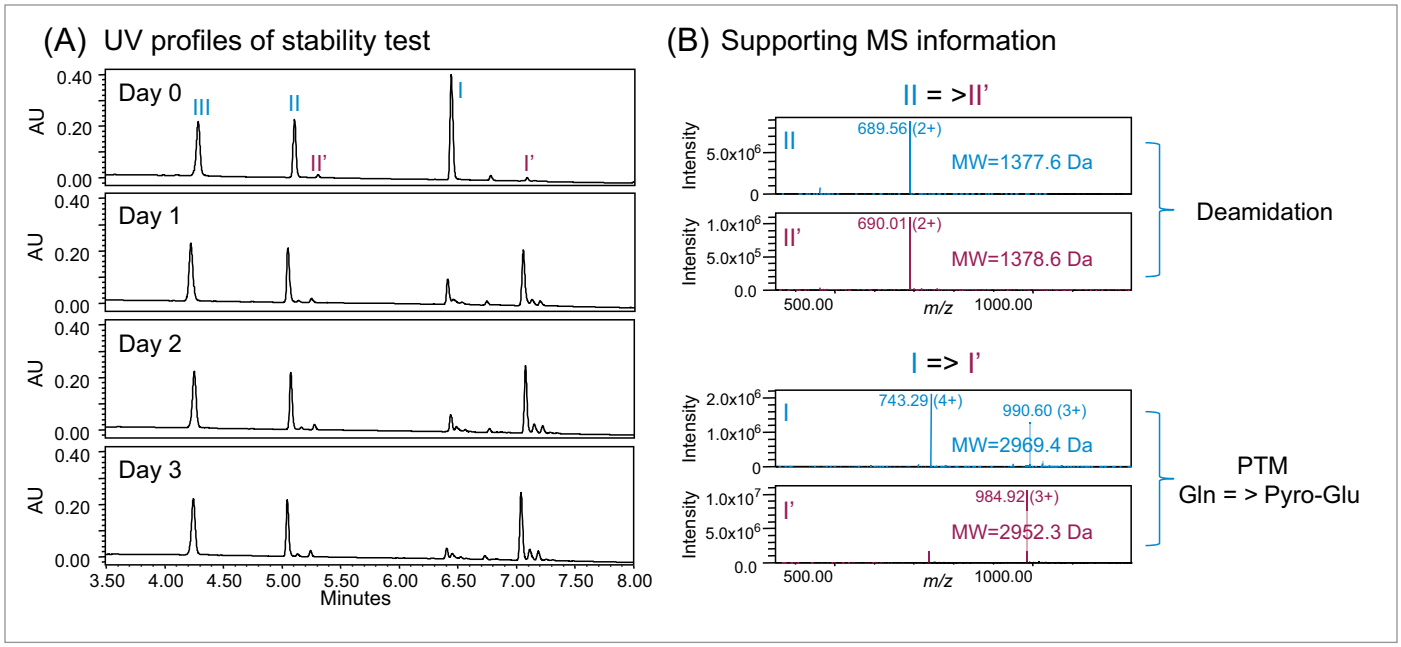

Figure 6. Stability test of insulin human digests. (A) UV profiles of stability test from day 0 to day 3. (B) Supporting MS information. The mass differences suggest that the degradation of Peak II and I might be from deamidation, and spontaneous transfer of glutamine to pyro-glutamic acid on peptide terminals, respectively.

CONCLUSIONS

This application note demonstrates how the addition of an ACQUITY QDa Mass Detector to LC-UV based workflows can improve the analysis of biotherapeutics. Using DFA as mobile phase additive, an LC-UV/MS-based platform method was developed as an identity test for insulin analogues with high chromatographic resolution and MS sensitivity. Purity and stability tests were also performed on intact or digested insulin analogues, showing improved specificity using orthogonal MS data. Together, the data showed how the addition of the ACQUITY QDa can improve the confidence and productivity when carrying out routine analysis of biotherapeutics.

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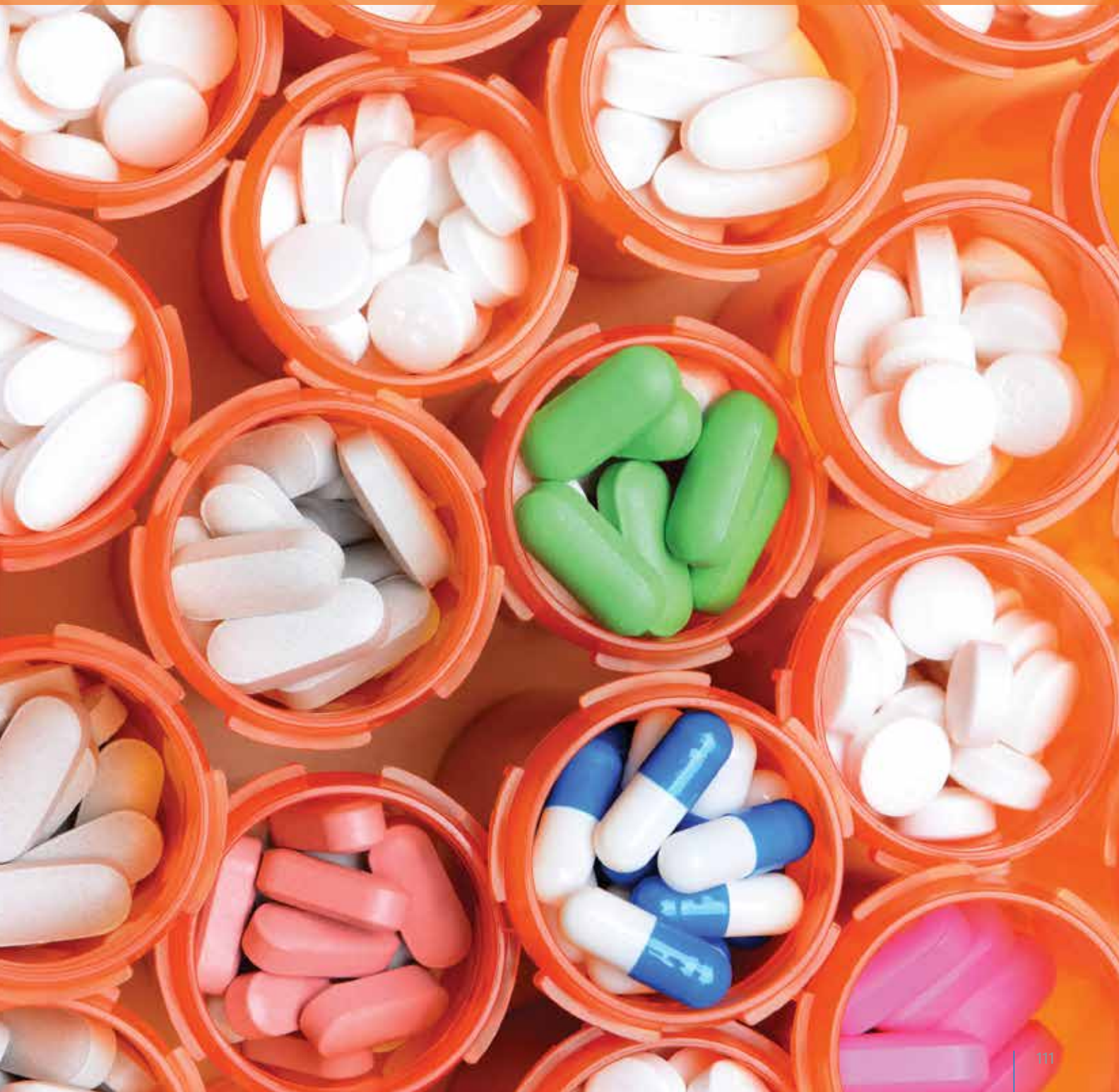

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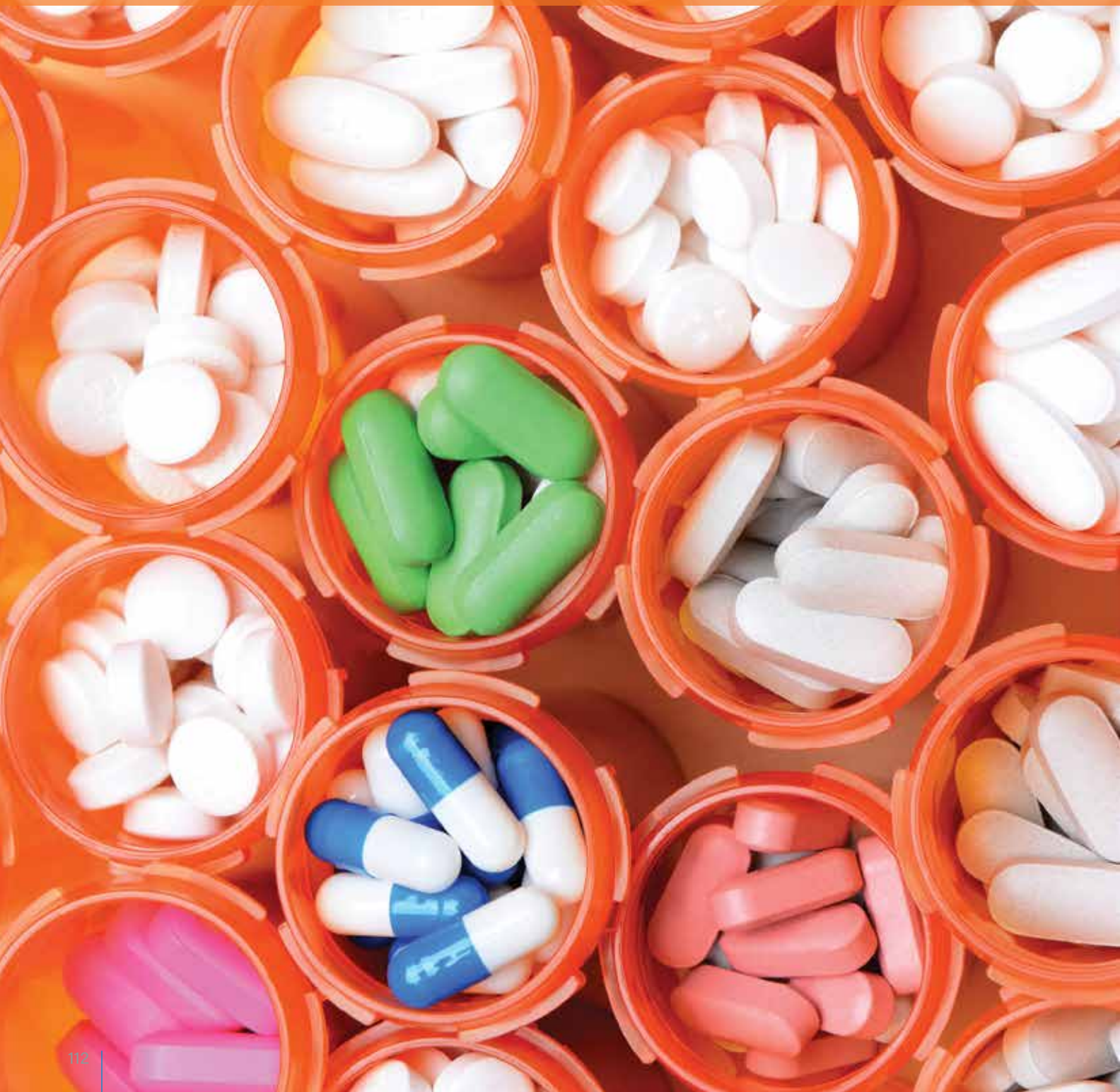

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Developing Analytical Chromatographic Methods for Pharmaceutical Stability Investigations

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APPLICATION BENEFITS

- Understand factors, responses, response criteria, and strategies to consider when doing chromatographic method development.
- Learn the benefits and limitations of Quality by Design software assisted chromatographic method development.

WATERS SOLUTIONS

Empower® 3 CDS with
S-Matrix Fusion QbD Software

ACQUITY Arc™

XBridge® BEH *XP* Column

XSelect® HSS *XP* Column

PDA Detector

ACQUITY® QDa® Mass Detector

KEYWORDS

Empower 3 CDS, Fusion QbD, Quality by Design (QbD), ACQUITY Arc, Method Validation Kit (MVK)

INTRODUCTION

An important aspect of pharmaceutical development is the determination of stability and shelf life for new pharmaceutical products. The ICH Quality Guidelines[1] specify the type and duration of stability investigations expected prior to drug registration in the European Union, Japan, and the United States. These are long term, accelerated, and stress studies where pharmaceutical companies use different exposure environments to evaluate drug quality over time.

The first two types of stability investigations assess shelf life. The temperatures and humidities used are, respectively, similar to and somewhat above that typically experienced by the drug product after manufacture. These studies require an analytical stability indicating method (SIM). This is a method capable of measuring drug and degradants in the presence of excipients and additives expected to be in the drug product formulation. Typically, SIMs are chromatographic methods. They are used to regularly monitor the time course appearance of impurities during long term and accelerated stability examinations.

The third type of study, stress, explores stability under harsher settings. Also called forced degradation studies, these investigations use reactive conditions (thermal, oxidative, photolytic, high/low pH, etc.) to produce "worst case" mixtures of drug and degradants. Method development with such difficult samples helps ensure the resulting analytical SIM has sufficient resolving power for the simpler mixtures from long term and accelerated stability investigations.

This application describes a case study where a Quality by Design (QbD) approach was used to develop an analytical stability indicating method for monitoring degradation of amoxicillin powder for oral suspension.

EXPERIMENTAL

Sample preparation

Amoxicillin Powder for Oral Suspension (APOS)

Amoxicillin Powder for Oral Suspension consists of the active pharmaceutical ingredient (API), amoxicillin, plus inactive excipients, and flavorants. The drug product dosage level is expressed as a target number of milligrams of amoxicillin per milliliter of aqueous suspension. This dosage level concentration is achieved when the powder is suspended in the requisite amount of water.

In this study, we used APOS manufactured at the 400 mg/mL dosage level. To prepare the stressed (forced degradation) sample required for this study, we suspended 40 mg APOS in 960 µL HPLC grade water. This mixture, in a sealed glass vial, was heated with magnetic stirring for 2 h at 90 °C. The resulting homogeneous solution was cooled to room temperature and then filtered (0.45 µm PDVF syringe filter). The filtered stock solution of stressed sample was stored frozen at -30 °C. As needed, the stock solution was thawed to remove a 40 µL aliquot. This aliquot was then diluted with 160 µL HPLC grade water to produce the working stressed sample solution of APOS for analysis.

Data management

Empower 3 Chromatographic Data System (Empower CDS) and Fusion QbD

Method conditions

System:	ACQUITY Arc with Quaternary Solvent Manager (QSM-R), Sample Manager (FTN-R), Column Manager (CHC with 3 column select valve), PDA Detector, QDa Mass Detector, Isocratic Solvent Manager (ISM for QDa makeup flow)
Columns[2]:	XBridge BEH C_{18} ***XP***, 2.5 µm, 3.0 x 50 mm (p/n: 186006033) (CV = 233 µL) XBridge BEH Shield RP18 ***XP***, 2.5 µm, 3.0 x 50 mm (p/n: 186006057) (CV = 233 µL) XSelect HSS T3 ***XP***, 2.5 µm, 3.0 x 50 mm (p/n: 186006153) (CV = 233 µL)
Mobile phase A:	20 mM aq formic acid (Weak Solvent 1, WS1)
Mobile phase B:	Acetonitrile (Strong Solvent 1, SS1)
Mobile phase C:	Methanol (Strong Solvent 2, SS2)
Mobile phase D:	20 mM aq ammonium formate (Weak Solvent 2, WS2)
On-line pH blending:	Mobile phases A and D were blended in the following ratios to achieve set pH values A/D = 100/0 gives pH 2.75 A/D = 80/20 gives pH 3.16 A/D = 40/60 gives pH 3.88 A/D = 5/95 gives pH 5.05 A/D = 0/100 gives pH 6.95
QSM-R flow rate:	1.10 mL/min
Profile:	Equilibrate at 0% SS for 2.60 min (12.3 CV) Isocratic at 0% SS for 0.46 min (2.2 CV). Gradient from 0% to 25% SS for the following gradient times (normalized gradient slopes) 5.88 min (0.90% SS/CV) 8.82 min (0.60% SS/CV) 11.77 min (0.45% SS/CV) 14.71 min (0.36% SS/CV) 17.65 min (0.30% SS/CV) Isocratic at 25% SS for 1.27 min (6.0 CV) Gradient from 25% to 50% SS for 0.5 min (10.5 % SS/CV) Isocratic at 50% SS for 1.27 min (6.0 CV). *Note: The % Strong Solvent plus the % Weak Solvents sum to 100%.*
Column temp.:	30 °C
Detection (UV):	214 nm
Injection volume:	4.0 µL working solution
ISM flow rate:	0.1 mL/min (using 0.1% formic acid in 1:1 methanol/water)
ACQUITY QDa split ratio:	1:5
ACQUITY QDa settings:	Ionization mode: positive and negative ESI Cone voltage: 15 V Capillary voltage: 1.5 kV (positive) and 0.8 kV (negative) Mass range: 50–1000 *m/z*

BACKGROUND

Amoxicillin is a bactericidal antibiotic that functions by irreversibly binding to the protein that catalyzes bacterial cell wall cross-linking, via opening of the strained beta-lactam ring. This capability makes a potent and selective drug but the resulting lability presents challenges to manufacturers who need to maintain (and confirm) good drug product storage stability. An analytical SIM capable of measuring the amoxicillin and its breakdown impurities is therefore imperative.

Figure 1 depicts amoxicillin degradation pathways. Prominent breakdown modes are hydrolysis of the four membered ring and of the central amide linkage. Subsequent self-reaction, decarboxylation and/or cyclization sequences lead to a variety of breakdown products. Amoxicillin is a polar amino acid and the typical degradants are also polar with many having both acidic and basic functionality. The polarity limits the mobile phase options to weaker solvent blends. The contrasting chargeable groups emphasize the need for optimal choice of mobile phase pH.

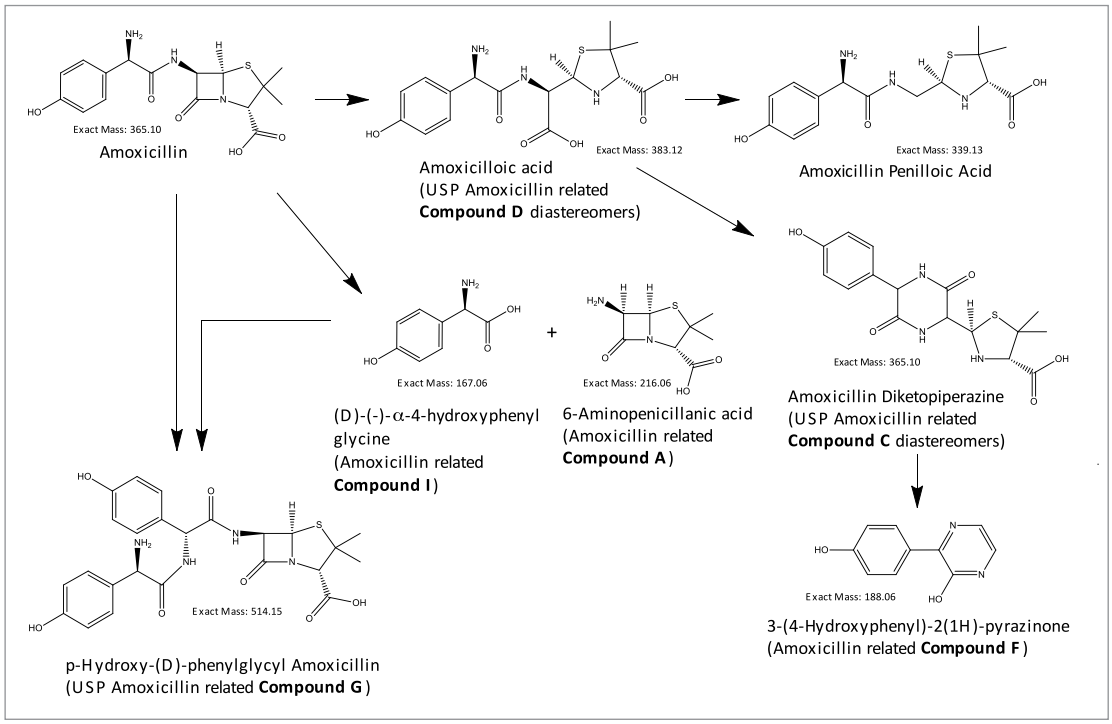

Figure 1. Amoxicillin and some degradation pathways.

The development of an analytical chromatographic method has three discreet stages: Set up, screen, and optimize. In the set up stage, the analyst selects the factors to vary, the responses to measure, the response criteria to assess progress, and the strategy to follow. Factors are the "inputs" to method development that cause analyte selectivity and resolution changes. Some factors, like the strong solvent, pH, and column stationary phase, have stronger effects whereas others, like gradient slope[3] and column temperature, have weaker effects. Furthermore, some factors are numeric and continuously variable, such as column temperature, pH, and gradient slope. Other factors are "categorical" or non-numeric, like column stationary phase. For each factor selected, the factor values are then set, such as which exact columns, solvents, and pH values to use.

Responses are the "outputs" to method development, as measured from chromatograms. Some responses are specific peak properties such as retention time, resolution, and tailing for specific components. Other responses are chromatogram properties from peak counting (aggregation), as in the total number of peaks in a chromatogram or the number of peaks with a specific desirable result.

Response criteria are used to compare the quality of different factor combinations. Examples of chromatogram property response criteria include "total # of peaks in the chromatogram = # of components known or believed to be present" or "maximize # of peaks found". For peak property response criteria, these include retention, tailing, and resolution in a specific range.

Figure 2 lists four method development strategies. The simplest strategy to execute but the least comprehensive is "One Factor At a Time" or OFAT, where the analyst alternates between picking one factor and value to test and assessing the responses from that test. The most comprehensive and labor/time intensive strategy uses a Full Factorial Screen where all combinations of factor values are tested to determine the best combination. A hybrid of these two approaches is the Tiered Screen. Here, all factors except one are locked to a specific value (e.g. a single specific column stationary phase, strong solvent, temperature, etc.). The one factor allowed to vary is changed to assess its affect on the responses. For example, the pH may be varied between a low and a high value. Based on how well response criteria are met, the one factor varied in the first tier is fixed to a single value. Moving to the second tier, some of the original locked factors are now systematically varied in full factorial fashion. This strategy is a balance between being comprehensive and being fast.

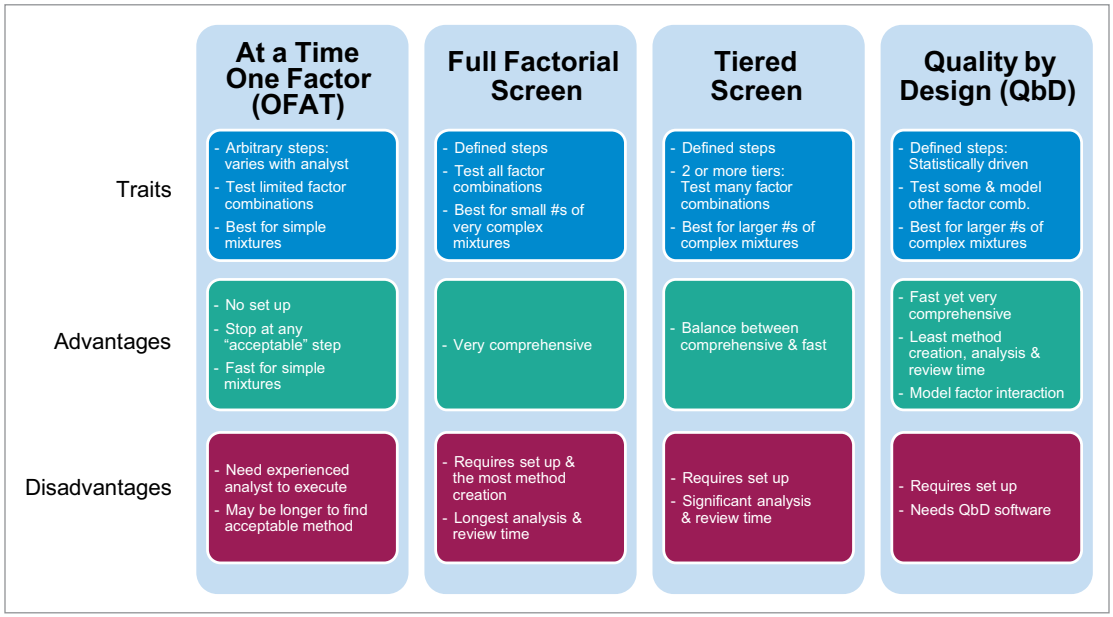

Figure 2. Comparison of different method development strategies.

These three strategies are examples of "analyst only" method development strategies. More recently, "analyst with software assist" strategies have appeared. These use specialized software to model the relationships between the varied factors and the measured responses. Such software falls into one of three categories: Statistical Based Modeling, Structure Based Modeling, and Chromatographic Theory Based Modeling. Statistical Based Modeling uses the Design of Experiments (DoE) approach and Fractional Factorial design to model Full Factorial space. In the pharmaceutical industry, this is called Quality by Design or QbD. Structure Based Modeling uses knowledge of analyte structures and functional group properties to create a model of analyte, stationary phase and mobile phase interactions. Chromatographic Theory Based Modeling uses a framework such as solvophobic theory to also create a model of analyte, stationary phase, and mobile phase interactions.

The "analyst only" strategies could be applied to the development of a SIM but each has disadvantages for complex samples that a stability indicating method may have to deal with. Following an "analyst with software assist" course generally gives a more comprehensive understanding of the chromatographic space with fewer actual conditions run. It has the further benefit of specialized method development software to manage the investigation and to review/interpret the data. The structure based modeling software is not very appropriate for the stress samples used in SIM development since the structures of all components are not known. Either the statistical based or the chromatographic theory based modeling categories are suitable for the development of a SIM.

QbD has an advantage in that it excels at modeling the comprehensive understanding afforded by a Full Factorial approach at low "cost". The "cost" in this case is a smaller but representative number of factor values and combinations that are run. Figure 3 shows a generic schematic of runs (factor combination points) needed to perform a generic Full Factorial screen with four factors.

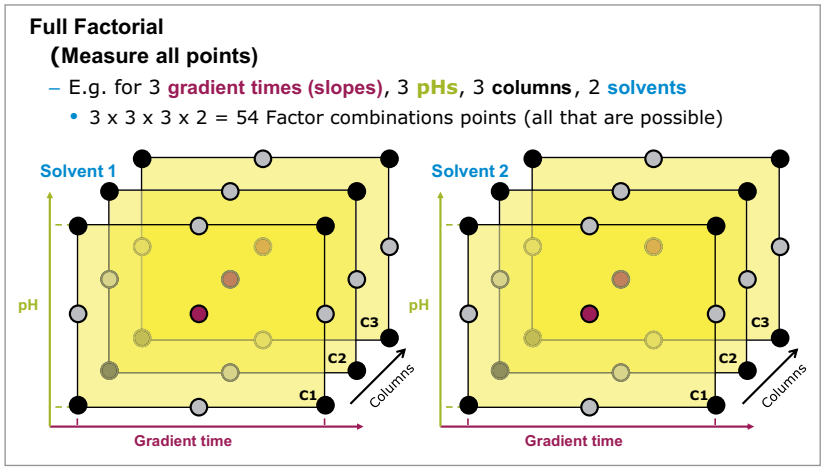

Figure 3. Generic schematic example for Full Factorial measurements at all factor combination points.

Figure 4 graphically depicts the same factor combination space but uses the Fractional Factorial screen of QbD wherein some factor combination points are not run but are still inferred due to appropriate sampling.

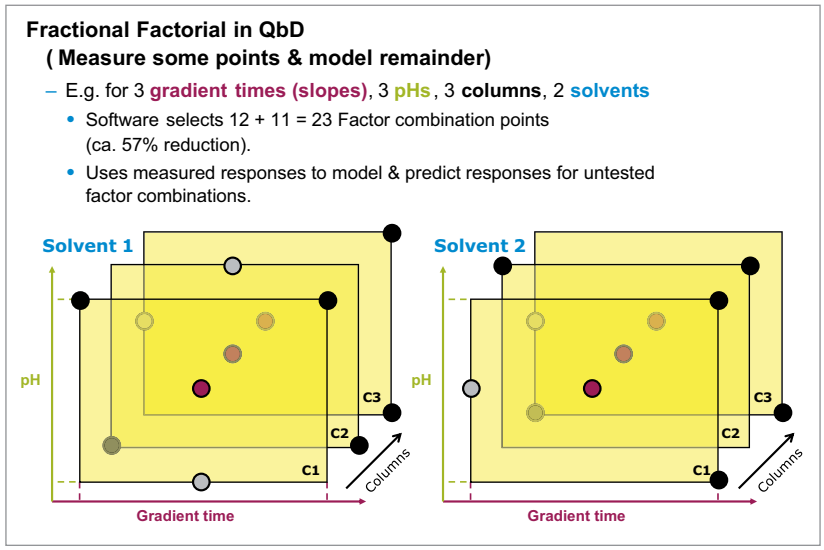

Figure 4. Generic schematic example for Fractional Factorial measurements at balanced and orthogonally selected factor combination points.

Where the statistical based modeling software is Fusion QbD by S-Matrix, there is the additional advantage of tight integration with the Empower CDS. The Fusion QbD Software manages all aspects of the method development. These include study design with balanced and orthogonal selection of factor values and combinations, automatic acquisition method creation in Empower, retrieval and processing of chromatographic data and responses from Empower, and scoring of results plus graphical display of factor values and combinations vs. responses. For these reasons, we used the Quality by Design approach in this application note.

RESULTS AND DISCUSSION

Our study set up began by selecting all categorical factors and their values to screen since such factors are modeled differently from numerical factors and need to be specified first. In this case, our categorical factors were column stationary phase and strong solvent. For the column stationary phase, we first picked XBridge BEH C_{18} for good all around reverse phase selectivity. We then chose XBridge BEH Shield RP18 which offers additional interactions from the embedded polar group and XSelect HSS T3 which provides additional silanol interactions. Both are potentially beneficial for the analysis of polar compounds. The strong solvents were acetonitrile and methanol. Since we used the ACQUITY Arc, a UHPLC class instrument, it was important to match the instrument dispersion and pressure capabilities to the appropriate column diameter and particle size. Also a short column length is desirable for the creation of rapid higher flow methods. These considerations led us to select a column geometry of 3.0 x 50 mm with 2.5 µm particles.

We also selected some numerical factors with specific values to screen. The mobile phase pH was picked since it is the numerical factor with the strongest effect on analyte selectivity and peak shape when acidic and basic functional groups are present. As stated above, this is especially important when developing an amoxicillin SIM. The five pH values in the Experimental section, for the pH range of 3 to 7, allowed use of all three columns. A second numerical factor of gradient slope was also selected (see the five specific values in the Experimental section). Examining this weaker effect was important to assess how this "method speed" factor[4] can impact the measured responses. The other "method speed" factor of flow rate was set to a "ballistic" value that achieves ca. 80% of the maximum instrument pressure (ca. 7600 out of 9500 psi) using the more viscous methanol strong solvent. This and other fixed factor values are in the Experimental section.

Using three columns, two solvents, five pHs and five gradient slopes, the Full Factorial number of combinations to run is 150 (3x2x5x5). To get the benefit of this rich data space at a fraction of the analysis (and analyst) time "cost", we selected the "analyst with software assist" strategy of Statistical Based Modeling using Fusion QbD Software. Figure 5 contains a Fusion QbD screen image specifying the strong solvents and the on-line blending to produce the pH values. Figure 6 depicts the set up for the gradient slopes and the columns.

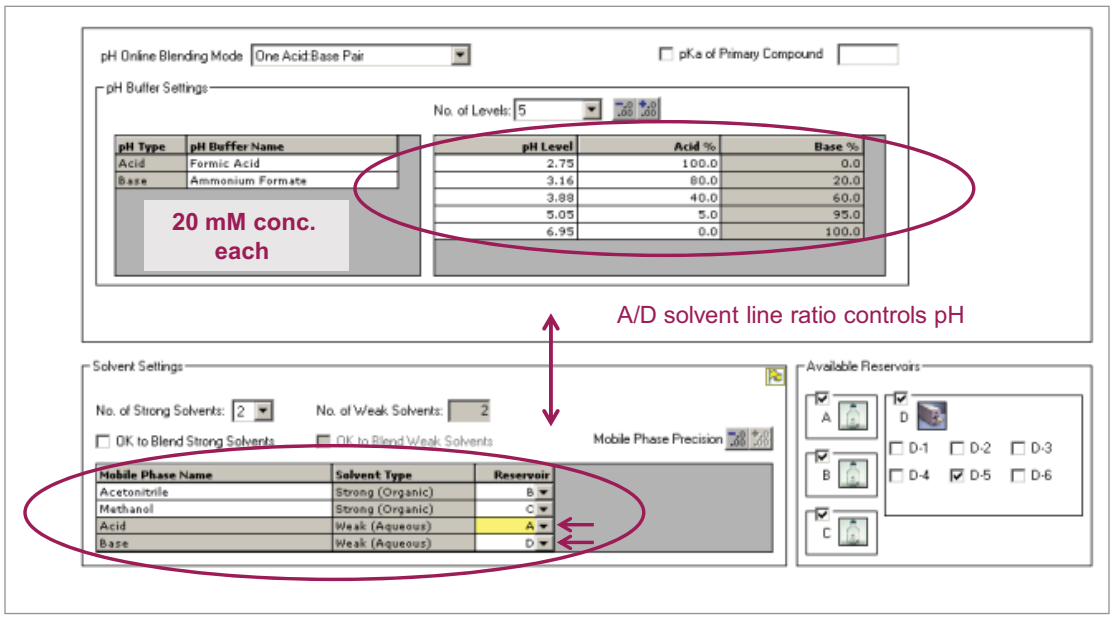

Figure 5. Fusion QbD set up of solvents and pH on-line blending.

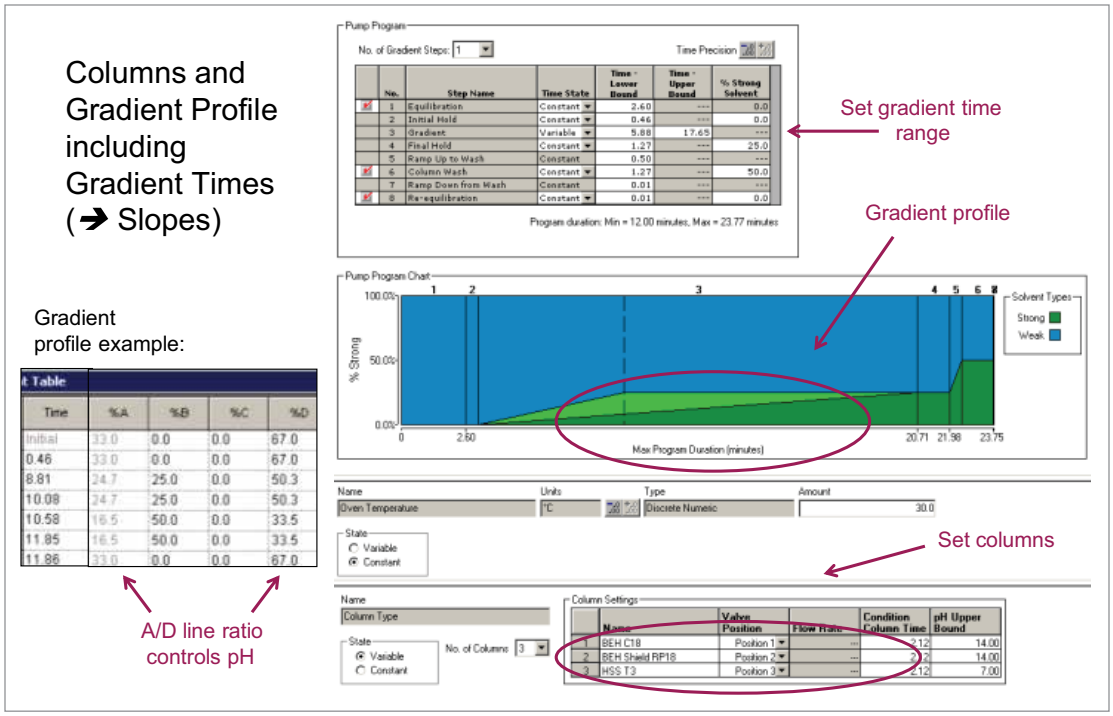

Figure 6. Fusion QbD set up of gradient slopes and columns.

This established four factors and their values to screen. Next, we set the responses to measure and the response criteria to drive towards. For chromatogram property responses, we used the "Total Number Of Peaks Found" and the "Number Of Peaks With A USP Resolution of ≥1.50 and ≥2.00." The response criteria were to maximize these peak counts.

For peak property responses, we used "Max Peak #1 USP Resolution" and "Max Peak #1 USP Tailing." In Fusion QbD parlance, "Max Peak #1" is the largest peak observed in a chromatogram, in our case, the API. So here, the "Max Peak #1 USP Resolution" response measured specific API peak separation quality and the "Max Peak #1 USP Tailing" measured specific API peak shape quality. The response criterion for "Max Peak #1 USP Resolution" was ≥1.50 and for "Max Peak #1 USP Tailing" was = 1.00 ± 0.10.

From these factors and factor values, Fusion QbD selected 38 combinations to run. Of these, 29 factor combinations modeled the full factorial space and 9 provided replicates to assess the measurement uncertainties. This eliminated ca. 80% of the injections required for full factorial screening and greatly shortened the acquisition and data review time. Even this reduced set of work required 67 instrument methods and 67 method sets to handle column conditioning, equilibration, and the actual runs. Fortunately, Fusion QbD created all of these Empower methods plus the one sample set method needed to orchestrate these acquisitions.

Empower used the acquisition methods created by Fusion QbD to screen the APOS forced degradation sample under the Quality by Design selected conditions. The stressed conditions generated many degradants as Figure 7 illustrates in a representative chromatogram. Five Empower processing methods, one for each gradient slope/time, were used to objectively find and integrate peaks. All processing methods applied the ApexTrack™ integration algorithm and had the same peak integration parameters. The methods differed by having [1] increased apex detection peak width and [2] extended chromatogram end times as the gradient slope decreased (gradient time increased).

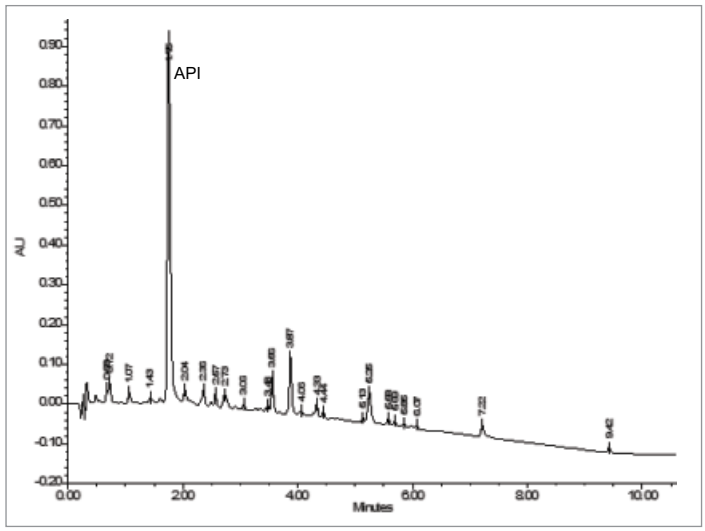

Figure 7. Representative APOS stress sample chromatogram.

This accounted for broadened peaks and longer acquisitions at shallower gradients. To focus only on the more abundant degradants, a minimum peak area was set equal to 1% of the average API peak area.

Fusion QbD fetched the processed data from Empower and used the measured responses to calculate models for each categorical factor combination. For each such model, a Cumulative Desirability Result score was calculated. This score (0 to 1 scale) assesses the likelihood that each combination will meet the response criteria. Table 1 summarizes these study results. The high score for the combination of acetonitrile as the strong solvent and the BEH C_{18} as the column stationary phase made clear each was the best choice for these categorical factors.

With the column and strong solvent categorical factor values selected, the Fusion QbD model provided a "performance map" for the numerical factors of gradient time and pH. A performance map segments numerical factor space into regions of predicted better response (closer to target criteria) and worse response (further from target criteria). It is built by sequentially plotting the effect of the numerical factors on each response.

Figure 8a shows a blank map where no responses are considered. The entire gradient time vs pH space is "white" because any such combination is acceptable when it doesn't matter what the responses are. Considering the chromatogram property response of "total number of peaks found", we set a threshold such that about a third of the factor space is colored and therefore rejected as lower performance (fewer peaks found), Figure 8b. In our case, this is somewhat arbitrary and up to the analyst's discretion since, in a stress sample, we have no way of knowing how many peaks "should" be present.

The chromatogram property responses "Number of Peaks With USP Rs ≥1.50 and ≥2.00" were considered next. Again, rather arbitrary thresholds were applied, Figure 8c and Figure 8d, to exclude underperforming factor combinations. The excluded regions had some overlap with the first chromatogram property but they mostly eliminated the higher pH region.

Table 1. Cumulative Desirability Result scores for strong solvent and column stationary phase categorical factor combinations.

Cumulative Desirability Result (scale of 0 to 1)	BEH C_{18}	BEH Shield RP18	HSS T3
Acetonitrile	0.9360	0.7476	0.3826
Methanol	0.7735	0.5279	0.7480

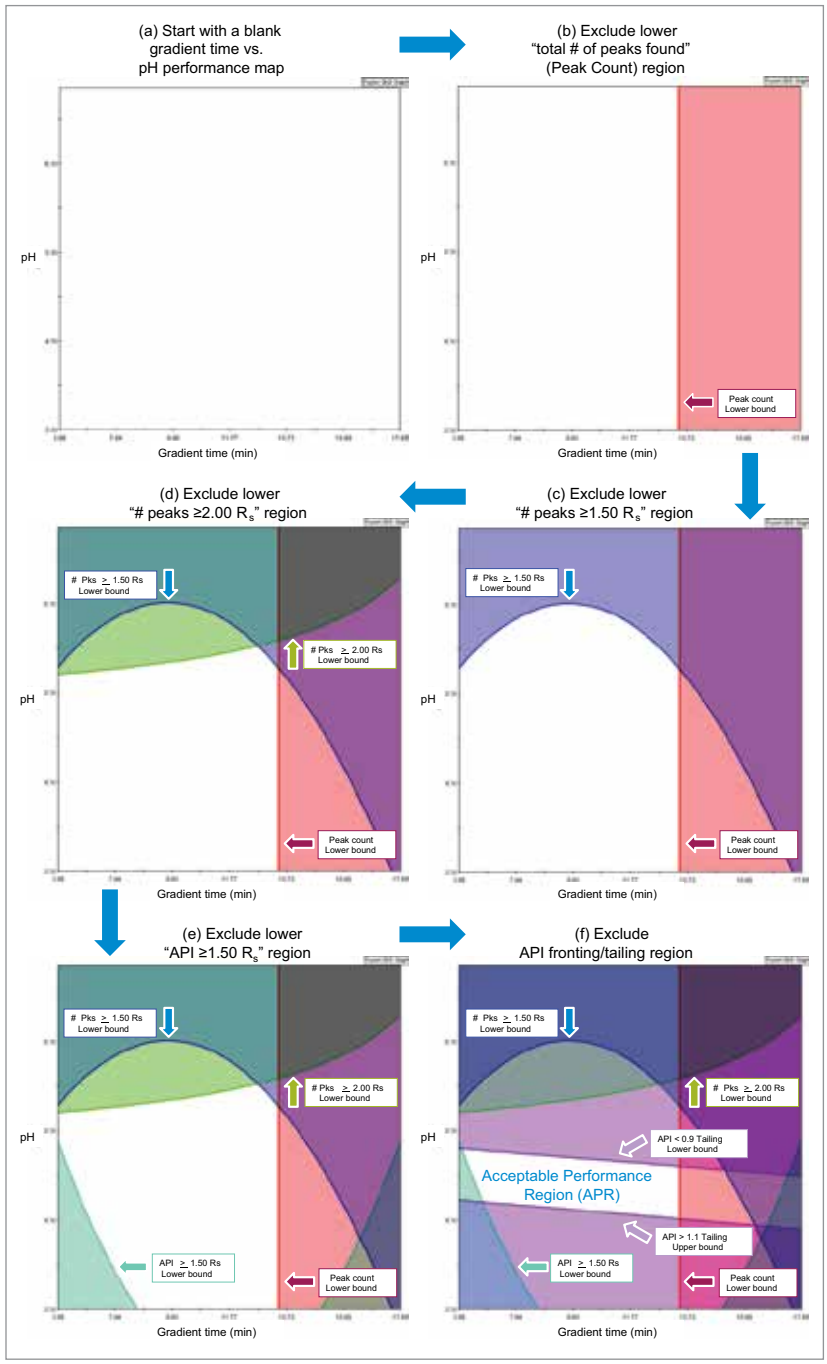

Figure 8. Construction of the Fusion QbD performance map.

Unlike the chromatogram property responses, which involved peak counting for an unknown number of degradants, specific peak property responses had well defined expectations. In Figure 8e, we applied the threshold "Max Peak #1 USP Resolution ≥1.50" response criterion. This rejected the lower left and right corners with low pH and both low and high gradient times. Finally, we used the threshold "Max Peak #1 USP Tailing = 1.00 ± 0.10" which eliminated all combinations except those in a relatively narrow pH range, Figure 8f. The unrejected white space is called the modeled Acceptable Performance Region (APR). This is the region in and around which verification test runs are next performed and where method optimization then occurs.

Figure 9 shows a verification chromatogram obtained at the lower end of the gradient time range and at the middle of the pH range defined by the APR. The measured API resolution was acceptable and consistent with the model. However, the resolution that is being optimized is a "before peak" resolution and it is obvious that this chromatogram has a poor "after peak" resolution from a component that closely follows the API. Also there are some larger peaks that coelute.

Verification testing above the APR, Figure 10, found the API resolution lower than the model predicted (observed USP Rs = 0.46; expected ≥1.50) but the larger peaks were more favorably spread out. This suggested that a pH somewhat outside of what the model indicated may provide useful separation for some peaks.

Testing below the APR, Figure 11, we found the resolution and tailing as expected (≥1.50 and >1.1, respectively), the peak counts were somewhat improved and the larger peaks were more spread out. These results illustrate the importance of doing verification runs in the vicinity of the APR, as software assistance provides only a guide on where to focus one's efforts.

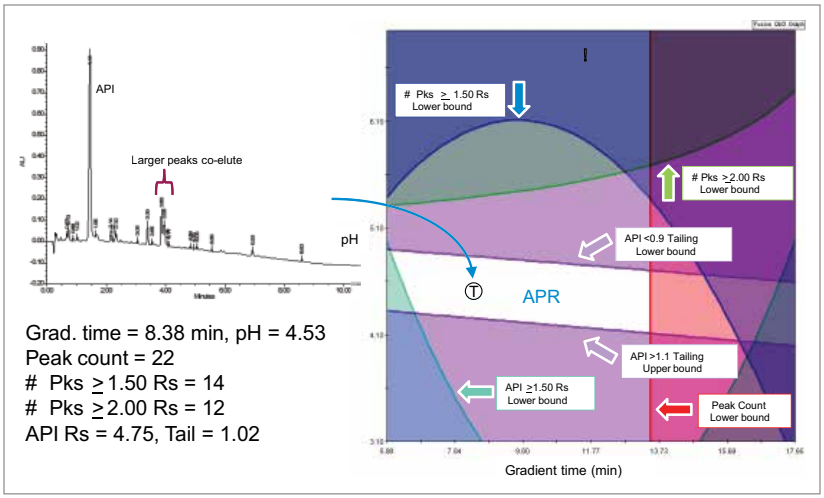

Figure 9. Example chromatogram within the modeled Acceptable Performance Region (APR).

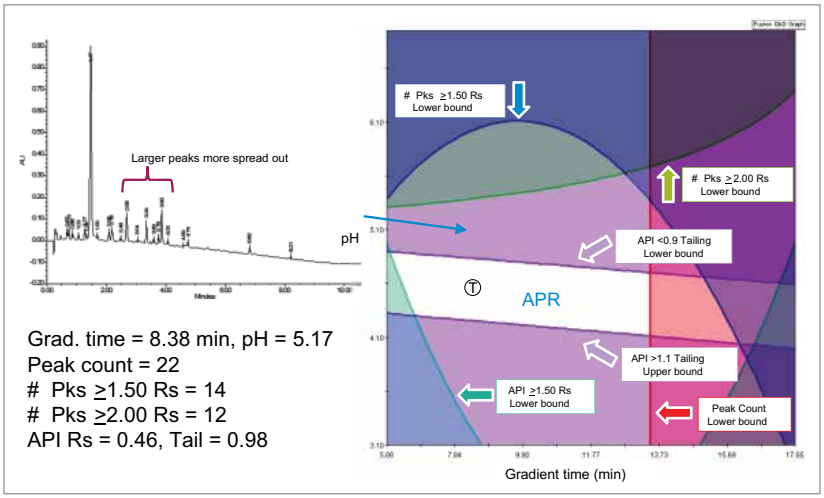

Figure 10. Example chromatogram above the modeled Acceptable Performance Region (APR).

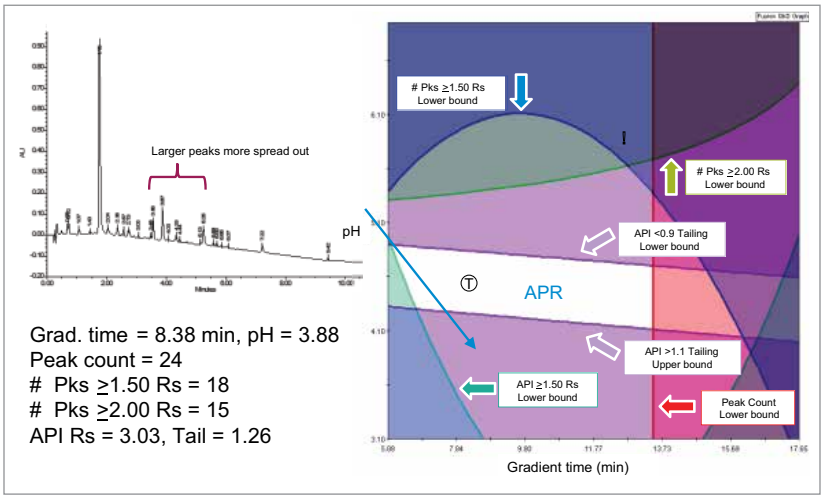

Figure 11. Example chromatogram below the modeled Acceptable Performance Region (APR).

The combination of the modeled APR plus the results from verification test runs allowed us to set an optimization region. In this study, the APR had a rather wide gradient time range but a relatively narrow pH range. The "width" of the optimization region was therefore set at the lower end of the APR gradient times to generate a faster final method. The "height" was set somewhat above and below the pH range defined by the APR for the reasons given above. Figure 12 depicts the optimization region.

For specific optimization factor values, we selected gradient times of 6.80, 8.35, and 9.90 min and pH values of 4.01, 4.49, and 5.04. Additionally, we added optimization factor values for column temperatures of 30, 35, and 40 °C. Some example chromatograms from the optimization are given in Figure 13. Coelution of degradants in the latter part of or just after the API peak was a common theme. Both situations are "invisible" to Fusion QbD.[5] The former condition can only be observed using the Empower Mass Analysis window tool (shown in the insets of these figures) whereas the latter can be found by the analyst on visual inspection of the chromatogram. Coelutions among degradants were also observed during optimization. This also presents a challenge, since Fusion QbD does not track individual degradant peaks. As a result, the optimization stage of method development may not benefit as much from the current generation of software assistance compared to the great advantages such help affords during screening.

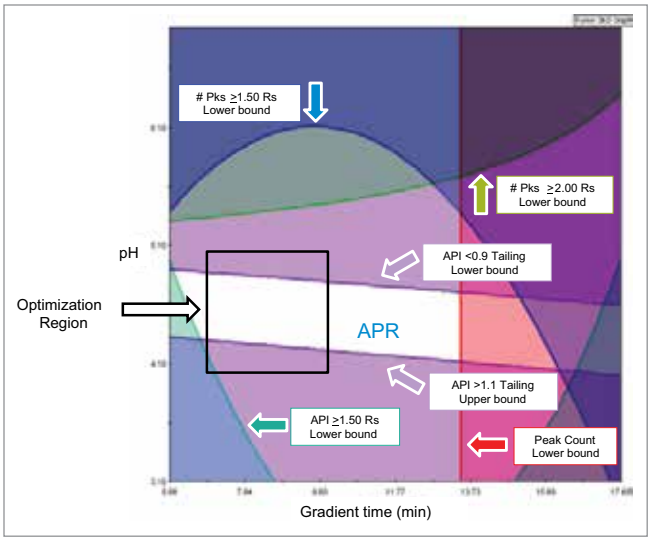

Figure 12. Fusion QbD Performance Map with the optimization region.

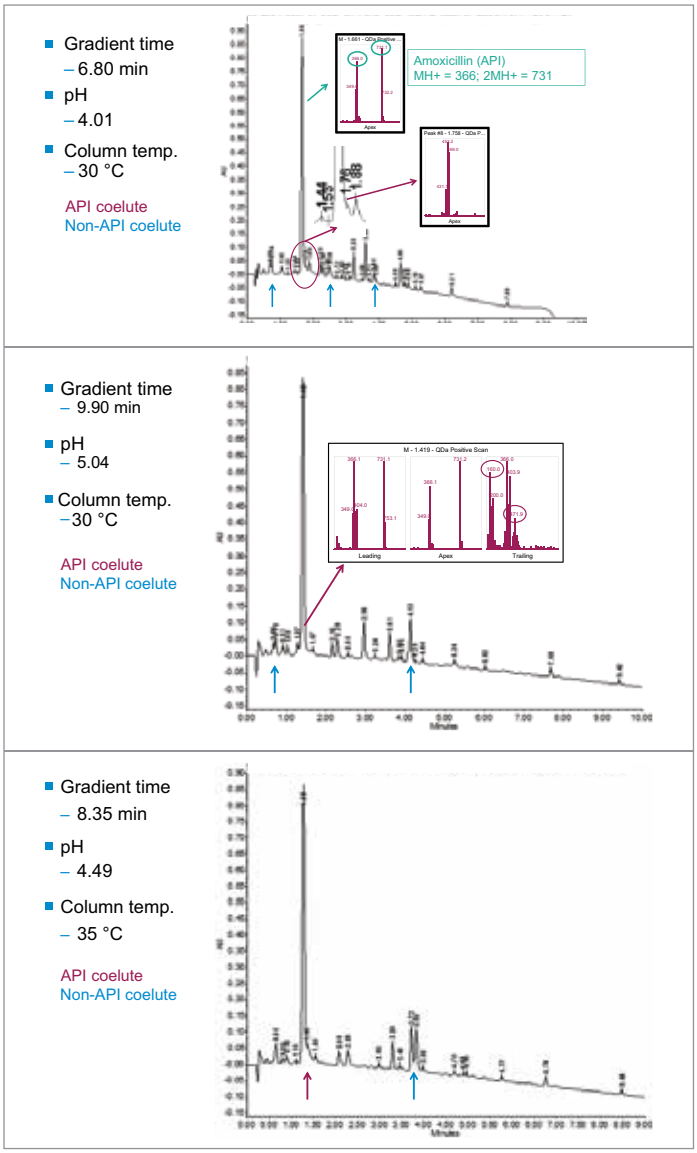

Figure 13. Example optimization chromatograms.

Figure 14 lists the optimized factor values for gradient time, pH, and column temperature and shows the resulting chromatogram. The Empower Mass Analysis window indicated good peak purity (homogeneity) for the API peak and there was acceptable resolution for the degradant peaks. Using these conditions, standard injections confirmed the identity of the indicated components.

The final chromatographic SIM after QbD software assisted screening and optimization is displayed in Figure 15. The best pH was outside of the APR and near the lower edge of the Optimization Region. This reinforces the need to treat the software assistance as a guide that must be verified. The best column temperature was found during the Optimization stage.

Once a final method is created, it is a good idea to validate that method on different column batches. This allows assessment of the method robustness to any small changes in different manufacturing lots of stationary phase particles or variations in column packing. After optimization, we therefore used an

XBridge BEH C_{18} ***XP***, 2.5 µm, 3.0 x 50 mm Method Validation Kit (MVK) (p/n: 186006199) and compared our method results from the different column batches with the results from the original column. As demonstrated in Figure 16, equivalent chromatograms were observed for all the columns.

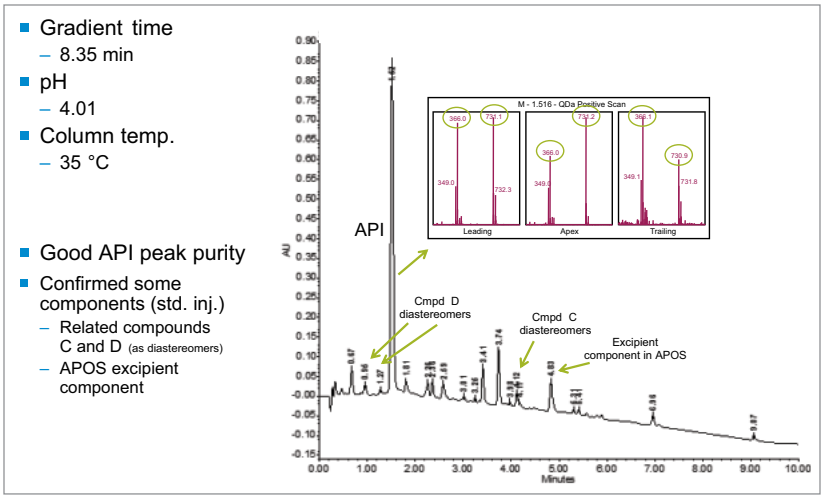

Figure 14. Chromatogram using best optimization conditions.

System:	ACQUITY Arc with PDA and QDa detection, 3 column positions
Column:	XBridge BEH C_{18} ***XP***, 2.5 µm, 3.0 x 50 mm
Mobile phase A:	20 mM aq formic acid (for online pH blending)
Mobile phase B:	Acetonitrile
Mobile phase D:	20 mM aq ammonium formate (for online pH blending)
Flow rate:	1.10 mL/min
Gradient:	See Table
Column temp.:	35 °C
UV detection:	214 nm
Injection volume:	4.0 µL

Time (min)	%A	%B	%D	#CV	Slope (%B/CV)
0.00	33.0	0.0	67.0	–	–
0.46	33.0	0.0	67.0	2.2	–
8.81	24.7	25.0	50.3	39.4	0.64
10.08	24.7	25.0	50.3	6.0	–

CV = Column Volume
(from the Waters Columns Calculator)

Listed Constant Mobile Phase A/D Ratio Sets pH = 4.0

Figure 15. Final Stability Indicating Method for analysis of APOS.

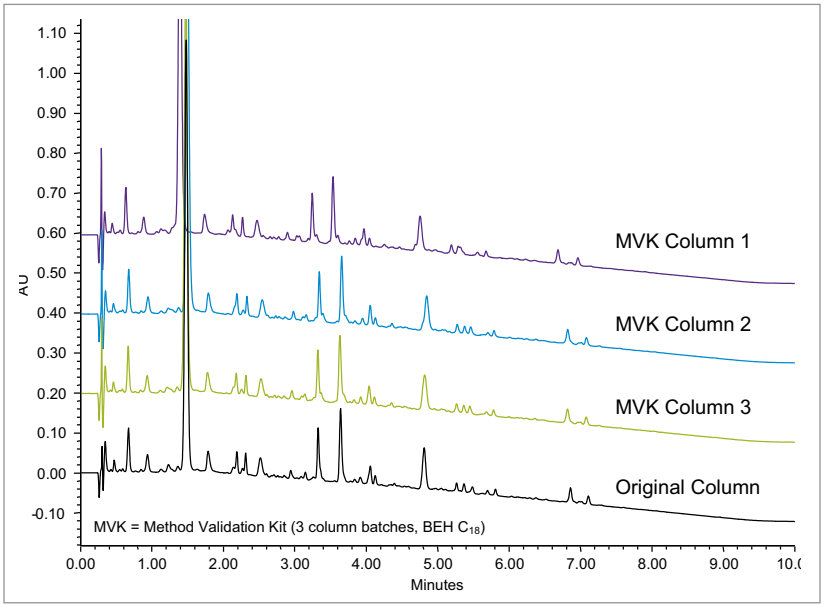

*Figure 16. Final SIM using a Method Validation Kit compared to the original XBridge BEH C_{18} **XP** Column.*

CONCLUSIONS

This application describes how factors affecting analyte separations can be examined in an orderly and efficient fashion to create an analytical chromatographic method. A Quality by Design approach was illustrated, leveraging the software assistance of Fusion QbD to select factor value combinations, create acquisition methods, and evaluate modeled responses. The resulting Stability Indicating Method has sufficient resolving power to separate the worst case mixture of analytes afforded by a forced degradation sample. This gives confidence that this SIM will be able to separate whatever degradants arise during long term and accelerated pharmaceutical stability investigations.

References

1. International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, Stability Sections Q1-A though Q1-E.
2. The packed column volume, CV (in µL), is calculated using the following relationship: $CV = \varepsilon_t L\pi(D/2)^2$. The terms L and D are the column length and diameter (in mm), respectively. The term ε_t is the total column porosity which is the fraction of the column taken up by the mobile phase in the space between particles and in the internal pores of the particles. A nominal value of total column porosity is generally applied in such calculations. In well packed columns, $\varepsilon_t = 0.66$ has been used for fully porous particles and $\varepsilon_t = 0.49$ has been used for solid-core particles. For example, see the Waters Columns Calculator at http://www.waters.com/waters/support.htm?lid=134891632&type=DWNL.
3. Gradient slope can be described in various terms. In this study, the % strong solvent change is a fixed parameter so the gradient time is sufficient to communicate the changing factor of gradient slope. However, it is useful to think of gradient slope in normalized terms, specifically the % strong solvent change per unit of column volume. In this way, the analyst can easily compare different gradient profiles independent of the column geometry and method parameters such as flow rate. Both descriptions of gradient slope are used herein.
4. "Method speed" factors are those that directly affect how quickly a method can be executed. The gradient slope and the flow rate are two such factors. A steeper gradient slope and/or a higher flow rate will provide a method that takes less time to perform and is hence "faster".
5. To help mitigate these challenging situations, Fusion QbD version 9.8.0 SR2 Build 858 and later have peak property responses and response criteria for resolutions before AND after a "Max Peak" such as the API. This makes it easier to find chromatographic conditions with adequate resolutions both before AND after large peaks such as the API.

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The Direct Quantification of a Mutagenic Impurity, Methyl Amino Crotonate, Using ACQUITY UPC² and QDa Detector

Janet Hammond[1] and Mark Wrona[2]
[1]Waters Corporation, Wilmslow, UK, [2]Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Highly selective, sensitive, and robust mass detection analysis using the ACQUITY® QDa® Detector
- ACQUITY UPC²® provides high resolution and throughput with a short analysis time of 2.5 minutes
- Alternative technology for the analysis of aqueous-sensitive compounds

WATERS SOLUTIONS

ACQUITY UPC²

ACQUITY UPLC Column Manager

ACQUITY QDa Detector

MassLynx® Mass Spectrometry Software
(also available with Empower® control)

KEYWORDS

UPC², QDa, mutagenic impurities, quantification, mass detection

INTRODUCTION

Mutagenic impurities, formerly known as genotoxic (GI) or potential genotoxic impurities (PGI), are compounds that have the potential to modify DNA, and as a consequence can cause cancer. It is important that impurities potentially present in the marketed drug are evaluated early in the drug development process. To that end, analytical methods must be developed that are sensitive and specific enough to determine the levels in both drug substance and product.

The International Conference on Harmonization published ICH M7 guidelines, which highlight the requirements for assessment and control of DNA-reactive impurities to ensure the safety of pharmaceutical products.[1] The European Medicine Evaluation Agency (EMEA), U.S. FDA, and the Asia regulatory agencies all follow these guidelines. They require that any mutagenic impurities in a drug substance or drug product must be below the Threshold of Toxicological Concern (TTC) of 1.5 µg per day based upon the maximum daily dosage of the pharmaceutical compound over a lifetime. For example, for a dosage of 1 g of Active Pharmaceutical Ingredient (API) per day, any impurity must be less than 1.5 ppm (1.5 µg). This is orders of magnitude lower than for general pharmaceutical impurities analysis, which is at the 500 ppm level and governed by Q3B(R).[2]

Pharmaceutical analysis is typically performed using LC with UV detection for non-volatile compounds, or GC with FID detection for volatile compounds. However, the low levels of detection required for mutagenic impurities present a significant challenge. In these situations, MS detection is required in order to achieve the desired sensitivity. Some of these methods are required to provide support during the whole life cycle of a drug from early development through to manufacturing quality control. Typical reverse-phase (RP) (where the majority of separations are done on C_{18} stationary phases) and normal-phase (NP) chemistries can be used; this opens up a wide range of selectivity choices to help develop successful separations.

Convergence chromatography (CC) is a chromatographic technique similar to HPLC, but instead of the weak mobile phase being aqueous it is replaced with supercritical carbon dioxide (CO_2). Supercritical CO_2 can be paired with a large number of different co-solvents to increase the solvating power. CO_2 is miscible with the whole range of the eluotropic series opening up a large choice of solvent selectivity – with methanol, IPA, ethanol, and acetonitrile being the most commonly used co-solvents.

Methyl-3-aminocrotonate (MAC) is a Michael-reactive receptor and a starting material in a number of different cardiovascular drug products. The API used is an active substance from a proprietary drug product; therefore, only the partial structure is shown in Figure 1. MAC flags up a positive from the mutagenic structural alerts. This compound would typically be analyzed by static head space (SHS) GC-MS after derivatization with trifluoroacetic anhydride to increase the volatility (Figure 2).[3] When MAC (underivatized) was analyzed by UPLC®-MS nothing was seen; this was thought to be due to its poor stability in aqueous solvents.

In this type of trace analysis where there is a large amount of matrix it would be advantageous if chemical derivatization of the mutagenic impurity can be avoided for the following reasons:

- The formation of acylation derivatives can be difficult to prepare
- Reaction bi-products can occur, which could add more complexity to the matrix
- The extra derivatization step would require extra validation to be completed

To avoid the above issues, this application note discusses the work carried out to investigate the use of Waters® UltraPerformance Convergence Chromatography™ (UPC²) and MS detection using the ACQUITY QDa as an alternative technology for the analysis of MAC without the prior need of derivatization, for the detection and quantification in an active pharmaceutical ingredient (API).

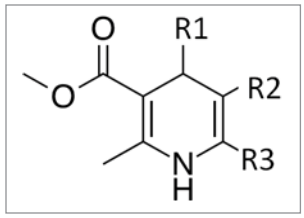

Figure 1. Partial structure of drug product.

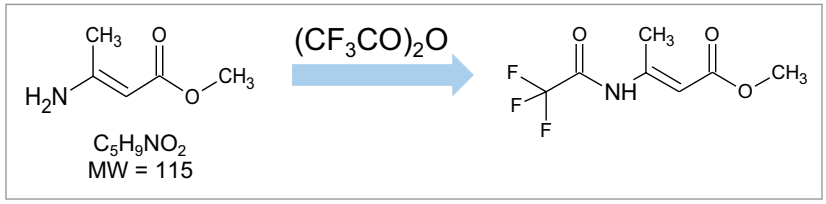

Figure 2. Reaction scheme for the derivatization of MAC, typically used for GC analysis.

EXPERIMENTAL

Sample preparation

10, 5, 2.5, 1, and 0.5 ppm standards of MAC (with respect to 1 mg/mL API) were prepared in methanol.

UPC² method conditions

System:	ACQUITY UPC²
Column:	ACQUITY UPC² BEH, 1.7 µm, 2.1 mm x 100 mm (P/N 186006560)
ABPR:	1700 psi
Column temp.:	40 °C
Sample temp.:	15 °C
Injection volume:	1 µL
Flow rate:	1.5 mL/min
Mobile phase A:	CO_2
Mobile phase B:	MeOH
Gradient:	5% to 95% B at 1.5 mins, held until 2.1 mins then 5% B
Run time:	2.5 mins
Make up solvent:	MeOH, 2% H_2O and 0.1% formic acid
Make up flow:	0.6 mL/min

MS conditions

MS system:	ACQUITY QDa Detector
Ionization mode:	ESI positive
Single ion recording (SIR):	*m/z* 116.1 Da $[M+H]^+$
Capillary voltage:	0.8 kV
Sampling frequency:	5 Hz
Probe temp.:	600 °C
Cone voltage:	8 V

Data management

MassLynx Software v4.1

RESULTS AND DISCUSSION

The structure of MAC is shown in Figure 2. It has a nominal molecular weight of 115 Da. Full scan analysis on the ACQUITY QDa Detector detected the expected $[M+H]^+$ ion at *m/z* 116.1 Da.

METHOD DEVELOPMENT

A number of different mobile B eluents were tried – including methanol, methanol with 0.1% formic acid, and methanol with 20 mM ammonium formate. The final method resulted in an elution time of 0.7 minutes for the MAC and 1.5 minutes for the API (Figure 3).

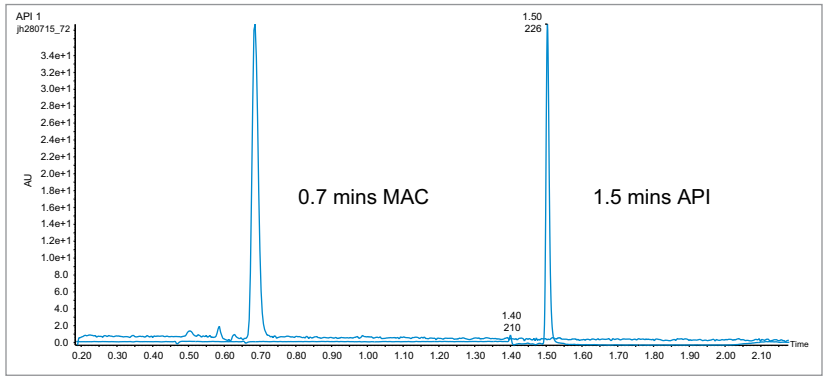

Figure 3. MS TIC of m/z 116 from MAC and DAD of API from UPC² analysis.

ACQUITY QDa Detector probe temperature and cone voltage conditions were optimized for maximum sensitivity for the MAC analysis.

MATRIX EFFECTS

Selectivity issues can arise during trace analysis because the target analyte is at low levels in the presence of a large concentration of API, a counter ion, or – in the case of drug products – excipients. It is important when carrying out this type of analysis that a series of samples of API or drug product are spiked with the corresponding mutagenic impurity. This will indicate if there are any issues relating to stability, ion suppression, or enhancement effects. In this analysis, samples were prepared by spiking into the API a 1 ppm MAC standard, then analyzed. The result of this experiment showed that the areas for the unspiked and spiked standard were comparable. This implies the matrix does not have an effect on the MS response of this analysis. The areas of both spiked and unspiked samples are overlayed as shown in Figure 4.

LINEARITY AND SENSITIVITY

The linearity of the method was evaluated with five standards of 0.5, 1.0, 2.5, 5, and 10 ppm of MAC in methanol. The method showed good linear correlation between the peak areas and the ppm concentration with a correlation coefficient of $R^2 = 0.9985$ (Figure 5). The signal-to-noise for the LOQ standard is more than 10 to 1 (Figure 6), and signal-to-noise ratio is 3 to 1 at the LOD standard (Figure 7). The percentage standard deviation of the six individual injections of all the five standards was less than 4%.

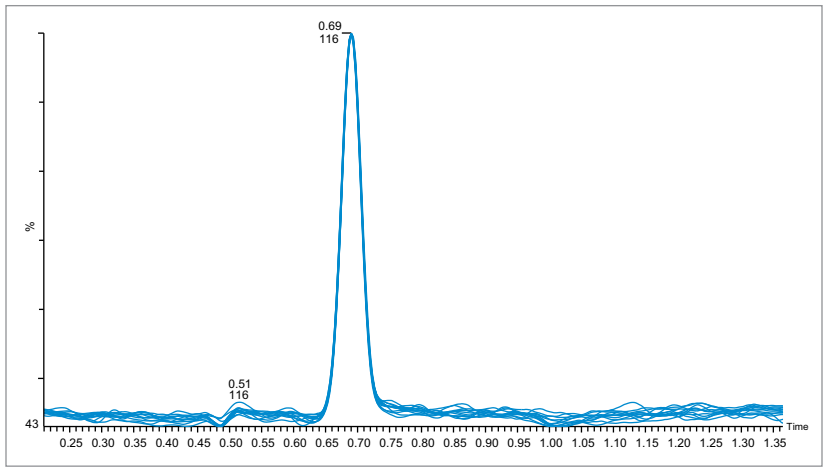

Figure 4. An overlay of TIC chromatogram of m/z 116 from six injections of 1 ppm standard MAC and a 1 ppm MAC standard spiked into an API.

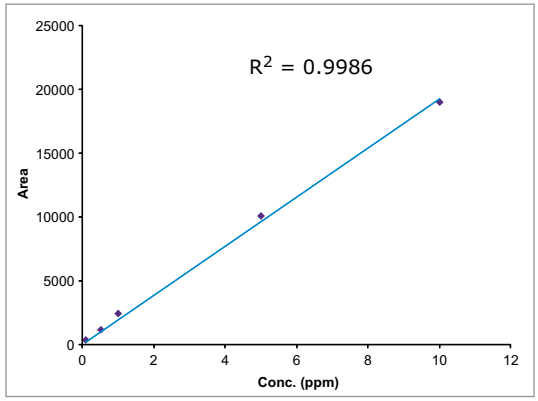

Figure 5. Calibration graph of 0.5, 1, 2.5, 5, and 10 ppm standards of MAC.

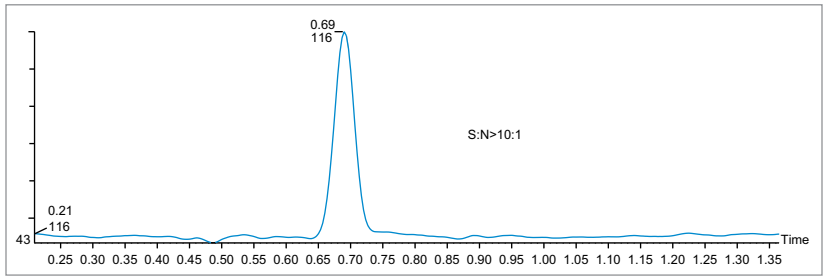

Figure 6. SIR trace for the LOQ (1 ppm) of the MAC standard.

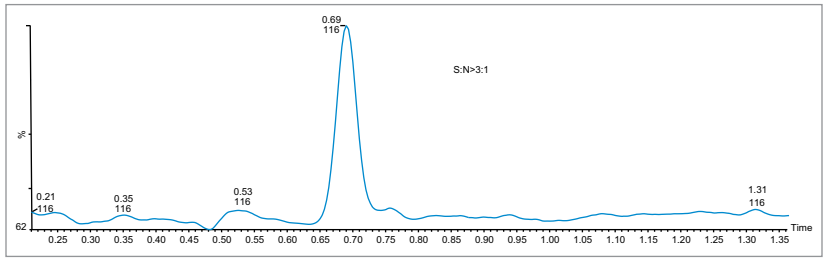

Figure 7. SIR trace for the LOD (0.5 ppm) of the MAC standard.

ANALYSIS OF THE API

Three different batches of a 1 mg/mL solution of API in methanol were analyzed, and the results showed that they all contained less than 1.0 ppm of MAC. The overlay of a typical batch with a MAC 1 ppm standard is shown in Figure 8.

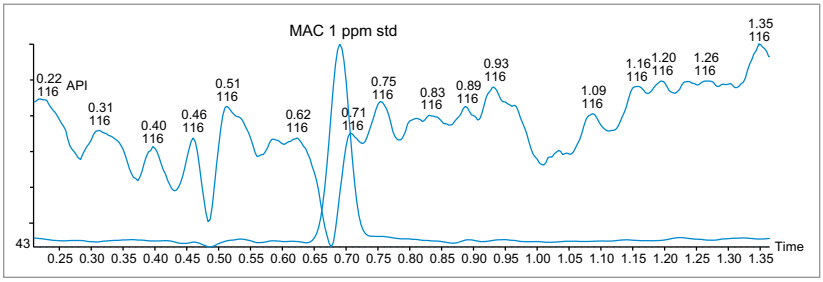

Figure 8. TIC of m/z 116 from a typical batch of API and a 1 ppm MAC standard.

CONCLUSION

- The ACQUITY UPC² and ACQUITY QDa Detector with SIR provide a highly specific and sensitive method for the analysis of MAC down to a LOQ of 1.0 ppm related to 1 mg/mL API in solution
- ACQUITY UPC² and ACQUITY QDa Detector combination is an excellent opportunity for high-sensitivity trace analysis, and should be included as part of the toolkit for the analysis of mutagenic impurities
- ACQUITY UPC² and ACQUITY QDa Detector can be used through all stages of drug development and into a QC environment, if required
- Fast analysis time because no derivatization and less validation was required
- Alternative analysis for aqueous-sensitive components

References

1. ICH M7, Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk, International Conference on Harmonization, 23 June 2014.
2. Impurities in New Drug Products Q3B(R) August 2005.
3. Knapp D.R., Handbook of Analytical Derivatization. pg. 10.

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Waters ACQUITY UPLC H-Class Coupled with an ACQUITY QDa Detector to Provide a Highly Sensitive and Specific Solution for Cleaning Validation

Chris Henry,[1] Mark Wrona,[2] Jayne Kirk,[1] and Richard Ladd[1]
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[2]Waters Corporation, Milford, MA, USA

TECHNOLOGY BENEFITS

- Sensitive qualitative and quantitative analysis for the detection of low level analytes using ACQUITY® QDa®
- Provides true UPLC® separation combined with easy to use mass detection for greater confidence in compound identification
- Simple, convenient mass detection which compliments UV approaches by improving sensitivity limits and the ability to detect compounds lacking a strong chromophore
- Integrated UV-PDA-UPLC technology running class leading CDS software for compliant environments (GMP)

WATERS SOLUTIONS

Waters® ACQUITY UPLC® H-Class System coupled with photodiode array and ACQUITY QDa Mass Detector

Empower® 3 Software

Waters ACQUITY UPLC BEH C_{18} Column, 75 mm x 2.1 mm, 1.7 µm (p/n 186005604)

KEYWORDS

Cleaning validation, UPLC, mass detection, Empower, regulatory, quality control

GOAL

To demonstrate that the Waters ACQUITY UPLC H-Class combined with a PDA (Photodiode Array detector) and ACQUITY QDa Mass Detector, can be utilised for cleaning validation to provide confident confirmation, as well as a highly specific and high sensitivity quantitative analysis for poorly UV absorbing or non-chromaphoric compounds. When controlled by the industry leading CDS software, Empower, this provides a solution well suited for rigorously regulated environments especially areas which require a high degree of data integrity, SOP adherence and compliance management.

BACKGROUND

Cleaning validation is a required activity in many companies, including pharmaceutical, biological, nutritional supplement, and medical device industries. From both a regulatory and industry standpoint, cleaning validation is recognised as an important activity that ensures that product cross-contamination does not adversely affect patient safety and product quality.[1]

Cleaning validation is a vital part of Quality Control (QC) workflow in the pharmaceutical industry and is defined as the process of providing documented evidence that the cleaning methods employed within a facility consistently monitors and controls for the potential carryover of product (including intermediates and impurities), cleaning agents, and extraneous material into subsequent product batches to a level which is below established safety levels.[2] It is critical that a reactor or vessels do not contain any materials which may introduce contamination into subsequent batches, this can both be a dangerous and costly mistake.

Depending on the process need, cleaning validation is performed using a number of analytical methods including HPLC-UV detection and/or total organic carbon analysis (TOC), each having unique pros and cons. HPLC-UV is restricted to chromophore containing compounds and tends to be a targeted method analysis specific only for compounds within the scope of the method and may miss other sources of contamination, or may be susceptible to baseline drift/interference. HPLC-UV also can lack the necessary sensitivity for low level analysis. TOC conversely is a non-specific technique that will detect any source of organic carbon.

While this is useful for detecting a wide range of contamination sources, TOC may not convey any qualitative data and will often require further testing (for example by LC-MS) for substance identification[3] to establish the appropriate cleaning protocol. This need for an additional stage of testing results in further costly downtime for the production plant.

For determination of drug residues for cleaning verification purposes, it is often necessary to have the capability of detecting trace amounts of drug, down to nanogram or even picogram levels.[4]

The combination of ACQUITY UPLC H-Class System and ACQUITY QDa Mass Detector (along with PDA) provides any analytical chemist with a powerful, synergistic tool for quantitative and qualitative separation. The Waters ACQUITY UPLC H-Class is complimented with a wide range of sub-2-µm column chemistries affording excellent peak shape, peak capacity and significantly faster runtimes for a wide range of compounds and mixtures.

The ACQUITY QDa Mass Detector can be integrated as a complimentary technique to UV detection. Mass detection brings trace level detection below the range of UV detection of at least 10-fold. This results in significantly lower limits of quantification and detection which are important parameters in all areas of analysis but especially in cleaning validation protocols for high toxicity drugs which have stringent limits for maximum allowable carryover (MACO). Additionally, rapid identification of components using mass allows the corrective action to be applied or the process be revisited and optimized more quickly if needed.

These technologies are all controlled by Empower 3 Chromatography Data Software, a compliance ready package providing secure data from acquisition to reporting/distribution.

DISCUSSION AND RESULTS

In practice, a cleaning validation procedure would involve a swabbing stage e.g. reaction vessels followed by an extraction process. This application note relates to the methodology that occurs after the extraction procedure, i.e. once the extracted product has been transferred to a sample vial.

A rapid 5 minute method was developed for five generic compounds (naphazoline HCl, lidocaine HCl, amitriptyline HCl, loperamide HCl, and tolazamide) using an PDA detector coupled with the ACQUITY QDa Mass Detector with data acquisition and instrument control performed by Empower 3 CDS Software (Table 1).

The compounds were prepared (n=6) over twelve concentrations ranging between 0.01 and 1000.0 ng/mL. Diluent consisted of 50% deionized water and 50% methanol.

The lidocaine HCl data was chosen as an exemplar of the ACQUITY QDa's crucial role in the analysis of poorly UV absorbing compounds. ACQUITY QDa data acquisition was initially run in full scan mode 200–1000 *m/z* to establish the protonated species. Once established, the ACQUITY QDa was run in SIR (Single Ion Recording) mode where the DC (Direct Current) and RF (Radio Frequency) of the quadrupole are set to allow ions of a specific *m/z* value through to the detector which greatly reduces noise and increases sensitivity. The analytical method demonstrated linearity over the range 0.01 to 1000.0 ng/mL achieving an R^2 value of 0.997 (Figure 1). Limit of detection was achieved at approximately 0.005 ng/mL for lidocaine HCl using SIR based on ICH Q2, (R1) guidelines, 'Validation of Analytical Procedures: Text and Methodology' for signal to noise ratios, equating to 0.025 pg on column (Figure 2/Table 2).

The results of the other four compounds are summarized (Table 3).

The PDA acquiring data at 220 nm achieved limits of detection of <1000.0 ng/mL for lidocaine HCl based on guidelines laid out in ICH Q2, (R1) for signal to noise ratios, equating to <5000 pg on column. Linearity was therefore not calculated.

The extended dynamic range of the ACQUITY QDa when compared to the PDA, maintains excellent precision at low concentration levels providing a powerful analytical tool for low level analysis enabling a linearity range for lidocaine HCl of at least five orders of magnitude (Figure 1/Table 4).

LINEARITY AND PRECISION

Lidocaine HCl was measured reliably down to approximately 0.02 ng/mL using ACQUITY QDa mass detection (Table 3), compared to the UV which provided an LOQ of 1000 ng/mL. Lidocaine, a compound with poor UV absorbance, yielded a MS method which was 50,000 times more sensitive than UV at 220 nm and also provided a high degree of confidence that the correct peak was measured with the confirmation of mass (Figure 6).

Method precision was carried out for lidocaine HCl from 0.01ng/mL to 1000 ng/mL (n=6). The system exhibited excellent precision over the full range with %RSD's ranging from 0.7 to 10.6 ng/mL (Table 4).

Signal-to-noise (s/n) values for the 0.01 ng/mL concentration is 5.28. Limit of detection is 3 x s/n according to ICH Q2, (R1) guidelines. The limit of detection based on this criterion can be said to be slightly greater than 0.005 ng/mL based on this data. The limit of quantitation (10 x s/n) can be estimated at around 0.02 ng/mL based on these results.

A blank was run at the end of the analytical sequence to assess potential carryover. Results show no significant levels of analyte in the blank (0.006% wrt 1000 ng/mL sample – approximately limit of detection).

Table 1. Method conditions.

Parameter	Value		
Mobile phase A (MPA)	2 mM Ammonium formate/0.1% formic acid		
Mobile phase B (MPB)	Acetonitrile/0.1% formic acid		
Flow rate	0.5 mL/min		
Injection volume	5 µL		
Gradient conditions	Time (min)	% MPA	% MPB
	0	95	5
	1.5	5	95
	3.0	5	95
	3.6	95	5
	5.0	95	5
Column	ACQUITY BEH 75 mm x 2.1 mm I.D., 1.7 µm		
Column temperature	40 °C		
PDA (± 1 nm)	220 nm (lidocaine HCl, loperamide HCl) 225 nm (naphazoline HCl, tolazamide) 254 nm (amitriptyline HCl) Full scan 210–400 nm		
ACQUITY QDa mass detection positive ESI (electrospray ionization) SIR positive mode *m/z* $(M+H)^+$	211 (naphazoline HCl) 235 (lidocaine HCl) 278 (amitriptyline HCl) 312 (tolazamide) 477 (loperamide HCl) Full scan 200–1000 *m/z*		

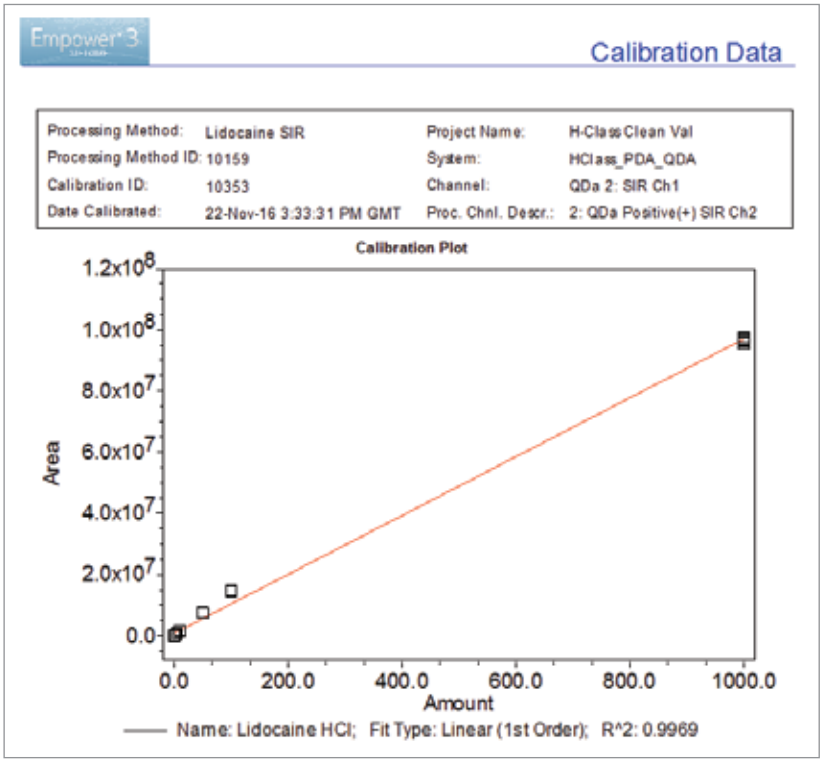

Figure 1. The calibration curve for from 0.01 to 1000.0 ng/mL (n=6). Excellent linearity was observed over 5 orders of magnitude i.e. R^2 = 0.997 using linear fit no weighting.

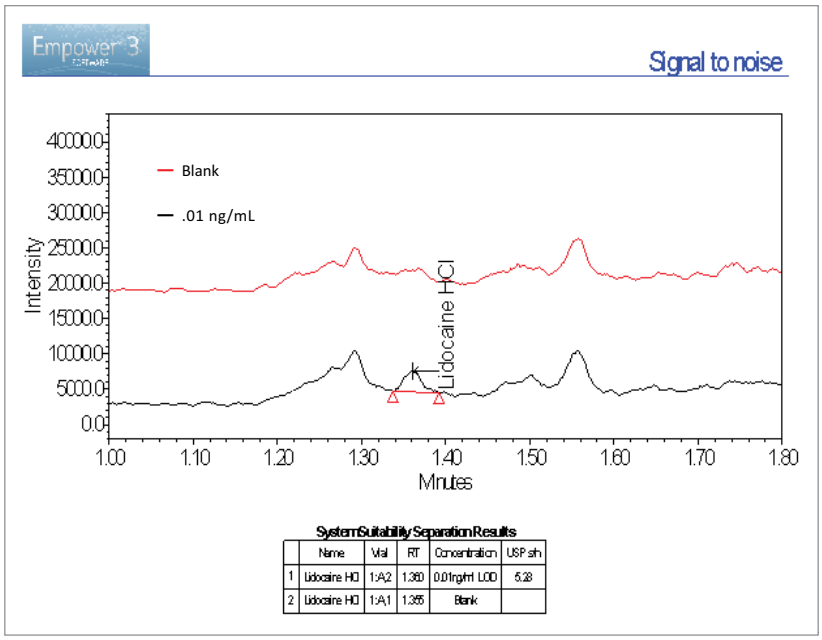

Figure 2/Table 2. Injection of Lidocaine HCl at a concentration in the region of LOD analyzed using the ACQUITY QDa Mass Detector in SIR mode.

Table 3. Summary of results for all five compounds.

Compound	Linear range (ng/mL)	Orders of magnitude	R^2	Equivalent amount on column (pg)	%RSD range	Approx. LOD (ng/mL)	Approx. LOQ (ng/mL)
Lidocaine HCl	0.01–1000	5	0.997	0.05–5000	0.7–10.6	0.005	0.02
Naphazoline HCl	0.01–1000	5	0.997	0.05–5000	0.6–17.3	0.005	0.02
Amitriptyline HCl	0.1–1000	4	0.996	0.5–5000	0.5–2.9	0.2*	0.6*
Loperamide HCl	0.01–1000	5	0.998	0.05–5000	09–21.2	0.01	0.03
Tolazamide	0.01–1000	5	1	0.05–5000	0.6–9.9	0.005	0.02

**Calculated using blank subtraction due to background interference.*

For naphzoline HCl, loperamide HCl, and tolazamide linearity was demonstrated over 0.01 ng/mL and 1000 ng/mL yielding LOQ's of between 0.02 and 0.03 ng/mL.

For amitriptyline HCl linearity over the range 0.1–1000 ng/mL with LOQ calculated at approximately 0.6 ng/mL using this method.

Analysing all the compounds using UV at the wavelengths specified in Table 1 achieved LOD's greater than 20 ng/mL with amitriptyline HCl proving the most sensitive. Accurate linearity was not possible due to lack of sensitivity over the concentration range.

Table 4. Precision (n=6) was between 0.7 and 10.6 %RSD over the range.

Concentration (ng/mL)	Mean (n=6)	%RSD
0.01	5418	10.6
0.05	34778	2.6
0.1	28609	6.1
0.2	50008	2.4
0.5	79997	1.4
1.0	151201	1.3
2.0	297201	1.4
5.0	817375	1.4
10	1538119	1.5
50	7469796	0.7
100	14634333	1.1
1000	96601107	0.7

ACQUITY QDa MASS DETECTION

All five compounds were run in full scan mode to confirm the protonated species as $(M+H)^+$ (Figure 4).

With the protonated species established for each compound an appropriate SIR acquisition method was set up in Empower.

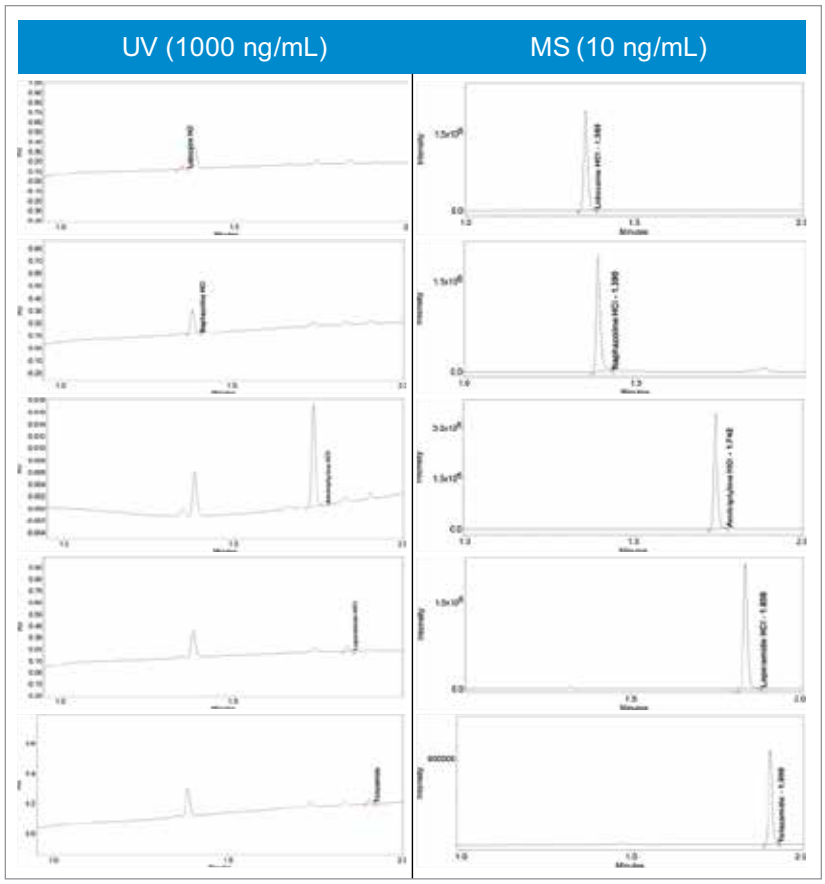

Figure 3. Comparison of the five compounds at their respective optimum UV wavelengths against analysis using SIR.

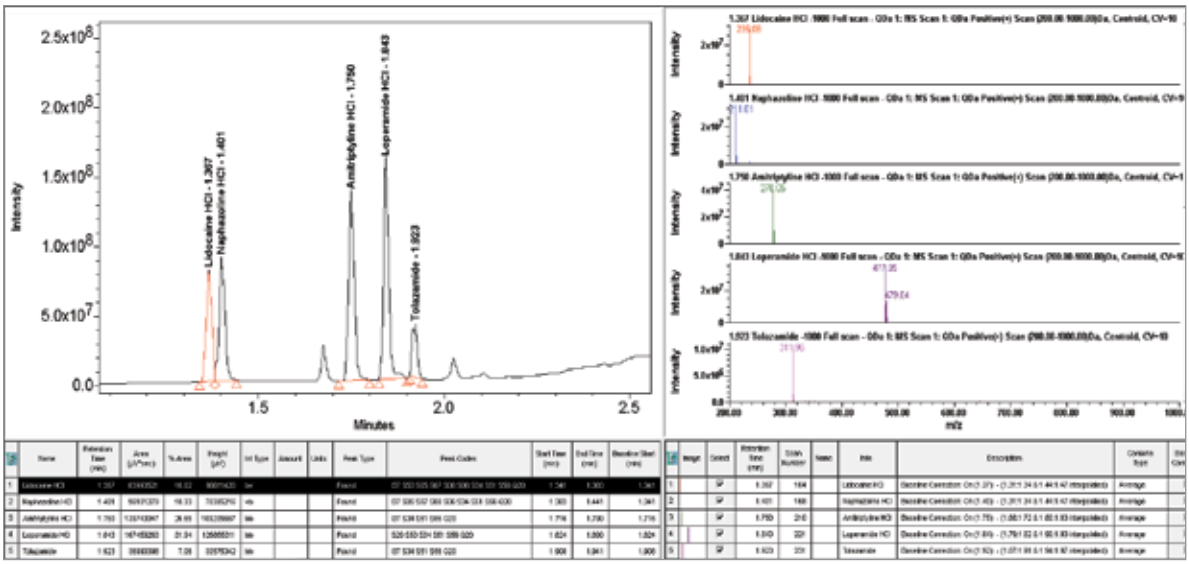

Figure 4. Main review window in Empower showing full scan data.

Slight co-elution with lidocaine HCl and naphazoline HCl evident, however SIR capability of the ACQUITY QDa enables specific, accurate and precise quantitation of both analytes (acquired in separate data channels) over the full range of concentrations (Table 3).

The compounds were analysed using six replicates of each to establish precision (Figure 5).

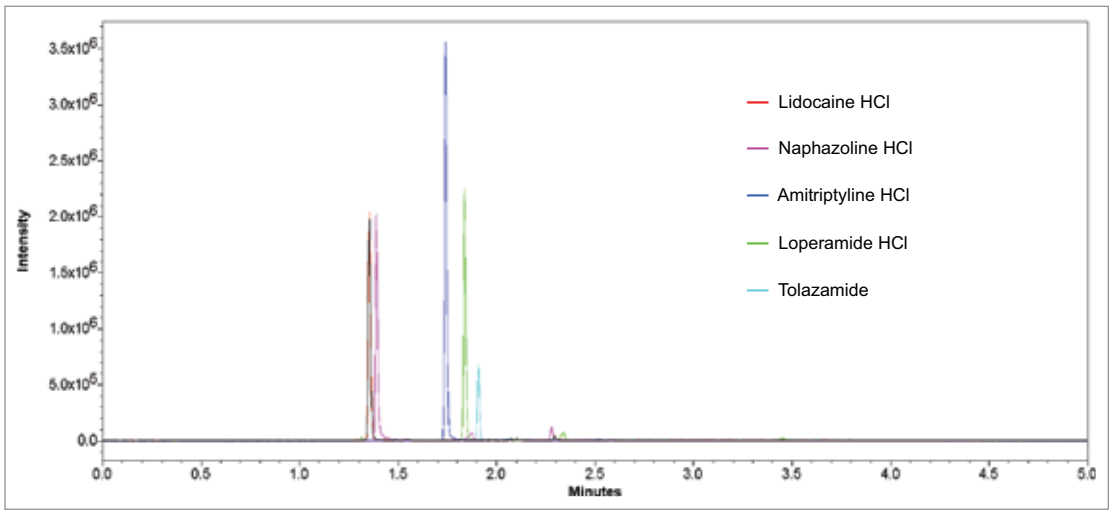

Figure 5. Overlay of SIR's (n=6) for all five compounds at 10 ng/mL.

BENEFITS OF ORTHOGONAL DETECTION

The ability to combine PDA data with mass detection equips the analyst with a powerful orthogonal solution to peak identification and a broader scope for peak detection. The ACQUITY QDa Mass Detector provides a significant increase in sensitivity beyond that of UV detection to enable confident and consistent quantification of analytes.

The integration of mass detection into UV workflow not only brings enhanced sensitivity but also offers convenient access to a more confident peak identification capability.

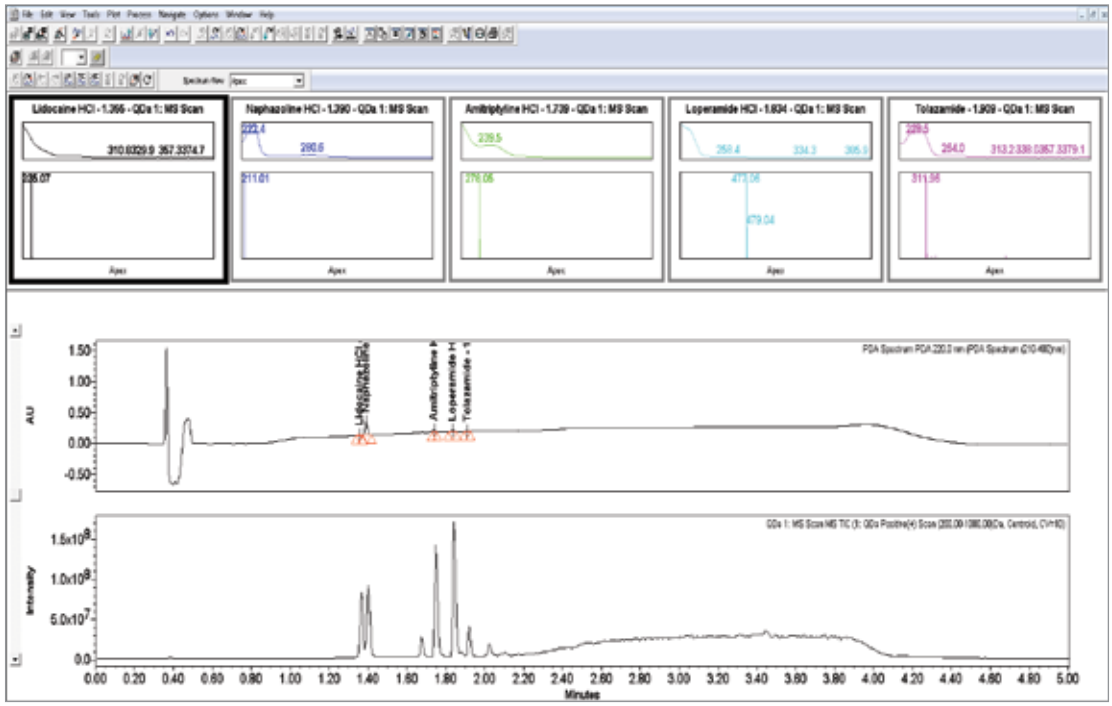

Figure 6. 'Mass Analysis Window' feature in Empower 3.

Empower 3 provides UV spectral data and MS data in one convenient screen giving easy access to increased peak ID confidence through mass confirmation (Figure 6). The loperamide HCl mass analysis (light blue) exhibits two isotopes (477.06/499.09) separated by two mass units, typical of single chlorine containing compounds.

CONCLUSIONS

The ability to bring ACQUITY QDa mass detection to cleaning validation enables the pharmaceutical industry to access unprecedented sensitivity and selectivity to an increasingly scrutinised area of drug production, providing improved throughput, accuracy and data confidence.

- Waters ACQUITY UPLC H-Class coupled to PDA and ACQUITY QDa Mass Detector provides increased peak ID confidence.
- QC analysts can now be equipped with accessible mass information enabling routine trace level analysis of poorly UV absorbing compounds by easily integrating the ACQUITY QDa Mass Detector into the QC workflow.
- The ability to control integrated mass detection using Empower 3 ensures results generated are fully compliant with the data integrity demands of the QC environment.
- SIR mass detection enables low level quantitation even for co-eluting peaks.
- With increasingly potent compounds and therapeutics being developed, technologies such as ACQUIY QDa can be utilized to address the analytical challenge of low detection thresholds typical of these compounds.

Additional Information

In addition to ACQUITY UPLC H-Class, ACQUITY QDa, and Empower CDS, Waters also provides a large number of products and consumables to support cleaning validation through Waters, ERA catalog. Follow link:

https://www.eraqc.com/Catalogs/catalogid/1-4GU8H7/categoryid/1-4GU8H8 OR

http://www.eraqc.com/LifeSciences/catalogid/1-4GU8H7

References

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3. McHale W, Wallace B, Lawson S. *Why Total Organic Carbon (TOC) Is Often Superior to HPLC for Cleaning Validation Sample Analysis: A Real World Example* (Teledyne Technologies).
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[APPLICATION NOTE]

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Benefits of Using Mass Detection for Assessing Quality and Purity of Cetrimonium Bromide Pharmaceutical Raw Material

Margaret Maziarz,[1] Mark Wrona,[1] Dominic Moore,[2] and Chengxia O'Shea[2]
[1]Waters Corporation, Milford, MA, USA
[2]Genzyme A Sanofi Company, Northborough, USA

APPLICATION BENEFITS

- Easy and direct technique for analysis of non-chromophoric raw materials with the ACQUITY® QDa® Detector with confirmation and quantitation
- Accurate identification of components by mass detection with the ACQUITY QDa Detector
- Eliminate complex and time-consuming sample preparation procedures
- Compatibility with existing UPLC systems and methodologies

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY UPLC Columns

ACQUITY QDa Detector

Empower® 3 CDS Software

KEY WORDS

UPLC, QDa, non-chromophoric compounds, pharmaceutical raw materials, cetrimonium bromide, sub-2-µm particles column, mass spectrometry

INTRODUCTION

Pharmaceutical raw materials are substrates or elements used for manufacturing different drug products. Raw materials include active pharmaceutical ingredients (API), excipients, and other inactive ingredients. Excipients and inactive ingredients generally have no pharmacological effect, yet they are essential components that function as fillers, binders, disintegrants, lubricants, coloring agents, and preservatives.[1] The quality and purity of excipients and inactive ingredients are critical to the safety of the final drug product and must be controlled.[2] Excipients with low purity or containing contaminants may often compromise the safety and efficacy of the end pharmaceutical product. It is therefore essential to have reliable methods for accurate assessment of quality and purity of the pharmaceutical raw materials.

Rapid and accurate analysis of components that lack UV chromophores or have low UV-extinction coefficient can be challenging. As these compounds cannot be directly detected by UV, alternative methods must be employed to accurately identify and measure them. In the case of cetrimonium bromide, a non-chromophoric material, the assay method listed in the United States Pharmacopeia (USP) Monograph[3] is based on a titrimetric analysis using a multistep liquid-liquid extraction with chloroform. This is a complex and time-consuming procedure, not ideal for routine testing within the QC laboratory. Mass detection enables quick, direct, and accurate analysis of non-chromophoric compounds, eliminating the need for complex sample preparation procedures.

In this application note, we present a robust and quick UPLC method for analysis of cetrimonium bromide raw material. This methodology was developed in partnership with Genzyme A Sanofi Company. The UPLC method utilizes an ACQUITY QDa Detector for fast, information-rich, and accurate assessment of quality and purity of raw materials. We demonstrate the system suitability and linearity of the method achievable with mass detection. This method was then used to confirm identity of, assess purity of, and assay cetrimonium bromide raw material purchased from three different suppliers.

Overall, mass detection enables quick identification and accurate analysis of pharmaceutical raw materials, which makes this technology suitable for routine testing in the QC laboratory.

EXPERIMENTAL

Solutions preparation

Standards

Cetrimonium bromide USP reference standard (USP p/n: 1102974) stock solution was prepared in water at a concentration of 0.5 mg/mL. The stock solution was then diluted with water (Fisher Scientific, Optima) to a working concentration of 20.0 µg/mL. This standard solution was used to prepare linearity standards by dilution with water. Linearity standards were prepared at the following concentrations: 0.10, 0.25, 0.50, 0.75, 1.0, 2.0, and 3.0 µg/mL.

Raw material samples

Cetrimonium bromide raw materials tested in this study were purchased from three different suppliers:

- Sigma-Aldrich, product number: H9151-25G
- Alfa Aesar, product number: A15235
- TCI, product number: H0081

Stock sample solutions were prepared at 0.5 mg/mL concentration and then diluted with water to a concentration of 1.0 µg/mL.

UPLC conditions

LC system:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC CSH™ C_{18}, 2.1 x 50 mm, 1.7 µm
Column temp.:	40 °C
Flow rate:	0.6 mL/min
Injection volume:	1.0 µL
Solvent A:	1% Formic acid in water
Solvent B:	Water
Solvent C:	Acetonitrile

Separation: Gradient

Step	Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Curve
1	Initial	10.0	40.0	50.0	Initial
2	2.0	10.0	0.0	90.0	6
3	2.5	10.0	0.0	90.0	6
4	2.6	10.0	40.0	50.0	6
5	4.5	10.0	40.0	50.0	6

Purge/sample wash:	50:50 water/acetonitrile
Seal wash:	90:10 water/acetonitrile
UV detector:	ACQUITY UPLC PDA: 200–500 nm

MS conditions

Mass detector:	ACQUITY QDa (performance option)
Ionization mode:	ESI+
MS acquisition range:	150–350 Da
SIR:	284.3 Da
Sampling rate:	10 pts/sec
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Probe temperature:	600 °C
Data format:	Centroid

Data Management

Empower 3 FR2 Chromatography Data System (CDS) Software

RESULTS AND DISCUSSION

Cetrimonium bromide is a quaternary ammonium salt that lacks chromophores required for UV detection (Figure 1). Thus, it cannot be directly detected by UV. However, it is readily ionizable, producing a robust MS signal on the ACQUITY QDa Detector. Chromatographic data for analysis of cetrimonium bromide is displayed in Figure 2. The UV trace at 215 nm (Figure 2a) shows that no peaks were detected, as expected. The ACQUITY QDa data collected across the mass range (150–350 Da, Figure 2b) is referred as the total ion chromatogram (TIC) and shows that one major peak is present with a mass of 284.3 Da, corresponding to cetrimonium bromide. A specific mass of interest can be extracted from the TIC data to generate an extracted ion chromatogram (XIC) as illustrated in Figure 2c. For targeted assay testing, the data can be collected using single ion recording (SIR) scanning mode, which records the signal intensity for a specific ion of interest. SIR can simplify the analysis and quantitation, further increase signal-to-noise ratio (S/N), and is suitable for targeted assays (Figure 2d).

System suitability

The performance of the developed UPLC method was verified by evaluating repeatability of six replicate injections of the USP reference standard at 2.0 µg/mL (Figure 3) according to the specifications defined in the USP General Chapter, <621>, Chromatography.[4] The UPLC system suitability results were processed using SIR scanning mode at 284.3 Da (Figure 4). The retention times and area repeatability were well within the USP specifications of less than 2.0% RSD.

Cetrimonium bromide

- Molecular formula: $C_{19}H_{42}BrN$
- Average mass: 364.45 Da
- Monoisotopic mass: 363.25 Da
- Free base monoisotopic mass: 284.33 Da
- Detected mass: M^+ = 284.3 Da

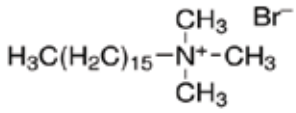

Figure 1. Structure and molecular information of cetrimonium bromide.

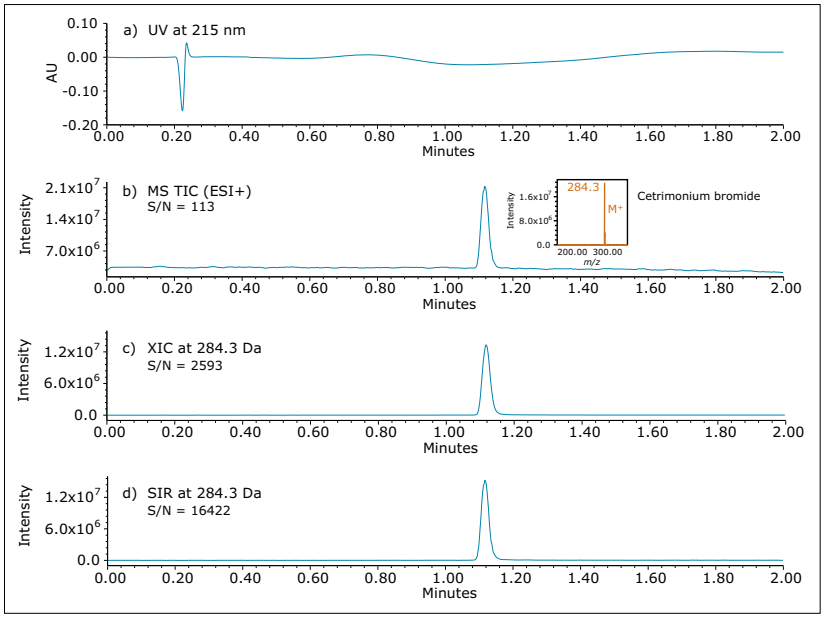

Figure 2. UV and MS chromatographic data for cetrimonium bromide acquired using the ACQUITY UPLC H-Class System with ACQUITY PDA and ACQUITY QDa Detector.

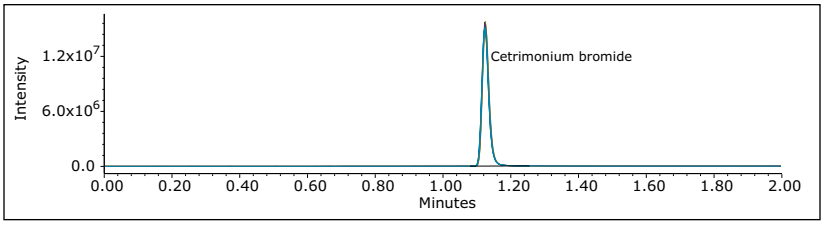

Figure 3. Overlay of six replicate injections of cetrimonium bromide USP reference standard, SIR at 284.3 Da.

System Suitability_Report

Sample Set ID: 10071
Result Set ID: 13076
Channel Name: QDa 2: SIR Ch1

Name: Cetrimonium Br

	Name	Inj	RT	Area	USP Tailing	K Prime
1	Cetrimonium Br	1	1.124	21573313	1.2	3.5
2	Cetrimonium Br	2	1.124	21848031	1.2	3.5
3	Cetrimonium Br	3	1.125	21937619	1.2	3.5
4	Cetrimonium Br	4	1.125	21780348	1.2	3.5
5	Cetrimonium Br	5	1.126	21776335	1.2	3.5
6	Cetrimonium Br	6	1.125	21495826	1.2	3.5
Mean			1.125	21735245	1.2	3.5
Std. Dev.			0.001	167994.075		
% RSD			0.06	0.77		

Figure 4. System suitability results for six replicate injections of cetrimonium bromide USP reference standard, SIR at 284.3 Da.

Linearity

Linearity of the method for cetrimonium bromide with SIR at 284.3 Da was evaluated over 7 concentrations ranging from 0.1 to 3 µg/mL. The method showed acceptable linearity with a correlation coefficient (R^2) ≥0.9969 (Figure 5). In addition, the percent deviation of the calculated X values or concentrations were less than 10% except for the 0.2585 µg/mL standard, which was less than 18.0% (Figure 6).

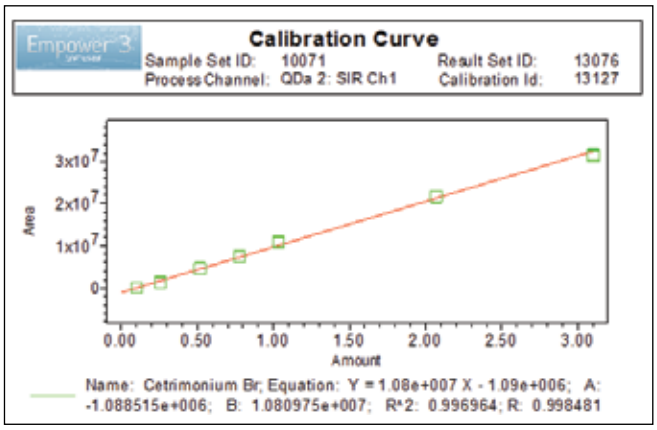

Figure 5. Linearity of the method for cetrimonium bromide. Data processed using SIR at 284.3 Da.

Empower 3 SOFTWARE

Cal Curve %Deviation_Accuracy

Sample Set ID: 10071 Result Set ID: 13076

Channel Name: QDa 2: SIR Ch1

Peak: Cetrimonium BrX Value (ug/mL): 0.10340

	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	1	103451	0.10340	0.11027	6.642
2	2	110301	0.10340	0.11090	7.255
3	3	94141	0.10340	0.10941	5.809

Peak: Cetrimonium BrX Value (ug/mL): 1.03400

	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	1	10724680	1.03400	1.09283	5.689
2	2	10704598	1.03400	1.09097	5.510
3	3	11081568	1.03400	1.12584	8.882

Peak: Cetrimonium BrX Value (ug/mL): 0.25850

	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	1	1266680	0.25850	0.21788	-15.715
2	2	1220622	0.25850	0.21362	-17.363
3	3	1292652	0.25850	0.22028	-14.785

Peak: Cetrimonium BrX Value (ug/mL): 2.06800

	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	1	21569343	2.06800	2.09606	1.357
2	2	21569445	2.06800	2.09607	1.357
3	3	21684750	2.06800	2.10673	1.873

Peak: Cetrimonium BrX Value (ug/mL): 0.51700

	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	1	4658207	0.51700	0.53162	2.829
2	2	4578027	0.51700	0.52421	1.394
3	3	4662449	0.51700	0.53202	2.905

Peak: Cetrimonium BrX Value (ug/mL): 3.10200

	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	1	31475067	3.10200	3.01243	-2.888
2	2	31625936	3.10200	3.02638	-2.438
3	3	31234155	3.10200	2.99014	-3.606

Peak: Cetrimonium BrX Value (ug/mL): 0.77550

	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	1	7480280	0.77550	0.79269	2.217
2	2	7513669	0.77550	0.79578	2.615
3	3	7333195	0.77550	0.77908	0.462

Figure 6. Calibration curve data processed using SIR at 284.3 Da. The percent deviation of the calculated x values or concentrations were less than 10% except for the 0.2585 µg/mL standard, which was less than 18.0%.

Sample analysis

Cetrimonium bromide purchased from three different suppliers was analyzed using UPLC-MS. The ACQUITY QDa Detector enabled quick confirmation of peak identity by mass detection. For peak purity assessment, we used ACQUITY QDa TIC data collected across a mass range (150–350 Da). We evaluated the MS TIC data for presence of any peaks with mass different than 284.3 (±0.2) Da, which corresponds to the mass of cetrimonium bromide. As shown in TIC traces (Figure 7), only one major peak was detected in the sample injections with a mass specific for cetrimonium bromide. In addition, the MS spectral data at the leading, apex, and tailing edge of the peak shows presence of one mass which corresponds to cetrimonium bromide. This demonstrates that cetrimonium bromide is not coeluting with other peaks. Overall, the TIC mass data demonstrated that no contaminants were present in the cetrimonium bromide pharmaceutical raw materials tested in this study.

For assay testing, we compared cetrimonium bromide materials purchased from three different suppliers against the USP reference standard. The assay results generated using SIR at 284.3 Da (Figure 8) ranged from 98.4 to 100.4 % for all raw materials, which meet the USP acceptance criteria of 96.0 to 101.0 % defined in the USP Monograph for cetrimonium bromide.

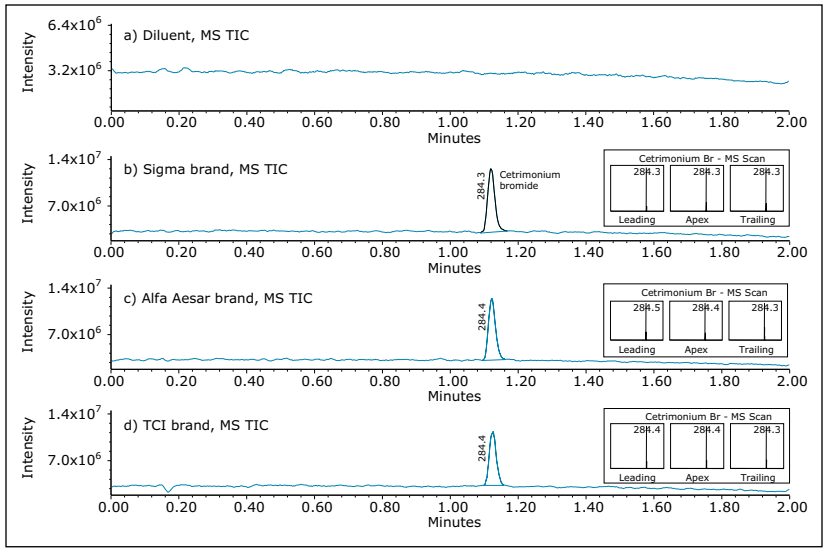

Figure 7. Cetrimonium bromide raw material purchased from three different suppliers. TIC MS and mass spectral data at the leading, apex, and tailing edge of the peak.

Sample_Analysis_Report

Sample Set ID: 9461
Result Set ID: 13201
Channel Name: QDa 2: SIR Ch1

SampleName: Sigma brand

	SampleName	Name	RT (min.)	Area	Calibration Id	Calculated Amount (ug/mL)	Target Amount (ug/mL)	Assay (%)
1	Sigma brand	Cetrimonium Br	1.124	10684061	13127	1.0891	1.0850	100.38

SampleName: Alfa Aesar brand

	SampleName	Name	RT (min.)	Area	Calibration Id	Calculated Amount (ug/mL)	Target Amount (ug/mL)	Assay (%)
1	Alfa Aesar brand	Cetrimonium Br	1.122	9244808	13127	0.9559	0.9660	98.96

SampleName: TCI brand

	SampleName	Name	RT (min.)	Area	Calibration Id	Calculated Amount (ug/mL)	Target Amount (ug/mL)	Assay (%)
1	TCI brand	Cetrimonium Br	1.125	9496532	13127	0.9792	0.9950	98.41

Figure 8. Assay results for cetrimonium bromide raw materials purchased from three different suppliers, SIR at 284.3 Da.

CONCLUSIONS

Mass detection using the ACQUITY QDa Detector coupled with UPLC enables quick and easy analysis of non-chromophoric cetrimonium bromide raw material. The new method eliminates the need for a complex titrimetric procedure, hence improving productivity. Furthermore, the LC-MS method provides improved confidence associated with sample confirmation and accurate assessment of purity and assay of cetrimonium bromide pharmaceutical raw material. The system suitability and linearity of the method calculated using mass data were excellent.

Overall, the ACQUITY QDa Detector is a robust and simple-to-use mass detector that can be added as an orthogonal detection technique to the UV detection. It provides accurate and reliable results, making this technology ideal for routine testing of pharmaceutical raw materials in the QC laboratory.

References

1. Overview of pharmaceutical excipients used in tablets and capsules, October 24 2008.
2. The United States Pharmacopeia (USP) Monograph Excipients Introduction.
3. USP Monograph, Cetrimonium Bromide, USP37-NF32, The United States Pharmacopeia Convention, official August 1, 2014.
4. USP General Chapter, <621>, Chromatography, USP37-NF32, The United States Pharmacopeia Convention, official August 2, 2014.

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[APPLICATION NOTE]

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The Use of the ACQUITY QDa Detector for a Selective, Sensitive, and Robust Quantitative Method for a Potential Genotoxic Impurity

Janet Hammond
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- High selective, sensitive, and robust analysis using the ACQUITY® QDa® Detector
- ACQUITY UPLC® H-Class System provides high resolution and throughput with a short analysis time of 3 minutes
- The option of either MassLynx® Mass Spectrometry Software or Empower® Chromatography Data Software control allows the system to be deployed in many different analytical laboratories
- Facility to divert the LC flow to waste preventing contamination of the source by high concentrations of API

WATERS SOLUTIONS

ACQUITY UPLC H-Class System fitted with an ACQUITY UPLC Column Manager

ACQUITY QDa Detector

Empower or MassLynx Software

KEY WORDS

UPLC,® QDa, genotoxic impurities, quantitation

INTRODUCTION

Genotoxic impurities (GTI) are compounds that have the potential to modify DNA and as a consequence cause cancer. It is important that drug manufacturers identify the presence of these impurities early in the drug development process, and develop analytical methods that are sensitive and specific enough to determine the levels in both drug substance and drug product.

Maximum allowable levels of GTIs are based on a Threshold for Toxicological Concern (TTC) of 1.5 µg per day, corresponding to 1 ppm or lower. This is orders of magnitude lower than for general pharmaceutical impurities analysis which is at the 500 ppm level.

Analytical instrumentation used routinely in pharmaceutical analysis such as liquid chromatography (LC) with ultraviolet (UV) detection for non volatile compounds, or gas chromatography (GC) with flame ionization detection (FID) for volatile compounds are preferred. However, the low levels of detection required for genotoxic impurities present a significant challenge. In these situations, MS detection is required in order to achieve the desired sensitivity, specificity, and robustness. Some of these methods are required to provide support during the whole life cycle of a drug from early development to manufacturing quality control.

Here, we describe an analytical method for the detection and quantitation of a GTI, 1-phenylpiperazine in an active pharmaceutical ingredient (API) at a limit of quantitation (LOQ) of 0.5 ppm relative to the API. It uses the ACQUITY QDa Detector, which is a small, simple to use robust mass detector and the ACQUITY UPLC H-Class System fitted with an ACQUITY UPLC Column Manager.

EXPERIMENTAL

UPLC method conditions

LC system:	ACQUITY UPLC H-Class with ACQUITY UPLC Column Manager
Column:	ACQUITY UPLC HSS, 1.8 µm, 2.1 x 50 cm
Column temp.:	40 °C
Sample temp.:	10 °C
Injection vol.:	1 µL
Flow rate:	0.5 mL/min
Mobile phase A:	0.05% formic acid in water
Mobile phase B:	0.05% formic acid in acetonitrile
Gradient:	5 to 95% B at 1.5 min held until 2.1 min then 5% B
Run time:	3 minutes

MS conditions

MS system:	ACQUITY QDa Detector
Ionization mode:	ESI positive
Single ion recording (SIR):	*m/z* 163.1 Da $[M+H]^+$
Capillary voltage:	0.8 kV
Sampling frequency:	5 Hz
Probe temp.:	600 °C
Cone voltage:	10V

Data management

MassLynx Software v4.1

Sample preparation

10, 5, 1, 0.5, and 0.1 ppm standards of 1-phenylpiperazine (with respect to 1 mg/mL API) were prepared in 10% acetonitrile: 90% water.

RESULTS AND DISCUSSION

The structure of 1-phenylpiperazine is shown in Figure 1. It has a nominal molecular weight of 162 Da. Full scan analysis of 1-phenylpiperazine on the ACQUITY QDa Detector detected the expected $[M+H]^+$ ion at 163.1 Da.

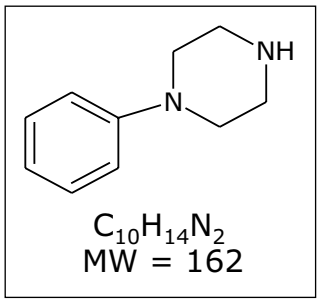

Figure 1. Structure of 1-phenylpiperazine.

Reconfiguration of the ACQUITY UPLC Column Manager

In order to avoid contamination issues and poor recoveries during trace analysis involving MS, it is preferable where possible to exclude the matrix and API from entering the MS source. Reconfiguring the ACQUITY UPLC Column Manager is one of the available options which allows flow to be diverted away from the ACQUITY QDa Detector. The ACQUITY UPLC Column Manager tubing configuration on the ACQUITY UPLC H-Class System facilitates the diversion of the solvent flow to waste by timed events using the instrument control software method events table. As shown in Figure 2, the outlet of the ACQUITY UPLC Sample Manager (FL or FTN) connects directly to the active column pre heater of the column that is being used for the experiment. The outlet of the column connects to the centre of the outlet switching valve (OSV). The 'W' port on the OSV (position 1) connects to waste. The '1' port on the OSV (position 2) is connected to the detector. Using the instrument control software method events table during the run, the column manager is used to move the OSV between positions 1 (waste) and 2 (detector) at the desired time intervals.

Method development

The UPLC method was optimised to ensure that 1-phenylpiperazine eluted before the API. The final method resulted in an elution time for 1-phenylpiperazine of 1.2 min and of 1.5 min for the API. An event time of 1.3 min was used to divert the API to waste as seen in Figure 3.

ACQUITY QDa Detector probe temperature and cone voltage conditions were optimised for maximum sensitivity for the 1-phenylpiperazine standards.

Matrix effects

Selectivity issues can arise during these very low levels of analysis because the target analyte (GTI) is at very low levels in the presence of a large concentration of API, a counter-ion or in the case of drug products, excipients. It is important when carrying out these types of analyses that a series of samples of API or drug product spiked with the corresponding GTI are also analysed. This will indicate if there are any issues relating to stability, ion suppression, or enhancement effects. In this analysis, samples were prepared by spiking into the API at 0.1, 0.5, and 10 ppm of 1-phenylpiperazine and analyzed. The result of this experiment showed that the areas of five replicates were 28% lower when compared to the areas of the unspiked standards. This implies the matrix does have an effect on the response of 1-phenylpiperazine. The areas of the 0.5 ppm standard of both spiked and unspiked samples are shown in Figure 4.

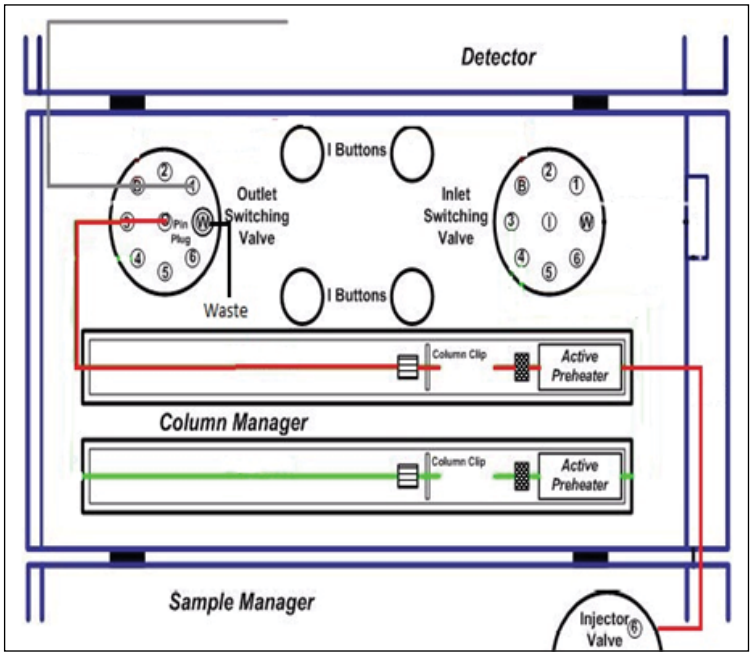

Figure 2. Configuration of the ACQUITY UPLC Column Manager to facilitate the diversion of the solvent flow to waste.

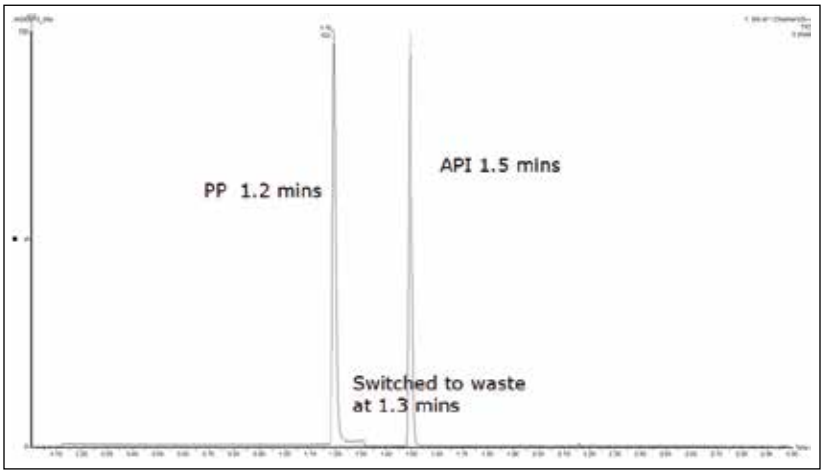

Figure 3. SIR traces for MH^+ from both 1-phenylpiperazine and the API.

Sample number	Area
1	802
2	791
3	847
4	827
5	810

A

Sample number	Area
1	1152
2	1182
3	1142
4	1139
5	1162

B

Figure 4. A) Areas from 5 injections of 0.5 ppm std of 1-phenylpiperazine spiked into the API. B) Areas from 5 injections of an unspiked 0.5 ppm standard of 1-phenylpiperazine.

Linearity

The linearity of the method was evaluated with five standards of 0.1, 0.5, 1.0, 5, and 10 ppm of 1-phenylpiperazine in a 1 mg/mL API solution. The method showed good linear correlation between the peak areas and the ppm concentration (Figure 5). The signal to noise for the LOQ standard is more than 10 to 1 and signal to noise ratio is 3 to 1 at the limit of detection (LOD) standard (Figures 6A and 6B). The percentage standard deviation of the six individual injections of all the five standards was less than 4%.

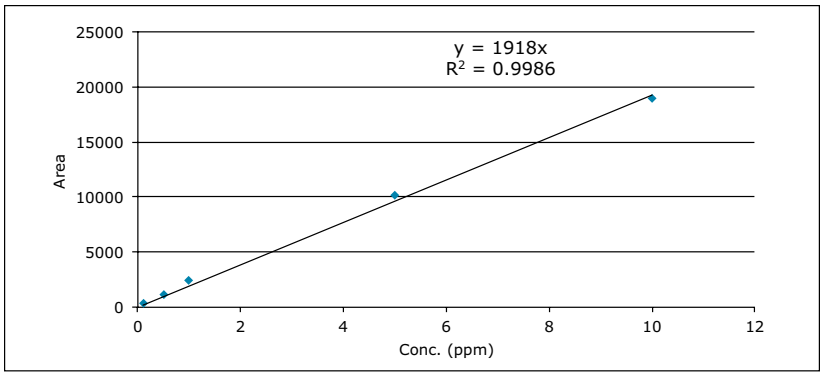

Figure 5. Calibration graph showing the linearity across the 5 1-phenylpiperazine standards.

Analysis of the API

Three different batches of a 1 mg/mL solution of the API in 10% acetonitrile: 90% water were analysed and the results showed that all contained less than 0.5 ppm of 1-phenylpiperazine. The result from a typical batch is shown in Figure 7.

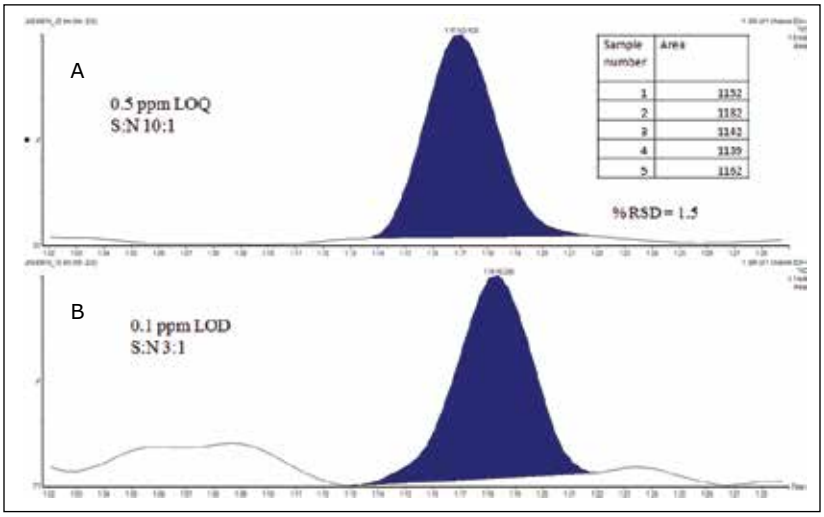

Figure 6. A) SIR trace for the LOQ (0.5 ppm) of the 1-phenylpiperazine standard. B) SIR trace for the LOD (0.1 ppm) 1-phenylpiperazine standard.

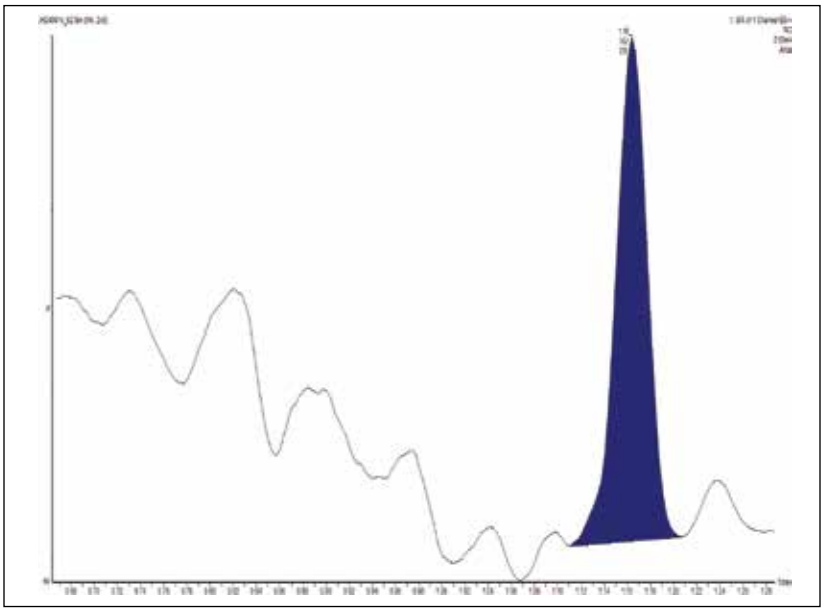

Figure 7. Analysis for 1-phenylpiperazine in a typical batch of API.

CONCLUSION

The ACQUITY UPLC H-Class System and ACQUITY QDa Detector provide an excellent solution for analysis of 1-phenylpiperazine in the presence of an API.

- The use of the ACQUITY QDa Detector with SIR achieves high specificity and sensitivity to provide a method for analysis of 1-phenylpiperazine down to the LOQ of 0.5 ppm related to 1 mg/mL API in solution.
- UPLC provides high resolution and high throughput, which delivers high efficiency with an analysis time of 3 min.
- ACQUITY UPLC Column Manager allows the facility to switch the solvent flow to waste before the API elutes, preventing contamination of the source.
- Peak area reproducibility showed % RSD for all the 1-phenylpiperazine standards was less than 4%.
- The calibration graph was linear for a set of 1-phenylpiperazine standards from 0.1 to 10ppm.
- The option of either MassLynx or Empower Software control allows the system to be deployed in many different analytical laboratories.
- Can be used throughout all stages of drug development and QC environments.

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Benefits of Using ACQUITY QDa Mass Detection for Quantitative Analysis of Non-Chromophoric Memantine HCl in Tablet Formulation

Margaret Maziarz, Mark Wrona, and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Easy and direct technique for analyzing non-chromophoric compounds with the ACQUITY® QDa® Detector
- Accurate identification of components by mass detection with the ACQUITY QDa Detector
- Eliminate the need for complex, time-consuming derivatization procedures
- Compatibility with existing UPLC systems and methodologies

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY UPLC Columns

ACQUITY QDa Detector

Empower® 3 CDS Software

Syringe filters

KEY WORDS

UPLC, QDa, non-chromophoric compounds, memantine HCl, sub-2-µm particles column, mass spectrometry, ApexTrack™ integration

INTRODUCTION

The analysis of compounds that lack UV chromophores or that have low UV-extinction coefficients can be challenging. Because these compounds cannot be directly detected by UV, their identification and measurement must depend on alternate methods. For all pharmaceutical products, it is particularly important to correctly identify its components, for failing to do so can compromise the drug's safety, efficacy, or both.

For components that exhibit poor UV absorbance or none, samples often require pre-or post-column derivatization, for UV detection. In the case of memantine hydrochloride, a non-chromophoric drug compound, several methods are found in the literature for quantitative determination in dosage. Few methods for determining memantine HCl in drug formulations have been reported, including HPLC methods with UV-detection by means of a pre-column derivatization technique[1,2] and GC-FID methods.[3,4] While effective and sensitive, these methods are not ideal for routine testing in a quality control laboratory. They require tedious and complex pre-column derivatization procedures or additional instrumental analysis such as gas chromatography. The lack of straightforward methods can lead to additional undesirable variability in response. This often requires additional method-development time, robustness testing, user training, and monitoring in order to improve the quality of the assay. Mass detection, on the other hand, enables quick and accurate determination of non-chromophoric compounds, and can eliminate the need for complex sample-preparation procedures.

In this application note, we describe the use of the ACQUITY QDa module, a small but robust, simple-to-use mass detector, to quantitate non-chromophoric memantine HCl, a drug commonly used to treat dementia often associated with Alzheimer's disease. Here, we present a UPLC method coupled with the ACQUITY QDa Detector to quantitatively determine memantine HCl in the drug tablet formulation. We will demonstrate the system suitability, method linearity, and specificity achievable with mass detection for routine assays.

Overall, mass detection provides quick identification and analysis of non-chromophoric compounds, accurate and reliable results. Thus, mass detection is complementary to UV techniques and suitable for routine testing in the QC laboratory.

EXPERIMENTAL

UPLC method conditions

LC system:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC CORTECS® C_{18+}, 2.1 x 50 mm, 1.6 µm
Column temp.:	45 °C
Flow rate:	0.6 mL/min
Injection volume:	1.0 µL
Solvent A:	125 mM Formic acid in water
Solvent B:	Water
Solvent C:	Acetonitrile
Separation:	Gradient

Step	Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Curve
1	Initial	10.0	85.0	5.0	Initial
2	2.5	10.0	42.5	47.5	6
3	2.6	10.0	0.0	90.0	6
4	3.1	10.0	0.0	90.0	6
5	3.2	10.0	85.0	5.0	6
6	5.0	10.0	85.0	5.0	6

Purge/Sample wash:	50:50 water/methanol
Seal wash:	90:10 water/acetonitrile
UV detector:	ACQUITY UPLC PDA: 210–400 nm (derived at 210 nm)

MS conditions

Mass detector:	ACQUITY QDa (Extended Performance)
Ionization mode:	ESI+
MS Acquisition range:	100 – 300 Da
Single Ion Recording:	180.2 Da
Sampling rate:	10 pts/s
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Probe temperature:	600 °C
Data:	Centroid
System Control, Data Acquisition, and Analysis:	Empower 3 FR2 CDS Software

Solutions preparation

Standard solutions

The memantine HCl stock solution was prepared in methanol at a concentration of 1.0 mg/mL. The stock solution was then diluted with standard diluent (10:90 methanol/water) to a working concentration of 0.005 mg/mL. The working standard solution was used to prepared linearity standards by dilution with standard diluent (10:90 methanol/water). Linearity standards were prepared at these concentrations: 0.05, 0.10, 0.15, 0.25, 0.50, 0.75, and 1.00 µg/mL.

Tablet sample solutions

Stock sample solutions were prepared by dissolving tablets containing 10 mg of memantine HCl in 50:50 0.1 N HCl/ethanol,* to a concentration of 1.0 mg/mL. The solutions were sonicated and centrifuged, at 3500 rpm, for 30 minutes. Finally, the solutions were filtered through 0.2-µm GHP membrane syringe filters* and diluted with sample diluent (0.1N HCl) to the working concentration of 0.75 µg/mL.

*Final method, other dissolution and filtering conditions tested and presented in this application note.

RESULTS AND DISCUSSION

Memantine HCl is a tricyclic amine that lacks the chromophores required for UV detection (Figure 1). Thus it cannot be directly detected by UV. Nevertheless, it is readily ionizable, producing a robust MS signal on the ACQUITY QDa Detector. Figure 2 shows several detector traces. As expected, the UV trace at 210 nm for memantine at 1 µg/mL shows no discernible peak. (Figure 2a.)

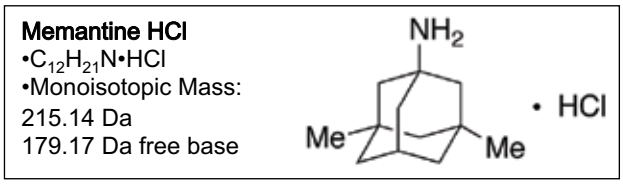

Figure 1. Chemical information and structure of Memantine HCl.

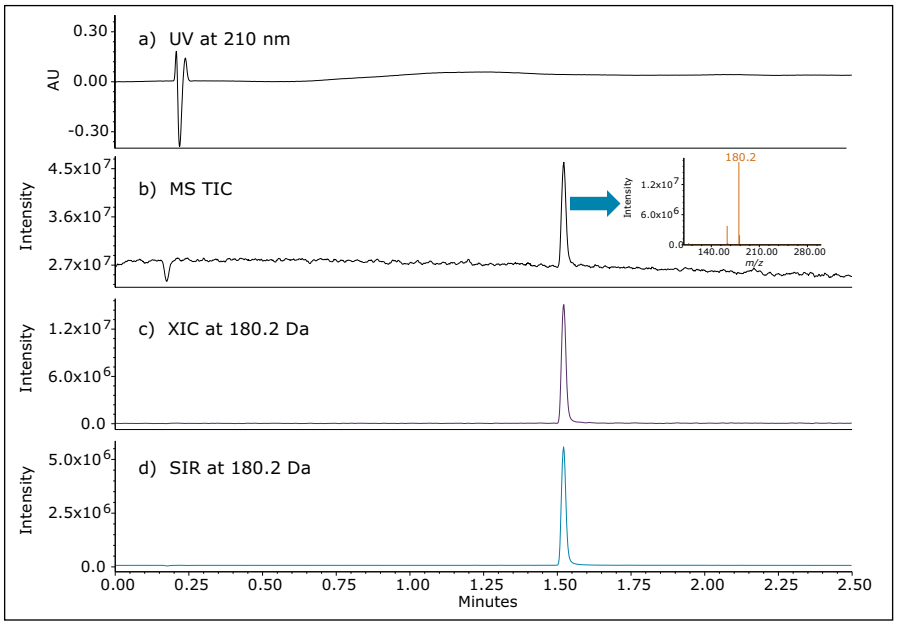

Figure 2. UV and MS chromatographic data for memantine HCl acquired using the ACQUITY UPLC H-Class System with PDA and ACQUITY QDa Detector.

System Suitability

Performance of the UPLC method was verified by evaluating repeatability of five replicate injections of the 1 µg/mL standard (Figure 3) made according to the specifications defined in the USP General Chapter, <621> "Chromatography".[5] The UPLC system suitability results, processed using SIR mass data at 180.2 Da, are shown in Figure 4. The retention times and area repeatability were well within the USP specification of less than 2% RSD.

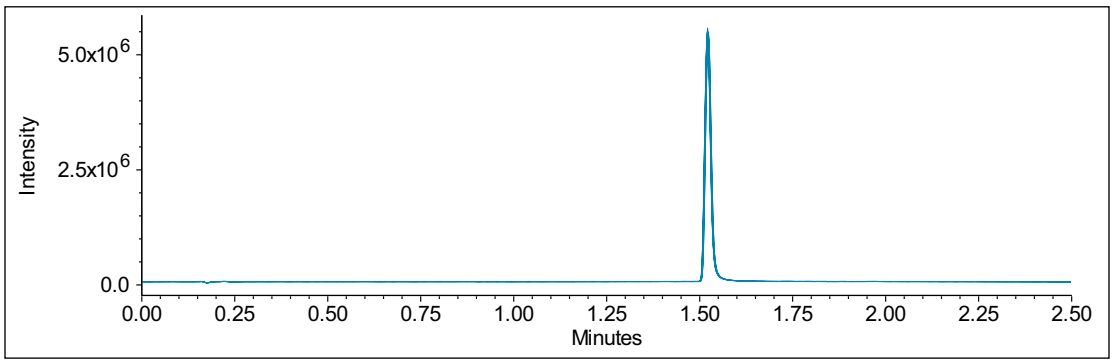

Figure 3. Overlay of five replicate injections of Memantine HCl standard: SIR mass data at 180.2 Da.

System Suitability_Report

Sample Set ID: 12773
Result Set ID: 14455
Channel Name: QDa 3: SIR Ch1

Name: Memantine

	Name	SampleName	Inj	RT	Area	USP Tailing	K Prime
1	Memantine	Memantine SS: 1ug/mL	1	1.523	6071507	1.2	5.1
2	Memantine	Memantine SS: 1ug/mL	2	1.522	6076992	1.2	5.1
3	Memantine	Memantine SS: 1ug/mL	3	1.521	6107702	1.2	5.1
4	Memantine	Memantine SS: 1ug/mL	4	1.521	6139821	1.2	5.1
5	Memantine	Memantine SS: 1ug/mL	5	1.521	6157180	1.2	5.1
Mean				1.522	6110640	1.2	5.1
Std. Dev.				0.001	37714.946		
% RSD				0.06	0.62		

Figure 4. System suitability results for five replicate injections of standard solution: SIR mass data at 180.2 Da.

Linearity

Linearity of the method for memantine HCl with mass detection was evaluated over seven concentrations ranging from 0.05 µg/mL to 1.0 µg/mL. The method showed good linear correlation between the peak areas and concentrations of memantine HCl, with correlation coefficients (R^2) ≥0.998 (Figure 5). In addition, the percent deviation of the calculated x values or concentrations were less than 7.0% (Figure 6).

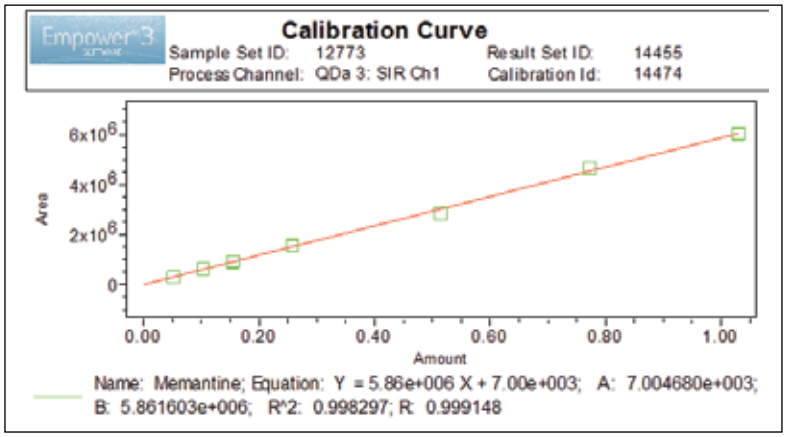

Figure 5. Linearity of the method for memantine HCl. Data processed using SIR mass data at 180.2 Da.

Empower 3

Calibration Curve %Deviation

Sample Set ID: 12773 Result Set ID: 14455

Channel Name: QDa 3: SIR Ch1

Peak MemantineX Value (ug/mL): 0.05150

	Name	Level	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	Memantine	1	1	305873	0.05150	0.05099	-0.995
2	Memantine	1	2	309364	0.05150	0.05158	0.161
3	Memantine	1	3	305437	0.05150	0.05091	-1.139

Peak MemantineX Value (ug/mL): 0.10300

	Name	Level	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	Memantine	2	1	635819	0.10300	0.10728	4.152
2	Memantine	2	2	625800	0.10300	0.10557	2.493
3	Memantine	2	3	639816	0.10300	0.10796	4.814

Peak MemantineX Value (ug/mL): 0.15450

	Name	Level	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	Memantine	3	1	920154	0.15450	0.15578	0.832
2	Memantine	3	2	917046	0.15450	0.15525	0.488
3	Memantine	3	3	911865	0.15450	0.15437	-0.084

Peak MemantineX Value (ug/mL): 0.25750

	Name	Level	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	Memantine	4	1	1561034	0.25750	0.26512	2.959
2	Memantine	4	2	1566506	0.25750	0.26605	3.322
3	Memantine	4	3	1579787	0.25750	0.26832	4.202

Peak MemantineX Value (ug/mL): 0.51500

	Name	Level	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	Memantine	5	1	2837467	0.51500	0.48288	-6.237
2	Memantine	5	2	2843904	0.51500	0.48398	-6.023
3	Memantine	5	3	2856054	0.51500	0.48605	-5.621

Peak MemantineX Value (ug/mL): 0.77250

	Name	Level	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	Memantine	6	1	4662628	0.77250	0.79426	2.817
2	Memantine	6	2	4642911	0.77250	0.79089	2.381
3	Memantine	6	3	4640096	0.77250	0.79041	2.319

Peak MemantineX Value (ug/mL): 1.03000

	Name	Level	Injection	Response	X Value (ug/mL)	Calc. Value (ug/mL)	% Deviation
1	Memantine	7	1	6015896	1.03000	1.02513	-0.473
2	Memantine	7	2	6042378	1.03000	1.02965	-0.034
3	Memantine	7	3	6041853	1.03000	1.02956	-0.043

Figure 6. Calibration curve data processed using SIR mass data at 180.2 Da. The percent deviation for the measurements is less than 7.0%.

Sample Analysis

The UPLC/MS method was then used to analyze commercially available memantine HCl tablets, to demonstrate specificity for routine assay. To develop a sample preparation procedure for the tablets, various sample diluents and filters were considered, with a goal of meeting the acceptance criteria for recovery, defined in the USP Monograph for Memantine Hydrochloride Tablets.[4] According to that monograph, "Memantine HCl Tablets contain an amount of memantine hydrochloride equivalent to NLT 90.0% and NMT 110.0% of the labeled amount of memantine hydrochloride ($C_{12}H_{21}N{\cdot}HCl$)."

Sample diluents investigated during the study are shown in Table 1. Separate sample solutions were prepared by dissolving tablets using all diluents and then sonicating and centrifuging the stock solutions. They were then filtered through 0.2 µm GHP syringe filters before undergoing dilution to the working concentration. The data show that preparation in diluent containing 50:50 0.1 N HCl/ethanol resulted in the highest recovery of memantine HCl: 99.9%.

Tablet prep.	Stock solution diluent	Working solution diluent	% Recovery
1	H_2O	90:10 H_2O/methanol	67.0
2	0.01 M H_3PO_4	0.01 M H_3PO_4	89.4
3	0.05 M H_3PO_4	0.05 M H_3PO_4	91.9
4	0.1 M H_3PO_4	0.1 M H_3PO_4	91.9
5	0.1 N HCl	0.1 N HCl	90.1
6	50:50 0.05 M H_3PO_4/ethanol	0.05 M H_3PO_4	93.7
7	50:50 0.1 N HCl/ethanol	0.1 N HCl	99.9

Table 1. Diluent study for development of a sample preparation procedure for memantine HCl tablets.

In addition to diluent, the effect on recovery of filter type was also evaluated. Particulates or insoluble materials present in the sample solution can interfere with chromatography or effect a poor recovery. Removal of particulates through sample filtration can often improve the recovery. The effect on recovery of three different filter membranes, as compared with no filtration (control), was studied (Table 2). Stock sample solutions prepared in 50:50 0.1 N HCl/ethanol were filtered through three, 0.2-µm, syringe filters. As shown in Table 2, filtration through the GHP-membrane syringe recovered the memantine HCl from the tablet formulation most effectively. Significantly, recovery of the compound from the unfiltered sample solution was lower than it was from the solution filtered through the GHP filter. These results show that sample filtration and prudent selection of filter type are necessary to maximize recovery.

Syringe filter	Waters P/N	Stock solution diluent	Working solution diluent	% Recovery
unfiltered	N/A	50:50 0.1N HCl/Ethanol	0.1N HCl	94.5
GHP	WAT200562	50:50 0.1N HCl/Ethanol	0.1N HCl	99.8
Nylon	WAT097962	50:50 0.1N HCl/Ethanol	0.1N HCl	93.3
PVDF	WAT200804	50:50 0.1N HCl/Ethanol	0.1N HCl	98.1

Table 2. Syringe-filter study for development of a sample preparation procedure for memantine HCl tablets.

For sample analysis, three separate preparations of tablets were tested for the assay of memantine HCl. An example of the chromatographic data of sample diluent and tablet sample solution acquired using the ACQUITY QDa Detector with SIR at 180.2 Da is displayed in Figure 7. The average percent recovery of memantine HCl for the three preparations ranged from 96.0 to 100.1% (Figure 8), which meets the USP acceptance criteria of 90.0–100.0%, defined in the USP Monograph for memantine HCl tablets.

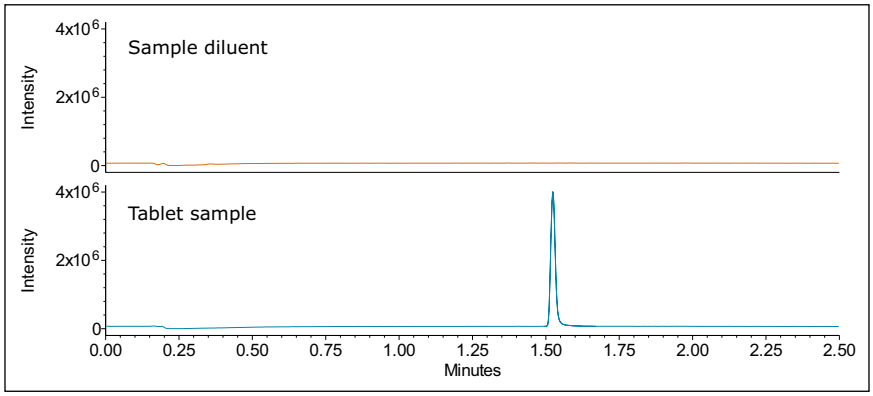

Figure 7. Tablet sample solution analysis for memantine HCl assay, SIR mass data at 180.2 Da.

Sample %Recovery Report

Sample Set ID: 12660
Result Set ID: 14518
Channel Name: QDa 3: SIR Ch1

Sample Name: Memantine Tablet Prep 1

	SampleName	Inj	Cal Id	Calculated Amount (ug/mL)	Target Amount (ug/mL)	% Rec	Pass?
1	Memantine Tablet Prep 1	1	14474	0.7178	0.7500	95.70	Pass
2	Memantine Tablet Prep 1	2	14474	0.7231	0.7500	96.41	Pass
3	Memantine Tablet Prep 1	3	14474	0.7200	0.7500	96.00	Pass
Mean						96.0	
Std. Dev.						0.4	
% RSD						0.37	

Sample Name: Memantine Tablet Prep 2

	SampleName	Inj	Cal Id	Calculated Amount (ug/mL)	Target Amount (ug/mL)	% Rec	Pass?
1	Memantine Tablet Prep 2	1	14474	0.7533	0.7500	100.44	Pass
2	Memantine Tablet Prep 2	2	14474	0.7470	0.7500	99.60	Pass
3	Memantine Tablet Prep 2	3	14474	0.7514	0.7500	100.19	Pass
Mean						100.1	
Std. Dev.						0.4	
% RSD						0.43	

Sample Name: Memantine Tablet Prep 3

	SampleName	Inj	Cal Id	Calculated Amount (ug/mL)	Target Amount (ug/mL)	% Rec	Pass?
1	Memantine Tablet Prep 3	1	14474	0.7334	0.7500	97.79	Pass
2	Memantine Tablet Prep 3	2	14474	0.7348	0.7500	97.98	Pass
3	Memantine Tablet Prep 3	3	14474	0.7275	0.7500	97.00	Pass
Mean						97.6	
Std. Dev.						0.5	
% RSD						0.53	

Figure 8. Percent recovery of memantine HCl from the tablet samples, SIR mass data at 180.2 Da. The USP acceptance criteria for %Recovery specified in the USP Monograph for Memantine HCl tablets are: 90.0–100.0%.

CONCLUSION

Mass detection using the ACQUITY QDa Detector enabled detection and quantitative determination of non-chromophoric memantine HCl. System suitability and method linearity calculated using mass data were excellent. For analysis of tablets, a non-derivatization sample preparation procedure for quantitative determination of memantine HCl was developed, eliminating the need for a complex and tedious pre-column derivatization protocol before the analysis. The use of SIR allowed the selection of a target mass, reducing interferences in the analysis of formulated samples.

Overall, the ACQUITY QDa Mass Detector is a robust, simple to use, orthogonal, detection technique to UV detection. It provides accurate and reliable results, making this technology ideal for routine testing in the QC laboratory.

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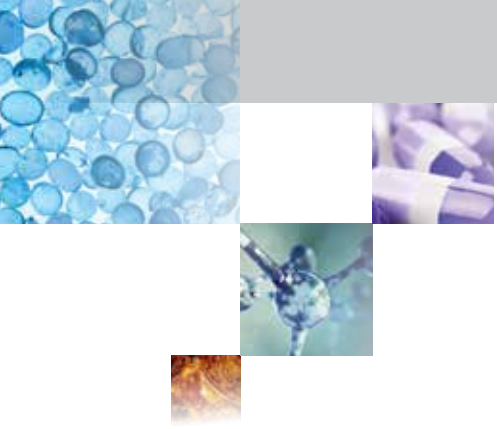

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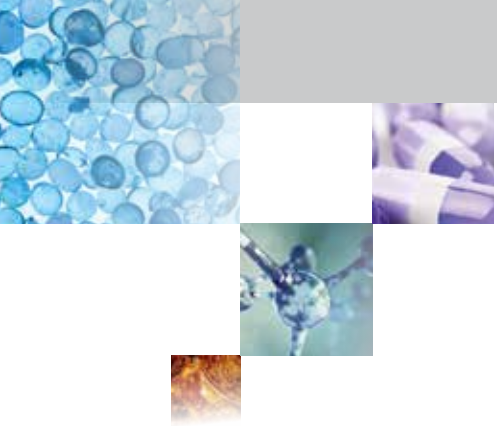

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