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Ionization Efficiency for Environmentally Relevant Compounds Using Atmospheric Pressure Photoionization Versus Electrospray Ionization

For solution-phase samples, the world of mass spectrometry defaults to electrospray ionization (ESI). ESI is used for the analysis of a broad variety of compounds, ranging from polar to moderately nonpolar. However, ESI possesses limitations that prevent the ionization of certain analytes—particularly nonpolar compounds. This study aims to compare the ionization efficiency of complementary ionization techniques, and demonstrate that multiple methods can improve the analytical results with respect to limits of detection and matrix tolerance. Atmospheric pressure photoionization (APPI) is an ionization method that complements ESI, excelling in the analysis of nonpolar and moderately polar analytes. For this study, we optimized methods using APPI and ESI for the detection and quantitation of pharmaceuticals frequently detected in the environment, including antibiotics, beta-blockers, and selective-serotonin reuptake inhibitors, and tested their matrix tolerance relative to artificial wastewater. While most of these compounds ionized preferentially by ESI, some performed significantly better using APPI.

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Every day in the world of analytical chemistry, we strive to improve our analytical methods to achieve lower limits of detection, have broader dynamic ranges, or tolerate greater interference, for example. Along with the need for improved methods of quantitation, there is pressure on the field of mass spectrometry (MS) to be able to successfully detect multitudes of analytes of interest from single injections, which poses the question, How do we improve the detection of analytes? However, sometimes using a single injection reduces performance by only using a single ionization method. When you can optimize not only your separation, but also your ionization method, your method may truly improve. The basic principle of MS is to ionize molecules under study into gaseous ions, separate these ions based on their mass to charge (m/z) ratio, and detect them (1). Today's commercial instruments are capable of transferring 97–99% of ions successfully from the source to the detector, so the greatest improvements in detection and methods today are focused on the source. The ultimate question then becomes, When ionizing a sample, will the use of complementary ionization techniques improve the figures of merit of the individual analytes?

ESI and APPI: Complementary Ionization Techniques

By choosing the appropriate ionization source, liquid chromatography–tandem mass spectrometry (LC–MS/MS) can be used for the detection of trace levels of contaminants like

Table I: Physicochemical characteristics of antibiotics and endocrine disruptor compounds (EDCs) (24)

Class	Compound	Chemical Formula	Molecular Weight (g/mol)	Water Solubility (mg/mL)	pK_a	Log K_{ow}
Beta-lactam antibiotics	Ceftriaxone	$C_{18}H_{18}N_8O_7S_3$	554.6	0.105	2.5	0.68*
	Cephalexin	$C_{16}H_{17}N_3O_4S$	347.4	0.297	5.2	0.65*
	Ampicillin	$C_{16}H_{19}N_3O_4S$	349.4	10.1	2.5	1.35*
	Penicillin G	$C_{16}H_{18}N_2O_3S$	334.4	0.285	2.74	1.83*
Macrolide antibiotics	Erythromycin	$C_{37}H_{67}NO_{13}$	733.9	2	8.9	3.06
	Tylosin	$C_{46}H_{77}NO_{17}$	916.1	5	7.73	3.5
Sulfonamide antibiotics	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.3	0.61	1.6	0.89*
	Trimethoprim	$C_{14}H_{18}N_4O_3$	290.3	0.4	7.12	0.91*
Tetracycline antibiotics	Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.4	0.313	9.5	-0.92*
	Tetracycline	$C_{22}H_{24}N_2O_8$	444.4	0.231	3.3	-1.37*
Nitroimidazole antibiotics	Metronidazole	$C_6H_9N_3O_3$	171.16	9.5	14.58	-0.02*
	1,2 dimethyl-5-nitroimidazole	$C_5H_7N_3O_2$	141.13	18.3	2.81	0.31*
β1-selective beta-blocker	Acebutolol	$C_{18}H_{28}N_2O_4$	336.43	0.259	9.57	1.77*
β1-selective beta-blocker	Metoprolol	$C_{15}H_{25}NO_3$	267.36	0.402	9.68	1.95*
β -adrenergic blocker	Propranolol	$C_{16}H_{21}NO_2$	259.34	0.0794	9.45	3.48
β1-selective beta-blocker	Atenolol	$C_{14}H_{22}N_2O_3$	266.34	0.429	9.6	0.16*
SSNRI	Venlafaxine	$C_{17}H_{27}NO_2$	277.40	0.230	10.09	3.20
SSRI	Citalopram	$C_{20}H_{21}FN_2O$	324.39	0.00588	9.78	3.74
SSRI	Paroxetine	$C_{19}H_{20}FNO_3$	329.37	0.00853	9.6	1.37*

* A compound with Log K_{ow} less than 2.5 means the compound is hydrophilic and readily found in the aqueous phase.
(SSNRI stands for selective serotonin and norepinephrine reuptake inhibitor)
(SSRI stands for selective serotonin reuptake inhibitor)

antibiotics and endocrine disruptor compounds (EDCs) from environmental samples. The electrospray ionization (ESI) source has been used as a powerful soft ionization technique for the analysis of a wide array of sample types, ranging from polar to nonpolar by MS, and can also be used for the analysis of thermolabile molecules of high molecular weight (2). Although ESI is popularly used for the analysis of environmental pollutants, it may not be able to ionize all contaminants efficiently. Certain contaminants are either poorly ionized, or not ionized at all. ESI is limited to analytes that are of low to high polarity, and moderate to high molecular weight (3).

Atmospheric pressure photoionization (APPI), introduced in 2000, is also a soft ionization technique. APPI was found to have success with the analysis of compounds with low to no polarity, and compounds of low to moderate molecular weight, but cannot be used on thermolabile compounds. These parameters are what make APPI and ESI complementary to each other (4). This opens new doors for studies already utilizing the ESI method, because APPI can be used for complementary analysis of compounds that may not be detected by ESI. Additionally, APPI has shown tolerance to matrix components beyond what ESI has, due to its ionization pathway (5). APPI can be used for the analysis of a wide range of compounds, including drugs, human endogenous compounds, lipids, natural compounds, pesticides, synthetic organics, and petroleum derivatives (6). There are very few research papers reporting comparative studies of ionization efficiencies in MS for the detection of antibiotics and EDCs (7–9).

A triple quadrupole mass analyzer was used in this study utilizing both the full scan mode—optimization and multiple reaction monitoring (MRM)—quantitation. Full scan mode can give qualitative analysis of a sample's composition under study, and MRM mode is a highly selective mass monitoring mode with a wider linear dynamic range, improved limit of quantitation (LOQ), increased sensitivity, and superior accuracy. The advantage of the

MRM scan mode is improved signal-to-noise ratio due to removal of nonanalyte ions and isobaric precursors by monitoring fragments.

There are different MS acquisition parameters that affect the signal intensity of ions. The Agilent MassHunter Data Acquisition Software used in this study sets a default value for all acquisition parameters for each ionization source (see table S1 in the supplemental information). There is a sheath gas flow chamber in the electrospray ionization source that is absent in the atmospheric pressure photoionization source. As a result, the sheath gas temperature and sheath gas flow rate parameters are present only for ESI, while APPI has an additional vaporizer parameter that is not present in ESI. Fragmentor voltage, collision energy, cell accelerator voltage, gas temperature, vaporizer, gas flow (L/min), nebulizer (psi), sheath gas temperature, and sheath gas flow rate were all optimized for each analyte prior to data acquisition in this study.

Analytes of Interest

The current global population is growing at the annual rate of 1.09%. This increase in population means that pharmaceuticals are continuing to be prescribed and consumed at an alarming rate. In 76 countries across the globe, antibiotic consumption as described in defined daily doses (DDD) increased by 65%—from 21.1 billion doses in 2000 to 34.8 billion doses in 2015—and the overall antibiotic consumption rate has increased by 39% (10). In addition to antibiotics, beta blockers and antidepressants are two classes of pharmaceuticals gaining popularity. Beta blockers are a class of drugs frequently used to treat hypertension, heart disease, and other cardiovascular events. Although the true nature of their efficacy has been questioned in certain studies, beta blockers are still highly prescribed, due to the diverse range of clinical symptoms they can successfully treat (11–12). Per the National Center for Health Statistics (NCHS) in 2017, the rate of antidepressant use in America has increased by 65% since 1999 (13). Unfortunately, this increase in pharmaceutical use means more pharmaceutical waste is likely to end up in the environment. Although there is an urgency to know the exact harm this excess will cause, the priority is to harness the ability to detect as many pharmaceuticals in environmental samples as possible. This will then allow for proper removal techniques to be employed before the harmful substances have a chance to further contaminate the environment (14).

Low Concentrations, Large Impact

Numerous studies have shown that some pharmaceuticals are not completely removed during the wastewater treatment, and ultimately enter the environment in low concentrations. The adverse effects of pharmaceuticals entering the environment in low concentrations are

Table II: Ionization source efficiency for antibiotics and endocrine disruptor compounds (EDCs)

Class	Antibiotics	ESI	APPI	Efficient Ionization Source
		LOQ (ppb)	LOQ (ppb)	
Beta lactams	Ampicillin	<0.001	<0.001	-
	Ceftriaxone	<0.001	-	ESI
	Cephalexin	<0.001	<0.001	-
	Penicillin G	139.1	<0.001	APPI
Macrolides	Erythromycin	1.158	162.5	ESI
	Tylosin	5.337	3401	ESI
Sulfonamides	Sulfamethoxazole	226.1	70.87	APPI
	Trimethoprim	81.1063	<0.001	APPI
Tetracyclines	Oxytetracycline	<0.001	-	ESI
	Tetracycline	<0.001	<0.001	-
Nitroimidazoles	Metronidazole	<0.001	2.945	ESI
	1,2 Dimethyl-5-nitroimidazole	169.2	19.91	APPI

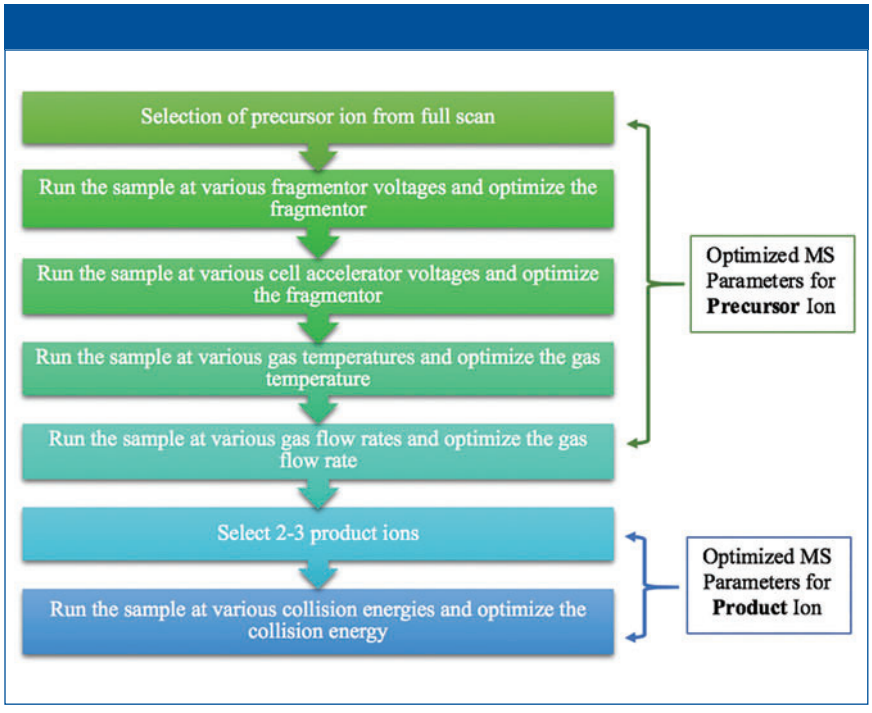

Figure 1: Flowchart of the ionization optimization strategy used in this study.

antibiotic resistance, genotoxicity, acute or chronic toxicity, and endocrine disruption (15). Antibiotics and EDCs are emerging pollutants detected throughout the world, yet they remain unregulated by the United States Environmental Protection Agency (EPA) (16).

Sir Alexander Fleming, the British bacteriologist, discovered penicillin in 1928 from the fungus *Penicillium notatum,* and what followed was an era of novel antibiotics derived from microorganisms and antibiotic synthesis (17). The ability of antibiotics to eradicate a wide range of bacterial infections led to their increased use over time. Unfortunately, bacteria have developed mechanisms to combat the actions of antibiotics. Thus, the overprescribing of antibiotics, along with a lack of patient knowledge regarding the importance of correct antibiotic administration, has become an insidious issue that is known as *antibiotic resistance*. Antibiotic resistance arises as microorganisms develop the ability to survive the action of antibiotics, meaning that when antibiotic resistant bacteria infect animals and humans, the antibiotic regimen that would normally eradicate the bacteria becomes useless. This is the reason being able to successfully detect antibiotics from wastewater samples is so important. In this study, five different classes of antibiotics were used: beta-lactams, macrolides, nitroimidazoles, sulfonamides, and tetracyclines.

Antibiotics can either be bacteriostatic, which means they prevent the growth of bacteria, or they can be bactericidal, meaning they actively kill bacteria. However, antibiotics' mechanism of action is more important when considering treatment options. Beta-lactams inhibit the biosynthesis of bacterial cell walls by making penicillin-binding proteins unavailable for new peptidoglycan synthesis, which causes the lysing of bacteria. The beta-lactams used in this study are ampicillin, ceftriaxone, cephalexin, and penicillin G. Macrolides inhibit protein synthesis during translocation in bacteria by dissociating peptidyl-tRNA from the middle of the 23S rRNA of the ribosome's 50S subunit,

Table III: Ionization source efficiency for endocrine disruptor compounds (EDCs)

Class	EDCs	ESI	APPI	Efficient Ionization Source
		LOQ (ppb)	LOQ (ppb)	
Beta blockers	Acebutolol	14.81	38.37	ESI
	Atenolol	9.68	22.44	ESI
	Propranolol	<0.001	<0.001	-
	Metoprolol	8.690	21.700	ESI
Selective serotonin reuptake inhibitors	Paroxetine	<0.001	<0.001	-
	Citalopram	166.2	<0.001	APPI
	Venlafaxine	<0.001	3788	ESI

Table IV: Matrix effect on the analysis of antibiotics using ESI and APPI

Antibiotics	ESI			APPI		
	With Matrix LOQ (ppm)	Without matrix LOQ (ppm)	Matrix Effect (%)	With Matrix LOQ (ppm)	Without Matrix LOQ (ppm)	Matrix Effect (%)
Trimethoprim	<LOD	0.0811	-	0.0357	<LOD	-
Ampicillin	0.0144	<LOD	-	<LOD	<LOD	-
Ceftriaxone	<LOD	<LOD	-	-	-	-
Cephalexin	0.166	<LOD	-	<LOD	<LOD	-
Erythromycin	0.00976	0.00116	742.92	0.988	0.163	507.62
Oxytetracycline	<LOD	<LOD	-	-	-	-
Penicillin G	0.00796	0.139	-94.28	<LOD	<LOD	-
Sulfamethoxazole	<LOD	0.226	-	0.939	0.0709	1224.93
Tetracycline	<LOD	<LOD	-	<LOD	<LOD	-
Tylosin	0.0293	0.00534	448.47	0.00629	3.40	-99.82
Metronidazole	<LOD	<LOD	-	0.00233	0.00295	-20.77
1,2 Dimethyl-5-nitroimidazole	0.0268	0.169	-84.18	0.0285	0.0199	43.13

causing early detachment of unfinished peptide chains. The macrolides used in this study are erythromycin and tylosin, an antibiotic popularly used in farm animals. Tetracyclines prevent the attachment of aminoacyl t-RNA to the A site in bacterial ribosomes by acting on the 16S rRNA of the 30S subunit inhibiting protein synthesis. Oxytetracycline and tetracycline were the tetracyclines used in this study. Sulfonamides prevent the multiplication and growth of bacteria by inhibiting certain steps in the metabolism of folic acid. Sulfamethoxazole and trimethoprim were the sulfonamides used in this study. Nitroimidazole antibiotics inhibit nucleic acid synthesis that occurs in bacterial cells by disruption of the DNA in microorganisms. Metronidazole and 1,2 dimethyl-5-nitroimidazole were the nitroimidazoles used in this study (18).

EDCs are natural compounds or synthetic chemicals that mimic natural hormones in the body and interfere with the action of the natural hormones (19). These compounds most profoundly cause adverse effects on reproduction, developmental, neural and immune systems of human beings and animals. Research suggests that EDCs reduce fertility and the increase the risk of cancer, diabetes, obesity, and endometriosis (20). Among various EDCs, beta-blockers (acebutolol, atenolol, metoprolol and propranolol) and SSRI antidepressants (citalopram, paroxetine and venlafaxine) were used in this study.

Make or Break for Successful Analysis: Matrix Effects and Wastewater

Properly dealing with impurities is a necessary complication in every field of research. In MS, the problem with matrix is variability in ionization efficiency of analytes of interest as coeluted species serve to either enhance or inhibit the ionization process for an analyte. This issue becomes increasingly problematic when trying to discern analytes of interest from wastewater. Water that is obtained as a byproduct of agricultural, industrial, domestic, and commercial activity is termed *wastewater*. Wastewater contains nutrients such as calcium, iron, nitrogen, phosphorus, potassium, and components such as fats, sugars, and proteins. Synthetic wastewater was made to mimic the wastewater from the influent of a typical wastewater treatment plant with its composition designed to imitate the dissolved inorganic solids and dissolved organic solids of real wastewater. The synthetic wastewater prepared in this study was from H. E. Gray (2012) (21).

APPI has been found to be less susceptible to matrix effects compared to ESI. This is likely due to the fact that APPI is more selective in ionization, because the photon emitter krypton lamp at 10.6 eV can ionize analytes, but not the matrix component, meaning that the difference in how a sample is ionized

can be the difference between more or less matrix interference (22). Using APPI involves the ejection of an electron from the analyte molecule to produce the gaseous radical cation (23). It is also possible, however, that the matrix component can act as a dopant and ionize sample components with high ionization energy through electron transfer leading to signal enhancement.

Although matrix effects cannot be removed completely, they can be minimized by optimizing the sample preparation procedure and LC–MS parameters. Solid-phase extraction (SPE) with an appropriate sorbent can reduce the matrix effect by eliminating interfering matrices. The formula for calculation of the matrix effect is:

$$\text{Matrix effect} = \left[\left(\frac{\text{analyte in matrix}}{\text{same conc.of analyte in neat solvent}}\right) - 1\right] \times 100 \quad [1]$$

Physicochemical characteristics of antibiotics and EDCs help to determine the environmental fate of these compounds. Table I contains important physicochemical characteristics of antibiotics and EDCs under study including solubility, pK_a, and log K_{ow}. A compound with a log K_{ow} value <2.5 is hydrophilic and readily found in the aqueous phase.

Methods

Specific Analytes Used

A total of 12 antibiotics and 7 EDCs were analyzed in this study. Ceftriaxone sodium salt hemi(heptahydrate) and erythromycin were purchased from Acros Organics with a purity of >98%. Propranolol hydrochloride (99%), metronidazole (99%), acebutolol hydrochloride, metoprolol tartrate (98+%), tetracycline hydrochloride (96%), and oxytetracycline hydrochloride were purchased from Alfa Aesar. TCI Co. was the main supplier of chemicals: cephalexin monohydrate (>98%), sulfamethoxazole (>98%), penicillin G potassium salt (>98%), atenolol (98%), venlafaxine hydrochloride (>98%), trimethoprim (>98%), citalopram hydrobromide (>98%), and 1,2 dimethyl-5-nitroimidazole. Ampicillin sodium salt was procured from Affymetrix Inc. Tylosin tartrate (95+%) and paroxetine hydrochloride (98+%) were bought from Ark Pharm Inc. All antibiotics and EDCs were used without further purification. All the solvents used in the analysis are of HPLC grade and purchased from Fischer Chemical. Potassium phosphate monobasic (99.8%) was purchased from EK Industries Inc. Sodium acetate trihydrate (100.7%), magnesium sulfate heptahydrate (99.9%), ammonium chloride (99.7%), and calcium chloride dihydrate (99.9%) were bought from Fischer Scientific. All solvents and chemicals were used without further purification.

Optimized Parameters

The parameters were optimized for MS as follows: 1.00 ppm sample of each analyte was analyzed for the selection of the precursor ion, optimization of fragmentor voltage, optimization of cell accelerator voltage, optimization of gas temperature, optimization of gas flow rate, and optimization of collision energy. All the ions formed were analyzed for intensity. Ions with *m/z* values equal to and greater than the molecular weight of the analyte were considered to determine the precursor ion. See Figure 1 for the flowchart of the optimization strategy.

A calibration curve for each analyte was obtained by the internal calibration method using the optimized MS parameters. Calibration was performed in the range of 1.00 ppt to 10.0 ppm for each analyte under study. Each of the standard solutions for antibiotics was spiked with the mixture of internal standards of antibiotics (azithromycin d_3, cephalexin d_5, ciprofloxacin d_8, penicillin G d_5, sulfamethoxazole d_4, and trimethoprim d_3) to produce a final concentration of 100 ppb of each internal standard. Each of the standard solutions for EDCs was spiked with the mixture of internal standards of EDCs (metoprolol d_7 and paroxetine d_6) to produce a final concentration of 10.0 ppb of each internal standard.

A synthetic wastewater matrix solution was prepared by dissolving potassium phosphate monobasic, sodium acetate trihydrate, magnesium sulfate heptahydrate, ammonium chloride, and calcium chloride dihydrate in MilliQ water. The concentration and quantity of reagents used for synthetic wastewater matrix preparation is given in supplemental table S2.

Analysis of the antibiotics and EDCs was performed using an Agilent Technologies 1290-6460 Triple Quadrupole LC–MS/MS instrument using two ionization sources: an Agilent Jet Spray ESI source and an Agilent APPI source operated in positive mode. Full scan mode was used for the optimization of MS parameters, and MRM mode was used for calibration and analysis of wastewater. Data interpretation was performed using Agilent's MassHunter Workstation Software. HPLC parameters for analysis are given in supplemental table S3.

Calibration was performed in the range of 1.00 ppb to 10.0 ppm in matrix for all the analytes under study. Internal standards were added as described previously.

A setup of Waters Oasis Prime HLB cartridges and a SPE vacuum manifold was used for off-line SPE to extract antibiotics and EDCs from the synthetic wastewater matrix calibration sample. Waters Oasis Prime HLB cartridges, 1 mL barrel syringe with 30 mg universal polymeric reversed-phase sorbent, were employed. SPE pretreatment was performed by washing the column with 2 mL of HPLC-grade methanol, 2 mL of Millipore deionized water, and 2 mL of Millipore deionized water at pH 2 under gravity. Then the samples were loaded on the column under vacuum at 10–20 mL/min rate.

Washing and Elution Step for Antibiotics Sample

After loading the sample on the column, the cartridge was washed with 2 mL Millipore deionized water for the antibiotics sample. The column was then washed first with 2 mL of methanol, and then with 1 mL of methanol: acetone (1:1) under gravity, and collected and combined in test tubes.

Table V: Matrix effect on EDCs using ESI and APPI

Antibiotics	ESI			APPI		
	With Matrix LOQ (ppm)	Without matrix LOQ (ppm)	Matrix Effect (%)	With Matrix LOQ (ppm)	Without Matrix LOQ (ppm)	Matrix Effect (%)
Acebutolol	0.0382	0.0148	158.11	0.135	0.0384	252.95
Atenolol	6.53	0.00968	67432.40	0.330	0.0224	1369.77
Citalopram	<LOD	166.2	-	<LOD	<LOD	-
Metoprolol	1.04	0.00869	11865.13	0.115	0.0217	428.97
Paroxetine	<LOD	<LOD	-	<LOD	<LOD	-
Propranolol	0.249	<LOD	-	<LOD	<LOD	-
Venlafaxine	0.104	<LOD	-	3.71	3.79	-2.07

Table VI: Antibiotic detected in real wastewater sample using ESI and APPI

Antibiotics	With Matrix (ppm)		Without Matrix (ppm)	
	ESI	APPI	ESI	APPI
Trimethoprim	1.36	1.08	6.20	6.92
Ampicillin	0.125	<LOD	<LOD	<LOD
Ceftriaxone	0.860	-	8.92	-
Cephalexin	0.13*	11.2*	0.0747*	4.04*
Erythromycin	6.23	0.316	4.96	0.819
Oxytetracycline	0.317	-	2.58	-
Penicillin G	0.168*	4.62*	0.562	0.939
Sulfamethoxazole	0.748*	0.181*	2.89*	0.949*
Tetracycline	0.311	>LOL	3.13	>LOL
Tylosin	0.496	0.273	<LOD	0.889
Metronidazole	0.0330	0.0170	0.309	0.0200
1,2 Dimethyl-5-nitroimidazole	0.0857	<LOD	0.136	0.00310

*t-test p-value < 0.05

Washing and Elution Step for EDCs Sample

EDCs sample cartridges were washed with 1 mL of methanol:water (5:95) solvent. After the first washing step, the column was dried for 15–30 min under vacuum. The column was washed first with 1 mL of ethyl acetate:methanol (9:1) under gravity (this eluate was collected in a test tube labeled as fraction 1), then washed with 1 mL of 5% methanol:2% acetic acid in water, then with 1 mL of 5% methanol:2% NH_4OH in water under vacuum, and dried for 10–15 min under vacuum. After drying, the column was eluted with 1 mL of 2% NH_4OH in methanol, and combined with eluate present in the test tubes labeled as fraction 1.

Wastewater Sample

Wastewater samples were collected from the influent of the aeration treatment at the Environmental Resources Training Center (ERTC), a training center for drinking water and wastewater treatment at Southern Illinois University Edwardsville (SIUE). The samples were analyzed for the presence of antibiotics and EDCs to demonstrate the effectiveness of the method development on real wastewater samples.

The wastewater samples were sequentially filtered through VWR 417 (40 µm) filter paper, then through VWR 696 (1.2 µm) glass microfiber filter paper, and then through an Ahlstrom 193 (0.7 µm) microfiber glass filter. The filtrate was separated into 6 bottles each with 250 mL of filtrate, 3 samples for analysis of antibiotics and 3 for EDCs. All samples were spiked with appropriate internal standards as described previously.

Samples were adjusted to pH 3.0 using 6.0 M sulfuric acid before performing the SPE. Waters Oasis Prime HLB cartridges (6 mL, 200 mg universal polymeric reversed-phase sorbent) were used for wastewater sample analyte extraction. The SPE method for wastewater was identical to the synthetic matrix sample preparation except the quantity of reagent solvents used was five times greater due to the increased volume and cartridge bed mass.

Results and Discussion

The optimized MS parameters for the precursor ion of antibiotics for ESI as an ionization source can be found in supplemental tables S4 and S5, while parameters for APPI are shown in S6 and S7. Ions with the highest intensity peak with a m/z equal to or greater than the molecular weight of the analyte were selected as potential precursor ions and MS parameters were optimized using these ions.

The optimized MS parameters for the product ion of antibiotics for ESI as an ionization source can be found in supplemental tables S8 and S9, while parameters for APPI are shown in S10 and S11. At most, three ions with m/z less than the molecular weight of the analyte and with the highest ion abundance were optimized to determine the optimized collision energy of the product ions.

The regression equations for antibiotics and EDCs without matrix were selected such that they were equivalent for calibration curve performed with

Table VII: Antibiotic detected in real wastewater sample using ESI and APPI

Antibiotics	With Matrix (ppm)		Without Matrix (ppm)	
	ESI	APPI	ESI	APPI
Acebutolol	0.235	2.78	0.0344	0.925
Atenolol	<LOD	0.0234	<LOD	<LOD
Citalopram	<LOD	0.0388	0.144*	0.419*
Metoprolol	0.0830	0.0006	0.167	<LOD
Paroxetine	<LOD	<LOD	0.229	7.67
Propranolol	0.474*	0.932*	0.906	2.50
Venlafaxine	0.0503*	2.48*	0.0271*	4.00*

*t-test p-value < 0.05

and without matrix. The calibration curve correlation coefficient (R^2) criteria was established as higher than 0.99 for all the antibiotics and EDCs without matrix using ESI, shown in supplemental tables S12 and S13. Ampicillin, ceftriaxone, cephalexin, sulfamethoxazole, oxytetracycline, tetracycline, and metronidazole have limits of detection (LODs) <1 ppt using ESI and sulfamethoxazole has the highest LOQ among the antibiotics analyzed (226.1 ppb). All standard calibration curves are shown in supplemental table S20. Paroxetine, propranolol, and venlafaxine have a LOD <1 ppt using ESI, and acebutolol has the highest LOQ(14.81 ppb).

Ampicillin, cephalexin, penicillin G, trimethoprim, and tetracycline have LOD values <1 ppt using APPI as the ionization, and tylosin has the highest LOQ among the antibiotics analyzed (3,401 ppb), as shown in supplemental table S14. Citalopram, paroxetine, and propranolol have LODs <1 ppt using APPI as the ionization source and venlafaxine has the highest LOQ (3,788 ppb) as shown in supplemental table S15.

The efficiency of both ionization sources was determined by comparing the LOQ for each of the pharmaceuticals obtained using ESI and APPI. Limits of quantitation <1 ppt in both the ionization sources means the most efficient ionization source could not be determined. Any compounds that are thermolabile will degrade using APPI, meaning the compound will not be detected by APPI, and ESI was the ionization source that was used. Erythromycin, tylosin, and metronidazole ionized efficiently by ESI based on the comparative LOQ result. This hypothesized to be due to the pK_a of each compound (Table I) being greater that the pH of the mobile phase (3.80) used in the analysis of antibiotics which allows it to protonate easily. Acebutolol, atenolol, metoprolol, and venlafaxine were ionized efficiently by ESI based on the comparative LOQ results obtained from the calibration curve performed with and without matrix. A complete breakdown of this analysis can be found in Tables II and III.

Penicillin G, sulfamethoxazole, and 1,2 dimethyl-5-nitroimidazole have lower LOQs when ionized by APPI, so APPI is the preferred ionization source for these analytes. The pK_a values of these compounds (Table I) are less than the pH of the mobile phase (3.80) leading them not to be protonated in solution. In addition, sulfamethoxazole and 1,2 dimethyl-5-nitroimidazole each have a high degree of conjugation in their structures facilitating the absorption of photons and molecular radical ion formation ($M^{+\bullet}$) (24). This specific trend was not observed in the case of trimethoprim indicating that some other preferred ion formation pathway must be present. Citalopram also has a higher degree of conjugation in its structure which facilitates the absorption of photons and molecular radical ion formation ($M^{+\bullet}$) making the APPI source highly efficient for the analysis of these compounds.

Two-way paired t-tests were conducted at a significance level (α) of 0.05 on the data of the calibration curve performed with an artificial matrix and without a matrix. Supplemental table S16 provides the p-values of the test for both the ESI and APPI ionization sources for analysis of antibiotics and shows that all population means are equal, therefore there is no significant difference between data obtained with or without a matrix. Supplemental table S17 shows the population means are also equal between the data obtained with and without artificial matrix.

The matrix effects on antibiotics and EDCs using ESI and APPI was calculated using the limit of quantitation (LOQ) obtained from calibration curves performed with and without the matrix. The trends can be seen in Tables IV and V.

Real wastewater samples from the ERTC were analyzed for the detection of antibiotics and EDCs. Table VI shows the concentration of antibiotics in real wastewater samples and Table VII shows the EDCs calculated using the calibration curve equation obtained from calibration curve performed with and without synthetic matrix using ESI and APPI.

Paired t-tests were used to compare the concentration of antibiotics and EDCs in sample calculated using calibration curve performed with artificial matrix and without matrix. Two-way paired t-tests were conducted at a significance level (α) of 0.05 and the significance difference between the concentration of antibiotics and EDCs on the real wastewater sample collected from ERTC wastewater treatment plant using the equation obtained from calibration curve performed with artificial matrix and without a matrix were determined. Only three of the p-values shown in supplemental tables S18 and S19 demonstrated a statistical difference between using the matrix calibration

curve versus the water curve illustrating that while important for some analytes, overall it had insignificant effect in this specific study.

Conclusion

There are few studies comparing the efficiencies of ionization sources for the analysis of pharmaceutical analytes. While many researchers are limited to using ESI as their only ionization source, the use of complementary ionization techniques produces better results for the quantitation of analytes, and should thus be considered for future studies. When purchasing an instrument costing $250,000 and up, the addition of a $25,000 additional ion source in order to improve analyte coverage in one's analysis should be viewed as nearly doubling the analytical capabilities of the instrument in terms of analyte coverage.

It was found that ESI is preferable for the analysis of pharmaceuticals such as antibiotics, beta-blockers, and SSRI antidepressants. However, ESI is not suitable for the ionization of all the pharmaceuticals with high sensitivity. APPI is an excellent complement to ESI; it is highly efficient in the ionization of analytes that ESI is unable to ionize.

There was no significant difference observed in the presence of a matrix at very low analyte concentrations. With higher concentrations of analytes, however, matrix effects should be taken into consideration when using these methods given the significant difference observed. Since the limit of quantitation of most of the analytes was <1 ppt, further study is needed to determine the ionization efficiency of ESI and APPI for these compounds by calibrating at lower concentrations.

By determining the ionization energy of analytes using the appropriate software, it can be predicted which compounds will ionize by APPI or ESI preferentially. This determination will aide in the analysis of other classes of environmental pollutants, including other groups of pharmaceuticals like statins and pesticides.

Using ESI and APPI as complementary ionization techniques yields a more complete picture of what compounds are present when analyzing in full scan mode and better quantitation of analytes when appropriately optimized. This information holds value because employing multiple ionization techniques is an easy fix that creates a cost-effective method for analyte detection that can improve the outcome of research in future studies.

Supplemental Information

The supplemental tables can be found online in the issue archives.

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Recent Advances in Hyphenated Chromatography and Mass Spectrometry Techniques and Their Impact on Late-Stage Pharmaceutical Development

This article reviews the changing role of mass spectrometry (MS) hyphenated to reversed-phase liquid chromatography (LC) and alternative separation techniques in late-stage pharmaceutical development. The impact of the changing portfolios within the pharmaceutical industry is discussed as the industry moves from a traditional small-molecule model to a more diverse portfolio. A new generation of high-resolution mass spectrometers and ion mobility mass spectrometers operating as orthogonal separation techniques has greatly increased the ability to resolve impurities and increase the level of knowledge gained from a single experiment. The continued impact and innovation of gas chromatography–mass spectrometry (GC–MS) in late-stage development is also discussed.

Tony Bristow and Andrew Ray

The introduction of small, compact mass spectrometers has widened the potential uses for this technique (1) These mass spectrometers may be considered as cheaper options for open access systems, and are used as supplementary and complementary detectors to UV for peak tracking and forced degradation studies, or as quantitative detectors for potentially mutagenic impurities, or for analytes without chromophores. The use of mass spectrometry (MS) to confirm the identity of an impurity during (accelerated) stability analysis and route development activities gives the analyst greater confidence in the data, and potentially highlights issues earlier than when using UV detection alone (for example, for the identification of coeluting peaks). The smaller size of these systems makes it much easier to take the mass spectrometer to the sample, for example, for on-line reaction monitoring (2); this has enabled self-optimizing routines to be used where the mass spectrometer is identifying when optimum conditions are reached (2,3).

Recent years have seen an increase in the use of different separation techniques, moving from traditional reversed-phase high performance liquid chromatography (HPLC) and gas chromatography (GC) to ultrahigh-pressure liquid chromatography (UHPLC) with shorter run times, hydrophilic interaction liquid chromatography (HILIC), supercritical fluid chromatography (SFC), and ion chromatography (IC). These can be a challenge to the mass spectrometer as a result of the need for faster scan speeds or issues with interfacing. In SFC–MS, the pressure reduces as the eluent leaves the column, the CO_2 can potentially boil off, and analytes can potentially precipitate. To overcome these challenges, the eluent flow can be split before the back pressure regulator, or the eluent can be mixed with a solvent miscible with CO_2. The use of a back pressure regulator alone can compromise the chromatographic integrity (4). SFC–MS has been shown to be applicable to a wide range of pharmaceutical compounds (5), including analysis from dosage forms (6), for chiral analysis (7), and preparative chromatography (8). SFC–MS has also been operated as an open access system in support of an academic MS facility (9). Capillary electrophoresis (CE)–MS has also been shown to have advantages in some instances (10).

The range and capability of mass analyzers available has continued to evolve. An increased number of these systems are capable of high mass resolution; as resolution increases, the mass accuracy and specificity increases such that it becomes easier to make structural assignments. The high resolution also offers an alternative to more traditional MS/MS experiments for quantitative analysis, where the specificity is gained by removing nominally isobaric impurities through mass resolution rather than the formation of different fragment ions (11). The robustness of modern analyzers and their ease of use has to some extent moved the operation of these instruments from MS specialists into the hands of analytical scientists.

The potential for application of ion mobility-mass spectrometry (IM-MS) within the pharmaceutical industry was first demonstrated by Eckers and co-workers in 2007 (12). The use of collisional cross-section (CCS) as an additional characteristic of an impurity, in addition to its retention and molecular weight, has significant potential as a tool within the pharmaceutical industry (Figure 1, reference [13]). The peer-reviewed literature contains abundant examples from academic research groups of the application of many different types of ion mobility techniques interfaced to MS for pharmaceutical analysis. The potential impact of the technology is illustrated by the 2018 review by Iain Campuzano and Jennifer Lippens (14), which discusses innovations in ion mobility technology and how they have been applied within research in the pharmaceutical industry. The review outlines the theory of different ion mobility technologies and describes applications to small molecules, metabolites, lipids, peptides, proteomics, proteins, and antibody–drug conjugates (ADCs). The authors note and reflect that ion mobility has seen broad acceptance and adoption within the academic community. However, within the pharmaceutical industry, it is still seen as a niche and specialist technique, which is reflected in its slower uptake and the resulting limited examples of applications originating from industrial research within the peer-reviewed literature.

An area of particular interest in the pharmaceutical industry is enantiomeric analysis of small molecules and this has been explored by IMS-MS. A recent example is the publication by Donald and co-workers, where differential ion mobility spectrometry (DMS)–MS was explored for the rapid and quantitative chiral recognition of small molecules (tryptophan and phenylalanine) using a chiral selector (N-tert-butoxycarbonyl-O-benzyl-L-serine [BBS]) that formed proton bound diastereomeric complex ions (15). The formation of gas-phase charge isomers (protomers) has been shown by Sobott and co-workers to be an additional complication during ion mobility analysis because multiple peaks are observed for the same molecule (16); this has also been observed by Hines and associates (13).

The biggest challenge to the analytical chemist or MS specialist working in late-stage pharmaceutical development is the now immense diversity of molecular entities that are being developed as drug molecules, with a notable shift towards larger molecules (17); these may be peptides, oligonucleotides, or drug delivery systems such as ADCs. This shift can require adoption of new techniques or a retraining in old techniques that have to some degree fallen out of favor (CE and size-exclusion chromatography [SEC], for example). These molecules provide challenges, especially around the identification and quantification of impurities. For example, CE–MS has shown some complementarity with LC–MS for the analysis of peptides through orthogonal separation (18).

Oligonucleotides present a particular challenge as a result of the large number of chiral isomers. The complex structure and multistep synthesis and purification lead to a broad range

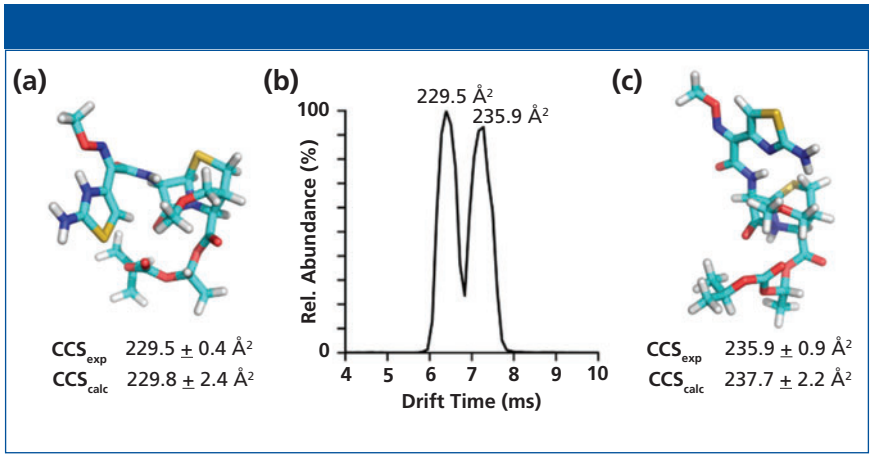

Figure 1: (a) Conformation of cefpodoxime proxetil, obtained through molecular modeling, which had a theoretical CCS value 0.65%, different to that of lower experimental CCS value; (b) bimodal arrival time distribution of cefpodoxime proxetil annotated with the experimental CCS values; (c) conformation of cefpodoxime proxetil, obtained through molecular modeling, which had a theoretical CCS value 0.97%, different to that of the higher experiment CCS value. Adapted with permission from Hines et al., *Anal. Chem.* **89**, 9023 (2017), copyright 2017 American Chemical Society (13).

of impurities such as *N* - 1 and *N* + 1 shortmers and longmers where the impurities have either one less or one more nucleotide (and the similarity between the main component and the impurities). The separation of these molecules are typically based around ion-pair chromatography (19,20), but the presence of coeluting impurities means that MS is used to quantify the purity of the main peak. The importance of therapeutic oligonucleotides is clearly reflected in their increasing prevalence within the peer-reviewed literature. The potential impact of oligonucleotides was illustrated in the 2011 review paper by Niessen and van Dongen, which discussed bioanalytical LC–MS of therapeutic oligonucleotides (21). This review recognized the increasing importance of LC–MS to characterize the parent oligonucleotide and its metabolites in biological fluids. The extensive review covers many of the key aspects of LC–MS of oligonucleotides, including chromatographic retention, ionization efficiency, ion-pair chromatography, pH, organic modifiers, the distribution of multiple charges, and fragmentation efficiency. Bartlett and co-workers have been notably active and this is reflected in two recent publications. A review published in 2018 focuses on the application of chromatographic techniques (including ion-pair reversed phase–HPLC–MS) for the determination of a broad range of oligonucleotide impurities and degradation products (22). The review also describes in detail the vast range of impurities and their synthetic origin. The importance of the characterization of the impurities and understanding their origin in the context of both process optimization and design of commercial synthetic processes is highlighted. In addition to this thorough review, Bartlett and associates have also recently described the application of IP–reversed-phase LC–MS/MS for the in-depth characterization of the degradation products formed from four different antisense oligonucleotides under stressed conditions (different pH values and temperatures) (23). There have been a number of recent examples of research in the area of oligonucleotide characterization originating directly for the pharmaceutical industry. Smith and Beck at GlaxoSmithKline described the application of LC–MS and 31P NMR to quantify a low-level coeluting impurity in a modified oligonucleotide (24), and Breda and co-workers at Aptuit have published a validated (10–10000 ng/mL) bioanalytical ion pair LC–MS/MS assay for the quantification of a 13-mer oligonucleotide in rat plasma to support a four-week toxicology study (25). Though less prevalent within drug project portfolios, therapeutic peptides are of increasing interest within analytical science. This has been reflected in the growing market for counterfeit biopharmaceuticals and the impact on analytical science has been investigated by Vanhee and associates (26). Their 2015 paper discusses the analysis of illegal peptide biopharmaceuticals frequently encountered by controlling agencies. It describes the development of a general screening method employing LC–MS/MS for both the identification and quantitation of illegal injectable peptide preparations that covers a range of therapies including oncology. The method was selective for the characterization of 25 different peptides (based on MS/MS fragmentation), and also validated for quantitation according to ISO-17025.

Many peptide separations can require buffers, salts, or additives that render them incompatible with MS. Hao Luo and colleagues at Merck have sought to overcome this challenge by developing two-dimensional (2D)-LC as an online desalting tool to allow peptide identification directly from these MS-unfriendly HPLC methods (27). Their method employs a heart-cutting 2D-LC system coupled to a quadrupole time-of-flight (QTOF)-MS. Fractions separated in the first dimension using an MS-incompatible mobile phase are transferred to the second dimension, where fast desalting with an MS-compatible phase allows subsequent MS characterization of impurities. In a novel method, Gammelgaard and associates have investigated the use of selenium as an elemental label for the quantification of the cell-penetrating 16 amino acid peptide penetratin (28). Using the labeling method in combination with flow injection combined with inductively coupled plasma–mass spectrometry (ICP-MS) (for total Se), LC–ICP-MS (for quantitative peptide uptake), and liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) (for the characterization of degradation products) provided detailed information of the peptide cellular uptake.

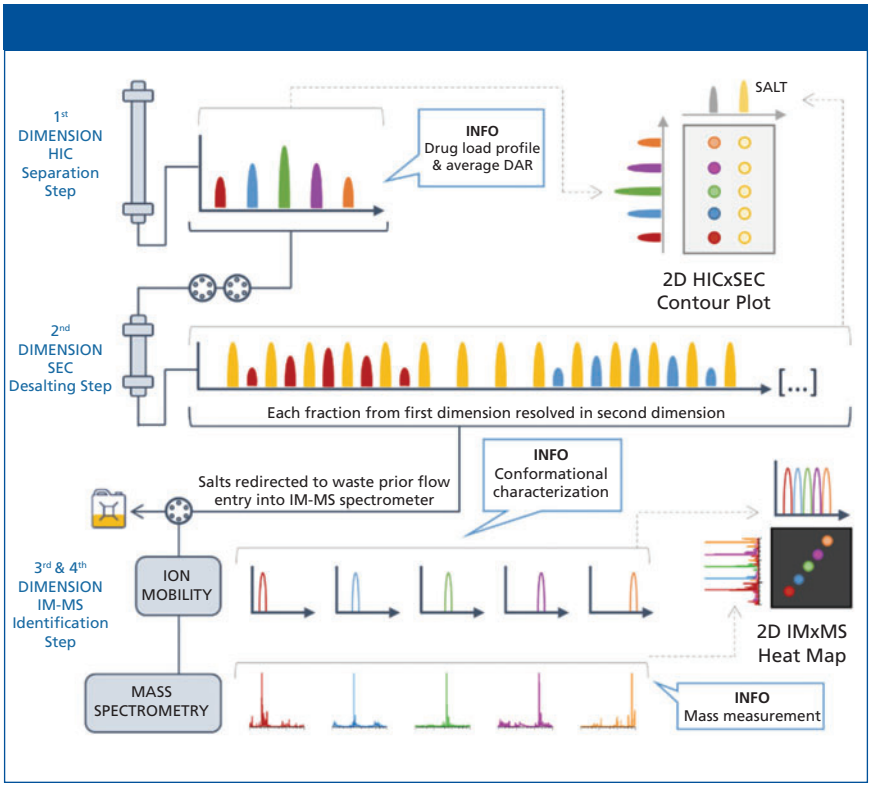

Figure 2: Flow chart of the analysis from brentuximab vedotin. Adapted with permission from Ehkirch and associates, *Anal. Chem.* **90**, 1578 (2018), copyright 2018 American Chemical Society (30).

Another class of compound that is becoming increasingly prevalent is the ADC. The challenges involved in the mass spectrometric analysis of these compounds have been investigated by Friese and co-workers (29). For characterization of ADCs, Cianferani and colleagues have described a proof of concept study on the application of an on-line four-dimensional hydrophobic interaction chromatography (HIC)×SEC×ion mobility-mass spectrometry (IM-MS) methodology (Figure 2). The approach allows several critical quality attributes required for process and formulation development, lot characterization, and stability testing to be monitored in a single analysis (30).

Polymeric materials have long played an important role in the pharmaceutical industry, for example as excipients in oral solid-dose drug product formulations. Fiebig and colleagues from Boehringer Ingelheim have taken a novel approach to characterizing the regularly used formulation constituents, polyethylene glycol 400 and polysorbate 80. Their publication describes the application of traveling wave ion mobility spectrometry (TW-IMS) quadrupole time-of-flight high resolution mass spectrometer (QTOF-HRMS) and the use of both the collision cross-section and accurate mass for this characterization challenge (31). The methodology was applied to in vivo metabolite studies allowing rapid identification of the formulation constituents.

More recently polymeric materials are being developed as nanocarriers for targeted drug delivery in biomedicine. Examples include nanoparticles that encapsulate an active pharmaceutical ingredient (API) and dendrimer drug conjugates, where a number of API molecules are attached to the surface of a hyperbranched polymer (32). As a result of their relatively recent emergence and novelty, reports on the characterization of dendrimers is limited, however poly(amidoamine) (PAMAM) dendrimers have found some focus, notably by Fernandez-Alba and colleagues in 2013 (33,34). The group have described the application LC–ESI-MS and LC–ESI-MS/MS (using both QTOF and hybrid quadrupole–linear ion trap) to the characterization (accurate mass MS/MS) and quantitation (SRM) of PAMAM dendrimers (generations G0 to G3) in simple aqueous media and more biorelevant urine. The quantitative method was validated and shown to have sensitivity in the micromolar range.

Finally, we should not lose sight that GC–MS remains an essential tool within the pharmaceutical industry for many qualitative and quantitative applications. Continued innovation in GC–MS technology has been demonstrated by the introduction of a number of high-resolution GC–MS systems (35). The authors of this article have themselves demonstrated the capability of GC coupled to an orbital mass spectrometer for structural characterization to deliver process development and understanding (36). Accurate mass GC–electron ionization (EI)-MS and GC–chemical ionization (CI)-MS data were used to characterize key impurities of a synthetic building block for an important drug substance that was under development. Such characterization and impurity tracking of small synthetic building blocks is an essential aspect of process development and design for long-term product quality and patient safety. The quantitative potential of GC with orbital trap MS was also evaluated.

GC–MS plays an important role in the characterization and quantitation of extractables and leachables that may result from devices used within the pharmaceutical industry. GC coupled with HRMS has proved particularly effective in extractable and leachable analysis (37,38).

A recent example of this is the report by Lacorte and associates who have assessed the migration of plasticizers from poly(vinyl chloride) and infusion bags both qualitatively and quantitatively using selective extraction and GC–MS (39). PVC is widely used in the pharmaceutical industry for the manufacture of a wide range of medical devices, including tubes, probes, bags, and primary packaging. Therefore, the characterization of the migration potential of plastic additives (for example, phthalates, various phenols, and benzophenone) is of great importance in the

context of patient safety and adherence to international regulations.

Summary

The use of mass spectrometry in all areas of the pharmaceutical industry has increased markedly over the last ten years as instruments become smaller and cheaper, or smaller with increased resolution. The changes in the project portfolios across the pharmaceutical industry with novel (larger) molecules and complex drug delivery devices means that there are many challenges where mass spectrometry will be the analytical technology of choice. However, there is also a requirement to shift to differing separation techniques in front of the mass spectrometer or for ion mobility mass spectrometry, after the ionization has occurred. It is clear that mass spectrometry coupled to a wide range of separation technologies continues to play an essential role throughout the pharmaceutical industry, from discovery to development, to supporting a long-term supply of essential medicines to patients. The continuing evolution of MS technologies will only further strengthen the future impact and importance of MS in the pharmaceutical industry. LC–MS is still a predominant technique and its impact will not only continue, but will be enhanced over the coming years.

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Quantitative Analysis of PFAS in Drinking Water Using Liquid Chromatography Tandem Mass Spectrometry

Per- and polyfluoroalkyl substances (PFAS) are chemicals found in firefighting foams and consumer products requiring water-resistant and stain-repellent properties. As a result of their unique chemical properties and long-term widespread usage, these chemicals are an emerging human health concern. The US Environmental Protection Agency (EPA) first released analytical methods for PFAS measurement in 2009, and revised these methods in November of 2018. In this article, data generated using these methods with allowed analytical modifications is presented, and demonstrates robustness and reproducibility, while achieving low level detection limits in drinking water.

Emily Parry and Tarun Anumol

Per- and polyfluoroalkyl substances (PFAS) are a class of man-made compounds widely used in industry and manufacturing because of their uniquely desirable chemical properties. These compounds are used in non-stick cookware, food contact materials, fire-fighting foams, surfactants, and many other applications. Their chemistry makes these compounds extremely persistent, bioaccumulative, and potentially toxic to animals and humans (1). As a result of their widespread usage over the last few decades, they are now ubiquitous in the environment.

There are more than 4500 PFAS commercially manufactured, but only very few have been monitored in the environment. The most commonly measured PFAS classes in the environment are the perfluorocarboxylic acids (PFCAs), such as perfluorooctanoic acid (PFOA), and perfluorosulfonic acids (PFSAs), such as perfluorooctanesulfonate (PFOS). Some of these PFAS compounds are currently the subject of regulation, and much public and research attention (2).

The US Environmental Protection Agency (EPA) indicates a drinking water health guidance for PFOA and PFOS at a combined 70 ng/L, while several US states have guidelines for PFOA, PFOS, and other PFAS (PFNA, GenX) at low ng/L levels. In Europe, the drinking water directive recommends levels of lower than 0.1 µg/L for individual PFAS, and 0.5 µg/L for total PFAS, while several member countries have guidelines for PFAS in the ng/L range in drinking water. PFOS and its salts have been listed as priority pollutants to be phased out from use under the Stockholm Convention. With PFOA and PFOS banned or in the process of being phased out by manufacturers globally, alternative compounds are being used resulting in emerging classes of PFAS now being detected in the environment.

The measurement of these compounds at ng/L levels is quite challenging. Therefore, the need for standard methods to measure them in the environment is critical for establishing baselines and future regulatory decisions. In 2009, the US EPA established EPA Method 537 for the quantification of 14 PFAS in drinking water, using solid-phase extraction (SPE) and liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) (3). In late 2018, the US EPA revised this method (EPA 537.1) to include four emerging PFAS, including hexafluoropropylene oxide dimer acid (HFPO-DA aka GenX), ADONA, 9Cl-PF3ONS, and 11Cl-PF3ONS (components of F-53B; replacement for PFOS) (4).

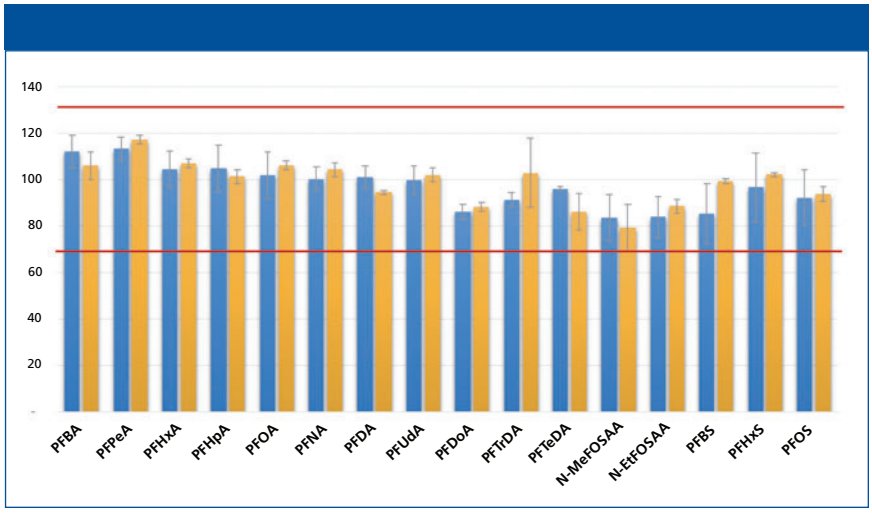

Figure 1: The average spike recoveries of PFAS in ultrapure and finished drinking water using SPE..

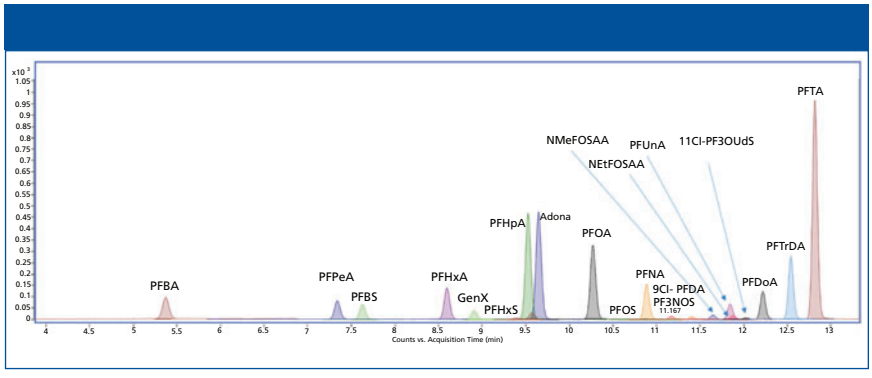

Figure 2: Chromatogram of EPA 537.1 analytes with the addition of PFBA and PFPeA.

This article aims to provide a simple SPE procedure for the extraction of PFAS in drinking water analyzed in EPA Method 537, along with a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the analysis of PFAS listed in EPA Method 537.1 to achieve the required low ng/L levels in drinking water.

Experimental

Chemicals

Standards were purchased from Wellington Laboratories, Inc. and calibration standards diluted to a desired concentration in 96:4 methanol:water.

Instrumental

Five µL of the standard sample were introduced for analysis into the LC–MS/MS system. Instrument sensitivity allowed for the reduction of 10 µL cited in the EPA 537 method. LC separation was performed on an Agilent 1260 Infinity II Prime LC system with a 3.0 × 50 mm, 1.8-µm Zorbax Eclipse Plus C18 column (Agilent). A 4.6 × 50 mm, 3.5-µm Zorbax Eclipse Plus C18 delay column (Agilent) was used after the binary pump to separate background PFAS introduced from the solvent, tubing, and the degasser from the desired analytes.

The Agilent Jet Stream Technology Ion Source (AJS) was used for maximum ionization. Source parameters were the same as can be seen in reference (5), with the exception of the increase of drying gas flow to 7 L/min. The Agilent Ultivo Triple Quadrupole LC–MS (LC-TQ) was operated in dynamic multiple reaction monitoring (MRM) mode to optimize sensitivity through maximizing dwell time. For most analytes, two transitions were acquired to provide quantitation and qualification ratios. MRM parameters are noted in Table I. EPA Method 537.1 now requires the use of 80 mass-to-charge ratio (*m/z*) for PFHxS and PFOS to reduce bias between linear and branched isomers and this was implemented.

Solid-Phase Extraction

Six replicates of 250 mL ultrapure water and finished drinking water samples were spiked at 4 ng/L for each PFAS. The samples were then extracted using a weak anion exchange (WAX, 150 mg, 6 cc) SPE cartridge (Agilent), as in the procedure described in EPA Method 537. Details for the specific SPE procedure can be found in reference (6). The eluate was evaporated to a final volume of 1 mL constituting ~96:4 methanol:water. Figure 1 shows that the extraction recoveries of all PFAS compounds were 70–130% and ranging from 79 to 112% in both ultrapure and drinking water. The relative standard deviations (RSDs) for all compounds was <15% (within acceptable parameters for the EPA method), demonstrating that the cartridge is effective at extracting low-level PFAS from drinking water samples with high efficiency.

Results and Discussion

Background Contamination Elimination

In this study, a delay column was installed in between the pump mixer and the injection port to time resolve any background PFAS coming from the solvents or the tubing of the LC system itself.

Chromatographic Separation and Method Performance

The analysis and separation of the 18 PFAS in EPA Method 537.1 were performed with all analytes achieving good peak shapes and peak widths between 6–10 s. Figure 2 shows a representative chromatogram of the 14 analytes in EPA Method 537, four of the emerging PFAS (GenX, ADONA, 9Cl-PF3OUdS, and 11Cl-

Table I: PFAS compound optimized transitions and estimated limit of detection on the LC-TQ system

Analyte	RT	Transition	Fragmentor	Collision Energy	Estimated Instrument Level of Detection (Pg on Column)
PFBA[1]	Ceftriaxone	$C_{18}H_{18}N_8O_7S_3$	554.6	0.105	0.68
PFPeA[1]	7.3	263 > 218.9	60	6	0.06
PFBS	7.6	298.9 > 80.1 298.9> 99.1	100	34 22	0.11
PFHxA	8.5	312.9 > 119.1 312.9 > 269	70	14 6	0.7
HFPO-DA	8.9	285.1 > 169	100	0	0.44
PFHpA	9.5	362.9 > 169 362.9 > 319	72	9 0	0.18
PFHxS[2]	9.5	398.9 > 80.1 398.9 > 99.1	100 70	37 34	0.31
ADONA	9.65	377.1 > 251.1 377.1 > 84.9	95	0 30	0.04
PFOA	10.2	412.9 > 169 412.9 > 369	69	13 3	0.08
PFOS[2]	10.8	498.9 > 80.1 498.9 > 99.1	100	38 38	1.30
PFNA	10.9	462.9 > 169 462.9 > 418.9	66	13 3	0.26
9Cl- PF3ONS	11.2	531 > 351.1	90	20	1.35
PFDA	11.4	512.9 > 219 512.9 > 468.9	100 81	3 12	1.51
NMeFOSAA	11.7	570>482.9 570>418.9	115	15 12	0.47
PFUnA	11.6	562.9>219 562.9>519	100 73	15 4	1.17
NEtFOSAA	11.9	584>525.9 584 > 418.9	115	15 15	1.01
11CL-P3OUdS	12.0	631 > 451	70	30	1.32
PFDoA	12.2	612.9> 269 612.9 > 568.9	100 79	15 4	0.50
PFTrDA	12.6	662.9 > 169 662.9 > 618.9	100 91	23 7	0.18
PFTA	12.8	712.9 > 669 712.9 > 169	100	7 23	0.11

[1]Not included in EPA Method 537 or EPA Method 537.1.
[2]EPA Method 537.1 requires that the 80 *m/z* product ion must be used to reduce bias between linear and branched isomers.

PF3OUdS) added to EPA Method 537.1, and the addition of PFBA and PFPeA. PFBA and PFPeA were added to show the good chromatographic separation and peak shapes of the early PFAS eluters, even though these are not present in the EPA method. The mobile phase was 5 mM ammonium acetate in water and 5 mM ammonium acetate in 95:5 methanol:water, instead of the 20 mM used in the EPA methods. The EPA's method flexibility allows changes in the LC separation. However, the EPA notes that reduced RT stability was observed over time with lower concentrations. Reduced stability at the lower concentration has not been observed so far. The gradient run time was reduced from 37 min in EPA Method 537 to 19.5 min (14 min gradient and a 5.50 min post time).

Figure 3 shows representative calibration curves for PFOA and PFOS from 0.1–50 parts per billion (ppb) in the final extract. Calibra-

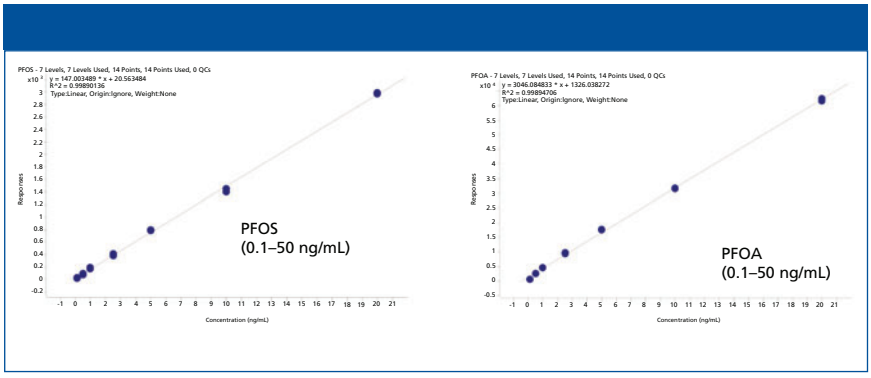

Figure 3: Linear calibration curves for PFOA and PFOS; 7-point calibration curve in duplicate (14 points) from 0.1–50 ppb in the extract.

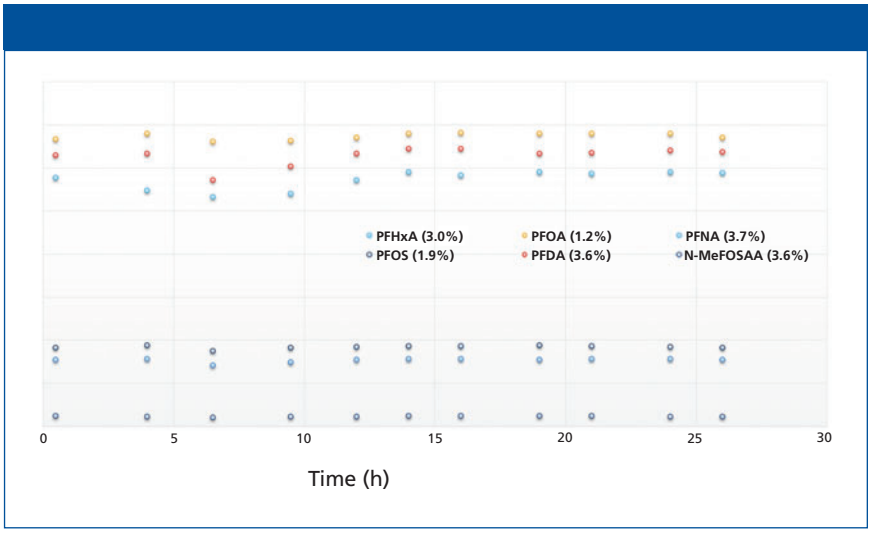

Figure 4: Raw response deviation for six PFAS in the continuous calibration standards run across a 26 h batch; the number in brackets is the percent RSD.

tion curves were linear with $R^2 >$ 0.99. Complete details of the analytical method including method optimized parameters and method verification along with linearity, robustness, and analysis of real-world drinking water samples can be found in reference (5).

Robustness and Reproducibility

US EPA Method 537 requires sensitive analysis of PFAS and robustness of the data across samples and batches. For example, the method calls for the injection and analysis of a continuing calibration standard in a batch every 10 samples to monitor system performance and variability. In this study, this method was evaluated by following the raw response of the PFAS standards run as continuous calibration standards every 10 samples across a batch of samples over a 26 h worklist. The standards were prepared in drinking water extracts at 1 ppb in the vial (~2.5 ng/L in sample equivalent). All PFAS analytes had response variation less than 5% RSD except N-EtFOSAA (5.6%). Figure 4 illustrates the response stability of the calibration standards across the 26 h batch and shows that the relative response, uncorrected by internal standards (ISs), was stable across the 11 CCV samples analyzed over 26 h.

Conclusion

The analysis of PFAS at extremely low levels in drinking water is required for adequate baseline monitoring and regulatory determination. This article provides a sample extraction protocol for PFAS in the US EPA method that achieves high recoveries in the target matrix, and a robust LC–MS/MS method for excellent separation, low level detection, and reliable and robust quantification of PFAS.

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Novel Methods Using Mass Spectrometry for Food Safety—From Contamination to Nutrition

Modern eating habits have led to the further diversification of an already complex food supply chain. The public confidence in the food supply is not only impacted by publicized crises involving contaminations, but also through the misreporting of nutritional information. Food analysis is integral to the whole supply chain, be it through a rapid response to a food crisis, such as the fipronil egg scandal, the continued monitoring of pesticides that could harm an unsuspecting public, or accurately reporting nutritional information to provide the information the public needs to make an informed decision about the food they eat. Each event is fraught with difficulties, but by developing new methods of analysis, crises in the food industry can be avoided, or their effects mitigated. This article highlights three events that require new method development to meet various detection needs, ranging from the detection of pesticides (such as fipronil and glyphosate), to the detection and quantification of fat-soluble vitamins.

Ashley Sage, Jianru Stahl-Zeng, and Philip Taylor

Modern diets have resulted in unprecedented growth and diversification of the food supply chain. The necessity of a continual supply of food means crops are commonly being treated with pesticides that are essential to reduce the risk of failed harvests. These chemicals are toxic to both insects and humans, and as such the public must be protected from unsafe concentrations of pesticides. Regulations imposed by food standard agencies dictate pesticide maximum residue limits (MRLs), which ensure the food on supermarket shelves is safe for consumption. In addition to ensuring food is free from contaminants, it is also essential to provide the public with accurate nutritional information to help enable the healthy growth of the global population.

To meet these goals, emphasis is placed on food content analysis to ensure quality and consistency between batches. Characterization and detection techniques, such as mass spectrometry (MS), offer manufacturers and producers the ability to screen large quantities of samples in a timely fashion, while guaranteeing reliable, repeatable measurements. Approaches to analysis are generally twofold—proactive monitoring of essential nutrients and possible contaminants, and crisis response, where widespread contamination requires the rapid development and deployment of analytical methods and equipment.

Rapid Response: Fipronil Egg Contamination

One recent example highlighting the need for a crisis response is the fipronil contamination of eggs, which resulted in the recall of millions of eggs. Fipronil is an insecticide, belonging to the phenylpyrazole family of chemicals, developed in the 1980s (1). Its high toxicity makes fipronil useful for controlling levels of insects including fleas, mites, and cockroaches, and is even useful against pests resistant to various insecticides (2). However, in July 2017, fipronil made news headlines after it was found to be present in eggs across Europe. This crisis was so widespread that, by the end of August, fipronil-contaminated eggs were detected in 15 European countries and as far afield as Hong Kong and China.

Fipronil treatment for crops used in the food chain is prohibited, and the US Environmental Protection Agency (EPA) has labeled fipronil as possibly carcino-

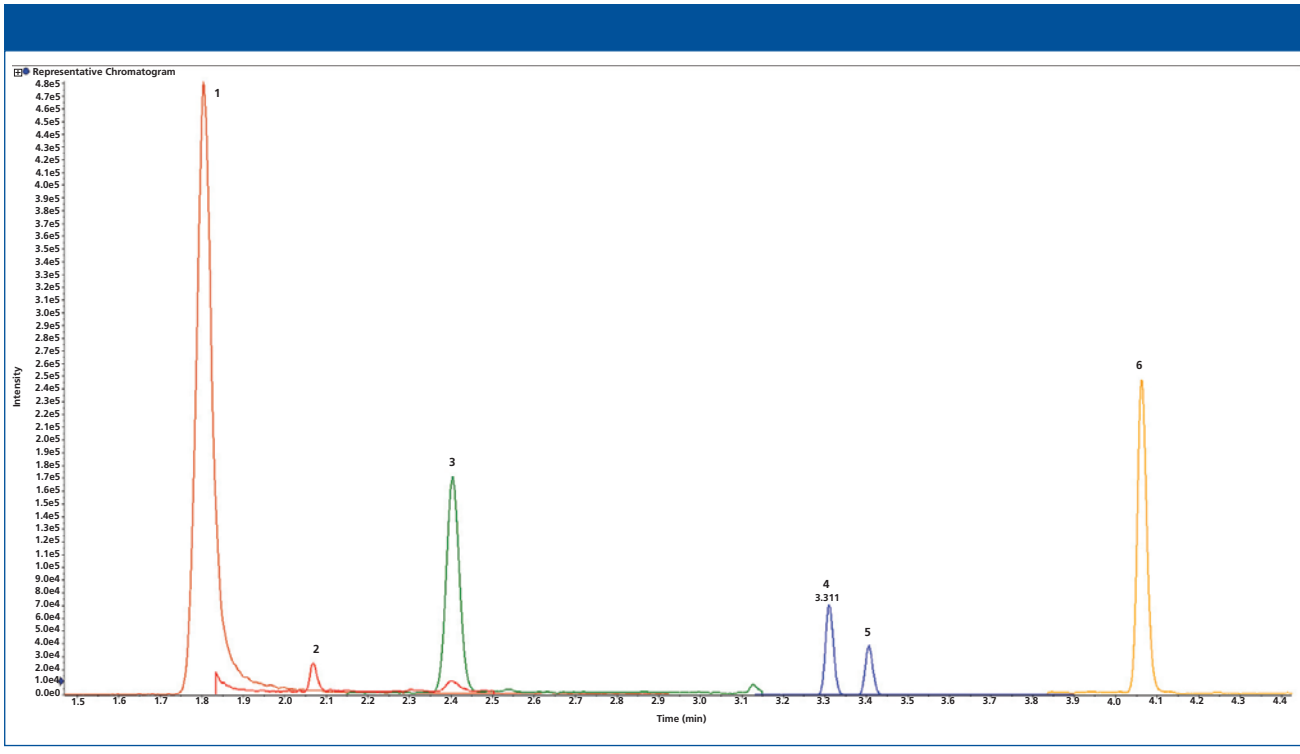

Figure 1: A chromatogram showing the clear separation of analytes, identifying fipronil and amitraz (another insecticide) and their related metabolites. 1. DPMF, 2. DMA, 3. DMF, 4. fipronil, 5. fipronil sulfone, 6. amitraz.

genic, prohibiting its presence in the food chain (3). Furthermore, the European Food Safety Authority (EFSA) dictates fipronil concentrations of under 5 µg/kg to be safe for human consumption (4). Complying with concentrations dictated by regulation is therefore vital for manufacturers and producers to ensure the continued safety and confidence in food supplied to the public. Detection methods form one essential part of this process, identifying and quantifying potential contaminations prior to distribution in the food chain. There are several plausible methods of detecting contaminants such as fipronil in samples of food. The most common detection methods include liquid chromatography–tandem mass spectrometry (LC–MS/MS) and gas chromatography–mass spectrometry (GC–MS), with both requiring different methods of sample preparation prior to analysis.

The challenge arises in detecting fipronil concentrations at the low level stipulated by government regulation, but novel methods capable of detecting fipronil and its associated metabolite, fipronil sulfone, have been developed. Since the fipronil contamination scandal began, demand for a fast, sensitive detection method has only continued to grow. One such detection method involves a modified Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) sample preparation technique prior to detection, using a triple quadrupole instrument and electrospray ionization (ESI). The high sensitivity of detection and ability to analyze two compounds in the same run enable regulation-compliant detection in a timely manner. This developed method can detect fipronil, as well as its major metabolite fipronil sulfone, to the MRL level of 5 µg/kg, as demonstrated in Figure 1 (5).

In responding to a food contamination crises, it is also prudent to develop methods that can screen for multiple contaminants at once, potentially preventing contamination from other, unexpected sources. Using nontargeted approaches, such as sequential window acquisition of all theoretical mass spectra (SWATH-MS) acquisition, contaminant detection is not limited to the chosen molecule, in this case enabling the analysis of fipronil and other contaminants such as pesticides and polyaromatic hydrocarbons (6). Combining instrumentation that provides linear, reproducible contaminant detection to regulation-specific concentrations, with novel sample preparation methods, is essential to avoid repeated events, and helps improve public confidence in the integrity of the food supply chain.

Continual Monitoring: Glyphosate Pesticide

While fipronil is known to be hazardous for health, regulatory advice on other pesticides, such as glyphosate, is conflicted. Glyphosate is a widely used broad-spectrum systemic herbicide and crop desiccant. While it has recently made headlines for its potentially hazardous nature to humans as a possible carcinogen, its impact on human health is contested, and, therefore, the use of glyphosate as a farming pesticide is still permitted (7). In cases where potentially harmful pesticides are being used, continual monitoring is required to ensure chemical concentrations in

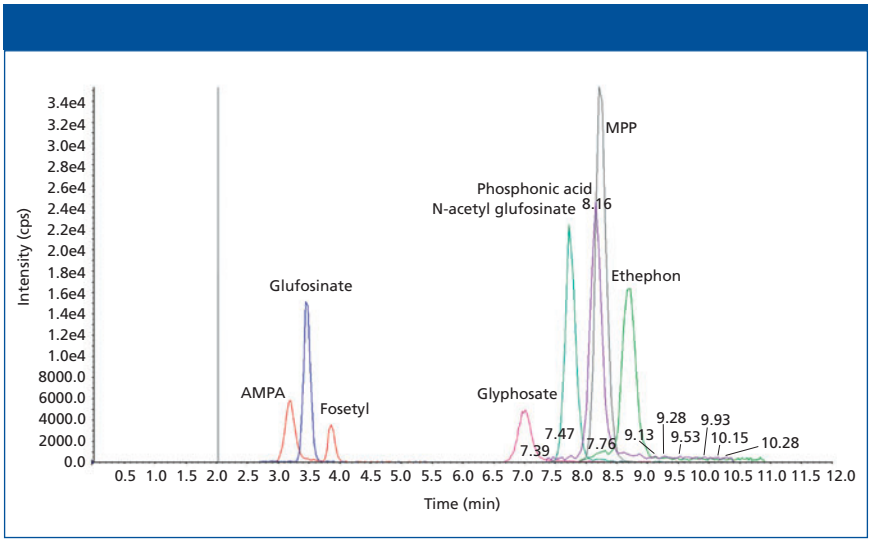

Figure 2: MS spectra provide the ability to clearly identify and distinguish glyphosate and its metabolite AMPA and other pesticides, such as glufosinate.

foods are safe. Glyphosate is used globally and has been detected at trace levels, along with related metabolites, in 45% of European topsoils (8), and in samples of milk (9).

The controversy surrounding the use and potential contamination of glyphosate has placed greater emphasis on data collection and analysis methods to ensure levels of glyphosate in food samples fall below the safe maximum residue level (MRL) (reported by the EFSA as 50 µg/kg) (10). However, while the analysis of glyphosate and its associated metabolites is essential, its detection presents different sample preparation and analysis challenges that must be overcome. The high polarity of glyphosate and its related metabolites previously made sample extraction and LC analysis difficult. To overcome retention issues, derivatization is employed using a method with fluorenylmethyloxycarbonyl chloride (FMOC-Cl) as the derivatization reagent to convert glyphosate into an analogue that can then be analyzed. However, while this approach enables detection, it is both complicated and time-consuming, and fails to detect pure glyphosate and its metabolites.

Currently, the detection of underivatized glyphosate can be achieved using new methods of extraction coupled with instrumentation. One such underivatized method of detection starts by using the Quick Pesticide Preparation (QuPPe) extraction method to prepare samples (11). Using a combination of this approach with sensitive MS instruments, accurate quantification of glyphosate and its metabolites can be achieved. By combining the LC–MS/MS method with differential mobility separation (DMS) technology, interferences can be removed from analyses to improve the signal-to-noise ratio and, consequently, increase confidence in quantification results. These samples are then analyzed using LC–DMS–MS/MS to quantify and identify those contaminants present, as shown in Figure 2.

Vitamin Detection in Food

General food composition monitoring is not limited to the detection of harmful contaminants. It also forms an essential component of the accurate reporting of nutritional information for packaging labels. Vitamins are vital nutrients that are essential for an individual's growth and development. Deficiency in any vitamin is detrimental to health, and is linked to a multitude of health issues. For instance, deficiency in vitamin D leads to the bone disorder known as rickets, and has been associated with other health problems, including heart disease and cancer (12). While most vitamins can be obtained through natural means—exposure to sunlight is the best source of vitamin D—this is not always possible for some. Vitamin supplements are one option, and are commonly incorporated into food (for example, infant formula). It is therefore imperative to report accurate nutritional information on food packages to ensure an individual's vitamin needs are met, and for manufacturers to correctly advertise the benefits of their products.

Vitamins broadly separate into two categories: water-soluble (vitamins B and C), and fat-soluble (vitamins A, D, E, and K). Detection of water-soluble vitamins is relatively easy, with analysis possible using MS. Conversely, analysis of fat-soluble vitamins is difficult, owing to the challenges associated with MS detection. These problems originate from the presence of lipids in fat-soluble vitamin samples that cause an effect known as *ion suppression* (13), negatively affecting the detection, precision, and capability of a mass spectrometer. The ease in the detection of water-soluble vitamin samples is a direct result of the absence of lipids, enabling clean detection of vitamin B and C in samples (14). No uniform solution to ion-suppression exists, but its effects can be circumvented by the removal of lipids.

The detection of vitamins provides a specific detection challenge and, until recently, it was difficult to detect fat-soluble vitamins using LC–MS methods. Food samples contain various concentrations of vitamins, ranging from parts per billion to parts per million. To obtain clean, analyzable detection of fat-soluble vitamins, the lipid content should be removed from food samples. This reduces the ion suppression, and enables robust MS analysis within a single chromatogram that identifies multiple vitamins (see Figure 3). Combining sensitive MS methods with associated expertise in methodology allows universal application of the sample preparation and a simple analysis of vitamin concentrations in various food samples, meeting the specific requirements of the customer.

Summary

Identification and quantification of potential contaminants and nutri-

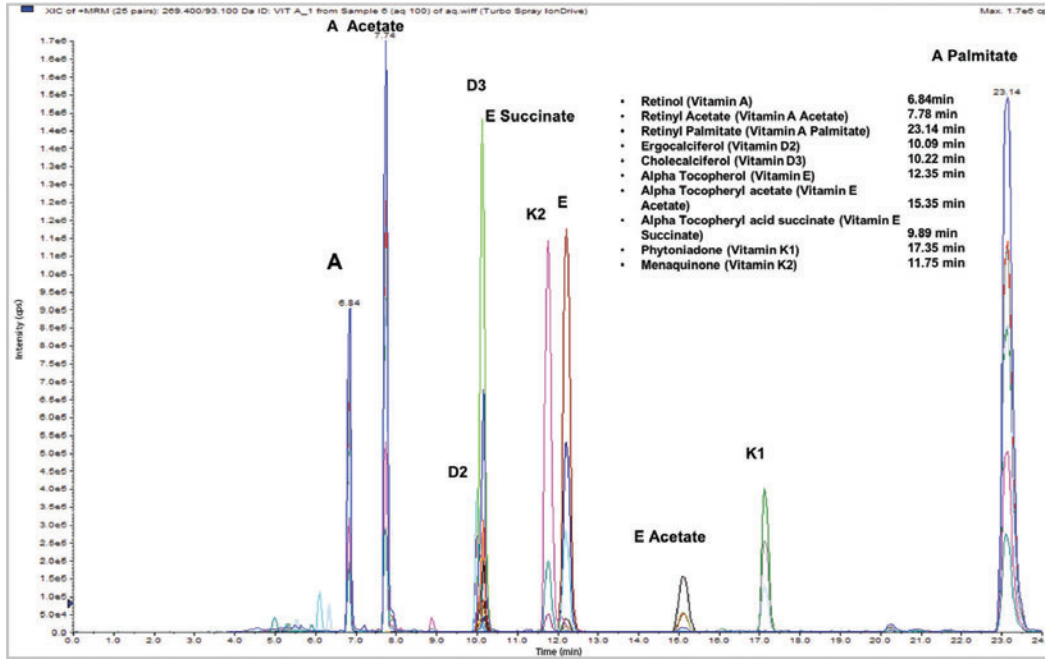

Figure 3: A single chromatogram identifying the various vitamins that can be identified within a single mass spectrum run.

ents are essential to maintaining the integrity of the food supply chain. By avoiding unnecessary contamination of pesticides and providing clear labeling information, food manufacturers can be confident that food is safe for public consumption, while also delivering accurate nutritional information. Continual methodological and instrumentation development will enable more sensitive and timely detection to continue for years to come.

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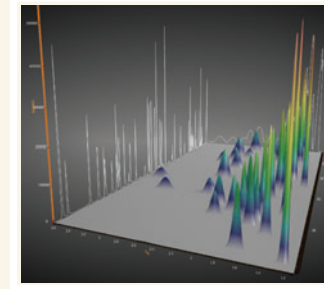
PRODUCTS & RESOURCES

Solid-phase extraction disks

Empore solid-phase extraction disks from CDS Analytical are designed as an efficient alternative to liquid–liquid extraction. According to the company, a proprietary process is used to entrap adsorbent particles into a matrix of inert polytetrafluoroethylene to create a mechanically stable sorbent disk. The disks are used for purification and concentration of analytes from aqueous samples.
CDS Analytical, Oxford, PA. www.cdsanalytical.com

Software for GCxGC–TOF MS



ChromSpace, a GCxGC software package from SepSolv Analytical, is designed to provide streamlined instrument control and data processing with an intuitive interface. According to the company, the software allows 1D and 2D data from a range of detectors to be imported, enabling processing to be unified on a single platform.
SepSolv Analytical Ltd., Peterborough, UK.
http://chem.sepsolve.com/software

Mass spectrometer

Thermo Fisher's Orbitrap Eclipse Tribrid mass spectrometer is designed with advancements that improve system sensitivity and speed over previous generations of platforms through its high performance and flexibility. According to the company, the system extends structural analysis up to *m/z* 8000, enabling the isolation and selective dissociation of protein complexes into their individual components.
Thermo Fisher Scientific,
San Jose, CA. www.thermofisher.com

Hydrogen laboratory server

The Proton OnSite hydrogen laboratory server is designed to use a protonexchange membrane, electricity, and deionized water to produce up to 18.8 standard liter per min (SLM or SLPM) of ultrahigh-purity hydrogen gas per day. According to the company, the unit senses demand and adjusts production accordingly.
Proton OnSite,
Wallingford, CT.
www.protononsite.com

GC–MS thermal desorption system

Gerstel's MPS TD system is designed as a dedicated sampler for automated thermal desorption, thermal extraction, and dynamic headspace analysis. According to the company, the system can process up to 240 samples and is operated with one integrated method and one sequence table.
Gerstel, Inc.
Linthicum, MD.
www.gerstel.com

HPLC columns

HPLC columns from Hamilton are available with both silica-based and polymeric supports. According to the company, columns include 17 polymeric HPLC columns for reversed-phase, anion exchange, cation exchange, and ion-exclusion separations, and two silica-based columns for reversed-phase separations.
Hamilton Company,
Reno, NV.
www.hamiltoncompany.com

Air valves

Restek's RAVEqc quick connect air valves are designed as a tool-free alternative to bellows or diaphragm valves. According to the company, the air valves reduce the time and variability associated with connecting air canisters to other devices.
Restek Corporation,
Bellefonte, PA.
www.restek.com

MALDI-TOF mass spectrometer

Shimadzu's MALDI-8020 benchtop mass spectrometer is designed for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). According to the company, using linear TOF, the system enables fast, low-level detection of proteins, peptides, and polymers, among other analytes.
Shimadzu Scientific Instruments,
Columbia, MD.
www.ssi.shimadzu.com

THE **APPLICATION** NOTEBOOK

Call for **Application Notes**

LCGC is planning to publish the next issue of *The Application Notebook* special supplement in **February**. The publication will include vendor application notes that describe techniques and applications of all forms of chromatography and capillary electrophoresis that are of immediate interest to users in industry, academia, and government. If your company is interested in participating in these special supplements, contact:

Edward Fantuzzi, Publisher,
(732) 346-3015

Brianne Molnar, Sales Manager,
(732) 346-3034

Application Note Preparation

It is important that each company's material fit within the allotted space. The editors cannot be responsible for substantial editing or handling of application notes that deviate from the following guidelines:

Each application note page should be no more than 500 words in length and should follow the following format:

- **Title:** short, specific, and clear
- **Abstract:** brief, one- or two-sentence abstract
- **Introduction**
- **Experimental Conditions**
- **Results**
- **Conclusions**
- **References**
- **Two graphic elements:** one is the company logo; the other may be a sample chromatogram, figure, or table
- **The company's full mailing address, telephone number, fax number, and Internet address**

All text will be published in accordance with *LCGC*'s style to maintain uniformity throughout the issue. It also will be checked for grammatical accuracy, although the content will not be edited. Text should be sent in electronic format, preferably using Microsoft Word.

Figures

Refer to photographs, line drawings, and graphs in the text using arabic numerals in consecutive order (Figure 1, etc.). Company logos, line drawings, graphs, and charts must be professionally rendered and submitted as .TIF or .EPS files with a minimum resolution of 300 dpi. Lines of chromatograms must be heavy enough to remain legible after reduction. Provide peak labels and identification. Provide figure captions as part of the text, each identified by its proper number and title. If you wish to submit a figure or chromatogram, please follow the format of the sample provided below.

Tables

Each table should be typed as part of the main text document. Refer to tables in the text by Roman numerals in consecutive order (Table I, etc.). Every table and each column within the table must have an appropriate heading. Table number and title must be placed in a continuous heading above the data presented. If you wish to submit a table, please follow the format of the sample provided below.

References

Literature citations must be indicated by arabic numerals in parentheses. List cited references at the end in the order of their appearance. Use the following format for references:

(1) T.L. Einmann and C. Champaign, *Science* **387**, 922–930 (1981).

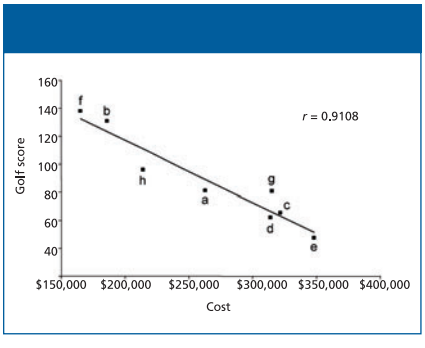


Figure 1: Chromatograms obtained using the conditions under which the ion suppression problem was originally discovered. The ion suppression trace is shown on the bottom. Column: 75 mm × 4.6 mm ODS-3; mobile-phase A: 0.05% heptafluorobutyric acid in water; mobile-phase B: 0.05% heptafluorobutyric acid in acetonitrile; gradient: 5–30% B in 4 min. Peaks: 1 = metabolite, 2 = internal standard, 3 = parent drug.

Table I: Factor levels used in the designs

Factor	Nominal Value	Lower Level (−1)	Upper Level (+1)
Gradient profile	1	0	2
Column temperature (°C)	40	38	42
Buffer concentration	40	36	44
Mobile-phase buffer pH	5	4.8	5.2
Detection wavelength (nm)	446	441	451
Triethylamine (%)	0.23	0.21	0.25
Dimethylformamide	10	9.5	10.5

The deadline for submitting application notes for the February issue of *The Application Notebook* is:

January 10, 2020

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EPA TO-17 Volatile Organic Compounds Analysis Using Thermal Desorption

Xiaohui Zhang, CDS Analytical

The performance of the CDS 7550S coupled to a GC–MS was demonstrated for the EPA TO-17 volatile organic compounds (VOCs), from dichlorodifluoromethane to naphthalene.

An automatic CDS 7550S Thermal Desorption System, featured for its 72-position sample rack, Peltier-focusing trap, pre-heat function, internal standard (ISTD) addition, inert-coated flow path, and design for high temperature, was coupled to an Agilent 6890 GC with 5975B MS. The adsorptive packing for the focusing trap was Tenax TA, while the adsorbent for the ¼ "OD × 3.5" length sample tubes was CAMSCO Carbograph 2/Carbograph 1/Carboxene 1000.

The TO-17 calibration gas mix standards were loaded to the sample tubes with a device having selectable sample loop volume from 1-mL, 2-mL, and 5-mL, each with a 5-mL gaseous ISTD. The chromatographic separation was performed in a Restek Rtx-VMS 30.0 m x 250.00 um x 1.40 um column with helium as carrier gas. Thermal desorption conditions were summarized as below.

Tube Heater rest:	38 °C
Tube desorb:	300 °C
Dry:	38 °C, 0.5 min
IS loop:	5.0 mL
IS fill:	1 min
IS transfer:	1 min
Trap type:	Tenax TA
Trap rest:	-20 °C with Peltier
Trap	pre-heat: 15 s
Trap desorb:	300 °C
Oven:	275 °C
Transfer line:	250 °C

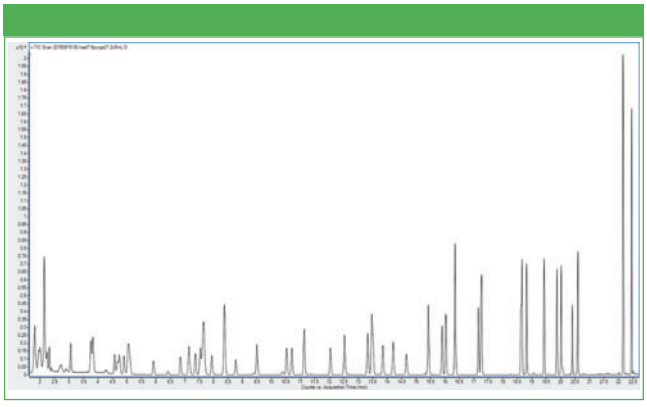

Figure 1: Chromatogram obtained with oven temperature ramping from 35 °C to 250 °C in 25 min with a split ration of 5:1. The shape of the peaks were optimized with a proper 15 s focusing trap pre-heat time.

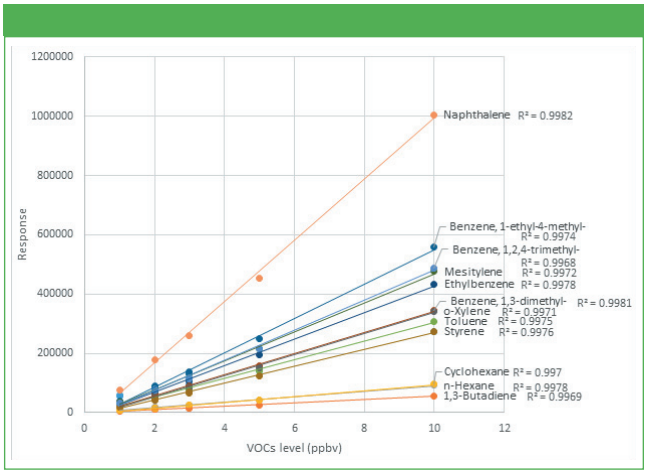

Figure 2: Calibration for hydrocarbons

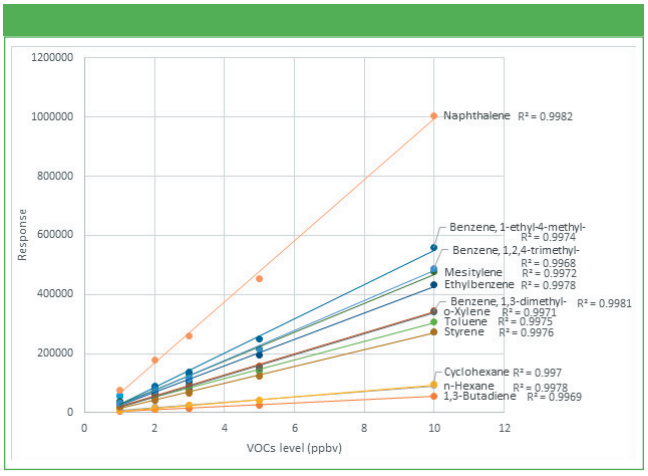

Figure 3: Calibration for halogenated hydrocarbons

The TO-17 standards chromatogram is shown in Figure 1.

The linearities of different types of compounds are shown in Figure 2 and 3, as examples.

Table I: Internal Standard RSD

ISTD	R_t	Peak Area
Bromochloromethane	0.06%	2.07%
1,4-Diflourobenzene	0.04%	1.83%
Chlorobenzene-d5	0.03%	1.86%

The reproducibility of the ISTD addition is shown in Table I as relative standard deviations of peak areas, all below 3%.

Automating Metabolic Stability Assays and Analyses Using a Robotic Autosampler and LC–MS/MS Platform

Fredrick D. Foster, John R. Stuff, Laurel A. Vernarelli, and Jacqueline A. Whitecavage, Gerstel, Inc.

The in vitro metabolic stability of drug candidates is routinely examined at an early stage of drug discovery. Automating the entire metabolic stability assay and subsequent liquid chromatography—tandem mass spectrometry (LC–MS/MS) analysis can provide the high-throughput necessary for use in drug metabolism and pharmacokinetic (DMPK) laboratories. The Gerstel MPS robotic autosampler performs syringe transfer of all liquids involved in the metabolic stability procedure as well as temperature-controlled incubation of the samples for defined time periods. Additional sample preparation steps are performed as needed. The PrepAhead function enables parallel LC–MS analysis and preparation of the following sample, thereby increasing throughput while ensuring that samples receive identical treatment. The resulting extracts were automatically introduced into an Agilent Ultivo LC–MS/MS instrument for analysis immediately after being prepared for best possible reproducibility. Linear calibration curves resulting in R^2 values of 0.99 or greater were achieved for the complete automated procedure. Time-course studies for model drug compounds in microsomes were examined and are presented.

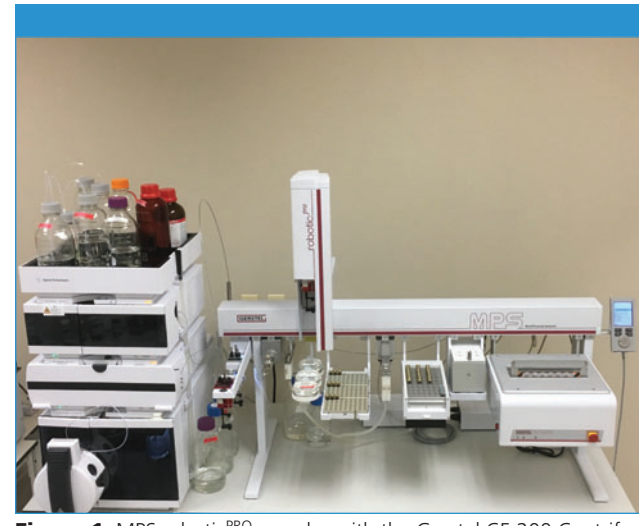

Figure 1: MPS robotic^{PRO} sampler with the Gerstel CF-200 Centrifuge option.

Experimental

Materials: Stock solutions were purchased from Cerilliant. Individual substrate samples for each compound examined were prepared at a concentration of 5 mM each, respectively, in DMSO. Male, CD-1, mouse liver microsomes (20 mg/mL), male, Sprague Dawley, rat liver microsomes (20 mg/mL), and NADPH Regenerating System Solutions A and B were purchased from Corning Discovery Labware, Inc. The NADPH Regenerating Solution A contains 26 mM NADP+, 66 mM glucose-6-phosphate, and 66 mM magnesium chloride in water. The NADPH regenerating solution B contains 40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate. When combined, solutions A and B can be used for NADPH requiring oxidase assays. All other reagents and solvents used were reagent grade.

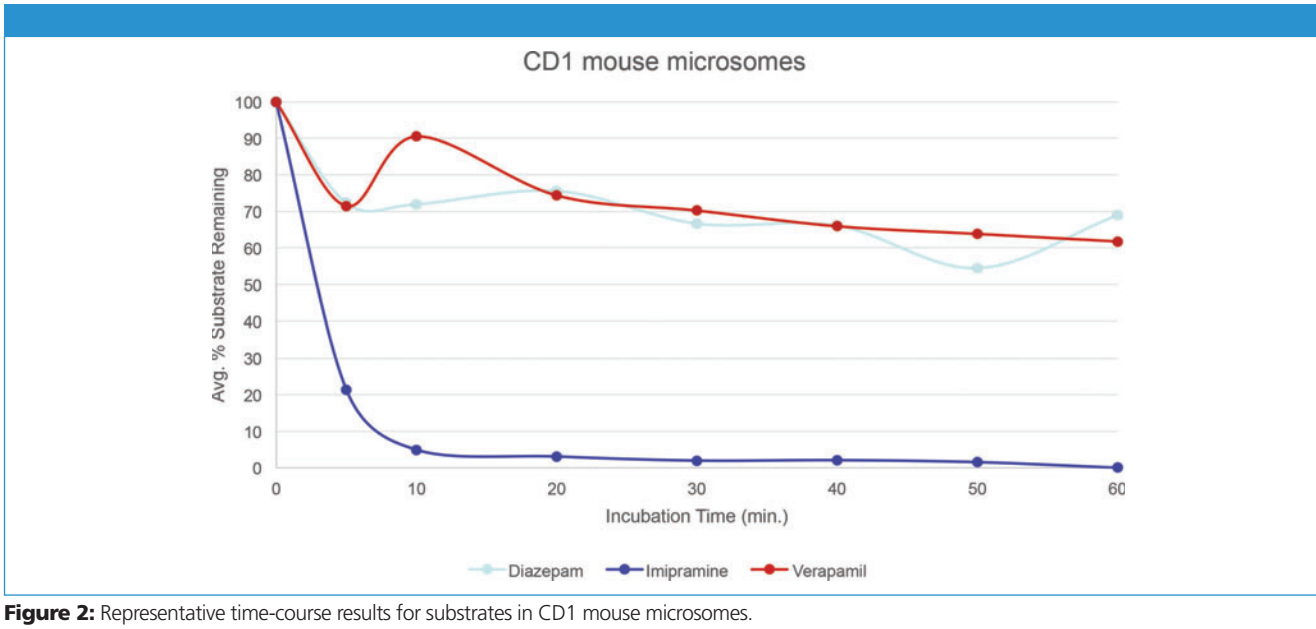

Figure 2: Representative time-course results for substrates in CD1 mouse microsomes.

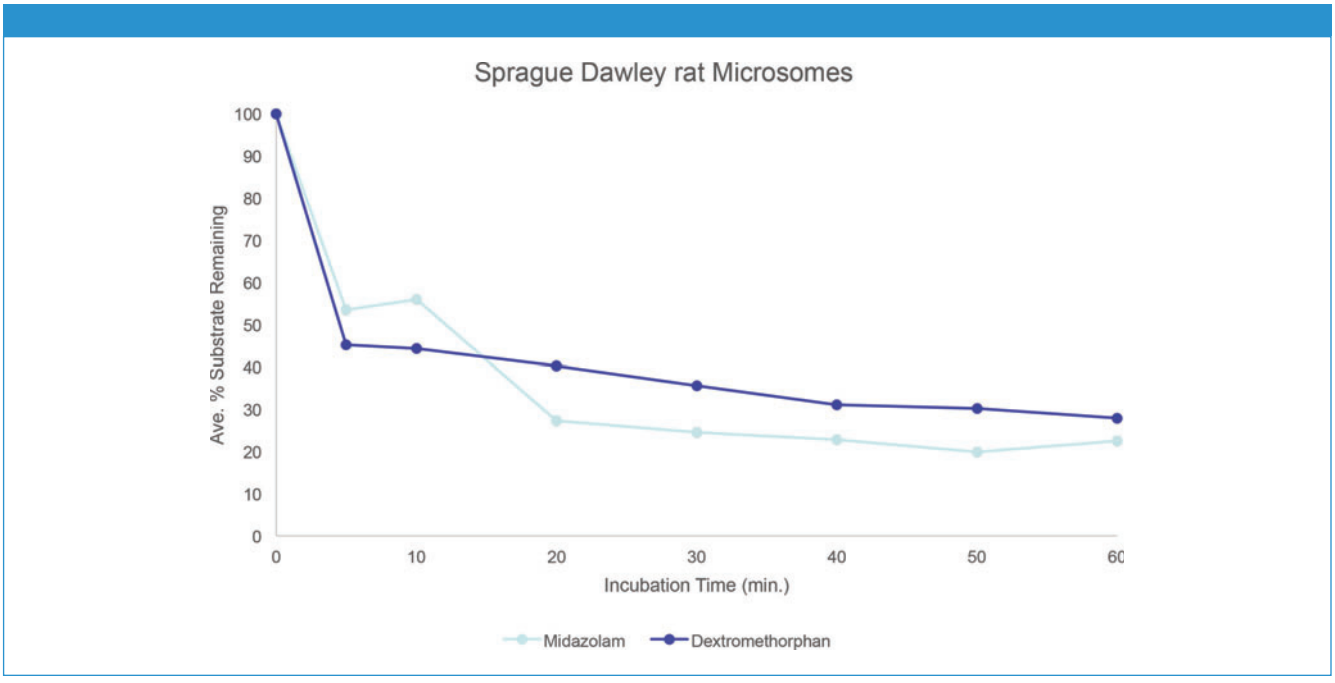

Figure 3: Representative time-course results for substrates in Sprague Dawley rat microsomes.

Instrumentation

All automated PrepSequences were performed using an MPS robotic^{PRO} sampler with the Gerstel CF-200 centrifuge option and heated agitator as shown in Figure 1. All analyses were performed using an Agilent 1260 HPLC system with an Agilent Poroshell 120 EC-C18 column, (3.0 × 50 mm, 2.7 um) and an Agilent Ultivo triple quadrupole mass spectrometer with Jet stream electrospray source. Samples, stop solution, substrates, microsomes, and NADPH regeneration solutions were stored within a Peltier-cooled tray at 4 °C throughout the automation. Sample injections were made using the Gerstel LC–MS tool into a six-port (0.25 mm) Cheminert C2V injection valve outfitted with a 2-uL stainless steel sample loop.

Automated Prep Sequence: The automated microsomal stability experiment followed industry standard experimental conditions (1), including a range of liquid addition steps. Following the sample preparation procedure, the MPS robotic injects the prepared extract into the LC–MS/MS for analysis. For more details, please consult Gerstel App Note 206. Negative controls were performed using the same steps, minus the cofactors, in order to exclude substrate disappearance due to causes other than those induced by the presence of cofactors.

Results and Discussion

Figures 2 and 3 show representative time-course results for various substrates in either mouse or rat liver microsomes from the automated microsomal stability assays performed. These data provide evidence that the automated microsomal stability assays and their subsequent LC–MS/MS analyses can be readily automated using the Gerstel MPS robotic^{PRO} sampler.

Conclusions

- Automated microsomal stability assays are readily automated using the Gerstel MPS robotic^{PRO} sampler including subsequent LC–MS/MS analysis using an Agilent Ultivo triple quadrupole mass spectrometer.
- Linear calibration curves resulting in R^2 values 0.99 or greater were achieved for the compounds being analyzed.
- The LC–MS/MS method proved to be accurate and precise. accuracy data averaged 95.6% (range: 73.8%–113%), and precision data averaged 2.78 %RSD (range: 1.48%–5.29%) for all compounds analyzed.

Reference

(1) Corning Discovery Labware Inc., Mammalian Liver Microsomes, Guidelines for Use, TF000017 Rev. 2.0, retrieved April 2019 from https://certs-ecatalog.corning.com/life-sciences/product-descriptions/452701.pdf.

(2) Gerstel app note 206, http://www.gerstel.com/pdf/AppNote-206.pdf

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LC–MS/MS Analysis of Mycotoxins in Peanut Powder in 5.5 Min

Restek Corporation

- Fast analysis for higher sample throughput
- Excellent separation improves accuracy for 12 regulated mycotoxins
- Quick and easy sample preparation (dilute-filter-shoot)

Certain fungi that can grow on agricultural products produce toxic metabolites known as mycotoxins. Modern food processing procedures cannot completely remove these compounds if they are present, so strict monitoring protocols have been established. Although a universal method for the analysis of mycotoxins would allow highly efficient screening, it is very challenging to develop such a method, due to differences in physiochemical properties of mycotoxins, extraction efficiencies, and matrix effects. Zhang and associates published a multi-lab study (1) aimed at providing labs with an analytical procedure that could be broadly applied to the analysis of a variety of mycotoxins in many different matrices. Using that work as inspiration, we developed the following LC–MS/MS method that resolves 12 FDA regulated mycotoxins within the pressure limits of traditional HPLC instruments.

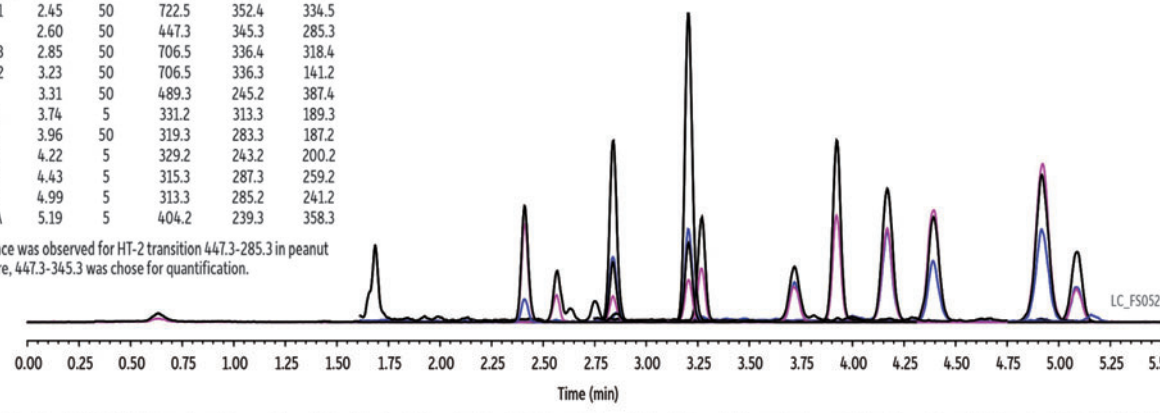
In this example, mycotoxins were analyzed in a peanut powder matrix. The use of a relatively short column format, the selectivity of the Biphenyl stationary phase, and the efficiency of 2.7-µm Raptor superficially porous particles provided excellent separations in a fast 5.5-min analysis (total cycle time of 7 min). A coeluting matrix compound that shared the most abundant MRM transition for mycotoxin HT-2 (447.3-285.3) was observed, so a less abundant transition (447.3-345.3) was selected for quantitation. To increase sensitivity, an ammonium buffer was used to promote better ionization of mycotoxins. The Raptor Biphenyl column worked very well for the 12 mycotoxins studied in the cited work, but for longer compound lists containing isobaric mycotoxins with similar structures, the Raptor FluoroPhenyl phase may be necessary to provide adequate chromatographic resolution. The selectivity of the Raptor FluoroPhenyl column is demonstrated in an analysis of 20 mycotoxins that can be found by visiting www.restek.com and entering LC_FS0511 in the search.

This method showed excellent precision and accuracy for the 12 FDA regulated mycotoxins that were evaluated during a validation study that covered a variety of matrices (including multiple sources of cornmeal and brown rice flour, in addition to the peanut powder example shown here).

Restek would like to thank Dr. Zhang for his technical support during this project.

Peaks	tR (min)	Conc. (ng/g)	Precursor Ion	Product Ion 1	Product Ion 2
1. Deoxynivalenol	0.62	50	297.3	249.3	231.2
2. Fumonisin B1	2.45	50	722.5	352.4	334.5
3. HT-2	2.60	50	447.3	345.3	285.3
4. Fumonisin B3	2.85	50	706.5	336.4	318.4
5. Fumonisin B2	3.23	50	706.5	336.3	141.2
6. T2	3.31	50	489.3	245.2	387.4
7. Aflatoxin G2	3.74	5	331.2	313.3	189.3
8. Zearalenone	3.96	50	319.3	283.3	187.2
9. Aflatoxin G1	4.22	5	329.2	243.2	200.2
10. Aflatoxin B2	4.43	5	315.3	287.3	259.2
11. Aflatoxin B1	4.99	5	313.3	285.2	241.2
12. Ochratoxin A	5.19	5	404.2	239.3	358.3

Matrix interference was observed for HT-2 transition 447.3-285.3 in peanut powder. Therefore, 447.3-345.3 was chose for quantification.



Column: Raptor Biphenyl (cat.# 9309A52); Dimensions: 50 mm x 2.1 mm ID; Particle Size: 2.7 µm; Pore Size: 90 Å; Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252); Temp.: 40 °C; Inj. Vol.: 5 µL; **Mobile Phase:** A: Water, 2 mM ammonium formate, 0.1% formic acid; B: Methanol, 2 mM ammonium formate, 0.1% formic acid; **Gradient (%B):** 0.00 min (30%), 0.6 min (30%); 0.7 min (50%); 3.00 min (70%); 4.5 min (75%); 5.0 min (90%); 5.2 min (90%); 5.21 min (75%); 6.00 min (75%); 6.01 min (30%); 7.00 min (30%); **Flow:** 0.5 mL/min; **Detector:** MS/MS; Ion Mode: ESI+; Mode: MRM; **Instrument:** UHPLC; **Notes:** Weighed 1.00 gram of peanut powder in a 50 mL centrifuge tube and added 2.00 mL of water. Vortexed at 3000 rpm for 5 min followed by the addition of 4.0 mL of extraction solvent (50:50 water:acetonitrile, v/v). The tube was then vortexed at 3000 rpm for 5 min followed by centrifugation for 15 min at 4200 rpm. 475 µL of the supernatant was filtered through a Thomson SINGLE StEP Nano filter vial (0.2 µm, cat.# 25882). The sample was then fortified with 25 µL of a standard solution prepared in water at 1000 ng/mL (100 ng/mL for aflatoxins and ochratoxin A) as part of the matrix-matched calibration curve. Vortexed at 3000 rpm for 1 min prior to analysis.

Raptor Biphenyl LC Columns (USP L11)

Length	2.1 mm cat.#	3.0 mm cat.#	4.6 mm cat.#
1.8 µm Columns			
30 mm	9309232	—	—
50 mm	9309252	930925E	—
100 mm	9309212	930921E	—
150 mm	9309262	—	—
2.7 µm Columns			
30 mm	9309A32	9309A3E	9309A35
50 mm	9309A52	9309A5E	9309A55
100 mm	9309A12	9309A1E	9309A15
150 mm	9309A62	9309A6E	9309A65
5 µm Columns			
30 mm	—	930953E	—
50 mm	9309552	930955E	9309555
100 mm	9309512	930951E	9309515
150 mm	9309562	930956E	9309565
250 mm	—	—	9309575

Reference

(1) K. Zhang, M.R. Schaab, G. Southwood, E.R. Tor, L.S. Aston, W. Song, B. Eitzer, S. Majumdar, T. Lapainis, H. Mai, K. Tran, A. El-Demerdash, V. Vega, Y. Cai, J.W. Wong, A.J. Krynitsky, T.H. Begley, *J Agr Food Chem*, **65**(33), 7138–7152 (2017). https://www.ncbi.nlm.nih.gov/pubmed/27983809.

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Recent Addition

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Direct Thermal Extraction-GC/MS Analysis of Food Packaging Material for crème-filled cookies, cheese-filled crackers and soft and chewy candy.

Recent Addition

Thermal Extraction requires almost no sample preparation and is well suited for trace analysis of migrating compounds in food packaging material. The packaging of three products was analyzed and benzaldehyde was quantified in one case.

Fully Automated Determination of 3-MCPD and Glycidol in Edible Oils by GC–MS Based on the Commonly Used Methods ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10)

Automated determination of 3-MCPD and glycidol in edible oils by GC–MS. An evaporation step helps reach the required LODs using a standard MSD, while removing excess derivatization reagent for improved uptime and stability.

Automated determination of Acrylamide in Brewed Coffee samples by Solid Phase Extraction (SPE)–LC–MS/MS

A manual SPE method used for the determination of acrylamide in brewed coffee was automated. Calibration standards prepared in freshly brewed green (unroasted) coffee produced good linearity and precision.

Qualitative Analysis of Coconut Water Products Using Stir Bar Sorptive Extraction (SBSE) combined with Thermal Desorption-GC–MS

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