HUMAN HEALTH

ENVIRONMENTAL HEALTH

MEASURING HAS NEVER BEEN EASIER IN LC/MS.

Flexar SQ 300 MS

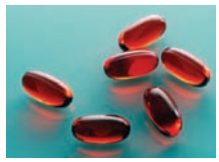
A Reference Notebook of LC/SQ MS Applications—Second Edition, 2011

PerkinElmer®
For the Better

Table of Contents

INTRODUCTION AND OVERVIEW

The coupling of liquid chromatography (LC) with mass spectrometry (MS) has created a powerful tool in the verification, quantification and characterization of compounds. These integrated technologies enable laboratories to take advantage of the popular and practical separation capabilities of LC while enhancing the quality of their data with the unsurpassed sample insight of MS.



With the Flexar™ SQ 300 MS, PerkinElmer has designed the ultimate single quadrupole MS detector, a cutting-edge instrument that features the latest ion source technologies.

This document is intended to provide an overview of the innovations behind the Flexar SQ 300 MS, and to show how the instrument extends the flexibility of the Flexar family of LC platforms. It also includes a look at the instrument's application-oriented Chromatography Data System (CDS)—Chromera®—and features a series of sample applications to highlight many of the performance capabilities and advantages of the system.

The Flexar SQ 300 MS. No matter what your industry, application, or sample type, it's the ideal tool to help you verify, quantify, simplify.

The Flexar SQ 300 MS accurately measures to ± 0.1 u of the nominal mass.

Instrument Capabilities Overview	
Mass Range:	20-3,000 u
Mass Accuracy:	± 0.1 u/24 hr; ± 3 °C
Mass Axis Stability:	± 0.1 u/24 hr; ± 3 °C
Max Scan Rate:	10,000 u/sec
Resolution (FWHM):	0.6 u ± 0.1
Polarity Switching:	Standard
Quantitative Dynamic Range:	10^5

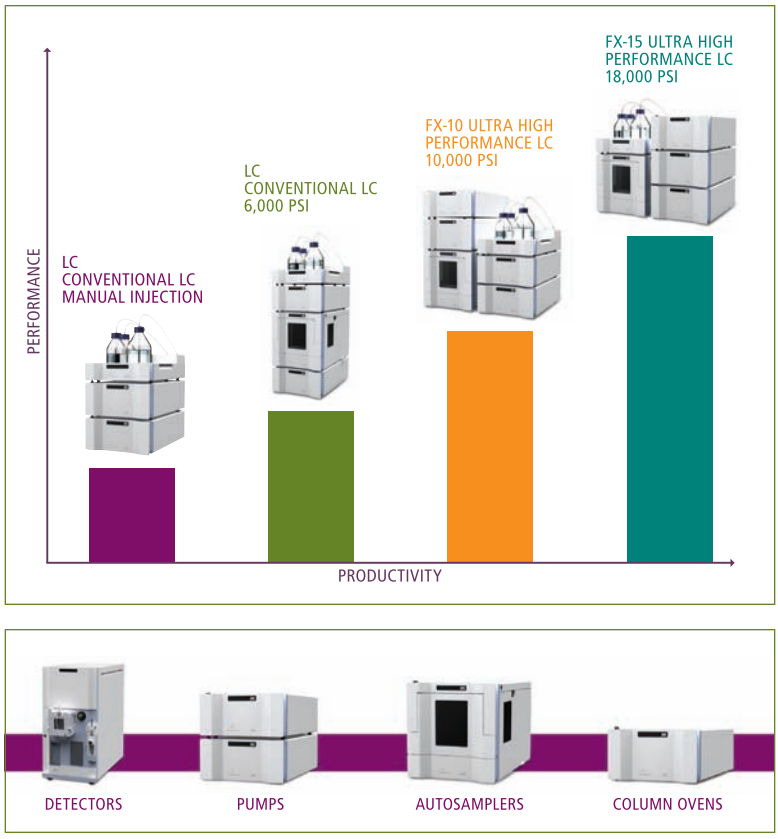
Front-end Chromatographic Separation.

Today's High Performance Liquid Chromatography (HPLC) and Ultra-High Performance (UHPLC) systems can be used for qualitative and quantitative analysis of most non-volatile compounds—including organics, biomolecules and polymers.

With such a broad array of application possibilities the Flexar family features a series of stackable, modular liquid chromatography technologies that can be seamlessly integrated to create the ideal configuration for a specific analytical need.

Components include the industry's most rugged, reliable pumps and precision autosamplers which offer best-in-class cycle times and excellent injection reproducibility with incredibly low carryover for enhanced sample throughput and efficiency. Flexar ovens deliver precise temperature control for better column performance and more repeatable retention times. Additionally, a wide array of detectors provide the speed and sensitivity to shed light on every detail of your samples.

With the widest range of operating pressures available (6,000-18,000 psi), Flexar pumps offer the flexibility, throughput and resolution required to create the perfect front-end LC solution to complement the sensitivity, speed and sample insight of the Flexar SQ 300 MS.



Mix and match components to create the ideal configuration to suit your performance/ productivity requirements.

DECOUPLING TECHNOLOGIES DELIVERS SUPERIOR RESULTS

The design of the Flexar SQ 300 MS can be divided into three distinct areas—ion source, ion transfer and mass analyzer. Each is completely decoupled to deliver complete and efficient control of their specific processes.

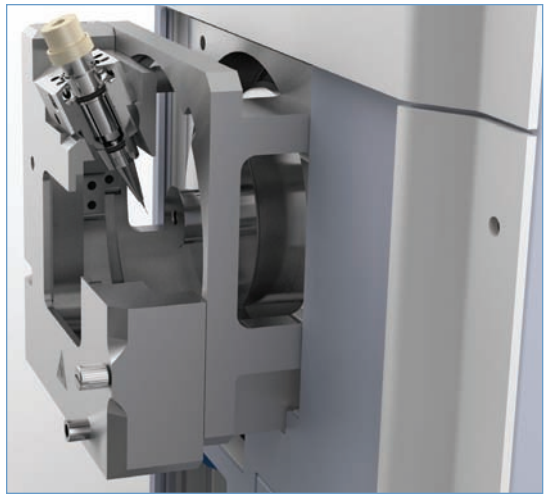
Ion Source.

Since the technique's inception, the greatest challenge in LC/MS has been extracting and charging analytes dissolved in the mobile phase and efficiently transferring them into the mass spectrometer.

Today, multiple ionization sources are available to facilitate this process, the two most popular of which are Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI).

The Flexar SQ 300 MS is available with both source types to suit a wide range of applications or sample matrices. Ion sources can easily be interchanged without using any tools or shutting down the vacuum for quick and efficient switching between applications.

The instrument can be configured with the latest PerkinElmer ion source technology: Ultraspray™ ESI, dual-probe Ultraspray2™ ESI for the simultaneous connection of multiple analytical channels, or a groundbreaking Field-Free APCI option. All three operate with the standard polarity switching capability, and each one has a built-in source transponder that ensures the system is configured with the correct parameters for the attached source type.



Source housings may be removed and cleaned without shutting down the vacuum for unsurpassed instrument uptime.

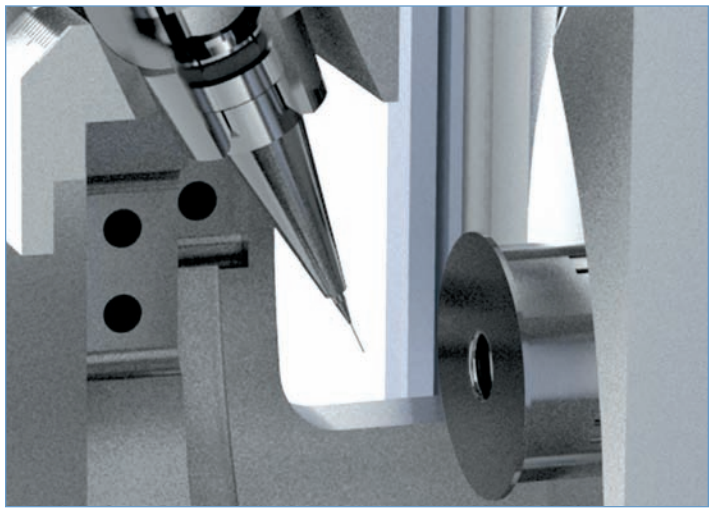
Ultraspray/Ultraspray2 Electrospray Ionization (ESI).

Used to analyze molecules over a wide mass range and solutions covering a wide spectrum of pH, ESI is a concentration-dependent ionization technique most commonly used with polar molecules that can easily be charged.

ESI is typically used with a mobile phase flow rate of 0.2-1.4 ml/min. The process starts with the LC eluent being pneumatically nebulized to accelerate the evaporation of the mobile phase. The ions are drawn away from the grounded sprayer needle toward the capillary entrance by a strong electric field generated by voltages applied to the capillary entrance, cylinder lens and endplate. Counter-current drying nitrogen gas assists with the evaporation of the solvent, freeing charged molecules from the droplets.

Sensitivity Specifications
ESI(+) SIM S/N >120:1 RMS for 1 pg/µl reserpine injected @ 400 µl/min MeOH/H_2O
ESI(–) SIM S/N >100:1 RMS for 2 pg/µl *p*-nitrophenol injected @ 400 µl/min MeOH/H_2O

Source Flow Rate Capabilities
ESI 0.004–1.4 ml/min (no split)



The patented angled design of the Flexar SQ 300 MS probe enhances the desolvation process and provides charged molecules with additional momentum to reach the capillary entrance.

Engineered by the team that pioneered the development of ESI technology, PerkinElmer's Ultraspray sources deliver industry-leading desolvation and ionization capabilities. A unique, angled probe projects charged molecules toward the entrance of the mass spectrometer facilitating superior transmission. Fully X, Y, Z-adjustable, the patented probe can be precisely positioned to sample from the edge of the spray plume where the droplets are smaller and require less energy to ionize. This facilitates super-soft ionization, enabling even the weakest molecular structures to be preserved for analysis.

Quickly and easily interchangeable, individual snap-in probes can be assigned to specific applications or users, minimizing cross-contamination and maximizing productivity in open-access laboratories. With no wires to connect or current to carry, the zero-potential probes eliminate the possibility of redox reactions occurring within the needle. The grounding also ensures counter ions only deposit at the probe tip to prevent residual chemical products forming along the analytical path after switching ion polarities.

Patented Ultraspray2 ESI sources feature all the technological innovations of standard Ultraspray interfaces but with a unique, dual-probe design. With two analytical channels simultaneously connected, Ultraspray2 gives users the option of having a dedicated calibrant inlet, eliminating the time required to flush lines and preventing contamination of the sample probe.

Once efficiently desolvated and ionized in the source, ions are drawn into the capillary entrance by the pressure differential between the source housing (atmospheric pressure) and the first vacuum stage of the internal ion path (~2.5 millibar).

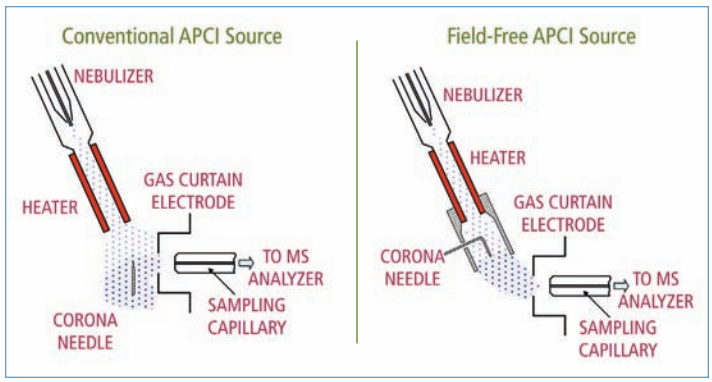
Field-Free Atmospheric Pressure Chemical Ionization (APCI).

Typically used for the analysis of molecules of low polarity and those that have no strong acidic or basic sites (including hydrocarbons, steroids, polyaromatic hydrocarbons, carbamates, aldehydes and ketones) or for those separated using normal phase HPLC, APCI is a mass flow-dependent technique.

Usually capable of a wider dynamic range than ESI, it is superior for samples and sample matrices where ion suppression is a problem using ESI due to a competition for charge. However, it is more challenging to use on analytes with a mass-to-charge ratio approaching 1,000 or higher.

Sensitivity Specifications
APCI(+) SIM S/N >100:1 RMS for 1 pg/µl reserpine injected @ 1.0 ml/min MeOH/H_2O
APCI(–) SIM S/N >100:1 RMS for 20 pg/µl *p*-nitrophenol injected @ 1.0 ml/min MeOH/H_2O

Source Flow Rate Capabilities
APCI 0.05–1.5 ml/min (no split)



By housing the corona needle inside the probe, PerkinElmer has created a unique Field-Free APCI source that delivers unsurpassed sensitivity and more reliable results.

PerkinElmer has developed an innovative APCI design to extend the domain of (U)HPLC/MS applications for compounds with low polarity. In traditional APCI sources, the corona discharge needle must be adjusted to be positioned directly in front of the entrance to the MS. There, the vaporized liquid sample is exposed to a corona discharge and reagent ions ionize analyte molecules—primarily through proton exchange—prior to being guided into vacuum for mass spectrometric analysis.

The new, patent-pending, Field-Free APCI source on the Flexar SQ 300 MS is configured to shield the intense potential field generated at the corona discharge needle tip from defocusing the ion trajectory towards the orifice into vacuum. This is accomplished by having the corona needle fixed in position within the APCI probe itself. Using this Field-Free APCI source geometry, the ionization step and the sample ion-focusing step can effectively be decoupled and thus optimized independently. This design allows unmatched levels of sensitivity, even at very low flow rates and excellent peak shape in the most demanding UHPLC separations.

Cleaner Source. Superior Sensitivity.

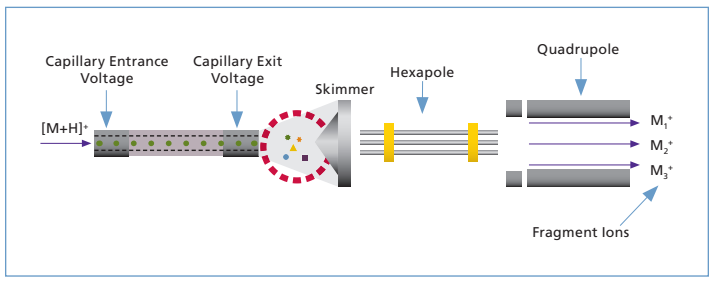
With its choice of source types and patented angled probe design, the Flexar SQ 300 MS can handle a wide range of solvent compositions, including a broad array of non-volatile buffers. When such buffers are required, the clean ionization environment can be maintained by using a dedicated probe and by cleaning the removable capillary cone, an easily accessible contamination shield protecting the capillary entrance.

Ion Transfer.

To ensure optimum sensitivity in any LC/MS system, ion transmission must be maximized between the source and the detector. Today, manufacturers take varying approaches to achieve this optimization on their instruments, often with very different results.

The Flexar SQ 300 MS features a proprietary dielectric capillary connecting the ion source and ion optics that allows a complete decoupling of the two regions. This separation allows the settings for each area to be controlled and optimized more easily for a particular application.

In particular, the capillary exit voltage can be modulated for effective and reliable front-end Collision Induced Dissociation (CID) without modifying settings in the ion source. In addition, due to the exceptional desolvation efficiency of the source, vapor is prevented from entering the capillary, ensuring a more reliable CID process and more reproducible fragmentation.

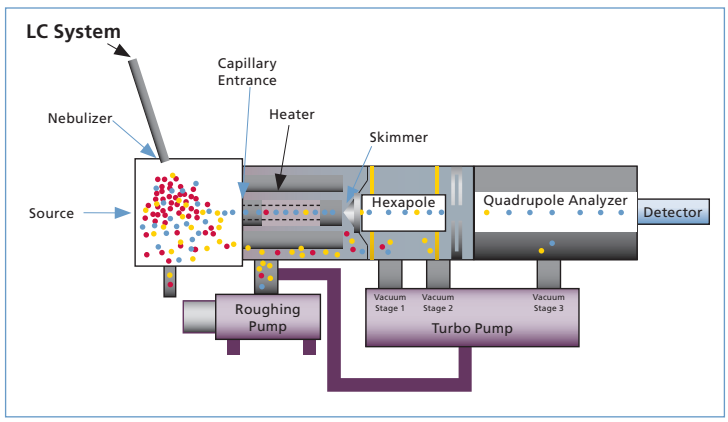


Fragmentation zone on the Flexar SQ 300 MS where CID is performed to obtain detailed structural information.

Ions seeded in gas pass through the capillary at Mach 2 (315 meters/sec) before being accelerated through the skimmer (as the gas expands) at supersonic speeds. As the pressure drops at the entrance to the transfer hexapole, ions are slowed and have zero kinetic energy, then reaccelerated and precisely focused by voltages applied to the hexapole rods.

The hexapole ion guide is unique in that it traverses 2 vacuum regions while delivering the ion beam to the analytical quadrupole. This patented ion guide technology significantly improves ion transmission across pressure boundaries, improving the overall sensitivity of the system.

The Flexar SQ 300 MS features a 4-stage vacuum system that goes from atmospheric pressure to ~2.5 millibar at the capillary exit. The 3-stage turbo molecular pump then gradually drops the pressure from ~2.5 millibar to ~$5x10^{-6}$ millibar in the analyzing quadrupole region.



The multi-stage ion path of the Flexar SQ 300 MS enhances ion transmission for exceptional sensitivity and speed.

Mass Analyzer.

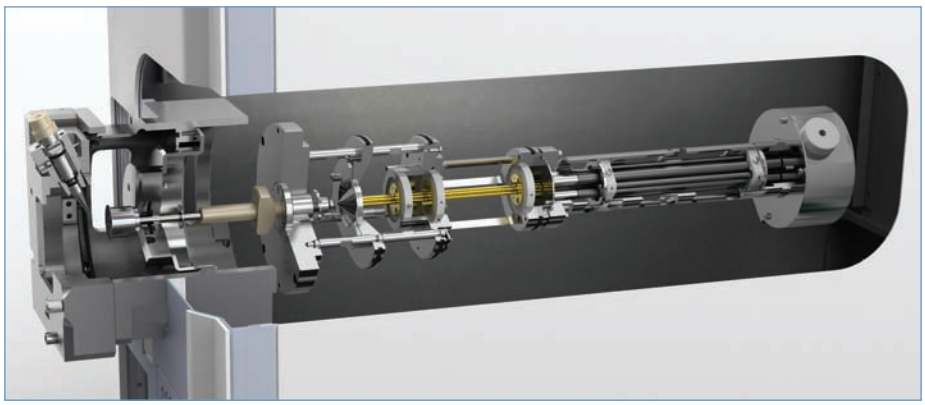
The role of the analyzing quadrupole in a LC/MS detector is to transmit as many ions of a desired mass-to-charge ratio as possible while filtering out interfering ions. Essentially, the quadrupole acts as a mass filter, enabling users to specify a "window" through which a certain mass may pass while all others are ejected.

The four rods of the analyzing quadrupole have an applied DC voltage and a superimposed RF voltage (amplitude) with a fixed RF frequency (typically between 0.8 and 1.5 MHz) applied to them. One pair of opposing rods features a positive DC voltage and RF amplitude, while the other pair has an equal and opposite negative set of conditions. By adjusting the DC and RF amplitudes, the quadrupole performs its mass filtering, allowing only ions with a specific mass-to-charge ratio to maintain a stable trajectory and pass through to the detector.

The analyzing quadrupole on the Flexar SQ 300 MS offers exceptionally fast scanning (10,000 u/sec). This exceptional speed allows the instrument to be run in multiple acquisition modes—SIM (Selected Ion Monitoring), Scan, or any combination—for detailed confirmatory analysis and superior quantitative sensitivity. Multi-channel and positive/negative acquisition modes may also be employed to produce high quality spectra with unknown samples.

For additional performance and sensitivity, the Flexar SQ 300 MS features a set of pre-quad filters that act as an additional focusing lens, narrowing the beam of ions and channeling it into the center of the quadrupole.

The final step in generating information-rich data is to count the number of analyte ions that make it through the analyzing quadrupole. With a best-in-class digital pulse-counter detector capable of exceptionally fast data acquisition rates, the Flexar SQ 300 MS ensures superior chromatographic peak resolution and complete spectral integrity even when the instrument is being used in conjunction with challenging UHPLC separations.



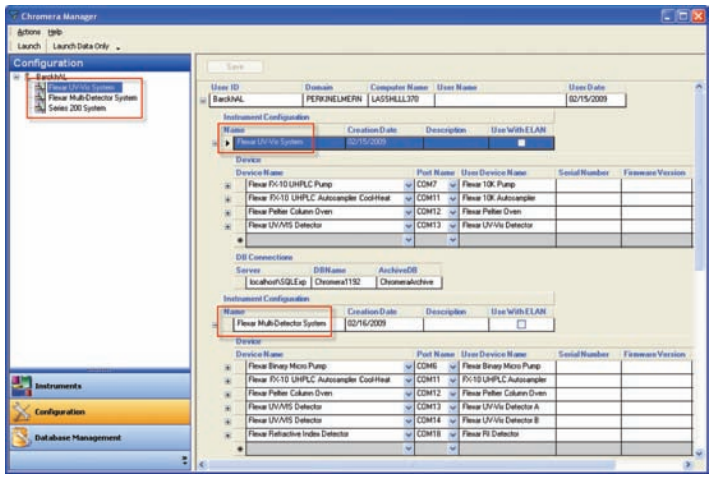
The patented, state-of-the-art hexapole ion guide, traversing multiple vacuum stages, enhances ion transmission to the fast-scanning analyzing quadrupole and best-in-class detector.

CHROMERA CHROMATOGRAPHY DATA SYSTEM

Any laboratory instrumentation is only as good as the software behind it. For maximum productivity and long-term return on investment (ROI), a Chromatography Data System (CDS) needs to be intuitive, application-focused and scalable. And when chromatography is being used in combination with mass spectrometry, the software also needs to provide complete control of both techniques and to allow the smooth integration of data from the two systems.

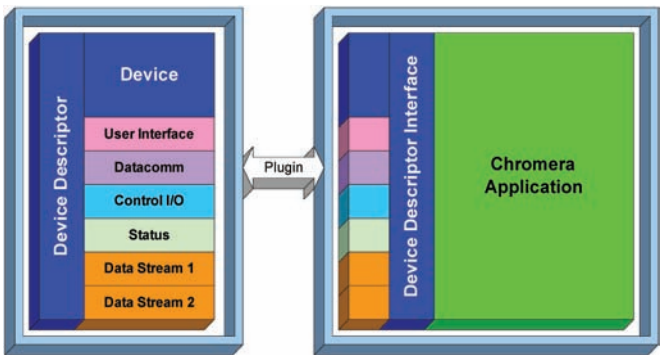
PerkinElmer's Chromera® CDS was specifically developed for chromatographers, but built to provide full mass spectrometer control and spectral data handling. This unparalleled integration enables the software to smoothly transition from one analytical technique to the other and to seamlessly merge data from the two instrument types.

Chromera allows users to build and continually adapt a LC/MS system to suit their specific needs. By using unique, patented Instrument Device Descriptors, users can quickly and easily create custom configurations on the fly.

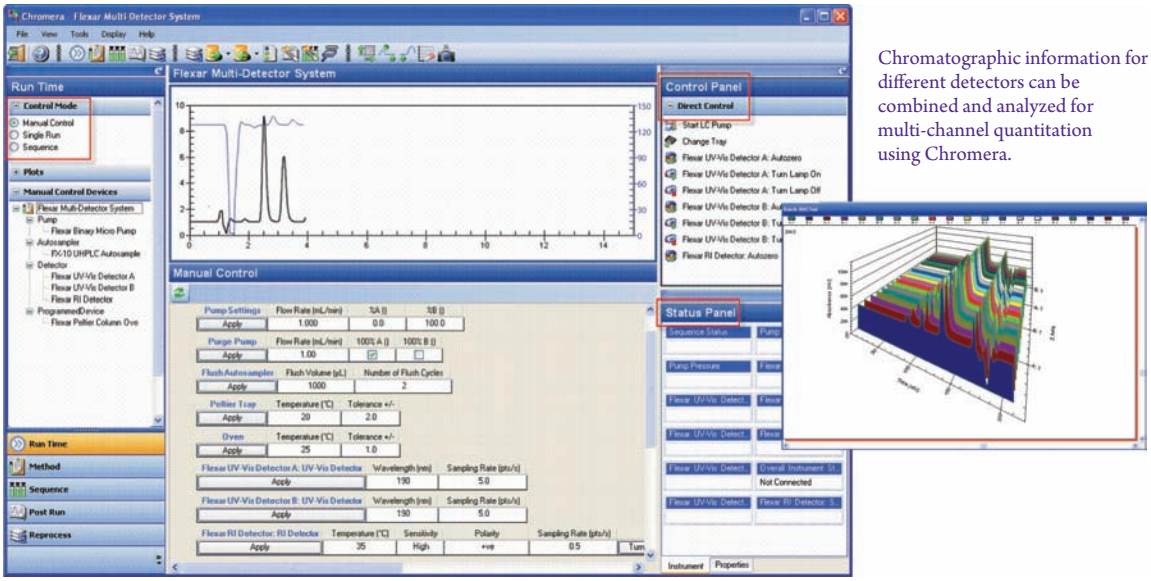


The Chromera Manager assembles device modules into instrument configurations. Shown here: two of three instrument configurations—note the different number of devices for each.

Instrument Device Descriptors are in charge of controlling the devices as well as channeling recordable information from the device modules to Chromera. Not just detector signals, but also flows, pressures, temperatures and other data acquired over the length of an experiment. The Device Descriptors also "describe" additional parameters of the analytical modules: behavior, rules, graphical user interface. These parameters populate the sequence and method editor user interface in Chromera allowing the presentation of only configuration-related, relevant information and communicating the behavior of every module to the CDS.



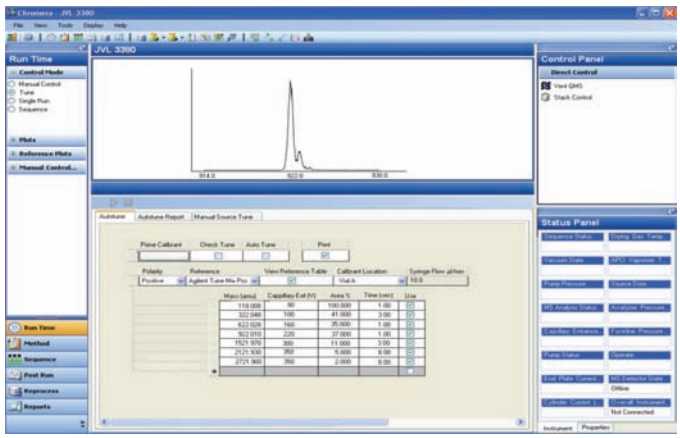
This flexibility in device integration includes the option to mix and match multiple detectors and simultaneously display their results within the same user interface. This enables different detector types to be used in conjunction with each other—e.g. UV with the Flexar SQ 300 MS—for additional confirmatory analysis and application flexibility.



Chromatographic information for different detectors can be combined and analyzed for multi-channel quantitation using Chromera.

With a clear, intuitive workflow, Chromera simplifies each and every step of a quantitative analysis. All tools and information are never more than a couple of clicks away so you can quickly and easily control every aspect of your Flexar SQ 300 MS system and generate information-rich data specifically targeted to your application. A full diagnostic suite also displays the status of mass spectrometer functions in real-time.

Chromera CDS also features a unique one-click Autotune for the fastest, easiest instrument tuning and calibration in both polarities. Tuning can be performed across the entire mass range of the instrument (20-3,000 mass units) or limited to a user-specified mass range for superior speed and results.



One-click Autotune ensures reliable day-to-day operation and reproducibility.

Parameters can be changed during a sequence, enabling you to do everything from adding or deleting samples to changing acquisition modes or modifying calibration standards. Checkpoints can also be activated at certain steps in a sequence (real-time or post run) to automatically pause the system, allowing you to review data or results before the instrument proceeds to the next step.

For added flexibility and more application-specific data generation, Chromera features customizable report functionality. Users can select exactly the type and style of information they want displayed for simpler, clearer, more relevant results.

Index of Applications

FOOD/NUTRACEUTICAL

PHARMACEUTICAL

CHEMICAL/INDUSTRIAL

ENVIRONMENTAL

CLINICAL

Index of Analytes

Continued on next page.

Index of Analytes

Analysis of Water Soluble Vitamins Using UHPLC/SQ MS

FOOD/NUTRACEUTICAL

Water soluble vitamins are essential nutrients for the human body and are naturally present in food or added as supplements to food and beverages. Traditionally, hydrophilic compounds such as water soluble vitamins are separated on column using ion pair reagents. We describe a UHPLC/SQ MS method to separate, identify, and quantify some essential water soluble vitamins without the use of ion pair reagents.

Experimental Conditions			
Target Analytes:	Thiamine, niacinamide, pyridoxine, pantothenate, riboflavin		
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee Supra™ Aqueous C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: 10 mM ammonium formate, pH 3		
	B: acetonitrile containing 10 mM ammonium formate, pH 3		
Flow Rate:	0.8 ml/min with a post column split of 0.4 ml/min into MS		
Injection Volume:	1 µl in partial fill mode		
Gradient:	Time (min)	%A	%B
	0	100	0
	1	100	0
	0.1	80	20
	2	40	60
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
The $[M+H]^+$ ions were monitored in two different time periods:			
Time Period 1: (0-1.1 min) SIM ions 265.1, 123.1, 170.1 for thiamine, niacinamide and pyridoxine respectively; dwell time of 100 ms each			
Time Period 2: (1.1-2.5 min) SIM ions 220.1 and 377.2 for pantothenic acid and riboflavin respectively; dwell time of 120 ms each			
Capillary Exit Voltage:	Optimized for each vitamin, ranged from 50 to 120 V		

Results.

The water soluble vitamins were analyzed in SIM mode (Figure 1). Calibration curves for the vitamins pyridoxine, niacinamide and thiamine are shown in Figure 2. Instrument detection limits (S/N > 3) were as low as 1-2 ng/ml for all of the analytes.

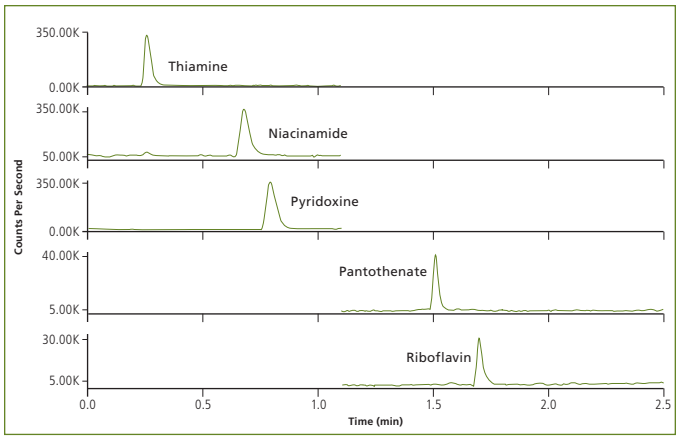


Figure 1. SIM chromatograms of water soluble vitamins.

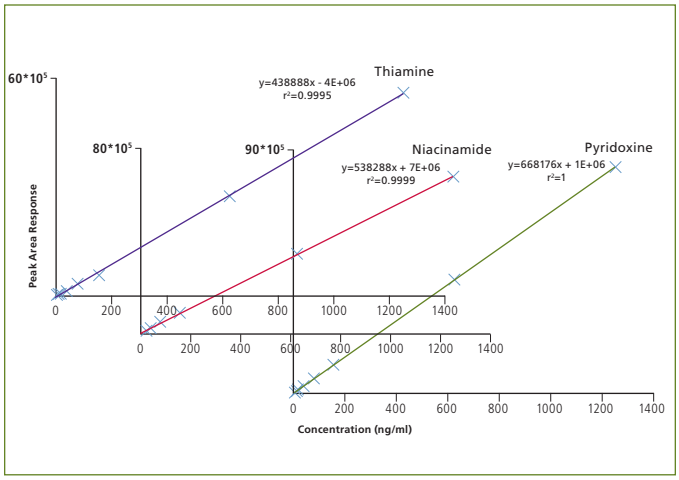


Figure 2. Calibration curves of the water soluble vitamins thiamine, niacinamide and pyridoxine.

Analysis of Quinolones by UHPLC/ Fluorescence/SQ MS

FOOD/NUTRACEUTICAL

Quinolones are structurally related antibiotics that are used in human and veterinary medicine. European Union (EU) has set tolerance levels for quinolones while FDA has banned the use of some fluoroquinolones in products of animal origin. We present a method to measure nine quinolones including some fluoroquinolones using UHPLC for separation followed by detection with a single quadrupole mass spectrometer and a fluorescence detector (Figures 1 and 2).

Experimental Conditions			
Target Analytes:	Norfloxacin, ofloxacin, ciprofloxacin, enoxacin, cinoxacin, nalidixic acid, pipemidic acid, enrofloxacin, flumequine		
Standard Concentrations:			
Concentration of ciprofloxacin, nalidixic acid and flumequine were each at 2.5 µg/ml, while the remaining quinolones were at 4 µg/ml.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee Supra™ C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water with 0.1% formic acid B: acetonitrile with 0.1% formic acid		
Flow Rate:	0.8 ml/min with a post column split of 0.4 ml/min each into fluorescence and MS detectors		
Injection Volume:	1 µl in partial fill mode		
Gradient:	Time (min)	%A	%B
	0	90	10
	3	85	15
	2	80	20
	0.3	60	40
	0.7	60	40
Fluorescence Detector Conditions:			
Excitation:	275 nm		
Emission:	400 nm		
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	200-400 m/z		
Scan Rate:	400 u/sec		
Capillary Exit Voltage:	100 V		

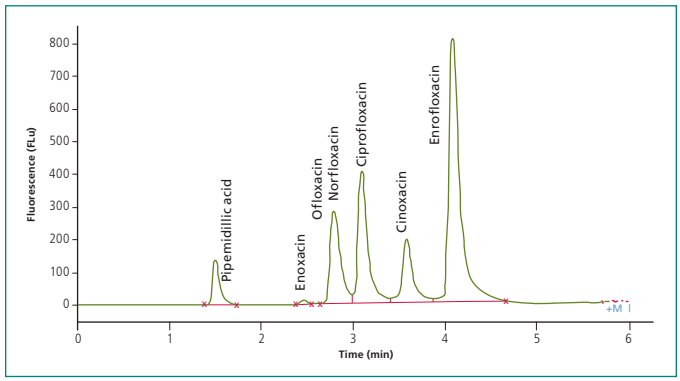


Figure 1. Separation and fluorescence detection of quinolones.

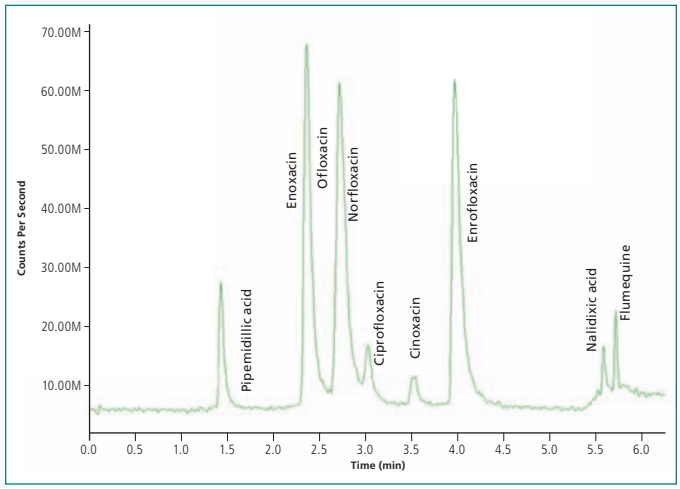


Figure 2. TIC of quinolones. Note the response of nalidixic acid and flumequine by MS detection was significantly higher compared to fluorescence detection of these analytes.

Results.

All nine quinolones were separated by UHPLC except for norfloxacin and ofloxacin which coeluted. Due to coelution, norfloxacin and ofloxacin cannot be quantified individually by fluorescence detection. However, it is possible to quantitate the coeluting quinolones by mass spectrometry (Figure 3) since selected ions unique to the compounds can be monitored.

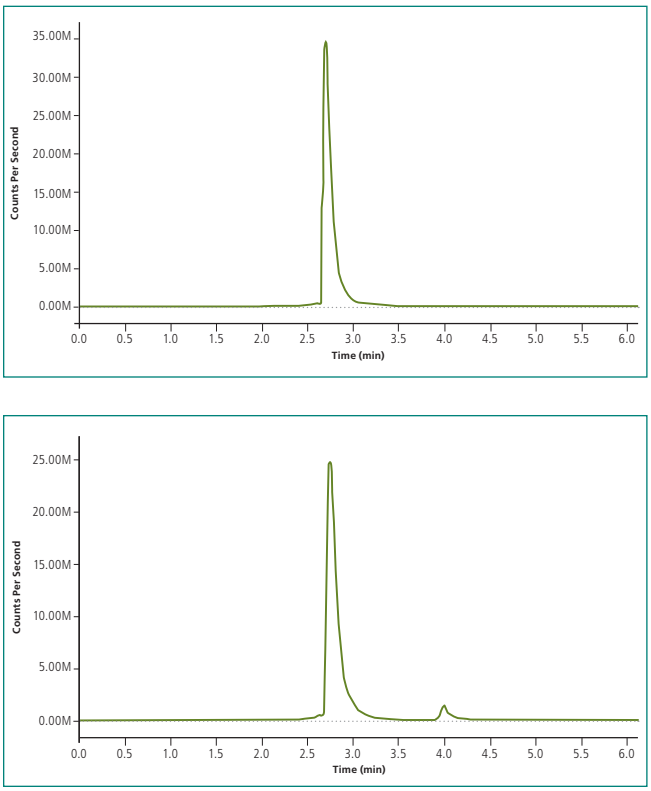


Figure 3. Extracted ion chromatograms (EICs) by MS detection of the coeluting compounds norfloxacin (320.1 m/z shown above) and ofloxacin (362.2 m/z shown here).

The sensitivity of MS detection can be improved by monitoring in SIM mode for the $[M+H]^+$ ion for each target analyte. Selectivity by MS detection can be further improved by monitoring for the product ion along with the precursor ion produced by front-end CID. A representative calibration curve is shown for enrofloxacin generated by monitoring in SIM mode for the $[M+H]^+$ ion and the fragment ion resulting from loss of CO_2 (Figure 4). Enrofloxacin can be quantitated in an unknown sample by monitoring, both for the precursor and product fragment which gives additional confirmation of the presence of the analyte in a given matrix.

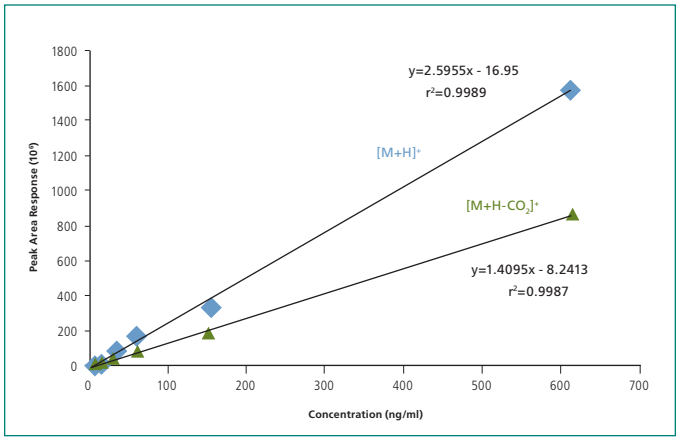


Figure 4. Calibration curve for enrofloxacin. Both $[M+H]^+$ ion (360.2) and fragment $[M+H\text{-}CO_2]^+$ (316.2) were monitored (dwell time of 150 ms each).

Determination of Aflatoxins in Raw Peanuts by UHPLC/SQ MS

FOOD/NUTRACEUTICAL

Aflatoxins are harmful or fatal to livestock and are considered carcinogenic to animals and humans. They are secondary metabolites produced by *Aspergillus Flavus* and *Aspergillus Parasiticus* fungi. Aflatoxins can accumulate in agricultural products such as cereals, peanuts, dried fruits, wine, etc. due to inadequate storage conditions. Several aflatoxins have been identified and the most ubiquitous ones are B1, B2, G1 and G2. We describe a UHPLC/SQ MS method to identify these aflatoxins in ground peanuts using immunoaffinity cartridges for sample clean up.

Experimental Conditions			
Target Analytes:	Aflatoxins B1, B2, G1, G2		
Sample Preparation Conditions:			
A fine powder of raw peanuts (5g) was spiked with aflatoxin G2 (20 ng/g) and aflatoxins B1, B2 and G1 (5 ng/g). A methanol/water (60:40) mixture (25 ml) was added to the sample and mixed thoroughly for 10 min. A control sample of ground peanuts containing no spike of aflatoxins was prepared similarly. The samples were centrifuged at 2,000 RPM for 10 min. The supernatant was passed through a 0.45 µm filter and an aliquot of the filtered solution (5 ml corresponding to 1 g of peanuts) was mixed with an equal volume of water. The sample was loaded on an immunoaffinity cartridge (Alfaprep, R-Biopharm, Darmstadt, Germany) at a flow of 1 ml/min. The immunoaffinity cartridges contained a gel suspension of monoclonal antibodies specific for aflatoxins B1, B2, G1 and G2. The cartridge was then washed with water (5 ml). Aflatoxins were eluted from the cartridge with methanol (1 ml). The cartridge was backflushed during elution to improve recovery. The sample was brought to about 0.5 ml and reconstituted to 1 ml with water and injected on the analytical system.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee™ Hres C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water with 0.1 % formic acid B: acetonitrile with 0.1 % formic acid		
Flow Rate:	0.5 ml/min		
Injection Volume:	3 µl in partial fill mode		
Gradient:	Time (min) 0 2.5 1	%A 75 60 60	%B 25 40 40
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
The $[M+H]^+$ ions for each of the aflatoxins were monitored in three different time periods:			
Time Period 1: (0-2.1 min) SIM ion 331.0 for G2; dwell time of 300 ms			
Time Period 2: (2.1-2.7 min) SIM ions 329.0 and 315.0 for G1 and B2 respectively; dwell time of 200 ms each			
Time Period 3: (2.7-3.5 min) SIM ion 313.0 for B1; dwell time of 300 ms			
Capillary Exit Voltage:	100 V		

Results.

This application's detection limits for each of the aflatoxins B1, B2 and G1, G2 by UHPLC/SQ MS was estimated at 0.4 ng/ml (S/N > 3). Calibration curves of all the aflatoxins showed excellent linearity for a concentration range of 0.5 ng/ml. A representative calibration curve for aflatoxin B1 is shown in Figure 1. The recovery of aflatoxin G2 (20 ppb) spiked in peanut samples as internal standard and purified over the immunoaffinity cartridge was estimated at 72 ± 10.7% (n = 3). The recovery of spiked aflatoxins B1, B2 and G1 in the peanuts using G2 as internal standard was estimated in the range of 90-128%. Figure 2 shows the SIM ions of the aflatoxins analyzed in the peanut samples with and without spike of each of these analytes.

The FDA guidelines recommend an action level of 20 ppb of total aflatoxins in peanuts and other cereals for both human and animal consumption. With the Flexar SQ 300 MS it was possible to easily quantify 5 ppb of aflatoxins in peanuts using immunoaffinity clean up, fully addressing FDA requirements reaching sensitivity similar to fluorescence detection.

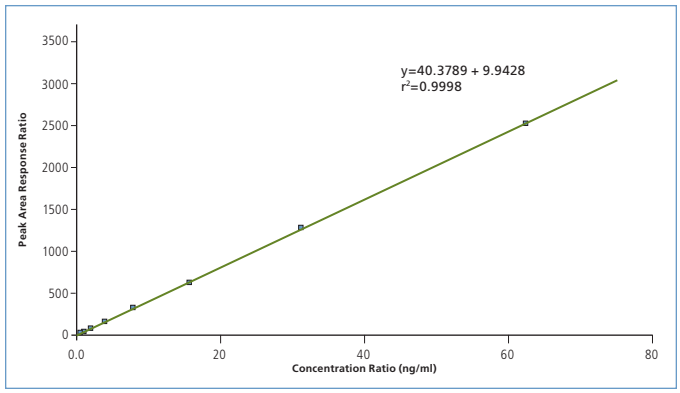


Figure 1. Calibration curve for aflatoxin B1 for a concentration range of 0.5 - 63 µg/ml.

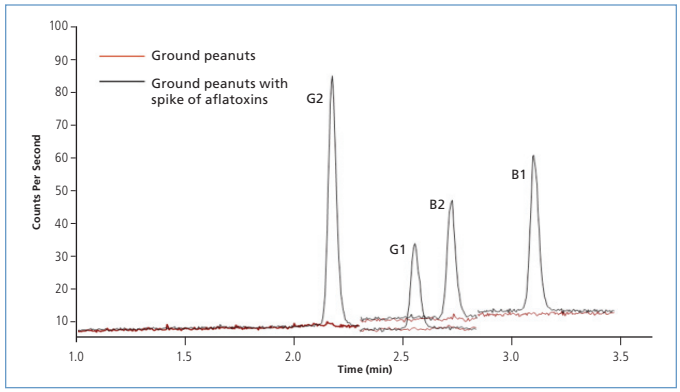


Figure 2. Overlaid SIM chromatograms of $[M+H]^+$ ions of aflatoxins B1, B2, G1 in ground peanuts spiked at 5 ppb level with aflatoxin G2 used as internal standard (spiked at a concentration of 20 ppb). A control sample of ground peanuts with no spike of aflatoxins is also shown.

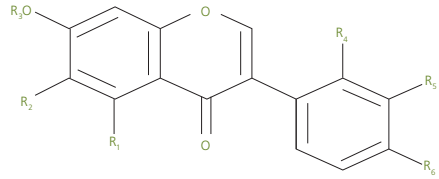
Quantitative Analysis of Soy Isoflavone Compounds Using UHPLC/SQ MS

FOOD/NUTRACEUTICAL

Isoflavones are non-steroidal compounds with estrogenic effects and can be found in many foods; the best known source is the soy bean (*Glycine max*). Recent studies have demonstrated that isoflavones have potent antioxidant properties which can reduce the long-term risk of cancer by preventing free radical damage to DNA. They can also be used for treating menopause and osteoporosis.

A quantitative UHPLC/SQ MS method is presented to detect isoflavone aglycones (genistein, diaidzein and glycitein) and isoflavone glycosides (genistin, diaidzin and glycitin) using a single quadrupole MS detector and its front-end Collision Induced Dissociation (CID) capability.

Experimental Conditions			
Target Analytes:	Diaidzin, glycitin, genistin, diaidzein, glycitein, genistein		
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee™ Analytical C18 column (2.1 mm x 100 mm, 3 µm)		
Mobile Phase:	A: acetonitrile with 5 mM acetic acid B: water with 5 mM acetic acid		
Flow Rate:	0.5 ml/min		
Injection Volume:	2.5 µl		
Gradient:	Time (min) 0 6	%A 15 45	%B 85 55
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive and Negative modes		
Selected Ion Monitoring (SIM) Mode:	Reported in Figure 1; dwell time of 100 ms each		
Capillary Exit Voltage:	100 V		



	R_1	R_2	R_3	R_4	R_5	R_6	$[M-H]^-$	$[M+H]^+$
Diaidzin	H	H	Glucose	H	H	OH	415	417
Glycitin	H	OCH_3	Glucose	H	H	OH	445	447
Genistin	OH	H	Glucose	H	H	OH	431	433
Diaidzein	H	H	H	H	H	OH	253	255
Glycitein	H	OCH_3	H	H	H	OH	283	285
Genistein	OH	H	H	H	H	OH	269	271

Figure 1. Structures of isoflavones.

Results.

A 6 minute UHPLC/SQ MS method was developed for measurement of 6 isoflavone compounds (Figure 2). This study determined that negative ion mode is more suitable than positive ion mode for 4 out of 6 isoflavone compounds (Figure 3) analyzed (genistein, genistin, diaidzein and diaidzin). The other two compounds (glycitein and glycitin), which contain a methoxy group, have comparable sensitivity in both ionization modes. Using this optimized method, the detection limit of 6 isoflavone compounds ranged from 0.56 to 2.29 pg.

Reference.

ASMS 2010 Poster: Effect of Ion Polarity on the Determination of Specific Isoflavone Compounds Using LC-MS

Avinash Dalmia; et al.

PerkinElmer Inc., Shelton, CT 06484

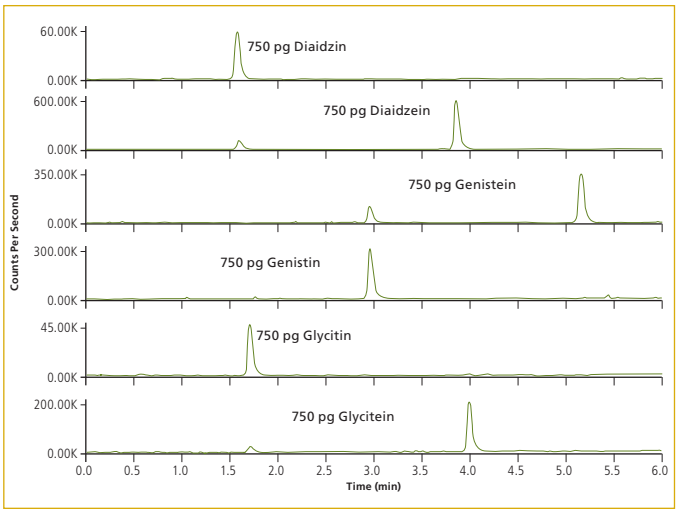


Figure 2. SIM chromatograms of isoflavones in negative ion mode.

Negative vs. Positive Ion Sensitivity				
Compound	S/N Positive Ion Mode	S/N Negative Ion Mode	LOD (pg) Positive Ion Mode	LOD (pg) Negative Ion Mode
Genistein	1282	2318	1.75	1.03
Diaidzein	1329	3023	1.69	0.74
Glycitein	1054	1008	2.13	2.23
Genistin	1016	3978	2.21	0.56
Diaidzin	668	1084	3.38	2.07
Glycitin	861	982	2.61	2.29

Figure 3. Negative ion mode is more sensitive than positive ion mode for 4 out of 6 isoflavone compounds. The other two compounds (glycitein and glycitin) have comparable sensitivity in both ionization modes. S/N has been calculated for a concentration of 750 pg each.

Measurement of the Active Compound (P57) in *Hoodia Gordonii* Plant Extract by UHPLC/SQ MS

FOOD/NUTRACEUTICAL

Hoodia gordonii is an herb that is traditionally used in Southern Africa for its appetite suppressant properties. Its active principle is an oxypregnane glycoside known as P57. *Hoodia gordonii* is listed as an endangered species and is in great demand; hence, the potential for the product to be adulterated is very high. It is therefore important to chemically characterize the active compound present. We describe herein a UHPLC/SQ MS based method to identify and quantify P57 in a herbal extract.

Experimental Conditions			
Target Analyte:	Oxypregnane glycoside (P57)		
Sample Preparation Conditions:			
A methanolic extract of *Hoodia gordonii* was obtained from the Food and Drug Administration (FDA), Silverspring, MD. The extract was injected without further purification to analyze in Full Scan mode and subsequently diluted 1:40 in 70% acetonitrile/water for SIM (Selective Ion Monitoring) mode. A standard solution of P57 (1mg/ml) was purchased from ChromaDex, Inc (Irvine, CA) and was diluted to generate a calibration curve.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee™ Hres C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water with 0.05% formic acid B: acetonitrile with 0.05% formic acid		
Flow Rate:	0.5 ml/min		
Injection Volume:	1 µl in partial fill mode		
Gradient:	Time (min) 0 15	%A 70 60	%B 30 40
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	250-1300 m/z		
Scan Rate:	1000 u/sec		
Selected Ion Monitoring (SIM) Mode:	$[M+Na]^+$ of P57 monitored at 901.5 m/z; dwell time of 300 ms		
Capillary Exit Voltage:	100 V for Scan data and 70 V for SIM data		

Results.

The TIC of the *Hoodia gordonii* extract showed several compounds eluting in the range of 250-1300 m/z. However, the extracted ion chromatogram (EIC) of the $[M+Na]^+$ ion of P57 showed 6 compounds at various retention times. Of these 6 compounds, the compound at 10.57 min had a spectrum and retention time which matched the standard of P57 shown in Figure 2. The spectrum of P57 contained a complete mass spectral fingerprint of all the major components of the molecule, including the loss of the various sugars attached to the aglycone, water, and the tigloyl moiety from the $[M+H]^+$ ion.

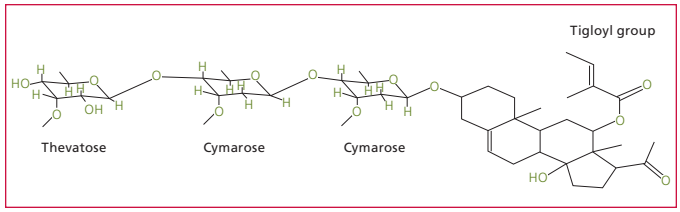


Figure 1. Molecular structure for P57.

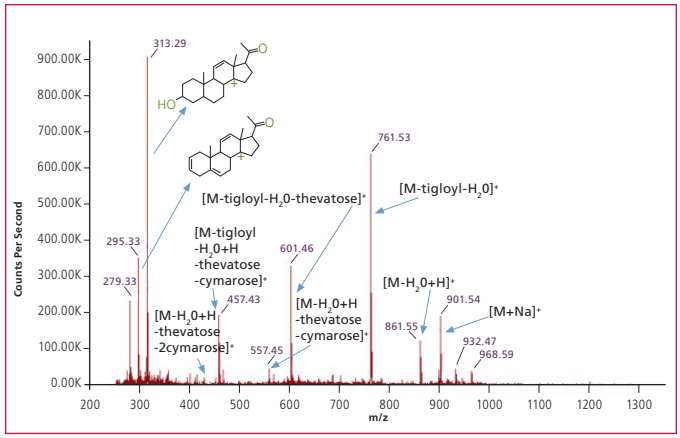


Figure 2. The mass spectrum of P57 was obtained by use of the front-end CID capability with a capillary exit setting of 100 V. The spectrum shows the $[M+Na]^+$ of P57 and the loss of the various fragments from the molecule.

The amount of P57 in the extract was determined by external calibration using the standard of P57. The method detection limits for the $[M+Na]^+$ adduct ion (901.5 m/z) of P57, monitored in SIM mode was estimated at 5 pg on column (S/N > 3). The calibration curve shows an excellent linearity (Figure 3, r^2=0.9998). The *Hoodia gordonii* sample was injected multiple times (n=6) and the concentration of P57 was calculated off the calibration curve. Figure 4 shows the overlays of all 6 injections with excellent reproducibility in retention time and in area response. The average concentration of P57 in the sample was estimated at 30.92 µg/ml of extract.

Conclusions.

We were able to obtain an excellent mass spectral fingerprint for the appetite suppressant molecule P57 in the herb *Hoodia gordonii* using the front-end CID capability of the Flexar SQ 300 MS. Using the SIM capability, we were also able to quantify P57 in an extract of *Hoodia gordonii*.

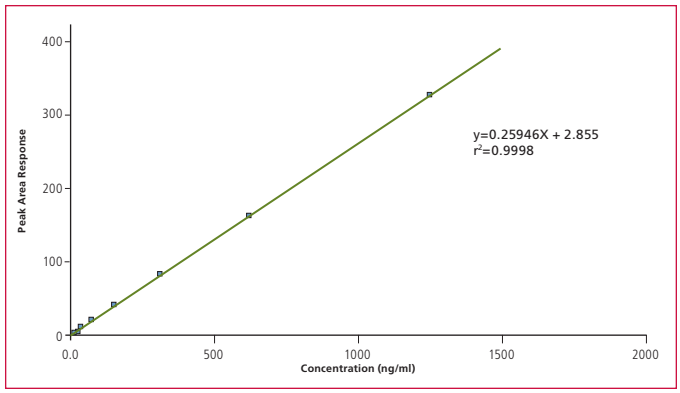


Figure 3. Calibration curve for P57, in the range of 5-1250 ng/ml. The Flexar SQ 300 MS operated in SIM mode (dwell time 300 ms).

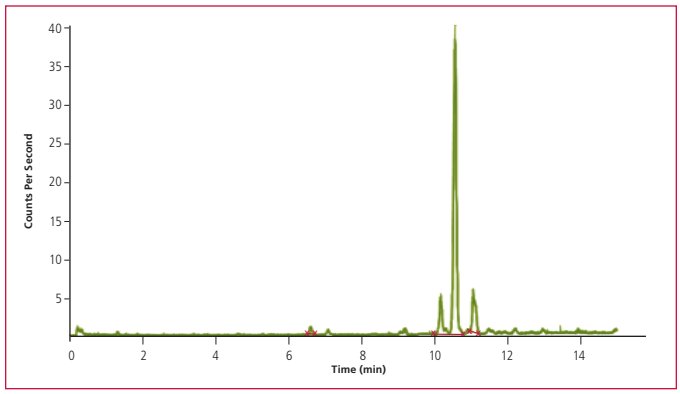


Figure 4. Chromatogram overlay of 6 consecutive injections of *Hoodia gordonii* extract, diluted 1:40 and analyzed for P57 in SIM.

Transferring a HPLC Fluorescence Method for Analyzing Ergot Alkaloids to a UHPLC/SQ MS Method

FOOD/NUTRACEUTICAL

Ergot alkaloids are mycotoxins produced by fungi such as *Claviceps* that live on grass and grain. Cattle that consume these alkaloids in grass can experience severe poisoning that can affect their central nervous system, immune and reproductive systems. In the United States alone, ergot alkaloid poisoning results in a billion dollars of annual damage to livestock. We describe a UHPLC/SQ MS method for measuring 8 ergot alkaloids. The method was transferred from HPLC to UHPLC conditions using a PerkinElmer Flexar UHPLC pump with a sub-2 micron column.

Experimental Conditions			
Target Analytes:	Lysergic acid, ergonovine, lysergol, ergovaline, ergotamine, ergocornine, α-ergocryptine, ergocristine		
Standard Concentrations:			
A sample of ergot alkaloids, 10 µg/ml of each standard in methanol, was diluted in mobile phase A to a concentration of 2 µg/ml each.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee™ HRes C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: 2.5 mM ammonium bicarbonate in water, pH 7.7 B: acetonitrile		
Flow Rate:	0.5 ml/min		
Injection Volume:	2 µl in partial fill mode		
Gradient:	Time (min)	%A	%B
	0	90	10
	1	90	10
	0.5	70	30
	4	30	70
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	150-700 m/z		
Scan Rate:	1000 u/sec		
Capillary Exit Voltage:	80 V		

Results.

A HPLC method for analyzing ergot alkaloids uses a PerkinElmer Series 200 pump with a PerkinElmer fluorescence detector on a Phenomenex C18 column (4.6 mm x 150 mm, 3 µm). The analysis time including column equilibration was 30 min (Figure 1). The method transferred to UHPLC conditions resulted in a 9 min analysis time including column equilibration time (Figure 2; concentrations injected different from Figure 1 sample). A total savings of 21 min per run was achieved by the UHPLC separation. The flow rate used for the HPLC sample analysis was 1 ml/min as opposed to a flow of 0.5 ml/min used for UHPLC separation, resulting in a solvent saving of ~26 ml per sample analysis.

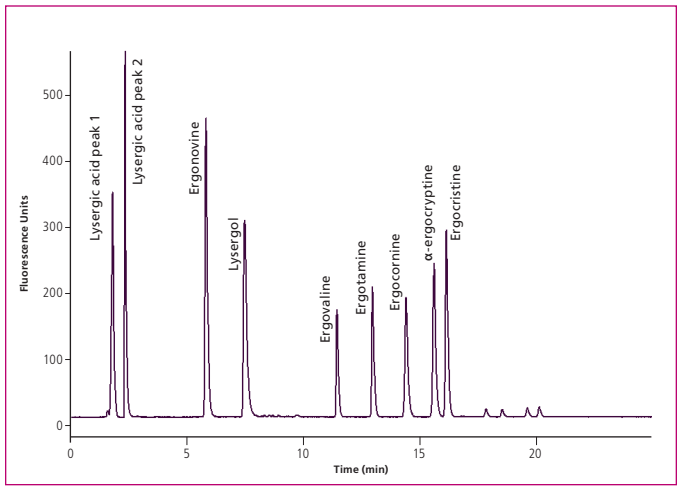


Figure 1. HPLC separation of ergot alkaloids on a 4.6 mm x 150 mm, 3µm column (Phenomenex) with fluorescence detection. The analysis time including equilibration time was 30 min.

Conclusions.

The mass spectrometer detector offers excellent specificity and sensitivity, as it is based on measuring ions characteristic of the analytes. The extracted ion chromatograms (EICs) of the $[M+H]^+$ ions of each of the alkaloids are shown in Figure 3. When further sensitivity is required Selective Ion Monitoring (SIM) is the acquisition mode of choice.

By transferring a HPLC method to UHPLC conditions, we can significantly scale down analysis time, solvent consumption and improve overall sample throughput.

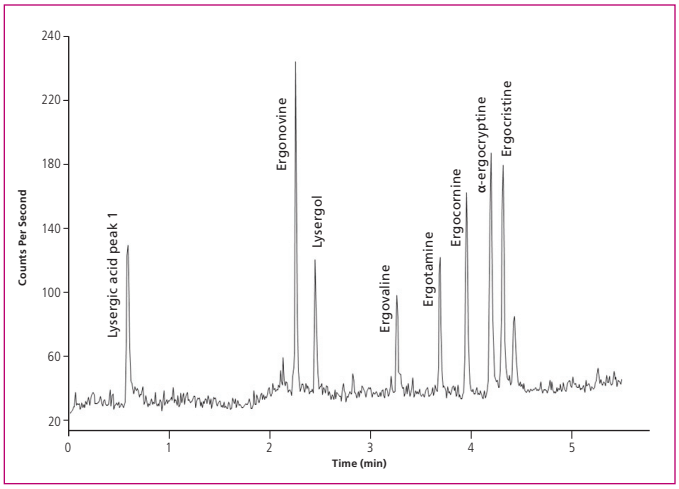


Figure 2. UHPLC separation of ergot alkaloids on a 2.1 mm x 50 mm, 1.9 µm column (PerkinElmer, Brownlee HRes C18) with MS detection in Full Scan mode. The analysis time including equilibration time was 9 min.

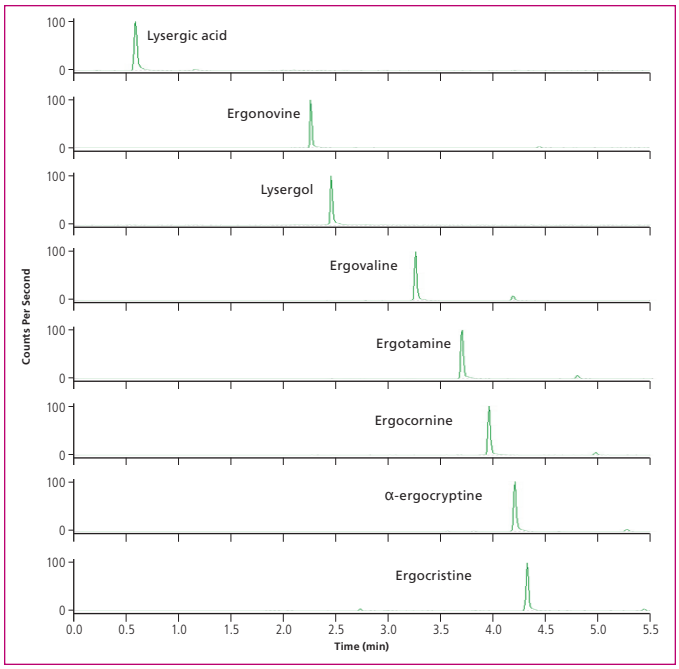


Figure 3. Extracted ion chromatograms of the molecular ion of each of the ergot alkaloids.

Rapid Determination of Sudan Dyes by UHPLC/SQ MS

FOOD/NUTRACEUTICAL

Sudan dyes are azo dyes that are used legally in the textile and leather industries for coloring purposes. However, these carcinogenic dyes have been illegally added as coloring agents in food, especially spices. We present a UHPLC/SQ MS method to detect four Sudan dyes using the Selected Ion Full Ion (SIFI) method. A SIFI experiment is a powerful tool to simultaneously obtain both Full Scan data—giving full spectral information—and Selected Ion Monitoring (SIM) data, to achieve the best sensitivity.

Experimental Conditions			
Target Analytes:	α-Naphthyl red, 4-phenylazophenol, Sudan II, Sudan IV		
Standard Concentrations:			
A 10 µg/ml concentration of each of the Sudan dyes was made in acetonitrile and used for the SIFI experiment. A calibration curve for the dyes was set up for concentration ranges between 5-500 ng/ml.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	Knauer BlueOrchid175 C18 column, (2 mm x 50 mm, 1.8 µm)		
Mobile Phase:	A: water containing 0.1% formic acid B: acetonitrile containing 0.1% formic acid		
Flow Rate:	1 ml/min with a post column 50/50 split into MS		
Injection Volume:	1 µl in partial fill mode		
Gradient:	Time (min) 0 0.5 1 0.5	%A 30 30 0 0	%B 70 70 100 100
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	100-390 m/z		
Scan Rate:	2500 u/sec		
Selected Ion Monitoring (SIM) Mode:	SIM ions 199.2, 248.2, 277.2, 381.3 were each monitored for phenyl-azo-phenol, α-naphthyl red, Sudan II and Sudan IV respectively; dwell time 120 ms each. Full Scan and SIMs acquired simultaneously in SIFI mode.		
Capillary Exit Voltage:	160 V was set for Scan mode to fragment the parent ions. In SIM mode, the voltage was optimized for each analyte and was in the range of 90-115 V.		

Results.

The separation of the four Sudan dyes was achieved within two min. Figure 1 shows the SIM ions of the individual Sudan dyes collected during a SIFI experiment along with a scan. The scan was performed at high capillary voltage to generate Collision Induced Dissociation (CID) to obtain complete mass spectral information during the run. The two peaks associated with α-naphthyl red were most likely isomers since they had identical spectra.

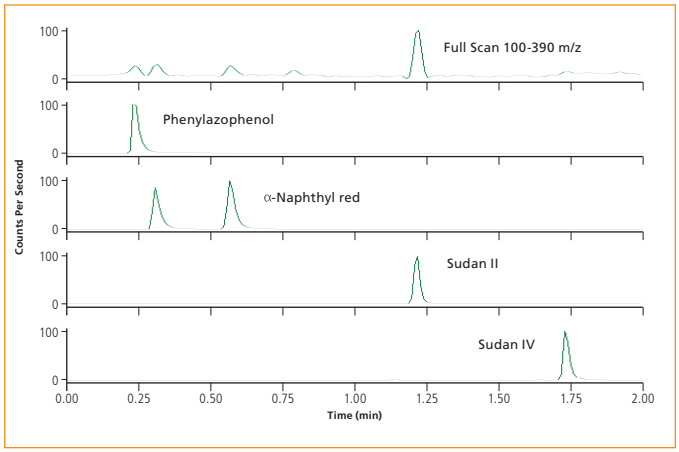


Figure 1. SIFI experiment of Sudan dyes by UHPLC/SQ MS. The scan experiment was performed at high capillary exit voltage to induce fragmentation and simultaneously the SIM ions of phenylazophenol (199.2 m/z), α-naphthyl red (248.2 m/z), Sudan II (277.2 m/z) and Sudan IV (381.3 m/z) were monitored at capillary exit voltages optimal for each SIM ion.

The spectrum of Sudan II resulting from the SIFI experiment is shown in Figure 2. The calibration curve (5-500 ng/ml) for Sudan II is shown in Figure 3. All of the Sudan dyes analyzed had detection limits of 5-10 pg on column.

Conclusions.

Using a SIFI method in UHPLC/SQ MS provides complete mass spectral data to confirm the presence of analytes and show the presence of non-target species, while simultaneously acquiring SIM ions to get the best detection limits for target compound quantification.

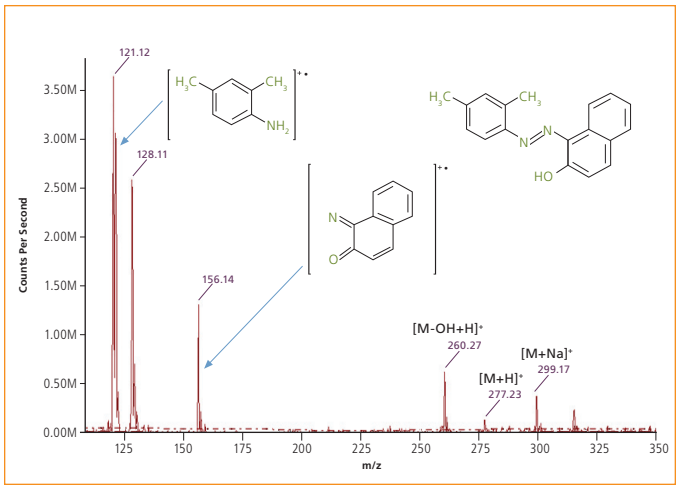


Figure 2. Mass spectrum of Sudan II obtained during the SIFI experiment confirming presence of Sudan II.

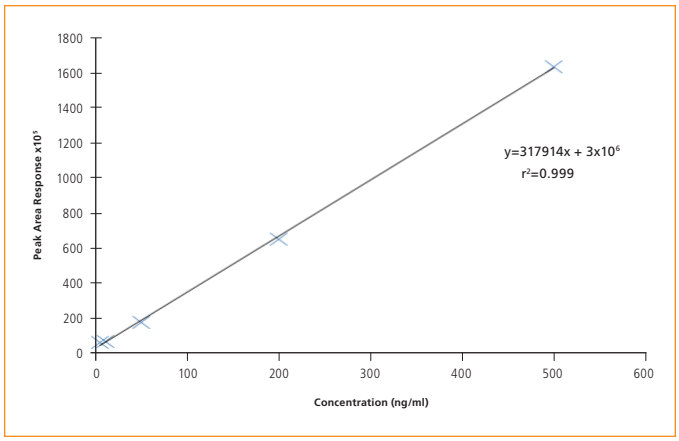


Figure 3. Calibration curve for Sudan II in the concentration range of 5-500 ng/ml.

Detection of Acrylamide in Potato Chips Using UHPLC/SQ MS

FOOD/NUTRACEUTICAL

Acrylamide is a known carcinogen found in heated and fried foods. The most common methods for acrylamide analysis are GC/MS, GC/FID, GC with a nitrogen specific detector or LC/MS/MS.

For GC analysis, acrylamide is often brominated to facilitate extraction into an organic solvent. We describe a UHPLC/SQ MS method that does not require a tedious derivatization and is a less expensive alternative to the LC/MS/MS assay.

Experimental Conditions			
Target Analyte:	Acrylamide		
Sample Preparation Conditions:			
Potato chips obtained from a grocery store were ground to a fine powder in a food processor. Water (10 ml) was added to the chips (1 g), spiked with 1,2,3,C13-acrylamide as the internal standard (50 ng/ml) and was gently shaken for ~20 min to extract acrylamide from the chips. The resulting solution was centrifuged at ~8000 RPM for 30 min. Three layers were observed from centrifugation. The middle aqueous layer (1 ml) was gently removed to avoid sampling any of the upper oily layer. The extract was loaded on a mixed mode MCAX SPE cartridge (Supelco, DSC-MCAX, 300 mg/3 ml) stacked in a series with the C18 cartridge (Supelco, DSC-18, 1g/6 ml) using SPE tube adaptors. Prior to sample loading, the cartridges were conditioned with methanol (2 ml) followed by DI water (2 ml). The sample loaded on the preconditioned cartridges was pulled through with vacuum followed by a water wash (1 ml). The MCAX cartridge was disposed of along with the eluate. The acrylamide on the C18 cartridge was eluted with 2 ml of methanol. The methanol eluate was then concentrated under a stream of nitrogen at 30°C to ~500 µl and reconstituted to 1 ml with methanol. The samples were filtered through 0.45 µm filters prior to UHPLC/SQ MS analysis. Three separate extractions of potato chips were analyzed to obtain an average concentration of acrylamide in the chips.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	Thermo Hypercarb (2.1 mm x 100 mm, 3 µm)		
Mobile Phase:	A: water containing 0.1 % formic acid B: acetonitrile containing 0.1% formic acid		
Flow Rate:	0.25 ml/min		
Injection Volume:	3 µl/min in partial fill mode		
Isocratic Conditions:	Time (min) 0-5	%A 98	%B 2
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Selected Ion Monitoring (SIM) Mode:	$[M+H]^+$ of acrylamide and 1,2,3,C13-acrylamide monitored at 72.1 m/z and 75.1 m/z respectively; dwell time of 250 ms each		
Capillary Exit Voltage:	100 V		

Results.

A calibration curve for acrylamide was run over the range of 3.9-250 ng/ml with 1,2,3,C13-acrylamide as internal standard (50 ng/ml). Figure 1 shows the SIM ion trace of the lowest standard of acrylamide (S/N > 3 RMS) and the spiked internal standard of 1,2,3,C13-acrylamide. The instrument detection limit for acrylamide on the Flexar SQ 300 MS was determined to be 3.9 ng/ml.

The recovery of the internal standard 1,2,3,C13-acrylamide taken through the solid phase extraction procedure was estimated at 75.4% (RSD of 9.5%, n=3).

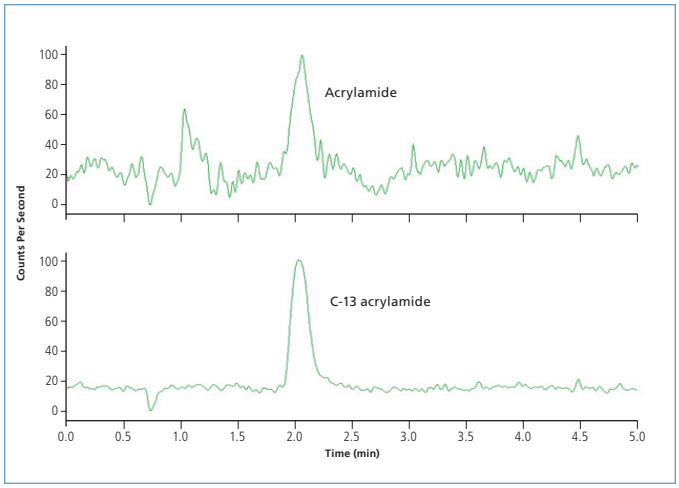


Figure 1. SIM ion trace of the lowest standard of acrylamide (3.9 ng/ml, S/N = 10) and SIM ion trace of the internal standard 1,2,3,C13-acrylamide (50 ng/ml) were monitored simultaneously.

Figure 2 shows SIM ion traces of acrylamide and its internal standard in a potato chip extract. The summary for three separate analyses of potato chip extracts is shown in Figure 3.

Conclusions.

A method has been developed for the measurement of acrylamide using off-line SPE extraction followed by UHPLC/SQ MS analysis with the Flexar SQ 300 MS. The ESI source is able to handle ~100% water and the instrument detection limits of 3.9 ng/ml were achieved for acrylamide analysis.

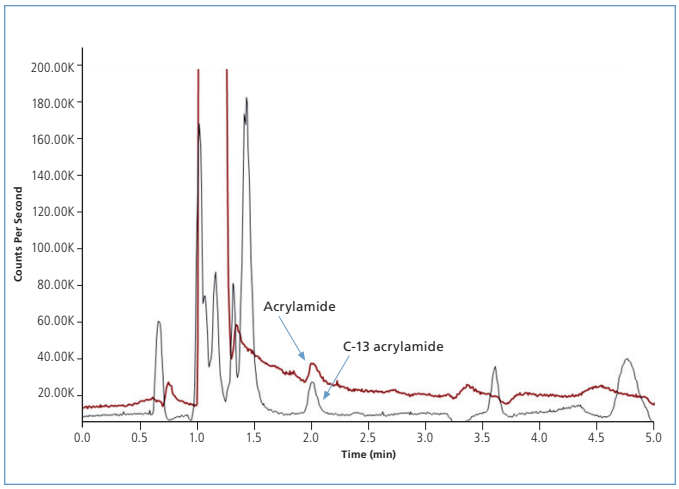
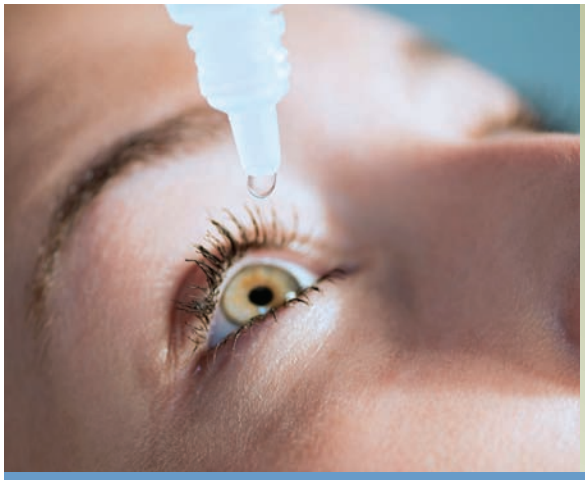


Figure 2. SIM chromatograms of a potato chip sample.

Sample	µg/Kg
Extract 1	63.2
Extract 2	71.3
Extract 3	63.9
Average concentration of acrylamide	66.1
% RSD	6.8

Figure 3. Results of quantitative analysis on potato chip extracts.



Analysis of an Antibiotic Ophthalmic Solution by UHPLC/SQ MS

PHARMACEUTICAL

An expired ophthalmic solution of trimethoprim and polymyxin B sulphates was analyzed for degradants using UHPLC/SQ MS. The ophthalmic solution is used to treat bacterial eye infections and is active against a variety of gram negative and gram positive bacteria. An assay to detect degradants formed during storage beyond the expiration date of this diluted antibacterial ophthalmic solution is described.

Experimental Conditions			
Target Analytes:	Polymyxin B1, B2		
Sample Preparation Conditions:			
An expired ophthalmic solution containing a mixture of polymyxin B sulphates and trimethoprim was diluted 1:10 in HPLC grade water and injected on column.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	Grace® VisionHT™-HL column (2.1 mm x 50 mm, 1.9 µm)		
Flow Rate:	0.8 ml/min with a post column split of 0.4 ml/min into MS		
Injection Volume:	1 µl in partial fill mode		
Mobile Phase:	A: water with 0.1% formic acid		
	B: acetonitrile with 0.1% formic acid		
Gradient:	Time (min)	%A	%B
	0	90	10
	1	80	20
	5	50	50
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	100-1500 m/z		
Scan Rate:	2000 u/sec		
Capillary Exit Voltage:	100 V and 150 V		

Results.

Polymyxin B is a cyclic peptide antibiotic which is usually present as a mixture of B1 and B2 sulphates. The structure of polymyxin B1 sulphate is shown in Figure 1.

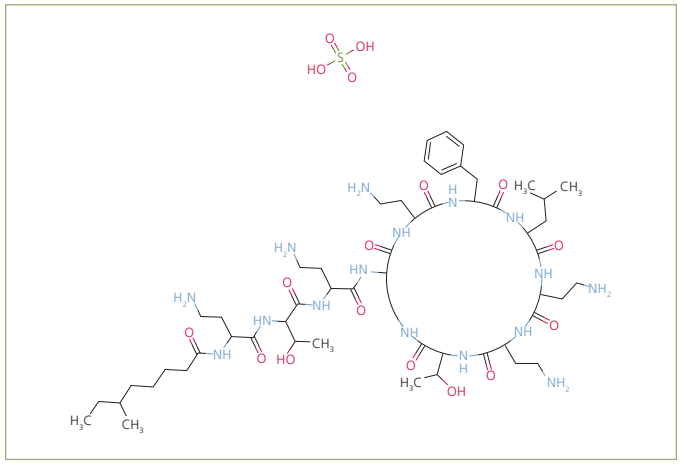


Figure 1. Structure of polymyxin B1 sulphate. Polymyxin B1 has a terminal 6-methyloctanoyl group and polymyxin B2 sulphate has a terminal 6-methylheptanoyl group.

The mass spectrum of polymyxin B1 at a low capillary exit voltage showed the intact molecule with multiple charges on it (Figure 2). Multiple charge information is very useful for this type of biomolecule to further confirm the molecular weight information.

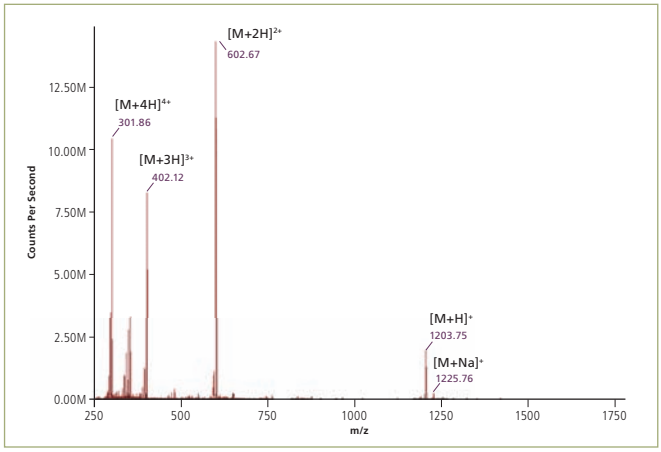


Figure 2. Mass spectrum of polymyxin B1 showing intact molecule with multiple charges analyzed at a low capillary exit voltage of 100 V. The observed $[M+H]^+$ of the intact molecule matches the calculated monoisotopic $[M+H]^+$ of 1203.75.

The mass spectrum of polymyxin B1 analyzed at a higher capillary exit voltage resulted in fragmentation of the cyclic antibiotic to yield characteristic fragments of the molecule (Figure 3).

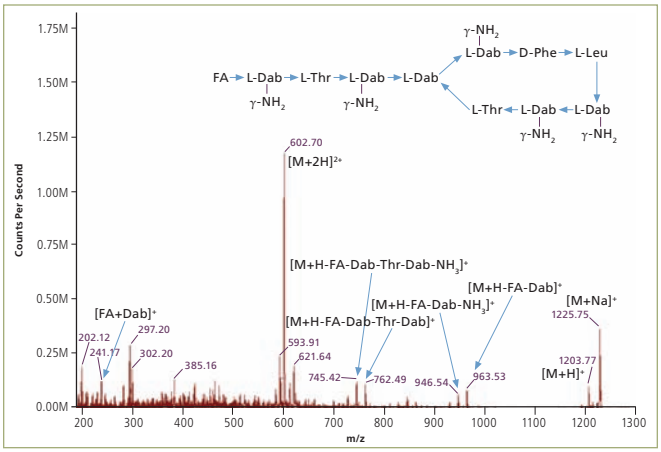


Figure 3. Collision Induced Dissociation (CID) spectrum of polymyxin B1 obtained at a higher capillary exit voltage of 150 V. Annotations: FA is the fatty acid 6-methyloctanoyl group; Dab is α, γ-diaminobutryic acid; amino acids are represented as three letter notations.

The extracted ion chromatogram of polymyxin B1 molecular ion showed the antibiotic to elute at different times during the chromatographic run. However, the spectra of polymyxin B1 was identical at these different retention times suggesting they are most likely stereoisomers formed by isomerization of L with D amino acids. A similar scenario was observed with polymyxin B2. To determine if these stereoisomers are formed at higher temperatures, the sample was heated to 40 °C overnight and then analyzed by UHPLC/SQ MS. The results showed increased formation of some of the stereoisomers of polymyxin B1 and B2 (data not shown).

Conclusions.

UHPLC/SQ MS analysis of an expired ophthalmic solution containing diluted concentrations of cyclic antibiotics of polymyxin B showed several stereoisomers of B1 and B2. These isomers increased upon heating of the sample, suggesting racemic mixtures can be formed in the ophthalmic solution during storage under non-refrigerated conditions.

The Flexar SQ 300 MS ionization capabilities allowed us to obtain molecular ion information even on a labile structure like that of polymyxin B. Multi-charged species were also obtained confirming the molecular ion.

Monitoring Basic Hydrolysis By-Products of Loratadine by UHPLC/SQ MS

PHARMACEUTICAL

Drugs can undergo degradation during storage. We studied the breakdown of the antihistamine drug loratadine, under accelerated degradation conditions and identified the hydrolysis products using UHPLC/SQ MS. Loratadine is commercially used in tablet or syrup form and contains an ester bond that is susceptible to hydrolysis.

Experimental Conditions			
Target Analytes:	Desloratadine, loratadine		
Sample Preparation Conditions:			
A 10 mg tablet of a commercial product containing loratadine was crushed into a 50 ml volumetric flask and brought to volume with ethanol. The flask was shaken, vortexed for ~2 min, 1 ml of supernatant was filtered through a 0.45 micron filter. The filtered supernatant was diluted 1:10 in ethanol and 2 µl injected on column. Additionally, filtered supernatant (1 ml) was base hydrolyzed in NaOH (final concentration of NaOH was 0.25 N) overnight at ~95 °C and analyzed by UHPLC/SQ MS.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	Grace® VisionHT™-HL column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water containing 0.1% formic acid B: acetonitrile containing 0.1% formic acid		
Flow Rate:	0.8 ml/min with a post column split of 0.4 ml/min into MS		
Injection Volume:	1 µl in partial fill mode		
Gradient:	Time (min) 0 5 3	%A 90 50 50	%B 10 50 50
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	100-500 m/z		
Scan Rate:	2000 u/sec		
Capillary Exit Voltage:	100 V		

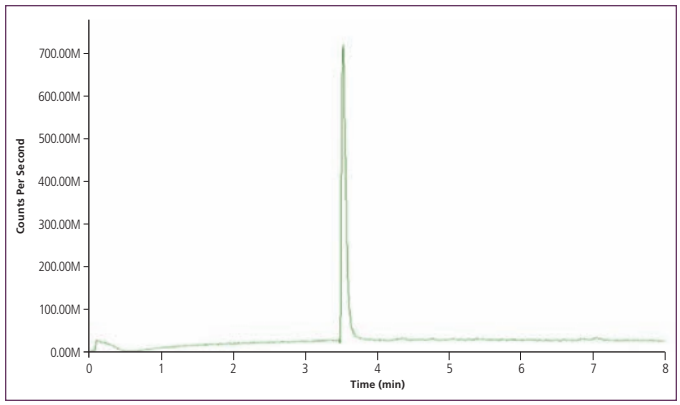


Figure 1. TIC of loratadine sample.

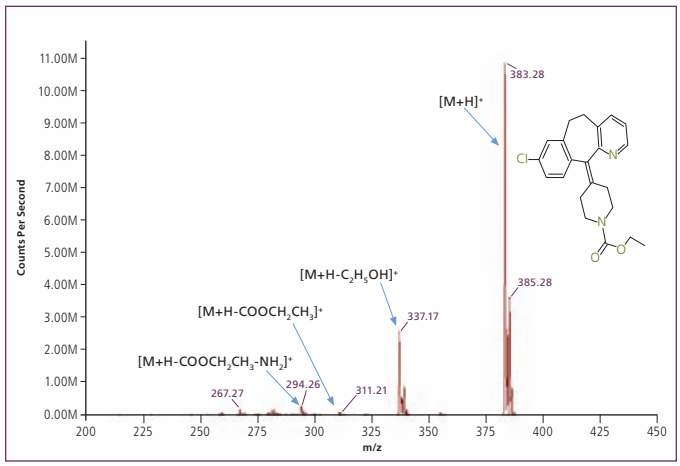


Figure 2. Mass spectrum of loratadine eluting at 3.6 min.

Results.

Figure 1 shows the TIC of an over-the-counter tablet containing loratadine. The mass spectrum of the major peak eluting at 3.6 min shows an electrospray mass spectrum consistent with the loratadine structure (Figure 2).

The loratadine sample subjected to basic hydrolysis showed two by-product peaks at retention times ~1 and 2.7 min (Figure 3A). The peak at ~1 min shows a mass spectrum that matched a known degradation product called desloratadine (Figure 3B) obtained from the parent drug. The peak at ~2.7 min corresponded to a mass spectrum of a proposed structure (Figure 4) which may most likely form with rearrangement reactions.

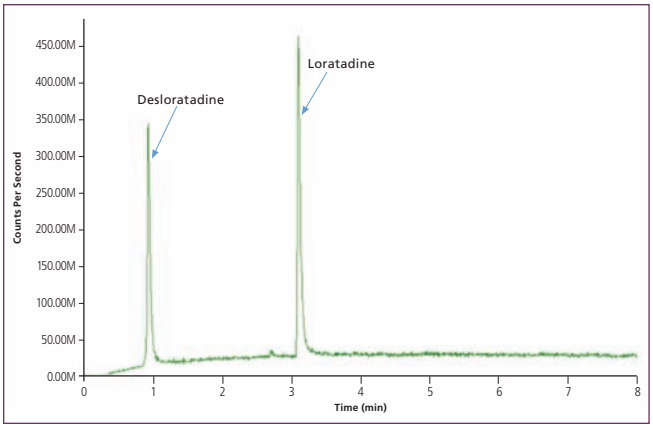


Figure 3A. TIC of the basic hydrolyzed loratadine sample.

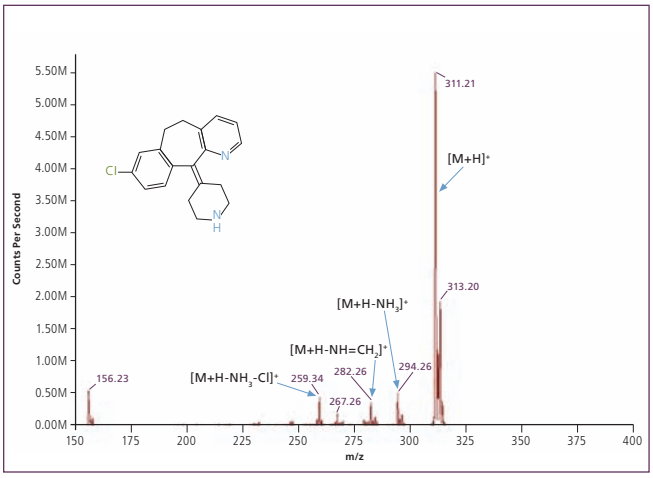


Figure 3B. Mass spectrum of desloratadine, a by-product of basic hydrolysis of loratadine eluting at retention time ~1 min.

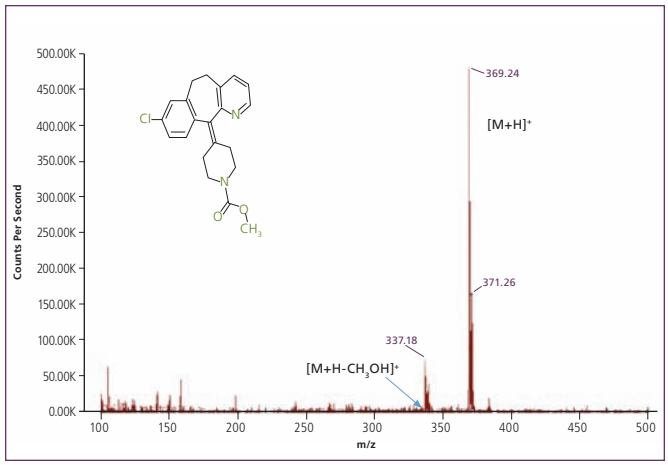
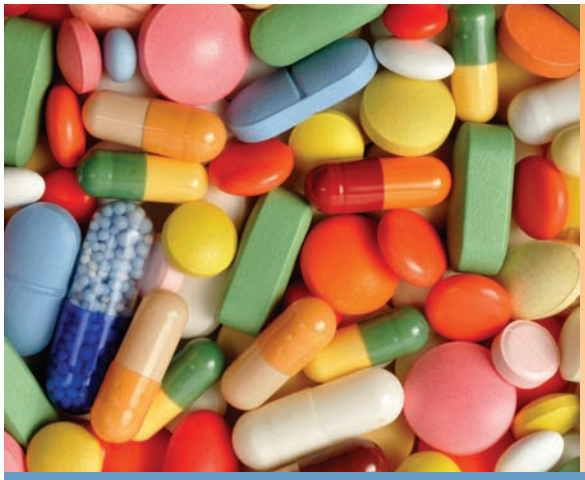


Figure 4. Mass spectrum of a by-product of basic hydrolysis of loratadine eluting at retention time of ~2.7 min. The proposed structure (methyl 4-(8-chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-ylidene) -1-piperidinecarboxylate) based on the mass spectrum may form with rearrangement reactions.



Analysis of Tetracyclines by UHPLC/SQ MS

PHARMACEUTICAL

Tetracyclines are broad-spectrum antibiotics indicated for use against many bacterial infections. Tetracyclines act by interfering with the ability of a bacterium to produce certain vital proteins, thus inhibiting the growth of bacterial organisms in a bacteriostatic manner. These anti-microbial compounds have a common basic structure and are either isolated directly from several species of *Streptomyces* bacteria or produced semi-synthetically.

In addition to treating infections, tetracyclines have been widely applied as feed additives for food-producing animals to prevent disease and increase nutritional output.

Here we demonstrate a quick and robust method for analysis of tetracyclines and their metabolites by UHPLC/SQ MS using the Flexar SQ 300 MS as an alternative to UV-based detectors commonly used for this type of analysis.

Experimental Conditions			
Target Analytes:	Monocycline, tetracycline, chlortetracycline, doxycycline		
Sample Preparation Conditions:			
A standard mixture of four tetracycline class antibiotics was prepared in 50% acetonitrile at a concentration of 1 mg/ml. The stock solution was further diluted to a working concentration of 10 µg/ml in 15% acetonitrile. Reverse phase UHPLC was used to separate them and the mass spectrometer operated in Scan mode for initial analysis.			
LOQ and LOD values were determined from data obtained in SIM (Selected Ion Monitoring) mode analysis.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-10		
Column:	PerkinElmer Brownlee™ C18 column (2.1 mm x 100 mm, 3.0 µm)		
Mobile Phase:	A: 100% water containing 0.1% formic acid		
	B: 100% acetonitrile containing 0.1% formic acid		
Flow Rate:	2 ml/min		
Injection Volume:	1 µl		
Gradient:	Time (min)	%A	%B
	10	85	15
	8	45	55
	3	0	100
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	100-550 m/z		
Scan Rate:	1000 u/sec		
The $[M+H]^+$ ion of each of the analytes were monitored in three different time periods:			
Time Period 1: (0-3.2 min) SIM ion 458.2 for monocycline; dwell time of 200 ms			
Time Period 2: (3.2-5.2 min) SIM ion 445.2 for tetracycline; dwell time of 200 ms			
Time Period 3: (5.2-8.2 min) SIM ions 479.2 and 445.2 for chlortetracycline and doxycycline respectively; dwell time of 200 ms each			
Capillary Exit Voltage:	80 V		

Results.

The separation of the four tetracycline compounds was accomplished by reverse phase UHPLC with an 8 min gradient. Each compound in the mixture was unambiguously identified by its mass spectral signature producing $[M+H]^+$ ion as the main ion species. LOQ and LOD values were established as 5 pg and 2 pg respectively. Chromatographic, spectral and SIM mode information for the mixture analysis is shown in Figures 1 through 4 (below and continued on the following page).

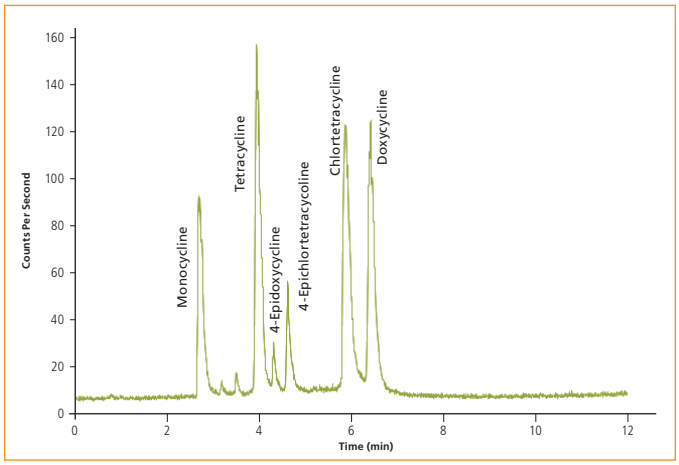


Figure 1. Chromatogram of tetracyclines by UHPLC/SQ MS in Full Scan mode. 4-epidoxycycline and 4-epichlortetracycoline are degradation products of chlortetracycline and doxycycline respectively.

Conclusions.

LC/SQ MS is continuing to be the method of choice for the accurate measurement of analytes across a broad spectrum of industrial applications. The powerful combination of HPLC/UHPLC and the Flexar SQ 300 MS enables users to develop quick, sensitive and robust methodologies for the analysis of a wide variety of compounds. As shown in this application, simultaneously operating in Full Scan and SIM mode, the Flexar SQ 300 MS overcomes the limitations of conventional detectors by providing important molecular weight and structural information, from Full Scan, while still reaching the highest sensitivity in SIM mode.

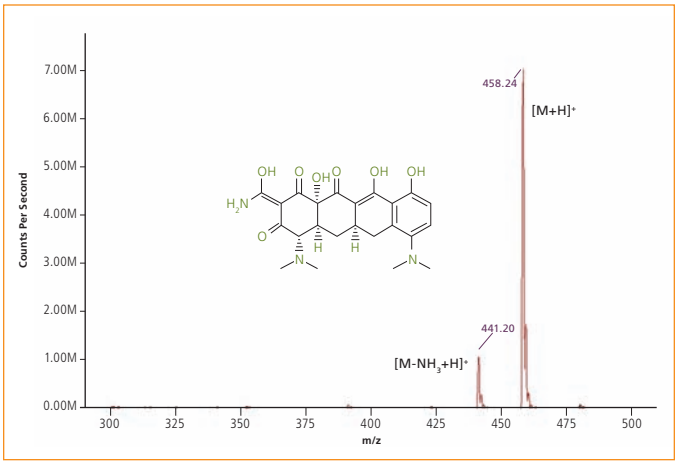


Figure 2. Mass spectrum of monocycline.

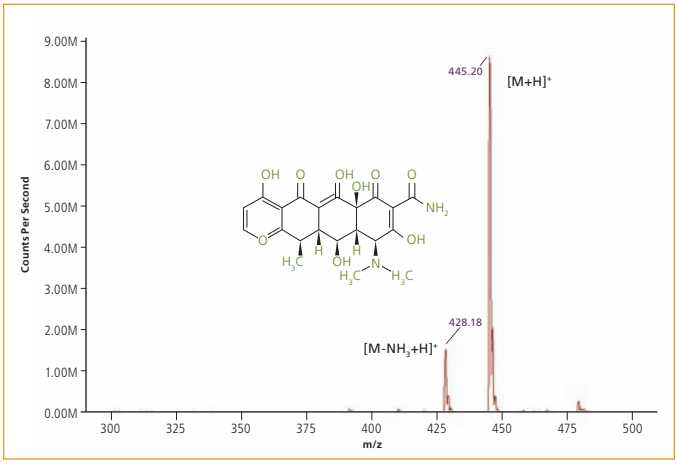


Figure 3. Mass spectrum of doxycycline.

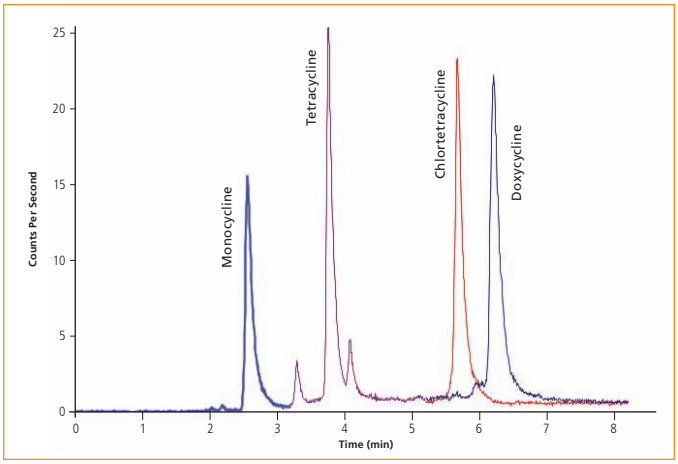


Figure 4. Overlaid chromatograms obtained in SIM mode of the 4 target compounds. 50 pg of each was injected on the column.

Following the Synthesis of a Sulfa Drug Using UHPLC/SQ MS

CHEMICAL/INDUSTRIAL

Sulfonamide drugs were the first antimicrobial drugs, discovered in 1935, that led to the antibiotic revolution in medicine. Sulfonamides are sulfa-related antibiotics which are used to treat bacterial and some fungal infections. Sulfonamides are prepared by the reaction of a sulfonyl chloride with ammonia or an amine. Thousands of molecules containing the sulfanilamide structure have been created since its discovery, yielding improved formulations with greater effectiveness and less toxicity.

Experimental Conditions			
Target Analytes:	Acetanilide, *p*-acetamidobenzene sulfonamide and sulfanilamide		
Sample Preparation Conditions:			
Stage 1—Synthesis of *p*-acetamidobenzene sulfonamide from acetanilide			
Acetanilide in an Erlenmeyer flask was heated to melt and spread in a thin film over the flask. The flask was then cooled in an ice bath. Chlorosulfonic acid was added into flask. The flask was connected to a trap to collect the HCl produced during the reaction. The flask was then heated on a steam bath for ~15 minutes to dissolve the solid, resulting in an oily substance. The flask was then removed from heat and ice added to the product. A thick white precipitate of *p*-acetamidobenzenesulfonyl chloride was observed to form. The white precipitate was filtered after mixing with ammonium hydroxide solution. The mixture was warmed in a steam bath and then cooled. A white pasty solid of *p*-acetamidobenzene sulfonamide was observed to form. ~1 mg of the wet precipitate was dissolved in MeOH, diluted 1:100 in water and analyzed by UHPLC/ SQ MS to identify the intermediate compound formed.			
Stage 2—Synthesis of sulfonamide from *p*-acetamidobenzene sulfonamide			
The white precipitate of *p*-acetamidobenzene sulfonamide from Stage 1 was filtered and washed with water into a 50 ml flask. HCl was added to the precipitate. The flask was connected to a reflux water condenser and heated on a steam bath for ~20 min.			
An aliquot of the mixture was retrieved from the flask every 5 min through the reaction, neutralized with $NaHCO_3$ and analyzed by UHPLC/SQ MS.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee™ HRes PFPP column (2.1 mm x 100 mm, 1.9 µm)		
Mobile Phase:	A: water containing 0.1% formic acid		
	B: acetonitrile containing 0.1% formic acid		
Flow Rate:	0.4 ml/min		
Injection Volume:	2 µl in partial fill mode		
Gradient:	Time (min)	%A	%B
	0	100	0
	2	100	0
	3	95	5
	2	70	30
	4	30	70
	3	30	70
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	65-400 m/z		
Scan Rate:	1000 u/sec		
Capillary Exit Voltage:	100 V		

Results.

A qualitative UHPLC/SQ MS method is presented to detect and identify the reaction products of a multi-step chemical synthesis (Figure 1) using a single quadrupole MS detector and its front-end Collision Induced Dissociation (CID) capability.

The soft ionization provided by the Flexar SQ 300 MS Ultraspray ESI, combined with the low capillary exit voltage values used, allowed the molecular ion and fragmentation information of the intermediate product *p*-acetamidobenzene sulfonamide (Figure 2) and the final product sulfanilamide (Figure 3) to be obtained.

This method has also been used for a real-time monitoring of the formation of the sulfanilamide during the acid refluxing-2nd stage synthesis (Figure 4).

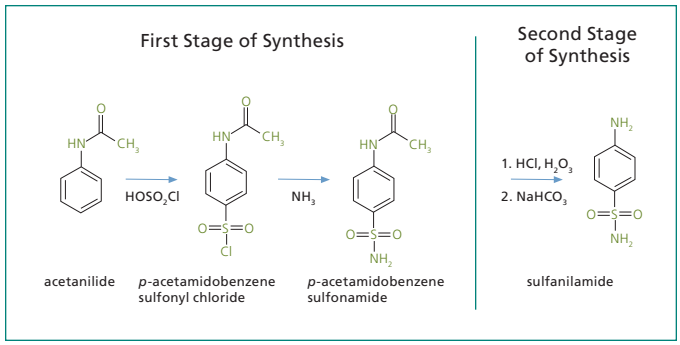


Figure 1. Schematic of the two stage synthesis steps for the formation of the sulfa drug, sulfanilamide.

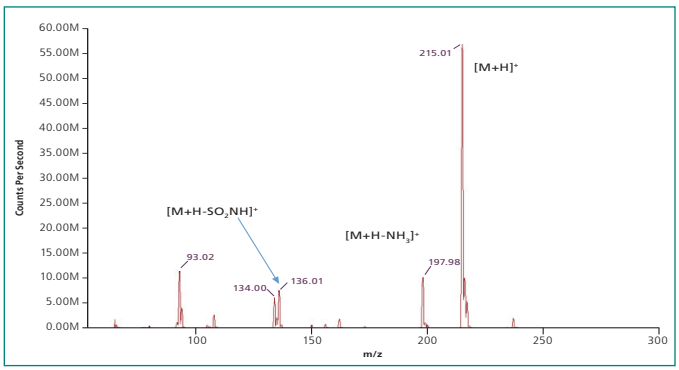


Figure 2. Mass Spectrum of *p*-acetamidobenzene sulfonamide after first stage of synthesis. The same spectrum has been observed in the three chromatograms shown in Figure 4 for the peak eluting at ~8.5 min.

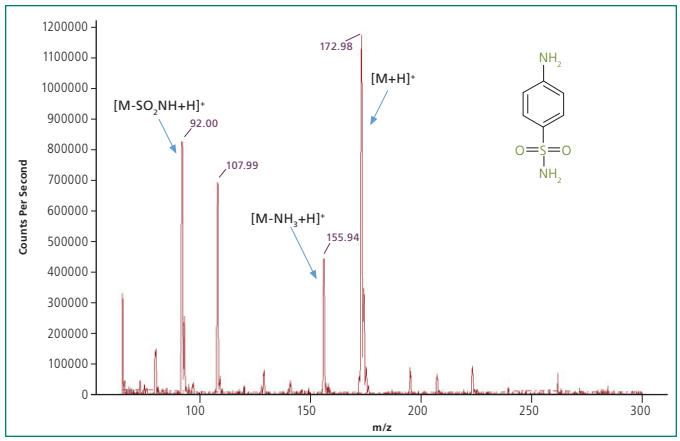


Figure 3. Mass spectrum of the synthesized sulfanilamide.

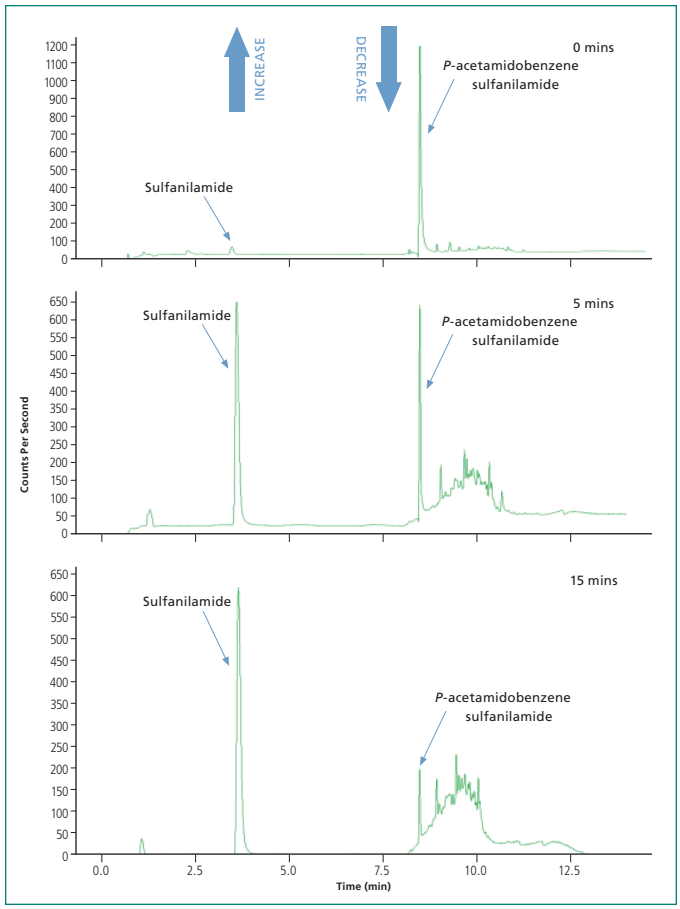


Figure 4. Real time monitoring by UHPLC/SQ MS of formation of sulfanilamide upon acid refluxing of *p*-acetamidobenzene sulfonamide during the second stage of synthesis. Formation of sulfanilamide (eluting at ~4 min) occurred with degradation of *p*-acetamidobenzene sulfonamide (eluting at ~8.5 min).

QA/QC Analysis of Parabens in Hand Lotion Using UHPLC/SQ MS

CHEMICAL/INDUSTRIAL

Parabens are esters of *p*-hydroxybenzoic acid which are often added to cosmetics and other personal care products including shampoos, moisturizers, etc. Parabens are considered good preservatives for their broad antimicrobial spectrum but also for their relatively low toxicity, low volatility and high stability.

Recent studies suggest parabens may be carcinogenic and can cause estrogenic disrupting activity, thereby disputing the notion of low toxicity for these compounds. We present an LC/MS assay to identify several of the parabens found in hand lotion.

Experimental Conditions			
Target Analytes:	Methyl paraben, ethyl paraben, *n*-propyl paraben, isopropyl paraben, *n*-butyl paraben, isobutyl paraben		
Sample Preparation Conditions:			
Hand lotion (100 mg) obtained from a grocery store was spiked with isopropyl paraben as internal standard (280 ng) and extracted in two different solvents of varying hydrophobicity (1 ml of methanol or ethyl acetate). The samples were vortexed and centrifuged for 10 min at 4000 RPM to separate the undissolved solids from the liquid. The extracts were then diluted in methanol (1:25) and analyzed by UHPLC/SQ MS.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee™ Hres C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water containing 0.1% formic acid B: 50/50 acetonitrile/methanol containing 0.1% formic acid		
Flow Rate:	0.5 ml/min		
Injection Volume:	2 µl in partial fill mode		
Gradient:	Time (min) 0 2 2.5	%A 70 50 43	%B 30 50 57
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Negative mode		
$[M-H]^-$ ions of each of the analytes were monitored in two different time periods:			
Time Period 1: (0-3 min) SIM ions 151.0, 165.0, 179.1 for methyl, ethyl, *n*-propyl and isopropyl paraben, respectively; dwell time of 120 ms each			
Time Period 2: (3-4.5 min) SIM ion 193.0 for *n*-butyl and isobutyl paraben; dwell time of 120 ms			
Capillary Exit Voltage:	-50 V		

Results.

Figure 1 shows the separation and detection of parabens in SIM mode by UHPLC/SQ MS. Calibration curves for each of the parabens were developed using isopropyl paraben (11 ng/ml) as internal standard (Figure 2—next page—shows the calibration curve for methyl paraben).

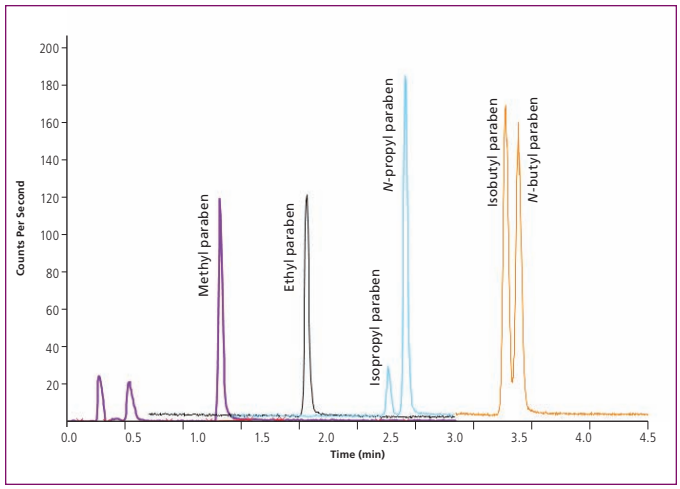


Figure 1. Overlaid chromatograms of the $[M-H]^-$ SIM ions of the various parabens analyzed by UHPLC/SQ MS.

Hand lotion extracted for parabens showed slightly better recoveries (not significantly different) in ethyl acetate than methanol. The recovery of internal standard taken through the extraction procedure showed 110% ± 0.9% (n=3) recovery.

Figure 3 shows that methyl paraben was the predominant paraben found in the ethyl acetate extract of hand lotion, at 7.8 µg/g (%RSD=10.5, n=3). Very low concentrations of *n*-propyl paraben and *n*-butyl paraben were also observed in the lotion.

These results were consistent with the ingredient methyl paraben listed on the bottle. The European Directive 76/768/EEC and its amendments limit the use of a single ester of paraben to 0.4% (w/w) in a cosmetic product. We detected 0.001% of methyl paraben in hand lotion by UHPLC/SQ MS.

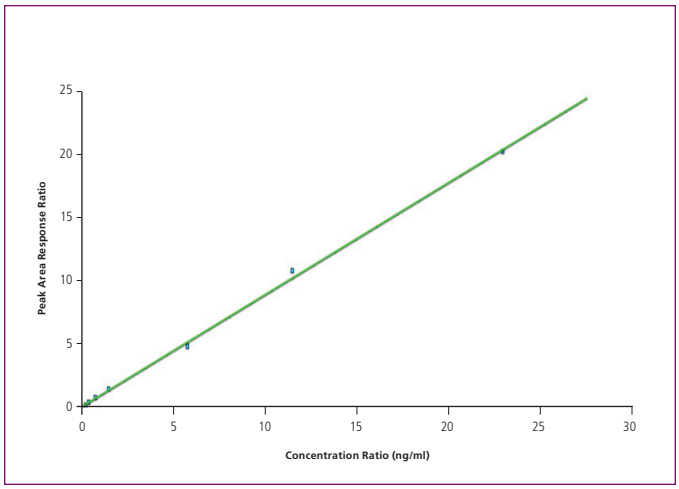


Figure 2. Calibration curve for methyl paraben with isopropyl paraben used as the internal standard (concentration range of methyl paraben 1.95-250 ng/ml, r^2=0.9987).

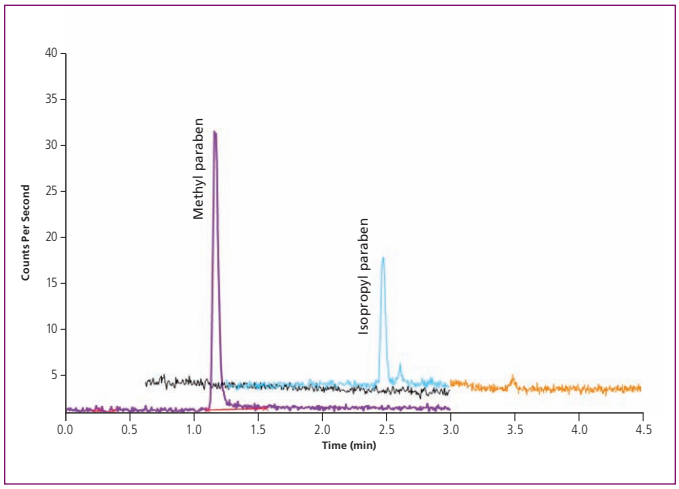


Figure 3. Hand lotion extracted in ethyl acetate and spiked with isopropyl paraben as the internal standard shows the majority of parabens in the sample to be methyl paraben.

Determination of Fatty Acids in Biodiesel Fuel by UHPLC/SQ MS

CHEMICAL/INDUSTRIAL

Due to the depletion of fossil fuels, biodiesel fuel is gaining more importance and becoming an appealing alternative. Since biodiesel fuel is produced from plant oils and animal fats, fatty acids are a primary component of the fuel. The amount of fatty acids affects the efficiency of the esterification process which is necessary for the fuel to be usable. The presence of fatty acids in biodiesel fuel that have not been transesterified can cause engine degradation and produce hazardous emissions.

Here we present a method that is fast and sensitive for the monitoring of individual fatty acids in a biodiesel fuel sample using ultra high performance liquid chromatography (UHPLC) and a single quadrupole mass spectrometer (SQ MS). Other lipid species found in the oils such as triglycerides and phospholipids can also be monitored using this method without esterification and minimal sample preparation. Quick and sensitive analysis of fatty acids in biodiesel using UHPLC/SQ MS followed by an intuitive and easy-to-use data analysis software package provides a powerful and robust method for monitoring biodiesel quality.

Experimental Conditions			
Target Analytes:	Palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoelic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), and gadoleic acid (C20:1)		
Sample Preparation Conditions:			
50 µl of biodiesel fuel was extracted with 950 µl of 2:1 dichloromethane:methanol solution. The sample was vortexed for 10 sec and then centrifuged for 10 min at 12000 RPM. The sample was diluted 1:100 with 2:1 dichloromethane:methanol solution prior to injection.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee™ HRes C18 column (2.1 mm x 100 mm, 1.9 µm)		
Mobile Phase:	A: 40/60 water/acetonitrile with 12 mM ammonium acetate B: 90/10 isopropanol/acetonitrile with 12 mM ammonium acetate		
Flow Rate:	0.5 ml/min		
Column Temp:	55 °C		
Injection Volume:	1 µl		
Gradient:	Time (min) 0 10 1 1	%A 40 0 0 40	%B 60 100 100 60
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Negative mode for fatty acids Ultraspray ESI—Positive mode for triglycerides and phospholipids		
Scan Range:	100-1000 m/z		
Scan Rate:	2500 u/sec		
Selected Ion Monitoring (SIM) Mode:	Reported in Figure 1		
Capillary Exit Voltage:	-100 V for negative Scan data; 100 V for positive Scan data; 100V for SIM data		

Analyte	SIM m/z
C16:0	255.1
C16:1	253.1
C18:0	283.1
C18:1	281.1
C18:2	279.1
C18:3	277.1
C20:0	311.1
C20:1	309.1

Figure 1. $[M\text{-}H]^-$ of each analyte in SIM mode.

Results.

The total ion chromatogram shows that there are many peaks found in the biofuel oil sample shown in Figure 2. Extracted ion chromatograms allow for the identification of fatty acids of interest. After identification of the fatty acid retention times in Scan mode, Selected Ion Monitoring (SIM) can be used to monitor the fatty acids for quantitation at lower concentrations shown in Figure 3. The increased saturation of the fatty acids correlates with the increased retention times. ESI positive Full Scan mode acquisition shows additional lipid species such as triglycerides that can be extracted from the TIC (Figure 4). Future experiments will include quantitation of fatty acids in biodiesel fuel.

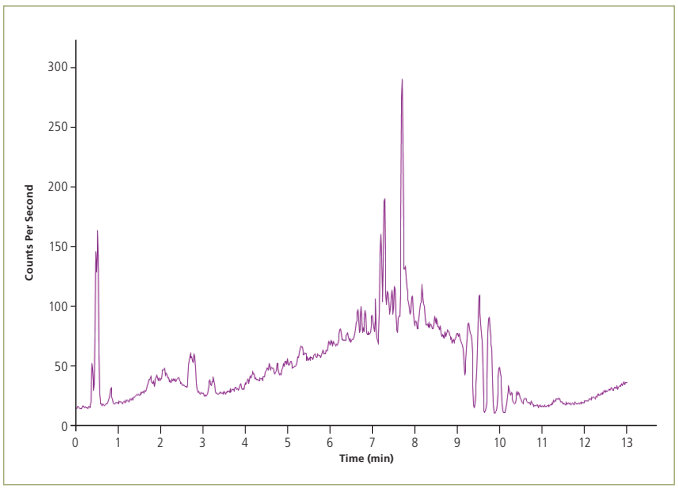


Figure 2. ESI negative mode chromatogram of the oil sample.

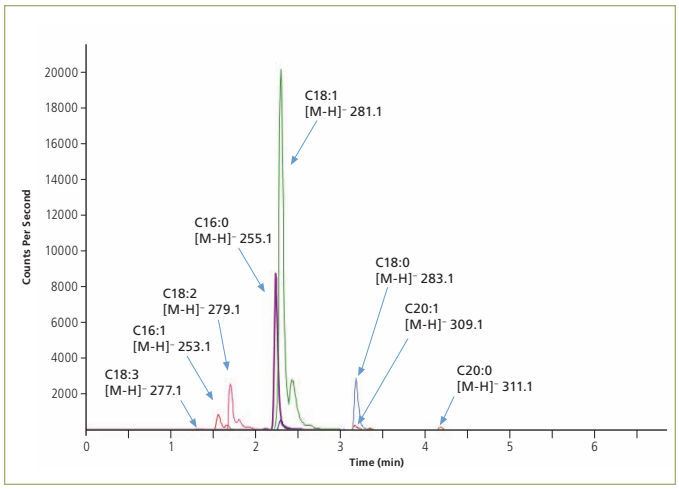


Figure 3. Overlaid chromatograms of [M-H]⁻ ions for 8 individual fatty acids in ESI negative SIM mode.

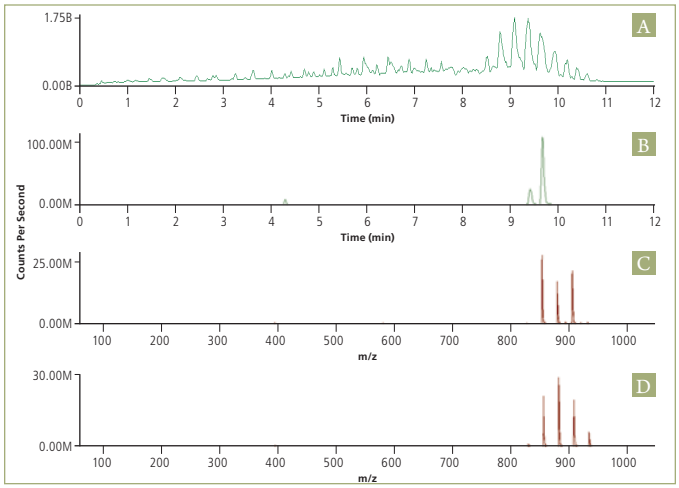


Figure 4. A) ESI positive mode Full Scan chromatogram of biofuel extract.
B) Extracted ion chromatogram of 850.8 m/z.
C) Average mass spectrum of a triglyceride under peak 1.
D) Average mass spectrum of a triglyceride under peak 2.

Analysis of Synthetic Steroids by UHPLC/SQ MS

ENVIRONMENTAL

Synthetic steroids and naturally occurring hormones are potent endocrine disruptors that may cause an adverse effect on aquatic species once they enter the environment through water systems. We present a UHPLC/SQ MS method for separating and detecting these compounds.

Experimental Conditions			
Target Analytes:	Norethindrone, d_6-norethindrone, tiblone, drospirenone, norgestrel, medroxyprogesterone, dehydrogesterone, cyproterone-acetate, progesterone, d_9-progesterone and medroxyprogesterone-acetate		
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee™ Hres C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water with 0.1% acetic acid		
	B: acetonitrile with 0.1% acetic acid		
Flow Rate:	0.5 ml/min		
Injection Volume:	2 µl volume in partial fill mode		
Gradient:	Time (min)	%A	%B
	0	60	40
	2.9	54	46
	2	54	46
	1	40	60
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Negative mode		
The $[M-H]^-$ ions of each of the steroids were measured in three different time periods:			
Time Period 1: (0.0-2.0 min) SIM ions 299.2, 305.4, 313.2 for norethindrone, d_6-norethindrone and tiblone respectively; dwell time of 200 ms each			
Time Period 2: (2.0-3.5 min) SIM ions 313.2 for norgestrel and dehydrogesterone, 345.26 and 367.2 for medroxyprogesterone and drospirenone respectively; dwell time of 200 ms each			
Time Period 3: (3.5-5.9 min) SIM ions 315.21, 324.3, 387.28, 417.22 for progesterone, d_9-progesterone, medroxyprogesterone-acetate and cyproterone-acetate respectively; dwell time of 150 ms each			
Capillary Exit Voltage:	100 V		

Results.

To obtain optimal sensitivity the $[M-H]^-$ ions were monitored in SIM mode in three different time periods (Figure 1). The detection limits were in the range of 2.5 ng/ml for majority of the analytes (S/N > 3).

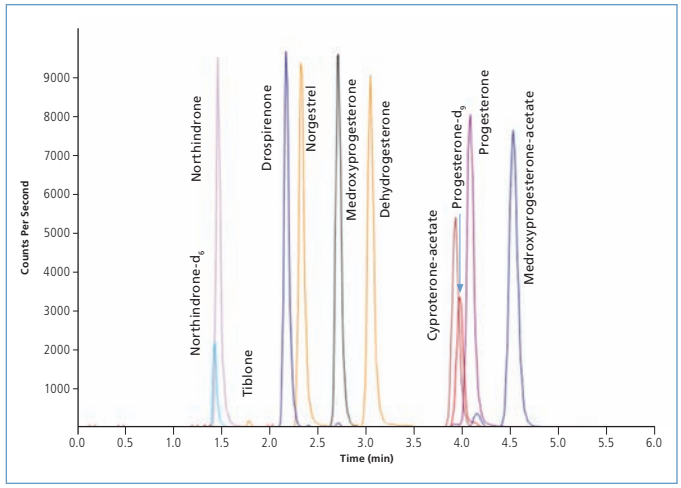
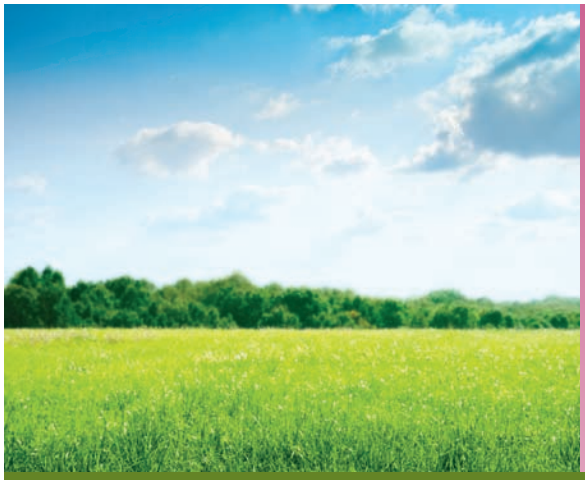


Figure 1. Overlaid chromatograms of $[M-H]^-$ ions for each of the steroids in SIM mode measured in three different time periods.



Analysis of Pesticides by UHPLC/SQ MS

ENVIRONMENTAL

LC/MS provides an excellent alternative to conventional LC with UV, PDA or Fluorescence detection or GC/MS to measure polar pesticides in food matrices and in the environment. Many of these pesticides are not very volatile and have to be derivatized prior to detection. Also, many of these pesticides are labile compounds and may get degraded easily in GC. We present a UHPLC/SQ MS separation and detection method for a mixture of organophosphorus pesticides, carbamates, triazines and chlorinated pesticides.

Experimental Conditions			
Target Analytes:	Acephate, oxamyl, mevinphos, pyrazon, dimethoate, aldicarb, simazine, dichlorvos, prometon, carbofuran, atrazine, chlorotoluron, carbaryl, prometryn, methiocarb, azinophos methyl, malathion, diazinon, ethion		
Standard Concentrations:			
A mixture of 20 pesticides (organophosporous pesticides, carbamates, chlorinated pesticides and triazines) at a concentration of 1 mg/ml each in acetonitrile was obtained from SPEX Certiprep group (Metuchen, NJ). The stock solution was diluted to 10 µg/ml in acetonitrile and injected on column.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee Supra™ Aqueous C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water containing 0.1% formic acid B: acetonitrile containing 0.1% formic acid		
Flow Rate:	0.4 ml/min		
Injection Volume:	2 µl volume in partial fill mode		
Gradient:	Time (min)	%A	%B
	0	90	10
	3	80	20
	3.5	70	30
	5.4	35	65
	5	10	90
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	60-410 m/z		
Scan Rate:	1000 u/sec		
Capillary Exit Voltage:	50 V		

Results.

The separation of the 20 pesticides was accomplished within 13 min (Figure 1). Extracted ion chromatograms allowed good selectivity and accurate quantitation even at low concentration levels (Figure 2).

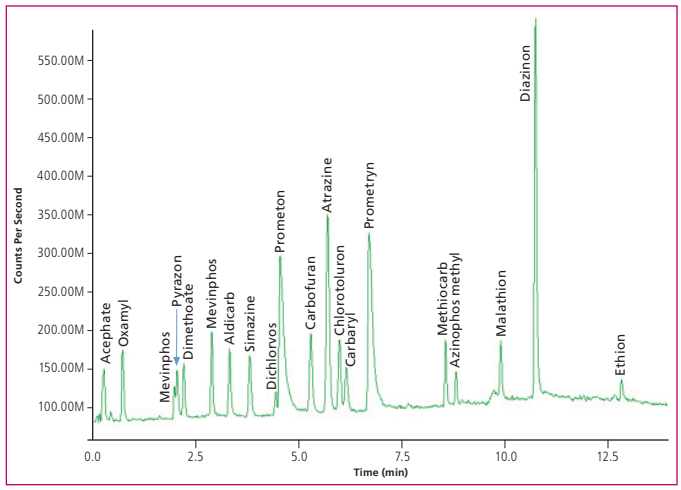


Figure 1. TIC of pesticides by UHPLC/SQ MS.

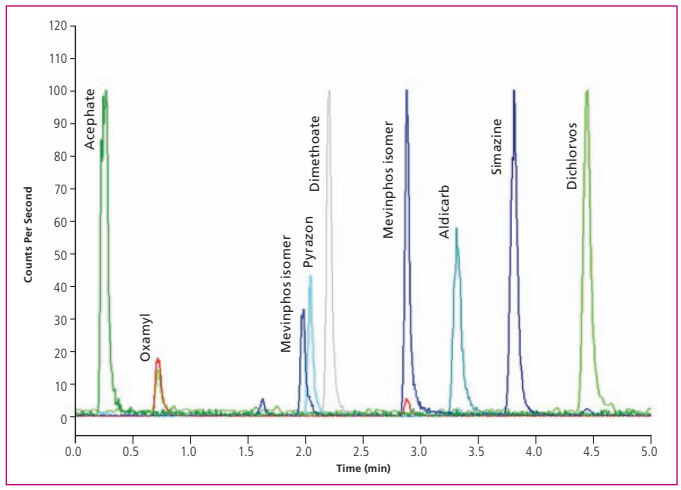


Figure 2. Extracted ion chromatogram (EIC) from 0-5 min.

The soft ionization provided by the Flexar SQ 300 MS combined with the low capillary exit voltage values used, allowed for excellent sensitivity while at the same time obtaining molecular ion information (Figures 3 and 4).

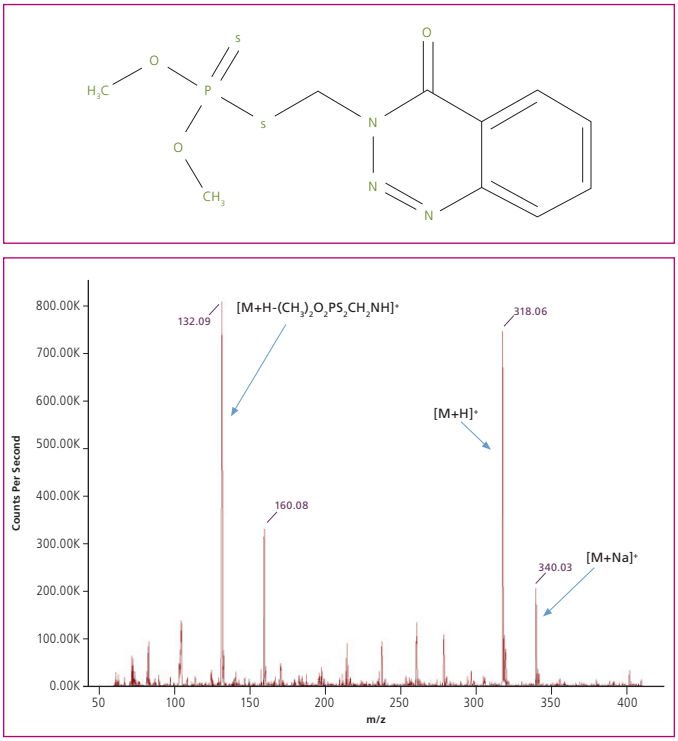


Figure 3. Molecular structure and mass spectrum of azinophos methyl.

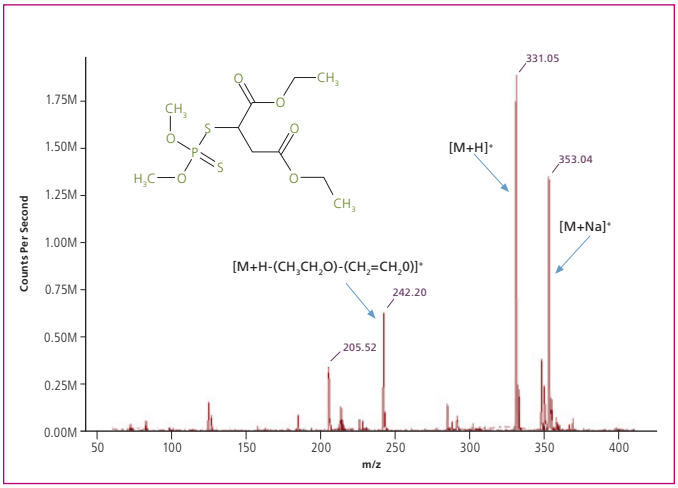


Figure 4. Mass spectrum of malathion.

Determination of Carbamates by UHPLC/SQ MS

ENVIRONMENTAL

Carbamates are organic compounds derived from carbamic acid (NH_2COOH). They are generally highly poisonous chemicals when ingested at high concentrations. They're not considered carcinogenic but there are some studies about the possible formation of cancerous chemicals when they mix with other elements in the stomach.

Carbamate insecticides have been used in agricultural pest control since the 1960's.

In this application brief, we describe a UHPLC/SQ MS method for the determination of carbamates and carbamate-based insecticides that could be used—after the proper sample preparation—on different sample matrices.

Experimental Conditions			
Target Analytes:	Aldicarb sulfone, aldicarb sulfoxide, oxamyl, methomyl, carbofuran-3-hydroxy, aldicarb, proproxur, carbofuran, 1-naphthyl-n-methylcarbamate, mercaptodimethur (methiocarb)		
Standard Concentrations:			
A 100 µg/ml carbamates standard in methanol purchased from Sigma was diluted 1:100 in acetonitrile to 1 µg/ml (1 ppm). A calibration curve for the carbamates was prepared with a concentration ranging from 0.05 ppb to 19.53 ppm.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	Restek Ultra II® Carbamate (2.1 mm x 100 mm, 3 µm)		
Mobile Phase:	A: water containing 10 mM ammonium acetate B: methanol containing 10 mM ammonium acetate		
Flow Rate:	0.5 ml/min		
Column Temp:	35 °C		
Injection Volume:	2 µl		
Gradient:	Time (min) 6 9 1	%A 90 10 90	%B 10 90 10
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
Scan Range:	50-300 m/z		
Scan Rate:	2500 u/sec		
Selected Ion Monitoring (SIM) Mode:	Reported in Figure 1; dwell time of 150 ms each		
Capillary Exit Voltage:	45 V for Scan data; reported in Figure 1 for SIM data		

Peak	Pesticide	SIM Ion	Time Period	Capillary Exit Voltage
1	Aldicarb sulfone	223.1	0-4.5 min	80 V
2	Aldicarb sulfoxide	207.1	0-4.5 min	60 V
3	Oxamyl	237.1	0-4.5 min	35 V
4	Methomyl	163	0-4.5 min	40 V
5	Carbofuran-3-hydroxy	238.1	4.5-5.1 min	70 V
6	Aldicarb	208.1	4.5-5.1 min	30 V
7	Proproxur	210.1	5.1-5.8 min	40 V
8	Carbofuran	222.1	5.8-6.8 min	65 V
9	1-Naphthyl-n-methylcarbamate	202.1	5.8-6.8 min	40 V
10	Mercaptodimethur (methiocarb)	226.1	6.8-11.0 min	75 V

Figure 1. $[M+H]^+$ of each carbamate in SIM mode.

Results.

The mass spectrum of each target analyte has been identified by initial experiments in Full Scan UHPLC/SQ MS (Figure 2). Selected Ion Monitoring (SIM) was used to achieve optimal separation and sensitivity (Figures 3 and 4). In each case, the molecular ion is the only one used for quantitation because of its high abundance in each carbamate mass spectrum.

The calculated detection limits for each of the carbamates are:
0.05 ppb for proproxur, carbofuran, 1-Naphthyl-N-methylcarbamate, Mercaptodimethur
1.25 ppb for Aldicarb sulfoxide, methomyl, carbofuran-3-hydroxy, aldicarb
6.25 ppb for aldicarb sulfone, oxamyl

Conclusions.

In this application brief, carbamates separated by UHPLC are detected with MS achieving sensitivity similar to conventional post-column derivatization methods followed by Fluorescence detection.

When compared to these methodologies, the Flexar SQ 300 MS provides additional flexibility and applicability to multiple applications, thus representing a better return on investment.

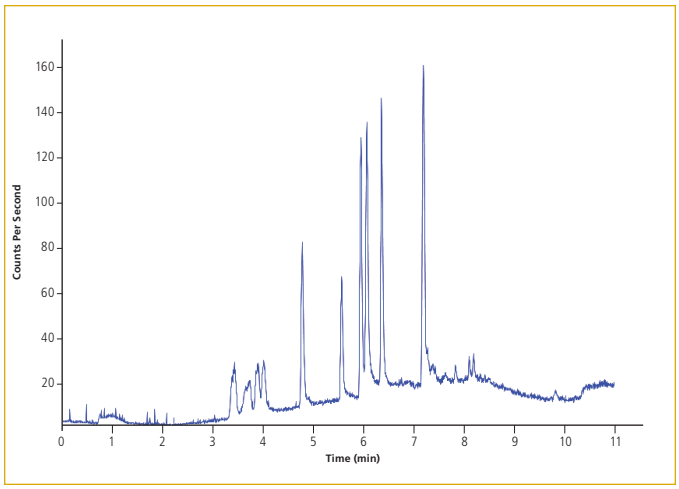


Figure 2. Full Scan separation of the carbamates mixture at 5 ppm.

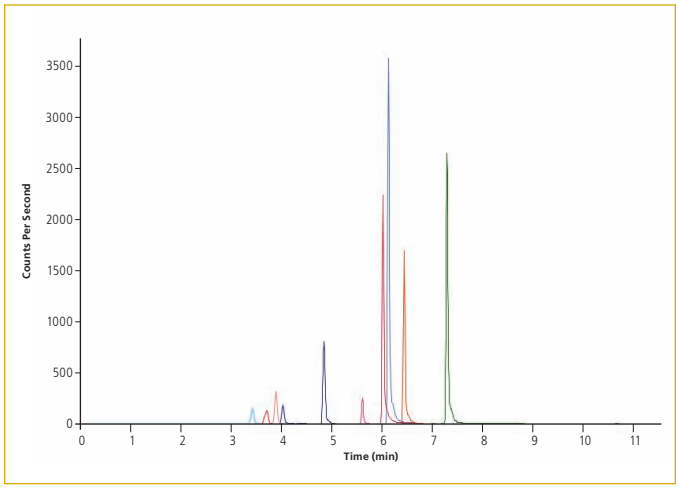


Figure 3. Overlaid chromatograms of $[M+H]^+$ ions for each of the carbamates in SIM mode.

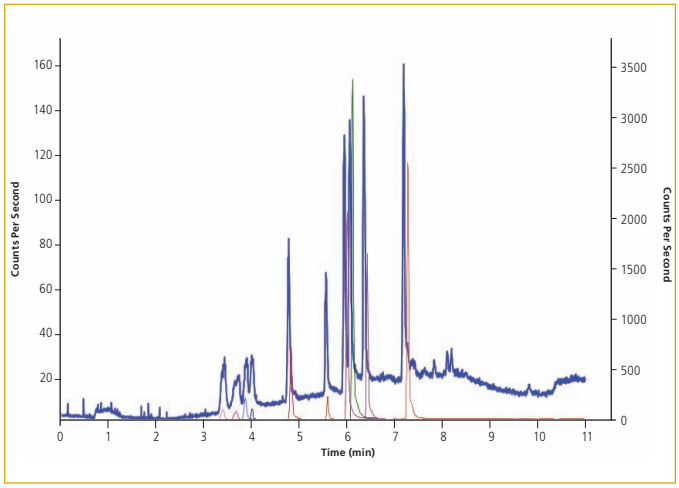


Figure 4. Full Scan mode versus SIM mode.

Analysis of Nitro Compounds in Sheep Rumen Fluid Using UHPLC/SQ MS

ENVIRONMENTAL

Chemically synthesized nitro aromatic compounds including pesticides, explosives, pharmaceuticals, etc. can enter the environment and cause both short- and long-term damage to living organisms. It is important to understand the biological fate of these compounds so that appropriate remedial measures can be adopted to clean up the environment.

One innovative and cost-effective cleanup of explosive nitro compounds—such as TNT, HMX and RDX—involves the use of ruminant animals, including sheep, which possess a highly anaerobic intestinal environment. The anaerobic bacteria in these ruminant animals can reduce the highly toxic compounds to more benign, non-toxic molecules. We present a study of the analysis of HMX spiked in sheep rumen fluid using UHPLC/SQ MS.

Experimental Conditions			
Target Analyte:	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocane (HMX)		
Sample Preparation Conditions:			
A standard solution of HMX was prepared by diluting mobile phase A. A calibration curve was set up in the range of 5 to 200 ng/ml. Sheep rumen fluid spiked with HMX (6 µg/ml) was diluted 1:100 in mobile phase A prior to analysis.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee Supra™ Aqueous C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water containing 0.1% acetic acid B: 80/20 methanol/acetonitrile containing 0.1% acetic acid		
Flow Rate:	0.4 ml/min		
Injection Volume:	2 µl in partial fill mode		
Gradient:	Time (min)	%A	%B
	0	80	20
	1	80	20
	2	50	50
	1	50	50
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Negative mode		
Scan Range:	40-365 m/z		
Scan Rate:	1000 u/sec		
Selected Ion Monitoring (SIM):	SIM ion 355.0 for HMX; dwell time of 200 ms		
Capillary Exit Voltage:	-50 V		

Results.

The mass spectrum of the target analyte has been acquired by initial experiments in Full Scan UHPLC/SQ MS. Figure 1 shows both the acetate adduct and the chloride adduct of the HMX molecule.

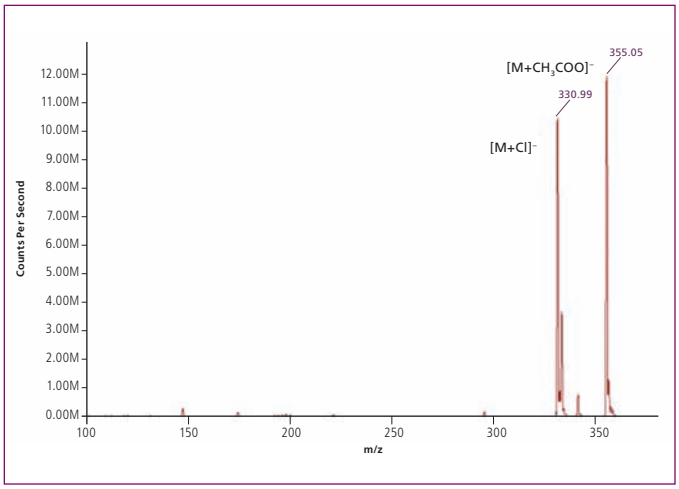


Figure 1. Mass spectrum of HMX showing acetate and chloride adducts of analyte.

Subsequently, using SIM mode, a calibration curve was established for the acetate adduct of HMX (355 m/z). Excellent linearity was observed in the range of 5 to 200 ng/ml of HMX (r^2 = 0.999, Figure 2). Figure 3 shows the SIM chromatogram of the HMX acetate adduct spiked in sheep rumen fluid. The HMX was separated on column in less than 4 minutes using UHPLC with a Flexar SQ 300 MS detector at pg sensitivity.

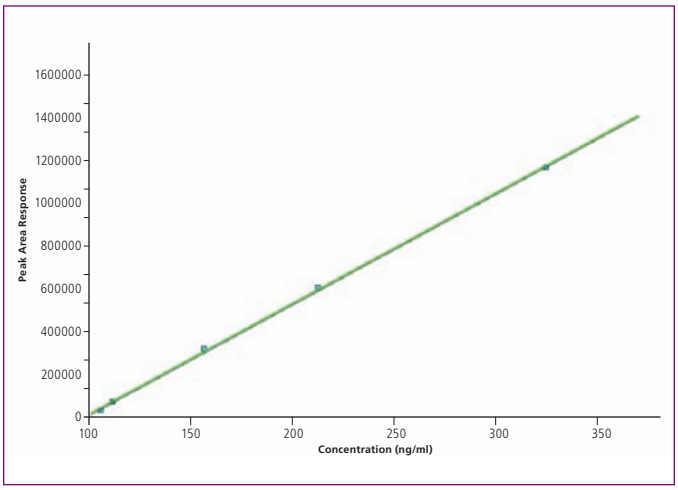


Figure 2. Calibration curve of HMX (range 5 to 200 ng/ml, r^2 = 0.999).

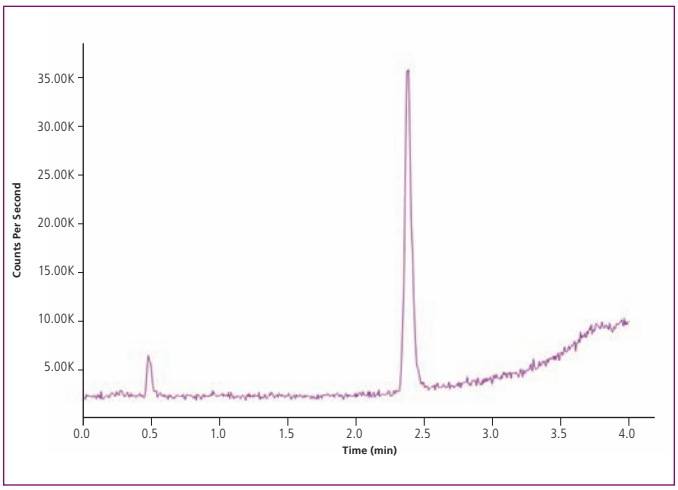


Figure 3. Shows the 355.0 m/z, SIM ion of HMX spiked in sheep rumen fluid.

UHPLC/SQ MS Analysis of Glyphosate After Derivatization with FMOC

ENVIRONMENTAL

Glyphosate is a widely used herbicide for both agricultural and urban landscape management applications. It has been shown to have an endocrine disrupting capability that led the U.S. Environmental Protection Agency (EPA) to require testing for glyphosate in drinking and surface water.

We present a method for the analysis of glyphosate and its major degradation product, aminomethylphosphonic acid (AMPA), using UHPLC/SQ MS. Both of these molecules were derivatized using 9-fluorenylmethoxycarbonyl chloride (FMOC) to increase the sensitivity of response by mass spectrometry. Figure 1 (see next page) shows the derivatization reactions of FMOC with glyphosate and AMPA. A post-column diverter valve was also used to redirect the salts eluting in the void volume (0-2 min) and the excess derivatizing agent and its byproducts (11-13 min) through the waste line, maintaining a cleaner LC/MS system.

Experimental Conditions			
Target Analytes:	Glyphosate (N-(phosphonomethyl)glycine), AMPA (aminomethylphosphonic acid)		
Sample Preparation Conditions:			
A borate buffer (50 µl of 5% tetraborate solution) was added to 100 µl of a series of standard solutions (containing glyphosate, AMPA and ^{13}C, ^{15}N glyphosate at various concentrations). The derivatizing agent FMOC (50 µl of 10 mg/ml of fluorenylmethyloxycarbonyl chloride in acetonitrile) was added to these solutions.			
The standard solutions were incubated overnight at room temperature in the dark. The following day, the reaction in each of the standards solutions was stopped by the addition of phosphoric acid (500 µl of 2% solution). The excess FMOC that had precipitated in the solutions upon adding the acid was re-dissolved back into the solution by the addition of methanol (300 µl). The final standard solutions contained concentrations of glyphosate and AMPA ranging from 5 ng/ml to 500 ng/ml for each analyte and internal standard ^{13}C, ^{15}N glyphosate (at a concentration of 100 ng/ml).			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	Phenomenex, Prodigy C18 (3.2 mm x 150 mm, 5 µm)		
Mobile Phase:	A: 5 mM ammonium formate in water B: acetonitrile		
Flow Rate:	0.4 ml/min		
Injection Volume:	4 µl in partial fill mode		
Gradient:	Time (min)	%A	%B
	2	80	20
	7	60	40
	1	10	90
	5	10	90
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Negative mode		
The $[M-H]^-$ ions for each of the analytes were monitored in two different time periods: Time Period 1: (0-8.0 min) SIM ions 390.0 and 392.0 for glyphosate and ^{13}C, ^{15}N respectively; dwell time of 200 ms each Time Period 2: (8.0-11.0 min) SIM ion 332.0 for AMPA; dwell time of 150 ms			
Capillary Exit Voltage:	-70 V		

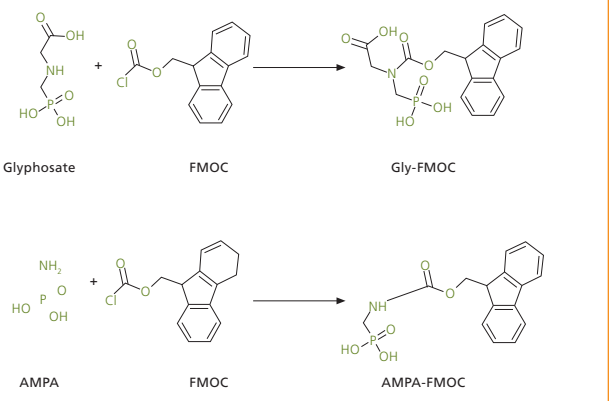


Figure 1. Derivatization reactions of glyphosate and AMPA with FMOC.

Results.

The derivatized FMOC products of glyphosate and its degradation product, AMPA, were well separated from each other by UHPLC (Figure 2). The calibration curves for glyphosate and AMPA are plotted as a ratio to the internal standard and show excellent linearity for the concentration range of 5-500 ng/ml (r^2 = 0.998 for both), Figures 3 and 4 respectively. We were easily able to detect 5 ppb concentrations of both glyphosate and AMPA using UHPLC in tandem with the Flexar SQ 300 MS detector.

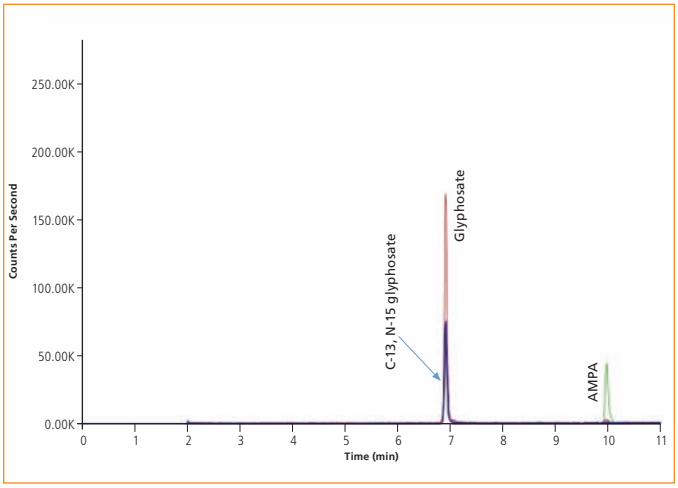


Figure 2. UHPLC/SQ MS elution profile of glyphosate (red trace), ^{13}C, ^{15}N glyphosate (blue trace) and AMPA (green trace).

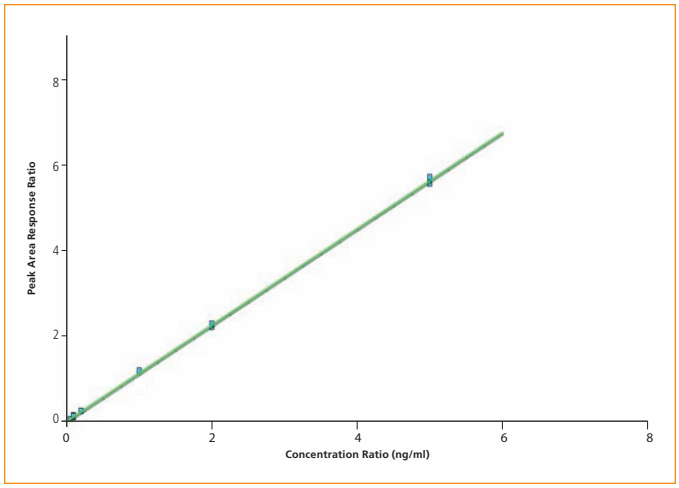


Figure 3. Calibration curve of glyphosate (5-500 ng/ml, n=3) plotted as a ratio to the internal standard, ^{13}C, ^{15}N glyphosate (r^2 = 0.998).

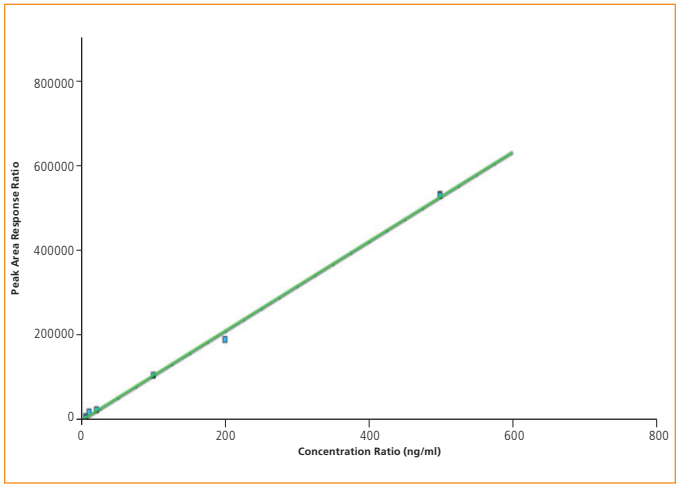


Figure 4. Calibration curve of AMPA (5-500 ng/ml, n=3, r^2=0.998) plotted as a ratio to the internal standard ^{13}C, ^{15}N glyphosate.

High Throughput HPLC/SQ MS Analysis with Dual-Probe Ultraspray™ 2 ESI Source

ENVIRONMENTAL

The time required for the LC separation and column equilibration can be a bottleneck for speed, method development and sample analysis productivity in high-throughput environments, such as pharmaceutical and environmental analysis laboratories. We present an application brief showing increased sample throughput using two identical columns with a dual-probe Ultraspray™ 2 ESI source for HPLC/SQ MS analysis using the Flexar SQ 300 MS.

The configuration contains two pumps: one for sample analysis and one for column regeneration. A 6-port pre-column switching valve was employed to switch the two columns between the analytical and regeneration pumps. A post-column valve diverted the effluent from the regenerating column away from the mass spectrometer (Figure 1).

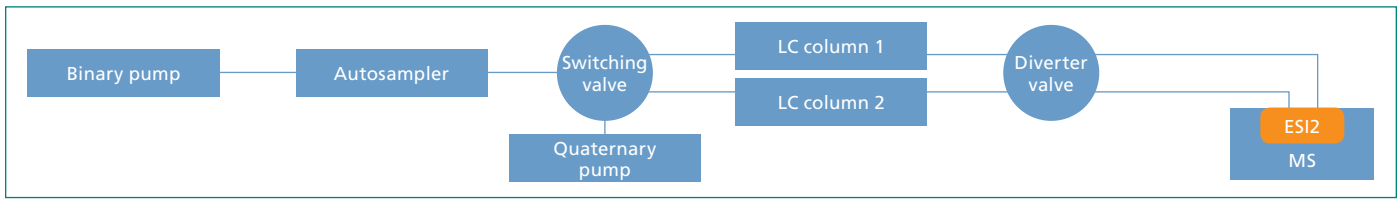


Figure 1. Schematic of instrument configuration.

Experimental Conditions							
Target Analytes:	Carbaryl, carbofuran, isoprocarb, methiocarb and methomyl						
Standard Concentrations:							
A mixture of the five carbamate pesticides (125 ng/ml) in 50/50 methanol/water mixtures was analyzed by LC/SQ MS.							
Liquid Chromatography Conditions:							
Pump Type:	Flexar Binary LC Pump as the analytical pump, Flexar Quaternary LC Pump as the regenerating pump						
Columns:	PerkinElmer Brownlee™ C18 column (2.1 mm x 100 mm, 3 µm)						
Mobile Phase: (Flexar Binary Pump)	A: water containing 0.1% formic acid B: acetonitrile containing 0.1% formic acid			Mobile Phase: (Flexar Quaternary Pump)	A: water containing 0.1% formic acid B: acetonitrile containing 0.1% formic acid		
Flow Rate:	0.3 ml/min						
Injection Volume:	4 µl in partial fill mode						
Gradient: (Flexar Binary Pump)	Time (min) 0 8 3	%A 80 20 20	%B 20 80 80	Isocratic: (Flexar Quaternary Pump)	Time (min) 0 11	%A 100 100	%B 0 0
Mass Spectrometer Conditions:							
Ionization:	Dual-probe Ultraspray 2 ESI—Positive mode						
The $[M+H]^+$ ions of each of the analytes were monitored: SIM ions 202.1, 222.1, 194.1, 226.1 and 163.1 were monitored for carbaryl, carbofuran, isoprocarb, methiocarb and methomyl respectively; dwell time of 100 ms each							
Capillary Exit Voltage:	70 V						

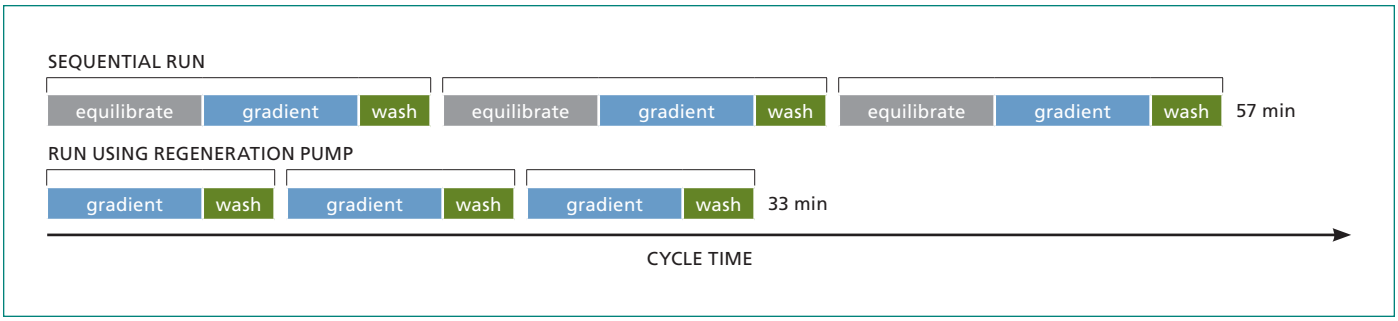


Figure 2. Cycle time for three sequential samples analyzed on a single column (top) and the cycle time for samples analyzed alternatively on two columns using a regenerating pump (below). The latter configuration allows a 42% time saving.

Results.

An increase in sample throughput of 42% was achieved by alternately regenerating one column while the other column was used for analysis (Figure 2).

While samples were being separated on one column and analyzed by the MS detector, the other column was being regenerated and the effluent diverted to waste.

The chromatographic overlay of samples (n = 4) analyzed on each column is shown in Figure 3. The precision in retention time for each of the carbamates analyzed on the two columns varied between 0.1-1.25%. The precision in area for each of the carbamates was in the range of 1.1-3.5%. The position of the dual spray probe was adjusted so that the signal intensity of a test analyte (niacinamide) was equivalent through each probe.

To ensure the connection between the regenerating pump and column, a small PEEK tubing connection between positions 4 and 5 on the switching valve was made (Figures 4A and 4B on next page). This only slightly increased the path volume during the regeneration step (<2µl). The positions of the pre-column switching valve and the waste diverter valve were programmed into the Instrument Method section of PerkinElmer's Chromera CDS. Two separate instrument methods—containing the two valve combinations—were created and used in the sequence.

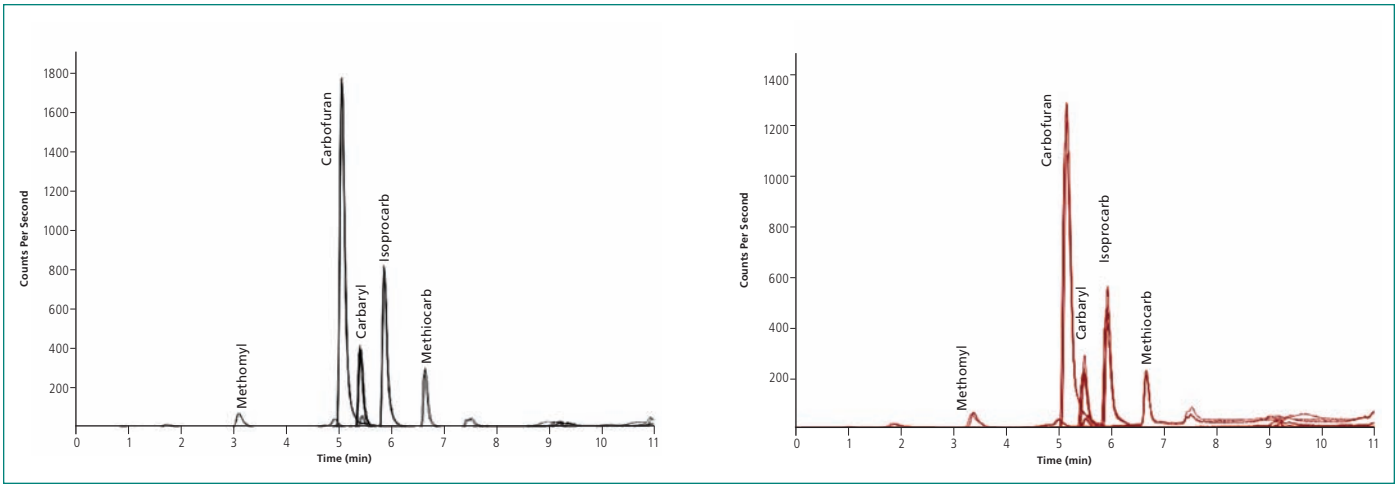


Figure 3. The chromatographic overlays (n=4) of the SIM $[M+H]^+$ ions of carbamates on column 1 (black trace) and column 2 (red trace) are shown. Retention time and area reproducibility are affected by the two different column's performance (theoretical plates).

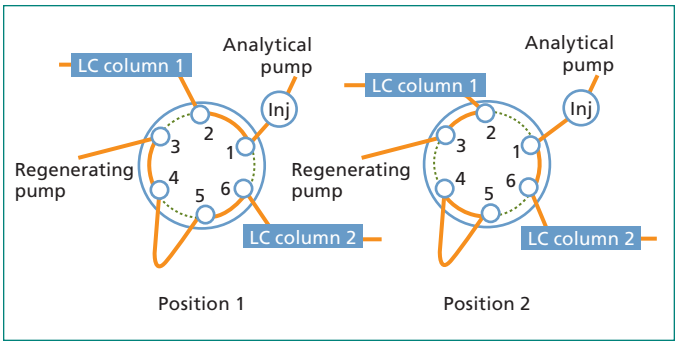


Figure 4A. Positions of pre-column switching valve.

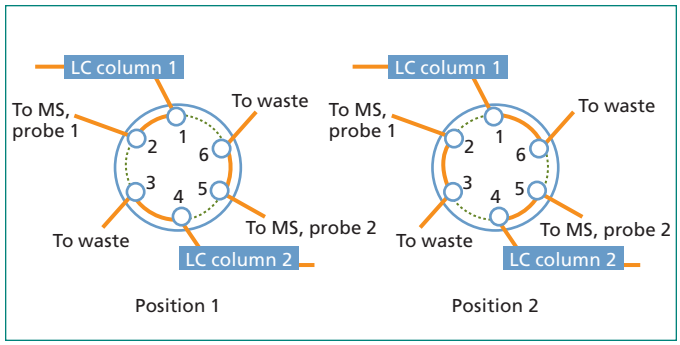


Figure 4B. Positions of waste diverter valve.

Conclusions.

We were able to significantly increase sample throughput by using the Ultraspray™ 2 ESI source with dual spray probes and a switching valve.

An additional increase in sample throughput could have been achieved by using the regeneration pump to also perform the wash step. A similar configuration could also be set up when two different solvent systems are being used for analysis of compounds potentially requiring two different columns.

The use of a dual spray probe eliminates the possibility of cross contamination between the two systems and also allows an immediate method change without spending time switching solvents, conditioning the LC/MS system and running multiple blanks.

Analysis of Hippuric Acid and Related Compounds in Human Urine Using UHPLC/SQ MS

CLINICAL

Exposure to volatile organic compounds (VOCs) including toluene, xylenes, styrenes and ethylbenzene can result in adverse health effects. Occupational exposure of workers in certain industries to these compounds can be measured by quantifying the metabolites of VOCs in their urine. The major metabolites of these VOCs are hippuric acid, mandelic acid, phenylglyoxylic acid and methyl hippuric acid. We describe a UHPLC/SQ MS method to identify and quantify these compounds in human urine.

Experimental Conditions			
Target Analytes:	Hippuric acid, mandelic acid, phenylglyoxylic acid, methyl hippuric acid, d_3 muconic acid		
Sample Preparation Conditions:			
Human urine was diluted 1:100 in methanol for analysis.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-15		
Column:	PerkinElmer Brownlee Supra™ Aqueous C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water containing 0.1% formic acid B: acetonitrile containing 0.1% formic acid		
Flow Rate:	0.5 ml/min		
Injection Volume:	1 µl in partial fill mode		
Gradient:	Time (min) 0 2 2	%A 95 95 60	%B 5 5 40
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Negative mode		
The $[M-H]^-$ ions of each of the analytes were monitored in two different time periods:			
Time Period 1: (0-1.5 min) SIM ions 145 and 149 for d_3 muconic acid and phenylglyoxylic acid, respectively; dwell time of 200 ms each			
Time Period 2: (1.5 -4 min) SIM ions 151, 178, and 192 for mandelic acid, hippuric acid and methyl hippuric acid respectively; dwell time of 150 ms each			
Capillary Exit Voltage:	Optimized to maximize intensity for each analyte, values in the range of -70 to -100 V		

Results.

The separation of the analytes was achieved within a 4 min run time (Figure 1). Except for phenylglyoxylic acid (PGA), most of the analytes had a detection limit of 0.004 mg/L (S/N > 3). PGA showed a higher detection limit, at 0.01 mg/L (S/N > 3). Human urine samples were analyzed for the various VOC biomarker compounds.

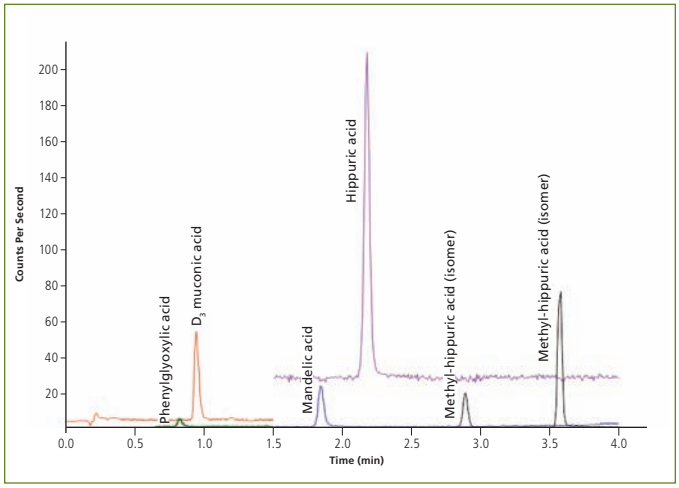


Figure 1. Overlay of $[M-H]^-$ SIM ions of the standard PGA, mandelic acid, hippuric acid and methyl hippuric acid. D_3 muconic acid was used as the internal standard.

Figure 2 shows the presence of hippuric acid in urine which was determined at 0.48 g/L of urine in the sample analyzed. The SIM ion trace for the molecular ion of mandelic acid (151.0 m/z) in a urine sample showed several peaks. However, only one of the peaks had a retention time matching mandelic acid, which was confirmed by spiking experiments (Figure 3).

This UHPLC/SQ MS method to determine occupational exposure to aromatic solvents offers superior sensitivity and selectivity compared to a UV detector (measuring absorbance at 240 nm) and is faster, less expensive, and easier than a GC/MS method which requires sample derivatization prior to analysis.

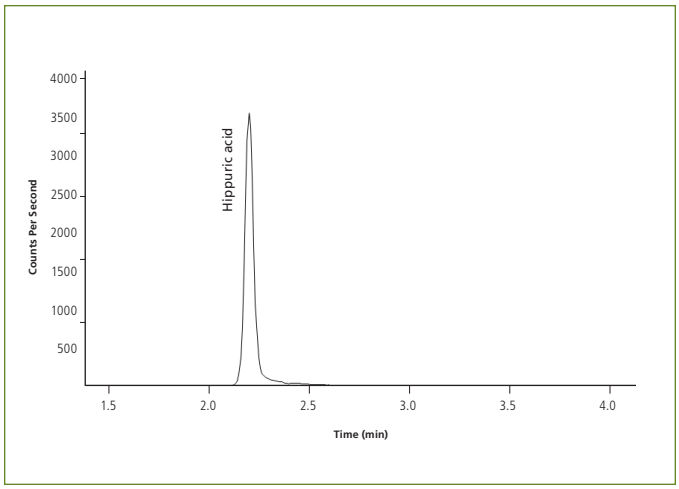


Figure 2. The SIM ion trace of hippuric acid measured in a human urine sample.

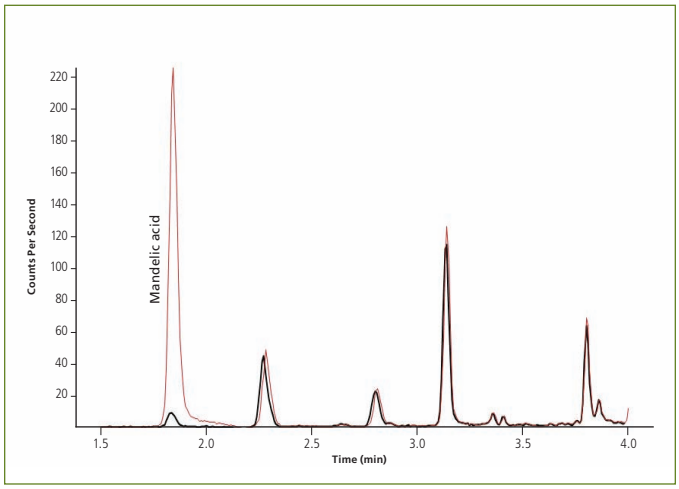


Figure 3. Overlay of SIM ion traces of mandelic acid in human urine sample (black line) and of urine sample spiked with a standard of mandelic acid (red line). The spiking experiment confirms peak eluting at R_t of 1.84 min is indeed mandelic acid.

Analysis of 25-Hydroxyvitamin D_2 and D_3 in Serum Using UHPLC/SQ MS

CLINICAL

Vitamin D (where D refers to D_2 or D_3) is a fat-soluble pro-hormone that when metabolized into its active form targets less than 200 human genes in a wide variety of tissues in the body. In addition to the major physiological function of vitamin D metabolites to maintain calcium and phosphate homeostasis, vitamin D status has been associated with a variety of disease states including cancer, cardiovascular disease, diabetes, multiple sclerosis, osteoporosis, rheumatoid arthritis, and chronic pain.

Vitamin D_3 (cholecalciferol) is formed in the skin upon exposure to sunlight and vitamin D_2 (ergocalciferol) is obtained from the ultraviolet irradiation of plant materials (Figure 1). Natural sources of vitamin D_3 include oily fish (such as salmon or mackerel), cod liver oil, and fortified food (such as milk, orange juice, butter, cheeses, and breakfast cereals). Prescription vitamin D preparations contain ergocalciferol (50,000 IU/capsule) while over-the-counter supplements contain cholecalciferol (400, 800, 1000, and 2000 IU/capsule).

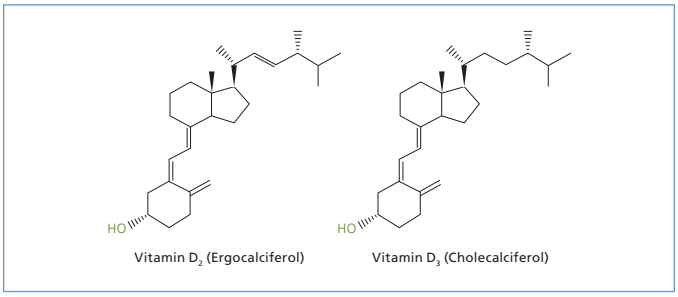


Figure 1. Structures of Vitamin D_2 (ergocalciferol) and Vitamin D_3 (cholecalciferol).

Upon penetration of the skin with ultraviolet B radiation, vitamin D_3 is synthesized from 7-dehydroxycholesterol to form previtamin D_3 which is rapidly converted by a thermally-induced process to vitamin D_3. Vitamin D_3 is converted into its metabolically active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) by two subsequent hydroxylations, consisting of 25-hydroxylation in the liver followed by 1α-hydroxylation in the kidney (Figure 2). Both hydroxylations are carried out by specific cytochrome P450-containing enzymes, the first by vitamin D 25-hydroxylase (CYP27) and the second by 25-hydroxyvitamin D 1α-hydroxylase (CYP1α).

25-Hydroxyvitamin D is the metabolite measured in the blood to determine the vitamin D status of patients. Methodologies for measuring serum 25(OH)D include competitive protein binding assays (CPBA), radioimmunoassay (RIA), enzyme-linked immunoassay (ELISA), high-performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrometry (LC/MS), and random access automated assay (RAAA) based on chemiluminescence assay technology. Conventional techniques for vitamin D analysis, based on immunoassay or LC/UV, often lack adequate sensitivity, specificity, and speed; thus, interest in UHPLC/MS methods is growing. An advantage of UHPLC/MS technology is the ability to differentiate between $25(OH)D_2$ and $25(OH)D_3$ which is particularly useful when monitoring the vitamin D levels of patients being treated with vitamin D_2.

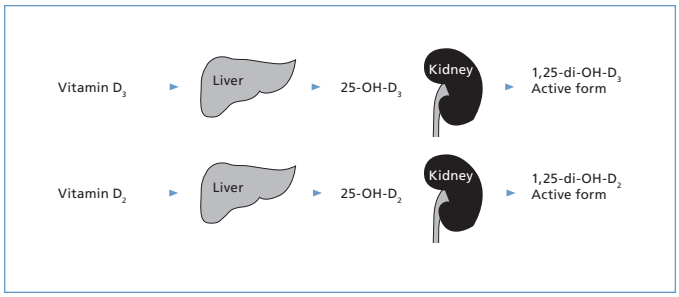


Figure 2. Formed in the skin, vitamins D_3 and D_2 are converted into their metabolically active forms by subsequent reactions in the liver and kidneys.

Experimental Conditions			
Target Analytes: 25-Hydroxyvitamin D_2 and D_3			
Sample Preparation Conditions:			
Human serum controls and patient unknown samples were obtained. 150 µl serum was taken and 50 µl internal standard, 25-Hydroxyvitamin D_3-d_6, added and vortex. 150ul 0.2M $ZnSo_4$ was added and again vortex. Finally, 750 ml Hexane was added to the mixture and centrifuged at 8000 RPM for 7 min. The top layer, organic phase, was removed and blown down under nitrogen and reconstituted in 100 µl MeOH/H_2O and analyzed by HPLC/SQ MS.			
Liquid Chromatography Conditions:			
Pump Type:	Flexar FX-10		
Column:	PerkinElmer Brownlee™ HRes Analytical DB C18 column (2.1 mm x 50 mm, 1.9 µm)		
Mobile Phase:	A: water containing 2mM Ammonium Acetate and 0.1% formic acid		
	B: methanol containing 2mM Ammonium Acetate and 0.1% formic acid		
Flow Rate:	0.3 ml/min		
Injection Volume:	8 µl in micro liter pickup mode		
Isocratic Conditions:	Time (min)	%A	%B
	0	7	93
	6	7	93
Mass Spectrometer Conditions:			
Ionization:	Ultraspray ESI—Positive mode		
The $[M+H]^+$ ions of each of the analytes were monitored:			
Time Period 1: (0-6 min) SIM ions 401.4, 407.4, 413.4 were monitored for 25-Hydroxyvitamin D_3, 25-Hydroxyvitamin D_3-d_6 and 25-Hydroxyvitamin D_2 respectively; dwell time of 150 ms each			
Capillary Exit Voltage:	45 V		

Results.

UHPLC/SQ MS allows for increased specificity for detection as compared to competitive protein binding assays (CPBA), radioimmunoassay (RIA), enzyme-linked immunoassay (ELISA), high-performance liquid chromatography (HPLC) as it can monitor both D_2 and D_3 levels separately as opposed to total vitamin D. This is important especially for patients being treated with vitamin D_2.

Measurement of both 25-hydroxyvitamin D_2 and D_3 is possible using the Flexar SQ 300 MS with a simple protein precipitation/LLE method. The conditions used here for routine vitamin D testing by UHPLC/SQ MS resulted in highly symmetric peaks with a run time of 6 min which is much faster than competing techniques. We were able to detect and quantify patient samples below 4 ng/ml.

According to current industry definitions, severe deficiency can be defined as <6 ng/ml, deficiency as 16 ng/ml, insufficiency as <20 ng/ml and as normal >20 ng/ml[1].

Therefore, the Flexar SQ 300 MS is fully capable of detecting and quantifying within the clinical significant range for vitamin D.

[1]*Mosekilde L et al, Ugeskr Læger 167/1, 2005, 29-33*

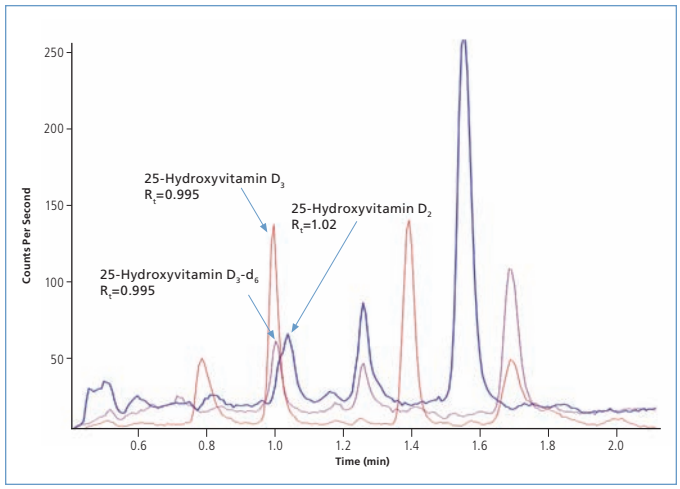


Figure 3. Patient sample with separation and detection of 25-hydroxyvitamin D_2 (blue trace), 25-hydroxyvitamin D_3 (red trace) and 25-hydroxyvitamin D_3-d_6 (purple trace).

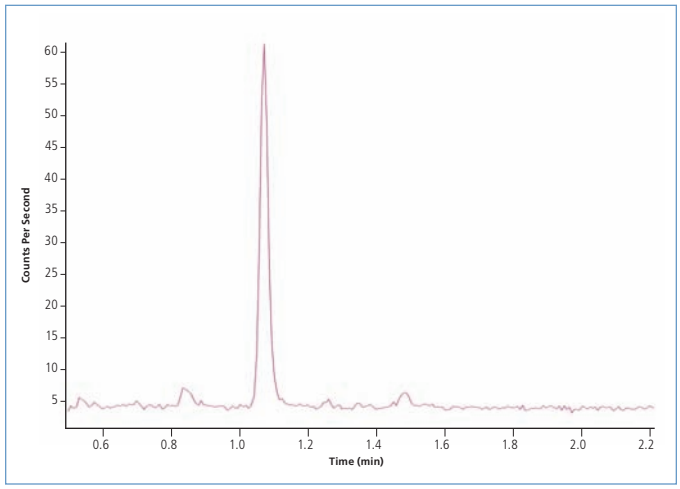


Figure 4. Standard injection of 25-hydroxyvitamin D_3-d_6 at 3 ng/ml.

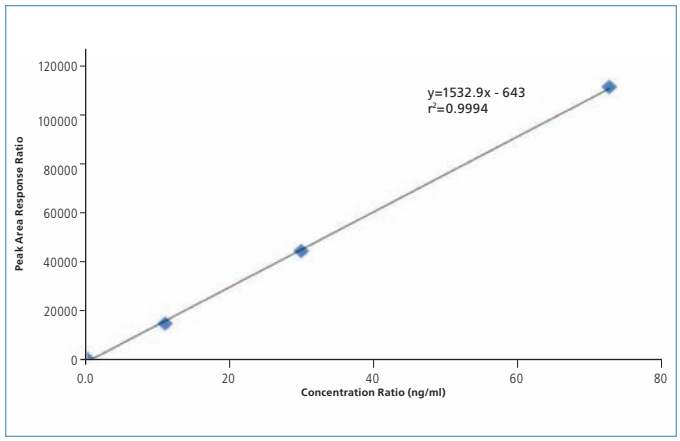


Figure 5. Calibration curve for 25-hydroxyvitamin D_3 concentration range from blank to 73 ng/ml.

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