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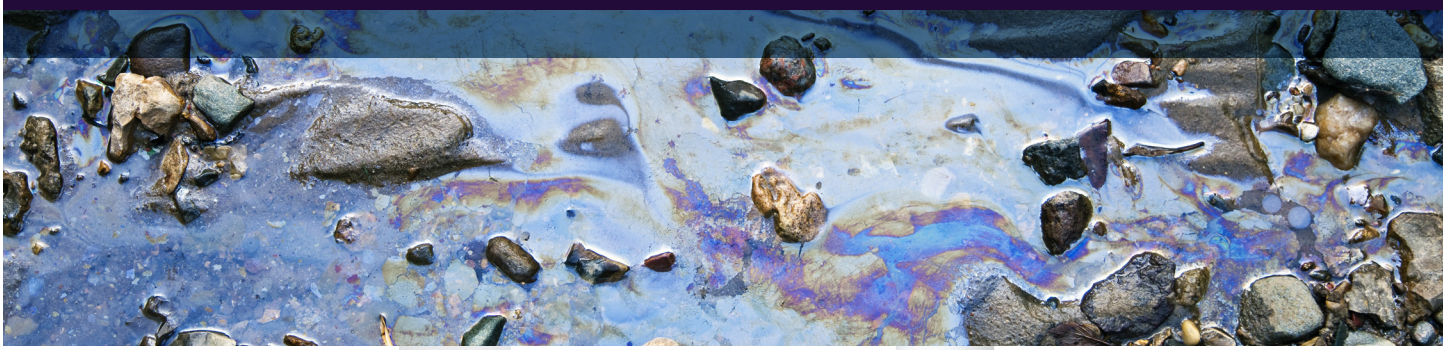
Application Compendium



Identify Emerging Contaminants in Air, Water, and Soil

Today's environmental analysis must be done more reliably, more efficiently, and with higher quality results than ever before. Unfortunately, pharmaceuticals, PFAS, pesticides, and other potentially toxic compounds can be difficult to detect in environmental and biological matrices.

To complicate matters, several thousand chemicals present in the environment are currently unknown and unidentified. Many of these chemicals could be toxic, but are not regulated in the environment, due to the absence of toxicological data.

Harness the power of accurate mass for identifying emerging (or evolving) contaminants

Accurate mass high-resolution instruments from Agilent—along with simple, yet powerful software tools—provide a complete workflow for identifying emerging and unknown chemicals in the environment.

Agilent 6500 Series LC/Q-TOF and 7200 Series GC/Q-TOF systems give you a complete chemical profile of your samples. So you can characterize a wider range of contaminants. What's more, these high-resolution instruments can be operated at fast acquisition rates to collect molecular ion and fragment information in MS/MS mode. This maintains mass accuracy and isotope fidelity for confident compound identification at environmentally relevant concentrations.

Take the first step in identifying environmental toxicity

Along with regulated contaminants, unknown and unregulated chemicals can put wildlife and humans at risk.

Agilent SeaHorse analyzers and cell analysis measure cellular functions that are affected by exposure to environmental toxins. This provides toxicity information in the environmental samples. You can then identify chemicals that cause toxicological effects using advanced mass spectrometry. This combination of biological toxicity testing and identification is called "effects-directed" analysis.

What's inside?

In this compendium, you'll find high-resolution mass spectrometry applications for compounds such as pesticides, PFASs, VOCs, SCCPs, and pharmaceuticals. These applications include the identification of new and previously unknown environmental contaminants.

And as always, our product and application experts are available to help you maximize productivity. They can provide simple workflows for identifying emerging chemicals while maintaining stringent standards for regulated compounds.

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Exposure in Environmental Matrices

Nontargeted screening of suspect and unknown contaminants

Exposure to chemical contaminants in water, air, and soil present potential health risks for biota and humans. Concern about emerging and unknown environmental contaminants has increased efforts to identify the scope and origin of trace chemicals. Global screening initiatives have also been implemented for soil contamination, wastewater treatment, and drinking water purification. Successful, effects-directed characterization of biologically and chemically relevant contaminants requires suspect screening and nontargeted identification using advanced MS technology.

Agilent provides proven end-to-end solutions for emerging contaminant research. Our LC and GC/Q-TOF systems let you characterize a sample's complete chemical profile. In addition, our simple, yet powerful software tools and databases make identifying nontargeted chemicals efficient. We also offer tools for suspect and nontargeted screening that deliver **speed to results** by reducing false positives—without compromising data quality and spectral resolution.

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LC/Q-TOF Workflows for Comprehensive Micropollutant Analysis

Targeted Quantification, Suspect Screening, and Unknown Compound Identification

Application Note

Environmental

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Abstract

This application note presents three complementary LC/Q-TOF workflows designed to provide comprehensive analysis of micropollutants in surface waters:

- Targeted quantification
- Suspect screening with and without MS/MS spectra
- Unknown compound identification

The first two workflows rely on the Agilent All Ions MS/MS accurate mass capabilities of the Agilent LC/Q-TOF system, Agilent MassHunter Qualitative Analysis software, and Agilent Personal Compound Database and Libraries (PCDLs) to detect and confirm compound identities, with or without reference standards. Agilent Molecular Structure Correlator (MSC) software is shown to aid in the identification of unknown compounds, in this case transformation products (TPs).

The targeted quantification workflow was validated using 32 reference standards. The workflow detected and quantified 25 compounds in at least one of the 51 surface water samples tested. The suspect screening workflow generated an expanded list of 85 possible pollutants, of which 73 were subsequently positively identified with an authentic standard (67 compounds) or by matching MS/MS spectra (six compounds). The unknown compound identification workflow identified five TPs that had not been identified using the targeted quantification or suspect screening workflows.

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Introduction

Comprehensive analysis of micropollutants in waste, surface, and drinking waters is necessary to confidently assess exposure and risk. Traditional targeted screening workflows, such as triple quadrupole MS methods, monitor and quantify a predefined list of compounds using analytical reference standards. However, a targeted approach will miss pollutants not on the target list, and may underestimate exposure when unexpected pollutants are present.

While triple quadrupole LC/MS systems are well suited for targeted analysis, their methods require standards that are not always easily obtained. In addition, they are not useful when trying to identify new or unknown compounds in the sample. High-resolution, accurate-mass (HRAM) quadrupole time-of-flight (Q-TOF) LC/MS analysis allows complementary suspect screening and unknown compound identification workflows, which, when used together with targeted quantification, provide a more complete picture of the chemical profile of the sample analyzed (Figure 1).

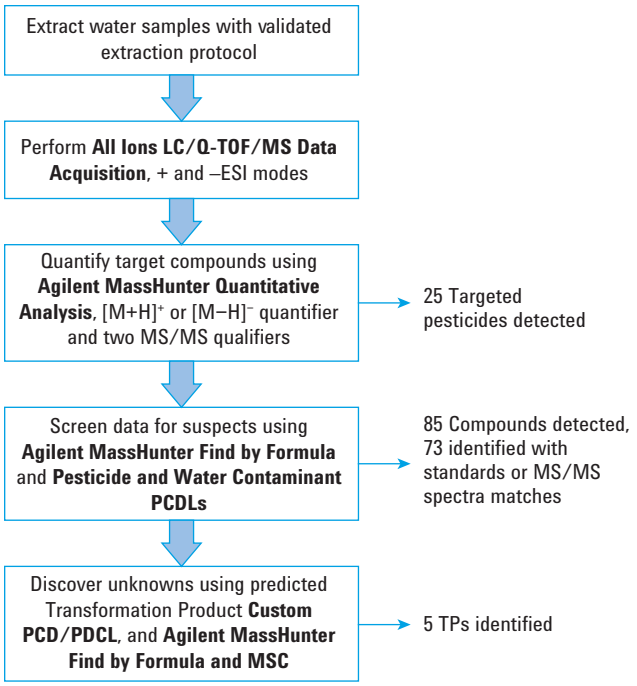


Figure 1. Complementary targeted quantification, suspect screening, and unknown compound identification LC/Q-TOF workflows for comprehensive micropollutant analysis.

For compounds not identified through targeted analysis, the suspect screening workflow, which uses Agilent All Ions MS/MS and Personal Compound Database and Libraries (PCDLs), allows accurate mass determination and MS/MS fragment confirmation of compounds. The screening workflow allows the analyst to presumptively identify compounds without analytical reference standards with a high degree of confidence. The unknown compound identification workflow attempts to identify compounds for which there are not MS/MS fragment entries in the selected PCDLs, nor are analytical standards readily available. In this workflow, Agilent Molecular Structure Correlator (MSC) software compares MS/MS fragments for compounds of interest to plausible candidate structures in additional databases such as Chemspider, Pubchem, or custom databases. MSC calculates scores based on the quality of the matches of the experimental fragments with those predicted.

This application note describes the use of targeted quantification, suspect screening, and unknown compound identification with LC/Q-TOF data acquisition and analysis workflows to achieve broader, more confident characterization of micropollutants, including transformation products (TPs), in surface water samples. The study *LC- and GC-QTOF-MS as Complementary Tools for a Comprehensive Micropollutant Analysis in Aquatic Systems* provides a detailed description of the analytical results and their implications for environmental monitoring [1]. Because not all of the compounds studied are amenable to LC/MS analysis, the research also describes a complementary GC/Q-TOF workflow that provides a comprehensive chemical profile of the sample.

Experimental

Target compounds and standards

For the targeted quantification workflow, 32 LC/MS-amenable pesticides were chosen for analysis of the surface water samples (Table 1). The target compounds were chosen to include compounds used in the area of the waters sampled, and to represent pesticides of different classes and physicochemical properties. Seventeen of the targets had better sensitivity in positive ESI mode, while 15 had better sensitivity in negative ESI mode. Eleven isotopically-labeled internal standards were used for accurate quantification.

For method validation and quality control analyses, reference standards for the target compounds were prespiked before extraction and post-spiked before injection. Procedural blanks were also used to look for blank contamination.

Table 1. Target Pesticides and Targeted Quantification Results

Target analyte	ESI mode	Matrix factor[1]	Method detection limit (ng/L)[2]	Absolute recovery (%)[3]	Accuracy (%)[4]	Precision (%)[5]
2,4-D	–	2.6	2.6	95 %	160 %	18 %
2-Phenylphenol	–	1.2	1.2	75 %	170 %	6 %
Azoxystrobin	+	1.7	0.2	95 %	128 %	1 %
Boscalid	–	1.6	0.3	97 %	111 %	3 %
Chlorantraniprole	+	2.7	2.7	95 %	87 %	3 %
Clomazone	+	2.7	1.3	76 %	190 %	1 %
Cyprodinil	+	3.1	0.3	91 %	118 %	3 %
DEET	+	2.2	0.2	76 %	78 %	4 %
Difenoconazole	+	1.7	0.9	95 %	104 %	1 %
Dimethoate	+	3.8	0.9	92 %	62 %	5 %
Diuron	–	1.7	0.2	92 %	102 %	1 %
Fipronil	–	1.2	0.1	97 %	96 %	6 %
Fipronil-desulfinyl	–	1.2	0.1	101 %	78 %	4 %
Fipronil-sulfide	–	1.1	0.1	97 %	74 %	18 %
Fipronil-sulfone	–	1.2	0.1	96 %	113 %	1 %
Hexazinon	+	3.0	0.3	91 %	117 %	3 %
Imidacloprid	–	4.2	2.1	93 %	152 %	5 %
MCPA	–	2.9	1.1	96 %	112 %	1 %
Methomyl	+	2.7	13	93 %	104 %	2 %
Methoxyfenozide	–	1.3	0.1	99 %	72 %	4 %
Metolachlor	+	1.7	0.2	80 %	108 %	2 %
Novaluron	–	1.2	0.6	74 %	91 %	2 %
Pendimethalin	+	1.8	1.8	71 %	74 %	3 %
Propanil	–	1.1	1.2	98 %	138 %	4 %
Propoxur	+	2.6	1.3	76 %	83 %	1 %
Pyriproxyfen	+	2.3	0.2	89 %	98 %	7 %
Simazine	+	7.2	1.8	89 %	77 %	3 %
Thiacloprid	+	4.1	1.0	93 %	97 %	6 %
Thiamethoxame	+	2.0	1.0	92 %	108 %	3 %
Thiobencarb	+	1.6	1.6	77 %	99 %	2 %
Triclocarban	–	1.4	0.1	92 %	97 %	1 %
Triclosan	–	1.2	1.3	89 %	89 %	2 %

Equation 1.
$$\text{Matrix factor} = \frac{\text{Area STD 100 ng/mL}}{\text{Area extract post spiked 100 ng/mL} - \text{Area extract unspiked}}$$

Equation 2.
$$\text{Method detection limit (ng/L)} = \frac{\text{Instrument detection limit (ng/mL)} \times \text{Matrix factor (–)}}{\text{Concentration factor (mL/L)}}$$

Equation 3.
$$\text{Absolute recovery (\%)} = \frac{\text{Area prespiked extract} - \text{Area unspiked extract}}{\text{Area post spiked extract} - \text{Area unspiked extract}}$$

Equation 4.
$$\text{Accuracy (\%)} = \frac{\text{Concentration prespiked extract} - \text{Concentration unspiked extract}}{\text{100 ng/mL}}$$

Equation 5.
$$\text{Precision (\%)} = \frac{\text{Standard deviation concentration prespiked extract (triplicate)}}{\text{Average concentration prespiked extract (triplicate)}}$$

Sample preparation

Fifty-one 1-L surface water samples collected from the Sacramento-San Joaquin River Delta in Northern California were extracted using a mixed-mode solid phase extraction (SPE) cartridge. The cartridges were eluted sequentially with 6 mL of 50/50 methanol/ethyl acetate with 0.5 % ammonia, 3 mL of methanol/ethyl acetate with 1.7 % formic acid, and 2 mL of methanol, per Moschet; *et al.* [2]. The eluent was evaporated to 0.2 mL and reconstituted to 1 mL with nanopure water to obtain a 20 %/80 % methanol/water ratio for injection into the LC/MS system.

LC/Q-TOF analysis

LC/MS analysis of the reference standards and sample extracts was performed using an Agilent 1260 Infinity LC coupled to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system equipped with an Agilent Jet Stream dual electrospray ionization (ESI) source. The HPLC system included a binary pump, Agilent 1260 Autosampler, and an Agilent ZORBAX Eclipse Plus C-18, 2.1 × 100 mm, 1.8 µm column (959758-902). Table 2 lists the LC parameters.

Table 2. LC Parameters. (+): Positive ESI Mode; (-): Negative ESI Mode

Parameter	Value
Liquid chromatograph	Agilent 1260 Infinity Binary LC
Analytical column	Agilent ZORBAX Eclipse Plus C-18, 2.1 × 100 mm, 1.8 µm
Injection volume	20 µL
Column temperature	30 °C
Mobile phase	A) Water + 0.1 % formic acid (+)/ Water + 1 mM ammonium fluoride (–) B) Acetonitrile + 0.1 % formic acid (+)/ Acetonitrile (–)
Flow rate	0.35 mL/min
Gradient	Time (min) %A 0.0 98 1.5 98 16.5 0 21.5 0
Equilibration time	3.0 minutes

The Q-TOF mass spectrometer was operated in both positive and negative ESI modes to maximize compound detection. Agilent MassHunter Workstation software was used to acquire data (version B.07.00). The All Ions MS/MS acquisition mode with collision energies (CEs) of 0, 10, 20, and 40 V was used to obtain both precursor and fragment ion data for all species. The All Ions MS/MS acquisition mode simultaneously collects high- and low-CE scans. The low CE scans allow the user to obtain precursor information, while the higher CE scans provide fragment information that enhances compound identification and confirmation when comparing experimental spectra to those in the PCDL. Table 3 lists the Q-TOF mass spectrometer parameters.

To facilitate identification of TPs not identified in the targeted quantification or suspect screen workflows, the samples with the highest abundances of plausible TPs were rerun in targeted MS/MS mode with the collision energy set to 20 V.

Table 3. Q-TOF Mass Spectrometer Parameters

Parameter	Value
Mass spectrometer	Agilent 6530 Accurate Mass Q-TOF-LC/MS with Agilent Jet Stream Technology
Ionization mode(s)	Positive (+) and negative (–) ESI
Instrument mode	2 GHz extended dynamic range
Mass range	50–1,050 *m/z*
Gas temperature	300 °C
Drying gas flow	12 L/min
Nebulizer	25 psig
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Capillary voltage	3,500 V (+), 3,000 V (–)
Fragmentor voltage	110 V
Scan speed	4.0 spectra/sec
Collision energies	Agilent All Ions MS/MS: 0, 10, 20, 40 V Targeted MS/MS: 20 V
Reference ions*	(+) 121.0509 and 922.0098 (–) 112.9855 and 1033.9881

*The positive mass reference ions were not used in some instances because of interferences in some of the samples. The user can check for interferences and use alternate reference ions if required.

Data processing and analysis

Target compounds were quantified using Agilent MassHunter Quantitative Analysis software (version B.07.00). The $[M+H]^+$ or $[M-H]^-$ ion within the exact mass window of ±10 ppm was used as the quantifier ion. The two most abundant unique MS/MS fragments for each compound were selected from the MS/MS library spectra in the Agilent PCDLs to use as qualifiers. The Agilent Pesticide PCDL for TOF or Q-TOF LC/MS systems (1,684 compounds, 914 with MS/MS spectra) and the Agilent Water Screening PCDL (1,451 compounds, 1,157 with MS/MS spectra) were used.

The suspect screening workflow used the Agilent Pesticide and Water Screening PCDLs, in combination with the MassHunter Qualitative Analysis software (version B.07.00) Find by Formula algorithm. Find by Formula automatically extracts precursor ions from the All Ions MS/MS data using the accurate-mass database in the PCDLs. When available in the accurate mass library, the corresponding MS/MS fragments are also extracted from the data. Precursor and corresponding fragment ion peaks are plotted to score the quality of their correlation for each compound. The suspect screening workflow data analysis parameters used are provided in Figure 2.

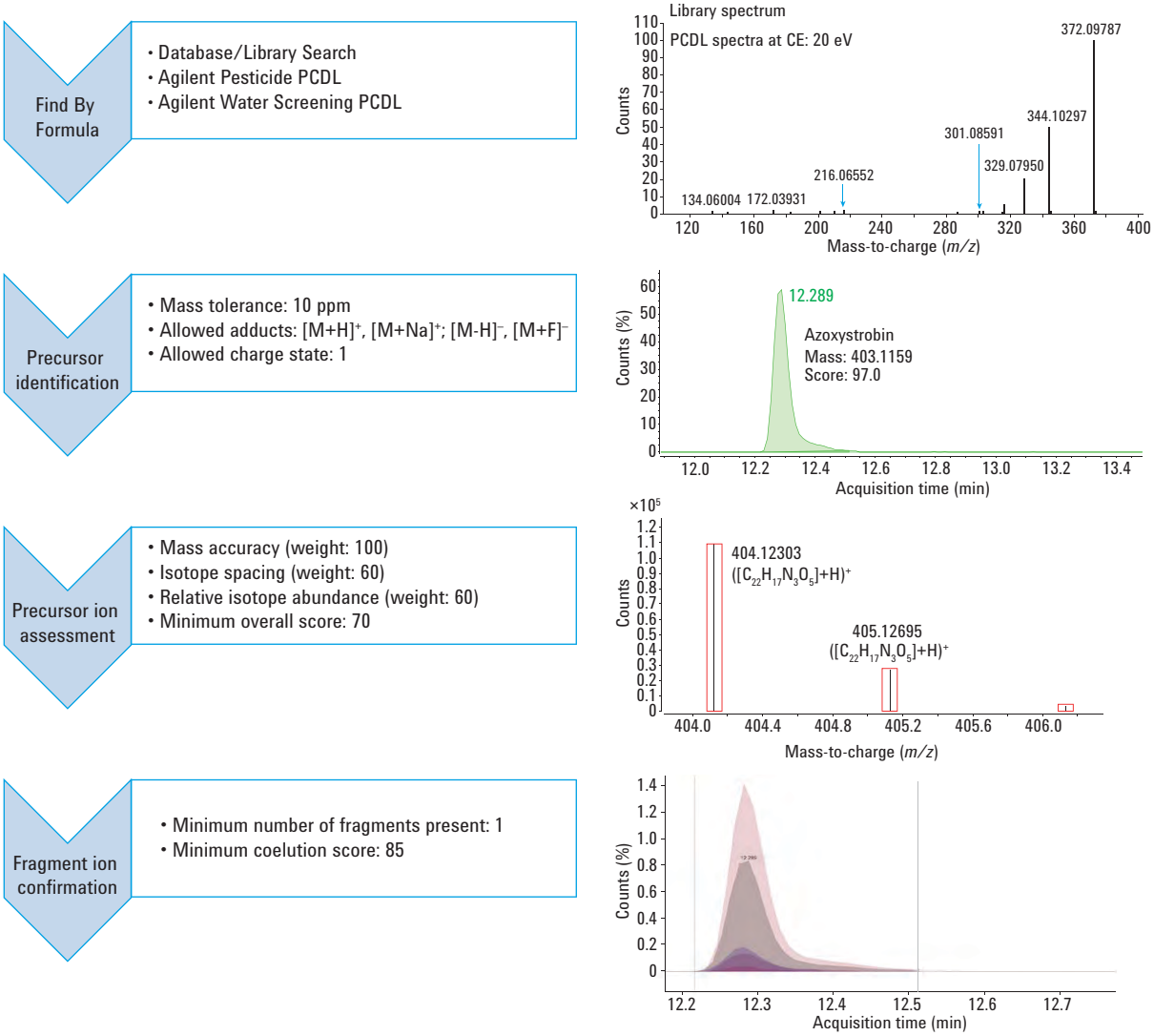


Figure 2. Suspect screening workflow: data analysis parameter settings and example. The precursor mass of the fungicide azoxystrobin (403.1168 m/z) is found at retention time 12.3, and its fragment ions are confirmed.

The unknown compound identification workflow can be used to identify compounds without *a priori* knowledge using accurate mass and fragment data along with *in silico* fragment prediction solfware. In this case, it relied on a custom PCD of potential TPs, which contained accurate masses and molecular formulas. The Eawag Pathway Prediction System (EAWAG-PPS) [3] was used to generate 1,409 possible TP structures for the pesticides detected in the study. After eliminating 71 structures due to the implausibility of being ionized by ESI, the molecular formulas of the plausible structures were added to build the custom PCD. The MassHunter Qualitative Analysis software Find by Formula feature was used to screen the data for these plausible molecular formulas. Because there were no MS/MS spectra for these compounds in the custom PCD, the exact mass, isotope score, number of detections across the 51 samples, and retention time (RT) were used to produce a short list of plausible matches.

Water samples with the highest abundances of the short list of plausible candidates were rerun in targeted MS/MS mode to obtain MS/MS spectra for processing by MSC software (version, B.07.00). MSC software was used to search the custom PCD for compounds with the same exact mass as the isolated mass. MSC software automatically compares predicted *in silico* fragments of the structures in the custom PCD (or in a web-based database such as ChemSpider or PubChem) with the measured MS/MS spectra. All measured MS/MS fragments that can be explained by each structure were listed and scored based on a weighted match. It is important to note that this workflow requires that the matching structures are present in the custom PCD. The structures can be manually uploaded into the PCD using mol-files.

To aid in the identification of unknown TPs, CFM-ID (http://cfmid.wishartlab.com/predict) was used to predict the MS/MS spectra of the plausible TPs [4].

Results and Discussion

Targeted quantification

Table 1 shows the LC/Q-TOF positive and negative ESI quantification results for the 32 target pesticide standards. The pesticide standards were run on the LC/Q-TOF system to obtain method validation parameters and to determine method suitability for targeted quantification. The method validation parameters, including method detection limits, absolute recovery, accuracy, precision, and matrix factors, are presented. The parameter details and their calculation have been described by C. Moschet; *et al.* [1].

Method detection limits for all target analytes ranged from 0.1 and 13 ng/L in water. All targets had absolute recoveries between 70–110 %. The precision, calculated as the relative standard deviation (%RSD) of triplicate injections on the LC/Q-TOF system, was <10 % for 30 of the target analytes, while the accuracy for 26 analytes was between 70 and 130 %.

Targeted screening detected 25 of the target compounds among the 51 water samples tested (Figure 1).

Suspect screening

The All Ions MS/MS sample data collected in both positive and negative ESI modes were screened for compounds present in the Agilent Pesticide and Water Screening PCDLs using the Find by Formula algorithm. Based on specified adducts (Figure 2), the software automatically searched the acquired data for the presence of the precursor ions of compounds stored in the PCDL, and assigned a cumulative score to matches based on mass accuracy, isotopic spacing, and relative isotope abundance.

A score threshold of >70 was chosen (Figure 2) for compounds to be considered for further evaluation. Compound matches with higher scores were subsequently evaluated for the presence of MS/MS fragment ions matching the compounds' MS/MS spectra in the PCDL (when MS/MS spectra were present in the PCDL). Compounds without MS/MS spectra in the PCDL were tentatively identified.

The validity of fragment ion matches was evaluated by scoring their coelution with their corresponding precursor ions (coelution score). Using the spiked analytes, it was determined that the presence of one fragment with a coelution score >85 was sufficient to identify compounds with a low false positive rate, while producing a manageable amount of data to process and review (Figure 2). Figure 2 also provides an example of the identification of the fungicide azoxystrobin using the suspect screening workflow. Figure 3 provides a more detailed comparison of the theoretical (in the PCDL) and measured isotope pattern, as well as the coelution of the main fragments (from the PCDL) of the herbicide fluridone.

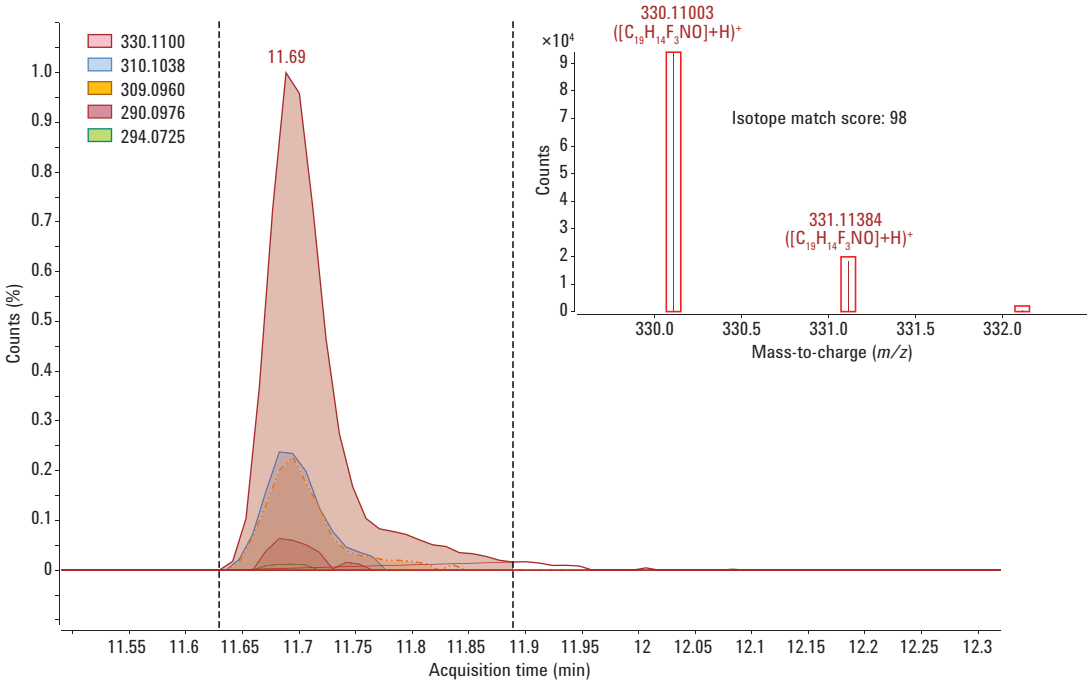


Figure 3. Details of the theoretical and measured isotope pattern, along with molecular and fragment ions, identified for fluridone using All Ions MS/MS and the PCDL.

After manual inspection, the suspect screening workflow detected 85 compounds (53 in positive ESI, 26 in negative ESI, and six in both positive and negative ESI). Of these, 67 could be confirmed unambiguously by a reference standard, six could be confirmed tentatively with high confidence by matching MS/MS spectra, and 12 compounds were rejected because they were not confirmed by a reference standard or due to implausible MS/MS fragments. The latter was the case when there were no MS/MS spectra in the PCDL for the suspected compound.

Unknown compound identification

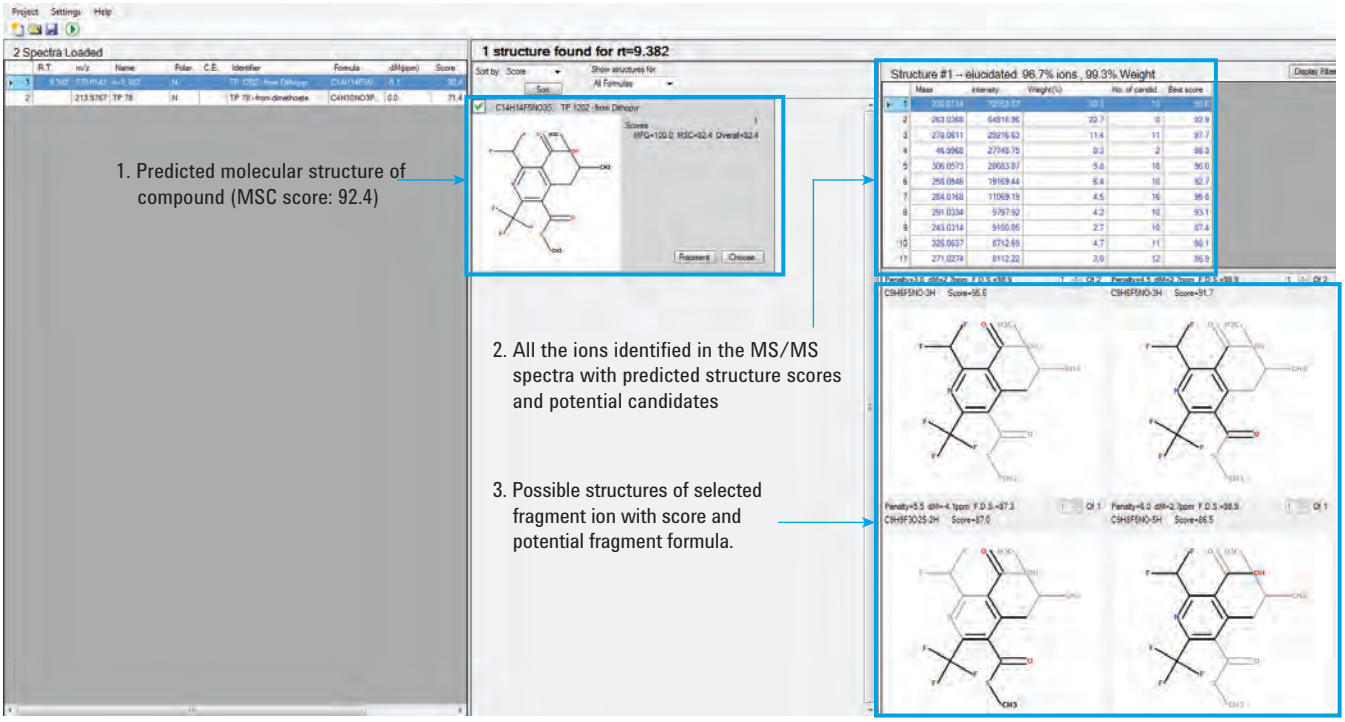
The MassHunter Qualitative Analysis software Find by Formula feature was used to identify TPs in the water sample data using plausible TP structures that had been added to the custom PCD. This step yielded 110 matches. Inspection of peak shape, signal-to-noise ratio (S/N), RT plausibility, and whether the detected compound is theoretically ionizable in the selected ionization mode left 33 plausible compounds.

Comparison of the abundance pattern of the 33 plausible TPs with the concentration pattern of their potential parent compounds in the water samples reduced the list of those tentatively identified to 14.

After rerunning the samples in targeted MS/MS mode, evaluating the MS/MS spectra using the MSC software, comparing measured fragments to those predicted by CFM-ID and manually inspecting the results, seven compounds were eliminated for having implausible MS/MS spectra (that is, having fragments that could not be explained by the molecular structure). Thus, seven plausible TPs were identified using the workflow. Two of them were already detected and confirmed using the targeted quantification or suspect screening workflows. Therefore, five new TPs could uniquely be identified by the unknown compound identification workflow. Three of them were able to be confirmed unambiguously by a reference standard, and two of them remained tentatively identified because no reference standard was commercially available.

Figure 4 shows an example of a transformation product of the herbicide dithiopyr (CAS 128294-56-4), which was tentatively identified with high confidence in several samples. This compound, along with several MS/MS fragment ion structures, was identified using MSC (score of 92.4). MSC was able to identify and elucidate structures for 96.7 % of the ions in the MS/MS spectra for this TP.

A



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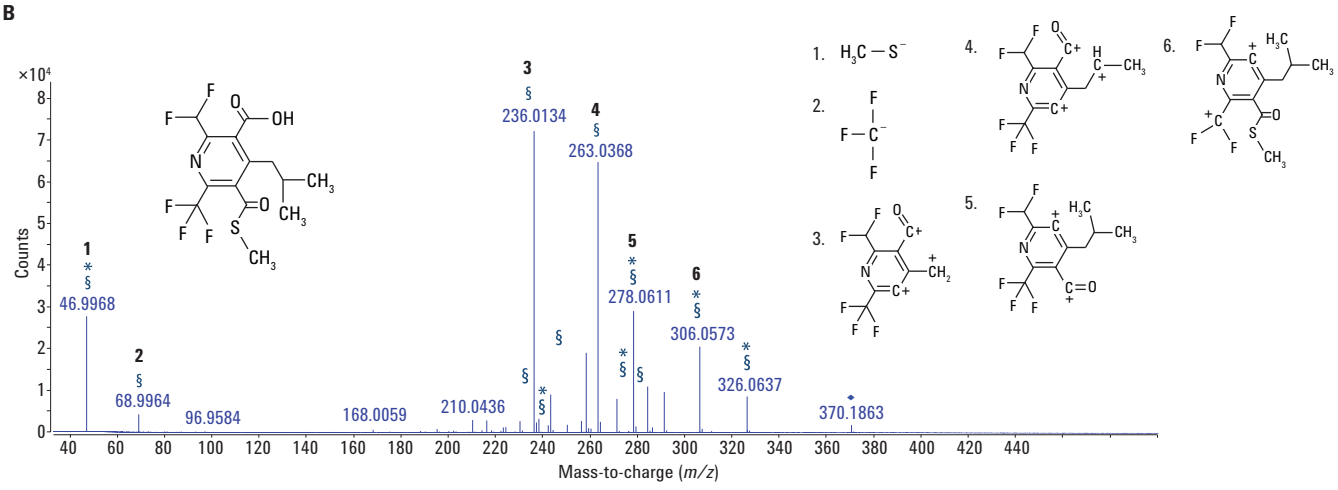

Figure 4A. Agilent MSC software window showing MS/MS fragments of a dithiopyr TP with CAS 128294-56-4 (MSC score 92.6). 1) predicted by MSC software; 2) list of all ions identified with predicted structure scores and candidates; 3) possible structures and formulas of selected fragment ions. B) Mass spectrum of a dithiopyr TP (§) predicted by MSC software; (*) predicted by CFM-ID.

Conclusions

LC/Q-TOF targeted quantification, suspect screening, and unknown compound identification workflows were applied to the analysis of surface water samples. The targeted quantification workflow was validated using 32 pesticide standards. Twenty-five of them were detected among the samples tested using the targeted quantification workflow. The suspect screening workflow generated an expanded list of 85 possible pollutants, 73 of which were subsequently positively identified. When applied to herbicide and pesticide TPs, the unknown compound identification workflow identified five plausible TPs not identified using the targeted quantification or suspect screening workflows. Compared to targeted analysis alone, the complementary workflows enabled by the Agilent 6530 Accurate-Mass Q-TOF LC/MS system provided more comprehensive, higher confidence characterization of the micropollutants present, including potential pesticide and herbicide TPs. Several more compounds were identified that would have been missed if only a targeted approach were used.

The Agilent Pesticide and Water-Screening PCDLs, combined with the Agilent All Ions MS/MS accurate mass capabilities of the Q-TOF LC/MS system and Agilent MassHunter Qualitative Analysis software, enabled presumptive matching of acquired spectra with library spectra to confirm compound identities, without the need to source standards. Agilent Molecular Structure Correlator software aided identification of TPs by correlating the unknown MS/MS spectrum against multiple candidate structures in compound databases.

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Application Note

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Identification of Unknowns in Ground and Surface Water by LC/Q-TOF

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Abstract

Accurate mass analysis by LC/Q-TOF with a series of accurate mass tools is used in this study to find target, suspect, and unknown pharmaceuticals and their degradation products in surface and groundwater along the South Platte River near Denver, Colorado. The ability to detect unknown pharmaceuticals is used to examine removal and transport of pharmaceuticals from a major metropolitan area (Denver) to the South Platte River and eventually to alluvial groundwater nearby. This study is a good example of using unknown identification to understand transport and removal of pharmaceuticals in groundwater and surface water systems.

Introduction

The importance of wastewater as a source of pharmaceuticals in surface water has been studied extensively since the late 1990s with a famous review of the problem reported in 1998[1]. This review was followed by a study of pharmaceuticals in surface water by the U.S. Geological Survey[2]. Both studies found that sewage wastewater was the major source of pharmaceuticals in water samples. Since then, thousands of papers have been published on the occurrence of pharmaceuticals in surface water and wastewater, including many review articles[3,4]. However, the occurrence of pharmaceuticals in groundwater is much less studied or reviewed[4], despite the earliest documented report of pharmaceuticals in water being for groundwater impacted by sewage[3].

This study describes the analytical workflow and the set of analytical tools with accurate mass that have successfully been used to identify pharmaceuticals and their degradants using liquid chromatography and quadrupole time-of-flight mass spectrometry (LC/Q-TOF-MS). In addition, using these workflows, both suspect and unknown pharmaceuticals found in the South Platte River near Denver, Colorado were reported. Finally, these pharmaceuticals and their degradants in alluvial groundwater wells affected by the South Platte River were measured using LC/Q-TOF-MS analysis to better understand transport and removal of pharmaceuticals. This Application Note is a compilation of the many studies carried out using the Agilent 6500 Series of LC/Q-TOF-MS for the discovery of pharmaceuticals in surface and groundwater.

Experimental

Samples

Ten alluvial wells along the South Platte River were sampled multiple times over a three-month period in 2016. Standard ground and surface water sampling methods were used, with both a peristaltic and bladder pump[5]. Sampling of the South Platte River and two alluvial wells was also carried out in 2009–2010, sampling monthly over one year. All of the alluvial wells were in hydrologic contact with the South Platte River. The South Platte River itself was sampled by grab sample. Figure 1 shows the location of the alluvial wells along the river.

Analytical methods

The separation of the analytes was carried out using an Agilent 1290 Infinity II LC equipped with a reversed-phase C8 analytical column of 150 mm × 4.6 mm and 3.5 µm particle size (Agilent ZORBAX Eclipse XDB-C8). Column temperature was maintained at 25 °C. The injected sample volume was 10 µL. Mobile phases A and B were water with 0.1 % formic acid, and acetonitrile, respectively. The optimized chromatographic method held the initial mobile phase composition (10 %A) constant for five minutes, followed by a linear gradient to 100 %A after 30 minutes.

The HPLC system was connected to an Agilent 6545 LC/Q-TOF equipped with an Agilent Jet Stream source. The data recorded were processed using Agilent MassHunter software.

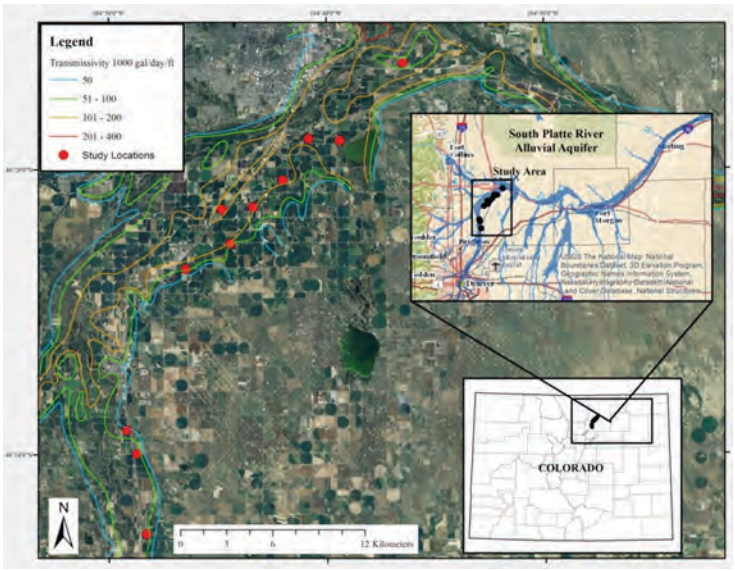

Figure 1. Location of groundwater wells along the South Platte River approximately 30 miles north of Denver, Colorado[5].

Sample preparation

Samples were concentrated using an offline solid phase extraction (SPE) technique that allowed the sample to be analyzed multiple times for MS analysis, MS/MS analysis, and to reach the required ng/L concentration level. A 100 mL water sample was processed using SPE on a Gilson robotic system. The sample was eluted with 5 mL of methanol, and was then evaporated under nitrogen to 0.5 mL for a final concentration factor of 200 fold. This method is a generic method that has been used successfully for antidepressants in surface waters and wastewaters[6].

Results and discussion

Analytical workflow and tools used

The analysis of groundwater and surface water samples required the adoption of a workflow method that allows for both suspect and unknown detection of pharmaceuticals. Table 1 shows the set of tools used for suspect and unknown analysis, while Figure 2 shows the workflow system that allows for a thorough analysis of unknown samples using an iterative system. The four types of tools include:

- Hardware
- Software
- Ion chemistry
- Physical, chemical, and biological tools related to surface and groundwater chemistry

This approach is highly effective at the process of suspect and unknown identification in surface and groundwater samples for pharmaceuticals.

Table 1. The tools used for unknown and suspect analysis.

Hardware tools

- High resolution and accurate mass[7–8, 25]
- All Ions, Auto MS/MS, Pseudo MS[3 (22–24)]

Software tools

- Databases, libraries[14]
- Kendrick mass, mass structure correlator, elemental forcing routines, isotope calculator[14–16, 26]

Ion chemistry tools

- Adduct formation (rule of 5), fragmentation tools (diagnostic and fragment ions), rules of fragmentation (N-rule and odd and even electron ion rules)[8,9,16,17]

Physical and biochemical tools—thinking outside the box

- Biochemical pathways[18,19]
- Biodegradation pathways[6,19]
- UV degradation and chemical oxidation[20,21]
- Correlation pathways

Tools used

The workflow in Figure 2 begins with a suspect list being searched in the accurate mass file acquired in TOF mode. The suspect list differs from a targeted list in that only the exact mass is known for a suspect (sometimes a retention time may be known but no standard is currently available, which is the *de facto* definition of a suspect or nontarget list). A targeted compound will have retention time and several confirmation ions using accurate mass as a confirmation based on a concept of identification points[7]. The suspect/nontarget list used was a list of 100 common pharmaceuticals that have been found in surface water and wastewater[8].

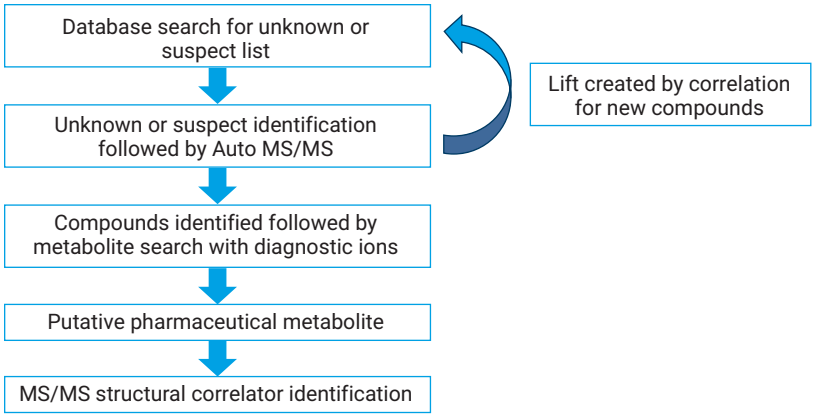

Figure 2. Analytical workflow chart for unknown and suspect identification.

Two approaches are possible, either an Excel database (CSV file) or an Agilent database search, called a Personal Compound Database (PCD.cdb) file. A personal compound database and library (PCDL.cdb) file will also contain MS/MS spectra, in the library portion. The PCD.cdb file may also include a structure as a mol file that can readily be imported into the Molecular Structure Correlator software for fragment verification. Both are easy to make and use.

The 22 compounds detected and confirmed in this study include:

- Beta blocker pharmaceuticals for blood pressure control
- Antibiotics
- Antidepressants
- Caffeine
- Sucralose, a low-calorie sweetener

The beta blockers, antibiotics, and antidepressants were:

- Atenolol
- Azithromycin
- Bupropion
- Clarithromycin
- Codeine
- Dextrorphan
- Diltiazem
- Diphenhydramine
- Erythromycin anhydrate
- Gabapentin
- Lamotrigine
- Metoprolol
- Oxycodone
- Sulfamethoxazole
- Thiabendazole
- Trimethoprim
- Venlafaxine
- Various metabolites of these pharmaceuticals

In addition to using the tools in Table 1 and the workflow in Figure 2, metabolites were found using different tools, such as diagnostic ions (Box 3, Figure 2). The concept of diagnostic ions means that pharmaceuticals of a specific chemical family will often have similar fragment ions or ions that are diagnostic of their structure. The publications by Ferrer and Thurman[8,9] list approximately three fragment ions that may be used as diagnostic ions for the identification of related pharmaceuticals and pesticides either in the same family or metabolites of detected compounds.

A good example of this tool is the discovery of lamotrigine glucuronide, which was found in the South Platte River using several tools, including both a chlorine filter and diagnostic fragment ion[10]. The diagnostic ion in this case was the *m/z* 256 ion, which is the same mass as the protonated molecule of the parent compound, lamotrigine. This compound was eventually identified after MS/MS and standard matching[10].

The Molecular Structure Correlator shown in Box 5 in Figure 2, is another useful tool. Before a standard is purchased, it is possible to carry out MS/MS. The accurate mass fragments can then be considered using the structure correlator, which, if possible, assigns the correct accurate mass to each of the fragments. This tool is a favorite for suspect analysis, since it is rare that two compounds have the same fragment ions, though it does occasionally happen[11], as was the case with an identification of tramadol and a metabolite of venlafaxine.

Finally, Box 6 in Figure 2 shows the power of iterative use of the workflow chart. There can be correlation, or lift, between compounds. Lift is a marketing term indicating sales increase with a specific advertisement. Here, we use lift to mean that a compound is present due to the sale of this compound as either a mixture or given with another compound. These compounds do not correlate in water samples by concentration due to different chemistries and removal processes during water treatment and transport. Thus, the presence of one may add a lift or give an opportunity for the other compound to be present. This is the case where two compounds are related by use but do not necessarily correlate in a 1:1 sense. A good example is the detection of sulfamethoxazole in the South Platte River (Table 2), which is commonly taken as an antibiotic for urinary infections, and is combined with trimethoprim. When the accurate mass for trimethoprim is searched, it is found at a low intensity, below the cutoff of the suspect search by database, but the compound is present at trace levels. It is preferentially removed by wastewater treatment but may still be present at just above the limit of detection of the analysis method, which shows the power of using the workflow chart, for each compound detected in the suspect analysis or unknown identification.

Pharmaceuticals in alluvial groundwater

Eight compounds were frequently found in the alluvial groundwater 100–500 m from the South Platte River. These compounds were:

- Bupropion
- Caffeine
- Carbamazepine
- Gabapentin, a metabolite of carbamazepine
- Lamotrigine
- Sucralose
- Sulfamethoxazole

This was a decrease from the 22 compounds confirmed in the South Platte River. The 14 compounds that were removed by bank filtration or diluted below detection levels as the river water flowed to the alluvial wells included:

- Atenolol
- Azithromycin
- Clarithromycin
- Codeine
- Dextrorphan
- Diltiazem
- Diphenhydramine
- Erythromycin anhydrate
- Metoprolol
- Oxycodone
- Thiabendazole
- Trimethoprim
- Venlafaxine and its metabolite

In a 2009–2010 sampling of a groundwater well much closer to the South Platte River, approximately 50 m, atenolol, diphenhydramine, venlafaxine, and its metabolite were also detected, which shows minimal transport for these compounds.

Table 2. LC and MS and MS/MS instrument conditions used in this study.

LC conditions for the 1290 Infinity II LC	
Column	ZORBAX Eclipse XDB-C8 150 mm × 4.6 mm and 3.5 µm (p/n 963967-906)
Column temperature	25 °C
Injection volume	10 µL
Mobile phase	A) Acetonitrile B) 0.1 % formic acid in water
Run time	30 minutes
Flow rate	0.6 mL/min
Gradient	90 %B at time 0, hold for 5.0 minutes, gradient to 100 %B at 30 minutes, then 10 minutes post run time
MS conditions positive ion mode	
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Gas temperature	250 °C
Desolvation gas flow-rate	10 L/min
Nebulizer pressure	45 psi
Capillary voltage	3,500 V
Nozzle voltage	0 V
Skimmer voltage	65 V
Octopole RF	750 V
Accurate mass spectral range	50–1,000 *m/z*
Fragmentor voltage	190 V
Auto MS/MS conditions	
Quadrupole isolation width	1.3 *m/z*
Collision energies	15, 30 eV
MS mass range	100–1,000 *m/z*
MS acquisition rate	4 spectra/second
MS/MS mass range	40–700 *m/z*
MS/MS acquisition rate	4 spectra/second
Threshold	25,000 counts
Relative threshold	0.01 %
Active exclusion	Enabled, excluded after 1 second, released after 0.2 minutes
Static exclusion	118–123 *m/z* 700–1,000 *m/z*
Model	Common organic molecules, only singly charged precursors, sort precursors by abundance, scan speed varied on precursor abundance, with a target of 50,000 counts/spectrum
Purity	100 % with a purity cutoff of 30 %

Figure 3 shows a good example of conservative transport (that is, nonremoval), which includes both a pharmaceutical and a sweetener. They are gabapentin and sucralose. The concentration of these two compounds in the South Platte River were either identical or decreased only by 50 % when moving through alluvial groundwater, even at distances of up to 500 m from the river.

Sucralose was 50 % of the concentration found in the South Platte River, while gabapentin reached the same concentration as in the river. These two compounds can then be compared to the other pharmaceuticals found by LC/Q-TOF-MS analysis. For example, Figure 4 shows the removal of atenolol and diphenhydramine relative to the South Platte River. There is 90 % removal of atenolol, a blood pressure regulator, and 99 % removal of diphenhydramine, an over-the-counter antihistamine medication. Both compounds are thought to be adsorbed to the sediments of the alluvial aquifer.

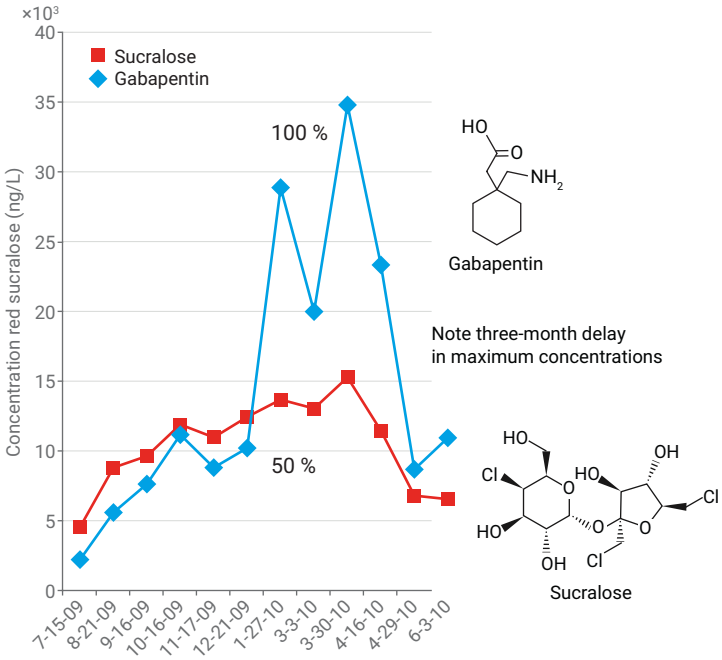

Figure 3. Conservative tracers of alluvial groundwater flow with a pharmaceutical, gabapentin, and a sweetener, sucralose.

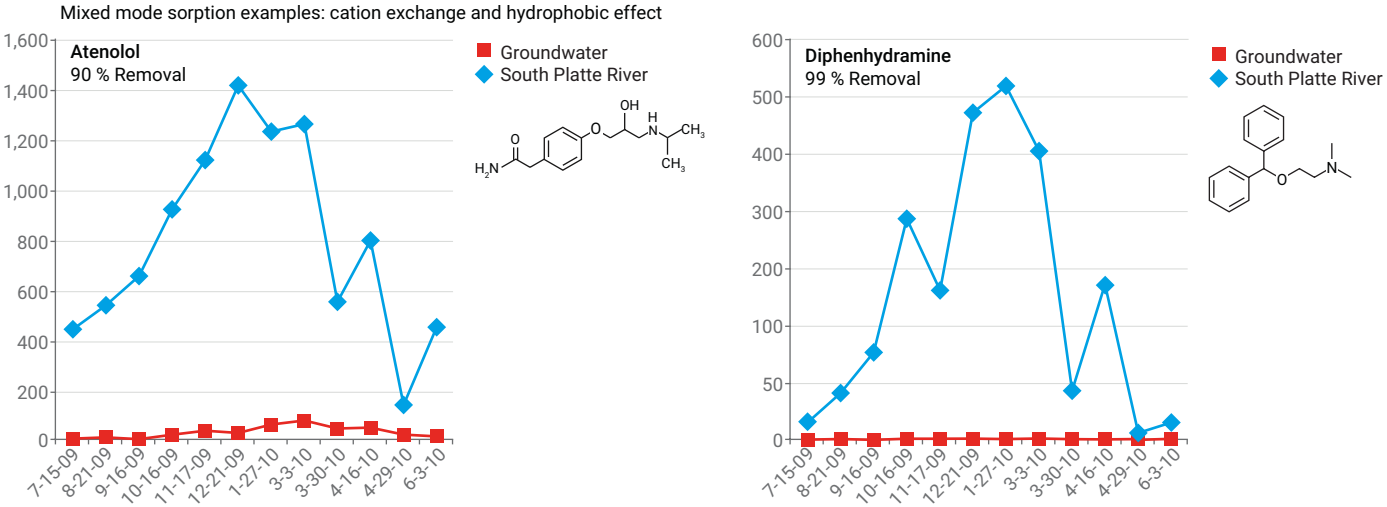

Figure 4. Removal of atenolol and diphenhydramine in alluvial groundwater relative to the South Platte River.

Although it is not clear why there is removal, it appears both sorption caused by the hydrophobic effect and by cation exchange could be important mechanisms in the removal of these pharmaceuticals as they transport through groundwater. Figure 5 shows another example of this removal process of pharmaceuticals from alluvial groundwater for venlafaxine and its metabolite, desmethylvenlafaxine.

In this example, venlafaxine and its major metabolite are 60 % removed during transport to alluvial wells close to the South Platte River. The amine group present may be partially protonated at the pH of groundwater (~7.5). Thus, cation exchange and the hydrophobic effect are in play to remove this antidepressant and its metabolite from groundwater. These two compounds are also important in that they are sources for N-nitrosodimethylamine (NDMA) during water treatment when using chloramines for water purification[12]. Apparently, the dimethylamine group reacts with chloramine to form NDMA[12].

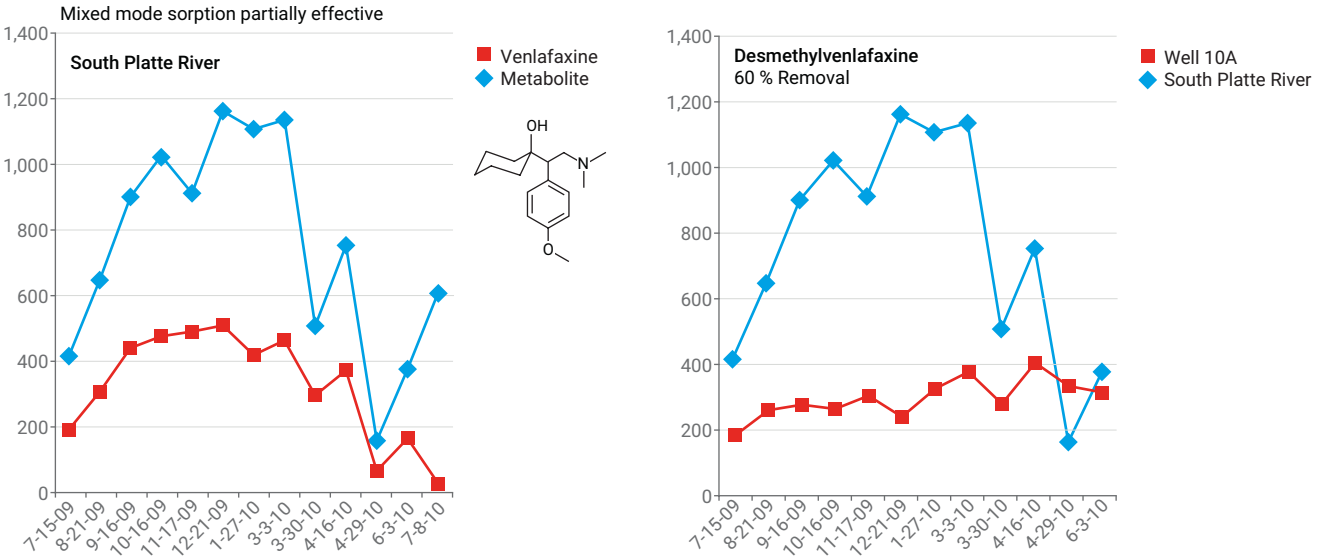

Figure 5. Venlafaxine and desmethylvenlafaxine in alluvial groundwater within 50 m of the South Platte River.

Figure 6 shows three pharmaceuticals that were nearly always found in alluvial groundwater 100–500 m from the South Platte River. The compounds include carbamazepine, lamotrigine, and sulfamethoxazole. They are two antidepressants and one antibiotic, respectively. The compounds may vary radically in concentration over time, with lamotrigine always being of the highest concentration, a trend noted in the literature for wastewater[10]. These three compounds are definitely important for future studies of groundwater and pharmaceuticals. It is also important to realize that concentration levels may vary quickly because of the river water source, which is affected by wastewater output from the Denver area, in this case, and by the seasonal uses of many pharmaceuticals.

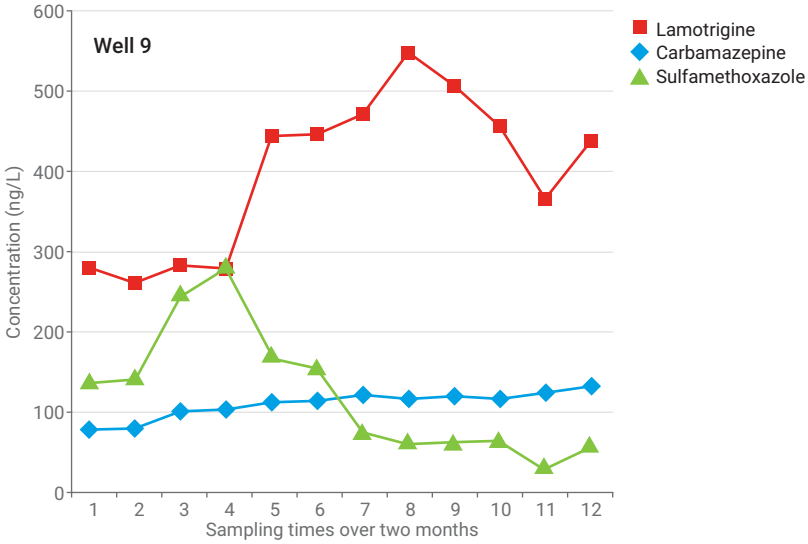

Figure 6. The concentration of three pharmaceuticals in groundwater over a two-month period.

Finally, one of the important uses of alluvial groundwater is as a drinking water source, especially along rivers in the arid western United States. In fact, the South Platte River is a good example since it flows from Denver to near Kansas City, MO before joining the Missouri River. Along this 600 mile stretch, alluvial groundwater is a drinking water source for many communities in Nebraska and eastern Colorado. Thus, the results reported here have important impact for drinking water standards, and shows how important the analysis of suspect and unknown compounds are for water quality in the arid west where water re-use is important. Water re-use in this sense means that wastewater may be purified by infiltration to groundwater and later consumed as a drinking-water source.

Conclusions

The identification of unknowns requires a complex set of tools, which are described in this Application Note and are shown in Table 1. There are four major categories of tools: hardware, software, ion-chemistry, and physical and biochemical tools. The reference set included in this Application Note and shown in Figure 2 are a guide for examples of how to apply these various tools. Both hardware and software tools continue to evolve with improved resolving power and mass accuracy. The use of auto MS/MS is useful since the evolution of accurate mass databases. The application of ion chemistry tools and physical and biochemical tools is an area of research on accurate mass that develops around the goal of the user. The type of problem to be solved, for example, a pharmaceutical class such as opiates, will dictate the ion chemistry and biochemical pathways. These tools are enhanced by software, but are driven by the user's application. Finally, the study of unknowns will continue to develop as more applications and problems are addressed by LC/Q-TOF analysis.

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Environmental Profiling of River Water Using Q-TOF LC/MS and Mass Profiler Software

Application Note

Environmental

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Abstract

The demand for the detection and identification of emerging contaminants in the environment requires a new approach to environmental screening that employs accurate mass LC/MS analysis and rigorous data analysis with a statistical software package. This method uses an Agilent 6550 iFunnel Q-TOF LC/MS with Agilent MassHunter Profiler software to investigate river water samples taken upstream and downstream from a water treatment plant. The presence of contaminants in the downstream versus the upstream sample indicates that contamination is taking place. These contaminants were extracted from the downstream water sample data and submitted to an accurate mass database and library for identification. Ultimately, 890 compounds were detected in the downstream sample, and 21 were successfully identified.

Introduction

Emerging contaminants in the environment have been of increasing concern to environmental scientists and the public in many parts of the world. Pesticides, herbicides, pharmaceuticals, corrosion inhibitors, and flame retardants are contaminants that pose a threat to the quality of surface, groundwater, and drinking water. They also adversely affect aquatic ecosystems and public health[1,2]. However, less than 0.1 % of oxidative stress on cells from water samples could be linked to the effects of known contaminants[3]. This clearly demonstrates the demand for further screening approaches.

The detection and quantitation of known contaminants in the environment using mass spectrometry is well established and understood, but these techniques fail to detect unexpected contaminants. As a result, new tools and techniques have been developed for a truly untargeted approach using high resolution mass spectrometry and chemometric (statistical) analysis.

Agilent Technologies

The full approach uses an Agilent 6550 iFunnel Q-TOF LC/MS system, Agilent Mass Profiler software, and Agilent MassHunter Qualitative analysis software to analyze, extract, and identify unanticipated contaminants in river water samples.

Experimental

Water samples were collected from the Ammer River, Germany, in locations upstream and downstream from a wastewater treatment plant. Enrichment was performed using solid phase extraction (HLB and ENBV+) at a preconcentration factor of 500.

Separation was carried out using an Agilent 1260 Infinity HPLC system consisting of an Agilent 1260 Infinity Binary Pump (G4220A), an Agilent 1260 Infinity High Performance Autosampler (G4226A), and an Agilent 1260 Infinity Thermostatted Column compartment (G1316C).

An Agilent 6550 iFunnel Q-TOF was operated with MassHunter Acquisition rev. B.05.01 in 2 GHz extended dynamic range mode with an acquisition rate of 1 scan/sec in MS mode and 3 scans/sec in MS/MS mode with mass-dependent collision energy setting for a targeted MS/MS inclusion list.

Experimental parameters		
UHPLC column	Phenomenex Synergi Polar-RP, 150 × 3 mm, 4 µm at 35 °C	
Mobile phase	A) water + 0.1% formic acid B) methanol + 0.1% formic acid	
Gradient program	Min	% B
	0	5
	1	5
	10	95
	17	95
Stop time	17 min	
Flow rate	0.3 mL/min	

Results and Discussion

The data were analyzed in MassHunter Qualitative Analysis rev. B.06.00 using the Molecular Feature Extractor (MFE) algorithm. MFE links MS data derived from a particular molecule of interest, and compiles the result into a chromatogram. Once the molecules (features) in a particular sample are extracted, the data are submitted to the Mass Profiler (MP) for statistical analysis.

In the water sample taken downstream from the water treatment plant and extracted in triplicate, 1,564 features were detected. Most of these were unique to the downstream sample (1,043 features), revealing the considerable impact of wastewater treatment plant effluents on surface water quality. Data reduction included alignment of retention time and mass data, blank subtraction, and the occurrence in all three replicates. The remaining 1,043 features attributed to wastewater effluents were further examined for potential identification. The accurate mass for the features of interest were used by Molecular Formula Generator (MFG) software to calculate and propose molecular formulae and were assessed for accuracy using a score derived from the isotopic pattern, isotopic spacing, and the difference between the theoretical exact mass of the assigned formula and the acquired accurate mass for the feature. For example, *m/z* 264.1967 was given a score of 97.1 for $C_{16}H_{25}NO_2$ based on combined scores of 94.5 for the mass difference, 99.7 for the isotopic pattern, and 99.4 for the isotopic spacing. A total of 894 sum formulae could be assigned to the measured accurate masses using the elements C, H, N, O, S, P, and Cl at a mass accuracy of 5 ppm.

The preliminary identification of the significant features in the downstream water samples by searching the sum formulae in databases yielded 18 hits with a homemade database of more than 450 contaminants relevant for the aquatic environment, 144 hits with an Agilent Forensic Toxicology Personal Compound Database, and 26 with an Agilent Pesticide Personal Compound Database. Relevant compounds were selected for

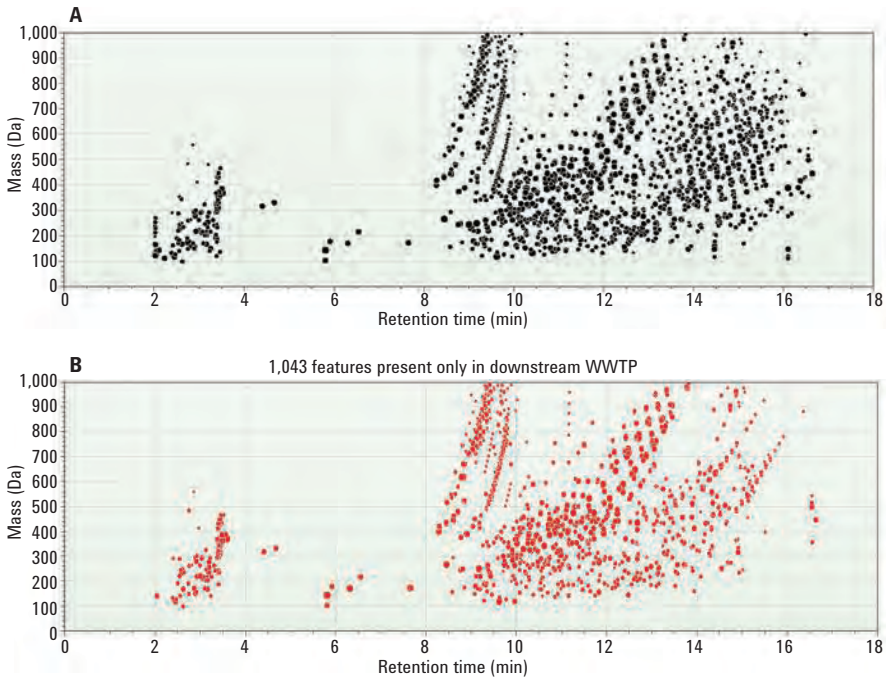

Figure 1. Features found by MFE in river water samples downstream of a WWTP. A) all features, B) features unique to the input of a WWTP.

further investigation and identification. These compounds were compiled into a compound list, where precursor ions were automatically transferred to a list for MS/MS analysis (MS/MS inclusion list) in a further Q-TOF LC/MS measurement.

The resulting accurate MS/MS data were then submitted to an Agilent Forensic Toxicology Personal Compound Database and Library for identification. Figure 2 shows an example of an identification of carbamazepine, an anticonvulsive and mood-stabilizing drug, by perfect matching of the acquired and library mass spectra. This result reveals the importance of the availability of comprehensive mass spectral libraries with accurate mass fragmentation information[4].

For compounds not found in the accurate mass databases or libraries, a search of the sum formulae in general chemical databases such as ChemSpider and PubChem was performed. These searches resulted in several hundreds of hits for possible structures. To narrow down and rank the positive hits for one sum formula, *in silico* fragmentation was performed using Agilent Molecular Structure Correlator (MSC) software. The input data are the molecular formula and the measured accurate mass MS/MS data for the unknown compound. The MSC software then submitted the sum formula to a ChemSpider search and ranked the positive hits based on matching calculated with measured mass fragments. The most probable chemical structures were selected from the high ranked hits of MSC, as the example of iopamidol reveals (Figure 3). The ultimate unequivocal identification should be validated by an authentic standard.

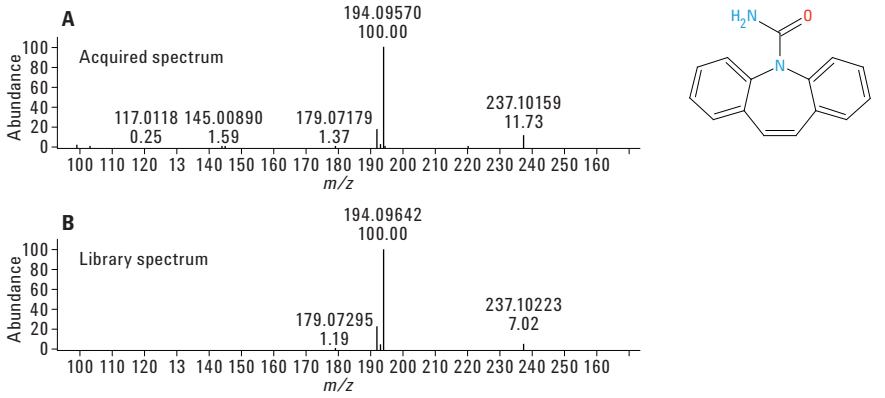

Figure 2. Structure, acquired spectrum (A) and library spectrum (B) for carbamazepine, a drug detected in the downstream water sample.

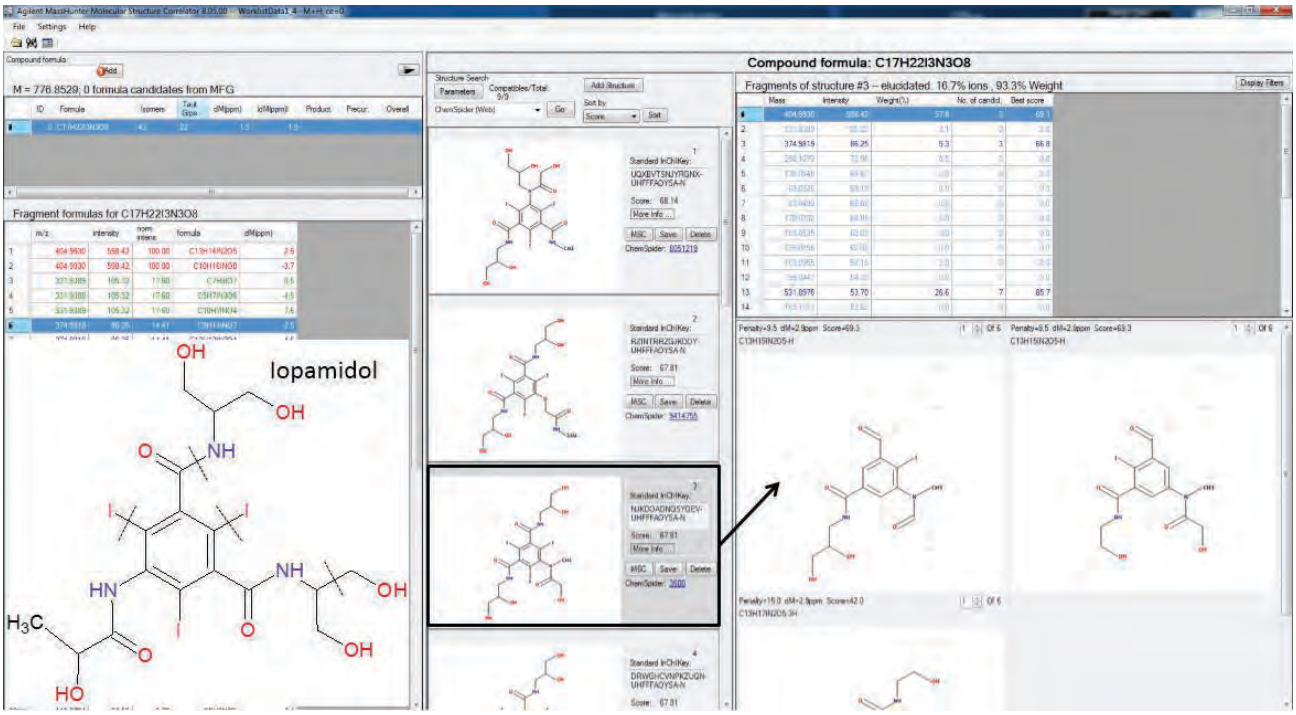

Figure 3. Identification of iopamidol using accurate mass data for precursor and products ions, Agilent MassHunter Molecular Structure Correlator (MSC) software, and ChemSpider.

Conclusions

River water samples taken upstream and downstream from a water treatment plant were analyzed using an Agilent 6550 iFunnel Q-TOF LC/MS and a suite of Agilent MassHunter statistical and qualitative analysis software packages. The result was an untargeted screening method that detected more than 890 unknown compounds. Compounds of interest were submitted for identification using accurate mass databases resulting in 150 suggested contaminants. MS/MS analysis with library matching identified 21 contaminants, and 32 additional contaminants were confirmed with authentic standards. For the compounds which were not present in the database or library, *in silico* fragmentation was used, along with a ChemSpider search. These results will be used to further expand compounds present in the accurate mass database and library for future studies.

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Agilent Technologies

Screening for Emerging Chemical Contaminants in Water Using LC/Q-TOF and Mass Profiler Professional Software

Application Note

Environmental

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USA

Abstract

The Agilent 6540 Q-TOF LC/MS and Agilent Mass Profiler Professional Software (MPP) were used to sensitively characterize the complex chemical composition of municipal wastewater. The processing of Q-TOF high-resolution MS data using the MPP multivariate statistical analysis package revealed changes in the occurrence patterns of organic chemicals during water treatment. Using this technique, we were able to determine which organic contaminants were attenuated, resilient, and formed during ozonation of recycled water. Heat maps created in MPP provide patterns that can be used to assess subtle changes in water quality and to identify emerging contaminants unique to a particular water or treatment process.

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Introduction

A plethora of reports have shown trace levels of unregulated contaminants (aka emerging contaminants) in water supplies [1] and in drinking water [2]. Those chemicals detected represent a very small portion of the approximately 7,500 chemicals listed in the US Environmental Protection Agency (EPA) Contaminant Candidate List universe (http://water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfm) and an even smaller portion of the nearly 85 million chemicals assigned CAS numbers (https://www.cas.org/). Numerous chemicals exist in water that have not yet been identified, and largely have an unknown impact on environmental and public health.

Specifically, regional water resources are facing unprecedented stress due to water shortages resulting from rapid population growth and relocation [3]. Many metropolitan areas are seeking additional resources to augment drinking water supplies [3]. The most likely new resource is water reuse, involving recycling wastewater into drinking water. Considering the daunting number of chemicals identified and potentially present in municipal wastewater, monitoring programs struggle to select those indicator compounds that are most representative of treatment efficacy. Perhaps more difficult is the nearly unlimited number of transformation products that may form when organic contaminants are subjected to oxidative or biological treatment processes. In some cases, byproducts of treatment are more toxic than the initial contaminants [4]. A survey method is needed that can monitor all of these processes to ensure selection of the most efficient and cost-effective treatments for wastewater reuse.

Accurate-mass quadrupole time-of-flight (Q-TOF) spectrometry is an excellent platform for detecting and resolving trace levels of thousands of different organic compounds in water, making it ideal as a monitoring tool for wastewater treatment. This application note describes a method that has been developed to profile the organic contaminants in wastewater before and after ozonation, a technology commonly used for disinfection and organic contaminant attenuation. The method developed employs ultra high performance liquid chromatography (UHPLC) and accurate-mass spectrometry, using an Agilent 1290 Infinity LC System and an Agilent 6540 Q-TOF LC/MS to separate and detect thousands of organic compounds in water. Clusters of compounds with similar fate during a given treatment process were elucidated, and some of these compounds could be used as indicators of the efficiency of the oxidation process. Finally, should a contaminant become of particular interest in the future, the Q-TOF data will constitute an archive that could be mined again, providing historical data without the necessity of storing or reanalyzing any sample.

Experimental

Reagents and standards

All solvents used were of highest purity available and suitable for LC/MS analysis. Methyl tertiary butyl ether (MTBE), methanol, and HPLC grade water used for solid phase extraction were procured from Fisher Scientific. Acetonitrile and HPLC grade water used for chromatography were obtained from Burdick and Jackson, while the additive formic acid was procured from Sigma-Aldrich.

Instruments

This study was conducted using an Agilent 1290 Infinity LC System coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS system. The instrument conditions are shown in Table 1.

Table 1. LC and Q-TOF MS Conditions

LC run conditions			
Column	Agilent ZORBAX Eclipse Plus 50 × 2.1 mm, 1.8 µm (p/n 959757-902)		
Column temperature	35 °C		
Injection volume	3 µL (three replicate injections of each sample)		
Mobile phase	A) 0.1% formic acid in water v/v B) 0.1% formic acid in acetonitrile v/v		
Linear gradient	**Time (min)**	**%A**	**%B**
	0	95%	5%
	1.5	95%	5%
	10	0%	100%
	13	0%	100%
Flow rate	0.4 mL/min		
Q-TOF MS conditions			
Ion mode	ESI positive, MS only		
Nebulizer gas	40 psi		
Capillary voltage	4,000 V		
Acquisition mode	2 GHz		
Acquisition range	25–3,200 *m/z*		
Acquisition speed	2.5 spectra/sec		
Resolving power	20,000 at *m/z* = 400		
Accuracy	< 2 ppm		

Sample preparation

Samples were collected from a wastewater treatment plant located in Arizona, where some of the secondary wastewater effluent was partially diverted through an ozonation pilot. Samples were collected after treating the secondary wastewater with 0, 1.5, 3, 4.5, or 5.6 mg/L of ozone.

Each sample was then filtered through a glass fiber filter (Whatman GF/F). Solid phase extraction was performed using cartridges preconditioned with 5 mL of MTBE, 5 mL of methanol and 5 mL of reagent grade water. The water sample (375 mL) was loaded onto the cartridge, which was then rinsed with 5 mL of reagent grade water and dried for 30 minutes under a nitrogen stream. The cartridge was then sequentially eluted with 5 mL of methanol and 5 mL of methanol/MTBE (10/90). The eluate was evaporated down to 0.5 mL under a nitrogen stream.

Data analysis

The data were processed using Molecular Feature Extractor (an algorithm detecting compounds based on isotopic ion clusters) in the Agilent MassHunter software suite, followed by compound alignment using the Agilent Mass Profiler Professional (MPP) multivariate statistical analysis package. To discard artifacts, the aligned features were then subjected to recursive analysis using the Find Compound by Formula tool in MassHunter, followed by a second round of compound alignment in MPP. Statistical analysis to identify profile differences between sample types was also performed using MPP.

Results and Discussion

Feature extraction and compound alignment

While total ions chromatograms (TICs) of triplicate injections overlap, and some differences can be observed corresponding to the different levels of ozone treatment, they are too complex for direct interpretation (Figure 1). Feature extraction is necessary to extract compounds from each chromatogram and establish a detailed profile of each sample. However, between two injections, a compound might have an insignificant shift in retention time that would cause MassHunter to identify it as two different entities. To correct for this, after features were extracted in MassHunter they were subjected to compound alignment using MPP. This process identified a total of 24,779 compounds. Initial filtering by occurrence (blank subtraction) identified 23,574 compounds detected only in wastewater samples. Of these, 13,996 compounds were detected at least twice (either in different replicates or in different samples), and were used for recursive analysis to further eliminate any false positives.

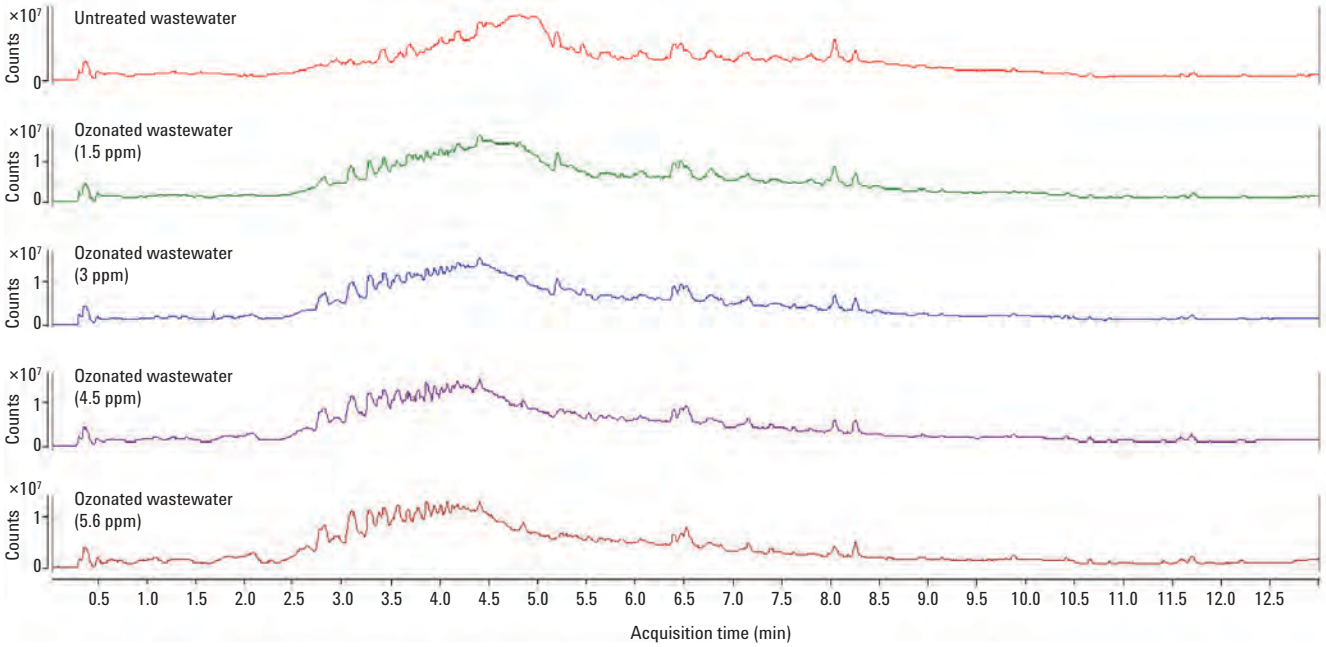

Figure 1. Total ions chromatograms (TICs) for untreated wastewater and wastewater treated with various doses of ozone.

Recursive analysis

The 13,996 compounds that passed the initial filtering were exported in a CEF file and imported into MassHunter Qualitative Analysis Software. The chromatograms were examined again to search particularly for these compounds using the Find Compound by Formula tool, and then the resulting compounds were imported back into MPP for a second round of compound alignment and filtering. A total of 12,889 compounds passed this recursive analysis. Of these, only those compounds that did not appear in blanks (extracted HPLC grade water) and were detected in 100% of the triplicate analyses for at least one of the ozone dose levels were selected for statistical analysis, leaving a total of 9,493 compounds.

Principal component analysis

Principal Component Analysis (PCA) was performed on the data using MPP to determine if the samples could be distinguished based on the ozone dose that they received. Figure 2 shows that every ozone level could be distinguished from the others, and the triplicate samples taken at each level cluster very closely, indicating excellent reproducibility.

Hierarchical clustering analysis

To further characterize and clearly observe the impact of ozonation on compounds present in wastewater, Hierarchical Clustering Analysis (HCA) was performed on the samples, revealing several groups of compounds that differed in their response to ozonation (Figure 3). Two groups, (A and B) were removed by ozone, while three others (C, D, and E) were formed by ozone treatment. Several compounds were actually resistant to ozone treatment (F). Additional detail can be observed in the HCA. For example, differences in the level of ozone required to remove compounds can be seen in Group B, revealing three subgroups (B1, B2, and B3, Figure 4).

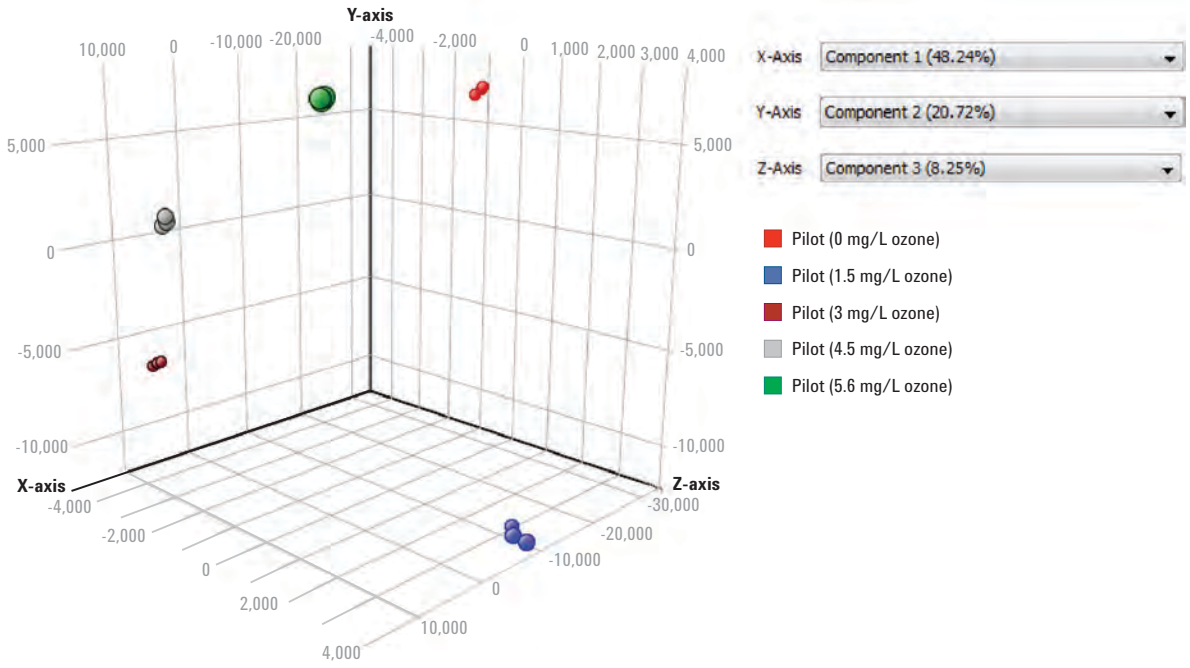

Figure 2. PCA reveals that samples treated with different doses of ozone can be clearly differentiated. The triplicates in each sample group also cluster very closely together.

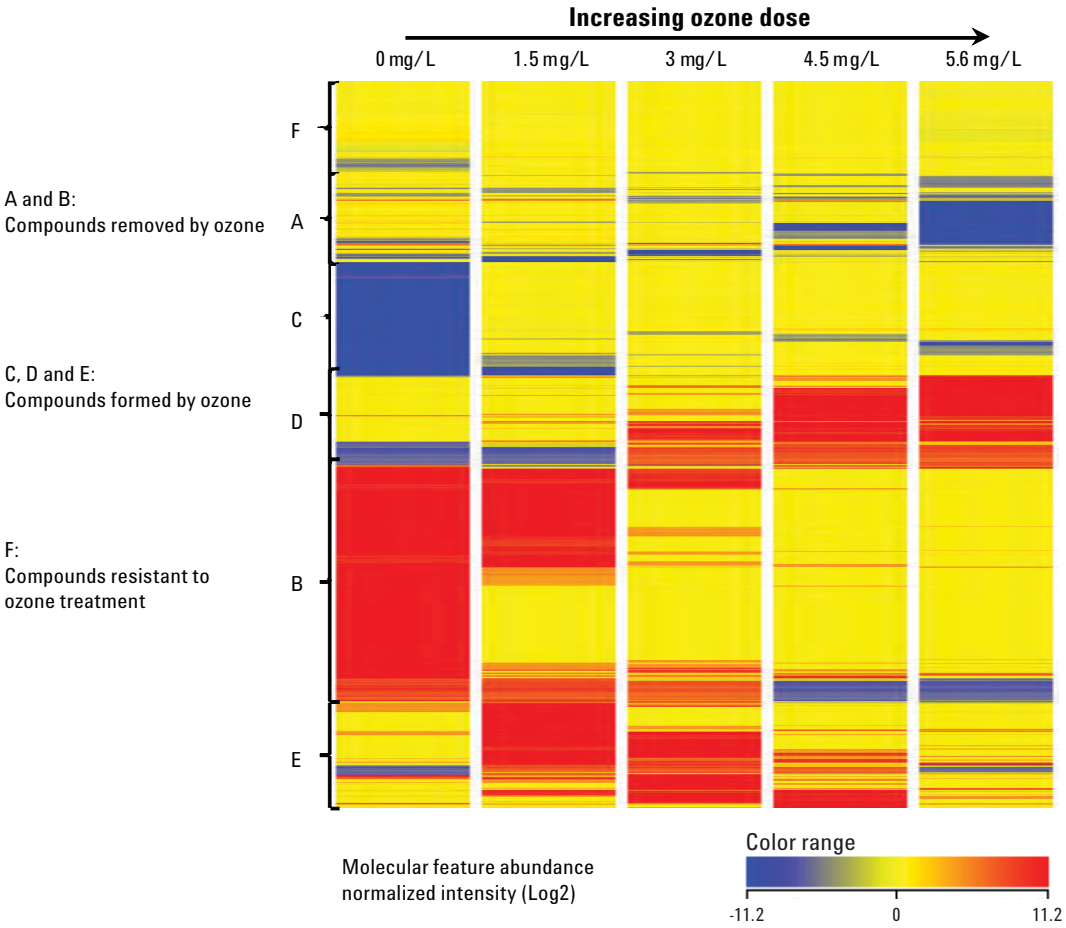

Figure 3. HCA, revealing groups of compounds that respond differently to ozone treatment.

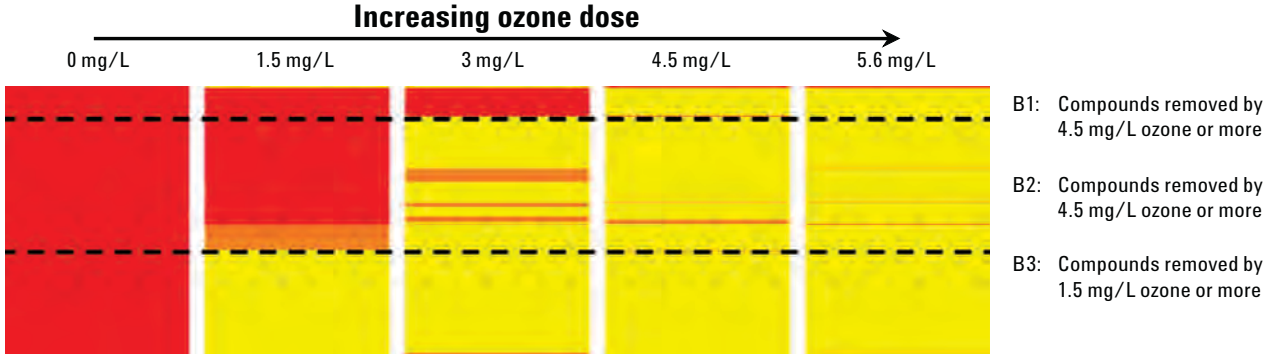

Figure 4. Expanded view of Group B from Figure 3, showing the three subgroups (B1, B2, and B3) that differ in the level of ozone required to remove them from the wastewater.

Analysis of variance

To further understand the impact of various ozone treatments on wastewater, an analysis of variance (ANOVA) was performed in combination with Tukey's test to identify those compounds whose abundance varied significantly between the various ozone doses applied. The ANOVA identified 8,244 compounds out of 9,493 that had a significant variation of abundances (p value <0.05) dependent on the ozone dose. Tukey's test provided a table comparing each sample treated by a different ozone dose, indicating the number of compounds that did or did not significantly vary in abundance (Table 2).

Table 2. Number of Compounds That Vary in Abundance with Ozone Dose (ANOVA with p value < 0.05)

		Ozone dose (ppm)				
		0	**1.5**	**3.0**	**4.5**	**5.6**
Ozone dose (ppm)	**0**	8244	4548	5524	6083	6150
	1.5	3736	8244	3083	4299	4843
	3.0	2720	5161	8244	2476	3395
	4.5	2161	3945	5768	8244	2226
	5.6	2094	3401	4849	6018	8244

Compounds that vary in abundance

Compounds that do not vary in abundance

Comparison of one sample group to itself

Identification of compounds

It is not feasible to monitor all compounds in water to assess their removal. Therefore, an alternative is identifying one compound in each cluster (B1, B2...) that could be used as an indicator to assess the fate of the other chemicals with similar behavior. This can be done using ID Browser in MPP and a database of suitable compounds. For example, when randomly selecting a compound in the subgroup B3, the Find Similar Entities tool in MPP identified 953 compounds that had a similar fate during ozonation with a correlation coefficient $0.95 < R^2 < 1$. This list was exported to ID Browser, which searched the Agilent METLIN Personal Compound Database and Library (PCDL) for matches. One of the compounds was identified as warfarin, the most widely prescribed anticoagulant drug in North America (Figure 5). Therefore, it could be used as an indicator for the fate of the 952 other compounds that are also easily removed by ozone (subgroup B3).

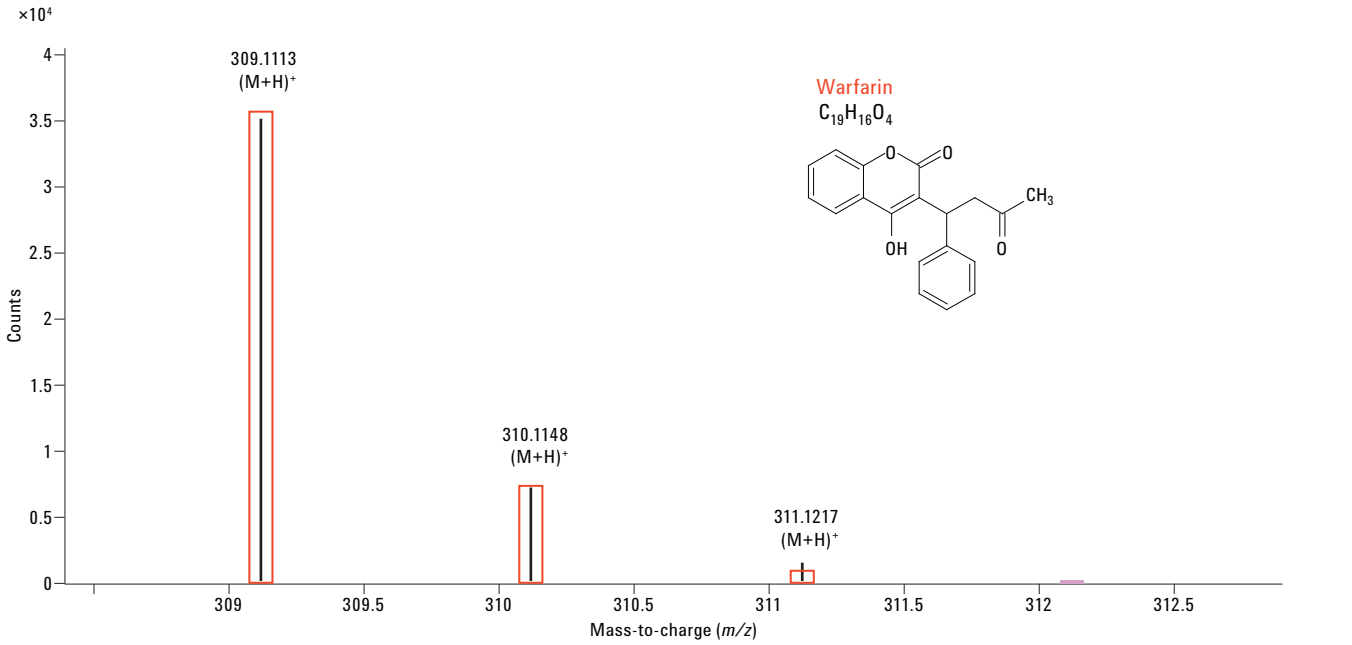

Figure 5. One of the compounds in subgroup B3 identified as warfarin using MPP ID Browser and the Agilent METLIN PCDL database. The identification score was 97 (of a maximum of 100), and the red rectangles represent the theoretical ion cluster for warfarin.

Conclusions

Wastewater contains a very large number of compounds, most of which are not monitored in routine analysis. Using untargeted LC/Q-TOF analysis on the Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS system and statistical analysis with Agilent Mass Profiler Professional software can differentiate groups of compounds with different vulnerability to ozone. Some compounds are resilient to ozonation, others are more or less easily attenuated depending on the ozone dose and, finally, some are formed by ozonation. Therefore, the method presented in this application note can be used as a sensitive monitoring tool for the changes in water quality that occur during water treatment.

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Screening and Identification of Emerging Contaminants in Wastewater Treatment Plant Effluents Using UHPLC/Q-TOF MS and an Accurate Mass Database and Library

Application Note

Environmental, Emerging contaminants, Water, Accurate mass screening, pesticides, PPCPs

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Abstract

This application note describes the creation of an accurate mass library for relevant environmental contaminants and its application for the screening of pesticides, pharmaceuticals and personal care products, their metabolites, and transformation products in effluents of municipal waste water treatment plants (WWTPs). An Agilent 1290 Infinity LC System coupled to an Agilent 6550 iFunnel Q-TOF LC/MS System was operated in positive and negative electrospray mode using Dual Spray Agilent Jet Stream Technology. Accurate mass spectra were acquired for a large collection of potential environmental contaminants in either one or both ionization modes, and for all relevant ion species.

Four WWTPs in central Europe were sampled over 3.5 months, and samples were analyzed using a Target Screening approach including reference standards for 390 contaminants. In addition, an extended suspect screening was done using all compounds included in the Agilent MassHunter Water Screening Personal Compound Database and Library (PCDL) as the suspect list. Samples were measured using All Ions MS/MS as well as auto MS/MS acquisition with an inclusion list. The results clearly show the value of both acquisition modes combined with an efficient data analysis workflow, as well as the inherent value of the Water Screening PCDL for the surveillance screening of potential contaminants in complex environmental samples.

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Introduction

Environmental regulations throughout the world currently focus on monitoring a limited number of well-known compounds that are assumed to be responsible for significant ecological and human health related risks [1]. As a consequence, there is a tendency to further reduce the maximum allowable concentrations of the environmental quality standards (EQS) for those priority pollutants. In Europe, for example, EU directive 2013/39/EU specifies EQS for 41 priority substances or substance groups amongst which are pesticides, perfluorocarbons (PFCs), nonyl- and octylphenol, polybrominated flame retardants, dioxins and PCBs, polyaromatic hydrocarbons (PAHs), and heavy metals [2]. However, these priority pollutants represent only a small fraction of the anthropogenic chemicals that are used, and consequently found in the environment. Apart from the legal requirement for surveillance screening for potential contaminants in surface waters in the EU, there is a growing interest to collect occurrence data for contaminants of emerging concern. This is reflected in a number of country-specific government regulations, such as the EPA method 1694, on the analysis of pharmaceuticals and personal care products in water, soil, and sediments [3], and in the US EPA Contaminant Candidate List (CCL) fourth edition from 2015 [4].

Most anthropogenic contaminants enter the aquatic environment as a result of incomplete removal in wastewater treatment plants (WWTPs). At the same time, degradation and transformation products are formed during biological wastewater treatment. These products are typically not monitored, and often are not even known [5,6]. Surface waters downstream of WWTPs contain innumerable anthropogenic contaminants and their transformation products at trace levels. When river water is subsequently used as the source for drinking water production, referred to as unplanned indirect potable reuse, comprehensive data on the chemical quality of the water are essential. This becomes even more important as planned indirect and direct potable reuse becomes a common practice [7] due to climate change, population growth, and water scarcity.

Targeted analytical methods are increasingly complemented by untargeted acquisition methods using high resolution accurate mass Q-TOF LC/MS due to comprehensive screening requirements in current environmental regulations as well as increasing interest in the occurrence of contaminants of emerging concern. Agilent Q-TOF LC/MS instruments allow for full spectrum acquisition with high sensitivity and at a very high data rate. This is essential to obtain information on molecular ions, isotope patterns, and fragments with a single large volume direct injection of water samples. For most of the LC/MS amenable compounds specified in the EU Water Framework Directive, the EU Drinking Water Directive, or the EPA Clean Water Act, method detection limits in the low ng/L range can be achieved with this type of Q-TOF LC/MS method [8].

Three different approaches towards substance identification are used in the environmental context in combination with accurate mass LC/MS: Target, Suspect, and Non-target Screening.

In Target screening, a reference standard is measured with the same analytical method and within the same worklist as the unknown sample, so that retention time, accurate mass, and fragment information can be directly compared, and (semi-)quantitative results are obtained.

Suspect screening describes a workflow where compound database and MS/MS library information of expected contaminants are used to screen for the suspected substances. While no reference standard is available, identification of compounds with increasing confidence is obtained by comparing accurate mass and isotope patterns, retention times, and coeluting fragments, or by a spectral MS/MS library comparison.

Non-target screening aims at the identification of all remaining components detected in a sample where no structural information is available. Often, non-target screening includes the statistical comparison of two or more samples or sample groups for data reduction. A full identification considers the accurate mass, isotope pattern, and MS/MS spectrum information, and includes spectral library comparison for known compounds, and spectral similarities and in-silico fragmentation tools such as Agilent MassHunter Molecular Structure Correlator (MSC) for unknowns.

Compound databases containing accurate mass MS/MS spectral data for all expected contaminants, as well as those theoretically predicted, and their transformation products have been proven to assist in the identification of potentially relevant compounds and support efficient data analysis in all three screening workflows. The Agilent MassHunter Water Screening Personal Compound Database and Library (PCDL) contains a relevant list of more than 1,400 environmental contaminants including pesticides, pharmaceuticals, personal care products, large volume industrial chemicals, and their transformation products. This PCDL includes all compounds currently regulated in the US, EU, and China, and further compounds that have either been previously detected in the environment or are likely to be detected due to their production amount or widespread use.

Searchable fields containing compound information including compound class and regulation tags make it easy to create subsets of the Water Screening PCDL for target and suspect screening. The Water Screening PCDL contains more than 1,000 compounds with accurate mass MS/MS spectra, which directly supports the Agilent All Ions MS/MS workflow for Target and Suspect screening, and greatly increases confidence in the identification of potential contaminants [9].

This application note describes the screening and (semi-)quantification of contaminants in WWTP effluents using an Agilent 1290 Infinity UHPLC coupled to the highly sensitive Agilent 6550 iFunnel Q-TOF LC/MS system. Four WWTPs located in agricultural and rural areas in Switzerland were sampled over 3.5 months, covering the main pesticide application period from March to late June. Comprehensive screening of potential water contaminants revealed the chemical characteristics of the different treatment and different catchment areas. A subset of the Water Screening PCDL, containing roughly 390 compounds, was used for Target screening and (semi-)quantification. For Suspect screening, the entire comprehensive Water Screening PCDL was used to find and identify compounds with high confidence. Examples of the two different screening strategies used are shown. First, the Q-TOF was operated in the All Ions MS/MS mode with three collision energies. The All Ions technique features an easy setup of the acquisition method with verification of the potential contaminants using the MS/MS spectral library to produce chromatographic coelution of the precursor and product ions. An additional approach was used for compounds for which either no library spectrum was available, or which were not verified due to a low coelution score. Their precursor masses were added to an inclusion list for an Auto MS/MS method using a second injection. The obtained spectra were compared to the MS/MS library for compound identification. This workflow can also be combined with a discovery run operated in TOF mode to obtain the target list of suspects.

Experimental

Reagents and standards

All reagents and solvents were HPLC or LC/MS grade. Acetonitrile, methanol, and acetic acid were purchased from Fluka (Sigma-Aldrich, Buchs, Switzerland). Ammonium acetate was purchased from VWR International (Darmstadt, Germany). Ultrapure water was produced using a Milli-Q Integral system equipped with a 0.22-µm point-of-use membrane filter cartridge (EMD Millipore, Billerica, MA, USA). For acquisition of accurate mass MS/MS spectra, most individual reference compounds were purchased from Sigma-Aldrich (Buchs, Switzerland) or from VWR International (Darmstadt, Germany). Reference standards for transformation products and residues were purchased from Ehrenstorfer (LGC Standards, Wesel, Germany). Estimation of retention times was performed using mixed standard solutions of pesticides, pharmaceuticals, and drugs of abuse, which were part of the LC/MS Pesticide Comprehensive Test Mix (p/n 5190-0551), the LC/MS Forensic Toxicology Comprehensive Test Mix (p/n 5190-0555), or were provided by research groups.

Stock standard solutions were prepared by dissolving the reference compounds in isopropanol, acetonitrile, methanol, water, or mixtures thereof, depending on the physicochemical properties of the substance. For calibration and spiking experiments, the comprehensive test mixtures for pesticides, pharmaceuticals, and drugs of abuse were combined to a multi-analyte working solution. Stock standard solutions as well as the multi-analyte working solution were stored until use at –20 °C. Calibration samples were prepared by dilution of the working solution with tap water.

Sample preparation

Effluents of four different wastewater treatment plants in central Europe were collected as 14-day composite samples over 3.5 months (March to June). The catchment areas of three of the WWTPs are agriculturally dominated (AG, AI, AL), whereas the other one is located in an urban area (AZ). One of the WWTPs also receives some industrial wastewater (AL). Efficient nitrification-denitrification is observed in three of the WWTPs (AG, AL, AZ) and, thus, a better elimination of trace contaminants can be expected.

Samples were filtered using glass fiber filters, and were stored at –20 °C. Immediately before measurement, samples were thawed, and an aliquot was transferred to an HPLC vial.

LC/Q-TOF MS analyses

Separation was carried out using an Agilent 1290 Infinity UHPLC system consisting of:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity High Performance Autosampler (G4226A) equipped with a large volume injection kit (G4216-68711)
- Agilent 1290 Infinity Thermostatted Column compartment (G1316C)

The UHPLC system was coupled to an Agilent G6550A iFunnel Quadrupole Time-of-Flight LC/MS System equipped with a Dual Spray Agilent Jet Stream electrospray ionization source. The Q-TOF LC/MS instrument was operated with:

- Agilent MassHunter Acquisition rev. B.06.01 in 2 GHz extended dynamic range mode with positive or negative electrospray ionization (ESI) with two different methods:
    - All Ions MS/MS acquisition with 3 scans/sec with two discrete collision energies
    - Auto MS/MS acquisition with a data rate of 5 scans/sec in MS and 5 scans/sec in MS/MS

The use of two collision energies in the All Ions acquisition resulted in alternating spectra with a low energy channel containing the precursor ion, and two high energy channels containing the precursor and product ions.

Reference mass ions were delivered using an Agilent Infinity 1260 Isocratic pump (G1310B) operated with a flow rate of 1.0 mL/min, and using a 1-in-100 flow splitter (G1607-60000). The final flow rate going to the reference sprayer was 10 µL/min.

Table 1 shows the chromatographic conditions, and Table 2 shows the major MS conditions.

Data were evaluated using MassHunter Qualitative Analysis software B.07.00. Positive identifications of water contaminants were reported if the compound was detected in the accurate mass MS data by the Find-by-Formula data mining algorithm with a mass error below 5 ppm, and with a sufficient score (including isotope abundance and isotope spacing). A retention time window of ±1 minute was specified for peak detection to compensate for retention time shifts due to matrix variability.

Table 1. Chromatographic Conditions

Parameter	Value	
UHPLC column	Agilent ZORBAX RRHD SB-Aq, 2.1 × 150 mm, 1.8 µm (p/n 859700-914)	
Column temperature	40 °C	
Mobile phase	A) 1 mM NH_4 acetate + 0.1% acetic acid B) 0.1% acetic acid in acetonitrile	
Gradient program	Time (min)	% B
	0.0	0
	2.0	0
	14.0	98
	16.0	98
	19.0	0
	19.5	0
Stop time	20.0 minutes	
Post time	3.0 minutes	
Flow rate	0.40 mL/min	
Injection volume	100 µL	

Table 2. Major MS Conditions

Parameter	Value	
Gas temperature	160 °C	
Gas flow	16 L/min	
Nebulizer	30 psig	
Sheath gas temperature	350 °C	
Sheath gas flow	12 L/min	
	Positive	**Negative**
Capillary voltage	4,500 V	3,500 V
Nozzle voltage	500 V	1,000 V
Reference mass correction	121.05087 922.00980	112.98559 1,033.98811
All ions MS/MS		
Mass range	50 to 1,200 amu	
Scan rate	3 spectra/s	
Collision energies	0–20–40 V	
Auto MS/MS		
MS Mass range	100 to 1,200 amu	
MS/MS Mass range	50 to 1,200 amu	
Scan rate	5 spectra/s (MS) 5 spectra/s (MS/MS)	
Collision energy	20 V	

Creation of the Agilent MassHunter Water Screening PCDL

Accurate mass spectra of single-analyte solutions were acquired with flow injection or using a short column in targeted MS/MS mode with collision energies of 10, 20, and 40 V. If precursor ion stability required higher collision energies, additional spectra were acquired in a second run. Typically, MS/MS spectra were acquired for the $[M+H]^+$ and $[M-H]^-$ ion species for each analyte. If highly abundant additional adduct ion species were observed, accurate mass MS/MS spectra were also acquired for the $[M+NH_4]^+$, $[M+Na]^+$, or $[M+Cl]^-$ species. In either positive or negative ionization mode, meaningful MS/MS spectra were acquired for more than 1,000 relevant water contaminants. For many compounds, MS/MS library spectra were captured in both ionization modes, and for more than one precursor ion species. To eliminate mass assignment errors, fragment masses in the acquired spectra were compared to the theoretical fragment formulas, and all ion peaks were corrected to their theoretical masses. All MS/MS spectra were curated for spectral noise, and a minimum base peak threshold was applied to ensure good ion statistics for all fragment ions. The corrected spectra were included in the Agilent Water-Screening Personal Compound Database and Library (G6882CA).

The Water Screening PCDL was then used for the screening and identification of environmental contaminants in the effluents of four WWTPs. Moreover, by analyzing mixed standard solutions of pesticides, pharmaceuticals, and drugs of abuse with the given UHPLC method, retention time information was added to 390 compounds, thus adding in retention time to the identification score and increasing identification confidence.

Figure 1 shows a screen capture of the MassHunter PCDL Manager software along with the accurate mass MS/MS spectrum of the antiviral drug aciclovir acquired in negative ionization mode with a collision energy of 20 V.

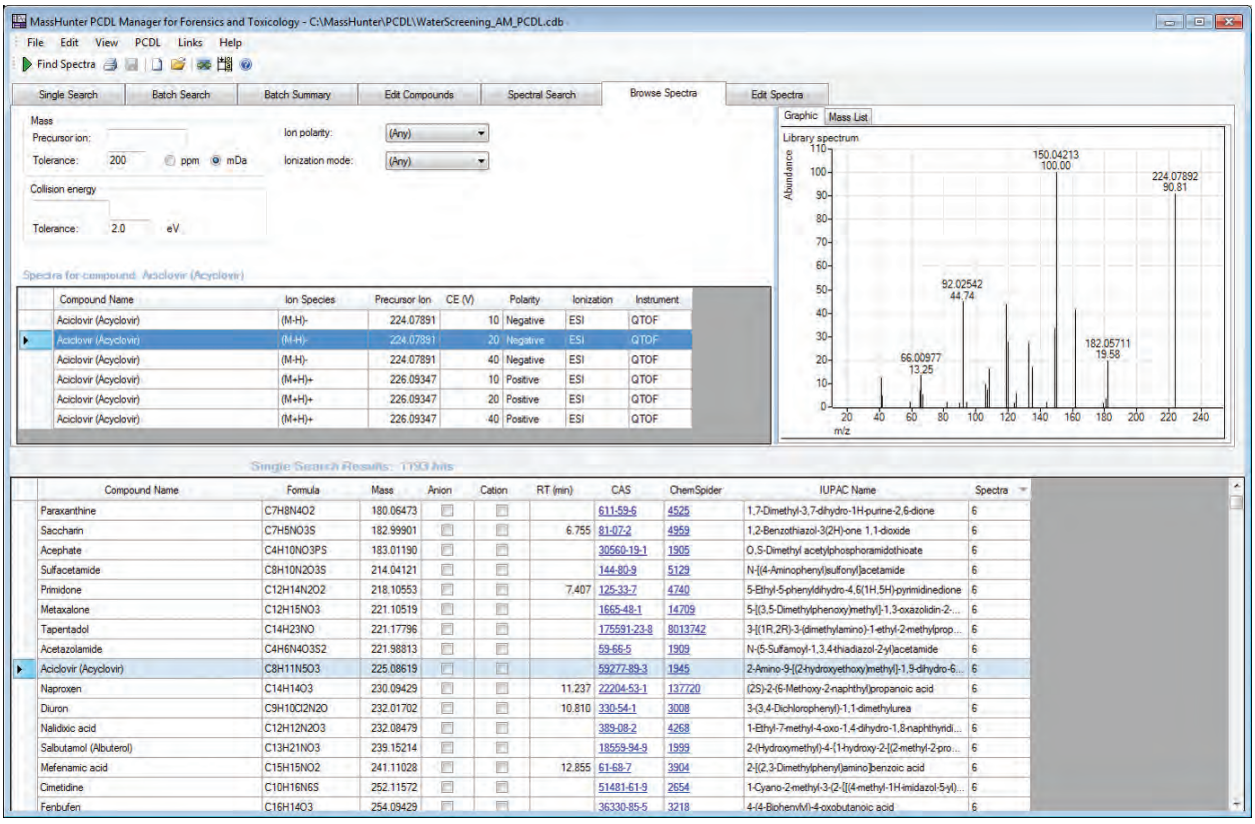


Figure 1. Agilent MassHunter PCDL Manager software showing the Agilent MassHunter Water Screening PCDL and the accurate mass MS/MS spectrum of aciclovir acquired in negative ionization mode with a collision energy of 20 V.

Results and Discussion

Target screening for pesticides, pharmaceuticals, and personal care products

For the target screening and (semi-)quantification of pesticides, pharmaceuticals, and personal care products in the WWTP effluents, samples were measured in positive and negative All Ions MS/MS mode with collision energies of 0, 20, and 40 V. A subset PCDL with 390 entries was created from the Water Screening PCDL including all compounds for which reference standards were available. Data for the calibration samples were initially evaluated in the MassHunter Qualitative Analysis Software (B.07.00) using the Find-by-Formula data mining algorithm with a mass error of ± 5 ppm and a retention time window of ± 0.5 minutes compared to the expected retention time. In positive mode, $[M+H]^+$ and $[M+NH_4]^+$ species, and in negative mode, $[M-H]^-$ species have been considered as charge carriers since they are represented by the majority of accurate mass MS/MS spectra included in the PCDL. Identification was done by Fragment Confirmation using the Water Screening PCDL as the fragment ion source, and evaluating the five most specific ions from the MS/MS spectral library. In the All Ions MS/MS software workflow, fragment ions that show perfect coelution with the molecular ions were identified and the ideal collision energies and relative ratios were detected. This information was passed to the MassHunter Quantitative Analysis software for (semi-)quantification and batch review using accurate mass for molecular ions and fragments as well as isotope pattern matching for compound identification with high confidence.

From the 390 targeted compounds, 315 were detected in positive mode, and 75 were detected in negative mode. With a direct injection of 100 µL of water into the UHPLC Q-TOF MS system, more than 60% of the compounds could be quantified at or below 10 ng/L in the spiked tap water samples. Another 35% of the compounds were detected between 10 and 100 ng/L, and just 5% of the compounds were only detected at concentrations of 200 ng/L or higher. For most targeted compounds, one or more specific fragment ions could be used as qualifier ions, and generally mass accuracy of the molecular ions and fragments was better than 5 ppm. Figure 2 shows the extracted ion chromatograms, MS peak spectra and calibration curves for some examples from the lists of priority pollutants (EU) and US EPA method 1694.

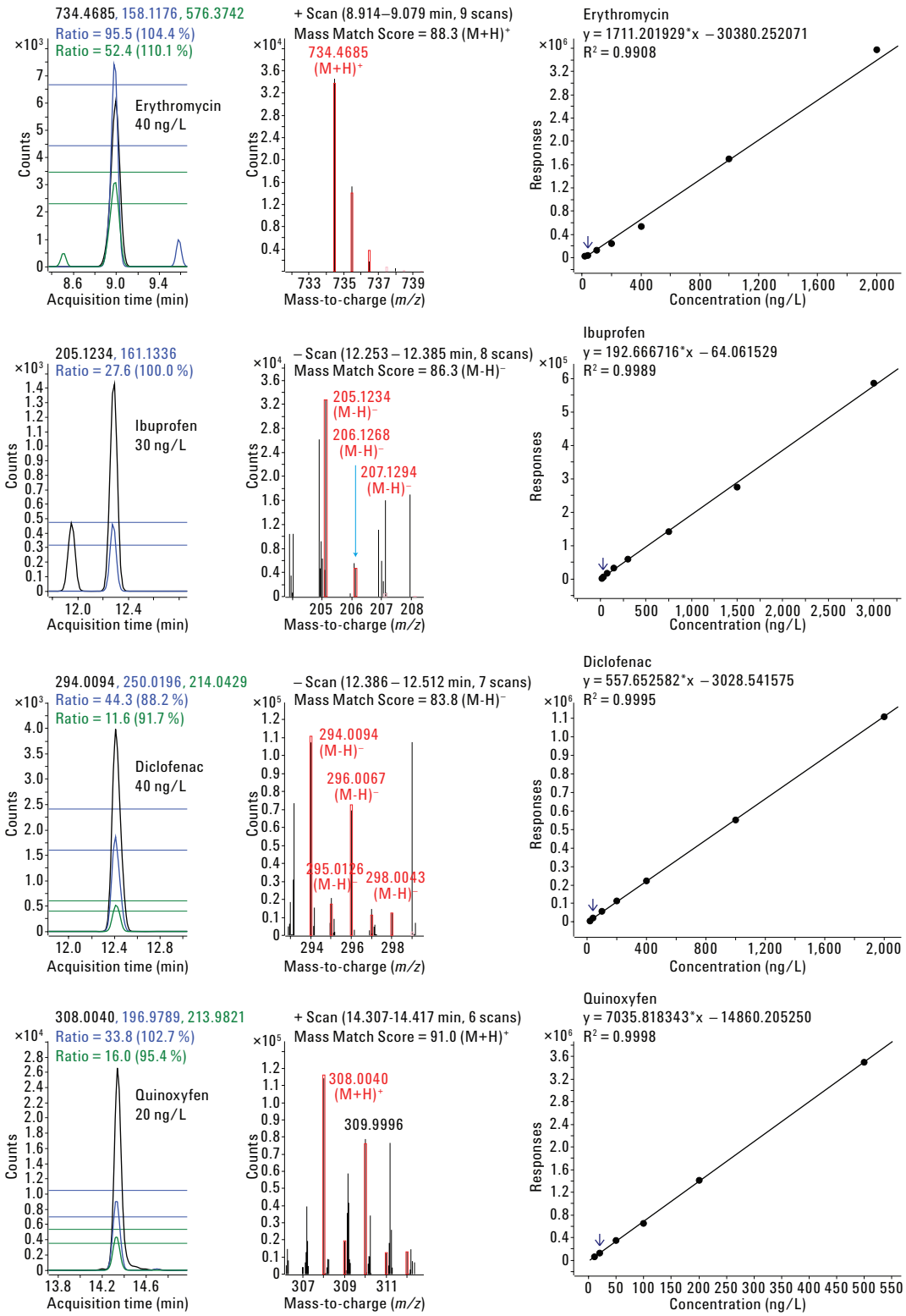


Figure 2. EIC chromatograms of molecular ion and fragments, MS peak spectra and calibration curves for erythromycin (positive mode), ibuprofen (negative mode), diclofenac (negative mode), and quinoxyfen (positive mode).

Applying this target screening method to the samples from the WWTP effluents allowed the (semi-)quantification of a large variety of environmental contaminants in a concentration range of a few ng/L up to several µg/L. Pharmaceutical residues were found to dominate the effluents of the larger WWTPs (AG, AL, AZ), which all receive wastewater from about 50,000 population equivalents, while AI receives wastewater from 7,100 population equivalents. In all wastewater treatment plant effluents, X-ray contrast media were identified with estimated concentrations of up to 7 µg/L for iomeprol, and up to 2 µg/L for iopromide. Other common pharmaceuticals were amisulprid (up to 500 ng/L), atenolol (up to 1.7 µg/L), metoprolol (up to 470 ng/L), and tramadol (up to 2 µg/L) as well as carbamazepine, diclofenac, ibuprofen, naproxen, and sulfamethoxazole. Metabolites were identified for carbamazepine, sulfamethoxazole, and metamizole. In total, 33 pharmaceuticals and metabolites of pharmaceuticals were identified in the WWTP effluents. As expected, the largest number of these compounds were found in the effluent of AZ, which has an urban catchment area. However, concentrations were highest in effluents of AI, probably due to the limited elimination efficiency of the simple treatment.

In the effluents of all WWTPs, a total of 46 pesticides were detected. The insecticide diethyltoluamide (DEET) was found in all WWTPs with a concentration of 14 to 770 ng/L. Also present in samples of all WWTPs were the herbicides metolachlor (up to 1.1 µg/L) and isoproturon (up to 450 ng/L). The largest number of pesticides were found in the effluent of AI, which has an agriculturally-dominated catchment area. Major crops grown there include cereals, vegetables, corn, beetroot, and potatoes. The pesticides found most often, and with the highest concentrations in the effluent of this WWTP were azoxystrobin, flufenacet, linuron, metamitron, methomyl, metribuzin, propamocarb, spiroxamin, and terbuthylazine. All of these pesticides are mainly used for the above listed crops.

Figure 3 shows the normalized chromatograms for the antiepileptic drug carbamazepine and the pesticide azoxystrobin in the effluent samples from WWTP AI, spanning a time period from March to end of June. While the concentration of the pharmaceutical is more or less constant over the whole sampling period, the concentration of the pesticide increased over the growing season. This example demonstrates the different contaminant profiles for compounds that are continuously introduced, compared to those that enter the water cycle within a specific period of time.

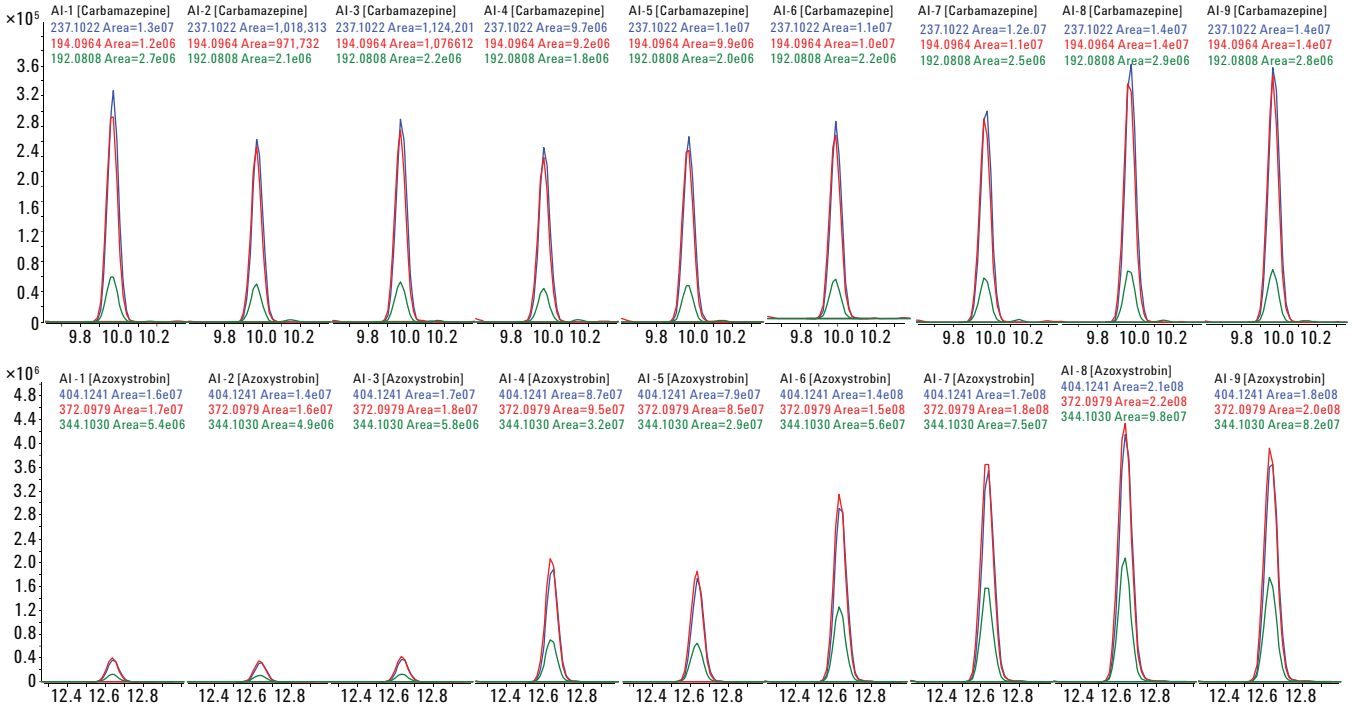


Figure 3. Normalized EIC chromatograms of molecular ions and fragments for the antiepileptic drug carbamazepine and the pesticide azoxystrobin over the course of the sampling period (March to end of June).

Extended Suspect screening using the comprehensive Agilent MassHunter Water Screening PCDL

Based on the results from the target screening, the samples from the WWTPs AI and AZ were selected for an extended Suspect screening looking for all remaining compounds included in the Water Screening PCDL. The availability of accurate mass MS/MS information is key for the identification of potential candidates, and is either used in the All Ions MS/MS workflow for the extraction and alignment of EICs of the molecular ion and characteristic fragments, or for the library matching of an acquired accurate mass MS/MS spectrum against the reference spectra in the PCDL. Both workflows are shown in Figure 4 for some of the examples identified in the WWTP effluents.

All Ions MS/MS screening workflow

In the All Ions MS/MS workflow, accurate mass data is collected without fragmentation in a low energy channel. Virtually at the same time, using two high energy channels, compounds are fragmented with two different collision energies without precursor selection in very fast sequential steps, and accurate mass fragment data are recorded for both channels. When the data is analyzed using the Find-by-Formula algorithm, the Water Screening PCDL provides the precursor formula information, and compound chromatograms are extracted for all specified ion species. For putative identifications, chromatograms are automatically extracted from the high energy channels for a specified number of the most abundant fragments from the MS/MS spectra stored in the PCDL. As an example, Figure 4A shows the accurate mass library spectrum of valsartan from the Water Screening PCDL, compared to the cleaned high energy spectrum from an effluent sample from the WWTP AI (Figure 4B). The red triangles indicate the fragment ions that have been selected from the library spectrum for evaluation.

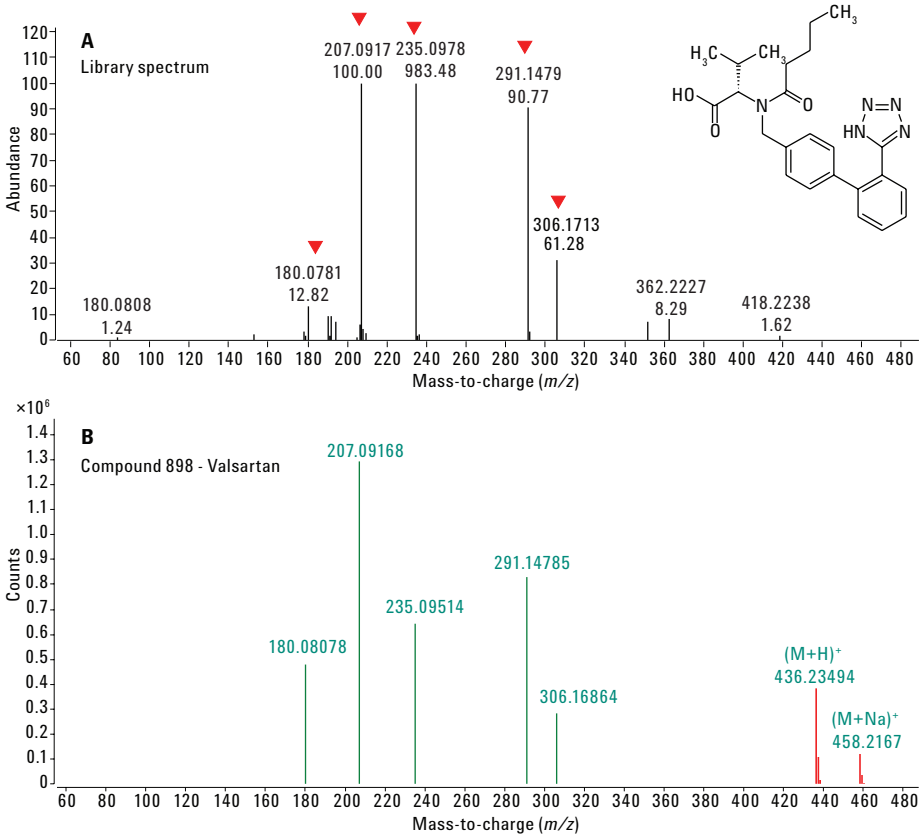
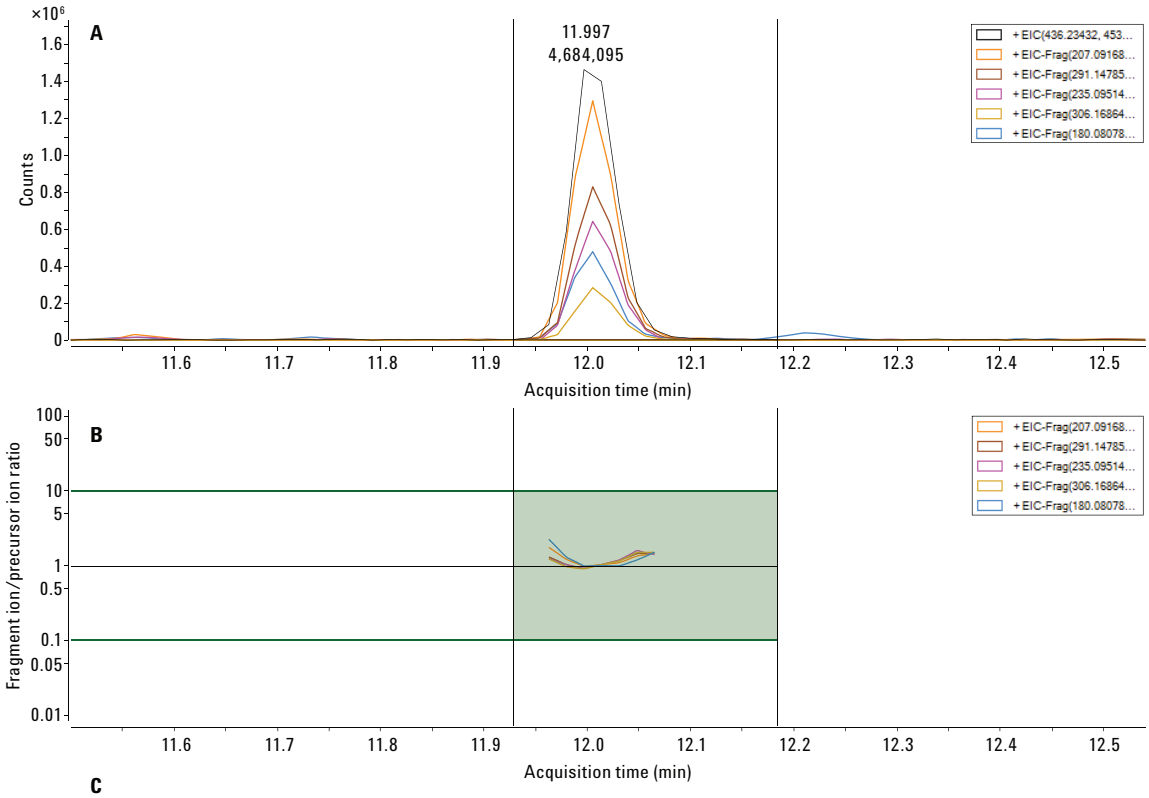


Figure 4. Accurate mass library spectrum for valsartan at a collision energy of 20 V (A) in comparison to the acquired high energy spectrum (B) from an effluent sample of the wastewater treatment plant AI (cleaned spectrum). The red triangles in the library spectrum (A) indicate automatically selected ions for the All Ions MS/MS evaluation.

While the library spectrum is based on a collision energy of 20 V, the cleaned high energy spectrum combines information from both high energy channels acquired with 20 and 40 V.

By overlaying chromatograms for both precursor and fragment ions, and the calculation of a coelution score, the identity of the angiotensin receptor blocker (ARB) valsartan was confirmed. The coelution score takes into account factors such as abundance, peak shape (symmetry), peak width, and retention time. The normalized intensity ratios are plotted and made available to the user for inspection in a coelution plot. Figure 5A shows the overlay of the molecular ion chromatogram with the fragment chromatograms from the high energy channels. All five fragment chromatograms showed coelution with the precursor ion. This is also demonstrated in the coelution plot in Figure 5B. The detailed compound identification results, including the coelution scores, are shown in the compound table in Figure 5C.

Identification with high confidence is achieved when the EICs of the molecular ion and at least one or two fragments show perfect coelution, which is expressed by a coelution score of > 90 (out of 100), and the mass accuracy for the peak spectrum for both molecular ions and fragments is better than 5 ppm. Based on this rule, additional pharmaceuticals were identified in the effluent of WWTP AI, namely the ARBs candesartan, irbesartan, and losartan.



Best	Name	Formula	Score	m/z	Mass	Mass (Tgt)	Diff (ppm)	Score (Tgt)	RT
◉	Valsartan	C24 H29 N5 O3	99.09	436.23494 453.25752 458.21671	435.22762	435.22704	-1.34	99.09	11.997

Coelution Score	CE	Flags(FIs)	FV	Height	m/z	Compound Name	RT	RT Diff	SNR
99.4	20	Qualified		1293470.5	207.09168	Valsartan	12.005	0.009	221.1
98.9	20	Qualified		829727.9	291.14785	Valsartan	12.005	0.009	131.4
98.5	20	Qualified		642690	235.09514	Valsartan	12.005	0.009	72.9
98.6	20	Qualified		283336.3	306.16864	Valsartan	12.005	0.009	183
99.1	40	Qualified		479184.1	180.08078	Valsartan	12.005	0.009	28.2

Figure 5. Overlay of precursor and fragment ion traces for valsartan in an effluent sample of the wastewater treatment plant AI (A), coelution plot (B) and compound identification results including the coelution score (C).

Other pharmaceuticals and personal care products that were not targeted in the previous workflow (target screening using a reference standard), including the compounds clarithromycin, fexofenadine, sitagliptin, celiprolol, and phenylbenzimidazole sulfonic acid were also identified. Moreover, nine further pesticides (napropamid, pyrimethanil, fenamidone, lenacil, dimethenamid, boscalid, dinoseb, fludioxonil, and penconazole) as well as perfluorooctanoic acid (PFOA) and several organophosphates (triethyl phosphate, tris(2-chloroethyl)phosphate, tributylphosphate, and triphenylphosphate) were also found. In the AZ samples, valsartan, candesartan, and irbesartan, as well as fexofenadine, clarithromycin, venlafaxine, and its metabolite desmethyl venlafaxine, citalopram, cetirizine, clopidogrel, and ritonavir were found. In addition to PFOA, perfluorononanoic acid was also found. No further pesticides were detected and confirmed in the effluent of the WWTP AZ.

Suspect screening and verification using targeted MS/MS

The verification of compounds by matching accurate mass MS/MS spectra against reference spectra from an accurate mass MS/MS library sometimes offers advantages over the All Ions MS/MS workflow. This is the case if contaminants are present only at trace concentrations and, thus, signal intensities of the fragment ions are low, or if interferences occur between the low mass fragment ions and ions from the matrix. Moreover, accurate mass MS/MS library comparison after precursor isolation is considered to be the gold standard for compound verification. In cases where there is no library spectrum available, for example, for newly identified compounds or suspected transformation products, accurate mass MS/MS spectra can be compared to theoretical fragmentation of a compound in the MassHunter Molecular Structure Correlator (MSC) software.

Typically, this workflow starts with an All Ions MS/MS acquisition discovery run, and the tentative identification of suspected compounds using the Find-by-Formula data mining algorithm with the fragment confirmation. In cases when no fragment ions can be identified or if only one unspecific fragment is observed, the compounds are selected from the MassHunter Qualitative Analysis software, and exported into a target list. In a consecutive run using the same chromatography, accurate mass MS/MS spectra for the targeted precursors are acquired. If utmost sensitivity is required, it might be beneficial to operate the Q-TOF in TOF mode for the discovery run. Using TOF mode results in a higher number of suspects and, therefore, more precursor masses for the consecutive targeted MS/MS or auto MS/MS run. However, in this instance, more of the low abundant contaminants will be detected. Data analysis for the targeted MS/MS or auto MS/MS run starts with data mining using the Find by MS/MS (target or auto) feature finding algorithm followed by library searching using the MassHunter Water Screening PCDL.

Figure 6 shows the compound chromatogram and MS1 level peak spectrum for the antidiabetic drug metformin found in the effluent of wastewater treatment plant AZ. Due to the low mass of the molecular ion and the even lower masses of the specific fragments, the compound could not be verified in the All Ions MS/MS workflow, but was confidently identified with the targeted MS/MS approach. The predominant ion species for metformin was $[M+H]^+$, and the measured *m/z* was in good agreement with the calculated mass (–0.7 ppm). The red boxes around the mass signals show the expected isotope ratio. The measured intensities for the monoisotopic mass signal, and the [M+1] isotope signal are in very good agreement with the theoretical pattern. However, there was an interference observed for the [M+2] signal, and therefore the target score was only 72.7 (out of 100).

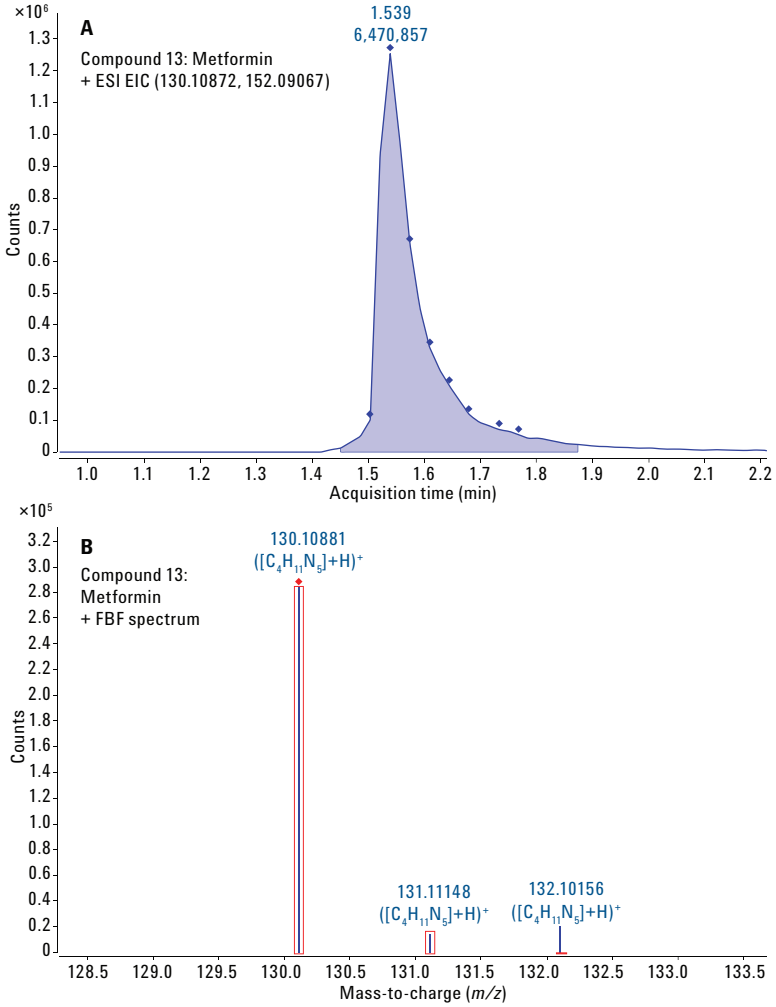


Figure 6. Compound chromatogram and cleaned peak spectrum obtained by the Find-by-Formula algorithm for the antidiabetic drug metformin found in the effluent of wastewater treatment plant AZ.

The red diamond in Figure 6 indicates that MS/MS spectra have been acquired for that particular *m/z*. MS/MS spectra were extracted automatically over the peak range, and were matched against the library spectra contained in the MassHunter Water Screening PCDL. Figure 7 shows the MS/MS spectrum for metformin acquired in the wastewater treatment plant effluent (7A) in comparison to the library spectrum from the PCDL (7C). A mirror representation of the difference spectrum is shown in panel 7B. All major fragment ions listed in the library spectrum of metformin were found in the measured spectrum within a narrow mass extraction window and in a similar ratio as in the reference spectrum for a collision energy of 20 V. Thus, the reverse search against the accurate mass library resulted in a score of 95.4 out of 100, and verified the presence of metformin in the sample. MS/MS scores were required to be above 60 for verification.

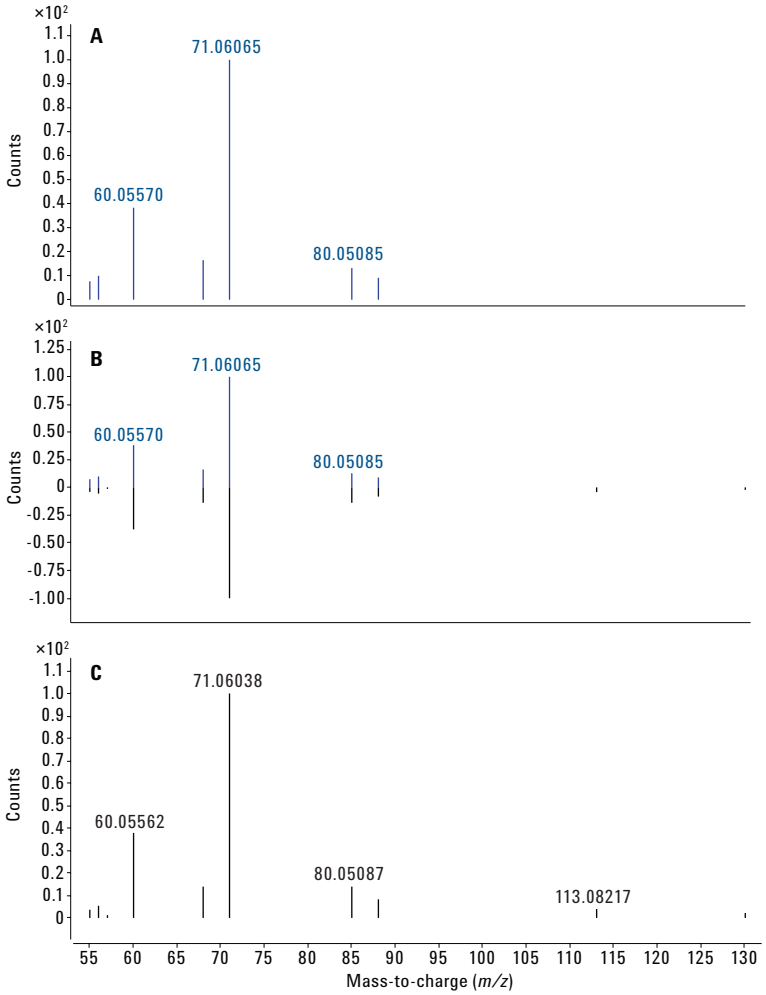


Figure 7. Comparison of the measured spectrum of metformin in the WWTP effluent sample (A) with the reference spectrum from the Agilent MassHunter Water Screening PCDL (C), and a mirror representation of the difference spectrum (B).

Figure 8 shows other compound examples that could be verified by using the targeted MS/MS workflow. Melamine was found in the effluent of AZ with a library score of 85.3. It has several industrial uses, and is formed as a metabolite of the pesticide cyromazine. Denatonium was found in the effluent of AI with a library score of 91.5. It is the most bitter compound known, and is used as bitterant in personal care products to prevent inappropriate ingestion.

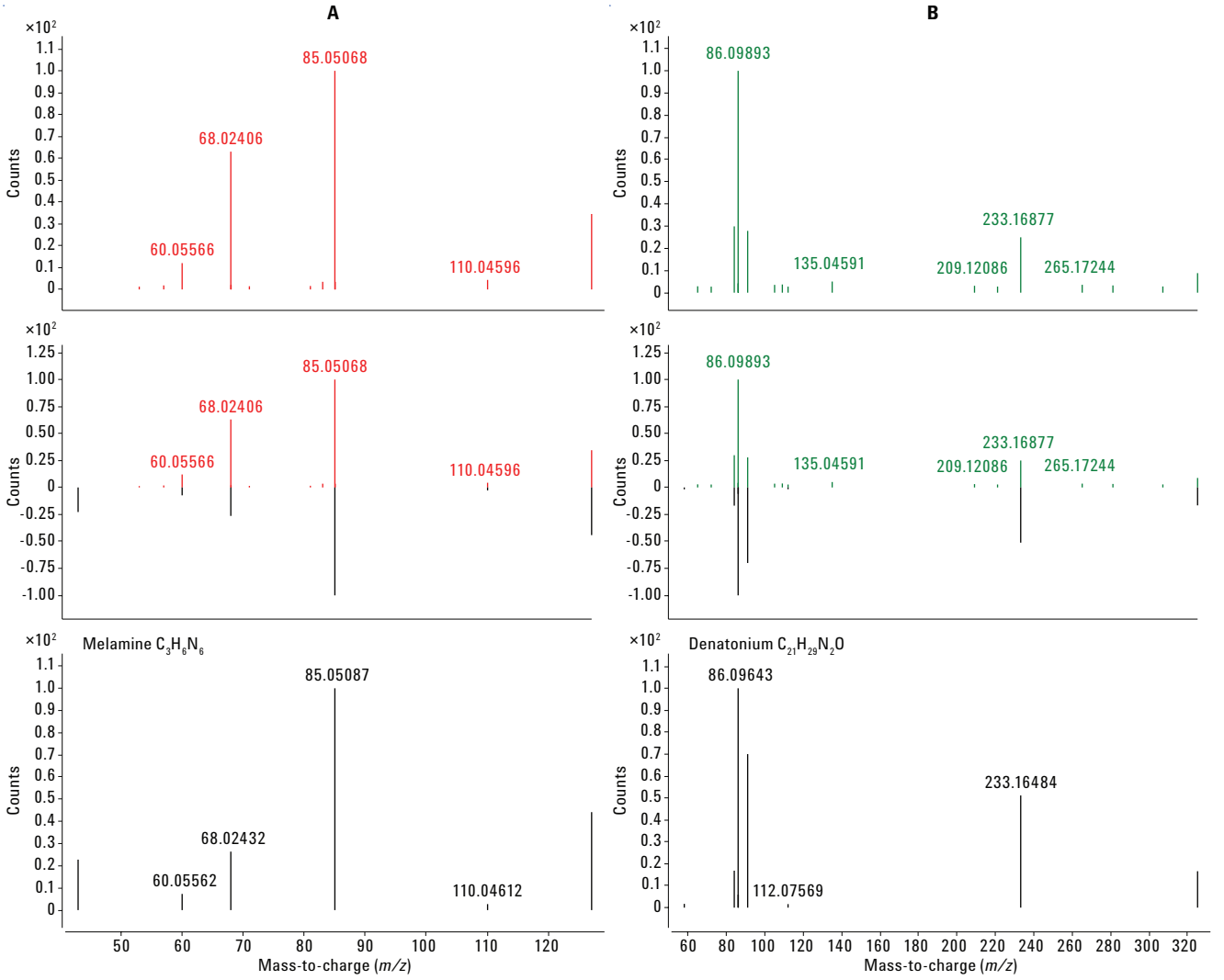


Figure 8. Comparison of the measured spectrum of melamine in the WWTP effluent sample AZ (A) and of denatonium in the effluent sample AI (B) with the reference MS/MS spectra from the Agilent MassHunter Water Screening PCDL.

Conclusions

A method for the screening and (semi-)quantification of environmental contaminants in water samples was developed and applied to WWTP effluents. This method takes full advantage of the low delay volumes of the Agilent 1290 Infinity LC, and its ability to handle high backpressures in UHPLC separations to increase the chromatographic resolution. The method benefits from the sensitivity of the Agilent 6550 iFunnel Q-TOF, and from the versatile ionization capabilities of the Agilent Jet Stream ionization source.

The Agilent MassHunter Water Screening PCDL is an ideal complement for the Target and suspect screening workflows in the Agilent MassHunter Qualitative and Quantitative software. In Target Screening and (semi-)quantification, the PCDL is used to define the suite of compounds and to identify selective qualifier ions. While in suspect screening, identification with high confidence is achieved by fragment confirmation or library searching. Applying both workflows to the WWTP effluent samples revealed the characteristics of the treatment technology as well as the catchment area by the different chemical inventory of trace contaminants.

Auto MS/MS acquisition with an inclusion list and a single collision energy combined with library matching resulted in similar verification rates as the Agilent All Ions MS/MS acquisition with fragment coelution. However, it can be seen that the precursor isolation in auto MS/MS acquisition improved the identification compared to the All Ions MS/MS workflow in heavy matrix and for low mass contaminants. Conversely, All Ions MS/MS acquisition is very fast, and allows the differentiation of closely eluting isomers. Another important feature of the All Ions MS/MS workflow is that the data can be re-interrogated at a later time for compounds that were not in the scope of the analysis during measurement, allowing for retrospective data analysis for new emerging contaminants without the need to reacquire data from old samples.

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Accurate Mass Analysis of Hydraulic Fracturing Waters: Identification of Polyethylene Glycol Surfactants by LC/Q-TOF-MS

Application Note

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Abstract

A combination of UHPLC followed by LC/Q-TOF-MS was used to detect suites of polyethylene glycol compounds (PEGs) that occur in flowback water samples from hydraulic fracturing. The Kendrick Mass Defect was applied to differentiate the various adducts within a suite of PEGs. A database of the accurate masses along with their retention times by UHPLC has been designed to enable rapid and accurate analysis of either groundwater or flowback samples from hydraulic fracturing. Forty PEGs and their various adducts and multiply-charged ions can be identified in less than 2 minutes of computer time.

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Introduction

Hydraulic fracturing (fracking) extracts oil and gas by forcing fluids into oil and gas rich shale deposits. The fracking fluids contain a mixture of proppants (sand), surfactants, biocides, inorganic salts, and other compounds intended to facilitate the release of the trapped gas. More than 7,000 wells have been drilled in Colorado alone, and reports of groundwater contamination have occurred most notably in Wyoming, New York, and Pennsylvania [1]. The first water that returns from the fracking process (flowback) has the potential to contaminate nearby aquifers or surface water. Care is taken to recycle the flowback water, or to dispose of it properly, although the Safe Drinking Water Act does not apply to hydraulic fracturing solutions [2]. Thus, there is a demand for tracer compounds that are indicative of the presence of contamination by hydraulic fracturing and do not occur in the subsurface [2].

In this application note, a combination of ultra high performance liquid chromatography (UHPLC) followed by quadrupole time-of-flight tandem mass spectrometry (Q-TOF-MS/MS) was used to detect a bimodal distribution of polyethylene glycols that may be used as indicator compounds for groundwater or surface water impacted by flowback waters. The Kendrick Mass Defect [2,3] was applied in a novel application to differentiate the various adducts of a suite of glycols, in particular, the proton, ammonium, and sodium adducts of each of the chains of glycols. The database of the accurate masses along with their retention times by UHPLC is needed to provide a rapid and accurate analysis of either groundwater or surface water samples using Agilent MassHunter Software.

Experimental

Instruments

Separation of the analytes was carried out using an Agilent 1290 Infinity LC System coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS system equipped with electrospray Jet Stream Technology. The instrument conditions are shown in Table 1.

Sample collection

The flowback sample was collected on October 14, 2014 and obtained from Weld County, Colorado, with the help of James Rosenblum, University of Colorado, Department of Environmental Sustainability.

Table 1. LC and Q-TOF MS Conditions and Analysis Parameters

LC run conditions	
Column	Agilent ZORBAX Eclipse XDB-C8, 4.6 × 150 mm, 3.5 µm
Column temperature	25 °C
Injection volume	10 µL
Mobile phase	A) 0.1% formic acid in water v/v B) Acetonitrile
Linear gradient	10% B for 5 minutes; 10% B to 100% B over 25 minutes
Flow rate	0.6 mL/min
Post run	12 minutes
Q-TOF MS conditions	
Ion mode	ESI, positive
Drying gas	10 L/min
Gas temperature	325 °C
Nebulizer gas	45 psig
Sheath gas	11 L/min at 350 °C
Capillary voltage	4,000 V
Nozzle voltage	1,000 V
Fragmentor voltage	190 V
Skimmer voltage	45 V
Octopole RF	750 V
Mass range	*m/z* 50–1,000
Detector rate	2 GHz
Resolving power	30,000 at *m/z* 1,522

Data analysis

The data were processed with Agilent MassHunter Software. Accurate mass measurements of each peak from the total ion chromatograms were obtained by means of an automated calibrant delivery system using a low flow of a calibrating solution (calibrant solution A, Agilent Technologies, Inc.) that contained the internal reference masses purine *m/z* 121.0509 and HP-921 at *m/z* 922.0098. The instrument provided a typical mass resolving power of 30,000 at *m/z* 1522.

Results and Discussion

UHPLC/Q-TOF-MS analysis of hydraulic fracturing waters

The positive ion electrospray total ion current (TIC) chromatogram for the analysis of the flowback water exhibits two distinct zones, or a bimodal distribution (Figure 1). First is a polar series of peaks at a retention time of 4–12 minutes, and second is a less-polar zone that elutes from 12–14 minutes. The series of peaks in both zones are separated by a nominal mass of 44 mass units, which suggest a glycol structure consisting of CH_2-CH_2-O (Table 2). Furthermore, the accurate mass data in Table 2 show that the differences for the first seven peaks are 44.0266, on the average. A similar result was also observed for the second region between 12–14 minutes in Figure 1 (data not shown). There is an apparent repeatable relationship among the chromatographic peaks, which makes it is possible to apply the Kendrick mass scale [3].

Kendrick mass scale

Kendrick used a filtering technique (scaling factor) named after him to better separate and understand a series of hydrocarbons that differed in mass by a methylene group, CH_2. The Kendrick mass scale has been applied to other series, but only recently to the polyethylene glycol structures and linear alkylethoxylates in flowback and produced waters from hydraulic fracturing by Thurman *et al*. in 2014 [2].

The application of the Kendrick mass scale first involves the determination of the Kendrick mass scaling factor, which is determined as the ratio of the nominal mass of the repeating glycol unit, (CH_2CH_2-O), divided by the exact calculated mass of the same glycol unit. This is equal to 44/44.026214748, which gives a scaling factor of 0.999404559. When this scaling factor is multiplied by the measured accurate mass, the resulting mass is called the Kendrick mass [2]. For example, Table 2 shows the Kendrick masses for some of the chromatographic peaks in Figure 1.

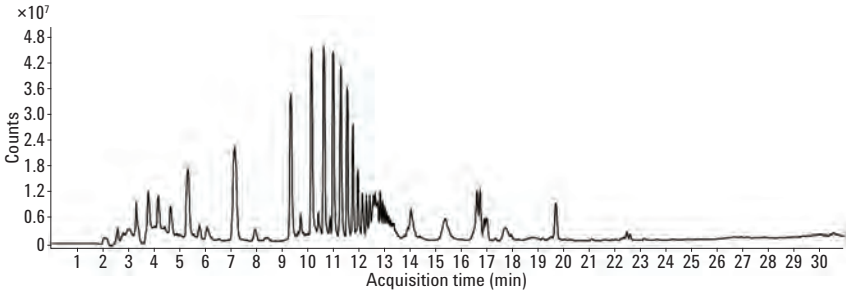

Figure 1. UHPLC/Q-TOF-MS chromatogram of a flowback sample from a recently hydraulic fractured well, which shows a bimodal distribution of polymers that differ by 44.026 mass units, a hydrophilic zone at 4–12 minutes and a second less-polar zone at 12–14 minutes.

*Table 2. Kendrick Mass for a Suite of Polyethylene Glycols Found in Flowback Water with an Average Mass Difference of 44.0266**

Retention time (min)	Ion (*m/z*) measured accurate mass	Kendrick mass	Ion adduct
3.5	173.0776	172.975	Na + PEG-EO-3
4.2	217.1048	216.975	Na + PEG-EO-4
5.4	261.1309	260.975	Na + PEG-EO-5
7.3	305.1586	304.975	Na + PEG-EO-6
9.5	349.1830	348.975	Na + PEG-EO-7
10.2	393.2095	392.975	Na + PEG-EO-8
10.7	437.2373	436.977	Na + PEG-EO-9
10.7	432.2830	432.026	NH_4 + PEG-EO-9
11.0	476.3067	476.023	NH_4 + PEG-EO-10
11.3	520.3333	520.023	NH_4 + PEG-EO-11

*JR-5 sample from Weld County, Colorado, based on a scaling factor of 0.999404559.

The concept of the Kendrick mass defect can then be applied, which is that if two compounds have the same repeating chemical structure, that is, (CH_2CH_2-O), then there will be an addition of the exact mass of CH_2-CH_2-O to each new compound in a chromatogram. Thus, when the scaling factor is multiplied by the measured masses, all the ions that differ by the CH_2-CH_2-O group will have exactly the same mass defect (within the error of accurate mass measurement, which is typically ±0.001 mass units). These results are shown in Table 2.

For example, Table 2 shows that the measured accurate masses for ions at retention times of 3.5 to 10.7 minutes all had different mass defects ranging from 0.0776 to 0.3333. However, after multiplying by the appropriate Kendrick mass scaling factor for the glycol unit (CH_2CH_2-O) of 0.999404559, only two mass defects, 0.975 and 0.023, were found for this suite of 9 ions (Table1). The fact that all of the Kendrick mass defects for each type of ion adduct are nearly identical means that each of the compounds increases by one ethylene glycol unit, or an accurate mass of 44.0262. Thus, it is only necessary to obtain the correct formula and structure for one of the glycols, and then all others can be calculated as either one unit longer or shorter depending on the gain or loss of the 44.0262 mass unit.

Using the measured mass of 305.1586 (a sodium adduct in Table 2), which also shows a proton adduct at *m/z* 283.1753 in Figure 2, a formula can be determined using MassHunter Software, as shown in Figure 2. The proton adduct at 283.1753 is first highlighted, followed by using the **Generate Formula** option to determine the best formula, shown in Figure 2 as $C_{12}H_{26}O_7$, which is the neutral molecular formula.

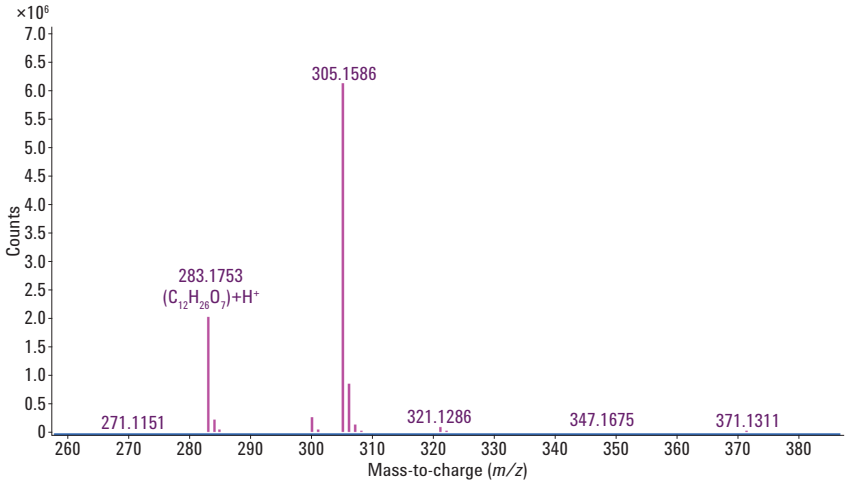

Figure 2. Q-TOF-MS scan from 7.060 to 7.409 minutes, showing the proton adduct at m/z 283.1753 along with the formula generated in Agilent MassHunter Software. The sodium adduct of the ion is also shown at m/z 305.1586.

Analysis by MS/MS of the *m/z* 283.1753 can determine if the spectrum will match a polyethylene glycol structure, as shown in Figure 3. The mass spectrum consists of a series of 44.026 mass unit losses that translate into a simple PEG structure [4]. The polyethylene glycol unit is equal to $HO\text{-}(CH_2CH_2O)_n\text{-}H$, which means that the chain length of the unit must be n = 6, since this length provides the correct formula of $C_{12}H_{26}O_7$. Table 2 can then be used to assign PEG-7 and PEG-8 to the higher mass ions, as well as PEGs -3, -4, and -5 to the lower mass ions, since all of the compounds with the same Kendrick mass differ by one glycol unit.

Figure 4 shows the mass spectrum for PEG-9 at a retention time of 10.6 minutes with three major ions at *m/z* 415.2539, 432.2830, and 437.2378. The mass differences among these three ions are 17 and 22, which indicate that the proton adduct is at 415.2539. What is important to note about the PEG-9 is that the major adduct ion for PEG-9 is no longer the sodium adduct, but rather the ammonium adduct (compare Figures 2 and 4). The mobile phase does not contain ammonium; thus, the ammonium adduct is formed from trace levels of ammonium present in the mobile phase and sample [2]. This shift to the ammonium adduct at PEG-9 has been attributed to a cage-like structure that surrounds the ammonium ion more readily (that is, energetically) and favors the ammonium ion over the sodium ion adduct [2]. The same is true for ammonium adducts of PEG-10 and PEG-11.

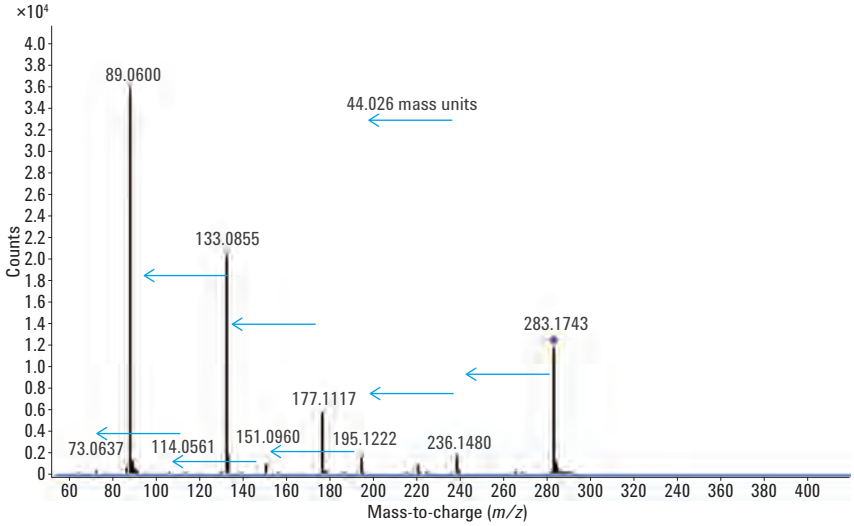

Figure 3. MS/MS spectrum of the m/z 283.1743 proton adduct from Figure 2.

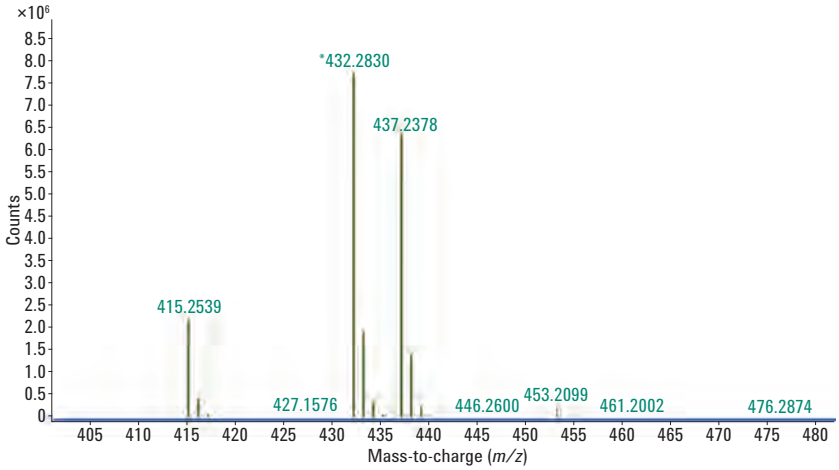

Figure 4. The mass spectrum at 10.6 minutes for PEG-9, with the major ion being the ammonium adduct at m/z 432.2830.

An accurate mass database of PEG compounds

After the application of this Kendrick mass scale, it was possible to identify proton, sodium, and ammonium adducts of the various PEGs shown in Figure 1. These enabled the construction of an accurate mass database of these PEG ions and adducts. It also allowed subsequent use of the MassHunter Software to find the > 40 different PEGs shown in Figure 1, as well as their various ion adducts, in less than 2 minutes of computer time. Identification of these PEGs may enable their use as indicators of contamination of aquifers or surface water by hydraulic fracturing.

Conclusions

The Agilent 1290 Infinity LC System and Agilent 6540 LC/Q-TOF-MS enabled the UHPLC/MS/MS characterization of two sets of representative polyethylene compounds from hydraulic fracturing flowback samples. Applying the Kendrick mass scale to the accurate masses of these PEGs and other ethoxylated surfactants [2] facilitates their identification using Agilent MassHunter Software, and the construction of a database of such compounds present in flowback samples. This database can, in turn, enable the use of these compounds as unique tracers of hydraulic fracturing.

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Using a Chlorine Filter for Accurate-Mass Data Analysis of Environmental Samples

Application Note

Environmental

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Abstract

A chlorine mass-filter is used to screen both LC/TOF-MS and LC/QTOF-MS data files in order to discover compounds that contain chlorine. The chlorine filter uses Mass Hunter software to generate formulae of chlorine containing compounds. Examples are shown in the analysis of an environmental water sample (the South Platte River after wastewater discharge) for pharmaceuticals. The chlorine filter is a useful data analysis tool for complex sample analysis in the field of environmental chemistry.

Introduction

Data files generated by LC/TOF-MS and LC/QTOF-MS contain literally thousands of individual ions that are difficult to evaluate by manual techniques. It is important to have software that makes data analysis rapid and effective. One such data analysis tool that we report in this application note is the chlorine mass filter. Chlorine appears in many pesticides and pharmaceutical products that are important to environmental analysis. Because chlorine contains two isotopes, Cl^{35} and Cl^{37}, there is a distinctive A+2 isotope pattern that is generated by a single chlorine atom in a molecule. Furthermore, there is a isotopic mass defect that occurs with chlorine-37 that makes the identification of chlorine in a molecule relatively easy [1]. More than one chlorine atom in a molecule generates an A+2 and A+4 isotopic pattern, which is characteristic and commonly shown in all mass spectrometry books as a key to compound identification of chlorinated compounds [2].

In this application note, we have automated the MassHunter software to generate formulae that contain chlorine from a data file of a water sample from surface water that is contaminated with wastewater. Furthermore, the automated report includes a database search using either a forensic or pesticide database for compound identification.

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At this point the user needs only to check the data report for quality control and quality assurance purposes. The chlorine mass filter will work on any .d data file from environmental samples, such as food for pesticides or water samples for pharmaceuticals.

Experimental Conditions

Any of the Agilent accurate mass analysis instruments, the Model 6200 or 6500 series, may be used to generate data files for this analysis procedure. We applied the 6220 to generate data files of pharmaceuticals in surface water, which is from the South Platte River and is affected by the Denver Metropolitan area and wastewater from Denver. The data files, as .d files, are ready for software analysis using the MassHunter software package with Qualitative Analysis. The same Qualitative Analysis software is available on all of the accurate mass instruments, the Model 6200 or 6500 series.

Results and Discussion

Applying the Chlorine Mass Filter

The chlorine filter is applied by an eight-step procedure that begins with first opening the Qualitative Analysis software package and opening the .d file of interest.

Step 1. Open the .d file of interest with the Qualitative Analysis software package, the green icon. Go to the Find Compounds Menu (see Figure 1) and open the Find by Molecular Feature tab. Figure 1 shows the blue arrow that activates this program. The tab open is called compound filters and is set for a relative height of 1.5% and absolute height of 10,000 counts. To obtain detection of even lower abundance compounds, the relative height might also be left unchecked. However, these settings may need to be adjusted for the noise levels of both individual instruments and data sets. These three settings allow for a filter of a complicated sample and take full advantage of the software's ability to find small peaks that contain chlorine.

The other settings needed are *ion species* at H+ and Na+ for the two positive ion species. In negative ion, the setting would be H-. *Extraction* is for small molecules (chromatographic) and use peaks with height greater than 1000 counts. Again, this setting may need to be adjusted for individual instrument background and sample set noise, *Charge State* is set for peak spacing of 0.0025 plus 7 ppm, isotope model is common organic molecules, and limit assigned charge state to a maximum of one. The *Mass Filter* is blank and the *Mass Defect* is blank. The *Results* is to extract the EIC, highlight all compounds, and delete previous results. These settings should result in a clean run of the program at this point, that is, the Molecular Feature.

Step 2. Run the Find by Molecular Feature program by clicking the **arrow** shown in Figure 1. This program identifies all of the ions in the .d file and groups ions together that are related, such as isotopic clusters and sodium adducts. It does not group fragment ions, but considers them as different compounds.

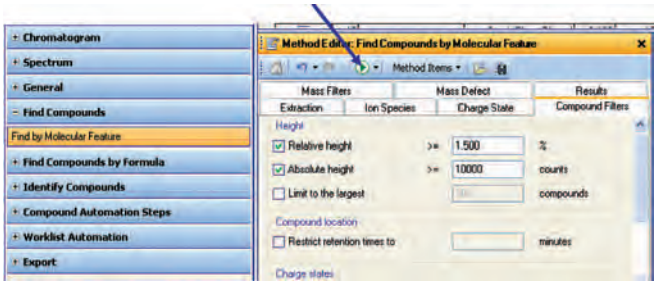


Figure 1. Find by Molecular Feature.

Step 3. Open the Identify Compounds tab (Figure 2) and click the **Generate Formulas** tab. This opens the window shown in Figure 2, and here, one specifies two chlorine atoms and at least one chlorine atom in the table (see Figure 2 with arrows). Note that this example filters the compounds for only 1–2 chlorines but one could select as many chlorines as desired. *Limits* tab and *Charge State* tabs are shown in Figure 3 with a setting that will give good results. Copy these values.

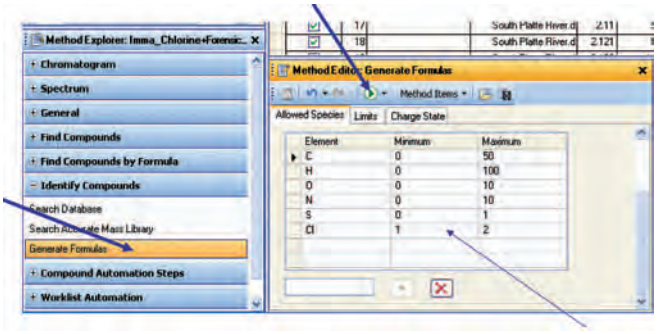
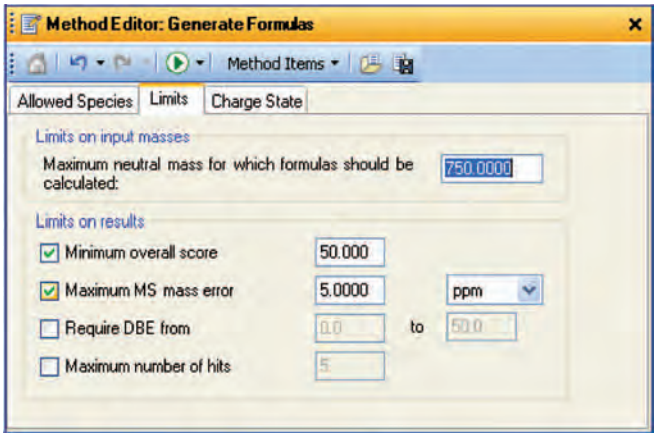


Figure 2. Identify Compounds and Generate Formulas.



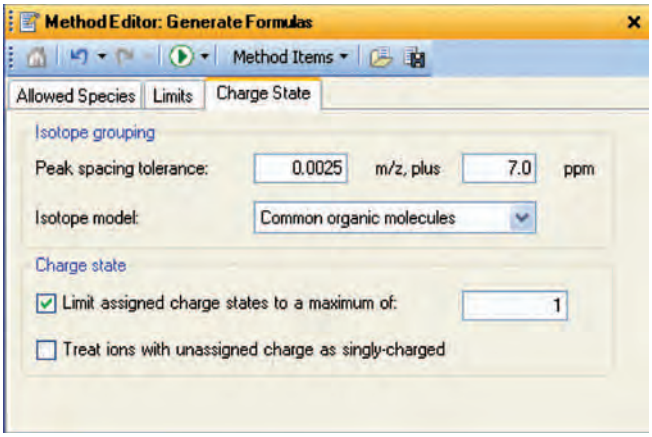


Figure 3. Other tab settings.

Step 4. Run the Generate Formulas program with the forced chlorine atoms. This generates formula for all of the features that were identified in Step 2 and will include only formulae that contain 1 to 2 chlorines.

Step 5. Open the **Search Database** pane and open the tab called Search Criteria. Select **Molecular Formula** as shown in Figure 4. This will force the search to report only those compounds that match the found formulae (and will only contain 1 to 2 chlorines). Next select the **Database**. Here specify the **Forensic** or **Pesticide Database** (Figure 5). The tab settings are: + Ions set at +H, *-Ions* set at –H, *Search Results* set at Blank, *Peak Limits* set at 5, and *Search Criteria* set at Mass and 5 ppm. These settings will work well to identify compounds. The Forensic database contains over 7,500 compounds including pharmaceuticals and pesticides. The chlorinated compounds are grouped under a column labeled *MFG Formula* or molecular formula generator.

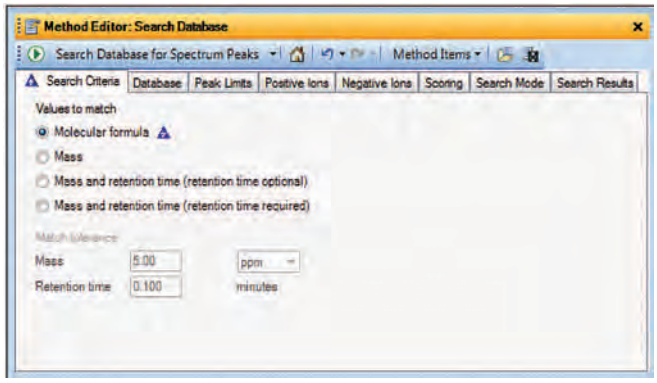


Figure 4. Select search criteria by Molecular formula.

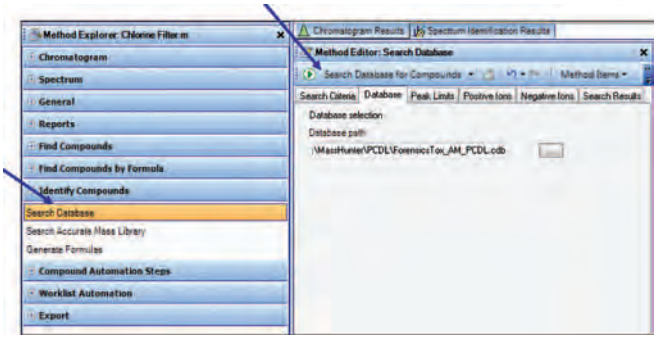


Figure 5. Search Database program using forensics database of 7500 compounds.

Step 6. Run the Search Database Program. This program now goes in and checks all of the formulas that were generated in Step 4 and assigns a name to the compound if it appears in the database. If the formula is not in the database, the Name is left blank in the final report. In this case, the best fit formula is printed in the column labeled Molecular Formula Generator for all chlorinated compounds.

Step 7. Print the Report. The report contains the following columns: Compound label, Retention Time, Mass of Neutral compound, Name (if available in the database), Molecular Formula Generator (MFG) elemental formula, MFG difference in mass accuracy, Database Formula (DB), and DB difference in mass accuracy. The report for the river sample is shown in Figure 6.

Qualitative Compound Report

Data File	South Platte River.d	**Sample Name**	
Sample Type	Sample	**Position**	P1-C6
Instrument Name	Instrument 1	**User Name**	
Acq Method	Multiresidue_Pesticides.m	**Acquired Time**	8/10/2009 7:09:22 PM
IRM Calibration Status	Success	**DA Method**	Chlorine Filter Automated.m
Comment			

Compound Table

Compound Label	RT	Mass	Name	Formula	MFG Formula	MFG Diff (ppm)	DB Formula	DB Diff (ppm)
Cpd 464: Lamotrigine	13.641	255.0077	Lamotrigine	C9 H7 Cl2 N5	C9 H7 Cl2 N5	0.54	C9 H7 Cl2 N5	0.54
Cpd 499: Ketamine	13.858	237.0923	Ketamine	C13 H16 Cl N O	C13 H16 Cl N O	-0.89	C13 H16 Cl N O	-0.89
Cpd 637: C9 H10 Cl N	15.265	167.0502		C9 H10 Cl N	C9 H10 Cl N	-0.39	C9 H10 Cl N	
Cpd 640: C13 H20 Cl N O	15.266	241.1233		C13 H20 Cl N O	C13 H20 Cl N O	0.33	C13 H20 Cl N O	
Cpd 735: C19 H27 Cl N6 O2 S	16.37	438.1611		C19 H27 Cl N6 O2 S	C19 H27 Cl N6 O2 S	-1.36	C19 H27 Cl N6 O2 S	
Cpd 775: C14 H34 Cl2 N10 O2	16.768	444.2245		C14 H34 Cl2 N10 O2	C14 H34 Cl2 N10 O2	-0.5	C14 H34 Cl2 N10 O2	
Cpd 889: C18 H29 Cl N2 O3 S	18.264	388.1583		C18 H29 Cl N2 O3 S	C18 H29 Cl N2 O3 S	1.18	C18 H29 Cl N2 O3 S	
Cpd 1015: Losartan	19.635	422.1629	Losartan	C22 H23 Cl N6 O	C22 H23 Cl N6 O	-1.7	C22 H23 Cl N6 O	-1.7
Cpd 1137: C18 H29 Cl N2 O6 S	21.105	436.1439		C18 H29 Cl N2 O6 S	C18 H29 Cl N2 O6 S	-0.9	C18 H29 Cl N2 O6 S	
Cpd 1159: C28 H41 Cl N4 O2	21.311	500.2929		C28 H41 Cl N4 O2	C28 H41 Cl N4 O2	-2.1	C28 H41 Cl N4 O2	
Cpd 1194: C18 H33 Cl N2 O4	21.64	376.2118		C18 H33 Cl N2 O4	C18 H33 Cl N2 O4	2.91	C18 H33 Cl N2 O4	

Figure 6. Printout of Report for database search of forensics to determine for the first time an antidepressant in riverwater [3].

Step 8. QA/QC Manual Report. Finally, it is necessary for the operator to manually check the assigned chlorinated compounds. This is done by manually calling up the ion at the correct retention time and checking the isotopic signature of the ion. Figure 7 shows an example of this for lamotrigine found in the South Platte River that received wastewater from a nearby effluent. This is an antidepressant drug that was detected in the sample at a retention time of 13.4 minutes [3]. The measured mass of the ion was *m/z* 256.0150 and shows an isotopic signature for both an A+2 and an A+4 isotope at masses of *m/z* 258.0121 and *m/z* 260.0092. It is important to note the relative isotopic mass defect of both the A+2 and the A+4 isotope for this compound. The relative mass defects are -0.0029 and -0.0029 for both peaks. These values are quite close to the theoretical value of -0.0030 and are excellent evidence for the presence of two chlorine atoms in the molecule [1]. This step is the critical step of the chlorine filter and is a manual step at this time. Because the molecular formula generator looks at mass only, it is possible for some formulae to be generated that

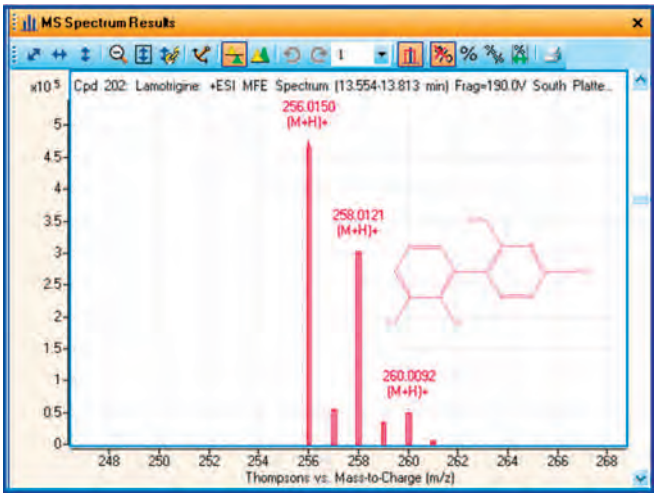


Figure 7. Mass spectrum for lamotrigine.

do not fit the isotopic signature. In this example from the South Platte River, we had approximately 100 possible chlorinated peaks of which 20 were identified to contain chlorine based on the relative mass defect and the isotopic signature.

Of these 20 different ions, there were 15 different components and the remaining five ions were fragment ions based on the retention time matching of the ions. For example, Figure 8 shows the structure of cetirizine, which was matched by the forensics database at 18.3 minutes with a protonated molecule at *m/z* 389.1626 and a fragment ion at the same retention time with a *m/z* 201.0466. The combination of mass accuracy, database matching, and identifying a fragment ion shows the power of using the chlorine mass-filter to find and identify trace chlorinated substituents in water samples impacted by wastewater. The application shown here of the chlorine mass-filter will work equally well for food samples contaminated with pesticides or other similar environmental samples.

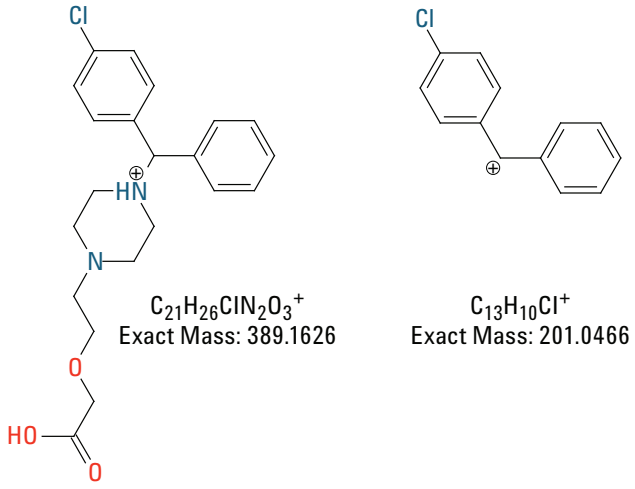


Figure 8. Cetirizine example showing the two ions that were found at the same retention time of 18.3 minutes.

Conclusions

Using accurate mass LC/MS with the Agilent 6200 or 6500 series TOF or Q-TOF and MassHunter Qualitative analysis version 5.0, a chlorine filter is described that effectively mines single MS data for all compounds that contain 1 to 2 chlorines. The process can be modified to contain any specified number of halogens or for that matter any specific elements, for example a fluorine filter or iron. The process can be automated using Worklist Automation and then manually QC'ed as described.

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Identification of Unknown Microcystins in Alberta Lake Water

Application Note

Environmental

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Abstract

Detection, characterization, and tentative identification of very low levels of unknown microcystins in lake water are possible in the absence of analytical standards using a combination of triple quadrupole LC/MS and LC/Q-TOF analysis and a Personal Compound Database (PCD) compiled from the Word Health Organization (WHO) list of microcystins.

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Introduction

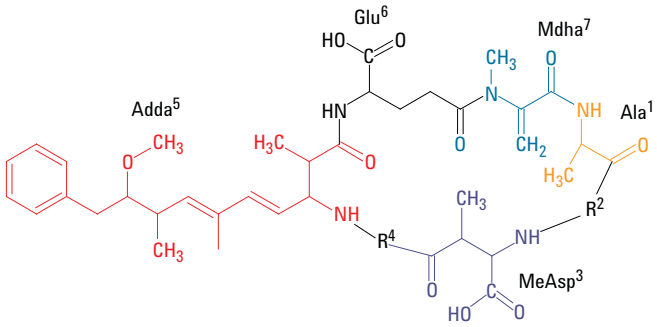
The occurrence of cyanobacterial toxins in Canadian fresh waters is a serious environmental and public health concern [1]. Microcystins (MCs) are a class of common cyanobacterial toxins present in Canadian lakes, and they are potent inhibitors of eukaryotic protein phosphatases [2]. Microcystins are also powerful hepatotoxins, and may promote tumor development in mammals, presenting a serious threat to livestock and human drinking water sources. These cyanobacterial toxins have been detected in every province in Canada, often at levels above maximum guidelines for recreational water quality [2].

The MCs are cyclic peptides containing seven amino acids. They have the general structure shown in Figure 1. Table 1 gives the chemical structures for eight microcystins surveyed in Alberta lakes. The most frequently reported MC is LR, containing leucine and arginine at positions R^2 and R^4, respectively.

The Alberta Centre for Toxicology (ACFT) conducted a comprehensive study of microcystins in Alberta lakes, and it continues to monitor fresh water sources using liquid chromatography and triple quadrupole mass spectrometry. While this method tests for eight microcystins, a nontargeted compound was also detected in a sample with the same transition qualifier ion as MC YR (*m/z* 1045), but it had an incorrect retention time (RT) and qualifier ion ratio.

This application note describes two methods developed through a collaboration between Alberta Centre for Toxicology (ACFT) and Vogon Laboratory Services to provide sensitive detection of a wide range of microcystins and establish the identity of this newly observed compound. A confirmation method was first developed using an Agilent 1290 Infinity LC System and an Agilent 6460 Triple Quadrupole LC/MS, with a different retention time pattern from the reference ACFT method. Accurate mass determination on an Agilent 6540 Q-TOF LC/MS system was then used to provide tentative identification of the unknown peak as desmethylated microcystin HtyR.

All microcystins were detected at 0.1 ng/mL, which is well below the 2007 Canadian Drinking Water Guideline of 1.5 µg/L. Using a Personal Compound Database (PCD) of 52 compounds created in the Agilent MassHunter software suite from the World Health Organization (WHO) list of microcystins, the Q-TOF data enabled the tentative identification of an additional seven microcystins.



General microcystin structure

Position	Abbreviation	Amino acid
R^1	Ala^1	Alanine
R^2	Leu^2 (L)	Leucine
R^3	$MeAsp^3$	Methylaspartic acid
R^4	Arg^4 (R)	Arginine
R^5	$Adda^5$	3-amino-9-methoxy-2,6,6-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid
R^6	Glu^6	Glutamic acid
R^7	$Mdha^7$	N-methyldehydroalanine

Figure 1. General microcystin (MC) structure.

*Table 1. Eight Microcystins Surveyed in Alberta Lakes**

Microcystin	R^2	R^4	Formula	Neutral mass
LR	Leucine	Arginine	$C_{49}H_{74}N_{10}O_{12}$	994.5488
Desmethyl LR	Leucine	Arginine	$C_{48}H_{72}N_{10}O_{12}$	980.5331
RR	Arginine	Arginine	$C_{49}H_{75}N_{13}O_{12}$	1037.5658
YR	Tyrosine	Arginine	$C_{52}H_{72}N_{10}O_{13}$	1044.5280
LA	Leucine	Alanine	$C_{46}H_{67}N_7O_{12}$	909.4848
LW	Leucine	Phenylalanine	$C_{54}H_{72}N_8O_{12}$	1024.5270
LF	Leucine	Tryptophan	$C_{52}H_{71}N_7O_{12}$	985.5161
HtyR	Homotyrosine	Arginine	$C_{53}H_{74}N_{10}O_{13}$	1058.5437

*See Figure 1 for the general microcystin structure

Experimental

Instruments

The confirmation method was developed on an Agilent 1290 Infinity LC System equipped with an Agilent G4226A Autosampler and coupled to an Agilent 6460 Triple Quadrupole LC/MS System. The instrument conditions are listed in Table 2.

The method for tentative identification of unknown microcystins was developed on a 1290 Infinity LC system equipped with a G4226A Autosampler and coupled to an Agilent 6540B Q-TOF LC/MS System with Jet Stream electrospray source. The instrument conditions are listed in Table 2.

Table 2. LC and Triple Quadrupole MS Run Conditions

LC conditions		
Column	Agilent Poroshell SB-C18, 3.0 × 100 mm, 2.7 µm (p/n 685975-306)	
Column temperature	50 °C	
Injection volume	20 µL	
Mobile phase	A) 1 mM ammonium fluoride in water (HPLC grade) B) 20% isopropanol in acetonitrile (LC/MS grade)	
Autosampler temperature	5 °C	
Flow rate	0.6 mL/min	
Gradient	Time (min)	% B
	0	20
	3.0	30
	5.0	50
	6.0	100
Stop	7 minutes	
Post time	2 minutes	
Triple quadrupole MS conditions		
Ionization mode	ESI with Agilent Jet Stream Technology	
Drying gas temperature	350 °C	
Drying gas flow	12 L/min	
Nebulizer pressure	40 psig	
Sheath gas temperature	400 °C	
Sheath gas flow	11 L/min	
Capillary voltage	4,000 V	
Nozzle voltage	1,000 V	
EMV	400 V	
Q-TOF MS conditions		
Mode	Targeted MS/MS	
Acquisition	Profile and centroid; 2 GHz	
Range	100–1,700 amu	
Acquisition rate (MS)	3 scans/s	
Acquisition rate (MS/MS)	1 scan/s	
Reference masses	121.0509 and 922.0098	

Analysis parameters

The 6460 Triple Quadrupole LC/MS multiple reaction monitoring (MRM) analysis parameters are shown in Table 3.

Table 3. MRM Analysis Parameters[a] for the Target Compounds Using a Triple Quadrupole LC/MS

Microcystin	Precursor[b] (*m/z*)	Product ion (*m/z*)	Collision energy (V)
LR	995.6	135.2	80
		213.2	80
Desmethyl-LR	981.5	135.2	80
		213.2	80
RR	520.0	135.2	30
		213.2	40
YR	1045.5	135.2	80
		213.2	70
LA	910.5	135.2	70
		213.2	70
LY	1002.5	135.2	80
		213.2	70
LW	1025.5	135.2	80
		213.2	60
LF	986.5	135.2	70
		213.2	50
HtyR	1059.5	135.2	80
		213.2	70

[a] The fragmentor and cell acceleration voltages were 150 V and 2 V, respectively, for all transitions.

[b] All precursors are singly charged, except RR which is doubly charged.

Sample preparation

Water samples (10 mL) were taken through three freeze/thaw cycles, then sonicated for 5 minutes, followed by filtration through 0.2-µm cellulose filters directly into autosampler vials.

Results and Discussion

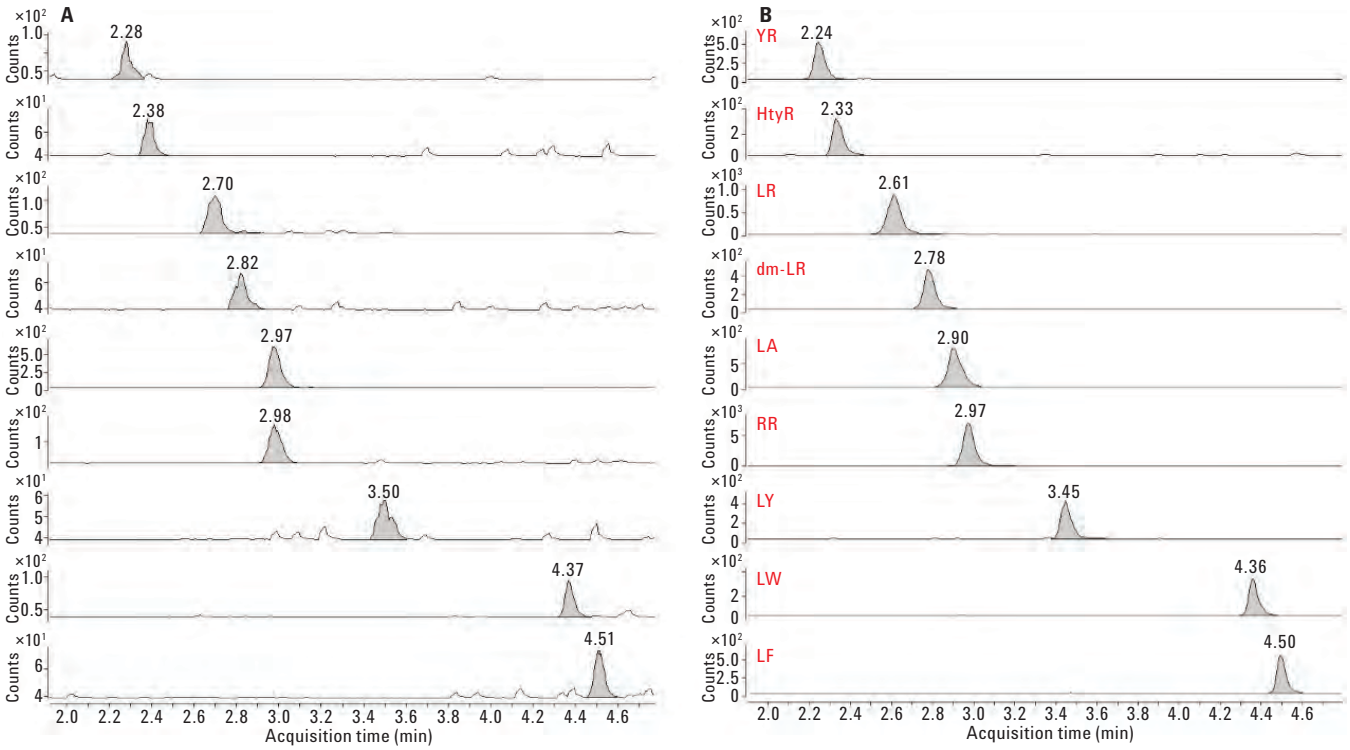
Identifying an unknown microcystin

One sample analyzed using the ACFT method revealed a compound with the same transition and qualifier ion as YR (*m/z* 1045), but at the wrong retention time (RT) and qualifier ion ratio. It was tentatively identified as desmethyl-HtyR, based on the loss of 14 amu from HtyR (*m/z* 1059).

To confirm the identification of this unknown MC, a triple quadrupole LC/MS confirmation method was developed, which provided a different retention time pattern from the reference ACFT method. Additional microcystin analytes were added to the method to help characterize compounds. Finally, samples were also analyzed using an LC/Q-TOF method to confirm the identity of the unknown compound.

Confirmation method

The triple quadrupole LC/MS confirmation method was optimized to provide a minimum detection level at approximately 10% of the Canadian Drinking Water Guidelines (CDWG), which is 1.5 µg/L, based on MC LR. The lowest level calibrators used for the method were 0.1 µg/L for MCs YR, LR, and RR, and 0.2 µg/L for all others. Figure 2 illustrates the complete separation achieved for nine MCs with the high performance liquid chromatography (HPLC) chromatographic method at both the lowest calibrator levels and calibrator levels near the CDWG.



Lowest level calibrator

0.1 µg/L for YR, LR, and RR
0.2 µg/L for all others

Calibrator near DW guidelines

0.9 µg/L for YR, LR, and RR
1.9 µg/L for all others

Figure 2. Example total ions chromatograms (TICs) for nine microcystins at concentrations approximately 10% (A) and 100% (B) of the CDWG of 2007.

Quantitation calibration coefficients (R^2) were excellent, ranging from 0.9978 to 0.9995 for the six MCs using a minimum calibrator level of 0.2 µg/L and a maximum calibrator level of 50 µg/L (Table 4). The R^2 values ranged from 0.9992 to 0.9997 for the three MCs using a minimum calibrator level of 0.1 µg/L and a maximum calibrator level of 25 µg/L. A representative calibration curve for MC LR is shown in Figure 3, illustrating excellent linearity even at extremely low concentrations. Accuracy ranged from 84% to 121% for the six MCs with a minimum calibrator level of 0.2 µg/L, and 110% to 119% for the three MCs with a minimum calibrator level of 0.1 µg/L (Table 4).

Analyzing the unknown

Using the confirmation method to analyze the unknown MC from an Alberta lake water sample showed a peak with the same transition and qualifier ions as YR, but with an RT of 1.91 minutes, rather than 2.24 minutes. The qualifier ion ratio (*m/z* 213.2/135.2) was also too low to be YR (2% versus 24%). The low abundance of the *m/z* 213.2 peak indicates possible desmethylation of the N-methyldehydroalanine (Mdha) moiety in the YR structure. Unfortunately, desmethyl HtyR is not commercially available. Therefore, Q-TOF for accurate mass analysis was used to further confirm the desmethylation hypothesis.

Table 4. Calibration Coefficients and Accuracy of Recovery

Calibration range	MC	R^2	Accuracy[a] (%)
0.2 to 50 µg/L	Hty R	0.9995	95
	Desmethyl-LR	0.9993	84
	LA	0.9988	121
	LY	0.9998	92
	LW	0.9989	118
	LF	0.9978	119
0.1 to 25 µg/L	YR	0.9993	118
	LR	0.9997	110
	RR	0.9992	119

[a] Quantitative accuracy is listed for calibrator 1 (0.1 or 0.2 µg/L)

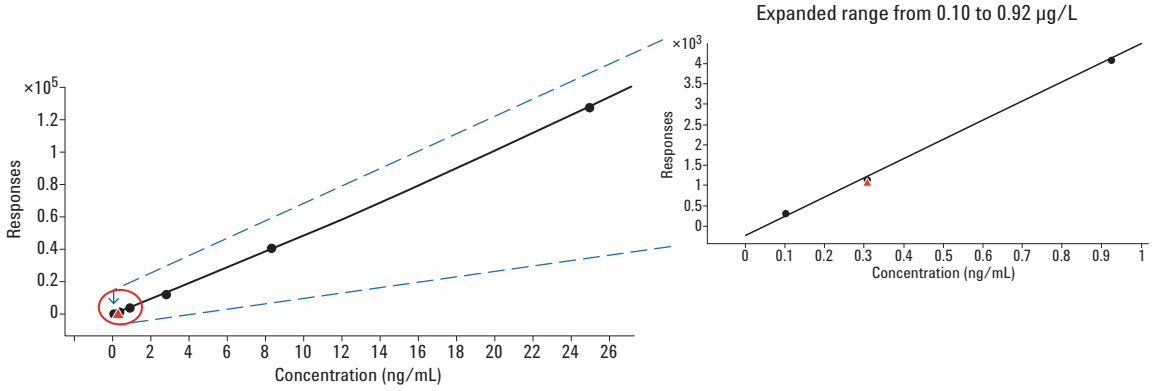


Figure 3. A calibration curve for MC LR using three-fold serial dilutions from 0.1 to 25 µg/L, including the expanded range from 0.10 to 0.92 µg/L, to illustrate excellent linearity.

LC/Q-TOF analysis

The accurate mass determined for the precursor ion for the unknown MC using liquid chromatography/Q-TOF MS (LC/Q-TOF) was 1059.5500, which matches the actual mass for desmethylated HtyR with a mass error of 5.0 ppm. This further supports the hypothesis that the unknown is formed by the desmethylation of HtyR. Examination of the transition ions reveals that both the unknown and YR contain the two *m/z* 135 nominal mass ions characteristic for microcystins (Figure 4). The actual masses of the two ions are 135.0804 and 135.1168, formed by different fragmentation patterns of the Adda group. Both ions are well separated in both microcystins at mass resolution 14,000.

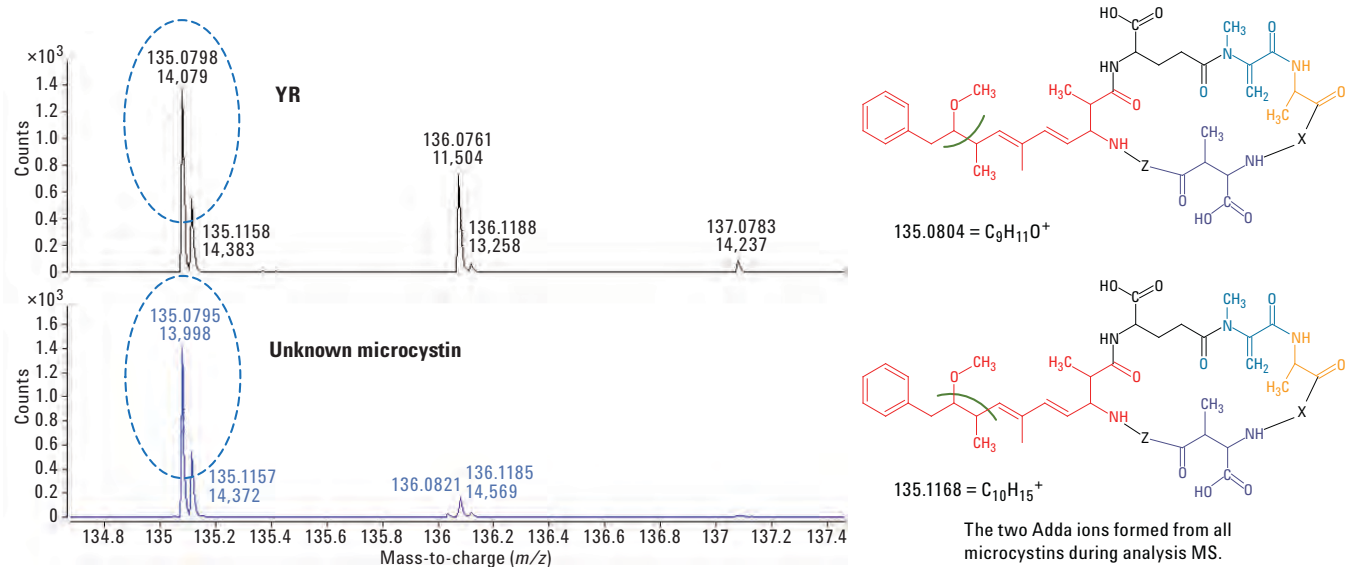


Figure 4. The two ions derived from the Adda group and characteristic of all microcystins are present and well separated in both YR and the unknown microcystin, using LC/Q-TOF.

However, the *m/z* 213 ion formed from cleavage of the Glu + Mdha groups from the microcystins is present as expected in MC YR, but present at only trace levels in the unknown MC (Figure 5). This indicates that the unknown MC is not YR. Desmethylation of the Glu + Mdha group would be expected to produce an ion with an accurate mass of 199.0713 (Figure 6). This ion is observed in the spectra from the unknown MC, but not in the spectra of MC YR. These results also support the hypothesis that the unknown MC is dm-HtyR.

A previous structural LC/MS/MS characterization of microcystins had identified eight major ions designated a-h [3]. Two of these, ions f and h, can be used to confirm the identity of the unknown MC. Ion f contains the R^7 and R^2 moieties (Figure 1), which should be dm-Mdha (Dha) and Hty, respectively for the microcystin dm-HtyR. Ion h contains the R^2 moiety, as well as a second possible desmethylation site, MeAsp[3]. Depending on the identities of R^7 and R^2 and the presence or absence of desmethylation at position R^3, the accurate masses of these two ions will vary and be diagnostic of the microcystin structure.

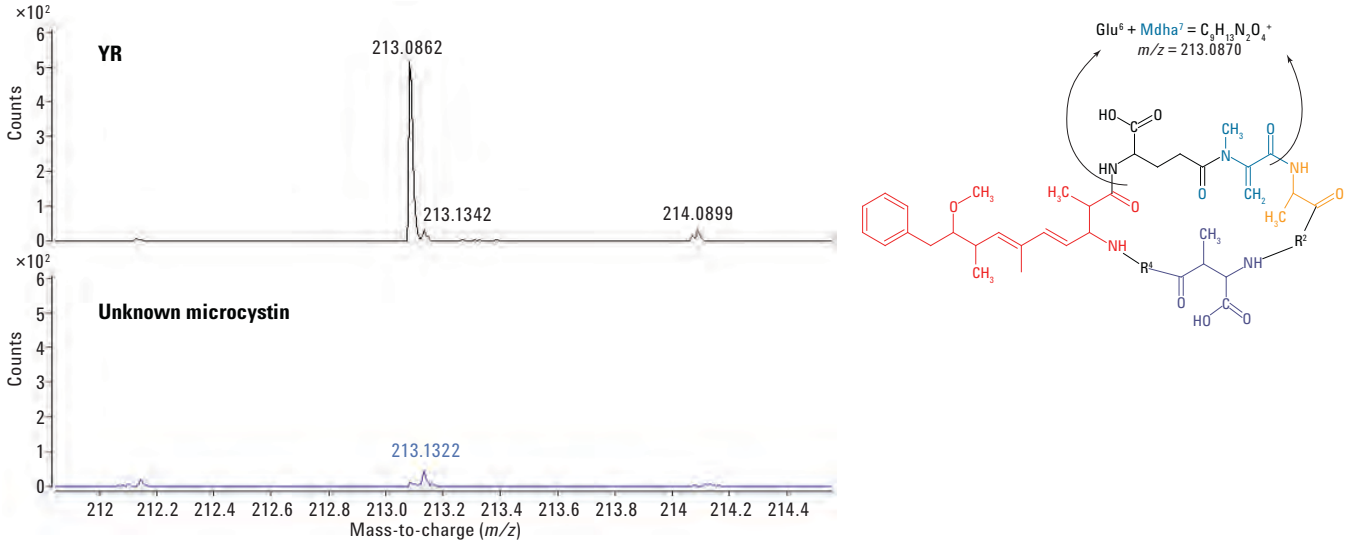


Figure 5. Q-TOF spectra of YR and the unknown microcystin in the m/z range from 212 to 214.4. The m/z 213 ion characteristic of the Glu + Mdha group is present in microcystin YR but at low abundance in the unknown MC.

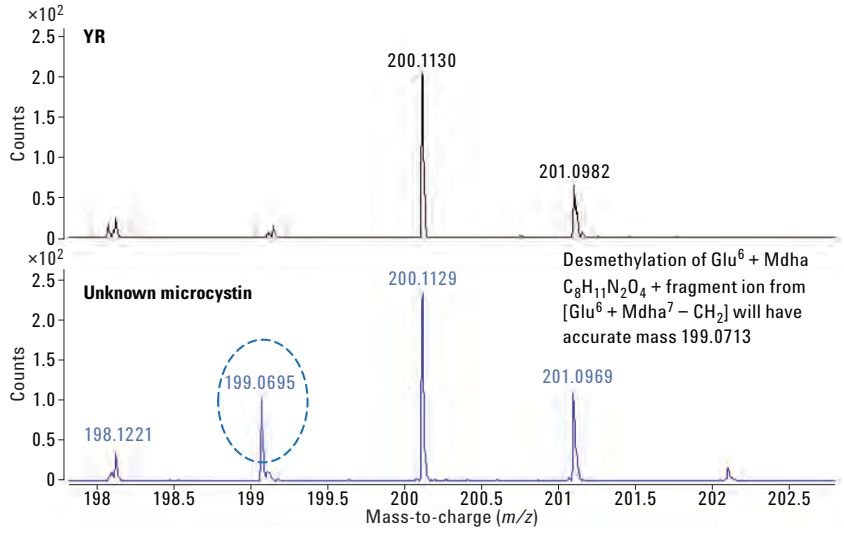
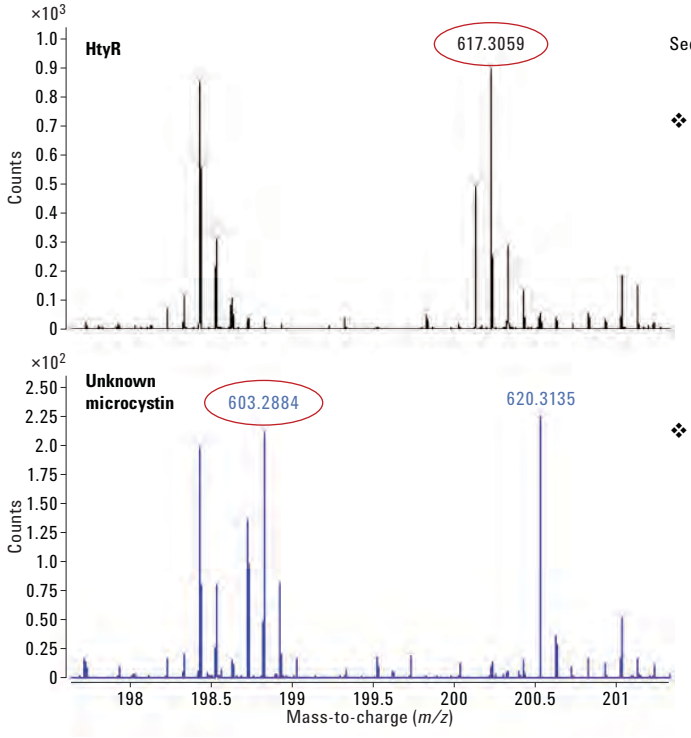


Figure 6. Q-TOF spectra of YR and the unknown microcystin in the m/z range from 198 to 202. The m/z 199 peak is present in the unknown microcystin, indicating desmethylation of the Glu + Mdha group. This peak is not present in YR.

Analysis by Q-TOF of ion f in the unknown microcystin revealed accurate masses that confirmed the presence of Dha at position 7 and Hty at position 2, consistent with the identification of the unknown as dm-HtyR. Analysis of the HtyR standard also gave the correct accurate mass for ion f (Figure 7).



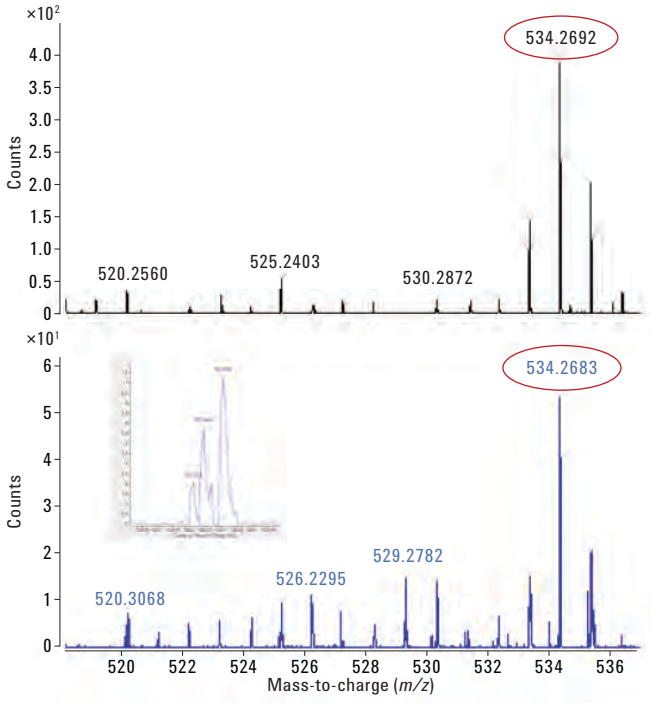
Sequence for Ion f: R^7–Ala^1–R^2–MeAsp^3–Arg^4

❖ Structure of MC HtyR:
- R^7 = Mdha; R^2 = Hty
- Calc *m/z*: 617.3042
- Measured: 617.3059 (2.8 ppm mass error)

❖ Proposed structure (dm-HtyR) for the unknown compound, with desmethylation of Mdha^7 to Dha^7:
- R^7 = Dha; R^2 = Hty
- Calc *m/z*: 603.2885
- Measured: 603.2884 (0.2 ppm mass error)

Figure 7. Q-TOF spectra of ion f for HtyR and the unknown microcystin. The accurate mass for the f ion observed in HtyR (upper) matched the calculated mass for the ion containing Hty at position 7 and Mdha at position R^3. In contrast, the mass observed for ion f in the unknown MC matched the calculated mass for the ion containing Hty at position 7 and Dha at position R^3.

The spectra of the h ion confirmed that minimal desmethylation is occurring in the R^3 position in the unknown MC, as its accurate mass matched the calculated mass for methylated aspartic acid in position 3, in both the unknown and the HtyR standard (Figure 8).



Sequence for Ion h: Ala^1–R^2–$MeAsp^3$–Arg^4

❖ Structure of MC HtyR:

- R^2 = Hty
- Calc *m/z*: 534.2671
- Measured: 534.2692 (2.3 ppm mass error)

❖ Proposed structure (dm-HtyR) for the unknown compound, with no desmethylation at position 3:

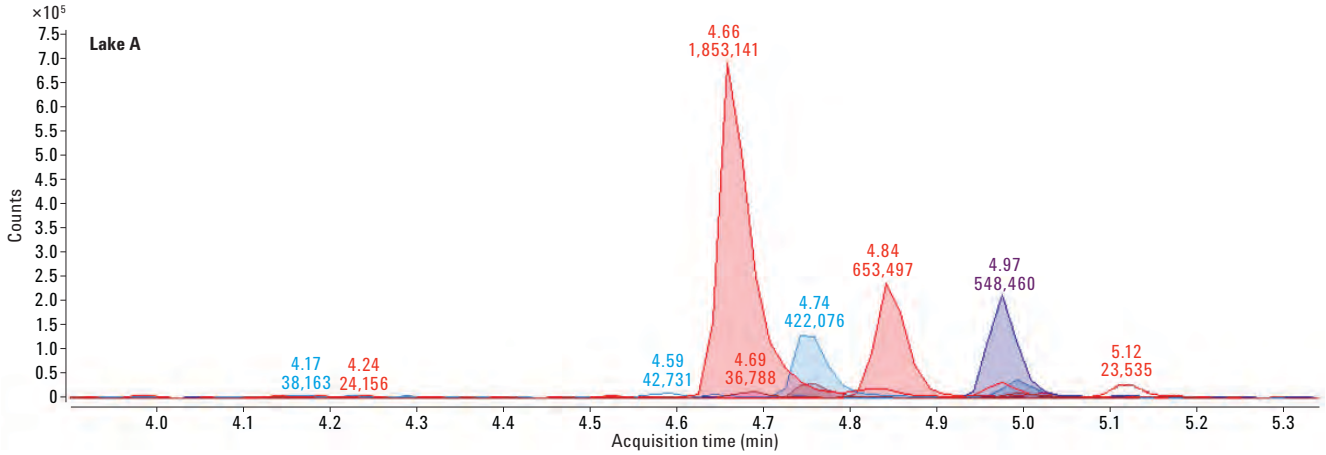
- R^7 = Hty
- Calc *m/z*: 534.2671
- Measured: 534.2683 (4.0 ppm mass error)

Figure 8. Q-TOF spectra of ion h for HtyR and the unknown microcystin. The accurate mass for the h ion observed in both HtyR (upper) and the unknown MC matched the calculated mass for the ion containing Hty at position R^7 and Mdha at position R^3. In contrast, only a very small peak was observed at nominal mass m/z 520 in both microcystins, indicating that little or no desmethylation was occurring at position R^3.

Using a Personal Compound Database to identify multiple unknown microcystins

The accurate mass capabilities of the Q-TOF can be used to tentatively identify other microcystins that may be present in Alberta lakes, based only on the mass of their H+ adducts. The first step in the process is to build a Personal Compound Database (PCD) using the Agilent MassHunter PCDL Manager Software, which enables the user to create and edit a customizable PCD, including compounds, accurate-mass, and retention time information. One of the advantages of accurate mass scan data is the ability to retrospectively search acquired data for new compounds using such a database.

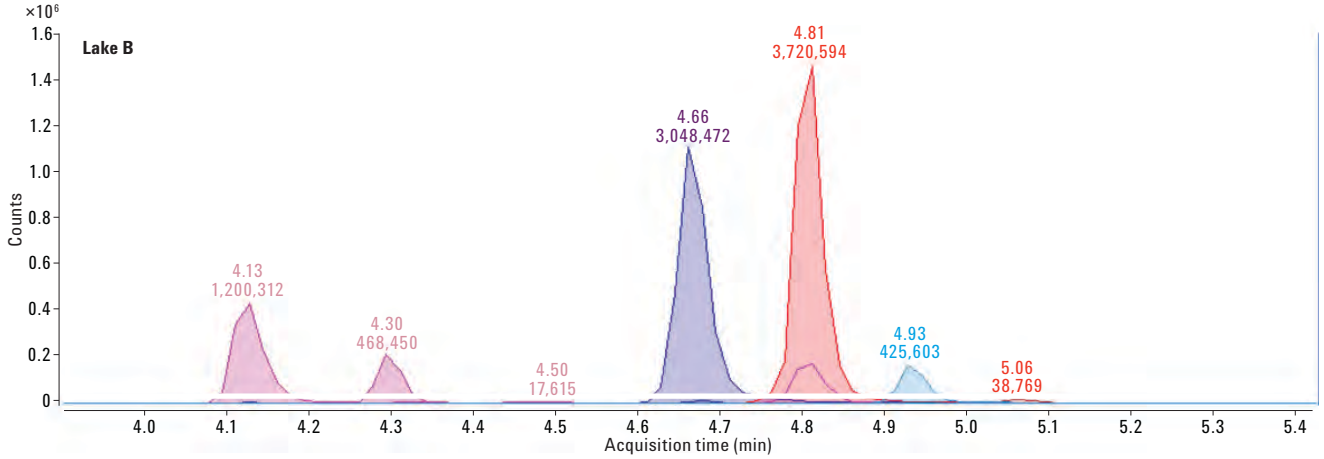
In this study, the WHO list of microcystins [4] was used to enter the formulas for 52 microcystins into a PCD, which generates accurate masses for the H+ adducts. The Find by Formula tool in MassHunter was then used to search for these accurate masses in the sample total ions chromatogram (TIC) data file. Using this process with the Q-TOF, seven unknowns were tentatively identified in samples from Lake A and Lake B, in addition to the desmethyl HtyR (Figures 9 and 10). Additional analysis by Q-TOF MS/MS and comparison to analytical standards is needed to confirm the presence and identity of these MCs. Work is on-going to develop an exact-mass calculator model to help identify nontargeted MCs and their variants.



RT	Compound name	Formula	*m/z*	Area	Score	Mass diff (ppm)*
4.66	MC-YR	C52 H72 N10 O13	1045.5346	1,853,141	98.5	-0.7
4.69	MC-LR	C49 H74 N10 O12	995.5549	36,788	90.4	-0.1
4.74	MC-HtyR-DAsp3-Dha7	C51 H70 N10 O13	1031.5185	422,076	96.8	-0.7
4.76	MC-HtyR	C53 H74 N10 O13	1059.5501	94,974	98.4	-0.3
4.84	MC-DesMe-LR	C48 H72 N10 O12	981.5405	653,497	99.1	-0.1
4.97	MC-HphR-Dha7	C52 H72 N10 O12	1029.5400	548,460	99.6	-0.2
4.99	MC-LR-DAsp3-Dha7	C47 H70 N10 O12	967.5236	116,668	94.3	-1.5
4.99	MC-LR	C49 H74 N10 O12	995.5559	32,185	92.7	-0.6

*Mass difference determined by subtracting the theoretical mass of the compound from the calculated mass derived from the Q-TOF analysis, expressed in ppm.

Figure 9. Extracted ion chromatograms of a water sample from Lake A, and a table including retention time (RT), compound name, calculated m/z, and difference of the calculated mass from the theoretical mass for each unknown tentatively identified using the Find by Formula tool in MassHunter and the personal compound database (PCD) created for the microcystins in the WHO list. A mass tolerance of 5 ppm and a minimum match score of 70 were used to make the identification of H+ adducts.



RT	Compound name	Formula	*m/z*	Area	Score	Mass diff (ppm)*
4.13	MC-HtyR-DAsp3-Dha7	C51 H70 N10 O13	1031.5193	1,200,312	99.5	-0.4
4.30	MC-LR-DAsp3-Dha7	C47 H70 N10 O12	967.5246	468,450	99.7	-0.3
4.50	MC-LR-DAsp3-Dha7	C47 H70 N10 O12	967.5256	17,615	86.4	0.7
4.66	MC-HtyR-DAsp3-ADMAdda5-Dhb7	C53 H72 N10 O14	1073.5305	3,048,472	99.4	0.4
4.68	MC-LR	C49 H74 N10 O12	995.5549	18,836	97.7	-1.1
4.81	MC-LR-DAsp3-ADMAdda5-Dhb7	C49 H72 N10 O13	1009.5359	3,720,594	99.6	0.5
4.93	MC-LY	C52 H71 N7 O13	1002.5181	425,603	99.4	-0.3
5.06	MC-LR-ADMAdda5	C50 H74 N10 O13	1023.5505	38,769	95.9	-0.6

*Mass difference determined by subtracting the theoretical mass of the compound from the calculated mass derived from the Q-TOF analysis, expressed in ppm.

Figure 10. Extracted ion chromatograms of a water sample from Lake B, and a table including retention time (RT), compound name, calculated m/z, and difference of the calculated mass from the theoretical mass for each unknown tentatively identified using the Find by Formula tool in MassHunter and the personal compound database (PCD) created for the microcystins in the WHO list. A mass tolerance of 5 ppm and a minimum match score of 70 were used to make the identification of H+ adducts.

Conclusion

When analytical standards are available, the Agilent 1290 Infinity LC System and an Agilent 6460 Triple Quadrupole LC/MS provides an excellent platform for the targeted analysis of microcystins. Analysis by triple quadrupole LC/MS using the Agilent 6540 Q-TOF LC/MS System can confirm suspect compounds using the accurate mass of the molecular ion adducts, as well as MS/MS fragments. The combination of these two technologies supported the hypothesis that dm-HtyR was present in an Alberta lake water sample. Databases can be compiled using the Agilent MassHunter PCDL Manager Software to include the chemical formula for additional microcystins based on reported analogues in the literature. Using MassHunter Find by Formula, previously acquired data files can be retrospectively searched against PCDL databases and libraries for these additional compounds as they become known.

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Identification and Fragmentation of Sucralose Using Accurate-Mass Q-TOF LC/MS and Molecular Structure Correlator Software

Application Note

Food and Environmental

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Abstract

The use of Accurate-Mass Q-TOF LC/MS and MS/MS, in both positive and negative electrospray ionization (ESI) modes, was evaluated for the identification of sucralose in water. Response and fragmentation pathways were investigated. Sucralose responded well using Q-TOF LC/MS in either the positive or negative ion ESI modes. The overall signal intensity obtained in positive ion mode was approximately twice that of negative ion mode.

In positive ion mode, sucralose was detected by its sodium adduct $[M+Na]^+$ at *m/z* 419.0038. Accurate mass MS/MS measurements provided structural confirmation of the sodiated fragments obtained (*m/z* 221.0187 and *m/z* 238.9848). In negative ion mode, the deprotonated molecule was observed ($[M-H]^-$ at *m/z* 395.0073). Fragmentation by MS/MS yielded one characteristic fragment ion (*m/z* 359.0306). Agilent MassHunter Molecular Structure Correlator (MSC) software was used to draw and investigate the fragmentation pathways for the negative and positive ion MS/MS analyses. The MSC software proved to be a useful tool in assisting with the characterization of the fragment ion structures.

Agilent Technologies

Introduction

Due to its intense sweetness, noncaloric properties, low bioaccumulation potential, low toxicity, and the dietary requirements of many consumers, sucralose has become one of the most popular artificial sweeteners used worldwide. Because the human body does not metabolize sucralose, it ends up in wastewater and surface water. Current wastewater treatment technologies do not address sucralose, so it is now ubiquitous in the environment. This is a point of significant concern. A recent study revealed the biological effects of sucralose in the aquatic environment, which may have important toxicological consequences [1]. For these reasons, there is growing interest in measuring sucralose in drinking water, groundwater, surface water, wastewater, and aquatic environments.

Due to its solubility, sucralose is readily analyzed by LC/MS. It contains three chlorine atoms, which produce a distinctive chlorine signature when analyzed by MS. Based on many papers published describing the analysis of sucralose, LC/MS/MS with multiple reaction monitoring (MRM) in negative ion mode is the most popular method. However, the MRM transitions used are not selective enough to identify sucralose in water with the same confidence as with accurate mass. The transitions are not discriminatory because they involve a chlorine loss, which can be present in many other common organic molecules.

This application note evaluates the use of an Agilent 6540 Accurate-Mass Q-TOF LC/MS system in both positive and negative ESI modes for the unequivocal identification of sucralose in water. Response and the usefulness of molecular structure correlation software were investigated. The complementary study of Analytical Methodologies for the Detection of Sucralose in Water in Analytical Chemistry [2] provides a detailed comparison of Q-TOF LC/MS and LC/MS/MS for the detection of sucralose in environmental water samples.

Experimental

A detailed description of the experimental procedures can be found in the complementary journal article published in Analytical Chemistry [2].

Standard preparation

Sucralose was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of sucralose (1,000 µg/mL) was prepared in water and stored at –18 °C. From this solution, working standard solutions were prepared by dilution with methanol and water.

Instrumentation

The standard was analyzed using an Agilent 1290 Infinity Binary LC System coupled to an Agilent 6540 Accurate-Mass Q-TOF LC/MS system with Agilent Jet Stream technology for electrospray ionization.

The HPLC was equipped with a binary pump with an integrated vacuum degasser (G4220A) and an autosampler (G4226A). The HPLC parameters are shown in Table 1.

Table 1. HPLC Parameters

Instrument	Agilent 1290 Infinity Binary LC System
Mobile phases	(A) acetonitrile (B) 0.1% formic acid in water
Gradient	Linear: Initial mobile phase composition was 10% A, held constant for 1.7 minutes, followed by a linear gradient to 100% A, for a total run time of 10 minutes.
Flow rate	0.4 mL/min
Column	Agilent ZORBAX Eclipse Plus reversed phase C18 analytical column, 50 × 2.1 mm, 1.8 µm particle size (p/n 959741-902)
Column temperature	25 °C
Injection volume	20 µL

Q-TOF MS accurate mass spectra were recorded across the range 30–1,000 *m/z* at 2 GHz. Polarity switching was not used; samples were injected twice, one under positive ion mode and the other under negative ion mode. MS/MS experiments were also carried out in both positive and negative ion modes. The Q-TOF MS and MS/MS parameters are shown in Table 2.

Table 2. Q-TOF MS and MS/MS Parameters

Instrument	Agilent 6540 Accurate-Mass Q-TOF LC/MS
Ionization mode	Positive and negative ESI with Agilent Jet Stream technology
Mass range	30–1,000 *m/z* at 2 GHz
Drying gas temperature	250 °C
Drying gas flow rate	10 L/min
Sheath gas temperature	350 °C
Sheath gas flow rate	11 L/min
Nebulizer gas	45 psi
Skimmer voltage	65 V
Octopole RF	750 V
Fragmentor	190 V
Capillary	3,500 V
Nozzle	1,500 V (negative mode) or 0 V (positive mode)
MS/MS parameters	
Targeted MS/MS	Precursors: sodiated (positive ion) and deprotonated molecule (negative ion)
Isolation width	Medium (~4 *m/z*)
Collision energies	10, 20, and 40 eV

A reference solution containing the internal reference masses (purine ($C_5H_4N_4$) at *m/z* 121.0509 and HP-921 [hexakis-($^1H,^1H,^3H$-tetrafluoro-pentoxy)phosphazene] ($C_{18}H_{18}O_6N_3P_3F_{24}$) at *m/z* 922.0098 in positive ion mode, and *m/z* 119.0363 and 966.0007 (formate adduct) in negative ion mode), was delivered by an external quaternary pump.

Stability of mass accuracy was checked daily, and if values went above 2 ppm error, the instrument was recalibrated.

Data analysis

The accurate mass Q-TOF MS and MS/MS data was processed using Agilent MassHunter Workstation Software. Agilent MassHunter Molecular Structure Correlator (MSC) Software was used to draw and investigate the fragmentation pathways for the negative and positive ion MS/MS analyses.

Results and Discussion

Q-TOF LC/MS results

Figure 1 shows the positive and negative ion ESI mass spectra obtained for the sucralose standard using Q-TOF LC/MS. In negative ion mode (Figure 1A), the sucralose molecule lost a proton, forming the base peak with the exact mass of *m/z* 395.0073 (mass error 0.0 ppm). The ions at *m/z* 397.0045 and 399.0018 correspond to the Cl-37 isotopes of sucralose.

In positive ion mode (Figure 1B), the sucralose molecule adducted a sodium ion, forming the ion with a measured accurate mass of *m/z* 419.0040. The measured mass was within 0.5 ppm of the exact mass of *m/z* 419.0038. In this case, a protonated molecule was not formed. The two chlorine isotopes were found at *m/z* 421.0012 and 422.9988.

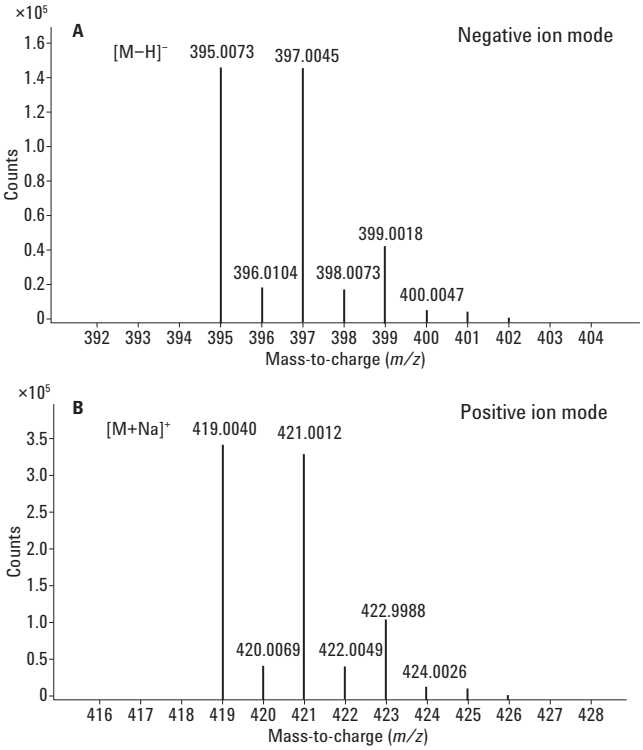

Figure 1. Results from Q-TOF LC/MS analysis of sucralose in (A) negative ion mode and (B) positive ion mode, showing measured accurate masses. In positive ion mode, sucralose was detected as its sodium adduct $[M+Na]^+$ at m/z 419.0040. In negative ion mode, the deprotonated molecule $[M–H]^–$ was observed at m/z 395.0073.

Because the sucralose molecule is hydrogen-rich, allowing a proton shift as needed, the charged sodium can undergo a neutral loss. This mode of fragmentation can be called a sodium migration fragmentation, which is quite rare since sodium adducts are known to be quite difficult, if not impossible, to fragment to produce structurally significant ions [2]. This phenomenon is not commonly reported for ESI of sodium adducts, and has been underestimated for the detection of sucralose using positive ESI.

Figure 2 shows the difference in signal obtained from the sucralose standard using positive and negative ESI Q-TOF LC/MS. The signal intensity obtained in positive ion mode was approximately twice that of negative ion mode. Still, the ratio of signal-to-noise was slightly better in negative ion mode because background ions were less abundant.

Figure 3 shows the accurate mass spectrum obtained by applying Q-TOF MS/MS fragmentation to the sodium adduct of sucralose (positive ion mode). Two characteristic masses were obtained: *m/z* 221.0190 and 238.9853.

Proposed MS/MS fragmentation pathways

Figure 4 shows the proposed fragmentation pathways for negative and positive ion MS/MS. In positive ion mode, the sodium adducted sucralose ion splits into two saccharide fragments, each of which retains the sodium ion. The sodium ion migrates to either the glucose or fructose side of the molecule, giving rise to the ions at *m/z* 221.0187 and 238.9848. In negative ion analyses, sucralose fragments by the loss of HCl, which gives rise to the ion at *m/z* 359.0306. The chemical structures were easily confirmed by the Q-TOF accurate mass measurements with the help of the MSC software.

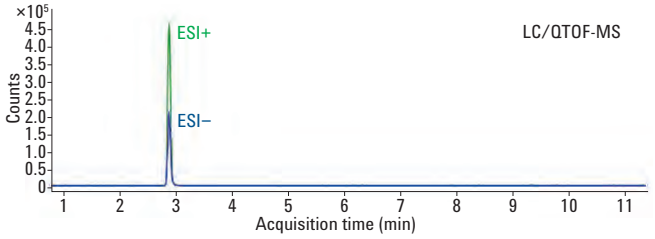

Figure 2. Q-TOF LC/MS analysis of the sucralose standard, comparing the signal intensity obtained in positive and negative ion modes.

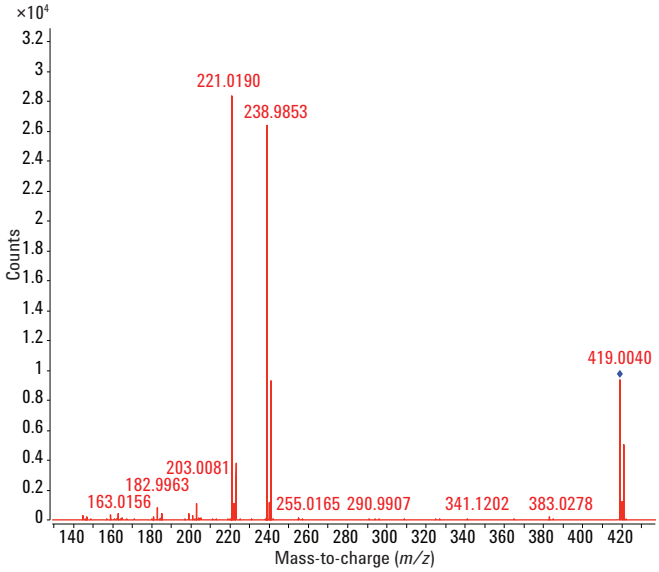

Figure 3. Accurate mass MS-MS spectrum of the sodium adduct of sucralose (positive ion mode).

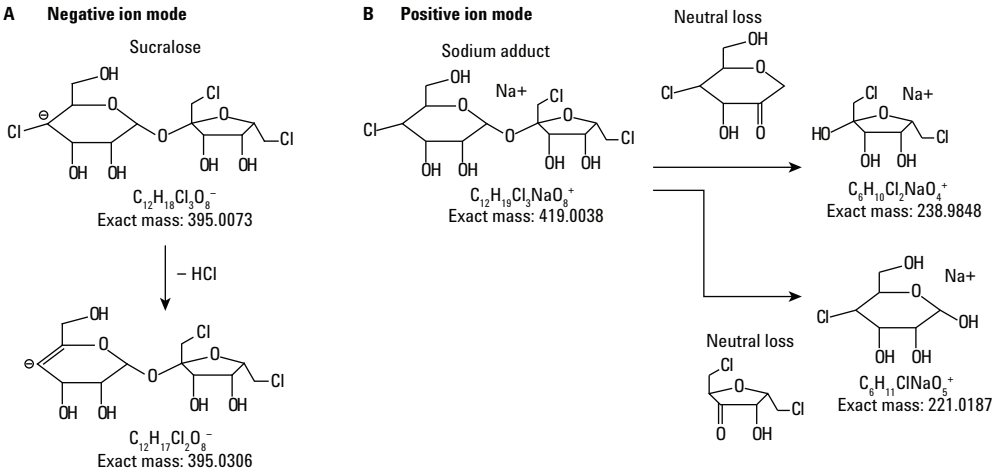

Figure 4. Proposed fragmentation pathways for negative and positive ion MS/MS of sucralose showing exact masses.

As shown in Figures 5 and 6, the fragmentation pathways were investigated using the MSC software. The software correlates accurate mass MS/MS fragment ions of the compound of interest with one or more proposed molecular structures for that compound. MSC accomplishes this by trying to explain each observed fragment ion into the proposed structure using a "systematic bond-breaking"approach.

The input for the MSC software is an accurate mass MS/MS fragment spectrum, a molecular formula for the compound of interest, and candidate molecular structures. The user can input a molecular formula or structure manually, or select the most probable structure from the possible molecular formulas that the MSC calculates using the accurate mass MS and MS/MS information. The MSC then uses the selected formula, retrieves one or multiple possible structures from a .mol file, an .sdf file, a MassHunter compound database (PCD, PCDL), or ChemSpider (over the Internet), and scores how well each candidate structure correlates with the MS/MS spectrum.

Because the MSC software does not currently handle sodiated ions, it was necessary to draw the structure of sucralose with the sodium on the glucose ring; the upper structure shown in the middle Figure 5. The fragment ion is shown on the far right. The row in the table on the far right, highlighted in blue, shows the measured mass of *m/z* 221.0189 that corresponded to the proposed structure for that fragment ion.

The structure of sucralose with sodium on the fructose ring was also drawn using the MSC software (Figure 6). The highlighted portion of the sucralose molecule in the far right box shows the only proposed structure of the fragment ion at *m/z* 238.9852 (the blue highlighted row in the table above the fragment structure.)

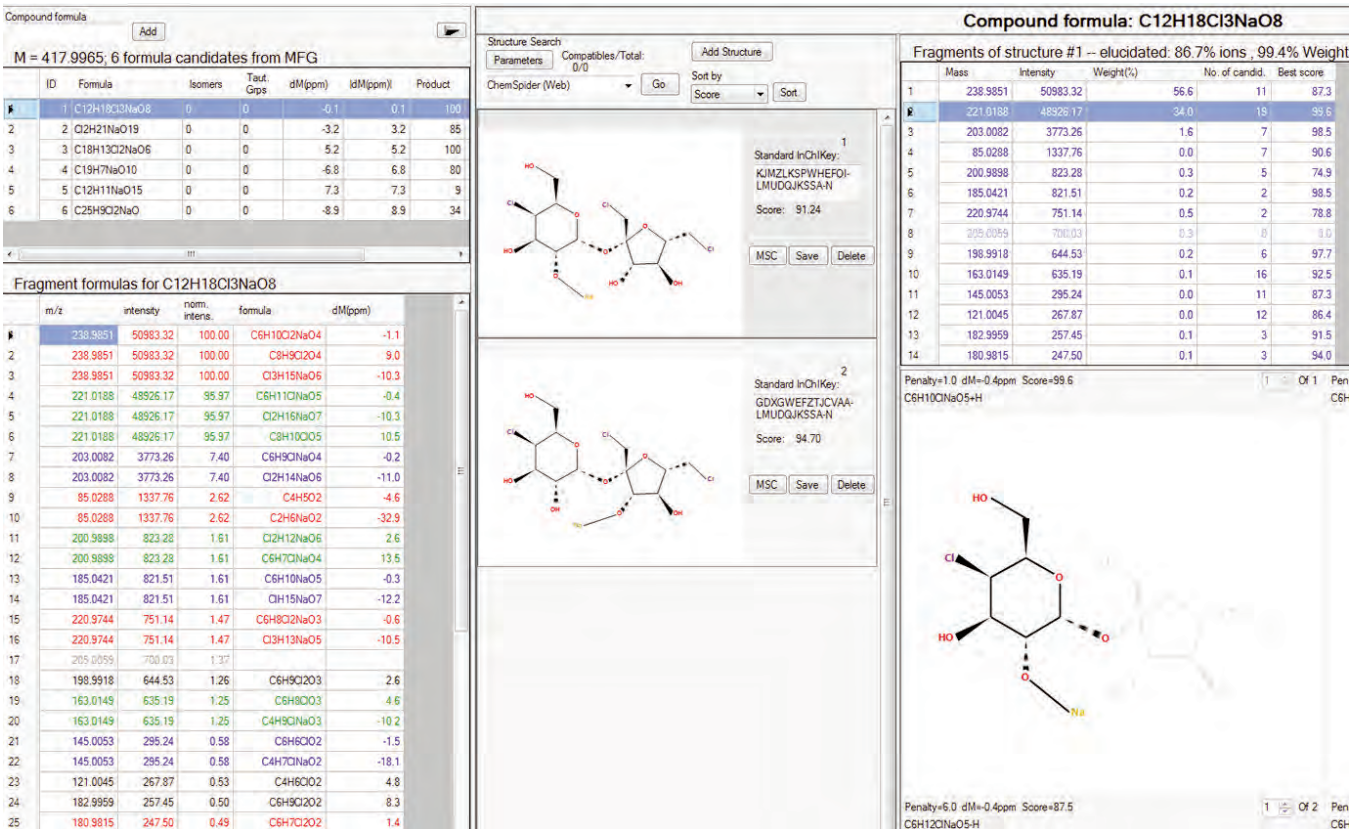

Figure 5. MSC analysis showing the fragment of the sodiated molecule of sucralose with the sodium on the glucose ring.

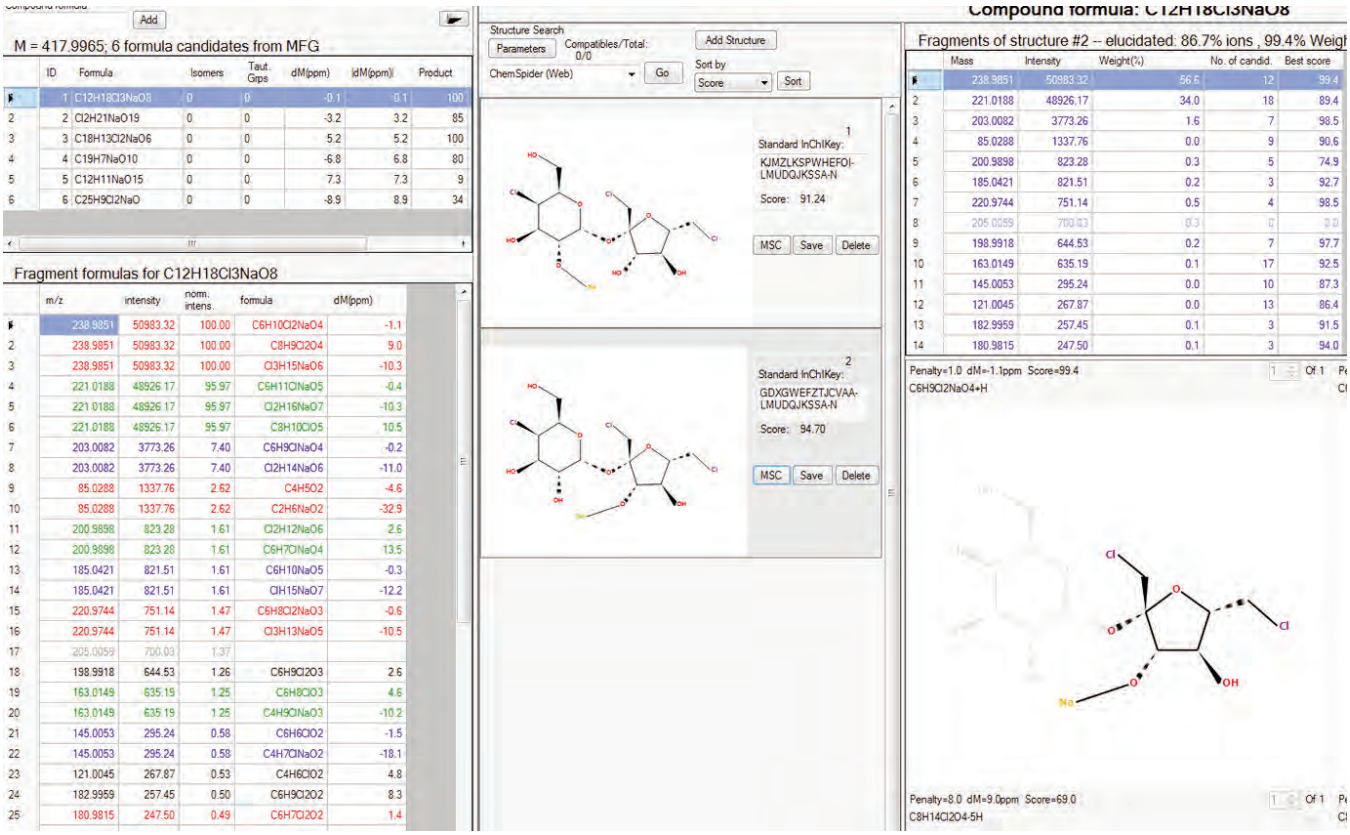

Figure 6. MSC analysis showing the fragment of the sodiated molecule of sucralose with the sodium on the fructose ring.

Figure 7 shows the Q-TOF MS/MS spectrum for sucralose in negative ion mode. The ion at *m/z* 359.0307 represents the loss of HCl, and is a major fragment ion for this compound. The ion at *m/z* 231.9874 is a complex re-arranged sucralose fragment.

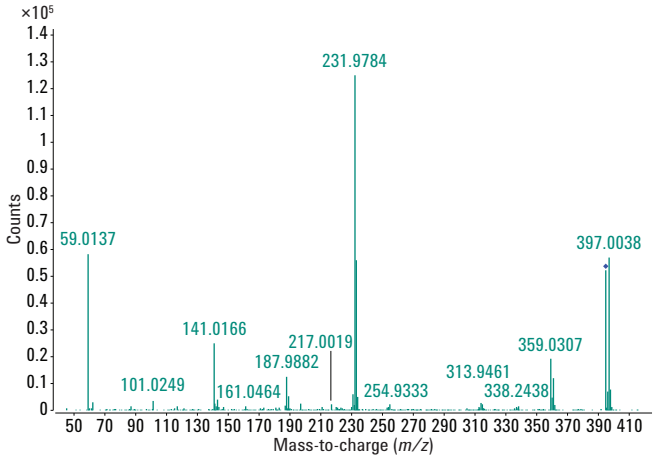

Figure 7. MS/MS spectrum of sucralose (negative ion mode.)

Figure 8 shows the MSC analysis of the fragment ion at *m/z* 359.0307. Both structures are compatible with the loss of HCl. Many of the structures in ChemSpider match that of sucralose; however, for sucralose to be the number one hit in the MSC software, the structures need to be sorted by number of literature references, not the compatibility score (red arrow in Figure 8.)

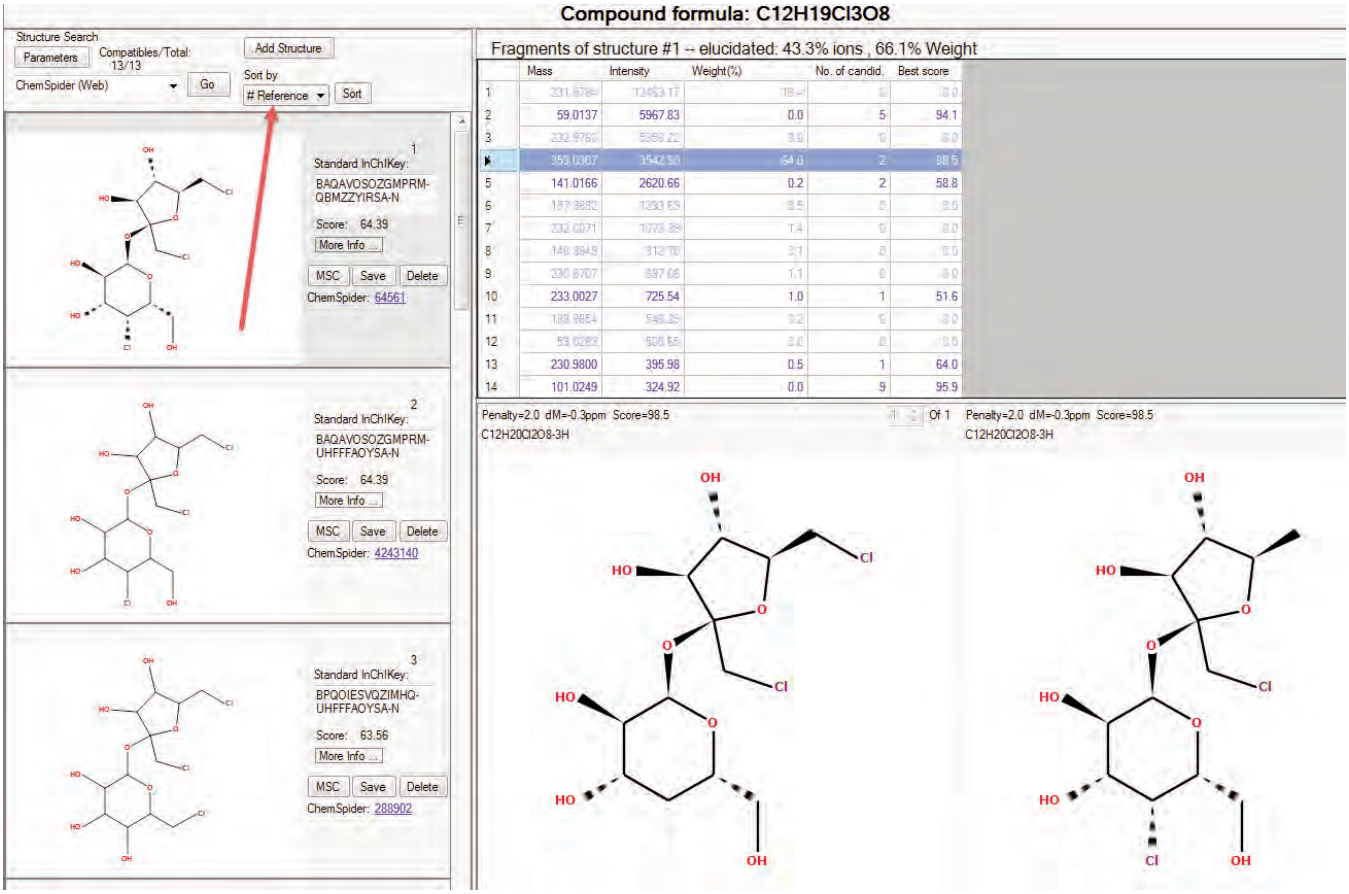

Figure 8. MSC analysis of the Q-TOF MS/MS spectrum obtained in negative ion mode. When searching ChemSpider, all structures have same score. Sorting by number of literature references (# references) raises sucralose to the first (top) position (see red arrow).

Conclusions

Sucralose responded well using Q-TOF LC/MS when operated in either the positive or negative ESI mode. Sucralose formed a strong sodium adduct in positive ion mode and readily lost a proton in negative ion mode. The overall signal intensity obtained in positive ion mode was approximately twice that of negative ion mode. As demonstrated in the complementary study of detection methodologies for sucralose in water, for the triple quadrupole LC/MS MRM method, sensitivity was higher in the positive ion mode (using the two transitions shown in this application note), than in the negative ion mode [2].

Contrary to what is commonly reported for ESI analyses of sodium adducts, the strong sodium adduct formed in the positive ion mode was easily fragmented by MS/MS. The two characteristic accurate mass fragments produced can be used to identify sucralose unequivocally. The MSC software is a useful tool to assist with the characterization of fragment ion structures.

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1. A.K.E Wiklund, M. Breitholtz, B.E. Bengtsson, and M. Adolfsson-Erici "Sucralose – An ecotoxicological challenger?" *Chemosphere* 2012, **86**, 50–55.

2. I. Ferrer, J.A. Zweigenbaum, and E.M. Thurman "Analytical Methodologies for the Detection of Sucralose in Water" *Analytical Chemistry* 2013, **85**, 9581-9587.

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Sensitive Screening of Pharmaceuticals and Personal Care Products (PPCPs) in Water Using an Agilent 6545 Q-TOF LC/MS System

Application Note

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Abstract

As a follow up to the Application Note on detection of pharmaceuticals and personal care products (PPCPs) in water using the Agilent 6495 Triple Quadrupole Mass Spectrometer[8], this Application Note describes two methods to screen and quantitate PPCPs in water at part per trillion (ppt) levels using the Agilent 6545 Q-TOF LC/MS System. Similarly, the methods were divided into positive ion mode and negative ion mode due to the unique mobile phases used for the two methods. The precise and accurate screening and quantitation of 118 compounds in positive ion mode and 22 compounds in negative mode was accomplished on the 6545 Q-TOF LC/MS using the Swarm tune parameters optimized for small fragile organic molecules. The high sensitivity slicer mode was selected to maximize instrument sensitivity. Most of the PPCPs could be detected without tedious analyte enrichment such as solid phase extraction (SPE). The extent of sample preparation included filtering approximately 3 mL of sample, adding internal standards to a 1.0 mL aliquot of the filtered sample, and injecting 40 µL of sample for analysis by Q-TOF LC/MS with reporting limits for the majority of analytes at 10 ppt. The limit of detection (LOD) and lower limit of quantitation (LLOQ) for most of the analytes are much lower than 10 ppt.

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Introduction

Pharmaceuticals and Personal Care Products (PPCPs) are comprised of thousands of chemical substances, including prescription and over-the-counter therapeutic drugs, veterinary drugs, fragrances, and cosmetics. Several studies have shown that pharmaceuticals and their metabolites are present in our waterbodies[1,2]. PPCPs in surface waters can eventually enter drinking water systems when treatments are insufficient. Governmental agencies, such as the EPA and the European Water Framework, have proposed regulations to monitor water supply systems[3,4]. High performance liquid chromatography (HPLC) in combination with high resolution Q-TOF mass spectrometry is gaining traction to investigate the occurrence and fate of PPCPs in water systems. There are several advantages associated with the analysis of PPCPs by Q-TOF LC/MS:

- Screening of a large number of analytes within one run
- Retrospective data mining for new analytes
- No need for individual standards for fragmentation information
- Structure confirmation by MS/MS fragments

Compared with targeted analysis (for example, triple quadrupole), Q-TOF LC/MS has the added benefit of nontargeted or semitargeted screening for unknowns.

The detection limits for PPCPs in drinking water are typically in the low part per trillion (ppt) levels. This poses significant challenges in analytical methodology and instrumentation. Sample enrichment by solid phase extraction (SPE) is often performed to reach these levels in drinking water samples[5]. SPE requires large sample quantities, high consumption of solvents, and laborious procedures. PPCPs analysis also has the complexity of significant contamination, such as urban surface water sources, where some of the PPCPs can be found above part per billion (ppb) levels. In addition to higher analyte concentrations, total organic carbon levels in these samples also increase. This can add substantial interferences to the analytes. The instrumentation required for PPCPs analysis must have, not only a broad dynamic range, but also provide precise and accurate screening and quantitation through excellent mass accuracy and resolution.

The Agilent 6545 Q-TOF LC/MS, in combination with the Agilent Jet Stream Ionization source, meets the dynamic analytical demands for the occurrence and fate of PPCPs in water along with the convenience of direct sample injection. Several modifications associated with the 6545 Q-TOF LC/MS have resulted in higher analytical performance compared to previous model. Some of these improvements include:

- A new slicer design with the option to operate in high sensitivity or high resolution mode
- A new high performance high voltage power supply, along with a new pulser to improve mass accuracy and resolution
- A new enhanced gain-shifted detector that provides much better instrument robustness
- A new front end ion optics for increased precursor ion transmission

The most noteworthy change is the new Particle Swarm Optimization technology. For the first time, the Particle Swarm Optimization technology is used to optimize the 6545 Q-TOF LC/MS mass spectrometers (called Swarm autotune). Swarm autotune provides many choices to maximize sensitivity or mass resolution. First, it can optimize ion transmission at particular mass ranges (for example, 50–250 *m/z*, 50–750 *m/z*, or 50–1,700 *m/z*) based on application needs. Secondly, the improvements in ion transmission for small molecules has also resulted in enhanced mass accuracy below 100 *m/z*. Lastly, instrument parameters can be tuned according to the fragility of analytes, which requires milder ion transmission parameters to preserve their molecular masses. In combination with the modifications, and the ability to select the high sensitivity slicer mode, a substantial increase in signal response compared with the previous generation of the instrument has been achieved[6].

Experimental and Instrumentation

Reagents and chemicals

All reagents and solvents were HPLC-MS grade. Acetonitrile was purchased from Honeywell (015-4). Ultrapure water was obtained from a Milli-Q Integral system equipped with a LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak). Ammonium acetate, 5 M solution, was purchased from Fluka (09691-250ML). Acetic acid was purchased from Aldrich (338828-25ML). The PPCP standards and some of the internal standards were acquired from an outside collaborator. The list of analytes and their internal standards are listed in Table 1 for the positive ion mode method and Table 2 for the negative ion mode method. Personal Compound Database Libraries (PCDLs) for analytes were created using the Agilent PCDL Manager (B.07.00) with retention time acquired with standards.

Table 1. Analytes and internal standards in positive ion mode method.

Compound	Mass	RT (min)	Compound	Mass	RT (min)
10,11-Dihydro-10-hydroxycarbamazepine	254.10553	5	MDMA	193.11028	3.91
6-Acetylmorphine	327.14706	3.72	MDMA-D5	198.14166	3.9
6-Acetylmorphine-D6	333.18472	3.71	Mefenamic acid	241.11028	8.15
Acebutolol	336.20491	4.39	Mefenamic acid-D3	244.12911	8.15
Acetaminophen	151.06333	2.92	Meperidine	247.15723	4.98
Acetaminophen-D4	155.08844	2.92	Meperidine-D4	251.18234	4.97
Albuterol	239.15214	2.77	Meprobamate	218.12666	5.15
Amitriptyline	277.18305	6.67	Meprobamate-D7	225.17059	5.14
Amitriptyline metabolite	293.17796	5.06	Metformin	129.10145	1
Amitriptyline-D3	280.20188	6.66	Methadone	309.20926	6.74
Amphetamine	135.1048	3.6	Methadone-D9	318.26576	6.71
Amphetamine-D5	140.13618	3.57	Methamphetamine	149.12045	3.82
Aripiprazole	447.14803	7.29	Methamphetamine-D11	160.18949	3.78
Aripiprazole-D8	455.19825	7.14	Methotrexate	454.17132	3.26
Atenolol	266.16304	2.88	Methotrexate-D3	457.19015	3.26
Atenolol-D7	273.20698	2.87	Methylphenidate	233.14158	4.65
Atorvastatin	558.253	7.51	Methylphenidate-D9	242.19807	4.64
Atrazine	215.09377	7.03	Metoprolol	267.18344	4.53
Atrazine-D5	220.12516	7	Mevastatin	390.24062	9.42
Benzoylecgonine	289.13141	4.01	*m*-Hydroxybenzoylecgonine	305.12632	3.73
Benzoylecgonine-D3	292.15024	4.01	Modafinil	273.08235	5.68
Buprenorphine	467.30356	8.07	Modafinil-D10	283.14512	5.65
Buprenorphine-D4	471.32867	7.72	Monoethylglycinexylidide	206.14191	3.8
Bupropion	239.10769	5.33	Montelukast	585.21044	10.88
Caffeine	194.08038	3.6	Morphine	285.13649	2.4
Caffeine-$^{13}C_3$	197.09044	3.6	Morphine-D3	288.15532	2.39
Carbamazepine	236.09496	6.28	Nifedipine	346.11649	7.57
Carbamazepine 10,11 epoxide	252.08988	5.47	Nifedipine oxidized	344.10084	7.48
Carbamazepine-D10	246.15773	6.22	Norfentanyl	232.15756	4.21
Carisoprodol	260.17361	6.75	Norfentanyl-D5	237.18895	4.19
Carisoprodol-D7	267.21754	6.72	Norfluoxetine	295.1184	6.55
Chlorpheniramine	274.12368	5.47	Norfluoxetine-D6	301.15606	6.53
Clenbuterol	276.07962	4.6	Normeperidine	233.14158	4.9
Clenbuterol-D9	285.13611	4.59	Normeperidine-D4	237.16669	4.89
Clopidogrel carboxylic acid	307.04338	4.69	Norquetiapine	295.11432	5.82
Cocaethylene	317.16271	5.42	Norsertraline	291.05815	6.87
Cocaethylene-D3	320.18154	5.41	Norsertraline-$^{13}C_6$	297.07828	6.71
Cocaine	303.14706	4.96	Norverapamil	440.26751	6.48
Cocaine-D3	306.16589	4.95	Omeprazole	345.11471	5.92
Codeine	299.15214	3.4	Oxazepam	286.05091	6.52
Codeine-D6	305.1898	3.39	Oxcarbazepine	252.08988	6.47
Cotinine	176.09496	3.69	Oxycodone	315.14706	3.68
Cotinine-D3	179.11379	3.38	Oxymorphone	301.13141	2.65
DEET	191.13101	7.1	Oxymorphone glucuronide	477.1635	1.13
DEET-D6	197.16867	7.06	Oxymorphone glucuronide-D3	480.18233	1.12
Dehydroaripiprazole	445.13238	6.87	Oxymorphone-D3	304.15024	2.63
Desmethylcitalopram	310.14814	5.81	Paroxetine	329.14272	6.22
Desmethylcitalopram-D3	313.16697	5.81	Paroxetine-D6	335.18038	6.21

Compound	Mass	RT (min)	Compound	Mass	RT (min)
Desmethylvenlafaxine	263.18853	4.6	Phenmetrazine	177.11536	3.74
Desmethylvenlafaxine-D6	269.22619	4.23	Phentermine	149.12045	3.97
Dextromethorphan	271.19361	5.69	Phentermine-D5	154.15183	3.94
Dextromethorphan-D3	274.21244	5.68	Phenylpropanolamine	151.09971	2.93
Diltiazem	414.16133	6.14	Phenylpropanolamine-D3	154.11854	2.93
Diphenhydramine	255.16231	5.88	Pioglitazone	356.11946	7.72
Diphenhydramine-D3	258.18114	5.88	Pregabalin	159.12593	2.73
Disopyramide	339.23106	4.87	Pregabalin-D6	165.16359	2.76
Donepezil	379.21474	5.65	Primidone	218.10553	4.43
Duloxetine	297.11873	6.47	Propranolol	259.15723	5.52
Duloxetine-D3	300.13757	6.47	Propranolol-D7	266.20117	5.5
Ecgonine methyl ester	199.12084	1.15	Pseudoephedrine	165.11536	3.3
Ecgonine methyl ester-D3	202.13967	1.15	Pseudoephedrine-D3	168.13419	3.29
EDDP	277.18305	6.31	Quetiapine	383.16675	6.27
EDDP-D3	280.20188	6.31	Quetiapine-D8	391.21696	6.17
Erythromycin	733.46124	5.78	Ritalinic acid	219.12593	3.78
Erythromycin-$^{13}C_2$	735.46795	5.78	Ritalinic acid-D10	229.1887	3.75
Erythromycin-anhydro	715.45068	6.3	Sertraline	305.0738	6.88
Escitalopram	324.16379	5.92	Sertraline-D3	308.09264	6.87
Famotidine	337.04493	2.89	Sildenafil	474.20492	6.65
Fentanyl	336.22016	5.9	Simvastatin	418.27192	10.4
Fentanyl-D5	341.25155	5.88	Sotalol	272.11946	2.93
Fluoxetine	309.13405	6.7	Sulfamethazine	278.08375	4.45
Fluoxetine-D6	315.17171	6.69	Sulfamethazine-$^{13}C_6$	284.10388	4.45
Fluticasone propionate	500.18443	9.05	Sumatriptan	295.13545	3.5
Gabapentin	171.12593	2.75	Tadalafil	389.13756	6.86
Gabapentin-D10	181.1887	2.72	Temazepam	300.06656	7.2
Glyburide	493.14382	8.27	Temazepam-D5	305.09794	7.16
Hydrocodone	299.15214	3.84	Thiabendazole	201.03607	5.18
Hydrocodone-D6	305.1898	3.84	Thiabendazole-$^{13}C_6$	207.0562	5.19
Hydromorphone	285.13649	2.9	Tramadol	263.18853	4.6
Hydromorphone-D3	288.15532	2.89	Tramadol-^{13}C-D3	267.21071	4.58
Hydroxybupropion	255.10261	4.62	Trazadone	371.15129	5.9
Hydroxybupropion-D6	261.14027	4.61	Trazadone-D6	377.18895	5.81
Ketoprofen	254.09429	6.42	Triamterene	253.10759	4.12
Lamotrigine	255.00785	4.73	Trimethoprim	290.13789	3.95
Lamotrigine-$^{13}C^{15}N_4$	259.99935	4.74	Trimethoprim-$^{13}C_3$	293.14795	3.94
Lamotrigine-$^{13}C_3$	258.01792	4.73	Tylosin	915.51915	6.12
Levorphanol	257.17796	4.43	Valsartan	435.22704	5.97
Lidocaine	234.17321	4.51	Venlafaxine	277.20418	5.19
Loratadine	382.14481	9.38	Venlafaxine-D6	283.24184	5.19
Lorazepam	320.01193	6.67	Verapamil	454.28316	6.63
Lorazepam-D4	324.03704	6.67	Zolpidem	307.16846	6.02
MDA	179.09463	3.73	Zolpidem phenyl-4-carboxylic acid	337.14264	3.93
MDEA	207.12593	4.18	Zolpidem-D7	314.2124	5.98

Table 2. Analytes and internal standards in negative ion mode method.

Compound	Mass	RT (min)	Compound	Mass	RT (min)
(±)11-Nor-9-carboxy-*delta*-THC	344.19876	6.568	Diclofenac 4-hydroxy	311.0116	5.067
$^{13}C_{12}$ Triclosan	299.99142	6.535	Fenbufen	254.09429	5.317
$^{13}C_{3}$ Ibuprofen	209.14074	5.965	Furosemide	330.00772	4.712
$^{13}C_{6}$ Diclofenac 4-hydroxy	317.03173	5.066	Gemfibrozil	250.15689	6.32
$^{13}C_{6}$ Methylparaben	158.06747	4.216	Hydrochlorothiazide	296.96447	3.341
$^{13}C_{6}$ *n*-Butylparaben	200.11442	5.458	Ibuprofen	206.13068	5.958
$^{13}C_{6}$ Sulfamethoxazole	259.07224	4.096	Methylparaben	152.04734	4.21
$^{13}C_{6}$ Triclocarban	319.99818	6.512	Modafinil acid	274.06637	4.619
Bezafibrate	361.10809	5.257	Naproxen	230.09429	5.225
Celecoxib	381.07588	5.967	*n*-Butylparaben	194.09429	5.451
Chloramphenicol	322.01233	4.15	Phenobarbital	232.08479	4.184
D10 Phenytoin	262.15265	4.58	Phenytoin	252.08988	4.6
D4 Diclofenac	299.04179	5.87	Pravastatin	424.2461	4.326
D5 Chloramphenicol	327.04371	4.14	Sulfamethoxazole	253.05211	4.1
D5 Phenobarbital	237.11618	4.175	Triclocarban	313.97805	6.519
D6 Gemfibrozil	256.19456	6.304	Triclosan	287.95116	6.535
D9 (±)11-Nor-9-carboxy-*delta*-THC	353.25525	6.546	Warfarin	308.10486	5.532
Diclofenac	295.01668	5.88			

Instrumentation and conditions

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Standard Autosampler (G4226A) and sample cooler (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

UHPLC conditions are listed in Table 3 for positive ion mode, and Table 4 for negative ion mode.

Table 3. Agilent 1290 Infinity UHPLC conditions for the positive ion mode method.

Parameter	Value
Column	Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902)
Column temperature	40 °C
Injection volume	40 µL
Speed	Draw 100 µL/min; Eject 200 µL/min
Autosampler temperature	6 °C
Needle wash	5 seconds (80 % MEOH/20 % water)
Mobile phase	A) Water with 5 mM ammonium acetate + 0.02 % acetic acid B) Acetonitrile
Flow rate	0.3 mL/min
Gradient program	Time %B 0 5 0.5 5 11 100 13 100 13.1 5
Stop time	15 minutes
Post time	1 minute

MS detection

An Agilent 6545 Q-TOF LC/MS with an Agilent Jet Stream electrospray ionization source was used.

Jet Stream ionization source parameters are critical for the sensitive detection of analytes[7]. For multiple analyte applications, parameters are typically weighted towards hard-to-detect analytes. In this case, source parameters were accessed based on the triple quadrupole data and other studies on the particular compounds[8]. Mass spectrometer source conditions are listed in Table 5 for the positive ion mode method, and Table 6 for the negative ion mode method.

Software

- Agilent MassHunter data acquisition for Q-TOF mass spectrometer, Version B.06.01
- Agilent MassHunter Qualitative Software, Version B.07.00 Build 7.0.7024.0
- Agilent MassHunter Quantitative Software, Version B.07.00 Build 7.0.457.0

Table 4. Agilent Infinity 1290 UHPLC conditions for the negative ion mode method.

Parameter	Value
Column	Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902)
Column temperature	40 °C
Injection volume	40 µL
Speed	Draw 100 µL/min; Eject 200 µL/min
Autosampler temperature	6 °C
Needle wash	5 seconds (80 % MEOH/20 % water)
Mobile phase	A) Water with 0.005 % acetic acid B) Acetonitrile
Flow rate	0.3 mL/min
Gradient program	Time %B 0 5 0.5 5 6 100 8 100 8.1 5
Stop time	10 minutes
Post time	1 minute

Table 5. Agilent 6545 Q-TOF LC/MS source parameters for positive ion mode method.

Parameter	Value
Mode	2 GHz Extended dynamic range; high sensitivity slicer mode
Tune	50–250 *m/z*; Fragile ions
Drying gas temperature	150 °C
Drying gas flow	10 L/min
Sheath gas temperature	375 °C
Sheath gas flow	11 L/min
Nebulizer pressure	35 psi
Capillary voltage	3,500 V
Nozzle voltage	200 V
Fragmentor	125 V
Skimmer	45 V
Oct1 RF Vpp	750 V
Acq mass range	100–1,000 *m/z* (MS only)
Acq rate	3 spectra/s
Ref mass ions	121.050873, 922.009798

Dilutions

Stock solutions for analyte standards and internal standards were prepared at 25 ppb in acetonitrile for each compound. All samples were fortified with internal standards at a constant concentration of 100 ppt, while calibration standards were spiked at 10 ppt, 25 ppt, 50 ppt, 100 ppt, 250 ppt, 500 ppt, and 1,000 ppt (seven levels) in Milli-Q water.

Two of the three unknown samples were from an outside collaborator. One was from a remote site removed from significant anthropogenic sources, and one was from an urban surface water source. Another sample was freshly collected local tap water (Santa Clara, USA). All samples were fortified with internal standards at 100 ppt after filtration.

Results and Discussion

System stability

System stability was evaluated using 300 continuous injections of reserpine samples at 100 ppb in 70 % acetonitrile with a gradient of 1.5 minutes. The acquisition was set to 2 spectra per second in the presence of internal reference masses (*m/z* 121.0509 and 922.0098). The mass accuracy was obtained by Agilent MassHunter Qualitative Analysis. For all 300 injections, mass accuracy remained very stable, within 0.25 ppm, as illustrated by Figure 1. The area %RSD for 300 injections was 2.56 % with three separate sample preparations.

Table 6. Agilent 6545 Q-TOF LC/MS source parameters for negative ion mode method

Parameter	Value
Mode	2 GHz Extended dynamic range; high sensitivity slicer mode
Tune	50–250 *m/z*; Fragile ions
Drying gas temperature	200 °C
Drying gas flow	12 L/min
Sheath gas temperature	375 °C
Sheath gas flow	12 L/min
Nebulizer pressure	35 psi
Capillary voltage	4,000 V
Nozzle voltage	2,000 V
Fragmentor	110 V
Skimmer	40 V
Oct1 RF Vpp	750 V
Acq mass range	100–1,000 *m/z* (MS only)
Acq rate	2 spectra/s
Ref mass ions	119.03632, 966.000725

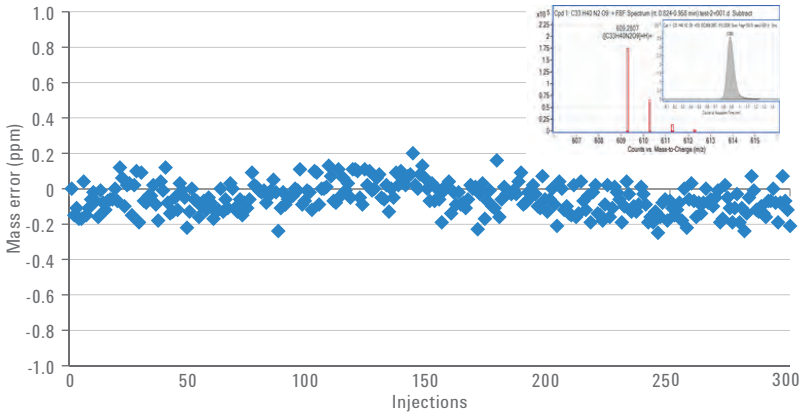

Figure 1. Excellent system stability. Mass accuracy was maintained within 0.25 ppm for 300 continuous injections of reserpine samples with area %RSD of 2.56 % .

Increased method performance

The sensitivity of the 6545 Q-TOF LC/MS was maximized by tuning the instrument to the 50–250 *m/z* range for this PPCPs application, and setting the slicer to high sensitivity mode. It is revolutionary that the user can optimize the ion transmission based on an analyte's *m/z*, especially for midrange mass spectrometers. The fragile ion option also prevents organic compounds from degrading during ion transmission. All these factors contribute to the sensitive detection of PPCPs at low ppt levels without tedious sample enrichment. Databases for the positive ion mode compounds and negative ion mode compounds were created using the PCDL manager (B.07.00) with retention time for the analytes and isotope-labeled internal standards. Data were initially evaluated in the Agilent MassHunter Qualitative Analysis Software (B.07.00) using Find by Formula with a mass error of 5 ppm and a retention time window of ± 0.5 minutes. Figure 2 shows the responses of the 118 analytes in positive ion mode, and Figure 3 shows the 22 analytes in negative ion mode at 25 ppt.

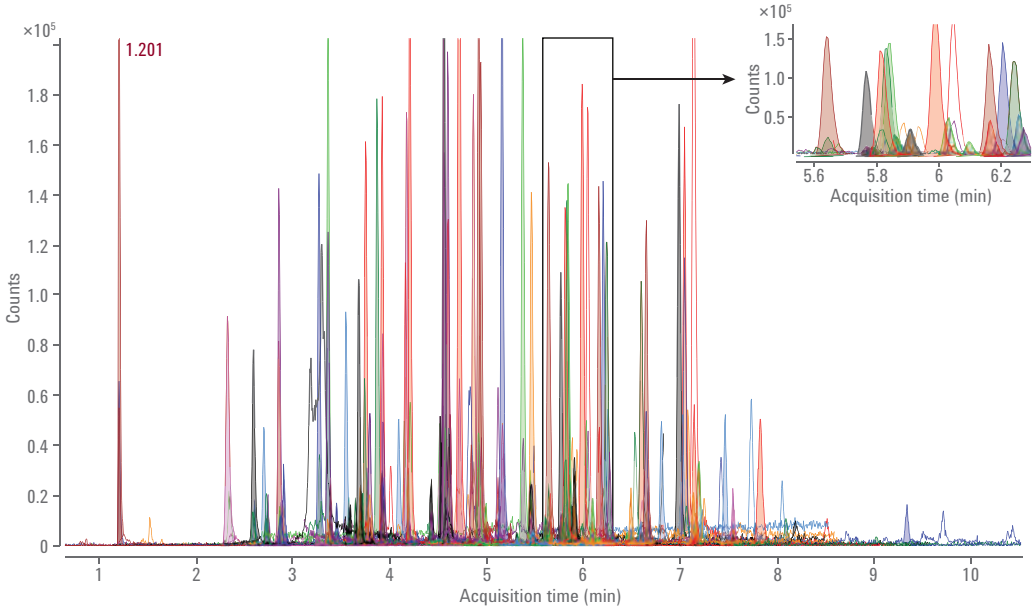

Figure 2. Signal response in positive ion mode (25 ppt at 40 µL direct injection).

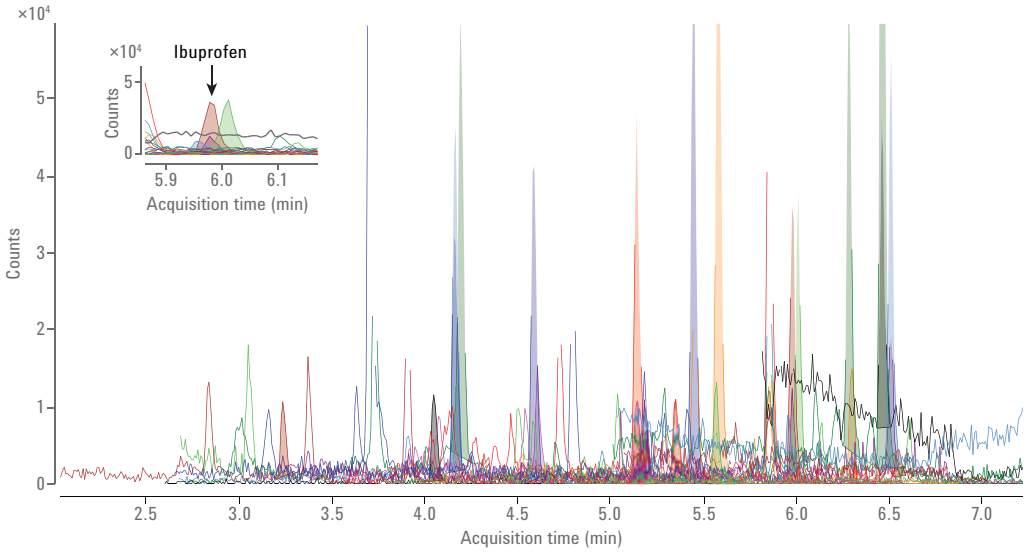

Figure 3. Signal response in negative ion mode (25 ppt at 40 µL direct injection).

Most of the compounds can be detected at a concentration much lower than 10 ppt without sample enrichment. Figure 4 shows the number of compounds that could be quantified, lower limit of quantitation (LLOQ), at each concentration level with accuracy between 80–120 % for at least three out of five replicates. There were 140 compounds, 118 in positive ion mode and 22 in negative ion mode. Norsertraline was not detected in all spiked concentrations, probably due to degradation since the stock standard was over three months old. Of the 44 compounds that failed to be quantified at 10 ppt, approximately 43 % failed due to quantitation accuracy beyond 80–120 %.

Due to the improvement in mass accuracy and the increased sensitivity as well as innate quantitation accuracy of the 6545 Q-TOF LC/MS, high confidence compound identification was achieved based not only on mass accuracy but also on isotopic abundance and spacing. An example is presented in Figure 5. 6-Acetylmorphine was identified with an overall target score of 93.43 out of 100 at 25 ppt in the presence of ~1,000x coeluting ions.

Calibration curves

Calibration curves were assessed with PPCPs spiked in Milli-Q water covering a concentration range from 10 ppt to 1,000 ppt. Some of the analytes had corresponding isotope-labeled internal standards. All samples were fortified with internal standards at a constant concentration of 100 ppt. Calibration curves were generated using a quadratic fit with a weighting factor of 1/x, including the origin. The correlation coefficients (R^2) for most of the target analytes in both polarities were equal to or greater than 0.99; most were greater than 0.995, except for methotrexate (R^2 = 0.978) and thiabendazole (R^2 = 0.984). The calibration curves for cotinine in positive ion mode and ibuprofen in negative ion mode are shown as examples in Figure 6.

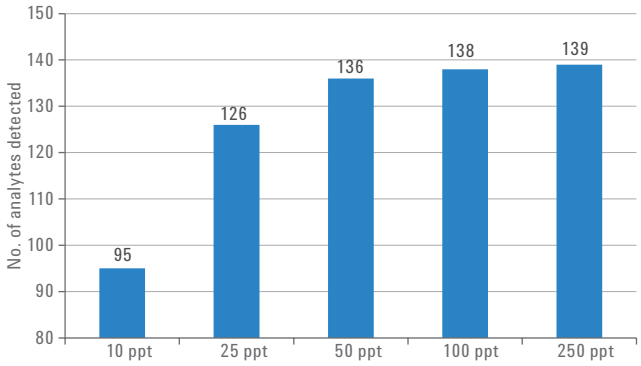

Figure 4. Number of compounds that could be quantified at each concentration level with 40 µL direct injection of water samples.

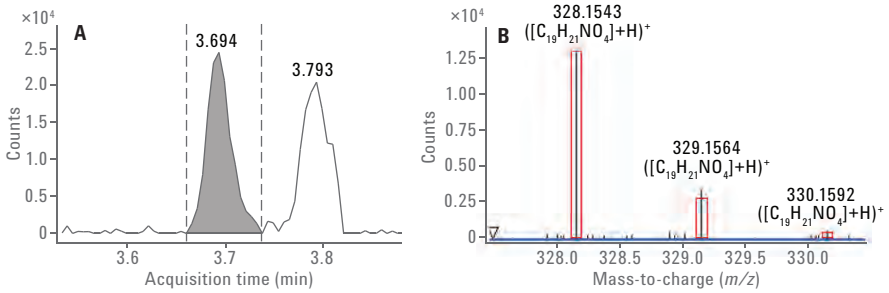

Figure 5. Identify with confidence. 6-acetylmorphine at 25 ppt can be detected with high confidence (target score 93.43 out of 100) in the presence of ~1,000x coeluting ions.

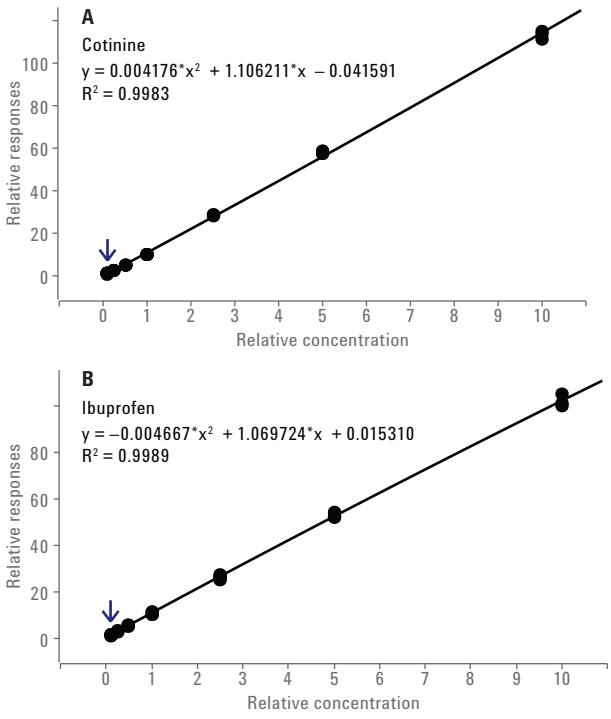

Figure 6. Calibration curves of cotinine (positive) and ibuprofen (negative) in Milli-Q water.

Precision and accuracy

The %RSD calculation was based on five replicate injections of 138 compounds made from 10 ppt to 100 ppt (95 compounds at 10 ppt, 31 compounds at 25 ppt, 10 compounds at 50 ppt, and two compounds at 100 ppt). The %RSD results are shown in Figure 7. About 79 % of the compounds could be quantified with a %RSD of less than 10 %. Only four compounds had elevated %RSD of 20–25 %. These results clearly demonstrate the precise quantitative ability of the 6545 Q-TOF LC/MS due to the modifications, the high sensitivity slicer mode, and fast data acquisition.

Quantification accuracy on the 6545 Q-TOF LC/MS is exceptional due to excellent mass accuracy and mass resolution. This is reflected by the number of compounds that can be quantified at low ppt levels without sample enrichment. One requirement for the analytes to be considered detectable is that the concentration accuracy of at least three of the five replicates had to be within 80–120 %. At 10 ppt, 43 % of 44 compounds failed due to quantification accuracy beyond 80–120 % even though the signal-to-noise for these analytes was much greater than 5. Quantification accuracy was affected more drastically at lower levels mainly due to slight background influence on peak integration.

Real-world samples

Three samples were tested. The first was freshly collected from local tap water. The other two samples were from an outside collaborator, one from a remote site removed from significant anthropogenic sources, and the other from an urban surface water source. Duplicate injections were run on each sample. The compound was reported if the average concentration of the two runs was greater than 10 ppt. The results are listed in Tables 7–10. Figure 8 and Figure 9 represent the chromatographs for the two unknown samples with only 2–3 PPCPs identified.

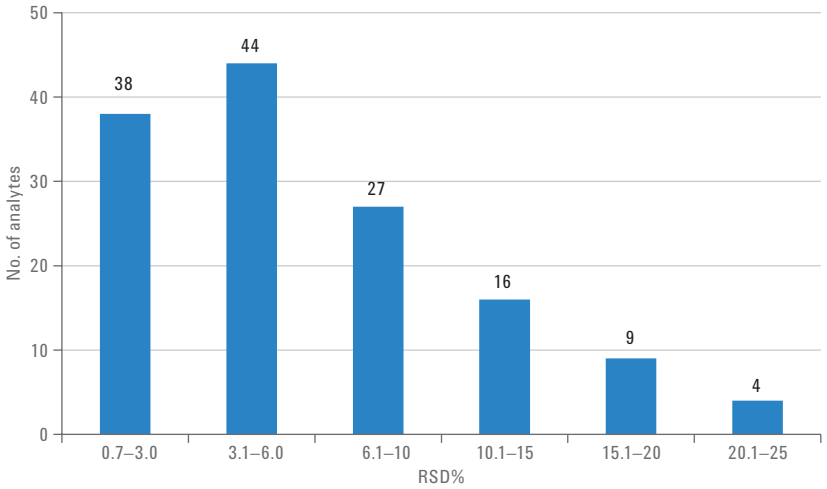

Figure 7. Measurement precision of five replicates at LLOQ levels from 10 ppt to 100 ppt; 79 % of analytes had %RSD less than 10 %.

Table 7. Compounds found in local tap water with positive ion mode method.

Name	Inj 1 (ppt)	Inj 2 (ppt)	Avg. (ppt)
Normeperidine	28.4	30.4	29.4
Temazepam	12.4	13.9	13.2

Table 8. Compounds found in remote source sample with positive ion mode method.

Name	Inj 1 (ppt)	Inj 2 (ppt)	Avg. (ppt)
6-Acetylmorphine	17.7	18.9	18.3
DEET	106.7	107.6	107.1
Temazepam	17.9	18.6	18.2

Table 9. Compounds found in an urban surface water sample with positive ion mode method.

Name	Inj 1 (ppt)	Inj 2 (ppt)	Avg. (ppt)	Name	Inj 1 (ppt)	Inj 2 (ppt)	Avg. (ppt)
10,11-Dihydro-10-hydroxycarbamazepine	82.5	82.3	82.4	MDMA	18.5	14.1	16.3
Acebutolol	21.1	20.5	20.8	Meprobamate	105.6	116.5	111.0
Amitriptyline	33.9	35.3	34.6	Metformin	2796.1	2774.4	2785.3
Atenolol	1590.3	1476.8	1533.5	Methadone	42.6	42.4	42.5
Atrazine	40.1	40.2	40.1	Methamphetamine	250.8	249.5	250.1
Bupropion	167.9	160.2	164.0	Metoprolol	426.4	425.3	425.9
Caffeine	719.4	660.1	689.8	Modafinil	21.0	19.8	20.4
Carbamazepine	211.2	219.2	215.2	Monoethylglycinexylidide	44.5	52.3	48.4
Carbamazepine 10,11 epoxide	62.1	56.0	59.1	Norquetiapine	56.6	59.5	58.0
Carisoprodol	31.0	29.9	30.5	Oxazepam	25.6	24.1	24.8
Chlorpheniramine	30.7	29.9	30.3	Oxycodone	94.1	94.6	94.4
Clopidogrel carboxylic acid	144.2	142.8	143.5	Oxymorphone	32.5	31.8	32.2
Cotinine	10.0	10.8	10.4	Phentermine	124.5	121.9	123.2
DEET	564.9	567.2	566.1	Pregabalin	209.3	220.5	214.9
Dehydroaripiprazole	37.1	39.3	38.2	Propranolol	57.8	57.1	57.5
Desmethylcitalopram	100.3	96.8	98.6	Pseudoephedrine	110.8	104.8	107.8
Desmethylvenlafaxine	809.2	834.5	821.9	Ritalinic acid	112.8	123.0	117.9
Dextromethorphan	53.7	49.0	51.4	Sertraline	48.5	49.1	48.8
Diltiazem	76.1	79.4	77.7	Sildenafil	29.6	31.8	30.7
Diphenhydramine	163.5	164.3	163.9	Sotalol	79.3	76.6	78.0
Disopyramide	13.9	14.2	14.0	Temazepam	115.9	110.9	113.4
EDDP	322.8	312.8	317.8	Thiabendazole	76.4	51.9	64.1
Erythromycin	38.5	39.8	39.2	Tramadol	907.5	859.8	883.6
Erythromycin-anhydro	94.1	86.8	90.5	Trazadone	28.6	27.5	28.0
Escitalopram	226.8	225.7	226.3	Triamterene	108.5	113.1	110.8
Fluoxetine	34.3	33.6	33.9	Trimethoprim	269.9	278.1	274.0
Hydrocodone	30.6	31.9	31.3	Tylosin	50.1	48.9	49.5
Hydroxybupropion	165.9	142.1	154.0	Venlafaxine	397.9	405.9	401.9
Levorphanol	184.7	180.3	182.5	Verapamil	29.5	29.4	29.4
Lidocaine	377.8	375.5	376.7	Zolpidem phenyl-4-carboxylic acid	48.9	46.3	47.6
Loratadine	17.3	18.4	17.8				

No compounds were found in the local tap water or the remote source water samples with the negative ion mode method, however, warfarin was detected at borderline in the remote source water sample. The compounds found in the urban surface water sample in negative ion mode are listed in Table 10.

The two surface water samples were also tested on the Agilent 6495 Triple Quadrupole LC/MS[8], however, the two studies were separated by several months during which the sample might have degraded. Even so, most compounds detected in both the targeted UHPLC-Triple Quadrupole method and the untargeted UHPLC-Q-TOF method overlapped well in terms of identified compounds and corresponding concentrations. This Application Note clearly demonstrates that the Agilent mass spectrometer portfolio can be used as a complete solution in environmental testing.

Conclusion

Fast and simple Q-TOF LC/MS methods for the screening of PPCPs in water have been developed. The methods leverage the full advantage of the sensitivity improvement provided by the hardware change of the Agilent 6545 Q-TOF LC/MS System and Swarm autotune on small fragile molecule ion transmission. The sensitivity can be further improved by the selection of the high sensitivity slicer mode. It has been demonstrated that low ppt level LLOQs can be achieved for the quantitation of trace contaminants in water through direct injection. With these design enhancements, tedious sample enrichment and cleanup processes can be avoided. This will increase sample throughput significantly.

Table 10. Compounds found in an urban surface water sample with negative ion mode method.

Name	Inj 1 (ppt)	Inj 2 (ppt)	Avg. (ppt)
Celecoxib	40.2	36.6	38.4
Chloramphenicol	8.9	11.9	10.4
Diclofenac	277.2	235.2	256.2
Diclofenac 4-hydroxy	10.0	10.0	10.0
Furosemide	309.3	307.9	308.6
Gemfibrozil	223.7	225.6	224.7
Hydrochlorothiazide	532.7	539.8	536.3
Ibuprofen	47.5	46.8	47.2
Methylparaben	78.6	83.4	81.0
Naproxen	175.4	177.0	176.2
n-Butylparaben	10.2	12.5	11.3
Phenobarbital	43.3	26.2	34.7
Phenytoin	666.4	956.1	811.3
Sulfamethoxazole	649.8	599.2	624.5
Triclocarban	28.2	25.9	27.0
Triclosan	36.4	37.6	37.0

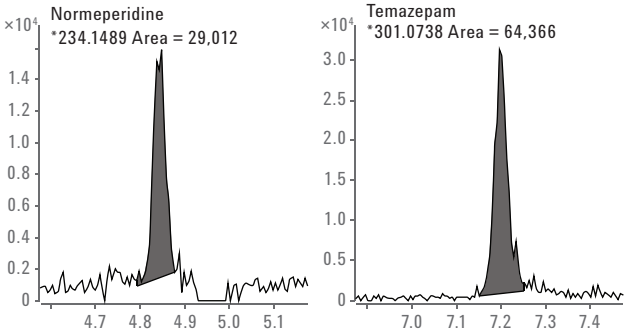

Figure 8. Chromatographs of PPCPs found in local tap water with positive ion method.

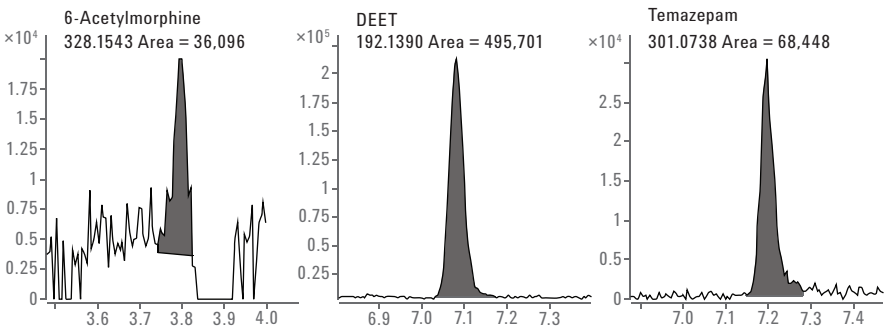

Figure 9. Chromatographs of PPCPs found in remote source sample with positive ion mode method.

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Agilent Technologies

The Use of High Resolution Accurate Mass GC/Q-TOF and Chemometrics in the Identification of Environmental Pollutants in Wastewater Effluents

Application Note

Environmental

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Abstract

A GC/Q-TOF method employing the Agilent 7200 series GC/Q-TOF system and chemometric analysis tools in Agilent Mass Profiler Professional software has been used to effectively identify environmental pollutants in complex effluent samples from multiple wastewater treatment sites and track their transformation during the treatment process.

Agilent Technologies

Introduction

Efforts to characterize the fate of environmental pollutants during wastewater treatment are hampered by the large number of compounds present in various wastewater streams [1]. Untargeted analysis of pesticides and other environmental pollutants in wastewaters using gas chromatography/mass spectrometry (GC/MS) can benefit from comprehensive EI libraries available for this technique, such as NIST 14 (containing over 200,000 EI spectra with retention index (RI) values for over 80,000 compounds). However, the highly complex chromatograms and very large data sets characteristic for this workflow represent a substantial analytical challenge [2,3].

While deconvolution of unit mass electron ionization (EI) data followed by a mass spectral library search is the most typical workflow used for the identification of environmental pollutants, this approach does not provide enough confidence in compound identification, especially in case of poor library matching. Using the high resolution, accurate mass capability of GC/Q-TOF provides analysts the required tools for reliable compound identification.

This application note presents a novel combined untargeted and targeted approach that uses high resolution accurate mass quadrupole-time-of-flight mass spectrometry (Q-TOF MS) to increase the efficacy of identification of large numbers of unknown compounds in wastewater. Furthermore, chemometric techniques using Agilent Mass Profiler Professional (MPP) software are then used for statistical analysis and data interpretation to ascertain the fate of environmental pollutants during wastewater treatment.

Experimental

Instruments

This study was performed on an Agilent 7890B GC system coupled to an Agilent 7200 series GC/Q-TOF system. The instrument conditions are listed in Table 1.

Table 1. GC and Mass Spectrometer Conditions

GC run conditions	
Column	Agilent DB-5 MS Ultra Inert, 30 m × 0.25 mm, 0.25 µm film (p/n 122-5532UI)
Injection volume	1 µL
Split ratio	10:1
Split/Splitless inlet temperature	300 °C
Oven temperature program	70 °C for 2 minutes 25 °C/min to 160 °C 3 °C/min to 205 °C 8 °C/min to 280 °C, 7.5 minutes hold 40 °C/min to 325 °C, 2 minutes hold
Carrier gas	Helium at 1.5 mL/min constant flow
Transfer line temperature	300 °C
MS conditions	
Ionization mode	EI
Source temperature	280 °C
Quadrupole temperature	150 °C
Mass range	50 to 600 *m/z*

Sample preparation

Settled primary and final effluent samples from three wastewater treatment works in South Wales, UK, were collected over a period of a few days. Five replicates of final effluent and primary effluent samples from each of the sites, as well as blanks, were extracted with dichloromethane, and concentrated to low volume. Internal standard deuterated phenanthrene (D_{10}) was added to each sample prior to extraction.

Data processing and statistical analysis

The data were processed by chromatographic deconvolution using the Unknowns Analysis tool in Agilent MassHunter Quantitative Analysis software (version B.07), followed by tentative compound identification by comparison to the NIST 14 mass spectral library. The identification of environmental contaminants was further confirmed using the accurate mass tools available in the MassHunter Qualitative Analysis software (version B.07). A set of approximately 200 putative contaminants of potential interest was then selected from the list of identified components, and semiquantitation was performed using MassHunter Quantitative Analysis.

The results from quantitation analysis were subsequently imported and processed in the multivariate statistical package Mass Profiler Professional (MPP, version 13) to evaluate the transformation of environmental pollutants in the wastewater treatment works. Figure 1 outlines the data analysis workflow.

Figure 1. Data analysis workflow.

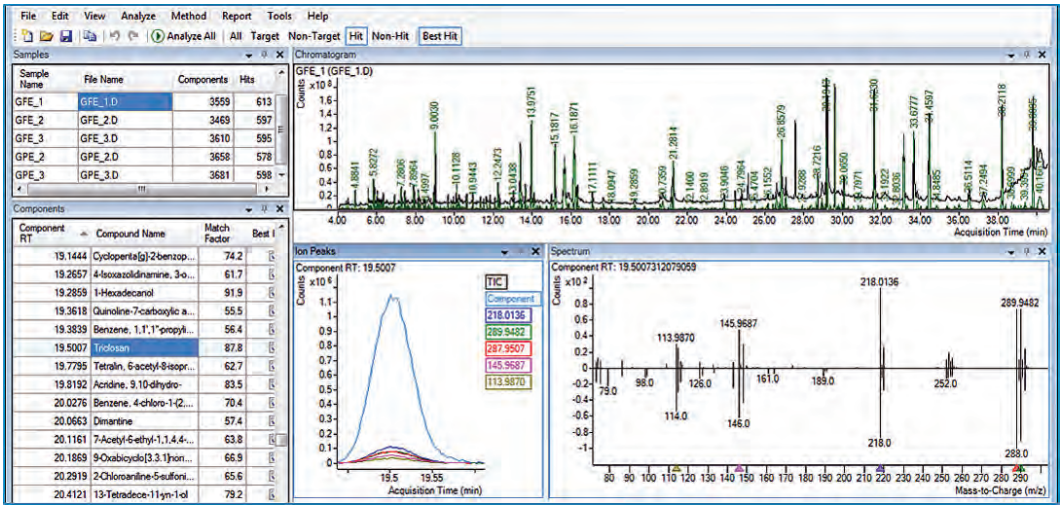

Figure 2. The Unknowns Analysis tool was used to perform deconvolution and the NIST library search. The lower middle panel shows deconvoluted ions selected for the component. They all have the same peak shape, confirming that they all belong to the same component, and thus aiding in its identification (triclosan in this case).

Results and Discussion

Chromatographic deconvolution, library search and accurate mass confirmation

Using chromatographic deconvolution and NIST library search, approximately 600 components were tentatively identified in each sample (Figure 2). Compound identity was further confirmed using accurate mass information, relative isotope abundance information, and MassHunter accurate mass tools, including Molecular Formula Generator (MFG) (Figure 3). Excellent mass accuracy and small isotope abundance error facilitated confirmation of tentative hits (Table 2). The data were further processed in MassHunter Quantitative Analysis software using peak areas normalized to the internal standard.

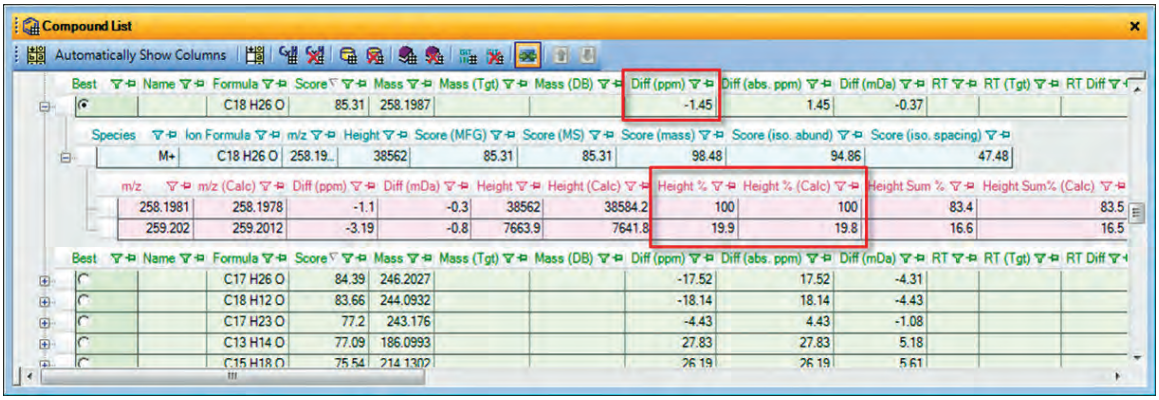

Figure 3. Molecular Formula Generator results.

Table 2. Mass and M+1 Molecular Ion Isotope Abundance Error for Some of the Compounds in the Final Effluent of Site 3

Compound	Formula	Absolute *m/z*	Calculated *m/z*	Mass error for MI* (ppm)	M+1 Abundance error (%)
p-Dichlorobenzene	$C_6H_4Cl_2$	145.9684	145.9685	-0.69	0.3
1,2,4-Trithiolane	$C_2H_4S_3$	123.9469	123.947	-0.81	-0.7
2,6-Dichlorophenol	$C_6H_4Cl_2O$	161.963	161.9634	-2.47	-1.2
Benzothiazole	C_7H_5NS	135.0142	135.0137	3.70	-0.1
3,5-Dichloroaniline	$C_6H_5Cl_2N$	160.9795	160.9794	0.62	0.8
Chloroxylenol	C_8H_9ClO	156.0336	156.0336	-0.30	0.7
5-Methylbenzotriazole	$C_7H_7N_3$	133.0636	133.0634	1.00	0.9
2,3,4-Trichloroaniline	$C_6H_4Cl_3N$	194.9404	194.9404	0.02	-1.5
4-Methyl-1H-benzotriazole	$C_7H_7N_3$	133.0634	133.0634	-0.20	-0.5
Crotamiton	$C_{13}H_{17}NO$	203.131	203.1305	2.46	2.2
Benzophenone	$C_{13}H_{10}O$	182.0733	182.0726	3.84	0.1
Tonalide (ANTH)	$C_{18}H_{26}O$	258.1981	258.1978	1.16	0.1
Average				**1.44**	**0.76**

*MI = Molecular ion

Statistical analysis

A target list of putative contaminants of potential interest was chosen, and the quantitation results were subsequently imported into and processed in the multivariate statistical package MPP to evaluate the transformation of pollutants in the wastewater treatment plants. The data analysis workflow is outlined in Figure 1.

Principal component analysis

Principal Component Analysis (PCA) is a frequently employed unsupervised multivariate statistical analysis technique for data dimensionality reduction. PCA analysis revealed distinct data clusters that represented differences in composition and abundance between all three wastewater treatment plant (WWTW) sites, as well as differences between the primary and final effluents of each site (Figure 4). The grouping of data points along the Z-axis suggests chemical similarity in the primary effluents for all three sites.

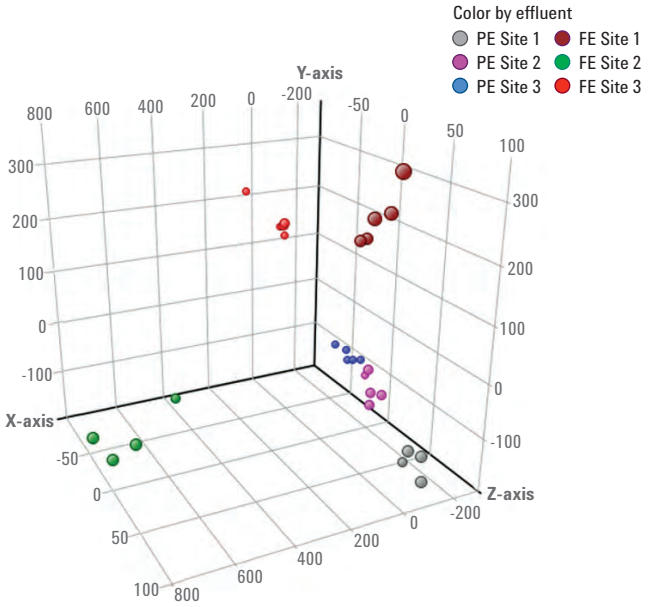

Figure 4. PCA plots confirmed the existence of distinct clusters of compounds for the replicate samples of each type of effluent. Primary Effluent (PE); Final Effluent (FE).

Heatmap analysis

A heatmap was created in MPP to display the transformation of the environmental pollutants between the primary and final effluents of the three WWTW plants (Figure 5). A few of these compounds are shown in the detailed view on the right. The abundance of some compounds decreased in the final effluent relative to the primary effluent. For example, caffeine and tonalide (AHTN) were significantly decreased in the final effluents of all three sites while cashmeran (DPMI) did not significantly change in relative abundance for Sites 1 and 2. Other compounds actually increased in abundance in the final effluent versus the primary effluent, such as 2,3,4-trichlorophenol at Site 3.

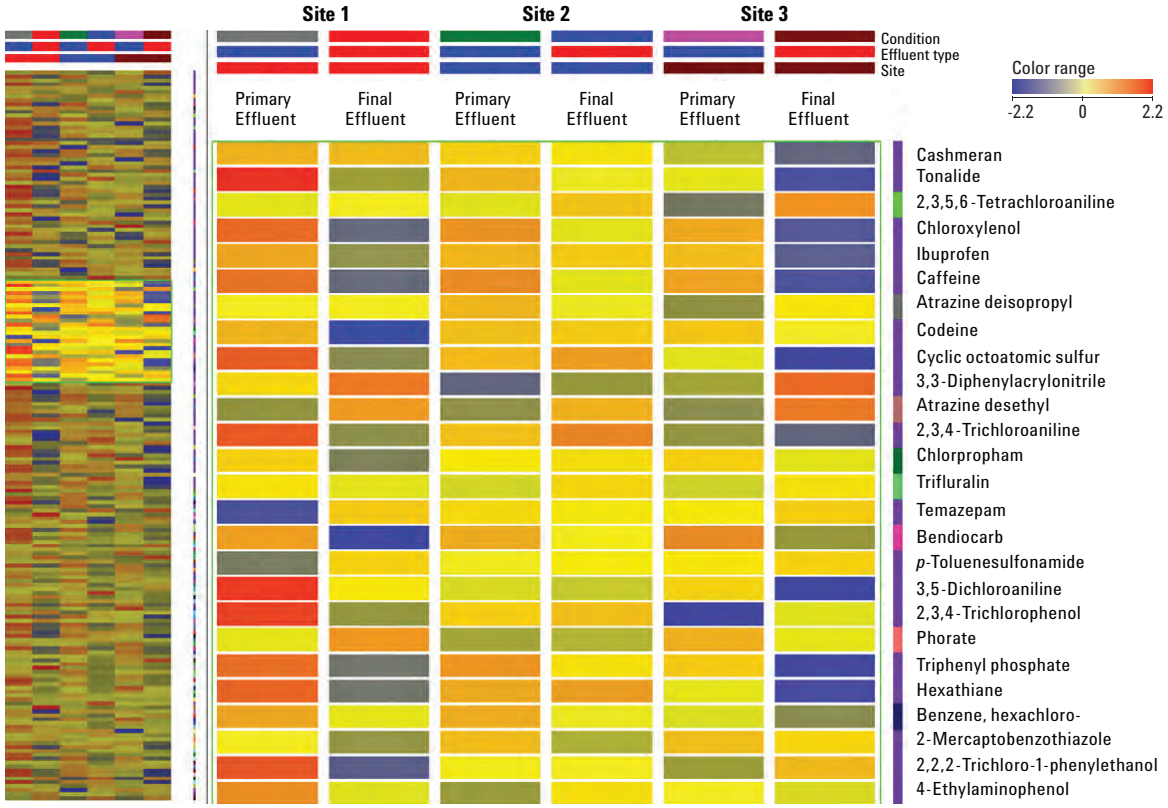

Figure 5. MPP Heatmap summary (left) and detailed view (right). Columns represent effluent type and treatment site, and the rows represent compounds.

K-means clustering analysis

The K-means clustering tool in MPP divides entities (compounds in this case), into groups (clusters) based on similarity of their behavior under different conditions (primary and secondary effluents in this case). K-means clusters are constructed so that the average behavior (increase or decrease in abundance measured by degree of fold change) in each group is distinct from any of the other groups. Figure 6 illustrates this analysis for two groups of compounds analyzed at Site 3. In the left panel, all members of the group decrease in abundance in a similar manner in the final effluent with respect to that of the primary effluent. Conversely, all members of the group in the right panel increase in abundance in a similar manner in the final effluent. In this way, several groups of compounds with similar changes in abundance were identified.

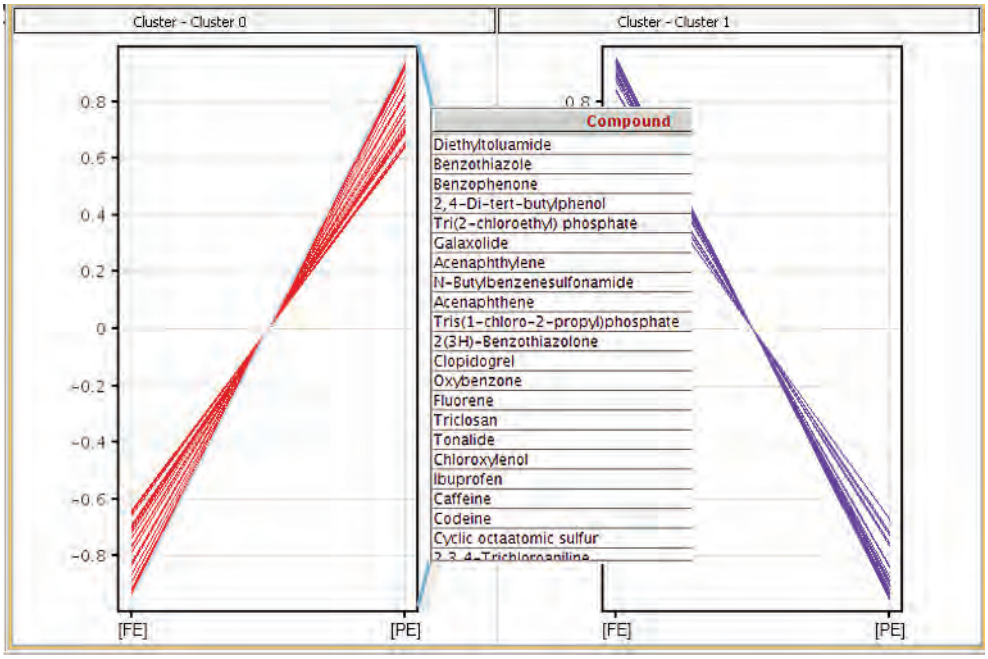

Figure 6. K-means clustering of two representative groups of compounds from Site 3 that display similar degree of fold change in abundance between primary effluent (right side of each graph) and final effluent (left side of each graph). Some of the compounds present in the left hand cluster are shown in the text box.

Venn diagram

To visually compare treatment sites with respect to the number of shared and unique compounds removed or accumulated during the course of the treatment, Venn diagrams were used (Figure 7). For all the treatment sites, the number of compounds removed from primary effluents (left side of Figure 7) was larger than the number accumulated.

Conclusions

The combination of gas chromatography, high resolution Q-TOF mass spectrometry, and chemometrics techniques was successfully used to characterize and identify environmental pollutants in complex effluent samples from multiple wastewater treatment sites. Data analysis, using both targeted and untargeted approaches, revealed a number of compounds, including pharmaceuticals, benzothiazole-based corrosion inhibitors and polycyclic musks that were specifically present in the primary effluents, but were reduced to significantly lower concentrations in final effluents.

The statistical analysis tools in Agilent Mass Profiler Professional enabled easy and rapid visualization of the results using multiple statistical approaches that revealed similarities and differences in treatment modalities between treatment sites as well as between groups of compounds. This approach can thus facilitate our understanding of the effectiveness of wastewater treatment for the removal of trace organic pollutants.

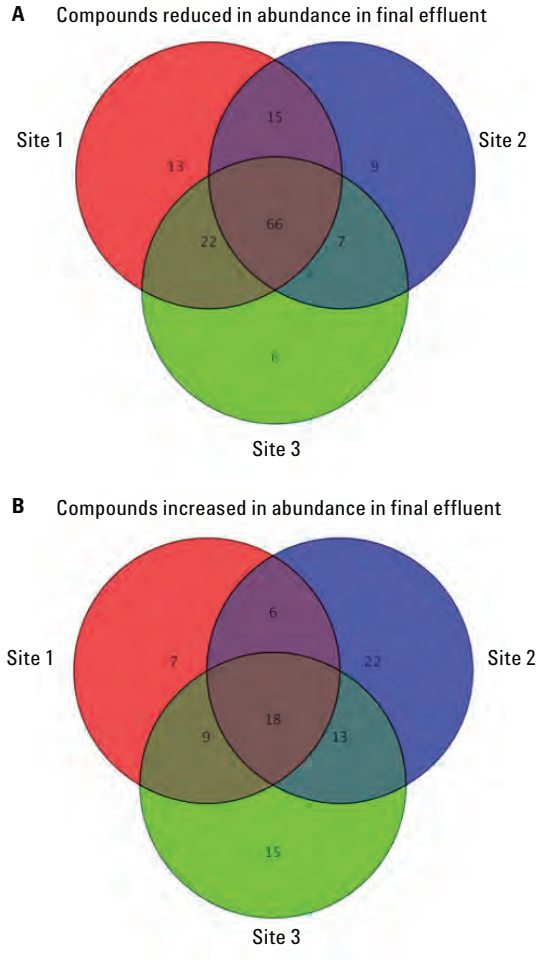

Figure 7. Venn diagrams showing compounds present at significantly higher levels in primary effluents as compared to the final effluents (A), and those accumulated in final effluents as compared to the primary effluents (B). The numbers of compounds shared by multiple sites are depicted by the overlap in the diagrams.

References

1. T. Portoles, E. Pitarch, F. J. Lopez, J. V. Sancho, F. J. Hernandez. "Methodical approach for the use of GC-TOF MS for screening and confirmation of organic pollutants in environmental water." *J. Mass Spectrom*. **42(9)**, 1175-85 (2007).
2. T. Portoles, E. Pitarch, F. J. Lopez, F. Hernandez. "Development and validation of a rapid and wide-scope qualitative screening method for detection and identification of organic pollutants in natural water and wastewater by gas chromatography time-of-flight mass spectrometry." *J. Chromatogr. A* **1218(2)**, 303-15 (2011).
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Analysis of Combustion Byproducts on Firefighter Protection Equipment Using a Novel High-Resolution GC/Q-TOF

Application Brief

Author

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Introduction

Cancer is a leading cause of fire service morbidity and mortality [1,2]. During a fire, firefighters are exposed to smoke and elevated levels of organic chemicals, such as flame retardants, originating from furniture, carpets, and so forth, as well as their combustion by-products. A high-resolution MS used in full spectrum acquisition mode is extremely advantageous for the untargeted analysis of the environmental contaminants in complex matrices, especially for identification of trace compounds. To identify polycyclic aromatic hydrocarbons (PAHs) and polybrominated diphenyl ethers (PBDEs) and other combustion products, we used a novel high-resolution Agilent 7250 GC/Q-TOF.

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Experimental

Sample collection and extraction method

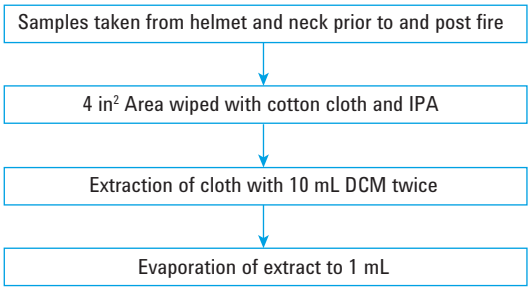


Figure 2. Sample collection and extraction workflow.

Analysis

GC/MS analysis was performed using an Agilent 7890B GC system coupled to a novel high-resolution (25,000 at *m/z* 272) Agilent 7250 GC/Q-TOF, equipped with a low-energy capable EI source.

Table 1 shows the instrument parameters.

Table 1. Instrument Parameters for Agilent 7250 GC/Q-TOF Analysis

Parameter	Value
Column	Agilent DB-5MS, 0.25 mm × 30 m, 0.25 µm
Injection volume	1 µL
Injection mode	Splitless
Split/Splitless inlet temperature	280 °C
Oven temperature program	50 °C for 3 minutes, 10 °C/min to 300 °C, 7 minutes hold
Carrier gas	Helium at 1.5 mL/min, constant flow
Transfer line temperature	300 °C
Ionization mode	Standard EI at 70 eV Low energy EI at 15 eV and 12 eV
Source temperature, 70 eV/15 eV or less	240 °C/200 °C
Quadrupole temperature	150 °C
Mass range	50 to 1,200 *m/z*
Spectral acquisition rate	5 Hz

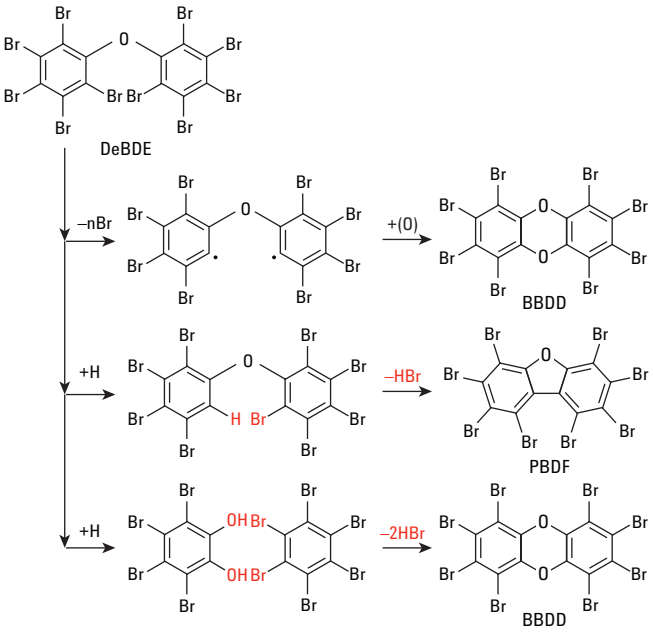


Figure 1. Decomposition of PBDEs, and formation of dioxin and furan structures.

Data analysis

The data were acquired using a 7250 accurate mass high-resolution GC/Q-TOF system. First, the data were processed using the feature detecting algorithm SureMass in Unknowns Analysis B.08.00. Initial compound identification was performed by spectrum comparison with NIST14 EI library and confirmed by retention index (RI) matching when possible (Figure 3). Based on Unknowns Analysis results, a Quant method was created in Agilent MassHunter Quantitative Analysis software B.08.00 for semiquantification of PAHs as well as PAH-like compounds (Figure 4). PBDEs and molecular ions of unknown brominated compounds were identified with the help of low electron energy compound spectra. Molecular ions were confirmed by evaluating the entire isotopic cluster for *m/z*, relative isotope abundance, and isotope ratios using Molecular Formula Generator (MFG) of MassHunter Qualitative Analysis B.08.00.

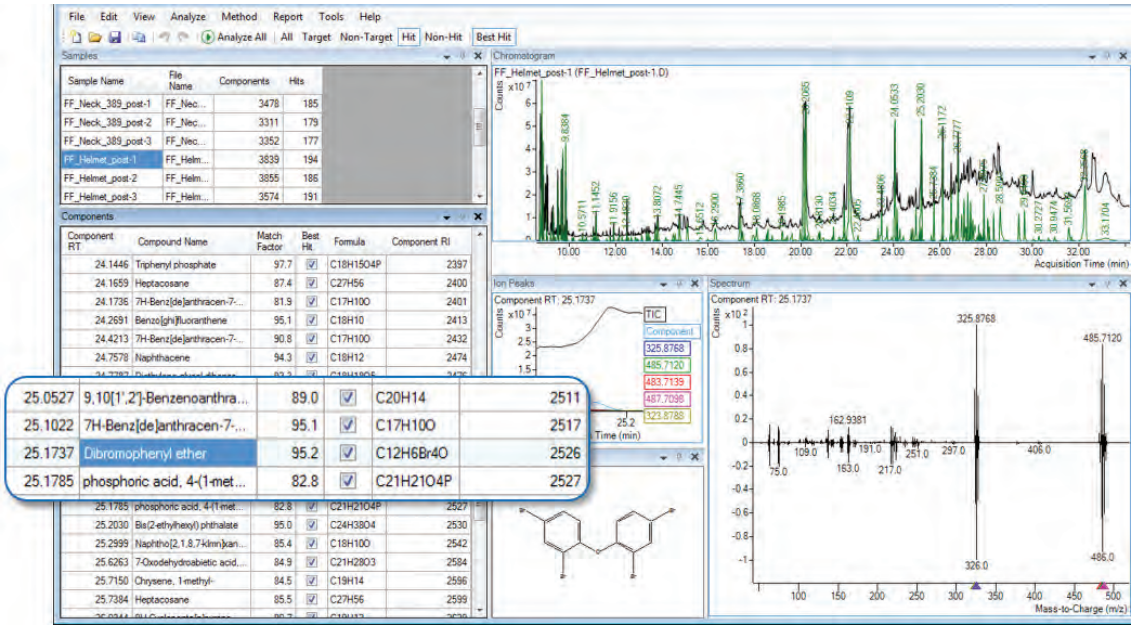


Figure 3. Unknowns Analysis B.08.00 and spectrum comparison with NIST14.

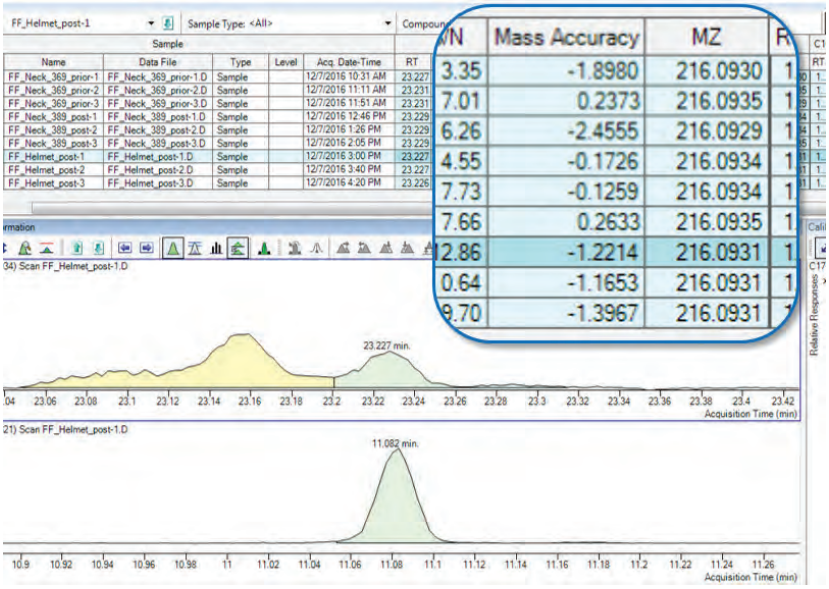


Figure 4. Quantitation of PAHs using Agilent MassHunter Quant B.08

Results and Discussion

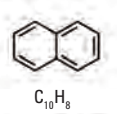
PAH Identification

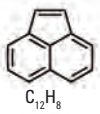
PAHs were identified in all post fire samples. Over 50 PAH species were identified on the helmet post fire. Regulated PAHs that were identified using NIST14 were confirmed by retention index. Table 2 summarizes examples of identified PAHs and PAH-like compounds on the helmet post fire.

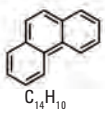
Potential structures of the identified formulas are suggested in Table 2. These are similar to the PAHs that were detected by Fernando, *et al.* [3] in the air and on the skin of firefighters post fire.

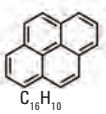
Table 2. Combined List of Identified PAHs ***Note:*** *For Most of the PAH and PAH-like Formulas, Multiple Isomers were Identified*

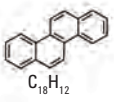
Compound/formula	*m/z*	Avg mass error (ppm)
Regulated PAHs		
Naphthalene [$C_{10}H_8$]	128.0626	1.01
Acenaphthylene [$C_{12}H_8$]	152.0626	0.82
Acenaphthene [$C_{12}H_{10}$]	154.07825	0.35
Fluorene [$C_{13}H_{10}$]	166.07825	1.15
Phenanthrene [$C_{14}H_{10}$]	178.07825	1.25
Anthracene [$C_{14}H_{10}$]	178.07825	0.96
Fluoranthene [$C_{16}H_{10}$]	202.07825	1.06
Pyrene [$C_{16}H_{10}$]	202.07825	1.51
Benz[a]anthracene [$C_{18}H_{12}$]	228.0939	1.27
Chrysene [$C_{18}H_{12}$]	228.0939	1.07
Benzo[b]fluoranthene [$C_{20}H_{12}$]	252.0939	1.81
Benzo[k]fluoranthene [$C_{20}H_{12}$]	252.0939	2.23
Benzo[a]pyrene [$C_{20}H_{12}$]	252.0939	1.79
Indeno[1,2,3-cd]pyrene [$C_{22}H_{12}$]	276.0939	1.65
Dibenz[a,h]anthracene [$C_{22}H_{14}$]	278.10955	1.55
Benzo[ghi]perylene [$C_{22}H_{12}$]	276.0939	1.73

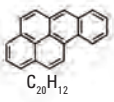


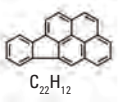


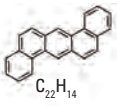




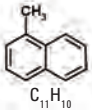


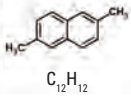


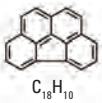




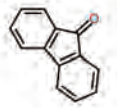
Compound/ formula	*m/z*	Avg mass error (ppm)
Other PAHs		
$C_{10}H_{12}$	132.0934	0.91
$C_{11}H_{14}$	146.109	1.07
$C_{11}H_{10}$	142.0777	0.55
$C_{12}H_{16}$	160.1247	1.22
$C_{12}H_{12}$	156.0934	1.07
$C_{13}H_{12}$	168.0934	1.14
$C_{14}H_{14}$	182.109	0.59
$C_{15}H_{12}$	192.0934	0.53
$C_{15}H_{10}$	190.0777	2.57
$C_{18}H_{22}$	238.1716	1.07
$C_{18}H_{18}$	234.1403	1.04
$C_{17}H_{12}$	216.0934	0.87
$C_{18}H_{10}$	226.0777	0.92
$C_{20}H_{14}$	254.109	1.05
$C_{19}H_{14}$	242.109	0.58
$C_{19}H_{12}$	240.0934	2.17
$C_{22}H_{14}$	278.10955	0.91
$C_{24}H_{18}$	306.1403	1.67
$C_{22}H_{12}$	276.0939	2.25

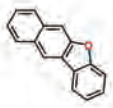


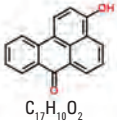


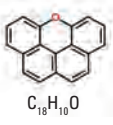


Compound/ formula	*m/z*	Avg mass error (ppm)
O-containing PAHs		
$C_{13}H_8O$	180.057	0.90
$C_{16}H_{10}O$	218.0726	1.47
$C_{17}H_{10}O$	230.0726	1.99
$C_{17}H_{10}O_2$	246.0675	1.27
$C_{18}H_{10}O$	242.0726	2.03
$C_{18}H_{10}O_2$	258.0675	1.15
$C_{20}H_{12}O$	268.0883	1.68

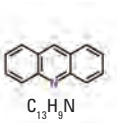


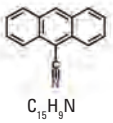






Compound/ formula	*m/z*	Avg mass error (ppm)
N-containing PAHs		
$C_{13}H_9N$	179.073	1.24
$C_{12}H_9N$	167.073	1.55
$C_{15}H_9N$	203.073	1.52
$C_{17}H_{11}N$	229.0886	0.63
$C_{19}H_{11}N$	253.0886	1.08





Based on relative abundances, fluoranthrene, pyrene, and the larger PAHs were the predominant PAHs measured on the helmet post fire. Figure 5 illustrates the abundances of PAHs and PAH-like compounds on the helmet post fire.

Figure 6 shows the sum of PAHs, O-containing PAHs, and N-containing PAHs in all samples. The PAHs on the helmet post fire exceeded the concentration on the skin. The post fire skin samples exhibited greater concentrations of all groups of PAHs.

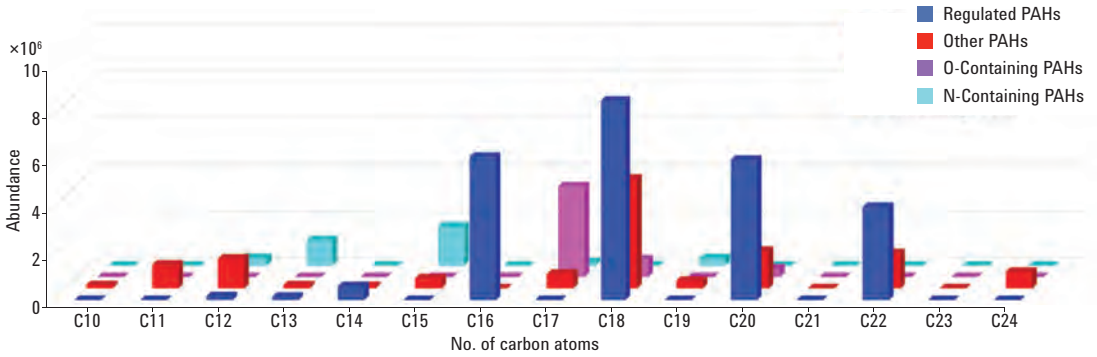


Figure 5. PAHs and PAH-like compounds on post fire wipe of helmet.

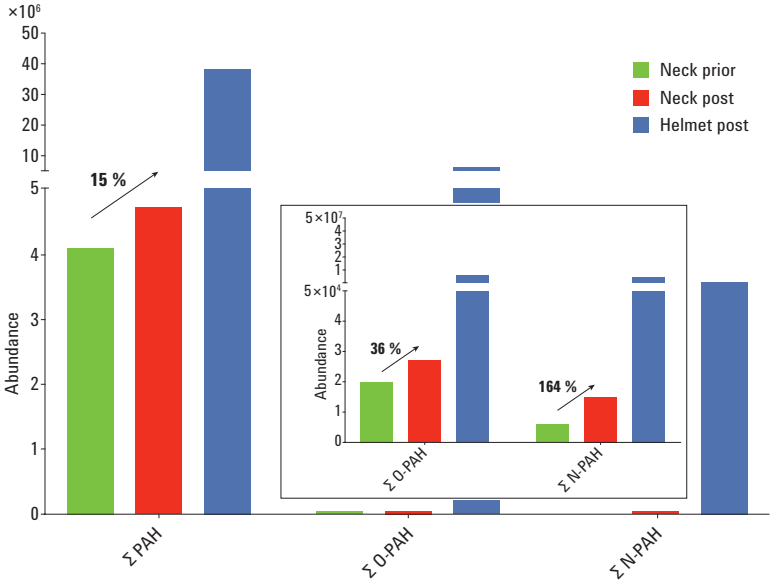


Figure 6. Total PAHs identified in pre and post fire wipes.

Brominated compounds

Brominated compounds were detected only in post fire samples, indicating that they originated from exposure to smoke.

Isomers of tri-BDE, tetra-BDE, penta-BDE, and hexa-BDE originating from burned material containing flame retardants were detected on the neck and helmet post fire. Table 3 summarizes the relative abundances. The retention times were verified using native standards.

Other Br-containing compounds were found only on the helmet post fire, but not on the neck. These are potential PBDE combustion byproducts (for example, $C_6H_4Br_2O$) and brominated PAHs (for example, $C_{16}H_9Br$). Figures 7 and 8 illustrate the mass spectra of a few brominated compounds at different electron energies. Low electron energies facilitated the identification of molecular ions of the unknown compounds.

Table 3. List of Identified PBDE and Other Brominated Compounds Identified in Post Fire Samples

Formula	*m/z*	Mass error (ppm)*	Resolving power	MFG score	Verified by standard	Retention time (min)	Neck: Prefire, % abundance	Neck: Post fire, % abundance	Helmet: Post fire, % abundance
$C_6H_4Br_2O$	249.8623	1.7	27336	98.34		13.15	n.d.	n.d.	15.26
$C_{14}H_{21}BrO$	284.077	0.92	26911	90.92		16.409	n.d.	n.d.	31.17
$C_7H_8Br_2N_2$	277.9049	0.22	27495	88.15		19.891	n.d.	n.d.	5.72
$C_7H_6Br_2N_2O$	291.8841	0.99	26072	90.08		20.832	n.d.	n.d.	13.84
$C_{10}H_9BrN_2$	235.9944	1.27	27136	97.41		21.122	n.d.	n.d.	1.53
$C_{16}H_9Br$	279.9882	1.69	26187	98.71		24.609	n.d.	n.d.	4.39
$C_{15}H_{14}Br_2O_2$	383.9355	0.25	29880	95.36		25.88	n.d.	n.d.	0.32
$C_{12}H_7Br_3O$	403.8047	0.45	31609	90.67	Tri-BDE	22.687	n.d.	1.1	0.29
		0.23	29157	96.13		23.063	n.d.	1.41	0.4
$C_{12}H_6Br_4O$	481.7152	1.36	30425	91.87	Tetra-BDE	24.835	n.d.	0.9	0.4
		2.62	31364	95.40		25.175	n.d.	58.84	13.09
		1.15	27322	93.01		25.507	n.d.	0.69	0.3
$C_{12}H_5Br_5O$	559.6257	2.59	30367	95.05	Penta-BDE	26.684	n.d.	8.99	2.74
		2.03	30813	94.57		27.159	n.d.	25.29	9.26
		1.24	29448	91.29		27.974	n.d.	1.1	0.35
$C_{12}H_4Br_6O$	637.5357	0.7	30817	91.38	Hexa-BDE	28.335	n.d.	0.83	0.51
		1.7	31299	94.73		29.058	n.d.	0.85	0.43

* Mass error is calculated as a weighted average mass error for the entire isotopic cluster.

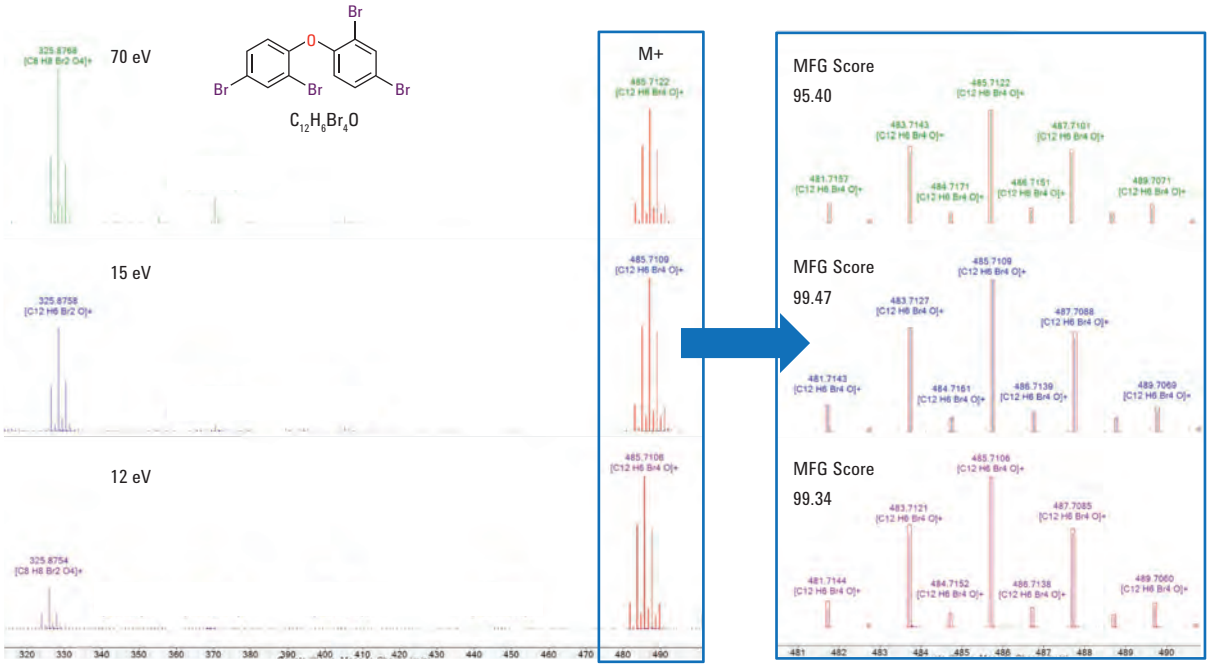


Figure 7. Example of PBDE detected on neck wipe post fire. MFG score is based on mass accuracy, isotope spacing as well as relative isotope abundance ratios.

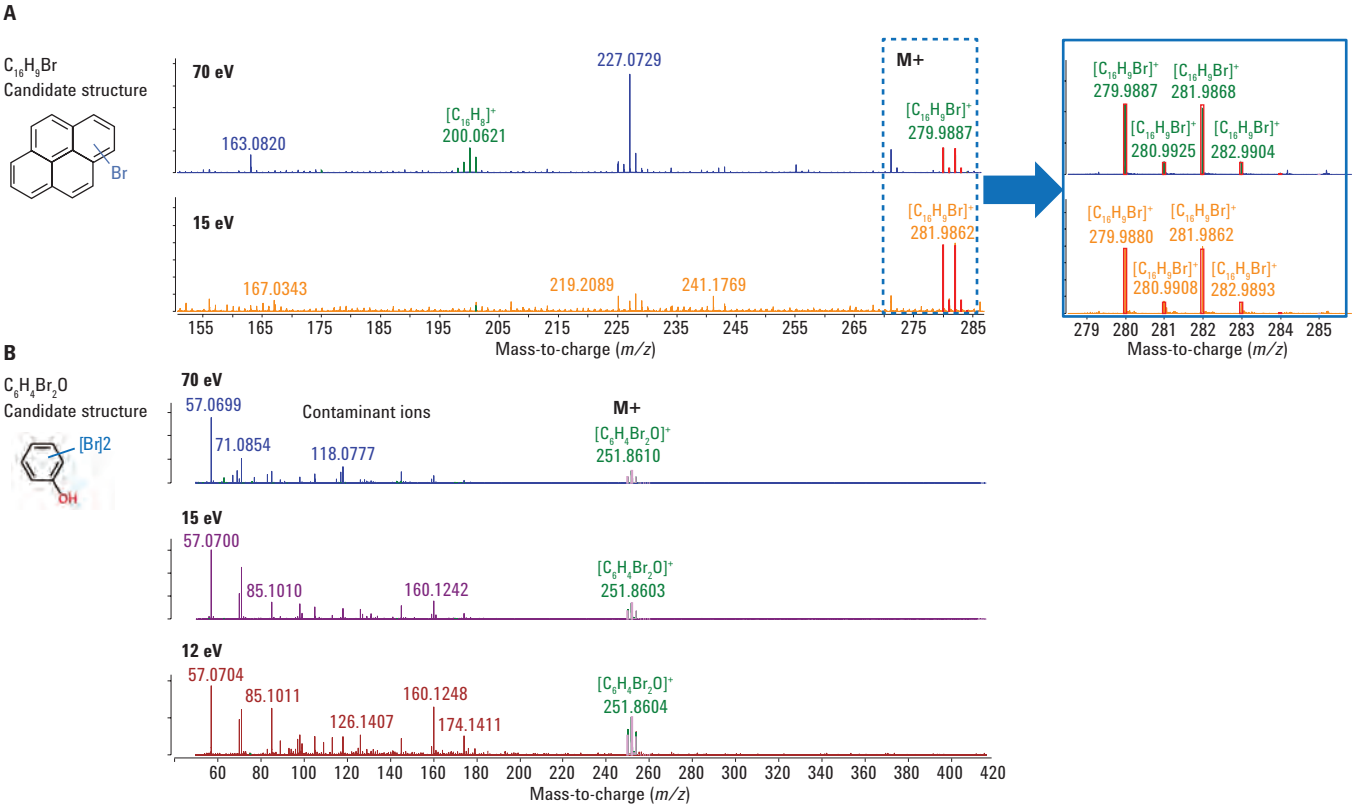


Figure 8. Examples of the unknown brominated compounds spectra at different electron energies. Spectra are annotated using MFG tool with Fragment Formula Annotation. Unannotated ions are likely contaminant ions originated from coeluting compounds. Candidate structures based on 70 eV and low electron energy spectra are shown on the left.

Conclusions

Neck wipes collected post fire showed that the hood did not fully protect from smoke-related contaminants. PAHs as well as PBDEs and other brominated compounds were found on the neck and helmet of firefighters post fire. Due to their low detection limits, brominated compounds can act as biomarkers of exposure of firefighters to smoke.

The applied GC/Q-TOF method proved to be very sensitive and selective in detecting numerous PAHs, PBDEs, and other brominated combustion byproducts.

A low-energy-capable EI source facilitated identification of the molecular ions of the unknown brominated compounds.

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Application Note

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GC/Q-TOF workflows for comprehensive pesticide analysis

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Abstract

High-resolution GC/Q-TOF mass spectrometry optimizes simultaneous qualitative and quantitative screening, providing benefits in throughput and characterization of environmental samples.

This Application Note presents three complementary GC/Q-TOF workflows for the comprehensive analysis of pesticides and related compounds in environmental samples:

- Target quantification
- Suspect screening using high-resolution accurate mass GC/Q-TOF data
- Nontarget screening using spectral deconvolution and library searching

An Agilent 7200 GC/Q-TOF was used to analyze 51 water samples taken from the Sacramento/San Joaquin River delta in California before, during, and after two rain events. After filtering, contaminants in the water extracts were isolated by solid phase extraction and concentrated by solvent evaporation. The filters were extracted to recover contaminants bound to particulates. A quantitative analysis method was validated for 21 target pesticides analyzed by the GC/Q-TOF in negative chemical ionization mode. Sixteen of these target pesticides were found in at least two of the water extracts. Samples were then re-analyzed using electron ionization (EI). These data files were processed using Agilent MassHunter Qualitative Analysis Software using the Find by Formula (FBF) workflow. Samples were screened for approximately 750 pesticides and related compounds contained in the Agilent Pesticide Personal Compound Database and Library (PCDL) for GC/Q-TOF. Accurate mass data provide high selectivity while retention time locking helped to reduce false positive results. Forty-one additional suspects were identified through this technique, with most being confirmed by the analysis of standards. Of these 41, 24 were also found by LC/Q-TOF, and 17 compounds were uniquely detected by GC-EI-Q-TOF.

Nontarget screening used the Agilent MassHunter Unknowns Analysis Software. This software first deconvolutes the spectra in the chromatogram, and searches the deconvoluted components against a mass spectral library of choice: NIST14 and the Agilent GC/Q-TOF Pesticides PCDL. Five pesticides and one transformation product (TP) not found by the first two GC-Q/TOF approaches were tentatively identified in Unknowns Analysis. In addition, several halogenated and nonhalogenated organophosphorus flame retardants, several phenolic antioxidants, and various organohalogen compounds were tentatively identified.

Introduction

To assess exposure and risk, it is necessary to monitor micropollutants in waste, surface, ground, and drinking water. Traditionally, this has been done by GC/MS in scan or selected ion monitoring (SIM) mode. However, for complex environmental samples, a single quadrupole instrument is not sufficiently selective. A GC triple quadrupole MS operating in Multiple Reaction Monitoring (MRM) mode offers much better selectivity, but this technique is optimal for targeted compounds only. Compounds not on the target list will be missed no matter what their concentration. This restricted approach can lead to inaccuracies in exposure and risk assessment. An ideal scheme would quantify target compounds for which standards are available. It would also look for a broad range of other contaminants that can be detected and quantified once standards become available.

This Application Note describes three GC/Q-TOF workflows that have been used to identify nonpolar and semipolar micropollutants (for example, pesticides, and so forth) in surface waters. The first approach (target method) is to quantify compounds for which standards are available. In this case, the Q-TOF was operated in negative chemical ionization mode (NCI), as most of the 21 target compounds were halogenated, thereby providing optimal sensitivity in NCI mode. The second approach (suspect screening) uses EI data with Agilent MassHunter Qualitative analysis tools and the Agilent GC/Q-TOF Pesticides Personal Compound Database and Library (PCDL). The Find by Formula[a] workflow extracts chromatograms for the most significant ions for each compound at its known retention time. The number of ions extracted along with the retention time and mass windows are user-settable, as are the requirements for compound identification. This approach allows users to presumptively identify compounds without the need for analytical reference standards.

[a] In the most recent version of Agilent MassHunter Qualitative Analysis Software (B.08.00 Workflows), this process is called Find by Fragments.

The third approach (nontarget screening) uses Agilent MassHunter Unknowns Analysis. This software deconvolutes spectra over the whole chromatogram, and finds individual components with cleaned spectra—that is, spectra where interferences have been identified and removed. Each of the components is searched against a large mass spectral library. This work queried the NIST14 unit mass spectral library with spectra for more than 240,000 compounds, and the Agilent GC/Q-TOF Pesticides PCDL. Figure 1 shows the overall workflow used.

This combined target and suspect pesticide screening GC/Q-TOF workflow was applied to 51 surface water samples collected from the Cache Slough in the Sacramento-San Joaquin River Delta in Northern California. To get an idea of other compounds that could be found in the extracts, six of the samples were analyzed using Unknowns Analysis.

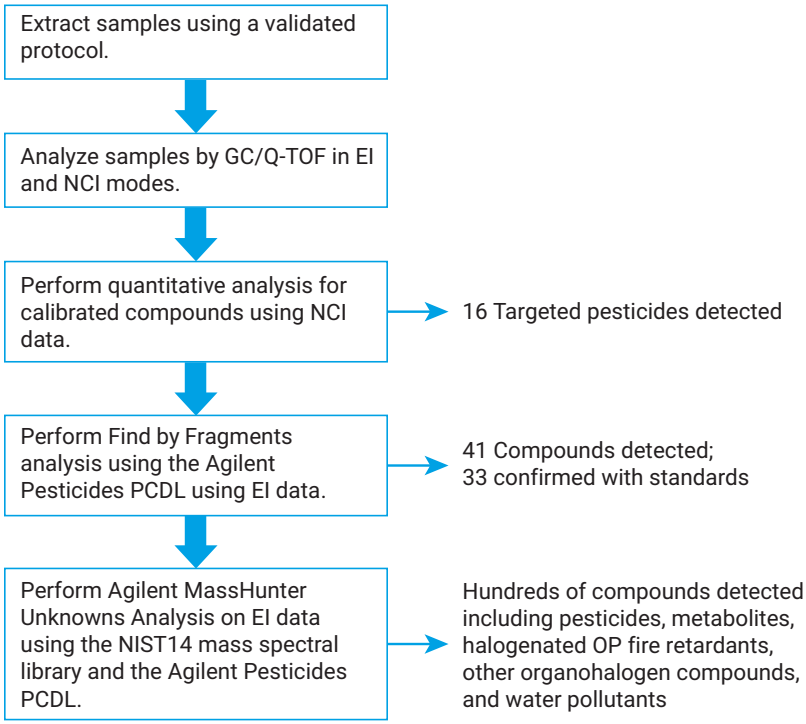

Figure 1. GC/Q-TOF workflow for 1) quantifying target pesticides, 2) screening for hundreds of pesticides using the Agilent Pesticides PCDL, and 3) screening for other compounds found in the NIST mass spectral library.

Samples were collected before, during, and after two rain events. An article entitled *LC- and GC-Q-TOF-MS as Complimentary Tools for a Comprehensive Micropollutant Analysis in Aquatic Systems*[1] describes, in detail, the quantitative target method and the qualitative suspect screening method. Because not all the compounds studied are amenable to GC/MS analysis, the research article also describes a complementary LC/Q-TOF workflow[1,2] that provides a comprehensive chemical profile of the samples.

Experimental

Target compounds and standards

Twenty-one GC-amenable pesticides (Table 1) were included in the targeted GC/Q-TOF workflow. Most were pyrethroids, and most contained halogens, making them good candidates for analysis by NCI. One internal standard (4,4'-dibromooctafluorobiphenyl) and two surrogates (chlorpyrifos D10 and etofenprox D5) were used. For method validation and quality control, prespiked (before extraction), post spiked (before injection), and procedural blank (extracted from ultrapure water) samples were run in triplicate.

Table 1. Target pesticides with their validation results for extracts of water and the filters.

Compound	CASRN	MDL (ng/L)	Absolute recovery water extraction (%)	Absolute recovery filter extraction (%)	Accuracy (%)	Precision (n = 3) (%)
Bifenthrin	82657-04-3	0.2	73 %	82 %	106 %	1 %
Bioallethrin	28434-00-6	0.1	76 %	72 %	111 %	0 %
Chlorothalonil	1897-45-6	0.6	94 %	0 %	103 %	7 %
Chlorpyrifos	2921-88-2	0.1	80 %	62 %	108 %	0 %
Cyfluthrin[1]	68359-37-5	1.0	–	–	–	–
Cyhalothrin	91465-08-6	0.1	82 %	82 %	110 %	1 %
Cypermethrin	65731-84-2	1.0	85 %	62 %	120 %	1 %
Cyphenothrin	39515-40-7	0.5	48 %	81 %	113 %	2 %
Deltamethrin	52918-63-5	1.0	96 %	66 %	123 %	1 %
Esfenvalerate	66230-04-4	0.1	93 %	80 %	118 %	1 %
Fipronil	120068-37-3	0.5	92 %	77 %	105 %	3 %
Fipronil amide	–	0.1	98 %	82 %	116 %	0 %
Fipronil-desulfinyl	205650-65-3	0.2	77 %	96 %	87 %	1 %
Fipronil-desulfinyl amide	–	0.2	88 %	74 %	247 %	0 %
Fipronil-sulfide	120067-83-6	0.1	79 %	89 %	74 %	1 %
Fipronil-sulfone	120068-36-2	0.2	91 %	85 %	102 %	3 %
Novaluron	116714-46-6	0.05	48 %	91 %	96 %	3 %
Permethrin	52645-53-1	2.0	84 %	80 %	113 %	2 %
Phenothrin	26002-80-2	5.0	47 %	75 %	123 %	2 %
Prallethrin	23031-36-9	0.1	299 %	36 %	81 %	6 %
Tetramethrin	7696-12-0	5.0	80 %	205 %	106 %	1 %

[1] Not determined, as reference standard was acquired after the validation experiments.

Sample preparation

Fifty-one 1-L samples were collected at a depth of approximately 30 cm from the Sacramento-San Joaquin River Delta in Northern California before, during, and after two different rain events. All the samples were cooled during transport, and stored at 4 °C in the dark until extraction. Water samples (1 L) were passed through a GF/F filter, and the filtrate was spiked with the two surrogates before being passed through a polymeric solid phase extraction (SPE) cartridge. After drying for one hour, the cartridges were eluted with 10 mL of ethyl acetate. To account for losses due to sorption of pyrethroids to the glass wall, the 1-L containers were rinsed with dichloromethane (3 × 4 mL). The combined extracts were reduced to 0.2 mL. The GF/F filters were extracted by sonicating them with 1:1 hexane/acetone (2 × 20 mL), and the combined filter extracts were reduced to 0.2 mL. All samples were spiked with 10 ng of 4,4'-dibromooctafluorobiphenyl. A 10-point calibration curve between 0.1 and 250 ng/mL (in ethyl acetate) was generated using the same ISTD and surrogate concentrations.

Sample analysis

Samples were analyzed on an Agilent 7890B GC coupled to an Agilent 7200B Q-TOF MS, once by NCI with methane reagent gas, and once by EI. Table 2 lists the instrument conditions.

Table 2. Instrumentation and conditions for analysis.

GC-NCI-MS Method	
Injection volume	2.5 µL
Injection mode	Splitless
Purge flow to split vent	33 mL/min at 0.75 minutes
Inlet temperature	280 °C
GC Settings	
Column	Agilent HP-5MS (30 m × 0.25mm, 025 µm)
Initial oven temperature	100 °C, hold 1 minute
Ramp 1	15 °C/min to 200 °C
Ramp 2	3.8 °C/min to 290 °C
Ramp 3	10 °C/min to 300 °C, hold 4 minutes
He Flow	1.35 mL/min, constant flow
Transfer line temperature	300 °C
MS Settings	
N_2 Collision gas	1.5 mL/min
Reagent gas (methane)	40 %
Source temperature	200 °C
Emission current filament	90 µA
Electron energy	70 eV
Acquisition range	35–1,000 *m/z*
Acquisition speed	3 spectra/sec
Mass calibration	Automated mass calibration after every second sample
GC-EI-MS Method	
Injection volume	2.5 µL
Injection mode	Splitless
Purge flow to split vent	33 mL/min at 0.75 minutes
Inlet temperature	280 °C
GC Settings	
Column	Agilent HP-5MS (30 m × 0.25mm, 025 µm)
Initial oven temperature	60 °C, hold 1 minute
Ramp 1	40 °C/min to 120 °C
Ramp 2	5 °C/min to 310 °C
Optimized He flow for RT locking	0.776 mL/min, constant flow
Transfer line temperature	280 °C
MS Settings	
N_2 Collision gas	1.5 mL/min
Source temperature	300 °C
Emission current filament	35 µA
Electron energy	70 eV
Scan range	35–1,000 *m/z*
Scan speed	4 spectra/sec
Mass calibration	Automated mass calibration after every second sample

Results and Discussion

Target method data processing and analysis

Target pesticides in all sample extracts were quantified using Agilent MassHunter Quantitative Analysis Software (B.07.00) applied to the NCI data. The main NCI fragment was used as the quantifier, and two additional fragments were used as qualifiers.

Validation results for the 21 GC-NCI-Q-TOF target compounds showed that 17 had absolute recoveries >70 % in the water extracts, while 15 of the filter extracts had recoveries >70 %. Nineteen had accuracies between 70 and 130 %; all 21 had precisions <10 %. Eighteen had MDLs <1 ng/L (Table 1). All of the compounds with recoveries less than 70 % (phenothrin, cyphenothrin, and prallethrin) were synthetic pyrethroids that contained no halogens, so they would be expected to have lower responses in the NCI mode.

Suspect screening using GC/Q-TOF with the Pesticides PCDL

The water extracts were rerun in EI mode to perform a suspect screening for additional pesticides and related compounds. The Find By Formula (FBF) workflow within MassHunter Qualitative Analysis Software (B.07.00) was applied to screen for the 750 compounds[b] in the Agilent Pesticides PCDL for the GC/Q-TOF. The PCDL contains curated exact mass spectra for all the compounds, along with locked retention times, for the EI GC/MS method shown in Table 2. Locked retention times are available for one 20-minute method and one 40-minute method using two 15-m columns configured for backflushing[3]. A third available method uses a backflushing configuration with a 5-m column followed by a 15-m column with locked retention times for a 20-minute run.

[b] A more recent version of the Pesticides PCDL for GC/Q-TOF contains entries for more than 850 compounds.

The GC retention times were first adjusted to be close to the PCDL values by adjusting the column flow rate. Then, retention time locking was performed by making five runs: one at the nominal flow rate, and four more at ±10 % and ±20 % of the nominal flow rate. MassHunter Acquisition Software automatically creates a calibration curve relating the column flow rate to the retention time of the locking standard. Chlorpyrifos, a pesticide that elutes near the middle of the chromatogram, was used as the locking standard. Using the calibration curve, it does a final adjustment of the flow rate to bring the RTs for all compounds within 0.2 minutes of their PCDL values.

The FBF data mining tool in MassHunter Qualitative Analysis Software (B.07.00) used the Pesticides PCDL with the setpoints shown in Table 3. In this case, five of the most specific ions for each compound were extracted from the chromatogram inside a ±0.2-minute window around the compound's locked retention time and within a specified mass extraction window. The software automatically chooses one EIC as a reference for the RT and peak shape. It then compares the RT and peak shape of the other four EICs to see if they fall within the method's qualification criteria. In this case, the reference ion and two more ions must be qualified for the compound to be listed as a hit. Figure 2 shows the five EICs for boscalid, a fungicide that was found in all 51 samples of river water.

Table 3. Parameters for suspect screening by GC/Q-TOF-MS.

Parameter	Value
Software	Agilent MassHunter Qualitative Analysis (B.07.00)
Workflow	Find Compounds by Formula
Values to match	Mass and retention time (retention time optional)
Library	Agilent GC/Q-TOF – Pesticide PCDL (including retention times). 750 compounds
Extraction algorithm	Agile 2
Match tolerance masses	±20 ppm
Retention time tolerance	±0.2 minutes
Allowed adducts	pos: - electron
Allowed charge state	1
Isotope model	Common organic molecules
Peak spacing tolerance	0.0025 *m/z*, plus 7 ppm
Scoring (weight)	Mass score: 100 Isotope abundance score: 60 Isotope spacing score: 50 Retention time score: 100
Find by formula score	>70 (out of 100)
Absolute height	>1,000 counts
Confirm with fragment ions	Molecular ion optional
Number of most specific ions from MS/MS library	5
RT Difference	±0.1 minutes
S/N	Not applied
Coelution score	>85 (out of 100)
Minimum number of qualified fragments	2

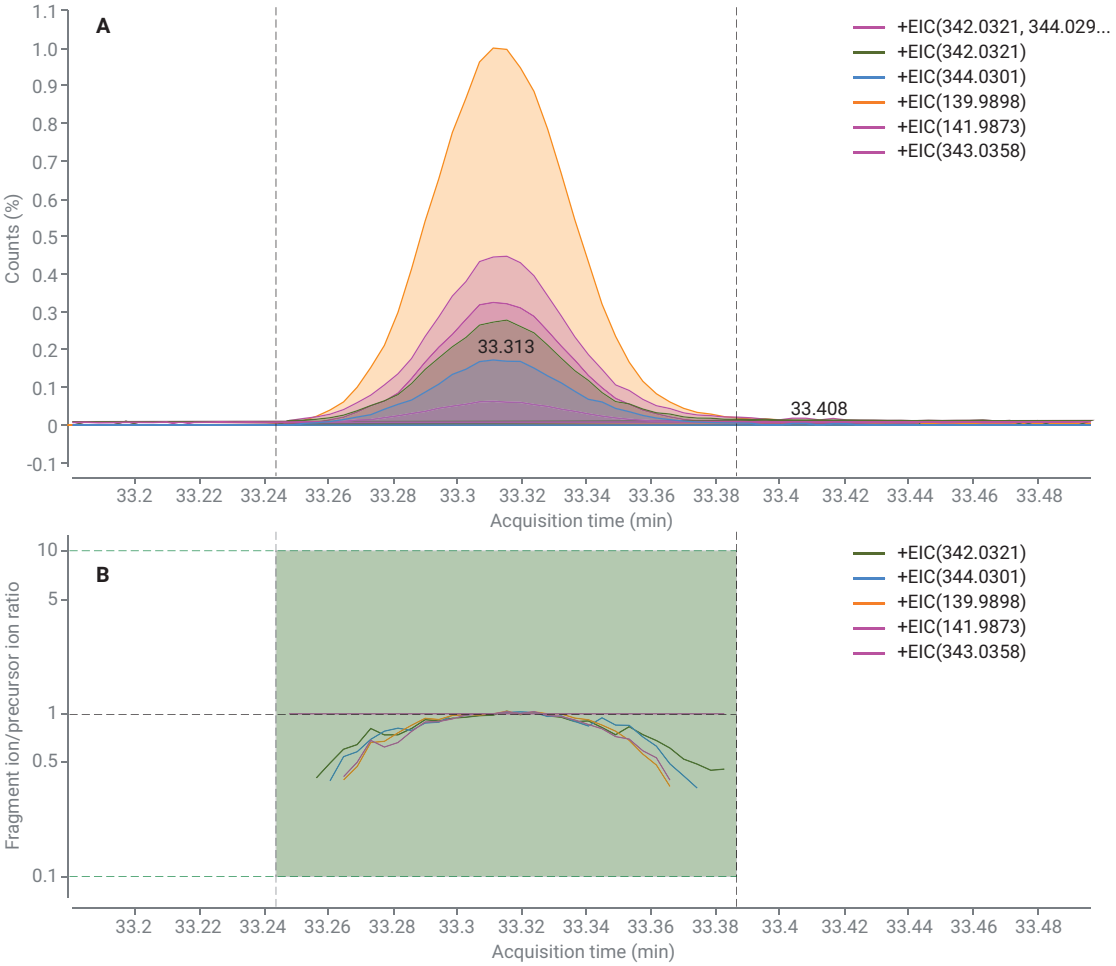

Figure 2. A) Extracted ion chromatograms for boscalid found in a sample of river water extract. The normalized coelution plot (B) shows how the peak shape of the four EICs match the shape of the reference ion. If their peak shapes were the same, the plot would be a horizontal straight line.

Figure 3 shows more information provided by the FBF software including:

- The coelution scores (out of 100) for each fragment
- The difference between the measured and theoretical monoisotopic molecular ion mass
- The difference between the measured retention time and the value recorded in the PCDL for boscalid

In cases where there is a measurable molecular ion, the software compares the theoretical isotope spacing and abundance to the measured values (Figure 4). The molecular ion is not necessary for compound identification, as the molecular ion is often not dominant in GC-EI-MS spectra (see the *Confirm with fragment ions* setting in Table 3)

The FBF approach identified 41 suspect compounds (Table 4) that were not on the target list shown in Table 1. Of these, 33 were unambiguously confirmed by analyzing a reference standard. For the additional eight compounds, no reference standard was available, and they remain tentatively identified.

The suspect screening approach using the FBF workflow allows one to presumptively identify any of the compounds contained in the PCDL without having an authentic standard. Multiple fragment ions as well as retention time matching requirements minimize the possibility of false positive identifications. This expands the scope of the analysis from the relatively few compounds that have standards to the many hundreds of compounds found in the PCDL. However, unambiguous confirmation and quantification of these tentatively identified compounds still requires an authentic standard.

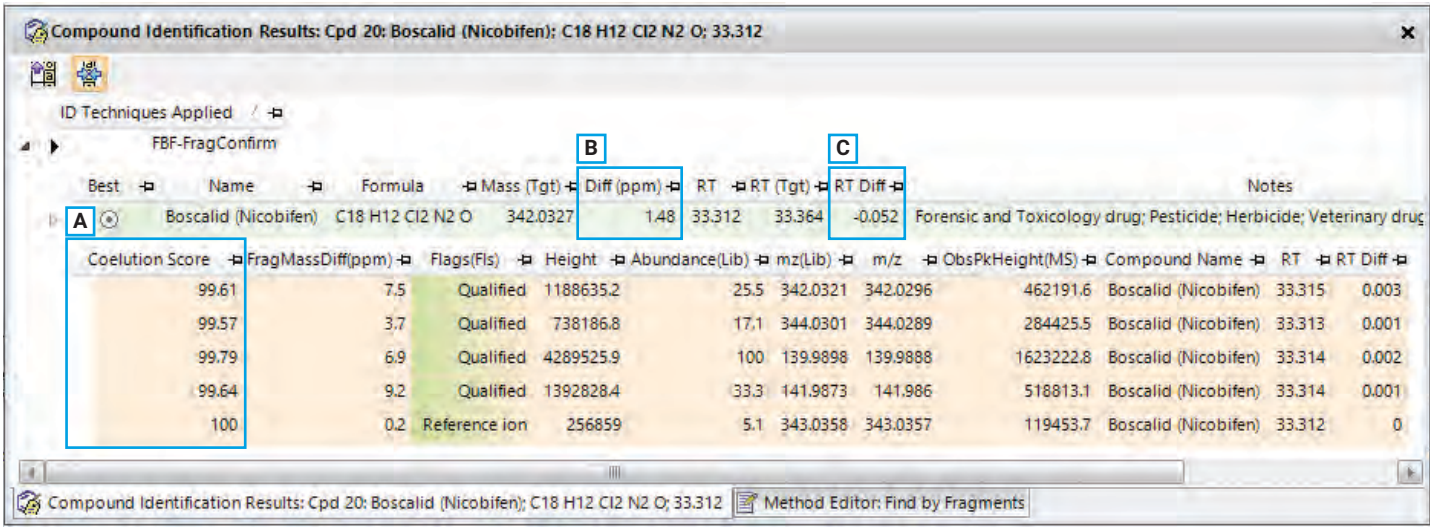

Figure 3. FBF results for boscalid showing: A) coelution scores, B) difference between the measured and theoretical monoisotopic molecular ion mass, and C) difference between the measured and database retention times.

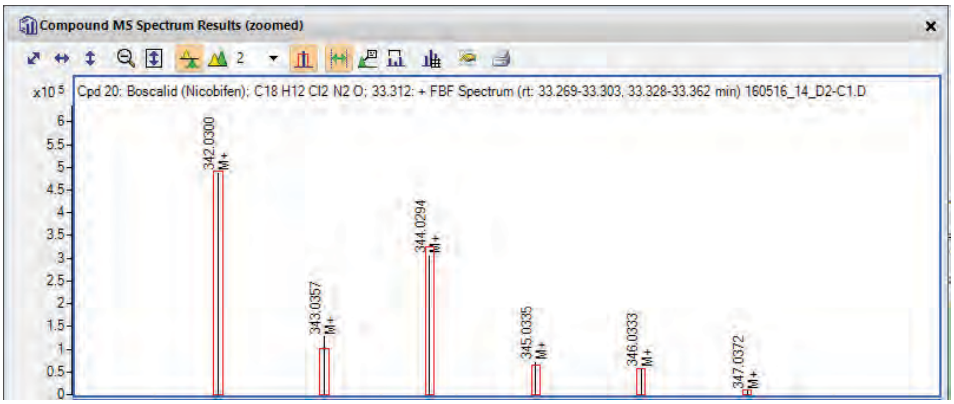

Figure 4. Theoretical (red rectangles) and measured molecular ion isotope pattern for boscalid found in a Cache Slough water extract.

Nontarget screening using MassHunter Unknowns Analysis software

Having screened the samples for all 750 pesticides in the PCDL, it may be desirable to determine whether there are any other compounds of interest in the extract. MassHunter Unknowns Analysis (UA) is designed to perform this nontarget screening. Unknowns Analysis deconvolutes the mass spectral data to isolate cleaned spectra from a complex mixture of overlapping spectra. These component spectra are then searched against a library, and a list of hits is generated. Since there is no large comprehensive accurate mass library available, the NIST unit mass library was used to generate the broadest possible list of hits. A new version of the pesticides PCDL (B.08.00), containing more than 850 compounds, was also used since it does contain exact mass spectra. Unknowns Analysis, using deconvolution, is a different data mining process than screening using the FBF workflow, and might identify some compounds missed by the FBF process. Table 5 shows the most important setpoints used for the Unknowns Analysis.

To determine if there might be other pollutants of interest in the water extracts, Unknowns Analysis was applied to six of the 51 samples. Depending on the extract analyzed, the number of deconvoluted components ranged from approximately 1,500 to 4,500. Of these, between 90 and 325 had NIST library match factors >70, and approximately 60–120 had match factors >80. An efficient way to sort through all the hits was by scrolling down from one hit to the next while looking at the molecular structure. When a compound of interest was observed, the hit was scrutinized to see if the spectral match looked good and if the extracted ions appeared to coelute with good peak shape.

Table 4. Suspect compounds identified by the FBF algorithm.

Compound	Use	CAS No.
2,4,6-Tribromophenol[1]	Different uses	118-79-6
2,4-Dimethylphenol (2,4-xylenol)	Different uses	105-67-9
2-Methylphenol	Different uses	95-48-7
4-Methylphenol	Different uses	106-44-5
Azoxystrobin	Fungicide	131860-33-8
Boscalid	Fungicide	188425-85-6
Bromacil	Herbicide	314-40-9
Carvone[1]	Insect repellent	99-49-0
Chlorthal-dimethyl (Dacthal or DCPA)	Herbicide	1861-32-1
Cyprodinil	Fungicide	121552-61-2
DEET	Insect repellent	134-62-3
Diazinon (Dimpylate)	Insecticide	333-41-5
Dichlobenil	Herbicide	1194-65-6
Dimethenamid (SAN 582H)	Herbicide	87674-68-8
Dimethoate	Insecticide	60-51-5
Diphenylamine[1]	Fungicide	122-39-4
Dithiopyr	Herbicide	97886-45-8
Diuron metabolite [3,4-Dichlorophenylisocyanate] [1]	Herbicide TP	–
Eugenol[1]	Insect attractant	97-53-0
Fluridone	Herbicide	59756-60-4
Hexazinone	Herbicide	51235-04-2
Iprodione (Glycophen)	Fungicide	36734-19-7
Malathion	Insecticide	121-75-5
Mepanipyrim[1]	Fungicide	110235-47-7
Metolachlor	Herbicide	51218-45-2
Napropamide	Herbicide	15299-99-7
Norflurazon	Herbicide	23576-24-1
Norflurazon-desmethyl	Herbicide TP	23576-24-1
Omethoate	Insecticide TP	1113-02-6
Oxadiazon	Herbicide	19666-30-9
Oxyfluorofen	Herbicide	42874-03-3
p,p'-DDE[1]	Insecticide TP	72-55-9
Pendimethalin	Herbicide	40487-42-1
Pentachlorophenol (PCP)	Different uses	87-86-5
Prodiamine	Herbicide	29091-21-2
Propiconazole	Fungicide	60207-90-1
Propyl cresol[1]	Different uses	–
Sulfentrazone	Herbicide	122836-35-5
Tebuthiuron	Herbicide	34014-18-1
Triclosan	Biocide	3380-34-5
Trifluralin	Herbicide	1582-09-8

[1] Tentatively identified (no reference standard comparison available)

The software calculates a component peak shape quality value (max = 100) and all but a few of the reported hits had a component shape quality >60. When using the Pesticides PCDL as the target library, retention times had to be close to the library value to report a hit, giving further confidence in the peak assignment.

Using the UA procedure, 25 pesticides, three pesticide transformation products (TPs), six organophosphates (three chlorinated flame retardants), and 13 other water pollutants (for example, phenolic antioxidant compounds) were found and tentatively identified in the six samples. Five pesticides and one TP were identified by UA that were not originally targeted by the GC/Q-TOF suspect screening procedure: 2,6-diisopropylnaphthalene, 3,4-dichloroanaline, fenbuconazole, fluorpyram, fluxapyroxad, and simazine. The first two were not included in the earlier version of the Pesticides PCDL, and the latter four were targeted by LC/Q-TOF suspect screening.

Table 5. Key setpoints for the method used with Unknowns Analysis.

Tab/Parameter[1]	Setpoint
Peak detection	
Peak detection	Deconvolution
SNR Threshold	0
Area filters	Absolute area ≥1,000
Deconvolution	
RT Window size factor	25, 50, 100, 200
Left *m/z* delta	0.3 amu or 50 ppm
Right *m/z* delta	0.7 amu or 50 ppm
Use integer *m/z* values	Checked for unit mass, unchecked when using 50 ppm
Component shape	Use base peak shape checked
Sharpness threshold	75 %
Library search	
Libraries	NIST14.L and Pesticides PCDL (ver. B.08.00)
Adjust score	Checked
Remove duplicate hits	Unchecked
Use RT match	Unchecked for NIST, checked for PDCL
RT Penalty function	Gaussian (30 seconds)
RT Mismatch penalty	Multiplicative
Max RT penalty	20
Compound identification	
Max hit count	1
Min match factor	70 for NIST14.L, 20 for PCDL
Min *m/z*	30
Library search type	Spectral Search
Target match	Not applied
Blank subtraction	
Perform blank subtraction	Unchecked

[1] Parameters not listed used the default values

Figure 5 shows the Unknowns Analysis results for *tris*(2,3-dichloropropyl) phosphate, a flame retardant that was found in several samples. Because many organophosphates have been used as plasticizers or fire retardants, they are widely distributed in the environment, and can show up in procedural blanks.

We found triphenyl phosphate, tributyl phosphate, *tris*(2-chloroethyl) phosphate, and *tris*(2,4-di-*tert*-butylphenyl) phosphate in at least one of two procedural blanks processed by UA.

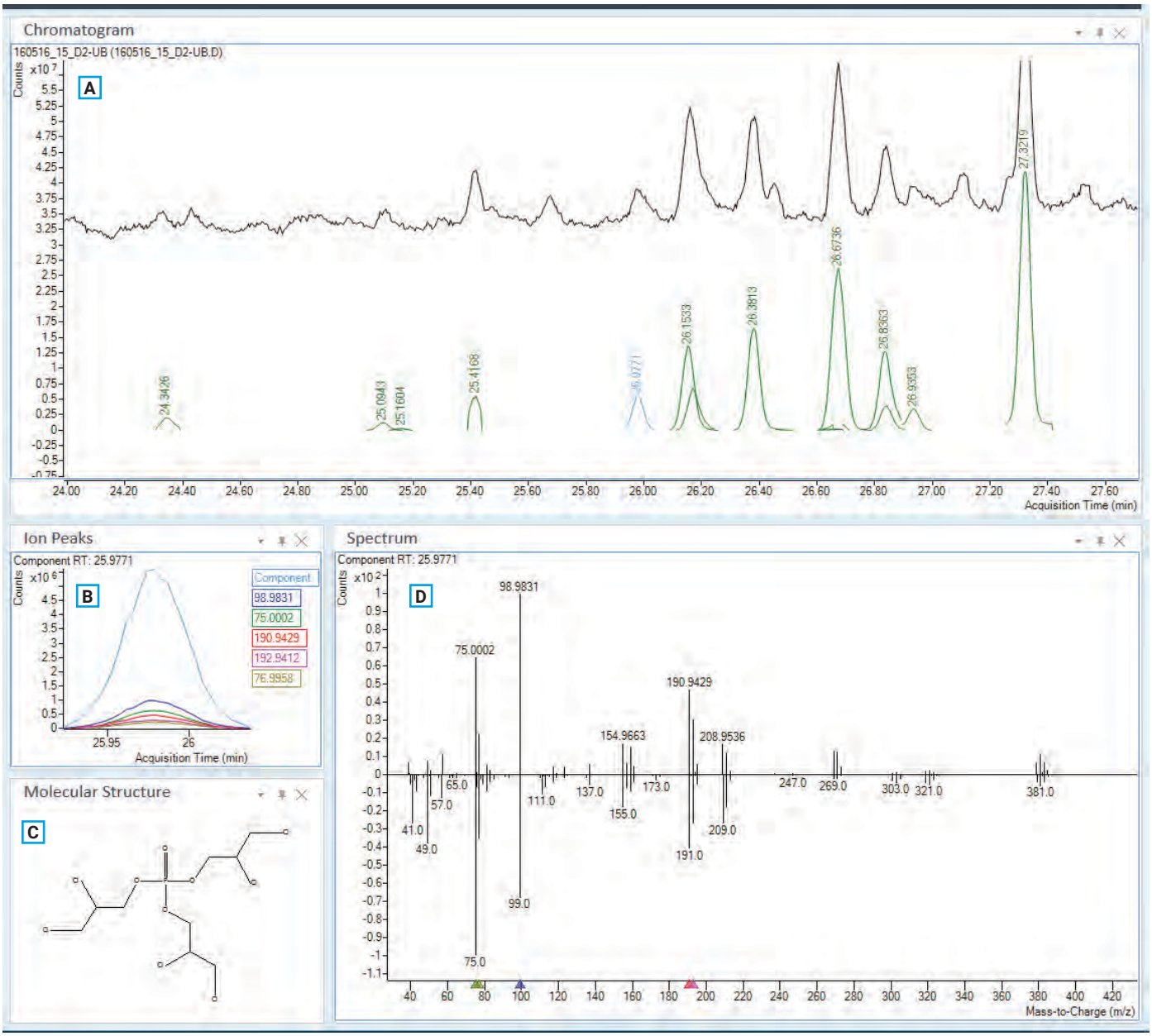

Figure 5. Agilent MassHunter Unknowns Analysis results for a Cache Slough water extract, showing the fire retardant *tris*(2,3-dichloropropyl) phosphate. A) TIC (black) and deconvoluted components [green (blue, peak for which results are displayed)]; B) plots of significant EICs overlaid with the component plot; C) molecular structure; D) component spectrum (top) positioned head-to-tail with the library spectrum. The compound had a NIST library match score of 84, and a component shape quality of 82.

Among the other compounds detected using this approach, were an isomer of dichloronitrobenzene, *bis*(dichloromethyl) ether, cyanomelamine, dibenzazepine, and a trichloronaphthalene isomer. These compounds remain tentatively identified because no authentic standard was available for unambiguous confirmation.

Table 6 lists the compounds found using UA. The first 37 compounds listed in Table 6 were identified with increased confidence since their retention times matched the values in the PCDL. The remaining nine compounds were tentatively identified based only on their NIST library match score.

Table 6. Pesticides, TPs, and other water pollutants tentatively identified by UA.

Retention time confirmed w/ PCDL
2-Methylphenol[1]
4-Methylphenol[1]
Trichloronaphthalene isomer
2,6-Diisopropylnaphthalene
3,4-Dichloroaniline (propanil TP)
Azoxystrobin[1]
Boscalid[1]
Bromacil[1]
Chlorothalonil[2]
Chlorpyrifos[2]
Cyprodinil[1]
DEET[1]
Desmethylnorflurazon
Diazinon[1]
Dimethenamid[1]
Dimethoate[1]
Dithiopyr[1]
Diuron mnetabolite (3,4-dichlorophenylisocyanate)[1]
Fenbuconazole
Fipronil[1]
Fluorpyram
Fluridone[1]
Fluxapyroxad
Hexazinone[1]

Retention time confirmed w/ PCDL
Metolachlor[1]
Oxadiazon[1]
Oxyfluorfen[1]
Propiconazole[1]
Simazine
Sulfentrazone[1]
Tributyl phosphate
Trifluralin[1]
tris(2-Butoxyethyl)phosphate
tris(2,4-Di*ert*butyl)phosphate
tris(2.3-Dichloropropyl)phosphate
tris(2-Chloroethyl)phosphate
tris(2-Chloroisopropyl)phosphate
Compounds with NIST library search >80 but not confirmed with RT
Benzothiazole
Dichloronitrobenzene isomer
Benzenesulfonamide, N-butyl-
Benzonitrile
p-Cresol
Cyanomelamine
bis(dichloromethyl) ether
Octodrylene
Dibenzazepine (iminostilbene)

Key: Pesticides | Transformation products | Water pollutants | Organophosphates

[1] Found by GC/Q-TOF suspect screening workflow
[2] Found by GC/Q-TOF target analysis

Conclusions

The Agilent GC/Q-TOF is an ideal tool for performing both target and nontarget analysis. River water extracts were analyzed in NCI mode for 21 targets (mostly pyrethroids) at sub-ng/L levels, and 16 of them were found in concentrations ranging from 0.6 to 33 ng/L. The Find by Formula approach paired with the Agilent Pesticides PCDL for GC/Q-TOF tentatively identified an additional 41 suspect compounds, most of which were confirmed by analyzing standards. Five of the most significant ions were extracted around the locked retention time for each compound in the database. Hits required that a reference ion and two additional ions meet the specified requirements for a hit (Table 3) and that the retention time matches the PCDL value. Six of the extracts were subjected to a nontarget screening using Agilent MassHunter Unknowns Analysis, which uses deconvolution to pick out cleaned spectra for hundreds of individual components. These were then searched against the NIST14 mass spectral library and the Agilent Pesticides PCDL. Twenty-five pesticides, three pesticide TPs, six organophosphates, antioxidants, several halogenated compounds, and other pollutants were found. Most could be tentatively identified by matching their retention time to the PCDL.

With the GC-Q/TOF workflows (target, suspect, and nontarget), it was possible to identify nearly 80 semipolar to nonpolar water contaminants. Running the same samples by LC/Q-TOF identified approximately 100 polar to semipolar water contaminants[1,2]. Although approximately 30 chemicals from the middle of the polarity range were detected on both instruments, this result shows that GC-Q/TOF and LC-Q/TOF are complementary, and it is necessary to rely on both platforms to obtain the full contaminant profile in environmental samples.

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5991-9132EN

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Analysis of Extractable and Leachable (E&L) Compounds Using a Low-Energy EI-Capable High-Resolution Accurate Mass GC/Q-TOF

Application Brief

Authors

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Introduction

Accurate compound identification is critical to the study of extractables and leachables (E&L) [1]. The complexity of E&L extracts, containing chemicals with a wide range of classes and concentrations, poses challenges for compound identification [2]. The GC-amenable portion of E&L studies is conventionally carried out with a unit mass GC/MS in standard EI full scan mode, with compound identification through NIST GC/MS library searching. Limited knowledge can be obtained from this technique for those compounds detected without a convincing library match score.

This work presents a novel tool to study E&L compounds with enhanced flexibility and confidence using a high-resolution accurate mass GC/Q-TOF equipped with a low-energy EI capable ion source.

Figure 1. Agilent 7250 Series GC/Q-TOF system.

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Experimental

Instrumental analysis

The sample extracts and controls were analyzed by an Agilent 7250 Series GC/Q-TOF system (Figure 1), with operational conditions listed in Table 1. An injection of *n*-alkanes was used to calibrate the retention index (RI) of the acquisition method.

Table 1. Agilent 7250 GC/Q-TOF Operational Conditions

Parameter	Value
Column	Agilent DB-5 MS UI, 15 m × 0.25 mm, 0.25 µm
Inlet	S/SL, 310 °C
Carrier gas	1.5 mL/min Helium
Oven program	50 °C for 5 minutes 10 °C/min to 320 °C, 10 minutes
Transferline	280 °C
Source mode	EI, 70 eV, 10-15eV
Source temperature	200 °C
Quad temperature	150 °C
Spectral range	50 to 1,000 *m/z*

Data analysis

Compound identification started with Agilent MassHunter Unknowns Analysis B.08.00 using SureMass signal processing [3] and matching against the NIST 14 GC/MS library (Figure 2). The formulas of identified compounds were studied by comparing the standard EI and low energy EI spectra. Agilent MassHunter Qualitative Analysis B.08.00 was used to review MS and MS/MS mass spectra when necessary. The MS/MS spectra-based structure elucidation of the candidates for the unknowns was performed using Agilent MassHunter Molecular Structure Correlator B.08.00. Agilent Mass Profiler Professional (MPP) B.13 was used for differential analysis among sample groups.

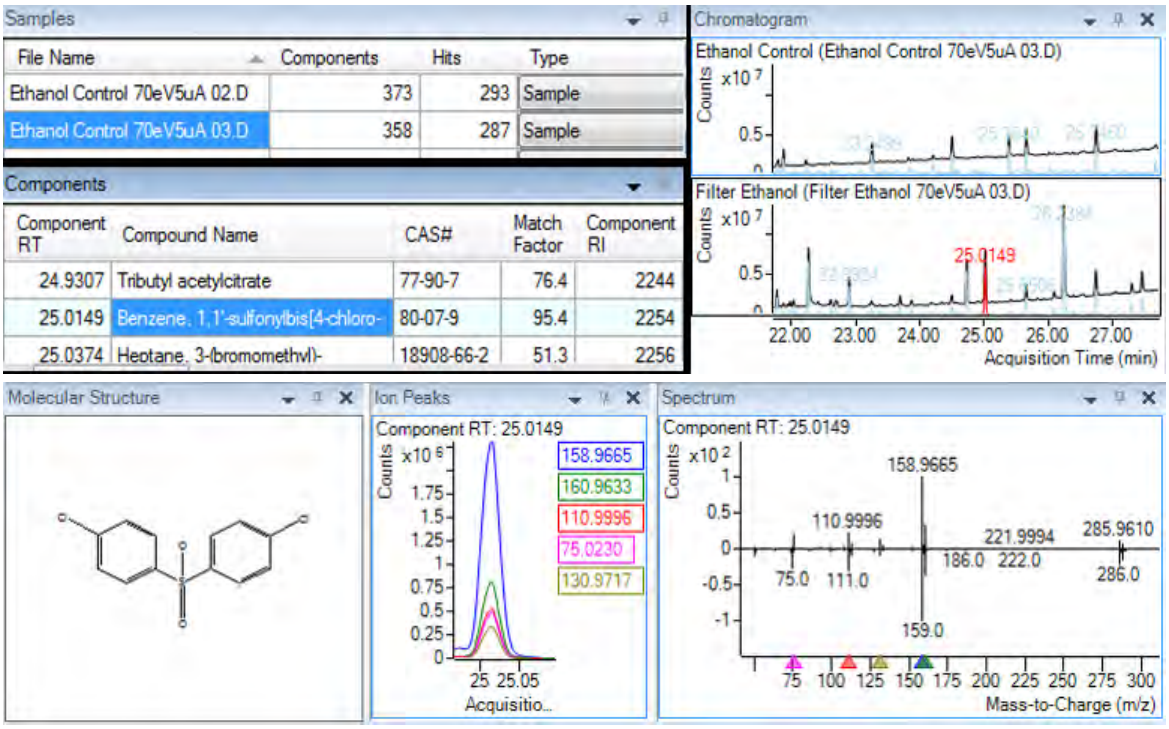


Figure 2. Agilent MassHunter Unknowns Analysis software for SureMass peak detection and library matching.

Sample preparation

A fully assembled single-use bioprocessing system was extracted using flow-through extraction with saline solution at 37 °C for 72 hours. The saline solution was prepared by adding one phosphate buffered saline tablet (Sigma) to each 200 mL of distilled water, resulting in a 137 mH NaCl, 2.7 mM KCl, 10 mM phosphate buffer solution (pH 7.4 at 25 °C). The filter of the device was extracted using ethanol and water/ethanol (1:1) solutions to demonstrate the difference between extraction solvents. Control blanks were prepared for all the extraction experiments. Each extract solution (except ethanol) was extracted with equal volume of dichloromethane, then concentrated 10 times for GC/Q-TOF analysis.

Results and Discussion

Saline extract versus control blank

We used MPP software to perform the differential analysis between sample and control, with saline extract results shown as a representative data set. The results indicate that 113 compounds present in saline extract of the complete device with a fold change ≥3 and a p-value ≥0.05 compared to the control blank (Figure 3). Table 2 shows the most abundant components.

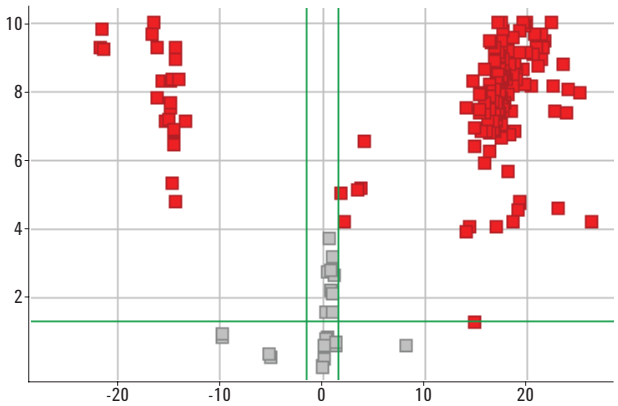


Figure 3. Volcano plot revealing compounds significantly present in the saline extract (upper right).

Table 2. Compound Identification List of Saline Extract (Top List)

Compound	Formula*	RI	Mass diff. (mDa)
Caprolactam	$C_6H_{11}NO$	1,268	0.2
Phenol	C_6H_6O	978	0.0
Tri(1,2-propyleneglycol), monomethyl ether	$C_{10}H_{22}O_4$	1,315	0.0
Dowanol 62b isomer 1	$C_{10}H_{22}O_4$	1,291	-0.2
Dowanol 62b isomer 2	$C_{10}H_{22}O_4$	1,294	-0.2
Dowanol 62b isomer 3	$C_{10}H_{22}O_4$	1,289	0.0
Tentative ID compound	$C_9H_{12}O_4$	1,572	0.5
Dowanol 62b isomer 4	$C_{10}H_{22}O_4$	1,286	-0.1
Benzoic acid, 4-ethoxy-, ethyl ester	$C_{11}H_{14}O_3$	1,527	0.1
Tentative ID compound	$C_{12}H_{15}N_3O_3$	1,659	0.2
Vanillin	$C_8H_8O_3$	1,399	-0.1
Hexanamide	$C_6H_{13}NO$	1,144	-0.2
Tentative ID compound	$C_8H_{12}O_3$	1,403	0.1
7,9-Di-*tert*-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	1,908	-0.2
Tentative ID compound	$C_{15}H_{22}O$	1,476	0.4
Ethylparaben	$C_9H_{10}O_3$	1,522	0.2
2-Pyrrolidinone, 1-methyl-	C_5H_9NO	1,040	0.3
2,4-Di-*tert*-butylphenol	$C_{14}H_{22}O$	1,507	0.0
Tentative ID compound	C_8H_8O	1,069	-0.2
2-Imidazolidinone, 1,3-dimethyl-	$C_5H_{10}N_2O$	1,109	0.3
Acetamide, N-cyclohexyl-	$C_8H_{15}NO$	1,292	0.2
Butoxyethoxyethanol	$C_8H_{18}O_3$	1,187	-0.2
Di-*t*-butylhydroquinone	$C_{14}H_{22}O_2$	1,467	0.0
2-Phenylisopropanol	$C_9H_{12}O$	1,088	-0.3
Tentative ID compound	$C_5H_{12}O_2$	1,014	0.1
Benzothiazole	C_7H_5NS	1,232	0.2
Dimethyl phthalate	$C_{10}H_{10}O_4$	1,452	0.1
Tentative ID compound	$C_{13}H_{20}O_2$	1,349	0.5

* Formulae of identified compounds were confirmed (or proposed for tentative ID compounds) by comparing the spectra from standard EI and low-energy EI modes.

Impact of extraction solvent

The filter extracts were evaluated to study the impact of using different extraction solvents on the overall extractable profile (Figure 4). The Venn diagram enables the easy visualization of these results, and shows both the unique as well as common extractables detected in each extract.

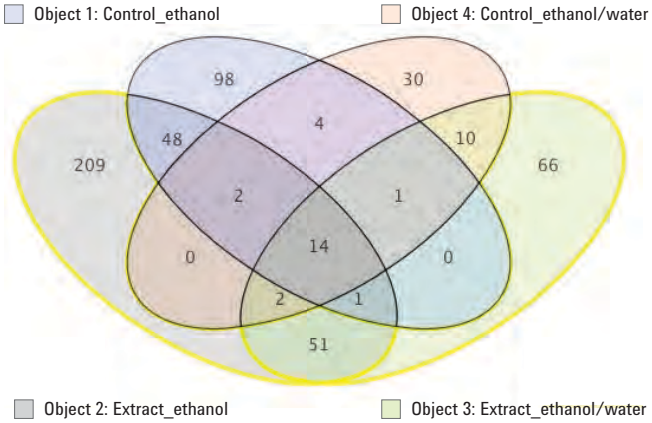


Figure 4. Venn diagram of extractable compounds from the filter of the device extracted by different solvents.

Low-energy EI investigation

Low-energy EI experiments increase the possibility of preserving or confirming the molecular ion (M^+) in the spectrum, as shown in Figure 5. These experiments can offer additional insights into identifying tentative compounds when the library search result is not promising.

Figure 6 illustrates the workflow to study an unknown compound (common between two solvent extraction groups) with low-energy EI and Q-TOF MS/MS. The possible candidate is a benzenemethanol derivative.

Figure 7 shows that the low-energy EI spectra also helped to confidently identify many alkane compounds unique to the ethanol extract.

Conclusions

- Low-energy EI increases the possibility of preserving or confirming M^+, and accurate mass MS/MS spectra provide valuable insights into structure elucidation of unknown compounds.
- Accurate mass measurements and RI calibration can enhance confidence in compound identification.
- Differential analysis facilitates the comparison of E&L compounds among sample groups.

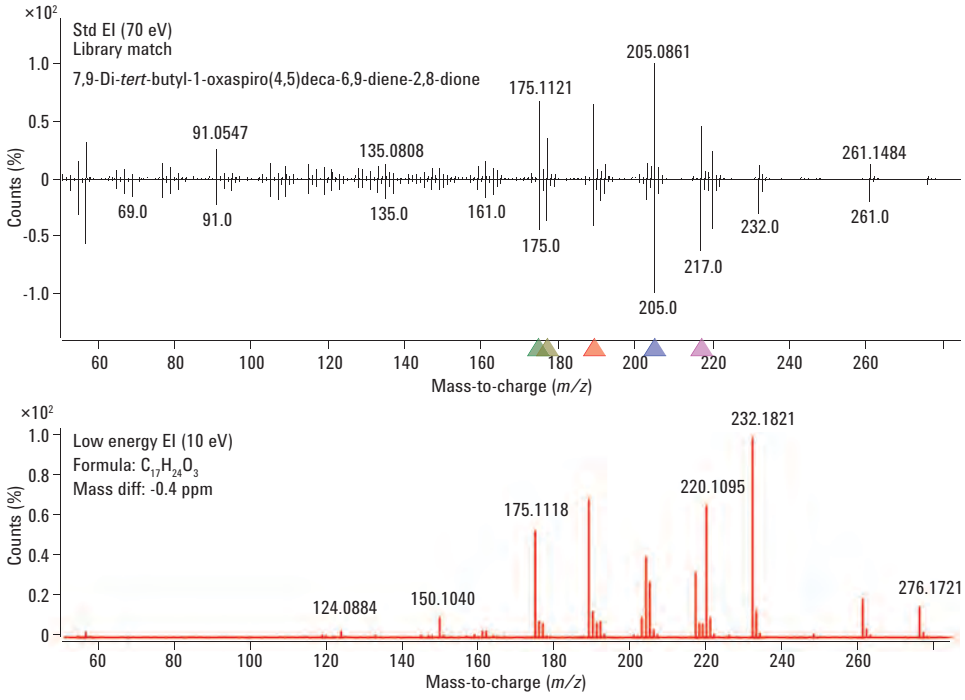
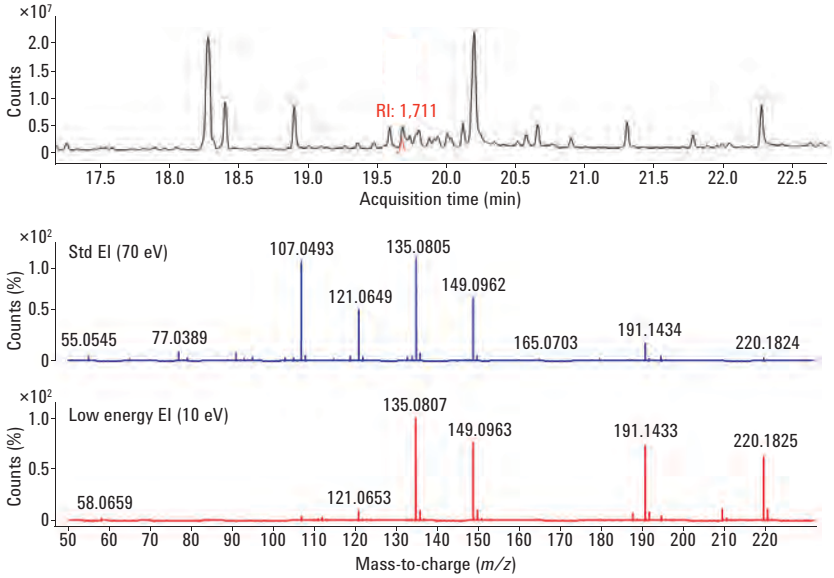
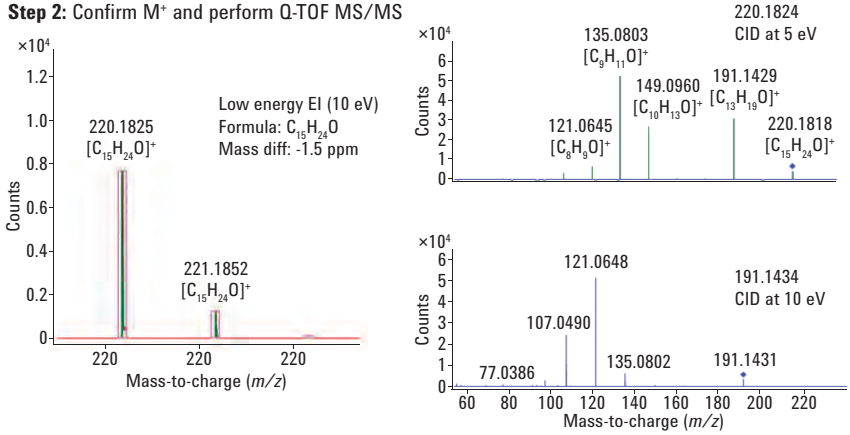


Figure 5. Low-energy EI increases the relative abundance of M^+ in the spectrum of a compound confidently identified with match score of 92.6 (RI: 1908).

Step 1: Measure unknowns with low energy EI source



Step 2: Confirm M^+ and perform Q-TOF MS/MS



Step 3: Structure elucidation on possible candidate

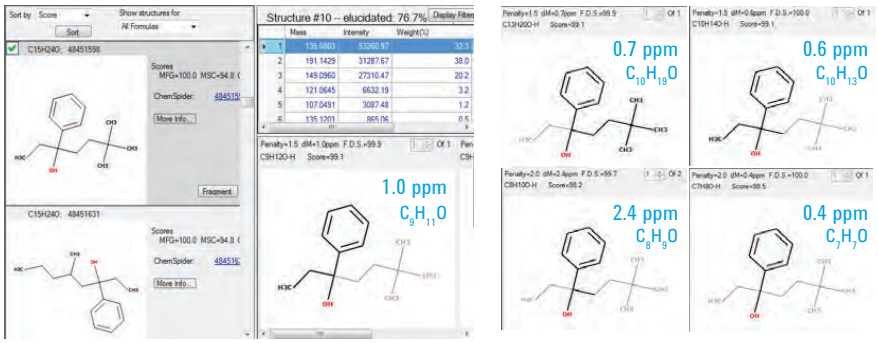


Figure 6. Study of an unknown compound with low-energy EI and structure elucidation on a possible candidate using Agilent MassHunter Molecular Structure Correlator.

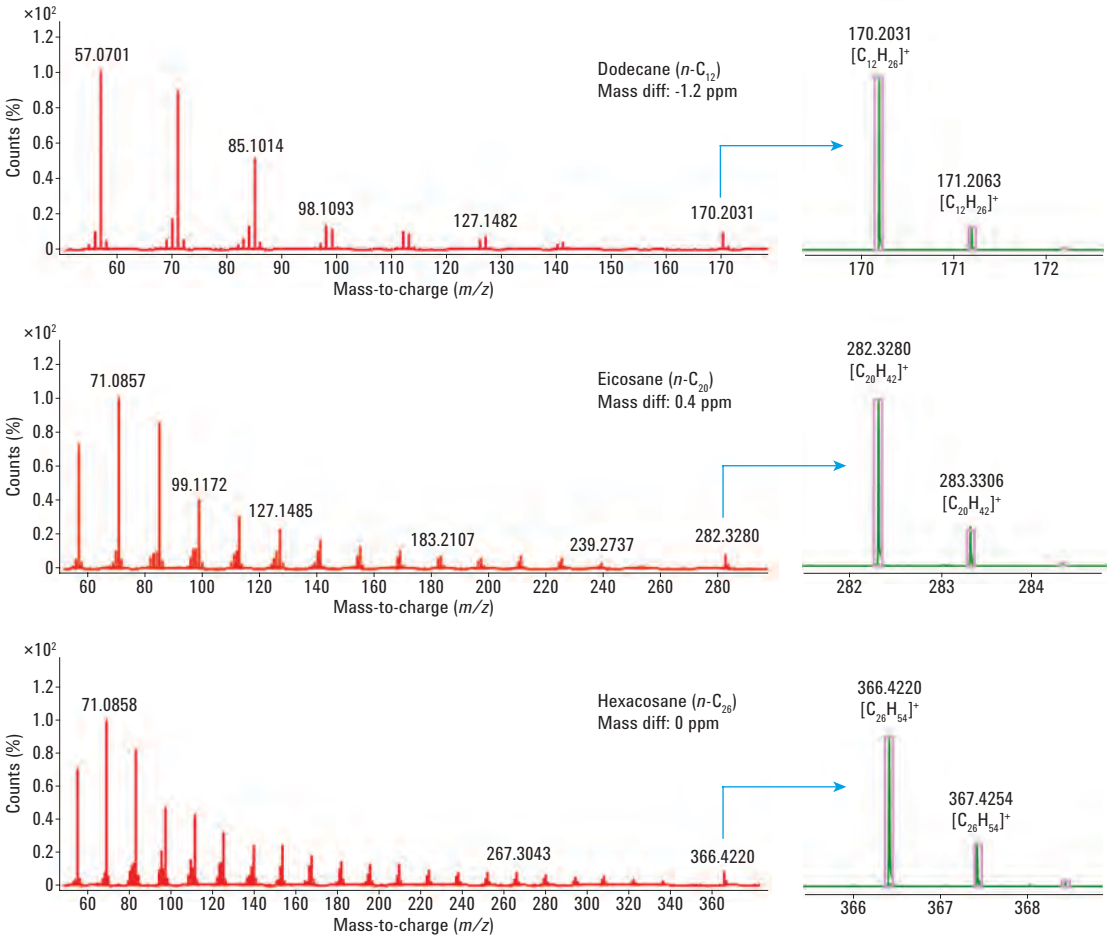


Figure 7. Low-energy EI (12 eV) spectra of n-alkanes. M^+ clusters show good mass accuracy and isotopic fidelity.

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Screening of Semivolatile Organic Compounds (SVOCs) on Aerosol Particles Using the Agilent 7200 Series GC/Q-TOF System

Application Note

Environmental

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Abstract

The composition of organic compounds absorbed in aerosol particles can provide important clues concerning aerosol-based air pollution studies. The full acquisition electron ionization (EI) mode of the Agilent 7200 Series Accurate Mass GC/Q-TOF MS, in combination with Agilent MassHunter Software tools, enabled a nontargeted workflow to screen a large variety of compounds in a complex particle extract. The EI-MS/MS capability was used to study the structure of unknown compounds based on the accurate mass of product ion fragments.

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Introduction

Semivolatile organic compounds (SVOCs) exhibit a wide range of molecular structures, and correlate to the formation and health-related effects of aerosol particles [1,2]. Due to a constantly growing interest in pollution by fine aerosol particles and an increasing diversity of absorbed organic compounds, screening of SVOCs has become a more demanding and complex task that requires enhanced selectivity, sensitivity, and a nontargeted workflow for data analysis.

An accurate-mass approach to the analysis of the organic content of aerosol particles using quadrupole time-of-flight mass spectrometry (Q-TOF) offers more reliable identification, and allows for a virtually unlimited number of SVOCs to be screened simultaneously. It provides an ideal analytical tool that can be used to screen and confirm both target and unknown compounds in complex aerosol particle extracts.

This application note demonstrates a nontargeted screening workflow for SVOCs absorbed in aerosol particles using a high-resolution Agilent 7200 Series GC/Q-TOF system. Compound hits were obtained by using deconvoluted mass spectra that were searched against a NIST nominal mass spectral library. The accurate mass of molecular ion or fragment ions was used in the confirmation of the compound formula. As an added advantage, the GC/Q-TOF system can be operated in MS/MS mode to investigate structures of unknown compounds.

Experimental

Instruments

This study was performed using an Agilent 7890B GC system coupled to an Agilent 7200A Q-TOF system. The instrument configuration is shown in Figure 1, and the instrument conditions are listed in Table 1. The GC operation enabled retention time locking (RTL) with the constant flow midcolumn backflush full screening method included in the Agilent Pesticides and Environmental Pollutants (P&EP) MRM database 3.0 (p/n 9250AA).

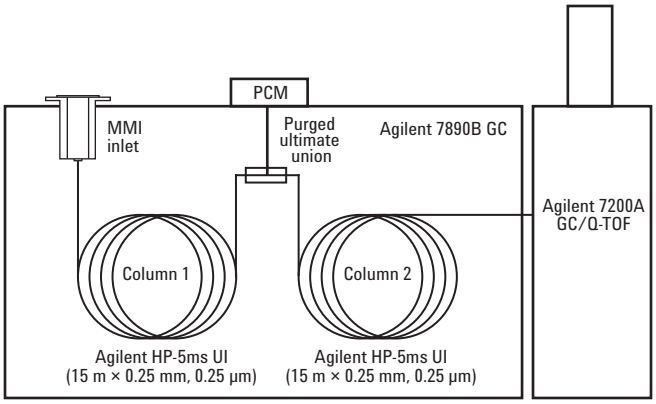

Figure 1. Agilent 7200 GC/Q-TOF System configuration depicting midcolumn backflush. The Agilent 7890B GC was coupled to the Agilent 7200A Q-TOF.

Table 1. Agilent 7890B GC and Agilent 7200A GC/Q-TOF Mass Spectrometer Conditions

GC conditions	
Columns	Agilent HP-5ms UI, 15 m × 0.25 mm, 0.25 µm film (two each)
Carrier gas	Helium
Column 1 flow	1.0 mL/min
Column 2 flow	1.2 mL/min
Inlet temperature	280 °C
Injection mode	Splitless
Injection volume	2 µL
Oven temperature program	60 °C for 1 minute, 40 °C/min to 120 °C, 0 minutes, 5 °C/min to 310 °C, 10 minutes
Run time	50.5 minutes
Backflush	5 minutes (Post run)
Transfer line temperature	310 °C
Q-TOF MS conditions	
Ionization mode	EI
Source temperature	300 °C
Quadrupole temperature	180 °C
Mass range	50 to 500 *m/z*
Spectral acquisition rate	5 Hz, collecting both in centroid and profile modes

Sample preparation

Aerosol particles (PM2.5) were collected on quartz fiber filters (QFF, Whatman, 5 inch × 8 inch), using samplers (Guangzhou, China) at a flow rate of 300 L/min. The QFFs were equilibrated at 20 °C and 40 % relative humidity (RH) in a temperature and humidity-controlled cleanroom chamber for 24 hours before and after sampling. The mass of particles on each QFF was determined by an electronic microbalance (Sartorious, IL, US) with 0.001 mg sensitivity. The filter (half) was extracted by Soxhlet with 50 mL of dichloromethane/hexane (1:1, v/v) at 70 °C for 48 hours. The extract was filtered with a filter device. After concentration by rotary evaporator, the extract was further concentrated to 2 mL under a pure N_2 stream, with solvent exchanged to *n*-hexane.

Data analysis

The data were processed by chromatographic peak deconvolution using the Unknowns Analysis tool in Agilent MassHunter Quantitative Analysis Software (B.07.01), followed by compound identification by comparison with the NIST 14 mass spectral library. The identities of deconvoluted peaks can be further confirmed using accurate mass information and the accurate mass tools in MassHunter Qualitative Analysis Software (B.07.01). Molecular Structure Correlator (MSC) Software was used to further study the structures of tentatively identified compounds.

Results and Discussion

Chromatographic peak deconvolution and library search

Data were processed using chromatographic peak deconvolution in the Unknowns Analysis Software with a 100 parts per million (ppm) accurate mass Extraction Window setting and variable Retention Time Window Size Factor of 50–200 to find the highest number of components (Figure 2). The comparison with the NIST library with a Match Factor score > 50 identified approximately 2,600 components including alkanes, hopanes, ketones, polycyclic aromatic hydrocarbons (PAHs), oxygenated polycyclic aromatic hydrocarbons (O-PAHs), esters, and heterocyclic compounds. The Molecular Formula Generator and Formula Calculator tools were used to confirm the identity of each compound found by deconvolution. Screening results of PAHs and O-PAHs are shown in the following text as examples (Figures 3 and 4). A similar workflow can be applied to screen for other chemical groups.

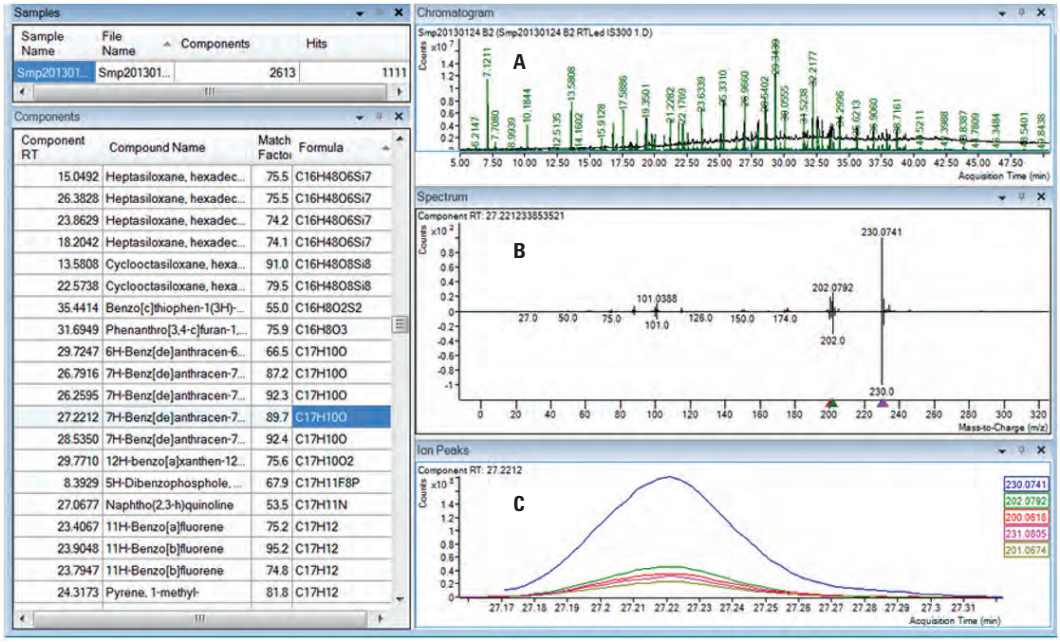

Figure 2. Unknowns Analysis software was used to perform chromatogram deconvolution. The Total Ion Chromatogram (TIC) (A), mirror plot of component and library hit spectra (B), and overlaid Extracted Ion Chromatograms (EICs) of the component (C), are depicted.

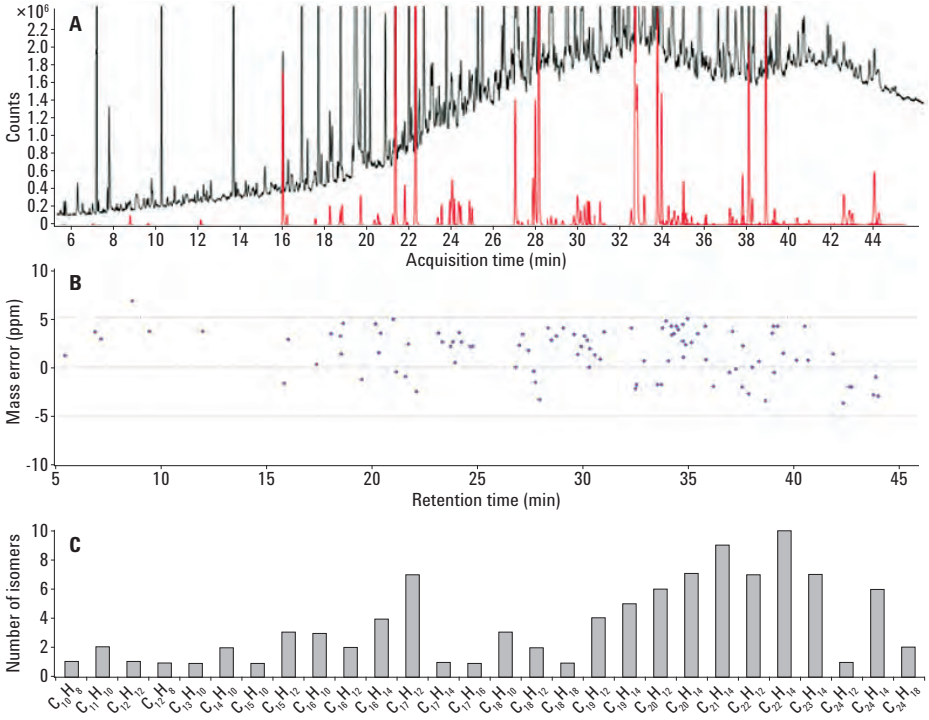

Figure 3. Screening results of PAHs. The chromatogram (A) overlays the TICs (black) and the component profiles of identified PAHs (red), while the mass errors of molecular ions of the identified PAHs calculated by the Formula Calculator are shown in B, and formula distribution is shown in C.

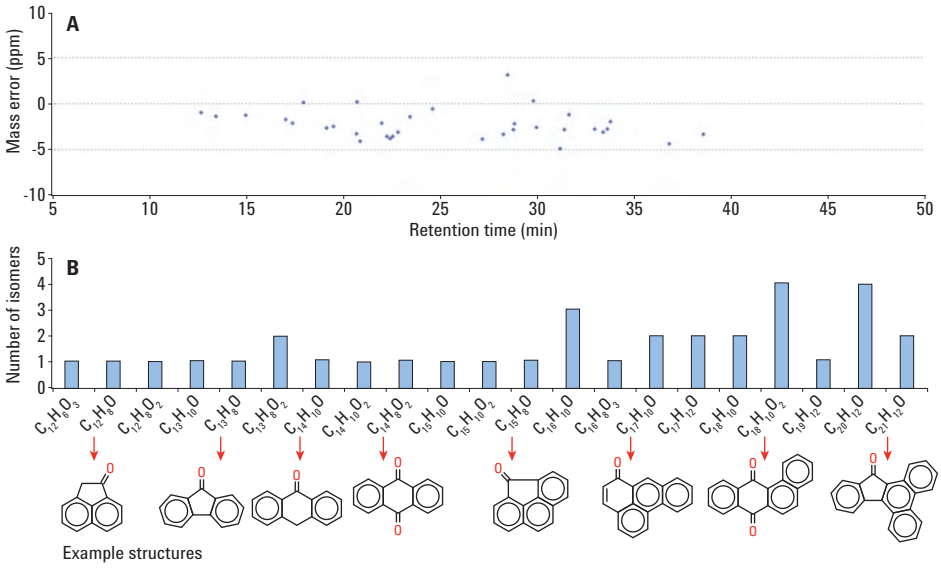

Figure 4. Confirmation of O-PAHs using accurate mass information. The mass errors of molecular ions of the identified O-PAHs calculated using the Formula Calculator are shown in A, while formula distribution and example structures are shown in B.

Identification of PAHs and O-PAHs

Unknowns Analysis was able to identify a large number of PAHs that coeluted in the unresolved complex mixture, as shown in Figure 3. The accurate mass information was used to confirm approximately 100 PAHs, with mass errors of less than 5 ppm. The P&EP MRM database contains retention times (RTs) for several of the PAHs, and was used to further confirm hits. The RT differences between database and sample were all within a window of 0.03 minutes. The good RT match further verifies the use of accurate-mass information as a confirmation tool. The formula distribution shows a wide range of PAHs in the extract of aerosol particles, with carbon numbers from 10 to 28.

Similarly, O-PAHs were also identified in the extract of aerosol particles, with 34 components confirmed by the accurate mass information. Figure 4 indicates the mass errors and the formula distribution of all identified O-PAHs. The structures for some typical O-PAHs are also displayed in Figure 4.

Structure of an unknown compound proposed by MS/MS

The chromatographic peak deconvolution was able to discover unknown compounds, as shown in Figure 5. The closest match for this spectrum in the NIST library was anthra[1,9-*cd*]pyrazol-6(2*H*)-one, with a formula of $C_{14}H_8N_2O$. However, this tentative match could be readily rejected based on mass accuracy alone, since the error on the molecular ion is 48.62 ppm. This highlights the advantage of accurate mass data obtained from a Q-TOF versus a unit mass instrument.

Using accurate mass information, the proposed formula for this unknown compound was $C_{15}H_8O_2$, with a mass error of 2.83 ppm. However, no compound with this formula was found in the NIST MS library. One of the other advantages of the 7200 GC/Q-TOF is the ability to perform accurate mass MS/MS experiments, which are very valuable for structural elucidation of unknowns.

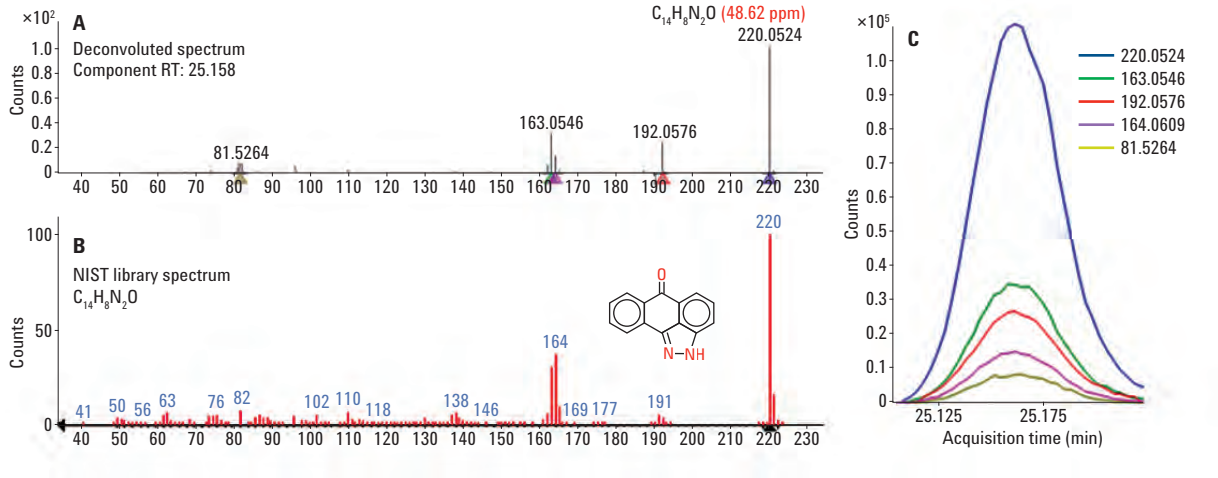

Figure 5. Comparison of mass spectrum between an unknown compound and a tentative NIST library match (A,B). The coelution profiles of deconvoluted ions (C) confirm that they all belong to the same component. However, this compound can be readily rejected based on mass accuracy alone, since the error on the molecular ion is 48.62 ppm.

Figure 6 shows the workflow using the MS/MS mode with accurate mass fragments to propose the structure of this unknown compound. The Formula Generator tool was used to assign an accurate empirical formula to the molecular and major fragment ions. To propose the structure of this unknown compound, the spectrum was imported into Molecular Structure Correlator (MSC) software as a CEF file, and MSC searched the ChemSpider database to find all possible structural isomers. Although this type of confirmation is not completely unambiguous, it provides additional validation for this tentatively identified O-PAH. Figure 7 illustrates a proposed fragmentation pathway based on fragments listed in MSC software.

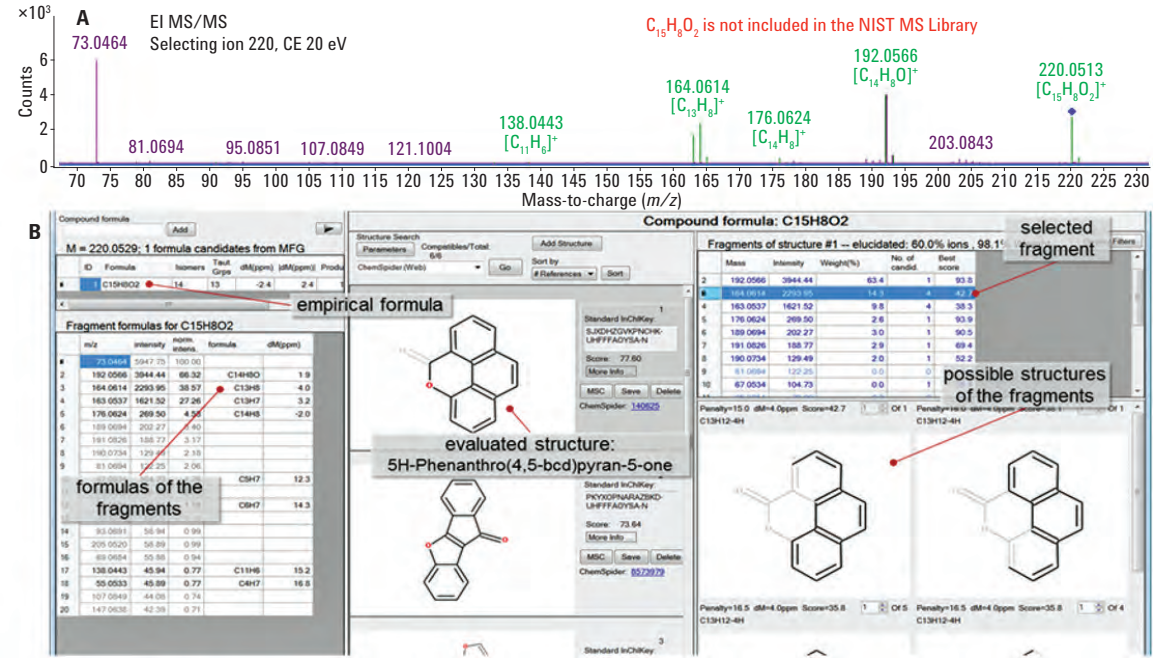

Figure 6. Empirical formulas generated from a MS/MS spectrum using the Formula Generator tool (A), and structure elucidation results of the compound with an empirical formula of $C_{15}H_8O_2$ using MSC software (B). Each individual fragment ion is ranked based on mass error corresponding to the proposed formula, along with a penalty based on how many bonds needed to be broken to generate that proposed formula.

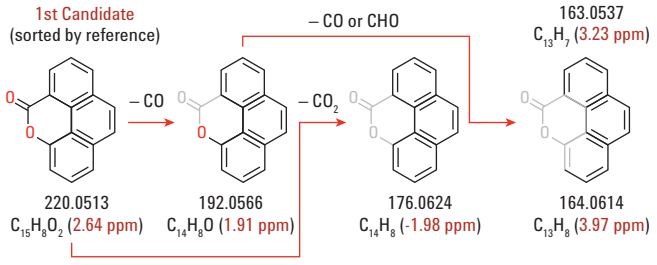

Figure 7. Fragmentation pathway of $C_{15}H_8O_2$ candidate based on fragments listed in MSC software.

Conclusion

Studies of SVOC compounds absorbed in aerosol particles can be greatly enhanced by using the multiple features of the Agilent 7200 GC/Q-TOF such as accurate mass information, high sensitivity in full spectrum mode, and MS/MS capabilities. The use of Agilent MassHunter Software capabilities such as deconvolution, automatic fragment formula annotation, and structure elucidation enabled the nontargeted approach in SVOC screening. Compound confirmation from library searches and structure suggestions for unknown compounds are also important investigative tools.

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Analysis of Biomarkers in Crude Oil Using the Agilent 7200 GC/Q-TOF

Application Note

Petrochemical and Environmental

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Abstract

The analysis of biomarkers such as (alkyl-) dibenzothiophenes, hopanes, and steranes in crude oil is used in many petrochemical applications, including the characterization of oil sources and the identification of sources of oil spillage. The analysis is normally done by GC-MS after complex sample preparation and fractionation.

Using a high resolution time-of-flight mass spectrometer, a diluted sample can be analysed without fractionation and the biomarkers of interest can be measured by exploiting the high selectivity of ion extraction at accurate mass.

The excellent sensitivity of the system allows the selective detection of dibenzothiophene, alkylated dibenzothiophenes and hopanes. Using the Agilent GC/Q-TOF system in MS/MS mode, low levels of steranes could be selectively detected.

Agilent Technologies

Introduction

Biological markers or biomarkers include a large group of hydrocarbons, including alkanes, polycyclic aliphatics, and polycyclic aromatic hydrocarbons, that are persistent in the environment. These biomarkers can be used in many petrochemical applications including the characterization of oil sources, as indicators of oil maturity or oil weathering. The biomarkers can also be used to identify sources of environmental pollution by oil spillage [1,2].

Typical biomarkers are heterocyclic polycyclic aromatic hydrocarbons, such as alkyl-dibenzothiophenes, pentacyclic triterpanes such as hopanes, and sterol-derived polycyclic alkanes such as steranes (for example cholestane). Usually, the analysis of these biomarkers is performed by GC-MS. Prior to the analysis, sample fractionation by liquid-liquid extraction, column chromatography, and/or solid phase extraction is used to isolate alkane and aromatic fractions. Finally, the extracts are separated by GC, and detection is performed by MS operated in selected ion monitoring (SIM). Since a large number of markers are monitored, often multiple runs, each monitoring a group of markers might be necessary.

In this application note, the Agilent 7200 Q-TOF instrument was used to analyze dibenzothiophenes (DBTs), hopanes, and steranes in a crude oil by direct injection of a diluted solution of the oil. The time-of-flight instrument combines high sensitivity and resolution with accurate mass determination. This offers unique selectivity for the detection of trace analytes in a complex matrix. The GC/Q-TOF approach is also not limited to a group of preselected analytes (as in SIM or MRM operation using single quadrupole or triple quadrupole systems), but the different classes of biomarkers can be detected, identified and quantified by using extracted ion chromatograms at accurate masses of selective ions. In addition, the possibilities of MS/MS operation also enables additional selectivity at trace level if the selectivity in full scan mode is not sufficient.

Experimental

Chemicals and Sample

A reference solution NIST SRM 2260a (LGC, Molsheim, France) containing dibenzothiophene was used to check instrument performance. The test sample was diluted 10 times in hexane. The final concentration of dibenzothiophene was 0.38 ng/µL.

A crude oil was obtained from Total, France. From the crude oil, 100 mg was weighed and extracted in 10 mL hexane using sonication. The solution was centrifuged and an aliquot of the clear supernatant was diluted 10 times in hexane (final oil concentration: 1 mg/mL)

GC and MS conditions

An Agilent 7890A GC System, equipped with a SSL, combined with a 7200 Q-TOF system was used.

The analytical conditions are summarized in Table 1.

Table 1. GC/Q-TOF Conditions

Injection	Inlet type	Split/splitless
	Mode	Splitless
	Temperature	300 °C
	Volume	1 µL
Column	DB-5MS, 30 m x 0.25 mm, 0.25 µm	
Carrier	1.5 mL/min, helium, constant flow	
GC oven	50 °C (1 min) - 10 °C/min - 320 °C (8 min)	
Detection	Ionization mode	EI
	MS mode	scan 40–500 Da
	Acquisition rate	5 Hz
	MS/MS mode	scan 40–500 Da
		CE : 10 eV
	Source temp	280 °C
	Quad temp	150 °C

Results and Discussion

First a reference sample, containing 0.38 ng/µL dibenzothiophene was analyzed. The chromatogram (elution window 5–23.5 minutes) is shown in Figure 1. DBT elutes at 16.3 minutes. The mass spectrum is shown in Figure 1B. The most abundant ion, corresponding to the molecular ion, is detected at *m/z* 184.0338. The mass error was less than 2 ppm as compared to the exact mass of molecular ion ($C_{12}H_8S$, $M^{\bullet+}$ = 184.0341).

Next, the crude oil sample was analyzed using the same method. The total ion chromatogram is shown in Figure 2a. The profile is characterized by the typical homologue series of n-alkanes. The elution time of dibenzothiophene is indicated by an arrow. Using an extracted ion chromatogram at *m/z* 184 ± 0.5 amu, as is typically done in single quadrupole MS systems, dibenzothiophene can be detected, as shown in Figure 2b. However, several other compounds are also detected, especially in a time window between 14–18 minutes. These compounds (probably C4-naphthalenes, $C_{14}H_{16}$, MW = 184) can potentially interfere with the selected biomarker.

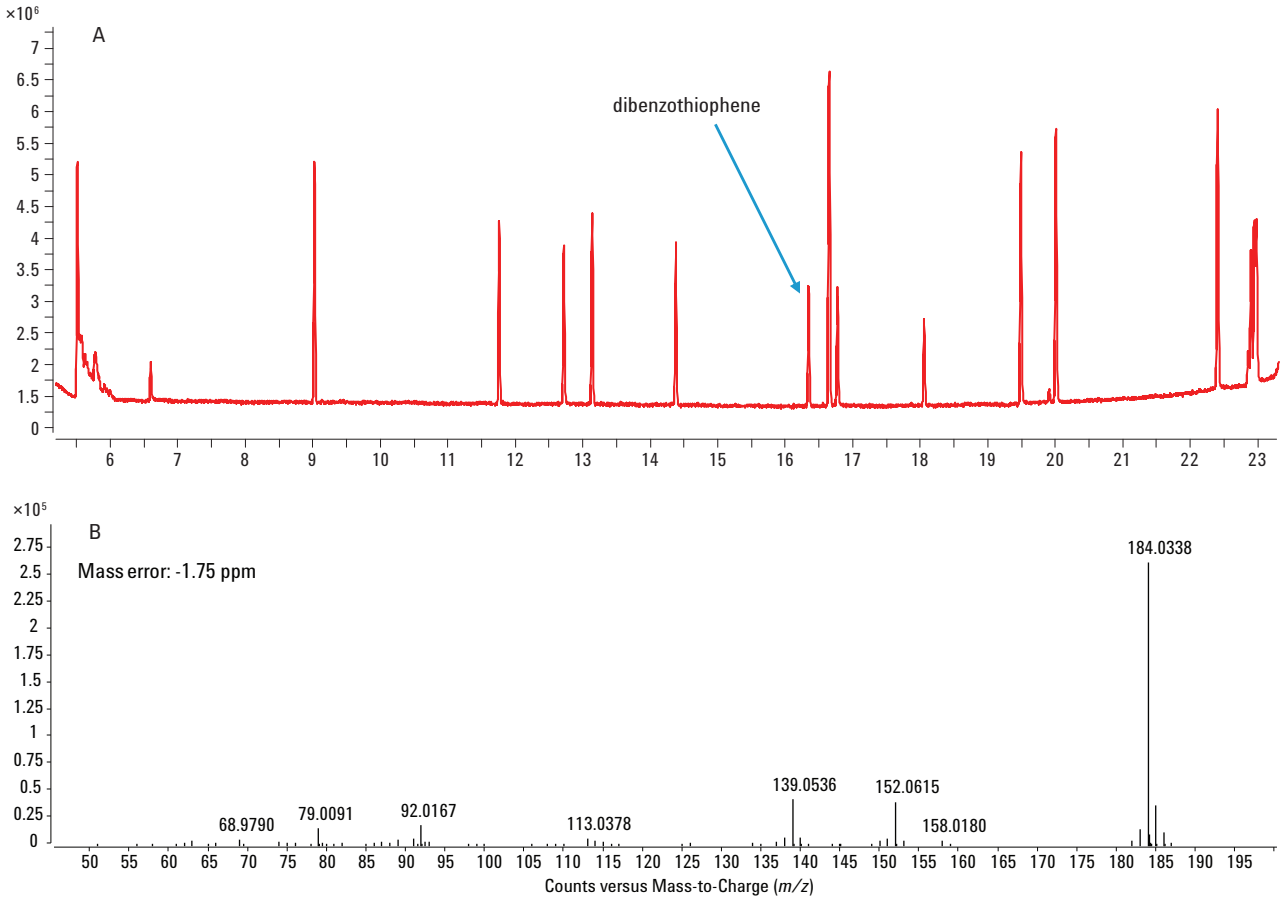

Figure 1. GC/Q-TOF analysis of aromatic hydrocarbon standard mixture. The spectrum obtained for 0.38 ng dibenzothiophene($C_{12}H_8S$, $M^{\bullet+}$ = 184.0341) is shown in B.

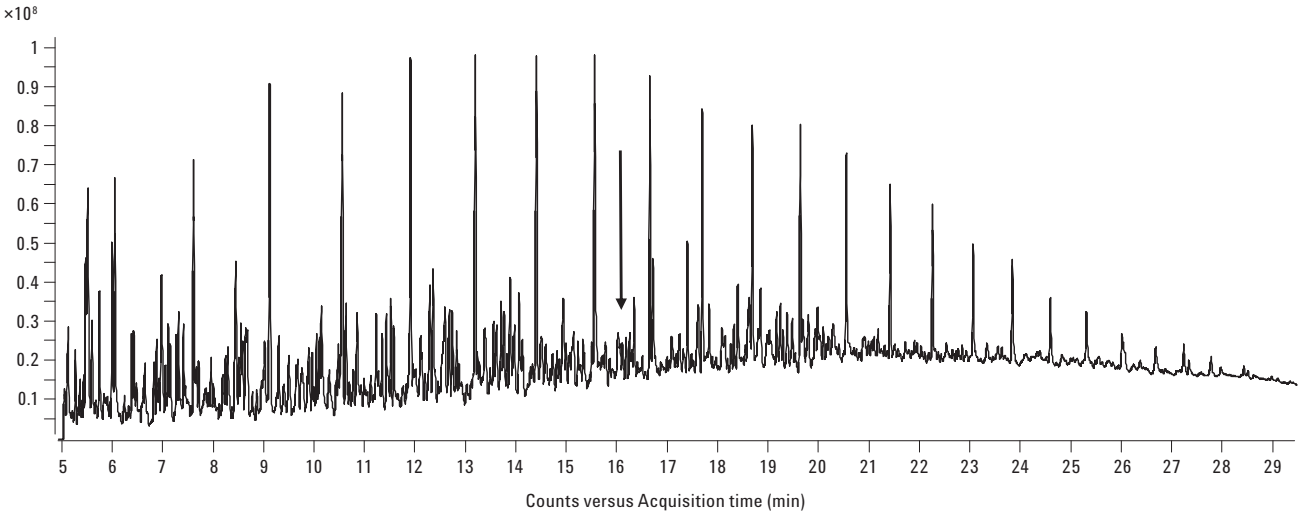

Figure 2a. Total ion chromatogram of crude oil, elution time of DBT is indicated by arrow.

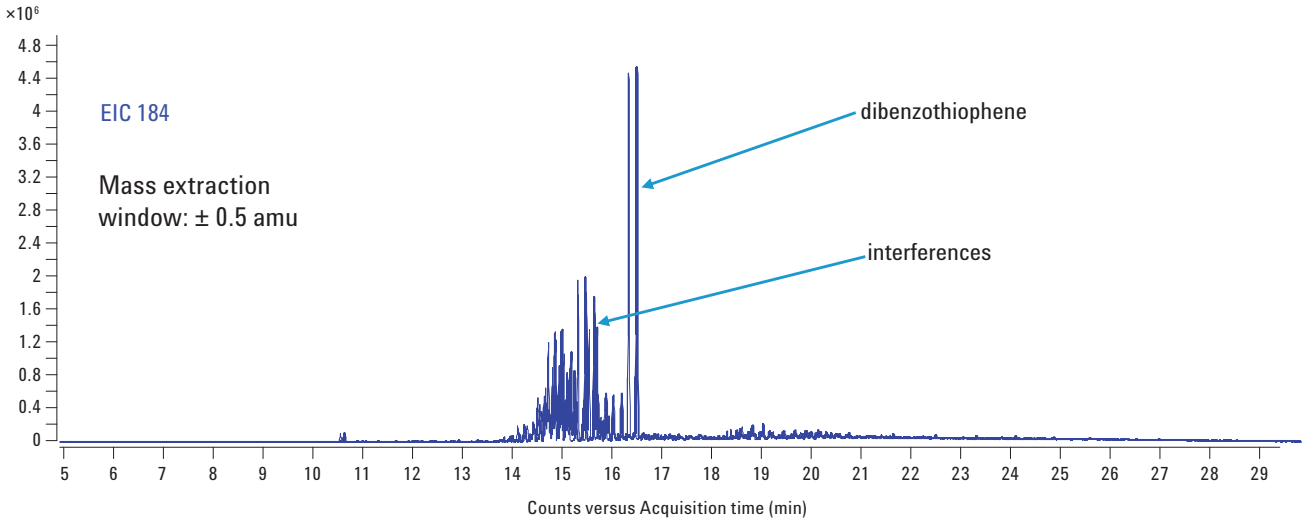

Figure 2b. Extracted ion chromatogram at 184 ± 0.5 amu.

Using an extracted ion chromatogram at exact mass (184.0341 ± 5 ppm), a much higher selectivity is obtained allowing to eliminate all the interferences as shown in Figure 2c. The mass spectrum acquired at 16.32 minutes is shown in Figure 2d. The mass accuracy obtained for DBT in the complex matrix is not significantly affected (*m/z* 184.0339) with a mass error below 2 ppm.

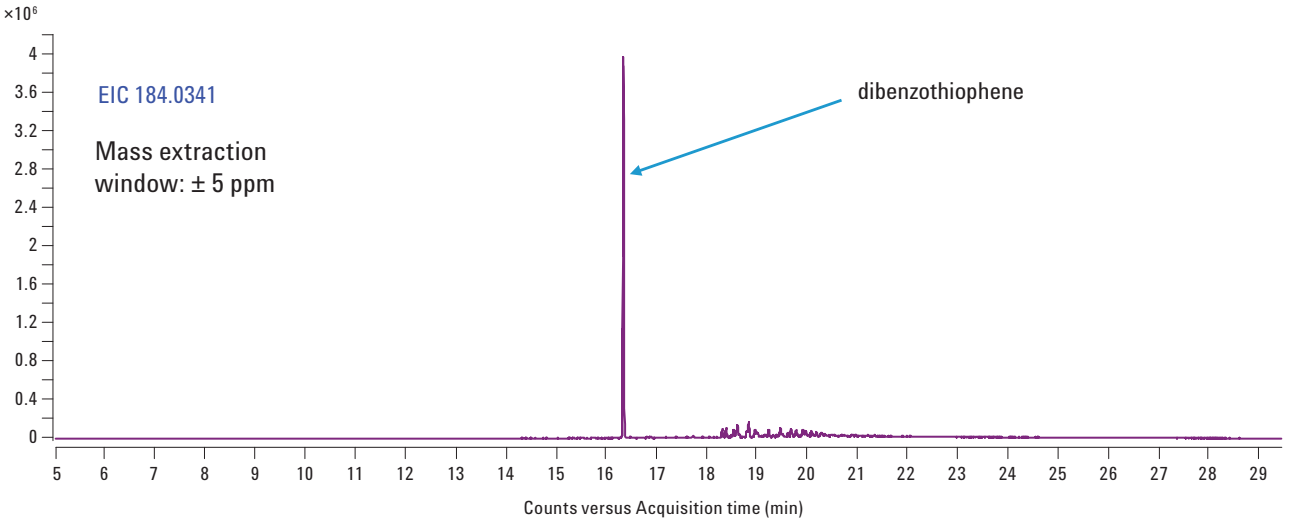

Figure 2c. Extracted ion chromatogram at 184.0341 ± 5 ppm.

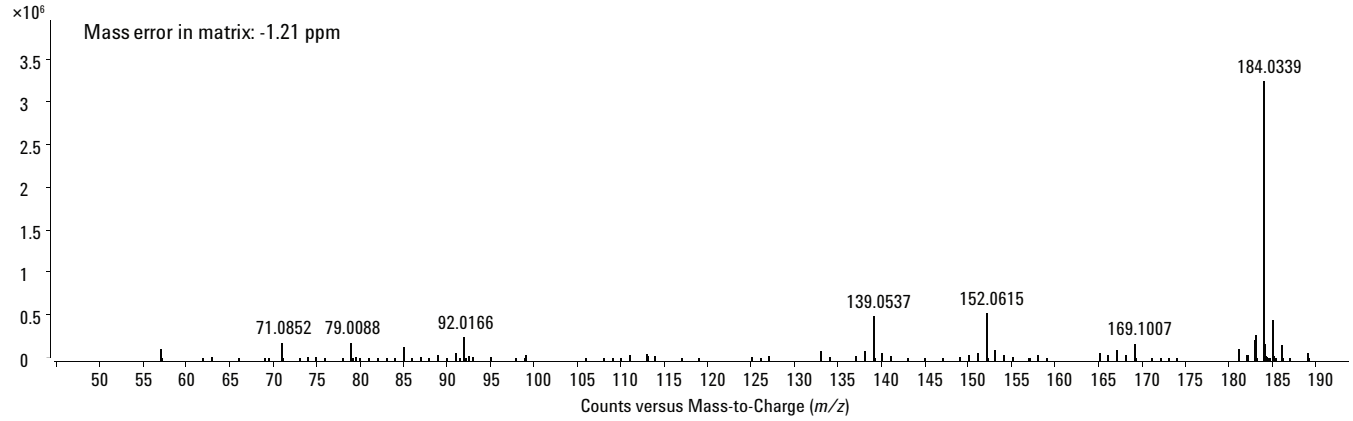

Figure 2d. Mass spectrum of dibenzothiophene in crude oil matrix.

In the same way, it was possible to extract ion chromatograms at *m/z* 198.0498 for methyl-dibenzothiophenes (C1-DBT, 4 isomers, only three chromatographically resolved) and at *m/z* 212.0645 for C_2-dibenzothiophenes. These DBT biomarkers are easily detected as shown in Figure 3.

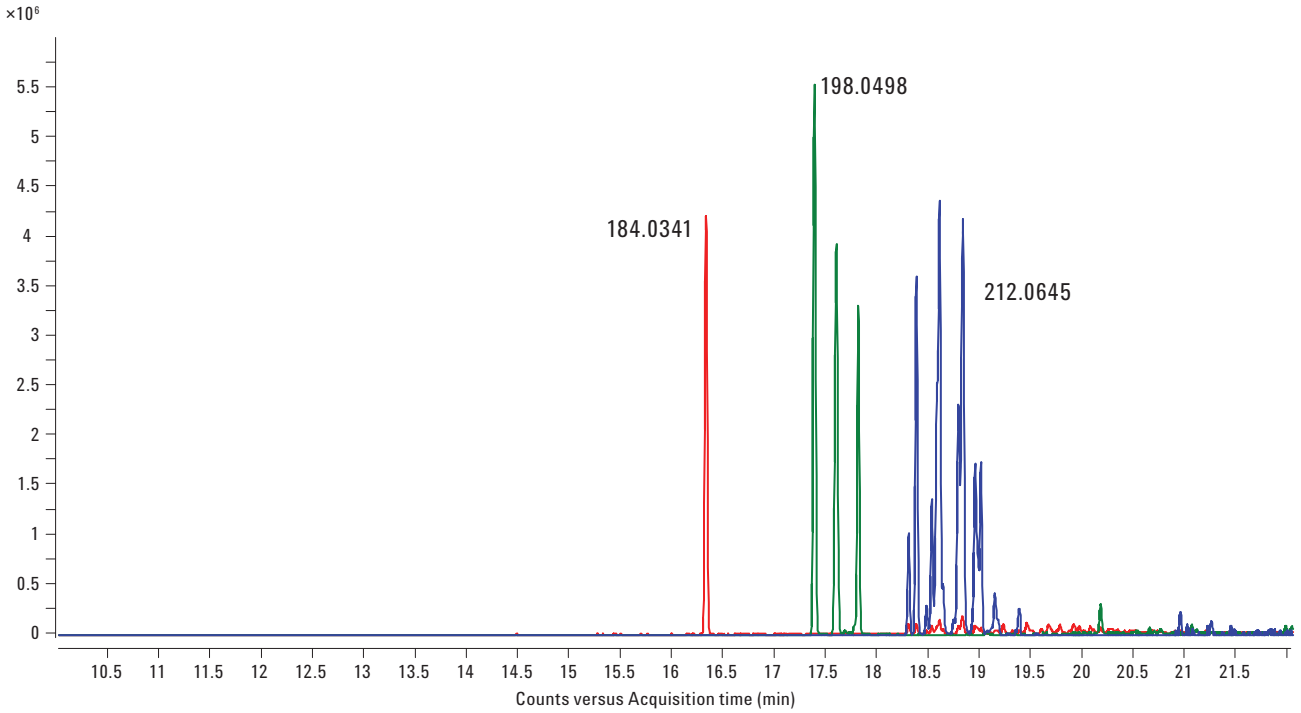

Figure 3. Extracted ion chromatograms at accurate mass (± 5ppm) for the detection of DBT (ion 184.0341), methyl-dibenzothiophenes (ion 198.0498) and C_2-dibenzothiophenes (ion 212.0645).

Besides the S-containing PAHs, hopanes and steranes are also important biomarkers. In the same way, extracted ion chromatograms at accurate mass can be used to selectively detect these analytes in the complex crude oil matrix. In Figure 4, the extracted ion chromatograms at 191 ± 0.5 amu (top) and at 191.1794 ± 10 ppm (bottom) are compared. Much higher selectivity and, consequently, higher signal-to-noise are clearly obtained by using accurate mass detection. Several hopanes could be detected in the elution range between 26 and 30 minutes. Main peaks (27–28 minutes) probably correspond to nor-hopanes ($C_{29}H_{50}$, MW=398).

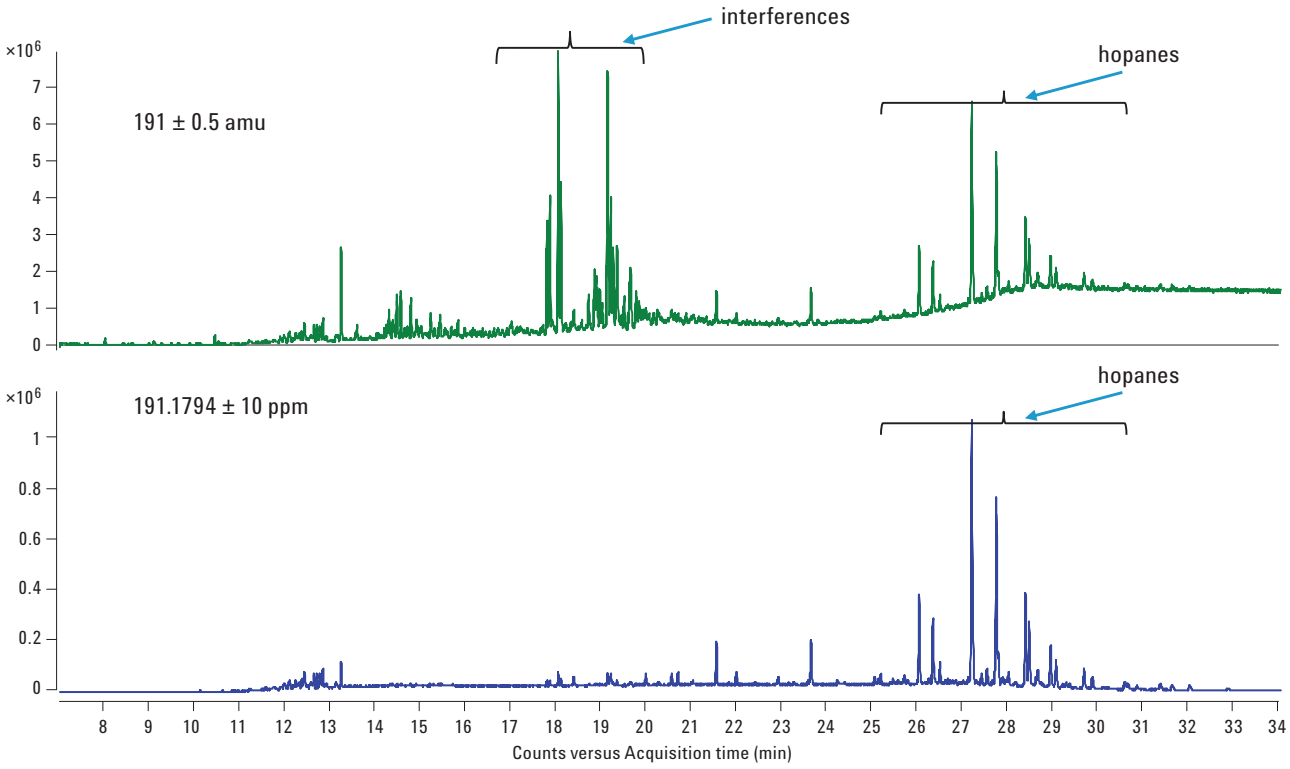

Figure 4. Extracted ion chromatograms at 191 ± 0.5 amu (top) and at accurate mass (191.1794 ± 10ppm) for the detection of hopanes.

Finally, specific ions for steranes were extracted. As these analytes are present at lower concentration in this sample and interfered with by matrix ions even when using accurate mass EICs, as illustrated in Figure 5. Since the GC/Q-TOF also allows the operation in MS/MS mode, the analysis was repeated using ion 400 ($M^{\bullet+}$ for $C_{29}H_{52}$ = ethylcholestane) as a precursor ion. The EIC of product ion at 217.1951 now shows improved selectivity of detection for the ethylcholestane steranes, as shown in Figure 6.

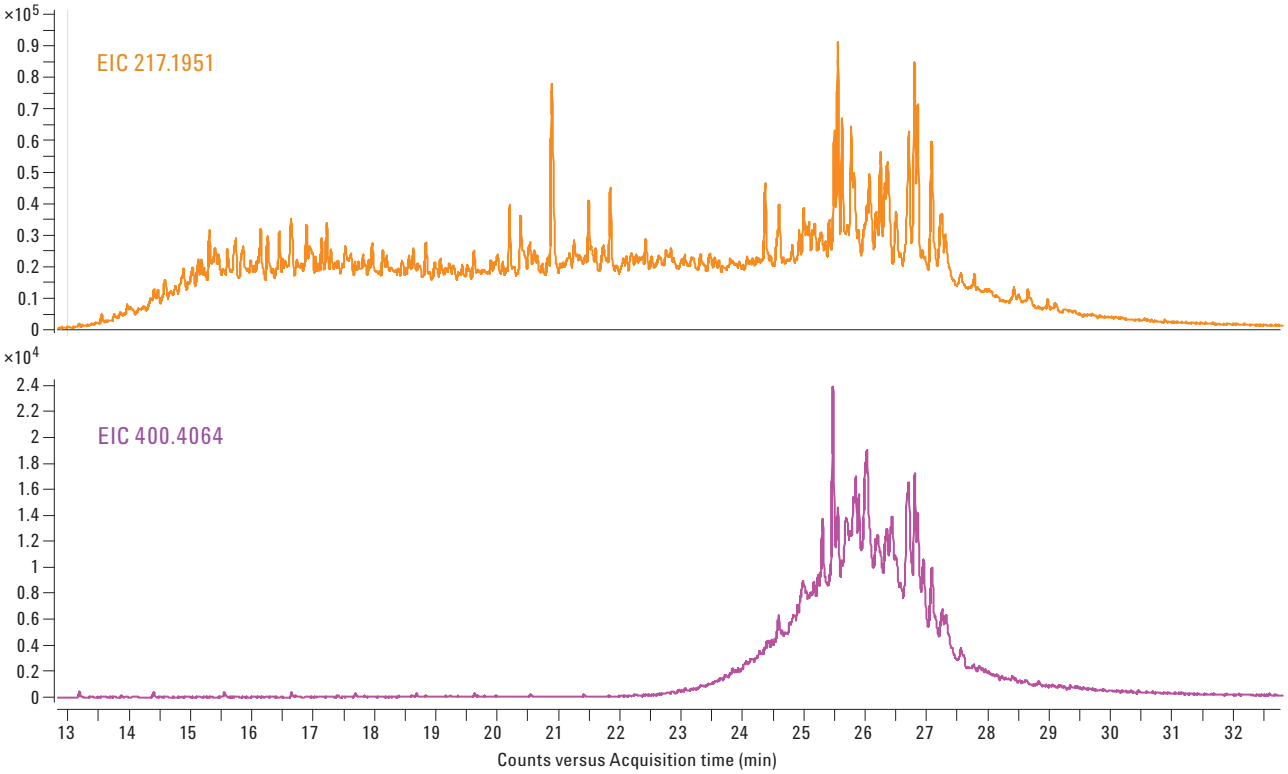

Figure 5. Extracted ion chromatograms at accurate masses (217.1951 ±10ppm and 400.4064 ± 10 ppm) for the detection of steranes.

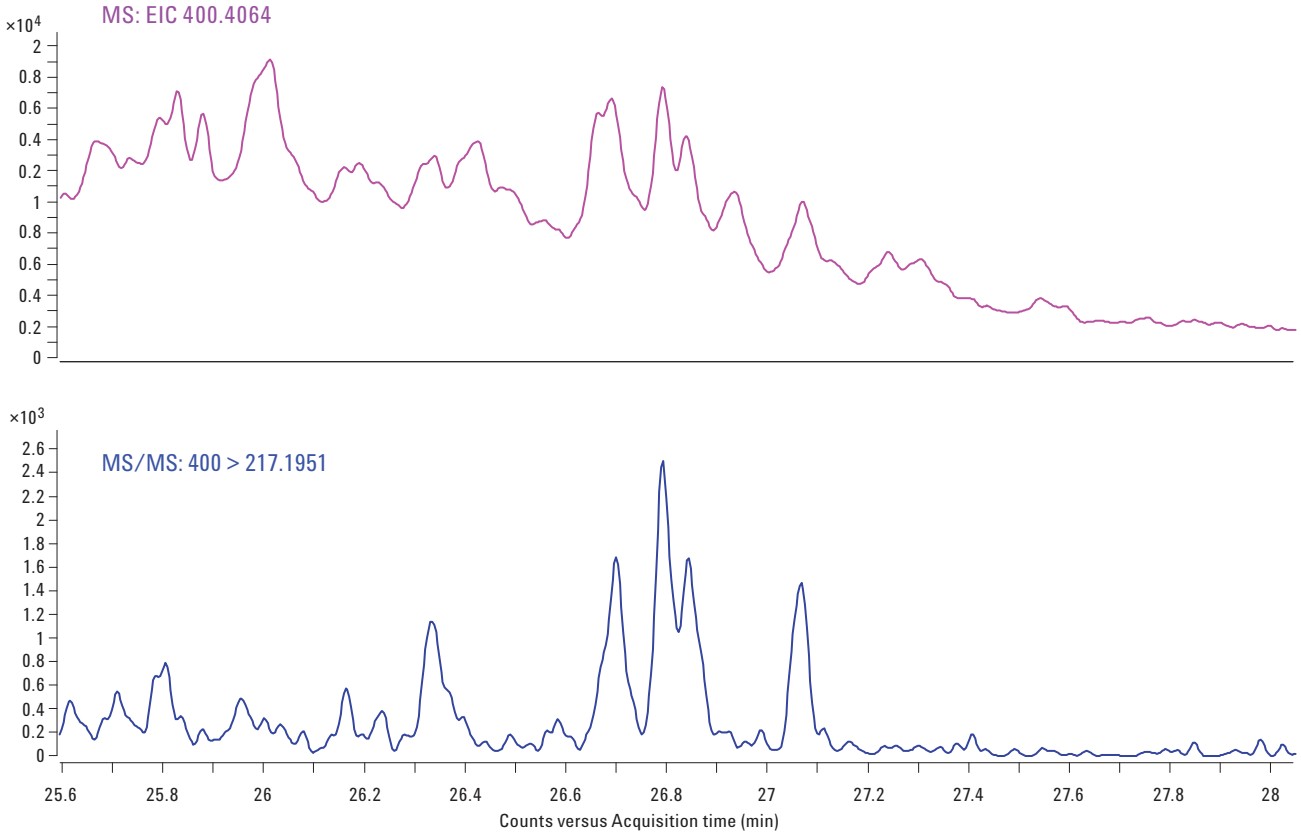

Figure 6. Comparison of extracted ion chromatogram at accurate mass (400.4064 ± 10 ppm) obtained in Full Scan mode (top) with the Extracted Product Ion (400 > 217.1951 ± 10 ppm) obtained in MS/MS mode (bottom) for the detection of steranes.

Conclusions

The Agilent 7200 Q-TOF instrument allows the analysis of a wide range of biomarkers in crude oil without the need for pre-fractionation. The diluted crude oil is directly analyzed and biomarkers such as dibenzothiophenes and hopanes could be selectively monitored by using extracted ion chromatograms at exact masses and using a narrow extraction window.

The 7200 Q-TOF system operated in MS/MS mode also allowed the selective detection of traces of steranes.

In summary, the Agilent 7200 GC/Q-TOF system can be efficiently utilized in targeted and untargeted biomarker analysis in petroleum characterization.

References

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A New Approach to the Analysis of Chlorinated Paraffins by Gas Chromatography Quadrupole Time-of-Flight Mass Spectrometry

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Abstract

Chlorinated paraffins (CPs) are industrial products produced and used in bulk for various purposes. However, the analysis of CPs is challenging, as they are complex mixtures of compounds and isomers. This study develops an analytical method for the analysis of short-chain CPs (SCCPs) and medium-chain CPs (MCCPs) using gas chromatography coupled with quadrupole time-of-flight high-resolution mass spectrometry operated in negative chemical ionization mode (GC-NCI-Q-TOF-HRMS). The linear relationship between chlorination and the CP total response factors was applied to quantify the CP content and the congener group distribution patterns. In a single injection, 24 SCCP formula groups and 24 MCCP formula groups were quantified. Extraction of accurate masses using TOF-HRMS allowed the SCCPs and MCCPs to be distinguished, with interference from other chemicals (for example, PCBs) being effectively avoided. The SCCP and MCCP detection limits were 24–81 ng/mL and 27–170 ng/mL, respectively. Comparison of the results with those obtained through gas chromatography coupled with low-resolution mass spectrometry operated under the same ionization mode (GC-NCI-LRMS) indicated that the developed technique was a more accurate and convenient method for the analysis of CPs in samples from a range of matrices.

Introduction

Chlorinated paraffins (CPs), also known as polychlorinated *n*-alkanes, have been widely used for decades in commercial products[1,2,3]. The commercial CP mixtures can be divided into three categories:

- Short-chain chlorinated paraffins (SCCPs) C_{10}–C_{13}
- Medium-chain chlorinated paraffins (MCCPs) C_{14}–C_{17}
- Long-chain chlorinated paraffins (LCCPs) C >17.

Among these, SCCPs have drawn significant attention due to their high toxicity[2]; however, as MCCPs and SCCPs coexist in the environment, and MCCPs can be transformed into SCCPs through environmental processes such as combustion, the issue of MCCP analysis should also be addressed.

The quantification of CPs in environmental samples is challenging[4] due to the complexity of the industrial mixtures and self-interference among the CPs. A number of different methods have been developed for the determination of SCCPs and MCCPs in a range of environmental matrices[5-9]. However, these methods encounter several challenges such as high cost and the risk of interference between other chlorinated pollutants and CPs with the same nominal mass. Interference related to mass overlap between SCCP and MCCP congeners must also be addressed, and fragmentation patterns should be studied to allow more accurate quantification of CPs. With these challenges in mind, this Application Note describes a published study on the development of a novel analytical approach based on the GC-NCI-Q-TOF-HRMS system to simultaneously analyze SCCPs and MCCPs in a single injection[10].

High-resolution TOF scan mode was used to directly quantify SCCPs, and avoid possible interference by MCCPs in environmental samples. Twenty-four different SCCP formula groups (C_{10}–C_{13} with 5–10 chlorine atoms) and 24 MCCP formula groups (C_{14}–C_{17} with 5–10 chlorine atoms) were analyzed by extracting accurate masses. CPs bearing fewer chlorine atoms and shorter chain lengths were also studied. Samples from a range of environmental matrices were analyzed using the developed method, proving that it is a more accurate and convenient method for the analysis of CPs in environmental samples.

Experimental

Reagents and Standards

Pesticide analytical grade solvents were purchased from J.T. Baker (Phillipsburg, NJ, USA). Solutions of the SCCP mixtures (100 ng/µL, C_{10}–C_{13} with 51 %, 55.5 %, and 63 % chlorination, 100 % purity) and MCCP mixtures (100 ng/µL, C_{14}–C_{17} with 42 %, 52 %, and 57 % chlorination, 100 % purity) in cyclohexane and ε-hexachlorocyclohexane (ε-HCH, solution in cyclohexane, 10 ng/µL, 99.9 % purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 1,5,5,6,6,10-Hexachlorodecane ($^{13}C_{10}$ -,100 ng/µL, solution in cyclohexane, ≥98 % purity) and 1,5,5,6,6,10-hexachlorodecane (unlabeled, 100 ng/µL in cyclohexane, ≥98 % purity) were purchased from Cambridge Isotope Laboratories (Andover, USA).

Instrument

GC Conditions	
GC System	Agilent 7890B, coupled with a CTC autosampler;
Column	Agilent HP-5MS UI, 30 m × 0.25 mm, 0.25 µm (p/n 19091S-433 UI)
Carrier gas	Helium
Oven temperature program	100 °C hold 1 minute, at 5 °C/min to 160 °C hold 2 minutes, at 30 °C/min to 310 °C hold 10 minutes
Flow rate	1.0 mL/min
Inlet temperature	280 ℃
Injection volume	2 µL
Injection mode	Splitless, purge on after 1.5 minutes
Transfer line temperature	280 °C
Q-TOF MS Conditions	
MS System	Agilent 7200 GC-Q-TOF
Ionization mode	Negative Chemical Ionization (NCI)
Source temperature	150 °C
Quadrupole temperature	150 °C
Mass range	50 to 600 *m/z*
Spectral acquisition rate	5 Hz, collecting both in centroid and profile modes
Acquisition mode	4 GHz high resolution

Sample Preparation

To test the performance of the NCI-TOF-HRMS method, samples from several environmental matrices were analyzed for both SCCPs and MCCPs. Air samples were obtained using a passive air sampler (Xpress-Application Developer, XAD). The industrial CP products were kindly provided by manufacturers. Food samples were purchased from several well-known fast food outlets. Sample pretreatment was based on the previously reported method[11,12] with some minor modifications. Briefly, frozen dried samples (1 g) were mixed with diatomaceous earth (5 g), and spiked with the $^{13}C_{10}$-1,5,5,6,6,10-hexachlorodecane (10 ng) as surrogate internal standard and quantitative internal standard prior to accelerated solvent extraction (ASE). The extract was concentrated to approximately 1 mL by rotary evaporation. The extract was then cleaned and fractionated on a 1.5 cm silica-Florisil composite column packed with Florisil (3 g), neutral silica gel (2 g), acidic silica gel (5 g, 30 %), and anhydrous sodium sulfate (4 g) (packed from bottom to top). The column was conditioned with *n*-hexane (50 mL), and the sample was eluted with *n*-hexane (40 mL) (fraction 1 contained polychlorinated biphenyls and toxaphenes), followed by dichloromethane (50 mL) and *n*-hexane (50 mL) (fraction 2 contained CPs and HCHs). The second fraction was concentrated to approximately 2 mL by rotary evaporation, and further concentrated to close to dryness under a gentle stream of N_2. The fraction was then reconstituted in cyclohexane (200 µL). Prior to MS analysis, a ε-HCH (10 ng) was added as injection internal standard to determine the sample recoveries. Instrumental blanks were composed of pure cyclohexane. No CPs were observed following injection of the blanks.

Results and Discussion

Quantification Method Workflow and Auto-Integration Procedure

Figure 1 describes the workflow for the chlorination response factor-based quantification method.

In the NCI-LRMS method, manual integration was traditionally applied to compare the peak shapes and retention times with the reference standards. In the NCI-TOF-MS method, the observed extracted ion chromatography (EIC) peak was comparable to that of the standard, as high-resolution MS removed interference from the matrix. Auto integration was applied using Agilent MassHunter Quantitative Analysis B.07.

The accurate masses of the SCCPs and MCCPs and quantitative and qualitative ions, along with their retention times (Table 1), were added to the method. New batch files were built, and the data files were imported. The integration results could directly transfer to customer's homemade excel table to calculate the subsequent results listed in Figure 1. The quantitative method, based on the linearity of the response factor and chlorination, compensated for the difference in response factors between the reference CP mixtures and the real samples[8]. Figure 2 shows the linear relationship between the response factor (RF: the ratio of internal standard adjustment response to the CP content) and calculated chlorination (%) for MCCPs and SCCPs.

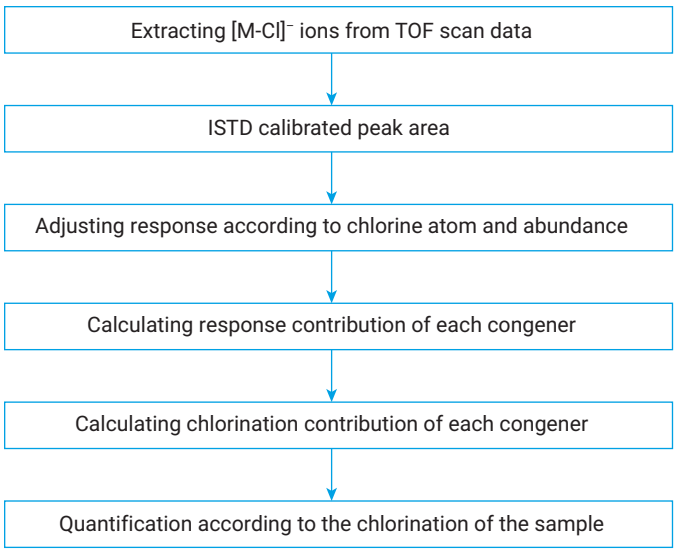

Figure 1. The quantification method workflow for CPs. For detailed information about the quantification method please see reference 10.

Table 1. Accurate mass of quantitative and qualitative $[M\text{-}Cl]^-$ ions for SCCPs and MCCPs, average molecular mass, retention time, and limit of detection of each single formula group (continued next page).

SCCP and MCCP congeners (*n, z*)	Quantitative ions		Qualitative ions		Average molecular mass	Retention time (min)	LOD (ng/mL)
	m/z $[M\text{-}Cl]^-$	Abundance	*m/z* $[M\text{-}Cl]^-$	Abundance			
SCCP							
$C_{10}H_{17}Cl_5$	279.0006	37.60%	277.0009	29.40%	314.5	9.5–14	11.8
$C_{10}H_{16}Cl_6$	312.9671	35.60%	314.9641	22.70%	349	11–13.5	7.5
$C_{10}H_{15}Cl_7$	346.9281	32.30%	348.9251	25.90%	383.5	11.5–14	5.2
$C_{10}H_{14}Cl_8$	380.8891	28.60%	382.8862	27.50%	418	12.5–14.5	4.78
$C_{10}H_{14}Cl_9$	416.8472	27.80%	414.8501	24.80%	452.5	12.5–14.5	3.2
$C_{10}H_{12}Cl_{10}$	450.8082	27.10%	448.8112	21.20%	487	11.5–16	1.1
$C_{11}H_{19}Cl_5$	293.0217	37.20%	291.0246	29.10%	328.5	10 –14	9.05
$C_{11}H_{18}Cl_6$	326.9437	35.20%	328.9798	22.50%	363	11–14	6.5
$C_{11}H_{17}Cl_7$	360.9437	32.00%	362.9408	25.60%	397.5	12–14.5	1.6
$C_{11}H_{16}Cl_8$	394.9048	28.30%	396.9018	27.20%	432	12–14.5	0.75
$C_{11}H_{15}Cl_9$	430.8628	27.50%	428.8658	24.50%	466.5	12.5–14.5	0.75
$C_{11}H_{14}Cl_{10}$	464.8239	26.70%	462.8268	20.90%	501	13.5–15.5	0.75
$C_{12}H_{20}Cl_5$	307.0373	36.80%	305.0403	28.70%	342.5	11–14	5.55
$C_{12}H_{19}Cl_6$	340.9984	34.80%	342.9954	22.30%	377	11.5–14	5.15
$C_{12}H_{18}Cl_7$	374.9594	31.70%	376.9564	25.30%	411.5	12.4–14.6	1.45
$C_{12}H_{17}Cl_8$	408.9204	28.00%	410.9175	26.90%	446	12.5–15	1.2
$C_{12}H_{16}Cl_9$	444.8785	27.10%	442.8814	24.20%	480.5	13–15	1
$C_{12}H_{15}Cl_{10}$	478.8395	26.40%	476.8425	20.70%	515	13.5–16	1
$C_{13}H_{22}Cl_5$	321.053	36.30%	319.0059	28.40%	356.5	11.5–14.5	10
$C_{13}H_{21}Cl_6$	355.0123	34.40%	357.0111	22.00%	391	12.2–15	8.7
$C_{13}H_{20}Cl_7$	388.975	31.30%	390.9721	25.00%	425.5	12.5–14.5	3.5
$C_{13}H_{19}Cl_8$	422.9361	27.70%	424.9331	26.60%	460	13–15.5	2
$C_{13}H_{18}Cl_9$	458.8941	26.80%	456.8971	24.00%	494.5	12.5–17	2
$C_{13}H_{17}Cl_{10}$	492.8552	26.10%	490.8581	20.40%	529	14–17	1.75

Table 1. Accurate mass of quantitative and qualitative $[M\text{-}Cl]^-$ ions for SCCPs and MCCPs, average molecular mass, retention time, and limit of detection of each single formula group.

SCCP and MCCP congeners (*n, z*)	Quantitative ions		Qualitative ions		Average molecular mass	Retention time (min)	LOD (ng/mL)
	m/z $[M\text{-}Cl]^-$	Abundance	*m/z* $[M\text{-}Cl]^-$	Abundance			
MCCP							
$C_{14}H_{25}Cl_5$	335.0686	37.60%	333.0716	29.40%	370.5	12.2–14.2	9.3
$C_{14}H_{24}Cl_6$	369.0697	35.60%	371.0267	22.70%	405	12.4–14.6	2.6
$C_{14}H_{23}Cl_7$	402.9907	32.30%	404.9877	25.90%	439.5	12.8–15.2	5.5
$C_{14}H_{22}Cl_8$	436.9517	28.60%	438.9488	27.50%	474	13.6–15.8	7.5
$C_{14}H_{21}Cl_9$	472.9098	27.80%	470.9127	24.80%	508.5	14–16.8	3.5
$C_{14}H_{20}Cl_{10}$	506.8708	27.10%	504.8738	21.20%	543	15–18	3.1
$C_{15}H_{27}Cl_5$	349.0843	37.20%	347.0872	29.10%	384.5	12.2–14.4	7.7
$C_{15}H_{26}Cl_6$	383.0453	35.20%	385.0424	22.50%	419	12.5–15.5	10
$C_{15}H_{25}Cl_7$	417.0063	32.00%	419.0034	25.60%	453.5	13.8–15.2	38
$C_{15}H_{24}Cl_8$	450.9674	28.30%	452.9644	27.20%	488	13.5–16.8	5.6
$C_{15}H_{23}Cl_9$	486.9254	27.50%	484.9284	24.50%	522.5	14.6–18	4.6
$C_{15}H_{22}Cl_{10}$	520.8865	26.70%	518.8894	20.90%	557	15.5–19.5	2.1
$C_{16}H_{29}Cl_5$	363.0999	36.80%	361.1029	28.70%	398.5	12.5–15.5	9.6
$C_{16}H_{28}Cl_6$	397.061	34.80%	399.058	22.30%	433	13.5–15.5	11.7
$C_{16}H_{27}Cl_7$	431.022	31.70%	433.019	25.30%	467.5	13.8–15.8	7.9
$C_{16}H_{26}Cl_8$	464.983	28.00%	466.9801	26.90%	502	14.4–17.4	2.3
$C_{16}H_{25}Cl_9$	500.9411	27.10%	502.9381	24.20%	536.5	15.5–19.5	1.6
$C_{16}H_{24}Cl_{10}$	534.9021	26.40%	532.9051	20.70%	571	16.5–21	0.9
$C_{17}H_{31}Cl_5$	377.1156	36.30%	375.1185	28.40%	412.5	12.5–15	8.6
$C_{17}H_{30}Cl_6$	411.0766	34.40%	413.0737	22.00%	447	13.4–15.2	9.3
$C_{17}H_{29}Cl_7$	445.0376	31.30%	447.0347	25.00%	481.5	13–17.5	2.7
$C_{17}H_{28}Cl_8$	478.9987	27.70%	480.9957	26.60%	516	14.5–19	1
$C_{17}H_{27}Cl_9$	514.9567	26.80%	512.9597	24.00%	550.5	16.5–20.5	1.2
$C_{17}H_{26}Cl_{10}$	548.9178	26.10%	546.9207	20.40%	585	18–23	1.3

Limit of Detection (LOD) and Linearity Range

The instrumental LOD was determined as the standard deviation of the signal intensities from the five replicate injections multiplied by Student's T-value at a 95 % confidence level. In real samples, detection of a congener group was defined as both *m/z* values of the quantitative and qualitative ions being detected above their respective LODs, and where the LOD of the congener group was equal to the LOD of the least sensitive of the two monitored *m/z* values. The LOD for the SCCPs and MCCPs was defined as detection of the most abundant congener group. Results showed that the LOD of the MCCPs was in the range of 27–170 ng/mL, while that of the SCCPs was in the range of 24–81 ng/mL. Table 1 provides the LOD of each formula group. The linearity of the NCI-Q-TOF-HRMS method was determined by fitting the intensities obtained from the standard solutions of 55.5 % Cl SCCP, 52 % Cl MCCP, and 57 % Cl MCCP mixtures against their concentrations ranging from 0.25 to 100 ng/ µL using weighted linear regression. Figure 3 shows the corresponding fitting curves. It was found that the linearity ranges for both SCCP and MCCP can reach three orders of magnitude, which are higher than that of the NCI-LRMS method[8]. This relatively good linearity performance for the CPs was due to no isomer reaching its upper limit, even at high total concentrations.

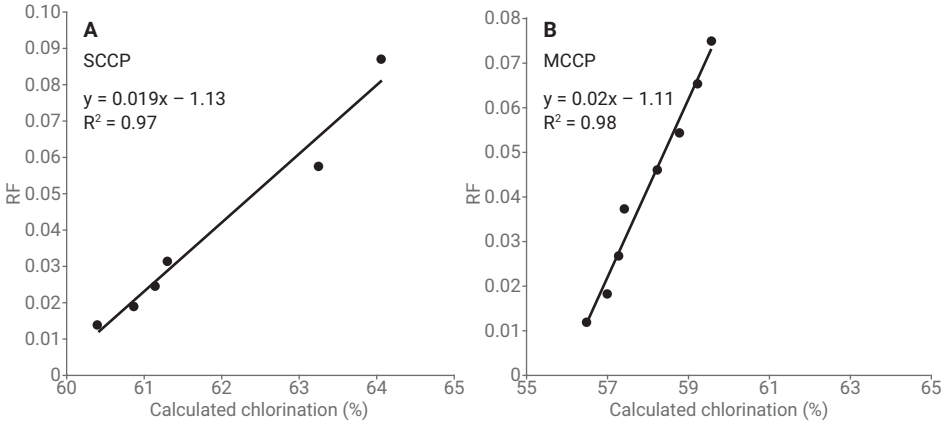

Figure 2. Linear relationship between the response factor (RF: the ratio of internal standard adjustment response and the CPs content) and calculated chlorination (%) for MCCPs and SCCPs. A) Standard curve of SCCPs at 10 ng/µL (different chlorination obtained by mixing 51.5 % Cl, 55.5 % Cl, and 63 % Cl SCCP standards). B) Standard curve of MCCPs at 10 ng/µL (different chlorination obtained by mixing 42 % Cl, 52 % Cl, and 57 % Cl MCCP standards).

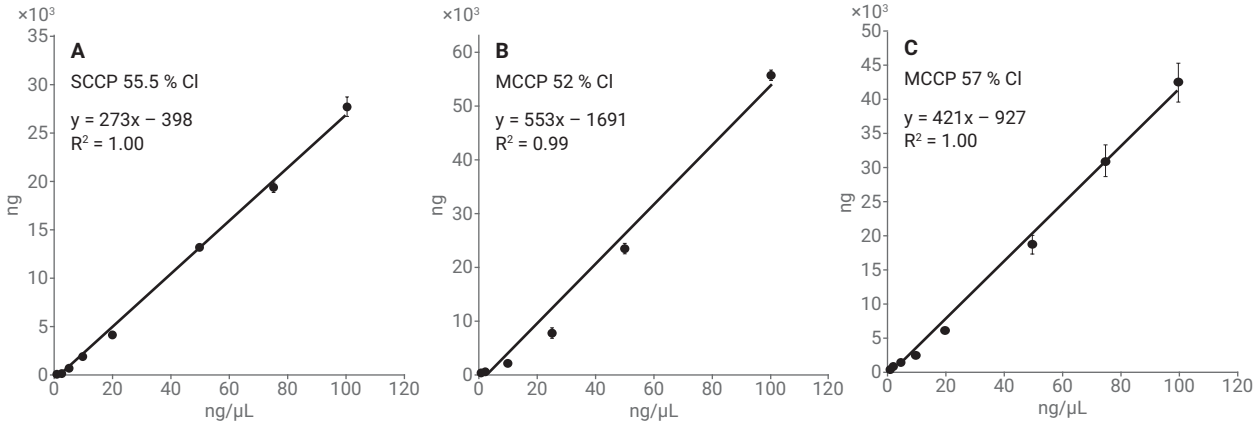

Figure 3. A) Linearity of 55.5 % chlorinated SCCP mixtures (0.25–100 ng/ µL). B) Linearity of 52 % chlorinated MCCP mixtures (0.25–100 ng/ µL). C) Linearity of 57 % chlorinated MCCP mixtures (0.25–100 ng/ µL).

Accuracy and Repeatability

Accuracy was calculated as the ratio between the average measured concentration (n = 5) and the reference SCCP and MCCP mixture standards at different chlorination percent (51.5 % Cl SCCP, 55.5 % Cl SCCP, 63 % Cl SCCP, 52 % Cl MCCP, and 57 % Cl MCCP). Table 2 shows the results.

With the NCI-TOF-MS method, the relative accuracies for SCCPs and MCCPs can be acquired within the range of 86–124 % and 114–129 %, respectively. When using the binary mixture standards of SCCP and MCCP, larger positive bias was observed than that for the single mixture standard.

Repeatability can be determined by the standard deviation of repeated injections (n = 18, spiked at 1, 10, and 100 ng/L of both 55 % Cl SCCP and 52 % Cl MCCP) over a single day (intra-day) and across several days (inter-day). The relative standard deviations (RSDs) of SCCP for the inter-day injections obtained by NCI-TOF-MS at the three concentration levels were 2.55 %, 1.95 %, and 3.58 %, respectively. For the MCCP, the corresponding RSDs were 12.3 %, 7.37 %, and 0.97 %, respectively.

Table 2. Accuracy and repeatability of the NCI-TOF-HRMS method.

Performance test	Reference conc. (ng/µL)	Calculated conc. (±error) (ng/µL)	Accuracy[a]	Binary mix	Reference conc. (ng/µL)	Calculated con. (±error) (ng/µL)	Accuracy[a]
SCCP Test							
51 % Cl SCCP	5.00	4.30 (±0.41)	86 %				
55 % Cl SCCP	10.00	10.00 (±0.19)	100 %	55 % Cl SCCP and 57 % Cl MCCP (1:1, v/v 20 ng/µL)	10.00	12.43 (±4.6)	124 %
63 % Cl SCCP	10.00	12.05 (±0.14)	120 %				
MCCP Test							
52 % Cl MCCP	10.00	12.13 (±0.89)	121 %				
57 % Cl MCCP	10.00	11.36 (±0.71)	114 %	55 % Cl SCCP and 57 % Cl MCCP (1:1, v/v 20 ng/µL)	10.00	12.89 (±0.27)	129 %

[a] Accuracy is defined as the percentage ratio of the calculated concentration of CPs and the reference concentration of CPs.

Influence of Resolution in CP Analysis

The relationship between resolution and deviation of mass (DM) is defined by Equation 1.

$$\text{Resolution} = \frac{M}{DM}$$

Equation 1.

M is the *m/z* ratio of the fragment ions, DM is the mass distance between two adjacent peaks.

Equation 1 shows that the resolution of a signal is related to the mass of the species. In this case, the majority of CP target ions were in the *m/z* range of 300 to 500, where TOF resolutions of 10,000–15,000 could theoretically yield mass accuracies of 5–10 ppm. For the 96 quantitation and qualification fragments, a minimum resolution of 3,000 was required for separation of the two closest *m/z* values for the $C_{12}H_{16}{}^{35}Cl_7{}^{37}Cl_2$ (478.839 Da) and $C_{17}H_{28}{}^{35}Cl_6{}^{37}Cl$ (478.9987 Da) fragments (Table 3). The ion source temperature of 150 °C was selected to minimize fragmentation patterns other than $[M-Cl]^-$[5,11]. Indeed, if $[M-Cl]^-$ could be considered the main fragmentation pattern, the resolution requirement would be 8,000 ($C_{12}H_{16}{}^{35}Cl_7{}^{37}Cl_2$ = 478.839 Da, and $C_{17}H_{28}{}^{35}Cl_6{}^{37}Cl$ = 478.9987 Da). Thus, the TOF-HRMS method applied in this study with a resolution of 12,000–15,000 was suitable for resolving all congener groups of SCCPs and MCCPs.

Table 3. Accurate masses of MCCP and SCCP formulation groups that generated fragmentation ions with the same nominal mass, and the D-value between the two ions.

Nominal mass	Formula group	Accurate mass	Formula group	Accurate mass	D-value (ppm[a])
417	$C_{10}Cl_9$	416.8472	$C_{15}Cl_7$	417.0063	382
451	$C_{10}Cl_{10}$	450.8082	$C_{15}Cl_8$	450.9674	353
431	$C_{11}Cl_9$	430.8628	$C_{16}Cl_7$	431.022	369
465	$C_{11}Cl_{10}$	464.8239	$C_{16}Cl_8$	464.983	342
445	$C_{12}Cl_9$	444.8785	$C_{17}Cl_7$	445.0376	358
479	$C_{12}Cl_{10}$	478.8395	$C_{17}Cl_8$	478.9987	333

[a] Part per million

Furthermore, matrix interference was found to exist even after following thorough sample pretreatment procedures[14]. Under the NCI-LRMS system, SIM combined the retention time window to eliminate self-interference. However, this approach did not yield satisfactory results (Figure 4: EIC at ±0.5 amu), as baseline separation of the components could not be achieved. Figure 4 shows that interference from the matrix along with CP self-interference (for example, *m/z* 451 generated by $C_{10}Cl_{10}$ and $C_{15}Cl_8$) could be avoided, to a large extent, with a mass tolerance of 50 ppm upon extracting the accurate mass. In Figure 4, MCCPs were treated as interference, while SCCPs were regarded as the targets.

Analysis of Environmental Samples and Comparison Between Two Methods

To assess improvements in the quality of the CP environmental measurements[13] (Figure 5), it is essential to compare the of results from the current HRMS method with the LRMS method previously reported[11,12]. The NCI-TOF-HRMS method was evaluated to quantify SCCPs and MCCPs in industrial products, food, and XAD-based air samples (Figure 5). The SCCP concentration ranged from 70 to 73,172 ng/g dw for the food samples. In addition, the SCCP concentration in the XAD-based air samples ranged from 0.04–29 ng/m^3, and finally, for the technical products, the SCCP content ranged from 54 to 1,651 ng in the CP-52 products at a concentration of 10 ppm. The SCCP contents and chlorination values obtained using the two different MS methods were also compared (Figure 5).

In the XAD-based air samples, the concentrations obtained using the NCI-TOF-HRMS method were prevalently lower than those obtained by the NCI-LRMS method, with the exception of two cases (an extremely low content (13 bz) and an extremely high content (14 dppl)). The SCCP concentrations determined by NCI-TOF-HRMS differed from those obtained by NCI-LRMS by factors of 0.19–0.92.

Conversely, the results obtained for the food samples varied due to different matrix effects. The SCCP concentrations determined by NCI-TOF-HRMS differed from those obtained by NCI-LRMS by factors of 0.16–2.55.

For the industrial CP products, the concentrations obtained using the NCI-TOF-HRMS method were generally higher than those obtained using the NCI-LRMS method, with the exception of CP8, which had a very low SCCP content. The SCCP concentrations measured by NCI-TOF-HRMS differed from those measured using NCI-LRMS by factors of 3.79–6.05.

To further investigate the reasons for the differences in results obtained using the two methods, individual formula group contents of the SCCPs obtained using both the NCI-TOF-HRMS method and the NCI-LRMS method were compared (Figure 6). This comparison showed that, in air, SCCPs containing fewer chlorine atoms and shorter chain lengths were predominant, whereas the reverse was true for the technical products. At high resolutions, the obtained content of CPs containing fewer chlorine atoms was lower. Therefore, for the lighter components found in XAD-based air samples, the content determined by NCI-TOF-HRMS was higher, while for the heavier components found in technical products, the content determined by NCI-TOF-HRMS was lower (Figure 6). However, the differences in the absolute amounts obtained did not represent significant deviations from the true values. As discussed above, CPs with varying chlorine contents exhibited various response patterns related to different instrumental conditions, which mainly resulted from the varying degrees of chlorination.

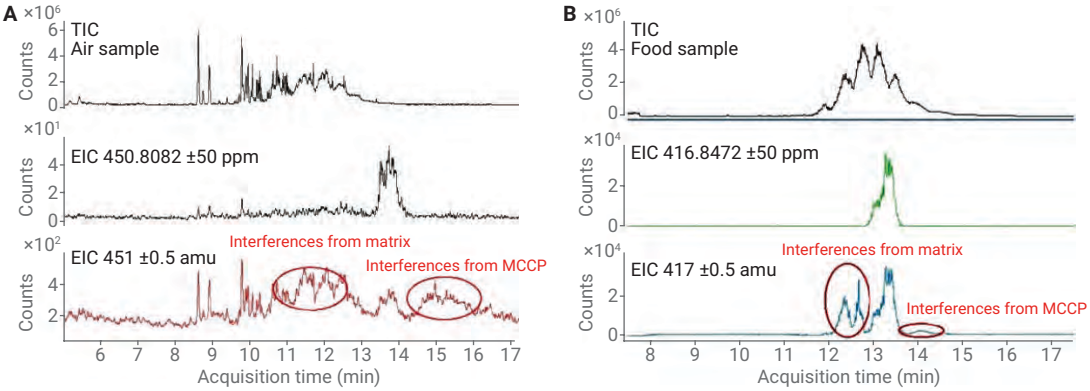

Figure 4. Elimination of matrix effects in samples by accurate mass extraction, presented by EIC comparison. A) Total ion chromatogram (TIC) of an air sample and the corresponding EIC extraction ($C_{10}Cl_{10}$) at different mass tolerances (50 ppm and 0.5 amu); B) TIC of a food sample and the corresponding EIC extraction ($C_{10}Cl_9$) at different mass tolerances (50 ppm and 0.5 amu).

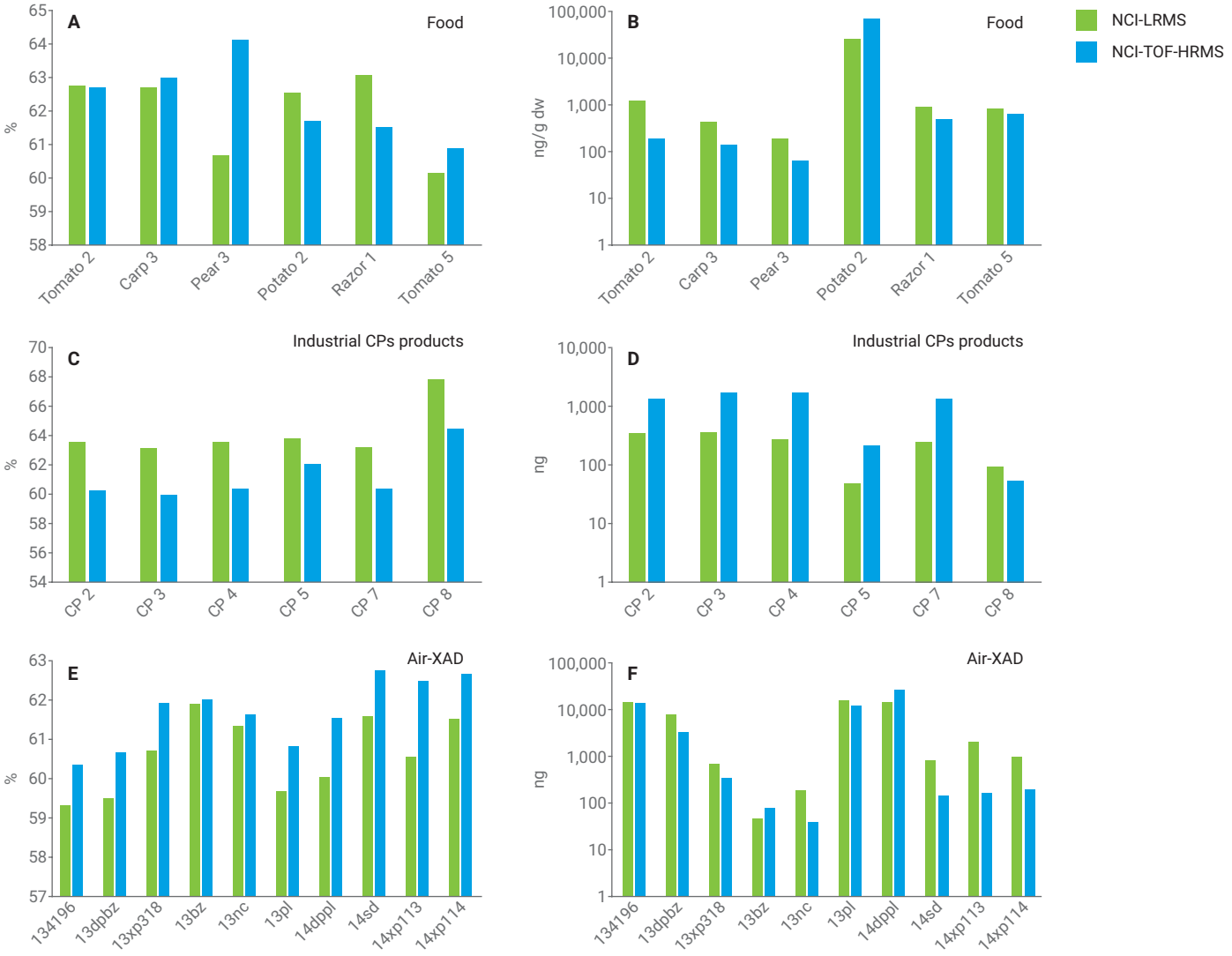

Figure 5. Comparison of calculated chlorination and content of SCCPs obtained with the NCI-TOF-HRMS method (blue bars) and the NCI-LRMS method (green bars) for (A) calculated chlorination comparison in food samples, (B) content comparison in food samples, (C) calculated chlorination comparison in industrial CP products, (D) content comparison in industrial CPs products, (E) calculated chlorination comparison in XAD-based air samples, and (F) content comparison in XAD-based air samples.

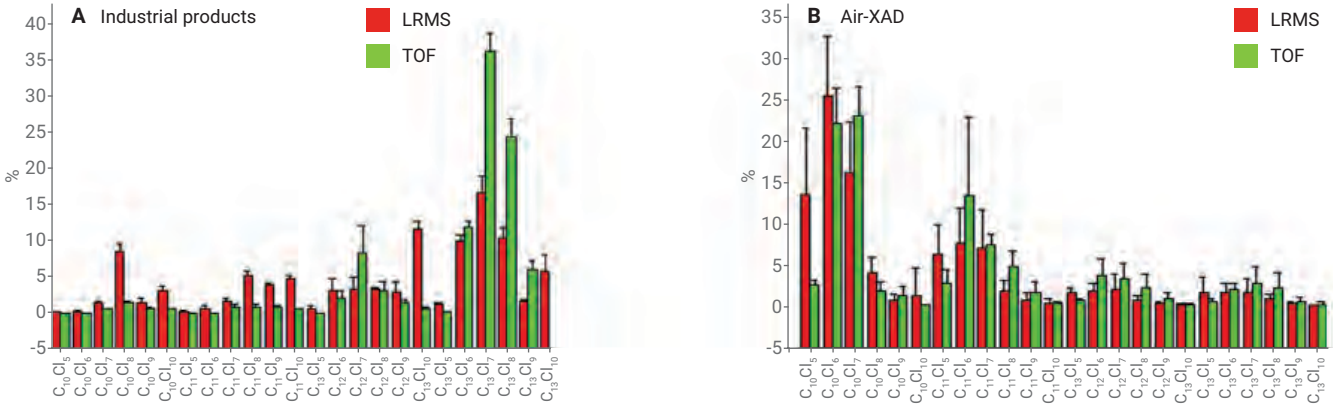

Figure 6. Comparison of SCCP individual congener contents obtained using the NCI-TOF-HRMS method (green bars) and the NCI-LRMS method (red bars) for (A) congener group distribution of SCCPs in industrial products, and (B) congener group distribution of SCCPs in XAD-based air samples.

The calculated chlorine content obtained using the NCI-TOF-HRMS method were generally higher than the degrees of chlorination calculated using the NCI-LRMS method, with the exception of industrial CP products and two food samples (potato 2 and razor 1). This variation could be accounted for the low content of CPs bearing fewer chlorine atoms (CPs that generate ions with *m/z* ~300), as determined under a high resolution (in the SIM of the LRMS, interference occurred at *m/z* ~300).

In this study, analyses of MCCPs were conducted using only the NCI-Q-TOF-HRMS system. Due to a shortage of available data in the literature, no inter-lab comparison results were available for MCCPs. Results from this study showed that the MCCP concentration in air was lower compared to that in other matrices, with the contents ranging from 0.04 to 0.89 ng/m^3 obtained for the XAD-based samples. In the food samples, the MCCP levels were between 603 ng/g and 7,478 ng/g.

For the industrial products, the concentration of LCPs (SCCPs + MCCPs) were in the range of 3,796–6,235 ng in six CP-52 products (CP2, CP3, CP4, CP5, CP7, and CP8) of 10 ng/µL (in which the total amount of LCPs should be 2,000 ng), indicating that the results were overestimated. It is possible that the calculated degree of chlorination of the industrial products were at the low end of the calibration curve of chlorine content versus MS response. The degree of chlorination was inversely correlated with the quantification results, and the lower chlorine content of the industrial products relative to the environmental matrices might result in the overestimation of CP concentrations. The results implied that more specific reference standards with a wider chlorination range should be synthetized to build more accurate quantified and qualified CP methods for different matrices.

Time Efficiency and Suitability for Routine Analysis

Unlike the earlier LRMS method[11,12] that required four separate runs to acquire all necessary SIM ions for identification and quantification, the new HRMS approach only required one injection. The higher selectivity afforded by the HRMS approach allowed effective use of automatic peak integration without significant interference instead of the time-consuming manual integration required for LRMS data. This combination reduced turnaround time on samples from a few months to a few days.

Conclusions

The novel GC-Q-TOF-MS method offers a number of benefits over established GC/NCI-LRMS methods for the analysis of CPs in environmental samples. This method was especially efficient in the simultaneous analysis of SCCPs and MCCPs in complex environmental samples, and was efficient in eliminating CP self-interference by accurate mass extraction. The results obtained for different environmental samples showed that the high-resolution TOF-MS method was capable of reducing interferences from different matrices. In addition, the GC-Q-TOF-MS method shows a comparable linear dynamic range and detection limits to previous methods, along with improved accuracy. Moreover, this method is suitable for high-throughput analyses of large sets of samples due to its efficiency in both analysis time and quantification processes. Further application of this GC-Q-TOF-MS method should be considered to achieve more accurate analyses of CPs in different matrices.

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Application Note

Environmental

Analysis of Per- and Polyfluoroalkyl Substances (PFASs) in Biological Fluid Using a Novel Lipid Removing Sorbent and LC-MS/MS

Authors

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Abstract

Efficient sample preparation prior to LC-MS/MS analysis of per- and polyfluoroalkyl substances (PFASs) is an important consideration for environmental contamination research laboratories performing multiresidue analysis. Phospholipids (PPLs) have been identified as a major cause of matrix effects in the LC-MS/MS analysis of plasma samples. This Application Note describes plasma sample preparation and LC-MS/MS analysis of PFASs using in-well PPT followed by PPL removal using the Agilent Captiva EMR—Lipid cartridge. Captiva EMR—Lipid cartridges produced cleaner eluents, with removal of over 99 % of unwanted PPLs from the plasma matrix, and over 75 % recovery of target analytes, with RSDs <14 %. Analysis of PFASs at 5 ng/mL yielded ideal peak shapes with good signal-to-noise (S/N). Calibration curves for all PFASs from 0.1–50 ng/mL were linear, with an R^2 >0.992.

Introduction

Per- and polyfluoroalkyl substances (PFASs) are man-made compounds widely used as surfactants, fire-retardants, waterproofing, and nonstick and nonstain agents. Their unique properties also make them persistent and ubiquitous in the environment and in animals. Research suggests that PFASs can cause reproductive and developmental problems such as liver, kidney, and immune effects, tumors, and changes in cholesterol. When PFASs are ingested by drinking or eating, they are readily absorbed, but slowly cleared, and can accumulate in animal tissue. Studies have shown that PFASs with carbon chains longer than seven carry the most risk for bioaccumulation[1].

Efficient sample preparation prior to LC-MS/MS analysis of PFASs is an important consideration for multiresidue analysis. Sample preparation is used to reduce system contamination, improve data integrity and method selectivity, and to enhance analytical sensitivity. Two of the major interferences found in plasma are proteins and phospholipids (PPLs). PPLs have been identified as a major cause of matrix effects in LC-MS/MS bioanalyses due to competition for space on the surface of droplets formed during electrospray ionization (ESI)[2].

Common sample preparation techniques for plasma, serum, and whole blood in research laboratories include protein precipitation (PPT), solid phase extraction (SPE), liquid-liquid extraction (LLE), and supported liquid extraction (SLE). Each technique has advantages and disadvantages in terms of speed, cost, and quality of data generated. For example, PPT, LLE, and SLE do not remove PPLs, and SPE is more time-consuming and complicated to perform. However, of these techniques, PPT is most widely used. Using PPT, proteins are easily removed by adding an organic crash solvent such as ACN or MeOH into bio-fluid samples in a prescribed ratio. As the proteins are denatured, they form a precipitate that can be removed by filtration or centrifugation. PPLs are not removed by PPT because they are soluble in the organic crash solvent.

A sample preparation method that eliminates certain sample preparation steps, including off-line PPT, centrifugation, and dilution, while allowing streamlined in-well PPT and PPL removal, is highly desirable. This Application Note describes an approach that relies on Agilent Captiva EMR—Lipid to remove interferences, particularly PPLs, without analyte loss, in a simple pass-through format. The resulting extract is cleaner, reducing potential ion suppression, and column and mass spectrometer contamination.

Extraction of PFASs from plasma was performed using in-well PPT followed by PPL removal using a Captiva EMR—Lipid cartridge. Subsequent quantitative analysis was performed using an Agilent 6495 Triple Quadrupole LC/MS system. Efficiency of PPLs removal was evaluated. Method reproducibility and recovery for the PFASs evaluated were also determined.

Experimental

Reagents and Chemicals

Table 1 lists the PFASs analyzed. All standards and internal standards were purchased from Wellington Laboratories (Guelph, ON, Canada). LC-MS grade ammonium acetate was purchased from Sigma-Aldrich. All solvents were LC-MS grade or higher, and were obtained from Burdick and Jackson (Muskegon, MI, USA).

Table 1. PFASs and IS analyzed, with corresponding triple quadrupole MRM acquisition parameters.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Retention time (min)
PFTrDA	663	618.7	8	3.16
PFDoA	613	268.7	20	2.92
PFDoA-$^{13}C_2$	614.9	570	5	2.92
N-EtFOSAA	584	526	20	3.40
10:2 FTA	577	463	6	2.51
N-MeFOSAA	570	511.8	24	3.23
N-MeFOSAA d_3	573	515	24	3.23
PFUdA	563	519	8	2.70
PFDA	513	468.6	8	2.48
PFDA	513	218.7	16	2.48
PFDA-$^{13}C_2$	514.9	469.9	5	2.48
PFOS	498.9	99	50	2.61
PFOS	498.9	80	50	2.61
PFOS-$^{13}C_4$	502.9	80	50	2.61
8:2 FTA	477	393	14	2.12
PFNA	462.9	418.9	5	2.25
PFNA	462.9	169	17	2.25
PFOA	412.9	368.9	5	2.00
PFOA-$^{13}C_4$	416.0	371.9	5	2.00
PFHxS	398.9	99	45	2.10
PFHxS-$^{13}C_3$	401.9	79.8	52	2.10
6:2 FTA	377	293	18	1.51
PFHpA	362.9	319	5	1.67
PFHxA	313	268.6	4	1.07
PFHxA-$^{13}C_2$	314.9	269.9	5	1.07
PFBS	298.9	98.9	29	1.15
PFPeA	263	218.7	0	0.63
PFBA	213	168.7	4	0.47
PFTeDA	712.9	668.5	8	3.33

Solutions

A combined standard working solution of PFASs was made at 10 ug/mL in methanol. The isotopically-labeled PFASs were combined in a working solution at 10 ug/mL in methanol, and used as internal standard (IS). All working solutions were stored in polypropylene vials with snap caps and polypropylene-lined septa to prevent the PFASs from sticking to the glass and to avoid contamination.

Calibration Standards and Quality Control Samples

Prespiked quality control (QC) samples were fortified with standard working solution to the appropriate concentrations in replicates of seven. The QC samples were low QC (LQC), middle QC (MQC), and high QC (HQC), corresponding to 1, 5, and 20 ng/mL in plasma, respectively. The IS was spiked at 10 ng/mL at each QC level.

Blank matrix after cleanup by Captiva EMR—Lipid was post-spiked with a corresponding working solution to yield 1, 10, and 20 ng/mL concentrations of PFASs. The IS was spiked to a final concentration of 10 ng/mL, in replicates of five.

Matrix-matched calibration curves were prepared with the standard working solution. Blank matrix after Captiva EMR—Lipid was post-spiked to correspond to 0.1, 1, 5, 10, 25, and 50 ng/mL in plasma. The IS was spiked at 10 ng/mL at each calibration level.

Equipment and Instrumentation

Table 2 provides the list of the equipment and instrumentation used to perform the analysis.

LC-MS/MS Analysis

An Agilent 1290 Infinity II LC System coupled to an Agilent 6495 Triple Quadrupole LC/MS system was used for the LC-MS/MS analysis. Tables 3 and 4 provide the LC and MS conditions. The sample extracts (4 µL) were directly injected into the LC system.

Table 1 provides the triple quadrupole dynamic multiple reaction monitoring (DMRM) acquisition parameters for each PFAS compound monitored. To evaluate PPL removal by Captiva EMR—Lipid, 11 major PPL compound precursor ions, and the product ion fragment at *m/z* 184 were monitored, as shown in Table 5.

Table 2. Equipment and instrumentation used for sample preparation and analysis.

Component	Part number
Sample Preparation	
Agilent Captiva EMR—Lipid, 1 mL cartridge	5190-1002
Agilent Vac Elut SPS 20 Manifold with collection rack for 13 × 100 mm test tubes	12234101
VWR 13 × 100 mm culture tubes 8 mL polypropylene	
Eppendorf pipettes and repeater pipettor (VWR, NJ, USA)	
Liquid Chromatography System	
Agilent 1290 Infinity II LC System	
Agilent ZORBAX Eclipse Plus 95Å C18, 4.6 × 50 mm, 3.5 µm (delay column)	959943-902
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm	699775-902T
Agilent 1290 Infinity inline filter 0.3 µm	5067-6189
Crimp/snap-top polypropylene vials, 1.0 mL, 100/pk	5182-0567
Crimp/snap caps with polypropylene septa, 100/pk	5182-0542
Mass Spectrometry System	
Agilent 6495 Triple Quadrupole LC/MS system with iFunnel Technology	
Agilent MassHunter Software (Ver. 08.00)	

Table 3. LC conditions.

Parameter	Value
Flow rate	0.5 mL/min
Colum temperature	50 °C
Autosampler temperature	5 °C
Injection volume	4 µL
Mobile phase	A) 5 mM Ammonium acetate in water B) Acetonitrile
Needle wash: Multiwash	S1) H_2O S2) H_2O:ACN (50:50) S3) ACN 10 seconds each wash
Gradient	Time (min) %B 0.0 30 0.5 30 3.5 90 4.5 100
Stop time	5.0 minutes
Post time	1.5 minutes

Table 4. MS conditions.

Parameter	Value
Ionization mode	Negative ESI
Gas temperature	130 °C
Gas flow	15 L/min
Nebulizer	35 psi
Sheath gas heater	375 °C
Capillary voltage	2,000V
Vcharging	500
Delta electron multiplier voltage (EMV)	200
Polarity	Negative

Table 5. Triple quadrupole MRM acquisition parameters for PPLs.

Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
808.4	184.4	30
806.4	184.4	30
786.4	184.4	30
784.4	184.4	30
760.4	184.4	30
758.4	184.4	30
704.4	184.4	30
524.4	184.4	30
522.4	184.4	30
520.4	184.4	30
496.4	184.4	30

Agilent MassHunter Software (Ver. 08.00) was used for instrument control, and for qualitative and quantitative data processing and analysis. Reproducibility and recovery of the method for PFASs were determined.

Sample Preparation Procedures

PFAS Extraction from Plasma

1. Add 400 µL of ACN (1 % FA) to the Captiva EMR—Lipid 1 mL cartridge.
2. Add 100 µL of spiked or blank human plasma, prespun.
3. Perform in-well mixing.
4. Pull a low vacuum of 2–4 psi for a controlled flow rate of 1 drop per 3–5 seconds.
5. Collect the extract in polypropylene test tubes.
6. Inject directly onto the LC-MS/MS system using polypropylene autosampler vials.

Because MeOH forms smaller precipitant particles than ACN, ACN is recommended to maximize PPT and avoid gelation prior to Captiva EMR—Lipid treatment. A ratio range of 1:3 to 1:5 (sample/solvent) is recommended. Plasma sample is added after the crash solvent. Acid (formic acid) helps break up proteins, and minimizes protein binding.

Preferably, active in-well mixing is done using wide-bore pipette tips. The vacuum initiates flow through the Captiva EMR—Lipid cartridge. A controlled flow rate of one drop per 3–5 seconds is recommended for optimal lipid removal. After sample elution off the cartridge, higher vacuum is applied to maximize sample recovery. Polypropylene collection tubes and autosampler vials are highly recommended to prevent PFAS loss due to sticking on glass surfaces.

PPL Removal Evaluation, PPT Only

1. Add 400 µL of ACN (1 % FA) to a test tube.
2. Add 100 µL of blank plasma, prespun.
3. Vortex on a Heidolph Multi Reax at 800–1,000 rpm for 5 minutes.
4. Centrifuge at 5,000 rpm for 5 minutes.
5. Pipette the supernatant into a polypropylene autosampler vial for LC-MS/MS analysis.

Results and Discussion

Unwanted Lipid Matrix Removal

The EMR—Lipid approach is simple and universally applicable to reducing matrix effects and improving analyte recoveries for the analysis of polar, midpolar, and nonpolar target analytes, in research laboratories. The EMR—Lipid sorbent selectively traps lipids by size exclusion and hydrophobic interaction (Figure 1). Unbranched hydrocarbon chains (lipids) enter the pores of the sorbent, but bulky analytes do not. Lipid chains that enter the sorbent are then trapped by hydrophobic interactions.

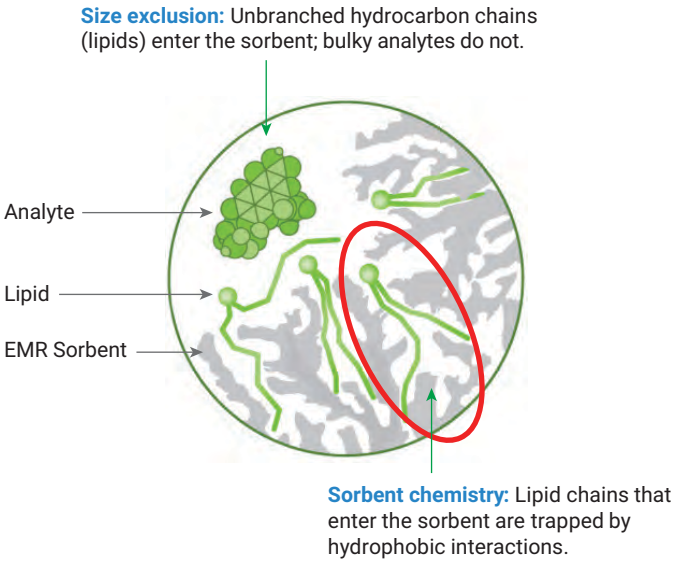

Figure 1. EMR—Lipid mechanism: size exclusion and hydrophobic interactions.

Though the PFAS structures shown in Figure 2 contain a long straight carbon chain, the carbon is attached to fluorine atoms, which are sterically larger than unbranched hydrocarbon chains. Therefore, they are sterically hindered from entering into the pores of EMR—Lipid sorbent.

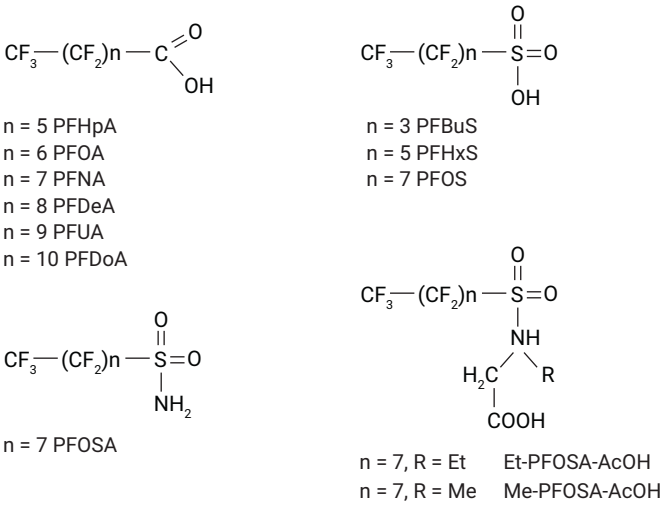

Figure 2. Molecular structures of PFASs.

Chromatographic Performance

The MRM chromatogram of plasma spiked at 5 ng/mL (Figure 3) shows the chromatographic performance that can be obtained using the EMR—Lipid protocol. Even at the 5 ng/mL level, ideal peak shapes due to reduced matrix effect and interferences resulted in good separation and signal-to-noise (S/N) for accurate integration. Using the 6495 Triple Quadrupole LC/MS system, accurate detection and quantification at levels of 0.1 ng/mL and lower can be achieved when performing analysis of PFASs in plasma.

PPL Removal

PPLs are the main constituents of cell membranes and the main class of compounds that cause significant matrix effect[3,4]. Glycerophophocholines and lysophosphatidylcholines represent 70 % and 10 % of the total plasma PPLs, respectively[5], and are the major source of matrix effects. To determine the efficiency of PPL removal from plasma using Captiva EMR—Lipid cartridge cleanup, 11 naturally occurring PPL compounds were monitored. Specifically, the ion fragment at *m/z* 184 was used to monitor the PPLs in plasma extract after PPT and Captiva EMR—Lipid removal.

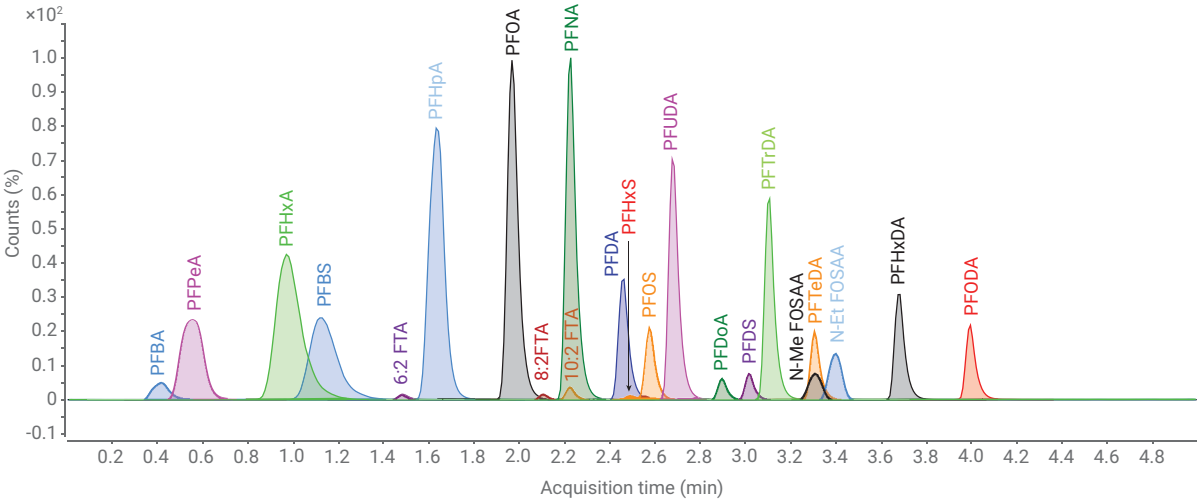

Figure 3. MRM chromatogram of plasma spiked at 5 ng/mL.

As shown in Figure 4, approximately 99 % (based on peak area comparison) of the PPLs monitored were eliminated from the extracted plasma samples compared to PPT alone, some of which would have coeluted with the target analytes. The high relative abundance of PPLs shown in Figure 4 (red trace) subjects the detector to potential saturation and could impact the quality of quantification. In addition, a high abundance of PPLs can contaminate an LC-MS system over time.

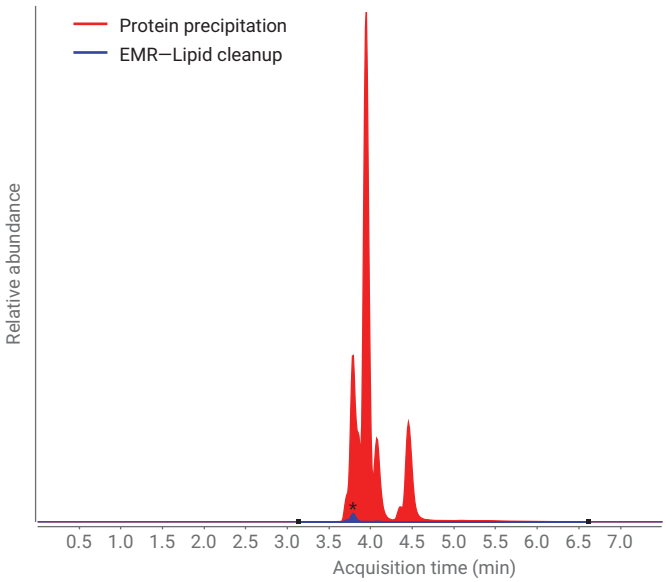

Figure 4. Overlay of MRM chromatograms of 11 PPLs monitored at *m/z* 184 after PPT only (red) and after Agilent Captiva EMR—Lipid cleanup (blue).

Quantitative Performance

Calibration curve linearity was evaluated. Figure 5 shows the calibration curves for PFOA and PFOS. Good linearity of response was observed at the six concentration levels tested (0.1 to 50 ng/mL) on the three separate occasions that they were generated. The average coefficient of determination (R^2) for all PFASs studied was greater than 0.992, with linearity from 0.1 to 50 ng/mL, linear fit, 1/x weighting.

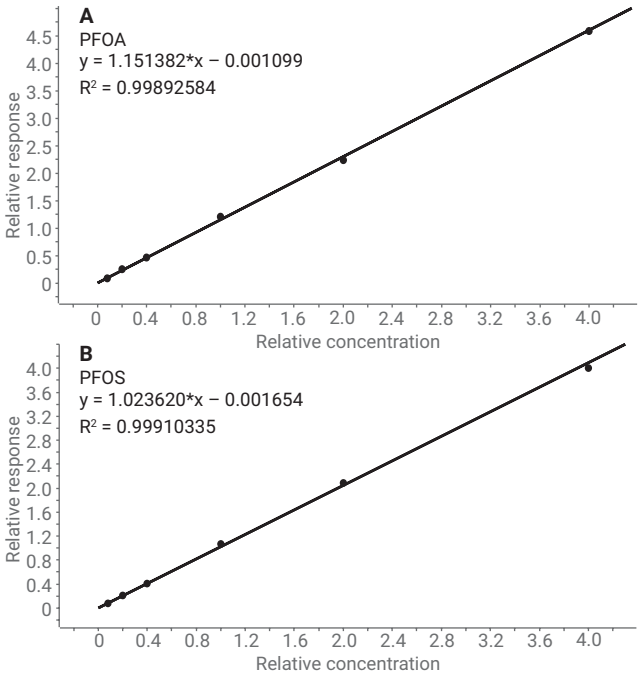

Figure 5. Calibration curves. A) PFOA, B) PFOS. Concentration range 0.1–50 ng/mL in plasma using protein precipitation followed with Agilent Captiva EMR—Lipid cleanup.

Method recovery and reproducibility (RSDs) for the 22 PFASs were determined by spiking the standard into plasma at 5 and 20 ng/mL in replicates of five. Overall recoveries were excellent and between 75 and 125 % (Figure 6). Most PFASs had recoveries of 90–110 %. The widely studied PFASs, PFOS and PFOA, had average recoveries of 92.7 ±6.6 % and 93.1 ±5.0 %, respectively, at both spiking levels in plasma. Relative standard deviations were acceptable, and ranged from 0.8 to 14 % at the 5 and 20 ng/mL levels.

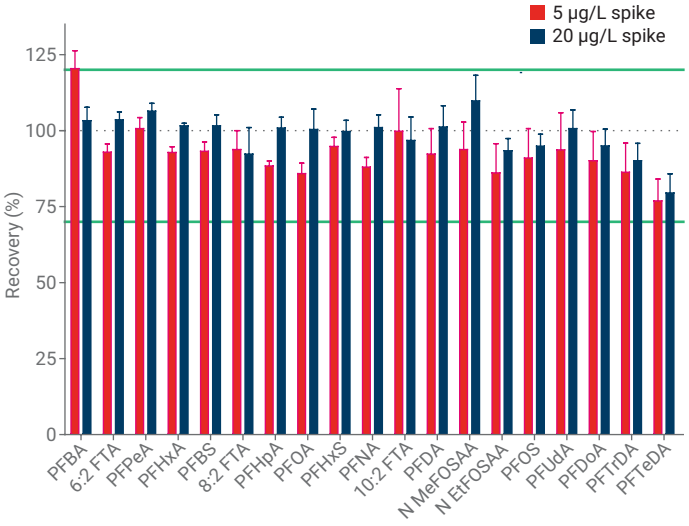

Figure 6. Recovery and RSD for the PFASs evaluated at 5 and 20 ng/mL.

Conclusion

This Application Note presents a simple and rapid workflow to prepare plasma samples for LC-MS/MS analysis of PFASs. Extraction of 22 PFASs from plasma was performed using in-well PPT followed by PPL removal using an Agilent Captiva EMR—Lipid cartridge in a pass-through format. Captiva EMR—Lipid efficiently removed 99 % of the unwanted PPLs from the plasma matrix, with excellent recovery of target analytes. The sample extract was cleaner than using PPT alone, thereby reducing the potential for ion suppression and LC-MS/MS system contamination and downtime. In-well PPT has the benefit of less sample handling and transfer.

Analysis of PFASs at 5 ng/mL yielded ideal chromatographic peak shapes and good S/N. Response for PFASs over six concentration levels (0.1–50 ng/mL) was linear, with an R^2 greater than 0.992. Recoveries were excellent at 75 % or higher, and RSDs less than 14 % for the PFASs tested. The results showed the method to be acceptable for multiresidue extraction and analysis of PFASs.

Captiva EMR—Lipid methodology can readily be incorporated into existing research laboratory workflows, and does not require additional sample preparation devices or glassware. In either 96-well plate or 1 mL cartridge formats, Captiva EMR—Lipid is compatible with automation, enabling high-throughput applications. The frit design provides easy and efficient elution of samples without clogging.

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Using the Blood Exposome to Discover Causes of Disease

Technical Overview

Clinical Research

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Introduction

Of the 52.8 million world-wide deaths in 2010, approximately two-thirds were caused by chronic diseases, mainly cardiovascular disease (> 15 million) and cancer (> 7 million) [1]. Thus, it is reasonable to ask whether chronic diseases are attributable to genetic factors, exposures, or some combination of the two. Data compiled by the Swedish Family-Cancer Database indicate that genetic (G) risks for 15 common cancers were 10% or less [2]. This suggests that approximately 90% of cancer risks result from exposures (E) or G×E interactions.

Despite the relatively small genetic risks for cancer and other chronic diseases, exquisite tools are available to investigate G factors in studies of human diseases. In fact, genome-wide-association studies (GWAS) currently measures more than one million single nucleotide polymorphisms in 2,000–20,000 subjects. In contrast, individuals' exposures are still inferred from personal interviews or self-administered questionnaires [3] much as they were a century ago. Since this disparity in characterizing G and E makes it impossible to thoroughly investigate the GxE matrix, technologies and methods for data-driven analysis of exposures must be developed [4].

Agilent Technologies

The exposome represents the sum of all chemical exposures received by an individual over a lifetime from both exogenous sources (food, pollutants, ionizing radiation, drugs, lifestyle factor, infections) and endogenous sources (human and microbiota metabolism, oxidative stress, lipid peroxidation, infections, and preexisting disease). "As such, the exposome is everything that is 'not the genome' and serves as an umbrella for all of the traditional omes" [5]. Figure 1 illustrates the placement of the exposome in the continuum between states of human health and disease.

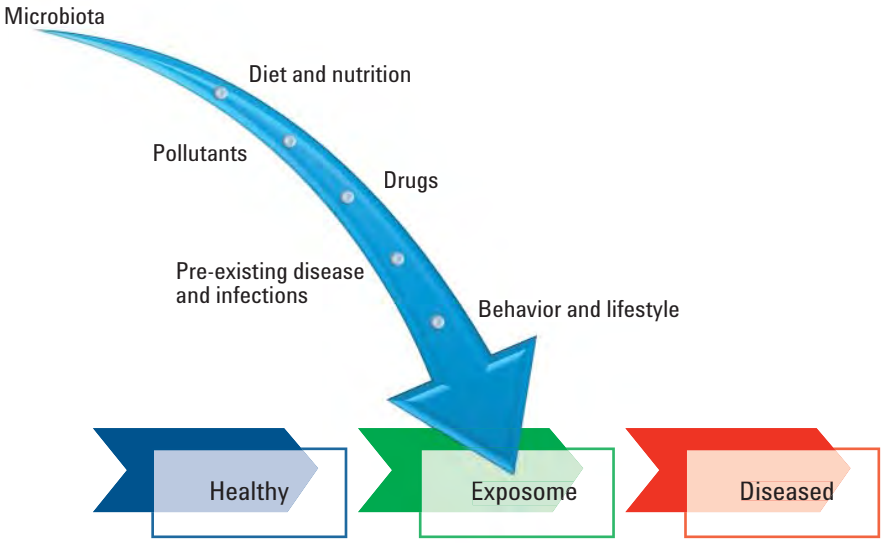


Figure 1. Exposure contributions to chronic human disease.

A Two-stage Strategy for Discovering and Targeting Causal Exposures

By focusing upon all circulating chemicals in the human body, the blood exposome motivates studies of nongenetic causes of chronic diseases in much the same manner that GWAS explores genetic causes [6,7,4]. To this end, Stephen M. Rappaport, Director of the Center for Exposure Biology at the University of California, Berkeley, describes a two-phase approach for interrogating the blood exposome to discover important exposures in disease cases and controls and then to target these exposures in follow-up studies with large populations [7].

Professor Rappaport functionally defines the exposome as roughly 200,000 circulating chemicals in human blood, including metals, small molecules, proteins, and foreign DNA. By comparing untargeted profiles of blood exposomes between diseased and healthy subjects, he suggests that we perform exposome-wide association studies (EWAS) to pinpoint discriminating chemicals [7]. After identifying these key chemicals and verifying their disease associations in independent samples of cases and controls, the chemicals can be used as biomarkers of exposures or disease progression in targeted analyses of blood from large populations. Thus, a successful strategy for discovering and reducing harmful exposures requires an initial data-driven investigation (EWAS), to find promising biomarkers, followed by knowledge-driven studies that use the biomarkers to elucidate exposure-response relationships (biochemical epidemiology), sources of exposure and human kinetics (exposure biology) and mechanisms of action (systems biology). Prof. Rappaport stresses that this two-step strategy will lead to reduced exposures, improved public health, early diagnosis of diseases, and personalized medical interventions. Figure 2 illustrates the two-step strategy of EWAS followed by targeted studies.

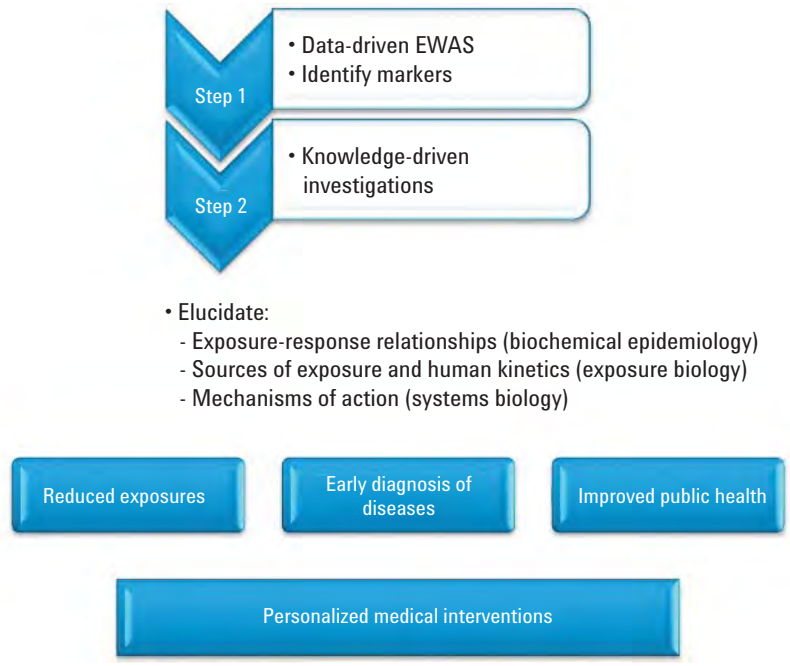
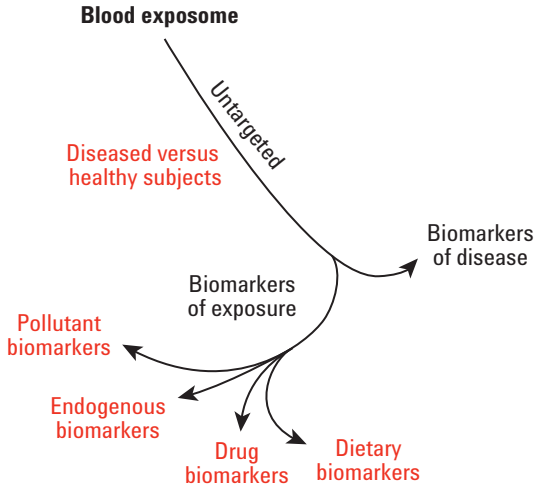


Figure 2. Two-phase EWAS targeted analytical strategy to identify and reduce harmful exposures.

Measuring the Exposome

Enormous technical challenges must be confronted to achieve the necessary combination of extreme multiplexing, sensitivity, and throughput required for EWAS and follow-up studies. It is desired to conduct untargeted EWAS with 10–50 µL of blood, serum, or plasma from each of a few hundred subjects, and to perform targeted analyses of biomarkers in equivalent volumes of blood or serum from thousands of subjects. A relevant analytical platform for many EWAS would wed high-resolution liquid chromatography (LC) or gas chromatography (GC) with a time-of-flight (TOF) mass spectrometer (MS) having excellent sensitivity and mass accuracy. Follow-up investigations of promising biomarkers could then employ robotics and triple quadrupole MS with multiple-reaction monitoring. Through rigorous selection of specimens from prospective cohort studies, it is possible to differentiate biomarkers of causal exposures from biomarkers of disease progression.



Based on:
S. Rappaport, *Biomarkers*, 2012, **17**(6), 48: 3-9

Figure 3. EWAS of blood offers differentiation of biomarkers of exposure (causal pathways) from biomarkers of disease (reactive pathways).

Proof-of-concept studies have already identified unknown biomarkers of exposure for colorectal cancer and cardiovascular disease and biomarkers of disease progression for diabetes [7].

Analytical Strategies

Clearly, dedicated platforms of nanoflow LC/TOF and LC/Triple Quad and GC/TOF, GC/Triple Quad are essential to ensure the necessary sensitivity and precision for both data-driven EWAS and targeted, high-throughput follow-up studies. To this end, Ivanisevic and Zhu, *et al*. (2013) [8] reported the ability to measure more than 30,000 unique features from a single 100 µL sample of serum or plasma in an untargeted analysis using reversed-phase LC and hydrophilic interaction chromatography (HILIC) in both positive and negative electrospray modes. The analytical methodology used an Agilent 1200 Series LC system coupled to an Agilent 6538 UHD Accurate-Mass Q-TOF LC/MS system, and is the approach needed for a small-molecule EWAS.

Figure 4. The Agilent 1200 Series LC system and an Agilent 6500 Series Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system.

Another relevant analytical approach involves modifying the Agilent-Fiehn GC/MS methodology [9] to incorporate an Agilent 7890B GC with an Agilent 7200 Q-TOF system for investigating serum extracts derivatized with methoxyamine hydrochloride and MSTFA. In post-acquisition processing, an Agilent retention-time-locked spectral library of over 1,000 chemical entities can be used to annotate unknown features. The data can be used independently or to orthogonally compliment and corroborate LC/MS data.

Figure 5. The Agilent 7890A GC with an Agilent 7200 Q-TOF system.

Once biologically relevant biomarkers are annotated and verified, targeted high-throughput analyses are needed to measure biomarkers of causal exposures and biomarkers of disease progression. A relevant example of such targeted analyses involves measurement of endocrine disrupting chemicals, using the Agilent 7890B GC with an Agilent 7000C Series Triple Quadrupole system [10]. Similar applications of the Agilent GC/MS system have been used in clinical research laboratories to measure estrogen biomarkers in breast cancer studies at extremely low levels (for example, 17β-estradiol can be detected at 0.12 pg/mL ($4.4E^{-7}$ µM)) [11].

Once data are collected, sophisticated bioinformatic software is required to compare biomarker levels across populations and to investigate covariates. Agilent Mass Profiler Professional (MPP) Software provides advanced statistical analysis and visualization tools for GC/MS, LC/MS, CE/MS and ICP-MS data analysis that can be used to identify key features and perform global analyses. The analyst can further map these to biological pathways using the Pathway Architect tools.

Reactive Electrophiles

Reactive electrophiles represent an important class of toxic chemicals that is produced from metabolism of xenobiotic and endogenous precursor molecules, oxidative stress, and lipid peroxidation [12]. Although too reactive to measure directly in blood, levels of these electrophiles can be inferred by measuring adducts from reactions with prominent blood proteins such as human serum albumin (HSA). Prof. Rappaport's laboratory has proposed untargeted MS approaches for profiling adducts of HSA at the nucleophilic hotspot, Cys34, as part of EWAS [13] and for identifying prominent features by high resolution mass spectrometry [14].

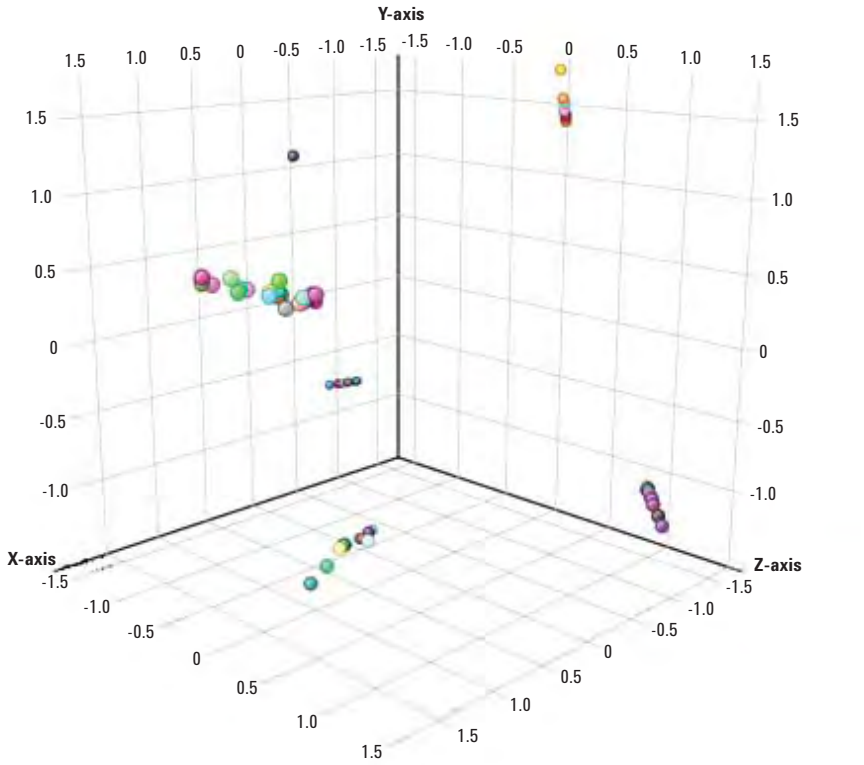


Figure 6. Mass Profiler Professional illustrating separation of different chemotypes from GC/TOF data using PCA analysis of ANOVA results.

Conclusion

Exposomics is a collaborative paradigm that brings together epidemiology, environmental toxicology, analytical chemistry, nutrition, and microbiology in an open access milieu. Since exposomics applies multiple analytical techniques (chromatography, spectrometry, spectroscopy, sensor-array technologies) and bioinformatics to characterize individuals' exposomes, it requires development of novel technologies to achieve the demands of high resolution, high sensitivity and high throughput for EWAS and follow-up investigations. The current state of exposome research could be compared to that of genomics in the early nineties. By integrating several omic technologies (metallomics, metabolomics, proteomics, and metagenomics) with one unified objective, the nascent field of exposomics may well provide the missing links to disease causality and personalized medicine.

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Analysis of Polycyclic Aromatic Hydrocarbons (PAH) and Hydroxylated PAH Metabolites in Plasma and Urine Using High-Resolution GC/Q-TOF

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are products of incomplete combustion of organic materials and present ubiquitously in the environment. PAHs are well-know carcinogens, and the primary sources of human exposure to PAHs are environmental, dietary as well as occupational [1]. Hydroxylated metabolites of the PAHs are present at trace levels in biological matrices, and can be used as biomarkers of the recent exposure to these compounds [1]. Because of high analytical sensitivity requirements from the analytical methods for PAH metabolite analysis, Triple Quadrupole GC/MS instruments are preferred for their targeted analysis [2]. To add capability for untargeted analysis in addition to a highly sensitive targeted approach for detection of the persistent organic pollutants and their metabolites, use of a high resolution accurate mass 7250 GC/Q-TOF system was evaluated in the current study. Thus, we have applied a targeted approach for the detection of PAHs and PAH metabolites, and an untargeted approach to discover other biologically relevant compounds in human urine and plasma extracts.

Experimental

Six milliliters of pooled plasma and urine samples were extracted with an equal volume of hexane/acetone (1:1, v/v). To these, 1.1 g magnesium sulfate and 1.1 g sodium chloride was added to assist in phase separation. The extracts were spiked with various concentrations of PAH and hydroxy-PAH standards, ranging from 0.2 ppb to 2 ppm, as well as deuterated internal standards. Dried samples were derivatized with a mixture of MSTFA/pyridine and analyzed using an Agilent 7890B GC system coupled to a high resolution 7250 GC/Q-TOF, equipped with an Electron Ionization (EI) source allowing low-energy ionization (Figure 1). Instrument parameters are shown in Table 1.

The data were processed using MassHunter Qualitative Analysis (B.08) as well as Quantitative Analysis (B.09) software. Unknowns Analysis was further used for the untargeted identification of additional PAH-like compounds and other compounds of potential interest not found in the target list.

Experimental

Figure 1. Agilent 7250 GC/Q-TOF

GC and MS Conditions:	
Column	DB-5MS UI, 30 m, 0.25 mm, 0.25 µm
Injection volume	1 µL
Injection mode	Splitless
Split/Splitless inlet temperature	270°C
Oven temperature program	70°C for 1 min 20°C/min to 270°C, 10°C/min to 307°C, 40°C/min to 325°C, 4 min hold
Carrier gas	Helium at 1.2 mL/min constant flow
Transfer line temperature	280°C
Ionization mode	Standard EI at 70 eV Low Electron Energy EI at 15 eV and 12 eV
Source temperature	280°C (200°C for low electron energy)
Quadrupole temperature	150°C
Mass range	50 to 650 m/z
Spectral acquisition rate	5 Hz

Table 1. GC/Q-TOF conditions

Quantitation and qualitative screening approach

As a first step, an accurate mass Personal Compound Database and Library (PCDL) containing PAHs and hydroxylated PAHs in their derivatized form, (Table 2) was constructed (Figure 2) and used for automated creation of a quantitative method.

Compound name	RT	Formula of derivatized compound	m/z of derivatized compound
Naphthalene-d8	5.485	C10D8	136.1123
Naphthalene	5.507	C10H8	128.0621
Acenaphthylene	7.366	C12H8	152.0621
Acenaphthene-d10-IS	7.54	C12D10	164.1405
Acenaphthene	7.58	C12H10	154.0777
Fluorene	8.216	C13H10	166.0777
1-Hydroxynaphthalene	7.838	C13 H16 O Si	216.0965
1-Hydroxynaphthalene-d7	7.799	C13H9D7OSi	223.1404
2-Hydroxynaphthalene	7.99	C13 H16 O Si	216.0965
Phenanthrene-d10	9.37	C14D10	188.1405
Phenanthrene	9.4	C14H10	178.0782
Anthracene	9.46	C14H10	178.0782
Fluoranthene	10.86	C16H10	202.0777
Pyrene	11.14	C16H10	202.0777
3-Hydroxyfluorene	10.18	C16H18OSi	254.1121
2-hydroxyfluorene-d9	10.24	C16H9D9OSi	263.1686
2-hydroxyfluorene	10.29	C16H18OSi	254.1121
4-Hydroxyphenanthrene	10.76	C17H18OSi	266.1121
1,6-Dihydroxynaphthalene	9.75	C16H24O2Si2	304.1309
3-Hydroxyphenanthrene-d9	11.038	C17H9D9OSi	275.1686
3-Hydroxyphenanthrene	10.94	C17H18OSi	266.1121
2,7-Dihydroxynaphthalene	9.87	C16H24O2Si2	304.1309
1-Hydroxyphenanthrene	11.083	C17H18OSi	266.1121
9-hydroxyphenanthrene-d8	10.899	C17H10D8OSi	274.1624
9-Hydroxyphenanthrene	11.12	C17H18OSi	266.1121
Benzo(a)anthracene	12.796	C18H12	228.0934
Chrysene-d12	12.81	C18D12	240.1687
Chrysene	12.849	C18H12	228.0934
1-hydroxypyrene-d9	12.761	C19H9D9OSi	299.1686
1-Hydroxypyrene	12.816	C19H18OSi	290.1121
Benzo(b)fluoranthene	14.484	C20H12	252.0934
Benzo(k)fluoranthene	14.529	C20H12	252.0934
Benz(a)pyrene	14.981	C20H12	252.0934
Perylene-d12	15.06	C20D12	264.1687
3-Hydroxychrysene-d11	14.521	C21H9D11OSi	327.1968
6-Hydroxychrysene	14.08	C21H20OSi	316.1278
Indeno(1,2,3-cd)pyrene	16.557	C22H12	276.0934
Dibenz(a,h)anthracene	16.595	C22H14	278.1091
Benzo(ghi)perylene-d12	16.924	C22D12	288.1687
Benzo(ghi)perylene	16.97	C22H12	276.0934
3-Hydroxybenzo(a)pyrene-d11	16.614	C23H9D11OSi	351.1968
9-Hydroxybenzo(a)pyrene	16.634	C23H20OSi	340.1278
7,8-Hydroxybenzo(a)pyrene	17.89	C26H28O2Si2	428.1622

Table 2. List of targeted compounds

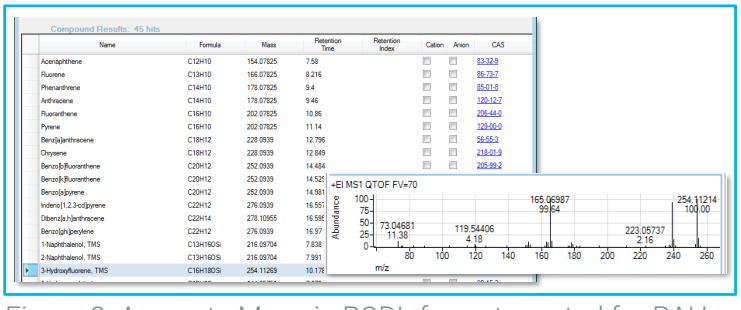

Figure 2. Accurate Mass in PCDL format created for PAHs and hydroxylated PAH metabolites.

The examples of matrix-matched calibration curves for PAHs and their hydroxylated metabolites are shown in Figure 3.

In most cases, the calibration curves were linear up to 2000 ng/mL. However, in a few cases, non-linearity was observed above approximately 1000 ng/mL.

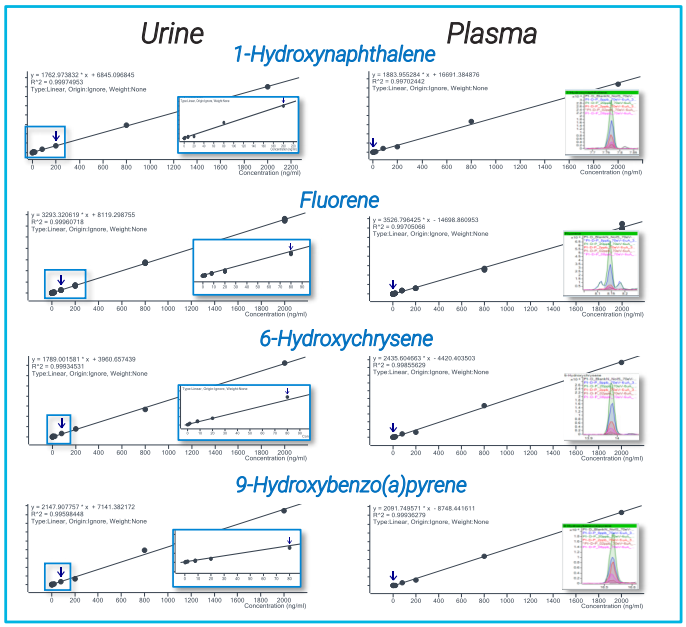

Figure 3. Calibration curves examples for both urine and plasma matrices (0.2-2000 ng/mL). EIC overlay for concentrations 0.2-20 ng/mL is shown on the right.

Mass accuracy (Table 3) is shown for PAH and OH-PAH in plasma matrix across a concentration range of 2-2000 ppb and on average was below 1 ppm.

LOD for both urine and plasma matrices were calculated based on 5 replicate injections and are shown in Table 4.

In addition to quantitation, the PCDL was also utilized in quick qualitative screening using MassHunter Qualitative Analysis (shown in Figure 4).

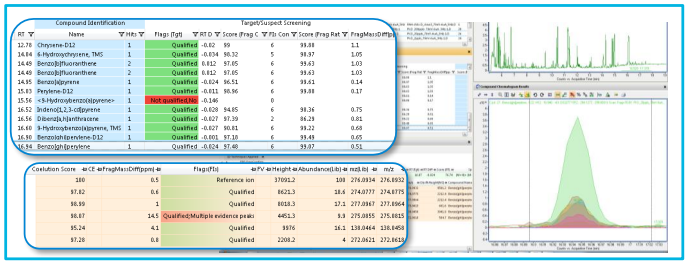

Figure 4. Example of screening results using PCDL MassHunter Qulitative software

Results and Discussion

Unknowns identification and confirmation of tentative hits

The untargeted workflow using Unknowns Analysis and NIST 17.L library identified several additional PAH-like compounds and other xenobiotics, including 1-methyl-2-(phenylmethyl) benzene, benzophenone, pentochlorophenol, Escitalopram, octocrylene, 1,12-dimethyl benz(a)anthracene and dibenz(a,j)acridine in urine and cotinine (biomarker of exposure to tobacco smoke [3]) and 1-naphthoic acid in plasma (Figure 5A). Accurate mass information as well as retention indices were utilized to confirm the candidate hit's identity. A proprietary Electron Ionization (EI) source design allowed for low energy ionization to assist in the confirmation of candidate molecular ions (Figure 5B).

	Mass Accuracy (ppm) per concentration						
Compound/concentration, ng/mL	2	8	20	80	200	800	2000
Naphthalene	0.47	0.56	0.47	1.02	0.55	1.48	0.84
Acenaphthylene	0.63	0.50	0.18	0.40	0.19	0.45	0.79
Acenaphthene	0.93	0.52	0.97	1.85	1.37	2.16	3.37
1-Hydroxynaphthalene	0.74	0.49	0.48	0.52	0.50	0.74	0.86
2-Hydroxynaphthalene	0.95	0.51	0.54	0.47	0.43	0.76	1.07
Fluorene	0.92	1.23	1.07	0.39	0.77	0.96	1.45
Phenanthrene	0.64	0.45	0.88	1.19	1.34	0.64	0.61
Anthracene	0.50	0.44	0.84	1.35	1.28	0.80	0.46
1,6-Dihydroxynaphthalene	0.73	1.26	0.62	0.36	0.62	0.40	0.56
2,7-Dihydroxynaphthalene	0.35	0.55	0.78	1.05	0.86	0.41	0.53
3-Hydroxyfluorene	0.59	0.59	0.79	0.82	0.43	0.40	0.34
2-hydroxyfluorene	0.96	0.54	0.66	0.73	0.51	0.49	0.34
4-Hydroxyphenanthrene	0.70	0.90	0.34	0.36	0.34	0.38	0.59
Fluoranthene	0.41	0.56	0.37	0.52	0.73	0.73	0.71
3-Hydroxyphenanthrene	0.78	0.39	0.38	0.36	0.43	0.21	0.38
1-Hydroxyphenanthrene	0.46	0.37	0.47	0.55	0.59	0.37	0.35
9-Hydroxyphenanthrene	0.52	0.65	0.37	0.74	0.46	0.26	0.45
Pyrene	0.35	0.25	0.36	0.40	0.42	0.70	0.51
Benzo(a)anthracene	0.58	0.48	0.33	0.58	0.59	1.11	1.71
Chrysene	0.93	0.66	0.41	0.56	0.44	0.92	1.51
6-Hydroxychrysene	0.37	0.25	0.62	0.53	0.36	0.20	0.60
Benzo(b)fluoranthene	1.14	0.46	0.49	0.70	0.74	0.92	0.98
Benzo(k)fluoranthene	0.59	0.62	0.93	0.75	0.99	0.21	0.55
Benz(a)pyrene	0.53	0.91	0.32	0.28	0.42	0.44	0.76
Indeno(1,2,3-cd)pyrene	0.29	0.43	0.48	0.62	0.44	0.47	0.78
Dibenz(a,h)anthracene	0.57	0.42	0.50	0.47	0.47	0.98	1.06
9-Hydroxybenzo(a)pyrene	0.68	0.67	0.84	0.65	0.49	0.45	0.51
Benzo(ghi)perylene	0.88	0.76	0.61	0.65	0.43	0.61	0.35
7,8-Hydroxybenzo(a)pyrene	2.57	1.17	0.98	0.57	0.81	0.48	0.93

Table 3. Mass accuracy in plasma observed for PAHs and hydroxylated PAH metabolites across concentrations of 2-2000 ppb.

LOD (ng/mL) per matrix		
Compound name	Urine	Plasma
Naphthalene	0.52	0.38
Acenaphthylene	0.42	0.74
Acenaphthene	0.98	1.28
1-Hydroxynaphthalene	0.48	0.47
2-Hydroxynaphthalene	0.28	0.20
Fluorene	1.12	0.26
Phenanthrene	0.35	0.18
Anthracene	0.36	0.26
1,6-Dihydroxynaphthalene	0.34	0.16
2,7-Dihydroxynaphthalene	0.13	0.10
3-Hydroxyfluorene	0.22	0.36
2-hydroxyfluorene	0.25	0.21
4-Hydroxyphenanthrene	0.39	0.42
Fluoranthene	0.30	0.19
3-Hydroxyphenanthrene	0.60	0.27
1-Hydroxyphenanthrene	0.34	0.30
9-Hydroxyphenanthrene	0.50	0.33
Pyrene	0.42	0.10
Benzo(a)anthracene	0.16	0.23
Chrysene	0.26	0.09
6-Hydroxychrysene	0.14	0.16
Benzo(b)fluoranthene	0.38	0.26
Benzo(k)fluoranthene	0.38	0.27
Benz(a)pyrene	0.61	0.30
Indeno(1,2,3-cd)pyrene	0.65	0.35
Dibenz(a,h)anthracene	0.51	0.09
9-Hydroxybenzo(a)pyrene	0.47	0.34
Benzo(ghi)perylene	0.89	0.36
7,8-Hydroxybenzo(a)pyrene	6.65	3.63

Table 4. LOD calculated for PAH and PAH metabolites in plasma and urine matrices based on 5 replicate injections.

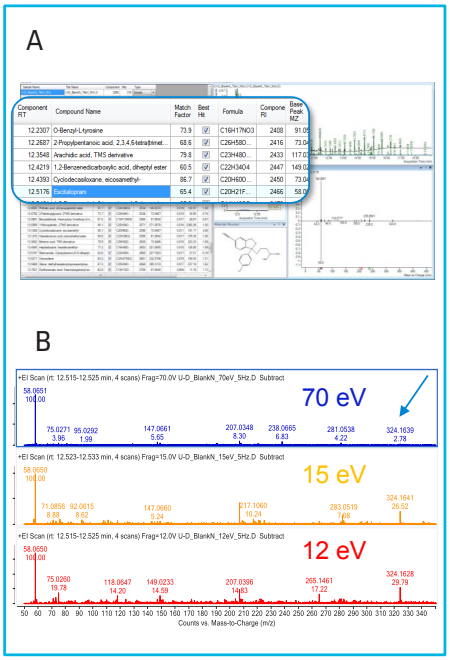

Figure 5. Unknowns Analysis results showing an example of a tentatively identified compound in urine matrix using NIST17.L (A) as well as 70 eV and low electron energy spectra for the tentatively identified compound (B).

Conclusions

Targeted quantification and untargeted screening for PAHs, their metabolites, and other substances in complex biological matrices was successfully performed using an HRMS GC/Q-TOF.

References

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