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LCMSMS Food Applications Notes

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Consumer safety is the driving-force behind food analysis, with mass spec being a common characterization tool used to detect many different classes of residual contaminants in food. One such class is mycotoxins, which are toxins produced by fungi and molds in foods. Mycotoxin analysis also helps illustrate how mass spec technology has, and will continue to, evolve with advances and innovation in instrumentation and methodology.

Originally, mycotoxin detection relied on a targeted analysis, with mass spec only analyzing a panel of characterized mycotoxins which may be present in the sample. This legacy approach of detection and quantitation of a target suite was both time-consuming in method development, and limited in the scope of what answers could be reported. While this is still a dominant approach for mycotoxins and other residues analysis, advancements in technology continue to expand the capabilities of these analytical methods.

Nowadays, innovation has advanced mass spec technology from targeted analysis to a non-targeted or screening analysis. This change has begun through two simultaneous changes in technology. First, instrumentation has become more sensitive, meaning lower-abundance mycotoxins can be identified and the library of known toxins expanded. Secondly, a non-targeted analysis overcomes the need to analyze each toxin individually. Scientists can instead use instrumentation specifically designed to screen samples and identify unknown analytes. For instance, the ATL LCMSMS system has been successfully applied to multiple analyte screening methods detailed in this compendium.

“Consumer safety is the drivingforce behind food analysis, with mass spec being a common characterization tool used to detect many different classes of residual contaminants in food.”

However, this decades-long journey from targeted analysis, to screening analyses and onto metabolite detection is not limited to the food industry nor mycotoxin detection. In fact, there are a multitude of other instances including veterinary drug detection, pesticide identification and environmental analysis that can benefit from non-targeted analysis. It is becoming increasingly apparent that mass spec has a fundamental role in any future food analysis.

But, innovation is not limited to the present day. Metabolomics of food is an area gaining popularity amongst food researchers. Once these toxins have entered foodstuffs and have metabolized, it is possiblefor scientists to identify and quantify these analytes, which are often present at ultra-low concentrations. The connection between metabolomics research and food analysis is novel to this community. Although full integration of these two disciplines will take time, foodomics highlights an area of potential collaboration and will be an area of impending food research and analysis over the coming years.

An Overview of ATL Technology Notes

Simultaneous Analysis of 25 Mycotoxins in Grain by LC-MS/MS

Produced by fungi, mycotoxins are capable of causing health issues and death if consumed through contaminated food and agricultural commodities. This risk has led to many countries implementing strict regulations controlling mycotoxin concentrations To ensure the highest accuracy and reliability, LCMS/MS is rapidly becoming the method of choice for such analyses. However, the many different classes of mycotoxins necessitate standardizing sample preparation techniques, which can be time consuming.

To address this issue, ATL researchers have developed a new, fast purification method, which allows up to 25 mycotoxins in the same sample to be detected using the ATL Food Application LC-MS/MS system.

Simultaneous analysis of 12 food allergens in baked and raw food products using the LC-MS/MS Food Application system

Food allergies are the leading cause of anaphylaxis, a severe and potentially deadly allergic reaction. As there is currently no cure for allergies, those who suffer from them must rely on the accurate testing and labelling of food products to avoid health issues.

Although they are the most commonly used tests for screening allergens, enzyme-linked immunosorbent assays (ELISAs) have limited selectivity and can produce false positive or false negative results.

To combat the potential risk associated with mislabeled allergens, ATL researchers developed a method using the Food Application LC-MS/MS system that detects and screens 12 separate allergenic proteins simultaneously in a single injection.

Improving Identification and Quantification of Polar Herbicides by CESI-MS

Glyphosate is a common herbicide that has been associated with various health risks. As a result, more stringent regulation has been introduced to restrict its presence in the food chain to safe-for-consumption levels, most recently by the European Union. However, current LC-MS methods of analysis can have difficulty distinguishing between different degradation products of these herbicides. Thus, ATL researchers endeavored to develop a new, more effective CESI-MS method for this separation and identification.

The new CESI-MS method not only demonstrates an excellent ability to distinguish between glyphosate and its degradants, but also between similar degradation products of another widely-used herbicide, fosetyl aluminum.

Combining Non-Targeted SWATH MS/MSALL Acquisition with Highly Selective MRMHR for the Analysis of Veterinary Drugs in Tissue Using the ATL LCMSMS System

Due to the associated risks of antimicrobial resistance and possible allergenic reactions, the European Union has strict guidelines concerning veterinary drugs in animal products.

The ATL LCMSMS system is a powerful instrument capable of performing the sensitive analysis of veterinary drugs in complex matrices. When analyzing veterinary drugs in a liver extract, the ATL system displays the mass errors of the precursor and fragment ions and the ion ratio as a traffic light system. This allows users to review of large volumes of data simply and be confident in the identification of a detected signal, which meets the European Union's criteria of identification points.

Pesticide Residues in Produce Analyzed by Targeted MRMHR "FullScan" Acquisition and Processing

Food and environmental sample analysis is a field of great importance to both local and worldwide economies. Positive hits or results above tolerance limits can lead to the delay or destruction of products, with massive impacts on the import, export, sale or distribution of goods, and millions of dollars at stake.

For such tests on pesticides the standard method for many organizations has been LC-MS/MS coupled with Multiple Reaction Monitoring (MRM). While this practice has a high degree of sensitivity, ATL researchers explored potential improvements by incorporating High Resolution Accurate Mass (HRAM) mass spectrometry technology. The research effort concluded that MRMHR provides high resolution monitoring of known ion transitions as well as full scan product ion spectrum collection.

Quantitation of Oregon List of Pesticides and Cannabinoids in Cannabis Matrices by LC-MS/MS

The increasing legal use of cannabis in the now requires a more robust and reproducible analytical method, quantifying both residual pesticides as well as psychotropic cannabinoid content.

In an effort to contribute to the creation of such a method, ATL researchers analyzed cannabis samples with two different ATL Triple Quad platforms to detect all the pesticides compromising the Oregon Pesticide List (the most comprehensive list of pesticides allowed in plant products in the).

This method is superior to previous practices in multiple ways. For one, the ATL vMethod can analyze ten cannabinoids within the same sample. When verified, the method was shown to offer a simpler form of sample preparation and optimized LC-MS conditions.

In addition, the final version of the vMethod is accompanied by a disc that contains a comprehensive a quantitation methods and reporting template that may be directly loaded on to the instrument. This convenient feature can allow laboratories to become fully operational for pesticide and cannabinoid analysis in a matter of days.

LCMSMS System with SWATH Acquisition for Pesticide Residue Screening in Fruits and Vegetables

Agriculture is often subjected to excessive and potentially toxic levels of chemical fertilizers, pesticides, and herbicides. The most commonly used method for detecting these compounds is used on the system and high resolution TOF-IDA-MS/MS technology.

In an attempt to provide an even better service, ATL researchers used the ATL LCMSMS System with SWATH Acquisition to screen for pesticide residues in six varieties of vegetables and fruits. By the end of the analysis it was found that although pesticide residues were extremely high, they could be easily washed off with detergent.

Ultimately, the experiment's goal was achieved and SWATH screening was established as a highly accurate method for scanning the residues of the 190 most commonly used types of pesticides according to the Ministry of Agriculture.

A Selective and Robust LC-MS/MS Method for Multiple Meat Speciation and Authentication on the Food Application System

Following the identification of horsemeat in certain beef products in early 2013 and its subsequent publicity, the Food Safety Authority (FSA) and Department for Environment Food & Rural Affairs (DEFRA) set the threshold for undeclared meat species in meat products to 1%.

Thus, it is imperative that the previously adequate analytical methods, such as polymerase chain reactions (PCR) and enzyme-linked immunosorbent assays (ELISA), are superseded by methods that offer greater sensitivity and accuracy.

One such method, as presented by ATL researchers, is an LC-MS/MS method, using the Food Application LC-MS/ MS system, that can detect and screen pork, beef, lamb, chicken, duck and horse to a threshold limit of 1% simultaneously, in a single injection

A Robust and Sensitive Method for the Direct Analysis of Polar Pesticides in Food and Environmental Samples Without Derivatization

In recent years, multi-residue LC-MS/MS analyses have become the minimum requirement for the quantification of pesticides in food and environmental samples. However, some highly polar compounds can only be analyzed using single-residue methods, which often involve derivatization – a time consuming technique used to transform a chemical compound into a product – to improve detection.

However, NofaLab, an independent Dutch sampling laboratory, has developed a method that can analyze many of these highly polar pesticides in a single analysis without derivatization. Along with a technique that utilized the ATL + QTRAP mass spectrometer, the methods were found to be considerably more robust and sensitive than contemporary approaches and have achieved the target limits of detection required to meet existing and proposed regulations.

Use of LCMSMS for Monitoring Unexpected Additives in Nutritional Supplements

Nutritional supplement manufacturers often claim that their products can support an individual's recovery from illness. But in order to maximize these functions, the companies may add related drugs to the supplement to increase its efficacy without including them as a listed ingredient. This uncertainty creates a potential risk to consumer's health

The ATL LCMSMS high-resolution mass spectrometry system can qualitatively confirm the presence of over 50 additives, and provides an efficient means for rapid, high-throughput monitoring of nutritional supplements for additives.

To prove this efficacy, this ATL study randomly selected 19 nutritional supplements commonly found on the market. Screening results showed that blood pressure-lowering and glucose-lowering products commonly contained additives, especially those products advertised to use medicine extracts to lower blood sugar. Many of the additives detected were present in amounts several times greater than therapeutic doses. Thus, they could be quite hazardous to consumers' health.

Analyzing Different Compositions of Polygala from Different Regions using the LCMSMS System

Authentic herbs from regions have commonly been used as herbal medicines. There is now interest in studying these herbal medicines, including Polygala, to identify and analyze compounds, and further understand their pharmacodynamic efficacy.

However, this is currently difficult to achieve owing to the need for easy identification of active ingredients and differentiate authentic medicines from fraudulent herbs. To make identification more efficient, this study analyzed 24 different Polygala samples from 4 regions. Using the LCMSMS mass spectrometer and MarkerView software, the different compounds were easily identified, obtaining high resolution spectrometric data that support identification of medicinal components.

SOFTWARE

Delivering clear, accurate and concise results for Food testing laboratories around the world.

Whether your laboratory is doing high throughput regulatory analysis of pesticides, mycotoxins and veterinary drugs or you are leading the way in foodomics research, Software enables you to process your acquired sample data in a quickly and efficiently without the need for time consuming manual integrations and spectral interpretation.

Features

- Next Generation Calculation Algorithms
- Data Processing and Acquisition Capabilities
- Tools to Help Maintain Regulatory Compliance
- Full Support, from Start to Finish
- Quick to Learn, Simple to Master
- Minimize Mouse Clicks and Reduce Data Bottlenecks
- Quality with Less Effort

Simultaneous Analysis of 25 Mycotoxins in Grain by LCMS/MS

Mycotoxins are secondary metabolites produced by a wide range of fungi known to contaminate a variety of food and agricultural commodities worldwide and has been recognized as a potential health threat to humans and animals. Many countries have regulations in place for mycotoxin detection and identification and their permissible limits. The limits of mycotoxins in certain products are regulated by GB 2761 and in EU, mycotoxin limits are harmonized in the regulation for contaminants in foodstuffs EC 1881/2006 and the amended regulation EC 1126/2007. Regulations on food and environmental analysis require the analysis of contaminants using confirmatory techniques. Thus, there is a demand for powerful and rapid analytical methods that can detect very low concentrations of mycotoxins in a variety of sample matrices. In recent years, LCMS/MS has gained popularity of becoming the method of choice, leveraging its ability to analyze a wider range of compounds in a single analysis coupled together with the high selectivity and sensitivity of Multiple Reaction Monitoring (MRM).

Figure 1. Accuracy and LOQ values shown for the panel of mycotoxins. Limits of Quantitation (LOQ) of all mycotoxins were found between 0.1 ng/g and 5 ng/g. Accuracy assessed over three concentrations ranged from 82% to 118%. These measurements of performance demonstrate excellent sensitivity and accuracy for this assay.

Traditionally, different classes of mycotoxins required different sample preparation techniques, making the process laborious and time consuming. Presented is an integrated workflow to analyze 25 compounds simultaneously in one sample. This includes a simplified extraction procedure that does away with additional clean-up steps by immunoaffinity columns and couples it to high resolution LC separation and high sensitivity MS detection.

Key Assay Attributes

- A fully integrated LC-MS/MS solution is presented to analyze 25 common mycotoxin residues simultaneously in relevant grain samples. Polarity switching ensures best coverage of relevant analytes.
- Simplified extraction procedure is described which does away with additional clean-up steps, saving time and labor at the front end of analysis.
- The method was validated for performance including sensitivity and robustness in different grain matrices.
- Limits of Quantitation (LOQ) of all mycotoxins were found between 0.1µg/kg and 5µg/kg. All LOQ meet the requirements of the GB methods.

Experimental

Sample Preparation

Sample preparation was carried out in accordance to the vMethod SOP (P/N 5060674). Grain samples (corn, rice, wheat etc.) were first homogenized and 2.5g of sample was extracted using a mixture of acetonitrile and water. Once sonicated and centrifuged, the supernatant was passed through a Cleanert MC SPE Cartridge (Agela Technologies, P/N ZS-MYT10-B) which contains a sorbent chemistry specially optimized for mycotoxins. The filtrate was then dried down and reconstituted for LC-MS analysis.

LC Conditions

Liquid chromatography analysis was performed using a ATL LC TM AD UHPLC system. 20µL was injected onto a Phenomenex Kinetex C18 column (100mm X 2.1 mm, 1.7µm, P/N 00D-4475-AN). Mobile phase A contained water with 0.1% formic acid and mobile phase B contained methanol with 0.1%.

Table 1. LC Gradient time program. Flow rate at all steps was 0.3 mL/min, and the total run time was 13 minutes including reequilibration.

Time (min)	%B
1.0	3
2.0	10
4.0	50
9.0	80
9.1	99
11.0	99
11.1	3
13.0	3

MS/MS Conditions

Electrospray ionization was carried out on a ATL QTRAP Food Application system with fast polarity switching. The Turbo V™ source was kept at a temperature of 500°C and the Scheduled MRM™ algorithm was used to analyze grain samples for 25 mycotoxins in a single injection by multiplexing the detection of multiple MRM transitions for signature fragments.

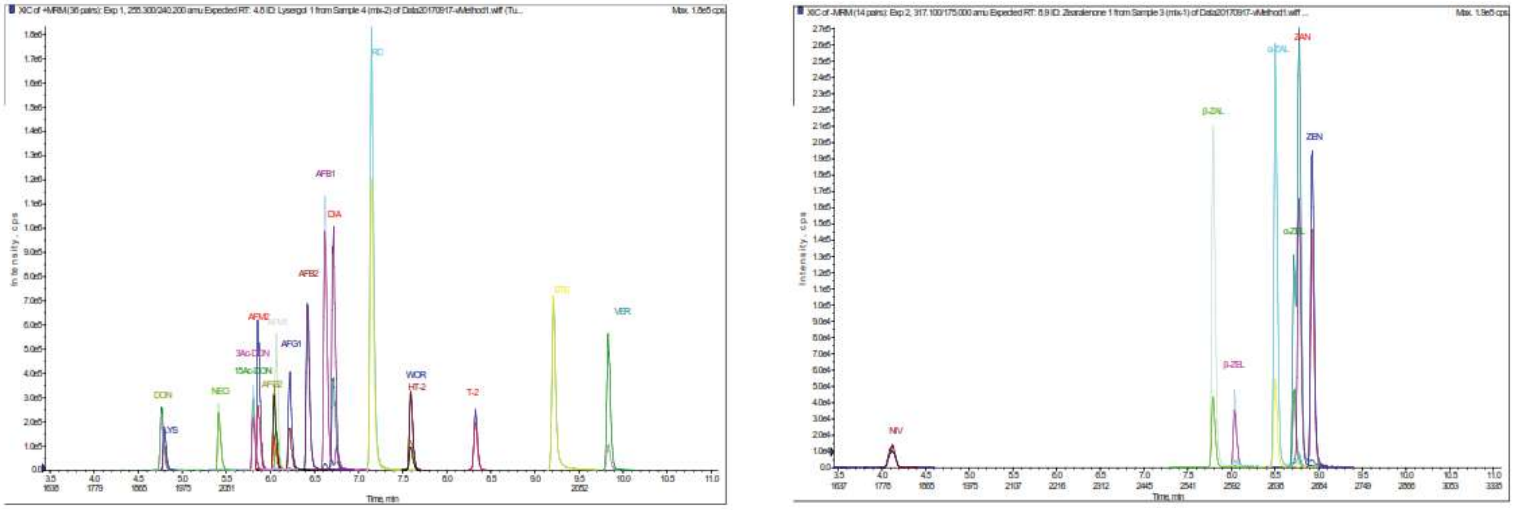


Figure 2. Chromatographic profile is shown for those 18 mycotoxins collected in ESI positive mode (top) and those 7 mycotoxins collected in ESI negative mode (bottom).

Both positive and negative modes were analyzed simultaneously during a single sample injection, allowing all 25 mycotoxins to be analyzed in one data acquisition method.

Results and Discussions

For each analyte, two signature MRM transitions were chosen to ensure confidence in the identification of each mycotoxin (Table 2). To monitor many MRM transitions during a single injection, the Scheduled MRM algorithm was employed, where individual MRM transitions were monitored for a short time window during their expected retention time. Thus, at any one point in time, the number of concurrent MRM transitions were significantly reduced resulting in much higher duty cycles for each analyte. Combining Scheduled MRM with fast polarity switching further allowed extending the target list of mycotoxins while maintaining sample throughput by eliminating need for multiple injections. Typical chromatograms of solvent standard were shown in Figure 2. The total target cycle time of 0.6 sec ensured the collection of at least 12 data points across the LC peak resulting in excellent accuracy and reproducibility. The system suitability was tested with the concentration of 5 or 50ng/mL standards (some compounds spiked at 5 and some at 50 depending on relative sensitivity) and the standard solution was injected three times. The %CV of each analyte peak was calculated to less than 15%.

For sample preparation, a simplified sample clean-up method was developed. Instead of immunoaffinity columns, one mycotoxin specialized solid phase extraction (SPE) column (Cleanert MC, Agela) was used. This column proved advantageous in that it doesn't need to be activated, washed, and eluted. It not only shortened the sample preparation time, but also saved cost. Figure 3 shows the comparison of the sample clean-up step before and after. Combing with LC-MS/MS analysis it could be quantified more accurately.

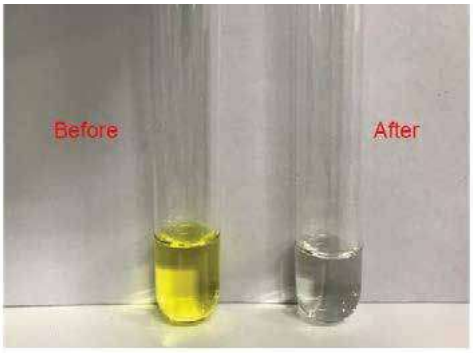


Figure 3. Sample preparation and clean up. Visual comparison of a grain sample before and after the Cleanert SPE column clean-up step. Cleaning up the sample can provide reduction of matrix interferences as well as help in maintaining instrument performance.

The limit of quantitation and matrix matched linearity were evaluated. Because of the matrix inhibitory effects, the matrix matched curves were used to quantify the unknown samples. For AFB1 and DON as example, the method was found to be good reproducibility, linear regression coefficient was found to be greater than 0.99 (Figure 4). According to the different sensitivity of each compound on the instrument, the LOQ of all target mycotoxins were from 0.1ng/g to 5ng/g. The accuracy of low, medium and high concentration spiked sample was between 80% and 120%
(Figure 1).

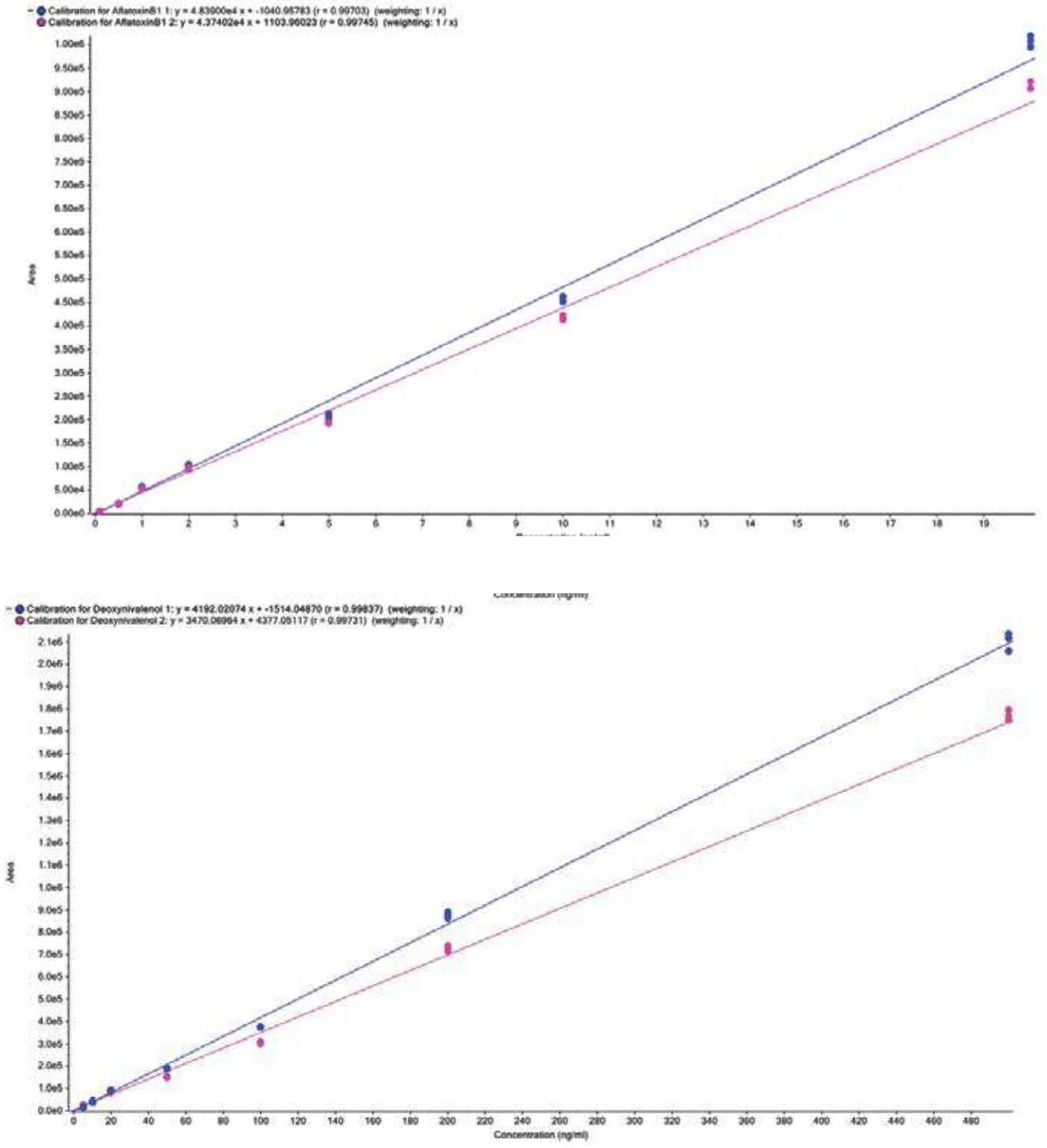


Figure 4. Calibration lines of AFB1 (top) and DON (bottom) from 5 to 500 ng/mL. Two MRM transitions were monitored: fragment 1 (blue) and fragment 2 (pink). R-values shown for both transitions for both representative analytes are >0.99, demonstrating excellent linear range and response for the assay.

CONCLUSIONS

A fast, robust, and reliable method, for the detection 25 mycotoxins in the matrix grain was developed and validated. A fast purification method was used to cover the 25 kinds of mycotoxins. High resolution LC using a small particle size column was combined with high sensitivity detection using a ATL Food Application LC-MS/MS system. Multiple Reaction Monitoring (MRM) was used because of its high selectivity and sensitivity. The Scheduled MRM™ algorithm used automatically optimized dwell times and cycle times for best sensitivity and reproducibility. The method was validated in different grain matrices. Limits of Quantitation (LOQ) of all mycotoxins were found between 0.1µg/kg and 5µg/kg. All LOQ meet the requirements of the GB methods.

Table 2. MRM transitions and retention times are provided for two transitions for each mycotoxin in the 25-analyte panel. Shown are the 18 compounds analyzed in positive ion mode.

Compounds name	RT(min)	MRM (primary, quantifier)	MRM (secondary, qualifier)
AflatoxinB1(AFB1)	6.62	313.1>285.1	313.1>241.1
AflatoxinB2(AFB2)	6.43	315.1>287.1	315.1>259.1
AflatoxinG1(AFG1)	6.22	329.1>243.2	329.1>214.9
AflatoxinG2(AFG2)	6.05	331.1>245.1	331.1>189.1
AflatoxinM1(AFM1)	6.07	329.0>273.1	329.0>268.9
AflatoxinM2(AFM2)	5.86	331.1>273.1	331.1>285.1
T-2 toxin(T-2)	8.32	484.2>305.3	484.2>185.1
Verruculogen(VER)	9.84	534.3>392.3	534.3>191.1
Neosolaniol(NEO)	5.41	400.2>185.1	400.2>305.2
Wortmannin(WOR)	7.59	447.2>345.2	447.2>285.2
Roquefortine C(RC)	7.13	390.3>193.1	390.3>322.2
Sterigmatocysin(STE)	9.19	325.1>310.1	325.1>281.0
Lysergol(LYS)	4.8	255.3>240.2	255.3>197.2
Diacetoxyscirpenol (DIA)	6.7	384.2>307.2	384.2>105.1
HT-2 Toxin(HT-2)	7.59	442.1>263.1	442.1>215.0
Deoxynivalenol(DON)	4.76	296.9>249.1	296.9>231.1
3-Acetyl Deoxynivalenol (3-AcDON)	5.8	339.0>231.0	339.0>203.0
15-Acetyl Deoxynivalenol (15-AcDON)	5.8	339.1>321.3	339.1>137.2

Concurrent Quantitation, Library Searching, and High-Confidence ID Confirmation

Food and environmental sample analysis represents an impossibly large universe of potential matrices and hundreds of potential contaminant residues, including chemically alike (even isomeric) species, as well as those which may be widely chemically diverse. In addition to robust routine quantitation, testing laboratories are increasingly tasked with confirmation of positive detections. In addition to the paramount importance of protecting consumers and the environment, positive hits or above-tolerance limit results can also lead to the delay or destruction of products, with massive impacts to import, export, sale or distribution, and millions of dollars, at stake.
Application of LC-MS/MS with multiple reaction monitoring (MRM) has represented the principal workflow for pesticide residues analyses due to the high degree of sensitivity and selectivity imparted by the monitoring of unique MRM transitions. The work presented explores the additional advantages gained when leveraging High Resolution Accurate Mass (HRAM) mass spectrometric technology.

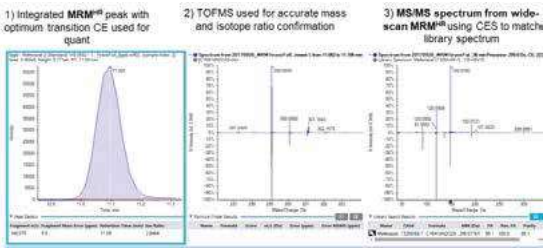


Figure 1. Simplified Quantitation and Confirmation Combined. An MRM experiment type was employed to collect pesticide data in food matrices. MRM acquisition allows monitoring of both optimized transitions as well as full-scan product ion spectrum collection. This approach provides the capability for concurrent quantitation (using a highly specific MRM transition optimized for maximum sensitivity) and identity confirmation by MSMS spectral matching, with a single acquisition method and a single processing step. In this example, an MRM peak for Mefenecet is shown, including its retention time error and fragment mass error, alongside confirmatory TOFMS spectrum and MSMS library matched spectrum with a Purity score of 99.

The ATL LCMSMS system and software combined provide the ability to perform both routine targeted quantitation as well as screening. The key advantages of this HRAM approach are realized in the streamlined MRM workflow which achieves sensitive and selective quantitative MRM data collection and processing with practical, concurrent collection and searching of MSMS data. Application of LC-MS/MS with multiple reaction monitoring (MRM) has represented the principal workflow for pesticide residues analyses due to the high degree of sensitivity and selectivity imparted by the monitoring of unique MRM transitions. The work presented explores the additional advantages gained when leveraging High Resolution Accurate Mass (HRAM) mass spectrometric technology.

Key Advantages of MRM Analysis

- Data acquisition with MRM in conjunction with the simultaneous collection of TOF MS data provides access to multiple approaches for achieving accurate and sensitive quantitative analyses.
- MRM takes advantage of monitoring a transition for specificity. Defining optimized voltages for each transition maximizes sensitivity. MRM specificity leads to reduced background and increased signal to noise ratios. Retention time scheduling allows data collection only during known elution windows for best peak quality.
- Full scan MSMS can be collected in MRM mode, and the resulting spectra can be searched against a compound library for qualitative ID.
- High confidence in compound identification is achieved through multiple points of matching including accurate precursor ion mass, isotope pattern matching, accurate fragment mass, ion ratio, chromatographic retention time, and library matching.

Experimental

Sample Preparation

The iDQuant Standards Kit for Pesticide Analysis includes 209 well characterized pesticides. Here we present example data where we used the iDQuant Kit to screen for, quantify, and identify pesticides in extracts of fruits and vegetables using Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) with an AB ATL 5500 system. Organic produce samples were extracted using QuEChERS. The iD Quant Kit

Pesticides mixture, containing 209 characterized pesticides, was used as a spiking solution in some samples and to build standard calibrators for external quantitation.

HPLC Conditions:

Analytical liquid chromatography (LC) separation was achieved using a ATL LC AD system and a Phenomenex Kinetex XB-C18 LC Column (100 x 3 mm) with mobile phases consisting of A) Water + 5 mM ammonium formate + 0.1% formic acid and B) Methanol + 5 mM ammonium formate. Column oven temperature was 50°C and a 20 µL injection was used. Gradient conditions were used with a run time of 21 minutes for the full gradient with a flow rate of 0.4 mL/min. An example elution profile of the MRM transitions is shown in Figure 2.

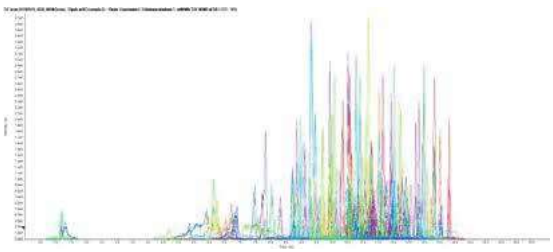


Figure 2. Chromatographic profile of MRM transitions in a standard solution of the 209-pesticide mixture in the iD quant kit. Separation was achieved using Phenomenex Kinetex XB-C18 column and a 21-minute LC gradient.

MS Conditions:

The ATL LCMSMS system with the Turbo V source was operated in positive mode electrospray ionization (ESI). Source parameters are listed in Table 1. The TOF MS scan was conducted over a range of 50 to 1000 m/z. Two different MS acquisition methods are demonstrated. Targeted analysis of the pesticide panel was conducted using an MRM experiment including two transitions monitored for each analyte. Additionally, retention time (RT) values were specified for each MRM transition, with RT tolerance values of 15 s for each, and the Extended Linear Dynamic Range feature was turned on (Figure 3).

Table 1. Ion Source Parameters. Electrospray Ionization (ESI) conducted in positive ion mode.

Parameter	Setting
Curtain Gas (CUR)	30
Collision Gas	10
Ion Spray voltage (IS)	5500
Temperature (TEM)	650
Nebulizer Gas (GS1)	50
Heater Gas (GS2)	50

The second acquisition method demonstrated was the Data Independent Acquisition known as SWATH Acquisition. TOF MS scan parameters were identical to the MRM method. Variable window SWATH acquisition was employed to cover the precursor mass ranges from 50 to 800 m/z. A total of 20 nominal mass SWATH windows were defined, and total scan time for this acquisition method was approximately 1.7 seconds.

MRM Data Acquisition and Full-scan MSMS Collection

For each target transition in the acquisition method, the nominal mass precursor ion was defined for the target analyte, and a mass range was defined which would encompass the expected fragment ion. Optimized declustering potential (DP) and collision energy (CE) voltages were designated for the primary transition, around which a narrow (20 Da) TOF mass range was defined. A second MRM transition was also defined for each target, with the same nominal mass precursor ion, but which collects a “full scan” range of product ion masses from 40 to 1000 m/z. A generic CE (35 V) with Collision Energy Spread (CES) of 15 V was defined to achieve a more robust MSMS spectrum for searching against database spectra. Additionally, scan scheduling was applied to all transitions by assigning the known retention time to each; in this mode of operation, data for each transition will only be acquired within the defined chromatographic time window, this preserving total instrument cycle time to maintain peak quality, sensitivity, and ability to potentially add large numbers of additional transitions. Figure 3 shows a portion of the MS acquisition method in the software, highlighting the differences between the two defined transitions for each compound.

Figure 3. MRM Data Acquisition for Combined Quantitation and Library Matching. This MS method setup in software includes each pesticide compound with two MRM transitions with different acquisition parameters. Example components for this type of workflow are shown, and the columns utilized to set up the method. From left to right, these are: Compound ID, Group Name, Precursor Ion, TOF Start Mass, TOF Stop Mass, Accumulation Time, Declustering Potential, Collision Energy, Collision Energy Spread, and Retention Time. “Apply Scan Schedule” is checked, so that data acquisition of each compound occurs only around its known RT. The first MRM transition of each compound includes a narrow TOF range for product ion collection, an optimized CE, and CES of 0. The second MRM transition includes a generic CE of 35 and a CES of 15 to generate a robust MSMS spectrum.

Quantitation with TOFMS and MRM

Matrix interferences are an obstacle and confidence in identification of residues is paramount. The increased specificity of monitoring an MRM transition is one approach which can be utilized to reduce matrix background, baseline, or interferences which may be observed in the TOFMS data trace. However, the signal intensity and peak quality of the transition relies on the efficient formation of the monitored fragment ion. Reduction in signal during precursor transmission and fragmentation results in a lower absolute intensity observed when monitoring an MRM transition versus extracted TOFMS ions. Despite this, reduced baseline can still provide greater perceived sensitivity due to drastically reduced baseline and subsequently increased signal to noise ratio. In the presented MRM acquisition method, both scans happen simultaneously in a single injection, and processing can utilize either or both, thus reducing or eliminating the need for multiple confirmatory injections or re-injections.

Method Performance:

Table 2 shows some example method performance data for a subset of pesticides, comparing quantitation achieved using extracted TOFMS data and MRM transitions. In general, the sensitivity achieved for most pesticides in the iD Quant Kit mixture was <0.1 ng/mL in neat solvent and most analytes also exhibited >3.5 orders of linear dynamic range.

Table 2. Method performance measurements for a small set of analytes using TOF MS data for quantitation. XIC width around theoretical mass of 0.02 Da was used.

Analyte	~LLOQ, ng/mL (S/N > 10)	CV % LLOQ	CV % 10x LOQ	Cal Range (ng/mL)	Dynamic Range(log[ULOQ/ LLOQ])
Quinoxyfen	0.05	6%	5%	0.05 – 500	4
Carboxim	0.01	22%	11%	0.01 – 100	4
Isoproturon	0.05	2%	4%	0.05 – 500	4
Tebuconazole	0.1	13%	1%	0.1 - 500	3.7

Method performance measurements for a same subset of analytes, shown for quantitation using MRM data.

Analyte	~LLOQ, ng/mL (S/N > 10)	CV % LLOQ	CV % 10x LOQ	Cal Range (ng/mL)	Dynamic Range (log[ULOQ/ LLOQ])
Quinoxyfen	0.05	9%	3%	0.05 – 500	4
Carboxim	0.01	22%	1%	0.01 – 100	4
Isoproturon	0.05	6%	9%	0.05 – 500	4
Tebuconazole	0.1	7%	12%	0.1 - 500	3.7

When comparing the method performance of extracted TOFMS ions to MRM transitions in a complex matrix such as a plant extract, three scenarios represent the most commonly observed behavior. Identifying which compounds in a panel exhibit which of these three behaviors can help in assessing which type of scan is best used for optimal quantitation method performance. The three potential observed behaviors are:

1. Despite a higher absolute signal in TOFMS data, the MRM data provides a reduced baseline, increased signal to noise, and results in greater observed sensitivity in matrix.
2. TOFMS data is drastically more sensitive than MRM data. Poor fragmentation is a potential reason for this, and the result is that the greater signal for TOFMS peak provides improved sensitivity and method performance over the MRM peak.
3. Isobaric matrix peaks which elute close to or overlapping with the target analyte make peak integration in the TOFMS trace challenging and impact the accuracy and reproducibility of the quantitation; the MRM trace, however, does not show the interferences and therefore has improved sensitivity and quantitative method performance.

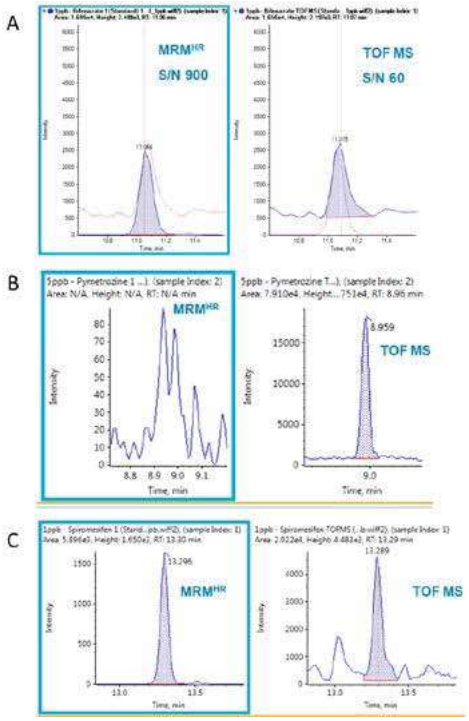


Figure 4. Example behavior of different analytes comparing MRM data and TOFMS data. A.) Bifenezate example. The baseline is greatly reduced in the MRM data compared to TOFMS, leading to a higher signal to noise ratio observed for the MRM peak. B.) Pymetrozine example. No signal is observed in the MRM trace at all at the 5ppb concentration level, however, for this same concentration a distinct TOFMS peak can be observed. For this analyte, TOFMS signal is vastly improved over MRM. C.) Spiromesifen example. Interfering peaks and background in the TOFMS data make integration challenging; these a greatly reduced and both baseline and integration improvement can be seen in the MRM data for this analyte in this matrix.

In an analyte panel which can be very diverse (such as a pesticide suite) and a matrix or matrices which can be very complex and have high concentrations of endogenous background species, there is potential for these differing behaviors to be observed not only between analytes (for example, some analytes do not provide sensitive fragments) but also between different types of matrix (i.e., not all matrices will produce the same interfering peaks at the same masses). It may be important, then, to consider assessing quantitative method performance of both TOFMS data and MRMHR data until a better understanding of the behaviors in the desired panels/matrices is attained. Table 3 breaks down some of the pesticides in the iD quant kit mixture by which of these behaviors

each of them demonstrates in the QuEChERS arugula extract. A subset of these examples can also be seen in Figure 4.

Ion Ratios:
Many triple quadrupole- based MRM quantitative workflows include the reporting of signal ratios between multiple MRM transitions. To do so, however, requires the collection of a secondary MRM transition during data acquisition, adds to the number of transitions in the method and which, without stringent method optimization, can impact method parameters such as cycle time, data points collected across a peak, and ultimately sensitivity and reproducibility. Utilizing the described data acquisition approach of monitoring two MRM channels per compound, there are multiple ways in which ion ratios can be derived and reported to gain further confirmation in analyte detection and identification. Multiple MRM traces can be generated without having multiple specific transitions defined during acquisition, because the full- scan product ion range in the second monitored MRM channel allows for extraction of any fragment or fragment within that range. Additionally, the extracted TOFMS peak, when grouped together with an MRM transition, can also produce ion ratio values which can be reported (Figure 5).

Table 3. Comparing MRM data to extracted accurate mass from the TOFMS data reveals differences in the optimum type of monitoring for each analyte. For some compounds, MRM is an improvement over TOFMS due to reduction of interferences or lowered baseline. These behaviors might also be expected to differ when observed in a variety of matrices.

MRM Baseline reduction from TOFMS	Much greater sensitivity for TOFMS	Shows interferences in TOFMS but not in MRM
Bifenezate	Acibenzolar-S-methyl	Bupirimate
Amitraz	Alanycarb	Diclobutrazol
Benfuracarb	Ametryn	Dimoxystrobin
Bitertanol	Dioxacarb	Fenbuconazole
Carbetamide	Ethiofencarb	Flusilazole
Cycluron	Fenoxycarb	Ipconazole
Fenarimol	Fenpropimorph	Prometryn
Fenuron	Hydramethylnon	Spiromesifen
Fluometuron	Imazalil	Terbutryn
Iprovalicarb	Indoxacarb	
Isoprocarb	Mandipropamid	
Metalaxyl	Omethoate	
Methamidophos	Oxadixyl	
Methiocarb	Phenmedipham	
Methoxyfenozide	Prometon	
Metribuzin	Propham	
Nitenpyram	Pymetrozine	
Propamocarb	Pyrimethanil	
Propargite	Spiridiclofen	
Pyracarbolid	Sulfentrazone	
Tebufenozide	Tebufenpyrad	
	Terbumeton	
	Thiofanox	
	Triadimefon	

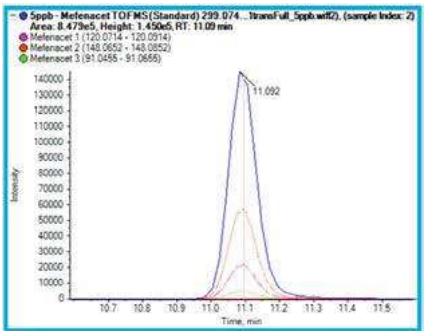


Figure 5. Ion ratios. Ion ratios for compound identity confirmation can be generated for multiple MRM transitions. In this example for Mefenacet, the TOFMS peak is overlaid with the MRM peaks extracted for three transitions, the first of which comes from the optimized acquisition channel and the last two extracted from the full- scan acquisition channel.

Library Searching and Confirmation of Compound ID from MRM Acquisition

Collection of full MSMS spectrum allows for spectral library searching and matching, without performing a separate sample acquisition. Use of the Collision Energy Spread (CES) ensures that the collected MSMS spectrum includes an enriched range of fragment masses collected over multiple collision energy values, which can be searched against a compound library or database for more dependable spectral matching. Data processing methods were built in the software which incorporated both the integration and quantitation parameters for the primary MRMHR transitions, but also dictated that MSMS library searching be performed on the processed data. The results table displays, for review, the chromatographic peak for quantitation; the TOF MS mass spectrum and isotopic distribution; and the MSMS product ion spectrum mirrored with the matching database spectrum for confirmation (Figure 1).
Identification of these pesticides in unknown samples were achieved with high confidence by leveraging HRAM analysis to provide multiple points of matching using accurate mass of the precursor ion, MRM transition monitoring (including accurate mass of the fragment ion), isotope pattern matching, ion ratio, and chromatographic retention time (Figure 7). This extremely high degree of confidence in analyte identification provides failsafe against reporting false positive hits, by ensuring that multiple points of independent confirmation are satisfied.

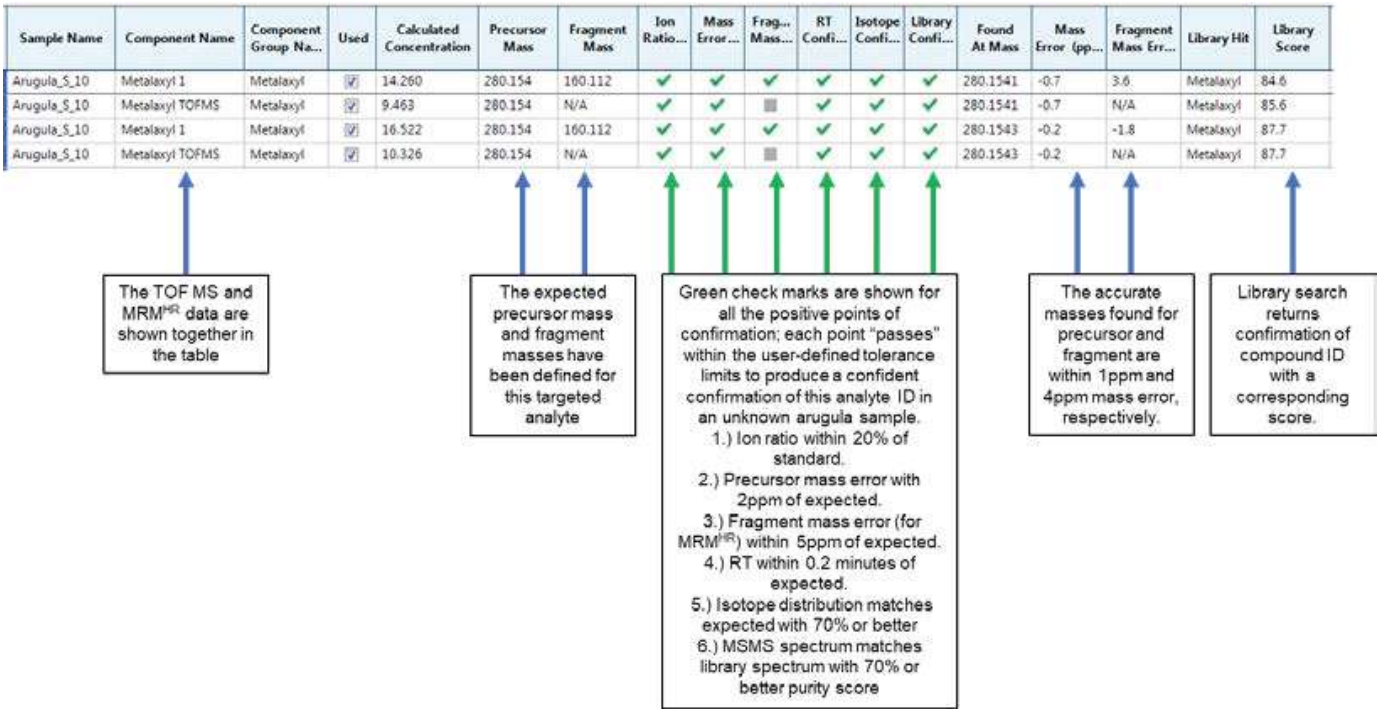


Figure 7. Target Identification Points of Confirmation: Some example rows from OS results table are shown. Identification and quantitation of pesticides in unknown samples can be achieved with high confidence by utilizing the breadth of information available for processing from MRM full scan acquisition.

LCMSMS System with SWATH Acquisition for Pesticide Residue Screening in Fruits and Vegetables

Introduction

It is widely accepted that modern agriculture has a long history of excessive chemical fertilizer, pesticide, and herbicide use, and this has not only resulted in reduced nutrient content in food, but also in a variety of chemical residues that have harmed human health. It has been confirmed that pesticide residues may interfere with the body's endocrine effects on the immune system and hematopoietic system, and can even cause in-vivo fetal visceral hypoplasia or deformity in pregnant women. Weighed against the benefits of consuming more fruits and vegetables, people have recently become more heavily concerned about the widespread existence of pesticide residues and the excessive damage they can cause to the human body.

With the continued development and popularization of liquid chromatography / mass spectrometry, more and more pesticide residue detection technologies are being developed based on the LC-MS/MS system. The most commonly used pesticide residue screening method includes the MRM-IDA-EPI system, which is based on QTRAP system and high resolution TOF-IDA-MSMS technology. While the SWATH technology is based on high-resolution systems, it also combines the advantages of IDA and MRM by dividing the mass range of the parent ion into multiple mass windows and allowing all ions in each window to collide with each other and fragment, resulting in fragmentation information for all ions in the entire mass range. SWATH technology's measurement of second-order fragmentation differs from the IDA, in which only the selected ions are triggered, ensuring the continuity of all ion debris and achieving SWATH's second degree of quantification. By customizing the unique variable window settings, the size of the mass window is automatically adjusted according to the quantity of ions, ensuring the collection of high-quality data.

The ATL High Resolution Mass Spectrometry LCMSMS system provides high resolution, high accuracy, high sensitivity and high linearity range scan speeds, making ATL SWATH technology not only popular for protein macromolecules but also for small molecule pesticide residue screening. The LCMSMS system uses newly designed software to achieve an all-in-one whole process analysis with instrument control, data acquisition and data processing. The software has the built-in SWATH method of setup and powerful automatic deconvolution capabilities. This simple and convenient design meets food safety field use requirements.

Experimental considerations

1. Collect and process samples of fruits and vegetables, and measure the actual SWATH data
2. Prepare Standard Curve, Test 190 Pesticide Standard SWATH data
3. Screening of Pesticide Residues in Vegetables and Fruits
4. The pesticide residue was quantified at two levels

Sample treatment

- Weigh10g of mashed sample into a clean tube
- Add 10mL of Acetonitrile with 1% acetic acid, votex for 1min
- Add 1.5g of NaAC, 6g of MgSO4, votex, then Centrifuge for 5 mins
- Precipitate 8mL of supernatant with Agela clean package
- Centrifuge for 5 mins, transfer supernatant for analysis

The QuEChERS method was used to pretreat received samples: 1 leek, 2 cauliflower, 3 bean, 4 jujube (after washing), 5 jujube (not cleaned), 6 pear.

Chromatographic Methods

Chromatography column: Phenomenex Kinetex C18, 100*2.1 mm, 2.6μm
Mobile phase: A: Contains 5mM ammonium acetate in water; B: Contains 5mM ammonium acetate in methanol gradient elution
Flow rate: 0.4mL/min
Column temperature: 40°C
Input volume: 10μL

Time (min)	B%
0	3
1	3
2	45
19	95
22	95
22.1	5
25	5

Mass Spectrometry Method

Scanning method: SWATH Acquisition methods
Ion source: ESI+source CDS automatic calibration

Table 1: Ion source parameters

IS Voltage: 5500V	Air curtain gas CUR: 35psi
Atomizing gas GS1: 55 psi	Auxiliary gas GS2: 55 psi
Source Temperature TEM: 550°C	Collision Gas CAD: 7
Collision energy CE ± CES: 35 ±15V	Air curtain gas CUR: 35psi

Data acquisition and SWATH setup process

IDA (Information Dependent Acquisition) uses TOF/MS Survey Scan to pre-scan. When a peak ion is successfully triggered and detected, the scan mode is switched to Q1 and the parent ion is selected to acquire a high sensitivity MS/MS secondary spectrum of the target ion. SWATH distributes all the ions into successive windows, and all the ions in each window are transferred to the collision chamber and broken into secondorder MS/MS debris and then traced back to the parent ion through the software's powerful de-convolution function. Thus all of the second-order fragments of all abundant ions can be obtained through this technique, which ensures that the secondary information of the low-content target is included, allowing the trace residue screening to become more complete and accurate.

Unique intelligent variable windows, according to the distribution of ions in the sample, set narrow windows in the high density distribution areas and set up wider windows in the regions with fewer ions to ensure high-quality secondary mass spectra are collected for all ions

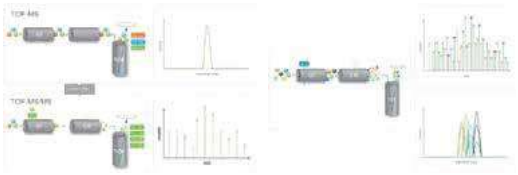


Figure 1 Left IDA schema;
Right SWATH schema

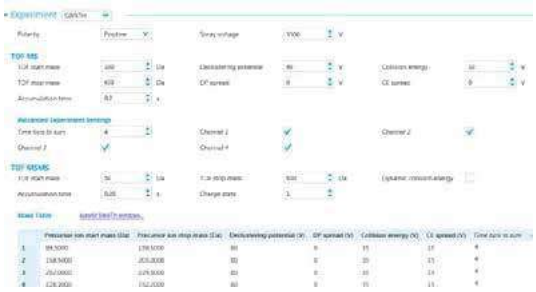


Figure 2 SWATH method settings

In the Software, choose “Experiment” and then pick the SWATH Acquisition mode. The software then automatically lists the required parameters for the SWATH mode. Mass Table is for the Q1window.

The Variable Window Calculator can be based on TOF/MS‘s parent ion to automatically calculate the SWATH smart variable window. The mode can be established by copying and pasting to the Mass Table, which is a method that is simple, rapid, and easy to use.

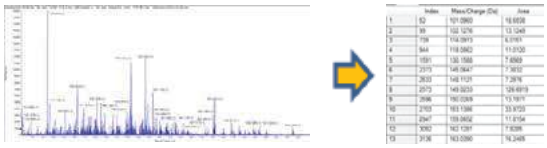


Figure 3 TOF/MS’s parent ion

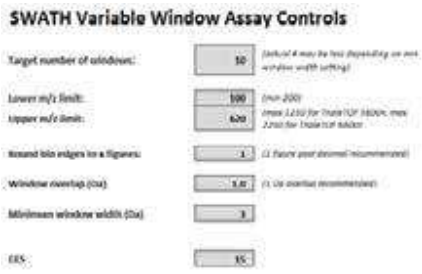
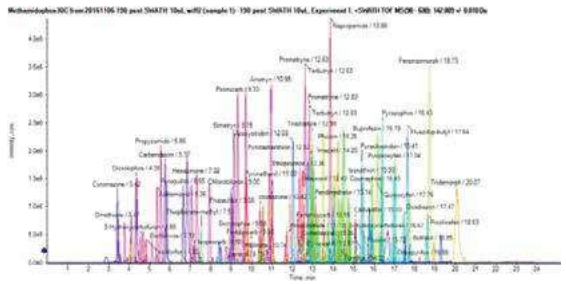


Figure 4 Variable
Window Calculator

99.5	149.1	15
148.1	170	15
169	203	15
202	221.6	15
220.6	236.4	15
235.4	252.2	15
251.2	273.8	15
272.8	293.6	15
292.6	306.6	15
305.6	318.8	15
317.8	331.3	15
330.3	345.6	15
344.6	364.4	15
363.4	413.8	15
412.8	610.5	15

Q1 Window

Establish SWATH
Acquisition method and initial test of 190 varieties of standard pesticide products.



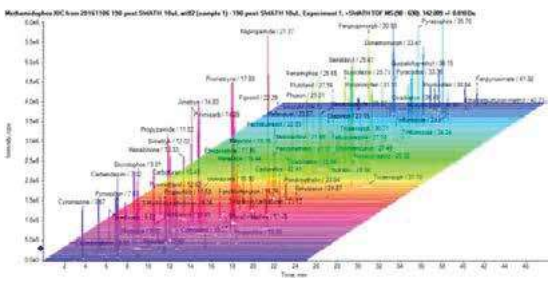
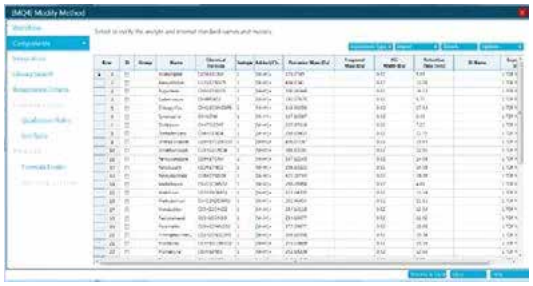


Figure 5 Chromatogram of 190 varieties of pesticide residue standard products collected by SWATH.

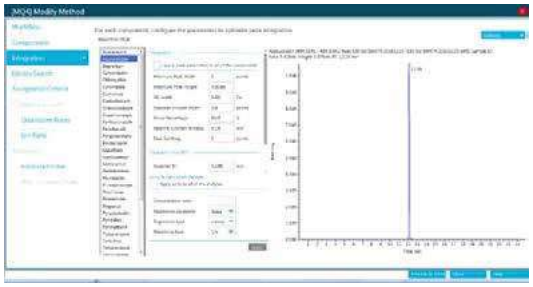
Data Analysis

1. Qualitative screening

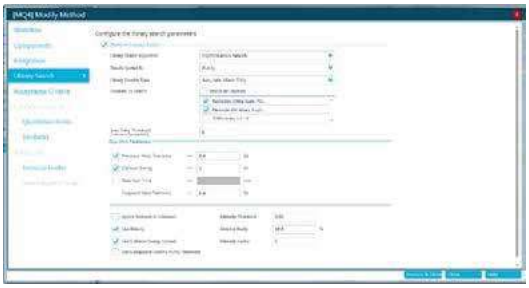
Test SWATH data of 1 (leek), 2(cauliflower), 3 (kidney beans), 4 (jujube, washed), 5 (winter jujube, unwashed), and 6 (pear). Use Software to perform data analysis by passing four confidence conditions: mass accuracy, retention time, isotope distribution and secondary library matching to screen pesticide residues in the 6 samples.



1. Select the standard product data to establish screening methods; import the screening list



2. Set the quantitative integration parameters



3. Set the library search criteria

4. Set the screening confidence conditions

5. One time import of all standard product and samples' SWATH data to perform screening

6. Filter results through the Mass error, RT, Isotope, Library

7. Obtain he results of screening for each sample

2. SWATH second degree quantification

Using TOF/MS's first degree quantitative data in complex matrix samples has disadvantages such as high baseline noise and a narrow linear range, etc. The Software in the LCMSMS system can be used to directly copy and paste the ion pairs of compounds when a quantitative method is established, obtaining the results of the second quantification by using the MRM method to process SWATH data.
Preparation of 190 kinds of pesticide on the standard curve1ng/mL~100ng/mL established the second degree quantitative SWATH method to obtain second degree quantitative linear relationships, see Fig. 6.

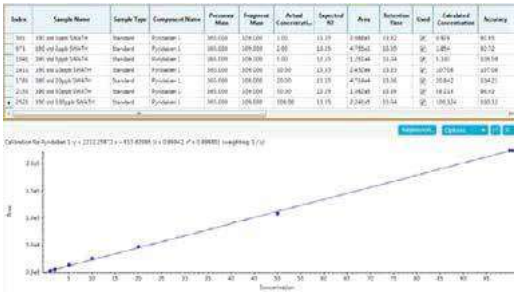


Figure 6 Quantitative linearity using pyridaben as an example

For use of the high sensitivity and high selectivity SWATH second degree quantitative method to quantify the pesticide residues contained in leek, cauliflower, kidney bean, winter jujube (washed), winter jujube (unwashed), and pear, please see the table below for the pesticide residues contained in the above samples.

Detected pesticides (unit: ng / mL)

	Leek	Cauliflower	Kidney Bean	Winter Jujube (washed)	Winter Jujube (unwashed)	Pear
Carbendazim	8.7		1		1.4	
Insecticide	4.5					
Methylpyrimidine	3.5					
Prometryn	33					
Pyrimethanil	23		270			
Thiophanate-methyl	2.1					
Imidacloprid		1.2			580	3.4
Propoxur		50	13.7			
Tebuconazole		1.1	4.5			3.4
Acetamiprid			3.7		5.1	86
Kresoxim			8.2		22	
Streptozotocin			35	15	74	
Buprofezin				18	160	
Fenpyroximate					61	
Paclobutrazol					140	
Triadimefon					23	

A Robust and Sensitive Method for the Direct Analysis of Polar Pesticides in Food and Environmental Samples Without Derivatization

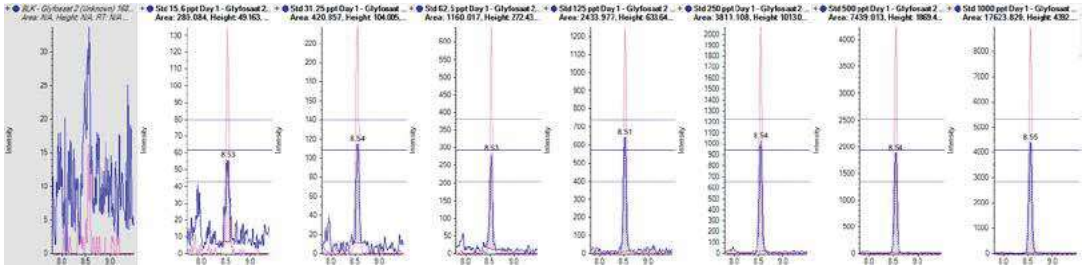
The Challenge of Polar Pesticides

The prevalence of multi-residue LC-MS/MS analyses for the quantification of pesticides in food and environmental samples has been steadily increasing for many years, and they are now considered to be a minimum requirement of most laboratories working in these fields. Modern tandem quadrupoles are capable of detecting such regulated compounds at very low levels with minimal sample preparation, such as QuEChERS, thereby enabling labs to process large numbers of samples for many analytes with a fast turnaround. However, some very polar compounds which are not amenable to the extraction procedure, chromatographic method or are poor ionizers require additional single-residue methods which involve time-consuming preparation and separation and often involve derivatization to improve detection.

Key Advantages Presented

• All analytes were well retained, allowing detection of the majority of background components which could otherwise interfere. Separation between the analytes was also sufficient to allow unambiguous identification, and retention times were reproducible. Sensitivity in spiked environmental waters was found to be similar to that in standards, and the target limit of detection of 20 ng/L was easily achieved with real drinking water samples.

• Matrix effects were largely eliminated in both the NofaLab method for food sample extracts and the modified method for direct injection of water samples. Use of QTRAP is expected to confirm positive results by their full-scan MS/MS spectra, but future work will investigate different or additional clean-up.



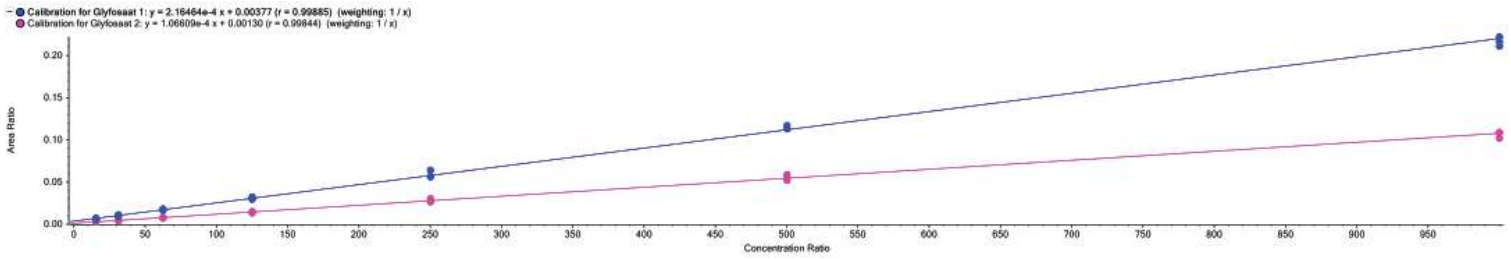


Figure 1. Method sensitivity and linearity of glyphosate. Calibration standards in concentrations from 15.6 to 1000 ng/L of glyphosate achieved using the modified method for water samples. Ion ratios were all well within the specified ±20% tolerance.

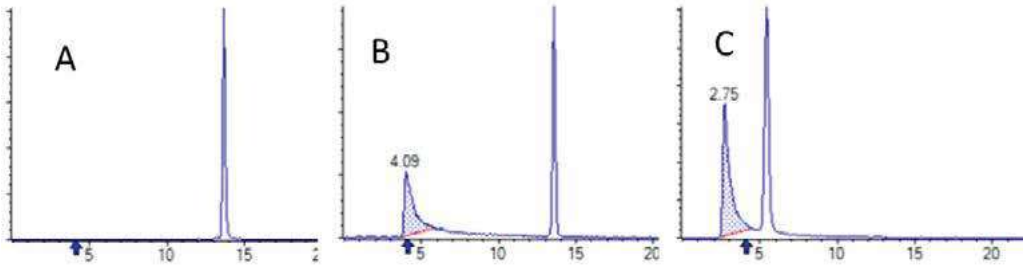


Figure 2. Use of a preferred column means: Install, Prime, Repeat, and finally Replace. Image A shows the performance of the preferred column after installation, no glyphosate peak is present. Image B shows the same column after it has been conditioned with 30 spinach extracts, a glyphosate peak can be detected at 4.09 mins. Image C shows retention time (RT) drift of the glyphosate peak on the same column after 100 injections.

Growing Concerns

Recent increase in public concern regarding the presence of glyphosate has significantly increased the requirement to analyse it and its metabolites in food, feed and the environment, so has accelerated the need for a more efficient and robust analytical method. The extraction and chromatography of these compounds is well described in the EURL-QUPPE method, but the separation is not robust in practice, so system and method maintenance are intensive. Several different HPLC or HILIC based methods have failed to address the issues of reproducibility and sensitivity, so FMOC derivatization prior to analysis is often still employed for glyphosate, AMPA and glufosinate. Although possible to automate, this procedure is still time consuming or expensive, and is not applicable to the other polar pesticides of interest.

Creating a High Throughput Method

NofaLab is an independent sampling and testing laboratory based near Rotterdam, Netherlands, specializing in the fields of food, feed and environmental safety. The increasing pressure to provide fast, quantitative analysis has driven NofaLab to add to their portfolio of LC-MS/MS instrumentation and develop a new method which covers as many of these polar pesticides in a single analysis as possible. Ion chromatography has been shown to be beneficial for separation, but the need for a suppressor is detrimental to MS analysis and the inefficiencies of changing inlet systems on a heavily used mass spectrometer makes it impractical in a busy lab performing primarily reverse-phase LC.

So, the final method, presented here, makes use of an LC column in a method-switching reverse phase (RP) system with MS amenable mobile phases at around pH 9. Such conditions configure glyphosate ideally for MS detection with good retention and separation of the other analytes and matrix interferences. The method meets the DG-SANTE [1] requirements of reproducibility (<20%) and recovery (80-110%), and the LOD of the method is below 0.01 mg/kg. Excellent long-term stability and robustness were achieved throughout the validation of this method for food samples extracted by the QUPPE procedure.

Where environmental samples require testing, the regulatory limits are much lower5 and interference from matrix more problematic in traditional analyses with a short retention time, so derivatization is often the only option. However, since glyphosate is well retained in this new method, the potential to further develop it for direct large-volume injection was investigated in collaboration with ATL. By modifying the gradient conditions and optimizing the injection parameters, a second method specific to environmental water samples has been developed. Although the large volume injection (LVI) is more susceptible to changes in pH (for example, due to evaporation of mobile phase) robustness has been shown to be similarly good, and allows detection of the same suite of analytes with a LOD of <0.02 ng/l.

Experimental Considerations

Food samples

The QuPPe method for extraction of polar pesticides from samples of plant and animal origin developed by Anastassiades et al. at CVUA Stuttgart[2] are well described and have undergone several revisions. Since the analytes are water soluble, it is based on aqueous extraction with addition of methanol and formic acid to improve efficiency.

The addition of internal standards is essential to compensate for the shifting retention times in most chromatographic method and helps to counter matrix effects where present. This was particularly important for grain and seed samples, where chromatographic performance deteriorates, and the MS source becomes dirty, losing sensitivity quickly, so dispersive C18 cleanup as described in the QuPPe-AO3 method was attempted before finalizing on a push-through method with two sorbents using SPE filters.

Various chromatographic methods have been investigated and found to have several limitations. Figure 2 illustrates the common practice of extensive conditioning prior to analysis, which after relatively few (typically 30-50) sample injections in order to maintain peak shape and retention time Ion chromatographic methods showed most promise, but the eluents' incompatibility with electrospray ionization sources requires the use of a suppressor, which is detrimental to peak width. However, by employing a polyvinyl alcohol based column with quaternary ammonium groups and using an ammonium bicarbonate buffer prior to detection by a very sensitive quadrupole mass spectrometer, the need for a suppressor is removed.

Lists of Validated Commodities	
A	Fruit and Vegetables
B	Seeds
C	Vegetable oil, Fat and Fatty Acids
D	Grain
E	Herbs and spices
F	Meat and Seafood
G	Animal Oil, Fat and Fatty Acids
H	Eggs and Eggs products
I	Milk and Milk products
V	Fatty acids

Water samples

Environmental and drinking water samples varied widely in the degree of comprised particulate matter, which causes difficulties for LC injection and is detrimental to reproducibility. However, minimal sample preparation is desirable in a high throughput laboratory situation and SPE type clean-up would add significant time and financial cost. In order to overcome these challenges, a simple filtration step using Chromacol 17-SF-02 (RC) from 17 mm syringe filters was performed when transferring samples to the LC vials. Internal standards to a final concentration of 1ppb were added to samples and standards, and QC samples in tap water were prepared in a similar fashion. Experiments were also performed using standard addition to the samples to investigate any potential matrix effects.

Separation was achieved using a Shimadzu Nexera UHPLC system comprising LC-30AD pumps, a SIL-30AC autosampler fitted with a 500µL loop and a CTO-20A column oven. An injection volume of 500µL was employed in a chromatographic method similar to that used for the food samples. During verification of the method, the primary focus was on achieving stable peak shapes and retention times for all analytes. Loop size (irrespective of injection volume), initial conditions, gradient and pH of the mobile phase had very significant effects, so the final optimized method should be fixed, and fresh mobile phases prepared regularly.

Method verification was performed with real drinking water samples, testing for both AMPA and Glyphosate, a LOQ of 20ng/L could be reached.

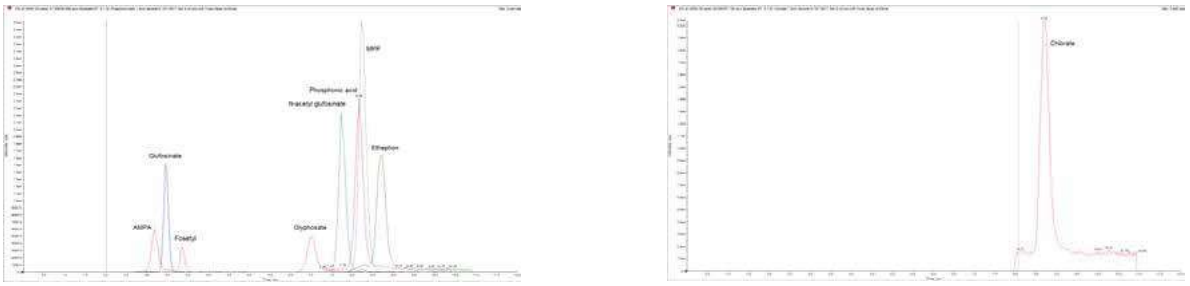


Figure 3. Example chromatograms shown for polar pesticides suite. Chromatographic separation using the hypercarb column was an integral component of the described method.

Table 3. List of analytes with MRM transitions employed. Internal standards are crucial to this method and must be used.

Analyte	Q1 m/z	Q3 m/z
Glyphosate 1	167.9	150.0
Glyphosate 2	167.9	78.8
Glyphosate 3	167.9	62.8
Ethephon 1	142.9	106.8
Ethephon 2	142.9	79.0
N-ac Glufosinate 1	222.0	136.0
N-ac Glufosinate 2	222.0	62.8
N-ac Glufosinate 3	222.0	59.1
AMPA 1	110.0	81.2
AMPA 2	110.0	79.1
AMPA 3	110.0	62.9
Glufosinate 1	180.0	136.0
Glufosinate 2	180.0	95.0
Glufosinate 3	180.0	85.0
Glufosinate 4	180.0	63.1
3-MPPA 1	151.0	132.9
3-MPPA 2	151.0	107.0
3-MPPA 3	151.0	63.1
Phosphonic Acid 1	81.0	62.9
Phosphonic Acid 2	81.0	79.0

Results and Discussion

Food samples

Chromatographic performance using both the NofaLab method for QuPPe extracts of food samples and the modified method for water samples achieved good separation between the analytes and from matrix interferences, and excellent repeatability in terms of peak profile and retention time. The EU maximum residue limits for these compounds in food samples range from 10 to 2000 µg/kg, depending on the commodity and compound[4], so the target for each is variable. Although water regulations are under discussion, a detection limit of 20 ng/L for environmental samples is desirable in anticipation of future regulation. Some analyte/matrix combinations proved to be particularly difficult, but these target concentrations were easily achieved for all samples in the verification of the methods. Over 1000 food samples from a variety of commodities were analyzed at NofaLab without maintenance of the system, and the stability in terms of retention time, peak width, peak area and tailing factor was found to be excellent. Figure 1 shows several measures of reproducibility based on the glyphosate internal standard.

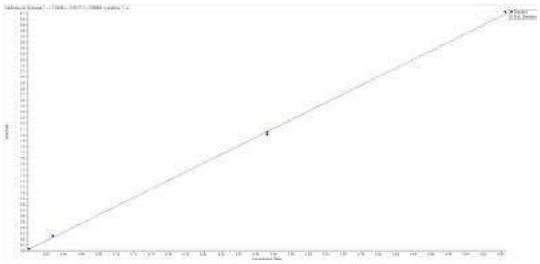


Figure 4. Glyphosate calibration standards. Linear calibration regression for glyphosate with 1/x weighting, showing r-value of 0.9997 and excellent precision for duplicate calibrators.

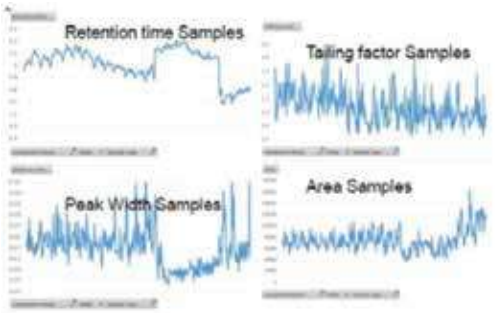


Figure 5. Reproducibility data for glyphosate IS. NofaLab method for food samples, tested over 1000 injections of extracts from fruit/veg, seeds, veg oil/fat, grains, herbs/spices, meat/fish, animal oil/fat, eggs/egg products, milk/milk products and other fatty acids.

Table 4. Summary of Limits of Detection achieved in various food matrices using the NofaLab method. Shown along with their EU Maximum Residue Limits[1].

Product	Glufosinate sum			Fosetyl sum			Glyphosate			Chlorate			Ethephon		
	LOD	MRL	%RSD at MRL	LOD	MRL	%RSD at MRL	LOD	MRL	%RSD at MRL	LOD	MRL	%RSD at MRL	LOD	MRL	%RSD
Fruit and Vegetables	16	30	11%	25	2000	13%	5	100	15%	8	10	15%	18	50	at MRL
Seeds	12	30	12%	90	2000	15%	8	100	15%	3	10	10%	6	50	11%
Vegetable oil, Fat and Fatty Acids	15	30	19%	40	2000	12%	7	100	22%	2	10	6%	3	50	14%
Grain	18	30	12%	71	2000	14%	8	100	7%	7	10	14%	9	50	7%
Herbs and spices	25	100	8%	87	2000	13%	23	100	6%	8	10	15%	8	100	6%
Meat and Seafood	19	30	15%	23	100	12%	9	50	23%	4	10	8%	4	50	16%
Animal Oil, Fat and Fatty Acids	14	30	20%	51	100	11%	9	50	25%	10	10	16%	7	50	10%
Eggs and Eggs products	18	30	12%	33	100	11%	4	50	13%	12	10	9%	6	50	12%
Milk and Milk products	17	30	9%	20	100	6%	8	50	22%	5	10	12%	5	50	17%
Fatty acids	21	100	14%	70	1000	14%	3	100	18%	4	10	9%	3	100	13%

Water samples

To achieve the target sensitivity for environmental water samples, it was necessary to inject increase the amount of sample, so trials with increasing injection volume and different loop sizes were carried out. With each incremental change, the composition of eluent in the loop was altered, thereby changing initial conditions of the analysis and the retention times and peak shapes of the analytes. To compensate, modification of the stating composition of the mobile phase was required, but when final parameters had been fully developed, the method was found to be as stable and robust as the NofaLab method for food samples. All analytes were well retained, allowing detection after the majority of background components which could otherwise interfere had eluted. Separation between the analytes was also sufficient to allow unambiguous identification, and retention times were reproducible. Sensitivity in spiked environmental waters was found to be similar to that in standards, and the target limit of detection of 20 ng/L was easily achieved with real drinking water samples. In order to verify the results, analyses with standard addition of the target compounds were also performed.

Matrix effects were largely eliminated in both the NofaLab method for food sample extracts and the modified method for direct injection of water samples. However, MRM ion ratios were found to be outside of the normal ±20% tolerance in some very complex sample matrices. Use of the will be advantageous to confirm positive results by their full-scan MS/MS spectra, but future work will investigate different or additional clean-up of samples in order to remove background interferences.

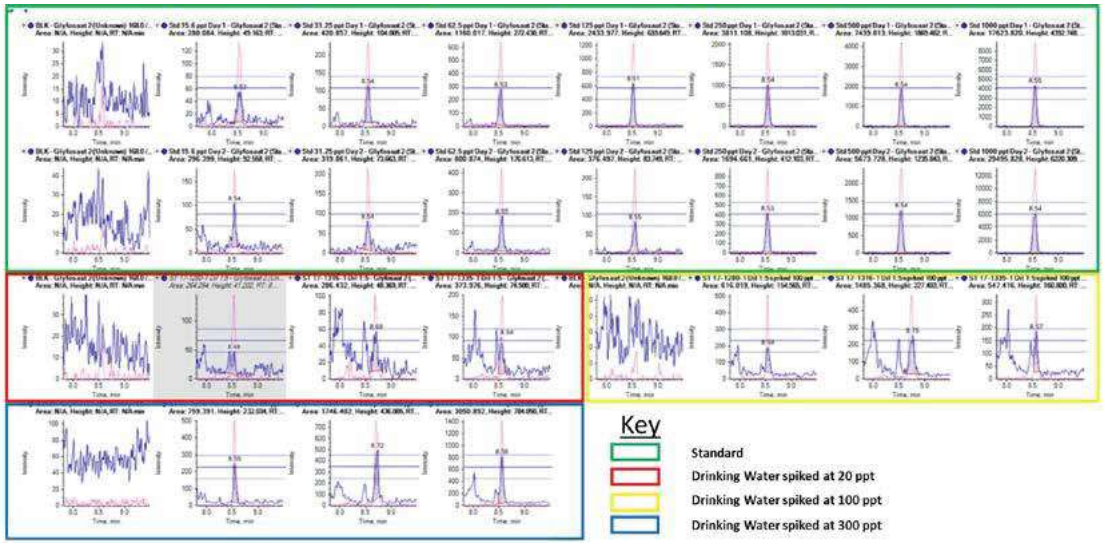


Figure 6. Example chromatography from drinking water samples using the modified water method.

Quantitation of Oregon List of Pesticides and Cannabinoids in Cannabis Matrices by LC-MS/MS

Overview

Increased legalization of cannabis for medical and recreational use substantiates the need for a standardized robust and reproducible method for quantitation of pesticide residues and relevant psychotropic cannabinoids in cannabis products. A fully verified method is presented using two different ATL triple quadrupole platforms for the analysis of those pesticides comprising the Oregon Pesticide List. The QET Food Application presents a cost-effective platform for achieving the majority of the Oregon List Maximum Residual Limits (MRL) in cannabis flower matrix.

The highly sensitive Triple Quad/ + is capable of meeting the MRLs for the full list in cannabis flower matrix. Cannabis flower demonstrated the most severe matrix-induced ion suppression on our target analytes and, therefore, the performance of this method in flower represents performance in the most difficult matrix. The ATL vMethod utilizes dilution with six pesticide deuterated internal standards and two cannabinoid internal standards in its sample preparation method to maximize recoveries for the most analytes as well as to correct for analyte recovery efficiency. A 16 minute gradient maximizes separation of endogenous isobaric interferences for pesticide analysis. A five-minute gradient separates all isobaric cannabinoids from each other and ensures precision of quantitative analysis.

Introduction

Pesticide application in agricultural industries is intended to protect crop yield from pests or pathogens. Insecticides, acaricides, fungicides or other protective chemical reagents on crops pose potential health risks both to field employees via exposure as well as consumers through consumption. Pesticides and pesticide action levels may be regulated differently by state.

Currently, the most comprehensive list of pesticides and their respective MRLs allowed in plant products is known as the Oregon List of Pesticides.

Several pesticides on the Oregon List have been historically monitored by GC-MS including complicated sample preparation with derivatization and relatively long sample run times. The ATL vMethod Application for Quantitation of Pesticide
Residues in Cannabis Matrices 1.0 presents a simplified sample preparation protocol complete with analysis of all 59 compounds using electrospray ionization (ESI) and LC-MS/MS. Additionally, the method can be used to analyze ten cannabinoids with the same sample extract using a 5 minute acquisition method that utilizes our triple quadrupole's fast polarity switching to monitor targets in both negative and positive polarities.

Experimental

Standards and Internal Standards (IS)
Pesticide standards were purchased from RESTEK (Bellefonte, PA). The complete list of pesticides monitored can be found in the ATL vMethod Application for Quantitation of Pesticide
Residues in Cannabis Matrices 1.0. Deuterated internal standards were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).
Cannabinoid standards and deuterated internal standard were purchases from Cerilliant (Round Rock, TX). The complete list of cannabinoids monitored can be found in ATL vMethod Application for Quantitation of Pesticide Residues in Cannabis
Matrices 1.
Acetonitirile, methanol, water, formic acid, acetone and ammonium formate were purchased from Sigma-Aldrich (St.Louis, MO).

Sample preparation

Calibrators and quality controls were made in acetonitrile and then diluted with 75:25 (v/v) methanol:water.

Unknown cannabis matrices were analyzed using 0.2 gram of cannabis flower or 0.02 gram of cannabis concentrates diluted in 5mL of acetonitrile which was sonicated, vortexed and centrifuged.

The extract was then diluted in 1:6 (v/v) using 75:25 (v/v) methanol and water.

LC-MS/MS injection volumes are 20µL for a Food Application system and 25µL for a + system. The maximum injection volume for this method is 25 µL in order to maintain symmetrical peak profiles of early eluting Daminozide and Acephate.

The sample extract was also used for cannabinoid potency analysis by further diluting 1:2000 (v/v) serially. The suggested LC-MS/MS injection volumes are 5 µL for a Food Application system and 1 µL for a + system

An outline of the sample preparation procedure is shown inFigure 2A for pesticides and 2B for cannabinoid analysis.

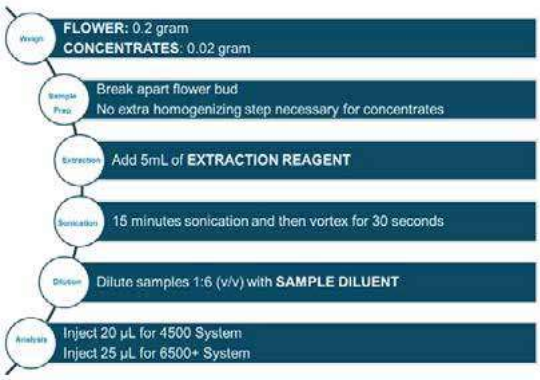


Figure 2A An overview of sample preparation for cannabis flower and concentrates for pesticide residue analysis

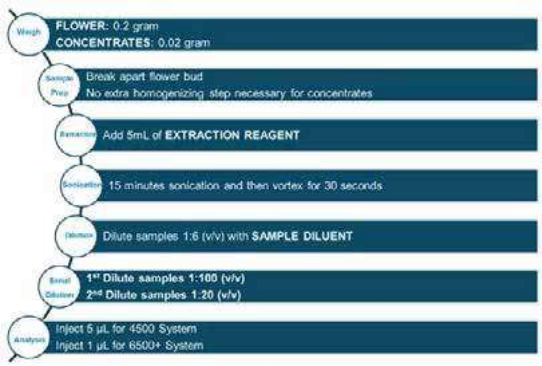


Figure 2B An overview of sample preparation for cannabis flower and concentrates for cannabinoid analysis.

HPLC System

Chromatographic separation was achieved using Shimadzu LC20AD binary pumps or with a ATL LC AC LC system and a Phenomenex Kinetex Biphenyl Column (2.6µm, 4.6 x150mm) at flow rate of 1 mL/min.

The analytical column is heated to 30°C for analysis using the CTO-20AC integrated column oven for pesticide analysis and 35°C for cannabinoid testing respectively. The eluents used for the separation are shown in Table 1 and the gradient profile is shown in Figure 3A for pesticide residue testing and Figure 3B for cannabinoid testing.

LC Reagent	Composition
Mobile Phase A	5mM Ammonium Formate (100:0.1, Water: Formic Acid)
Mobile Phase B	5mM Ammonium Formate (98:2, Methanol: Water)
Autosampler Wash	(70:20:10) (Acetonitirile: Isopropanol: Acetone)

Table 1: LC reagents for LC-MS/MS analysis

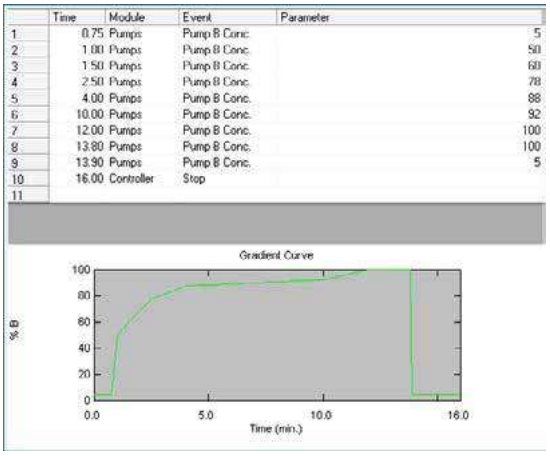


Figure 3A: LC Gradient is detailed using % Mobile Phase B as the parameter

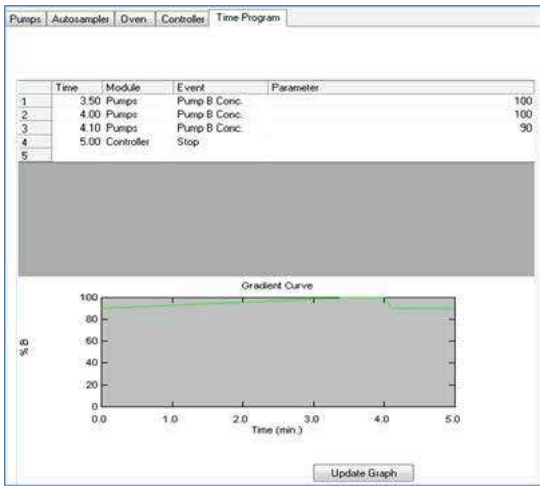


Figure 3B: LC Gradient is detailed using % Mobile Phase B as the parameter

MS/MS Detection

Optimized source parameters for a ATL QET Food Application(Tables 2A and Table 2B) and a +(Tables 3A and 3B) coupled to a TurboV and IonDrive source respectively are detailed below.

Figure 4: ATL TurboV source(left) and ATL IonDrive source(right) have different ion source temperatures that are optimal for each model.

Table 2A. Ion source parameters for QET Food Application - pesticide analysis

Parameter	Value
Curtain Gas (CUR)	35 psi
IonSpray voltage (IS)	3450 V
Temperature (TEM)	500°C
Nebulizer Gas (GS1)	60 psi
Heater Gas (GS2)	60 psi

Table 2B. Ion source parameters for QET Food Application - cannabinoid analysis

Parameter	Value
Curtain Gas (CUR)	35 psi
IonSpray voltage (IS)	3450 V/-3450
Temperature (TEM)	600°C
Nebulizer Gas (GS1)	60 psi
Heater Gas (GS2)	60 psi

Table 3A. Ion source parameters for Triple Quad/ + pesticide analysis

Parameter	Value
Curtain Gas (CUR)	35 psi
IonSpray voltage (IS)	3450 V
Temperature (TEM)	400°C
Nebulizer Gas (GS1)	60 psi
Heater Gas (GS2)	60 psi

Table 3B. Ion source parameters for Triple Quad/ + cannabinoid analysis

Parameter	Value
Curtain Gas (CUR)	35 psi
IonSpray voltage (IS)	3450 V/-3450
Temperature (TEM)	500°C
Nebulizer Gas (GS1)	60 psi
Heater Gas (GS2)	60 psi

Two MRM transitions were monitored for each analyte while one transition was monitored for each of the internal standards. In the pesticide panel, the Scheduled MRM algorithm was activated to monitor compounds during a 60 second expected retention time window to maximize dwell times and optimize the cycle time such that all analytes have at least 12 scans across the baseline of the peak. For a complete list of all target analytes monitored, refer to ATL vMethod Application for Quantitation of Pesticide Residues in Cannabis Matrices 1.0. Due to the variable ionization efficiencies of the different pesticide groups and the commercial standards being at the same concentration, a 9-point calibration curve is coupled with 2 quality controls to ensure accuracy for quantitation analysis. (Table 4A).

Cannabinoid results are reported as % by weight and the calibration level for each standard as well as quality control in solvent are listed in Table 4B below.

STANDARD	Concentration (ppb or ng/mL)
STD 1	0.075
STD 2	0.25
STD 3	1
STD 4	2
STD 5	3
STD 6	5
STD 7	9
STD 8	12.5
STD 9	15
QC 1	0.125
QC 2	7.5

Table 4A. Calibration and quality control scheme for pesticide residue analysis

STANDARD	% by weight
STD 1	0.3
STD 2	1.5
STD 3	6
STD 4	15
STD 5	24
STD 6	30
QC 1	0.75
QC 2	22.5

Table4B. Calibration and quality control scheme for cannabinoid analysis

Quantitation was performed using MultiQuant 3.0.2 using 1.5 Gaussian smoothing and 1/x weighted variable quadratic or linear regression for the QET Food Application. The detector on the + allows for a greater dynamic range compared to the QET Food Application, therefore all calibration curves are analyzed with 1/x weighted linear regression.

Several pesticides containing different isomers were integrated with a peak split factor of 10 and a noise percentage level of 50% in MultiQuant 3.0.2. Examples of this integration are found in Figures 5-7 for Propiconazole, Cyfluthrin, and Cypermethrin.

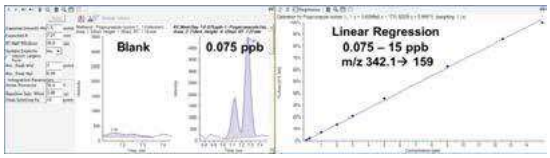


Figure 5: Integration parameters in MultiQuant 3.0.2 for Propiconazole acquired on a + system with a 25µL injection showing multiple isomers.

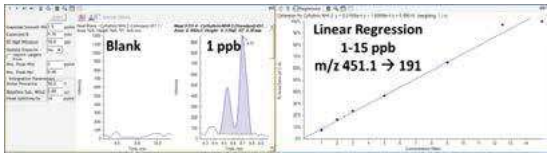


Figure 6: Integration parameters in MultiQuant 3.0.2 for Cyfluthrin acquired on a + system with a 25µL injection showing multiple isomers.

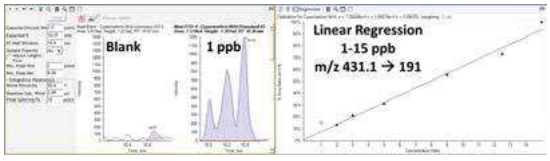


Figure 7: Integration parameters in MultiQuant 3.0.2 for Cypermethrin acquired on a + system with a 25µL injection showing multiple isomers.

Results and Discussion

Chromatography

The biphenyl column chemistry provides retention of early eluting pesticides as well as chromatographic separation of endogenous pyrethrin-like compounds found in cannabis flower. A representative elution profile of target analytes in solvent can be found in Figure 8A for pesticides and Figure 8B for cannabinoids. An example of the isobaric interferences surrounding Pyrethrin, Pyrethrin I and II are detailed in Figure 9 when comparing a solvent standard to standards spiked into flower extract. The ability to chromatographically separate isobaric interferences allows for both easier visual and quantitative analysis of the pyrethrins in an unknown sample.

Carryover analysis was done by analyzing the highest calibrator standard, followed a solvent blank. The absence of any analyte peaks ≥20% of the low calibrator areas demonstrated that the method is free from carryover.

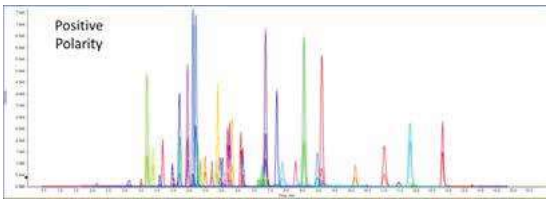


Figure 8A: Elution profile of target analytes in solvent

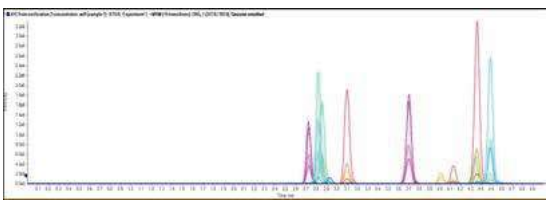


Figure 8B: Elution profile of target cannabinoid analytes in solvent usingthe same mobile phases and analytical column as the pesticide panel

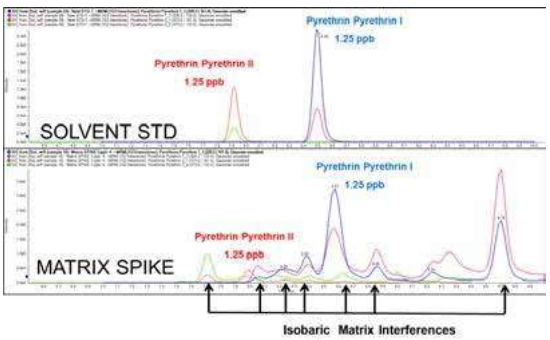


Figure 9: Comparison of standards in solvent compared to Pyrethrin Pyrethrin I and II spiked into cannabis flower extract showinginterferences with similar ion ratios of both quantifier and qualifier MRMtransitions.

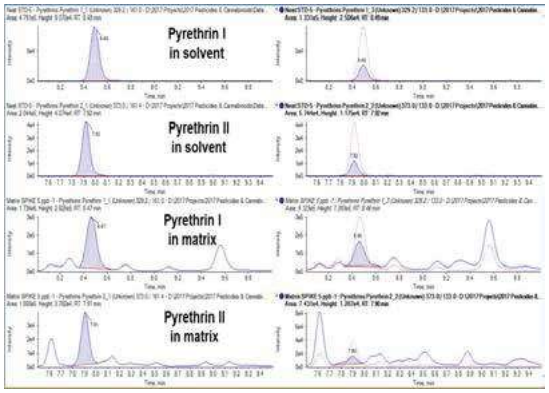


Figure 10: Example chromatograms in MultiQuant 3.0.2 showing chromatographic separation of Pyrethrin Pyrethrin I and II in cannabis flower extract. The chromatograms on the left are the quantifier ions while the chromatograms on the right are the qualifier ions. The qualifier ions also show overlaid quantifier ions for ion ratio analysis (pink trace).

Matrix Recovery

Matrix induced ion suppression was observed in cannabis flower more so than the three concentrates tested (shatter, kief/pollen and hash). To correct for ion suppression, deuterated internal standards were assigned to each pesticide based on a combination of retention time, chemical structure and backcalculated concentrations from solvent calibration curves. A table outlining the recoveries from solvent standards can be found in Table 1 in the Appendix for targeted pesticides. Several pesticides showed recoveries greater than ±20% deviation from the target concentration, potentially because the compound did not have its own deuterated internal standard to correct for suppression or ion enhancement.

Limit of Quantitation Analysis

Solvent LOQs were determined by analyzing 5 solvent spiked replicates over the course of two days. The parameters for determining LOQ was %CV of ≤ 20% and a %Recovery of 80 to 120% of the target spike concentration.

The ATL vMethod for pesticide analysis outlines the concentrations of calibration standards to be used, with the lowest of these at a concentration of 0.075 ppb. The instrument LOQ for the majority of pesticides is lower than this concentration, both in solvent as well as spiked into cannabis flower matrix. A complete table of the LOQ analysis for solvent using the + can be found in Table 1 in the Appendix. The LOQ tables for pesticides in cannabis flower matrix acquired on the + are found in Table 2 in the Appendix.

The cannabinoid analysis in the ATL vMethod has six calibration standards that range from 0.3-30% by plant weight. The %CV of the ten cannabinoids ranged from 6.24-19.09% at the first calibration level (0.3% by weight). The %Recovery of the LOQ standard range from 82-116%

Linear Dynamic Range

The dynamic range was established across five calibration curves acquired through method verification. All curve fittings used a linear regression with 1/x weighting. Calibration points below the LOQ of the method were excluded. Figures 11-15 show examples of dynamic range for some representative pesticide analytes Refer to Figures 16-17 for representative calibration curves of cannabinoid analytes.

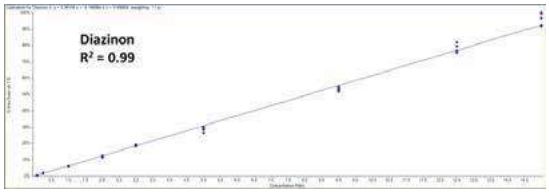


Figure 11: 5 calibration curve replicates for Diazinon from 0.75-15 ppb on a + system.

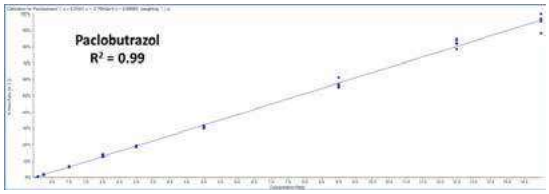


Figure 12: 5 calibration curve replicates for Paclobutrazol from 0.75-15 ppb on a + system.

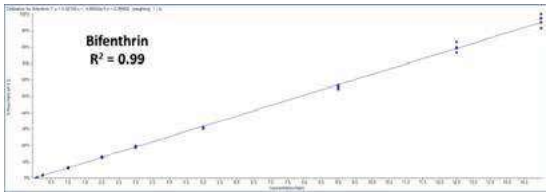


Figure 13: 5 calibration curve replicates for Bifenthrin from 0.75-15 ppb on a + system.

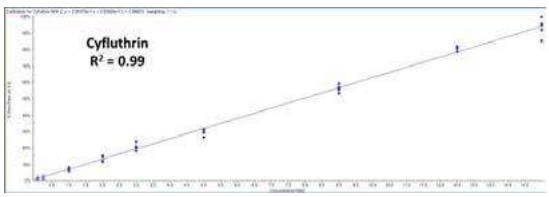


Figure 14: 5 calibration curve replicates for Cyfluthrin from 1-15 ppb on a + system.

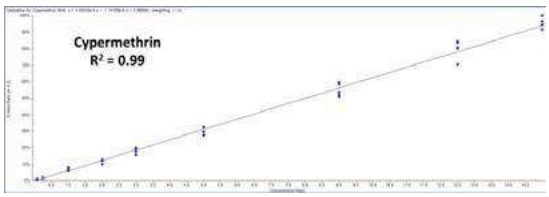


Figure 15: 5 calibration curve replicates for Cypermethrin from 0.75-15 on a + system.

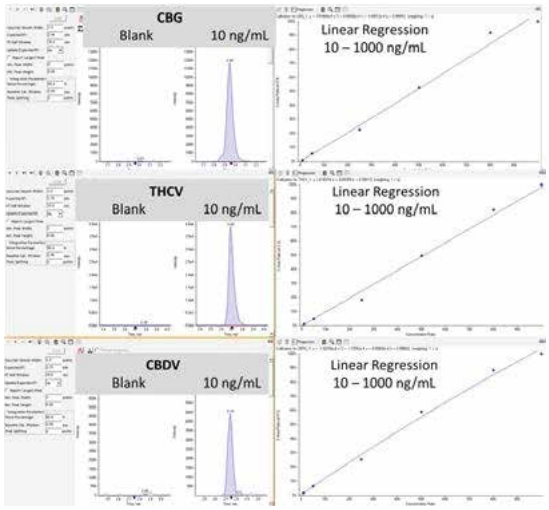


Figure 16: Representative solvent blanks, first LOQ standard and calibration linearity of CBG, THCV and CBDV.

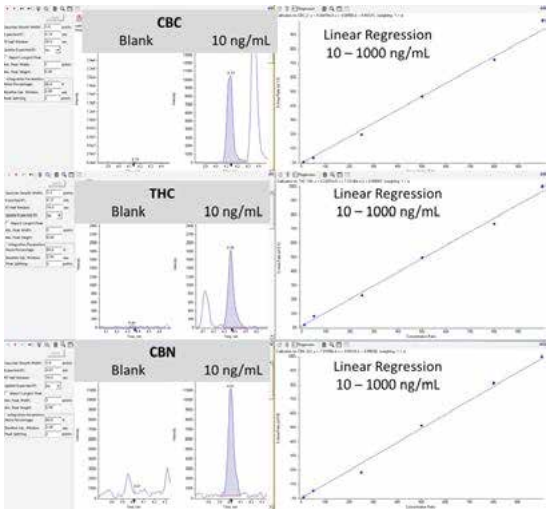


Figure 17: Representative solvent blanks, first LOQ standard and calibration linearity of CBC, THC and CBN. The first calibrator also shows separation of isobaric CBC and THC.

APPENDIX

Appendix Table 1: Solvent LOQ analysis on a Triple Quad/ + System. Pesticides annotated with * is based on the most abundant isomer.

Compound	LOQ (ppb)	%CV	%Recovery
Abamectin*	0.25	6.84%	107.33%
Acephate	0.075	6.63%	98.89%
Acequinocyl	0.25	17.54%	104.00%
Acetamiprid	0.075	2.88%	94.44%
Aldicarb	0.075	6.48%	101.11%
Azoxystrobin	0.075	7.11%	92.22%
Bifenazate	0.075	6.63%	98.89%
Bifenthrin	0.075	2.88%	94.44%
Boscalid	0.075	9.07%	90.00%
Carbaryl	0.075	6.63%	98.89%
Carbofuran	0.075	9.78%	103.33%
Chlorantraniliprole	0.25	7.96%	107.33%
Chlofenapyr	2	16.00%	96.58%
Chlorpyrifos	0.075	8.39%	105.56%
Clofentezine	0.075	8.39%	105.56%
Cyfluthrin	1	13.96%	103.83%
Cypermethrin	1	13.54%	104.83%
Daminozide	3	9.12%	103.73%
Diazinon	0.25	13.54%	120.00%
Dichlorvos	0.075	7.40%	98.67%
Dimethoate	0.075	7.40%	98.67%
Ethoprophos	0.075	7.40%	98.67%
Etofenoprox	0.075	6.21%	96.00%
Etoxazole	0.075	2.83%	97.87%
Fenoxycarb	0.075	1.99%	94.67%
Fenpyroximate	0.075	5.73%	104.00%
Fipronil	0.25	15.96%	109.28%
Flonicamid	0.075	6.43%	97.87%
Fludioxinil	0.25	13.39%	103.36%
Hexythiazox	0.075	3.20%	94.93%
Imazalil	0.075	10.58%	102.93%
Imidacloprid	0.075	6.96%	108.40%
Kresoxim-methyl	0.125	7.25%	106.56%
Malathion	0.075	2.12%	95.47%
Metalaxyl	0.075	2.35%	86.13%
Methiocarb	0.075	6.90%	91.20%
Methomyl	0.075	2.40%	104.00%
MGK 264*	0.075	6.07%	101.07%
Myclobutanil	0.075	18.22%	88.53%
Naled	0.075	10.82%	96.80%
Oxamyl	0.075	2.32%	94.40%
Parathion Methyl	0.075	13.76%	92.27%
Permethrins*	≠	8.47%	83.80%
Phosmet	2	8.87%	92.00%
Piperonyl Butoxide	0.075	11.32%	84.27%

Appendix Table 2:. LOQ analysis spiked into cannabis flower extracts and analyzed against a solvent calibration curve on a +.

*Analytes is based on the most abundant isomer.

≠Analytes have %recoveries that can be improved using their deuterated internal standards.

Compound	LOQ (ppb)	%CV	%Recovery
Abamectin*≠	0.25	13.00%	135.00%
Acephate	0.25	4.00%	119.00%
Acequinocyl	1	7.00%	88.00%
Acetamiprid	0.25	4.00%	96.00%
Aldicarb	0.25	9.00%	105.00%
Azoxystrobin	0.25	5.00%	109.00%
Bifenazate	0.25	5.00%	112.00%
Bifenthrin	0.25	13.00%	116.00%
Boscalid	1	3.00%	110.00%
Carbaryl	1	10.00%	94.00%
Carbofuran	0.25	19.00%	97.00%
Chlorantraniliprole	0.25	13.00%	98.00%
Chlofenapyr	5	22.00%	104.00%
Chlorpyrifos	1	2.00%	108.00%
Clofentezine ≠	0.25	12.00%	75.00%
Cyfluthrin	5	16.51%	100.32%
Cypermethrin	2	10.32%	113.24%
Daminozide ≠	5	4.19%	70.58%
Dichlorvos	0.25	9.00%	86.00%
Diazinon	0.25	11.00%	113.00%
Dimethoate	0.25	3.00%	91.00%
Ethoprophos ≠	1	6.00%	60.00%
Etofenoprox	0.25	2.00%	94.00%
Etoxazole	0.25	1.00%	90.00%
Fenoxycarb≠	0.25	6.42%	129.12%
Fenpyroximate	1	3.00%	91.00%
Fipronil	1	14.00%	90.00%
Flonicamid	0.25	2.00%	90.00%
Fludioxonil	0.25	2.00%	105.20%
Hexythiazox ≠	0.25	4.76%	75.36%
Imazalil	0.25	3.00%	94.00%
Imidacloprid ≠	0.25	6.16%	124.56%
Kreosim-methyl	0.25	18.00%	113.00%
Malathion ≠	0.25	6.66%	73.92%
Metalaxyl	0.25	2.29%	104.88%
Methiocarb	0.25	3.28%	127.52%
Methomyl	0.25	4.46%	116.72%
MGK 264*≠	0.25	10.10%	53.68%
Myclobutanil	0.25	18.00%	112.00%
Naled	0.25	17.89%	111.68%
Oxamyl	0.25	5.67%	88.32%
Paclobutrazol	0.25	5.42%	91.28%
Parathion Methyl≠	1	19.87%	72.36%
Permethrins*	0.25	5.62%	108.96%
Phosmet	0.25	6.43%	86.12%

Improving Identification and Quantification of Polar Herbicides by CESI-MS

Overview

Who Should Read This: Senior Scientists, Lab Directors

Focus: Advantages of CESI-MS for separating, identifying and quantifying the polar herbicides glyphosate and fosetyl aluminum, and their degradation products.

Goals: Develop an effective CESI-MS method for separating, identifying and quantifying polar herbicides and compare the selectivity, accuracy and reproducibility of that method to those of an approved, currently-used LC-MS method.

Problem: Concerns about the safety of glyphosate-based herbicides (GBHs) have made it essential to be able to detect glyphosate in foods (especially fruits and nuts) and distinguish it from other alternative herbicides such as fosetyl aluminum.
Current LC-MS methods have significant limitations, including ion suppression, retention time instability and problems in distinguishing between degradation products of these herbicides (Figure 1). Both glyphosate and fosetyl aluminum are regulated but false positive identification (ID) and inaccurate quantitation of their degradation products, phosphate and phosphonate, is possible using current LC-MS methods. Therefore, a method is needed that provides accurate ID and quantitation of these degradation products.

Results: The developed CESI-MS method demonstrated an excellent ability to distinguish between glyphosate and its degradants, and between similar degradation products of another widely-used herbicide, fosetyl aluminum. It also demonstrated better migration/retention time stability and quantitative linearity than the LC-MS method.

Key Challenges

• Separation of highly polar molecules by LC requires either time-consuming analyte derivatization prior to reverse-phase LC, or reliance on less reliable LC techniques, e.g. HILIC or anion exchange chromatography
• LC-MS methods suffer from a variety of issues, including:
derivatization selectivity, ion suppression due to matrix effects, and retention time instability
• LC-MS methods frequently have difficulty resolving phosphate and phosphonate, the final degradation products of glyphosate and fosetyl aluminum, respectively

Key Features

• Capillary electrophoresis is well suited to the separation of polar herbicides
• The CESI-MS method provided excellent specificity, easily resolving and identifying glyphosate, fosetyl aluminum, and many of their degradation products
• The CESI-MS method demonstrated very good migration time stability over more than 160 runs
• The CESI-MS exhibited excellent quantitative linearity when analyzing phosphonate, the degradation product of fosetyl aluminum, in matrices

Differentiating Glyphosate, Other Herbicides and Their Degradation Products

Glyphosate is a common, broad-spectrum, systemic herbicide widely used to kill weeds that compete with crops. Concerns about the safety of glyphosate have led to increasing restrictions on glyphosate-based herbicides (GBHs), most recently in the European Union. As such, it is increasingly important to develop robust analytical methods with the sensitivity and selectivity to identify and quantify glyphosate and its degradation products in foods and differentiate them from other herbicides such as fosetyl aluminum.

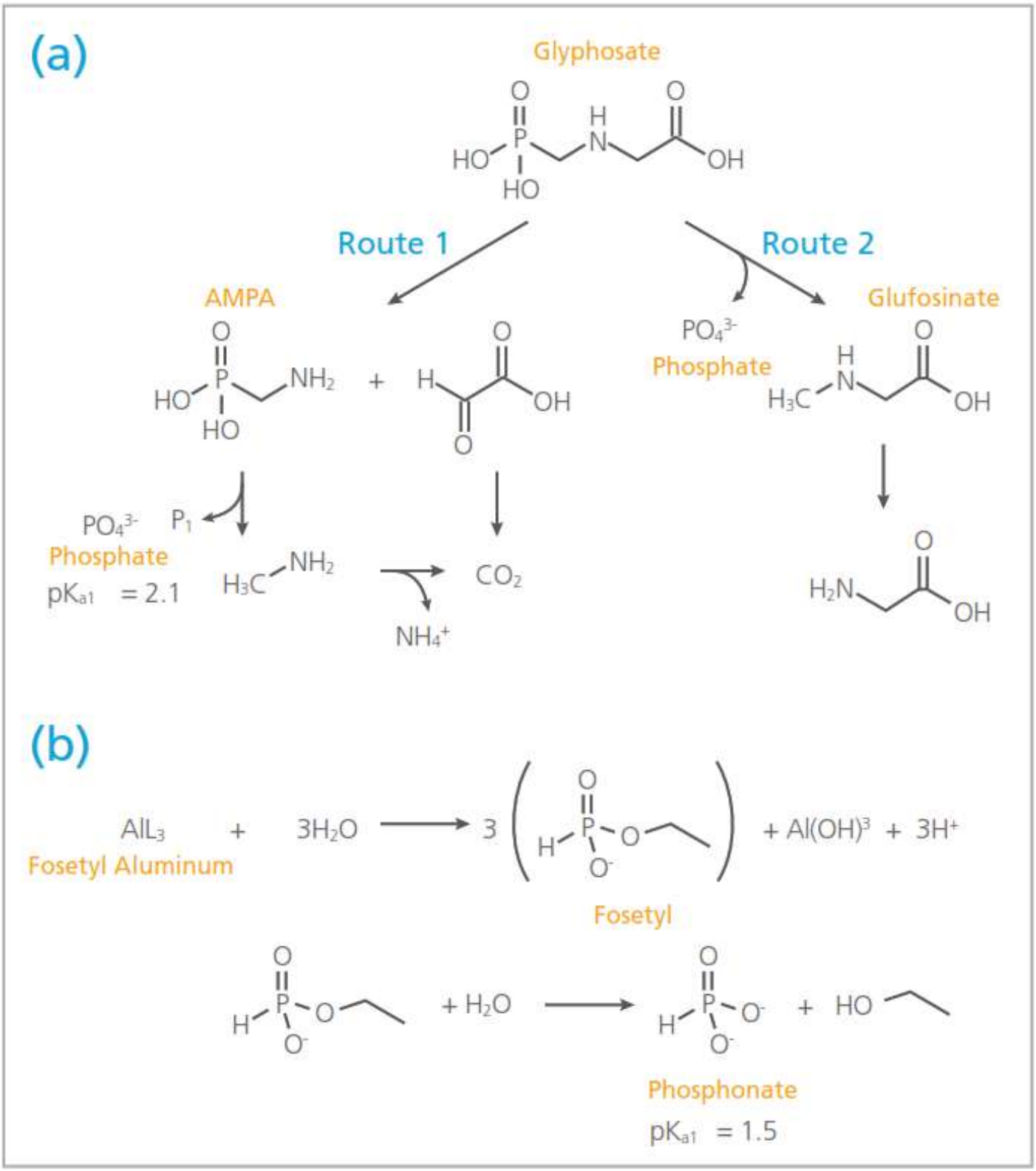


Figure 1: Degradation pathways for (a) glyphosate and (b) fosetyl aluminum.

Limitations of LC-MS Methods

Several LC-MS -based methods are currently used to analyze glyphosate and its degradation products, with many listed in the Quick Polar Pesticides (QuPPe) Method document created by the EU Reference Laboratories for Residues of Pesticides.

The methods use anion-exchange, porous-graphitized carbon, or HILIC liquid chromatography coupled with mass spectrometry. Reverse-phase LC methods have also been used but require analyte derivatization with fluorenylmethyloxycarbonyl chloride (FMOC-Cl) before sample analysis.

While LC-MS methods generally can differentiate glyphosate and fosetyl aluminum (Figure 1), these methods can suffer from derivatization selectivity, matrix effects, ion suppression, and poor retention time reproducibility. Additionally, LC-MS methods are generally not suitable for resolving phosphate and phosphonate, the final degradation products of glyphosate and fosetyl aluminum, especially in real-world matrices.

Advantages of CESI-MS

Capillary electrophoresis (CE) is well-suited to the analysis of polar ions and has already proven useful in the analysis of pesticides.

The mechanism of separation is by differences in pKa and hydrodynamic radii. In the case of phosphate and phosphonate, they differ in pKa by 0.6 units (Figure 1).

Integration of capillary electrophoresis and electrospray ionization (CESI) into a single dynamic process facilitates the mass spectrometric use of CE detection and analysis. The developed CESI-MS method readily differentiated glyphosate, fosetyl aluminum and their degradation products (Figure 2). It demonstrated far better migration/retention time stability than a corresponding LC-MS method (Figure 3). Finally, quantitative CESI-MS/MS analysis of phosphonate in real-world nut extracts showed exceptional linearity while the corresponding anion-exchange LC-MS/MS method exhibited significant ion suppression due to matrix effects (Figure 4).

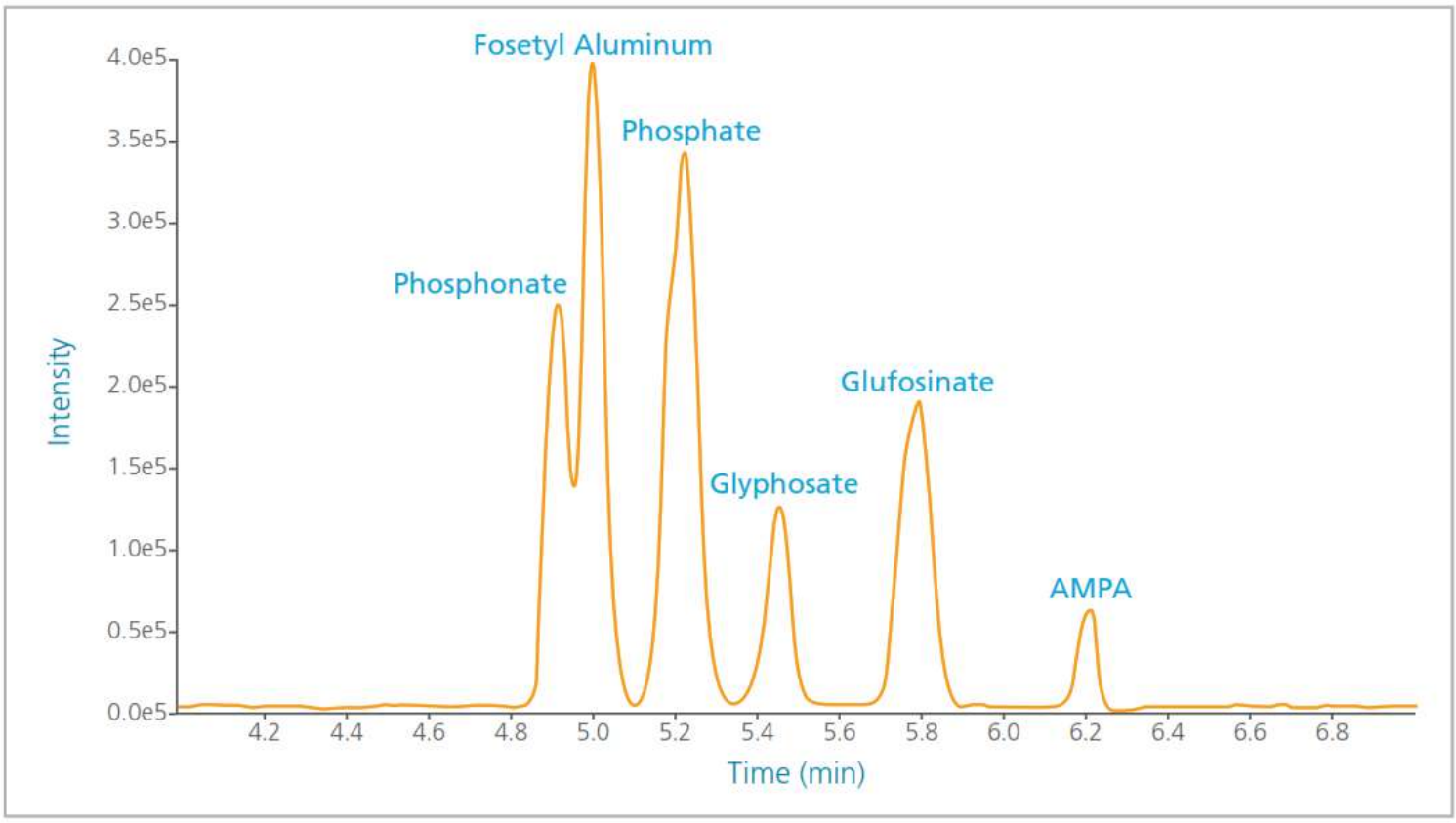


Figure 2: CESI-MS provides clear separation and detection of glyphosate and three of its degradation products: glufosinate, AMPA and phosphate, along with fosetyl aluminum and one of its degradation products, phosphonate.

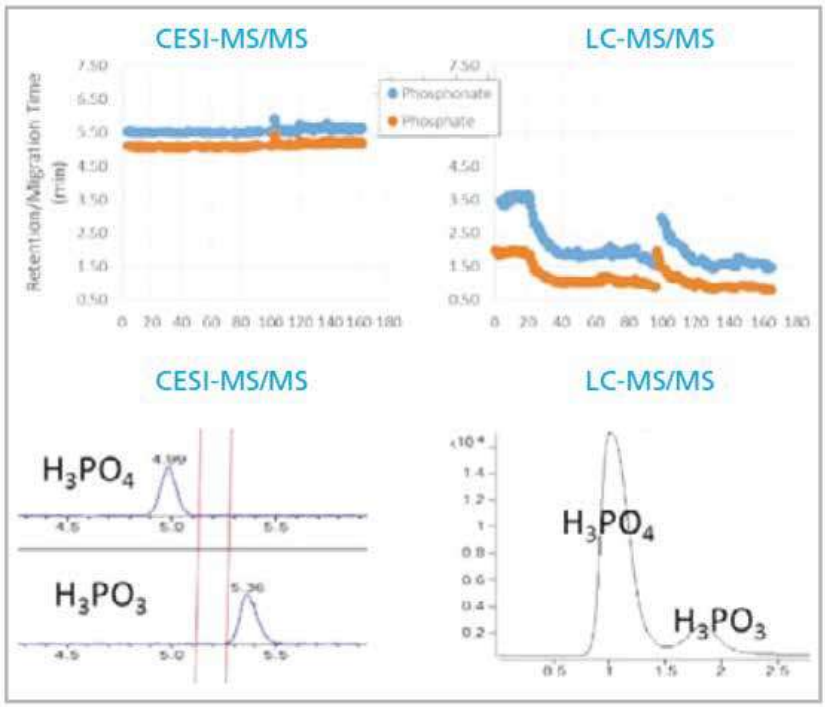


Figure 3: Migration/retention times for phosphonate and phosphate across more than 160 analyses, with sample electropherograms (CESI-MS/MS) and chromatograms (LC-MS/MS). CESI-MS proved far more stable over time and baseline separation was achieved only in the CESI-MS analysis.

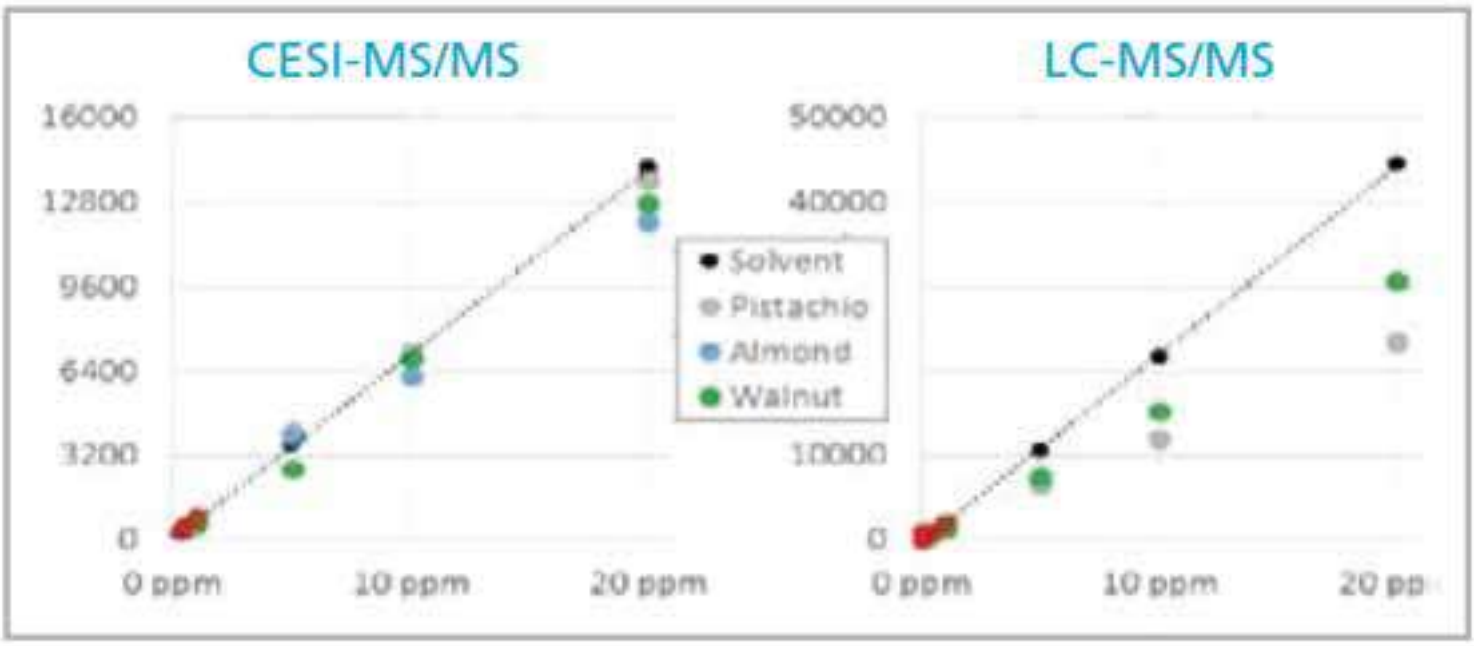


Figure 4: CESI-MS/MS and anion-exchange LC-MS/MS quantification of phosphonate spiked into 0.1% formic acid (solvent) and nut extracts. Significant ion suppression due to matrix effects is clearly visible in the LC-MS/MS results, but not in the CESI-MS/MS results. Fit lines are for the solvent curves only.

Combining Non-Targeted SWATH MS/MSALL Acquisition with Highly Selective MRM for the Analysis of Veterinary Drugs in Tissue Using the ATL LCMSMS System

Overview

A highly flexible, selective and sensitive LC-MS/MS method for the analysis of veterinary drugs in liver extract is presented, using the ATL LCMSMS high resolution mass spectrometer together with the ATL OS software for a combined non-targeted and targeted screening workflow.

Introduction

Veterinary drugs are commonly used in livestock breeding to prevent or treat infections of the animals and to ensure their optimal growth. Legal regulations define waiting periods between the application of active pharmaceutical ingredients and the release of the animals for food manufacturing. Veterinary drugs which still find their way into human nutrition represent a potential risk to human health, e.g. in terms of possible allergenic reactions or reproductive dysfunctions. Furthermore, abuse of antibiotics in animals may also contribute to the development of antimicrobial resistance.

Therefore, European guidelines require to carefully and sensitively control residues of veterinary drugs in animal products [1]. Here we present a versatile and sensitive workflow on the ATL LCMSMS system which combines a nontargeted screening workflow using SWATH data acquisition looped with highly selective MRM acquisition. Confident identification of veterinary drug residues according legal requirements [2] is achieved by accurate precursor and fragment mass measurement and their compound specific ion ratios, as reported in the software.

Materials and Methods

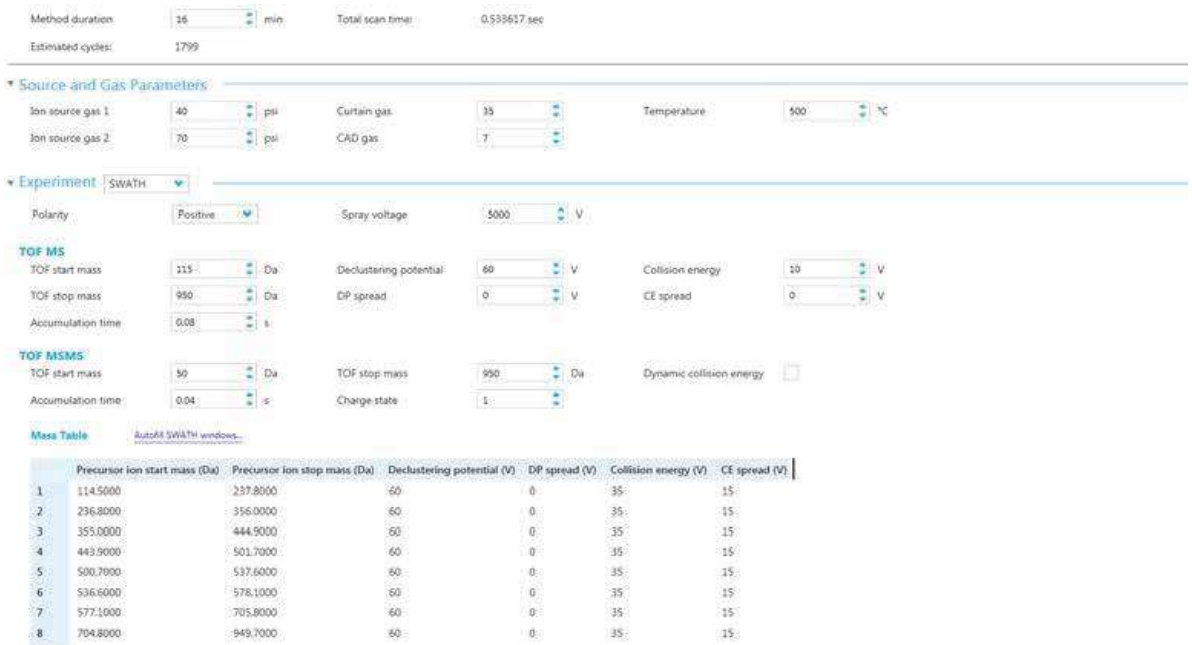
Sample Preparation

Liver tissue was mixed with extraction solution (acetonitrile, water, formic acid) and homogenized. Following centrifugation for 5 minutes, a 5 mL aliquot from the supernatant was concentrated under nitrogen flow. After addition of 2.5 mL of solvent A, the extract was vortexed, centrifuged and filtered prior to injection. Aliquots of the extracts were spiked with a standard solution yielding final concentrations of 0.2, 1, 5, 10, and 50 ng/mL (corresponding to 0.08, 0.4, 2, 4, and 20 µg/kg liver).

LC Method

Veterinary drugs were chromatographically separated on a ATL LC AD UHPLC system, using a Phenomenex Kinetex C18 column (150 x 2.1 mm, 2.6 µm). Mobile phase A was water with 5% acetonitrile and 0.3% formic acid. Mobile phase B was acetonitrile with 5% water and 0.3% formic acid. Chromatographic separation was achieved using the gradient below. Oven temperature was set to 30 °C. Injection volume was 5 µL.

	A [%]	B [%]	Flow [mL/min]
0.0 min	100	0	0.4
2.0 min	100	0	0.4
7.0 min	70	30	0.4
11.0 min	0	100	0.4
11.1 min	0	100	0.8
12.5 min	0	100	0.8
12.6 min	100	0	0.4
14.0 min	100	0	0.4



Experiment MRM HR

TOF MSMS

Mass Table | Apply fragment ion mass | Apply TOF start/stop mass | Apply Scan Schedule | Import and autofill... | Sort by precursor ion

	Compound ID	Group name	Precursor ion (Da)	Fragment ion (Da)	Accumulation time (sec)	Declustering potential (V)	Collision energy (V)	Retention time (min)	Retention time tolerance (+/- sec)
1	Metronidazol_MRM	Metronidazol	172.07	128.0449	0.0500	105	16	2.39	8
2	Sulfamerazin_MRM	Sulfamerazin	265.08	156.0114	0.0500	80	20	5.42	8
3	Danofloxacin_MRM	Danofloxacin	358.16	340.1461	0.0500	145	28	6.28	10
4	Clenbuterol_MRM	Clenbuterol	277.09	203.0141	0.0500	130	20	6.25	8
5	Azithromycin_MRM	Azithromycin	749.52	591.4173	0.0500	140	38	7.07	8
6	Oxolinsäure_MRM	Oxolinsäure	262.07	244.0608	0.0500	100	25	8.16	12
7	Clotrimazol-frag_MRM	Clotrimazol	277.08	165.0689	0.0500	40	24	9.15	8
8	Rifampicin_MRM	Rifampicin	823.41	791.3882	0.0500	45	22	9.70	8
9	Salinomycin NH4_MR...	Salinomycin NH4	768.53	733.4874	0.0500	80	25	12.37	8

Figure 2: MS Method

MS Method

The ATL LCMSMS system was operated in positive mode with electrospray ionization. Data acquisition was performed using TOF-MS mode looped with eight SWATH MS/MS experiments and scheduled MRM acquisition. Variable SWATH Q1 windows were used, calculated with the ATL SWATH Variable Window Calculator. MRMHR experiments were acquired in fragment mode with a TOF scan window of 20 Da. Figure 2 shows the MS method as displayed in SOftware. Data processing was done in Software version 1.3.

Results & Discussion

Quantitative Results

On the ATL LCMSMS system, TOF-MS mode is the standard acquisition mode for quantitation, providing nontargeted data collection which can be subsequently processed in software using a list of targeted compounds. For the 27 analytes of interest, TOF-MS mode provides excellent sensitivity in the standard solution at 1 ng/mL, as shown in figure 3.

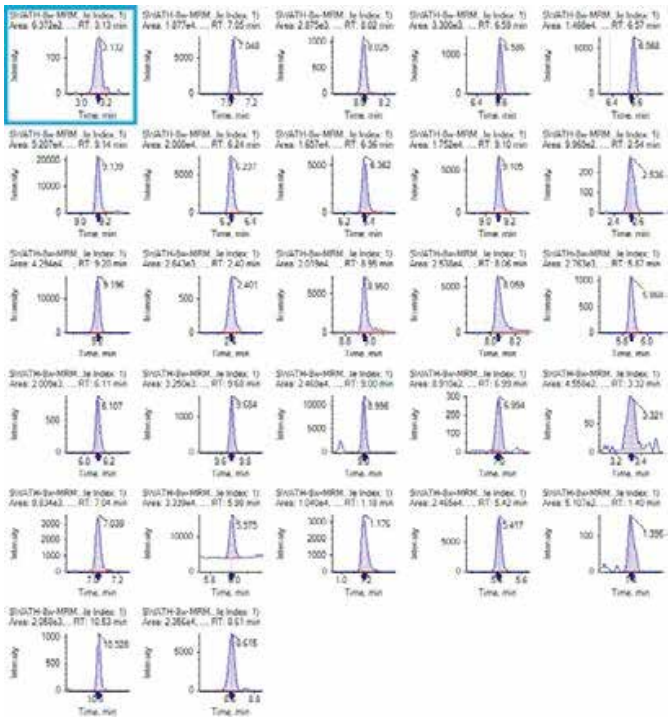


Figure 3: Extracted ion chromatograms of a standard solution of veterinary drugs at 1 ng/mL. 1 Amoxicillin. 2 Azithromycin. 3 Ceftiofur. 4 Chlortetracycline. 5 Clenbuterol. 6 Clotrimazole. 7 Danofloxacin. 8 Enrofloxacin. 9 Flumequine. 10 HMMNI. 11 Josamycin. 12 Metronidazole. 13 Nalidixic acid. 14 Oxolinic acid. 15 Oxytetracycline. 16 Penicillin G. 17 Rifampicin. 18 Roxythromycin. 19 Spiramycin. 20 Sulfacetamide. 21 Sulfachlorpyridazine. 22 Sulfadimidine. 23 Sulfagunidine. 24 Sulfamerazine. 25 Sulfanilamide. 26 Triclabendazolesulfone. 27 Tylosin A.

However, in very complex matrices such as liver extracts, interferences may hamper the sensitive detection of certain analytes. For example, the signal for azithromycin in matrix spiked at 0.2 ng/mL shows a shoulder from a matrix interference which is not chromatographically resolved, and which makes an accurate integration and thus quantitation difficult (left panel in figure 4). In such a case, quantitation can be alternatively performed using the comprehensive MS/MS traces from SWATH acquisition, a unique – as low matrix interfered –MS/MSALL technology. Using the MRM-like higher selectivity of SWATH fragments, the interference observed in the TOF-MS trace can be removed (middle panel in figure 4). If even higher selectivity and sensitivity is needed, true MRM provides even better signal-to-noise ratios (right panel in figure 4). The increaseof signal-to-noise performance is due to the fact that MRM uses compound specific collision energy, CE, and declustering potential, DP, voltages, while SWATH is a generic method. Furthermore, transmission of the precursor ion as well as the fragment ion on their way through the mass spectrometer is optimized. Finally, the high selectivity in MRM decreases the noise in the chromatogram to its minimum.

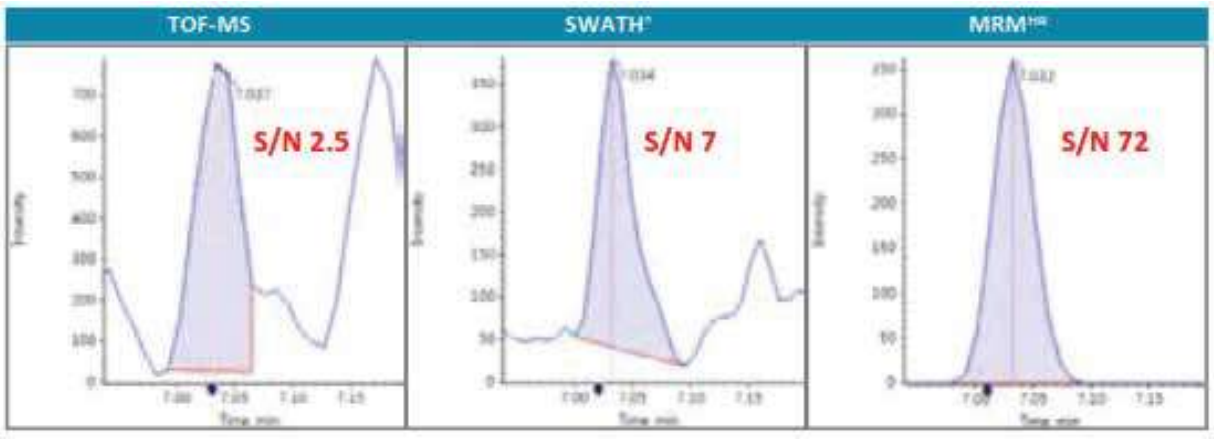


Figure 4: Extracted ion chromatograms of azithromycin spiked at 0.2 ng/mL in liver extract from different acquisition experiments. Left panel: TOF-MS (m/z 749.5158). Middle panel: SWATH -MS/MS (749.52 > 591.4215). Right panel: MRMHR (749.52>591.4215).

Qualitative Results

Software displays several parameters allowing the confident identification of a detected signal, meeting the European Union criteria of identification points [2]. First, it calculates mass errors of the precursor ion as well as of the fragment ions. Second, the ion ratio measured in unknown samples is compared to the one calculated from standards. Both the mass error and the ion ratio confidences are clearly displayed with a traffic light system, using a green checkmark for signals which meet the identification criteria. This allows the user to easily review large data sets and filter for positively detected compounds (figure 5).

Typically, the ion ratio can be calculated from the area of the precursor ion and the area of one fragment. Alternatively, if the TOF-MS trace is disturbed by interferences, two MS/MS fragments can be used. MS/MS fragments can be taken either from the SWATH experiment or, if higher selectivity is needed, from a looped MRM experiment.

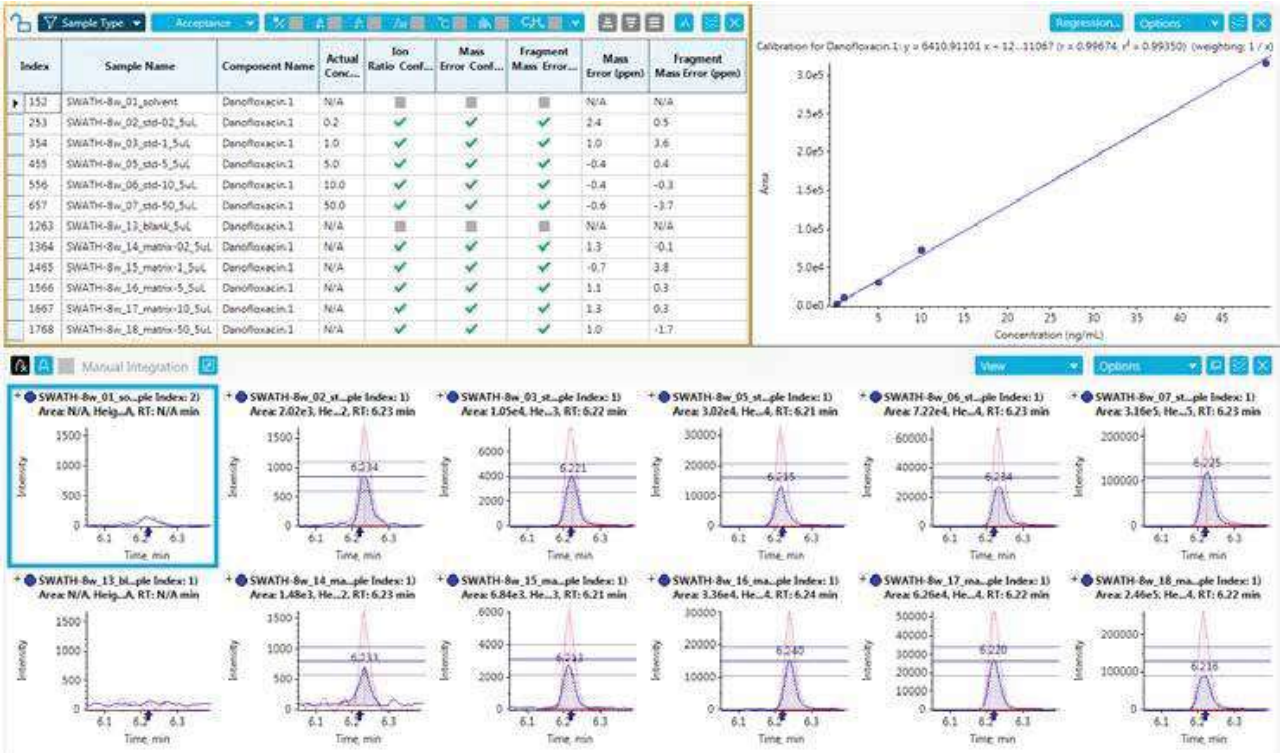


Figure 5: Quantitative and qualitative results for Danofloxacin as shown in Software. Upper left panel: Results table with confidence display for ion ratio and mass errors of precursor and fragment. Upper right panel: Calibration curve. Lower Panel: Extracted ion chromatograms of standard solutions and matrix samples. Quantifier (TOF-MS) is displayed in pink. Qualifier (MS/MS fragment from SWATH) is displayed in blue. Expected ion ratio is shown as blue solid line, tolerances (±30%) as dotted line.

Conclusion

The ATL LCMSMS system is a powerful instrument for the sensitive analysis of veterinary drugs in complex matrices, with a unique combination of versatile acquisition modes for different requirements:

1) TOF-MS data as standard trace used for quantitation.

2) Concurrent acquisition of untargeted SWATH MS/MS data, used for identification with the help of accurate fragment masses and compound specific ion ratios as required by official guidelines. Furthermore, SWATH MS/MS fragment can be used for quantitation, if the TOF-MS trace shows interferences.

3) Concurrent acquisition of targeted MRM data increased selectivity for analytes which show interferences both in TOF-MS and SWATH MS/MS mode.

Simultaneous analysis of 12 food allergens in baked and raw food products using the LC-MS/MS Food Application system

Introduction

A food allergy is an immune-mediated, adverse reaction to an antigenic protein. Even limited exposure to an antigen can provoke a significant reaction in sensitive individuals, causing rashes, itching and swelling in the mouth, nausea, vomiting, and asthma. Additionally, food allergies are the leading cause of anaphylaxis, an acute, potentially deadly allergic reaction. The prevalence and severity of food allergies are rising, with approximately 150 million people suffering from food allergies worldwide.

Presently, there is no cure for food allergies, and sufferers must rely on the correct labeling of foods to avoid consuming allergens. Hence, the development of sensitive and accurate analytical methods to screen for the presence of allergens in food products is necessary for the prevention of potentially life-threatening health problems for allergy sufferers.

Enzyme-linked immunosorbent assays (ELISA) are the most commonly used tests for screening allergens. Although relatively quick and simple to perform, ELISA tests are limited in selectivity and susceptible to cross-reactivity, which can lead to false positive or false negative results. Additionally, most ELISA tests are capable of detecting only one allergen at a time, requiring multiple tests to screen for more than one allergen in a food sample. Therefore, a method that can unambiguously confirm and identify multiple allergens would be invaluable for food screening.

Herein, we present an LC-MS/MS method using the Food Application LC-MS/MS system that detects and screens 12 separate allergenic proteins simultaneously in a single injection. The allergens detected in this method were selected from the guidelines presented in the Codex Alimentarius, a resource developed by the United Nations' Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to

The Codex recommends eight allergenic food groups be declared on the labels of pre-packaged foods: grains, shellfish, eggs, fish, legumes, milk, sulfite, tree nuts.
Five of these allergens are detected with this method including eggs, milk, peanuts, soy beans, and tree nuts (almonds, Brazil nuts, cashew nuts, hazelnuts, pecans, pine nuts, pistachios, and walnuts).
To evaluate a range of food products (both raw and bakery goods) for their allergenic content, several unique signature peptides specific to each allergen were identified from tryptic digests of food homogenate extracts. A mixture of 12 allergens was added to bakery product food matrices (either bread or cookie) over a range of known concentrations, and several MRM transitions corresponding to allergenic signature peptides were evaluated simultaneously using the Scheduled MRM algorithm. Presently, this method can detect allergenic peptides from five of the major classes of allergenic foods at a detection limit of 10 ppm in a variety of food matrices.

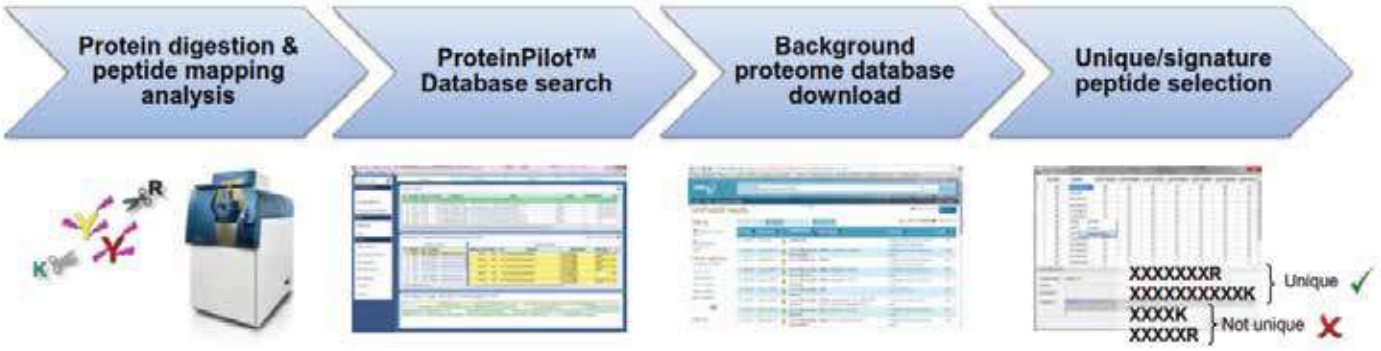


Figure 1. Signature peptide selection workflow using the ATL TripleTOF 6600 system and ProteinPilot software

Experimental

Sample Preparation

To prepare bread and cookie homogenates, unbaked gluten-free bread or cookie mixes (100 g) were supplemented with 10 to 500 ppm (by weight) of each of the following 12 allergenic foods: eggs, milk, peanuts, soy, almonds, Brazil nuts, cashew nuts, hazelnuts, pecans, pine nuts, pistachios, and walnuts. The fortified foods were then cooked according to manufacturer's specifications. The food samples (raw nuts, baked goods) were finely homogenized using a coffee grinder. Each homogenate (1 g) was defatted by extracting twice with hexane and dried by evaporation in the fume hood. Extraction buffer (4 mL) was added to the defatted homogenates, which were then centrifuged prior to the removal of supernatants (500 µL). Reducing reagent (50 µL) was added to supernatants at 60°C for 1 hr. After cooling (25°C), samples were alkylated using a cysteine blocking reagent(25 µL). Trypsin (20 µg) was added to modified proteins (3 to 12 h) in calcium chloride/ammonium bicarbonate buffer to obtain tryptic peptides for signature peptide analysis prior to neutralization with formic acid (30 µL). Digested samples

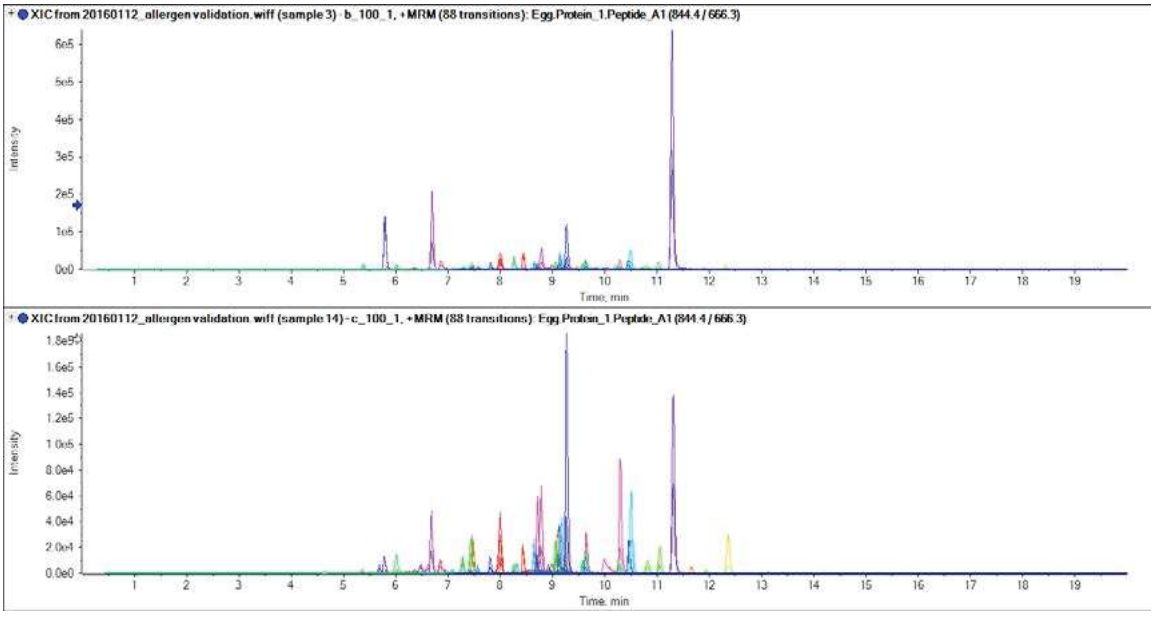


Figure 2. Extracted ion chromatograms (XIC) from LC-MS/MS analysis of bread (top) and cookie (bottom) homogenates fortified with egg, milk, peanut, soy, and nut proteins at100 ppm. Multiple peaks corresponding to allergenic tryptic peptides are displayed.

(500 µL) were centrifuge-filtered using a 10 kDa MWCO filter prior to LC-MS/MS analysis.

LC Separation

Tryptic peptides (30 µL injection volume) were chromatographically separated using a Shimadzu Prominence UFLC$_{XR}$ system equipped with a Phenomenex Kinetex C18 column (2.6 µm, 100 x 3 mm). A linear gradient was employed over 12 min at a flow rate of 300 µL/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

MS/MS Detection

To identify signature peptides for allergen screening, peptide maps of various allergenic foods (eggs, milk, peanuts, soy beans, and tree nuts) were acquired using a LC-MS/MS System (Figure 1). The strategy for the selection of signature peptides can be found in more detail in the Results and Discussion.

To screen foods for allergens, a ATL TRAP Food Application system with Turbo V source in positive ESI mode was employed using an ion source temperature of 500°C. The Scheduled MRM algorithm was used to analyze food samples for 12 allergens in a single injection by multiplexing the detection of multiple MRM transitions for allergenic signature peptides.

Result and Discussion

Signature peptides were chosen for each allergen based on: 1) their uniqueness compared to background proteins; and 2) their sensitivity of detection. Further details on peptide sequences, their relative abundance, and possible post-translational modifications were generated using the ProteinPilot software's protein database search features after LC-MS/MS analysis of peptides on a System (Figure 1). The list of selected peptides was refined by removing peptide sequences susceptible to further reaction (e.g., post translational modification, Maillard reaction) during food processing or baking.

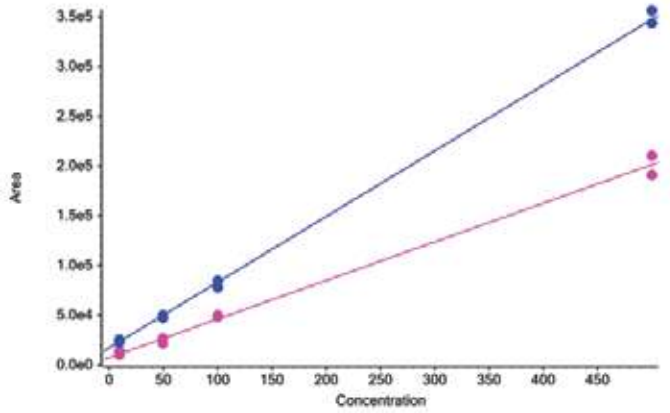


Figure 3a. Calibration lines of a hazelnut peptide from 0 to 500 ppm. Two MRM transitions were monitored: fragment 1 (blue) and fragment 2 (pink)

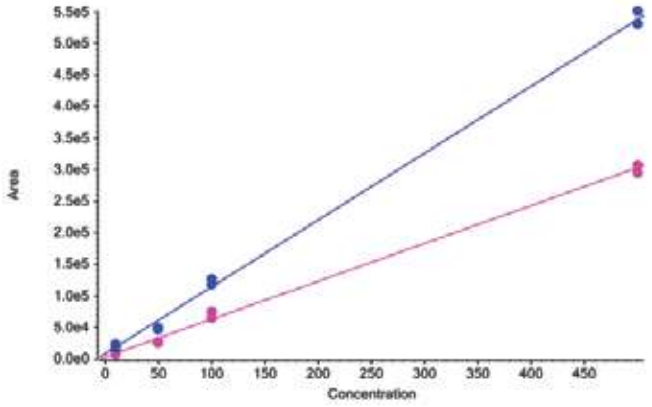


Figure 3b. Calibration lines of a peanut peptide form 0 to 500 ppm. Two MRM transitions were monitored: fragment 1 (blue) and fragment 2 (pink)

For each allergen, two unique proteins, two unique peptides per protein, and two MRM transitions per peptide were chosen to ensure confidence in the identification of an allergen. To monitor many MRM transitions during a single injection, the Scheduled MRM Algorithm was employed, where individual MRM transitions were monitored for a short period during their expected retention time, decreasing the total number of concurrent MRM experiments during a cycle and allowing cycle time and dwell time to be maintained. This approach maximized the S/N for signature peptide detection and allows the method to be expanded as new allergenic markers are identified.

To identify multiple allergens in the same food sample, a total of 88 MRM transitions corresponding to 44 allergenic peptides, from eggs, milk, peanuts, soy beans, and tree nuts, were characterized (Figure 2). Of these 44 peptides, 40 transitions corresponded to peptides with unique sequences not shared by background proteins. The LC-MS/MS-based screening method deployed here simultaneously detected 12 allergenic proteins from 5 major classes of food allergens (egg, milk, peanut, soy and tree nuts) that had been fortified into bakery products at varying concentrations

To show that signature peptide signals were linear in response to increasing allergen levels, calibration curves for each peptide and its three transitions were generated over a wide dynamic range

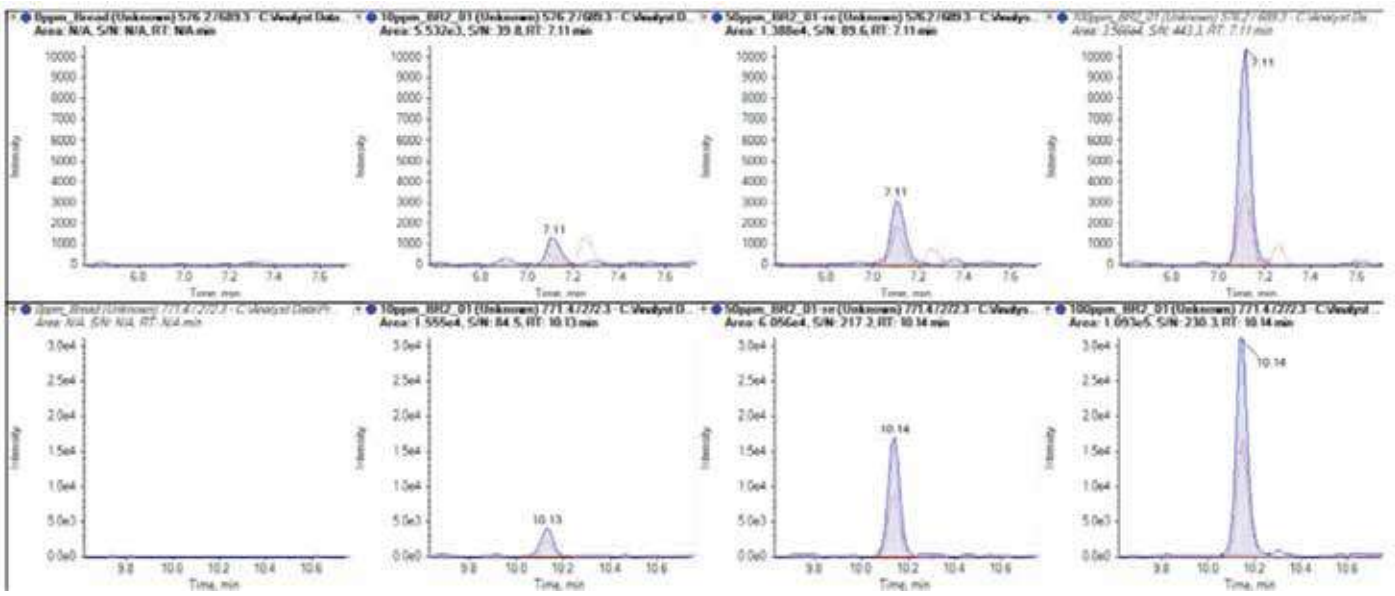


Figure 4. Extracted ion chromatograms for the signature peptide, protein 1 peptide 1, from hazelnut (top) and peanut (bottom). Varying concentrations of allergen (0, 10, 50 and 100 ppm) were added to bread samples. Two different MRM transitions for protein 1, peptide 1 are shown (blue, and pink traces).

(0 to 500 ppm) with good reproducibility in matrix (Figures 3a and 3b). MRM transitions were linear over a broad dynamic range and resulted in regression values over 0.95 for all allergens.

All allergenic peptides were detected at concentrations as low as10 ppm (Figure 4) and generated signals proportional to the quantity of supplemented allergen.

One advantage of the LC-MS/MS method over ELISA-based detection methods is that multiple allergens can be detected in the same sample with one injection. To ensure that a high standard of performance was maintained as throughput increased with the multiplexed LC-MS/MS method, two separate allergen detection methods were directly compared. Signature peptides for select allergens (hazelnut and peanut) were analyzed using two separate ELISA kits and with the LC-MS/MS based method. In general, there was good correlation between the calculated concentrations obtained from ELISA and LCMS/MS with $r^2 \geq 0.99$ (Figure 5). However, results from the ELISA-based tests underestimated the concentrations of hazelnut and peanut supplements in bread and cookie matrices, especially at higher concentrations.

To verify the effectiveness of the LC-MS/MS method for detecting allergens in commercial food samples, bakery products (cookies) containing a variety of allergens were screened using the signature peptide method (Figure 6). Allergen-related signals were not detected in cookie samples that were egg-, milk- and nut-free. However, cookies and bread products that listed hazelnuts and peanuts as ingredients tested positive using the LC-MS/MS method. Other allergens were identified, including egg and milk. unique signature peptides for each allergen and multiplexing their detection into a single injection. In total, there are 88 MRM transitions representing peptides from the egg, milk, peanut, soy, and tree nut allergen groups. Unlike ELISA methods, this LC-MS/MS analysis detects multiple peptides from each allergic protein, thus improving method specificity and minimizing the potential for false positive and false negative results. Using only a single sample preparation method and a multiplexed data acquisition, more allergens than previously reported[4] were screened and differentiated from other food ingredients contained in baked food matrix.

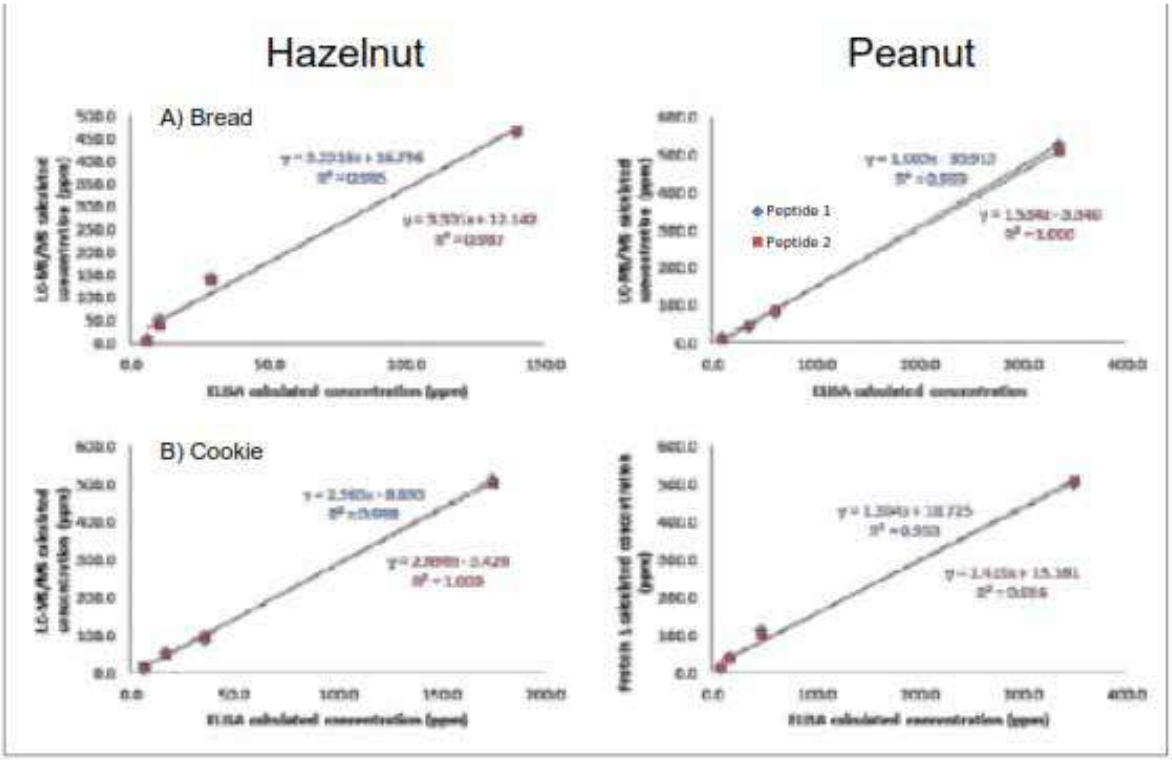


Figure 5. Comparison of allergen concentrations detected using ELISA vs. LC-MS/MS methods for two peptides (blue and orange) and two matrices, bread (top) and cookie (bottom)

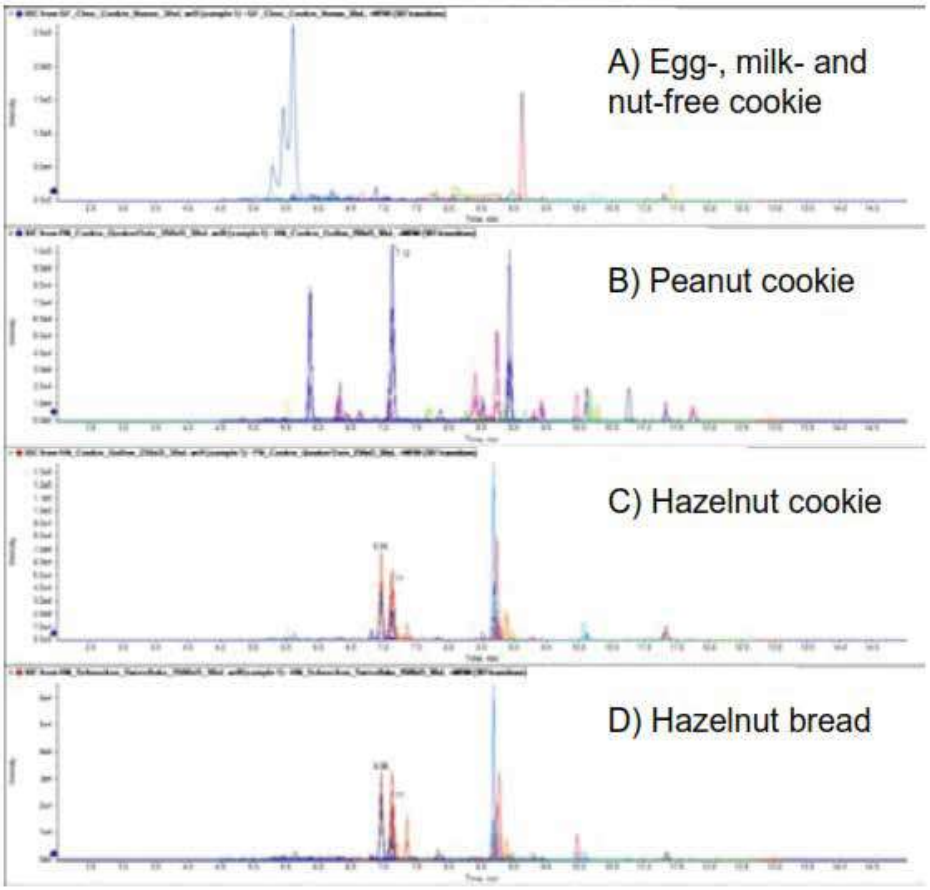


Figure 6. Extracted ion chromatograms of (A) Egg-, milk-, and nut-free cookie, (B) peanut cookie, (C) hazelnut cookie, and (D) hazelnut bread.

A Selective and Robust LC-MS/MS Method for Multiple Meat Speciation and Authentication on the Food Application System

Introduction

In early 2013, horse and pig DNA were identified in beef products sold in several supermarket chains. Further testing across Europe and beyond had revealed widespread incidences of such contamination. This type of contamination not only misleads the consumers, but also has health, religious, and ethical implications. In response to this, the Food Safety Authority (FSA) and Department for Environment Food & Rural Affairs (Defra) have set the threshold for undeclared meat species in meat products to 1% (w/w).Therefore, it is imperative that analytical methods are sensitive and accurate enough to screen for the presence of meat adulteration in food products.

Traditionally, polymerase chain reaction (PCR) and enzymelinked immunosorbent assays (ELISA) are used for meat speciation. PCR amplifies fragments of DNA extracted from food samples and demonstrates good sensitivity in unprocessed products.

However, DNA can be easily disrupted or removed during food processing and manufacturing, thus limiting the use of PCR for processed or cooked meat products.

ELISA is relatively quick and simple to perform, but has poor selectivity and is susceptible to cross-species reactivity which can lead to false positive or false negative results. Moreover, most ELISA tests lack multiplexing capabilities. Hence, LC-MS/MS provides an excellent alternative to these methodologies to identify and confirm different meat species with more accuracy and reliability.

Herein, we present a robust and sensitive LC-MS/MS method using the Food Application LC-MS/MS system that detects and screens pork, beef, lamb, chicken, duck and horse

simultaneously in a single injection. The optimized sample preparation procedure is easy to follow and can be used for analyzing raw, cooked and processed meat products. Signature marker peptides unique to each species were identified and verified to ensure that they do not present any cross-species reactivity. Presently, this method can detect peptides from each meat species at a threshold detection limit of 1% w/w

Sample Preparation

Meats or meat products (10 g) were frozen for 1 hour and grounded using a food processor or a coffee grinder. As an optional step, each grounded meat (1 g) was defatted with hexane and dried under a gentle flow of nitrogen. Extraction buffer was added to each defatted meat sample and the mixture was homogenized at high speed using a probe homogenizer to extract the proteins. Standard samples were prepared by combining different amounts of pork, beef, lamb, chicken, duck and horse homogenates to final concentrations of 0% and 1% (w/w) for each meat species (single-point calibration). The mixed meat homogenates (2 mL) were centrifuged and 0.4 mL of supernatant was diluted with ammonium bicarbonate buffer.

Reducing reagent was added and the samples were incubated at 60°C for 1 hour. After cooling to room temperature, samples were alkylated using a cysteine blocking reagent. The modified proteins were digested with trypsin (4 to 12 hours). After which, the enzymatic activity was quenched with formic acid. Digested samples were desalted and concentrated using Agela

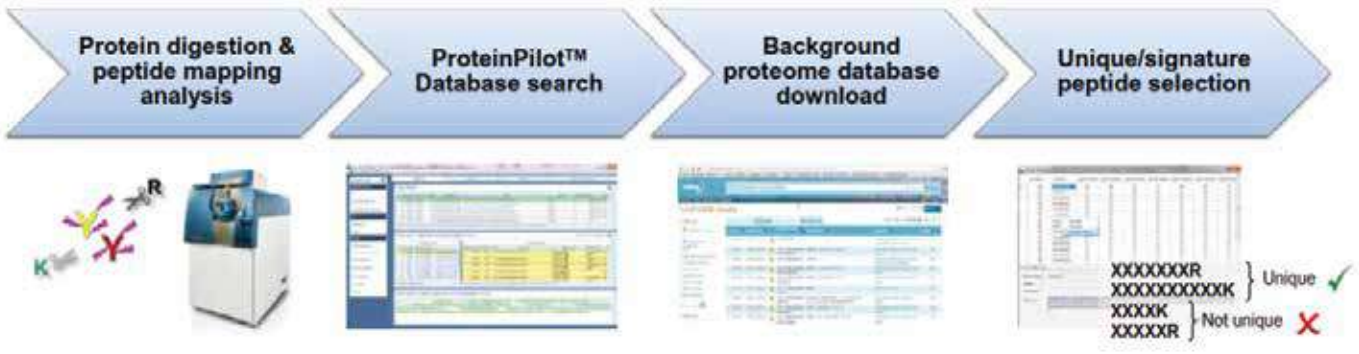


Figure 1. Signature peptide selection workflow using the ATL system and ProteinPilot software

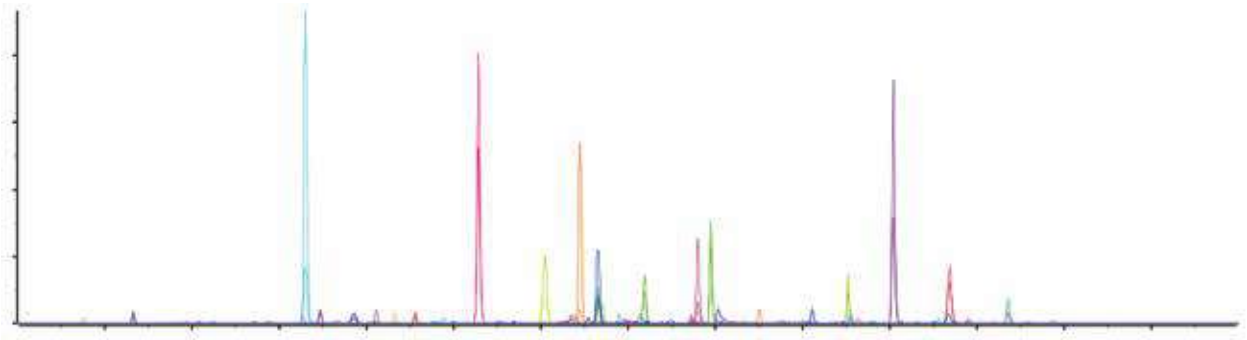


Figure 2. Extracted ion chromatograms from the LC-MS/MS analysis of raw meat mixture containing pork, beef, lamb, chicken, duck and horse (10, 20, 20, 20, 20 and 10% w/w, respectively). Multiple peaks corresponding to tryptic marker peptides are displayed.

Technologies Cleanert PEP SPE cartridges (60 mg/3 mL). The SPE eluents containing the peptides were dried and reconstituted for LC-MS/MS analysis.

LC Separation

Analytes (10 µL injection volume) were chromatographically separated using a LC AC system equipped with a Phenomenex Kinetex C18 column (2.6 µm, 100 x 4.6 mm i.d.). A linear gradient was employed over 15 min at a flow rate of 500 µL/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

MS/MS Detection

Ion-dependent acquisitions (IDA) on a LC- MS/MS System were performed to identify the proteins and peptides representative of pork, beef, lamb, chicken, duck and horse meats (Figure 1). The strategy for the selection of signature peptides can be found in more detail in the Results and Discussion.

Meat speciation and screening analysis was performed on a ATL Food Application system with Turbo V source in positive ESI mode using an ion source temperature of 650 °C. The Scheduled MRM algorithm was used to analyze food samples for 6 meats in a single injection by multiplexing the detection of multiple MRM transitions for unique signature peptides.

Results and Discussion

Comprehensive information of protein/peptide IDs was generated using the ProteinPilot™ software's protein database search features after LC-MS/MS analysis of digested meat samples on a System (Figure 1). Selections of signature peptides for each meat species were performed using the Skyline software and NCBI Protein BLAST to ensure that the shortlisted peptides were unique and not found in other common livestock.

Signature peptides were finalized for each meat based on their:

1) specificity for each meat species; 2) uniqueness compared to the cross-species background; 3) sensitivity of detection; and 4) ability to be detected in both raw and cooked or processed meat samples.

For each meat species, two unique proteins, two unique peptides per protein, and two unique MRM transitions per peptide were chosen to ensure confidence in positive identification (Table 1). This corresponds to 24 marker peptides or a total of 48 MRM transitions representing pork, beef, lamb, chicken, duck and horse, for the simultaneous identification of multiple meat species in the same food sample (Figure 2). To monitor many MRM transitions during a single injection, the Scheduled MRM™ algorithm was employed, where each MRM transition was monitored for a short period during its expected retention time, decreasing the total number of concurrent MRM experiments during a cycle and allowing cycle time and dwell time to be maintained. This approach maximized the sensitivity for signature peptide detection and allows the method to be expanded as markers from other meats are identified.

LC-MS/MS analyses of raw and cooked (pan-fried) meat mixtures were performed to evaluate the thermal stability of the marker peptides. As shown in Figure 3, each meat marker peptide was detected without significant changes in sensitivity before (raw) and after cooking.

To demonstrate that signature peptide signals were linear in response to increasing meat concentrations, calibration curves for each peptide were generated over a wide dynamic range (0 to 100% w/w) with good reproducibility in combined meat matrix.

For all meat species tested (pork, beef, lamb, chicken, duck and horse), MRM transitions were linear over a broad dynamic range with correlation coefficient values of over 0.99 for both MRM transitions. Figures 4 and 5 show examples of pork and beef with good linear response in meat matrix.

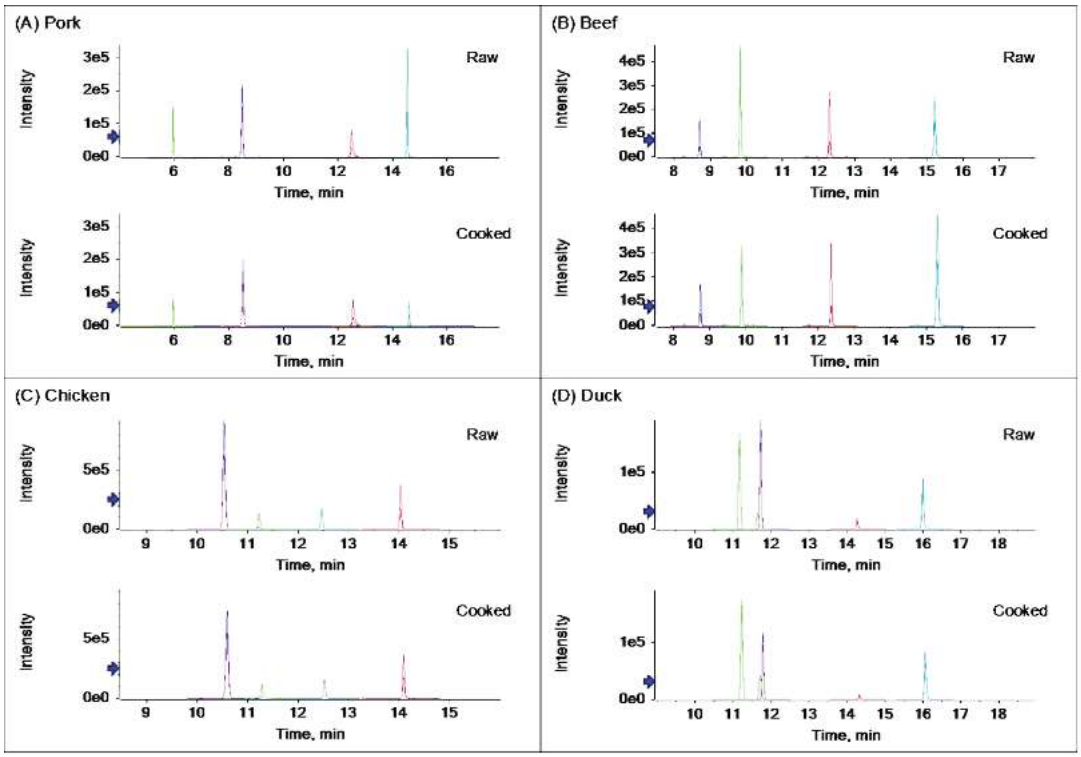


Figure 3. Extracted ion chromatograms (XIC) from the LC-MS/MS analysis of raw (top) and cooked (bottom) meat mixture containing pork, beef, chicken, duck and lamb (data not shown).

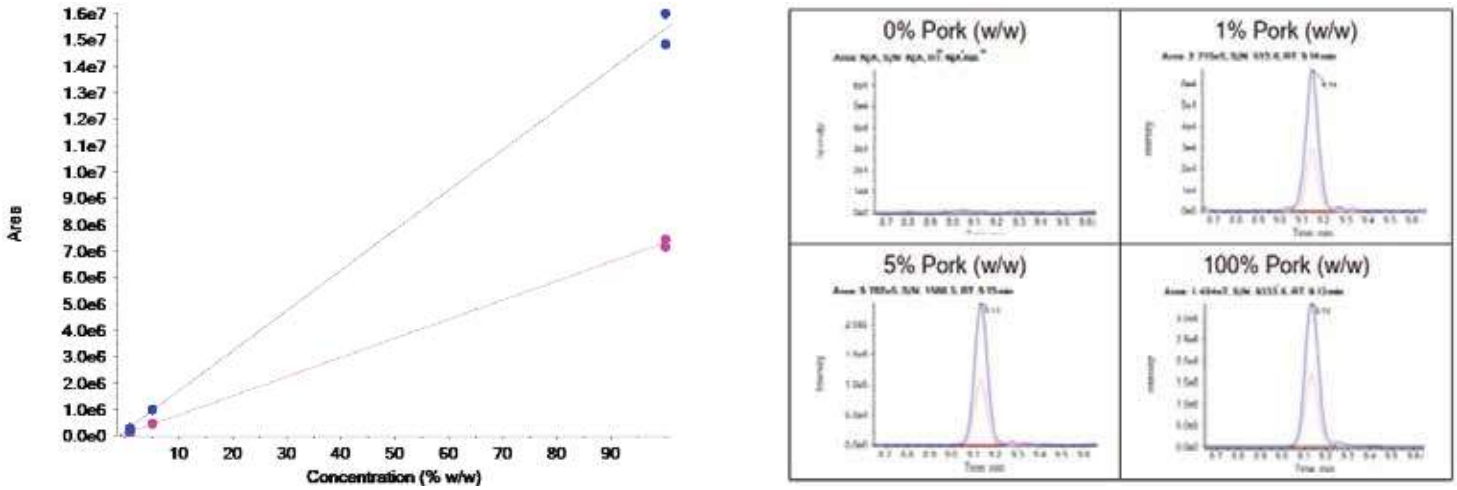


Figure 4. Calibration curves and XICs of Protein_1.Peptide_A from 0 to 100% raw pork (w/w). Two MRM transitions, fragment 1 (blue) and fragment 2 (pink), were monitored.

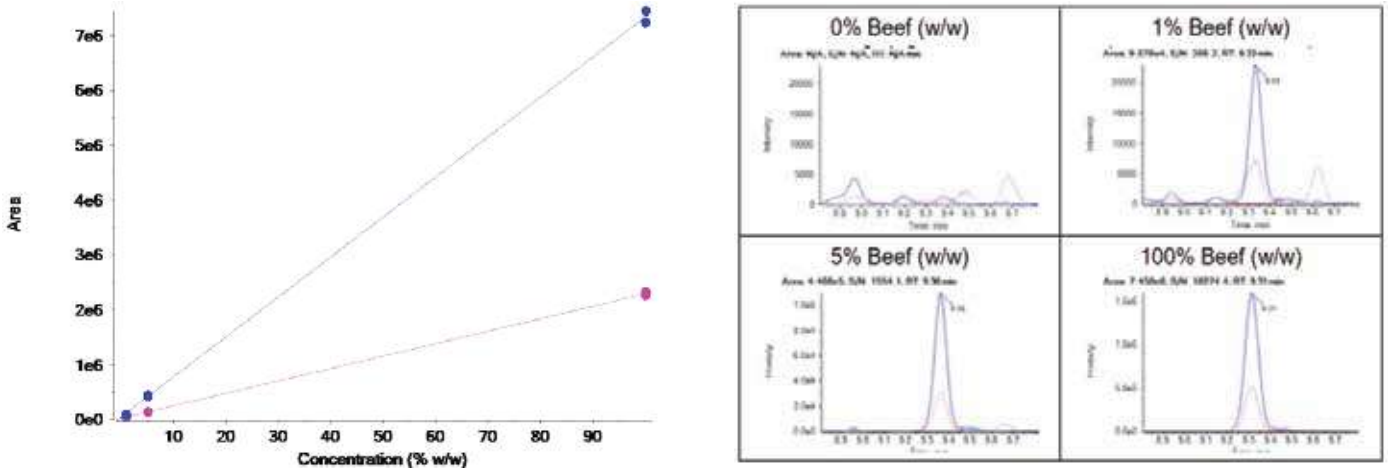


Figure 5. Calibration curves and XICs of Protein_1.Peptide_A from 0 to 100% raw beef (w/w). Two MRM transitions, fragment 1 (blue) and fragment 2 (pink), were monitored.

The 1% (w/w) detection threshold limit of meat species in the combined meat matrix was verified on a ATL Food Application system by analyzing the 0% and spiked 1% (w/w) meat species in meat matrix. All marker peptides for each meat species were reliably detected at 1% spiked and no interference signals were observed in the background matrix (0%). Figures 5 and 6 show example XICs of quantifier ion (Protein_1.-Peptide_A1) for each meat in 0% and 1% (w/w) samples, demonstrating high sensitivity and reliability of detection. It's worth noting that 0.1% (w/w) detection threshold limit of meat can also be achieved with a ATL + system (data not shown).

To verify the effectiveness of the method for detecting meat contamination or adulteration, various raw and processed food products purchased from supermarkets were screened. As an example in Figure 7, no significant pork marker peptides were detected in the halal certified products. Pork was tested positive only in products that had this meat labeled as one of the ingredients.

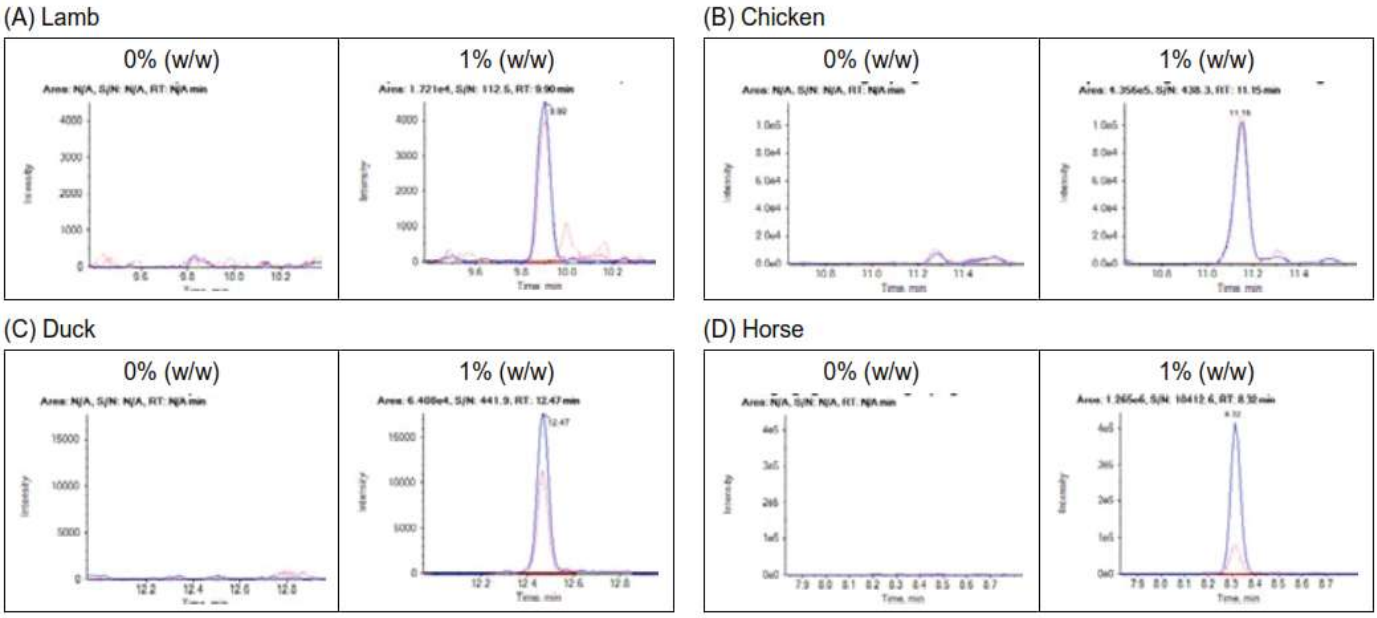


Figure 6. XICs of Protein_1.Peptide_A for 0 and 1% (w/w) of lamb, chicken, duck and horse in combined meat matrix (refer to Figure 5 for detection of pork and beef at 0 and 1% w/w). Two MRM transitions, fragment 1 (blue) and fragment 2 (pink), were monitored for each marker peptide.

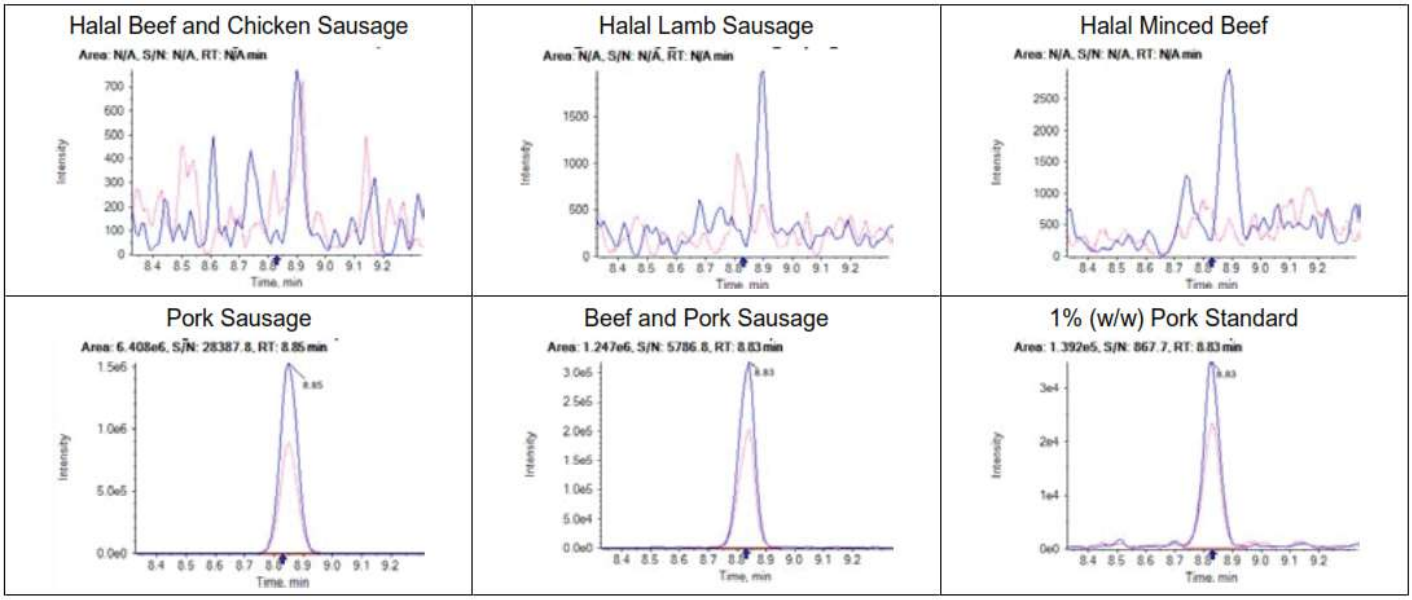


Figure 7. XIC of Pork.Protein_1.Peptide_A in commercial sausage products. Two MRM transitions, fragment 1 (blue) and fragment 2 (pink), were monitored for the marker peptide.

Use of LCMSMS for Monitoring Unexpected Additives in Nutritional Supplements

Introduction

Nutritional supplements can supplement necessary nutrients and are believed to support recovery from illness. Generally, these products promote a particular effect or claimed function; thus, in typical use, people often incorrectly believe they have a definite treatment efficacy. They are often linked to the alleviation of certain illnesses. In order to maximize these functions, manufacturers may add related drugs in order to increase their efficacy without including them as a listed ingredient. According to reports and discoveries from actual monitoring cases, unexpected additives to nutritional supplements are generally selected because they relate to the health product effects or address the additive side effects or functions; the additive usually takes the form of one or more drug additives, drug derivatives, etc.

Because these additives are generally high-dose, drug interactions can be unclear. Thus, a great potential hazard exists for human health; the Food and Drug Administration (CFDA) “Health product potential illegal additives list” clearly stipulates monitoring processes for additives in 6 different types of nutritional supplements: those with weight loss, blood sugar reduction, blood pressure reduction, anti-fatigue, sleep improvement, and immune strengthening functions. The purpose is to protect consumers’ health.

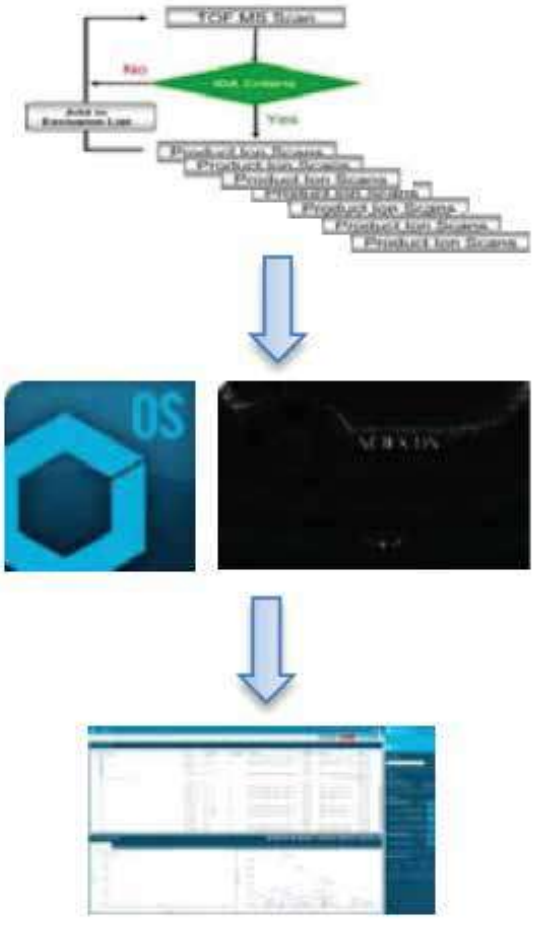
ATL’s LCMSMS high resolution mass spectrometry system can be used for rapid monitoring of additives in nutritional supplements; after sample injection, a first order mass accuracy number and second order fragmentation spectrum are simultaneously obtained. Currently, over 50 additives can quickly be qualitatively confirmed in this way. Matrix interference in complex matrices can be overcome for specific screening of additives; preprocessing is even simpler and more convenient.

The new software fully integrates instrument control, data collection, data handling, and other processes. The workflow is more intuitive and smarter; this method provides an efficient means for rapid, high-throughput monitoring of nutritional supplements for additives.

Experimental Process

1. Collect samples of 6 types of nutritional supplements currently on the market - those with weight loss, blood sugar reduction, blood pressure reduction, anti-fatigue, sleep improvement, and immune strengthening functions. Perform simple preprocessing.
2. Use TOF MS-IDA MS/MS mode for data collection; after sample injection, obtain first order ion and second order ion fragmentation spectrograms.
3. The mass accuracy number, isotope distribution, retention time, and standard library alignment are used for positive verification of samples and checking the accuracy of sample monitoring results.
4. Monitoring reports systematically summarize sample screening results; the report content can be tailored to specific requirements.

LCMSMS high-resolution mass spectrometry screening workflow



1. Both TOF-MSIDA-MS/MS And TOF-MS/MS datagathered in the same injection

2. integral software used to perform this analysis

3. Screening results and report generation

Preprocessing Method

1. Use tablets ground into a powder, granules from inside capsules, or liquid samples; weigh accurately a 1.0gsample, and place in a 10mLcentrifuge tube;
2. Add 5mL acetonitrile and agitate 2 min;
3. Vortex 2 min;
4. Centrifuge at 4ºC at 10000 Rpm for 15min;
5. Dissolve the supernatant 1-fold;
6. Pass through a 0.22μm filter and directly inject sample;

Liquid Phase Conditions

Chromatographic Column: Phenomenex Kinetex C18, 2.1*100mm, 2.6μm;

Elution gradient

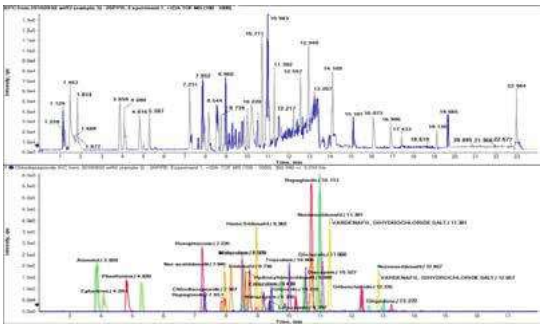
Time (min)	A%	B%
0	95	5
5.0	55	45
15.0	20	80
20.0	5	95
25.0	5	95
25.1	95	5
30	95	5

Positive ion mode: A: 0.1% Formic acid Water; B: 0.1% Formic acid Acetonitrile;
Negative ion mode: A: Water; B: Acetonitrile;
Flow rate: 0.3mL/min;
Column temperature: 40ºC;
Amount inserted: 10 μL;
Mass Spectrometry Method
Scanning method: TOF MS-IDA MS/MS
Ion source: ESI source
Scanning range: m/z 50-2000
CUR gas: 30 PSI
Collision gas CAD: 7

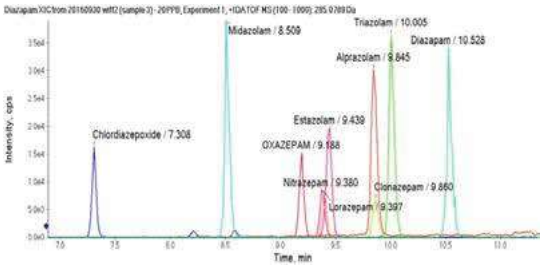
IS voltage: 5500V/-3450V
Source temperature: 600ºC
Atomizing gas GAS1: 55 PSI
Auxiliary gas GAS2: 70 PSI
DP voltage: ± 60V
Collision energy: 35 ± 15V

Unexpected Additive Screening Method

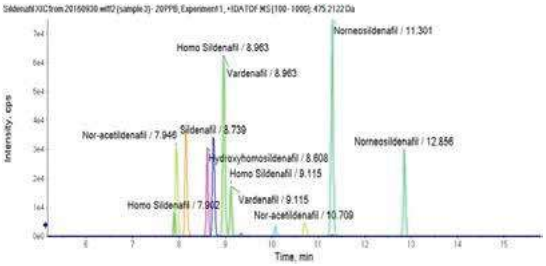
Injection of a single sample simultaneously monitors for over 50 unexpected additives:



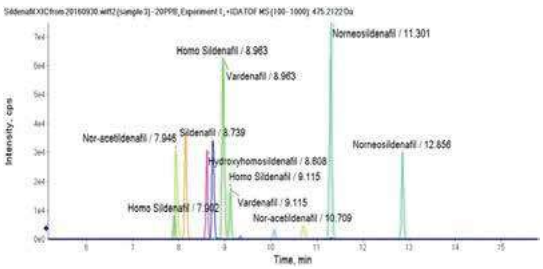
1. 10 sedative-hypnotic mixtures (20ppb), ion extraction flow diagram (XIC) appears below:



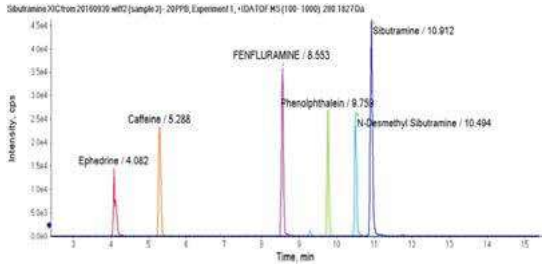
2. 7 blood glucose-lowering drugs (concentration 20ppb); ion extraction flow diagram (XIC) appears below:



3. 8 impotence drug mixtures (20ppb), ion extraction flow diagram (XIC) appears below;



4. 6 weight loss drug mixtures (20ppb), ion extraction flow diagram (XIC) appears below;



5. 5 blood pressure-lowering drug mixtures (20ppb), ion extraction flow diagram (XIC) appears below;

Sample Information

Following the CFDA's "Health product potential illegal additives list" 6 different nutritional supplements were randomly selected, including those for weight loss, blood sugar reduction, blood pressure reduction, anti-fatigue, sleep improvement, and immune strengthening. Samples came from 19 different brands;

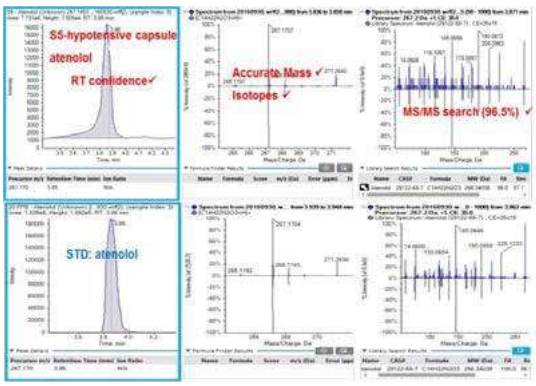
Sample No	sample type	name
Sample 1	sleeping	epiphysis pacify
Sample 2	hypoglycemic action	glycolipids safe
Sample 3	hypoglycemic action	hypoglycemic extract
Sample 4	anti-hangover	prime power
Sample 5	hypotensive	hypotensive capsule
Sample 6	sleeping	pacify syrup
Sample 7	hypoglycemic action	hypoglycemic TCM
Sample 8	slimming	slimming capsule
Sample 9	hypotensive	Hypotensive pill
Sample 19	.....	.....

Experimental Results

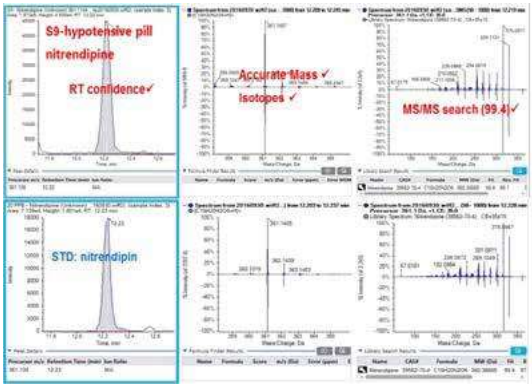
Blood Pressure-Lowering Drugs

1. Sample no. 5 - atenolol positive

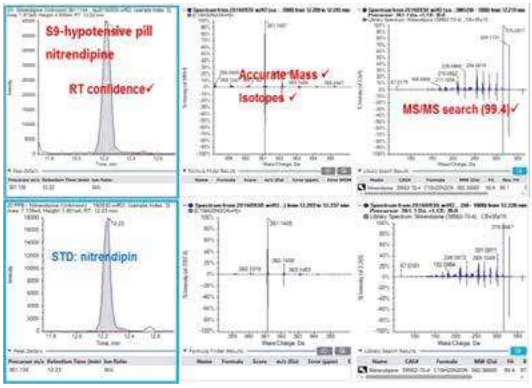
Sample no. 5 is a blood pressure-lowering capsule; it claims to have a rapid effect and prolonged use can control blood pressure.



Screening with the LCMSMS system showed Sample no. 5 contains large amounts of the additive atenolol. Prolonged use of high-dose atenolol can lead to serious side effects including decreased vision, breathing difficulties, weakness, depression, unexplained rash and ankle swelling and other symptoms.



2. Sample no. 9 - nitrendipine positive Sample no. 9 is from a brand of blood pressure-lowering tablet; screening shows a definite quantity of nitrendipine. The product claims to contain pure and natural extracts with no side effects,but prolonged oral nitrendipine can cause diseases like allergic hepatitis, rash, and even exfoliative dermatitis.

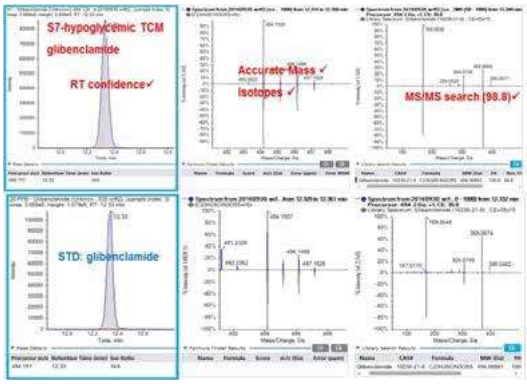


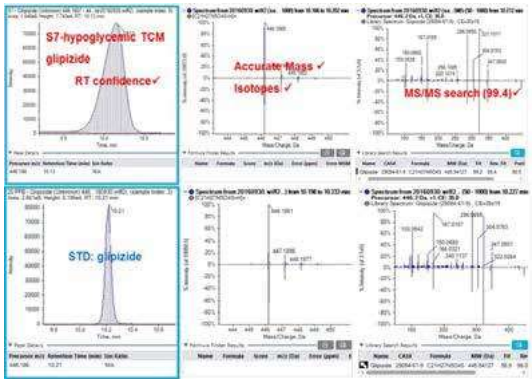
3. Sample no. 17 - nifedipine positive Sample no. 17 is from a brand of blood pressure-lowering medicine; screening shows a nifedipine additive. It claims to lower blood pressure with medicine, falsely advertising an anti-hypertensive effect.

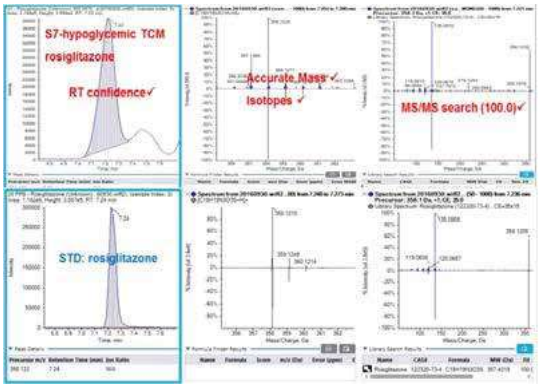
Glucose-Lowering Drugs

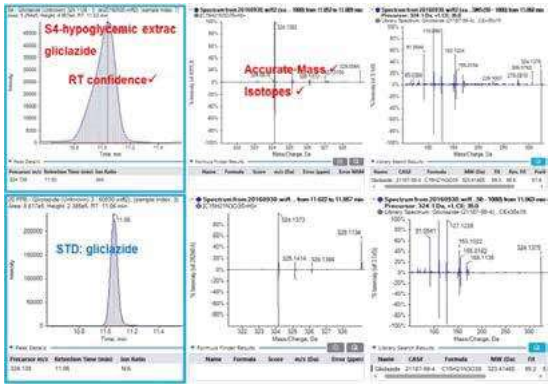
1. Sample no. 7 - glibenclamide, glipizide, rosiglitazone positive

Sample no. 7 is a brand of glucose- and lipid-lowering capsule; test results show sample no. 7 contains the 3 glucose-lowering drugs glibenclamide, glipizide, and rosiglitazone as additives. Improper use of sulfonylureas such as glibenclamide and glipizide can cause hypoglycemia; patients can rarely develop rash, erythema multiforme, edema, and liver and kidney damage. Thiazolidinediones like rosiglitazone can cause slight hypersensitivity and mild headache when used incorrectly or atimproper doses.









2. Sample no. 4 - Gliclazide positive

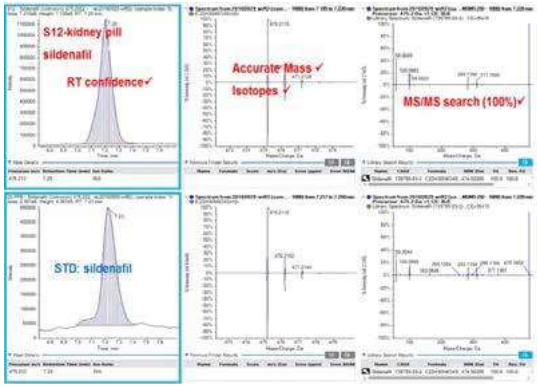
Sample no. 4 is a brand of plant extract; it is mainly used to stabilize blood sugar. Screening results show an addition of glicazide, which produces a definitive glucose-lowering effect.

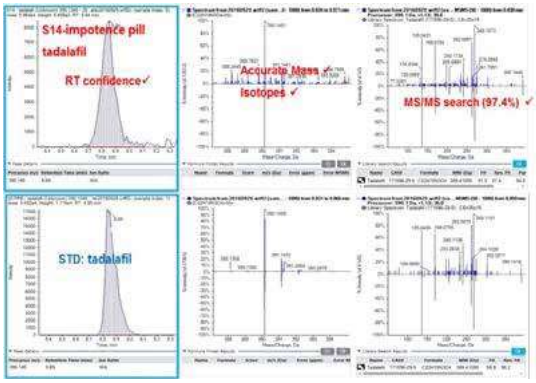
Glucose-lowering drugs are low-cost; they are common “functional components” added to nutritional supplements. These chemical drugs are often used to treat diabetes, as they have a clear hypoglycemic effect. However, their side effects are also quite evident; prolonged use can lead to hypoglycemia and kidney damage, even leading to death.

Anti-Fatigue/Impotence

1. Sample no. 12 - sildenafil positive

Sample no. 12 is a kidney health product for the elderly; its description states it is pure medicine and contains several flavors of medicine. Screening shows an addition of large quantities of sildenafil in order to achieve its claimed kidney effects.





2. Sample no. 14 - tadalafil positive

Sample no. 14 is a brand of impotence health product. Impotence products are the most frequently found to contain additives. In order to accelerate the speed of onset, additives are generally used in large quantities; screening results showed sample no. 14 contained tadalafil.

When not used under the guidance of a specialized physician, prolonged use of nutritional supplements containing “impotence” additives can severely harm the body. Side effects can include dizziness, fainting, and even hearing loss.

Screening results appear in the table:

1. The problem of additives in nutritional supplements is widespread; additives appear in many samples;
2. Blood sugar- and pressure-reducing products contain many different additives; they generally take the form of multiple drugs, and use of medicine is especially serious.
3. Anti-fatigue and impotence health care products generally contain large amounts of additives;

Sample No	sample name	positvie results
Sample 1	epiphysis pacify	--------
Sample 2	glycolipids safe	--------
Sample 3	hypoglycemic action	--------
Sample 4	hypoglycemic extrac	gliclazide
Sample 5	hypotensive capsule	atenolol
Sample 6	pacify syrup	--------
Sample 7	hypoglycemic TCM	glipizide、rosiglitazone、glibenclamide
Sample 8	slimming capsule	--------
Sample 9	hypotensive pill	nitrendipine
Sample 12	kidney pill	sildenafil
Sample 14	impotence pill	tadalafil
Sample 17	hypoglycemic extrac	nifedipine

Analyzing Different Compositions of Polygala from Different Regions Using the LCMSMS System

Introduction

Authentic herbs come from specific locations that are traditionally known for these high-quality products. Authentic herbs have become synonymous with traditional medicine and form a comprehensive material standard for evaluating the quality of herbal medicines. Authentic herbs thus play a unique and important role in authentication and quality control of herbal preparations. Authenticity of medicine has become an important guarantee of high herbal quality.

Polygala is one of the main herbal medicines, one of 85 traditional herbal medicine exports, and one of 42 species of level 3 protected wild products

The 2010 "Pharmacopoeia" divides Polygala herbs into those derived from the plant leaves of Polygalaceae and those made from dried Polygala leaves and roots. They have the properties of sedation, promoting heart and kidney circulation, acting as an expectorant, and decreasing swelling. They are used to treat insomnia, excessive dreaming, forgetfulness, and fear caused bypoor heart and kidney circulation

The commercial Polygala industry depends on the Polygala supply, which is found in an area bounded by the desert to the south and the Yangtze River to the north. It is grown mainly in Shanxi, Shaanxi, Henan, and Hebei, under the traditional notion of "Shanxi - large quantity, Shaanxi - high quality"

Currently, the identification and analysis of herbal medicine components is quite challenging. These componentsunderlie the pharmacodynamic efficacy of medicinal products. Herein lies the key to modernizing medicine.How to quickly identify the active ingredient and its structure, aswell as how to identify the differences between the active ingredients of authentic and inauthentic herbs, are urgent problems that must be solved.

This study used the ATL high resolution LCMSMS mass spectrometer for data acquisition and used the accompanying MarkerView analytic software to statistically analyze differences between components. This study involved medicine (e.g., Polygala) that includes components from different regions. This method makes component identification more effective, faster and a better reflection of the integrity and unique nature of the sample tested. In turn, it highlights the differences

between Polygala components from different sources and provides a new framework for quality evaluation of herbal medicines.

The high resolution LCMSMS hardware design, including N-type ion path technology, time of flight tube design, and a stable and durable Turbo V ion source, ensures that under routine testing conditions, sample identification is more stable, higher quality, and more reliable for the long term. The LCMSMS high-sensitivity, high-resolution analysis and accurate mass-to-charge ratio analysis, combined with the intelligent TOF-MS-IDA-MS/MS acquisition mode, truly achieve the goal of collection of high-quality, accurate primary and secondary mass spectrometry data by single injection, and quickly provide the most accurate qualitative screening results.

ATL LCMSMS mass spectrometry system with LC liquid chromatography system and workstation

Study Design

1. Samples of Polygala herbs from different sources were obtained and assigned to groups, each containing 6 samples.
2. TOF-IDA-MS/MS mode was used for data acquisition; one injection allowed simultaneous collection of various components' primary ions and secondary daughter ions.
3. The MarkerView Software was used to analyze differences in components and identify statistically significant differences between groups for use as markers.
4. After entering mass spectrometry data on primary ions and secondary daughter ions into Software, the components were matched with the ATL high resolution MS/MS medicine database or the ChemSpider online database; differences in components were identified.

Study Design Workflow

Materials and Methods

This study collected Polygala herbs from 4 regions:
Chengcheng, Shaanxi; Shangluo, Shaanxi; Shanxi; and Hebei. After the samples were dried, they were cut into small pieces and dried in the oven at 40 degrees C for 18 h. After removal, they were crushed and filtered through a 20 mesh sieve, placed in the dryer, and then used.

Sample Preparation

Carefully weigh out about 1.0 g of Polygala powder of consistent weight, add 50 mL of 70% methanol aqueous solution, ultrasonicate 30 min., centrifuge for 10 min at 13000 rpm, and take the supernatant for injection.

Chromatographic Conditions

Chromatographic Column: Phenomenex Kinetex F5, 100*3.0 Mobile phase: A is ultra-pure water/B is acetonitrile;
Gradient elution was performed as shown below:

Time (min)	A%	B%
0.0	95	5
5.0	90	10
15.0	85	15
20.0	80	20
25.0	75	25
30.0	70	30
35.0	65	35
40.0	10	90
45.0	10	90
45.1	90	5
50.0	90	5

Flow rate: 0.4 mL/min；
Column temperature: 40 ℃；
Amount inserted: 5 μL

Mass Spectrometry Method

Scanning method: TOF-IDA MS/MS qualitative screening;
Ion source: ESI source
Mass spectrum parameters are established in 4 steps:

Chromatogram

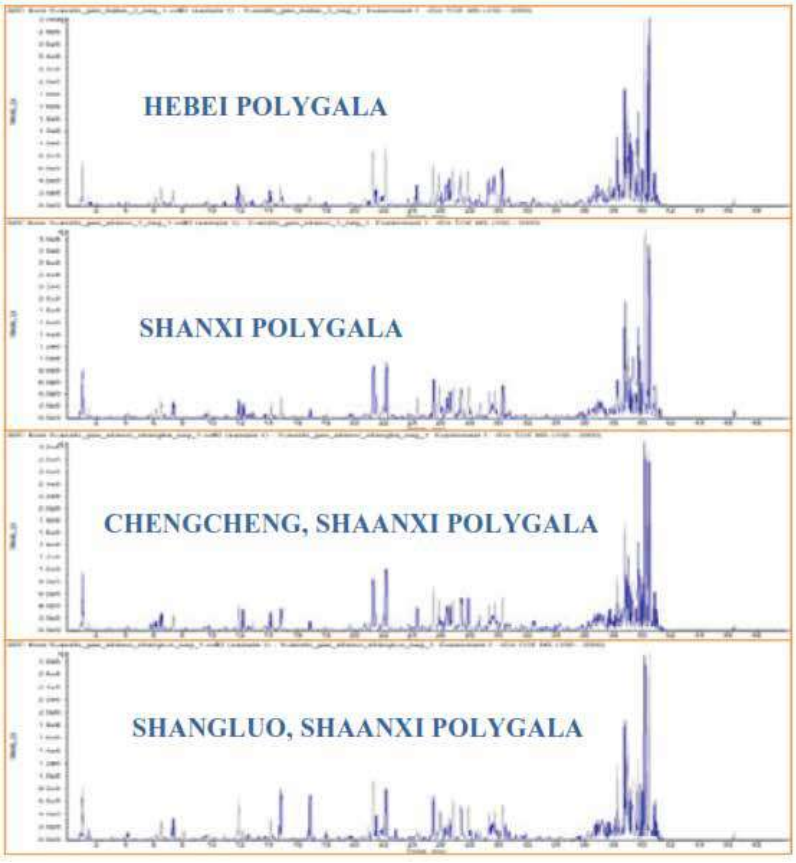


Figure 1. Typical BPC for four polygala samples from different sources

Typical ion base peak chromatograms (BPC) for four groups of polygala samples from different sources; see fig. 1 below:
Chromatographic peak retention reproducibility was very good among the four Polygala samples from different sources. Many baseline analysis separation peaks were obtained on the BPC, showing good chromatographic separation.

MarkerView Data Processing

The MarkerView Software was used for preliminary data extraction of chromatographic peaks. Identification and integration were performed on chromatographic peaks with a retention time of 0 - 50 min; the three-dimensional data was transformed into a two-dimensional data matrix, including variables (m/z_RT), number observed (24 samples), and the integral area. This study found 994 variables (m/z_RT).

PCA-DA processing and Library Database Search

All samples underwent supervised PCA analysis, and their Score and Loading chart is as shown in Fig.2:

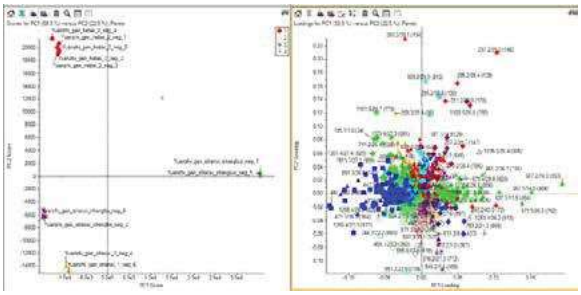


Figure 2 A) Polygala samples from different sources, PCA Score Plot; B) Polygala samples from different sources, PCA Loading Plot;

Fig. 2 Score Plot shows Polygala samples from the 4 different areas are well separated, meaning that there are large differences between groups.
Using Polygala products sourced from different areas, take m/z 667.2 (RT=16.9 min) as an example. For m/z 667.2 in the figure below, showing content differences in samples from different areas, the line plot shows that Chengluo, Shaanxi Polygala has a Tenuifoliside B2 content that is approximately 5 times that of the 3 other areas, as in Fig. 3:

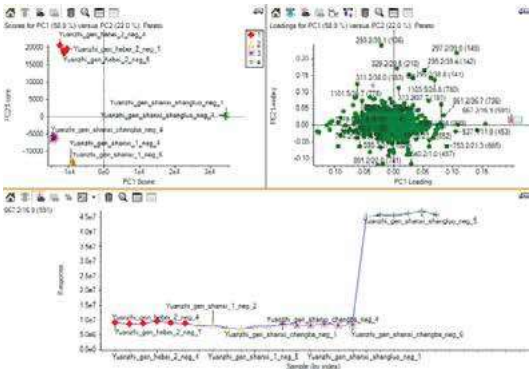


Figure 3 Polygala Marker: m/z (667.2), (RT=16.9 min)

Polygala characteristic marker m/z 667.2, retention time 16.9 min, Software identification of the marker is: Tenuifoliside B2, C30H36O17, m/z (MS)= 667.1875, m/z (MS/MS) = 461.1288, 367.1035, 239.0557, 205.0498, 190.0265. Using Library search, identification results in Fig. 4-1 shows that secondary fragment matching is good, with the main fragment structural analysis shown in Fig. 4-2:

Figure 4-1. Polygala Marker m/z 667.2 via structural attribution results

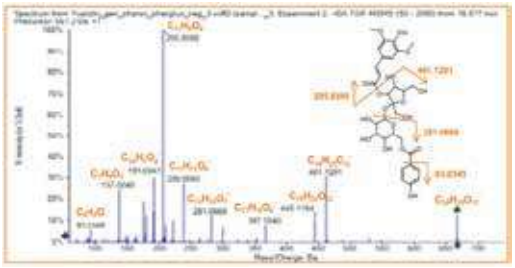


Figure 4-2. Polygala Marker m/z 667.2 secondary fragment attribution and main fragment structural analysis

T-test data processing

All samples underwent T-test data processing; results are in Fig. 5. Fig. A is the volcano plot, expressed as log fold change vs. pvalue; as the X axis is approached, more ions are located at both ends of the X axis, indicating a greater difference between them. Fig. B is a line plot, and Fig. C is a box plot, showing the content relationships between the samples.

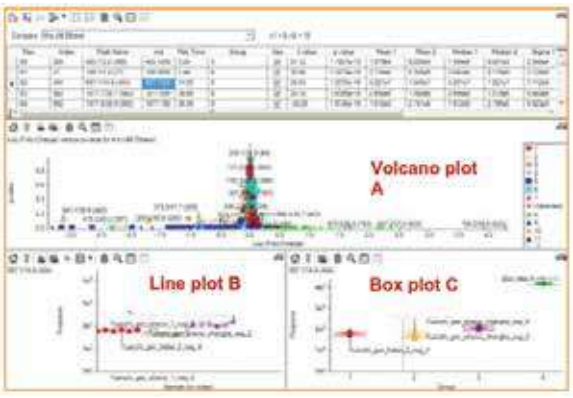


Figure 5 Log (Fold Change) versus p-values data processing

T-experimentally ($p < 0.005$) differentiated ion scans appear in line plot B and box plot C. Compound m/z 567.1 (RT 14.8 min) is significantly different in the Shangluo, Shaanxi Polygala, so it is used as a marker. Its structure is identified with software's ChemSpider online structural identification for markers. Results are in Fig. 6:

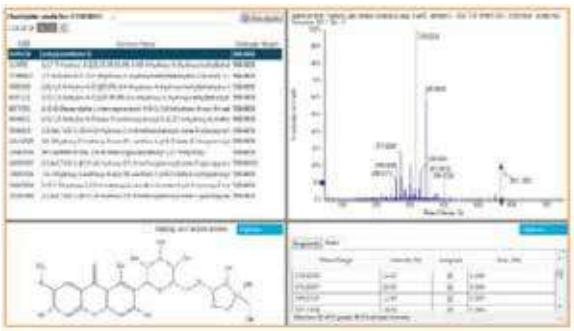


Figure 6-1. Polygala Marker: m/z (567.1), (RT 14.8 min)

Marker identified as: Polygalaxanthone III , $C_{25}H_{28}O_{15}$, m/z (MS)= 567.1359 , m/z (MS/MS) = 345.0608, 315.0510, 399.0724, 271.0247; its online secondary fragment matching is good.

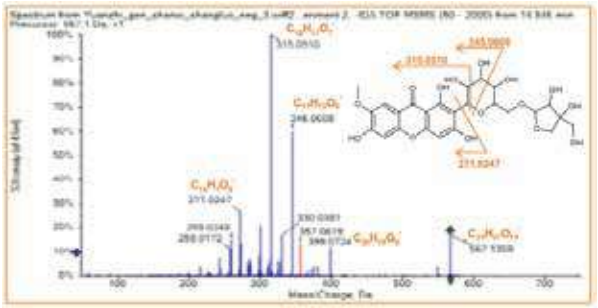


Figure 6-2. Polygala Marker m/z 567.1 secondary fragment attribution and main fragment structure analysis

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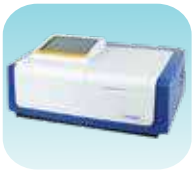

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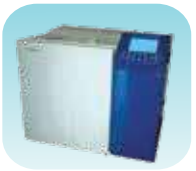

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Corporate & Regd. Office:
Analytical House, # E67 & E68,
Ravi Park, Vasna Road, Baroda,
Gujarat 390 015. INDIA

T: +91 265 2253620
+91 265 2252839
+91 265 2252370
F: +91 265 2254395

E: info@hplctechnologies.com
info@multiplelabs.com
info@analyticalgroup.net

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