RADAR and PICS Compendium

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RADAR and PICS Compendium

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UNDERSTANDING SAMPLE COMPLEXITY

IMPROVING QUANTITATIVE DATA QUALITY

Uncertainty in chromatographic method development and quantitative experiments often arises when co-eluting unknown interferences reduce performance. In particular, targeted multiple reaction monitoring (MRM) analysis of compounds in complex matrices is a difficult task and it is often impossible to separate all the interfering compounds present in matrix from the target analytes. It is likely that an analyst performing a routine MRM experiment would be unaware if interferences were present or if they were changing from sample to sample as subtle changes in analysis results are difficult to detect.

Some key issues for quantitative methods are:

- Calculating compound concentrations in addition to the detection of unexpected contaminants.
- Characterization of the background matrix for every sample, increasing data quality.
- Detection of analytes that are not in a targeted MRM screening method.
- Improving method development by discovering more matrix components.

In order to address these challenges a tandem quadrupole instrument must be able to rapidly switch between MS qualitative scanning and MS/MS quantitative modes so as not to affect the duty cycle time of the instrument. This is a key requirement when performing UPLC®, UPC²® or GC separations which produce narrow peak widths offering less time to acquire data for the sample component peak as it arrives at the mass analyser. Within this narrow time window it is still important to maintain sensitivity and mass resolution as well as providing reproducible data from injection to injection. To maintain high quality reproducible data, RADAR™ was introduced on the Xevo® TQ MS in 2008 and has since become a well-established technology available on the Xevo TQD, Xevo TQ-S micro, Xevo TQ-S, and Xevo TQ-XS instruments. In RADAR mode, it is possible to monitor for matrix interferences, metabolites, impurities, and degradants in a sample while accurately quantifying target compounds without losing sensitivity or performance.

Understanding the matrix of individual samples and monitoring changes in matrix across different samples will lead to continuous improvement of quality of the services provided by the laboratory.

RADAR – PRINCIPLE OF OPERATION

Innovative T-Wave™ collision cell design permits the use of RADAR which is operated by rapidly switching between MRM (MS/MS) mode and MS full scan acquisition. Switching between the two modes occurs in only 5 ms ensuring that the duty cycle time is kept to a minimum and maximizing data points across the peak. Setting up a RADAR experiment is a simple process as the analyst can add an MS scan function to any MRM method and use the automatic dwell time feature to create optimal MRM channel dwell times. Figure 1 shows the two modes of acquisition that traditionally would require two separate injections but can now operate together.

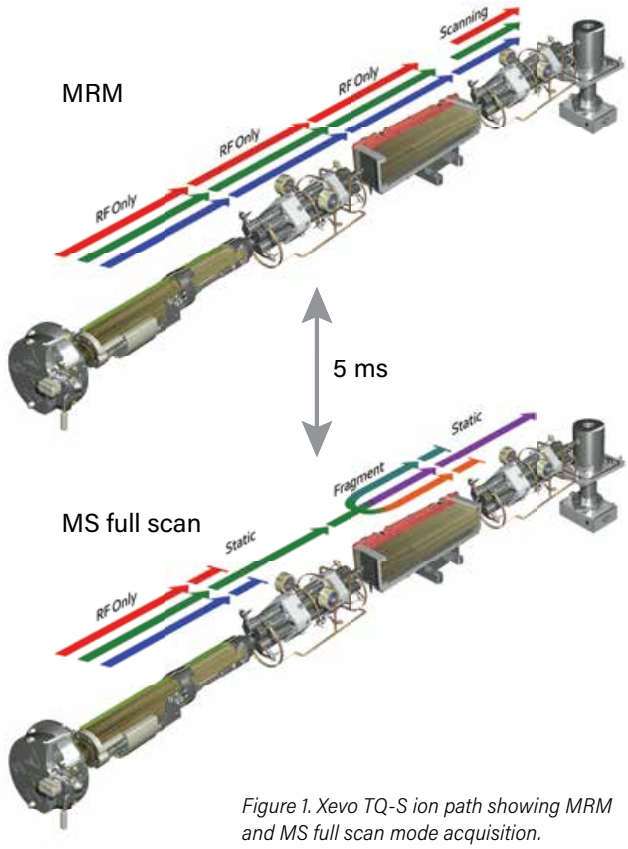

Figure 1. Xevo TQ-S ion path showing MRM and MS full scan mode acquisition.

PICs

CONFIDENCE IN YOUR PEAK IDENTITIES

Product Ion Confirmation Scanning (PICS) is a technique that allows the analyst to collect MS/MS product ion spectra to confirm the identity of an analyte without compromising the quantitative MRM data quality. This ensures the very best MRM quantification data for demanding, high speed, and high resolution UPLC-MS/MS assays. It is very simple to add PICS to an MS method as it can be enabled with a simple checkbox for each compound. The technologies that make PICS possible are:

- The time it takes to switch from MRM to MS/MS product ion scanning is very short
- The PICS acquisition is timed so that the data collection begins after the MRM peak apex and finishes before the peak has eluted (see Figure 2).

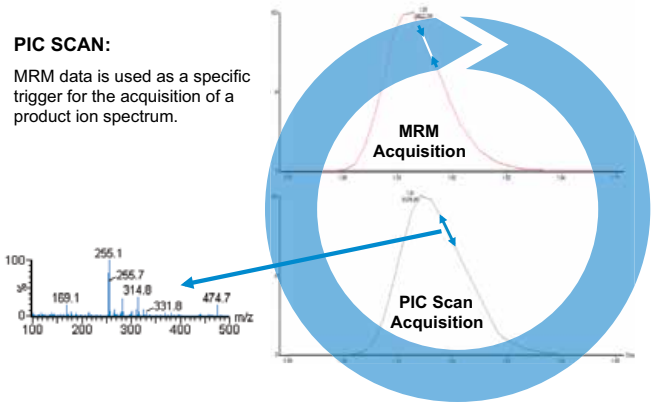

Figure 2 . MS/MS spectral enhancement provided by ScanWave.

SCANWAVE – BETTER SPECTRAL DATA FROM TANDEM QUADRUPOLE MS

Conventional tandem quadrupole MS instruments are sensitive enough when used to monitor targeted compounds in MRM mode, but they are significantly less sensitive when used to acquire data in spectral mode.

Introduced on the Xevo TQ MS and implemented on the Xevo TQ-S and Xevo TQ-XS, ScanWave™ ensures that the PICs spectra are of a good quality even at low concentrations.

ScanWave Technology allows ions within the collision cell to be accumulated and separated according to their mass-to-charge ratio (*m/z*). Synchronizing these ions with the scanning of the second quadrupole significantly enhances product ion spectra, enabling you to more easily confirm the identities and structures of your analytes of interest (see Figure 3).

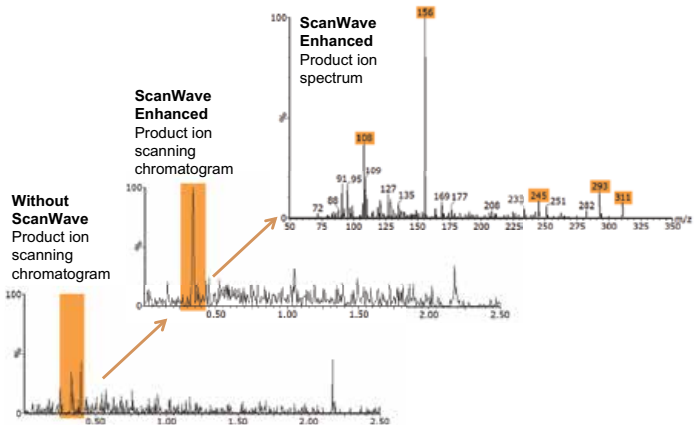

Figure 3. Data for an injection of sulphadimethoxine showing the benefit of using ScanWave for low level identifications, performing a daughter scan of m/z 311.1.

Chemical Materials

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Enantioseparation and Detection of Triazole Fungicides in Wheat Grain and Wheat Straw using the ACQUITY UPC^2 System and Xevo TQ-S

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APPLICATION BENEFITS

- Enantiomeric detection and quantitation of triazole fungicides at parts per trillion levels (ppt).
- Improved enantiomeric resolution and shorter analysis times using SFC compared with normal-phase HPLC chiral separations resulting in higher sample throughput.
- Reliable and reproducible measurement of the enantiomer ratios for use in enantioselective degradation studies.

WATERS SOLUTIONS

ACQUITY® UPC2® System

Xevo® TQ-S

Oasis® Sample Extraction Products

DisQuE™ Dispersive Sample Preparation

Sep-Pak SPE

MassLynx® Software

KEY WORDS

Chiral quantitation of pesticides, enantiomer, chiral separation, uniconazole, tebuconazole, diniconazole, flutriafol, fenbuconazole, triazole fungicide, SFC, supercritical fluid chromatography, UPC^2, enantioselective degradation studies.

INTRODUCTION

The safe use of crop protection products is of paramount importance to the agricultural chemicals manufacturing industry. Extensive studies and trials are carried out in support of product registration. These studies ensure that any risks associated with using the product are characterized and properly understood so that it can be safely applied to the field. When a crop protection active ingredient (AI) contains one or more stereogenic centers in its structure the enantioselective behavior must be studied, since it is known that enantiomers can exhibit different bioactivities.[1,2] Analytical methods used to evaluate the influence of stereochemistry on the degradation dynamics, environmental fate, and final residue levels help to establish a more accurate risk assessment of crop protection products.

Liquid chromatography (LC) on chiral stationary phases (CSPs), such as polysaccharide stationary phases including amylose and cellulose, has been the most commonly used chiral separation technique.[3-6] More recently, there has been an increasing adoption of using supercritical fluid chromatography (SFC) on CSPs for chiral separation.[7,8] The properties of a supercritical fluid, its high diffusivity and low viscosity in particular, enable high efficiency chiral separations with shorter run times. For example, triazole fungicides, such as tebuconazole, structure shown in Figure 1, are a commonly used group of pesticides due to their potent activity against a broad spectrum of crop diseases. Using HPLC, the analysis times for the enantiomeric resolution of tebuconazole ranged from 13 to 45 min.[3-6] Similar resolutions were achieved for tebuconazole using SFC, but the analysis times were reduced to 10 min.[8]

UltraPerformance Convergence Chromatography™ (UPC^2) applies the performance advantages of UPLC® to SFC, combining the use of supercritical CO_2 with sub-2-µm particle columns.[9,10] UPC^2 is an orthogonal analytical technique to reversed-phase LC and can be used to solve complex separations challenges. The detection sensitivity and specificity offered by tandem MS/MS is advantageous for determining trace levels of pesticides in complex matrices like field crops or soil.[11-14]

EXPERIMENTAL

SFC conditions

SFC system:	ACQUITY UPC^2
Chiral separation:	Diniconazole, fenbuconazole, flutriafol, tebuconazole
Column:	Chiralpak IA-3, 4.6 x 150 mm, 3.0 µm
Co-solvent (B):	Methanol with 2% water and 0.1% formic acid
ABPR:	1990 psi/137 bar
Flow rate:	2.5 mL/min
Column temp.:	40 °C
Injection volume:	4 µL
UPC^2 conditions:	0 min 20% B, 2.5 min 20% B, 2.6 min 30% B, 5 min 30% B, return to initial conditions.
Chiral separation:	Uniconazole
Column:	Chiralpak IA-3 4.6 x 150 mm, 3.0 µm
Co-solvent (B):	50:50 2-propanol/ethanol with 2% water and 0.1% formic acid
ABPR:	1990 psi/137 bar
Flow rate:	2.5 mL/min
Column temp.:	15 °C
Injection volume:	4 µL
UPC^2 conditions:	0 min 15% B, 4 min 15% B, 4.1 min 35% B, 5 min 35% B, return to initial conditions.

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI+
Capillary voltage:	2.8 kV
Cone voltage:	See Table 1
Desolvation temp.:	600 °C
Source temp.:	150 °C
Collision energy (eV):	See Table 1
MS scan range:	100 to 800 *m/z*

An AgileSLEEVE™ 30 cm x 1/16" I.D. tubing heater (Analytical Sales and Services Inc. Pompton Plains, NJ) set to 65 °C was used to heat the transfer line to the MS system. All compounds were automatically tuned by direct infusion using IntelliStart™ prior to the analysis. A summary of the optimized MRM transitions and voltages is shown in Table 1.

In this application note, ACQUITY UPC^2 and tandem quadrupole mass spectrometry were used for the trace level enantioanalysis of five triazole fungicides (Figure 1) in wheat grain and/or wheat straw. A QuEChERS (quick easy cheap effective rugged and safe) extraction modified for dry commodities was performed followed by solid phase extraction using Oasis MCX. Chiral separations using a 3.0 µm chiral CSP followed by multiple reaction monitoring (MRM) detection allowed concentrations of part per trillion (ppt) levels to be reproducibly detected and quantified.

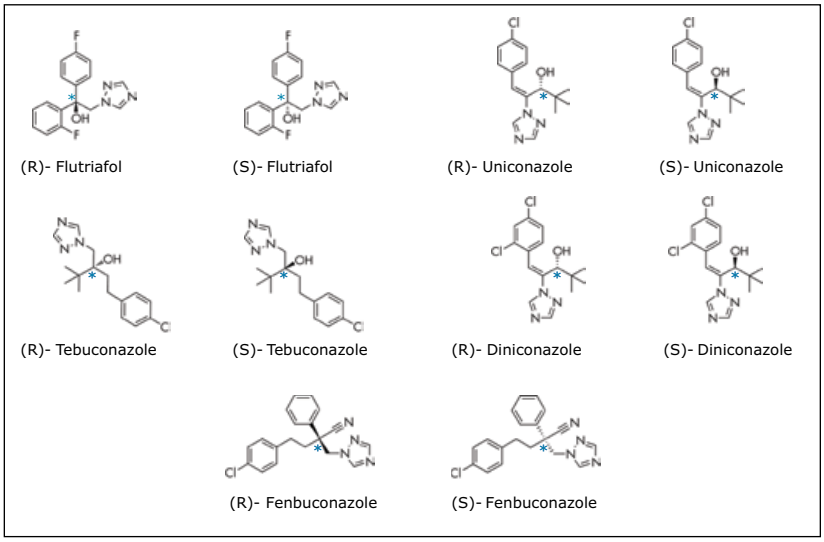

Figure 1. Structures of the target triazole fungicide enantiomers. The asterisks denote the stereogenic centers.

Instrumentation

All separations were performed on a Waters® ACQUITY UPC^2 System. Detection was by positive ion electrospray mass spectrometry (MS) using a Xevo TQ-S tandem quadrupole mass spectrometer. MassLynx Software was used for data acquisition, and TargetLynx Application Manager was used for data processing.

Sample preparation

Initial extraction (QuEChERS):

Triturated wheat straw (1 g) or wheat grain (5 g) were placed in a 50-mL polypropylene centrifuge tube. The volume of water added to the wheat straw was 9 mL with 5 mL of water added to the wheat grain, followed by phosphoric acid (100 µL) and acetonitrile (10 mL). The mixture was shaken for 20 minutes. A DisQuE Pouch for the European Committee for Standardization (CEN) QuEChERS method (Part No. 186006813) was added to the tube and shaken vigorously for 1 minute. Centrifugation at 4000 rpm followed to produce a liquid partition with a clear acetonitrile top layer. The top acetonitrile layer (5 mL) was transferred to a clean 50-mL centrifuge tube and diluted with water (45 mL) for cleanup using an Oasis MCX 3 cc, 60 mg Cartridge (Part No. 186000254).

Oasis MCX sample cleanup

Oasis MCX 3 cc, 60 mg Cartridges were conditioned with 3 mL of methanol and equilibrated with 3 mL of water. The samples were loaded in reverse phase mode into Sep-Pak 60 cc Reservoirs (Part No. 186005587) at a flow rate of 1 to 3 mL/min. After sample loading was completed the cartridge was washed with 2% formic acid in water (3 mL) followed by 100% methanol (3 mL). A collection vessel was installed and elution was achieved using 2 mL 2% ammonium hydroxide in methanol. The base containing eluent from the elution step was blown down to dryness and reconstituted in neat methanol (5 mL).

Standard and sample preparation

Working standard solutions were prepared by sequential dilution of the stock solution using acetonitrile. The working standards were spiked (in triplicate) on to the dry wheat straw/wheat grain at levels of 1, 5, and 10 ng/g. The samples were allowed to equilibrate for 30 min prior to extraction. Matrix-matched standard curves were prepared with blank matrix extracted using the same protocol.

Wheat straw and wheat grain samples were obtained from online vendors.

Table 1. MRM transitions and instrument settings for the analysis of the triazole fungicides. The primary quantitation transition is listed (top) with the confirmatory transition (bottom).

Analyte	MRM transitions	Cone voltage (V)	Collision energy (eV)
Diniconazole	326>70	16	20
	326>159	16	30
Fenbuconazole	337>70	14	18
	337>125	14	26
Flutriafol	302>70	16	16
	302>123	16	26
Tebuconazole	308>70	40	18
	308>125	40	36
Uniconazole	292>70	20	22
	292>125	20	24

RESULTS AND DISCUSSION

Enantioseparation of the five triazole fungicides

A Chiralpak IA-3, 4.6 x 150 mm, 3.0 µm was used to perform enantioseparation of the five triazole fungicides. Resolution was achieved for diniconazole, fenbuconazole, flutriafol, and tebuconazole using methanol as the co-solvent; while the chiral resolution of uniconazole was improved using a mixture of ethanol and 2-propanol (50:50 v/v). Water (2%) and formic acid (0.1%) were added directly to the co-solvents to promote ionization. A chromatogram of wheat grain directly spiked at a level of 1 ng/g and extracted using QuEChERS followed by sample cleanup using Oasis MCX is shown in Figure 2. All triazole AI's were enantiomerically resolved in less than 3.5 minutes. The United States Pharmacopeia (USP) resolution (Rs) ranged from 1.73 to 6.83.

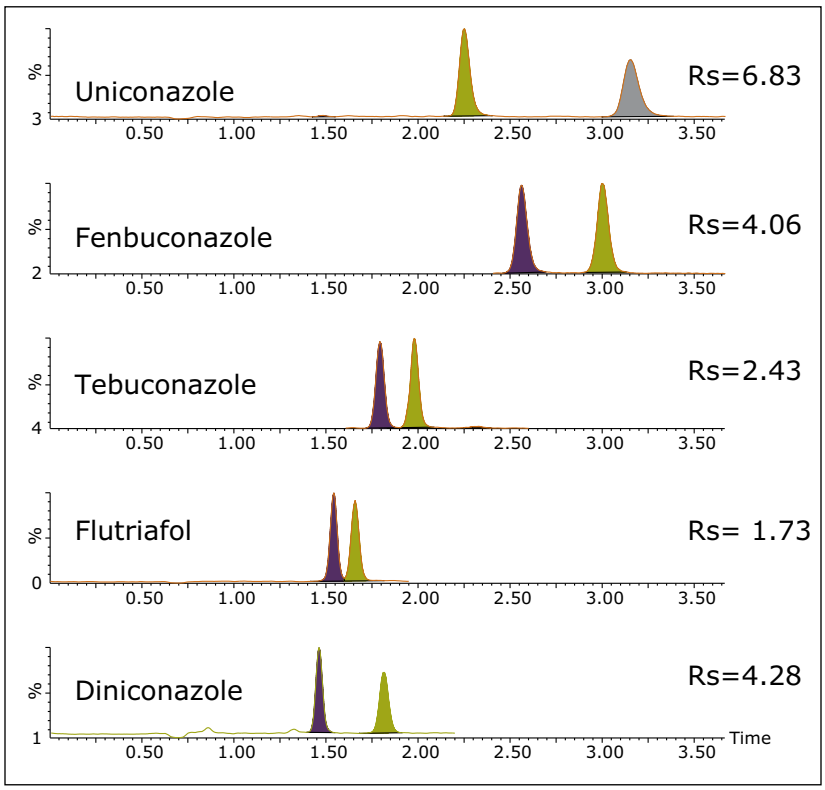

Figure 2. ACQUITY UPC²-MRM chromatograms showing the enantioseparation of five triazole fungicides pre-spiked onto wheat grain at a level of 1 ng/g and extracted using QuEChERS with Oasis MCX sample cleanup.

Linearity, accuracy, and sensitivity

The five triazole fungicides were post spiked into the wheat grain and/or wheat straw extracts. The spiked extracts were sequentially diluted with blank matrix extract to produce a series of matrix-matched curves and QC samples ranging in concentration from 0.005 to 50 ng/mL. Examples of the quantitation curves for each flutriafol enantiomer spiked into blank wheat grain extract, and for the fenbuconazole enantiomers spiked into blank wheat straw extract are shown in Figures 3 and 4 respectively. Linear calibration curves (R^2 >0.998) for each enantiomer of the target fungicides were obtained.

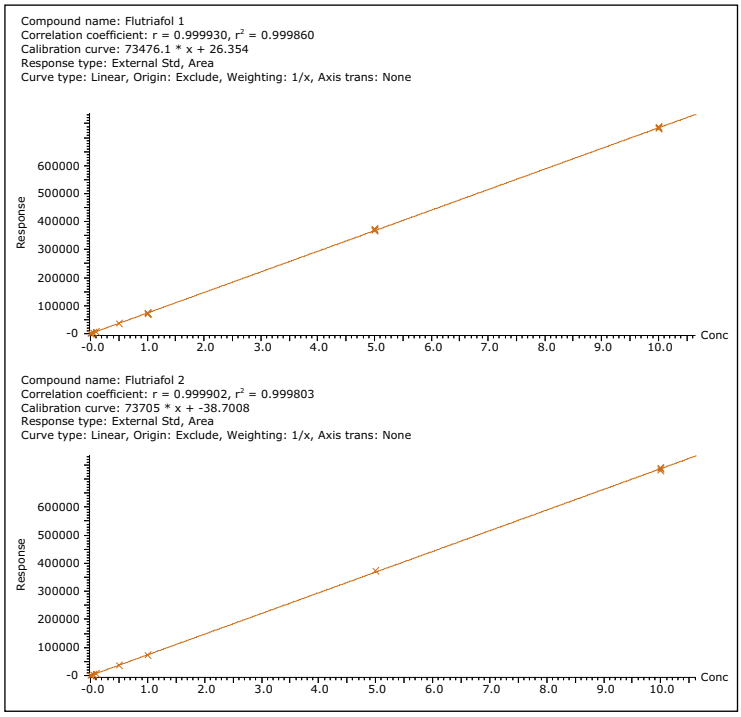

Figure 3. Wheat grain matrix-matched quantitation curves for each flutriafol enantiomer analyzed in triplicate 0.005 to 10 ng/mL.

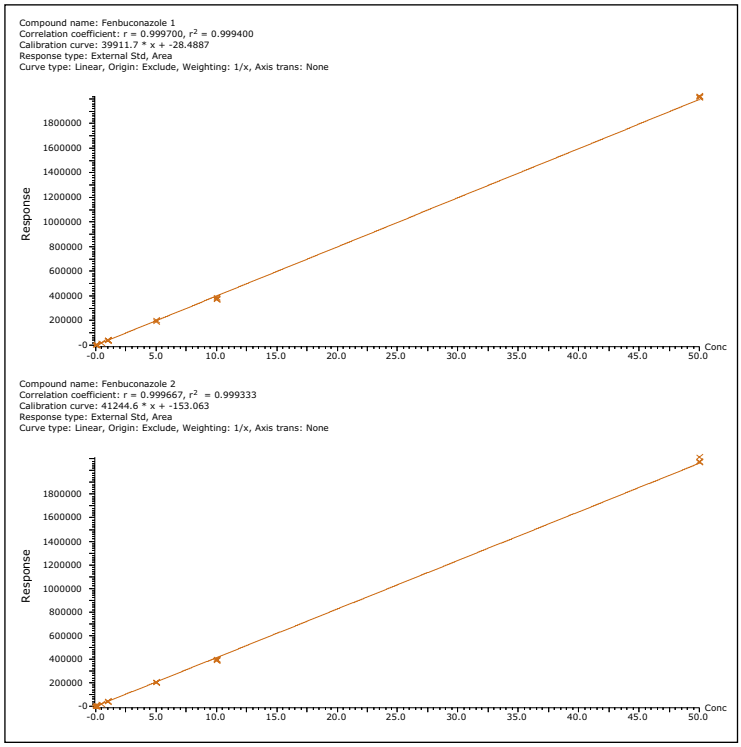

Figure 4. Wheat straw matrix-matched quantitation curves for each fenbuconazole enantiomer analyzed in triplicate 0.01 to 50 ng/mL.

To assess the accuracy of the method, quality control (QC) samples were made up in the blank extracted matrices at four concentration levels: 0.016, 0.16, 1.66, and 16.66 ng/mL. Three concentration levels were analyzed against the curves. The calculated concentrations for the QC samples were within +/- 15% of the known concentration for each enantiomer in both the wheat and straw matrices.

Examples of the blank, 0.01 ng/mL and 0.05 ng/mL level for all compounds spiked into extracted wheat grain matrix are shown in Figure 5.

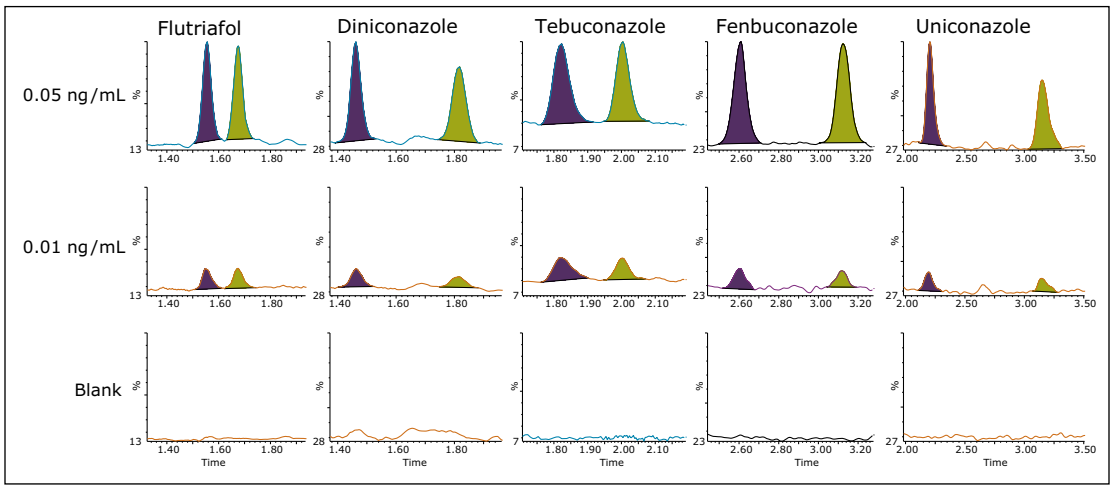

Figure 5. ACQUITY UPC²-MRM chromatograms showing the enantioseparation of five triazole fungicides spiked into blank wheat grain matrix extracted using QuEChERS, followed by cleanup using Oasis MCX. The blank, 0.01 ng/mL, and 0.05 ng/mL levels (equivalent to 0.02 ng/g and 0.1 ng/g if spiked directly on to the wheat grain pre-extraction) are shown; 4 µL injection.

Reproducibility

The precision of the technique was determined by a repeatability study (n=4) using four concentration levels 0.05 ng/mL, 1 ng/mL, 5 ng/mL, and 10 ng/mL of matrix-matched wheat grain standards, as shown in Table 2.

Table 2. Table shows the %RSD (n=4) for each enantiomer of the triazole fungicides at four concentration levels 0.05, 1, 5, and 10 ng/mL (4 µL injection).

		%RSD (n=4)			
	Enantiomer	0.05 ng/mL	1 ng/mL	5 ng/mL	10 ng/mL
Flutriafol	Peak 1	1.2	1.5	1.0	1.2
	Peak 2	2.9	1.9	0.6	2.5
Tebuconazole	Peak 1	1.3	1.3	2.8	2.0
	Peak 2	1.8	2.2	1.7	2.0
Fenbuconazole	Peak 1	1.9	0.8	2.5	1.3
	Peak 2	2.5	1.3	1.7	1.0
Diniconazole	Peak 1	2.4	2.9	2.1	1.1
	Peak 2	2.6	3.9	2.9	2.8
Uniconazole	Peak 1	1.5	1.3	1.9	1.7
	Peak 2	2.6	1.1	1.5	1.5

Internal standards were not available for the study; however the RSD's ranged from 0.6% to 3.9%. These results illustrate the reliability of the method reproducibility over a range of concentration levels.

Matrix effects and recovery

A series of standard solutions was prepared in methanol at the same concentration levels as the matrix-matched curves. The analyte response and slopes from both curves were compared. The matrix effects were calculated to within +/- 10% for each enantiomer of the target fungicides.

The average extraction recoveries from three samples fortified at 1 ng/g, 5 ng/ g, and 10 ng/g (n=3) in wheat were calculated. Recoveries in excess of 75% were obtained for each enantiomer of the pesticides analyzed in the study.

Simultaneous qualitative tools: RADAR™ and PICs

Depending on the chromatographic conditions target analytes can co-elute with endogenous matrix components which can lead to matrix effects and decreased method robustness. The Xevo TQ-S employs a proprietary scanning technology known as RADAR from which full scan (MS) and MRM (MS/MS) data can be acquired simultaneously. RADAR provides a convenient way to monitor the background matrix using its full-scan MS function. Co-eluting components can be identified at an earlier stage of the method development process.

In addition Product Ion Confirmation Scan (PICs) can be activated, which facilitate the collection high quality full-scan spectra during MRM acquisition, and provide an additional means of chromatographic peak identification based on MS or MS/MS spectra. Activated by a single check box in the method editor, PICS automatically triggers a product ion scan when a peak is detected by MRM.

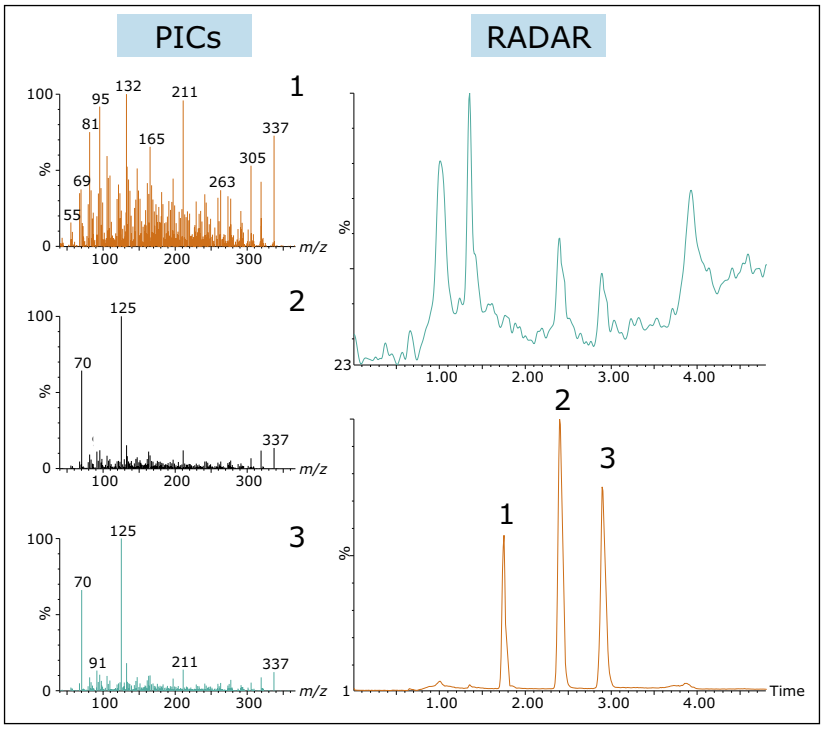

Figure 6. ACQUITY UPC²-MRM (lower trace) and RADAR chromatograms using gradient elution showing the resolution of fenbuconazole enantiomers pre-spiked onto wheat straw.

During method development for the analysis of fenbuconazole enantiomers in wheat straw the RADAR and PICs chromatograms shown in Figure 6 were acquired simultaneously with the MRM function. Peak 1 in the MRM chromatogram was isobaric (*m/z* 337) with fenbuconazole and shared a common fragment. Enantiomers were differentiated using the MS/MS PIC spectrum. The RADAR data acquired simultaneously identified components eluting closely to the analytes (Peaks 2 and 3). Changes were made to the SPE cleanup and isocratic elution was employed, which resulted in a lower spectral background and the removal of closely eluting matrix components.

CONCLUSIONS

The study of pesticide enantiomers is important as they can exhibit different bioactivities. Analytical methods that can rapidly provide information about each enantiomer at trace concentration levels can lead to a more accurate assessment of the influence of stereochemistry on the degradation dynamics, environmental fate, and final residue levels of crop protection chemicals.

In this study, the enantioseparation of five triazole fungicides was performed in less than 3.5 minutes. The Xevo TQ-S was used for detection of the rac-triazole fungicides in wheat grain and wheat straw. The results from the chiral UPC^2-MRM analysis show that trace level detection (ppt) can be achieved with good precision and accuracy over at least 3.5 orders of magnitude using this technique.

The use of RADAR, where full-scan data can be acquired simultaneously with MRM data can help identify co-eluting components that could potentially decrease the assay's robustness.

When complex matrices are analyzed, despite the specificity of MRM, matrix components give rise to signals that can be misidentified as an analyte peaks. PICs data provides an added qualitative element to the acquisition, which is useful for achieving higher selectivity, and greater confidence for peak assignment and confirmation.

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Food & Environmental

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Dilute and Shoot Method for the Determination of Tobacco-Specific Nitrosamines (TSNAs) in Smokeless Tobacco Products by UPLC-MS/MS

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APPLICATION BENEFITS

- Sensitive and robust method for analysis of TSNAs in smokeless tobacco products including snus, moist snuff, dry snuff, chewing tobacco, and raw tobacco.
- Rapid separation of TSNAs within 7 minutes using an ACQUITY UPLC® BEH C_{18} Column.
- Wide linear dynamic range for TSNAs (NNN, NNK, NAT–0.25 to 128 ng/mL and NAB–0.0625 to 32 ng/mL).
- Faster method development using RADAR™ Technology to overcome sample matrix effects.
- Simplified workflow using sample dilution and smaller injection volume to eliminate the need for SPE cleanup.

WATERS SOLUTIONS

ACQUITY UPLC H-Class System

Xevo® TQD

ACQUITY UPLC BEH C_{18} Column

Oasis® SPE Cartridge

MassLynx® MS Software

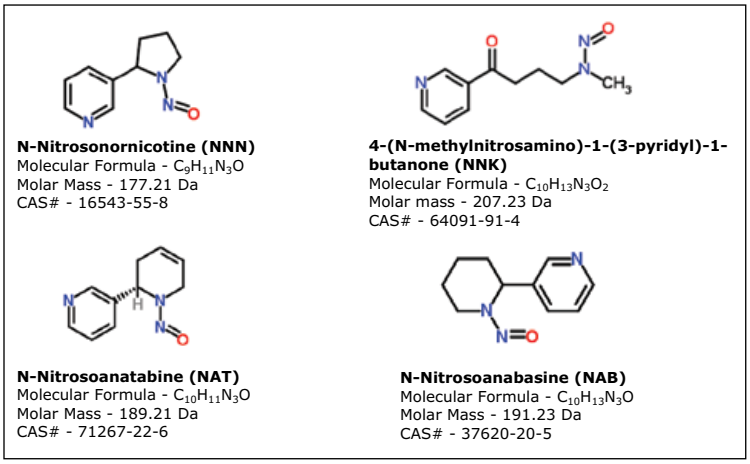
TargetLynx™ Application Manager

KEY WORDS

Tobacco specific nitrosamines (TSNAs), NNN, NNK, NAT, NAB, tobacco, snus, snuff, chewing tobacco

INTRODUCTION

Tobacco specific nitrosamines (TSNAs) are a group of carcinogenic compounds found in tobacco and tobacco smoke. Four different TSNAs (Figure 1) are monitored in tobacco and smoke emissions: N-nitrosonornicotine (NNN), 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB).[1] These harmful constituents are formed from nicotine and related alkaloids by a nitrosation reaction that occurs during the curing and processing of tobacco.



N-Nitrosonornicotine (NNN)
Molecular Formula - $C_9H_{11}N_3O$
Molar Mass - 177.21 Da
CAS# - 16543-55-8

4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
Molecular Formula - $C_{10}H_{13}N_3O_2$
Molar mass - 207.23 Da
CAS# - 64091-91-4

N-Nitrosoanatabine (NAT)
Molecular Formula - $C_{10}H_{11}N_3O$
Molar Mass - 189.21 Da
CAS# - 71267-22-6

N-Nitrosoanabasine (NAB)
Molecular Formula - $C_{10}H_{13}N_3O$
Molar Mass - 191.23 Da
CAS# - 37620-20-5

Figure 1. Structure and chemical properties of TSNAs.

Global tobacco product regulations require tobacco companies to disclose the contents of tobacco products, smoke emissions, and changes to their products. Several priority toxicants including TSNAs have been identified in tobacco and smoke emissions that need to be accurately measured and reported to regulatory bodies. The development of robust and reliable analytical methods for quantitative measurement of priority tobacco and smoke constituents is critical to global tobacco harm reduction initiatives.

Standardized methods for the analysis of TSNAs in tobacco products exist from CORESTA and ISO TC/126, developed through inter-laboratory collaborative studies.[2–4]

EXPERIMENTAL

UPLC conditions

UPLC system:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	45 °C
Injection volume:	5 µL
Flow rate:	0.45 mL/min
Mobile phase A:	10 mM ammonium acetate in water
Mobile phase B:	0.1% acetic acid in methanol (v/v)
Weak needle wash:	50/50 water/methanol (v/v)
Strong needle wash:	10/90 methanol/water (v/v)
Seal wash:	90/10 water/methanol (v/v)
Analysis time:	7 min

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.45	99	1	6
3.00	0.45	10	90	6
4.00	0.45	10	90	6
4.01	0.45	1	99	6
5.00	0.45	99	1	6
7.00	0.45	99	1	6

Table 1. UPLC gradient for TSNA analysis.

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI+
Capillary voltage:	2.5 kV
Desolvation temp.:	550 °C
Desolvation gas flow:	1000 L/Hr
Source temp.:	150 °C

MRM transitions (Table 2) for TSNAs and labeled internal standards (NNN-D4 and NNK-D4), were optimized using IntelliStart™ and monitored in the analysis of calibration standards and sample extracts. The first and second MRM transitions of target analytes were used for quantification and confirmation, respectively. The data was acquired and processed using MassLynx Software with TargetLynx Application Manager.

TSNAs can be measured using gas chromatography with a thermal energy analyzer (GC-TEA), or liquid chromatography coupled to tandem quadrupole mass spectrometry (LC-MS/MS). LC-MS/MS methods for TSNA analysis provide significant advantages over GC-TEA as they offer high sensitivity, high selectivity and high sample throughput (simplified workflow).

In this application note, we describe a UPLC®-MS/MS method for TSNA analysis in tobacco products that has been developed based on the CORESTA standard (CRM-72), and provides further improvements in sensitivity, linear dynamic range, sample workflow, and overall analysis throughput. This method is applicable to the determination of TSNAs in tobacco and smokeless tobacco products including snus, moist snuff, dry snuff, and chewing tobacco.

Compound	Retention Time (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
NNN	2.10	178.06	148.02	24	10
		178.06	105.01	24	16
NNN-D4	2.10	182.10	152.10	24	10
NNK	2.22	208.07	122.01	28	12
		208.07	79.03	28	32
NNK-D4	2.22	212.15	126.07	28	12
NAT	2.41	190.06	160.08	18	10
		190.06	79.01	18	26
NAB	2.48	192.07	162.10	28	12
		192.07	133.07	28	20

Table 2. Optimized MRM conditions for TSNAs and labeled internal standards using IntelliStart on the Xevo TQD.

Standards

TSNA reference standards (1 mg/mL) were purchased from Cerilliant (Round Rock, Texas). Isotopically labeled internal standards (NNN-D4 and NNK-D4) were purchased from the Toronto Research Chemicals (Toronto, Canada). An intermediate stock solution consisting of 10 µg/mL of NNN, NNK, NAT, and 2.5 µg/mL of NAB, was prepared in acetonitrile and used to prepare calibration standards in the range of 0.25 to 128 ng/mL for NNN, NNK, NAT, and 0.0625 to 32 ng/mL for NAB. The calibration standards and internal standard spiking solution were prepared in 100 mM ammonium acetate in water. NNN-D4 was used as the internal standard for NNN quantification, while NNK-D4 was used as the internal standard for quantification of NNK, NAT, and NAB. For accurate quantification of TSNAs in different tobacco matrices, the use of all four labeled internal standards is recommended.

Tobacco samples

CORESTA Reference Products (CRP) for smokeless tobacco were provided by North Carolina State University (Raleigh, NC) including snus (CRP-1), moist snuff (CRP-2), dry snuff (CRP-3), and loose-leaf chewing tobacco (CRP-4). Cigarette tobacco was purchased from a local retail store.

Sample preparation

Recommendations on sampling and grinding tobacco products can be found in CRM-72.[3] The tobacco sample was weighed (1±0.05 gram) and extracted using 30 mL of 100 mM ammonium acetate in water. The amount of labeled internal standards was kept constant in the sample extracts and calibration standards to facilitate accurate quantification. The samples were shaken for 30 minutes on a hand motion shaker (Model EL680.Q, Eberbach Corporation, Ann Arbor, MI) at 350 rpm. Sample extracts were filtered using a 0.45-µm PTFE syringe filter and diluted 10-fold with 100-mM ammonium acetate in water prior to UPLC-MS/MS analysis.

Sample analysis

Method optimization studies were conducted to evaluate chromatographic separation and matrix effects using RADAR acquisition mode which enables simultaneous full-scan (MS) and MRM acquisitions. The method was also evaluated with respect to linearity, sensitivity, accuracy and precision. For linearity, calibration curves were created using solvent standards in the range of 0.25 to 128 ng/mL for NNN, NNK, NAT, and 0.0625 to 32 ng/mL for NAB. The method sensitivity was evaluated using a diluted 0.05 ng/mL TSNA standard. Accuracy of the method was evaluated by determining recoveries for TSNAs from five different tobacco matrices, spiked (n=3) at 50 ng/g for NNN, NNK, NAT, and 12.5 ng/g for NAB.

RESULTS AND DISCUSSION

Method optimization – RADAR (full scan MS and MRM)

To optimize the chromatographic separation for TSNAs and to improve the data quality, RADAR acquisition mode was utilized on Waters® Xevo TQD to characterize the tobacco matrix. RADAR enables simultaneous acquisition of full scan MS and MRM data, a unique capability that can both simplify and accelerate development of robust methods. Two different mobile phase B solvents were evaluated: 10 mM ammonium acetate in methanol and 0.1% acetic acid in methanol. While both mobile phases provided improved chromatographic resolution for TSNA separation compared to the CORESTA standard CRM-72,[3] significant matrix suppression was observed for NNN using 10 mM ammonium acetate in methanol. The full scan data from RADAR revealed that the NNN peak co-eluted with the high-intensity nicotine peak (Figure 2A). The use of 0.1% acetic acid in methanol as the mobile phase B improved separation between nicotine and TSNAs while minimizing matrix suppression for the NNN peak (Figure 2B).

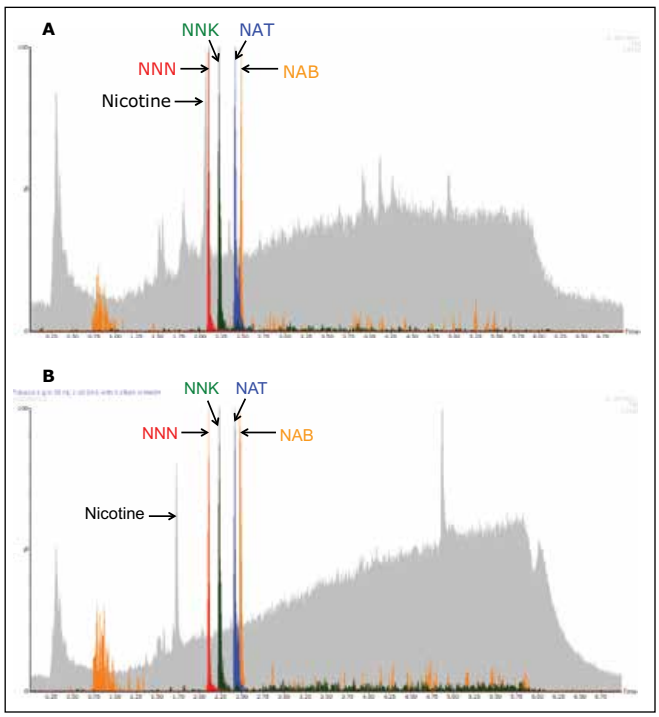


Figure 2A. Full scan TIC (in grey) and MRM chromatogram of TSNAs (in color) using 10 mM ammonium acetate in methanol as mobile phase B.

Figure 2B. Full scan TIC (in grey) and MRM chromatograms of TSNAs (in color) using 0.1% acetic acid in methanol as mobile phase B.

The tobacco extracts (1 gram in 30 mL of 100 mM ammonium acetate in water) were analyzed directly and also with a 10-fold dilution to evaluate the method sensitivity for detection of TSNAs. This comparison showed that there was adequate instrument sensitivity for the detection of TSNAs using 10-fold diluted tobacco extracts (data not shown). The advantage of the dilution step is that matrix effects are reduced and instrument maintenance and downtime is minimized. Alternatively, the CORESTA method[3] provides a sample cleanup procedure using Waters Oasis HLB SPE Cartridges to reduce matrix effects observed in TSNA analysis.

Linearity

The TSNAs showed excellent linearity with R^2 values >0.999 (Figure 3) for the calibration range of 0.25 to 128 ng/mL for NNN, NNK, NAT, and 0.0625 to 32 ng/mL for NAB. The wide calibration range evaluated in this study exceeds the calibration range used in CRM-72 (0.5 to 100 ng/mL for NNN, NNK, NAT, and 0.125 to 25 ng/mL for NAB) and enables determination of TSNAs in different tobacco products using a single sample preparation procedure.

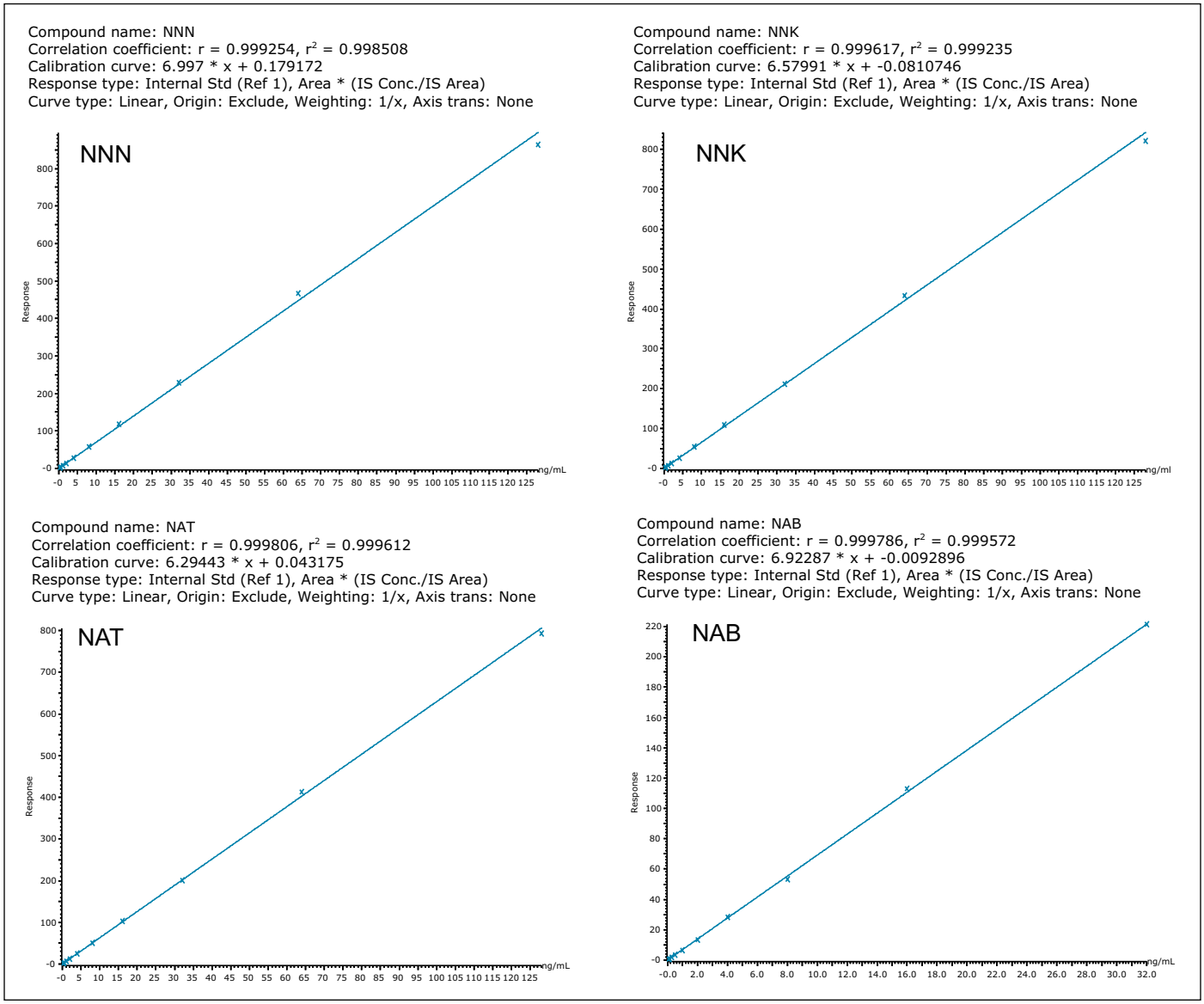


Figure 3. Calibration curves of TSNAs solvent standards.

Sensitivity

Method sensitivity was evaluated by determining analyte response in diluted TSNAs solvent standards, below the lowest calibration standard of 0.25 ng/mL for NNN, NNK, NAT, and 0.0625 ng/mL for NAB. As shown in Figure 4, the signal-to-noise (S/N) ratio calculated by peak-to-peak noise approach for a 0.05 ng/mL TSNA standard was >10 using a Xevo TQD System. The ability to detect and quantify TSNAs at such low levels allows users to inject diluted sample extracts to minimize ion suppression from tobacco matrix and eliminate the need for time-consuming SPE cleanup procedures.

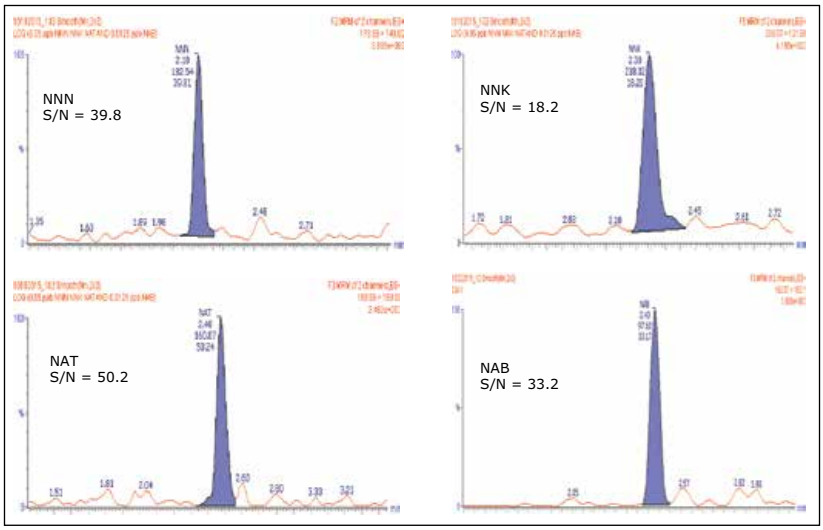


Figure 4. Signal-to-noise (S/N) ratio of diluted TSNA solvent standard (0.05 ng/mL) analyzed using the Xevo TQD.

Recovery

In the absence of a blank tobacco matrix, TSNA recoveries were determined by standard additions method. Tobacco sample extracts with incurred nitrosamines were fortified with 50 ng/g of NNN, NNK, NAT, and 12.5 ng/g of NAB in triplicates. Both tobacco and fortified tobacco extracts were analyzed and quantified against solvent calibration curves. The calculated recoveries for TSNAs after subtracting the incurred levels from tobacco matrices are shown in Figure 5. The recoveries for TSNAs were acceptable and ranged from 105% to 119% in the different tobacco matrices evaluated. The error bars represent percent relative standard deviation (%RSD) that ranged from 0.1% to 7.2%. NNN and NNK showed relatively lower mean recoveries of 109% and 108% from different tobacco matrices using respective isotopically labeled internal standards (NNN-D4 and NNK-D4). The mean recoveries for NAT and NAB were relatively higher (117% and 116%) as NNK-D4, a structural analogue, was used as the internal standard for their quantification. For more accurate quantification in tobacco matrices, the use of all four labeled internal standards is recommended.

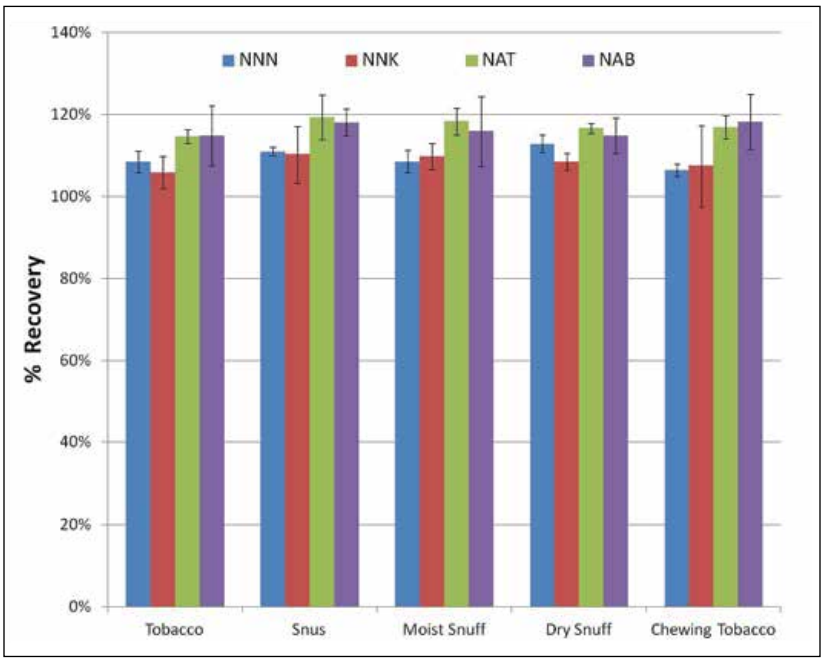


Figure 5. %Recovery of TSNAs from different tobacco product matrices.

Tobacco analysis

The raw tobacco and smokeless reference tobacco products were analyzed in triplicate following the optimized UPLC-MS/MS method. The results from the UPLC-MS/MS method were compared to results from HPLC-MS/MS methods used in the CORESTA inter-lab study (n=3 replicates, 11 labs) in which TSNA data was generated following CRM-72 (Figure 6). The TSNA yields from the UPLC-MS/MS method are within the range of CORESTA inter-lab study results. The error bars represent ± one standard deviation. As observed from the individual and total TSNA yields, the TSNA content varies significantly between the different tobacco products. The differences in TSNA yields among the various tobacco products are attributed to differences in tobacco blend, curing and processing methods, and storage conditions.

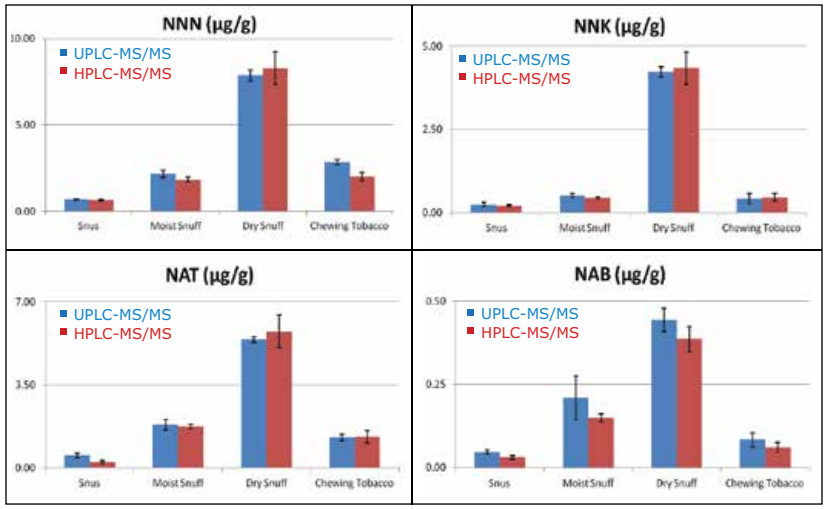


Figure 6. Comparison of TSNA yields from analysis of smokeless tobacco products using the UPLC-MS/MS method and the HPLC-MS/MS methods used in the CORESTA inter-lab study.[5]

CONCLUSIONS

A rapid, sensitive and robust UPLC-MS/MS method has been developed for the determination of TSNAs in tobacco and smokeless tobacco products. The UPLC-MS/MS method further improves the performance of industry standard CRM-72 by utilizing a UPLC column to improve chromatographic resolution and reduce analysis time. The key benefits of UPLC-MS/MS method for TSNA analysis are compared to HPLC-MS/MS based on CORESTA method in Table 3.

Method parameter	HPLC-MS/MS standardized method[3]	UPLC-MS/MS method	Key benefits
Calibration range	2–80 ng/mL for NNN, NNK, NAT and 0.125 to 25 ng/mL for NAB	0.25-128 ng/mL for NNN, NNK, NAT and 0.0625 to 32 ng/mL for NAB	Extended calibration range
Sample cleanup	SPE recommended	10-fold dilution	Simplified workflow
Injection volume	10 µL	5 µL	Reduced matrix load
Analytical column	XTERRA® MS C_{18}, 2.1 x 50 mm, 2.5 µm	ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm, 1.7 µm	Higher chromatographic resolution
Analysis time	10 min	7 min	Reduced analysis time

Table 3. Key benefits of UPLC-MS/MS method for TSNA analysis compared to HPLC-MS/MS method.

The dilute and shoot approach for TSNA analysis eliminates the need for sample cleanup and minimizes instrument maintenance and downtime.

- This method is suitable for the determination of TSNAs in various tobacco products including snus, moist snuff, dry snuff, chewing tobacco, and raw tobacco.
- The UPLC-MS/MS method provides higher sensitivity, selectivity, and reduced analysis time (7 min) for the determination of TSNAs compared to CRM-72.
- RADAR helps understand sample complexity and leads to faster and robust method development.
- Dilution of tobacco extracts and use of a lower sample injection volume reduces matrix effects, and eliminates the need for SPE cleanup step without compromising data quality.

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5. *CORESTA STS Technical Report*: Smokeless Reference Tobacco Product Analysis. 2014.

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The Development of a Sensitive Multi-Residue LC-MS/MS Method for the Quantitative Determination of Mycotoxins in Animal Feedstuffs and Silage Using Xevo TQ-S

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[3] Ghent University, Ghent, Belgium

APPLICATION BENEFITS

- Provides a quantitative LC-MS/MS method for the simultaneous determination of 33 mycotoxins in animal feedingstuffs and silage.
- Suitable for use as sensitive screening assay for the determination of low level mycotoxin contamination in animal feedingstuffs.
- Reduces complex matrix effects by incorporating a simple extract dilution step prior to analysis.

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System

Xevo® TQ-S

TargetLynx™ Application Manager

Quanpedia™ Database

KEY WORDS

mycotoxin, feedingstuffs, silage, tricothecene, beauvercin, enniatin, fumonisin, ochratoxin, T2, HT-2, alternaria, tandem quadrupole,

INTRODUCTION

There are now over 400 recognized mycotoxins that may be found in animal feedings materials and it has been reported that as much as 25% of the world's cereal grains may be contaminated with mycotoxins.[1]

The analysis of animal feedingstuffs including silage represents a major technical challenge due to the complexity and in-homogeneity of these matrices. Although permitted limits for mycotoxins are set at relatively high (µg kg^{-1}) concentrations in the EU,[2,3] toxic effects such as immunotoxicity and feed uptake problems in certain species (poultry and porcine) are often observed at sub µg kg^{-1} concentrations.[4] For this reason there is often a requirement to achieve low detection limits in feedingstuffs. There is also a potential for co-contamination due to pre- and post- harvest infestation resulting in the occurrence of tricothecenes, beauvercin and enniatins, fumonisins, ochratoxin, T2, HT-2, and alternaria toxins for example within a single feed sample.[5]

In this application note, we report the development of a quantitative method for the determination of 33 relevant mycotoxins in a variety of animal feed and silage extracts. A Waters® ACQUITY UPLC I-Class System coupled to a Xevo TQ-S was used for rapid, high quality, and ultra-sensitive analysis of multiple mycotoxins in feed extract. Our goal was to investigate the effect of matrix dilution and enhanced instrument sensitivity to overcome common analytical challenges such as ion suppression and to reduce the effects of matrix variability.

EXPERIMENTAL

Extract preparation

The extracts of different animal feedingstuffs and silage were kindly provided by RIKILT, The Netherlands and Ghent University for the purposes of this study. A generic and simplified sample extraction protocol based on 84:16 (v/v) acetonitrile: acidified water for the recovery of mycotoxins from the variety of feedingstuffs and silage was used.[6] Briefly, the feed samples were mechanically homogenized in the presence of the extraction solvent followed by a centrifugation step. An aliquot of the supernatant was removed and placed in autosampler vial for subsequent LC-MS/MS analysis.

UPLC conditions

LC system:	ACQUITY UPLC I-Class
Column:	BEH C_{18}, 2.1 x 100 mm, 1.7 µm
Temp.:	40 °C
Injection volume:	5 µL
Flow rate:	0.4 mL/min
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid

Time	A	B	Curve
0	90	10	
3	90	10	
10	30	70	
10.1	10	90	6
12	10	90	
12.1	90	10	
15	90	10	

Table 1. UPLC gradient.

MS conditions

MS System:	Xevo TQ-S
Ionization mode:	ES +/- switching
Capillary voltage:	3.4 kV
Source temp.:	150 °C
Desolvation temp.:	400 °C
Cone gas flow:	150 L/hr
Desolvation gas flow:	800 L/hr

Table 2. MS/MS conditions optimized using Quanpedia for the multi-mycotoxin analysis method for feedingstuffs and silage using the Xevo TQ-S.

Mycotoxin	MRM transition	Dwell time (s)	Cone voltage (V)	Collision energy (eV)	Polarity
Aflatoxin B1	Q 313.1 > 241.1 q 313.1 > 285.1	0.02	25	50 30	Pos
Aflatoxin B2	Q 315.1 > 259.1 q 315.1 > 287.1	0.02	25	40 35	
Alfatoxin G1	Q 329.1 > 243.1 q 329.1 > 311.1	0.02	20	33 20	
Alfatoxin G2	Q 331.1 > 245.1 q 331.1 > 313.1	0.037	25	18 19	
DON	Q 297.1 > 231.1 q 297.1 > 249.1	0.103	20	30 36	
Enniatin A	Q 682.6 > 99.9 q 682.6 > 210.3	0.108	70	30 34	
Enniatin A3	Q 668.5 > 99.9 q 668.5 > 210.0	0.052	64	30 36	
Enniatin B	Q 640.5 > 85.9 q 640.5 > 195.9	0.052	64	30 36	
Enniatin B1	Q 654.5 > 85.9 q 654.5 > 195.9	0.02	70	55 50	
Fumonisin B1	Q 722.4 > 334.3 q 722.4 > 352.3	0.023	25	55 50	
Fumonisin B2	Q 706.4 > 336.2 q 706.4 > 318.2	0.023	50	5 12	
HT-2	Q 425.2 > 245.1 q 425.2 > 263.1	0.021	14	32 30	
OTA	Q 404.2 > 245.1 q 404.2 > 263.1	0.021	10	5 5	
T-2	Q 467.3 > 245.1 q 467.3 > 305.1	0.052	20	23 19	
Zearalenone	Q 319.1 > 185 q 319.1 > 187	0.038	78	70 28	
Beauvericin	Q 784.5 > 133.9 q 784.5 > 244.0	0.052	32	43 30	
Tentoxin	Q 415.2 > 131.9 q 415.2 > 312.1	0.038	17	12 7	
Pencillic acid	Q 171.1 > 125.0 q 171.1 > 153.0	0.163	28	25 25	
Citrinin	Q 251.1 > 191.0 q 251.1 > 205.1	0.023	25	30 25	
Alternariol	Q 259.0 > 185.1 q 259.0 > 213.2	0.02	10	20 8	
NIV	Q 313.7 > 175.1 q 313.7 > 295.1	0.163	14	15 7	
α- Zearalenone	Q 323.0 > 277.1 q 323.0 > 305.2	0.023	14	15 7	
β- Zearalenone	Q 323.0 > 277.1 q 323.0 > 305.2	0.023	20	39 36	
Sterigimatocystin	Q 325.1 > 253.1 q 325.1 > 281.1	0.05	22	20 26	
Cyclopiazonic acid	Q 337.1 > 182.0 q 337.1 > 196.0	0.163	18	19 12	
3-acetyl-DON	Q 339.1 > 137.0 q 339.1 > 231.0	0.03	14	12 10	
15-acetyl-DON	Q 339.1 > 261.0 q 339.1 > 279.1	0.03	20	20 13	
Fusarenon-X	Q 355.2 > 229.1 q 355.2 > 247.1	0.03	14	10 10	
Diacetoxyscirpenol	Q 367.2 > 289.1 q 367.2 > 307.2	0.02	17	27 20	
Neosolaniol	Q 383.1 > 185.1 q 383.1 > 215.1	0.03	27	28 19	
Roquefortin	Q 390.2 > 193.0 q 390.2 > 322.2	0.032	25	42 30	
Ergotamine	Q 587.3 > 208.1 q 587.3 > 187.5	0.02	35	40 30	
Alternariol monomethyl ether	Q 271.2 > 228.2 q 271.2 > 256.2	0.031	42	30 10	Neg

Figure 1 shows the MRM transitions and automated time window scheduling functionality to obtain a minimum of 12 points across each chromatographic peak generated by Quanpedia. This method is available in the Quanpedia database.

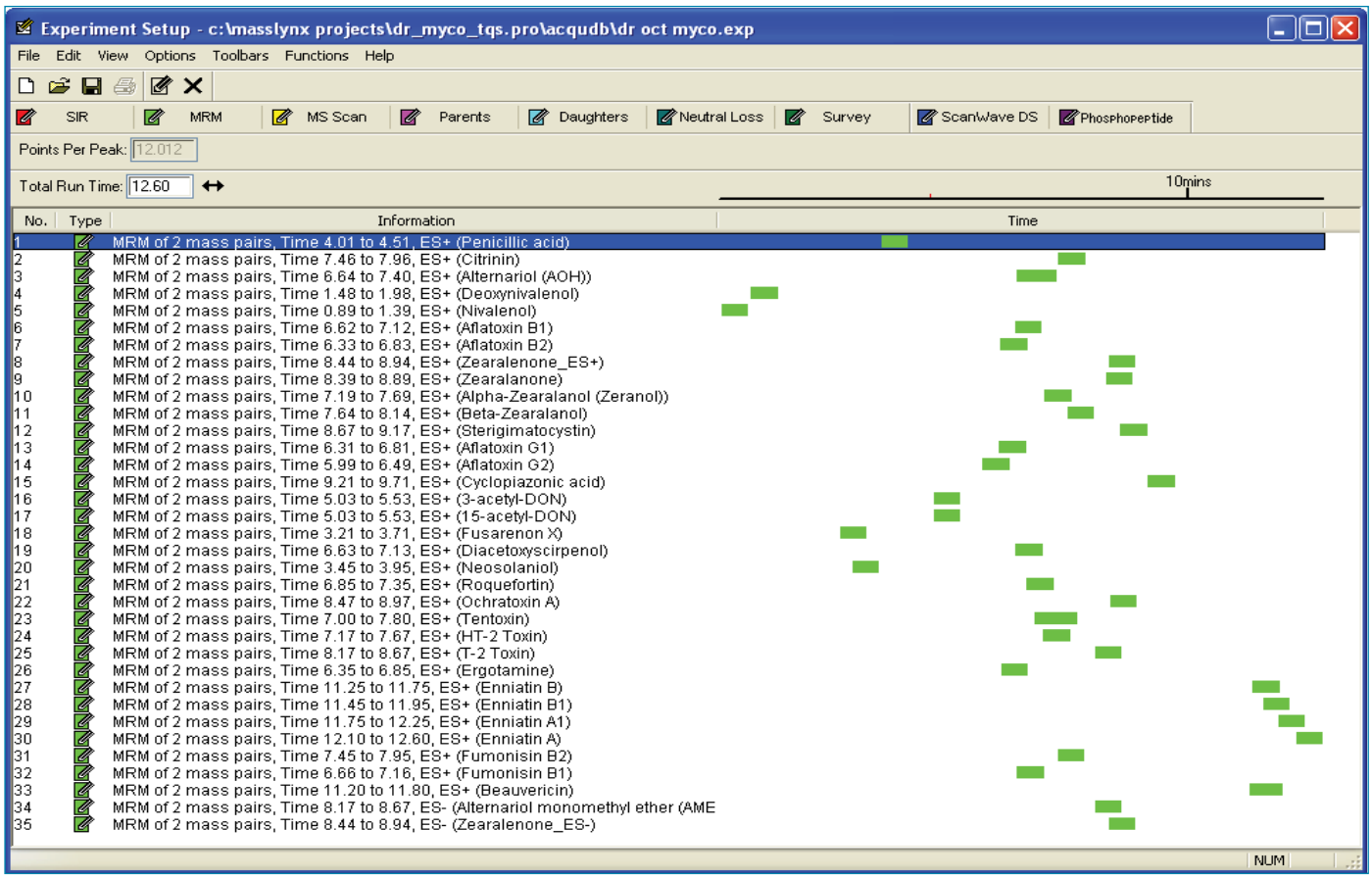

Figure 1. MRM transitions for 33 mycotoxins (in ESI + and - modes) and automated time window scheduling functionality generated by the Quanpedia Database.

RESULTS AND DISCUSSION

The feed and silage samples were analyzed as neat and diluted extracts and quantified against either solvent or matrix matched standards (as available). As anticipated, the matrix interference profile was found to be highly complex and variable between samples as determined via the RADAR functionality. Figure 2 shows the LC-MS/MS spectra obtained for a neat extract of a porcine feed. The Base Peak Intensity (BPI) spectrum obtained in full scan mode and the simultaneously acquired MRM transitions for five mycotoxins identified in the sample (displayed in the Figure inset) were found to elute in a region of high matrix background. The presence of high concentrations of matrix background can result in variable ion suppression effects for the analytes of interest and can affect the overall analytical performance. For this reason, matrix matched calibrants, standard addition, and isotopically labelled internal standards are approaches typically used for the quantitative analysis of mycotoxins in complex matrices to overcome the matrix effects and improve the quantitative accuracy and precision.

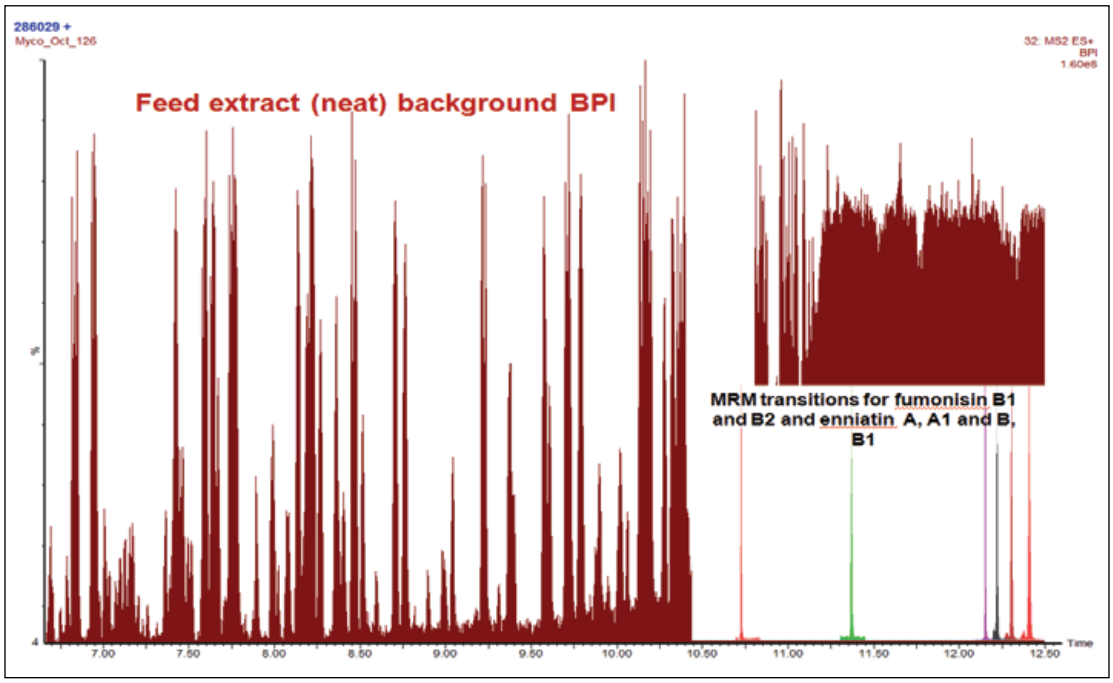

Figure 2. Base peak intensity (BPI) chromatogram obtained for naturally contaminated porcine feed extract showing the background matrix profile monitored in full scan mode (MS function 2, RADAR) and the MRM transitions (MS function 1) for fumonisins B1 and B2 and enniatins A, A1, B, and B2.

In this study, we investigated the use of a simple dilution step coupled to the enhanced sensitivity of the Xevo TQ-S to reduce the matrix contribution and improve measurement repeatability and accuracy between different sample types. Table 3 shows the repeatability data obtained for six different extracts of feed spiked with six mycotoxins diluted 1:10 prior to injection. The intra-day method precision (expressed as %RSD) was found to be good at less than 23% for all the spiked mycotoxins.

The same dilution factor (1:10) was also applied to a wider selection of feedingstuffs (12) and silage (10) sample extracts and analyzed according to the optimized Xevo TQ-S conditions monitoring for 33 mycotoxins and quantified against an appropriate calibration series. Figure 3 shows the TargetLynx report generated showing the linearity and sensitivity of the method and calculated concentrations for the unknown samples.

Table 3. The repeatability data generated for a selection of six mycotoxins spiked into to a variety of different animal feeds (n=6), the extracts were diluted 1:10 prior to analysis on the Xevo TQ-S.

Feed Type	Measured concentration in animal feed extract diluted 1:10 (ng/g)*					
	Enniatin A	Enniatin A1	Enniatin B	Enniatin B1	Fumonisin B1	Fumonisin B2
Maize Gluten	305.9	165.6	48.0	86.1	14.0	17.1
Pig feed	275.5	160.5	43.6	56.5	17.0	17.5
Rye	258.5	136.1	61.8	62.1	18.7	19.3
Oats	221.9	115.5	41.4	62.0	22.7	20.1
Sunflower seed	197.4	117.7	48.2	77.1	13.0	13.2
Cattle feed	193.9	102.2	32.0	51.3	14.0	14.8
Mean	242.2	133.0	45.8	65.9	16.6	17.0
SD	45.1	25.7	9.8	13.1	3.7	2.6
%RSD	19	19	21	20	22	15

**Mycotoxins spiked into feed samples prior to extraction*

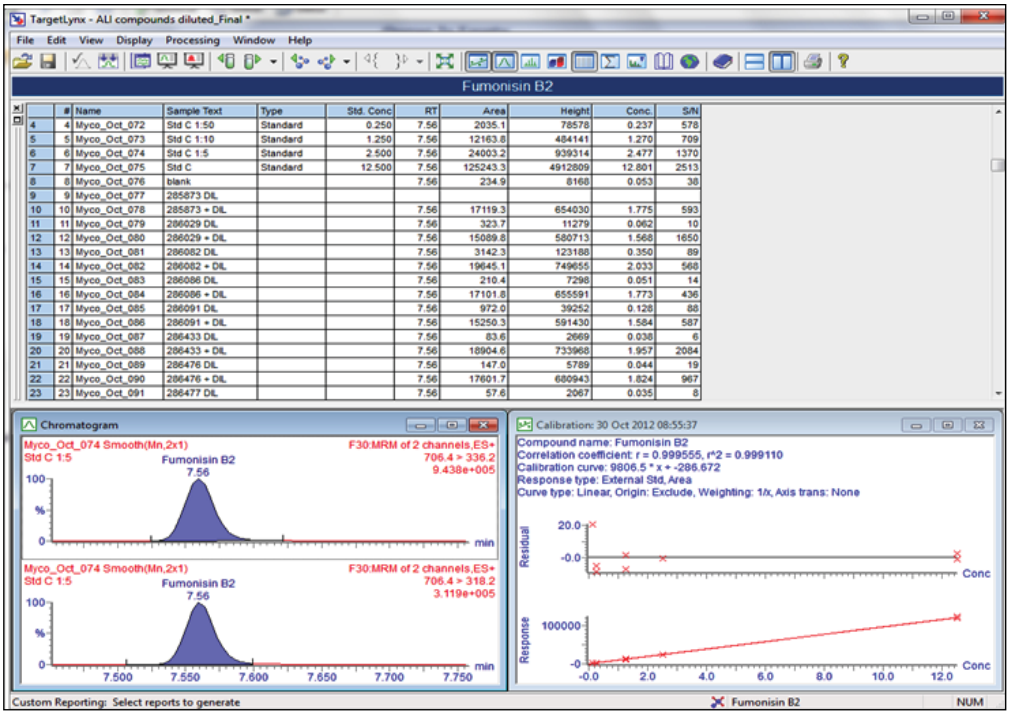

Figure 3. TargetLynx report showing linearity and sensitivity of standards down to 0.25 µg kg for fumonisin B2 using the Xevo TQ-S.

Tables 4 and 5 show the measured concentrations of the mycotoxins identified (monitoring two MRM transitions) in naturally contaminated feeds and silage samples, respectively along with the calculated LODs (S:N of ≥1:3). The LOD values were found to be in the ppt to low ppb range in both solvent and matrix for all the mycotoxins identified. The naturally contaminated feed samples were found to contain multiple mycotoxins ranging from 3 to 12 of the 33 potential mycotoxins monitored for and estimated to be present at concentrations equivalent sub-1 to circa 300 µg kg^{-1}. The method was considered to be suitable for use as a highly sensitive presence/absence screen as the contaminant concentrations were determined against a solvent standard calibration series, therefore matrix effects may still affect the quantitative performance.

Table 4. The measured concentrations for a range of mycotoxins identified using two MRM transitions in 12 different samples of animal feedingstuffs diluted 1:10 prior to analysis on the Xevo TQ-S.

Mycotoxin	LOD (ng/g)	Measured concentration in animal feed extract diluted 1:10 (ng/g)*											
		Animal feed sample identity and type											
		U1 / cattle feed	U2 / pig feed	U3 / maize gluten	U4 / Diva L vital pig feed	U5 /Alpha maximal pig feed	U6 / rye	U7 / barley	U8 / wheat	U9 / oats	U10 / maize	U11 / sunflower oil	U12 / pig feed
15-acetyl-deoxynivalenol	0.50	*nd*	*nd*	152.8	*nd*	*nd*	*nd*	13.2	33.4	*nd*	*nd*	*nd*	*nd*
Aflatoxin B1	0.05	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	0.2	*nd*
Aflatoxin B2	0.05	*nd*	*nd*	0.8	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	0.1	*nd*
Aflatoxin G1	0.05	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	0.1	*nd*
Aflatoxin G2	0.05	0.3	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Alternariol	0.06	*nd*	3.2	*nd*	*nd*	*nd*	5.3	*nd*	*nd*	7.6	2.6	10.0	*nd*
DON	0.13	*nd*	21.2	283.6	13.2	18.4	*nd*	*nd*	*nd*	4.8	*nd*	0.3	*nd*
Enniatin A	0.10	59.3	6.3	1.4	15.7	39.9	9.7	11.7	0.4	3.2	*nd*	*nd*	50.5
Enniatin A1	0.10	148.6	17.1	3.2	40.1	19.0	14.2	34.1	0.5	4.9	*nd*	*nd*	122.4
Enniatin B	0.10	125.2	43.3	5.8	65.3	53.3	92.8	52.9	0.4	9.0	*nd*	*nd*	116.1
Enniatin B1	0.10	263.0	41.8	5.5	72.1	32.3	42.8	64.0	0.5	9.9	*nd*	*nd*	238.2
Fumonisin B1	0.10	0.3	0.7	18.9	*nd*	4.0	*nd*	*nd*	*nd*	*0.4*	92.8	*nd*	1.7
Fumonisin B2	0.10	0.1	*nd*	3.1	*nd*	0.8	*nd*	0.2	*nd*	*nd*	16.0	*nd*	0.3
HT-2 Toxin	0.25	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	3.9	*nd*	*nd*	*nd*
Ochratoxin A	0.06	0.1	*nd*	*nd*	0.1	*nd*	0.2	2.8	*nd*	*nd*	*nd*	*nd*	0.1
Roquefortine	0.10	*nd*	0.3	0.3	0.2	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Sterigmatocystin	0.10	*nd*	0.1	0.4	0.2	*nd*	10.7	*nd*	*nd*	*nd*	*nd*	0.1	0.2
Zearalenone	0.20	*nd*	1.6	84.0	*nd*	4.9	31.2	*nd*	6.1	*nd*	*nd*	*nd*	*nd*
Number of mycotoxins found		*8*	*10*	*12*	*8*	*9*	*8*	*7*	*6*	*8*	*3*	*6*	*8*

**Concentration determined against a solvent calibration series.*

Fewer mycotoxins were detected in the silage samples (ranging from 0 to 3 of the 33 potential mycotoxins monitored for) by comparison with the compound feed samples. Figure 4 shows the chromatographic separation achieved for an extract of spiked silage following 1:10 dilution. The calculated concentrations ranged from circa 20 to 260 µg kg^{-1} The recovery values determined from the spiked control sample ranged from 89% to 108% for 17 mycotoxins with a mean value of 96%.

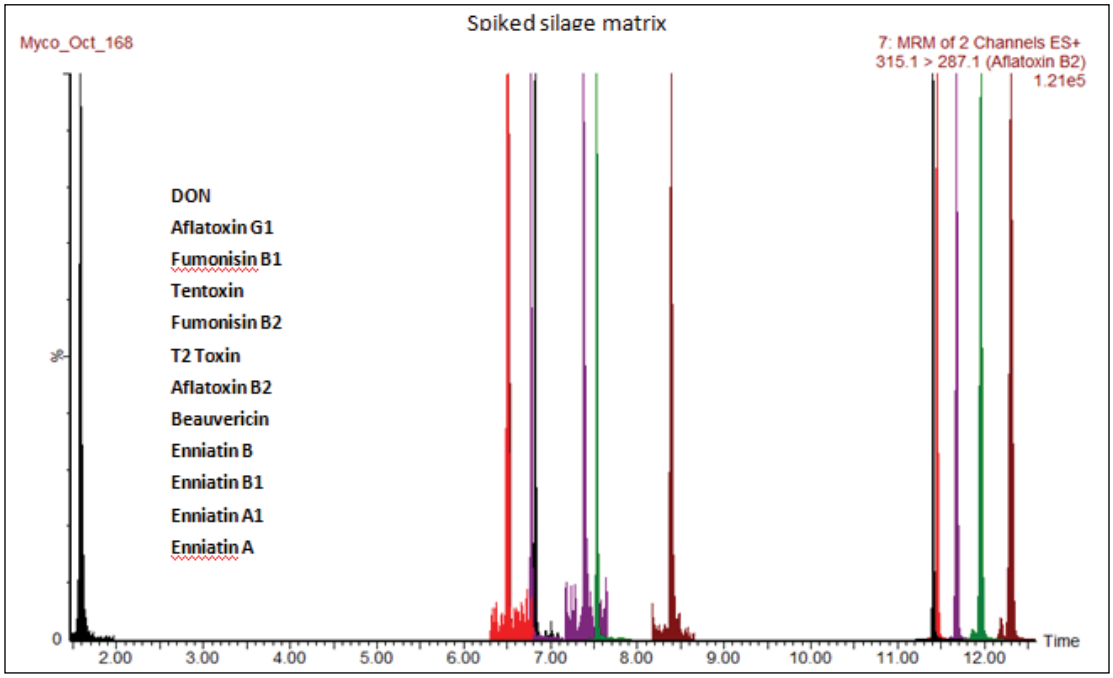

Figure 4. Chromatographic separation achieved for a spiked silage extract using the ACQUITY UPLC I-Class System with Xevo TQ-S.

Table 5. The measured concentrations and %recovery values obtained for a range of mycotoxins identified using two MRM transitions in 10 different samples of silage diluted 1:10 prior to analysis on the Xevo TQ-S.

Mycotoxin	LOD (ng/g)	Measured concentration in silage samples diluted 1:10 (ng/g)*										
		Silage sample identity										
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Spike control %recovery
Aflatoxin B1	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	95
Aflatoxin B2	0.2	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	104
Aflatoxin G1	0.6	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	93
Aflatoxin G2	0.4	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	94
DON	0.9	958.1	1310.0	158.4	550.8	435.4	359.8	216.4	*nd*	261.7	*nd*	100
Enniatin A	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	94
Enniatin A1	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	94
Enniatin B	0.1	*nd*	*nd*	64.0	72.4	50.9	108.4	204.1	*nd*	82.7	*nd*	89
Enniatin B1	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	94
Fumonisin B1	2.0	*nd*	*nd*	*nd*	256.3	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	93
Fumonisin B2	0.4	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	95
HT-2 Toxin	0.8	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	36.2	*nd*	*nd*	*nd*	97
Ochratoxin A	0.8	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	103
T2 Toxin	5.0	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	96
Zearalenone	2.5	170.2	140.2	108.4	42.5	*nd*	84.2	76.1	*nd*	76.3	*nd*	108
Beauvericin	0.2	*nd*	72.5	23.2	*nd*	*nd*	28.4	22.5	*nd*	*nd*	*nd*	96
Tentoxin	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	92
Number of mycotoxins found		2	3	4	4	2	4	5	0	3	0	17

CONCLUSIONS

- A quantitative LC-MS/MS method applicable for the simultaneous determination of 33 mycotoxins in animal feedingstuffs and silage has been developed with the Waters ACQUITY UPLC I-Class System coupled to the Xevo TQ-S using the Quanpedia database.
- Due to the enhanced sensitivity of the TQ-S instrument it has been possible to incorporate a simple extract dilution step prior to analysis to reduce the complex matrix effects associated with these challenging samples and achieve good method repeatability and accuracy.
- The observed intra-day repeatability (%RSD) was found to be ≤22%, and the mean recovery for 17 mycotoxins in silage was found to be 96%.
- The optimized method has been used to identify the presence and determine the concentrations of a range of mycotoxins present in a wide variety of samples.

Acknowledgments

RIKILT, Institute of Food Safety, Wagnenigen, The Netherlands and the Ghent University, Ghent, Belgium for the provision of the animal feed and silage extracts and analytical standards.

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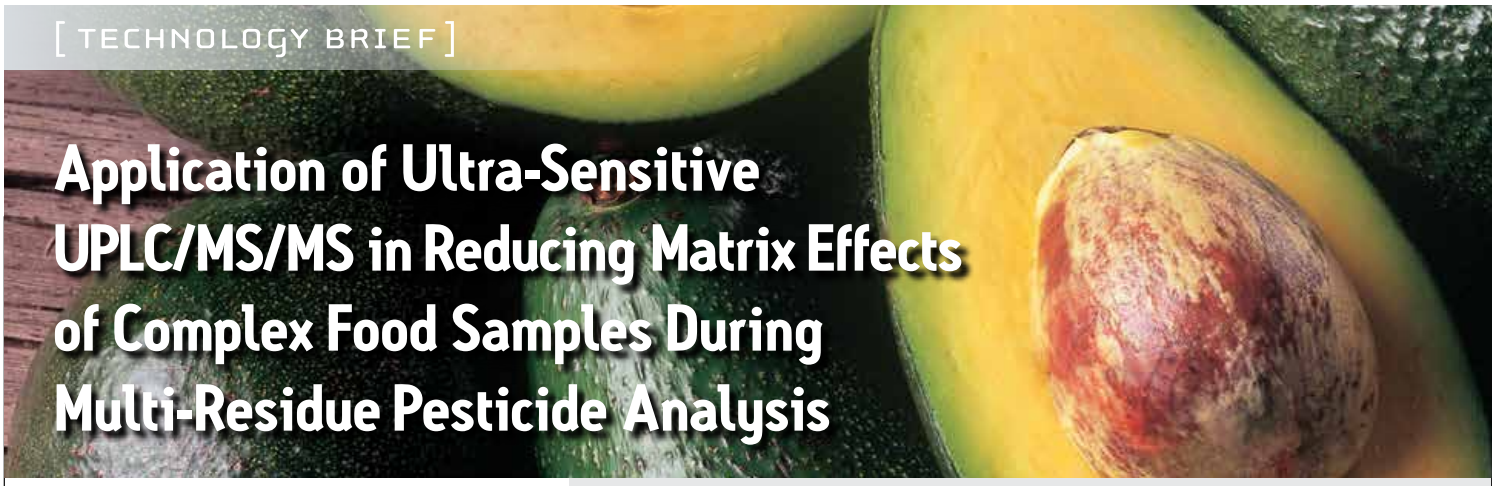

Application of Ultra-Sensitive UPLC/MS/MS in Reducing Matrix Effects of Complex Food Samples During Multi-Residue Pesticide Analysis

GOAL

To utilize the ultra-sensitive detection of pesticides to reduce matrix effects in complex food samples.

BACKGROUND

One of the biggest challenges in ensuring the safety of our food supplies is measuring hazardous ultra-trace components in the presence of a highly complex sample matrix. For the analysis of pesticides in food matrices, the increased use of liquid chromatography systems coupled with tandem quadrupole mass spectrometers has allowed progress in reducing the problems caused by the sample matrix. However, difficulties remain when trying to discriminate against matrix components that exhibit similar physiochemical properties.

Problems caused by matrix can include disruption to chromatography, increased chemical noise, and ion suppression. In complex matrices, such as spices, these problems can be found in combination, making determinations of pesticide residue concentrations very difficult.

In addition to problems caused by the sample matrix, there are also pesticides that by nature are more difficult to analyze using LC/MS/MS due to a poor (relative) response factor. Successful analysis of these compounds to the regulatory concentration limits is difficult when considering the practicality of increasing sample amount and the balance of extracted matrix concentration. A much more practical solution is to use a more sensitive instrument to maximize performance at these required concentrations.

With Xevo TQ-S, ultra-sensitive analysis allows detection of the most difficult compounds and limits the impact of the sample matrix.

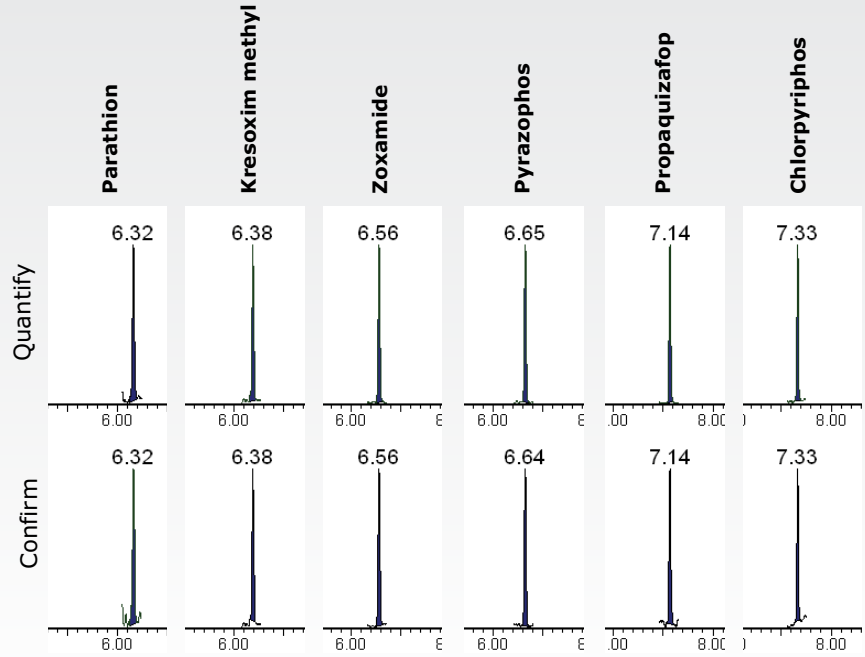

Figure 1. Avocado spiked to 0.005 mg/kg with extracted MRM chromatograms for a selection of pesticides. Both quantitative and confirmatory transitions are detected. This concentration equates to 10 times below the European MRL except zoxamide, which is 4 times below.

In this technical brief, we describe the use of Waters® Xevo™ TQ-S for the ultra-sensitive detection of multiple pesticide residues in food samples, and the application of that sensitivity to matrix reduction strategies.

THE APPROACH

The Waters DisQuE™ Dispersive SPE Kit and ACQUITY UPLC® System, coupled with Xevo TQ-S were applied to the high sensitivity determination of multiple pesticide residues in grape, avocado, marjoram, and ginger. Typical reversed-phase conditions were used for the ACQUITY UPLC separations with formic acid modified mobile phase to aid positive ion electrospray. Multiple reaction monitoring (MRM) mass spectrometer parameters were generated by the Waters Quanpedia™ Database for the simultaneous determination of 86 pesticides. Xevo TQ-S was operated with RADAR,™ an information-rich acquisition approach that allows you to track your target analytes with extreme precision in MRM mode, while simultaneously scanning the background for all other components.

For a laboratory to report results around the European Maximum Residue Limits (MRLs) it is imperative that high quality measurements are possible even for the most challenging compounds. Comfortable quantitation and confirmation below target MRLs allow high confidence in the analytical data produced, if samples are encountered that are close to the limit. Figure 1 shows quantitative and confirmatory MRM chromatograms for a selection of pesticides (including some challenging compounds) in avocado spiked at 0.005 mg/kg. This concentration is equivalent to 10 times below the European MRL, except zoxamide, which is four times below.

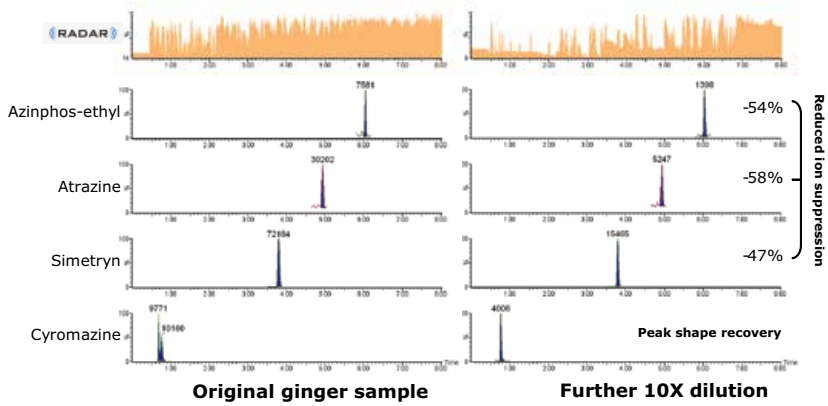

Figure 2. RADAR full scan chromatograms with selected pesticide MRM chromatograms for a ginger sample (0.1 g/mL matrix) at 0.01 mg/kg and a subsequently 10 times diluted sample.

For particularly challenging matrices the mass of the sample taken for extraction can be limited for practical reasons, as well for controlling the complexity of the co-extracted matrix. Some matrices require ten times less sample to be extracted which means that higher sensitivity is required from the analytical system to compensate.

A good example of this problem is the food commodity ginger. The matrix complexity is very high, even when using relatively low matrix concentrations (0.1 g/mL as opposed to the usual 1 g/mL using QuEChERS). The matrix is complex enough that problems with pesticide chromatography and ion suppression can be observed. However, with ultra-sensitive detection, complex samples can be diluted to reduce matrix problems while maintaining detection. Figure 2 shows RADAR full scan chromatograms with selected pesticide MRM chromatograms for a ginger sample at 0.01 mg/kg and a subsequently 10 times diluted sample. Matrix reduction is clearly visible in the RADAR traces and as a consequence, ion suppression is reduced and peak shape recovery is possible.

SUMMARY

The ultra-sensitive pesticide analysis capability of Xevo TQ-S minimizes the impact of more challenging matrices that laboratories may face. This extra sensitivity also allows a better chance of detecting the most challenging compounds in these matrices. In addition, large multi-residue methods are possible without compromising required detection levels. This can ultimately lead to continuous improvement in sample turnaround, quality, and performance that translates to business activities reliant on the laboratory.

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Meeting Challenging Requirements for the Quantitation of Regulated Growth Promoters Dexamethasone and Betamethasone in Liver and Milk

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APPLICATION BENEFITS

- Highly selective and sensitive analysis for dexamethasone and betamethasone in complex matrices.
- European MRLs and confirmation requirements (2002/657/EC) can be comfortably achieved using Xevo TQ MS.
- The high chromatographic resolution of the ACQUITY UPLC System facilitates the critical separation of the epimers dexamethasone and betamethasone.
- Additional spectral information can be obtained by data directed Product ion confirmation scan (PICS), which could help confirm the presence of banned substances.

WATERS SOLUTIONS

ACQUITY UPLC with Xevo TQ MS

KEYWORDS

Glucocorticoids, epimeric compounds, veterinary drugs, food safety

INTRODUCTION

Ensuring consumer safety is a priority for governments, international regulatory bodies and organizations that process and handle products prior to consumption. Food safety issues arising from commodity products often become globally reported and have the potential to impact consumer confidence and trade at international levels.

Dexamethasone and betamethasone are synthetic glucocorticoids widely used in animal husbandry[1]. These epimeric compounds are licensed for therapy in veterinary practice, while their use as growth promoters is banned within the European Union (corticosteroids are listed in Annex I of European Council 96/23 - group B2f)[2].

In order to protect consumer safety, Maximum Residue Limits (MRLs) have been fixed for both molecules by the European Community in several matrices, for instance: 0.3 µg/L (ppb) in bovine milk and 2.0 µg/kg (ppb) in liver from different species[3].

The major challenge in the analysis of dexamethasone and betamethasone consists of performing an efficient separation of both epimers and detecting and identifying these molecules at the required maximum residue limit (MRL). Malone *et al.*[4] and Li *et al.*[5] have illustrated efficient methods for the separation of both epimers.

This application note describes the use of Waters® Xevo™ TQ MS for the high sensitivity determinations of dexamethasone, at the European MRL level in food. In addition, the use of PICS acquisitions for further presence confirmation is explored.

EXPERIMENTAL

Sample preparation

Sample extraction and purification procedure in liver is described in detail elsewhere[6]. Initial sample preparation for milk included a protein precipitation step which then followed the same procedure as liver (Figure 1).

LC conditions

LC system:	ACQUITY UPLC® System
Runtime:	7.0 min
Column:	ACQUITY® UPLC BEH C_{18} Column 1.7 µm, 2.1 x 100 mm
Mobile phase A:	0.5% acetic acid dissolved in water
Mobile phase B:	acetonitrile
Flow rate:	0.6 mL/min
Injection volume:	2 µL

MS conditions

MS system:	Xevo TQ MS
Ionization mode:	ESI negative
Capillary voltage:	3 kV
Source temp:	150 °C
Desolvation temp:	500 °C
Desolvation gas:	920 L/H
Collision gas flow:	0.15 mL/min

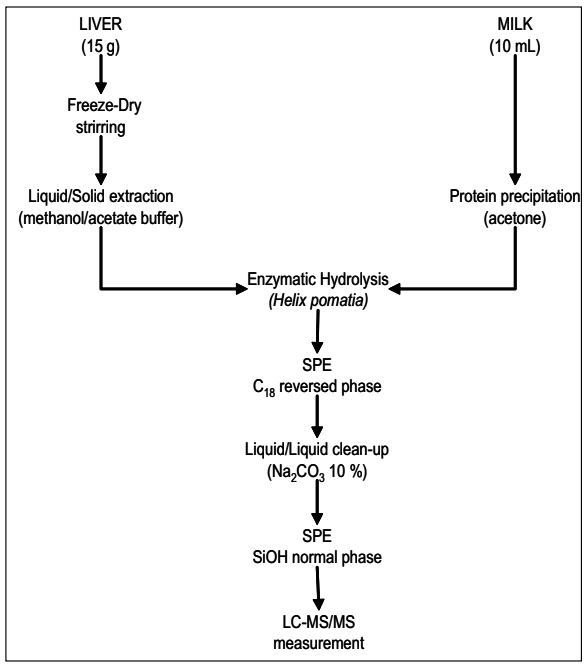

Figure 1. Overview of sample preparation for liver and milk samples.

	Time (min)	Flow rate (mL/min)	%A	%B
1.	Initial	0.6	75	25
2.	4.0	0.6	75	25
3.	4.3	0.6	0	100
4.	5.0	0.6	0	100
5.	5.1	0.6	75	25
6.	7.0	0.6	75	25

MRM method parameters

Diagnostic MRM transitions were first generated using Waters' IntelliStart™ Technology. All the parameters (detailed in the following table) were then optimized individually for each diagnostic signal.

Compound name	Parent (m/z)	Daughter (m/z)	Dwell (s)	Cone (V)	Collision (eV)
Fludrocortisone (2nd internal standard)	349.2	295.1	0.094	40	22
		313.2	0.094	40	20
Fluorometholone (external standard)	355.2	255.1	0.094	34	14
d4-dexamethasone (1st internal standard)	363.2	309.1	0.094	40	20
		327.2	0.094	40	18
Dexamethasone and betamethasone	451.2	361.2	0.094	20	18
		307.2	0.094	20	30
	361.2	307.2	0.094	40	18
		325.2	0.094	40	20

RESULTS AND DISCUSSION

Critical separation of dexamethasone from betamethasone

In order to obtain accurate determinations for these particular growth promoters, it is essential that chromatographic separation of the epimers dexamethasone and betamethasone is achieved. An efficient separation of betamethasone (t_R=3.14 min) from dexamethasone (t_R=3.25 min) was observed using isocratic gradient in the range [0-4 min]. These UPLC® conditions allow identification of molecules from their relative retention time (Figure 1).

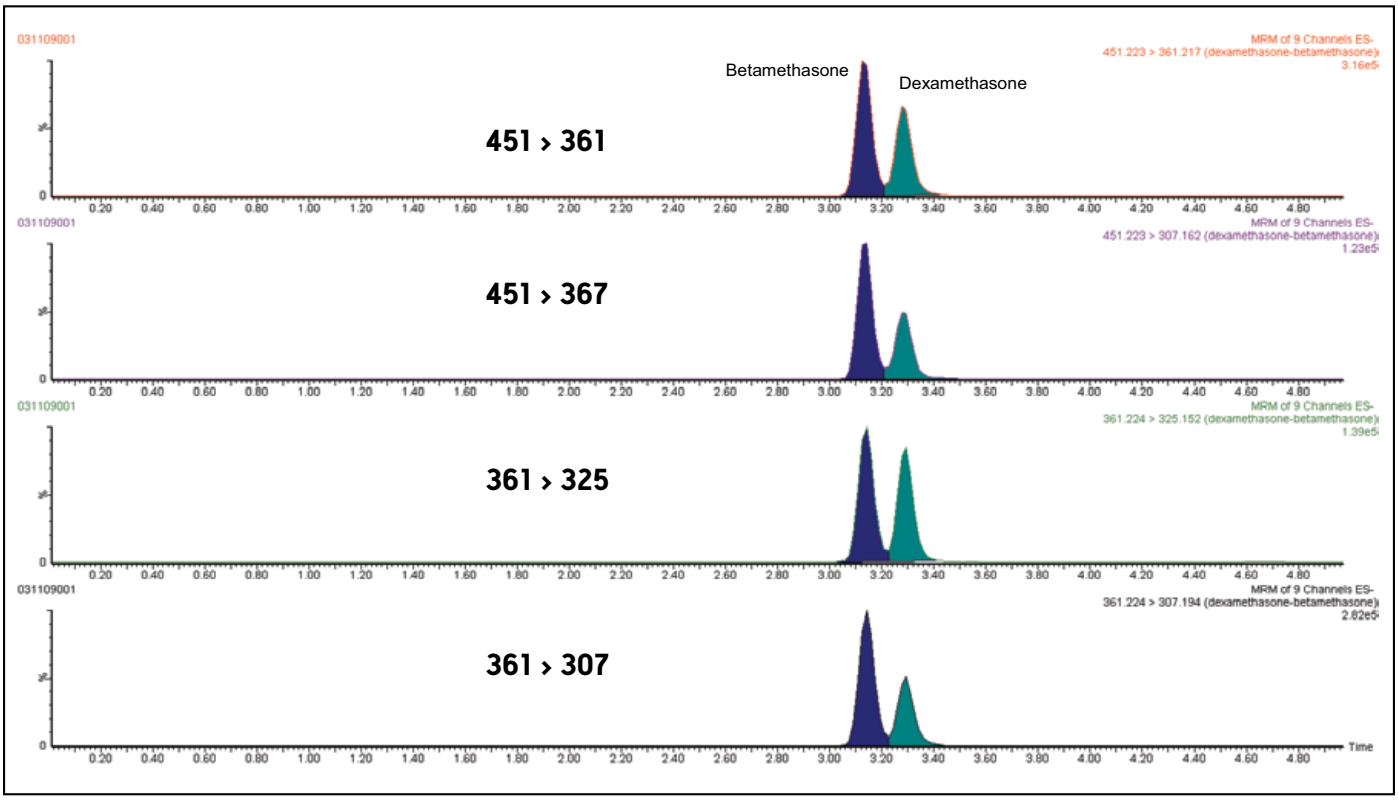

Figure 2. UPLC (ESI-)/MS/MS MRM diagnostic signals of dexamethasone, betamethasone (451 > 361, 361 > 307, 361 > 325, and 451 > 307) obtained from a standard solution at 1 ng/mL.

Regulatory target analysis of dexamethasone and betamethasone in milk

Four diagnostic MRM transitions were set for the identification of dexamethasone and betamethasone in order to fulfil the regulatory requirements of the European Commission Decision 2002/657:EC[7]: these four diagnostic transitions (451 > 361, 361 > 307, 451 > 307, and 361 > 325) were selected in the MRM transition mode in order to perform unambiguous identification of the compounds. Moreover, d_4-dexamethasone (363 > 309, t_R= 3.56 min) was used as internal standard because of its mimetic properties with dexamethasone.

Blank milk sample chromatograms are shown in Figure 3a with no positive response for dexamethasone and betamethasone at the expected retention time. This demonstrates the selectivity of the methodology with the combination of chromatographic resolution and instrumental selectivity. Extracted MRM chromatograms corresponding to milk samples fortified at 0.3 µg/L (MRL) and 0.075 µg/L (4 times below MRL) are shown in Figures 3b and 3c. High instrument sensitivity allows highly confident identification of both substances to be performed (Identification points=10), even at concentrations that are 4 times below the European MRL.

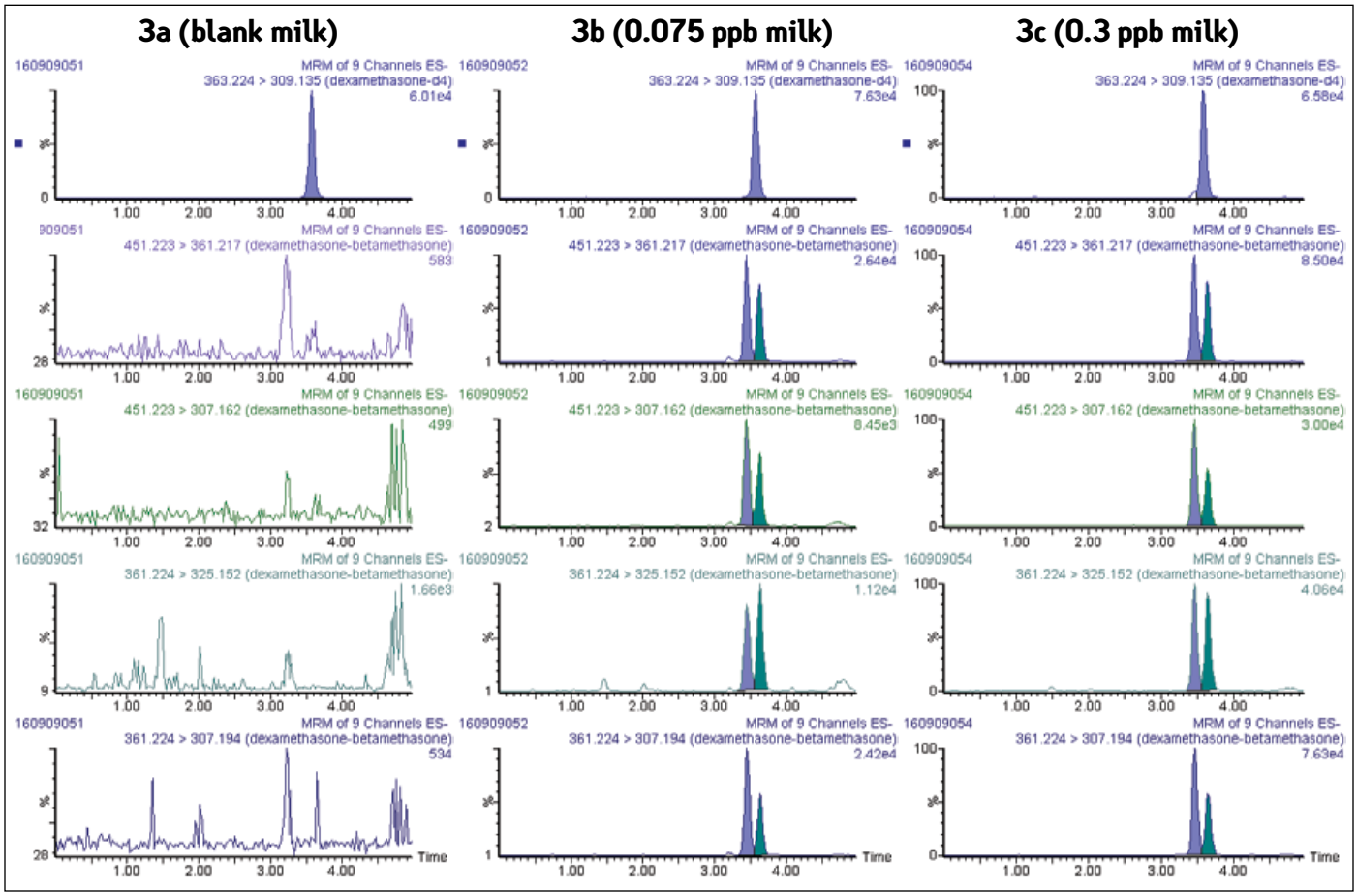

Figure 3. UPLC (ESI-)/MS/MS SRM diagnostic signals of dexamethasone, betamethasone (451 > 361, 361 > 307, 361 > 325 and 451 > 307) and d_4-dexamethasone (363 > 309) obtained from a blank milk sample (3a) and spiked milk samples: 0.075 ppb (3b), and 0.3 ppb (3c).

Regulatory target analysis of dexamethasone in liver

To test performance around the regulatory limits, fortified liver samples were analyzed at twenty times below, four times below, and at the European MRL for dexamethasone (2.0 ppb). The extracted MRM chromatograms for each level are shown in Figure 3. Positive identification according to regulatory requirements (2002/657/EC) was comfortably achieved with 10 identification points (IP=10) for concentrations around the MRL (3 IPs are mandatory with 4 IPs reserved for illegal substances). As with previous determinations in milk, the method selectivity was such that the blank liver samples did not show any response (were s/n > 3).

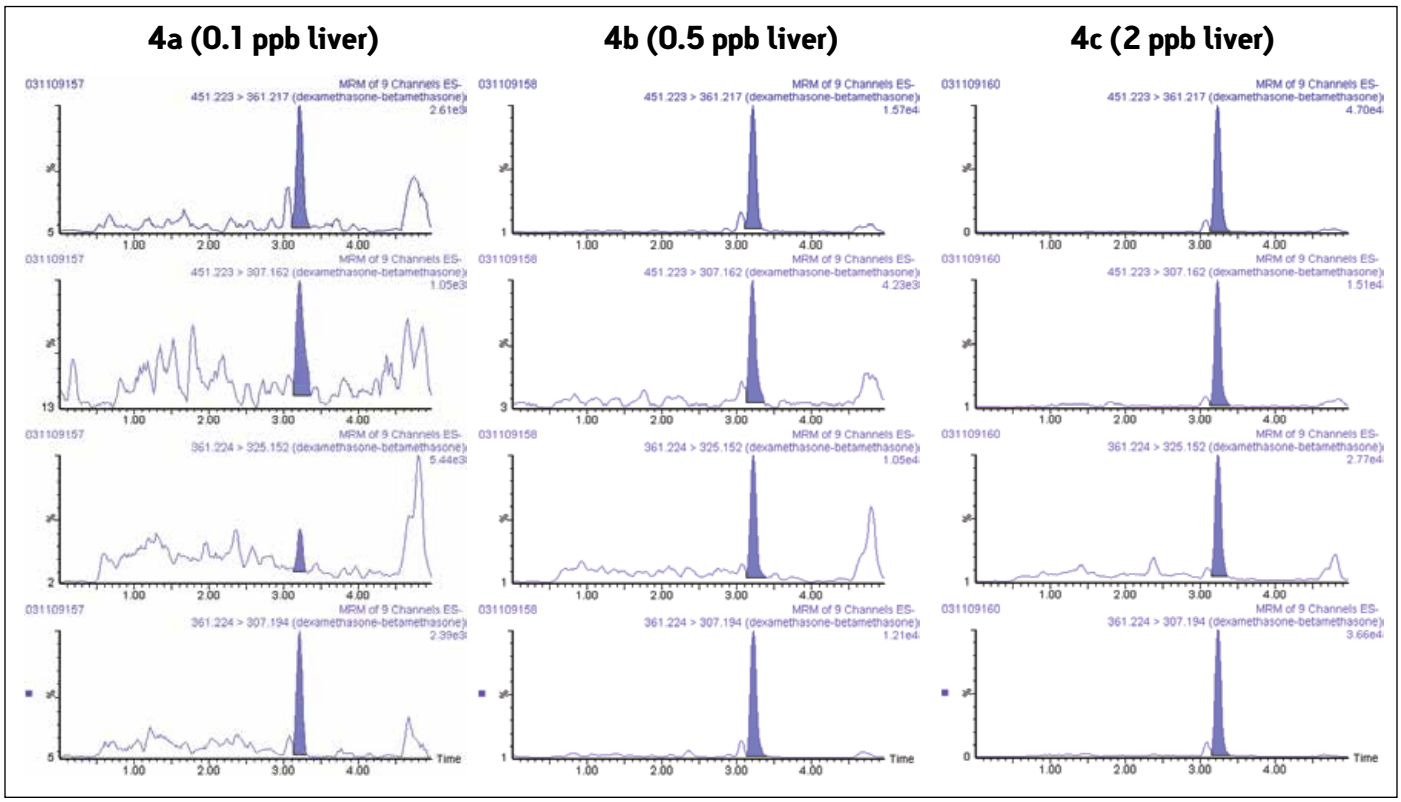

Figure 4. UPLC (ESI-)/MS/MS MRM diagnostic signals of dexamethasone, (451 > 361, 361 > 307, 361 > 325 and 451 > 307) obtained from the following various spiked liver samples: 0.1 ppb (4a), 0.5 ppb (4b), and 2.0 ppb (4c).

Method validation

The stability of the transition ratios was also evaluated in the different matrices for all concentrations. Signals obtained were very repeatable even at very low concentration levels (MRL/4 for milk: 0.075 ppb and MRL/20 for liver: 0.1 ppb) with an unambiguous identification of the compound (IP=10).

Concerning the liver matrix, linearity was evaluated by plotting relative peak height ratios in the range [0 to 10 µg/kg]. The correlation coefficients of the calibration curve were above 0.99. At the MRL level, the relative standard deviations for signal responses were calculated to 6.4% and 6.1% for betamethasone and dexamethasone. At the same level, the relative standard deviations for relative retention times were 0.16% and 0.20% for betamethasone and dexamethasone respectively.

CCα (decision limit) and CCβ (detection limit) were determined in accordance with European Decision 2002/657. CCα were 2.31 and 2.35 while CCβ were 2.57 and 2.63 for dexamethasone and betamethasone respectively.

Additional residue confirmation using Product Ion Confirmation Scan (PICS)

Data directed acquisition allows spectral data to be collected when a particular parameter is detected above a certain threshold. Very rapid switching between quadrupole static mode and scanning mode is essential for this to happen in real time whilst a chromatographic peak is eluting. This capability allows additional information when trying to confirm presence of residues in a sample and is an additional step that can be taken to help identify and investigate false positive results.

Product ion confirmation scan (PICS) allows a product ion scan to be acquired which is triggered by a selected MRM transition reaching a critical threshold. This capability is especially relevant when applied to supporting the identification of a target substance. Figure 4 shows extracted MRM chromatogram for dexamethasone at European MRL in a liver sample which has PICS enabled. Figure 4 also shows the product ion spectrum from the automatically triggered scan from the 361 > 307 transition. This spectrum can then be compared to an MS/MS library to provide additional evidence for presence of dexamethasone.

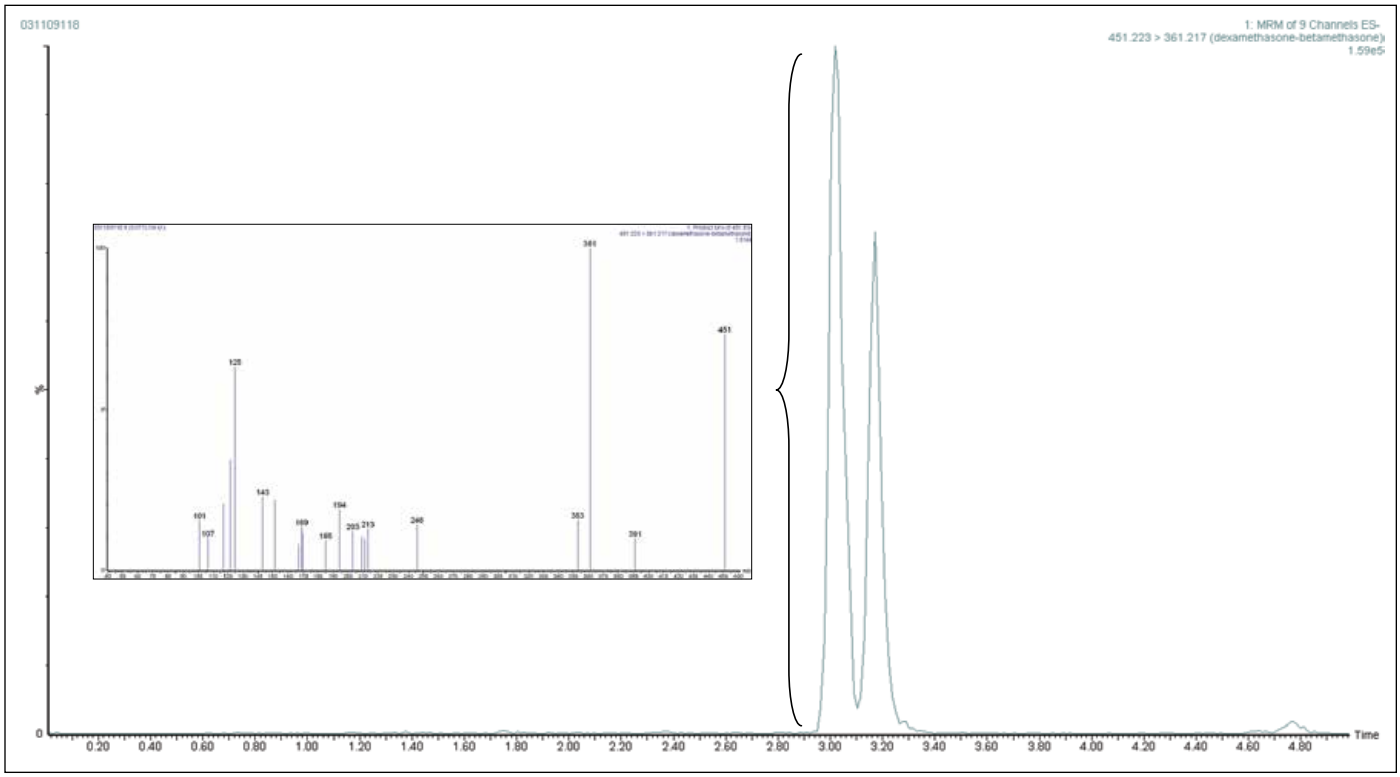

Figure 5. UPLC (ESI-)/MS/MS MRM for dexamethasone (451 > 361) with triggered ScanWave™ product ion scan spectrum for a liver sample spiked at twice the MRL (4 ppb).

CONCLUSIONS

- Xevo TQ MS allowed highly selective and sensitive analysis for dexamethasone and betamethasone in complex matrices.
- European MRLs and confirmation requirements (2002/657/EC) were comfortably achieved using Xevo TQ MS.
- The high chromatographic resolution of the ACQUITY UPLC System facilitated the critical separation of the epimers dexamethasone and betamethasone.
- Additional spectral information can be obtained by data directed PICS which in turn could help confirm the presence of banned substances

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Routine UPLC-MS/MS Quantification of Pesticide Residues in Okra with Simultaneous Acquisition of Qualitative Full-Spectrum MS and MS/MS Data

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APPLICATION BENEFITS

- Multiple pesticide residues can be detected simultaneously at legislative limits in okra samples using the ACQUITY UPLC® H-Class System coupled with Xevo® TQD MS.
- Quantitative and qualitative information can be achieved in a single injection.
- RADAR™ technology enables simultaneous full-scan data to be acquired, providing important information on matrix background ions that could potentially interfere with the analysis.
- PICS (product ion confirmation scan) provide additional confirmation for compound identification through acquisition of MS/MS spectra in the same injection.

WATERS SOLUTIONS

ACQUITY UPLC H-Class System

Xevo TQD

ACQUITY UPLC HSS T3 Column

MassLynx® Software

KEY WORDS

Pesticides, okra, QuEChERS, food safety

INTRODUCTION

Okra is an important vegetable of the tropical countries and a popular diet component in several countries including India. According to the Food and Agriculture Organization of the United Nations (FAO),[1] India is one of the largest okra producers in the world and it produced approximately 5,800 tons of okra in 2010 and 2011. Okra is susceptible to a variety of pests and diseases[2] and a wide-range of pesticides are used to treat okra plants in India. Legislative limits are in place for the presence of pesticides in domestically produced, imported, or exported okra.[3] It is, therefore, very important to monitor the presence of commonly used pesticides in okra at legislative limits.

According to the PRiF (Pesticide Residues in Food) report, import controls under regulation (EC) No 669/2009 have been increased for okra imported from India because of the frequent detection of pesticide residues, mainly monocrotophos. The consignment is supposed to be rejected if it is non-compliant with MRLs (Maximum Residue Limits). Since July 1, 2012, the frequency of testing consignments has been increased from 10% to 50%. With this frequent testing, monocrotophos, triazophos, and acetamiprid were found at 0.02 mg/kg in okra samples from India, while the MRL for these compounds is 0.01 mg/kg.[4]

In this application note, a multi-residue analysis method for the detection of 212 pesticides in okra is presented. For a complete list of all pesticides, see Appendix A.

Methods

A multi-residue MS method for the pesticides was created using Waters® Xevo TQD Quanpedia™ database. All of the pesticides were analyzed under ESI+ or ESI- mode using rapid polarity switching. Full-scan data were acquired in order to assess any matrix effects and the use of two MRMs and product ion confirmation scans were acquired to confirm and quantify the pesticide residues.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC H-Class
Column:	ACQUITY HSS T3 2.1 X 100 mm, 1.8 µm
Column temp.:	45 °C
Injection volume:	10 µL
Flow rate:	0.45 mL/min
Mobile phase A:	10 mM ammonium acetate (pH 5) in water
Mobile phase B:	10 mM ammonium acetate (pH 5) in methanol
Weak needle wash:	50/50 Water/methanol (v/v)
Strong needle wash:	10/90 Methanol/water (v/v)
Seal wash:	90/10 water/methanol

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.450	98	2	6
0.25	0.450	98	2	6
12.25	0.450	1	99	6
13.00	0.450	1	99	6
13.01	0.450	98	2	6
17.00	0.450	98	2	6

Table 1. UPLC method for pesticide analysis.

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI+/ESI-
Capillary voltage:	3 kV
Desolvation temp.:	500 °C
Desolvation gas flow:	1000 L/Hr
Source temp.:	150 °C

Standard preparation

Pesticide standards were purchased either from Sigma-Aldrich, Fisher Scientific, or AccuStandard. A mix of all pesticides at 400 ng/mL was prepared in acetonitrile and stored at 4 °C.

Sample preparation

QuEChERS is a popular method worldwide for the multi-residue analysis of pesticides in fruits and vegetables. The AOAC official method 2007.01, was used to prepare okra samples that were purchased at a local supermarket. Briefly, okra samples were homogenized in water and 15 grams of homogenate was collected into a 50-mL centrifuge tube. Samples were extracted with acidified acetonitrile and mixed with $MgSO_4$ and NaCl (Tube 1). The tube was shaken for a minute and centrifuged at 1500 rcf for 1 minute. After centrifugation, the matrix cleanup was accomplished by dispersive solid phase extraction (d-SPE) by using 50 mg of primary secondary amine (PSA), 50 mg of C_{18} bonded silica, 150 mg of $MgSO_4$, and 7.5 mg of graphitized carbon black (GCB).[5] 1 mL of supernatant from Tube 1 was added to d-SPE cleanup tube and centrifuged at 1500 rcf for 1 minute. 1 mL of this extract was evaporated to dryness and reconstituted in 200 µL of 40/60 acetonitrile/water spiked with internal standard.

RESULTS AND DISCUSSION

All of the pesticides were successfully detected at 10 ppb (0.01 mg/kg) in okra sample. For all of the pesticides, Appendix A lists the ionization mode, retention time, and whether or not the compound was detected in a pre-spike 1 ppb sample, as well as the 10 ppb pre-spike sample. Figure 1 shows an overlay of the total ion chromatogram (TIC) of all the pesticides at 10 ppb in okra sample.

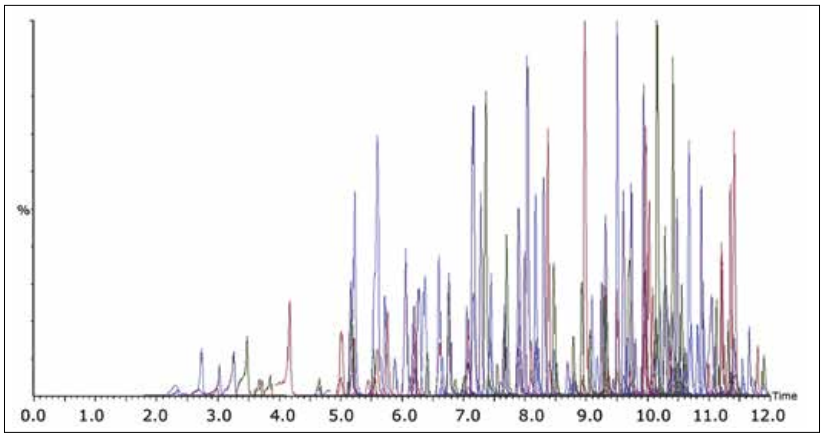

Figure 1. Overlay of MRM chromatograms of all pesticides at 10 ppb (0.01 mg/kg) in okra.

Solvent and matrix match spiked calibration (MMS) curves were prepared at concentrations that equated to the range 1 ppb to 50 ppb (*i.e.* 0.001 to 0.05 mg/kg of okra) and injected in triplicate. The majority of the compounds showed linearity with R^2 values greater than 0.99 in both the solvent and MMS curves. Ethoxyquin, milbemectin A3, and A4, oxadiazon, spiromesifen, and terbufos showed R^2 values greater than 0.970 for both solvent and MMS curves. However, fipronil, phorate, and thiabendazole showed R^2 values greater than 0.970 in MMS curves only. Figures 2 and 3 show calibration curves and residuals for an example compound (triazophos) in solvent and matrix respectively.

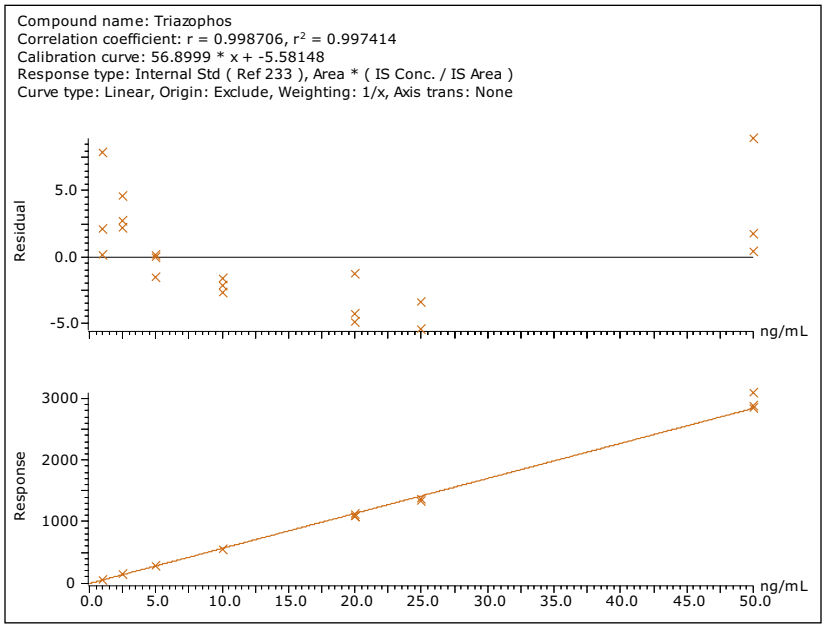

Figure 2. Calibration curve of triazophos in solvent from 1 ppb to 50 ng/mL (0.001 to 0.05 mg/kg).

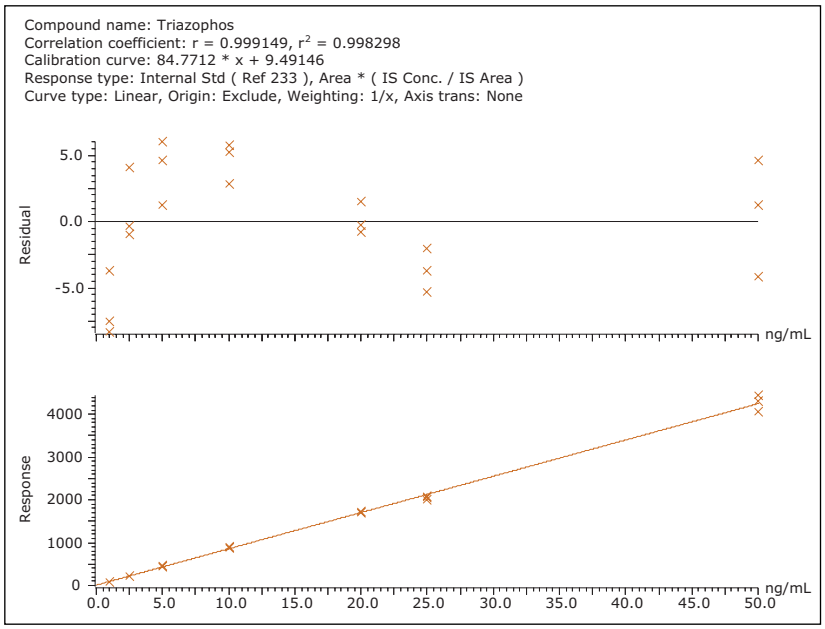

Figure 3. Matrix-match spiked calibration curve of triazophos in okra sample from 1 ppb to 50 ppb (0.01 to 0.05 mg/kg).

To evaluate the recovery, accuracy, and precision of the method, studies were carried out on spiked samples. Okra samples were pre-spiked with all the pesticides at 10 ppb (0.01 mg/kg) in triplicate, extracted, and quantified against the MMS calibration curve. Recoveries were calculated using TargetLynx™ Software. The recoveries reported are without any internal standard correction. As shown in Figure 4 (A, B, C, and D), recoveries for all of the pesticides ranged from 25% to 150%. Relative standard deviations (RSDs, shown as error bars in Figure 4) for most compounds were <20%. The RSDs for 34 compounds were found to be higher than 20%. Use of an internal standard would be likely to significantly improve repeatability for those analytes.

Figure 4. %Recovery for 212 pesticides in okra sample at 10 ppb (0.01 mg/kg).

Matrix effects

Matrix effects for all of the pesticides were calculated by taking the ratio of the slope of the MMS calibration curve to the slope of solvent calibration curve. A percent variation of + 20% was considered as no matrix effect as this variation is close to the repeatability values.[6] Values between + 20% to + 50% were considered as a medium matrix effect, and a strong matrix effect was considered to be values greater than + 50%.[7] Figure 5 shows levels of the matrix effect that were observed in the analysis of okra for all pesticides. A strong matrix effect was observed for the majority of compounds, demonstrating that the analysis of okra samples poses a challenge in regards to high matrix complexity. Even with these high matrix effects, all compounds can easily be detected at legislative limits and quantified using the matrix-matched calibration curve.

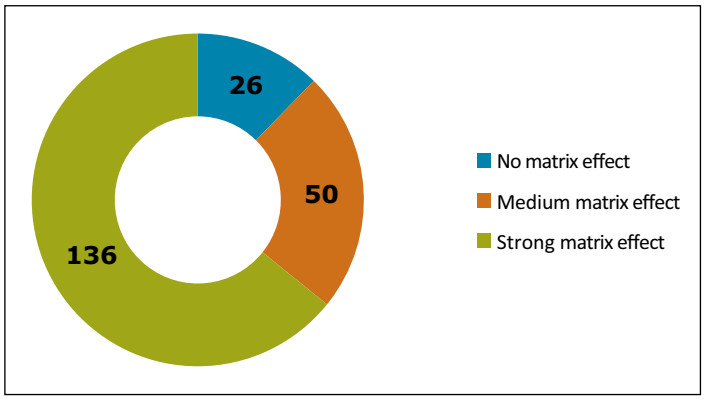

Figure 5. Matrix effects observed for okra sample.

Understanding matrix effects – RADAR

To further understand the impact of co-eluting matrix components that can compete with an analyte of interest during the ionization process, RADAR technology enables the simultaneous acquisition of full spectrum data during quantitative MS/MS analysis. Figure 6 shows an example of the use of RADAR technology. In Figure 6A, the base peak intensity (BPI) chromatogram from the full-scan background data for the okra sample is shown. At 5.08 minutes, close to the retention time of dimethoate (Figure 6B and 6C), high matrix interference was observed. The spectrum at 5.08 minute showed an intense ion at *m/z* 217.1 (Figure 6D).

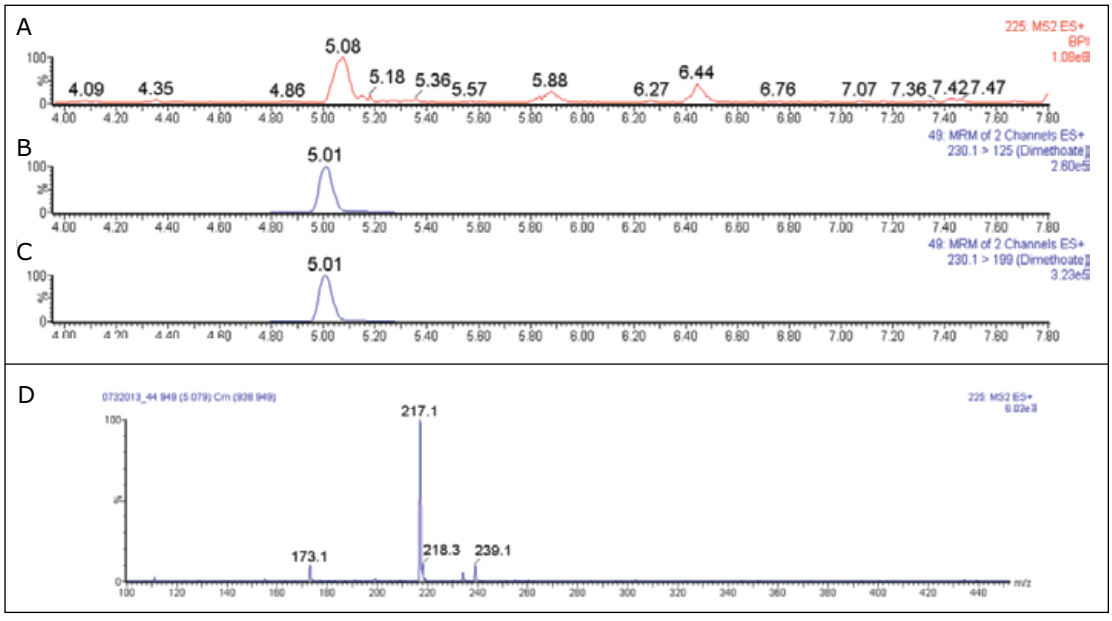

Figure 6. Use of RADAR Technology: (A) Full scan background data for okra sample, (B) and C) MRM transitions of dimethoate, (D) spectrum at retention time of dimethoate.

This interferent potentially has a large impact on the detection of dimethoate and a 48% ion suppression effect was observed for dimethoate. In the case of aldicarb, however, matrix interference was minimal (0.4%) and the RADAR data (Figure 7) showed no evidence of interferences at the retention time of aldicarb (6.13 minutes). The spectrum at the retention time of aldicarb has been expanded and zoomed in the inset (Figure 7D), clearly demonstrating that there was a much higher response from co-extracted matrix ions at the retention time of dimethoate compared to aldicarb. These data clearly demonstrate the usefulness of RADAR technology in assessing the matrix background and its potential effect on ion enhancement or suppression.

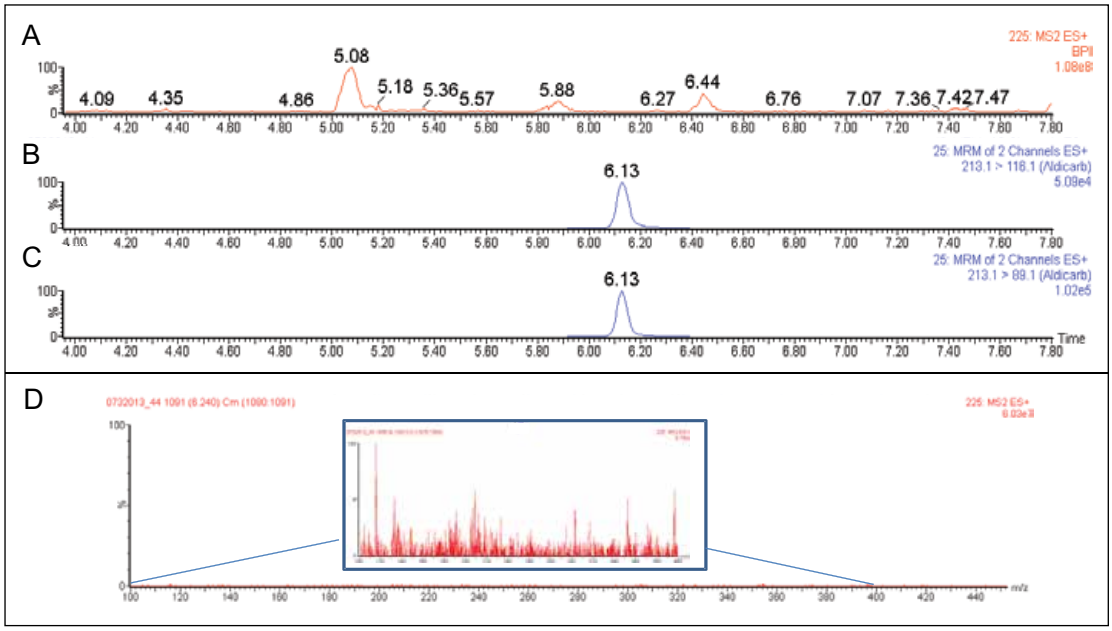

Figure 7. Use of RADAR technology. (A) Full-scan background data for okra sample, (B) and (C) MRM transitions of aldicarb, (D) Spectrum at retention time of aldicarb. The inset has been zoomed to show lower level response compared to the spectrum at the retention time of dimethoate.

Product ion confirmation (PICs)

In complex matrices, situations arise where closely-related compounds such as metabolites or matrix interferences show responses for the target compounds of interest, even in MRM mode. This can lead to ambiguity and may require an additional qualitative experiment. An alternative is to employ a product ion confirmation scan (PICs) within the quantitative MRM experiment. PICs can be used to confirm peak identity through automatic acquisition of an MS/MS spectrum after the apex of the peak has eluted. PICs, in combination with TargetLynx, provides additional confirmation of the compounds of interest through comparison of the acquired MS/MS spectrum to a reference spectrum. Figure 8 shows the TargetLynx results from the comparison of the atrazine MS/MS spectrum obtained from PICS in an okra sample versus the reference spectrum, which was obtained from MS/MS analysis of the standard in solvent.

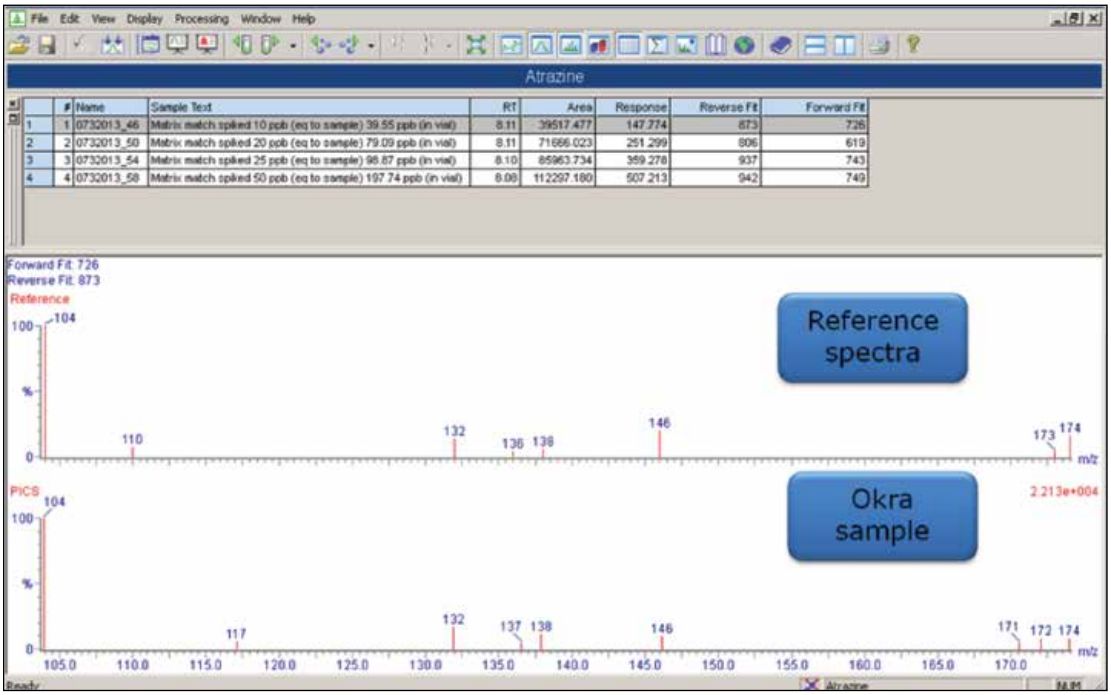

Figure 8. Product ion confirmation (PICs) data for atrazine in okra sample.

CONCLUSIONS

- The combination of ACQUITY UPLC H-Class System with the Xevo TQD tandem mass spectrometer can detect pesticides below the legislative limit in okra samples.
- Even though a strong matrix effect was observed for many compounds, detection and quantification at the legislative limit was achieved.
- Simultaneous acquisition of MRMs and RADAR full-scan data provides quantitative and qualitative information in single injection.
- Product ion confirmation (PICs) increases confidence in compound assignments, which proves highly useful when working with complex matrices.

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Appendix A

In order to determine that the method was fit-for-purpose for the analytes listed, the analysis of pre-spiked samples at 1 ppb (0.01 mg/kg) and 10 ppb (0.01 mg/kg) was undertaken. All compounds were detected at 10 ppb. Those compounds that were also detected at 1 ppb are indicated in the fourth column. Some early eluting compounds showed compromised peak shapes, owing to the sample diluent (40% acetonitrile). Signal-to-noise improvements (and therefore lower LODs) can be gained from reducing the organic content of the sample diluent, however, some risk lies with ensuring that non-polar analytes remain in solution. For this work 40% organic was utilized. Atrazine desethyldesisopropyl, dinotefuran, methamidophos, and oxydemeton methyl showed compromised chromatographic peaks. In addition, for seven compounds, the second transition peak was not apparent at the lowest level. These compounds are shown by an asterisk in the table below.

Name	Ionization mode	Retention time (minute)	Pre-spike level detected
2,4-D	ESI -	6.10	10 ppb
6-Benzyl Adenine*	ESI +	6.63	1 ppb
Acephate	ESI +	2.33	10 ppb
Acetachlor	ESI +	9.80	10 ppb
Acetamiprid	ESI +	5.19	1 ppb
Acifluorfen*	ESI -	8.49	10 ppb
Aldicarb	ESI +	6.16	1 ppb
Aldicarb sulfone	ESI +	3.27	1 ppb
Aldicarb Sulfoxide	ESI +	3.00	1 ppb
Allethrin	ESI +	11.73	1 ppb
Atrazine	ESI +	8.10	1 ppb
Atrazine desethyldesisopropyl	ESI +	1.81	10 ppb
Atrazine desisopropyl	ESI +	4.33	1 ppb
Atrazine-desethyl	ESI +	5.61	1 ppb
Azoxystrobin	ESI +	8.98	1 ppb
Barban/Barbamate*	ESI +	9.25	1 ppb
Bendiocarb	ESI +	7.19	1 ppb
Benalaxyl	ESI +	10.41	1 ppb
Benfuracarb	ESI +	11.20	1 ppb
Bensulfuron methyl	ESI +	8.51	1 ppb
Bifenazate	ESI +	9.53	1 ppb
Bitertanol	ESI +	10.50	1 ppb
Boscalid	ESI +	9.19	1 ppb
Bromacil	ESI +	7.03	1 ppb
Bromodialone	ESI +	10.11	10 ppb
Buprofezin	ESI +	11.37	1 ppb
Butachlor	ESI +	11.43	1 ppb
Carbaryl	ESI +	7.42	1 ppb
Carbendazim	ESI +	5.61	1 ppb

Name	Ionization mode	Retention time (minute)	Pre-spike level detected
Carbofuran	ESI +	7.18	1 ppb
Carbofuran 3 keto	ESI +	6.22	1 ppb
Carbofuran-3-hydroxy	ESI +	5.23	1 ppb
Carbosulfan	ESI +	12.46	1 ppb
Carboxin	ESI +	7.46	1 ppb
Chlorantraniliprole	ESI +	8.73	1 ppb
Chlorfenvinphos	ESI +	10.51	1 ppb
Chlorimuron ethyl	ESI +	7.94	1 ppb
Chlorpyriphos /Dursban	ESI +	11.52	1 ppb
Chlorsulfuron	ESI +	5.46	1 ppb
Clothianidin	ESI +	4.68	1 ppb
Coumachlor	ESI +	8.57	1 ppb
Coumatetralyl	ESI +	7.49	1 ppb
Cruformate	ESI +	9.98	1 ppb
Cyazofamide/ cyazofamid	ESI +	9.81	1 ppb
Cycloxidim	ESI +	10.17	1 ppb
Cymoxanil	ESI +	5.55	1 ppb
Cyprazine	ESI +	8.19	1 ppb
Cyproconazole I	ESI +	9.36	1 ppb
Cyproconazole II	ESI +	9.52	1 ppb
Diafenthiuron	ESI +	11.89	10 ppb
Diazinon	ESI +	10.43	1 ppb
Dichlofluanid	ESI +	9.64	10 ppb
Dichlorvos	ESI +	6.89	10 ppb
Diclofop methyl	ESI +	11.19	10 ppb
Difenconazole I	ESI +	10.35	10 ppb
Difenconazole II	ESI +	10.74	1 ppb
Difenoxuron	ESI +	8.32	1 ppb
Diflubenzuron	ESI +	10.15	1 ppb
Diflufenican	ESI +	10.82	1 ppb
Dimethoate	ESI +	5.04	1 ppb
Dimethomorph I	ESI +	9.09	1 ppb
Dimethomorph II	ESI +	9.29	1 ppb
Diniconazole	ESI +	10.62	1 ppb
Dinotefuran	ESI +	2.99	10 ppb
Dioxathion	ESI +	11.25	1 ppb
Diuron	ESI +	8.20	1 ppb
DMSA	ESI +	6.23	1 ppb
Edifenfos	ESI +	10.28	1 ppb

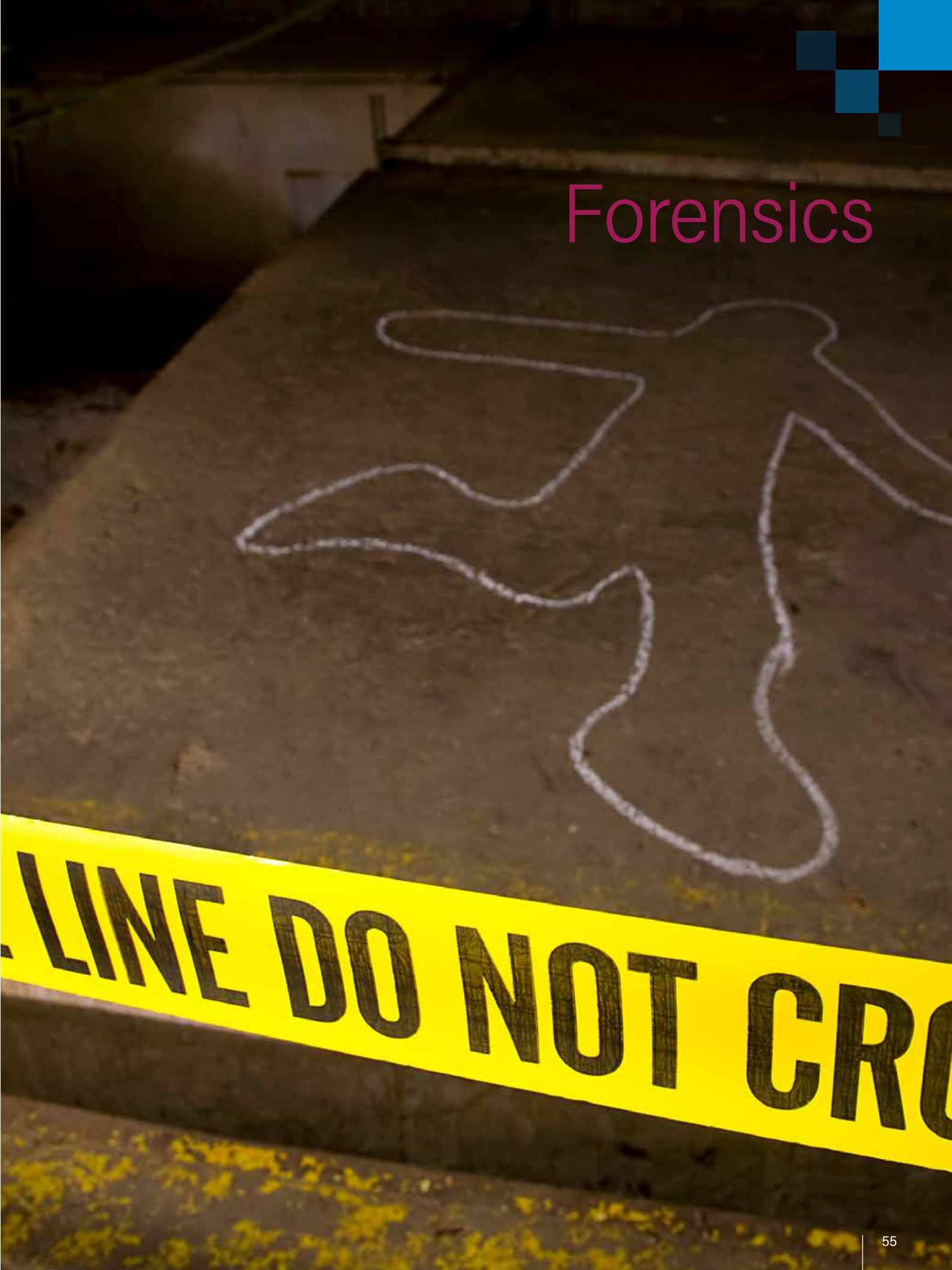
Name	Ionization mode	Retention time (minute)	Pre-spike level detected
Emamectin Benzoate	ESI +	11.81	1 ppb
Ethiofencarb	ESI +	7.68	1 ppb
Ethion	ESI +	11.42	1 ppb
Ethoxyquin	ESI +	9.79	10 ppb
Ethoxysulfuron	ESI +	7.73	1 ppb
Etrimphos	ESI +	10.25	1 ppb
Famoxadone	ESI +	10.37	1 ppb
Fenamidone	ESI +	9.11	1 ppb
Fenamiphos	ESI +	9.97	1 ppb
Fenarimole	ESI +	9.64	1 ppb
Fenazaquin	ESI +	12.11	1 ppb
Fenchlorphos-oxon	ESI +	9.61	10 ppb
Fenobucarb	ESI +	8.79	1 ppb
Fenoxaprop-p-ethyl	ESI +	11.16	1 ppb
Fenoxycarb	ESI +	9.95	1 ppb
Fenpropathrin	ESI +	11.79	1 ppb
Fenpyroximate	ESI +	11.91	1 ppb
Fenthion	ESI +	10.21	10 ppb
Fenthion sulfoxide	ESI +	7.45	1 ppb
Fenthion-sulfone	ESI +	7.67	1 ppb
Fipronil*	ESI +	10.01	1 ppb
Fipronil carboximide	ESI -	8.54	1 ppb
Fipronil desulfinyl	ESI -	9.81	1 ppb
Fipronil sulfone	ESI +	8.77	10 ppb
Fipronil sulphide	ESI -	10.12	1 ppb
Flonicamid	ESI +	3.69	1 ppb
Fluazifop	ESI +	7.65	1 ppb
Fluazifop-p-butyl	ESI +	11.24	1 ppb
Flubendazole	ESI +	8.42	1 ppb
Flufenacet	ESI +	9.76	1 ppb
Flufennoxuron (flufenoxuron)	ESI +	11.66	1 ppb
Flufenzine *	ESI +	10.06	1 ppb
Fluopicolide	ESI +	9.32	1 ppb
Fluopyram	ESI +	9.61	1 ppb
Flusilazole	ESI +	9.94	1 ppb
Halosulfuron-methyl	ESI +	6.82	1 ppb
Haloxyfop	ESI +	8.85	10 ppb
Hexaconazole	ESI +	10.37	1 ppb
Hexazinone	ESI +	7.17	1 ppb

Name	Ionization mode	Retention time (minute)	Pre-spike level detected
Hexythiazox	ESI +	11.54	1 ppb
Imazalil	ESI +	10.06	1 ppb
Imazaquin	ESI +	5.28	10 ppb
Imazosulfuron	ESI +	6.69	1 ppb
Imidachloprid	ESI +	4.65	1 ppb
Indoxacarb	ESI +	10.81	1 ppb
Iodosulfuran-methyl	ESI +	6.64	1 ppb
Iprobenfos	ESI +	10.15	1 ppb
Iprodione	ESI +	9.91	10 ppb
Iprovalicarb	ESI +	9.72	1 ppb
Isoprothiolane	ESI +	9.32	1 ppb
Isoproturon	ESI +	8.18	1 ppb
Linuron	ESI +	8.83	1 ppb
Lufenuron	ESI -	11.28	1 ppb
Malaoxon	ESI +	7.37	1 ppb
Malathion	ESI +	9.33	1 ppb
Mandipropamid	ESI +	9.25	1 ppb
Mesosulfuron methyl	ESI +	7.31	1 ppb
Metaflumizone	ESI -	11.08	10 ppb
Metalaxyl	ESI +	8.38	1 ppb
Methabenzthiazuron	ESI +	8.09	1 ppb
Methamidophos	ESI +	1.76	10 ppb
Methidathion	ESI +	8.47	1 ppb
Methiocarb	ESI +	8.92	1 ppb
Methomyl	ESI +	3.71	1 ppb
Metolachlor + S-metolachlor	ESI +	9.94	1 ppb
Metoxuron	ESI +	6.30	1 ppb
Metribuzin	ESI +	7.08	10 ppb
Metsulfuron methyl	ESI +	5.19	1 ppb
Mevinphos I	ESI +	5.22	1 ppb
Mevinphos II	ESI +	5.88	1 ppb
Milbemectin A3*	ESI +	12.26	10 ppb
Milbemectin A4 *	ESI +	12.53	10 ppb
Molinate	ESI +	9.37	1 ppb
Monocrotophos	ESI +	4.18	1 ppb
Monolinuron	ESI +	7.55	1 ppb
Mycobutanil	ESI +	9.38	1 ppb
Novaluron	ESI +	10.99	1 ppb
Omethoate	ESI +	2.73	1 ppb

Name	Ionization mode	Retention time (minute)	Pre-spike level detected
Oryzalin	ESI +	9.69	10 ppb
Oxadiargyl	ESI +	10.52	10 ppb
Oxadiazon	ESI +	11.38	1 ppb
Oxamyl	ESI +	3.49	1 ppb
Oxycarboxin	ESI +	5.56	1 ppb
Oxydemeton methyl	ESI +	3.78	10 ppb
Oxyfluorfen	ESI +	8.83	1 ppb
Paclobutrazole	ESI +	9.26	1 ppb
Parathion ethyl	ESI +	9.98	10 ppb
Paraxon methyl	ESI +	6.42	1 ppb
Penconazole	ESI +	10.16	1 ppb
Pencycuron	ESI +	10.67	1 ppb
Pendimethalin	ESI +	11.57	10 ppb
Phenthoate	ESI +	10.09	1 ppb
Phorate	ESI +	5.36	10 ppb
Phorate sulfone	ESI +	8.04	1 ppb
Phorate sulfoxide	ESI +	7.93	1 ppb
Phosalone	ESI +	10.56	1 ppb
phosmet	ESI +	8.70	1 ppb
Phosphamidon	ESI +	6.77	1 ppb
Picoxystrobin	ESI +	10.02	1 ppb
Pirimiphos methyl	ESI +	10.65	1 ppb
Pretilachlor	ESI +	11.04	1 ppb
Primicarb	ESI +	8.06	1 ppb
Prochloraz	ESI +	10.55	10 ppb
Profenofos	ESI +	11.11	1 ppb
Propanil	ESI +	8.81	1 ppb
Propetamphos	ESI +	9.44	1 ppb
Propiconazole (Tilt)	ESI +	10.36	1 ppb
Propoxur	ESI +	7.09	1 ppb
Pyraclostrobin	ESI +	10.48	1 ppb
Pyridalyl	ESI +	12.91	1 ppb
Pyrimethanil	ESI +	8.97	10 ppb
Pyriproxyfen	ESI +	11.40	1 ppb
Pyrithiobac sodium	ESI +	7.01	10 ppb
Quinalphos	ESI +	10.11	1 ppb
Quizalfop free acid	ESI +	8.53	10 ppb
Quizalfop-p-ethyl	ESI +	11.14	1 ppb
Rimsulfuron	ESI +	5.79	1 ppb

Name	Ionization mode	Retention time (minute)	Pre-spike level detected
Simazine	ESI +	7.09	1 ppb
Spinosad A	ESI +	12.40	1 ppb
Spinosad D	ESI +	12.62	1 ppb
Spiromesifen	ESI +	11.76	10 ppb
Spirotetramat	ESI +	9.75	10 ppb
Spiroxamine	ESI +	10.20	1 ppb
Sulfosulfuron	ESI +	6.26	1 ppb
Tebuconazole	ESI +	10.21	1 ppb
Temephos	ESI +	11.33	1 ppb
Terbufos	ESI +	11.26	10 ppb
Tetraconazole	ESI +	9.68	1 ppb
Tetradifon	ESI +	9.40	1 ppb
Thiabendazole	ESI +	6.38	1 ppb
Thiacloprid	ESI +	5.73	1 ppb
Thiobencarb	ESI +	10.59	1 ppb
Thiodicarb	ESI +	7.87	1 ppb
Thiomethoxam (Thiamethoxam)	ESI +	3.87	1 ppb
Thiophanate methyl	ESI +	7.09	10 ppb
Tralkoxydim	ESI +	10.52	1 ppb
Triademefon	ESI +	9.41	1 ppb
Triademenol	ESI +	9.52	1 ppb
Triallate	ESI +	11.61	10 ppb
Triazophos	ESI +	9.50	1 ppb
Trichlorfon	ESI +	5.04	1 ppb
Tricyclazole	ESI +	6.07	1 ppb
Tridemorph	ESI +	12.84	1 ppb
Trifloxystrobin	ESI +	10.88	1 ppb
Triflumizole	ESI +	10.94	1 ppb
Triticonazole	ESI +	9.72	10 ppb
Vamidathion (Vamidothion)	ESI +	5.24	1 ppb

Forensics



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Forensic Toxicology Screening Using the ACQUITY UPLC I-Class System with the Xevo TQD

Mark Roberts and Michelle Wood
Waters Corporation, MS Technologies Centre, Manchester, UK

APPLICATION BENEFITS

Two complementary methodologies for comprehensive toxicological screening using the latest generation of instrumentation. The ACQUITY UPLC® I-Class System and Xevo® TQD MS:

- A targeted MRM method utilizing two transitions per compound that can screen for a panel of 178 key analytes with excellent sensitivity and selectivity
- A full scan MS method incorporating a spectral library for over 950 toxicologically relevant substances that can be easily appended by the user

WATERS SOLUTIONS

ACQUITY UPLC I-Class System

Xevo TQD Mass Spectrometer

RADAR Technology ™

PIC® Scanning

ACQUITY UPLC HSS C_{18} Column

Waters® Total Recovery Vials

KEY WORDS

Forensic toxicology, targeted analysis, non-targeted screening, toxicants, UPLC®/MS/MS

INTRODUCTION

Forensic toxicology laboratories require broad screening techniques that can detect toxicants in highly complex biological matrices, such as ante- and post-mortem specimens. Two alternative toxicological screening methods, each utilizing the ACQUITY UPLC System with ACQUITY® TQD, have been previously described.[1-3] These two complementary approaches allow the user to take full advantage of the benefits associated with full scan data acquisition and the improved sensitivity associated with targeted MRM screening.[4]

These methods have been successfully implemented in over one hundred laboratories worldwide, including those with little or no previous LC/MS experience. With the availability of a new generation of instruments offering improved functionality and performance, there is an interest in applying these powerful and proven screening methods to the new systems. This application note presents a preliminary evaluation of the applicability of the existing two screening methods to the ACQUITY UPLC I-Class System and Xevo TQD Mass Spectrometer.

The goals of this study were to evaluate the utility of the ACQUITY UPLC I-Class System and Xevo TQD for forensic toxicology screening and to assess the applicability of the existing chromatographic method combined with the existing full scan MS and MRM methodologies[1-3] on this new platform.

EXPERIMENTAL

Sample Description

Drug standards were obtained from Sigma-Aldrich® (Poole, Dorset, UK) and LGC Standards (Teddington, Surrey, UK) and were either solid chemicals or certified solutions at concentrations of 1 mg/mL. Ammonium formate and formic acid were from Sigma-Aldrich. Acetonitrile was obtained from Greyhound Chromatography (Birkenhead, UK). Authentic urine samples were obtained from collaborators for routine screening.

Authentic urine samples were extracted by liquid/liquid extraction and transferred to Waters Total Recovery Vials. Extracts were injected onto both the ACQUITY UPLC I-Class/Xevo TQD and the original configuration of the classic ACQUITY UPLC/TQD.

UPLC conditions

System:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS C_{18}, 2.1 x 150 mm, 1.8 µm, part number 186003534
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	5 µL (MRM) or 10 µL (full scan)
Needle wash solvent:	5 mM ammonium formate, pH 3.0
Purge solvent:	5 mM ammonium formate, pH 3.0
Flow rate:	400 µL/min
Mobile phase A:	5 mM ammonium formate, pH 3.0
Mobile phase B:	0.1% formic acid in acetonitrile
Gradient:	13% mobile phase B increasing to 95%, with 15-minute analysis time

These are the same conditions that were previously used with the ACQUITY UPLC System.[1,2]

MS conditions

Mass spectrometer:	Xevo TQD
Ionization mode:	ESI positive (and ESI negative in full scan)
Capillary voltage:	3.0 kV
Cone voltage:	Varies according to method
Collision energy:	Varies according to method
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Cone gas:	20 L/h
Acquisition mode:	Multiple Reaction Monitoring (MRM) or full scan MS
Data management:	MassLynx® incorporating TargetLynx™ and ChromaLynx™ application managers

These are the same conditions that were previously used on the ACQUITY TQD.[1,2]

INNOVATIVE TECHNOLOGIES

The ACQUITY UPLC I-Class System with Flow-Through Needle (FTN)[5] design, shown in Figure 1, ensures that the sample comes into contact with only the needle and the direct flow path to the UPLC column. The sample is not transferred to a loop, which minimizes sample carryover; therefore, confidence in results is improved. In addition, the system volume has been reduced to 100 µL, which produces less analyte dispersion and improves peak shape. The column heater has also been improved with the inclusion of the active pre-heater (APH) reducing gradient delay and extra-column band-spreading.

The latest Xevo TQD,[6] shown in Figure 1, incorporates additional instrument features which could be highly advantageous to forensic analysis, such as RADAR and PICs. RADAR offers the capability to acquire full scan information while performing MRM analysis, which is a very useful tool for method development and troubleshooting. Product Ion Confirmation scanning (PICs) provides the option to automatically trigger a product ion scan when a particular MRM peak is detected. This allows the analyst to view additional confirmatory data and improve analyte identification.

Figure 1. The ACQUITY UPLC I-Class with Xevo TQD Mass Spectrometer.

RESULTS AND DISCUSSION

Overview of the MRM and full scan MS techniques

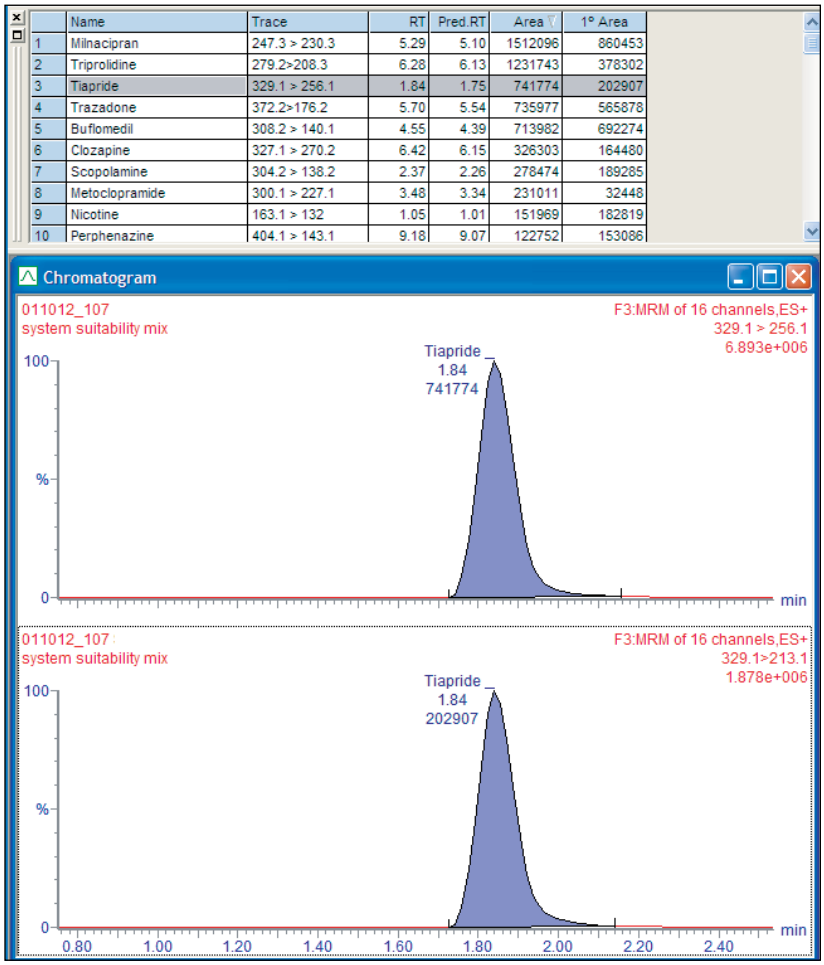
The original MRM method screened for 178 commonly encountered substances. The acquisition method was arranged into 30 time windows over the chromatographic elution range in order to improve the efficiency of data collection and to ensure sufficient data points for peak characterization. Each MRM time window was configured so that the start of the window was 0.5 minutes before the first eluting compound, and the end of the window was 0.5 minutes after the last eluting compound. Therefore, a key element of this study was an evaluation of the transferability of retention time (RT) and to assess whether the original MRM time windows were still applicable on the ACQUITY UPLC I-Class System.

The original full scan MS method generated a comprehensive catalogue of mass spectral data by acquiring data at seven different cone voltages (+20 V, +35 V, +50 V, +65 V, +80 V, +95 V, and -20 V). This data was automatically compared to a library comprised of spectral data for more than 950 substances using the ChromaLynx Application Manager.

In this study, ChromaLynx was used to compare spectral data acquired on the new MS system with the spectra contained in the original ACQUITY TQD library.

System suitability mixture injections using MRM and full scan acquisition modes

It is good laboratory practice to verify the performance of any analytical system prior to acquiring authentic sample data. This is commonly achieved by injecting a system suitability mixture (SSM) that contains a combination of substances that elute over the entire chromatographic range.



	Name	Trace	RT	Pred.RT	Area	1º Area
1	Milnacipran	247.3 > 230.3	5.29	5.10	1512096	860453
2	Triprolidine	279.2>208.3	6.28	6.13	1231743	378302
3	Tiapride	329.1 > 256.1	1.84	1.75	741774	202907
4	Trazadone	372.2>176.2	5.70	5.54	735977	565878
5	Buflomedil	308.2 > 140.1	4.55	4.39	713982	692274
6	Clozapine	327.1 > 270.2	6.42	6.15	326303	164480
7	Scopolamine	304.2 > 138.2	2.37	2.26	278474	189285
8	Metoclopramide	300.1 > 227.1	3.48	3.34	231011	32448
9	Nicotine	163.1 > 132	1.05	1.01	151969	182819
10	Perphenazine	404.1 > 143.1	9.18	9.07	122752	153086

Figure 2. TargetLynx browser displaying the results obtained with a typical SSM following targeted MRM screening using an ACQUITY UPLC I-Class/Xevo TQD. The RTs for all ten components detected were within 0.3 minutes of the expected RT and well within the original MRM time windows. This indicates successful transfer of the chromatography method to the ACQUITY UPLC I-Class System.

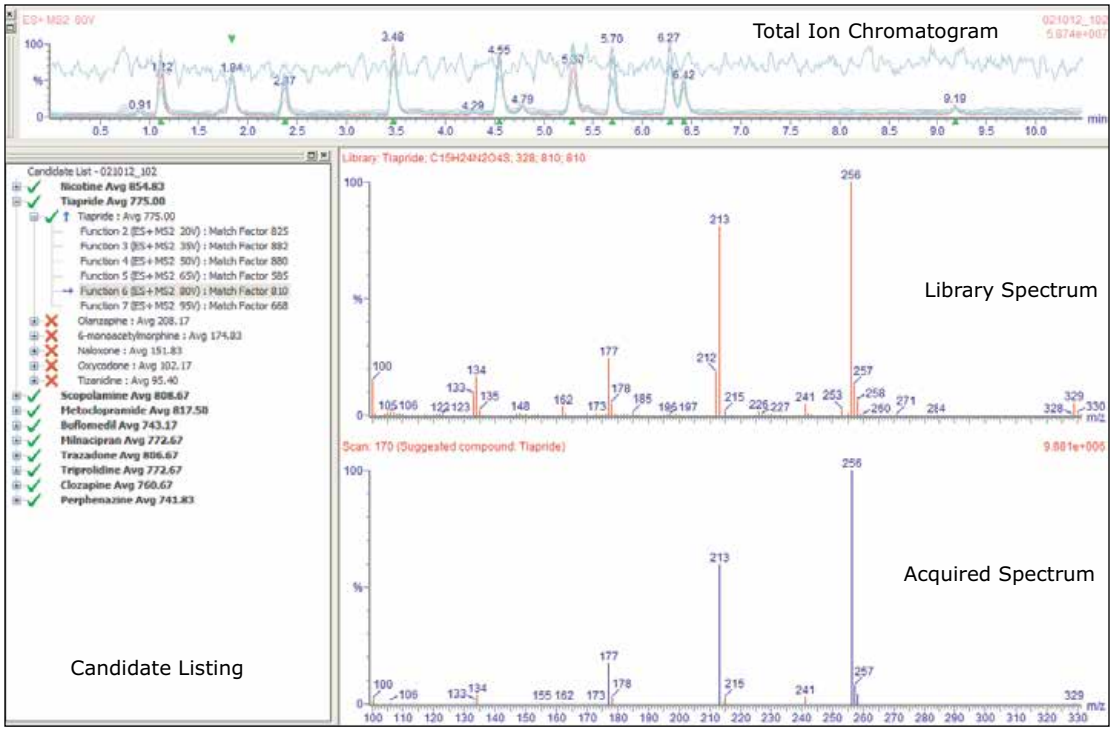


Figure 3. ChromaLynx browser displaying results of the full scan screening analysis of the SSM. Again, all ten components were detected, each one with a high average (Avg) match factor, indicating excellent agreement with the existing spectral library.

Analysis of an authentic urine sample by targeted MRM screening

The results for a urine sample submitted for routine forensic toxicological screening are shown in Table 1. The RTs of all identified substances were within 0.19 minutes of the expected RT, again supporting excellent transferability of the existing chromatographic method to the ACQUITY UPLC I-Class System.

The identification of drug metabolites within a biological specimen can be highly beneficial for the following reasons: they can be used to extend the window of drug detection; provide additional confirmation of drug intake; and generally assist in data interpretation. In this sample, methadone and its metabolite EDDP, as well as cocaine and its metabolite, benzoylecgonine, were identified by both systems. Figure 4 shows the TargetLynx browser detailing the results from the ACQUITY UPLC I-Class/Xevo TQD MRM analysis. Some additional substances were detected with the new system configuration suggesting enhanced sensitivity; this was also supported by the observation that the peak areas obtained with the new configuration were generally larger than those seen with the ACQUITY UPLC/TQD. This may be as a result of the new ACQUITY UPLC I-Class FTN, designed to minimize peak dispersion and maximize peak response.

		ACQUITY UPLC/TQD (original configuration)		ACQUITY UPLC I-Class/Xevo TQD (new configuration)	
Compound Name	**Predicted RT**	**Found RT**	**Peak Area**	**Found RT**	**Peak Area**
Methadone	8.61	8.77	434734	8.80	463948
EDDP	7.46	7.67	145310	7.65	149118
Paracetamol	1.50	1.58	5703	1.55	55966
Cocaine	4.61	4.80	11644	4.75	11702
Benzoylecgonine	2.97	3.13	10500	3.09	11261
Nicotine	1.01	1.02	3284	1.05	6143
Caffeine	2.10	2.19	1499	2.16	3866
Temazepam	9.34	–	–	9.46	3683
Oxazepam	8.07	8.17	1853	8.18	2528
Theophylline	1.46	–	–	1.45	2255
Nordiazepam	9.14	–	–	9.31	1297

Table 1. Results for an authentic urine sample analyzed by MRM targeted screening using both original and newer instrument configurations.

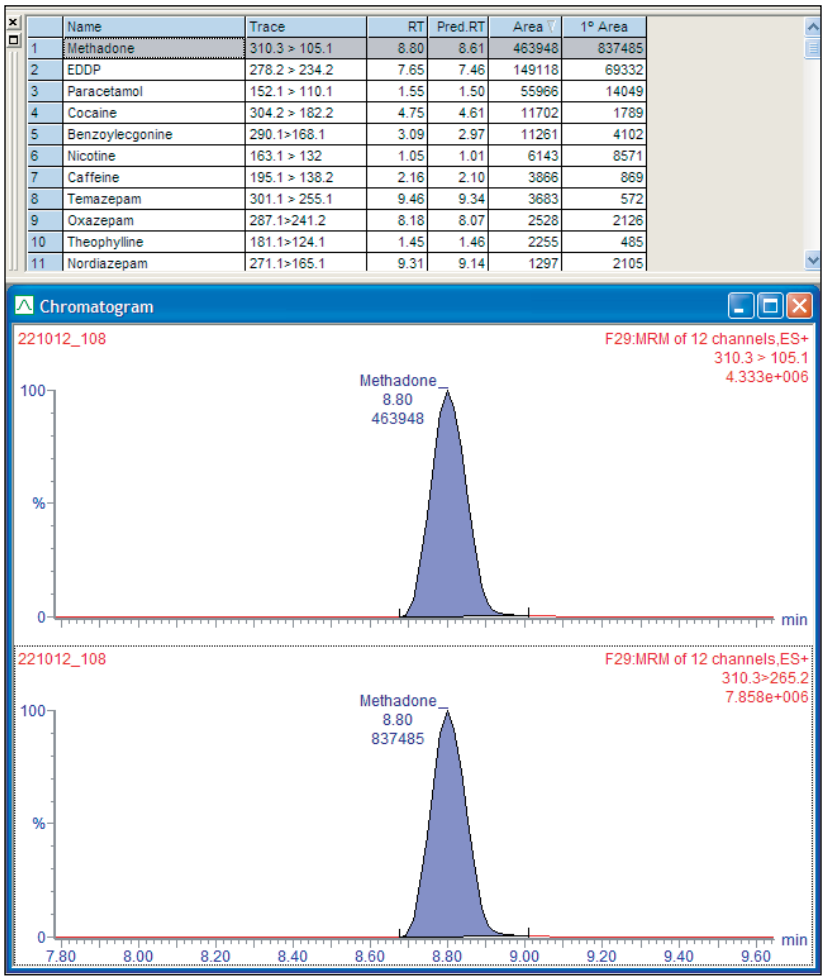


Figure 4. TargetLynx browser displaying processed results from a urine sample extract showing result for methadone.

Analysis of an authentic toxicology urine sample by full scan MS

A major advantage of the full scan MS approach is the ability to simultaneously screen for extremely large numbers of substances (limited only by the size of the spectral library). This is in contrast to the targeted MRM approach which is restricted to a panel of key analytes. At this time, a library comprising spectral data for more than 950 substances is available from Waters. Additionally, this library can be very easily expanded by the user. Alternatively, new libraries may be created and multiple libraries searched using the ChromaLynx Application Manager. Another benefit of this approach is that the data is not restricted to specific channels; thus the complete data set is available for retrospective analysis.

Figure 5 shows the results for another urine sample screened using the full scan MS method on the new system. In this sample the metabolite mirtazapine N-desmethyl was found, as well as the parent compound, mirtazapine. The spectrum window within the ChromaLynx browser clearly shows a good match for the fragments of mirtazapine N-desmethyl compared with the library spectrum that was originally acquired using the ACQUITY TQD. This indicates that the existing library acquired on ACQUITY TQD can be used with the newer Xevo TQD.

The full scan MS method also detects another compound, xylometazoline. This nasal decongestant would not be identified by the targeted MRM method because the substance is not currently included in the targeted panel.

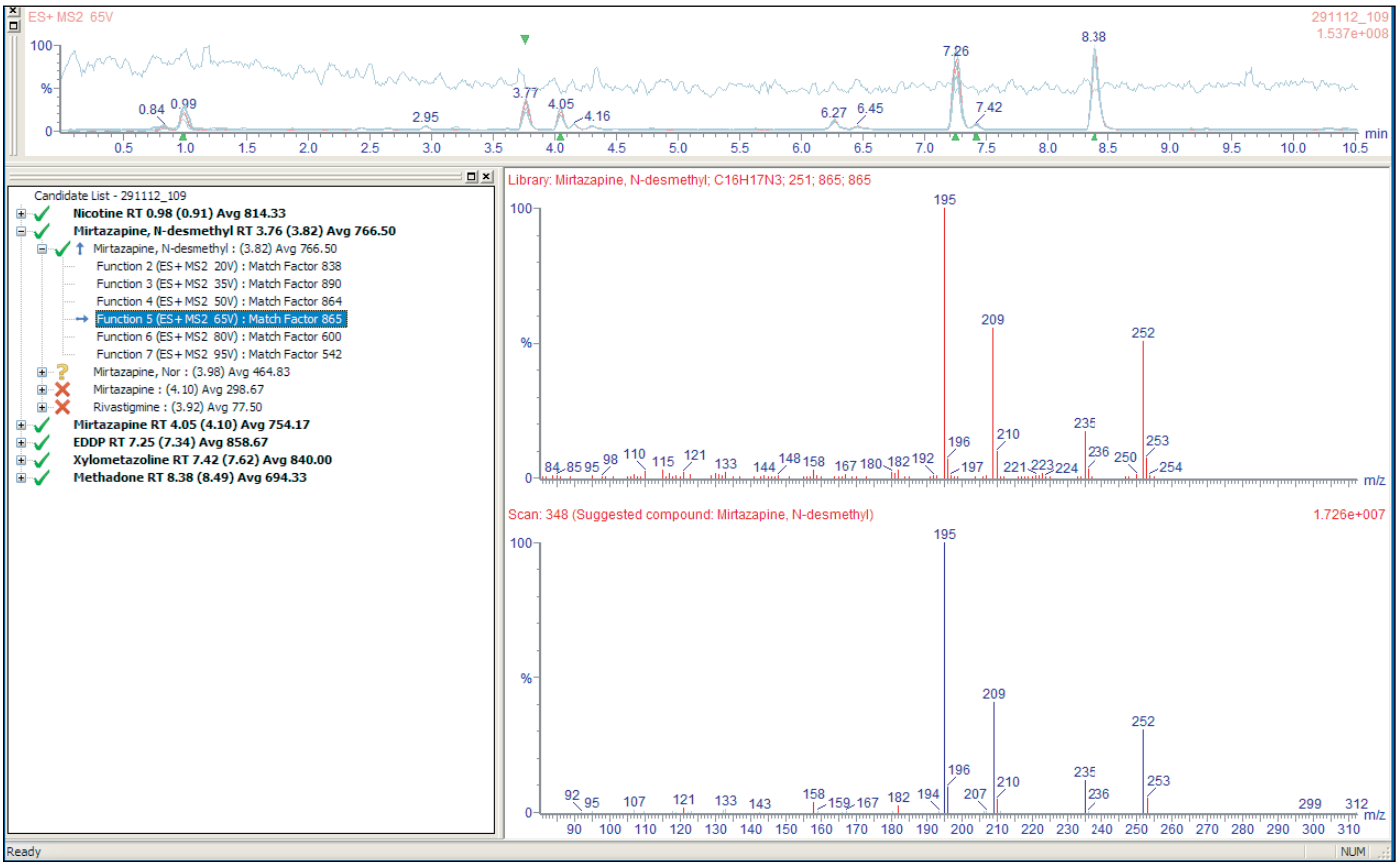


Figure 5. ChromaLynx browser displaying results from an analysis of urine sample extract showing results for the N-desmethyl metabolite of mirtazapine.

Using PICs to distinguish between hydromorphone and morphine

A useful feature of the new Xevo TQD is the ability to trigger data-directed Product Ion Confirmation scanning (PICs) during MRM analysis. This enables very similar compounds to be distinguished from one another by the pattern of their fragmentation from precursor into product ions.

Figure 6 shows the data obtained following analysis of hydromorphone using the MRM screening method. Responses were obtained in the two MRM channels for hydromorphone but also for morphine. This is not surprising, as these two substances are isomers sharing several MRM transitions. Furthermore, they elute within 0.2 minutes of one another using the chromatography described in this application note. Data generated from PIC scanning, however, may be used to provide additional information, which may assist in the differentiation of similar substances.

In this example, the PICs data produced a better match with a previously saved reference spectrum for hydromorphone than the one for morphine. The analyst is able to select both reference spectra from within the TargetLynx method to allow visual comparison of the spectra.

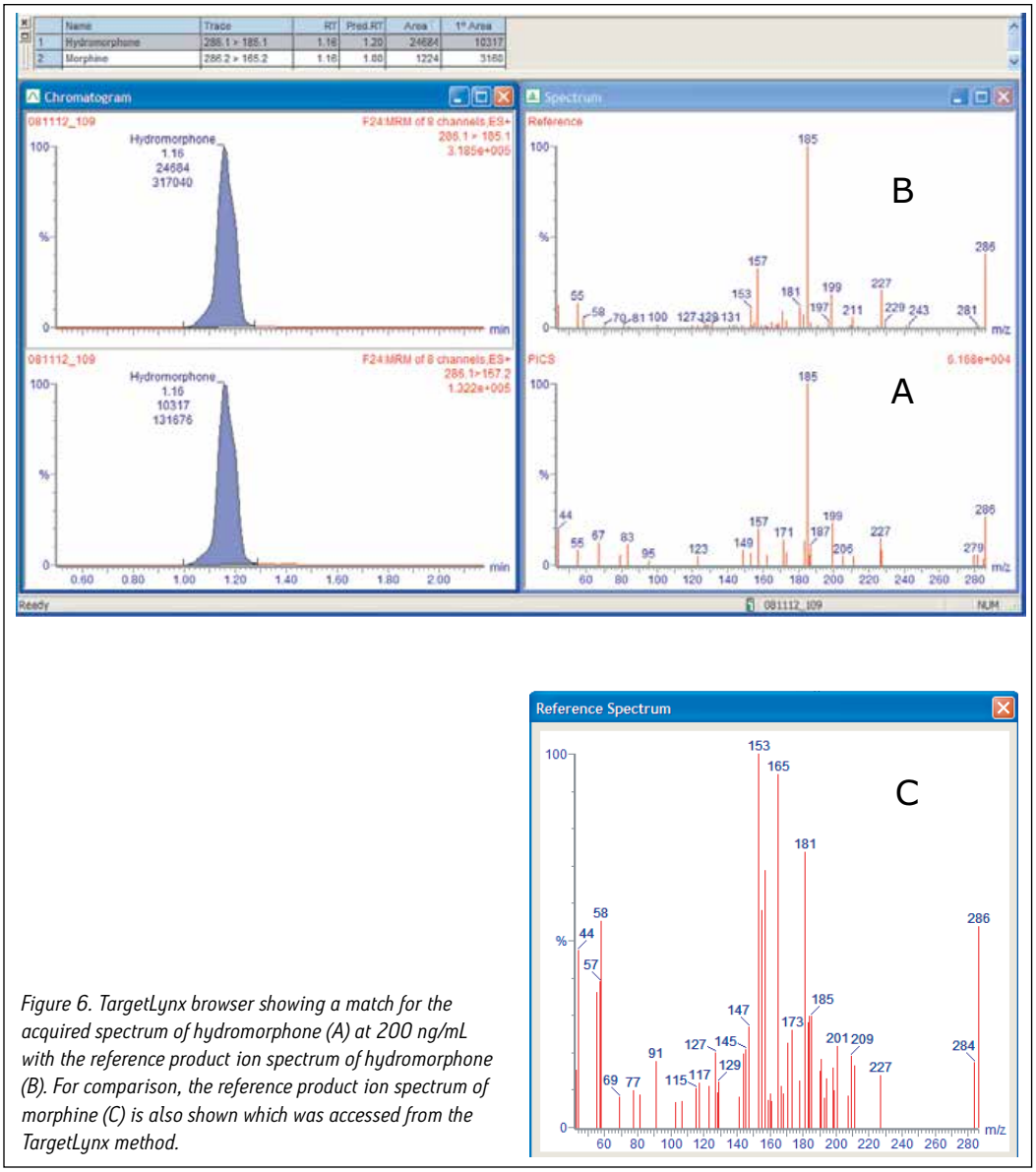


Figure 6. TargetLynx browser showing a match for the acquired spectrum of hydromorphone (A) at 200 ng/mL with the reference product ion spectrum of hydromorphone (B). For comparison, the reference product ion spectrum of morphine (C) is also shown which was accessed from the TargetLynx method.

Using RADAR to indicate the presence of extra analytes

The urine sample analyzed by full scan MS was also analyzed using the MRM targeted screening method with RADAR enabled. This allows full scan data to be collected while performing conventional MRM analysis and will show peaks that would potentially be missed if the analytes were not in the MRM method. Figure 7 shows the full scan data from this analysis with a selection of the MRM data that was simultaneously acquired. The full scan peaks for the metabolites N-desmethyl mirtazapine, desmethyl citalopram, and the drug, xylometazoline, are clearly visible at 3.79, 6.47, and 7.42 minutes, respectively; however, they were not detected by the MRM method as these substances were not included in this targeted assay. Figure 8 shows the mass spectra of the two metabolite peaks acquired at a cone voltage of 30 V. This extra information can be very useful, particularly in complex biological specimens, as it can indicate the presence of unknown components that would typically remain undetectable in a targeted screening scenario. Moreover, it can be an invaluable tool for troubleshooting during method development and validation as it may be used to identify and resolve issues with co-eluting compounds.

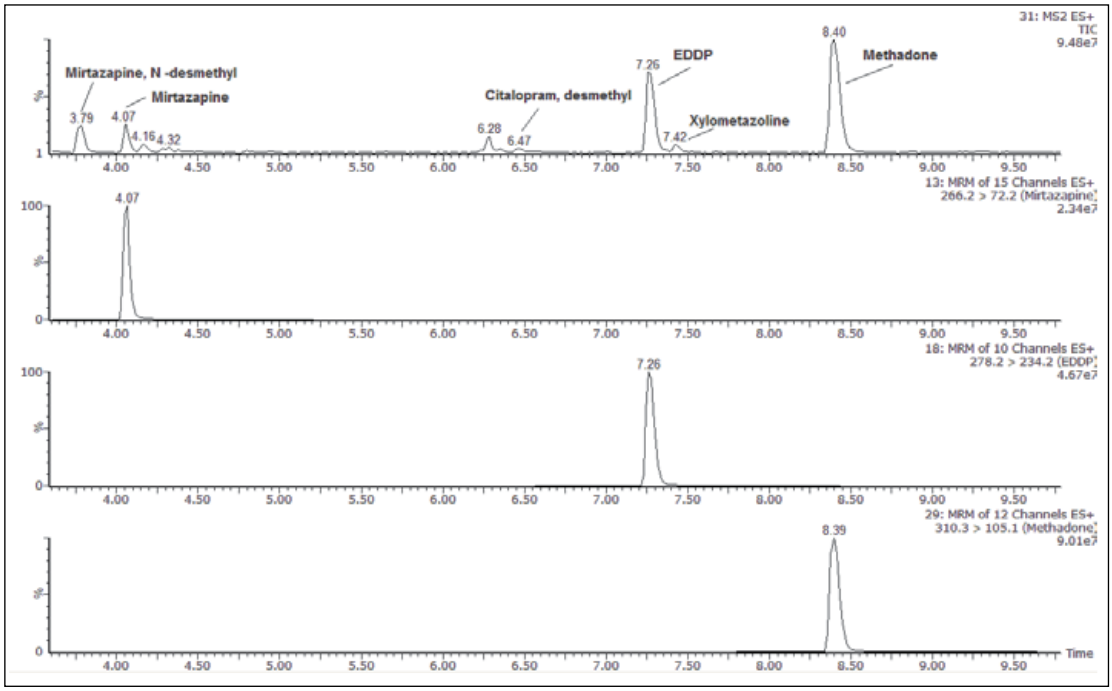


Figure 7. Full scan data (A) that was acquired simultaneously with MRM data for the hydrolyzed urine sample using RADAR. The full scan peak at 3.79 minutes was N-desmethyl mirtazapine, at 6.47 minutes desmethyl citalopram, and at 7.42 minutes was xylometazoline which are not currently included in this targeted MRM method.

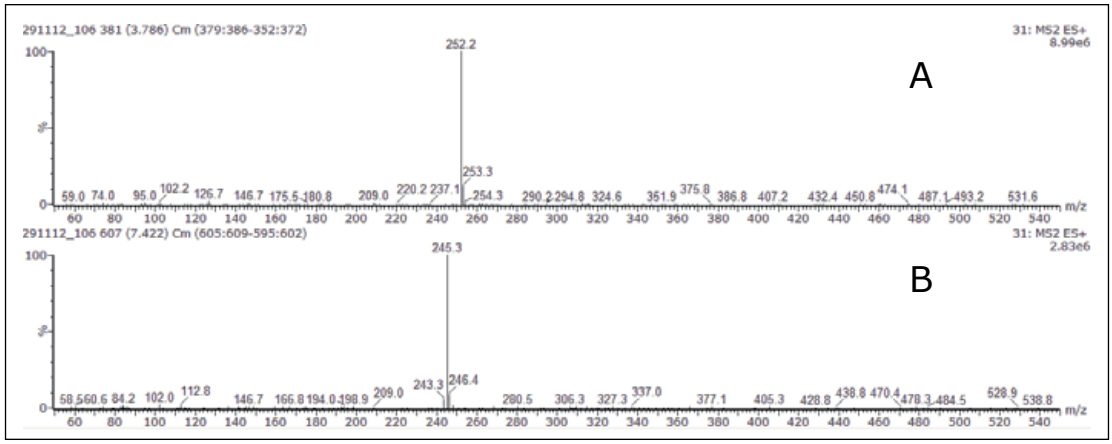


Figure 8. Full scan mass spectra (cone voltage 30 V) collected in RADAR mode for N-desmethyl mirtazapine at 3.79 minutes (A) and xylometazoline at 7.42 minutes (B).

CONCLUSIONS

Successful transfer of the toxicology screening methods to the next generation of the UPLC/MS system enables forensic analysts to access the latest analytical tools for their laboratories. The ACQUITY UPLC I-Class System has reduced peak dispersion and small system volume, thereby improving the sensitivity of the MS used in the assay. The Xevo TQD has the new features of RADAR and PICs, which can be used to enhance the information that is available to the analyst.

Starter projects are available that contain all the necessary methods to both acquire and process the data. The preconfigured methods contain a large number of the most commonly encountered toxicants and are ready for laboratory implementation with minimal user intervention.

The methods are fully customizable and can be easily appended to meet the scientists' needs. For example, additional compounds can be added to the databases. This ensures that the methods are versatile and will remain relevant for the future.

A full validation by the user would be necessary prior to adoption in a laboratory.

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Rapid Detection and Quantification of Selected Microsomal Substrates and Metabolites Using the Waters ACQUITY UPLC I-Class System and Xevo TQ-S micro

Kerri M. Smith and Paul D. Rainville
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- The Xevo® TQ-S micro offers excellent sensitivity and expansive linear range with fast MRM and polarity switching that can accommodate multiple transitions on UPLC®-scale analyses
- Discrete assays were combined into a single method, reducing cycle time and enabling high throughput
- Limits of quantification (LOQs) as low as 0.200 nM were obtained for analytes

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System

Xevo TQ-S micro Mass Spectrometer

ACQUITY UPLC 1.7 µm BEH Technology™ C_{18} Column

MassLynx® Mass Spectrometry Software and TargetLynx™ Application Manager

KEY WORDS

Xevo TQ-S micro, mass spectrometry, triple quadrupole, UPLC, bioanalysis, microsomes

INTRODUCTION

High-throughput absorption, distribution, metabolism, and excretion (ADME) screening for the properties of drug candidates have become an essential part of modern drug development. Such screening provides a basis for better information and, consequently, better decision-making in the drug discovery and development process.[1] As part of these studies, incubating candidate compounds together with liver microsomes containing cytochrome CYP450 is relied upon extensively to determine compound metabolism, stability, and possible drug-drug interactions (DDI).

The use of CYP450 to determine drug-drug interactions is carried out through inhibition and induction studies and is of particular importance because as the number of prescribed drugs increases, the greater the probability that an adverse drug reaction could exist. Also, it has been stated that there could be up to a 40% probability of a DDI when a patient is administered ten drugs or more.[2] In a standard CYP450 inhibition and induction assay, multiple test compounds are evaluated to determine their ability to alter or influence the metabolism of known CYP450 specific substrates.

In the work presented here, we show the development and validation of a rapid and sensitive LC-MS method for the simultaneous detection and quantification of multiple, selected CYP450 substrates and metabolites.

Figure 1. Waters ACQUITY I-Class UPLC and Xevo TQ-S micro.

EXPERIMENTAL

Method conditions

LC conditions

LC system:	ACQUITY UPLC I-Class
Vials:	Waters Maximum Recovery
Column:	ACQUITY UPLC BEH C_{18}, 3.0 x 50 mm, 1.7 µm
Column temp.:	35 °C
Sample temp.:	Ambient
Injection volume:	5 uL
Flow rate:	0.600 mL/min
Mobile phase A:	2 mM NH_4CH_4COOH, 0.1% NH_4OH in H_2O
Mobile phase B:	2 mM NH_4CH_3COOH, 0.1% NH_4OH in CH_3OH
Gradient:	1% to 90% B over 2 minutes; curve 4

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI+
Capillary voltage:	1 kV
Acquisition mode:	MRM

Data management

MassLynx Mass Spectrometry Software

Compound	Transition	Dwell time ms	Cone voltage	Collision energy
Acetaminophen	152>110	5	20	13
Acetaminophen-d4	156>114			
Hydroxymephenytoin	235>150	5	25	14
Hydroxymephenytoin-d3	238>150			
Hydroxybupropion	256>131	5	30	26
Hydroxybupropion-d6	262>131			
Dextorphan	258>199	5	20	29
Dextorphan-d3	261>199			
Hydroxytestosterone	305>269	5	20	11
Hydroxytestosterone-d3	308>272			
Hydroxydiclofenac	312>231	5	20	17
Hydroxydiclofenac-13C6	318>237			
Hydroxymidazolam	342>203	5	30	23
Hydroxymidazolam-d4	346>203			
6a-Hydroxypaclitaxel	870>286	5	30	4
6a-Hydroxypaclitaxel-d5	875>291			

RESULTS AND DISCUSSION

The number and diversity of new drug candidates entering the pharmaceutical pipeline has increased, necessitating strategies such as rapid ADME assays for obtaining information that can be used to better qualify candidates. The rapid determination of potential DDIs is of particular importance. If they are discovered late in the development process or after the drug goes to market, DDIs can cost pharmaceutical companies millions of dollars in lost revenue. Figure 2 depicts the separation of two substrates and six metabolites routinely monitored in testing for potential DDIs through inhibition and induction CYP450 assays. Good separation is shown with base widths of chromatographic peaks on the order of three seconds. These results were obtained under generic conditions, illustrating the ease and time for method development.

Figure 3 shows the calibration curve for the CYP450 isoform 1A2 substrate acetaminophen. It is linear well beyond three orders of magnitude, and it produces an R^2 value of 0.998935 (Table 1 shows the R^2 values for the other compounds used in this assay.) Figure 3 also shows a lower limit of quantification (LLOQ) of 0.2 nM, with greater than 14 times the signal-to-noise ratio, as measured from the baseline of the separation.

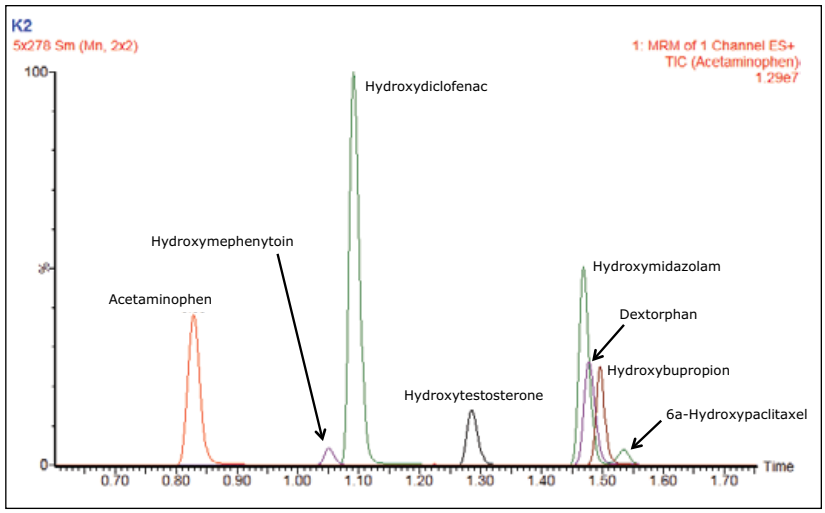

Figure 2. Rapid separation of CYP450 substrates and metabolites with the Waters ACQUITY UPLC I-Class and Waters Xevo TQ-S micro.

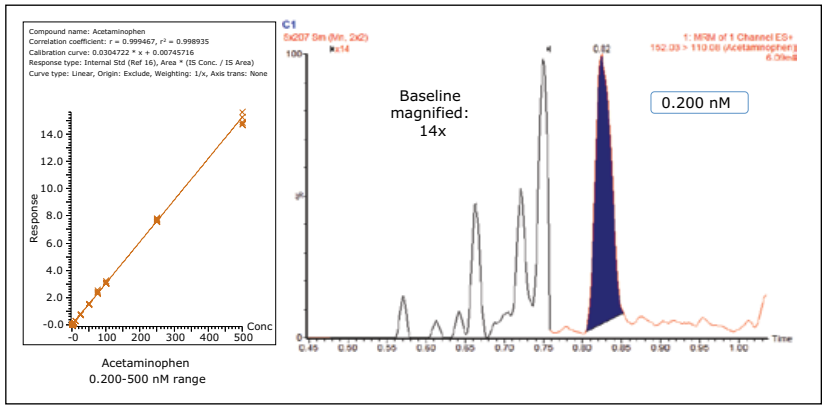

Figure 3. Calibration curve and LLOQ for acetaminophen.

Compound	Calibration Curve R^2 Value
Hydroxymephenytoin	0.996465
Hydroxybupropion	0.993304
Dextorphan	0.998533
Hydroxytestosterone	0.995952
Hydroxydiclofenac	0.996227
Hydroxymidazolam	0.997798
6a-Hydroxypaclitaxel	0.997247

Table 1. Calibration curve R^2 values of selected CYP450 substrate and metabolites utilized in this study.

The developed method was then tested for accuracy and precision by LC-MS, as prescribed in the USFDA guidance on bioanalytical method validation. Five determinations were made for 10 concentrations ranging from 0.2 to 5000 nM. The data in Table 2 show that the accuracy and precision for all of the analytes tested falls well within the 15% CV limit (3).

	Acetaminophen			Hydroxymephenytoin			Dextorphan			6α-Hydroxypaclitaxel		
[Nominal]nM	[Mean]	Accuracy %	%CV	[Mean]	Accuracy %	%CV	[Mean]	Accuracy %	%CV	[Mean]	Accuracy %	%CV
0.5	0.456	-11	10				0.446	-11	9	0.534	7.6	8
1	1.05	5.1	5	1.05	4.6	8	0.944	-5.7	5	1.06	5.5	7
2	1.98	-1.1	4	1.93	-3.4	13	1.97	-1.3	5	1.96	-2.2	6
5	4.89	-2.2	2	4.68	-6.4	12	5.17	3.3	3	4.82	-3.7	12
10	10.2	1.6	4	10.3	2.7	11	10.5	5	5	9.61	-3.9	10
25	24.9	-0.55	2	24.9	-0.38	6	25.5	2	3	24.5	-1.9	9
50	50.4	0.73	2	51.3	2.5	4	50.8	1.6	4	48.8	-2.5	8
75	80	6.7	4	73.7	-1.8	3	76.5	2	5	75.8	1.1	7
100	101	1.5	2	102	1.7	4	103	2.6	4	103	3	10
250	252	0.94	1	253	1.3	4	254	1.6	4	251	0.23	4
500	491	-1.8	3	495	-0.93	7	490	-2	2	498	-0.43	4

	Hydroxymidazolam			Hydroxytestosterone			Hydroxydiclofenac			Hydroxybupropion		
[Nominal]nM	[Mean]	Accuracy %	%CV	[Mean]	Accuracy %	%CV	[Mean]	Accuracy %	%CV	[Mean]	Accuracy %	%CV
0.5	0.484	-0.1	12	4.85	-3	12	0.454	13.4	9	0.872	-13	6
1	0.93	-7	7	10.4	3.7	10	0.8	0.04	9	1.95	-2.4	11
2	2.17	8.3	5	20.1	0.45	7	1.93	-3.5	8	4.94	-1.2	4
5	5.12	2.3	11	50.1	0.13	6	4.01	0.2	11	9.96	-0.38	1
10	10.4	4.3	9	102	2.3	8	7.85	-1.9	10	27.7	11	4
25	24.7	-1.1	12	252	0.69	9	19.4	-2.8	9	51.5	3	2
50	48.9	-2.2	8	508	1.5	6	39.6	-1	8	79.2	5.6	3
75	73.4	-2.2	7	813	8.5	8	97.7	-2.3	9	96.8	-3.2	8
100	98.4	-1.6	5	1045	4.5	7	195	-2.4	10	258	3.1	2
250	240	-3.8	5	2528	1.1	4	302	0.57	8	487	-2.5	3

Table 2. Accuracy and precision for selected CYP450 substrates and metabolites.

The sensitivity of a bioanalytical LC-MS assay can depend on matrix suppression, chromatographic peak shape, and the duration over which the mass spectrometer samples the chromatographic peak (or peaks) of interest. The sensitivity in this work can also be attributed to the incubation conditions: longer incubation times or higher enzyme concentrations can lead to the development of greater amounts of detected, metabolized substrate. The narrow peak widths produced by the sub-2-µm LC separation require a sufficient number of points across the chromatographic peak for good quantification. Figure 4 shows that the Xevo TQ-S micro is fully capable of acquiring more than 20 points across these narrow peak widths while subsequently monitoring multiple MRM transitions.

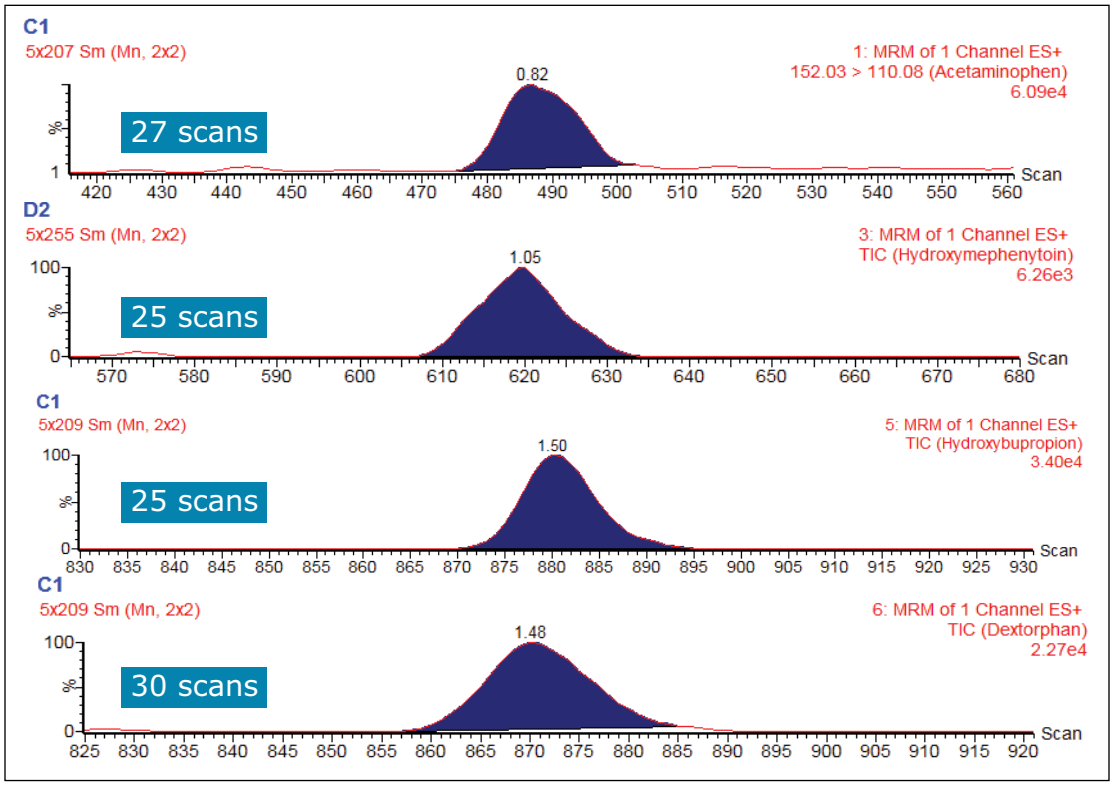

Figure 4. Replicate injections of acetaminophen illustrating the ability of the Xevo TQ-S micro to acquire high scan rates with narrow chromatographic peak widths as produced by UPLC.

The Xevo TQ-S micro can also be operated in RADAR™ acquisition mode. This allows the simultaneous acquisition of full-scan and MRM data in a single experiment. In Figure 5, we observe the data from the MRM channels monitored in this experiment and also the data from a full MS scan from *m/z* 50–600. We also observe co-elution of the target analyte with components in the matrix. Thus, the ability to monitor MRM channels and full-scan data can aid in the rapid development of robust methods that minimize the potential for co-elutions and possible matrix suppression. The capability of monitoring full-scan and MRM-channel data could be exploited for drug stability or metabolite identification studies. Though certain chemical entities would lay outside the specific MRM channels, they would nevertheless be subject to detection during full-scan acquisition. Data obtained in this way could then be further verified to determine positive identification.

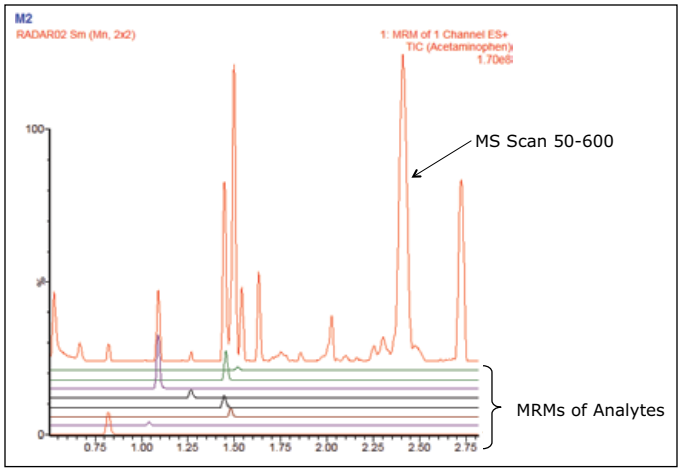

Figure 5. Chromatogram illustrating RADAR capability of the Xevo TQ-S micro. Here a full MS scan and multiple MRM channels can be simultaneously monitored.

CONCLUSIONS

The combination of the Xevo TQ-S micro and ACQUITY UPLC I-Class produced a rapid, sensitive, and robust method for the separation and detection of multiple, selected CYP450 substrates and metabolites often utilized in DDI studies. The accuracy and precision of the data produced from the method was well within the limits designated by the USFDA guidance for bioanalytical method validation. Linear responses for the calibration curves were obtained, revealing an LLOQ for the analytes of 0.2 nM. Further, the RADAR acquisition mode of the Xevo TQ-S micro enables the acquisition of full-scan and MRM data in a single run; a feature that can aid in method development and other applications.

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Advantages of Online Two-Dimensional Chromatography for MRM Quantification of Therapeutic Monoclonal Antibodies in Serum

Catalin Doneanu, Paul Rainville, and Robert S. Plumb
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The 2D LC/MRM assay can provide up to a three-fold increase in sensitivity compared to 1D LC/MRM. High pH/low pH RP/RP 2D LC separations can significantly reduce analyte suppression in protein bioanalysis.

WATERS SOLUTIONS

ACQUITY UPLC® System with 2D Technology

Oasis® MCX SPE 96-well Plate

ACQUITY UPLC BEH130 C_{18} Column

Xevo® TQ-S Mass Spectrometer

KEY WORDS

Trastuzumab, 2D LC/MRM, high pH/low pH RP/RP separation, two-dimensional chromatography, serum digest

INTRODUCTION

Quantification of therapeutic proteins in serum without analyte pre-fractionation can offer some advantages in terms of reducing the assay costs and simplifying the sample preparation workflow. Analyte isolation (typically performed by immunoaffinity) requires additional purification steps and uses expensive isotopically labeled protein standards to account for analyte recovery.

An alternative approach is the use of an LC system with greater chromatographic resolution, such as multi-dimensional LC. The multiple reaction monitoring (MRM) assays designed for measuring protein therapeutics in complex serum digests produced without analyte fractionation can be enhanced by multi-dimensional chromatography.

In particular, two-dimensional reversed phase/reversed phase (RP/RP) chromatography has been of significant interest in the bioanalysis community in recent years.[1-5] The major driving force behind the adoption of 2D-chromatographic methods is demonstrated improvement in the separation of the analyte of interest from other sample components in order to reduce suppression of the analyte signal. 2D-chromatographic techniques are responsible for increased sensitivities compared to one-dimensional LC methods using the same amount of sample.

Trastuzumab (herceptin) is a humanized IgG1 *kappa* monoclonal antibody (mAb). The antibody was obtained through genetic engineering[6,7] by joining the constant regions of the human monoclonal antibody with the complementarity-determining regions (CDRs) of a mouse monoclonal antibody able to bind human epidermal growth factor receptor 2 proteins (HER2) receptors. These HER2 receptors belong to a family of human oncoproteins expressed in approximately 25% of invasive breast cancers. Trastuzumab was approved in 1998 by the U.S. Food and Drug Administration (FDA) for the treatment of HER2-overexpressing breast cancers. Trastuzumab is administered by intravenous infusions in clinical doses to produce saturation of the HER2 receptor. For a conventional 4 mg/kg loading dose, followed by 2 mg/kg weekly doses, the mean maximum concentration of trastuzumab in plasma of 22 patients was approximately 70 µg/mL.[8]

In this application note, we report the development of a highly sensitive 2D LC/MRM assay for trastuzumab in human serum, employing gradient separation at pH 10.0 in the first RP chromatographic dimension, followed by gradient RP separation at pH 2.5 in the second dimension. We demonstrate that two-dimensional high pH/low pH RP/RP chromatography is able to significantly reduce ion suppression in protein bioanalysis.

EXPERIMENTAL

Sample Description

A stock solution of trastuzumab (150 kDa monoclonal antibody) was spiked with the internal standard ($^{13}C^{15}N$-isotopically labeled extended peptide GRFTISADTSK), and digested using trypsin to produce a stock solution containing 5 µM of digested trastuzumab and 10 µM of internal standard peptide (FTISADTSK). In parallel, 400 µL of human serum was dispensed in 10 Eppendorf vials (40-µL serum/vial), and digested using trypsin following the same procedure, to produce 200 µL of human serum digest in each vial. The digestion protocol involved sample denaturation (with 0.05% RapiGest at 80 °C for 10 min), disulfide bond reduction (in the presence of 20 mM dithiothreitol (DTT) for 60 min at 60 °C), cysteine alkylation (10 mM iodoacetamide (IAM) for 30 min at room temperature in the dark), and overnight digestion using porcine trypsin (25:1 (w/w) protein to enzyme ratio). Following digestion, half of the sample (100 µL of peptide digest) was diluted 1:1 with a solution containing 4% H_3PO_4, then loaded onto an Oasis mixed-mode SPE Elution Plate (10 mg/well of 30 µm MCX particles p/n 186000259). Digests were washed with 500 µL of 2% FA and 500 µL of 5% methanol before being eluted with 2 x 200 µL aliquots of 25% ACN in 1.5% NH_4OH (pH 10).

Matrix-free digests were prepared by diluting the 5-µM trastuzumab digest with 20 mM ammonium formate (pH 10) to prepare the following concentrations: 0.1, 1.0, 5.0, and 50.0 nM.

The same trastuzumab concentrations (0.1, 1.0, 5.0, and 50.0 nM) were prepared in 20 mM ammonium formate (pH 10) by diluting the stock trastuzumab digest in the human serum digest (using more than 80% of the serum digest matrix for each dilution).

LC/MS conditions

1D LC/MRM

An ACQUITY UPLC I-Class System equipped with an ACQUITY UPLC BEH300 C_{18} 2.1 x 150 mm, 1.7 µm Column (p/n 180003687) was used. The column temperature was maintained at 35 °C, and the flow rate was 0.3 mL/min. Mobile phases contained 0.1% (v/v) formic acid (FA) in water (A) and 0.1% (v/v) FA in acetonitrile (B). Peptides were eluted with a linear gradient from 0% to 35% B in 10 min.

2D LC/MRM

An ACQUITY UPLC H-Class System with 2D Technology configured in the heart-cutting mode was used for two-dimensional chromatography. Peptide separations were performed by RP/RP chromatography using the pH of the mobile phases to change the selectivity of the separation.[9,10] A diagram of the 2D-LC system is shown in Figure 1. The first dimension separation was performed on a 1.0 x 50 mm column packed with 2.5-µm XBridge™ $C_{18\ pa}$ particles, kept at 35 °C, and operated at 100 µL/min. Mobile phases contained 20 mM ammonium formate in water, pH 10.0 (Solvent A), and 20 mM ammonium formate in 90% ACN (Solvent B). Peptides were eluted with a linear gradient from 0% to 40% B in 5 min, as shown in Figure 1A. The analyte of interest (FTISADTSK peptide from trastuzumab), eluting between 6.6 and 6.9 min in the first chromatographic dimension, was transferred to the second chromatographic dimension using a switching valve, as shown in Figure 1B. Shortly after analyte transfer (~ 0.1 min later), the gradient for the second chromatographic separation began, as shown in Figure 1C. This separation was performed on a 2.1 x 50 mm column, packed with BEH300 1.7-µm particles, maintained at 35 °C, and operated at 300 µL/min. Mobile phases for low pH separations contained 0.1% formic acid (FA) in water (Eluent A) and 0.1% FA in ACN (Eluent B). Peptides were eluted with a linear gradient from 0% to 30% B in 5 min (gradient started 7 min after sample injection on the first dimension). The total run time for the entire 2D LC method was 15 min.

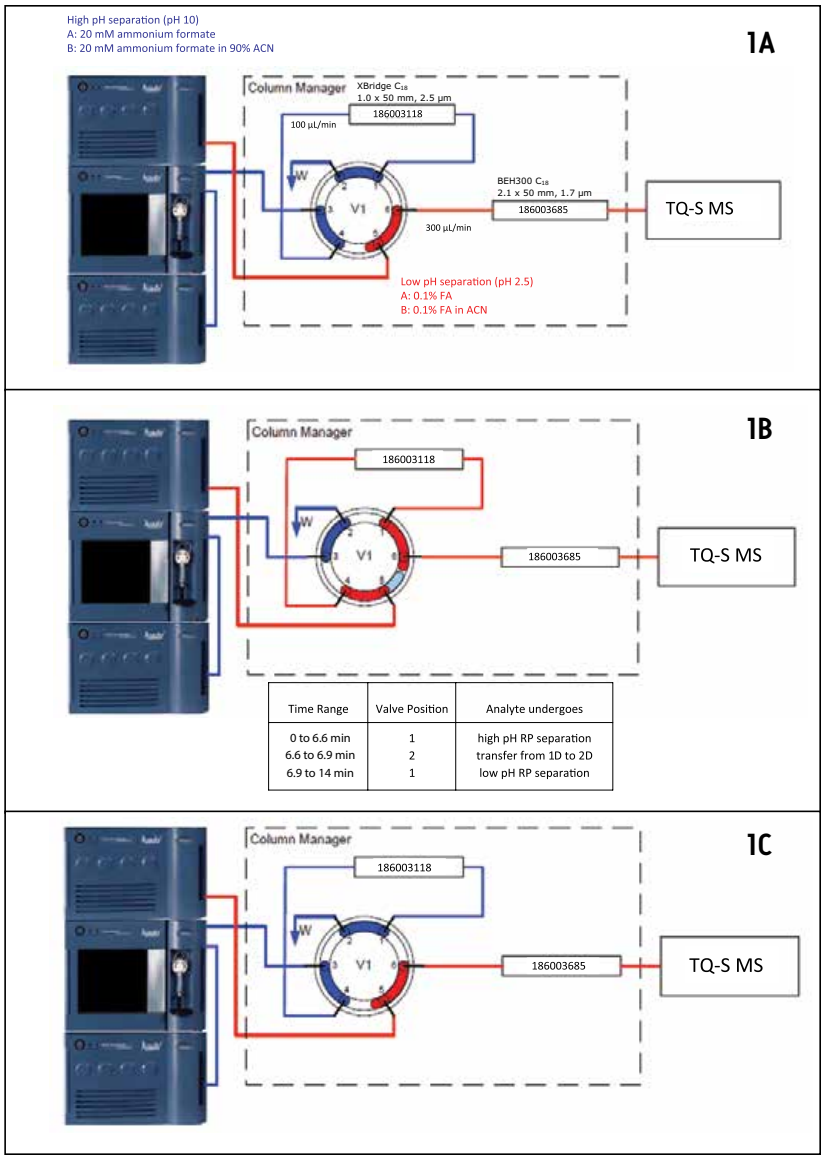

Figure 1. Heart-cutting configuration for two-dimensional chromatography: (A) Sample loading and first dimension separation under basic conditions (pH 10.0); (B) Analyte transfer from the first dimension to the second dimension; (C) Separation in the second dimension under acidic conditions at pH 2.5.

Mass Spectrometry

Assays were performed on a Xevo TQ-S Mass Spectrometer operated in MRM positive ion electrospray mode. The operating parameters were as follows: ESI potential 3.5 kV, CV 28 V, source temperature 120 °C, MS1/MS2 isolation window 0.75 Da (FWHM), 28 eV collision energy. Four MRM transitions were monitored continuously throughout the 2D LC/MRM assay using a dwell time of 100 ms: two MRMs monitored the endogenous signature peptide FTISADTSK from trastuzumab (485.2 → 721.4 for peptide quantification; 485.2 → 608.3 for peptide confirmation), while the other two MRM channels monitored the corresponding 13C15N-isotopically labeled internal standard peptide FTISADTSK (489.2 → 729.4 for peptide quantification; 489.2 → 616.3 for peptide confirmation). In addition to the assays designed for MRM monitoring only, several experiments were performed with RADAR and MRM monitoring. The scans for RADAR monitoring were performed in positive ESI mode over a mass range of m/z = 400 to 1400 with 1 s scans.

RESULTS AND DISCUSSION

Figure 2 shows the 2D LC/MRM chromatograms of FTISADTSK, the signature peptide from trastuzumab, recorded at four digest concentrations in the range of 0.1 to 50.0 nM. The digest was diluted with 20 mM ammonium formate (pH=10.0) in the absence of serum digest matrix. The assay linearity over the dynamic range investigated (500-fold) is clearly demonstrated by the peak areas displayed in Figure 2. The reproducibility of the 2D assay is shown in Figure 3A where peak areas were recorded for replicate injections (n=4) of the digest containing 5 nM trastuzumab and 10 nN $^{13}C^{15}N$-isotopically labeled peptide standard. The overall assay reproducibility was also very good, with the average peak area RSD for all concentrations tested greater than 2%, when four replicate injections were performed for each trastuzumab concentration.

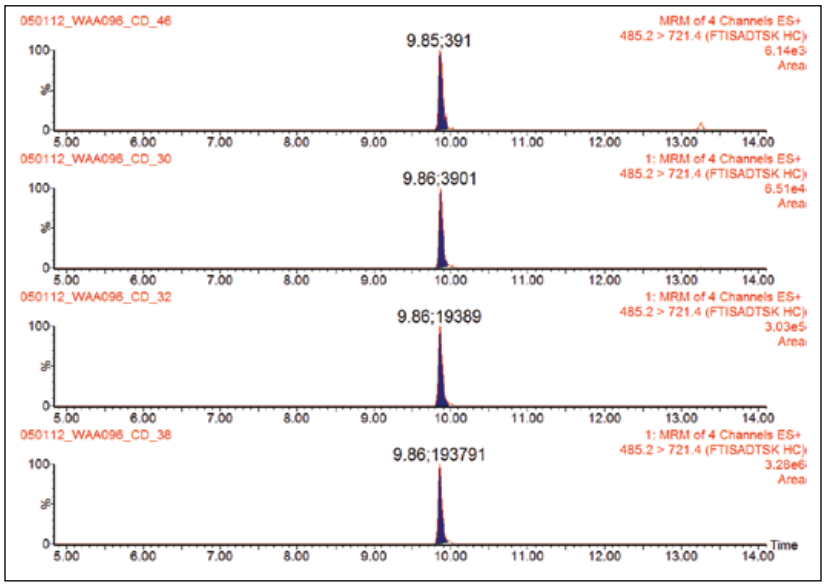

Figure 2. 2D LC/MRM chromatograms of the trastuzumab peptide FTISADTSK showing the linearity of the assay. Four digest concentrations covering a dynamic range of 500 fold (0.1 to 50.0 nM trastuzumab) were prepared in 20 mM ammonium formate (pH 10) and analyzed in replicate (n=4). The RSD for this experiment was greater than 2%.

The 2D LC/MRM assay, in the absence of the serum digest matrix, is very sensitive, with the lowest detected trastuzumab concentration determined to be 0.1 nM or 15.0 ng/mL, which is at minimum three orders of magnitude lower than the mean maximum trastuzumab concentration of 70 µg/mL measured in patients' plasma.[8]

The sensitivity of the MRM assay was also investigated in the presence of the complex serum digest matrix. Trastuzumab digests were spiked in SPE-cleaned human serum digests and analyzed by 2D LC/MRM, as shown in Figure 3B. This figure reveals that the signals corresponding to the native FTISADTSK peptide, as well as the signals produced by its isotopicallly labeled analogue, were clearly suppressed by co-eluting compounds from serum digest. However, the ratio between the native peptide and the isotopically labeled peptide was not affected by the complex matrix. This is an important observation, as the quantification method is based on comparing the peak area obtained for an unknown trastuzumab concentration to the peak area produced by the IS peptide spiked at a known concentration in the serum sample. Peak area RSDs were greater than 5%, as shown in these chromatograms in Figure 3B. In conclusion, in the 2D LC/MRM experiment, the analyte/IS suppression due to the complex serum digest background was only ~25%.

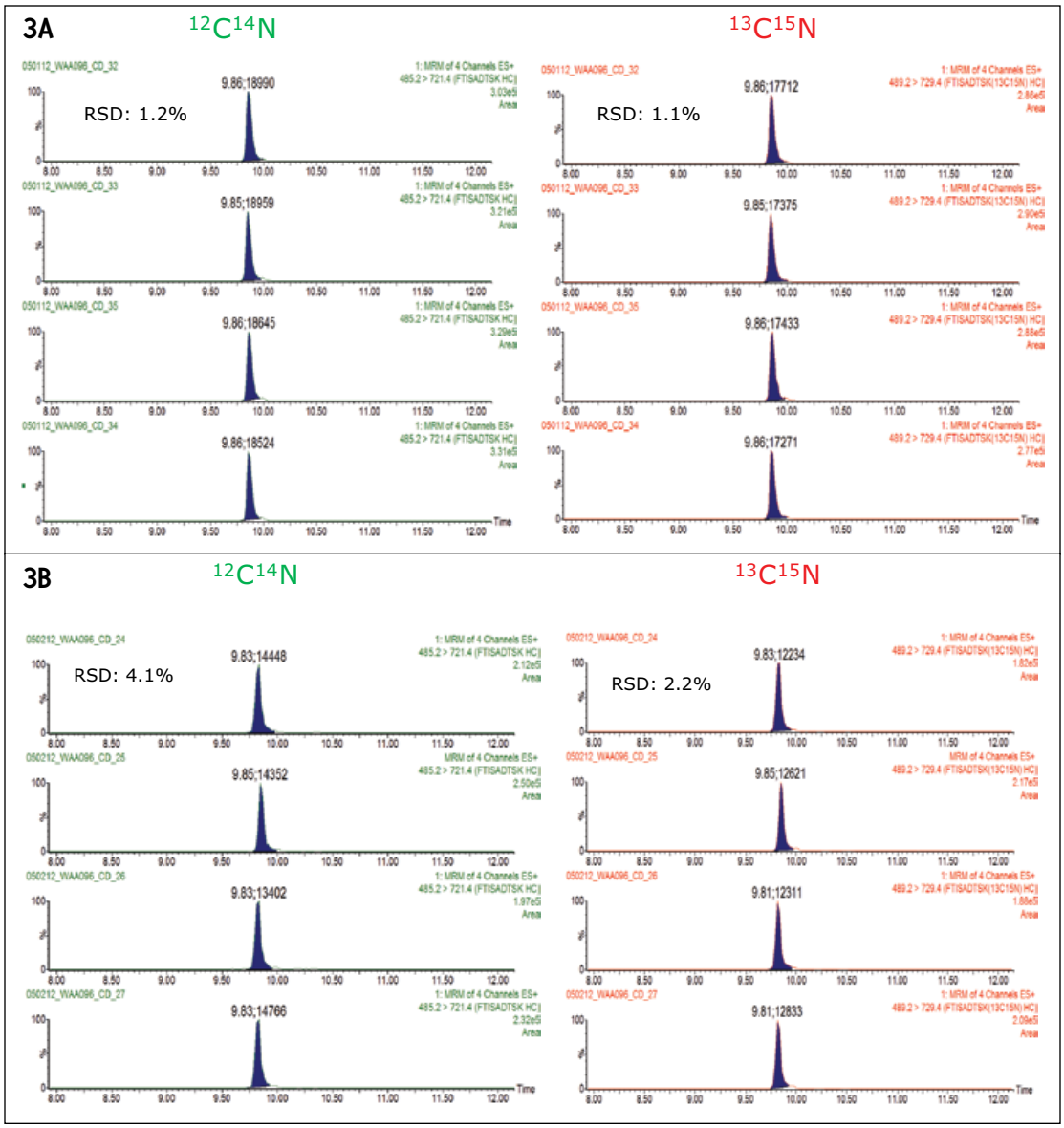

Figure 3. Reproducibility of the 2D LC/MRM assay: (A) in the absence of the serum digest background; (B) in the presence of the serum digest matrix. Figure 3A shows a sample containing 5 nM trastuzumab and 10 nM $^{13}C^{15}N$ labeled peptide prepared in 20 mM ammonium formate (pH 10). Figure 3B shows the sample containing 5 nM trastuzumab and 10 nM $^{13}C^{15}N$ peptide spiked into SPE-cleaned human serum digest.

When the trastuzumab digests spiked in SPE-cleaned human serum were analyzed by 1D LC/MRM, the analyte suppression was four-fold higher, as illustrated by the graphs shown Figure 4. The average peak areas of the native and isotopically labeled peptide IS are displayed in this figure for 1D- and 2D-MRM experiments performed for 5 nM trastuzumab digest prepared in neat solvent (20 mM ammonium formate, pH=10.0), as well as in SPE-cleaned human serum. These results clearly indicate that, relative to the quantification of therapeutic proteins in serum, the 2D LC/MRM method can provide up to a three-fold increase in sensitivity compared to conventional 1D LC/MRM.

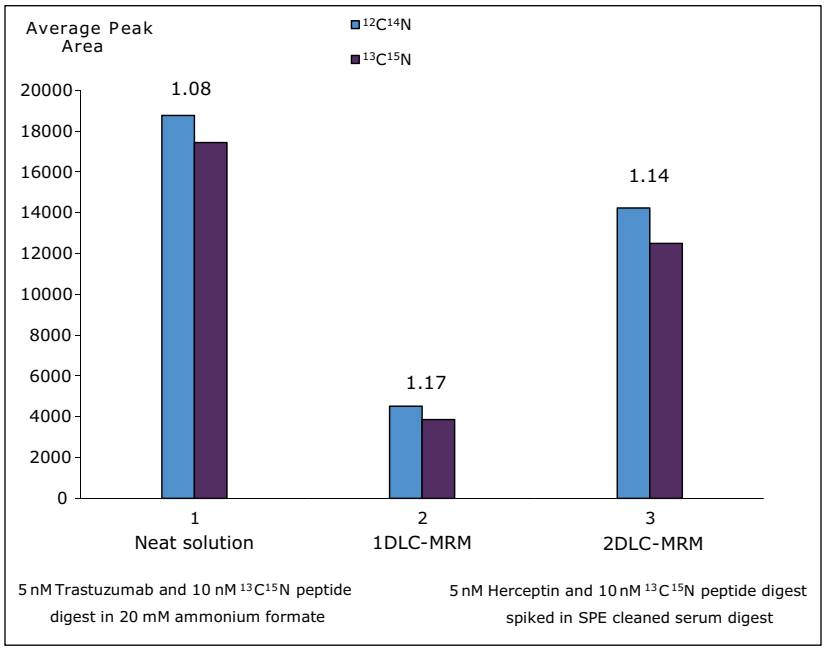

Figure 4. Evaluation of signal suppression. Comparison of average peak areas recorded for native and isotopically labeled trastuzumab peptide FTISADTSK in the absence and presence of the serum digest matrix. A sample containing 5 nM trastuzumab digest and 10 nM $^{13}C^{15}N$ labeled peptide was spiked into SPE-cleaned human serum digest.

The increase in sensitivity of the 2D LC/MRM method is usually explained by the ability of extensive chromatographic separations that remove some of the co-eluting compounds responsible for analyte/IS suppression. To verify this hypothesis, the presence of background peptides from the serum digest was monitored by full scan MS scans (RADAR scans) during 1D- and 2D-MRM separations. These RADAR scans were scheduled throughout the chromatographic run (1 s scans), and they were performed at the end of each MRM cycle containing the four transitions (50 ms each) used for trastuzumab quantification. The data collected during these experiments are illustrated in Figure 5. During the 1D LC/MRM run, the chromatogram recorded with full scan MS acquisitions (RADAR chromatogram), shown in Figure 5A, reveals the true complexity of the serum digest sample. The inset shows the MRM chromatograms corresponding to the native/IS peptides. The signals of these peptides are significantly reduced (~four-fold) by the presence of the "heavy" peptide background contained in the SPE-cleaned sample. Figure 5B shows the RADAR chromatogram produced by the

2D high pH/low pH RP/RP chromatography with heart-cutting. The MRM chromatograms, shown in the inset, indicate that most of the background components that were co-eluting with the analyte in 1D chromatography no longer co-elute after two-dimensional chromatography. Data in Figure 5 provide a clear explanation for the increase in assay sensitivity observed in the 2D-MRM method.

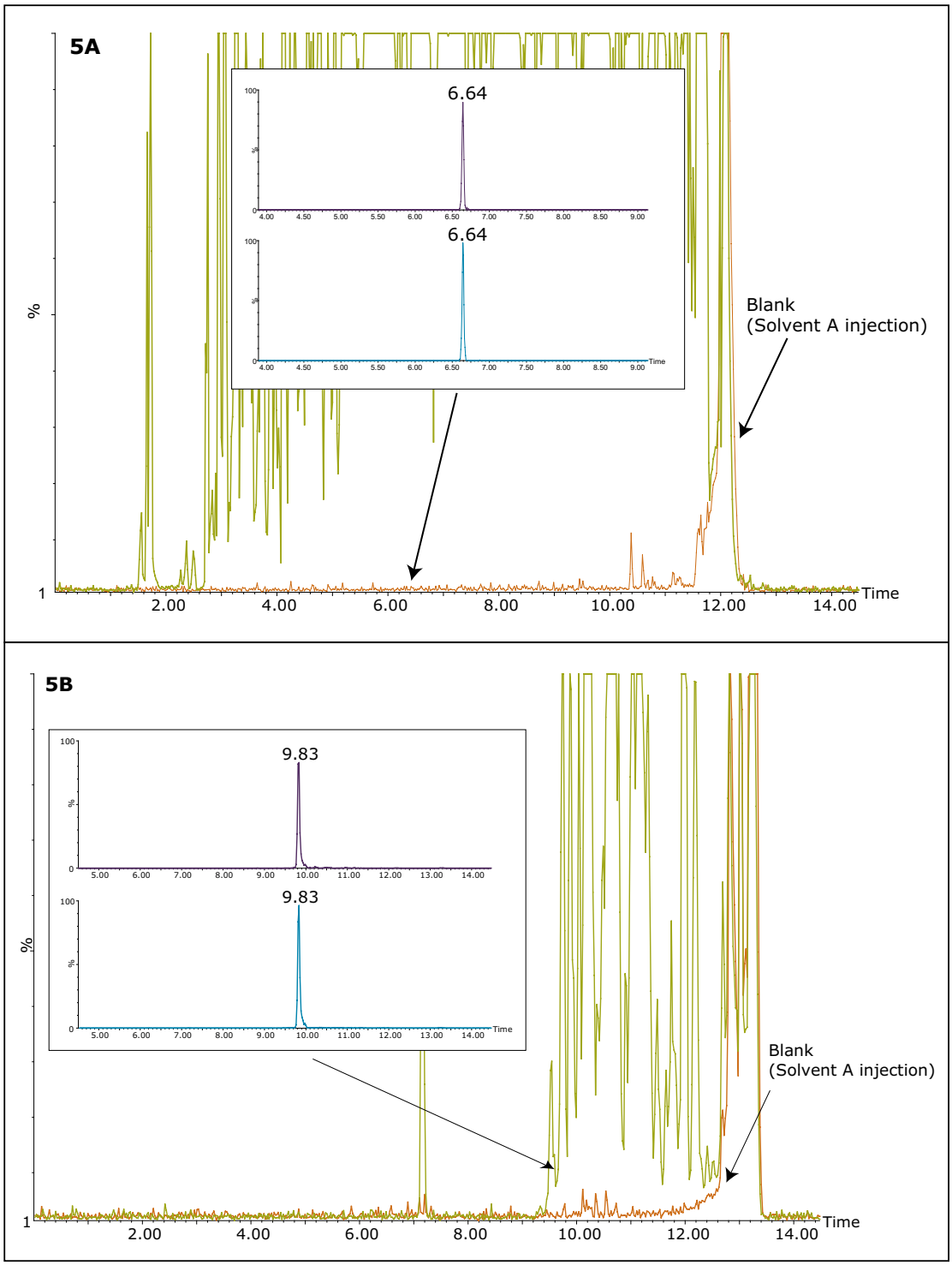

Figure 5. RADAR monitoring of SPE-cleaned samples containing 5 nM trastuzumab digest: (A) 1D LC/MRM separation under acidic conditions (pH 2.5); (B) 2D LC/MRM separation with heart cutting. The sample was prepared by spiking a trastuzumab digest (5 nM trastuzumab and 10 nM $^{13}C^{15}N$ labeled peptide) into SPE-cleaned human serum digest.

CONCLUSIONS

- A high sensitivity 2D LC/MRM method has been tested for the analysis of trastuzumab in human plasma digest using a signature peptide.
- The ratio of 12C/13C peptides was not significantly affected by the serum matrix.
- High pH/low pH RP/RP 2D LC separation can significantly reduce analyte suppression in protein bioanalysis.
- The 2D LC/MRM assay can provide up to a three-fold increase in sensitivity compared to 1D LC/MRM.

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KEY WORDS

IntelliStart, genotoxins, impurities

INTRODUCTION

Alkyl sulfonic acids, particularly methanesulfonic acid, benzenesulfonic acid, and p-toluenesulfonic acid, are a common class of alkylating agents used in the pharmaceutical industry as alkylating reagents, catalysts, and in purification steps in the chemical synthesis of an API. In addition, these sulfonic acids are often used as the final salt form of the drug due to improved chemical properties or bioavailability.

The presence of any residual alcohols from synthetic reaction or re-crystalization steps may result in the formation of alkyl esters of the sulfonic acids. Many of these mesylate, besylate, or tosylate esters are known to be genotoxic while others are potentially genotoxic, requiring monitoring in the drug substance and drug product.

Typical methods utilized in the past for the analysis of these akyl sulfonate esters have been based on GC/MS or HPLC/UV/MS, with derivatization typically using run times in the order of 20 to 30 minutes. We have previously demonstrated how good results can also be achieved using UPLC®/MS with run times of less than five minutes.

In this application note, we show how the presence of genotoxic impurities at Threshold of Toxicological Concern (TTC) can be quickly and easily detected using the Xevo TQD, a tandem quadrupole detector, with an Atmospheric Pressure Solids Analysis Probe (ASAP Probe).

EXPERIMENTAL

The Xevo TQD was tuned to each of the three impurity standard solutions using the on-board fluidics and IntelliStart™ allowing the instrumental conditions for the tablet analysis to be chosen quickly and easily.

MS conditions

MS system:	Xevo TQD

ASAP positive ion

Polarity:	API+
Corona:	0.50 µA
Corona:	1.5 kV
Cone:	30.00 V
Extractor:	3.00 V
Source temp.	150 °C
Probe temp.	450 °C
Desolvation gas flow:	400 L/Hr

ASAP negative ion

Polarity	API-
Corona:	0.80 µA
Corona:	1.5 kV
Cone:	30.00 V
Extractor:	3.00 V
Source temp.	150 °C
Probe temp.	450 °C
Desolvation gas flow:	400 L/Hr

RESULTS AND DISCUSSION

A single 10-mg amlodipine besylate tablet was crushed and solubilized in 5 ml of acetonitrile. The supernatant was removed and diluted 1:1 with water to give a 1 mg/mL solution of amlodipine besylate. Standard solutions of methyl- and ethyl- benzene sulfonates and ethyl-toluene sulfonate were prepared at 1 mg/mL in acetonitrile, and diluted to 15 µg/mL with water. These were spiked into the 1 mg/mL amlodipine solution using a 1:100 dilution, which equates to final impurities concentration of 1.5 µg (0.015%) per 10 mg tablet or the TTC when based on a single 10 mg per day dose of amlodipine besylate.

The ASAP probe was dipped into the spiked tablet solution, placed into the source and analyzed directly in multiple reaction monitoring (MRM) mode with product ion confirmation (PIC)[1] enabled. The Xevo TQD can be used to perform quantification of a sample with simultaneous characterization of the MRM peak as it elutes from the chromatographic system – or as shown in this case the ASAP MRM peak.

This eliminates the need for separate injections when qualitative confirmation of MRM peaks is required and reduces the total analysis time in these situations. When used routinely, PIC increases user confidence in qualitative results from complex matrixes, and thus reduces the need for re-analysis.

The presence of each of the impurities was confirmed in the spiked tablet solution, and the identities of the impurities confirmed using PIC.

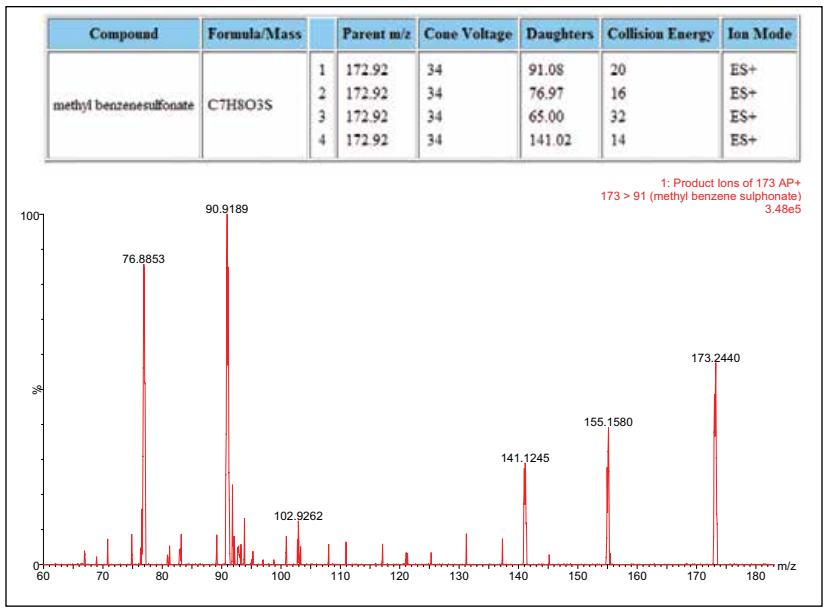

Figure 1. Methyl benzenesulfonate.

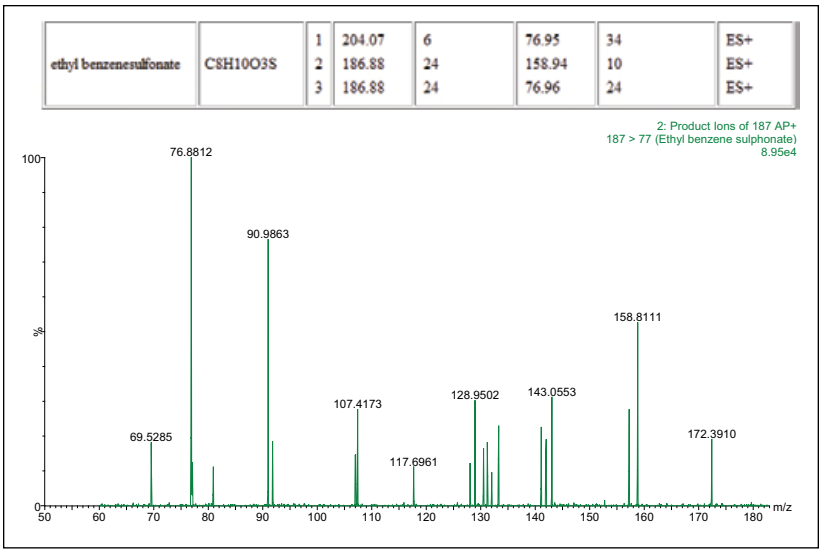

Figure 2.
Ethyl benezenesulfonate.

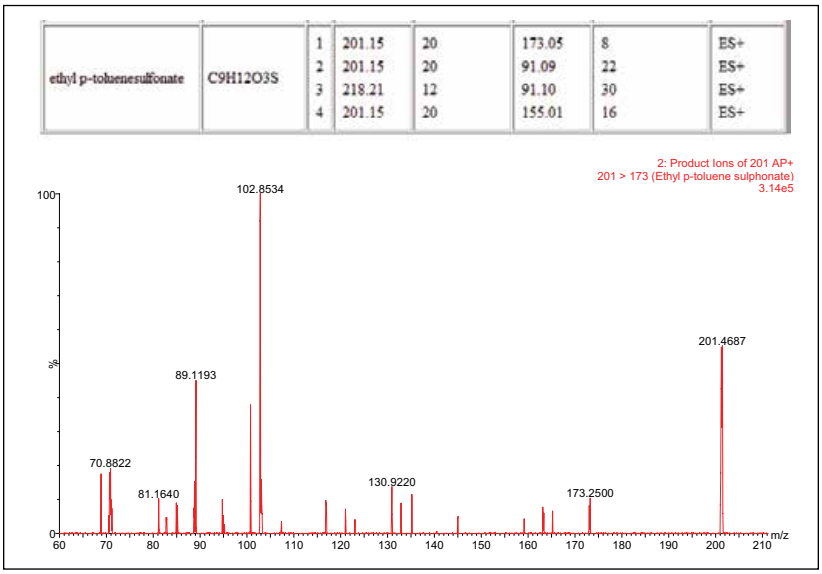

Figure 3. Ethyl
p-toluenesulfonate.

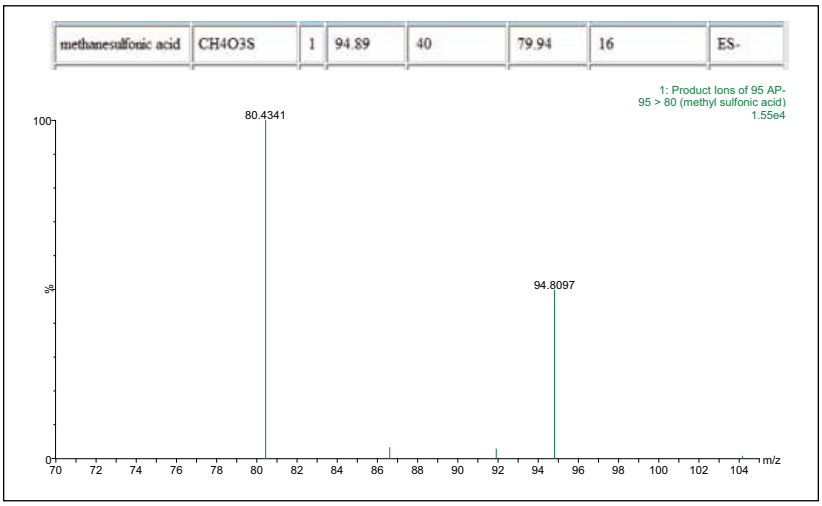

Figure 4.
Methanesulfonic acid.

CONCLUSIONS

Genotoxic impurities at the Threshold of Toxicological Concern (TTC) can easily be identified using the Xevo TQD when used in a qualitative manner with the ASAP probe and PIC. This allows for rapid check for the presence of genotoxic and other identified impurities and also allows confirmation of identity through the use of PIC. This rapid, simple, and powerful approach allows productivity gains into a routine laboratory setting that has not been possible before.

Reference

1. Confirming Peak Identification in Bioanalytical Studies Utilizing Xevo TQ MS Product Ion Confirmation. Waters Application Note. 2009: 720002858en.

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