Laboratory Procedure Manual

Analyte: **PHYTOESTROGENS: Daidzein, *o*-Desmethylangolensin, Equol, Enterodiol, Enterolactone, and Genistein**

Matrix: **Urine**

Method: **HPLC-APCI-MS/MS**

Method No.:

Revised:

as performed by: *Toxicology Branch*
Division of Laboratory Sciences
National Center for Environmental Health, CDC

Contact: *Dr. Dana Barr*
1-770-488-7886

Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 1999–2000 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label (and SI units)
Lab26	URXDAZ	Daidzein (ng/mL)
	URXDMA	*o*-Desmethylangolensin (O-DMA) (ng/mL)
	URXEQU	Equol (ng/mL)
	URXETD	Enterodiol (ng/mL)
	URXETL	Enterolactone (ng/mL)
	URXGNS	Genistein (ng/mL)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Phytoestrogens are plant compounds that can effect in vivo estrogen signaling. These 'plant estrogens' have been shown to affect the human endocrine system and thus may affect human health. Consumption of foods rich in phytoestrogens is associated with many positive health outcomes: reduced risk for cancer and heart disease, reduction of menopausal symptoms and modulation of osteoporosis. The potential of these compounds to impact human health has led to the need for rapid, sensitive and precise assays for phytoestrogen metabolites in physiological matrices. Previous methods for phytoestrogen quantitation used less selective and/or less sensitive techniques.

Many laboratories use HPLC coupled with UV detection (1, 2) due to its low cost, although these methods are lacking selectivity and sensitivity. A novel approach using HPLC with coulometric array detection (3) shows promise for increased selectivity and sensitivity, but selectivity depends greatly on redox potential, resulting in inadequate sensitivity for some important phytoestrogens in complex matrices such as serum. The application of capillary electrophoresis-tandem mass spectrometry to the detection of phytoestrogens shows promise of improved sensitivity and specificity, but this technique has yet to be fully utilized due to difficulties in handling very small sample volumes. GC-MS methods provide needed selectivity and sensitivity for the analysis of phytoestrogens in biological matrices (4, 5), but requires cumbersome derivatization steps for these nonvolatile compounds. HPLC-MS does not require derivatization, and therefore results in much higher throughput. Barnes, et. al. (6) developed an HPLC-MS/MS method for the detection of phytoestrogens in serum and urine; however, the method did not chromatographically resolve all of the major phytoestrogens and included only one internal standard.

Here we present an HPLC-MS/MS method that builds on the work by Barnes, et. al. (6) to result in a method with improved selectivity, sensitivity and precision for the quantitative detection of phytoestrogens in human urine and serum. The method uses enzymatic deconjugation of the phytoestrogens followed by solid phase extraction and reverse phase HPLC to resolve the analytes. The phytoestrogens are detected using a Sciex API III heated nebulizer-atmospheric pressure chemical ionization (HN-APCI) interface coupled with MS/MS. This method allows for rapid detection of the major isoflavones and lignans in human serum and urine with limits of detection in the low ppb range. Selectivity of the method is insured by the combination of tandem mass spectrometry and chromatographic resolution. Internal standards for each of the analytes improved the precision of the assay.

2. SAFETY PRECAUTIONS

a. Reagent Toxicity Or Carcinogenicity

Some of the reagents used are toxic. Special care should be taken to avoid inhalation or dermal exposure to the reagents necessary to carry out the procedure.

b. Radioactive Hazards None

c. Microbiological Hazards

The possibility of being exposed to various microbiological hazards through serum exists. Appropriate measures should be taken to avoid any direct contact with the specimen (i.e., utilize gloves, chemical or biological hoods, etc.). A Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues. Laboratory personnel handling human fluids and tissues are required to complete the CDC "Human Blood-Borne Pathogen Training Course" to insure proper compliance with CDC safe work place requirements.

d. Mechanical Hazards

There is only minimal mechanical hazard when performing this procedure using standard safety practices. Laboratorians should avoid any direct contact with the electronics of the mass spectrometer unless all power to the instrument is off. Generally, electronic maintenance and repair should only be performed by qualified technicians. Following proper instrument procedures will limit the potential for

burns due to the heated nebulizer or the heated gate valve. Care must also be taken o avoid puncture wounds from the corona discharge needle when removing the APCI interface.

e. Protective Equipment

Standard safety protective equipment should be utilized when performing this procedure. This includes lab coat, safety glasses, durable gloves, and a chemical fume hood.

f. Training

Training and experience in the use of a triple quadrupole mass spectrometer should be obtained by anyone using this procedure. Formal training is not necessary; however, personnel should be trained appropriately by an experienced operator of the instrument and are required to read the operation manuals. Laboratory personnel handling human fluids and tissues are required to complete the CDC "Human Blood-Borne Pathogen Training Course" to insure proper compliance with CDC safe work place requirements. To insure proper compliance with CDC waste disposal requirements, laboratory personnel were required to attend annual hazardous waste disposal courses.

g. Personal Hygiene

Care should be taken in handling any biological specimen. Routine use of gloves and proper hand washing should be practiced. No food or drink was allowed in sample preparation and analysis areas.

h. Disposal of Wastes

Solvents and reagents are disposed of in an appropriate container clearly marked for waste products and temporarily stored in a flame resistant cabinet. Containers, glassware, etc., that come in direct contact with the specimen were either autoclaved or decontaminated with 10% bleach. Contaminated analytical glassware was bleach sterilized, washed and reused; disposable labware was autoclaved prior to disposal. To insure proper compliance with CDC requirements, laboratory personnel were required to attend annual hazardous waste disposal courses.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Software and Knowledge Requirements

All samples were queued for analysis using PE-SCIEX RAD application. This menu-driven interface allowed the user to control all aspects of the data acquisition. Information pertaining to a particular specimen was entered into RAD manually (sample identification number, notebook number associated with the sample preparation, sample type, standard number). This header information linked the mass spectral data to the sample prep data. All raw mass spectral data is archived for future reference.

All raw data files are analyzed using the PE-SCIEX McQuan application, which allows manual peak selection and area integration. These raw data (peak area, peak height, retention time, analyte name, MRM name) are exported to a Microsoft Excel spreadsheet where the values are properly arranged in contiguous lines to allow for import into RBASE databases (using copy and paste functions). Data is stored in two databases (PHYTOSRM for serum and PHYTO2 for urine) using R:Base 4.5+ (Microrim Inc., Redmond, WA). This database is used for storage, retrieval, and analysis of data from the phytoestrogens analyses. These databases are stored on the EHLS-PC Network (N:\apps\dbfiles) as well as in several archive locations. Statistical analysis of data, programming, and reporting is performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). Knowledge of and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

b. Sample Information

All samples were queued for analysis using PE-SCIEX RAD application. This menu-driven interface allowed the user to control all aspects of the data acquisition. Information pertaining to a particular specimen was entered into RAD manually (sample identification number, notebook number associated with the sample preparation, sample type, standard number). This header information linked the mass spectral data to the sample prep and demographic data. After data file preparation in Excel, the data was saved as a comma delimited file and imported into a RBASE 4.5 database using a command file as described later in this document.

c. Data Maintenance

All sample and analytical data are checked after being entered into the database for transcription errors and overall validity. The database is routinely (at least once weekly) backed up onto a computer hard drive and onto a network magnetic tape. Data from completed studies are saved as flat files on CD-ROM, hard drive and Jaz media. Additionally, final reported data is saved as paper copy as an official government record.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Sample Collection

Urine specimen are collected from subjects in standard urine collection cups. Samples should be refrigerated as soon as possible. The specimen should be transferred to specimen vials within 4 hours of collection. A minimum of five milliliters of urine is collected, and can be stored frozen in borosilicate glass or polypropylene vials or specimen cups. Teflon coated stoppers are used to plug vial and the vial is sealed with an aluminum seal. The specimen are then labeled and frozen immediately to -20 °C, and stored on dry ice for shipping. Special care must be taken in packing to protect bottles from breakage during shipment. All samples should be stored at -20 °C until analysis.

b. Sample Handling

Samples are thawed, sonicated, aliquoted, and the residual specimen is again stored at -20 °C until needed.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this method

6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

a. Reagents and Sources

Methanol, acetonitrile, acetic acid, 96% ethanol, dimethyl sulfoxide (DMSO), and other solvents used were analytical grade or HPLC grade (Aldrich). Enterolactone, enterodiol, equol, O-desmethylangolensin (O-DMA), and matairesinol were purchased from Kristina W≅h≅l≅ (University of Helsinki, Finland). Internal standards [$^2H_6$]-enterolactone, [$^2H_6$]-enterodiol, [$^2H_6$]-matairesinol, [$^2H_4$]-equol, [$^2H_5$]-O-DMA, and [$^2H_4$]-genistein were also purchased from Kristina W≅h≅l≅. Genistein, coumestrol, and daidzein were purchased from Indofine Chemical (Somerville, NJ). Stable isotope-labeled daidzein ([$^2H_3$]-daidzein) was supplied by Cambridge Isotope Laboratory. Fetal bovine serum, hexestrol, 4-methylumbelliferone

glucuronide, 4-methylumbelliferone sulfate and β-glucuronidase/sulfatase (Helix pomatia, H1) were obtained from Sigma (St. Louis, MO).

b. Working Solution

(1) 1.0 M ammonium acetate solution pH 5.0

(2) 1.0 M ammonium acetate solution pH 5.0

(3) 250 mM ammonium acetate pH 5.0

(4) Resuspension Buffer: 70% ammonium acetate 10 mM, 30% acetonitrile:methanol (1:1), 200 ng p-nitrophenol.

(5) β-glucoronidase solution: 1mg enzyme/sample in a solution with a concentration of 2 mg/mL in 1.0 M ammonium acetate pH 5.0

b. Standards Preparation

(1) Stock Solutions

Standard stock solutions were prepared by dissolving solid standards (3.0 – 5.0 mg) in DMSO (0.2 mL) followed by the addition of ethanol (96 %, to 25 mL). Working standard solutions containing all analytes were prepared by serial dilutions in ethanol from stock solutions to final concentrations that covered the linear range of the assay. Stock solution from each analyte are prepared by weight and diluting in 96% ethanol. Store stock solutions sealed at -70 °C.

(2) Phytoestrogen Analytical Standards Mix

10 mL of each standard mix are prepared from the stock solutions as outlined below and stored as 1 ml aliquots. The working standard mix solutions are kept at -20 °C, while others are kept at -70°C until use.

Table 1. Standard #8 preparation.

Analyte	Amount (ng)	Stock (ppm)	Volume (µL)
Enterodiol	3.0	16.2	18.6
Enterolactone	0.5	154	50
Matairesinol	0.8	8	10
Daidzein	2.0	100	20
Equol	0.5	14	5
ODMA	0.5	16	3.14
Genistein	0.8	11.6	6.90
Coumestrol	2.0	20.0	10

Table 2. Standard #7 preparation.

Analyte	Amount (ng)	Stock (ppm)	Volume (µL)
Enterodiol	6.0	16.2	37.0
Enterolactone	20.0	154	13.0
Matairesinol	1.6	8	20.0
Daidzein	20.0	100	20.0
Equol	6.0	14	43.0
ODMA	2.0	16	12.5
Genistein	4.0	11.6	34.6
Coumestrol	4.0	20	20.0
4methylumbelliferone	60.0	432	13.9

Table 3. Standard #6 preparation.

Analyte	Amount (ng)	Stock (ppm)	Volume (µL)
Enterodiol	20.0	16.2	124.0
Enterolactone	50.2	154	32.6
Matairesinol	4.0	8	50.0
Daidzein	40.0	100	40.0
Equol	16.0	14	114.0
ODMA	6.0	16	37.6
Genistein	10.0	11.6	86.2
Coumestrol	10.0	20	50.0
4methylumbelliferone	120.0	432	27.8

Table 4. Standard #5 preparation.

Analyte	Amount (ng)	Stock (ppm)	Volume (µL)
Enterodiol	50.2	162	31.0
Enterolactone	80.0	154	52.0
Matairesinol	10.0	80	12.5
Daidzein	70.0	100	70.0
Equol	30.0	140	21.4
ODMA	16.0	16	100.0
Genistein	30.2	116	26.0
Coumestrol	30.0	20	150.0
4methylumbelliferone	180.6	432	41.8

Table 5. Standard #4 preparation.

Analyte	Amount (ng)	Stock (ppm)	Volume (µL)
Enterodiol	100.2	162	61.8
Enterolactone	120.2	154	78.0
Matairesinol	20.0	80	25.0
Daidzein	130.0	100	130.0
Equol	60.2	140	43.0
ODMA	40.0	160	25.0
Genistein	60.0	116	51.8
Coumestrol	80.0	200	40.0
4methylumbelliferone	198.0	432	45.8

Table 6. Standard #3 preparation.

Analyte	Amount (ng)	Stock (ppm)	Volume (µL)
Enterodiol	199.2	162	123.0
Enterolactone	160.2	154	104.0
Matairesinol	40.0	80	50.0
Daidzein	250.2	1220	20.5
Equol	120.2	140	85.8
ODMA	110.0	160	68.8
Genistein	160.0	116	138.0
Coumestrol	199.0	630	31.6
4methylumbelliferone	240.0	432	55.6

Table 7. Strandard #2 preparation.

Analyte	Amount (ng)	Stock (ppm)	Volume (µL)
Enterodiol	599.4	162	370.0
Enterolactone	499	154	324.0
Matairesinol	80.0	80	100.0
Daidzein	500.2	1220	41.0
Equol	299.6	140	214.0
ODMA	300.8	160	188.0
Genistein	501.1	116	432.0
Coumestrol	400.6	630	63.6
4methylumbelliferone	270.4	432	62.6

Table 8. Standard #1 preparation.

Analyte	Amount (ng)	Stock (ppm)	Volume (µL)
Enterodiol	1799.8	162	1111.0
Enterolactone	2002.0	154	1300.0
Matairesinol	170.4	80	213.0
Daidzein	1200.4	1220	98.4
Equol	999.6	140	714.0
ODMA	800.0	160	500.0
Genistein	1999.8	116	1724.0
Coumestrol	1209.6	630	192.0
4methylumbelliferone	300.0	432	69.4

*Standard # 9 is prepared taking an aliquot of 50µL of standard #8, and after dryness resuspended in 100 µL of resuspension buffer. Concentrations of analytes are half of the levels presented for standard #8.

(3) Internal Standard Mix

Stock solutions were prepared by weight and diluted in 96% ethanol. Aliquots from stock solutions were transferred to a 10 mL volumetric flask and diluted with deionized water.

Table 9. Internal standard mix preparation

Analyte	Stock Concentration (ng/ µL)	Volume (µL)	Amount[1] (ng)
D6-Enterodiol	112	1428	80
D6-Enterolactone	91	1100	50
D6-Matairesinol	72	486	18
D3-Daidzein	1867	204	161
D4-Equol	126	1588	100
D5-O-DMA	100	1130	57
D4-Genistein	63	794	25
Hexestrol	1000	100	50

[1]Amount in ng of the labeled analyte spiked to the unknown samples and standard mix.

(4) Deconjugation Internal Standard Mix

4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide are use as deconjugation standards to quantify the thoroughness of the enzymatic reaction. Deconjugation standard was prepared adding 240 µg of 4-methylumbelliferyl glucuronide and 200 µg of 4-methylumbelliferyl sulfate diluted to 10 mL with ethanol.

(5) Mass-Spec Operational Check Standard

4- Nitrophenol is used as a mass spec external standard. All the samples and standards are resuspended in the resuspension buffer that includes 4-nitrophenol (200 ng). This standard is used to determine if the mass spec is functioning properly.

(6) Proficiency Testing Standards (PT)

Table 10. Urine proficiency testing standards.

Analyte	Target ng/mL PT1	Target ng/mL PT2	Target ng/mL PT3
Enterodiol	71	100	157
Enterolactone	217	600	1121
Matairesinol	5.1	12	18
Daidzein	218	600	1765
Equol	35	160	329
ODMA	76	400	1702
Genistein	100	400	818
Coumestrol	9.1	25	37

.

Table 11. Serum proficiency testing standards.

Analyte	Target ng/mL PT1	Target ng/mL PT2	Target ng/mL PT3
Enterolactone	20	300	600
Enterodiol	20	250	499
Matairesinol	4.0	40	80
Daidzein	25	250	503
Equol	25	150	300
O-DMA	10	150	301
Genistein	15	250	501
Coumestrol	10	200	401

d. Other Materials

(1) C18 SPE column (Varian, 500 mg, 3 mL, 120 micron pore size)

(2) Oasis cartridges (60 mg HLB, 3mL, Waters Scientific, Beverly, MA)

(3) Solid phase extraction vacuum manifold, Visiprep DLJ, and disposable flow control valve liners (Supelco, Bellefonte, PA)

(4) Disposable centrifuge 15 mL tubes (Pyrex or Kimax, Scientific Services, CDC), and phenolic screw caps with Teflon interseals (Corning, Scientific Services, CDC)

(5) Vortex mixer (Scientific Industries Inc., Springfield, MA)

(6) 10 µL AC@ micro/pettor7 and glass capillaries (Scientific Manufacturing Industries, Berkeley, CA)

(7) Tip ejector variable volume micropipetters (Wheaton, Millville, NJ) and pipet tips (Raining Instrument Co., Woburn, MA)

(8) HPLC autosampler vial (conical bottom, polypropylene, crimp top).

(9) Sartorius research balance (Sartorius, Westbury, NY)

(10) Sartorius top-loading balance (Sartorius, Westbury, NY)

(11) pH indicator paper strips pH 0-14 (Whatman International Ltd.,Germany)

(12) pH meter

(13) Magnetic stirrer and magnetic stir bars (Fisher Scientific, Pittsburgh, PA)

(14) Keystone Prism column (3.0 mm i.d., 50 mm length, 5 µm particle size) and guard column.

(15) Assorted glassware

e. Instrumentation

(1) Mass Spectrometer Configuration

Mass spectrometric analyses were conducted with the API III tandem mass spectrometer (Perkin Elmer-Sciex) in the heated nebulizer, APCI interface mode. Nitrogen was used as nebulizing gas (80 psi) and laboratory-grade air used for auxillary gas (1 L/min). The heated nebulizer was set at 500 °C to produce a spray at approximately 120 °C. Collision cell settings were constant for all analytes: collision gas thickness (argon, 375 units) and collision energy (71.6 eV). Ionization parameters were also constant for all analytes: corona discharge voltage (–5 V) and orifice potential (-60 V). Resolution of 0.8 was used for both quadrupole one and quadrupole three. The state file containing all recorded mass spectrometric parameters are shown in Table 12. The mass spectrometer was interfaced to a Keystone Prism column (3.0 mm i.d., 50 mm length, 5 µm particle size) and guard column. A Hewlett-Packard model 1090 liquid chromatograph delivered gradient solvent flow and precise sample injection from an autosampler. The entire system from sample injection to data acquisition was computer controlled with standard Sciex software.

Table 12. Mass spectrometric parameters for the analysis of serum phytoestrogens.

Parameter	Setting	Parameter	Setting
DM3	0.068	DI	-5
RX	10	IN	-650
R3	5	OR	-60
L9	150	R0	-30.0
FP	50	M1	1000
MU	4000	RE1	124.0
CC	1	DM1	0.070
Di µa	4.9	R1	-26
CGT	375.9	L7	-3
IS v	4895.0	R2	2
UV	102.6	M3	1000
		RE3	121.5

(2) Mass Spectrometer Control Program (PHYTO-ONE)

A set of samples was analyzed by queuing the sample header information in the SCIEX RAD software. Starting this program initiated the multiple reaction monitoring (MRM) experiment by loading the PHYTO-ONE state file which contained the mass spectral settings shown above in Table 12. It sets the

instrument to centroid acquisition mode and the ion polarity to negative ions. It scans the specific ions for the analytes. The parent/daughter ions of the respective analytes are summarized in Table 19. In addition, it sets the mass spectrometric parameters presented in the previous section, and turns on electron multiplier. While PHYTO-ONE runs in the SCIEX API III, the HPLC 1090 system operates using the 10 mM ammonium acetate, acetonitrile/methanol gradient described in Table 14. The HPLC was controlled using the stand alone key pad, with the two instruments communicating by contact closure.

(3) HPLC Configuration

Table 13. HPLC configuration.

PARAMETERS	SETTING
Column Type	C18 A Keystone Prism reverse phase (3.0 mm i.d., 50 mm length, 5 µm particle size) column with guard column was used
Mobile Phase A	Methanol: Acetonitrile (1:1)
Mobile Phase B	10mM ammonium acetate buffer pH 6.5
Injection Volume	25µL

(4) HPLC Mobile Phase Gradient

Table 14 . Mobile phase gradient.

Time (min)	0	2.0	4.0	5.0	7.0	7.5
Solvent acetonitrile:methanol (1:1)	35%	35%	50%	55%	95%	35%
10 mM ammonium acetate, pH 6.5	65%	65%	50%	45%	5%	65%

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. Mass Spectrometer

The Sciex API III mass spectrometer was calibrated and tuned using polypropylene glycol (PPG) solution according to the instructions contained in the operator's manual.

b. Calibration Curve

Calibration curves were prepared daily from the ion ratios produced by freshly analyzed standards. Linear regression analysis of relative response factor (i.e., area native/area label) versus standard concentration were performed. Correlation coefficients must be greater than 0.90. The use of a secondary calibration curve, based on a less abundant daughter ion, extended the upper end of the linear range of the assay to the levels shown in table 15. The lowest point on the calibration curve is at the measurable detection limit and the highest point is above the expected range of results for most samples.

Samples with values exceeding the highest point in the calibration curve were completely reanalyzed using less sample material. The LOD was defined as larger of the following numbers:

- the lowest measurable standard
- three times the standard deviation at zero concentration ($3S_0$).

c. Calibration Verification (CV)

Since the calibration curve was prepared daily with fresh standards verification of the calibration curve is not necessary .

d. Proficiency Testing (PT)

(1) Because no standard reference materials exist for phytoestrogen serum analysis, PT samples were prepared in-house as described in the standard preparation section and encompass the entire linear range of the method. Characterization of PT materials requires at least 15 separate determinations. Once the PT pools are characterized this data will be forwarded to an EHLS representative responsible for executing the PT program (Sam Caudill). These PT samples will then be blind-coded by the PT administrator and returned to the laboratory for storage. When proficiency testing is required, the PT administrator will be notified and will provide the blinded reference numbers for the 5 PT samples to be analyzed.

(2) Proficiency testing should be performed a minimum of once per 6 months. The PT administrator will randomly select five of the PT materials for analysis. Following analysis, the results will be forwarded directly to the PT administrator for comparison with the values previously characterized. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the administrator. The PT administrator will notify our laboratory of its PT status (i.e. pass/fail).

(3) All proficiency results shall be appropriately documented.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Sample Preparation

Removed serum or urine samples from the freezer (-20 °C) and let them thaw. Measure sample (2.0 mL urine or 1.0 mL of serum) into vial (disposable borosilicate glass tube with Teflon-lined screw cap). Spike the sample with 5µL of internal standard and 10 µL of deconjugation standard mix. Additionally, in the case of urine spike 100 ng of hexestrol, the internal standard for coumestrol. Dilute serum with 1 mL of ammonium acetate 250 mM, pH 5. Gently mix the sample by rocking. Allow sample to stand room temperature while preparing the β-glucoronidase/sulfatase solution. Prepare a separate mixture of β-glucoronidase/sulfatase (1mg/sample) at a concentration of 2 mg/mL in 1M ammonium acetate pH 5. Gently rock the vial to dissolve the enzyme. Add 500 µL of buffered enzyme solution to each sample. Gently mix the sample by rocking and incubate overnight at 37°C. The next step after incubation is the Cleanup Procedure.

(1) Extraction of phytoestrogens from urine

After incubation, add 300 µL of methanol to each sample and mix. Make sure that the propylene inserts in the manifold are new to minimize carry over. Prepare SPE (Varian Bond Elut LRC C18 200 mg) by first adding approximately 2 mL of methanol to wet the sorbent. Use the vacuum manifold to create a slight vacuum to pull the solvent into the SPE at a rate of approximately 2 drops/sec. Stop this process while sorbent is wet with methanol. Complete SPE equilibration by adding approximately 3 mL of wash solution (10% methanol, 0.1% acetic acid). Use vacuum to draw wash solution through the SPE, stopping the process while the sorbent bed still wet with wash solution. Add the sample to each labeled SPE cartridge and slowly use the vacuum to pull sample into the SPE bed. Rinse sample vial with approximately 1mL of wash solution and add the rinsate to the respective SPE cartridge. Once all the sample is drawn into the sorbent bed, add approximately 5mL of wash solution and pull this through the sorbent bed with a slight vacuum. Leave vacuum on high for five seconds to remove most of the wash solution. Elute phytoestrogens with 2mL of methanol collecting the eluant in centrifuge tubes (15 mL, 16 x 125 mm). Aliquot standards (100 µL of each Stds 1-8 in duplicate) into disposable centrifuge tube and add internal standard (5 µL deuterated standard mix per tube). Dry samples and standards in a speed vac at t 50 °C. Resuspend the samples and standards with 100 uL injection solvent (70%ammonium acetate 10 mM, 30% 1:1 ACN:MeOH, 200ng of 4 nitrophenol (stock 100 ng/µL; 2 µL per sample and 98 µL 70%ammonium acetate 10 mM 30% 1:1 ACN:MeOH) of mass spec external standard. Vortex mix

for 15sec and transfer to HPLC autosampler vial (conical bottom, polypropylene, crimp top). Analyze by HPLC-MS/MS method.

(2) Extraction of phytoestrogens from serum

After overnight incubation, prepare SPE (Oasis 60 mg cartridge, small reservoir) first adding 3 mL of methanol to wet the sorbent. Use the vacuum manifold to create a slight vacuum to pull the solvent into the SPE at a rate of approx 2 drip/secs. Complete the equilibration process by adding 3 mL of water. Use the vacuum to pull the solvent. Add sample to each labeled oasis cartridge and slowly use the vacuum to pull the sample into the cartridge. Rinse sample vial with approx 1mL of wash solution (0.10%ammonium hydroxide in 10% MeOH) and add this rinsate to the respective cartridge. Once all of the sample is drawn into the sorbent bed, add approx 3 mL wash solution and pull this through the sorbent bed with a slight vacuum. Leave vacuum on high for a five seconds to remove most of wash solution. Elute phytoestrogens using 2 mL of MeOH and low vacuum to pull the solvent through the sorbent bed. Collect eluant in disposable centrifuge tubes (15 mL). Aliquot standards (100 μL of each Stds 1-8 in duplicate) into disposable centrifuge tube and add internal standard (5μL deuterated standard mix per tube). Dry samples and standards in a Turbo Vap evaporator at 55°C under nitrogen. Resuspend the samples and standards with 100 uL injection solvent (70%ammonium acetate 10 mM, 30% 1:1 ACN:MeOH, 200 ng of 4 nitrophenol (stock 100 ng/μL; 2 μL per sample and 98 μL 70%ammonium acetate 10 mM 30% 1:1 ACN:MeOH) of mass spec external standard. Vortex mix for 15sec and transfer to HPLC autosampler vial (conical bottom, polypropylene, crimp top). Analyze by HPLC-MS/MS method.

b. Analysis

(1) Preliminary System Setup and Performance Check

(a) Make sure that the APCI probe is attached and connect to the API ion source assembly.

(b) Open the Gage valve this will open the gases. Check that the nebulizing gas is at 80psi and the auxiliary gas at 1 L/min. The vacuum meter should read close to 2 x 10^{-5} torr.

(c) Turn the heated nebulizer on. The temperature should read 500°C.

(d) Turn CID gas on from the computer controller.

(e) In the HP 1090 HPLC system, make sure the MeOH and ammonium acetate (pH 6.5, 10 mM) solutions are fresh.

(f) Turn HPLC pump on. Let the system equilibrate for about 15 min before starting a run. To check the performance of the system, inject phytoestrogen standard mix containing mass spec external standard, 4-nitrophenol is run under the same conditions as samples. The S/N and area counts for standard and labeled standard are assessed. If the minimum S/N and area counts are met, the HPLC 1090-SCIEX API III system is ready to start a run.

(2) Final Setup and Operation

Place the sample vials in the HPLC autosampler. Make sure to add one extra vial with MeOH after the sample vials. If the instrument is going to be used manually use the following instructions: Press SEQU to get a display (SEQU 0 100 100 1). The numbers displayed correspond to the labels beneath the display. The first number (0) is the line #. The cursor is above METH # (method number). The others correspond to WAIT TIME, FIRST VIAL, LAST VIAL and # OF INJECT (number of injections), respectively. Type in method number and use 0 for wait time. Vials 0 to 99 are in autosampler. When the correct entries are in the display, press ENTER to display the next line of the sequence. Type in method number use the rinse vial number for last vial entries, and press ENTER. Make sure the last line of the sequence table has PUMP=0 (turn off the pump). When the correct entries are in the display, press START and ENTER to begin the analysis. When

the HPLC injects, it sends a contact closure statement to the mass spectrometer to signal the start of data acquisition.

Alternatively the HPLC 1090 system can be controlled with the Agilent Chemstation software using a personal computer. If this is the case, load HPLC method PHYTO ONE which will have the optimum gradient conditions for the chromatographic separation of the phytoestrogens studied. Open the sequence option, write the vial range of the samples to be analyzed starting from vial #0. Make sure that the method loaded for each vial is the appropriate (PHYTO ONE). The last vial should contain methanol and the method loaded should be (PHYTOWASH).

(3) System Shutdown

To turn off the Sciex API III mass spectrometer:

(a) Turn off the heated nebulizer temperature probe and let the mobile phase (acetonitrile: methanol) flow through until the temperature drop close to 100°C.

(b) Turn HPLC pump off.

(c) Close the gate valve to turn off all the gases.

(d) Turn off sample pump.

(e) Turn off electronic one.

(f) Prepare the instrument for recycle. Normal recycle for 10 hours, weekend recycle from 48 to 60 hours. Minimum recycle to thaw the cold fingers is 6 hours.

c. Processing data

(1) Quantification

All raw data files are analyzed using McQuan application, which allows manual peak selection and area integration. These integrated values and retention times are copied and pasted into a Microsoft Excel spreadsheet. All blank rows, spaces in fields, and nonsense characters are then removed and the file is saved in comma-delimited format (*.csv, where * is the YYYYMMDD of the acquisition).

(2) Importation of Data into Database

The comma-delimited file is copied to a temporary file (n:\apps\dbfiles\phytosrm.txt), which is imported into the MSPEC table of the phytoestrogen database using a command file (n:\apps\dbfiles\phyto.cmd). To import the data, a password is required.

(3) Statistical Analysis and Interpretation of Data

Data is exported from the database to a fixed ASCII text file and imported into SAS. SAS programs for standard curve generation, QC analysis, blank analysis, limit of detection determination, unknown calculations, and data distribution have been created and may be executed in SAS when this information is needed.

d. Replacement and periodic maintenance of key components

(1) Daily Maintenance

(a) API III Sciex Mass Spectrometer

(i). Cleaning of the spray shield and the entrance end of the heated capillary, described in the Sciex API III System manuals, is performed at the end of each operating day or before starting a new run

(ii). The gate valve/front plate is clean daily before starting a new run. First wash with a solution of water: methanol (1:1) and finally with 100% methanol. Then clean the gate valve using

three polishing cloth with different grid (4000, 6000, 8000), finishing with the higher grid polishing cloth. Wash with methanol between each different cloth.

(iii). The pump oil is changed approximately every three months.

(b) HP 1090L HPLC

No routine maintenance is necessary. Maintenance may be necessary if there is a general decrease in instrument performance (see below).

(2) Performance Maintenance

In general, these maintenance procedures are performed after detecting a decrease in the system performance (sensitivity and/or S/N ratio) without any other apparent technical reasons.

(a) SCIEX API III Mass spectrometer

Maintenance of the API interface, L1x lenses and the analyzer assembly requires venting the system; maintenance of the APCI ion probe does not. After venting, the system will usually require around 4-6 hours to reestablish high vacuum.

When a partial blockage of the vacuum is detected the orifice is probe with a syringe-cleaning wire.

(b) HP 1090L HPLC

(i) The HPLC column and HPLC guard column are replaced if analyte resolution begins to fail. Specifically the peak shape of matairesinol was used; when matairesinol begin to tail badly, the column was replaced.

(ii) If high pressure (> 400 bar) error messages are observed, the guard column, analytical column frit, HPLC lines, needle seat, or injector components may need replacement.

(iii) Reestablishment of performance and calibration.

Each time the system is disturbed for cleaning or maintenance, a mass spec operational check standard is analyzed to assess the HPLC and MS performance. For the mass spectrometer, a retune of the system may or may not be necessary. If the instrument did not pass this test then the instrument was retuned using polypropylene glycol as described previously.

9. REPORTABLE RANGE OF RESULTS

The linear range of the standard calibration curves and the method limit of detection (LOD) determine the reportable range of results. The reportable range must be within the range of the calibration curves. However, samples with analytical data values exceeding the highest reportable limit may be diluted, re-extracted, and reanalyzed so that the measured value will be within the range of the calibration.

a. Linearity Limits.

The calibration curve is linear for all analytes (R^2>0.95) over three orders of magnitude. The limit on the linearity is determined by the highest standard analyzed in the method. Due to the wide variation of phytoestrogen levels found in humans, we set our highest standard near the high end of the linear range. The low end of the linear range is limited by the method LOD. Unknown (urine or serum) samples whose concentrations exceed this range must be reanalyzed using a smaller aliquot. Samples whose concentrations are below the method LOD are reported as non-detectable concentrations.

Table 15. Calibration curve parameters for each phytoestrogen measured.

Analyte	Linear range (μg/mL)
Enterodiol	0.02-18.0
Enterolactone	0.003-20.0
Matairesinol	0.008-1.7
Daidzein	0.01-12.0
Equol	0.06-10.0
O-DMA	0.005-8.0
Genistein	0.008-20.0

b. Analytical Sensitivity.

The limits of detection (LOD) were defined for each analyte by repetitive analysis of low level standards as described by Taylor (7) for the calculation of the standard deviation at zero concentration (S_o). The formal limit of detection was then defined as 3 x S_o. The functional LOD was equal to the formal LOD unless the lowest point in the calibration curve was higher, then the functional LOD was defined as the lowest standard concentration used in the calibration curve. Table 16 summarizes the LOD determined for each analyte in urine and serum. Limits of detection in the low ppb range were obtained for most analytes. Daidzein and equol had higher LOD due to significant amounts of native signal in the internal standard. Reduction of this isotopic contamination would significantly improve the LOD for these analytes. The limit of detection in extracted matrix is in the range of the low's ppb.

Table 16. Limit of detection for phytoestrogens in serum and urine.

Analyte	Serum LOD (ng/mL)	Lowest Standard Concentration (serum, ng/mL)	Urine LOD (ng/mL)	Lowest Standard Concentration (urine, ng/mL)
Enterolactone	0.1	0.3	3.3	10.0
Enterodiol	1.1	1.5	0.5	3.0
Matairesinol	0.5	0.4	0.2	0.8
Daidzein	0.5	1.0	9.3	10.0
Equol	2.4	6.0	1.1	3.0
O-DMA	0.3	0.3	0.3	1.0
Genistein	0.6	0.4	4.2	2.0
Coumestol	-	-	1.5	2.0

c. Accuracy

The accuracy of this method was determined using aliquots of phytoestrogen-free fetal bovine serum or synthetic urine spiked with known amounts of phytoestrogen standards at different levels of concentration and extracted using Oasis SPE cartridges. Internal standard was added after the extraction, and the resulting ion ratios compared with unextracted, spiked standards. The recoveries for serum in the concentration lower level (range from 4-50 ppb) were from 88-101%. Recoveries in the higher level (range from 80-600 ppb) were from 87-101%. In the case of urine the recoveries were determined spiking 100ng of each standard in syntheric urine. The recoveries range from 92-104%. These data confirm the accuracy of the method.

d. Precision

The precision of this method is reflected in the variance of quality control samples over time. The average coefficient of variation (CV) of two different quality control pools were used. In the case of serum QC pool,

QC low contained 4-25 ppb of the seven phytoestrogens, while QC high contained 80-600 ppb of the analytes. The coefficient variation for serum QC low was less than 10% for most of the analytes, with only equol and genistein exceeding 10%. For serum QC high samples the CV was less than 7.5%. For urine the QC low concentration was from 5.1 to 218 while for the QC high the concentration range was from 18 to 1765. The coefficient variation for urine QC low was less than 10% for most of the analytes, except for matairesinol and coumestrol (12.6% and 13.8%, respectively). For urine QC high samples the CV was less than 10% except for enterodiol and coumestrol (10.5 % and 12.9 %, respectively). In the case of enterolactone, daidzein, and O-DMA off scale values were obtained due to improvement of sensitivity after the QC high was formulated.

Table 17. Approximate urinary phytoestrogen levels in the different QC pools.

Analyte	QC Low Concentration (ppb)	QC High Concentration (ppb)
Enterolactone	217	1121
Enterodiol	71	157
Matairesinol	5.1	18
Daidzein	218	1765
Equol	35	329
O-DMA	76	1702
Genistein	100	818
Coumestrol	9.1	37

Table 18. Approximate serum phytoestrogen levels in the different QC pools.

Analyte	QC Low Concentration (ppb)	QC High Concentration (ppb)
Enterolactone	20	600
Enterodiol	20	499
Matairesinol	4.0	80
Daidzein	25	503
Equol	25	300
O-DMA	10	301
Genistein	15	501
Coumestrol	10	401

e. Analytical Specificity.

This is a highly selective method that requires that the phytoestrogens and their respective labeled internal standards: 1) coelute; 2) elute at a specific retention time; 3) have precursor ions with specific mass/charge ratios; 3) have two specific daughter ions formed from the precursor ion with specific mass/charge ratios.

Table 19. Phytoestrogen parent and daughter ion combinations for MS/MS specificity.

Native				Internal standards		
Compound	Parent	Quant	Conf	Compound	Parent	Quant
Enterodiol	301	253	241	D_6-Enterodiol	307	259
Enterolactone	297	107	93	D_6-Enterolactone	303	110
Matairesinol	357	83	147	D_6-Matairesinol	363	125
Daidzein	253	223	117	D_3-Daidzein	256	226
Equol	241	135	119	D_4-Equol	245	123
O-DMA	257	108	148	D_4-O-DMA	261	111
Genistein	269	133	224	D_4-Genistein	273	137
Deconjugation Internal Standard				4-Methylumbelliferone	175	133

f. Deconjugation Optimization

Accurate quantitation of the levels of a phytoestrogen assumes complete hydrolysis of its conjugated forms. Therefore, the deconjugation was optimized to identify the time period required for enzyme mediated deconjugation. Therefore a time course experiment was conducted with urine and serum containing conjugated phytoestrogens. This experiment revealed that the incubation of a sample with β-glucoronidase/ sulfatase for eight hours resulted in quantitative deconjugation. Overnight deconjugation (12-16 hours) ensured completion of hydrolysis, and was found not to induce artifactual changes to the levels of analyte.

10. QUALITY CONTROL (QC) PROCEDURES

a. Quality Control Materials

The quality control materials used for each unknown run were enriched with known amounts of each phytoestrogen. The matrix used was appropriate for the matrix analyzed: human serum analysis (spiked and characterized fetal calf serum) or human urine analysis (spiked and characterized human urine).

b. Quality Control Pools

Two quality control pools were prepared and used in each run of unknown samples:

(1) Serum: Quality control samples were identified as QC low and QC high. QC low contained 4-25 ppb of the seven phytoestrogens, while QC high contained 80-600 ppb of the analytes. The QC materials were then frozen at –70 °C until needed.

(2) Urine: Quality control samples were identified as QC low and QC high. QC low contained 5.1 to 218 ppb of the eight phytoestrogens, while QC high contained 18 to 1765 ppb of the analytes. The QC materials were then frozen at –70 °C until needed.

c. Characterization of QC Materials.

The QC serum and urine samples were characterized over an 11 week period. Using the data from these runs, the mean and upper and lower 99th and 95th confidence intervals were established. The confidence intervals were determined so they were adjusted according to the number of each QC material analyzed in each run.

d. Use of Quality Control Samples.

During each analytical run, four QC low and four QC high were analyzed. One QC high and one QC low urine were analyzed with each extraction manifold batch of samples. These QC samples were analyzed alongside unknown samples to monitor for accuracy and precision throughout the analysis batch.

Table 20. Urine QC concentration values with the respective confidence limits with *n* = 4 for each of the phytoestrogens analyzed.

ANALYTE	QC MATERIAL	Lower 99%	Lower 95%	Mean	Upper 95%	Upper 99%
Enterolactone	QC High	-	-	-	-	-
	QC Low	180.1	188.2	213.9	239.6	247.7
Equol	QC High	277.8	292.1	335.0	377.9	392.1

	QC Low	28.3	30.0	35.1	40.2	41.9
Matairesinol	QC High	15.3	16.6	20.4	24.2	25.5
	QC Low	3.7	4.2	5.5	6.9	7.3
O-DMA	QC High	-	-	-	-	-
	QC Low	68.1	69.5	74.2	78.9	80.4
Coumestrol	QC High	26.7	29.3	37.0	44.8	47.3
	QC Low	5.9	6.7	9.0	11.4	12.2
Daidzein	QC High	-	-	-	-	-
	QC Low	179.9	188.4	215.4	242.4	251.0
Enterodiol	QC High	108.0	120.0	156.3	192.5	204.6
	QC Low	52.2	56.8	70.7	84.7	89.3
Genistein	QC High	662.7	695.9	796.0	896.2	929.4
	QC Low	83.6	87.5	99.3	111.1	115.1

Table 21. Urine QC range of concentration value with the corresponding confidence limits for *n* = 4 for all the phytoestrogens analyzed.

ANALYTE	QC MATERIAL	MEDIAN	95%	99%
Enterolactone	QC High	-	-	-
	QC Low	18.1	33.1	40.2
Equol	QC High	41.3	76.2	93.7
	QC Low	5.1	9.3	11.5
Matairesinol	QC High	2.3	4.2	5.2
	QC Low	0.7	1.3	1.6
O-DMA	QC High	-	-	-
	QC Low	7.8	14.3	17.4
Coumestrol	QC High	6.3	11.5	14.2
	QC Low	1.5	2.8	3.4
Daidzein	QC High	-	-	-
	QC Low	18.7	34.3	41.6
Enterodiol	QC High	11.4	21.1	26.0
	QC Low	7.1	13.2	16.7
Genistein	QC High	82.5	152.2	187.3
	QC Low	9.3	17.2	21.2

Table 22. Serum QC concentration values with the corresponding confidence interval limits for each of the phytoestrogens analyzed.

ANALYTE	QC MATERIAL	Lower 99%	Lower 95%	Mean	Upper 95%	Upper 99%
Genistein	QC High	429.4	444.1	488.2	532.4	547.1
	QC Low	12.8	13.8	16.7	19.7	20.7
Daidzein	QC High	410.4	424.6	467.3	510.0	524.1
	QC Low	20.3	21.5	25.1	28.7	29.8
Enterodiol	QC High	529.5	547.1	600.2	653.4	671.0
	QC Low	20.2	20.6	21.7	22.9	23.3
Enterolactone	QC High	395.0	414.5	473.1	531.8	551.2
	QC Low	20.7	21.2	22.5	23.8	24.3
Equol	QC High	239.8	252.3	290.3	328.2	340.8
	QC Low	19.4	20.8	25.1	29.4	30.8
Matairesinol	QC High	67.1	70.1	79.2	88.3	91.3
	QC Low	3.7	3.9	4.2	4.6	4.7
O-DMA	QC High	244.6	254.0	282.4	310.8	320.2

QC Low	9.8	10.2	11.4	12.5	12.9

Table 23. Serum QC range of concentration values with the corresponding confidence limits for *n* = 4 for each of the phytoestrogens analyzed.

ANALYTE	QC MATERIAL	MEDIAN	95%	99%
Genistein	QC High	49.1	90.6	111.5
	QC Low	6.7	12.4	15.2
Daidzein	QC High	31.2	57.6	70.8
	QC Low	4.4	8.2	10.1
Enterodiol	QC High	56.0	103.4	127.2
	QC Low	2.3	4.3	5.3
Enterolactone	QC High	41.3	76.3	93.9
	QC Low	2.0	3.6	4.5
Equol	QC High	32.9	60.7	74.6
	QC Low	6.8	12.5	15.4
Matairesinol	QC High	8.8	16.3	20.0
	QC Low	0.7	1.3	1.6
O-DMA	QC High	29.5	54.4	66.9
	QC Low	1.6	2.9	3.6

e. Final evaluation of Quality Control Results.

An analytical run is considered “out-of-control” if the mean QC value or QC range values (for multiple QCs) are outside the 99% confidence intervals. If two consecutive mean QC values or QC range values are outside the 95% confidence intervals, both of those runs is considered “out-of-control”. Any data generated from a run that is not in control are not reported. If more than 8 consecutive QCs are on the same side of the mean of the characterized QC material, all operations were suspended until it is determined whether a bias is present in the method. This is a preventative measure only; the run is not considered “out-of-control”.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the QC systems or the calibrations failed to meet acceptable criteria, all operations are suspended until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, for instance, failure of the mass spectrometer or a pipeting error, the problem is immediately corrected. Otherwise, fresh reagents are prepared and the mass spectrometer is cleaned. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure) are reanalyzed. After calibration or quality control has been reestablished, analytical runs may be resumed.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. Urine volume

Because the solid-phase extraction column has a limited capacity, no more than 2 mL of urine may be used during for extraction. Occasionally, a 2 mL urine sample may be too concentrated to use as well. This is evident by the low recovery of the isotope during the extraction. In this case, a smaller aliquot of urine can be used to successfully extract the phytoestrogens. Most likely, the LOD is not higher in this case because of the concentrated nature of the urine.

b. Internal Standard purity

The isotopic purity of the isoflavonoid internal standards, especially daidzein and equol, was less than ideal. This led to a significant amount of native signal in the internal standard itself. This problem was

minimized by adding minimal amounts of internal standard, but still raised the LOD of the assay for these analytes.

13. REFERENCE RANGES (NORMAL VALUES)

Table 24. Urinary phytoestrogen levels (ppb) in NHANES III call-back urine specimen.

Analyte	Range	Mean£	Literature mean	n (n.d.)	Median	LOD¥	Low std (ng/mL)
Enterolactone	n.d.-5583	512	375	187 (10)	209	3.3	10.0
Enterodiol	n.d.-2508	62.8	62	191 (7)	27.0	0.5	3.0
Matairesinol	n.d.-91.8	14.5	4†	194 (5)	8.6	0.2	0.8
Daidzein	n.d.-15946	317	48	169 (30)	73.8	9.3	10.0
Equol	n.d.-4135	35.8	78	154 (45)	8.9	1.1	3.0
O-DMA	n.d.-1840	49.3	19	159 (40)	4.6	0.3	1.0
Genistein	n.d.-7285	129	40	179 (20)	34.8	4.2	2.0
Coumestrol	n.d.-13.1	5.8§	71†	9 (190)	n.d.	1.5	2.0

† Based on ≤ 4 small studies in the literature.

£ Calculate n.d. as LOD/2 (the actual assay LOD was the larger value of the lowest standard or (3 x S_o).

§ Calculated as the mean of the 9 detects only.

¥ Calculated as 3 x S_o.

Table 25. Serum phytoestrogen levels (ppb) in NHANES III serum specimen.

Analyte	Range	Mean£	Literature mean†	n (n.d.)	Median	LOD¥	Low std (ng/mL)
Enterolactone	n.d.-112	3.6	10.1	189 (19)	1.5	0.14	0.25
Enterodiol	n.d.-19.0	1.8	0.8	116 (92)	1.6	1.14	1.50
Matairesinol	n.d.-3.33	< LOD	0.1	3 (205)	n.d.	0.45	0.40
Daidzein	n.d.-162	3.9	1.6	179 (29)	1.3	0.45	1.00
Equol	n.d.-8.18	< LOD	0.4	2 (206)	n.d.	2.40	6.00
O-DMA	n.d.-28.5	1.0	0.2	138 (70)	0.6	0.29	0.25
Genistein	n.d.-203	4.7	2.1	116 (92)	0.7	0.60	0.40

†Based on 14 omnivorous Finnish women (8).

£ Calculate n.d. as LOD/2 (the actual assay LOD was the larger value of the lowest standard or (3 x S_o).

§ Calculate n.d. as 0 due to very few detects for this analyte.

¥ Calculated as 3 x S_o.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Insufficient data exist to correlate urinary phytoestrogen values with serious health effects. Therefore, critical call values have not been established.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Urine samples may be stored overnight in refrigeration to expedite thawing prior to aliquoting the sample. The urine extracts are stored in autosampler vials in a –20°C freezer after analysis. Stability studies suggest that the extracts remain stable at room temperature for up to five days.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMEN IF TEST SYSTEM FAILS

Alternate procedures do not exist for the measurement of urinary phytoestrogens.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Once the validity of the data has been established by the QC/QA sytem outlined above and has been verified by an EHLS statistician, one hardcopy and one electronic copy (ASCII format) of the data will be generated. This data, a cover letter, and a table of method specifications and reference range values will be routed through the appropriate channels for approval (i.e. supervisor, branch chief, division director). Once approved at the division level, they will be sent to the contact person who requested the analyses.

18. TRANSFER OR REFERRAL OF SPECIMEN; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

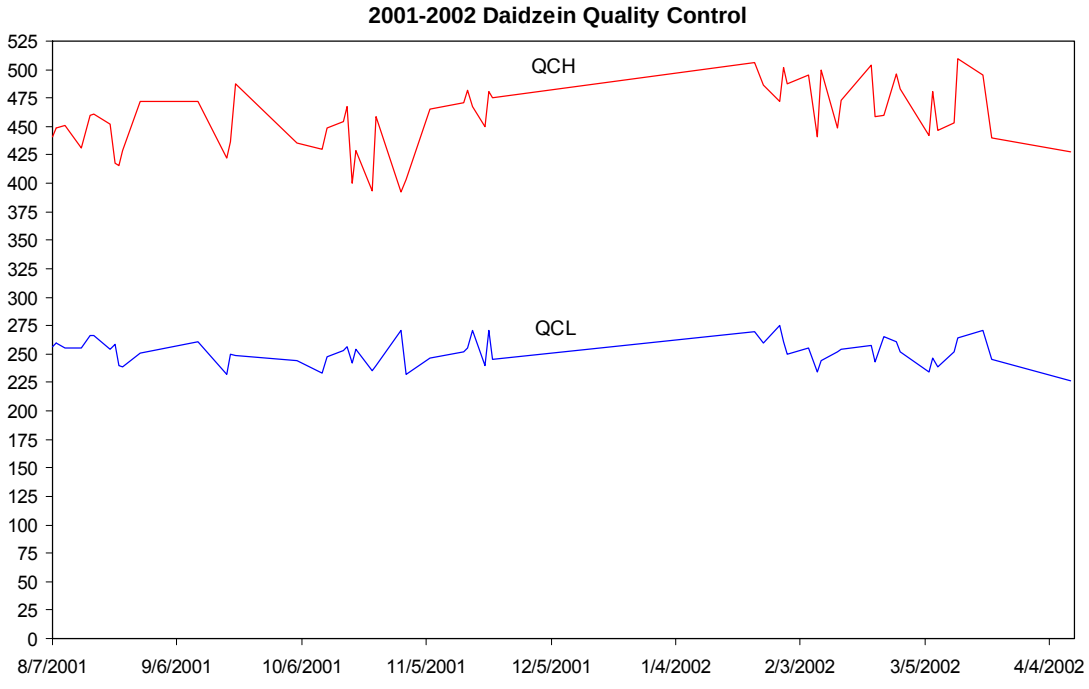
Standard record keeping systems (i.e. notebooks, sample logs, data files, creatinine logs, demographic logs) should be employed to keep track of all specimen. Specimen will only be transferred or referred to CLIA certified laboratories.

19. SUMMARY STATISTICS AND GRAPHS

a. Daidzein

Summary Statistics for Daidzein by Lot

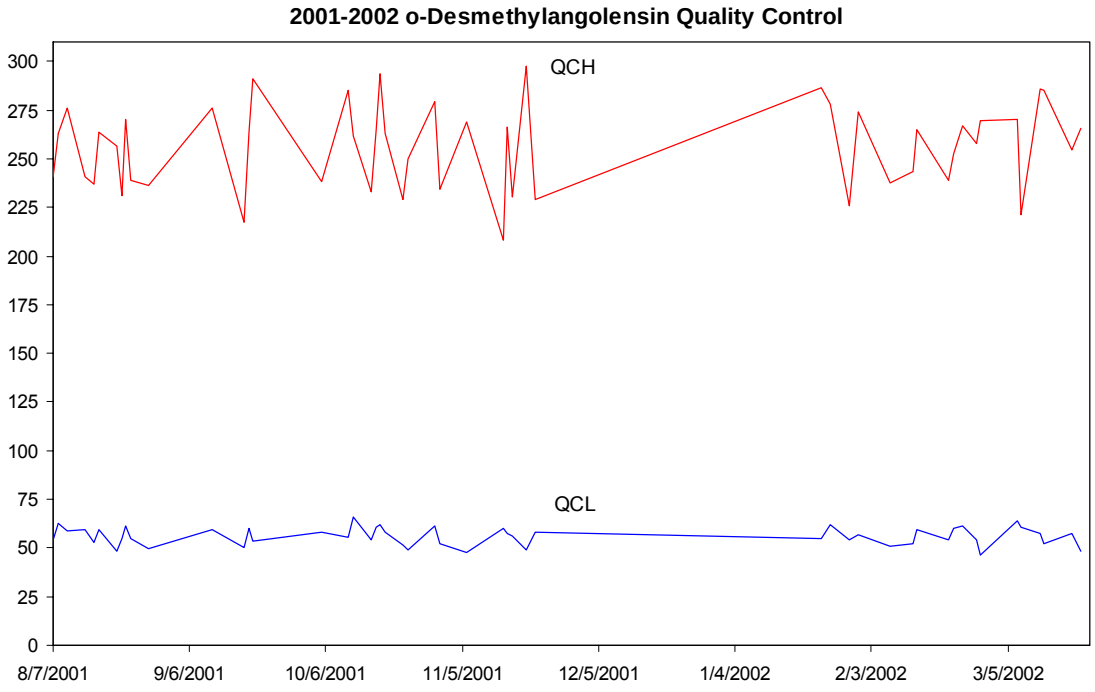
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL	59	8/7/2001	4/9/2002	251.79	11.76	4.67
QCH	59	8/7/2001	4/9/2002	457.75	29.06	6.35



b. *o*-Desmethylangolensin

Summary Statistics for 0-Desmethylangolensin by Lot

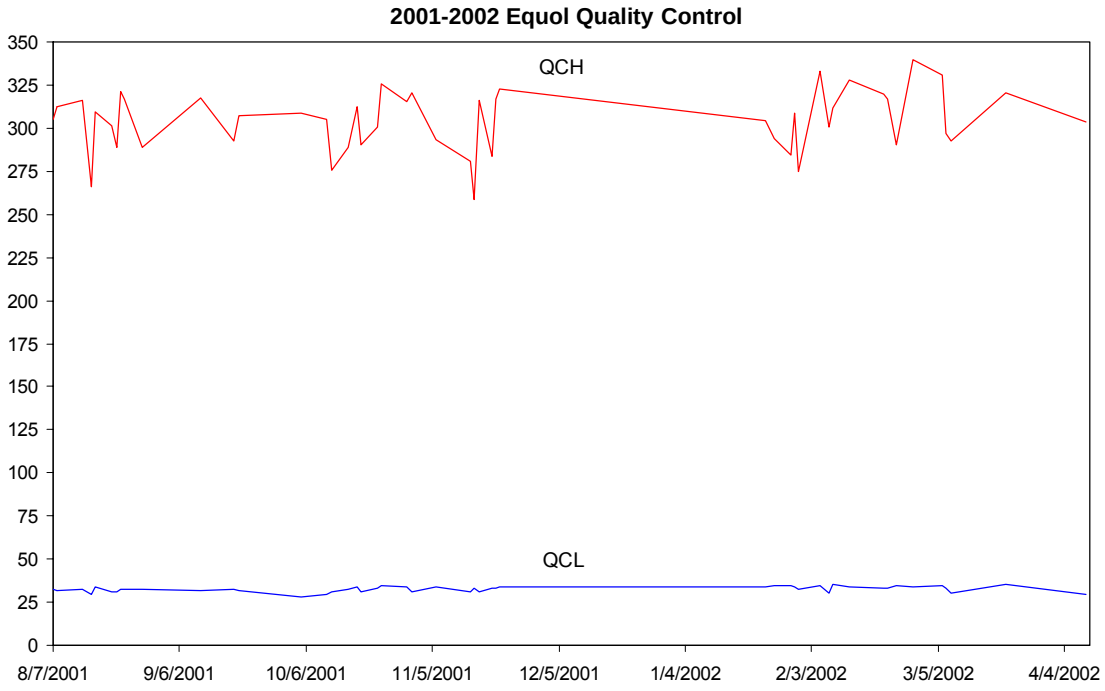
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL	53	8/7/2001	3/21/2002	56.16	4.68	8.33
QCH	53	8/7/2001	3/21/2002	256.12	22.34	8.72



c. Equol

Summary Statistics for Equol by Lot

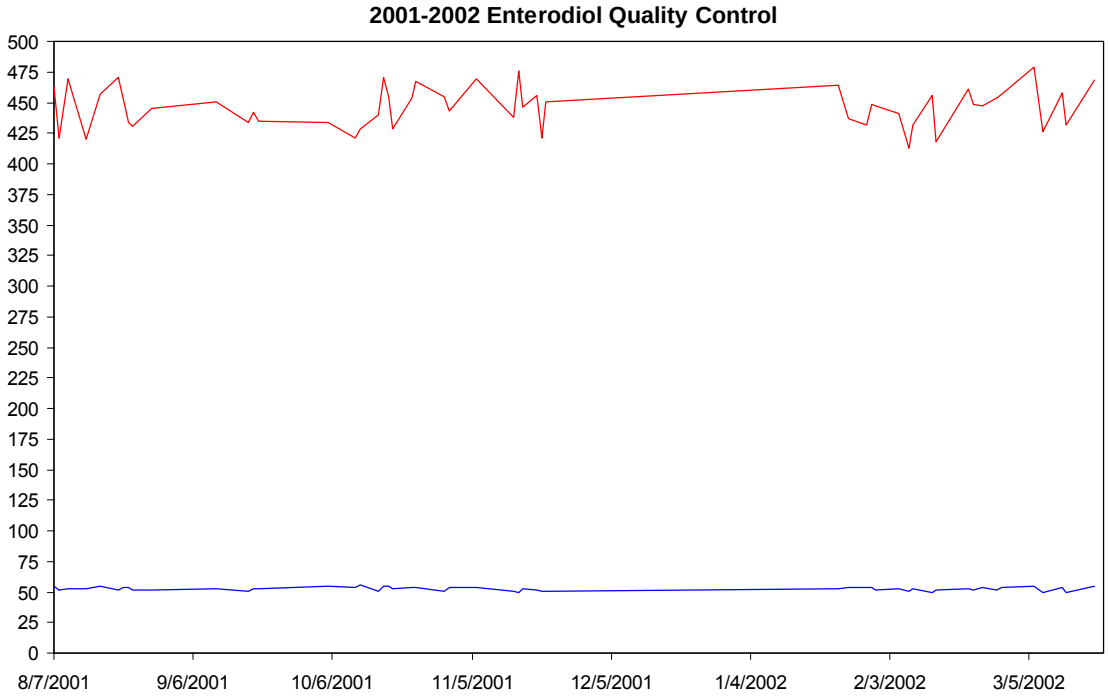
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL	49	8/7/2001	4/9/2002	32.51	1.78	5.47
QCH	49	8/7/2001	4/9/2002	304.04	17.69	5.82



d. Enterodiol

Summary Statistics for Enterodiol by Lot

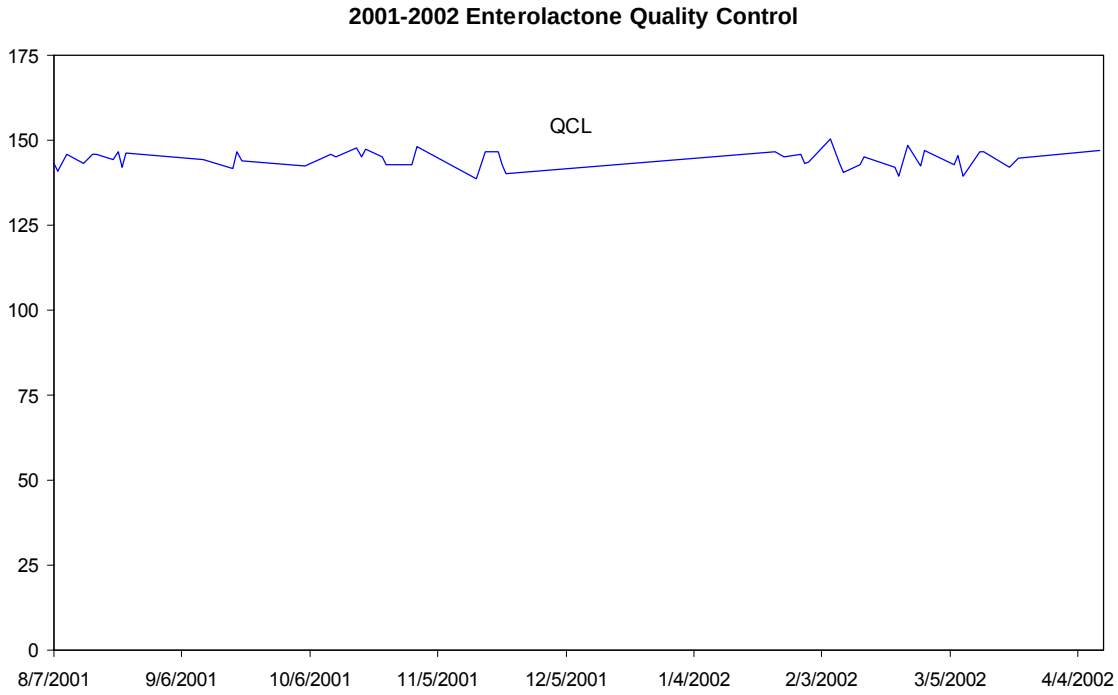
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL	55	8/7/2001	3/19/2002	52.52	1.59	3.02
QCH	55	8/7/2001	3/19/2002	446.35	16.72	3.75



e. Enterolactone

Summary Statistics for Enterolactone by Lot

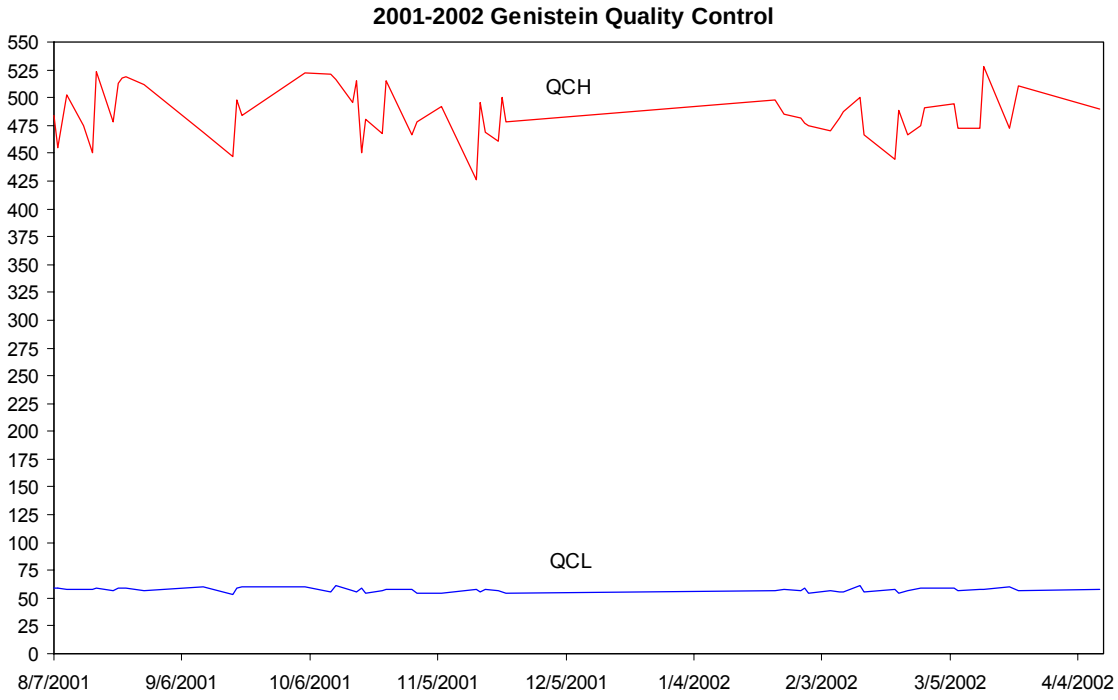
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL	55	8/7/2001	4/9/2002	144.27	2.61	1.81



f. Genistein

Summary Statistics for Genistein by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL	57	8/7/2001	4/9/2002	57.59	1.90	3.29
QCH	57	8/7/2001	4/9/2002	485.16	22.57	4.65



20. REFERENCES

(1) Obermeyer, W. R., Musser, S. M., Casey, R. E., Pohland, A. E., Page, S.W. Proc Soc Exp Biol Med. 1995,208:6–12.

(2) Franke, A. A., Custer, L. J. J Chromatogr. B Biomed Appl. 1994,662:47–60.

(3) Gamache, P. H., Acworth, I. N. Proc Soc Exp Biol Med. 1998,217:274–280.

(4) Joannou, G. E., Kelly, G. E., Reeder, A. Y., Waring, M., Nelson, C. J Steroid Biochem Mol Biol. 1995,54:167–184.

(5) Messina, M., Barnes, S., Setchell, K. D. Lancet. 1997,350:971–972.

(6) Barnes, S., Coward, L., Kirk, M., Sfakianos, J. Proc Soc Exp Biol Med. 1998,217:254–262.

(7) Taylor, J. K. Quality Assurance of Chemical Measurements, Lewis Publishers, CRC Press: Boca Raton, Florida, 1987.