Food Compendium

Volume 2

SCIEX

The Power of Precision

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SCIEX Food Compendium Volume 2

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Perfect Balance to Elevate your Lab's Performance

Using the X500R QTOF System and SCIEX OS Software to Quickly Identify Unknowns in Food Samples

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Overview

Here we present results using a new method to identify unexpected chemical residues and contaminants in food using the SCIEX X500R QTOF System. Samples were extracted using a QuEChERS method and analyzed by LC-HR-MS/MS.

Unknown compounds were automatically identified by using a non-target peak finding algorithm followed by sample-control-comparison to separate matrix and sample specific signals from true contaminations. TOF-MS and MS/MS data for ions of interest were automatically processed using formula finding and searched against mass spectral libraries and online databases, such as ChemSpider, for identification. The SCIEX OS Software offers an easy to use and intuitive workflow to tentatively identify unexpected chemicals in food.

Introduction

Hybrid LC-MS/MS systems like quadrupole-quadrupole Time-of-Flight (QTOF) provide the ability to perform targeted and non-targeted screening in food samples on a routine basis.

The SCIEX X500R QTOF System is a robust, high performance high resolution MS/MS system designed for routine use providing:

- Sensitivity to easily detect compounds at relevant concentrations
- Resolving power to remove interference from complex food matrices
- Linearity over up to 3 orders of magnitude to identify compounds at different concentration levels
- Mass accuracy to identify compounds following regulatory guidelines
- Confident identification using MS/MS spectra and ion ratios
- Industry leading robustness of Turbo V™ source and Curtain Gas™ interface

Full scan chromatograms are very rich in information and easily contain thousands of ions from any chemical present in the sample, including the food matrix itself. Powerful software is

needed to explore the high resolution MS/MS spectra generated to get answers and results from these complex data.

The SCIEX OS Software is a single platform for MS control, data processing and reporting, and provides:

- Simple software workflows that deliver reliable results
- Automated identification of unknowns
- Quick data review and reporting utilizing customizable flagging and filtering of results

Experimental

Sample preparation

Food samples from a local supermarket were extracted using a QuEChERS procedure following guideline EN 15662/2007. Sample extracts were diluted 10x to minimize possible matrix effects.

LC Separation

LC separation was performed using a SCIEX ExionLC™ AC System with 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).

The injection volume was 5 µL.

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Table 1. Gradient conditions used for unknown screening

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

MS/MS Detection

The SCIEX X500R QTOF System with Turbo V™ source and Electrospray Ionization (ESI) was used.

Mass calibration was achieved using the integrated calibrant delivery system (CDS) with the TwinSprayer probe (dual ESI needle).

High resolution data were acquired using an IDA method consisting of a TOF-MS survey (100-1000 Da for 100 msec) and up to 20 dependent MS/MS scans (50-1000 Da for 35 msec). MS/MS fragmentation was achieved using CE of 35 V with a collision energy spread (CES) of ±15 V.

Dynamic background subtraction (DBS) was activated for best MS/MS coverage, and no inclusion list was used to also allow retrospective unknown identification without the need for a second injection to acquire MS/MS data.

Data Acquisition and Processing

All data were acquired and processed using SCIEX OS Software version 1.0, which showcases a thoughtfully designed user interface that is fast to learn and delivers improved lab productivity.

Results and Discussion

X500R Performance Characteristics

Resolution > 20,000 (at full width half height) and mass accuracy <5 ppm are often sufficient to separate the analytes of interest from interfering matrices and, thus, are identified as the set requirements for compound identification in various guidelines.[1, 2]

The X500R QTOF system utilizes N-optics design to maximize resolution while maintaining benchtop design and a minimized footprint (Figure 1). Its resolving power increases with mass range providing ~30000 to 40000 for the typical molecular weight range of pesticides.[3]

The 4 mm orifice leading into the TOF accelerator delivers resolution without compromise in sensitivity. The sensitivity of the X500R QTOF system is comparable to a SCIEX QTRAP® 5500 System operated in MRM mode, allowing extract dilution to minimize ion suppression while detecting easily at 10 µg/kg levels.[3]

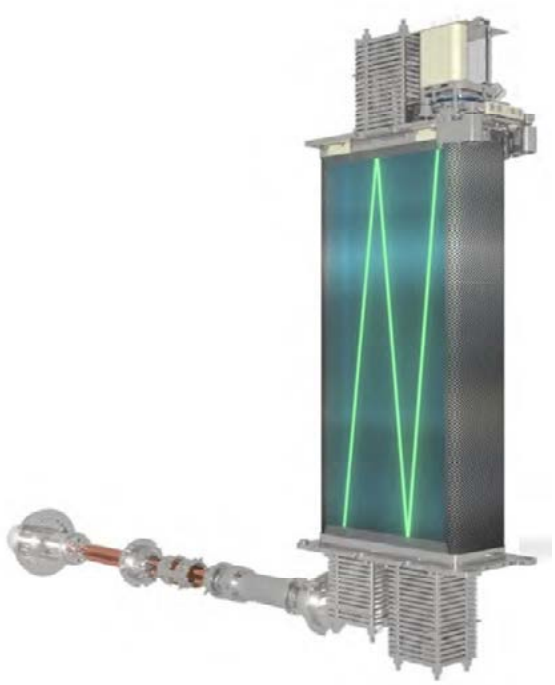

Figure 1. N-optics design of the X500R QTOF system to maximize resolution while maintaining benchtop design and a minimized footprint, 6 heater drones are integrated into the TOF path to maintain mass accuracy and robustness

The X500R QTOF system achieves stable mass accuracy of less than 2 ppm by using a heated TOF configuration, with 6 heater drones throughout the TOF path to maintain mass accuracy and robustness. In addition, the integrated CDS with the TwinSprayer probe provides an independent calibrant

delivery path for reliable auto-calibration. The CDS setup maintains mass accuracy over long periods of time by automatically calibrating in batch mode (it is recommended to infuse a calibrant standard every hour or two).

Furthermore, the X500R QTOF's mass accuracy is supplemented by legendary dynamic transmission control and dynamic background calibration, introduced in 2010 with the TripleTOF® system and optimized over time.

While accurate mass measurement of the molecular ion is important for empirical formula finding, this is not the only information available. Combining all available accurate mass MS and MS/MS information is crucial to minimize the list of potential formulae. Figures 2, 3 and Table 2 illustrate that the number of formulae can be reduced from over 200 to a single match by not only using the accurate mass of the molecular ion but also including the isotope pattern and MS/MS matching in the formula-finding algorithm.

Using the combined scoring of MS and MS/MS matches, SCIEX OS lists the most likely chemical formula at the top of results table. Also, SCIEX OS downloads a ChemSpider hit count for each calculated formula which further assists in identifying the correct result (Figure 2).

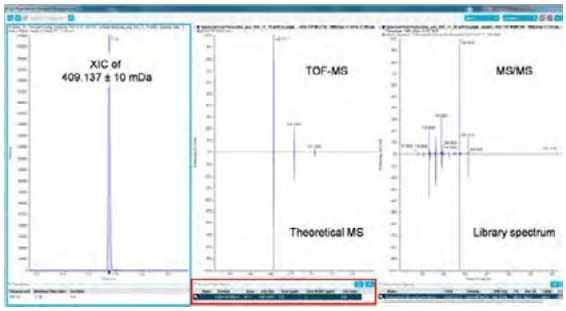

Figure 2. TOF-MS and MS/MS spectra used for empirical formula finding, results are ranked by a combined score using MS and MS/MS information, and when combined with the ChemSpider hit count, can be used to quickly find the correct match

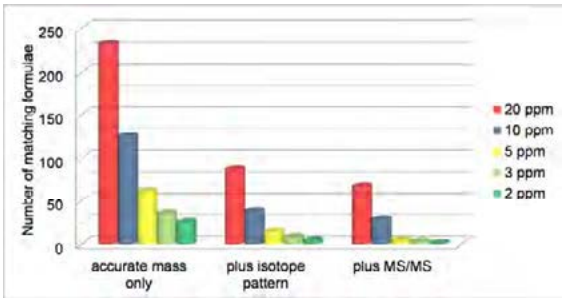

Figure 3. Number of matching molecular formulae depending on the information and mass accuracy used for empirical formula finding (elements allowed $C_{49}H_{75}Br_3Cl_5F_3I_3N_{10}O_{10}PS_3$)

Table 2. Ranking of matching formulae using MS and MS/MS information collected for Trifloxystrobin, the MS rank combines mass accuracy and isotope pattern matching and the MS/MS rank combines mass accuracy and number of ions (n)

Hit	Formula	MS Rank	ppm	MS/MS Rank	ppm (n=11)
1	$C_{20}H_{19}F_3N_2O_4$	2	0.3	2	2.0
2	$C_{21}H_{15}F_3N_6$	9	-2.9	4	3.0
3	$C_{18}H_{16}N_8O_4$	4	0.9	6	4.8
4	$C_{15}H_{17}FN_8O_5$	11	-1.9	5	4.8
5	$C_{16}H_{13}FN_{12}O$	7	-5.2	10	9.0
6	$C_{14}H_{20}F_3N_6O_3P$	22	2.8	1	2.0
7	$C_{16}H_{21}N_6O_5P$	7	-3.1	11	9.4
8	$C_{23}H_{18}F_2N_2O_3$	9	3.1	14	9.4
9	$C_{21}H_{23}F_2O_4P$	1	-0.9	24	22.1
10	$C_{19}H_{21}FN_2O_7$	16	-8.4	12	9.4

In addition to more efficient formula finding, MS/MS spectra are also needed for structural elucidation. Without MS/MS spectra it is impossible to conclude a correct structure from a molecular formula alone.

The example shown in Figure 4 highlights the need of fragment ion detection to confidently differentiate between isomers Prometon and Terbumeton.

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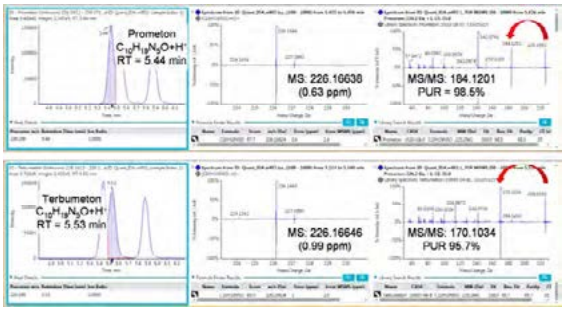

Figure 4. Confident identification of isomers Prometon and Terbumeton using characteristic MS/MS fragment ions and MS/MS library searching

Processing Workflow for Unknown Identification in SCIEX OS Software

Extracted Ion Chromatograms (XIC) are generated using a non-target peak finding algorithm. No masses or retention times are provided to find chromatographic features. Sample-control-comparison is used to separate matrix and sample-specific signals from true contaminations.

High resolution TOF-MS and MS/MS data of ions of interest are automatically processed using:

- MS/MS library searching to identify compounds already present in existing libraries
- Empirical formula finding based on TOF-MS and MS/MS
- ChemSpider searching
- Comparison of structures retrieved from ChemSpider against the acquired HR-MS/MS spectra

The method editor in SCIEX OS Software to setup parameters and criteria for unknown identification is shown in Figure 5a-c.

Figure 5a. Method editor in SCIEX OS Software for unknown identification, selection of sample and control-sample for non-target peak finding

Figure 5b. Method editor in SCIEX OS Software for unknown identification, configuration of library search parameters

Figure 5b. Method editor in SCIEX OS Software for unknown identification, configuration of formula finding options

SCIEX offers true HR-MS/MS spectral libraries for over 2500 compounds, including pesticides, veterinary drugs, toxins, fluorochemicals, pharmaceuticals, and illicit drugs.

Results of Unknown Identification

Two samples of bell pepper, including an organic pepper, were extracted and analyzed using the developed LC-HR-MS/MS method in positive and negative polarity. Both samples were processed using the described non-target workflow.

A total of 2358 (positive polarity) and 1563 (negative polarity) chromatographic features were identified using the non-target peak finding algorithm. Less than 50 features were found to be characteristic for the contaminated bell pepper after sample-control-comparison using an area ratio of 10.

Results can be sorted and filtered for easy data review after performing sample-control-comparison. Library searching and formula finding results and scores are listed in the result table. More details and a visual display of XIC, TOF-MS and MS/MS for both samples can be found in peak review (Figure 6).

Contents

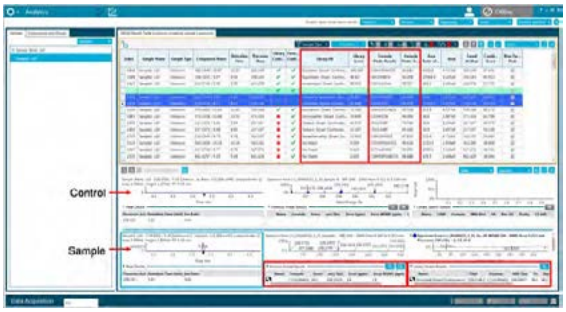

Figure 6. Results display after non-target screening, library searching and formula finding results are displayed in the table (top) and chromatograms and spectra with result details can be reviewed (bottom)

Formula finding results are displayed below the TOF-MS spectrum in the peak review window. Results are automatically ranked by mass accuracy (MS and MS/MS) and the matching of the isotope pattern. In addition the ChemSpider hit count is listed to quickly identify the correct match. The formulae can be searched against ChemSpider. Structural information from ChemSpider will be automatically compared against the acquired MS/MS spectrum to provide feedback for a quick identification.

Examples of tentatively identified pesticides in the bell pepper sample are shown in Figures 7, 8 and 9.

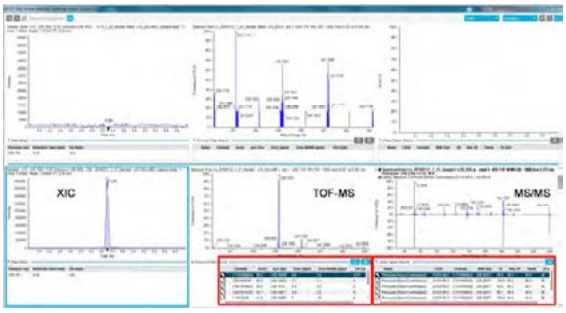

Figure 7a. Review of XIC of m/z 239.150 at RT 5.3 min and spectra with a found formula of $C_{11}H_{18}N_4O_2$

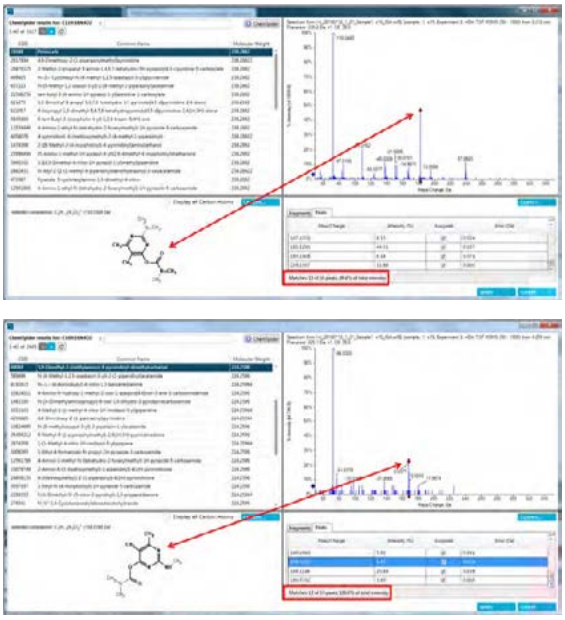

Figure 7b. The ChemSpider search and automatic elucidation of the MS/MS spectrum led to the tentative identification of Pirimicarb (top) and also of its metabolite Desmethyl-pirimicarb (bottom), both compounds were confirmed by MS/MS library searching

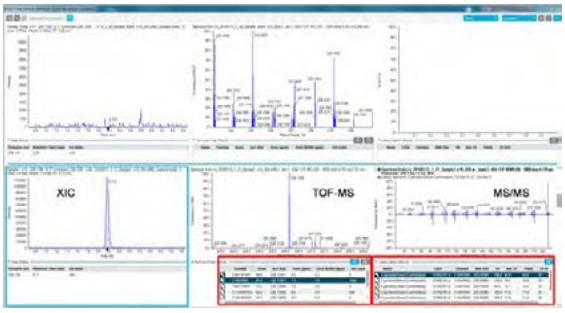

Figure 8a. Review of XIC of m/z 226.134 at RT 8.2 min and spectra with a found formula of $C_{14}H_{15}N_3$, although ranked second based on mass accuracy the high ChemSpider hit count revealed the correct match

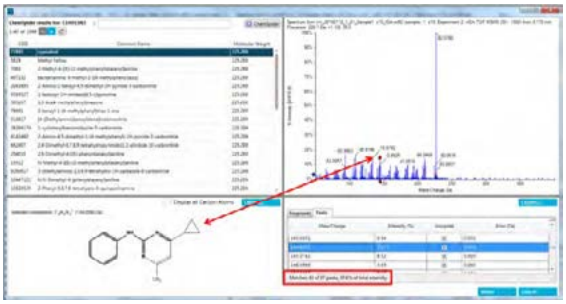

Figure 8b. The ChemSpider search and automatic elucidation of the MS/MS spectrum led to the tentative identification of Cyprodinil, this compound was confirmed by MS/MS library searching

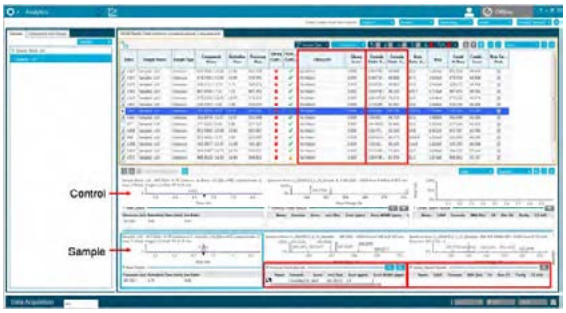

Figure 9a. Results display after non-target screening of the negative polarity data, review of XIC of m/z 367.203 at RT 6.7 min and spectra with a found formula of $C_{22}H_{28}N_2O_3$

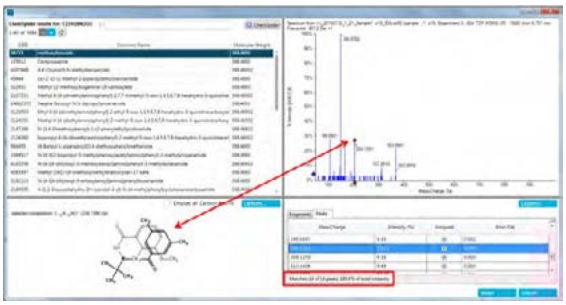

Figure 9b. The ChemSpider search and automatic elucidation of the MS/MS spectrum led to the tentative identification of Methoxyfenozide

Summary

A new method to identify unexpected chemical residues and contaminants in food samples was developed using the SCIEX X500R QTOF System. Store-bought food samples were extracted using a QuEChERS procedure and analyzed by LC-HR-MS/MS.

Data processing was performed in SCIEX OS Software. The processing workflow consists of peak finding using a non-target algorithm (no masses or retention times were provided to find chromatographic features). Automatic sample-control-comparison was used to separate matrix and sample specific signals from true contaminations. In a final step, tools such as empirical formula finding, MS/MS library searching and online database searching was used for identification.

The method was successfully applied to tentatively identify pesticide residues in vegetable samples.

References

1. EU Commission Decision 'concerning the performance of analytical methods and the interpretation of results' #2002/657/EC
2. EU Commission Guidance Document: 'on analytical quality control and method validation procedures for pesticides residues analysis in food and feed' #SANTE/11945/2015
3. André Schreiber et al.: 'Using the X500R QTOF System and SCIEX OS Software to Identify and Quantify Food Residues' Application Note SCIEX (2016) # RUO-MKT-02-3760-A

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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 a 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.

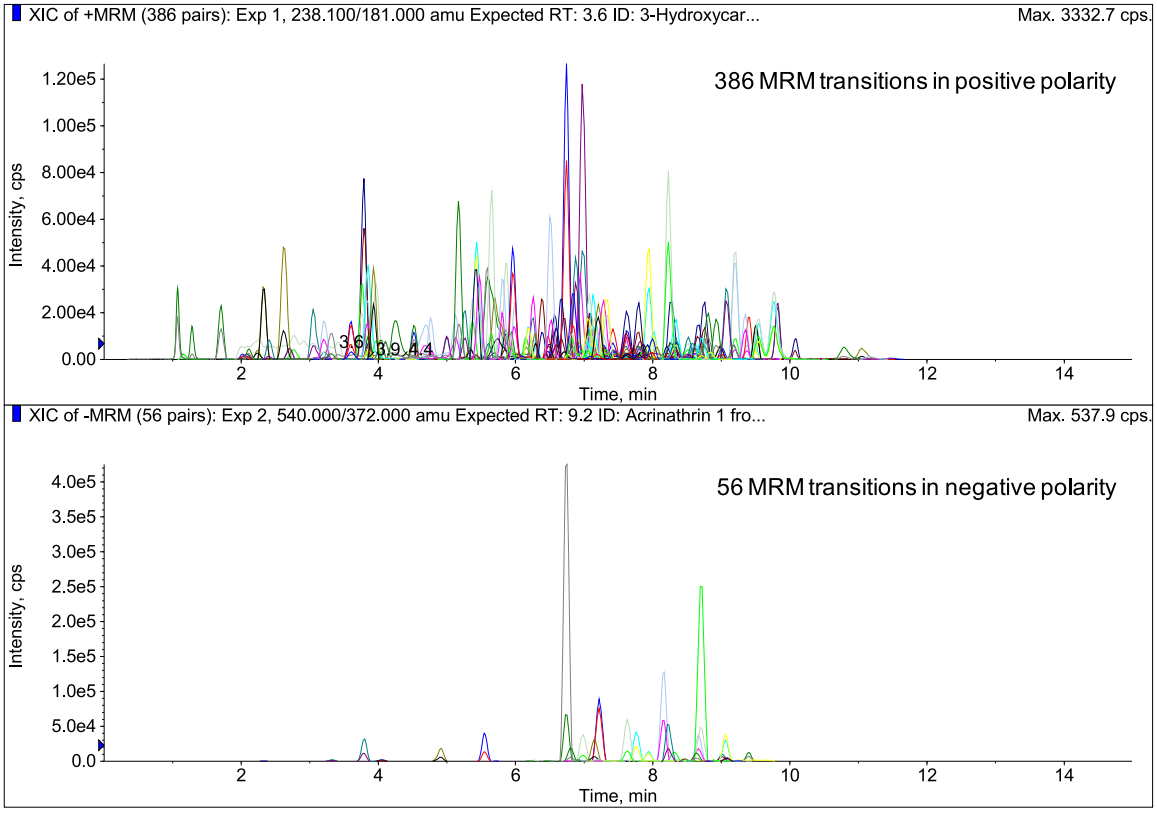

Figure 1. Detection of pesticides at a concentration of 1 ng/mL by monitoring 442 MRM transitions in positive and negative polarity using the *Scheduled* MRM™ algorithm and fast polarity switching

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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.

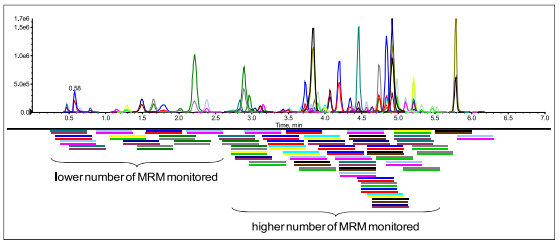

Figure 2. The *Scheduled* MRM™ Algorithm uses the knowledge of the elution of each analyte to monitor MRM transitions only in a short retention time window. This allows many more MRM transitions to be monitored in a single LC run, while maintaining maximized dwell times and optimized cycle time.

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.

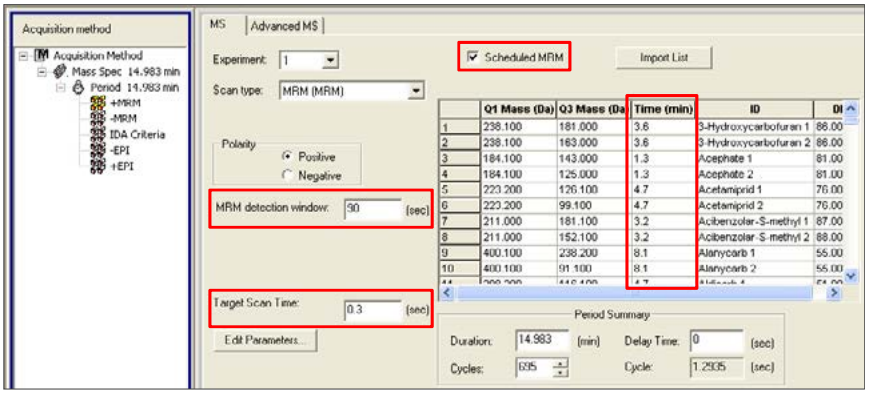

Figure 3. Acquisition method interface for *Scheduled* MRM™, in addition to traditional MRM parameters, the user provides retention times of all analytes, an MRM detection window, and a Target scan time. The software then automatically designs and optimizes the *Scheduled* MRM™ acquisition method.

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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.

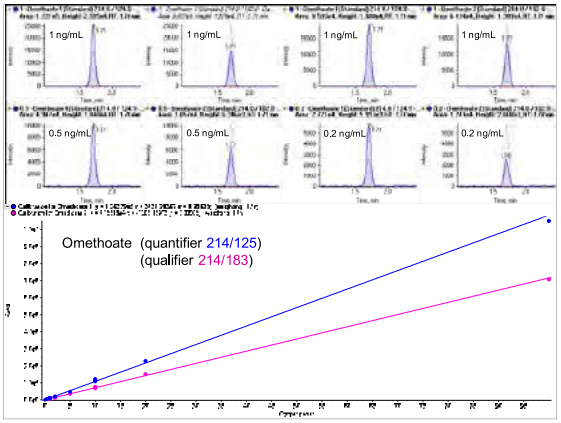

Figure 4a. Calibration lines of the quantifier and qualifier MRM transition of Omethoate from 0.1 to 100 ng/mL

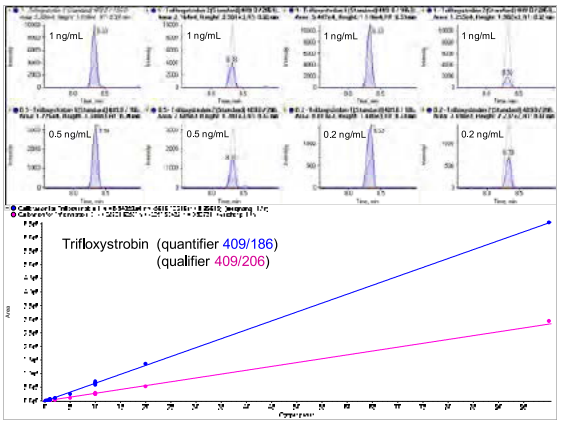

Figure 4b. Calibration lines of the quantifier and qualifier MRM transition of Trifloxystrobin from 0.1 to 100 ng/mL

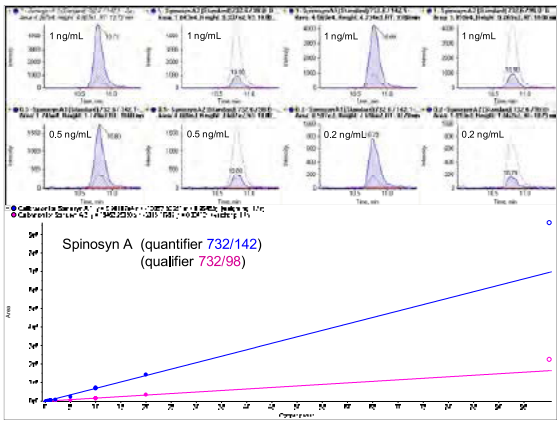

Figure 4c. Calibration lines of the quantifier and qualifier MRM transition of Spinosyn A from 0.1 to 100 ng/mL

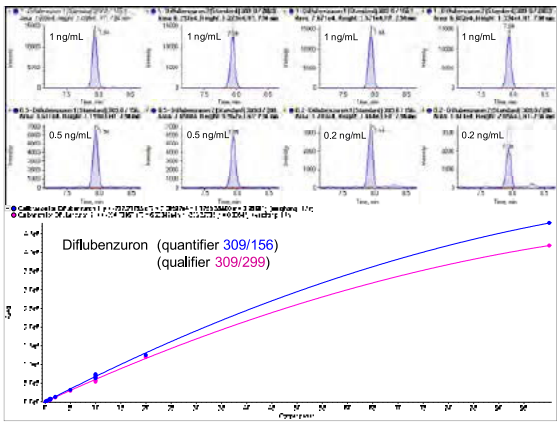

Figure 4d. Calibration curves of the quantifier and qualifier MRM transition of Diflubenzuron from 0.1 to 100 ng/mL

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.

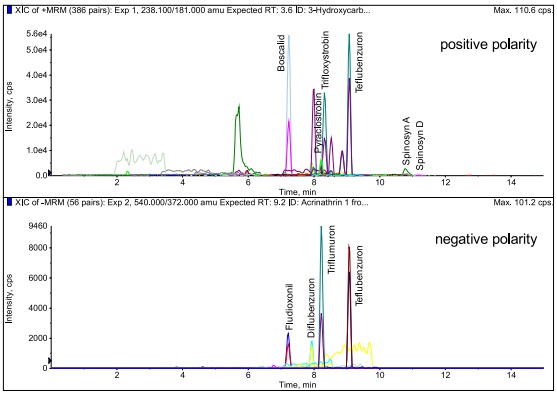

Figure 5a. Pear sample (extract 10x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

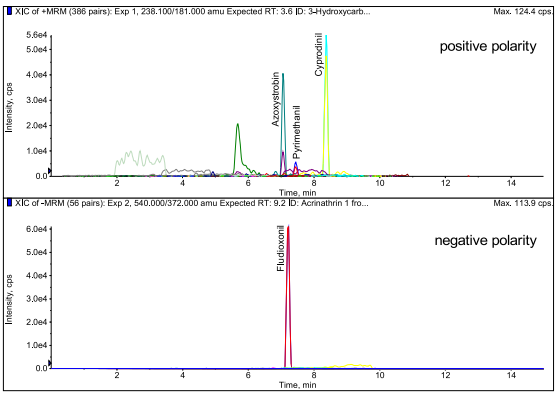

Figure 5b. Organic raspberry sample (extract 10x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

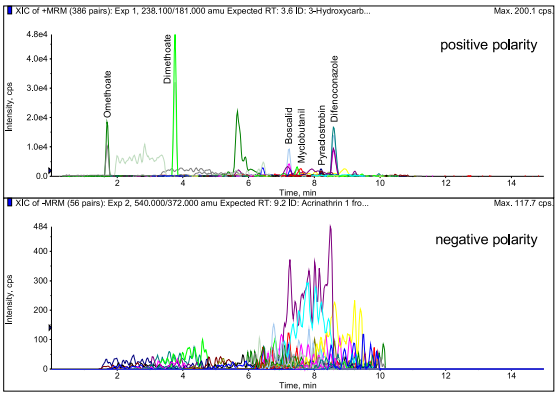

Figure 5c. Carrot sample (extract 10x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

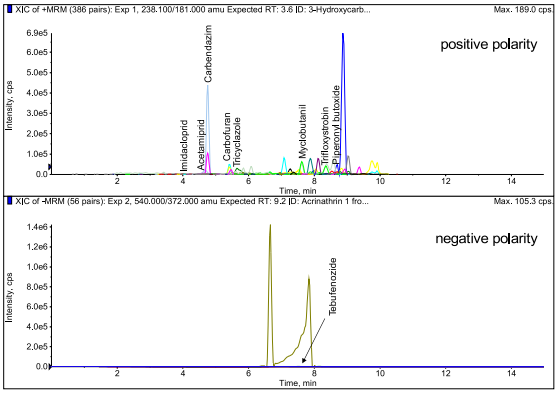

Figure 5d. Curry powder sample (extract 50x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

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

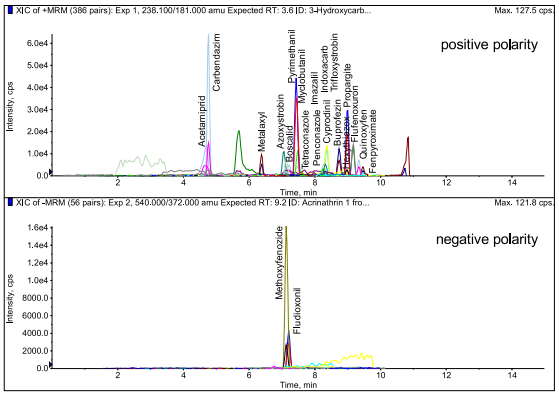

Figure 5e. Raisin sample (extract 20x diluted) screened for pesticides using *Scheduled* MRM™ and fast polarity switching, identified and quantified pesticides are summarized in Table 1

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.

Contents

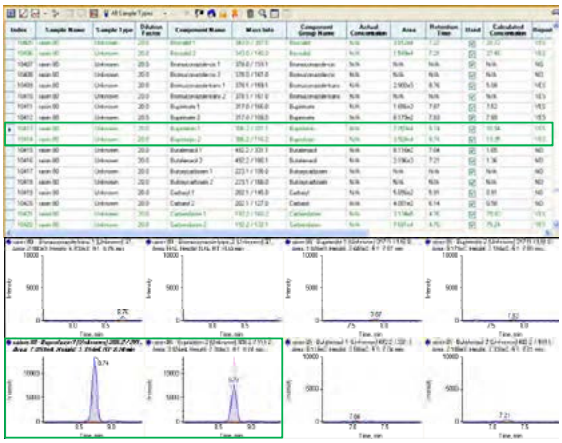

Figure 6. Results and peak review after running the 'Multicomponent' query in MultiQuant™ Software, shown here is an example from raisins, of pesticides detected above an MRL of 10 µg/kg and positively identified by automatic MRM ratio calculation (compare to Figure 5d and Table 1 for complete results).

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.

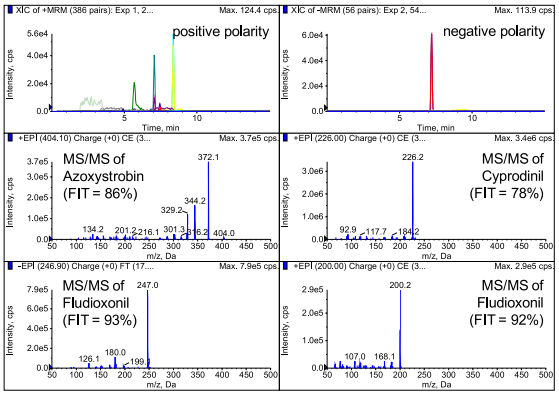

Figure 7a. Organic raspberry sample (extract 10x diluted) screened for pesticides with MS/MS library search results for additional confidence in compound identification

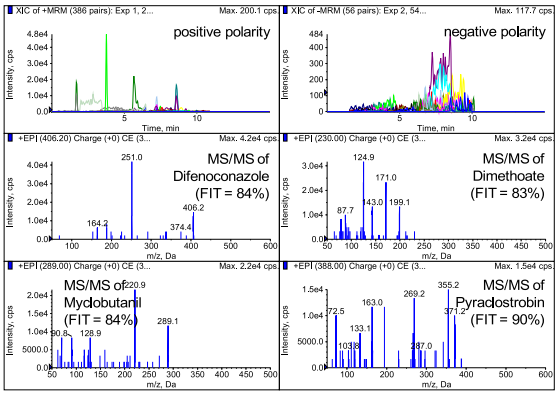

Figure 7b. Carrot sample (extract 10x diluted) screened for pesticides with MS/MS library search results for additional confidence in compound identification

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.

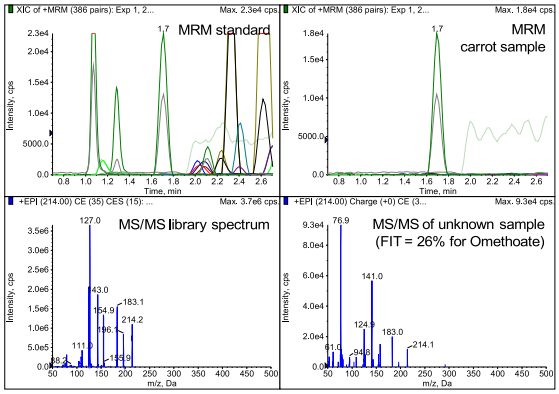

Figure 8. False positive finding identified by MS/MS library searching, standard and carrot sample have identical retention times of 1.7 min and MRM ratio of 0.6 but MS/MS spectra differ and the search results clearly prove the false positive

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 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 quantitation, samples may be prepared through an SPE clean-up procedure optimized for Carbendazim.[2]

LC

LC separation was achieved using the SCIEX 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.

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MS/MS

The SCIEX QTRAP® 4500 and 5500 systems are highly suitable for this analysis allowing simultaneous quantitation 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 QTRAP® 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.

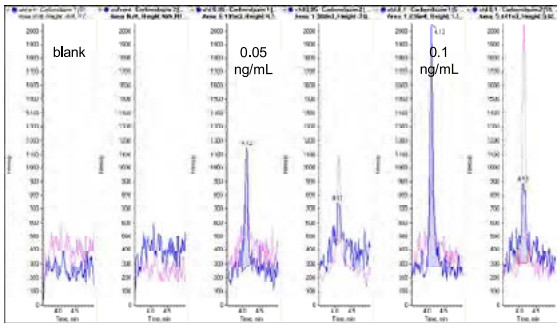

Figure 1. Determination of LOD and LOQ of detection of Carbendazim, LOD was found at 0.05 ng/mL and LOQ at 0.1 ng/mL using the SCIEX QTRAP® 4500 system

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]

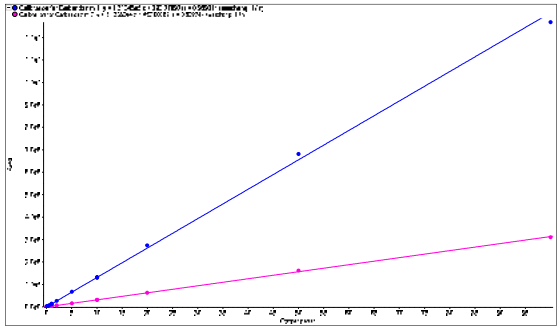

Figure 2. Linear range of the detection of Carbendazim from 0.05 to 100 ng/mL with an r > 0.999 for both MRM transitions

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.

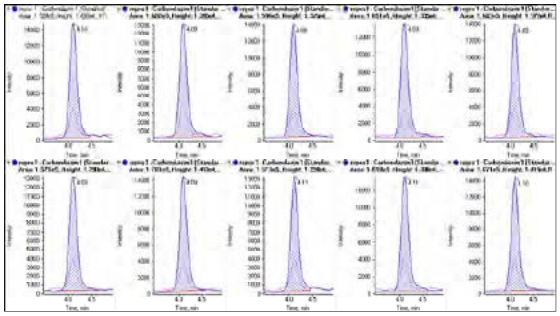

Figure 3. Reproducibility at 1 ng/mL with a %CV of 3.6 and 5.7%, respectively, for both MRM transitions

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

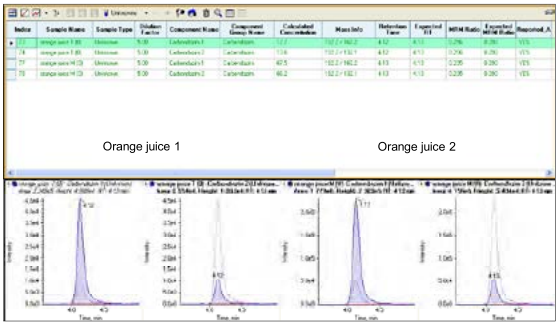

Figure 4. Quantitation and identification of Carbendazim in store bought orange juice using 'Multicomponent' query in MultiQuant™ Software

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.

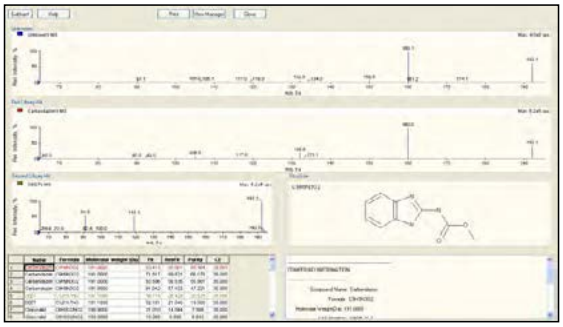

Figure 5. Library search of automatically collected EPI spectra of the orange juice sample 1 identifying 13 ng/mL Carbendazim with a library FIT of 93%

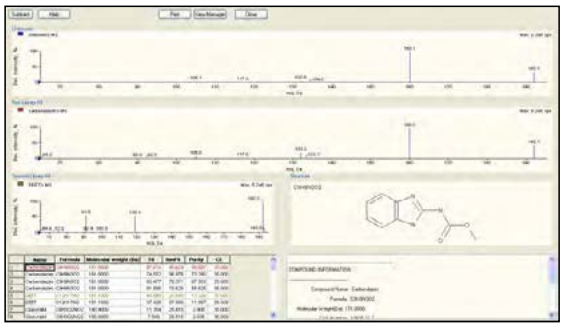

Figure 5. Library search of automatically collected EPI spectra of the orange juice sample 2 identifying 67 ng/mL Carbendazim with a library FIT of 97%

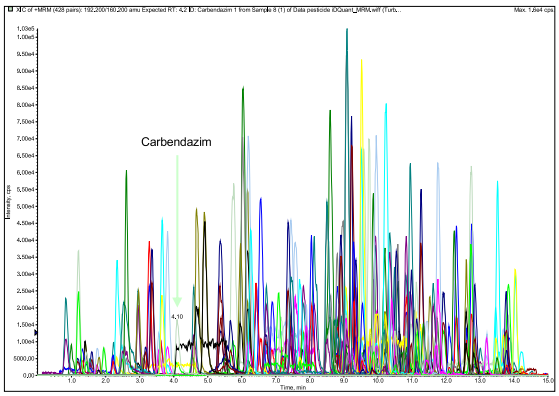

Figure 7. Comprehensive pesticide screening using LC-MS/MS and the iDQuant™ standards kit for pesticide analysis

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 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

Publication number: 4980212-02

Contents

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 SCIEX MicroLC 200 System in combination with a LC-MS/MS method developed on a 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 SCIEX MicroLC 200 System

The LC system used for these tests was the SCIEX 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 u[illegible] 0.5 x 50 mm Halo C18 column held at 50 ºC and with th

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

SCIEX 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® 4500SLC-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 SCIEX 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.

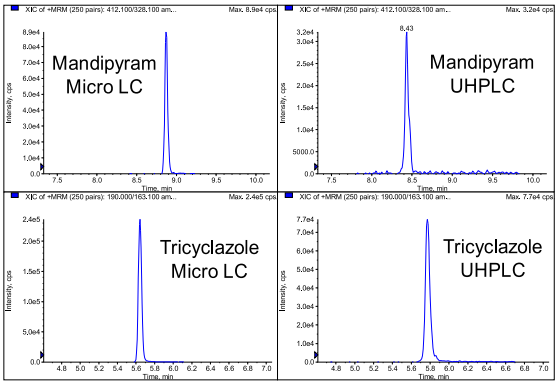

Figure 1. A comparison of micro flow LC and high flow LC

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%.

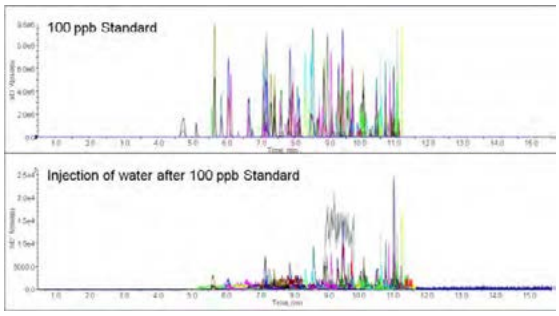

Figure 2. The top pane shows a 100 ppb calibration standard injected using the micro flow LC MS/MS set-up. The bottom pane shows water injected directly after this standard showing very low carryover.

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.

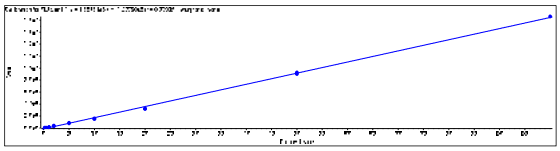

Figure 3. Example of a calibration line for one of the pesticides, Flutolanil, from 0.2 to 100 ppb. The fit used was Linear and the 'r' value obtained was greater than 0.999.

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.

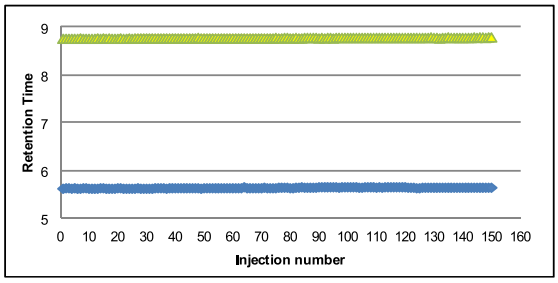

Figure 4. In this graph, retention time of two pesticides, Flutolanil (top) and Tricyclazole (bottom) were plotted against the injection number. The graph shows that the retention times obtained are rock solid with little or no variation between injections, confirming the low dead volume of the system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

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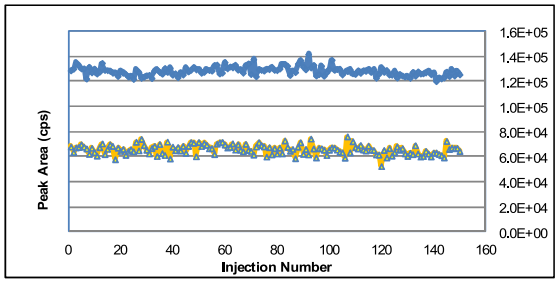

Figure 5. This graph shows the peak areas of two pesticides, Flutolanil (bottom) and Tricyclazole (top), which elute at different times during the run. It shows that the robustness is excellent with no deterioration in response even after 150 injections of a crude spice extract.

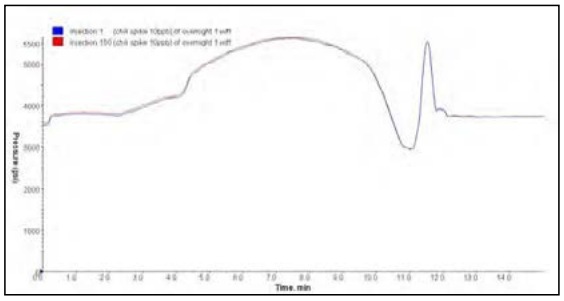

Figure 6. This figure compares the pressure profiles obtained from two injections of chili extract, 150 injections apart.

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.

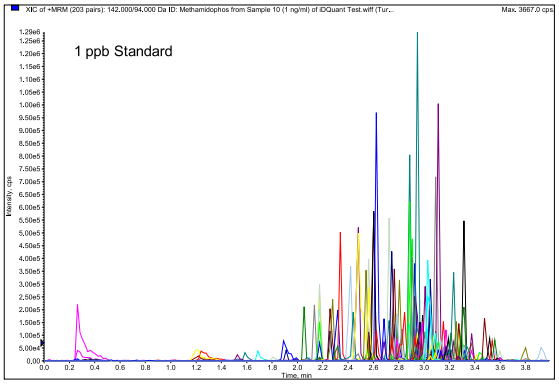

Figure 7. An example of the rapid gradient conditions that can be achieved using micro flow LC for pesticide residue analysis.

The method using the SCIEX 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.

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1 M. Anastassiades et al.: 'Fast and Easy Multiresidue 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

2 K. Mriziq et al.: 'Higher Sensitivity and Improved Resolution Microflow UHPLC with Small Diameter Turbo V™ Source Electrodes and Hardware for use with the ExpressHT™-Ultra System' Technical Note SCIEX (2011) # 4590211-01

Publication number: 6330212-01

Comprehensive Quantitation and Identification of Pesticides in Food Samples Using the SCIEX UltraLC 100 and the 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 SCIEX 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 SCIEX 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 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 SCIEX ultraLC 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 SCIEX 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 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.

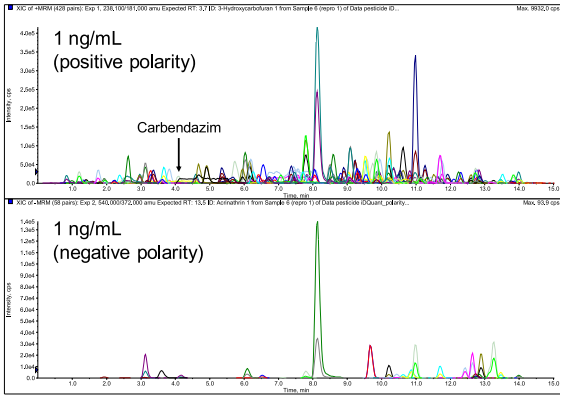

Figure 1. Comprehensive pesticide screening using the *Scheduled* MRM™ algorithm and fast polarity switching, ~500 MRM transition were detected with a total target cycle time of 0.7 sec

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.

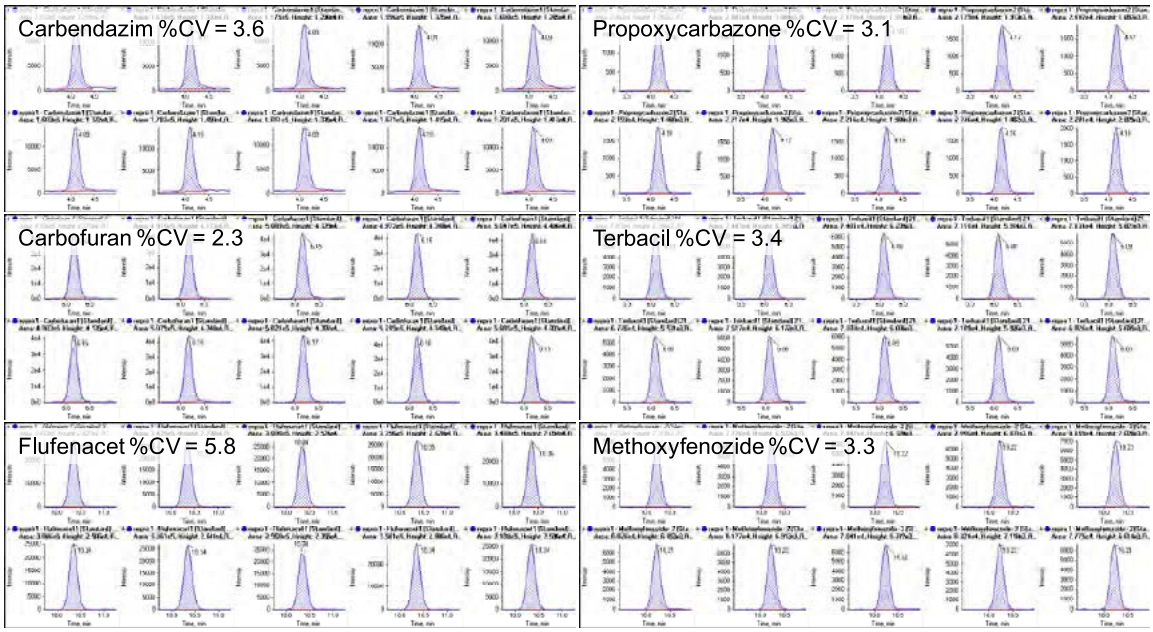

Figure 2. Repeat injections of pesticides at a concentration of 1 ng/mL detected in positive (left) and negative (right) polarity in a single run using *Scheduled* MRM™ and fast polarity switching (Carbendazim and Propoxycarbazone at 4.1 min, Carbofuran and Terbacil at 6.1 min, and Flufenacet and Methoxyfenozide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

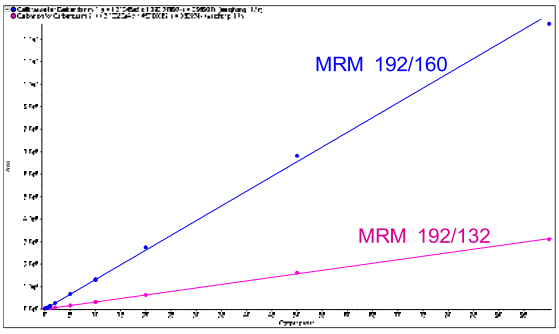

Figure 3. Calibration lines of both MRM transitions of Carbendazim

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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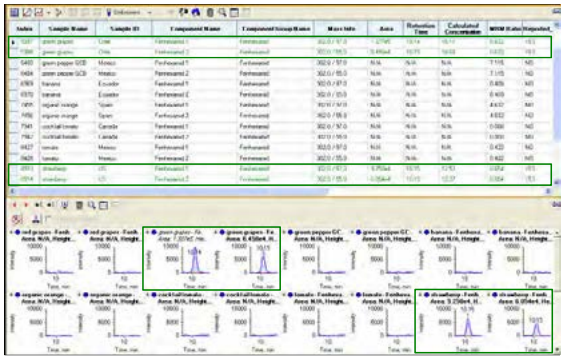

Figure 4. Automatic reporting of pesticides using the 'Multicomponent' query in MultiQuant™ Software, Fenhexamid was positively identified using MRM ratio calculation in two samples and quantified in green grapes at 18.1 µg/kg and in strawberry at 12.5 µg/kg, respectively

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

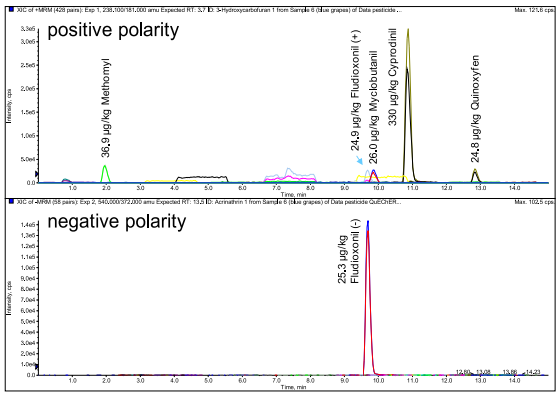

Figure 5a. Pesticides identified and quantified in a red grape sample

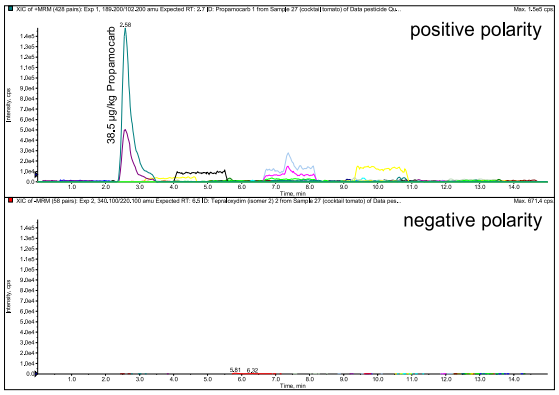

Figure 5b. Pesticides identified and quantified in a cocktail tomato sample

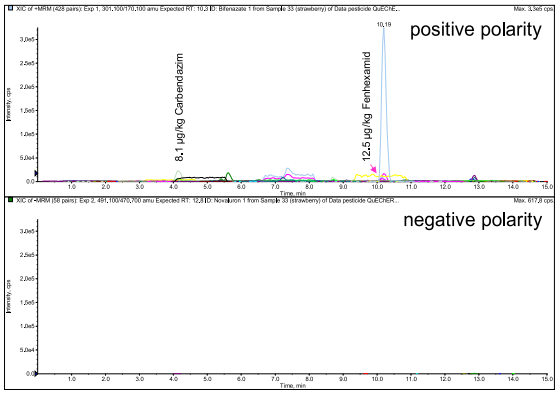

Figure 5c. Pesticides identified and quantified in a strawberry sample

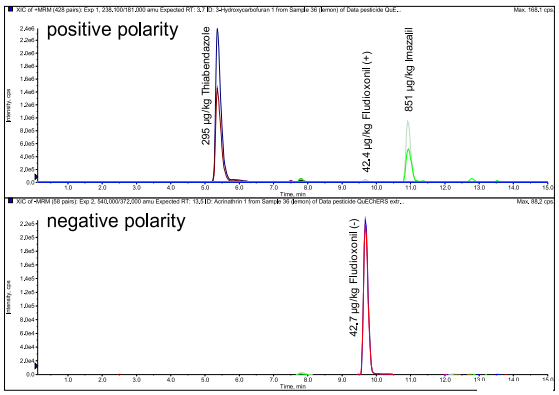

Figure 5d. Pesticides identified and quantified in a lemon sam

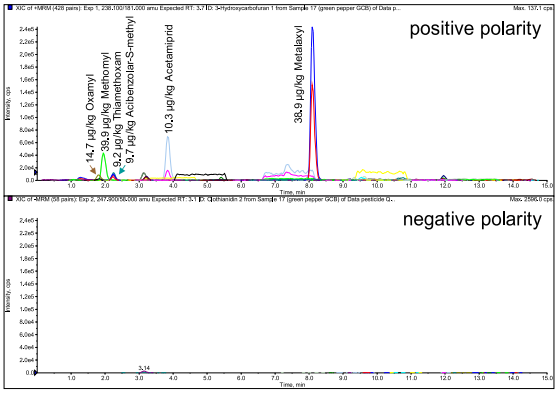

Figure 5e. Pesticides identified and quantified in green pepper sample

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)

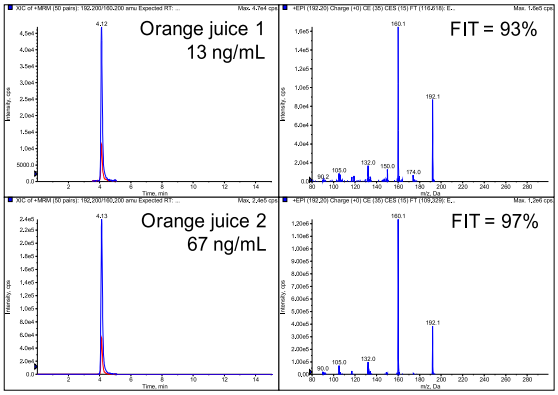

Figure 6. Identification of Carbendazim in two orange juice samples using MS/MS library searching, the samples were injected directly after 5x dilution, FIT values above 90% clearly confirm the identity of Carbendazim

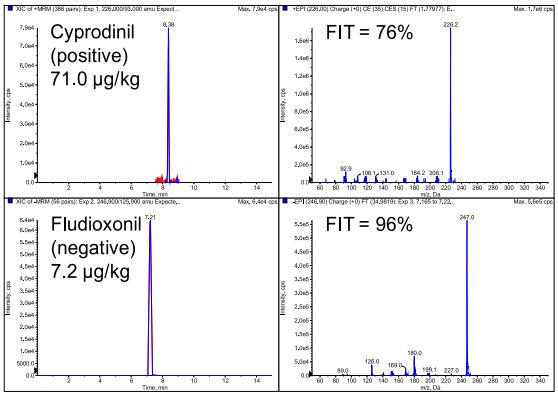

Figure 7. Identification of Cyprodinil and Fludioxonil in a raspberry sample using MS/MS library searching, the samples were injected after QuEChERS extraction and 5x dilution, MS/MS spectra were acquired in positive polarity and negative polarity

Summary

This new and unique LC-MS/MS method using the SCIEX ultraLC 100 and QTRAP® 4500 systems 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 (2010) 1282310-01

Publication number: 5090212-01

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

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[1]SCIEX Concord, Ontario (Canada); [2]GERSTEL Linthicum, Maryland (USA); [3]University of South Carolina, Department of Chemistry and Biochemistry, Columbia, South Carolina (USA)

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 a SCIEX QTRAP® 4500 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 a 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 iDQuant™ 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			

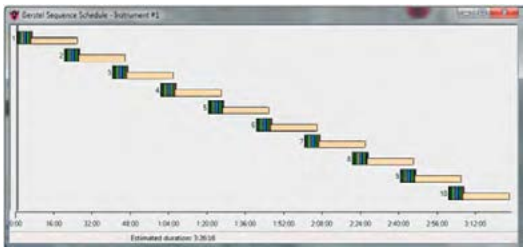

Figure 2. Example sample preparation sequence for automated DPX-QuEChERS LC-MS/MS analysis

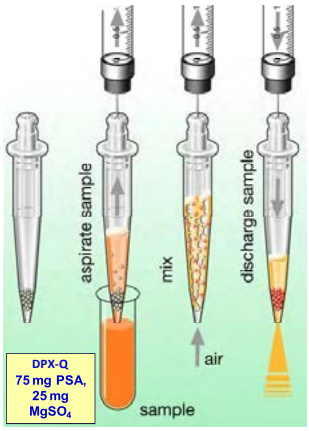

Figure 3. Example sample preparation sequence for automated DPX-QuEChERS LC-MS/MS analysis

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.

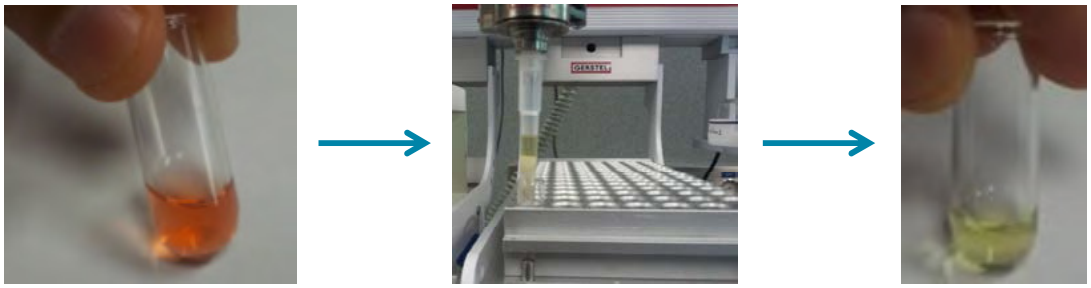

Figure 4. Schematic of the automated DPX-QuEChERS procedure, 500 µL of apple extract (left) and after DPX-QuEChERS cleanup (right)

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.

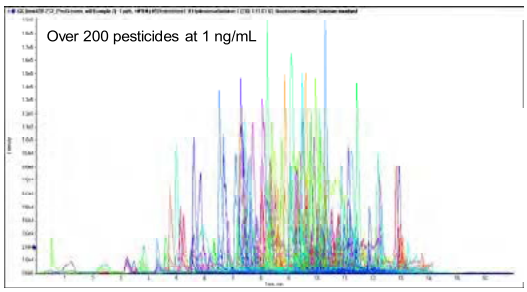

Figure 5. Detection of over 200 pesticides in a fortified cucumber sample at 1 ng/mL

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.

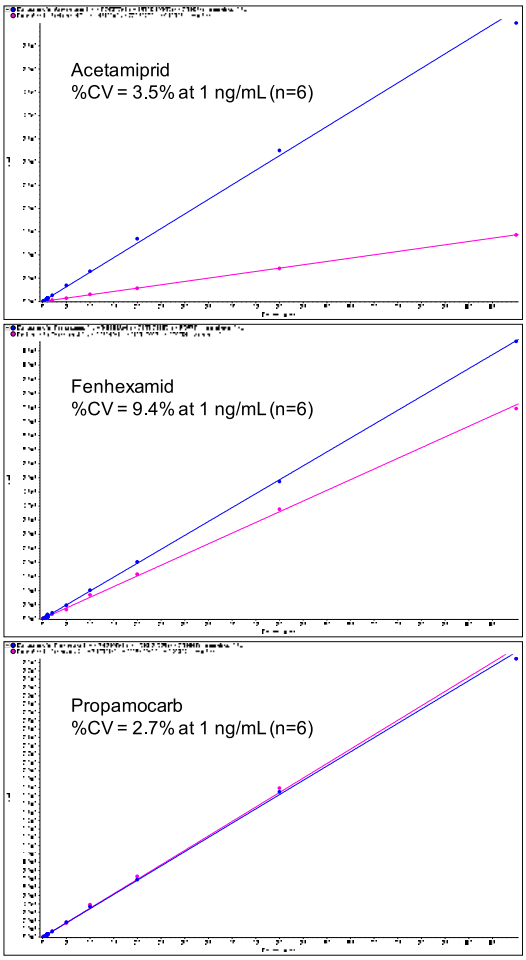

Figure 6. Representative calibration lines for Carbendazim, Propiconazole, and Thiacloprid from 0.1 to 100 ng/mL with an regression coefficient $r^2 > 0.997$ with excellent repeatability of %CV < 10%

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.

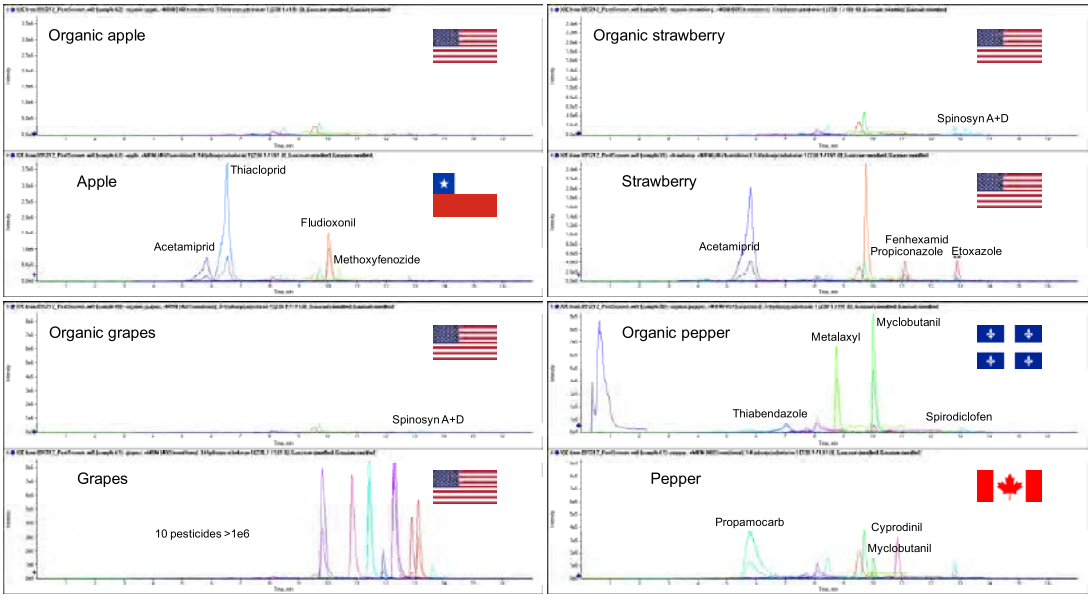

Figure 7. MRM chromatograms of pesticides identified in different food samples, including organic produce, from a local super market

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

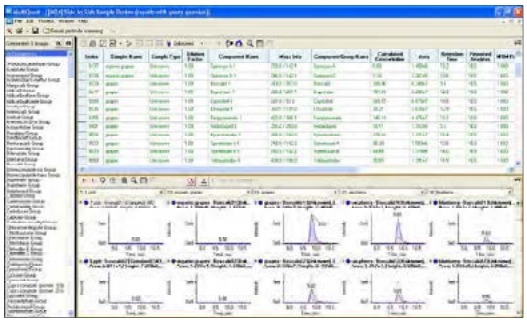

Figure 8a. Results review in MultiQuant™ Software after using the 'Multicomponent' query with peak review for the pesticide Boscalid (bottom): 1 ng/mL standard, organic grapes, grapes, raspberry, and blueberry

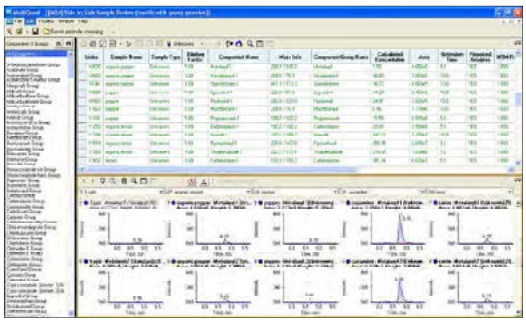

Figure 8b. Results review in MultiQuant™ Software after using the 'Multicomponent' query with peak review for the pesticide Metalaxyl (bottom): 1 ng/mL standard, organic pepper, pepper, cucumber, and raisin

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 a 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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Publication number: 8013613-01

Contents

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

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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.

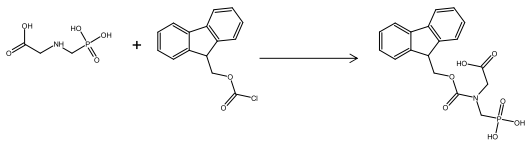

Figure 1. Derivatization of glyphosate using FMOC-Cl

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.

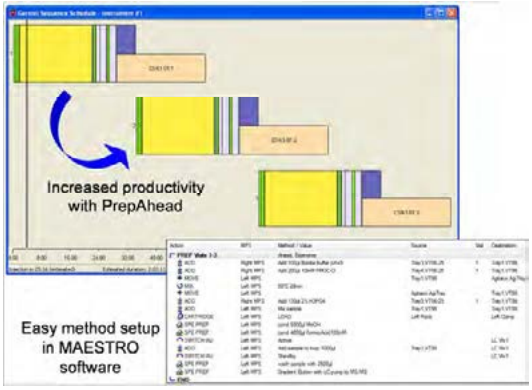

Figure 3a. Sequence of scheduled events in the Maestro software for online SPE: green - adding buffer and FMOC-Cl, yellow - derivatization, light blue - online SPE, orange LC-MS/MS analysis, dark blue washing of the autosampler, the PrepAhead function increases productivity by simultaneously preparing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

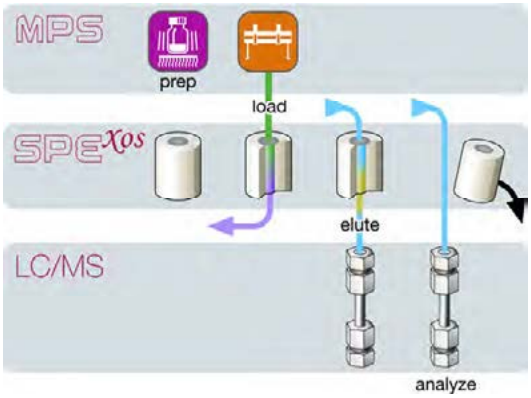

Figure 3b. Sequence of scheduled events when using the automated workflow of FMOC-derivatization, SPE cleanup, and LC-MS/MS detection

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-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.

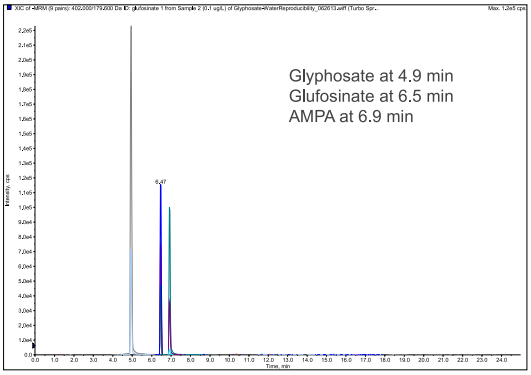

Figure 4. Standard chromatogram at a concentration of 10 ng/mL

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).

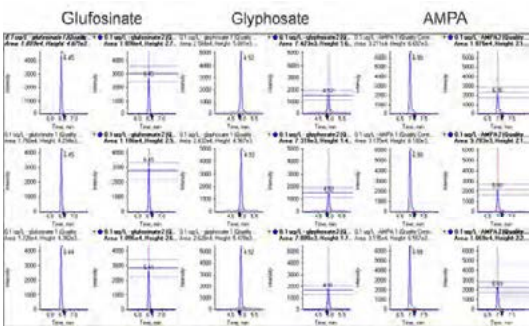

Figure 5. Triplicate analysis of polar pesticides in a spiked water sample at 0.1 µg/L (injection volume of 10 µL), ion ratios for compound identification were calculated automatically in MultiQuant™ Software version 3.0

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%)

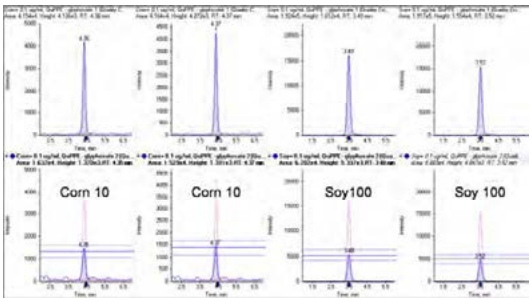

Figure 6. 10 and 100 µg/kg of glyphosate spiked into corn and soy and analyzed using automatic derivatization, dilution, and cleanup followed by LC-MS/MS, ion ratios for compound identification were calculated automatically in MultiQuant™ Software version 3.0

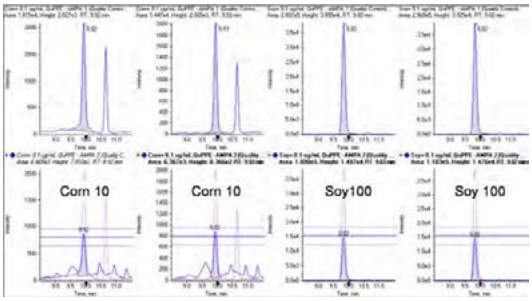

Figure 7. 10 and 100 µg/kg of AMPA spiked into corn and soy and analyzed using automatic derivatization, dilution, and cleanup followed by LC-MS/MS, ion ratios for compound identification were calculated automatically in MultiQuant™ Software version 3.0

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.

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).

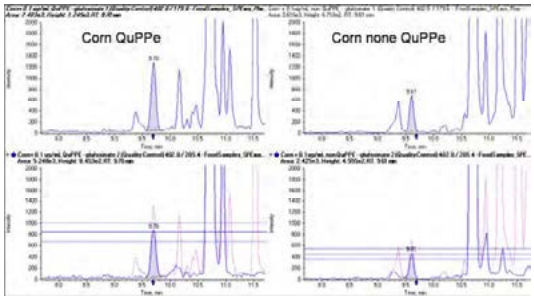

Figure 8. Corn analyzed for glufosinate using the QuPPe and a none QuPPe extraction procedure with higher recoveries but more matrix interferences when using the QuPPe protocol

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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2 http://ec.europa.eu/food/plant/protection/evaluation/existactive/list1_glyphosate_en.pdf

3 http://www.crl-pesticides.eu/library/docs/srm/meth_QuPPe.pdf

4 C. Miles and A. Moye: 'Extraction of glyphosate herbicide from soil and clay minerals and determination of residues in soils' J. Agric. Food Chem. (1988) 486-491

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

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

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Introduction

Recent regulations on food analysis require screening for pesticides 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. 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.

Challenges for pesticide residue laboratories at the moment are the request to test for more compounds, in a wider range of samples, all without sacrificing data quality.
The SCIEX QTRAP 6500 LC/MS/MS system uses multi component IonDrive™ 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 the patented and proven Linear Accelerator™ trap technology.

- 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 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 s 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.

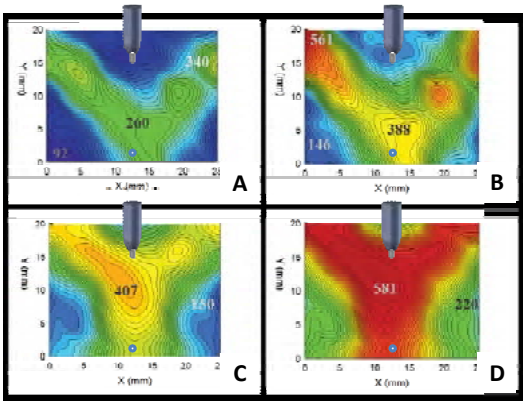

Figure 1. Temperature maps of the spray region of the traditional Turbo V™ source (top) and the new IonDrive™ Turbo V source (bottom). A and C show the source operated at 500°C and B and D at 700°C with Gas 2 set to 70 psi.

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.

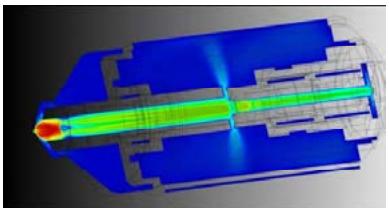

Figure 2. Computed gas flow model of the dual stage QJet™ ion guide

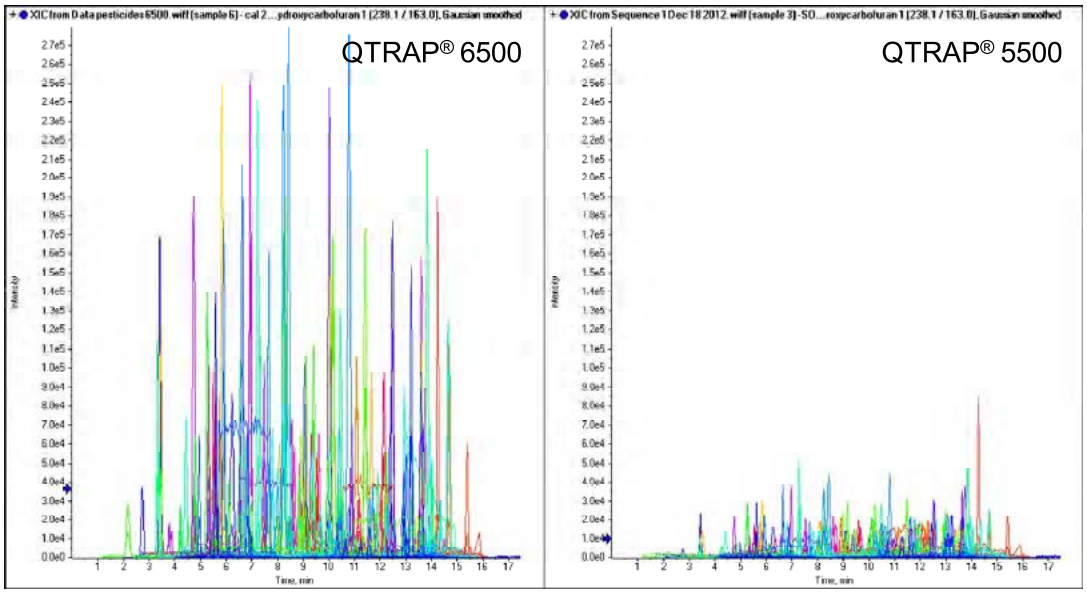

Figure 3. Sensitivity comparison of a 0.1 ng/mL standard analyzed using the QTRAP® 6500 system (left) and QTRAP® 5500 system (right)

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.

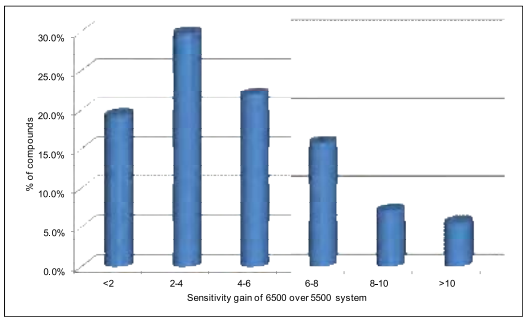

Figure 4. Distribution of sensitivity gain for multi-pesticide analysis with an average gain of 4.7

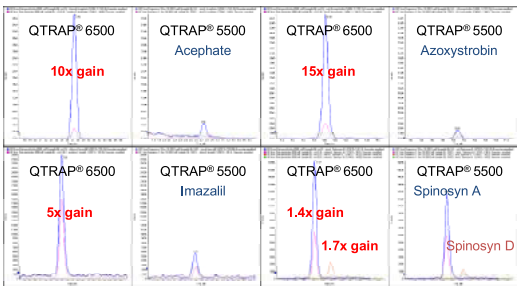

Figure 5. Compound specific sensitivity gains for selected pesticides, including Acephate, Azoxystrobin, Imazalil, and Spinosad, sensitivity gains are compound dependent and can be influenced by ion source temperature

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.

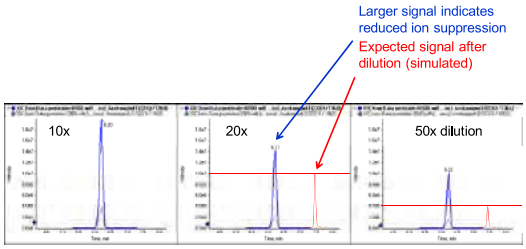

Figure 6. Dilution of peppermint tea extract spiked with Acetamiprid, the increase in sensitivity over the expected (simulated) peak demonstrates successful dilution of matrix effects

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%.

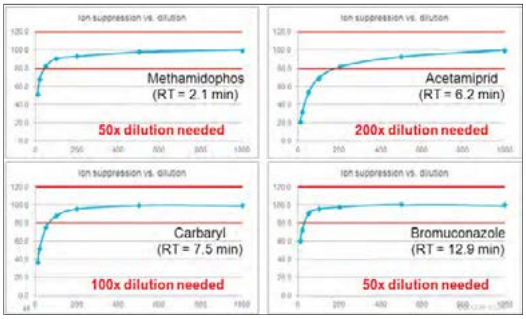

Figure 7. Ion suppression caused by peppermint tea was successfully reduced by dilution of 50 to 200x

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.

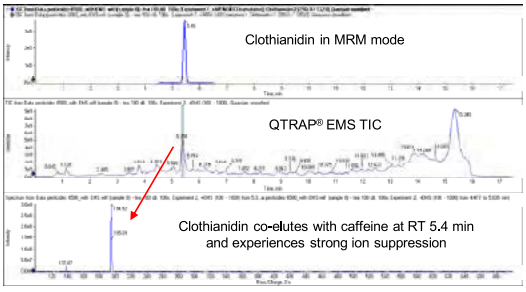

Figure 8. Monitoring of matrix effects by simultaneous acquisition of target MRM transitions and EMS scans, the strong ion suppression observed for Clothianidin can be explained by co-elution with caffeine at a much higher concentration

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.

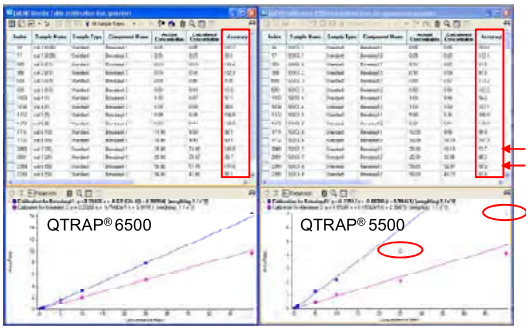

Figure 9a. Quantitation of Benalaxyl, a high sensitivity pesticide, 0.05 to 50 ng/mL, no points had to be excluded when the HED IonDrive™ detector was used due to extended pulse counting

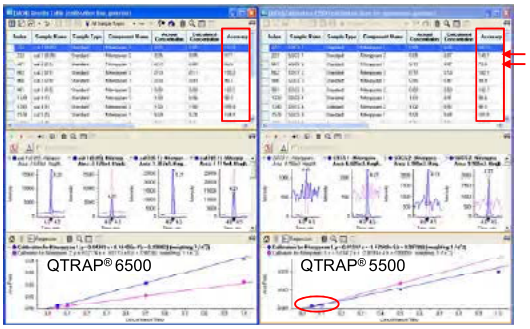

Figure 9b. Quantitation of Nitenpyram, a low sensitivity pesticide, 0.05 to 50 ng/mL, no points had to be excluded when the HED IonDrive™ detector was used due to increased sensitivity

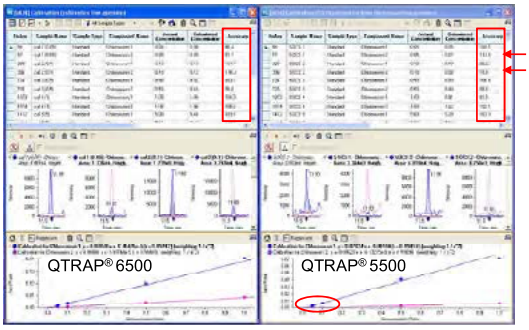

Figure 9c. Quantitation of Chloroxuron, a pesticide with a weak qualifier ion, 0.05 to 50 ng/mL, no points had to be excluded when the HED IonDrive™ detector was used to extended pulse counting and increased sensitivity

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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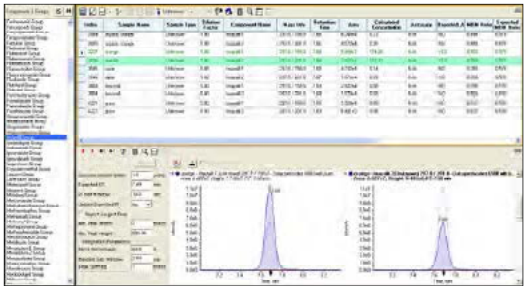

Figure 10. Automatic reporting of pesticides using the 'Multicomponent' query in MultiQuant™ Software: Imazalil was flagged in the result table because of a concentration above a user defined threshold and positive identification using the MRM ratio

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).

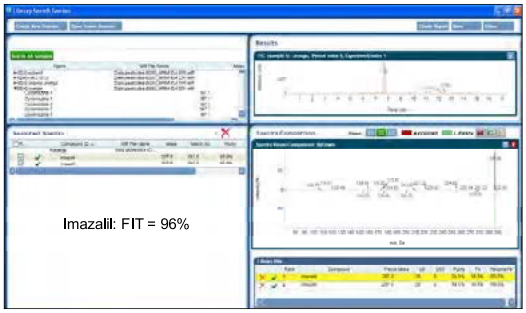

Figure 11. Review of MS/MS search results in LibraryView™ Software

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

1 Carmen Ferrer et al.: 'Overcoming Matrix Effects Using the Dilution Approach in Multiresidue Methods for Fruits and Vegetables' J. Chromatogr. A 1218 (2011) 7634-7639

2 Michael Jarvis: 'Ultra-Sensitive Analysis of Aldosterone in Serum Using the SCIEX Triple Quad™ 6500 System' Application Note 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 SCIEX TripleTOF® 4600 System and Intuitive Data Processing Tools' Application Note SCIEX (2012) 5680212-01

Publication number: 7580413-01

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

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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 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 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 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 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).

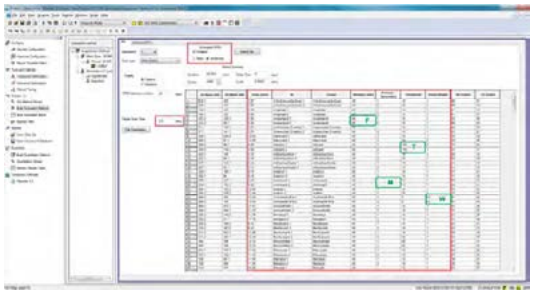

Figure 1. *Scheduled* MRM™ Pro algorithm allowing: Flexible Window Width (F), Dynamic Window Extension (T), MRM-triggered MRM (M, T), Dwell Time Weighting (W)

- 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.

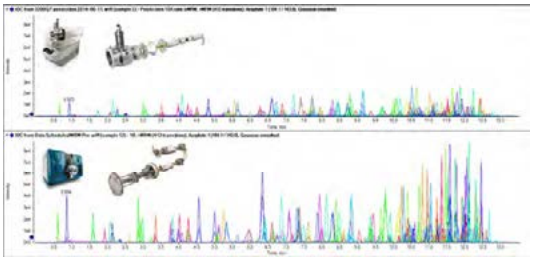

Figure 2. Sensitivity comparison of a 10 ng/mL standard analyzed using the API 3200™ system (top) and Triple Quad™ 3500 system (bottom) with an average sensitivity gain of 3x

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.

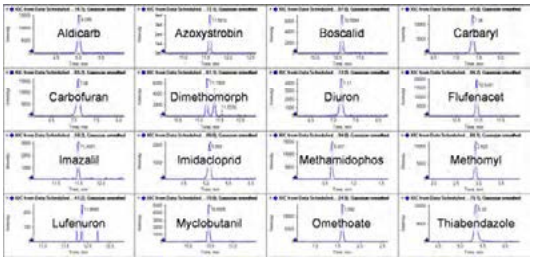

Figure 3. Sensitivity of selected pesticides detected at a concentration of 5 ng/mL using the Triple Quad™ 3500 system

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

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).

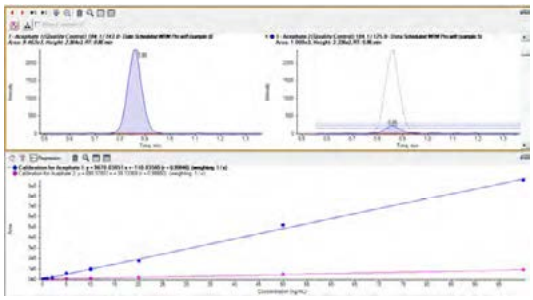

Figure 4. Peak review quantifier-qualifier ratio of Acephate at 1 ng/mL and calibration line from 0.1 to 100 ng/mL with %CV of 2.9% and 3.2% at 1 and 10 ng/mL, respectively, and.

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.

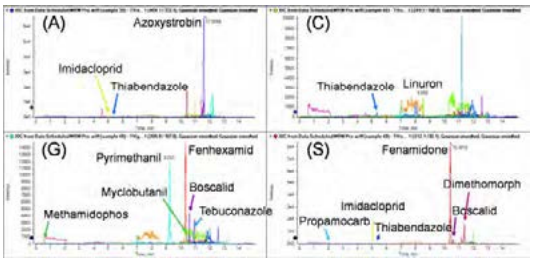

Figure 5. Detection of pesticides in filtered QuEChERS extracts of avocado (A), carrot (C), grapes (G), and spinach (S)

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]

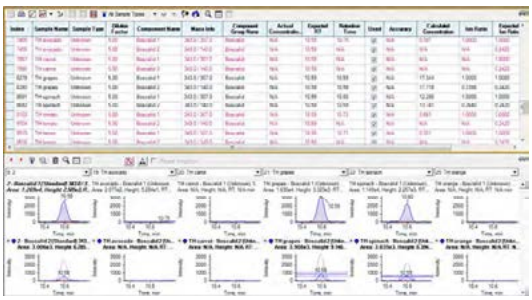

Figure 6. Quantitation and identification based on MRM ratios in MultiQuant™ Software, the example shows the side-by-side peak review for Boscalid with positive findings in grapes and spinach samples

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.

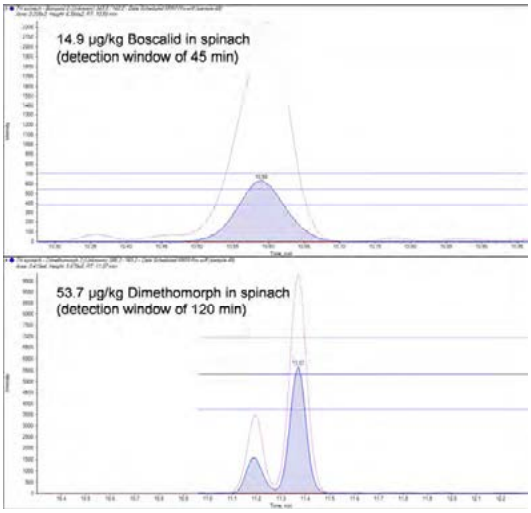

Figure 7. Examples of using the Flexible Window Width in a *Scheduled* MRM™ Pro method: the window for Boscalid was set to 45 sec and Dimethomorph was detected using a wider window to detect both isomers together

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.

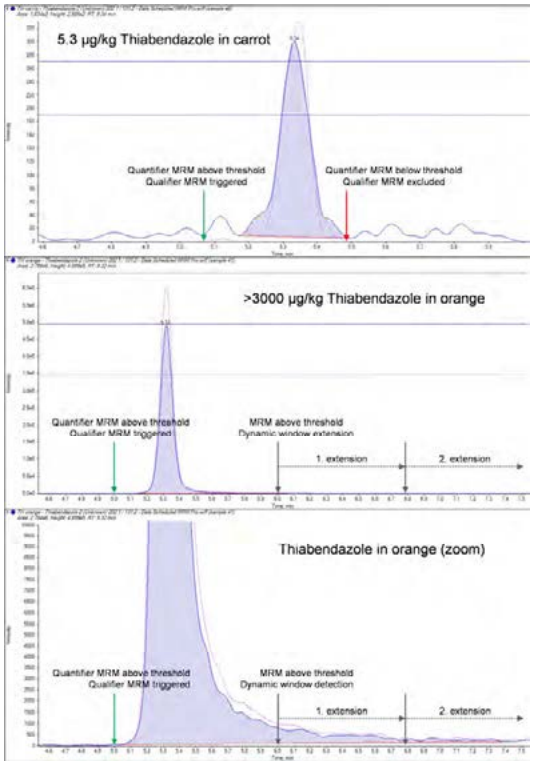

Figure 8. Examples of MRM-triggered MRM and Dynamic Window Extension: the qualifier MRM transition is automatically triggered when the quantifier MRM transitions exceeds the threshold set in the *Scheduled* MRM™ Pro method, the detection window is automatically extended if the MRM signal is above the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

References

1 M. Anastassiades et al.: 'Fast and easy multiresidue 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

2 St. Lehotay: 'Determination of Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate: Collaborative Study' J. AOAC Int. 90 (2007) 485-520

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 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 SCIEX QTRAP® 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 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

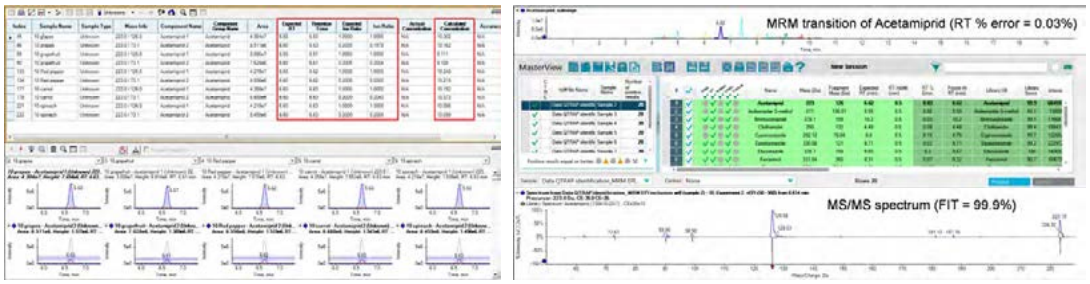

Figure 1. Quantitation, identification and confirmation using a dual injection approach using two complementary LC-MS/MS methods utilizing the *Scheduled* MRM™ Pro algorithm with automatic MRM ratio calculation and *Scheduled* MRM™-IDA-MS/MS followed by MS/MS library searching

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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%).

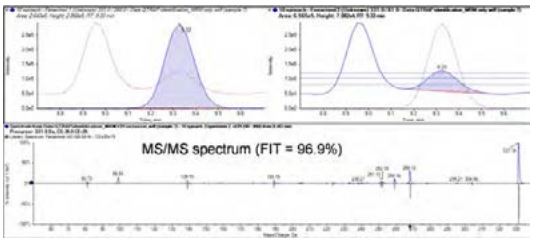

Figure 2. Detection of Fenarimol in spiked spinach: the MRM ratio was slightly out of the 30% tolerance due to high background and a closely eluting interfering matrix peak, but MS/MS library searching confirmed the presence of the detected pesticide.

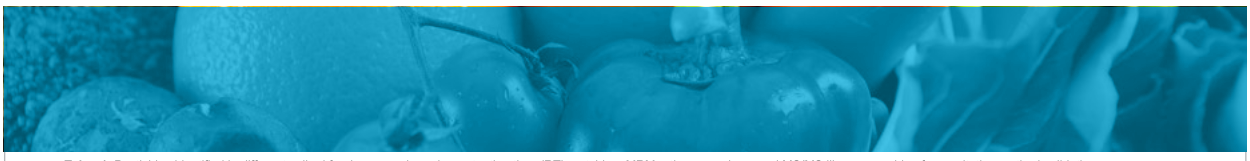

Table 1. Pesticides identified in different spiked food samples based on retention time (RT) matching, MRM ratio comparison, and MS/MS library searching for qualitative method validation

	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).

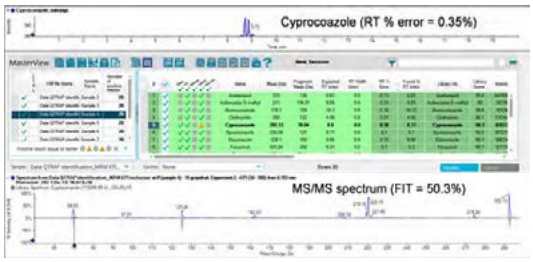

Figure 9. Processing of *Scheduled* MRM™ and MS/MS data in MasterView™ Software, compound identification is achieved through automatic retention time (RT) matching and MS/MS library searching

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 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 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.'

Publication number: 10390214-01

Contents

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

André Schreiber[1] and Lauryn Bailey[2]

[1]SCIEX Concord, Ontario (Canada); [2]AB SCIEX Framingham, Massachusetts (USA)

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

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- 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 i*DQuant*™ 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)

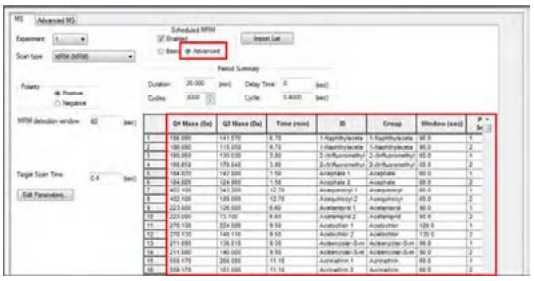

Figure 1. Acquisition method editor to build a method using the *Scheduled* MRM™ Pro algorithm

- 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.

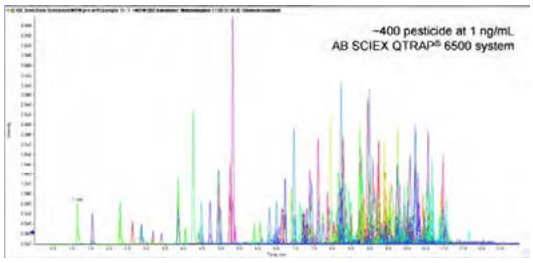

Figure 3. Approximately 400 pesticides detected using 800 MRM transition with the *Scheduled* MRM™ Pro algorithm

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

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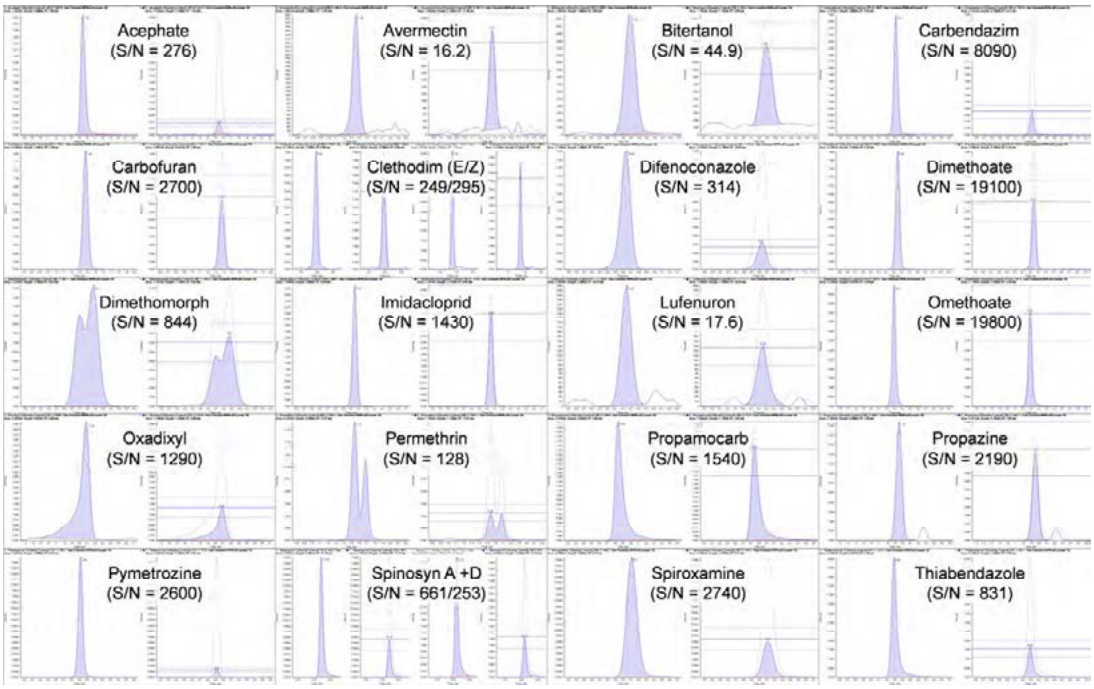

Figure 4. Quantifier and qualifier MRM transitions of selected pesticides with S/N at a concentration of 1 ng/mL, the MRM ratio tolerance of 30% is displayed in the MultiQuant™ Software peak review (SANCO/12571/2013)

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

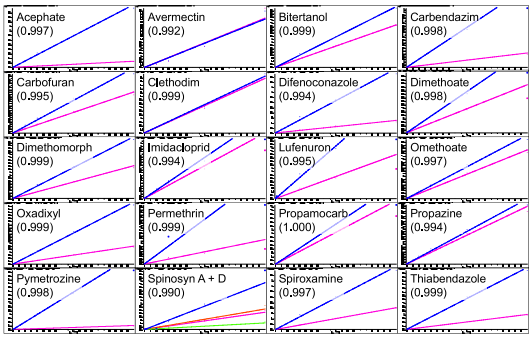

Figure 5. Calibration lines of selected pesticides from 0.1 to 100 ng/mL

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.

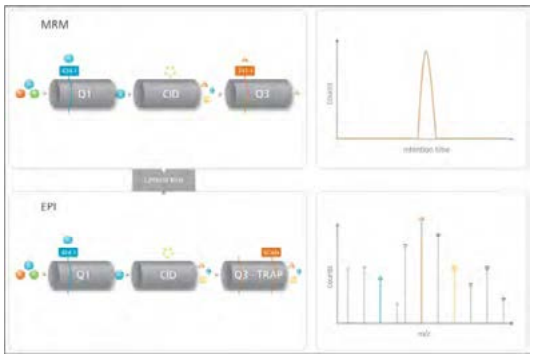

Figure 6. Information Dependent Acquisition (IDA) of MS/MS spectra using an MRM survey scan on a QTRAP® system

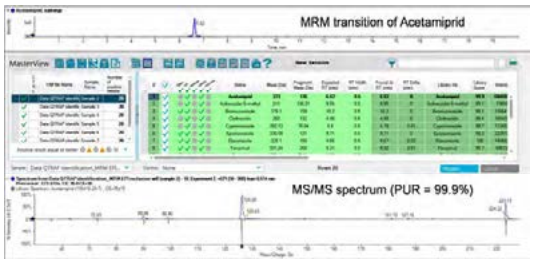

Figure 7. Processing of *Scheduled* MRM™ and MS/MS data in MasterView™ Software, compound identification is achieved through automatic retention time matching and MS/MS library searching

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

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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

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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.

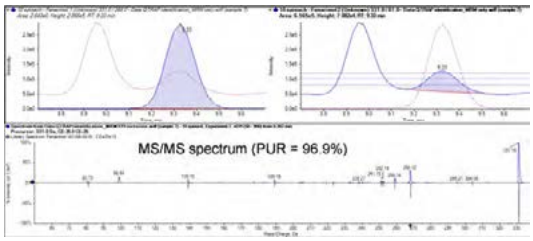

Figure 8. Detection of Fenarimol in spiked spinach: the MRM ratio was slightly out of the 30% tolerance due to high background and a closely eluting interfering matrix peak, but MS/MS library searching confirmed the presence of the detected pesticide.

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).

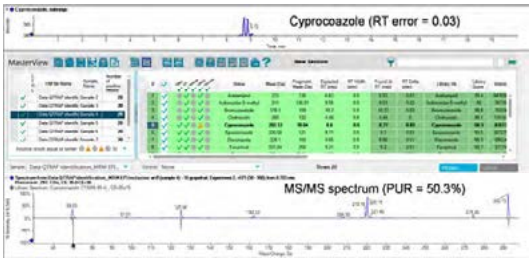

Figure 9. Detection of Cyproconazole in grapefruit: the MS/MS library search resulted in a PUR value of 50.3% only, however, review of spectra revealed in isobaric matrix interference, the MRM ratio error of 6.9% further confirmed the presence of the pesticide.

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).

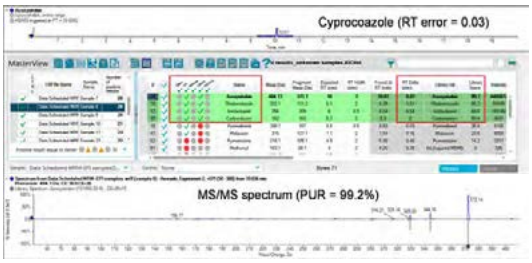

Figure 9. Identification of Azoxystrobin, Imidacloprid, Thiabendazole, and Carbendazim in an avocado sample based on retention time matching and MS/MS library searching, results were confirmed using MRM ratio calculation (note: Thiabendazole and Carbendazim were present below 5 µg/kg)

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.

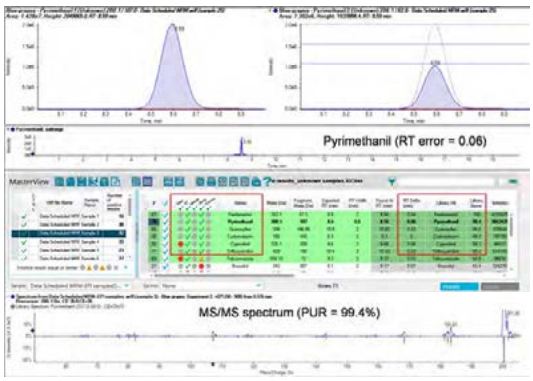

Figure 10. Fenhexamid, Pyrimethanil, Quinoxyfen, and Trifloxystrobin were identified based on MRM ratios and quantified in a grapes sample, the MRM ratio of Pyrimethanil were slightly outside the 30% tolerance (top), however, second analysis using MS/MS library searching confirmed the presence of Pyrimethanil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

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

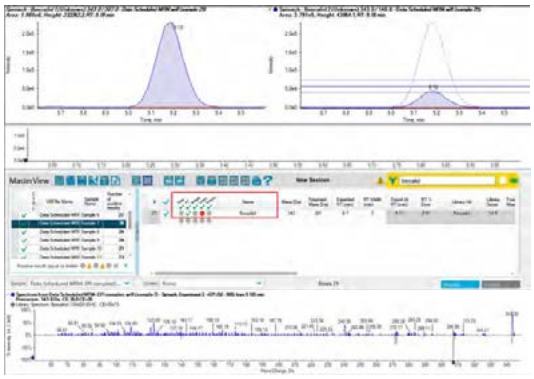

Figure 11. Boscalid was detected in a spinach samples with a concentration of 14.9 µg/kg, the ion ratio of 21.3 is inside the 30% tolerance (top), however, the MS/MS library searching with a PUR of 14.9% indicated strong matrix interference and suggest that Boscalid is not present in the sample (bottom)

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.

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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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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).

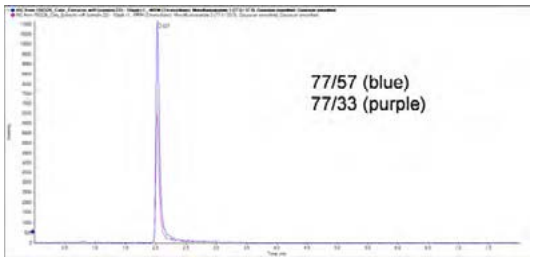

Figure 1. Example chromatogram of a 10 ng/mL standard of sodium fluoroacetate

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.

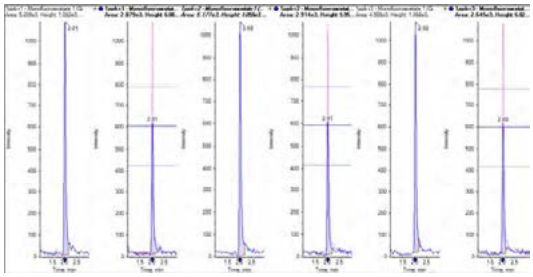

Figure 2. Repeat analysis at 1 ng/mL, 2 MRM transitions were monitored and the ratio of quantifier and qualifier transition (alternating from left to right, respectively) was used for compound identification (displayed MRM tolerances are 30%).

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).

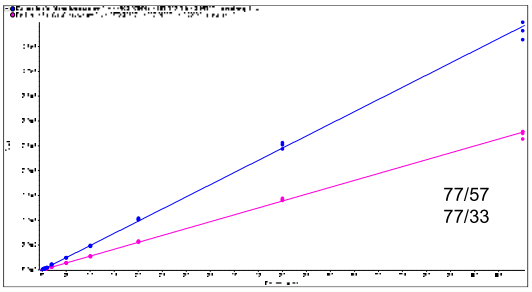

Figure 3. Calibration lines (0.1 to 100 ng/mL) for sodium fluoroacetate

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

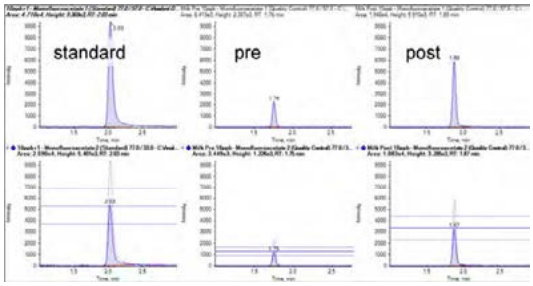

Figure 4. Chromatograms of standard at 10 ng/mL in comparison to the pre-extraction and post-extraction spike of milk at 10 ng/mL (the quantifier ion results are shown on the top row and the qualifier ion on the bottom row)

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

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

Stephen Lock
SCIEX Warrington (UK)

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 SCIEX 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 a SCIEX 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.

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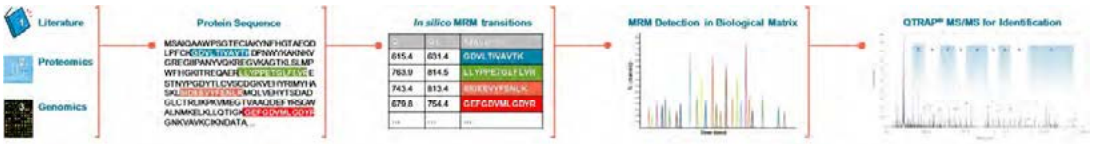

Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

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 a SCIEX 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 a SSCIEX QTRAP® 5500 LC-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.

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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

Contents

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.

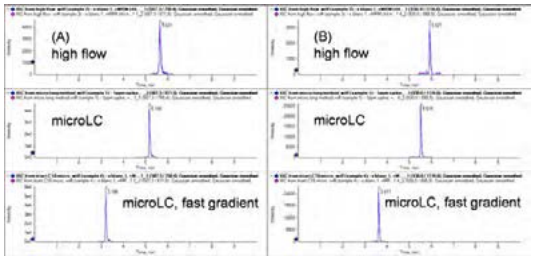

Figure 2. Comparison of high flow vs. microLC using a 1 ppm protein spike in white wine. A milk peptide is shown on the left (A) and an egg peptide is shown on the right (B).

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

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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.

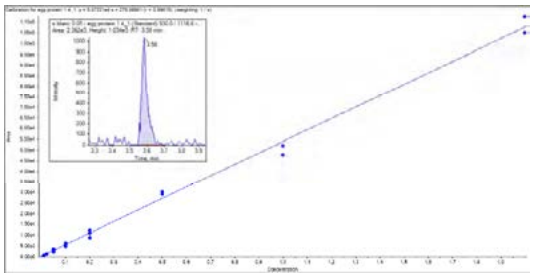

Figure 3. Calibration line from a peptide from egg which had been spiked into a sauvignon blanc wine (0.01 to 2 ppm) and chromatogram of the 50 ppb spike sample. The linearity is provided without the use of any internal standards.

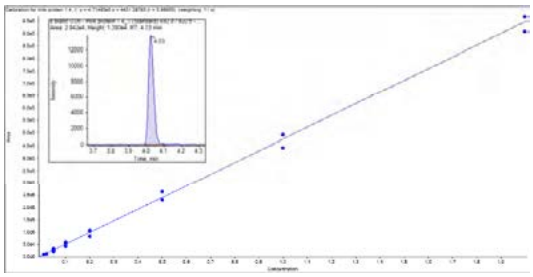

Figure 4. Calibration line from a peptide from milk which had been spiked into a sauvignon blanc wine (0.01 to 2 ppm) and chromatogram of the 50 ppb spike sample. The linearity is provided without the use of any internal standards.

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.

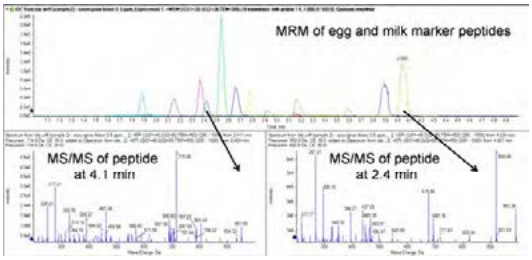

Figure 5. Micro flow LC-MS/MS analysis of 0.5 ppm spike of egg and milk proteins into a sauvignon blanc sample analyzed using the MIDAS™ workflow. The top pane shows the extracted ion chromatogram for the peptides of milk and egg and the bottom two panes show examples of MS/MS spectra for target peptides

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]).

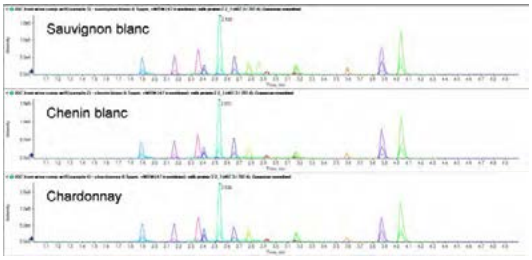

Figure 6. microLC-MS/MS analysis of 0.5 ppm spiked samples of egg and milk proteins into 3 different white wines

Summary

MicroLC-MS/MS using the SCIEX MicroLC 200 System coupled to a 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 microLC-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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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.

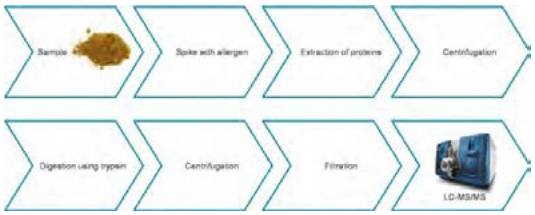

Figure 1. Sample preparation workflow

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.

Contents

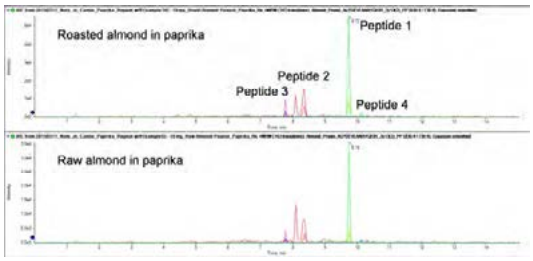

Figure 2a. Detection of almond in extracts of paprika (spiked at 10 mg/g)

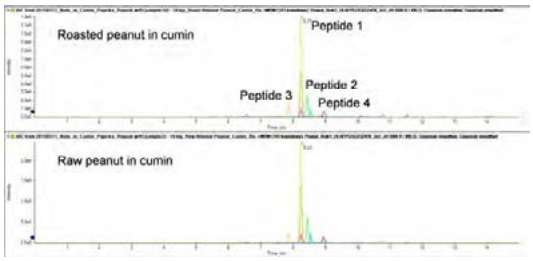

Figure 2b. Detection of peanut in extracts of cumin (spiked at 10 mg/g)

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%.

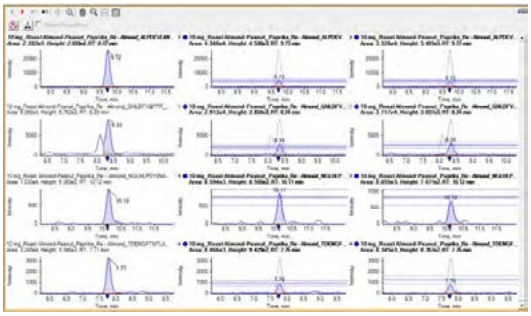

Figure 3a. Identification of almond in a paprika extract based on multiple MRM ratios

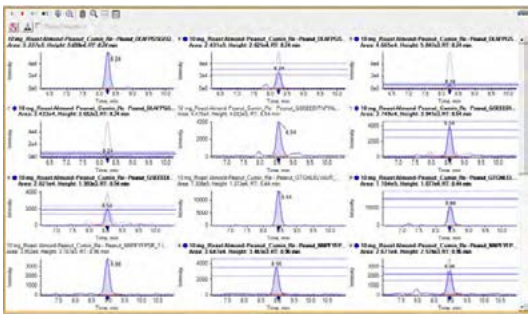

Figure 3b. Identification of peanut in a cumin extract based on multiple MRM ratios

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.

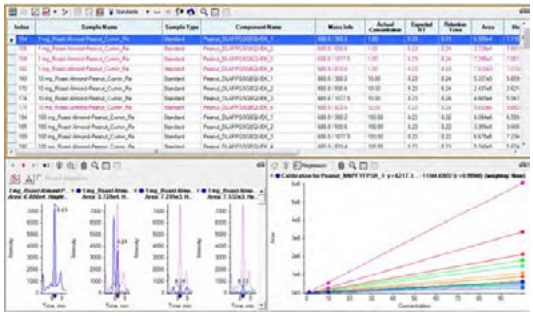

Figure 4a. Quantitative results of analyzing almond spiked into paprika powder

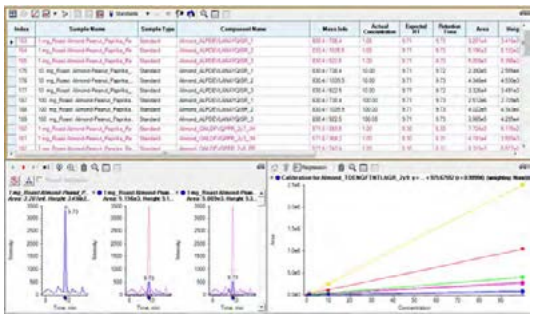

Figure 4b. Quantitative results of analyzing peanut spiked into cumin powder

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.

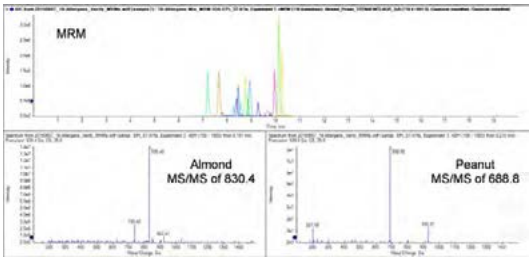

Figure 5. High confidence in identification using MS/MS full scan

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.

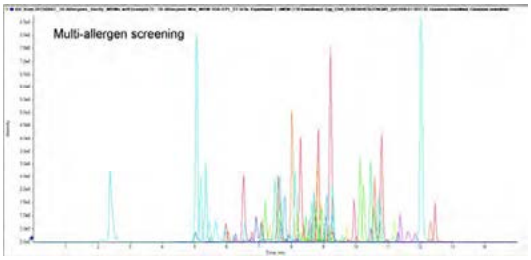

Figure 6. Multi-allergen screening by LC-MS/MS, detection of a total of 18 allergens in a single analysis

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

Contents

Analysis of Nitrofuran Metabolites in Honey Using the SCIEX Triple Quad™ 3500 System

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Overview

A LC-MS/MS method for the simultaneous quantification of four Nitrofuran Metabolites (3-amino-2-oxazolidinone (AOZ) for furazolidone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) for furaltadone, 1-aminohydantoin (AHD) for nitrofurantoin and semicarbazide (SEM) for nitrofurazone) on SCIEX Triple Quad™ 3500 was developed to detect Nitrofuran residues in honey samples, The method showed adequate linearity with correlation coefficients above r≥0.99 for all four analytes. The Minimum Required Performance Limit (MRPL) for Nitrofuran Metabolites in Honey was 1µg/kg.

Introduction:

Nitrofurans are broad spectrum antibacterial agents which were used in the treatment of bacterial infections in bee colony health. Nitrofurans have been prohibited in food-producing animals in the European Union and most other Countries for public health and safety concerns. The nitrofurans are unstable and easily metabolized within a few hours but Nitrofuran metabolites are highly stable in nature. Several methods have been described in the analysis of nitrofuran metabolite in honey samples by incubation period for derivatization with nitrobenzaldehyde in overnight or 16 hours at 37 ºC.

The LC-MS/MS method developed on SCIEX Triple Quad™ 3500 described here for the quantitation of nitrofuran metabolites in honey was found to meet the regulatory requirements of 1µg/kg.

Figure 1: SCIEX Triple Quad™ 3500

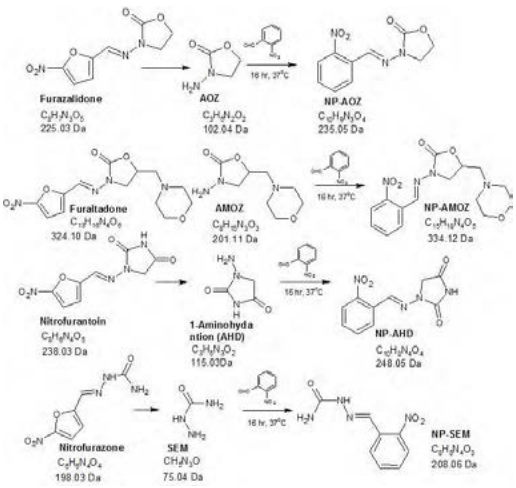

Figure 2: Structures of Nitrofuran, Nitrofuran metabolites and Nitrophenyl derivatives.

Materials and Methods

Chemicals

Nitrofuran metabolite standards were purchased from clearsynth and 2-Nitrobenzaldehyde was purchased from Sigma Aldrich ≥99% Purity. All other chemicals used were of LC-MS grade, commercially available.

Honey samples

Honey samples were procured from local market of Delhi and Gurgaon, India and were stored at room temperature until end of analysis.

Sample Preparation

Honey sample (1gm) was mixed with 3ml of HCl (0.1M) and 50mM of 2-Nitrobenzaldehyde (0.3ml), vortexed and incubated on ultrasonic bath for 16hr added 0.6ml of 1M K2HPO4 solution and added 10 ml of ethyl acetate, vortexed it, followed by centrifugation at 4000 rpm. The supernatant was evaporated to dryness, reconstituted with 1ml of Methanol: water (5:95) and 10µl is used for LC-MS/MS analysis.

LC Conditions

LC separation was achieved using the Shimadzu prominence system with an Eclipse plus C18 (4.6×150 mm) 5 µm column with a gradient of 1mM ammonium acetate as mobile phase A and Methanol as mobile phase B at flow rate of 0.4 mL/min. The injection volume was set to 10 µL. Gradient profile is given Table1.

Time (min)	Mobile phase A%	Mobile phase B%
0.01	95	5
0.50	45	55
3.50	45	55
4.00	95	5
12.00	Controller	Stop

Table 1: Mobile Phase Gradient

MS/MS Conditions

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in positive polarity. Two selective MRM transitions were monitored for all nitrofuran metabolites using the Analyst® 1.6.2 Software and MultiQuant™ Software version 3.0.2. MRM transition is given in Table 2.

Compound	Precursor ion	Product ion Quantifier	Product ion Qualifier
AOZ	236.0	104.0	78.0
AMOZ	335.0	291.1	128.2
SEM	209.0	166.0	192.0
AHD	249.1	134.0	104.0

Table 2: MRM transitions

Results and Discussions

The results of repeatability data obtained for nitrofuran metabolites in the honey matrix is given in table 3 at different levels.

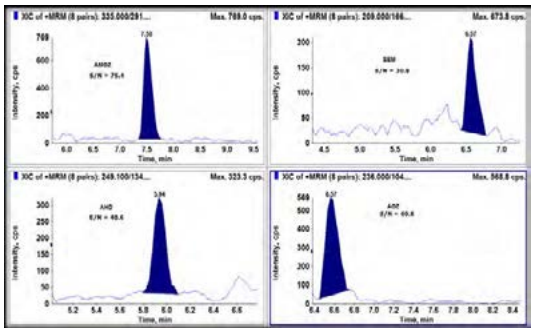

Figure 3: Signal to noise of AMZO, AHD, SEM and AOZ at MRPL level (1.0 ng/ml) in honey matrix sample

For all four Nitrofuran metabolites in honey the matrix based calibration curve shows excellent linearity (0.50 to 20.0 ppb), with a correlation coefficient r≥0.99 using linear regression and weighing factor 1/X. The SCIEX Triple Quad™ 3500 was found to be capable of analyzing concentrations well below the MRPL required by EU. The signal to noise ratio for all four nitrofuran metabolites at 1.0 ppb is ≥ 30. The signal to noise ratios and calibration curves are shown in Figure 3 and Figure 4.

Analyte	Repeatability			Recovery (n=6)		
	½ MRPL (0.5ppb)	MRPL (1.0ppb)	1.5MRPL (1.5ppb)	½ MRPL (0.5ppb)	MRPL (1.0ppb)	1.5MRPL (1.5ppb)
AOZ	6.01	7.00	4.28	113.47	95.05	89.89
AMOZ	12.16	4.46	4.33	83.80	103.88	96.11
SEM	4.49	7.31	9.44	109.67	98.13	91.33
AHD	4.96	7.58	8.22	114.90	105.40	105.00

Table 3: Repeatability (%CV) and recovery statistics in honey sample

Analyte	Calibration Range (ppb)	Linearity (r)	CCα	CCβ
AOZ	0.5 -20	0.9981	0.58	0.63
AMOZ	0.5 -20	0.9963	0.62	0.70
SEM	0.5 -20	0.9974	0.56	0.60
AHD	0.5 -20	0.9987	0.57	0.16

Table 4: Summary of CCα, CCβ and linearity in honey sample

Recovery experiments were performed in honey samples at ½ MRPL, MRPL and 1.5 MRPL level (n=6). The recovery of all nitrofuran metabolites was ≥ 80%. The recovery data for nitrofuran metabolites are shown in Table 3. The retention time (RT) of the AHD, AOZ, SEM, AMOZ, were 5.94, 6.57, 6.57 and 7.50 min, respectively.

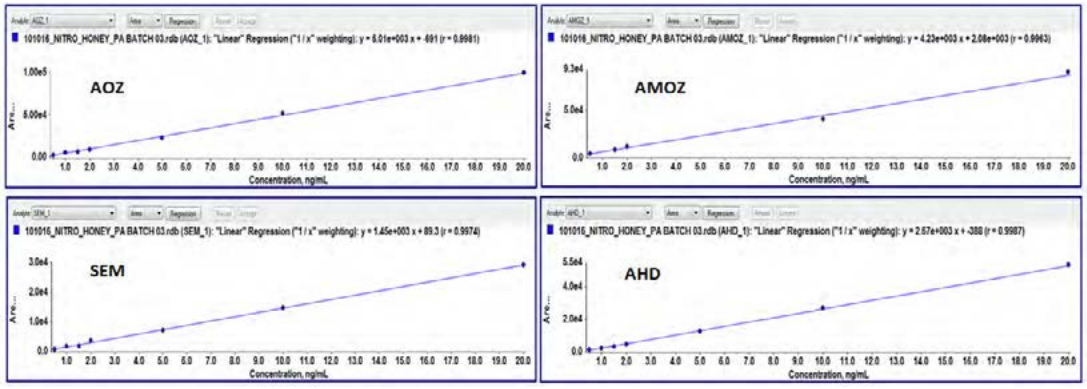

Figure 4: Matrix based calibration curve AOZ, AMOZ, SEM and AHD in Honey sample showing r = >0.99

Decision limit (CCα) and detection capability (CCβ) were calculated for all the four derivatives of Nitrofuran in Honey samples. The calculation was based on using linear regression model analyzing spiked honey samples at below MRPL level (Van Loco et al, 2007).

The calculated value of CCα and CCβ are given in table 4. The decision limit (CCα) and detection capability (CCβ) of all the metabolites were well below the MRPL.

Conclusions

- The developed quantitative method of Nitrofurans in honey on SCIEX Triple Quad™ 3500 was sensitive, linear, and reproducible.
- Trueness (Average recovery %) for this method found to be ≥ 80% at various MRPL levels.
- The method and data presented in the application note showcase the fast and accurate solution for the quantitation and identification of nitrofuran metabolites in honey samples for quality control check

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Contents

Analysis of Streptomycin and Its Metabolite in Honey Using the SCIEX Triple Quad™ 3500 System

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Overview

Streptomycin and Dihydrostreptomycin quantification method was developed in honey samples using SCIEX Triple Quad™ 3500 Liquid chromatography tandem mass spectrometry (LC-MS/MS) system. A simple sample preparation protocol was used for method development. The method showed adequate linearity with correlation coefficients above r≥0.98 for both analytes with the dynamic range of 0.25–20 ng/ml. The average accuracies for both the analytes were between88 to 117%. The Minimum Required Performance Limit (MRPL) for Streptomycin and Dihydrostreptomycin in Honey was 10µg/kg.

Introduction

Streptomycin (STR) and its metabolite dihydrostreptomycin (DHSTR) are the aminoglycoside antibiotics which work against gram-negative systemic bacterial infections. These antibiotics are commonly applied for crop protection and by bee keepers to eliminate disease among honeybees.

Safety of food and feed is one of the main objectives in consumer health policy. Honey is widely used as food and medicine. Many different antibiotics are used in Apiculture to keep bees away from various bacterial infections. Accumulation of antibiotic residues in the honey leads to adverse health effects during human consumption. Hence it become necessary for analyzing antibiotic residues in Honey as a part of its quality check.

There are several reports of Streptomycin (STR) and its metabolite dihydrostreptomycin (DHSTR) residue analysis in honey using LC-MS/MS. Streptomycin (STR) and its metabolite dihydrostreptomycin (DHSTR) belong to Aminoglycosides class of antibiotic. Aminoglycosides are a broad class of antibiotic having more than two amino sugars linked by glycosidic bonds to an aminocyclitol component.

Therefore, streptomycin and dihydrostreptomycin are highly soluble in water so the sample extraction, cleanup and

chromatographic method become very challenging in honey sample.

Figure 1. SCIEX Triple Quad™ 3500

SCIEX Triple Quad™ 3500 LC-MS/MS System takes the best features and enhances them with modern engineering and advanced eQ™ electronics. The proven design of Turbo V™ source and Curtain Gas™ interface provide exceptional robustness and ruggedness.

In this application note, we describe a sensitive, reproducible quantitative method for streptomycin (STR) and dihydrostreptomycin (DHSTR) in honey sample.

Contents

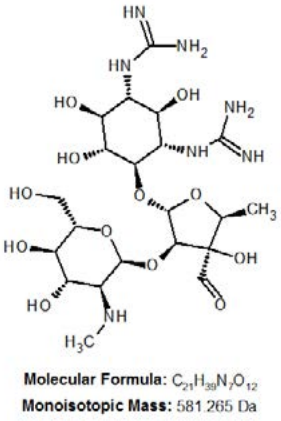

Figure 2. Structure of Streptomycin

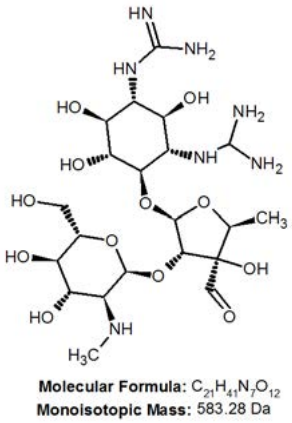

Figure 3. Structure of Dihydrostreptomycin

Materials and Methods

Chemicals

Streptomycin and Dihydrostreptomycin were purchased from Sigma Aldrich ≥99% Purity and SPE Cartridges were purchased from Agela. All other chemicals used were of LC-MS grade, commercially available.

Honey samples

Honey samples were procured from local market of Delhi and Gurgaon, India and were stored at room temperature until end of analysis.

Sample Preparation

Accurately weighed 1.0g of honey sample, mixed with 3ml of 100mM Ammonium acetate, vortexed for 5min and loaded into Cleanert (PWCX-SPE 30mg/ml) cartridge. Washed with 2ml of Methanol: water (80:20) and eluted with 1ml of methanol containing 5% formic acid. The eluent was evaporated to dryness under Nitrogen. Reconstituted in 1ml of Acetonitrile: water: Formic acid (5:95:0.2%) and subjected to LC-MS/MS analysis.

LC Conditions

LC separation was achieved using the Shimadzu prominence system with ZORBAX SB-C18 (4.6×150 mm) 5 µm column with a gradient (Table 1) of 0.1% Heptafluorobutyric acid (A) and Acetonitrile (B) at flow rate of 0.8 mL/min. The injection volume was 25 µL.

Time (min)	Mobile phase A%	Mobile phase B%
0.01	75	25
1.50	75	25
4.00	90	10
5.30	75	25
10.00	Controller	Stop

Table 1. Gradient Time Program

MS/MS Conditions

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with Electrospray Ionization (ESI) probe in positive polarity. Two selective MRM transitions were monitored (Table 2).

Compound	Precursor ion	Product ion Quantifier	Product ion Qualifier
Streptomycin	582.3	263.4	246.2
Dihydrostreptomycin	584.3	263.1	246.2

Table 2. MRM Transition for Streptomycin and Dihydrostreptomycin

Results and Discussions

The matrix based calibration curve for Streptomycin and its metabolites Dihydrostreptomycin showed excellent linearity (0.25 x MRPL to 2 x MRPL level), with a correlation coefficient r≥0.99 using linear regression and weighing factor 1/X2. The developed method in honey was found to be capable of analyzing concentrations well below the MRPL (10µg/Kg) required by EU.

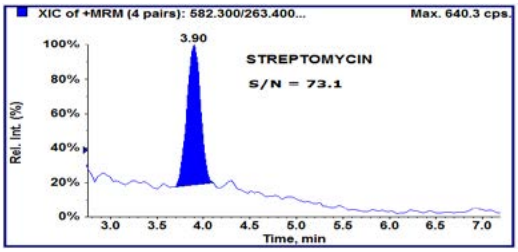

Figure 4. Signal to Noise (S/N) of Streptomycin at 10 ppb in matrix based samples.

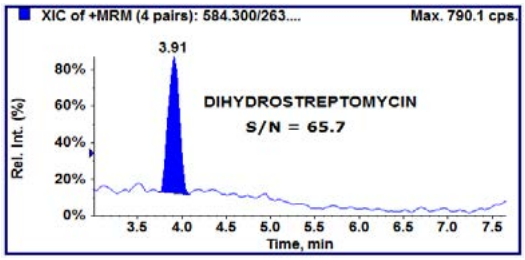

Figure 5. Signal to Noise (S/N) of Dihydrostreptomycin at 10 ppb in matrix based samples.

The signal to noise ratio for streptomycin and dihydrostreptomycin is ≥ 73.1 and ≥ 65.7, respectively at MRPL level and the recovery and repeatability (%CV) data obtained for streptomycin and dihydrostreptomycin in the honey matrix is given in Table 3. The signals to noise ratio chromatogram at MRPL are shown in Figure 4 and 5

Analyte	Repeatability (%CV, n=6)		Recovery (n=6)
	½ MRPL (5.0ppb)	MRPL (10.0ppb)	MRPL (10.0ppb)
STR	9.34	6.96	101.75
DHSTR	10.21	9.51	99.73

Table 3. Recovery and Repeatability (%CV) statistics of Streptomycin and Dihydrostreptomycin in the honey matrix (10ppb).

Recovery experiment was performed in honey samples at 10ppb MRPL level (Six replicates). The Recovery of streptomycin and dihydrostreptomycin were 101.75 and 99.73% respectively. The retention time of streptomycin and dihydrostreptomycin were 3.90 min and 3.91 min, respectively. A representative chromatogram obtained from a standard mixture of the Streptomycin and Dihydrostreptomycin is given in Figure 6 showing the qualifier and quantifier.

Repeatability experiment was evaluated at the MRPL level of 10 ppb (n=6) gives %CV of ≤ 10.0.

Contents

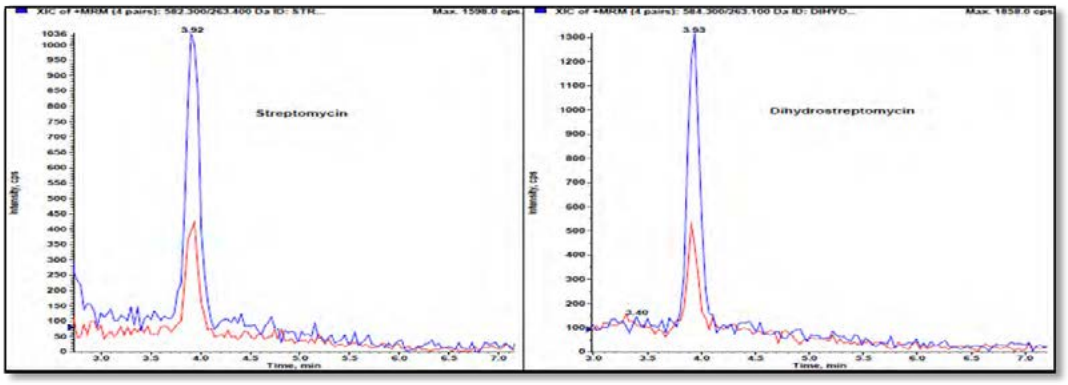

Figure 6. Representative chromatogram of Streptomycin and Dihydrostreptomycin (Quantifier & Qualifier) at 20ppb

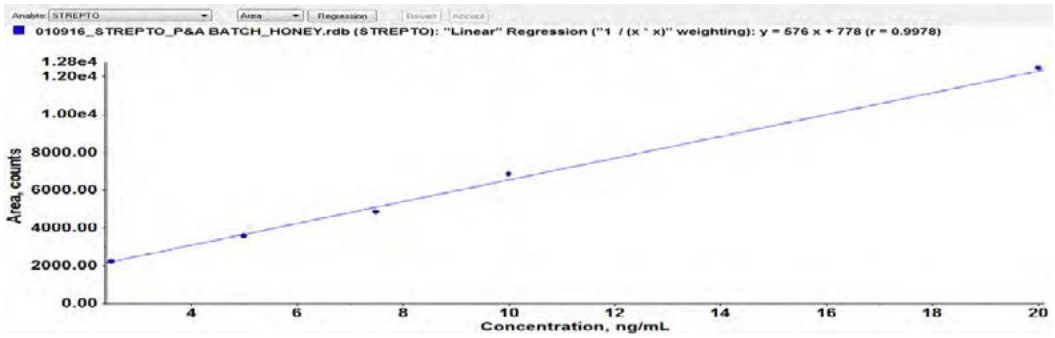

Figure 7. Linear range of the detection of Streptomycin from 2.50 to 20.0 ppb (r ≥ 0.99).

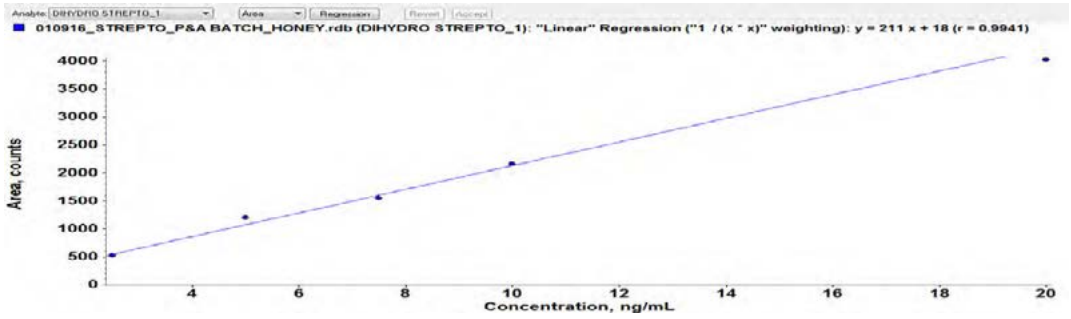

Figure 8. Linear range of the detection of Dihydrostreptomycin from 2.50 to 20.0 ppb (r ≥ 0.99).

Conclusions

- The developed quantitation method on SCIEX Triple Quad™ 3500 was simple, sensitive, linear, and reproducible for Streptomycin and its metabolite
- Better separation and reproducibility of Streptomycin and Dihydrostreptomycin was achived using ion pairing reagent in Honey sample.
- Average recovery for this method found to be ≥ 95% meeting the requirement of EU/SANCO regulation of 70-120%.
- This method exhibited excellent linearity from 0.25x MRPL to 2x MRPL, with a correlation coefficient r≥0.99.
- Method developed on SCIEX Triple Quad™ 3500 can be used to check the presence of streptomycin and dihydrostreptomycin in honey sample for quality control purpose.

References

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Analysis of Chloramphenicol in Honey Using the SCIEX Triple Quad™ 3500 System

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[1]SCIEX, Udyog Vihar, Phase IV, Gurgaon - 122015, Haryana, India; [2]SCIEX, Darmstadt, Germany

Overview

The combination of liquid chromatography with tandem mass spectrometry (LC-MS/MS) allows identification and quantification of trace amount of chloramphenicol in complex food matrices due to the specificity and sensitivity associated with this technique. 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. The method development was performed according to the criteria established by the EC Commission Decision 657/2002[1]. This method described here demonstrates the ability of SCIEX Triple Quad™ 3500 to carry out quantitation and the confirmation of the chloramphenicol in honey at the required minimum required performance limit (0.3ng/ml).

Figure 1. SCIEX Triple Quad™ 3500

Introduction

Chloramphenicol has a wide spectrum of antimicrobial activity. It is effective against Gram-positive and Gram-negative cocci and bacilli (including anaerobes), Rickettsia, Mycoplasma, Chlamydia, among others. It is widely used as a human antibiotic and also as a veterinary drug. A triple quadrupole based method for the quantitation of Chloramphenicol in honey was developed using selective Multiple Reaction Monitoring (MRM). The ratio of quantifier and qualifier transition was used for compound identification. Sensitivity of detection met existing regulatory requirements, such as Codex Alimentarius' minimum required performance limit was 0.3ng/ml. The method was successfully applied to the analysis of store bought honey samples.

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.

Figure 2. Re-engineered quadrupole to maximize efficiency

Contents

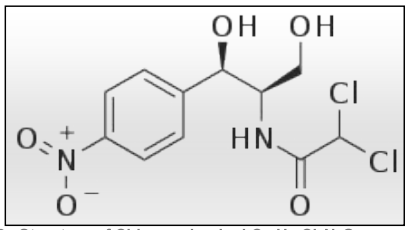

Figure 3. Structure of Chloramphenicol $C_{11}H_{12}Cl_2N_2O_5$ 322.0123 g/mol

Unique Features

1. A sensitive, specific, rugged and reproducible LC-MS/MS method was developed for Chloramphenicol using simple Extraction technique for the sample preparation.
2. Accuracy and Precision for Chloramphenicol in honey samples found to be between 85-115%
3. Reproducibility of results for Chloramphenicol in terms of % CV in honey samples is less than 5%
4. Average recovery of Chloramphenicol in honey using the developed extraction method is more than 84%

Materials and Methods

Chemicals

Standard Chloramphenicol (with chemical purity ≥ 99%) was purchased from Clearsynth. All other chemicals used were of LC-MS grade.

Honey samples

Honey samples were procured from the local market of Delhi & Gurgaon in India and were kept at 2 - 8 °C until end of analysis.

Sample Preparation

1. Weighed 1 gm honey added 5 ml water and vortex well for 2 min.
2. Add 10 ml of Acetonitrile and 1gm of sodium chloride. Mix well.
3. Centrifuge for 5 min at 4000 rpm. Transfer the acetonitrile layer and evaporate under nitrogen steam.
4. Reconstitute with 1 ml diluent (water: acetonitrile 90:10), and used for LC-MS/MS analysis.

LC Conditions

LC separation was performed on a Shimadzu instrument using Synergy Fusion RP 18e (50 X 2.6) mm 2.5µ and a fast gradient of water (Mobile Phase A) and acetonitrile (Mobile Phase B) from 85% aqueous to 85% organic in 5 minutes at a flow rate of 0.4ml/min and injection volume of 20µl is used to obtain a good peak shape.

Time (min)	Mobile phase A%	Mobile phase B%
0.01	85	15
0.30	85	15
0.50	75	25
1.00	70	30
1.50	15	85
3.00	15	85
4.00	85	15
5.00	Controller	Stop

Table 1. Mobile Phase Gradient

MS/MS Conditions

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe negative ionization mode at 2800 ion spray voltage, with Declustering potential(DP) -85V and Collision Energy(CE) -15V and -23V respectively. Two selective MRM transitions were monitored and ion ratio is calculated automatically by software for compound identification. Analyst® 1.6.2 Software was used for method development and data acquisition. LC-MS/MS data was processed using the MultiQuant™ Software version 3.0.1

Contents

Compound	Precursor ion	Product ion Quantifier	Product ion Qualifier
Chloramphenicol	320.8	151.8	256.8

Table 2. Selected MRM transitions

Results and Discussions

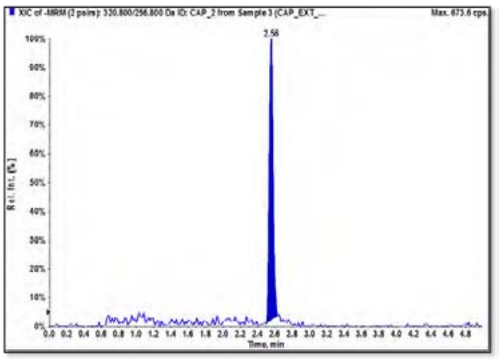

Figure 4. Representative Chromatogram of Chloramphenicol (0.1ng/ml) in Honey Matrix.

The calibration curves were made with standard levels ranging from 0.1ng/ml to 5.0ng/ml spiked concentration; 0.1ng/ml is set as the lowest linearity calibration point with the regression co-efficient (r): 0.99 by using weighing factor 1/X2.

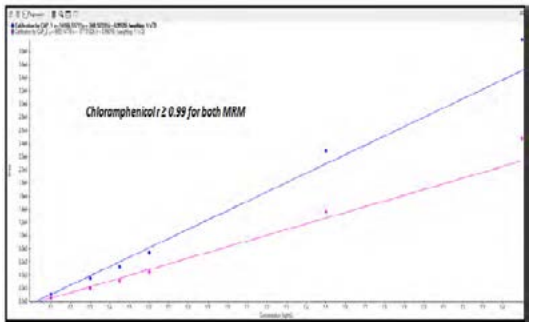

Figure 5. Linear range of the detection of Chloramphenicol from 0.1 to 5.0ng/mL (r = 0.99)

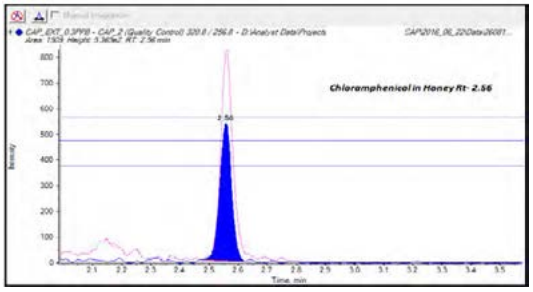

Figure 6. MRM Ratio of Chloramphenicol at Rt- 2.56 was ≤ 1.0

All Sample Types

Sample Name	Sample Type	Component Name	Area	Actual Concentration	Calculated Concentration	Accuracy	Retention Time	Used	MRM Ratio
CAP_EXT_BLK	Blank	CAP_2	N/A	N/A	N/A	N/A	N/A	☑	0.000
CAP_EXT_0.1PPB	Standard	CAP_2	544	0.10	0.1	106.04	2.55	☑	0.474
CAP_EXT_0.3PPB	Standard	CAP_2	1975	0.30	0.3	90.21	2.56	☑	0.563
CAP_EXT_0.45PPB	Standard	CAP_2	3094	0.45	0.4	88.73	2.55	☑	0.584
CAP_EXT_0.6PPB	Standard	CAP_2	4394	0.60	0.5	91.48	2.55	☑	0.586
CAP_EXT_1PPB	Standard	CAP_2	13696	1.50	1.6	107.93	2.55	☑	0.599
CAP_EXT_5PPB	Standard	CAP_2	24750	2.50	2.9	115.62	2.55	☑	0.622

Table 3. Accuracy data obtained for Chloramphenicol in the Honey matrix

Contents

Recovery was assessed by performing tests in honey samples in replicates (n=6) at MRPL concentration respectively. The Recovery of Chloramphenicol was ≥ 84% at RT of 2.56 minutes with minimum background noise in 5.0 minutes chromatographic run.

Replicates (n=6)	Chloramphenicol MRPL
1	0.300
2	0.250
3	0.250
4	0.250
5	0.270
6	0.260
Average Conc. (ng/mL)	0.263
Original Conc. (ng/mL)	0.300
% Recovery	87.78

Table 4. Recovery of Chloramphenicol in the Honey matrix at MRPL concentration level

Summary

The method and data presented here showcase the fast and accurate solution for the quantitation and identification of Chloramphenicol in honey samples by LC-MS/MS which meets the regulatory requirements. The SCIEX Triple Quad™ 3500 System provide excellent sensitivity and selectivity, with minimal sample preparation allowing maximized throughput for the analysis of many samples in a short time period. Automatic MRM ratio calculation in MultiQuant™ Software can be used for confirmation in compound identification.

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Analysis of Sulfonamides in Honey Using the SCIEX Triple Quad™ 3500 System

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Introduction

Honey is widely consumed as food and medicine. Many different antibiotics are used in Apiculture to keep bees away from various bacterial infections. Accumulation of antibiotic residues in the raw material from bees, lead to adverse health effects during human consumption.

According to European Union regulations, honey is considered as a natural product and must be free of chemicals. Antibiotics used in honey and other bee products production are usually veterinary medicines. Beekeepers use high doses of antibiotics to prevent and treat bacterial infections in honey. Antibiotic residues have a relatively long half-life and may have direct toxic effects on consumers. Extensive use of antibiotics and its accumulation makes the trade of honey difficult globally. To reduce the chances of health risks to consumers, regulatory legislations were laid down for various antibiotic classes for honey. Minimum required performance limit (MRPLs) of antibiotics have been set to levels as small as parts-per-billion (ppb).

The LC-MS/MS method was developed using the Multiple Reaction Monitoring (MRM) that detects antibiotics as per European Union regulatory guidelines with the consideration of two Transitions to one analyte (ratio of quantifier and qualifier ion) for all the nine sulfonamides (Sulfamerazine, Sulfadiazine, Sulfamethazine, Sulfadimethoxine, Sulfamethoxypyridazine, Sulfamethoxazole, Sulfadoxine, Sulfathiazole, and Sulfapyridine) on SCIEX Triple Quad™ 3500 LC/MS/MS System with minimum required performance limit (10.0 ng/ml).

Figure 1. SCIEX Triple Quad™ 3500

Contents

Compound	Precursor ion	Product ion Quantifier	Product ion Qualifier
Sulfamerazine	265.0	155.9	107.9
Sulfadiazine	251.0	156.0	91.9
Sulfamethazine	279.0	186.0	124.0
Sulfadimethoxine	311.0	156.1	92.0
Sulfamethoxypyridazine	280.9	91.9	107.9
Sulfamethoxazole	254.0	155.8	92.0
Sulfadoxine	310.9	155.9	92.0
Sulfathiazole	256.3	156.1	92.0
Sulfapyridine	249.9	155.7	108.1

Table 1. MS Transition for Sulfonamides

Materials and Methods

Chemicals

Sulfonamides Standards were purchased from Sigma Aldrich. All other chemicals used were of LC-MS grade, commercially available.

Honey samples

Honey samples were purchased from local market of Delhi and Gurgaon, India and were stored at room temperature for analysis.

Sample Preparation

Accurately weighed 1 g of honey, mixed with 2ml of 0.1 M HCl, sonicated for 30 min. Followed by the addition of 3ml of 0.3M Citric acid (final vol. around 5 ml) and Vortex well. Then 10 ml Acetonitrile with 0.1% Formic Acid (FA) was added and mixed well for 10 min. followed by the addition of 2 g Sodium chloride (NaCl), vortexed again, centrifuged, collected supernatant and evaporated at 500C. The residue was reconstituted in 1 ml Methanol: Water (80:20) with 0.1% Formic Acid (FA) and transfer into vial for analysis.

LC Conditions

LC separation was achieved using the Shimadzu prominence system with a Zorbax SB C-18 (4.6×150 mm) 5 µm column with a gradient of water containing 0.1% formic acid (mobile phase A) and Acetonitrile containing 0.1% formic acid(mobile phase B) at flow rate of 0.5 mL/min. The injection volume was set to 10 µL.

Time (min)	Mobile phase A%	Mobile phase B%
0.01	98	2
5.50	2	98
6.00	2	98
8.00	98	2
11.00	Controller	Stop

Table 2. Gradient method

MS/MS Conditions

The SCIEX Triple Quad™ 3500 LC/MS/MS System was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in positive polarity. Two selective MRM transitions were monitored for all sulfonamides using the ratio of quantifier and qualifier ion for compound identification. Analyst 1.6.2 Software was used for method development and data acquisition. LC-MS/MS data was processed using the MultiQuant™ Software version 3.0.2

Results and Discussions

The calibration curve shows excellent linearity, with a correlation coefficient greater than 0.98 for nine sulfonamides using linear regression and weighing factor 1/X2. Matrix based calibration curves were made with standard levels ranging from 1.0 ng/ml to 100 ng/ml spiked concentration; linear graph was obtained with regression co-efficient (r) ≥0.99 for all the nine sulfonamides. The calibration curve was shown in Figure 3 and a representative chromatogram was shown in Figure 4 & 5

The retention times of the analytes were ranging from 5.50 min to 7.00 min. A representative chromatogram obtained from a standard mixture of the sulfonamides with minimum background noise in 11.0 minutes chromatographic run.

The method demonstrated good precision and accuracy batch. No interferences with the peaks of interest were observed throughout the chromatographic run.

The recovery study was carried out by spiking the honey samples with 10 ng/ml concentration of Sulfonamides and found the recoveries ≥ 86% at MRPL level. The recovery was performed with six replicates (n=6) respectively. The recovery data for sulfonamides are shown in Table 4.

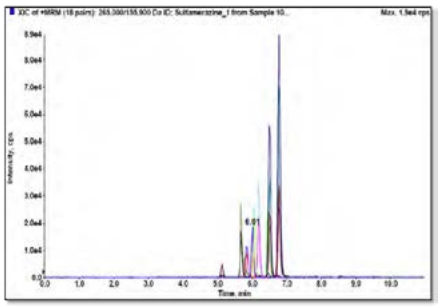

Figure 3. Representative chromatogram of Sulfonamide (Sulfamerazine) at MRPL Level (10ng/ml) Concentration.

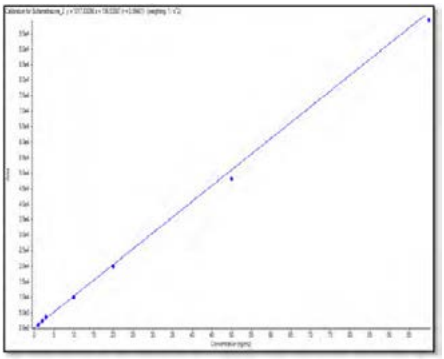

Figure 2. Linear range of the detection of Sulfamethazine from 1.0 to 100ng/mL (r ≥ 0.98)

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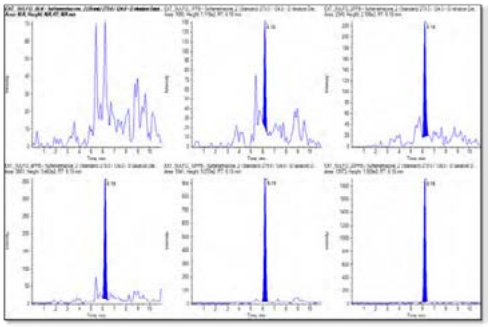

Figure 4. Representative Calibration Linearity chromatogram of Sulfonamide (Sulfamerazine) from 1.0 – 20.0ng/ml.

All Sample Types

Sample Name	Sample Type	Component Name	Mass Info	Actual Concentration	Calculated Concentration	Accuracy	Retention Time	Used	MRM Ratio
EXT_SULFO_BLK	Blank	Sulfamethazine_2	279.0 / 124.0	N/A	N/A	N/A	N/A	☑	0.000
EXT_SULFO_1PPB	Standard	Sulfamethazine_2	279.0 / 124.0	1.00	0.92	91.69	6.18	☑	0.970
EXT_SULFO_2PPB	Standard	Sulfamethazine_2	279.0 / 124.0	2.00	2.15	107.72	6.18	☑	0.742
EXT_SULFO_3PPB	Standard	Sulfamethazine_2	279.0 / 124.0	3.00	3.46	115.32	6.19	☑	0.778
EXT_SULFO_10PPB	Standard	Sulfamethazine_2	279.0 / 124.0	10.00	9.61	96.13	6.19	☑	0.812
EXT_SULFO_20PPB	Standard	Sulfamethazine_2	279.0 / 124.0	20.00	19.47	97.35	6.18	☑	0.825
EXT_SULFO_50PPB	Standard	Sulfamethazine_2	279.0 / 124.0	50.00	47.17	94.34	6.19	☑	0.791
EXT_SULFO_100PPB	Standard	Sulfamethazine_2	279.0 / 124.0	100.00	97.46	97.46	6.19	☑	0.826

Table 3. Accuracy data obtained for sulfonamides (Sulfamethazine) with MRM Ratio

Compound	% Recovery 10 ppb
Sulfamerazine	91.33
Sulfadiazine	96.93
Sulfamethazine	89.33
Sulfadimethoxine	93.02
Sulfamethoxypyridazine	86.52
Sulfamethoxazole	91.05
Sulfadoxine	91.58
Sulfathiazole	97.35
Sulfapyridine	90.43

Table 4. Recovery of sulfonamides at MRPL (10ng/ml) in honey matrix

Repeatable injections (n= 06) at MRPL gives the % relative standard deviation of ≤5.0%.

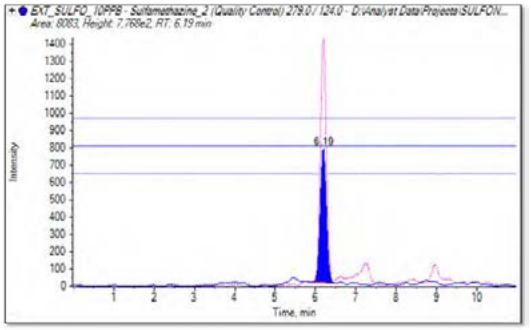

Figure 5. MRM Ratio of sulfonamides (Sulfamethazine) at RT-6.19 was ≤ 1.0

Summary

A SCIEX Triple Quad™ 3500 LC/MS/MS System reduces analysis time and improves sensitivity and resolution, detecting and quantifying several classes of sulfonamides drugs. Nine sulfonamide analytes were determined with a single extraction and the proposed method could be applied in routine analysis.

The method and data presented here showcased the fast and accurate solution for the quantitation and identification of Sulfonamides in honey samples by LC-MS/MS.

Matrix interferences study was conducted to understand the matrix effects. Automatic MRM ratio calculation in MultiQuant™ Software can be used for confirmation in compound identification.

References

[1]Method validation and quality control procedures for pesticide residue analysis in food and feed Document NO. SANCO/12495/2011, Implemented by 01/01/2012

[2]Guidelines for the design and implementation of National regulatory food safety assurance programme associated with use of veterinary drugs in food producing animals CAC/GL 71-2009

[3]Rongyuan Liu, Pingli He, Zhen Li, and Ruiguo Li; Simultaneous Determination of 16 Sulfonamides in Animal Feeds by UHPLC–MS–MS. Journal of Chromatographic Science, (2011) Vol. 49 (8):640-646

[4]Neha Bhasin, Prasanth Joseph, Praveen Sharma, Manoj Pillai, Jens Dahlmann, André Schreiber and Christopher Borton; Targeted Multi-Residue LC-MS/MS Method for Sulfonamide and Nitroimidazole Antibiotics in Honey. (2015), AOAC poster

Document number: RUO-MKT-02-4797-A

Quantitation Method for Nitrofuran Metabolites in Milk Using SCIEX Triple Quad™ 3500 System

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Overview

A liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantification of Nitrofuran metabolites in milk was developed. The method presented adequate linearity with correlation coefficients above r ≥0.99 for both analytes in the dynamic range of 0.50–20.0 µg/kg, with average accuracies for matrix based recovery were in the range 85%–120%. The results qualified the method for the quantification and confirmation of the analytes in milk at concentrations lower to the established Minimum Required Performance Limit (1.0µg/kg).

Introduction

Nitrofurans are synthetic chemotherapeutic agents which have a broad spectrum of bacteriostatic activity. Nitrofurans mainly inhibit the enzymes involved in the carbohydrate metabolism. These bacteriostatic compounds are prohibited in livestock production by EU which is listed in Annexe IV of EC Council Regulation 2377/90. No MRLs have been established for Nitrofurans hence it is necessary to have sensitive confirmatory analytical methods for the detection of nitrofuran residues in food commodities. Further the detection of Nitrofurans has been shown to be difficult as they are quickly metabolized. Therefore the analysis of the protein bound, solvent extractable metabolites of Nitrofurans have been reported as the ideal choice of analysis. Analytically, residues are checked only for marker metabolites of the 4 nitrofuran chemicals, in particular: 3-amino-2-oxazolidinone (AOZ) for furazolidone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) for furaltadone,1-aminohydantoin (AHD) for nitrofurantoin and Semicarbazide (SEM) for Nitrofurazone (Figure 2).

Figure 1. SCIEX Triple Quad™ 3500

In general the study of nitrofuran metabolites in food samples requires incubation period for derivatization with nitrobenzaldehyde for 16hr at 37ºC in dark. The quantitative and confirmatory determination of nitrofuran metabolites was performed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) in positive ion mode, according to European Decision 2002/657/EC. The MRPL for nitrofuran metabolites (individual) is 1.0µg/kg as per RMP/EU/2016-17.

The present application note describes a method which is sensitive and selective enough to meet the global guidelines analyze the nitrofuran metabolites in milk using SCIEX Triple Quad™ 3500 LC-MS/MS System.

Contents

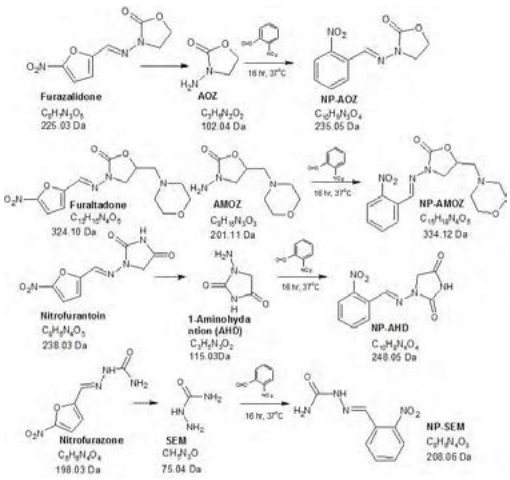

Figure 2. Structures of Nitrofuran, Nitrofuran metabolites and Nitrophenyl derivatives.

Materials and Methods

Chemicals

Nitrofuran metabolites Standards were purchased from Clearsynth and 2-Nitrobenzaldehyde was purchased from Sigma Aldrich ≥99% Purity. All other chemicals used were of LC-MS grade.

Sample Preparation

Milk sample (3 ml) was mixed with 1ml of HCl (0.1M) and 50mM of 2-Nitrobenzaldehyde (0.3ml), vortexed and incubated on ultrasonic bath for 16hr added 0.6ml of 1M K2HPO4 solution and 10 ml of ethyl acetate, vortexed, followed by centrifugation at 4000 rpm, the supernatant was evaporated to dryness reconstituted with 1ml of Methanol: water (5:95) and 10µl is used for LC-MS/MS analysis.

Experimental Conditions

LC Conditions

LC separation was performed on a Shimadzu instrument using Zorbax Eclipse Plus C18(150 X 4.6)mm 5.0µ and a fast gradient of 1mM Ammonium acetate(Mobile Phase A) and Methanol(Mobile Phase B) at a flow rate of 0.4ml/min (Table 1).

Time (min)	Mobile phase A%	Mobile phase B%
0.01	95	5
0.50	45	55
3.50	45	55
4.00	95	5
12.00	Controller	Stop

Table 1: Mobile Phase Gradient

MS/MS Conditions

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The TurboV™ source was used with an Electrospray Ionization (ESI) probe in positive ionization mode at 5500 ion spray voltage. Two selective MRM transitions were monitored and ion ratio was calculated automatically by software for compound identification (Table 2). Analyst® 1.6.2 Software was used for method development and data acquisition. LC-MS/MS data was processed using the MultiQuant™ Software version 3.0.2

Compound	Precursor ion	Product ion Quantifier	Product ion Qualifier
AOZ	236.0	104.0	78.0
AMOZ	335.0	291.1	128.2
SEM	209.0	166.0	192.0
AHD	249.1	134.0	104.0

Table 2: MRM transitions

Results and Discussions

Sensitivity, Reproducibility, Linearity and Accuracy

The developed method showed signal-to-noise ratio > 23 for all the analytes with sample extracted at a level of 1.0 µg/kg (Spiked) which meets the regulatory criterion (Figure 3)

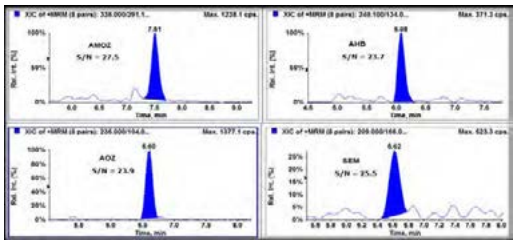

Figure 3: Chromatogram (1.0 µg/kg) showing signal to noise

Matrix based Calibration curve was plotted, found linear in the range of 0.50 µg/kg (ppb) to 20.0µg/kg (ppb) and correlation regression co-efficient r > 0.98 for both quantifier and qualifier ions by applying weighing factor of 1/X2 (Table 4).

Repeatability at three levels (1/2 MRPL, MRPL, 1.5MRPL) were evaluated for 6 injections and %relative standard deviation (%CV) was observed to be less than 10 (Table 3). Accuracies observed were in the range from 85% to 120%.

Analyte	Repeatability			Recovery (n=6)		
	½ MRPL (0.5ppb)	MRPL (1.0ppb)	1.5MRPL (1.5ppb)	½ MRPL (0.5ppb)	MRPL (1.0ppb)	1.5MRPL (1.5ppb)
AOZ	3.41	5.04	1.72	98.83	105.00	107.89
AMOZ	5.98	2.89	3.49	94.37	92.82	101.67
SEM	8.70	8.84	3.30	92.37	102.75	108.33
AHD	7.12	5.86	8.00	92.57	86.93	96.56

Table 3: Repeatability (%CV) and recovery statistics and in Milk

Analyte	Calibration Range (ppb)	Linearity (r)	CCα	CCβ
AOZ	0.5 -20	0.9994	0.54	0.57
AMOZ	0.5 -20	0.9994	0.57	0.61
SEM	0.5 -20	0.9964	0.59	0.65
AHD	0.5 -20	0.9992	0.58	0.63

Table 4: Summary of CCα, CCβ and linearity in milk Sample

Decision limit (CCα) and detection capability (CCβ) were calculated for AOZ, AMOZ, SEM and AHD derivatives of Nitrofuran in milk samples. The calculation was based on using linear regression model analyzing spiked milk samples at below MRPL level (Van Loco et al, 2007).

The calculated value of CCα and CCβ are given in Table 4. The decision limit (CCα) and detection capability (CCβ) of all the compounds were well below the MRPL.

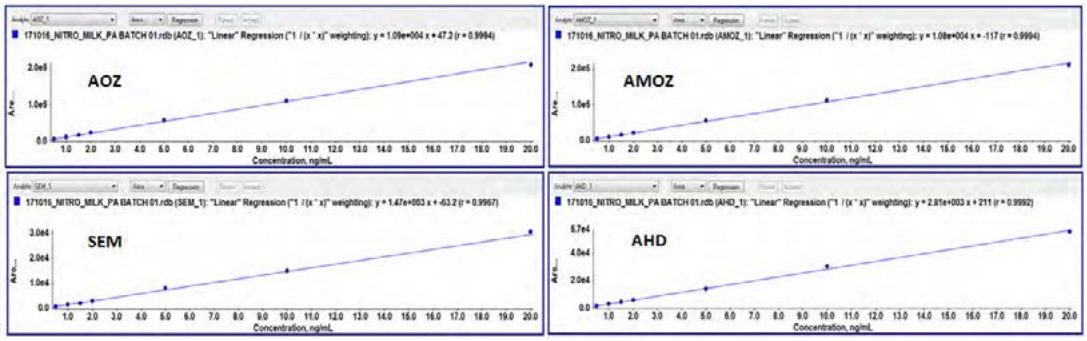

Figure 4: Matrix based calibration curve AOZ, AMOZ, SEM and AHD

Conclusion

The method and data acquired here gives sensitive and accurate solution for the quantitation and confirmation of Nitrofuran metabolites in Milk samples by LC-MS/MS. The SCIEX Triple Quad™ 3500 System provides good sensitivity and selectivity for this analysis, allowing maximum output for the analysis of a bigger batch of samples in a short time period. Automatic ion ratio calculation in MultiQuant™ Software can be used for confirmation of compound. The method showed acceptable accuracies (85%-120%), linearity with r >0.99 for both quantifier and qualifier, repeatability (%CV) observed was less than 10. The method allows high throughput, selective, rapid and sensitive LC-MS/MS identification and quantitation of banned Nitrofuran metabolites meeting EU MRPL of 1.0 µg/kg.

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1. FAO-Dairy Production and products: milk production facts, Food and Agriculture Organization of United Nations, Rome.-2013 http://fao.org/agriculture/ dairy-gateway/meat-production/en.
2. Method validation and quality control procedures for pesticide residue analysis in food and feed: SANCO/12495/2011
3. FSSAI manual of methods of analysis of food. http://fssai.gov.in/Portals/0/Pdf/15Manuals/ANTIBIOTICS%20AND%20RESIDUES.pdf
4. 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
5. Neha Bhasin, Prasanth Joseph, Praveen Sharma, Manoj Pillai, Jens Dahlmann, André Schreiber and Christopher Borton; Targeted Multi-Residue LC-MS/MS Method for Sulfonamide and Nitroimidazole Antibiotics in Honey, Poster, AOAC conference (2015)
6. Tomasz Śniegocki, Andrzej Posyniak, And Jan Żmudzki, Determination of Nitrofuran Metabolite Residues In Eggs By Liquid Chromatography-Mass Spectrometry. Bull Vet Inst Pulawy 52, 421-425 (2008)
7. J. Van Loco, A.Janosi, S. Impens, S. Fraselle, V. Cornet, J.M. Degroodt, Calculation of the decision limit (CCα) and the detection capability (CCβ) for banned substances: The imperfect marriage between the quantitative and the qualitative criteria. Analytica Chimica Acta Volume 586, Issues 1–2, 14, Pages 8–12(2007)

Document number: RUO-MKT-02-5032-A

Analysis of Sulfonamides in Milk Using the SCIEX Triple Quad™ 3500 System

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Overview

A LC-MS/MS method for the simultaneous quantification of nine sulfonamides (sulfamerazine, Sulfadiazine, Sulfamethazine, Sulfadimethoxine, Sulfamethoxypyridazine, Sulfamethoxazole, Sulfadoxine, Sulfathiazole, and Sulfapyridine) on SCIEX Triple Quad™ 3500 was developed with a simplified sample preparation to detect veterinary residues, The method presented here demonstrated adequate linearity with correlation coefficients above r≥0.99 for all the nine sulfonamides analyzed.

Introduction

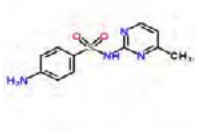
Sulfonamides (SAs) are used to treat a wide variety of bacterial and protozoal infections in animals. The presence of these antimicrobials can be a potential risk for consumers health if present above the allowed limits. Sulfonamides are illegally used as additives in animal feed as a growth promoters and thus they can generate serious threats in human health such as allergic or toxic reactions, carcinogenic.

For the purpose of monitoring the presence of these residues, an LC-MS/MS method was established to identify and quantify the nine sulfonamide residues in milk with a very simple sample preparation and shorter runtime. The Committee for Veterinary Medicinal Products considers that the sum of all substances belonging to the sulfonamide group in bovine milk should not exceed 100 µg/kg (EMEA, 1995a)

Figure 1. SCIEX Triple Quad™ 3500

Figure 2. Re-engineered quadrupole to maximize efficiency

Contents



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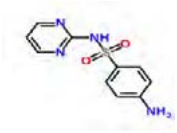
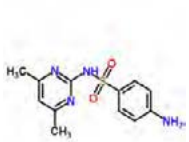
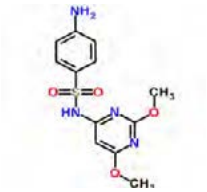


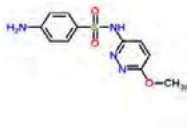
2.Sulfadiazine



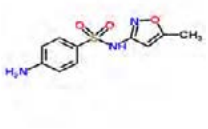
3. Sulfamethazine



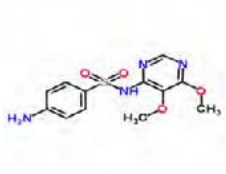
4.Sulfadimethoxine



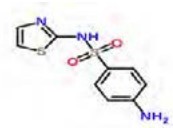
5. Sulfadimethoxypridazine



6. Sulfamethoxazole



7. Sulfadoxine



8.Sulfathiozole

9. Sulfapyridine

Figure 3: Structures of Sulfonamide Analytes.

Materials and Methods

Chemicals

Sulfonamides Standards were purchased from Sigma Aldrich ≥99% Purity. All other chemicals used were of LC-MS grade, commercially available.

Milk samples

Milk samples were procured from local market of Delhi and Gurgaon, India and was stored at 2−8 °C until end of analysis.

Sample Preparation

1. 1 ml Milk sample is mixed with 5ml of acidified acetonitrile
2. Add 1 gm of Sodium Chloride and Vortex followed by centrifugation at 4000 rpm
3. Transfer the supernatant and evaporate with N2 steam to dryness
4. Reconstitute with 1ml of Methanol: water: Formic Acid (80:20:0.1%) and use it for LC-MS/MS analysis.

LC Conditions

LC separation was achieved using the Shimadzu prominence system with a Zorbax SB C-18 (4.6×150 mm) 5 µm column with a gradient of water with (0.1% formic acid) as mobile phase A and Acetonitrile with (0.1% formic acid) as mobile phase B at flow rate of 0.5 mL/min. The injection volume was set to 10 µL.

Time (min)	Mobile phase A%	Mobile phase B%
0.01	98	2
5.50	2	98
6.00	2	98
8.00	98	2
11.00	Controller	Stop

Table 1. Gradient Time Program

MS/MS Conditions

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in positive polarity. Two selective MRM transitions were monitored for all sulfonamides using the Analyst® 1.6.2 Software and MultiQuant™ Software version 3.0.2.

Compound	Precursor ion	Product ion Quantifier	Product ion Qualifier
Sulfamerazine	265.0	155.9	107.9
Sulfadiazine	251.0	156.0	91.9
Sulfamethazine	279.0	186.0	124.0
Sulfadimethoxine	311.0	156.1	92.0
Sulfamethoxypyridazine	280.9	91.9	107.9
Sulfamethoxazole	254.0	155.8	92.0
Sulfadoxine	310.9	155.9	92.0
Sulfathiazole	256.3	156.1	92.0
Sulfapyridine	249.9	155.7	108.1

Table 2. MS Transition for the nine sulfonamides.

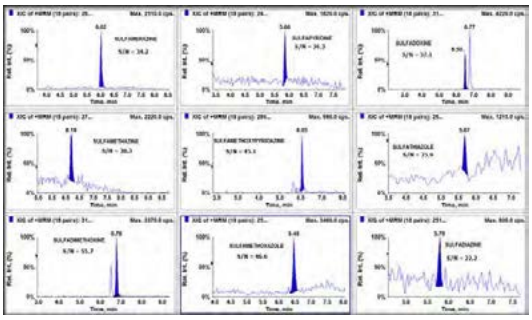


Figure 4: Signal to Noise (S/N) of all sulfonamides at 5 ng/ml concentration.

Results and Discussions

The matrix matched calibration curve shows excellent linearity (5 to 300ng/ml), with a correlation coefficient r≥0.98 for all sulfonamides in milk using linear regression and weighing factor 1/X2. The lowest calibration point for quantitation of sulfonamides was 5 ng/ml. The SCIEX Triple Quad™ 3500 was found to be capable of analyzing concentrations well below the MRPL required by EU. The signal to noise ratio for all sulfonamides compound at 5 ng/ml is ≥ 60.

The calibration curves and chromatographs are shown in Figure 4 and Figure 5.

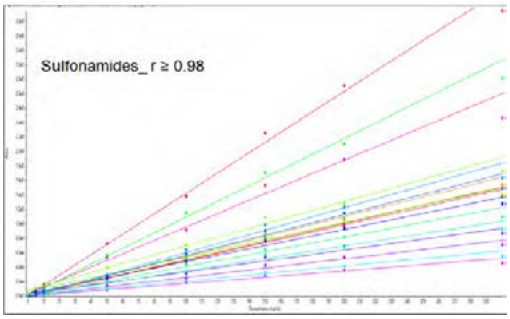


Figure 5. Linear range of the detection of sulfonamides from 5 to 300 ng/mL (r ≥ 0.98)

The Results of accuracy data obtained for Sulfonamides in the milk matrix is given in Table 3.

Recovery was assessed by performing tests where fortified milk

All Sample Types

Sample Name	Sample Type	Component Name	Mass Info	Actual Concentration	Area	Retention Time	Used	Calculated Concentration	Accuracy
EXT_SULFO_BLK	Blank	SULFADOXINE_1	311.0 / 155.9	N/A	754.34	6.51	☑	0.08	N/A
EXT_SULFO_5PPB	Standard	SULFADOXINE_1	311.0 / 155.9	5.00	6148.68	6.50	☑	5.13	102.59
EXT_SULFO_10PPB	Standard	SULFADOXINE_1	311.0 / 155.9	10.00	10710.14	6.50	☑	9.40	93.96
EXT_SULFO_50PPB	Standard	SULFADOXINE_1	311.0 / 155.9	50.00	56054.20	6.50	☑	51.81	103.61
EXT_SULFO_100PPB	Standard	SULFADOXINE_1	311.0 / 155.9	100.00	105314.54	6.51	☑	97.88	97.88
EXT_SULFO_150PPB	Standard	SULFADOXINE_1	311.0 / 155.9	150.00	171744.02	6.50	☑	160.01	106.68
EXT_SULFO_200PPB	Standard	SULFADOXINE_1	311.0 / 155.9	200.00	222819.90	6.50	☑	207.79	103.89
EXT_SULFO_300PPB	Standard	SULFADOXINE_1	311.0 / 155.9	300.00	293796.37	6.51	☑	274.17	91.39

Table 3. Accuracy data obtained for sulfonamides (Sulfadoxine)

Contents

samples at the MRL level were analyzed (n=6) respectively. The recovery of all sulfonamides was ≥ 93%. The recovery data for sulfonamides are shown in Table 4.

Compound	% Recovery 10 ng/ml
Sulfamerazine	93.93
Sulfadiazine	101.34
Sulfamethazine	95.13
Sulfadimethoxine	99.43
Sulfamethoxypyridazine	100.95
Sulfamethoxazole	94.78
Sulfadoxine	100.17
Sulfathiazole	102.26
Sulfapyridine	98.84

Table 4. Recovery of sulfonamides in the milk matrix at (10ng/ml).

The retention times of the analytes were ranging from 5.50 min to 7.00 min. A representative chromatogram obtained from a standard mixture of the sulfonamides with minimum background noise in 11.0 minutes chromatographic run.

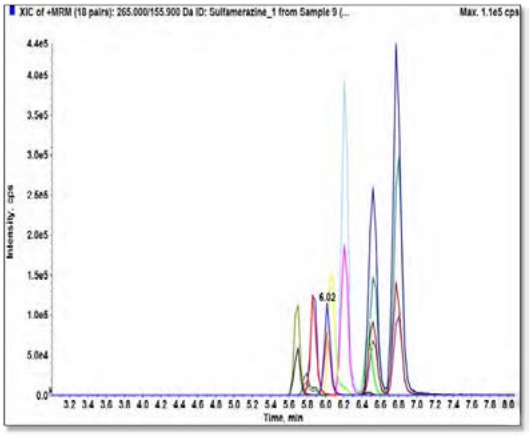


Figure 6. Representative chromatogram of Sulfonamides at 100ng/ml

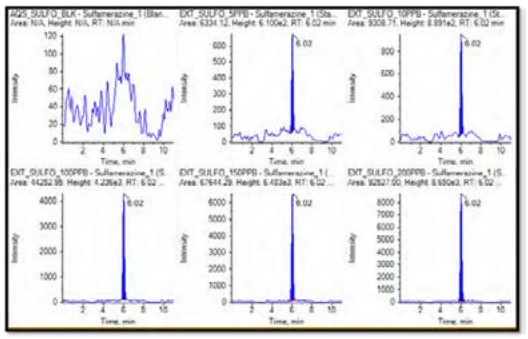


Figure 6. Representative chromatogram of Sulfonamide (Sulfamerazine; 5ng/ml to 200ng/ml)

Repeatability experiment was evaluated by 06 repeated injections at the lowest calibration point (5 ng/ml). Repeatable injections (n= 06) at 5ng/ml level gives the % relative standard deviation of ≤ 5.0%.

Conclusions

- The developed method on SCIEX Triple Quad™ 3500 was simple, sensitive and reproducible.
- This method found to be simple, linear, reproducible and rugged.
- Trueness (Average recovery %) for this method found to be ≥ 93%.

Summary

A SCIEX Triple Quad™ 3500 reduces analysis time and improves sensitivity and resolution, detecting and quantifying several classes of sulfonamides drugs. Nine sulfonamide analytes were determined with a single extraction and the proposed method could be applied in routine analysis. The method and data presented here showcase the fast and accurate solution for the quantitation and identification of Sulfonamides in milk samples by LC-MS/MS.

References

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- Elizabeta Dimitrieska Stojkovic, Hajrulai Musliu Zehra, Biljana Stojanovska Dimzoska and Risto Uzunov; Screening of veterinary drug residues in milk from individual farms in Macedonia. Macedonian Veterinary Review 34(1):5-13, May 2011
- N.Furusava and K.Kishida; High performance liquid chromatographic procedure for routine residue monitoring of seven sulfonamides in milk. Fresenius J Anal Chem (2001) 371: 1031
- EC (2010): Council Regulation 37/2010/EU of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of European Union, L15, 1-72.

Document number: RUO-MKT-02-4798-A

Analysis of Streptomycin and Its Metabolite in Milk Using the SCIEX Triple Quad™ 3500 System

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Overview

A Liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantification of streptomycin and dihydrostreptomycin residues in milk was developed. A simple sample preparation was followed by the LC-MS/MS analysis. The method presented adequate linearity with correlation coefficients above r≥0.99 for both analytes in the dynamic range of 10–1000 ng/ml, with average accuracies for matrix based recovery calibration was between 85–105%. Method selectivity was verified by the absence of interfering peaks in the retention time of the analytes, the results qualified the method for the quantification and confirmation of the analytes in milk at concentrations inferior to the established Maximum Residue limit (200ng/ml).

Introduction

Antimicrobial agents are widely used in dairy cattle management. Improper administration for disease therapy and as growth promoting agents can result in antibiotic residues in milk and dairy products and can contribute to the development of microbial drug resistance and the spread of resistant bacteria, including those with serious health consequences in animals. Aminoglycosides like Streptomycin and dihydrostreptomycin are protein synthesis inhibitors. These are widely used in veterinary medicine for the treatment of gram-negative bacterial infection in clinical and sub-clinical mastitis in cattle. These are administered with combination of penicillin and tetracycline. The Maximum Residual limit (MRL) for streptomycin and dihydrostreptomycin in milk was 200ng/ml (1). Due to the harmful effects of veterinary medicinal residues, surveillance systems are enforced in the European Union pursuant to the requirement (EU).

The accurate detection of low levels of aminoglycosides residues in milk is of great importance for the dairy industry and also for farmers; the development of highly selective method for the detection of Streptomycin and dihydrostreptomycin using the SCIEX Triple Quad™ 3500 was performed

The SCIEX Triple Quad™ 3500 System takes the best features 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.

Figure 1. SCIEX Triple Quad™ 3500

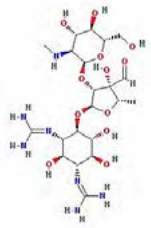

Figure 2: Structure of Streptomycin

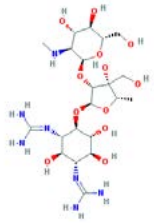

Figure 3: Structure of Dihydrostreptomycin

Contents

Unique Features

1. A sensitive, specific, rugged and reproducible LC-MS/MS method was developed for Streptomycin using simple extraction technique for the sample preparation.

2. Streptomycin analysis at 10.0 ng/ml level gave an S/N ratio > 142:1 with good accuracy and precision (n=6) in milk.

3. Dihydrostreptomycin analysis at 10.0 ng/ml level showed S/N ratio > 191.8:1 with good accuracy and precision (n=6) in milk.

4. Accuracy and Precision for Streptomycin and Dihydrostreptomycin in milk samples (matrix based) found to be between 80-120%.

5. Reproducibility of matrix based results for Streptomycin and Dihydrostreptomycin in terms of % CV in milk samples is less than 5%.

6. Average recovery of Streptomycin and Dihydrostreptomycin in milk using the developed extraction method is more than 88%.

Materials and Methods

Chemicals

Standard Streptomycin and Dihydrostreptomycin was purchased from Sigma Aldrich ≥99% Purity. All other chemicals used were of LC-MS grade, commercially available.

Milk samples

Milk samples were procured from the local market of Delhi & Gurgaon in India and were kept at 2 - 8 °C until end of analysis.

Sample Preparation

Milk sample (1ml) was taken and 10% Trichloroacetic acid (0.4ml) added and vortexed for 5 minutes followed by centrifugation at 4000 rpm for 5 minutes. Supernatant collected and filtered through 0.2µ syringe filter and injected for LC-MS/MS analysis.

LC Conditions

LC separation was achieved using the ExionLC™ AC with a Phenomenex, Synergi Hydro RP 150 x 4.6 mm, 4u column with a gradient of pump (A): Water: hepta fluoro butyric acid and pump (B): Acetonitrile at flow rate of 0.8 mL/min. The injection volume was set to 20 µl.

Time (min)	Mobile phase A %	Mobile phase B %
0.01	95	5
0.50	95	*5*
1.00	*70*	*30*
2.00	*70*	*30*
3.00	*95*	*5*
3.50	*95*	*5*

Table 1: LC conditions

MS/MS Conditions

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in positive polarity. Two selective MRM transitions were monitored for Streptomycin and Dihydrostreptomycin, The LC-MS/MS data was processed using Analyst 1.6.2 Software and MultiQuant™ software version 3.0.1.

Analyte	Q1	Q3 (Quantifier	Q3 (Qualifier)
Streptomycin	582.3	246.2	*263.4*
Dihydrostreptomycin	584.3	*263.1*	*246.2*

Table 2: MRM transitions

Results and Discussions

The SCIEX Triple Quad™ 3500 System was found to perform at lower levels than the required MRL. For this method matrix based calibration at 10ng/ml for Streptomycin had S/N>142 and for Dihydrostreptomycin showed an S/N>191.8 which is a much lower concentration than the MRL.

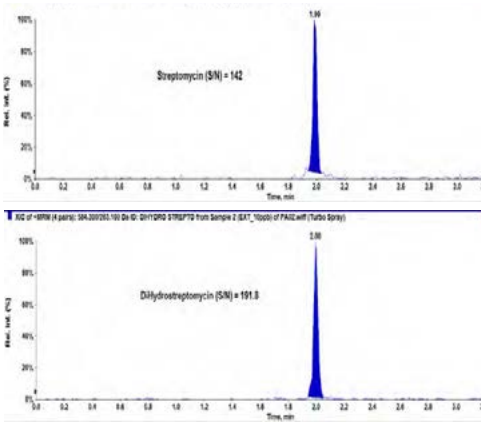

Figure 4: Signal to Noise (S/N) of Streptomycin and Dihydrostreptomycin (10ng/ml)

Matrix based calibration curves were made with standard levels ranging from 10.0ng/ml to 1000 ng/ml spiked concentration. Linear graph was obtained with regression co-efficient (r): 0.9993 and 0.9994 by using weighing factor $1/X^2$ for Streptomycin and dihydrostreptomycin respectively.

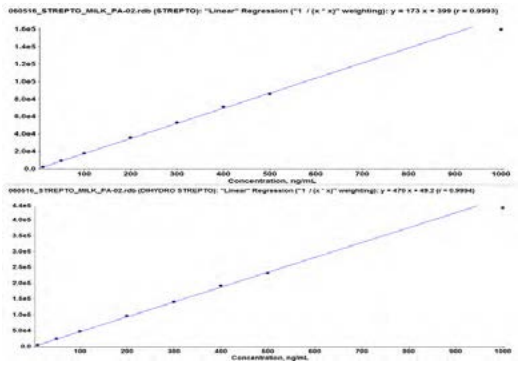

Figure 5: Linear range of the detection of Streptomycin and Dihydrostreptomycin from 10.0 to 1000 ng/mL (r = 0.9993, 0.9994)

Results of accuracy data obtained for Streptomycin and Dihydrostreptomycin in the milk matrix is given in Table 3

	Sample Name	Sample ID	Sample Type	Analyte Peak Area (counts)	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)	[Custom] Qualifier	MRM Ratio
1	EXT_10ppb	Streptomycin	Standard	2110.	10.0	9.92	99.2	1040.	0.493
2	EXT_50ppb	Streptomycin	Standard	9118	50.0	51.7	103	5898	0.647
3	EXT_100ppb	Streptomycin	Standard	17700.	100.	100.	100.	9120.	0.515
4	EXT_200ppb	Streptomycin	Standard	35700.	200.	204.	102.	19500.	0.547
5	EXT_300ppb	Streptomycin	Standard	52300.	300.	304.	101.	28100.	0.537
6	EXT_400ppb	Streptomycin	Standard	71100.	400.	410.	102.	40200.	0.565
7	EXT_500ppb	Streptomycin	Standard	91500.	500.	496.	99.1	45100.	0.595
8	EXT_1000ppb	Streptomycin	Standard	161000.	1000.	923.	92.3	84500.	0.530

Table 3: Accuracy data obtained for Streptomycin in the milk matrix

	Sample Name	Sample ID	Sample Type	Analyte Peak Area (counts)	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)	[Custom] Qualifier	MRM Ratio
1	EXT_10ppb	Dihydro Streptomycin	Standard	4710	10.0	9.92	99.2	1800	0.307
2	EXT_50ppb	Dihydro Streptomycin	Standard	24200	50.0	51.4	103	7900.	0.327
3	EXT_100ppb	Dihydro Streptomycin	Standard	47500	100.	102.	102	15300	0.319
4	EXT_200ppb	Dihydro Streptomycin	Standard	96200.	200.	205.	102.	30600.	0.318
5	EXT_300ppb	Dihydro Streptomycin	Standard	144000	300.	299.	99.7	44200	0.314
6	EXT_400ppb	Dihydro Streptomycin	Standard	192000.	400.	409.	102.	62600.	0.326
7	EXT_500ppb	Dihydro Streptomycin	Standard	231000.	500.	493.	98.5	73600.	0.318
8	EXT_1000ppb	Dihydro Streptomycin	Standard	436000.	1000.	972.	93.2	140100.	0.320

Table 4: Accuracy data obtained for Dihydrostreptomycin in the milk matrix

Recovery was assessed by performing tests where fortified milk Samples at 0.5, 1 and 1.5 times the MRL level were analyzed (Six replicates, respectively). The Recovery of Streptomycin in matrix based was ≥ 90% and Dihydrostreptomycin ≥ 90%

	Streptomycin		
Replicates (n=6)	**50% of MRL**	**MRL**	**150% of MRL**
1	105.00	196.00	*295.00*
2	100.00	*191.00*	*283.00*
3	*107.00*	*197.00*	*284.00*
4	*105.00*	*198.00*	*271.00*
5	*102.00*	*205.00*	*264.00*
6	*104.00*	*200.00*	*273.00*
Average Conc (ng/mL)	*103.88*	*197.83*	*278.33*
Target Conc (ng/mL)	*100.00*	*200.00*	*300.00*
% Recovery	*103.88*	*98.92*	*92.78*

Table 5: Recovery of Streptomycin in the milk matrix

Contents

	Dihydrostreptomycin		
Replicates (n=6)	50% of MRL	MRL	150% of MRL
1	*102.000*	*198.000*	*292.000*
2	*102.000*	*188.000*	*274.000*
3	*102.000*	*192.000*	*274.000*
4	*107.000*	*199.000*	*262.000*
5	*105.000*	*197.000*	*265.000*
6	*103.000*	*206.000*	*266.000*
Average Conc (ng/mL)	*103.500*	*196.667*	*272.167*
Target Conc (ng/mL)	*100.000*	*200.000*	*300.000*
% Recovery	*103.50*	*98.33*	*90.72*

Table 6: Recovery of Dihydrostreptomycin in the milk matrix

Streptomycin and Dihydrostreptomycin eluted at RT of 1.99 and 2.00 minutes with minimum background noise in 3.50 minutes chromatographic run.

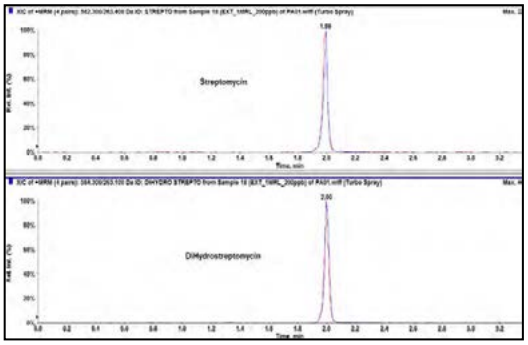

Figure 6: Representative chromatogram of Streptomycin and Dihydrostreptomycin at MRL (200ng/ml)

Conclusions

- The developed method on SCIEX Triple Quad™ 3500 was simple, sensitive and reproducible which can meet the regulatory requirements.
- Trueness (Average recovery %) for this method found to be ≥ 90%.

Summary

The method and data presented here showcase the fast and accurate solution for the quantitation and identification of Streptomycin and Dihydrostreptomycin in milk samples by LC-MS/MS. The SCIEX Triple Quad™ 3500 System provide excellent sensitivity and selectivity, with minimal sample preparation allowing maximized throughput for the analysis of many samples in a short time period.

References

1. EC (1997): Commission Decision 97/747/EC of 27 October 1997 on fixing the levels and frequencies of sampling provided for by Council Directive 96/23/EC for the monitoring of certain substances and residues thereof in certain animal products. Official Journal of European Union L303, 12-15.

2. Juan, C., Moltó, J.C., Mañes, J., Font, G. (2010): Determination of macrolide and lincosamide antibiotics by pressurized liquid extraction and liquid chromatography tandem mass spectrometry in meat and milk. Food Control 21 1703-1709

3. EC (2010): Council Regulation 37/2010/EU of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of European Union, L15, 1-72

4. EMEA (2002b): Streptomycin, summary report (3) committee for veterinary medicinal products, EMEA. EMEA/MRL809/01 final, February 2002.

5. Renata Cabrera de Oliveira1,*, Jonas Augusto Rizzato Paschoal1, Marcela Sismotto1, Flávia Pereira da Silva Airoldi2, and Felix Guillermo Reyes Reyes1 Determination of Streptomycin and Dihydrostreptomycin Residues in Milk

Document number: RUO-MKT-02-4750-A

Quantitation of Chloramphenicol in Milk using SCIEX Triple Quad™ 3500 LC-MS/MS System

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Overview

Chloramphenicol (CAP) is a Broad-Spectrum antibiotic used for the treatment of a number of bacterial infections. The use of CAP for the treatment of food producing animals is prohibited in several countries (e.g. European Union, Canada, United States, and most Asian counties). In India, the judgement referred to "Executive summary on National survey on milk adulteration" released by FSSAI said that at national level, 68.4% of milk being sold is adulterated. The Commission Decision 2002/657/EC Annexure II requires control of CAP residues in edible tissues, meat, seafood, eggs, honey, milk, and milk products. The Minimum Required Performance Limit (MRPL) for CAP in milk was 0.3µg/kg. An LC MS/MS method for the quantitation of Chloramphenicol in milk which meets the regulatory requirements is described in this article.

Introduction

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 MRPL have been established around the world, and agencies monitor the food supply to ensure that antibiotic residue concentrations do not exceed these levels. The accurate detection of low levels of antibiotic residues in milk is of great importance for the dairy industry. The development of sensitive and selective method for the quantitation of Chloramphenicol in milk which meets the regulatory requirements was done using SCIEX Triple Quad™ 3500. The SCIEX Triple Quad™ 3500 System has the legendary Turbo V™ ion source, efficiently ionizes compounds and virtually eliminates cross-contamination for reliable quantitation over a wide range of flow rates. The proprietary Curtain Gas™ interface reduces the need for routine maintenance and ensures maximum productivity by protecting your mass spec from contamination. In addition the Curved LINAC® collision cell design improves data quality and helps in achieving optimal sensitivity for all compounds. The method development was performed as per the regulatory guidelines described in EU/SANCO/12495 directive recommendations

Figure 1: SCIEX Triple Quad™ 3500

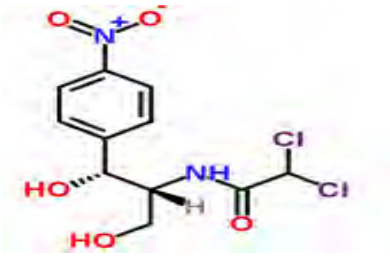

Figure 2: Structure of Chlormaphenicol (C11H12Cl2N2O5; MW:322.012)

Materials and Methods

Standard Chloramphenicol was purchased from Clearsynth. All other chemicals used were of LC-MS grade, commercially available. Milk samples were purchased from the local market of Delhi, and Gurgaon and stored in refrigerator at 2 to 8ºC till the analysis was completed.

Contents

Sample Preparation

A generalized extraction procedure was performed in which, 1ml of milk was vortexed with acetonitrile, water mixture (4:1) v/v), followed by the addition of NaCl and mixed well. This solution was centrifuged, supernatant was evaporated to dryness, reconstituted, filtered and 20µl was used for the LC MS/MS analysis.

LC Conditions

LC separation was performed on a ExionLC™ AC instrument using Synergy Fusion RP 18e (50 X 2.6) mm 2.5µ and a fast gradient of water (Mobile Phase A) and acetonitrile (Mobile Phase B) from 85% aqueous to 85% organic in 5 minutes at a flow rate of 0.4ml/min and injection volume of 20µl is used to obtain a good peak shape.

Time (min(	Mobile phase A%	Mobile phase B%
0.01	85	15
0.30	85	15
0.50	75	25
1.00	70	30
1.50	15	85
3.00	15	85
4.00	85	15
5.00	85	15

Table 1: Mobile Phase Gradient

MS/MS Conditions

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The TurboV™ source was used with an Electrospray Ionization (ESI) probe in negative ionization mode at 2800 ion spray voltage, with Declustering potential (DP) -85V and Collision Energy(CE) -15V and -23V for the MRM transitions 320.8/151.8 and 320.8/256.8 respectively. Analyst 1.6.2™ Software was used for method development and data acquisition. LC-MS/MS data was processed using the MultiQuant™ Software version 3.0.2.

Results and Discussions

Sensitivity, Reproducibility, Linearity and Accuracy

The SCIEX Triple Quad™ 3500 System showed very good sensitivity for chloramphenicol analysis in milk. The experimental data was acquired in accordance with EU SANCO/12495 directive recommendations. The matrix based method for the chloramphenicol analysis was set for a Minimum Required Performance Limit (MRPL) of 0.3µg/kg level. Chloramphenicol eluted at Retention time 2.90 min with minimal background noise in 5 minutes gradient run. The signal-to-noise ratio obtained was 88.8 for extracted 0.1µg/kg spiked sample. Repeatability at MRPL level (0.3ug/kg) was evaluated (n=6) and %CV was found < 5.0.

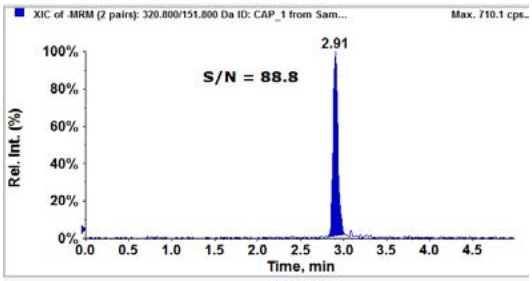

Figure 3: Chromatogram showing S/N ratio at 0.1µg/kg concentration

Extracted matrix based linearity curve plotted with a linear dynamic range of 3 orders was made from a set of standard dilutions in the range from 0.1µg/kg to 10.0µg/kg correlation regression co-efficient r is found > 0.99 for both quantifier and qualifier ions by using weighing factor of 1/ X2. Recovery of the extracted method is evaluated by spiking the milk samples at different concentrations.

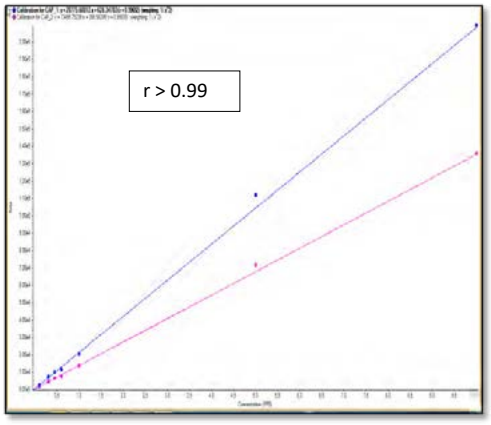

Figure 4: Matrix Based Calibration Curve

Sample Name	Sample Type	Component Name	Mass Info	Used	Accuracy	MRM Ratio
CAP_EXT_BLK	Blank	CAP_1	320.8 / 151.8	☑	N/A	0.589
CAP_EXT_0.1PPB	Standard	CAP_1	320.8 / 151.8	☑	99.70	0.650
CAP_EXT_0.3PPB	Standard	CAP_1	320.8 / 151.8	☑	106.42	0.631
CAP_EXT_0.45PPB	Standard	CAP_1	320.8 / 151.8	☑	101.75	0.640
CAP_EXT_0.6PPB	Standard	CAP_1	320.8 / 151.8	☑	87.81	0.659
CAP_EXT_1PPB	Standard	CAP_1	320.8 / 151.8	☑	96.55	0.672
CAP_EXT_5PPB	Standard	CAP_1	320.8 / 151.8	☑	107.15	0.642
CAP_EXT_10PPB	Standard	CAP_1	320.8 / 151.8	☑	100.62	0.649
CAP_EXT_BLK	Blank	CAP_1	320.8 / 151.8	☑	N/A	0.753
CAP_EXT_0.3PPB	Quality Control	CAP_1	320.8 / 151.8	☑	104.33	0.636
CAP_EXT_0.3PPB	Quality Control	CAP_1	320.8 / 151.8	☑	107.79	0.660
CAP_EXT_0.3PPB	Quality Control	CAP_1	320.8 / 151.8	☑	99.95	0.659
CAP_EXT_0.3PPB	Quality Control	CAP_1	320.8 / 151.8	☑	106.97	0.651
CAP_EXT_0.3PPB	Quality Control	CAP_1	320.8 / 151.8	☑	109.92	0.620
CAP_EXT_0.3PPB	Quality Control	CAP_1	320.8 / 151.8	☑	107.86	0.624
CAP_EXT_0.6PPB	Quality Control	CAP_1	320.8 / 151.8	☑	85.95	0.674
CAP_EXT_0.6PPB	Quality Control	CAP_1	320.8 / 151.8	☑	95.96	0.649
CAP_EXT_0.6PPB	Quality Control	CAP_1	320.8 / 151.8	☑	101.11	0.694
CAP_EXT_0.6PPB	Quality Control	CAP_1	320.8 / 151.8	☑	105.01	0.643
CAP_EXT_0.6PPB	Quality Control	CAP_1	320.8 / 151.8	☑	107.29	0.655
CAP_EXT_0.6PPB	Quality Control	CAP_1	320.8 / 151.8	☑	108.06	0.626
CAP_EXT_5PPB	Quality Control	CAP_1	320.8 / 151.8	☑	102.15	0.649
CAP_EXT_5PPB	Quality Control	CAP_1	320.8 / 151.8	☑	111.06	0.656
CAP_EXT_5PPB	Quality Control	CAP_1	320.8 / 151.8	☑	107.67	0.652
CAP_EXT_5PPB	Quality Control	CAP_1	320.8 / 151.8	☑	106.52	0.656
CAP_EXT_5PPB	Quality Control	CAP_1	320.8 / 151.8	☑	110.29	0.642
CAP_EXT_5PPB	Quality Control	CAP_1	320.8 / 151.8	☑	113.05	0.639

Table 2: Accuracy and MRM Ratio using Multiquant™

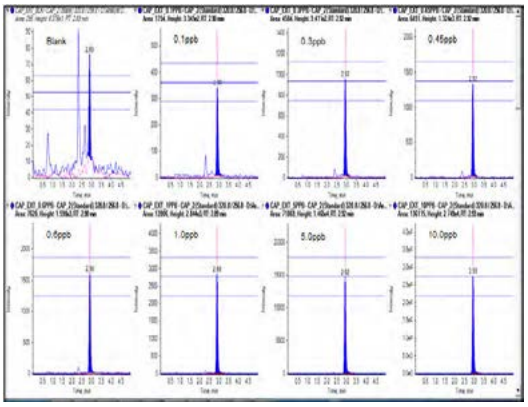

Figure 5: Ion Ratio Tolerance Chromatograms

Findings in Milk Samples

The samples (1) and (4) obtained from the market were found to contain chloramphenicol which is evident from the retention time as well as ion ratio, however samples (2) and (3) are possible false positives. The analysis was done in duplicate for each sample. Figure 6 shows chromatogram of different milk samples analyzed for the quantitation of chloramphenicol.

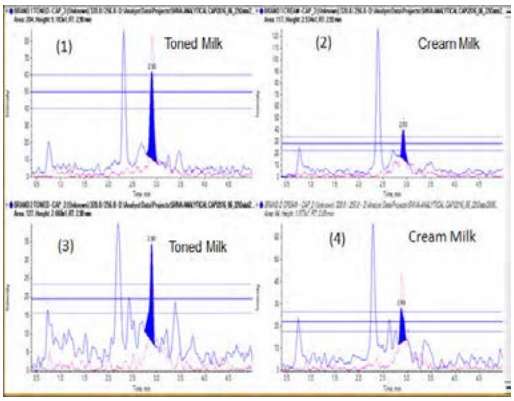

Figure 6: XIC's of different commercial Milk samples

Replicate injections	Chloramphenicol(320.8/151.8) 0.3µg/kg
1	0.313
2	0.323
3	0.300
4	0.321
5	0.330
6	0.324
Average conc	0.318
Original conc	0.300
%Recovery	106.14
%CV	3.32

Table 3: Recovery data for Matrix based samples

Conclusions

The method developed as per EU /SANCO/12495 directive recommendations showed acceptable accuracies (85%-120%) for matrix based recovery samples, linearity with r > 0.99 for both the transitions, repeatability was< 5. No significant matrix interferences observed. The method allows high throughput, rapid and sensitive LC-MS/MS identification and quantitation of banned antibiotic Chloramphenicol meeting EU MRPL of 0.3µg/kg level.

Contents

Summary

1 The method and data presented here shows fast and accurate solution for the quantitation and confirmation of Chloramphenicol in milk samples by LC-MS/MS.

2. The SCIEX Triple Quad™ 3500 System provides excellent sensitivity and selectivity for this analysis, with minimal sample preparation allowing maximized throughput for the analysis of a bigger batch of samples in a short time period.

3. Automatic MRM ratio calculation in MultiQuant™ Software can be used for confirmation of compound.

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3. 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 SCIEX (2013) #8160213-01

Document number: RUO-MKT-02-4751-A

Rapid, Sensitive, Quantitation method for Chloramphenicol in Meat using SCIEX Triple Quad™ 3500 LC-MS/MS System

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Overview

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been widely used for the identification and quantification of trace amount of antibiotic residues like chloramphenicol in complex food matrices due to the specificity and sensitivity associated with this technique. 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 like achieving the low level sensitivity along with reproducibility and method ruggedness. The Minimum Required Performance Limit (MRPL) set by the European Union for Chloramphenicol in meat and meat products was 0.3µg/kg. The method of analysis for chloramphenicol in meat described here was developed and performed according to the criteria established by the EC Commission Decision 657/2002.

Introduction

Chloramphenicol (CAP) is a highly effective bacteriostatic compound. Due to the findings of chloramphenicol residues in animal derived foods intensive surveillance had been conducted by regulatory authorities like EU or USFDA. Most of the countries like EU, USA, Canada and Asian countries etc. have banned the usage of CAP in food products and implemented stringent regulations to keep a check on the entry of such products contaminated with Chloramphenicol into supermarkets. The purpose of this study was to develop a quantitative method for the determination of chloramphenicol in meat as per the regulatory requirements using the SCIEX Triple Quad™ 3500 System.

The SCIEX Triple Quad™ 3500 System takes the best features and enhances the response with modern engineering and electronics. The proven legendary design of Turbo V™ source and Curtain Gas™ interface provides 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-residue analysis. Compound identification was based on the criteria of directive 2002/657/EC9 (retention time tolerance of ± 2.5% and maximum tolerances for ion ratios of ± 20 to 50% depending on the ratio).

Figure 1: SCIEX Triple Quad™ 3500

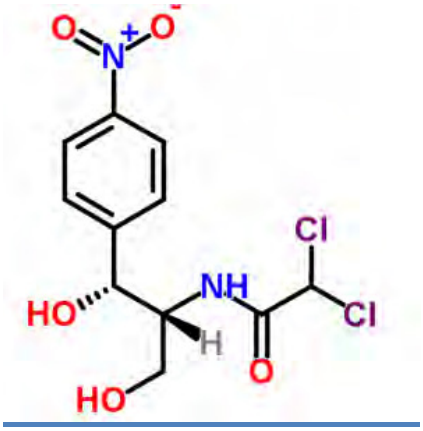

Figure 2: Structure of Chloramphenicol (C11H12Cl2N2O5 MW: 322.012)

Contents

Materials and Methods

Chemicals

Standard Chloramphenicol Purity(≥99%) was purchased from Clearsynth, India. All other chemicals used were of LC-MS grade.

Sample Preparation

Accurately weigh 2.0g of homogenized chicken meat sample, 6ml of Ethyl acetate was added vortexed, centrifuged and the supernatant was evaporated to dryness. Add 1ml of Hexane and Carbon Tetrachloride (1:1 v/v), mix well and 1ml of Mobile phase, filtered using 0.22 µM PTFE filter and 20µl was used for LC-MS/MS.

LC Conditions

LC separation was performed on an ExionLC™ AC instrument using Synergy Fusion RP 18e (50 X 2.6) mm 2.5µ and a fast gradient of water(Mobile Phase A) and Acetonitrile(Mobile Phase B) from 85% aqueous to 85% organic in 5 minutes at a flow rate of 0.4ml/min.

Time (min)	Mobile phase A %	Mobile phase B %
0.01	85	15
0.30	85	*15*
0.50	*72*	*25*
1.00	*70*	*30*
1.50	*15*	*85*
3.00	*15*	*85*
4.00	*85*	*15*
5.00	*85*	*15*

Table 1: Mobile phase gradient

MS/MS Conditions

The SCIEX Triple Quad™ 3500 was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in negative ionization mode at 2800 ion spray voltage, with Declustering potential(DP) -85V and Collision Energy(CE) -15V and -23V for 320.8/151.8 and 320.8/256.8 respectively. Two selective MRM transitions were monitored and ion ratio is calculated automatically by software for compound identification. Analyst 1.6.2™ Software was used for method development and data acquisition. LC-MS/MS data was processed using the MultiQuant™ Software version 3.0.1

Results and Discussions

Sensitivity, Reproducibility, Linearity and Accuracy

The sensitivity for the chloramphenicol analysis in meat was achieved below the required limit set by the regulations using the SCIEX Triple Quad™ 3500. The sensitivity of the method using matrix matched recovery study showed S/N ratio of 110.2 at 0.01µg/kg chloramphenicol level.

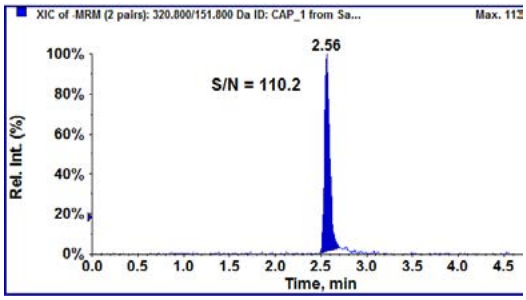

Figure 3: Chromatogram showing S/N at 0.01µg/kg level

Matrix matched Calibration curve plotted was found linear in the range of 0.01µg/kg (ppb) to 1.0µg/kg (ppb) with 2 orders of linear dynamic range and correlation regression co-efficient r > 0.99 for both quantifier and qualifier ions by applying weighing factor of $1/X^2$.

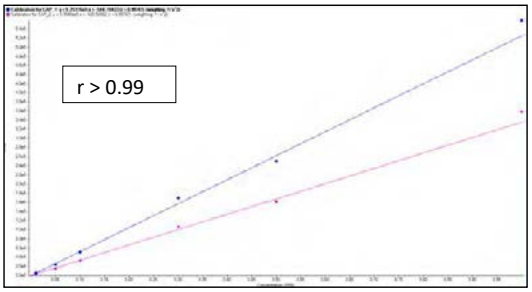

Figure 4: Matrix Matched Calibration curve ranging 0.01µg/kg to 1.0 µg/kg with r > 0.99 for both transitions

Repeatability at 0.01µg/kg level was evaluated for 6 replicate injections and percentage relative standard deviation (%CV) was observed to be < 5. Accuracies observed were in the range from 85% to 120%. Chloramphenicol eluted at RT of 2.56 min in 5

minutes run. Quantifier and qualifier ion ratio shown by MultiQuant™ Software in figure attached below.

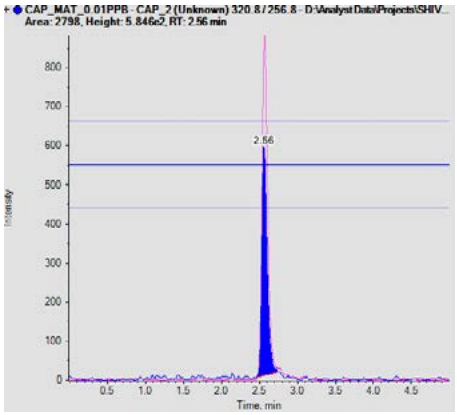

Figure 5: Representative Chromatogram at 0.01µg/kg level showing Ion Ratio Tolerance set at ± 20%.

Replicate Injections	Chloramphenicol(320.8/151.8)		
	0.01µg/kg	0.1µg/kg	0.3µg/kg
1	0.011	0.094	0.311
2	0.010	0.091	0.314
3	0.010	0.092	0.304
4	0.011	0.094	0.308
5	0.011	0.094	0.309
6	0.011	0.092	0.317
Average conc. (µg/kg)	0.011	0.093	0.310
Original conc. (µg/kg)	0.010	0.100	0.300
%Recovery	109.57	93.02	103.50
%CV	3.44	1.56	1.44

Table 2: Recovery data for matrix matched samples spiked at MRPL and Lower than MRPL (0.01µg/kg & 0.1µg/kg)

Conclusions

The quantitation method described here meets the regulatory requirements and the lowest point in the calibration curve was below the limits set by the EU to 0.01µg/kg with adequate linearity and reproducibility. The method showed acceptable accuracies, linearity with r >0.99 for both quantifier and qualifier, repeatability (%CV) observed was < 5. This method utilizes a generic sample preparation protocol which meets the validation parameters as per the recovery % requirements set by the global regulations (85%-120%). The method allows high throughput, selective, rapid and sensitive LC-MS/MS identification and quantitation of banned antibiotic Chloramphenicol in meat samples.

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1. FAO-Dairy Production and products: milk production facts, Food and Agriculture Organization of United Nations, Rome.- 2013 http://fao.org/agriculture/dairy-gateway/meat-production/en.

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4. Determination of the antibiotic chloramphenicol in meat and seafood products by liquid chromatography – electrospray ionization tandem mass spectrometry. Pascal Mottier, Veronique Parisod, Eric Gre,aud, Philippe A Guy, and Richard H. Stadler, Journal of Chromatography A, 994 (2003) 75-74.

Document number: RUO-MKT-02-4753-A

Simultaneous Analysis of Chloramphenicol and Tetracycline Antibiotics in Food Samples Using the SCIEX Triple Quad™ 3500 System

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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.

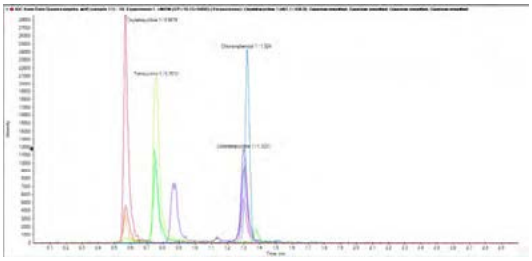

Figure 1. LC separation and detection in MRM mode of three tetracyclines and Chloramphenicol at 10 ng/mL

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.

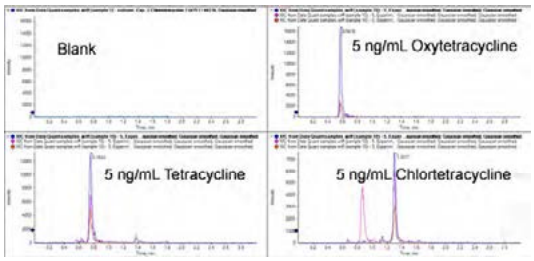

Figure 2. Sensitivity of a 5 ng/mL standard of tetracyclines (injection volume of 10 µL)

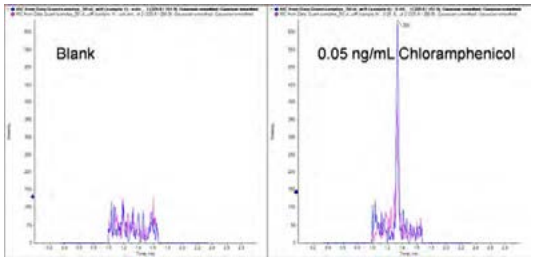

Figure 3. LOQ for Chloramphenicol of less than 0.05 ng/mL with an injection volume of 50 µL, allowing 10x dilution of matrix extracts

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.

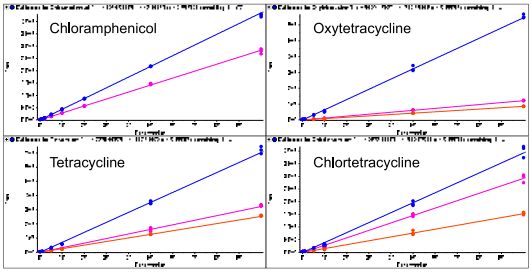

Figure 4. Calibration lines for all 4 compounds analyzed in this study

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).

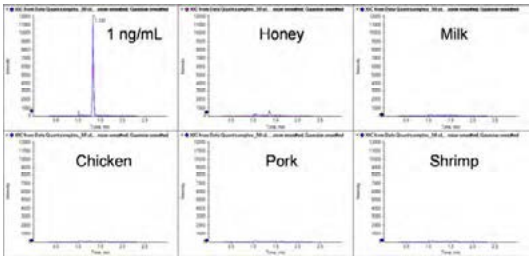

Figure 5. Blank matrices tested for Chloramphenicol (50 µL injection), the honey sample had a trace contamination with Chloramphenicol below the LOQ of 0.05 ng/mL (0.25 µg/kg in matrix after 5x dilution)

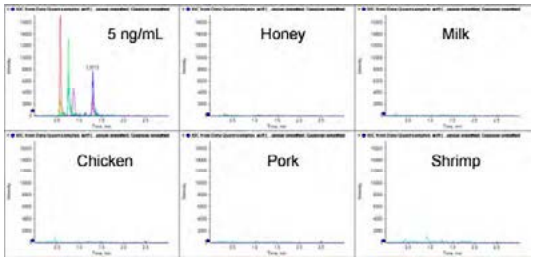

Figure 6. Blank matrices tested for tetracyclines (10 µL injection)

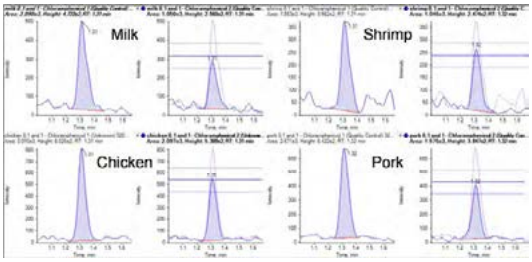

Figure 7. Different food extracts spiked with Chloramphenicol at 0.1 µg/kg (50 µL injection), the MRM ratio tolerances are displayed in the peak review window

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]

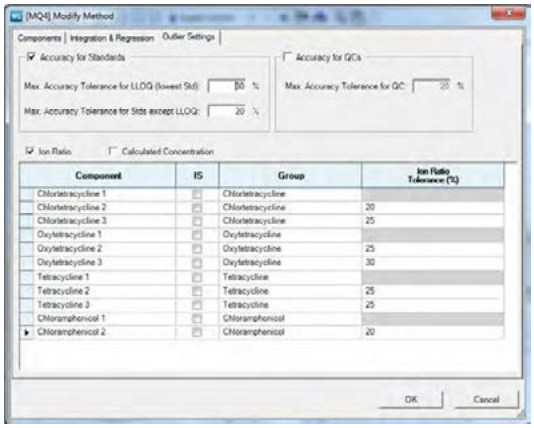

Figure 6. MRM ratio tolerances setup in the method editor of MultiQuant™ Software

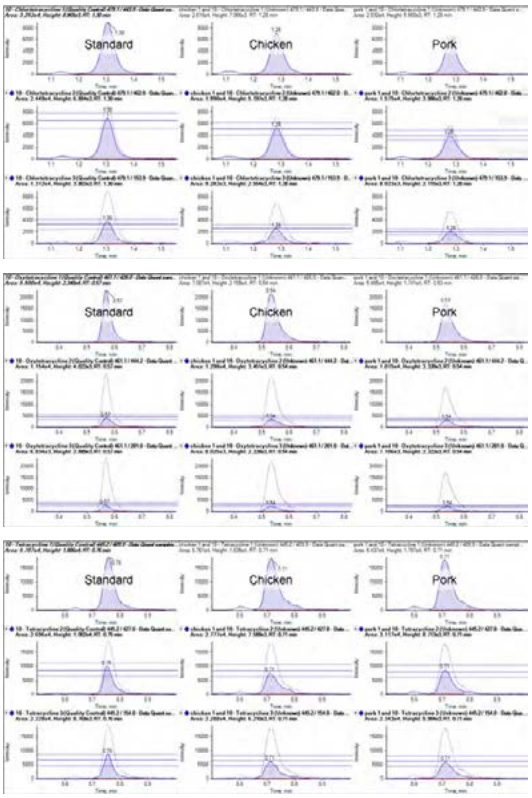

Figure 8. Side-by-side peak review of a standard injection (left) and spiked meat extracts (middle and right) with automatic calculation of MRM ratios, the MRM ratio tolerances are displayed in the peak review window

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 SCIEX (2013) #8160213-01

Publication number: 10641514-01

Contents

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 a 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				

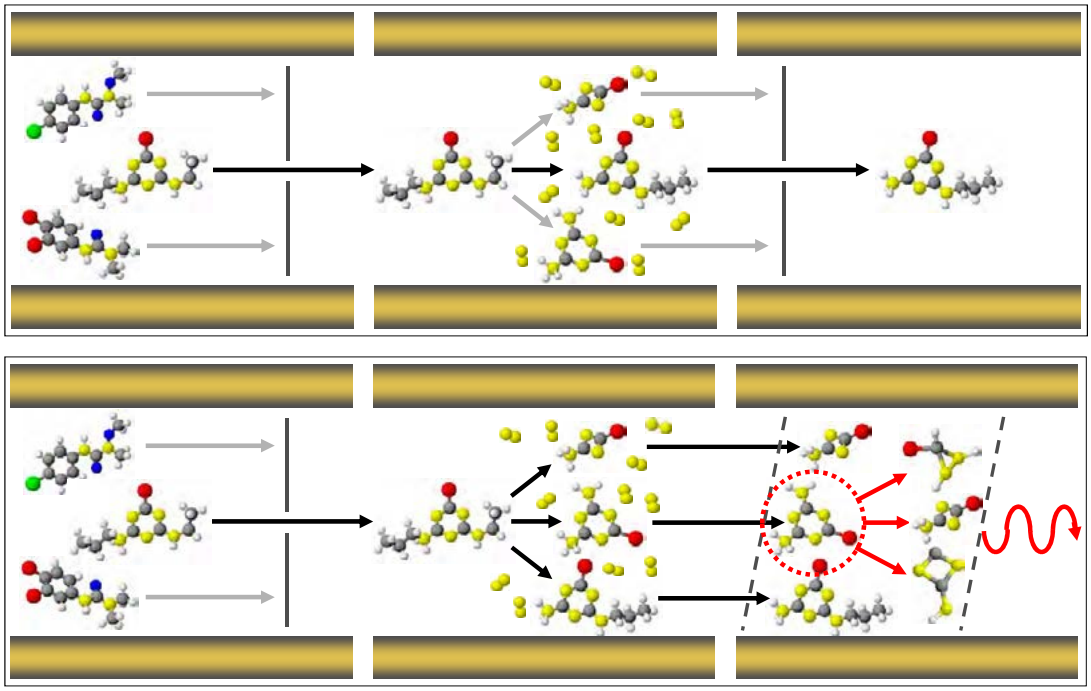

Figure 1. Operation of a QTRAP® system in MRM (top) and MRM^3 (bottom) modes

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.

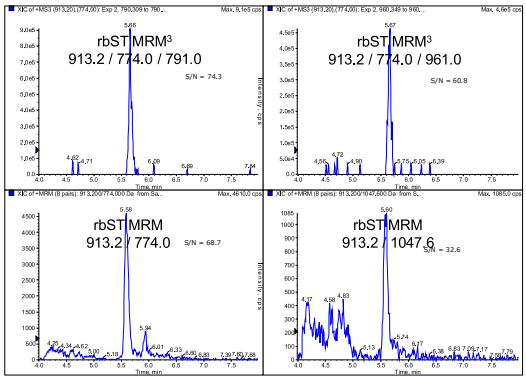

Figure 2. XIC of milk samples spiked with 10 ppb of rbST and purified using solid phase extraction: MRM^3 data (top) and MRM data (bottom)

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.

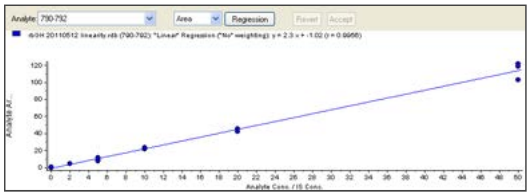

Figure 3. Calibration line for extracts of milk samples spiked with rbST at different levels. The calibration line is from the MRM^3 experiment 913.2/774.0/791.0

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.

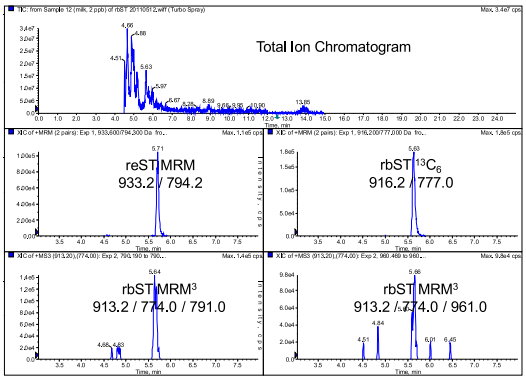

Figure 4. Typical chromatogram for a 2 ppb spiked milk calibration standard

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 SCIEX 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

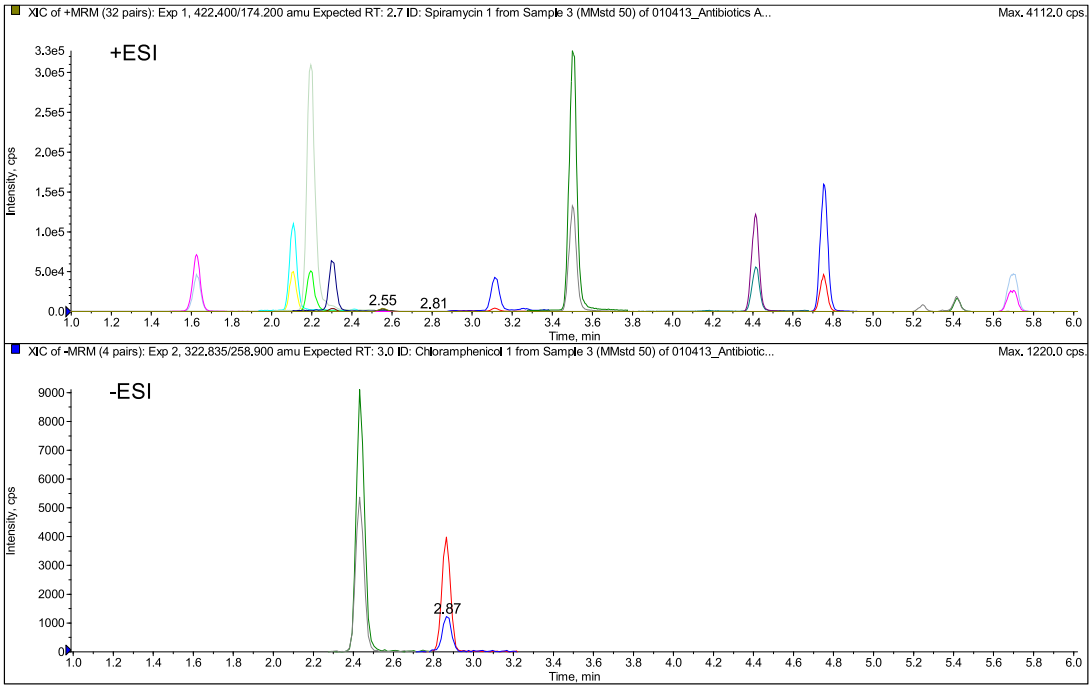

Figure 1. Detection of antibiotics and insecticides in a single run by LC-MS/MS using *Scheduled* MRM™ with polarity switching. Positive mode (+ESI) MRM transitions shown in top pane, and XIC of all negative mode (-ESI) MRM transitions shown at bottom. Peaks are identified by retention time in Table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

Contents

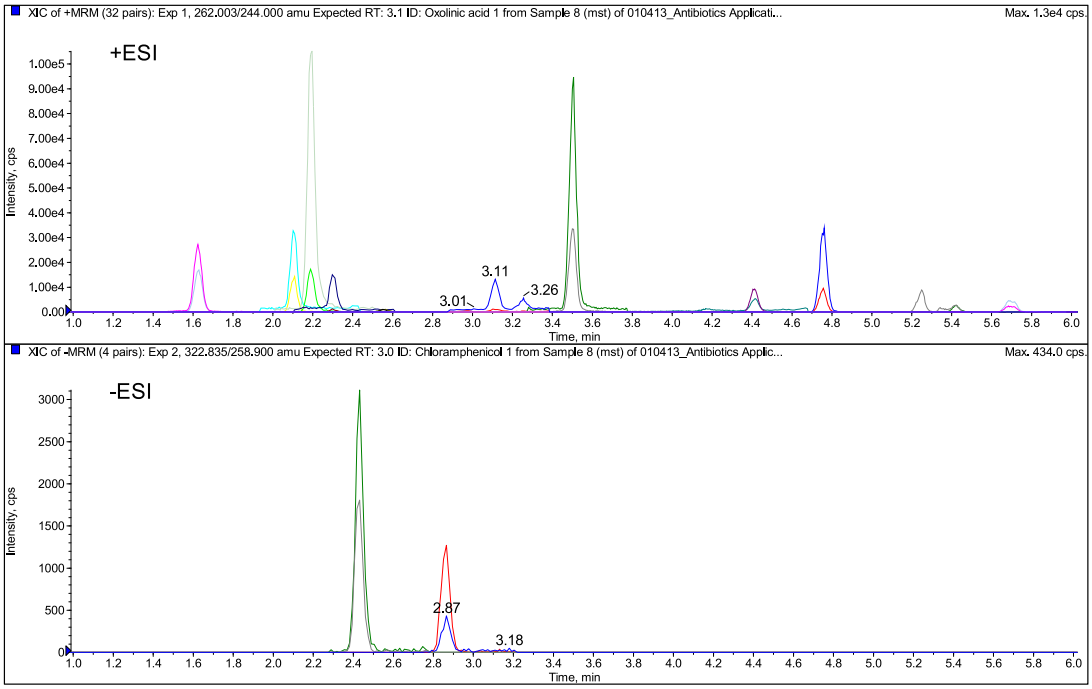

Figure 2. Poultry feed sample fortified at 40 µg/kg in sample (20 µg/mL in extract).

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 a SCIEX 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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Publication number: 7180213-01

The Use of Micro Flow LC Coupled to MS/MS in Veterinary Drug Residue Analysis

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SCIEX Warrington (UK)

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 a SCIEX 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 a SCIEX 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).

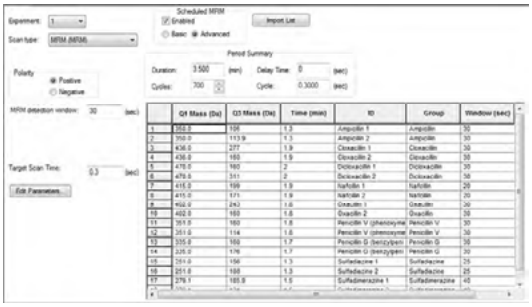

Figure 1. Method editor in Analyst® Software version 1.6.2 used to setup the *Scheduled* MRM™ Pro experiment

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).

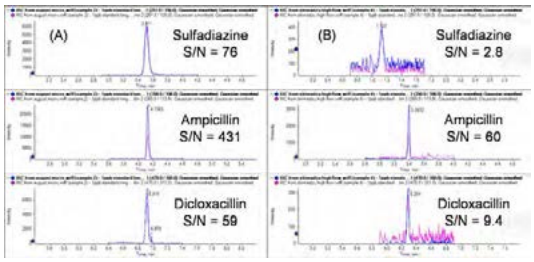

Figure 2. Comparison of microLC (A) with traditional high flow LC (B) using a 1 ng/mL standard.

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).

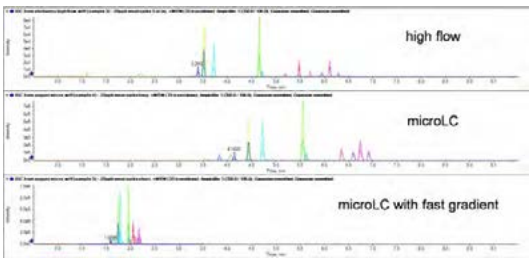

Figure 3. Comparison of meat sample spiked at 20 µg/kg and analyzed by traditional high flow LC and micro flow LC-MS/MS,. In this example analysis time was decreased from 10 min to 3.5 min using micro flow LC and by speeding up the gradient. In all methods peak widths at the base were 3 seconds or less.

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).

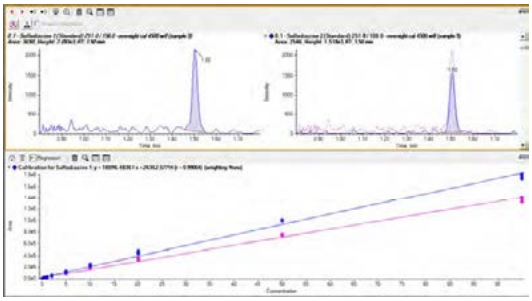

Figure 4a. Quantifier, qualifier MRM transition at 0.1 ng/mL (top), and calibration line of sulfadiazine from 0.1 to 100 ng/mL (bottom), the linearity is provided without the use of any internal standards

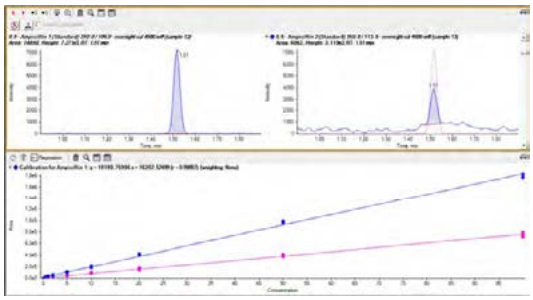

Figure 4b. Quantifier, qualifier MRM transition at 0.5 ng/mL (top), and calibration line of ampicillin from 0.1 to 100 ng/mL (bottom), the linearity is provided without the use of any internal standards

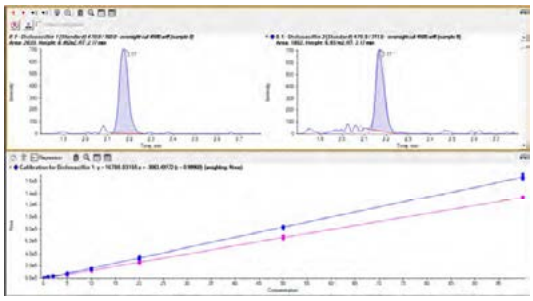

Figure 4c. Quantifier, qualifier MRM transition at 0.1 ng/mL (top), and calibration line of dicloxacillin from 0.1 to 100 ng/mL (bottom), the linearity is provided without the use of any internal standards

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 a SCIEX 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.

References

1. COMMISSION REGULATION (EU) No 37/2010 'on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin' December 2009
2. K. Mriziq et al.: 'Higher Sensitivity and Improved Resolution Microflow UHPLC with Small Diameter Turbo V™ Source Electrodes and Hardware for use with the ExpressHT™-Ultra System' Technical Note SCIEX (2011) # 4590211-01
3. Stephen Lock: 'The Use of Micro Flow UHPLC in Pesticide Screening of Food Samples by LC-MS/MS' Application Note SCIEX (2012) # 6330212-01

Publication number: 8880414-01

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 a SCIEX MicroLC 200 UHPLC System coupled with a QTRAP® 5500 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

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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 SCIEX 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 (SCIEX) 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 an SCIEX 5500 QTRAP® 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.

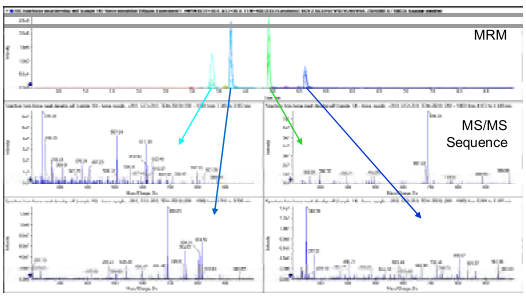

Figure 2. MRM initiated acquisition of MS/MS spectra to sequence characteristic proteins for horse meat

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

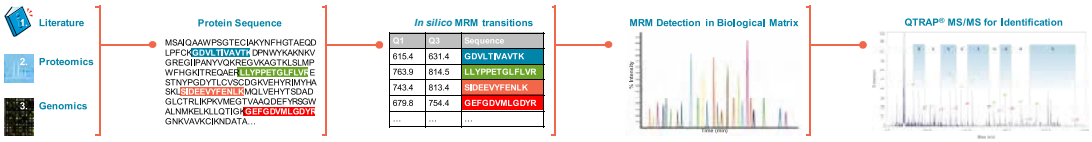

Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

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.

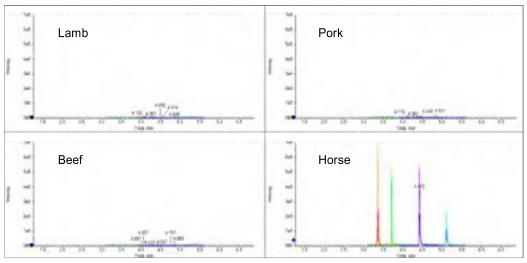

Figure 3. A comparison of the analysis of extracts from different types of meat. These initial results were obtained during the development of the method.

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.

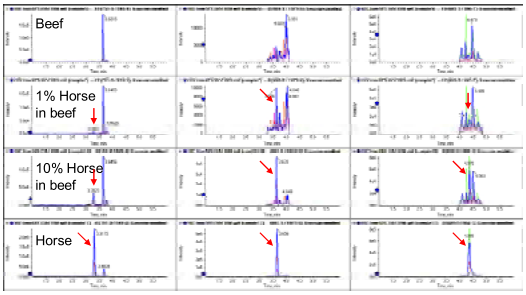

Figure 4. Detection of peptides characteristic for horse meat in beef at different levels, it shows that horse meat can be detected at a 1% level

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.

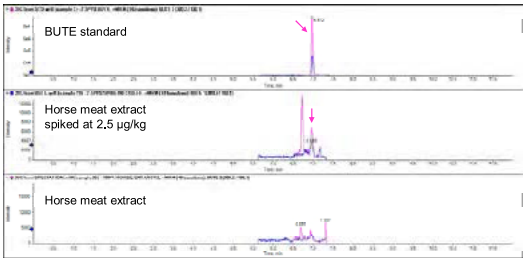

Figure 5. A comparison of the analysis of extracts from different types of meat. These initial results were obtained during the development of the method.

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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1 http://www.food.gov.uk/enforcement/monitoring/horse-meat/

2. K. Mriziq et al.: 'Higher Sensitivity and Improved Resolution Microflow UHPLC with Small Diameter Turbo V™ Source Electrodes and Hardware for use with the ExpressHT™-Ultra System' Technical Note SCIEX (2011) # 4590211-01

3 http://www.absciex.com/products/methods/imethod-applications-for-food-and-beverage/imethod-application-for-antibiotic-screening-version-13-for-cliquid-software

Publication number: 7580213-01

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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[1]SCIEX Darmstadt (Germany); [2]SCIEX Warrington, (UK)

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.

Contents

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

An SCIEX Triple Quad™ 5500 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 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.

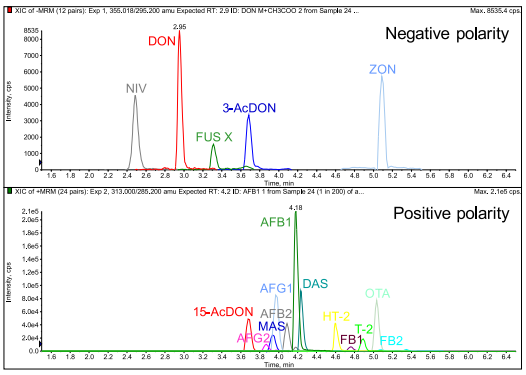

Figure 2. TIC of all mycotoxins analyzed in a single method with negative and positive polarity switching and the *Scheduled* MRM™ algorithm

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]

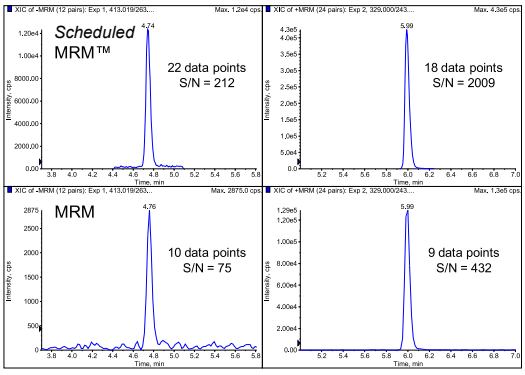

Figure 3. Comparison of a positive and negative switching experiment with and without *Scheduled* MRM™, FUS X in negative polarity (left) and AFG1 in positive polarity (right)

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.

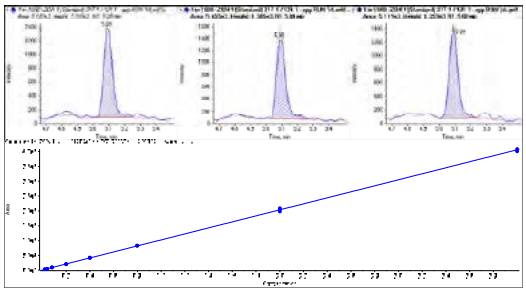

Figure 4. Triplicate injections of ZON at 0.04 ng/mL (top) and calibration line from 0.02 to 4 ng/mL with r = 0.9998 (bottom)

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.

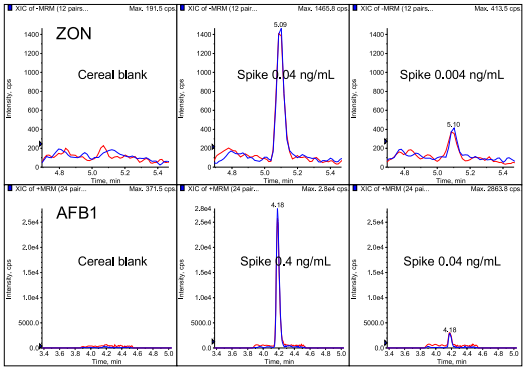

Figure 5. Chromatograms of ZON and AFB1 spiked into baby cereal compared with a matrix blank. The samples had been diluted 20 times.

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).

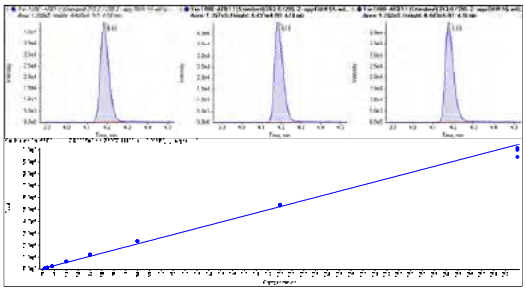

Figure 6 Triplicate injections of AFB1 at 0.4 ng/mL (top) and calibration line for ZON from 0.2 to 40 ng/mL with r = 0.9969 (bottom)

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

LC-MS/MS Analysis of Emerging Food Contaminants

Identification of Artificial Colors and Dyes in Food Samples using LC-HR-MS/MS

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Overview

Here we present a novel LC-HR-MS/MS method that was used to identify artificial colors and dyes in food samples.

High resolution MS and MS/MS data were collected using a SCIEX X500R QTOF System in negative Electrospray Ionization (ESI). Non-target peak finding, sample-control-comparison followed by identification based on empirical formula finding and ChemSpider database searching was performed in SCIEX OS Software. In addition, statistical data processing was done in MarkerView™ Software.

Introduction

Artificial colors and dyes are used in food to make it visually more appealing and “flavorful” since people associate certain colors with certain flavors.

However, some dyes are banned because they are toxic and carcinogenic. Other dyes are approved for use in foods and regulated by Codex Alimentarius, the US-FDA, EFSA etc. Nature derived color additives (pigments derived from vegetables, minerals or animals) are exempt from certification.[1-3]

Recent research shows a link between the presence of artificial colors in food and behavioral problems of children.[4-5] These findings have resulted in public concern about the use of artificial dyes.

Analytical methods used to test for the presence of banned colors and dyes in food include TLC-UV/VIS, LC-UV/VIS, and LC-MS. Such methods have limited selectivity and sensitivity and are therefore only used for target analysis. Recent advancements in LC-HR-MS technology provide the ability to perform targeted and non-targeted screening in food samples on a routine basis. The exact mass and MS/MS data provided by these instruments contain enough information to confidently identify known food ingredients and contaminants and also to identify unknown chemicals that may also be present in the sample.

Artificial colors and dyes in food samples were identified using the SCIEX X500R QTOF System. MS detection was performed

using information dependent acquisition (IDA) to simultaneously collect accurate mass MS and MS/MS information.

Compounds were automatically identified. SCIEX OS was used to automatically process the data using a non-target peak finding algorithm and sample-control-comparison to locate unique peaks in the sample. MarkerView™ Software and statistical data processing was used to separate matrix and sample specific signals from true contaminants. TOF-MS and MS/MS data of ions of interest were automatically processed using empirical formula finding and searched against online databases, such as ChemSpider, for identification. The SCIEX OS Software offers an easy to use and intuitive workflow to tentatively identify unknown chemicals in food.

Experimental

Samples

Store-bought “Icing Colors” were diluted 10,000x using a sugar solution prepared by dissolving 10 g of sugar in 10 mL water (LC grade) to mimic the icing sugar matrix typically used for baking.

LC Separation

LC separation was performed using a SCIEX ExionLC™ AD System with a Phenomenex Luna Omega 1.6 µm Polar C18 (50 x 2.1 mm) column and a fast gradient of water and methanol with

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5 mM ammonium formate buffer at a flow rate of 0.5 mL/min (see Table 1 for the gradient profile). The injection volume was 5 µL.

Table 1. Gradient conditions used for the identification of food dyes

Step	Time (min)	A (%)	B (%)
0	0.0	90	10
1	1.0	90	10
2	6.0	10	90
3	7.0	10	90
4	7.1	90	10
8	10.0	90	10

MS/MS Detection

The SCIEX X500R QTOF System with Turbo V™ source and Electrospray Ionization (ESI) was used in negative polarity.

Mass calibration was achieved using the integrated calibrant delivery system (CDS) with the TwinSprayer probe (dual ESI needle).

High resolution data were acquired using an IDA method consisting of a TOF-MS survey scan (100-1000 Da for 200 msec) and up to 10 dependent MS/MS scans (50-1000 Da for 50 msec). Declustering Potential (DP) was set to -80 V and MS/MS fragmentation was achieved using a Collision Energy (CE) of CE of -35 V with a collision energy spread of ±15 V.

Dynamic background subtraction (DBS) was activated for best MS/MS coverage. No inclusion list was used, which allowed non-target identification without the need for a second injection to acquire MS/MS data.

Data Acquisition and Processing

All data were acquired and processed using SCIEX OS Software version 1.0, which showcases a thoughtfully designed user interface that is fast to learn and delivers improved lab productivity. In addition, MarkerView™ Software version 1.3 was used for statistical processing using Principal Components Analysis (PCA).

Results and Discussion

X500R Performance Characteristics and Data Acquisition Workflows

The X500R QTOF system utilizes an N-optics TOF design to maximize resolution while maintaining benchtop design and a minimized footprint. Its resolving power increases with mass range providing ~30000 to 40000 resolution for the typical molecular weight range of ingredients and contaminants in food (Figure 1).

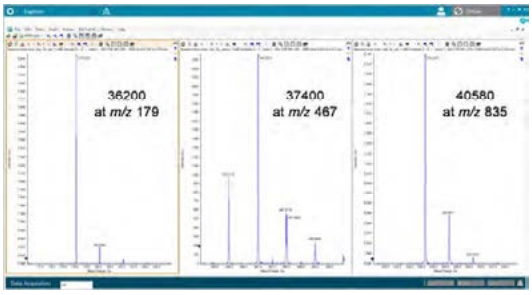

Figure 1. Resolution of different chemicals detected in negative polarity ESI in diluted dye samples

The X500R QTOF system achieves stable mass accuracy of less than 2 ppm by using a heated TOF configuration, with 6 heater drones throughout the TOF path and by using the dynamic background calibration software algorithm. The X500R QTOF's mass accuracy is supplemented by legendary dynamic transmission control and dynamic background calibration, introduced in 2010 with the TripleTOF® system and optimized over time.

In addition, the integrated CDS with the TwinSprayer probe provides an independent calibrant delivery path for reliable auto-calibration (Figure 2), maintaining mass accuracy over long periods of time by automatically calibrating in batch mode.

Figure 2. TwinSprayer ESI probe showing the independent inlet for LC and calibrant

The accurate mass measurement of a molecular ion is insufficient for compound identification. Single stage MS information can only be used for empirical formula finding. Because many different chemicals have identical molecular formulas accurate mass MS/MS data are absolutely necessary to identify chemical structures based on the molecular fingerprint observed in the MS/MS spectrum.

Using IDA, simultaneous acquisition of TOF-MS and MS/MS into a single data file for each sample was possible. Up to 10 MS/MS spectra were automatically collected for each chromatographic data point (Figure 3).

Figure 3. Simultaneous acquisition of TOF-MS and MS/MS using IDA, the example shows spectra of sucrose and sucrose dimer of the matrix

Processing Workflow for Non-Target Identification in SCIEX OS Software

Full scan chromatograms are very rich in information and easily contain thousands of ions from chemicals present in the sample, including the food matrix itself. Powerful software is needed to explore the high resolution MS and MS/MS spectra generated to get answers and results from these complex data.

Figure 4 shows Total Ion Chromatograms (TIC) of samples analyzed. It can be seen that the TIC are dominated by matrix components (sugars) eluting at ~0.3 min. The main dyes in the red and blue sample can be found, but minor components and ingredients in the yellow and brown sample are not visible.

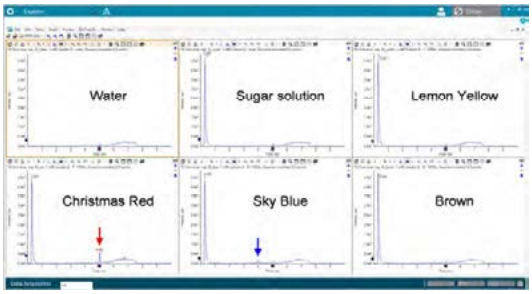

Figure 4. TIC of a blank (water), matrix (sugar solution) and for dyes samples, peak finding without software tools is very complicated or even impossible

SCIEX OS Software a single platform for MS control, data processing and reporting, and provides:

- Simple software workflows that deliver reliable results
- Automated feature detection based on non-target peak finding followed by sample-control-comparison
- Automated compound identification using empirical formula finding followed by library and online database searching
- Quick data review and reporting utilizing customizable flagging and filtering of results

The workflow to setup non-target data processing is illustrated in Figures 5a and b.

Figure 5a. Selecting unknown sample(s) and control sample for non-target data processing and sample-control comparison in SCIEX OS Software

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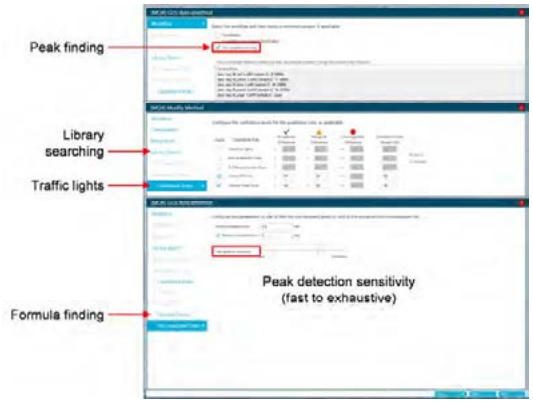

Figure 5b. Setup of non-target peak finding criteria and identification tools, including MS/MS library searching and empirical formula finding, criteria for traffic lights are set for later data review and filtering

Data Review during Non-Target Identification in SCIEX OS Software

After non-target peak finding and sample-control-comparison the results are displayed (Figure 6). The results table can be sorted and filter using the traffic lights. The Peak review will automatically provide XIC, TOF-MS and MS/MS data for both the sample and control sample.

Figure 6. Results display after non-target peak finding and sample-control comparison, results were filtered by formula finding score (>70%) and sorted by intensity

Zooming into the TOF-MS spectrum provides details of formula finding, including mass error in TOF-MS and MS/MS and the number of structures found in ChemSpider for each possible formula (Figure 7).

Figure 7. Zoom into TOF-MS to display detailed results of formula finding, the most likely formula has a mass error of 0.3 ppm in TOF-MS an average mass error of 1 ppm of all fragments in MS/MS and 5 matching structures in ChemSpider

From the TOF-MS display the formula can be linked to ChemSpider. The ChemSpider display will list all matching structures, automatically sorted by number of references. The selected structure is automatically fragmented in-silico and compared against the accurate mass MS/MS spectrum.

Using this workflow the main ingredient in the red food coloring was quickly identified as Erythrosine (Figure 8).

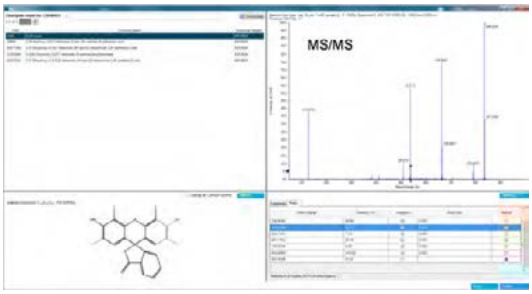

Figure 8. ChemSpider search results and in-silico fragmentation assisting to quickly identify Erythrosine in red food coloring

Results of Analyzing Food Coloring

Identified artificial dyes and by-products are summarized in Table 2. Figures 9, 10 and 11 show further examples of identification based in ChemSpider searching and MS/MS elucidation.

Table 2. Artificial dyes and by-products identified in samples

Sample	*m/z* - RT	Area %*	Formula	Formula Finder Score (%)	Mass error (ppm)	MS/MS error (ppm)	Identification
Red	834.6480 / 5.02	88.5	C20H8I4O5	94.5	0.3	1.0	**Erythrosine**
	708.7515 / 4.91	6.1	C20H9I3O5	93.4	0.5	0.9	Erythrosine-I
	890.6733 / 5.47	1.0	C23H12I4O6	92.0	0.8	0.8	Erythrosine+C3H4O
	407.0012 / 2.76	0.6	C16H12N2O7S2	96.2	0.1	1.0	**Sunset Yellow**
	582.8543 / 4.87	0.4	C20H10I2O5	92.1	0.1	2.1	Erythrosine-I2
Yellow	197.9867 / 0.53		$C7H4NO4S^-$	88.9	0.7	2.0	in-source fragment
	466.9974 / 0.53	90.3	C16H12N4O9S2	92.0	0.2	2.0	**Tartrazine**
	224.0134 / 0.53		$C8H6N3O3S^-$	93.6	0.7	0.5	in-source fragment
	501.9503 / 0.56	9.7	contains 2 Cl^-				not identified
Blue	747.1508 / 4.01	90.1	C37H36N2O9S3	86.9	0.3	3.3	**Brilliant Blue**
	577.1473 / 4.49	5.4	C30H30N2O6S2	92.5	0.2	1.7	Brilliant Blue - C7H6O3S (by-product)
	184.9909 / 0.52	4.5	C7H6O4S	78.1	2.1	2.3	3-Formylbenzene-sulfonic acid (by-product)
Brown	407.0012 / 2.76	33.9	C16H12N2O7S2	93.3	0.2	1.6	**Sunset Yellow**
	451.0277 / 3.31	24.8	C18H16N2O8S2	86.6	0.5	3.0	**Allura Red**
	834.6480 / 5.02	19.1	C20H8I4O5	92.8	0.1	1.9	**Erythrosine**
	501.9503 / 0.56	9.3	contains 2 Cl^-				not identified

* Area % includes monoisotopic peak, isotopes, adducts (i.e. Na+), multiply charged ions and in-source-fragments

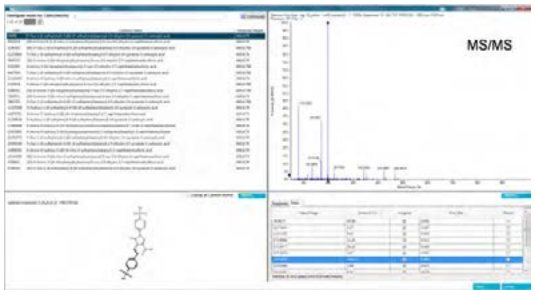

Figure 9. ChemSpider search results and in-silico fragmentation assisting to quickly identify Tartrazine in yellow food coloring

Figure 10. ChemSpider search results and in-silico fragmentation assisting to quickly identify Brilliant Blue in blue food coloring

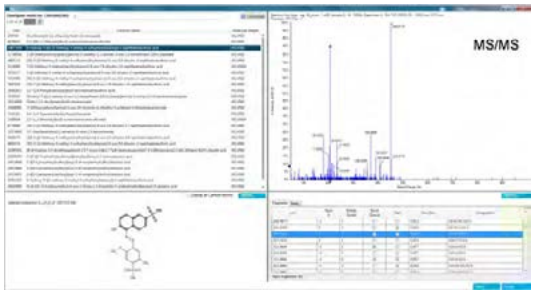

Figure 11. ChemSpider search results and in-silico fragmentation assisting in the quick identification of Allura Red in the brown food coloring

Statistical Data Analysis to Identify Unknowns

Statistical data analysis is an alternative to simple sample-control-comparison. Tools, such as Principal Components Analysis (PCA), can be used to identify characteristic markers in complex samples and at lower levels. Figure 12 shows an example of PCA performed in MarkerView™ Software to find ingredients in food dyes.

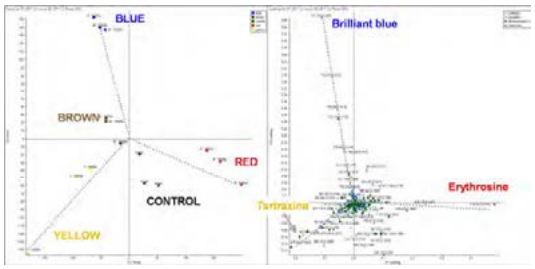

Figure 12. PCA as an alternative to sample-control comparison quickly finds differences between samples (score plot shown left) and helps to identify characteristic *m/z*-RT using the above described tools

Summary

A new non-target LC-HR-MS/MS based approach to quickly identify artificial colors in food samples was developed using the SCIEX X500R QTOF System.

Negative polarity ESI TOF-MS and MS/MS data acquired using information dependent acquisition were processed in SCIEX OS and MarkerView™ Software. Characteristic *m/z*-RT were further processed using empirical formula finding and ChemSpider searching. The major compounds in food coloring were quickly identified using automated and intuitive software workflows in SCIEX OS.

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Publication number: RUO-MKT-02-4482-A

Profiling and Identification of Hop-Derived Bitter Compounds in Beer Using LC-HR-MS/MS

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Overview

Here we present a method to study the profile of hop-derived bitter compounds using LC coupled to high resolution mass spectrometry.

Diluted beer samples were injected directly into the SCIEX X500R QTOF System. Data were processed using both a targeted list of accurate masses of molecular ions of known hop-derived compounds in SCIEX OS Software and a non-targeted analysis by performing statistical data processing (Principal Components Analysis, PCA) in MarkerView™ Software.

Introduction

Beer is the world's most widely consumed beverage (after tea and water) and probably the oldest alcoholic beverage.[1] Beer has attracted consumers over centuries due to its refreshing character, attractive aroma, and typical bitter taste.

The production of beer is called brewing, which involves the fermentation of starches, mainly derived from cereal grains (most commonly malted barley, although wheat, corn, and rice are widely used). Most beer is flavored with hops, which add bitterness and act as a natural preservative, though other flavorings such as herbs or fruit may occasionally be included.[2]

Aroma-active volatiles as well as nonvolatile bitter compounds of beers have been thoroughly investigated in recent decades.

The typical hop-derived beer bitterness is caused by adding cones, pellets, or extracts of hop during wort boiling. A number of isomerization processes have been identified to be of major importance for bitter taste development in the final beer product. The so-called isoxanthohumol (Figure 1), identified as a bitter compound in beer, was found to be generated from the hop-derived xanthohumol, during wort boiling. Moreover, *trans*- and *cis*-iso-α-acids have been identified as the major bitter contributors in beer and were demonstrated to be generated upon a rearrangement reaction of their hop-derived precursors, the α-acids. Following the α-acids, the second major constituents of hop are the β-acids (Figure 1), but there are almost no data available on the direct contribution of these

compounds to beer bitterness or on their role in the generation of bitter-tasting conversion products during wort boiling.[3, 4]

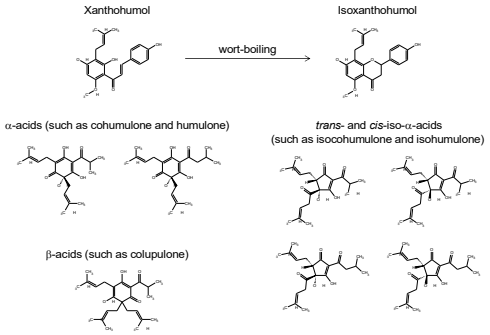

Figure 1. Hop-derived bitter compounds and their isomerization processes during the wort boiling

Here an LC-HR-MS/MS based method is presented using the SCIEX X500R QTOF System to identify hop-derived bitter compounds in beer based on accurate mass MS and MS/MS data after information dependent acquisition (IDA) followed by

Contents

targeted and non-targeted data processing in SCIEX OS and MarkerView™ Software.

Experimental

Beer Samples

- Store-bought samples from the Liquor Control Board of Ontario (LCBO)
- Degassed and diluted 2x with water
- Injection of 5 µL

LC Separation

- ExionLC™ AD system
- Phenomenex Luna Omega 1.6 µm Polar C18 (50 x 2.1 mm)
- Gradient of water/methanol + 5 mM ammonium formate at a flow of 0.5 mL/min (Table 1)

Table 1. Gradient conditions used for the identification hop-derived bitter compounds

Step	Time (min)	A (%)	B (%)
0	0.0	90	10
1	1.0	90	10
2	6.0	10	90
3	7.0	10	90
4	7.1	90	10
8	10.0	90	10

MS/MS Detection

The SCIEX X500R QTOF System with Turbo V™ source and Electrospray Ionization (ESI) was used in positive polarity. Ion source temperature was set to 450ºC and IS voltage was set to 5500 V.

Mass calibration was achieved using the integrated calibrant delivery system (CDS) with the TwinSprayer probe (dual ESI needle).

High resolution data were acquired using an IDA method consisting of a TOF-MS survey (100-1000 Da for 200 msec) and up to 10 dependent MS/MS scans (50-1000 Da for 50 msec). Declustering Potential (DP) was set to 80 V and MS/MS fragmentation was achieved using a Collision Energy (CE) of 35 V with a Collision Energy Spread (CES) of ±15 V.

Dynamic background subtraction (DBS) was activated to achieve the most complete MS/MS coverage. No inclusion list was used which allowed non-target identification without the need for a second injection to acquire MS/MS data.

Data Acquisition and Processing

All data were acquired and processed using SCIEX OS Software version 1.0, which showcases a thoughtfully designed user interface that is fast to learn and delivers improved lab productivity. In addition, MarkerView™ Software version 1.3 was used for statistical processing using Principal Components Analysis (PCA).

Results and Discussion

X500R Performance Characteristics and Data Acquisition Workflows

The X500R QTOF system utilizes N-optics design to maximize resolution while maintaining benchtop design and a minimized footprint. Its resolving power increases with mass range providing ~30000 to 40000 resolution for the typical molecular weight range of ingredients and potential contaminants in beer (Figure 2).

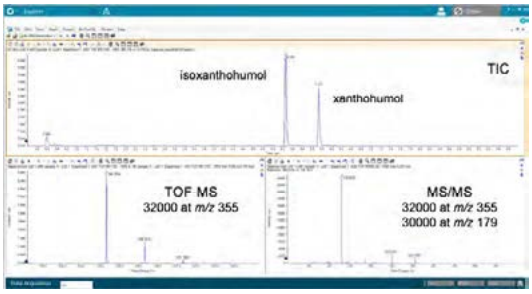

Figure 2. Resolution in TOF-MS and MS/MS mode achieved for isoxanthohumol

The X500R QTOF system achieves stable mass accuracy of less than 2 ppm by using a heated TOF configuration, with 6 heater drones throughout the TOF path and by using the dynamic background calibration software algorithm. The X500R QTOF's mass accuracy is supplemented by legendary dynamic transmission control and dynamic background calibration, introduced in 2010 with the TripleTOF® system and optimized over time.

In addition, the integrated CDS with the TwinSprayer probe provides an independent calibrant delivery path for reliable auto-calibration (Figure 3), maintaining mass accuracy over long periods of time by automatically calibrating in batch mode.

Figure 3. TwinSprayer ESI probe showing the independent inlet for LC and calibrant

The accurate measurement of a molecular ion is insufficient for compound identification. Single stage MS information can only be used for empirical formula finding, and accurate mass MS/MS data are absolutely necessary to identify chemical structures based on the molecular fingerprint saved into the pattern of fragment ions.

Using IDA, simultaneous acquisition of TOF-MS and MS/MS data in a single data file for each sample was possible. Up to 10 MS/MS spectra were automatically collected for each chromatographic data point (Figure 4).

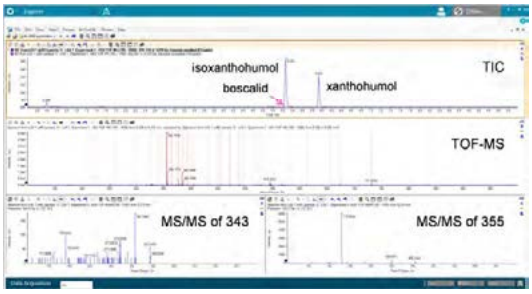

Figure 4. Simultaneous acquisition of TOF-MS and MS/MS spectra using IDA, the example shows spectra of isoxanthohumol (355) and the pesticide boscalid (343)

Processing Workflow for Target Identification in SCIEX OS Software

A targeted processing method was built to profile for 26 known hop-derived bitter compounds (Figure 5). Retention times were established by comparing the chromatographic profiles of beer samples to results published in literature.[3]

During targeted processing, Extracted Ion Chromatograms (XIC) of all analytes are generated based on user input (chemical formula and expected retention time). MS and MS/MS information is automatically evaluated if an XIC signal is detected and compounds are identified by matching retention time, accurate mass and isotope pattern of the molecular ion.

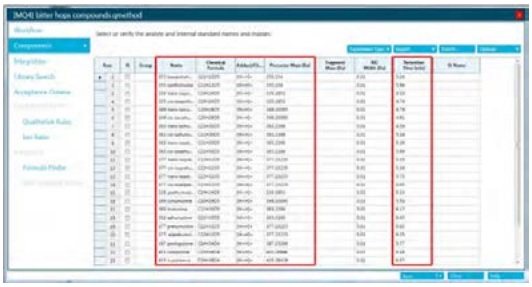

Figure 5. Targeted processing method to profile known hop-derived bitter compounds, XIC will be generated based on provided chemical formula and retention time)

During this project a small HR-MS/MS library was generated to assist future identification by also utilizing the MS/MS fragmentation (Figure 6).

Figure 6. Updating HR-MS/MS libraries in SCIEX OS Software to assist future compound identification

Results of Analyzing 40 Beer samples

The amounts of xanthohumol (X) and isoxanthohumol (IX) in a beer can provide information about how hops were used during the beer making process. Figure 7 shows the intensity for IX and X in 3 different beers. The observed X/IX ratio of the pilsner and Weissbier is in line with published data.[3]

Figure 7. Intensity of isoxanthohumol (IX) and xanthohumol (X) and the X/IX ratio in different beers (left to right German pilsner, German Weissbier of the same brewery and dry-hoped IPA produced in Ontario, Canada)

The concentrations of IX, X and X/IX from the beer samples are summarized in Figure 8. This data reflectins different styles of beer making. It is obvious that the high X/IX ratio in Pale Ales and India Pale Ale (IPA) is caused by the late addition of hops during the boiling and fermentation process.[4]

Figure 8. Intensity of isoxanthohumol (IX) and xanthohumol (X), top, and the X/IX ratio, bottom, for 40 different beers including different styles. The data reflect the differences of beers produced in different countries

Isoxanthohumol (IX), isocohumulone (ICH), and isohumulone (IH) were the 3 most abundant hop-derived bitter compounds detected in beer. ICH and IH and other iso-α-acids were present in their *trans*- and *cis*- form (Figure 9). The intensity ratio of *trans*/*cis* acids varied between 0.02 to 0.15 (American Lagers < European Pilsners < American Pal Ales and IPA).

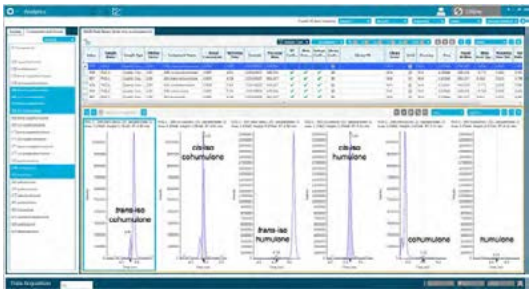

Figure 9. Intensity of *cis*- and *trans*-isocohumulone, isohumulone, cohumulone, and humulone in a Czech pilsner, the *trans*/*cis* ratio was 0.06

The measure of bitterness in beer is the IBU (International Bittering Units). A plot of the sum of the peak areas of IX, ICH and IH against the IBU for different beers is shown in Figure 10.

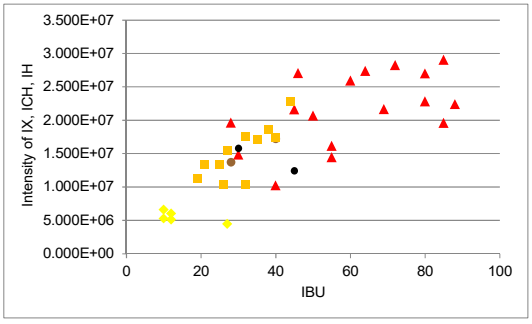

Figure 10. Correlation of intensity of IX, ICH and IH against the published IBU for different beer styles (yellow: American lagers, orange: European pilsners, red: Pale Ales and India Pale Ales, brown: Bock, and black: Stout)

Beer Profiling using Statistical Data Analysis

Statistical data analysis, such as Principal Components Analysis (PCA), can be used to profile and compare different beverage samples. Figure 11 shows the PCA scores plot for 40 different beers. Beers of similar style group together. The location of a sample in the plot indicates a specific flavor or color (i.e. lighter beer vs. a more hoppy/bitter beer). The PCA loadings plot assists in finding characteristic markers (*m/z*-RT). Once these markers are found, the corresponding chemical can be identified using formula finding based on accurate mass MS and MS/MS followed by ChemSpider searching and MS/MS elucidation.

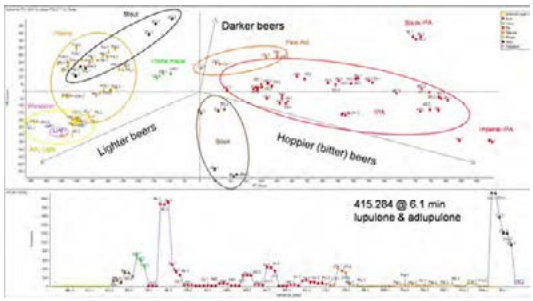

Figure 11. PCA scores plot used to profile and map different beer styles, the loading plot (not shown here) assisted to find characteristic *m/z*-RT

The β-acids lupulone and adlupulone were found in higher concentrations in darker beers, such as stout and black IPA, as the profile plot (bottom trace) in Figure 11 shows. Both compounds were identified using the described automated software tools in SCIEX OS Software (Figure 12).

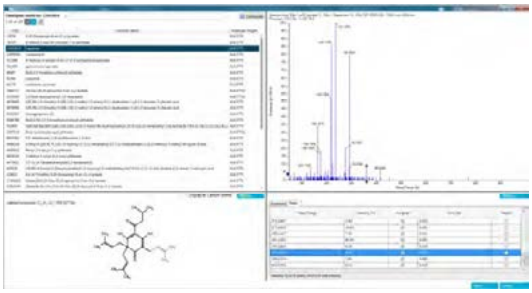

Figure 12. Identification of lupulone and adlupulone in an Irish stout based on formula finding followed by ChemSpider searching and MS/MS elucidation

Summary

The SCIEX X500R QTOF System was used to analyze 40 different beer samples in positive polarity ESI using information dependent acquisition of MS and MS/MS spectra. Samples were processed using a targeted approach to profile 26 known hop-derived bitter compounds in SCIEX OS Software. Samples were also processed using PCA in MarkerView™ Software.

This study shows that LC-HR-MS/MS is a valuable tool to study flavor and aroma profile in food and beverage samples.

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Contents

Chemical Components Identification of Cistanche Deserticola Using the X500R QTOF System

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Introduction

Cistanche Deserticola was first recorded in the "*Shen Nong Materia Medica*"; it is also known as "*Dayun*," "*Rousong Rong*," and "*Zong rong*." As traditional herbal medicine, it has the properties of nourishing the kidney yang, improving bloodflow, acting as a laxative, immune stimulation, and other effects[1]. In 1983, the Japanese scholar H. Kobayashi and others began to study the chemical composition of *Cistanche Deserticola*[2], and since then it has become a popular topic in Chinese medicine research that has generated great interest both domestically and abroad over the last 30 years. *Cistanche Deserticola* belongs to the class of plants containing phenolic glycosides, iridoids and their glycosides, and lignans and their glycosides.

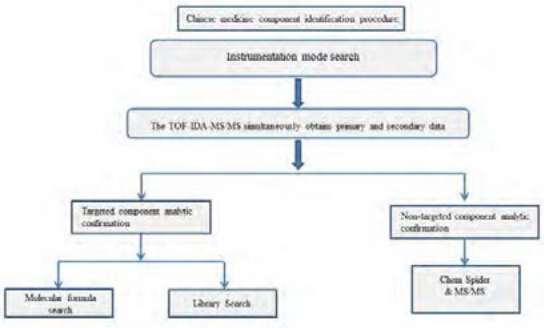
Quadrupole time-of-flight (QTOF) mass spectrometry is a sensitive and specific tool for identification of Chinese medicine components that has gradually become indispensable to research. This technology has overcome traditional technical challenges with retrospective analyses of single injections that permit extraction of important data and the most comprehensive acquisition of sample information. Using exact mass and high resolution TOF-MS and TOF-MS/MS data allows for simultaneous, highly specific targeted and non-targeted qualitative analysis. However, the complexity of instrument operation and software use have vastly limited the spread and development of this technology. Here we introduce a new QTOF system that uses a revolutionary N-type geometry-based TOF path, intuitive software, and accurate molecular weight techniques that are easier to use in Chinese medicine component identification.

The benefits of this method are as follows:

1. The X500R uses the durable, industry-approved Turbo V™ ion source and air curtain gas interface design, which has a strong anti-contamination feature;
2. SCIEX OS Software has integrated acquisition, processing, and reporting functions on a single platform; the interface is intuitive, easy to master, and has one-touch auto-adjust correction to ensure that analysts with any degree of expertise can obtain high quality, reliable data;
3. Using SCIEX OS Software to process data to identify Chinese medicine components is simple and permits rapid extraction of useful information, thus improving efficacy;
4. It derives more accurate and reliable identification results from Chinese medicine libraries containing MS/MS spectra;
5. High-resolution MS/MS Chinese medicine databases are based on the "Chinese pharmacopeia" Part 1 TCM active ingredients; including component references in the pharmacopeia and active ingredients in the herbs, there are nearly 900 compounds.

Experimental Process

1. Using TOF-MS-IDA MS/MS mode, inject a sample and simultaneously obtain primary precursor ions and corresponding secondary spectra;
2. Using SCIEX OS Software targeted screening, confirmation of target compounds, and secondary spectra along with screening of Chinese medicine standards and matching methods can increase accuracy and work productivity.
3. SCIEX OS Software's non-targeted identification workflow uses library searches and complete unknown searches in ChemSpider to verify results, ensuring more components are identified with a simpler workflow.



Samples and Preprocessing Method

Sample source:

Purchased from Shanghai pharmacies in sliced form

Preprocessing method:

1. Slices were crushed to form powder;
2. 0.9mg was weighed and immersed in 3mL methanol for 40 min;

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3. The sample from step 2) was ultrasonicated 1 h;
4. Centrifugation and removal of the supernatant to use as a sample were performed.

Liquid Phase Conditions

Chromatographic Column: XSelect HSS T3, 2.1*150mm, 3.5μm;

Mobile phase: Gradient elution was used

Mobile phase: A is 0.1% formic acid water-2mM NH4FA
B is 95% acetonitrile-5% water-2mM NH4FA

Flow rate: 0.5mL/min

Column temperature: 40ºC

Amount inserted: 5μL

Mass Spectrometry Method

Scanning method: TOF MS-IDA-15 MS/MS qualitative screening

ESI ion source parameters:

Air curtain gas CUR: 35psi; IS voltage: 5500V/-4500V

Source temperature: 600ºC

Atomizing gas GAS1: 55psi; Auxiliary gas GAS2: 60psi

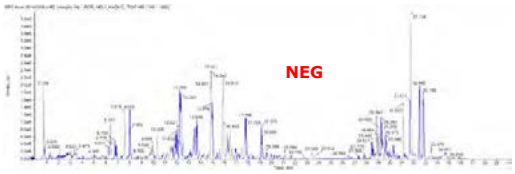
Instrumentation mode search:

One-touch (select MS Check on the lower right), fully automated TOF-MS and TOF-MS/MS correction mode ensures that analysts of any expertise level can obtain accurate, reliable, reproducible data.

Ion Data Plots

Positive and negative ion mode BPC's:

POS

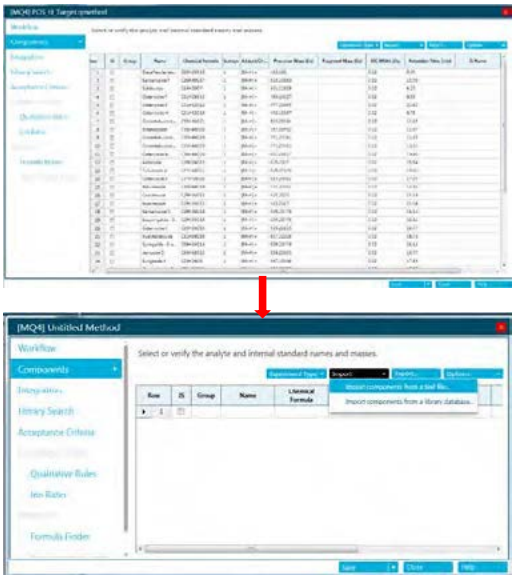


Simple Chinese Medicine Component Identification Procedure

1. Targeted component identification workflow

1.1: Molecular formula search

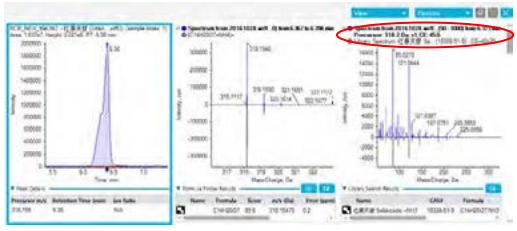
Only the chemical compound name and molecular formula are required; these can be input directly or imported using Excel's copy and paste function to create a processing method.



Once a results table is created, quantitative and qualitative results can be viewed in the same window. A red/green indicator system is used to indicate mass accuracy, retention time, isotope type, and confidence in identification by database matching.

SCIEX OS Software lets users filter results and display only those compounds meeting acceptance criteria and falling within confidence intervals defined by the user. It can quickly find targeted results in large databases.

The TCM database of MS/MS spectra allows for secondary matching and yields more reliable results (grey color in the database indicates MS/MS data).



The literature contains names and molecular formulas of phenolic glycoside active ingredients. Using the above process for the identification of target components, it was determined that the sample contains 39 types of phenolic glycosides:

Component Name	Formula	Found At Mass (NH4)	RT (min)	Mass Error (ppm)
Veroascose glycoside	C20H30O12	480.208	6.17	0.951
Kankanoside F	C26H40O17	642.26	6.16	-0.749
Salidroside	C14H20O7	318.155	6.38	0.241
Cistanoside F	C21H28O13	506.187	6.74	0.898
Cistanoside E	C21H32O12	494.223	8.04	0.449
Cistanoside H	C21H32O13	510.219	7.24	0.997
Cistanche tubulosa glycoside C	C35H46O21	820.287	10.3	-0.413
Echinacea glycoside	C35H46O20	804.291	12.21	-0.733
Cistanche tubulosa glycoside A	C35H46O19	788.296	13.62	-0.982
Cistanche tubulosa glycoside B	C35H46O19	788.296	13.62	-0.898
Cistanoside A	C36H48O20	818.307	13.84	-0.55
Calamus glycosides	C29H36O15	642.239	14.83	-0.6
Tubuloside A	C37H48O21	846.301	14.94	-0.965
Isoacteoside	C29H36O15	642.239	14.83	-0.6
Kankanoside G	C29H36O14	626.244	16.42	-0.871
Isosyringalide -3-a-l-rhamnopyranoside	C29H36O14	626.244	16.42	-0.871
Cistanoside C	C30H38O15	656.255	17.11	0.739
Acetylfuran glycoside	C31H38O16	684.249	17.77	-0.723
Syringalide -3-a-l-rhamnopyranoside	C29H36O14	626.244	16.42	-0.871
Jionoside D	C30H38O15	656.255	17.11	0.739
Phenylethyl glycoside B	C29H36O13	610.249	18.22	-0.789
Tubuloside B	C31H38O16	684.249	17.77	-0.723
Tubuloside E	C31H38O15	668.254	19.55	-0.638
Salsaside D/F	C31H38O15	668.254	19.55	-0.638
Salsaside E	C32H40O16	698.267	19.93	0.997
Cistansinenside A	C32H40O16	698.267	14.83	-0.6
Cistanoside G	C20H30O11	464.213	14.83	-0.6
2-acetylacteoside	C31H38O16	684.249	16.42	-0.871
Cistanche tubulosa glycoside B2	C35H46O19	788.295	16.42	-0.871
Lipedoside A1 Isosyringalide 3-rhamnoside	C29H36O14	626.244	17.11	0.739
campneoside I	C30H38O16	672.249	17.77	-0.723
campneoside II	C29H36O16	658.234	16.42	-0.871
crenatoside	C29H34O15	640.224	18.22	-0.789
Tubuloside C	C43H54O24	972.334	17.77	-0.723
Tubuloside D	C43H54O23	956.339	19.55	-0.638
Cistanoside I	C21H28O12	490.193	19.55	-0.638
Cistantubulose A1	C27H38O18	668.24	6.19	0.585
Cistantubulose A2	C27H38O17	652.246	7.63	0.894
Kankanoside H1/H2	C37H48O20	830.307	16.3	-0.977

Besides the phenolic glycoside active components, *Cistanche* also contains a large number of compounds such as iridoids, glycosides and lignans. The identification results are as follows:

The iridoids, glycosides and lignans identification list:

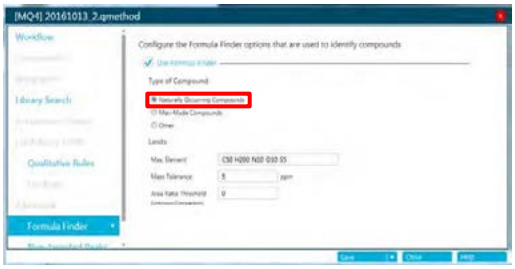
Component Name	Formula	Found At Mass (NH4)	RT (min)	MassError (ppm)
mussaenoside acid/8-epiloganic acid	C16H24O10	394.17	3.16	-0.934
Gluroside	C15H24O8	350.181	7.97	-0.509
Kankanoside A/O/P	C16H26O8	364.196	10.42	-0.402
Leonuride/Kankanoside L	C15H24O9	366.176	5.02	-0.014
8-epideoxyloganic acid	C16H24O9	378.176	9.21	0.306
6-deoxycatalpol	C15H22O9	364.16	6.3	-0.764
catalpol	C15H22O10	380.156	3.27	0.985
bartsioside/antirrhide	C15H22O8	348.165	7.54	-0.175
Kankanoside B / phelypaeside	C15H24O10	382.171	2.68	-0.368
adoxoside acid	C17H26O10	408.187	4.29	0.995
Kankanoside D	C15H26O7	336.202	11.22	0.891
Kankanoside N	C16H28O8	366.212	11.95	-0.986
(+)-pinoresinol-O-β-D-glucopyranoside	C26H32O11	538.228	15.52	-0.786
(+)-syringaresinol-O-β-D-glucopyranoside	C28H36O13	598.249	16.2	-0.952
liriodendrin	C34H46O18	760.302	13.01	-0.64
syringin	C17H24O9	390.176	8.14	0.896

1.2: Database Search:

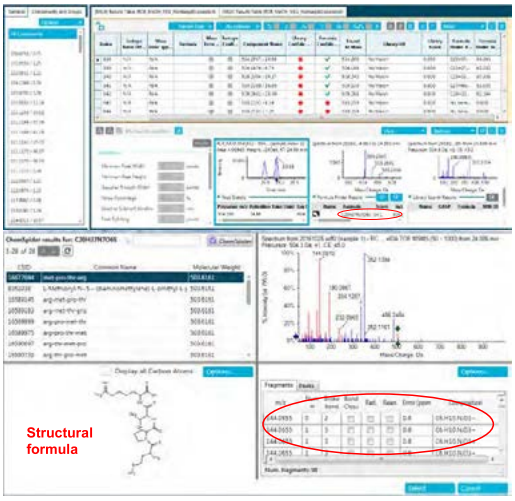
53 compounds were obtained from matching identification in the TCM database: 43 positive ions, 22 negative ions, and 12 repeats, listed below. Besides active phenolic glycosides, iridoids, glycosides and lignans, Cistanche also contains mannitol, leucine, and geniposidic acid. See appendix for list.

2. Non-targeted component analysis

Non-targeted component identification can be performed with the built-in ChemSpider search function to determine the classification and type of unknowns. For non-targeted component identification, simply choose the “non-targeted” mode, and the molecular formula search will develop a processing method. The workflow is as follows:



For complete unknowns, molecular formula search results are shown in a peak browser window in the lower part of the TOF-MS mass spectrum; with ChemSpider database search, results are listed by priority and the structural information obtained in ChemSpider is automatically compared with the MS/MS spectrum obtained, providing secondary feedback for rapid identification.



Summary

1. Rapid high-resolution data acquisition; a single injection yielded high-resolution TOF MS and MS/MS data, with 39 identifiable phenolic glycoside active components and 16 iridoids, lignans and glycosides;
2. A TCM database of secondary spectra provides additional matching information, and the software automatically provides a database match score. Using the score, one can easily, quickly, and accurately identify Chinese medicine components;
3. The device is simple and has one-touch auto-adjust correction to ensure that analysts with any degree of expertise can obtain high quality, reliable data;
4. The new SCIEX OS Software version integrates data acquisition, processing (quantitative and qualitative), display, reporting, and database management. It solves the difficulties that many users face with an intuitive and easy-to-use interface;
5. Both the targeted and non-targeted screening workflows are simple, and the built-in method guide helps users accurately and rapidly create methods.

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Document number: RUO-MKT-02-6091-A

Analysis of the Vitamin B Complex in Infant Formula Samples by LC-MS/MS

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[1]SCIEX Warrington, Cheshire (UK), [2]SCIEX Concord, Ontario (Canada)

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.

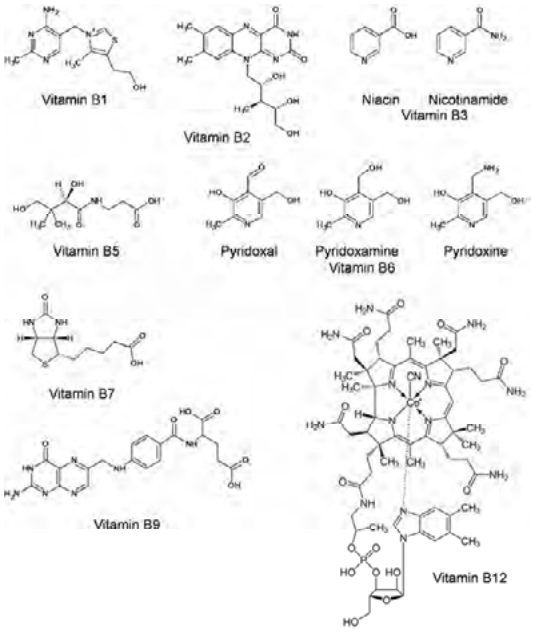

Figure 1. Chemical structures of B vitamins

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.

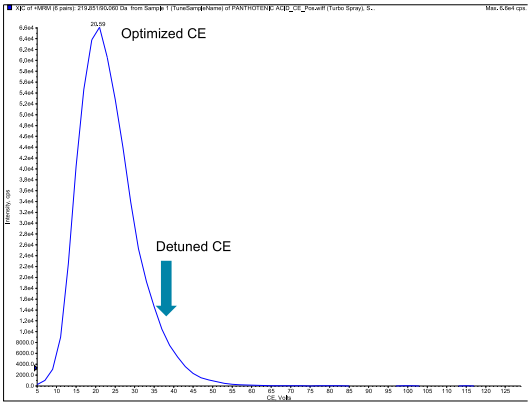

Figure 2. This is a typical ramp of the collision energy (CE) for a vitamin B5 fragment ion. Using this approach the more sensitive vitamins that showed a non-linear response at higher concentrations were detuned for a lower response by choosing non-optimal collision energies.

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.

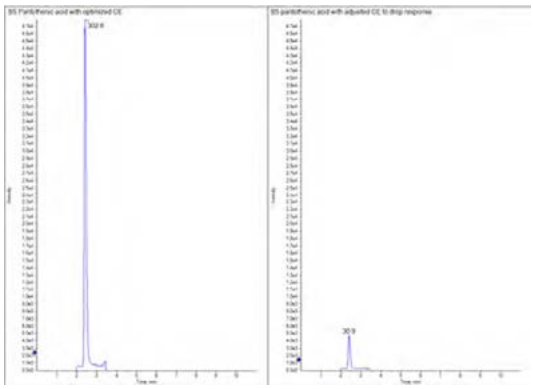

Figure 3. The effect of changing the collision energy on the response of vitamin B5

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.

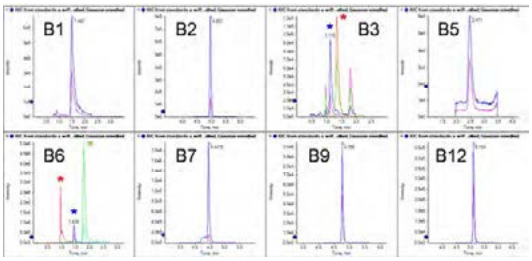

Figure 4. Example of a 5 ng/mL solvent standard of B vitamins, quantifier and qualifier ions are shown

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

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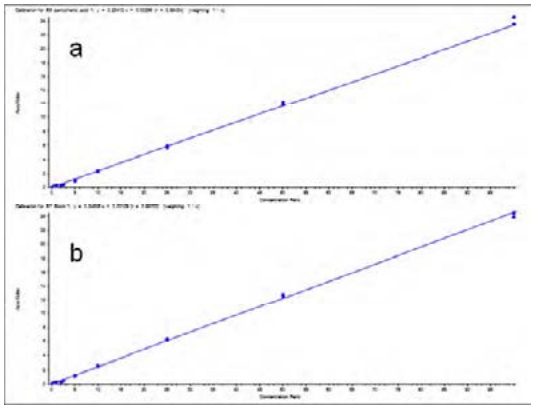

Figure 5. Calibration lines for two vitamins, an early eluting vitamin B5 (a) and a late eluting vitamin B7 (b)

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

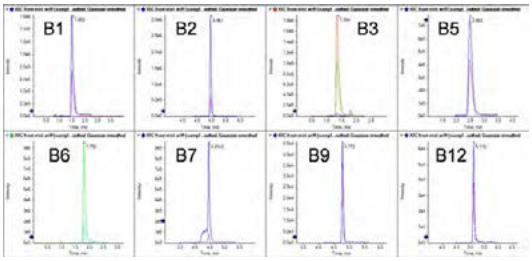

Figure 6. Example of an extract from NIST 1849A reference material showing chromatograms for the fortified vitamins. Vitamin B3 was present as nicotinamide and vitamin B6 as pyridoxine.

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.

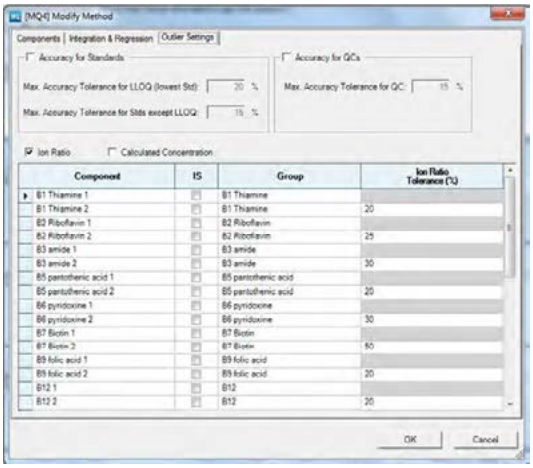

Figure 7. Query settings in the quantitation method editor of MultiQuant™ Software to calculate ion ratios

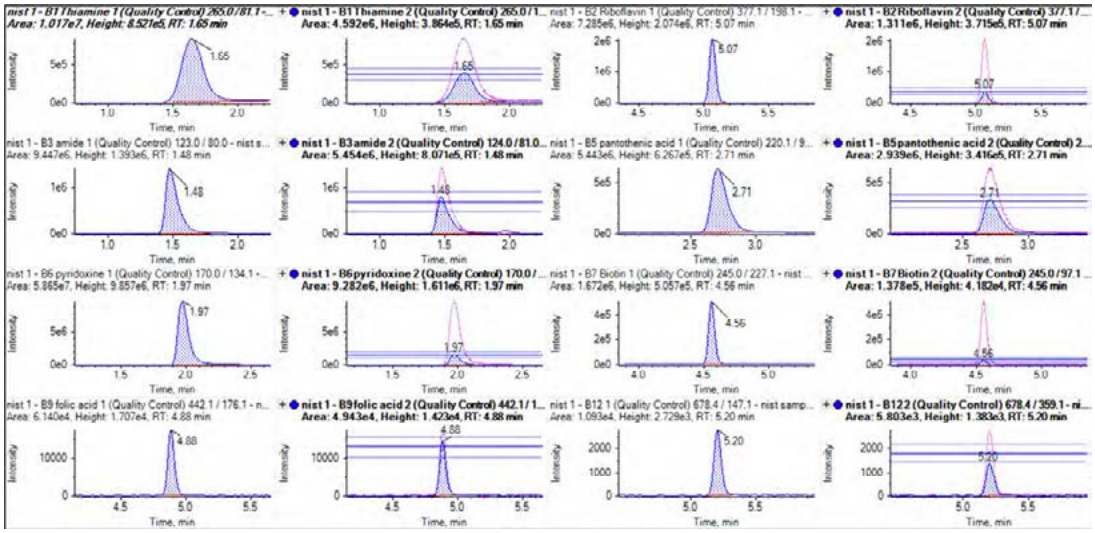

Figure 8. Peak review with ion ratio tolerances of an extract from NIST 1849A reference material

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

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 a 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

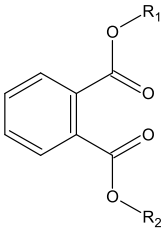
The SCIEX QTRAP® 5500 System was used with Turbo V™ source and Electrospray Ionization (ESI) source. Two selective MRM transitions were monitored for each targeted analyte (Table 1). MRM^3 was used to differentiate between isomers and to increase selectivity to reduce interferences.

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).

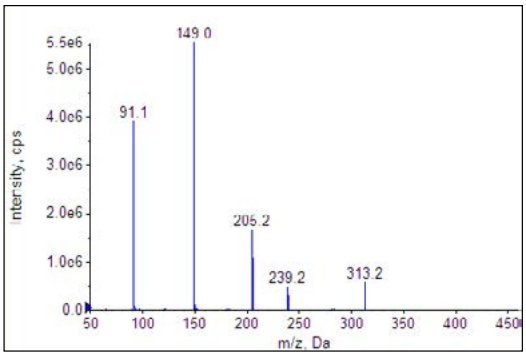


Figure 1. EPI spectrum of BBP, the molecular fingerprint saved into the MS/MS spectrum was used for compound identification with highest confidence

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

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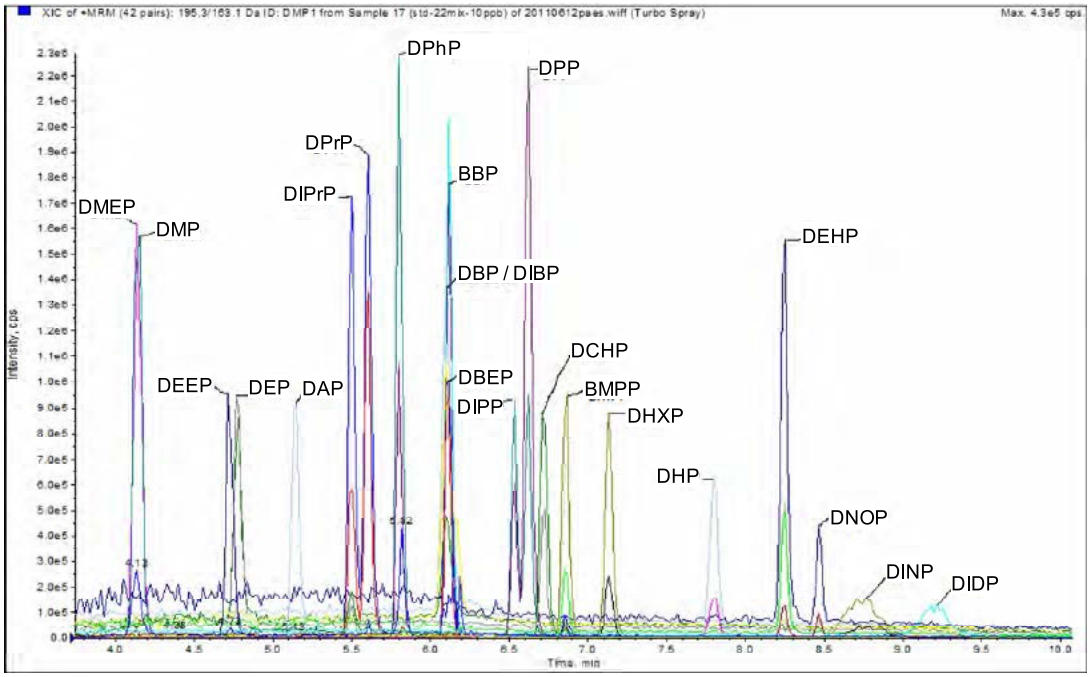

Figure 2. Example LC-MS/MS chromatogram showing the separation and detection of 22 phthalates at a concentration of 10 ng/mL

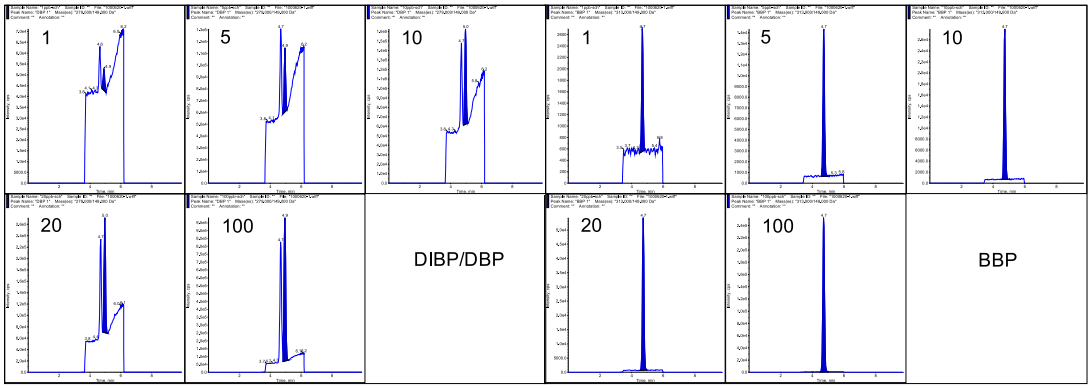

Figure 3a. MRM chromatograms of the high priority phthalates DBP and BBP at 1, 5, 10, 20, and 100 ng/mL

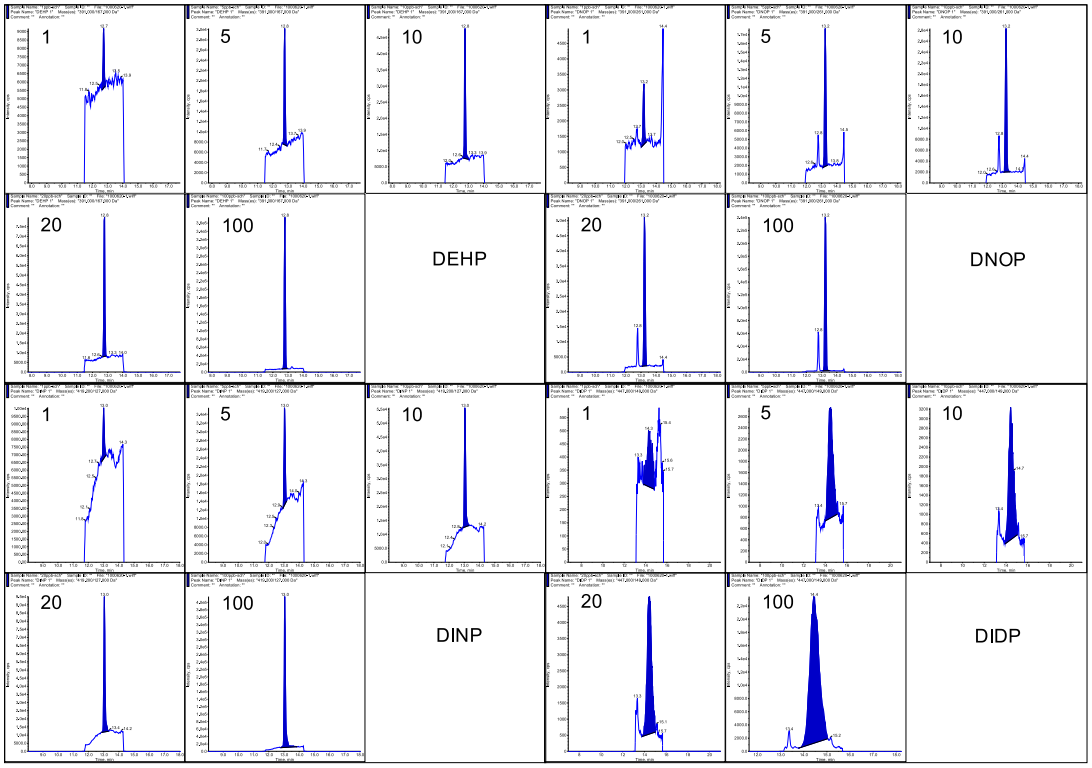

Figure 3b. MRM chromatograms of the high priority phthalates DEHP, DNOP, DINP, and DIDP at 1, 5, 10, 20, and 100 ng/mL

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.

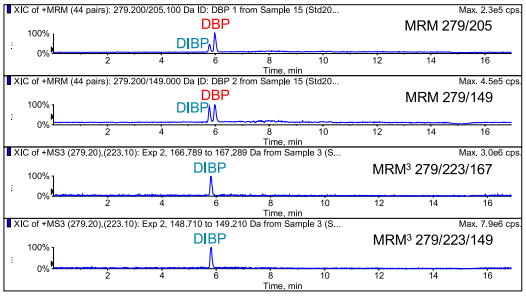

Figure 4. Differentiation of DIBP and DBP using the different fragmentation pattern in MRM^3 mode in comparison to MRM mode

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.

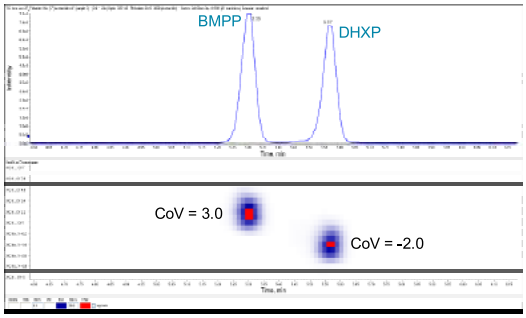

Figure 5a. Separation of the isomers BMPP and DHXP, both phthalates can be separated in the LC and DMS space resulting in increased selectivity

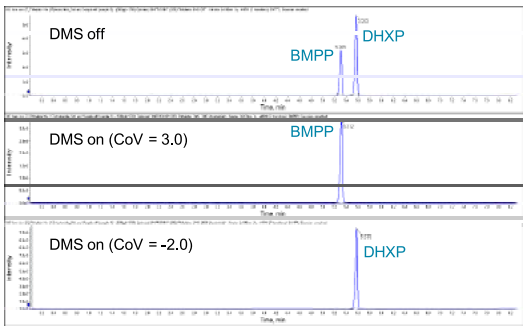

Figure 5b. Selective detection of BMPP and DHXP by compound specific CoV for each analyte, acetonitrile was introduced as chemical modifier

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 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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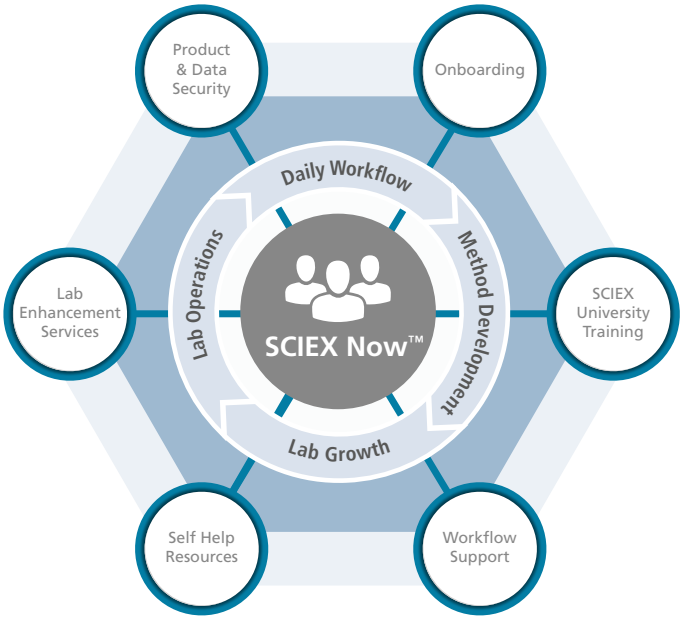

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