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Quantification of Five Isoflavones and Coumestrol in Various Solid Agroenvironmental Matrices Using $^{13}C_3$-Labeled Internal Standards

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We developed and validated three different sample preparation and extraction methods followed by HPLC−MS/MS (negative electrospray ionization) analysis for the quantification of estrogenic isoflavones (formononetin, daidzein, equol, biochanin A, and genistein) and coumestrol in red clover, soil, and manure. Plant and manure samples were solid−liquid extracted, whereas soil was extracted with accelerated solvent extraction. Absolute recoveries were between 80 and 93%, 20 and 30%, and 14 and 91% for plant, soil, and manure samples, respectively. Relative recoveries ranged from 75 to 105% for all matrices, indicating that isotope-labeled internal standards ($^{13}C_3$-formononetin, $^{13}C_3$-daidzein, $^{13}C_3$-equol, $^{13}C_3$-biochanin A, and $^{13}C_3$-genistein) were capable to compensate for losses during analysis. The limits of detection in red clover, soil, and manure were 3−9 $\mu g/g_{dryweight(dw)}$, 0.6−8.2 ng/g_{dw}, and 34.2 ng/g_{dw} to 17.0 $\mu g/g_{dw}$, respectively. Formononetin was the most dominant compound in red clover plants (up to 12.5 mg/g_{dw}) and soil (up to 3.3 $\mu g/g_{dw}$), whereas equol prevailed in manure (up to 387 $\mu g/g_{dw}$).

KEYWORDS: Endocrine disruptors; micropollutants; emerging contaminants; legumes; sewage sludge; biowaste

INTRODUCTION

Phytoestrogens, such as isoflavones and coumestrol, are weakly nonsteroidal estrogenic polyphenols (*1*) that are produced in plants. These compounds are present in particularly high concentrations in legumes, such as clover (*Trifolium* ssp.), soybeans (*Glycine max*), and alfalfa (*Medicago sativa*). Red clover (*Trifolium pratense*) is a common pasture and forage crop known for its high content of formononetin and biochanin A (*2*). In contrast, the main compounds in soybeans are genistein and daidzein (*3*). In the intestinal track of humans and husbandry animals, formononetin and biochanin A are demethylated to daidzein and genistein, respectively. Daidzein is further reduced to equol, which is not produced *in planta* (*4*, *5*). Coumestrol is produced in alfalfa (*3*), but also in lower concentration in clover and soybeans (*6*). **Figure 1** shows the chemical structures of these five isoflavones and coumestrol.

The content of isoflavones and coumestrol in feed and food has been intensively studied (reviewed in refs *7*, *8*). In contrast, solid agroenvironmental matrices such as soil (*9*, *10*) and manure (*11*−*13*) have so far only been poorly investigated for phytoestrogens. Ozan et al. (*9*) reported that formononetin and biochanin A spiked to nonsterile soil disappeared after three weeks by 95% and 100%, respectively. Burnison et al. (*11*) examined hog manure that was several months old and found equol concentration in the mg/L range. Tucker et al. (*13*) investigated the fecal and urinary extraction of seven phytoestrogens from Holstein heifers. They showed that equol excretion surpassed those of other phytoestrogens by at least a factor of 10. Isoflavones were detected in influents of four Swiss wastewater treatment plants, but in the effluents the concentrations were below the detection limits (*14*). Most likely, phytoestrogens are easy degradable and additionally tend to sorb to organic carbon like sewage sludge flocks (log K_{ow}'s of isoflavones range from 2.5 to 3.7 (*14*)). However, digested sewage sludge has not yet been investigated for phytoestrogens. Overall, agricultural fields cultivated with phytoestrogen-producing crops and receiving applications of manure or digested sewage sludge could be potential input sources of phytoestrogens to the agricultural environment, not only to surface waters (*14*) but also especially to soil. To accurately and precisely quantify the amounts of isoflavones and coumestrol expected to reach agricultural soils via fodder crops, manure, and sewage sludge, sensitive and robust analytical methods for these matrices are required.

Different methods for the extraction, separation, and quantification of isoflavones and coumestrol in red clover were already reported in the literature (reviewed in refs *15*, *16*). The following extraction techniques were mainly applied: Soxhlet, liquid−liquid,

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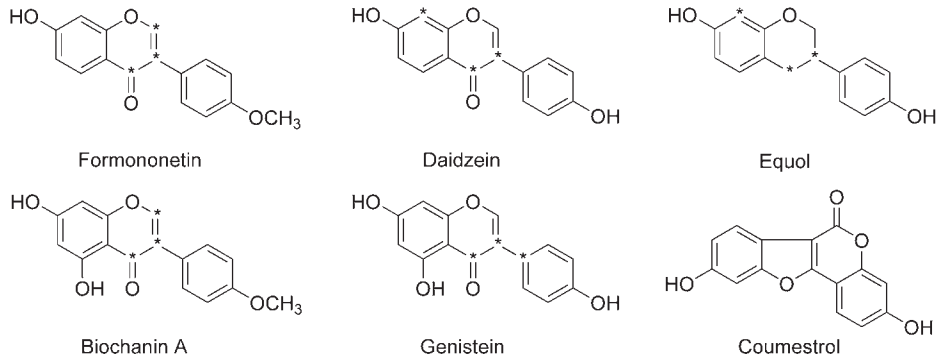

Figure 1. Chemical structures of five isoflavones and coumestrol. Asterisks indicate the positions of ^{13}C-atoms in the isotope-labeled internal standards.

and solid−liquid. Separation and detection techniques were performed on GC−MS, HPLC with UV/vis, DAD, fluorescence, or MS(/MS) detection. For compound identification mostly comparison to pure standard solutions was applied. The use of internal standards was not mentioned in the reviews, but two publications referred to the use of injection standards (4-hydroxy-1-naphthalene sulfonic acid (*17*); 6-methoxyflavanone (*18*)). Isoflavones in soils were extracted with boiling methanol, separated and detected with HPLC−UV (*9*). Shaw and Hooker (*10*) determined flavonoid sorption isotherms with HPLC-DAD. Isoflavones were identified in both cases by comparing retention times and spectra with those of pure isoflavones in methanol. For phytoestrogens in manure, liquid−liquid (*13*) or solid phase extraction (*11*, *12*) was performed. These samples were separated with HPLC followed by GC−MS or HPLC−MS/MS (*11*−*13*). Only in Tucker et al. (*13*), samples were spiked with an isotope-labeled internal standard (ILIS) (d_4-genistein). To date, no analytical method exists for the quantification of isoflavones and coumestrol in red clover and soil samples based on HPLC−MS/MS using ILIS. As mentioned before, such a method was established for manure but with only one ILIS (d_4-genistein) for quantification of seven phytoestrogens (*13*). In recent years, HPLC−MS/MS became the state-of-the-art technique for the quantification of phytoestrogens in various environmental samples (ref *19* and references therein (*13*)). In general, the crucial step in HPLC−MS/MS is the ionization of analytes. Ion suppression is already a difficulty in aqueous samples (*20*). This problem may even be worse in extracts from solid matrices containing considerable amounts of coextracted matrix compounds which affect the ionization of the analytes (*21*). Thus far, the only effective way to achieve precise and accurate results in the presence of matrix compounds is the use of ILIS, in our case $^{13}C_3$-formononetin, $^{13}C_3$-daidzein, $^{13}C_3$-equol, $^{13}C_3$-biochanin A, and $^{13}C_3$-genistein. One important objective of this work is to demonstrate that ILIS can help to overcome matrix related problems and to compensate for sample losses during the analytical procedure.

In this study, we present three different sample preparation and extraction methods followed by HPLC−MS/MS (negative electrospray ionization (ESI-)) analysis for the quantification of five isoflavones and coumestrol in various environmentally relevant types of samples (plant, soil, and manure). To our knowledge, this is the first time that $^{13}C_3$-labeled internal standards have been systematically used for the quantification of these compounds in these matrices. Analytical methods were optimized and validated for plant material, soil, and manure. Their application is demonstrated with the quantification of isoflavones in a series of red clover varieties, in soils from a local field study at our research station (Agroscope ART), and in manure samples from selected Swiss dairy farms.

MATERIALS AND METHODS

Chemicals. Biochanin A (≥97%), coumestrol (≥95%), daidzein (≥98%), equol (≥99%), formononetin (≥99%), and genistein (≥98%) were supplied by Fluka AG (Buchs, Switzerland). The ILIS [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), acetonitrile (MeCN), acetone, ethanol, ethyl acetate, toluene, cyclohexane, and *n*-heptane were of multisolvent quality and from Scharlau (Sentmenat, Spain). Dichloromethane (HPLC-grade), ammonium acetate (puriss p.a.), hydrochloric acid (37%) (puriss p.a.), and sea sand were supplied by Fluka AG (Buchs, Switzerland). Deionized water was further cleaned with a Milli-Q gradient A10 water purification system from Millipore (H_2O) (Volketswil, Switzerland). High-purity nitrogen (N_2) (99.99995%) was obtained from PanGas (Dagmarsellen, Switzerland). Individual isoflavone and coumestrol stock solutions holding concentrations of 500 μg/mL 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 compound. The ILIS solution was prepared in MeOH and held all five ILIS (0.9−5.8 μg/mL). Aqueous calibration standards were prepared in H_2O/MeCN (80/20, v/v) from the methanolic mulicomponent stock solutions holding all five isoflavones and coumestrol equivalent to the concentration rage of 0.5−100 ng/mL. To all these calibration standards, 50 μL of ILIS was added. For the isoflavone equol in manure samples, a matrix matched calibration was prepared. In this case ILIS was not added. All compounds were stored as methanolic solutions at −20 °C.

Sample Collection, Extraction, Extract Processing, and Quality Assurance. *Plant Material.* For method development and validation, clover plant materials collected in 2006 were provided from the fodder-crop breeding research group of Agroscope ART. For method application, red clover plant samples were collected manually from randomly selected locations over a 0.2 ha field study site at Agroscope ART several times before and in between crop cutting within a time period from April 2007 to April 2010. The field was cultivated with a red clover-grassland-mixture (UFA-300 CH with red clover variety Milvus). Additionally, a selection of red clover varieties was provided from the fodder-crop breeding research group of Agroscope ART. All samples were stored at −20 °C. Before extraction, all samples were freeze-dried until weight constancy was achieved (but at least for 48 h). After drying, all samples were ground and sieved to 2 mm using a SM 2000 cutting mill (Retsch GmbH, Germany). Method application samples were analyzed within 48 h after drying.

For red clover plants, several extraction methods were already reported in the literature (*15*). To account for aglycons and glycosides, our extraction method was adapted from Tsao et al. (*22*). Before extraction, the ground red clover powder was homogenized with a Turbula (Willy A. Bachofen AG, Basel, Switzerland) for 15 min. Solid−liquid extraction of 0.25 g plant material with 20 mL of ethanol/H_2O (70:30, v/v) was carried out for 16 h on a SM-30 orbital shaker at 200 rpm (Edmund Bühler GmbH, Hechingen, Germany). Different solvents (MeOH, MeCN, H_2O/MeCN (80:20, v/v), acetone, and ethyl acetate) and extraction durations (6 h, 8 h, and 16 h) were tested.

After extraction, the suspended material was let to sediment. An aliquot of 100 μL was diluted with H_2O to 25 mL (for subsequent quantification of daidzein and genistein). From this, 500 μL was further diluted with H_2O to 5 mL (for subsequent quantification of formononetin and biochanin A). Analyte-specific dilution was indicated, because the concentrations in the extracts varied and were generally much higher than the accessible linear range of the HPLC−MS/MS (*23*). Afterward, 1 mL of each extract was transferred into a 5 mL conical microreaction vial (Supelco, Bellefonte, PA, USA). ILIS solution (50 μL) and hydrochloric acid (0.5 mL, 2M) were added. The vial was capped and gently swirled and then placed in a preheated oven for 2 h at 85 °C. Hot hydrolysis was performed as already described in refs *17*, *22*. In the following, the extract was evaporated to dryness (50 °C, N_2), reconstituted in 500 μL of H_2O/MeCN (80:20, v/v) and mixed on a Vortex Genie (Scientific Industries, Bohemia, NY, USA). The solution was transferred into a HPLC vial and analyzed with HPLC−MS/MS within 48 h.

Our own data (not shown) showed that different drying techniques (freeze-drying, oven drying, or fresh) did not lead to different isoflavone contents. A red clover sample was re-extracted to test the extraction performance. To examine the stability of the analytes in dried plant material, a red clover sample was extracted five times within 21 days. The stability of ethanolic extracts was investigated by repeatedly hydrolyzing and measuring an aliquot of a red clover extract (eight times) within 27 days. The extract was kept in a closed vial at 4 °C. To assess possible degradation in processed samples, one final red clover extract (stored at room temperature) was analyzed six times within eight days.

Soil. For method development and validation, topsoil (0−10 cm) samples from the field study at Agroscope ART were collected with a split tube core sampler (2.5 cm diameter). Fifteen to twenty samples were taken from randomly selected locations over the field and pooled to one composite sample. All these samples naturally contained phytoestrogens; additional spiking was not necessary. This composite topsoil sample was immediately dried at 40 °C until weight constancy was achieved (but at least for 48 h). For method application, composite soil samples from the field study at Agroscope ART were taken (as described above) in depths of 0−10 cm and 10−20 cm within a time period from April 2007 to April 2010. Samples were stored at −20 °C. Before extraction, all samples were dried at 40 °C until weight constancy was achieved (but at least for 48 h), ground, and sieved to 2 mm using a ZM1 centrifuge mill (Retsch GmbH, Germany). Method application samples were analyzed within 48 h after drying.

Three different extraction methods were tested for soil: solid−liquid extraction, Soxhlet and accelerated solvent extraction (ASE200, Dionex Corporation, Sunnyvale, CA, USA). Soxhlet and ASE extraction exhibited similar extraction rates and were superior to solid−liquid extraction. ASE was preferred to Soxhlet extraction, because less solvent was used and the extraction times were shorter. Before extraction, the soil sample was homogenized with a Turbula for 15 min. Afterward, 5 g of soil sample was filled in a ASE cell, and this cell was filled up to the top with sea sand. Different solvents and mixtures thereof (MeOH, MeCN, *n*-heptane, ethyl acetate, acetone, toluene, MeOH/MeCN 1:3 (v/v), MeOH/MeCN 1:1 (v/v), MeOH/MeCN 3:1 (v/v), MeOH/ethyl acetate 1:1 (v/v), MeCN/ethyl acetate 1:1 (v/v), MeOH/*n*-heptane 4:1 (v/v), MeCN/*n*-heptane 4:1 (v/v), ethyl acetate/*n*-heptane 4:1 (v/v), acetone/toluene 1:1 (v/v)), different numbers of static cycles (one, two, three), different durations of static cycle (5 min, 10 min, 15 min), different flush volumes (75%, 100%, 115%), different pressures (1000 psi, 1500 psi, 2000 psi, 2500 psi), and different temperatures (40 °C, 60 °C, 80 °C, 120 °C, 150 °C, 180 °C, 200 °C) were successively tested to optimize the ASE extraction (i.e., using always the optimized conditions of the respective preceding parameters). This order and procedure was selected because it was reported in literature (e.g. refs *24*, *25*) that the solvent type seems to influence the extraction efficiency more than pressure, temperature, and any other ASE parameters. After the ASE extraction, an aliquot of 10% of the total extract volume was transferred in a 5 mL conical microreaction vial, and ILIS solution (100 μL) was added. In the following, the extract was evaporated to dryness (50 °C, N_2). The dried extract was reconstituted in 500 μL of H_2O/MeCN (80:20, v/v) and mixed on a Vortex Genie. The solution was transferred into a HPLC vial and analyzed with HPLC−MS/MS within 48 h.

A soil sample was re-extracted to test the extraction performance. To investigate if substantial amounts of isoflavones and coumestrol were present in a conjugated form, soil extracts were hydrolyzed as described for plant materials. To verify the stability of isoflavones and coumestrol over time, the sample used for validation was extracted repeatedly over a period of three weeks.

Manure. For method development and validation, 100 L of manure (cattle/swine, 3/1 (v/v)) was obtained from a farmer located near Agroscope ART. The received manure naturally contained phytoestrogens; additional spiking was not necessary. For method application, manure samples were collected from selected Swiss dairy farms. The samples were collected from the beginning of February to the beginning of April 2010. Before sampling, the liquid manure storage facility at the selected farms was homogenized for 30 min using a stirring unit. A sample was taken with a sampling probe (PVC pipe, length 2.4 m, diameter 4.5 cm) for vertical sampling. The bottom end of the pipe was closed with a rubber ball connected to a cord. Ten vertical subsamples (each subsample around 1 L) were filled in a 40 L pail to form a composite sample. One liter of this composite sample was taken for analysis. During the transportation to Agroscope ART samples were put in a cooler and afterward stored at 4 °C until the samples were treated further. Because of the same origin and its similar composition, liquid manure/slurry (urine and feces) and dung (feces and straw material) were handled in the same way. In this study, the term manure stands for both liquid manure and dung. Before extraction, all samples were dried at 105 °C until weight constancy was achieved (but at least for 96 h), ground, and sieved to 2 mm using a ZM1 centrifuge mill. Application samples were analyzed within 48 h after drying.

Solid−liquid extraction was selected for manure because Soxhlet extraction led to very dirty extracts with oily residues that made further concentration steps almost impossible and caused clogging of ASE capillaries. Before extraction, the ground manure powder was homogenized with a Turbula for 15 min. Solid−liquid extractions of 0.25 g (for equol) and 0.05 g (for formononetin, daidzein, biochanin A, genistein, and coumestrol) manure, respectively, with 20 mL of different solvents (H_2O, MeOH, MeCN, ethyl acetate, acetone, toluene, clyclohexane, H_2O/MeOH 1:1 (v/v), MeOH/acetone 1:1 (v/v), MeOH/toluene 1:1 (v/v), MeOH/dichloromethane 1:1 (v/v), ethanol/H_2O 70:30 (v/v)) were carried out on a SM-30 orbital shaker at 200 rpm. Additionally, the extraction duration was varied from 30 min over several hours (3 h, 6 h, 16 h, and 24 h) up to 4 days. ILIS solution (100 μL) was added prior to extraction to 0.05 g manure. For the analysis of equol in manure no ILIS was added because of the very high concentration of equol found in manure, which would require an equivalent addition of ILIS.

After extraction, the suspended material was allowed to sediment. For equol, an aliquot of 50 μL was taken from the 0.25 g manure extract and diluted with H_2O to 10 mL. Afterward, 1 mL of diluted extract was transferred into a 5 mL conical microreaction vial. Further dilution was indicated, because the concentrations of equol in the extracts were much higher than the accessible linear range of the HPLC−MS/MS (*23*). The sample was evaporated to dryness (50 °C, N_2), reconstituted in 500 μL of H_2O/MeCN (80:20, v/v) and mixed on a Vortex Genie. For the other isoflavones and coumestrol, the extract (20 mL) was centrifuged with a Labofuge 200 (Heraeus Sepatech, Hanau, Germany) at 5000 rpm for 10 min and afterward stepwisely transferred to a 10 mL conical microreaction vial. In between, the volume of the sample was reduced by evaporating the solvent (50 °C, N_2) until the final volume of the extract reached 4 mL. Hydrochloric acid (0.5 mL, 2 M) was added. The vial was capped, gently swirled, and placed in a preheated oven for 2 h at 85 °C. In the following, the extract was evaporated to dryness (50 °C, N_2). The dried extract was reconstituted in 500 μL of H_2O/MeCN (80:20, v/v) and mixed on a Vortex Genie. The solution was transferred into a HPLC vial and analyzed with HPLC−MS/MS within 48 h.

Manure extracts were not stable over a longer time period, hence the extracts were analyzed within 24 h after extraction. To investigate if substantial amounts of isoflavones and coumestrol were present in a conjugated form, manure extracts were hydrolyzed as described for plant materials.

Digested Sewage Sludge. A dried and milled digested sewage sludge sample (as described in ref *21*) was extracted with Soxhlet, ASE and solid−liquid extraction with different solvents. Unfortunately, with all three extraction methods no isoflavones and coumestrol were detected and therefore the method was not further optimized. It is likely that isoflavones were degraded and that no significant absorption to sludge took place.

Table 1. Retention Times, Precursor and Product Ions, and Collision Energies for Isoflavones and Coumestrol and Their Corresponding $^{13}C_3$-Labeled Internal Standards

compound	retention time (min)	precursor ion (m/z) → product ion (collision energy (eV))[a]
$^{13}C_3$-daidzein	7.84	**256 → 134 (35 eV)**; 256 → 226 (35 eV); 256 → 210 (35 eV)
daidzein	7.85	**253 → 132 (40 eV)**; 253 → 223 (40 eV); 253 → 195 (40 eV)
$^{13}C_3$-genistein	12.08	**272 → 135 (35 eV)**; 272 → 161 (35 eV); 272 → 185 (35 eV)
genistein	12.09	**269 → 133 (35 eV)**; 269 → 180 (35 eV); 269 → 157 (35 eV)
coumestrol[b]	12.45	**267 → 182 (45 eV)**; 267 → 166 (45 eV); 267 → 211 (45 eV)
$^{13}C_3$-equol	12.67	**244 → 120 (20 eV);** 244 → 123 (20 eV); 244 → 138 (20 eV)
equol	12.67	**241 → 119 (20 eV)**; 241 → 121 (20 eV); 241 → 135 (20 eV)
$^{13}C_3$-formononetin	16.34	**270 → 254 (35 eV)**; 270 → 225 (35 eV); 270 → 197 (35 eV)
formononetin	16.34	**267 → 252 (25 eV)**; 267 → 223 (35 eV); 267 → 195 (40 eV)
$^{13}C_3$-biochanin A	23.99	**286 → 270 (45 eV)**; 286 → 213 (45 eV); 286 → 241 (45 eV)
biochanin A	23.99	**283 → 268 (30 eV)**; 283 → 239 (35 eV); 283 → 211 (45 eV)

[a] Precursor and product ions used for quantification (in bold); first qualifier; second qualifier. [b] $^{13}C_3$-Genistein used as internal standard.

In fact, Kang and Price (*26*) showed that most phytoestrogens were eliminated by biological treatment using activated sludge.

Chromatographic Separation and Mass Spectrometric Detection. Analyte separation, detection, and quantification by HPLC−MS/MS (ESI-) was performed on a Varian 1200 L HPLC−MS instrument (Varian Inc., Walnut Creek, CA, USA) as described in Erbs et al. (*23*). Briefly, the isoflavones and coumestrol were separated on an XTerra MS C18 column (2.1 × 100 mm, 3.5 μm; Waters Corp., Milford, MA, USA) with a guard column of the same type (2.1 × 20 mm, 3.5 μm; Waters Corp.). The following optimized elution gradient was applied at room temperature: 0 min, 21% eluent B (79% eluent A); 3 min, 21% B; 4 min, 26% B; 26.5 min, 47% B; 27 min, 95% B; 29 min, 95% B; 30 min, 21% B; 35 min, 21% B with eluent A consisting of H_2O/MeCN (95:5, v/v) and eluent B of H_2O/MeCN (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 with tandem mass spectrometry. Interface parameters for the HPLC−MS/MS system were as follows: needle voltage, −4500 V; nebulizing gas (compressed air), 3.45 bar; drying gas (N_2, 99.5%, generated online using a high-purity nitrogen generator (Nitrox UHPLCMS18, Domnick Hunter Ltd., Gateshead, U.K.)), 300 °C and 1.31 bar; and shield voltage, −600 V. The collision cell gas (Ar, 99.999%) pressure was 2.66×10^{-6} bar, and the detector voltage was set to −2000 V. The total run time for a single sample was 35 min. Retention times, precursor and product ions, and collision energies are reported in **Table 1**. Further detailed information about the method is given in Erbs et al. (*23*).

Method Validation Parameters. The linearity of the methods was determined from the individual matrix matched calibrations. Ion suppressions were evaluated by comparing the analyte signals obtained from injection of the same amount (0.25, 0.5, 1.25, 2.5, and 5 ng, respectively) of analyte dissolved in the final extract from the various matrices (i.e., matrix matched calibrations) and in the respective pure solvent. The ion suppression (expressed in percentage) was quantified as 1 minus the ratio between the slope of the curve obtained for the extracted matrix and the slope of the curve for the pure solvent. One replicate for each concentration (corresponding to a total of $N = 5$) was prepared for plant and soil, three replicates (corresponding to a total of $N = 9$) for manure.

Absolute recoveries over extraction, cleanup, and quantification were determined for all analytes in all described matrices. Soil and manure samples were spiked directly on the dried matrices 24 h prior to extraction with different amounts of isoflavones and coumestrol that yielded final extract concentrations similar to those of matrix matched calibrations (see above). For plants the diluted extracts instead of the dried samples were spiked with different amounts of isoflavones that yielded final extract concentrations similar to those of the matrix matched calibrations (see above). Three replicates were prepared for plant material, five for soil, and six for manure. ILIS was added prior to the analysis by HPLC−MS/MS. The absolute method recovery was defined as the ratio between the quantified and the spiked amount. Native amounts as determined in respective blank samples were accounted for. For equol in manure extracts no ILIS was used. Therefore, the absolute recovery was calculated by the ratio of the standard addition to the final matrix matched extracts and standard addition to the diluted manure extracts. The equol concentrations added to both extracts for the standard addition were 30 μg/g, 60 μg/g, and 90 μg/g.

Relative recoveries over extraction, cleanup, and quantification were obtained for the three matrices. Addition of analytes and replicates was performed as mentioned in the section before, but the analytes and the ILIS were spiked directly on the dry material 24 h prior to extraction or in the dilutions. The relative recoveries were defined as the ratio of the quantified and the spiked amounts.

Method precision was defined as the relative standard deviation of five replicates at chosen concentration levels. The instrument precisions were determined by injecting a sample five times out of the same sample vial in direct succession. For all compounds, the limits of detection (LOD) were defined as three times the absolute standard deviation of five replicates of a low spiked level.

Statistics. Statistical evaluations were performed with S-Plus-software. A pairwise t test after Bonferroni was performed to compare between group levels with corrections for multiple testing. A simple variance analysis (one way ANOVA) was used to compare means of two or more samples.

RESULTS AND DISCUSSION

Extraction. *Plant Material*. **Figure 2** shows that shaking dry plant material in aqueous ethanol (70%) or methanol (100%) at room temperature for 16 h resulted in comparable formononetin concentrations, whereas biochanin A was more efficiently extracted with aqueous ethanol (70%). The same observation was made for daidzein and genistein (data not shown). Neither the use of hot solvent (80 °C) nor additional sonication for 15 min at room temperature led to higher concentrations. After 6 h extraction, the maximum extractable concentration was achieved for formononetin, daidzein, and biochanin A. Genistein reached this maximum after 8 h. Overall, these optimized conditions are similar to Tsao et al. (*22*) and only differ in the hydrolyzing step which takes place in our case after the extraction and dilution. The residual amount of analytes in the re-extracted sample was determined to be less than 2% of the initially determined amount. Isoflavones and coumestrol were stable in dried plant material at room temperature for at least 21 days, in extraction solvents for at least 27 days, and in final extracts for at least eight days.

Soil. The ASE parameters were optimized based on formononetin, because this compound made up more than 60% of all analytes and was the single most dominant compound in soil. In general, biochanin A paralleled the results of formononetin

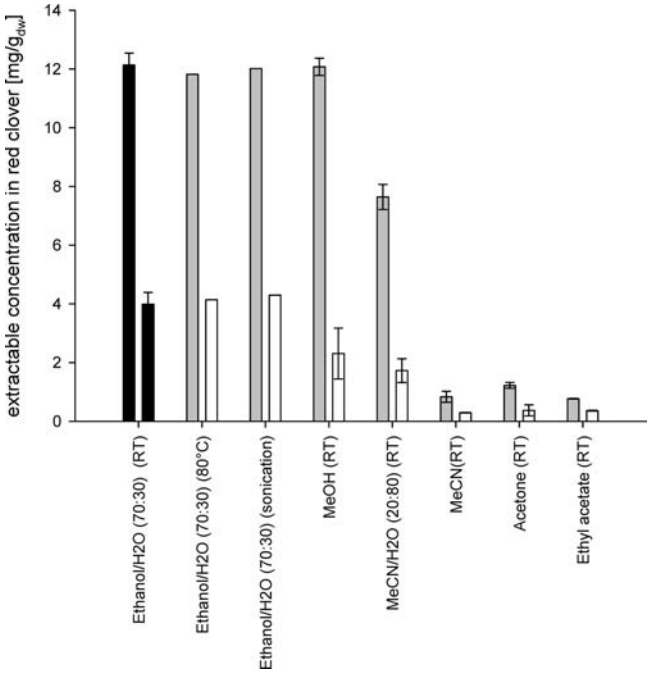

Figure 2. Concentrations of formononetin (gray bars) and biochanin A (white bars) in red clover for several solvents. $N = 3$, except $N = 1$ for hot ethanolic extraction and ethanolic extraction with sonication. Error bars represent standard deviations of replicate analyses. H2O = Milli-Q water; MeOH = methanol; MeCN = acetonitrile; dw = dry weight; RT = room temperature; black bars = chosen solvent.

(data not shown), whereas daidzein, equol, genistein, and coumestrol were mostly not detected. Of all solvent compositions tested, the highest and least variable formononetin concentration was extracted with MeOH/MeCN (1:1, v/v) (**Figure 3A**). Comparing this result with the calculated Hildebrand solubility parameters (*27*) for these compounds showed that this solvent mixture was pretty unexpected. The Hildebrand solubility parameters suggested mixtures of alcohols (like MeOH) with apolar solvents (like toluene) or esters (like ethyl acetate) to be best for extraction. However, the Hildebrand approach is not taking into account the nature of the matrix. With one static cycle of 5 min the highest formononetin yield was obtained, although variations of these two parameters did not show significant differences (pairwise *t* test, *p*-value = 1 for both variables) (**Figure 3B**, **3C**). Significantly higher extraction efficiencies were obtained with flush volumes of 100% and 115%, as compared to 75% (pairwise *t* test, *p*-value < 0.05) (**Figure 3D**), whereas the extraction efficiency was largely independent of pressure (pairwise *t* test, *p*-value = 1) (**Figure 3E**). In both cases the settings of the parameters which resulted in the highest mean values of extractable concentration in soils were chosen (**Figures 3D**, **3E**). The highest concentration of formononetin was extracted at 200 °C (highest possible temperature of ASE; pairwise *t* test, *p*-value < 0.05) (**Figure 3F**). This experiment further showed that the isoflavones were not heat sensitive. For ASE extractions employment of high temperature (above 100 °C) and high pressure (above 100 bar) is often reported (e.g. (*24*, *25*, *28*)).

Under these optimized conditions the residual amount of analytes in the re-extracted sample was determined to be around 9% and 4% for formononetin and biochanin A, respectively, of the initially determined amount. Additional hydrolysis of the extracts did not result in higher extracted amounts. This indicates that only a minor, if any, fraction of isoflavones is present in soil in a conjugated form. Contrarily, equol was nearly completely degraded during this process. Consequently, we decided to omit the hydrolysis step for soil samples. Isoflavones were stable in dried soil samples for at least three weeks, as tested by repeated analysis of an individual sample (one-way ANOVA, *p*-value < 0.05).

Manure. The manure extraction was optimized for equol (in diluted extract of 0.25 g sample) and formononetin (in concentrated extract of 0.05 g sample), because these compounds dominated in the respective extracts. By far the highest equol and formononetin concentrations were extracted with ethanol/H_2O (70:30, v/v), as for plant materials (**Figure 4**). The highest concentrations of equol and formononetin were reached after 16 and 6 h of extraction, respectively.

The quantified concentrations proofed to be independent of the extracted amount of manure (pairwise *t* test, *p*-value > 0.05). Hence, we decided to reduce the sample weight for manure (to 0.05 g), so that ILIS could be added from the beginning of the extraction to compensate for analyte loss. Extract hydrolysis caused an almost complete degradation of equol. Conversely, this process resulted in much higher extraction concentrations (more than 20 times) of the other five compounds. Equol is microbially produced in the rumen and mainly excreted in the free form (*4*, *5*). Contrarily, fodder crop plant debris is a regular constituent of manure, which makes it plausible that isoflavones are present in this matrix also in conjugated form. For these reasons, we decided to omit the hydrolysis for equol, but to hydrolyze the extracts of all the other compounds.

Method Validation Parameters. *Plant Material*. Matrix matched calibration curves were linear ($0.98 < R^2 < 1.0$) within the working range from 50 $\mu g/g_{dw}$ to 10 mg/g_{dw} for formononetin and biochanin A, and from 5 $\mu g/g_{dw}$ to 1 mg/g_{dw} for daidzein and genistein, respectively. The ion suppression for the isoflavones in red clover samples is presented in **Table 2**. Considering that we were working with dilution factors of 5000 to 50000 in red clover extracts the ion suppression is remarkable in the case of daidzein, indicating the necessity to apply ILIS for compensation even in these presumably "easy" samples. The absolute and relative recoveries were satisfactory with numbers above 80% (**Table 2**). Hence, no significant analyte losses occurred during hydrolysis and reconstitution. Absolute recoveries were comparable to those found in the literature (*29*). The method precision (3.3−9.7%) for plant samples was lower than the instrument precision (1.4−2.5%), indicating that the instrument was more robust than the method (**Table 2**). Unfortunately, in literature no values for method or instrument precisions were reported. Only Krenn et al. (*18*) mentioned that their precision was satisfactory. The values for method precision correspond well with overall method uncertainties as calculated from error propagation at these concentration levels (*30*). The LODs of all isoflavones in plants were in the lower $\mu g/g_{dw}$ range (**Table 2**), which is reasonable considering the mg/g concentrations in plants. These values were comparable to de Rijke et al. (*17*), but higher than those of Ramos et al. (*29*). Hence, although our method is similar to those already published and its overall performance is not necessarily superior to them, it is the overall high degree of harmonization between all the methods presented here that renders the generated data for the different matrices more comparable and trustworthy (see Method Applications).

Soil. Matrix matched calibration curves were linear ($0.98 < R^2 < 1.0$) within the working range from 5 ng/g_{dw} to 100 ng/g_{dw} for all isoflavones. The ion suppression for isoflavones and coumestrol in soil ranged from 40 to 85% (**Table 2**). Daidzein, genistein, and coumestrol had a factor of 2 higher ion suppression than formononetin, equol, and biochanin A. A reason for that might be the fact that these three compounds were eluting first from the

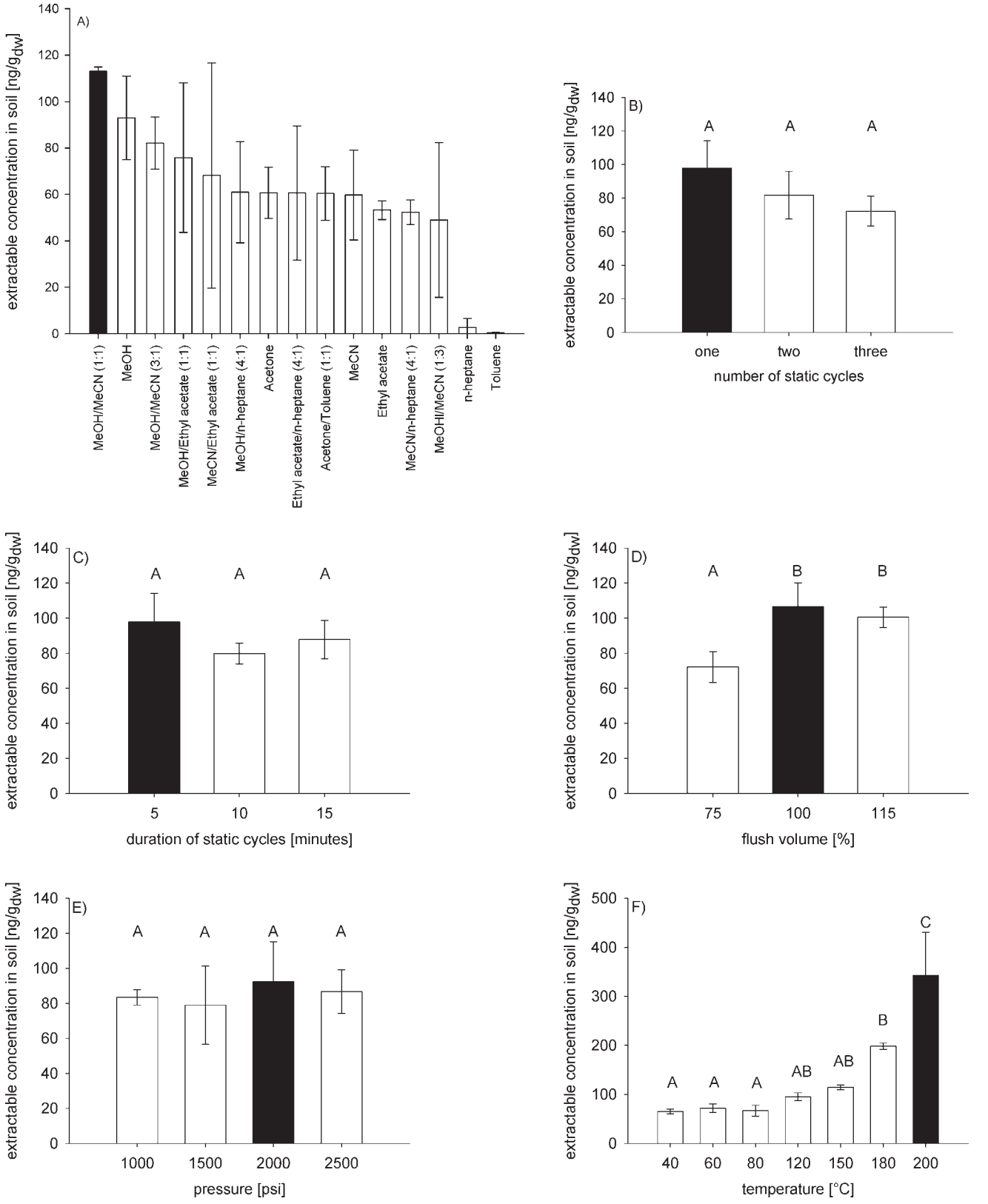

Figure 3. Concentrations of formononetin in soil for (**A**) different extraction solvents and mixtures thereof. $N = 2$, except $N = 3$ for the MeOH/MeCN mixtures. MeOH = methanol; MeCN = acetonitrile; (**B**) different numbers of static cycles ($N = 3$); (**C**) different durations of static cycles ($N = 3$); (**D**) different flush volumes ($N = 3$); (**E**) different pressures ($N = 3$); (**F**) different temperatures ($N = 3$). Error bars represent standard deviations of replicate analyses. dw = dry weight; black bars = chosen parameters. Capital letters in different graphs indicate significance (pairwise *t* test after Bonferroni).

HPLC column. Although considerable, such ion suppression values are not unusual, and for instance also found in WWTP (*31*). The absolute recoveries of isoflavones and coumestrol spiked 24 h before extraction were below 30% (**Table 2**). In contrast, addition of isoflavones and coumestrol to soil followed by extraction after 30 min led to a complete extraction of spiked formononetin, daidzein, equol, and coumestrol (129%, 100%, 92%, and 90%, respectively), but still only 13% and 8% of the total

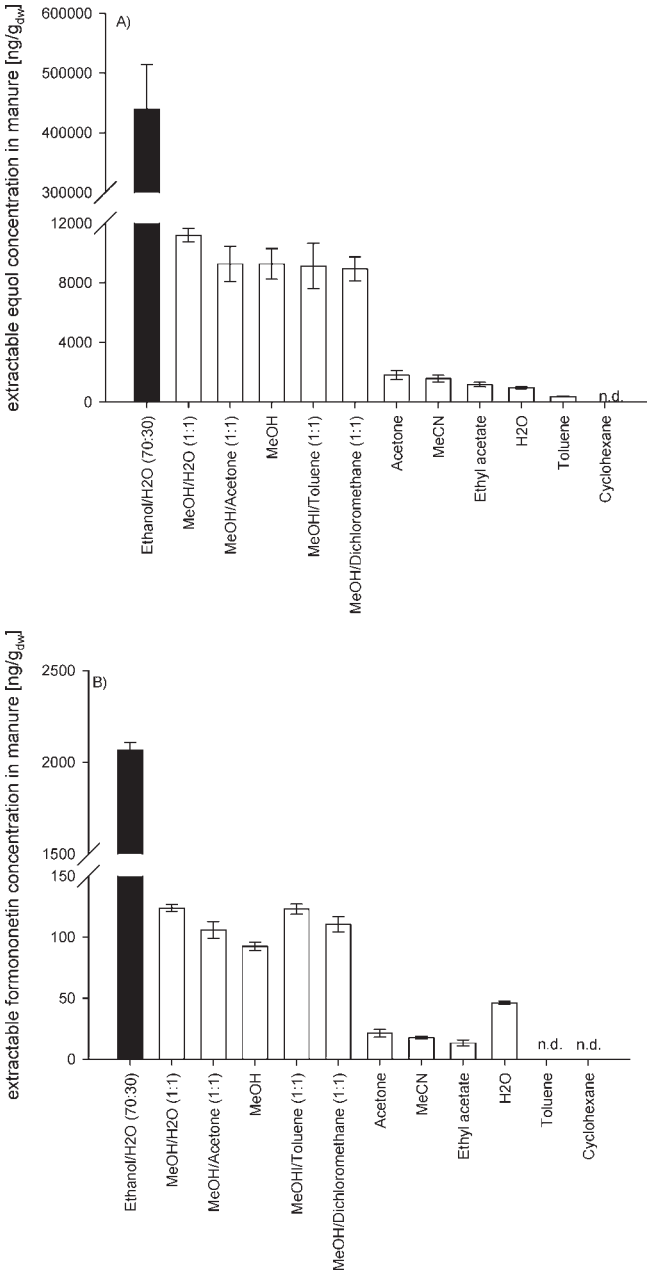

Figure 4. Concentrations of equol (**A**) and fomononetin (**B**) in manure for different extraction solvents and mixtures thereof. $N = 3$, except $N = 2$ for ethanol/H_2O (70:30, v/v). Error bars represent standard deviations of replicate analyses. H2O = Milli-Q water; MeOH = methanol; MeCN = acetonitrile; dw = dry weight; n.d. = not detected; black bars = chosen solvent.

spiked amounts of biochanin A and genistein were recovered, respectively. This is in accordance with 95% recovery of fomononetin extracted immediately after spiking a nonsterile soil (*9*). In contrast to our findings, in the same publication a biochanin A recovery of 95% was reported (*9*). Shaw and Hooker (*10*) extracted lower concentration of formononetin (71%) immediately after spiking to a nonsterile soil. One of the most plausible hypotheses for increasingly severe analyte losses over time is the sequestration of these isoflavones and coumestrol to some soil constituents. This effect was already observed for the analysis of antibiotics in soils without a more satisfactory explanation (*28*). In a review on pesticide nonextractable (bound) residues in soil, Barriuso et al. (*32*) outlined that the persistence of the analyte in the matrix after extraction can exceed 70% for some pesticides. In addition to sorption to humic acid (*32*), also cation exchange and hydrophobic interactions seem to be plausible mechanisms causing nonextractable residues in soil (as shown for pesticides in ref *33*). In contrast, relative recoveries from soil (extracted 24 h after spiking with both analytes and ILIS) were satisfying (**Table 2**). Relative standard deviations of absolute and relative recoveries paralleled the degree of sequestration, with values twice as high for biochanin A, genistein, and coumestrol than for formononetin, daidzein, and equol. The method precision for soil was lower than the instrument precision, indicating that the instrument was more robust than the method. The values for method precision correspond well with overall method uncertainties as calculated from error propagation at these concentration levels (*30*). The LODs of all isoflavones and coumestrol in soil were in the lower ng/g_{dw} range. Compounds with higher ion suppression (i.e, daidzein, genistein, and coumestrol) also had higher LODs.

Manure. Matrix matched calibration curves were linear ($0.98 < R^2 < 1.0$) within the working range from 5 ng/g_{dw} to 1 μg/g_{dw} for formononetin, daidzein, biochanin A, genistein, and coumestrol and from 40 ng/g_{dw} to 800 μg/g_{dw} for equol, respectively. The ion suppression in manure ranged from 40 to 61% (**Table 2**). The numbers were very similar for isoflavones and somewhat higher for coumestrol. The absolute recoveries for the four isoflavones and coumestrol were between 14 and 45%. Losses probably occurred during the extract concentration from 20 mL to 500 μL. For equol the absolute recovery was 91% (**Table 2**). The relative recoveries were between 95 and 105% (**Table 2**). Tucker et al. (*13*) reported similar relative recoveries for manure between 88 and 116% (using another solid−liquid extraction method than one presented here). In contrast to our method, they calculated the recoveries with the help of only one internal standard (d_4-genistein). Furuichi et al. (*12*) reported relative recoveries for genistein and equol of 84% and 88%, respectively, performing solid phase extraction. The method precision for manure was lower than the instrument precision, indicating that the instrument was more robust that the method (**Table 2**). The values for method precision correspond well with overall method uncertainties as calculated from error propagation at these concentration levels (*30*). The LODs of all isoflavones and coumestrol in manure were in the ng/g_{dw} to μg/g_{dw} (for equol) range. The equol value (LOD 17 μg/g_{dw}) was comparable to those of isoflavones in plants (see above). The two methods which reported LODs in manure had both limits of detection in the lower ng/mL concentration range (both methods used liquid extractions) (*12*, *13*).

Method Applications. We are currently using the presented analytical methods to study the input and distribution of isoflavones and coumestrol on a field study at Agroscope ART. Additionally, red clover varieties and manure samples from different Swiss dairy farms were investigated. A summary of measured isoflavone and coumestrol concentrations in different matrices are reported in **Table 3** and **Table 4** and are discussed in the following paragraphs.

Plant Material. In **Table 3** the isoflavone concentrations of different red clover varieties are reported. Formononetin was analyzed with two methods: a fluorometric assay reference method used at Agroscope ART (*34*) and with the here presented HPLC−MS/MS method. The concentrations measured with HPLC−MS/MS were systematically higher than those obtained with the fluorometric method (linear regression with slope of 1.67). The differences between the methods were larger with higher formononetin concentration. Several reasons may lead to such a difference in these two methods. The quantification of the fluorometric assay is based on the fluorescence of conjugated

Table 2. Method Validation Parameters: Ion Suppressions (Negative Values Corresponding to Enhancement) (with Relative Standard Deviation), Absolute and Relative Recoveries (with Relative Standard Deviation), Method and Instrument Precisions, and Limits of Detection (LOD) for Plant, Soil, and Manure, Respectively[a]

			Plant[b]			
analyte	ion suppression [%] (N = 1 for C = 5)	abs recov [%] (N = 3 for C = 5)	rel recov [%] (N = 3 for C = 5)	meth precision [%] (N = 5 for C = 2)	instrum precision [%] (N = 5 for C = 2)	LOD [$\mu g/g_{dw}$]
formononetin	−1	92 (8)	89 (5)	3.3	1.4	9
daidzein	22	93 (6)	91 (4)	9.7	1.8	3
biochanin A	7	85 (3)	91 (3)	4.6	1.8	4
genistein	−7	80 (9)	86 (4)	5.0	2.5	5
			Soil			
analyte	ion suppression [%] (N = 1 for C = 5)	abs recov [%] (N = 5 for C = 5)	rel recov [%] (N = 5 for C = 5)	meth precision [%] (N = 5 for C = 2)	instrum precision [%] (N = 5 for C = 2)	LOD [ng/g_{dw}]
formononetin	44	29 (8)	101 (12)	13.7	4.1	0.6
daidzein	84	30 (14)	102 (14)	15.0	5.2	6.7
equol	41	23 (9)	92 (8)	14.4	7.9	1.2
biochanin A	40	20 (32)	75 (27)	14.1	7.0	1.0
genistein	77	25 (30)	85 (19)	19.5	6.1	7.3
coumestrol	79	22 (25)	85 (32)	10.5	5.8	8.2
			Manure			
analyte	ion suppression [%] (N = 3 for C = 3)	abs recov [%] (N = 6 for C = 3)	rel recov [%] (N = 6 for C = 3)	meth precision [%] (N = 5 for C = 2)	instrum precision [%] (N = 5 for C = 2)	LOD [ng/g_{dw}]
formononetin	40 (7)	28 (15)	105 (3)	15.2	2.4	38.6
daidzein	48 (2)	45 (11)	95 (6)	7.8	2.7	77.6
equol	n.d.	91 (4) (N = 3)	n.d.	n.d.	n.d.	17 $\mu g/g_{dw}$
biochanin A	45 (5)	14 (10)	95 (4)	16.3	3.2	34.2
genistein	48 (3)	27 (11)	102 (5)	9.0	2.0	66.5
coumestrol	61 (4)	24 (10)	95 (9)	7.1	5.5	82.8

[a] N: number of replicates per concentration level. C: number of different concentration levels tested. dw = dry weight. n.d. = not determined. [b] Equol and coumestrol were not present and not detected, respectively, in red clover.

Table 3. Concentrations of Formononetin Measured with the Fluorometric Assay and the Concentrations of Isoflavones in Red Clover Varieties Measured with HPLC−MS/MS[a]

	fluorometric assay	HPLC−MS/MS			
clover variety	formononetin [mg/g_{dw}]	formononetin [mg/g_{dw}]	daidzein [mg/g_{dw}]	biochanin A [mg/g_{dw}]	genistein [mg/g_{dw}]
Tedi	8.25	12.5	0.16	4.96	0.66
Larus oven-dried	5.03	9.36	1.09	2.98	1.06
Larus freeze-dried	4.67	8.54	0.84	2.68	0.90
Milvus	n.a.	8.33	0.14	3.75	0.68
TP9915	5.37	6.76	0.19	3.24	0.55
TP0045	4.20	6.53	0.16	3.51	0.75
TP0185	3.16	3.59	0.10	3.39	0.70
AberRuby	1.70	1.69	0.05	5.80	0.73

[a] For each variety two samples were analyzed. dw = dry weight. n.a. = not analyzed.

and nonconjugated formononetin as a whole mixture (daidzein fluoresces under the same conditions, but is present in much lower concentrations in clover) (*35*). It is assumed that the fluorescence yield of formononetin is not affected by the sugar substituent, which in fact might not be entirely correct. Additionally, other matrix effects may have an influence (e.g., self-absorption of excitation energy, or fluorescence quenching due to quenching agents present in the plants (*36*)). In contrast, the HPLC−MS/MS method is considered more robust because ILIS compensate for losses and matrix effects, and the chromatographic separation step combined with the triple quadrupole mass analyzer allows for maximum selectivity and specificity.

Formononetin concentration varied in the different varieties between 1.69 and 12.5 mg/g_{dw} (**Table 3**). For the other isoflavones the concentration range was much smaller. Tedi, Larus, and Milvus had the highest formononetin concentrations, Aber Ruby and TP0185 the lowest. Only in one variety (Aber Ruby) the biochanin A concentration exceed the formononetin concentration. Similar isoflavone concentrations were reported in a wider variety of red clover cultivars (*2*, *22*, *29*).

Soil. Isoflavone and coumestrol concentrations in soil samples from the field study at Agroscope ART are reported in **Table 4**. Generally, higher isoflavone concentrations were quantified in the topsoil (0−10 cm). In both depths, fomononetin was detected at highest concentrations (up to 3350 ng/g_{dw} in 0−10 cm, and 251 ng/g_{dw} in 10−20 cm). In the topsoil, the concentration pattern of the isoflavones (except equol) paralleled those of Milvus red clover variety cultivated on the field, but not so in subsurface soils, where daidzein surpassed biochanin A. Equol and genistein were found in the topsoil, but not in 10−20 cm depth. Coumestrol

Table 4. Concentrations of Isoflavones and Coumestrol in Soil of the Field Study at Agroscope ART and from Manure of the Swiss Dairy Monitoring Program[a]

compound	mean [ng/g_{dw}]	min [ng/g_{dw}]	max [ng/g_{dw}]
Soil: 0−10 cm (N = 7)			
formononetin	791	188	3350
daidzein	55.8	37.2	74.2
equol	6.70	6.70	6.70
biochanin A	83.5	19.6	258
genistein	80.5	det	80.5
coumestrol	n.d.		
Soil: 10−20 cm (N = 5)			
formononetin	104	55.4	251
daidzein	41.0	36.4	53.2
equol	n.d.		
biochanin A	21.5	10.3	38.2
genistein	n.d.		
coumestrol	n.d.		
Manure (Predominant Feed Type: Grass and Corn Silages; N = 3)			
formononetin	4090	2970	6180
daidzein	2390	904	4310
equol	225 $\mu g/g_{dw}$	106 $\mu g/g_{dw}$	387 $\mu g/g_{dw}$
biochanin A	1330	597	2370
genistein	480	225	809
coumestrol	773	486	919
Manure (Predominant Feed Type: Hay; N = 5)			
formononetin	1460	655	2510
daidzein	675	238	1850
equol	60.1 $\mu g/g_{dw}$	det	181 $\mu g/g_{dw}$
biochanin A	313	96	581
genistein	196	102	327
coumestrol	410	165	555

[a] dw = dry weight. det = detected. n.d. = not detected.

was the only compound which was not detected in any of these soil samples.

Comparing the ratio between the degradation product daidzein and its parent compound formononetin in Milvus and in the two soil depths, there was an increase of this ratio from 2% to 7% to 39%, respectively. For the ratio between genistein and its parent compound biochanin A, these values were 18% to 96% (no value for 10−20 cm depth), respectively. These results suggest that dissipation of the parent compound isoflavones takes place as soon as they are emitted into the environment. Thereby, biochanin A seemed to dissipate fastest, which is in accordance with its half-life in water being shortest (*14*). Higher metabolite-to-parent ratios (daidzein/formononetin = 16%; genistein/biochanin A = 47%) were also observed in drainage water from the very same field (*23*).

Ozan et al. (*9*) and Shaw and Hooker (*10*) confirmed our finding of isoflavone dissipation in natural soil. According to their lab studies, both formononetin and biochanin A can be degraded in soil, but degradation products were not identified. Although equol is not produced *in planta* (*4*, *5*), this compound was found in the topsoil, indicating that probably microbial degradation in the soil took place. Until now only anaerobic degradation of formononetin and daidzein to equol was reported (*4*, *5*), conditions that may be rather unlikely in topsoil though. Again, the findings in soils were in accordance with our earlier work with drainage water, in which we quantified equol up to 121 ng/L (*23*).

Manure. Isoflavone and coumestrol concentrations in manure samples from different Swiss dairy farms are reported in **Table 4**. Since the feeding regime has an influence on the isoflavone and coumestrol concentrations in manure (*13*), the results are listed separately for two groups with different dominant feeds, i.e., grass and corn silages, and hay. When feeding grass and corn silages, the concentrations were generally higher than when feeding hay. In manure from both feed types, equol exhibited the highest concentrations (up to 387 $\mu g/g_{dw}$ and 181 $\mu g/g_{dw,}$ for grass/corn silages and hay, respectively). However, the differences in isoflavone and coumestrol concentrations were not significant in the two feeding regimes (pairwise t test, p-value >0.05 for all compounds). Tucker et al. (*13*) reported equol concentration in manure of Holstein heifers fed with red clover of around 85 $\mu g/g_{dw}$ (assuming 34 L of excretion per day, and a dw of 5%). This concentration is similar to ours. Much lower concentrations were found for formononetin (up to 6.2 $\mu g/g_{dw}$), daidzein (up to 4.3 $\mu g/g_{dw}$), and biochanin A (up to 2.4 $\mu g/g_{dw}$) in grass/corn silage feeding and for formononetin (up to 2.5 $\mu g/g_{dw}$) and daidzein (up to 1.9 $\mu g/g_{dw}$) in hay feeding (**Table 4**). In contrast to soil and red clover samples, coumestrol was quantified in all manure samples (up to 919 ng/g_{dw}). Therefore, we assume that the source of this compound in manure originated from other feed components (e.g., alfalfa, concentrated feed).

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