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

Keywords: Daidzein, Endocrine Disruptors, Environment, Genistein, GX-271 ASPEC™, LC/TOF-MS, Phytoestrogens, Solid Phase Extraction, SPE, Water, Wastewater

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Introduction

Phytoestrogens are a group of nonsteroidal 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).

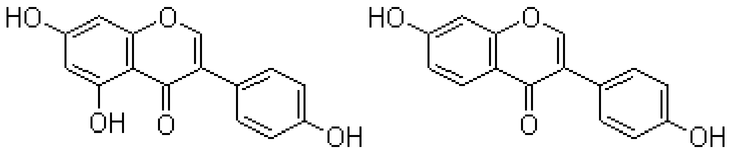

Figure 1. Chemical structures of two common phytoestrogens found in plants—from left to right is genistein and diadzein. *Chemical structures were obtained from ChemBlink at www.chemblink.com.*

There is a great deal of 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 (Figure 2).

Figure 2. Gilson GX-271 ASPEC System with 406 Single Syringe Pump (Part no. 2614007).

Experimental Conditions

Materials

All solvents were distilled in glass suitable for GC, HPLC, pesticide residues analysis and spectrophotometry. HPLC solvents were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from Fluka (Buchs, Switzerland). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA) was used throughout the study to obtain HPLC-grade water. Analytical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and 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. The SPE procedure used Oasis™ HLB (200 mg, 6 mL) obtained from Waters Corporation (Milford, MA, USA).

Sample Preparation of Water Samples

Wastewater samples were collected from effluent locations downstream from a soy processing plant as well as upstream and downstream from a municipal wastewater treatment plant. Extraction was performed using the Gilson (Middleton, WI, USA) GX-271 ASPEC System.

SPE Hardware

The Gilson GX-271 ASPEC System was configured as follows:

Description	**Part Numbers**
GX-271 ASPEC w/ Single 406 Syringe Pump	2614007
25 mL Syringe	25025346
SPE Pressure Reg. Assembly and plumbing package for gas + 25 mL Plumbing Package	25051376, 2644703 and 2644702
221x1.5x1.1 BV Tapered Probe and Guide Assembly for 1.5 mm Probes	27067374 and 26046228
Rinse Stations	26034551 and 26034555
Locator Tray for three 200-series Racks	26041035
Rack Code 211 for 9 – 48 x 113mm (125 mL) bottles	2504611
Rack Code228 for 4 – (500mL or 700mL) bottles and pkg of four 700 mL bottles	2504628 and 543701700
Rack Code 306 for 20 – (6 mL) SPE cartridges and Disposable Sealing Caps for 6 mL SPE cartridges	2504306 and 2954730
Glass collection tubes for 6 mL DECs, 10 mL (15 x 85mm), pkg of 1000	2954729
Viton tubing, .313 ID x .438 OD, 20 ft	4701438630
Safety Shield Assembly, GX27X	2604706
TRILUTION® LH Software Package	21063020, 210630R20 and ORACLE10GXE

Solid Phase Extraction (SPE) Protocol

The SPE procedure used 6 mL Waters Oasis™ HLB (200 mg) Cartridges. The cartridges were sealed using Gilson 6 mL Sealing Caps.

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 2).

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

The details of each step are as follows:

- Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack (Figure 3) and probe rinsed with 10% methanol
- Condition SPE cartridge with 4 mL of methanol at a flow rate of 1 mL/min
- Condition SPE Cartridge with 6 mL of water at 1 mL/min
- 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.
- Move the Gilson Mobile SPE Rack over the collection tubes
- Elute the analytes of interest with 5 mL methanol at 1 mL/min
- 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 3. Gilson Mobile Rack. This rack allows for easy method development.

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) equipped with a reversed phase C_8 analytical column of 150 mm x 4.6 mm and 5 μm particle size (Zorbax Eclipse XDB-C8). Column temperature was maintained at 25°C. The injected sample volume was 50 μL. Mobile phases A and B were acetonitrile and water with 0.1% formic acid, respectively. The optimized chromatographic method held the initial mobile phase composition (10% A) constant for 5 min, followed by a linear gradient to 100% A in 25 min. The flow rate used was 0.6 mL/min. A 10-min post-run time was used after each analysis. 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. The data recorded was processed with 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 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,3H-tetrafluoro-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

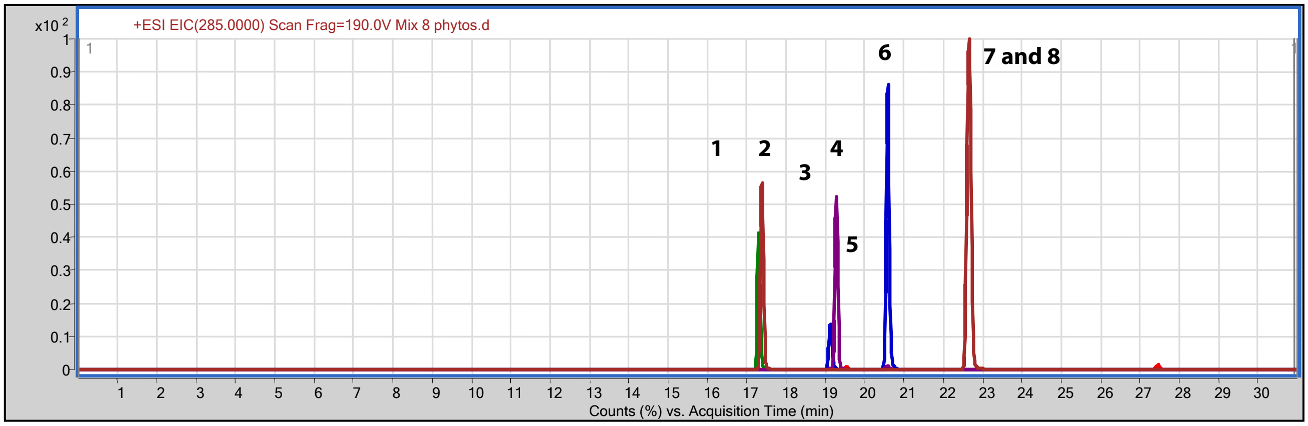

Figure 4. LC/TOF-MS ion chromatogram for a mixture of phytoestrogens. Compound labels: 1 = Daidzein, 2 = Glycitein, 3 = Coumestrol, 4 = Genistein, 5 = Equol, 6 = Formononetin, 7 and 8 = Biochanin A and Prunetin.

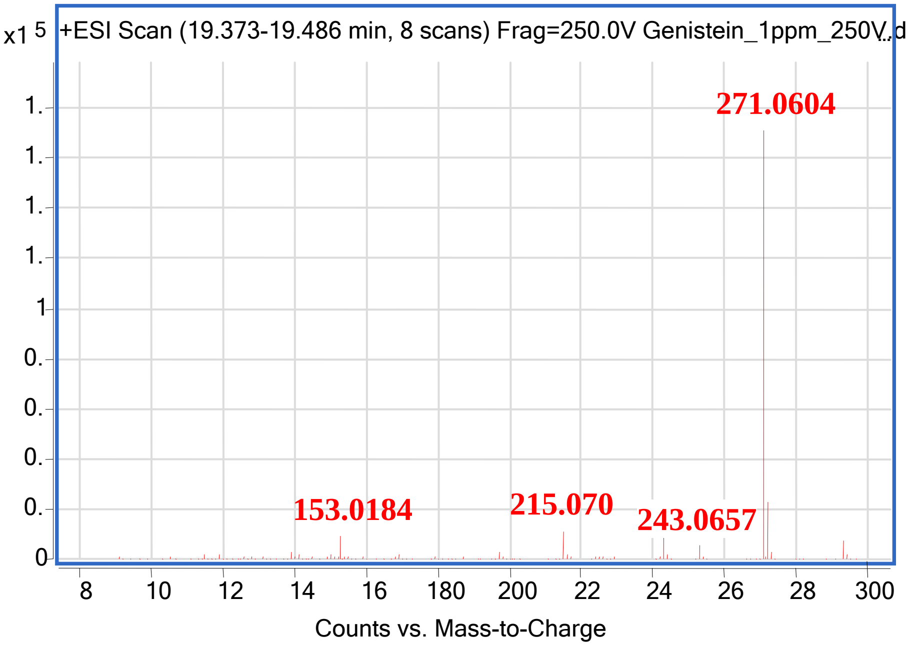

Figure 5. Mass Spectrum for Genistein.

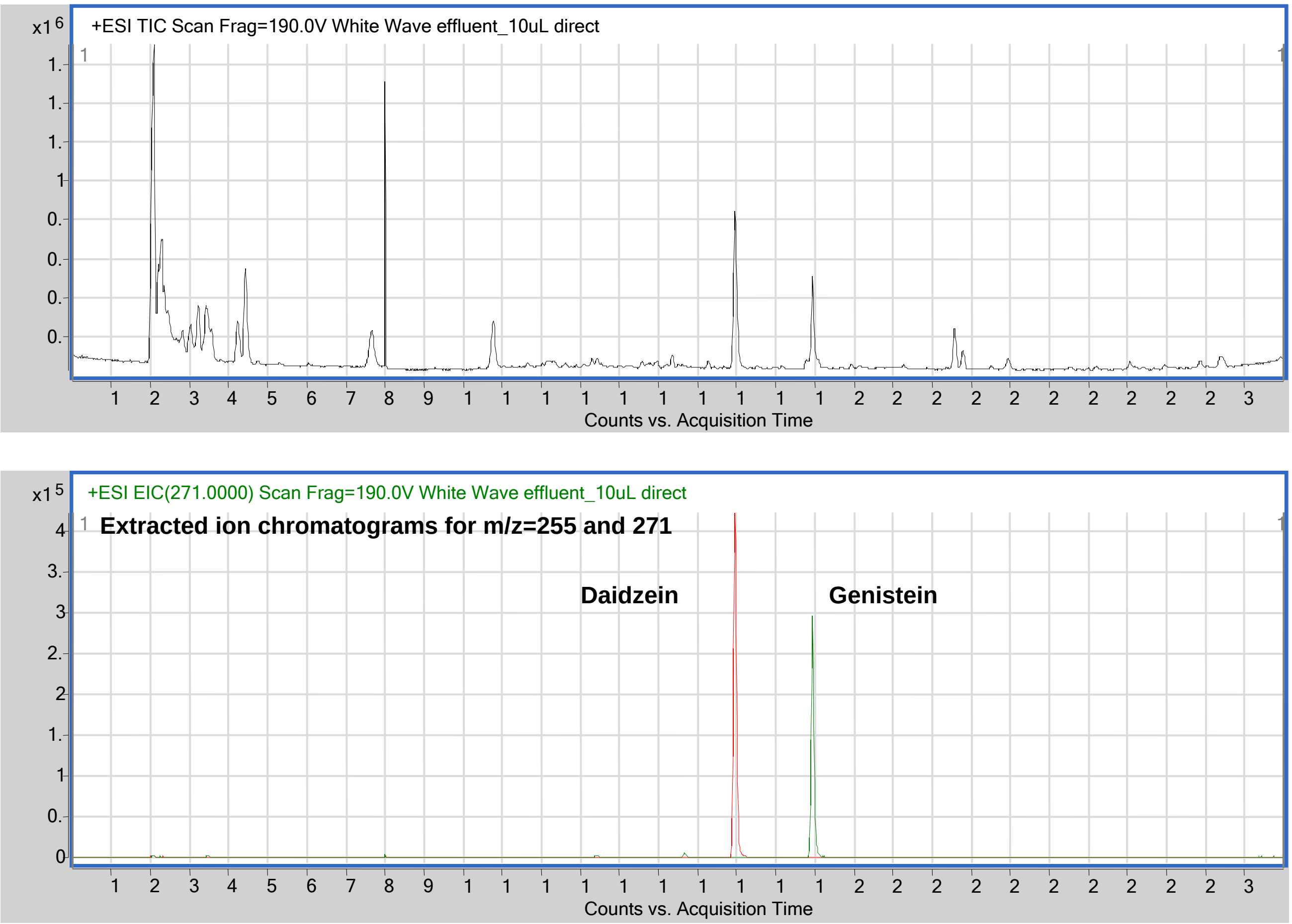

Figure 6. LC/TOF-MS chromatogram of wastewater effluent from soy processing plant.

Table 4. 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	Wastewater Treatment Plant Effluent
Genistein	50,000	2000	20	<1
Daidzein	15,000	500	20	<1
Glycitein	200	50	<1	<1

Conclusion

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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