Food Compendium

Volume 1

SCIEX

The Power of Precision

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Simultaneous Analysis of 14 Mycotoxins and 163 Pesticides in Crude Extracts of Grains by LC-MS/MS

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Overview

Multi-component methods for the detection of different compound classes, such as mycotoxins or pesticides, have been established and are widely used to analyze a broad range of food or feed. However, there is a continuing demand to test for a larger number of compounds in shorter times. The development of a combined method for different compound classes can help to meet those new challenges. In this paper we present a fast, robust, and reliable method, which has been validated for the detection of 14 mycotoxins and 163 pesticides in the matrix grain. The LC-MS/MS method using the Scheduled Multiple Reaction Monitoring (Scheduled MRM™ algorithm) detects all mycotoxins with Limits of Quantitation (LOQ) between 1µg/kg and 10µg/kg. The LOQ for pesticides were found to be 10µg/kg and less. All LOQ meet the requirements of the EU.

Introduction

Pesticides and mycotoxins are known to harm the health of humans and animals. Many of these compounds are known either as carcinogenic, cytotoxic, or ecotoxic. Therefore, different countries have set regulations on pesticides and mycotoxins. For example, in the EU, maximum residue levels of pesticides in or on certain products are regulated by EC/396/2005 and the amended regulation EC/839/2008 and, in Japan, by the Japanese Positive List Syoku-An No.0124001 January 14, 2005 and amendments May 26, 2006. Mycotoxin limits are harmonized in the regulation for contaminants in foodstuffs EC 1881/2006 and the amended regulation EC 1126/2007 in the EU.[1-6]

Regulations on food and environmental analysis require the analysis of contaminants using confirmatory techniques, such as GC-MS and LC-MS/MS. More than 1000 pesticides are used worldwide and, along with their metabolites and degradation products, are present in food and the environment. Thus, there is a demand for powerful and rapid analytical methods that can detect very low concentrations of pesticides in mycotoxins in a variety of sample matrices.

Over the last years, LC-MS/MS replaced traditional GC and LC methods for the screening of pesticides and mycotoxins because of its ability to analyze a wider range of compounds in a single analysis and the unmatched selectivity and sensitivity of Multiple Reaction Monitoring (MRM).

Traditionally, mycotoxins and pesticides require different sample preparation. A simplified extraction procedure was established to analyze the two compound classes simultaneously in one sample, without additional cleanup steps by SPE or immunoaffinity columns. This new simplified sample preparation in combination with high resolution LC, and sensitive MRM detection allows detecting pesticides and mycotoxins faster and less labor-intensive and time-saving.

Experimental

Sample Preparation

10g of grain sample was extracted using a mixture acetonitrile/water. The extract was filtered and diluted with water + 5 mM ammonium acetate to optimize LC peak shape.[7]

LC

A Shimadzu Prominence LC system with an Agilent ZORBAX Eclipse XDB C18, 100x4.6 mm, 1.8µm column at 40°C with a gradient of eluent A water/methanol (80/20) + 5 mM ammonium

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acetate and eluent B water/methanol (10/90) + 5 mM ammonium acetate was used at a flow rate of 500 µL/min. The injection volume was set to 100 µL.

MS/MS

A SCIEX API 4000™ LC-MS/MS System with Turbo V™ source and Electrospray Ionization (ESI) probe was used. A number of 14 mycotoxins and 163 pesticides were detected using 2 MRM transitions per compound to allow quantitation and identification based on the ratio of quantifier and qualifier transitions as defined by regulation 2002/657/EC. The *Scheduled* MRM™ algorithm was used for best accuracy and reproducibility (Figure 1). Every sample was injected twice in positive and negative polarity.

Results and Discussion

A method for quantitation and identification of 9 fusarium toxins: Nivalenol (NIV), Deoxynivalenol (DON), Fusarenon X (FUS X), 3-Acetyldeoxynivalenol (3-AcDON), 15-Acetyldeoxynivalenol (15-AcDON), Diacetoxyscirpenol (DAS), HT-2 toxin, T-2 toxin, Zearalenon (ZON), and Ochratoxin A (OTA) was developed (Figure 1). This method was extended to also detect aflatoxins B1, B2, G1, and G2 (Figure 2). The complete method was validated for the analysis of wheat, barley, corn, and oat samples (Table 1).[7-8]

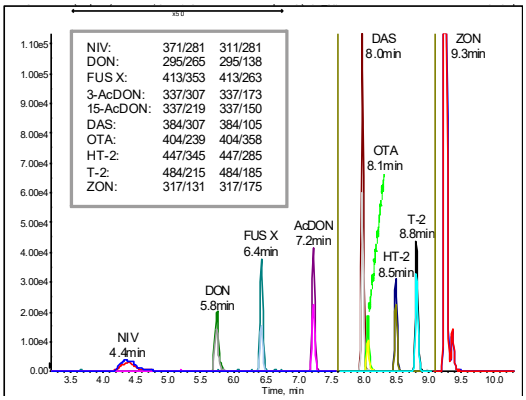

Figure 1. Detection of fusarium toxins and Ochratoxin A by LC-MS/MS

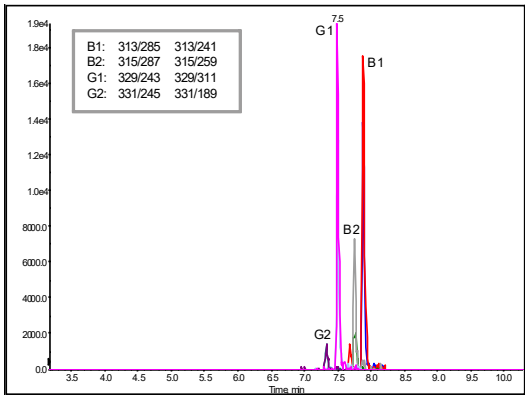

Figure 2. Detection of aflatoxins by LC-MS/MS

Table 1. LOQ and linear range of detected mycotoxins

Mycotoxin	LOQ (µg/kg)	Linear Range (µg/kg)	EU MRL[#]
3-AcDON	10	400	(1)
15-AcDON	10	150	(1)
DON	10	10000	1750* 1250** (2)
FUS X	10	2000	(1)
DAS	10	400	(1)
NIV	10	4000	(1)
OTA	1	>10	5***
HT-2	5	200	(2)
T-2	5	1000	(2)
ZON	5	80	100*** (2)
Aflatoxin B1	1	>20	2
Aflatoxins	1	>20	Σ=4

Footnotes to Table 1:

EC 1881/2006 and the amended EC 1126/2007

* Unprocessed durum wheat and oats

** Unprocessed cereals other than durum wheat and oats

*** Unprocessed cereals

(1) Due to co-occurrences and as "generally low" considered levels no MRL was estimated

(2) Appropriateness of setting a maximum level should be considered by 1 July 2008

The developed method was recently updated to also quantify and identify 163 pesticides (Figure 3). The use of the *Scheduled* MRM™ algorithm allows the monitoring of such a large panel of analytes without sacrificing sensitivity and reproducibility. The method was validated in different grain matrices. Limits of Quantitation (LOQ) of all mycotoxins were found between 1 µg/kg and 10 µg/kg. Pesticides were quantified at 10 µg/kg and less. All LOQ meet the requirements of the EU. Positive findings in two selected grain samples are shown in Figure 4.

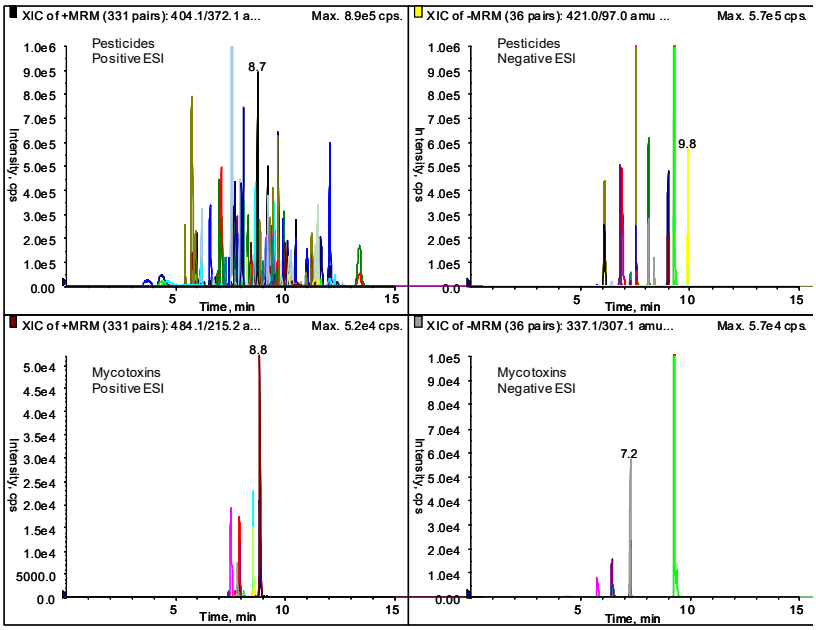

Figure 3. Detection of 14 mycotoxins and 163 pesticides using LC-MS/MS in two injections (positive and negative polarity) using the Scheduled MRM™ algorithm for best sensitivity and reproducibility

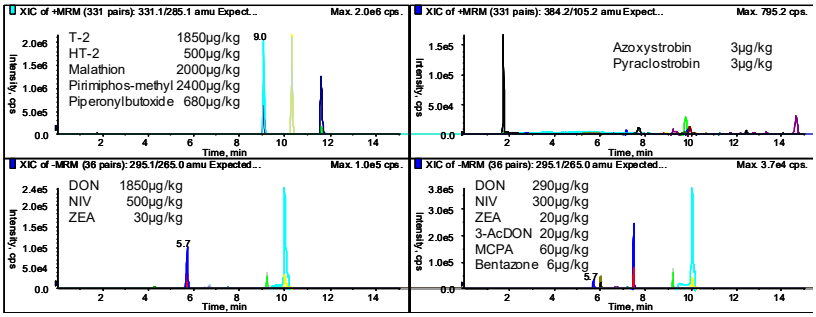

Figure 4. Detection of mycotoxins and pesticides in a durum wheat sample (left) and a barley sample (right)

Summary

A fast, robust, and reliable method, for the detection 14 mycotoxins and 163 pesticides in the matrix grain was developed and validated. A generic extraction procedure followed by a dilution step was used to cover the large panel of analytes. High resolution LC was combined with high sensitivity detection using a SCIEX API 4000™ LC-MS/MS System. Multiple Reaction Monitoring (MRM) was used because of its high selectivity and sensitivity. With the *Scheduled* MRM™ algorithm activated for accuracy and reproducibility.

The method was validated in different grain matrices. Limits of Quantitation (LOQ) of all mycotoxins were found between 1µg/kg and 10µg/kg. Pesticides were quantified at 10µg/kg and less. All LOQ meet the requirements of the EU.

References

[1] D. Elbert et al.: presentation at AOAC conference (2008) in Dallas

2 A. Voller et al.: presentation at AOAC conference (2009) in Philadelphia

Publication number: 2110210-01

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Target and Non-Target Screening for Pesticide Residues in Food Samples using the SCIEX TripleTOF™ 5600 System.

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Overview

The new SCIEX TripleTOF™ 4600 system was used to screen for chemical residues in extracts of fruit and vegetable samples. The mass spectrometer was operated using fast Information Dependent Acquisition (IDA) combining a TOFOF-MS survey scan followed by dependent TOF-MS/MS scans s. . TOF-MS data were used to accurately quantify targeted compounds and the additionally collected MS/MS spectra were used to confidently identify detected compounds with highest confidence by mass spectral library searching. The acquired MS and MS/MS information was mined retrospectively to identify non-ta targeted and unexpected compounds. Novel software tools embedded into the PeakView® software were used for automatic sample-control-comparison during the data processing.

Introduction

LC-MS/MS is a powerful analytical tool for the analysis of a wide molecular weight range of polar, semi-volatile and thermally labile compounds. Triple quadrupole-based mass analyzers are popular for targeted quantification of hundreds of food contaminants in a single analysis because of their extra degree of selectivity and sensitivity when operated in Multiple R Reaction Monitoring (MRM) mode.

Advancements in LC-MS/MS technology, including hybrid systems like quadrupole-quadrupole Time-of-Flight (TripleTOF™), now provide the ability to perform targeted and non-targeted screening on a routine basis. High resolution and accurate mass MS and MS/MS information is acquired into full scan chromatograms which enables screening for virtually every known chemical and identify and quantify undesirable contaminants quickly and easily.

Full scan chromatograms are very rich in information and easily contain thousands of ions from any compounds of interest present in the sample as well as from the sample matrix itself. Thus, powerful software tools are needed to explore the high resolution and accurate mass data generated.

Method Details

- Store-bought fruit and vegetable samples, including organic produce for sample-control-comparison
- QuEChERS extraction following guideline EN 15662/2007 using Restek kits (extraction with Q110 and dSPE with Q210, Q212, Q213) followed by 10x dilution to minimize possible matrix effects
- Quantitation using the iD*Quant*™ standards kit for pesticide analysis
- UHPLC using a Shimadzu UFLC$_{XR}$ system with a Restek Ultra Aqueous C18 (100 x 2.1 mm) 3 µm column and a gradient of water and methanol with 10 mM ammonium formate at a flow rate of 0.5 mL/min
- Injection volume of 10 µL
- SCIEX TripleTOF™ 4600 system with DuoSpray™ source operated in electrospray ionization
- Continuous recalibration between injections using the Calibrant Delivery System (CDS)
- Information Dependent Acquisition (IDA) using a TOF-MS survey scan 100-1000 Da (100 ms) and up to 10 dependent TOF-MS/MS scans 50-1000 Da (100 ms) using Collision

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Energy (CE) of 35 V with Collision Energy Spread (CES) of ± 15 V

- Qualitative and quantitative data processing using PeakView® version 1.2 with XIC Manager and MultiQuant™ software version 2.1

Results and Discussion

Qualitative screening and identification

High resolution and accurate mass LC-MS/MS chromatograms contain comprehensive information of all molecules present in the sample that are amenable to the ionization technique and polarity used. Narrow extracted ion chromatograms (XIC) can be generated to selectively screen for targeted compounds. Resolution > 20,000 (at full width half height) and mass accuracy <5 ppm is often sufficient to separate the analytes of interest from interfering matrices and, thus, is a requirement for compound identification in various guidelines.[1,2]

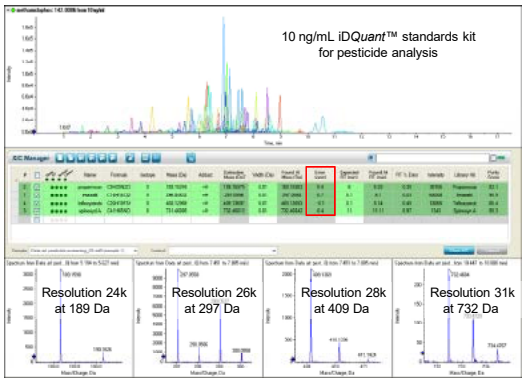

Figure 1. Resolution and mass accuracy across the mass range for selected pesticides of the iDQuant™ standards kit

The SCIEX TripleTOF™ 4600 system with Accelerator TOF™ Analyzer provides high resolution of up to 35,000 dependent on the mass detected (Figure 1), and stable mass accuracy of ~2 ppm at fast acquisition speed in MS and MS/MS mode. This allows generating narrow XIC for the best Signal-to-Noise (S/N) when screening for a large set of targeted chemical residues in complex samples (Figure 2).

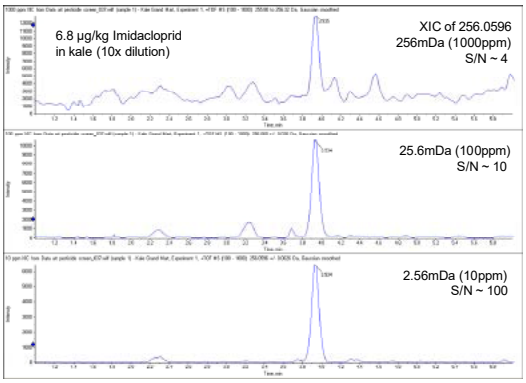

Figure 2. Increasing selectivity and S/N using narrow extracted ion chromatograms (XIC)

The XIC Manager software was used to screen for and identify pesticides in fruit and vegetable samples. The XIC Manager consists of a table for defining a list of masses or formulae to generate XIC, and the ability to review the results for the identification of the detected compounds. High confidence in results is based upon retention times, accurate mass, isotopic pattern and MS/MS library searching. Confidence data of compound identification is visualized using 'traffic lights'.[3]

Examples of automatic identification of pesticides in clementine and kale extracts are shown in Figures 3 and 4.

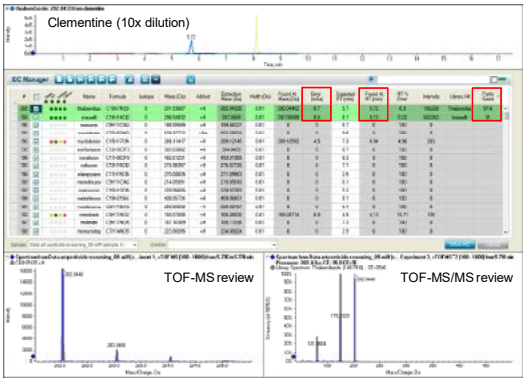

Figure 3. Identification of Imazalil and Thiabendazole in a clementine sample, retention time, mass accuracy, isotopic pattern, and MS/MS library match were reported automatically and visualized using 'traffic lights'

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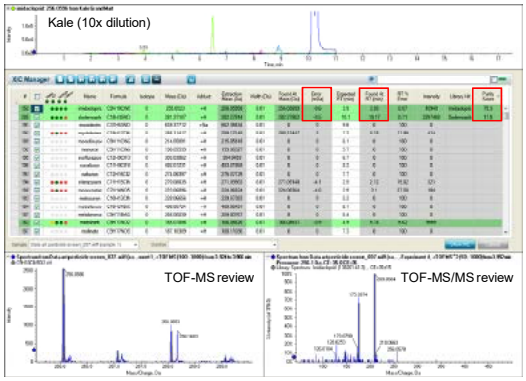

Figure 4. Identification of Imidacloprid in a kale sample, retention time, mass accuracy, isotopic pattern, and MS/MS library match were reported automatically and visualized using 'traffic lights'

Figure 5 shows the identification of Dodemorph in kale based on retention times, accurate mass, and isotopic pattern. However, the low MS/MS library match (dictated by the low numeric purity score) and the MS/MS review clearly show that Dodemorph was not present in the extract. This example highlights the importance of MS/MS information for identification. Relying on high resolution MS alone can result in false positive results.

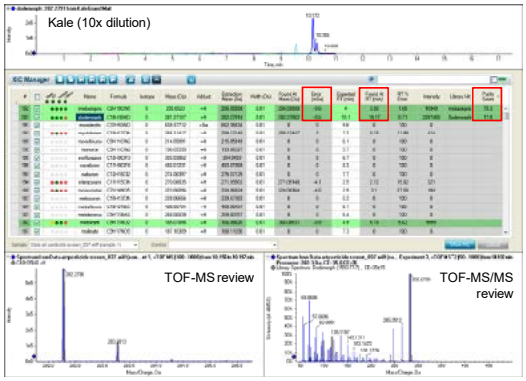

Figure 5. The MS/MS library search clearly proves that Dodemorph was not present in the kale sample although retention time, mass accuracy, and isotopic pattern matched

Quantitative analysis

The XIC manager can also quantitatively compare samples to highlight identified compounds above a target concentration. The software compares the sample data with a standard injection and automatically highlights all findings above a user defined threshold.

Figure 6 shows an example of identification of Imazalil and Thiabendazole in an orange sample at a concentration greater than 5 µg/kg.

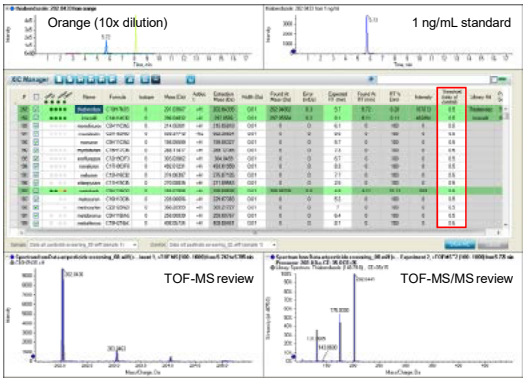

Figure 6. Quantitative comparison of an orange sample (10x diluted extract) and a pesticide standard, the XIC Manager software automatically identified Imazalil and Thiabendazole and highlights that both pesticides have a signal of more than 50% than the standard, which corresponds to a concentration greater than 5 µg/kg

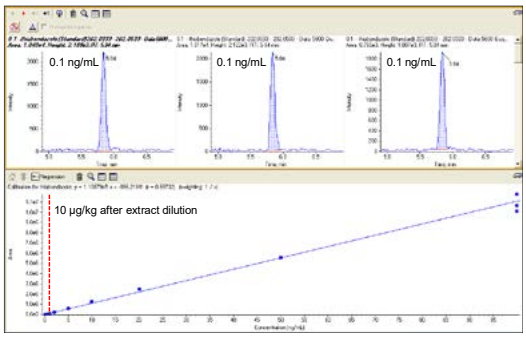

Figure 7. Chromatograms at 0.1 ng/mL and calibration line for Thiabendazole (0.1 to 100 ng/mL) using linear fit with 1/x weighting

Furthermore, the LC-MS/MS data can be exported into the MultiQuant™ software for external or internal quantitation using standard injections at different concentration levels. Figure 7 shows an example calibration line for Thiabendazole from 0.1 to 100 ng/mL. Accuracy and reproducibility data are summarized in Table 1. Pesticide findings are summarized in Table 2.

Table 1. QC parameters for Thiabendazole

Concentration (ng/mL)	# of injections	Accuracy (%)	%CV (%)
0.1	3	96.2	7.9
0.2	1	85.0	N/A
0.5	1	86.8	N/A
1	3	96.1	1.7
2	1	100.1	N/A
5	1	106.4	N/A
10	3	112.8	1.8
20	1	112.5	N/A
50	1	100.3	N/A
100	3	97.8	8.0

Table 2. Pesticide findings in food samples above 5 µg/kg (n.d. = no pesticides were detected)

Sample	Pesticide	RT error (%)	Mass error (mDa)	PUR (%)
Organic orange	n.d	-	-	-
Orange	Imazalil	0.3	0.2	91.6
	Thiabendazole	0.1	0.5	97.9
Clementine	Imazalil	0.3	0.5	91.0
	Thiabendazole	0.2	0.7	97.6
Orange juice	n.d.	-	-	-
Carrot	n.d.	-	-	-
Pepper	Cyromazine	0.2	-0.1	71.5
Broccoli	Imidacloprid	1.7	-0.3	82.9
	Metalaxyl	0.4	0.2	96.4
	Spirotetramat	2.2	-0.5	96.3
	Thiabendazole	1.1	-0.8	96.5
Kohlrabi	Imidacloprid	1.0	-0.5	85.4
	Methoxyfenozide	0.5	0.0	85.6
Kale	Imidacloprid	0.9	-0.6	75.3

Non-Target Screening and Unknown Identification

Another feature of the XIC Manager software is the ability to perform sample-control-comparison for non-target screening, or general unknown screening. Figure 8 shows an example of comparing an organic orange to a non-organic orange. All ions with 20 times higher sensitivity in the sample than in the control sample are reported and automatically searched against the iMethod™ application - Meta Library, which contains MS/MS spectra of over 2400 chemicals, including pesticides, mycotoxins, veterinary drugs, pharmaceuticals, drugs of abuse, etc. The two fungicides Imazalil and Thiabendazole were successfully identified with a library match of over 90%.

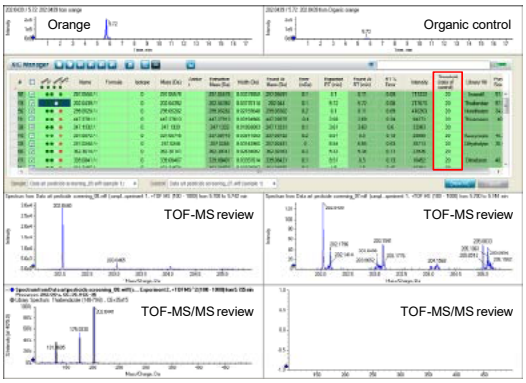

Figure 8. Identification of Imazalil and Thiabendazole using a non-targeted sample-control-comparison and MS/MS library searching, the software automatically reports all ions which are 20 times higher in the sample versus the organic control

The example presented in Figure 5 illustrates the importance of MS/MS information for high confidence compound identification. MS/MS library searching was used to clearly prove that Dodemorph was not present in the sample extract.

As a last step we used the new formula finder in PeakView® software version 1.2 to tentatively identify the molecular formula and structure of the unknown compound.

The formula finder uses high resolution accurate mass information of the molecular ion, adducts, isotopic pattern, and fragment ion information to empirically calculate potential molecular formulas for the detected compound. Only a single molecular formula only can explain the detected compound when combining all available MS and MS/MS data. The calculated formula of $C_{18}H_{35}NO$ was then automatically searched against ChemSpider to find a total of 6 possible matching structures (Figure 9).

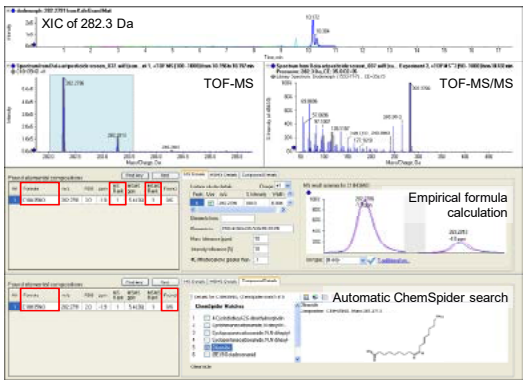

Figure 9. Combined empirical formula calculation and ChemSpider search in PeakView® software, accurate mass, isotopic pattern and MS/MS data was used to calculate the molecular formula and then automatically searched against online databases, this resulted in one possible molecular formula with a total of 6 potential structures

One of these hits was Oleamide, (Z)-Octa-9-decenamide, an amide of the fatty acid oleic acid. This was a very likely hit since Oleamide is an endogenous substance with E and Z isomers explaining the double peak in the chromatogram.

The structures of Oleamide and Dodemorph were imported into the fragmentation prediction tool of PeakView® software. This tool automatically compares the experimental MS/MS pattern with a theoretical fragmentation pattern using the proposed structures. In this case, 100% of the observed fragment ions were explained by the structure of Oleamide but only 71% of the 36 ions were explained by the Dodemorph structure, excluding the two characteristic high mass fragment ions (Figures 10 and 11). This suggests again that Dodemoph was not present in the kale sample and the detected compound was tentatively identified as Oleamide.

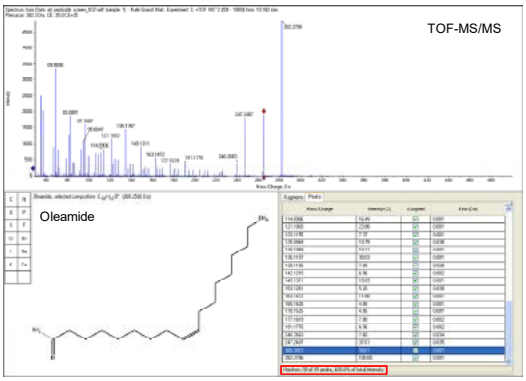

Figure 10. MS/MS fragment ion prediction for Oleamide, 100% of MS/MS ions are explainable by the structure of Oleamide

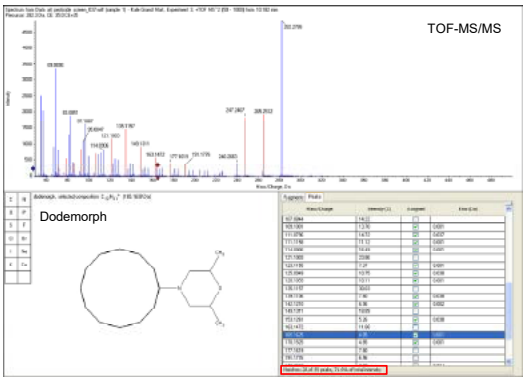

Figure 11. MS/MS fragment ion prediction for Dodemorph, many MS/MS ions cannot be explained by the structure suggesting that Dodemorph was not present in the kale sample

Summary

A straightforward procedure using generic extraction and high resolution accurate mass LC-MS/MS was developed and successfully applied to screen for and identify chemical residues in food samples using the SCIEX TripleTOF™ 4600 system. The high sensitivity, resolution, and unmatched scan speed of the Accelerator TOF™ analyzer enabled reproducible and accurate quantification at regulated maximum residue levels. Extract dilution was possible to minimize possible matrix effects.

Data was processed using PeakView® with XIC Manager and MultiQuant™ software to identify and quantify targeted compounds in food samples using retention time, accurate mass, isotopic pattern, and MS/MS library searching. MS/MS library searching was found to be particularly crucial to minimize potential false positive results.

The PeakView® software was also used to perform sample-control comparison to find unexpected non-targeted compounds which were further identified using empirical formula calculation and automatic ChemSpider search.

References

1 EU Commission Decision 'concerning the performance of analytical methods and the interpretation of results' #2002/657/EC

2 SANCO Document: 'Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed' #SANCO/10684/2009

3 A. Schreiber and D. Cox: 'Using PeakView® Software with the XIC Manager for Screening and Identification with High Confidence based on High Resolution and Accurate Mass LC-MS/MS' Application Note AB SCIEX (2011) #2170811-03

Publication number: 5680212-01

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The Detection of Acidic Herbicides and Phenyl Ureas by LC-MS/MS with Large Volume Injection and Automated Column Switching

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Introduction

Acidic herbicides like dicamba are used to kill broadleaf weeds before and after sprout. They control annual and perennial weeds in grain crops and highlands, are used to control brush and bracken in pastures, and in combination they are also used in pastures, range land, and non-crop areas (fence rows, roadways, and wastage) to control weeds. Phenyl urea pesticides such as Linuron are used as selective herbicides for pre- and post-emergence weed control in vegetables including potatoes, peas, carrots and beans; also on wheat, celery, parsnip and parsley. Both classes of pesticides are toxic to wildlife and some are suspected hormone-disrupting substances.

The provision of clean, uncontaminated drinking water is of paramount importance to the water industry. In recent times the requested limits of detection for such pesticides have been decreasing as methodologies improve. Typically water companies need to be able to have limits of quantification for pesticides between 0.1 – 1 µg/L (100 – 1000 part-per-trillion, ppt) which often means that methods should have limits of detection for certain pesticides in the range of 10 – 50 µg/L.

These low levels have often meant that water samples have to be prepared either by liquid/liquid or solid phase extraction in order to concentrate these contaminants to such a level where they can be detected using traditional techniques such as GC-MS or HPLC with UV detection. Where GC-MC is used an additional derivatization step is often required before sample analysis. This sample pre-treatment used for traditional techniques can often be time consuming and add additional cost to the analyses. Therefore in this work the direct injection of filtered samples was used for sample analysis, to reduce both cost and speed up the sample throughput.

Experimental

Sample Preparation

River and ground water samples (10 mL) were filtered through a Chromfil PET 20/25, 0.2 µm 25 mm filter. The filter was washed by acetonitrile (0.85 mL) with the filter wash added directly into the sample. This filtered sample was directly injected onto the LC-MS/MS system.

Chromatography

Samples (200 µL) were directly injected and separated by reversed-phase HPLC using a Dionex Ultimate 3000 system. A Gemini 3 µm, 150 x 2.0 mm C18 and a LUNA 3 µm C18 (2), 150 x 3 mm column from Phenomenex were used to analyze acid herbicides and phenyl ureas respectively. Both columns were kept at 40ºC and gradients from water containing 0.1% acetic acid to acetonitrile containing 0.1% acetic acid were used to separate analytes. Automated column switching, involving a 10 port Valco switching valve, was used to switch between the column for acidic herbicide and the one for phenyl urea analysis (the gradient profiles are shown in Table 1).

Contents

Table 1. Gradient profiles used for the separation of acidic herbicides and phenyl ureas

Acidic herbicides			Phenyl ureas		
Time (min)	Flow (mL/min)	% B	Time (min)	Flow (mL/min)	% B
0.0	10	0.4	0.0	10	0.2
1.5	10	0.4	5.0	10	0.2
10.0	95	0.7	9.0	100	0.3
18.0	95	0.7	16	100	0.3
18.5	10	0.4	17	10	0.2
18.6	10	0.4			

Mass Spectrometry

Analysis was performed on an SCIEX API 4000™ LC/MS/MS system with Turbo V™ source electrospray ionisation (ESI) probe in negative polarity (acidic herbicides) and positive polarity (phenyl ureas). The MRM transitions for acidic herbicides and phenyl ureas are shown in Table 2.

Results and Discussion

Examples of calibrations for both acidic herbicides and phenyl ureas are shown in Figures 1, 2 and 3. For both classes of pesticides linear responses were obtained over the range tested with 'r' values never less than 0.998 (Table 3).

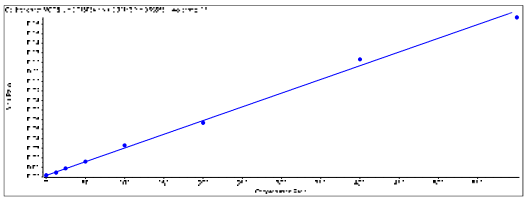

Figure 1. Calibration for MCPB from 12.5 – 600 ng/L

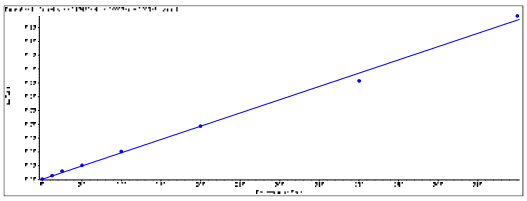

Figure 2. Calibration for Dicamba from 12.5 – 600 ng/L

Table 2. MRM transitions to detect acidic herbicides and phenyl ureas using the SCIEX API 4000™ system

Pesticide	Q1	Q3	DP	CE
MCPA	199.0	141.1	-55	-20
Clopyralid A	189.9	146.0	-20	-12
Clopyralid B	191.9	148.0	-20	-12
2,4-D	218.9	161.1	-20	-20
Dicamba	218.9	175.1	-20	-8
2,4-DB	246.9	161.0	-20	-18
Dichlorprop	232.9	161.1	-25	-18
Bromoxynil	275.8	81.0	-50	-45
Ioxynil	369.7	127.0	-55	-50
Bentazone	239.0	132.0	-50	-36
MCPB	227.1	141.1	-35	-25
MCPP	213.0	141.1	-30	-22
Triclopyr	253.9	196.0	-20	-16
Fluroxypyr	253.0	195.0	-35	-20
Benazolin	242.0	170.1	-25	-20
Aminopyralid	204.8	160.8	-55	-14
2,4-DPA (S)	203.1	159.1	-35	-12
4-CAA (IS)	169.0	125.0	-20	-12
2,B-4,C-phenol (IS)	195.9	78.9	-45	-32
Isoproturon A	207.1	134.2	45	35
Isoproturon B	207.0	72.0	56	35
Diuron	233.0	72.0	71	35
Isoproturon	207.0	72.0	56	35
Monolinuron	215.0	126.0	56	25
Chlorotoluron A	215.0	182.9	51	11
Chlorotoluron B	213.0	72.0	51	15
Metoxuron	229.0	72.0	106	35
Fenuron	165.2	72.0	86	29
Pencycuron	329.0	124.8	90	39
Linuron	249.0	159.9	51	27
Isoproturon	207.1	134.2	45	35

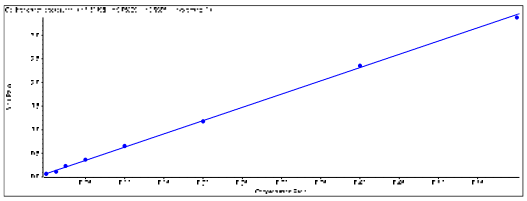

Figure 3. Calibration for Isoproturon from 12.5 – 600 ng/L

Table 3. Signal-to-noise (S/N)* of the lowest calibration standard and r' values taken from calibration lines 12.5 – 600 ng/L

Pesticide	S/N at 12.5 ng/L	'r' value
MCPA	86.7	0.99967
Clopyralid#	25.1	0.99769
2,4-D	51.3	0.99963
Dicamba	25.5	0.99856
2,4-DB	25.8	0.99936
Dichlorprop	76.5	0.99934
Bromoxynil	50.3	0.99956
Ioxynil	148.5	0.99932
Bentazone	368.1	0.99888
MCPB	15.3	0.99868
MCPP	102.1	0.99968
Triclopyr	27.6	0.99871
Fluroxypyr	22.3	0.99846
Benazolin	26	0.99876
Aminopyralid	100.7	0.99955
Diuron	41.2	0.99816
Isoproturon	39.5	0.99864
Monolinuron	32	0.99904
Metoxuron	54.9	0.99882
Fenuron	53.1	0.99913
Pencycuron	167.9	0.99982
Linuron	26.2	0.9993
Chlorotoluron	50.5	0.99921

* S/N was calculated in MultiQuant™ software version 2.0.1

S/N of Chlopyralid at 25 ng/L

It can also be seen that every compound with the exception of Clopyralid gave a good signal-to-noise (> 15:1) from the lowest standard 12.5 ng/L (Table 3). Clopyralid, the least sensitive of all the compounds, gave a signal to noise of 25:1 at its lowest standard level of 25 ng/L. There was no carryover observed for either method.

This method has been validated and used routinely for testing water samples as part of surveillance exercises. Normally such tests produce negative results but in certain instances positive results can be observed which normally result from the illegal disposure of pesticides.

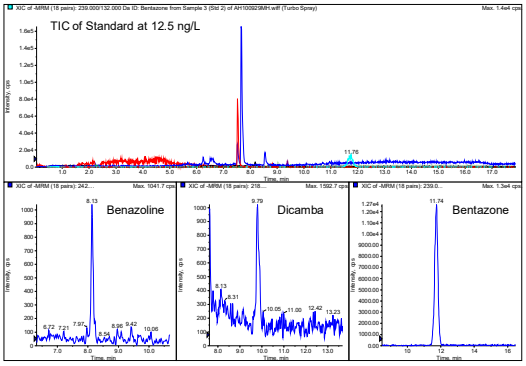

Figure 4a. 12.5 ng/L standard in negative polarity

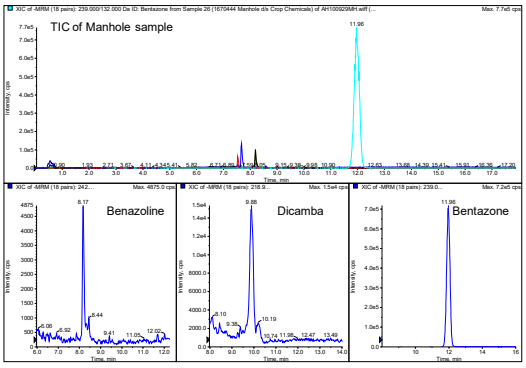

Figure 4b. Manhole sample in negative polarity

Figures 4 and 5 show examples of where this method has detected both the presence of certain acidic herbicides and phenyl ureas in samples of water from manholes. In each example the amount of pesticide detected varies with analyte and is in the parts per trillion range but exceeds the lowest calibration standard.

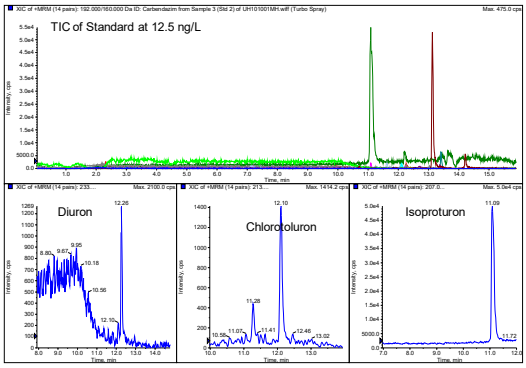

Figure 5a. 12.5 ng/L standard in positive polarity

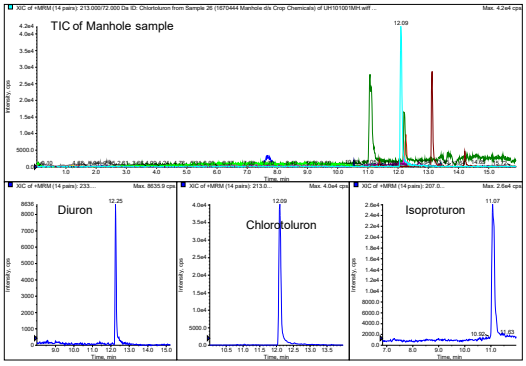

Figure 5b. Manhole sample in positive polarity

Conclusion

The results show that both acidic herbicides and phenyl ureas can be detected at the required limits set by the water industry in the UK. The sample preparation used involved a simple filtration step which removed the cost and time associated with solid phase extraction and/or liquid liquid extraction traditionally used for GC-MS analysis. Acidic herbicides and phenyl urea pesticides ionise under different polarities and require different HPLC conditions to obtain their best sensitivity. Using conventional LC and a timed switching valve samples can be run under the optimised LC conditions for either class of compounds, without supervision. The automated column switching enables researchers to optimise the pH of the mobile phase and column chemistry to produce the best sensitivity for both compound classes.

Such a method has been shown to be robust and sensitive enough to be applied to surveillance work, in the UK, needed to maintain a safe water supply.

Publication number: 3370611-01

Comprehensive Quantitation and Identification of Pesticides in Food Samples using LC-MS/MS with *Scheduled* MRM™, Fast Polarity Switching, and MS/MS Library Searching

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Overview

Liquid Chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a widely used analytical tool for the screening of food residues and contaminants. Here we present a new and unique method using QuEChERS extraction, separation using a polar embedded C18 phase, and MS/MS detection with highly selective and sensitive Multiple Reaction Monitoring (MRM) on an SCIEX QTRAP® 5500 system. The *Scheduled* MRM™ algorithm was used to obtain the best data quality and combined with fast polarity switching to cover the broadest range of pesticides possible. In addition MS/MS spectra were acquired to enable compound identification with highest confidence based on mass spectral library matching.

Introduction

LC-MS/MS is a powerful analytical tool capable of screening samples for numerous compounds. MRM is typically used because of its excellent sensitivity, selectivity, and speed. As LC-MS/MS technology continues to be adapted demands are made to detect and quantify an increasing number of compounds in a single run.

The development of generic extraction procedures, like QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) and LC methods using polar embedded C18 phases with good resolution and excellent peak shape made it possible to detect pesticides of a wide variety of compound classes and chemical properties in each sample.[1-3]

Modern LC-MS/MS systems make it possible to detect hundreds of pesticides and other food residues in a single run. The Turbo V™ source with Curtain Gas™ interface to reduce chemical noise, and the LINAC® collision cell to allow fast MS/MS scanning, are key technologies that make these high-throughput experiments possible. In addition, advanced software tools like the *Scheduled* MRM™ algorithm intelligently uses information of retention times to automatically optimize MRM dwell time of each transition and total cycle time of the experiment resulting in highest data quality. To further increase confidence in analytical results QTRAP® technology is used to automatically acquire fast and sensitive MS/MS spectra in Enhanced Product Ion (EPI) mode and search them against mass spectral libraries for compound identification. The information of the complete molecular fingerprint saved into EPI spectra significantly reduces the risk of false positive results.[4-6]

Additionally, for a comprehensive screening of pesticides it is necessary to employ both positive and negative Electrospray Ionization (ESI).

Here we present a new and unique LC-MS/MS method utilizing the *Scheduled* MRM™ algorithm in combination with fast polarity switching and acquisition of MS/MS spectra for compound identification. The method was successfully applied to quantify and identify pesticides in a number of QuEChERS extracts of fruit, vegetables, and spices.

Method Details

- Different fruit and vegetable samples were extracted using a modified QuEChERS procedure and diluted 10 to 50 times with water to optimize chromatographic peak shape and minimize possible matrix effects and interferences.

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- The SCIEX iD*Quant*™ Standards Kit for Pesticide Analysis was used for method setup and preparation of calibration standards. Additional pesticides were added to cover all compounds of interest.
- LC separation was achieved on a Shimadzu UFLC$_{XR}$ system with a Restek Ultra Aqueous C18 3 µm (100x2.1 mm) column and a 15 min gradient of water and methanol with ammonium formate buffer at a flow rate of 0.5 mL/min. The injection volume was set to 10 µL.
- The SCIEX QTRAP® 5500 system was operated with Turbo V™ source and Electrospray Ionization (ESI) probe.
- A total of 386 transitions in positive and 56 transitions in negative polarity were monitored with an MRM pause time of 2 ms. Optimized transitions for all compounds were obtained through the MRM catalogue of the iMethod™ Test for Pesticide Screening version 2.1.
- The *Scheduled* MRM™ algorithm was used with an MRM detection window of 90 s and a target scan time of 0.3 s in Analyst® 1.6 Software
- A settling time of 50 ms was used for polarity switching.
- For increased confidence in compound identification EPI spectra at a scan speed of 10000 Da/s were acquired using a dynamic fill time for optimal MS/MS quality.
- EPI spectra were generated using standardized Collision Energy (CE) of ±35 V with Collision Energy Spread (CES) of 15 V to ensure a characteristic MS/MS pattern independently on compound's fragmentation efficiency. MS/MS spectra were search against the iMethod™ Pesticide Library version 2.1.
- MultiQuant™ 2.1 Software was used for quantitative data processing.

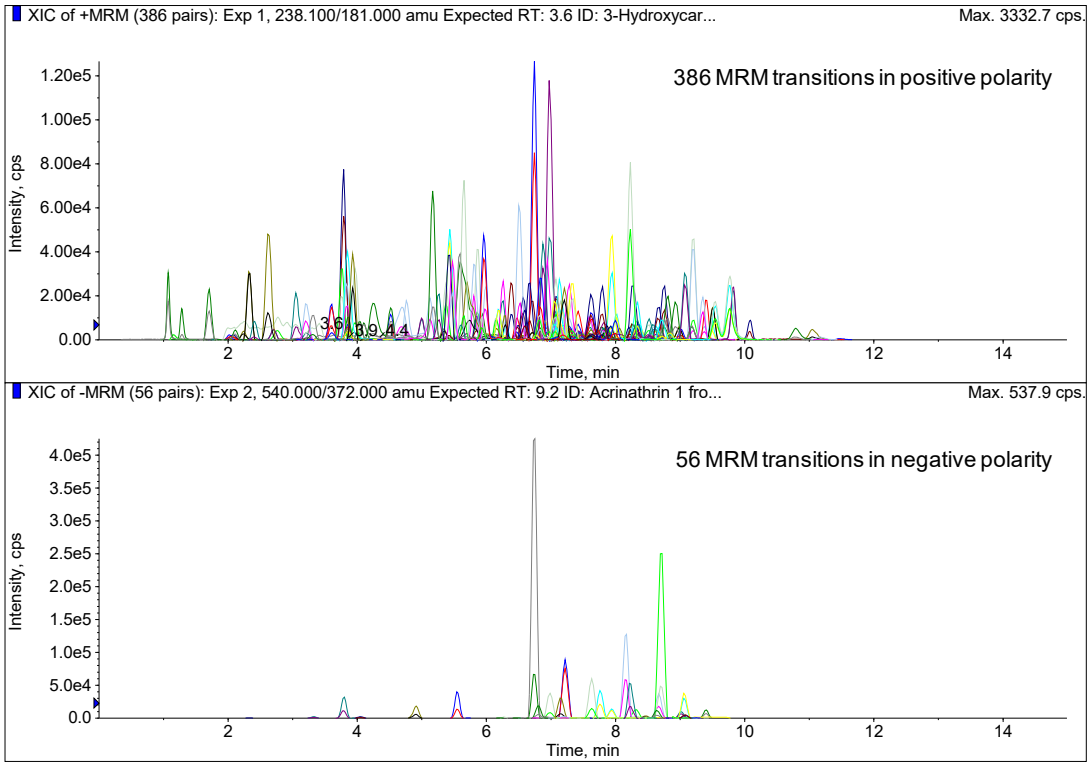

Figure 1. Detection of pesticides at a concentration of 1 ng/mL by monitoring 442 MRM transitions in positive and negative polarity using the *Scheduled* MRM™ algorithm and fast polarity switching

Contents

Results

Scheduled MRM™ with Fast Polarity Switching

The *Scheduled* MRM™ algorithm uses knowledge of the retention of each analyte to monitor the MRM transition only in a short time window. Thus at any one point in time, the number of concurrent MRM transitions are significantly reduced resulting in much higher duty cycles for each analyte. The software computes maximum dwell times for the co-eluting compounds while still maintaining the desired cycle time for best signal-to-noise (S/N), accuracy, and reproducibility. As a result *Scheduled* MRM™ allows the monitoring of many more MRM transitions in a single acquisition without compromising data quality (Figure 2).[4]

The enhanced version of the *Scheduled* MRM™ algorithm offered in Analyst® 1.6 software also allows to combine MRM scheduling with fast polarity switching to further extend the panel of compounds by covering substances with a wider range of chemical properties.

Easy Method creation

A key advantage of the *Scheduled* MRM™ algorithm is the ease with which powerful quantitative MRM acquisition methods can be created. The user is required to specify a few key parameters (Figure 3):[1]

- MRM transition: (Q1, Q3) and any compound dependent parameters in both polarities
- Expected retention time for each MRM transition
- MRM detection window must be wide enough to allow the MRM peak to stay entirely within the window across all injections
- Target scan time for each polarity to adjust the total cycle time
- MRM ID, like compound name, for easier data processing and reporting

The software algorithm then automatically builds an acquisition method that schedules the appropriate MRM transitions to be monitored and the required polarity switches at the appropriate times over the chromatographic analysis.

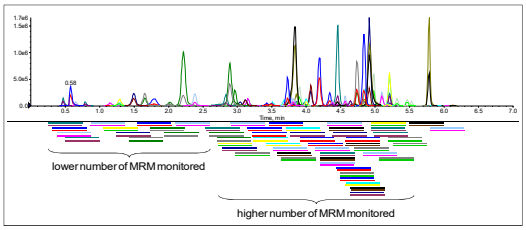

Figure 2. The *Scheduled* MRM™ Algorithm uses the knowledge of the elution of each analyte to monitor MRM transitions only in a short retention time window. This allows many more MRM transitions to be monitored in a single LC run, while maintaining maximized dwell times and optimized cycle time.

Good Chromatography is the Key to the Best LC-MS/MS Data using the *Scheduled* MRM™ Algorithm

The key to the highest order multiplexing and optimal MS/MS performance is high quality and highly reproducible LC separation.

One of the user inputs to the software to automatically create the *Scheduled* MRM™ method is the MRM detection window. This is an estimate of the LC peak width and chromatographic reproducibility expected, and should therefore reflect the time window around the supplied retention time which will contain the entire LC peak plus any shifts in chromatography. The narrower the peak widths and the more reproducible the elution, the tighter this MRM detection window can be and, thus, less concurrent MRM transitions are monitored. Reduced concurrency also means that higher dwell times will be used for each MRM, improving the data quality.

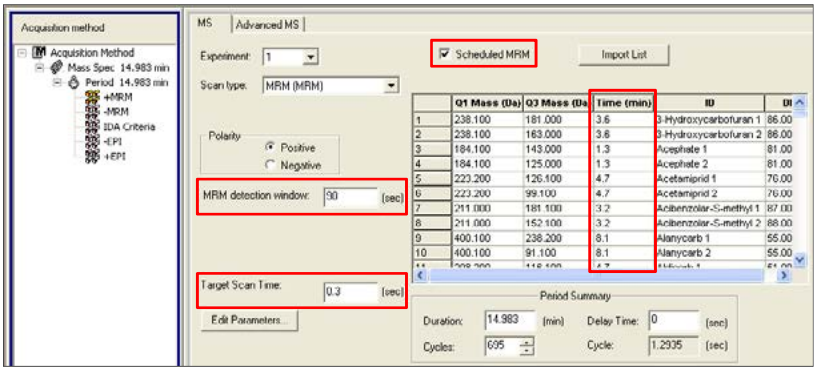

Figure 3. Acquisition method interface for *Scheduled* MRM™, in addition to traditional MRM parameters, the user provides retention times of all analytes, an MRM detection window, and a Target scan time. The software then automatically designs and optimizes the *Scheduled* MRM™ acquisition method.

Quantitative Performance

The developed LC-MS/MS method delivered excellent quantitative data. Calibration standards were injected over the range of 0.1 to 100 ng/mL. For a maximum residue level of 10 µg/kg, the limit of quantitation (LOQ) will depend on the dilution factor of the extract. Here we used a dilution factor of 10x, 20x, or 50x, respectively, depending on the matrix to be analyzed. Therefore, an LOQ of at least 0.2 ng/mL was required for the 50x dilution. Example chromatograms of pesticides detected at 0.2 ng/mL using two MRM transitions are shown in Figures 4a-d.

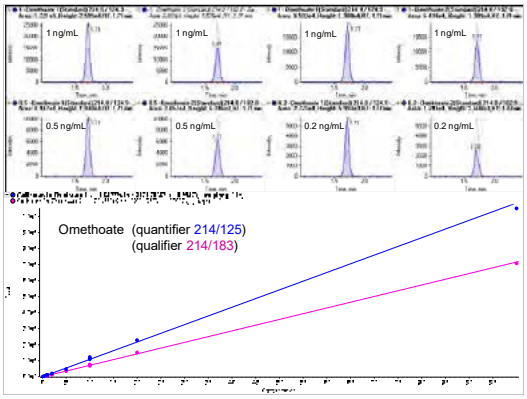

Figure 4a. Calibration lines of the quantifier and qualifier MRM transition of Omethoate from 0.1 to 100 ng/mL

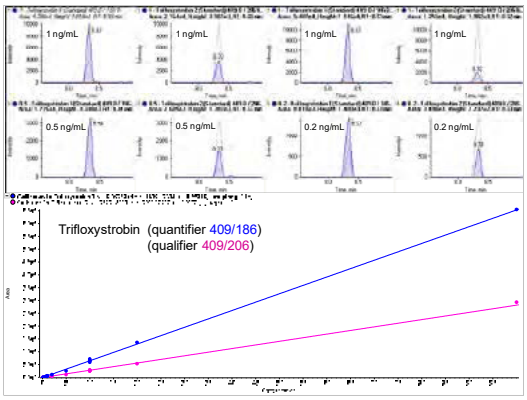

Figure 4b. Calibration lines of the quantifier and qualifier MRM transition of Trifloxystrobin from 0.1 to 100 ng/mL

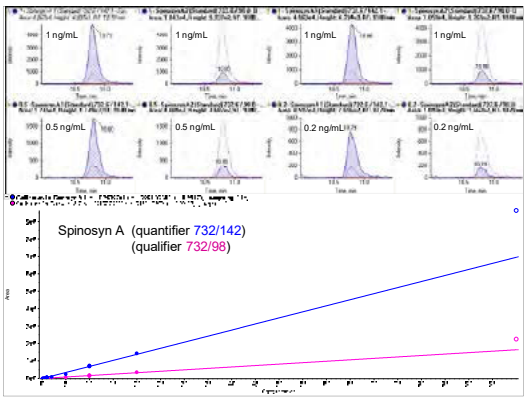

Figure 4c. Calibration lines of the quantifier and qualifier MRM transition of Spinosyn A from 0.1 to 100 ng/mL

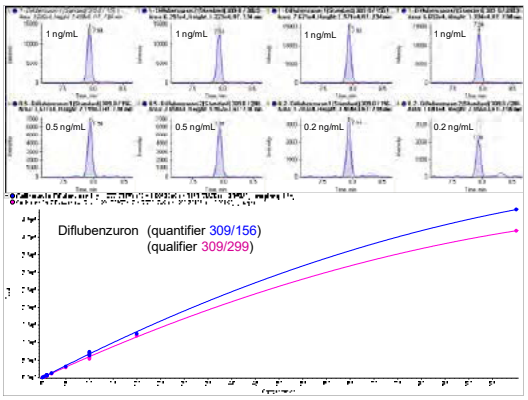

Figure 4d. Calibration curves of the quantifier and qualifier MRM transition of Diflubenzuron from 0.1 to 100 ng/mL

Calibration standards were injected from 0.1 to 100 ng/mL (Figure 4a-d). Accuracy between 80 and 120% were achieved for all targeted pesticides over the entire calibration range. Data points of the lowest or highest standards were excluded for a few pesticides with weak or strong ionization, respectively.

Reproducibility was investigated by repeat injections at 1 and 10 ng/mL (n = 5). The coefficients of variation (%CV) were typically found to be much below 10% for both MRM transitions.

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These excellent quantitative results highlight the advantage of combining *Scheduled* MRM™ with fast polarity switching for a comprehensive multi-target quantitative screen.

Findings in Fruit and Vegetable Samples

The developed method was applied to the quantitation of pesticides in real food extracts. Example chromatograms are shown in Figures 5a-e. The findings are also summarized in Table 1.

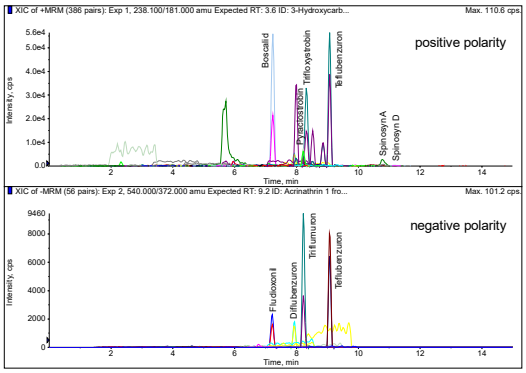

Figure 5a. Pear sample (extract 10x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

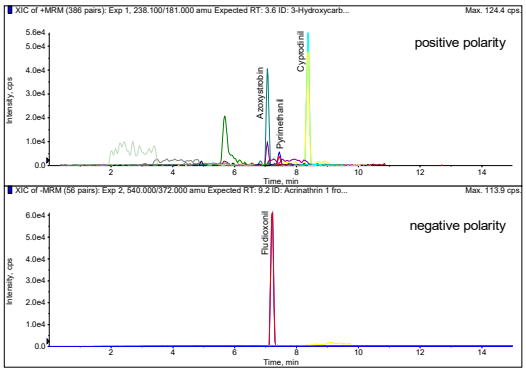

Figure 5b. Organic raspberry sample (extract 10x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

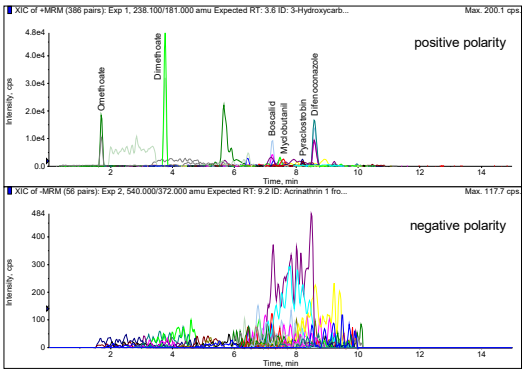

Figure 5c. Carrot sample (extract 10x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

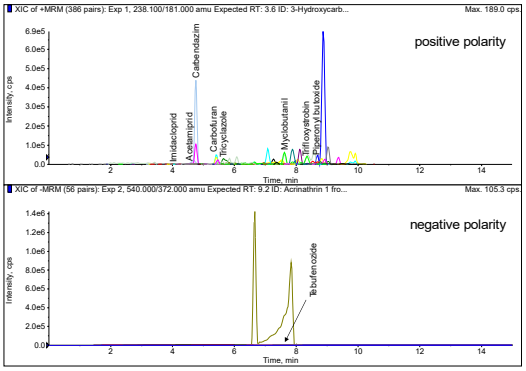

Figure 5d. Curry powder sample (extract 50x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

Table 1. Summary of pesticide findings in real samples above 1 µg/kg (findings above the MRL of 10 µg/kg are highlighted)

Sample	Pesticide	Concentration (µg/kg)
Pear	Boscalid	**150**
	Diflubenzuron	1.3
	Pyraclostrobin	7.0
	Spinosyn A	7.3
	Spinosyn D	4.2
	Teflubenzuron	**16**
	Trifloxystrobin	**32**
	Triflumuron	1.3
Organic raspberry	Azoxystrobin	**38**
	Cyprodinil	**71**
	Fludioxonil	7.2
	Pyrimethanil	**26**
Carrot	Boscalid	**26**
	Difenoconazole	**24**
	Dimethoate	**16**
	Myclobutanil	**11**
	Omethoate*	8.5
	Pyraclostrobin	5.4
Curry powder	Acetamiprid	**59**
	Carbendazim	**1300**
	Carbofuran	**51**
	Imidacloprid	5.4
	Myclobutanil	**960**
	Piperonyl butoxide	**39**
	Tebufenozide	4.9
	Tricyclazole	**45**
	Trifloxystrobin	**18**
Raisin	Acetamiprid	**20**
	Azoxystrobin	**21**
	Boscalid	**29**
	Buprofezin	**11**
	Carbendazim	**76**
	Cyprodinil	1.7
	Fenpyroximate	8.7
	Fludioxonil	1.0
	Flufenoxuron	**36**
	Hexythiazox	**10**
	Imazalil	**10**
	Indoxacarb	**58**
	Metalaxyl	7.9
	Methoxyfenozide	**11**
	Myclobutanil	**65**
	Penconazole	**17**
	Propargite	**100**
	Pyrimethanil	**417**
	Quinoxyfen	**10**
	Tetraconazole	**10**
	Trifloxystrobin	**14**

* identified as false positive by MS/MS library searching

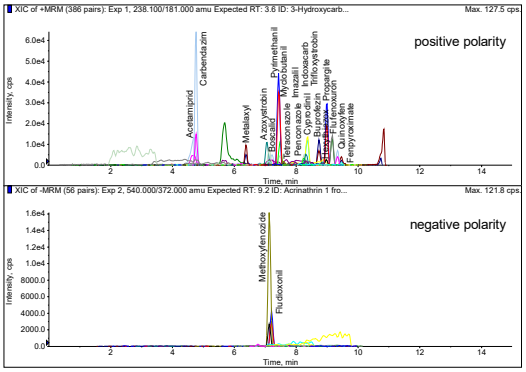

Figure 5e. Raisin sample (extract 20x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

Sample data was processed using MultiQuant™ software version 2.1 with the 'Multicomponent' query. Query files are customizable commands to perform custom querying of the result table. Here we used the 'Multicomponent' query to automatically calculate and compare MRM ratios for compound identification and to highlight concentrations above a specified maximum residue level. An example of the results and peak review after running the query file is shown in Figure 6.

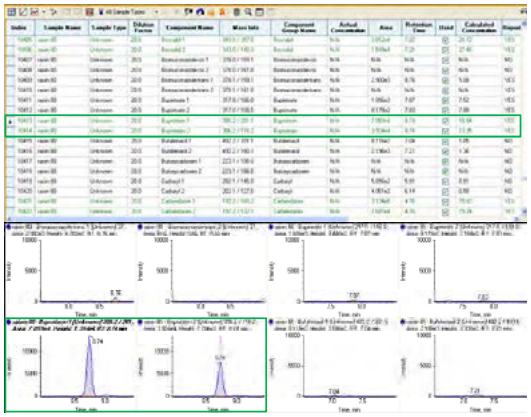

Figure 6. Results and peak review after running the 'Multicomponent' query in MultiQuant™ software, shown here is an example from raisins, of pesticides detected above an MRL of 10 µg/kg and positively identified by automatic MRM ratio calculation (compare to Figure 5d and Table 1 for complete results).

Compound Identification using MS/MS Library Searching

Despite the high selectivity of MRM detection, there is always a risk of false positive findings due to interfering matrix signals. Typically a second MRM is monitored per analyte and the ratio of quantifier to qualifier transition is calculated for each unknown sample and compared to the MRM ratio of standards for identification. However, it has been reported that relying only on MRM ratios for identification can result in a significant number of false positive results for compound identification, especially if the targeted analytes have a low fragmentation efficiency (many low intensity product ions).[7-9]

For improved accuracy, identification can be performed using full scan MS/MS experiments and library searching to compare the unknown with a standard spectrum. Here MS/MS spectra acquired in the EPI mode of the QTRAP® 5500 system and mass spectral library searching were used to increase the confidence of detection. Example spectra and library search FIT values using a new and improved MS/MS library search algorithm are shown in Figure 7.

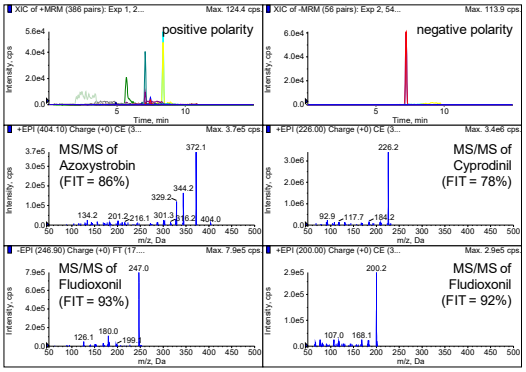

Figure 7a. Organic raspberry sample (extract 10x diluted) screened for pesticides with MS/MS library search results for additional confidence in compound identification

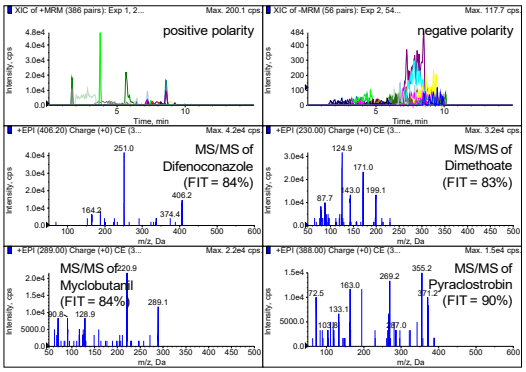

Figure 7b. Carrot sample (extract 10x diluted) screened for pesticides with MS/MS library search results for additional confidence in compound identification

Contents

The additional experiment carried out using MS/MS scanning and library searching allowed the identification of a false positive result for the carrot sample. Omethoate was not present in the sample, although the retention time and MRM ratio of Omethoate was identical to the found peak in the extract. Figure 8 shows a comparison of MRM chromatograms and MS/MS spectra.

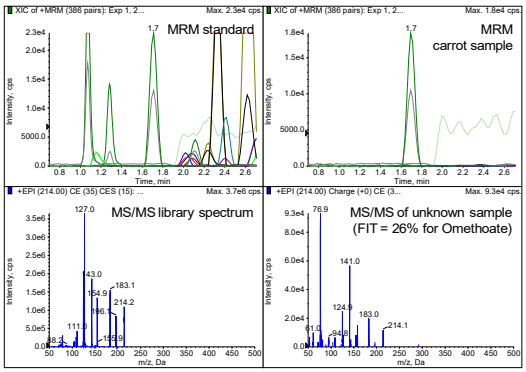

Figure 8. False positive finding identified by MS/MS library searching, standard and carrot sample have identical retention times of 1.7 min and MRM ratio of 0.6 but MS/MS spectra differ and the search results clearly prove the false positive

Summary

This new and unique LC-MS/MS method utilizing the *Scheduled* MRM™ algorithm in combination with fast polarity switching and acquisition of MS/MS spectra for compound identification has significant advantages. The method was successfully used to quantify and identify pesticides covering a broad range of chemical properties, including the acquisition of positive and negative polarity spectra.

The automatic method setup based on the Scheduled MRM™ algorithm resulted in excellent quantitative data. LOQ were measured for all pesticides at 0.1 ng/mL or below. This allows the dilution of sample extracts by up to 50x, significantly reducing matrix effects and interferences. Accuracies were typically found between 80 and 120% with %CV of less than 10%.

Different samples of fruits, vegetables, and spices were analyzed after QuEChERS extraction and dilution.

Results were processed using MultiQuant™ software with the 'Multicomponent' query. This query automatically highlights findings above a user specified threshold (like the MRL) and when identification based on MRM ratio comparison was positive.

In addition full scan MS/MS spectra were acquired using the QTRAP® 5500 system. MS/MS spectra contain the complete molecular fingerprint of each analyte and searched against a spectral library reduce the possibility of false positive and negative results. This procedure helped to identify and correct a false positive finding in one of the samples.

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Publication number: 3370411-01

Rapid Quantitation and Identification of Carbendazim in Orange Juice Using the New SCIEX QTRAP® 4500 LC-MS/MS System

Fast method development in response to contaminated orange juice imports to the U.S.

André Schreiber[1] and Lauryn Bailey[2]

[1]SCIEX Concord, Ontario (Canada); [2]SCIEX Framingham, Massachusetts (U.S.)

Introduction

Recent issues surrounding the presence of the fungicide Carbendazim in orange juice samples imported to the U.S. from Brazil, the biggest orange juice exporter in the world, have heightened the need for regulatory agencies and food manufacturers to begin proactive testing of orange juice to ensure product compliance to U.S. regulatory standards and overall consumer safety.

Carbendazim (a fungicide used to treat citrus trees in Brazil against diseases such as black spot), while approved for use in some countries, is not approved by the U.S. Environmental Protection Agency for use on oranges. The United States reportedly imports 15 percent of its orange juice supply, the majority of which comes from Brazil.[1] Given this volume of product imported, the detection of this substance has created cause for investigation and increased testing of orange juice shipments to the U.S. and throughout the world.

A fast, easy, and sensitive LC-MS/MS method was developed for the detection of Carbendazim in orange juice samples. The method utilizes a simple dilute-and-shoot approach, with UHPLC analysis using a Phenomenex Synergi-Fusion 2.5 µm column. This method, with minor adjustments, can be adapted for analysis using different SCIEX mass spectrometers, including the QTRAP® 4500 and 5500 LC-MS/MS systems.

Additionally, the acquisition method is amenable to extension for screening of up to 204 additional commonly used pesticides through incorporation of the iD*Quant*™ standards kit for pesticide analysis.

Experimental

Sample Preparation

The sensitivity and selectivity of the SCIEX QTRAP® systems allow minimal sample preparation for this analysis. Orange juice samples were simply centrifuged at high speed, an aliquot of the supernatant was diluted 5-fold with water, and the sample was ready for LC-MS/MS analysis.

However, to achieve even lower limits of quantification, samples may be prepared through an SPE clean-up procedure optimized for Carbendazim.[2]

LC

LC separation was achieved using the Eksigent ekspert™ ultraLC 100 with a Phenomenex Synergi-Fusion 2.5 um (2 x 50 mm) column with a gradient of water and methanol containing 10 mM ammonium formate at a flow rate of 0.5 mL/min. The injection volume was set to 10 µL.

Contents

MS/MS

The SCIEX QTRAP® 4500 and 5500 systems are highly suitable for this analysis allowing simultaneous quantification using Multiple Reaction Monitoring (MRM) and identification based on Enhanced Product Ion (EPI) scanning with library searching. The Turbo V™ source was used with an Electrospray Ionization (ESI) source. Two selective MRM transitions were monitored for Carbendazim as outlined in Table 1. EPI spectra were acquired using dynamic fill time and Collision Energy Spread (CES) for highest spectral quality.

Table 1. MS/MS Parameters for Carbendazim using the SCIEX Q TRAP® 4500 system

MRM	Q1/Q3	DP (V)	CE (V)
Carbendazim 1	192/160	56	27
Carbendazim 2	192/132	56	41

Results and Discussion

First, limit of detection (LOD), limit of quantitation (LOQ), linearity, and reproducibility were evaluated using injections of the iD*Quant*™ Standards Kit for Pesticide Analysis ranging in concentration from 0.05 to 100 ng/mL.

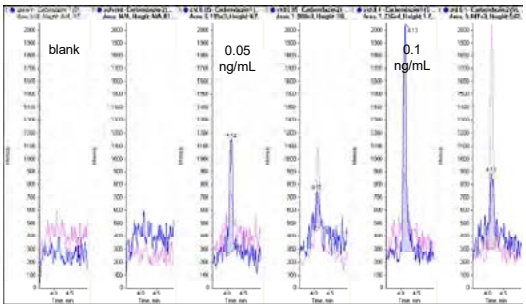

Figure 1. Determination of LOD and LOQ of detection of Carbendazim, LOD was found at 0.05 ng/mL and LOQ at 0.1 ng/mL using the SCIEX QTRAP® 4500 system

The LOD was determined based on Signal-to-Noise (S/N) calculated with an algorithm using 3x standard deviation. The S/N at a concentration of 0.05 ng/mL was 5. The LOQ was determined based on reproducibility. The coefficient of variation (%CV) at 0.1 ng/mL was 7.0% (Figure 1 and Table 2).

This level of sensitivity allows the direct injection of orange juice samples without using time-consuming and extensive sample cleanup. Juice samples were injected directly after centrifugation and a simple dilution to minimize any possible matrix effects.

The linearity obtained for both MRM transitions for Carbendazim are shown in Figure 2. Results showed linearity with regression coefficients of > 0.999, sufficient to analyze for Carbendazim in juice samples, particularly at the FDA action level of 10 parts per billion (ppb)[3] and the EU maximum residue level of 200 mg/kg.[4-5]

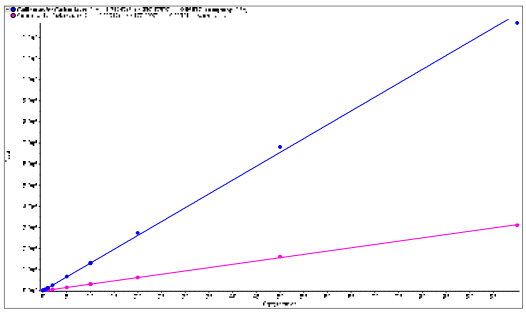

Figure 2. Linear range of the detection of Carbendazim from 0.05 to 100 ng/mL with an r > 0.999 for both MRM transitions

Table 2. Reproducibility and accuracy over the entire linear range when quantifying Carbendazim

Concentration (ng/mL)	# of injection	Accuracy (%)	% CV
0.050	1	88.6	-
0.100	3	98.0	7.0
0.200	1	109.0	-
0.500	1	100.1	-
1.000	10	98.0	3.6
2.000	1	104.7	-
5.000	1	104.0	-
10.00	3	100.1	0.4
20.00	1	104.5	-
50.00	1	104.1	-
100.0	1	96.8	-

Reproducibility was investigated by repeated injections of spiked juice at a concentration of 1 ng/mL. Both MRM transitions showed excellent %CV as shown in Figure 3.

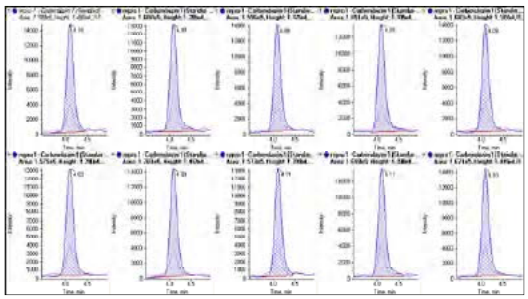

Figure 3. Reproducibility at 1 ng/mL with a %CV of 3.6 and 5.7%, respectively, for both MRM transitions

Several orange juice samples were purchased from a local store and analyzed by the method described. The MRM chromatograms of two samples are shown in Figure 4. When quantified against the standard calibration curve and corrected for dilution, the samples were determined to contain 13 ng/mL and 67 ng/mL of Carbendazim, respectively.

The MRM ratio of quantifier and qualifier transitions was used to identify Carbendazim in both samples. The 'Multicomponent' query in MultiQuant™ software automatically calculates and compares MRM ratios for identification and flags samples with a concentration of the targeted analytes above a specific concentration

Figure 4. Quantitation and identification of Carbendazim in store bought orange juice using 'Multicomponent' query in MultiQuant™ software

To further confirm the identification of Carbendazim in both samples, the automatically collected EPI spectra were evaluated with a search against our pesticide MS/MS library (iMethod™ application pesticide LC-MS/MS library version 1.1). The results revealed a library FIT of 93% and 97%, respectively, for the MS/MS spectrum (Figures 5 and 6), further verifying the presence of Carbendazim in the juice sample, adding an extra level of confidence in the results.

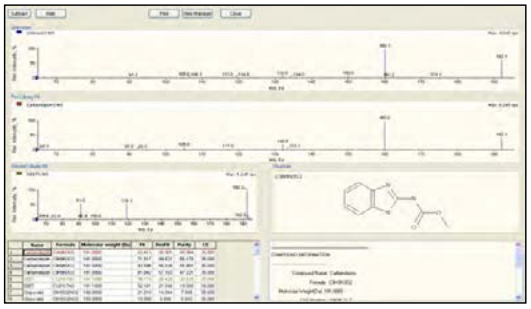

Figure 5. Library search of automatically collected EPI spectra of the orange juice sample 1 identifying 13 ng/mL Carbendazim with a library FIT of 93%

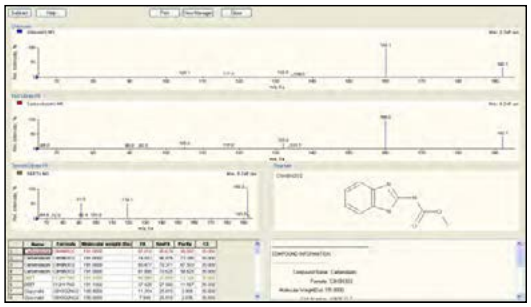

Figure 5. Library search of automatically collected EPI spectra of the orange juice sample 2 identifying 67 ng/mL Carbendazim with a library FIT of 97%

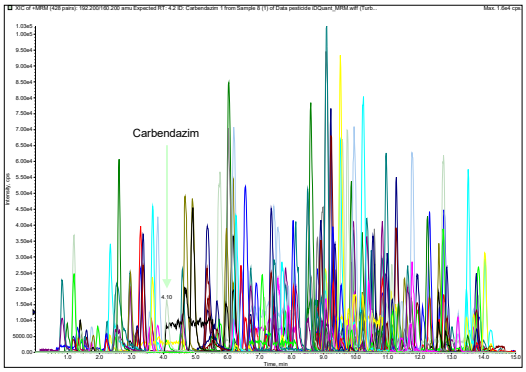

Figure 7. Comprehensive pesticide screening using LC-MS/MS and the iDQuant™ standards kit for pesticide analysis

Summary

The method and data presented here showcase the fast, easy, and accurate solutions for the analysis of Carbendazim in orange juice by LC-MS/MS. The SCIEX QTRAP® 4500 and 5500 systems provide excellent sensitivity and selectivity for this analysis, with minimal sample preparation allowing maximized throughput for the analysis of many samples in a short time period.

The approach also lends itself to be extended for the screening of many different pesticides through the use of the iD*Quant*™ kit for pesticide analysis and MS/MS library searching, which would be ideal to identify any additional potential contaminants that could arise in the future (Figure 7).

References

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3 http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/FruitsVegetablesJuices/ucm287783.htm

4 Regulation (EC) No 396/2005 'on maximum residue levels of pesticides in or on food and feed of plant and animal origin' with amendment (EC) No 299/2008

5 Regulation (EC) No 149/2008 'Annexes II setting maximum residue levels' with amendment (EC) No 559/2011

Additional resources and information

For more details on free iMethod™ applications for the analysis of Carbendazim in orange juice, as well as for details on iMethod™ applications for general pesticide screening, visit www.sciex.com or contact us as support@absciex.com.

Publication number: 4980212-02

The Use of Micro Flow UHPLC in Pesticide Screening of Food Samples by LC-MS/MS

Reduce costs without sacrificing analytical performance by the use of micro LC

Stephen Lock
SCIEX Warrington (UK)

Introduction

Traditionally in pesticide screening of food, samples are prepared using generic extraction procedures, like QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)[1, 2] and then analyzed by LC-MS/MS or GC-MS/MS. Usually in LC-MS/MS analysis, LC flow rates exceed 400 µL/min and are used in combination with small particle size HPLC columns with high pressures to maintain sharp peaks and fast chromatography. These flow rates produce excellent peak shapes and results, but have a draw back in that they require higher volumes of organic solvents. The consumption of HPLC organic solvents, such as acetonitrile and methanol, is a growing cost of analysis, and their disposal can have an adverse environmental impact. Therefore, new approaches to reduce solvent consumption in pesticide residue testing will be beneficial to the environment while also reducing the running costs of a testing lab.

Here we present new data using Eksigent ekspert™ microLC 200 System in combination with a LC-MS/MS method developed on an SCIEX QTRAP® 4500 system and utilizing the *Scheduled* MRM™ algorithm to maximize the number of data points across each peak. This approach was applied to a screen of over 100 pesticides in QuEChERS food extracts, and for the majority of these tests, the method was applied to an extract from chili powder, a matrix notorious for producing dirty extracts.

Materials and Methods

Sample Preparation

For linearity and sensitivity tests, calibration standards were prepared in water from concentrations 0.2 – 100 parts-per-billion (ppb). Chili powder and fresh basil were extracted using a QuEChERS method supplied with a kit from Supelco. Herb or spice (5 g) was mixed with water (10 mL) and acetonitrile (10 mL containing 0.05% acetic acid) in a 50 mL PTFE tube. Dispersive SPE (dSPE) $MgSO_4$ QuEChERS salts were added and the tube shaken (1 min) and centrifuged (5 min, 3500 rpm). The top layer (6 mL) was mixed with a dSPE PSA/C18 clean-up mixture and shaken (1 min) and centrifuged (5 minutes, 3500 rpm). The supernatant (100 µL) was diluted with water (900 µL) and injected (2 µL).

LC Conditions for Eksigent ekspert™ microLC 200 System

The LC system used for these tests was the Eksigent ekspert™ microLC 200. The system was run at 40 µL/min, which is at least 10 times lower than conventional LC separations using a 4.6 mm ID column. The separation of the 2 µL injection was done using a 0.5 x 50 mm Halo C18 column held at 50 ºC and with the

gradient profile shown in Table 1 where A = water and B = methanol, with both phases containing 2 mM ammonium acetate and 0.1% formic acid.

LC Conditions for UHPLC

The LC system used for comparative tests was a Shimadzu UFLC$_{XR}$ system consisting of two Shimadzu LC20AD pumps, SIL 20AC autosampler and a CTO20A column oven. The system was run at 400 µL/min with a conventional 4.6 x 5.0 mm Kinetex 2.6 µm core shell HPLC column held at 50ºC for a direct comparison. The same injection volume of 2 µL and gradient separation (Table 1) was used with the same mobile phases as with the micro flow LC analysis.

Table 1. Gradient conditions used for separation

Eksigent ekspert™ microLC 200			UHPLC		
Time (min)	A %	B %	Time (min)	A %	B %
0.0	98	2	0.0	98	2
2.0	98	2	2.0	98	2
9.5	30	70	9.0	30	70
10.5	5	95	10.5	5	95
11.0	5	95	11.5	5	95
11.5	98	2	11.5	98	2
15.0	98	2	15.0	98	2

M/MS Conditions

In this work, the SCIEX QTRAP® 4500 LC/MS/MS system (Figure 1) was used in positive mode with an IonSpray voltage (IS) of 5500 V. The method was set-up to detect 125 pesticides (250 MRM transitions), in a single injection, taken from the list contained in the SCIEX iDQuant™ Standards kit. Data was acquired using the *Scheduled* MRM™ algorithm.

For the high flow injection using the Shimadzu UHPLC, a standard electrospray electrode and Turbo V™ probe was used with a source temperature of 550 ºC, gas 1 (nebulizer gas) setting of 50 psi and a gas 2 (heater gas) settings of 60 psi. When the micro LC was used, the electrode was changed to a micro LC hybrid electrode (50 µm ID).[3] The installation of the micro LC electrode was fast and simple, requiring only the replacing of the standard electrode, taking approximately one minute for the exchange. The micro LC electrode is a hybrid PEEKSIL/stainless steel tip electrode, designed for low dead volume to eliminate peak dispersion and improve peak shape. The source settings were set-up for low flows, utilizing a lower source temperature and lower gas flow settings; however, the MRM settings were the same as used in the high flow method. This enables easy transfer of methods from a traditional high flow HPLC to the new Eksigent ekspert™ microLC 200 system.

Results and Discussion

In this work, all data was acquired and processed using Analyst® software version 1.6 and MultiQuant™ software version 2.1. The aim of this work was to test the micro flow LC applicability for routine food testing and compare the sensitivity and performance with a traditional, higher flow method already established for pesticide analysis. In this study, the chromatography was not optimized for speed, although the micro flow LC methods could be optimized to reduced run times, if desired (described briefly at the end of this application note). To compare the micro flow LC method with a higher flow analysis, a 2 ppb standard was injected. Extracted ion chromatograms comparing 2 pesticides eluting at different regions of the chromatograms are shown in Figure 1.

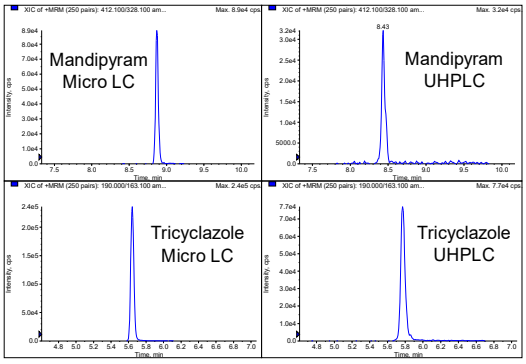

Figure 1. A comparison of micro flow LC and high flow LC

This result shows that the micro flow LC produces similar peak shapes when compared to normal flow rates due to the very low dead volume of the system. The comparative sensitivities are shown in Table 2, where a list of 10 pesticides spanning the run was compared. The results clearly demonstrate the increases in response, which ranged from a 3 fold to > 10 fold increase across the chromatographic separation (signal / noise values were taken directly from the MultiQuant™ software).

Table 2. Comparison of the signal / noise observed from a 2 µL injection of a 2 ppb standard using micro flow LC versus high flow LC

Pesticide	Retention time (min)	Signal / Noise micro LC	Signal / Noise UHPLC
Monocrotophos	4.05	1083.5	229
Tricyclazole	5.62	758.4	56.8
Simetryn	6.18	414.8	126.3
Monolinuron	6.89	432.6	40.2
Isoproturon	7.57	613.5	65.7
Terbutryn	8.03	883.7	92.5
Flutolanil	8.77	416.9	80.7
Fenoxycarb	9.44	99.8	16.7
Pyridaben	10.62	903.7	22.9

To confirm that the carryover between injections was very low, a 100 ppb standard was injected (producing a saturated response for most of the pesticides) followed by a water blank (Figure 2). For the majority of the pesticides, no carryover was observed in the water blank, with overall carryover estimated at < 0.1%.

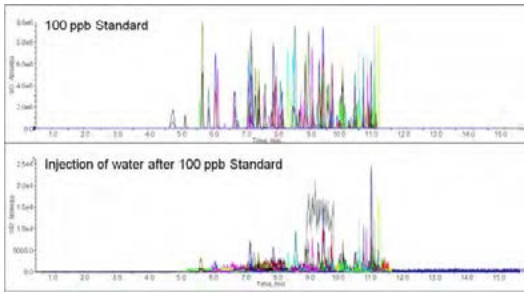

Figure 2. The top pane shows a 100 ppb calibration standard injected using the micro flow LC MS/MS set-up. The bottom pane shows water injected directly after this standard showing very low carryover.

The linearity of response for Flutolanil, analyzed using micro flow LC, is shown in Figure 3. This curve clearly demonstrates that the linearity of the method is preserved using micro flow LC, and this result is typical of what was observed for other pesticides in this analysis.

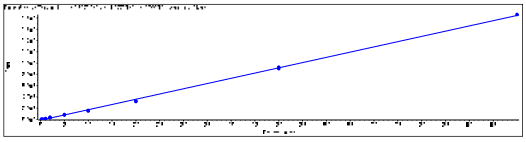

Figure 3. Example of a calibration line for one of the pesticides, Flutolanil, from 0.2 to 100 ppb. The fit used was Linear and the 'r' value obtained was greater than 0.999.

The robustness of the micro flow LC was also evaluated. In these tests, the system was stressed by repeatedly injecting unfiltered diluted QuEChERS extract of chili powdered (totaling over 150 injections). The retention time stability (Figure 4), response (Figure 5), and pressure curves (Figure 6) were then compared to see if the system had been affected by the large number of crude samples injected. The results showed outstanding reproducibility for the duration of the 150 injections, showing that micro flow LC is very robust and capable of withstanding long analytical runs that include 'dirty matrix' samples.

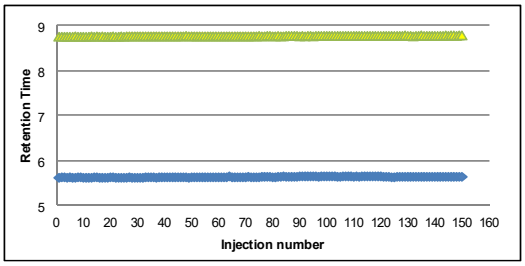

Figure 4. In this graph, retention time of two pesticides, Flutolanil (top) and Tricyclazole (bottom) were plotted against the injection number. The graph shows that the retention times obtained are rock solid with little or no variation between injections, confirming the low dead volume of the system and that fast equilibration times are possible.

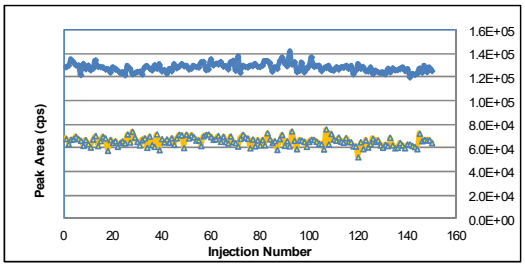

Figure 5. This graph shows the peak areas of two pesticides, Flutolanil (bottom) and Tricyclazole (top), which elute at different times during the run. It shows that the robustness is excellent with no deterioration in response even after 150 injections of a crude spice extract.

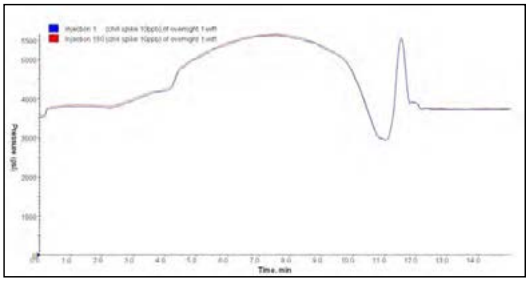

Figure 6. This figure compares the pressure profiles obtained from two injections of chili extract, 150 injections apart.

Finally, an additional advantage of micro flow LC is the ability to shorten the run times due to the low dead volume of the system. An example of this is shown in Figure 7 where the run time has been shortened from 15 minutes to less than 5 minutes. In this example, 6 µL of a 1 ppb pesticide standard containing over 200 pesticides was injected at 30 µL / min onto the same type HALO C18 column used in the above chilli extract analysis. The sensitivity was excellent, and the peak heights for some of the pesticides exceeded 1 million cps.

Conclusions

This study has clearly demonstrated that using micro flow LC is a valid approach in residue analysis in food samples.

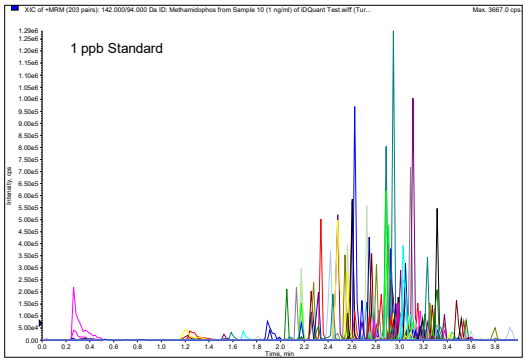

Figure 7. An example of the rapid gradient conditions that can be achieved using micro flow LC for pesticide residue analysis.

The method using the Eksigent ekspert™ microLC 200 system was quick, sensitive, robust and reproducible but also provides a huge cost saving to labs. With LC grade acetonitrile running at a cost of £100/L, this 3 day study could have cost about £ 100 with convention chromatography (0.6 mL/min running for 24 hours per day) and less than £10 with micro flow LC. Over one year, this corresponds to a savings of over £4000 (£90 x 50 weeks) in solvent consumption alone.

In addition, due to the very low dead volume of the micro flow LC, run times can easily be reduced by speeding up the gradient, greatly improving throughput for high volume testing laboratories. Finally, a great added benefit of micro flow LC analysis is the improvement in sensitivity, allowing greater dilution of sample extracts and the use of lower injection volumes to reduce matrix effects and improve robustness of the whole analysis.

References

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Publication number: 6330212-01

Quantitation and Identification of Organotin Compounds in Food, Water, and Textiles Using LC-MS/MS

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Overview

Organotin compounds are chemicals composed of tin linked to hydrocarbons, used in industrial materials and various biocides and fungicides. As a result, organotin compounds can enter the environment through a number of channels, and can often be found in seawater, seafood, fruits and vegetables, and consumer goods. Due to the toxicity of these compounds, there is a need for analytical methods allowing accurate quantitation and identification. Here we present an LC-MS/MS method to measure tributyltin, fentin, cyhexatin, and fenbutatin oxide in different matrices. Triphenyl phosphate was used as the internal standard.

Spiked apple, potato, synthetic seawater, and textile samples were prepared using a quick and easy acetonitrile extraction. Organotin compounds were detected using an SCIEX 4000 QTRAP® system with Electrospray Ionization (ESI) using Multiple Reaction Monitoring (MRM). Detection limits were determined to be well below regulated levels, enabling extra dilution of the sample extract to minimize possible matrix effects.

Introduction

Organotin (organostannic) compounds are chemical compounds comprised of tin with hydrocarbon substituents. Organotin compounds are widely used as additives in plastic material, wood preservatives, marine biocides, and agricultural pesticides.

Tri-substituted organotin compounds were previously widely used as antifouling agents in paints on ships. However, such paints were found to release organotin compounds into the aquatic environment, where they can accumulate in sediments and organisms or degrade to less substituted toxic compounds. Studies have shown that trace amounts of organotin compounds can have significant detrimental effects on aquatic organisms. For instance, tributyltin (TBT), present in sea water at ng/L levels, has been identified as an endocrine disruptor promoting harmful effects on aquatic organisms. Therefore, the use of organotin compounds in antifouling paints is prohibited or restricted in many countries.[1-3]

The use of organotin compounds in consumer products, such as textiles, footwear, wall and floor coverings, etc., has been found

to pose a risk to human health, particularly for children. Therefore, the use of tri-substituted and di-substituted organotin compounds, including TBT, tributyltin (TPhT), dibutyltin (DBT), and dioctyltin (DOT) in consumer products is restricted.[4-5]

Finally, organotin compounds enter the human diet through contaminated seafood and the use as agricultural pesticides. International maximum residue limits (MRL) have been established by Codex Alimentarius and the EU for many food commodities, with some MRL as low 50 µg/kg.

Traditionally gas chromatography coupled to mass spectrometry (GC-MS) was used for analysis of organotin compounds. However, the analysis by GC requires time consuming derivatization, because of poor compound volatility, and long chromatographic run times. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) allows simplifying sample preparation and shortening run times due to increased selectivity and sensitivity and, thus, is evolving as a preferred technique for the analysis of organotin compounds.

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Method Details

Sample Preparation

TBT chloride, fentin acetate, cyhexatin and fenbutatin oxide were purchased from Sigma-Aldrich and spiked into four matrices (apple, potato, synthetic seawater (drinking water with 35 g salt per liter), and textile material). Triphenyl phosphate (TPP) was used as the internal standard.

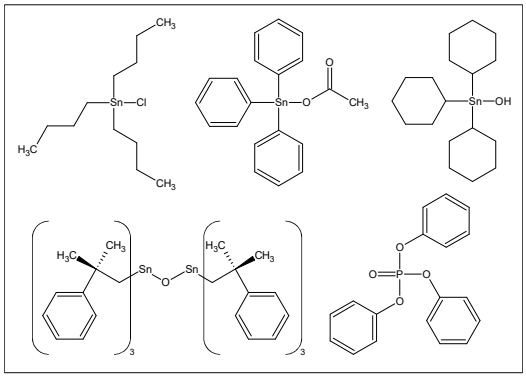

Figure 1. Target organotin compounds: TBT chloride, fentin acetate, cyhexatin, fenbutatin oxide, and internal standard triphenyl phosphate (top left to bottom right)

Spiked samples were extracted using acetonitrile and diluted 10x with LC grade water prior to LC-MS/MS analysis. The spiked synthetic seawater was directly injected for detection of organotin compounds. Note that additional dilution is possible depending on required limits of detection to reduce possible matrix effects (Figure 2).

UHPLC Separation

A Shimadzu UFLC$_{XR}$ system was used with a Phenomenex Kinetex 2.6u C18 50x3mm column at 40ºC. A gradient of water with 2% formic acid + 5 mM ammonium formate and methanol with 2% formic acid + 5 mM ammonium formate at a flow rate of 800 µL/min resulted in a total run time of 12 minutes.

The injection volume was set to 20 µL for apple and potato extracts and 50 µL for textile extracts and synthetic seawater.

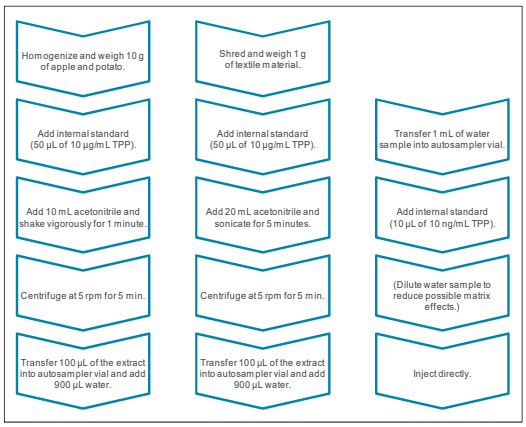

Figure 2. Sample preparation protocols for the analysis of organotin compounds in fruit and vegetable, textiles, and water

MS/MS Detection

The SCIEX 4000 QTRAP® LC/MS/MS system with Turbo V™ source and ESI probe was used. All the analytes and internal standard were detected in positive polarity using MRM for best selectivity and sensitivity. Two MRM transitions were monitored for each compound to allow quantification and identification using the characteristic MRM ratio. The *Scheduled* MRM™ algorithm was activated for best data quality (Table 1).

The data was processed in MultiQuant™ software version 2.1.

Table 1. MRM transitions and retention times (RT) of targeted organotin compounds

Organotin compound	Q1 (amu)	Q3 (amu)	RT (min)
TBT 1	291.0	123.0	3.8
TBT 2	291.0	235.1	3.8
Fentin 1	351.0	120.0	3.0
Fentin 2	351.0	197.0	3.0
Cyhexatin 1	369.0	205.0	5.3
Cyhexatin 2	369.0	287.1	5.3
Fenbutatin oxide 1	519.1	351.0	6.2
Fenbutatin oxide 2	517.1	349.0	6.2
TPP (internal standard)	326.9	152.1	4.4

Results and Discussion

Chromatography conditions were important for successful determination of organotin compounds by LC-MS/MS. Organotin compounds are known for strong interaction with reversed phase material resulting in peak broadening. A strong acidic mobile phase was used to reduce this effect and to optimize peak shape.[8]

Two chromatographic interferences were observed for TBT in all matrices. Thus, stable retention times and good separation was important. A core-shell column (Phenomenex Kinetex) was used for improved UHPLC performance while operating at reduced column pressure (Figure 3).

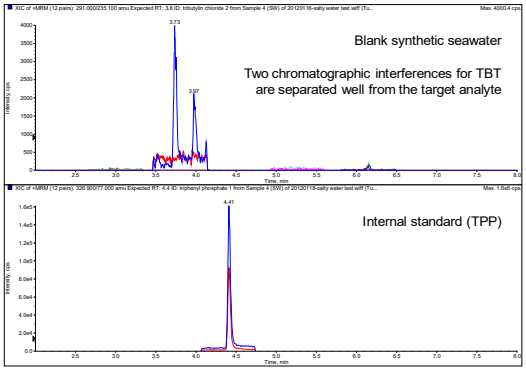

Figure 3. Blank synthetic seawater, two chromatographic interferences for TBT are separated well from the target analyte (top) and internal standard (bottom)

Apple, potato, textile, and synthetic seawater samples were spiked at different concentrations, extracted, and analyzed using the fast LC-MS/MS method. Example chromatograms are shown in Figures 4 and 5.

The achieved Signal-to-noise (S/N) ratios are listed in Table 1. S/N values were measured in MultiQuant™ software after applying a 2x Gaussian smooth. S/N values were used to estimate limits of quantitation (LOQ) for all analytes in each matrix.

Table 2. Signal-to-noise (S/N) in different matrices

Organotin compound	Apple (2 µg/kg)	Potato (2 µg/kg)	Textile (0.1 mg/kg)	Seawater (50 ng/L)
TBT 1	105	71	93	53
Fentin 1	355	315	209	186
Cyhexatin 1	240	197	51	133
Fenbutatin oxide 1	339	377	66	176

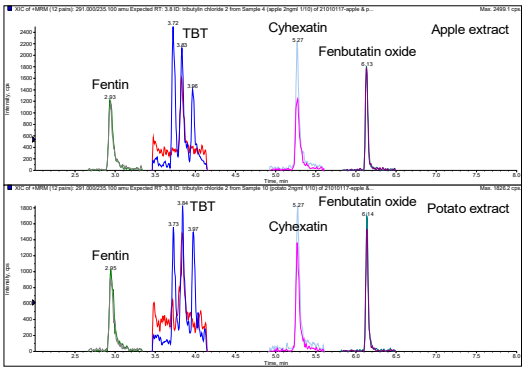

Figure 4. Apple (top) and potato (bottom) sample spiked at 2 µg/kg and diluted 10x after extraction

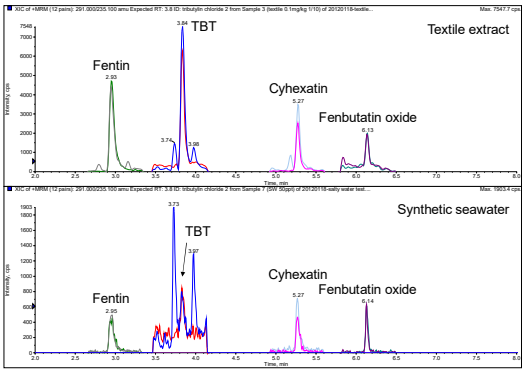

Figure 5. Textile material spiked with 0.1 mg/kg and diluted 10x after extraction (top) and synthetic seawater spiked at 50 ng/L and analyzed by direct injection (50 µL)

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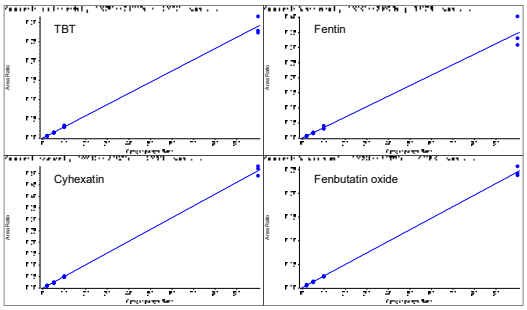

Figure 6. Calibration lines of organotin compounds in apple matrix (2 to 100 µg/kg)

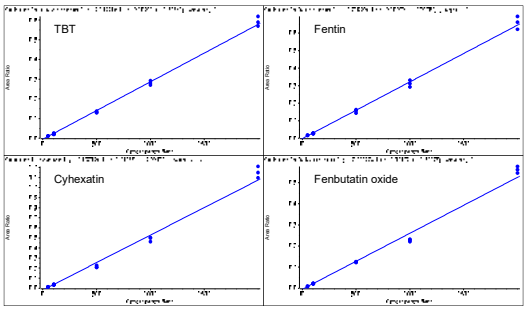

Figure 7. Calibration lines of organotin compounds in synthetic seawater (50 to 2000 ng/L)

Table 3. Estimated limits of quantitation (LOQ) in different matrices based on S/N of 10

Organotin compound	Apple µg/kg	Potato (µg/kg)	Textile (µg/kg)	Seawater (ng/L)
TBT	0.2	0.3	10	10
Fentin	< 0.1	< 0.1	< 10	< 10
Cyhexatin	0.1	0.1	20	< 10
Fenbutatin oxide	< 0.1	< 0.1	15	< 10

The linear dynamic range was evaluated from 2 to 100 µg/kg for apple and potato, from 0.1 to 1 mg/kg for textiles, and from 50 to 2000 ng/L for seawater. Example calibration lines of all four organotin compounds in apple and synthetic seawater are shown in Figures 6 and 7.

Repeatability was found to be less than 15% coefficient of variation (%CV) and accuracy between 85 and 115% for all compounds at all concentrations (Table 4).

Table 4. Repeatability (%CV) and accuracy of organotin compounds at the lowest point of the calibration line

	Apple (2 µg/kg)		Potato (2 µg/kg)		Textile (0.1 mg/kg)		Seawater (50 ng/L)	
Organotin compound	%CV	Accuracy (%)	%CV	Accuracy (%)	%CV	Accuracy (%)	%CV	Accuracy (%)
TBT	10.0	97.0	13.9	86.4	7.3	95.6	6.3	113.1
Fentin	9.9	101.4	12.4	96.8	4.7	95.8	7.9	112.6
Cyhexatin	5.9	108.5	2.4	88.4	3.6	93.3	4.2	115.0
Fenbutatin oxide	11.4	104.4	11.8	99.5	13.2	97.3	3.6	107.4

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Compound identification was achieved using the 'Multicomponent' query in MultiQuant™ software. This query automatically calculates and compares MRM ratios for identification and highlights concentrations above a user specified residue level. Examples of the result table and peak review after running the query file are shown in Figures 8 and 9.

Figure 8. Automatic compound identification using the 'Multicomponent' query (example cyhexatin in potato)

Figure 9. Automatic compound identification using the 'Multicomponent' query (example fentin in textile)

Summary

A quick, easy, and robust LC-MS/MS method for the determination of different organotin compounds in food, seawater, and textile materials was developed. The method allows accurate and reproducible quantification using the selectivity and sensitivity provided by the SCIEX 4000 QTRAP® system operated in MRM mode. Detection limits well below regulated levels allow sample extract dilution to minimize possible matrix effects. Confident compound identification was achieved through the automatic calculation of MRM ratios using the 'Multicomponent' query in MultiQuant™ software.

References

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6 http://www.codexalimentarius.org/standards/pesticide-mrls/en/

7 Council Directive 'maximum levels for pesticide residues' 96/32/EC

8 EU Reference Laboratory for Single Residue Methods: 'Analysis of Organotin Compounds via QuEChERS and LC-MS/MS – Brief Description' www.crl-pesticides.eu

Publication number: 6690212-01

Comprehensive Quantitation and Identification of Pesticides in Food Samples Using the New Eksigent ekspert™ ultraLC 100 and the SCIEX QTRAP® 4500 System

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Overview

Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) is a widely used analytical tool for the screening of food residues and contaminants. Here we present a new and method using QuEChERS extraction, separation using the new Eksigent ekspert™ ultraLC 100 system with a Phenomenex Synergi™ 2.5u Fusion-RP column, and the SCIEX QTRAP® 4500 system. The mass spectrometer was operated in highly selective and sensitive Multiple Reaction Monitoring (MRM) mode using the *Scheduled* MRM™ algorithm was used to obtain the best data quality and combined with fast polarity switching to cover the broadest range of pesticides possible. In addition MS/MS spectra were acquired to enable compound identification with highest confidence based on mass spectral library matching.

Introduction

LC-MS/MS is a powerful analytical tool capable of screening samples for numerous compounds. MRM is typically used because of its excellent sensitivity, selectivity, and speed. As LC-MS/MS technology continues to be adapted demands are made to detect and quantify an increasing number of compounds in a single run.

Generic extraction procedures, like QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) and ultra high performance LC systems combined with polar embedded C18 phases with small particles, providing good resolution and excellent peak shape, made it possible to detect pesticides of a wide variety of compound classes and chemical properties in each sample.[1-3]

State-of-the-art LC-MS/MS systems make it possible to detect hundreds of pesticides and other food residues in a single run.

The new Eksigent ekspert™ ultraLC 100 is a UHPLC system designed specifically for use with SCIEX mass spectrometers sustaining pressure of up to 18000 psi at any flow rate of up to 5 mL/min. It contains a unique injector valve to maximize column life time, a side port injector needle for increased ruggedness, and the programmable needle wash greatly reduces carry-over.

The new SCIEX QTRAP® 4500 system combines the legendary sensitivity, reproducibility, and accuracy of the 4000 series with the speed and trapping capabilities of the QTRAP® 5500 system. The Turbo V™ source and Curtain Gas™ interface provide exceptional robustness and successfully reduce chemical noise. The advanced eQ™ electronics and Qurved LINAC® collision cell was designed for unparalleled speed of MRM detection and fast polarity switching for comprehensive multi-component analysis.

In addition, advanced software tools like the *Scheduled* MRM™ algorithm intelligently uses information of retention times to automatically optimize MRM dwell time of each transition and total cycle time of the experiment resulting in highest data quality.

To further increase confidence in analytical results QTRAP® technology is used to automatically acquire fast and sensitive MS/MS spectra in Enhanced Product Ion (EPI) mode and search them against mass spectral libraries for compound identification. The information of the complete molecular fingerprint saved into EPI spectra significantly reduces the risk of false positive results.

Here we present a new LC-MS/MS method utilizing the ekspert™ ultra LC 100 and the QTRAP® 4500 system using the *Scheduled* MRM™ algorithm in combination with fast polarity switching, and acquisition of MS/MS spectra for compound identification. The method was successfully applied to quantify

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and identify pesticides in a QuEChERS extracts of fruit and juice samples.

Method Details

- Different fruit and samples were extracted using Restek QuEChERS kits (Q110, Q210 and Q213) and diluted 5 times with water to optimize chromatographic peak shape and minimize possible matrix effects and interferences. Juice samples were injected directly after centrifugation and 5x dilution. The injection volume was set to 10 µL.
- The SCIEX iD*Quant*™ Standards Kit for Pesticide Analysis was used for method setup and preparation of calibration standards. Additional pesticides were added to cover all compounds of interest.
- LC separation was achieved on the new Eksigent ekspert™ ultraLC 100 with a Phenomenex Phenomenex Synergi-Fusion 2.5u 50x2 mm column and a fast gradient of water and methanol with 10 mM ammonium formate buffer at a flow rate of 0.5 mL/min.
- The new SCIEX QTRAP® 4500 system was operated with Turbo V™ source and Electrospray Ionization (ESI) probe.
- Approximately 500 MRM transitions were monitored in both positive and negative polarity. Optimized transitions for all compounds were obtained through the MRM catalogue of the iMethod™ Test for Pesticide Screening version 2.1.
- The *Scheduled* MRM™ algorithm was used in combination with fast polarity switching using Analyst® 1.6.1 Software.
- For increased confidence in compound identification EPI spectra were acquired at a scan speed of 10000 Da/s using dynamic fill time for best spectral quality and Collision Energy Spread (CES) to ensure a characteristic MS/MS pattern independently on compound's fragmentation efficiency. MS/MS spectra were search against the iMethod™ Pesticide Library version 2.1.
- MultiQuant™ 2.1 Software was used for quantitative data processing.

Results

Sensitivity, Reproducibility, Linearity and Accuracy

The *Scheduled* MRM™ algorithm uses knowledge of the retention of each analyte to monitor the MRM transition only in a short time window. Thus at any one point in time, the number of concurrent MRM transitions are significantly reduced resulting in much higher duty cycles for each analyte. The software computes maximum dwell times for the co-eluting compounds while still maintaining the desired cycle time for best data quality.[3] Combining *Scheduled* MRM™ with fast polarity switching further allows extending the target list of pesticides while maintaining throughput.

An example chromatogram of a solvent standard at 1 ng/mL is shown in Figure 1. Approximately 500 MRM transitions were monitored in both polarities throughput the entire chromatographic run. The total target cycle time of 0.7 sec ensures the collection of at least 12 data points across the LC peak resulting in excellent accuracy and reproducibility.

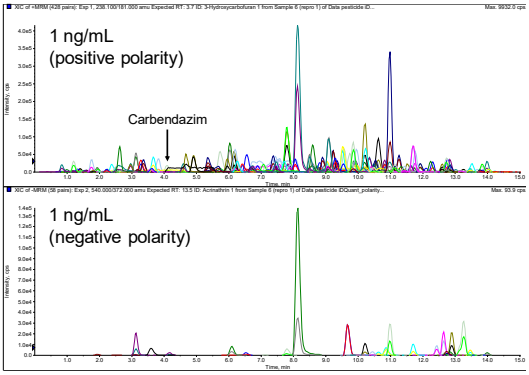

Figure 1. Comprehensive pesticide screening using the *Scheduled* MRM™ algorithm and fast polarity switching, ~500 MRM transition were detected with a total target cycle time of 0.7 sec

Figure 2 shows example chromatograms of 10 repeat injections at 1 ng/mL of early to late eluting pesticides in both polarities. The %CV values of 10% or less highlight the speed and effectiveness of *Scheduled* MRM™ combined with fast polarity switching. The developed method enables quantitation of all target pesticides with an LOD of at least 1 ng/mL and, thus, allowing sample extract dilution to minimize possible matrix effects.

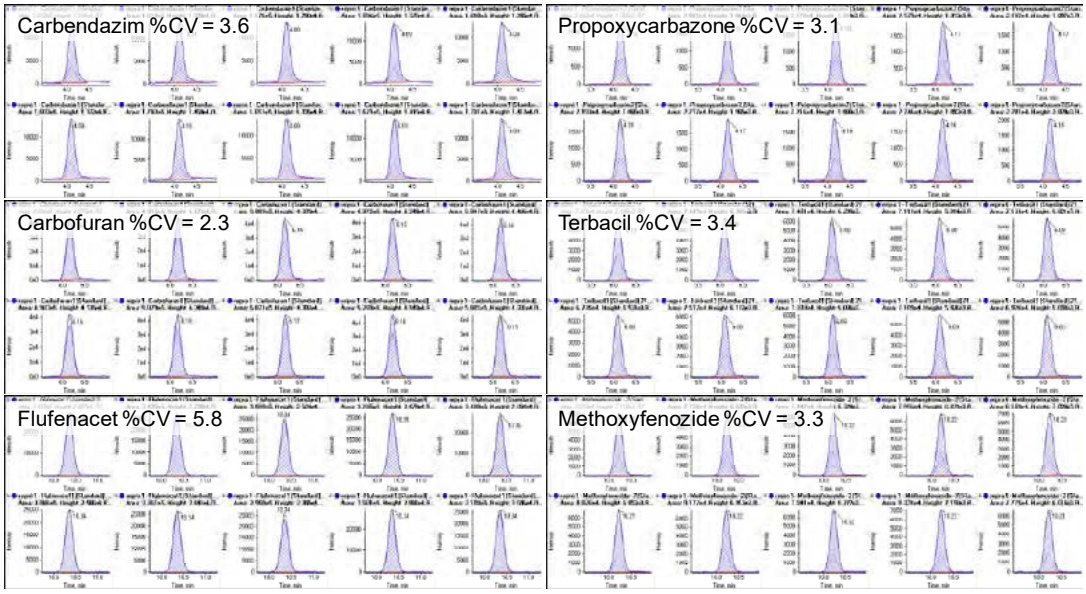

Figure 2. Repeat injections of pesticides at a concentration of 1 ng/mL detected in positive (left) and negative (right) polarity in a single run using *Scheduled* MRM™ and fast polarity switching (Carbendazim and Propoxycarbazone at 4.1 min, Carbofuran and Terbacil at 6.1 min, and Flufenacet and Methoxyfenozide at 10.3 min)

Linearity was obtained for most pesticides over 4 orders of magnitude (0.1-100 ng/mL). An example calibration line of Carbendazim is shown in Figure 3. Both MRM transitions have a regression coefficient of > 0.999 with accuracies between 97 and 109%.

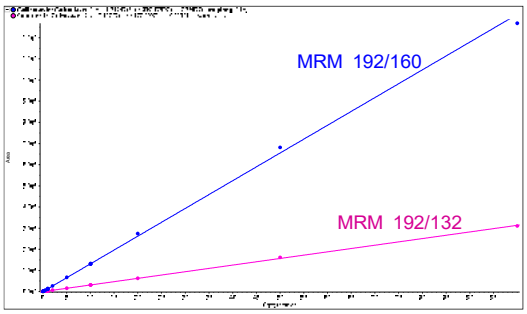

Figure 3. Calibration lines of both MRM transitions of Carbendazim

Accuracy between 80 and 120% were achieved for all targeted pesticides over the entire calibration range. Data points of the lowest or highest standards were excluded for a few pesticides with weak or strong ionization, respectively.

Findings in Fruit and Vegetable Samples

The developed method was applied to the quantitation and identification of pesticides in real food extracts. QuEChERS extracts of fruits and vegetables were diluted 5x prior LC-MS/MS analysis. Juice samples were injected directly after centrifugation and 5x dilution.

Sample data was processed using MultiQuant™ software version 2.1 with the 'Multicomponent' query. Query files are customizable commands to perform custom querying of the result table. The 'Multicomponent' query automatically calculates and compares MRM ratios for compound identification and highlights concentrations above a user specified maximum residue level. An example of the results and peak review after running the query file is shown in Figure 4.

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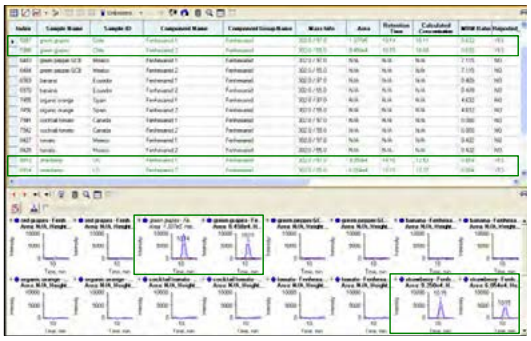

Figure 4. Automatic reporting of pesticides using the 'Multicomponent' query in MultiQuant™ software, Fenhexamid was positively identified using MRM ratio calculation in two samples and quantified in green grapes at 18.1 µg/kg and in strawberry at 12.5 µg/kg, respectively

Example chromatograms of analyzed samples are shown in Figures 5a-e. The findings are also summarized in Table 1.

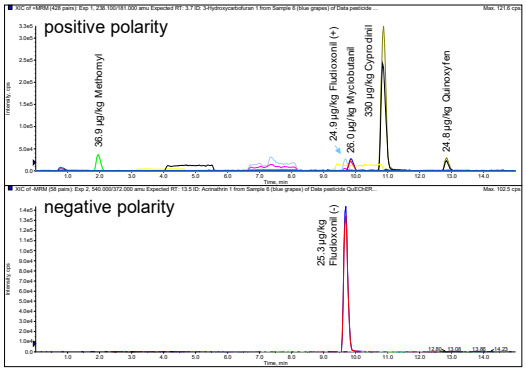

Figure 5a. Pesticides identified and quantified in a red grape sample

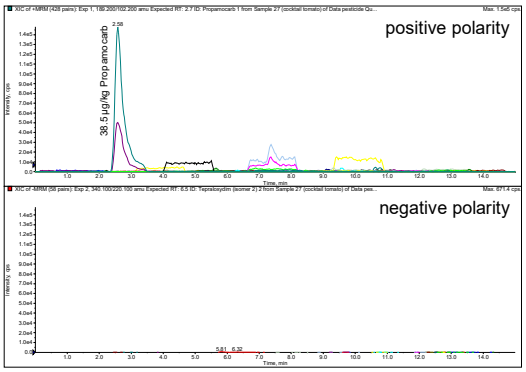

Figure 5b. Pesticides identified and quantified in a cocktail tomato sample

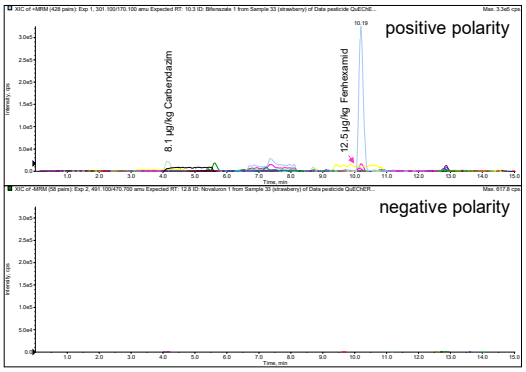

Figure 5c. Pesticides identified and quantified in a strawberry sample

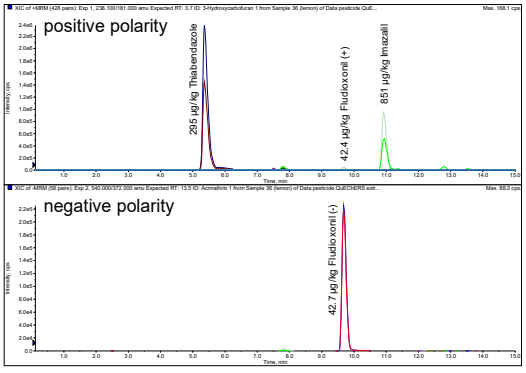

Figure 5d. Pesticides identified and quantified in a lemon sample

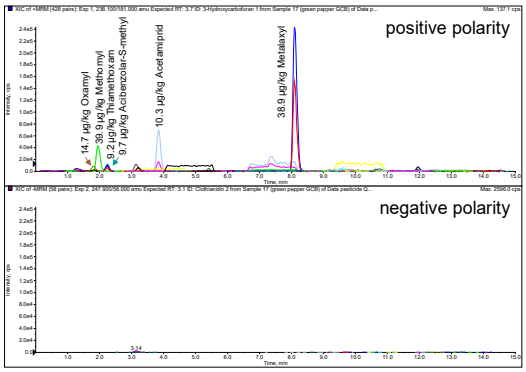

Figure 5e. Pesticides identified and quantified in green pepper sample

Table 1. Summary of pesticide findings in store bought food and orange juice samples above a concentration of 5 µg/kg

Sample	Pesticide	Concentration (µg/kg)
Red grapes	Cyprodinil	330
	Fludioxonil	24.9
	Methomyl	36.9
	Myclobutanil	26.0
	Quinoxyfen	24.8
Cocktail tomato	Propamocarb	38.5
Strawberry	Carbendazim	8.1
	Fenhexamid	12.5
Lemon	Fludioxonil	42.4
	Imazalil	851
	Thiabendazole	295
Green pepper	Acetamiprid	10.3
	Acibenzolar-S-methyl	9.7
	Metalaxyl	38.9
	Methomyl	39.9
	Oxamyl	14.7
	Thiamethoxam	9.2
Banana	Imazalil	40.7
	Thiabendazole	18.5
Clementine	Imazalil	1250
Green grapes	Boscalid	10.8
	Fenhexamid	18.1
	Imidacloprid	32.0
	Myclobutanil	7.2
	Quinoxyfen	12.5
Organic orange	no pesticides detected above 5 µg/kg	
Raspberry	Azoxystrobin	35.5
	Cyprodinil	71.0
	Fludioxonil	7.2
	Pyrimethanil	22.7
Red pepper	Flutriafol	44.0
Tomato	Difenoconazole	61.0
	Buprofezin	97.8
Orange juice 1	Carbendazim	13.0 ng/mL
Orange juice 2	Carbendazim	67.0 ng/mL

Compound Identification using MS/MS Library Searching

Despite the high selectivity of MRM detection, there is always a risk of false positive findings due to interfering matrix signals. Typically a second MRM is monitored per analyte and the ratio of quantifier to qualifier transition is calculated for each unknown sample and compared to the MRM ratio of standards for identification. However, it has been reported that relying only on MRM ratios for identification can result in a significant number of false positive results for compound identification.

To increase confidence in identification full scan MS/MS experiments can be performed and unknown spectra can be searched against mass spectral libraries. Here MS/MS spectra acquired in the EPI mode of the QTRAP® 4500 system were searched against the iMethod™ pesticide library (version 1.1). Example spectra and library search FIT values to identify Carbendazim in orange juice samples and Cyprodinil and Fludioxonil in a raspberry sample are shown in Figures 6 and 7. These examples highlight that MS/MS library searching increases confidence in identification, especially if the targeted analytes have low fragmentation efficiency (many low intensity product ions)

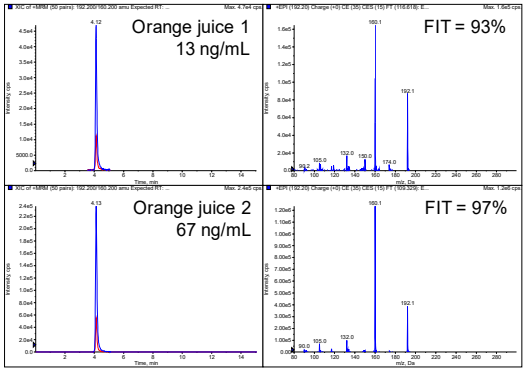

Figure 6. Identification of Carbendazim in two orange juice samples using MS/MS library searching, the samples were injected directly after 5x dilution, FIT values above 90% clearly confirm the identity of Carbendazim

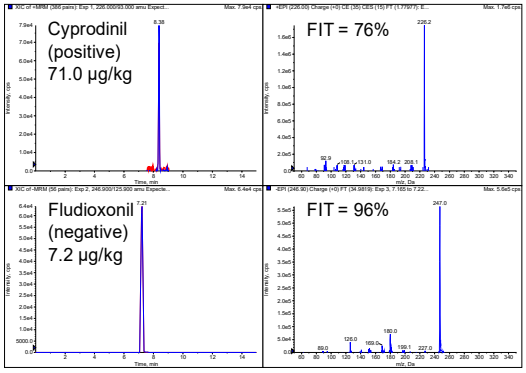

Figure 7. Identification of Cyprodinil and Fludioxonil in a raspberry sample using MS/MS library searching, the samples were injected after QuEChERS extraction and 5x dilution, MS/MS spectra were acquired in positive polarity and negative polarity

Summary

This unique LC-MS/MS method using the Eksigent ekspert™ ultraLC 100 and SCIEX QTRAP® 4500 system utilizing the *Scheduled* MRM™ algorithm in combination with fast polarity switching and acquisition of MS/MS spectra for compound identification has significant advantages. The method was successfully used to quantify and identify pesticides covering a broad range of chemical properties, including the acquisition of positive and negative polarity spectra.

The automatic method setup based on the Scheduled MRM™ algorithm resulted in excellent quantitative data. LOQ were measured for all pesticides at 0.1 ng/mL or below. This allows the dilution of sample extracts to significantly reduce possible matrix effects and interferences. Accuracies were typically found between 80 and 120% with %CV of less than 10%.

Different food and juice samples were analyzed after QuEChERS extraction and dilution to minimize possible matrix effects.

Results were processed using MultiQuant™ software with the 'Multicomponent' query. This query automatically highlights findings above a user specified threshold and when identification based on MRM ratio comparison was positive.

In addition full scan MS/MS spectra were acquired using the QTRAP® 4500 system. MS/MS spectra contain the complete molecular fingerprint of each analyte and searched against a spectral library reduce the possibility of false positive and negative results.

References

1 M. Anastassiades, et al.: J. AOAC Int. 86 (2003) 412-431

2 J. Wong et al.: J. Agric. Food Chem. 58 (2010) 5897-5903

3 A. Schreiber et al.: Application Note AB SCIEX (2010) 1282310-01

Publication number: 5090212-01

Contents

Quantitation of Antibiotics and Insecticides in Poultry Feed using Liquid Chromatography Tandem Mass Spectrometry

Ryan Picou[1], Sarah Ruiz[1], Cheryl Stephenson[1], John Reuther[1], Seyed Sadjadi[2], Sky Countryman[2], and Lauryn Bailey[3]

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Overview

An LC-MS/MS assay has been developed for the analysis of multiclass antibiotics and insecticides in poultry feed.

Introduction

For decades, antibiotics have been added to livestock feeds in low doses to serve as growth promoters. [1] Antibiotics have recently been shown to accumulate in poultry feathers, which is significant because poultry feathers serve as a high protein ingredient in animal feed, such as poultry feed.[1]

The continued use of these antibiotics as feed additives has inadvertently created antibiotic-resistant micro-organisms, which has caused human health concerns.[2] The types and quantities of antibiotics administered to livestock in the U.S. are not reported by the FDA.[1] In 2012, a federal judge ordered to withdraw the approval for the use of common antibiotics in animal feed because overuse could create antibiotic-resistant micro-organisms.[2]

Plant protection products may be introduced into animal feeds through several means, but the most common source of residues is through the legitimate use of pesticides (herbicides, insecticides and fungicides) in the production of crops used in preparation of feeds. Various grains and related glutens are frequently utilized in animal feeds. Animal feeds can in fact contain many nutritional ingredients and additives, including but not limited to proteins, fats, carbohydrates, antimicrobials, emulsifiers, binders, pH control agents, pelleting agents and preservatives.[3, 4] The inherent complexity of the sample matrix demands an efficient extraction and cleanup and a highly sensitive mass spectrometer to accurately quantify low levels of common antibiotics and insecticides in animal feeds in a single method.

In this work, a method has been developed to analyze for nine antibiotics, which included fluoroquinolones, sulfonamides, amphenicols, macrolides and quinolones, and four insecticides in poultry feed.

The preparative method involves a three-part extraction, sample cleanup with Phenomenex® Strata™-XL-CW solid phase extraction (SPE) cartridges and analysis by LC-MS/MS on an Eksigent ekspert™ UltraLC 100-XL with an SCIEX QTRAP® 5500 system utilizing Multiple Reaction Monitoring (MRM) with the *Scheduled* MRM™ algorithm and fast polarity switching. For the work presented here, accuracy and reproducibly are demonstrated by evaluating poultry feed samples fortified in triplicate.

Contents

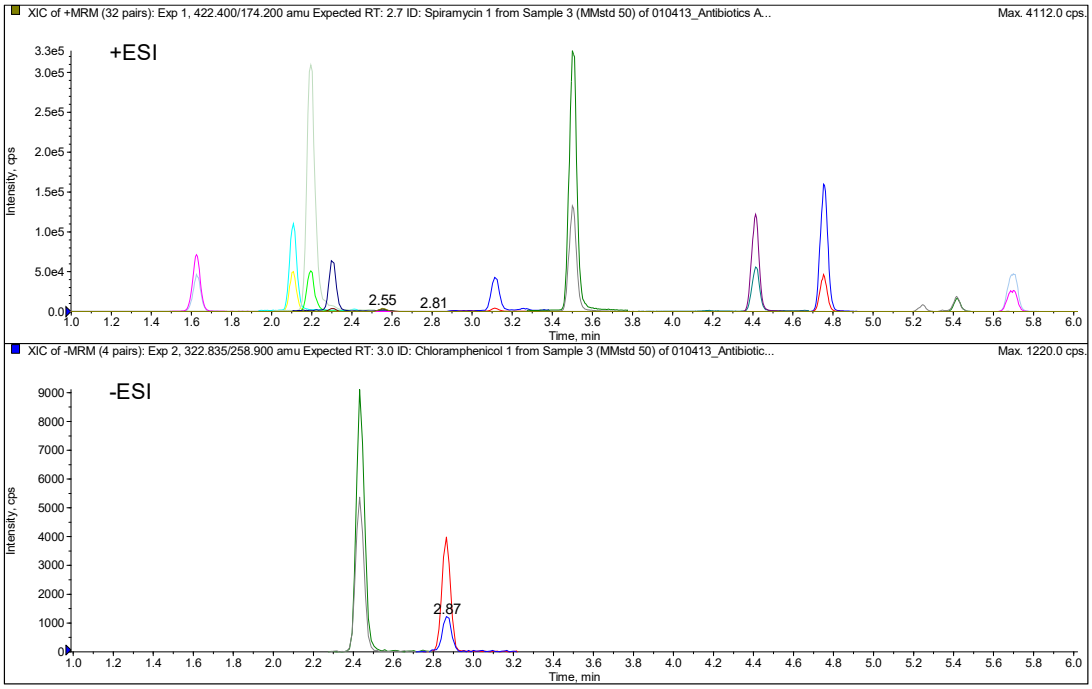

Figure 1. Detection of antibiotics and insecticides in a single run by LC-MS/MS using *Scheduled* MRM™ with polarity switching. Positive mode (+ESI) MRM transitions shown in top pane, and XIC of all negative mode (-ESI) MRM transitions shown at bottom. Peaks are identified by retention time in Table 2.

Experimental

Standards

All targeted analytes were available commercially and were either purchased as pure solid material or as high concentration analytical solutions. To prepare stock solutions of the solid materials, 10 mg of pure material was brought to either 10 or 100 mL with solvent to prepare 1 or 0.1 mg/mL solutions, respectively. The concentration of each stock solution was dependent on it solubility.

Sample Preparation

1) Extraction

Approximately 1.25 g of poultry feed sample were added to a 50 mL polypropylene centrifuge tube. Fortified samples were spiked into the dry sample for an in-sample concentration of 40 µg/kg. The sample was wetted with 10 mL of HPLC water and blended on a horizontal wrist-action shaker for 20 minutes. Three extractions were performed. The first extraction was performed with 5 mL of 1.5 mM EDTA and 5 mL of 1% TCA. The second extraction was performed with 10 mL of 75% methanol in water. The third extraction was performed with HPLC water. Between each extraction step, the sample was vortexed, shaken for 15 minutes on a wrist action shaker, sonicated for 10 minutes and centrifuged at 10,000 rpm for 5 minutes. All extracts were combined and brought to 50 mL with HPLC water.

2) SPE Cleanup

Sample cleanup was performed with Phenomenex® 200 mg Strata™-XL-CW SPE cartridges. This cartridge was selected based on the sorbent's weak cation exchange functionality to extract basic compounds from the poultry feed extract. Moreover, the large particle size of the XL (100 µm) allowed high volume loading and fast flow of the extract through the sorbent without the need to pre-filter the extract.

The final methanol percentage in this combined extraction was 15%, which was optimized for the SPE cleanup by performing a breakthrough study with various methanol percentages ranging

from 0 to 100%. It was determined that at 15% methanol concentration, all the targeted analytes retained on the sorbent during the loading step. At ≥25% methanol, some of the analytes would fail to be retained on the sorbent in the loading step, particularly oxolinic acid, florfenicol and chloramphenicol (data not shown).

The cartridge was conditioned with methanol followed by HPLC water. A 20 mL aliquot of the extract was loaded onto the SPE cartridge and sent to waste. The cartridge was washed with 10 mL of 15% methanol. The cartridge sorbent was dried under a light vacuum after the washing steps and prior to eluting the analytes. A 5 mL aliquot of 5% formic acid in methanol was used to elute the analytes.

3) Concentration/Reconstitution

Samples were evaporated to dryness under a gentle stream of nitrogen on a heating block (≤35°C). It was determined that these conditions resulted in no significant loss of analyte. The samples were reconstituted in 1 mL of 70% methanol in water, which was vortexed and filtered through a 0.22 µm syringe filter into an autosampler vial for analysis. The sample dilution factor was 2x.

LC Separation

The chromatography was performed on an Eksigent ekspert™ UltraLC 100-XL system with a Phenomenex® column configuration that used two Silica SecurityGuard™ cartridges, followed by a Luna® Silica (2) mixer column (30 x 2 mm, 5 µm). A Gemini® 3 µm NX-C18 (50 x 2 mm) served as the analytical column. The column compartment was maintained at 30°C. The gradient is listed in Table 1. Mobile Phase A was HPLC water with 0.1% formic acid and Mobile Phase B was 10 mM ammonium formate in methanol with 0.1% formic acid.

Table 1. LC gradient

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.0	0.7	100	0
5.0	0.7	5	95
7.0	0.7	5	95
7.1	0.7	100	0
10.0	0.7	100	0

MS/MS Detection

Analysis was performed on an SCIEX QTRAP® 5500 LC/MS/MS system using electrospray ionization (ESI) and *Scheduled* MRM™ in which each analyte's MRM is monitored across a user defined time window around each analyte's expected retention time, maximizing sensitivity. Each analyte's MRM and retention time are listed in Table 2. Most analytes are ionized in positive mode (+ESI) with the exception of florfenicol and chloramphenicol which are ionized in negative mode (-ESI). In order to achieve a single run, polarity switching was used in conjunction with the *Scheduled* MRM™ algorithm. The use of short pause times (2-3 ms) proved to be necessary to achieve optimal peak shapes and sensitivity to quantify the narrow UPLC peaks (FWHM = 3 to 4 s) particularly during polarity switching.

Table 2. Analytes, retention times (RT) and MRM transitions with collision energies (CE)

Analyte	RT (min)	Q1 (amu)	Q3 (amu)
Trimethoprim	1.63	291.2/261.2 (34)	291.2/230.2 (31)
Ciprofloxacin	2.11	332.0/314.0 (27)	332.0/230.9 (51)
Enrofloxacin	2.20	360.1/342.0 (29)	360.1/286.0 (47)
Sarafloxacin	2.30	386.1/368.2 (27)	386.1/348.1 (43)
Florfenicol	2.43	357.9/337.9 (-14)	357.9/184.8 (-46)
Spiramycin	2.55	442.4/174.2 (29)	422.4/101.1 (26)
Chloramphenicol	2.87	332.8/258.9 (-16)	322.8/151.9 (-24)
Oxolinic Acid	3.12	262.0/244.0 (23)	262.0/216.0 (39)
Flumequine	3.50	262.0/243.9 (25)	262.0/201.8 (45)
Diflubenzuron	4.42	311.2/158.1 (18)	311.2/141.1 (42)
Emamectin	4.75	886.7/158.2 (42)	886.7/82.3 (107)
Abamectin	5.42	891.0/305.1 (33)	891.0/568.1 (19)
Ivermectin	5.70	893.3/570.2 (21)	893.3/307.1 (33)

Results and Discussion

Figure 1 shows the extracted ion chromatograms (XIC) of a 10 µL injection of a matrix matched standard at 50 µg/mL.

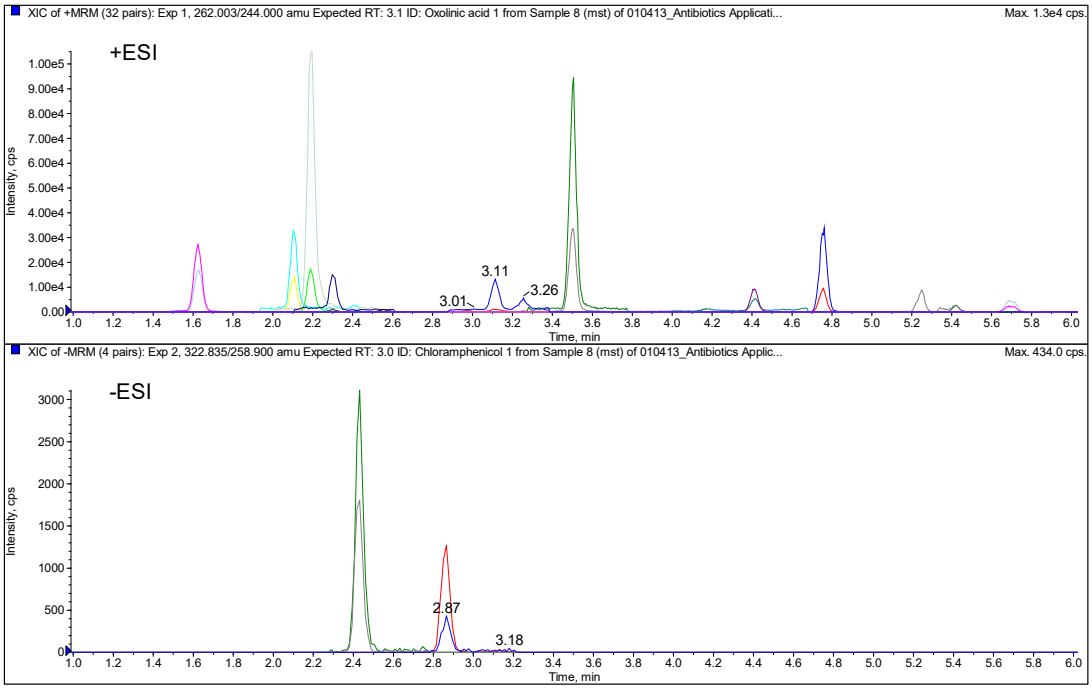

Figure 2. Poultry feed sample fortified at 40 µg/kg in sample (20 µg/mL in extract).

Figure 2 shows the extracted ion chromatograms (XIC) of a 10 µL injection of a poultry feed sample fortified at 40 µg/kg level (20 µg/mL in extract after 2x dilution).

The recoveries for each analyte are shown in Table 3. Given the complexity of the sample matrix and the inherent chemical differences between the target analytes, most analytes were reasonably recovered with the described extraction and cleanup. The method proved to be precise with %RSDs generally less than 5%. Recoveries could potentially be improved with the use of internal standards; however, absolute recoveries are a more accurate approach to assessing the effectiveness of a preparative method.

Table 3. Quantitation and Recovery Data based on MRM 1. Four point calibration using 5, 10, 50 and 100 µg/mL matrix matched standards.

Analyte	r^2	Average recovery (%) ± % RSD
Trimethoprim	0.999	89 ± 4 %
Ciprofloxacin	0.997	60 ± 0 %
Enrofloxacin	0.999	73 ± 4 %
Sarafloxacin	0.996	47 ± 4%
Florfenicol	1.000	85 ± 1 %
Spiramycin	1.000	70 ± 3 %
Chloramphenicol	1.000	77 ± 2 %
Oxolinic Acid	1.000	64 ± 1 %
Flumequine	0.998	64 ± 3 %
Diflubenzuron	1.000	20 ± 5 %
Emamectin	0.999	52 ± 7 %
Abamectin	0.999	40 ± 5 %
Ivermectin	1.000	24 ± 3 %

Contents

Summary

A single method has been developed to quantify a wide class of antibiotics and insecticides in poultry feed. The poultry feed extract was cleaned by SPE on a Phenomenex® Strata™-XL-CW prior to analysis utilizing an Eksigent ekspert™ UltraLC 100-XL system with a Phenomenex® Luna® Silica mixer column in series with a Gemini® NX-C18 analytical column with an SCIEX QTRAP® 5500 system for detection. *Scheduled* MRM™ in combination with fast polarity switching was used to maximize sensitivity while achieving a single run for all analytes. Analyte recoveries and precision from triplicate fortified poultry feeds were acceptable, given the complexity of the sample matrix and the generic approach to the extraction, and cleanup procedure required to simultaneously test such a variety of analytes.

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4 Prince Agri Products: http://www.princeagri.com/Phibro/Products/Catalog/Premixes-Feed-Ingredient-For-Poultry.html

Publication number: 7180213-01

Automated Sample Preparation and Analysis Workflows for Pesticide Residue Screening in Food Samples using DPX-QuEChERS with LC-MS/MS

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Overview

QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation methods have been developed to help monitor pesticides in a range of food samples. These methods require many manual steps, such as shaking, centrifugation, and dispersive SPE cleanup, making it a quite labor-intensive process. There is a need for automating parts of QuEChERS extraction in order to improve laboratory productivity for monitoring pesticide residue in foods.

In this publication, we describe an automated sample preparation and analysis workflow for the screening of over 200 pesticides in different food matrices by LC-MS/MS. The automated cleanup of the QuEChERS extracts was performed using Disposable Pipette Extraction (DPX) with a GERSTEL MultiPurpose Sampler (MPS) 2XL interfaced to an SCIEX QTRAP® 4500 LC/MS/MS system. The sensitivity and selectivity of the LC-MS/MS system enabled us to identify and quantify with limits of detection which meet acceptance criteria for reporting Maximum Residue Levels (MRL) as established by regulatory agencies. The ability to automate the dispersive SPE cleanup of QuEChERS extracts followed by direct LC-MS/ MS analysis resulted in improved laboratory productivity by streamlining the complete analytical process.

Introduction

QuEChERS protocols are widely used to prepare samples for the monitoring of pesticide residues in food. These methods require many manual steps, such as shaking, centrifugation, and dispersive SPE cleanup, making it a quite labor-intensive process. A laboratory's productivity and efficiency can be greatly improved by automating parts of the QuEChERS procedure, the dispersive SPE cleanup step and subsequent dilution prior LC-MS/MS analysis.

A simpler and more practical way to perform the dispersive SPE cleanup method is to use DPX tips. These tips have a screen that retains loose sorbent material inside the pipette tip. The DPX tips used for this project contain anhydrous magnesium sulfate ($MgSO_4$) and primary and secondary amine (PSA) as cleanup sorbents and are denominated "QuEChERS Tips".

Here we present a new automated sample preparation and analysis workflow for pesticide residue screening of food samples using DPX-QuEChERS with LC-MS/MS. The use of QuEChERS tips has been reported previously[1-3] and has been found to provide comparable results to those obtained using manual methods based on dispersive SPE. A GERSTEL MPS 2XL equipped with DPX option coupled to an SCIEX QTRAP® 4500 system was used for the automated cleanup of QuEChERS extracts and extract dilution. The LC-MS/MS method utilized the *Scheduled* MRM™ algorithm to obtain the best data quality in combination with fast polarity switching to cover the broadest range of pesticides possible. In addition, QTRAP® full scan MS/MS spectra were acquired to allow library searching in order to increase confidence in identification.

The method was successfully applied to identify and quantify over 200 pesticides in QuEChERS extracts of fruit, vegetable, herb and spice samples.

Contents

Figure 1. GERSTEL MPS 2XL with DPX option coupled to an QTRAP® 4500 system

Experimental

Materials

- Fruit, vegetable, herb, and spice samples, including organic produce, from a local supermarket
- SCIEX iD*Quant*™ standards kit for pesticide analysis plus additional pesticides of interest (Table 1)
- Serial dilutions to prepare calibration standards with concentration of 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 ng/mL
- Acetonitrile extracts of blank matrix samples, incurred samples and fortified samples using commercial QuEChERS kits following the AOAC method 2007.1
- DPX QuEChERS tips provided by DPX labs containing PSA (75 mg), $MgSO_4$ (25 mg) and GCB (12.5 mg) for dispersive SPE cleanup

QuEChERS Pretreatment

1. Pipette 1 mL of the acetonitrile extract obtained following the 1st centrifugation step of the QuEChERS sample preparation method, into an autosampler vial.
2. Place the sample onto a tray on the dual head GERSTEL MPS XL configured for automated DPX-QuEChERS LC-MS/MS analysis.

Automated QuEChERS Sample Preparation Sequence[5]

1. MPS transfers 500 µL of QuEChERS extract to an open test tube.
2. DPX-QuEChERS tip is picked up and transported to the test tube for sample cleanup.
3. Sample is aspirated into the tip, mixed for 30 sec and discharged to test tube. Repeat 3 times.
4. MPS transfers 50 µL of cleaned extract to a sealed vial, where it is diluted with 450 µL of mobile phase A.
5. The diluted cleaned extract is injected to the LC-MS/MS for analysis.

A schematic of the automated DPX-QuEChERS procedure is shown in Figure 2.

Table 1. Pesticides monitored using the automated DPX-QuEChERS-LC-MS/MS method

3-Hydroxycarbofuran	Acephate	Acetamiprid	Acibenzolar-S-methyl	Alanycarb	Aldicarb
Aldicarb sulfone	Aldicarb sulfoxide	Aspon	Avermectin B1a	Avermectin B1b	Azadirachtin
Azoxystrobin	Benalaxyl	Bendiocarb	Benfuracarb	Benoxacor	Benthiavalicarb
Benzoximate	Bifenazate	Bifenthrin	Bitertanol	Boscalid	Bromuconazole
Bupirimate	Buprofezin	Butafenacil	Butocarboxim	Butoxycarboxim	Cadusafos
Carbaryl	Carbendazim	Carbetamide	Carbofuran	Carboxin	Carfentrazone-ethyl
Chlordimeform	Chlorfenvinphos	Chlorfluazuron	Chlortoluron	Chloroxuron	Clethodim
Clofentezine	Clothianidin	Coumaphos	Cumyluron	Cyanazine	Cyanophos
Cyazofamid	Cycluron	Cymoxanil	Cyproconazole	Cyprodinil	Cyromazine
D_{10}-Diazinon	D_6-Dichlorvos	D_6-Dimethoate	D_6-Diuron	D_6-Linuron	D_6-Malathion
Daimuron	Dazomet	Deltamethrin	Diazinon	Dichlorvos	Dicrotophos
Diethofencarb	Difenoconazole	Diflubenzuron	Dimethenamid	Dimethoate	Dimethomorph
Dimoxystrobin	Diniconazole	Dinotefuran	Dioxacarb	Disulfoton	Dithiopyr
Diuron	Dodemorph	Fenpyroximate	Emamectin B1a	Emamectin B1b	Epoxiconazole
Eprinomectin B1a	EPTC	Esprocarb	Ethidimuron	Ethiofencarb	Ethion
Ethiprole	Ethirimol	Ethofumesate	Ethoprophos	Etobenzanid	Etofenprox
Etoxazole	Famoxadone	Fenamidone	Fenarimol	Fenazaquin	Fenbuconazole
Fenhexamid	Fenoxanil	Fenoxycarb	Fenpropathrin	Fenpropimorph	Fenuron
Flonicamid	Flucarbazone	Fludioxonil	Flufenacet	Flufenoxuron	Flumetsulam
Flumioxazin	Fluometuron	Fluquinconazole	Flusilazole	Fluthiacet-methyl	Flutolanil
Flutriafol	Forchlorfenuron	Formetanate	Fuberidazole	Furalaxyl	Furathiocarb
Heptenophos	Hexaconazole	Hexaflumuron	Hexythiazox	Hydramethylnon	Imazalil
Imazapyr	Imibenconazole	Imidacloprid	Indanofan	Indoxacarb	Ipconazole
Iprovalicarb	Isocarbamid	Isofenphos	Isopropalin	Isoproturon	Isoxaben
Isoxaflutole	Kresoxim-methyl	Lactofen	Leptophos	Linuron	Lufenuron
Mandipropamid	Mefenacet	Mepanipyrim	Mepronil	Metalaxyl	Metconazole
Methabenzthiazuron	Methamidophos	Methiocarb	Methomyl	Methoprotryne	Methoxyfenozide
Metobromuron	Metribuzin	Mevinphos	Mexacarbate	Molinate	Monocrotophos
Monolinuron	Moxidectin	Myclobutanil	Neburon	Nitenpyram	Norflurazon
Novaluron	Nuarimol	Omethoate	Oxadixyl	Oxamyl	Paclobutrazol
Penconazole	Pencycuron	Phenmedipham	Picoxystrobin	Piperonyl butoxide	Pirimicarb
Pirimicarb-desmethyl	Pirimicarb-desmethyl-formamide	Prochloraz	Promecarb	Prometon	Prometryn
Propachlor	Propamocarb	Propargite	Propazine	Propham	Propiconazole
Propoxur	Pymetrozine	Pyracarbolid	Pyraclostrobin	Pyridaben	Pyrimethanil
Pyriproxyfen	Quinoxyfen	Rotenone	Sebuthylazine	Secbumeton	Siduron
Simazine	Simetryn	Spinosyn A	Spinosyn D	Spirodiclofen	Spiromesifen
Spiroxamine	Sulfentrazone	Tebuconazole	Tebufenozide	Tebufenpyrad	Tebuthiuron

Table 1. (cont.)

Teflubenzuron	Temephos	Terbumeton	Terbutryn	Terbuthylazine	Tetraconazole
Tetramethrin	Thiabendazole	Thiacloprid	Thiamethoxam	Thiazopyr	Thidiazuron
Thiobencarb	Thiofanox	Thiophanate-methyl	Triadimefon	Triadimenol	Trichlamide
Trichlorfon	Tricyclazole	Trifloxystrobin	Triflumizole	Triflumuron	Triticonazole
Uniconazole	Vamidothion	Zoxamide			

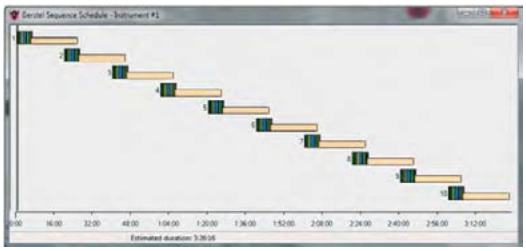

Figure 2. Example sample preparation sequence for automated DPX-QuEChERS LC-MS/MS analysis

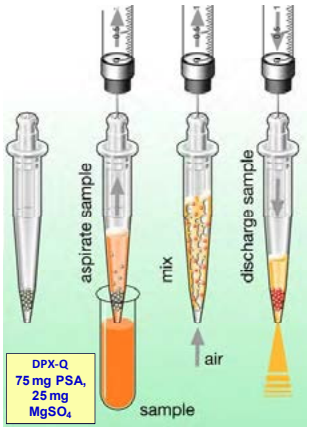

Figure 3. Example sample preparation sequence for automated DPX-QuEChERS LC-MS/MS analysis

Figures 3 and 4 show the automated sample preparation sequence used to perform DPX-QuEChERS.

Preparation of Solvent Standards and Matrix Matched Standards

1. Transfer 100 µL of previously extracted matrix blank or 100% acetonitrile into an empty autosampler vial.
2. Transfer 250 µL of mobile phase A into the vial.
3. Transfer 150 µL of the respective standard stock solution into the vial and mix.

LC-MS/MS Analysis

All analyses were performed using an Agilent 1200 Series LC system and a GERSTEL MPS MPS 2XL equipped with DPX option and a 10 µL stainless steel loop with active wash station.

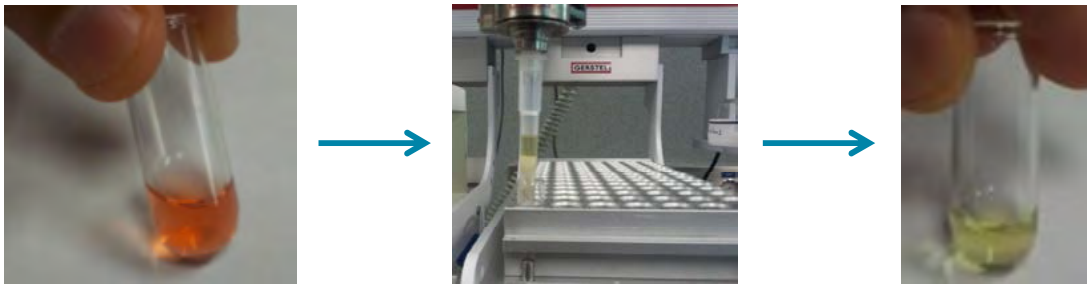

Figure 4. Schematic of the automated DPX-QuEChERS procedure, 500 µL of apple extract (left) and after DPX-QuEChERS cleanup (right)

A Phenomenex Synergi-Fusion 2.5u (50x2 mm) column was used with a gradient of water / methanol + 5 mM ammonium formate at a flow rate of 0.4 mL/min was used with a total run time of 20 min.

The SCIEX QTRAP® 4500 system was used with Turbo V™ source and Electrospray Ionization (ESI) probe operated in both positive and negative polarity. The *Scheduled* MRM™ algorithm was used for enhanced Signal-to-Noise (S/N), accuracy and reproducibility.[6]

Optimized MRM transitions for all pesticides were obtained through the MRM catalogue of the iMethod™ application for pesticide screening version 2.1. Two MRM transitions were monitored for each target pesticide to allow quantitation and identification using the MRM ratio. In addition, QTRAP® full scan MS/MS spectra were acquired to allow library searching in order to increase confidence in identification.

MultiQuant™ and LibraryView™ software was used for qualitative and quantitative data processing.

Results and Discussion

The automated DPX-QuEChERS cleanup method was performed to automatically remove matrix components from sample extracts prior to LC-MS/MS analysis. The removal of water ($MgSO_4$) and fatty acids (PSA) is necessary to ensure reproducible peak intensities for quantitative analysis. GCB is used to remove pigments, particularly chlorophyll and carotenoids.

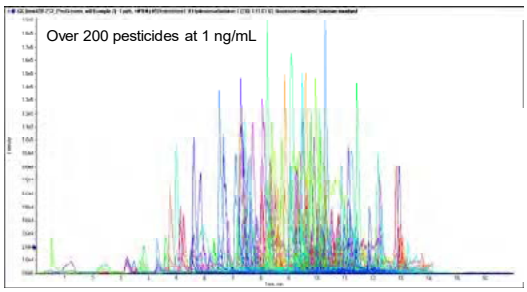

Figure 5. Detection of over 200 pesticides in a fortified cucumber sample at 1 ng/mL

Figure 5 shows a representative MRM chromatogram from a pesticide-fortified cucumber sample QuEChERS extract at 1 ng/mL. Over 200 pesticides were successfully detected in this sample matrix using the automated DPX-QuEChERS LC-MS/MS method.

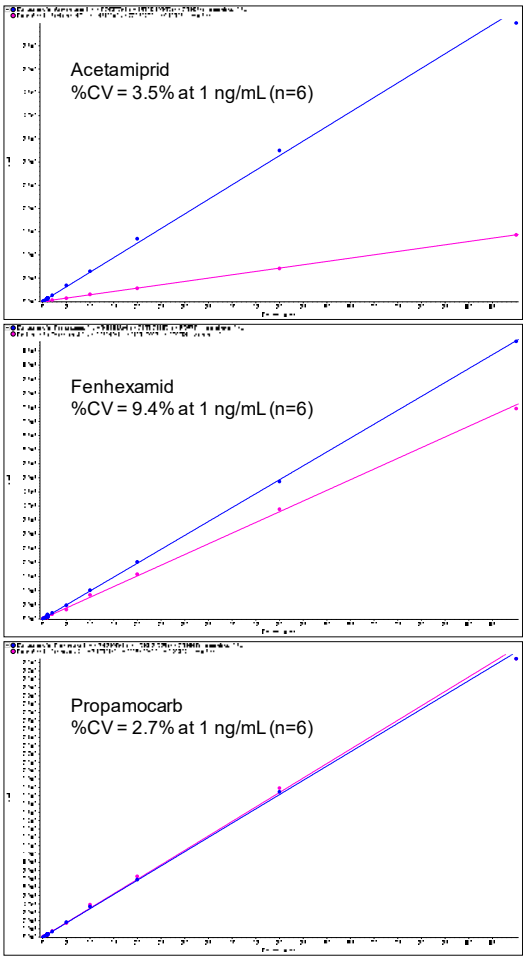

Figure 6. Representative calibration lines for Carbendazim, Propiconazole, and Thiacloprid from 0.1 to 100 ng/mL with an regression coefficient $r^2 > 0.997$ with excellent repeatability of %CV < 10%

Figure 6 shows calibration curves obtained using automated solvent standards. The resulting calibration curves were shown to be linear from at least 0.1 to 100 ng/mL with excellent repeatability for the pesticides monitored.

Contents

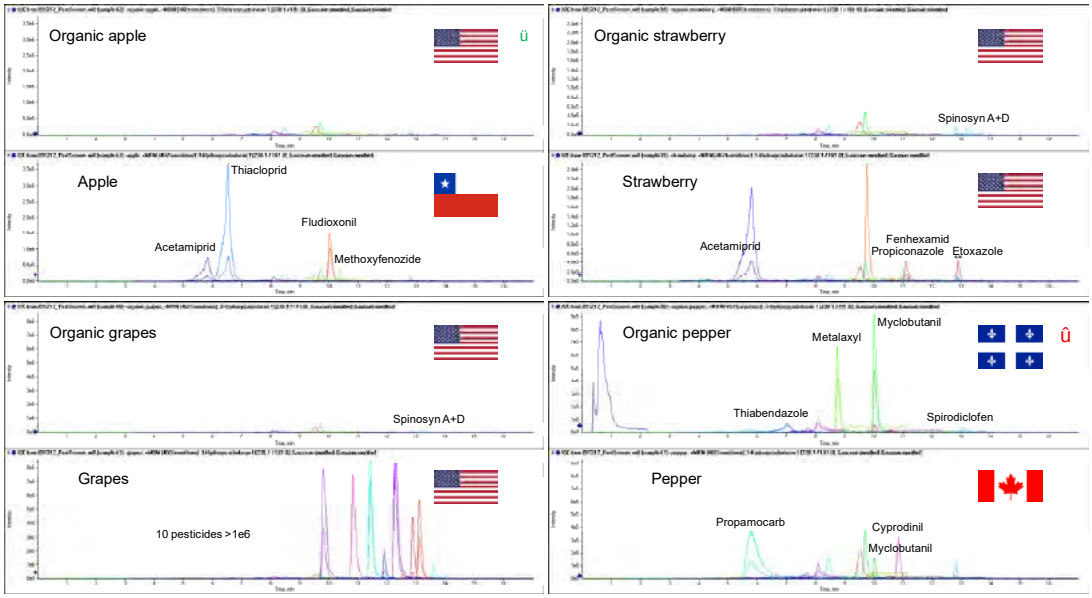

Figure 7. MRM chromatograms of pesticides identified in different food samples, including organic produce, from a local super market

The developed method was applied to the detection of pesticides in extracts of real food samples obtained from a local supermarket. QuEChERS extracts were cleaned using the DPX-QuEChERS method and diluted 10x for LC-MS/MS analysis (Figure 7).

Sample data was processed using MultiQuant™ software with the 'Multicomponent' query. Query files are customizable commands to perform custom querying of the result table. Figures 8a and b show examples of using the 'Multicomponent' query to flag pesticides present in sample extracts above a user

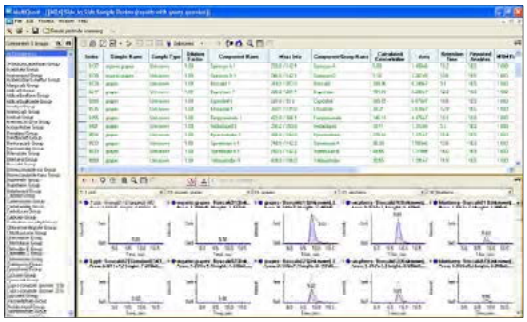

Figure 8a. Results review in MultiQuant™ software after using the 'Multicomponent' query with peak review for the pesticide Boscalid (bottom): 1 ng/mL standard, organic grapes, grapes, raspberry, and blueberry

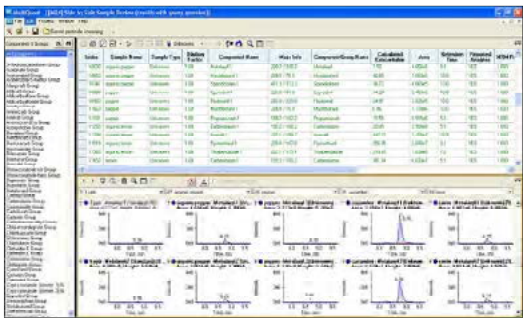

Figure 8b. Results review in MultiQuant™ software after using the 'Multicomponent' query with peak review for the pesticide Metalaxyl (bottom): 1 ng/mL standard, organic pepper, pepper, cucumber, and raisin

specified concentration level and with positive identification using the MRM ratio.

To increase confidence in identification additional full scan MS/MS experiments were performed and spectra were searched against the iMethod™ pesticide library. Extracted spectra and library search Purity score values using the LibraryView™ software are shown in Figures 9a and b for an paprika and tarragon with low analyte concentrations.

Figure 9a. Automated library identification for Pyrimethanil butoxide detected in a paprika extract after DPX-QuEChERS LC-MS/MS

Figure 9b. Automated library identification for Desmethyl-pirimicarb detected in a paprika extract after DPX-QuEChERS LC-MS/MS

Summary

As a result of this study, we were able to show:

- The described DPX-QuEChERS LC-MS/MS workflow using. the GERSTEL MPS 2XL equipped with DPX option coupled to an SCIEX QTRAP® 4500 system enabled automated cleanup and analysis of QuEChERS extracts for screening and confirmation of over 200 pesticides in a single LC-MS/MS run.
- Quantitative analysis was performed in the same run allowing for both quantitation and qualitative data to be collected simultaneously. Linear calibration curves resulting in r^2 values of 0.99 or greater were achieved for the samples analyzed.
- With this configuration a 15 min/sample cycle time is achieved, including "just-in-time" PrepAhead sample preparation, for LC-MS/MS analysis of QuEChERS extracts.

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2 L. Podhorniak: 'Tubes or Tips?' Poster presentation at Florida Pesticide Residue Workshop (2010) St. Pete Beach, FL (USA)

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6 A. Schreiber et al.: 'Comprehensive Quantitation and Identification of Pesticides in Food Samples using LC-MS/MS with *Scheduled* MRM™, Fast Polarity Switching, and MS/MS Library Searching' Application Note AB SCIEX (2011) 3370411-01

Publication number: 8013613-01

Contents

Automated Derivatization, SPE Cleanup and LC-MS/MS Determination of Glyphosate and Other Polar Pesticides

André Schreiber[1] and Oscar G. Cabrices[2]

[1]SCIEX Concord, Ontario (Canada) and [2]GERSTEL Linthicum, Maryland (USA)

Overview

Glyphosate and glufosinate are widely used herbicides and, thus, there is an interest in the reliable and sensitive determination of glyphosate in water and food. These pesticides are difficult to extract and analyze because of their high polarity. Here we describe an automated workflow for the FMOC-derivatization, sample cleanup, and LC-MS/MS detection using a GERSTEL Multi Purpose Sampler (MPS) 2XL configured with an online solid phase extraction (SPE^{XOS}) module coupled to a SCIEX QTRAP® 4500 system for the identification and quantitation of glyphosate, its major metabolite AMPA, and glufosinate in water and food samples.

Introduction

Glyphosate (N-phosphonomethyl glycine) and glufosinate [ammonium (S) -2-amino-4-[hydroxyl (methyl) phosphinoyl] butyrate] are non-selective post emergence herbicides used for the control of a broad spectrum of grasses and broad-leaf weed species in agricultural and industrial fields. Aminomethyl-phosphonic acid (AMPA) is the major metabolite of glyphosate and also included into the pesticide residue definition.[1, 2]

There is interest in the reliable and sensitive determination of residues of these pesticides in water and food. Due to their high polarity it is difficult to extract these pesticides from samples and to retain them on LC phases. Derivatization with fluorenylmethyloxycarbonyl chloride (FMOC-Cl) is a common procedure to improve extraction and separation for the analysis of glyphosate and related compounds. Previously reported methods using derivatization with FMOC-Cl have inherent limitations, such as long derivatization times, long LC run times, and often suffer from lack of repeatability and reproducibility.

Here we present an automated workflow to derivatize and analyze water and food samples for glyphosate, glufosinate and AMPA by LC-MS/MS using a GERSTEL Multi Purpose Sampler (MPS) 2XL with SPE^{XOS} coupled to a SCIEX QTRAP® 4500 system (Figure 2).

Water samples were injected directly into the LC-MS/MS system providing sufficient sensitivity to identify and quantify targets at sub 100 µg/L concentrations. Food samples can be injected directly after automatic derivatization followed by extensive dilution or can be cleaned up using online SPE prior LC-MS/MS analysis. Target compounds can be easily identified and quantified at 10 µg/kg levels with excellent repeatability.

Experimental

Derivatization and Sample Preparation

Water samples were analyzed directly and food samples were extracted using the QuPPe (Quick Polar Pesticides) method developed by the EU Reference Laboratories for Residues of Pesticides.[3] QuPPe results were compared to results obtained when using an extraction method reported by Miles et al.[4]

Derivatization and cleanup was performed using the GERSTEL MPS 2XL with SPE^{XOS} system configured for automatic sample handling, derivatization, and online SPE. The Gerstel system is fully controlled using the GERSTEL MAESTRO version 1.4 coupled to Analyst® software version 1.6.

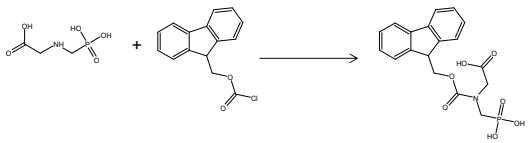

Figure 1. Derivatization of glyphosate using FMOC-Cl

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Figure 2. GERSTEL MPS 2XL with SPE^{XOS} coupled to an QTRAP® 4500 system

Automated Derivatization Procedure (Figure 1)

1. Add 100 µL of borate buffer (pH=9) to 1 mL of sample.
2. Add 200 µL of 10mM FMOC-Cl solution.
3. Agitate for 20 min at 50°C.
4. Cool to bring to ambient temperature.
5. Add 130 µL 2% H_3PO_4.

Water samples were derivatized and injected directly (10 µL) into LC-MS/MS.

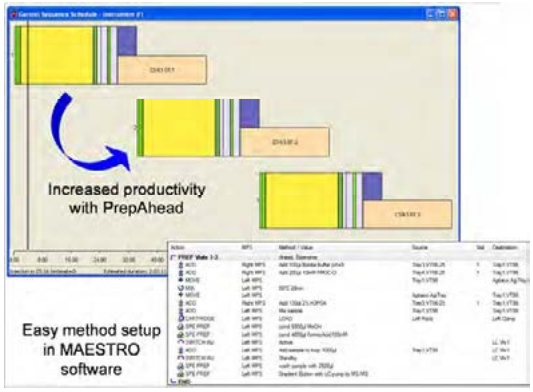

Figure 3a. Sequence of scheduled events in the Maestro software for online SPE: green - adding buffer and FMOC-Cl, yellow - derivatization, light blue - online SPE, orange LC-MS/MS analysis, dark blue washing of the autosampler, the PrepAhead function increases productivity by simultaneously preparing the following sample while perming LC-MS/MS analysis of the previous sample

Automated Online-SPE Cleanup Procedure

1. Condition GERSTEL SPE^{XOS} C8EC-SE (18.5 mg) cartridge with methanol and water + 100 mM formic acid.
2. Load 1 mL of derivatized sample onto SPE.
3. Wash with water + 100 mM formic acid.
4. Elute with LC pump gradient

Food sample extracts were diluted extensively to minimize possible matrix effects and automatically cleaned up using SPE^{XOS} system. Here we injected 1 mL of the diluted sample extract onto the SPE cartridge. (Figures 3a and 3b)

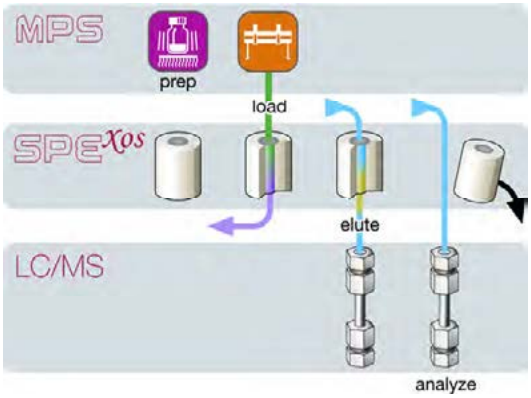

Figure 3b. Sequence of scheduled events when using the automated workflow of FMOC-derivatization, SPE cleanup, and LC-MS/MS detection

LC Separation

The analyses were performed using a Phenomenex Gemini 3µ C18 (150 x 2 mm) column with a gradient of (A) 50 mM ammonium acetate adjusted to pH= 9 and (B) Acetonitrile. The gradient conditions are listed in Table 1.

Table 1. LC gradient used for separation

Time (min)	Flow (mL/min)	A (%)	B (%)
0	0.25	80	20
10	0.25	5	95
15	0.25	5	95
15.1	0.25	80	20
25	0.25	80	20

MS/MS Detection

The analyses were performed on a SCIEX QTRAP® 4500 LC/MS/MS system using the Turbo V™ source operated in electrospray ionization and negative polarity with an IS voltage of -4200 V.

The Curtain Gas™ interface (CUR) was set to 30 psi, nebulizer gas (Gas 1) set to 50 psi, drying gas (Gas 2) set to 70 psi, and the source temperature set to 400ºC.

The MRM transitions used for the detection of pesticides are shown in the table below. Each MRM was monitored with a dwell time of 100 ms.

Table 2. MRM transitions used for detection

Compound	Q1	Q3	CE (V)
Glyphosate	390	168, 150	-18, -34
Glufosinate	402	180, 206	-16, -20
AMPA	322	110, 136	-12, -22

Analyst® version 1.6.1 was used for data acquisition and MultiQuant™ version 3.0 software was used for qualitative and quantitative processing.

Results and Discussion

A standard chromatogram after automatic derivatization is shown in Figure 4.

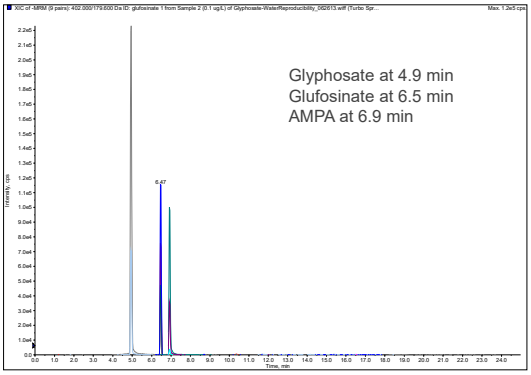

Figure 4. Standard chromatogram at a concentration of 10 ng/mL

A drinking water sample was spiked at 0.1 and 10 µg/L, automatically derivatized, and analyzed in triplicates. The method allowed accurate quantitation of all target compounds well below 0.1 µg/L with excellent repeatability (Figure 5 and Table 3).

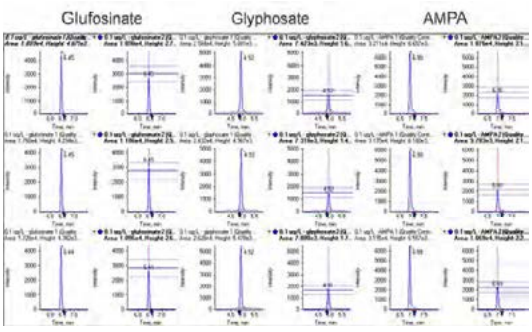

Figure 5. Triplicate analysis of polar pesticides in a spiked water sample at 0.1 µg/L (injection volume of 10 µL), ion ratios for compound identification were calculated automatically in MultiQuant™ software version 3.0

Table 3. Triplicate analysis of polar pesticides in a spiked water sample at 0.1 µg/L (injection volume of 10 µL)

Compound	Concentration (µg/L)	%CV of MRM 1	%CV of MRM 2
Glyphosate	0.1	4.0	3.9
	10	7.7	8.9
Glufosinate	0.1	2.3	4.5
	10	4.6	5.4
AMPA	0.1	1.4	5.3
	10	5.1	5.4

Different food matrices (corn and soy bean) where spiked with glyphosate, glufosinate, AMPA at 10 and 100 µg/kg and extracted using the QuPPe (Quick Polar Pesticides) method:

1. Add 10 mL water to 5 g of homogenized sample, shake and soak for 10 min.
2. Add 10 mL of acidified methanol (1% formic acid).
3. Shake vigorously for 1 min and centrifuge (at 3000 rpm) for 10 min.
4. Load 1 mL onto the Gerstel MPS 2XL system for automated dilution, derivatization, and SPE cleanup followed by LC-MS/MS analysis.

Corn and soy samples were spiked at 10 and 100 µg/kg and analyzed in triplicates using the automated derivatization and cleanup procedure. The method allowed accurate quantitation of all target compounds well below the target concentration of 100 µg/kg with excellent repeatability (Table 4, Figures 6 and 7).

Table 4. Triplicate analysis of polar pesticides spiked into corn and soy samples 100 µg/kg

Compound	Concentration (µg/kg)	%CV of MRM 1	%CV of MRM 2	Ion ratio (%RSD)
Glyphosate	100 (in corn)	3.6	6.0	0.36 (1.9%)
	100 (in soy)	5.1	5.9	0.31 (1.9%)
Glufosinate	100 (in corn)	1.6	12.5	0.71 (8.9%)
	100 (in soy)	5.2	7.7	0.67 (3.9)%
AMPA	100 (in corn)	5.7	4.8	0.43 (0.9%)
	100 (in soy)	5.3	6.2	0.38 (2.2%)

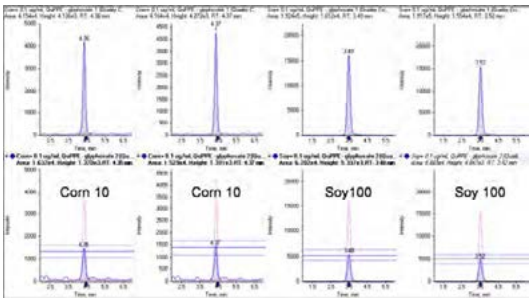

Figure 6. 10 and 100 µg/kg of glyphosate spiked into corn and soy and analyzed using automatic derivatization, dilution, and cleanup followed by LC-MS/MS, ion ratios for compound identification were calculated automatically in MultiQuant™ software version 3.0

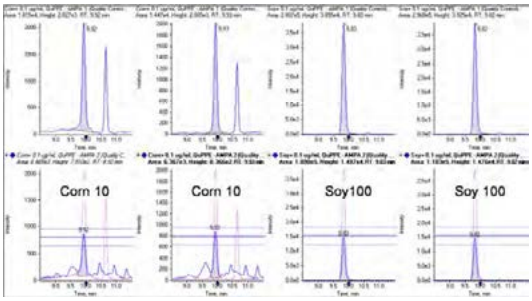

Figure 7. 10 and 100 µg/kg of AMPA spiked into corn and soy and analyzed using automatic derivatization, dilution, and cleanup followed by LC-MS/MS, ion ratios for compound identification were calculated automatically in MultiQuant™ software version 3.0

Ion ratios for compound identification where automatically calculated in the result table in MultiQuant™ software version 3.0. The quantifier and qualified ratio was found to be a valuable tool to identify all target pesticides in matrix samples with excellent reproducibility and values well in between ± 20% (Table 4).

The slightly higher %RSD of the ion ratio of glufosinate in corn can be explained by interfering matrix signals (Figure 8). Stable LC separation was essential for confident identification and accurate quantitation of glufosinate.

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The results using the QuPPe extraction where compared to results obtained when using the none QuPPe procedure based on extraction with 0.1 M HCl.[8] In general, recoveries were between 70-120% for both matrices when using the QuPPe protocol with slightly better recoveries in corn due to the lower protein content.

Recoveries using the none QuPPe extraction where found to be lower in all cases. However, in the case of corn this extraction resulted in cleaner MRM chromatograms for glufosinate (Figure 8).

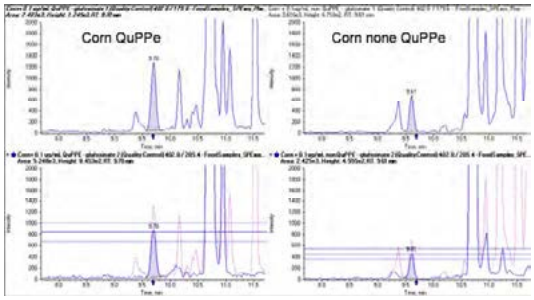

Figure 8. Corn analyzed for glufosinate using the QuPPe and a none QuPPe extraction procedure with higher recoveries but more matrix interferences when using the QuPPe protocol

The total cycle time per sample for the automated sample derivatization and online SPE was approximately 25 minutes, enabling "just in time" sample preparation using the GERSTEL MAESTRO software PrepAhead function. Using this automated procedure for derivatization, extraction and analysis over 55 samples can be processed per day.

Future studies will include the use of isotopically labeled standards to compensate for possible matrix effects. Also retention time shifts were observed when analyzing glyphosate in matrix samples with high protein content. The use of an internal standard will increase confidence in identification using relative retention times.

Summary

As a result of this study, we were able to show:

- Glyphosate, glufosinate, and AMPA can be detected after automatic derivatization using FMOC-Cl at relevant concentration in drinking water and food samples[5-7]
- The described workflow using the GERSTEL MPS 2XL with SPE^{XOS} coupled to a SCIEX QTRAP® 4500 system enabled automated derivatization, dilution, and SPE cleanup and analysis of water and QuPPe extracts of food for LC-MS/MS of polar pesticides.
- The method is highly repeatable with %CV well below 10% due to the automation of sample handling and derivatization.
- Sensitivity was sufficient to inject water samples directly and detect all target compounds below 0.1 µg/L. Food samples can be diluted prior SPE cleanup using the online SPE to monitor at 10 µg/kg.

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5 Regulation (EC) 'concerning the placing of plant protection products on the market' No 1107/2009

6 Commission Regulation (EU) 'regards maximum residue levels' No 441/2012

7 Council Directive 'concerning the quality of water intended for human consumption' 98/83/EC

8 O. Cabrices et al.: 'Automated Derivatization, SPE Cleanup and LC-MS/MS Determination of Glyphosate and Other Polar Pesticides' Application Note Gerstel (2013) #AN9/2013

Publication number: 8013813-01

Contents

Using the SCIEX QTRAP 6500 System to Quantify and Identify Pesticides in Complex Food Samples

André Schreiber and Yun Yun Zou
SCIEX Concord, Ontario (Canada)

Introduction

Recent regulations on food analysis require screening f foor pesticides using confirmatory techniques, such as GC-MMS and LC-MS/MS. More than 1000 pesticides are used worldwwideide and, along with their metabolites and degradation products, aar re present in food. There is a demand for powerful and rappid id analytical methods that can identify pesticides with high h confidence in a broad range of food matrices and quanttiify them at low concentrations with good accuracy and reproduc cibibility.

Challenges for pesticide residue laboratories at the momment are the request to test for more compounds, in a wider rangenge of samples, all without sacrificing data quality.

The SCIEX QTRAP® 6500 LC/MS/MS system uses multi-component *Ion*Drive™ technology to:

- Improve ionization efficiency using the new IonDrive™ Turbo V ion source
- Increase robustness using a reengineered curtain gas interface acting as a better barrier against neutrals and micro droplets
- Increase sensitivity using the new IonDrive™ QJet ion guide with dual stage design
- Extend the linear dynamic range for quantitation using the HED IonDrive™ detector.

In addition, the SCIEX QTRAP® 6500 system uses patented and proven Linear Accelerator™ trap technology to:

- Acquire full scan MS and MS/MS spectra with high selectivity, sensitivity, and speed.

A new method for the quantitation and identification of hundreds of pesticides in food samples was developed and successfully applied to the analysis of complex food samples using t the SCIEX QTRAP® 6500 system. Results are compared to QTRAP® 5500 data. The increased sensitivity was used to extensively dilute sample extracts to eliminate ion suppression caused by matrix components and the extended linear dynamic range allowed quantifying more pesticides across a wider range

of chemical properties. QTRAP® scanning was used to investigate the presence of matrix components and to identify targets with high confidence through library searching. Quantitative and qualitative results were generated using MultiQuant™ 2.1 and LibraryView™ 1.0 software.

Experimental

Standards and Sample Preparation

- The iD*Quant*™ standard kit for pesticide analysis containing 204 pesticides was used for method setup and analysis. A few more pesticides of interest were added.
- A QuEChERS protocol was used for sample extraction followed by extensive dilution to eliminate ion suppression.

UHPLC

- Separation was achieved on a Shimadzu UFLC$_{XR}$ system with a RESTEK Ultra Aqueous C18 (100 x 2.1 mm) 3 µm and a gradient of water/methanol containing 10 mM ammonium formate and 0.1% formic acid (Table 1).
- A flow rate of 350 µL/min was used.
- The injection volume was set to 10 µL.

MS/MS Detection

- The SCIEX QTRAP® 6500 system was operated with IonDrive™ Turbo V ion source using the electrospray ionization probe.
- The ion source temperature was optimized to 450°C.
- A total of 493 Multiple Reaction Monitoring (MRM) transitions (2 transitions per pesticide plus 1 transition for the internal standard D_{10}-Diazinon) were detected to allow quantitation and identification of all target pesticides using the MRM ratio.
- The *Scheduled* MRM™ algorithm was activated to achieve highest data quality. The MRM detection window was set to 120 sec and a target scan time of 0.7 sec was used.
- In addition, Enhanced MS (EMS) and Enhanced Product Ion (EPI) scanning features were explored to monitor matrix effects and to increase confidence in identification by MS/MS library searching.

Table 1. Gradient conditions used for separation

Time	Flow (mL/min)	A (%)	B (%)
0	0.35	95	5
5	0.35	40	60
12.5	0.35	5	95
14.5	0.35	5	95
14.6	0.35	95	5
17.5	0.35	95	5

Results and Discussion

Method Setup

An existing pesticide screening method optimized for use on a QTRAP® 5500 system was transferred to the QTRAP® 6500 system without adjusting compound dependent parameters, such as Declustering Potential (DP) and Collision Energy (DP) values.

The new IonDrive™ Turbo V ion source has larger heaters (11 mm) and an optimized geometry transfers heat more efficiently resulting in improved ionization. The source temperature was optimized from 350 to 700°C with steps of 50 K to investigate best settings for a multi-pesticide screening method. Most compounds gave best Signal-to-Noise (S/N) at 450 or 500°C. A temperature of 450°C was used in the final method not to compromise sensitivity of low stability analytes. To achieve similar ionization 550°C were used in the original method of the QTRAP® 5500 system.

Figure 1 shows temperature maps of the spray region at different temperature settings visualizing the efficiency of heat transfer and the wider 'sweet' spot making probe optimization less crucial to gain maximum sensitivity and reproducibility.

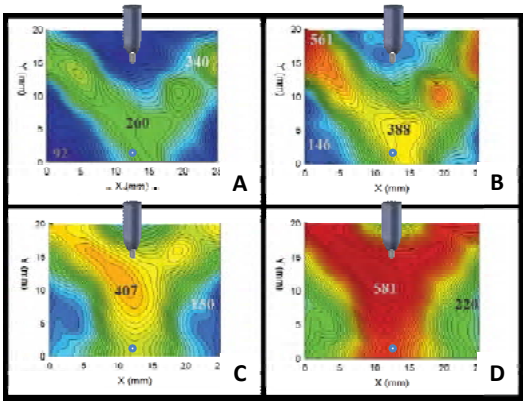
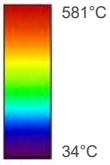


Figure 1. Temperature maps of the spray region of the traditional Turbo V™ source (top) and the new IonDrive™ Turbo V source (bottom). A and C show the source operated at 500°C and B and D at 700°C with Gas 2 set to 70 psi.

The increased heat transfer and wider 'sweet' spot for ionization of the IonDrive™ Turbo V source is clearly depicted in the maps C and D.



Increased Sensitivity

The new design of the ion source and the dual stage design of the IonDrive™ QJet ion guide result in increased sensitivity.

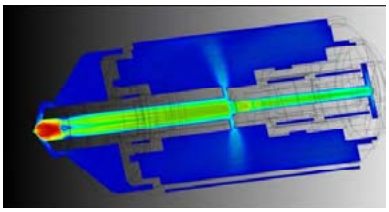


Figure 2. Computed gas flow model of the dual stage QJet™ ion guide

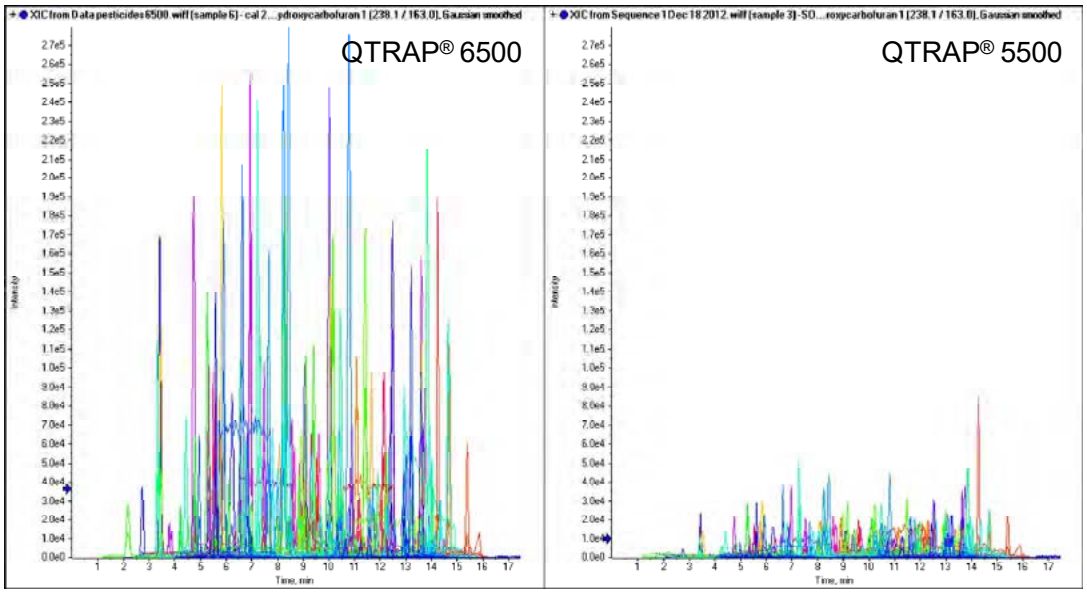


Figure 3. Sensitivity comparison of a 0.1 ng/mL standard analyzed using the QTRAP® 6500 system (left) and QTRAP® 5500 system (right)

The injection of a 0.1 ng/mL (100 parts-per-trillion) standard into the QTRAP® 6500 and QTRAP® 5500 systems is shown in Figure 3. An average gain in sensitivity by a factor of 4.7 was observed. Over 51% of all detected pesticides showed a sensitivity gain larger than 4x (Figure 4).

Results for selected pesticides spanning the entire range of chemical properties are presented in Figure 5. The sensitivity gain for specific compounds can be fine-tuned and mostly depends on ion source temperature.

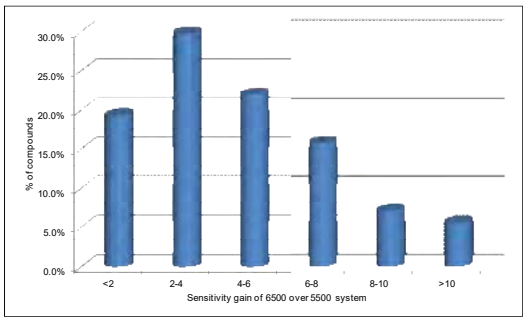


Figure 4. Distribution of sensitivity gain for multi-pesticide analysis with an average gain of 4.7

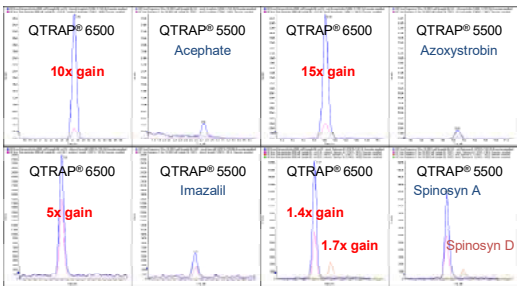


Figure 5. Compound specific sensitivity gains for selected pesticides, including Acephate, Azoxystrobin, Imazalil, and Spinosad, sensitivity gains are compound dependent and can be influenced by ion source temperature

Extensive Extract Dilution to Eliminate Matrix Effects

Matrix effects, like ion suppression and ion enhancement, are caused by co-elution of target analytes with matrix components. While matrix effects can be compensated with co-eluting internal standards or by standard addition, both techniques have limited use for multi-residue analysis since they are expensive and time consuming, respectively. In addition, compensating matrix effects using these techniques has the risk of false negative findings in case the analyte signal is completely suppressed when analyzing complex matrices. Dilution has been shown to be a valuable tool to overcome the problem of matrix effects.[1]

Here we used the increased sensitivity of the QTRAP® 6500 system to dilute QuEChERS extract extensively (up to 1000x) to eliminate matrix effects even in the most challenging matrices.

Figure 6 shows an example of reduced ion suppression of Acetamiprid spiked into a peppermint tea at 100 µg/kg. The 20 and 50x dilution did not result in the expected signal decrease by a factor of 2x or 5x indicating a successful reduction of matrix effects.

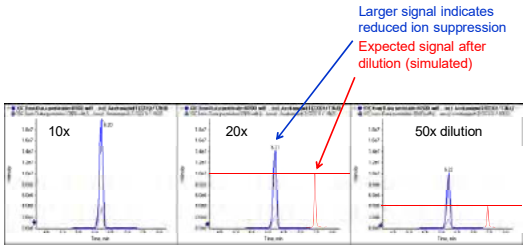


Figure 6. Dilution of peppermint tea extract spiked with Acetamiprid, the increase in sensitivity over the expected (simulated) peak demonstrates successful dilution of matrix effects

Figure 7 shows results of dilution experiments for 4 selected pesticides spiked into peppermint tea. It can be seen that matrix effects are different for each analyte due to different matrix components eluting at the respective retention time. Also different dilution factors are needed to eliminate matrix effects for each compound. A dilution factor of 50 to 200 was required to reduce ion suppression for the selected pesticides to less than 20%.

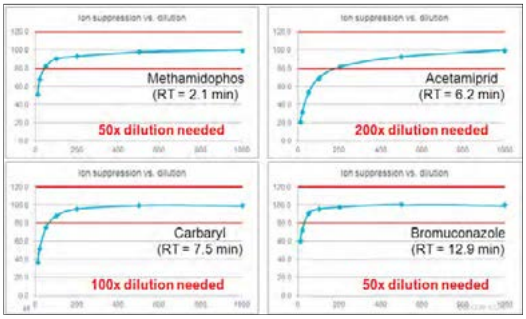


Figure 7. Ion suppression caused by peppermint tea was successfully reduced by dilution of 50 to 200x

Monitoring of Matrix Effects using QTRAP® EMS Scanning

Acquiring full scan MS chromatograms is a valuable tool to monitor and understand matrix effects. Figure 8 shows an example of combining an MRM experiment and Enhanced MS (EMS) scanning when analyzing a black tea extract.

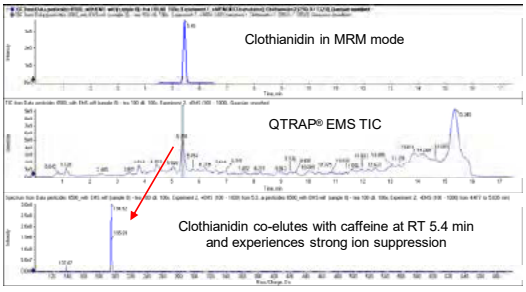


Figure 8. Monitoring of matrix effects by simultaneous acquisition of target MRM transitions and EMS scans, the strong ion suppression observed for Clothianidin can be explained by co-elution with caffeine at a much higher concentration

Extended Linear Dynamic Range

The new HED IonDrive™ detector allows taking advantage of sensitivity gains not at the expense of the dynamic range for quantitation. The detector enables ultra-fast pulse counting up to 10^8 cps without compromising data quality of low sensitivity ions. Up to 6 orders of magnitude linear dynamic range were reported.[2]

The extended linear dynamic range of the QTRAP® 6500 system can also be beneficial when quantifying larger panels of compounds covering a wide range of chemical properties (low sensitivity analytes to high sensitivity analytes). Examples of calibration lines obtained from the QTRAP® 6500 and QTRAP® 5500 systems are presented in Figures 9 a-c.

The extended linear dynamic range allowed easier and faster data processing and review since fewer points had to be excluded from the calibration line.

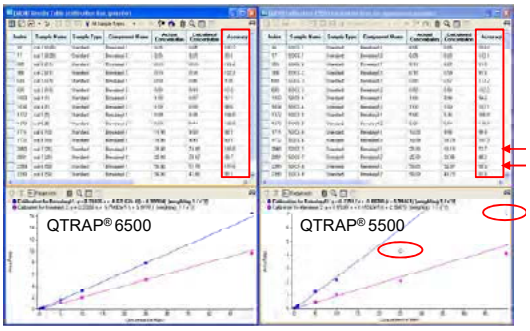


Figure 9a. Quantitation of Benalaxyl, a high sensitivity pesticide, 0.05 to 50 ng/mL, no points had to be excluded when the HED IonDrive™ detector was used due to extended pulse counting

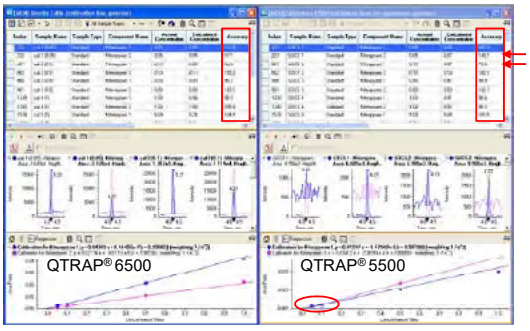


Figure 9b. Quantitation of Nitenpyram, a low sensitivity pesticide, 0.05 to 50 ng/mL, no points had to be excluded when the HED IonDrive™ detector was used due to increased sensitivity

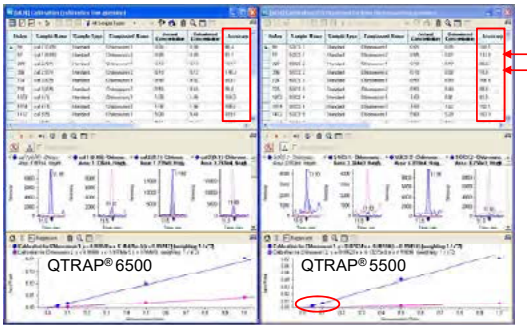


Figure 9c. Quantitation of Chloroxuron, a pesticide with a weak qualifier ion, 0.05 to 50 ng/mL, no points had to be excluded when the HED IonDrive™ detector was used to extended pulse counting and increased sensitivity

Automatic Compound Identification using MRM Ratios and Full Scan MS/MS Library Searching

Guidelines for food residue analysis require the identification of MRL exceeding compounds and unusual residues.[3]

LC-MS/MS can be used in different ways to acquire the mass spectrometric information needed to identify compounds with high confidence, including ratio of quantifier and qualifier MRM transition, full scan product ion spectra (i.e. Enhanced Product Ion (EPI) scanning using QTRAP® functionality), or accurate mass measurements.[4]

Sample data was processed using MultiQuant™ software version 2.1 with the 'Multicomponent' query. Query files are customizable commands to perform custom querying of the result table. The 'Multicomponent' query automatically calculates and compares MRM ratios for compound identification and highlights concentrations above a user specified maximum residue level. An example of the results and peak review after running the query file is shown in Figure 10.

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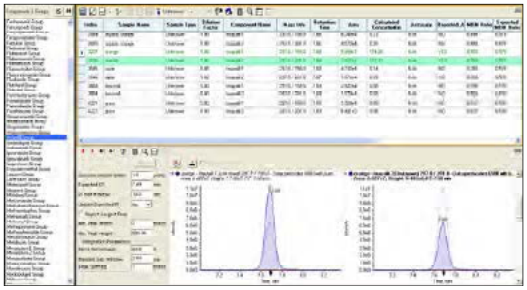

Figure 10. Automatic reporting of pesticides using the 'Multicomponent' query in MultiQuant™ software: Imazalil was flagged in the result table because of a concentration above a user defined threshold and positive identification using the MRM ratio

Despite the high selectivity of MRM detection, there is always a risk of false positive findings due to interfering matrix signals. Identification based on full scan MS/MS data searched against mass spectral libraries significantly increases confidence in identification. Here MS/MS spectra acquired in the EPI mode of the QTRAP® 6500 system were searched against the iMethod™ pesticide library (version 1.0 for LibraryView™ software). Library searching was performed in LibraryView™ software for easy data review and reporting (Figure 11).

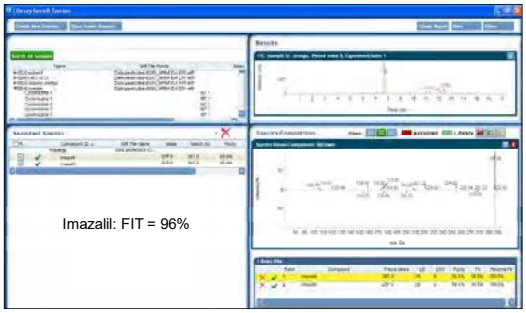

Figure 11. Review of MS/MS search results in LibraryView™ software

Summary

The SCIEX QTRAP® 6500 system was used for multi-pesticide quantitation and identification in complex food samples. The increased sensitivity was used to extend the scope of the method and to dilute matrix extracts extensively to eliminate matrix effects. The extended linear dynamic range allowed easier and faster data processing and review while monitoring high sensitivity and low sensitivity pesticides in a single method. QTRAP® scanning was used to investigate the presence of matrix components and to identify targets with high confidence through library searching.

Acknowledgement

The authors like to thank Jon Wong (FDA, College Park, MD, USA) and Paul Yang (Ministry of Environment, Etobicoke, ON, Canada) for providing the LC method and sample extracts.

References

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2 Michael Jarvis: 'Ultra-Sensitive Analysis of Aldosterone in Serum Using the AB SCIEX Triple Quad™ 6500 LC/MS/MS System' Application Note AB SCIEX (2012) 5730212-01

3 Document N° SANCO/12495/2011 'Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed'

4 André Schreiber et al.: 'Target and Non-Target Screening for Chemical Residues in Food Samples using the AB SCIEX TripleTOF® 4600 System and Intuitive Data Processing Tools' Application Note AB SCIEX (2012) 5680212-01

Publication number: 7580413-01

Contents

Analysis of Pesticides in Food Samples Using the SCIEX Triple Quad™ 3500 System

André Schreiber
CIEX Concord, Ontario (Canada)

Overview

Pesticides are widely used in agriculture to protect crops and to improve efficiency of production. Pesticide residues may pose a potential threat to human health. Modern analytical techniques, such as LC-MS/MS allow the screening for hundreds pesticide residues in food samples quickly, efficiently, and with excellent sensitivity and selectivity to meet global food trade guidelines and regulations.[1-3]

Mass spectrometers are typically considered to be expensive and complex instruments. However, the SCIEX Triple Quad™ 3500 system, combined with an extensive compound MRM catalog, provides labs with robust and reliable mass spec technology and method starting points, at an affordable price.

Here we present a method using QuEChERS extraction with Phenomenex roQ kits, filtration with Thomson filter vials, separation using a Kinetex Biphenyl 2.6u (50 x 2.1mm) column, and the SCIEX Triple Quad™ 3500 system. The mass spectrometer was operated in highly selective and sensitive Multiple Reaction Monitoring (MRM) mode. The *Scheduled* MRM™ Pro algorithm was used to obtain the best data quality. Compound identification and quantitation was achieved by monitoring two MRM transitions for each pesticide. The MRM ratio was automatically evaluated in MultiQuant™ software.

Introduction

LC-MS/MS is a powerful analytical tool capable of screening samples for numerous compounds. MRM is typically used because of its excellent sensitivity, selectivity, and speed.

Generic extraction procedures, like QuEChERS, ultra high performance LC systems combined with core-shell particle columns, providing good resolution and excellent peak shape, made it possible to detect pesticides of a wide variety of compound classes and chemical properties in each sample. State-of-the-art LC-MS/MS systems make it possible to detect hundreds of pesticides and other food residues in a single run.

The SCIEX Triple Quad™ 3500 system takes the best features of the API 3200™ system and enhances them with

modern engineering and electronics. The proven design of Turbo V™ source and Curtain Gas™ interface provide exceptional robustness and ruggedness. The advanced eQ™ electronics and the curved LINAC® collision cell were designed for ultra-fast speed of MRM detection and fast polarity switching for comprehensive multi-component analysis.

Advanced software tools like the *Scheduled* MRM™ Pro algorithm intelligently uses information of retention times to automatically optimize MRM dwell time of each transition and total cycle time of the experiment resulting in best data quality. Two MRM transitions were monitored for each pesticide to use the ratio of quantifier and qualifier ion for compound identification.

Experimental

- The SCIEX iD*Quant*™ standards kit for pesticide analysis was used for method setup and preparation of calibration standards.[4]
- Store-bought fruit and vegetable samples were extracted using Phenomenex roQ QuEChERS kit buffer-salt mix and dSPE kits following the European standard method 15662.[5]
- Extracts were diluted 5 times with water in Thomson filter vials, filtered using the 0.45 µm PVDF membrane and directly

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placed into the autosampler for LC-MS/MS analysis. The injection volume was set to 2 µL.

- LC separation was achieved using a Phenomenex Kinetex Biphenyl 2.6u (50 x 2.1mm) column and a fast gradient of water and methanol with 5 mM ammonium formate buffer at a flow rate of 0.5 mL/min (see Table 1 for the gradient profile).

Table 1. Gradient conditions used for the separation of pesticides

Step	Time (min)	A (%)	B (%)
0	0.0	90	10
1	0.5	90	10
2	2.0	70	30
3	9.0	40	60
4	11.0	20	80
5	12.0	5	95
6	15.0	5	95
7	16.0	90	10
8	20.0	90	10

- The SCIEX Triple Quad™ 3500 system was operated with Turbo V™ source and Electrospray Ionization (ESI) probe set to 400°C.
- Approximately 400 MRM transitions were monitored in positive polarity. Optimized transitions for all compounds were obtained through the MRM catalogue of the iMethod™ application for Pesticide Screening version 2.1.
- The *Scheduled* MRM™ Pro algorithm was used with a target cycle time of 0.5 sec and compound dependent detection windows and thresholds (Figure 1).

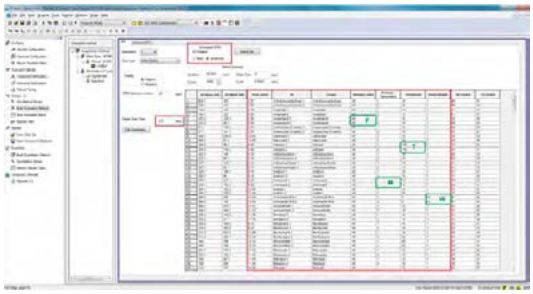

Figure 1. *Scheduled* MRM™ Pro algorithm allowing: Flexible Window Width (F), Dynamic Window Extension (T), MRM-triggered MRM (M, T), Dwell Time Weighting (W)

- MultiQuant™ software version 3.0 was used for quantitative and qualitative data processing.

Results and Discussion

Sensitivity, Reproducibility, Linearity and Accuracy

Chromatograms of a solvent standard at 10 ng/mL analyzed using the API 3200™ and Triple Quad™ 3500 are shown in Figure 2. An average gain in sensitivity of 3x was observed.

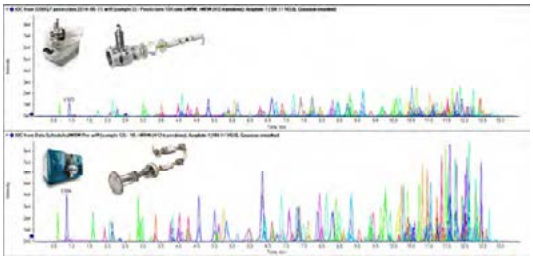

Figure 2. Sensitivity comparison of a 10 ng/mL standard analyzed using the API 3200™ system (top) and SCIEX Triple Quad™ 3500 system (bottom) with an average sensitivity gain of 3x

Most pesticides were detectable at a concentration below 1ng/mL and all pesticides had a limit of detection (LOD) of 2 ng/mL or lower. Example chromatograms at a concentration of 5 ng/mL are shown in Figure 3. The achieved sensitivity allows sample extract dilution by 5x to minimize possible matrix effects.

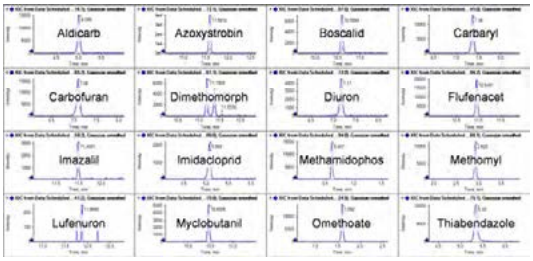

Figure 3. Sensitivity of selected pesticides detected at a concentration of 5 ng/mL using the Triple Quad™ 3500 system

Linearity was obtained over 3 to 4 orders of magnitude for most pesticides with accuracies between 80 and 120%. Data points of

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the lowest or highest standards were excluded for a few pesticides with weak or strong ionization, respectively. Repeatability was studied at 1 and 10 ng/mL (n=5). The coefficient of variation (%CV) was typically below 10%.

An example calibration line of Acephate is shown in Figure 4. Both MRM transitions had a regression coefficient of > 0.998 and excellent repeatability of 2.9 and 3.2% at 1 and 10 ng/mL respectively (n=5).

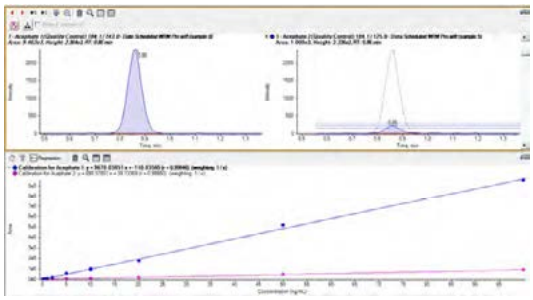

Figure 4. Peak review quantifier-qualifier ratio of Acephate at 1 ng/mL and calibration line from 0.1 to 100 ng/mL with %CV of 2.9% and 3.2% at 1 and 10 ng/mL, respectively, and.

Findings in Fruit and Vegetable Samples

The developed method was applied to the quantitation and identification of pesticides in real food extracts. Different dispersive SPE kits of Phenomenex (roQ KS0-8913, 8914, 8915, 8916) were used for sample cleanup depending on the type of matrix following the European standard method 15662. Extracts were diluted 5 times with water to minimize possible matrix effects. The diluted extracts were filtered using the Thompson 0.45 µm PVDF membrane and directly placed into the autosampler for LC-MS/MS analysis.

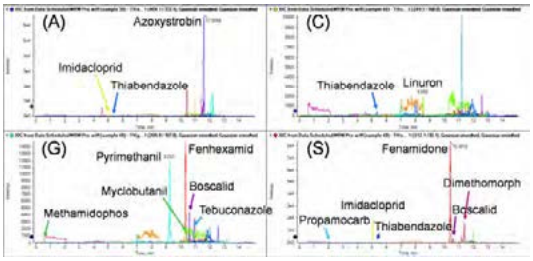

Figure 5. Detection of pesticides in filtered QuEChERS extracts of avocado (A), carrot (C), grapes (G), and spinach (S)

Example chromatograms of different type of food samples with detected compounds are presented in Figure 5. Qualitative and quantitative results are summarized in Table 2. Compound identification was based on the criteria of SANCO/12571/2013 (retention time tolerance of ± 0.02 min and maximum tolerances for ion ratios ± 30%). All quantitative and qualitative results were automatically calculated in MultiQuant™ software (Figure 6).[6]

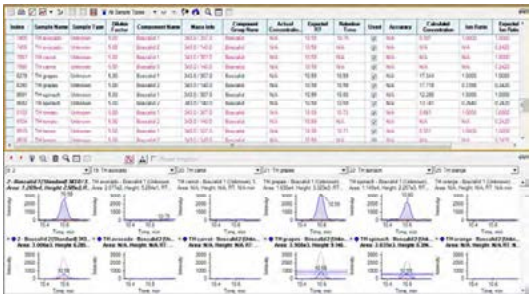

Figure 6. Quantitation and identification based on MRM ratios in MultiQuant™ software, the example shows the side-by-side peak review for Boscalid with positive findings in grapes and spinach samples

Table 2. Summary of pesticide findings in store bought food above a concentration of 1 µg/kg

Sample	Pesticide	Concentration (µg/kg)	RT Error (min)	MRM Ratio (Expected)
Avocado	Azoxystrobin	55.0	0.01	0.146 (0.126)
	Imidacloprid	6.2	0.03	0.823 (0.818)
	Thiabendazole	2.9	0.06	1.035 (0.820)
Carrot	Linuron	14.3	0.00	0.613 (0.742)
	Thiabendazole	5.3	0.04	0.995 (0.820)
Grapes	Boscalid	17.3	0.00	0.240 (0.242)
	Fenhexamid	363	0.04	0.973 (1.053)
	Methamidophos	1.2	0.01	0.873 (0.698)
	Myclobutanil	14.2	0.02	0.811 (0.830)
	Pyrimethanil	687	0.05	0.482 (0.435)
	Tebuconazole	7.1	0.03	0.030 (0.261)
Grapefruit	Imazalil	899	0.07	0.410 (0.348)
	Imidacloprid	1.3	0.03	1.052 (0.993)
	Thiabendazole	7.6	0.03	0.812 (0.820)
Lemon	Imazalil	981	0.06	0.266 (0.348)
	Thiabendazole	7.6	0.04	0.782 (0.820)
Orange	Imazalil	1830	0.06	0.282 (0.348)
	Thiabendazole	>3000	0.04	0.812 (0.820)
Spinach	Boscalid	12.3	0.00	0.264 (0.242)
	Dimethomorph	53.7	0.08	0.537 (0.541)
	Fenamidone	755	0.01	0.749 (0.672)
	Imidacloprid	217	0.03	0.907 (0.993)
	Propamocarb	3.1	0.06	0.260 (0.336)
	Thiabendazole	3.6	0.05	0.917 (0.820)

Improving data acquisition quality with Scheduled MRM Pro algorithm

Figures 7 and 8 show results of pesticides detected in food samples to explain different features of *Scheduled* MRM™ Pro algorithm.

The detection window can be set differently for each compound depending on LC peak width and potential retention time shifts. This allows a more effective scheduling of MRM transitions resulting in better data quality. The example in Figure 7 shows Boscalid detected with a window of 45 sec, while the window of Dimethomorph was set to 120 sec to detect both isomers together.

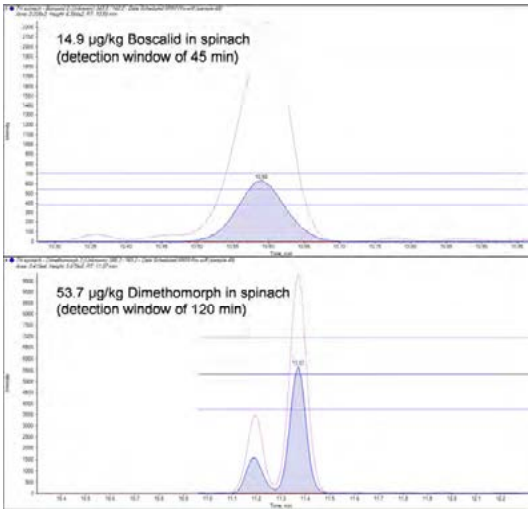

Figure 7. Examples of using the Flexible Window Width in a *Scheduled* MRM™ Pro method: the window for Boscalid was set to 45 sec and Dimethomorph was detected using a wider window to detect both isomers together

The *Scheduled* MRM™ Pro algorithm also allows automatic triggering of qualifier MRM transitions when a quantifier transitions is present (Figure 8). This feature further optimizes the MRM scheduling. The threshold is also used to automatically extend the detection window if an MRM signal is still present at the end of the default detection window.

Figure 8 shows an example of dynamic window extension for the detection of Thiabendazole in an orange sample. The sample contained Thiabendazole at more than 3000 µg/kg resulting in peak tailing. The automatic extension of the detection window enabled to capture the complete peak area for accurate quantitation and identification based on the MRM ratio.

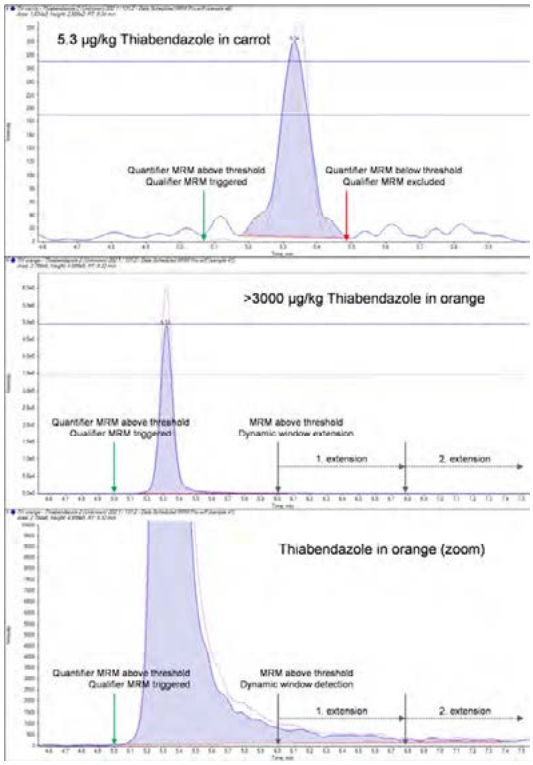

Figure 8. Examples of MRM-triggered MRM and Dynamic Window Extension: the qualifier MRM transition is automatically triggered when the quantifier MRM transitions exceeds the threshold set in the *Scheduled* MRM™ Pro method, the detection window is automatically extended if the MRM signal is above the threshold at the end of the detection window

Summary

A new LC-MS/MS method for the identification and quantitation of pesticides was developed and successfully applied to fruit and vegetable samples.

Samples were extracted using a QuEChERS protocol following the European standard method 15662 with Phenomenex roQ kits. Sample extracts were diluted 5x to minimize potential matrix effects and filtered using Thomson filter vials. The SCIEX Triple Quad™ 3500 system operated in MRM mode and utilizing the *Scheduled* MRM™ Pro algorithm was used for detection. Two MRM transitions were monitored for each analyte and the ratio of quantifier and qualifier transition was used for identification.

Qualitative and quantitative data processing was performed in MultiQuant™ software. Criteria of SANCO/12571/2013 were used for identification. All pesticides had an LOD of 2 ng/mL or lower and good linearity of 3-4 orders of magnitude with repeatability well below 10%.

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3 J. Wong et al.: 'Development and Interlaboratory Validation of a QuEChERS-Based Liquid Chromatography–Tandem Mass Spectrometry Method for Multiresidue Pesticide Analysis' J. Agric. Food Chem. 58 (2010) 5897-5903

4. A. Schreiber et al.: 'Using the iD*Quant*™ Standards Kit for Pesticide Analysis to Analyze Residues in Fruits and Vegetable Samples' Application Note AB SCIEX (2011) #3370211-01

5 CSN EN 15662: 'Foods of plant origin - Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS-method' (2008)

6 SANCO/12571/2013: 'Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed.'

Publication number: 10641314-01

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Using Your QTRAP® LC/MS/MS System at Full Potential

Verification of Qualitative Method Performance using MRM Ratios and MS/MS Library Searching

Overview

This document summarizes the results of method verification utilizing LC-MS/MS with MRM ratios and MS/MS library searching for pesticide identification in food samples. A SCIEX QTRAP® 6500 LC/MS/MS system was used in this study to analyze spiked extracts of different fruit and vegetable samples.

Key findings of this study:

- **MRM quantitation** – powerful approach to quantify hundreds of pesticides in food samples with high selectivity and sensitivity, especially in combination with the *Scheduled* MRM™ Pro algorithm
- **MRM ratio identification** – established technique for compound identification, however, ion ratio calculation can result in false positive and negative results
- **Identification using MS/MS library searching** – alternative approach for compound identification providing increased confidence because of the detection of multiple fragments (beyond just 2 MRM transitions)
- **Improved data processing** – dual injection approach with automatic quantitation, identification and confirmation using MasterView™ software and MultiQuant™ software

The QTRAP® Data Processing Workflow in MultiQuant™ and MasterView™ Software

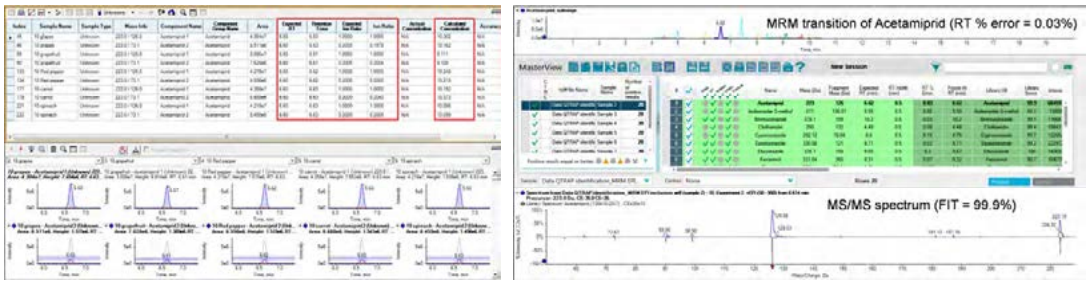

Figure 1. Quantitation, identification and confirmation using a dual injection approach using two complementary LC-MS/MS methods utilizing the *Scheduled* MRM™ Pro algorithm with automatic MRM ratio calculation and *Scheduled* MRM™-IDA-MS/MS followed by MS/MS library searching

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Experimental

Additional method details and results are described in an additional application note published by SCIEX.[1]

Sample Preparation

Store-bought food samples were extracted using a QuEChERS procedure based on the European standard method 15662.[2]

Mix D of the SCIEX iD*Quant*™ kit for pesticide analysis, containing 20 compounds, was spiked into food samples and used to verify method performance for identification and confirmation.[3]

LC Separation

LC separation was achieved using a Phenomenex Kinetex Biphenyl (100 x 2.1 mm, 2.6u) column using a gradient of water/methanol with 5 mM ammonium formate and a total run time of 15 min. The injection volume was set to 10 µL.

MS/MS Detection

Samples were analyzed using two separate methods using the SCIEX QTRAP® 6500 system with IonDrive™ Turbo V ion source using the electrospray ionization probe.

Method 1 utilized the *Scheduled* MRM™ Pro algorithm to monitor approximately 800 transitions to quantify and identify ~400 pesticides based on the ratio of quantifier and qualifier transition.

Method 2 utilized the *Scheduled* MRM™-IDA-MS/MS workflow to collect additional MS/MS information for identification based on library searching. MS/MS spectra were acquired using information dependent acquisition (IDA) and collision energy settings of CE = 35 V with CES = 15 V

Results and Discussion

The method provide sufficient speed, sensitivity and linearity to detect all ~400 pesticides at a concentration of 1 µg/kg in 10x diluted QuEChERS extract of food samples. Good linearity was observed for most compounds from 0.1 to 100 ng/mL with repeatability at 1 ng/mL typically well below 10% coefficient of variation.[1]

Mix D of the SCIEX iD*Quant*™ kit for pesticide analysis, containing 20 compounds, was spiked into carrot, grapes, grapefruit, red pepper, and spinach extract at 10 µg/kg.

Example screenshots of identification and quantification of Acetamiprid are shown in Figure 1. Identification in MultiQuant™ software (left) was based on an MRM tolerance of 30% following SANCO/12571/2013 guideline.[4] MS/MS library searching was performed in MasterView™ software. A PUR value of 70% or higher was used for positive identification. The retention time tolerance was set to 0.2 min

The results of identification based on retention time matching, MRM ratio comparison, and MS/MS library searching are summarized in Table 2. All 20 pesticides were confidentially identified in all 5 spiked samples. The average retention time error ranged from 0.008 to 0.024%, the average MRM ratio error from 5.09 to 6.30%, and the average MS/MS PUR from to 95.9 to 98.5%.

However, very few pesticides required confirmatory analysis since the identification criteria were slightly outside of tolerance levels.

For example Fenarimol was detected in all samples with matching retention time but the MRM ratio was outside or very close to the 30% tolerance due to high background and a closely eluting interfering matrix peak (Figure 2). But the analysis of a second sample extract to acquire MS/MS spectra confirmed the presence of Fenarimol with excellent library PUR well above 90% (94.4 to 99.7%).

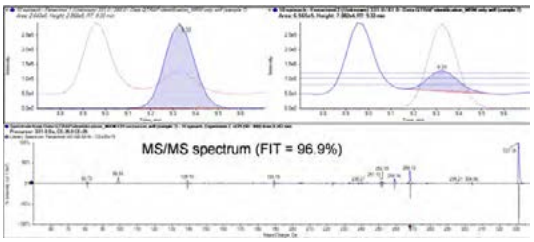

Figure 2. Detection of Fenarimol in spiked spinach: the MRM ratio was slightly out of the 30% tolerance due to high background and a closely eluting interfering matrix peak, but MS/MS library searching confirmed the presence of the detected pesticide.

Table 1. Pesticides identified in different spiked food samples based on retention time (RT) matching, MRM ratio comparison, and MS/MS library searching for qualitative method validation

	Carrot					Grapes					Grapefruit					Red Pepper					Spinach				
Pesticides	**RT (min)**	**RT Error**	**MRM Ratio**	**Ratio Error**	**PUR (%)**	**RT (min)**	**RT Error**	**MRM Ratio**	**Ratio Error**	**PUR (%)**	**RT (min)**	**RT Error**	**MRM Ratio**	**Ratio Error**	**PUR (%)**	**RT (min)**	**RT Error**	**MRM Ratio**	**Ratio Error**	**PUR (%)**	**RT (min)**	**RT Error**	**MRM Ratio**	**Ratio Error**	**PUR (%)**
Acetamiprid	6.63	0.01	0.20	1.7	97.7	6.64	0.02	0.20	1.3	98.0	6.63	0.01	0.20	0.0	99.4	6.63	0.01	0.20	0.2	99.5	6.61	0.01	0.20	0.0	99.6
Acibenzolar-S-methyl	9.56	0.01	0.35	6.5	62.7	9.59	0.04	0.39	4.9	96.1	9.53	0.02	0.40	5.1	80.0	9.55	0.00	0.41	8.3	71.4	9.57	0.02	0.34	8.9	95.5
Bromuconazole	10.20	0.00	0.16	9.2	99.5	10.23	0.03	0.13	7.5	98.6	10.22	0.02	0.14	5.2	99.8	10.20	0.00	0.14	5.0	99.1	10.21	0.01	0.13	10.1	98.4
Clothianidin	4.48	0.00	0.35	5.6	98.1	4.49	0.01	0.36	2.4	97.4	4.48	0.00	0.36	2.2	98.1	4.49	0.01	0.35	3.5	98.0	4.47	0.01	0.36	1.3	98.7
Cyproconazole	8.84	0.04	0.58	8.4	100.0	8.81	0.01	0.61	14.2	99.0	**8.77**	**0.03**	**0.57**	**6.9**	**50.3**	8.88	0.08	0.61	14.8	98.9	8.75	0.05	0.54	1.3	99.7
Epoxiconazole	9.73	0.02	0.35	5.2	95.6	9.75	0.04	0.33	0.2	74.6	9.70	0.01	0.34	2.3	99.5	9.72	0.01	0.35	6.7	96.5	9.70	0.01	0.33	0.1	99.8
Etaconazole	9.68	0.03	0.17	3.2	99.6	9.69	0.04	0.16	1.3	97.7	9.66	0.01	0.17	1.8	99.3	9.66	0.01	0.18	7.0	99.2	9.67	0.02	0.17	0.7	89.6
Fenarimol	**9.30**	**0.01**	**0.26**	**36.7**	**99.7**	**9.33**	**0.02**	**0.25**	**33.3**	**99.3**	**9.30**	**0.01**	**0.24**	**27.8**	**99.7**	**9.30**	**0.01**	**0.25**	**33.4**	**94.4**	**9.31**	**0.00**	**0.25**	**32.0**	**96.9**
Flutriafol	8.04	0.01	0.59	6.0	99.8	8.06	0.03	0.56	1.7	100.0	8.04	0.01	0.62	11.3	100.0	8.04	0.01	0.57	3.7	99.9	8.03	0.00	0.56	1.4	99.4
Imazalil	9.98	0.01	0.57	1.8	97.9	10.01	0.02	0.58	3.6	98.8	9.99	0.00	0.60	7.5	98.8	9.98	0.01	0.59	6.7	98.0	9.99	0.00	0.63	13.1	98.8
Imidacloprid	6.04	0.00	0.81	0.9	98.7	6.05	0.01	0.81	0.7	98.7	6.04	0.00	0.79	1.5	99.5	6.05	0.01	0.80	0.0	99.1	6.03	0.01	0.82	2.0	97.9
Metribuzin	6.97	0.01	0.43	2.6	100.0	6.98	0.02	0.43	3.2	100.0	6.96	0.00	0.46	10.4	100.0	6.97	0.01	0.42	1.7	100.0	6.96	0.00	0.44	4.7	100.0
Myclobutanil	9.04	0.00	0.76	7.5	99.5	9.05	0.01	0.78	11.0	100.0	9.05	0.01	0.72	1.5	99.6	9.04	0.00	0.70	1.6	99.8	9.04	0.00	0.72	1.7	99.9
Nitenpyram	4.38	0.00	0.86	3.2	94.3	4.39	0.01	0.85	1.3	95.2	4.38	0.00	0.84	0.5	95.6	4.39	0.01	0.84	1.0	95.9	4.38	0.00	0.85	1.5	97.0
Paclobutrazol	8.41	0.01	0.19	6.5	100.0	8.44	0.04	0.17	4.9	100.0	8.40	0.00	0.16	8.3	100.0	8.40	0.00	0.17	4.6	100.0	8.42	0.02	0.18	1.4	100.0
Pyrimethanil	8.57	0.00	0.53	3.2	99.5	8.60	0.03	0.51	7.8	99.5	8.56	0.01	0.55	0.5	99.5	8.57	0.00	0.54	1.9	99.5	8.58	0.01	0.55	0.5	99.5
Thiacloprid	7.43	0.01	0.11	3.7	99.8	7.44	0.02	0.12	10.7	99.8	7.42	0.00	0.11	1.7	100.0	7.43	0.01	0.12	4.3	100.0	7.42	0.00	0.12	5.5	99.8
Thiamethoxam	4.97	0.00	0.35	1.1	98.8	4.98	0.01	0.34	2.1	99.3	4.97	0.00	0.34	3.4	98.5	4.98	0.01	0.34	3.6	99.2	4.96	0.01	0.34	2.6	99.3
Triadimenol	8.46	0.00	0.38	0.4	100.0	8.50	0.04	0.39	2.3	99.2	8.45	0.01	0.36	6.3	99.7	8.45	0.01	0.36	6.8	100.0	8.49	0.03	0.34	12.2	100.0
Triticonazole	9.14	0.02	0.07	3.3	98.6	9.15	0.03	0.09	11.7	99.7	9.12	0.00	0.08	4.2	100	9.14	0.02	0.08	4.7	99.0	9.12	0.00	0.08	1.0	100.0
Average		0.009		5.84	96.99		0.024		6.30	97.55		0.008		5.42	95.87		0.012		5.98	97.37		0.011		5.09	98.5

Bold and green = positive identification (RT error < 0.2 min, ratio error <30%, MS/MS PUR >70%

Bold and yellow = questionable identification (MS/MS PUR <70%),

Bold and red = no identification (ratio error >30%)

Cyproconazole was identified in the grapefruit sample with matching retention time but the MS/MS PUR value was below the tolerance level (50.3%). Figure 9 shows the MS/MS review in MasterView™ software which helped to identify an isobaric matrix interference causing the low library search PUR. The analysis of a second sample extract confirmed the presence of Cyproconazole by MRM ratio matching (0.569 vs. theoretical 0.532).

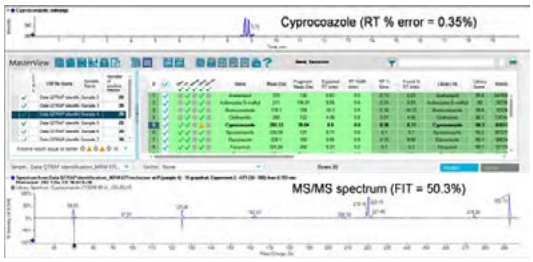

Figure 9. Processing of *Scheduled* MRM™ and MS/MS data in MasterView™ software, compound identification is achieved through automatic retention time (RT) matching and MS/MS library searching

These two data examples highlight the complementary nature of identification using MRM ratios and MS/MS library searching. Both methods, utilizing the *Scheduled* MRM™ Pro algorithm and *Scheduled* MRM™-IDA-MS/MS, are suitable to quantify and identify pesticides in food samples. However, matrix interferences and high background can result in questionable identification. The analysis of a second sample extract using the alternative approach greatly enhances identification making it a viable tool for confirmation. Such a confirmation method is especially important if the target pesticide is not amenable to an orthogonal method, such as GC-MS.

Summary

A QuEChERS and LC-MS/MS based method for the analysis of approximately 400 pesticides in food samples was developed.

The method used the SCIEX QTRAP® 6500 system utilizing the *Scheduled* MRM™ Pro algorithm and information dependent acquisition of full scan MS/MS spectra allowing quantitation and confident identification.

20 pesticides spiked into different food samples at 10 µg/kg and diluted extracts were analyzed using both methods. All 20 compounds were confidentially identified in all samples. Very few pesticides required confirmatory analysis since the identification criteria were slightly outside of tolerance levels (MRM ratio tolerance of 30% or library PUR value of 70%). However, these results highlight the complementary nature of MRM ratios and MS/MS full scan offering a possibility for confirmatory analysis.

Automatic data processing was performed in MultiQuant™ and MasterView™ software.

References

1. A. Schreiber and Lauryn Bailey: 'Advanced Data Acquisition and Data Processing Workflows to Identify, Quantify and Confirm Pesticide Residues' Application Note AB SCIEX (2014) #09270214-01
2. CSN EN 15662: 'Foods of plant origin - Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS-method' (2008)
3. A. Schreiber et al.: 'Using the iD*Quant*™ Standards Kit for Pesticide Analysis to Analyze Residues in Fruits and Vegetable Samples' Application Note AB SCIEX (2011) #3370211-01
4. SANCO/12571/2013: 'Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed.'

For additional support on implementing this workflow in your own lab, or for support on other SCIEX products, visit our website or email us at support@absciex.com.

Publication number: 10390214-01

Advanced Data Acquisition and Data Processing Workflows to Identify, Quantify and Confirm Pesticide Residues

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Overview

Pesticides are widely used in agriculture to protect crops and to improve efficiency of production. Pesticide residues may pose a potential threat to human health. Modern analytical techniques, such as QuEChERS extraction followed by LC-MS/MS, allow screening for pesticides in a variety of food matrices.[1-3]

Here we present a new and powerful workflow to identify, quantify and confirm the presence of 400 pesticides utilizing generic QuEChERS extraction and LC-MS/MS analysis with the SCIEX QTRAP® 6500 system using the *Scheduled* MRM™ Pro algorithm and Information Dependent Acquisition (IDA) of full scan MS/MS spectra. High confidence in identification and confirmation was achieved by automatically calculating the ratio of quantifier and qualifier ions and searching MS/MS spectral libraries in MultiQuant™ and MasterView™ software. Qualitative method performance was verified using guideline SANCO/12571/2013 guideline.[4]

Introduction

Pesticides are widely used in agriculture to protect crops and to improve efficiency of production. After application pesticides may remain on agricultural products or accumulate in the environment, posing a potential threat to human health. Consequently, government agencies, food producers and food retailers have the duty to ensure that pesticide residues occurring in food are below established maximum residue limits set by Codex Alimentarius, the European Union, the US EPA, or by the Japanese Ministry of Health, Labour and Welfare.

There is a demand for powerful and rapid analytical methods that can identify pesticides with high confidence in a broad range of food matrices and quantify them at low concentrations with good accuracy and reproducibility.

A new analytical workflow was developed to screen for 400 pesticides in fruit, vegetable, tea and spices utilizing generic QuEChERS extraction, UHPLC separation using a core-shell particle column, and MS/MS detection with the SCIEX QTRAP® 6500 system. The *Scheduled* MRM™ Pro algorithm was used to acquire ~800 MRM transitions to accurately quantify target pesticides and identify them based on the characteristic

ratio of quantifier and qualifier ions. The *Scheduled* MRM™ data were also used to automatically acquire full scan MS/MS spectra to allow data to be searched against spectral libraries. The data processing in MultiQuant™ and MasterView™ software was used as a confirmatory tool to enhance confidence in quantitative and qualitative results.

Experimental

Sample Preparation

A pesticide standard containing ~400 compounds was used for method development and sample analysis.

Store-bought food samples were extracted using a QuEChERS procedure based on the European standard method 15662.[5]

- 10 g of frozen homogenized sample
- Addition of water to increase the water content of the sample to approximately 10 g
- Addition of 10 mL acetonitrile and internal standard
- Extraction by vigorous shaking for 1 min
- Addition of Phenomenex roQ™ QuEChERS kit buffer-salt mix (KS0-8909) and immediate vigorous shaking for 1 min
- Centrifugation for 10 min at 9000 rpm

Contents

- Transfer of a 1 mL aliquot of the sample extract into a tube containing Phenomenex roQ™ dSPE kit (KS0-8916, 8913, 8914 or 8915 depending on sample type)
- Cleanup by vigorous shaking for 30 sec
- Transfer of 100 µL of the cleaned sample extract into an autosampler vial
- 10x dilution with water prior LC-MS/MS analysis

Mix D of the SCIEX iD*Quant*™ kit for pesticide analysis, containing 20 compounds, was spiked into food samples and used to verify method performance for identification and confirmation.

LC Separation

- Separation using a Phenomenex Kinetex Biphenyl (100 x 2.1 mm, 2.6u) column
- Gradient water/methanol with 5 mM ammonium formate with a total run time of 15 min (Table 1)
- Injection volume of 10 µL

Table 1. LC gradient conditions at a flow rate of 500 µL/min

Step	Time	A (%)	B (%)
0	0.0	90	10
1	10	10.0	90
2	13	10	90
3	13.1	90	10
5	15	90	10

MS/MS Detection

Samples were analyzed with two separate methods utilizing the SCIEX QTRAP® 6500 system with IonDrive™ Turbo V ion source using the electrospray ionization probe. The following gas settings were used: CUR 30 psi, Gas1 50 psi, Gas2 65 psi, CAD high.

The ion source temperature was set to 300°C to avoid degradation of thermally fragile pesticides such as Avermectin.

Method 1: *Scheduled* MRM™ Pro algorithm monitoring 2 transitions for each target pesticide (Figure 1)

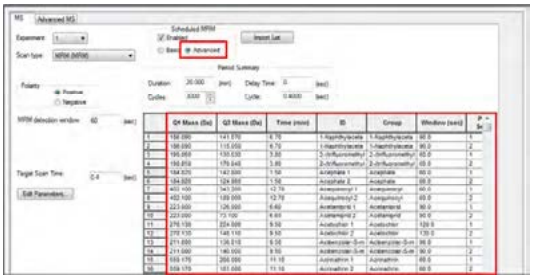

Figure 1. Acquisition method editor to build a method using the *Scheduled* MRM™ Pro algorithm

- Compound dependent detection window to match LC peak width and shape
- Compound dependent threshold for dynamic window extension and MRM-triggered MRM
- Target scan time of 0.4 sec to monitor ~800 transitions

Method 2: *Scheduled* MRM™-IDA-MS/MS to collect additional MS/MS information for identification (Figure 2)

- Information dependent acquisition of the most intense precursor ion detected in the MRM survey
- Dynamic background subtraction with a threshold of 1000 cps in methods without using an inclusion list (screening methods)
- Dynamic background subtraction with a threshold of >1000000 cps in methods when using an inclusion list, threshold of 100 cps for every compound in the inclusion list (confirmatory methods)

Figure 2. Acquisition method editor to build a method using (IDA)

MS/MS spectra were acquired in Enhanced Product Ion (EPI) scanning mode using a scan speed of 10000 Da/s. Dynamic fill time was used to achieve good quality spectra of compounds present at low and high concentrations. Highly characteristic MS/MS spectra were achieved using a collision energy (CE) of 35 V with collision energy spread (CES) of 15 V.

Data Processing

MultiQuant™ software version 3.0 was used for quantitative analysis and automatic MRM ratio calculation. MasterView™ software version 1.1 was used for MS/MS library searching.

MS/MS spectra were searched against the MS/MS spectra were search against the iMethod™ Pesticide Library version 2.1.

Results and Discussion

Compound Coverage

An example chromatogram of a solvent standard at 1 ng/mL is shown in Figure 3.

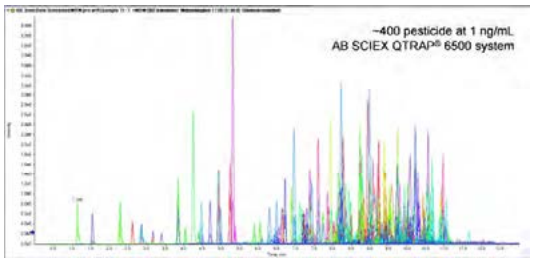

Figure 3. Approximately 400 pesticides detected using 800 MRM transition with the *Scheduled* MRM™ Pro algorithm

Approximately 800 MRM transitions were monitored using the *Scheduled* MRM™ Pro algorithm. This allows quantitation and identification of 400 pesticides in a single LC-MS/MS run while using the ratio of quantifier and qualifier transitions. Further optimization of the gradient profile is planned to spread late eluting compounds more evenly through the chromatogram to extend the method to a total of 500 compounds (1000 MRM transitions).

The example chromatograms shown in Figure 4 highlight the advantage of setting compound dependent detection windows to match LC peak width and shape. Pesticides with wider peaks or partly separated isomers were detected using a longer window, while narrow peaks were detected using a shorter window to enhance scheduling of transitions for best data quality.

Quantitative Results

Solvent standards were injected at a concentration ranging from 0.1 to 100 ng/mL. Example calibration lines are shown in Figure 5. Linear regression with 1/x weighting was used and points with accuracy values outside 80 to 120% were excluded. The coefficient of regression was typically higher than 0.99.

All target compounds had limits of quantitation (LOQ) of at least 1 ng/mL, for most compounds the estimated LOQ was much lower than 0.1 ng/mL (Signal-to-Noise, S/N >10). Example chromatograms and S/N at 1 ng/mL are shown in Figure 4 and Table 1.

Table 1. Signal-to-Noise (S/N) and Coefficient of Variation (%CV) for selected pesticides at a concentration of 1 ng/mL

Pesticide	S/N at 1 ng/mL	%CV at 1 ng/mL
Acephate	276	1.18
Avermectin	16.2	6.16
Bitertanol	44.9	6.12
Carbendazim	8090	1.70
Carbofuran	2670	1.52
Clethodim E	249	4.18
Clethodim Z	295	2.02
Difenoconazole	314	8.65
Dimethoate	19100	0.98
Dimethomorph	844	1.71
Imidacloprid	1430	0.49
Lufenuron	17.6	4.79
Omethoate	19800	1.22
Oxadixyl	1290	2.39
Permethrin	128	5.91
Propamocarb	1540	0.44
Propazine	2190	1.92
Pymetrozine	2600	1.66
Spinosyn A	661	3.10
Spinosyn D	253	4.47
Spiroxamine	2740	2.62
Thiabendazole	831	2.32

Contents

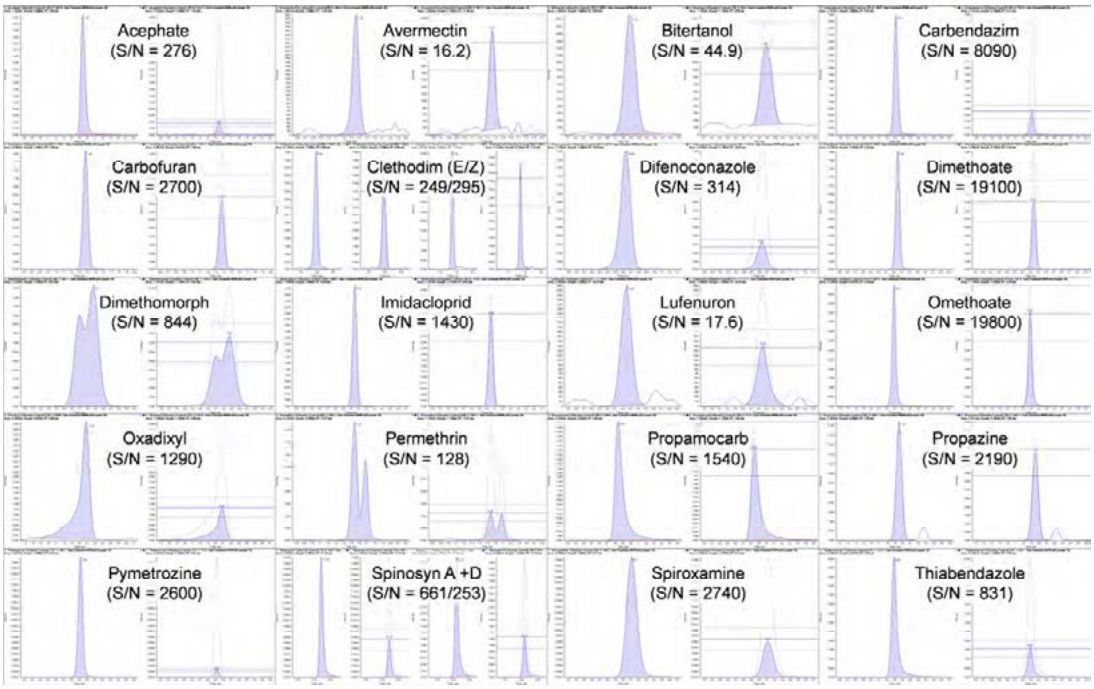

Figure 4. Quantifier and qualifier MRM transitions of selected pesticides with S/N at a concentration of 1 ng/mL, the MRM ratio tolerance of 30% is displayed in the MultiQuant™ software peak review (SANCO/12571/2013)

Replicate injections at 1 ng/mL (n=5) were used to evaluate repeatability. The results are summarized for selected compounds in Table 1.

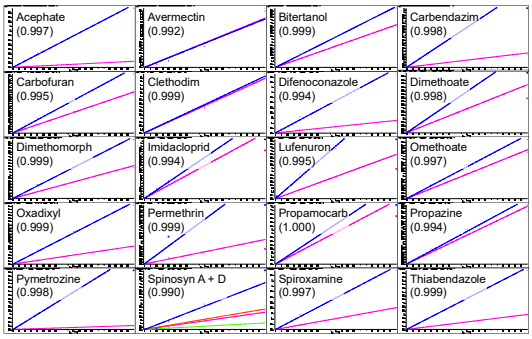

Figure 5. Calibration lines of selected pesticides from 0.1 to 100 ng/mL

As a result the developed method provides sufficient sensitivity to dilute matrix extracts by a factor of 10 or more while quantifying and identifying pesticides at 10 µg/kg.

Qualitative Results

Compound identification is typically performed by retention time matching and calculating the ratio of quantifier and qualifier MRM transition. The ion ratio of unknown samples is compared to standard samples and tolerance levels are applied to decide if a result is positive. These tolerance levels are defined by a number of guidelines.[4, 6]

MRM ratios were automatically calculated in MultiQuant™ software. The ratio of quantifier and qualifier transition in unknown samples is automatically compared to the average ratio of all included standard samples for compound identification. Tolerance levels are displayed in the peak review window (Figure 4). Here we used a generic tolerance of 30% following SANCO/12571/2013 guideline.

Despite the high selectivity of MRM detection, there is always a risk of false positive or negative findings due to interfering matrix signals. To increase confidence in identification or to confirm MRM ratio results, highly sensitive MS/MS spectra can be acquired on QTRAP® systems and searched against mass spectral libraries. Full scan MS/MS spectra contain more structural information of a detected compound resulting in a more confident identification.

Full scan spectra were acquired using and *Scheduled* MRM™-IDA-MS/MS method (Figure 6). This way quantitative (MRM peak area) and qualitative information (MRM ratio and MS/MS full scan spectrum) can be collected at the same time. Data processing was performed in MasterView™ software. A library PUR value of 70% or higher was used for positive identification.

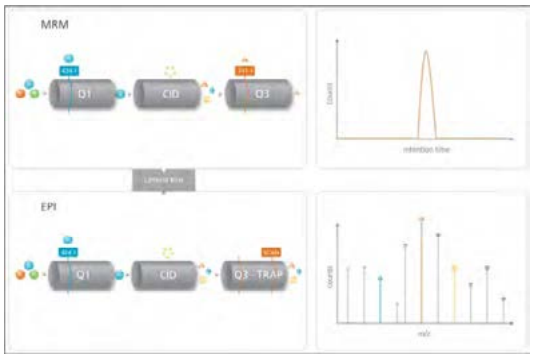

Figure 6. Information Dependent Acquisition (IDA) of MS/MS spectra using an MRM survey scan on a QTRAP® system

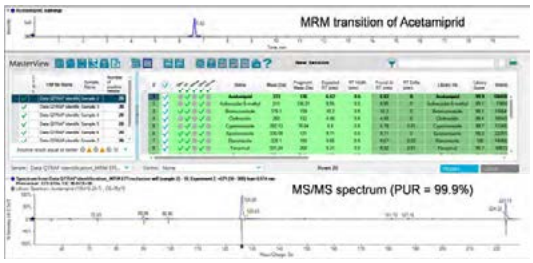

Figure 7. Processing of *Scheduled* MRM™ and MS/MS data in MasterView™ software, compound identification is achieved through automatic retention time matching and MS/MS library searching

Verification of Qualitative Method Performance

Mix D of the SCIEX iD*Quant*™ kit for pesticide analysis, containing 20 compounds, was spiked into carrot, grapes, grapefruit, red pepper, and spinach extract at 10 µg/kg.[7]

The results of identification based on retention time matching, MRM ratio comparison, and MS/MS library searching are summarized in Table 2. All 20 pesticides were confidentially identified in all 5 spiked samples. The average retention time error ranged from 0.008 to 0.024%, the average MRM ratio error from 5.09 to 6.30%, and the average MS/MS PUR from to 95.9 to 98.5%.

Table 2. Pesticides identified in different spiked food samples based on retention time (RT) matching with a tolerance of 0.2 min, MRM ratio comparison, and MS/MS library searching for qualitative method validation

Pesticides in Carrot	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.63	0.01	0.20	1.7	97.7
Acibenzolar-S-methyl	9.56	0.01	0.35	6.5	62.7
Bromuconazole	10.20	0.00	0.16	9.2	99.5
Clothianidin	4.48	0.00	0.35	5.6	98.1
Cyproconazole	8.84	0.04	0.58	8.4	100.0
Epoxiconazole	9.73	0.02	0.35	5.2	95.6
Etaconazole	9.68	0.03	0.17	3.2	99.6
Fenarimol	**9.30**	**0.01**	**0.26**	**36.7**	**99.7**
Flutriafol	8.04	0.01	0.59	6.0	99.8
Imazalil	9.98	0.01	0.57	1.8	97.9
Imidacloprid	6.04	0.00	0.81	0.9	98.7
Metribuzin	6.97	0.01	0.43	2.6	100.0
Myclobutanil	9.04	0.00	0.76	7.5	99.5
Nitenpyram	4.38	0.00	0.86	3.2	94.3
Paclobutrazol	8.41	0.01	0.19	6.5	100.0
Pyrimethanil	8.57	0.00	0.53	3.2	99.5
Thiacloprid	7.43	0.01	0.11	3.7	99.8
Thiamethoxam	4.97	0.00	0.35	1.1	98.8
Triadimenol	8.46	0.00	0.38	0.4	100.0
Triticonazole	9.14	0.02	0.07	3.3	98.6
Average		0.009		5.84	96.99

Contents

Pesticide in Grapes	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.64	0.02	0.20	1.3	98.0
Acibenzolar-S-methyl	9.59	0.04	0.39	4.9	96.1
Bromuconazole	10.23	0.03	0.13	7.5	98.6
Clothianidin	4.49	0.01	0.36	2.4	97.4
Cyproconazole	8.81	0.01	0.61	14.2	99.0
Epoxiconazole	9.75	0.04	0.33	0.2	74.6
Etaconazole	9.69	0.04	0.16	1.3	97.7
Fenarimol	**9.33**	**0.02**	**0.25**	**33.3**	**99.3**
Flutriafol	8.06	0.03	0.56	1.7	100.0
Imazalil	10.01	0.02	0.58	3.6	98.8
Imidacloprid	6.05	0.01	0.81	0.7	98.7
Metribuzin	6.98	0.02	0.43	3.2	100.0
Myclobutanil	9.05	0.01	0.78	11.0	100.0
Nitenpyram	4.39	0.01	0.85	1.3	95.2
Paclobutrazol	8.44	0.04	0.17	4.9	100.0
Pyrimethanil	8.60	0.03	0.51	7.8	99.5
Thiacloprid	7.44	0.02	0.12	10.7	99.8
Thiamethoxam	4.98	0.01	0.34	2.1	99.3
Triadimenol	8.50	0.04	0.39	2.3	99.2
Triticonazole	9.15	0.03	0.09	11.7	99.7
Average		0.024		6.30	97.55

Pesticide in Grapefruit	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.63	0.01	0.20	0.0	99.4
Acibenzolar-S-methyl	9.53	0.02	0.40	5.1	80.0
Bromuconazole	10.22	0.02	0.14	5.2	99.8
Clothianidin	4.48	0.00	0.36	2.2	98.1
Cyproconazole	**8.77**	**0.03**	**0.57**	**6.9**	**50.3**
Epoxiconazole	9.70	0.01	0.34	2.3	99.5
Etaconazole	9.66	0.01	0.17	1.8	99.3
Fenarimol	**9.30**	**0.01**	**0.24**	**27.8**	**99.7**
Flutriafol	8.04	0.01	0.62	11.3	100.0
Imazalil	9.99	0.00	0.60	7.5	98.8
Imidacloprid	6.04	0.00	0.79	1.5	99.5
Metribuzin	6.96	0.00	0.46	10.4	100.0
Myclobutanil	9.05	0.01	0.72	1.5	99.6
Nitenpyram	4.38	0.00	0.84	0.5	95.6
Paclobutrazol	8.40	0.00	0.16	8.3	100.0
Pyrimethanil	8.56	0.01	0.55	0.5	99.5
Thiacloprid	7.42	0.00	0.11	1.7	100.0
Thiamethoxam	4.97	0.00	0.34	3.4	98.5
Triadimenol	8.45	0.01	0.36	6.3	99.7
Triticonazole	9.12	0.00	0.08	4.2	100
Average		0.008		5.42	95.87

Pesticide in Red Pepper	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.63	0.01	0.20	0.2	99.5
Acibenzolar-S-methyl	9.55	0.00	0.41	8.3	71.4
Bromuconazole	10.20	0.00	0.14	5.0	99.1
Clothianidin	4.49	0.01	0.35	3.5	98.0
Cyproconazole	8.88	0.08	0.61	14.8	98.9
Epoxiconazole	9.72	0.01	0.35	6.7	96.5
Etaconazole	9.66	0.01	0.18	7.0	99.2
Fenarimol	**9.30**	**0.01**	**0.25**	**33.4**	**94.4**
Flutriafol	8.04	0.01	0.57	3.7	99.9
Imazalil	9.98	0.01	0.59	6.7	98.0
Imidacloprid	6.05	0.01	0.80	0.0	99.1
Metribuzin	6.97	0.01	0.42	1.7	100.0
Myclobutanil	9.04	0.00	0.70	1.6	99.8
Nitenpyram	4.39	0.01	0.84	1.0	95.9
Paclobutrazol	8.40	0.00	0.17	4.6	100.0
Pyrimethanil	8.57	0.00	0.54	1.9	99.5
Thiacloprid	7.43	0.01	0.12	4.3	100.0
Thiamethoxam	4.98	0.01	0.34	3.6	99.2
Triadimenol	8.45	0.01	0.36	6.8	100.0
Triticonazole	9.14	0.02	0.08	4.7	99.0
Average		0.012		5.98	97.37

Pesticide in Spinach	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.61	0.01	0.20	0.0	99.6
Acibenzolar-S-methyl	9.57	0.02	0.34	8.9	95.5

Bromuconazole	10.21	0.01	0.13	10.1	98.4
Clothianidin	4.47	0.01	0.36	1.3	98.7
Cyproconazole	8.75	0.05	0.54	1.3	99.7
Epoxiconazole	9.70	0.01	0.33	0.1	99.8
Etaconazole	9.67	0.02	0.17	0.7	89.6
Fenarimol	**9.31**	**0.00**	**0.25**	**32.0**	**96.9**
Flutriafol	8.03	0.00	0.56	1.4	99.4
Imazalil	9.99	0.00	0.63	13.1	98.8
Imidacloprid	6.03	0.01	0.82	2.0	97.9
Metribuzin	6.96	0.00	0.44	4.7	100.0
Myclobutanil	9.04	0.00	0.72	1.7	99.9
Nitenpyram	4.38	0.00	0.85	1.5	97.0
Paclobutrazol	8.42	0.02	0.18	1.4	100.0
Pyrimethanil	8.58	0.01	0.55	0.5	99.5
Thiacloprid	7.42	0.00	0.12	5.5	99.8
Thiamethoxam	4.96	0.01	0.34	2.6	99.3
Triadimenol	8.49	0.03	0.34	12.2	100.0
Triticonazole	9.12	0.00	0.08	1.0	100.0
Average		0.011		5.09	98.5

Bold and green = positive identification (RT error < 0.2 min, ratio error <30%, MS/MS PUR >70%

Bold and yellow = questionable identification (MS/MS PUR <70%),

Bold and red = no identification (ratio error >30%)

However, very few pesticides required confirmatory analysis since the identification criteria were slightly outside of tolerance levels.

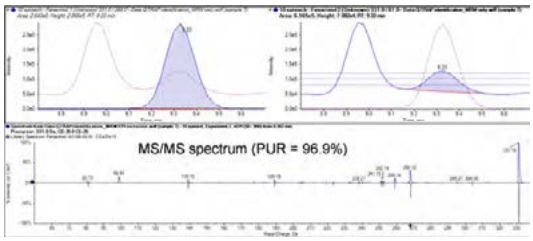

Figure 8. Detection of Fenarimol in spiked spinach: the MRM ratio was slightly out of the 30% tolerance due to high background and a closely eluting interfering matrix peak, but MS/MS library searching confirmed the presence of the detected pesticide.

For example Fenarimol was detected in all samples with matching retention time but the MRM ratio was outside or very close to the 30% tolerance due to high background and a closely eluting interfering matrix peak (Figure 8). But the analysis of a second sample extract to acquire MS/MS spectra confirmed the presence of Fenarimol with excellent library PUR well above 90% (94.4 to 99.7%).

Cyproconazole was identified in the grapefruit sample with matching retention time but the MS/MS PUR value was below the tolerance level (50.3%). Figure 9 shows the MS/MS review in MasterView™ software which helped to identify an isobaric matrix interference causing the low library search PUR. The analysis of a second sample extract confirmed the presence of Cyproconazole by MRM ratio matching (0.569 vs. theoretical 0.532).

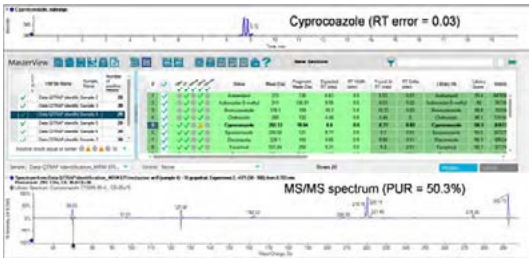

Figure 9. Detection of Cyproconazole in grapefruit: the MS/MS library search resulted in a PUR value of 50.3% only, however, review of spectra revealed in isobaric matrix interference, the MRM ratio error of 6.9% further confirmed the presence of the pesticide.

These two data examples highlight the complementary nature of identification using MRM ratios and MS/MS library searching. Both methods, utilizing the *Scheduled* MRM™ Pro algorithm and *Scheduled* MRM™-IDA-MS/MS, are suitable to quantify and identify pesticides in food samples. However, matrix interferences and high background can result in questionable identification. The analysis of a second sample extract using the alternative approach greatly enhances identification making it a viable tool for confirmation. Such a confirmation method is especially important if the target pesticide is not amenable to an orthogonal method, such as GC-MS.

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Application to Incurred Food Samples

Store-bought food samples were extracted using a QuEChERS procedure. Extracts were diluted 10x to minimize possible matrix effects and analyzed by LC-MS/MS using the two described methods utilizing the *Scheduled* MRM™ Pro algorithm and the *Scheduled* MRM™-IDA-MS/MS approach.

Results are summarized in Table 3.

Table 3. Pesticides identified in different incurred food samples based on retention time matching, MRM ratio comparison, and MS/MS library searching

Sample	Pesticide	Conc. (µg/kg)	RT Error	% Ratio Error	MS/MS PUR (%)
Avocado	Azoxystrobin	55.0	0.07	3.9	99.2
	Imidacloprid	6.2	0.01	0.6	95.2
Banana	Bifenthrin	26.8	0.12	9.4	73.0
	Fenpropimorph	12.2	0.08	4.6	99.7
	Imazalil	120	0.08	4.2	97.0
	Thiabendazole	37.3	0.00	0.7	100
Carrot	Linuron	14.3	0.07	1.9	95.1
Grapefruit	Fenbuconazole	5.1	0.05	9.8	75.4
	Imazalil	900	0.08	7.3	97.7
	Thiabendazole	269	0.01	2.3	100
Grapes 1	Fenhexamid	711	0.04	10.4	100
	Pyrimethanil	**226**	**0.06**	**32.8**	**99.4**
	Quinoxyfen	5.9	0.02	7.8	99.4
	Trifloxystrobin	16.2	0.03	4.0	99.2
Grapes 2	Boscalid	15.9	0.07	8.9	78.7
	Fenhexamid	363	0.05	11.4	100
	Myclobutanil	14.2	0.05	0.86	70.7
	Pyrimethanil	687	0.07	28.2	99.5
	Spirotetramat metabolite	6.0	0.04	7.1	not in library
	Tebuconazole	7.1	0.33	11.6	75.4
Lemon	Imazalil	981	1.00	0.8	98.8
	Thiabendazole	7.6	0.20	0.59	99.5
Onion	no pesticides detected				
Orange	Imazalil	1830		4.4	
	Thiabendazole	3110		13.2	
Pepper 1	Acetamiprid	8.9	0.04	3.4	98.6
	Boscalid	9.8	0.06	7.2	82.8
	Clothianidin	6.0	0.00	7.6	87.2
	Imidacloprid	9.1	0.05	0.7	80.8
	Myclobutanil	17.3	0.03	9.0	86.4
	Pyriproxyfen	11.7	0.00	2.4	87.6
	Thiamethoxam	10.6	0.02	0.9	83.5
Pepper 2	Boscalid	47.6	0.06	4.2	87.2
	Pyraclostrobin	21.5	0.03	0.6	80.2
Spinach	**Boscalid**	**14.9**	**0.07**	**21.3**	**14.9**
	Dimethomorph	53.7	0.17	6.2	79.0
	Fenamidone	755	0.02	5.9	99.2
	Imidacloprid	217	0.04	0.8	98.0
	Permethrin	**1060**	**0.10**	**1.4**	**17.0**
Tomato	no pesticides detected				

Four pesticides were identified in the avocado samples based on retention time matching and MS/MS library searching. Confirmatory analysis and quantitation was performed using the *Scheduled* MRM™ Pro method and MRM ratio calculation (Figure 9).

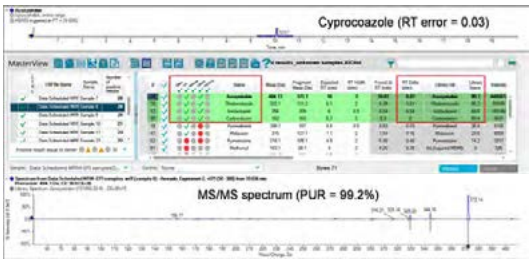

Figure 9. Identification of Azoxystrobin, Imidacloprid, Thiabendazole, and Carbendazim in an avocado sample based on retention time matching and MS/MS library searching, results were confirmed using MRM ratio calculation (note: Thiabendazole and Carbendazim were present below 5 µg/kg)

Four pesticides were identified and quantified in the grapes samples using the *Scheduled* MRM™ Pro method. The example presented in Figure 10 shows the results for Pyrimethanil. It can be seen in the Peak Review window that the MRM ratio is outside the 30% tolerance.

We performed confirmatory analysis of a second sample extract using the *Scheduled* MRM™-IDA-MS/MS approach. Figure 10 shows the excellent MS/MS library match with a PUR 99.4% confirming the presence of Pyrimethanil.

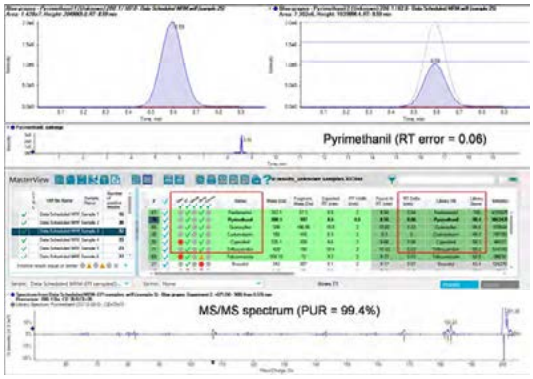

Figure 10. Fenhexamid, Pyrimethanil, Quinoxyfen, and Trifloxystrobin were identified based on MRM ratios and quantified in a grapes sample, the MRM ratio of Pyrimethanil were slightly outside the 30% tolerance (top), however, second analysis using MS/MS library searching confirmed the presence of Pyrimethanil (bottom)

Figure 11 and 12 highlight the complementary nature of MRM ratio and MS/MS library searching for identification.

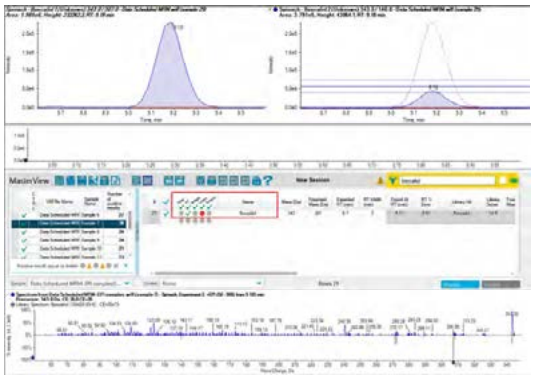

Figure 11. Boscalid was detected in a spinach samples with a concentration of 14.9 µg/kg, the ion ratio of 21.3 is inside the 30% tolerance (top), however, the MS/MS library searching with a PUR of 14.9% indicated strong matrix interference and suggest that Boscalid is not present in the sample (bottom)

Boscalid was detected in spinach. The ion ratio was inside the 30% tolerance, however, the MS/MS library searching with a PUR of 14.9% indicated strong matrix interference and suggested that Boscalid was not present in the sample.

Figure 12. Permethrin was detected in the spinach sample at a high concentration of 1060 µg/kg, the identification using MRM ratio was positive but the MS/MS library searching indicates strong matrix interferences, manual searching in LibraryView™ software confirms the presence the presence of both characteristic ions in the MS/MS spectrum, further confidence is gained through the presence of characteristic isomers in the LC profile

Permethrin was detected in the spinach sample at a high concentration of 1060 µg/kg (above the MRL of 50 µg/kg set by the EU[8]). MRM ratio and library searching are in disagreement for compound identification. Manual evaluation of the MS/MS spectrum in LibraryView™ software confirms the presence of both characteristic fragment ions in the MS/MS spectrum suggesting that Permethrin is present in the sample. The characteristic LC profile of Permethrin isomers further helps compound identification (Figure 12). Since the high level detected is a violation of the maximum residue level additional confirmation is recommend, which can be achieved by using an alternative LC separation setup and the acquisition of additional confirmatory MRM transitions using the *Scheduled* MRM™ Pro algorithm.

Summary

A QuEChERS and LC-MS/MS based method for the analysis of approximately 400 pesticides in food samples was developed.

The method used the SCIEX QTRAP® 6500 system utilizing the *Scheduled* MRM™ Pro algorithm and information dependent acquisition of full scan MS/MS spectra allowing quantitation and confident identification.

The method provide sufficient speed and sensitivity to quantify all ~400 pesticides at a concentration of 1 µg/kg in 10x diluted QuEChERS extract of food samples. Good linearity was observed for most compounds from 0.1 to 100 ng/mL with coefficient of variation typically well below 10%.

Qualitative method performance was verified by 20 compounds, into 5 different matrices at a concentration of 10 µg/kg. All compounds were confidentially identified in all samples using the dual method approach. Retention time errors observed were well below the 0.2 min tolerance. Very few pesticides required confirmatory analysis since the identification criteria were slightly outside of tolerance levels (MRM ratio tolerance of 30% or library PUR value of less than 70%). However, these results highlight the complementary nature of MRM ratios and MS/MS full scan offering a possibility for confirmatory analysis.

Last but not least store-bought food samples were analyzed. Automatic identification, quantitation, and confirmation were performed in MultiQuant™ and MasterView™ software.

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6 2002/657/EC: European Commission Decision 'concerning the performance of analytical methods and the interpretation of results'

7. A. Schreiber et al.: 'Using the iD*Quant*™ Standards Kit for Pesticide Analysis to Analyze Residues in Fruits and Vegetable Samples' Application Note AB SCIEX (2011) #3370211-01

8 http://ec.europa.eu/sanco_pesticides

Publication number: 10390414-01

Contents

LC-MS/MS Analysis of Emerging Food Contaminants

Detection of Pesticide 1080 (Sodium Fluoroacetate) in Milk and Infant Formula

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Introduction

Recently (November 2014), threats in the form of letters were sent to farming and dairy industry leaders in New Zealand. The letters were accompanied by small packages of milk powder that were shown to contain a concentrated form of the pesticide 1080 (sodium fluoroacetate). The sender demanded that the New Zealand government stop using 1080 for pest control. Sodium fluoroacetate is used to protect New Zealand's native flora and fauna against introduced pests like possums and ferrets. Opponents, however, argue that it also kills native animals and contaminates the environment.[1-2]

Such criminal threats are a potential danger and weaken consumers' trust in the food supply chain. Accurate and reliable analytical methods are needed to monitor food ingredients and final products to ensure food safety in light of this threat.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is an ideal analytical technique to detect polar analytes in complex food samples.

Here we present first results of method development to detect sodium fluoroacetate in milk and infant formula. The sample preparation protocol consists of a simple acetonitrile extraction and defatting using hexane. LC separation was achieved using a HILIC column in normal phase mode. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode. In MRM mode the transition of a molecular ion into a characteristic fragment ion is monitored. The monitoring of more than a single fragment ion allows not only quantitation but also highly confident identification based on the ratio between quantifier and qualifier transitions.

Initial studies show that sodium fluoroacetate can be detected at concentrations below 1 ng/mL (below 10 ng/mL in matrix) using the SCIEX QTRAP® 4500 system, with good accuracy and repeatability. Linearity for quantitation was achieved over 3 orders of magnitude (0.1 to 100 ng/mL). Future experiments are planned to further increase sensitivity, simplify sample preparation and to include an internal standard to correct low recoveries and matrix effects.

Experimental

Standards

Sodium fluoroacetate (Pestanal, analytical standard, Sigma-Aldrich #31220) was purchased from Sigma Aldrich.

Future studies will include the use of an internal standard which was not available at the time this study was conducted.

Sample preparation

10 g of infant formula was thoroughly mixed with 100 mL of water. Ready-to-feed samples were extracted directly.

Samples were extracted with acetonitrile and defatted using hexane. After pH adjustment the extract was phase-separated using QuEChERS salts, diluted and analyzed by LC-MS/MS.

LC Separation

LC separation was performed using a Shimadzu UFLC$_{XR}$ system with an Amide column (100 x 2.1 mm, 1.7 µm) and a normal phase gradient consisting of water with ammonium formate and acetonitrile. The injection volume was 50 µL.

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MS/MS Detection

The SCIEX QTRAP® 4500 system with Turbo V™ source was operated using an ESI probe in negative polarity. The MRM transitions monitored were 77/57 and 77/33. Ion source parameters were set to the following values: CUR = 30 psi; Gas1 = 40 psi; Gas2 = 60 psi; TEM = 600°C; and IS = -4500 V.

Results and Discussion

An example chromatogram is shown in Figure 1. The selected LC conditions guaranteed separation from matric components (retention time > 2 min) to minimize potential matrix effects (i.e. ion suppression).

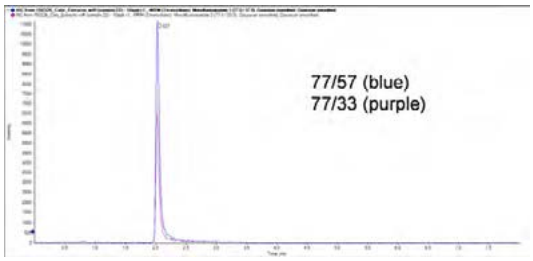

Figure 1. Example chromatogram of a 10 ng/mL standard of sodium fluoroacetate

Sodium fluoroacetate was accurately and reproducibly identified and quantified. The repeat analysis of a 1 ng/mL standard (n= 3) is shown in Figure 2.

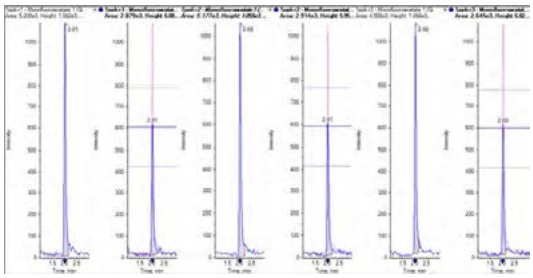

Figure 2. Repeat analysis at 1 ng/mL, 2 MRM transitions were monitored and the ratio of quantifier and qualifier transition (alternating from left to right, respectively) was used for compound identification (displayed MRM tolerances are 30%).

Identification was achieved using the ratio of quantifier and qualifier ion. The MRM ratio tolerances were well within the tolerance levels of 30% set by food testing guidelines (i.e. SANCO/12571/2013).

The MRM ratio is automatically calculated on MultiQuant™ software (version 3.0.2) and tolerance levels are displayed in the peak review window for easy data review (Figure 2).

Calibration lines for both MRM transitions are shown on Figure 3. The accuracy of all injections was between 92 and 109%.

Repeatability was excellent at all concentration levels and well below 10%, with the exception of 0.1 ng/mL for the quantifier MRM 77/33 (12.3%). Both coefficients of regression were larger than 0.999 using linear fit with 1/x weighting (Figure 3).

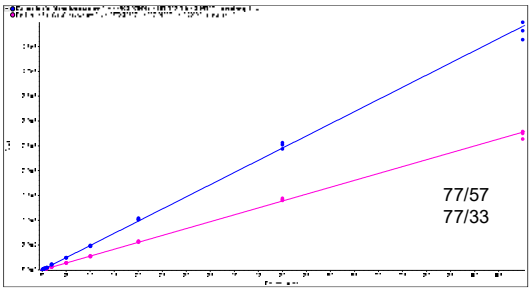

Figure 3. Calibration lines (0.1 to 100 ng/mL) for sodium fluoroacetate

Initial studies show that the developed method can detect sodium fluoroacetate in matrix samples at 10 ppb.

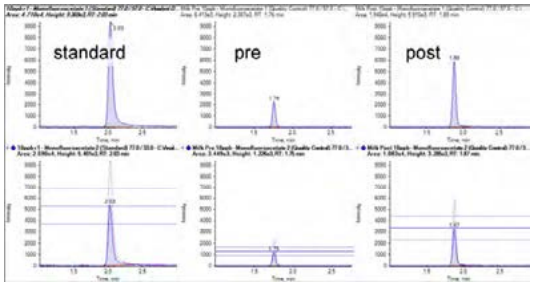

Figure 4. Chromatograms of standard at 10 ng/mL in comparison to the pre-extraction and post-extraction spike of milk at 10 ng/mL (the quantifier ion results are shown on the top row and the qualifier ion on the bottom row)

Figure 4 shows the pre-extraction and post-extraction spike of 1080 into milk at 10 ng/mL. The post-extraction spike indicates ion suppression of ~40% and the pre-extraction spike an additional recovery loss of 30%.

Summary

First results of method development were presented to detect sodium fluoroacetate by LC-MS/MS using the SCIEX QTRAP® 4500 system. Samples were prepared by simple acetonitrile extraction and defatting using hexane. LC separation was achieved using a HILIC column and normal phase chromatography. The MS/MS was operated in MRM mode, enabling detection limits below 1 ng/mL (below 10 ng/mL in matrix). Good accuracy, repeatability, and linearity for quantitation were achieved over 3 orders of magnitude.

Future experiments are planned to increase sensitivity, simplify sample preparation and to include an internal standard to correct low recoveries and correct for matrix effects.

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Publication number: 221015-01

The Detection of Allergens in Bread and Pasta by Liquid Chromatography Tandem Mass Spectrometry

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the simultaneous detection of four major food allergens peanut, milk, wheat and egg. Peptides of allergens were detected at low parts-per-million (ppm) levels after simple homogenization, digestion with trypsin and SPE cleanup.

Introduction

The prevalence of food allergies in the United States is estimated at around 6% for children and 3.7% for adults[1], and reports suggest that the number of food allergies is rising.[2] Allergens themselves come from a variety of sources and are a complex mix of different chemicals but include proteins from buckwheat, egg, peanut, cereals containing gluten, tree nuts, crustaceans, fish, soybean, sesame, mustard and celery but can also be chemicals such as sulphites.[3] Allergic reactions can range from mild to severe and during the period 1999-2006, 48 fatal allergic reactions were recorded in the United Kingdom.[4] Currently, the only therapy available for food allergy is avoidance, and self-treatment with epinephrine[5] so there is a need amongst food producers and regulators for specific and sensitive methods to detect allergens at trace levels.

The Codex Alimentarius, the food standards commission for the United Nations Food and Agriculture Organization and the World Health Organization, recommends that eight potential allergens should always be declared on pre-packaged foods: peanuts, tree nuts, eggs, milk, cereals containing gluten, shellfish, fish, and sulphites.

Screening for allergens in food is traditionally performed using enzyme-linked immunosorbent assay (ELISA), which employ antibodies raised against proteins specific for the allergenic food.[3, 6] Qualitative and quantitative analyses regularly generate variable results, together with false positives and false negatives, constituting a severe limitation of this technique; additionally, each target allergen requires a separate ELISA test kit. Another approach is the use of real-time polymerase chain reaction (PCR). This has the drawback of being an indirect method where the presence of the allergen is not monitored only the presence

of material from the organism, which can produce false negatives and positives. Therefore, a method that could unambiguously confirm the identification of multiple allergenic proteins simultaneously would be invaluable for allergen screening in food.[7-8]

Our original research into using liquid chromatography with tandem mass spectrometry (LC-MS/MS)[9] used an extraction method described by Careri et al.[10] This method was time consuming and when applied to the extraction of real samples lead to a coefficient of variation (CV) of >20% at low allergen levels. Here we present some new data using a modified and shorter sample preparation method incorporating solid phase extraction (SPE) to simplify the procedure which has been developed using information provided by a food testing laboratory.[11-12]

Contents

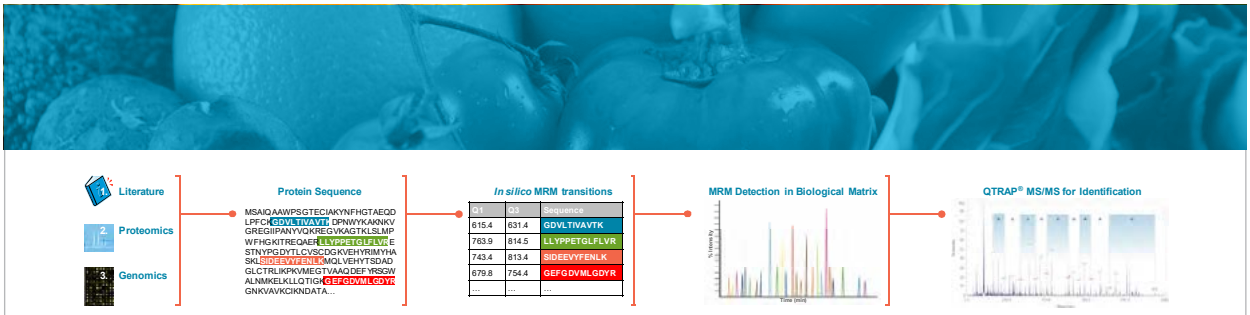

Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

Experimental

Standards

For the initial development work some of the target allergens were commercially available and therefore purchased. Where allergens were not available the unprocessed food, e.g. peanuts, were purchased and the allergens extracted[9], these extracts were then used for method development.

Sample Preparation

The test sample, bread or pasta, was homogenized using a food processor and then the required amount of allergen protein was added to the sample to produce a spiked sample. Powdered spiked sample (5g) was mixed with the extraction buffer containing ammonium bicarbonate, urea and dithiothreitol. The mixture was broken up by shaking and agitated further using a roller mixer.

This mixture was centrifuged and 1 mL of the top liquid layer was mixed with iodoacetamide, incubated in the dark for 20 min, and digested by addition of a digestion buffer containing ammonium bicarbonate, acetonitrile and trypsin. After overnight incubation at 37°C the sample was acidified and filtered.

The filtrate was purified using a conventional conditioned polymeric SPE cartridge from Phenomenex. The peptides were extracted from the cartridge using acetonitrile and the extract was evaporated to dryness and reconstituted in acidified aqueous acetonitrile.

LC

Initial method development was carried out using an Eksigent Technologies Tempo™ LC system with 75mm x 150 mm C18 reversed phase HPLC column (LC Packings) at 300 nL/min using a gradient of water and acetonitrile where both solvents contained formic acid. This HPLC system was used to determine what MRM transitions were suitable for allergen detection.

Final extracted samples were separated over a 12 minute gradient from water to acetonitrile, by reversed-phase HPLC on a polar end capped column running at a flow of 300 µL/min, using a Shimadzu UFLC System. Both the water and acetonitrile mobile phases contained formic acid and trifluoroacetic acid.

MS/MS

All analyses were performed on an SCIEX 4000 QTRAP® LC/MS/MS system using electrospray ionization (ESI).

Initial method development was carried out using a NanoSpray® source at a flow rate of 300 nL/min. MRM Pilot™ software was used with the MIDAS™ workflow (MRM-initiated detection and sequencing).

Using the MIDAS™ workflow, a set of MRM transitions were predicted from the known protein sequence and then used as a survey scan to trigger the acquisition of full scan hybrid triple quadrupole linear ion trap (QTRAP®) MS/MS spectra (Figure 1). This data was then submitted to a database search engine for confirmation of peptide identification and confirmation of the feasibility of the MRM transition for allergen detection. With this workflow MRM transitions were designed without the need for synthetic peptides which was essential where commercial available allergen proteins were not available.

The final LC-MS/MS method to detect allergens in food samples was performed on a SCIEX 4000 QTRAP® system equipped with Turbo V™ source and ESI probe at a flow rate of 300 µL/min.

Results and Discussion

In the method development care was taken to make sure that peptides chosen were unique to the allergen. The list was further consolidated by removing peptides that could be susceptible to modification during food processing, e.g. undergo post translational modification or the Maillard reaction. This reduced the number of peptides used as triggers for detection and generation of peptide finger prints. For each allergen multiple triggers were used.

Contents

Figure 2 shows the total ion chromatogram for the MRM transitions used for the detection of peanut, milk, egg and wheat proteins. Here a total of 55 MRM transitions corresponding to 19 unique peptides for the allergens are shown.

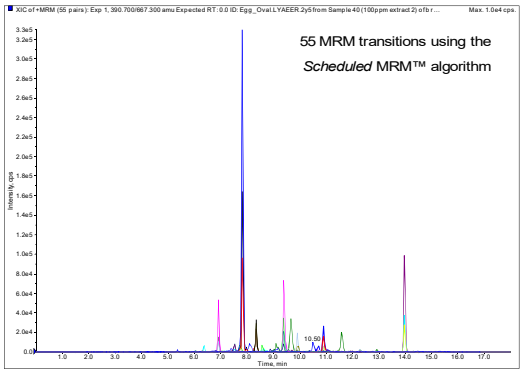

Figure 2. *Scheduled* MRM™ screen for peanut, milk, egg and wheat allergens in a bread sample spiked with 100 ppm milk and egg proteins

The *Scheduled* MRM™ algorithm was used in this method. Using this approach each MRM is monitored only across its expected retention time, decreasing the number of concurrent MRM transitions at any one time and maintaining both the cycle time and the dwell time.[6] This approach maximizes sensitivity but will also enable the easy addition of additional allergen markers as the method expands in the future.

This final list of MRM transitions was used as a survey scan to trigger the acquisition of QTRAP® MS/MS spectra. These spectra can be submitted to database search engines, providing confirmation of peptide identification.

Examples of this are shown in Figure 3a and 3b, here a pasta and a bread sample were spiked at 100 ppm with allergens of milk and egg, extracted and analyzed.

The extraction of both spiked pasta and bread yielded identical MS/MS spectra for the same peptides from egg and milk. This additional MS/MS information together with MRM ratio data gave multiple points of identification of allergen contamination in food and, as these peptides are unique, false positive allergen detection was dramatically reduced.

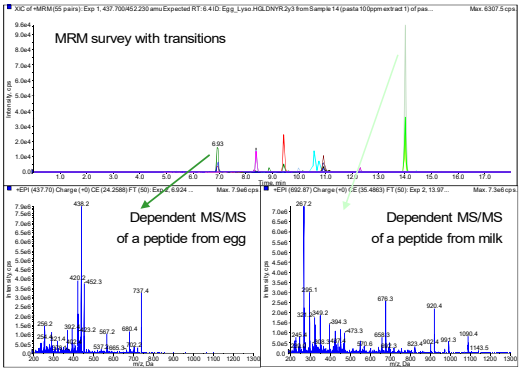

Figure 3a. MIDAS™ workflow for the detection of allergens in pasta. Analysis of an extract from pasta spiked at 100 ppm with egg and milk allergens. The top pane shows the total ion chromatogram for all MRM transitions; the bottom left pain shows the QTRAP® MS/MS spectrum which has been automatically generated by an egg peptide, and the bottom right pain is the spectrum generated by a milk peptide

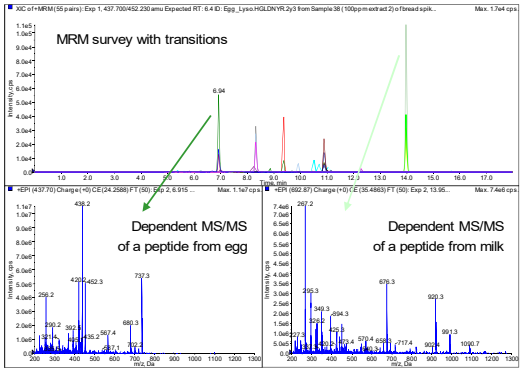

Figure 3b. MIDAS™ workflow for the detection of allergens in bread. Analysis of an extract from bread spiked at 100 ppm with egg and milk allergens. The top pane shows the total ion chromatogram for all MRM transitions; the bottom left pain shows the QTRAP® MS/MS spectrum which has been automatically generated by an egg peptide, and the bottom right pain is the spectrum generated by a milk peptide

Figure 4 shows a comparison of the tryptic peptide maps of 3 of the 4 investigated allergens.

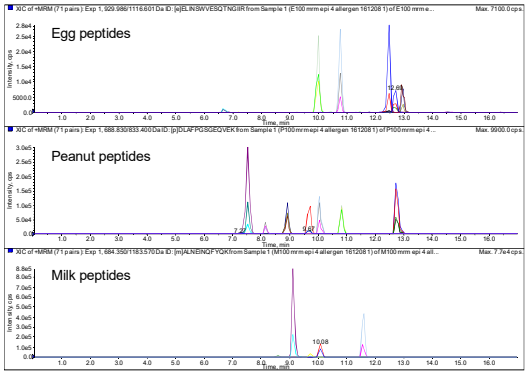

Figure 4. MRM transitions of a 100 ppm standard for egg (top), peanut (middle) and milk (bottom) allergens

This shows that each allergen protein produces a different peptide map with different intensities. The fact that some allergen peptides are of lower intensity will mean that detection limits will vary between different allergens. In Figure 4 egg peptides produce lower intensity signals compared to peanut and milk will therefore have a higher limit of detection.

To fully evaluate this approach bread samples were spiked at different concentrations with milk and egg proteins (highest and lowest sensitivity of the 4 allergens). Samples were spiked in duplicate and analyzed in triplicate to assess both linearity and robustness of the method. In this instance internal standards were not available so all results are without the positive effect of internal standardization. Results therefore show the reproducibility of the LC-MS/MS method as well as the extraction protocol.

Figures 5a and 5b show both egg and milk peptides give a linear response. In these tests milk peptides were detected at less than 2 ppm whereas egg peptides had a limit of detection between 5 and 10 ppm.

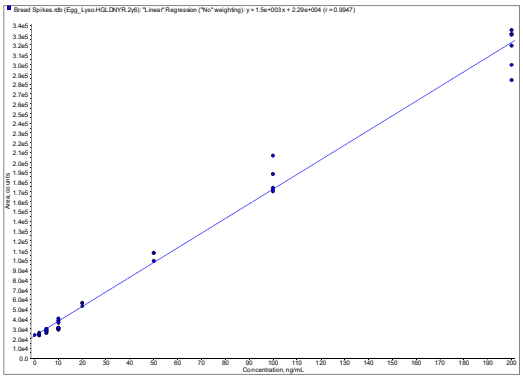

Figure 5a. Example of a calibration line obtained for an egg peptide

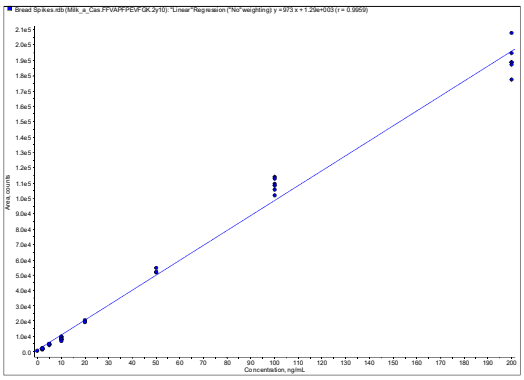

Figure 5b. Example of a calibration line obtained for a milk peptide

Milk peptide CVs were less than 5% at 100 ppm and less than 10% at 10ppm showing that the full procedure was reproducible (Table 1).

Table 1. Examples of reproducibility from the duplicate extraction and triplicate injection of a 10 and 100 ppm spike of milk proteins into bread

Extract	Injection	Calculated concentration (ppm)	
		Milk spiked at 10 ppm	Milk Spiked at 100 ppm
1	*1*	7.76	102.7
1	*2*	9.67	114.9
1	*3*	8.89	113.7
2	*1*	7.42	106.5
2	*2*	7.71	110.3
2	*3*	6.64	109.2
	Mean	8.02	109.5
	Std Deviation	1.09	4.58
	CV	9.3%	3.9%

Summary

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the simultaneous detection of four major food allergens peanut, milk, wheat and egg. The initial sample preparation has been significantly simplified. The detection of allergens in processed foods was possible at low part per million levels.

Sensitivities achieved were equivalent to sensitivities of some currently available methods based on ELISA and real-time PCR, but the CV without any internal standards were better than have been previously reported by users[9] and were significantly better than those that can be obtained at low levels by ELISA. The LC-MS/MS approach has the additional advantage of being a multi allergen screen unlike ELISA where individual allergens are detected by separate kits. By using the MIDAS™ workflow full scan QTRAP® MS/MS spectra were obtained at the same time as quantitative information, confirming peptide identification and reducing the occurrence of false positives associated with other techniques.

ACKNOWLEDGEMENTS

We would like to acknowledge Dr Bert Pöpping and Julia Heick (Eurofins) for their continued contribution to this research area.

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Publication number: 1830610-01

Allergen Detection in Wine by Micro Flow Liquid Chromatography Tandem Mass Spectrometry microLC-MS/MS

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the simultaneous detection of milk and egg proteins in white wine. The method utilizes a simplified sample preparation protocol, the Eksigent ekspert™ microLC 200, and the SCIEX QTRAP® 5500 system with the *Scheduled* MRM™ algorithm to detect below 0.1 ppm casein in wine.

Introduction

In wine production fining a wine eliminates any appearance of cloudiness by removing sediment. In this process fining agents, such as casein, are stirred into barrels of wine where they act as magnets by picking up the sediment in the wine and depositing it at the bottom of the barrel. Once the wine has been clarified, racking of the wine is done to separate the wine from the sediment.

In 2011 EFSA concluded that wines fined with casein, caseinate and milk products may trigger adverse reactions in susceptible individuals following a survey of wine where the detection of casein was reported in trace amounts (<2 mg/L [2 parts-per-million]) in two (out of 32) experimental wines without bentonite treatment and in three (out of 61) commercial wines with unknown treatment.[1, 2] This fact together with a new European Union legislation (that states that wine after 30 June 2012 must disclose on the label if fining reagents such as casein and egg ovalbumin have been used in processing)[3] has driven the need for methods which are capable of detecting casein products in wine at low levels.

Here we present new data using micro flow LC in combination with an LC-MS/MS method developed on an Eksigent ekspert™ microLC 200 and SCIEX QTRAP® 5500 system utilizing the *Scheduled* MRM™ algorithm which detects casein in wine at sub ppm levels. The method utilizes a simple protein digestion of the wine followed by dilution and injection and has been designed to limit extensive sample preparation and perform all protein modification in the same Eppendorf tube. In this paper we will discuss the benefits of micro flow LC over higher flow rate separations.

Experimental

Standards

For this work the target proteins were commercially available as well as reagents used for alkylating, reducing and digesting the samples and all were purchased from Sigma Aldrich. Wine for spiking experiments was obtained from a local supermarket.

Sample Preparation

The wine samples (0.5 mL) were reduced by adding TCEP (tris(2-carboxyethyl)phosphine, 0.2 M, 50 µL) and agitating using a thermal mixer for 60 minutes at 60°C. The samples were cooled to room temperature and alkylated by adding a solution of MMTS (S-methyl methanethiosulfonate, 0.2 M, 100 µL in iso-propanol) and storing protected from light for 30 minutes at ambient temperature. This process cleaves the disulfide bridges of the allergenic proteins and then alkylates the free cysteine residues preventing reformation of the bridges and aids trypsin digestion. The extracts containing the modified proteins were diluted 1 in 4 with a ammonium bicarbonate buffer and rapidly digested over a one hour period using trypsin and thermal mixing (60 minutes at 40°C). After 1 hour digestion the samples were further diluted 1 in 2 with 0.1% formic acid to deactivate the trypsin and stop the digestion and prepare the sample for LC-MS/MS analysis.

Contents

Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

LC

The initial high flow LC analysis used a Shimadzu UFLC$_{XR}$ system and the conditions shown in Table 1 where A = water and B = acetonitrile both containing 0.1 % formic acid. A volume of 10 µL of sample was injected onto a Phenomenex Kinetex 2.6 um XB-C18 100A (2.1 x 50 mm) column held at 40°C.

Table 1. Gradient conditions used for 'high flow' LC separation at a flow rate of 300 µL/min

Step	Time	A (%)	B (%)
0	0	98	2
1	2	98	2
2	8	60	40
3	8.2	2	98
4	9.0	2	98
5	9.1	98	2
6	10	98	2

Table 2. Gradient conditions used for micro flow LC separation at a flow rate of 25 µL/min

Step	Time	A (%)	B (%)
0	0	98	2
1	0.3	98	2
2	4	60	40
3	4.1	5	95
4	4.3	5	95
5	4.4	98	2
6	5.5	98	2

All micro flow LC method development and analysis was done using an Eksigent ekspert™ microLC 200 system. Final extracted samples (10 µL) were separated over a 5.5 minute gradient (Table 2) of A = water and B = acetonitrile both containing 0.1 % formic acid. Peptides were separated on a reversed-phase YMC Triart C18 2.7 µm (50 x 0.5 mm) column held at 40ºC.

MS/MS

All analyses were performed on SCIEX QTRAP® 5500 LC/MS/MS system using a TurboV™ source, with a standard electrospray ionization (ESI) probe used with the high flow LC system and for micro flow LC analysis the ESI electrode was changed to a micro LC hybrid electrode (50 µm ID).[4]

The initial method development was carried out using the MIDAS™ workflow (MRM-initiated detection and sequencing). MIDAS uses a set of predicted MRM transitions from the known protein sequence as a survey scan to trigger the acquisition of QTRAP® full scan MS/MS spectra (Figure 1). This data was then submitted to a database search engine for confirmation of peptide identification and of the feasibility of the MRM transition for casein, milk, and egg product detection in wine. With this workflow MRM transitions were designed without the need for synthetic peptides.

In the final micro flow LC method the following Turbo V™ source conditions were used: Gas 1, Gas 2, and the CUR set at 30 psi, the ion source temperature (TEM) at 350°C and IS voltage of 5500 V. The peptides were detected in Multiple Reaction Monitoring (MRM) mode for best selectivity and sensitivity using the *Scheduled* MRM™ algorithm with an MRM detection window of 40 sec and a target scan time of 0.30 sec. Q1 resolution was set to low and Q3 resolution was set to unit. A total of 44 MRM transitions (Tables 3 and 4) were evaluated for over 16 target peptides from milk and egg. This meant that there is plenty of scope to add further markers in the future.

Source conditions of the high flow method were optimized for 300 µL/min, but all other setting were identical.

Contents

Table 3. MRM transitions and retention times (RT) of peptides for the detection of egg and milk protein in wine

Peptides for the detection of egg protein					
Identity	**RT (min)**	**Q1 (amu)**	**Q3 (amu)**	**DP (V)**	**CE (V)**
egg protein 1 1_1	3.2	563.3	631.3	100	29
egg protein 1 1_2	3.2	563.3	732.4	100	29
egg protein 1 2_1	2.9	791.4	951.4	76	39
egg protein 1 2_2	2.9	791.4	1052.5	96	43
egg protein 1 3_1	3.2	845.0	860.4	161	47
egg protein 1 3_2	3.2	845.0	1007.5	136	47
egg protein 1 4_1	3.6	930.0	1116.6	186	49
egg protein 1 4_2	3.6	930.0	888.5	166	49
egg protein 1 4_3	3.6	930.0	1017.3	216	49
egg protein 1 5_1	1.9	390.7	667.3	90	20.9
egg protein 1 5_2	1.9	390.7	504.2	90	20.9
egg protein 1 5_3	1.9	390.7	433.2	90	20.9
egg protein 2 1_1	1.9	437.7	452.2	90	31
egg protein 2 1_2	1.9	437.7	680.3	90	27
egg protein 2 1_3	1.9	437.7	737.4	90	27
egg protein 2 2_1	2.4	714.8	1152.5	139	37
egg protein 2 2_2	2.4	714.8	951.5	139	38
egg protein 2 2_3	2.4	714.8	804.4	139	39
Peptides for the detection of milk protein					
milk protein 1 1_1	3.2	587.3	758.4	91	27
milk protein 1 1_2	3.2	587.3	871.5	76	27
milk protein 1 1_3	3.2	587.3	790.4	81	29
milk protein 1 2_1	3.9	634.4	771.5	80	37
milk protein 1 2_2	3.9	634.4	934.5	80	37
milk protein 1 2_3	3.9	634.4	991.6	80	37
milk protein 1 3_1	2.8	598.3	911.5	81	25
milk protein 1 3_2	2.8	598.3	456.3	71	27
milk protein 1 3_3	2.8	598.3	266.2	76	49
milk protein 1 4_1	4.0	692.8	920.5	91	29
milk protein 1 4_2	4.0	692.8	991.5	106	31
milk protein 1 4_3	4.0	692.8	1090.6	106	29
milk protein 1 5_1	3.2	880.5	436.2	211	49
milk protein 1 5_2	3.2	880.5	663.0	206	51
milk protein 1 5_3	3.2	880.5	408.2	236	55

Table 3. continued

Identity	RT (min)	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)
milk protein 2 2_1	2.6	467.3	707.4	101	21
milk protein 2 2_2	2.6	467.3	608.3	101	25
milk protein 2 2_3	2.6	467.3	379.2	101	33
milk protein 3 1_1	2.7	348.7	421.2	80	22
milk protein 3 1_2	2.7	348.7	550.2	80	22
milk protein 4 1_1	2.2	415.7	563.3	80	26
milk protein 4 1_2	2.2	415.7	660.4	80	26
milk protein 4 1_3	2.2	415.7	759.4	80	26
milk protein 4 2_1	2.4	390.8	471.3	80	25
milk protein 4 2_2	2.4	390.8	568.4	80	25
milk protein 4 2_3	2.4	390.8	681.4	80	25

Results and Discussion

Before analyzing a batch of wine samples the micro flow LC method was first compared to a high flow method that had previously been developed for allergen detection in baked goods.[5]

A spiked sample at a concentration of 1 ppm in white wine was analyzed using a Phenomenex Kinetex 2.6 µm column at a flow rate of 300 µL/min and then compared to the result obtained using a YMC Triart C18 2.7 µm column with micro flow LC at 25 µL/min. The gradient conditions were kept the same as was the injection volume and column temperature for both separations, and the results are shown in Figure 2.

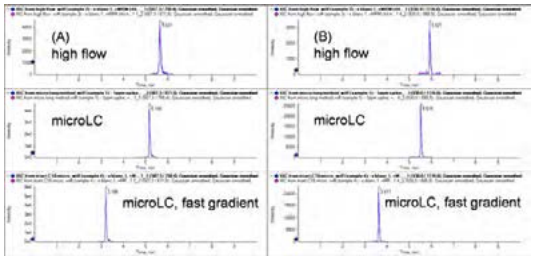

Figure 2. Comparison of high flow vs. microLC using a 1 ppm protein spike in white wine. A milk peptide is shown on the left (A) and an egg peptide is shown on the right (B).

Figure 2 and Table 4 show that moving to micro flow LC increases sensitivity by typically a factor of 4 to 13 fold in signal-to-noise (S/N), compared to the high flow LC method. Further to this the runtime could be halved without any detrimental effect on S/N.

Table 4. Signal-to-noise (S/N) improvements when using microLC and microLC with a faster gradient over the traditional high flow LC method

	Milk peptide	Egg peptide
S/N high flow LC	41.5	65.0
S/N microLC	539.5	260.6
S/N gain	**13x**	**4.2x**
S/N microLC with fast gradient	381.5	354.4
S/N gain	**9.2x**	**5.7x**

These results demonstrated the low gradient delay volume of the microLC system which enables rapid gradients even at flow rates ranging from 10 to 40 µL/min. The sensitivity increase was not only due to improved peak shape (peak width of 6 sec using micro flow LC and 8 sec for high flow LC) but was mainly down to the improved ionization efficiency which is possible at these lower flow rates, a fact that nanoLC has taken advantage of historically in proteomics applications.

The ionization efficiency gains of microLC are not as great as those seen in nanoLC, which runs at sub µL/min, but microLC has the advantage over nanoLC that runtimes can be a lot

shorter (< 6 minutes, Table 2) compared to a traditional nanoLC run which can take from 40 minutes to over 1 hour.[6] Also as microLC uses the TurboV™ source this technique has been shown to be very robust.[7]

To assess the sensitivity of this approach egg and milk proteins were spiked into white wine from 0.05 to 2 ppm concentrations. Figures 3 and 4 demonstrate that both egg and milk could be detected in wine at 50 ppb or below and that the response was linear over the 2 orders tested. This linearity of response is typical for LC-MS/MS which can easily exceed 3 orders of linearity which is far greater than commercial ELISA techniques.

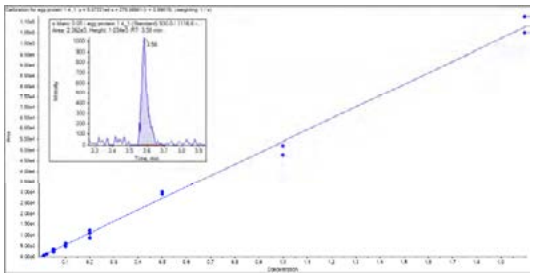

Figure 3. Calibration line from a peptide from egg which had been spiked into a sauvignon blanc wine (0.01 to 2 ppm) and chromatogram of the 50 ppb spike sample. The linearity is provided without the use of any internal standards.

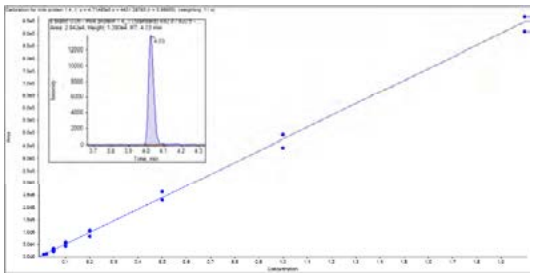

Figure 4. Calibration line from a peptide from milk which had been spiked into a sauvignon blanc wine (0.01 to 2 ppm) and chromatogram of the 50 ppb spike sample. The linearity is provided without the use of any internal standards.

One of the big advantages that LC-MS/MS has over other techniques used for allergen detection, such as ELISA and PCR, is its ability to acquire multiple points of identification. This is clearly shown in Figure 5 where MRM transitions are used to trigger the acquisition of full scan data. In this figure the SCIEX QTRAP® 5500 system was used to analyze a wine sample which had been spiked at 0.5 ppm. At this level multiple peptides for egg and milk were detected which were used to trigger full scan MS/MS spectra given unambiguous identification of these proteins in samples.

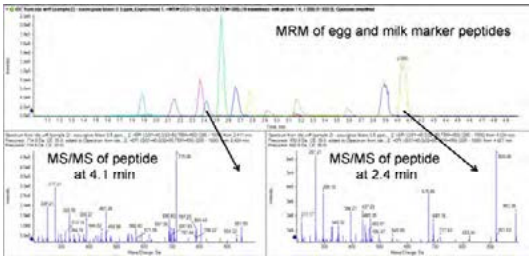

Figure 5. Micro flow LC-MS/MS analysis of 0.5 ppm spike of egg and milk proteins into a sauvignon blanc sample analyzed using the MIDAS™ workflow. The top pane shows the extracted ion chromatogram for the peptides of milk and egg and the bottom two panes show examples of MS/MS spectra for target peptides

Finally the effect of the white wine variety was tested by spiking 0.5 ppm of the proteins into different white wine samples. Figure 6 shows that the white wine variety did not have a major effect on response of the peptides or the peptide profile. However, for accurate quantitation the addition of internal standard of the proteins into wine would be recommended or the use of standard addition (as done previously in baked goods[4]).

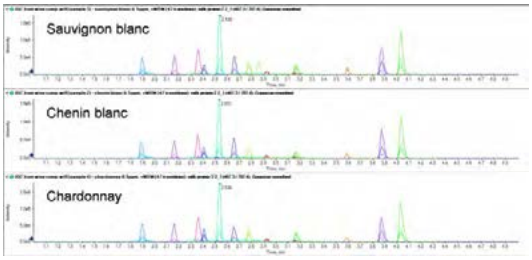

Figure 6. microLC-MS/MS analysis of 0.5 ppm spiked samples of egg and milk proteins into 3 different white wines

Summary

MicroLC-MS/MS using the Eksigent ekspert™ microLC 200 system coupled to SCIEX QTRAP® 5500 system has been shown to offer a rapid, robust, sensitive and specific assay for the simultaneous detection of a series of milk and egg markers in white wine. A simple sample preparation was used with the complete extraction procedure in the same Eppendorf tube. The method is capable of providing detection levels below 100 ppb.

Sensitivities achieved were equivalent to sensitivities of some currently available methods based on ELISA and real-time PCR methods. The micro LC-MS/MS approach has the additional advantage of being a potential multi-allergen screen unlike ELISA where different allergens, like egg and milk, are detected by separate kits. Using the MIDAS™ workflow full scan QTRAP® MS/MS spectra were obtained at the same time as quantitative information, confirming multiple peptide target identification and reducing the occurrence of false positives associated with other techniques.

Micro flow LC has been able to show that analysis times can be halved and sensitivities increased by upwards of a factor of 10 with also the additional reduction in solvent consumption which leads to the added benefit of a cost saving for the allergen analysis.

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Publication number: 8880213-01

Contents

LC-MS/MS Analysis of Emerging Food Contaminants

Detection of Peanut and Almond Allergens in Spices

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[1]SCIEX Singapore (Singapore), [2]SCIEX Redwood City, California (USA), [3]SCIEX Concord, Ontario (Canada)

Introduction

Recent findings (in February 2015) of allergens in spices caused the recall of many food products in North America and Europe. The US Food and Drug Administration (FDA) advised people who are highly allergic to peanuts to consider avoiding products that contain ground cumin or cumin powder, because some shipments of these products have tested positive for undeclared peanut protein. The Food Standards Agency (FSA) has issued a further allergy alert following confirmation that a batch of paprika was the most likely source of undeclared almond protein in three food products which had been recalled. According to the European Rapid Alert System for Food and Feed (RASFF) portal, additional food products containing Cayenne pepper and Pilli-Pilli powder were found to contain undeclared traces of peanuts. Another recall of cumin containing product was triggered by the Canadian Food Inspection Agency (CFIA).[1-4]

This was the latest in a string of spices being recalled for possible nut protein findings. It remains unclear whether the contamination is accidental or deliberate.

It is important that consumers know food is safe and authentic. Potential weaknesses in the food supply chain need to be identified and counter measures need to be taken to strengthen consumer protection. Accurate and reliable analytical methods are needed to monitor the food supply chain and to allow correct labeling of food products.

Traditionally enzyme-linked immunosorbent assay (ELISA) based methods are used for food allergen testing. However, it is well known that ELISA can generate variable results, including false negative and false positive results that can occur due to the technique's limited sensitivity and selectivity. In addition, each allergen requires a separate test kit for the identification of an individual allergen. A multi-allergen screening method would be invaluable to increase the throughput and efficiency in allergen testing.

Here we present a method to detect the presence of peanut and almond in spices. Samples were extracted and then the allergenic proteins were reduced, alkylated and digested using trypsin. The extract containing peptides from the digested

proteins were filtered and analyzed by LC-MS/MS using a reverse phase chromatography and positive polarity electrospray ionization (ESI). The SCIEX QTRAP® 4500 system used for this study was operated in Multiple Reaction Monitoring (MRM) mode to achieve high selectivity of detection. In MRM mode characteristic transitions of peptides breaking into compound-specific fragment ions are monitored. At least 12 transitions (3 transitions for 4 peptides) were monitored per allergen to minimize potential false positive results caused by matrix interferences. The QTRAP® 4500 system also allows the acquisition of full scan MS/MS spectra which can be searched against mass spectral libraries to further increase the confidence in identification.

Experimental

Samples

Samples of cumin and paprika were obtained from local supermarkets. Store-bought roasted and raw peanuts and almonds were used for spiked experiments.

Sample preparation

The sample preparation method was based on previous work of Lock et al. The complete protocol is available in the iMethod™ Application for Allergens in Baked Goods (version 1.0).[5-6]

Contents

The analytical workflow is shown in Figure 1.

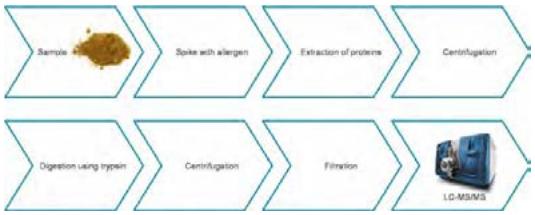

Figure 1. Sample preparation workflow

LC Separation

A Shimadzu UFLC$_{XR}$ system was used for analysis. Separation was achieved using a Phenomenex Kinetex 2.6u XB-C18 100A (30 x 1.0 mm) column with a mobile phase consisting of water and acetonitrile containing 0.1% formic acid and a 15 min gradient from 98/2 to 2/98 (A/B%). The LC column was held at 30°C. The flow rate was set to 300 µL/min and the injection volume to 30 µL.

MS/MS Detection

A SCIEX QTRAP® 4500 system with Turbo V™ source with ESI probe was used in positive polarity. The ion source temperature was set to 500°C.

MRM transitions were obtained from *in-silico* and protein ID experiments. Specificity and cross reactivity was evaluated by injecting extracts of roasted and raw almonds and peanuts as well as spiked extracts of spices. The final list of MRM transitions used in this study is shown in Table 1.

Table 1. Retention times (RT) and MRM transitions used for the detection of almond and peanut

Allergen (Peptide)	RT (min)	Q1	Q3
Almond (Peptide 1)	9.7	830.4	738.4
Almond (Peptide 1)	9.7	830.4	1035.5
Almond (Peptide 1)	9.7	830.4	922.5
Almond (Peptide 2)	8.3	571.8	369.2
Almond (Peptide 2)	8.3	571.8	858.5
Almond (Peptide 2)	8.3	571.8	743.4
Almond (Peptide 3)	7.7	698.3	732.4
Almond (Peptide 3)	7.7	698.3	879.5
Almond (Peptide 3)	7.7	698.3	936.5
Almond (Peptide 4)	10.1	780.8	1154.7
Almond (Peptide 4)	10.1	780.8	848.5
Almond (Peptide 4)	10.1	780.8	1186.7
Peanut (Peptide 1)	8.2	688.8	300.2
Peanut (Peptide 1)	8.2	688.8	930.6
Peanut (Peptide 1)	8.2	688.8	1077.5
Peanut (Peptide 1)	8.2	688.8	833.4
Peanut (Peptide 2)	8.4	564.4	686.6
Peanut (Peptide 2)	8.4	564.4	557.5
Peanut (Peptide 3)	8.5	793.9	827.5
Peanut (Peptide 3)	8.5	793.9	612.4
Peanut (Peptide 3)	8.5	793.9	726.4
Peanut (Peptide 4)	8.9	571.3	913.5
Peanut (Peptide 4)	8.9	571.3	669.3
Peanut (Peptide 4)	8.9	571.3	506.3

Results and Discussion

Qualitative Allergen Screening using MRM

Example chromatograms of spiked extracts are presented in Figure 2.

Figure 2a shows the results for 10 mg of roasted and raw almond spiked into 1 g of paprika, and Figure 2b shows the results for 10 mg of roasted and raw peanut spiked into 1 g of cumin.

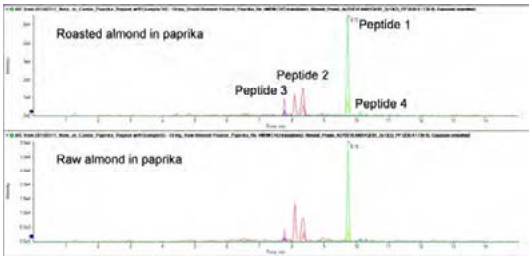

Figure 2a. Detection of almond in extracts of paprika (spiked at 10 mg/g)

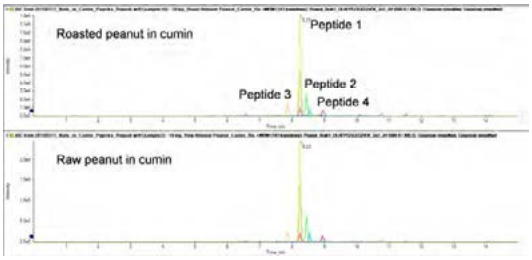

Figure 2b. Detection of peanut in extracts of cumin (spiked at 10 mg/g)

Identification of target compounds or peptides is typically based on MRM ratio calculation when utilizing LC-MS/MS.

There is the possibility of protein modification during food product, transportation, storage, and processing. The monitoring of 12 MRM transitions corresponding to 4 different peptide fragments per allergen provides high confidence in identification since different characteristic peptides of the allergen are monitored simultaneously. This procedure greatly reduces the possibility of false negative results.

MultiQuant™ software automatically calculates MRM ratios and MRM ratio tolerances. MRM transitions outside the tolerance will be flagged to identify outliers quickly. The MRM tolerances are also displayed in the Peak Review (see Figures 3a and 3b).

The MRM ratio measured from raw and roasted almonds and peanuts spiked into spices was typically well below 30%.

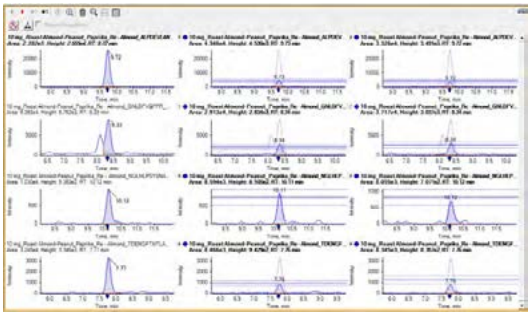

Figure 3a. Identification of almond in a paprika extract based on multiple MRM ratios

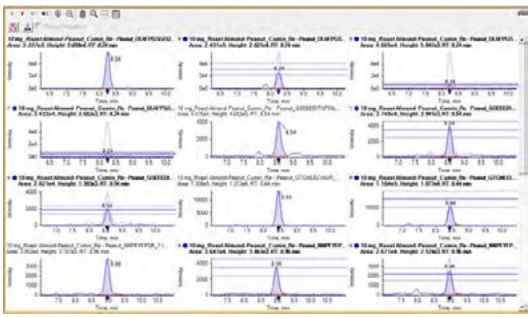

Figure 3b. Identification of peanut in a cumin extract based on multiple MRM ratios

Quantitation of Allergens in Spices

LC-MS/MS is a well know technique for the accurate and reproducible quantitation.

In this study initial quantitative results were obtained by spiking almond and peanut in spices (1, 10, and 100 mg/g) and analyzing samples following the complete sample preparation and LC-MS/MS workflow.

Example calibration lines are presented in Figure 4.

Figure 4a shows the results for roasted almond spiked into paprika and Figure 2b shows the results for roasted peanut spiked into cumin. Good accuracy and coefficients of correlation >0.999 were achieved for all transitions.

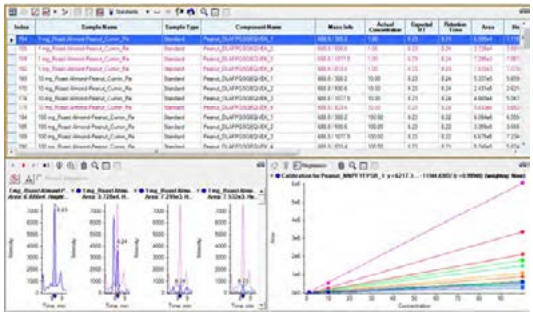

Figure 4a. Quantitative results of analyzing almond spiked into paprika powder

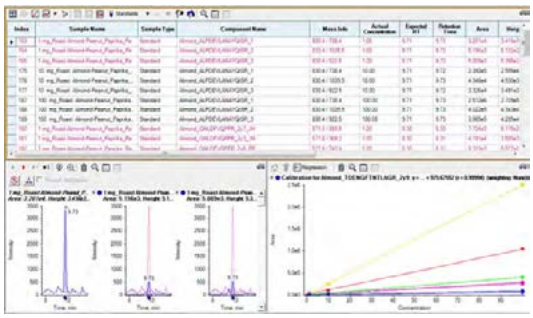

Figure 4b. Quantitative results of analyzing peanut spiked into cumin powder

Identification using MS/MS Scanning

The SCIEX QTRAP® 4500 system allows collecting MRM and MS/MS full scan data simultaneously using information dependent acquisition (IDA).

An example chromatogram with acquired MS/MS spectra for two peptides of peanut is presented in Figure 5. The spectra can be searched against mass spectral libraries which increases the confidence in identification when analyzing complex food samples.

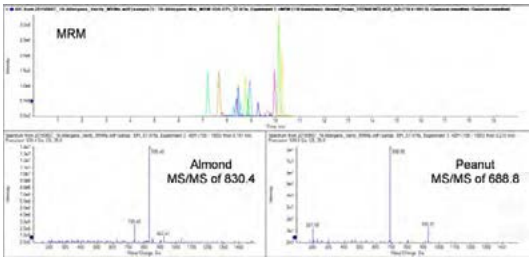

Figure 5. High confidence in identification using MS/MS full scan

Multiplexing of Allergens by LC-MS/MS

LC-MS/MS has the additional advantage of performing multi-allergen screening, unlike ELISA where different allergens are detected by separate kits.

In our laboratory LC-MS/MS was successfully applied to simultaneously screen for multiple food allergens, including egg, milk, gluten, peanut, tree nuts, soy, sesame, and mustard. An example of detecting a total of 18 allergens with a single analysis is presented in Figure 6.

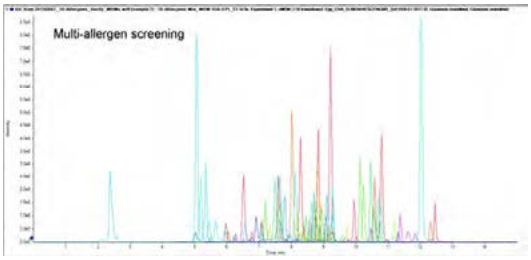

Figure 6. Multi-allergen screening by LC-MS/MS, detection of a total of 18 allergens in a single analysis

Summary

An LC-MS/MS method for the detection of almond and peanut in spices was presented.

Samples were extracted and then the allergenic proteins were reduced, alkylated and digested using trypsin. The digested extract was filtered and analyzed by LC-MS/MS using a SCIEX QTRAP® 4500 system operated in MRM mode.

Good linearity for quantitation was achieved when analyzing almond and peanut spiked into paprika and cumin at different concentrations.

Allergen identification was achieved through the monitoring of 12 characteristic MRM transitions per allergen. MRM ratios were calculated automatically using MultiQuant™ software and MRM ratios were typically well below 30%. The QTRAP® 4500 system also allows the acquisition of full scan MS/MS spectra which further increase the confidence in identification.

LC-MS/MS has the additional advantage of performing multi-allergen screening, unlike ELISA where different allergens are detected by separate kits.

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Publication number: 221115-01

Multiplexing Two Different Food Residue Methods using HILIC and Reversed Phase Chromatography in the Same LC-MS/MS Run

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Overview

Multiplexing liquid chromatography (LC) systems, and synchronizing to a single tandem mass spectrometer (MS/MS), can generate the high throughput needed by maximizing the efficiency of the MS/MS detector. An integrated multiplexing system has been specifically designed to synchronize two LC systems and an SCIEX mass spectrometer, allowing injection of samples into two LC streams in parallel. The overlapping LC runs and efficient use of MS detection, achievable with the SCIEX MPX™-2 High Throughput system, is shown to result in an overall higher throughput for common routine analyses.

Here two applications are presented to analyze milk extracts for chemical residues in the same LC-MS/MS run. The applications are examples of two opposite extremes usage in mobile phase. The first stream was used to implement an antibiotic screening with reversed phase (RP) setup while the second stream was used to analyze melamine with a hydrophilic interaction chromatography (HILIC) setup. The MPX™-2 High Throughput System can be easily configured and controlled to multiplex the two different analyses in the same run and achieve an overall higher throughput for common routine analyses.

Introduction

The incidences regarding the determination of melamine and cyanuric acid in wheat gluten as the cause of animal deaths and more recently hospitalization of thousands of children as a result of consumption of melamine contaminated milk products have highlighted the need for accurate analytical techniques to quantify and identify melamine in food. This is to allow manufacturers and regulatory agencies to pro-actively ensure consumer product safety. With the fast growing numbers of food items to analyze and having to maintain the fast turnaround times, so as to minimize the cost impact to manufacturers, a system is required to increase throughput of the analysis.

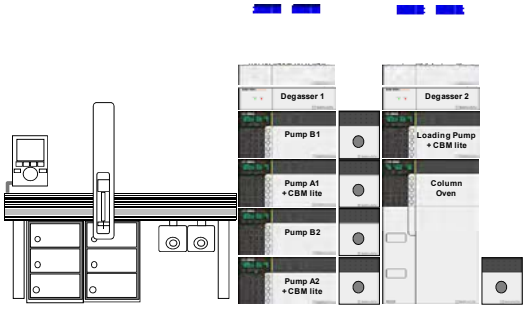

Figure 1. SCIEX MPX™-2 SP High Throughput system: configuration and module arrangement

HILIC separation with MS/MS detection is a fast, selective, and sensitive method for the analysis of Melamine in food samples after simple extraction.[1-3]

Antibiotics are used against infectious diseases with great success and are part of modern agriculture for many years. The beta-lactam, macrolide, sulfonamide, tetracycline, and other antibiotics help to maintain the health of the animal. However, the residues of antibiotics remain in animal-derived human foods may pose potential human health hazards. In addition, the widespread use of antibiotics has resulted in the emergence of drug-resistant bacteria.

Many countries have built a series of regulations to the use, dosage, and withdrawal times for many of these antibiotics in animal production. While there are several methods to determine antibiotic residues, LC-MS/MS using RP conditions is used more widely because of its higher specificity and sensitivity, which lead to better detection and identification. [4-6]

Contents

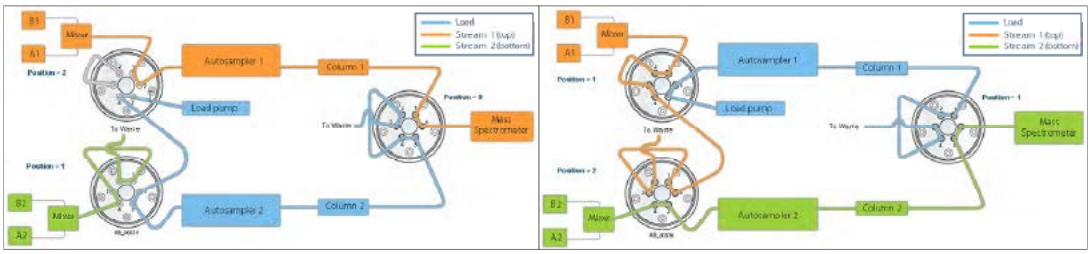

Figure 2. Solvent flow paths at typical states of a multiplex run (left: Stream 1 into MS/MS, right: Stream 2 into MS/MS)

HILIC is a chromatographic technique used for the separation of polar and hydrophilic compounds. As opposed to RP chromatography in which the stationary phase is non-polar in nature; the column packing that is used for HILIC separations is very polar. This requires the use of high organic mobile phase composition in HILIC separations, the opposite of the required starting conditions of an RP setup.

Here we multiplex a screening method for seven classes of antibiotics, using RP chromatography and a HILIC method to test for melamine using the SCIEX MPX™-2 High Throughput system to demonstrate the ability of the system to not only maximize throughput of two different analyses that differ in both run time and mobile phase conditions while maintaining data integrity for both analyses.

Experimental

Multiplex Hardware Setup

An integrated multiplex LC-MS/MS system was used consisting of an SCIEX 4000 QTRAP® system, two Shimadzu UFLC$_{XR}$ LC systems, a CTC PAL autosampler with DLW (dynamic load and wash) option, a pump containing a four solvent selection valve for sample loading and 5 switching valves for flow path control (Figure 1). The two chromatographic channels were not independent as they share a single high pressure loading pump which provides additional flexibility for injection and loading solvent composition.

All hardware modules were controlled by Analyst® software 1.5.1 with MPX® driver 1.1 add-on. The MPX™ driver was designed to control a two-stream LC system in various configurations in combination with any SCIEX mass spectrometer. It synchronizes sample injections in staggered LC runs, allows the user to create multiplex LC method, and enables targeted MS/MS data acquisition in a pre-determined retention time window using the parallel LC streams. Precise timing for the switching valves allows each LC stream to perform interleaved injection and LC gradient elution. Figure 2 shows common flow paths for a typical LC-MS/MS analysis.

Multiplex Software Configuration and Operation

After running the multiplex software installer, the user may easily configure and activate the multiplex option from the 'Hardware Configuration' dialog within Analyst® software. The MPX® driver provides an easy-to-use interface to modify system configuration in the 'Settings Pane' (Figure 3 A), to create or update multiplex LC methods in the 'Method Pane' (Figure 3 B), or to monitor the real-time acquisition and system status (including pressure, flow rate, temperature, flow path, and system state for both LC streams) in the 'Status Pane' (Figure 3 C).

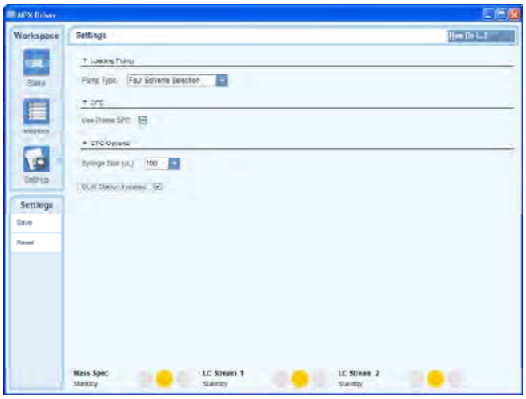

Figure 3 A. Settings Pane to modify system configuration

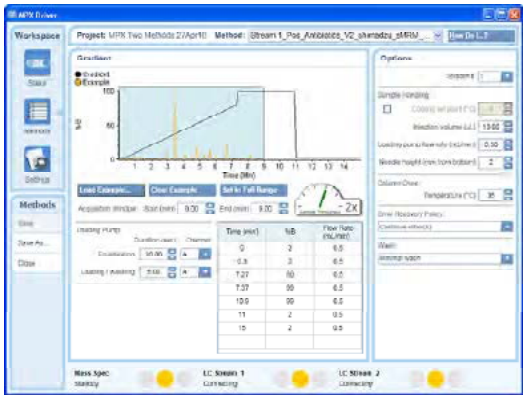

Figure 3 B. Method Pane to create or update multiplex LC methods, the blue shaded area in the chromatogram defines the MS/MS acquisition window

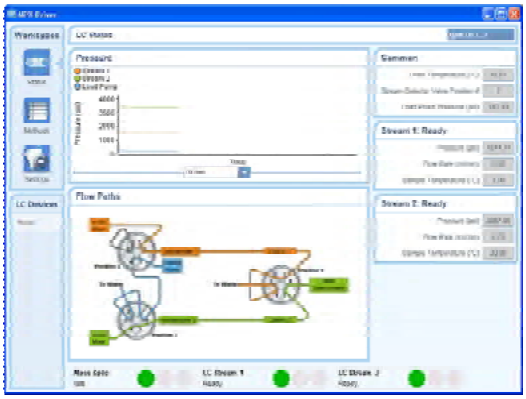

Figure 3 C. Status Pane to monitor the real-time acquisition and system status

Automated multiplex data acquisition for a batch of samples is very similar to performing a regular LC-MS/MS acquisition by creating and submitting a batch in either Analyst® or Cliquid® software. During a multiplex run, as illustrated in Figure 4, a staggered injection timing schedule is calculated in real-time by the MPX® driver. All switching valves and synchronization between LC components and the mass spectrometer are completely controlled by the MPX® driver.

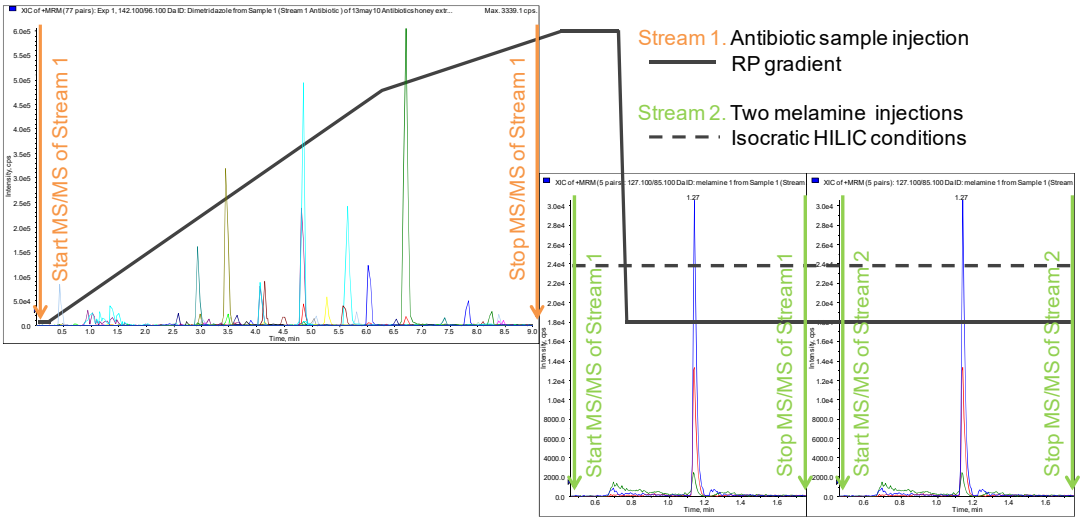

Figure 4. Multiplexing two methods that differ in their LC run times and chromatographic conditions

Results and Discussion

The SCIEX MPX™-2 High Throughput system has been designed to support two parallel LC streams into a single MS/MS. This arrangement allows minimizing MS/MS redundancy time during LC column equilibration and dead volume and improve throughput by as much as 2 times.

The MPX™-2 High Throughput system uses a loading pump consisting of a four solvent selection valve. The inclusion of the valve allows the flexibility of choosing between four different solvents in which to load the sample onto the column. As this loading valve is shared between the two streams, different analyses can be run on opposite streams. The system is therefore capable of multiplexing two analyses that differ in their LC run times and mobile phase conditions in the same run. An example in which such a workflow is effective is when a method with a long equilibration time, in which no analytes are eluting, is multiplexed with a method that has a short LC run time. To demonstrate this, an RP method (antibiotic screening) and a HILIC method (melamine quantitation) were multiplexed.

The antibiotic method used a non-polar C18 stationary phase (Phenomenex Gemini 3u C18 110 Á, 100x2 mm) with a polar mobile phase (water/methanol + 0.1% formic acid) as opposed to the HILIC method in which the stationary phase is very polar (Phenomenex Luna 3u HILIC 200 Á, 100x2 mm) with a less polar mobile phase (acetonitrile/water + 50 mM ammonium formate (80/20) acidified with HCl to pH 3.2). Multiplexing these two methods therefore resulted in switching quickly between solvents of wide differences in organic content. This had to be done while still maintaining chromatographic performance and data integrity for both analyses.

Antibiotic Screening

The antibiotic screening method was used to screen for a total of 77 compounds of seven classes of antibiotics, including beta-lactam, tetracycline, sulfonamide, macrolide, amphenicol, fluoroquinolone, and flunixin, in milk extracts.

The method consisted of a Multiple Reaction Monitoring (MRM) survey to automatically trigger Enhanced Product Ion (EPI) scans to identify each analyte based on their molecular fingerprint with high confidence. An example of the workflow is shown in Figure 5 which shows an MRM chromatogram and an example EPI spectrum generated from a spiked milk sample during the multiplexing of the antibiotics screening method with the melamine quantitation method.

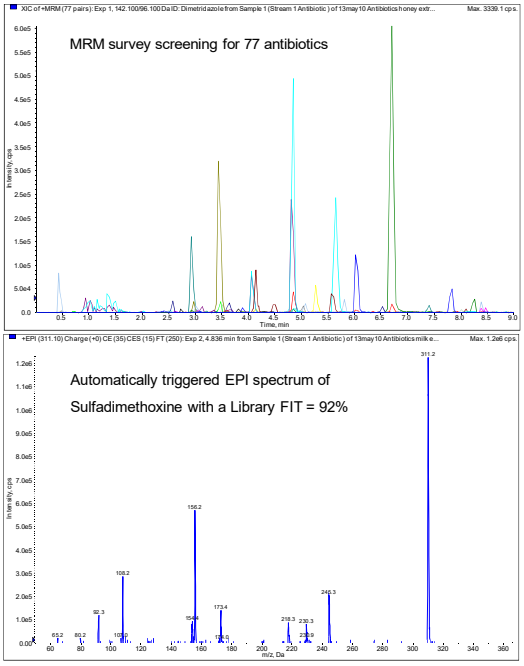

Figure 5. MRM survey and example EPI spectrum of the antibiotic screening which was multiplexed with the melamine quantitation method

Melamine Quantitation and Identification

During the same LC-MS/MS run samples were also tested for melamine. A number of three MRM transitions were monitored to quantify melamine and to perform compound identification based on MRM ratio calculation (Figure 6). The generated calibration curves highlight that the analytical performance was not compromised by multiplexing this methods with the antibiotic screening using the MPX™-2 High Throughput system (Figure 7 and Table 1).

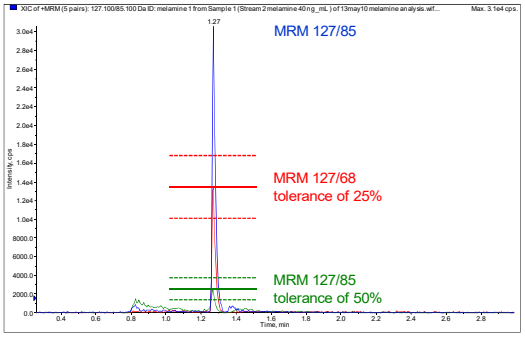

Figure 6. Chromatogram of melamine spiked into milk with MRM ratio tolerances for compound identification as defined by the European guideline 2002/657/EC[7]

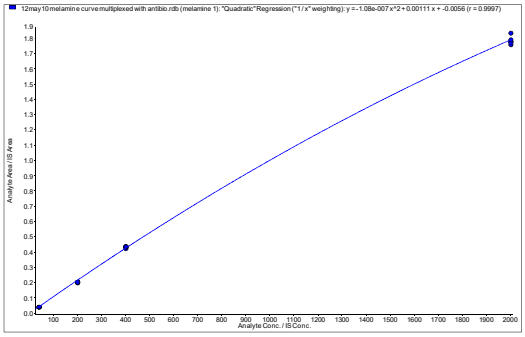

Figure 7. Calibration curve of melamine spiked into a blank milk with an r^2 of 0.9997 after quadratic regression with 1/x weighting

Table 1. Accuracy and reproducibility of quantifying melamine in milk

Concentration (ng/mL)	% CV	Accuracy in %
40	1.96	102.4
200	0.97	95.2
400	1.12	102.5
2000	1.94	99.9

Summary

Multiplexing LC systems, and synchronizing to a single MS/MS, can generate the high throughput needed by modern day laboratories to analyze increasing numbers of samples.

The SCIEX MPX™-2 High Throughput system has the capability of multiplexing two analyses that differ in their LC run times and mobile phase conditions in the same run and achieve an overall higher throughput system for common routine analyses. This allows for considerable time savings for analytical runs.

References

1 Sherri Turnipseed et al.: FDA Laboratory Information Bulletin #4421 (2008)

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3 Takeo Sakuma et al.: Application note AB SCIEX (2008) #1283110-01

4 A.A.M. Stolker and U.A.Th. Brinkman: J. Chromatogr. A 1067 (2005) 15-53 – A Review

5 E. Daeseleire et al.: Rapid Commun. Mass Spectrom. 14 (2000) 1404-1409

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7 Commission decision 2002/657/EC

Publication number: 1550210-01

The Quantitation and Identification of Coccidiostats in Food by LC-MS/MS using the SCIEX 4000 QTRAP® System

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Introduction

Coccidiostats are antiprotozoal agents that act upon parasites. In animal production, particularly in intensive animal rearing coccidiostats are used to treat infections and as such meat, chicken, egg and milk are regularly tested for these compounds. Recently maximum levels for these compounds (due to unavoidable carry-over of authorized coccidiostats to non-target feed) were set by the EU in Commission Regulations [(EC) No 124/2009][1] so methods for their detection were required. This work compares the traditional approach to sample preparation of solid phase extraction (SPE) followed by separation on a conventional 5µm particle column with that of the quicker and simpler QuEChERS[2-3] technique followed by separation with a newer 2.6 µm particle column and shows how liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) can be used to detect coccidiostats including Narasin, Diclazuril and Monensin in milk.

Experimental

1) Conventional Approach

Sample Preparation

Milk (2.0 g) in a Polypropylene Tube was mixed with acetonitrile (2 mL) and vortexed for 40 seconds. Another 2 mL of acetonitrile was added and the the tube was sealed, shaken by hand and then continually mixed using a head over head mixer for 15 minutes. The sample was then centrifuged for 15 minutes (3600 g at 4°C). The supernatant was removed and water (16 mL) and ammonia solution (1 mL, 25%) were added and this mixture was shaken. The whole extract was loaded onto an OASIS HLB SPE cartridge (3 cm^3, 60 mg) which previously had been conditioned with methanol (3 mL) and water (3 mL). The cartridge was washed with ammonia (5 mL, 1.25%) dried for 2 minutes under vacuum and eluted with methanol (5 mL). The eluent was evaporated to dryness, the sample was reconstituted in methanol/water (1 mL, 50/50), vortexed, and sonicated for 5 minutes before injection.

LC

Column: Agilent Zorbax Eclipse XDB-C8, 5 µm, 150 x 4.6 mm
Flow rate: 400 µL/min
Oven temperature: 40 ºC
Injection Volume: 40 µL
Mobile Phase A: water + 0.2% acetic acid
Mobile Phase B: methanol + 0.2% acetic acid

Table 1. LC gradient profile of conventional approach

Step	Time (min)	A (%)	B (%)
1	0.5	100	0
2	1.5	20	80
3	10	10	90
4	13	0	100
5	18	0	100
6	18.5	100	0
7	23	100	0

Contents

2) New Approach

Sample Preparation

The sample extraction was based on a QuEChERS method by Anastassiades et al. and Lehotay et al.[2-3] Milk in a polypropylene tube (50 mL) was roller mixed with acetonitrile. To this mixture anhydrous magnesium sulfate and sodium acetate were added and samples were shaken vigorously and centrifuged. Anhydrous magnesium sulfate, PSA and C18 were added to an aliquot (2 mL) of the upper layer and these samples were shaken by hand. This mixture was centrifuged and the supernatant transferred into an autosampler vial for analysis.

LC

Column: Phenomenex Kinetex C8, 2.6 µm, 100 x 4.6 mm
Flow: 600 µL/min
Oven temperature: 40 ºC
Injection Volume: 40 µL
Mobile Phase A: water + 0.2% acetic acid
Mobile Phase B: methanol + 0.2% acetic acid

Table 2. LC gradient profile of new approach with a Phenomenex Kinetex column using 2.6 µm core-shell particles for increased efficiency and improved performance

Step	Time (min)	A (%)	B (%)
1	1.0	100	0
2	2.5	20	80
3	5.0	10	90
4	7.5	0	100
5	9.2	0	100
6	9.5	100	0
7	11.5	100	0

MS/MS

The SCIEX 4000 QTRAP® system was used with Turbo V™ source and Electrospray Ionization (ESI probe. The source was heated to 600ºC with 45 psi nebulizer and heater gas.

Negative and positive polarities were used with polarity switching during, the chromatographic run, to cover all target analytes.

For best selectivity and sensitivity Multiple Reaction Monitoring (MRM mode was used for detection. Two MRM transitions were detected per compound to allow quantitation and identification by MRM ratios (Table 4. However, since detection in MRM mode only can lead to false positive results full scan MS/MS spectra were additionally acquired to increase confidence in compound identification using mass spectral library searching. In this mode an information dependent acquisition (IDA) experiment was used to automatically trigger the MS/MS spectra acquisition when a chromatographic MRM signal exceeded a threshold of 1000 cps.

Results and Discussion

The maximum residue limits for the coccidiostats vary with analyte (Table 3). The analysis is further complicated by the fact that Diclazuril ionizes in negative polarity so to maximize sensitivity the method contains periods, so it switches from positive to negative and back to positive as shown in Figure 1.

Table 3. Maximum Residue limits (MRL) for some coccidiostats[1]

Coccidiostats	MRL in milk (µg/kg)
Diclazuril	5
Lasalocid	1
Maduramycin	2
Monensin	2
Narasin	1
Robenidine	5
Salinomycin	2

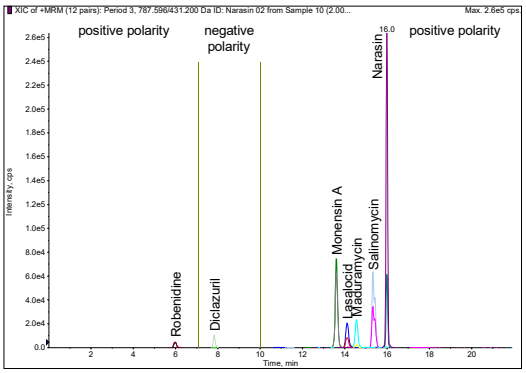
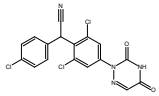
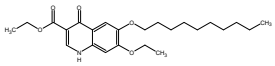
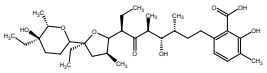
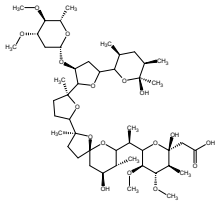
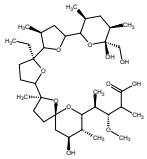
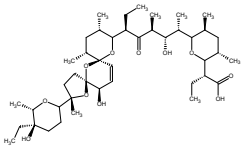
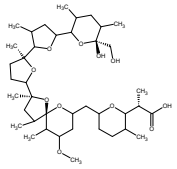
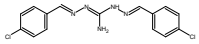
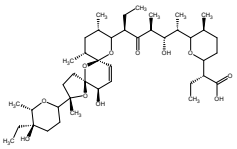


Figure 1. Example of an LC-MS/MS chromatogram from a milk matrix matched calibration standard (concentration of coccidiostats ranging from 2 to 10 µg/kg) prepared and analyzed using the conventional approach

Table 4. Targeted coccidiostats with retention times, polarity, and detected MRM transitions using the Phenomenex Kinetex C8 column

Coccidiostats	CAS	Structure	RT (min)	Polarity	Q1 (amu)	Q3 (amu)
Diclazuril	101831-37-2		5.3	negative	405 407	334 336
Decoquinate	18507-89-6		6.4	positive	418	204 372
Lasalocid	25999-31-9		7.1	positive	613	377 595
Maduramycin	84878-61-5		7.2	positive	939	877 895
Monensin A	17090-79-8		7.0	positive	693	461 479
Narasin	55134-13-9		6.8	positive	787	279 431 531
Nigericin	28643-80-3		7.8	positive	747	703 501
Robenidine	25875-51-8		4.6	positive	334	138 111
Salinomycin	53003-10-4		6.6	positive	773	431 531 265
Decoquinate D5 (internal standard)			6.4	positive	423	377

The conventional approach using a 5 µm column, as shown in Figure 1, produced peaks with peak widths in the range of 12 to 30 seconds and a run time of 23 minutes. When this method was switched to the Kinetex core-shell particle column the peak widths were reduced to between 7 and 12 seconds and the run time could be reduced to 11.5 minutes (Figure 2).

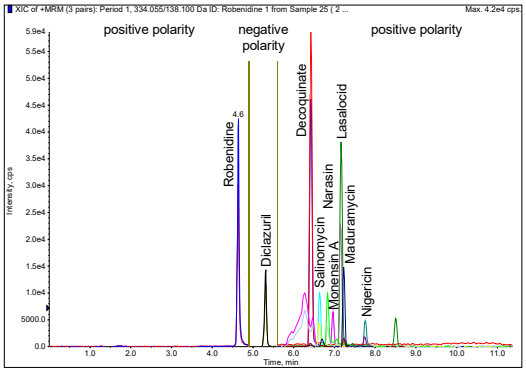


Figure 2. Example of an LC-MS/MS chromatogram from a milk matrix matched calibration standard (concentration of coccidiostats ranging from 2 to 10 µg/kg) prepared and analyzed using the new approach

To further speed up the analysis the off-line SPE was replaced by the simpler QuEChERS sample preparation technique, which is commonly used in pesticide residue analysis. The resulting simplification of the extraction produced dirtier extracts but the background interferences did not co-elute with analytes so this approach was shown to be a feasible alternative.

To assess the sensitivity of the developed method the coccidiostats were spiked into milk and extracted using the QuEChERS procedure. The results showed that this technique was capable of detecting all the coccidiostats reproducibly in milk at concentrations below 1 µg/L.

When both approaches, the conventional using SPE and the new one using QuEChERS, were compared both showed coefficients of variation (% CV) of less than 10% at or below the LOD levels needed except for Robenidine whose CV was 19% using the SPE methodology (Table 5). This showed that both methods could be applied to food samples. Both approaches produced linear responses and r values > 0.985 (see examples in Figure 3). This included the QuEChERS method which used spiked calibration standards whose concentration ranged from 0.2 to 50 µg/L with the exception of Decoquinate whose fit was quadratic over this range. The internal standard Decoquinate D5 was later used to correct the non linearity and additional internal standards could further improve these results.

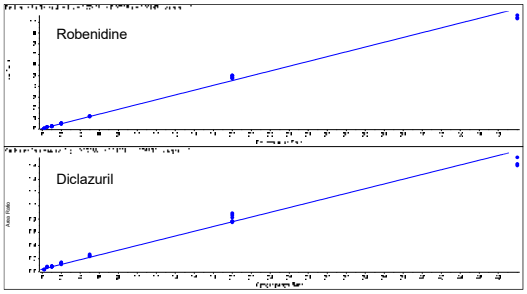


Figure 3. Calibration line for Robenidine (top) and Diclazuril (bottom) 0.2 to 50 µg/L in milk using the new approach with QuEChERS extraction and fast chromatography

Table 5. Reproducibility from the repeat analysis of a low spiked matrix matched standard

Coccidiostats	Concentration of spiked SPE extract (µg/L)	% CV (4 replicates) using the conventional approach	Concentration of QuEChERS extract (µg/L)	% CV (4 replicates) using the new approach
Diclazuril	1.25	2.6	1	7.7
Lasalocid	0.25	6.3	0.5	5.1
Maduramycin	0.5	0.7	0.5	3.5
Monensin A	0.5	2.9	0.5	3.8
Narasin	0.25	4.7	0.5	4.7
Robenidine	1.25	18.8	1	7.9
Salinomycin	0.5	3.6	0.5	7.9

Contents

There are known cases, especially in food analysis, when MRM ratios can be misleading and produce false positive results therefore additional information for identification is beneficial.

So in addition to collecting MRM data there is the possibility of automatically acquiring full scan MS/MS spectra when an MRM signal exceeds a defined threshold. These full scan MS/MS spectra [Enhanced Product Ion (EPI) spectra] are highly characteristic and sensitive using this unique scan function of a Q TRAP® system. Figure 4 shows two examples of how MRM triggered EPI spectra further aids identification of coccidiostats in food samples.

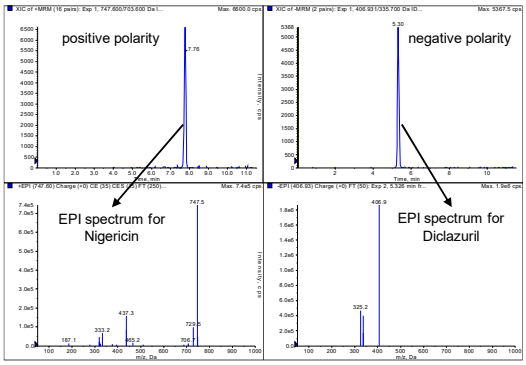


Figure 4. Example of an LC-MS/MS chromatogram from a 2 µg/L matrix matched calibration standard run in positive polarity with an EPI spectrum of Nigericin (left) and an LC-MS/MS chromatogram from the same sample run in negative polarity where a spectrum of Diclazuril has been automatically acquired

Summary

The LC-MS/MS approaches discussed in this work have been shown to be suitable for the detection of coccidiostats in food at the required sanctioned levels.

When the sample preparation was simplified using a QuEChERS procedure and a core-shell particle column was used the additional sensitivity of this assay enabled the detection of these residues below the MRL required but at over twice the speed of the conventional method which enables a reduction in cost of the analysis.

References

1 Commission Regulation (EC) No 124/2009 'Setting maximum levels for the presence of coccidiostats or histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed'

2 M. Anastassiades et al.: 'Fast and easy multi-residue method employing acetonitrile extraction/partitioning and dispersive solid-phase extraction for the determination of pesticide residues in produce' J. AOAC Int. 86 (2003) 412-431

3 S. J. Lehotay et al.: 'Validation of a fast and easy method for the determination of residues from 229 pesticides in fruits and vegetables using gas and liquid chromatography and mass spectrometric detection' J. AOAC Int. 88 (2005) 595-614

Publication number: 4482011-01

The Quantitation of Recombinant Bovine Somatropin by QTRAP® LC-MS/MS Operated in MRM and MRM^3 Mode

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the detection of recombinant bovine somatropin (rbST) using an MRM^3 approach. This growth hormone, which can be present in low levels in milk, has been shown to be detected at low parts-per-billion (ppb) levels by this method.

Introduction

In dairy farming rbST is used to treat cows in order to increase their milk output or as a growth promoter.[1,2] This growth hormone is banned in many countries[3] but is commonly used in the United States since it's authorization by the FDA in 1994.[4] To date most methods used to detect this hormone involve immunoassays[5,6] but the problem is that the native and the recombinant version of this hormone can not be differentiated by this approach. However, both native and recombinant forms do differ by one amino acid at the N-terminal end.[2] This slight difference means that a method based on mass spectrometry is a viable alternative and would have several advantages including specificity and sensitivity. rbST is usually only present at low ppb (ng/mL) amounts[1,2] in milk so any technique developed should be able to detect the hormone at this level.

Previously methods[1,7,8] have been developed to detect this hormone in plasma, but in this work we show the detection of this hormone at this level in milk, a more complex matrix, and also show how MRM^3 can help reduce the effect of the matrix on the results obtained.

Experimental

Sample Preparation

Internal standard [equine hormone (reST) used as a measure of whole protein recovery was added to bovine milk (10 mL) which was loaded onto a C4 SPE cartridge. The cartridge was washed with water containing 0.1% trifluoroacetic acid (TFA) followed by 30/70 mixture of acetonitrile / 0.1% TFA. The rbST was then eluted with an 80/20 mixture of acetonitrile / 0.1% TFA (7mL) and evaporated down to a volume of 1 mL. Cold methanol was

added to induce precipitation, the sample was centrifuged, and the supernatant dried down. This residue was reconstituted with ammonium bicarbonate buffer (120 µL) and digested overnight, at 37°C, with trypsin. The digest was evaporated to dryness and reconstituted with 30/70 acetonitrile / 0.2% formic acid and a C13 internal standard for the N-terminal peptide for rbST was added prior to injection.

LC-MS/MS Analysis

Final extracted samples were separated over a 25 minute gradient from 90% water / 10% acetonitrile to 10% water / 90% acetonitrile with both phases containing 0.1% formic acid. The separation occurred by reversed-phase HPLC on a 150x2.1 mm C18 Interchrom QS Uptisphere 3HDO HPLC column, at ambient temperature running at a flow of 300 µL/min on a Shimadzu UFLC$_{XR}$ system. MS detection was performed on an SCIEX QTRAP® 5500 system equipped with Turbo V™ source and electrospray ionization probe set at an IonSpray voltage of 3500 V. The conditions of the Multiple Reaction Monitoring (MRM) and MRM^3 experiments are shown in Table 1.

Contents

Table 1. MS conditions used for each peptide in MRM and MRM^3 mode

		MRM			MRM^3			
Hormone	N-Terminal Peptide sequence	Transitions	DP (V)	CE (V)	Transitions	DP (V)	CE (V)	AF2 (mV)
rbST	MFPAMSLSGLFANAVLR	913.2/774.0 913.2/1047.6	35	37	913.2/774.0/791.0 913.2/774.0/961.0	35	37	0.2
reST	MFPAMPLSSLFANAVLR	933.2/794.2	35	38				
rbST $^{13}C_6$	MFP(A^{13}C)MS(L^{13}C)SG(L^{13}C)F(A^{13}C)N(A^{13}C)V(L^{13}C)R	916.2/777.0	35	37				

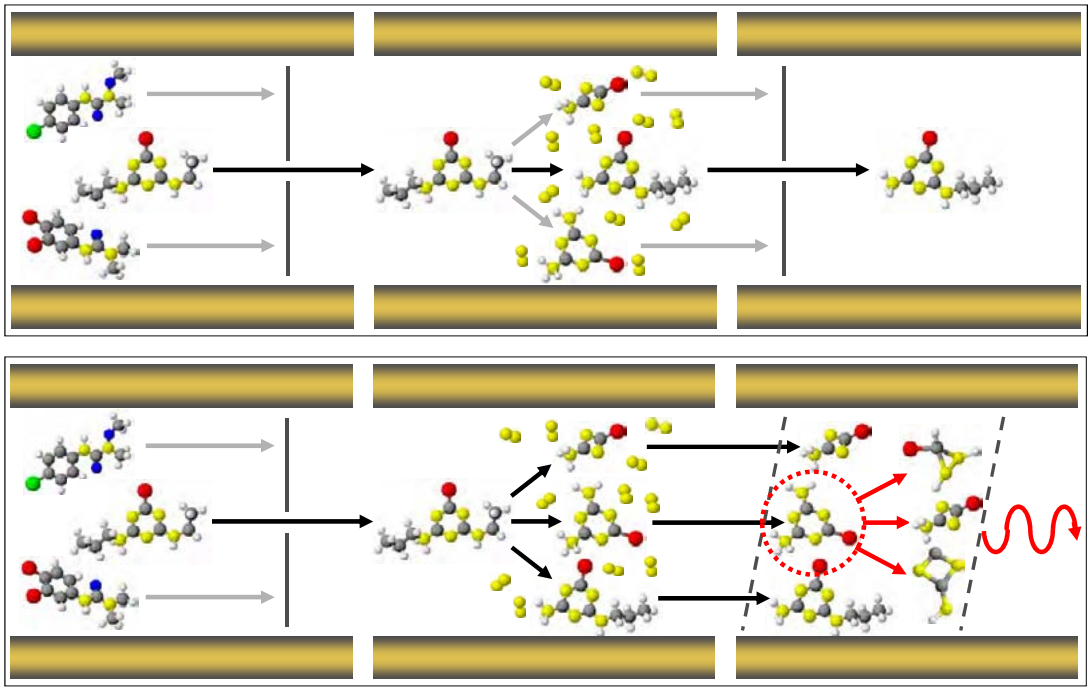

Figure 1. Operation of a QTRAP® system in MRM (top) and MRM^3 (bottom) modes

Results and Discussion

MRM^3 is a unique detection mode of hybrid triple quadrupole linear trap (QTRAP®) technology which is especially useful on the QTRAP® 5500 system because of sensitivity and speed enhancements in comparison to legacy instruments.

The QTRAP® 5500 system enables MRM^3 quantitation with a cycle time of 100 ms per scan providing scan speeds faster than LC demands and gives the ability to run in parallel several of these experiments in a single run or to combine MRM^3 and MRM experiments.

MRM^3 experiments enable higher specificity by first fragmenting precursor ions in the collision cell (Q2) and detecting the first set

of product ions (as in MRM experiment) but then the most intense of these product ions is trapped, isolated and fragmented again in the linear ion trap (Q3). These second generation fragment ions are then used for quantitation (Figure 1).

MRM^3 has a special advantage when analyzing dirty or complex samples for example food extracts. The increased selectivity allows the removing of matrix interferences and thus improves the signal-to-noise (S/N) ratio.

For this work MRM was directly compared to MRM^3 using a low ppb spike of rbST in milk (Figure 2). From this example it can be seen that MRM^3 has reduced matrix interference and an improved S/N for quantitation at low concentrations.

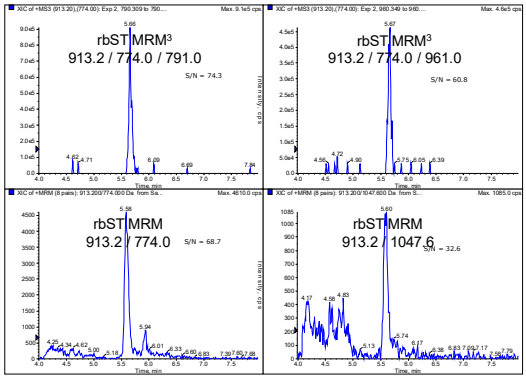

Figure 2. XIC of milk samples spiked with 10 ppb of rbST and purified using solid phase extraction: MRM^3 data (top) and MRM data (bottom)

As the MRM^3 experiment was shown to have advantages over the MRM experiment in this instance, milk was spiked at several different concentrations to generate a calibration line. The calibration line obtained can be seen in Figure 3.

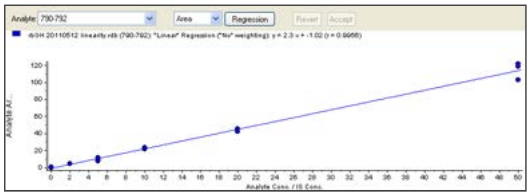

Figure 3. Calibration line for extracts of milk samples spiked with rbST at different levels. The calibration line is from the MRM^3 experiment 913.2/774.0/791.0

A typical 2 ppb spike into milk (a level which can be seen in milk) is shown in Figure 4. The top pane shows the total ion chromatogram, the middle pane shows the chromatograms of the MRM experiments for the internal standards and the bottom two panes show the two MRM^3 transitions for rbST.

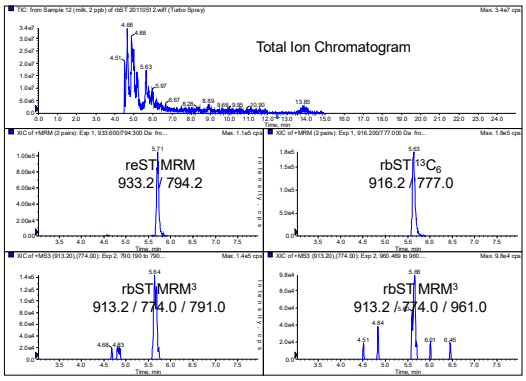

Figure 4. Typical chromatogram for a 2 ppb spiked milk calibration standard

Summary

From the results presented it can be seen that the application of more selective techniques for both sample preparation and mass spectrometric determination have improved the performance of the method for the detection of rbST in milk. These results show that we can unambiguously detect rbST in milk unlike the conventional methods which use immunoassay based technologies. This is now the first step to the development of a sensitive method for the efficient control of somatotropin abuse in milking cows.

Moving forward further improvements are still necessary, especially in the sample preparation as non-specific loss of rbST has been seen to occur, probably resulting from instability and adsorption issues, which have reduced the % recovery. Further to this additional optimization of the trypsin digestion step may well increase recoveries and provide even lower limits of detection. With regards to MS detection technologies the use of differential ion mobility is still to be investigated as a way to further increase specificity and improve detection limits.

Acknowledgement

We would like to acknowledge Prof. Bruno Le Bizec and his group at LABERCA for their continued contribution to this research area.

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Publication number: 4650211-01

Quantitation of Antibiotics and Insecticides in Poultry Feed using Liquid Chromatography Tandem Mass Spectrometry

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Overview

An LC-MS/MS assay has been developed for the analysis of multiclass antibiotics and insecticides in poultry feed.

Introduction

For decades, antibiotics have been added to livestock feeds in low doses to serve as growth promoters. [1] Antibiotics have recently been shown to accumulate in poultry feathers, which is significant because poultry feathers serve as a high protein ingredient in animal feed, such as poultry feed.[1]

The continued use of these antibiotics as feed additives has inadvertently created antibiotic-resistant micro-organisms, which has caused human health concerns.[2] The types and quantities of antibiotics administered to livestock in the U.S. are not reported by the FDA.[1] In 2012, a federal judge ordered to withdraw the approval for the use of common antibiotics in animal feed because overuse could create antibiotic-resistant micro-organisms.[2]

Plant protection products may be introduced into animal feeds through several means, but the most common source of residues is through the legitimate use of pesticides (herbicides, insecticides and fungicides) in the production of crops used in preparation of feeds. Various grains and related glutens are frequently utilized in animal feeds. Animal feeds can in fact contain many nutritional ingredients and additives, including but not limited to proteins, fats, carbohydrates, antimicrobials, emulsifiers, binders, pH control agents, pelleting agents and preservatives.[3, 4] The inherent complexity of the sample matrix demands an efficient extraction and cleanup and a highly sensitive mass spectrometer to accurately quantify low levels of common antibiotics and insecticides in animal feeds in a single method.

In this work, a method has been developed to analyze for nine antibiotics, which included fluoroquinolones, sulfonamides, amphenicols, macrolides and quinolones, and four insecticides in poultry feed.

The preparative method involves a three-part extraction, sample cleanup with Phenomenex® Strata™-XL-CW solid phase extraction (SPE) cartridges and analysis by LC-MS/MS on an Eksigent ekspert™ UltraLC 100-XL with a SCIEX QTRAP® 5500 system utilizing Multiple Reaction Monitoring (MRM) with the *Scheduled* MRM™ algorithm and fast polarity switching. For the work presented here, accuracy and reproducibly are demonstrated by evaluating poultry feed samples fortified in triplicate.

Contents

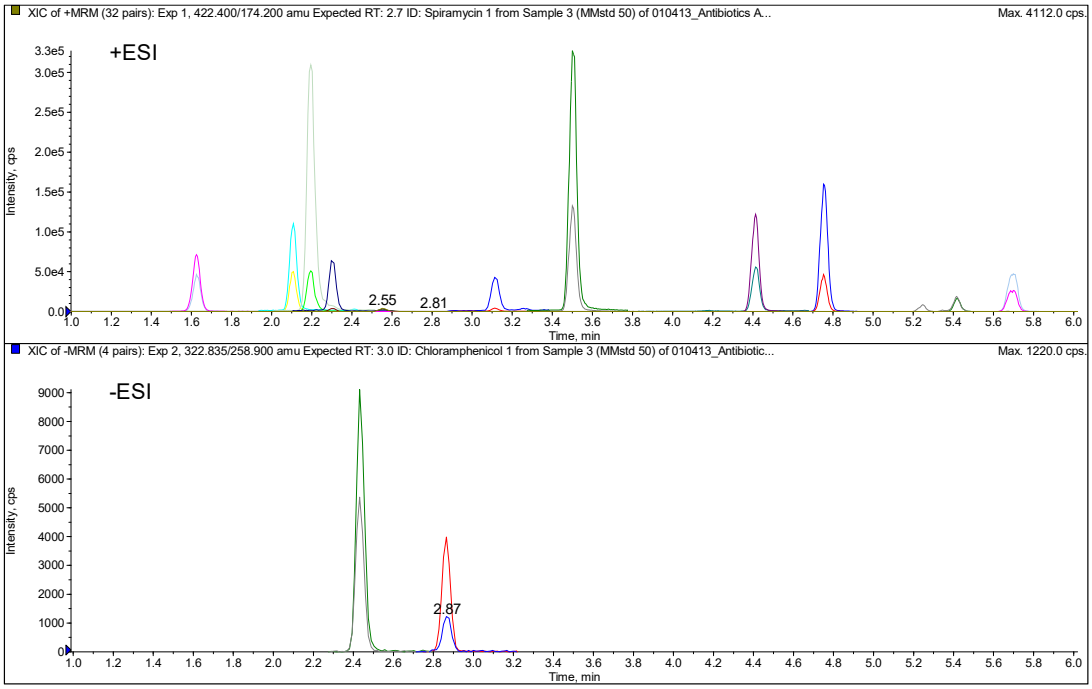

Figure 1. Detection of antibiotics and insecticides in a single run by LC-MS/MS using *Scheduled* MRM™ with polarity switching. Positive mode (+ESI) MRM transitions shown in top pane, and XIC of all negative mode (-ESI) MRM transitions shown at bottom. Peaks are identified by retention time in Table 2.

Experimental

Standards

All targeted analytes were available commercially and were either purchased as pure solid material or as high concentration analytical solutions. To prepare stock solutions of the solid materials, 10 mg of pure material was brought to either 10 or 100 mL with solvent to prepare 1 or 0.1 mg/mL solutions, respectively. The concentration of each stock solution was dependent on it solubility.

Sample Preparation

1) Extraction

Approximately 1.25 g of poultry feed sample were added to a 50 mL polypropylene centrifuge tube. Fortified samples were spiked into the dry sample for an in-sample concentration of 40 µg/kg. The sample was wetted with 10 mL of HPLC water and blended on a horizontal wrist-action shaker for 20 minutes. Three extractions were performed. The first extraction was performed with 5 mL of 1.5 mM EDTA and 5 mL of 1% TCA. The second extraction was performed with 10 mL of 75% methanol in water. The third extraction was performed with HPLC water. Between each extraction step, the sample was vortexed, shaken for 15 minutes on a wrist action shaker, sonicated for 10 minutes and centrifuged at 10,000 rpm for 5 minutes. All extracts were combined and brought to 50 mL with HPLC water.

2) SPE Cleanup

Sample cleanup was performed with Phenomenex® 200 mg Strata™-XL-CW SPE cartridges. This cartridge was selected based on the sorbent's weak cation exchange functionality to extract basic compounds from the poultry feed extract. Moreover, the large particle size of the XL (100 µm) allowed high volume loading and fast flow of the extract through the sorbent without the need to pre-filter the extract.

The final methanol percentage in this combined extraction was 15%, which was optimized for the SPE cleanup by performing a breakthrough study with various methanol percentages ranging

from 0 to 100%. It was determined that at 15% methanol concentration, all the targeted analytes retained on the sorbent during the loading step. At ≥25% methanol, some of the analytes would fail to be retained on the sorbent in the loading step, particularly oxolinic acid, florfenicol and chloramphenicol (data not shown).

The cartridge was conditioned with methanol followed by HPLC water. A 20 mL aliquot of the extract was loaded onto the SPE cartridge and sent to waste. The cartridge was washed with 10 mL of 15% methanol. The cartridge sorbent was dried under a light vacuum after the washing steps and prior to eluting the analytes. A 5 mL aliquot of 5% formic acid in methanol was used to elute the analytes.

3) Concentration/Reconstitution

Samples were evaporated to dryness under a gentle stream of nitrogen on a heating block (≤35°C). It was determined that these conditions resulted in no significant loss of analyte. The samples were reconstituted in 1 mL of 70% methanol in water, which was vortexed and filtered through a 0.22 µm syringe filter into an autosampler vial for analysis. The sample dilution factor was 2x.

LC Separation

The chromatography was performed on an Eksigent ekspert™ UltraLC 100-XL system with a Phenomenex® column configuration that used two Silica SecurityGuard™ cartridges, followed by a Luna® Silica (2) mixer column (30 x 2 mm, 5 µm). A Gemini® 3 µm NX-C18 (50 x 2 mm) served as the analytical column. The column compartment was maintained at 30°C. The gradient is listed in Table 1. Mobile Phase A was HPLC water with 0.1% formic acid and Mobile Phase B was 10 mM ammonium formate in methanol with 0.1% formic acid.

Table 1. LC gradient

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.0	0.7	100	0
5.0	0.7	5	95
7.0	0.7	5	95
7.1	0.7	100	0
10.0	0.7	100	0

MS/MS Detection

Analysis was performed on a SCIEX QTRAP® 5500 LC/MS/MS system using electrospray ionization (ESI) and *Scheduled* MRM™ in which each analyte's MRM is monitored across a user defined time window around each analyte's expected retention time, maximizing sensitivity. Each analyte's MRM and retention time are listed in Table 2. Most analytes are ionized in positive mode (+ESI) with the exception of florfenicol and chloramphenicol which are ionized in negative mode (-ESI). In order to achieve a single run, polarity switching was used in conjunction with the *Scheduled* MRM™ algorithm. The use of short pause times (2-3 ms) proved to be necessary to achieve optimal peak shapes and sensitivity to quantify the narrow UPLC peaks (FWHM = 3 to 4 s) particularly during polarity switching.

Table 2. Analytes, retention times (RT) and MRM transitions with collision energies (CE)

Analyte	RT (min)	Q1 (amu)	Q3 (amu)
Trimethoprim	1.63	291.2/261.2 (34)	291.2/230.2 (31)
Ciprofloxacin	2.11	332.0/314.0 (27)	332.0/230.9 (51)
Enrofloxacin	2.20	360.1/342.0 (29)	360.1/286.0 (47)
Sarafloxacin	2.30	386.1/368.2 (27)	386.1/348.1 (43)
Florfenicol	2.43	357.9/337.9 (-14)	357.9/184.8 (-46)
Spiramycin	2.55	442.4/174.2 (29)	422.4/101.1 (26)
Chloramphenicol	2.87	332.8/258.9 (-16)	322.8/151.9 (-24)
Oxolinic Acid	3.12	262.0/244.0 (23)	262.0/216.0 (39)
Flumequine	3.50	262.0/243.9 (25)	262.0/201.8 (45)
Diflubenzuron	4.42	311.2/158.1 (18)	311.2/141.1 (42)
Emamectin	4.75	886.7/158.2 (42)	886.7/82.3 (107)
Abamectin	5.42	891.0/305.1 (33)	891.0/568.1 (19)
Ivermectin	5.70	893.3/570.2 (21)	893.3/307.1 (33)

Results and Discussion

Figure 1 shows the extracted ion chromatograms (XIC) of a 10 µL injection of a matrix matched standard at 50 µg/mL.

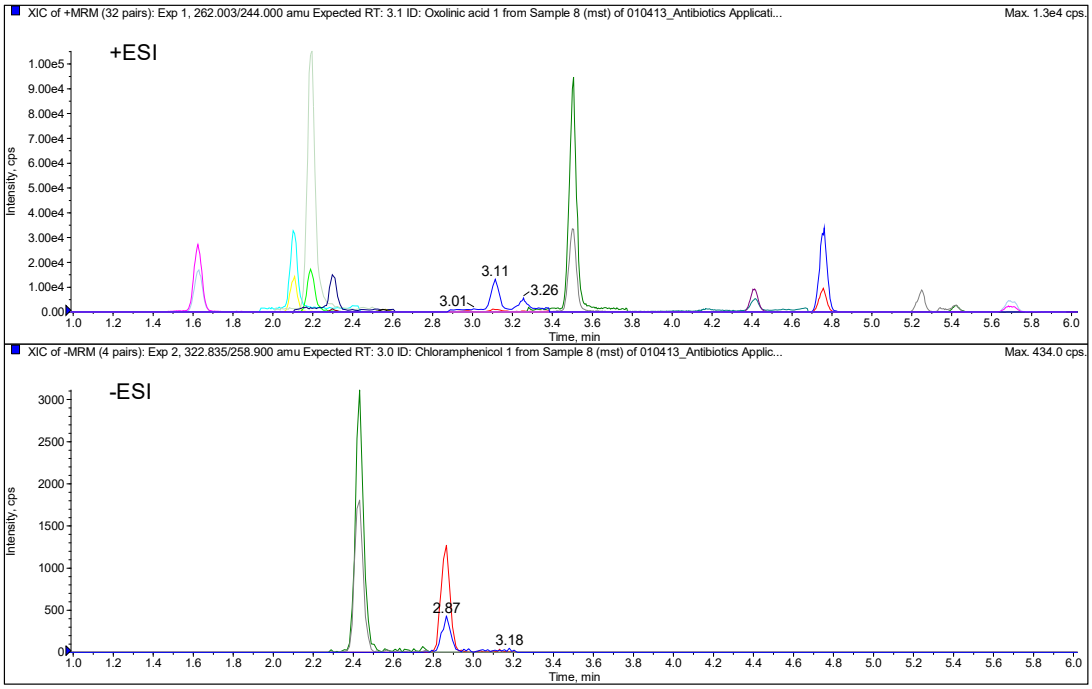

Figure 2. Poultry feed sample fortified at 40 µg/kg in sample (20 µg/mL in extract).

Figure 2 shows the extracted ion chromatograms (XIC) of a 10 µL injection of a poultry feed sample fortified at 40 µg/kg level (20 µg/mL in extract after 2x dilution).

The recoveries for each analyte are shown in Table 3. Given the complexity of the sample matrix and the inherent chemical differences between the target analytes, most analytes were reasonably recovered with the described extraction and cleanup. The method proved to be precise with %RSDs generally less than 5%. Recoveries could potentially be improved with the use of internal standards; however, absolute recoveries are a more accurate approach to assessing the effectiveness of a preparative method.

Table 3. Quantitation and Recovery Data based on MRM 1. Four point calibration using 5, 10, 50 and 100 µg/mL matrix matched standards.

Analyte	r^2	Average recovery (%) ± % RSD
Trimethoprim	0.999	89 ± 4 %
Ciprofloxacin	0.997	60 ± 0 %
Enrofloxacin	0.999	73 ± 4 %
Sarafloxacin	0.996	47 ± 4%
Florfenicol	1.000	85 ± 1 %
Spiramycin	1.000	70 ± 3 %
Chloramphenicol	1.000	77 ± 2 %
Oxolinic Acid	1.000	64 ± 1 %
Flumequine	0.998	64 ± 3 %
Diflubenzuron	1.000	20 ± 5 %
Emamectin	0.999	52 ± 7 %
Abamectin	0.999	40 ± 5 %
Ivermectin	1.000	24 ± 3 %

Summary

A single method has been developed to quantify a wide class of antibiotics and insecticides in poultry feed. The poultry feed extract was cleaned by SPE on a Phenomenex® Strata™-XL-CW prior to analysis utilizing an Eksigent ekspert™ UltraLC 100-XL system with a Phenomenex® Luna® Silica mixer column in series with a Gemini® NX-C18 analytical column with a SCIEX QTRAP® 5500 system for detection. *Scheduled* MRM™ in combination with fast polarity switching was used to maximize sensitivity while achieving a single run for all analytes. Analyte recoveries and precision from triplicate fortified poultry feeds were acceptable, given the complexity of the sample matrix and the generic approach to the extraction, and cleanup procedure required to simultaneously test such a variety of analytes.

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Publication number: 7180213-01

The Use of Micro Flow LC Coupled to MS/MS in Veterinary Drug Residue Analysis

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Overview

A rapid, robust, sensitive and specific LC-MS/MS method has been developed for the simultaneous detection of veterinary drug residues in milk and meat. The method uses an Eksigent ekspert™ microLC 200 and the SCIEX QTRAP® 4500 system utilizing the *Scheduled* MRM™ algorithm with a simplified sample preparation to detect veterinary residues below EU screening requirements.

Introduction

Traditionally, in veterinary drug residue screening of food samples, samples are extracted and analyzed by LC-MS/MS usually at LC flow rates in excess of 500 µL/min and in combination with smaller particle size LC columns result in high UHPLC pressure separations. These conditions result in short chromatographic run times with excellent efficiency and peak shape, but have a drawback in that they require higher volumes of mobile phase. The consumption of organic LC solvents, such as acetonitrile and methanol, is a growing cost of analysts and its disposal has an environmental impact. Therefore, ways to reduce solvent consumption in food residue testing will be beneficial to the environment and reduce running costs of a testing laboratory.

Here we present new data using micro flow LC, running below 40 µL/min, in combination with a LC-MS/MS method developed on a SCIEX QTRAP® 4500 system which utilizes the *Scheduled* MRM™ Pro algorithm. Initially this approach has been applied to a screen of veterinary residues including sulfonamides and beta-lactam antibiotics to show its applicability in food analysis. Data presented shows a comparison of micro flow LC-MS/MS with traditional high flow LC-MS/MS and show that low limits of detection (LOD) below legislated levels[1] are easily possible by this approach.

Experimental

Standards and Samples

For this work the target compounds were commercially available and purchased from Sigma Aldrich. Milk and meat samples for spiking experiments were obtained from a local supermarket.

Sample Preparation

The milk samples (2 mL) was simply mixed with acetonitrile (8 mL) and roller mixed for 20 minutes. After mixing the sample extracts was centrifuged for 5 minutes at 2500 rpm. The supernatant (4 mL) was evaporated to dryness (Eppendorf vacuum concentrator at 60°C) and then reconstituted into 0.1% formic acid in water(2 mL). The reconstituted sample was centrifuged for 1 min at 13,000 and the top layer was decanted into plastic HPLC vials ready for LC-MS/MS analysis.

Contents

For meat samples the extraction protocol was exactly the same except the initial extraction solvent was acetonitrile/water (87.5/12.5).

LC

All microLC method development and analysis was done using an Eksigent ekspert™ microLC 200 UHPLC system. Final extracted samples (5 µL) were separated over a 3.5 minute gradient (shown in Table 1 where A = water and B = acetonitrile both containing 0.1 % formic acid) on a reversed-phase Triart C18 2.7 µm (50 x 0.5 mm) column (YMC) at 30 µL/min and at a temperature of 60ºC.

For the high flow LC comparison a Shimadzu UFLC$_{XR}$ system was used at a flow rate of 600 µL/min using a Kinetex 2.6 µm XDB-C18 (50 x 2.1 mm) column (Phenomenex). The gradient conditions are shown in Table 2.

Table 1. Gradient conditions used for micro flow LC separation at a flow rate of 30 µL/min

Step	Time	A (%)	B (%)
0	0	98	2
1	0.5	98	2
2	1.7	35	65
3	1.8	0	100
4	2.3	0	100
5	2.4	98	2
6	3.5	98	2

Table 2. Gradient conditions used for traditional high flow LC separation at a flow rate of 600 µL/min

Step	Time	A (%)	B (%)
0	0	98	2
1	2	98	2
2	7	40	60
3	7.2	5	95
4	8	5	95
5	8.1	98	2
6	10	98	2

MS/MS

All analyses were performed on a SCIEX 4500 QTRAP® system using the Turbo V™ source in electrospray ionization (ESI) mode. For micro flow LC analysis the electrode was changed to a microLC hybrid electrode (50 µm ID) designed for micro flow rates.[2] In the final micro flow LC method the ion source conditions used were Gas 1, Gas 2 and the Curtain Gas™ interface was set to 30 psi, the temperature (TEM) was set at 350°C and the IS voltage was set to 5500 V.

The veterinary drugs were analyzed using Multiple Reaction Monitoring (MRM) using the *Scheduled* MRM™ algorithm to obtain high selectivity, sensitivity, accuracy and reproducibility. The *Scheduled* MRM™ Pro algorithm in Analyst® software version 1.6.2 allows setting the MRM detection window separately for each compound based on the LC peak width for more efficient scheduling of dwell time (Figure 1).

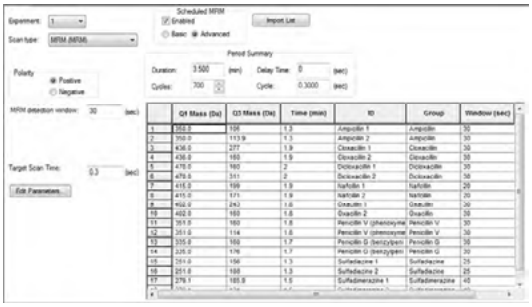


Figure 1. Method editor in Analyst® software version 1.6.2 used to setup the *Scheduled* MRM™ Pro experiment

A total of 32 MRM transitions (Table 3) were monitored to quantify and identify 15 veterinary drug residues and internal standards over a 3.5 minute run time. Only a small set of residues were tested in this project but there is scope to add more compounds to this method. In all the analyses Q1 and Q3 resolution were set to unit.

Table 3. MRM transitions and retention times (RT) of veterinary drug residues investigated in this

Compound	RT (min)	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)
Ampicillin	1.3	350	106, 114	56	23, 41
Cloxacillin	1.9	436	277, 160	51	19, 17
Dicloxacillin	2	470	160, 311	66	19, 21
Nafcillin	1.9	415	199, 171	61	19, 47
Oxacillin	1.8	402	243, 160	46	19, 17
Penicillin V	1.8	351	160, 114	50	19, 45
Penicillin G	1.7	335	160, 176	50	15, 19
Sulfadiazine	1.3	251	156, 108	66	26, 30
Sulfadimerazine	1.5	279	186, 124	80	23, 31
Sulfadimethoxine	1.7	311	156, 92	71	29, 45
Sulfamerazine	1.4	265	108, 92	80	33, 35
Sulfamethaxazole	1.55	254	156, 92	120	21, 35
Sulfamethazine	1.5	279	186, 124	120	23, 31
Sulfaquinoxaline	1.9	301	156, 108	80	27, 37
Sulfathiazole	1.4	256	156, 92	80	19, 33

Results and Discussion

Before the micro LC was used for residue analysis the method was compared against a traditional high flow method that had previously been developed for residue detection in meat and milk. A 1 ng/mL standard of a mixture of different veterinary residues was prepared and analyzed (Figure 2).

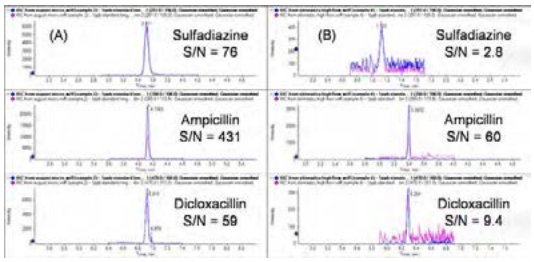


Figure 2. Comparison of microLC (A) with traditional high flow LC (B) using a 1 ng/mL standard.

For the high flow separation a Kinetex 2.6 µm XDB-C18 column at a flow rate of 600 µL/min was used and a Triart C18 column was used for microLC at 25 µL/min. The gradient conditions (Table 2) were kept the same as was the injection volume and column temperature. The results showed sensitivity increases of factors greater than 4 fold to over 10 fold for the veterinary drugs tested with none of the compounds showing a sensitivity loss.

The gradient on the microLC was then adjusted and the flow rate increased to 30 µL/min, to shorten the run time down to 3.5 minutes (Figure 3).

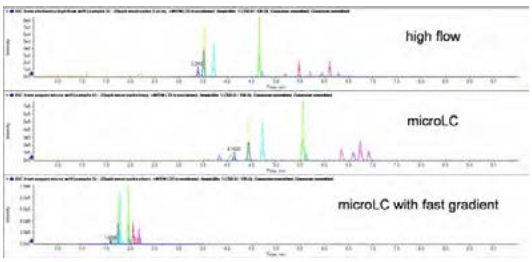


Figure 3. Comparison of meat sample spiked at 20 µg/kg and analyzed by traditional high flow LC and micro flow LC-MS/MS,. In this example analysis time was decreased from 10 min to 3.5 min using micro flow LC and by speeding up the gradient. In all methods peak widths at the base were 3 seconds or less.

Table 4. Results for the calibration lines for a selection of veterinary drug residues and the repeat analysis of spiked milk and meat samples. Displayed are the coefficient of regression (r), coefficient of variation (CV), and signal-to-noise (S/N) obtained. Linearity ranged from 0.1 to 100 ng/mL with linear fit and no weighting used except for sulfamerazine where linear fit and 1/x weighting was used.

Compound	r	CV (%) at 20 µg/kg spiked into milk (n=20)	S/N at 2 µg/kg spiked into milk	S/N at 20 µg/kg spiked into milk	CV (%) at 20 µg/kg spiked into meat (n=20)	S/N at 20 µg/kg spiked into meat
Ampicillin	0.999	5.8	67	712	3.6	285
Cloxacillin	0.999	4.7	94	934	9.1	591
Dicloxacillin	1.000	5.7	50	389	9.0	508
Nafcillin	0.999	2.7	39	379	10.2	800
Oxacillin	0.999	5.6	39	337	8.4	299
Penicillin V	0.999	4.3	101	1162	5.5	272
Penicillin G	0.991	5.8	19	150	14.0	175
Sulfadiazine	0.997	11.1	24	208	6.9	196
Sulfadimerazine	0.995	6.1	30	2131	8.3	1119
Sulfadimethoxine	0.999	4.2	152	1549	1.4	539
Sulfamerazine	0.996	3.5	44	366	3.0	333
Sulfamethaxazole	0.993	7.2	40	356	5.7	189
Sulfamethazine	0.997	10.4	55	662	2.8	357
Sulfaquinoxaline	0.998	4.8	25	275	3.7	705
Sulfathiazole	0.998	3.4	25	290	5.2	131

The results showed that for the late eluting compounds there was some sensitivity loss due to peak broadening but again sensitivity gains were also observed for early eluting compounds. Generally speaking increasing the speed of analysis three fold did not have a negative effect on the response observed for these veterinary residues.

Calibration standards were analyzed for all compounds using the shortened microLC method and three examples of calibration lines for different compounds are shown in Figures 4a to 4c. In each figure the calibration lines were linear and the residues could be detected at a level of 0.1 ng/mL or below (see peak review in each figure).

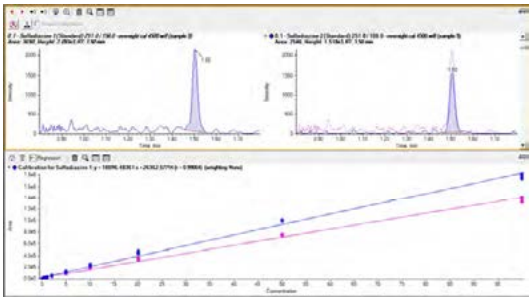
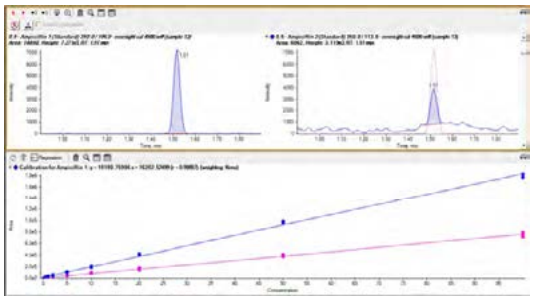
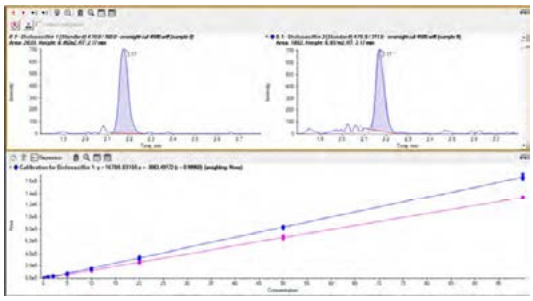


Figure 4a. Quantifier, qualifier MRM transition at 0.1 ng/mL (top), and calibration line of sulfadiazine from 0.1 to 100 ng/mL (bottom), the linearity is provided without the use of any internal standards





he linearity

is provided without the use of any internal standards

The calibration data for each compound is shown in Table 4. Following on from the assessment of linearity milk, meat samples were spiked and extracted and repeatedly analyzed to assess reproducibility with the results displayed in Table 4. For both the calibration lines and the spiking experiments no internal standards were used.

From the results displayed in Table 4 it can be seen that the method can easily provide detection limits which comply with current EU legislation. Linearity was excellent from 0.1 to 100 ng/mL with coefficients of regression greater than 0.99. The repeatability observed and signal-to-noise (S/N) measured varied with the matrix showing the need of internal standards to counter matrix effects from the simplified sample extraction protocol used. However, no coefficient of variation (CV) was over 15% which mirrored a previous study of pesticide residue analysis using microLC[3] with most generally below 10%. All S/N (calculated using 3x standard deviation algorithm in Analyst® software) were greater than 15/1 even in the 2 µg/kg spike into milk.

Summary

This study has clearly demonstrated that using microLC is a valid approach in veterinary residue analysis. The method developed using Eksigent ekspert™ microLC 200 and the SCIEX QTRAP® 4500 system was rapid, sensitive, reproducible, and easily reached the requirements of current EU legislation. Micro flow LC offers the opportunity to cut the analysis time by over half without a loss in performance and in the majority of cases a gain in signal by over a factor of 5 was observed.

Micro LC also provides huge cost saving to laboratories. With LC grade acetonitrile running at a cost of £100/L this 3 day study could have cost about £ 100 with conventional chromatography (0.6 mL/min running for 24hrs a day) and less than £10 with microLC. Over a year this amounts to savings of over £4000 (£90 x 50 weeks) in solvent consumption alone.

Although this method is still under development, with plans to expand the number of compounds in this screen, this work has shown the clear potential of Micro LC in this application area.

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Publication number: 8880414-01

Contents

Simultaneous Analysis of Chloramphenicol and Tetracycline Antibiotics in Food Samples Using the SCIEX Triple Quad™ 3500 System

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SCIEX Concord, Ontario (Canada)

Overview

Utilizing liquid chromatography with tandem mass spectrometry (LC-MS/MS) to analyze for antibiotic residues in a food samples offers many benefits to routine food testing labs, including the ability to screen for many compounds at once, the selectivity to meet regulatory guidelines, and the sensitivity to reduce sample preparation time to get to results faster. The SCIEX Triple Quad™ 3500 system enables labs performing antibiotic testing in foods to upgrade to LC-MS/MS and capitalize on its many benefits, at an affordable price.

Here we present a method using QuEChERS extraction (for the analysis of milk, meat and shrimp samples) with Phenomenex roQ kits and dilute-and-shoot (for honey samples), separation using a Kinetex Biphenyl 2.6u (50 x 2.1mm) column, and the SCIEX Triple Quad™ 3500 system for the detection of Chloramphenicol and Tetracyclines. The mass spectrometer was operated in highly selective and sensitive Multiple Reaction Monitoring (MRM) mode. Limits of detection (LOD) met regulatory limits. Compound identification and quantitation was achieved by monitoring two or three MRM transitions for each analyte. The MRM ratio was automatically evaluated in the MultiQuant™ software.

Introduction

Antibiotics are widely used as growth promoting agents and therapeutics against microbial infections. The presence of antibiotics in food of animal origin is of concern due to the potential of increasing bacterial resistance and to hypersensitivity for some individuals. Tolerance limits and maximum residue limits (MRL) have been established around the world and agencies monitor the food supply to ensure that antibiotic residue concentrations do not exceed these levels.

LC-MS/MS based methods for single-residue and single-class residues are used to monitor veterinary drugs in food. Recently multi-class multi-residue methods have been introduced to further increase monitoring efficiency.[1-3]

Generic extraction procedures[4-5], ultra high performance LC systems combined with core-shell particles columns, providing good resolution and excellent peak shape, made it possible to detect a variety of antibiotics in a single method. The LC-MS/MS system is typically used in MRM mode because of its excellent sensitivity, selectivity, and speed.

The SCIEX Triple Quad™ 3500 system takes the best features of the API 3200™ system and enhances them with modern engineering and electronics. The proven design of Turbo V™ source and Curtain Gas™ interface provide exceptional robustness and ruggedness. The advanced eQ™ electronics and the curved LINAC® collision cell were designed for ultra-fast speed of MRM detection and fast polarity switching for comprehensive multi-component analysis.

A triple quadrupole based method for the quantitation of Chloramphenicol and three selected tetracyclines was developed using selective Multiple Reaction Monitoring (MRM) with the *Scheduled* MRM™ algorithm activated. The ratio of quantifier and qualifier transition was used for compound identification. Sensitivity of detection met existing regulatory requirements, such as Codex Alimentarius' Maximum Residue Limits (MRL) of 200 µg/kg (tissue) and 100 µg/L (milk) for tetracyclines, the MRL

Contents

of 50 µg/kg set by Chinese government, and the Minimum Required Performance Limit (MRPL) for Chloramphenicol set by the European Union of 0.3 µg/kg.[6-8]

The method was successfully applied to the analysis of store-bought milk, meat, shrimp, and honey samples.

Experimental

- Store-bought food samples (milk, meat, shrimp) were extracted following the protocol of the European standard method 15662[5] using the Phenomenex roQ QuEChERS kit buffer-salt mix and the dSPE kit (#KS0-8913) containing 150 mg $MgSO_4$, 25 mg PSA, and 25 mg C18.
- QuEChERS extracts were diluted 10 times with water to minimize possible matrix effects.
- Honey samples were diluted with 5 times water and injected directly.
- The injection volume was set to either 10 or 50 µL, depending on targeted LOQ.
- LC separation was achieved using a Phenomenex Kinetex Biphenyl 2.6u (50 x 2.1mm) column and a fast gradient of water and acetonitrile with 0.1% formic acid at a flow rate of 0.5 mL/min (see Table 1 for the gradient profile).
- The SCIEX Triple Quad™ 3500 system was operated with Turbo V™ source and Electrospray Ionization (ESI) probe set to 500°C.
- Two MRM transitions were monitored for Chloramphenicol and three transitions were monitored for each tetracycline (Table 2).
- The *Scheduled* MRM™ algorithm was activated to achieve best data quality.
- Fast polarity switching of 50 msec was used. The IS voltage was to -4000 V and +5000 V, respectively.
- MultiQuant™ software version 3.0 was used for quantitative and qualitative data processing.

Table 1. Gradient conditions used for the separation

Step	Time (min)	A (%)	B (%)
0	0.0	80	20
2	4.0	5	95
3	7.0	5	95
4	7.1	80	20
5	10.0	80	20

Table 2. MRM transitions and retention times (RT) used for the detection of Chloramphenicol and tetracyclines

Compound	Polarity	RT (min)	Q1 (amu)	Q3 (amu)
Chloramphenicol 1	negative	1.32	321	152
Chloramphenicol 2	negative	1.32	321	257
Chlortetracycline 1	positive	1.30	479	444
Chlortetracycline 2	positive	1.30	479	462
Chlortetracycline 3	positive	1.30	479	154
Oxytetracycline 1	positive	0.57	461	426
Oxytetracycline 2	positive	0.57	461	444
Oxytetracycline 3	positive	0.57	461	201
Tetracycline 1	positive	0.76	445	410
Tetracycline 2	positive	0.76	445	427
Tetracycline 3	positive	0.76	445	154

Results and Discussion

Sensitivity, Reproducibility, Linearity and Accuracy

The LC-MS/MS chromatogram of a 10 ng/mL solvent standard is shown in Figure 1 highlighting the excellent separation and peak shape achieved using the Phenomenex Kinetex Biphenyl with a fast gradient of water and acetonitrile containing 0.1% formic acid. Fast polarity switching was required to detect all compounds in a single method since Chloramphenicol (negative polarity) and Chlortetracycline (positive polarity) are not chromatographically separated by this method.

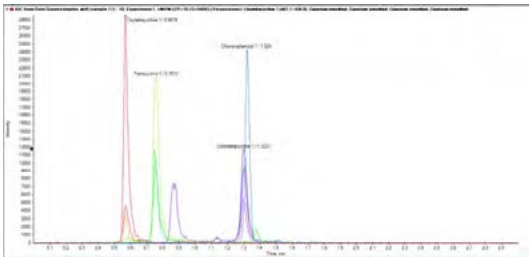

Figure 1. LC separation and detection in MRM mode of three tetracyclines and Chloramphenicol at 10 ng/mL

Figures 2 and 3 show the achieved sensitivity for all targeted antibiotics. Tetracyclines can be easily quantified at the target MRL using a small injection volume of 10 µL reducing the matrix

load for the mass spectrometer to increase robustness and to reduce potential ion suppression.

However, Chloramphenicol sometimes requires a larger injection volume to match the target MRPL while still allowing sufficient dilution to minimize potential matrix effects. In these cases, 50 µL injection volumes were utilized.

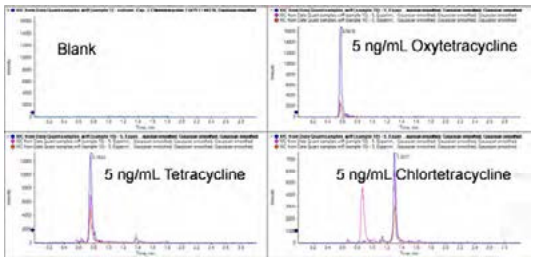

Figure 2. Sensitivity of a 5 ng/mL standard of tetracyclines (injection volume of 10 µL)

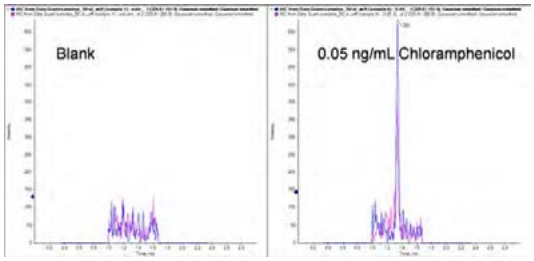

Figure 3. LOQ for Chloramphenicol of less than 0.05 ng/mL with an injection volume of 50 µL, allowing 10x dilution of matrix extracts

Calibration lines are shown in Figure 4, over the range of 0.05 to 100 ng/mL for Chloramphenicol and 0.1 to 100 ng/mL for tetracyclines, respectively, with a coefficient of regression > 0.997.

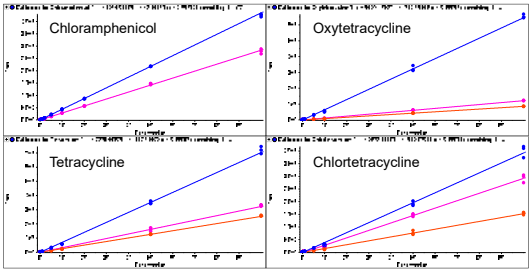

Figure 4. Calibration lines for all 4 compounds analyzed in this study

Accuracies for all calibration standards were between 80 and 120%, and repeatability was found to be better than 5% CV and 10% at the LOQ (n=3).

The achieved method performance allowed diluting sample extracts by a factor of 10 to reduce possible matrix effects. The additional use of isotope labeled internal standards is recommended to compensate matrix effects.

Findings in Food Samples

Figures 5 and 6 show matrix samples tested negative for Chloramphenicol and tetracyclines. The honey sample had a trace contamination with Chloramphenicol below the LOQ of 0.05 ng/mL (0.25 µg/kg in matrix after accounting for the 5x dilution during sample preparation).

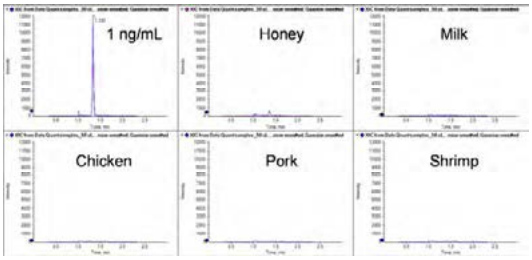

Figure 5. Blank matrices tested for Chloramphenicol (50 µL injection), the honey sample had a trace contamination with Chloramphenicol below the LOQ of 0.05 ng/mL (0.25 µg/kg in matrix after 5x dilution)

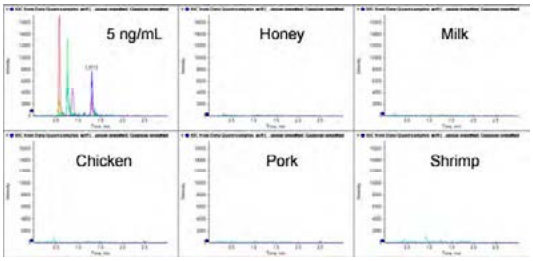

Figure 6. Blank matrices tested for tetracyclines (10 µL injection)

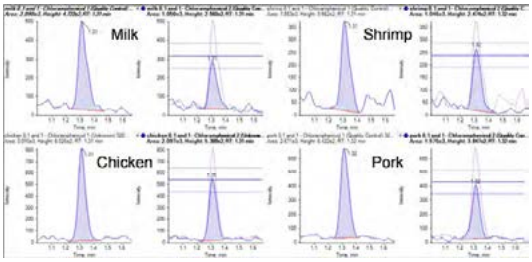

Figure 7. Different food extracts spiked with Chloramphenicol at 0.1 µg/kg (50 µL injection), the MRM ratio tolerances are displayed in the peak review window

Example chromatograms of different food samples spiked with antibiotics are presented in Figures 7 and 8. Compound identification was based on the criteria of directive 2002/657/EC[9] (retention time tolerance of ± 2.5% and maximum tolerances for ion ratios of ± 20 to 50% depending on the ratio). All quantitative and qualitative results were automatically calculated in MultiQuant™ software (Figure 6).[10]

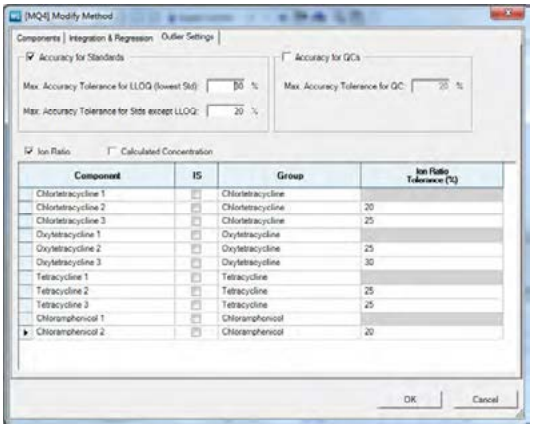

Figure 6. MRM ratio tolerances setup in the method editor of MultiQuant™ software

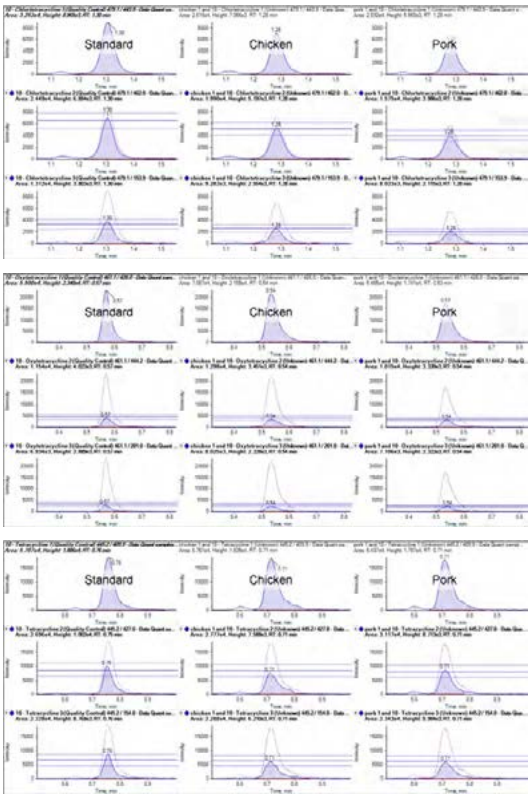

Figure 8. Side-by-side peak review of a standard injection (left) and spiked meat extracts (middle and right) with automatic calculation of MRM ratios, the MRM ratio tolerances are displayed in the peak review window

Summary

A new LC-MS/MS method for the identification and quantitation of antibiotics was developed and successfully applied to different food samples, including honey, milk, shrimp and meat.

The method consists of QuEChERS extraction followed by dilution to minimize possible ion suppression and a dilute and shoot approach for honey. The SCIEX Triple Quad™ 3500 system operated in MRM mode and utilizing the *Scheduled* MRM™ algorithm was used for detection. Limits of detection (LOD) met regulatory requirements. Two to three MRM transitions were monitored for each analyte and the ratio of quantifier and qualifier transition was used for identification. Data processing was performed in MultiQuant™ software. Identification criteria of directive 2002/657/EC were used for identification.

Acknowledgement

The author thanks Cheryl Stephenson (Eurofins Central Analytical Laboratories US) New Orleans, LA for providing standards.

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7 GB/T 21317-2007: 'Determination of tetracyclines residues in food of animal origin. LC-MS/MS method and HPLC method' (2007)

8 2003/181/EC: 'setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin' (2003)

9 2002/657/EC: 'concerning the performance of analytical methods and the interpretation of results' (2002)

10 A. Schreiber: 'MultiQuant™ Software Version 3.0 - Improving Data Quality and Processing Throughput with Better Peak Integration, Quantitative and Qualitative Compound Review for the Analysis of Food, Drinking Water, and Environmental Samples' Application Note AB SCIEX (2013) #8160213-01

Publication number: 10641514-01

Authenticity Assessment of Fruit Juices using LC-MS/MS and Metabolomic Data Processing

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Overview

Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS) with a SCIEX 4000 Q TRAP® system was used for comprehensive fingerprinting of several fruit juices. Metabolomic data processing tools were used for authentication, i.e. classification of juices and adulteration detection. The developed statistical model was able to reliably detect 25% of orange juice adulteration with apple or grapefruit juice. In addition, high resolution and accurate mass MS and MS/MS measurements using the SCIEX TripleTOF® 5600 system were performed to identify characteristic markers for fruit juice authenticity.

Introduction

The production of fruit juices represents an important and rapidly growing branch of the beverage industry. Besides orange juice, which is produced and consumed in the largest volume worldwide, other fruit juice types, such as those obtained from pomegranate and various types of berries, have become popular because of high levels of antioxidants resulting in positive health effects. Similarly to other highly prized food commodities, the economic value and large-scale production of juice made them a likely target for adulteration and fraud. The most frequent profit-driven fraudulent procedures applied, either alone or in combination, are dilution with water, addition of sugars or pulp wash, and extension of authentic juice with cheaper alternatives.[1, 2]

As the adulteration of fruit juices represents an ongoing problem, suitable analytical methods are needed to control authenticity parameters dictated by the legislation (Council Directive 2001/112/EC 2001). Until now, a number of methods have been developed to tackle various aspects of fruit juice authenticity. The most established approaches are based on profiling of carbohydrates, phenols, carotenoids, amino acids, or other organic acids using different chromatographic and spectroscopic methods.[3-5]

All of these methods are of targeted nature and can only be used to monitor one or few specific adulteration practices. However, it should be noted that fraud performers are usually one step ahead of the available testing methods, as new and more sophisticated adulteration practices are continuously developed. Therefore, analytical approaches for more comprehensive insight into chemical composition of fruit juices and its changes associated with adulteration are needed.

The field of metabolomics, a systematic study of the unique chemical fingerprints of samples, has recently found its application in many research areas including food quality and authenticity assessment. Advanced data mining tools are required to process and interpret complex data obtained within metabolomic-based studies.[6]

In this study, the feasibility of LC-MS/MS techniques employing QTRAP® and TripleTOF® systems for metabolomic-based authentication of fruit juices (including apple, blueberry, cranberry, grapefruit, orange, pomegranate, and their mixtures) was explored. Complex LC-MS/MS data were processed using Principal Components Analysis (PCA), Principle Components Variable Grouping (PCVG), and Linear Discriminant Analysis (LDA) to assess the suitability of the data to differentiate juice types and to detect their adulteration. In addition, high resolution and accurate mass MS and MS/MS data were acquired for characteristic marker compounds to empirically calculate their elemental formulas and for tentative identification.[7]

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Experimental

Sample Preparation

Different fruit juices made of apple (n = 16), blueberry (n = 1), cranberry (n = 1), grapefruit (n = 16), orange (n = 19), and pomegranate (n = 1) were purchased from Czech and Canadian supermarkets. Collected samples represented both freshly squeezed juices and juices prepared from concentrate, and were produced in various countries. Mixtures of different juices were prepared in various ratios to simulate adulteration.

Fruit juice samples were centrifuged to remove solid particles, 100x diluted, and transferred into autosampler vials for analysis.

LC

LC separation was achieved using an Agilent 1200 LC system with a Restek Ultra Aqueous C18 column (50 x 2.1 mm, 3 µm) and a gradient of water with 5 mM ammonium acetate and methanol with a total run time of 10 min. The injection volume was set to 10 µL.

MS/MS

The SCIEX 4000 Q TRAP® system equipped with Turbo V™ source and electrospray ionization (ESI) probe was used for metabolomic fingerprinting of juice samples. Full scan MS was acquired in EMS mode over a mass range of 100 to 1000 amu using dynamic fill time to avoid possible ion trap saturation for highly abundant compounds while enhancing sensitivity for compounds present at low concentrations. Information dependent acquisition (IDA) was used to automatically acquire MS/MS data when an MS signal exceeded a threshold of 3000 cps. The collision energy was set to 35 V with a spread of ±15 V.

Marker compounds were tentatively identified by processing MS and MS/MS data acquired using the SCIEX TripleTOF® 5600 system. The system was operated with the DuoSpray™ source. The ESI probe was used for sample analysis and the APCI probe was used to perform automatic mass calibration through the calibrant delivery system (CDS). TOF-MS (100 ms) and TOF-MS/MS (50 ms) acquisition were combined in an IDA method.

Results and Discussion

Chemometric Analysis

Juice samples were analyzed by LC-MS/MS in randomized order to avoid any possible effect of time-dependent changes in chemical fingerprints. Full scan MS chromatograms were processed using PCA and PCVG in MarkerView™ software.

PCA finds combinations of variables that explain the variance present in the data set. For each principal component (PC), every sample has a score, and every variable has a loading that represents its contribution to the combination. It is common practice to plot the scores and loadings for two PCs to visualize results and to identify characteristic marker compounds.

The scores plots for different juice samples analyzed in negative and positive polarity are displayed in Figures 1a and 1b. PCA of the data set revealed three separate clusters of apple, orange, and grapefruit juices samples, showing differences in LC-MS profiles associated with the fruit type. However, it is apparent that more pronounced clustering and significantly better resolution among sample clusters were obtained for positive ionization data. Therefore, only data recorded in positive ionization mode were further used in this study.

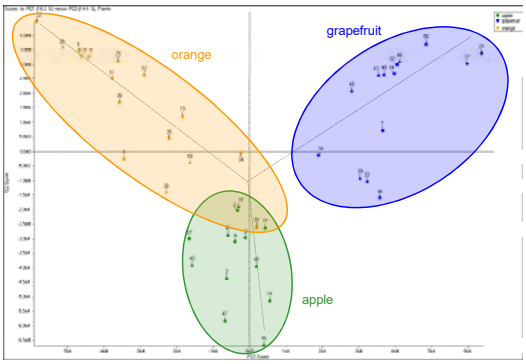

Figure 1a. Scores plot of PCA of apple, orange, and grapefruit juice samples analyzed using negative polarity LC-MS/MS

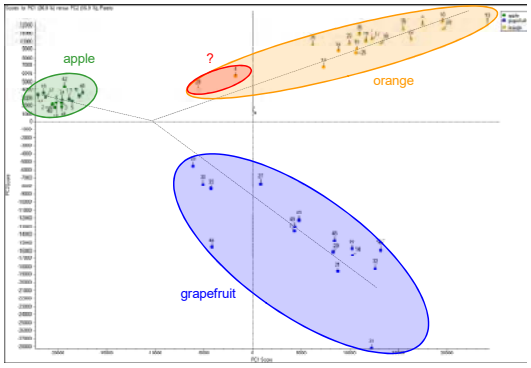

Figure 1b. Scores plot of PCA of apple, orange, and grapefruit juice samples analyzed using positive polarity LC-MS/MS

Note that two orange juice samples (# 4 and # 39) are located slightly separated from the main cluster of all other orange juice samples. This indicates a potential adulteration of these two juice products.

The corresponding loading plot in Figure 2 shows the variables that make the most difference in separating juice samples. It can be used to identify the molecular ion and retention time of characteristic marker compounds.

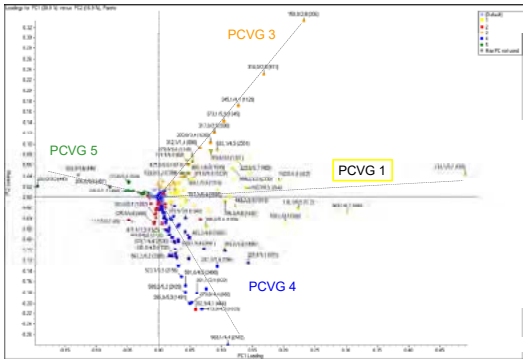

Figure 2. Loadings plot of PCA after PCVG of apple, orange, and grapefruit juice samples analyzed using positive polarity LC-MS/MS showing identified marker ions (m/z, retention time pairs)

Characteristic marker compounds of a group of samples are located in the same area of the loadings plot as the group is located in the scores plot. PCVG was utilized to automatically group variables to facilitate data interpretation. Four characteristic groups of variables were identified to be responsible for clustering of samples representing respective fruit juice types (apple group 5, grapefruit group 4, orange group 3, and all citrus fruits group 1).

Characteristic marker ions (m/z, retention time pairs) can be displayed in profile plots to verify the unique occurrence of marker ions in tested juice samples. Selected marker ions, i.e. 203 at 0.5 min for apple, 603 at 4.4 min for grapefruit, 633 at 4.5 min for orange, and 130 at 0.5 min, 144 at 0.7 min, and 160 at 0.5 min for citrus fruits are shown (Figure 3).

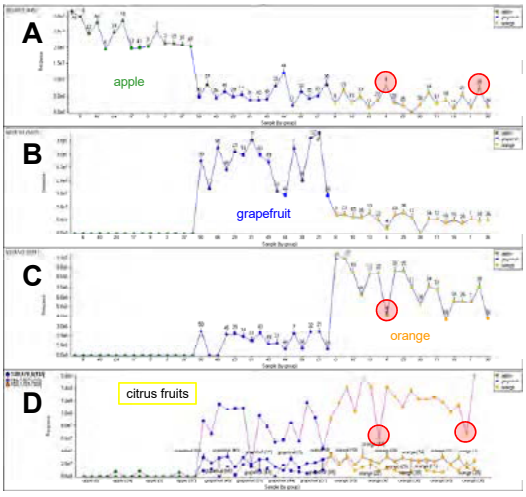

Figure 3. Profile plots of six selected marker ions for A) 203 at 0.5 min for apple, B) 603 at 4.4 min for grapefruit, C) 633 at 4.5 min for orange, and D) 130 at 0.5 min, 144 at 0.7 min, and 160 at 0.5 min for citrus fruits

The suspicious and potentially adulterated orange juice sample show slightly higher levels of the apple juice marker and lower level of characteristic markers for orange and citrus.

Contents

In a next step, the detection of adulterated orange juice was quantified by comparing laboratory prepared mixtures of orange juice with apple juice and grapefruit juice at different adulteration levels (Figures 4a and 4b). The LDA statistical model constructed with the use of statistiXL software (Nedlends, WA, Australia) was able to reliably detect 25% of orange juice adulteration with apple or grapefruit juice. Both recognition and prediction abilities of the model were 100%.[7]

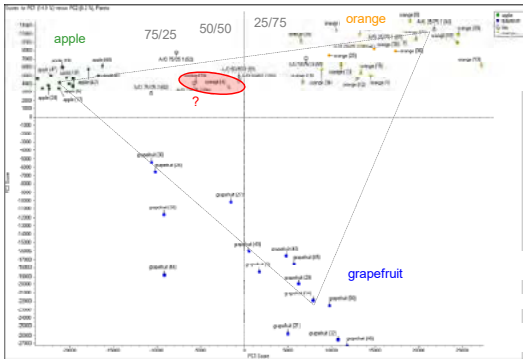

Figure 4a. Scores plot of PCA of apple, orange, and grapefruit juice samples and mixtures of apple and orange juice

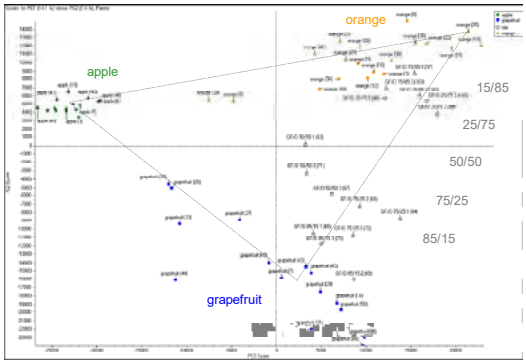

Figure 4b. Scores plot of PCA of apple, orange, and grapefruit juice samples and mixtures of grapefruit and orange juice

The model suggests an adulteration level of the suspicious orange juice samples (# 4 and # 39) of approximately 50% with apple juice (Figure 4a).

A similar experiment was carried out for other types of fruit juices, including apple, blueberry, cranberry, pomegranate, and their mixtures to simulate adulteration.

The loadings plot presented in Figure 5 shows that PCA can separate between these types of juices. However, only one juice sample was available per fruit type. Thus, the data set does not reflect the natural variability of the investigated fruits, but proves that LC-MS/MS with metabolomic processing seems to be applicable to these fruit juice types.

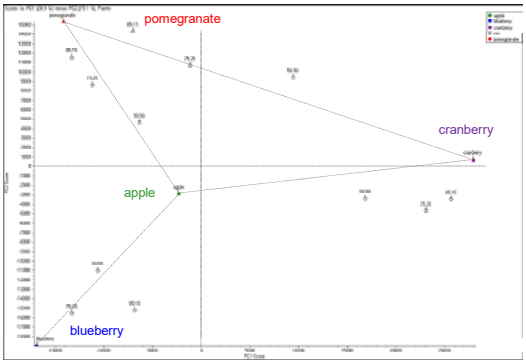

Figure 5. Scores plot of PCA of different juice samples and their mixtures

Tentative Identification of Marker Compounds

The identification of characteristic Marker compounds represents the most laborious and time-consuming step of the metabolomic workflow. Accurate mass MS and MS/MS measurements using the SCIEX TripleTOF® 5600 system were performed and data were processed using PeakView® software (version 1.2) to empirically calculate molecular formulas and to automatically perform online database searching for potential structures.

The formula finder uses high resolution accurate mass information of the molecular ion, adducts, isotopic pattern, and fragment ion information to empirically calculate potential molecular formulas for the detected compound. Furthermore, the calculated formulas are then automatically searched against online databases, like PubChem, Nist, and ChemSpider, to find possible matching structures.

The examples presented in Figures 6a and 6b show the tentative identification of the characteristic marker ions for orange (633 at 4.5 min) and grapefruit (603 at 4.4 min) as the flavones glycosides hesperidin and naringin. In both cases the molecular ion was automatically identified as Na-adduct.

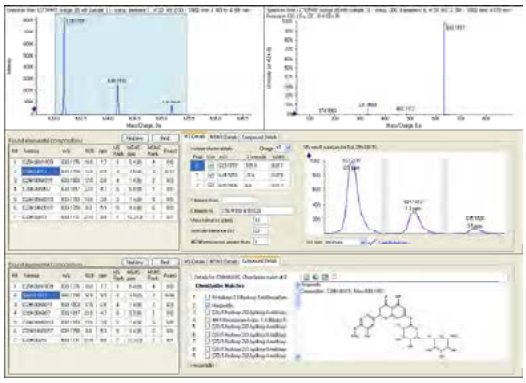

Figure 6a. Tentative identification of a marker ion characteristic for orange (633 at 4.5 min) as hesperidin based on empirical formula finding and automatic online database searching

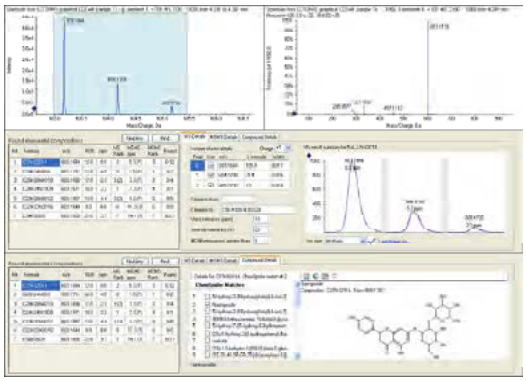

Figure 6b. Tentative identification of a marker ion characteristic for grapefruit (603 at 4.4 min) as naringin (naringoside) based on empirical formula finding and automatic online database searching

PeakView® software also allows comparing structures (imported mol-file obtained from online database search) with accurate mass MS/MS information to further increase confidence in identification.

The characteristic marker ions for citrus fruits (130 at 0.5 min, 144 at 0.7 min, and 160 at 0.5 min) were tentatively identified as N-methylproline, N,N-dimethylproline, and hydroxyl-N,N-dimethylproline. Figure 7 shows screenshots of the fragment prediction tool of PeakView® software. In all cases, 100% of the fragment ion intensity of the MS/MS of the precursor ion 130, 144, and 160 were explained by the structures of N-methylproline, N,N-dimethylproline (proleine betaine), and hydroxyl-N,N-dimethylproline (betonicine), respectively. The presence of betaines in citrus fruits was previously reported by Servillo et al.[8]

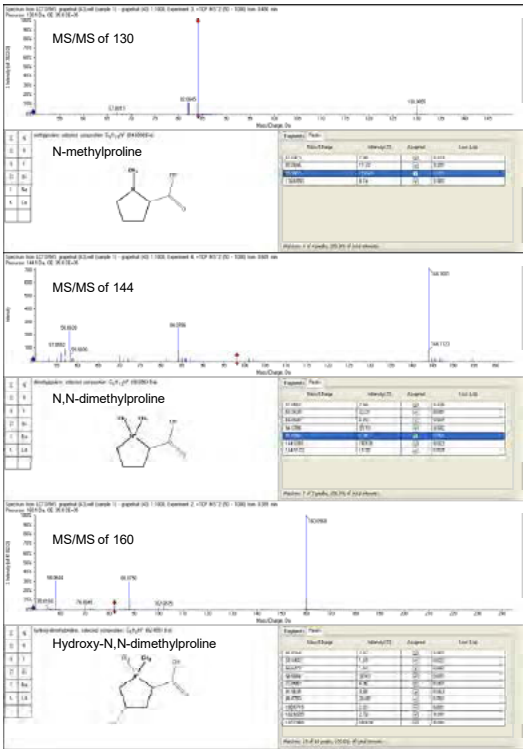

Figure 7. MS/MS fragment ion prediction for N-methylproline, N,N-dimethylproline, hydroxy-N,N-dimethylproline, 100% of MS/MS ions are explainable supporting the tentative identification in citrus fruits

The most characteristic marker compounds for apple juice were tentatively identified as hexose ($C_6H_{12}O_6$) and sugar alcohol ($C_6H_{14}O_6$). However, the existing LC-MS/MS data do not allow discrimination between the isomeric species of sugars and sugar alcohols.

Summary

Comprehensive, non-target LC-MS/MS with metabolomic data processing was demonstrated to be a powerful tool for fruit authenticity assessment. The SCIEX 4000 QTRAP® system was used to collect information-rich full scan data to discriminate different juices using statistical processing with PCA and PCVG. It was possible to reliably detect orange juice adulteration of 25% with apple or grapefruit juice. The feasibility of this approach for authentication of other highly prized fruit juices, such as pomegranate, blueberry or cranberry, was also shown.

Finally, characteristic marker compounds for each juice, pre-selected during PCA, were tentatively identified by processing of accurate mass MS and MS/MS data generated with a SCIEX TripleTOF® 5600 system. The formula finder integrated into PeakView® software automatically evaluates accurate mass information of the molecular ions, the isotopic pattern, adducts, and MS/MS fragment ions. Resulting molecular formulas are automatically searched against online databases to find matching chemical structures.

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Publication number: 6320212-01

Contents

Can LC-MS/MS Be Used in Horse Meat Detection?

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the simultaneous detection of horse meat at low % levels in beef and the banned substance phenylbutazone (BUTE) using peptides markers for horse proteins and specific MRM transitions for BUTE.

Introduction

Following the Food Standards Agency's (FSA) announcement in January that horse and pig DNA had been identified in beef products sold by several supermarket chains, further testing across Europe and beyond has revealed widespread incidences of such contamination.[1] However, most testing methods are based on detection of species-specific DNA in meat, using the polymerase chain reaction (PCR) – which does not detect or identify proteins. This is a concern because DNA can be easily disrupted or removed during standard meat processing and food manufacturing. As a result, horse tissue or other contaminants remain undetected in food samples, despite strong presence of the contaminating proteins. An alternative protein-based method, ELISA (enzyme-linked immunosorbent assay), can be used to complement DNA testing, but this method has limitations, including that it detects only one part of the protein and not multiple protein markers.

The LC-MS/MS-based method presented offers a more accurate and reliable approach to meat speciation than PCR or ELISA-based techniques or other indirect methods, and also allows for the detection of veterinary drug residues in the same analysis, which is not possible by ELISA or PCR.

The method was developed using an Eksigent ekspert™ microLC 200 UHPLC system coupled with a SCIEX QTRAP® 5500 LC/MS/MS system. The method uses multiple reaction monitoring (MRM) to detect peptide markers for horse and is capable of providing sequence information by acquiring an enhanced product ion (EPI) scan for each triggering MRM which can be used to further confirm the peptide's / proteins and therefore the species identity. This gives greater confidence for food testing when distinguishing between species; for example horse and beef proteins may differ by as little as one or two amino acids.

At the same time it is also possible to detect and quantify veterinary drug residues using the same extraction method and LC conditions by simply adding additional MRM transitions to the method. Here the nonsteroidal anti-inflammatory drug (NSAID) BUTE was detected in meat samples.

Method Details

Standards

For the initial development work some of the target proteins were commercially available and therefore purchased as well as commercially available reference materials of pork, beef, and horse meat and beef reference material which had been spiked at different levels with horse meat. A sample of lamb meat was obtained from a local supermarket.

A sigma standard of BUTE was not available at the time of this work so BUTE had to be extracted from a sample of horse medicine.

Sample Preparation

The meat sample was homogenized using a food processor and mixed (2 g) with an extraction buffer containing tris (2-amino-2-hydroxymethyl-propane-1,3-diol), urea and acetonitrile (10 mL). The meat was broken up by shaking, ultra sonication (15 min) and agitated further using a roller mixer (45 min). This mixture

was centrifuged and the top liquid layer (0.5 mL) was transferred to a 2mL Eppendorf tube. The protein markers were reduced in a thermal mixer with a solution of tris (2-carboxyethyl) phosphine (TCEP, 60 min, 60°C), alkylated by adding methyl methanethiosulfonate (MMTS, 30 min, room temperature in the dark) and digested in a thermal mixer by addition of a digestion buffer containing ammonium bicarbonate, calcium chloride and trypsin (60 min, 40°C).

The filtrate was purified using a conventional conditioned polymeric SPE cartridge from Phenomenex. The peptides were extracted from the cartridge using acetonitrile and the extract was evaporated to dryness and reconstituted in acidified aqueous acetonitrile.

LC Separation

All method development and analysis was done using an Eksigent ekspert™ microLC 200 UHPLC system. Final extracted samples (10 µL) were separated over a 11 minute gradient (Table 1) where A = water and B = acetonitrile both containing 0.1 % formic acid. Peptides were separated on a reversed-phase Halo C18 2.7 µm 90Å 50 x 0.5mm (Eksigent) column at 20 µL/min and at a temperature of 40ºC.

Table 1. Gradient conditions used for separation

Time (min)	A (%)	B (%)
0	98	2
2	98	2
6	60	40
7	2	98
8.5	2	98
8.7	98	2
11	98	2

MS/MS Detection

All analyses were performed on a SCIEX 5500 QTRAP® LC/MS/MS system using electrospray ionization (ESI).

Initial method development was carried out using the MIDAS™ workflow (MRM-initiated detection and sequencing, Figure 1) where the electrode was changed to a microLC hybrid electrode (50 µm ID) designed for MicroLC.[2] For MIDAS a set of predicted MRM transitions from the known protein sequence were used as a survey scan to trigger the acquisition of EPI spectra (Figure 2).

This data was then submitted to a database search engine for confirmation of peptide identification and of the feasibility of the MRM transition for meat speciation. With this workflow MRM transitions were designed without the need for synthetic peptides.

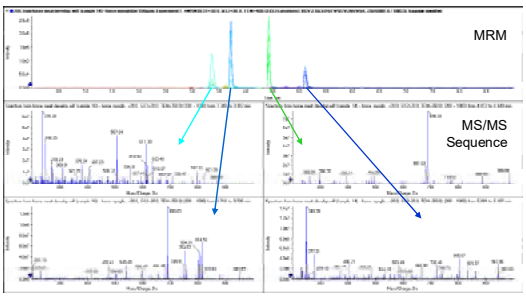

Figure 2. MRM initiated acquisition of MS/MS spectra to sequence characteristic proteins for horse meat

In the final method the Turbo V™ source conditions used were gas 1, gas 2 and the curtain gas set to 30 psi, the temperature of the source was set at 350°C and the IS voltage was 5500 V. The peptides and BUTE were analyzed using the *Scheduled* MRM™ algorithm with an MRM detection window of 50 s and a target scan time of 0.40 s. Q1 resolution was set to low and Q3 resolution was set to unit. A total of 56 MRM transitions were used over the 11 minute run time with 3 dedicated to BUTE, 12

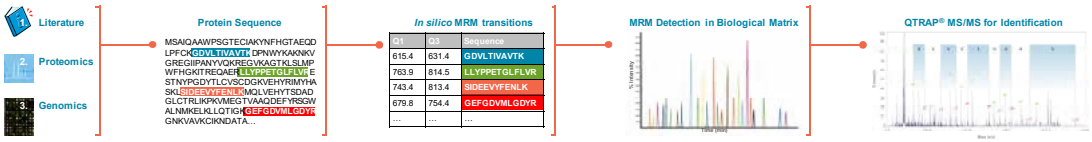

Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

for horse meat (4 peptides with 3 MRM transitions each) and the rest for other meat species peptides currently under evaluation.

The MRM conditions for the detection of BUTE were taken from the MRM catalogue of the iMethod™ application for Veterinary Antibiotic Screening 1.1 (Table 2).[3]

Table 2. MRM transitions for the detection of BUTE, taken from the iMethod™ application for Antibiotic Screening

MRM transition	DP (V)	CE (V)
309/160	120	28
309/120	120	32
309/188	120	22

Results and Discussion

In the method development care was taken to make sure that peptides chosen were unique to the meat species. The list was further consolidated by removing peptides that could be susceptible to modification during food processing, e.g. undergo post translational modification or the Maillard reaction (for future application to processed meat samples). This reduced the number of peptides used as triggers for detection and generation of peptide finger prints of species.

Figure 3 shows a comparison of horse, beef, pork and lamb extracts where 4 unique peptides for horse are shown from a method which contains additional markers for other species which are currently under evaluation. This confirmed the BLAST search results for the specific peptides chosen for horse meat were specific to horse and were not seen in beef, pork and lamb.

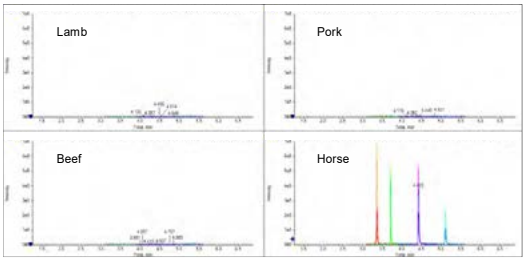

Figure 3. A comparison of the analysis of extracts from different types of meat. These initial results were obtained during the development of the method.

Figure 4 shows the comparison of beef and beef reference material which had been spiked at 10% and at 1% horse (current detection limit for PCR analysis).

In this figure the MRM transitions for 3 of the 4 peptides have been extracted and it shows clearly that horse meat can be detected at a 1% spike level. The fourth peptide was detected at 10% level it was below the LOD limit at 1% horse meat in beef. In order to confirm these results extraction of samples were performed multiple times and in each batch 1% horse meat could be detected in beef.

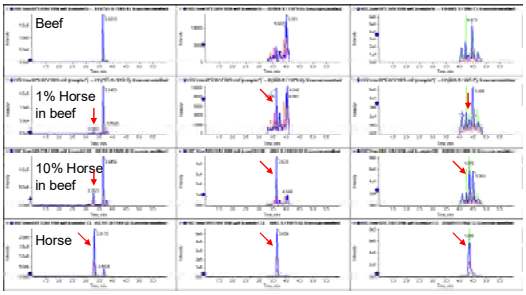

Figure 4. Detection of peptides characteristic for horse meat in beef at different levels, it shows that horse meat can be detected at a 1% level

Figure 5 shows an extracted ion chromatogram for BUTE in a standard, blank and a spiked sample of meat at a level below 10 µg/kg which had been extracted using the same protocol.

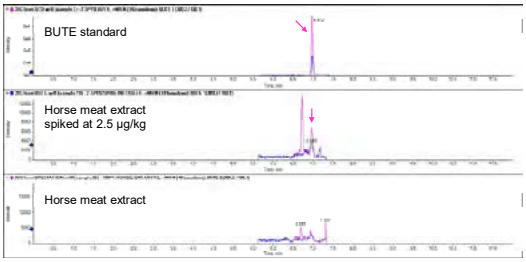

Figure 5. A comparison of the analysis of extracts from different types of meat. These initial results were obtained during the development of the method.

At the time of these initial tests the pure standard was not available so BUTE had been extracted from commercially available horse medicine. Levels in the extract were assumed to be lower than 10 µg/kg and this work is planned to be repeated using spiking experiments with analytical standard grade phenylbutazone. Also as this particular horse meat sample was just for speciation testing, the work will be repeated using beef which should be totally clear of BUTE.

Summary

LC-MS/MS has the potential to offer a rapid, robust, sensitive and specific assay for the simultaneous detection of a series of meat species as well as veterinary drug residues in a single analysis.

Sensitivities achieved were equivalent to sensitivities of some currently available methods based on ELISA and real-time PCR. The LC-MS/MS approach has the additional advantage of being a potential multi species screen unlike ELISA where individual meat species are detected by separate kits. By using the MIDAS™ workflow full scan QTRAP® MS/MS spectra can also be obtained at the same time as quantitative information, confirming multiple peptide target identification and reducing the occurrence of false positives associated with other techniques. Although this test is still qualitative quantitation is likely when internal standards can be used. Unlike PCR or ELISA LC-MS/MS has the ability to detect banned veterinary drug residues as well as meat speciation in the same analysis.

Acknowledgements

The author would like to acknowledge research scientists at the University of Münster (Prof. Dr. Hans-Ulrich Humpf, Dr. Jens Brockmeyer and Christoph von Bargen) who have independently verified these horse meat markers and also for their input in technical discussions which have supported this work.

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Publication number: 7580213-01

Are Pork Extracts Present in My Gummy Bears? Gelatin Speciation by LC-MS/MS

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[1]SCIEX Singapore, [2]SCIEX Warrington (UK)

Overview

The accidental or fraudulent blending of meat and animal products from different species is highly relevant for consumers with ethical concerns against eating species such as horse or pork in particular the Jewish and Muslim communities. In this work, we present the results from the initial development of an LC-MS/MS method utilizing SCIEX TripleTOF® 5600 and 4000 QTRAP® LC/MS/MS systems for the determination of the origin of gelatin used in food products and also pharmaceutical capsules.

Introduction

Following the Food Standards Agency (FSA)'s announcement in January that horse and pig DNA had been identified in beef products sold by several supermarket chains, further testing across Europe and beyond has revealed widespread incidences of such food contamination. This intended adulteration for financial gain or careless false declaration of meat products is a severe problem for consumers who have ethical or religious concerns about the consumption of pork or horse, more specifically the Muslim or Jewish communities who represent about 23 % of the worldwide population. As the tolerance level for porcine and equine content in foods is 0 %, for religious reasons, the limit of detection (LOD) needs to be as low as possible and so the continued development of more sensitive methods is necessary.

However, pork based products are not only used as the meat but can also be found in gelling agents in food (for example in candy, ice cream, and marshmallows) as well as in the cosmetic and pharmaceutical industry in the form of gelatin. Gelatin is made from collagen, a protein, which has been extracted from the skin, bones, and connective tissues of animals such as cows, chicken, pigs, and fish. After extraction the collagen is partially hydrolyzed to form the gelatin which is a mixture of peptides and proteins and is used in the form of sheets, granules or powder.

In the production of gelatin the protein hydrolysis normally occurs with hot water or under acidic conditions. The gelatin so produced is purified and used in food manufacturing and this process again may involve elevated temperatures. Under these conditions species-specific DNA present from the original animal is often denatured or removed making the use of the polymerase chain reaction (PCR), often used in species identification, difficult[1-3] or impossible.[4]

An alternative protein-based method, ELISA (enzyme-linked immunosorbent assay), has also been used for speciation[5] but this approach has limitations, including that it detects only one part of the protein and not multiple protein markers and so can pose a risk of producing false negatives and positives.

So an LC-MS/MS approach, detecting multiple tryptic peptides as markers for confirmation offers a more accurate and reliable approach to gelatin speciation than PCR or ELISA-based techniques. Initial identification of markers was by a shotgun proteomics approach using a high-resolution mass spectrometer, SCIEX TripleTOF® 5600 system, coupled to an Eksigent LC system. The method developed in this work uses the SCIEX 4000 QTRAP® system where multiple reaction monitoring (MRM) was used to detect markers which then automatically trigger the acquisition of enhanced product ion (EPI) scan to provide additional sequence information to further identify the peptides and proteins and therefore the gelatin species.

Contents

Experimental

Sample Preparation

Each sample (5mg) was dissolved in 600 µL of 50 mM ammonium bicarbonate buffer (37°C for 10-15 minutes). This extract was digested using trypsin (50 mM in ammonium bicarbonate buffer) in a trypsin to sample ratio of 1:100. Digestion took place either overnight at 37˚C (10-15 hours) or using a microwave burst technique where the samples are placed in ice and subject to 5 x 30 sec of microwave digestion, between each microwave burst the sample was shaken.

Once digested the samples were spun (12,000 rpm for 5 minutes). The top supernatant layer (500 µL) was removed carefully, not to disturb the bottom sediment, and centrifuged again (12,000 rpm for 5 minutes). The top portion of the supernatant (200 µL) was used for analysis.

LC-MS/MS

Initial identification of species specific peptides, from tryptic digests of porcine and bovine gelatin, was done by a shotgun proteomics approach using the high resolution and accurate mass SCIEX TripleTOF® 5600 system coupled to an Eksigent ekspert™ ultraLC 100-XL system. In these survey experiments a Phenomenex Aeris wide pore column was used for separation of the peptides using a 45 minutes gradient at a flow rate of 250 µL/min. An information dependent acquisition (IDA) method was used to automatically trigger 30 TOF-MS/MS spectra from the information in the TOF-MS survey scan. Principle components analysis within the MarkerView™ software was then used to identify species specific markers (Figures 1 and 2).

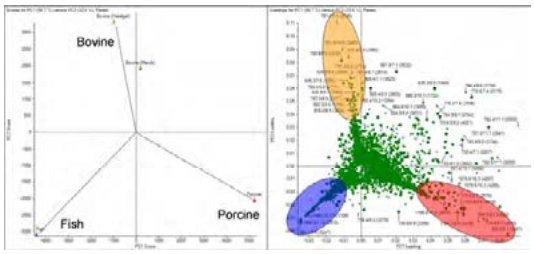

Figure 1. MarkerView™ software was used to identify characteristic markers for gelatin speciation, PCA Scores plot for bovine, porcine and fish (left) and PCA Loadings showing characteristic markers (right)

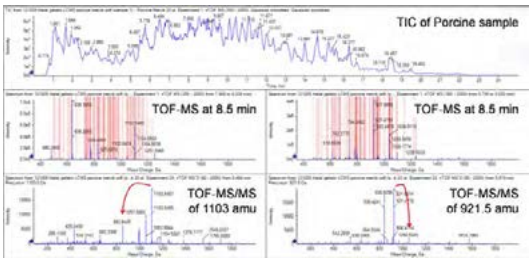

Figure 2. TOF-MS and TOF-MS/MS data of two selected marker compounds for porcine, TripleTOF® data was used to develop the MRM method

From this information seven markers were identified and the method was transferred to a SCIEX 4000 QTRAP® system where MRM transitions for each marker were optimized. In this final screening method samples were separated on a C18 column using the gradient shown in Table 1 where eluent A was water and eluent B was acetonitrile with both mobile phases containing 0.1% formic acid. The flow rate was set to 250 µL/min, column oven temperature to 40°C, and 20 µL of the sample volume was injected.

Table 1. LC gradient conditions used for separation at a flow rate of 250 µL/min

Step	Time	A (%)	B (%)
0	0.0	95.0	5.0
1	2.0	95.0	5.0
2	12.0	60.0	40.0
3	12.5	10.0	90.0
4	13.0	95.0	5.0
5	19.0	95.0	5.0

In the optimized method the Turbo V™ source conditions used were gas 1, gas 2 set at 30 psi and the curtain gas set to 25 psi, the temperature of the source was set at 450°C and IS voltage was 5500V.

MRM conditions for the most intense marker transitions are given in Table 2. In addition 3 qualifier transitions were monitored for each peptide marker. MRM transitions were acquired at a dwell time of 20 msec and were used as IDA triggers to automatically acquire full scan EPI spectra for identification of the gelatin marker.

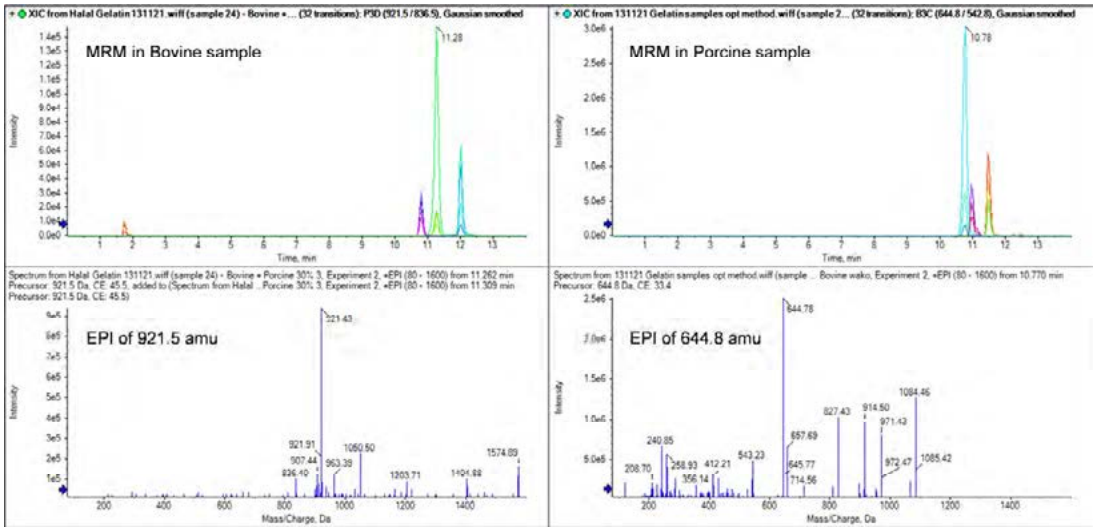

Figure 3. Comparison of a tryptic digest of a porcine (left) and a bovine (right) gelatin standard, MRM transitions were used to automatically acquire full scan EPI spectra for identification of the gelatin marker

Table 2. MRM transitions for the marker peptides of bovine (beef) and porcine (pork) gelatin, DP was set to 100 V for all transitions

Gelatin Marker	Q1 (amu)	Q3 (amu)	CE (V)
Pork gelatin 1	1103	850.9	57.5
Pork gelatin 2	486.2	786.4	26.4
Pork gelatin 3	921.5	1050.6	49.4
Pork gelatin 4	620.8	618.3	28.3
Beef gelatin 1	659.3	766.5	34
Beef gelatin 2	781.4	991.6	42.3
Beef gelatin 3	644.8	971.5	37.3

Results and Discussion

When burst microwave digestion was compared to the traditional overnight trypsin digestion results were identical, as this method was quicker this approach was used for this study. Alkylation and reduction of the proteins was also not necessary as the disulfide bridges and the secondary structure of the collagen had already been broken during extraction and purification of the gelatin. Trypsin extracts produced from beef and pork samples using this method were compared (Figure 3) and marker peptides which have different sequences and generated different MRM and fragmentation patterns could be produced.

Beef gelatin was then spiked with pork gelatin so that levels of contamination of beef with pork gelatin could be determined and a 1% contamination of bovine gelatin with porcine gelatin could be easily identified (Figure 4).

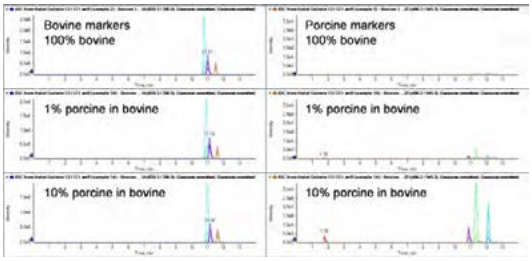

Figure 4. Results of spiking pork gelatin into beef gelatin, porcine markers were easily detected at 1% contamination

The method was then tested on extracts of gummy bears, fruit and chocolate candies as well as pharmaceutical capsules used for drug delivery and examples are shown in Figure 5. Here pork gelatin was detected but with no trace of bovine gelatin seen in the sweets and in the capsules only bovine gelatin was detected.

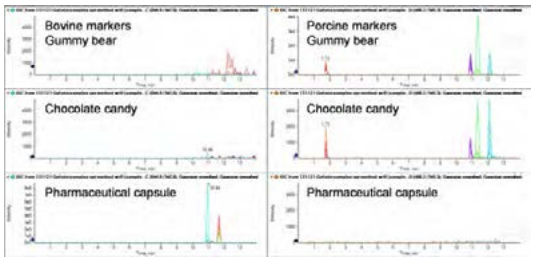

Figure 5. Results from the analysis of gummy bears, candy and pharmaceutical capsule for the presence of bovine (left) and porcine (right) markers. These examples show the presence of pork gelatin in gummy bear and chocolate candy. In the pharmaceutical capsule only bovine gelatin was detected.

Summary

In this study, we have identified 7 markers which are either specific for pork or beef gelatin and highlight the use of LC-MS/MS for gelatin speciation. These first results have shown that the gelatin ingredient can be extracted and analyzed in less than 1 hour and a 1% impurity of pork in beef gelatin can be detected. Further to this, this method can be used to detect the presence of pork gelatin in processed food such as sweets and also the animal source gelatin used in pharmaceutical capsules and offers multiple points of identification previously not available by ELISA analysis

In the future lower detection limits will be possible with the use of microLC[6] and more sensitive LC-MS/MS systems which mean that gelatin speciation at even lower levels is possible. This will help alleviate ethical concerns of the source of gelatin used in food manufacturing and pharmaceutical capsules used to deliver drugs.

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Publication number: 09270214-01

Fast, Robust and Reliable Method for the Identification and Quantitation of Sildenafil Residue in Honey using LC-MS/MS

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Overview

In recent years, natural products and herbal medicines are increasing in popularity all over the world. However, adulteration of natural products with synthetic adulterants is a serious concern and can impose deleterious health issues in human. Many reports suggest the adulteration of Sildenafil in honey based products with the intention of boosting the effects of products. A fast, robust and reliable LC-MS/MS method has been developed to identify, quantify and confirm traces of Sildenafil in honey samples using the SCIEX 4000 QTRAP® system. The method presented here can be routinely employed to screen for sildenafil in raw honey and herbal drug preparations.

Introduction

Honey is one of the precious food commodities from ancient times and there is high market demand for natural honey. According to the European Union (EU); international food standards Codex Alimentarius and other international honey standards - honey stipulates a pure product that does not allow for the addition of any other substance. Sildenafil citrate marketed as Viagra, a medicine for treatment of erectile dysfunction is very often added to honey based products and marketed to public in order to increase the popularity of these products. Adverse effects of Sildenafil especially cardiovascular risk are still under controversy. This highlights the need for herbal medicine manufactures to utilize a fast, reliable and unambiguous method to detect the low levels of Sildenafil residues in honey samples.

We developed an LC-MS/MS method using SCIEX 4000 QTRAP® system operated in Multiple Reaction Monitoring (MRM) mode to identify and quantify Sildenafil in honey with high selectivity and sensitivity.

The developed method was validated in-house as per European Commission Decision 2002/657/EC. Specificity, limit of detection and quantitation (LOD and LOQ), linear dynamic range, accuracy, repeatability, and limit of decision and detection capability (CCα and CCβ) were evaluated.

Multiple MRM transitions were monitored to use the ratio of quantifier and qualifier transition for identification of Sildenafil in samples. In addition, MRM-triggered MS/MS using the Enhanced Product Ion (EPI) mode was utilized to gain additional confidence in identification of positive findings. MS/MS spectra were acquired fully automatic using the logic provided by Information Dependent Acquisition (IDA), Dynamic Background Subtraction (DBS), and Dynamic Fill Time (DFT).

Full scan MS/MS spectra were interpreted using PeakView® software version 2.0, searched against mass spectral libraries using MasterView™ software version 1.1. Quantitative data were evaluated in MultiQuant™ software version 3.0.

Experimental

Chemicals and Honey Samples

Sildenafil Citrate certified reference material (CRM) was purchased from Sigma Aldrich. MS grade methanol was procured from J.T. Baker and formic acid from Fluka.

Honey samples were procured from the local markets of Punjab and Delhi, and were kept under at room temperature until completion of analysis.

Sample Preparation

Approximately 1 g of homogenized honey was weighed and fortified with 50 µL of the Sildenafil working standard to obtain

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dilutions of 0.1 to 1000 ng/mL. Spiked samples were extracted with 20 mL of methanol/water (80:20), vortexed to get homogenized mixture and sonicated for 5 minutes to achieve maximum extraction efficiency. Samples were centrifuged at 4000 rpm; the supernatant was collected and filtered through 0.45 µm filter. Filtered aliquots were transferred into the autosampler vials for LC-MS/MS analysis.

LC Separation

LC separation was achieved with a reverse phase C18 ACQUITY UPLC BEH column having particle size of 1.7 µm. Isocratic elution was employed over a short runtime of 4.5 min with an aqueous phase of 10 mM ammonium formate in water and an organic phase of methanol with addition 0.1% of formic acid at a ratio of 80:20. Optimized flow rate of 0.2 mL/min with column temperature maintained at 42°C was used for separation.

The injection volume was set to 20 µL.

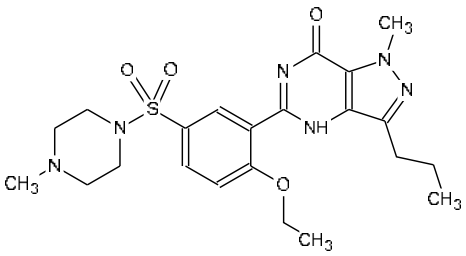

Figure 1. Chemical structure of sildenafil

MS/MS Detection

A SCIEX 4000 QTRAP® system equipped with Turbo V™ source was used, in positive Electrospray Ionization (ESI) mode. The presented method uses three MRM transitions: 475/100 as quantifier transition and 475/283 475/311 as qualifier transition.

Table 1. MRM transitions MS/MS Parameters for Sildenafil

Analyte	MRM transition	DP	CE
Sildenafil 1	475 / 100	40	54
Sildenafil 2	475 / 283	40	44
Sildenafil 3	475 / 311	40	44

Table 1 summarizes optimized compound dependent parameters such as Declustering Potential (DP) and Collision Energy (CE).

IDA was used to acquire MRM-triggered MS/MS spectra which aids in compound identification. CE was set to 35 V with a Collision Energy Spread (CES) of 15 V

Results and Discussion

A representative chromatogram of quantifier and qualifier MRM transitions is shown in Figure 2. MRM ratios were calculated automatically in MultiQuant™ software. The average MRM ratio of all standard injections with tolerance matching 2002/657/EC is displayed in the peak review window.

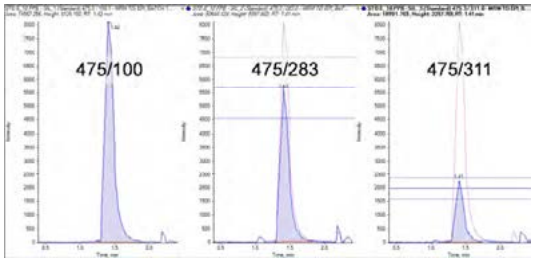

Figure 2. MRM transitions of Sildenafil at a concentration of 10 ng/mL, the MRM ratio was automatically calculated in MultiQuant™ software (Sildenafil 2: 0.686 ± 20%, Sildenafil 3: 0.210 ± 25%)

Lowest injected concentration of 0.1 ng/ml showed a signal-to-noise ratio (S/N) of 18.9 and was considered as the LOD whereas the LOQ was established at 0.5 ng/mL (Figure 3).

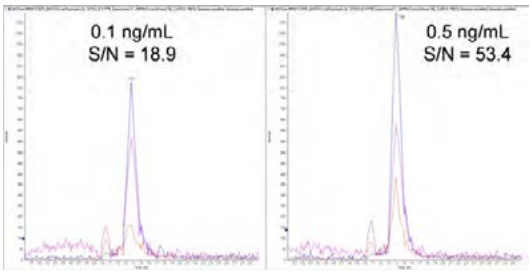

Figure 3. S/N for Sildenafil at the LOD (0.1 ng/mL) and LOQ (0.5 ng/mL)

Calibration lines were generated by using matrix matched calibration standards spiked within the linearity range of the 0.5 to 1000 ng/mL. Matrix matched calibration lines were found to be linear with correlation coefficient (r) of 0.995 or higher as shown in Figure 4.

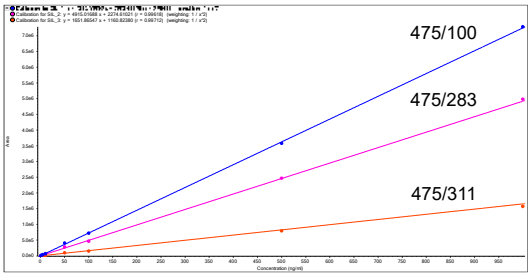

Figure 4. Calibration curve of Sildenafil from 0.5 to 1000 ng/mL with a regression > 0.995 for all three MRM transitions

Accuracy (% recovery) and precision (repeatability) were evaluated at four concentration levels of the LOQ (0.5 ng/mL), 2xLOQ, 5xLOQ and 10xLOQ with repeat injections (n=6). The mean recovery at the four levels was obtained above 85% as shown in Table 2.

Table 2. Accuracy data based on mean % recovery of 6 replicate injections at four different concentration levels

Analyte	Concentration (ng/mL)	Accuracy (%)
Sildenafil 1	0.5 (LOQ)	113.4
	1.0	89.6
	2.5	95.7
	5.0	94.2
Sildenafil 2	0.5 (LOQ)	117.6
	1.0	85.5
	2.5	94.5
	5.0	96.1
Sildenafil 3	0.5 (LOQ)	105.3
	1.0	109.9
	2.5	92.6
	5.0	105.8

Repeatability (precision) is defined in terms of the coefficient of variation (%CV). Repeatability of the method was determined using an independently spiked honey matrix at four different levels. In one day the set of four levels with six repetitions was measured to determine intra-day %CV. Two additional sets at same concentration levels with six repetitions were measured over the next two days for the determination of inter-day repeatability. Precision results were found satisfactory at all four levels of concentrations with %CV well below 15%. The results of intra-day and inter-day precision are summarized in Table 3.

Table 3. Repeatability data obtained by injecting 6 replicates over period of 3 days at four different concentration levels

Concentration (ng/mL)	Intra-day %CV	Inter-day %CV
0.5 (LOQ)	10.3	13.0
1.0	8.6	8.8
2.5	5.4	3.2
5.0	6.2	4.7

Both CCα (decision limit) and CCβ (detection capability) were determined following commission decision 2002/657/EC.

CCα was established by analyzing blank honey (n=60) at a level of 0.36 µg/kg. CCβ was established by analyzing blank honey spiked at 0.36 µg/kg (n=60) at a level of 0.44 µg/kg.

The developed method was subjected for screening and quantification of locally procured honey samples. Out of 15 samples analyzed, only one sample showed the traces of sildenafil and was quantified ~ 1.0 µg/kg.

Identification using QTRAP® Full Scan MS/MS Spectra and Mass Spectral Library Searching

Full scan MS/MS spectra were acquired for additional confidence in compound identification. During the method development step MS/MS spectra of Sildenafil were processed using the fragment prediction tool in PeakView® software (Figure 5). A tentative interpretation of the fragmentation pathway is shown in Figure 6.

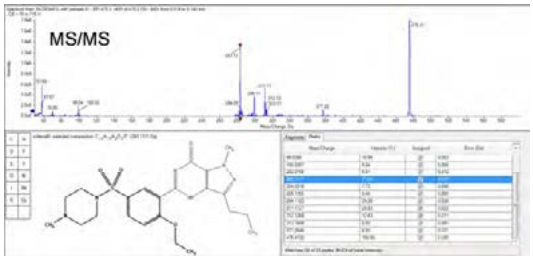

Figure 5. Automatic interpretation of the fragmentation pathway of Sildenafil in PeakView® software (CE ramp from 10 to110 V)

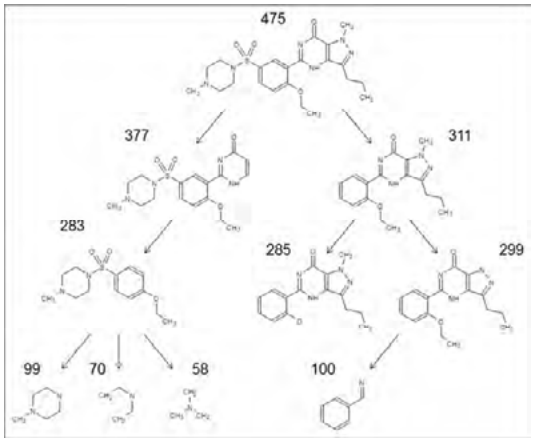

Figure 6. Tentative interpretation of the fragmentation pathway of Sildenafil based on QTRAP® full scan MS/MS data, proposed structures do not include the location of the charge and positions for double bond formation after H removal

An MS/MS spectrum of Sildenafil using the standardized settings for CE and CES was added to a mass spectral library.

Matrix spikes from 0.5 to 1000 µg/kg were analyzed using the MRM-triggered MS/MS approach. Confident identification of Sildenafil was achieved based on retention time matching and MS/MS library searching in MasterView™ software (Figure 8).

The retention error was well below 2.5% and the library search Purity above 95% with the exception of the 0.5 and 1000 µg/kg matrix spike. The MS/MS intensity at the lowest concentration was too low for library searching and some space charge was observed at the highest spiking level resulting in a change of the ion ratios and a lower Purity score of 74%.

Figure 8. Automatic interpretation of the fragmentation pathway of Sildenafil in PeakView® software (CE ramp from 10 to110 V)

Summary

The method and data presented here showcase the fast, simple, and accurate solutions for the analysis of sildenafil in honey using the SCIEX 4000 QTRAP® system. The sensitivity and selectivity of LC-MS/MS allows minimal sample preparation and high throughput.

The method was validated as per European Commission Decision 2002/675/EC for a quantitative method. The decision limit (CCα) and detection capability (CCβ) was established at 0.36 µg/kg and 0.44 µg/kg, respectively. The linear dynamic range for quantitation was over 3 orders of magnitude for all 3 MRM transitions monitored. Confident compound identification was achieved by retention time matching, MRM ratio calculation and QTRAP® MS/MS library search.

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Publication number: 10880214-01

Simultaneous Analysis of 10 Mycotoxins in Crude Extracts of Different Types of Grains by LC-MS/MS

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Overview

This application note presents a validated LC-MS/MS method to detect 9 Fusarium toxins and Ochratoxin A in diluted crude extracts of grain. The developed sample preparation procedure is quick, easy, robust, and inexpensive. The LC-MS/MS method in Multiple Reaction Monitoring (MRM) detects all compounds in a single run with Limits of Quantitation (LOQ) between 0.3 µg/kg and 10 µg/kg. The complete method was validated for the analysis of wheat, rye, barley, and oat samples.

Introduction

Mycotoxins are known to harm the health of humans and animals. They are known either as carcinogenic or cytotoxic and impair the immune system. Therefore, different countries have set regulations on mycotoxins. In the EU, mycotoxin limits are harmonized in the regulation for contaminants in foodstuffs (EC 1881/2006 of December 19, 2006) and the amended regulation (EC 1126/2007 of September 28, 2007). The focus of the legislation and the European monitoring program is on Fusarium toxins like Deoxynivalenol, Zearalenon, HT-2 and T-2 toxins because of their frequent and increasing occurrence in grain. In addition, the European Commission has decided to set maximum levels for T-2 and HT-2 toxin by July 1, 2008. The expected limit for the sum of both Fusarium toxins could be 50 µg/kg or less.[1-3]

Cereals and grains are often contaminated with Ochratoxin A and Fusarium toxins. Fusarium toxins can be found in all types of grains such as wheat, rye, maize, barley (malt), and oats. The contamination is dependent on climate conditions during growth, harvest, and storage. Because of bad weather conditions in 2007 high contaminations of Deoxynivalenol (DON) were found in wheat and T-2 and HT-2 toxin in wheat and oats.

Due to this, it is necessary to have a reliable, sensitive, robust, and fast method to analyze a high number of mycotoxins in grain. Methods used so far have not shown the required sensitivity. Thus necessary clean up steps with immunoaffinity columns have resulted in time consuming and expensive methods.

A method for the detection of 9 Fusarium toxins: DON, Zearalenon (ZON), 3-Acetyldeoxynivalenol (3- AcDON), 15-Acetyldeoxynivalenol (15-AcDON), HT-2, T-2, Fusarenon X (FUS X), Nivalenol (NIV), Diacetoxyscirpenol (DAS) and Ochratoxin A (OTA) was developed (Figure 1). Diluted crude extract were analyzed using Liquid Chromatography and tandem Mass Spectrometry (LC-MS/MS) in a single run on an API 4000™ LC/MS/MS system. No time consuming and possibly expensive sample preparation is needed. The method was validated for wheat, rye, barley and oat and applied for the analysis approximately 220 grain samples. The LOQ vary between 0.3 µg/kg and 10 µg/kg depending on the compound.

Contents

Method Details

Sample Preparation:

1) Mill and homogenize 10 g of grain sample.
2) Add 40 mL of acetonitrile/water (84/16).
3) Extract by mixing for 90 min (220 rpm).
4) Filter through Whatman S&S 1573 ½.
5) Dilute filtrate 1:10 with water + 5mM ammonium acetate.
6) Inject 100 µL into LC/MS/MS.

HPLC Conditions:

A Shimadzu Prominence LC system consisting of system controller, two pumps, degasser, autosampler, and column oven was used. Separation was performed on an Agilent ZORBAX Eclipse XDB C18, 100x4.6 mm (1.8 µm) column. The column oven temperature was set to 40°C. A gradient of eluent A: water + 5 mM ammonium acetate and eluent B: methanol + 5 mM ammonium acetate was used at a flow rate of 500 µL/min. Details of the gradient are given in Table 1. The injection volume was set to 100 µL.

MS/MS Conditions:

An API 4000™ LC/MS/MS system equipped with Turbo V™ source and Electrospray Ionization (ESI) probe was used. The method contained three periods with alternating polarities (0.0-7.6 min negative; 7.6-9.2 min positive; 9.2-16.0 min negative).

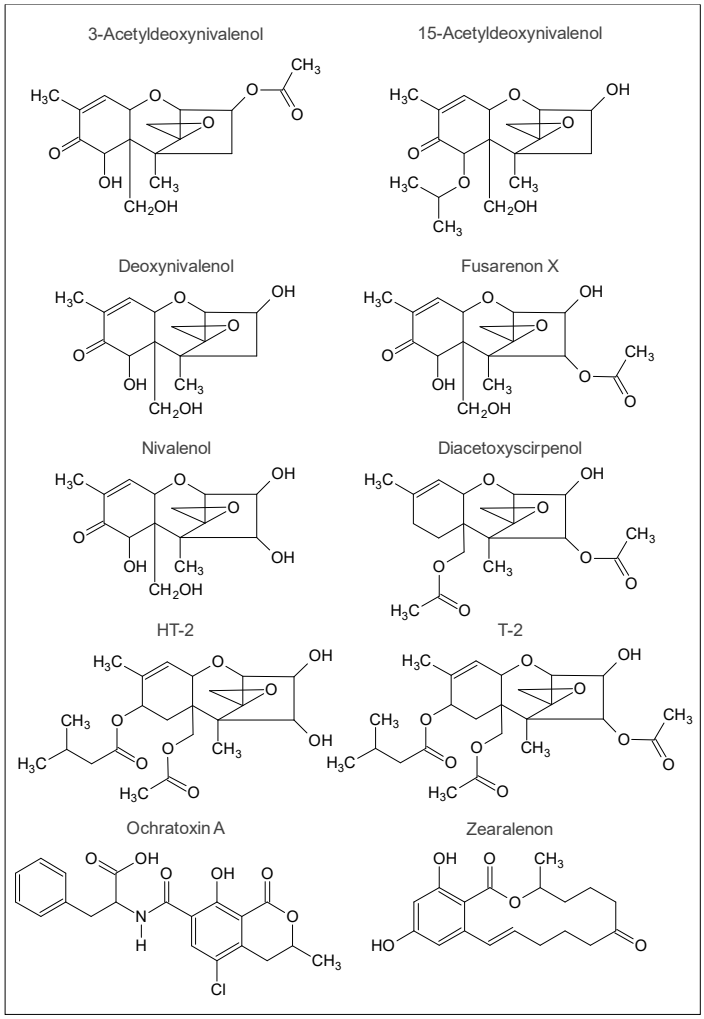

Figure 1. Priority mycotoxins analyzed by LC-MS/MS

The following ion source parameters were used: Temperature 600°C, Curtain Gas 25 psi, Gas1 50 psi, Gas2 70 psi, CAD Gas 6 (positive) or 10 (negative), and IS voltage +5000 V or -4000 V, respectively. All mycotoxins were detected using two MRM transitions in the following order: first period NIV, DON, FUS X, AcDON; second period DAS, OTA, HT-2, T-2; and third period ZON. Since 3-AcDON and 15-AcDON were not separated chromatographically, only compound specific transitions were used for detection. The used MRM transitions are listed in Table 2 and product ion spectra of 3-AcDON and 15-AcDON are shown in Figure 2.

Table 1. LC gradient to separate mycotoxins

Step	Time (min)	A (%)	B (%)
0	0.0	80	20
1	0.5	80	20
2	5.5	10	90
3	15	10	90
4	16	80	20

Table 2. MRM transitions to detect mycotoxins

Mycotoxin	Precursor Ion	MRM 1	MRM 2
3-AcDON	$[M-H]^-$	337/307	337/173
15-AcDON	$[M-H]^-$	337/219	337/150
DON	$[M-H]^-$	295/265	295/138
FUS X	$[M+CH_3COOH]^-$	413/353	413/263
NIV	$M+CH_3COOH]^-/[M-H]^-$	371/281	311/281
DAS	$M+H]^+$	384/307	384/105
OTA	$M+H]^+$	404/239	404/358
HT-2	$M+Na]^+$	447/345	447/285
T-2	$M+NH_4]^+$	484/215	484/185
ZON	$[M-H]^-$	317/131	317/175
3-AcDON	$[M-H]^-$	337/307	337/173

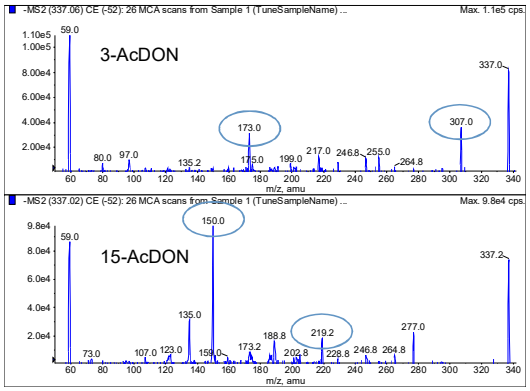

Figure 2. Product ion spectra of 3-AcDON and 15-AcDON (the in MRM detected product ions are highlighted)

Results and Discussion

Figure 3 shows a standard chromatogram of 9 Fusarium toxins at 50 µg/kg and Ochratoxin A at 10 µg/kg. During the evaluation it was shown that the sensitivity of the MRM transitions depends on the quality of the used solvents as well as on the analyzed matrices.

AcDON, DON, FUS X and NIV generally show good sensitivity for both the $[M+CH_3COO]^-$ and the $[M-H]^-$. However, in matrix samples the in Table 2 listed MRM transitions were used for better S/N, reproducibility, and recovery.

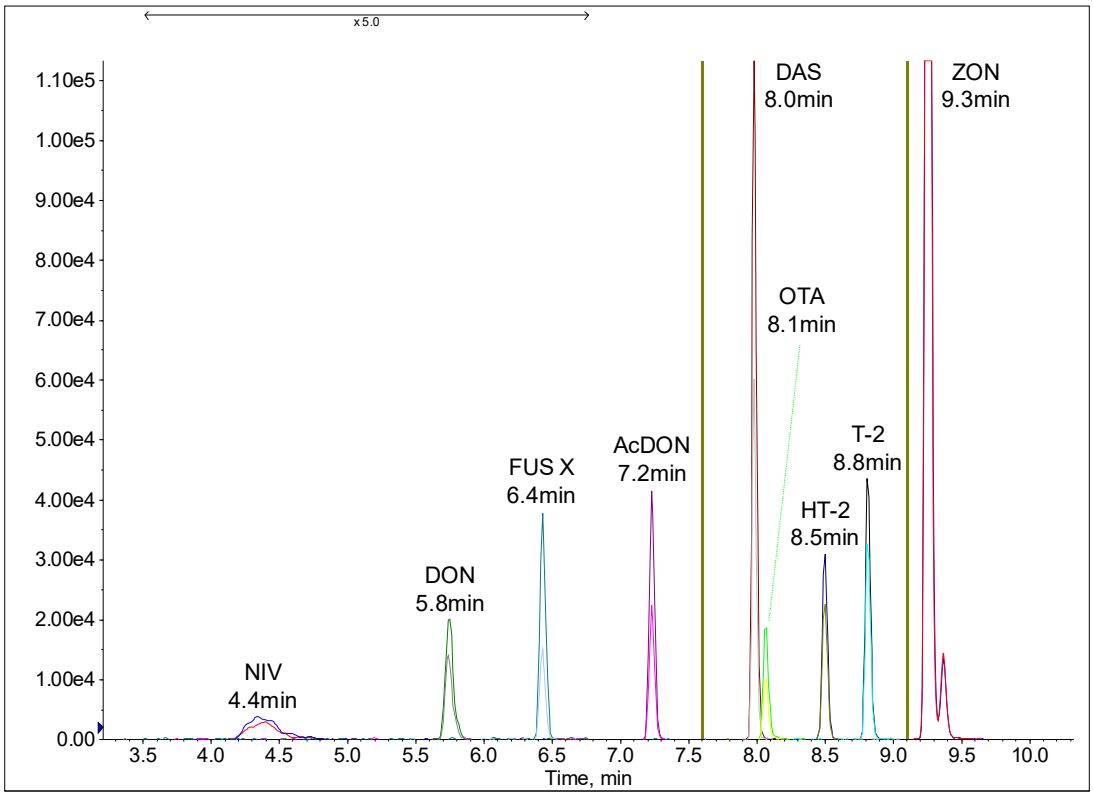

Figure 3. Mycotoxin standard analyzed using LC-MS/MS in MRM

A special characteristic of ionization was found for HT-2. The sensitivity of the MRMs either $[M+NH_4]^+$ or $[M+Na]^+$ differ in accordance to the analyzed matrices (Figure 4).

The LOQ were validated as 0.3 µg/kg for OTA, 5 µg/kg for HT-2, T-2 and ZON, and as 10µg/kg for AcDON, DON, FUS X, DAS and NIV.

The injection volume of 100 µL with a ten times diluted sample showed much better LOQ than 25 µL of a direct injected or 50 µL of a 1/5 diluted sample. Responsible for this finding are the specific initial chromatographic conditions needed for NIV. The acetonitrile in the sample, at the end of the extraction procedure, caused peak broadening for NIV. This could only be eliminated by diluting the sample in 100% water +5 mM ammonium acetate by a factor of 10.

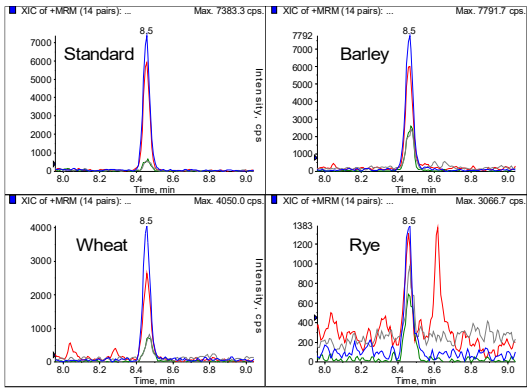

Figure 4. Comparison of MRM intensities of HT-2 in different matrices ($[M+Na]^+$: 447/345 blue, 447/285 red, $[M+NH_4]^+$: 442/263 green, 442/105 grey)

Table 3. Limits of Quantitation (LOQ) and linear dynamic range of detected mycotoxins

Mycotoxin	LOQ (µg/kg)	Linear Range (µg/kg)	EU MRL#
3-AcDON	10	400	(1)
15-AcDON	10	150	(1)
DON	10	10000	1750*; 1250** (2)
FUS X	10	2000	(1)
NIV	10	4000	(1)
DAS	10	400	(1)
OTA	0.3	> 10	5***
HT-2	5	200	(2)
T-2	5	1000	(2)
ZON	5	80	100*** (2)

Footnotes to Table 3:

\# EC 1881/2006 and the amended EC 1126/2007

* Unprocessed durum wheat and oats

** Unprocessed cereals other than durum wheat and oats

*** Unprocessed cereals

(1) Due to co-occurrences and as “generally low” considered levels no MRL was estimated

(2) Appropriateness of setting a maximum level should be considered by 1 July 2008

The calibration curves of all compounds were linear ranges differ highly. LOQ and upper ends of the linear dynamic range of all detected mycotoxins are shown in Table 3.

The recoveries were determined for ach mycotoxin in each matrix compared to the calibration curves without matrix (Table 4-6).

It was shown that the solutions of the extracted grains are stable over 36 hours under cool conditions (4°C).

A large carryover of OTA in the injection port was observed when injecting high standard concentrations, thus solvent blanks were injected after standard injections.

With the here presented validated method about 220 grain samples of the new harvest have been analyzed since July 2007.

The presented data are based on the European Grain Monitoring Program (EGM) and selected data are shown in Tables 4 to 6.

Table 4. Results in wheat / durum

Mycotoxin	n	LOQ (µg/kg)	Below LOQ	Above LOQ	Above EU limit	Recovery (%)
DON	175	10	33	13	66	100
ZON	169	5	138	29	2	60
NIV	168	10	101	67	-	100
T-2	168	5	166	2	-	85
HT-2	163	5	130	33	-	25
OTA	165	0.3	159	6	0	90

Table 5. Results in barley

Mycotoxin	n	LOQ (µg/kg)	Below LOQ	Above LOQ	Above EU limit	Recovery (%)
DON	21	10	5	16	0	82
ZON	20	5	16	4	0	40
NIV	21	10	7	14	-	100
T-2	21	5	10	11	-	100
HT-2	21	5	4	17	-	21
OTA	22	0.3	2	0	0	93

Table 6. Results in rye

Mycotoxin	n	LOQ (µg/kg)	Below LOQ	Above LOQ	Above EU limit	Recovery (%)
DON	25	10	12	12	1	98
ZON	25	5	22	2	1	45
NIV	24	10	22	2	-	100
T-2	24	5	24	0	-	88
HT-2	25	5	24	1	-	85
OTA	25	0.3	23	2	0	100

Summary

The developed method is appropriate for the analysis of 9 Fusarium toxins and OTA in one single LC-MS/MS run without time consuming sample preparation/enrichment. The LOQ were found at 0.3 µg/kg for OTA, 5 µg/kg for HT-2, T-2 and ZON and at 10 µg/kg for AcDON, DON, FUS X, DAS and NIV and meet the National and European law required detection limits. Recoveries were determined in the range of 21 to 100%.

References

1 EC 1881/2006 of December 19, 2006
2 Amended regulation EC 1126/2007 of September 28, 2007
3 http://www.mykotoxin.de/Gesetzgebung.htm

Publication number: 1121410-01

The Quantitation of Mycotoxins in Cereals Using a Simple Sample Extraction and LC-MS/MS with Fast Polarity Switching and the *Scheduled* MRM™ Algorithm

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the detection of several major classes of known toxic mycotoxins. The method uses a simple solvent extraction followed by a dilution and injection of extracts to achieve detection of mycotoxins below the regulatory requirements. Fast polarity switching and the *Scheduled* MRM™ algorithm were used with the SCIEX Triple Quad™ 5500 system to cover all mycotoxins of interest and to detect them with the best sensitivity, accuracy, and reproducibility.

Introduction

Mycotoxins are produced by several strains of fungi both in the field, during storage, mixing and delivery of grain, human and animal food. Mycotoxins are known to be toxic and harm humans and animals as they are carcinogenic or otherwise cytotoxic and impair the immune system. Mycotoxins fall into several major classes and those which can affect the health of humans or animals include the aflatoxins, ochratoxins, Fusarium toxins, including fumonisins, zearalenone (ZON), trichothecenes, and ergot alkaloids.[1]

Regulations for mycotoxin contamination for some of the major classes have been set in different countries. In the European Union the mycotoxin limits were harmonized in the regulation for contaminants in foodstuffs[2,3] and amended by regulations in September 2007.[4] Traditionally mycotoxin analyses have been carried out using multiple methods, each method just suitable for one single mycotoxin or a group of chemically similar compounds e.g. aflatoxins.[5] This has been due to the wide range of polarities and physical properties of these compounds. These single mycotoxin methods include two new analytical methods for measuring aflatoxin B1 (AFB1) and ZON in baby food which were adopted as European benchmark methods in July 2010.[6] Both methods are based on an immuno-affinity column cleanup of the sample followed by HPLC with fluorescence detection. However, it is possible that many different classes of mycotoxins could be present in the same sample of food or feed[7,8] and not just AFB1 and ZON.

In this work we show the ability to analyze AFB1 and ZON at comparable detection levels, to the benchmark methods, as well as implementing these two mycotoxins into an LC-MS/MS screening method. For these measurements the SCIEX Triple Quad™ 5500 system was used (Figure 1). In one single LC-MS/MS run of 13 minutes 17 compounds were detected; 12 of them in the positive ionization mode and 5 of them in the negative ionization mode. The crude extracts of different foods were diluted and injected without any extensive sample clean up or concentration steps. Detection limits of AFB1 and ZON were found to be comparable to the required values set by EN standards[6] and reproducibility was found to be better than 20% without the use of any internal standards. The method itself incorporates fast polarity switching using the *Scheduled* MRM™ algorithm, unlike previous work[9], and expands on the previous number of toxins detected.

Experimental

Sample Preparation

A very simplified sample preparation was used[10] similar to one that has been developed by SGS GmbH (Hamburg, Germany).[9] Homogenized sample (2 g) was mixed with acetonitrile/water (8 mL, 80/20) and roller mixed for 20 minutes. The sample was centrifuged for 10 minutes at 3500 rpm and filtered using a Phenomenex PHENEX filter (15 mm RC Membrane 0.45 µm). The filtrate was then diluted 1:4 with water containing 5 mM ammonium acetate prior to injection.

LC

Samples were injected onto a Shimadzu Nexera UHPLC system containing LC-30AD pumps, SIL-30AC autosampler and a CTO-20A column oven. The column used for the separation was a Phenomenex Kinetex 2.6 µm XB-C18 (50x2.1 mm) column and was run at a flow rate of 450 µL/min and at a temperature of 40°C using a gradient of water to methanol with both phases containing 5 mM ammonium acetate and 0.5% acetic acid (see Table 1 for the gradient profile). An injection volume of 30 µL was used.

Table 1. Gradient profile for mycotoxin analysis

Time (min)	Flow (µL/min)	A (%)	B (%)
0	450	98	2
2	450	98	2
5	450	20	80
5.2	450	2	98
8	450	2	98

MS/MS

A SCIEX Triple Quad™ 5500 LC/MS/MS System equipped with the Turbo V™ source and Electrospray Ionization (ESI) probe was used for MS/MS detection using the selective and sensitive Multiple Reaction Monitoring (MRM) mode.

Table 2 shows the MRM transitions corresponding to the analyzed compounds in a negative-positive switching method using the *Scheduled* MRM™ algorithm.

The following MS/MS parameters were kept constant during the whole acquisition: TEM: 550ºC; CUR: 25 psi; Gas 1: 60 psi; Gas 2: 70 psi; CAD: medium; IS (negative polarity): -4000V; IS (positive polarity): +5000V.

Figure 1. The SCIEX Triple Quad™ 5500 LC/MS/MS System

Data was acquired and processed using Analyst® software version 1.6 and MultiQuant™ software version 2.1.

Results and Discussion

With this study we wanted to investigate the possibility to analyze AFB1 and ZON at the defined baby food levels without sample concentration and implemented this into an LC-MS/MS screening method.

The studied mycotoxins are listed in Table 2. An example chromatogram with all mycotoxins is shown in Figure 2.

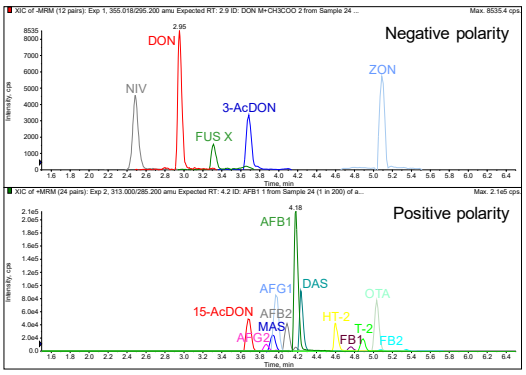

Figure 2. TIC of all mycotoxins analyzed in a single method with negative and positive polarity switching and the *Scheduled* MRM™ algorithm

Table 2. MRM transitions with their retention times (RT) used to detect target mycotoxins using a single method with negative and positive polarity switching

Mycotoxin	RT (min)	Polarity	Ion	MRM (quantifier)	MRM (qualifier)
15-Acetyldeoxynivalenol (15-AcDON)	3.7	positive	$[M+H]^+$	339/321	339/137
3-Acetyldeoxynivalenol (3-AcDON)	3.7	negative	$[M+CH3COO]^-$	397/307	397/59
		negative	$[M-H]^-$		337/307
Aflatoxin B1 (AFB1)	4.2	positive	$[M+H]^+$	313/285	313/128
Aflatoxin B2 (AFB2)	4.1	positive	$[M+H]^+$	315/287	315/259
Aflatoxin G1 (AFG1)	4	positive	$[M+H]^+$	329/243	329/200
Aflatoxin G2 (AFG2)	3.9	positive	$[M+H]^+$	331/313	331/245
Deoxynivalenol (DON)	3	negative	$[M+CH_3COO]^-$	355/295	355/59
Diacetoxyscirpenol (DAS)	4.2	positive	$[M+H]^+$	384/307	384/247
Fumonisin B1 (FB1)	4.8	positive	$[M+H]^+$	722/334	722/352
Fumonisin B2 (FB2)	5.1	positive	$[M+H]^+$	706/336	706/318
Fusarenon X (FUS X)	3.3	negative	$[M+CH_3COO]^-$	413/353	413/59
HT-2 toxin	4.6	positive	$[M+NH_4]^+$	442/263	442/105
Monoacetoxyscirpenol (MAS)	3.9	positive	$[M+H]^+$	342/265	342/307
Nivalenol (NIV)	2.5	negative	$[M+CH_3COO]^-$	371/281	371/59
Ochratoxin A (OTA)	5	positive	$[M+H]^+$	404/239	404/102
T-2 toxin	4.9	positive	$[M+NH_4]^+$	484/215	484/185
Zearalenon (ZON)	5.1	negative	$[M-H]^-$	317/131	317/175

To test the new method several products including baby cereals, adult cereals and a beer were prepared according to the documented sample preparation and standards were spiked into them. Internal standards have not yet been used.

In previous work we used chromatographic separation and a fixed switch between the negative and positive polarity experiments. Here we applied a single looped experiment containing both negative and positive polarity and combined it with the *Scheduled* MRM™ algorithm (Figure 2).

To test the effect of the *Scheduled* MRM™ algorithm two experiments were compared: one using polarity switching with traditional MRM mode and a second with polarity switching and *Scheduled* MRM™.

The use of *Scheduled* MRM™ not only increased the number of data points across the peak but also the signal-to-noise (S/N) observed (Figure 3). This increase in sensitivity was affected by retention time but was shown to be at least a factor of 3 fold for all mycotoxins. The scheduling of MRM transitions also allowed the addition of more mycotoxins to the method, so that a total of 17 mycotoxins were now detected in comparison to 10 in the previous method.[9]

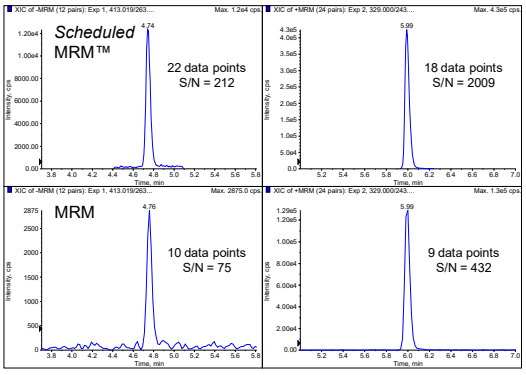

Figure 3. Comparison of a positive and negative switching experiment with and without *Scheduled* MRM™, FUS X in negative polarity (left) and AFG1 in positive polarity (right)

This method produced a linear regression coefficient greater than 0.99 for ZON (Figure 4). It was found to be reproducible with a %CV of 9.2 (Table 3), robust, and reliable for ZON with no major matrix effects like signal suppression or shift in retention times observed.

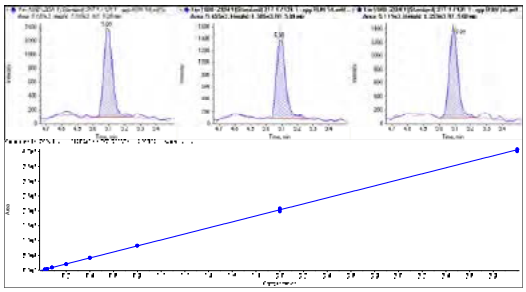

Figure 4. Triplicate injections of ZON at 0.04 ng/mL (top) and calibration line from 0.02 to 4 ng/mL with r = 0.9998 (bottom)

Diluted spiked matrix standards at several levels including 4 µg/kg (Figure 5) were injected three times and ZON was detected easily at these levels well below the EU legislation.

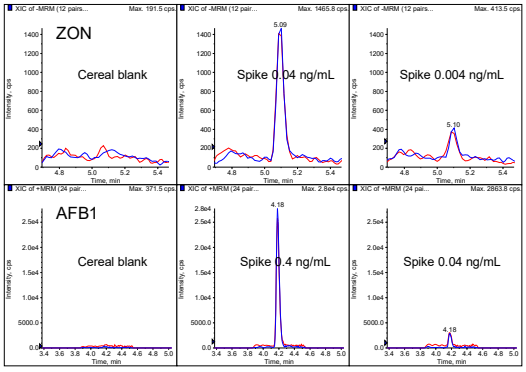

Figure 5. Chromatograms of ZON and AFB1 spiked into baby cereal compared with a matrix blank. The samples had been diluted 20 times.

Similarly AFB1 can be detected below the EU legislation. Figure 5 shows a spike into baby cereal of 0.040 ng/mL, which gave a S/N of 81. This clearly illustrated that AFB1 could be detected in baby food below the EU legislation limit of detection of 0.010 ng/mL (limit of detection of a method is normally defined as a signal to noise of 3 to 1 for the required analyte).

For AFB1 the method was found to be reproducible with a %CV of 4.2, robust and the linear regression coefficient was found to be greater than 0.99 (Figure 6).

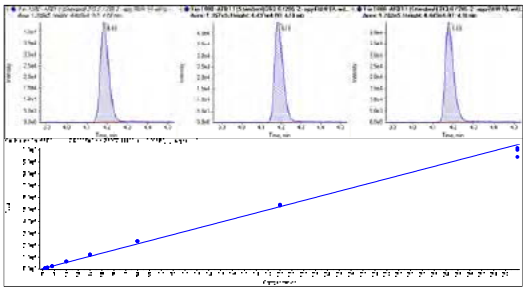

Figure 6 Triplicate injections of AFB1 at 0.4 ng/mL (top) and calibration line for ZON from 0.2 to 40 ng/mL with r = 0.9969 (bottom)

Table 3 shows the results for all the mycotoxins which all show good sensitivity, linearity and robustness.

Summary

The presented method has been tested on several cereal based samples and has been shown to be robust enough to detect these toxins below the required limits and met European Legislation.

The simple solvent extraction followed by dilution and the use of small particle size LC columns has meant that the method is fast and simple to apply. The use of polarity switching with *Scheduled* MRM™ acquisition has enabled shorter run times with an improvement in sensitivity, while extending the target list of compounds.

Table 3. Results from the multiple injections of standards for the 17 mycotoxins

Mycotoxin	Calibration range (ng/mL)	Linearity (regression coefficient)	Standard concentration (ng/mL)	S/N*	%CV
15-Acetyldeoxynivalenol (15-AcDON)	2 - 400	0.998	4	81	10.2
3-Acetyldeoxynivalenol (3-AcDON)	2 - 400	0.999	4	81	17
Aflatoxin B1 (AFB1)	0.2 - 40	0.997	0.4	621	4.2
Aflatoxin B2 (AFB2)	0.05 - 10	0.999	0.1	274.2	12.2
Aflatoxin G1 (AFG1)	0.2 - 40	0.998	0.4	573	8.4
Aflatoxin G2 (AFG2)	0.05 - 10	0.998	0.1	69	17
Deoxynivalenol (DON)	2 - 400	0.999	4	342	4.7
Diacetoxyscirpenol (DAS)	0.121 - 24.3	0.999	0.12	230	5.1
Fumonisin B1 (FB1)	0.2 - 40	0.993	0.4	36	15.1
Fumonisin B2 (FB2)	0.8 - 40	0.991	0.8	52	5.5
Fusarenon X (FUS X)	0.5 - 102	0.999	1.02	32	14.6
HT-2 toxin	0.4 - 80	0.999	0.8	148	5
Monoacetoxyscirpenol (MAS)	0.121 - 24.3	0.998	0.24	22	11.8
Nivalenol (NIV)	2 - 400	0.999	4	75	11.6
Ochratoxin A (OTA)	0.121 - 24.3	0.997	0.24	435	4.4
T-2 toxin	0.08 - 16	0.999	0.16	94	13.4
Zearalenon (ZON)	0.02 - 4	1.000	0.04	60	9.2

* Data calculated using signal / 1 x std dev noise

Acknowledgement

The authors like to acknowledge SGS in Hamburg (Germany) and IFA in Tulln (Austria) for their help and support in this important research area.

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Publication number: 4700211-01

Contents

Quantitative Analysis of Collagen in Meat Extracts using Liquid Chromatography and Tandem Mass Spectrometry

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Overview

Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals, including humans. In fact, it makes up about 25% to 35% of the total amount of protein in the body. Hydroxyproline is a major component of the protein collagen playing a key role for collagen's stability. Creatinine is a break-down product of creatine phosphate in muscle. These compounds determine how juicy and tender meat is.

Here we present a method using Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS) for the analysis of hydroxyproline and creatinine from collagen extracts. The samples were simply diluted and injected onto a Hydrophilic Interaction LC column (HILIC) coupled to an API 3200™ LC/MS/MS system operated in positive and negative polarity. Multiple Reaction Monitoring (MRM) was used for detection because of its high selectivity and sensitivity. The developed method had excellent Limits of Detection, linear range and reproducibility and was successfully applied to the analysis of meat extracts.

This analytical procedure can speed up the sample analysis for hydroxyproline and creatinine, which in turn, improves the whole processing of collagen products.

Introduction

Collagen is the main protein of connective tissue in animals and humans. The hydrolysis of collagen results in the formation of gelatin which is used in many food products, dietary supplements, pharmaceutical and cosmetic formulations, and many dental, orthopedic and surgical procedures, such as artificial skin substitutes in the management of severe burns.

Hydroxyproline, a major component of the protein collagen, and creatinine, a break-down product of creatine phosphate in muscle, are measured to determine the juiciness and tenderness of meat. Traditionally, colorimetric methods are used routinely in the meat and leather industries.[1-2]

However, these colorimetric methods require extensive sample preparation, and are subjected to interference with concomitant components in complex tendon extracts. Thus a more accurate and faster analytical method is required.

An LC-MS/MS method was developed to quantify both hydroxyproline and creatinine from meat extracts in one analysis with good sensitivity. The meat extracts were produced by adding hydrochloric acid to tendon in factory concentration tanks. These meat extracts are used to manufacture different meat products to satisfy tastes of consumers, soup flavoring and several meat-based ready-to-serve products.

Experimental

This method was developed using a Shimadzu Prominence LC system interfaced to a SCIEX API 3200™ LC/MS/MS system equipped with Turbo V™ source and Electrospray Ionization (ESI) probe. Ion source parameters are listed in Table 1. Targeted analytes were detected in Multiple Reaction Monitoring (MRM). MRM transitions for quantitation and compound identification are listed in Table 2.

LC separation was performed using a GL Sciences Inertsil HILIC column (5 µm) 3 x 150 mm and mobile phase A = acetonitrile + 10 mM ammonium acetate and B = water + 10 mM ammonium acetate (pH 6.7) at a flow rate of 0.5 mL/min. A mobile phase of

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(A/B) of 90/10 was used for 4 min and then ramped to 75/25 to 6 min before reequilibration.

Table 1. Ion source parameters using Electrospray Ionization

Parameter	Value
Curtain Gas (CUR)	25 psi
IonSpray Voltage (IS)	5000 V
Temperature (TEM)	500°C
Nebulizer Gas (GS1)	40 psi
Heater Gas (GS2)	60 psi

Table 2. MRM transitions in positive and negative polarity to detect hydroxyproline and creatinine

Analyte	Polarity	Q1	Q3	CE (V)
Hydroxyproline	positive	132.1	86.0	19
	positive	132.1	68.0	25
Hydroxyproline	negative	130.1	84.0	-23
	negative	130.1	82.0	-26
Creatinine	positive	114.1	44.0	27
	positive	114.1	86.0	15
Creatinine	negative	112.1	41.0	-35
	negative	112.1	68.0	-24

Due to high sample acidity (pH 3) the samples were diluted with a mixture of 45 mL acetonitrile, 1.25 mL of 1 M aqueous ammonium acetate solution and 3.75 mL of water. An aliquot of this sample was transferred to 1.7-mL auto-sampler vials for LC-MS/MS analysis.

In addition, the method was verified by analyzing bovine achilles tendon (Sigma-Aldrich, Lot 017K7018). 0.5 g of collagen was digested with a boiling solution of 6 N HCl (62 mL) for 6 hours and filtered through a 2.7-micron glass microfiber. The filtrate was transferred to a volumetric flask, and 6 N HCl was added to bring the total volume to 200 mL. An aliquot of this acidic solution was placed in an auto-sampler vial for LC-MS/MS analysis.

All quantitation data were processed using the MQ II algorithm within Analyst® Software (version 1.5).

Results and Discussion

Hydroxyproline and creatine can be detected in positive and negative polarity using Electrospray Ionization. However, positive polarity offers better sensitivity. An example chromatogram of the analysis of hydroxyproline and creatine in positive polarity is shown in Figure 1 highlighting the superior selectivity and sensitivity of LC-MS/MS operated in MRM.

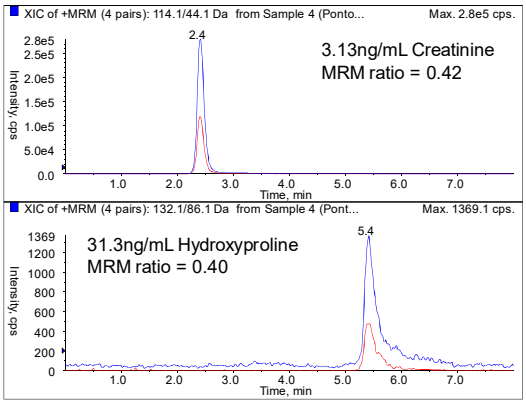

Figure 1. Standard of 3.13ng/mL creatinine and 31.3ng/mL hydroxyproline detected using an API 3200™ LC/MS/MS system

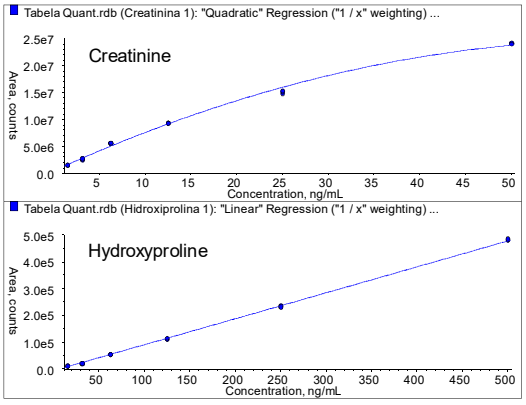

Figure 2. Calibration curves of creatinine (top) hydroxyproline (bottom)

Table 3. Reproducibility (n=3) and accuracy of the quantitation of creatinine and hydroxyproline

Analyte	Sample	Concentration (ng/mL)	Mean (ng/mL)	%CV	Accuracy (%)
Creatinine 1	Standard 6	1.56	1.50	3.2	95.9
	Standard 5	3.13	2.93	4.9	93.6
	Standard 4	6.25	7.10	1.2	113.5
	Standard 3	12.5	12.8	0.8	102.0
	Standard 2	25.0	23.1	2.3	92.5
	Standard 1	50.0	52.5	2.1	104.9
Creatinine 2	Standard 6	1.56	1.50	2.6	96.0
	Standard 5	3.13	2.95	4.1	94.3
	Standard 4	6.25	7.01	1.0	112.2
	Standard 3	12.5	12.8	1.1	102.2
	Standard 2	25.0	23.2	2.3	92.9
	Standard 1	50.0	52.2	4.4	104.4
Hydroxyproline 1	Standard 6	15.6	17.2	2.1	110.2
	Standard 5	31.3	28.3	4.0	90.4
	Standard 4	62.5	63.0	0.8	100.8
	Standard 3	125	123	1.8	98.4
	Standard 2	250	247	0.8	98.9
	Standard 1	500	505	0.6	101.1
Hydroxyproline 2	Standard 6	15.6	17.3	3.1	110.8
	Standard 5	31.3	27.7	1.9	88.5
	Standard 4	62.5	63.7	1.9	101.9
	Standard 3	125	124	1.5	98.8
	Standard 2	250	247	1.2	98.7
	Standard 1	500	505	0.5	101.1

Calibration curves (Figure 2) with excellent accuracies and coefficients of variation (%CV) were obtained in a dynamic range of 1.56 to 50.0 ng/mL for creatinine and 15.6 to 500 ng/mL for hydroxyproline.

Statistical data of both MRM transitions of calibration curves are listed in Table 3. %CV of both analytes over the whole calibration range was <5% with accuracies between 88 and 113%. The high sensitivity of the developed LC-MS/MS method allowed dilution of meat extracts greatly increasing robustness and reducing the risk of possible matrix effects.

Bovine achilles tendon collagen was digested, filtered, diluted, and analyzed by LC-MS/MS to verify method performance (Figure 3).

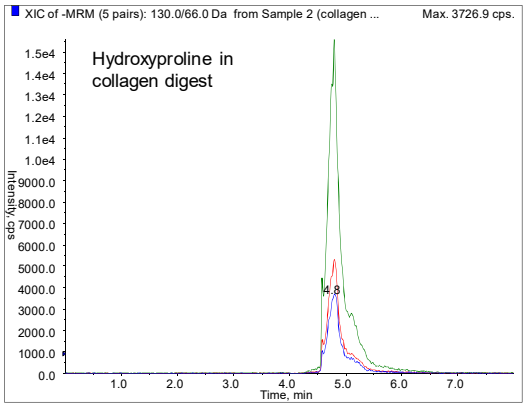

Figure 3. Detection of hydroxyproline in a collagen digest after simple filtration and dilution

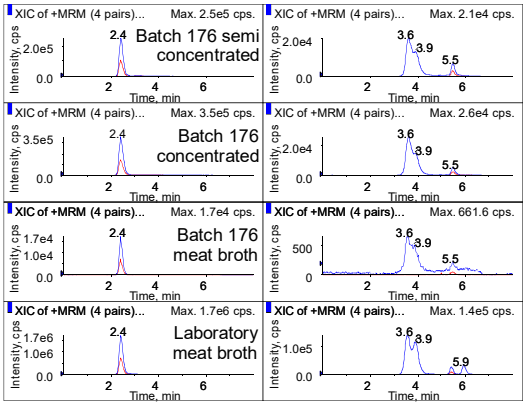

Figure 4. Concentrations of hydroxyproline and creatinine in meat extracts and sample broths, the ratios of quantifier and qualifier MRM transition were used for compound identification with a tolerance of +/- 20% (hydroxyproline 0.32-0.48 and creatinine 0.33-0.50)

Table 4. Concentrations of hydroxyproline and creatinine in meat extracts and sample broths, the ratios of quantifier and qualifier MRM transition were used for compound identification with a tolerance of +/-20% (hydroxyproline 0.32-0.48 and creatinine 0.33-0.50)

	Creatinine		Hydroxyproline	
Sample Name	**Concentration (ng/mL)**	**MRM ratio**	**Concentration (ng/mL)**	**MRM ratio**
Batch 176 semi concentrated meat broth	3.46	0.42	80.1	0.47
Batch 176 concentrated meat broth	5.45	0.41	61.4	0.47
Batch 176 meat broth	< LOD		8.56	0.43
Laboratory meat broth	35.8	0.42	289	0.44

Meat extract samples were analyzed with good selectivity and sensitivity. These extracts were sampled from factory tanks, which are used at the start of the meat extract concentration process. The samples were simply diluted and injected into the LC-MS/MS system, without any additional extraction or clean-up process (Figure 4). The concentrations of hydroxyproline and creatine ranging from 8.6 ng/mL to 289 ng/mL and 3.5 ng/mL to 36 ng/mL from, respectively, are listed in Table 4. The quantifier MRM transition was used to determine the concentration of the targeted analytes in the unknown samples. In addition, the ratio of quantifier and qualifier MRM transition was used to further identify hydroxyproline and creatinine. The MRM ratio of unknown samples was compared to an average of all standard injections with a tolerance of +/- 20%.

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Summary

We demonstrated that it is possible to detect and quantify hydroxyproline and creatinine in meat extracts with good detection and quantitation limits within an 8 min chromatographic run.

This LC-MS/MS method can replace the traditional colorimetric method used in the meat and leather industry. This method offers faster analysis time and more accurate data compared to the colorimetric method.

Acknowledgements

The authors would like to thank Mr. Wilson Duarte da Silva and Ms. Eládia R. S. Rodrigues Almeida from Bertin Food Division Ltda. for the provision of collagen meat extracts.

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Publication number: 0460410-01

The Quantitation and Identification of Artificial Sweeteners in Food and Drink by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

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Overview

Artificial sweeteners are food additives whose use has been controlled by European Parliament guidelines. The method described in this application note, shows how LC-MS/MS can be used to simultaneously detect and confirm the presence of several artificial sweeteners. The method is both quicker than conventional non LC-MS/MS methods and more sensitive. This has meant that these compounds can be detected in samples below ingredient levels using a simple dilute and shoot approach.

Introduction

As we aim to eat less sugar, many of us are turning more and more to alternative sweeteners. Intense sweeteners such as Acesulfame (E950), Aspartame (E951), Cyclamate (E952), Saccharin (E954), and Sucralose (E955) are very low in calories and are safer for teeth (Figure 1). As with all additives, sweeteners are thoroughly assessed for safety before they are permitted, and are only then permitted in a limited range of products. The European Parliament has set out guidelines for the labeling of food containing artificial sweeteners (Directive 94/35/EC 'on sweeteners for use in foodstuff' with several amendments 96/83/EC, 2003/115/EC, and 2006/52/EC) and it has deemed that the presence of Aspartame and Aspartame-Acesulfame salt should state that the food 'contains a source of phenylalanine'. In addition some sweeteners cannot be used in foods for infants and young children, mentioned in Directive 89/398/EC.

At present standard methods, for the detection of sweeteners in food, use LC with evaporating light scattering detection.[1] This work shows where LC-MS/MS can be used to detect seven commercially available artificial sweeteners in diet drinks and baby food which were obtained from local supermarkets. The method has several advantages over the existing methodology in that it is five times faster as well as more than 100 to 1000 fold more sensitive. In all cases, due to the sensitivity of the technique and the level of artificial sweeteners, the samples had to be diluted at least 100 fold before analysis thus reducing the effects of matrix on the analysis and simplifying sample preparation.

Experimental

Sample Preparation

Samples of soft drinks such as cola, orange flavored fizzy drink and lemonade were diluted 100 or 1000 fold in water. To test the method on baby food an 'off the shelf' sample of fruit was spiked with artificial sweeteners at 10 parts per million (ppm) and mixed with distilled water in a ratio of 1 part baby food to 9 parts water and shaken for one minute. The extract was centrifuged and then diluted 1 in 10 with water before LC-MS/MS analysis.

LC

Samples were separated by reversed-phase LC on a polar end capped column (4 µm, 150 x 2.1 mm), at 800 µL/min using a Shimadzu UFLC system. The gradient was over 6 minutes from 5% to 100% methanol in water. Both the water and methanol mobile phases had been modified by the addition of triethylamine and formic acid.

Contents

MS/MS

Analysis was performed using a SCIEX 3200 QTRAP® LC/MS/MS system fitted with a Turbo V™ source in Electrospray Ionization (ESI) mode and run in negative polarity. The detected Multiple Reaction Monitoring (MRM) transitions are listed in Table 1.

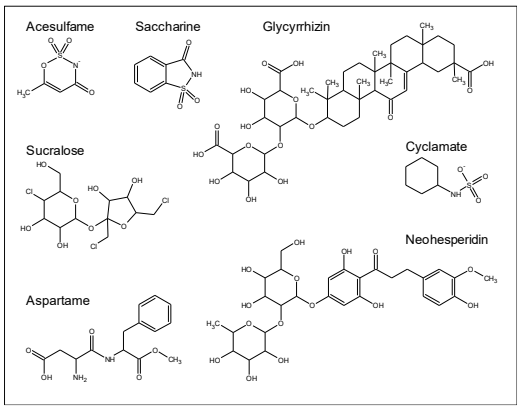

Figure 1. Structures for seven commercially available artificial sweeteners in the present method

Table 1. MRM transitions used in the method

Compound	Q1 (amu)	Q1 (amu)
Acesulfame	162	82
	162	78
Aspartame	293	200
	293	261
Cyclamate	178	80
	178	79
Glycyrrhizin	821	351
	821	113
Neohesperidin	611	303
	611	166
Saccharin	182	42
	182	106
Sucralose	395	359
	397	361

Confirmation of the identity of the compound has been further enhanced by the automatic generation of an Enhanced Product Ion (EPI) scan triggered by the MRM transition of a sweetener.

Results and Discussion

It can be seen that all the artificial sweeteners can be detected at concentrations of low parts per billion (ppb), Figure 2, with no carry over observed.

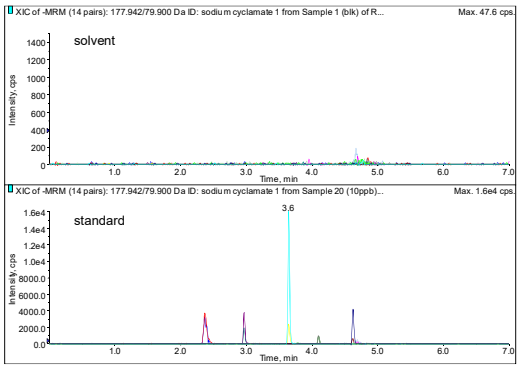

Figure 2. An example of the chromatogram obtained from a water blank (top) and a 10 ppb standard of artificial sweeteners in water (bottom)

When this method was applied to real samples it was found that drinks taken off supermarket shelves had to be diluted 100 or even a 1000 times to be within the range of the calibration standards (Figure 3). All the artificial sweeteners found in the samples corresponding to those which were listed on the ingredient labels. When this method was applied to a spiked baby food sample again all the sweeteners were observed at the spike level which was similar to the level used in drink manufacture.

From the peak heights shown in Figure 2 it can be seen that the sensitivity for the artificial sweeteners vary by over 2 orders of magnitude, with the acidic Cyclamate the most sensitive and Sucralose the least. This wide ranging sensitivity is down to the structural differences between these compounds which not only produces a wide range of different molecular weights but also a wide range pKa.

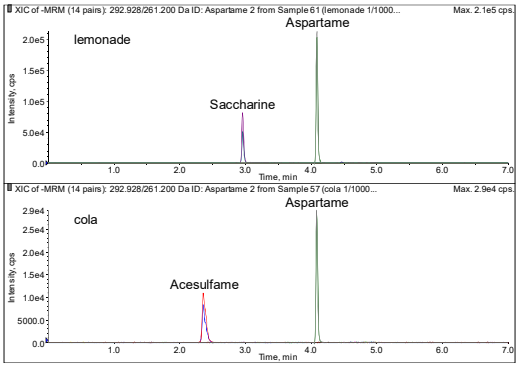

Figure 3. Chromatograms obtained from a 1000 dilution of a lemonade sample (top) and of cola sample (bottom). The two sweeteners detected corresponded to those listed on the drink's label.

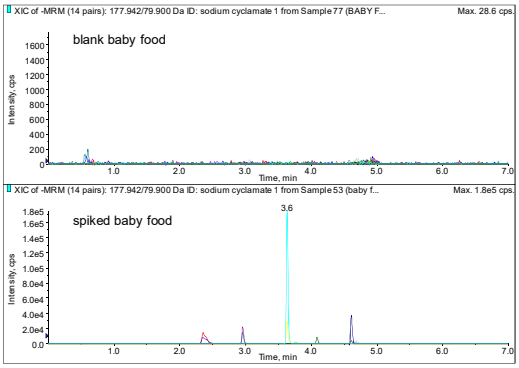

Figure 4. An example of the chromatogram obtained from a baby food sample (top) and 10ppm spike of sweeteners into baby food (bottom)

Little or no retention was found with standard reversed phase columns (C8 and C18) or a polar end-capped columns using a standard ammonium acetate buffered gradient making the use of an ion pairing reagent necessary.

The early elution and complex nature of some sweeteners also leads to some quadratic calibration curves (Figures 5). The non linearity has also been observed by other groups using ammonium acetate buffered LC conditions[2] and was improved in this work by the addition of triethylamine into the mobile phase. The non linearity starts below the point of normal detector saturation and seems to be a result of ionization efficiency and possibly the pH of sample and could probably be corrected further by the use of deuterated internal standards.

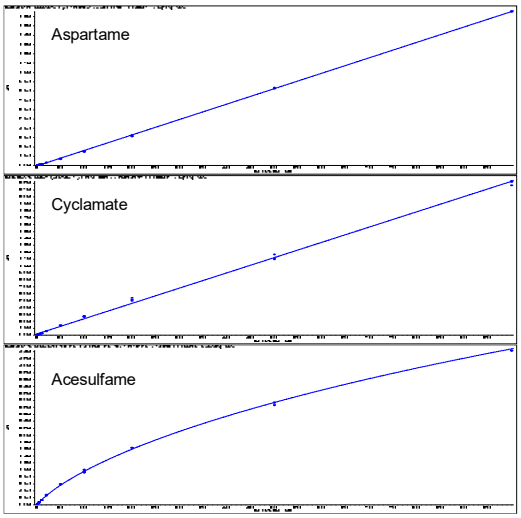

Figure 5. Examples of calibration curves for three commonly detected artificial sweeteners [Aspartame (top), Cyclamate (middle) and Acesulfame (bottom)], as it can be seen some compounds produce a non linear response over the range from 1 to 1000 ppb,

Even with the varying intensities and the complex nature of these compounds good robustness and reproducibility was observed. The coefficients of variation (%CV) observed from the repeat analysis of solvent standards are all less than 15% (except for Sucralose which was 15.2%) at 10 ppb and less than 10% at 100 ppb even with no internal standard present for any of the compounds (Table 2).

An additional advantage of using the SCIEX 3200 QTRAP® system is the possibility to confirm the identity of compounds based on automatically acquired EPI spectra. EPI spectra contain a complete molecular fingerprint of the detected analyte resulting in increased confidence of identification. An example of this is shown in Figure 6 where Acesulfame and Aspartame where identified using EPI spectra which were identical to those generated from standards.

		Concentration (ppb)	%CV
Acesulfame	1	10	8.0
	2	100	4.1
	1	10	3.9
	2	100	1.9
Aspartame	1	10	6.0
	2	100	5.4
	1	10	11.2
	2	100	4.0
Cyclamate	1	10	2.9
	2	100	3.2
	1	10	9.7
	2	100	3.9
Glycyrrhizin	1	10	6.7
	2	100	2.1
	1	10	9.4
	2	100	1.5
Neohesperidin	1	10	4.0
	2	100	4.7
	1	10	11.9
	2	100	8.0
Saccharin	1	10	5.6
	2	100	4.6
	1	10	5.7
	2	100	3.4
Sucralose	1	10	11.1
	2	100	2.9
	1	10	15.2
	2	100	4.6

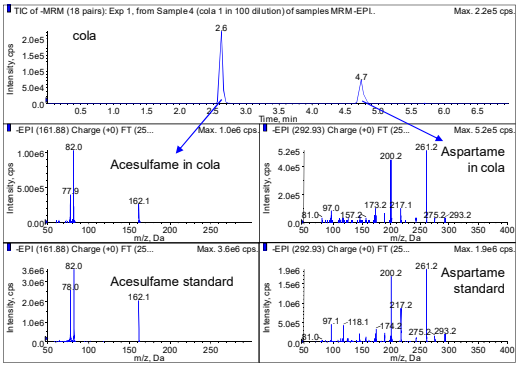

Figure 6. Examples of identification of sweeteners in a cola flavored drink by the automatic generation of EPI spectra

Summary

The work to date shows that artificial sweeteners can be easily detected in negative polarity LC-MS/MS using Electrospray Ionization and well below current levels used in the drink industry. The method is more than five times faster than non LC-MS/MS methods currently available and due to the high sensitivity a much reduced sample pre-treatment is possible.

References

1 Buchgraber and A. Wasik: 'Validation of an analytical method for the simultaneous determination of nine intense sweeteners by HPLC-ELSD' Report EUR 22726 EN (2007)

2 Christiane Barthel, Eurofins: (2010) personal communication

Publication number: 2170611-01

Analysis of the Vitamin B Complex in Infant Formula Samples by LC-MS/MS

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay using the SCIEX QTRAP® 6500 system has been developed for the simultaneous detection of all major forms of vitamin B complex. The method detects all currently used forms of vitamin B6 and vitamin B3 in infant formula and includes vitamin B12. The sample preparation allows the same extract to be used for Vitamin C detection and the LC-MS/MS conditions have been tuned so that the response for each vitamin is linear over the various required detection ranges.

Introduction

Vitamin B is a group of water-soluble vitamins that play important roles in cell metabolism. The absence of individual B vitamins in a diet can lead to several conditions including depression and high blood pressure so they are often added to foods, especially infant formula. Human daily nutritional recommendations for the members of the vitamin B complex vary considerably, for example from 6 µg of vitamin B12 to 20 mg of vitamin B3 (Table 1). The US Food and Drug Administration regulates food labels in the United States and food labeling is required for most prepared foods such as breads, cereals, canned foods, snacks, drinks, and especially for infant formula, which is highly regulated.[1]

Table 1. Daily required values (DV) of different B vitamins for a human adult as obtained from the FDA[1]

Vitamin		DV (mg)
Thiamine	B1	1.5
Riboflavin	B2	1.7
Niacin	B3	20
Pantothenic acid	B5	10
Pyridoxal	B6	2
Biotin	B7	0.3
Folic acid	B9	0.4
Cyanocobalamin	B12	0.006

Analysis of food samples can be challenging, as the matrices are complex and sensitive methods typically require highly selective sample clean up procedures. Vitamin B is a complex mixture of highly polar compounds (Figure 1) whose pK_a range from 0.5 to 10.2, making their analysis challenging.

Several methodologies exist to look at these analytes in separate classes, but relatively few analytical methods exist that examine the vitamin B complex as a whole, with high throughput capabilities, minimal sample preparation, and which have high sensitivity and specificity.

Here we present new data acquired by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) from a quantitative method that contains vitamins B1, B2, B3 (two forms), B5, B6 (three forms), B7, B12 and folic acid (vitamin B9).. Instrument detection levels for these vitamins using this method have been shown to be less than a ng/mL for the neat compounds using positive mode Electrospray Ionization (ESI) and the *Scheduled* MRM™ algorithm. The required limits of detection vary greatly between each vitamin, but all the B vitamins can be detected in infant formula, by adjusting the MS/MS voltages accordingly, even with detection limits having a 10,000-fold range .

The LC-MS/MS method utilizes a small particle size polar endcapped reversed phase (RP) column and an 11 min gradient. In this new iteration of the method very little sample preparation

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has been used to enable a high throughput suitable for routine food testing.

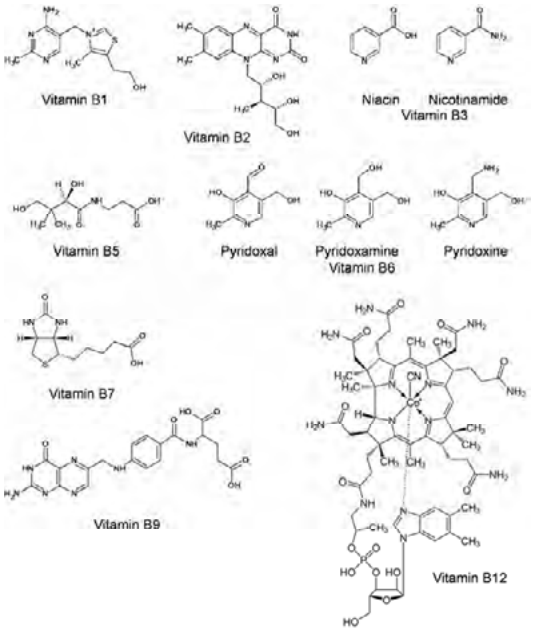

Figure 1. Chemical structures of B vitamins

Experimental

Standards

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and are commonly available. NIST SRM 1849a infant formula reference material (LGC, UK) was used to develop the method and verify the method performance.

Sample Preparation

Sample (1 g) was mixed with 50% acetonitrile in acidified water (containing an antioxidant) and internal standard solution was added. This was then shaken vigorously for 1 minute and roller mixed for 10 minutes (protected from light). After centrifugation the supernatant was filtered and the filtrate diluted 1 in 20 with water containing an ion paring reagent. The sample preparation was kept as simple as possible to reduce possible vitamin breakdown, with SPE no longer needed for the late eluting B7, B9, and B12 vitamins.

During the development work the effects of light, temperature, and acidity on standard stability were tested and it was found that the use of amber glass with a lower pH with the presence of an antioxidant helped stabilize the extracts.

LC Separation

Samples were separated by LC on a polar endcapped RP column using a Shimadzu UFLC$_{XR}$ system over an eleven minute gradient from acidified water to 100% methanol containing 0.1% formic acid (Table 2). The column temperature was maintained at 50°C and an injection volume of 20 µL was used. The separation was designed to allow retention of the early eluting vitamins until after the solvent front and to make sure that the late eluting vitamins were baseline resolved to help reduce possible ion suppression. Although the last vitamin B12, eluted at 5.2 minutes the column was washed and equilibrated for a further six minutes to make sure that retention times were stable between injections.

Table 2. Gradient conditions used for the separation of B vitamins

Step	Time (min)	Flow (µL/min)	A (%)	B (%)
0	0.0	500	100	0
1	2.0	500	100	0
2	2.5	500	75	25
3	5.0	500	57	43
4	5.5	500	2	98
5	5.6	500	2	98
6	6.0	1000	2	98
7	6.2	1000	2	98
8	6.3	1000	100	0
9	10.0	1000	100	0
10	10.5	500	100	0
11	11.0	500	100	0

MS/MS Detection

Analysis was performed on a SCIEX QTRAP® 6500 system. The source conditions were a standard set up of Curtain Gas™ interface of 35 psi, IonSpray™ source voltage = 5500V (positive polarity), gas 1 = 50 psi and gas 2 = 60 psi, source temperature = 550°C, and collision gas = 10 psi. The MRM conditions used

are shown in Table 3, with the resolution kept at unit for both Q1 and Q3. Two MRM transitions were monitored for each compound to use the ratio of quantifier and qualifier transition for compound identification. The *Scheduled* MRM™ algorithm was used to monitor a total of 28 transitions and acquire data with the best reproducibility and accuracy.

Table 3. Quantifier and qualifier MRM transitions and retention times (RT) for the detection of B vitamins

Compound	RT (min)	Q1 (amu)	Q3 (amu)
B1 1	1.5	265	81
B1 2	1.5	265	122
B2 1	5.1	377	172.2
B2 2	5.1	377	198.1
B3 niacin 1	1.2	124	53
B3 niacin 2	1.2	124	80
B3 nicotinamide 1	1.5	123	80
B3 nicotinamide 2	1.5	124	81
B5 1	2.7	220	98
B5 2	2.7	220	90
B6 pyridoxal 1	1.6	168	94
B6 pyridoxal 2	1.6	168	67
B6 pyridoxamine 1	0.9	169	134
B6 pyridoxamine 2	0.9	169	106
B6 pyridoxine 1	1.9	170	134
B6 pyridoxine 2	1.9	170	152
B7 1	4.6	245	227
B7 2	4.6	245	97
B9 1	4.9	442	176
B9 2	4.9	442	120
B12 1	5.2	678.4	147
B12 2	5.2	678.4	359
IS B1	1.5	268	125
IS B2	5.1	380	173
IS B3 niacin	1.2	127	80
IS B5	2.7	223	93
IS B7	4.6	249	231
IS B9	4.9	446	176

Some of the collision energies were modified to lower the responses and extend the linear ranges (Figure 2). This was done to accommodate the differences in fortification levels in infant formula and response factors of the B vitamins.

All results were processed in PeakView® software version 2.0 and MultiQuant™ software version 3.0.

Results and Discussion

Due to the extended dynamic range requirements and the large differences in limits of detection required for this class of vitamins, some responses had to be adjusted in order to maintain a linear response across the required concentration range. To this end, the collision energies (CE) were adjusted to decrease the vitamin responses as required using the information obtained by ramping the CE. The CE ramps were automatically generated during method development using the 'Compound Optimization' feature in Analyst® software. An example of this is shown in Figure 2.

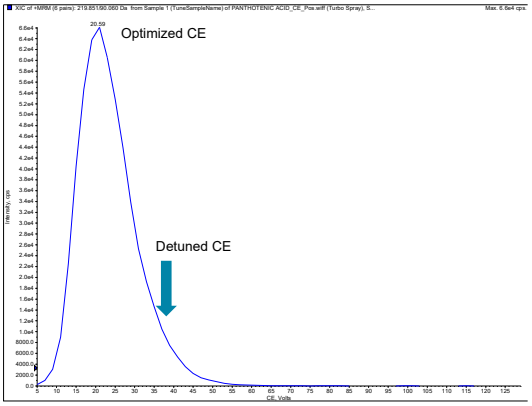

Figure 2. This is a typical ramp of the collision energy (CE) for a vitamin B5 fragment ion. Using this approach the more sensitive vitamins that showed a non-linear response at higher concentrations were detuned for a lower response by choosing non-optimal collision energies.

An example of signal reduction by detuning CE is shown in Figure 3 and the overall effect of adjusting the vitamins is shown in Table 4.

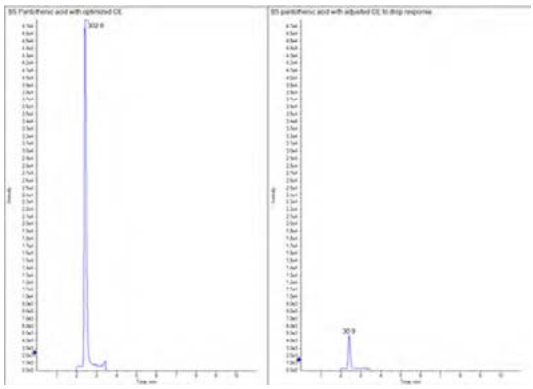

Figure 3. The effect of changing the collision energy on the response of vitamin B5

Table 4. The effect of adjusting the collision energy (CE) on reducing the overall response for different vitamins

Compound	CE (optimal)	CE (adjusted)	Response Decrease
B1	21	53	10x
B2	49	78	10x
B3 niacin	31	55	20x
B3 nicotinamide	29	50	10x
B5	21	38	10x
B6 pyridoxine	19	31	10x

Even though the responses were decreased by changing CE for some of the vitamins, a 5 ng/mL solvent standard (Figure 4) clearly shows that all the vitamins are easily detected at this level.

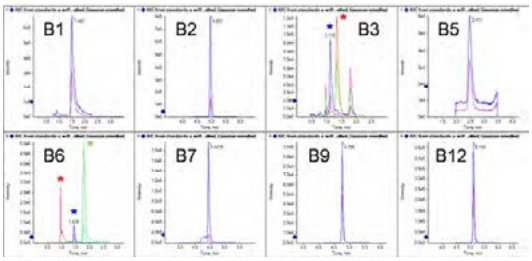

Figure 4. Example of a 5 ng/mL solvent standard of B vitamins, quantifier and qualifier ions are shown

Linearity was studied using solvent standards taken through the same sample preparation procedure as the reference material (equivalent to 0.1 to 100 mg/kg in matrix) for all the vitamins except B12 where the range was from 0.01 to 100 mg/kg. Linear fit with 1/x weighting was used for all target compounds resulting in coefficients of regression (r) between 0.994 and 0.999. Internal standards were used to achieve the best quantitative results (Table 5).

Table 5. Linear dynamic range (LDR) and coefficients of regression (r) for each vitamin

Compound	Internal Standard	LDR (mg/kg)	r
B1	B1 - D_3	0.1 - 100	0.997
B2	B2 - D_6	0.1 - 100	0.959
B3 niacin	B3 niacin - D_3	0.1 - 100	0.997
B3 nicotinamide	B3 niacin - D_3	0.1 - 100	0.998
B5	B5 - $^{13}CD_2$	0.1 - 100	0.994
B6 pyridoxal	B3 niacin - D_3	0.1 - 100	0.998
B6 pyridoxamine	B3 niacin - D_3	0.1 - 100	0.995
B6 pyridoxine	B3 niacin - D_3	0.1 - 100	0.997
B7	B7 - D_4	0.1 - 100	0.997
B9	B7 - D_4	0.1 - 100	0.996
B12	none	0.01 - 100	0.999

Examples of the calibration lines for vitamins B5 and B7 are shown in Figures 5a and 5b. This shows, in the case of B3, that linearity of response is obtained after the adjustment of CE and linear responses are obtained for early and late eluting vitamins

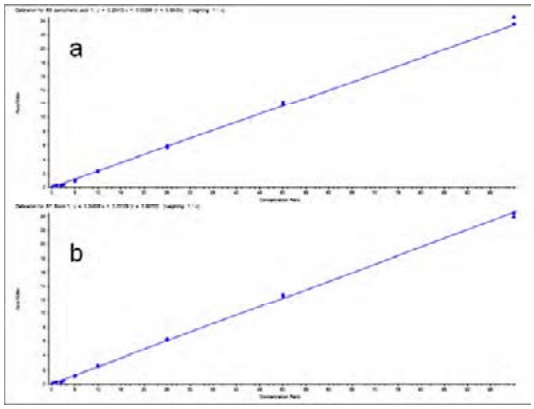

Figure 5. Calibration lines for two vitamins, an early eluting vitamin B5 (a) and a late eluting vitamin B7 (b)

Once each vitamin had their linear response verified for the desired dynamic range, extracts of the NIST 1849A infant formula reference materials were prepared. The results of these extracts are shown in Table 6 and example chromatograms are shown in Figure 6.

Table 6. Results from the repeat analysis of NIST reference material which had been extracted separately three times, with each extract injected seven times (in mg/kg)

Compound	NIST Reference Value	LC-MS/MS Value	CV (%)
B1	12.6	17.1	1.82
B2	20.4	16.5	2.22
B3 niacin	N/A*	N/A	N/A
B3 nicotinamide	109	105	3.01
B5	68.0	81.8	2.36
B6 pyridoxal	13.5	13.9	2.80
B6 pyridoxamine	N/A	N/A	N/A
B6 pyridoxine	N/A	N/A	N/A
B7	1.99	1.96	3.16
B9	2.29	2.45	4.79
B12	N/A	0.078	5.59

* N/A - compound not present in NIST material or not detected in sample

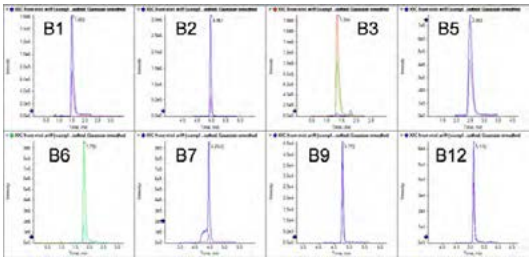

Figure 6. Example of an extract from NIST 1849A reference material showing chromatograms for the fortified vitamins. Vitamin B3 was present as nicotinamide and vitamin B6 as pyridoxine.

Built-in queries of MultiQuant™ software version 3.0 can be used to calculate ion ratios and flag outliers. Ion ratio tolerances for each analyte can be defined in the quantitation method editor (Figure 7). The peak review of an extract of NIST 1849A reference material with ion ratio tolerances is shown in Figure 8.

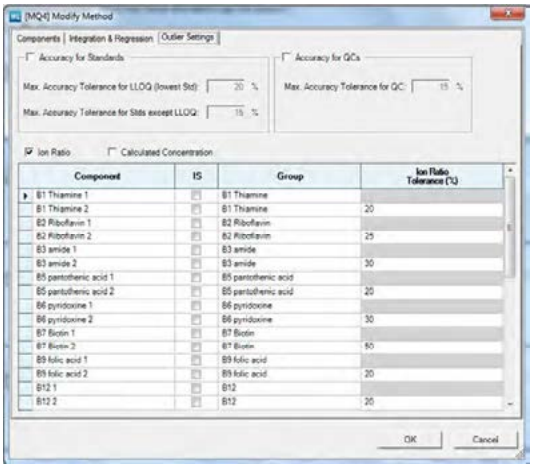

Figure 7. Query settings in the quantitation method editor of MultiQuant™ software to calculate ion ratios

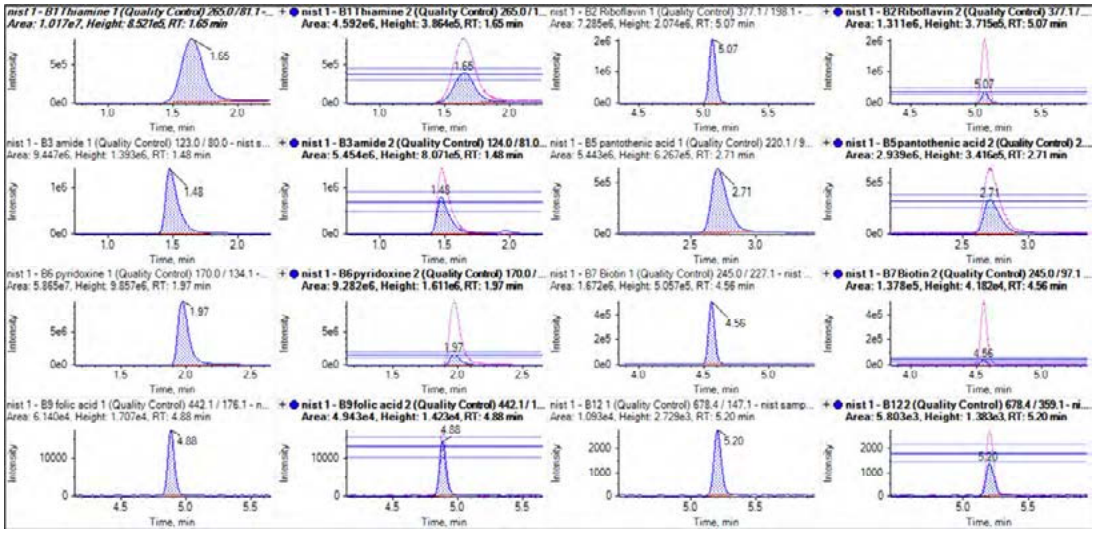

Figure 8. Peak review with ion ratio tolerances of an extract from NIST 1849A reference material

Summary

An LC-MS/MS method has been developed to detect the vitamin B complex in infant formula. Detection limits and linear dynamic range of quantitation were shifted into required ranges by adjusting (detuning) collision energies for some of the B vitamins.

Using a simple sample extraction followed by a 20-fold dilution has proved a valid approach to detect all B vitamins in infant formula. NIST 1849A infant formula reference material was analyzed for method verification. Results with excellent accuracy and reproducibility were achieved.

References

1 http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/labelingnutrition/ucm064928.htm

Publication number: 09340214-01

Quantitative Analysis and Identification of Migrants in Food Packaging Using LC-MS/MS

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Packaging improves the quality and safety assurance of food, especially from micro-organisms, biological and chemical contaminants. Packaging is therefore an essential component for the food industry and the manufacturing processes. However, over the last couple of years there has been a growth in the number of materials and substances used in food packaging so in order to improve food safety, migration studies for compounds that can migrate into food are critical.

Currently, an upper limit for the overall migration of 60 mg/kg or10 mg/dm2 has been set by the European Union (EU).[1] In the USA, the regulations for food packaging material are more complex, because the types of raw and processed foods, and conditions of use are separated.[2]

In this study three compounds: ITX, Irgacure, and TRP were investigated (Figure 1).

- ITX is a mixture of 2-Isopropylthioxanthone and 4-Isopropylthioxanthone.
- Irgacure contains Irgacure 819 (Phenylbis (2,4,6-trimethylbenzoyl)phosphine oxide. Both are used as photo-initiators in UV cured inks.
- TRP (Tri(propylene glycol) diacrylate is an ingredient of cured inks.

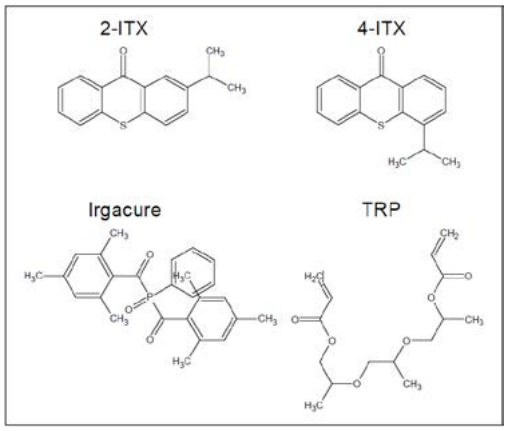

Figure 1. Investigated Migrants from Food Packaging.

The data presented discusses linearity of response, robustness and the use of the Multiple Reaction Monitoring combined with Enhanced Product Ion scanning (MRM-EPI) using a SCIEX QTRAP® 3200 LC-MS/MS System as a way of gaining additional information for the presence of these migrants.

Key Advantages of This Method

- Four common food packaging materials were identified and quantified by LC-MS/MS using the QTRAP® 3200 system
- The method provided sensitivity levels that enabled the detection of migrants at 0.01 mg/kg in extracts
- Utilization of the linear ion trap on the QTRAP system allowed for the collection of full scan MS/MS data that was compared to a library spectral database for confirmation of compound identification

Contents

Methods

Sample Preparation: Standards were prepared in the solvent composition at the start of the LC gradient (water/acetonitrile + 0.1% formic acid 70/30). Three sorts of real samples were analyzed: a packaging cap with only decoration (inks), a packaging cap with only varnish and a packaging cap with decoration and varnish. 1 dm^2 of each sample was extracted with acetonitrile. The extracted sample was evaporated and reconstituted in initial mobile phase before analysis.

Chromatography: An Agilent 1200 system with a binary pump flowing at 250 µL/min, an autosampler, and a column oven set at 20°C were used. Separation was performed on a Hypersil BDS C18 column (5 µm, 100 x 2 mm). 10 µL injections of standards and extracts were separated using a gradient (Table 1) of mobile phase A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile). 5 minutes column equilibration time was used between runs.

Table 1. LC Gradient.

Step	Time (min)	%A	%B
0	*5.0*	*70*	*30*
1	*2.0*	*5*	*95*
2	*7.0*	*5*	*95*
3	*7.1*	*70*	*30*
4	*12*	*70*	*30*

Mass Spectrometry: All experiments were performed on a SCIEX 3200 QTRAP® LC-MS/MS System with Turbo V™ source at 450°C using Electrospray Ionization (ESI) in positive polarity. The following source conditions were used:

- Curtain Gas (CUR) - 25 psi
- IonSpray Voltage (IS) - 5000 V
- Gas1 - 40 psi
- Gas2 - 50 psi
- CAD Gas - Medium
- Temperature - 450 °C

Analyses were based on two different Information Dependent Acquisition (IDA) experiments using Multiple Reaction Monitoring MRM) in the survey scan and dependent Enhanced Product Ion (EPI) scanning. MRM transitions were previously optimized (see Table 2). A dwell time of 100 msec was used for each transition and the pause time was set to 5 msec.

Table 2. MRM Acquisition Parameters.

Compound	Q1 Mass (amu)	Q1 Mass (amu)	DP	CE
ITX	*255.1*	*213.1*	*66*	*35*
	255.1	*184.1*	*66*	*61*
Irgacure	*419.2*	*147.2*	*21*	*23*
	419.2	*119.2*	*21*	*57*
TRP	*301.2*	*113.2*	*21*	*13*

Experiment 1 triggered three EPI scans at collision energies (CE) of 20; 35 and 50 V. Experiment 2 used a single dependent scan with a CE of 35 V and Collision Energy Spread (CES) of 15 V. CES was found to give more reproducible and richer MS/MS spectra, in comparison to dedicated and fixed collision energies, and thus greatly enhancing the quality of library searching (Figure 2). The scan speed of the EPI scans was 4000 amu/s and Dynamic Fill Time (DFT) was used for all EPI scans. In both experiments peaks were identified in the MRM survey using Dynamic Background Subtraction (DBS).

Data Processing: Identification of analytes in the real samples was based on searching against the mass spectral library created from MRM-EPI analyses of standards.

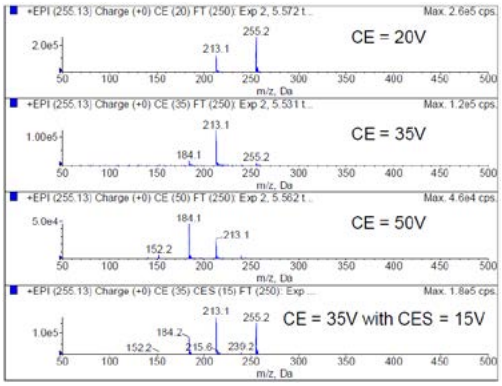

Figure 2. Optimizing Collision Energies. An example of the effect of collision energy on the EPI spectra of a migrant standard used for generating library data (10 ng/mL ITX standard).

Results

Standards at 10 ng/mL were used to build a mass spectral library. An example of reference spectra is shown in Figure 2. Standards were used over a range 0.1 to 1000 ng/mL to produce calibration lines.

Figure 3 shows calibration lines that were obtained from standards analyzed in MRM-EPI mode with each standard analyzed in duplicate. The 'r' values obtained from these calibration lines (0.5 – 500 ng/mL for ITX, 2 – 1000 ng/mL for Irgacure and 0.5 – 1000 ng/mL for TRP) were greater than 0.996 when a linear fit with 1/x weighting was applied.

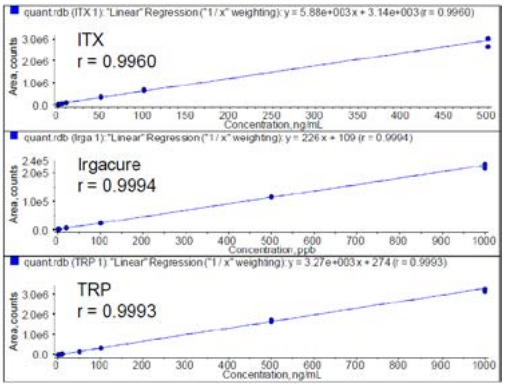

Figure 3. Quantitation Curves. Calibration lines obtained from ITX, Irgacure and TRP with r values > 0.996 (no internal standard used).

Repeatability and %CV were assayed by 5 repeat injections of a standard close to the limits of quantitation of each analyte and results are summarized in Table 3 with all coefficients of variation <10% (no internal standard was used).

Table 3. Reproducibility Data from 5 Replicate Injections.

Compound	Transition	Concentration (ng/mL	%CV 9n=5)
ITX	*255.1 / 213.1*	*0.5*	*8.2*
Irgacure	*419.2 / 147.2*	*0.844*	*5.2*
TRP	*301.2 / 113.2*	*0.515*	*9.5*

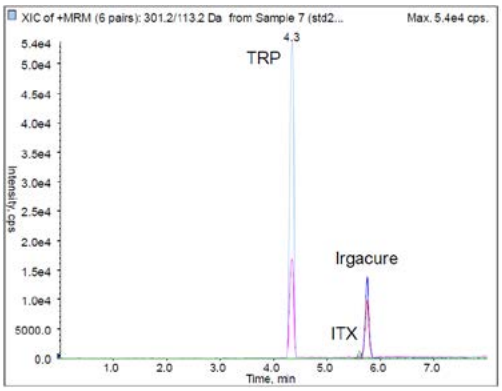

Figure 4. Chromatographic Separation. 10 µL injection of migrant standards in initial mobile phase.

Figure 4 shows a typical trace obtained from the analysis of migrant standard prepared in the initial mobile phase, all migrants were detected below 1 ng/mL as shown in Table 4 with Figure 5 giving the sensitivity of migrants at a concentration of 0.5 ng/mL (ITX and TRP) and 2 ng/mL (Irgacure).

Table 4. Estimates for Limits of Detection (LOD). Limits of Quantitation LOQ), and Linearity for Food Migrants.

Compound	S/N (at ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Linearity (ng/mL)
ITX	*40.4 (0.5)*	*0.04*	*0.12*	*0.12 - 500*
Irgacure	*18.4 (2.0)*	*0.33*	*0.5*	*0.5 - 1000*
TRP	*23.2 (0.5)*	*0.2*	*0.6*	*0.6 - 1000*

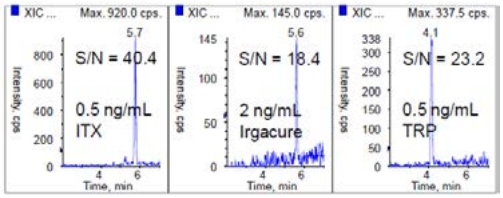

Figure 5. Signal to Noise (S/N) of Low Level Migrant Standards (S/N Calculated using Peak-to-Peak Algorithm).

This MRM data was then used to quantify migrants in cap extracts, examples of various extracts are given in Figures 6 and concentrations of migrants were summarized in Table 5.

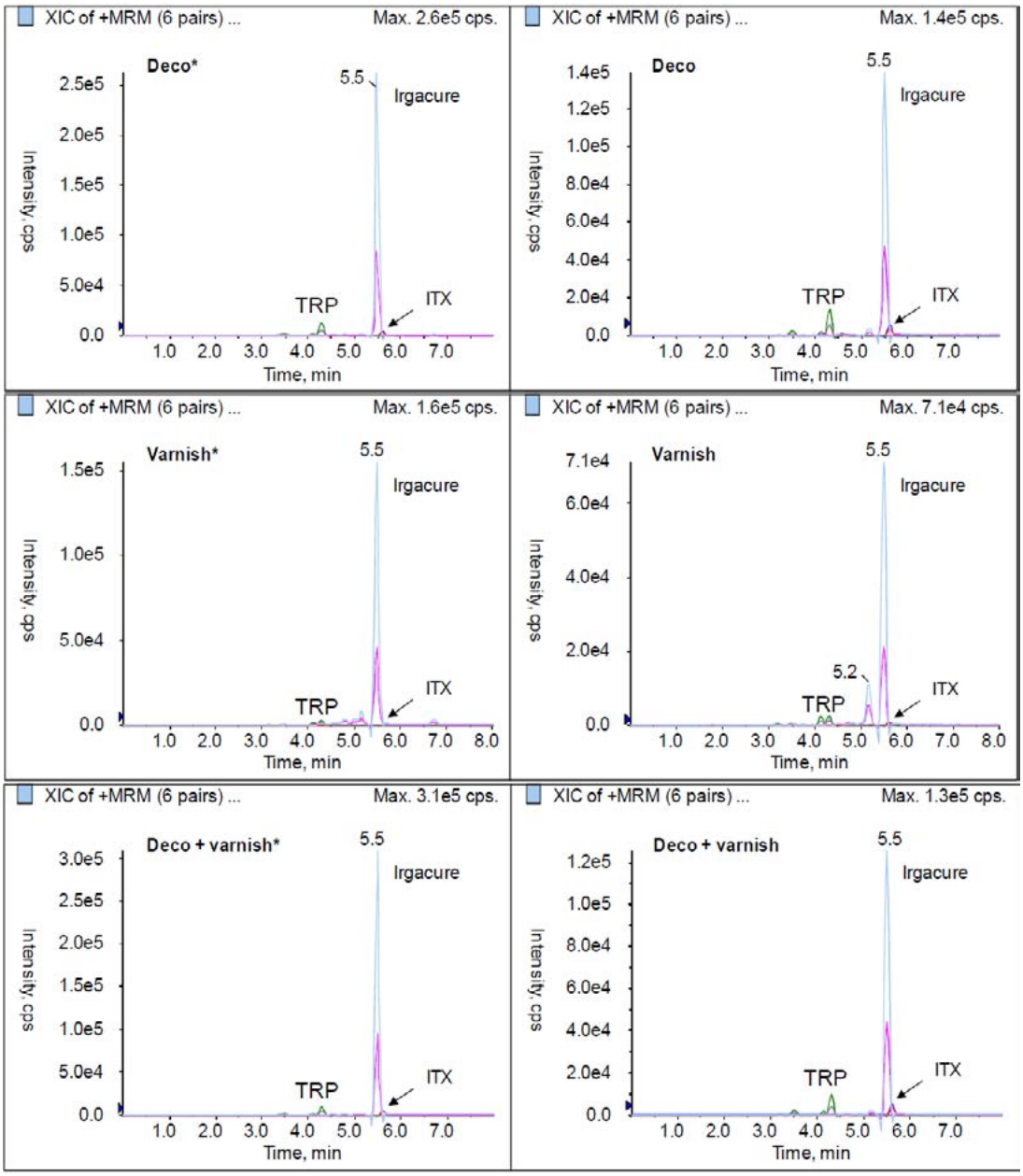

Figure 6. Sample Preparation Recoveries. A comparison of food packaging samples extracted with acetonitrile and where the acetonitrile extract of the same sample had been evaporated to dryness and reconstituted in mobile phase* (cap with decoration (top), cap sealed with varnish (middle), and cap with decoration and sealed with varnish (bottom)).

Table 5. Quantitation Results from Real Samples.

Extract	ITX (ng/dm²)	Irgacure (ng/dm²)	TRP (ng/dm²)
Deco	*4.43*	*6320*	*5.39*
*Deco**	*4.96*	*4347*	*5.77*
Varnish	*0.08*	*3940*	*0.67*
*Varnish**	*0.54*	*2100*	*0.69*
Deco + Varnish	*4.65*	*6750*	*3.97*
*Deco + Varnish**	*4.26*	*3687*	*3.99*

* sample was evaporated to dryness and reconstituted in the same volume of mobile phase A to improve HPLC peak shape.

To further identify the migrant the automatically acquired EPI spectra was searched against a mass spectral library previously created with spectra obtained from 10 ng/mL standards. DBS enabled the acquisition of high quality MS/MS spectra even for co-eluting compounds. The Purity Fit shown in Table 6 indicated if the spectrum, in the extract, was a good match for the library spectrum, generally a fit above 70% indicated a positive identification of the migrant in the extract.

Table 6. The Purity Fit (%) Results. Taken from the spectra obtained from contaminants in real samples when compared with those in a library of spectra of standards.

Extract	ITX (ng/dm²)	Irgacure (ng/dm²)	TRP (ng/dm²)
Deco	*78*	*88*	*81*
*Deco**	*87*	*28*	*31*
Varnish	*63*	*60*	*98*
*Varnish**	*34*	*81*	*44*
Deco + Varnish	*97*	*44*	*65*
*Deco + Varnish**	*91*	*57*	*95*

* sample was evaporated to dryness and reconstituted in the same volume of mobile phase A to improve HPLC peak shape.

Summary

The LC-MS/MS method developed can be used for quantitation of migrants in food packaging material. The sensitivity levels of the 3200 QTRAP® system were high enough to detect migrants at 0.01 mg/kg in extracts. A mass spectral library containing of EPI spectra at different standardized Collision Energy and Collision Energy Spread values can then be used to identify the compound at the required matrix detection levels, enabling direct injection analysis on extracts.

Acknowledgements

We acknowledge Mr Philippe Tourelle and Gilles Jarry of the society Impress Metal Packaging (France) for supplying extracts and samples.

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Publication number: 1830210-01

Increasing Selectivity and Confidence in Detection when Analyzing Phthalates by LC-MS/MS

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Overview

Recent issues with the determination of phthalates in food and beverages like yogurt, sport drinks and fruit juices have highlighted the need for both food manufacturers and regulatory agencies to utilize fast and accurate analytical techniques to proactively ensure product safety.

A fast and sensitive LC-MS/MS method was developed for the analysis of 22 phthalates utilizing a simple extraction, fast LC separation using a Phenomenex Kinetex™ C18 column with a run time of 10 minutes, and selective MS/MS detection using an SCIEX QTRAP® 5500 system operated in Multiple Reaction Monitoring (MRM) mode. Major challenges of method development were the presence of chemical background and matrix interferences. To address these challenges we successfully applied the unique MRM^3 mode to enhance detection selectivity by detecting second generation product ions and Enhanced Product Ion (EPI) scanning to increase confidence in identification using the molecular fingerprint of each target analyte saved into the MS/MS spectrum. In addition, the SCIEX SelexION™ technology was used to separate critical isomers using Differential Mobility Spectrometry (DMS).

Introduction

Phthalates are widely used industrial chemicals with an estimated annual production of over 8,000,000 tons. Phthalates are added to plastics to increases flexibility, transparency, and longevity. By weight, they contribute 10-60% of plastic products. Phthalates are used in a variety of products, including building materials (caulk, paint, adhesives), household products (vinyl upholstery, shower curtains, food containers and wrappers), and cosmetics.[1]

The use of various phthalates is restricted in many countries because of health concerns.[2-3]

In 2011, the illegal use of bis(2-ethylhexyl) phthalate (DEHP) and Diisononyl phthalate (DINP) in clouding agents for use in food and beverages has been reported in Taiwan.[4]

As a result fast and reliable methods for the detection of different phthalates in food and beverages are needed. Chromatographic techniques coupled to mass spectrometry are methods of choice because of their sensitivity and selectivity.[5]

Here we present a new and unique LC-MS/MS method using the SCIEX QTRAP® 5500 system operated in MRM, MRM^3, and EPI mode to detect 22 phthalates. In comparison to GC-MS the developed LC-MS/MS method has several advantages:

- Reduced sample preparation and no need for derivatization
- Superior quantitative results with shorter run times
- Higher degree of confidence due to the presence of the quasi-molecular ion and characteristic fragment ions

In addition, DMS was used to separate isomeric phthalates using the SCIEX SelexION™ technology.

Contents

Experimental

Sample Preparation

One gram sample was homogenized and extracted with 45 mL methanol using ultra sound for 30 min. An aliquot of 5 mL was transferred into a vial and centrifuged for 10 min (3500 rpm). The supernatant was further diluted for LC-MS/MS analysis.

LC Separation

LC separation was achieved using an Agilent 1200 system with a Phenomenex Kinetex C18 (100 x 4.6 mm; 2.6 µm) column and a fast gradient of water + 10 mM ammonium acetate and methanol at a flow rate of 500 µL/min.

MS/MS Detection

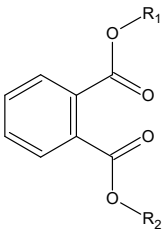
The SCIEX QTRAP® 5500 system was used with Turbo V™ source and Electrospray Ionization (ESI) source. Two selective MRM transitions were monitored for each targeted analyte (Table 1). MRM^3 was used to differentiate between isomers and to increase selectivity to reduce interferences.

DMS Separation

The SCIEX SelexION™ technology was used to selectively detect isomeric phthalates. A Separation voltage (SV) of 3800 V was used with acetonitrile as chemical modifier. The Compensation Voltage (CoV) was optimized for each target analyte specifically.

Results

Phthalates are esters of 1,2-benzenedicarboxylic acid.



Targeted analytes of this project are listed in Table 1.

All plastic material (i.e. pipette tips) was avoided when handling samples and making dilutions. All glassware was cleaned carefully to avoid contamination. Different organic solvents (LC and LC-MS grade) were evaluated and distilled water was used to minimize background interferences.

Solid Phase Extraction (SPE) is known to be a major source of phthalate contamination resulting in over-estimation and false positive results.[5] Thus, a simple and fast procedure using liquid extraction was developed and successfully applied to the analysis of food and beverage samples.

Different LC conditions were evaluated during method development. In general C18 material with a neutral buffer of ammonium acetate was found to give good separation. Methanol is organic modified was more efficient in separating isomers. The Phenomenex Kinetex C18 column was finally chosen because of its UHPLC like efficiency and resolution at significantly lower column pressure resulting in high robustness and long instrument up time.

The final gradient started at 50% methanol and included a cleanup step at 98% methanol at a flow rate of 1000 µL/min to reduce background levels.

In addition, a trap column was used between pump and autosampler to retain any phthalates originating from the HPLC system.

MRM transitions were fully optimized with $M+H^+$ as precursor ion and two compound dependent fragment ions. The dominating fragment ions were protonated phthalic acid (167), phthalic anhydride (149), and different esters of phthalic acid and phthalic anhydride (Figure 1).

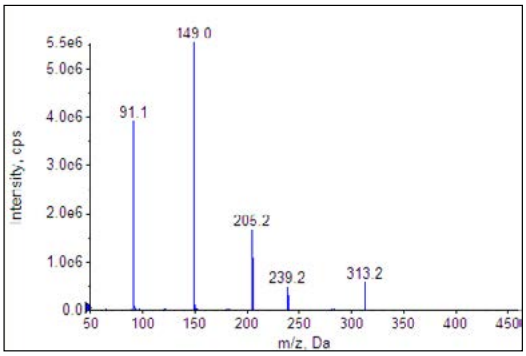


Figure 1. EPI spectrum of BBP, the molecular fingerprint saved into the MS/MS spectrum was used for compound identification with highest confidence

Table 1. Targeted phthalates, compound information, and optimized MRM transitions (Q1 and Q3 ions)

Phthalate		CAS	Formula	M.W.	Q1	Q3
Dimethyl phthalate	DMP	131-11-3	$C_{10}H_{10}O_4$	194.18	195	163 / 133
Diethyl phthalate	DEP	84-66-2	$C_{12}H_{14}O_4$	222.24	223	149 / 177
Diallyl phthalate	DAP	131-17-9	$C_{14}H_{14}O_4$	246.26	247	189 / 149
Dipropyl phthalate	DPrP	131-16-8	$C_{14}H_{18}O_4$	250.29	251	149 / 191
Diisopropyl phthalate	DIPrP	605-45-8	$C_{14}H_{18}O_4$	250.29	251	149 / 191
Dibutyl phthalate [EU, EPA]	DBP	84-74-2	$C_{16}H_{22}O_4$	278.34	279	149 / 205
Diisobutyl phthalate [EPA]	DIBP	84-69-5	$C_{16}H_{22}O_4$	278.34	279	149 / 205
Bis(2-methoxyethyl) phthalate	DMEP	117-82-8	$C_{14}H_{18}O_6$	282.29	283	207 / 59
Dipentyl phthalate [EPA]	DPP	131-18-0	$C_{18}H_{26}O_4$	306.40	307	219 / 149
Diisopentyl phthalate	DIPP	605-50-5	$C_{18}H_{26}O_4$	306.40	307	219 / 149
Bis(2-ethoxyethyl) phthalate	DEEP	605-54-9	$C_{16}H_{22}O_6$	310.34	311	221 / 149
Benzyl butyl phthalate [EU, EPA]	BBP	85-68-7	$C_{19}H_{20}O_4$	312.37	313	149 / 205
Diphenyl phthalate	DPhP	84-62-8	$C_{20}H_{14}O_4$	318.32	319	225 / 77
Dicyclohexyl phthalate	DCHP	84-61-7	$C_{20}H_{26}O_4$	330.42	331	167 / 249
Bis(4-methyl-2-pentyl) phthalate	BMPP	146-50-9	$C_{20}H_{30}O_4$	334.46	335	167 / 251
Dihexyl phthalate	DHXP	84-75-3	$C_{20}H_{30}O_4$	334.46	335	149 / 233
Di-n-heptyl phthalate	DHP	3648-21-3	$C_{22}H_{34}O_4$	362.51	363	149 / 233
Bis(2-n-butoxyethyl) phthalate	DBEP	117-83-9	$C_{20}H_{30}O_6$	366.45	367	101 / 249
Bis(2-ethylhexyl) phthalate [EU, EPA]	**DEHP**	**117-81-7**	**$C_{24}H_{38}O4$**	**390.56**	**391**	**167 / 279**
Di-n-octyl phthalate [EU, EPA]	DNOP	117-84-0	$C_{24}H_{38}O_4$	390.56	391	261 / 149
Diisononyl ortho-phthalate [EU, EPA]	**DINP**	**28553-12-0**	**$C_{26}H_{42}O_4$**	**418.61**	**419**	**275 / 149**
Diisodecyl ortho-phthalate [EU, EPA]	DIDP	26761-40-0	$C_{28}H_{46}O_4$	446.66	447	149 / 289

Bold Illegally used in food and beverages in Taiwan in 2011[4]
EU Restricted use in toys and childcare articles in Europe[2]
EPA Addressed in the phthalates action plan of the U.S. Environmental Protection Agency[3]

An example chromatogram of LC-MS/MS detection of 22 phthalates is shown in Figure 2.

Limits of detection (LOD), linearity and accuracy of quantitation were determined. Example chromatograms of six high priority phthalates (from 1 to 100 ng/mL) are shown in Figure 3a and 3b.

For all targeted phthalates an LOD of at least 1 ng/mL was achieved. Please note that the final LOD greatly depends on background interferences which can greatly vary from laboratory to laboratory.

Table 2. Accuracy and linearity of six high priority phthalates

Phthalate	Accuracy (%)	Regression
DBP	97-103	0.9998
BBP	91-108	0.9999
DEHP	88-108	0.9989
DNOP	85-113	0.9982
DINP	92-111	0.9998
DIDP	94-109	0.9931

Contents

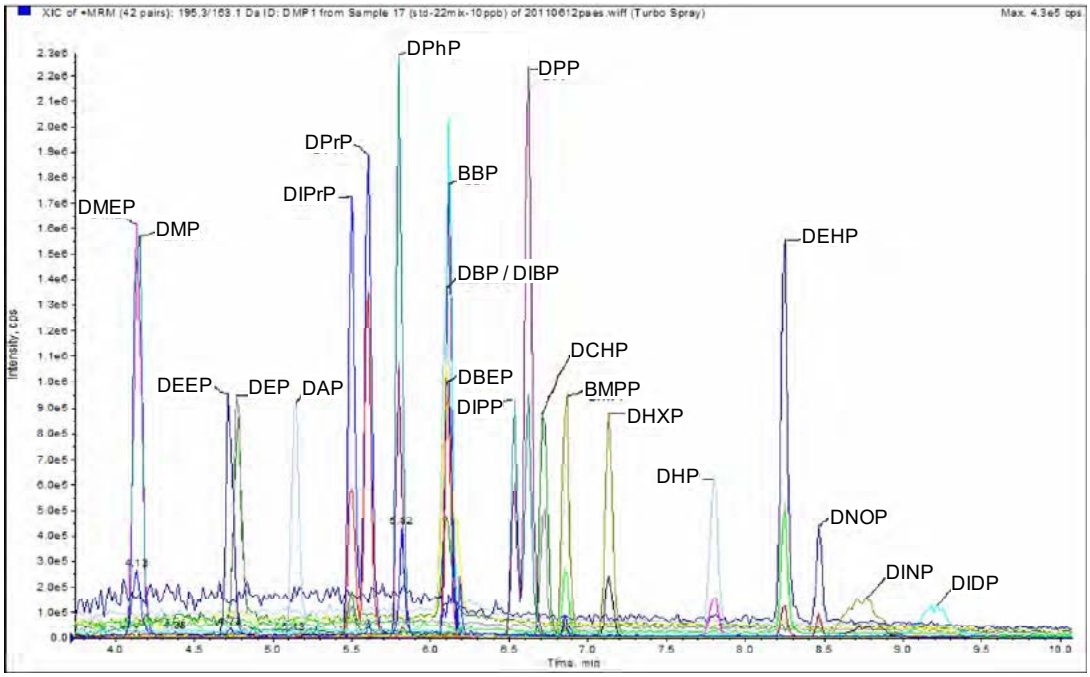


Figure 2. Example LC-MS/MS chromatogram showing the separation and detection of 22 phthalates at a concentration of 10 ng/mL

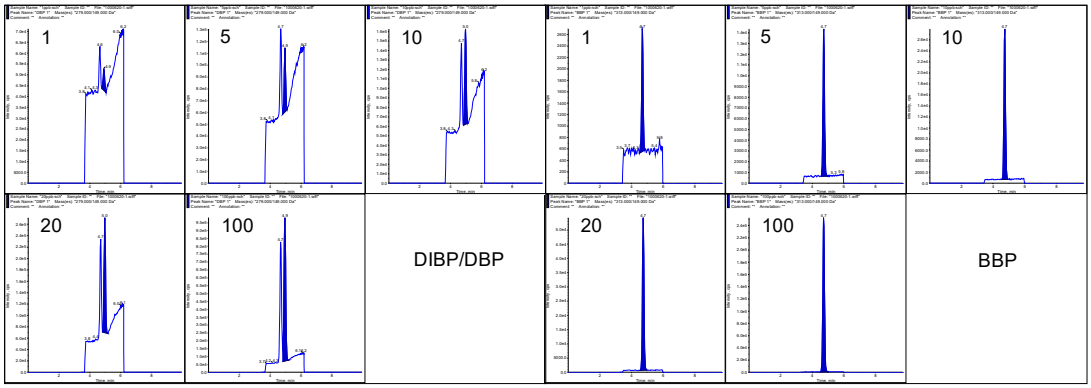


Figure 3a. MRM chromatograms of the high priority phthalates DBP and BBP at 1, 5, 10, 20, and 100 ng/mL

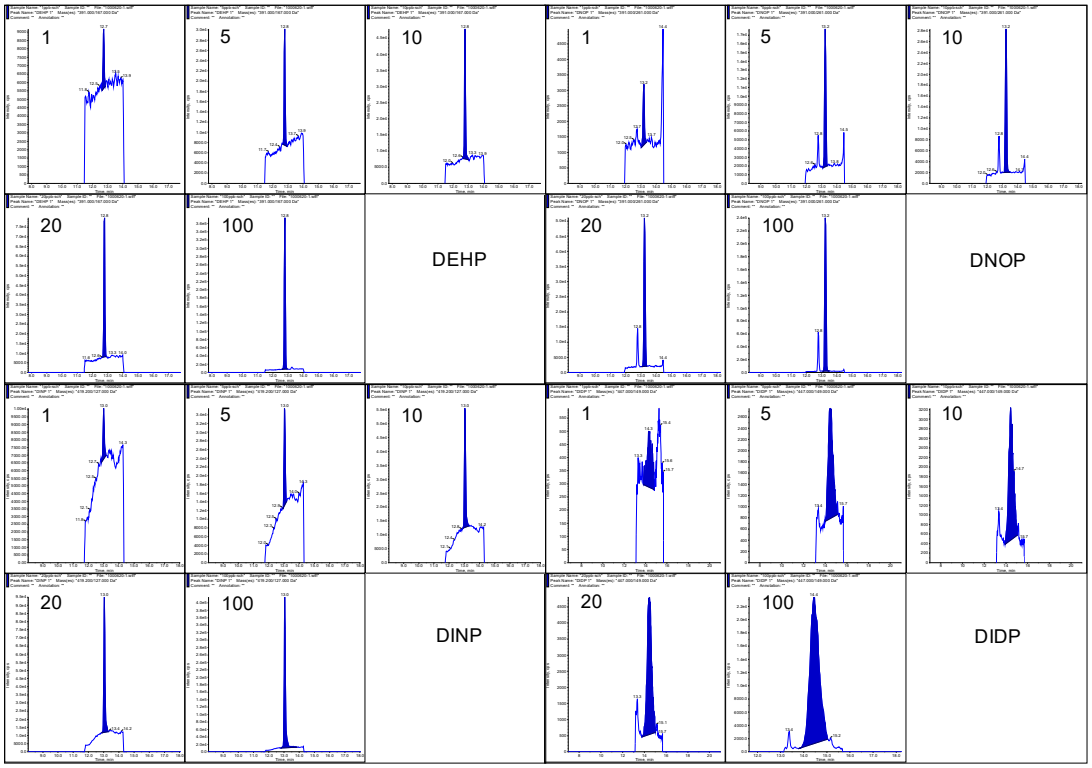


Figure 3b. MRM chromatograms of the high priority phthalates DEHP, DNOP, DINP, and DIDP at 1, 5, 10, 20, and 100 ng/mL

The accuracy was typically between 85 and 115% and quantitation was performed with linear regression and 1/x weighting. The coefficient of regression was above 0.999 for all analytes. Examples for accuracy and linearity are of six high priority phthalates are listed in Table 2.

The unique scan function of MRM^3 of the SCIEX QTRAP® 5500 system was investigated for its potential to differentiate isomeric species.

An example of successfully differentiating between the isomers DIBP and DBP using the different fragmentation pattern in MRM^3 mode is shown in Figure 4. Using traditional MRM mode both compounds had the exact same transitions and needed to be separated on the LC time scale. Thus, MRM^3 allows speeding up the LC method if throughput requires.

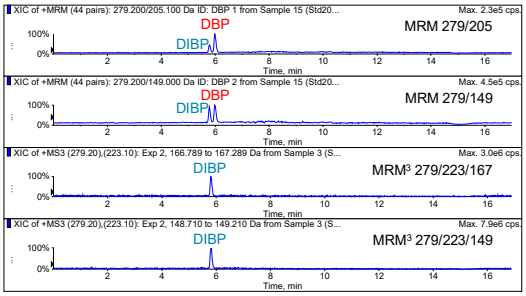


Figure 4. Differentiation of DIBP and DBP using the different fragmentation pattern in MRM^3 mode in comparison to MRM mode

Contents

Another possibility to enhance selectivity of detection is the use of Differential Mobility Spectrometry (DMS). The SCIEX SelexION™ technology uses a planar DMS cell attached between the curtain plate and orifice plate of the mass spectrometer. Ions are separated based on difference in their high field and low field mobility.SV and CoV are optimized to correct the trajectory of a desired ion. In addition, a chemical modifier can be introduced to alter separation characteristics.

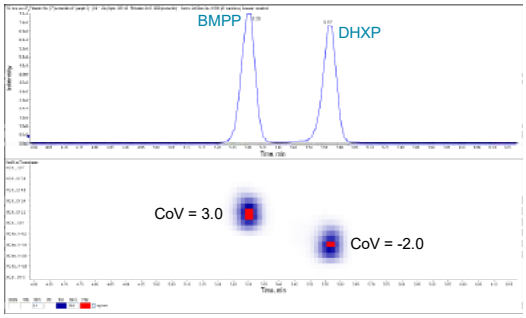


Figure 5a. Separation of the isomers BMPP and DHXP, both phthalates can be separated in the LC and DMS space resulting in increased selectivity

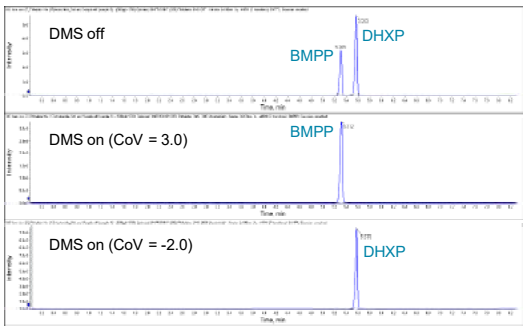


Figure 5b. Selective detection of BMPP and DHXP by compound specific CoV for each analyte, acetonitrile was introduced as chemical modifier

The example presented in Figure 5a and 5b highlights the unique selectivity achieved using DMS. The isomers BMPP and DHXP were separated using different CoV. Acetonitrile was introduced as chemical modifier to enhance separation.

Summary

A fast and sensitive LC-MS/MS method was developed for the detection of 22 phthalates in food and beverage samples. All possible precautions were taken to reduce chemical background. This included the avoidance of plastic material, careful handling of laboratory glassware, systematic evaluation of different LC solvents, a simple extraction procedure, and the use of a trap column inside the LC system.

All 22 phthalates were detected with an LOD of 1 ng/mL or lower, good accuracy, and linearity using two MRM transitions per analyte. Characteristic EPI spectra can be used to further increase confidence of compound identification based on characteristic MS/MS spectra and library searching.

In addition, the unique scan function MRM^3 of the QTRAP® 5500 system and the new SCIEX SelexION™ technology were successfully used to separate isomeric species enhancing the selectivity of LC-MS/MS detection.

Acknowledgement

The authors wish to thank Ching-Hsin Tung (Food and Drug Administration, Taiwan), Dr. Sheng-Che Lin (Tainan city health bureau, Taiwan) and Dr. Dunming Xu (CIQ Xiamen, China) for their assistance and advice during method development.

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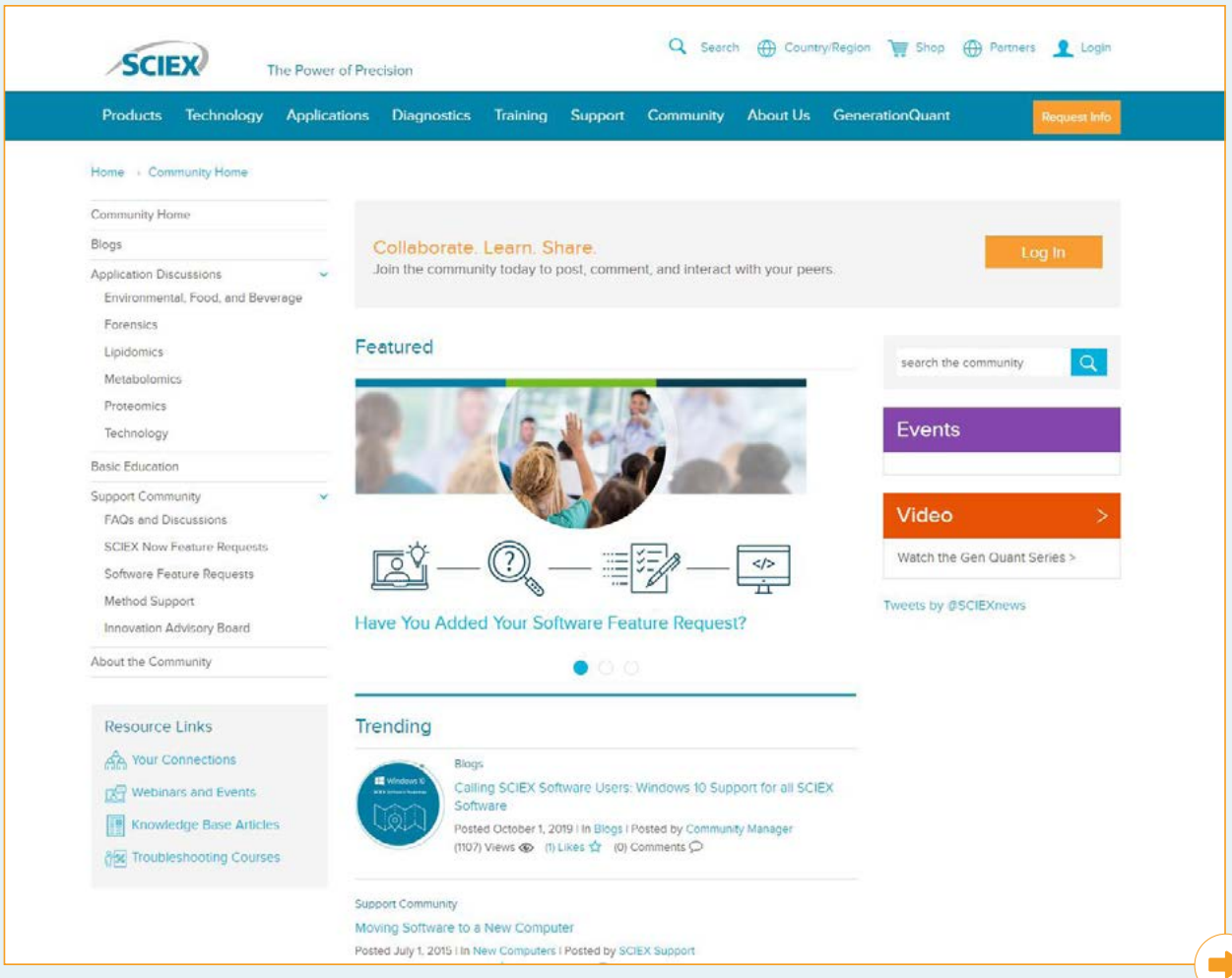

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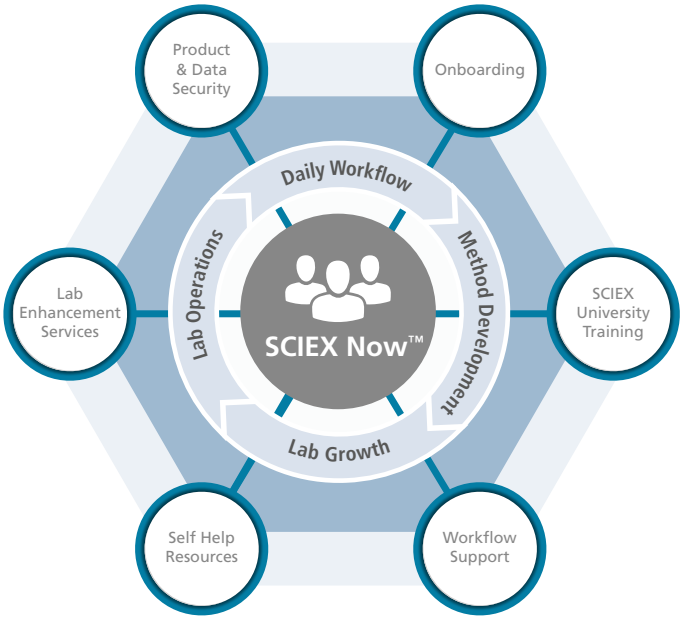
Increase your confidence with compliance services to help you safeguard your data, confirm data integrity, and ensure system modifications can be traced.

Onboarding

We register you to SCIEX Now Online, enroll you to your SCIEX University learning path, and send you a welcome email.



Lab Enhancement Services

SCIEX Lab Enhancement Services apply a holistic approach to your lab to increase productivity and reduce system downtime.

SCIEX University

Best-in-class content, personalized learning paths – delivered using the latest memory science techniques.

Self Help Resources

Our Knowledge Base and Community help you move your science forward and find the answers you need from SCIEX experts as well as your peers.

Workflow Support

Whatever your challenge, the SCIEX Support team is here to help you achieve your scientific goals quickly and efficiently.

Start Your Path to Success Now: sciex.com/sciexnow

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RUO-MKT-03-2692-A

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