Residues Application Notes Handbook

Gel Permeation Chromatography | Solid Phase Extraction | Liquid Handling

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Foreward: Residues Testing in the Laboratory

The testing of residues is a critical part of underlying safety with our natural resources and agricultural industries, impacting the global human and animal populations. Standardized and regulatory methods have been utilized for years as a routine practice for quality control. Research laboratories utilize these standardized methods as a starting point to push what is possible with today's chemistry and automation.

Often laboratories are looking for more efficient quantitative methods that focus on streamlined analysis or reducing the number of samples a laboratory would typically need to run on a daily basis. Over the last 20 years with the LC/MS-MS analysis of organic compounds in environmental and physiological matrices, one trend that has been prevalent with both disciplines is that scientists want trace level detection of many compounds (often 20+) in challenging matrices that cover a wide range of physicochemical properties (less polar parent compounds and some more polar degradation products in environmental, and less polar parent compounds with more polar metabolites for biomonitoring work) in a single analysis. The development of mixed mode SPE products and advancement of HPLC column technology have been keys to the success of these complex methods. Future enhancements in mixed mode SPE products will hopefully allow for better absolute recovery of target compounds (currently 40% to 60% for some compounds) to drive method detection limits down even further.

With the newer chemistries packed into Solid Phase Extraction (SPE) columns and High Pressure Liquid Chromatography (HPLC) columns combined with the increased sensitivity of today's Mass Spectrometry (MS) detectors AND the simplicity of benchtop automation systems, research is pushing the boundaries of what can be accomplished to keep safety at the forefront. For optimal sample throughput considerations, the implementation of using automated liquid handling equipment in place of manual procedures, the use of core shell column technology, and considering multiplexed HPLC (and UHPLC) systems have helped streamline sample preparation and analysis workflows. The bottleneck has now become management of the very large data sets that are being generated in less time.

This Application Notes Handbook is a collection of residues testing on food, beverages, water, and soil samples performed in a variety of laboratories focused on pushing the boundaries of what is possible using today's science and technology in the laboratory.

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Water & Soil Applications

Analysis of Sucralose in Water by Liquid Chromatography/Time-of-Flight Mass Spectrometry (LC/TOF-MS) After Automated Solid Phase Extraction

This study was performed by Imma Ferrer, Ph.D. and E. Michael Thurman, Ph.D. at the Center for Environmental Mass Spectrometry, Dept. of Civil, Environmental & Architectural Engineering, University of Colorado, Boulder, CO, USA

Featured Products: Gilson GX-271 ASPEC™ System with 406 Single Syringe Pump, TRILUTION® LH Liquid Handling Software.

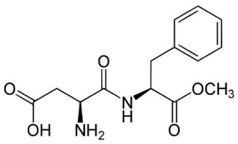
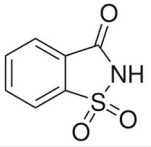
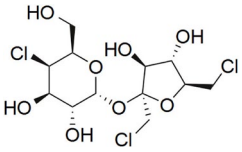
Introduction

Non-nutritive sweeteners are steadily growing in importance with increased awareness of diabetes and with more consumers becoming concerned about obesity and dental caries. Some of the most widely used non-nutritive sweeteners include saccharin, aspartame and sucralose (Table 1). Sucralose is a water soluble tri-chlorinated disaccharide that is 600 times sweeter than sucrose. It is highly stable and will not readily degrade at high temperatures (1). In 1991, Canada's Health Protection Branch became the first national regulatory agency to permit the use of sucralose in foods and beverages. In 1999, the U.S. Food & Drug Administration (FDA) approved permitting the use of sucralose as a general-purpose sweetener in a broad range of products. The European Union amended its Sweeteners Directive to permit the use of sucralose in a variety of foods and beverages in January 2004. Sucralose is now permitted for use in over 60 countries and has been consumed by millions worldwide.

In humans, the majority of orally ingested sucralose is eliminated through the gastrointestinal tract, while 11-27% is absorbed with a half life of 2 to 5 hours. Of the absorbed sucralose, only 20-30% is metabolized (2). As the majority of ingested sucralose passes unmetabolized, one would expect the presence of sucralose in wastewater effluent. While sucralose does not bioaccumulate in the environment, there is a lack of knowledge of its long-term biological effects in the aquatic environment. This has lead to an interest in monitoring levels of sucralose in water samples (3, 4, 5).

Recently, some methods employing liquid chromatography-mass spectrometry (LC/MS) have been published for the analysis of sucralose and other artificial sweeteners in water samples (6, 7). This study describes the analysis of several sweeteners in water using liquid chromatography coupled to time-of-flight mass spectrometry (LC/TOF-MS) as well as the automated extraction of these sweeteners from wastewater, surface water and groundwater utilizing the Gilson GX-271 ASPEC System (8).

Table 1. Elemental Composition, Base Peak Ions, and Chemical Structures of Aspartame, Saccharin and Sucralose.

Name	Elemental Composition	Base Peak	Chemical Structure
Aspartame	$C_{14}H_{18}N_2O_5$	$[M+H]^+$ $C_{14}H_{18}N_2O_5^+$ 295.1288	
Saccharin	$C_7H_5NSO_3$	$[M+H]^+$ $C_7H_5NSO_3^+$ 184.0063	
Sucralose	$C_{12}H_{19}Cl_3O_8$	$[M+Na]^+$ $C_{12}H_{19}Cl_3O_8^+$ 419.0038	

Experimental Conditions

Materials

- GX-271 ASPEC with 406 Single Syringe Pump
  - TRILUTION LH software
- SPE Cartridges: Oasis™ HLB 500 mg / 6 mL (Waters Corporation)
- HPLC system: Agilent Series 1200 (Agilent Technologies)
- HPLC column: Zorbax Eclipse XDB-C8, 150 mm x 4.6 mm, 5 µ (Agilent Technologies)
- HPLC Mobile phase
  - A: Acetonitrile with 0.1% formic acid
  - B: Water with 0.1% formic acid
- Time-of-Flight mass spectrometer: Agilent 6220 MSD TOF
  - MassHunter software
- Turbovap® Concentration Workstation (Caliper Life Sciences)
- Analytical standards:
  - Aspartame, Saccharin and Sucralose (Sigma-Aldrich)
  - Stock solutions (1000 µg/mL) in 100% methanol stored at -18 °C
- Water samples were collected from various locations around the USA
  - Wastewater: Effluent locations downstream from wastewater treatment plants
  - Surface water: Rivers and reservoirs
  - Groundwater: Wells

Method

The SPE steps are summarized with the schematic provided in the GX-271 ASPEC control software, TRILUTION LH (Figure 1).

1. Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack and probe rinsed with 10% methanol.
2. Condition SPE cartridge with 4 mL of methanol at a flow rate of 1 mL/min.
3. Condition SPE cartridge with 6 mL of water at 1 mL/min.

4. Load 200 mL of water sample at a flow rate of 10 mL/min. Follow this with an air push of 25 mL to remove any excess water.
5. Move the Gilson Mobile SPE Rack over the collection tubes.
6. Elute the analytes of interest with 5 mL methanol at 1 mL/min.
7. Evaporate to 0.5 mL with nitrogen at a temperature of 45 °C in a water bath with TurboVap.

Figure 1. TRILUTION LH SPE Tasks for Extraction of Sweeteners from Water.

LC/TOF-MS Analysis

Samples (50 µL) were injected onto the LC/TOF-MS and quantified (Tables 2, 3). Accurate mass measurements of each peak from the total ion chromatograms were obtained by means of an automated calibrant delivery system using a dual-nebulizer ESI source that introduces the flow from the outlet of the chromatograph together with a low flow of a calibrating solution (calibrant solution A, Agilent Technologies), which contains the internal reference masses (purine [$C_5H_4N_4$] at m/z 121.05 and HP-921 [hexakis-(1H,1H,3H-tetrafluoro-pentoxy)phosphazene] [$C_{18}H_{18}O_6N_3P_3F_{24}$] at m/z 922.01).

Table 2. HPLC Mobile Phase Gradient (Flow rate = 0.6 mL/min).

Step No.	Time (min)	%A	%B
1	0	10	90
2	5	10	90
3	30	100	0

Table 3. Mass Spectrometer Conditions (Capillary Voltage: 4000 V, Nebulizer Pressure: 45 psig, Drying Gas: 9 L/min, Gas Temp: 300 °C, Fragmentor Voltage: 190 V, Skimmer Voltage: 60 V, Octopole RF: 250 V, Mass Spectra Recorded 50-1000 m/z at 4 GHz) & Analyte Detection Specifications.

Analyte	Polarity	m/z	RT(min)
Saccharin	+	184	7.9
Aspartame	+	295	12.9
Sucralose	+	419	12.8

Results

Figure 2. LC/TOF-MS Ion Chromatograms for Saccharin (m/z 184, ret. time = 7.9 min), Aspartame (m/z 295, ret. time = 12.9 min) and Sucralose (m/z 419, ret. time = 12.8 min). The Concentration of Each Standard is 0.5 µg/mL.

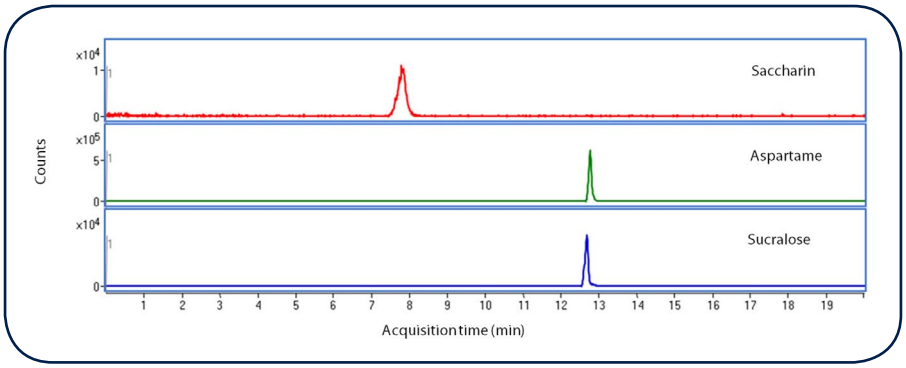


Figure 3. Mass Spectrum for Sucralose.

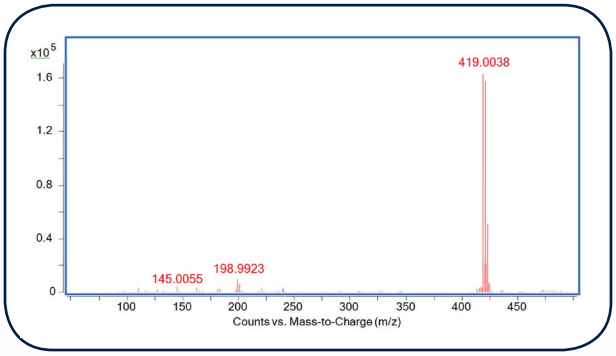


Table 4. Analytical Performance Parameters for Sweeteners in Water with SPE Followed by LC/TOF-MS.

Compound	External Calibration Range (µg/mL)	R^2	% Recovery (RSD)	Method LODs (µg/L)	Method LOQs (µg/L)
Aspartame	0.05 - 5	0.997	90 (6)	0.02	0.2
Saccharin	1 - 10	0.994	53 (8)	0.5	5
Sucralose	0.5 - 5	0.999	73 (5)	0.05	0.5

Summary

This application note describes the conditions necessary to automate the solid phase extraction of sucralose, saccharin and aspartame from water samples prior to analysis by LC/TOF-MS using the Gilson GX-271 ASPEC system. Extraction recoveries were 90% for aspartame, 53% for saccharin and 73% for sucralose (Table 4). The RSD for inter-day (n=5) values were between 5 and 8% showing good reproducibility of the methodology. The LC/TOF-MS limit of detection for sucralose was 0.05 µg/L.

Table 5. Analysis of Saccharin and Sucralose from Various Wastewater, Surface Water and Groundwater Locations in the USA. No Positive Findings for Aspartame Were Found. [n.d.=Below Level of Detection; <LOQ = Below Level of Quantification].

Samples	Saccharin (µg/L)	Sucralose (µg/L)
Wastewater Location 1 (CO)	5	1
Wastewater Location 1 (CO)	n.d.	1.8
Wastewater Location 2 (CO)	n.d.	0.8
Wastewater Location 3 (OH)	n.d.	1.5
Wastewater Location 3 (OH)	n.d.	1.8
Surface Water Location 2 (CO) (n=4)	n.d.	n.d.
Surface Water Location 4 (CO) (n=4)	n.d.	n.d.
Surface Water Location 5 (TX) (n=4)	n.d.	n.d.
Surface Water Location 6 (AL) (n=3)	n.d.	<LOQ
Surface Water Location 6 (AL)	n.d.	n.d.
Surface Water Location 7 (CO)	n.d.	0.8
Surface Water Location 7 (CO)	n.d.	1.6
Surface Water Location 7 (CO)	n.d.	1.0
Surface Water Location 7 (CO)	n.d.	1.8
Surface Water Location 8 (MN)	n.d.	<LOQ
Surface Water Location 8 (MN)	n.d.	n.d.
Ground Water Location 9 (CO)	n.d.	0.8
Ground Water Location 9 (CO)	n.d.	2.4
Ground Water Location 9 (CO)	n.d.	1.4
Ground Water Location 9 (CO)	n.d.	2.2
Ground Water Location 10 (CO)	n.d.	0.6
Ground Water Location 10 (CO)	n.d.	1.6
Ground Water Location 10 (CO)	n.d.	2.0
Ground Water Location 10 (CO)	n.d.	2.0

Sucralose was the most detected sweetener, showing up in wastewater, surface water and groundwater samples. Five wastewater samples from three different locations all showed positive detections for sucralose (Table 5). Eight surface water samples out of 22 were positive for sucralose and had no detections for the other two sweeteners. Eight alluvial groundwater samples from two locations were positive for sucralose. These are the first reports of sucralose in groundwater, which are most likely the effect of surface water being drawn into these alluvial wells during pumping.

The study data suggest that sucralose may be an excellent tracer of wastewater-contaminated surface water because of its widespread occurrence and its apparent stability in wastewater and surface water. Studies are currently underway to compare sucralose to caffeine as tracers of sewage wastewater in groundwater and drinking water supplies.

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Automated Solid Phase Extraction (SPE) of EPA Method 1694 for Pharmaceuticals and Personal Care Products in Large Volume Water Samples

This collaboration study was performed jointly by Gilson, Inc. and Curtis Hedman, Assistant Researcher with the Wisconsin State Laboratory of Hygiene.

Featured Products: Gilson Large-Volume Clean Water Solid Phase Extraction System (GX-274 ASPEC™ System Base With Special 1931-Series Accessories & Operated Using TRILUTION® LH Liquid Handling Software).

Introduction

Contamination of clean water sources is a constant concern because of its impact on our agricultural industry and natural resources; ultimately affecting both humans and animals. In the USA, the Clean Water Act (CWA) regulates surface water quality and pollutant discharges. The Environmental Protection Agency (EPA) has implemented pollution control programs and water quality standards under the CWA. EPA Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS-MS (December 2007). This was developed for use in CWA programs to test for common over-the-counter topical products, dietary supplements, human pharmaceuticals, veterinary drugs, and other consumer products and compounds labeled as Pharmaceuticals and Personal Care Products (PPCPs).

The U.S. Geological Survey in 2002 reported PPCP levels found in a variety of stream samples taken from across the USA. Using EPA Method 1694, laboratories today are monitoring PPCPs levels in a variety of clean water samples and linking these levels to any potential impact on humans and animals, even at low contamination levels.

EPA Method 1694 measures target analytes in large volume water samples by groups according to acid or basic solid phase extraction conditions and ionization mode. In this application, clean water samples of 1000 mL and 500 mL were prepared and run, comparing results of a suite of 45 target analytes (see Table 1) from manual acid solid phase extraction with results from the automated acid solid phase extraction (using the Gilson Large-Volume Clean Water Solid Phase Extraction System). ESI positive ionization mode HPLC/MS-MS analysis was used for final quantization and recovery. This application ultimately shows comparable research results for 1000 mL and 500 mL large volume water samples to address two common issues faced by many laboratories: 1) efficiency of the SPE process, and 2) data reproducibility.

Table 1. EPA Method 1694 Suite of 45 Target Acid Analytes.

Compound Name	Pharmacological Compound Description	Molecular Formula
Acetaminophen	Non-Narcotic Analgesic Antipyretic	$C_8H_9NO_2$
Ampicillin	Anti-Bacterial Agent	$C_{16}H_{19}N_3O_4S$
Atrazine	Herbicide	$C_8H_{14}ClN_5$
Azithromycin	Anti-Bacterial Agent	$C_{38}H_{72}N_2O_{12}$
Caffeine	Central Nervous System Stimulant Phosphodiesterase Inhibitor Purinergic Pt Receptor Antagonist	$C_8H_{10}N_4O_2$
Carbadox	Anti-Infective Agent Carcinogen Mutagen	$C_{11}H_{10}N_4O_4$
Carbamazepine	Non-Narcotic Analgesic Anticonvulsant Antimanic Agent	$C_{15}H_{12}N_2O$
Cefotaxime	Anti-Bacterial Agent	$C_{16}H_{18}FN_3O_3$
Clarithromycin		
Cloxacillin		
Codeine	Opoid Analgesic Antitussive Agent Narcotic	$C_{18}H_{21}NO_3$
Cotinine	Indicator and Reagent	$C_{10}H_{12}N_2O$
Digoxigenin	Metabolite of Digoxin, a Cardiotonic Drug	$C_{23}H_{34}O_5$
Diltiazem	Antihypertensive Agent Calcium Channel Blocker Cardiovascular Agent Vasodilator Agent	$C_{22}H_{27}ClN_2O_4S$
Paraxanthine	Central Nervous System Stimulant	$C_7H_8N_4O_2$
Diphenhydramine	Local Anesthetic Anti-Allergic Agent Antiemetic Histamine H1 Antagonist Hypnotic and Sedative	$C_{17}H_{21}NO$
Enrofloxacin	Antineoplastic Agent	$C_{19}H_{22}FN_3O_3$
Erythromycin	Anti-Bacterial Agent Gastrointestinal Agent Protein Synthesis Inhibitor	$C_{19}H_{67}NO_{13}$
Flumequine	Urinary Anti-Infective Agent	$C_{14}H_{12}FNO_3$
Fluoxetine	Second Generation Antidepressive Agent Serotonin Uptake Inhibitor	$C_{17}H_{18}F_3NO$
Lincomycin	Anti-Infective Agent Pretein Synthesis Inhibitor	$C_{18}H_{34}N_2O_6S$
Lomefloxacin	Anti-Infective Agent	$C_{17}H_{19}F_2N_3O_3$
Miconazole	14-alpha Demethylase Inhibitor Antifungal Agent	$C_{18}H_{14}Cl_4N_2O$
Norfloxacin	Anti-Bacterial Agent Enzyme Inhibitor Nucleic Acid Synthesis Inhibitor	$C_{16}H_{18}FN_3O_3$
Ofloxacin	Anti-Bacterial Agent Urinary Anti-Infective Agent Nucleic Acid Synthesis Inhibitor	$C_{18}H_{20}FN_3O_4$
Oxacillin	Anti-Bacterial Agent	$C_{19}H_{19}N_3O_5S$
Oxolinic acid	Urinary Anti-Infective Agent Nucleic Acid Synthesis Inhibitor	$C_{13}H_{11}NO_5$
Penicillin G	Anti-Bacterial Agent	$C_{16}H_{18}N_2O_4S$

Table 1, continued. EPA Method 1694 Suite of 45 Target Acid Analytes.

Compound Name	Pharmacological Compound Description	Molecular Formula
Penicillin V	Anti-Bacterial Agent	$C_{16}H_{18}N_2O_5S$
Roxithromycin	Anti-Bacterial Agent	$C_{41}H_{76}N_2O_{15}$
Sarafloxacin	Anti-Bacterial Agent - Veterinary Fluroinated Qunolone Anti-Bacterial	$C_{20}H_{17}F_2N_3O_3$
Sulfachloropyridazine	Urinary Anti-Infective Agent	$C_{10}H_9ClN_4O_2S$
Sulfadiazine	Anti-Infective Agent Antiprotozoal Agent Coccidiostats	$C_{10}H_{10}N_4O_2S$
Sulfadimethoxine	Anti-Infective Agent	$C_{12}H_{14}N_4O_4S$
Sulfamerazine	Anti-Bacterial Agent	$C_{11}H_{12}N_4O_2S$
Sulfamethazine	Anti-Infective Agent	$C_{12}H_{14}N_4O_2S$
Sulfamethizole	Anti-Infective Agent	$C_9H_{10}N_4O_2S_2$
Sulfamethoxazole	Anti-Infective Agent	$C_{10}H_{11}N_3O_3S$
Sulfanilamide	Anti-Bacterial Agent	$C_6H_8N_2O_2S$
Sulfathiazole	Anti-Infective Agent	$C_9H_9N_3O_2S_2$
Thiabendazole	Anthelmintics	$C_{10}H_7N_3S$
Trimethoprim	Urinary Anti-Infective Agent Antimalarial Folic Acid Antagonist	$C_{14}H_{18}N_4O_3$
Tylosin	Anti-Bacterial Agent	$C_{46}H_{77}NO_{17}$
Virginiamycin	Anti-Micobial Agent	$C_{28}H_{35}N_3O_7$

Experimental Conditions

Materials

Note: All solvents were distilled in glass suitable for GC, HPLC, pesticide residues analysis and spectrophotometry.

- Automated Solid Phase Extraction:
  - Gilson Large-Volume Clean Water Solid Phase Extraction System
    - GX-274 ASPEC System Base With Special 1931-Series Accessories & Operated Using TRILUTION LH v3.0 Liquid Handling Software
- Manual Solid Phase Extraction:
  - Vacuum manifold and vacuum pump
- SPE Cartridges: Waters Oasis™ HLB cartridges, 500 mg/6 mL using Gilson 6 mL Sealing Caps
- LC/MS-MS System:
  - Agilent 1100 HPLC System (Santa Clara, CA), consisting of an autosampler, binary pump, degasser, and column compartment.
  - Mass Spectrometer = AB/SCIEX API 4000 Triple Quadrupole Mass Spectrometer (Foster City, CA)
- HPLC Column: Phenomenex Synergi™ 4u MAX-RP, 4.6 x 250 mm
- Standards:
  - All standards, calibration solutions, matrix spiking solution and internal standards were prepared in accordance with the EPA Method 1694.
- Water Samples:
  - Water samples were taken from the Wisconsin State Laboratory of Hygiene (WSLH) water supply for all manual and automated tests.
  - System water blanks were run on the Gilson Large-Volume Clean Water Solid Phase Extraction System and analysis performed prior to running samples to determine if there was any compound bias present.

- All water samples used for this application were prepared by the WSLH in accordance with the EPA Method 1694 for aqueous sample acid extraction.

Automated Solid Phase Extraction Steps

The fractionation protocol is entirely automated using the Gilson Large-Volume Clean Water Solid Phase Extraction System. The SPE steps are summarized for the 1000 mL and 500 mL clean water samples, with the schematic provided using TRILUTION LH Software (Figure 1).

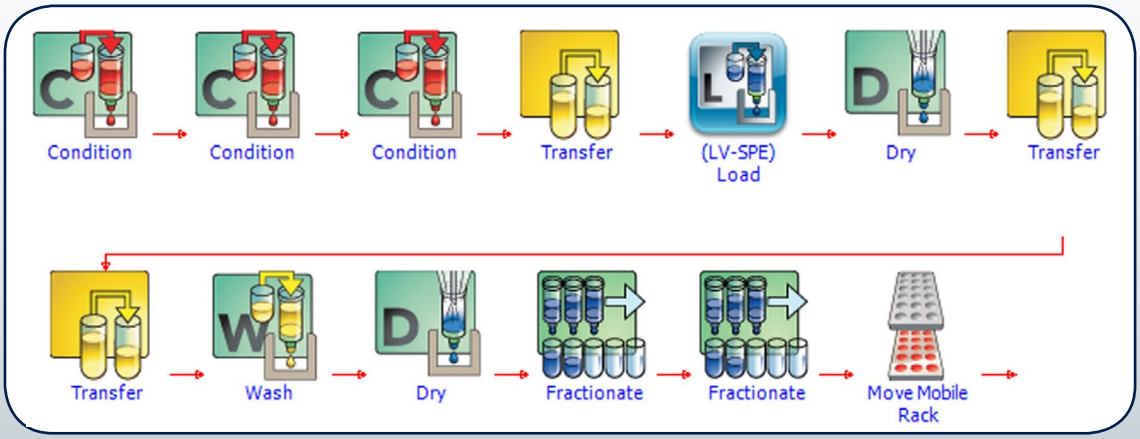
Automated Solid Phase Extraction Step*

1. Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack.
2. Prime solvent lines from VALVEMATE® II units.
3. Condition one 6 mL cartridge per sample with 20 mL methanol followed by an air push.
4. Condition the cartridge with 6 mL 18 Mohm/cm water followed by an air push.
5. Condition the cartridge with 6 mL of 18 Mohm/cm water @ pH 2 followed by an air push.
6. Prime Sample lines with sample (30mL).
7. Load 500 mL of sample to the cartridge at a dispense flow rate of 8 mL/min using a 0.7 minute air push.
8. Wash lines with Methanol (30mL).
9. Prime lines with Water (30mL).
10. Wash the cartridge with 10 mL 18 Mohm water.
11. Dry the cartridge for 5 minutes using an air purge.
12. Move the Gilson Mobile SPE Rack over the collection tubes.
13. Elute SPE cartridge into collect row 1 with 12 mL methanol at 3 mL/min followed by a 0.5 min air push.
14. Elute SPE cartridge into collect row 2 with 6 mL (1:1) acetone:methanol at 3 mL/min followed by a 0.5 min air push.
15. Move Mobile Rack.

Note: Offline concentration of the two fractions was performed in accordance with EPA Method 1694.

*Note: 500 mL samples were run in sequential mode according to the Automated Solid Phase Extraction Step method (Figure 1), using one 6 mL SPE cartridge per sample. Samples of 1000 mL were run in batch mode according to the same method, using two 6 mL SPE cartridges per sample.

Figure 1. TRILUTION LH SPE Tasks for Fractionation of Large Volume Water Samples.



HPLC/MS-MS Analysis

System performance and calibration was verified each working day. A mid-level calibration standard was run

after every 8 samples, along with duplicate methanol blanks before and after standard injections. An eight-point calibration curve was run at the beginning and end of each day.

Results

In this application, clean water samples of 1000 mL and 500 mL were prepared and run, comparing results of a suite of 45 target analytes (Figure 2 and Table 2) from manual acid solid phase extraction with results from the automated acid solid phase extraction (using the Gilson Large-Volume Clean Water Solid Phase Extraction System). As a summary of the full application, a smaller group selection of four target analytes, Erythromycin, Caffeine, Carbmazepine, and Fluoxetine, were randomly chosen to show that comparable research results.

Figure 2. Example 500 ng/mL Standard Chromatogram Showing 45 Target Analytes.

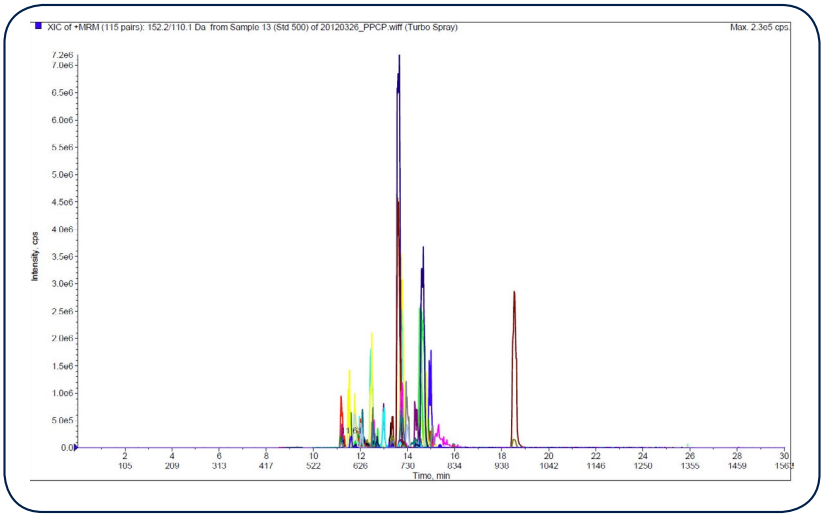


Table 2. HPLC/MS-MS Analysis Retention Times of 45 Target Analytes.

Target Analyte Name	Retention Time (Minutes)
Acetaminophen	11.6
Ampicillin	11.6
Atrazine-labeled	16.0
Azithromycin	12.4
Caffeine	12.3
Carbadox	12.5
Carbamazepine	14.9
Cefotaxime	12.1
Ciprofloxacin	12.1
Clarithromyclin	14.5
Cloxacillin	15.1
Codeine	11.3
Cotinine	11.2
Digoxigenin	13.4
Diltiazem	13.7
Paraxanthine	11.6
Diphenhydramine	13.6

Target Analyte Name	Retention Time (Minutes)
Enrofloxacin	12.3
Erythromycin	13.9
Flumequine	15.2
Fluoxetine	14.3
Lincomycin	11.5
Lomefloxacin	12.1
Miconazole	18.5
Norfloxacin	11.9
Ofloxacin	11.9
Oxacillin	15.1
Oxolinicacid	14.3
Penicillin G	14.5
Penicillin V	14.8
Roxithromycin	14.6
Sarafloxacin	12.5
Sulfachloropyridazine	13.3
Sulfadiazine	12.0

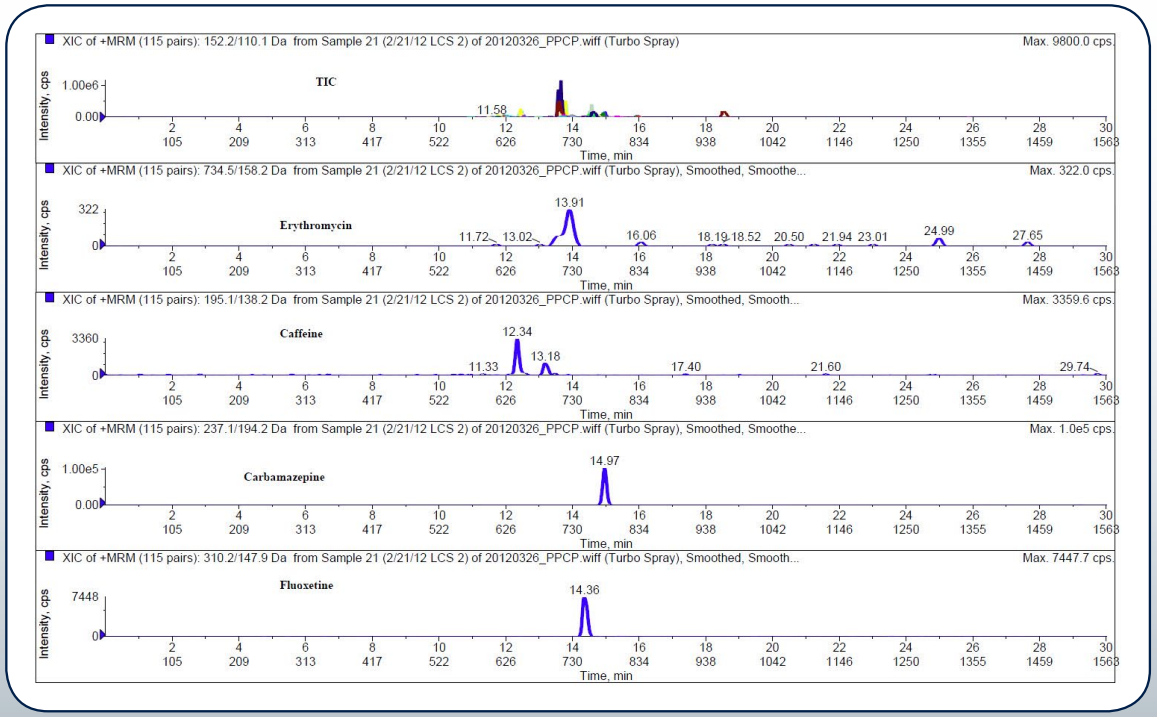
Target Analyte Name	Retention Time (Minutes)
Sulfadimethoxine	14.0
Sulfamerazine	12.6
Sulfamethazine	13.0
Sulfamethizole	12.7
Sulfamethoxazole	13.4
Sulfanilamide	9.4
Sulfathiazole	12.0
Thiabendazole	13.8
Trimethoprim	11.8
Tylosin	13.8
Virginiamycin	15.4

Recoveries of the four target analytes for the 1000 mL manual SPE samples ranged from 90.7% - 125.6%, with all recovery values within the expected range (Table 3 and Figure 3). For the automated Gilson SPE samples, the recovery range of the same four target analytes for the 1000 mL samples was 91.0% - 105.1%, with all recovery values within the expected recovery ranged listed.

Table 3. Manual vs. Automated SPE Recovery Results for 1000 mL Samples for Four Target Analytes.

	Erythromycin	Caffeine	Carbamazepine	Fluoxetine
Manual WSLH SPE - 1000 mL Samples Mean (n=4) (theoretical value=125ng/mL)	157.0	116.5	113.3	125.1
Manual WSLH SPE - 1000 mL Samples % Recovery (n=4)	125.6	93.2	90.7	100.1
Manual WSLH SPE - 1000 mL Samples %RSD (n=4)	37.3	16.9	20.5	14.9
Automated Gilson SPE 1000 mL Mean (n=4) (theo.=125ng/mL)	131.4	128	113.7	131.2
Automated Gilson SPE 1000 mL % Recovery (n=4)	105.1	102.4	91.0	105.0
Automated Gilson SPE 1000 mL % RSD (n=4)	35.4	33.4	4.8	13.1

Figure 3. Manual SPE HPLC/MS-MS Analysis Results for 1000 mL Samples for Four Target Analytes.



Recoveries of the four target analytes for the 500 mL manual SPE samples ranged from 101.3% to 171.6%, with Erythromycin and Floxetine showing higher results than the expected recovery range listed in the EPA Method 1694 (Table 4 and Figure 4). For the automated Gilson SPE samples, the recovery range of the same four target analytes for the 500 mL samples was 95.2% to 114.5%, with all recovery values within the expected recovery ranged listed.

Table 4. Manual vs. Automated SPE Recovery Results for 500 mL Samples for Four Target Analytes.

	Erythromycin	Caffeine	Carbamazepine	Fluoxetine
Manual WSLH SPE - 500 mL Samples Mean (n=4) (theoretical value=62.5ng/mL)	107	63.3	69.9	84.3
Manual WSLH SPE - 500 mL Samples % Recovery (n=4)	171.6	101.3	111.8	134.8
Manual WSLH SPE - 500 mL Samples %RSD (n=4)	52	26.7	17.8	52.8
Automated Gilson SPE 500 mL Mean (n=4) (theo.=62.5ng/mL)	59.5	71.5	67.2	71.6
Automated Gilson SPE 500 mL % Recovery (n=4)	95.2	114.5	107.5	114.5
Automated Gilson SPE 500 mL % RSD (n=4)	24.0	21.0	14.4	43.5

Automation of EPA Method 1694 included researching any carryover from the 45 target analytes. 1000 mL and 500 mL blank water samples were run through the Gilson Large-Volume Clean Water Solid Phase Extraction System. Mean sample values showed either no peaks detected or less than detectable reporting limits for all 45 target analytes.

Summary

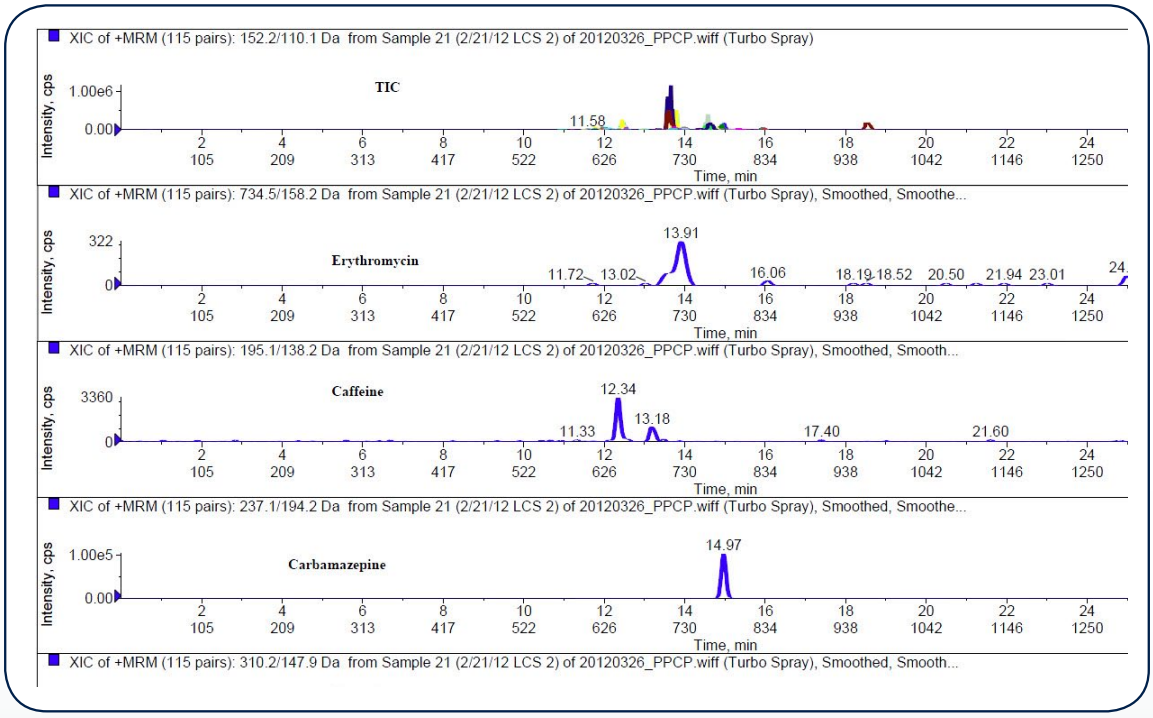
Efficiency with the SPE process for large volume water samples is addressed by comparing manual SPE to automated SPE, as well as comparing data that reduces the overall sample load volume and load time in half from 1000 mL to 500 mL. Recovery value for all 1000 mL automated SPE samples were within the expected EPA Method 1694 range, where the 500 mL manual SPE samples showed Erythromycin and Fluoxetine had reported values higher than the expected range. Comparability of mean recovery values between 1000 mL SPE samples and 500 mL SPE samples varies by less than 17% for the automated SPE samples, where the manual SPE samples vary by nearly three times from what the automated SPE samples reported, or up to 46% for the four target analytes when the sample volumes are compared.

Data reproducibility is a consideration when running samples. A robust method that eliminates environmental variables, technician variables, etc. can reduce the potential number of sample repeats performed. Comparing %RSD values from manual SPE samples and automated SPE samples provides a statistical representation of reproducibility. In all but one compound, % RSD values were lower for automated SPE samples vs. manual SPE samples. Caffeine reported from 1000 mL automated SPE samples showed nearly double the %RSD of the manual SPE samples reported for the same compound. Significant %RSD changes are visible with a five-fold reduction of

Carbamazepine with the 1000 mL SPE samples and the two-fold reduction of Erythromycin with the 500 mL SPE samples.

This application provides good insight into the simplicity of automating a manually intensive SPE process to provide efficiency in recovery and added efficiency with reduction of sample load volume with no negative impact on recoveries. Using the Gilson Large-Volume Clean Water Solid Phase Extraction System, carryover was tested, but not detected or seen for the 45 target analytes. With the exception of one analyte, the overall %RSD values show higher consistency with data generated from using automated SPE versus manual SPE. Research through this application has shown that altering the sample load volume from 1000 mL to 500 mL has no impact on detection of the target 45 analytes. Reducing the sample load volume speeds up the load time, allowing for higher daily throughput of samples by a typical laboratory.

Figure 4. Automated SPE HPLC/MS-MS Analysis Results for 500 mL Samples for Four Target Analytes.



References

1. Environmental Protection Agency (2007). EPA Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS-MS.

2. The Groundwater Foundation: http://www.groundwater.org/gi/.

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Automated Gel Permeation Chromatography (GPC) Clean-up of Soil Extracts Prior to Analysis for Semivolatile Organic Compounds by GC/MS (USEPA Method 8270)

This study was performed in collaboration with CT Laboratories of Baraboo, WI, USA.

Featured Products: Gilson GX-271 ASPEC™ System with 406 Single Syringe Pump Operated Using TRILUTION® LH Liquid Handling Software.

Introduction

Gel permeation chromatography (GPC) is a size-exclusion clean-up procedure that readily separates high molecular weight interferents from sample extracts. The procedure uses organic solvents and a porous hydrophobic gel (primarily a crosslinked divinylbenzene-styrene copolymer) that readily separates large molecular weight molecules from the smaller molecular weight analytes of interest. GPC cleanup is recommended for the elimination of lipids, polymers, copolymers, proteins, natural resins, cellular components and other high molecular weight compounds from a sample extract.

GPC clean-up is used extensively for numerous environmental analysis, especially for preparing sample extracts prior to semivolatile, pesticide, PCB, polyaromatic hydrocarbons (PAHs), dioxins and dibenzofuran analysis by gas chromatography (GC) or GC/MS. GPC clean-up is efficient at removing high boiling point materials that can condense in the injection port area of a GC or in the front of a GC column. GPC cleanup protects GC columns, improves accuracy, reduces GC maintenance costs and allows for lower detection limits. Laboratories that participate in the USEPA Contract Laboratory Program (CLP) or are following CLP protocols perform GPC clean-up according to USEPA Method 3640A.

This application note evaluates the Gilson Automated GX-271 GPC Clean-up System to perform the post-extraction clean-up of soil extracts prior to analysis for semivolatile organic compounds. Semivolatile organic compounds or SVOCs is a general term for solvent extractable organic compounds that can be determined by GC/MS. It includes chemical classes such as polyaromatic hydrocarbons (PAHs), phthalates, nitrosamines, cyclic ketones and nitroaromatics. Semivolatiles were extracted from soil using pressurized fluid extraction (also called accelerated solvent extraction or ASE) via USEPA Method 3545. The extracts were then purified using GPC clean-up. Analysis was performed by GC/MS. Recoveries for a variety of semivolatiles were determined.

Experimental Conditions

Materials

Note: All reagents were pesticide grade or higher. All chemicals were ACS grade quality.

- GX-271 GPC Clean-up System
  - TRILUTION LC software
  - Phenomenex (Torrance, CA) EnviroSep-ABC™ GPC Sample Clean-up column with guard column
    - The GPC clean-up column was calibrated using the method outlined in USEPA Method 3640A. The column flow rate was verified by collected the eluate in a graduated cylinder for 10 minutes and measuring the volume. The elution times for the corn oil, bis (2-ethylhexyl) phthalate, methoxychlor, perylene and sulfur were determined (See Figure 1).
  - 5 mL sample loop
  - Mobile Phase:
    - Flow rate: 5 mL/min
    - 100% Dichloromethane
- GPC Calibration standards:
  - Commercial GPC calibration mix standards were obtained from Restek Corporation (Bellefonte, PA) Semivolatile stock standards and surrogate standards were obtained from Restek Corporation (Bellefonte, PA) or Perkin Elmer (Waltham, MA)
  - GPC calibration standards were prepared according to USEPA Method 3640A and contained corn oil, bis (2-ethylhexyl) phthalate, methoxychlor, perylene and sulfur
  - GPC calibration standard dilutions were prepared in dichloromethane or methanol
- GC/MS Analysis:
  - Column: J&W Scientific DB 5.625 , 30mm x 0.25mm x 0.25um
  - Semivolatile organic compounds were analyzed by GC/MS using an Agilent 6890 GC with 7683 Autosampler and 5973 MSD
  - The carrier gas was Helium at a flow rate of 1.2 mL/minute
  - Injection volume: 0.5 µL of sample
  - Mode: Pulsed splitless mode with an injector temperature of 250 degrees C
  - The MS conditions were as follows:
    - MS Interface = 280 degrees C
    - MS Source = 230 degrees C
    - Mass Range = 35 – 500 amu
    - Scan Time = .317 sec/scan

Soil Extraction Method

Soil extraction was carried out utilizing pressurized solvent or accelerated solvent extraction (ASE) per USEPA Method 3545. Ten grams of soil was placed in a beaker and 2.5 mL of diatomaceous earth were added to the sample and then transferred to an extraction cell. Appropriate matrix spikes and matrix spike duplicate was added to each sample batch.

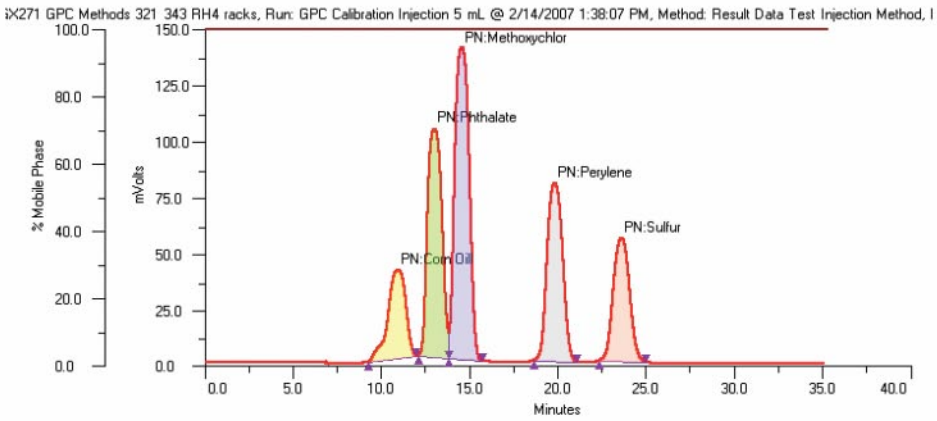
The samples were then extracted using a Dionex ASE 200 with the following conditions:

- Oven temperature = 100 degrees C; Pressure = 1500psi
- Static Time = 5 minutes; Heat = 5 minutes
- Flush Volume = 60%; Solvent A = 100%
- Nitrogen purge = 60 seconds at 150psi
- Extraction fluid = 1:1 dichloromethane:acetone

The sample extracts were then concentrated using Kuderna-Danish (K-D) apparatus and then reconstituted in dichloromethane prior to using the Gilson GX-271 GPC Clean-up System.

Information from the calibration standard allowed for the determination of appropriate collection times for the analytes of interest (See Figure 2). Column eluate collection was initiated just before the elution of bis (2-ethylhexyl) phthalate and after elution of the corn oil. Fraction collection was stopped after perylene elution but before sulfur elution. The collected fraction was then concentrated by K-D and reconstituted in appropriate solvent for GC/MS analysis.

Figure 1. TRILUTION LC Chromatogram of USEPA Method 3640A Calibration Standard Using a Phenomenex EnviroSep-ABC GPC Sample Clean-up Column.



Sample Table

Injection Number	Sample Name	Sample Location	Fraction Site(s)	Peak Name	Retention Time (min)	Area (uVmin/100)	Height (mV)	Front Resolution %	Back Resolution %
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Corn Oill	10.9771	4510346.6667	39.0269	0	93.54
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Phthalate	13.0603	9494656.2716	101.6768	93.54	85.61
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Methoxychlor	14.6066	13177787.6885	139.1887	85.61	245.64
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Perylene	19.8885	7983740.0291	79.4326	245.64	150.05
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Sulfur	23.6771	5779671.3132	54.9145	150.05	0

Figure 2. TRILUTION LC GPC Sample List Showing Methods Run and Fraction Collection Variables for Each Sample.

Sample Table

Injection Number	Sample Name	Sample Location	Fraction Site(s)	Peak Name	Retention Time (min)	Area (uVmin/100)	Height (mV)	Front Resolution %	Back Resolution %
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Corn Oill	10.9771	4510346.6667	39.0269	0	93.54
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Phthalate	13.0603	9494656.2716	101.6768	93.54	85.61
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Methoxychlor	14.6066	13177787.6885	139.1887	85.61	245.64
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Perylene	19.8885	7983740.0291	79.4326	245.64	150.05
1	Sample	Sample Zone,73,Reservoir,-1	<NONE>	Sulfur	23.6771	5779671.3132	54.9145	150.05	0

Results

Table 1. Recoveries of Semivolatiles From Soil Samples Run on Two Separate Days (n=7) Using Pressurized Fluid Extraction and GPC Clean-up.

Compound	Recovery (%) 0.1 mg/kg	Acceptable Soil Recovery Limits (%)
N-Nitrosodimethylamine	47.2	20 - 115
Pyridine	4.7	1 - 114
Aniline	21.2	1 - 81
Bis (2-chloroethyl)ether	68.6	40 - 105
Phenol	73.2	40 - 100
2-Chlorophenol	70.2	45 - 105
1,3 - Dichlorobenzene	65.5	40 - 100
1,4 - Dichlorobenzene	66.3	35 - 105
1,2 - Dichlorobenzene	64.1	45 - 95
Benzyl alcohol	54.0	20 - 125
Bis (2-chloroisopropyl)ether	72.0	20 - 115
2-Methylphenol	72.4	40 - 105
N-Nitrosopyrrolidine	85.2	52 - 119
Acetophenone	82.9	43 - 127
Hexachloroethene	60.1	35 - 110
N-Nitroso-di-n-propylamine	66.5	40 - 115
3 & 4 - Methylphenol	71.0	40 - 105
Nitrobenzene	68.4	40 - 115
Isophorone	70.8	45 - 110
2 - Nitrophenol	67.6	40 - 110
2,4 - Dimethylphenol	53.4	30 - 105
Bis (2-chlorethoxy)methane	69.5	45 - 110
2,4 - Dichlorophenol	71.1	45 - 110
1,2,4 - Trichlorobenzene	63.3	45 - 110
Benzoic acid	14.4	0 - 110
Napthalene	65.0	40 - 105
4 - Chloroaniline	31.5	10 - 95
2, 6 - Dichlorophenol	72.1	40 - 131
Hexachloropropene	66.1	1 - 158
Hexachlorobutadiene	65.4	40 - 115
4- Chloro 3- methylphenol	67.7	45 - 115
2 - Methylnapthalene	74.0	45 - 105
1 - Methylnapthalene	73.0	70 - 130
Hexachlorocyclopentadiene	78.6	1 - 141
1,2,3,4,5 - Tetrachlorobenzene	66.3	51 - 117
2,4,6 - Trichlorophenol	63.9	45 - 110
2,4,5 - Trichlorophenol	75.4	50 - 110
2 - Chloronapthalene	70.9	45 - 105
2 - Nitroaniline	73.6	45 - 120
Acenapthylene	69.8	45 - 105
Dimethylphtalate	72.4	50 - 110
2,6 - Dinitrotoluene	73.2	50 - 110

Table 1, continued. Recoveries of Semivolatiles From Soil Samples Run on Two Separate Days (n=7) Using Pressurized Fluid Extraction and GPC Clean-up.

Compound	Recovery (%) 0.1 mg/kg	Acceptable Soil Recovery Limits (%)
Acenapthene	63.6	45 - 115
3 - Nitroaniline	47.5	25 - 110
2, 4 - Dinitrophenol	50.4	15 - 130
Dibenzofuran	64.2	50 - 105
2, 4 - Dinitrotoluene	76.4	50 - 115
4 - Nitrophenol	70.9	15 - 140
2,3,4,6 - Tetrachlorophenol	94.2	70 - 130
Fluorene	65.3	40 - 115
4 - Chlorophenyl-phenyl ether	65.9	45 - 110
Diethyl phthalate	92.9	59 - 119
4-Nitroaniline	57.3	35 - 115
4,6 - Dinitro-2-methylphenol	70.2	30 - 135
N-Nitrosodiphenylamine & diphenylamine	65.2	50 - 115
Azobenzene & 1,2 - Diphenyl hydrazine	66.4	62 - 104
4 - Bromophenyl - phenyl ether	64.6	45 - 115
Hexachlorobenzene (HCB)	64.9	45 - 120
Pentachlorophenol	69.5	25 - 120
Phenanthrene	69.0	50 - 110
Anthracene	66.7	55 - 105
Carbazole	72.3	45 - 115
Di - n - butylphthalate	100.4	55 - 130
Fluoranthene	69.4	55 - 115
Benzidine	8.6	6 - 12
Pyrene	69.1	45 - 125
Butyl benzyl phthalate	80.8	50 - 125
3,3' - Dichlorobenzidine	74.6	10 - 130
Benzo (a) anthracene	72.5	50 - 110
Chrysene	69.0	55 - 110
Bis (2-ethylhexyl)phthalate	92.3	45 - 125
Di -n- octylphthalate	79.8	40 - 130
Benzo (b) fluoranthene	73.4	45 - 125
Benzo (k) fluoranthene	73.4	45 - 125
Benzo(a) pyrene	69.4	50 - 110
Indeno (1,2,3-cd) pyrene	59.4	25 - 135
Dibenzo (a,h) anthracene	59.6	40 - 125
Benzo (g,h,i) perylene	57.8	40 - 125

Figure 3. Soil Sample Chromatogram (GC/MSD) Performed Without GPC Clean-up and Spiked With the Semivolatile Organic Compounds Listed in Table 1. Note the Fronting and Tailing of Peaks Between 8 and 12 Minutes.

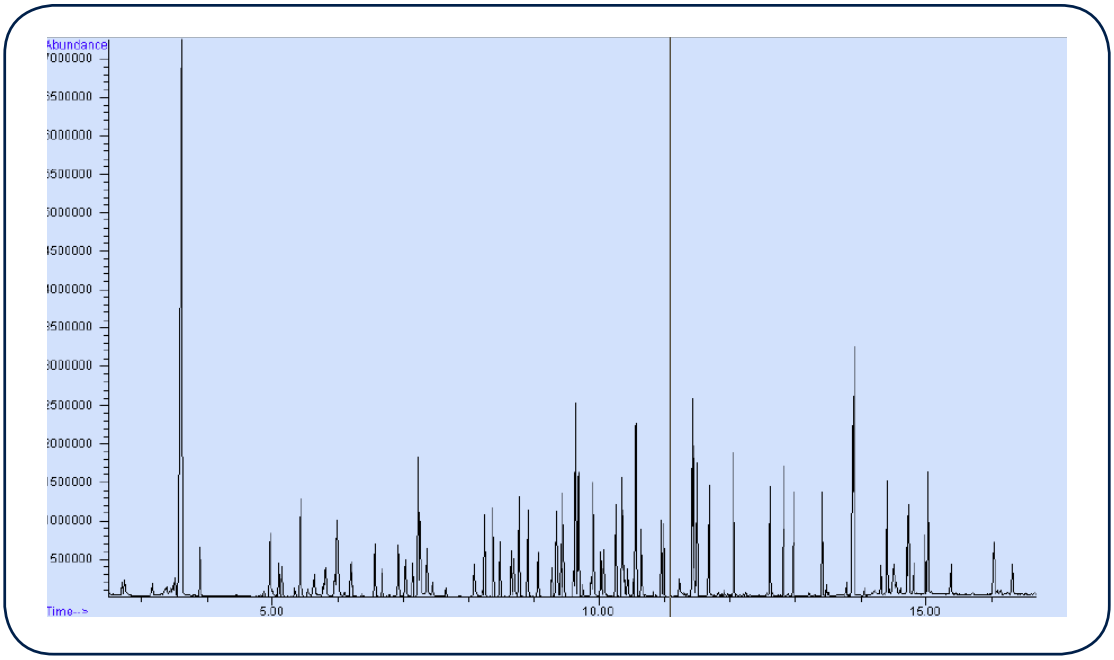
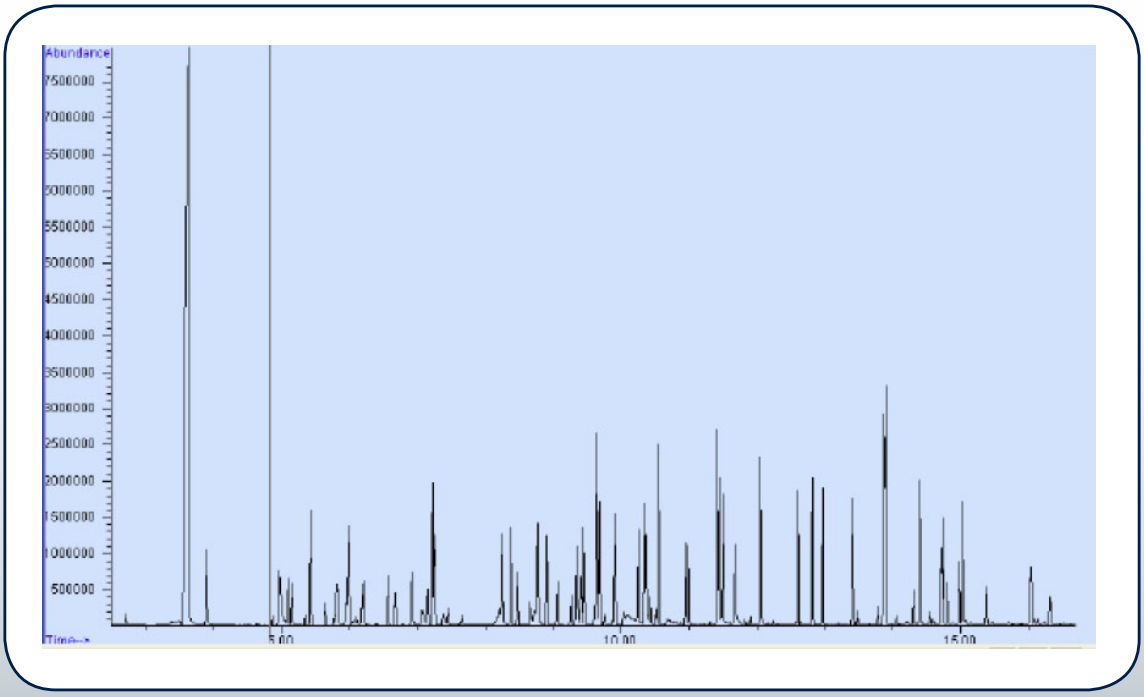


Figure 4. Soil Sample Chromatogram (GC/MSD) Performed With GPC Clean-up and Spiked With the Semivolatile Organic Compounds Listed in Table 1. Note the Improved Resolution of Peaks Between 8 to 12 Minutes Compared to the Chromatogram Without GPC Clean-up (Figure 3).



Summary

The use of GPC post-extraction clean-up improved peak resolution and reduced the presence of fronting and tailing for the analysis of semivolatiles from soils. Recovery data was adequate for semivolatile analysis and all compounds were within acceptable recovery limits. The use of GPC clean-up also reduced maintenance costs for the GC/MS systems since less contaminants were able to accumulate in the injection port area or on the front end of the column.

References

1. USEPA Method 3640A. Gel-Permeation Cleanup. Revision 1, September 1994. Available at http://www.epa.gov/epawaste/hazard/testmethods/sw846/pdfs/3640a.pdf.

2. USEPA Method 8270D. Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS). Revision 4, February 2007. Available at http://www.epa.gov/epawaste/hazard/testmethods/sw846/pdfs/8270d.pdf.

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Analysis of Lamotrigine and its Glucuronide Metabolite in Water by Liquid Chromatography/ Quadrupole Time-of-Flight Mass Spectrometry (LC/Q-TOF-MS) After Automated Solid Phase Extraction

This study was performed by Imma Ferrer, Ph.D. and E. Michael Thurman, Ph.D. at the Center for Environmental Mass Spectrometry, Dept. of Civil, Environmental & Architectural Engineering, University of Colorado, Boulder, CO, USA.

Featured Products: Gilson GX-271 ASPEC™ System with 406 Single Syringe Pump Operated Using TRILUTION® LH Liquid Handling Software.

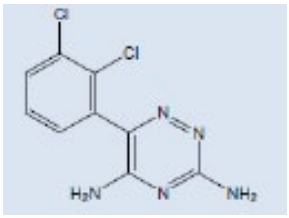
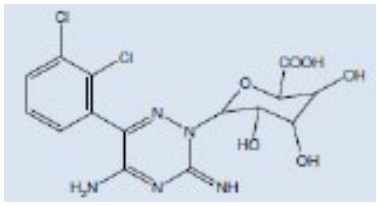
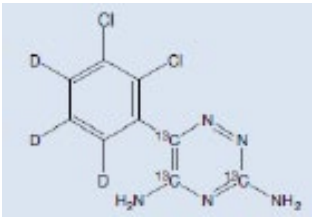
Introduction

Large quantities of pharmaceuticals are consumed each year throughout the world. A variety of pharmaceuticals has been detected in low concentrations in surface water, groundwater, drinking water and soil/sediments. Pharmaceutically active compounds, including drugs and their metabolites, are an important water-quality issue (Kolpin et al., 2002; Donn, J., 2009). There is an increased interest in measuring levels of pharmaceuticals in water due to their possible impact on humans, wildlife and fish (Schultz and Furlong, 2008: Vadja et al., 2008).

Lamotrigine, also known as Lamictal® (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine), is a widely prescribed and effective drug for the treatment of epilepsy and as a mood stabilizer for the treatment of bipolar disorder. Lamotrigine is primarily metabolized by the liver to form a glucuronide conjugate (Table 1). This metabolite is primarily excreted by the kidneys. It is less toxic than the parent compound, but can undergo hydrolysis back to the parent.

This study (Ferrer and Thurman, 2010) describes the analysis of lamotrigine and its 2-N-glucuronide metabolite in water using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC/Q-TOF-MS), as well as the automated extraction of these compounds from drinking water, groundwater, surface water and wastewater utilizing the Gilson GX-271 ASPEC System.

Table 1. Elemental Composition, Protonated Molecules, and Chemical Structures of Lamotrigine, its 2-N-Glucuronide and Lamotrigine Labeled Standard.

Name	Elemental Composition	Base Peak Ion	Chemical Structure
Lamotrigine	$C_9H_7Cl_2N_5$	$[M+H]^+$ $C_9H_8Cl_2N_5^+$ 256.0151	
N2-Glucuronide of Lamotrigine	$C_{15}H_{15}Cl_2N_5O_6$	$[M+H]^+$ $C_{15}H_{16}Cl_2N_5O_6^+$ 432.0472	
Lamotrigine-$^{13}C_3$-d_3	$C_6{}^{13}C_3H_4D_3Cl_2N_5$	$[M+H]^+$ $C_6{}^{13}C_3H_5D_3Cl_2N_5^+$ 262.0440	

Experimental Conditions

Materials

Note: All solvents used were HPLC grade or higher. All reagents were ACS grade or better. GX-271 ASPEC with single 406 Syringe Pump

- TRILUTION LH software

- SPE Cartridges: Oasis™ HLB 500 mg / 6 mL (Waters Corporation - Milford, MA, USA). The cartridges were sealed using Gilson 6 mL Sealing Caps.
- HPLC system: Agilent Series 1200 (Agilent Technologies)
- HPLC column: Zorbax Eclipse XDB-C8, 150 mm x 4.6 mm, 5 µm(Agilent Technologies)
- HPLC Mobile phase
  - A: Acetonitrile with 0.1% formic acid
  - B: Water with 0.1% formic acid
- Time-of-Flight mass spectrometer: Agilent 6220 MSD TOF
  - MassHunter software
- Analytical standards:
  - Lamotrigine and its 2-N-glucuronide were purchased from Sigma Aldrich (St. Louis, MO, USA) and from Carbosynth (Compton, UK) Deuterated standards were obtained from Cambridge Isotopes (Cambridge, MA, USA)
  - Lamotrigine-13C3-d3 labeled standard was purchased from Toronto Research Chemicals (North York, ON, Canada)
  - Individual stock solutions were prepared in pure methanol and stored at -18°C.
  - Working standards were prepared from stock solutions by dilution with acetonitrile and water.

Table 2. HPLC Mobile Phase Gradient (Flow rate = 0.6mL/min).

Step No.	Time (min)	%A	%B
1	0	10	90
2	5	10	90
3	30	100	0

Water Sample Collection

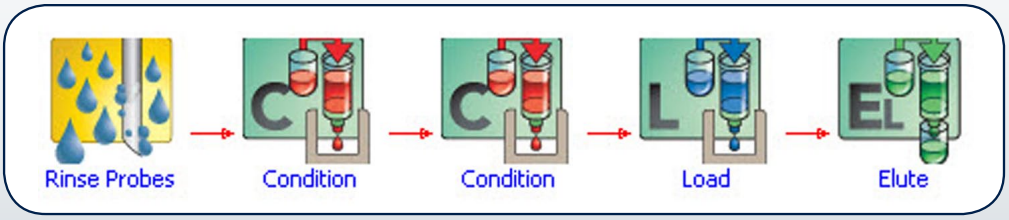
- Samples collection: baked glass 1 L amber bottles with Teflon®-lined cap
  - All water samples were stored at 4°C before analysis
  - Sample extraction was completed within seven days for all samples
- Samples:
  - Wastewater samples were collected at the out fall of a wastewater treatment plant
  - Source water river samples were collected according to U.S. Geological Survey (USGS) protocol (USGS, 2008)
  - Groundwater samples were collected from wells

Method

The SPE steps are summarized with the schematic provided in the GX-271 ASPEC control software, TRILUTION LH (Figure 2).

1. Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack.
2. Condition the cartridge with 4 mL of methanol at 1 mL/min.
3. Condition the cartridge with 6 mL HPLC grade water at 1 mL/min.
4. Load 200 mL of the sample onto the SPE cartridge at 10 mL/min.
5. Move the Gilson Mobile SPE Rack over the collection tubes.
6. Elute the analytes with 5 mL methanol at 1 mL/min.
7. Evaporate to 0.5 with nitrogen using a TurboVap® Concentration Workstation.
8. (Biotage, Charlottesville, VA).
9. Transfer to vial for analysis by LC/Q-TOF-MS.

Figure 1. TRILUTION LH SPE Tasks for Extraction of Lamotrigine and Metabolite from Water.



LC/TOF-MS Analysis

Following the automated SPE method, a sample volume of 50µL was injected onto the Agilent Series 1200 HPLC System. A 30 minute run time followed by a 10 minute post-run time was used for each sample analyzed.

The HPLC system was connected to an Agilent 6450 ultra high definition quadruple time-of-flight mass spectrometer equipped with electrospray Jet Stream Technology operating in positive ion mode. The operating

parameters were as follows: capillary voltage: 4000V; nebulizer pressure: 45 psig; drying gas: 10 L/min; gas temperature: 325°C; nozzle voltage: 1000V; fragmentor voltage: 190V; skimmer voltage: 60V and octapole RF at 750V. LC/MS accurate mass data were recorded across the range 50–1000 m/z at 4 GHz. The data recorded was 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, Santa Clara, CA, USA).

Method Validation

Method accuracy and precision were determined by recovery experiments with spiked samples. Drinking water, groundwater and surface water free of lamotrigine and its metabolite were spiked at 100 ng/L, extracted by SPE, and analyzed by LC/TOF-MS. Peak areas of the extracts were compared to peak area corresponding to a pure standard prepared in HPLC-grade water and recovery values were obtained. Peak areas, regression parameters, and concentrations were obtained by using Agilent MassHunter software.

Aliquots of standard solutions of analytes were added to water samples at seven different concentrations to obtain the standard calibration curves. All went through the SPE system and were treated like samples. To ensure accuracy, a calibration curve was developed for each type of matrix sample. An aliquot of 100 µL of surrogate labeled standard, lamotrigine-13C3-d3, was added to each calibration sample and to each environmental sample. The internal standard was used to account for recovery losses during SPE and any suppression from the matrix of the samples.

Results

Figure 2. LC/Q-TOF-MS Analysis of a Surface Water Sample Showing the MS-MS Spectrum of Lamotrigine.

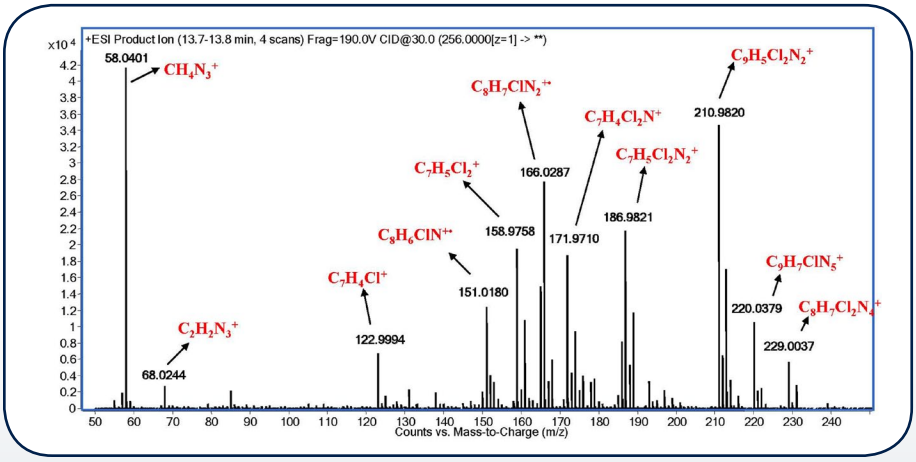


Table 3. Percent Recoveries and Standard Deviations (RSD) of Lamotrigine and its Glucuronide Metabolite from Drinking Water, Groundwater, Surface Water and Wastewater (N = 5).

Analyte	Drinking Water	Groundwater	Surface Water	Wastewater
Lamotrigine	91 (5)	95 (6)	82 (7)	75 (10)
Lamotrigine 2-N-Glucuronide	99 (7)	98 (8)	93 (7)	77 (8)

Table 4. Analysis of Wastewater, Groundwater, Surface Water and Drinking Water for Different Locations in the U.S. Showing Concentrations of Lamotrigine and Its 2-N-Glucuronide Metabolite.

Samples	Lamotrigine	Lamotrigine 2-N-Glucuronide
Wastewater (34 samples) Mean Concentration (ng/L) Percentage Detections (%)	 488 94	 209 21
Groundwater (15 samples) Mean Concentration (ng/L) Percentage Detections (%)	 324 93	 17 (below LOQ) 20
Surface water (62 samples) Mean Concentration (ng/L) Percentage Detections (%)	 108 47	 195 13
Drinking water (7 samples) Mean Concentration (ng/L) Percentage Detections (%)	 17 29	 Not detected Not detected

This application describes the conditions necessary to automate the solid phase extraction of lamotrigine and its 2-N-glucuronide metabolite from water samples prior to analysis by LC/Q-TOF-MS using the Gilson GX-271 ASPEC System (Figure 2). Extraction recoveries ranged from 75% to 99%. The RSD for inter-day (n=5) values were between 5% and 10%, which showed good reproducibility of the methodology (Table 3).

The LC/Q-TOF-MS limit of detection for lamotrigine and its metabolite were 1 ng/L and 5 ng/L. Automation of the SPE process allows one to reduce potential errors that may occur during manual extractions, increase lab efficiency, reduce solvent usage and increase sample throughput. Automation also allows one to optimize extraction conditions easily for different matrices and analytes.

Lamotrigine was detected in 94% of the 34 wastewater effluent samples tested and 93% of the 15 alluvial groundwater samples taken from down gradient of wastewater treatment plants (Table 4). Because of the widespread use of lamotrigine in the treatment of epilepsy and bipolar spectrum disorders, the wastewater plants are the principal source of lamotrigine and its glucuronide metabolite.

The 2-N-glucuronide was found in 21% of wastewater samples suggesting that certain conditions may exist in the wastewater treatment plant that prevent hydrolysis of the conjugated lamotrigine. Lamotrigine was also detected in 47% of the surface water samples tested and 29% of the drinking water samples (Table 4). These samples were collected from nine different states. The results suggest that the presence of this compound and its metabolite are widespread in environmental water samples. More studies are needed to determine the environmental impact of these compounds in the water supply.

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An Automated Method for the Fractionation of Extractable Petroleum Hydrocarbons (EPH) from Water and Soil Extracts Using the Gilson GX-274 ASPEC™ System

This study was performed by Patrick Sullivan and staff at TestAmerica Analytical Testing Corp., Westfield, MA, USA.

Featured Products: Gilson GX-274 ASPEC™ System with Two 406 Dual Syringe Pumps Operated Using TRILUTION® LH Liquid Handling Software.

Introduction

Crude and refined petroleum products contain a complex mixture of aliphatic and aromatic hydrocarbons as well as a variety of other compounds. The concentration of these different hydrocarbons will vary in different products such as crude oil, refined products and other residual oil products. Many of these hydrocarbons have been shown to pose a risk to human health or to aquatic life. Leaking underground storage tanks are a common source of groundwater and soil contamination.

It is important to determine the types of hydrocarbons that may be present in contaminated soil and water. Government agencies and other regulatory bodies have developed several methods for determining the types of aliphatic and aromatic hydrocarbons that may be found in contaminated soil or water. One such method is the "Method for the Determination of Extractable Hydrocarbons (EPH)" developed by the Massachusetts Department of Environmental Protection (MADEP, 2004). This method measures the collective concentrations of extractable aliphatic and aromatic petroleum hydrocarbons (EPH) that may be found in a soil or water sample. The MADEP EPH Method utilizes a solvent extraction step followed by a silica gel fractionation into two extracts – an aliphatic extract (C9–C18, C19–C36) and an aromatic extract (C11–C22). The two extracts are then concentrated and separately analyzed by capillary gas chromatography with a flame ionization detector (GC/FID).

The Silica Gel Cleanup and Fractionation step of the method requires a great deal of care and attention to detail to achieve satisfactory results. A high degree of recovery and reproducibility are required for success. The automation of this step will reduce the opportunity for human error, reduce the use of solvents and generation of hazardous wastes and decrease the overall cost per test. This study describes an automated protocol for the fractionation of EPH into aliphatic and aromatic fractions using a Gilson GX-274 ASPEC System.

Experimental Conditions

Materials

Note: All solvents were distilled in glass suitable for GC, HPLC, pesticide residues analysis and spectrophotometry.

- GX-274 ASPEC with two 406 Dual Syringe Pumps
    - TRILUTION LH software
- SPE Cartridges: Biotage ISOLUTE® Silica gel cartridges, 1g/6 mL (Part no. 460-0100C) were used to fractionate EPH sample extracts.
- using Gilson 6 mL Sealing Caps.
- GC/FID system: v (Agilent Technologies)
- GC column: Restek Rtx®-5 column (30 m x 0.25 mm, 0.5 µm)
- GC/FID Conditions:
    - Carrier gas was helium at a flow rate of 1.5 mL/min
    - Injector temperature was 290°C
    - FID temperature was 330°C
- Standards:
    - Stock solutions of target petroleum blends (aromatic and aliphatic) were purchased from Absolute Standards, Inc. (Hamden, CT), Accustandard (New Haven, CT) or Aldrich (Milwaukee, WI).
    - Surrogate standards, calibration solutions, matrix spiking solution and internal standards were prepared in accordance with the MADEP EPH Method.
    - A Fractionation Check Standard is required to monitor the fractionation.
    - efficiency of the silica gel columns and system. This ensures that the optimal amount of hexane is used to prevent breakthrough of the hydrocarbon aromatics into the aliphatic hydrocarbon fraction. Each new lot of SPE cartridge was monitored using the fractionation check standard.

Preparation of Samples Prior to Automated Silica Gel Fractionation Step

Water samples are prepared in accordance to USEPA Method 3510 (separatory funnel liquid-liquid extraction). Soil samples were prepared in accordance to USEPA Methods 3540 (Soxhlet extraction) or 3546 (Microwave extraction).

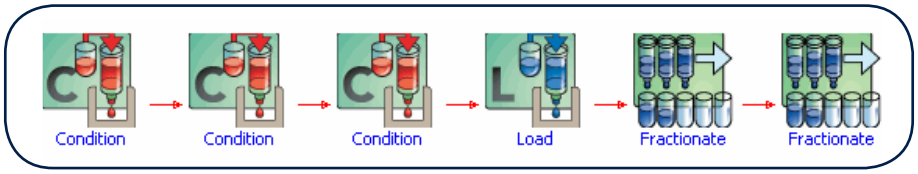
Automated Silica Gel Fractionation Step

The fractionation procedure used 1g/6 mL Biotage ISOLUTE Silica Gel Cartridges. The cartridges were sealed using Gilson 6 mL Sealing Caps. The fractionation protocol is entirely automated using the Gilson GX-271 ASPEC system. The SPE steps are summarized with the schematic provided in the GX-271 ASPEC control software, TRILUTION LH (Figure 1).

1. Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack.
2. Condition the cartridge with 5 mL acetone followed by an air push.
3. Condition the cartridge with 6 mL methylene chloride (dichloromethane) followed by an air push.
4. Condition the cartridge with 6 mL of hexane and allow the cartridge to stay moist (no air push).
5. Load 1 mL of sample and 100 µL of fractionation surrogate onto the cartridge at a low flow rate. *Note: Silica cartridges must not be overloaded with excessive mass of hydrocarbons. Limit loading to 5 mg total hydrocarbon per gram of silica gel.*
6. Move the Gilson Mobile SPE Rack over the collection tubes.
7. Elute the Aliphatic Fraction with 2400 µL of hexane. Allow to drip into the collection tube (15 x 85 mm glass tube) by gravity. *Note: The amount of hexane used in this step is critical. Excessive use of hexane may cause elution of aromatics into the aliphatic fraction. Insufficient hexane will cause low recoveries of the aliphatic fraction. Adjust the amount of hexane if necessary based on your QC results.*
8. Move the cartridges to the next set of collection tubes (Fractionate task) and elute the aromatic fraction with 4 mL of methylene chloride (dichloromethane). Allow to drip into the collection tube and then apply positive pressure to remove any excess solvent into the tube.

9. Concentrate the two fractions using a Caliper TurboVap or equivalent evaporation system to a final volume of 1 mL. Be careful not to concentrate below 1 mL.
10. The two fractions are then ready for analysis.

Figure 1. TRILUTION LH SPE Tasks for Fractionation of EPH Sample into Aliphatic and Aromatic Fractions.



GC/FID Analysis

A working calibration curve or calibration factor was verified each working day. A mid-level calibration standard was run after every 10 samples. The target compounds Naphthalene and 2-methylnapthalene were monitored in the Laboratory Control Sample (LCS) and Laboratory Control Sample Duplicate (LCSD) for breakthrough into the aliphatic fraction. If the concentration of either compound exceeded 5% of the total concentration (aromatic and aliphatic sum), fractionation was repeated.

Results

This application note describes the conditions necessary to automate the fractionation of EPH into aliphatic and aromatic fractions using the Gilson GX-274 ASPEC System with Biotage ISOLUTE Silica Gel Cartridges. Recovery of all analytes was excellent; ranging from 89 – 114% (Table 1). No aromatics were observed in the aliphatic fraction (Figures 2 and 3).

Automation of the fractionation process improved day-to-day reproducibility and increased sample throughput compared to results obtained using the manual fractionation method. This automated method has now been fully validated in our laboratory. Automation of the EPH fractionation process has the additional benefits of reducing solvent usage, lowering the cost per test and allowing scientists to spend more time developing new methods for the analysis of compounds of interest in the environmental laboratory.

Table 1. MADEP EPH Fractionation Check Standard Results.

Analyte	True Value (µg/mL)	STD Conc. (µg/mL)	% Recovery	Spike Limits
Napthalene	25.00	22.290	89	40-140
2-Methylnapthalene	25.00	22.529	90	40-140
Acenaphthylene	25.00	24.126	97	40-140
Acenaphthene	25.00	23.156	93	40-140
Fluorene	25.00	23.135	94	40-140
Phenanthrene	25.00	22.689	91	40-140
Anthracene	25.00	24.041	96	40-140
Fluoroanthene	25.00	22.650	91	40-140
Pyrene	25.00	22.873	91	40-140
Benzo(a)anthracene	25.00	23.470	94	40-140

Table 1, continued. MADEP EPH Fractionation Check Standard Results.

Analyte	True Value (µg/mL)	STD Conc. (µg/mL)	% Recovery	Spike Limits
Chrysene	25.00	24218	97	40-140
Benzo(b)fluoranthene	25.00	24.157	97	40-140
Benzo(k)fluoranthene	25.00	22.468	90	40-140
Benzo(a)pyrene	25.00	21.509	86	40-140
Indeno(1,2,3-cd)pyrene	25.00	22.484	90	40-140
Dibenzo(a,h)anthracene	25.00	22.699	91	40-140
Benzo(g,h,i)perylene	25.00	22.121	88	40-140
C9	25.00	28.542	114	40-140
C10	25.00	26.110	104	40-140
C12	25.00	24.054	96	40-140
C14	25.00	23.863	95	40-140
C16	25.00	23.528	94	40-140
C18	25.00	22.858	91	40-140
C19	25.00	24.286	97	40-140
C20	25.00	24.364	97	40-140
C22	25.00	23.476	94	40-140
C24	25.00	22.567	90	40-140
C26	25.00	25.495	102	40-140
C28	25.00	24.191	97	40-140
C30	25.00	22.953	92	40-140
C36	25.00	26.394	106	40-140

Figure 2. GC/FID Chromatogram of Aromatic Hydrocarbons after Automated Silica Gel Cartridge Fractionation of Water Extract Containing Fractionation Surrogate.

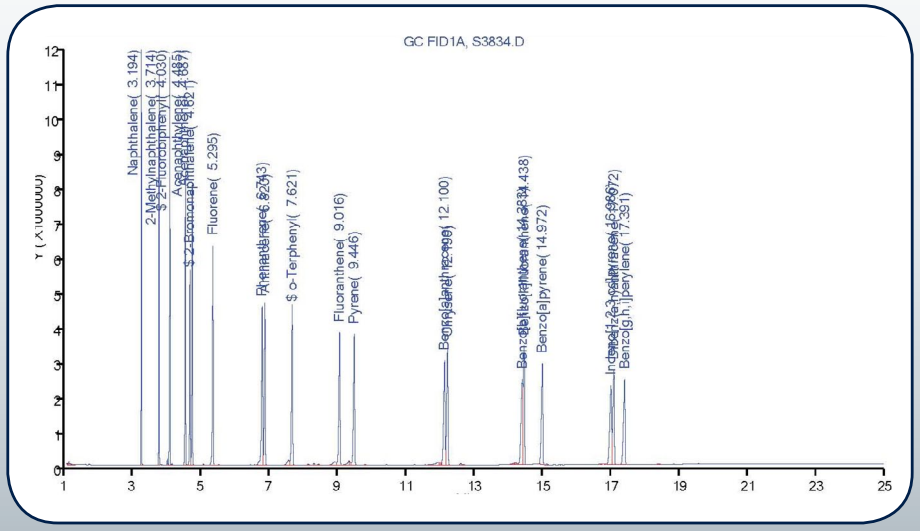
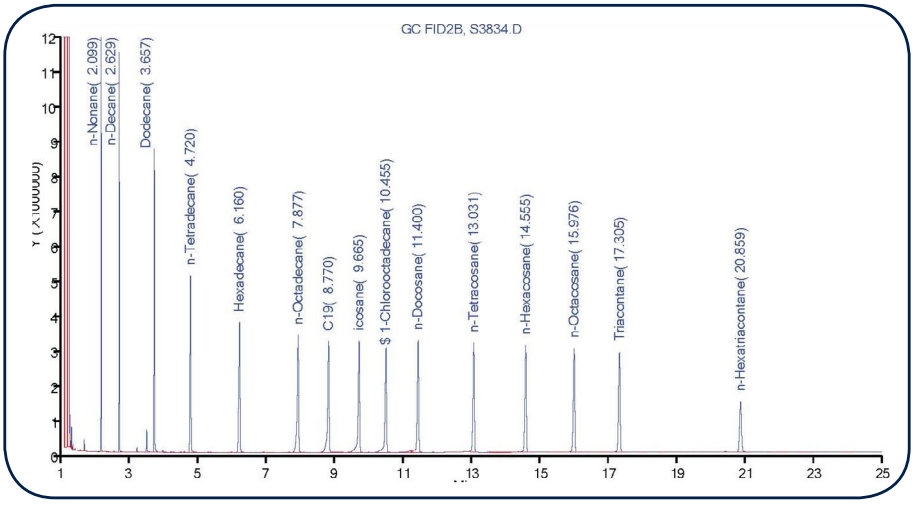


Figure 3. GC/FID Chromatogram of Aliphatic Hydrocarbons after Automated Silica Gel Fractionation of Water Extract Containing Fractionation Surrogate.



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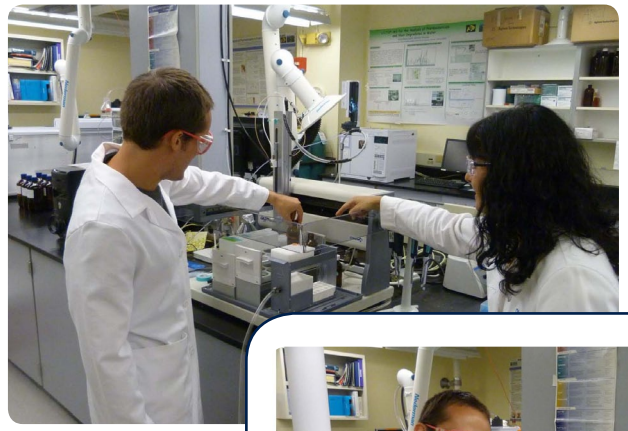
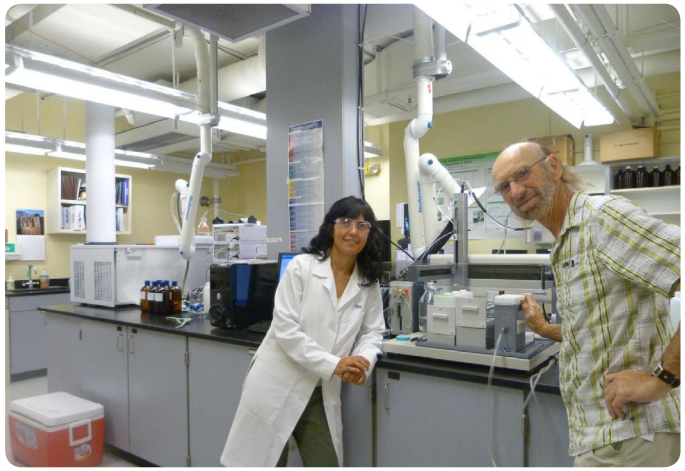
University of Colorado at Boulder

The Center for Environmental Mass Spectrometry (CEMS) focuses on the analysis of pesticides and pharmaceuticals in water, soil, plants, and food. The work in this center specializes in the analysis of water samples and unknowns, using high resolution and accurate mass analysis. Because water samples are so important to their research, the sample preparation process must be performed consistently.

Dr. E. Michael Thurman, Research Ph.D. (right), is a 30-year veteran of the United States Geological Survey (USGS), who has founded his research in water testing. Dr. Imma Ferrer, Research Ph.D. (left), is the chief analyst of CEMS, and is responsible for the highest quality accuracy measurements and operation of the laboratory. They stand in front of the Gilson GX-271 ASPEC™ within the CEMS.





At the CEMS, the Gilson GX-271 ASPEC is used on a routine basis by both Dr. Thurman and Dr. Ferrer. Their students also regularly use the Gilson system, controlled by the Gilson TRILUTION® LH Software, to prepare samples for analysis by mass spectrometry. Using the Gilson GX-271 ASPEC has provided very reliable and consistent results with little or no cross contamination, even at the low parts per trillion levels.

Dr. Thurman and Dr. Ferrer state: "We endorse the use of the ASPEC and appreciate the good service that Gilson has provided and the well-designed software for methods development and operation."

Analysis of Phytoestrogens in Wastewater by Liquid Chromatography/Time-of-Flight Mass Spectrometry (LC/TOF-MS) After Automated Solid Phase Extraction (SPE)

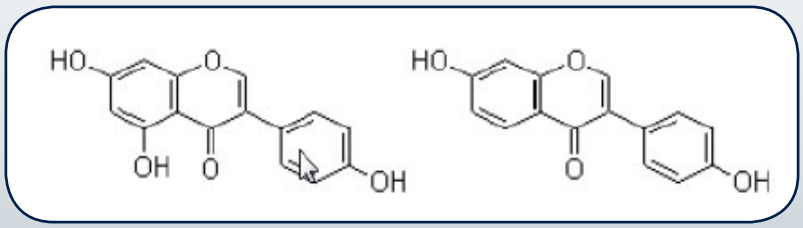
This study was performed by Imma Ferrer, Ph.D. and E. Michael Thurman, Ph.D. at the Center for Environmental Mass Spectrometry, Dept. of Civil, Environmental & Architectural Engineering, University of Colorado, Boulder, CO, USA

Featured Products: Gilson GX-271 ASPEC™ System with 406 Single Syringe Pump Operated Using TRILUTION® LH Liquid Handling Software.

Introduction

Phytoestrogens are a group of non steroidal polyphenolic compounds that occur naturally in a variety of plants such as soy, legumes, clover and alfalfa (Figure 1). Phytoestrogens can also be excreted by humans and livestock who consume these foods. This class of compounds has the ability to bind to estrogen receptors and thus disrupt the endocrine system in a variety of species including mice, humans and fish (Jefferson et al., 2007; Lampe, 2003; Lintelmann et al., 2003; Thorpe et al., 2003).

Figure 1. Chemical Structures of Two Common Phytoestrogens Found in Plants - Genistein (left) and Diadzein (right)1.



There is a growing interest in evaluating the effects of the exposure of fish and other aquatic organisms to phytoestrogens. Previous studies have demonstrated that phytoestrogens can decrease testosterone production and other hormones in fish and affect fish behavior (Lundgren and Novak, 2009). Thus, it is important to monitor

levels of phytoestrogens in water. Recently, a method employing gas chromatography coupled with ion trap mass spectrometry (GC/MS-MS) was developed for the identification of eight plant phytoestrogens in wastewater effluent from a soy processing plant and wastewater from a treatment plant (Ferrer et al, 2009).

Additional studies were performed utilizing LC/TOF-MS for analysis of phytoestrogens in wastewater (Ferrer and Thurman, 2009). LC/TOF-MS is an excellent screening tool and has the advantage of providing a full scan of data for the phytoestrogens of interest as well as data for metabolites and degradation products. This study describes the analysis of several phytoestrogens in wastewater using liquid chromatography coupled to time-of-flight mass spectrometry (LC/TOF-MS) as well as the automated extraction of these phytoestrogens from wastewater utilizing the Gilson GX-271 ASPEC System.

Experimental Conditions

Materials

Note: All solvents were distilled in glass suitable for GC, HPLC, pesticide residues analysis and spectrophotometry.

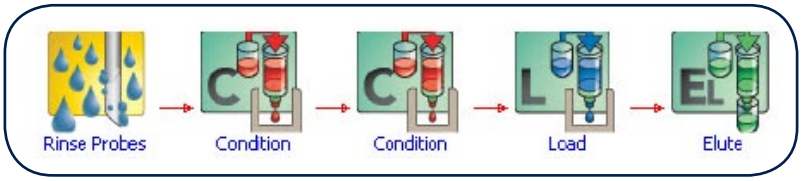
- GX-271 ASPEC with single 406 Syringe Pump
    - TRILUTION LH software
- SPE Cartridges: Oasis™ HLB 200 mg / 6 mL (Waters Corporation - Milford, MA, USA). The cartridges were sealed using Gilson 6 mL Sealing Caps.
- HPLC system: Agilent Series 1200 (Agilent Technologies)
- HPLC column: Zorbax Eclipse XDB-C8, 150 mm x 4.6 mm, 5 µm(Agilent Technologies)
- HPLC Mobile phase
    - A: Acetonitrile with 0.1% formic acid
    - B: Water with 0.1% formic acid
- Time-of-Flight mass spectrometer: Agilent 6220 MSD TOF
    - MassHunter software
- Analytical standards:
    - Standards were obtained from Sigma-Aldrich (St. Louis, MO, USA)
    - Deuterated standards were obtained from Cambridge Isotopes (Cambridge, MA, USA)
    - Individual stock solutions (500 µg/mL) were prepared in pure methanol and stored at -18°C
- HPLC solvents were obtained from Merck (Darmstadt, Germany)
- Formic acid was obtained from Fluka (Buchs, Switzerland)
- Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA) was used throughout the study to obtain HPLC-grade water
- Wastewater samples:
    - Collected from effluent locations downstream from a soy processing plant
    - Collected from upstream and downstream locations from a municipal wastewater treatment plant

Method

The SPE steps are summarized with the schematic provided in the GX-271 ASPEC control software, TRILUTION LH (Figure 2).

1. Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack and probe rinsed with 10% methanol.
2. Condition SPE cartridge with 4 mL of methanol at a flow rate of 1 mL/min.
3. Condition SPE Cartridge with 6 mL of water at 1 mL/min.
4. Load 200 mL of water sample at a flow rate of 10 mL/min. Follow this with an air push of 25 mL to remove any excess water.
5. Move the Gilson Mobile SPE Rack over the collection tubes.
6. Elute the analytes of interest with 5 mL methanol at 1 mL/min.
7. Evaporate to 0.5 mL with nitrogen at a temperature of 45 degrees C in a water bath using a TurboVap® Concentration Workstation (Caliper Life Sciences, Mountain View, CA, USA).

Figure 2. TRILUTION LH SPE Tasks for Extraction of Phytoestrogens from Water.



LC/TOF-MS Analysis

The separation of the selected phytoestrogens was carried out using an HPLC system (consisting of vacuum degasser, autosampler and a binary pump) (Agilent Series 1200, Agilent Technologies, Santa Clara, CA, USA). Column temperature was maintained at 25°C. The injected sample volume was 50 µL.

Table 1. HPLC Mobile Phase Gradient (Flow rate = 0.6mL/min).

Step No.	Time (min)	%A	%B
1	0	10	90
2	5	10	90
3	25	100	0

This HPLC system was connected to a time-of-flight mass spectrometer Agilent 6220 MSD TOF equipped with a dual electrospray interface operating in positive ion mode, using the following operation parameters: capillary voltage: 4000V; nebulizer pressure: 45 psig; drying gas: 9 L/min; gas temperature: 300°C; fragmentor voltage: 190V; skimmer voltage: 60V; octopole RF: 250V. LC/MS accurate mass spectra were recorded across the range 50–1000 m/z at 4GHz.

Accurate mass measurements of each peak from the total ion chromatograms were obtained by means of an automated calibrant delivery system using a dual-nebulizer ESI source that introduces the flow from the outlet of the chromatograph together with a low flow of a calibrating solution (calibrant solution A, Agilent Technologies), which contains the internal reference masses (purine ($C_5H_4N_4$ at m/z 121.0509 and HP-921 [hexakis-(1H,1H,3Htetrafluoro-pentoxy)phosphazene] ($C_{18}H_{18}O_6N_3P_3F_{24}$) at m/z 922.0098. The instrument worked providing a typical mass resolving power of 15000±500 (m/z 922).

Results

Figure 3. LC/TOF-MS Ion Chromatogram for a Mixture of Phytoestrogens. Compound labels: 1 = Daidzein, 2 = Glycitein, 3 = Coumestrol, 4 = Genistein, 5 = Equol, 6 = Formononetin, 7 and 8 = Biochanin A and Prunetin.

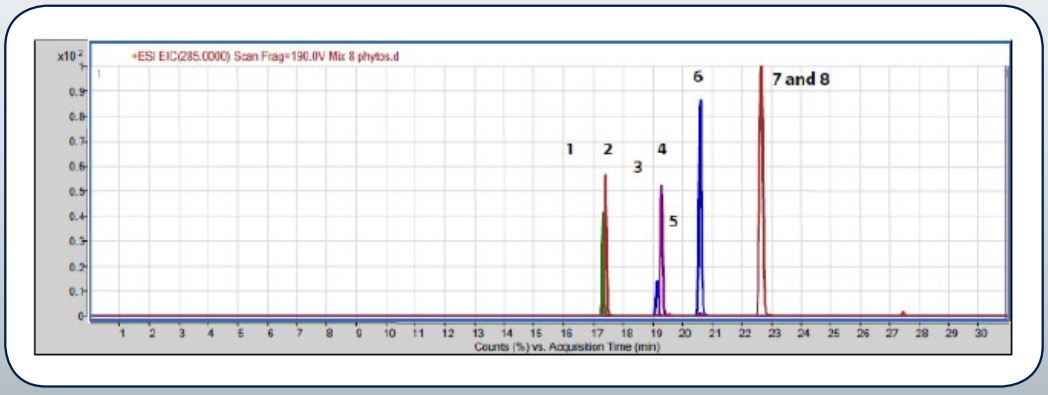


Figure 4. Mass Spectrum for Genistein.

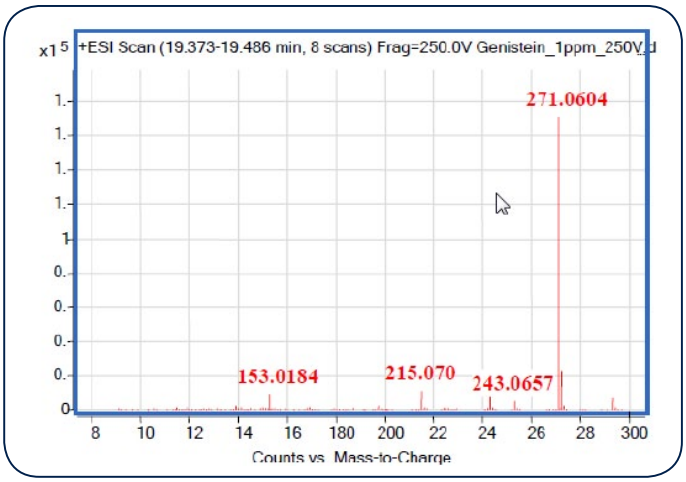


Figure 5. LC/TOF-MS Chromatogram of Wastewater Effluent from Soy Processing Plant. Extracted Ion Chromatograms for Diadzein and Genistein.

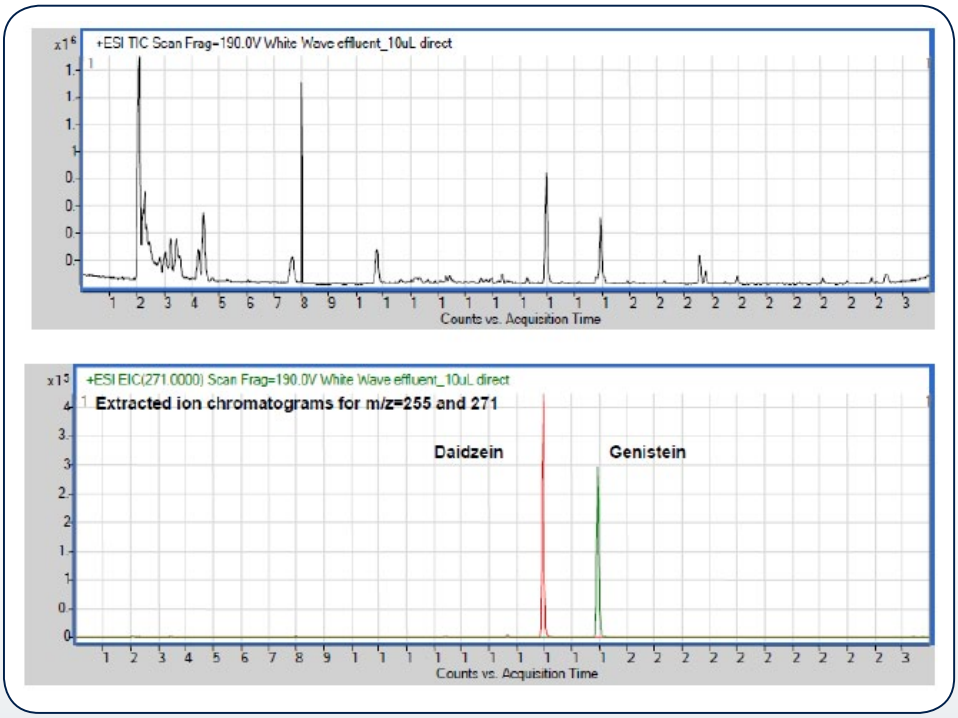


Table 2. Concentrations of Phytoestrogens (in µg/L) in Wastewater Samples Compared to Concentrations Found in Soy Milk.

Compound	Soy Milk	Soy Processing Plant Effluent	Wastewater Treatment Plant Influent	Watewater Treatment Plant Effluent
Genistein	50,000	2000	20	<1
Daidzein	15,000	500	20	<1
Glycitein	200	50	<1	<1

Summary

This application note describes the conditions necessary to automate the solid phase extraction of phytoestrogens from wastewater samples prior to analysis by LC/MS-TOF using the Gilson GX-271 ASPEC System. Automation of the SPE process allows one to reduce potential errors that may occur during manual extractions, increase lab efficiency, reduce solvent usage and increase sample throughput. Automation also allows one to easily optimize extraction conditions for different matrices and multiple classes of analytes.

Genistein and Daidzein were the only two phytoestrogens found in wastewater and are found in high quantities in soy milk, so one would expect to find these compounds in wastewater effluent from a soy processing plant. Concentrations in influent waters were 100 times lower than soy plant effluent. The data suggests that soy wastewater is not the only source for genistein and daidezein in the wastewater influent. Future studies will focus on the effects of genistein and daidzein on fish since they are the main compounds occurring in the wastewater influent.

References

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Preservation and Quantification of Hormones in Surface Water

This study was performed by Sonya M. Havens, James T. Schauer and other scientists in the Environmental Chemistry and Technology Program, University of Wisconsin – Madison and the Wisconsin State Laboratory of Hygiene, Madison, Wisconsin, USA (Havens et al., 2010).

Introduction

The contamination of aquatic environments with hormones can disrupt the reproductive and developmental function of aquatic organisms such as fish (Jensen, K.M. et al., 2006; Orlando, E.F. et al., 2004). One source of these hormones is the natural and synthetic hormones that are present in manure from large-scale livestock operations. Approximately 130 billion pounds of manure are produced annually in the United States (USEPA, 2000). The hormones that are present in manure and in crop fields that have been amended with manure can be readily transported to surface and shallow ground water with events such as rain or snowmelt. There is a great deal of interest in studying the distribution and fate of these hormones in surface water. Measuring the concentrations of natural and synthetic hormones in water can be challenging. Hormone degradation may occur during the storage of surface water prior to analysis in the laboratory (Baronti, C. et al., 2000; Vanderford, B.J. et al., 2006). This can result in the underestimation of hormone concentrations. Thus, a preservation protocol that inhibits the degradation of a large number of hormones and hormone metabolites during sample collection and storage is a necessary prerequisite for the accurate estimation of hormone occurrence in surface and ground water (Havens, S.M.et al., 2010).

This application note describes the use of different preservatives (sodium azide, hydrochloric acid and sulfuric acid) to inhibit the degradation of hormones in samples of surface water runoff from cattle manure-amended fields during storage at 4ºC. The hormones and hormone metabolites were extracted from surface water using the automated Gilson GX-271 ASPEC™ System (Figure 1). Details of this procedure are described below. Hormone and hormone metabolite levels were determined using HPLC tandem mass spectrometry (LC-MS/MS).

Figure 1. Gilson GX-271 ASPEC™ System with 406 Single Syringe Pump.

Experimental Conditions

Materials

The large suite of hormones chosen for the study included natural and synthetic estrogens, androgens and progestogens that have previously been detected or could be present in surface water (Table 1).

Table 1. Analytes Tested.

Analyte	Chemical Abstracts (CAS) Number	Isotope Analog
17 ß-Estradiol	50-28-2	17 ß-Estradiol-d5
Estrone	53-16-7	17 ß-Estradiol-d5
Estriol	50-27-1	Estriol-d3
α-Zearalenol	36455-72-8	α-Zearalenol-d4
Zearalenone	17924-92-4	α-Zearalenol-d4
Zearalenone	5975-78-0	α-Zearalenol-d4
Androsterone	53-41-8	Testosterone-d5
5α-Androstan-17ß-ol-3-one	521-18-6	Testosterone-d5
5α-Androstane-3, 17-dione	846-46-8	Testosterone-d5
4-Androstene-3, 17-dione	63-05-8	Testosterone-d5
1-Dehydrotestosterone	846-48-0	Testosterone-d5
17ß-Nortestosterone	434-22-0	Testosterone-d5
Testosterone	58-22-0	Testosterone-d5
17ß-Trenbolone	10161-33-8	17ß-Trenbolone-d3
Progesterone	57-83-0	Progesterone-d9
17,20-Dihydroxyprogesterone	1662-06-2	Progesterone-d9
Melengestrol	5633-18-1	Melengestrol-d3
Melengestrol acetate	2919-66-6	Melengestrol acetate-d3

All the analytical standards were of high purity (> 98%) and were obtained from Sigma-Aldrich (USA) with the exception of 17α-trenbolone, which was purchased from Hayashi Pure Chemical Inc. (Japan). Isotopically labeled standards were obtained from C/D/N Isotopes (Canada) or the European Union Reference Laboratory at the National Institute for Public Health and the Environments (RIVM, Bilthoven, The Netherlands). All solvents used were HPLC grade. All the runoff and ultra-pure water (control) samples were stored in 60 mL amber glass vials (I-CHEM, USA) that received a silanization treatment to deactivate the glass surface to prevent hormone adsorption to the vial wall. The silanization treatment included: one rinse with 5% dimethyldichlorosilane (intoluene; Supelco, USA), two rinses with toluene and three rinses with methanol.

Water Sample Collection, Preparation and Preservation

Three types of water were utilized for the study- ultra-pure water, a simulated fresh surface water runoff (a surrogate runoff), and an aged surface runoff sample collected from six edge-of field weirs at three anonymous cattle farms and stored for four months at 4ºC. The simulated fresh water runoff sample was prepared by suspending freshly collected cattle manure and soil into groundwater collected from a tile drain on a dairy farm. This mixture was brought to a final volume of 5 Liters with dechlorinated tap water and then filtered through a 1µm glass fiber filter to remove suspended particles. 50 mL aliquots of each sample type were distributed into silanized 60 mL amber vials prior to SPE extraction. For more details on preparation of these samples, see Havens et al., 2010.

Aged runoff, filtered simulated fresh water runoff and ultra-pure water received either no preservative, sulfuric acid (H_2SO_4, 90 µl, pH = 2), HCl (200µl, pH = 2) or sodium azide (NaAz; 1 g·L^{-}1). Isotopically labeled standards (ISTD) and target analytes (Target) were spiked at 50 µl of 1x103 ng·ml-1 (in methanol). Spiked and ambient analytes were

extracted (in triplicate) immediately (t = 0), after 24 hours (t=1 d) and after 14 days (t=14 d) of storage at 4ºC. The samples were sequentially extracted on the Gilson GX-271 ASPEC solid phase extraction system (Gilson Inc., USA). Simulated fresh water runoff samples were extracted first, followed by the aged surface runoff samples and then the ultra-pure water.

Automated Solid Phase Extraction

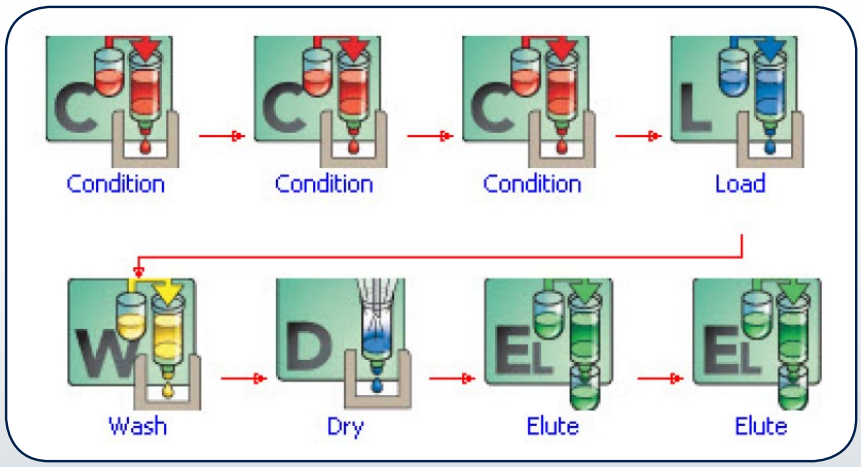
The fractionation procedure used 200 mg/ 6mL ISOLUTE™ + polypropylene solid phase extraction cartridges (Biotage, USA). The cartridges were sealed using Gilson 6 mL Sealing Caps. Note: for Bioassay studies, glass cartridges should be substituted for polypropylene cartridges to eliminate effects of hormone mimicking plasticizers. In this case, Gilson Special 1778 for 6 mL glass cartridges can be substituted for the standard 6 mL cartridge SPE racks. Risers may be necessary when using this rack with the 200-series racks.

The solid phase extraction protocol is entirely automated using the Gilson GX-271 ASPEC system. The SPE steps are summarized with the general schematic provided in the GX-271 ASPEC control software, TRILUTION® LH (Figure 3).

The summary of each step are as follows:

- Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack.
- Condition the cartridge with 3 mL of methanol:ethyl acetate (1:1, v/v) at 3 mL/min.
- Condition the cartridge with 3 mL of methanol at 3 mL/min.
- Condition the cartridge with 3 mL of ultra-pure water at 3 mL/min.
- Load 50 mL of sample onto the SPE cartridge at a flow rate of 3 mL/min.
- Wash the cartridge with 10 mL of ultra-pure water at a flow rate of 3 mL/min.
- Dry the cartridge with a stream of air for 5 minutes.
- Move the Gilson Mobile SPE Rack over the collection tubes.
- Elute with 4 mL of methanol at a flow rate of 0.5 mL/min.
- Elute with 4 mL of methanol: ethyl acetate (1:1, v/v) at a flow rate of 0.5 mL/min.
- Concentrate the fractions with a gentle stream of nitrogen gas to a volume of approximately 100 µL and reconstitute to a volume of 1.0 mL using methanol for HPLC-MS/MS analysis.

Figure 3. TRILUTION LH Basic SPE Tasks for Solid Phase Extraction of Hormones from Water.



LC-MS/MS Analysis

The hormone concentrations in the extracts were analyzed using high-performance liquid chromatography (Agilent Technologies 1100 HPLC, USA) with tandem mass spectrometric detection (Applied Biosystems/MDS SCIEX API 4000 USA; HPLC-MS/MS) operating in positive Atmospheric Pressure Chemical Ionization mode. A sample injection volume of 15 µL was applied to a 4 micron, 4.6 x 250 mm Synergi™ MAX-RP column (Phenomenex, USA)

and separated with a reversed phase binary mobile phase gradient (Havens et al., 2010) at 0.8 mL/min. Relevant multiple reaction monitoring (MRM) mass spectrometer settings include TurbolonSpray™ voltage at 5500 Volts, collision gas at 6 arbitrary units, curtain gas at 25 psig, nebulization gas at 40 psig, drying gas at 15 psig, corona discharge current of 3 volts and source temperature at 450°C.

In order to determine the concentration of each target analyte, normalized to ISTDs, the instrument was calibrated by generating a curve based on the relative response ratios of peak areas between variable target analyte concentrations (1.0, 2.0, 5.0, 10, 25, 50, 100, 250 and 500 ng/mL) and ISTDs added to each calibration point at a concentration of 50 ng/mL. This corresponded to the ISTD concentration spiked into all of liquid chromatography samples. Linear or quadratic regression with 1/x2 weighting was used to generate calibration curves for all analytes. The calibration coefficients always exceeded 0.990. The target analyte concentrations in all the sample extracts (spiked and ambient) were calculated by normalizing the relative response ratio in the sample extract to those in the calibration curve. Detection limits for the extracted samples are in the 1.0 ng/L range, but this is dependent upon the amount of interference due to co-eluted matrix components present in the sample.

Results

Table 3. The Recovery Range of Each Analyte Normalized to its Corresponding Isotopically Labeled Standard Insimulated Fresh Water Runoff (surrogate) with Either No Preservative, Sulfuric Acid or Sodium Azide after 14 days of Storage at 4°C.

Analyte	No Preservative	Sulfuric Acid	Sodium Azide
17 ß-Estradiol	<10%	80 - 120%	<10%
Estrone	<40%	40 - 59%	40 - 59%
Estriol	80 - 120%	80 - 120%	80 - 120%
α-Zearalenol	<20%	60 - 79%	60 - 79%
Zearalenone	<40%	60 - 79%	40 - 59%
Zearalenone	40 - 59%	60 - 79%	60 - 79%
Androsterone	non detected	60 - 79%	<40%
5α-Androstan-17ß-ol-3-one	<10%	60 - 79%	<10%
5α-Androstane-3, 17-dione	non detected	60 - 79%	<10%
4-Androstene-3, 17-dione	non detected	60 - 79%	80 - 120%
1-Dehydrotestosterone	non detected	60 - 79%	<10%
17ß-Nortestosterone	<10%	60 - 79%	<10%
Testosterone	non detected	60 - 79%	<10%
17ß-Trenbolone	40 - 59%	80 - 120%	60 - 79%
Progesterone	non detected	80 - 120%	<40%
17,20-Dihydroxyprogesterone	<10%	60 - 79%	60 - 79%
Melengestrol	60 - 79%	60 - 79%	60 - 79%
Melengestrol acetate	40 - 59%	60 - 79%	40 - 59%

Table 4. The Recovery Range of Each Analyte Normalized to its Corresponding Isotopically Labeled Standard in Aged Runoff Preserved with Either No Preservative, Sulfuric Acid or Hydrochloric Acid after 14 Days of Storage at 4ºC.

Analyte	No Preservative	Sulfuric Acid Preserved	Hydrochloric Acid Preserved
17 ß-Estradiol	<40%	60 - 79%	80 - 120%
Estrone	>160%	80 - 120%	80 - 120%
Estriol	<40%	80 - 120%	80 - 120%
α-Zearalenol	<40%	40 - 59%	60 - 79%
Zearalenone	40 - 59%	80 - 120%	80 - 120%
Zearalenone	>160%	60 - 79%	60 - 79%
Androsterone	<20%	80 - 120%	80 - 120%
5α-Androstan-17ß-ol-3-one	<20%	80 - 120%	80 - 120%
5α-Androstane-3, 17-dione	none detected	60 - 79%	80 - 120%
4-Androstene-3, 17-dione	<20%	60 - 79%	60 - 79%
1-Dehydrotestosterone	none detected	60 - 79%	60 - 79%
17ß-Nortestosterone	<10%	80 - 120%	80 - 120%
Testosterone	<20%	80 - 120%	80 - 120%
17ß-Trenbolone	40 - 59%	60 - 79%	60 - 79%
Progesterone	<10%	60 - 79%	60 - 79%
17,20-Dihydroxyprogesterone	<40%	80 - 120%	80 - 120%
Melengestrol	60 - 79%	60 - 79%	80 - 120%
Melengestrol acetate	60 - 79%	60 - 79%	60 - 79%

Conclusion

Significant degradation of estrogenic, androgenic and progestogenic hormones occurs in water samples within hours of sample collection. Much of this degradation is due to microbial activity (Havens et al., 2010). Adding sodium azide did not adequately inhibit androgen degradation at the concentration used. Acid preservation (HCl or H_2SO_4, pH = 2) stabilized the hormones in the water samples. Coupling acid preservation with the use of internal standards resulted in reliable and accurate recovery of a suite of androgens, estrogens and progestogens in surface water stored up to 14 days at 4ºC.

Using the Gilson GX-271 ASPEC for automation of the solid phase extraction (SPE) process increased sample throughput, reduced solvent usage and reduced the potential errors that may occur in during manual processing of samples. Automation also permitted scientists to spend more time planning scientific experiments and developing new methods for the analysis of compounds of interest to the laboratory.

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Automated Solid Phase Extraction of Pharmaceuticals and Personal Care Products (PPCPs) from Water

This application note was part of a collaboration with Drs. Imma Ferrer and Michael Thurman at the Center for Environmental Mass Spectrometry at the University of Colorado-Boulder, Boulder, CO, USA.

Featured Product: Gilson GX-271 ASPEC™ System with 406 Single Syringe Pump Operated Using TRILUTION® LH Liquid Handling Software.

Large quantities of pharmaceuticals and personal care products (PPCP) are sold and consumed each year throughout the world. A variety of PPCPs have been detected in low concentrations in surface water, ground water, and drinking water. Thus, there is a great deal of interest in measuring these compounds in water to determine their environmental impact. Solid phase extraction (SPE) is a common method for analyte pre-concentration and sample cleanup prior to determination of PPCPs from a water sample.

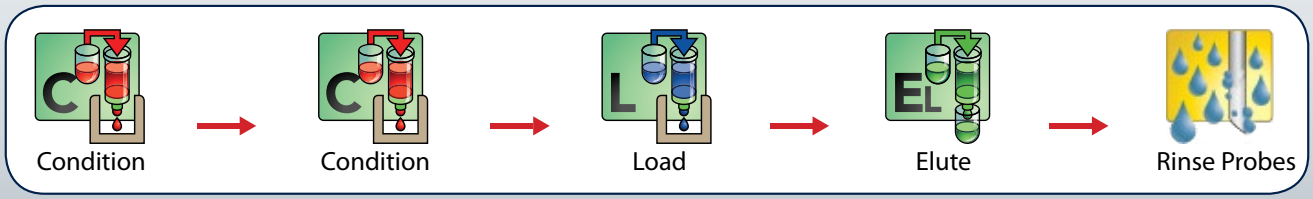
Experimental Conditions

Standard stock solutions of 17 PPCPs were prepared in LC-MS grade methanol at a concentration of 1 ppm. Water samples were ultra-pure water. No pH adjustment was performed on the water samples.

Solid Phase Extraction (SPE) Protocol

The SPE procedure used 6 mL Waters Oasis™ HLB (200 mg) Cartridges. The SPE protocol is entirely automated using the Gilson GX-271 ASPEC system. The SPE steps are summarized with the schematic provided in the GX-271 ASPEC control software, TRILUTION® LH (Figure 1).

Figure 1. TRILUTION LH SPE and Liquid Handling Tasks for Extraction of PPCPs from Water.



- Condition the cartridge with 4 mL of methanol at 1.0 mL/min.
- Condition the cartridge with 6 mL of water at 1.0 mL/min.
- Load 100 mL water sample at 7.5 mL/min.

- Move the Gilson Mobile SPE Rack over the collection tubes.
- Elute the analytes of interest with 10 mL of methanol.
- Evaporate to 0.5 mL with nitrogen at a temperature of 45° C in a water bath.

LC/TOF-MS Analysis

The separation of the PPCPs was carried out using an Agilent Series 1200 HPLC System equipped with a reverse phase C8 analytical column of 150 mm x 4.6 mm and 5 µm particle size (Zorbax Eclipse XDB-C8). The injected sample volume was 50 µL. Mobile phases A and B were acetonitrile and water with 0.1% formic acid, respectively. The flow rate was 0.6 mL/min.

The HPLC was connected to an Agilent 6220 MSD TOF equipped with a dual electrospray interface operating in positive ion mode. The data was processed with MassHunter software.

Results

Figure 2. TIC of Standard Versus Spiked Water Sample.

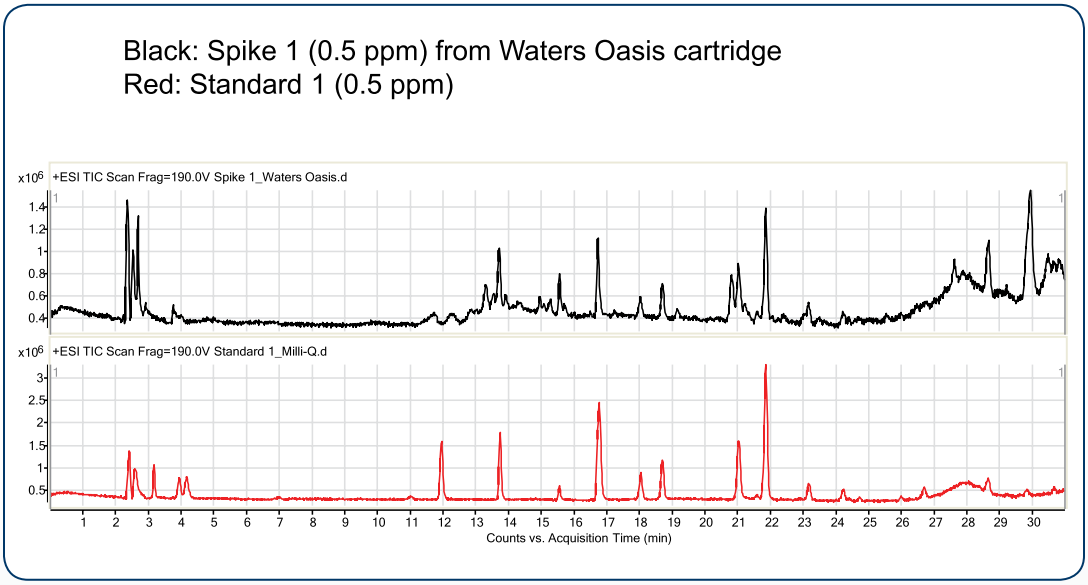


Table 1. Percent Recovery of Selected PPCPs in Water Using Oasis HLB Cartridge and 100 mL of Sample at a Loading Rate of 7.5 mL/min.

PPCPs	% Recovery
Acetominophen	154
Albuterol	89
Atenolol	99
Caffeine	132
Carbamazepine	106
Cotinine	67
DEET	91
Dehydronifedipine	93
Diclofenac	100

PPCPs	% Recovery
Diphenhydramine	119
Gemfibrozil	125
Ibuprofen	97
Metoprolol	88
Sulfadimethoxine	95
Sulfamethazole	98
Triclocarbon	36
Trimethoprim	104

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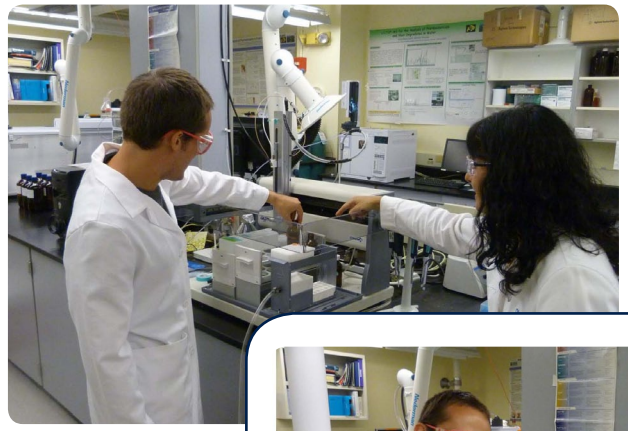
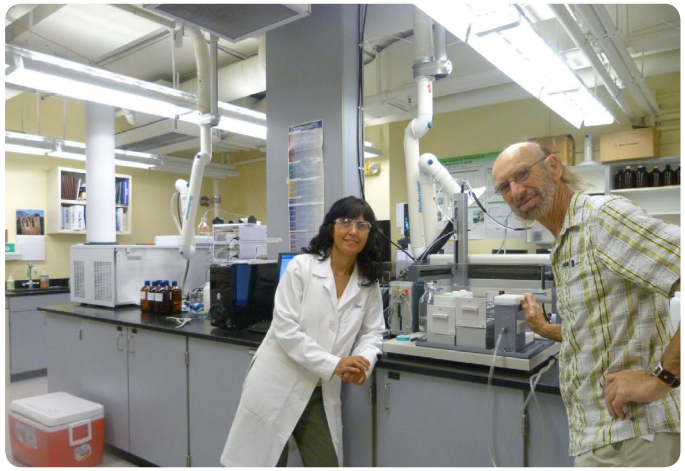
University of Colorado at Boulder

The Center for Environmental Mass Spectrometry (CEMS) focuses on the analysis of pesticides and pharmaceuticals in water, soil, plants, and food. The work in this center specializes in the analysis of water samples and unknowns, using high resolution and accurate mass analysis. Because water samples are so important to their research, the sample preparation process must be performed consistently.

Dr. E. Michael Thurman, Research Ph.D. (right), is a 30-year veteran of the United States Geological Survey (USGS), who has founded his research in water testing. Dr. Imma Ferrer, Research Ph.D. (left), is the chief analyst of CEMS, and is responsible for the highest quality accuracy measurements and operation of the laboratory. They stand in front of the Gilson GX-271 ASPEC™ within the CEMS.





At the CEMS, the Gilson GX-271 ASPEC is used on a routine basis by both Dr. Thurman and Dr. Ferrer. Their students also regularly use the Gilson system, controlled by the Gilson TRILUTION® LH Software, to prepare samples for analysis by mass spectrometry. Using the Gilson GX-271 ASPEC has provided very reliable and consistent results with little or no cross contamination, even at the low parts per trillion levels.

Dr. Thurman and Dr. Ferrer state: "We endorse the use of the ASPEC and appreciate the good service that Gilson has provided and the well-designed software for methods development and operation."

Food & Beverage Applications

Determination of Veterinary Drug Residues in Fish Using Automated Solid Phase Extraction Followed by HPLC

Featured Product: GX-271 ASPEC™ System with 406 Single Syringe Pump and Direct Inject Module.

Farm-raised seafood accounts for nearly half of seafood production worldwide. As the aquaculture industry has grown, there have been increasing problems associated with infectious diseases in fish and shellfish. These diseases can cause major economic losses to seafood farmers. As a result, fighting infections has led to the increased use of antibiotics and other antimicrobials. The use of these products is highly regulated. Recent reports have found the presence of prohibited antibiotics in farm-raised fish and shrimp. Some of these compounds include malachite green (a carcinogen), fluroquinolones, nitrofurans, chloramphenicol and other antibiotics. This note describes a simple and automated procedure employing solid phase extraction (SPE) to extract and concentrate some representative illegal residues from fish tissue prior to HPLC analysis.

Experimental Conditions

All solvents were HPLC grade or higher. All reagents were ACS grade or higher. Malachite green, naladixic acid and tetracycline were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fresh salmon was obtained from a local market.

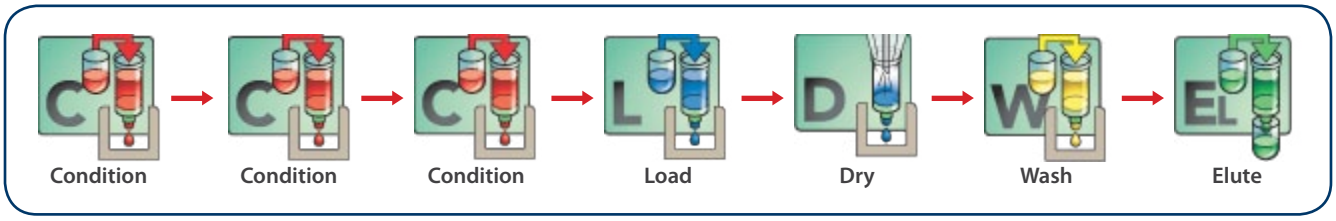
The following sample steps were performed prior to solid phase extraction:

- Mix 10g of ground salmon with 25 mL of ethyl acetate.
- Sonicate for 10 min and transfer the ethyl acetate extract to a separate container.
- Add an additional 25 mL of ethyl acetate to the ground fish and sonicate followed by removal of the ethyl acetate extract to the same container as last step.
- Repeat this process two more times. The total amount of ethyl acetate extract was 100 mL.
- Mix the ethyl acetate extract and filter using smooth fluted, 313 folded filter paper. Add 0.5 mL of acetic acid to filtrate and bring volume to 100 mL with ethyl acetate.

Solid Phase Extraction (SPE) Fractionation Protocol

The SPE procedure utilized 3 mL Macherey-Nagel Chromabond SA (500 mg) Cartridges. The SPE protocol is entirely automated using the Gilson GX-271 ASPEC System. The SPE steps are summarized with the schematic provided in the GX-271 ASPEC control software, TRILUTION® LH).

Figure 1. TRILUTION® LH SPE Tasks for Extraction of Select Residues in Fish.



Condition the cartridge with 2 x 3 mL n-hexane. Use an air push following the condition step to dry the cartridge.

- Condition the cartridge with 6 mL ethyl acetate containing 0.5% acetic acid. Do not dry the column.
- Load the sample extract onto the cartridge at a low flow rate (5 to 8 mL/min).
- Dry the cartridge with air for 10 minutes.
- Wash the cartridge with 10 mL of methanol.
- Move the Gilson Mobile SPE Rack over the collection tubes.
- Elute with 5 mL of 10% triethanolamine in methanol.
- The sample extract can now be analyzed directly onto the HPLC using the Direct Injection Module.

HPLC Conditions

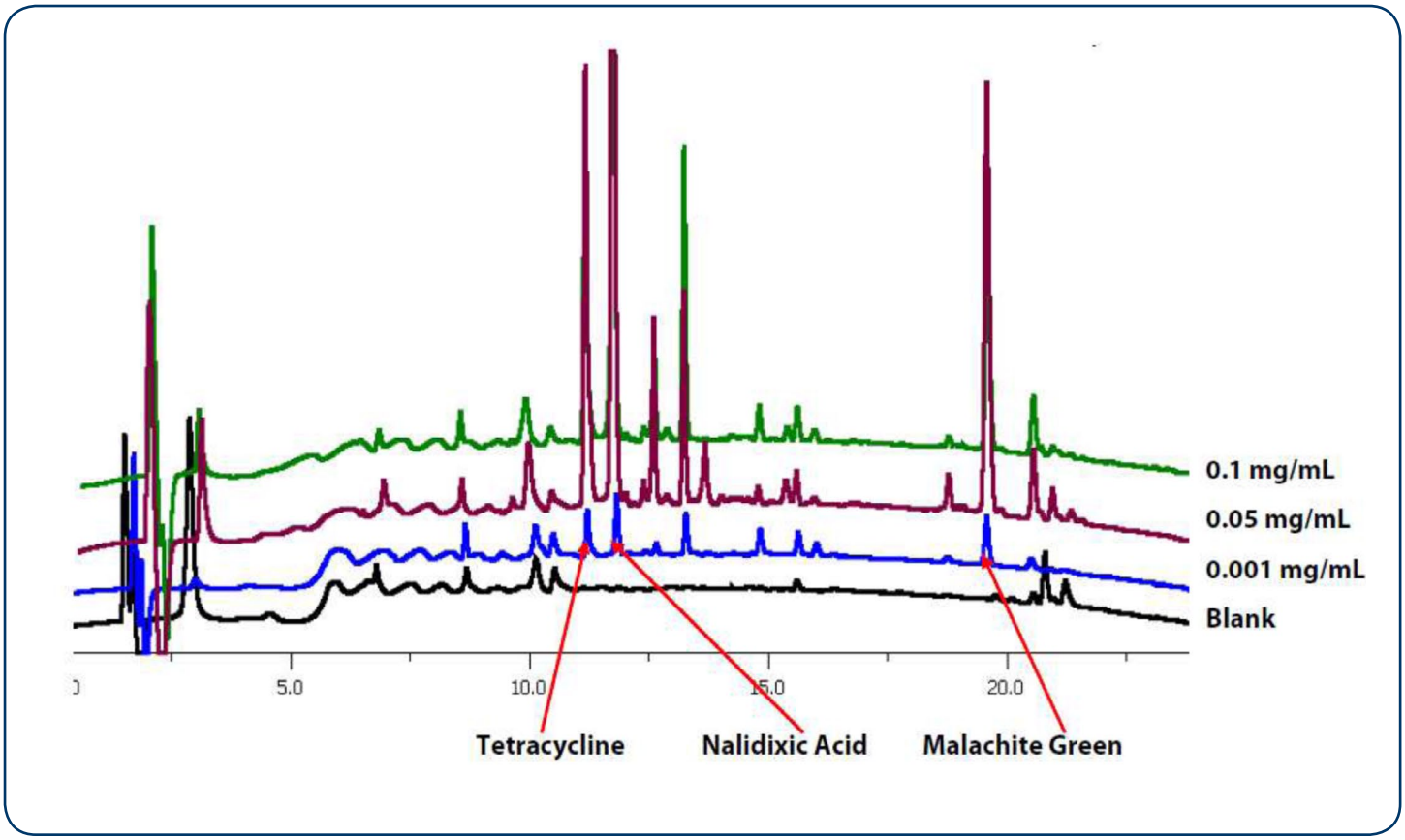
- Gilson GX-271 ASPEC with Direct Inject Module and 50µL Sample Loop
- Gilson 322 HPLC Pump with H2 Pump Heads
- Gilson 155 UV/VIS Detector, 210/254nm, Analytical flow cell (5 mm path length), Sensitivity Setting = 0.005/0.005
- Gilson TRILUTION Software for HPLC Control and Data Acquisition
- Column: Waters Atlantis® dC18, 5µm, 4.6 x 150mm
- Flow Rate of 1.5 mL/min, injection volume = 50µL, Mobile phase A; H2O with 0.1% TFA, Mobile Phase B: Acetonitrile with 0.1% TFA

Figure 2. HPLC Gradient Conditions.

Time (min)	% A (Aqueous)	% B (Organic)
0	95	5
1.3	95	5
26.5	5	95
30.0	5	95
31.5	95	5
34.0	95	5

Results

Figure 3. Chromatographic Analysis of Fortified Salmon.



Recovery values ranged from 85% to 104% and were consistent within (n=3) and between days (n =3).

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Post-Extraction Gel Permeation Chromatography (GPC) Clean-up of Fish Tissue Prior to PCB and PBDE Analysis

This application note was part of a collaborative study with the Wisconsin State Lab of Hygiene, Madison, WI, USA

Featured Product: Automated GX-271 GPC Clean-up System.

The determination of PCBs and PBDEs, as well as other environmental contaminants, in fish tissue, requires extensive sample clean-up prior to analysis by gas chromatography with an electron capture detector (GC/ECD) or GC/MS. The high fat content of fish tissue can cause buildup of nonvolatile materials on the GC injection port and the analytical column, giving poor analytical results and high instrument maintenance costs. GPC clean-up is often used for the clean-up of fish tissue extracts prior to analysis for halogenated compounds such as PCBs, PBDEs and chlorinated pesticides.

Experimental Conditions

GPC Standards were prepared according to USEPA Method 3640A. Stock solutions of PCB and PBDE congeners were obtained from Ultra Scientific.

Extraction Protocol

Weigh 10g of ground fish tissue into a beaker. Fortify with 1g of corn oil. Add 60g anhydrous sodium sulphate and 230 mL of dichloromethane. Spike with appropriate surrogate PCB/PBDE standards. Pour the mixture through a column containing Florisil® topped with a 1 mL layer of anhydrous sodium sulphate. Collect the eluent, and evaporate to near dryness using a gentle stream of nitrogen. Reconstitute in 5 mL GPC mobile phase.

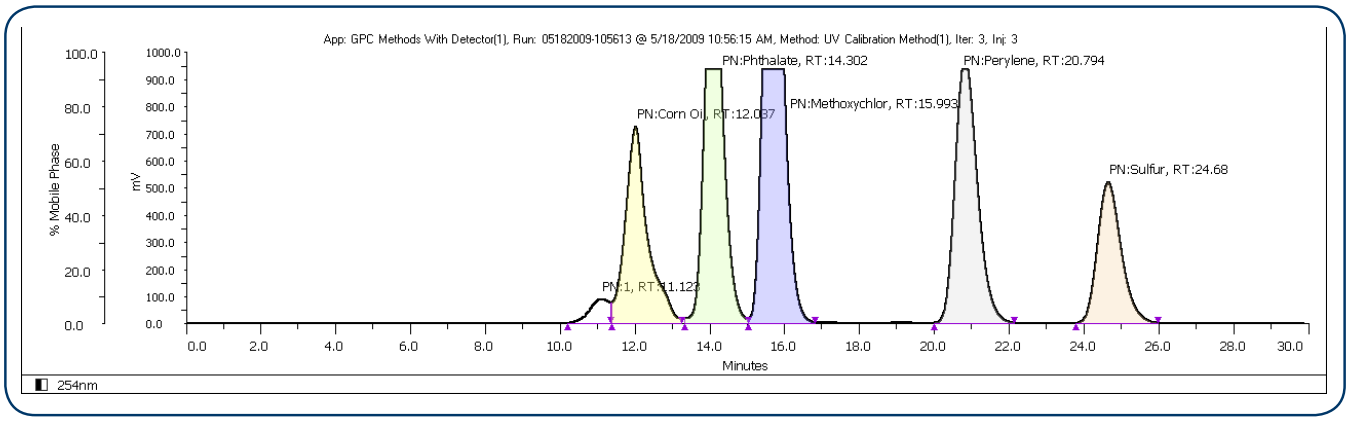
GPC Clean-up

Column calibration used a GPC calibration standard (as described on previous page), a Gilson 112 UV Detector set at 254 nm and Gilson TRILUTION® LC Software. Based on the UV trace, column eluent was collected just after bis(2-ethylhexyl) phthalate elution and stopped after perylene elution. For the columns listed in Table 1 below, the flow rate was 5 mL/min with an injection volume of 5 mL. The exception was with the OI Analytical Optima™ column, which used an injection volume of 1 mL.

Table 1. GPC Column Parameters for Fish Extract Clean-up.

Column	Dump Volume (mL)	Collect Volume (mL)	Total Run Time (min)	Column Lipid Loading Capacity (g)
OI Analytical Glass, 1:1 DCM/CYX	100	110	60	1
EnviroSep-ABC, 1:1 DCM/CYX	75	48	32	0.5
EnviroSep-ABC, 100% DCM	75	43	30	0.5
Optima Column 1:1 CYX/ethyl acetate	45	60	28	0.2

Figure 1. Chromatogram of a USEPA Method 3640A Calibration Standard Using an EnviroSep-ABC Column with a Mobile Phase of 100% Dichloromethane.

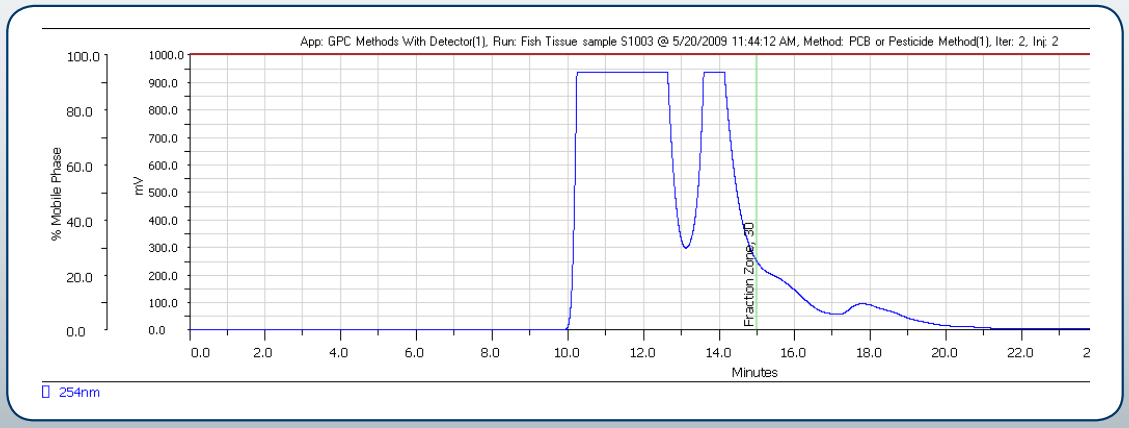


After GPC clean-up, collected fractions were put through further clean-up with silica gel to remove any pesticides and then concentrated with a gentle stream of nitrogen before they were reconstituted in appropriate solvent for GC analysis.

GC Analysis

PCBs and PBDEs were analyzed with an Agilent HP5890-II GC/ECD using a DB-5 column (60m x 0.25mm ID, 0.1 µM phase). PBDEs were confirmed using a DB-1 column (60m x 0.25mm ID, 0.1 µM phase).

Figure 2. Chromatogram of a Fish Tissue Extract During GPC Clean-up Using an EnviroSep-ABC Column with a Mobile Phase of 100% Dichloromethane and UV Detection at 254 nm.



Results

Table 2. PBDE Recovery in Fish Using a Phenomenex EnviroSep-ABC Column with a Mobile Phase of 100% Dichloromethane; Fish Spiked at 2 ng/g (n=3).

PBDE#	28	47	66	85	99	100	138	153	154
% Recovery	127.5	77.5	97.8	97.0	70.5	76.5	107.8	100.0	93.0

Table 3. PCB Recovery in Fish (n=3).

Column	Mobile Phase	PCB BZ #14 % Recovery	PCB BZ #65 % Recovery	PCB BZ #166 % Recovery
OI Analytical Glass 60g Environbeads SX-3	1:1 Dichloromethane/ Cyclohexane	74.7	81.6	81.5
EnviroSep-ABC	1:1 Dichloromethane/ Cyclohexane	80.5	86.8	92.9
EnviroSep-ABC	100% Dichloromethane	87.9	86.7	91
OI Optima Column 1:1 CYX/EA	1:1 Ethylacetate/ Cyclohexane	90	89	117.5

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Identification of Color Additives in Powdered Drink Mix using Solid Phase Extraction and Automation of AOAC Method 988.13

Featured Product: GX-274 ASPEC™ System with Agilent 8453 UV-visible Spectrophotometer.

The addition of synthetic color additives is regulated closely by the FDA and is examined from the manufacturing of the pigment itself, through to its use and appropriate product labeling. Color additives have come under scrutiny recently because of their potential adverse physical and mental health effects that may be linked to ingestion, especially in children.

AOAC method 988.13 qualitatively tests for the presence of eight synthetic color additives, one of which is now banned. These color additives are FD&C colors approved for use in food, drugs and cosmetics. Additives are extracted from the sample matrix using solid phase extraction (SPE), and then identified by spectrum analysis on a spectrophotometer.

In this application, AOAC method 988.13 was automated using a Gilson GX-274 ASPEC to perform the SPE process just prior to automated spectrum analysis using the Agilent 8453 UV-visible Spectrophotometer with the Agilent 8-position Multicell Transport. FD&C Yellow No. 5, FD&C Red No. 40 and FD&C Blue No. 1 were extracted from various powdered drink mixes. The full application note can be requested by emailing training@gilson.com.

Experimental Conditions

Tartrazine, Allura Red AC and Erioglaucine (Table 1) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Powdered drink mix (Kool-aid®) was obtained from a local supermarket.

Table 1. Alternate Names for FD&C Color Additives.

FD&C Name	E Number	Common Name
FD&C Yellow No.5	E102	Tartrazine
FD&C Red No.40	E129	Allura Red AC
FD&C Blue No.1	E133	Erioglaucine

Sample Preparation

Kool-aid: 1 g powder dissolved in 100 mL of NanoPure water. Solution filtering was automated with the GX-274 ASPEC on-bed using 8 mL Grace Alltech® Extract-Clean™ Filter Columns.

Solid Phase Extraction (SPE) Protocol

The SPE procedure used 6 mL Phenomenex Strata® C18-E (1000 mg) cartridges. The parameters were optimized for each sample (Tables 2 and 3) from the original schema in AOAC method 988.13.

Table 2. SPE Parameters Used for the Separation of Grape Kool-aid.

Step	Solvent	Volume (µL)	Air Push (µL)
Condition # 1	IPA	2000	1250
Condition # 2	1% Acetic Acid	2500	1750
Load	Kool-aid	2000	1200
Wash	2.5% IPA	3000	1500
Fractionate # 1	13% IPA	2000	1000
Fractionate # 2	20% IPA	3000	2000

Table 3. SPE Parameters Used for the Separation of Orange Kool-aid.

Step	Solvent	Volume (µL)	Air Push (µL)
Condition # 1	IPA	2000	1500
Condition # 2	1% Acetic Acid	2500	2000
Load	Kool-aid	2000	1200
Fractionate # 1	2.5% IPA	4000	2500
Fractionate # 2	13% IPA	3000	2000

Automated SPE Fraction Preparation Protocol for Absorbance Reading

Each fraction collected from the SPE process was then prepared for identification:

- The fraction was transferred to a clean test tube; volume transferred was 250 µL less than the amount of solvent used for elution in the Fractionate step.
- The fraction was diluted to 6 mL with appropriate IPA solution and volume.
- 2 mL was transferred to each of two sets of clean tubes.
- 1500 µL was transferred to the flow cells via the transfer ports, and an absorbance reading was taken on the neutral diluted fraction.
- A drop (23 µL) of concentrated hydrochloric acid was added to the second set of test tubes and the solution was mixed.
- 1500 µL was transferred to the flow cells via the transfer ports, and an absorbance reading was taken on the acidic fraction solution.
- A drop (23 µL) of 50% sodium hydroxide solution was added to the third set of test tubes and the solution was mixed.
- 1500 µL was transferred to the flow cells via the transfer ports, and an absorbance reading was taken on the basic fraction solution.

A blank of the appropriate IPA solution was taken prior to each set of absorbance readings. The readings were taken from 190 to 1100 nm, however the spectra were only analyzed from 350 to 750 nm, as specified in AOAC 988.13. The flow cells and lines were rinsed with 5 mL NanoPure water after each reading to eliminate carryover between samples.

Results

Standards were prepared for each of the color additives. Using these standards, the Fraction Preparation Protocol described above was followed to obtain representative spectra of each color additive for comparison purposes (Figures 1-3).

Figure 1. Standard Spectra for FD&C Yellow No. 5.

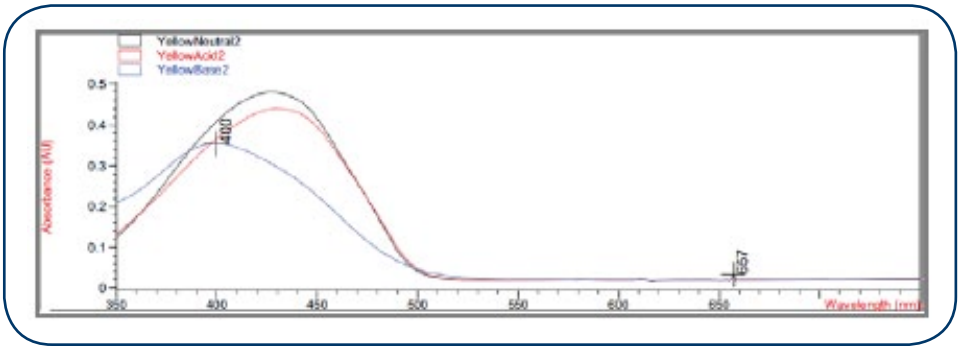


Figure 2. Standard Spectra for FD&C Red No. 40.

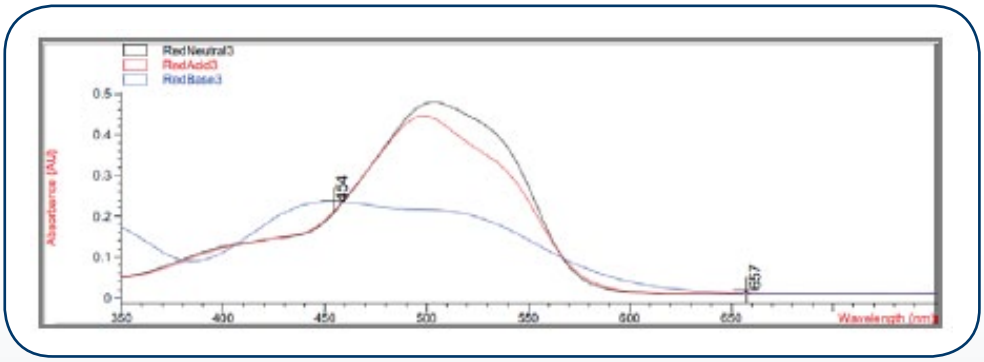
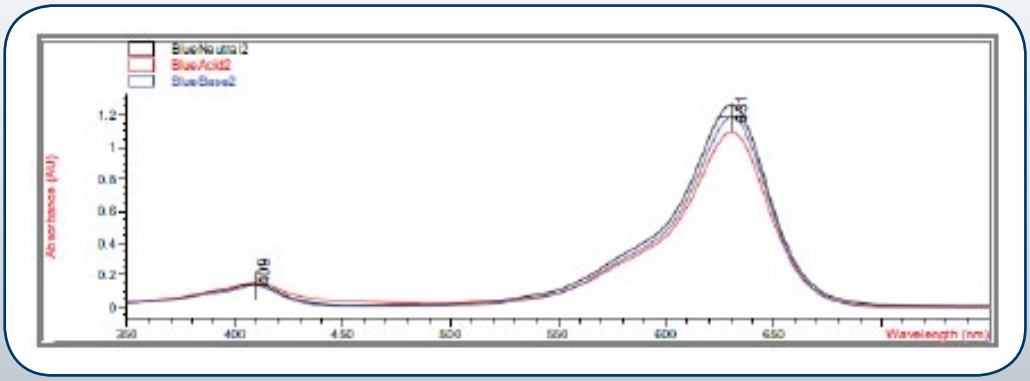


Figure 3. Standard Spectra for FD&C Blue No. 1.



For the powdered drink mixes, the identifying features of the spectra are visible, though some overlapping of colors is apparent. The Grape Kool-aid was confirmed to contain FD&C Red No. 40 (Figure 4) and FD&C Blue No. 1 (Figure 5). The average (n=4) absorbance values for the spectra can be found in Table 4. The %CV is relatively high due to extremely low absorbance values. This does not change the validity of the results for this qualitative method.

Figure 4. Representative FD&C Red No. 40 Spectra from Grape Kool-aid.

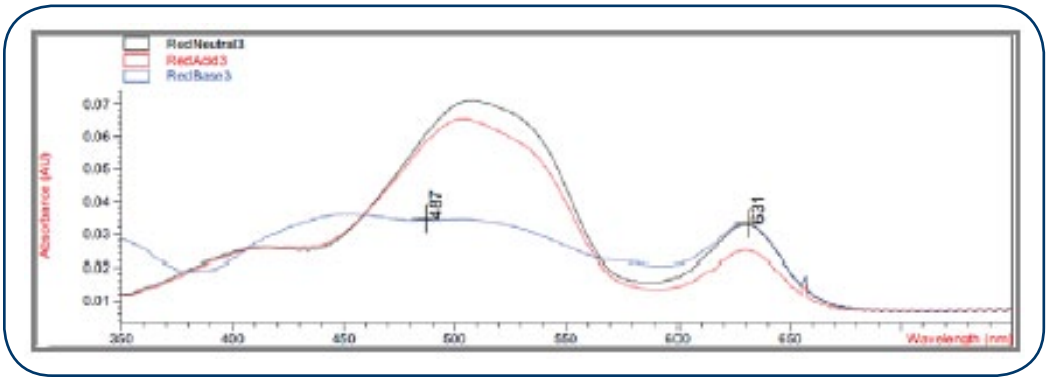


Figure 5. Representative FD&C Blue No. 1 Spectra from Grape Kool-aid.

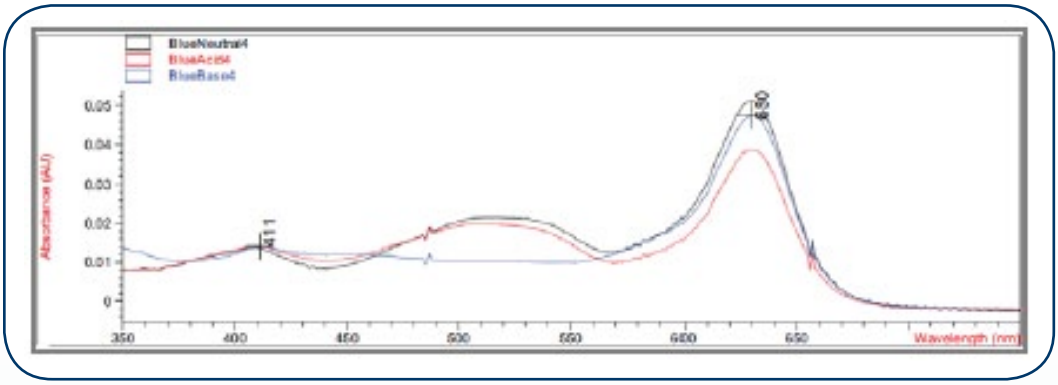


Table 4. Average (n=4) Absorbance Values for Grape Kool-aid.

Sample	λ (Neutral)	AU (Neutral)	λ (Acid)	AU (Acid)	Acid/Neutral	λ (Base)	AU (Base)	Base/ Neutral
FD&C Red No.40 Average	507.75	0.07085	503.50	0.06345	0.89547	450.0	0.03364	0.47369
FD&C Red No.40 %CV	0.10	6.24	0.11	6.76	2.19	0.26	12.03	6.73
FD&C Blue No.1 Average	630.0	0.05582	630.0	0.04278	0.76587	630.0	0.05033	0.89392
FD&C Blue No.1 %CV	0.00	7.93	0.00	10.05	3.91	0.00	8.66	7.32

The Orange Kool-Aid was confirmed to contain FD&C Yellow No. 5 (Figure 6) and FD&C Red No. 40 (Figure 7). Due to low concentration in the Orange Kool-aid, as well as the observance of some FD&C Yellow No. 5 remaining in the fraction, the absorbance for the FD&C Red No. 40 was not quantifiable, but could still be qualitatively identified. Average (n=4) absorbance values for the FD&C Yellow No. 5 spectra can be found in Table 5.

Figure 6. Representative FD&C Yellow No. 5 Spectra from Orange Kool-aid.

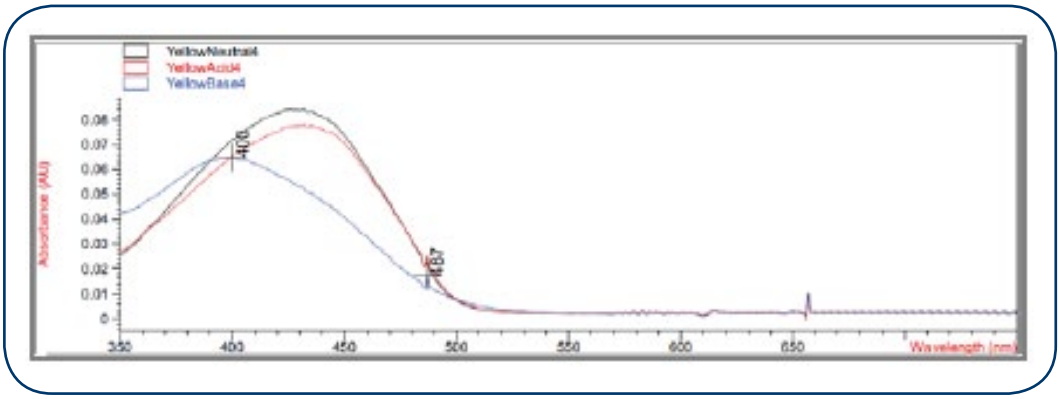


Figure 7. Representative FD&C Red No. 40 Spectra from Orange Kool-aid.

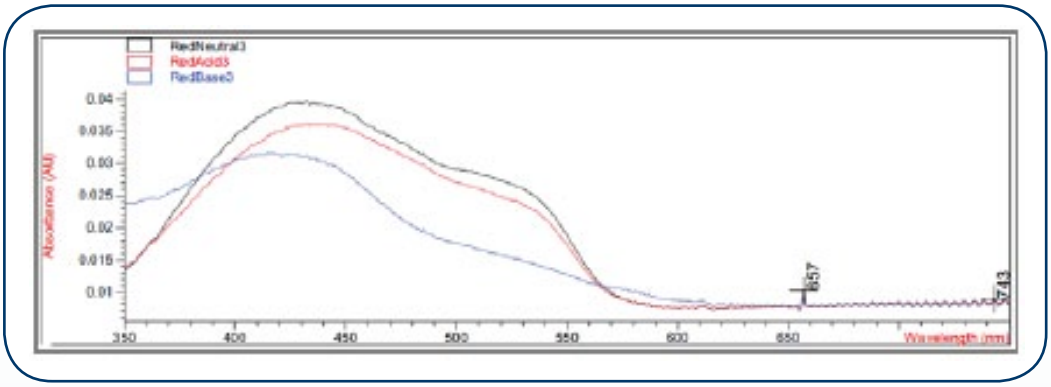


Table 5. Average (n=4) FD&C Yellow No. 5 Absorbance Values for Orange Kool-aid.

Sample	λ (Neutral)	AU (Neutral)	λ (Acid)	AU (Acid)	Acid/Neutral	λ (Base)	AU (Base)	Base/ Neutral
FD&C Yellow No.5 Average	430.5	0.08985	431.50	0.08329	0.92746	400.0	0.06807	0.75817
FD&C Yellow No.5 %CV	0.40	5.19	0.23	4.39	2.61	0.20	3.70	2.83

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Determination of Chlorinated Pesticides in Poultry Fat via AOAC 970.52, 984.21 and USDA-FSIS CHC3-19 Using GPC and Optional Alumina Clean-up with GC-ECD Detection

The data for this application note was in collaboration with Sean Linder, Ph.D. and Cheryl Fossler, M.S., Arkansas Livestock and Poultry Commission.

Featured Product: Automated Gilson GX-271 GPC Clean-up System.

Poultry fat is routinely monitored for the presence of chlorinated pesticides. This insures that consumers are not exposed to unacceptable levels of these pesticides and that unauthorized use of these pesticides is detected. Determination of chlorinated pesticides in poultry fat requires post-extraction clean-up steps to effectively remove lipids and other co-extractives prior to analysis by gas chromatography (GC) or GC/MS. Failure to remove these compounds results in decreased column life, contamination of the ion source and decreased analytical performance. This application note describes the use of the Gilson Automated GX-271 GPC Clean-up System to perform the post-extraction clean-up of poultry fat followed by an optional alumina clean-up step using a special filter rack placed on-line to the GPC eluent.

Gel permeation chromatography (GPC) is a common tool for the post-extraction removal of high molecular weight interferents prior to pesticide analysis. GPC removes these interferents via a size separation mechanism that uses organic solvents and a hydrophobic gel (a cross-linked divinylbenzene-styrene copolymer) to separate the interferents from the lower molecular weight compounds of interest. The interferents are discarded to waste and the fraction containing the pesticides is collected for further clean-up and analysis. GPC clean-up may be followed by additional clean-up procedures such as adsorption chromatography using alumina, Florisil™, or silica.

Experimental Conditions

Materials – GPC Clean-up Procedure

This application utilized the Gilson Automated GX-271 GPC Clean-up System with a low pressure glass column filled with 60 g Envirobeads S-X3 resin. The isocratic mobile phase consisted of a 1:1 dichloromethane:cyclohexane. Evaporation flasks fitted with fritted filter with grooved 24/40 joint (Adams & Chittenden part no. BUCH30C24G) were required, along with Gilson's collection rack special 1340.

Sample Preparation

Poultry fat was prepared according to AOAC International Methods 970.52, 984.21 and USDA-FSIS Method CHC3-19. Poultry fat was spiked with 10 ppb of the following compounds: Lindane, Heptachlor, Aldrin, Heptachlorepoxide, Alpha-Chlordane, Methoxychlor, Internal Standard 1: TCMX, and Internal Standard 2: DCBP.

Figure 1. Using Evaporation Flasks With Fritted Filters on the Automated Gilson GX-271 GPC Clean-up System.

General GPC Clean-up Procedure

- Isocratic mobile phase: 50:50 dichloromethane:cyclohexane
- Flow rate: 5 mL/min
- Start fraction collection: 24 min
- Fraction collection time: 26 min
- Total run time: 55 min
- Total injection of fat on column: 1 g
- Software: Gilson TRILUTION® LC with preinstalled GPC Clean-up Methods

Materials – Alumina Clean-up Procedure

A "keeper" (300 µL of 2% heavy paraffin oil in isooctane) was added to the GPC collection flasks prior to GPC

collection (A keeper solution is a small volume of a high boiling solvent used to prevent full evaporation to dryness and the occurrence of significant losses during the dry-down step.). The collected GPC fraction passes over an alumina bed (2 g bed weight of Alumina, Neutral Brockman Activity 1; 60-325 Mesh; [1344-28-1]-Fisher A950-500) as it elutes from the GPC column. The alumina-cleaned extract is transferred and dried down using a RapidVap® N2 System. It is then reconstituted in the appropriate mobile phase prior to GC analysis.

Materials & General Procedure– Gas Chromatography (GC) Analysis

- Agilent Series 5890 GC with ECD Detector
- Supelco Equity-5 column (30 m x 0.53 x 0.25)
- Splitless Injection, 3 µL
- Manual Flow Control

Results

Comparison analysis via GC-ECD was performed on poultry fat samples where no GPC Clean-up was performed, where GPC Clean-up was performed, and where GPC Clean-up + Alumina Clean-up was performed. The overlayed chromatograms displayed in Figure 5 represent the effectiveness GPC Clean-up + Alumina Clean-up has on reducing interferents from poultry fat samples. Recovery and reproducibility values for the pesticide compounds are presented in Table 2.

Figure 2. Overlayed Poultry Fat Analyses via GC-ECD.

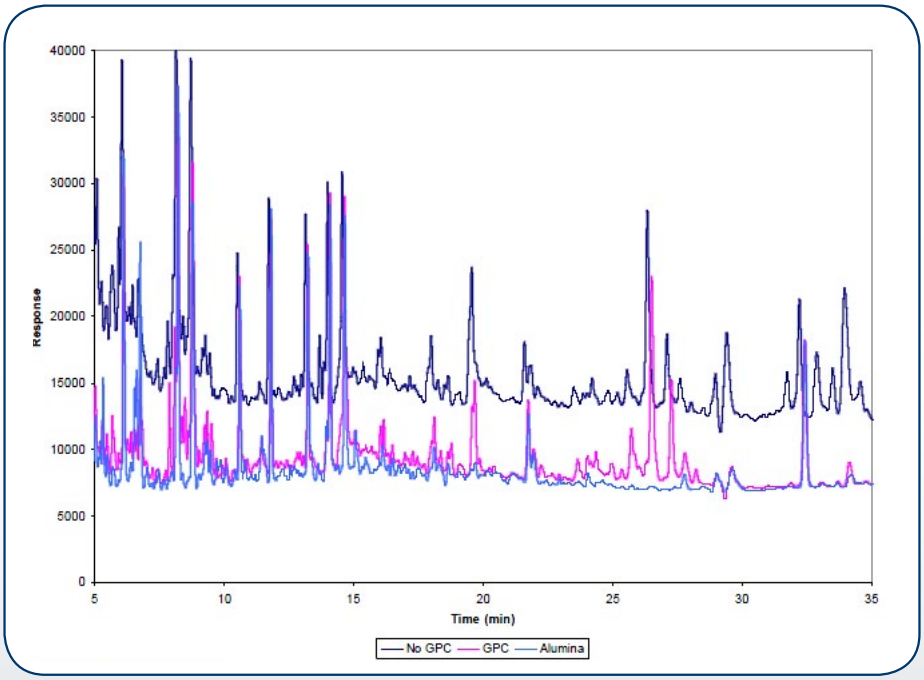

Table 1. Retention Times of Eluting Chlorinated Pesticides from GC-ECD Analysis.

Compound Name	Retention Time (min)	Compound Name	Retention Time (min)
TCMX (IS 1)	6.17	Gamma-Chlordane	14.18
Lindane	8.29	Alpha-Chlordane	14.74
Heptachlor	10.68	Methoxychlor	21.87
Aldrin	11.89	DCBP (IS 2)	32.59
Heptachlorepoxide	13.32		

Note: Analysis was performed on different days with manual flow control so slight variations in retention times occurred.

Table 2. Observed vs. Expected Compound Recoveries and Reproducibility Values.

Compound	Observed Recovery (%)	Observed Reproducibility %CV	CHC3 Expected Recovery (%)	CHC3 Reproducibility %CV
Lindane	108	3	70-120	20
Heptachlor	96	2	70-120	20
Aldrin	96	2	70-120	20
Heptachlorepoxide	98	1	70-120	20
Gamma-Chlordane	115	3	70-120	20
Edosulfan I	79	5	70-120	20
Alpha-Chlordane	101	2	70-120	20
Methoxychlor	93	3	70-120	20
2,4′-DDE	95	16	ND	ND
4,4′-DDE	97	14	70-120	20
2,4′-DDD	95	7	ND	ND
4,4′-DDD	93	6	70-120	20
2,4′-DDT	75	11	70-120	20
4,4′-DDT	93	10	70-120	20
TCMS (Internal Standard 1)	87	8	ND	ND
DCBP (Internal Standard 2)	103	4	ND	ND

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Comparison and Automation of Gel Permeation Chromatography (GPC), Solid Phase Extraction (SPE), and QuEChERS Extracting Organochlorine Pesticides from Olive Oil

This application, performed in collaboration with Julie Kowalski, Ph.D. at Restek in Bellefonte, PA, USA, investigates each of these separation techniques in separating pesticides from oil matrices and presents detailed information on the automation of each separation system.

Featured Products:

Gilson GPC Cleanup System:

Figure 1. Gilson GX-271 Platform with TRILUTION® LC Used with a 307 Pump and Gilson UV Detector for the GPC Cleanup Method.

Gilson SPE and QuEChERS System:

Figure 2. Gilson GX-271 Preparative Liquid Handler Fitted with the Gilson Orbital Shaker for Liquid Liquid Extraction (LLE) Were Used for the SPE and QuEChERS Methods.

Figure 1

Figure 2

Several techniques, such as GPC, SPE, or QuEChERS, are used today to investigate and isolate pesticides found in edible oils. Each technique provides strengths for its separation of pesticides. GPC has the ability to process large amounts of sample, SPE provides disposable cartridges with numerous sorbents to provide separation of the analyte from the matrix, and QuEChERS involves uncomplicated sample cleanup of pesticides in aqueous matrices.

GPC Cleanup Method

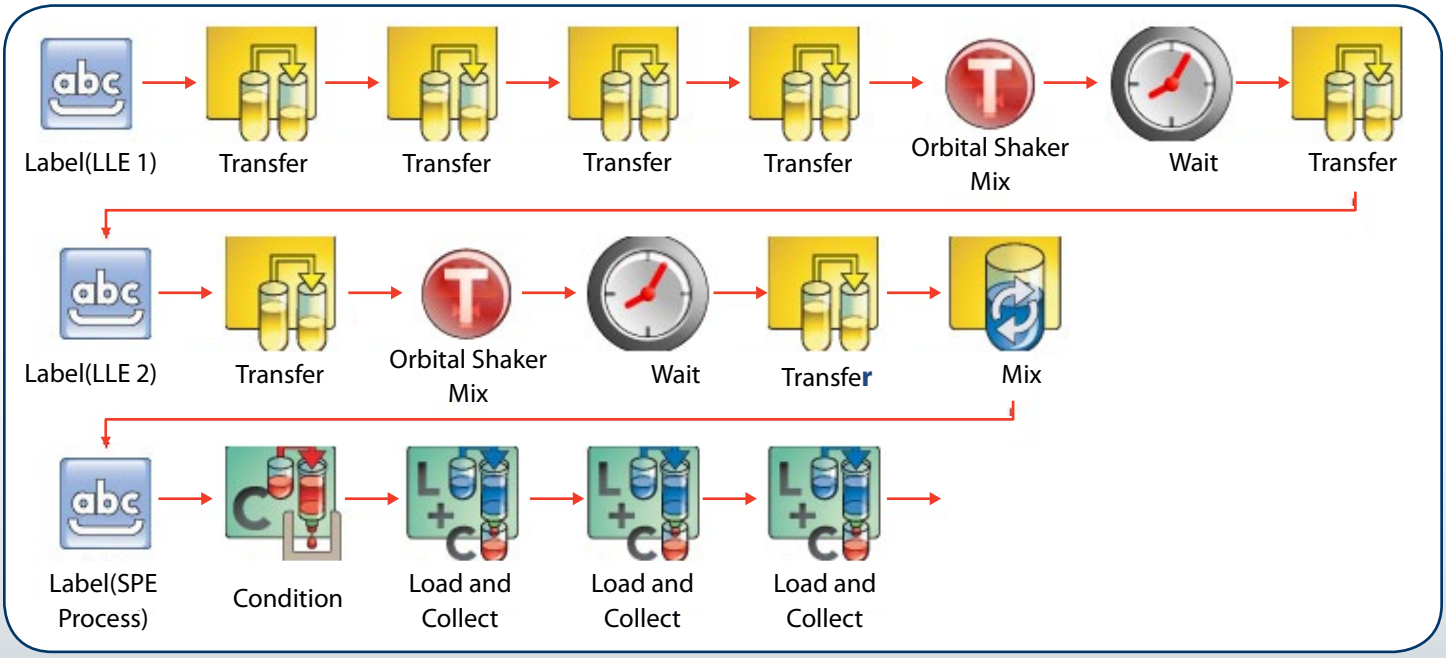
- Inject 1.5 mL of olive oil sample onto Gilson GPC Cleanup System using Phenomenex Envirosep ABC 350 x 21.20 mm GPC Column with 60x 21.20 mm GPC guard column.
- Mobile phase: 50% dichloromethane : 50% hexane at 12 mL/min.
- Start collecting fractions from end of oil peak (8 minutes) to 11 minutes; 6 fractions collected @ 6mL each.
- Combine fractions.

- Dry down eluent.
- Add 200 µL of hexane.
- Inject 1 µL sample onto GC outfitted with Rtx®-CL pesticides column.
- Starting temp 150°C and ramp 3 degrees per minute until 300°C is reached then hold for five minutes.

SPE and LLE Method

- Add 1.5 mL of olive oil sample to 1.5 mL of hexane.
- 3 mL mixture is placed in test tube and 6 mL ACN is added.
- Sample is mixed on an Orbital haker at 720 rpm for 30 minutes.
- Wait 20 minutes for sample to separate.
- Extract top layer (ACN).
- Repeat LLE with 6 mL of ACN.
- Wait 20 minutes for sample to separate.
- Mix ACN LLE fractions together.
- Condition SPE with 5 mL ACN.
- Extract 6 mL of ACN LLE mixture and place in SPE cartridge.
- Elute with 6 mL ACN.
- Collect eluent from sample load and elute.
- Repeat ACN rinse and collect.
- Dry down eluent.
- Add 200 µL of hexane.
- Inject 1 µL sample onto GC outfitted with Rtx-CL pesticides column.
- GC conditions - starting temp @ 150°C and ramp 3 degrees per minute until 300°C is reached; hold for five minutes.

Figure 3. TRILUTION® LH Method for SPE with LLE Process.

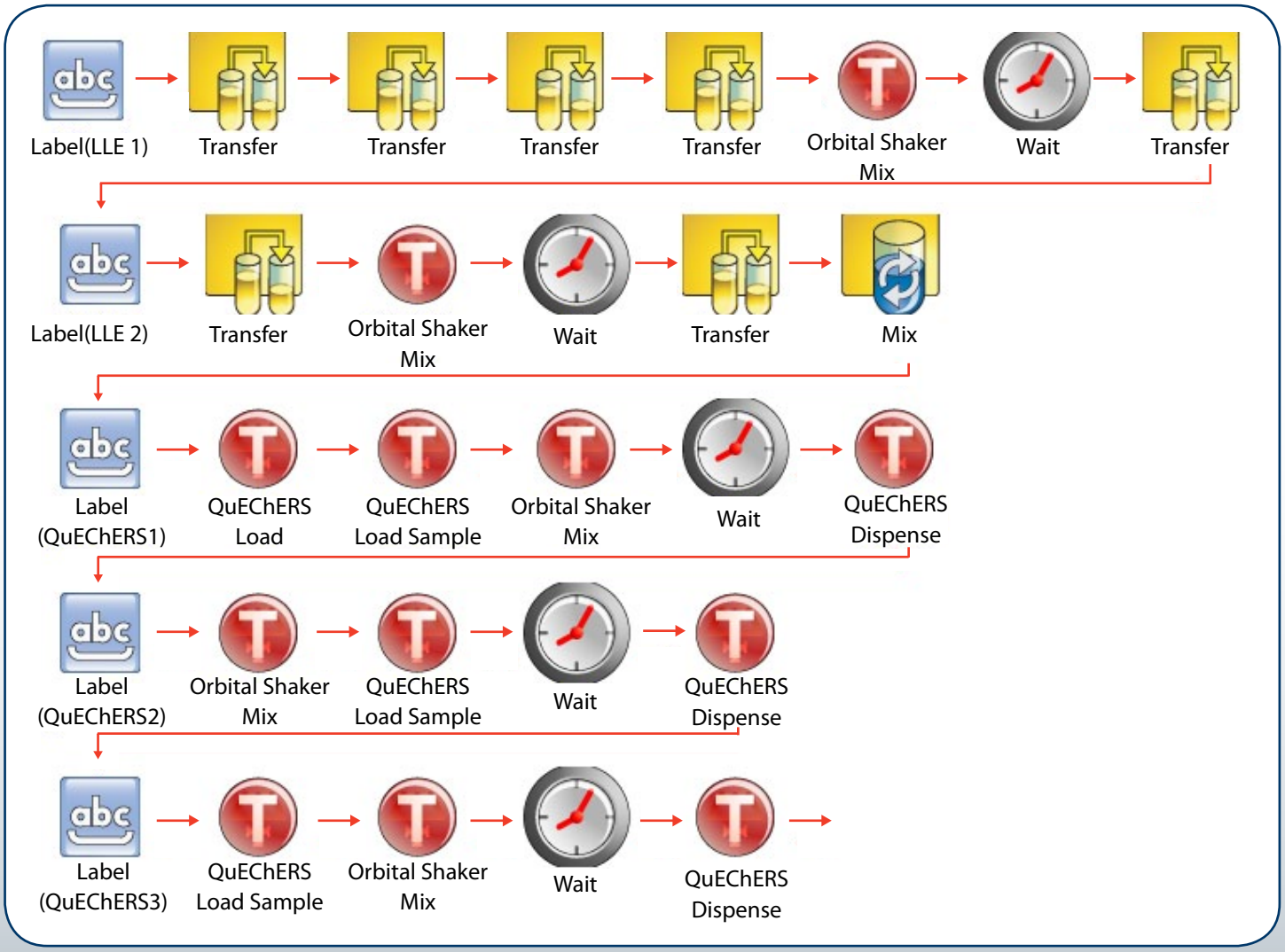


QuEChERS and LLE Method

- 1.5 mL of olive oil sample added to 1.5 mL of hexane.
- 3 mL mixture is placed in test tube and 6 mL ACN is added.
- Mix sample on Gilson Orbital Shaker at 720 rpm for 30 minutes.

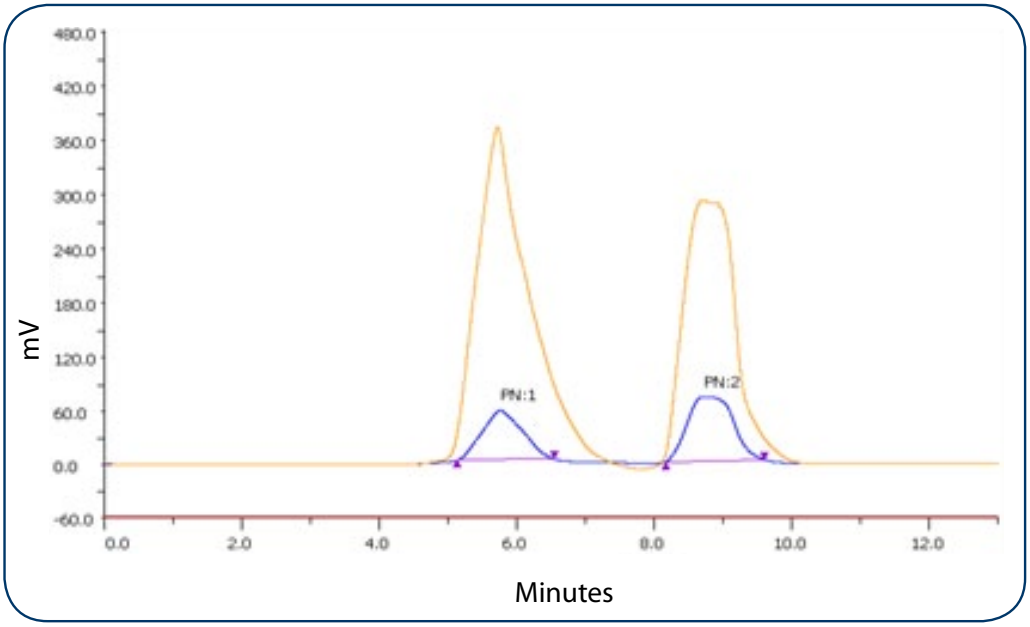
- Wait 20 minutes for sample to separate.
- Repeat LLE with 6 mL of ACN.
- Wait 20 minutes for sample to separate.
- Mix ACN LLE fractions together.
- Condition SPE with 5 mL ACN.
- Extract 1 mL of ACN mixture from the top layer and place in QuEChERS tube; shake on Orbital Shaker at 650 rpm for 2 minutes.
- Push through filter tube and collect eluent.
- Rinse with 2 mL ACN and mix for 5 minutes.
- Push through filter tube and collect eluent.
- Repeat ACN rinse and collect.
- Dry down eluent.
- Add 200 µL of ethyl acetate.
- Inject 1 µL sample onto GC outfitted with Rtx-CL pesticides column.
- GC conditions - starting temp @ 150°C and ramp 3 degrees per minute until 300°C is reached; hold for five minutes.

Figure 4. TRILUTION LH Method for QuEChERS with LLE Process.



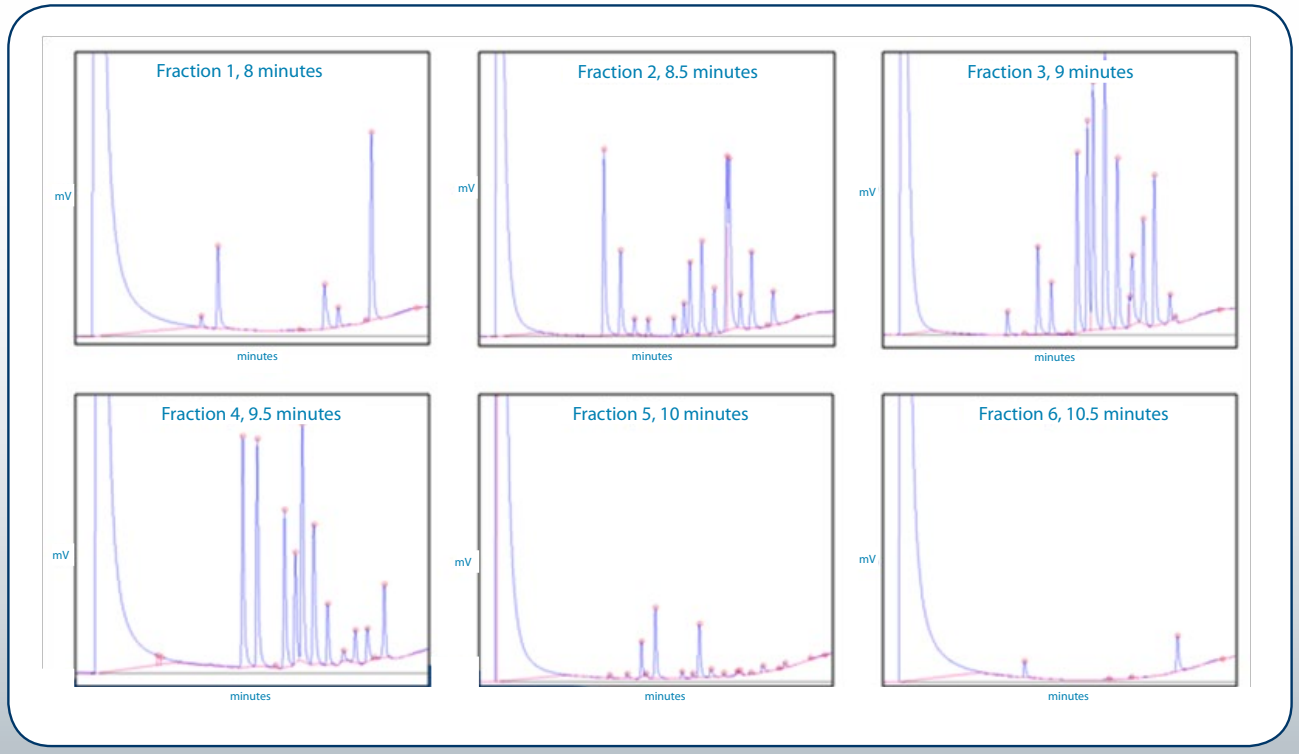
GPC Cleanup Results via UV

The first peak at 6 minutes is the olive oil and therefore was not collected. The second peak containing the pesticides was collected and subsequently analyzed via GC.



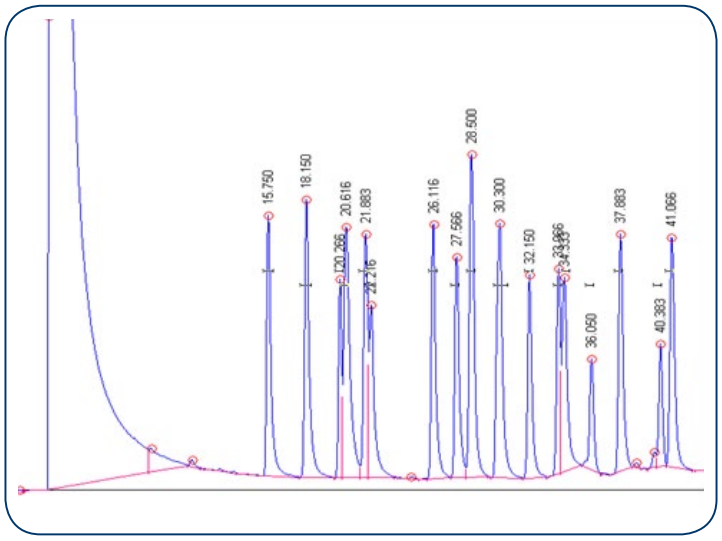
GPC Cleanup Results via GC

Each of the six fractions collected from 8 to 11 minutes (0.5 minute fractions) was analyzed individually to verify the collection region was appropriate prior to collecting another sample where fractions 1-6 were pooled and then analyzed by GC.



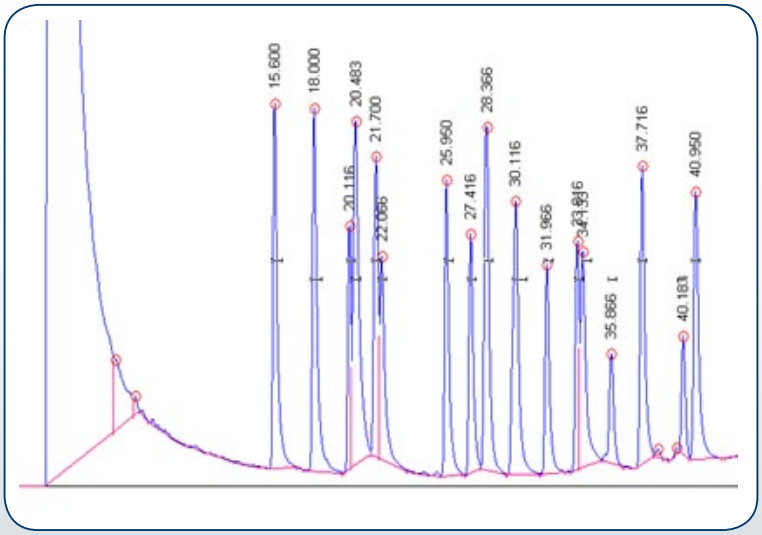
SPE Results via GC

The ability to perform method development of the solid phase extraction allowed for the recovery to be optimized. Sample was loaded to determine breakthrough of pesticides and several elution solvents were tested to determine the highest recovery percentage. To prevent the elution of interfering compounds, the SPE cartridge should not be over saturated with sample. Elution with a minimum of 10 mL of ACN provided highest recoveries.



QuEChERS Results via GC

In the optimization of the QuEChERS method the load of 1 mL was determined by the size of the cartridge and the amount of QuEChERS sorbent and magnesium sulfate within the cartridge. Several solvent amounts and types were tested rinsing the cartridge. Acetonitrile provided the highest recovery yield, and a rinse volume of 2 mL followed by another rinse of the same amount optimized this yield.



Conclusions

All methods attained levels of 25 µg/mL of each pesticide and extrapolated a possible low detectable limit of 5 µg/mL for QuEChERS, 1.7 µg/mL for SPE, and 400 ng/mL for GPC. GPC in this method attained the lowest detectable limits at 3.3 times that of SPE and 10 times that of the QuEChERS extraction method.

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Automated Solid Phase Extraction Method for the Determination of Pesticides in Tea using GC-MS/MS

The data for this application note was provided by K. Lichtmannegger, H. Unterluggauer, and S. Masselter, from the Austrian Agency for Health and Food Safety (AGES) GmbH, Competence Center for Residues of Plant Protection Products, Innsbruck, Austria

Featured Products: GX-271 Liquid Handler with 402 Dual With Tee Syringe Pump, TRILUTION® LH Liquid Handling Software.

Introduction

Tea crops have both worldwide economic impact and widespread consumption due to their specific aroma as well as the promised health benefits, especially for green tea. During cultivation and storage, various pesticides are widely used. Over the past years several reported cases of fungicide and herbicide trace level residues in green tea gained international attention, indicating the need for adequate analytical methods to allow for routine monitoring of this commodity. The method of choice so far for monitoring purposes in Austria is the multi residue method S19 by the Deutsche Forschungsgemeinschaft, German Research Foundation (DFG-S19); however drawbacks include laborious sample preparation and strong matrix interferences (1). Sample tea clean-up using Solid Phase Extraction (SPE) prior to the use of tandem mass spectrometry offers various advantages in selectivity and sensitivity at low quantities and especially in such complex matrices, where these techniques largely reduce these intrinsic matrix effects.

Experimental Conditions

A Gilson GX-271 Liquid Handler with 402 Dual With Tee Syringe Pump was used to automate a Solid Phase Extraction (SPE) method for the extraction of pesticides from tea samples. The extracted pesticide samples were then analyzed by GC-MS/MS (Figure 1). The scope of the investigation included roughly 80 relevant pesticides (organophophorous, organochlorine, pyrethroids, etc.), and the method has been tested in several tea varieties (green tea, mate tea, herbal tea, black tea and chamomile).

Materials

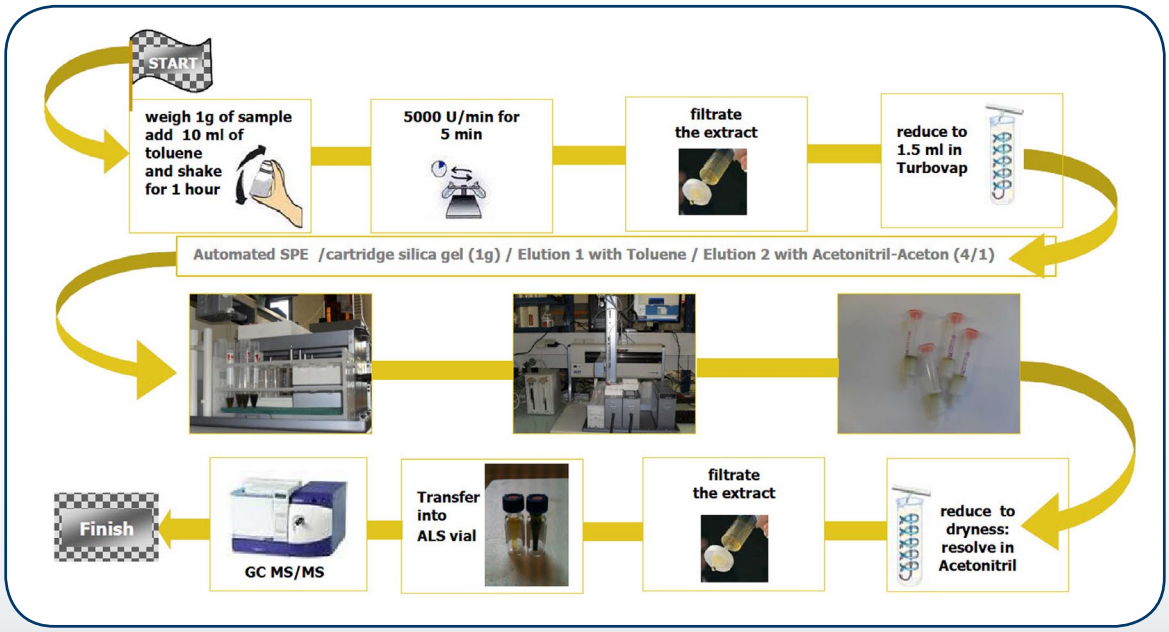
- GX-271 Liquid Handler with 402 With Tee Dual Syringe Pump
- SPE Cartridges: Phenomenex Strata™ SI-1 Silica (55 µM, 70 Å) 1 g/6 mL
- SPE Solutions

    - Toluene
    - Acetonitrile:Acetone (4:1)
- GC- MS/MS (Waters Quattro micro GC™)

Method

- Weigh 1 g of tea sample spiked with pesticide standard
    - Add 10 mL of toluene
    - Shake for 1 hour
- Spin at 5000 rpm for 5 minutes
- Filter the extract
- Reduce filtrate to 1.5 mL in Turbovap®
- Automated SPE (using Gilson)
    - Load: 1.5 mL sample extract
    - Elution 1: Toluene
    - Elution 2: Acetonitrile:Acetone (4:1)
- Reduce to dryness with Turbovap
- Reconstitute in Acetonitrile
- Filter the extract
- Transfer into ALS vial
- Analyze by GC-MS/MS

Figure 1. Extraction and Analysis of Pesticides in Tea Samples.



Results

Method validation calculations were performed on matrix samples spiked at two concentration levels each (near the LOD of 10 µg/kg and 100 µg/kg). The results clearly demonstrate good linearity, recoveries between 70-140% for the majority of analytes and adequate precision (average RSD of 10.6%), meeting the criteria of EU guidelines (SANCO/10684/ 2009) (Table 1). In addition, the presented method was successfully validated using a Food Analysis Performance Assessment Scheme (FAPAS®) tea sample from 2006 (Table 2).

Table 1. Validation Data for Representative Analytes in Tea Matrix.

Organic Group	Pesticide	RSD (%)	Recovery (%)
Organochlorine	Hexachlorbenzol	8	114
	Dieldrin	19	123
	Heptachlorepoxid	7	109
	4-4'-DDD	3	109
	2,4'-DDE	3	121
	Endosulfan-alpha	6	115
Pyrethroid	Bifenthrin	6	157
	Cypermethrin techn.	9	104
	Deltamethrin	4	114
	Lambda-Cyalothrin	14	96
	Permethrin	12	101
	Fenvalerate	7	121
Organophosphorous	Chlorpyriphos	6	111
	Ethion	5	111
	Fenitrothion	11	106
	Malathion	10	106
	Pirimiphos-methyl	23	77
	Triazaphos	8	118

Table 2. Validation of the Method Using FAPAS Reference Material.

FAPAS (PT 2006) - Tea Matrix		
	Assigned Value (mg/kg)	Analyzed Value (mg/kg)
p.p.-DDD	0.204	0.176
Ethion	0.332	0.386
Quintozene	0.094	0.082

The method was also tested for several real tea samples. In every sample batch, two spiked blank matrices (10 µg/kg and 100 µg/kg) were analyzed and used for calibration purposes. Only in cases of Maximum Residue Limit (MRL) violations was the alternative approach of standard addition for quantification performed. In the Chinese green tea, Fenvalerate was found to be nearly twice the MRL (Table 3).

Table 3. Analysis of Chinese Green Tea.

Compounds	Analyzed Value (mg/kg)	MRL (mg/kg)	% of MRL
Bifenthrin	0.197	5.00	3.9
Chlorpyriphos	0.009	0.10	9.0
Cypermethrin	0.048	0.50	9.6
Fenvalerate	0.093	0.05	186
1-Cyhatothrin	0.07	1.00	7.0
Endosulfane	0.092	30.00	0.3

Summary

The method presented demonstrates a rapid, sensitive and straightforward multi-residue approach for pesticide residue analysis in tea samples combining an automated extraction and clean-up procedure with the strength of GC-MS/MS tandem mass spectrometry and is thus suitable for food monitoring according to EU legal requirements. As a next step, the method has to be validated for its suitability in daily routine analysis - with the possibility to broaden the scope of investigation.

References

1. JMPR, 2004. Pesticide residues in Food 2004. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues, Rome, Italy, 20-29 September 2004. WHO and FAO, Rome 2004.

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Analysis of Acaricides from Honey Following Automated Solid Phase Extraction (SPE) Optimization Using the Gilson GX-271 ASPEC

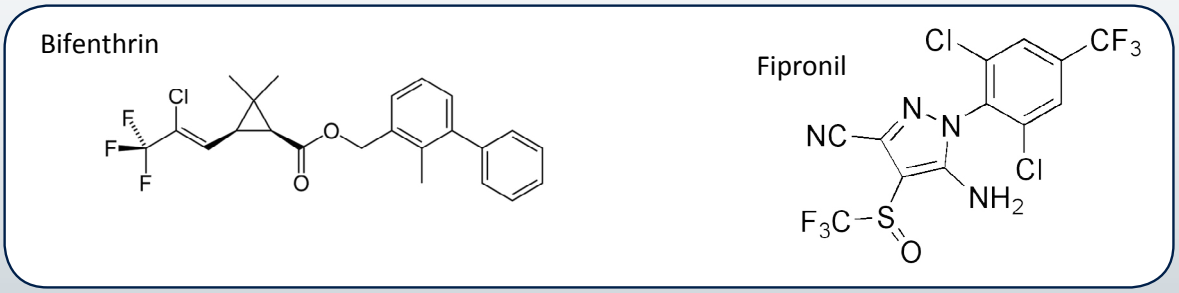
Featured Products: Gilson GX-271 ASPEC™ System with 406 Single Syringe Pump Operated Using TRILUTION® LH Liquid Handling Software (left). GX-271 Analytical HPLC System Operated Using TRILUTION® LC Liquid Chromatography Software (right).

Introduction

With the recent decline in hive bee populations, investigations into reasons why have focused on the presence of acaricides in the environment. Acaricides are insecticides used to aid agriculture, such as control of ticks and mites commonly found on cattle. Specifically, investigations are focusing on the quantity of acaricides in honey. Automation of this separation technique would provide laboratories with an efficient method to extract the acaricides for analytical analysis.

Bifenthrin and Fipronil are the acaricides of focus that are extracted from honey samples. Both are used as insecticides. Solid Phase Extraction (SPE) is the technique of choice used for automated sample preparation of these compounds from honey just prior to High Pressure Liquid Chromatography (HPLC). The Gilson GX-271 ASPEC was used to automate the solid phase extraction optimization and routine process. Depending on the detection limits required, additional research has indicated lower levels of detection can be achieved using mass spectrometry (LC/MS or GC/MS).

Figure 1. Chemical Structures of Bifenthrin and Fipronil.



Experimental Conditions

Materials

Note: All solvents used were HPLC grade or higher. All reagents were ACS grade or better.

- SPE System: GX-271 ASPEC with single 406 Syringe Pump
    - TRILUTION LH software
- SPE Cartridges: Strata® C18, 3 mL (Phenomenex, Torrance, CA, USA). The cartridges were sealed using 3 mL Gilson Sealing Caps.
- HPLC system: Gilson GX-271 Analytical HPLC System (GX-271 Liquid Handler, 402 Single Syringe Pump, 306 Pumps, 811D Analytical Mixer, 805 Manometric Module, 155 Dual Wavelength Detector)
- HPLC column: XTerra® 4.6 x 150 mm C18 (Waters Corporation, Milford, MA, USA)
- HPLC Mobile phase – 1.3 mL/min isocratic
    - A: Acetonitrile (85%)
    - B: Water (15%)
- Injection Volume: 100 µL
- Honey Sample: Obtained at a local grocery store
- Analytical Acaricide Standards:
    - Spiked at 0.25 µg each Acaricide
    - Fipronil and Bifenthrin (Obtained from Sigma Aldrich, St. Louis, MO, USA)

Extraction Method

Honey samples were prepared and extracted manually prior to automated solid phase extraction:

1. 10 grams of honey was weighed.
2. 20 mL of filtered water:ethanol (50:50 v:v) was added to weighed honey sample.
3. Sample mixture was shaken for 30 minutes.
4. Samples were spiked with Fipronil and Bifenthrin.

Automated SPE Optimization Method for Fipronil and Bifenthrin

1. SPE Cartridge Condition Optimization:
    a. Test different condition solvents: methanol, water, ethanol, and acetonitrile.
    b. Conditioning solvent was collected and tested for breakthrough.
2. Sample Load Optimization:
    a. Condition SPE cartridge using optimized conditioning solvent.
    b. Differing concentrations of Fipronil and Bifenthrin were tested.
    c. Load volume was collected and tested for breakthrough.
3. Wash Solvent & Volume Optimization:
    a. Condition SPE cartridge using optimized conditioning solvent.
    b. Load SPE cartridge using optimized load volume.
    c. Test different wash volumes and solvents.
    d. Wash volume was collected and tested for breakthrough of Fipronil, Bifenthrin, and any interfering compounds.
    e. Optimization volume was the volume that eluted interfering compounds but kept Fipronil and Bifenthrin on the SPE column.
4. Elute Solvent & Volume Optimization:
    a. Condition SPE cartridge using optimized conditioning solvent.
    b. Load SPE cartridge using optimized load volume.
    c. Wash SPE cartridge using optimized wash volume.
    d. Test different elute volumes and solvents.

e. Elute volume was collected and tested for recovery of Fipronil and Bifenthrin.
f. Optimization volume was the smallest volume that eluted Fipronil and Bifenthrin from the SPE column with the largest recovery.

Automated SPE Final Method for Fipronil and Bifenthrin

The SPE steps are summarized with the schematic provided in the GX-271 ASPEC control software, TRILUTION LH (Figure 2).

1. Condition SPE cartridge with 5 mL of methanol at a flow rate of 1 mL/min.
2. Condition SPE Cartridge with 5 mL of water at 1 mL/min.
3. Load extracted sample.
4. Wash twice.
   a. 2mL water at 1 mL/min.
   b. 2 mL water:ethanol (50:50 v:v) at 1 mL/min.
5. Dry SPE columns to remove any excess water.
6. Elute the analytes of interest with 4 mL ethyl acetate:dichloromethane (50:50 v:v) at 1 mL/min.
7. Evaporate to with nitrogen and bring back to 1 mL with acetonitrile:water (80:20 v:v).

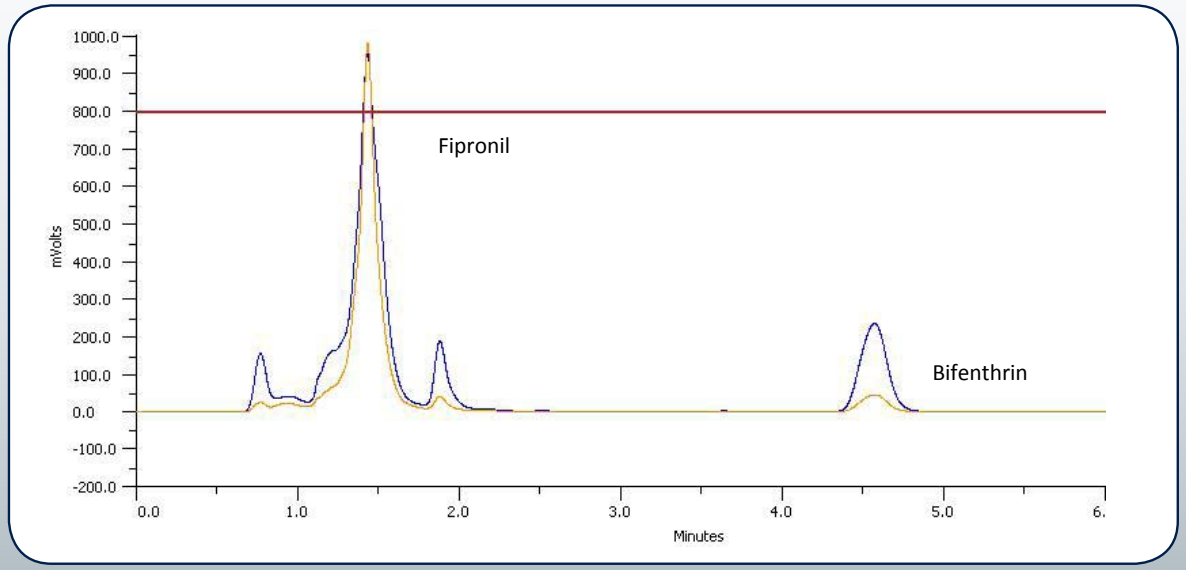
Figure 2. TRILUTION LH SPE Tasks for Extraction of Fipronil and Bifenthrin from Honey.

HPLC Analysis

Following the automated SPE method, a sample volume of 100µL was injected onto the Gilson GX-271 Analytical HPLC System. A 6 minute run time eluted both Fipronil and Bifenthrin.

Results

Figure 3. HPLC Analysis of Fipronil and Bifenthrin from Honey.



This application describes the importance of using Gilson GX-271 ASPEC automation to streamline the optimization procedure of solid phase extraction. Eliminating interferences and increasing recovery values can assist with obtaining low limits of detection (LOD). In this application, an LOD of 25 ng/gram of Fipronil and Bifenthrin were obtained in honey samples. Fipronil eluted from the Gilson GX-271 Analytical HPLC System at 1.9 minutes, while Bifenthrin eluted at 4.6 minutes.

As industry continues to look at the effect of using acaricides or alternatives for insecticides, the impact on bees is important to monitor. The environmental impact of these compounds on pollination, as well as honey production.

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Gel Permeation Chromatography (GPC) Clean-up of Plastic Additives from Edible Oils

Featured Product: Automated GX-271 GPC Clean-up System.

Plastics are ubiquitous in our society and are extensively used in a large variety of packaged foods. Plastics are manufactured by combining monomers into polymers under heat and pressure (polymerization). A variety of additives are often added to plastics. Some commonly used additives include plasticizers (such as phthalates) to improve flexibility, UV filters for protection from sunlight, brightening agents, coloring agents and preservatives. Some of these additives may migrate into food products that have been packaged using plastics. There is a great deal of interest in measuring the migration of these compounds into food products.

Gel permeation chromatography (GPC) is a size exclusion clean-up procedure that uses organic solvents and a hydrophobic gel to separate macromolecules. It is a highly effective method for the removal of high molecular weight interferences such as lipids from a fatty food prior to analysis for the compounds of interest. GPC clean-up has been used to clean-up fatty foods prior to analysis for plasticizers and other plastic additives.

This application note describes the use of GPC clean-up to separate a variety of plastics additives such as phthalates, Chimassorb® 81 (a UV absorber and stabilizer), Irganox® 1076 (an optical brightener) and Uvitex® OB (an antioxidant and thermal stabilizer) from a representative edible oil sample prior to analysis.

Experimental Conditions

All solvents were HPLC grade or higher. All reagents were ACS grade or higher. Phthalates and other plasticizer additives were obtained from Sigma-Aldrich (Table 1). Standards were prepared in 1:1 ethyl acetate/cyclohexane. Corn oil was obtained from a local market. GPC calibration standards were prepared according to USEPA Method 3640A in 1:1 ethyl acetate/cyclohexane (see Figure 1).

Table 1. Plasticizer Additives Used in the Study.

Compound	Trade Name/ Abbreviation	Formula Weight
Dimethyl phthalate	DMP	194.19
Diethyl phthalate	DEP	222.24
Dibutyl phthalate	DBP	278.35
2-ethyl hexyl phthalate	DEHP	390.56
Butyl benzyl phthalate	BBP	312.36
Diisobutyl phthalate	DIBP	278.35
Diisononyl phthalate	DINP	418.61
Diphenyl phthalate	DPP	318.33
Di-n-octyl phthalate	DNOP	390.56
Octadecyl 3- (3,5-di-tert-butyl-4-hydroxyphenyl) proprionate	Irganox® 1076	530.88
2-Hydroxy-4 (octyloxy)-benzophenone	Chimassorb® 81	326.4
2,5 - Bis (5-tert-butyl-2-benzoxazolyl) thiophene	Uvitex® OB	430.57

GPC Clean-up Protocol

GPC separation was achieved using the Automated GX-271 GPC Clean-up System and a Phenomenex EnviroSep-ABC™ GPC column with a mobile phase of 1:1 ethyl acetate/cyclohexane at a flow rate of 5 mL/min. The injection volume was 1mL. The column was calibrated using a GPC calibration standard (as described above), a Gilson 112 UV Detector set at 254 nm and Gilson TRILUTION® LC software (see Figure 1).

Results

Figure 1. Chromatogram of a USEPA Method 3640A Calibration Standard using a Phenomenex EnviroSep-ABC Column with a Mobile Phase of 1:1 Ethyl Acetate/Cyclohexane. Retention Times: Corn Oil = 12.6 min, DEHP = 15.6 min, Methoxychlor = 19.7 minutes and Perylene = 38.6 minutes.

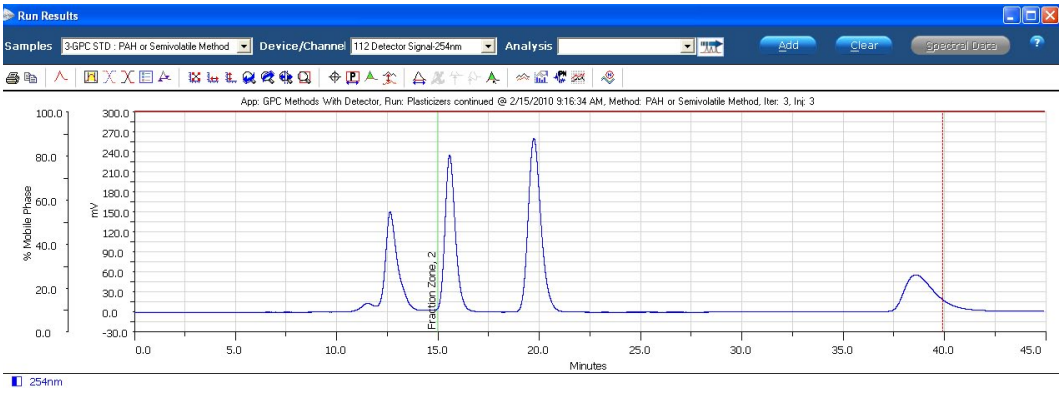


Figure 2. Chromatogram Showing Retention Time of Diisononyl phthalate (DINP).

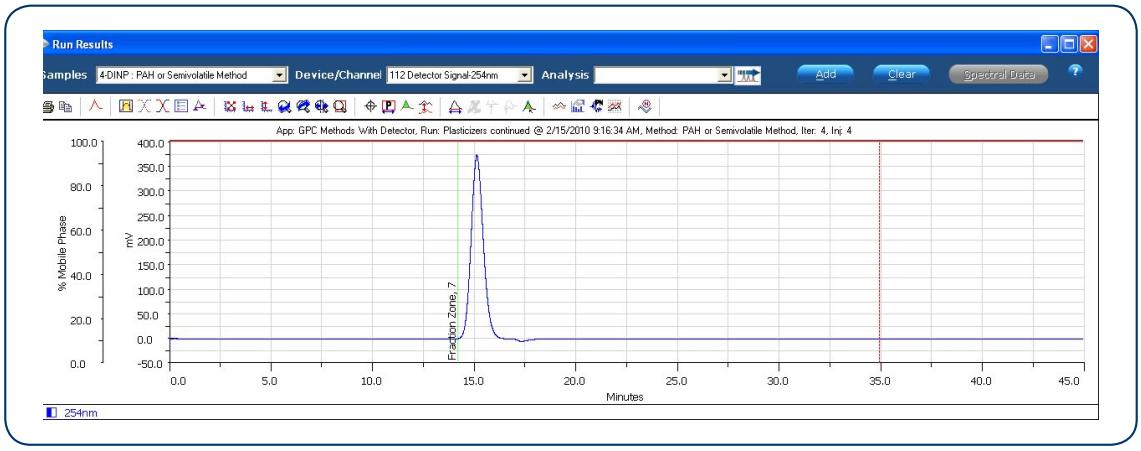
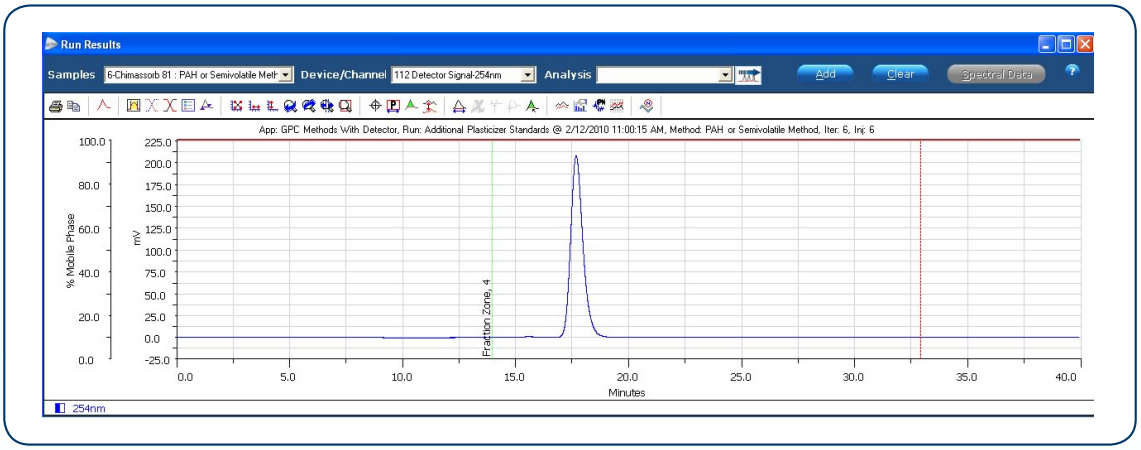


Figure 3. Chromatogram Showing Retention Time of Chimassorb 81.



All plastic additives separated well from the representative edible oil (corn oil) as shown in Table 2.

Table 2. Retention Times for Plasticizer Additives, Corn Oil and GPC Standards (For abbreviation key, see Table 1).

Additives	RT (min)
Corn Oil	12.6
Irganox® 1076	13.7
DINP	15.1
DNOP	15.5
DEHP	15.6
DIBP	17.1
Uvitex® OB	17.5
DBP	17.6

Additives	RT (min)
Chimassorb® 81	17.7
BBP	18.5
DEP	19.3
Methoxychlor (GPC Std)	19.7
DPP	19.9
DMP	21.0
Perylene (GPC Std)	38.6

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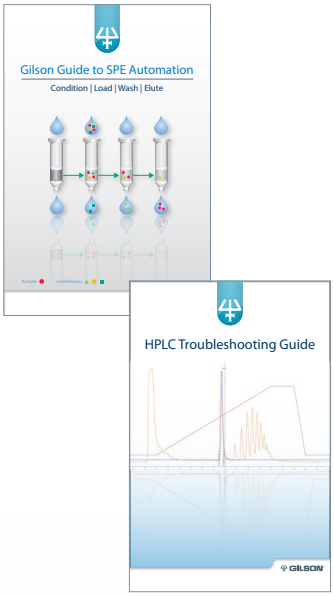

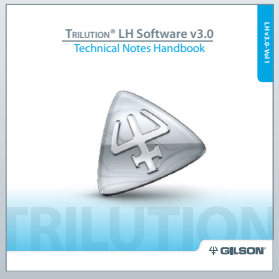

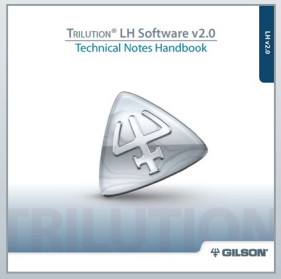

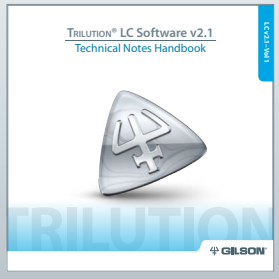

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