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Determination of Highly Polar Cationic Pesticides and Plant Growth Regulators in Food Using UPLC-MS/MS

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APPLICATION BENEFITS

Provides a direct, single extraction LC-MS/MS method for the analysis of various highly polar cationic pesticides and plant growth regulators in cereals, fruit, and vegetable commodities.

WATERS SOLUTIONS

ACQUITY™ UPLC™ I-Class System

Xevo™ TQ-S micro

MassLynx™ MS Software

TargetLynx™ XS Application Manager

ACQUITY UPLC BEH Amide Column

KEYWORDS

LC-MS/MS, HILIC, pesticides, plant growth regulators, residue analysis, MRL, QuPPe, SANTE guidelines

INTRODUCTION

The European Union Reference Laboratory for Pesticides Single Residue Methods (EURL-SRM) published the QuPPe (Quick Polar Pesticides)[1] methods for the simultaneous analysis of a number of highly polar pesticides. To meet the needs of analyzing highly polar pesticides by LC-MS/MS, details on a number of chromatographic methods have been provided including one based upon hydrophilic interaction liquid chromatography (HILIC)1-2 for the determination of a series of cationic and polar basic analytes.

Although several compounds included in this application note are approved for use in Europe (maximum residue limits (MRLs) are listed in Table 1), other pesticide/crop combinations are not and default MRLs apply.[3] As well as being a separate contaminant of interest, melamine4 is also a metabolite of cyromazine, although it is not yet part of the residue definition used for enforcement purposes.

In this application note, example performance data is provided from Waters™ ACQUITY UPLC I-Class System and Xevo TQ-S micro on three commodities which represent high water content and high starch, low water content sample types. Organic wheat flour, cucumber, and apple, were extracted following the QuPPe method[1] to assess various performance factors of the UPLC-MS/MS method such as calibration linearity, retention time stability, method precision, and trueness.

Table 1. Current MRLs[2-3] in the three representative matrices for the compounds included in this application note.

Compound		MRL (mg/kg)		
		Apple	Cucumber	Wheat
Difenzoquat	Herbicide	0.01	0.01	0.01
Propamocarb	Fungicide	0.01	5.0	0.01
Cyromazine	Growth regulator	0.05	2.0	0.05
Nereistoxin	Insecticide	0.01	0.01	0.01
Melamine	Contaminant	2.5	2.5	2.5
Chlormequat	Growth regulator	0.01	0.01	4.0
Mepiquat	Growth regulator	0.02	0.02	3.0
Trimethylsulfonium	Organic cation	0.05	0.05	5.0

EXPERIMENTAL

Sample preparation and extraction

Homogenized organic apple and cucumber were extracted using the QuPPe method[1] as shown in Figure 1. For wheat flour only 5 g of sample was taken and 10 mL of LCMS grade water was added to this before extraction with the acidified methanol. Before the centrifugation step, the wheat flour was placed in a freezer at -20 °C for 2 hrs. The supernatant from the QuPPe extracts were then filtered using a 0.45 µm PVDF filter, spiked with the pesticide mix and analyzed using the liquid chromatography, mass spectrometry method highlighted below. Method performance information for analyte recovery can be found in the QuPPe document.[1]

The performance of the LC-MS/MS step of the method was assessed using SANTE guidelines.[5] Solutions of matrix-matched standards were prepared over the range 0.002 to 0.200 mg/kg (1.0 to 100.0 ng/mL in vial concentration) in apple and cucumber, 0.004 to 0.400 mg/kg (1.0 to 100.0 ng/mL in vial concentration) in wheat flour. Replicate injections at two concentration levels were run between bracketed calibration curves to assess the performance of the method. No isotopically labelled standards were used for this analysis.

UPLC conditions

UPLC system:	ACQUITY UPLC I-Class with fixed-loop Sample Manager
Column:	ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 × 100 mm (p/n: 186004801)
Mobile phase A:	50 mM Ammonium formate (pH 2.9, adjusted with LCMS grade formic acid)
Mobile phase B:	Acetonitrile
Flow rate:	0.5 mL/min
Injection volume:	0.5 µL (partial loop needle overfill)
Weak wash solvent:	90:10 acetonitrile:water
Strong wash solvent:	10:90 acetonitrile:water
Column temp.:	40 °C
Sample temp.:	10 °C
Run time:	10 min

Gradient:

Time (min)	%A	%B	Curve
0.00	3.0	97.0	Initial
0.50	3.0	97.0	6
4.00	30.0	70.0	6
5.00	40.0	60.0	6
6.00	40.0	60.0	6
6.10	3.0	97.0	6
10.00	3.0	97.0	6

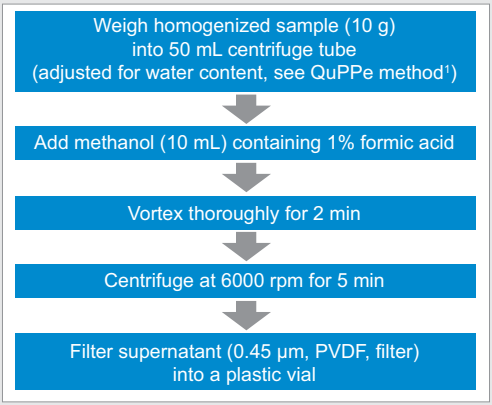

Figure 1. QuPPe sample extraction workflow for organic apple and cucumber.

MS conditions

MS system:	Xevo TQ-S micro
Ionization:	ESI+
Capillary voltage:	0.5 kV
Desolvation temp.:	600 °C
Desolvation gas flow:	1000 L/Hr
Source temp.:	150 °C
Cone gas flow:	150 L/Hr
Nebulizer gas pressure:	7 Bar

MRM transitions:

Compound	MRM transition	Dwell time (sec)	Cone voltage (V)	Collision energy (eV)
Difenzoquat*	249.3>130.2	0.081	20	40
Difenzoquat	249.3>193.2	0.081	20	25
Propamocarb	189.3>74.1	0.030	20	25
Propamocarb*	189.3>102.0	0.030	20	15
Cyromazine*	167.4>68.1	0.030	20	28
Cyromazine	167.4>85.1	0.030	20	18
Nereistoxin	150.1>61.0	0.249	20	25
Nereistoxin*	150.1>105.1	0.249	20	15
Melamine	127.1>68.1	0.030	20	22
Melamine*	127.1>85.1	0.030	20	17
Chlormequat*	122.1>58.1	0.030	20	20
Chlormequat	124.0>58.1	0.030	20	22
Mepiquat	114.2>58.2	0.029	20	20
Mepiquat*	114.2>98.2	0.029	20	20
Trimethylsulfonium	77.1>47.1	0.029	20	10
Trimethylsulfonium*	77.1>62.1	0.029	20	10

** denotes transition used for quantification.*

RESULTS AND DISCUSSION

The method was found to give good retention for all compounds, greater than two times the column void volume, as indicated in the SANTE guidelines.[5] Overall the method provided acceptable separation and excellent peak shapes for all compounds. Example chromatograms for chlormequat, mepiquat, and propamocarb in matrix at the 1 ng/mL in vial concentration level, are shown in Figure 2. Retention time stability was also assessed according to the SANTE guidelines (±0.1 min),[5] retention times within and between matrices were within 0.1 min for all compounds. Retention times for each compound in the representative matrices can be found in Figure 3.

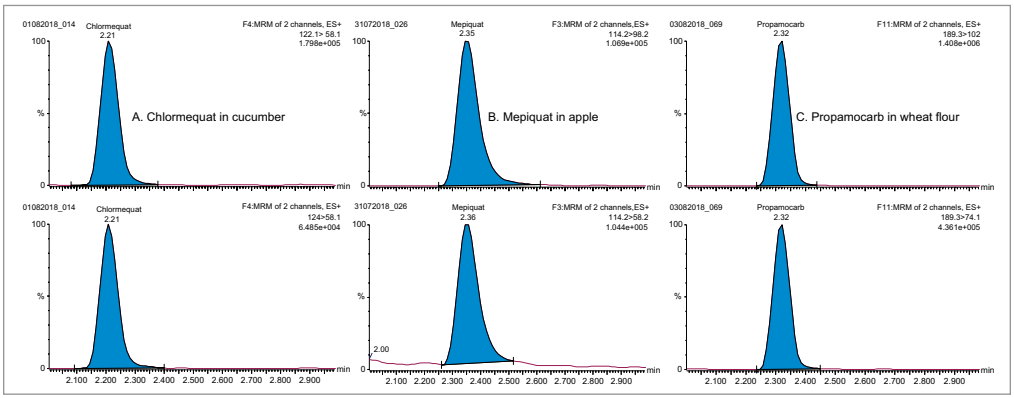

Figure 2. Example chromatograms for A. chlormequat in cucumber, B. mepiquat in apple, and C. propamocarb in wheat flour at 1.0 ng/mL in vial concentration. Excellent sensitivity for both MRM transitions for each compound was achieved with only a 0.5 µL injection volume.

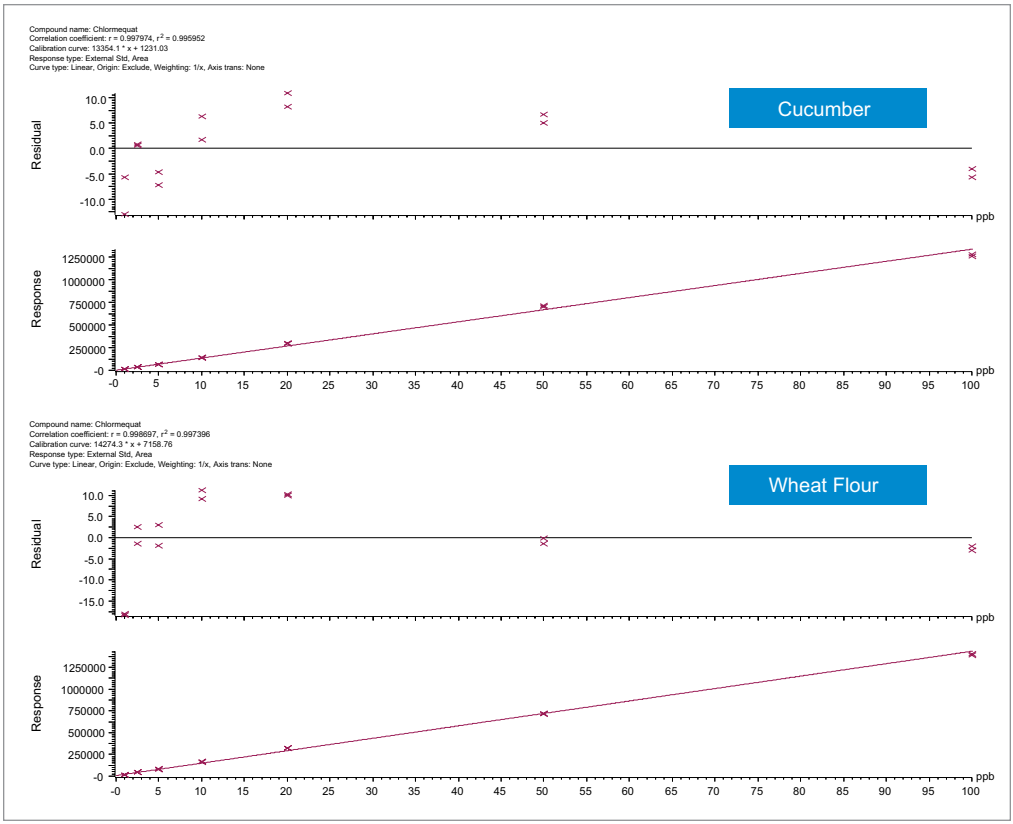

Figure 3. Bracketed matrix-matched calibration curves for cucumber and wheat flour for chlomequat, 1 ng/mL to 100 ng/mL in vial concentration.

The linearity of the method was assessed using bracketed matrix-matched calibration curves for each matrix, without the use of labeled internal standards; Figure 3 shows the calibration curves for chlormequat in organic cucumber and wheat flour. The linearity of response and calibration range in the tested matrices for all compounds assessed in this study, are shown in Table 2. The concentration levels take into account that only 5 g of wheat flour was taken for extraction. All compounds gave excellent linear response and residuals (back calculated concentrations) were within the 20% tolerance of the SANTE guidelines.[5]

Table 2. Matrix-matched calibration linearity of response and calibration range (mg/kg) for each compound in the three tested matrices. The calibration range for wheat flour takes into account that only 5 g of sample is used for the extraction.

Matrix	Compound	RT (min)	Calibration range (mg/kg)	Calibration R^2	Back calculated residuals <20%
Apple	Nereistoxin	1.06	0.002–0.200	0.998	Pass
	Difenzoquat	1.77	0.002–0.200	0.997	Pass
	Daminozide	1.91	0.002–0.200	0.998	Pass
	Chlormequat	2.2	0.002–0.200	0.998	Pass
	Cyromazine	2.22	0.002–0.200	0.998	Pass
	Propamocarb	2.3	0.002–0.200	0.998	Pass
	Mepiquat	2.35	0.002–0.200	0.998	Pass
	Trimethylsulfonium	2.61	0.002–0.200	0.998	Pass
	Melamine	2.88	0.002–0.200	0.998	Pass
Cucumber	Nereistoxin	1.05	0.002–0.200	0.999	Pass
	Difenzoquat	1.78	0.002–0.200	0.999	Pass
	Daminozide	1.93	0.002–0.200	0.997	Pass
	Chlormequat	2.21	0.002–0.200	0.996	Pass
	Cyromazine	2.27	0.002–0.200	0.998	Pass
	Propamocarb	2.31	0.002–0.200	0.998	Pass
	Mepiquat	2.36	0.002–0.200	0.997	Pass
	Trimethylsulfonium	2.62	0.002–0.200	0.996	Pass
	Melamine	2.91	0.002–0.200	0.998	Pass
Flour	Nereistoxin	1.06	0.004–0.400	0.999	Pass
	Difenzoquat	1.79	0.004–0.400	0.999	Pass
	Daminozide	1.92	0.004–0.400	0.999	Pass
	Chlormequat	2.22	0.004–0.400	0.997	Pass
	Cyromazine	2.28	0.004–0.400	0.999	Pass
	Propamocarb	2.32	0.004–0.400	0.998	Pass
	Mepiquat	2.37	0.004–0.400	0.998	Pass
	Trimethylsulfonium	2.62	0.004–0.400	0.997	Pass
	Melamine	2.92	0.004–0.400	0.998	Pass

Replicate (n=15) injections were run for two levels, 5.0 ng/mL and 20.0 ng/mL in vial concentrations. The calculated mean concentrations and precision for the tested compounds in all three matrices can be seen in Figure 4. Excellent accuracy and precision was achieved for all compounds, within 15% of the target concentration value and %RSD below 5%.

Ion ratios of the replicate injections agreed well with expected reference values and all were within the required tolerance[5] (±30%). An example of the ion ratios given by each of the (n=15) replicate levels for chlomequat in wheat flour can be seen in Figure 5.

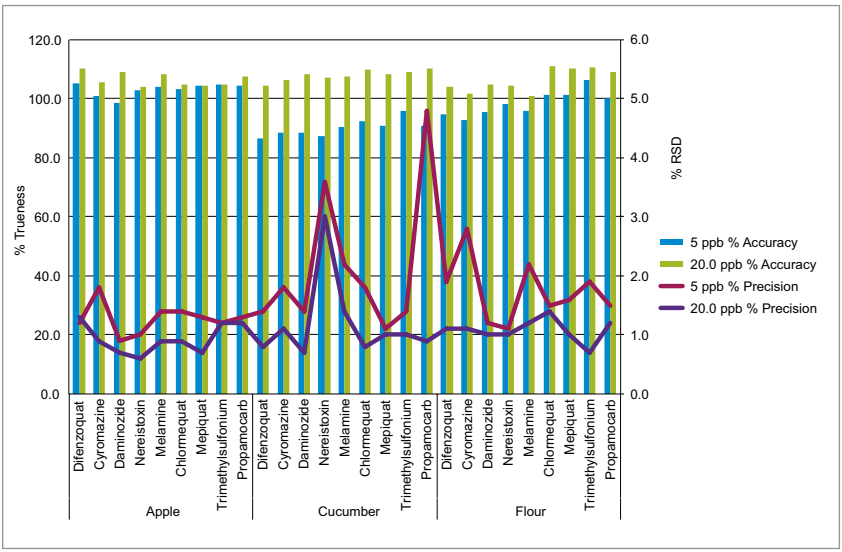

Figure 4. Replicate injection data for all tested compounds in apple, cucumber, and wheat flour. The primary axis is the mean %trueness to the target in vial concentration level and the secondary axis is the %RSD at each level (n=15).

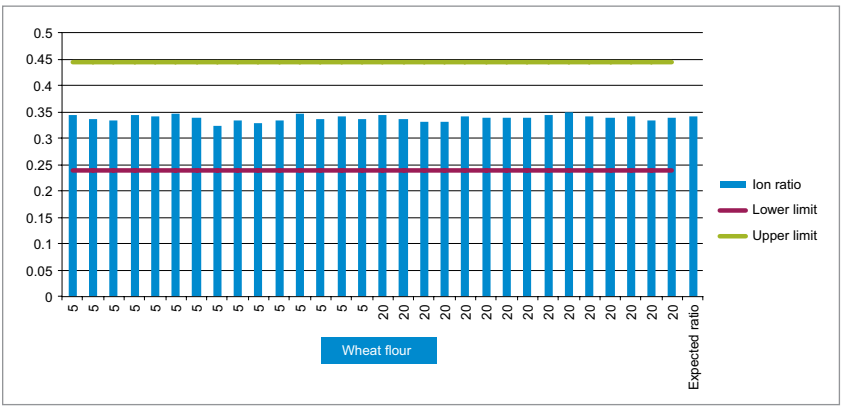

Figure 5. Calculated ion ratios (target/quan) for chlormequat at each replicate level (n=15) in wheat flour.

CONCLUSIONS

The aim of this study was to evaluate the combination of the ACQUITY UPLC I-Class System coupled with Xevo TQ-S micro for the determination of highly polar cationic pesticide residues and plant growth regulators in several food commodities. The Xevo TQ-S micro provided excellent, fit-for-purpose performance in terms of sensitivity, linearity, and calibration range for all of the tested matrices. The trueness and precision of this UPLC-MS/MS method determined at two matrix QC levels with 15 replicate injections was found to be acceptable for all compounds. Overall the performance data indicate that the configuration of the ACQUITY UPLC I-Class coupled with Xevo TQ-S micro, when used in combination with the ACQUITY UPLC BEH Amide Column and an established extraction protocol such as QuPPe, is suitable for checking MRL/tolerance compliance in routine laboratory testing for these target compounds.

References

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2. A Comprehensive Guide to HILIC: Hydrophillic Interaction Chromatography. (2014) Waters Corporation (p/n: 715002531). (Accessed online 30 August 2018).
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5. Document No. SANTE 11813/2017. Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticides Residues Analysis in Food and Feed European Union (2017).

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Determination of Anionic Polar Pesticides in Wheat Flour Extracts Using UPLC-MS/MS with the Torus DEA Column

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APPLICATION BENEFITS

This method is suitable for the determination of a range of polar anionic pesticides in wheat flour extract to facilitate monitoring of MRL/tolerance compliance. The method offers excellent chromatographic retention, selectivity, peak shape, and stability coupled with sufficient sensitivity to determine residues at concentrations as low as 0.01 mg/kg (10 ppb) levels in crude extracts without cleanup.

WATERS SOLUTIONS

Torus™ DEA Column

ACQUITY™ UPLC™ H-Class Bio System

Xevo™ TQ-XS

MassLynx™ MS Software

TargetLynx™ Application Manager

KEYWORDS

UPLC-MS/MS, anionic polar pesticides, targeted analysis, wheat flour, glyphosate, Quick Polar Pesticides (QuPPe) method

This Application Note was developed on a Torus DEA Column, but improved performance can now be achieved using the Waters Anionic Polar Pesticide Column. Please contact Waters Chemistry Technical Services with any questions www.waters.com/contact.

INTRODUCTION

Although various multi-residue LC-MS/MS methods are available to analyze food for pesticide residues, polar, anionic pesticides and their metabolites remain a considerable challenge. The QuPPe (Quick Polar Pesticides) method[1] allows the simultaneous extraction of many of these highly polar compounds. QuPPe is typically used with LC-MS/MS instruments offering high sensitivity in order to deal with the significant matrix effects associated with the crude extracts (no cleanup).

Previously, we have reported the results of the validation of a method based on QuPPe using Waters™ Torus DEA Column (p/n: 186007616) for the determination of polar pesticides and their metabolites in spinach.[2] The Torus DEA Column provides HILIC and WAX interactions, which has been shown to offer sufficient retention of these highly polar and ionic compounds while providing excellent retention time stability, selectivity, and peak shape.

In this application note, we report the performance data from an assessment of the slightly modified LC-MS/MS method for the determination of relevant anionic polar pesticides in wheat flour extracts, representative of commodities with high starch and/or protein content, low water and fat content. The previous LC-MS/MS method was developed on I-class. For similar chromatographic performance, method was transfer for H-class. Along with evaluating performance in a more complex and difficult matrix, this work also demonstrates how the method can be transferred to the ACQUITY UPLC H-Class Bio System. This system has the advantage of an inert flow path which reduces the unwanted interactions between some of these anionic analytes and metals in the UPLC system.

EXPERIMENTAL

Sample preparation and extraction

A sample of wheat flour was purchased from a retail outlet and stored frozen. Test portions were extracted using the EURL Quick Polar Pesticides (QuPPe) method,[1] modified by the addition of a freezing out step, prior to centrifugation. The details of the method are summarized in Figure 1.

The performance of the overall method was assessed as per in accordance with SANTE guidelines.[3] Solutions of matrix-matched standards were prepared over the range 0.005 to 0.250 mg/kg (5 to 250 ppb) and analyzed to determine the concentration of the anionic pesticides and metabolites in replicate injections of the standards at 0.1 mg/kg (using bracketed calibration) to realistically mimic the impact on the instrument of a routine batch of samples. Replicate injections of the 0.1 mg/kg spiked samples (n=15) were run to determine the reproducibility of the LC-MS/MS method.

UPLC conditions

Before use, the LC system and column requires simple cleaning and conditioning steps to remove metal ions that have been shown to interact with polar pesticides and cause poor peak shapes. Details can be found in the Waters Start-Up Guide[4] (p/n: 720006156EN).

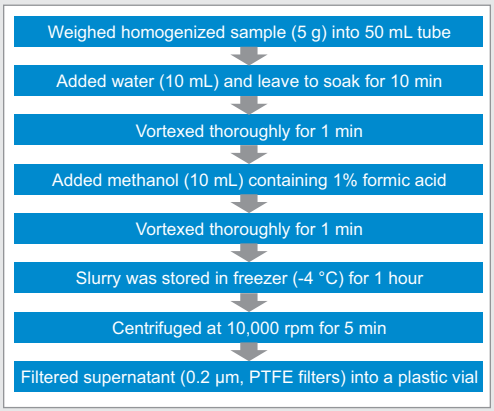

Figure 1. Schematic showing the modified Quick Polar Pesticides (QuPPe) method.

UPLC system:	ACQUITY UPLC H-Class Bio with FTN Sample Manager
Column:	Torus DEA 1.7 µm, 2.1 × 100 mm
Mobile phase A:	50 mM Ammonium formate + 0.9% formic acid
Mobile phase B:	Acetonitrile + 0.9% formic acid
Flow rate:	0.5 mL/min
Injection volume:	10 µL
Weak wash solvent:	90:10 acetonitrile:water
Strong wash solvent:	10:90 acetonitrile:water
Column temp.:	50 °C
Sample temp.:	10 °C
Run time:	20 min

Gradient:

Time (min)	%A	%B	Curve
0.00	10	90	–
4.00	60	40	2
5.00	90	10	6
8.5	90	10	1
15.5	10	90	1

MS conditions

MS system:	Xevo TQ-XS
Ionization:	ESI-
Capillary voltage:	2.5 kV
Ion counting threshold:	250
Desolvation temp.:	600 °C
Desolvation gas flow:	1000 L/Hr
Source temp.:	150 °C
Cone gas flow:	300 L/Hr
Collision gas flow:	0.14 mL/min
Nebulizer gas pressure:	7 Bar

Data was acquired using MassLynx MS Software v.4.2 and processed using TargetLynx XS Application Manager. The selection of MRM transitions and optimization of critical parameters was performed by infusion of individual solutions of each of the analytes and evaluation of the data by IntelliStart™ Software to automatically create acquisition and processing methods. Soft ionization mode was enabled for ethephon. Soft ionization mode is a function in the MS acquisition file that applies a shallower gradient of voltages to the ion transfer optics and improves transmission. Soft ionization is designed to reduce fragmentation and improve transmission of (fragile) compounds. As shown in Figure 2, increases in sensitivity (2X) and peak area (1.8X) were observed for Ethephon under soft ionization mode which ultimately improves sensitivity.

*Table 1. MRM parameters for anionic polar pesticides (quantitative transitions in **bold**).*

Compound	Retention time (min)	MRM	Cone (V)	CE (eV)	Dwell time (s)
Aminomethyl-phosphonic acid (AMPA)	2.02	**110>63**	35	13	0.125
		110>79	35	14	0.125
3-Methylphosphinico-propionic acid (MPPA)	2.44	**151>133**	20	11	0.050
		151>107	20	14	0.050
Glufosinate	2.58	**180>85**	30	16	0.050
		180>95	30	16	0.050
N-Acetyl glufosinate (NAG)	2.98	**222>136**	30	20	0.020
		222>69	30	15	0.020
Fosetyl aluminium	3.01	**109>81**	20	10	0.010
		109>63	20	16	0.010
Ethephon	3.03	**143>107**	15	7	0.090
		143>79	15	7	0.090
Glyphosate	3.16	**168>63**	25	18	0.120
		168>150	25	9	0.120
Phosphonic acid	3.56	**81>79**	25	11	0.010
		81>63	25	13	0.010
N-Acetyl glyphosate	5.59	**210>150**	25	13	1.100
		210>192	25	9	1.100

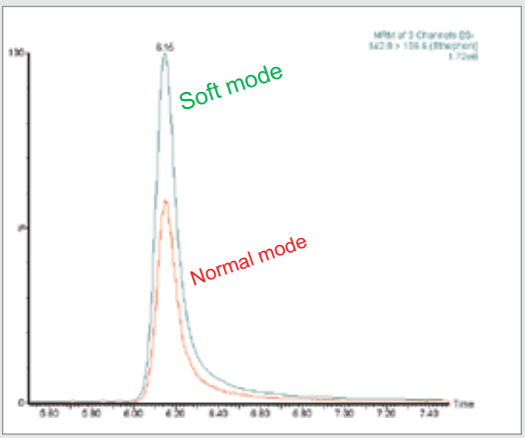

Figure 2. An overlay chromatograms of ethephon with soft ionization mode (green) and normal mode (red). Increased in intensity (2X) and peak area (1.8X) were observed for Ethephon with soft ionization mode.

RESULTS AND DISCUSSION

Excellent sensitivity and selectivity was demonstrated from the analysis of matrix-matched standards. Figure 3 shows the chromatography and response for the analytes at 0.01 mg/kg. The chromatgraphic separation of AMPA from Fosetyl AL, and Fosetyl AL from phosphonic acid was vital due to isobaric compounds and degradation issue respectively.

Calibration characteristics were assessed for the pesticides of interest through the use of bracketed calibration over a suitable concentration range, as shown in Figure 4. The coefficients of determination (r^2>0.99) and the residuals (referred to in the SANTE document as back-calculated concentrations; <20%)* were excellent, demonstrating good repeatability of the measurements, in the absence of labeled standards. Peak shapes remained stable without deterioration throughout the run. Replicate (n=15) injections of the matrix matched standard at 0.10 mg/kg (100 ppb) showed good precision, with RSDs <5% for all but fosetyl-Al (5.4% RSD) and AMPA (9.6% RSD).

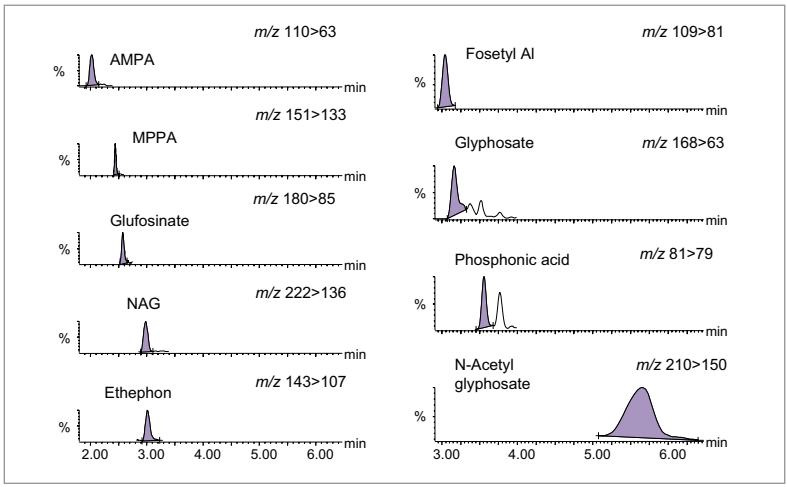

Figure 3. Typical chromatograms showing anionic polar pesticides from analysis of matrix-matched standard at 0.01 mg/kg (10 ppb) in wheat flour.

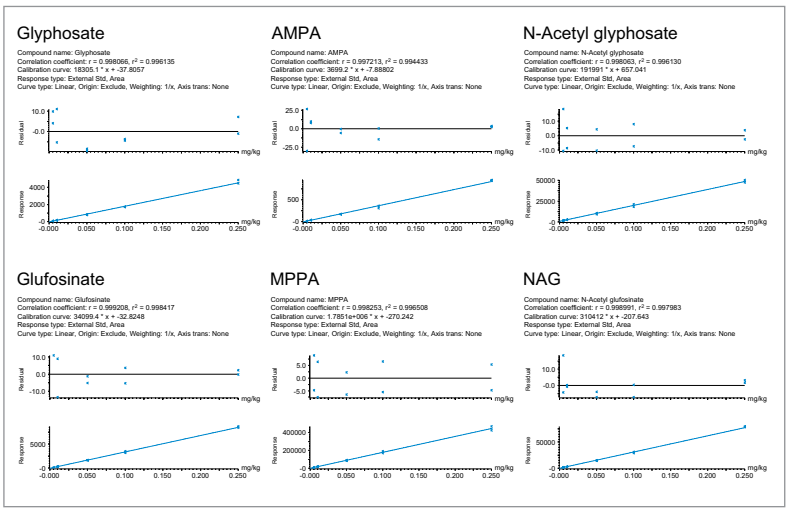

Figure 4. Calibration graphs for a selection of anionic polar pesticides, over the range in 0.005–0.250 mg/kg (5–250 ppb), in wheat flour.

Retention times from the matrix matched standards agreed well with reference values and most were within the required tolerances(+/- 0.1). Ion ratios were also within required tolerance (+/- 30%) for all matrix-matched standards. Overall all injections, samples and standards were mostly compliant with relevant tolerances. However, sensitivity/selectivity for the second transition was compromised for some of the compounds at concentrations ≤0.010 mg/kg (10 ppb).

Phosphonic acid was detected in the matrix used for preparation of the matrix matched standards. The concentration was calculated using standard addition feature of TargetLynx XS and found to be 0.002 mg/kg (2 ppb). Ion ratios and retention times were within the required tolerances of the SANTE guidelines.

The UPLC-MS/MS method performance has been determined to be suitable for monitoring MRL compliance of the target compounds in wheat flour. The scope of the analysis includes all of the components that make up the residue definition. Although the MRLs/tolerances for those pesticides approved for use on wheat vary across the globe, values tend to be in the ppm range (e.g. 10 and 30 mg/kg for glyphosate in the EU and U.S. respectively), so extracts can be diluted prior to analysis. This method also has been shown to have sufficient sensitivity to be used in combination with established extraction protocols for checking compliance with the EU default MRLs derived from the lower limit of analytical determination for these compounds (e.g. 0.03 mg/kg (30 ppb) for glufosinate in the EU).

CONCLUSIONS

The use of the Torus DEA Column provides excellent chromatographic performance for anionic polar pesticides and metabolites, even for the analysis of a complex and difficult matrix such as wheat flour. When coupled with the high sensitivity of the Xevo TQ-XS, these challenging compounds can be determined in a single analysis, without the need to use derivatization or specialized equipment. When used in combination with established extraction protocols, this method is suitable for checking MRL/tolerance compliance. Although we have shown data in a wheat flour extract that meet SANTE criteria, scientists must fully validate the method on their commodities of interest, in their own laboratories, to demonstrate that, when coupled with their extraction protocols, it is fit for purpose.

References

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Oasis PRiME HLB Cartridge for Clean-up of QuEChERS Extracts of Soybean Pods Prior to UPLC-MS/MS Determination of Free Acidic Herbicides

Kim Van Tran and Michxaael S. Young
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Efficient, time-saving multi-class/ multi-residue methodology
- Simple, rapid, and effective sample clean-up suitable for determination of acidic herbicides
- Simultaneous extraction and clean-up of neutral and basic pesticides
- Fast, sensitive UPLC™-MS/MS analysis

WATERS SOLUTIONS

ACQUITY™ UPLC I-Class System

Xevo™ TQ-XS Tandem Mass Spectrometer

Oasis™ PRiME HLB Cartridge for SPE clean-up

DisQuE™ Pouch for CEN QuEChERS

MassLynx™ MS Software

KEYWORDS

UPLC-MS/MS, acidic herbicides, multi-residues, pesticides, edamame, QuEChERS

INTRODUCTION

Acidic herbicides are commonly used for agricultural weed control. To help insure public health and safety, reliable analytical methods are necessary to determine residues of these herbicides in fruits and vegetables grown for human or animal consumption. For the analytical chemist, it is desirable to screen for multiple acidic herbicides with a single analytical method in order to maximize throughput and minimize costs. It is even more cost effective if the same single analytical extraction and clean-up method can be used to screen for acidic, neutral and basic pesticides. In this application note, a QuEChERS extraction and UPLC-MS/MS analysis method is demonstrated for multiresidue analysis of free (unbound) acidic herbicides in soybean pods. This vegetable (known as edamame) is a popular and nutritious foodstuff. However, this commodity is challenging for pesticide analysis; typically edamame is about 5–6% total fat and 0.3% phospholipid (lecithin) with significant amounts of pigments such as chlorophyll and carotenes. The presence of these co-extracted substances in the QuEChERS extract can lead to interference in the UPLC-MS analysis, contamination of the analytical column, and other components of the UPLC system, and contamination of the mass spectrometer itself. The Oasis PRiME HLB Cartridge is highly effective for removing fats, phospholipids, and chlorophyll from QuEChERS extracts of edamame. A QuEChERS extraction method has been successfully applied to the analysis of free acidic herbicides, but dSPE clean-up was not employed.[1] Common dispersive SPE methods (dSPE) using PSA sorbents for clean-up cannot be used for acidic herbicides because the acidic compounds are retained on the sorbent.[2] However, pass-through clean-up with the Oasis PRiME HLB Cartridge provides good recovery for acidic herbicides. Therefore, the same QuEChERS extract can be used to screen for acidic and non-acidic herbicides and other pesticides after a single pass-through clean-up using the Oasis PRiME HLB Cartridge.

This application note highlights a clean-up protocol, part of a multiresidue analytical method, suitable for unbound (free) acidic herbicides and also suitable for base/neutral herbicides. This is not a class specific method optimized for bound and unbound acidic herbicides; such an optimized method is currently under development. An application note or other publication will soon be presented for a class specific method for acidic herbicides and metabolites after basic hydrolysis.

EXPERIMENTAL

UPLC conditions

LC system: ACQUITY UPLC I-Class

Column: ACQUITY UPLC HSS T3, 1.8 µm, 2.1 × 100 mm

Mobile phase A: 0.02% Formic acid in water

Mobile phase B: Acetonitrile:MeOH (50:50)

Injection volume: 10 µL

Injection mode: Partial loop injection

Column temp.: 25 °C

Weak needle wash: 10:90 Acetonitrile:water (600 µL)

Strong needle wash: 50:30:40 Water:acetonitrile:IPA (200 µL)

Seal wash: 10:90 acetonitrile:water

Gradient:

Time (min)	Flow (mL/min)	%A	%B
0.00	0.400	95.0	5.0
5.00	0.400	5.0	95.0
6.00	0.400	5.0	95.0
6.10	0.400	50.0	50.0
6.50	0.500	50.0	50.0
6.80	0.500	95.0	5.0
7.00	0.400	95.0	5.0
8.00	0.400	95.0	5.0

MS conditions

MS system: Xevo TQ-XS

Ionization mode: ESI+

Source temp: 120 °C

Desolvation temp.: 300 °C

Desolvation gas flow: 1000 L/hr

Cone gas flow: 30 L/hr

Collision gas flow: 0.15 mL/min

Data management: MassLynx v4.2

Monitored transitions: see Table 1

Table 1. MRM transitions and instrument parameters used for this study; also presented in Table 1 are observed retention times (RT).

Name	MRM	Cone (v)	Collision (eV)	Retention time (min)
Compounds Analyzed in ES-				
2, 4-DP	233.0>161.0 233 .0>125.0	28	10 30	4.80
2,4-D	218.9>161.0 218.9>125.0	26	15 40	4.45
2,4-DB	246.9>160.9 246.9>125.0	12	10 10	4.97
2,4,5-T	252.8>194.9 252.8>158.9	19	14 36	4.86
2,4,5-TP (Silvex)	268.9>196.9 268.9>161.0	28	15 30	5.16
3,6-Dichloro-2-hydroxy benzoic acid (Dicamba metabolite)	204.9>160.9 204.9>124.9	14	11 11	3.77
4-CPA	185.0>127.0	28	16	3.93
Bentazone	239.0>132.0	30	30	4.06
Bromoxynil	275.8>80.8 275.8>78.8	48	30	4.34
Dicamba	218.8>174.8 218.8>145.0	9	9 9	3.69
Fenoxaprop-P	332>151.9 332>115.9	70	50 32	5.24
Fluazafop-P (butyl)	384.1>282.1 384.1>328.1	38	22 16	5.78
Fluroxypyr	254.9>208.8 254.9>180.8	28	16 12	3.82
Fomesafen	437.1>195.0 437.1>222.0	59	30 30	5.14
Imazaquin	310.0>266.0 310.0>233.0	20	16 25	4.01
Ioxynil	369.7>126.8 369.7>215.0	40	30 30	4.65
MCPA	199.2>140.9 201.0>143.0	20	10 8	4.48
MCPB	227.0>140.9	15	20	4.99
MCPP	213.0>141.0 213.0>118.8	21	14 14	4.81
Triclopyr	255.9>220.1 255.9>197.9	20	5 10	4.68
Compounds Analyzed in ES+				
Cycloxydim	326.0>280.0 326.0>180.0	34	16 22	5.82
Imazapyr	262.2>86.1 262.2>69.2	38	26 26	2.76
Imazethapyr	290.2>245.2 290.2>177.1	45	20 25	3.67
Haloxyfop	362.0>288.0 362.0>272.0	28	26 32	5.26
Imazosulfuron	413.0>152.8 413.0>155.9	7	12 18	4.77
Metosulam	418.0>175.0 418.0>140.0v	41	28 52	4.19
Metsulfuron methyl	382.0>167.0 382.0>198.9	28	16 22	4.03
Picloram	241.0>168.0 241.0>195.0	26	30 21	2.56
Quinmerac	222.2>204.2 222.2>141.1	17	15 30	3.24
Thifensulfuron methyl	388.0>167.0 388.0>56.0	25	15 15	3.93

Sample preparation

Initial extraction/precipitation

Place a 5 g homogenized sample into a 50-mL centrifuge tube. Add fortification standards if required and allow 30 min to equilibrate. Add 10 mL of water, vortex for 10 seconds, and add 10 mL of acetonitrile. Vortex for 30 seconds, and then add contents of DisQuE QuEChERS Pouch for CEN, p/n: 186006813. Vortex for 10 seconds and then place on mechanical shaker for 10 minutes. Centrifuge at 4000 rpm for 5 min. Portions of the supernatant (top layer) are taken for clean-up.

Note: The extraction/precipitation step gives good recovery of most compounds of interest but also extracts significant amounts of fat and phospholipids.

Oasis PRiME HLB clean-up (left) and no clean-up (right).

SPE clean-up

Mount an Oasis PRiME HLB 3 cc Vac Cartridge, 150 mg , p/n 186008717 on a pre-cleaned vacuum manifold. The vacuum is set to 1–2 psi. Approximately 0.7 mL of the QuEChERS supernatant is passed through the Oasis PRiME HLB Cartridge and discarded. After collection vessels are installed in the manifold, approximately 1.2 mL of the supernatant is passed through the cartridge and collected. Exactly 0.20 mL of the collected fraction is diluted with 0.40 of reagent water for UPLC-MS/MS analysis.

RESULTS AND DISCUSSION

Figure 1 shows the total method recovery data obtained from six replicate analyses of edamame samples spiked at 1, 10, and 100 ng/g. The chromatograms shown in Figure 2 demonstrate the effectiveness of the Oasis PRiME HLB Cartridge for removal of ≥95% of the phospholipids from the edamame extracts. The overall method recoveries are generally above 70% although lower recovery was observed for a few of the more polar acidic herbicides such as picloram and the dicamba metabolite. It is important to distinguish any recovery losses resulting from the SPE clean-up from losses resulting from the initial QuEChERS extraction. The graph presented in Figure 3 compares the total recovery (red bars) with the SPE recovery (blue bars) measured at the 100 ng/g spike level. The red data were obtained by spiking the sample before QuEChERS extraction and SPE clean-up; the blue data were obtained by spiking the extract after QuEChERS extraction and before SPE clean-up. For picloram, this shows that most of the recovery loss occurred during the QuEChERS extraction step and not from the Oasis PRiME HLB pass-through clean-up step.

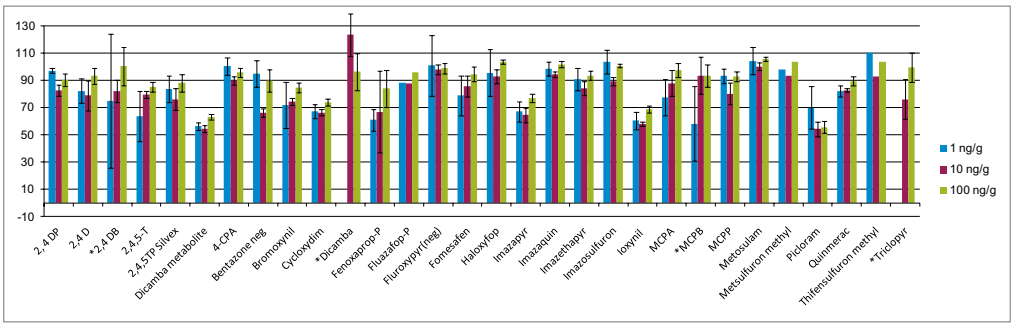

*Figure 1. Recovery of acidic herbicides from edamame after QuEChERS extraction and Oasis PRiME HLB pass-through clean-up (error bars indicate standard deviation for six replicate analyses, * indicates LOQ above 1 ng/g).*

QuEChERS methods have been widely accepted for pesticide analysis in many commodities. Among the hundreds of pesticides amenable to extraction using QuEChERS are many acidic herbicides. Traditional dispersive clean-up (dSPE) using PSA (primary/secondary amine silica) cannot be used for acidic herbicides because the compounds will be retained on the sorbent. Therefore, the analyst must prepare two separate aliquots of the QuEChERS extract for analysis with separate clean-up strategies for the acids and for base/neutrals. However, unlike PSA, Oasis PRiME HLB sorbent does not rely on ion-exchange mechanisms for removal of phospholipids and related contaminants and does not retain acidic pesticides. Therefore, a single aliquot of QuEChERS extract can be subjected to a rapid pass-through clean-up prior to analysis for both acids and base/neutrals.

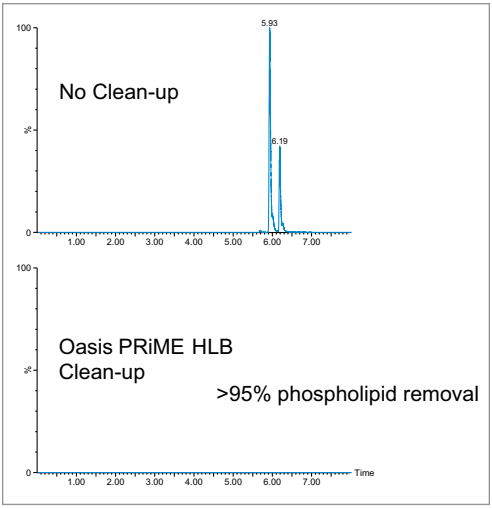

Figure 2. UPLC-MS/MS chromatograms showing effective removal of ≥95% of phospholipids from edamame QuEChERS extract (transitions monitored: 496.4, 520.0, 522.0, and 524.0 m/z, all to 184.4 m/z).

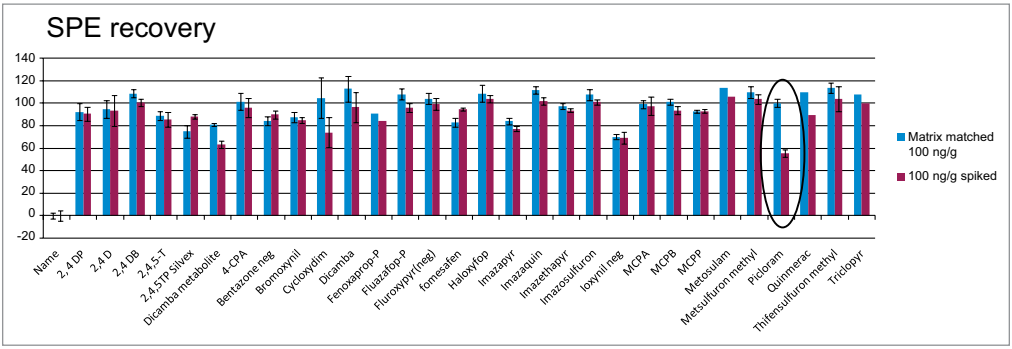

Figure 3. Comparison of total method recovery (red bars) with SPE recovery (blue bars) of acidic herbicides.

CONCLUSIONS

- Pass-through clean-up with the Oasis PRiME HLB Cartridge is effective for the removal of fats, phospholipids, and pigments from QuEChERS extracts of edamame.
- Good recoveries of acidic herbicides were obtained after pass-through clean-up with the Oasis PRiME HLB Cartridge; this is not possible using dSPE with PSA.
- Oasis PRiME HLB provides good cleanup for acid, base, and neutral pesticides in one step; dSPE with PSA cannot.

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Determination of 208 Pesticide Residues and their Metabolites in Foods Using Oasis PRiME HLB and Xevo TQ-GC

Defeng Huang,[1] Lauren Mullin,[2] Adam Ladak,[2] and Jonathan Fox[3]
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APPLICATION BENEFITS

Efficient workflows enable reliable determination of multiple residues across a variety of challenging food commodities. Waters offers a range of sample preparation techniques that provide improved accuracy for quantifying contaminants.

- Simple pass-through cleanup is readily incorporated into the QuEChERS workflow to maintain accuracy and precision in the quantitative performance, while improving overall method robustness.
- Easy method transfer, development, and updates.
- Fit-for-purpose to achieve Chinese National Standard Method regulatory requirements for GC-MS/MS pesticides.

WATERS SOLUTIONS

Xevo™ TQ-GC System

DisQuE™ QuEChERS Dispersive Solid Phase Extraction

Oasis™ PRiME HLB Plus Short Cartridge

TruView™ LCMS Certified Vials

MassLynx™ MS Software

Quanpedia™ Database

KEYWORDS

GC-MS, mass spectrometry software, QuEChERS, sample extraction, sample cleanup, pesticides, GB 23200.113-2018

INTRODUCTION

Gas chromatography-mass spectrometry (GC-MS) has been a common analytical method for pesticide measurement due to its high efficiency of separation, along with its qualitative and quantitative performance. As a milestone of pesticides analysis, Lehotay[1] and Nguyen, et al.[2] established a sample preparation method based on QuEChERS technology in 2015 for the simultaneous detection of multiple pesticide residues in vegetables and other foods using LC-MS/MS and GC-MS/MS. In recent years GC-MS/MS analysis has become the preferred method for pesticides analysis due to its advantages in selectivity, sensitivity, high throughput, and accurate quantitative performance.[3]

Recently, the first Chinese National Standard Method (GB 23200.113-2018)[4] for multiple pesticide residues using GC-MS/MS was released. For the first time in GB methodology, two efficient technologies have been adopted: QuEChERS for sample extraction, and GC-MS/MS for detection.

In this application note, foodstuffs of plant origin were further cleaned using Waters™ Oasis PRiME HLB following QuEChERS extraction and run on the Xevo TQ-GC to quantify 208 pesticides and their metabolites in fruits and vegetables. Rigorous method verification was carried out following the SANTE/11813/2017 guidance document,[5] which provided strong evidence that the method is fit for purpose and will achieve the method validation criteria set by the GB 23200.113-2018.

EXPERIMENTAL

Sample preparation

Cucumber, grape, and rice samples were purchased from local retail outlets and prepared using a modified version of QuEChERS sample preparation as reported in CEN method 15662.[6] The sample preparation used is summarized in Figure 1.

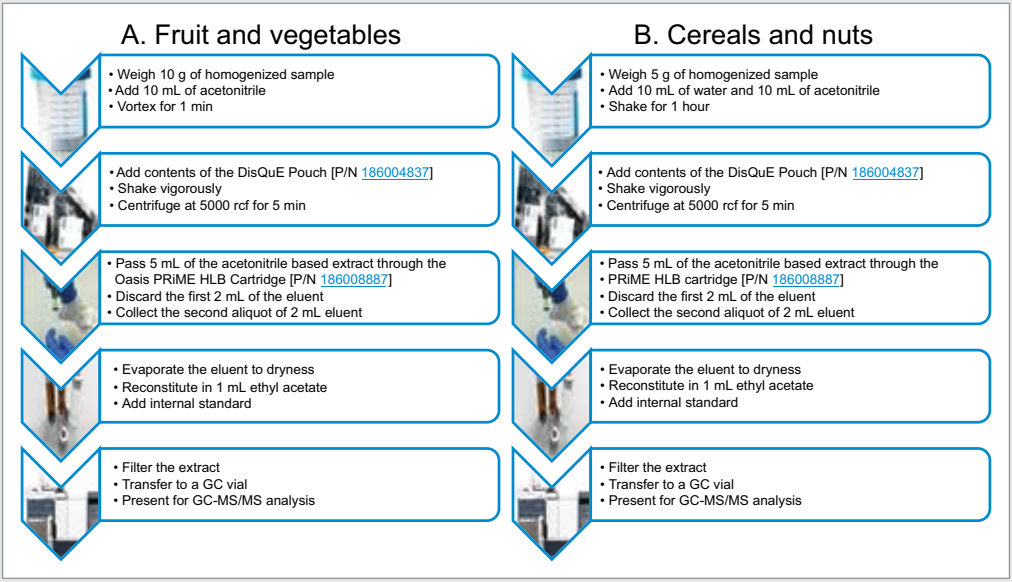

Figure 1. Sample preparation for A. fruits and vegetables, and B. cereal and nuts.

GC conditions

Column:	Rtx-1701 (30 m × 0.25 mm × 0.25 µm)
Carrier gas:	Helium
Gas flow rate:	1.0 mL/min
Injection type:	Pulsed splitless
Injection liner:	Gooseneck splitless, 4 mm × 6.5 × 78.5 (Restek)
Inlet temp:	280 °C
Pulse time:	1.0 min
Pulse pressure:	170 kPa
Purge flow:	30 mL/min
Septum purge flow:	3 mL/min
Wash solvent:	Hexane
Oven program:	80 °C (hold 1.1 min) to 120 °C at 40 °C/min, then to 240 °C at 5 °C/min, then 295 °C at 12 °C/min and hold 8 min
Run time:	38.68 min
Injection volum:	1 µL

MS conditions

MS system:	Xevo TQ-GC
Software:	MassLynx v4.2
Ionization mode:	EI, 70 eV
Source temp.:	250 °C
GC interface:	300 °C
MRM conditions:	All transitions were imported from the Waters™ Quanpedia Database. IntelliStart™ Custom Resolution settings were used.

RESULTS AND DISCUSSION

OPTIMIZATION OF SAMPLE PREPARATION

Typically for GC, pigments are undesirable because they can potentially contaminate the injection liner and the GC column. Graphitized carbon black (GCB) is commonly used to remove pigments. However caution is advised with the level of GCB used since it is both a reverse phase and an anion exchange sorbent and can potentially trap certain pesticides, especially for pesticides with planar structure. Therefore it is important to optimize the amount of GCB used to capture the maximum amount of pigment while maintaining good recovery of pesticides, which can be a time-consuming exercise. In this work GCB was not used, but instead a novel sorbent, Oasis PRiME HLB was employed. Oasis PRiME HLB has recently been used to quickly and efficiently remove co-extractives including fats and phospholipids, as well as pigments from food matrices, using a simple and fast pass-through protocol.[7] In this study, Oasis PRiME HLB provided excellent pigment removal, thus reducing the contamination of the GC inlet liner and extending the lifetime of the GC consumables.

QUANPEDIA FOR METHOD CREATION

GC-MS/MS methods for GB 23300.113-2018 were easily generated using Quanpedia Database. This provided the creation of the GC, MS/MS, and processing methods in three simple clicks, as shown in Figure 2. Quanpedia can greatly reduce time and lab resources employed for setting up new multi-residue methods.[8]

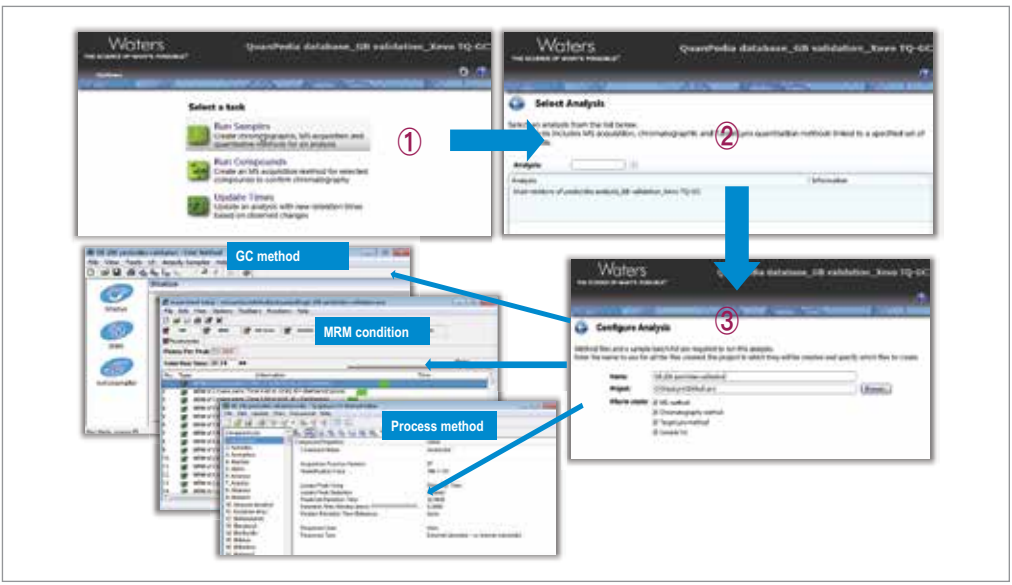

Figure 2. The complete GB method is available in the Quanpedia Database which can be set up with only three clicks.
Click 1: Run Samples. Click 2: Select Method. Click 3: Configure Analysis parameters required (GC, MS, and processing methods).

METHOD PERFORMANCE

In-house method verification was carried out to determine the overall method performance in accordance with the requirements of GB method 23300.113-2018, referencing the SANTE/11813/2017 guidance document and associated analytical and validation criteria.5 The method performance was assessed for trueness, reproducibility, quantification, and identification of 208 pesticides and associated metabolites in cucumber, grape, and rice. For each commodity (n=3), matrix matched calibration curves were generated and replicate spikes (n=6) were extracted at three concentrations (LOQ, 2x LOQ, and 5x LOQ. The results, as summarized in Table 1, were within the permitted tolerances of the required guidelines demonstrating that this method is fit for purpose.

Table 1. Summary of the in-house verification results for pesticides and associated residues in rice, cucumber, and grape at relevant concentrations (LOQ, 2x LOQ, and 5x LOQ).

Parameter	SANTE criteria	Rice	Grape	Cucumber	Criteria satisfied
Retention time	±0.1 minute	20.49–20.50	18.69–18.70	18.67–18.70	✓
Ion ratio	±30%	1.92–2.28	1.55–2.43	1.92–2.28	3
Residuals	±20%	≤20%	≤20%	≤20%	3
Recovery (trueness)	70 to 120%	103.6%	93.4%	96.9%	3
Repeatability (RSDr)	≤20%	2.6%	3.5%	2.8%	3
LOQ	≤MRL	0.02 mg/kg	0.01 mg/kg	0.01 mg/kg	3

TRUENESS AND REPRODUCIBILITY

Trueness and repeatability were assessed from the analysis of the three commodities: cucumber, grape, and rice. Each commodity was spiked at three concentration levels: LOQ, 2x LOQ, and 5x LOQ with five replicates (n=5) of each concentration prepared. In this study, the method performance is reported for each commodity spiked at the LOQ only, namely cucumber at 0.01 mg/kg, grape at 0.01 mg/kg, and rice at 0.02 mg/kg. These spiked concentrations were selected based on the LOQs defined in GB method 23200.113-2018. Figure 3 shows the chromatograms from some of the pesticides spiked at 0.01 mg/kg in rice, demonstrating that the sensitivity for these compounds is much lower than the required LOQ specified in the GB method.

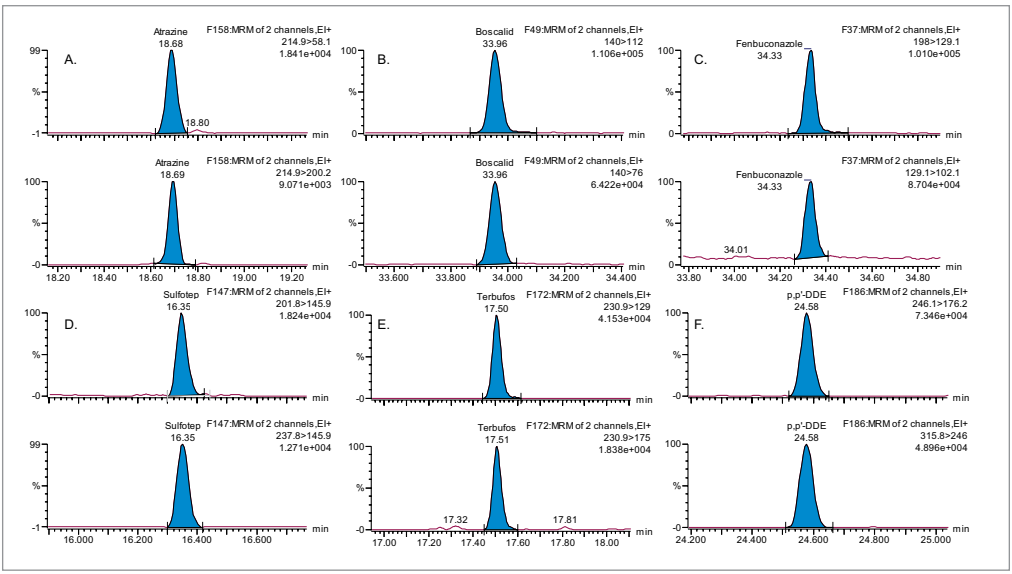

Figure 3. Two MRM transitions of A. atrazine, B. boscalid, C. fenbuconazole, D. sulfotep, E. terbufos, and F. p,p'-DDE spiked at 0.01 mg/kg (typical MRL) in rice.

Figure 4 shows the measured percentage recovery (trueness; between 70 and 120%) and repeatability (%RSD; <20%) for a representative selection of 15 pesticides in all of the commodities tested. Further details on recovery and repeatability for all 208 pesticides at the required LOQ across each commodity are summarized in Table 2, in the Appendix, which meet the acceptance criteria of the GB method.

QUANTIFICATION

Matrix-matched calibration curves allowed for accurate quantification of pesticides spiked in the commodity at the required LOQs. Calibrations were prepared over the concentration range of 0.005 mg/kg to 0.2 mg/kg for each target compound using internal standards. A weighted linear regression (1/x) was applied. Individual back-calculated concentrations were calculated automatically by TargetLynx™ Application Manager, and all were within the tolerance set in the SANTE guidelines (±20%). Figure 5 shows matrix-matched calibration plots for five representative pesticides.

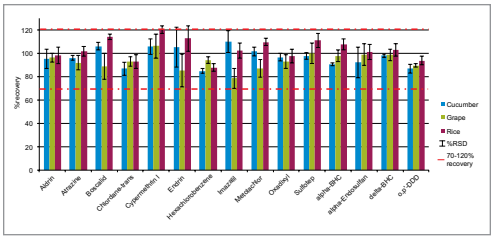

Figure 4. The measured recoveries (trueness) and repeatability (%RSD) of pesticides spiked at the required LOQ.

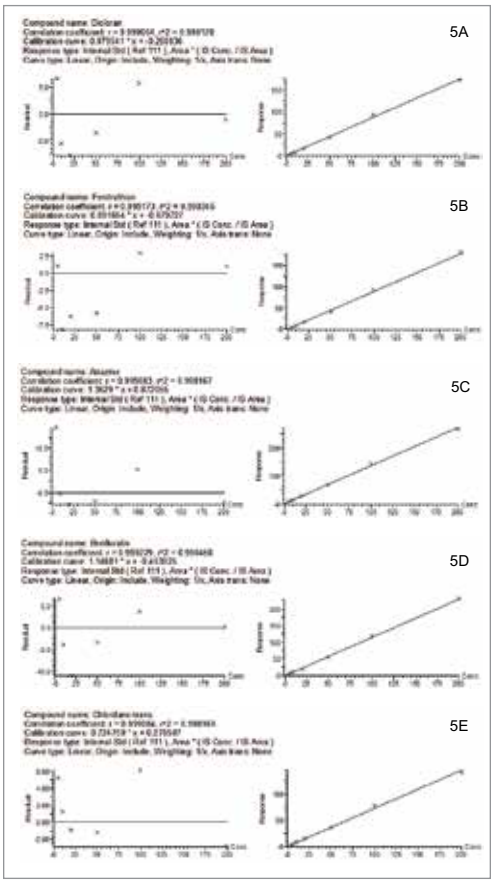

Figure 5. Examples of matrix-matched calibration graphs and residual plots for typical pesticides in the study generated automatically in a TargetLynx report (dicloran, fenitrothion, atrazine, benfluralin, and chlordane-trans).

IDENTIFICATION CRITERIA

The GC-MS GB Methods reference the SANTE requirements with respect to retention time and ion ratio tolerances. The guidelines state that the retention time of the analyte in the extract should be ±0.1 min to that of the calibration standard, and that ion ratios from sample extracts should be within ±30% of the reference (averaged calibration standards in the same sequence).

Using atrazine as an example, Figure 6 and Figure 7 show the plot of ion ratios and delta retention time, demonstrating that the analytical criteria within the guidelines were met.

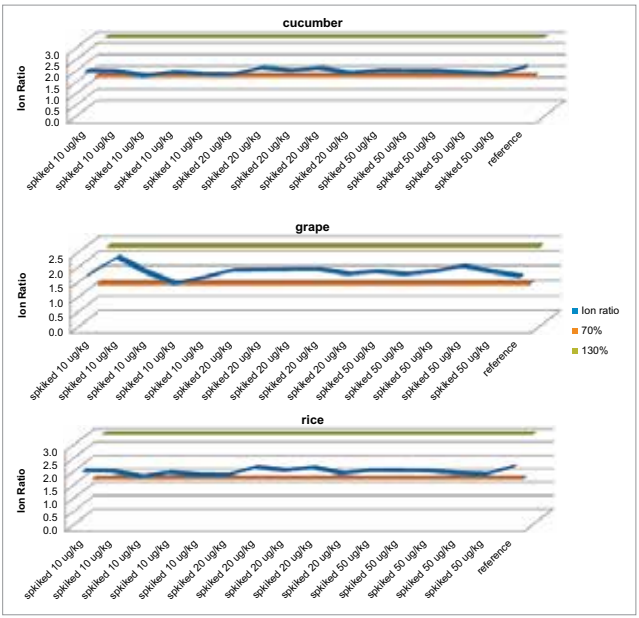

Figure 6. Plots of ion ratios for atrazine fortified in cucumber, grape, and rice showing that the ion ratios are within ±30%, per the SANTE guidelines.

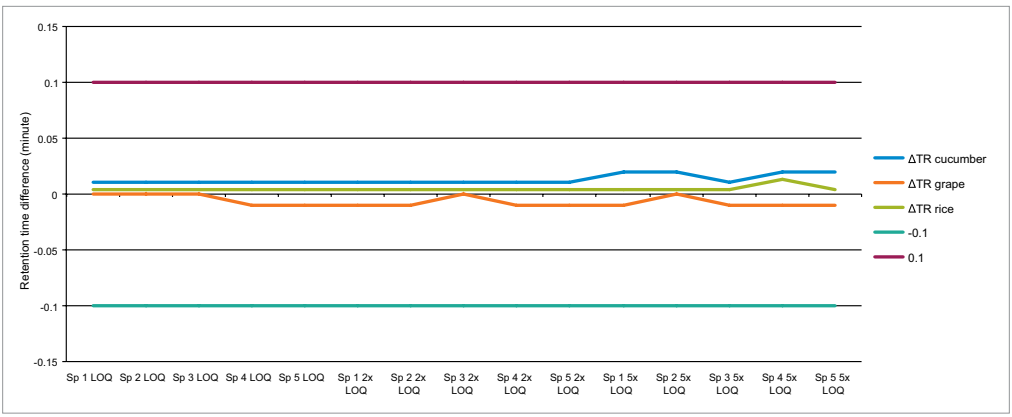

Figure 7. Plots showing retention time differences for atrazine fortified in cucumber, grape, and rice showing consistent retention times within ±0.1 min, meeting the SANTE guidelines.

CONCLUSIONS

The Xevo TQ-GC System is supplied with a Quanpedia method containing the appropriate GC conditions, MRM transitions, associated parameters, and processing methods that will facilitate implementation of GB method 23200.113-2018 in any food safety laboratory.

The addition of Oasis PRiME HLB Plus clean up to QuEChERS extraction, instead of dSPE, produced cleaner samples, allowing for a more robust analytical method.

More than 95% of the pesticides showed measured recoveries within the range of 70% to 120% range and repeatability (RSD) was <20% (n=5) for all compounds in all commodities.

The Xevo TQ-GC was able to easily meet the LOQs required by GB method 23200.113-2018, and in many cases surpassed them.

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Appendix

Table 2. The trueness (percentage recovery) and precision (%RSD) of pesticides spiked at LOQ levels.

	Cucumber 0.01 mg/kg		Grape 0.01 mg/kg		Rice 0.02 mg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Acetochlor	90.9	11.5	85.7	9.0	110.6	5.4
Aclonifen	87.1	18.5	82.4	10.7	97.5	2.7
Acrinathrin	106.8	4.8	96.9	7.3	109.7	3.8
Alachlor	102.1	5.8	84.5	7.1	116.1	5.7
Aldrin	95.3	8.3	96.3	3.8	98.3	7.1
Ametryn	103.7	5.3	72.9	9.1	91.0	3.0
Anilofos	96.1	6.0	94.7	3.8	110.7	5.2
Atratone	99.8	5.0	77.2	4.6	110.6	10.0
Atrazine	96.0	2.1	91.9	6.1	101.8	4.2
Atrazine-desethyl	92.5	9.3	87.4	11.6	102.8	6.3
Azinphos-ethyl	100.1	4.9	99.0	4.7	114.2	3.0
Beflubutamid	96.7	5.6	81.3	6.3	108.3	2.6
Benalaxyl	100.0	5.4	82.0	3.0	112.8	6.9
Benfluralin	91.6	3.2	89.8	8.4	113.1	3.3
Bifenox	103.3	13.0	98.2	6.7	101.9	8.1
Bifenthrin	89.2	3.3	84.2	11.8	106.7	2.9
Biphenyl	64.9	2.2	99.7	7.5	84.2	10.9
Boscalid	106.0	3.3	88.9	11.1	114.0	2.2
Bromacil	99.9	6.4	78.3	14.4	97.3	11.9
Bromfenvinfos	99.4	4.6	84.1	5.1	104.0	5.3
Bromophos	96.7	8.8	81.4	6.5	106.1	5.8
Bromophos-ethyl	92.9	5.5	80.8	2.9	98.9	4.4
Bromopropylate	96.7	4.9	91.6	8.6	100.4	1.6
Bupirimate	88.3	6.3	80.1	10.8	96.8	5.1
Butachlor	113.7	6.0	82.2	10.8	116.4	2.3
Butamifos	94.3	7.6	84.3	8.2	108.3	2.8
Carbofuran	110.3	7.0	99.8	6.9	114.5	7.1
Carbophenothion	85.1	6.0	77.9	12.0	94.6	5.0
Chlordane-trans	86.9	5.7	93.1	4.1	93.0	5.9
Chlorfenson	94.5	3.2	91.1	4.6	105.1	3.5
Chlorfenvinphos	98.0	5.9	79.5	7.1	105.2	5.1
Chloroneb	75.5	1.0	103.6	7.7	103.4	3.6
Chlorpropham	89.4	3.5	100.1	6.2	88.7	1.6
Chlorpyrifos	105.0	5.5	88.8	8.6	110.9	4.3
Chlorpyrifos-methyl	100.3	3.6	91.0	7.6	111.5	3.4
Chlorthiophos-1	99.3	6.4	83.7	12.4	99.7	4.7
Chlorthiophos-2	96.6	3.0	93.7	6.1	88.5	4.7
Clomazone	94.9	2.2	88.2	5.0	105.0	3.9
Coumaphos	99.2	4.1	94.9	5.5	111.8	3.6

	Cucumber 0.01 mg/kg		Grape 0.01 mg/kg		Rice 0.02 mg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Cycloate	90.2	4.4	99.2	9.8	105.6	3.7
Cyflufenamid	91.9	11.2	81.2	18.3	101.0	18.3
Cyfluthrin-1	106.0	2.7	110.3	10.1	120.0	5.6
Cyfluthrin-2	101.1	1.5	101.8	4.8	118.7	2.3
Cyfluthrin-3	92.9	2.5	94.8	7.0	117.8	4.4
Cyfluthrin-4	100.2	11.2	104.6	1.8	120.1	4.1
Cypermethrin -1	105.8	6.7	106.2	10.3	120.2	3.3
Cypermethrin -2	96.4	2.5	97.4	7.3	116.0	3.2
Cypermethrin -3	92.2	5.5	100.5	4.5	118.2	4.2
Cypermethrin -4	97.4	9.8	97.0	5.1	127.7	6.7
Cyproconazole-1	97.9	4.3	86.0	1.3	105.7	3.8
Cyproconazole-2	103.3	4.8	76.0	9.8	106.2	3.4
Cyprodinil	93.8	3.4	72.1	13.6	97.6	4.2
DEF	100.6	10.5	81.4	2.9	111.9	8.5
Deltamethrin	92.2	13.2	92.6	6.1	112.5	4.3
Desmetryn	94.9	3.2	70.6	6.9	107.3	4.5
Diazinon	94.4	3.2	89.2	5.7	114.8	6.2
Dichlofenthion	98.9	2.5	89.6	5.9	114.0	7.6
Dichlorobenzonitrile	79.9	1.6	100.8	9.2	89.9	13.6
Dichlorvos	102.9	6.4	105.4	4.3	94.6	11.0
Diclofop-methyl	96.3	4.6	85.8	6.2	105.5	3.9
Dicloran	97.8	7.5	86.3	4.4	108.3	4.5
Dicofol	95.5	2.0	84.6	6.4	104.3	3.6
Dicrotofos	103.2	2.4	75.3	9.7	112.5	6.5
Dieldrin	96.1	16.1	92.7	5.4	102.5	6.9
Difenoconazole-1	101.4	5.5	91.1	6.2	123.1	7.7
Difenoconazole-2	93.6	5.1	91.8	10.3	116.3	3.0
Diniconazole	96.6	6.8	85.3	6.6	107.7	4.8
Dioxathion	99.1	4.5	88.8	5.7	122.6	4.9
Diphenylamine	85.2	1.6	85.1	8.7	65.9	5.4
Dipropetryn	95.8	5.5	70.9	4.4	110.2	2.0
Ditalimfos	92.5	3.8	80.4	8.3	96.5	6.6
EPN	106.4	4.2	93.7	6.6	107.8	2.5
Edifenphos	99.9	1.6	82.2	7.0	108.0	4.7
Endrin	105.3	17.1	85.5	14.2	112.7	11.1
Epoxiconazole-1	101.8	5.9	88.0	6.6	111.1	1.3
Epoxiconazole-2	98.3	4.4	84.7	7.9	112.5	3.5
Ethalfluralin	99.3	5.8	96.0	7.8	115.0	8.1
Ethion	95.7	4.1	90.9	7.8	114.5	2.7
Ethofumesate	77.8	2.5	95.7	8.6	109.4	9.6
Ethoprophos	97.3	3.4	93.2	9.6	117.1	5.3
Etoxazole	90.0	9.5	91.2	8.4	109.7	7.6
Etridiazole	60.8	2.7	96.8	8.1	99.5	10.8
Etrimfos	94.3	6.4	94.4	11.6	113.9	4.0

	Cucumber 0.01 mg/kg		Grape 0.01 mg/kg		Rice 0.02 mg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Famphur	101.7	1.3	96.0	5.1	100.1	3.8
Fenamidone	92.2	1.7	88.6	2.3	105.6	2.9
Fenarimol	93.9	5.1	92.6	3.4	104.8	3.8
Fenbuconazole	100.2	2.5	97.7	4.6	110.1	2.5
Fenitrothion	102.3	6.5	92.8	5.9	103.5	8.9
Fenobucarb	111.2	2.5	99.1	6.8	126.9	7.2
Fenpropathrin	93.4	4.8	85.3	11.7	111.6	5.1
Fensulfothion	102.2	1.3	96.0	10.8	128.3	11.3
Fenthion	96.5	5.6	82.0	3.8	98.7	3.6
Fenthion sulfone	102.0	2.8	84.8	8.6	106.9	4.3
Fenthion sulfoxide	96.4	5.8	80.6	5.2	99.0	4.6
Fenvalerate-1	97.7	5.9	103.9	2.9	114.3	4.1
Fenvalerate-2	101.2	3.9	106.9	4.1	113.0	3.8
Fipronil	97.8	12.8	81.1	18.2	105.9	4.1
Fluazifop-butyl	95.6	5.0	79.8	7.9	103.9	3.4
Flucythrinate-1	100.9	3.7	101.4	3.8	113.6	2.8
Flucythrinate-2	102.9	4.7	112.5	1.3	112.9	2.9
Fludioxonil	97.8	3.6	124.8	10.3	100.0	3.2
Fluorodifen	92.5	6.9	88.5	3.0	104.9	4.0
Flutolanil	95.7	2.8	82.2	7.2	112.9	2.2
Fluvalinate-1	88.1	7.1	86.1	5.0	118.4	7.1
Fluvalinate-2	94.6	7.1	89.4	11.3	117.7	2.3
Fonofos	92.0	3.8	92.2	8.5	94.0	5.3
Formothion	95.6	4.7	87.6	15.6	64.4	3.9
Fosthiazate-1	97.6	7.7	92.3	10.8	118.5	12.4
Fosthiazate-2	102.4	9.7	87.3	3.2	120.2	2.8
Hexachlorobenzene	84.8	2.0	94.2	2.8	87.9	3.4
Hexaconazole	96.6	12.1	78.7	18.2	109.5	8.7
Hexazinone	94.4	2.2	92.3	2.3	100.9	2.5
Imazalil	110.1	9.4	78.6	8.6	102.2	6.4
Iprobenfos	106.0	2.4	98.4	9.6	121.4	5.2
Iprodione	103.2	7.9	101.0	6.4	103.2	6.5
Isazofos	99.6	1.9	93.7	11.9	116.8	4.1
Isocarbophos	101.7	3.4	82.1	4.3	106.5	4.1
Isofenphos	100.9	5.7	82.2	2.0	103.7	1.9
Isofenphos oxon	106.8	3.9	81.7	2.7	119.8	3.6
Isofenphos-methyl	103.1	5.4	90.5	5.3	108.9	3.7
Isoprocarb	116.0	2.3	94.1	6.1	112.0	4.9
Isoprothiolane	104.1	6.4	80.0	10.3	111.4	2.5
Kresoxim-methyl	92.9	2.0	90.0	8.4	111.0	3.8
Leptophos	74.4	6.5	101.1	5.9	99.7	3.0
Malaoxon	101.7	9.8	83.2	11.2	112.4	4.9
Malathion	99.9	2.8	89.7	9.0	111.0	3.6
Mefenacet	102.4	3.3	97.4	1.3	109.3	2.9

	Cucumber 0.01 mg/kg		Grape 0.01 mg/kg		Rice 0.02 mg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Mepanipyrim	97.2	4.6	81.6	1.9	92.4	5.2
Mephosfolan	97.5	3.8	75.1	4.3	110.6	4.9
Metalaxyl	107.3	5.7	80.1	11.9	101.1	7.9
Methacrifos	89.6	1.7	97.9	8.1	109.1	5.8
Methamidophos	92.8	3.2	67.2	5.0	76.9	12.7
Methidathion	97.1	1.3	78.6	7.1	106.4	3.9
Methoprene	83.6	15.7	77.8	10.4	111.8	7.2
Methoxychlor	102.7	6.0	86.2	8.0	100.6	5.4
Metolachlor	101.6	3.8	87.3	7.7	109.7	3.0
Metribuzin	86.4	8.3	75.4	7.6	94.4	4.6
Mevinphos	102.5	2.7	88.8	4.2	66.4	2.9
Molinate	73.4	2.0	95.1	5.0	94.7	5.0
Monocrotophos	106.9	4.2	77.4	10.9	118.5	8.6
Monolinuron	86.5	10.2	101.4	3.8	97.5	3.9
Myclobutanil	95.0	2.8	82.3	5.6	108.2	4.3
Napropamide	101.6	4.2	76.5	10.3	129.0	13.8
Nitrofen	98.7	4.4	91.5	5.4	100.4	2.5
Omethoate	104.5	4.9	72.7	9.3	108.3	16.5
Oxadiazon	95.9	5.5	88.1	7.5	109.4	3.0
Oxadixyl	96.6	3.3	93.1	6.0	97.6	5.9
Oxyfluorfen	99.1	13.2	87.9	7.5	95.5	8.4
Paclobutrazol	102.1	4.7	82.9	9.0	98.1	1.7
Paraoxon	103.0	7.1	87.2	7.8	50.0	14.2
Paraoxon-methyl	101.0	8.1	71.0	11.5	118.0	17.9
Parathion	92.2	1.9	81.1	8.3	108.3	4.8
Parathion-methyl	94.1	3.8	83.3	3.5	99.5	3.2
Penconazole	109.6	6.5	79.1	6.0	98.9	3.0
Pendimethalin	90.4	5.7	75.6	7.2	98.5	6.5
Pentachloroaniline	90.2	6.1	84.5	5.9	96.9	5.3
Pentachloronitrobenzene	102.8	6.6	94.6	2.3	106.0	5.5
Permethrin-1	86.5	13.1	84.8	10.7	121.1	9.1
Permethrin-2	83.7	7.4	107.9	3.4	103.2	9.0
Phorate	84.9	2.8	90.9	7.2	100.6	7.1
Phosalone	101.6	6.9	101.8	3.9	112.9	3.2
Phosfolan	97.2	4.8	81.4	4.2	105.6	4.2
Phosmet	100.2	1.8	106.3	5.4	105.0	3.2
Phosphamidon-1	100.0	9.1	90.0	14.0	99.2	11.0
Phosphamidon-2	104.8	6.3	93.3	8.2	113.7	2.3
Piperonyl butoxide	99.8	6.6	82.0	8.4	113.0	3.8
Piperophos	101.9	5.5	95.4	3.6	106.9	3.2
Pirimicarb	102.0	6.2	74.3	11.8	112.9	13.4
Pirimiphos-ethyl	97.4	3.4	74.8	6.9	107.9	6.3
Pirimiphos-methyl	106.1	5.2	77.4	3.8	101.8	3.9
Pretilachlor	103.2	1.7	80.7	14.5	112.8	1.2

	Cucumber 0.01 mg/kg		Grape 0.01 mg/kg		Rice 0.02 mg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Profenofos	94.8	8.1	87.2	6.0	115.5	2.8
Profluralin	86.5	12.3	87.3	5.5	102.8	12.0
Prometryn	94.7	5.8	78.0	9.8	104.0	4.2
Pronamide	97.4	2.5	84.2	10.7	115.8	4.9
Propanil	93.8	3.3	82.3	2.8	106.3	3.9
Propazine	99.3	3.3	98.0	10.3	110.1	5.1
Propetamphos	103.1	4.8	86.8	5.5	116.5	6.2
Propiconazole-1	101.8	3.3	82.5	6.1	114.1	3.0
Propiconazole-2	95.2	2.1	89.8	10.9	109.0	2.4
Propoxur	97.3	3.9	91.8	7.5	112.4	4.5
Prothiofos	90.7	6.7	76.4	2.7	104.6	5.8
Pyrazophos	108.0	7.1	100.1	5.6	112.4	2.8
Pyridaben	95.3	1.8	93.4	3.6	110.0	2.5
Pyridaphenthion	100.3	3.7	87.4	4.9	106.7	5.8
Pyrimethanil	117.7	5.3	82.6	5.3	109.5	2.4
Pyriproxyfen	94.5	3.0	88.6	11.9	105.8	4.5
Quinalphos	97.1	4.7	78.3	4.9	110.9	3.3
Quinoxyfen	84.9	5.7	71.1	9.2	92.1	3.9
Ronnel	90.0	1.5	82.5	6.2	107.3	6.6
Simazine	100.1	7.8	94.2	6.1	112.6	7.7
Sulfotep	97.8	2.8	100.1	8.8	111.0	6.0
Tebuconazole	97.9	11.1	91.4	9.4	110.1	4.0
Tebufenpyrad	94.0	6.2	91.6	5.2	98.0	4.6
Tebupirimfos	93.4	6.9	97.6	8.1	110.4	8.1
Tecnazene	80.6	1.2	100.0	3.0	95.0	4.4
Terbufos	91.2	3.4	95.3	4.1	109.6	6.5
Terbufos sulfone	99.2	4.4	84.4	6.7	110.9	1.8
Terbuthylazine	103.2	5.0	87.5	4.0	109.3	6.0
Terbutryn	97.2	6.7	76.7	4.2	96.6	8.2
Tetrachlorvinphose	103.2	2.4	81.3	6.1	106.4	3.5
Tetraconazole	99.8	6.8	80.7	8.2	109.8	1.8
Tetradifon	89.9	13.7	104.7	5.5	102.3	7.0
Tetramethrin-1	97.1	6.4	92.5	11.5	103.8	10.9
Tetramethrin-2	94.6	4.4	86.3	10.2	113.3	1.2
Thionazin	96.5	2.9	95.0	6.6	112.1	4.9
Tolclofos-methyl	99.2	2.9	88.3	6.6	111.6	2.1
Triadimefon	103.1	8.6	82.5	5.5	108.0	2.8
Triadimenol	98.5	8.6	80.0	3.5	110.9	2.2
Triallate	90.9	4.4	89.8	4.4	99.2	4.1
Triazophos	104.3	11.4	93.1	6.9	113.9	3.5
Trichloronat	93.6	6.0	80.8	8.0	102.8	5.7
Trifloxystrobin	96.7	2.2	78.4	11.6	109.3	4.6
Vinclozolin	96.9	5.7	91.1	6.3	112.0	5.6
alpha-BHC	90.6	1.3	97.9	5.2	107.7	4.7

	Cucumber 0.01 mg/kg		Grape 0.01 mg/kg		Rice 0.02 mg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
alpha-Endosulfan	92.1	13.0	99.0	9.3	101.0	6.4
beta-BHC	100.5	5.2	99.3	5.9	102.9	5.3
beta-Endosulfan	92.5	13.7	95.4	12.1	95.4	9.1
delta-BHC	98.1	1.5	98.6	4.9	102.8	5.4
gamma-BHC	89.9	4.3	102.2	5.0	102.8	4.3
lambda-Cyhalothrin-1	103.0	5.6	81.6	8.8	123.8	3.3
lambda-Cyhalothrin-2	98.9	2.8	100.5	2.9	113.1	4.0
o,p'-DDD	86.8	3.7	89.6	1.4	93.8	3.6
o,p'-DDE	79.1	1.0	91.4	1.6	89.1	2.3
o,p'-DDT	77.3	2.4	81.7	4.2	86.9	1.6
p,p'-DDD	90.0	1.7	83.2	3.2	91.0	2.5
p,p'-DDE	67.7	4.3	88.2	4.0	95.6	2.4
p,p'-DDT	77.9	5.7	78.5	6.7	85.2	2.5

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GC-MS/MS Pesticide Analysis in Green Tea in a Single Acquisition Using Xevo TQ-GC

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TECHNOLOGY BENEFITS

Concurrent MRM and full-scan data acquisition using RADAR™ Technology allows for the identification of co-extractives with minimum impact on the sensitivity of the method.

WATERS SOLUTIONS

Xevo™ TQ-GC System

DisQuE™ QuEChERS CEN extraction

Oasis™ PRiME HLB
Solid Phase Extraction (SPE) Sorbents

MassLynx™ MS Software

Quanpedia™ Database

TargetLynx™ Application Manager

KEYWORDS

Xevo TQ-GC, MRM, NIST Library, pesticides, QuEChERS, SPE

INTRODUCTION

Tea represents one of the most widely consumed beverages in the world, and pesticide analysis of teas is legally required to ensure consumer safety.[1] GC-MS/MS analyses using highly selective and specific MRM transitions are required for full coverage of all GC amenable pesticides at the trace levels required for many different method types. As green tea is a complex matrix, co-extractives, or matrix interferences, may impact MRMs. In this technical note, we demonstrate an easy method setup for targeted analysis of pesticides in green tea, as well as concurrent MRM/full scan acquisition for monitoring of matrix co-extractives.

Xevo TQ-GC.

RESULTS AND DISCUSSION

Green tea samples were prepared using Waters™ DisQuE QuEChERS CEN extraction followed by solid phase extraction (SPE) pass-thru clean-up using Oasis PRiME HLB. Following extraction and clean-up, a matrix matched calibration curve and QC samples were prepared for 1 µL injections using a splitless injection mode. Data were acquired using EI+ at a potential of 70 eV. Concentration determination is described here as both ppb (spike into volume of final extract, i.e. matrix matched), and mg/kg in tea, which is 0.005 × the ppb value, based on the sample preparation used (2 g of tea reconstituted as per QuEChERS extraction). The GC temperature program, MRM transitions and processing method were automatically generated using Quanpedia (Figure 1) which eliminated the potential of transcription error on input of the GC and MRM conditions. Utilizing Quanpedia also eliminates the need for extensive method development and simplifies the workflow for routine analysis.

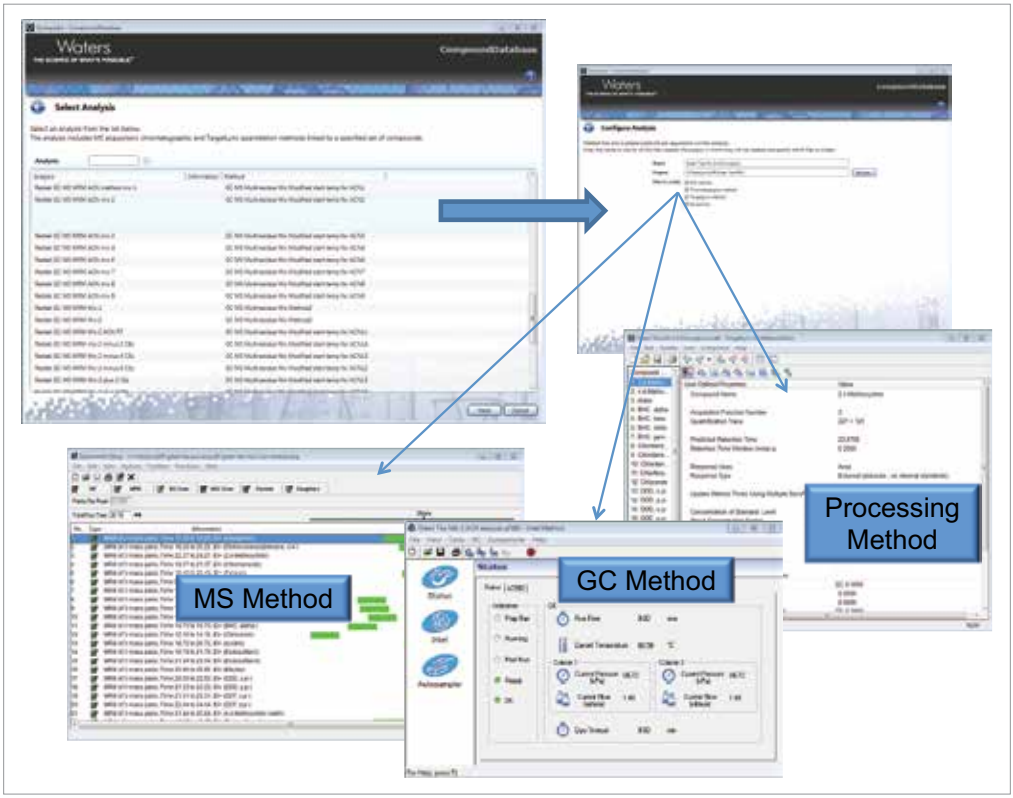

Figure 1. The use of Quanpedia Database for automatic method generation.

In order to assess the Xevo TQ-GC System's ability to meet regulatory recommendations, matrix matched calibration curves were prepared. As shown in Figure 2, the calibration curve and the QC injections met the key criteria of ion ratio conservation of ±30%, linearity of R^2 ≥0.998 (1 to 100 ppb, which calculates to 0.005 mg/kg to 0.5 mg/kg in tea matrix), back calculated residuals of <20%, and and calculated concentrations of the QCs within ±15% of the true value. Table 1 provides a summary of the QC sample data for various organochlorine pesticides.

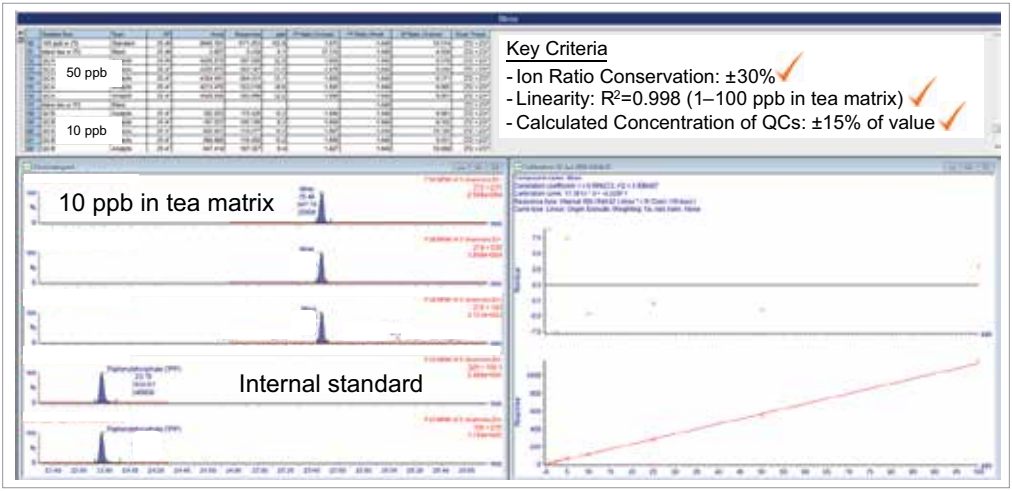

Figure 2. Calibration curve of mirex from 1 to 100 ppb (0.005 mg/kg to 0.5 mg/kg) in green tea matrix.

Table 1. Summary of the QC injections at 10 and 50 ppb (0.05 mg/kg and 0.25 mg/kg) in green tea matrix.

	Avg. calculated conc. (ppb) (mg/kg in tea)		%RSD (n=5)	
Analyte	10 (0.05)	50 (0.250)	10	50
Heptachlor	11.0	51.2	14.8	6.1
2,4-Methoxychlor	11.4	53.4	5.1	3.4
Chlorbenside	9.8	48.9	8.3	2.4
Fenson	10.5	51.6	3.7	0.9
Tetradifon	9.4	51.0	7.6	4.2
Chlorfenson	10.6	51.0	4.6	1.7
BHC, alpha-	10.7	50.6	11.0	4.8
BHC, beta-	9.8	52.9	6.3	4.9
BHC, delta-	10.1	49.9	6.5	2.5
BHC, gamma-	10.4	52.4	13.4	3.9
Chloroneb	11.3	50.9	7.1	7.1
Isodrin	11.1	54.2	14.2	5.9
Ethylene	10.5	49.8	9.0	2.6
DDD, o,p-	10.5	49.4	9.3	3.2
DDD, p,p-	11.0	52.5	15.9	1.6
DDT, p,p-	11.0	52.3	9.3	4.8
4,4-Methoxychlor olefin	11.2	52.2	6.8	5.5
Endosulphan ether	9.4	53.0	16.0	7.3
DDE, o,p-	10.9	52.3	11.8	1.9
DDE, p,p-	10.2	49.4	11.9	2.6
Pentachlorobenzene	10.6	51.1	2.9	3.7
Pentachloroanisole	10.7	52.1	7.3	3.7
Mirex	10.2	51.0	4.7	3.4
Endrin ketone	9.3	51.6	6.6	5.8

Following quantitative demonstration of the targeted MRMs, the use of concurrent MRM and full scan (RADAR) data was assessed. The full scan acquisition was from *m/z* 50–650 with a scan time of 0.5 sec. Green tea sample extracts were spiked at 0.1 mg/kg with more than 200 pesticides (in nine separate mixtures to prevent interactive effects as provided in the GC-MS Multiresidue Kit from Restek Corporation). The full scan data was interrogated to determine the identity of two large matrix interference peaks. In Figure 3 the identification of 1,2,3-benzenetriol and caffeine are shown using the NIST Library. Caffeine is a known stimulant which occurs naturally in green tea, and the phenolic compound 1,2,3-benzenetriol (also referred to as pyrogallol or pyrogallic acid) is also found in green tea.[2]

Using the same samples, an assessment of the loss in signal due to the combined acquisition of full scan and MRM was also performed. This was achieved by running one spiked sample with just MRM followed by the same sample with RADAR enabled.

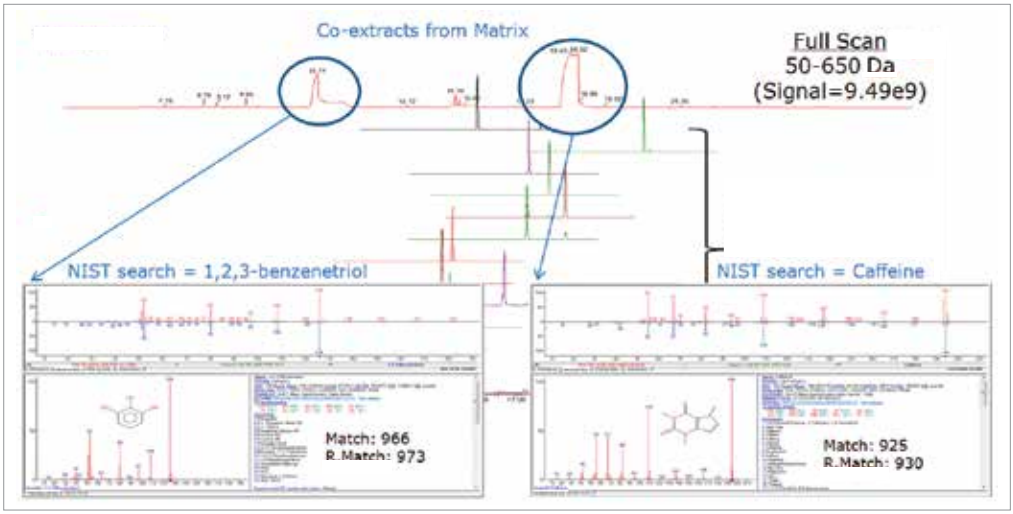

Figure 3. Utilizing RADAR Technology for the identification of interferences in the sample extracts coupled with NIST Library searching.

The loss of signal is dependent on the number of transitions contained in the method and when the transitions occur relative to one another. Figure 4 shows that the signal conservation when using RADAR ranged from 56 to 97% for the three illustrated compounds, representing the approximate range observed for the pesticides across mixes. This means that low limits of detection can still be achieved when utilizing RADAR which allows further sample information to be analyzed.

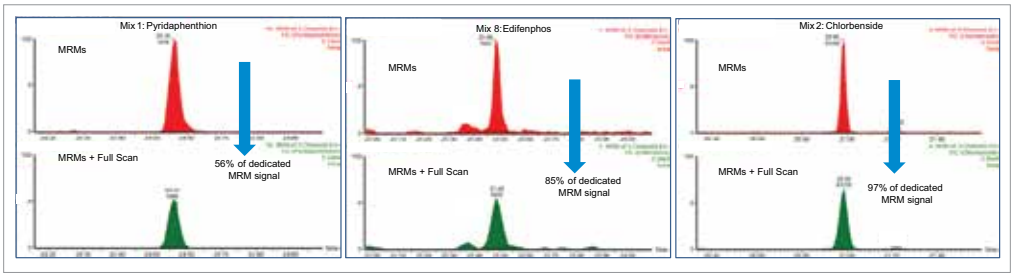

Figure 4. Assessment of the MRM signal maintained during RADAR acquisition.

SUMMARY

The Xevo TQ-GC has simplified workflows that enables users to produce results that meet regulatory recommendations. The added functionality of RADAR allows full scan and MRM data to be collected simultaneously. This data can be interrogated to identify change to understand extraction interferences at the time of the original analysis, or as part of a retrospective data mining investigation. The combination of MRM and Full scan in a single method acquisition does not impact sensitivity and is valuable for use in method optimization.

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UPLC and APGC Multi Residue Pesticide Analysis on a Single Tandem Quadrupole Mass Spectrometer Platform

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APPLICATION BENEFITS

Using the Xevo™ TQ-S micro Tandem Quadupole Mass Spectrometer with the Universal Source for pesticide analysis allows:

- UPLC™ and APGC analysis of the sample extracts on a single tandem quadrupole mass spectrometer.
- Analysis of large suites of pesticides in a single injection per chromatographic inlet.
- Analysis of fruit and vegetable matrices at legislatively relevant levels of 0.010 mg/kg.
- Easy generation of methods using the Quanpedia™ Database.

WATERS SOLUTIONS

ACQUITY™ UPLC H-Class System

Atmospheric Pressure Gas Chromatography (APGC)

Xevo TQ-S micro

DisQuE™ QuEChERS, AOAC Method Sample Preparation Kit, Pouches

MassLynx™ MS Software

Quanpedia Database

TargetLynx™ XS Application Manager

KEYWORDS

LC, GC, pesticide residue analysis, MRL, QuEChERS, GC-MS/MS, LC-MS/MS

AIM

Demonstrate analysis of a large suite of pesticides in fruit and vegetable extracts using both LC and GC on the same tandem quadrupole MS platform at legislatively relevant limits.

INTRODUCTION

Hundreds of pesticides are commercially available and approved for use on various fruit and vegetable plants, to prevent pest infestation and improve shelf-life of fresh produce. Maximum Residue Levels (MRLs) are set at the highest level of pesticide that the relevant regulatory body would expect to find in that crop when it has been treated in line with good agricultural practice. In the EU, if a pesticide is not explicitly mentioned in the MRL legislation, a default MRL is used for enforcement. This default value is set to be equal to the limit of quantification (LOQ) achievable with the analytical methods used for analysis. National authorities control and enforce MRLs by testing samples for pesticide residue levels using analytical surveillance programs. These programs check for compliance with MRLs, assess dietary exposure, and check for use of unauthorized pesticides. The food industry also carries out its own due diligence analyses.

Mass spectrometry coupled with both gas (GC) and liquid chromatography (LC) is needed to provide comprehensive analysis of a wide range of pesticide residues with sufficient sensitivity to meet global MRL regulations. The use of Quick, Easy, Cheap, Efficient, Rugged, and Safe (QuEChERS) sample extraction and clean up has streamlined analytical efficiencies for multi residue analyses.[1] The advantage of ultra performance liquid chromatography (UPLC) coupled with tandem quadrupole mass spectrometry (MS/MS) for multi residue pesticide analysis is widely reported.[2] More recently the use of GC-MS/MS operated at atmospheric pressure (APGC) has been shown to offer significant improvements in performance over electron impact (EI) for challenging pesticides, in terms of selectivity, specificity, and speed of analysis.[3,4]

The APGC source ionizes compounds using a corona discharge at atmospheric pressure in an APCI-like manner. Therefore, this ionization mechanism is a much softer technique than classic electron impact (EI) ionization and produces larger amounts of intact parent ions, especially in the case of fragile or easily fragmented compounds. APGC ionization can occur using two mechanisms; proton transfer (wet source) or charge transfer (dry source). In proton transfer ionization, $[M+H]^+$ ions are formed, whereas in charge transfer ionization, $M^{+\cdot}$ ions are formed.

In this application note, a single workflow for the multi residue analysis of pesticides is demonstrated on a variety of fruit and vegetable samples. Utilizing the universal source of Waters™ Xevo TQ-S micro allows for LC and GC analyses to be completed on the same tandem quadrupole MS instrument, with less than 30 minutes needed to switch between chromatographic inlets. The performance of the method will be highlighted in terms of sensitivity, repeatability, and linearity for both LC and GC in compliance with the SANTE guidelines (11945/2015) for pesticide analysis.[5]

EXPERIMENTAL

The LC and GC suites of pesticides analyzed in this study (listed in the Appendix) were chosen to cover a wide range of different pesticide classes and chemistries. The multi residue MS/MS methods were generated using Quanpedia, with separate databases utilized for generation of the LC and GC methods. Each database contains MRMs and retention time information for each compound. When the MS method is generated the MRM function windows are automatically set for each compound. For the UPLC method, a window of 1 minute was placed around each compound's expected retention time. For the APGC method, a window of 30 seconds was used due to the narrower peak widths exhibited in GC analysis. In addition to the MS methods, TargetLynx data processing methods and the LC inlet method were also generated through the Quanpedia Database.

Sample extraction and cleanup

Celery, lemon, corn, and kale samples were purchased at a local grocery store. Samples were chosen to be representative of different types of matrix complexity from different commodity groups, including high water content (celery and kale), high acid content (lemon), and high starch/protein with low water content (corn). Samples were immediately homogenized in a food processer and frozen until sample preparation was performed. QuEChERS extraction was performed according to the official AOAC method 2007.01 using the DisQuE QuEChERS, AOAC Method Sample Preparation Kit (p/n: 176002922)[6]. Figure 1 highlights the sample extraction.

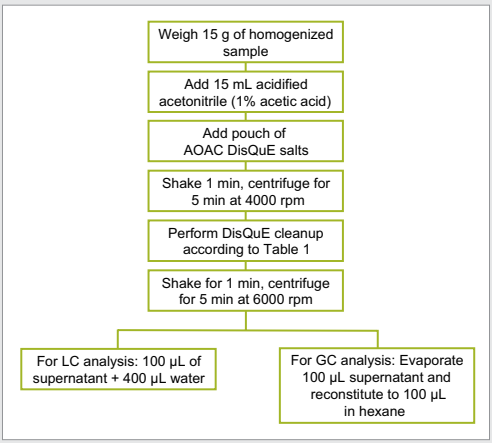

Figure 1. DisQuE sample extraction method.

Table 1. dSPE cleanup conditions used for each sample matrix.

Sample	$MgSO_4$	PSA	GCB	Volume	Part number
Celery	150 mg	25 mg	7.5 mg	1 mL	186004831 + 186004835
Lemon	150 mg	25 mg	–	1 mL	186004831
Corn	150 mg	25 mg	–	1 mL	186004831
Kale	900 mg	150 mg	150 mg	6 mL	186004833 + 186004835

LC-MS/MS conditions

LC system:	ACQUITY UPLC H-Class
Column:	ACQUITY BEH C_{18} 1.7 µm 2.1 x 100 mm
Column temp.:	45 °C
Injection volume:	5 µL
Flow rate:	0.45 mL/min
Mobile phase A:	Water + 10 mM ammonium acetate
Mobile Phase B:	Methanol + 10 mM ammonium acetate

Gradient:

Time (min)	%A	%B
0.00	98	2
0.25	98	2
12.25	1	99
13.00	1	99
13.01	98	2
17.00	98	2

MS system:	Xevo TQ-S micro
Ionization mode:	ESI+
Capillary voltage:	1 kV
Desolvation temp.:	500 °C
Desolvation gas flow:	1000 L/hr
Source temp.:	150 °C

GC-MS/MS conditions

GC system:	7890A
Autosampler:	CTC PAL
Column:	30 m x 0.25 mm x 0.25 µm Rxi-5MS
Carrier gas:	Helium
Flow rate:	2.0 mL/min
Injection:	Splitless
Injector temp.:	280 °C
Injection volume:	1 µL
Makeup gas:	Nitrogen at 250 mL/min
Transfer line temp.:	320 °C

Oven program:

Rate (°C/min)	Temp. (°C)	Hold (min)
–	80	1.00
25	150	0.00
8	270	0.00
20	320	4.10

MS system:	Xevo TQ-S micro
Ionization mode:	API+
Ionization mechanism:	Proton transfer (3 vials of water in source)
Corona current:	20 µA for first 3.5 min 3.0 µA for rest of run
Cone gas flow:	0 L/hr
Auxiliary gas flow:	250 L/hr
Source temp.:	150 °C

RESULTS AND DISCUSSION

METHOD MANAGEMENT USING THE QUANPEDIA DATABASE

Working with methods involving large numbers of compounds can be time consuming when done manually and is prone to errors when setting up time segmented acquisition. Quanpedia is a compound centric database, typically used for method generation, but can also function as a method management tool. Initial methods for this analysis were generated using existing UPLC and APGC databases (Figure 2). Retention time changes resulting from further method development or method changes wereupdated in the database. This allowed for immediate and automatic updates to be made in the MS and processing methods by just re-generating the methods in three simple clicks.

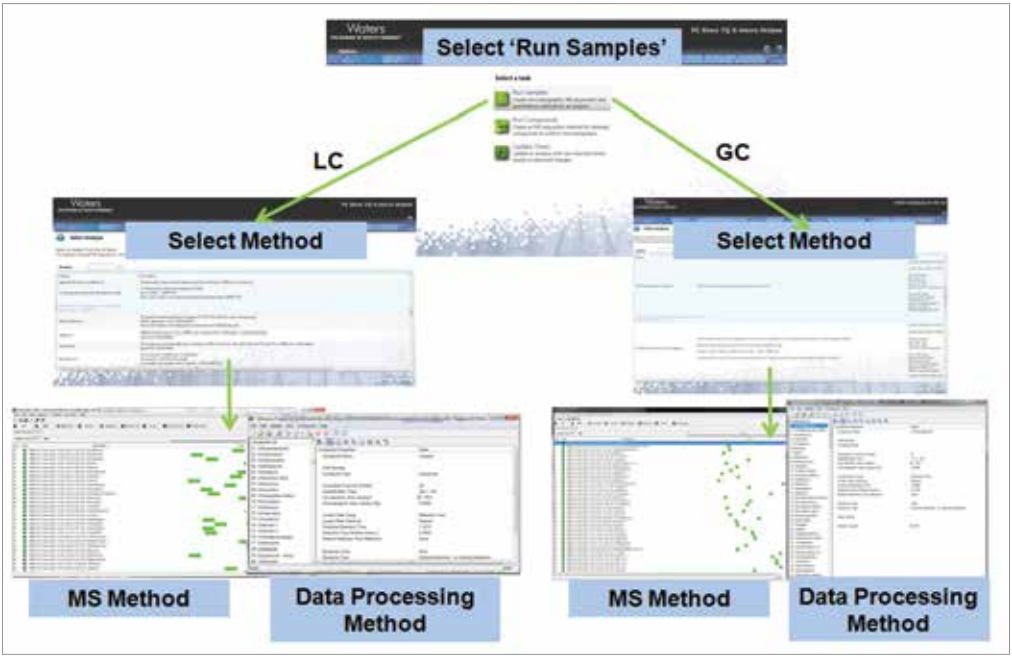

Figure 2. Quanpedia databases that were used to manage the methods for both UPLC and APGC analysis demonstrating the three click workflow of method generation.

RAPID AND ROBUST DATA AQUISITION

For successful analysis of large numbers of pesticides and their metabolites, it is important that the mass spectrometer can maintain sufficient sensitivity while acquiring MRM transitions with a fast scan speed to provide enough data points across each chromatographic peak (e.g. minumum of 12 points per peak). The fast scanning speeds of the TQ-S micro allow for this robust and rapid data acquisition while maintaining large retention time windows to accommodate any shift in retention time due to column maintenance (GC) or chromatography changes caused by the different matrices.[6] Figure 3 highlights one of the busiest sections of the APGC MS Method. In this example, flutolanil is just one of approximately 30 pesticides (set across 30 channels, each acquiring at least two transitions per compound) eluting in a 1.5 minute time window. The dwell time calculated by the autodwell function to collect a minimum of 12 points per peak was 0.006 s. The resulting chromatogram of three replicate injections of 0.010 mg/kg of flutolanil in celery matrix can be seen in Figure 3. Even with the fast scanning speed, 19 points were collected across the peak and the RSD of three consecutive injections in matrix was 5.2%. The same is true for the UPLC method used for this analysis.

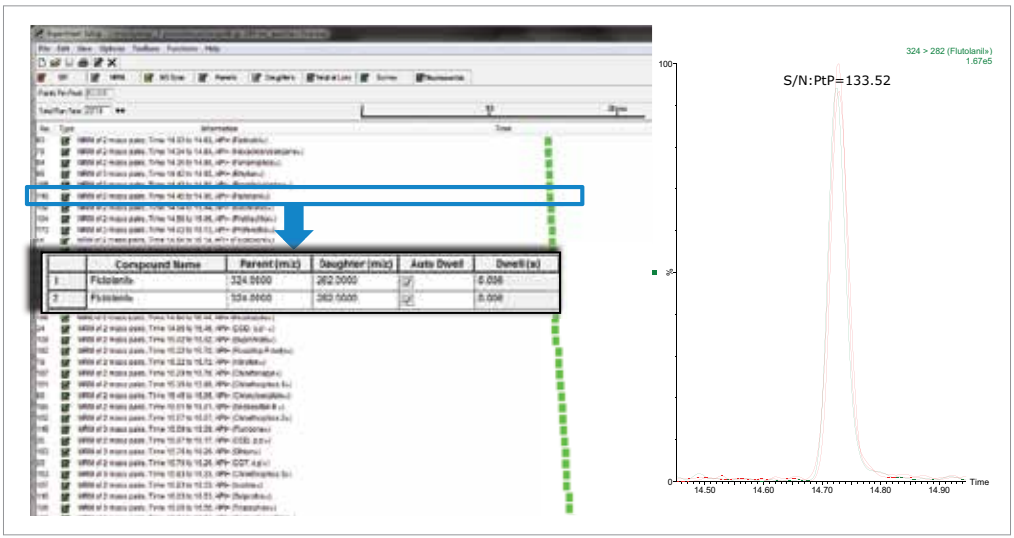

Figure 3. Demonstration of the fast scanning of the Xevo TQ-S micro demonstrating retention of peak quality at a fast scan time.

PESTICIDES IN MATRIX

Matrix matched standards were prepared in celery, lemon, corn, and kale over a range of 0.001 to 0.050 mg/kg and replicate injections made using the UPLC and APGC methods. A summed MRM overlay of a selection of pesticides can be seen in Figure 4, showing 0.010 mg/kg in celery extract from both the (A) APGC and (B) UPLC analyses. The data were fitted with the best fit calibration; for the UPLC data, the response was shown to be linear whereas the APGC response over the range investigated was non-linear and so was fitted with a quadratic calibration. The majority of the compounds in both analysis methods had correlation coefficient (R^2) values of 0.995 or greater. Figure 5 shows the matrix matched calibration curves and the peak response at 0.001 mg/kg of a representative pesticide from each analysis method in the four matrices. Residuals from triplicate injections at each calibration point were within ±20%. Ion ratios were also shown to be within 30% tolerance of the reference values.

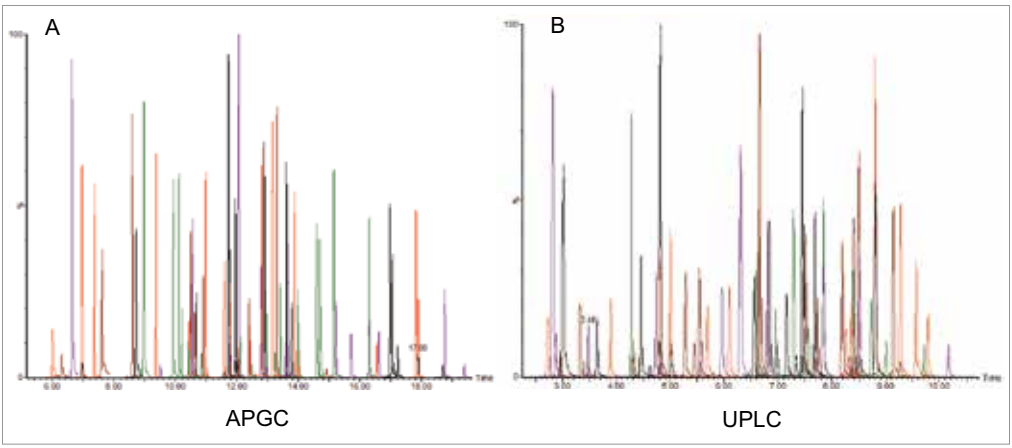

Figure 4. Overlay of a selection of pesticides at 0.010 mg/kg analyzed in a celery extract on A. APGC, and B. UPLC.

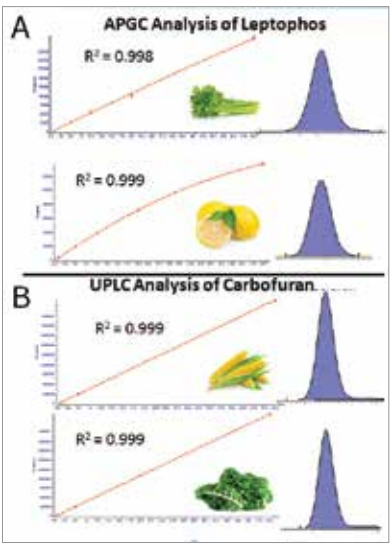

Figure 5. Matrix matched calibration curves and chromatograms for standards at 0.001 mg/kg for peaks from: A. APGC analysis of leptophos in celery and lemon; and B. UPLC analysis of carbofuran in corn and kale.

For convenience, all sample extracts were spiked at the default MRL of 0.010 mg/kg. Figure 6 demonstrates the percentage of pesticides in each method detected in the spiked matrices at 0.010 mg/kg. However many pesticides could also be detected at 0.001 mg/kg as demonstrated in Figure 5 showing leptophos (APGC compound) and carbofuran (UPLC compound) in the different matrices. The precision of the measurements was excellent with more than 90% of the detected pesticides exhibiting RSDs of peak area of less than 10% (n=3). The exception was the APGC analysis of the kale matrix which had more than 80% of pesticides exhibiting RSDs less than 10% (Figure 7).

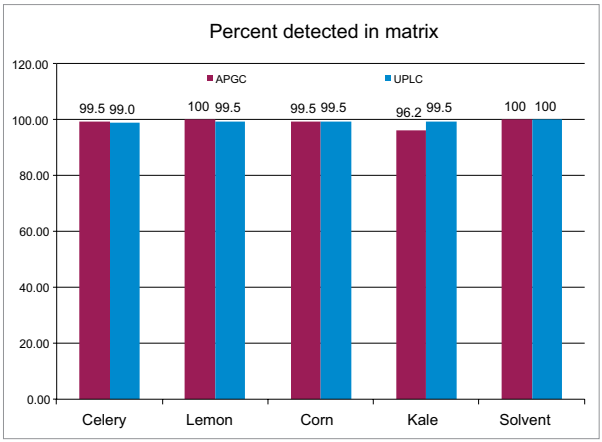

Figure 6. The percentage of pesticides detected in the 0.010 mg/kg standard for each matrix using both APGC and UPLC.

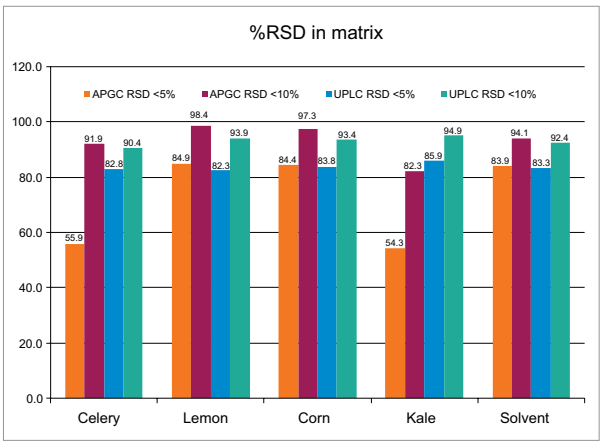

Figure 7. Percentage of compounds detected at 0.010 mg/kg in each matrix and associated RSDs.

CONCLUSIONS

Complex multi residue pesticide analysis was demonstrated using both UPLC and APGC analysis on the same tandem quadrupole instrument (Xevo TQ-S micro). Instrument methods were generated and maintained using Quanpedia databases making method generation and maintenance fast and simple. Although the multi residue methods contained approximately 200 compounds each, the reliable scanning speed of the Xevo TQ-S micro produced accurate and precise measurements. The performance for the determination of pesticide residues analyzed in four matrices of varying complexity complied with the SANTE guidelines for pesticide residue analysis. Detection at the EU default maximum residue limit of 0.010 mg/kg was easily achieved for >99% of pesticides analyzed with good precision (RSDs <10%) for most analytes in the food samples. Having the flexibility of the Universal Source architecture to provide access to both UPLC-MS/MS and GC-MS/MS on the same instrument, allows for an increase of laboratory efficiency, while maintaining required sensitivity and repeatability.

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Appendix

Pesticides in APGC Method

2-Phenylphenol	Diclobenil	Oxyfluorfen
4,4'-Methoxychlor olefin	Dicloran	Paclobutrazol
Acetochlor	Dimethachlor	Parathion
Acrinathrin	Diphenamid	Pebulate
Alachlor	Diphenylamine	Penconazole
Allidochlor	Edifenphos	Pendimethalin
Anthraquinone	Endosulfan ether	Pentachloroaniline
Atrazine	Endosulfan II	Pentachlorobenzonitrile
Azinphos-ethyl	Endosulfan sulfate	Pentachlorothioanisole
Azinphos-methyl	Endrin aldehyde	Permethrin, cis-
Benfluralin	EPN	Permethrin, trans-
Bifenthrin	Ethalfluralin	Phenothrin 1
Bioallethrin	Ethion	Phenothrin 2
Biphenyl	Ethylan	Phorate
Bromfenvinphos	Etofenprox	Phosalone
Bromfenvinphos-methyl	Etridazole	Phosmet
Bromophos-ethyl	Fenamiphos	Piperonyl butoxide
Bromophos-methyl	Fenarimol	Pirimiphos-ethyl
Bromopropylate	Fenchlorphos	Pirimiphos-methyl
Bupirimate	Fenitrothion	Prochloraz
Captafol	Fenpropathrin	Procymidone
Captan	Fenson	Prodiamine
Carbophenothion	Fenthion	Profenofos
Carfentrazone ethyl	Fenvalerate 1	Profluralin
Chlorfenapyr	Fenvalerate 2	Propachlor
Chlorfenvinphos	Fipronil	Propanil
Chlorobenzilate	Fluazifop-P-butyl	Propisochlor
Chloroneb	Fluchloralin	Propyzamide
Chlorothalonil	Flucythrinate 1	Prothiofos
Chlorpropham	Flucythrinate 2	Pyraclofos
Chlorpyrifos	Fludioxonil	Pyrazophos
Chlorpyrifos-methyl	Fluquinconazole	Pyridaben
Chlorthal-dimethyl	Flusilazole	Pyridaphenthion
Chlorthiophos 1	Flutolanil	Pyrimethanil
Chlorthiophos 2	Flutriafol	Pyriproxyfen
Chlorthiophos 3	Folpet	Quinalphos
Chlozolinate	Fonofos	Resmethrin 1
Clomazone	Hexachlorobenzene	Sulfotep
Coumaphos	Hexazinone	Sulprofos
Cycloate	Iodofenfos	tau-Fluvalinate 1
Cyfluthrin 1	Iprodione	tau-Fluvalinate 2
Cyfluthrin 2	Isazophos	Tebuconazole
Cyfluthrin 3	Isodrin	Tebufenpyrad
Cyfluthrin 4	Isopropalin	Tefluthrin
Cyhalothrin, lambda-	Lenacil	Terbacil
Cypermethrin 1	Leptophos	Terbufos
Cypermethrin 2	Linuron	Terbutylazine
Cypermethrin 3	Malathion	Tetrachloroaniline, 2,3,5,6-
Cypermethrin 4	Metalaxyl	Tetrachlorvinphos
Cyprodinil	Metazachlor	Tetradifon
DDD, o,p'-	Methacrifos	Tetramethrin 1
DDD, p,p'-	Methoxychlor	Tetramethrin 2
DDE, o,p'-	Methyl parathion	Tolclofos-methyl
DDE, p,p'-	Metolachlor	Tolylfluanid
DDT, o,p'-	Mevinphos	Transfluthrin
DDT, p,p'-	MGK 264 1	Triadimefon
Deltamethrin	MGK 264 2	Triadimenol
Diallate	Myclobutanil	Triallate
Diazinon	N-(2;4-Dimethylphenyl) formamide	Triazophos
Dichlofluanid	Nitralin	Triflumizole
Dichloroaniline, 3,4'-	Nitrofen	Trifluralin
Dichlorobenzophenone, 4,4'-	Oxadiazon	Vinclozolin

Pesticides in UPLC Method.

Abamectin	Etoxazole	Nuarimol
Acephate	Famoxadone	Omethoate
Acetamiprid	Fenamidone	Oxadixyl
Acibenzolar-S-methyl	Fenarimol	Oxamyl
Aldicarb	Fenazaquin	Paclobutrazol
Aldicarb sulfone	Fenbuconazole	Penconazole
Aldicarb sulfoxide	Fenhexamid	Pencycuron
Ametryn	Fenobucarb	Phenmedipham
Aminocarb	Fenoxycarb	Picoxystrobin
Amitraz	Fenpropimorph	Piperonyl butoxide
Azoxystrobin	Fenpyroximat	Pirimicarb
Benalaxyl	Fenuron	Procloraz
Bendiocarb	Fipronil	Promecarb
Benfuracarb	Flonicamid	Prometon
Benzoximate	Flufenacet	Prometryn
Bifenazate	Flufenoxuron	Propamocarb
Bitertanol	Fluomethuron	Propargite
Boscalid	Fluoxastrobin	Propham
Bromuconazole I	Fluquinconazole	Propiconazole
Bromuconazole II	Flusilazole	Propoxur
Bupirimate	Flutolanil	Prothioconazole
Buprofezin	Flutriafol	Pymetrozine
Butafenacil	Forchlorfenuron	Pyracarbolid
Butocarboxim	Formetanate HCL	Pyraclostrobin
Butoxycarboxim	Fuberidazole	Pyridaben
Carbaryl	Furalaxyl	Pyrimethanil
Carbendazim	Furathiocarb	Pyriproxifen
Carbetamide	Hexaconazole	Quinoxyfen
Carbofuran	Hexythiazox	Rotenone
Carbofuran-3-hydroxy	Hydramethylnon	Secbumeton
Carboxin	Imazalil	Siduron
Carfentrazone-ethyl	Imidacloprid	Simetryn
Chlorantraniliprole	Indoxacarb	Spinetoram
Chlorfluazuron	Ipconazole	Spinosad A
Chloroxuron	Iprovalicarb I	Spinosad D
Chlortoluron	Iprovalicarb II	Spirodiclofen
Clethodim I	Isocarbofos	Spirotetramat
Clofentezine	Isoprocarb	Spiroxamine I
Clothianidin	Isoproturon	Spiroxamine II
Cyazofamid	Kresoxim-methyl	Sulfentrazone
Cycluron	Linuron	Tebuconazole
Cymoxanil	Lufenuron	Tebufenozide
Cyproconazole I	Mandipropamid	Tebufenpyrad
Cyproconazole II	Mefenacet	Tebuthiuron
Cyprodinil	Mepanipyrim	Teflubenzuron
Cyromazine	Mepronil	Temephos
Desmedipham	Mesotrione	Terbumeton
Diclobutrazol	Metaflumizone	Terbutryn
Dicrotophos	Metalaxyl	Tetraconazole
Diethofencarb	Metconazole	Thiabendazole
Difenoconazole	Methabenzthiazuron	Thiacloprid
Diflubenzuron	Methamidophos	Thiamethoxam
Dimethoate	Methiocarb	Thidiazuron
Dimethomorph I	Methomyl	Thiobencarb
Dimethomorph II	Methoprotryne	Thiophanate-methyl
Dimoxystrobin	Methoxyfenozide	Triadimefon
Diniconazole	Metobromuron	Triadimenol
Dinotefuran	Metribuzin	Trichlorfon
Dioxacarb	Mevinphos I	Tricyclazole
Diuron	Mevinphos II	Trifloxystrobin
Emamectin benzoate	Mexacarbate	Triflumizole
Epoxiconazole	Monocrotophos	Triflumuron
Etaconazole	Monolinuron	Triticonazole
Ethiofencarb	Myclobutanil	Vamidothion
Ethiprole	Neburon	Zoxamide
Ethirimol	Nitenpyram	
Ethofumesate	Novaluron	

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Targeted and Sensitive Detection of Food Allergens in Complex and Processed Foodstuffs Using UPLC-MS/MS

Mélanie Planque,[1] Antonietta Wallace,[2] and Nathalie Gillard[1]
[1]CER Groupe, Marloie, Belgium; [2]Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Sensitive multi-allergen method using UPLC-MS/MS.
- Allergens monitored in this method were assessed from the recommended levels provided by VITAL (Voluntary Incidental Trace Allergen Labelling) and the AOAC SMPR for food allergens (2016.002).
- This multi-allergen detection method has the lowest limits of quantification available to date (expressed in total proteins and not soluble proteins).

WATERS SOLUTIONS

ACQUITY UPLC™ System

ACQUITY UPLC BEH130 BEH Column

Xevo™ TQ-S

MassLynx™ MS Software

KEYWORDS

Proteomics, allergens, LC-MS/MS, egg, peanut, milk, soybean

INTRODUCTION

Food allergy is a worldwide health problem affecting both adults and children. To avoid allergic reactions, allergens must be totally excluded from the diet. Consequently, allergic customers can only refer to mandatory labeling to try and avoid coming into contact with the food allergen. However, the undeclared presence of these allergens is still widespread.

To help food industries in the management of hidden allergens, sensitive, specific quantitative, and robust analytical methods need to be developed.

Traditionally techniques such as ELISA and PCR have been used for routine analysis, but in recent years, there has been increasing interest in the utility of LC-MS based methods. In March 2016, AOAC released the first standard method performance requirements (SMPR) specifically for the analysis of four food allergens using LC-MS/MS.[1] The detection levels tested are benchmarked against the levels stated in the AOAC SMPR 2016.002 and VITAL (Voluntary Incidental Trace Allergen Labelling)[2] reference doses.

In this application note, we describe the targeted analysis of four food allergens in a variety of matrices using Waters™ ACQUITY UPLC System and Xevo TQ-S.

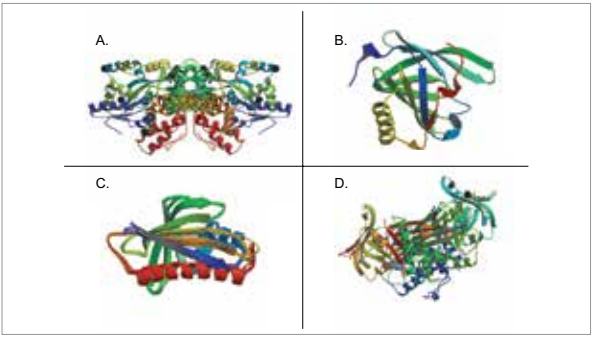

Figure 1.
1A. MBP-fusion protein of the major peanut allergen Ara h 2: DOI: 10.2210/pdb3ob4/pdb;
1B. Bovine allergen Bos d 2 in the trigonal space group P3221:DOI: 10.2210/pdb4wfu/pdb;
1C. NMR solution structure of soybean allergen Gly m 4: DOI: 10.2210/pdb2k7h/pdb;
1D. Crystal structure of uncleaved ovalbumin at 1.95 angstroms resolution: DOI: 10.2210/pdb1ova/pdb. Images courtesy of the RSCB Protein Data Bank.

EXPERIMENTAL

This method is based on a single protocol applicable to the different tested allergens and foodstuffs. Details on the sample preparation step are described elsewhere.[3]

The four allergens investigated in this method were milk (Bos Taurus), egg (Gallus gallus chicken), peanut (Arachis hypogaea) and soybean (Glycine Max (Glycine hispida).

The protocol was tested on processed and complex food matrices including chocolate, ice cream, tomato sauce, and cookies.

LC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH130, 2.1 x 150 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	20 µL
Flow rate:	0.2 mL/min
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Gradient:	0 to 1 min: 86% A; 1 to 16.5 min: 86% to 60% A; 16.5 to 16.6 min: 60% to 0% A; 16.6 to 21 min: 0% A; 21.0 to 21.1 min: 0% to 86% A; 21.1 to 24 min: 86% A

MS conditions

MS system:	Xevo TQ-S
Ionization mode :	ESI+ in MRM mode
Capillary voltage:	2.0 kV
Collision gas flow:	0.12 mL/min
Cone voltage:	35 V
Cone gas flow:	150 L/h
Desolvation flow:	1200 L/h
Source temp.:	150 °C
Desolvation temp.:	500 °C

Data solutions

Skyline (MacCoss Lab)

UniProt

MassLynx

Table 1. Multiple reaction monitoring (MRM) parameters for the identification of milk, egg, soybean, and peanut proteins by ACQUTIY UPLC and Xevo TQ-S.

Food	Peptide	RT* (min)	Precursor (charge state) (*m/z*)	Product ion (fragment)	Collision energy (eV)
Egg	GGLEPINFQTAADQAR	7.5	844.4 (++)	1331.7 (y12+)	26
				1121.5 (y10+)	28
				666.3 (y12+)	25
	LTEWTSSNVMEER	5.9	791.4 (++)	1052.5 (y9+)	31
				951.4 (y8+)	23
				864.4 (y7+)	23
	ISQAVHAAHAEINEAGR	2.3	887.5 (++)	1138.6 (y11+)	33
				1067.5 (y10+)	33
				996.5 (y9+)	32
	EALQPIHDLADEAISR	7.8	593.3 (+++)	761.4 (y7+)	19
				690.3 (y6+)	15
				668.8 (y12++)	15
	NIPFAEYPTYK	7.5	671.8 (++)	1115.5 (y9+)	15
				508.3 (y4+)	16
				558.3 (y9++)	29
	NIGELGVEK	4	479.8 (++)	731.4 (y7+)	12
				674.4 (y6+)	10
				545.3 (y5+)	19
	YLLDLLPAAASHR	10.4	480.6 (+++)	709.4 (y7+)	15
				582.3 (y11++)	10
				355.2 (y7++)	14
	NFLINETAR	6.2	539.3 (++)	816.5 (y7+)	14
				703.4 (y6+)	16
				590.3 (y5+)	16

(Table 1 continues on the next page.)

(Table 1 continued.)

Food	Peptide	RT* (min)	Precursor (charge state) (*m/z*)	Product ion (fragment)	Collision energy (eV)
Peanut	NTLEAAFNAEFNEIR	10.7	869.9 (++)	1139.5 (y9+)	27
				992.5 (y8+)	26
				878.4 (y7+)	26
	RPFYSNAPQEIFIQQGR	7.3	684.4 (+++)	748.4 (y6+)	20
				608.3 (y10++)	19
				836.4 (b7+)	17
	FNLAGNHEQEFLR	6.2	525.6 (+++)	692.4 (y5+)	20
				600.8 (y10++)	13
				565.3 (y9++)	14
	TANELNLLILR	11.2	635.4 (++)	983.6 (y8+)	21
				854.6 (y7+)	20
				741.5 (y6+)	22
Soybean	ISTLNSLTLPALR	10.5	699.9 (++)	984.6 (y9+)	23
				870.5 (y8+)	25
				783.5 (y7+)	25
	EAFGVNMQIVR	8.1	632.3 (++)	859.5 (y7+)	18
				760.4 (y6+)	17
				646.4 (y5+)	22
	ELINLATMCR	8.3	610.8 (++)	865.4 (y7+)	21
				751.4 (y6+)	21
				638.3 (y5+	17
	LITLAIPVNKPGR	7.9	464.6 (+++)	767.5 (y7+)	15
				583.4 (y11++)	9
				476.3 (y9++)	11

Food	Peptide	RT* (min)	Precursor (charge state) (*m/z*)	Product ion (fragment)	Collision energy (eV)
Milk	HQGLPQEVLNENLLR	8.1	587.3 (+++)	871.5 (y7+)	17
				758.4 (y6+)	16
				436.2 (b4+)	17
	FFVAPFPEVFGK	13.5	692.9 (++)	991.5 (y9+)	18
				920.5 (y8+)	18
				676.4 (y6+)	28
	YLGYLEQLLR	12.3	634.4 (++)	934.5 (y7+)	21
				771.5 (y6+)	20
				658.4 (y5+)	21
	NAVPITPTLNR	5.1	598.3 (++)	911.5 (y8+)	17
				456.3 (y8++)	14
				285.2 (b3+)	12
	VYVEELKPTPEGDLEILLQK	10.6	771.8 (+++)	912.0 (y16++)	19
				790.9 (y14++)	19
				627.9 (y11++)	20
	VLVLDTDYK	6.4	533.3 (++)	853.4 (y7+)	15
				754.4 (y6+)	14
				641.3 (y5+)	16
	LSFNPTQLEEQCHI	8.9	858.4 (++)	1254.6 (y10+)	26
	N-terminal peptide			928.4 (y7+)	27
				627.8 (y10++)	27

**Retention time (RT) is in sauce.*

RESULTS AND DISCUSSION

The software package Skyline, was used for in *silico* enzymatic digestion of food allergen proteins and to help produce potential MRMs for the experiment. From the list produced by Skyline, each MRM was analyzed using the ACQUITY UPLC System coupled to the Xevo TQ-S for sensitivity and reproducibility (in different food matrices).

In this method a total of 23 peptides and 69 MRMs were included as part of the analysis, although no regulations as yet state what determines a positive identification of an allergenic protein. (e.g. number of proteins and peptides to be monitored). For egg and milk, peptides representative of the different components of the egg: egg white (ovalbumin) and the yolk (vitellogenin), milk (casein), and whey (β-lactoglobulin), are included in the method.

METHOD SENSITIVITY

Current regulations address the analytical levels of detection for gluten, and so for the allergens monitored in this method, levels were assessed from the recommendation levels provided by VITAL and the AOAC SMPR for food allergens.

For each allergen, a single, common LOQ was determined for all targeted matrices (Figure 2). For each peptide, two MRM transitions in allergen-free matrices and incurred matrices were shown to demonstrate the specificity of the method and to confirm detection of the food allergens at the LOQ. The LOQ was defined as the minimum concentration giving a signal-to-noise ratio (S/N) of 10 for the most intense MRM transition of the targeted food allergen. The sensitivity of detection for the food allergen peptides was determined on the worst case, mainly processed cookies. The LOQs recorded are: 0.5 mg milk proteins/kg for caseins, 5 mg milk proteins/kg for whey, 3.4 mg egg proteins/kg for egg white, 30.8 mg egg proteins/kg for egg yolk, 2.5 mg/kg for peanut proteins, and 5 mg/kg for soybean proteins.

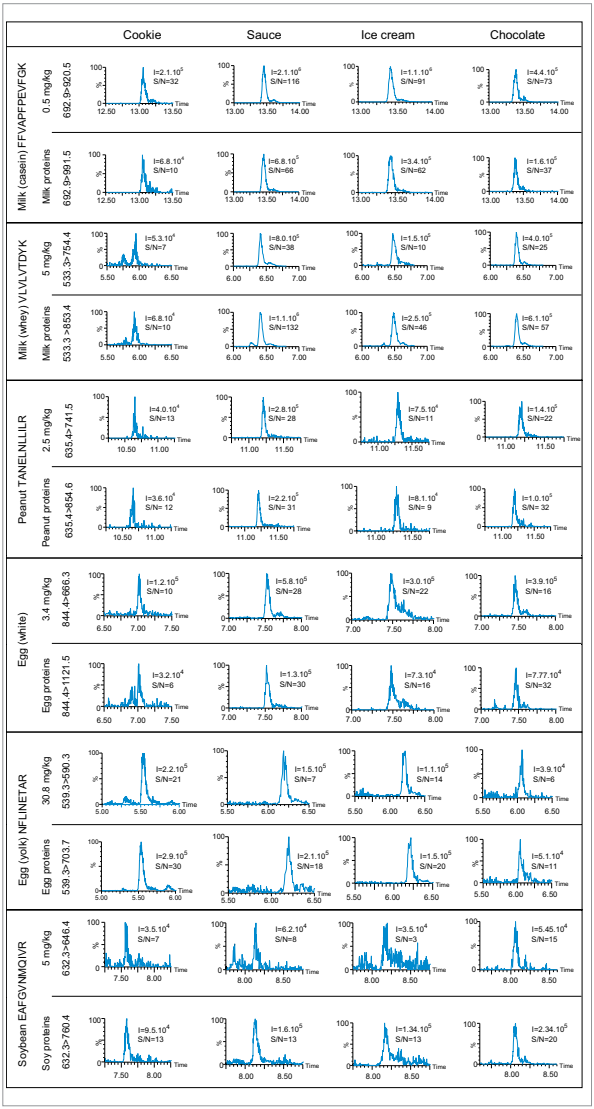

Figure 2. Chromatograms of the two higher MRM transitions of milk casein peptide FFVAPPEVFGK, whey milk peptide VLVLDTDYK, and peanut peptide TANELNLLILR. Egg white peptide GGLEPINFQTAADQAR, egg yolk peptide NFLINETAR, and soy peptide EAFGVNMQIVR in chocolate, ice cream, tomato sauce, and cookies. Data of incurred or processed matrices at the limit of quantification are presented without any data treatment.

METHOD LINEARITY

Linearity and matrix effects were tested by analyzing three independent foodstuff preparations (incurred chocolate and ice cream and processed cookies and sauce) that contained different concentrations of milk, egg, soy, and peanut food allergen proteins (Figure 3).

Although the matrix effect and the effect of the thermal process were not the same for both targeted peptides from the same food allergen, the linear coefficient of regression supported the reliability of the method even the absence of an internal standard.

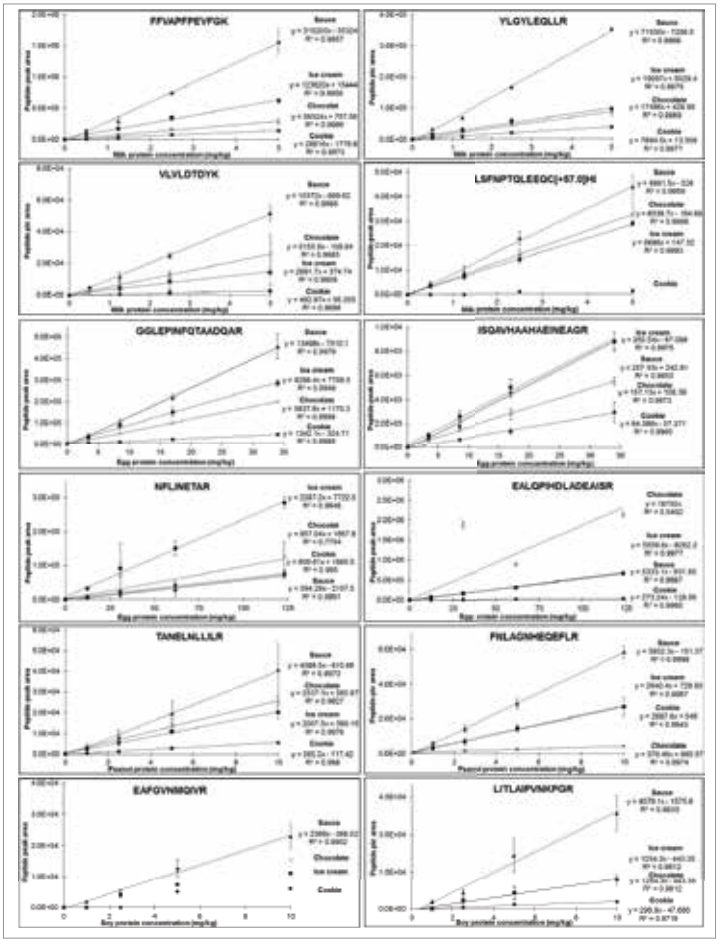

Figure 3. Linear regression of peptide peak area of the higher MRM in function of the concentration of food allergen proteins performed in three independent replicates in incurred tomato sauce, chocolate, ice cream, and processed cookies. The linearity was controlled for each food allergen: milk casein FFVAPFPEVFGK (692.9>920.5) and YLGYLEQLLR (634.4>771.5); whey milk VLVLDTDYK (533.3>853.4) and LSFNPTQLEEQC[+57] HI (858.4>928.4) (carbamidomethylation of cysteine amino acids by addition of iodoacetamide before an enzymatic digestion to block the onset of disulfur bridges); egg white GGLEPINFQTAADQAR (844.4>666.3) and ISQAVHAAHAEINEAGR (887.5>1067.5); egg yolk NFLINETAR (539.3>703.4) and EALQPIHDLADEAISR (593.3>668.8); peanut TANELNLLLR (635.4>741.5) and FNLAGNHEQEFLR (525.6>600.8; and soybean EAFGVNMQIVR (632.9>760.4) and LITAIPVNKPGR (464.6>583.4).

CONCLUSIONS

Sensitive detection of food allergens (milk casein, whey, egg white, egg yolk, peanut, and soybean) was achieved by analyzing food allergen peptides using the ACQUITY UPLC System coupled to the Xevo TQ-S.

In keeping with food production requirements, the targeted matrices were processed (tomato sauce, cookies) or incurred (chocolate, ice cream). This multi-allergen detection method has the lowest limits of quantification available to date (expressed in total proteins and not soluble proteins): 0.5 mg milk proteins/kg for caseins, 5 mg milk proteins/kg for whey, 3.4 mg egg proteins/kg for egg white, 30.8 mg egg proteins/kg for egg yolk, 2.5 mg peanut proteins/kg, and 5 mg soybean proteins/kg.

While matrix effects can be observed from the data shown, further work will involve the inclusion of internal standards in order to make the method quantitative.

References

1. AOAC SMPR 2016.002, Standard method performance requirements for Detection and Quantitation of Selected Food Allergens.
2. http://allergenbureau.net/vital/
3. M Planque, T Arnould, M Dieu, P Delahaut, P Renard, N Gillard. Advances in ultra-high performance liquid chromatography coupled to tandem mass spectrometry for sensitive detection of several food allergens in complex and processed foodstuffs. *J Chrom A.* 1464: 115–123, 2016.

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Identification and Quantitative Analysis of Egg Allergen Peptides Using Data Independent Ion Mobility Mass Spectrometry

Lee A Gethings,[1] Nathalie Gillard,[2] Antonietta Wallace,[1] and Valery Dumont[2]
[1]Waters Corporation, Wilmslow, UK; [2]CER Groupe, Marloie, Belgium

APPLICATION BENEFITS

- HDMS^E provides both qualitative and quantitative information in a single experiment.
- Utilizing ion mobility as part of the workflow provides enhanced specificity and therefore confidence of identifications returned.
- Method provides potential means for multi-allergen detection.

WATERS SOLUTIONS

SYNAPT™ G2-S*i*

ACQUITY UPLC™ M-Class System

Progenesis™ QI for Proteomics

KEYWORDS

Allergens, foodomics, proteomics, discovery omics, QTof, ion mobility

INTRODUCTION

Food allergies arise from an abnormal immunological response to certain foods. Proteins are the main candidates for triggering allergic reactions. Egg-based proteins are one of the most frequent causes of adverse reactions in food. Since many processed foods contain egg as a raw ingredient, the ability to assess changes in protein structure and detection through the manufacturing cycle is important.

Food allergen analysis using LC-MS/MS is a current hot topic for many food scientists and there are two approaches that can be used to generate a quantitative method. The first approach is to perform *in silico* digestion of proteins, based on fasta sequences available in databases (e.g. UniProt), providing a list of potential peptides and MRM transitions. This methodology requires further investigation to determine which MRMs are the most specific and sensitive at detectable response levels for post-food processing, sample treatment, and during the ionization process. The alternative is to perform a discovery omics experiment using a high resolution instrument, such as a QTof mass spectrometer and use the data observed from this experiment to generate a targeted method.

In this study, the second approach has been applied and focuses on identifying and quantifying known allergenic proteins from raw and cooked egg samples. Proteins extracted from raw and cooked egg samples were digested using trypsin and label-free protein expression data were acquired with Waters™ SYNAPT G2-S*i* using an ion mobility data independent approach (whereby the collision energy was switched between low and elevated energy states during alternate scans).

Utilizing ion mobility as part of the workflow provides enhanced specificity and therefore confidence of identifications returned, even in the presence of complex matrices, such as processed food samples. Precursor and product ions were associated by means of retention and drift time alignment. Although egg proteins were the focus of this work, other allergenic proteins that are also extracted using the sample preparation could be investigated, providing a potential means for multi-allergen detection.

The acquired data were processed using Progenesis QI for Proteomics and searched against a Gallus Gallus (Uniprot) database. The results generated allowed for relative quantification to be established. The results of this study showed that a significant proportion of proteins identified were expressed when comparing cooked and raw egg sample sets, which included known allergenic proteins (e.g. apovitellenin I). Peptides identified in both sample sets allowed for MRM transitions to be generated and a quantifiable value assigned.

EXPERIMENTAL

Sample preparation

Proteins were extracted from egg-based samples using phosphate buffer saline (PBS) and a BCA assay used to determine initial protein concentrations and afterward normalized proteins concentration to 1 mg/ml. Samples were reduced and alkylated before overnight digestion using trypsin. Prior to LC-MS analysis, samples were filtered using a 0.22 µm filter to remove any particulates and diluted appropriately using 0.1% formic acid (Figure 1).

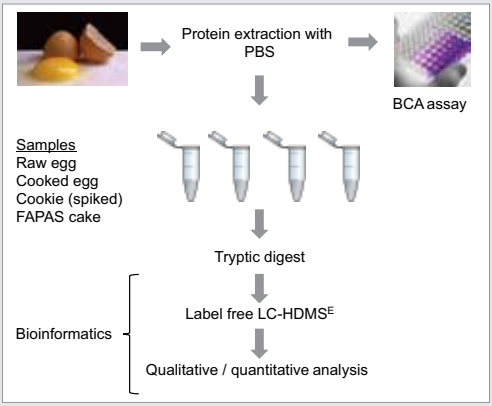

Figure 1. Experimental design study for egg allergen proteins.

LC-MS conditions

Label-free LC-MS was used for qualitative and quantitative peptide analyses. Experiments were conducted using a 90 min gradient from 5 to 40% acetonitrile (0.1% formic acid) at 300 nL/min using an ACQUITY UPLC M-Class System configured with an ACQUITY UPLC Peptide BEH C_{18} nanoACQUITY Column 10K psi, 130 Å, 1.7 µm, 75 µm X 150 mm, p/n: 186003543. Data were acquired in data independent analysis (DIA) utilizing a SYNAPT G2-S*i* Mass Spectrometer enabled with ion mobility functionality.

Bioinformatics

The LC-MS peptide data were processed and searched with Progenesis QI for Proteomics Software. A species specific Gallus Gallus (Uniprot) database was used. Fixed and variable modifications included carbamidomethyl C and met-oxidation respectively in addition to a protein false discovery rate of 4%.

ACQUITY UPLC M-Class System with the SYNAPT G2-Si.

RESULTS AND DISCUSSION

PROTEIN IDENTIFICATION AND QUANTIFICATION FOR RAW AND COOKED EGG

Proteins extracted from raw and cooked egg samples were analyzed to identify, quantify, and investigate the variance between potential allergenic markers. A total of 95 and 84 proteins were identified for raw and cooked respectively. A subset of those proteins identified are highlighted in Table 1 with their respective amounts found in both the raw and cooked egg extracts.

The SYNAPT G2-*Si* utilized data independent analysis with ion mobility (HDMS^E) enabled. The advantage of HDMS^E mode is that it maximizes the number of identified proteins through increased peak capacity and overall specificity. Example low energy (relating to precursor ions) and high energy (relating to fragment ions) are presented in Figure 2.

Ion mobility was enabled to provide enhanced specificity for the experiment. This results in cleaner spectra (important when analyzing complex food matrices), and provides the ability to separate similar species, as shown in Figure 3, where example spectra are shown with ion mobility deactivated (off) and activated (on).

Table 1. Typical allergenic proteins identified and quantified from raw and cooked egg extracts.

Allergenic protein	Raw (ng/µL)	Cooked (ng/µL)
P01005: Ovomucoid (OVM)	344.0	333.0
P01012: Ovalbumin (OVA)	44.3	43.8
P02659: Apovitellenin (APO)	12.7	5.2
P02789: Ovotransferrin (OVT)	29.7	5.8
P00698: Lysozyme (LYS)	6.5	1.0

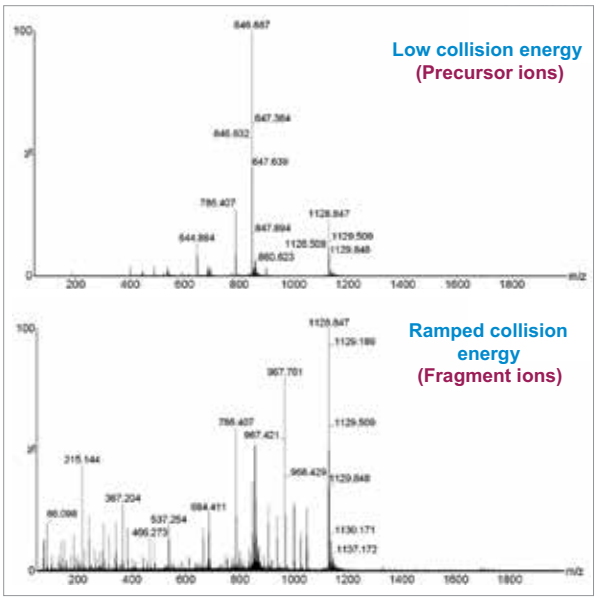

Figure 2. OVM diagnostic marker identifiable at 1 ppm (m/z 846.624, ELAAVSVDCSEYPKPDCTAEDRPLCGSDNK).

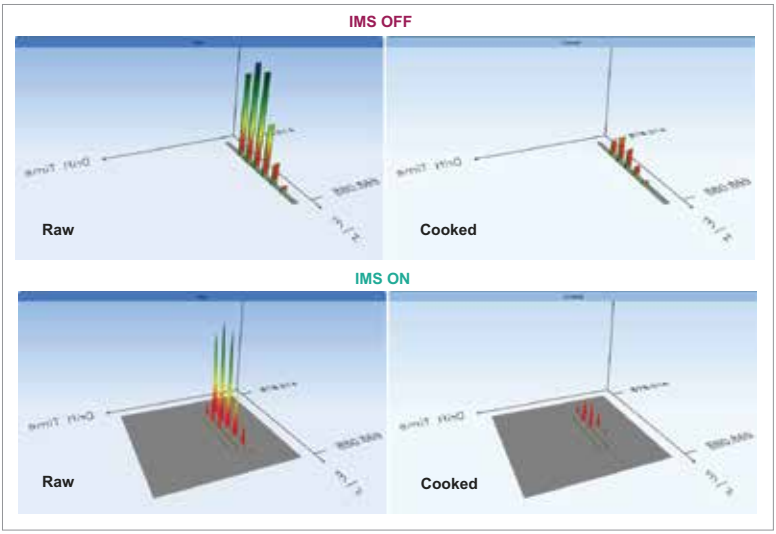

Figure 3. Effect of IMS separation demonstrated for the overlapping OVT peptide (m/z 878.7726, AIANNEADAISLDGG). Upper traces show overlapping species for both raw and cooked egg samples. The implementation of IMS (lower trace) allows separation of precursors with the same m/z, resulting in the identification of two distinct species.

Using the SYNAPT G2-S*i* in HDMS^E mode made it possible to obtain high peptide sequence coverage in the presence of cake matrix (Figure 4).

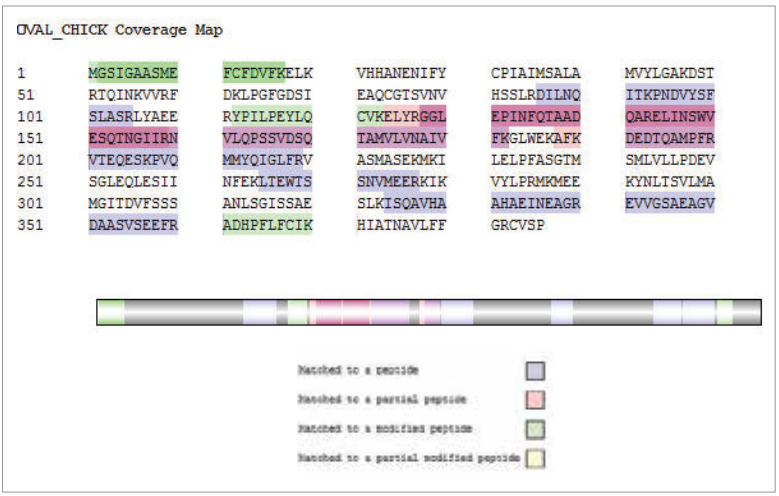

Figure 4. P01012, ovalbumin (OVA), and gallus gallus chicken peptides identified in the presence of cake matrix (51.8% sequence coverage).

UTILIZING ION MOBILITY TO REDUCE BACKGROUND MATRIX EFFECTS

To assess the capabilities and advantages of implementing ion mobility into the analytical workflow, a dilution series of cooked egg ranging from 500 to 1 ppm was spiked into cookie matrix, which was maintained at the same concentration throughout (Figure 5).

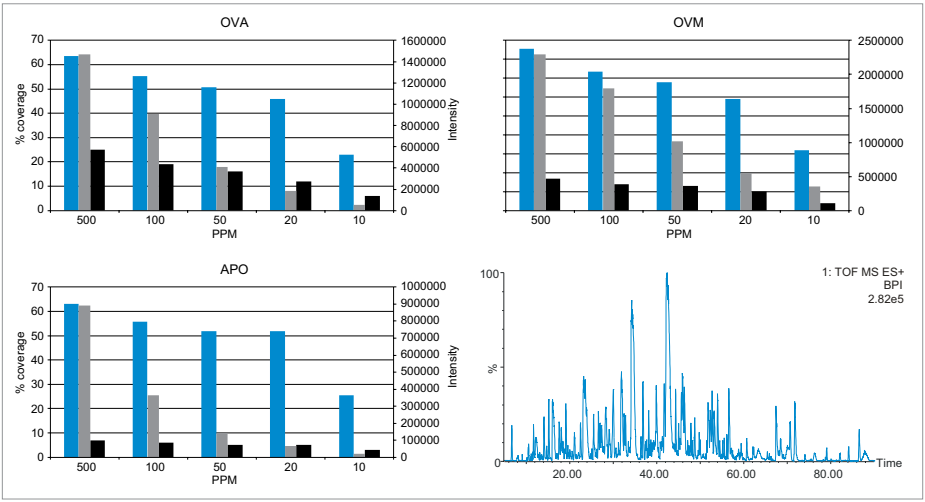

Figure 5. Identified allergenic proteins for serially diluted cooked egg in the presence of cookie matrix. Number of peptides (black), sum of intensity for the top three most abundant peptides (grey), and sequence coverage (blue). An example chromatogram is provided for 50 ppm of cooked egg in the presence of the cookie matrix.

FAPAS TEST SAMPLE – CAKE MIX

Test material was supplied for FAPAS Proficiency Test material T2770 in the form of a cake mix obtained from a retail source which was free from egg and milk but contained gluten. Royal icing sugar was used to introduce egg white protein.

The major allergen identified was ovalbumin (OVA), and the sequence coverage (percentage of peptides identified that make up the protein sequence) in the presence of cake matrix was high at 51.8%.

Initial results from the FAPAS proficiency study were conducted using ELISA, which quantified egg between 39.6 to 62.1 ppm (mean = 47.7 ppm). The LC-MS label-free experiments corresponded with the ELISA findings, quantifying at 58 ppm. The nature of HDMS^E also allows for multi-allergens to be detected and quantified as part of this experiment.

CONCLUSIONS

A discovery proteomic workflow has been applied to determine marker peptides that can be used for the quantitative analysis of allergenic proteins within food. A label-free proteomic approach has been applied for the analysis of egg-based allergens, by implementing HDMS^E to provide both qualitative and quantitative information in a single experiment.

Ion mobility as part of the workflow is shown to provide enhanced specificity and therefore confidence of identifications returned, even in the presence of complex matrices, such as processed food samples.

Although only egg proteins were the focus of this work, other proteins relating to other allergens were observed, providing a potential means for multi-allergen detection.

References

1. Commission Directive No 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC (Official Journal, L310, 28/11/2007, 001–0014).
2. Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ. Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. *Proteomics*. Mar;9(6):1696–719, 2009.

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Rapid, Simple, and Effective Clean-up of Bovine Liver Samples Prior to UPLC-MS/MS Multiresidue Veterinary Drugs Analysis

Michael S. Young and Kim Van Tran
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Efficient, timesaving multiclass/multiresidue methodology
- Simple, rapid, and effective sample clean-up suitable for a diverse range of analytes
- Fast, sensitive UPLC-MS/MS analysis

WATERS SOLUTIONS

ACQUITY UPLC™ I-Class System

Xevo™ TQ-XS Mass Spectrometer

Oasis™ PRiME HLB Cartridge for SPE Clean-up

KEYWORDS

UPLC-MS/MS, Oasis PRiME HLB Cartridges, veterinary drugs, beef liver

OVERVIEW

In order to ensure public health and safety, reliable analytical methods are necessary to determine veterinary drug residue levels in edible tissue samples such as beef liver. The compounds of interest range from highly polar water-soluble compounds to very non-polar fat-soluble compounds. In order to maximize throughput and minimize costs it is desirable to determine the widest possible range of veterinary drug residues in tissue samples with a single analytical method.

INTRODUCTION

Tissue samples, such as bovine muscle and liver, are typically extracted with an acetonitrile based solvent for LC-MS determination of veterinary drug residues. Among the most significant co-extracted substances are fats and polar lipids, particularly phospholipids (lecithin). A gram of bovine liver typically contains about 45 mg of fat, about half the amount usually present in muscle tissue, but still significant. Bovine liver is also a very good source of dietary lecithin (phospholipids); a gram of liver contains about 25 mg of phospholipids, about four times the amount typically found in muscle. Fats can be removed from the acetonitrile based tissue extracts by liquid extraction with hexane or with SPE with octadecyl silica (C_{18}). Although C_{18} is effective for removal of most non-polar lipids, it does not remove phospholipids. Excessive amounts of phospholipids can shorten LC column life, contribute to ion-suppression, and contaminate the mass spectrometer. In this study a novel reversed-phase sorbent, Oasis PRiME HLB, is used for highly effective removal of both phospholipids and fats from bovine liver extracts prior to LC-MS/MS analysis. With the new sorbent recoveries of veterinary drugs were similar to results obtained using C_{18} for clean-up. However, greater than 95% of phospholipids and greater than 85% of fats were effectively removed from the tissue extracts after the simple pass-through SPE procedure.

EXPERIMENTAL

UPLC conditions

LC system: ACQUITY UPLC I-Class with Fixed-Loop Sample Manager

Column: ACQUITY UPLC CSH™ C_{18}, 1.7 µm, 2.1 mm x 100 mm I.D.

Mobile phase: A: 0.1% formic in water

B: 0.1% formic acid in 50:50 acetonitrile/methanol

Injection vol.: 7 µL

Injection mode: partial loop injection

Column temp.: 30 °C

Weak needle wash: 10:90 acetonitrile:water (600 µL)

Strong needle wash: 50:30:40 water:acetonitrile: IPA (200 µL)

Seal wash: 10:90 acetonitrile: water

Gradient:

Time	Flow (mL/min)	%A	%B
0.00	0.400	99.0	1.0
4.00	0.400	80.0	20.0
5.00	0.400	50.0	50.0
7.00	0.400	1.0	99.0
10.00	0.400	1.0	20.0
10.10	0.400	99.0	1.0
12.00	0.400	99.0	1.0

MS conditions

Mass spectrometer: Xevo TQ-XS

Mode: Positive Ion Electrospray

Source temp.: 150 °C

Desolvation temp.: 400 °C

Desolvation gas flow: 1000 L/Hr

Cone gas flow: 30 L/Hr

Collision gas flow: 0.15 mL/Min

Data management: MassLynx™ v4.1

Table 1. MRM transitions (primary transition first) and instrument parameters used for this study; also listed are the observed retention times (RT) for the compounds.

Compound	MRM	Cone (V)	Collision (eV)	RT (min)
Amocixicillin	366.2>349.1, 366.2>114.1	30 30	8 20	2.46
Ampicillin	350.2>106.1, 350.2>160.1	30 30	18 12	4.14
Amprolium	243.3>150.2, 243.3>94.1	20 20	12 14	0.54
Bacitracin A	712.2>110.1, 712.2>191.1	68 68	70 40	5.72
Ceftiofur	524.3>241.1, 524.3>285.0	30 30	16 16	5.98
Chlorotetracycline	479.3>444.2, 479.3>462.2	15 15	22 18	5.28
Clopidol	192.1>100.9, 192.1>128.0	40 40	26 24	4.10
Clorsulon	378>342.0, 378>344.0	22 22	12 12	5.76
Cloxacillin	436.2 >160.0, 36.2>277.1	27 27	15 15	6.67
Danofloxacin	358.2>314.1, 358.2>96.0	38 38	20 25	4.65
Desethlylene Ciprofloxacin	305.9>268.1, 305.9>288.1	32 32	25 18	3.90
Erythromycin	734.7>158.1, 734.7>576.5	48 48	26 18	5.72
Eprinomectin	915.6>186.0, 915.6154.0	30 30	35 20	7.78
Famphur	326.0>217.0, 326.0>93.0	32 32	20 31	6.60
Fenbendazole	300.0>268.0, 300.0>159.0	40 40	23 24	6.52
Flunixin	297.2>264.1, 297.2>279.0	35 35	34 34	7.19
Ivermection	892.6>307.2, 892.6>569.4	15 15	14 25	8.18
Levamisole	205.0>123.0, 205.0>90.8	40 40	27 34	2.31
Melengestrol Acetate	397.4>337.3, 397.4>279.0	10 10	15 15	7.30
Monesin	693.7>675.3, 693.7>461.1	70 70	35 50	8.13
Morantel	221.2>186.1, 221.2>108.0	20 20	20 25	5.44
Moxidectin	640.0>528.4, 640.0>498.3	30 30	10 10	7.96
Noviobiocin	613.10>188.9, 613.1>396.0	45 45	20 15	7.45
n-methyl-1 3-propanediamine	89.1>72.2, 89.1>58.2	42 42	5 5	0.41
Oxfendazole	316.2>191.1, 316.2>284.0	40 40	18 18	5.76
Oxteracyline	461.4>426.2, 461.4>365.0	48 48	30 15	4.36
Penicillin G	335.2>289.1, 335.2 >158.1	40 40	25 25	5.54
Progesterone	315.2>109.0, 315.2>97.0	38 38	24 22	7.30
Ractopamine	302.2>164.1, 302.2>284.2	35 35	15 12	4.30
Sulfachlorpyridazine	285.0>156.0, 285.0>92.1	35 35	16 26	5.44
Sulfadimethoxine	311.1>156.0, 311.1>92.0	36 36	32 32	5.89
Sulfamethazine	279.1>186.0, 279.1>124.1	40 40	15 25	4.92
Sulfaquinoxaline	301.1>156.1, 301.1>92.2	32 32	16 30	5.93
Tetracycline	445.1>154.0, 445.1>410.1	40 40	26 22	4.43
Thiabendazole	202.0>175.0, 202.0>131.0	15 15	25 30	3.46
Tilmicosin	869.5 >174.2, 869.5>696.5	25 25	45 40	5.35
Tripelennamine	256.1>211.1, 256.1>91.0	21 21	17 33	3.87
Tylosin	916.5>174.1, 916.5>101.1	45 45	40 45	5.78
Zilpaterol	262.2>202.1, 262.2>185.1	25 25	18 22	0.79

Sample preparation

1. Initial Extraction/Precipitation:

A 2 g sample of tissue was placed into a 15 mL centrifuge tube containing ceramic homogenizer balls (a Bertin Technologies Precellys Evolution Homogenizer was used for this step). For standards or QC samples the samples were spiked with appropriate amounts of desired analytes. 10 mL 0.2% formic acid in 85:15 acetonitrile/water was added and the samples were homogenized/extracted for 1.5 minutes. The tubes were then centrifuged at 3200 rcf for 5 minutes.

Note: The extraction/precipitation step gives good recovery of most compounds of interest but also extracts significant amounts of fats and phospholipids.

2. Pass-through SPE clean-up:

An Oasis PRiME HLB Cartridge (6 cc, 200 mg) was mounted on a pre-cleaned vacuum manifold. Cartridge conditioning is NOT required, and was NOT performed. The vacuum was set to 2 psi. A 0.6 mL portion of the supernatant was passed-through the Oasis PRiME Cartridge and discarded. Collection tubes were then installed and a 1 mL portion of the supernatant was passed-through the Oasis PRiME Cartridge and collected. A 200 µL aliquot of the pass-through clean-up sample was taken and diluted with 400 µL of 10 mM ammonium formate buffer (pH 4.5) prior to UPLC-MS/MS analysis.

RESULTS AND DISCUSSION

Figure 1 shows the recovery data obtained from replicate analysis of spiked tissue samples (n = 6). Matrix effects averaged about 40%. The chromatograms shown in Figure 2 show the effectiveness of the Oasis PRiME HLB Cartridge for removal of ≥95% of phospholipids from the beef liver extracts. The cartridge also removes more than 90% of hexane extractable fat.

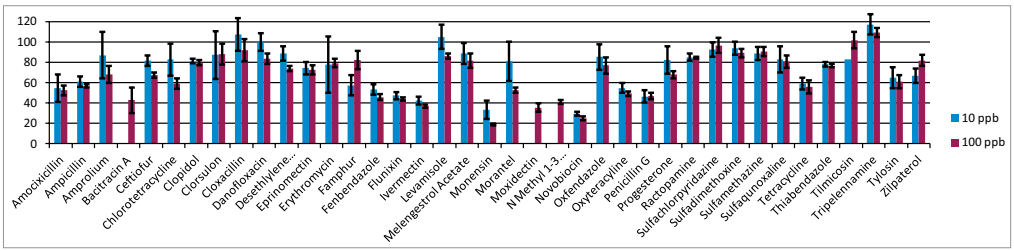

Figure 1. Recovery data from spiked beef liver sample for low level (10 ng/g in blue) and high level (100 ng/g) in red.

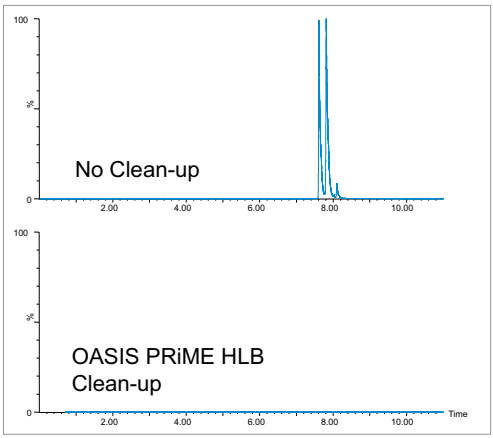

Figure 2. LC-MS/MS chromatograms showing effective removal of ≥95% of phospholipids from beef liver extract

The procedure utilized in this study was developed from methods presented previously.[1,2] Although the overall method recoveries averaged above 70 percent, lower recovery was observed for some of the more polar compound classes, such as tetracyclines. Unfortunately, no single solvent extraction step will be highly efficient for all target compounds. For most of the lower recovered compounds the signal response and reproducibility are acceptable for target screening analysis. It is important to understand the contribution of the sample cleanup to any observed recovery losses. The SPE recovery data shown in Figure 3 were obtained from beef liver samples spiked after solvent extraction and prior to SPE clean-up. These data indicate that, for most of the compounds, the Oasis PRiME HLB Cartridge clean-up contributes little to the observed recovery losses. However, for ivermectin, monensin, moxidectin, and novabiocin, the post extraction cleanup did introduce measurable recovery losses. More information on these analytes will be presented in future work.

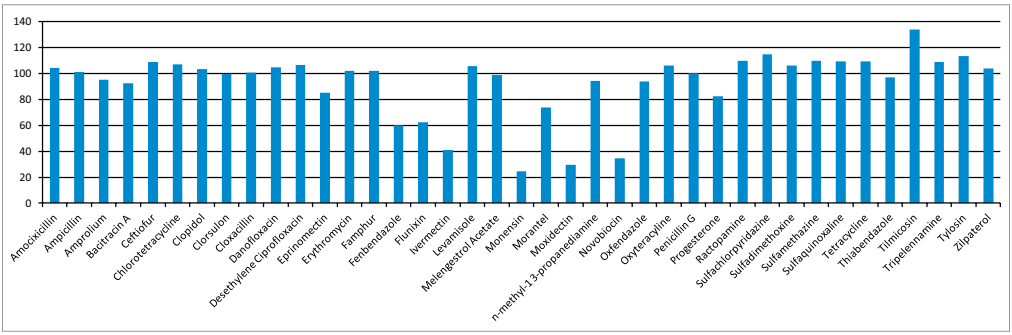

Figure 3. Recovery of veterinary compounds from blank beef liver extracts spiked after initial extraction and prior to Oasis PRiME HLB pass-through clean-up.

CONCLUSIONS

- A simple and effective extraction/protein precipitation procedure was developed for screening analysis of bovine liver tissue for a wide range of veterinary drugs
- A simple pass-through clean-up protocol using Oasis PRiME HLB Cartridges was employed to remove greater than 90% of fats and phospholipids from the initial extracts
- The sample preparation methodology produced an extract that was free of particulates and required no subsequent filtration prior to LC-MS analysis
- Consistent recoveries were observed for a wide range of veterinary drugs using the simple one-step pass-through clean-up protocol with Oasis PRiME HLB Cartridges

References

1. M. Young and K. Tran, "Oasis PRiME HLB Cartridge for Effective Clean-up of Meat Extracts Prior to Multi-Residue Veterinary Drug UPLC-MS Analysis", Waters Application Brief, 2015.
2. S. Lehotay, "High-Throughput Screening Analysis by UHPLC-MS/MS of >60 Veterinary Drugs in Animal Tissues", 125th AOAC Annual Meeting, Presentation 2303, 21 September, 2011.

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Rapid Detection of 7 Illegal Veterinary Additives in Animal Feed Using Oasis PRiME HLB Clean-up and UPLC-MS/MS

DeFeng Huang , Xia Geng, and Xiaowei He
Waters Technologies (Shanghai) Co., Ltd.

APPLICATION BENEFITS

- Efficient, time-saving total solution for muti-residue analysis of veterinary drugs in animal feed formula
- Simple and rapid sample preparation with Oasis™ PRiME HLB
- Fast and sensitive UPLC™-MS/MS analysis

WATERS SOLUTIONS

ACQUITY™ UPLC I-Class System

Xevo™ TQ-S micro Mass Spectrometer

Oasis PRiME HLB Cartridge

KEYWORDS

Olaquindox, neomycin sulfate, sulfaquinoxaline, dihydropyridine, Oasis PRiME HLB Cartridge, formula feed, UPLC, MS, veterinary drug

INTRODUCTION

When discussing illegal additives in the feed, we immediately think of clenbuterol. Since the scandal of clenbuterol was exposed in 2011, the Chinese government has established a strict standard for the use of additives, and also tightened regulation for the illegal use of additives in feed. Nevertheless, some feed producers still have not stopped their illegal behavior. The "CCTV 3.15 party in 2017" exposed this situation and aroused great concern from the public. A reporter's survey found the abuse of veterinary drugs including olaquindox, neomycin sulfate, sulfaquinoxaline, and dihdropyridine in animal breeding.

Olaquindox is an alternative to clenbuterol, that can promote growth, reduce the feed and meat ratio, improve body size, and improve feed intake. It tends to be accumulated in animal tissue and leads to chromosomal abnormalities in cells if added to animal feed over a long period of time. However, the residues of these compounds also pose a health risk to the consumers.

Currently, the determination of olaquindox in the Chinese national standard (GB)[1] is mainly based on LC-UV and LC-MS/MS methods. Accurate quantification of Olaquindox is a challenge because of the complex matrices and potential to decompose during sample preparation and when exposed to light.

In this application note, a simple clean-up protocol using a novel SPE device was introduced for the analysis Olaquindox and six other illegal veterinary additives in animal feed. The extract was cleaned up by pass-through SPE using the Oasis PRiME HLB Cartridge prior to UPLC-MS/MS analysis. The spiked samples were quantified using an external standards method, and the recovery and reproducibility for each compound met the regulatory requirements of the quantitative method. This method is simple, rapid, accurate, suitable for the analysis of the highlighted veterinary drugs in animal feed.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 x 100 mm
Temp.:	45 °C
Flow rate:	0.4 mL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in methanol
Run time:	9 min
Injection vol.:	2 µL

Gradient:

Time (min)	Flow rate (mL/min)	%A	%B
0.00	0.4	98	2
0.25	0.4	98	2
3.25	0.4	70	30
7.00	0.4	2	98
7.50	0.4	2	98
7.60	0.4	98	2
9.00	0.4	98	2

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI+
Capillary voltage:	3.0 kV
Desolvation temp.:	550 °C
Source temp.:	150 °C
Desolvation flow:	1000 L/h
Cone gas:	50 L/h

MRM conditions

Compound	Parent ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Olaquindox	264.1	143.0 212.1	32 32	30 23
Sulfaquinoxaline	301.0	92.0 155.9	32 32	30 13
Trimethoprim	291.1	123.0 230.1	40 40	27 28
Aminophylline	181.0	96.1 123.9	35 35	25 21
Diprophylline	255.1	123.9 181.0	35 35	35 22
Dexamethasone	393.2	355.2 373.2	20 20	10 10
Atropine	290.1	93.0 124.0	35 35	36 29

Sample preparation

Initial extraction

Step 1: Weigh 1 g of feed sample into a 50 mL centrifuge tube;

Step 2: Add 10 mL of extraction solvent (80% acetonitrile + 20% water) and shake well for 10 min;

Step 3: Centrifuge at 6000 rpm for 5 min

Pass-through SPE clean-up

Step 1: An Oasis PRiME HLB Cartridge (6 cc, 200 mg; p/n: 186008057) was mounted on a pre-cleaned SPE vacuum manifold. Cartridge conditioning is not required and is not performed.

Step 2: A 0.5 mL aliquot of the supernatant (sample extract) was passed through the Oasis PRiME HLB Cartridge and the eluant was discarded.

Step 3: Install the collection tubes. Another 1 mL of supernatant was passed through the cartridge, and the eluant was collected. The eluant was diluted 1:3 with water and injected into Xevo TQ-S micro for analysis.

RESULTS AND DISCUSSION

METHOD RECOVERY AND STABILITY

The analyte recovery was determined by spiking standards into the blank matrix, a 1:1 mixture of rice and corn powders. The analytes were spiked at concentrations of low, medium (5 times low spike) and high levels (10 times low spike). The lowest spike for olaquindox was 10 ug/kg, sulfaquinoxaline was 0.5 ug/kg, trimethoprim and atropine was 2.5 ug/kg and the lowest spike for aminophylline, diprophylline and dexamethasone was 5.0 ug/kg). Each level of spiking was repeated in five replicates. All samples were processed according to the method described previously. The concentrations were calculated using a matrix-matched calibration curve. The recovery range of the high, medium, and low level samples ranged from 70.6% to 112%. The precision range of the high and medium level spike samples was 0.88% to 4.2% and the precision range was 4.3% to 8.8% for the low spike samples.

MATRIX EFFECTS AND MATRIX MATCHED CALIBRATION CURVE

The matrix effect was measured by comparing the peak area of solvent standards and post spiked samples in chicken feed and swine feed samples, where the spiked level was equal to 5 µg/kg for atropine and diprophylline, and 1 µg/kg for the other compounds.

Calibration curves ranged from 0.01 to 1.00 µg/L for sulfaquinoxaline, from 0.1 to 10 µg/L for olaquindox, aminophylline, diprophylline, and dexamethasone, and 0.05 to 5.0 µg/L for trimethoprim and atropine.

Table 1. Matrix effects of each compound and the correlation coefficients of their matrix matched calibration curves.

Veterinary drugs	Matrix effects (%)	Matrix matched calibration curve R^2
Olaquindox	(9.0)	0.9998
Sulfaquinoxaline	(14.9)	0.9997
Trimethoprim	7.2	0.9998
Atropine	16.1	0.9994
Aminophylline	(0.5)	0.9995
Diprophylline	9.6	0.9992
Dexamethasone	(14.7)	0.9991

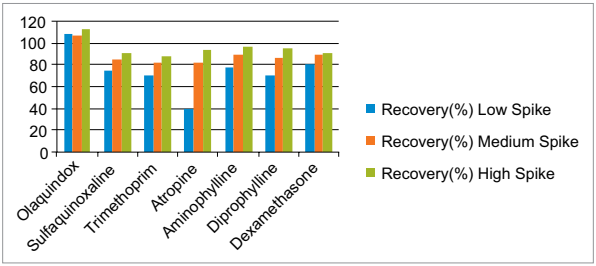

Figure 1. Summary of recoveries for spiked feed samples. The lowest spike for olaquindox was 10 ug/kg, sulfaquinoxaline was 0.5 ug/kg, trimethoprim and atropine was 2.5 ug/kg and the lowest spike for aminophylline, diprophylline and dexamethasone was 5.0 ug/kg).

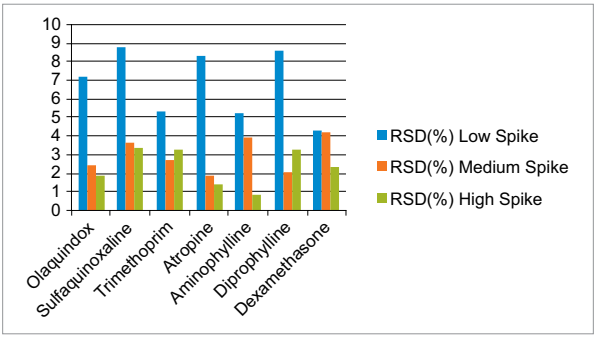

Figure 2. Precision of recoveries for spiked feed samples.

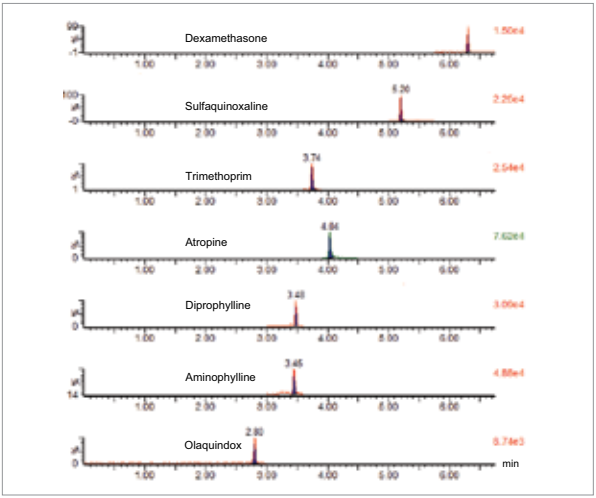

Figure 3. Typical chromatograms of spiked sample (sulfaquinoxaline spiked at 0.1 ppb; olaquindox, aminophylline, diprophylline, and dexamethasone spiked at 1.0 ppb; trimethoprim and atropine spiked at 0.5 ppb).

The established method was used for real sample analysis. Finally, an olaquindox content up to 1.9 to 18 mg/kg was detected in chicken feed and swine feed samples.

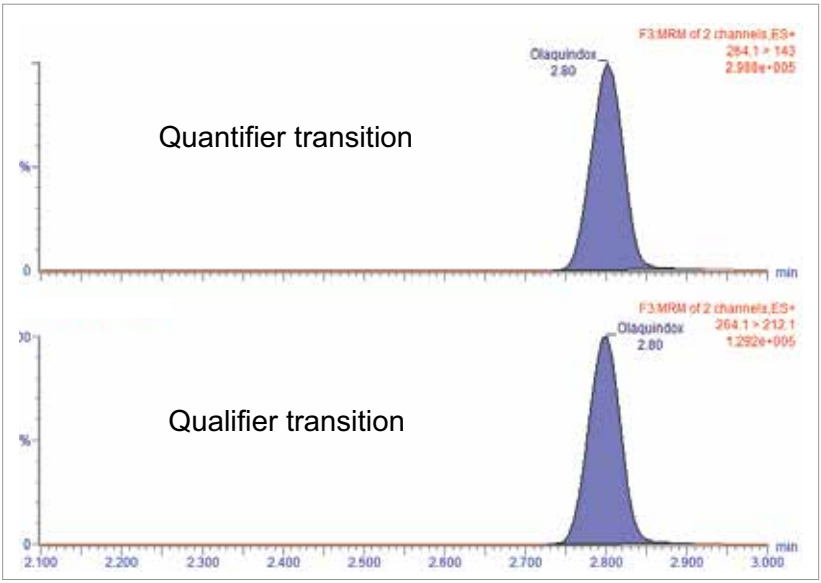

Figure 4. Chromatogram of olaquindox (1.9 mg/kg) in chicken feed.

CONCLUSIONS

- A simple and rapid analytical method was developed for the determination of seven illegal veterinary drug additives in animal feed. This method has been proven to achieve levels of detection that meet regulatory requirements.
- The Oasis PRiME HLB Cartridge provided effective clean-up and good recoveries for the target veterinary drugs in animal feeds.
- The ACQUITY UPLC I-Class System coupled with Xevo TQ-S micro offered good sensitivity and robust methodology.

Reference

1. Announcement No. 2086-5-2014 of the Ministry of Agriculture of the People's Republic of China: Determination of carbadox, mequindox, quinocetone and olaquindox in feeds – liquid chromatography – tandem mass spectrometry.

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Determination of Fipronil and its Metabolite Fipronil Sulfone in Eggs by Liquid Chromatography-Tandem Quadrupole Mass Spectrometry Using a Modified QuEChERS Method

Renata Jandova, Eimear McCall, Euan Ross, and Simon Hird, Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

A robust, cost-effective method for the determination of fipronil and fipronil sulfone in eggs, that meets requirements for both official control and food business operators' due diligence testing, at concentrations significantly lower than the EU MRL.

WATERS SOLUTIONS

Oasis™ PRiME HLB Column SPE Cartridge

DisQuE™ QuEChERS

ACQUITY™ UPLC™ I-Class System

Xevo™ TQ-XS Triple Quadrupole Mass Spectrometry

MassLynx™ MS Software

KEYWORDS

Fipronil, fipronil sulfone, UPLC-MS/MS, QuEChERS, egg, SANTE/11945/2015

INTRODUCTION

Fipronil is an insecticide used to protect seeds from insects, for professional pest control to combat infestation of insects such as cockroaches, as well as in veterinary medicine to protect dogs and cats from fleas, mites, and ticks. Fipronil is highly toxic and it is not authorized for use as a veterinary medicine, biocide, or pesticide around food producing animals. Hence it should never have found its way into a chicken coop. At the center of the recent food safety concern throughout Europe, fipronil has been found in eggs at concentrations above the maximum residue level (MRL).[1] With ongoing police investigations, product recalls, and destruction of many millions of eggs, increased analytical testing has ensued to ensure consumer safety throughout Europe and as far afield as Hong Kong.

Food safety authorities in the Member States of the European Union (EU) and the food industry have implemented significant monitoring of eggs for residues of fipronil, which is being conducted to ensure that the recall measures are protecting consumers. There is also interest in egg products, meat, and organs from laying poultry. In order to monitor fipronil abuse and ensure the safety of such foods, a simple, sensitive, reliable, and validated method for determining residues of fipronil in chicken egg is needed.

The default EU MRL for fipronil in eggs is set at 0.005 mg/kg with a residue definition of the sum of the parent fipronil and the metabolite fipronil sulfone, expressed as fipronil.[2] Fipronil and fipronil sulfone can be determined by either LC-MS/MS or GC-MS(/MS) after a generic extraction such as QuEChERS , followed by clean up with SPE, either in dispersive (dSPE) or in pass-through modes. When analyzing these compounds using LC-MS/MS with electrospray, consideration should be given to the impact of matrix effects from co-eluting co-extractives that can suppress the signal, reducing the sensitivity, accuracy, and robustness of the method. A balance must be struck between providing a rapid analytical method and the need for accurate quantification and robustness.

In this application note, we report the results of a validation of a modified QuEChERS method for the determination of fipronil and its metabolite fipronil sulfone in eggs by liquid chromatography-tandem quadrupole mass spectrometry, which meets the SANTE criteria (SANTE/11945/2015).[3]

EXPERIMENTAL

Extraction of egg samples

Eggs were purchased from a local shop and extracted using a modified QuEChERS method.[4] The sample preparation workflow employed in this method is summarized in Figure 1.

Validation of the method

The performance of the method was assessed using SANTE guidelines. To assess accuracy and precision of the method, test portions of eggs were spiked at two concentrations; 0.002 mg/kg and 0.02 mg/kg (n=5). Solutions of standards were prepared over the range 0.0005 to 0.05 mg/kg (0.5 to 50 ppb) in solvent and in egg extract (matrix matched), to determine the concentration of fipronil and fipronil sulfone in the spikes (using bracketed calibration) and to evaluate matrix effects.

UPLC conditions

UPLC system:	ACQUITY UPLC I-Class with FTN Sample Manager
Column:	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 × 100 mm
Mobile phase A:	2 mM Ammonium acetate (aq.)
Mobile phase B:	Acetonitrile (LC/MS grade)
Flow rate:	0.4 mL/min
Injection volume:	3 µL
Column temp.:	40 °C
Sample temp.:	10 °C
Runtime:	8.5 min

Gradient:

Time	%A	%B	Curve
Initial	95	5	Initial
0.5	95	5	6
5.0	2	98	6
7.0	2	98	6
7.5	95	5	6
8.5	95	5	1

MS conditions

MS system:	Xevo TQ-XS
Source:	Electrospray
Ionization mode:	ESI-
Capillary voltage:	2.0 kV
Desolvation temp.:	500 °C
Desolvation gas flow:	800 L/Hr
Source temp.:	150 °C
Cone gas flow:	150 L/Hr

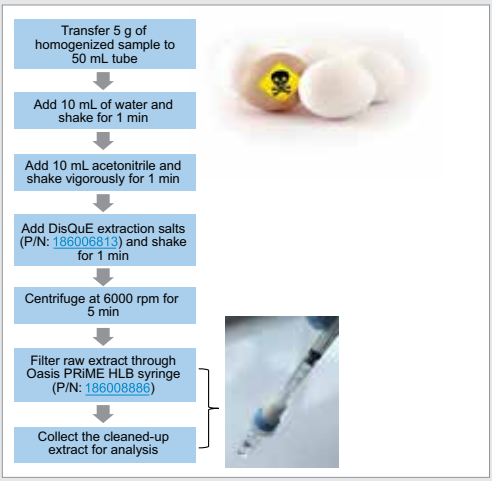

Figure 1. Sample preparation workflow: from sample homogenization to syringe filter into vial for analysis.

Table 1. Retention times and MRM parameters for fipronil and fipronil sulfone (quantitative transitions in bold).

Compound	Retention time	MRM transition	Cone (V)	CE (eV)
Fipronil	4.78	435>330 435>250	30 30	15 25
Fipronil sulfone	4.97	451>415 451>282	30 30	16 27

The two MRM transitions that showed the best selectivity were used for each of the analytes. Data were acquired using MassLynx MS Software (v4.2) and processed using TargetLynx™ XS Application Manager. The optimum dwell time was set automatically using the auto-dwell function based on 4 s wide peaks and 12 data points per peak.

RESULTS AND DISCUSSION

Within-laboratory method validation should be conducted to provide evidence that a method is fit for the purpose for which it is to be used. To meet the requirements of the SANTE guidelines this method has been tested to assess sensitivity, mean recovery (as a measure of trueness or bias), precision (as repeatability RSDr) and the method limit of quantification (LOQ).

A minimum of five replicates were required (to check recovery and precision) both at the targeted LOQ of the method and at least one other higher level. The lower concentration, the targeted LOQ, was set to 0.002 mg/kg to accommodate the residue definition for fipronil in eggs; the sum of fipronil and fipronil sulfone, expressed as fipronil. The higher concentration was set at 0.020 mg/kg, 10x the targeted LOQ.

Validation of the method demonstrated excellent performance for the identification and quantification of fipronil and fipronil sulfone in egg. These results are summarized in Table 2, showing that all of the relevant criteria set out in SANTE guidelines have been met. These analytical criteria and subsequent results are discussed, below, in more detail.

Table 2. Summary of method validation for the determination of fipronil and fipronil sulfone in eggs.

Parameter	SANTE criteria	Fipronil	Fipronil sulfone	Criteria satisfied
Retention time	±0.1 minute	4.77–4.78	4.97–4.97	4
Ion ratio	±30%	≤1%	≤1%	4
Residuals	±20%	3%	5%	4
Matrix effects	±20%	2%	0.2%	4
Recovery (trueness)	70 to 120%	95%	96%	4
Repeatability (RSDr)	≤20%	1.2%	1.4%	4
LOQ	≤MRL	0.002 mg/kg	0.002 mg/kg	4

Figure 2 shows the detection of fipronil and fipronil sulfone in a matrix-matched standard in eggs at 0.0005 mg/kg (0.5 ppb). This demonstrates the excellent sensitivity and selectivity of the method and its suitability for checking compliance with the EU MRL of 0.005 mg/kg as well as the potential for screening and quantification at much lower concentrations. Residues were detected in the egg sample chosen as the blank but at very low concentrations (estimated as ca. 0.02 ppb).

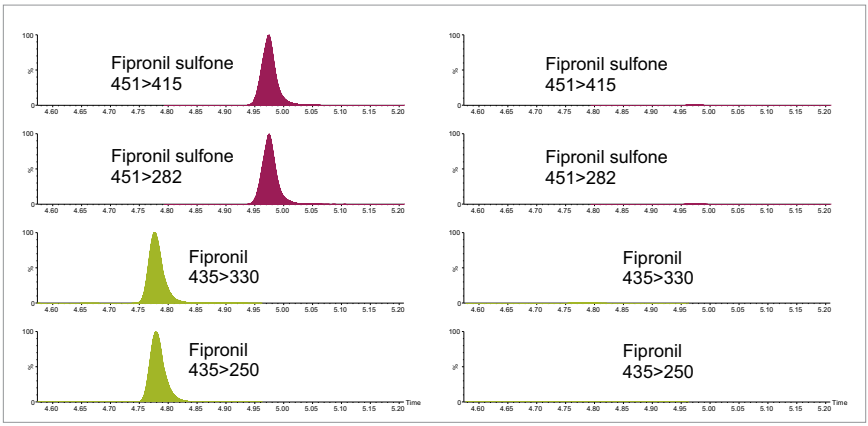

Figure 2. Chromatograms showing fipronil and fipronil sulfone from analysis of matrix-matched standard in eggs at 0.0005 mg/kg (0.5 ppb) and associated egg blank.

The linearity of response for fipronil and fipronil sulfone in egg matrix was evaluated using bracketed calibration over a suitable concentration range; 0.0005 to 0.05 mg/kg (0.5 to 50 ppb) as shown in Figure 3. The coefficients of determination and the residuals were satisfactory (r^2>0.999 and residuals <5%). Comparison of the slope of the matrix-matched calibration curve to the one prepared in solvent demonstrated that the use of Oasis PRiME HLB successfully removed any co-eluting co-extractives, as the matrix effects were observed to be minimal (ca. 2%).

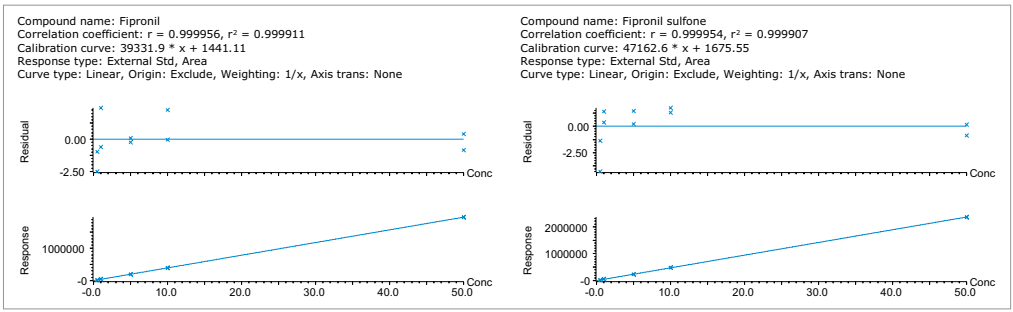

Figure 3. Calibration graphs for fipronil and fipronil sulfone in range of 0.0005 to 0.05 mg/kg (0.5 to 50 ppb) prepared in egg matrix. Two calibration curves, that bracketed the analytical run, are overlaid for each analyte.

When the Oasis PRiME HLB cleanup step was included, at least 95% of phospholipids were removed, as one can see by comparing the phospholipid response from analysis of egg extracts, before and after cleanup (Figure 4). As well as contributing to matrix effects, such endogenous material builds up in the LC-MS/MS system. Cleanup minimizes such contamination and the frequency of manual intervention and maintenance is dramatically decreased.

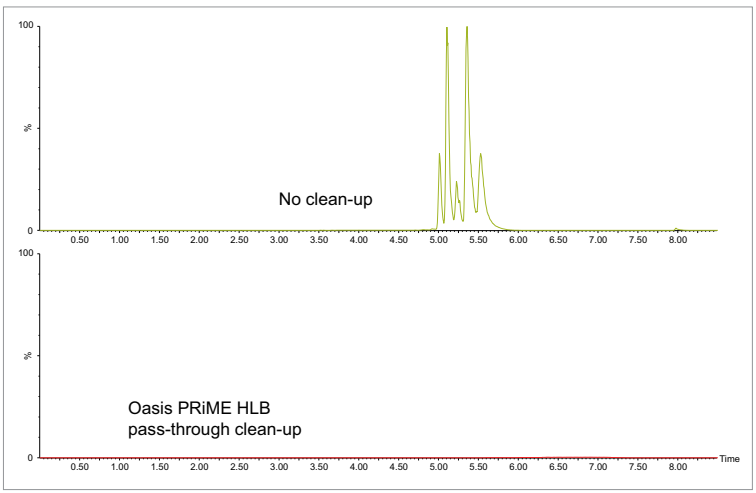

Figure 4. Chromatograms showing the phospholipid profiles from the analysis of QuEChERS (DisQuE) extracts of egg, before and after Oasis PRiME HLB cleanup.

To assess the accuracy and precision of the method, test portions of a blank egg sample were spiked at two concentrations, each with five replicates. Mean recovery and repeatability (RSDr) for fipronil and fipronil sulfone was 95% (1.2% RSD) and 96% (1.4% RSD), respectively.

Table 3 shows the concentrations of fipronil and fipronil sulfone determined in each spike. Ion ratio and retention times agreed well with the reference values derived from the matrix-matched standards and were well within the required tolerances.

Table 3. Concentration of fipronil and fipronil sulfone detected in the spiked egg samples.

Spike level	Measured concentration (mg/kg)	
0.002 mg/kg	Fipronil	Fipronil sulfone
Spike 1	0.00195	0.00199
Spike 2	0.00193	0.00197
Spike 3	0.00193	0.00195
Spike 4	0.00195	0.0020
Spike 5	0.00195	0.00195
0.02 mg/kg	Fipronil	Fipronil sulfone
Spike 1	0.01846	0.01871
Spike 2	0.01850	0.01865
Spike 3	0.01859	0.01870
Spike 4	0.01924	0.01932
Spike 5	0.01843	0.01861

CONCLUSIONS

- Waters™ ACQUITY UPLC I-Class System combined with the Xevo TQ-XS provides excellent sensitivity for the detection, identification, and quantification of fipronil and fipronil sulfone in eggs.
- This method can be implemented for both screening and confirmation for the purpose of official control and food industry due diligence.
- A modified QuEChERS method, using an Oasis PRiME HLB syringe filter (p/n: 186008886) type cleanup, provided effective extraction of the compounds of interest and the removal of more than 95% phospholipids, the source of significant matrix suppression during the analysis of contaminants in eggs.
- This method could easily be transferred to other Xevo tandem quadrupole MS/MS instruments and used for checking regulatory compliance.

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Utility of the ACQUITY UPLC I-Class System and Ion Mobility in a Routine Workflow to Understand the Challenge of Analyzing Fluoroquinolone Antibiotic Residues

Michael McCullagh, Ramesh Rao, and Sara Stead, Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Unique protomer collision cross section (CCS) values can be determined and used as an additional identification parameter in a routine screening workflow.
- Individual protomer spectra are generated along with proposed fragmentation pathways.
- The impact of matrix upon protomer ratios can be observed routinely using new ion mobility processing functionality within the UNIFI™ Scientific Information System.
- The ACQUITY™ UPLC™ I-Class System can be used in conjunction with ion mobility as a development tool to generate more robust analytical methods.
- Ability to perform retrospective UPLC and ion mobility data review.

WATERS SOLUTIONS

ACQUITY UPLC I-Class System

ACQUITY UPLC BEH C_{18} Column

SYNAPT™ G2-S High Definition Mass Spectrometry™ (HDMS™) System

MassLynx™ MS Software

UNIFI Scientific Information System

KEYWORDS

Protomer, collision cross section, CCS, ion mobility, spectral cleanup

INTRODUCTION

Across many application areas, the applicability of ion mobility to small molecule analysis continues to increase, along with the understanding of how this technology can help address current analytical challenges. The reason, challenge, and methods of achieving successful fluoroquinolones analysis were briefly discussed in a previous application note, where we described the use of the ACQUITY UPLC I-Class System combined with ion mobility mass spectrometry to show how fluoroquinolone class of compounds can form protomers.[1-5] Identification and characterization of the protomers of fluoroquinolones can now be routinely screened for using Waters™ UNIFI Scientific Information System. The software within UNIFI allows for the routine characterization of fragmentation pathways of the respective protomers to be visualized. In addition, it is possible to see the direct impact of the matrix upon protomer formation, and hence obtain a greater insight of the challenges of using MRM to perform residue analysis of fluoroquinolones.

Fluoroquinolones are a family of synthetic broad-spectrum antimicrobial agents that have been administered to livestock for different purposes, including the prevention and control of infections and for growth promotion. Due to concerns regarding the spread of resistant microorganisms in the human population, the U.S. Food and Drug Administration (U.S. FDA) introduced a ban on the use of enrofloxacin and ciprofloxacin in livestock production in September, 2005.[7-9] The use of antibiotic growth promoting agents (AGPs) in animal husbandry has been forbidden in the European Union (EU) since 2006.[10]

This application note explores the use of routine screening with UPLC and ion mobility to identify multiple protonation sites and different fragmentation patterns within the fluoroquinolone class of antibiotics. It can be used as an important method development tool to support the unequivocal identification of fluoroquinolone antibiotics in crude tissue extracts. UPLC and ion mobility have been utilized to analyze crude extracts of porcine muscle tissue to determine the presence of antibiotic residues including the fluoroquinolone class.

EXPERIMENTAL

Analytes:	Standards fluoroquinolones
Extracts:	Porcine tissue

UPLC conditions

UPLC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC BEH C_{18} 1.7 µm, 100 mm x 2.1 mm
Column temp.:	40 °C
Flow rate:	0.6 mL/min
Mobile phase A:	Water (0.1% formic acid)
Mobile phase B:	Acetonitrile (0.1% formic acid)
Injection volume:	10 µL

Gradient:

Time (min)	%A	%B
Initial	.0	5.0
1.00	95.0	5.0
8.00	5.0	95.0
9.00	95.0	5.0

MS conditions

MS system:	SYNAPT G2-S
Ionization mode:	ESI+
Capillary voltage:	2.0 kV
Cone voltage:	25 V
Desolvation temp.:	550 °C
Reference mass:	Leucine enkephalin $[M+H]^+ = 556.2766$
Acquisition range:	50 to 1200 Da
Acquisition rate:	4 spectra/sec
Collision energy:	15 to 45 eV
IMS T-Wave™ velocity:	900 m/s
IMS T-Wave pulse height:	40 V
IMS duty cycle:	10.8 ms
Drift gas:	N^2

The enhanced peak capacity provided by the combination of UPLC and ion mobility separation offers some unique advantages for profiling complex matrices. It uses a combination of high resolution mass spectrometry and high efficiency ion mobility-based measurements and separations. Ion mobility spectrometry (IMS) is a rapid, orthogonal, gas phase separation technique that allows another dimension of separation to be obtained within an LC timeframe. Compounds can be differentiated based on size, shape, and charge. In addition, both precursor ion and fragment ion information can be acquired in a single acquisition for all components.

A collision cross section (CCS) value is a robust and precise physicochemical property of an ion. CCS is an important distinguishing characteristic of an ion which is related to its chemical structure and three-dimensional conformation, where the shadow of a rotating three-dimensional ion, shown in Figure 1, represents the average collision cross section. Using CCS measurements can increase targeted screening specificity. CCS measurements generated have been entered into a scientific library within UNIFI. This allows the expected and determined CCS values to be utilized in order to screen and confirm fluoroquinolone protomer formation. Here we present CCS values (derived from ion mobility drift times) as a new identification parameter, which can distinguish protomers.

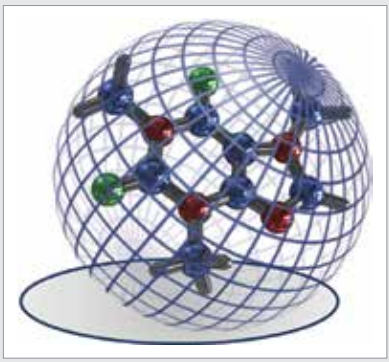

Figure 1. Illustration of rotating three-dimensional conformation of an ion and average collision cross section (shadow).

Extract preparation

Extracts of porcine muscle tissue were kindly provided by RnAssays BV for the purposes of this study. Briefly, known blank porcine muscle was fortified with 25 different antimicrobial compounds (from fluoroquinolone, tetracycline, and macrolide classes) at levels the relevant to the EU MRL concentrations prior to extraction. Tissue samples were mechanically homogenized in the presence of an aqueous/organic extraction solvent followed by a centrifugation step. An aliquot of the supernatant was removed and placed in an autosampler vial for subsequent LC-MS analysis.

RESULTS AND DISCUSSION

For the assay performed, MassLynx MS data were acquired and processed with the UNIFI Scientific Information System, allowing ion mobility data to be processed in a conventional workflow for non-targeted accurate mass screening applications.

UPLC ion mobility MS has been explored as an important method development tool to support the unequivocal identification of fluoroquinolone antibiotics in crude tissue extracts. With UNIFI, it has been possible to routinely identify and characterize protomers of nine fluoroquinolones standards in a routine screening workflow.[10] From the solvent standards analyzed, estimated CCS values of protomers formed for each fluoroquinolone were determined. The CCS values obtained have been incorporated into the UNIFI Scientific Library, which enabled the targeting of protomers.

The antibiotic ciprofloxacin was determined to elute at retention time 2.19 min using the generic gradient conditions employed. Figure 2 shows the base peak ion chromatogram with the UNIFI Component Plot Summary for nine of the identified fluoroquinolone antibiotics, which eluted in the region highlighted in the base peak intensity chromatogram. From review of the data using the Component Drift Plot Summary, 18 fluoroquinolone species were identified. Each fluoroquinolone is comprised of two protomers, i.e. protonation at two different sites on the molecule that have been mobility separated, as shown in Figure 3. Each retention time shows two dots on the Component Summary Drift Plot, which indicate two forms of each fluoroquinolone. The two protomers of ciprofloxacin have been highlighted. The CCS values determined are presented in the Component Summary table of Figure 4. The functionality illustrated is unique to the UNIFI Scientific Information System.

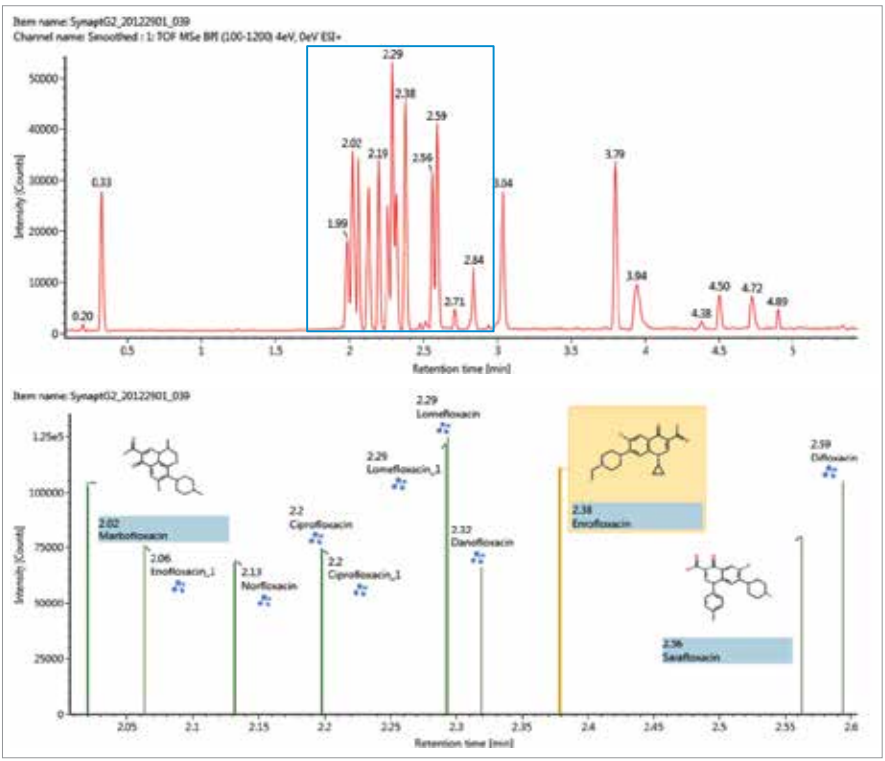

Figure 2. Base peak ion chromatogram for a mixture of 25 solvent standard antibiotic compounds, including nine fluoroquinolones. Also the Component Plot Summary is shown for 18 of the identified fluoroquinolone components between 2 and 2.6 minutes.

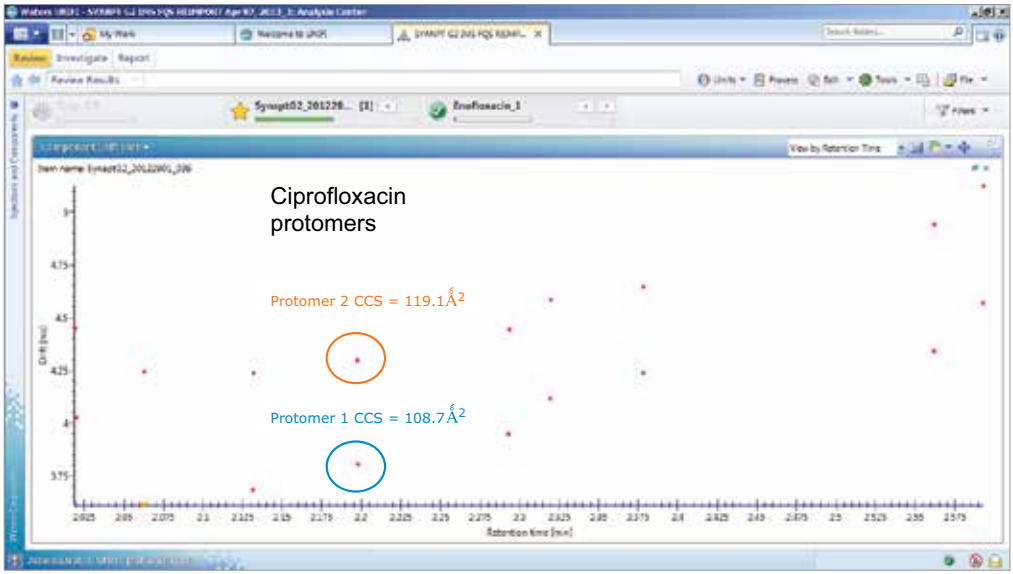

Figure 3. UNIFI Component Summary drift plot for nine pairs of fluoroquinolone protomers.

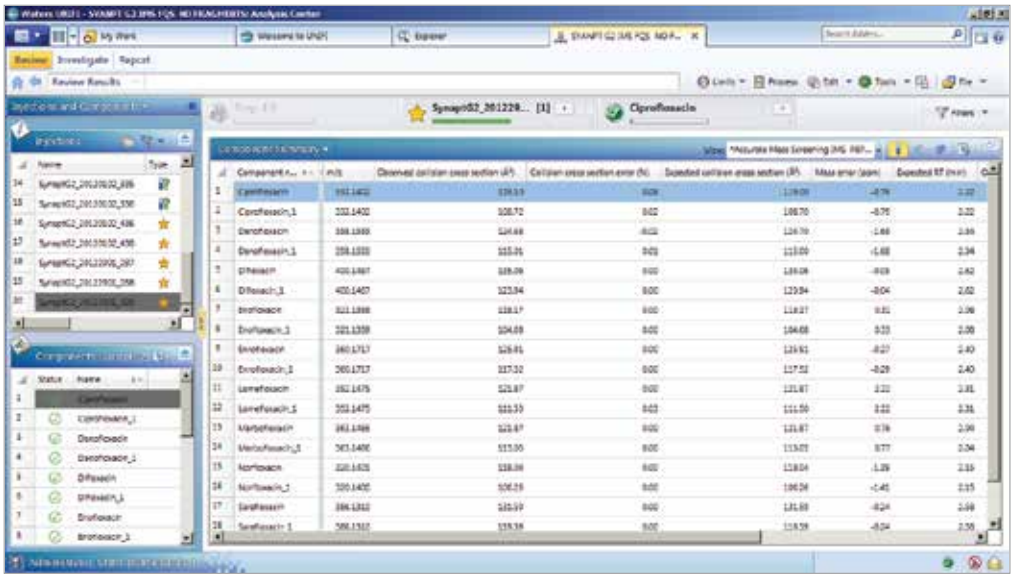

Figure 4. Component Plot Summary showing nine identified fluoroquinolone antibiotics and nine pairs of CCS values.

These protomer gas phase components, although they only differ with the site of protonation, have different collision cross sections. The example of ciprofloxacin is shown in Figure 5, and in this case a difference of >10 Å^2 (angstrom is a unit of length equal to 10–10 m, one ten-billionth of a meter), was observed in this ion mobility study.[11] For all fluoroquinolone protomers pairs observed, the respective difference between CCS pair values varied between 6 Å^2 and 12 Å^2 with respect to the protomer pairs. The mobility separation achieved enabled individual precursor ion and fragments of all nine fluoroquinolones to be obtained in one analysis. From a single component fragmentation spectra it was possible to determine that for ciprofloxacin, the two mobility separated species resulted from protonation taking place either on the acidic or the basic group. From method development with standards, specific CCS information was generated providing further specific information to be entered into the UNIFI scientific library. Using this information, veterinary drug residues can now be identified based on retention time, accurate mass, fragments, and CCS values. Ciprofloxacin's estimated CCS values of 108.7 Å^2 and 119.1 Å^2 have been determined. Ciprofloxacin fragments at *m/z* 314 and *m/z* 231 are shown in Figure 6. These fragments are hypothesized to form from a species where ionization has taken place on the acidic group. Fragments observed at *m/z* 288 and *m/z* 245 resulted from protonation of the basic group.

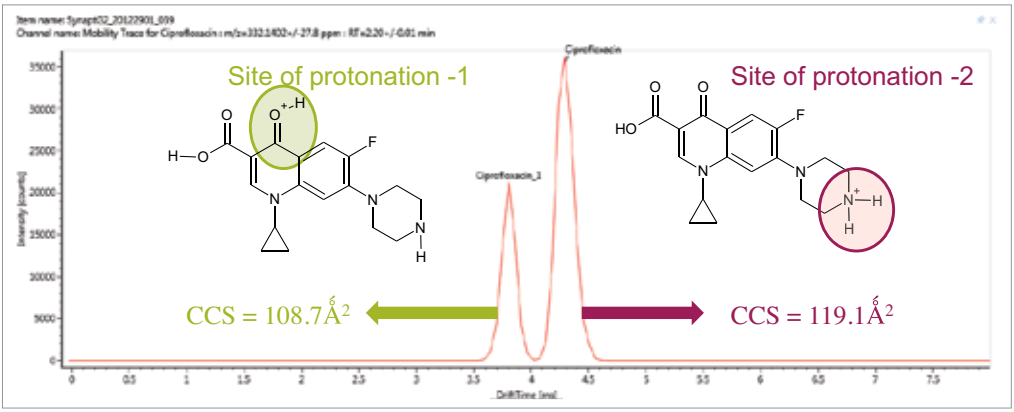

Figure 5. Mobility trace for protomers of ciprofloxacin with hypothesised respective sites of acid/basic group protonation highlighted and determined estimated CCS values.

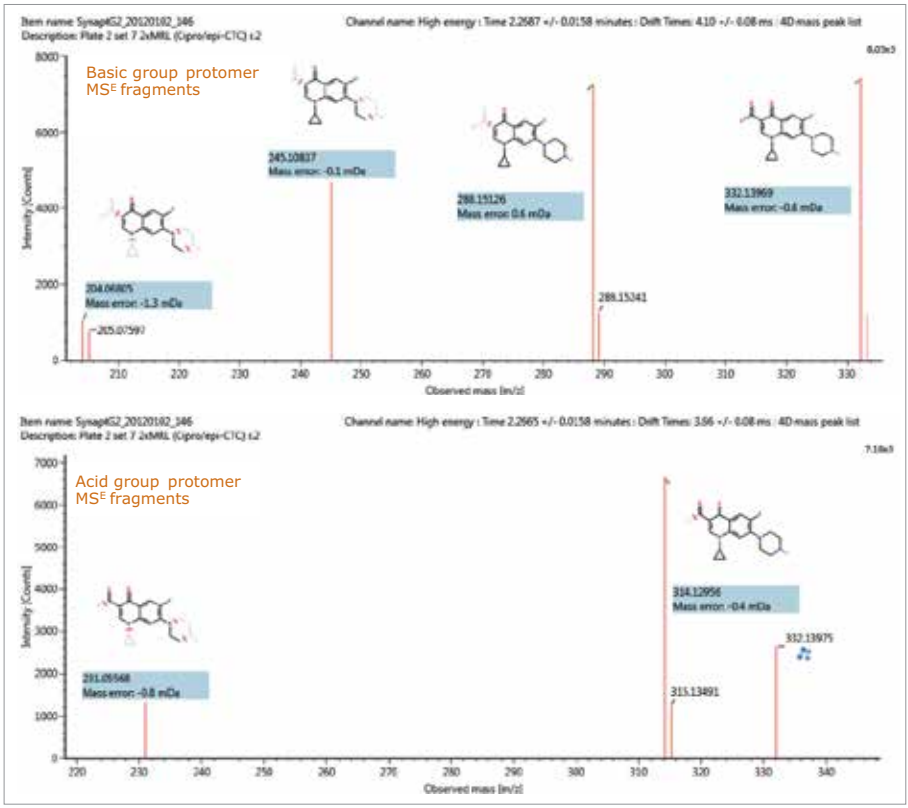

Figure 6. Ciprofloxacin acid and basic group single component fragmentation spectra generated using a UPLC ion mobility screening workflow in UNIFI.

Once the CCS values and fragments of the individual fluoroquinolones were entered into the UNIFI scientific library, a series of spiked porcine extracts were screened to determine the presence of fluoroquinolones. Examples of the screening results are shown in Figures 7 and 8, where the identification of two protomers of danofloxacin in porcine extract are presented. Here, the benefits of ion mobility resolution and the functionality of UNIFI are demonstrated, showing the resolved protomers, as well as the removal of the matrix background from the identified component danofloxacin. It can also be seen that for both protomers, mass accuracy <1 ppm was obtained, and that the CCS error was within 2% of the expected CCS values (124.7 $Å^2$ and 115.0 $Å^2$). The observed retention time was 2.36 min and the individual protomer precursor ion/fragmentation spectra have been obtained. This data further illustrates how confidence in true identifications can be increased when using UPLC ion mobility in conjunction with the functionality available within UNIFI Software.

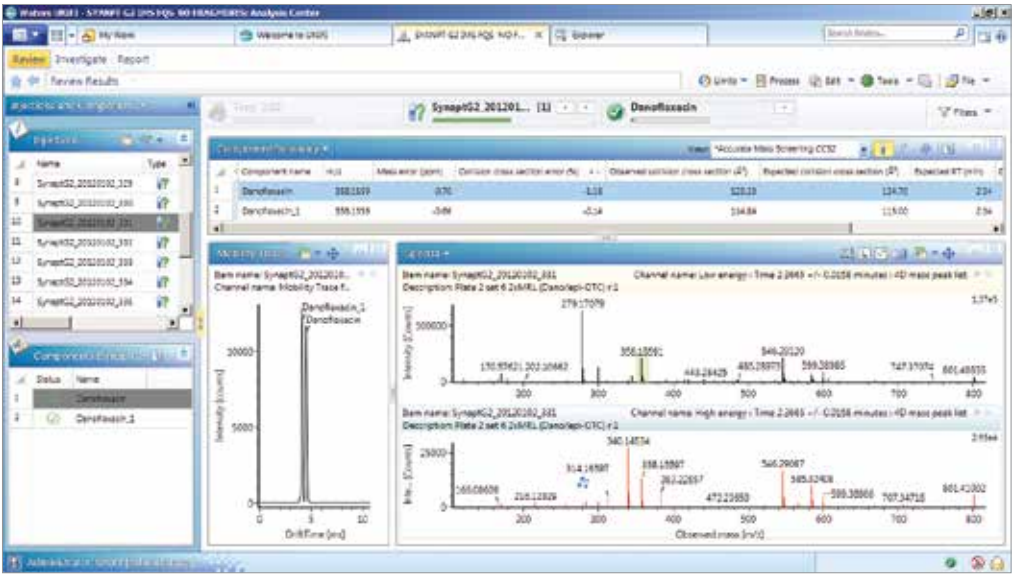

Figure 7. Identification of two danofloxacin protomers identified in porcine extract, where no ion mobility resolution spectral cleanup has been selected. Precursor ion and fragmentation with matrix background ions are shown for danofloxacin protomer, expected CCS 124.7 Å².

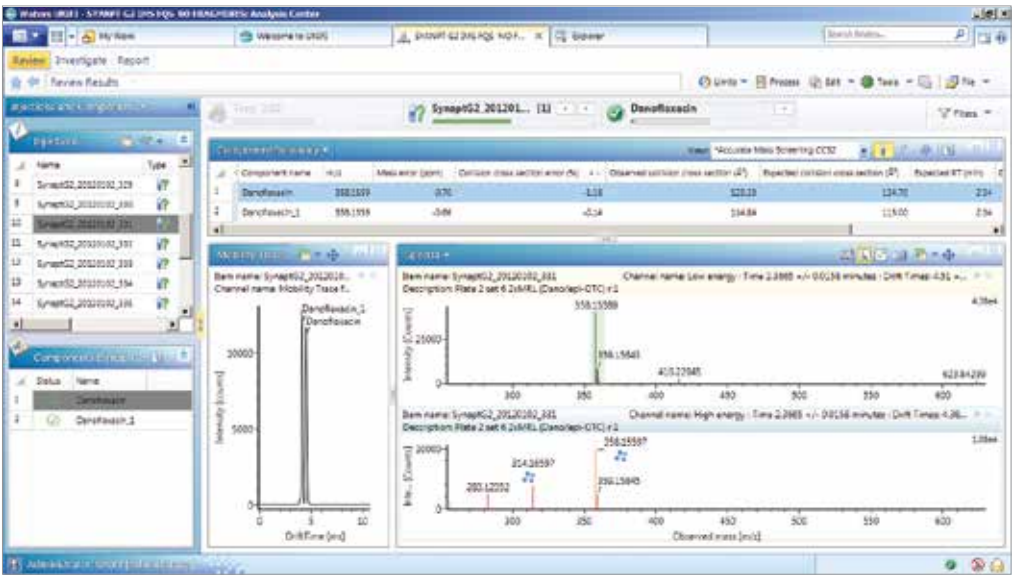

Figure 8. Identification of two danofloxacin protomers identified in porcine extract, where ion mobility resolution spectral clean up has been selected. Precursor and fragmentation ions with matrix background ions removed, are shown for the danofloxacin protomer with expected CCS 124.70 Å².

The impact of the matrix upon the gas phase intra-molecular protonation for ciprofloxacin is shown in Figure 9. The ability to routinely process ion mobility data within a workflow has made it possible to clearly observe fluctuations in the ratios of the fluoroquinolone protomers formed. As discussed, each protomer generates specific fragments, and from this data, an understanding can be obtained of why fluctuations in observed ion ratios can occur when monitoring MRM transitions.

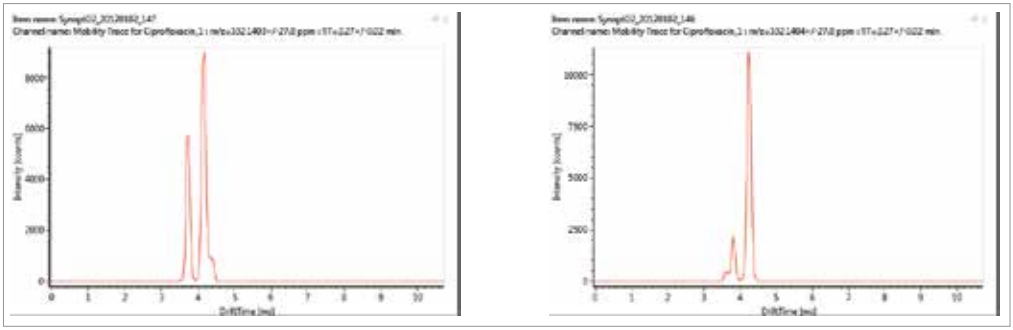

Figure 9. Consecutive acquisitions of replicate porcine extracts showing the impact of matrix upon the gas phase intra-molecular protonation for ciprofloxacin.

Multiple protonated species have been observed for the fluoroquinolone antibiotics screened. The extent of the protonation multiplicity and its experimental variation is still being investigated. This data confirms that further consideration should be given to method development and the means of analysis chosen, since the ratio and formation of the protomers can vary with the eluent flow rate, capillary voltage, cone voltage, and matrix.

If MRM is the method of choice, consideration of the experimental conditions used and the specific transitions selected is imperative. The data presented illustrate that consistency in MRM transitions in inter/intra laboratory studies could easily be misinterpreted within and between different laboratories, and demonstrates the challenges of achieving reproducible results for these types of compounds. Ion mobility can provide a valuable tool for method development in order to ensure method robustness and consistency of results.

The benefits of UPLC ion mobility mass spectrometry can be demonstrated over traditional 'shape selective' ion mobility-based separation techniques, such as Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS). FAIMS is typically used to transmit only ions of a particular mobility, essentially acting as a filter. Using the ACQUITY UPLC I-Class System with HDMS, the fastest, ion mobility separation can be performed for all components regardless of the sample complexity, maximizing the duty cycle of the analysis and the amount of information obtained. Having a compatible duty cycle available also ensures spectral integrity is retained.

The benefits of time-of-flight mass spectrometry and historical data review (retrospective data analysis) is well known. Such historical data review is also required with ion mobility mass spectrometry. The discovery and presentation of multiple sites of protonation occurring during analysis for fluoroquinolone antibiotics can only be possible if the ion mobility data is acquired for all of the components in a sample. Continued development of the UNIFI platform's functionality has enabled routine screening using ion mobility mass spectrometry, facilitating the opportunity to develop more reproducible, repeatable, and robust assays for a wide range application areas.

CONCLUSIONS

- Separation of different intra-molecular protonated species has been achieved uniquely using ion mobility.
- Single component precursor ion and fragmentation spectra can be generated for all components simultaneously.
- Multiple sites of protonation have been identified and confirmed from the individual fragmentation spectra of each protomer species.
- CCS values can be used as an identification point in addition to retention time, precursor ion accurate mass, and accurate mass fragmentation spectra.
- Ion mobility separations can be effectively utilized to resolve analyte peaks from matrix interferences and remove the need for complex sample cleanup.
- UPLC-ion mobility mass spectrometry observations have the potential to explain the differences sometimes observed in inter-laboratory studies, where participants report results obtained from monitoring specific MRM transitions.
- The UNIFI Scientific Information System enabled the routine interrogation of UPLC ion mobility MS data acquired using the SYNAPT G2-S and SYNAPT G2-S*i* HDMS systems.
- Based on the observations of characteristic ionization for fluoroquinolone antibiotics included in this study, the use of UPLC ion mobility for method development purposes is warranted.

Acknowledgements

Waters kindly acknowledges Aldert Bergwerff and Wouter de Keizer (RnAssays) for the provision of the samples analyzed.

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Quantifying Primary Aromatic Amines in Polyamide Kitchenware Using the ACQUITY UPLC I-Class System and Xevo TQ-S micro

Steven Haenen and Marijn Van Hulle
Waters Corporation, Brussels, Belgium

APPLICATION BENEFITS

- Single method for analysis of 23 PAAs
- No need for ion-pairing reagents, or the removal of acetic acid from the sample extract prior to analysis
- Sensitive detection at levels well below the EU guidelines with Xevo™ TQ-S micro Triple Quadrupole Mass Spectrometry

WATERS SOLUTIONS

ACQUITY UPLC™ I-Class System

Xevo TQ-S micro

ACQUITY UPLC HSS T3 Column

MassLynx™ MS Software

TargetLynx™ XS Application Manager

KEYWORDS

PAAs, primary aromatic amines, kitchenware, utensils, migration, food contact materials, FCMs

INTRODUCTION

Primary Aromatic Amines (PAAs) are a class of compounds of which the simplest form is aniline (Figure 1). PAAs are substances that are used, for example, in the production of certain colorants, so-called azo pigments, notably in the color range yellow – orange – red. Whereas a large number of PAAs are safe for human health, some PAAs are known human carcinogens. For kitchenware, paper napkins, baker's bags with colorful print and other printed items that come in contact with food, some PAAs may pose a health risk, if they are transferred to the food.

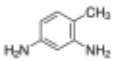
Compound	Mass	Structure
Aniline	93	
o-Toluidine	107	
2,4-Diaminotoluene	122	
o-Anisidine	123	

Figure 1. Chemical structures of some PAAs.

Because of the potential health risks, specific migration limits (SMLs) are put in place.[1] According to the regulation on plastics EU 10/2011: 'Plastic materials and articles shall not release primary aromatic amines, excluding those appearing in Table 1 of Annex I, in a detectable quantity into food or food simulant. The detection limit is 0.01 mg of substance per kg of food or food simulant. The detection limit applies to the sum of primary aromatic amines released'.

The provisions in Regulation 10/2011 state that for primary aromatic amine migration from polyamide kitchenware, only one migration test will be carried out, if this first extract is compliant with the summed SML (SML(T)) of 0.01 mg/kg . However, if this first simulant extract exceeds the permitted SML(T), two subsequent migration studies are required.[2] This PAAs migration testing is conducted with simulant B, 3% (w/v) acetic acid, as it has been demonstrated that this simulant represents the worst case for the migration of PAAs from polyamide kitchenware.[3]

PAAs are small, basic compounds, which are ionized with low pH. As a result of their basic properties and the 3% acetic acidic sample solvent, some PAAs don't focus well on the head of the column, resulting in poor peak shape and/or loss of retention. In order to improve chromatographic retention ion-pairing reagents are often used.[2] Unfortunately these reagents have a negative impact on the electrospray sensitivity and are to be avoided where possible.

In this application note we describe a LC-MS/MS method for the analysis of 23 common PAAs in kitchenware after migration using Waters™ ACQUITY UPLC I-Class System coupled to a Xevo TQ-S micro Mass Spectrometer. The described method does not use an ion-pair reagent to improve chromatographic retention.

EXPERIMENTAL

UPLC conditions

UPLC system:	ACQUITY UPLC I-Class
Sample manager:	Flow-through Needle
Column:	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 x 100 mm
Mobile phase A:	Water
Mobile phase B:	Methanol
Column temp.:	45 °C
Sample temp.:	10 °C
Flow rate:	0.4 mL/min
Run time:	15 min
Injection volume:	20 µL

Gradient:

Time (min)	%B
0	5
10	100
12	5
12.01	5
15	5

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI +
Capillary voltage:	2 kV
Desolvation temp.:	600 °C
Desolvation gas flow:	1200 L/hr
Source temp.:	150 °C
Acquisition:	Multiple Reaction Monitoring (MRM)

MS methods and data acquisition

Two MRM transitions were used, unless otherwise stated. The dwell times were chosen automatically using the built-in points-per-peak calculator in the MS method. The data were acquired using MassLynx v. 4.1 Software, and processed using TargetLynx XS Application Manager. Table 1 summarizes all MRM transitions. Figure 2 shows the retention time windows of the MRM method.

Table 1. Overview of MRM transitions for all 23 PAAs.

Compound	Transitions	Cone voltage (V)	Collision energy (eV)
Aniline	93.8>77.0	40	15
o-Toluidine	107.8>91.0 107.8>93.0	40 40	15 15
2,4-Diaminotoluene	122.8>106.2 122.8>108.3	40 40	15 18
o-Anisidine	123.9>65.0 123.9>109.0	40 40	20 15
4-Chloroaniline	127.8>93.1 129.8>93.1	40 40	18 18
3-Chloro-o-toluidine	140.8>77.1 140.8>95.1	40 40	10 10
2,4,5-Trimethyl aniline	135.9>91.0 135.9>121.0	40 40	20 15
2-Methoxy-5-methylaniline	137.8>78.1 137.8>123.1	40 40	25 15
4-Chloro-2-methylaniline	141.8>107.0 141.8>125.0	40 40	15 18
2-Amino naphthalene	143.8>117.1 143.8>127.0	40 40	20 20
2-Methyl-5-nitroaniline	152.8>107.0 152.8>121.0	40 40	15 10
4-Aminobiphenyl	169.9>92.0 169.9>152.1	40 40	20 25
2-Aminobiphenyl	169.9>92.0 169.9>152.1	40 40	20 25
Benzidine	184.9>167.1 184.9>168.1	40 40	25 18
4-Phenyl azoaniline	197.95>77.0 197.95>105.0	40 40	18 12
4,4'-Diamino diphenylmethane	199.0>77.1 199.0>106.0	40 40	22 22
4,4'-Oxydianiline	200.95>108.0 200.95>184.1	40 40	20 20
3,3'-Dimethyl benzidine	213.0>180.0 213.0>196.0	40 40	30 30
4,4'-Thiodianiline	216.95>124.0	40	20
o-Amino azotoluene	226.0>91.0	40	20
3,3'-Dimethyl-4,4'-diaminodiphenylmethane	227.0>120.2	40	20
3,3'-Dimethoxy benzidine	245.0>213.1 245.0>230.1	40 40	18 18
3,3'-Dichloro benzidine	252.9>182.1 252.9>217.0	40 40	25 20
4,4'-Methylene bis (2-chloroaniline)	266.9>140.1 266.9>231.1	40 40	25 22

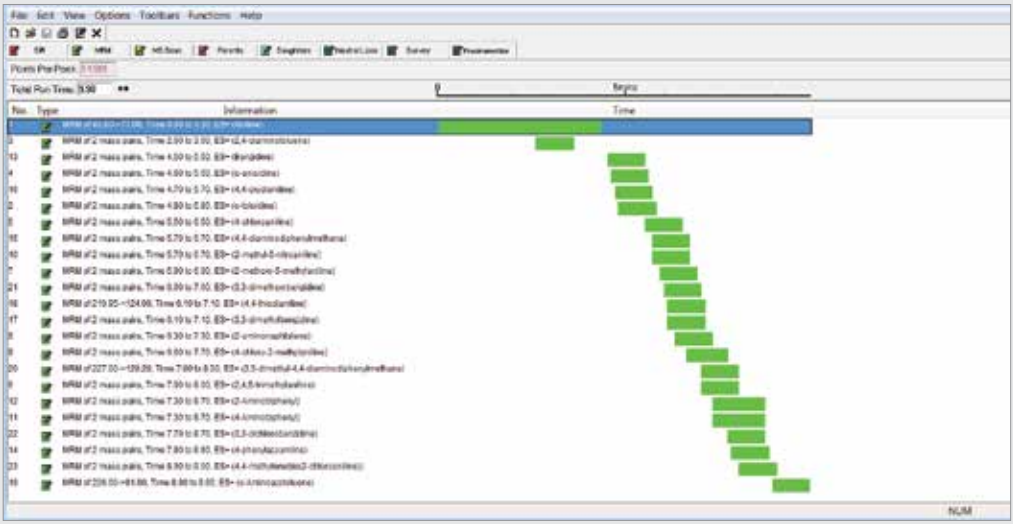


Figure 2. Retention time windows for the PAAs acquisition method.

Standards

A mixed standard solution containing all PAAs at a concentration of 100 µg/mL was used. The working standards were further diluted with the 3% acetic acid food stimulant solution. For the solvent calibration a dilution series starting at 100 ng/mL down to a level of 0.78 ng/mL was made.

Sample preparation

Nine polyamide kitchenware utensils were extracted with a 3% acetic acid solution according to the procedure described in the EU 10/2011 guidelines.[1]

RESULTS AND DISCUSSION

UPLC METHOD DEVELOPMENT

Because of the basic properties of PAAs, and the fact that acetic acid is used as a migration stimulant, some PAAs don't focus well on the head of the column, resulting in poor peak shape and/or loss of retention. Aniline elutes early and is therefore prone to this effect. As a result, some literature references cite the use of ion-pair reagents.[2] Adding ammmonium hydroxide to the 3% acetic acid samples prior to injection, the pH of the sample is increased and the polar and weakly basic PAAs such as aniline will be in their neutral form. A volume of 10 µL of a 25% NH_4OH solution was added to 1 mL of sample. This approach resulted in more robust results and is therefore preferred over the use of ion-pair reagent. Figure 3 shows a chromatogram of aniline with an unchanged pH (top) and adjusted pH (bottom). The neutralization of the pH drastically improves the peak shape of aniline, without the need for ion-pairing reagent.

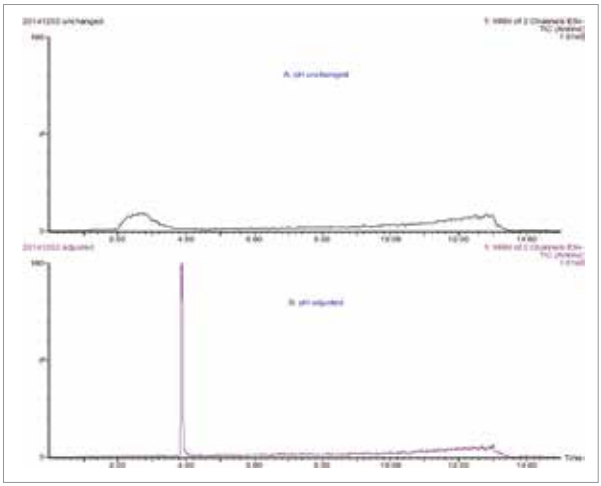


Figure 3. Chromatogram of aniline in 3% acetic acid food stimulant without (top) and with (bottom) pH adjustment.

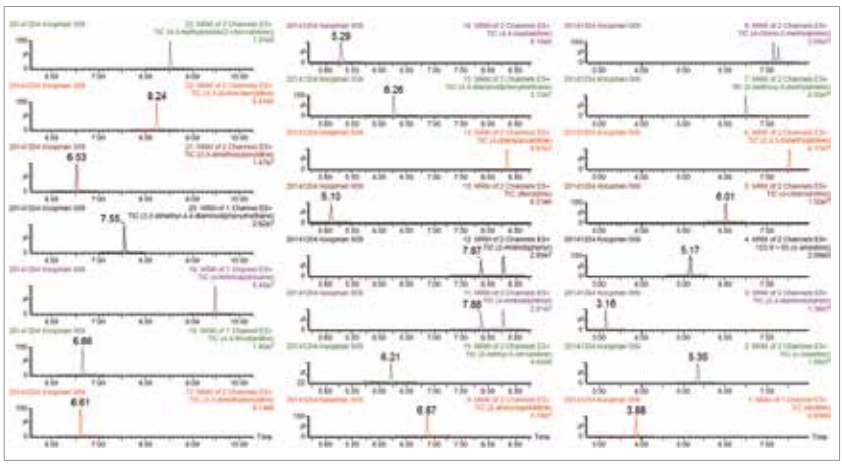


Figure 4. Chromatograms of all 23 PAAs.

LINEARITY

Calibration curves were prepared from 0.78 ng/mL to 100 ng/mL for all compounds. An example is given for aniline (Figure 5). For each calibration curve, a linear regression and a 1/X weighting was applied. All compounds show good linearity across the range of concentrations as well as excellent % residual values.

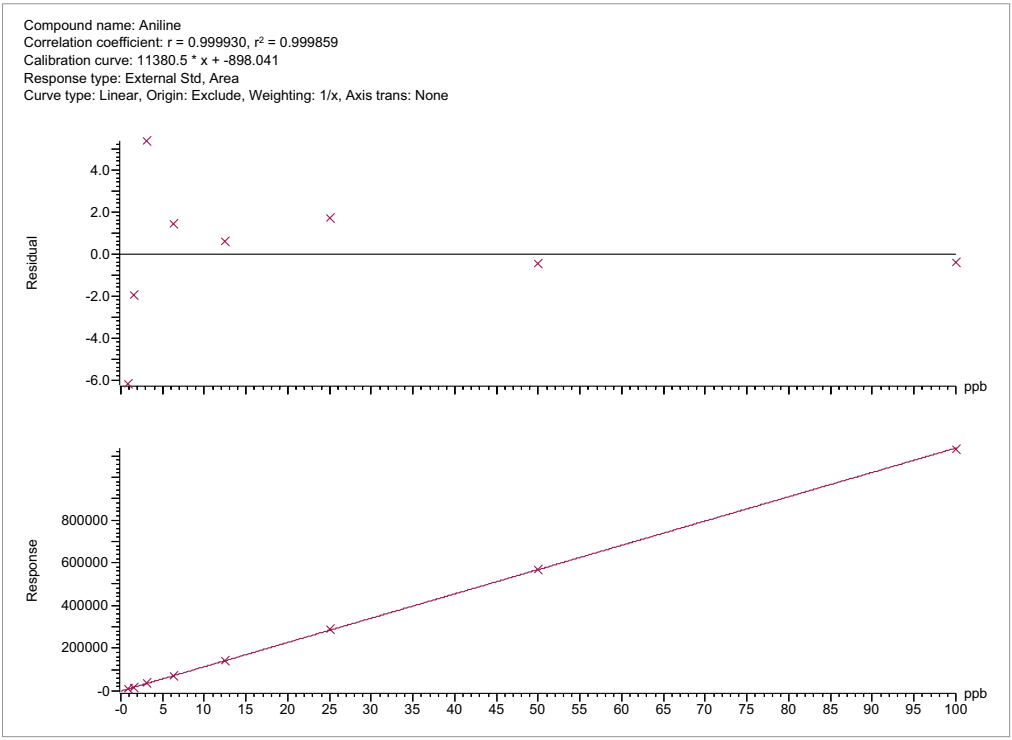


Figure 5. Calibration curve (bottom) and residuals plot (top) for aniline in the range 0.78 to 100 ng/mL.

Acidified mobile phases aid in the protonation of compounds and therefore improve the sensitivity in positive ion electrospray. As no acid was added to the mobile phases, we investigated whether a post-column addition (PCA) with formic acid would be beneficial. Using the Xevo TQ-S micro's built-in IntelliStart™ fluidics, a solution of 2% formic acid was infused at a constant flow rate of 20 µL/min into the UPLC™ flow exiting the column. As such the formic acid solution was diluted 20-fold with the mobile phase, resulting in a final concentration of 0.1% of formic acid going into the ESI source. Figure 6 shows how this PCA was configured in the acquisition method, while Figure 7 shows the chromatograms for a selection of PAAs with (top trace) and without (bottom trace) this post-column addition. For better interpretation, the intensity axes have been linked. As can be seen from the chromatograms, the sensitivity is significantly improved when formic acid is added to the eluent.

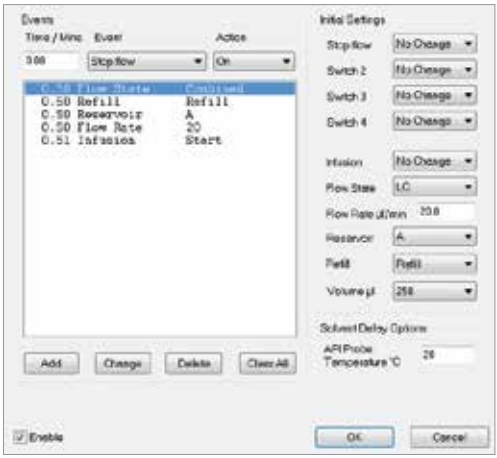


Figure 6. Post-column addition in the MS acquisition method.

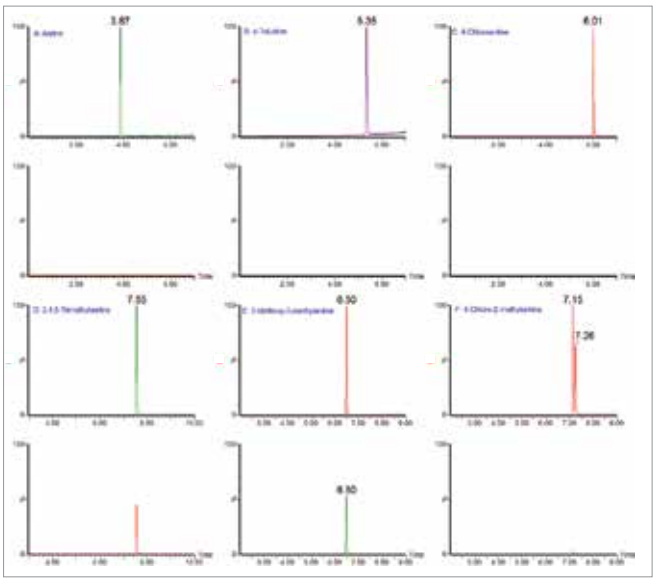


Figure 7. Increase in sensitivity with the use of a formic acid post-column addition (top), and without (bottom), illustrated for:
A. aniline,
B. o-Toluidine,
C. 4-Chloroaniline,
D. 2,4,5-Trimethylaniline,
E. 2-Methoxy-5-methylaniline, and
F. 4-Chloro-2-methylanaline.

Table 2 summarizes the quantitation limits (LOQ) for all compounds using this PCA approach. The LOQ is defined as the concentration giving rise to a signal-to-noise (S/N) value of 10:1. For the calculation of S/N, raw data was used and the peak-to-peak algorithm was applied. An extrapolation was made in most cases, as the reported S/N values were still significantly high, even at the lowest reported standard level of 0.78 ng/mL. Calculated LOQs below 20 pg/mL are not mentioned specifically but are cut off at this level. The reported LOQ concentrations range between 20 pg/mL and 300 pg/mL.

Table 2. Calculated S/N values at 0.78 ng/mL and estimated LOQ values for all 23 PAAs investigated.

Compound	S/N ratio	LOQ (ng/mL)
Aniline	377	0.02
o-Toluidine	768	<0.02
2,4-Diaminotoluene	52	0.15
o-Anisidine	89	0.09
4-Chloroaniline	323	0.03
2,4,5-Trimethyl aniline	693	<0.02
2-Methoxy-5-methylaniline	1444	<0.02
4-Chloro-2-methylaniline	3503	<0.02
2-Amino naphthalene	1858	<0.02
2-Methyl-5-nitroaniline	27	0.29
4-Aminobiphenyl	226	0.04
2-Aminobiphenyl	272	0.03
Benzidine	559	<0.02
4-Phenyl azoaniline	1931	<0.02
4,4'-Diamino diphenylmethane	1353	<0.02
4,4'-Oxydianiline	312	0.03
3,3'-Dimethyl benzidine	165	0.05
4,4'-Thiodianiline	2582	<0.02
o-Amino azotoluene	1746	<0.02
3,3'-Dimethyl-4,4'-diaminodiphenylmethane	1818	<0.02
3,3'-Dimethoxy benzidine	528	<0.02
3,3'-Dichloro benzidine	926	<0.02
4,4'-Methylene bis (2-chloroaniline)	1522	<0.02

MATRIX EFFECTS

Internal standards were not used in this method. Therefore it was investigated whether the food simulant extract leads to ion suppression. One of the samples was spiked to a final concentration of 10 ppb and this sample was compared with a standard dissolved in the same food stimulant solution. All spike recoveries were within 90% to 107%, indicating that matrix effects were low to non-existing for the 23 compounds under investigation.

KITCHENWARE SAMPLES

Using the external calibration curves, nine kitchenware samples were quantified. Except for aniline and 4,4'-diamino diphenylmethane found in all nine samples at levels between 0.4 to 1.1 ppb and 0.04 to 0.11 ppb, respectively, no other PAAs were detected. Figure 8 shows the chromatograms of aniline in the sample containing 0.4 ppb and of 4,4'-diamino diphenylmethane in the sample containing 0.04 ppb. As can be seen sensitivity was excellent at these sub ppb level.

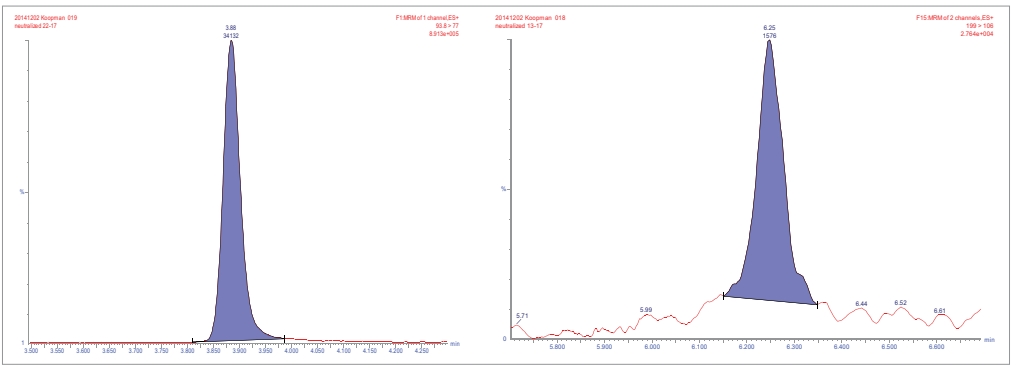


Figure 8. Chromatograms of aniline in kitchenware samples present at 0.4 ppb (left), and of 4,4'-Diamino diphenylmethane in the sample containing 0.04 ppb (right).

CONCLUSIONS

We have demonstrated a sensitive method for 23 PAAs with very easy sample preparation. The addition of ammonium hydroxide as neutralizing agent, and a post-column addition of formic acid into the Xevo TQ-S micro via IntelliStart's built-in fluidics – resulted in a very sensitive assay which could reach sub ppb levels. Linearity was observed over a large range and up to 100 ppb. The samples were all below detection limits except for aniline which was detected at 0.4 to 1.1 ppb, and 4,4'-diamino diphenylmethane which was detected at 0.04 to 0.11 ppb. The total PAAs content for all samples was below the SML(T) of 0.01 mg/kg as stipulated in the regulations EU 10/2011.

References

1. Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food.
2. LB-NA-24815-EN-N, Technical guidelines on testing the migration of primary aromatic amines from polyamide kitchenware and of formaldehyde from melamine kitchenware.
3. Analysis of primary aromatic amines (PAA) in black nylon kitchenware 2014. Selected samples from the Norwegian Market.

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Identification of Non-Intentionally Added Substances (NIAS) in Food Contact Materials Using APGC-Xevo G2-XS QTof and UNIFI Software

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Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Reliable GC-MS method for screening and structural elucidation of non-intentionally added substances (NIAS) in food packaging materials
- Atmospheric Pressure Gas Chromatography (APGC) is a soft ionization technique that produces lower levels of fragmentation than EI, enabling improved detection of challenging molecular ions and the avoidance of possible erroneous identification
- UNIFI™ Software provides customized workflows to streamline and simplify elucidation of unknown compounds from food packaging

WATERS SOLUTIONS

Atmospheric Pressure Gas Chromatography (APGC)

Xevo™ G2-XS QTof Mass Spectrometer

UNIFI Scientific Information System

KEYWORDS

High resolution mass spectrometry, HRMS, food contact materials, leachables, non-targeted analysis, GC-MS, migration, componentization, elucidation, electron ionization, EI, MS^E

INTRODUCTION

Food comes into contact with many materials and articles during its production, processing, storage, preparation, and serving before its eventual consumption. Such materials and articles are called food contact materials (FCMs). Recently, concern about the wholesomeness and safety of food products has increased dramatically. Most of the concern usually focuses on food additives, monomers, oligomers, and non-intentionally added substances (NIAS). A non-intentionally added substance is defined in the European Union (EU) Regulation No 10/2011 as "an impurity in the substances used or a reaction intermediate formed during the production process or a decomposition or reaction product."[1,2] FCMs can, therefore, be considered materials containing a complex mixture of substances of known or unknown identity/origin. Depending on their physico-chemical properties and chemical composition, FCMs may transfer some constituents, both Intentionally Added Substances (IAS) and NIAS to foodstuffs. This mass transfer phenomenon is called migration, and may lead to high exposure to certain chemicals, which might cause a risk for human health.[3] Therefore, migration must be evaluated and controlled. Furthermore, where migration brings about an unacceptable change in the composition of food or brings about deterioration in the organoleptic properties of the food, it must be avoided.[4]

Before performing a migration study, a screening analysis of the packaging material is required to identify the chemicals that are present in the material and those that are more likely to migrate. This initial step usually involves a strong extraction of the material with an organic solvent or a mixture of solvents. The extract is then injected via LC-MS and/or GC-MS for non-targeted screening analysis of non-volatiles, and volatiles/semi-volatiles, respectively. With respect to semi-volatiles and volatiles analyses, a GC coupled to a quadrupole mass spectrometer equipped with electron ionization using 70 eV in the ion source is typically employed, since it allows the analyst to use scientific libraries, such as NIST, for comparing acquired spectra with those in the library. However, the identification process becomes almost impossible when the compound of interest is not listed in the library, or when the sensitivity of the quadrupole MS is not sufficient for reliable mass confirmation. Waters™ Atmospheric Pressure Gas Chromatography (APGC) and Xevo G2-XS quadrupole time-of-flight (QTof) mass spectrometer, along with the UNIFI Scientific Information System provides an advantageous solution to overcome this hurdle.

APGC is a soft ionization technique which enables molecular ions to be observed.[5] Furthermore, the use of high resolution mass spectrometry (HRMS) and its proprietary MS^E mode[6] allows analysts to simultaneously acquire data containing the accurate mass of precursor and fragment ions. Finally, UNIFI's Discovery tool utilizes accurate mass and fragment information to facilitate the decision-making process towards the eventual identification of unknown compounds. To illustrate the benefits of APGC-QTof against electron ionization (EI)-single quadrupole MS, a polymer extracted sample was injected into both systems using the same chromatographic conditions in order to perform a comparative study of the chromatographic traces.

EXPERIMENTAL

Sample preparation

The sample, consisting of novel starch-based biopolymer pellets (0.5 g), was extracted three times with 2.5 mL of methanol in an ultrasonic bath for 1 hour at 40 °C. The total extraction solution (7.5 mL) was concentrated to 1 mL under a gentle nitrogen flow at room temperature before injection.

GC conditions

GC system:	Agilent 7890A
Autosampler:	7683B
Column:	DB-5MS, 30 m x 0.25 mm I.D. x 0.25 µm film thickness
Injection type:	1 µL pulsed splitless
Pulse time:	1.2 min
Pulsed pressure:	32 psi
Inlet temp.:	250 °C
Carrier gas:	He at 1 mL/min
Oven temp. program:	50 °C held for 2 min, ramp 50 to 300 °C 10 °C/min, 300 °C held for 10 min.

MS conditions

MS system:	Xevo G2-XS QTof, sensitivity mode
Scan range:	50 to 650 *m/z*
Corona current:	2.2 µA
Sample cone:	30 V
Source temp.:	150 °C
Cone gas flow:	140 L/h
Auxiliary gas flow:	225 L/h
Make-up gas:	N_2 300 mL/min at 300 °C
Collision ramp for MS^E:	20 to 30 eV
Lock mass:	Persistent column bleed peak, 207.0324 *m/z*
EI solvent delay:	4 min
Data management:	UNIFI Scientific Information System

RESULTS AND DISCUSSION

Data were acquired using dry conditions, where nitrogen charge transfer occurs and gives rise to the (radical cation) molecular ion $M^{+\cdot}$ information.

First, Total Ion Current (TIC) chromatograms acquired with EI (using an Agilent 6890N gas chromatograph with a MS 5975B detector) and APGC were compared. It is notable that APGC showed a higher number of peaks (Figure 1). This is due to the higher sensitivity of the QTof versus the single quadrupole, and to the intrinsic characteristics of the two different types of ionization techniques.

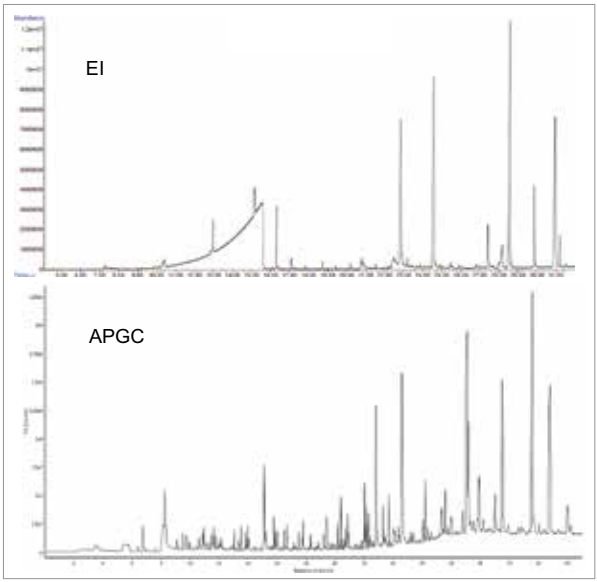

Figure 1. TIC chromatograms of the polymer extract acquired with EI (top), and with APGC at low collision energy (bottom).

BINARY COMPARISON

It is important to determine whether a peak comes from the tested material or from external contamination. Therefore, the analysis of a sample must always be accompanied by the analysis of its blank extract. UNIFI Software's Binary Compare feature allows direct comparison of the analysis results of an unknown sample with those of a reference (blank) sample, and to display the results in a mirror-image plot (Figure 2).

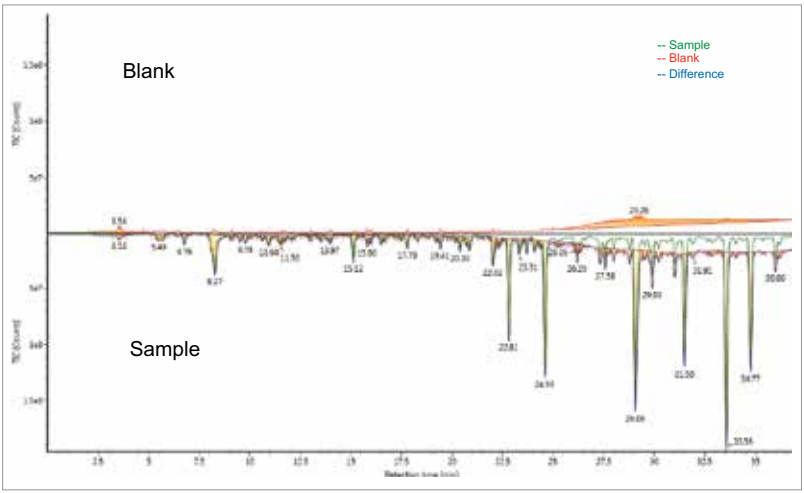

Figure 2. UNIFI's Binary Compare window shows the unknown sample and blank chromatographic profiles.

In addition, after specifying the mass tolerance, retention time tolerance, and intensity threshold of the unknown and reference samples in the comparison settings, UNIFI returns a Component Summary, where it is easy to identify the ions that are present in the unknown sample only, sorted by the intensity of response (Figure 3).

Component Summary

	Unknown component name	Unknown RT (min)	Unknown m/z	Match type	Unknown Intensity (Counts)	Unknown/Reference	Reference m/z
1	Candidate Mass 480.4893	34.78	480.4893	Unknown Unique	4817260		0.0000
2	Candidate Mass 421.1843	33.57	421.1843	Common	3952189	104.9904	421.1856
3	Candidate Mass 452.4577	31.50	452.4577	Common	3515446	430.0691	452.4577
4	Candidate Mass 401.2153	29.07	401.2153	Common	3353686	66.9177	401.2153
5	Candidate Mass 481.4697	34.79	481.4697	Unknown Unique	3079413		0.0000
6	Candidate Mass 450.1754	33.57	450.1754	Common	2865954	160.5685	450.1748
7	Candidate Mass 490.2064	29.06	490.2064	Common	2860768	112.7512	490.2060
8	Candidate Mass 420.1770	33.57	420.1770	Common	2702121	97.1917	420.1783
9	Candidate Mass 400.2085	29.06	400.2085	Common	2613383	60.3286	400.2082
10	Candidate Mass 435.1651	33.58	435.1651	Common	2361867	137.5356	435.1639
11	Candidate Mass 453.4619	31.50	453.4619	Unknown Unique	2163807		0.0000
12	Candidate Mass 256.2635	22.85	256.2635	Common	2119949	83.8248	256.2634
13	Candidate Mass 285.2981	34.60	285.2981	Common	2100727	208.3426	285.2978

Figure 3. Excerpt of Component Summary table.

UNIFI's Binary Compare function is particularly useful when the blank samples present a high level of contamination, as well as when some of the peaks are not perfectly resolved. Furthermore, some components were not visible in the TIC chromatogram due to the trace-level nature of some NIAS from the packaging materials. In these circumstances, UNIFI Software helps the user to determine the unique compounds in the sample extract despite their low intensity, which would be labelled as "unknown unique".

CONFIRMING IDENTIFICATION

The first step is testing the applicability of APGC for the confirmation of compounds that are associated to a candidate in the NIST library with a high *match* value. By way of example, the peak at retention time 16.3 min was identified by EI as 1,6-Dioxacyclododecane-7,12-dione (molecular formula $C_{10}H_{16}O_4$, monoisotopic molecular mass 200.1049 amu, CAS number 777-95-7) with a match of 917 (Figure 4A).

The same peak was processed via APGC, and its spectrum showed a base peak at *m/z* 201.1120, which is attributed to the $[M+H]^+$ ion (Figure 4B).

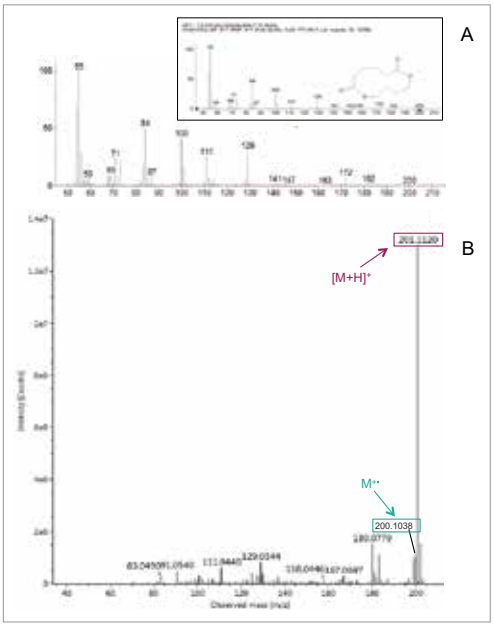

Figure 4. Comparison between the unknown and the reference for peak Rt = 16.2 min, showing (a) EI spectra, and (b) APGC low collision energy spectrum of the same chromatographic peak.

Using UNIFI's Mass Calculator feature, it is possible to obtain the exact mass of the adduct candidate molecular formula proposed by the EI library $[C_{10}H_{16}O_4+H]^+$. Hence, the mDa and ppm errors can be calculated. In the current example, the candidate molecular formula presents -0.14 mDa error and -0.7 ppm error. In APGC, the molecular ion $M^{+\cdot}$ at *m/z* 200.1038 is also present; in this case, the errors are -0.48 mDa and -2.4 ppm. Even though the presented APGC spectrum was obtained under dry conditions, protonation prevails over charge transfer because the structure of the investigated molecule favors accepting a proton, since even under dry conditions, the complete elimination of moisture in the ion source cannot be reached. The results demonstrate that the molecular formula of the candidate could be confirmed by the accurate mass of the molecular ion and the protonated adduct.

While linear adipates are usually employed as plasticizers in many plastic materials, 1,6-Dioxacyclododecane-7,12-dione is a cyclic adipate that was previously also found as a NIAS in biodegradable polyesters,[7] printing inks,[8] and polyurethane plastics.[9]

This example highlights the usefulness of APGC coupled with high resolution mass spectrometry when confirmation of the molecular formula is needed.

CORRECTING AN INCORRECT IDENTIFICATION

At the retention time 17.2 min in EI there was a very low intensity and broad peak that NIST attributed to 3,4-altrosan or beta-D-glucopyranose, 1,6-anhydro-, with a *match* value of 787. Both compounds have a molecular weight of 162 amu. However, by analyzing the same peak in APGC, a base ion peak at *m/z* 232.1817 appeared.

UNIFI Software allows users to create a customized workflow through the introduction of filters in order to get better visualization of data, and to save time by focusing on the most relevant components. For example, it is possible to select a specific Rt window to be analyzed and an ion intensity threshold. Applying this filter (Rt window 17.16–17.27 min and response >5000 counts) for peak Rt 17.2 min in APGC, UNIFI returns the component list that fits those settings. In this example, we displayed the processed and non-processed high collision energy spectra of the same component, shown in Figure 5. The processed spectrum appears "cleaner" because it focuses only on the component under investigation, without ions coming from other compounds that could partially coelute with the compound of interest.

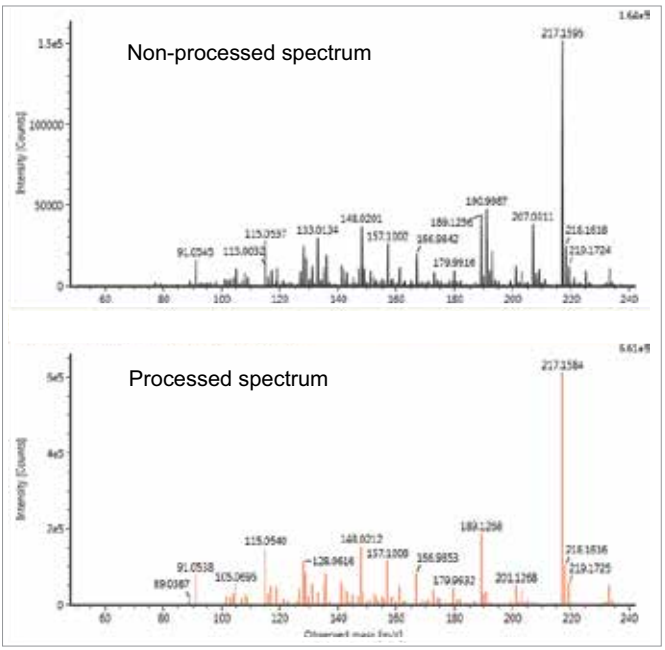

Figure 5. APGC high collision energy spectra of peak Rt 17.2 min. Non-processed spectrum (top) and processed spectrum based on component m/z 232.1817 (bottom).

UNIFI's filters, views, and workflow steps allow users to review data in a more timely, consistent, and accurate way. The componentization feature in UNIFI allows interrogation of entire datasets without having to interact with the raw data. Componentization also facilitates the selection of candidate components, which may represent unexpected substances within a sample; this is possible with UNIFI's 3D peak detection algorithm.[10]

When screening complex samples, the UNIFI Elucidation toolset can be used to investigate and potentially identify candidate components. The Elucidation toolset includes an elemental composition calculator that determines a number of possible formulas for an accurate mass peak. Elemental Composition uses an algorithm, i-FIT,™ to score each formula by the likelihood that the theoretical isotope pattern of the formula matches a cluster of peaks in the spectrum. To restrict the number of possible formulas, the i-FIT model can take into account fragment ion mass spectral peaks, the number of atoms of elements specified, valence state, the number of double bonds in a formula, the type of isotope pattern, and a series of chemical rules.

By applying the Elemental Composition tool to mass 232.1817 UNIFI proposed the molecular formula $C_{16}H_{24}O$ ($M^{+\cdot}$) with the lowest mDa error and the highest i-FIT confidence (%), as shown in Figure 6.

After searching ChemSpider, PubChem, and SciFinder, the suggested molecular formula was attributed to 1,2,3,4-tetrahydro-1-methoxy-1,6-dimethyl-4-(1-methylethyl) naphthalene (CAS number 60698-94-4). The Elemental Composition tool was also used to check the molecular formula of the most abundant fragments in the processed high collision energy spectrum, and to deduce their structures. In Figure 7 the proposed fragmentation pathway is shown, which confirmed the candidate structure of the molecular ion.

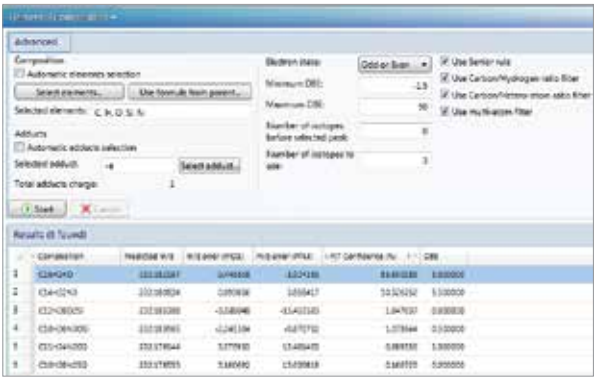

Figure 6. Results from UNIFI Software's Elemental Composition tool for the ion m/z 232.1817.

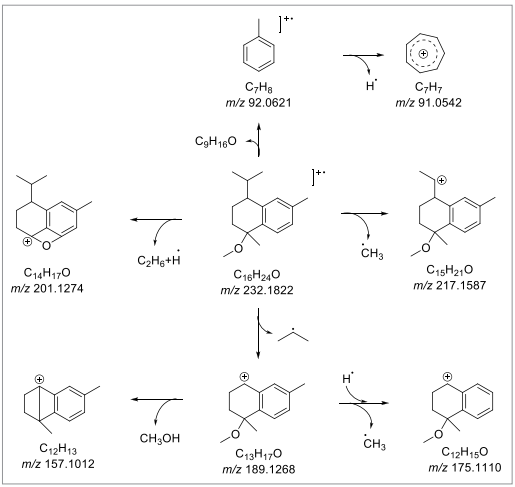

Figure 7. Proposed fragmentation pathway of the molecular ion $M^{+\cdot}$. Fragment ions are defined by their molecular formula and exact mass-to-charge ratio.

1,2,3,4-tetrahydro-1-methoxy-1,6-dimethyl-4-(1-methylethyl) naphthalene was also found in essential oil extracts of several plants, such as hops, pine and Japanese spicebush,[11-13] as well as in propoli extracts[14] as a component of the volatile profile.

Here, we were able to correct the EI identifications of components that presented a low match value or that were not listed in the libraries using APGC and UNIFI.

IDENTIFYING PREVIOUSLY NON-DETECTABLE PEAKS

Since the APGC-QTof MS system delivers enhanced sensitivity compared to EI-MS, APGC spectra lead to a significantly higher number of detected peaks. Consequently, it is possible to extend the identification process to a wider range of compounds. By way of example, the compound represented by the peak at Rt 27.3 min in the APGC spectrum was not present in the EI spectrum (Figure 8).

In this step, the Discovery tool in UNIFI was employed on the base ion peak *m/z* 410.3169.

In Figure 9 it can be noted that UNIFI attributed the component of interest to a predicted list of chemicals, recognized to be likely by an automatic search in ChemSpider. The table shows a list of possible compounds sorted by Predicted Intensity, i-FIT Confidence, Fragment Match, or number of citations.

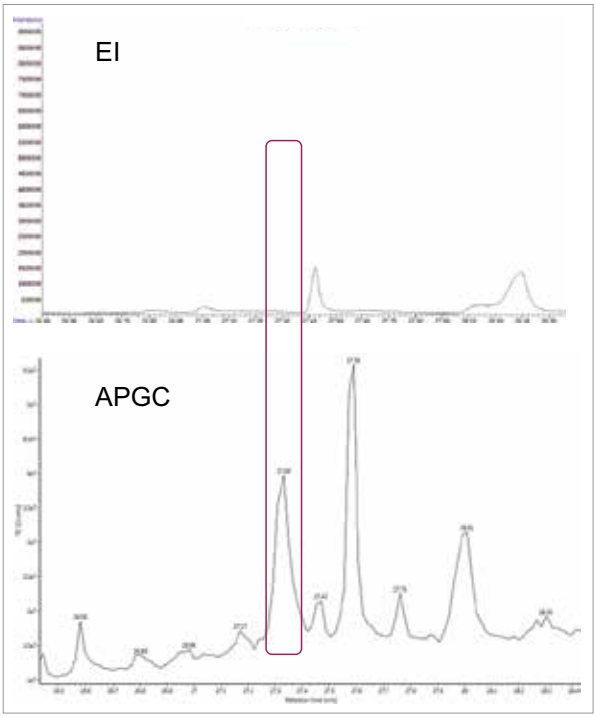

Figure 8. Comparison between the EI and APGC chromatograms within the range 26.4–28.4 min, highlighting the peak at 27.3 min in APGC, not detected with EI.

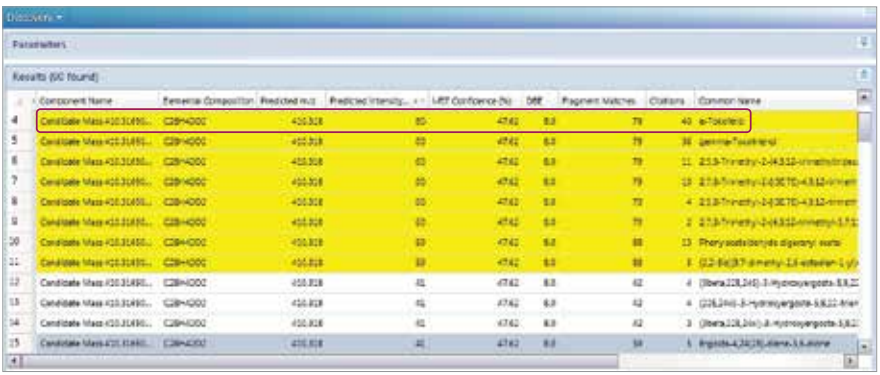

Figure 9. Results from UNIFI's Discovery tool for component m/z 410.3169 at Rt 27.33 min.

The candidates highlighted in yellow present a Predicted Intensity >50%. After analyzing the most important fragment ions, applying the common organic chemistry rules, and checking their molecular formula and mDa errors, the unknown compound was identified as e-tokoferol, more commonly called beta-tocotrienol, IUPAC name: [*R*-(*E*,*E*)]-3,4-dihydro-2,5,8-trimethyl-2-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-2H-1-benzopyran-6-ol (CAS number 490-23-3). In Figure 10, the Discovery information output is illustrated. On the left side of the figure there is a list of synonyms for the candidate, while on the right side, the software shows the chemical structure and the high collision energy mass spectrum, where the most important fragments are pointed out.

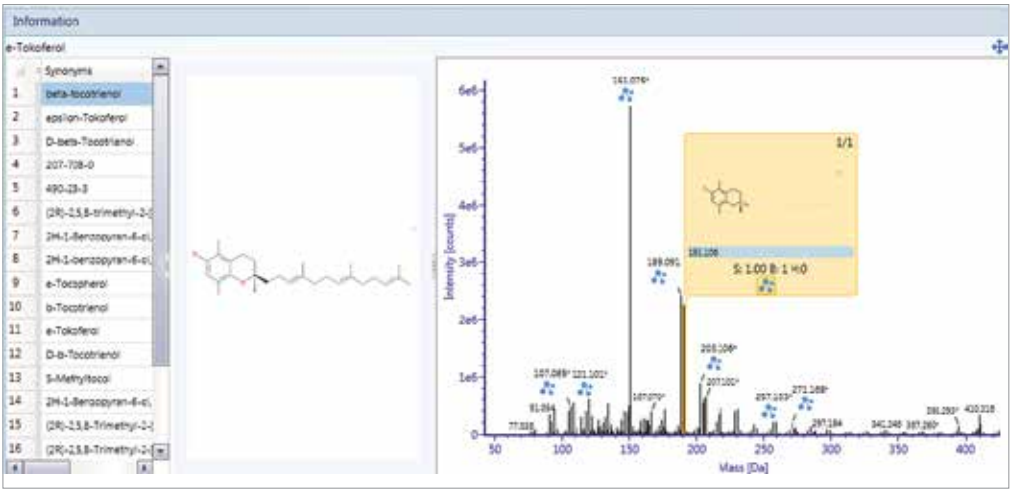

Figure 10. UNIFI's Discovery tool information output of beta-tocotrienol. Highlighted is one of the major fragments (m/z 191.1062).

It is possible to check out the molecule's cleavage points by clicking the fragment marker on the ion peak; the fragment *m/z* 191.1062 was chosen as an example.

Tocotrienols are members of the Vitamin E family, characterized by an unsaturated isoprenoid side chain (farnesyl isoprenoid tail) with three double bonds; their presence in the polymer could be due to their employment as antioxidant additives. In addition, tocotrienols are bioactive compounds normally present in many fatty foodstuff (such as vegetable oils), that have been used in many nutritional and pharmaceutical applications.[15]

UNIFI's Discovery tool saves analyst's time in the elucidation process and provides comprehensive high-quality information by sorting the possible candidates, based on several parameters set by the user. However, it should be noted that to reach a confidence level closer to 100% in the identification of an unknown compound, the candidate compound must be confirmed with a standard by verifying retention time, accurate mass, and common fragments.

CONCLUSIONS

Identifying unknown compounds in food contact materials is usually a challenging process. The UNIFI Scientific Information System simplifies the process by providing customizable workflows and achieving data containing accurate mass precursor and fragment ions information acquired by the MS^E functionality.

EI-MS and APGC-QTof MS systems have been proven to be complementary when the compounds of interest are described in commercially available libraries, whereas APGC-QTof MS is particularly advantageous when the elucidation is required for volatile and semi-volatile components not listed in the libraries, or for those at trace or ultra-trace levels. APGC-Xevo G2-XS QTof with UNIFI can determine possible erroneous identifications and also facilitate component identification for peaks that are not detected using an EI quadrupole MS system.

Finally, UNIFI componentization eases the burden of data interpretation for the analyst, reducing potential false-positive assignments, and allowing results to be presented clearly and concisely.

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Analysis of Ergot Alkaloids in Cereal Samples by Liquid Chromatography-Tandem Quadrupole Mass Spectrometry

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APPLICATION BENEFITS

Specific, targeted method for determination of a range of ergot alkaloids in cereal samples that is suitable for both official control and food business operators' due diligence testing.

WATERS SOLUTIONS

ACQUITY™ UPLC™ I-Class System

Xevo™ TQ-S

MassLynx™ MS Software

TargetLynx™ Application Manager

KEYWORDS

LC-MS/MS, ergot alkaloids, targeted analysis, cereal, mycotoxins, ergopeptine

INTRODUCTION

Ergot alkaloids (EAs) are mycotoxins produced by fungi including *Claviceps spp.* Among the cereal species, rye and triticale that have open florets are known to be especially susceptible, but wheat, barley, oats, and other cereal grains are also potential fungal hosts.[1] The fungus replaces the developing grain or seed with the alkaloid-containing wintering body, known as the ergot body or sclerotium. The sclerotia are harvested together with the cereals or grass and can thus lead to contamination of cereal-based food and feed products with EAs. Ingestion of contaminated products can cause a number of harmful effects in humans and livestock. Although ergotism has practically been eliminated as a human disease, it remains an important veterinary problem, particularly in cattle, horses, sheep, pigs, and chicken.[2] There are three main classes of EAs: short chain substituted amides of lysergic acid, clavine alkaloids, and ergopeptines, which are peptide EAs comprising (+)-lysergic acid and a tripeptide system containing L-proline. Structures of some of the major EAs are shown in Figure 1, including an example of epimerisation of an ergopeptine at the C8 position to form the corresponding ergopeptinine. Although the ergopeptinines are described as biologically inactive, interconversion can occur under various conditions so analytical methods should include the determination of both epimeric forms.

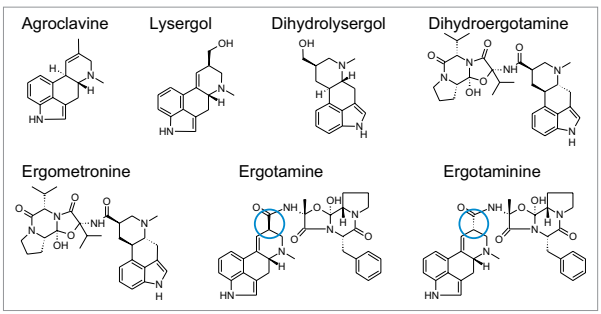

Figure 1. Representative structures of some of the ergot alkaloids included in this study.

Modern management of ergot is focused on limiting the presence of ergot sclerotia in cereal grain but compliance with maximum levels for ergot sclerotia does not necessarily guarantee the safety of food for the presence of EAs. Currently only a few countries have set limits for the individual EAs in feed (e.g. Canada); but no country has yet set limits in food as most do for other mycotoxins. In 2012, the European Commission started to consider setting maximum levels for EAs as the profile, concentration, and toxicity of EAs and their corresponding epimers vary considerably in different grains and batches of grain.[3] New legislation could be agreed upon by the end of 2017. In the meantime, the European Food Safety Authority (EFSA) has recommended that the major ergopeptines and their corresponding epimers be targeted for determination in food and feed to provide data to enable consumer exposure calculations.[4] The results of those analyses were used to estimate chronic and acute dietary exposure to EAs in humans and animals in Europe.[5] This interest in monitoring is also reflected globally with method development and results of analyses being reported in North America[6] and Asia.[7]

This application note describes a method for the determination of 25 EAs in cereals using a rapid and simple sample extraction protocol followed by LC-MS/MS on the ACQUITY UPLC System coupled to Xevo TQ-S.

EXPERIMENTAL

All of the cereal samples (rye flour, wheat flour, bread, and noodles) were purchased from local supermarkets and Taobao, the largest internet-based retailer in China. The FAPAS proficiency test sample of EAs in rye flour was obtained from Fera in the UK. The noodles, pasta, and bread were ground and 1.0 g of sample was weighed in a 50 mL polypropylene centrifuge tube, and 10 mL of acetonitrile and 3 mM ammonium carbonate (85:15, v/v) added. After shaking for 30 s, vortexing for 30 s, and centrifuging for 5 min at 9000 rpm below 4 °C, 5 mL of the supernatant was transferred to another tube that contained 150 mg of C_{18} sorbent (e.g. WAT035672) for dSPE clean-up. The mixture was vortexed for 30 s and centrifuged for 5 min at 9000 rpm below 4 °C, and then the upper layer was transferred to a vial for the UPLC-MS/MS analysis.

UPLC conditions

UPLC system:	ACQUITY UPLC I-Class with FL Sample Manager
Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 × 100 mm
Mobile phase A:	3 mM ammonium carbonate (*aq.*)
Mobile phase B:	Acetonitrile
Flow rate:	0.2 mL/min
Injection volume:	5 µL (partial loop mode from 10 µL loop)
Column temp.:	30 °C
Sample temp.:	4 °C
Run time:	16 min

Gradient:

Time (min)	%A	%B	Curve
0.00	75	25	–
1.00	60	40	6
5.00	40	60	6
8.00	22	78	6
10.50	10	90	6
11.00	10	90	6
13.00	75	25	6

MS conditions

MS system:	Xevo TQ-S
Source:	Electrospray
Ionization mode:	ESI+
Capillary voltage:	2.5 kV
Desolvation temp.:	500 °C
Desolvation gas flow:	700 L/Hr
Source temp.:	150 °C
Cone gas flow:	150 L/Hr
Cone voltage:	30 V
Collision gas flow:	0.14 mL/min
Nebulizer gas pressure:	7 Bar

Data management

Data were acquired using MassLynx MS Software (v4.1) and processed using TargetLynx XS Application Manager. The selection of MRM transitions and optimization of critical parameters was performed by infusion of individual solutions of all the analytes and evaluation of the data by IntelliStart™ Software to automatically create acquisition and processing methods. Table 1 summarizes conditions for all MRM transitions including the retention times. The dwell time was set to 20 ms for each MRM transition.

Table 1. MRM parameters for EAs (quantatitive transitions in bold).

Compound		Retention time (min)	MRM	CE (eV)
DErg	Dihydroergine	2.1	270.1>168.1	13
			270.1>210.1	22
Em	Ergometrine	2.2	326.2>208.1	28
			326.2>223.1	23
Cha	Chanoclavine	2.4	257.1>226.1	10
			257.1>168.1	21
Ely	Elymoclavine	2.4	255.1>196.1	19
			255.1>224.1	15
DLys	Dihydrolysergol	2.4	257.2>182.1	27
			257.2>208.1	23
Lys	Lysergol	2.5	255.1>197.1	22
			255.1>240.1	20
Erg	Erginine	2.7	268.1>208.1	20
			268.1>223.1	24
Emn	Ergometrinine	2.8	326.2>208.1	28
			326.2>223.1	23
Es	Ergosine	4.2	548.2>223.1	31
			548.2>268.1	23
DEt	Dihydroergotamine	4.4	584.3>253.1	53
			584.3>270.1	29
Et	Ergotamine	4.4	582.3>208.1	44
			582.3>223.1	34
Agr	Agroclavine	4.6	239.1>183.1	17
			239.1>208.1	17
DEco	Dihydroergocornine	4.7	564.3>168.1	53
			564.3>270.1	30
Fes	Festuclavine	5.0	241.1>154.1	32
			241.1>168.1	28
Eco	Ergocornine	5.1	562.3>223.1	37
			562.3>268.1	25
DEkr	Dihydroergocryptine	5.2	578.3>253.1	31
			578.3>270.1	31
DEcr	Dihydroergocristine	5.4	612.3>270.1	32
			612.3>350.2	25
α-Ekr	α-Ergocryptine	5.5	576.3>223.1	38
			576.3>268.1	25
b-Ekr	b-Ergocryptine	5.7	576.3>223.1	38
			576.3>268.1	25
Ecr	Ergocristine	5.7	610.3>223.1	35
			610.3>268.1	26
Esn	Ergosinine	6.3	548.2>223.1	31
			548.2>268.1	23
Etn	Ergotaminine	6.7	582.3>208.1	44
			582.3>223.1	34
Econ	Ergocorninine	7.2	562.3>223.1	37
			562.3>268.1	25
α-Ekrn	α-Ergocryptinine	7.7	576.3>223.1	38
			576.3>268.1	25
Ecrn	Ergocristinine	7.9	610.3>223.1	35
			610.3>268.1	26

RESULTS AND DISCUSSION

OPTIMIZATION OF THE LC CONDITIONS

The mobile phase composition for the chromatographic separation of the 25 EAs was based on previous work,[8,9,10] with minor modifications. Alkaline mobile phases are preferred for the analysis of EAs in order to minimize inter-conversion of the epimers, to avoid protonation, and to improve separation. The flow rate and column temperature were evaluated to obtain the optimum conditions for separation of the EAs with individual ergopeptines eluting immediately before the corresponding ergopeptinines. The resolution was highest when the column was maintained at 30 °C, with the flow rate set to 0.2 mL/min. All 25 EAs were well separated except α-ergocryptine and ergocristine but these can be distinguished by their different MRM transitions.

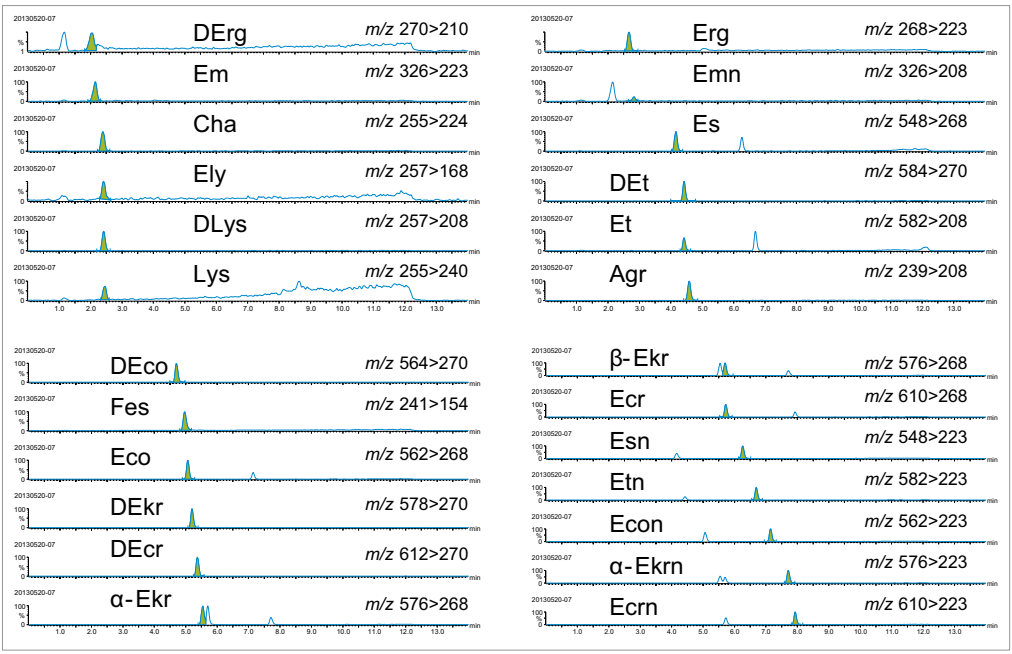

Figure 2. Chromatograms showing EAs from analysis of matrix-matched standards of rye flour prepared at 0.1 µg/kg.

OPTIMIZATION OF THE SAMPLE EXTRACTION AND CLEANUP STEPS

Optimization of the sample extraction and cleanup has been previously described.[7] An aprotic solvent, acetonitrile, was used to minimize epimerization during extraction. The method was modified from that used by Kokkonen and Jestoi[9] but with the introduction of a rapid and cost-effective C_{18} dSPE cleanup step.

UPLC-MS/MS PERFORMANCE

Excellent sensitivity and selectivity was demonstrated by the response for each of the analyte peaks detected from the analysis of a variety of different cereal samples (see Figure 2). No interfering compounds were detected at the retention times of the analytes in all the tested blank samples.

To compensate for matrix effects, calculated to be within the range of 78 to 122% in the commodities assessed, matrix-matched standards, at seven concentrations (0.05, 0.10, 0.25, 0.50, 1.0, 2.5, and 5.0 µg/kg), were used for calibration. The response was linear and the correlation coefficients (r) were >0.998 for all EAs tested with residuals <15%. Limits of detection (LOD) and quantification (LOQ) were determined as the analyte concentration corresponding to the mean of 3 and 10 standard deviations (SDs), respectively, of the response measured in an uninfected sample matrix. Although no regulatory limits are set for the content of EAs in food, the results presented in Table 2 indicate that the proposed method would be suitable for the detection of EAs for monitoring purposes.

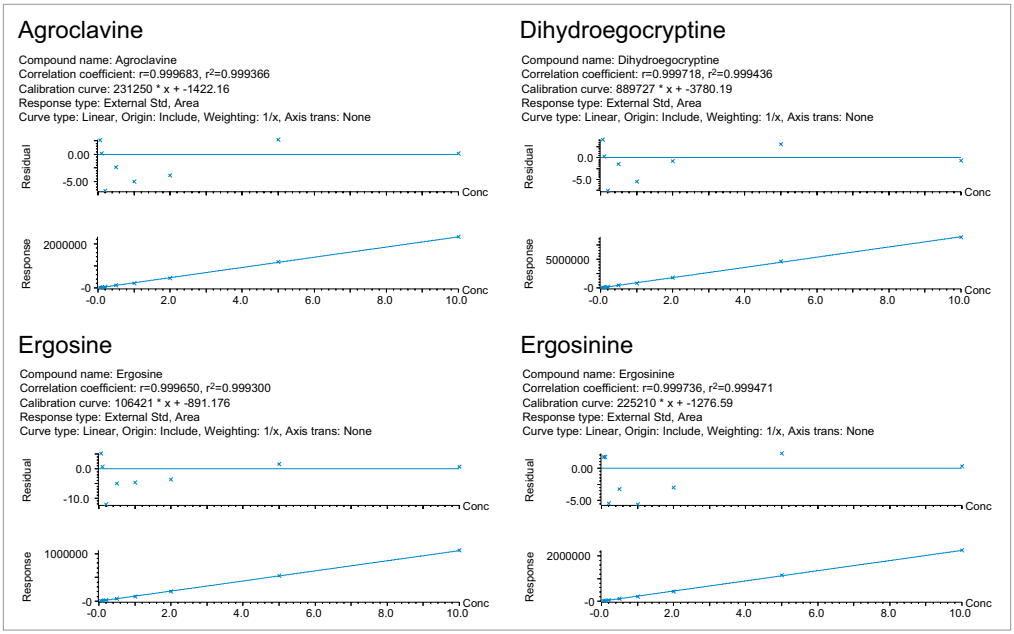

Figure 3. Calibration graphs for a selection of EAs prepared in rye flour extract.

RECOVERY, REPEATABILITY, AND ACCURACY

Recoveries were measured from the analysis of uninfected blank samples (wheat, rye, bread, noodle, and pasta) spiking with the EAs six times at three different concentrations: 0.1, 0.25, and 1.0 µg/kg. Unlike other mycotoxins regulated in the EU, there are currently no performance criteria set for the analysis of EAs. Recoveries were within the range 76.5 to 120% with RSDs <15%. A more detailed summary of the performance of the method for the analysis of EAs in rye is shown in Table 2.

Table 2. LOD, LOQ, recovery, and repeatability (%RSD) at three concentrations for the 25 EAs in rye flour.

Compound	LOD (µg/kg)	LOQ (µg/kg)	%Recovery (%RSD, n=6)		
			0.1 µg/kg	0.25 µg/kg	1.0 µg/kg
Dihydroergine	0.020	0.05	98.7 (4.1)	118 (11)	81.5 (8.4)
Ergometrine	0.010	0.04	97.4 (9.1)	102 (5.6)	93.3 (4.2)
Chanoclavine	0.020	0.05	95.6 (5.1)	102 (13)	86.3 (3.5)
Elymoclavine	0.005	0.02	77.5 (4.8)	111 (4.7)	82.2 (2.2)
Dihydrolysergol	0.005	0.02	115 (14)	112 (13)	85.4 (7.7)
Lysergol	0.020	0.05	84.2 (4.1)	76.5 (1.8)	84.6 (12)
Erginine	0.020	0.05	79.6 (13)	103 (8.8)	86.1 (1.4)
Ergometrinine	0.020	0.05	109 (10)	104 (5.1)	98.0 (6.2)
Ergosine	0.020	0.05	108 (12)	101 (5.8)	88.8 (3.4)
Dihydroergotamine	0.005	0.02	116 (6.7)	104 (0.9)	89.7 (2.3)
Ergotamine	0.005	0.02	107 (12)	97.5 (9.7)	91.2 (3.1)
Agroclavine	0.005	0.02	84.2 (9.3)	99.3 (6.6)	94.6 (2.9)
Dihydroergocornine	0.005	0.02	109 (4.5)	89.4 (2.2)	94.0 (3.6)
Festuclavine	0.020	0.05	93.7 (3.8)	95.3 (8.2)	86.8 (2.7)
Ergocornine	0.005	0.02	95.8 (6.5)	89.5 (5.5)	88.8 (2.9)
Dihydroergocryptine	0.005	0.02	97.3 (4.0)	85.5 (1.5)	88.4 (1.8)
Dihydroergocristine	0.005	0.02	108 (4.5)	104 (2.2)	90.3 (2.5)
α-Ergocryptine	0.005	0.02	113 (5.1)	102 (3.8)	90.8 (1.0)
α-Ergocryptine	0.005	0.02	108 (12)	107 (9.3)	92.3 (4.3)
Ergocristine	0.005	0.02	117 (7.1)	94.1 (3.8)	90.5 (3.9)
Ergosinine	0.005	0.02	120 (3.4)	106 (5.4)	92.3 (2.5)
Ergotaminine	0.005	0.02	97.5 (3.4)	87.7 (3.6)	93.1 (1.5)
Ergocorninine	0.005	0.02	103 (4.1)	107 (2.3)	90.9 (2.7)
α-Ergocryptinine	0.005	0.02	85.4 (7.0)	95.0 (3.2)	101 (3.6)
Ergocristinine	0.005	0.02	104 (5.0)	98.2 (2.3)	94.2 (4.5)

Method accuracy was evaluated by measuring the concentration of EAs in a FAPAS proficiency test sample (test no. 22013; EAs in rye flour). The performance of the method was considered "satisfactory" (Z score between +2 and -2) for almost all of the EAs with the exception of that for ergosinine, which was considered "questionable" (Z score between +3 and -3).

ANALYSIS OF CEREAL SAMPLES

The method was used to test 123 cereal samples purchased online (15) and at local supermarkets (108), including rye flours (9), wheat flours (52), wheat flour noodles (43), and breads (19). No EAs were detected in the flour samples and related food products purchased from supermarkets. Two rye and three whole wheat flour samples obtained online were found to contain 13 ergopepine and ergopepinine EAs at a concentration range of 1.01 to 593 µg/kg. The analysis of each positive sample was repeated six times to produce some repeatability data; RSDs were <6%. The profile of EAs varied between samples.

CONCLUSIONS

This application note describes the performance of a method for the analysis of 25 EAs by UPLC-MS/MS, after extraction and dSPE cleanup. The method is simple, time-saving, and inexpensive, providing fast and reliable quantification of EAs in various types of cereal samples. Although no regulatory limits are currently established for the content of EAs in food, these results indicate that the proposed method is likely to be suitable for the determination of EAs for monitoring purposes. Calibration characteristics, linearity, and residuals were excellent over the concentration range studied. This method was successfully validated using replicate spiked blank samples and a FAPAS proficiency test sample of EAs in rye flour and has been used for the surveillance of commercial samples of cereal flours, bread, and noodles in China.

Acknowledgements

This study was supported by the Science Research Foundation of the Ministry of Science and Technology of the People's Republic of China (Grant 2015FY111400).

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Sensitive Analysis of Nodularin and Microcystins of Concern in Drinking Water Using Simplified Sample Preparation

Stuart Oehrle, Douglas Stevens, Adam Ladak

GOAL

To show nodularin and the major microcystins of concern in drinking water can be analyzed with minimized sample preparation and increased confidence in results.

BACKGROUND

There is an increased interest in the monitoring of microcystins that are generated by blue-green algae in drinking water in order to protect the public from exposure.[1] EPA Method 544, for instance, monitors for six microcystins and nodularin, and utilizes solid phase extraction (SPE) and LC-MS/MS to reach the minimum reporting level of 1 µg/L.[2]

One major challenge in using some current methods is they involve SPE extraction of 500 mL of water that is subsequently concentrated down to 1 mL. This process is time consuming as the loading and evaporation of the extract required to meet necessary detection levels can take hours. However, with less sensitive instrumentation, this is the only way that the challenging regulatory limits can be met.

Increased sensitivity with reduced run time, minimized sample preparation and solvent consumption for microcystin analysis.

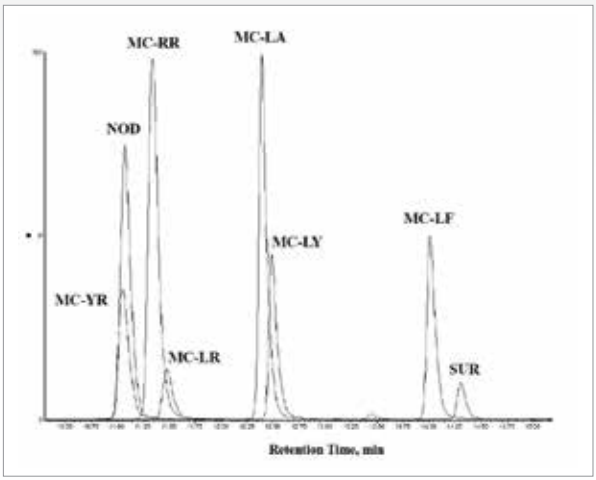


Figure 1. EPA Method 544 chromatographic separation example.2

Another challenge with the current method is the use of a single MRM transition for each analyte. This makes it difficult to confirm spurious results and can lead to re-analysis and delays in reporting results which are critical to ensure the public are not at risk from exposure. Having an analytical method that is more sensitive, with additional transitions and rapid run time provides multiple advantages in the targeted analysis of microcystins.

THE SOLUTION

In this work, the current EPA Method 544 was used as a starting point for method development. A Waters™ UHPLC column and the Waters Xevo™ TQ-S micro were used for this investigation. The CORTECS™ C8 90 Å, 2.7 µm, 2.1 mm x 100 mm Column (P/N 186008351) was used with a VanGuard™ C8 90 Å, 2.7 µm, 2.1 mm x 5 mm Cartridge (P/N 186008421) and holder (P/N 186007949) for the analysis. Chromatography was further optimized to improve separation between near eluting analytes. Table 1 shows the final chromatographic conditions utilized for this analysis. Figure 1 shows the separation defined in EPA Method 544 while Figure 2 shows the separation on the CORTECS™ Column. The method showed comparable separation to the current column used in EPA Method 544 and detection of the seven compounds of interest.

The seven compounds of interest were optimized on the Xevo TQ-S micro. An additional MRM transition was added for each compound. This allowed for further confirmation of the presence of the compound and verification of not only an additional transition but the ion ratios between the two transitions.

As the sensitivity of the Xevo TQ-S micro was excellent, no SPE or pre-concentration of drinking water was required for any of the work. While EPA Method 544 does not allow for the exclusion of SPE, this work does demonstrate that current generation tandem quads are able to meet the method's challenging detection requirements even without the enrichment provided by SPE sample preparation.

In order to assess the sensitivity of the method, a calibration curve was made of microcystin LR, YR, and RR compounds between 0.5 and 40 ppb in drinking water. The linearity and limit of detection were excellent as indicated by the R2 values of >0.99 and %RSDs of less than 15%. Figure 3 shows the linearity of microcystin LR and Figure 4 shows the detection of microcystin LR 0.5 ppb.

Time	Flow (mL/min)	%A 20 mM Ammonium formate	%B Methanol	Curve
–	0.3	90	10	–
2	0.3	90	10	6
16	0.3	20	80	6
16.1	0.3	10	90	6
22	0.3	10	90	6
22.1	0.3	90	10	6
26	0.3	90	10	6

Table 1. LC gradient utilized for method. (as published in EPA Method 544).

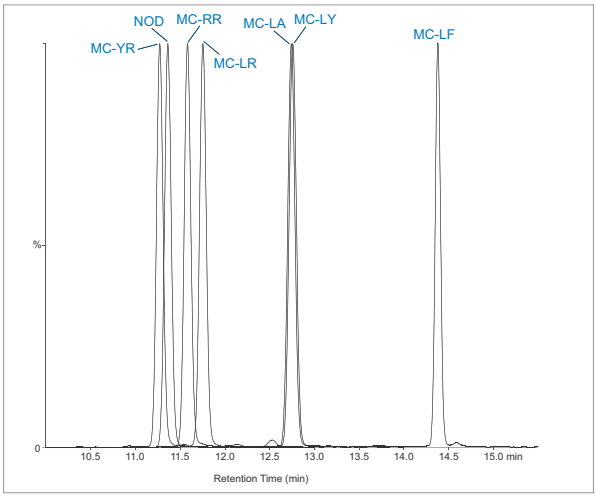


Figure 2. Standard between 40 and 60 µg/L showing separation of 6 microcystins and nodularin.

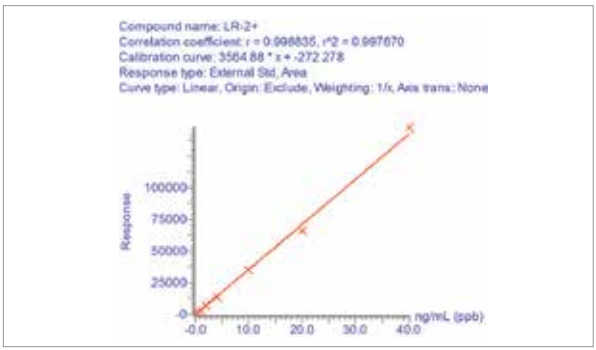


Figure 3. Linearity of microcystin LR between 0.5 µg/L and 40 µg/L .

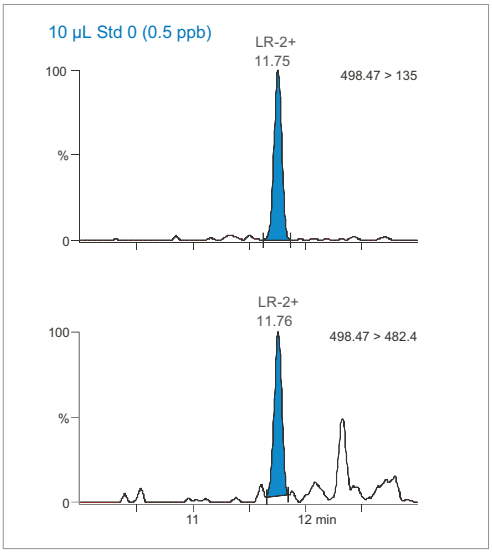
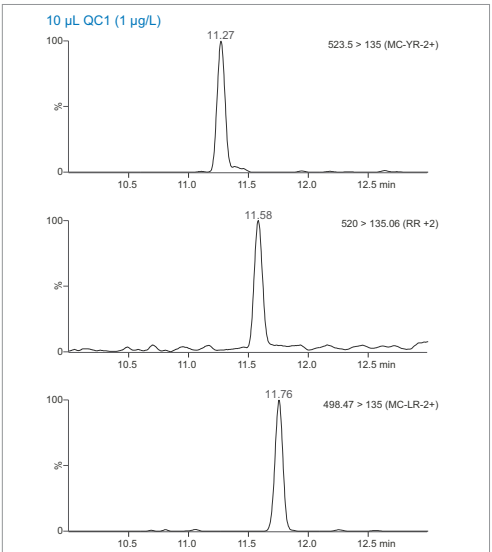


Figure 4. Detection of microcystin LR in drinking water at 0.5 µg/L with two transitions.

Finally, in order to ensure the method was reproducible, three example microcystins were spiked into a drinking water sample at 1 µg/L and injected 5 times. The % RSDs under 10% for the replicates fall within the requirements described in EPA Method 544.



Toxin	%RSD
LR	7.7
RR	5.5
YR	9.1

Figure 5. Reproducibility of microcystins at 1 ppb.

SUMMARY

The use of the CORTECS C_8 Column produces equivalent chromatographic separation within a shorter run time for the nodularin and the six microcystins investigated. Although EPA Method 544 does not allow for the exclusion of SPE, the increased sensitivity of the Xevo TQ-S micro allows the user to potentially eliminate SPE or use less water to concentrate while still meeting the challenging detection limit requirements for current analytical methods. The addition of a confirmatory MRM transition for each compound also ensures that the compound is accurately detected and reported.

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Rapid Determination of Cinnamomum Species Using the DART QDa System with LiveID for Ground Spice Authenticity Testing

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APPLICATION BENEFITS

- Real-time species level identification of cinnamon with minimal need for sample preparation and no chromatographic separation.
- Applicability for point of control qualitative testing with reduced sample manipulation giving results in seconds.
- Intuitive LiveID™ Software is accessible to non-expert users to develop and validate robust models for various food authenticity, integrity, and quality control challenges.

WATERS SOLUTIONS

DART QDa System with LiveID

MassLynx™ MS Software

Oasis™ HLB SPE Cartridge

KEYWORDS

Spices, food authenticity, food profiling, chemometrics

INTRODUCTION

Cinnamon is a popular, aromatic culinary spice; it is produced from the inner bark of the Cinnamomum tree which belongs to the genus *Cinnamomum* of the Laurel family *(Lauraceae)*.

Cinnamon has many species that differ in smell, taste, and color depending on the geographical region, but the species of most commercial importance are *Cinnamomum verum* and *Cinnamomum cassia*. *Cinnamomum verum* is known as Ceylon cinnamon or "true cinnamon" while C. *cassia* is the Chinese species. Both species have similar characteristics that exhibit a fragrant, sweet, and warm taste; however, the flavor of the Ceylon variety is more refined and subtle.[1] The unique properties of this spice come from its essential oils and compounds, in particular cinnamaldehyde. Cinnamaldehyde is the compound which gives cinnamon its flavor and aroma, and it is also proposed to be responsible for many of the health benefits associated with cinnamon ingestion. Strips of the inner bark are dried until they curl into rolls known as cinnamon sticks or quills. These can then be further ground into powder or made into an extract.

C. *cassia* is economically cheaper and more abundant than the C. *verum* species with its value depending on the percentage of cinnamaldehyde. C. *cassia* is also known to contain coumarin, cinnamyl acetate, cinnamic acid, phenylpropyl acetate, orthocumaric aldehyde, and tannic acid. The most common form of cinnamon adulteration is via the substitution or dilution of ground C. *verum* with C. *cassia*. European health agencies have warned against consuming high amounts of C. *cassia* due to the elevated amount of coumarin, a known blood-thinning agent which could damage the liver if taken in large amounts. Other bioactive compounds found in the bark, powder, and essential oils of C. *cassia* are cinnamaldehyde and styrene. In high doses these substances can also be toxic for humans.[2]

The IonSense™ Direct Analysis in Real Time (DART) is a form of ambient mass spectrometry which is defined as mass spectrometric analysis with minimal effort for sample preparation, using direct sampling and ionization at ambient conditions.[3] DART is an atmospheric pressure ionization related technique based on the thermal desorption of condensed phase analytes by a plasma discharge in a heated gas stream, typically helium or nitrogen. Metastable atoms generated from gas interact with ambient molecules, such as water to create gas-phase ionic reagents which in turn react and ionize analytes on a surface, or present as a vapor in the atmosphere. DART is capable of analyzing low to high polarity compounds (up to 1 kDa) in both negative ion and positive ion modes.

In this application note, we demonstrate the use of DART coupled to Waters™ ACQUITY™ QDa Mass Detector with chemometric modeling performed in real time with LiveID Software to rapidly determine the species level identity of cinnamon for label claim verification and authenticity purposes for the food industry.

EXPERIMENTAL

The chemometric model was trained using authentic ground samples of Ceylon cinnamon (C. *verum*) and C. *cassia* obtained from a national herb and spices supplier. Authentic samples of Ceylon cinnamon were mixed in variant percentage with C. *cassia* to investigate the capability of the method to detect mixtures of the cinnamon species in the case of adulteration by substitution or dilution.

Sample preparation

To detect compounds of interest 1 g homogenized sample of ground cinnamon/cassia was weighed into a 50-mL tube and mixed with 15 mL of EtOH:water 50:50 (v/v). The sample was mixed using vortex mixer for 30 s followed by sonication for 15 min. The samples were then centrifuged at 6000 rpm for 5 min, and 3 mL of supernatant was loaded onto an OASIS HLB Cartridge, 3 cc, 60 mg (p/n: WAT094226). Elution was performed using 1 mL of methanol. Two replicate extracts were prepared for each sample and stored at -20 °C. Prior to analysis, the extracts were spotted (3 µL) on the 12 positions of a QuickStrip card and allowed to dry under ambient conditions. Each QuickStrip card was then analyzed using DART QDa, thus generating 12 replicate Regions of Interest (ROIs) per card, per extract.

MS conditions

MS system:	ACQUITY QDa
MS source:	DART
Ionization mode:	Positive
Acquisition mode:	Full scan MS
Gas temp. (He):	150 °C
Sampling speed:	1.00 mm/sec
Sampling frequency:	2 Hz
Cone voltage:	10 V
Mass range:	100–600 *m/z* (continuum)

Data acquisition and processing

LiveID multivariate statistical software package (v1.2) was used as a chemometric model building and real time recognition tool.

RESULTS AND DISCUSSION

DART QDA CINNAMON AUTHENTICITY MODEL

The workflow illustrated in Figure 1 was followed for chemometric model training.

Combined spectrometric data (Figure 2) obtained from two different production lots of authentic C. *verum* (n=59) and *C. cassia* (n=60), and mixtures of the two species at portions representing 50:50% (n=63) and 10:90% (n=74) were used to train the chemometric model.

All chemometric models were calculated using the region of 100 to 300 *m/z*, as no significant features were observed above 300 *m/z*. For model training purposes, 10 PCA components and two LDA components were used. Class related clustering was apparent within the three-dimensional (3D) PCA scores plot using components 1, 2, and 3 (Figure. 3A). The combination of PCA (for data dimension reduction) and the supervised LDA generated four discrete class groupings within a five standard deviation outlier threshold are shown in Figure 3B.

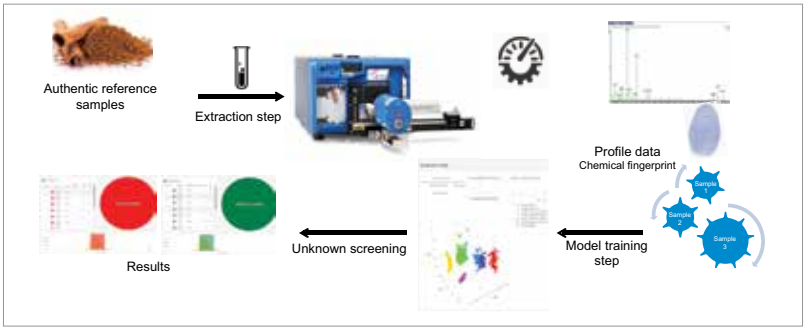


Figure 1. A schematic representation of the DART QDa LiveID workflow for chemometric modeling and real time recognition.

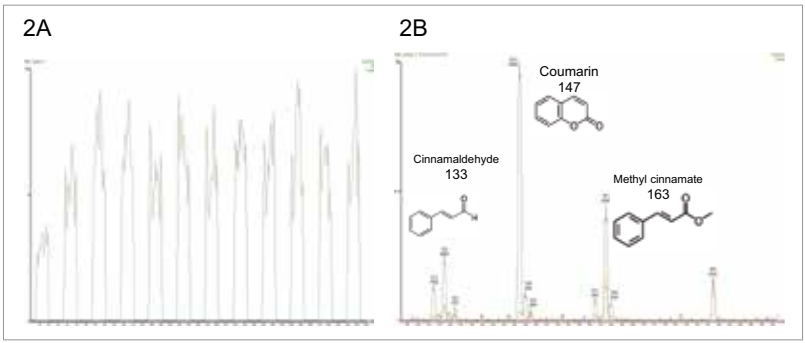


Figure 2. A. DART QDa Total Ion Chromatogram (TIC) generated from a QuickStrip card showing 12 replicate measurements; B. overlay of combined mass spectra from one Region of Interest (ROI) obtained from C. verum (red trace) and C. cassia (green trace) species in positive polarity.

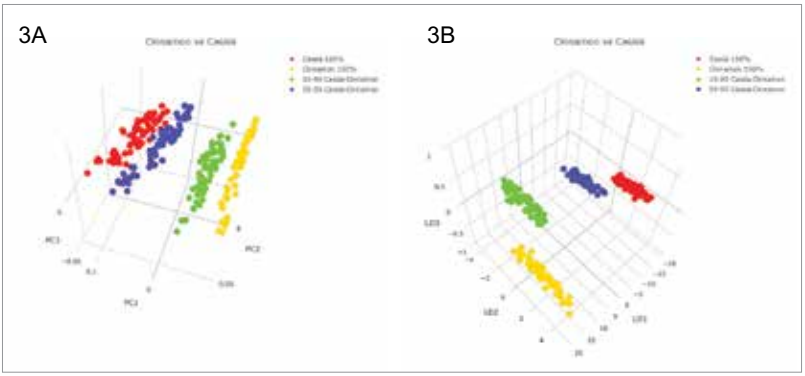


Figure 3. A. PCA and B. PCA/LDA scores plots generated in LiveID for the DART QDa cinnamon authenticity model.

The loadings plot (Figure 4) shows the significant ions contributing to the class level discrimination. The ion at 147 *m/z* (coumarin) is seen to be the major feature in PC 1 accounting for c. 84% of the variance. The ions at 133 (cinnamaldehyde), 163 (methyl cinnamate), 177 (cinnamyl acetate), and 183 *m/z* (unknown) are contributory features in PC 2 accounting for c. 6% of the variance.

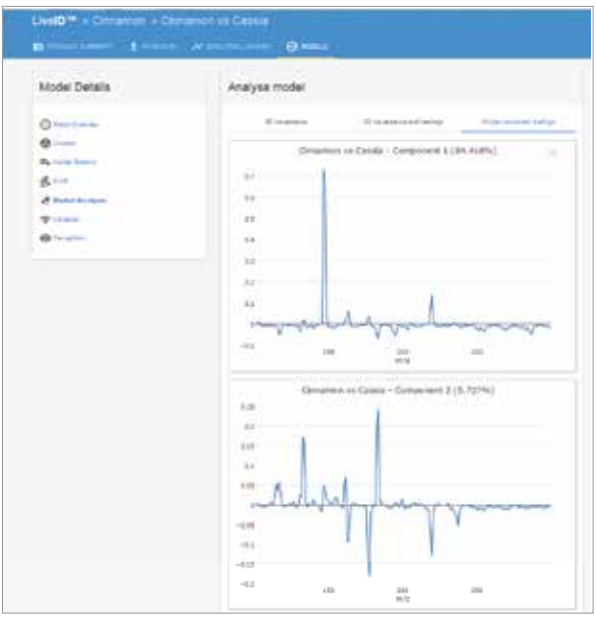


Figure 4. Loadings plots generated in LiveID for the DART QDa cinnamon authenticity model.

MODEL VALIDATION AND INTER-LABORATORY REPRODUCIBILITY

The cinnamon authenticity PCA/LDA model was subject to in silico cross validation using the "leave 20% out" method (Figure 5). The validation resulted in a 100% correct classification with no missed classifications and no outliers. The PCA/LDA model was also validated according to the "leave one file out" method, whereby each of the training data files was systematically excluded from the model and classified as an independent sample – which also resulted in a 100% correct classification rate.

An inter-laboratory validation experiment was performed whereby new extracts of the authentic *C. verum* and *C. cassia* samples used for model training were prepared and analyzed in a second laboratory (Queen's University Belfast) using a different DART QDa LiveID System. The mass spectral data (n=12 per sample) obtained was then classified using the LiveID model in playback recognition mode and the results were recorded (Tables 1A and 1B). All 12 replicate measurements of *C. verum* sample were accurately classified with high confidence scores, and 10 out of the 12 replicate measurements of *C. cassia* sample were accurately classified with two replicates being reported as "outliers".

As a further test of model robustness, a sample of ground spice labelled as Cinnamon, with ingredients declared as *C. cassia*, was purchased from a local retail outlet and analyzed using the described cinnamon authenticity method. The extract was analyzed using LiveID in real-time recognition mode. QuickStrip position 1 was used as a reagent blank and the results from 11 replicate measurements, shown in Figure 6, classified the retail sample as 100% *C. cassia* with a high confidence score.

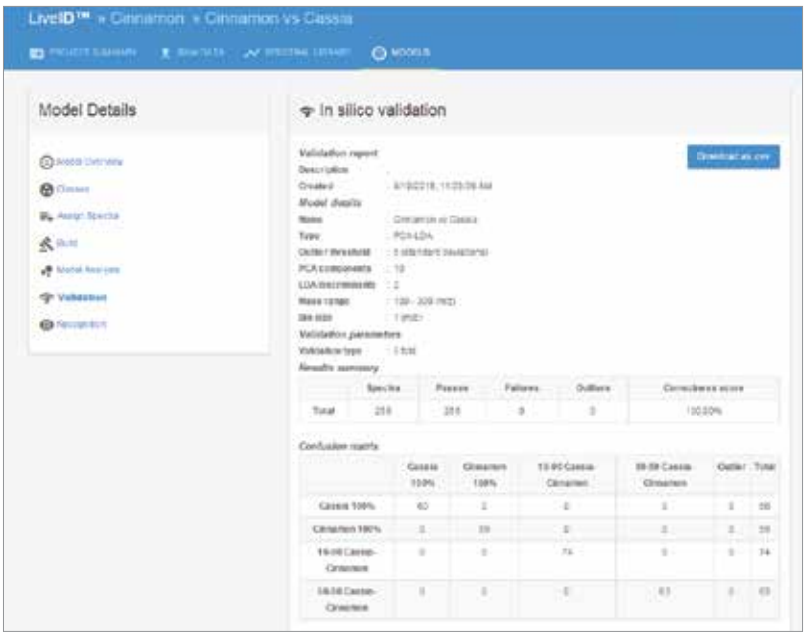


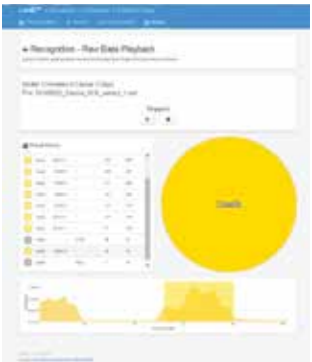
Figure 5. Cross validation (leave 20% out method) results for the DART QDa cinnamon authenticity model created from 256 spectra obtained from two different Cinnamomum species and prepared mixtures of the two species.

Table 1A. Inter-laboratory reproducibility showing the LiveID playback classification results for an authentic sample of 100% C. verum (n=12).

Region of interest	Classification result	% Confidence score	Scan
1	Cinnamon	100	7–18
2	Cinnamon	100	36–47
3	Cinnamon	100	66–78
4	Cinnamon	100	97–109
5	Cinnamon	100	126–139
6	Cinnamon	100	157–170
7	Cinnamon	100	187–200
8	Cinnamon	100	218–230
9	Cinnamon	100	248–261
10	Cinnamon	100	279–291
11	Cinnamon	100	309–322
12	Cinnamon	100	339–352

Table 1B. Inter-laboratory reproducibility showing the LiveID playback classification results for an authentic sample of 100% C. cassia (n=12).

Region of interest	Classification result	% Confidence score	Scan
1	Outlier	36.9	7–18
2	Cassia	100	36–49
3	Outlier	37.9	66–79
4	Cassia	99.4	97–109
5	Cassia	99.4	128–139
6	Cassia	100	157–170
7	Cassia	100	187–200
8	Cassia	100	217–230
9	Cassia	100	248–261
10	Cassia	99.6	279–292
11	Cassia	99.8	309–322
12	Cassia	99.7	339–352



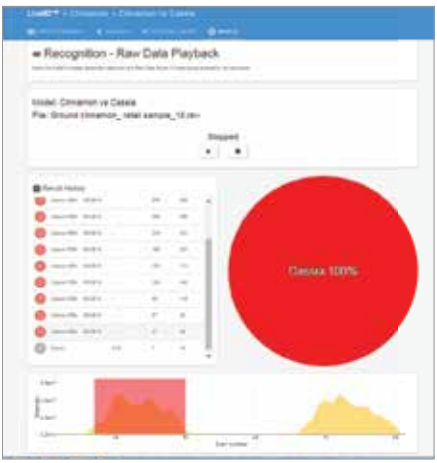


Figure 6. LiveID in real time recognition results recorded for the retail sample of ground cinnamon.

CONCLUSIONS

- DART QDa detected the key discriminatory ions present in crude extracts of ground powders of the Cinnamomum species (*C. verum* and *C. cassia*) used within this study.
- Using the full scan mass spectral data obtained from DART QDa, a chemometric model has been built using LiveID Software. The predictive accuracy of the model has been determined as 100% based on "leave 20% out" validation using the training set data.
- The LiveID model is able to detect adulteration via substitution or dilution of true cinnamon with *C. cassia* to levels of circa 10% as demonstrated in mixtures.
- The results of the inter-laboratory reproducibility study show that it is possible to generate data in a different laboratory location using a different instrumentation and obtain the correct classification from the model.
- DART QDa System with LiveID is fit-for-purpose as a rapid profiling technique and is capable of providing results for 12 samples within 3 minutes via the QuickStrip or Dip-IT introduction modes.
- It is also expected that this method can be optimized for applicability to other dried spice and herb commodities and used for authenticity, composition, and food QC testing purposes.

Acknowledgements

The authors kindly acknowledge John Hill, British Spice and Pepper Board, for the provision of the authentic *Cinnamomum* species samples.

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A Real-Time Lipidomics Approach for Detecting Fish Fraud Using Rapid Evaporative Ionization Mass Spectrometry and LiveID Software

Sara Stead,[1] Nathaniel Martin,[1] Connor Black,[2] Olivier Chevallier,[2] and Chris Elliott[2]
[1] Waters Corporation, Wilmslow, UK
[2]Institute for Global Food Security, Advanced ASSET Centre, Queen's University Belfast, Belfast, Northern Ireland, UK

APPLICATION BENEFITS

- Real-time species-level identification of genetically similar fish species without the need for sample preparation or chromatographic separation.
- Applicability for point of control qualitative testing with minimal sample manipulation.
- Develop and validate robust models for various food authenticity, integrity, and quality control challenges.
- Intuitive software accessible to non-expert users to develop and validate robust models for various food authenticity, integrity, and quality control challenges.

WATERS SOLUTIONS

Rapid Evaporative Ionization Mass Spectrometry (REIMS) with iKnife Sampling Device

Xevo™ G2-XS QTof

Progenesis™ QI Software

LiveID™ Sample Recognition Software

KEYWORDS

Lipidomics, foodomics, adulteration, food fraud, food authenticity, fish speciation, REIMS

INTRODUCTION

Economically motivated adulteration (EMA) of seafood products is a global issue occurring at alarmingly high rates, and it is estimated that on average 30% of commercial fish products sold are either misrepresented or mislabeled.[1] This equates to fraud of almost $120B within the global seafood industry, as the Food and Agriculture Organization of the United Nations (FAO) estimate the global seafood industry to be worth $400B annually, with global industry analysts expecting this value to rise to $430B by 2018.[2]

Genomics, proteomics, metabolomics and lipidomics are four alternative and in some cases complimentary systems biology approaches often employed for food fraud detection studies.[3] The majority of fish fraud detection studies utilize genomic profiling as DNA is found in all cells and organisms and can be analyzed in all types of tissue ranging from freshly caught fish to processed and cooked samples.[4] While very accurate qualitative and quantitative results are achievable using polymerase chain reaction (PCR), it comes at the expense of long and often complex sample preparation coupled with long assay running times which sometimes extend to more than a working day. In terms of managing fraud in fast moving supply chains, this is a substantial disadvantage.

Rapid Evaporative Ionization Mass Spectrometry (REIMS) is a form of ambient ionization mass spectrometry that, as is the case with many analytical innovations, was created for medical research purposes. It operates using an electrosurgical knife or bipolar forceps which create an aerosol (smoke) when cutting into a tissue sample. The aerosol is evacuated from the sample through a transfer line into the ionization source of a mass spectrometer where a heated collision surface is situated and the ionization process occurs. Although the majority of publications utilizing REIMS have centered on medical and bacterial identification applications,[5,6] there are early indications that it may also find applications in the detection of food fraud.[7] Results are obtained nearly instantaneously (2–3 seconds) and the technique can achieve results for solid samples without the need for any form of sample preparation.

In this application note, we demonstrate the use of REIMS with chemometric modeling performed in real time with LiveID Software to accurately determine the species level identification of five commercially

popular, visually and genetically similar white sea fish species: *Gadus morhua* (cod), *Pollachius virens* (coley), *Melanogrammus aeglefinus* (haddock), *Pollachius pollachius* (pollock), and *Merlangius merlangus* (whiting). Unlike most other analytical systems currently employed for species level identification in food, Waters™ REIMS Research System with iknife sampling device and LiveID has the capability to determine results in real time. This combination of mass spectrometric data and chemometric modeling is extremely beneficial to the food industry for the rapid identification of fish fraud including species level identification, capture method, geographical origin, and the potential for point-of-control testing.

EXPERIMENTAL

Sampling conditions

Sampling device:	iKnife (monopolar electrosurgical knife)
Diathermy generator:	Erbe VIO 50 C
Diathermy mode:	Autocut
Power setting:	30 W

The REIMS source was connected to a monopolar electrosurgical knife (Model PS01-63H, Hangzhou Medstar Technology Co, Ltd, Jiaxing City, China) through a 3 m long, 1 cm diameter ultra-flexible tubing (evacuation/vent line).

MS conditions

MS system:	Xevo G2-XS QTof, sensitivity mode
Source:	REIMS
Acquisition mode:	TOF MS
Ionization mode:	ESI-
Mass range:	200 to 1200 *m/z* continuum
Scan speed:	0.5 s/scan
Cone voltage:	30 V
Heater bias:	40 V

Instrument calibration and accurate mass correction

Prior to analysis, the Xevo G2-XS QTof Mass Spectrometer was calibrated using a 5 mM sodium formate solution (in 90% IPA) at a flow rate of 0.2 mL/min for 2 min. A lock mass solution of Leucine Enkephalin (Leu Enk) (*m/z* 554.2615) (2 ng/µL) in isopropanol (IPA) was infused using at a continuous flow rate of 0.1 mL/min to be used as a lock mass for accurate mass correction.

Model training samples

The model was trained using five commercially popular white fish species. All tissue samples (fillets, tails, and unspecified areas) of cod, coley, haddock, pollock, and whiting were sourced from trusted suppliers and stored at -80 °C. Prior to REIMS analysis, the samples were thawed at room temperature for 2 hours in the fume hood where the REIMS sampling took place.

iKnife sampling

Electrosurgical dissection in all experiments was performed using an Erbe VIO 50C generator (Erbe Medical UK Ltd, Leeds, UK). The generator was operated in Autocut mode with a power setting of 30 W. All samples were cut on the return electrode plate and a venturi gas jet pump driven by nitrogen (1 bar) evacuated the aerosol produced at the sample site towards a heated kanthal coil that was operated at 6.4 W (2.8 A at 2.3 V).

Depending on the size, each tissue sample was sampled between 8 and 12 times for repeatability with each cut lasting approximately 3 to 5 s. This enabled multiple locations on each tissue sample to be analyzed. The delay between sampling and appearance of a signal was approximately 2 s, with no carryover effects visible between each burn and/or sample.

LiveID chemometric modelling software

Multivariate statistical software package LiveID (v.1.1) was used as a model builder and recognition tool. To generate models from the untargeted profiling REIMS ToF MS data acquired in MassLynx™ MS Software (v.4.1) the following data pre-treatment steps were performed: lock mass correction applied using the Leu Enk ion at *m/z* 554.2615; all spectra contained within each "burn event" termed the region of interest (ROI) were combined to form a single continuum spectrum; Adaptive Background Subtraction (ABS) algorithm was applied to reduce the chemical background in the combined spectra; data resampling (binning to 0.5 Da) was performed to reduce the data dimensionality; the resulting spectrum was normalized using the Total Ion Chromatogram (TIC). All chemometric models were calculated using the mass region of 600–950 *m/z*. The peak detection threshold was automatically set within LiveID from file to file based on the minimum spectral intensity value plus 10% of the difference between the maximum and minimum intensities.

T = Intensity$_{Min}$ + 0.1*(Intensity $_{Max}$ – Intensity $_{Min}$)
Following data pre-treatment steps, a Principal Component Analysis (PCA)/Linear Discriminate Analysis (LDA) model was generated. First, an unsupervised PCA (Singular Value Decomposition algorithm) transform was applied to the spectral data calculating the scores and loadings plots; a supervised LDA transform was then applied to the scores calculated by the PCA transform. LDA is a transform that maximizes the inter-class variance, while minimizing the intra-class variance, resulting in a projection where examples from the same class are projected close to each other and, at the same time, the class centers (means) are as far apart as possible. Although it is not a true regularization technique, PCA-LDA is found to reduce the chance of over-fitting that may occur with a pure LDA model.

During the recognition step, the model transformed spectra acquired from test samples with an unknown classification into the associated model-space, after which, a classifier determined into which class (if any) the spectra belonged. The model classifier uses a multivariate normal distribution (MVN) for each model class. During the model building phase, these distributions are constructed by transforming the training spectra to generate scores for the n principal components/linear discriminants selected for the model.

The number of dimensions in the MVNs is also equal to n. The MVNs produced a likelihood measure for each class, and Bayes' rule was then applied to derive posterior probabilities.

In silico 5-fold stratified validation was performed to determine the predictive accuracy of the fish speciation model. The model building dataset was divided in five partitions (5-fold), each of which contains a representative proportion of each class within it (stratified). Four partitions (80%) of the dataset were used to build a model under the same conditions as the original model. This model was used to predict the classifications of the one partition (20%) of the training set that was left out. The cycle was repeated iteratively five times and each partition was predicted once by a model trained from the other four. The output of the validation details the total number of correct and incorrect classifications, as well as the number of outliers. Outliers were calculated according to the Mahalanobis distance[8] to the nearest class center. If this distance was greater than the outlier threshold, the sample was considered an outlier.

Additional and complementary statistical analyses were performed using Progenesis QI (NonLinear Dynamics, Newcastle, UK), EZInfo, and SIMCA-P (Umetrics Sartorius Stedim Biotech, Sweden) to determine the chemical identifications of candidate biomarkers and potential involvement of discrete biochemical pathways.

RESULTS AND DISCUSSION

REIMS FISH SPECIATION MODEL

Raw spectrometric data (Figure 1) obtained from authenticated samples of cod (n= 194), coley (n=51), haddock (n=133), pollock (n=50), and whiting (n=50) giving a total of 478 samples were pre-processed and subjected to multivariate analysis where PCA followed by supervised LDA were applied using LiveID.

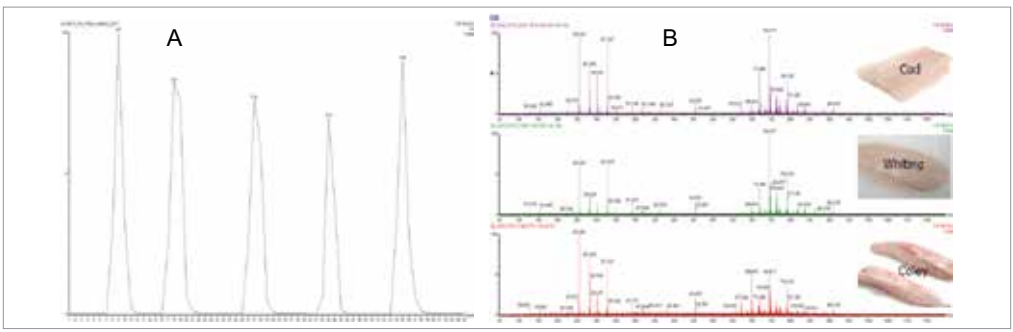

Figure 1. REIMS Total Ion Chromatogram (A) for replicate measurements of cod muscle tissue and combined mass spectral data (6 scans) (B) obtained from three different species of fish, cod, whiting and coley in negative polarity between m/z 50–1200.

80 PCA components and 4 LDA components were used to generate the chemometric models. Clustering was apparent within the three-dimensional (3-D) PCA scores plot using components 1, 2, and 3 which explained approximately 78% of the variance (Figure 2a). However, clear separation between the five species of fish was obtained within the 3-D PCA/LDA score plot using components 1, 2, and 3 (Figure 2b).

Figure 2. PCA (A) and the PCA/LDA (B) scores plots generated in LiveID for the REIMS multi-species fish classification model created from a training set of 478 biological replicates with 8–12 measurements per sample.

MODEL VALIDATION AND REAL-TIME RECOGNITION

The multi-species fish classification PCA/LDA model was subject to *in silico* cross validation using the "leave 20% out" method (Figure 3). The validation resulted in a 99.9% correct classification with no misclassifications, with only one cod and two coley samples classified as outliers. The PCA/LDA model was also validated according to the "leave one file out" method whereby each of the training data files was systematically excluded from the model and classified as an independent sample; in this case a 98.9% correct classification with no misclassifications was achieved. A higher number of outliers were observed (data not shown).

An independent validation was carried out to ensure the validity of the results from the *in silico* validation. The raw data acquired from a set of validation samples was subjected to a cross validation similar to that of the leave 20% out *in silico* validation. The model was created using a reduced training set of samples (n=379) excluding 99 samples assigned as the validation set. Each validation sample was then assigned a fish species classification. An overall correct classification rate of 98.9% was obtained in perfect agreement to the classification rate obtained using the LiveID cross-validation tool.

Figure 3. Cross validation (leave 20% out method) results for the REIMS fish model created from 2795 spectra obtained from 478 biological samples (8–12 replicate measurements) of authentic fish. An overall correctness score of 99.89% was obtained with only 1 replicate of cod and 2 replicates of coley classifying as outliers.

Following a successful build and validation, the PCA/LDA white fish model was used for real-time identification of fish samples. Raw data files were acquired and run live though the software providing a nearly instantaneous identification (Figure 4), excluding the delay between sampling appearance of a signal of approximately 2 s. A standard deviation of 5σ was used for class assignment. The spectral intensity limit was set at 1e8 counts thus ensuring that only the cuts were assigned a species classification and not any background noise. In all cases, the sample was correctly identified.

INTER-LABORATORY VARIABILITY

As a test of the inter-laboratory repeatability of the model, a subset of 68 of the training samples (representing approximately 14% of the total population) were sent to a second laboratory facility and analyzed using a different REIMS instrument. The second site's data was classified using the training set data generated at the primary site and resulted in a 95.6% correct classification rate which was due to three haddock samples being misclassified as cod.

REAL-TIME RECOGNITION OF SEABASS AND SEABREAM SAMPLES

To determine classification fidelity, raw spectrometric data obtained from authenticated samples of fish species not represented in the model [seabass *Dicentrarchus labrax* (n=6) and seabream *Sparas aurata* (n=8)] were run through the LiveID playback recognizer feature to obtain classification results. Of the 14 samples analyzed, 13 (92.8%) were correctly recognized as "outliers" with one sample being classified as both an outlier (with 66% predictive certainty) and coley (34% predictive certainty) within the multiple burn regions.

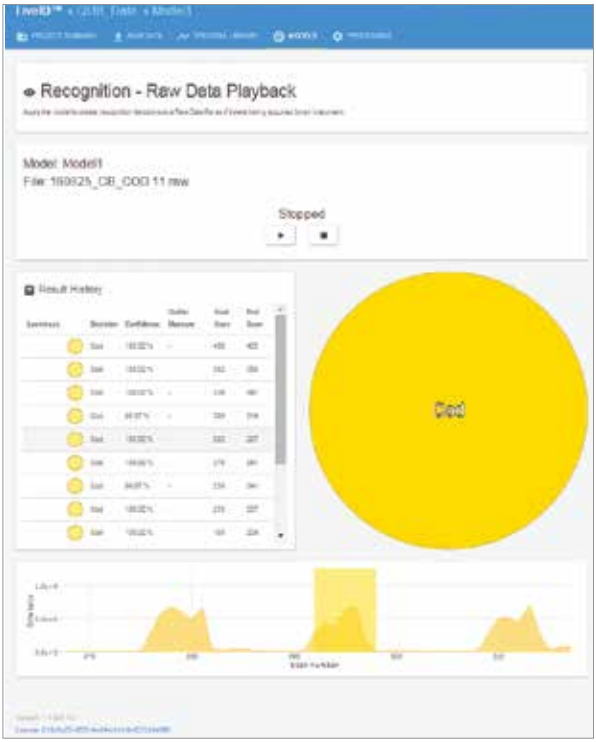

Figure 4. LiveID real-time recognition results (n=3 measurements) following challenge of the PCA/LDA model with an independent validation sample of cod.

PROGENESIS QI FOR BIOMARKER DISCOVERY

MS data files were processed through MassLynx Software's Sample List using the Progenesis Bridge application to convert the files that contained multiple sampling events (burn regions) into an individual file per burn region in the format of a Gaussian peak. Lock mass correction and ABS were also performed during this step. The pre-processed data files were subsequently imported into Progenesis QI Software (v.2.4) and a direct analysis workflow was followed to generate multivariate statistical models and feature abundance plots (Figure 5A).

EZInfo (v.3.0.0.0) was used to create a series of OPLS-DA S-plots to determine the significant ions responsible for species level separation in the PCA/LDA model. Ions present at the upper and lower extremity regions of the S-plots (highlighted in the red boxes Figure 5C) were deemed to be the significant ions involved in species classification and were selected for database searching within Progenesis QI using ChemSpider and LipidMaps databases (Figure 5B). Subsequent REIMS MS/MS experiments were performed whereby the precursor ion was isolated in the quadrupole region of the Xevo G2-XS QTof and a collision energy of 25 eV was applied to yield fragmentation spectra to assist with the chemical elucidation and tentative identification process (Figure 5D). Interpretation of the spectra revealed that members of the diacylglycerophosphoethanolamine (PE), phosphatidylinositol (PI), sphingomyelin (SM), and free fatty acid classes had a significant involvement in the differentiation of fish species.

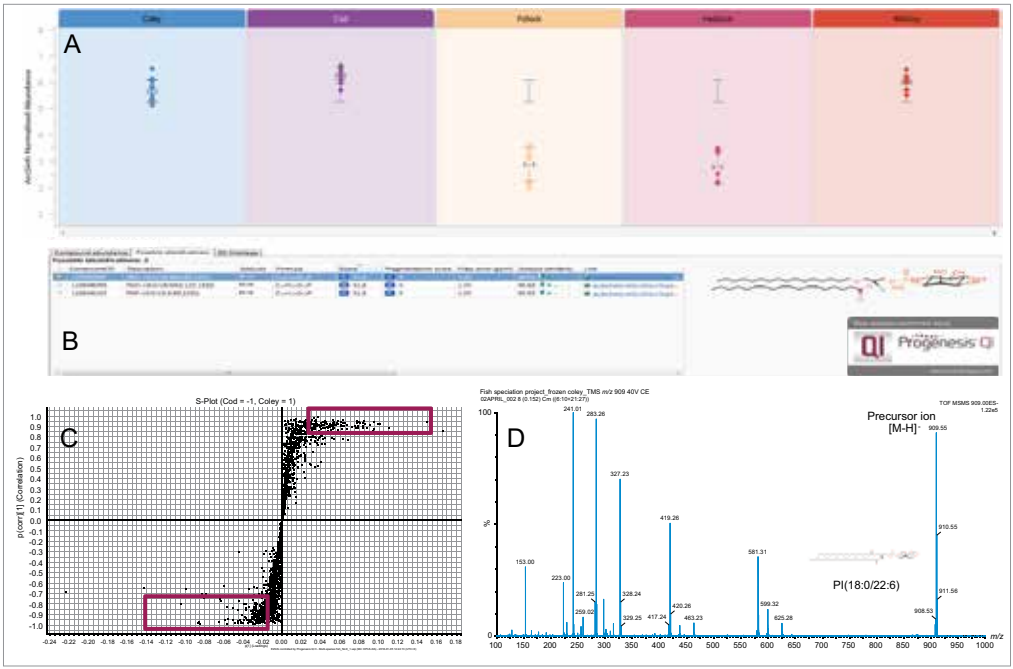

Figure 5. Relative abundance of the feature at m/z 909.5 across the five fish species (A), possible identifications following database searching against LipidMaps (B) OPLS-DA S-plot model for cod and coley species (C), and REIMS MS/MS fragmentation spectra obtained for m/z 909.5 (D).

CONCLUSIONS

REIMS with LiveID Technology requires no sample preparation and provides accurate and nearly instantaneous results. The reduced amount of time required for analysis and data interpretation that REIMS offers in contrast to the current PCR-based methods represents a significant improvement in operational efficiency. REIMS with LiveID has been demonstrated as a complementary technique for the detection of commercial fish fraud.

Along with speciation, REIMS is able to detect multiple aspects of fish fraud e.g. the separation of line and trawl caught haddock samples. By employing this technique, we may also be able to differentiate other aspects such as geographic origin and wild fish versus farmed fish; areas where genomic profiling alone would not be useful.

For further details on this study please refer to the journal article:

Connor Black, Olivier P, Chevallier, Simon A. Haughey, Julia Balog, Sara Stead, Steven D. Pringle, Maria V. Riina, Francesca Martucci, Pier L. Acutis, Mike Morris, Dimitrios S. Nikolopoulos, Zoltan Takats, Christopher T. Elliott. A real time metabolomic profiling approach to detecting fish fraud using rapid evaporative ionisation mass spectrometry. *Metabolomics* 2017, DOI 10.1007/s11306-017-1291-y.

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Automated 2-Dimensional Fingerprint Analysis for Routine Botanical Authentication Using the ACQUITY QDa Mass Detector

Jinchuan Yang and Paul Rainville
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Streamlined Empower™ Chromatography Data Software (CDS) processing is suitable for routine analysis
- Simple and affordable MS detection for routine analyses
- Highly selective and distinctive MS data is suitable for botanical authentication

WATERS SOLUTIONS

Empower 3 CDS Software

ACQUITY™ UPLC™ H-Class System

ACQUITY QDa™ Mass Detector

ACQUITY UPLC BEH C_{18} Column

KEYWORDS

Authenticity, LC-MS, botanical, dietary supplement, herbal medicine, black cohosh, MS library match, fingerprint analysis

INTRODUCTION

Botanical ingredients are widely used in dietary supplements, herbal medicines, cosmetics and personal care products. Potential contamination or misidentification of plants due to the lack of standardization of production has been a health concern to consumers. Ascertaining the authenticity of botanical ingredients and processed products is a challenging task due to their complex phytochemical constituents, the natural variation in their phytochemical profiles, and the similar phytochemical profiles of closely related species. Liquid chromatography (LC) coupled with mass spectrometry (MS) is one of the most effective analytical techniques[1-7] for authenticity evaluation. However, LC-MS has not been widely used in analytical labs for routine authenticity testing due to the relatively high cost of mass spectrometers and the high level of expertise needed. Recently, we have demonstrated the feasibility of using Waters™ ACQUITY UPLC H-Class System with the ACQUITY QDa Mass Detector for botanical authenticity testing.[8] The distinctive MS ion chromatogram and the specific mass spectrum for a marker compound were found to be very effective in differentiating North American (NA) black cohosh *(Actaea racemosa)* from other *Actaea* species.

Chemometric analysis techniques, such as Principle Component Analysis (PCA), Similarity Analysis, and Clustering Analysis are powerful data processing tools for the authentication and classification of botanicals.[9] However, data processing for these techniques is often sophisticated and time-consuming. In this application note, a simple 2-dimensional fingerprint analysis method is presented for NA black cohosh authenticity testing. The data processing was automatically handled by Empower CDS Software, which is convenient for routine analysis. The details of this 2-dimensional fingerprint analysis using Empower is described in this note.

EXPERIMENTAL

Samples

Three authentic NA black cohosh extracts (NA1-NA3), three Asian black cohosh (Actaea cimicifuga) extracts (A1-A3), and four commercial black cohosh samples (U1-U4) were provided by a collaborator. These extracts were diluted with 70% methanol to about 5 mg/mL. Four standards: cimifugin, cimiracemoside C (cimigenol-3-alpha-L-arabinoside), 27-deoxyactein (23-epi-26-deoxyactein), and actein were purchased from ChromaDex (Irvine, CA). These standards were prepared in 70% methanol at about 5 µg/mL. The standards' structures, CAS Registry numbers, and monoisotopic masses are shown in Figure 1. Home-made black cohosh samples: M-5 and M-10, were prepared by mixing NA black cohosh sample (NA1) and Asian black cohosh (A1) at 95:5 and 90:10 mass ratios, respectively. Sample solutions were filtered by 0.2 µm PTFE membrane prior to the analysis.

UPLC conditions

UPLC system:	ACQUITY UPLC H-Class
Column:	ACQUITY BEH C_{18}, 130 Å, 1.7 µm, 2.1 mm × 100 mm, (p/n: 186002352)
Column temp.:	50 °C
Eluent A:	De-ionized water with 0.1% formic acid
Eluent B:	Acetonitrile/methanol (v/v 7/3) with 0.1% formic acid
Injection volume:	10 µL
Run time:	9 min
Reconditioning:	2.5 min

Table 1. UPLC elution gradient.

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.40	80	20	Initial
0.5	0.40	80	20	6
4.0	0.40	35	65	6
6.0	0.40	0	100	6
9.0	0.40	0	100	6
9.1	0.40	80	20	6

MS conditions

MS system:	ACQUITY QDa (with Diverter Valve)
Software:	Empower 3
Detection:	ESI+, MS scan
Scan:	200 to 1000 Da
Capillary voltage:	1.5 kV
Cone voltage:	10 V
Probe temp.:	300 °C
Sampling rate:	5 Hz
Diverter valve events:	Switch on/Flow to QDa at 0.8 min; Switch off/Flow to waste at 9 min

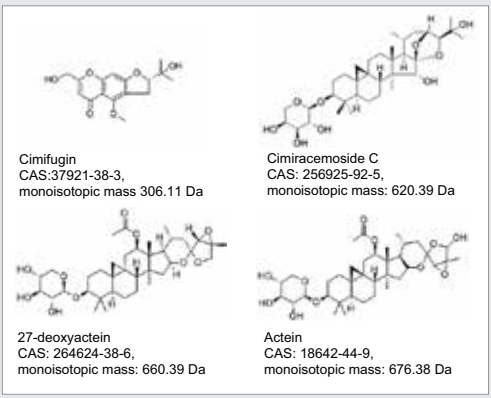

Figure 1. Structures, CAS Registry Numbers, and the monoisotopic masses of the standards.

RESULTS AND DISCUSSION

QUANTITATIVE PARAMETERS FOR AUTHENTICATION

In the black cohosh authenticity study,[8] cimiracemoside C was selected as the marker for the authentic black cohosh. The extracted ion chromatograms (XICs) at the marker's molecular ion mass-to-charge ratio (*m/z* 621 Da) from the NA black cohosh samples shared a simple and consistent pattern that was significantly different from the XICs from the Asian black cohosh samples (Figure 2). In addition, the mass spectra extracted at the marker's peak retention time (RT) 5.77 min showed a characteristic pattern, as shown in Figure 3. These patterns, or fingerprints, from two orthogonal dimensions provided the basis for this NA black cohosh authentication method.

Chromatographic pattern

The common feature in the XICs of the NA black cohosh was that there were two main peaks of about equal peak height, and the marker's peak was one of them (Figure 2, NA 1-NA 3). We used the marker peak's RT and its peak relative area as the quantitative parameters to characterize this chromatographic pattern. Additional parameters could be used, but these two parameters seemed to be effective enough to differentiate the NA black cohosh from the Asian black cohosh samples.

Mass spectral pattern

The top five abundant ions in the extracted mass spectra from NA black cohosh samples were used to characterize the pattern. These ions include the molecular ion (base peak, *m/z* 621 Da), a fragment ion (*m/z* 603 Da), the sodium adduct ion (*m/z* 643 Da), and the isotopic ions (*m/z* 622, 644 Da). Their *m/z* and relative intensity (relative to the base peak, or the molecular ion) values were used as the quantitative parameters for authentication. It should be noted that these mass spectra were obtained from the NA black cohosh samples, not from the cimiracemoside C standard. Therefore it is necessary to include those adduct and isotopic ions, in addition to the molecular ion and the fragment ion, in order to capture the overall spectral pattern of NA black cohosh samples at the UPLC RT 5.77 min.

MS Library Match

The extracted mass spectra (at RT 5.77 min) were stored in a customized NA black cohosh MS library, and were used for MS library search in the unknown black cohosh sample authenticity testing. One example of the Empower MS Library Match results is shown in Figure 3.

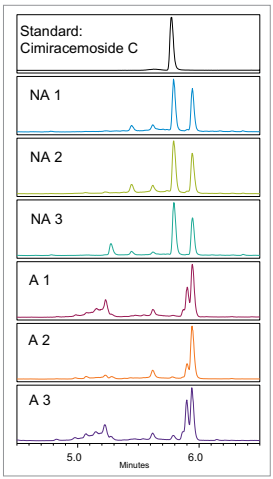

Figure 2. Extracted ion chromatograms of cimiracemoside C (m/z 621 Da) in standards, NA black cohosh (NA 1–NA 3), and Asian black cohosh (A 1–A 3) samples.

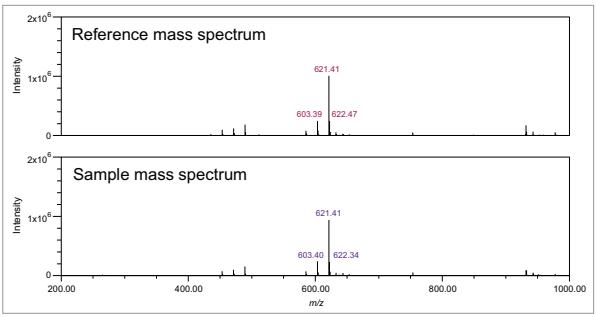

Figure 3. Empower Library Match results. The mass spectrum of the marker (cimiracemoside C) peak in a sample (bottom) matches one of the reference mass spectra in the NA black cohosh MS library (top).

THRESHOLD VALUES FOR AUTOMATED ANALYSIS

To determine the threshold values in those authentication parameters for the NA black cohosh, three NA black cohosh and three Asian black cohosh samples were measured in triplicate by UPLC-MS. These samples were measured in a randomized fashion to avoid artifacts related to injection order. Table 2 shows the statistical averages, the standard deviations (SD), and the threshold values for those authentication parameters. In Table 2, the upper and the lower limits in RT were set at the ±1% of the RT average. For the relative peak area (%Area), the limits were set at 3X the SD from the average. For the expected mass relative intensity, the lower limits were set at 3X the SD below the average. There was no upper limit used for the expected mass relative intensity. These threshold values were mainly chosen at the 3X the SD to cover the potentially wide variation in the NA black cohosh. A data processing Method Set was created in Empower Software to carry out the authentication process. Figure 4 shows the Empower data processing flow chart. Table 3 shows the Empower functions that were used in this method.

Table 2. Characteristics of the chromatographic pattern and the mass spectral pattern for NA black cohosh, and the threshold values in authentication parameters.

	Ion chromatogram pattern		Mass spectral pattern									
Parameters	RT (min)+	%Area	Expected mass 1		Expected mass 2		Expected mass 3		Expected mass 4		Expected mass 5	
			m/z (Da)	Intensity (%)	*m/z* (Da)	Intensity (%)	*m/z* (Da)	Intensity (%)	*m/z* (Da)	Intensity (%)	*m/z* (Da)	Intensity (%)
Average	5.771	50.3	621	97	643	75	622	34	644	23	603	18
SD	0.005	3.3		6		22		5		5		3
Upper limit++	5.829	60.0		–		–		–		–		–
Lower limit	5.713	40.0		79		9		17		7		8

+: ±1% of the RT is used for the upper and lower limits.
++: Upper limits for mass spectral pattern are not used.

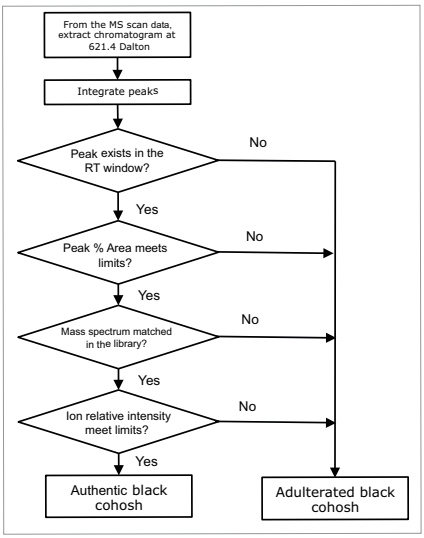

Figure 4. Authenticity data process protocol for black cohosh using a single marker's chromatographic pattern and its mass spectral pattern.

Table 3. The NA black cohosh authentication criteria and the related Empower functions and fields used in the Empower data processing Method Set.

Authentication criteria	Empower functions and fields
	▪ Derived channel for XIC at 621.4 Dalton; ▪ XIC peak integration
▪ Peak exist in the RT window? ▪ Peak %Area meets the limits?	▪ Peak found in the RT window that specified in the Component Table; ▪ %Area within the component suitability limits.
▪ Mass spectrum matched in the MS library? ▪ Ion relative intensity meet limits?	▪ Spectrum found in the Empower MS Library search; ▪ MS Expected Masses found, and the Expected Intensities are higher than the limits

+: ±1% of the RT is used for the upper and lower limits.
++: Upper limits for mass spectral pattern are not used.

ANALYSIS OF COMMERCIAL AND HOME-MADE BLACK COHOSH SAMPLES

Four commercial black cohosh samples (U1-U4) and two home-made black cohosh samples (M5 and M10) were tested by this method. Figure 5 shows an Empower report for these samples. Any nonconformity to the authentication criteria was automatically flagged in red by Empower Software. A summary of the test results was discussed in the previous application note.[8] Briefly, the authentic, inauthentic, and contaminated black cohosh samples were all correctly determined.

Sample	Name	RT	% Area	Peak Type	MS Match1 Spect. Name	Expected Mass 1 (Da)	Expected Intensity 1 (%)	Expected Mass 2 (Da)	Expected Intensity 2 (%)	Expected Mass 3 (Da)	Expected Intensity 3 (%)	Expected Mass 4 (Da)	Expected Intensity 4 (%)	Expected Mass 5 (Da)	Expected Intensity 5 (%)
U1	Black Cohosh	5.775	5	Found	BlackCohosh#07	621.4	9	645.5	10						
U2	BlackCohosh	5.770	48	Found	BlackCohosh#07	621.5	100	645.5	50	622.5	30	644.5	18	608.4	20
U3	BlackCohosh	5.771	43	Found	BlackCohosh#06	621.4	100	645.5	68	622.5	38	644.5	26	608.5	21
U4	Black Cohosh	5.766	4	Found	BlackCohosh#06	621.4	49	645.5	28	622.4	15	644.4	10		
M5	Black Cohosh	5.775	48	Found	BlackCohosh#06	621.4	31	645.5	100	622.4	13	644.5	28	608.5	6
M10	Black Cohosh	5.774	59	Found	BlackCohosh#08	621.5	36	645.5	100	622.4	9	644.5	39	608.4	6

Figure 5. Screen shot of an Empower Software authenticity test report for the commercial and home-made samples.

BENEFITS OF THIS NOVEL AUTHENTICATION APPROACH

Chemometric analyses are powerful tools for exploratory authenticity studies of botanicals. In the routine analysis environment, however, they are too sophisticated and cumbersome to be implemented. Here, we have demonstrated a novel authentication approach, in which a marker compound's 2-dimensional fingerprints are used to authenticate NA black cohosh. Because only the marker's chromatographic fingerprint and mass spectral fingerprint are processed, the amount of the data that need to be processed is relatively small, and the data handling is relatively simple. The whole data processing can be automated in Empower Software, which is suitable for laboratories performing routine analysis.

CONCLUSIONS

In this application note, details of an automated 2-dimensional fingerprint analysis for NA black cohosh authenticity are described. The key features, or patterns, in the chromatographic and the mass spectral fingerprints of a marker compound were characterized by a set of quantitative parameters, such as RT, peak relative area, *m/z*, and ion relative intensity. The threshold values of these parameters for NA black cohosh were determined and used in Empower Software's automated data processing. Using this UPLC-MS approach, we were able to differentiate NA black cohosh from Asian black cohosh samples, and detect Asian black cohosh contamination at 5 wt%. It should be noted that due to the limited number of reference or training samples used in method development, this black cohosh authenticity method may need to be further validated.

The key features of this UPLC-MS approach include the use of the ACQUITY QDa Mass Detector, the use of a marker's 2-dimensional fingerprints for authentication, and the automation of the whole data processing by Empower Software. The ACQUITY QDa is affordable, easy to learn, and use. Automated data processing using Empower Software is quick and objective. These features are suitable for routine authenticity testing, where the analyst's time and expertise may be limited. This new UPLC-MS approach could be easily implemented in analytical labs for the routine authentication, and/or quality control of botanical ingredients and finished products in dietary supplements, herbal medicines, cosmetics and personal care products to safeguard product quality and safety.

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Improving Quantitative Analysis of Red Wine Using the Xevo G2-XS QTof with SONAR Data Independent Acquisition (DIA)

Lauren Mullin,[1] Yunpeng Zhen,[2] and Rob Plumb[1]
[1]Waters Corporation, Milford, MA, USA; [2]Waters Corporation, Beijing, China

TECHNOLOGY BENEFITS

- Provides flexible targeted and non-targeted analysis of phenolic compounds of interest in red wine.
- Improved MS/MS spectral clarity afforded by SONAR™ without the need for method development or prior knowledge of the sample.
- Provides data for quantitation and identification in a single injection.

WATERS SOLUTIONS

Xevo™ G2-XS QTof

SONAR

UNIFI™ Scientific Information System

KEYWORDS

Wine, *trans*-resveratrol, *p*-coumaric acid, catechin, phenolic compounds

INTRODUCTION

In recent years, there has been an increased use of non-targeted approaches for the characterization of food and beverages to complement quantitative targeted analyses. Application of these techniques to the analysis of wine have been employed to assess previously unknown contents of wine lees,[1] patterns in glycosylated simple phenols across wine grape hybrid varieties,[2] and metabolic profiling of pest resistant genotypes against their susceptible ancestor vines.[3] In addition to generating quality high resolution mass spectrometry (HRMS) data, it is also important to ensure that the known analytes in these types of experiments can be quantified within relevant concentration ranges.

In this technology note we describe the use of a novel data independent acquisition (DIA) mode known as SONAR[4,5] for generation of highly selective HRMS spectra on Waters™ Xevo G2-XS QTof. Targeted quantitative assessment of this technique is shown for three known phenolic compounds present in red wine: *trans*-resveratrol, catechin, and *p*-coumarin, highlighting the diverse applicability of SONAR acquisition.

DISCUSSION

Eight red wine samples were purchased at a local retailer from various grape varieties were used in this study to assess linearity, LOD/Qs, and spectral clarity to aid in the identification of known compounds. Data was acquired using 10 µL injections and a SONAR window of 30 Da over the quadrupole mass scanning range of 100 to 700 *m/z*. Collision energy was set to 6 eV for ion transmission in the passive state, and a ramp of 20 to 45 eV for high energy. The Tof acquisition mass range was 50 to 1200 Da at an acquisition rate of 0.2 sec. Wine samples were prepared by initial 1:1 dilution with DI water, and centrifuged at 15,000 rpm followed by an addition 1:4 dilution with DI water. Diluted samples were then spiked with ^{13}C-isotopically labeled standards of +/- catechin 2,3,4, $^{13}C_3$, *p*-coumaric acid 1,2,3 $^{13}C_3$, resveratrol-(4-hydroxyphenyl-$^{13}C_6$), as internal standards and then as a matrix matched calibration curve.

Figure 1 shows a comparison of observed spectra for *p*-coumaric acid in its native form and as a labeled standard in a 1:1 wine dilution. Data is filtered using the Spectrum view in UNIFI Software for the SONAR acquired data. A much cleaner spectra was obtained using this mass filter acquisition approach as demonstrated when we compared the SONAR approach to traditional DIA. Data was acquired using the same parameters with the exception of using a non-resolving quadrupole for ion transmission. The spectral clarity afforded by SONAR is what would be expected from an MS/MS experiment without the need for method development or prior knowledge of the sample.

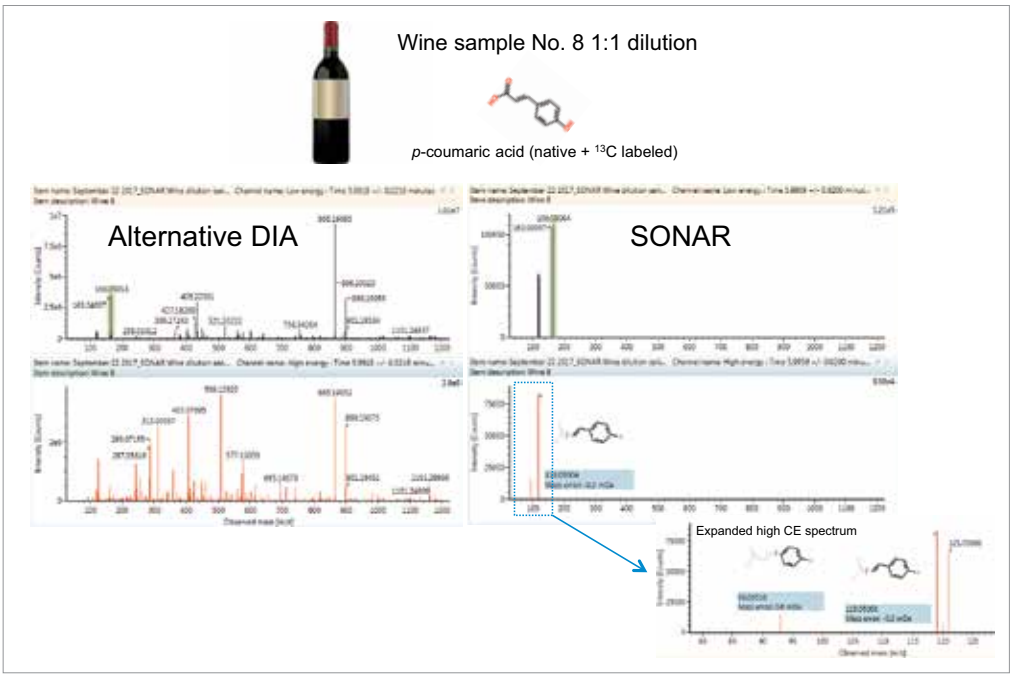


Figure 1. Spectra for p-coumaric acid (both native and labeled) in red wine sample with enhanced selectivity as compared to full-spectral acquisition. Explanded high collision energy (CE) spectrum shows exact mass fragment structures and mass errors.

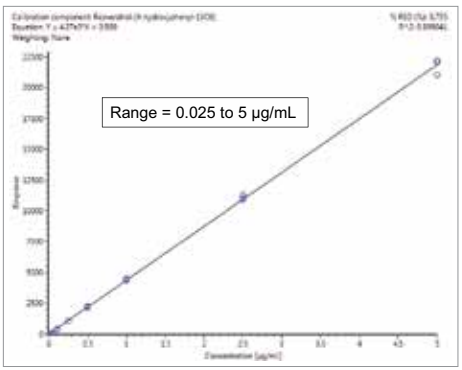
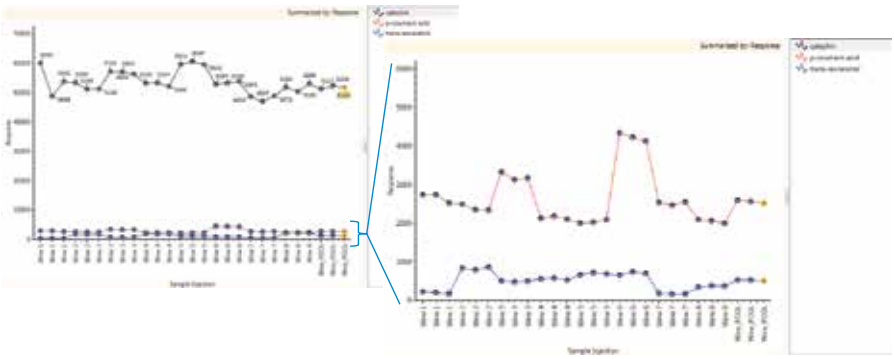


Figure 2. ^{13}C-isotopically labeled resveratrol standard dilution series and linearity in a matrix matched (diluted wine 1:5) calibration curve.

Calibration results for the labeled standards spiked into the wine samples as a matrix matched curve are summarized in Figure 2 for resveratrol-(4-hydroxyphenyl-$^{13}C_6$) and Table 1 for all compounds. Criteria for LOD/Qs were peak-to-peak signal-to-noise (S/N) ratios of 3 and 10 respectively, as well as a +/-5 ppm mass error tolerance.

Chromatographic peak widths were observed to be approximately 7 sec., with at least 12 points across the peak ensuring reliable quantification. Responses of the native forms of *trans*-resveratrol, catechin, and *p*-coumarin are shown in Figure 3. Measured concentrations from the labeled standard curves are shown as an average across the triplicate injections in each sample. As can be seen, the concentrations found in the diluted samples are generally within the calibration range, highlighting the relevance of the established quantification range achieved using SONAR for this application.



Average (n=3) concentrations (µg/mL)										
Analyte		*Wine 1*	*Wine 2*	*Wine 3*	*Wine 4*	*Wine 5*	*Wine 6*	*Wine 7*	*Wine 8*	*Wine pool*
Catechin	In-vial	2.81	2.57	3.00	2.65	3.34	2.68	2.29	2.56	2.56
	Actual	22.48	20.56	24.00	21.20	26.72	21.44	18.32	20.48	20.48
p-coumaric acid	In-vial	0.30	0.27	0.36	0.24	0.23	0.47	0.29	0.24	0.29
	Actual	2.40	2.16	2.88	1.92	1.87	3.76	2.32	1.92	2.32
trans-resveratrol	In-vial	<LOQ	0.11	0.05	0.07	0.09	0.09	<LOQ	0.03	0.06
	Actual	NA	0.88	0.40	0.56	0.72	0.72	NA	0.24	0.48

Figure 3. Responses of native phenolic species in wine samples with quantified concentrations against their isotopically-labeled calibration curves. Catechin responses were very high, across all wine samples, with the responses for p-coumarin and trans-resveratrol shown in the closeup. Concentrations in each wine are shown in the color coded table, with quantification possible for each analyte in every sample with the exception of trans-resveratrol in Wines 1 and 7.

Table 1. Summary of isotopically labeled standard calibration curve data for SONAR 30 Da window acquisition.

Compound	$[M-H]^-$	RT (min)	LOD (µg/mL)	LOQ (µg/mL)	Range (µg/mL)	Fit	R^2
+/- Catechin 2,3,4, $^{13}C_3$	292.0818	4.54	0.01	0.025	0.010–5	Quadratic	0.9997
p-coumaric acid 1,2,3 $^{13}C_3$	166.0501	6.00	0.10	0.200	0.100–5	Quadratic	0.9999
Resveratrol-(4-hydroxyphenyl-$^{13}C_6$)	233.0915	8.83	0.01	0.025	0.025–5	Linear	0.9990

SUMMARY

In addition to providing highly specific and selective spectra from a DIA approach, the SONAR acquisition parameters implemented in this work are suitable for the quantification of targeted phenolic compounds of interest in red wine. Combined, these highlighted benefits express flexibility in high quality assessments of sample composition for both targeted and non-targeted analyses.

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Determination of Vitamin D and Previtamin D in Food Products

Jinchuan Yang, Gareth Cleland, and Kari Organtini
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Determination of both previtamin D and vitamin D.
- More accurate and precise determination of total vitamin D analysis.

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class System

Xevo™ TQ-S micro Triple Quadrupole Mass Spectrometer

ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm Column

MassLynx™ Software v4.1

KEYWORDS

Vitamin D, previtamin D, cholecalciferol, ergocalciferol, D_3, D_2, PTAD, LC-MS

INTRODUCTION

Vitamin D is a fat-soluble vitamin that promotes calcium absorption and maintains adequate serum calcium and phosphate concentrations to enable normal mineralization of bone and to prevent hypocalcemic tetany.[1] The most common vitamin D compounds are vitamin D_3 (also known as cholecalciferol) and vitamin D_2 (ergocalciferol). Their structures are shown in Figure 1. Vitamin D can be produced endogenously when ultraviolet (UV) light strikes the skin and triggers vitamin D synthesis. Recent studies revealed that humans might not produce adequate supplies of vitamin D from exposure to sunlight alone, so it is important to supplement vitamin D intake through diet.[2] The U.S. Food and Drug Administration (FDA) revised the food labeling regulations in 2016 to make the vitamin D content a required item on the nutrition or supplement facts labels for conventional food and dietary supplements.[3] The change in labeling regulation is aimed to promote vitamin D awareness among consumers.

Existing standard methods for vitamin D analysis involve saponification, liquid-liquid extraction (LLE), sample clean-up, and liquid chromatography (LC)-UV determination. The most challenging aspect in vitamin D analysis is the diverse interferences from sample matrix. A large number of lipid-like compounds are co-extracted with the vitamin D, and even after extensive sample clean-up, there are still numerous interferences that co-elute and interfere with the vitamin D quantitation. Recently, to simplify the sample preparation and to improve the analysis, a derivatization reaction with 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) and mass spectrometry (MS) were adopted in a new AOAC standard method.[4] This new method has provided much better analytical performance for vitamin D analysis. However, previtamin D is not measured in this new standard.

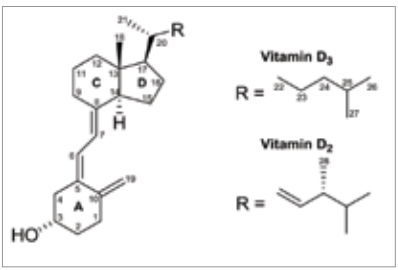

Figure 1. Structures of vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol).

It is known that vitamin D can thermally isomerize to previtamin D. This transformation is reversible (Figure 2), and both forms are biologically active. It has been reported that the relative content of previtamin D could be up to 22% of the total vitamin D at 80 °C.[5] Therefore, it is prudent to individually determine previtamin D and vitamin D contents in the analysis of vitamin D in foods. This application note demonstrates the determination of total vitamin D by individually measuring the vitamin D and previtamin D in food products.

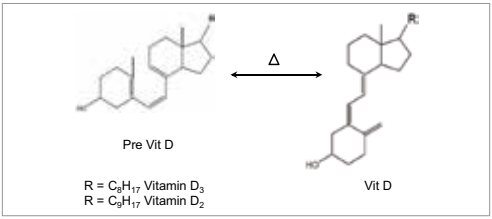

Figure 2. Reversible thermal isomerization of previtamin D to vitamin D. The equilibration constant and equilibration time depends on temperature.[5]

EXPERIMENTAL

Samples

Vitamin D_3 and vitamin D_2 were purchased from Sigma-Aldrich. Stable isotope labeled cholecalciferol (6,19,19-d_3) (SIL-D_3) was purchased from Cambridge Isotope Labs (Tewksbury, MA) and used as the internal standard (IS). Infant formula reference material NIST 1849a was purchased from National Institute of Science and Technology (NIST, Gaithersburg, MD). PTAD, potassium hydroxide (KOH), pyrogallol (or 1,2,3-trihydroxybenzene), butylated hydroxytoluene (BHT), formic acid, and absolute ethanol were purchased from Sigma-Aldrich. Food products, such as non-fat dry milk powder (fortified with A and D), infant formulas (milk based and soy based), oatmeal, and fish oil were purchased from local market. All sample preparation was carried out in subdued light and in amber glass vials.

Standard calibration solutions

The Vitamin D standards (vitamin D_3 and vitamin D_2) were dissolved in absolute ethanol to form 1 mg/mL stock solutions. Portions of these vitamin D stock solutions were used for the purity check using a UV/Vis spectrophotometer. Aliquots of the vitamin D stock solutions were mixed and diluted with acetonitrile (ACN) to form vitamin D mix stock solutions. The vitamin D mix stock solutions were spiked with IS (SIL-D_3), and diluted with ACN to obtain a series of standard calibration solutions ranging from 1 ppb to 500 ppb (or ng/mL). The IS level was kept at constant concentration in these standard solutions. These calibration solutions underwent the derivatization step as described below.

Sample solutions

About 0.5 g (weighted to 0.001 g) of samples were separately spiked with IS (20 ng SIL-D_3), mixed with 4 mL of water and 16 mL of pyrogallol ethanolic solution (2 g/100 mL). 8 mL of KOH (50%) solution was then added and mixed. The solutions turned black after mixing. The solutions were put in a hot water bath (75 °C) for 1 hour with periodical mixing every 30 min. After the solutions were cooled to room temperature in an ice bath, 12 mL of hexanes (with 12.5 mg/L BHT) was added, mixed, and centrifuged. The hexane layer portion was taken and washed with 8 mL water 4 times. A centrifuge (1500 rpm for 2 min) was used to aid the phase separation. These extracts underwent derivatization as described next.

Derivatization

100 µL of each standard calibration solution or 6 mL of the hexanes extract from each sample was dried with a gentle nitrogen stream at 30 °C, then mixed with 0.6 mL PTAD solution (1 mg/mL in ACN). The mixtures were kept at room temperature in the dark for 40 minutes. The derivatization reaction was quenched with 0.4 mL water and the sample was filtered with a 0.2 µm PTEF syringe filter before injection.

UPLC conditions

UPLC: System:	ACQUITY UPLC H-Class
Software:	MassLynx v4.1
Column:	ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm, 1.7 µm
Column temp.:	40 °C
Mobile phases:	A) Water (0.1% formic acid); B) ACN (0.1% formic acid)
Inj Vol.:	10 µL
Flow rate:	0.60 mL/min
Run time:	8.5 min

Table 1. Elution gradient.

	Time (min)	%A	%B	Curve
1	Initial	80	20	initial
2	0.25	80	20	6
3	2.75	0	100	6
4	6.5	0	100	6
5	6.6	80	20	6

MS conditions

MS system:	Xevo TQ-S micro
Polarity:	ESI+
Capillary (kV):	1.2
Source temp.:	150 °C
Desolvation temp.:	500 °C
Cone gas flow (L/Hr):	0
Desolvation gas flow (L/Hr):	1000

Table 2. MRM parameters.

	MRM	Dwell (secs)	Cone volt	Col. energy	Delay (secs)	Compound	Note
1	560.3>161.0	0.032	43	36	Auto	D_3:PTAD	Qualifier
2	560.3>298.1	0.032	43	19	Auto	D_3:PTAD	Quantifier
3	560.3>365.3	0.032	43	21	Auto	preD_3:PTAD	Qualifier
4	560.3>383.3	0.032	43	13	Auto	preD_3:PTAD	Quantifier
5	563.2>301.2	0.032	43	16	Auto	SIL-D_3:PTAD	Quantifier
6	563.2>386.3	0.032	43	11	Auto	preSIL-D_3:PTAD	Quantifier
7	572.3>311.8	0.032	43	15	Auto	D_2:PTAD	Qualifier
8	572.3>377.3	0.032	43	19	Auto	preD_2:PTAD	Qualifier
9	572.3>395.3	0.032	43	9	Auto	preD_2:PTAD	Quantifier
10	572.3>448.2	0.032	43	9	Auto	D_2:PTAD	Quantifier

RESULTS AND DISCUSSION

DETERMINATION OF VITAMIN D AND PREVITAMIN D

Figure 3 shows the typical MRM chromatograms of vitamin D and previtamin D in standard mixtures and in infant formula samples. The Retention times of the vitamin D derivatives (D_3:PTAD, D_2:PTAD, and SIL-D_3:PTAD) and the previtamin D derivatives (preD_3:PTAD, preD_2:PTAD, and SIL-preD_3:PTAD) are 3.50 min and 3.67 min, respectively.

There is no pure standard for previtamin D. In order to quantify the previtamin D, the relative response factors of the vitamin D over the previtamin D were determined in a simple experiment as follows. A solution with certain concentrations of the vitamin D_3, vitamin D_2, and SIL-D_3 was split into two portions. One portion was kept at room temperature, while the other portion was heated and maintained at 75 °C for 1 hour. Because of the isomerization equilibration, the heated portion would have increased previtamin D and decreased vitamin D contents than those in the unheated portion. Since the total vitamin D content in the two portions was the same, the relative response factor of the vitamin D over the previtamin D was calculated by the following equation:

$$\text{Rel. Response Factor} = \frac{\text{VitD Peak Area Unheated} - \text{VitD Peak Area heated}}{\text{PreD Peak Area Heated} - \text{PreD Peak Area Unheated}} \qquad \text{Eq. (1)}$$

The Rel. Response Factors for vitamin D_3, vitamin D_2, and SIL-D_3 were determined each time the samples were analyzed.

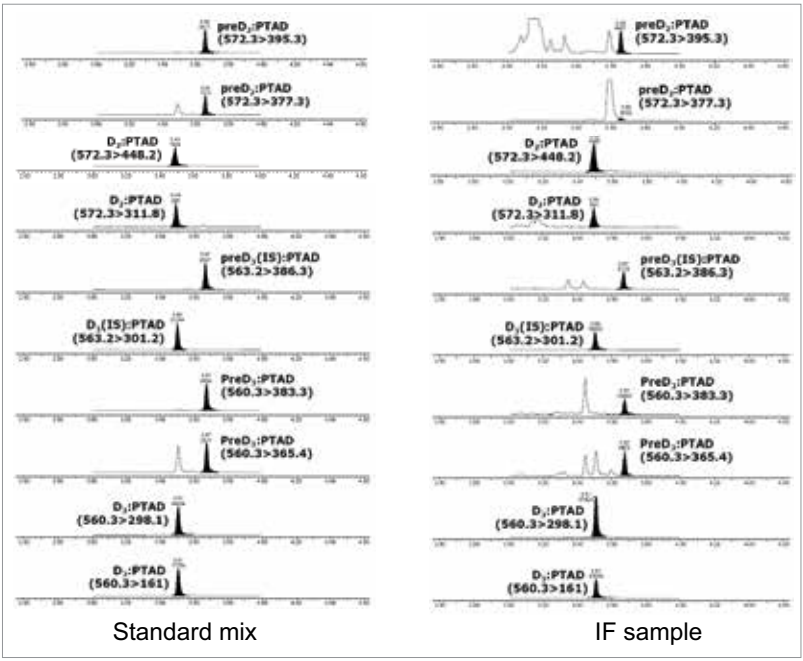

Figure 3. Typical MRM chromatograms of vitamin D and previtamin D in standard mix solutions and infant formula samples. Vitamin Ds (3.50 min) are separated from previtamin Ds (3.67 min).

METHOD OF CALIBRATION AND QUANTITATION IN VITAMIN D ANALYSIS

Total vitamin D is calculated as the sum of the previtamin D and the vitamin D contents. The total vitamin D peak area was calculated according to the following equation:

Total VitD Peak Area = VitD Peak Area + Rel. Response Factor x PreD Peak Area Eq. (2)

In the calibration process, the Total VitD Peak Area ratios of the analyte over the IS were plotted against their total vitamin D concentration ratios (analyte over IS). A linear regression through zero fitted the data points very well. Figure 4 shows a typical calibration plot. The R^2 values of 0.999 and 0.997 were obtained (Fig. 4) for vitamin D_3 and vitamin D_2, respectively.
The calibration ranges were 0.0004 mg/kg to 0.2 mg/kg for vitamin D_3, and 0.002 to 0.2 mg/kg for vitamin D_2. These ranges are comparable to the AOAC standard method.[4]

The total vitamin D (D_3 or D_2) content in samples was calculated using the following equation:

$$\text{Total Vitamin D} = \frac{\text{Total VitD Peak Area x mass of spiked IS in sample}}{\text{Total VitD Peak Area (IS) x Slope in Calibration Curve x mass of sample}} \quad \text{Eq. (3)}$$

where the *Total VitD Peak Area* is calculated according to equation 2. The IS concentration in calibration solutions were kept at 50 ppb (or 50 ng/mL), and the spiked mass of IS in samples were kept at 0.020 µg. The total vitamin D results are in mg/kg unit.

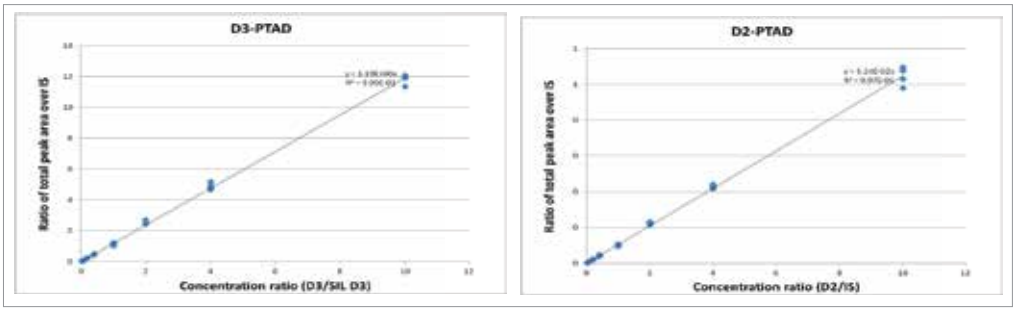

Figure 4. Calibration plots for vitamin D_3 and vitamin D_2.

METHOD PERFORMANCE AND ANALYSIS RESULTS

The limit of detection (LOD) and limit of quantitation (LOQ) were estimated based on the peak area standard deviation (SD) in oatmeal and in solvent at low concentrations near the LOQ (Table 3). The LOD was estimated at 3 times the SD in peak area and the LOQ was estimated at 10 times the SD in peak areas. The LOQ values for vitamin D_3 and vitamin D_2 were estimated at 0.01 mg/kg and 0.02 mg/kg in oatmeal, and 0.0003 mg/kg and 0.002 mg/kg in solvent, respectively. The LOQ values are comparable to the existing standard.[4]

Table 3. The estimated LOD and LOQ in the vitamin D analysis in oatmeal and in solvent.

	Oatmeal		Solvent	
	D_3	D_2	D_3	D_2
LOD (mg/kg)	0.003	0.006	0.0001	0.0007
LOQ (mg/kg)	0.01	0.02	0.0003	0.002

The NIST reference material 1849a was measured and the average value and relative standard deviation (RSD) were compared with the reference values (Table 4). Excellent accuracy (102.6%) and repeatability (RSD 2.4%) was obtained. A spiking experiment was performed on the infant formula (at 0.09 mg/kg) and the oatmeal (at 0.02 and 0.09 mg/kg) and results are shown in Table 5. The recovery of the two spiking levels ranged from 98% to 117%. Besides the infant formula, other types of foodstuff, such as non-fat dry milk powder fortified with vitamin D, soy based infant formula, chocolate fortified with vitamin D, oatmeal, and fish oil were tested. Table 6 shows the results of three replicate measurements of the total vitamin D contents of these food products. The mean and the RSD results for the vitamin D_3 and D_2 are listed in the table. The vitamin D values on nutrition or supplement facts sheet of these foods were converted to numbers in mg/kg and listed in Table 6 for comparison. The determined vitamin D concentrations for milk and oatmeal were in agreement with their label claim for vitamin D values (less than 9% in difference). The result for soy based infant formula was 52% higher than the label value, which is not uncommon for food product testing. The result for the fortified chocolate was high (70% higher than the label value), and the cause is unknown and needs further investigation. The nutrition fact information for the fish oil product was not available for comparison.

Table 4. Vitamin D analysis results for NIST 1849a reference material and comparison to its reference values.

	Measurements			Average			Ref. values	Accuracy
		Mean	SD	Mean	SD	RSD		
Vitamin D_3 (mg/kg)	1 2 3	0.116 0.107 0.118	0.003 0.002 0.003	0.114	0.003	2.4%	0.111 ± 0.017	102.6%

Table 5. Recovery data on infant formula and oatmeal samples.

	Infant formula		Oatmeal	
	D_3	D_2	D_3	D_2
Original (mg/kg) spike level 1 (0.02 mg/kg) spike level 2 (0.09 mg/kg)	0.116 N/A 116%	0.030 N/A 98%	0.000 100% 110%	0.000 102% 117%
Average	116%	98%	105%	110%

Table 6. Vitamin D analysis results for different food products. The total vitamin D values on the nutrition and supplement fact sheets on some food products are also listed.

Sample (mg/kg)	Non-fat dry milk fortified with Vitamin A and D		Infant formula (soy based)		Chocolate fortified with Vitamin D		Oatmeal		Fish oil	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
Vitamin D_3 Vitamin D_2	0.118 0.000	1.6% N/A	0.089 0.015	1.1% 4.3%	3.030 0.025	1.3% 28.5%	0.000 0.000	N/A N/A	0.190 0.011	7.1% 31.9%
Total vitamin D	0.118		0.103		3.055		0		0.200	
Label vitamin D	0.109		0.068		1.786		0		N/A	

BENEFITS OF MEASURING PREVITAMIN D IN THE VITAMIN D ANALYSIS

To emphasis the need to consider previtamin D in total vitamin D measurements, the same two sets of sample data were processed using two different methods of quantitation. A comparison of the methods is summarized in Table 7. In method A, total vitamin D was quantified without using the previtamin D peak area. This is the same data processing method that the standard method used.[4] In method B, total vitamin D was quantified using both the previtamin D and the vitamin D peak areas in the calibration and the quantitation, which is the new method that we propose to use in this study. One can see that in Table 7, method A allowed 11–12% difference for the standards prepared at different conditions (high temperature, HT, vs. room temperature, RT) while method B only had 1–2% difference. For samples with different saponification conditions (HT saponification vs. RT saponification), method A showed a larger difference (3–6%) than method B did (1–3%). Table 7 data proves that method B is less affected by the previtamin D concentration variation. The bottom line is that without measuring the previtamin D concentration, the total vitamin D analysis result could carry a large error that could be contributed to previtamin D formation during the manufacturing, transportation, or storage of food products.

Table 7. Comparison of two vitamin D methods in the event of different heating history.

	Method A[3]		Method B[3]	
	D_3	D_2	D_3	D_2
Standard (RT)[1]	0.0092	0.0092	0.0095	0.0096
Standard (HT)[1]	0.0103	0.0102	0.0097	0.0096
Difference between RT and HT treatment	12%	11%	2%	1%
Sample[2] (HT saponification)	0.303	0.191	0.299	0.189
Sample[2] (RT saponification)	0.285	0.185	0.303	0.194
Difference between RT and HT saponification	-6%	-3%	1%	3%

Note: 1) Standard was split into two parts. One is kept in RT. The other was heated at 75 °C for 1 hour (HT).
2) Samples from the same food product was split into two parts. One was saponified at 75 °C for 1 hour (HT saponification), the other was saponified at RT overnight (RT saponification).
3) Method A does not include the previtamin Ds. Method B includes the previtamin Ds in the total vitamin Ds. The results are in mg/kg unit.

CONCLUSIONS

This application note demonstrates an improvement in the current LC-MS method for vitamin D analysis. Previtamin D was directly measured in the total vitamin D analysis, eliminating the error that could arise from not measuring previtamin D. This method will be less affected by the heating history of food products. Therefore, potential errors due to conversion of vitamin D to previtamin D are eliminated when accidental situations occur during the manufacturing, transportation, or storage of food products.

The results of vitamin D analysis for the NIST reference sample showed excellent accuracy (102.6%), and repeatability (2.4%). The recovery data from oatmeal and infant formula ranged from 98% to 117%. The LOQs in oatmeal were 0.01 mg/kg and 0.02 mg/kg for vitamin D_3 and vitamin D_2, and 0.0003 mg/kg (D_3) and 0.002 mg/kg (D_2) in solvent. A variety of food products, such as non-fat dry milk power, infant formula (soy based), chocolate, oatmeal, and fish oil samples have been successfully tested with this method. Good agreement with the label values have been observed for the infant formula, dry milk powder, and oatmeal.

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Soft Drink Analysis Using the ACQUITY UPLC H-Class PLUS System

Margaret Maziarz
Waters Corporation, Milford, MA, USA

GOAL

Successful analysis of additives found in soft drink beverages using an ACQUITY™ UPLC™ H-Class PLUS System with UV detection and a Waters™ Beverage Analysis Kit.

BACKGROUND

Many of the soft drinks available on the market, especially diet formulations, can contain non-nutritive sweeteners such as acesulfame potassium (ASK), sodium saccharin, and aspartame; as well as preservatives, such as sodium benzoate and potassium sorbate. In case of energy formulations, caffeine may also be present. As a particular beverage can contain all or some of these six ingredients at varying concentrations, reliable and simple analytical techniques are required to accurately measure them during production and final packaging. These frequent measurements enable the quality control laboratories of the production facility to confirm that the six additives are present within the specified concentrations, which is essential for consistent product quality and taste.

The ACQUITY UPLC H-Class PLUS System with UV detection is a high-performance and reliable analytical system for quick and accurate analysis of soft drink additives.

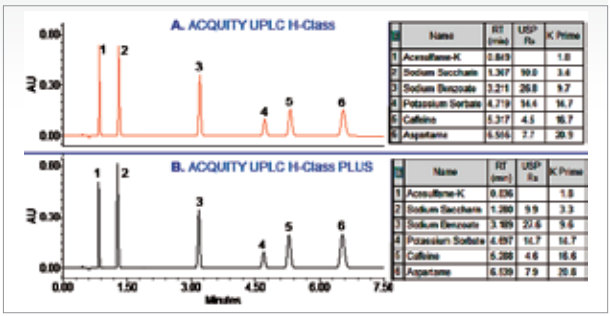


Figure 1. Chromatographic separation of beverage analysis standard acquired on an ACQUITY UPLC H-Class PLUS System with UV at 214 nm.

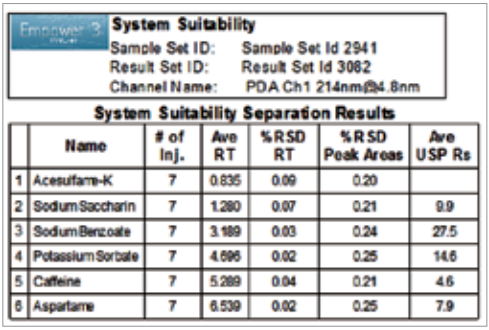
In this technology brief, we demonstrate that the ACQUITY UPLC H-Class PLUS System, coupled with UV detection and integrated with the Waters Beverage Analysis Kit,[1] is a highly robust, reliable performance analytical technique for the accurate analysis of additives in soft drink beverages.

THE SOLUTION

The ACQUITY UPLC H-Class PLUS System with UV detection and the Beverage Analysis Kit provides a quick and reliable analytical approach for accurate detection of sweeteners, preservatives, and caffeine in soft drinks. The Beverage Analysis Kit[1] minimizes the need for sample preparation by providing a pre-formulated mobile phase, wash reagent, standards, as well as detailed methodology.

The Beverage Analysis Standard with the six analytes (acesulfame potassium, sodium saccharin, sodium benzoate, potassium sorbate, caffeine, and aspartame) was analyzed on both the ACQUITY UPLC H-Class and the ACQUITY UPLC H-Class PLUS Systems. As displayed in Figure 1, the chromatographic separation acquired on both systems was comparable. The chromatographic resolution between all six analytes was excellent.

The standard mixture was injected seven times to evaluate performance of the method run on an ACQUITY UPLC H-Class PLUS System. The repeatability of the retention times and peak areas for all analytes are shown in Figure 2. Method exhibited excellent results with %RSD of retention times and peak areas ranging from 0.02 to 0.09% and 0.20 to 0.25%, respectively. The ACQUITY UPLC H-Class PLUS System delivered results with superior reproducibility, which is essential for manufacturers to monitor product quality during soft drink production.



System Suitability

Sample Set ID: Sample Set Id 2941
Result Set ID: Result Set Id 3082
Channel Name: PDA Ch1 214nm@4.8nm

System Suitability Separation Results

	Name	# of Inj.	Ave RT	%RSD RT	%RSD Peak Areas	Ave USP Rs
1	Acesulfame-K	7	0.835	0.09	0.20	
2	Sodium Saccharin	7	1.280	0.07	0.21	9.9
3	Sodium Benzoate	7	3.189	0.03	0.24	27.5
4	Potassium Sorbate	7	4.696	0.02	0.25	14.6
5	Caffeine	7	5.289	0.04	0.21	4.6
6	Aspartame	7	6.539	0.02	0.25	7.9

Figure 2. Seven replicate injections of Beverage Analysis Standard acquired on an ACQUITY UPLC H-Class PLUS System with UV at 214 nm.

SUMMARY

The ACQUITY UPLC H-Class PLUS System and the Waters' Beverage Analysis Kit provide a robust and reliable analytical methodology for the accurate analysis of the 'big six' additives (acesulfame potassium, sodium saccharin, sodium benzoate, potassium sorbate, caffeine, and aspartame) in soft drinks. Superior reproducibility of the system will enable manufacturers to accurately measure these ingredients during real-time process monitoring for continuous product quality. This will help to ensure that only the soft drinks batches that meet the desired specifications are delivered to the market.

References

1. Beverage Analysis Kit, Waters Care and Use Manual, 715003129, July 2015.

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