Analysis of Phytoestrogens and Polyphenols in Plasma, Tissue, and Urine Using HPLC with Coulometric Array Detection (44232)

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Abstract. **The study of phytoestrogens in food sources and their metabolism, effects, and mechanism of action in animals requires very selective and often sensitive analytical techniques. We have applied coulometric array detection, which uses a series of flow-through electrochemical sensors each providing 100% electrolytic efficiency, for measurement of a variety of phytochemicals in complex matrices. Recent work has involved the resolution of coumestrol (COM), daidzein (DE), daidzin (DI), diethylstilbestrol (DES), enterodiol (ED), enterolactone (EL), equol (EQ), estradiol (E2), estriol (E3), estrone (E), genistein (GE), and quercetin (QE). Binary gradient reversed-phase (C18) chromatography was used with a sodium acetate buffer (pH 4.8)–methanol–acetonitrile solvent system. Eight coulometric sensors were set at 260, 320, 380, 440, 500, 560, 620, and 680 mV (vs Pd reference). Compounds were resolved in 30 min *via* both their oxidation/reduction characteristics and chromatographic behavior. Respective maximal oxidation potentials (mV) were: COM = 380; DE = 500; DI = 620; DES = 440; ED = 620; EL = 620; EQ = 560; E2 = 560; E3 = 560; E1 = 560; GE = 500; and QE = 260 with limits of detection of 5–50 pg. Uterine tissue homogenates (30 mg/ml in Tris-EDTA) and plasma from Sprague-Dawley rats sacrificed 1 hr after sc injection with either vehicle, dimethylsulfoxide, 10 μg DES, or 1.0 mg EQ were analyzed before and after enzymatic hydrolysis with β-glucuronidase/sulfatase. Urine samples from humans receiving a Boston-area diet with or without soy protein isolate supplements were also analyzed. Ethanol extracts were evaporated and reconstituted in 20% methanol before HPLC analysis. DE, ED, EL, EQ, and GE were determined in urine with less than 5% (R.S.D.) intraassay imprecision and 85%–102% recovery. Levels (ng/ml) of GE (1.8), QE (11.2), and EQ (1.7) were found in control plasma before hydrolysis and GE (293), QE (183), and EQ (22) after hydrolysis. Higher concentrations, corresponding to sc injection, in free and total EQ were found in both tissue and plasma.**
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Plant-derived chemicals that influence the endocrine activity of animals have received a great deal of attention due to their possible beneficial as well as adverse effects. Of particular interest are the phytoestrogens that include some isoflavonoids, lignans, and coumestans. While much evidence suggests that these compounds may play a role in the prevention of certain hormone-dependent cancers (1, 2, 3), there is also concern over their potential toxicity (4, 5). Compounds from these groups are commonly ingested by animals, including humans, in relatively high amounts and are absorbed, metabolized, and excreted in varying degrees. These phytoestrogens and their metabolites may have biological activity at many levels including: effects at the estrogen receptor; alterations in the activity of certain enzymes; changes in absorption, metabolism, excretion and macromolecular binding of endogenous estrogens; and as dietary antioxidants (6). These and other potential effects in animals are also dependent on parameters such as sex and stage of development, dose and duration of exposure.

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There is great diversity in the occurrence of phytoestrogens and related chemicals in plants. The metabolism of these compounds is also quite variable and not completely understood. Many occur as glycosides in plants and are hydrolyzed to aglycones, further metabolized by intestinal microflora, and typically conjugated before or after absorption. The degree of metabolism, conjugation, absorption, and excretion has been shown to be highly variable within and between individuals with up to a 1527-fold interindividual variation in the urinary excretion of equol from human subjects having a similar diet (7).

Since the bioactivities of these compounds are quite diverse, it is important in both toxicological and therapeutic studies to conduct detailed investigations of the bioavailability, metabolism, pharmacokinetics, and fate of these compounds at target tissues in animals. We have applied high-performance liquid chromatography with coulometric array detection (8, 9, 10) for the analysis of these compounds in plant materials as well as in biological tissues. This technique is based on the use of multiple electrochemical detectors placed in series after the analytical column and maintained at different potentials. This technique has significant advantages in the detection of phenolic phytochemicals due to the inherent sensitivity, selectivity, and linear response range of electrochemical detectors. Also, because of the unique properties of the coulometric electrode, resolution of co-eluting solutes can be obtained based on small differences in their oxidation-reduction behaviors. This expands the analytical capabilities for multicomponent analysis and can minimize sample pre-purification steps when dealing with complex matrices such as urine. The utility of this approach for the analysis of plasma and urinary tea polyphenols has been previously reported (11). Other related areas of application of this coulometric array technology include the investigation of nucleic acid damage through measurement of tissue DNA adduct formation, the analysis of fat-soluble vitamins and antioxidants in serum, and the study of hydroxyl free radical production through measurement of dihydroxybenzoic acid formation after salicylate administration (12 and references within).

This paper describes the use of reversed phase gradient elution HPLC with 8-channel coulometric array detection. A method has been developed that resolves coumestrol, daidzein, daidzin, diethylstilbestrol, enterodiol, enterolactone, equol, estradiol, estriol, estrone, genistein, and quercetin standards in 30 min. This approach has been applied to the analysis of rat plasma, rat uterine tissue homogenates, and human urine. Some basic principles of this coulometric array technique and preliminary results from analysis of these animal tissues will be presented.

Materials and Methods

Apparatus. Analysis was performed using an 8-channel CoulArray Model 5600 HPLC detection system consisting of two Model 580 pumps, a high-pressure gradient mixer, a PEEK pulse damper, a Model 540 autoinjector, a CoulArray thermostatic chamber, an MD-150 column (150 × 3.0 mm i.d., C18 Hypersil, 3 μm), and a serial array of eight coulometric electrodes, all obtained from ESA Inc. (Chelmsford, MA). The system was controlled and data were acquired and processed using the CoulArray software on a Pentium-based computer.

Chemicals. Acetonitrile and methanol (MeOH) were HPLC grade (EM Science, Gibbstown, NJ). Sodium acetate was also obtained from EM Science. 100% Ethanol (EtOH) was obtained from Pharmco Products (Brookfield, CT). Ascorbic acid, diethylstilbestrol, estradiol, estriol, estrone, genistein, quercetin, and β-glucuronidase (from *Helix pomatia* 100,000 units/ml glucuronidase and 5000 units/ml sulfatase activity) were obtained from Sigma Chemical Co. (St. Louis, MO). Coumestrol, daidzein, daidzin, and equol were obtained from Indofine Chemical Co. (Somerville, NJ). Enterodiol and enterolactone solutions in EtOH were kindly provided by Dr. Barry Goldin, (Tufts University, Boston, MA).

Mobile Phases. Mobile phase A consisted of 50 m*M* sodium acetate, pH 4.8 with acetic acid-MeOH, 80:20 (v/v). Mobile phase B consisted of 50 m*M* sodium acetate, pH 4.8 with acetic acid-MeOH-acetonitrile, 40:40:20 (v/v/v).

Chromatographic and Detection Conditions. The gradient consisted of initial conditions of 20% mobile phase B and a linear increase to 100% B over 25 min followed by a 5-min hold at initial conditions. The flow rate was kept constant at 0.8 ml/min for the plasma and tissue studies and 0.6 ml/min for the urine studies. Samples were injected 1 min into the gradient. The detector settings were 260, 320, 380, 440, 500, 560, 620, and 680 mV (vs Pd).

Subjects, Sample Collection, and Storage. Female Sprague-Dawley rats were injected sc with either vehicle (dimethylsulfoxide), 10 μg diethylstilbestrol, 100 μg coumestrol, or 1.0 mg equol. A plasma sample was obtained 1 hr following injection; the animals were then sacrificed and uterine tissue collected. The uterine tissue from several rats was pooled and a 30-mg sample of tissue was homogenized in 1.0 ml of cold buffer (10 m*M* Tris, 1.5 n*M* EDTA, pH 7.4). Homogenates were stored at −70°C until analysis.

Twenty-four hr urine collections were obtained from female humans after consuming an ordinary Boston-area diet supplemented with a soy protein isolate. This supplement resulted in consumption of 15 mg of genistein and 7.0 mg of daidzein/day for 3 months. These samples were collected in 2.0 g of ascorbic acid, and 0.1% sodium azide was added prior to storage at −20°C. Random urine samples from five male humans whose ordinary Boston-area diet was not supplemented with isoflavonoids were processed immediately after collection. There were no dietary restrictions or other modifications for either the soy-supplemented or unsupplemented subjects.

Sample Preparation for Rat Plasma and Uterine Tissue. For determination of free metabolite levels, an aliquot (0.2 ml) of tissue homogenate, plasma, or standard mixture was vortex-mixed for 10 min with 1.2 ml of EtOH.

Following centrifugation (12,000g, 4°C, 10 min), 1.0 ml of supernatant was evaporated to dryness by vacuum centrifugation. Prior to analysis, the extracts were redissolved by sonication for 5 min in 0.1 ml of MeOH followed by dilution with 0.3 ml of water and analysis of 50 µl. Complete dissolution of analytes was confirmed by recovery studies with both augmented samples and aqueous standard mixtures and with the use of higher volumes of MeOH solvent (data not shown). For determination of total (free + conjugated) analyte levels, an aliquot (0.2 ml) of tissue homogenate, plasma, or standard mixture was mixed with 50 µl β-glucuronidase and 0.2 ml buffer [0.1 *M* sodium acetate, pH 5.0 containing 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA]. This mixture was incubated at 37°C for 3 hr before addition of 1.2 ml of EtOH. Following centrifugation (12,000g, 4°C, 10 min), 1.0 ml of supernatant was evaporated to dryness by vacuum centrifugation. Prior to analysis, the extracts were redissolved as above and 50 µl analyzed.

Sample Preparation for Human Urine. For determination of free analyte levels, aliquots of urine were diluted with an equal volume of mobile phase A, and after centrifugation (6,000g, 4°C, 10 min) 25 µl of supernatant were analyzed. For determination of total (free + conjugated) analyte levels, urine samples were processed as described above for total analyte levels in tissue and plasma with the exception that 25 µl of dissolved extract were analyzed.

Standard Preparation, Linearity, Limit of Detection, Precision and Recovery Studies. Individual stock standards (0.1–1.0 mg/ml) were made by dissolving the powders in EtOH and stored at −20°C. Working standard mixtures were prepared daily by dilution of stock standards in either 20% MeOH (v/v), for direct HPLC analysis, or in water to mimic the sample matrix. Water-based standards were included with every 10 samples for processing (i.e., extraction, hydrolysis) and were used for recovery studies. Response linearity was examined from duplicate injections of nine different levels of a mixed standard. The range studied was 25 pg to 10 ng of each analyte injected, and linearity was assessed as the correlation coefficient from least squares linear regression. Analytical recovery was studied by adding 1.0 ml of a 1.0 µg/ml standard mixture to 9.0 ml of a low-level urine sample. Another 9.0 ml of the same urine was diluted with 1.0 ml of water and used as the base value. Three aliquots of the base urine and 15 aliquots of the augmented urine were prepared as described above for total (free + conjugated) analyte levels in urine. Intraassay precision was calculated as the percentage relative standard deviation (%R.S.D.) from the 15 replicates of the augmented urine.

Quantitation. Single-point quantitation from peak height was performed using standard mixtures that were treated in the same manner as samples. Peaks were matched on the basis of retention time (±4%) as well as response ratio between adjacent channels (±20%).

Results and Discussion

Chromatographic conditions were similar to those described by Setchell and coworkers (13). Figure 1 shows a multichannel chromatogram obtained from injection of a standard mixture of 2.5 ng of each compound. Daidzin, the 7-glucoside of daidzein, eluted first due to the hydrophilic nature of the carbohydrate substituent. Daidzein, genistein, and coumestrol eluted in the same order as reported with other reversed phase HPLC methods (13, 14). Quercetin, not shown, co-eluted with enterodiol, but since its oxidation potential was 300–400 mV lower, it was clearly resolved voltammetrically (see below). The quercetin peak had a tendency to tail on this stationary phase, and for this reason alternative materials are currently under investigation. These gradient chromatographic conditions were developed to measure a variety of compounds ranging from the hydrophilic glycosides to the more hydrophobic estrogens. Therefore, this approach was chosen for general investigation of the occurrence of other electroactive metabolites with sample analysis time as a lower priority.

The technique of dual coulometric detection and multielectrode coulometric array detection has been extensively described elsewhere (8, 9, 10). Briefly, the coulometric detector uses a porous flow-through carbon graphite working electrode rather than the conventional thin-layer design (15). The thin-layer working electrode typically oxidizes (or reduces) less than 5% of an analyte's mass within a solute band under normal-bore chromatographic conditions due to inefficient diffusion of analyte to the electrode surface. This thin-layer design is highly subject to instability of response due to changes in flow rate and, more importantly, to loss of surface area from adsorptive effects. The coulometric electrode has a high surface area:volume ratio that allows high efficiency electrolysis. This results in a highly reproducible total peak area (coulombs) and very low susceptibility to loss of signal from adsorptive effects. The most significant

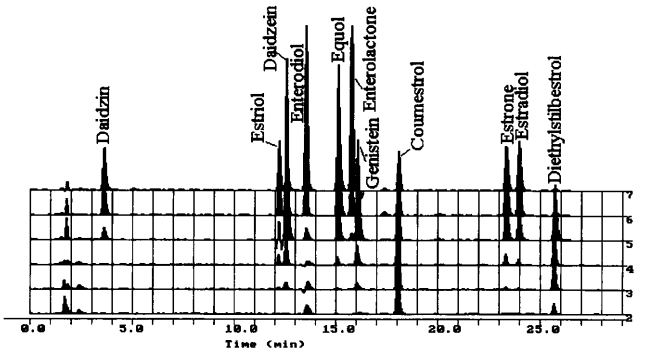

Figure 1. Multichannel chromatogram of a standard mixture representing 2.5 ng of each compound injected. Binary gradient elution was used with an MD-150 C18 column (150 x 3.0 mm i.d.; 3 µm) and a flow rate of 0.6 ml/min: A = 50 m*M* sodium acetate, pH 4.8 with acetic acid–MeOH, 80:20 (v/v), B = 50 m*M* sodium acetate, pH 4.8 with acetic acid-MeOH-acetonitrile, 40:40:20 (v/v/v); 20% B to 100% B linearly over 25 min and a 5-min hold at initial conditions. Detectors 2–7 are displayed at 2.0 µamp full scale with corresponding potentials of 320, 380, 440, 500, 560, and 620 mV (versus Pd).

difference between the coulometric and thin-layer amperometric detectors is obtained when more than one electrode is used in series. Since the coulometric electrode has high electrolytic efficiency, easily oxidized compounds can be detected at upstream electrodes whereas downstream electrodes are available for selective detection of higher oxidizing species. This results in an on-line means of resolving compounds in a manner that is orthogonal to the chromatographic separation. Up to 16 electrodes have been used in series with capabilities of resolving co-eluting compounds whose oxidation potentials differ by as little as 60 mV.

The voltammetric behavior of daidzein, coumestrol, enterolactone, equol, and estriol are shown in the hydrodynamic voltammograms (HDVs) in Figure 2. From this group coumestrol was most easily oxidized followed by daidzein, estriol, equol, and enterolactone. The differences in oxidation potentials of these analogous structures is related to their relative abilities to accommodate the loss of electrons *via* resonance or inductive charge stabilization. With eight coulometric sensors in an increasing oxidative array, stepwise oxidation along the current-voltage curve of an easily oxidized compound such as coumestrol takes place at the upstream electrodes until electrolytic conversion is complete. A compound with a higher oxidation potential, such as enterolactone, can then be detected selectively at the downstream sensors again in a stepwise fashion along the voltammetric curve. From examination of these HDVs, each analyte oxidizes over a range of 150–250 mV. With a 60-mV array, the majority of response for a particular compound then occurs across three adjacent sensors. The data analysis software defines this three-sensor response as a peak cluster. The sensor having the highest response within a cluster is defined as the dominant channel, the adjacent upstream sensor is termed the leading channel, and the adjacent downstream sensor is the following channel. The response ratios (height or area) between dominant/leading and dominant/following of a given cluster are therefore descriptive of an HDV for a particular compound. These ratios obtained from a pure standard can then be used to match a peak cluster of an unknown. Ratio accuracy is then calculated for the unknown as:

$$\frac{\text{Dominant response/leading (or following response) from the unknown}}{\text{Dominant response/leading (or following response) from the standard}}$$

where if the ratios are equivalent, then the ratio accuracy value = 1.

The chromatograms in Figure 3 show a comparison of the retention times and response profile obtained from a standard to that of a urine sample. For daidzein, the ratio accuracy obtained in this urine sample was 0.95 for channels 5/4 and 0.91 for channels 5/6. Ratio accuracy values, which are therefore a function of the voltammetric behavior of each compound, are useful and immediate indicators of the presence of co-eluting solutes. In the urine sample (Fig. 3), the genistein peak elutes near a lower oxidizing unknown component in the urine. The ratio accuracy values for genistein in this sample were above 0.9 indicating both the effectiveness of the electrode array in resolving this co-elution and the qualitative utility of the voltammetric data. Acceptable matches in both retention time (within 4%) and ratio accuracy (within 20%) were obtained for daidzein, enterodiol, equol, enterolactone, and genistein in all urine samples analyzed. The remaining analytes were either below the limit of detection or failed to meet the chromatographic or voltammetric criteria in one or more samples.

The lower limits of detection for each analyte, presented in Table I, were estimated from diluted standards using a signal to noise ratio of 3:1. Most analytes were detectable at 10 pg/injection. Values are also shown in ng/ml and n*M* concentrations after accounting for the sample dilution included in the sample preparation for total analyte levels in urine. Response was linear over three orders of magnitude for all analytes studied (Table I). Recovery from urine that was augmented with 100ng/ml of each analyte ranged from approximately 80%–100% relative to extracted standards (Table II). Daidzin, an early eluter, was excluded from the recovery and precision table after it was determined that an interference was present in urine analyzed with these conditions. Intraassay precision, as %R.S.D. (Table II) ranged from 1.66 for enterolactone to 10.2 for diethylstilbestrol.

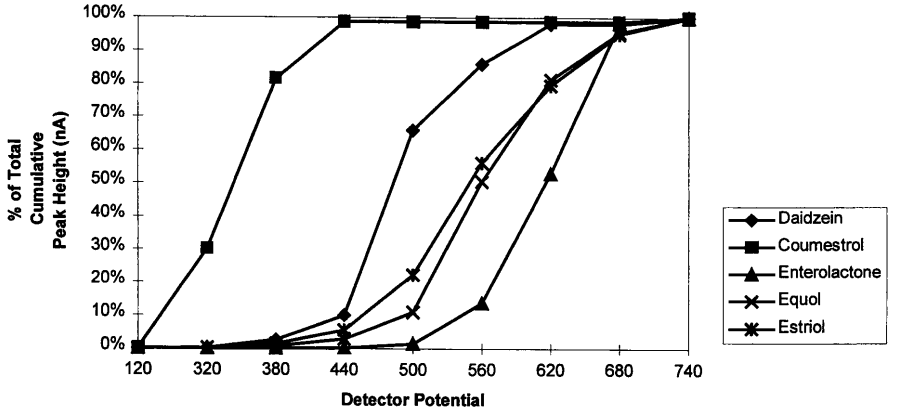

Figure 2. On-line hydrodynamic voltammograms obtained from Figure 1 data by plotting the percentage of total cumulative peak height response from each standard compound as a function of the potential across an array of 9 coulometric electrodes with potentials as indicated in (mV versus Pd).

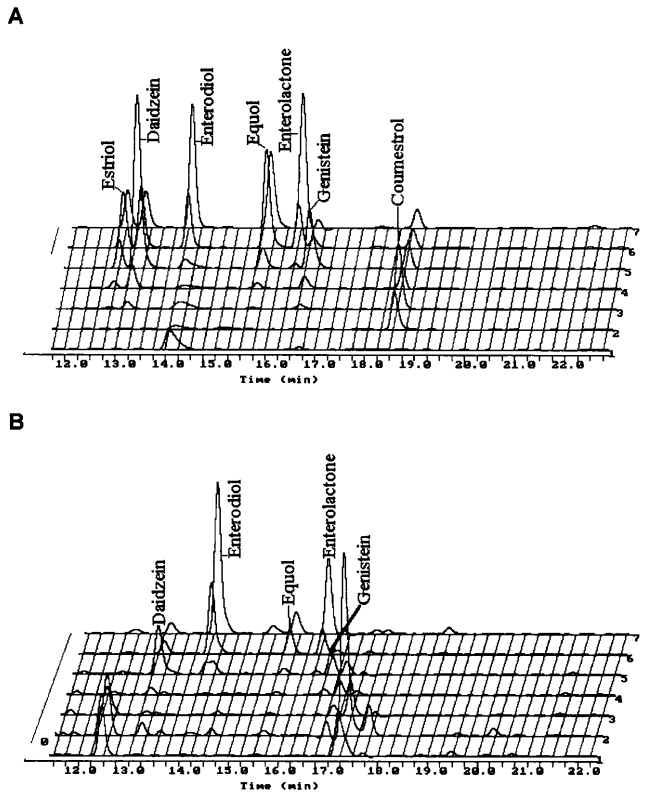

Figure 3. Segments of chromatograms obtained from an extracted standard (A) and urine (B) after enzymatic hydrolysis. Binary gradient elution was used with an MD-150 C18 column (150 x 3.0 mm i.d.; 3 µm) and a flow rate of 0.6 ml/min: A = 50 m*M* sodium acetate, pH 4.8 with acetic acid-MeOH, 80:20 (v/v), B = 50 m*M* sodium acetate, pH 4.8 with acetic acid-MeOH-acetonitrile, 40:40:20 (v/v/v), 20% B to 100% B linearly over 25 min and a 5-min hold at initial conditions. Detectors 1–7 are displayed at 1.0 (A) and 2.0 (B) µamp full scale with corresponding potentials of 260, 320, 380, 440, 500, 560, and 620 mV (versus Pd).

Table III is a summary of the data obtained from 24-hr urine collections from female humans after consuming an ordinary Boston-area diet supplemented with a soy protein isolate. This supplement resulted in consumption of 15 mg of genistein and 7.0 mg of daidzein/day for 3 months. Also shown is the mean and standard deviation obtained from random urine collections from five male humans whose ordinary Boston-area diet was not supplemented with isoflavonoids. These values of total urinary levels in unsupplemented subjects are generally comparable to those previously reported for omnivores living in the Boston area (1). Both the free and total (free + conjugated) values for daidzein, enterodiol, equol, enterolactone, and genistein are reported. These analytes demonstrated both acceptable retention time and ratio accuracy values in all samples. Although these urine samples were intended as an initial evaluation of this technique and are not corrected for rate of urinary output, some gross trends in the levels of these analytes are evident. The data from both groups show wide variability in the levels of all phytoestrogen metabolites. The automatic gain ranging capabilities of the data acquisition software, which encompasses eight orders of magnitude in response, enabled detection over a range of low pg to high µg levels

Table I. Response Linearity and Lower Limit of Detection for Phytoestrogens and Polyphenols

Analyte	Limit of detection[a]			Linearity[b]
	pg	ng/ml[c]	n*M*[c]	Correlation coefficient r^2
Coumestol	10	1.32	4.92	0.9922
Daidzein	10	1.32	5.19	0.9995
Daidzin	5	0.66	1.59	0.9997
Diethylstilbestrol	50	6.6	24.6	0.9905
Equol	10	1.32	5.45	0.999
Enterodiol	10	1.32	4.37	nd
Enterolactone	10	1.32	4.42	nd
Estradiol	10	1.32	4.85	0.9978
Estriol	10	1.32	4.58	0.9998
Estrone	10	1.32	4.88	0.9966
Genistein	15	1.98	7.33	0.9963

[a] Signal to noise ratio (peak height) of 3:1 on the dominant channel.
[b] r^2 from least squares regression analysis of peak height vs mass injected, range was 25 pg–10 ng, 9 levels, $n = 2$ each level.
[c] Limit of detection expressed as concentration obtained for hydrolyzed urine. An aliquot (0.2 ml) of standard mixture was incubated with 50 µl β-glucuronidase and 0.2 ml buffer [0.1 *M* sodium acetate, pH 5.0 containing 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA] before addition of 1.2 ml of EtOH. An aliquot (1.0 ml) of supernatant was evaporated to dryness, extracts were redissolved in 0.4 ml of 20% MeOH (v/v) and 25 µl analyzed.
nd = not done.

Table II. Analytical Recovery and Intra-Assay Precision for Urinary Phytoestrogens and Polyphenols

Analyte	Percent recovery[a]	Intra-assay precision[b] %R.S.D.
Coumestrol	95.43	3.74
Daidzein	94.59	2.43
Diethylstilbestrol	82.55	10.20
Enterodiol	91.26	1.86
Enterolactone	96.05	1.66
Equol	85.14	1.67
Estradiol	90.84	2.98
Estriol	101.71	6.97
Estrone	81.85	5.21
Genistein	90.23	4.07

[a] Sample was augmented with 100 ng of each analyte/ml of urine. Aliquots (0.2 ml) of augmented urine were incubated with 50 µl β-glucuronidase and 0.2 ml buffer [0.1 M sodium acetate, pH 5.0 containing 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA] before addition of 1.2 ml of EtOH. An aliquot (1.0 ml) of each supernatant was evaporated to dryness, extracts were redissolved in 0.4 ml of 20% MeOH (v/v) and 25 µl analyzed. Values represent the mean of 15 replicates calculated against extracted standards.
[b] Percent relative standard deviation for 15 replicates.

in the same injection without any loss of data. A 15.8-, 16.4-, and 5.8-fold higher level in the mean of total daidzein, equol, and genistein, respectively, is evident in the urine of the soy supplemented subjects. These differences in the mean values between supplemented and unsupplemented subjects are of similar magnitude to a previously reported 14.8–26.5-fold increase over a control period in

Table III. Lignan and Phytoestrogen Levels in Human Urine

		Concentration (n*M*)					
Urine Sample #		1	2	3	4	5	No Supplement[a]
Compound Name							
Daidzein	Free	36	33.5	75.5	309	11.8	NA
	Total	2100	6290	4020	9030	2140	298 (182)
Enterodiol	Free	20.1	133	36.1	55.6	15.7	NA
	Total	8000	820	157	1860	304	356 (355)
Equol	Free	46.2	55	704	97.4	35	NA
	Total	2250	5960	3630	1920	92	169 (84)
Enterolactone	Free	10.7	23	15.5	95.5	34.9	NA
	Total	3530	4110	1220	8090	3990	4870 (3090)
Genistein	Free	24.8	77	64.8	415	51.4	NA
	Total	3010	7210	6690	28000	2050	1610 (880)

Note. NA, not analyzed.
Urine samples 1–5 are 24-hr collections from female humans after consuming an ordinary Boston area diet supplemented with a soy protein isolate consisting of 15 mg genistein, 7.0 mg of daidzein/day for 3 months.
[a] Mean (and standard deviation) obtained from random urine collections from 5 male humans whose ordinary Boston diet was not supplemented with isoflavones.

daily urinary total isoflavonoid excretion reported for women after consuming comparable isoflavone supplements (16). Since, in our study, there were no restrictions on possible dietary sources of phytoestrogens in either group, these data reflect only a small sampling of the possible range of excretion rates of phytoestrogen metabolites. One of the five soy supplemented participants had a low urinary equol level. This is evidence of the interindividual polychotomy in equol excretion described elsewhere (7). There was little difference in the mean of enterolactone and a 6.2-fold higher level in enterodiol in the supplemented subjects although one of these samples would tend to skew these data. The percentage of free metabolite levels was generally 0.5%–5% of the total, in agreement with a previous report (17), although in some cases the percentage was as high as 20%. Although this was not a controlled study, these results do suggest a higher excretion rate for the isoflavonoid metabolites as expected from the soy supplements with little contribution of lignans.

Preliminary data show the feasibility of detecting daidzein, equol, genistein, and quercetin in plasma. In control rats, levels in ng/ml of genistein (1.8), quercetin (11.2), and equol (1.7) were determined before hydrolysis, whereas daidzein (63.6), equol (22), genistein (293), and quercetin (183) were found after hydrolysis. Higher concentrations, corresponding to sc injection, in free and total diethylstilbestrol and equol were found in plasma. Free plasma levels of equol increased ca. 100-fold and total increased ca. 5-fold 1 hr after sc injection of 1.0 mg of equol. Figure 4 shows the chromatograms obtained from an equol treated rat. An unknown, easily oxidized compound in the plasma which coeluted with equol was clearly resolved. All analytes reported, matched the authentic standard within 4% retention time and 20% ratio accuracy windows. Free and total plasma levels (ng/ml) of diethylstilbestrol (DES) were 3.22

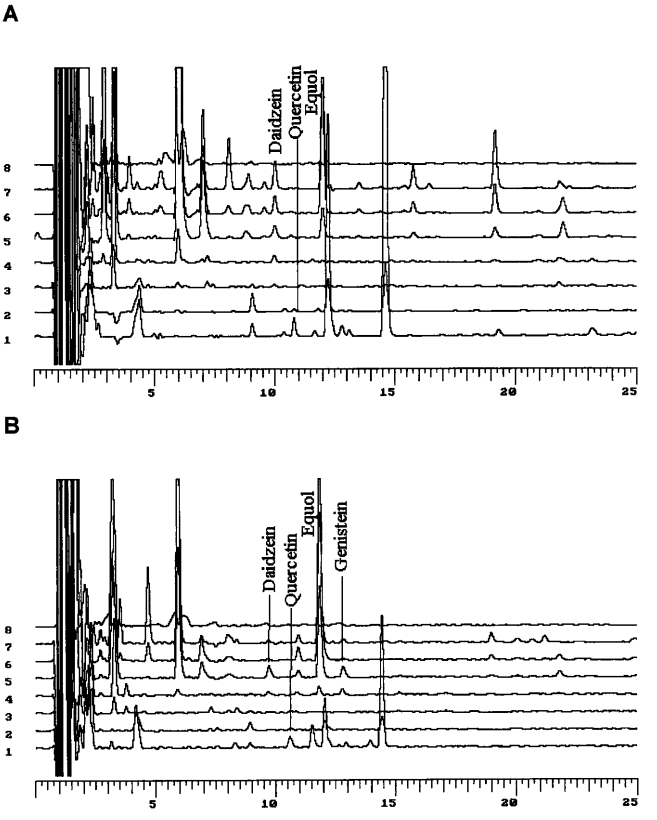

Figure 4. Chromatograms obtained from plasma of a female Sprague-Dawley rat injected sc with 1.0 mg Equol: (A) before enzymatic hydrolysis, (B) after enzymatic hydrolysis. Binary gradient elution was used with an MD-150 C18 column (150 x 3.0 mm i.d.; 3 µm) and a flow rate of 0.8 ml/min: A = 50 m*M* sodium acetate, pH 4.8 with acetic acid-MeOH, 80:20 (v/v), B = 50 m*M* sodium acetate, pH 4.8 with acetic acid-MeOH-acetonitrile, 40:40:20 (v/v/v); 20% B to 100% B linearly over 25 min and a 5-min hold at initial conditions. Detectors 1–8 are displayed at 0.2 (A) and 1.0 (B) µamp full scale with corresponding potentials of 260, 320, 380, 440, 500, 560, 620, and 680 mV (versus Pd).

and 37.8, respectively, 1 hr after sc injection of 10μg of this compound. No DES was found in controls. Equol was also measured in the rat uterine tissue homogenates. In the control, 0.1 of free and 0.33 of total nmole of equol per gram of tissue (wet weight) was found. One hour after sc injection of 1.0 mg equol both free and total tissue levels of equol increased ca. 10-fold. Although a small peak matching that of DES was evident in the uterine tissue of the DES-treated rats (1.20 nmole/g), these results require additional study.

These preliminary data show that coulometric array detection provides low pg sensitivity and a wide linear response range, suitable for the measurement of phytoestrogens and polyphenols in urine, plasma, and tissue. The voltammetric resolution obtained with the electrode array in conjunction with the chromatographic separation allows a high degree of selectivity. An additional benefit to the use of coulometric array HPLC is the on-line generation of voltammetric data for each electroactive component. These voltammograms can then be used qualitatively to examine peak purity and aid in structural identification of unknowns. This technique is easy to use with both isocratic and gradient elution chromatography and may provide an acceptable alternative to chromatographic methods that use mass spectrometry as a means of detection.

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