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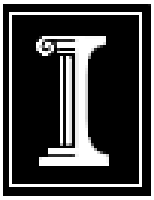
TR-042
March 2010
www.istc.illinois.edu



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March 2010

Submitted to the
Illinois Sustainable Technology Center
Institute of Natural Resource Sustainability
University of Illinois at Urbana-Champaign
www.istc.illinois.edu

The report is available on-line at:
http://www.istc.illinois.edu/info/library_docs/TR/TR-042.pdf

Printed by the Authority of the State of Illinois
Patrick J. Quinn, Governor

Acknowledgments

This research was funded in part by the Illinois Sustainable Technology Center (ISTC), a division of the Institute of Natural Resource Sustainability at the University of Illinois at Urbana-Champaign (Grant no. HWR06198).

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Abstract

Isoflavones are phytoestrogens that are abundant in the legume family. Little information exists on the presence of these compounds in the agricultural environment. We conducted a preliminary investigation of selected phytoestrogens in a small highly-cultivated watershed in central Illinois. Paired water and sediment samples were collected from 7 different locations in central Illinois during 2007 and analyzed for a suite of 14 phytoestrogens, including 12 isoflavones, coumestrol, and flavone (used as an internal standard). Seven of the phytoestrogens we measured were detected in low concentrations (parts per trillion) in water, whereas eight analytes were observed in sediment samples at higher concentrations (parts per billion) than in water samples. Our results suggested seasonality to the observed presence of these compounds. Based on the patterns observed in parent compounds and their metabolites, it appears that there are multiple sources of these phytoestrogens in the agricultural environment. Additional studies to further elucidate the patterns observed in this pilot study are warranted.

[*Editorial note: This project was funded by ISTC on a limited basis as a seed project. There were some analytical issues due to severe matrix suppression in LC/MS which may limit the usefulness of some of the results and these should be taken into consideration as readers view the report.*]

Introduction

Phytoestrogens are non-steroidal phenolic plant compounds that are abundant in the legume family, especially in soy, clover and alfalfa. Phytoestrogens are similar in structure to estradiol; thus, they may have estrogenic, antiestrogenic or antiandrogenic effects in animals (Jarred et al. 2003). Reproductive effects in mammals may include: reduced fecundity (Jefferson et al. 2007); feminization (Wisnieski et al. 2005) and suppression of sexual behavior in young males (Whitten et al. 1995); delayed puberty (Levy et al. 1995); vaginal acyclicity (Whitten et al. 1993); and changes in uterine weight and estrogen receptor levels (Medlock et al 1995). Exposure of fish to isoflavones has induced vitellogenesis (Pelissero et al. 1991), produced changes in gonadal development and feminization of males (Kiparissis et al. 2003), and reduced aggressive behavior in males (Clotfelter and Rodriguez 2006). Genistein has also been shown to arrest the development of frog embryos (Ingham et al. 2004). Flavone, a related plant secondary compound, produced changes in sex ratios, an increase in atresic oocytes, intersex, and other changes in the gonads of frogs (Mackenzie et al. 2003).

Little information exists regarding the presence of phytoestrogens, more specifically isoflavones, in the environment. Previously, these compounds have been detected in sewage treatment influent (Bacaloni et al. 2005), effluent (Bacaloni et al. 2005; Pawlowski et al. 2004; Spengler et al. 2001) and receiving streams (Bacaloni et al. 2005); pulp mill effluent (Kiparissis et al. 2001); and stored manure and runoff from manure-treated fields (Burnison et al. 2003). However, there have been no previous studies that have examined water and sediments in agricultural areas producing large volumes of soybeans.

Given the large acreages in central Illinois that are planted with soybeans, as well as the presence of cattle pastures in less-productive (i.e., lower soil fertility) or less easily cultivated locations, we hypothesized that we would detect selected phytoestrogens, especially the soy isoflavones, in a small watershed in this region. Additionally, because no established reference method existed, we worked to develop and evaluate a method for the extraction of isoflavones from sediment samples followed by clean-up of the extract before analysis by liquid chromatography electrospray ionization tandem mass spectrometry.

Methods

Sample Collections

Water and sediment samples were all collected in a highly agriculturalized portion of east-central Illinois. Water samples were collected from the middle of the water column into an acid-rinsed glass bottle with a Teflon lid-insert. Sediment samples were collected into an acid-rinsed glass jar with a Teflon lid-insert and included the top ten cm (approximately) of sediment and associated detritus.

A total of ten paired water and sediment samples were collected in 2007. Initially, two water and sediment samples were collected for preliminary methods development. These samples were collected from approximately 1.0 km downstream of the outflow of a small sewage treatment plant, and from a pond receiving water primarily from a small agricultural watershed. Four paired samples were collected for analysis during May 2007. A soil sample was collected from an early-season soybean field and a water sample was collected from a nearby well (FARM location). Additionally, water and sediment samples were collected from three locations within a small, highly agriculturalized watershed in east-central Illinois (Appendix A). Two of these locations, POND and CREEK, were sampled in May and July. The TILE outflow was sampled in May only. Two additional sites, the stream below the POND's dam (DAM) and a wetland (WETLAND) located on a nearby conservation area, were also sampled during July.

Mapping

A mapping component was conducted using ESRI ArcGIS version 9.2 GIS software. The boundaries of the watershed that included the TILE, CREEK and POND locations were determined by examining the contour lines of Digital Raster Graphics (DRG) images (scanned U.S. Geological Survey standard series 7.5 minute topographic maps), further adjusted with the Illinois statewide 30-meter shaded relief image, derived from the Illinois Statewide 30-Meter Digital Elevation Model of 2003. Field boundary information for cover-mapping was obtained from the USDA National Agriculture Statistics Service's 1:100,000-scale 2006 cropland data layer for Illinois. Field boundaries and this year's cropping patterns were further adjusted based on field reconnaissance. FARM fell just outside of the watershed but is included on the map; WETLAND was located several kilometers away and is not included.

Extraction of water samples

We followed closely the methods of Bacaloni et al. (2005) for the extraction of water samples. Approximately 1 liter water samples were collected in 1 L glass bottles and the entire sample volume was extracted before analysis. Waters Oasis HLB cartridges (6 cc, 200 mg, WAT106202) were pre-conditioned by washing sequentially with (1) 5 mL of methanol:water (50:50, v/v) containing 0.5 moles/L acetic acid, (2) 5 mL of methanol, and (3) 5 mL of deionized water. Samples were filtered through 47 mm GF/C glass fiber filters first and then passed through the Oasis solid phase extraction cartridges, fitted with 60 mL reservoirs, at a flow rate of approximately 15-20 mL/min or less. The cartridges were washed with 10 mL of nanopure water, followed by 1 mL of methanol:water (15:85, v/v) containing 0.5 moles/L acetic acid. Air was pulled through the cartridges for approximately 30 minutes to dry the cartridges. Subsequently, 150 µL of methanol was added to the cartridge to promote further drying. The elution was performed with 2 x 5 mL aliquots of methanol. The eluate was collected in 15 mL glass vials and the volume reduced to dryness by heating at approximately 37° C under nitrogen

flow. Analytes were redissolved in 0.5 mL of acetonitrile:water (25/75) and 25 µL of flavone (~1000 ng/mL) was added as an internal standard. Samples were shaken and sonicated for approximately 15 minutes, then filtered through 0.45 µm pore size nylon syringe filters directly into vials for liquid chromatography-tandem mass spectroscopy (LC/MS/MS) analysis.

Drying, Extraction and Clean-up of Soil Samples

There was no reference method for the extraction of soil samples. Therefore, we developed a method in our laboratory using spiked samples. Soils were dried at room temperature in glass dishes for several days. Samples were stirred and broken up periodically to facilitate drying. Soils were ground with a mortar and pestle and 10 g were transferred to 125 mL Erlenmeyer flasks fitted with ground glass stoppers. Forty mL of 25:70:5 acetonitrile:methanol:acetone were added to each. The samples were capped, shaken briefly, sonicated for 1 hour, and allowed to stand overnight. Samples were centrifuged and the solvent transferred to another container. The samples were reduced to approximately 2 mL with nitrogen flow and heating to approximately 37° C. Samples were quantitatively transferred to another container using 2 x ~2 mL methanol and 2 x 10 mL water washes. The volume was adjusted to approximately 200 mL for clean-up on the Waters Oasis HLB SPE cartridges as described in the water extraction section. The methanol eluates were taken to dryness with heating at 37° C under nitrogen flow. One mL of acetonitrile:water (50/50) containing 50 ng/mL flavone internal standard was added to each sample vial and the samples were analyzed by LC/MS/MS. Total organic carbon analysis was performed on each of the dried soil samples as well.

Analytical Instrumental Conditions

The chromatographic analysis was performed using a gradient solvent program. The flow rate was maintained at 0.25 mL/min through a Waters Symmetry C8 column, 2.1 x 100 mm, 3.5 µm particle size (WAT058961) fitted with a similar guard column, 2.1 x 10 mm (WAT106128). A Waters 2695 Separations Module was used for flow and gradient control and for making the 30 µL injections for analysis. The initial composition of the mobile phase was acetonitrile:water (18:82, v/v). The mobile phase was held at initial conditions for 0.1minute, then the concentration was changed to 100% acetonitrile in a convex curve (Waters curve 3), reaching this concentration at 15 minutes. The concentrations were changed back to initial conditions in a linear fashion over a 1 minute period, reaching initial conditions at 16 minutes and held for 6.5 minutes to allow for equilibration of the system before starting a new run. A Waters Micromass Quattro micro API Tandem Mass Spectrometer (MS) was used for MS and MS-MS detections, and Waters Mass Lynx V4.1 was used for both control of the MS and for data analysis.

The electrospray MS was operated in both positive and negative ion modes, utilizing the Mass Lynx program to switch as necessary for the optimal detection of the individual compounds of interest. The following settings were used:

Capillary Voltage:	3.5 kV
Cone Voltage:	see each analyte method
Extractor Voltage:	3.00 V
RF Lens Voltage:	0.2 V
Source Temperature:	125 °C
Desolvation Temperature:	350 °C
Cone Gas Flow:	25 L/Hr
Desolvation Gas Flow:	750 L/Hr

LM 1 Resolution:	12.0
HM 1 Resolution:	12.0
Ion Energy 1	0.5
Entrance	-5
Collision	see each analyte method
Exit 1	30
LM 2 Resolution:	12.0
HM 2 Resolution:	12.0
Ion Energy 2	2.0
Multiplier Voltage:	650 V
Gas Cell Pirani Pressure:	5.00e-3 mbar

Final data acquisition was performed using multiple reaction monitoring (MRM) mode. The functions, MRM masses, ion modes, retention times, cone voltages and collision energies are given in Table 1.

Table 1. Functions, channels, and parameters used for final data acquisition for analysis of flavonoids in water samples.

Compound	Function	Retention Time (Min)	MRM Masses (Da)	Ion mode	Cone (V)	Collision Energy (eV)
Daidzin	1	0.00 to 3.66	417.2 > 255.2	ES+	25	18
Glycitin	2	0.00 to 3.66	445.1 > 282.1	ES-	50	25
Acetylgenistin	3	0.00 to 7.00	269.1 > 133.1	ES-	50	30
Genistin	4	3.50 to 6.05	433.1 > 271.1	ES+	30	15
Daidzein	5	4.18 to 6.82	253.2 > 132.1	ES-	55	40
Acetyldaidzin	5	4.18 to 6.82	457.1 > 252.1	ES-	55	25
Equol	6	4.99 to 7.89	241.2 > 121.0	ES-	35	15
Coumestrol	6	4.99 to 7.89	267.2 > 91.0	ES-	55	40
Formononetin	6	4.99 to 7.89	267.2 > 223.1	ES-	40	30
Genistein	6	4.99 to 7.89	269.1 > 133.1	ES-	50	30
Trihydroxyiso-flavone	7	5.78 to 9.00	269.2 > 240.2	ES-	50	30
Flavone (IS)	8	6.00 to 9.00	223.2 > 121.1	ES+	50	28
Biochanin A	9	6.74 to 9.00	283.3 > 268.3	ES-	45	21
Malonylgenistin	3	5.00 to 7.00	269.1 > 133.1	ES+	50	30

Results and Discussion

Quality Assurance/Quality Control Data

The procedure spike recoveries from the solid phase extraction and concentration process were low; the recoveries averaged about 30% instead of the 50% that we anticipated in water samples (Table 2). The spike recoveries in a field sample (CREEK July) were even lower, less than 10% for most analytes. This could be caused by ion suppression due to a matrix problem. Analytical spike recoveries were in the 40-60% range, lower than usual range of 75% in HPLC-UV analysis.

For the first batch of soil samples using internal standard method for quantitation, the relative percent differences (RPDs) for analytical duplicates were good (Table 3). The analytical spike recoveries were satisfactory and they were slightly better than the water sample recoveries.

The second batch of soil samples were quantitated by an external standard method due to the high ion suppression we observed with the internal standard signal in these samples (10-20% of the average signal in the check standards) (Table 4). The soil matrix could have caused this to happen, but we were unable to explain the severe suppression in the second batch of samples. Possible factors include (a) differences in soil matrices between locations and (b) the clean-up work for second batch samples was not as good as for the first batch.

We also tried Accelerated Solvent Extraction (ASE; Dionex Corp., Sunnyvale, CA) for the first batch samples. However, the recoveries using this method were even lower. This may have been due to several factors (solvents, pressure, and temperature) in the extraction. We only tried one solvent mix (acetonitrile/methanol/acetone). Also phytoestrogens are heat sensitive and, therefore, they may have been degraded under the high temperature and pressure of the ASE extraction, giving low recoveries. Concentration of extracts by nitrogen gas can cause low recoveries for volatile compounds; however, phytoestrogens are not volatile so the loss should have been minimal. The heating (37° C) of samples might have caused some degradation of less thermally-stable compounds, but we did not measure such losses. Additionally, interference in the matrix could be from humus-continuing organics such as polyaromatic hydrocarbons (PAHs) or chelating organics.

Table 2. Water samples spike recoveries.

	Biochanin A	Equol	Formononetin	Daidzin	Acetylgenistin	Coumestrol	Trihydroxy-isoflavone
Analytical Spike Recovery (%)	63	41	67	30	22	81	54
SPE Procedure Spike Recovery (%)	35	13	32	180	19	36	30
CREEK May sample (%)	4	0.5	3	890	6	5	16

Table 3. Soil samples spike recoveries. First batch of samples using ISTD Quantitation.							
	Biochanin A	Equol	Formononetin	Daidzein	Acetylgenistin	Genistein	Trihydroxy-isoflavone
Analytical Duplicate POND July (RPD)	10	NA	NA	6	NA	NA	9
Analytical Spike Recovery FIELD May (%)	63	31	45	54	61	46	59

The RPDs for the analytical duplicates for the second batch were higher than the first batch, in the 20% ranges. The soil extraction spike recoveries were low, from <5- 24% range, but did meet the goals set in our QA/QC plan (±100%).

Overall, the LC-ESI-MS/MS method we developed is useable but matrix effects caused severe ion suppression in some samples. Matrix effects can be minimized in several ways including: (1) a better extraction and clean-up method to improve analyte isolation from the sample; (2) improved HPLC method to separate all individual analyte and interfering contaminants; and (3) reducing the amount of effluent entering the ESI chamber. The extraction efficiencies from water and soil samples were low and need to be improved.

Table 4. Soil samples spike recoveries. Second batch of samples using ESTD Quantitation.							
	Biochanin A	Equol	Formononetin	Daidzein	Acetylgenistin	Trihydroxy-isoflavone	Genistein
Analytical Duplicate (RPD) CREEK July	9	22	NA	19	NA	27	NA
Solvent Spike Recovery	23	76	140	190	170	30	83
Extraction Spike Recovery CREEK July	<5	7.0	24	24	6	<5	<5

Presence and Concentrations of Phytoestrogens in Water and Sediments
The following data are presented and interpreted as measured. The low analytical recoveries we experienced suggest that our results may be biased low, which would result in lower measured concentrations and fewer detections in samples.

Seven of the phytoestrogens we examined were detected in water samples, albeit at very low observed concentrations (Table 5). Daidzin, an important soy isoflavone, was present in one water sample collected in May and in water taken from several locations in July. Daidzin was present in the highest measured concentrations in July samples, but the sample spike recoveries were very high (~900%), indicating contaminants in the matrix caused signal enhancement. Therefore, the values could be biased high. Five of the phytoestrogens measured at POND were also present upstream at the creek site. Isoflavones were not present in measureable concentrations in well water or at water collected at TILE. Neither genistin, a major soy isoflavone, nor genistein, its aglycone, were observed in water samples, although the glucoside malonygenistin was detected in water samples collected in July.

Eight of the phytoestrogens we examined were detected in sediment samples at observed concentrations of an order of magnitude higher than in water (Table 6). Biochanin A, daidzein, and 4,6,7- trihydroxyisoflavone were observed in the greatest concentrations.
Although genistin was not detected in sediments and genistein was detected in only one sample at near detection limits, acetylgenistin, the acetylglycoside of genistin, was observed in sediments collected in May. The isoflavones we examined were not present in sediments collected in the stream immediately below the dam forming the pond (DAM) we sampled, nor in the sediments of WETLAND.

Interestingly, daidzin was not observed in sediments, though its aglycone, daidzein, was present in sediments in both months. The highest daidzein concentrations were observed in a soil sample from a soybean field and in sediment collected below a tile drainage outflow (Table 6). 4,6,7-trihydroxyisoflavone, an oxidative metabolite of daidzein, was present in sediments in both months.

Formononetin, found in clovers (especially red clover) and alfalfa, was present in one water sample and several sediment samples. Formononetin is converted to daidzein and equol by rumen microbes (Dickinson et al. 1988). Equol, a potent phytoestrogen from the standpoint of its ability to bind to estrogen receptors (Liwei et al. 2006), was present in several water samples collected in May and primarily in sediments samples collected in that month. Burnison et al. (2003) observed equol in stored, liquefied hog manure and attributed its presence to soybean products in hog feed. Storage of the manure under mostly anaerobic conditions likely contributed to the formation (beyond that formed in the gut of the hogs) and conservation of equol and, consequently, to the high concentrations observed. Although equol was detected in tile drain water after field application of the manure, the authors found its contribution to estrogenicity of that water was minimal compared to natural steroidal estrogens. The CREEK and POND sites are downstream of a cattle pasture, and cattle are left to forage in harvested cornfields upslope of these locations.

Table 5. Concentrations (pg/g) of selected phytoestrogens in water samples collected in a small watershed in central Illinois. ND = Not Detected.

	Biochanin A	Equol	Formononetin	Daidzein	Daidzin	Acetyldaidzin	Genistein	Genistin	Malonygenistin	Acetylgenistin	Glycitin	Flavone	Coumestrol	4,6,7-Trihydroxy-isoflavone
Location (month)														
POND (May)	4	10	14	ND	5	ND	ND	ND	ND	ND	ND	ND	8	ND
TILE (May)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CREEK (May)	7	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
WELL (May)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
POND (July)	ND	ND	ND	ND	50	ND	ND	ND	5	ND	ND	ND	ND	5
DAM (July)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	7
CREEK (July)	ND	ND	ND	ND	30	ND	ND	ND	6	ND	ND	ND	ND	15
WETLAND (July)	ND	ND	ND	ND	100	ND	ND	ND	8	ND	ND	ND	ND	ND

Table 6. Concentrations (ng/g) of selected phytoestrogens in sediment samples collected in a small watershed in central Illinois. ND = Not Detected.

	Biochanin A	Equol	Formononetin	Daidzein	Daidzin	Acetyldaidzin	Genistein	Genistin	Malonylgenistin	Acetylgenistin	Glycitin	Flavone	Coumestrol	4,6,7-trihydroxy-isoflavone
Location (month)														
FIELD (May)	0.4	1.0	2.4	18	ND	ND	0.4	ND	ND	0.8	ND	ND	3.0	ND
TILE (May)	19	0.6	0.8	20	ND	ND	<0.3	ND	ND	1.1	ND	ND	1.7	13
CREEK (May)	0.9	0.8	0.3	1.5	ND	ND	ND	ND	ND	ND	ND	ND	0.7	3.6
POND (May)	0.6	1.6	0.3	4.2	ND	ND	ND	ND	ND	0.5	ND	ND	0.7	1.7
CREEK (July)	0.7	0.9	0.6	1.0	ND	ND	ND	ND	ND	ND	ND	ND	0.9	1.2
POND (July)	0.9	<0.3	<0.3	0.4	ND	ND	ND	ND	ND	<0.3	ND	ND	<0.3	1.1
DAM (July)	<0.5	ND	<0.5	<0.5	ND	ND	<0.5	ND	ND	ND	ND	ND	<0.5	ND
WETLAND (July)	<0.5	ND	ND	<0.5	ND	ND	ND	ND	ND	<0.5	ND	ND	<0.5	ND

Biochanin A, found primarily in red clover, was detected in water and sediments at the CREEK and POND sites, both of which were located downstream of a cattle feedlot/pasture (Appendix A). This source does not account for the relatively high concentrations of this isoflavone observed in sediments of TILE in May. Biochanin A is not present in soybeans (Nakamura et al. 2001). Two homes, one with a horse pasture, are located 0.5 kilometers upslope of the sampling location. It is plausible that the source of biochanin A could be subterranean flow (within the tile drain system) from the home's septic system and pasture. Additional sources may include several homes and a large cattle lot located farther upslope. Curiously, genistein was not detected at TILE, as biochanin A can be demethylated in the gut to become genistein (Dickinson at al. 1988).

Coumestrol, the highest concentrations of which are found in clover and alfalfa (and is also present in soybeans) is a relatively potent phytoestrogen (Thigpen et al. 2004). Coumestrol was also present in the water and sediments of POND in May, as well in the sediments of TILE and CREEK, the latter in both May and July. Both CREEK and POND were located downstream of a cattle feedlot/pasture as well as two alfalfa fields. The highest coumestrol concentration was observed in soil from a soybean field.

Greater rainfall in the 5 days preceding collection in May (2.3 cm) than in July (0.0 cm) may have increased runoff and percolation, resulting in the presence of some compounds in samples during the May and not July. However, this cannot account for the presence of daidzin, malonylgenistin, and 4,6,7- trihydroxyisoflavone in water samples in July but not in those collected in May (with the exception of a very low concentration of daidzin in one sample). It is plausible that some compounds could become more concentrated in water during drought conditions. Also, it is unknown to what degree the seasonal growth of soybeans and leguminous forage crops might contribute to seasonal patterns of phytoestrogen presence and concentrations in the environment. Seasonal and daily pattern of feeding and pasture use by cattle might also be expected to impact the presence of phytoestrogens in the environment. Isoflavones are present not only in forage (e.g., alfalfa and clovers) but also in processed livestock feed, which often contain soy products.

Concentrations (and presence) of isoflavones in sediments at the CREEK site were similar between samplings. The same compounds were present in the POND as in the CREEK samples, although concentrations declined between May and July. Interestingly, concentrations were relatively high in sediments at TILE, located upstream of both CREEK and POND. The TILE site is below the outflow of approximately 623 hectares of subsurface tile drain. Thus, runoff directly from cattle pastures or feedlots would not have been responsible for the presence of phytoestrogens at this site. Percolation of phytoestrogen-containing water from soybean fields, residential drainage fields, and from field lots into the subterranean tile drainage system are potential sources.

Bacaloni et al. (2005) observed eight of the compounds that we examined in sewage treatment plant (STP) influent, effluent, or receiving waters. Daidzein and genistein were present in by far the highest concentrations (as high as 1,685 and 954 ng/L, respectively; both in STP influent). These values were considerably higher than we observed in our water samples. However, concentrations of 4,6,7-trihydroxyisoflavone, daidzein, genistein, coumestrol, formononetin, and biochanin A in our sediment samples were higher than or comparable to concentrations observed

in STP influent in that study. They also detected daidzin and genistin, which we did not, as well as glycitein (we looked for glycitin instead but did not detect it). Spengler et al. (2001) observed genistein in STP effluent at concentrations (median 2.7 ng/L) well above our detection limits for that compound in water. These concentrations were similar to the concentrations of malonygenistin, its glucoside, which we measured in water samples collected from 3 locations.

We developed and evaluated an extraction and clean-up method for these compounds in sediment samples that could be used for isoflavone analysis using liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS). Spike recoveries were low for some samples, partly due to matrix effects that caused severe ion suppression. The spike recoveries for daidzin in water samples collected in July were high, suggesting that the data for these samples could be biased high. Seven of the phytoestrogens we measured were detected in water samples at concentrations in the low parts per trillion range. Eight analytes were observed in sediment samples at an order of magnitude (parts per billion) higher than in water samples. Our results suggested seasonality to the observed presence of these compounds, which could be driven by rainfall and seasonal land-use (e.g., grazing) and plant growth patterns. Based on the spatial pattern of the presence of individual parent compounds or their metabolites, it appears that there are multiple sources of these phytoestrogens in the agricultural environment. Additional studies to further elucidate the patterns observed in this pilot study are warranted.

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Appendix A. Map of a small, highly-agriculturalized watershed in central Illinois that was sampled for isoflavones. Map shows watershed boundaries, field and other land use types, and sampling locations. Locations shown are TILE (T), CREEK (C), POND (P), and DAM (D). WETLAND and WELL were located outside of the watershed boundary and are not shown.