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Quantification of Six Phytoestrogens at the Nanogram per Liter Level in Aqueous Environmental Samples Using $^{13}C_3$-Labeled Internal Standards

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In light of the estrogenic potentials and the recent concentration levels found for six phytoestrogens in surface waters, detailed monitoring and assessment of potential input sources are required. An accurate, precise, and sensitive HPLC–MS/MS analytical method incorporating five $^{13}C_3$-labeled internal standards for the quantification of these plant estrogens in various aqueous environmental samples is presented here for the first time. The compounds investigated included biochanin A, daidzein, equol, formononetin, genistein, and coumestrol. The use of [$^{13}C_3$]biochanin A, [$^{13}C_3$]daidzein, [$^{13}C_3$]equol, [$^{13}C_3$]formononetin, and [$^{13}C_3$]genistein ensured an accurate quantification of the target analytes unaffected by matrix effects and analyte losses. Absolute method recoveries for all analytes ranged from 63 to 105%, from 63 to 99%, and from 73 to 133%, relative recoveries from 90 to 132%, from 89 to 139%, and from 89 to 115%, method detection levels from 0.5 to 2.7 ng/L, from 0.5 to 2.6 ng/L, and from 0.4 to 11.0 ng/L, and precision from 1 to 19%, from 1 to 16%, and from 1 to 11% in drainage water, river water, and WWTP effluent, respectively. The validated analytical method was applied in investigating the emission of the phytoestrogens via drainage water from a pasture containing 43% red clover (*Trifolium pratense*) and in monitoring their occurrence in Swiss surface waters. Isoflavone concentrations ranging from 4 to 157 ng/L and up to 22 ng/L were found in drainage and river water, respectively.

KEYWORDS: Phytoestrogen; biochanin A; daidzein; genistein; formononein; equol; coumestrol; isoflavones; isotope-labeled internal standard; LC–MS; endocrine disruption; red clover; *Trifolium pratense*

INTRODUCTION

Phytoestrogens are nonsteroidal weakly estrogenic polycyclic phenols that occur naturally in a wide range of plants. Isoflavones and coumestans are the most prevalent groups of estrogenic compounds, and they are present in particularly high concentrations in legumes such as soy, clover, alfalfa, lucerne, beans, and peas (*1, 2*). The supposedly beneficial health effects of dietary phytoestrogens on i.a. cancer prevention, menopausal symptoms, cardiovascular disease, and osteoporosis have received a great deal of attention over the past several years (*3*). Only in recent years has there been a growing recognition that phytoestrogens occur as endocrine-disrupting contaminants in the aquatic environment (*4–10*).

The aglycones genistein and daidzein and their 4′-methyl ether derivatives biochanin A and formononetin constitute the major isoflavones (**Figure 1**). Equol is not produced by the plant per se but is the major intestinal metabolite of daidzein (*11*). Coumestrol, the most common coumestan, is the phytoestrogen exhibiting the highest relative estrogenic potency followed by equol and genistein (*12–14*). The relative estrogenic potencies of the phytoestrogens differ by up to 2 orders of magnitude and generally display much weaker estrogenic effects than natural and synthetic steroidal estrogens (e.g., 17β-estradiol and 17α-ethinylestradiol). However, the phytoestrogens exhibit relative estrogenic potencies equal to those of several infamous and in surface water more frequently investigated xenoestrogens (e.g., bisphenol A, 4-nonylphenol, and methoxychlor). Hence, the recent reports of phytoestrogen levels found in surface waters throughout the developed world should raise concern (*5–10, 15*). Concentrations of biochanin A, daidzein, formononetin, and coumestrol ranging from 1 to 10 ng/L were found in rivers in Australia, Germany, and Italy (*5, 8–10*), whereas 43 μg/L daidzein and 143 μg/L genistein were detected in a Japanese river (*7*). Effluents from wastewater treatment plants (WWTP) and pulp mills as well as runoff from manure-treated soils have been identified as significant sources of input of these phytoestrogens into the aquatic environment (*4–6, 8, 9, 16*). Biochanin A, daidzein, genistein, and coumestrol were dis-

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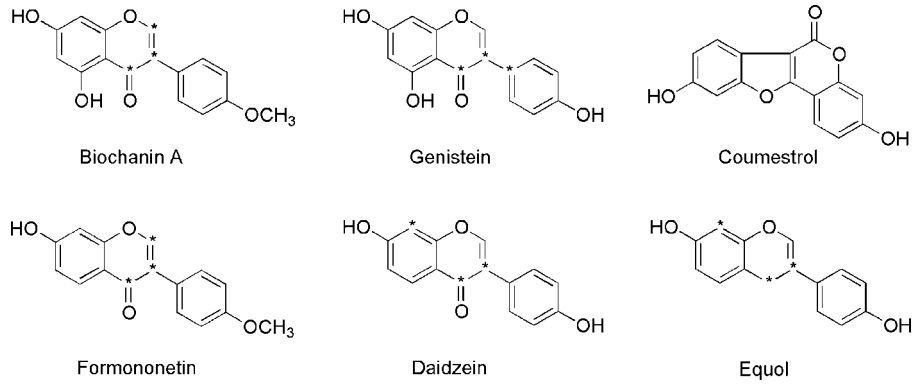

Figure 1. Chemical structures of the phytoestrogens. Asterisks indicate the positions of ^{13}C atoms in the isotope-labeled internal standards.

Table 1. Optimized Instrumental Parameters for the Phytoestrogens and Their Corresponding $^{13}C_3$-Labeled Internal Standards

compound	time segment	retention time (min)	precursor ion (*m*/*z*)	fragments (relative intensities)[a]	collision energies (eV)
[$^{13}C_3$]daidzein	1	7.84	256.0	**134** (100), 198 (50), 210 (71), 226 (100)	35, 35, 35, 35
daidzein	1	7.85	253.0	**132** (100), 195 (47), 208 (38), 223 (52)	40, 40, 40, 40
[$^{13}C_3$]genistein	2	12.08	272.0	**135** (100), 161 (26), 185 (20)	35, 35, 35, 35
genistein	2	12.09	269.0	**133** (100), 157 (17), 180 (25)	35, 35, 35, 35
coumestrol[b]	2	12.45	267.0	166 (89),[c] **182** (100), 211 (32), 239 (11)	45, 45, 45, 45
[$^{13}C_3$]equol	2	12.67	244.0	**120** (100), 123 (87), 138 (60)	20, 20, 20, 20
equol	2	12.67	241.0	**119** (100), 121 (74), 135 (60)	20, 20, 20, 20
[$^{13}C_3$]formononetin	3	16.34	270.0	134 (43), 197 (59), 225 (100), **254** (89)	35, 35, 35, 35
formononetin	3	16.34	267.0	132 (10), 195 (27), 223 (36), **252** (100)	40, 40, 35, 25
[$^{13}C_3$]biochanin A	4	23.99	286.0	134 (67), **213** (100), 241 (50), 270 (68)	45, 45, 45, 45
biochanin A	4	23.99	283.0	132 (19), 211 (30), 239 (44), **268** (100)	45, 45, 35, 30

[a] Fragments in bold were used for quantification. [b] [$^{13}C_3$]Genistein used as the internal standard. [c] Fragment used for quantification in WWTP effluent.

charged from WWTPs at concentrations ranging from 3 to 83 ng/L (*5*, *6*, *8*, *9*). Genistein (10 μg/L) was detected in treated pulp mill effluent (*4*), and equol (10–1300 ng/L) was released from an agricultural field fertilized with hog manure (*16*, *17*). Developmental and behavioral effects on aquatic wildlife exposed to such concentration levels of genistein and equol have been reported (*15*, *18*). Hence, despite their relatively low estrogenicity, even temporarily high loads of phytoestrogens discharging from WWTPs, pulp mills, and intensive crop farming may locally contribute to the overall estrogenic activity in the receiving waters and inflict permanent damage on biota, especially during vulnerable stages of development.

Apart from the secondary input sources mentioned above, another potentially relevant pathway for these phytoestrogens to enter the environment is a direct release from the cultivated plant. In analogy to other currently ongoing research activities in our group involving the emission of mycoestrogens (estrogens of fungal origin) from a *Fusarium*-infested crop field into the drainage system (*19*, *20*), it is reasonable to argue that plant estrogens also elute into surface waters via drainage water and surface runoff. The contribution of such primary input sources of plant estrogens to the aquatic ecosystem has never been investigated. Red clover (*Trifolium pratense*) is a common pasture and forage crop known for its high content of daidzein, genistein, formononetin, and biochanin A. Typical isoflavone concentrations range from 0.05 to 10 g/kg of dry matter with formononetin and biochanin A occurring at particularly high levels (*21*, *22*). Because of their similar structures and chemical properties and the low concentrations (nanograms per liter) that are expected, a highly selective and sensitive analytical method is required to monitor phytoestrogens in these aqueous samples.

HPLC–ESI-MS/MS has in recent years been established as the state-of-the-art technique for quantifying plant estrogens in aqueous environmental samples (*4*, *8–10*, *23*). However, signal suppression caused by co-extracted sample matrix components is frequently a major source of imprecision when quantifying low levels of organic compounds in environmental samples by LC–ESI-MS/MS (*24*, *25*). Ion suppression has a profound influence on accuracy, precision, and sensitivity. In the case of phytoestrogens in natural waters, it has been compensated by standard addition (*9*), by using a single nonlabeled (*8*) or a single deuterated internal standard (*10*). Despite the fact that matrix effects are notorious, only one of the currently reported LC–MS/MS methods applied for isoflavone analysis in water samples included a quantitative evaluation of ion suppression (*9*). The use of isotope-labeled internal standards (ILIS) is a powerful way of compensating for matrix effects occurring during the ionization process in the ion source of the MS/MS instrument as well as analyte losses during sample preparation. If ILIS are available, their application is a very time-effective alternative to standard addition or matrix-matched calibration. Some of the $^{13}C_3$-labeled isoflavones commercially available were used for quantitation of genistein, daidzein, and equol in human serum (*26*) but have not yet been applied as internal standards in environmental trace analysis.

In this study, we describe an accurate, precise, and sensitive HPLC–negative electrospray ionization (ESI)-MS/MS analytical method for the quantification of six phytoestrogens in various aqueous samples. The method comprises solid phase extraction for enrichment of the phytoestrogens and purification of the samples. To our knowledge, this is the first time $^{13}C_3$-labeled internal standards have been used for the quantification of these compounds in natural waters. The analytical method presented was validated for phytoestrogens in Milli-Q water, drainage water, river water, and WWTP effluent in terms of method recovery, precision, method detection limit (MDL), and linearity. Moreover, the influence of matrix effects on analyte signal intensities was investigated. The application of the validated analytical method was demonstrated in a field study on the

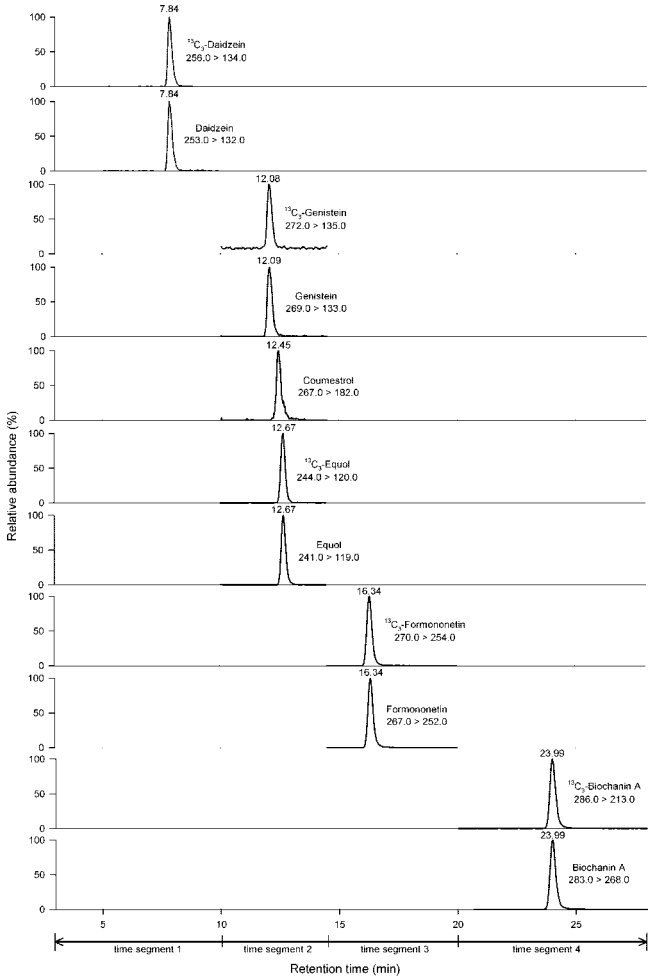

Figure 2. LC–MS/MS chromatograms of drainage water extracts spiked with 25 ng/L of each of the investigated phytoestrogens. Numbers below the analyte names indicate precursor–fragment ion transitions. For further details on the instrumental parameters, see the text and **Table 1**.

emission of these plant estrogens from a pasture containing red clover as well as in a surface water monitoring study.

MATERIALS AND METHODS

Chemicals. Biochanin A [491-80-5] (≥97%), coumestrol [479-13-0] (≥95%), daidzein [486-66-8] (≥98%), equol [94105-90-5] (≥99%), formononetin [485-72-3] (≥99%), and genistein [446-72-0] (≥98%) were supplied by Fluka AG (Buchs, Switzerland). For structures, see **Figure 1**. The [2,3,4-$^{13}C_3$]biochanin A (purity not available), [3,4,8-$^{13}C_3$]daidzein (100% pure), [3,4,8-$^{13}C_3$]equol (100% pure), [2,3,4-$^{13}C_3$]formononetin (100% pure), and [3,4,1′-$^{13}C_3$]genistein (100% pure) were obtained from STANDIL (St. Andrews, Fife, Scotland). Methanol (MeOH, 99.98%) and acetonitrile (ACN, 99.99%) were purchased from Scharlau (Barcelona, Spain). Ammonium acetate was supplied by Fluka AG. Deionized water was produced by a Milli-Q gradient A10 water purification system from Millipore (Volketswil, Switzerland). High-purity N_2 (99.99995%) and Ar (99.99999%) were obtained from PanGas (Dagmarsellen, Switzerland).

Individual phytoestrogen stock solutions holding concentrations of 500 mg/L were prepared in pure MeOH for all analytes. Multicomponent stock solutions were prepared in MeOH at concentrations of 10, 100, 1000, and 10000 ng/mL for each constituent. The ILIS solution was prepared in MeOH and held all five ILIS at a concentration (0.9–5.7 mg/L) required for collection of ILIS peak heights similar to the corresponding analyte peak heights at a calibration level of 50 ng/L when spiking 50 μL of the ILIS solution. Aqueous calibration standards holding all six phytoestrogens equivalent to the concentration range of 0.5–100 ng/L, and all five ILIS at 45–285 ng/L, were prepared in Milli-Q water from the methanolic multicomponent stock solutions. All compounds were stable when present in single-component and multicomponent methanolic stock solutions. All stock solutions were stored at −20 °C.

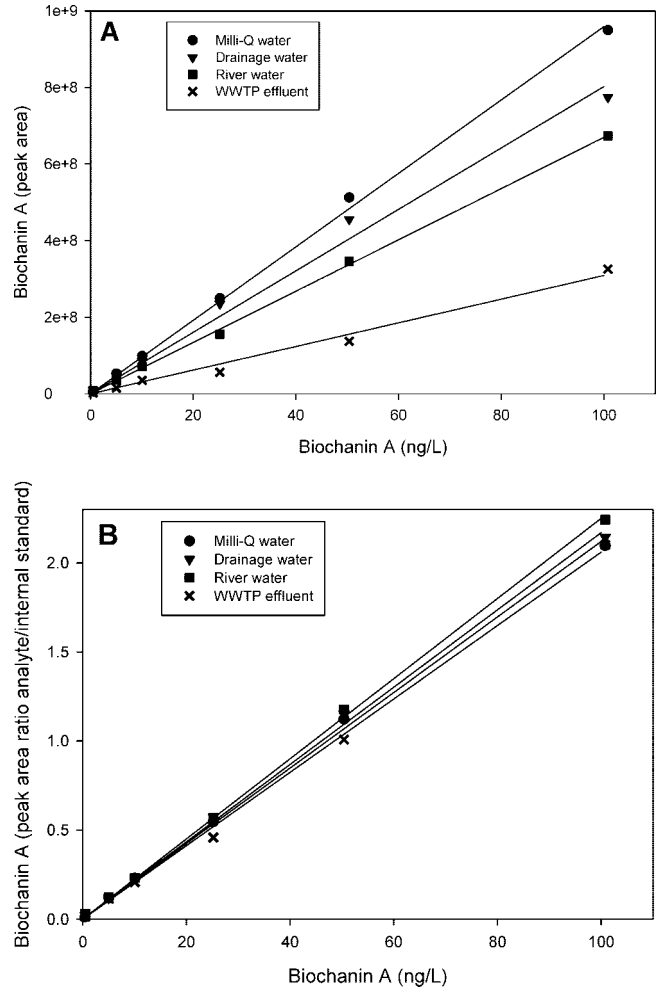

Figure 3. (**A**) Biochanin A peak area vs biochanin A concentration in Milli-Q water (●) and in matrix eluates [drainage water (▼), river water (■), and WWTP effluent (×)]. (**B**) Ratio of biochanin A peak area to [$^{13}C_3$]biochanin A peak area vs biochanin A concentration in Milli-Q water (●) and in matrix eluates [drainage water (▼), river water (■), and WWTP effluent (×)].

Field Site Management and Sample Collection. Flow-proportional drainage water samples were collected between February and March 2007 at our field study site at Reckenholz, Switzerland, using portable automatic flow-proportional samplers (Teledyne Isco Inc., Lincoln, NE). In July 2006, the field was sown with a mixture (standard seed mixture 200 CH) commonly cultivated as a grazing and forage legume in Switzerland consisting of 56% Italian ryegrass (*Lolium multiflorum*) and 43% red clover (*T. pratense*). A total of 3 kg of red clover and 6 kg of ryegrass were seeded in 0.2 ha which equals 1.5 g of red clover/m^2.

Surface water samples were collected from February to March 2007 at various sampling sites throughout Switzerland maintained by monitoring programs of the Swiss government [National Long-Term Surveillance of Swiss Rivers (NADUF)] and the Canton of Zurich [Office for Waste, Water, Energy, and Air (AWEL)]. WWTP effluent samples were collected in February 2007 at the Zürich-Werdhölzli (Zurich, Switzerland) facility. Raw water samples were transferred to 1 L glass bottles and stored at 4 °C until further processing. Stability experiments including unfiltered river water spiked with phytoestrogens and stored at 4 °C revealed that the phytoestrogens were transformed within a few weeks (half-lives of 8–41 days). Thus, the water samples were filtered (glass fiber filters, pore size of 1.2 μm, Millipore,

Table 2. Ion Suppression of Phytoestrogens in Various Aqueous Matrices

compound	drainage water (%)	river water (%)	WWTP effluent (%)
daidzein	63	70	79
genistein	53	65	85
coumestrol	62	70	82
equol	21	28	50
formononetin	27	34	60
biochanin A	20	34	68

Table 3. Absolute Recoveries[a] of the Phytoestrogens in Various Aqueous Matrices

compound	concentration (ng/L)	Milli-Q water (%)	drainage water (%)	river water (%)	WWTP effluent (%)
daidzein	5	96 (8)	103 (8)	99 (12)	111 (9)
	25	84 (6)	87 (5)	83 (3)	84 (7)
	100	86 (3)	84 (3)	84 (3)	88 (6)
genistein	5	80 (8)	87 (11)	92 (13)	97 (5)
	25	79 (3)	80 (7)	82 (6)	86 (6)
	100	81 (5)	77 (5)	68 (4)	74 (5)
coumestrol	5	80 (6)	80 (15)	72 (13)	133 (19)
	25	79 (8)	81 (4)	67 (6)	91 (8)
	100	73 (4)	73 (3)	71 (4)	87 (9)
equol	5	95 (10)	105 (5)	90 (7)	87 (10)
	25	87 (6)	93 (5)	85 (3)	81 (7)
	100	87 (3)	92 (2)	87 (3)	80 (3)
formononetin	5	90 (8)	91 (6)	87 (7)	87 (5)
	25	81 (5)	87 (3)	86 (3)	83 (2)
	100	86 (3)	88 (2)	89 (3)	85 (4)
biochanin A	5	69 (8)	69 (7)	69 (6)	79 (7)
	25	64 (2)	63 (2)	68 (2)	73 (2)
	100	69 (4)	66 (4)	63 (5)	77 (3)

[a] Absolute standard deviation (five replicates) in parentheses.

Volketswil, Switzerland) by vacuum filtration (Supelco, Bellefonte, PA) and prepared for analysis within 48 h. The exact volume of 1 L was spiked with 50 μL of the ILIS solution and shaken vigorously before further treatment.

Solid Phase Extraction (SPE). Filtered water samples were concentrated and purified by performing off-line reversed phase SPE (Oasis HLB cartridges, 6 mL, 200 mg; Waters Corp.). The SPE cartridges were conditioned with 5 mL of MeOH and 5 mL of Milli-Q water consecutively. Water samples (1 L) were drawn by vacuum through the cartridges at a maximal flow rate of 10 mL/min. The cartridges were subsequently washed with 5 mL of Milli-Q water and dried by using gentle syringe pressure. Finally, the analytes were eluted with 10 mL of MeOH into conical micro reaction vials and evaporated to dryness in an aluminum heating block at 50 °C using a gentle nitrogen gas stream for 30 min. The dried extracts were reconstituted in 300 μL of an 80/20 (v/v) Milli-Q water/ACN mixture and transferred into 350 μL amber glass vials. The samples were stored at 4 °C and analyzed within 48 h.

LC–MS/MS Analysis. The phytoestrogens were separated on an XTerra MS C18 column (2.1 mm × 100 mm, 3.5 μm; Waters Corp.) connected to an Xterra MS C18 guard column (2.1 mm × 20 mm, 3.5 μm; Waters Corp.). The following optimized elution gradient modified from that of Kang et al. (*10*) was applied at room temperature: 21.1% B (78.9% A) at 0 min, 21.1% B at 3 min, 26.3% B at 4 min, 47.4% B at 26.5 min, 94.7% B at 27 min, 94.7% B at 29 min, 21.1% B at 30 min, 21.1% B at 35 min [eluent A consisting of Milli-Q water and ACN (95/5, v/v) and eluent B of Milli-Q water and ACN (5/95, v/v)]. Both eluents were buffered with 10 mM ammonium acetate (pH 6.8). The injection volume was 50 μL and the mobile phase flow rate 0.2 mL/min.

Analyte detection was performed by means of negative electrospray ionization tandem mass spectrometry on a Varian 1200L LC–MS instrument (Varian Inc., Walnut Creek, CA). Nitrogen gas for the ESI was generated online using a high-purity nitrogen generator (Nitrox UHPLCMS18, Domnick Hunter Ltd., Gateshead, U.K.). Interface parameters for the LC–MS/MS system were as follows: needle voltage, −4500 V; nebulizing gas (compressed air), 3.45 bar; capillary voltage, −40 V; drying gas (N_2, 99.5%), 300 °C and 1.31 bar; and shield voltage, −600 V. The collision cell gas (Ar, 99.999%) pressure was 2.0×10^{-6} Torr, and the detector voltage was set to −2000 V. Detection of the phytoestrogens was performed in the ESI(−) mode using the mass transition reactions specified in **Table 1**. The total run time for a single sample was 35 min. To increase sensitivity, data acquisition was divided into four chronological scan segments: segment 1 (5.0–10.0 min), daidzein and [$^{13}C_3$]daidzein; segment 2 (10.0–14.5 min), genistein, coumestrol, equol, and the respective ILIS; segment 3 (14.5–20.0 min), formononetin and [$^{13}C_3$]formononetin; and segment 4 (20.0–35.0 min), biochanin A and [$^{13}C_3$]biochanin A (see **Figure 2**). Retention times for the phytoestrogens and their ILIS are given in **Table 1**. The analytes were quantified using external calibration, i.e., calibration standards in Milli-Q water containing the five ILIS (see above). Data processing was carried out using Varian MS Workstation (version 6.5).

Performance and Validation of the Analytical Method. Effects of the matrix on analyte ionization were evaluated by comparing the analyte signals obtained from injection of the same amount of analyte dissolved in pure Milli-Q water and an extracted matrix blank [Milli-Q water/ACN (80/20)]. For this purpose, 18 L of each matrix (drainage water, river water, and WWTP effluent) was filtered and subjected to SPE as described above. The 18 SPE eluates were combined and divided into six equal portions; 50 μL of internal standard solution was added to each portion. Standard addition to the matrix extraction eluates was carried out to produce concentrations equivalent to those of 1 L samples containing 0.5, 5, 10, 25, 50, and 100 ng of each analyte. Curves were obtained by plotting measured analyte peak areas against corresponding analyte concentration levels in pure Milli-Q water and in extracted matrix blank, respectively. Linear regression was performed for each curve. The ion suppression was quantified as the ratio between the slope of the curve for Milli-Q water and the slope of the curve obtained for the extracted matrix blank.

Absolute method recoveries were evaluated for all phytoestrogens in Milli-Q water, drainage water, river water, and WWTP effluent. One liter samples were spiked with phytoestrogens prior to SPE to produce three concentration levels: 5, 25, and 100 ng/L. Five replicates were prepared for every concentration level. A 1 L matrix blank was tested for native phytoestrogen content. Addition of ILIS did not yield any detectable amounts of native analytes. Sample residues were reconstituted in 250 μL of a Milli-Q water/ACN mixture (80/20, v/v) and 50 μL of an ILIS solution. The samples were otherwise treated and quantified as described above. The absolute method recovery was defined as the ratio between the quantified and spiked amount.

Relative method recoveries were determined for the matrices mentioned above. Samples were processed at the same three concentration levels each with five replicates as described above, the only difference being that both analytes and 50 μL of the ILIS solution were added to each sample prior to SPE. Again, a 1 L matrix blank was processed to correct for potential analyte background levels. The relative recovery was defined as the ratio between the quantified and spiked amount.

The precision of the analytical method was calculated as the mean relative standard deviation of five replicates at the spike concentration level of 5 ng/L. For all analytes, the MDL was defined as 3 times the mean absolute standard deviation of the five replicates at a concentration level of 5 ng/L. The linearity of the MS/MS detector was tested with Milli-Q water containing phytoestrogens at concentrations between 0.1 ng/L and 100 μg/L, corresponding to 5 pg and 5 μg at the detector, respectively.

RESULTS AND DISCUSSION

Chromatographic Separation and MS Detection. The reversed phase liquid chromatographic separation of the phytoestrogens was optimized in terms of LC column material, mobile phase, and gradient method. Due to the similar *m*/*z* ratios of precursor ions and fragments among the analytes and their ILIS (**Table 1**), a LC column was chosen which had previously been reported to be capable of separating formononetin from

Table 4. Relative Recoveries[a] (RR) and Precision of the Phytoestrogens in Various Aqueous Matrices

		Milli-Q water		drainage water		river water		WWTP effluent	
compound	concentration (ng/L)	RR (%)	precision (%)	RR (%)	precision (%)	RR (%)	precision (%)	RR (%)	precision (%)
daidzein	5	108 (10)	10	110 (6)	6	93 (13)	13	115 (13)	11
	25	103 (4)	4	112 (4)	4	102 (6)	6	109 (7)	7
	100	98 (2)	3	98 (5)	5	95 (5)	6	102 (2)	2
genistein	5	106 (7)	7	113 (14)	12	119 (15)	12	89 (4)	5
	25	103 (5)	4	113 (9)	8	106 (12)	11	93 (7)	8
	100	104 (6)	7	93 (10)	11	89 (9)	10	100 (10)	10
coumestrol	5	111 (13)	12	104 (20)	19	121 (19)	16	na[b]	na[b]
	25	103 (6)	6	110 (13)	12	139 (18)	13	113 (15)	14
	100	90 (6)	8	90 (14)	16	113 (13)	11	110 (11)	9
equol	5	111 (10)	9	107 (10)	10	111 (4)	4	95 (6)	7
	25	105 (3)	3	109 (5)	5	107 (4)	4	102 (5)	5
	100	103 (2)	4	98 (3)	4	100 (3)	3	102 (3)	3
formononetin	5	105 (4)	4	132 (3)	2	111 (5)	5	104 (3)	3
	25	102 (1)	1	113 (1)	1	112 (1)	2	106 (4)	4
	100	100 (3)	4	103 (1)	1	104 (2)	2	101 (1)	1
biochanin A	5	103 (2)	2	105 (4)	4	101 (3)	3	99 (6)	6
	25	96 (2)	2	111 (3)	3	103 (2)	2	104 (6)	6
	100	94 (2)	3	102 (2)	2	95 (1)	1	100 (4)	4

[a] Absolute standard deviation (five replicates) in parentheses. [b] Not available due to the higher method detection limit.

Table 5. Method Detection Limit (nanograms per liter)[a] of the Phytoestrogens in Various Aqueous Matrices

compound	Milli-Q water	drainage water	river water	WWTP effluent
daidzein	1.6	1.0	1.8	1.9
genistein	1.1	2.0	2.2	0.6
coumestrol	1.9	3.0	2.8	11.9[b]
equol	1.5	1.5	0.6	0.9
formononetin	0.6	0.5	0.8	0.5
biochanin A	0.4	0.7	0.5	0.9

[a] Three times the absolute standard deviation at 5 ng/L. [b] Three times the absolute standard deviation at 25 ng/L.

genistein and coumestrol (*10*). The detection of all analytes was optimized with respect to selectivity and sensitivity. Precursor and product ions as well as the collision energies required for fragmentation were optimized for each analyte and ILIS individually (**Table 1**). For all analytes, including ILIS, deprotonated molecules $[M - H]^-$ were the major precursor ions that were formed. The most selective or abundant product ion was used for quantification. At least three or four product ions were monitored for each analyte to ensure unquestionable identification and quantitation in matrix-loaded samples. Due to coeluting matrix compounds, a more selective but less intensive product ion had to be chosen for coumestrol in WWTP effluent (**Table 1**). In view of the fact that no ^{13}C-labeled coumestrol is commercially available, and since genistein and coumestrol have almost identical chromatographic retention times, [$^{13}C_3$]genistein was used as an internal standard for the quantitation of both genistein and coumestrol (see **Figure 2**).

Ion Suppression. The extent of ion suppression was quantified for all analytes in drainage water, river water, and WWTP effluent as the ratio between the slopes of calibration curves obtained in Milli-Q water and matrix. The impact of ion suppression on analyte signal intensity is graphically visualized for biochanin A in **Figure 3A**. The reduced slopes obtained for the environmental extracts equal ion suppression ranging from 20% in drainage water to 68% in WWTP effluent for biochanin A. Without exception, ion suppression (20–85%) occurred for all analytes in all investigated environmental matrices (see **Table 2**) and generally depends on the type of matrix. For all phytoestrogens, ion suppression was consistently higher in WWTP effluent than in drainage and river water. This is plausible considering the high output of organic material from WWTPs. Except for equol, ion suppression varied slightly with retention time; i.e., co-extracted interferences had a stronger influence on the signal response of daidzein, genistein, and coumestrol (time segments 1 and 2) than on those of formononetin and biochanin A (time segments 3 and 4). In contrast to genistein and coumestrol eluting in time segment 2 as well, equol had the lowest ion suppression in all environmental matrices. As seen in **Figure 3B**, the ratios of biochanin A to [$^{13}C_3$]biochanin A peak areas are constant within the analyte concentration range and identical for all matrices. Hence, ion suppression is effectively offset by normalization to ILIS. This holds true for all analytes and their corresponding ILIS, even for coumestrol which was quantified using [$^{13}C_3$]genistein as an internal standard, and basically legitimizes the use of ILIS-fortified calibration standards in Milli-Q water. The ion suppression values achieved in this study (20–85%) agree with the range of signal reduction in river water and WWTP influent (29–70%) reported previously (*9*).

Absolute Method Recoveries. Phytoestrogen extraction recoveries were optimized with respect to SPE material, elution solvent, and elution volume. Supelclean ENVI-18 cartridges, which we apply for the extraction of mycoestrogens from environmental aqueous samples (*20*), were less suitable for extracting the phytoestrogens from surface waters (results not shown). Highest absolute recoveries of all phytoestrogens were obtained when Oasis HLB cartridges were applied and samples were eluted with 10 mL of MeOH (**Table 3**). This is in line with previous observations (*9, 10*). The absolute recoveries were determined for all six phytoestrogens in Milli-Q water, drainage water, river water, and WWTP effluent at three different concentration levels (5, 25, and 100 ng/L). In natural waters, the absolute recoveries ranged from 69 to 105%, from 69 to 99%, and from 79 to 133% for drainage water, river water, and WWTP effluent, respectively, at 5 ng/L. At higher concentration levels, similar or slightly lower recovery rates (63–93%) were obtained for all six substances in the environmental matrices. These differences were in most cases not significant. Hence, within the concentration range investigated here, the absolute recoveries were largely independent of analyte concentration (**Table 3**).

Absolute recoveries were generally higher in WWTP effluent than in the other sample types, which is somewhat unexpected given that potentially interfering matrix constituents usually are

Table 6. Concentrations (nanograms per liter) of the Phytoestrogens Found in Drainage and Surface Water Monitored from February to March 2007[a]

	drainage water				river water			
compound	min–max	mean	no. of samples	detected in *x* samples	min–max	mean	no. of samples	detected in *x* samples
daidzein	5–30	15	8	8	d	d	23	9
genistein	d–14	7	8	8	nd	nd	23	0
coumestrol	nd	d	8	1	nd	nd	23	0
equol	34–121	80	8	8	d–22	3	23	13
formononetin	44–157	95	8	8	d–21	3	23	22
biochanin A	7–22	15	8	8	d–12	2	23	10

[a] d, detected but not quantifiable; nd, not detected.

most abundant in WWTP effluent. However, the pH values and the ionic strength varied for the investigated matrices and may have influenced the extraction efficiency of the phytoestrogens. Measured pH values were 6.2 for Milli-Q water, 6.8 for drainage water, 8.2 for river water, and 7.3 for WWTP effluent. The ionic strengths were not measured but were expected to be in the range of 5–20 mM for all environmental matrices that were investigated. Adjusting Milli-Q water samples to pH 5.0 and 8.0 while keeping the ionic strength constant did not reveal any increase in extraction recoveries at lower pH (results not shown). A similar study on the same group of phytoestrogens (equol not included) in natural waters compared SPE recoveries at pH 4 and 7 and found no difference (*23*). This is not surprising, when considering that pK_a values in the range of 8–11 have been estimated for the isoflavones and structurally similar analogues (*27*, *28*).

Overall, our results indicate that ionic strength and not pH influenced the extraction recoveries (**Table 2**). This is supported by a study of the correlation between sample ionic strength and extraction efficiency reported for the off-line SPE of triazines on Oasis HLB cartridges (*29*). The authors found that the addition of 5–15 mM NaCl increased extraction rates by 5–10% when compared to the rates of neat samples. The absolute recoveries found at spike levels of 5–100 ng/L in natural waters here (63–133%) are consistent with recoveries reported for the phytoestrogens extracted from creek water (89–95%), river water (82–100%), WWTP influent (80–100%), and effluent (86–99%) spiked with a concentration of 100–500 ng/L (*8–10*).

Relative Method Recoveries. ILIS were applied to countervail the highly variable and unpredictable matrix effects that impair SPE as well as analyte ionization. Ideally, an internal standard holds the same physical and chemical properties as its target analytes. This ensures similar SPE affinities and signal distortion for the analytes and their corresponding internal standards. In the case of phytoestrogens, this applies to their respective $^{13}C_3$-labeled analogues. The relative recoveries gained for all phytoestrogens by normalization to these ILIS were close to 100% (**Table 4**). This confirms that the ILIS were able to counterbalance analyte losses occurring throughout sample preparation and analysis. Hence, the ILIS are suitable internal standards. Altogether, our results demonstrate that applying ILIS is an effective way of compensating for analyte signal suppression (**Figure 3** and **Table 2**) and incomplete SPE recovery (**Table 3**) caused by matrix effects.

Precision, Method Detection Limits, Repeatability, and Linearity. The precision (see **Table 4**) of the analytical procedure was verified by multiple analyses of spiked matrix samples. The relative standard deviations (RSDs) were calculated at three concentration levels (5, 25, and 100 ng/L) from five replicates. Their values ranged from 2 to 12% for Milli-Q water, from 3 to 19% for drainage water, from 3 to 16% for river water, and from 3 to 11% for WWTP effluent at a concentration level of 5 ng/L. Due to the higher MDL (see below) for coumestrol in WWTP effluent, this RSD (14%) was calculated at 25 ng/L. The RSDs achieved here agree with the range of precision reported for the phytoestrogens in natural waters (*8–10*) and confirm the robustness of the method presented herein.

Aside from coumestrol, the method detection limit (MDL) was defined as 3 times the mean absolute standard deviation of the five replicates at the lowest spike level [5 ng/L (**Table 5**)]. The MDLs obtained in natural waters here (0.5–11 ng/L) are comparable to the MDLs previously reported for phytoestrogens in creek water, river water, WWTP influent, and WWTP effluent (*8–10*).

Interday instrument variation was assessed for a time period of 5 weeks. During this time, the same sample in Milli-Q water was quantified six times. The instrument repeatability (RSD) obtained in this manner was between 2 and 7% for all phytoestrogens. The MS/MS detector was linear between 5 pg and 50 ng, corresponding to 0.1 and 1000 ng/L, respectively.

The analytical figures of merit presented here are comparable to performance parameters published for analytical methods relying on plain standard addition (*9*) or a single nonlabeled (*8*) or a single deuterated internal standard (*10*). However, quantifying six analytes with an analytical methodology that incorporates five ILIS covering all chromatographic analyte-containing time segments is less time-consuming than standard addition and matrix-matched calibration and more reliable when it comes to analysis of unknown samples. When matrix effects are offset by the use of several ILIS, the results quantified for environmental samples are expected to be closer to the "true" values than when using just a single nonlabeled analogue. In this study, matrix effects had the same impact on coumestrol and [$^{13}C_3$]genistein signal intensity, and thus, the latter was suitable as an internal standard not only for genistein but also for coumestrol. In contrast, equol, which elutes in the same chromatographic time segment, would not have been quantified accurately by using [$^{13}C_3$]genistein since their ion suppression values differed significantly (**Table 2**).

Environmental Application. We are currently using the analytical method presented here to study the emission of the phytoestrogens via drainage water from a pasture consisting of 43% red clover and to monitor their occurrence in streams and rivers throughout Switzerland. The first results from these studies are compiled in **Table 6**. The high formononetin and biochanin A concentrations detected in drainage water are not surprising considering their high concentrations in red clover. In contrast, the high peak concentrations of equol of more than 100 ng/L recovered in drainage water are unexpected since equol is an intestinal metabolite of daidzein (*11*) and not produced by the plant itself like the other phytoestrogens. High levels of equol (6.9–16.6 mg/L) were found in hog manure stored for several months, and the release of equol from an agricultural field

fertilized with hog manure was also described (*16*). However, the fact that no manure was applied to our field implies that in this case equol probably is a microbial product originating from the field itself.

Biochanin A, daidzein, equol, and formononetin were found in the lower-nanogram per liter concentration range in 22–96% of all river water samples (**Table 6**). Compound instability and dilution may explain the lower concentration levels found in surface water versus drainage water. At present, we have not detected genistein and coumestrol in river water. Overall, our findings compare favorably with the isoflavone concentration range detected in surface waters previously (*6–10*). Detailed results from the currently ongoing field studies mentioned above will be published elsewhere.

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