

Division of Laboratory Sciences Laboratory Protocol

Analytes:	Phytoestrogens: Genistein, Daidzein, Equol, Enterodiol, Enterolactone, O-Desmethylangolensin
Matrix:	Urine
Method:	HPLC-ESI-MS/MS
Method Code:	4060.01
Branch:	Nutritional Biomarkers Branch

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Adopted: June 2004
date

Updated:
date

Director's Signature Block:

Reviewed:
signature date

Modifications/Changes: see Procedure Change Log

Procedure Change Log

Procedure: Urinary Phytoestrogens **DLS Method Code:** 4060.01

Date	Changes Made	By	Reviewed By (Initials)	Date Reviewed
July 2005	Changed write-up for instrument import of data into Access to more clearly explain steps involved.	Irene Williams		
10/20/05	The internal standard used for this method was changed from deuterium (² H) labeled compounds to carbon-13 (¹³ C) labeled. The ¹³ C labeled compounds are preferred (more pure as well as more stable than those with ² H labels). ² H IS have been used from 5/24/04 to 10/17/05. ¹³ C IS have been used from 10/20/05 on.	Daniel Parker		
10/24/05	The linear range of the calibration curve for enterodiol was extended to include the ninth (and highest) calibrator.	Daniel Parker		
07/27/06	Added a few sentences about calibration verification on p. 11.	Christine Pfeiffer		
10/17/06	Updates were made to the solid-phase extraction method to improve efficiency and reproducibility, including flow rate optimizations, reduction in the number of mix steps from 3 to 2, and increased the elution volume from 2 mL to 3 mL.	Daniel Parker		
10/17/06	Specific names of some methods (e.g. SPE methods, LCMS acquisition methods, quantitation methods) were removed as they are subject to change over the course of time. Specific method names and other related information can be found in the method SOP's in the Phytoestrogens QA book.	Daniel Parker		
5/22/07	Made changes associated with preparation of new analytical standards: preparation information, quantitation and data review, reportable range of results, and Table 1.	Daniel Parker		
10/25/07	Replaced existing API 4000 triple quadropole MS with a new API 4000 – Analyst software updated from version 1.4.1 to 1.4.2. New gas generator also installed.	Daniel Parker		
8/18/08	Replaced existing Agilent 1100 HPLC with Agilent 1200 HPLC with high performance 96-well plate autosampler.	Daniel Parker		
8/21/08	Began using a volume of 500-µL of urine for sample preparation, reduced from 1-mL – updated sections 4, 8, and 12 of this document, along with calibration points to include/exclude from each curve, to reflect this change.	Daniel Parker		
9/3/08	Updated the wording of section 8.b (Instrument Preparation) so as to include information related to the new autosampler and dilution factors.	Daniel Parker		



Laboratory Procedure Manual

Analyte: **Phytoestrogens: Genistein, Daidzein, Equol, Enterodiol, Enterolactone, O-Desmethylangolensin**

Matrix: **Urine**

Method: **HPLC-ESI-MS/MS**

Method No: 4060.01

Revised:

as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
National Center for Environmental Health (NCEH)

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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.

Urinary Phytoestrogens

NBB-DLS

DLS Method Code:

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Lab Number	Analyte	SAS Label (and SI units)
L06PHY_C	URXDAZ	Daidzein (ng/mL)
	URXDMA	α -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

a. Clinical relevance

Phytoestrogens, compounds naturally present in some plants, have estrogenic, anti-estrogenic, and anti-androgenic activity in animals and humans. Diet is the primary source of phytoestrogens. Isoflavones and lignans are two major groups of phytoestrogens. Plant sources of isoflavones include legumes, such as soybeans and soy based products, while the sources for lignans include whole grains, flax, and some fruits and vegetables. Genistein, daidzein, O-desmethyldangolensin (O-DMA), and equol are isoflavones; daidzein is metabolized to O-DMA by the tissues and to equol by intestinal bacteria. Matairesinol, enterolactone, and enterodiol are lignans; matairesinol is metabolized to enterolactone. Generally, phytoestrogens are less potent than endogenously produced estrogens, but they can be present in humans in much higher quantities. The consumption of foods rich in phytoestrogens has been associated with several beneficial health outcomes: reduced risk for breast and prostate cancer, reduction of menopausal symptoms, and modulation of osteoporosis. Although phytoestrogens are not acutely toxic in large dose animal tests, they have caused reduced reproductive capability in animals at chronic dietary doses; some studies suggest adverse effects on the immune system. After ingestion, the natural conjugated phytoestrogens are hydrolyzed to their aglycones (free form), absorbed, and glucuronidated in the intestine. The major circulating forms of the isoflavones are the glucuronidated species; glucuronidated forms also predominate in the urine.

b. Test principle

The test principle utilizes high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) for the quantitative detection of genistein, daidzein, equol, O-DMA, enterodiol, and enterolactone. Human urine samples are processed using enzymatic deconjugation of the glucuronidated phytoestrogens followed by solid phase extraction (SPE). The SPE eluate is concentrated, and the phytoestrogens are separated from other urine components by reversed phase HPLC, detected by ESI-MS/MS, and quantified by isotope dilution. Assay precision is improved by incorporating deuterium-labeled internal standards for each of the analytes, as well as a 4-methylumbelliferyl glucuronide and 4-methylumbelliferyl sulfate standards to monitor deconjugation efficiency. This selective method allows for rapid detection of six phytoestrogens in human urine with limits of detection in the low parts per billion (ppb; ng/mL) range.

2. Safety Precautions

Consider all urine specimens as potentially positive for infectious agents including HIV, hepatitis B and hepatitis C. We recommend the hepatitis B vaccination series for all analysts working with urine. Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method. Discard any residual sample material by autoclaving after analysis is completed. Place all disposable plastic, glassware, and paper (pipet tips, autosampler vials, gloves etc.) that contact urine in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Use disposable bench diapers during sample preparation and urine handling and discard after use. Also, wipe down all contaminated work surfaces with a 10% bleach solution when work is finished.

Handle acids and bases (which are used for preparation of ammonium acetate buffers) with extreme care; they are caustic and toxic. Handle organic solvents only in a well-ventilated area or, as required, under a chemical fume hood.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDSs) for all chemicals are readily available in the MSDS section as hard copies in the laboratory. MSDSs for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html> or at <http://intranet.cdc.gov/ohs>.

3. Computerization; Data System Management

- a. Mass spectrometry data are collected and stored using the Analyst Software of the Sciex API 4000 (Applied Biosystems) mass spectrometer workstation. During sample preparation and analysis, samples are identified by their Sample ID. The Sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample. All raw data is processed into results files (containing patient data as well as QC data) by using the Quantitation Wizard application in Analyst and then saved in the results folder in the directory. Results files allow for manual peak selection and integration when necessary. Data (including peak area, peak height, retention time, analyte name, and mass transition) from the results files are then exported into the Microsoft Access Network Database (FrontEnds) in the method described below:

Step 1 – Analyst – Import data file into ACCESS:

- Double click the ACCESS icon on desktop, password entry required
- [Add Sample Results to Database] (under Batch & X-Batch)
- [Import Instrument Data File] - Enter information (instrument, assay, date, time, analyst, study)
- [Import] – In “select data file” window, choose A: and import file number assigned. Check that sample ID’s are recognized.
- [Transfer]

Step 2 – Analyst – Review run in ACCESS:

- [Run Review] (under Batch & X-Batch) – Select assay
- [Show runs] – Cursor to desired run, enter sample set name and comments
- [QC Results] – Review QC results for transmission errors and whether they pass the 2S limits
- [Print Report] [Back]
- [Sample Results] – Review patient results to assure proper information transmission, enter appropriate comment codes on flagged samples
- [Set Final] results that are ready to be reported
- [Set Reviewed]
- [Print Report] [Back]

Step 3 – Analyst – Send email and run folder to QA Officer:

An e-mail is sent to the QA Officer including the following run information: Analysis date, Instrument, Study, Groups, File name, Batch ID, Run #, and QC Status. Noteworthy comments are included in the email. All printouts including raw data are submitted in a run folder to the QA Officer who reviews the Bench QC data via the ACCESS database as described below.

Step 4 – QA Officer – Review Bench QC via ACCESS:

- Double click the ACCESS icon on desktop, password entry required
- [Export QC to SAS] (under Batch & X-Batch) – Select Assay, Date range and Controls
- [Make QC Data Infile] – Save file to I:, appropriate subfolder for archival
- [Run SAS] – SAS will automatically open, [go], review each generated plot, print QC cover page and standard deviation plot, [Back]
- [Run Review] (under Batch & X-Batch) – Select assay
- [Show runs]
- [Sample Results]
- [Set Batch QC] – accept or reject
- [Set Reviewed]
- Forward email from Analyst to Second QA reviewer (for Blind QC review) specifying Bench QC status of the run.

Step 5 – Second QA Reviewer – Review Blind QC and other parameters in ACCESS:

- Double click the ACCESS icon on desktop, password entry required
- [Run Review] (under Batch & X-Batch) – Select assay, then desired run
- [Blind QC Results] – Review whether Blind QC results pass the 2S limits
- [Print Report] [Back]
- Check other parameters if applicable (i.e., background, calibration curve, repeat values, replicates, signal intensity)
- [Set RQC] – accept or reject
- Verify that appropriate comment codes have been applied and that final values have been set correctly
- [Set Ready] – Final results will be set ready to be exported
- [Set Reviewed]
- Forward email from QA Officer to Supervisor specifying Blind QC status of the run and other relevant comments.

Step 6 – Supervisor – Approval and Export of Results via ACCESS:

- Double click the ACCESS icon on desktop, password entry required
- [Run Review] (under Batch & X-Batch) – Select assay, then desired run
- Perform final review of Bench and Blind QC status, comment codes, repeat results
- [Set Reviewed]
- [Export/Report Results] (under Study Functions) – Select study, select analytes/panel, use selected panel
- [Generate Excel Spreadsheet] – Review file on I:\To be transmitted
- [Generate Export Text File and Set Results Exported] – Review file on I:\To be transmitted
- FTP file to Westat
- Send Westat an email that file was transmitted
- Move transmittal file from Q:\Nutritional\To Be Transmitted to Q:\Nutritional\Transmitted Data\Appropriate Year Folder.

For NHANES, data is transmitted electronically several times weekly to Westat's ISIS computer system, and transferred from there to NCHS. Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.

- b. The instrument raw and results files in Analyst are typically backed up to the CDC network on a daily basis and periodically backed up on CD. All sample, QC, and calibration data (both raw data and results) are stored on the network and are the responsibility of the analyst. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively.
- c. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

- a. We recommend that specimen donors fast prior to specimen collection, but fasting is not required.
- b. Specimens for phytoestrogen analysis are performed on fresh or frozen urine.
- c. 3-5 mL of urine is preferable to allow for repeat analyses. A volume of 500- μ L is required for each analysis.
- d. The appropriate amount of urine is dispensed into a Nalgene 5.0 mL cryovial or other plastic screw-capped vial labeled with the participants ID.
- e. Specimens collected in the field are frozen, and then shipped on dry ice by overnight carrier. Frozen samples are stored at least at -20°C , preferably at -80°C . Excessive freeze/thaw cycles might result in degradation of phytoestrogens in urine, however, phytoestrogens appear to be stable over the course of three freeze/thaw cycles.
- f. Specimens generally arrive frozen. Refrigerated samples may be used provided they are kept cold and brought promptly (within 2 hours) from the site of collection.
- g. Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q:/ITN/Nutrition Laboratory/CLIA). The protocol discusses collection and transport of specimens and the special equipment required. In general, plasma should be transported and stored at no more than -20°C . Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or plasma should be transferred into a sterile Nalgene cryovial labeled with the participant's ID.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this method.

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

a. Reagent Preparation

Prepare all reagents with deionized water with a resistance of at least 15 megaOhm/cm.

(1) Ammonium Acetate Buffer, pH 5.0 (1 M)

For 1 L, weigh 77.0 g of ammonium acetate into a 1-L beaker and add 800 mL of HPLC-grade water. Add 28.5 mL of glacial acetic acid and mix vigorously until ammonium acetate is dissolved. Add NH_4OH dropwise until pH 5.0. Transfer the solution to a 1-L volumetric flask and fill to mark using HPLC-grade water. Prepare monthly and store at 10°C or below.

(2) Ammonium Acetate Buffer pH 6.5 (1 M)

For 500 mL, weigh 38.5 g of ammonium acetate into a 500-mL beaker and add 400 mL HPLC-grade water. Mix vigorously until ammonium acetate is dissolved. Add glacial acetic acid dropwise until pH 6.5. Transfer the solution to a 500-mL volumetric flask and fill to mark using HPLC-grade water. Prepare monthly and store at 10°C or below.

(3) Reconstitution Solution

Use 30% organic mobile phase (1:1 methanol [MeOH]:acetonitrile [ACN]) and 70% aqueous mobile phase (10 mM ammonium acetate buffer pH 6.5). Prepare weekly and store at 10°C or below.

(4) β -Glucuronidase/Buffer Solution

Weigh 1 mg β -glucuronidase powder enzyme for each sample prepared and measure 0.5 mL ammonium acetate buffer pH 5.0 for each sample prepared (i.e. for a run of 40 samples, weigh 40 mg of enzyme and dissolve in 20 mL buffer). Extreme care should be taken during this process so as not to deactivate the enzyme; do not vortex or shake vigorously. To mix, use a gentle rocking motion. Prepare daily for each run.

(5) HPLC Mobile Phase (Aqueous)

For 1 L, add 10 mL of 1 M ammonium acetate buffer pH 6.5 and 990 mL of HPLC-grade water to a 1-L screw-cap bottle and shake vigorously. Prepare daily.

(6) HPLC Mobile Phase (Organic)

For 1 L, add 500 mL of MeOH and 500 mL of ACN to a 1-L screw-cap bottle and shake vigorously. Refill as needed.

(7) SPE Reservoir Solution

For 4 L, add 2 L of MeOH and 2 L of HPLC-grade water and shake vigorously. Refill as needed.

(8) Synthetic Urine

Prepare as needed and store at 10°C. For 1 L, add the following chemicals in the order shown:

500 mL	Water
3.8 g	Potassium Chloride
8.5 g	Sodium Chloride
24.5 g	Urea
1.03 g	Magnesium Sulfate (MgSO ₄ .7H ₂ O)
1.03 g	Citric Acid
0.34 g	Ascorbic Acid
1.18 g	Potassium Phosphate
1.4 g	Creatinine
0.64 g	Sodium Hydroxide (add slowly)
0.47 g	Sodium Bicarbonate
0.28 mL	Sulfuric Acid (conc.)
fill to 1 L with water	

b. Standards Preparation

1) Analytical Standard Stock Solutions

A stock solution of each phytoestrogen standard is prepared separately by dissolving 3-5 mg of the compound in 0.2 mL of DMSO (dimethylsulfoxide), and placing it in a 25-mL volumetric flask. The flask is then filled to volume with ethanol.

2) Mixed Working Analytical Standard Solutions

Nine mixed working solutions with increasing concentration of each phytoestrogen standard are prepared in 50-mL volumetric flasks by using appropriate volumes from each standard stock solution based on the concentrations needed to cover the linear range of the assay (see Table 1). Each flask is then filled to volume with the appropriate amounts of ethanol and water such that the final mixture is dissolved in 50% ethanol/water. Each mixed working solution is then dispensed in 100- μ L aliquots into 1.5-mL micro-centrifuge tubes and stored upright at -80°C.

3) Internal Standard Stock Solutions

Prepare a stock solution of each internal standard separately by adding 1-2 mg of each compound, dissolved in ethanol, to a 25-mL volumetric flask and fill to volume.

4) Mixed Working Internal Standard Solutions

A mixed working internal standard solution containing the appropriate concentration of each compound is prepared by pipetting the following amounts of each internal standard stock solution into a volumetric flask of appropriate size:

Using deuterium labeled compounds:

² H ₄ -Equol	24.5 mL
² H ₃ -Daidzein	18.75 mL
² H ₅ -o-Desmethylangolensin	1.25 mL
² H ₄ -Genistein	6.25 mL
² H ₆ -Enterolactone	24.5 mL
² H ₈ -Enterodiol	6.75 mL

Using carbon-13 labeled compounds:

¹³ C ₃ -Equol	15.79 mL
¹³ C ₃ -Daidzein	19.89 mL
¹³ C ₃ -o-Desmethylangolensin	3.13 mL

$^{13}\text{C}_3$ -Genistein	15.63 mL
$^{13}\text{C}_3$ -Enterolactone	15.18 mL
$^{13}\text{C}_3$ -Enterodiol	12.98 mL

The flask is then filled to volume with HPLC-grade water. The solution is dispensed in 1-mL aliquots into 1.5-mL micro centrifuge tubes and stored at -80°C (see Table 1).

5) Deconjugation Internal Standard Solution

4-methylumbelliferyl glucuronide and 4-methylumbelliferyl sulfate are used as deconjugation standards to quantify the extent of enzymatic reaction. The deconjugation standard is prepared by dissolving 1.20 mg of 4-methylumbelliferyl glucuronide and 1.00 mg of 4-methylumbelliferyl sulfate in ethanol and placing in a 50-mL volumetric flask and diluting to volume. The solution is dispensed in 1-mL aliquots into 1.5-mL micro-centrifuge tubes and stored at -80°C .

c. Preparation of Quality Control Materials

Low, medium, and high quality control pools are prepared by selecting and pooling urine containing the appropriate levels of all six phytoestrogens. For the low pool, urine is selected that contains levels of phytoestrogens near the 10th percentile for each analyte based on the January 2003 edition of the Second National Report on Human Exposure to Environmental Chemicals¹⁰. Urine selected for the medium pool contains levels of phytoestrogens near the 50th percentile for each analyte. Urine selected for the high pool has target levels of phytoestrogens near the 90th percentile (see Table 2).

Urine (3.5 mL) is aliquoted into 5.0-mL Nalgene cryovials, capped, and frozen. The QC pools are stored at -80°C and are stable for at least 3 years. Means plus range limits for all pools are established by analyzing duplicates for at least 20 consecutive runs.

d. Other Materials

- 1) Betasil C18 analytical column, 50x3mm, 5 μm (Thermo Electron Corp., Bellefonte, PA)
- 2) C18 (ODS) guard column, 4mm L x 2.0mm ID (Phenomenex, Torrance, CA)
- 3) Oasis HLB flangeless cartridges (60mg/3cc, Waters Corporation, Milford, MA)
- 4) 12x75mm disposable glass culture tubes (Corning Glassworks, Corning, NY)
- 5) 16x100mm screw-top disposable glass culture tubes (Corning Glassworks, Corning, NY)
- 6) 415/15 PTFE, rubber-lined screw caps (Corning Inc., Corning, NY)
- 7) 9" Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)
- 8) HPLC autosampler vials (2.0mL/12x32mm, National Scientific, Duluth, GA)
- 9) HPLC autosampler vial snap-caps (11mm, National Scientific, Duluth, GA)
- 10) HPLC autosampler vial inserts (200 μL , Kimble Glass, Vineland, NJ)
- 11) N-Dex nitrile examination gloves (Best Manufacturing Corporation, Menlo, GA)
- 12) Blue tips (50-1000 μL) for Eppendorf pipette (Brinkmann Instruments Inc., Westbury, NY)

- 13) Yellow tips (2-200 μ L) for Eppendorf pipettes (Brinkmann Instruments Inc., Westbury, NY)
- 14) Combitip plus (500 μ L) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)
- 15) 0.22 μ m PVDF filters (Millipore, Bedford, MA)
- 16) 5.0mL Polypropylene cryovials (Nalgene Company, Rochester, NY)
- 17) 1.5mL micro centrifuge tubes (VWR, Suwanee, GA)
- 18) Various glass beakers, volumetric flasks, graduated cylinders, and bottles, class A glassware.
- 19) Methanol, HPLC grade (Burdick & Jackson Laboratories, Muskegan)
- 20) Acetonitrile, HPLC grade (Burdick & Jackson Laboratories, Muskegan)
- 21) Ethanol, HPLC grade (Burdick & Jackson Laboratories, Muskegan, MI)
- 22) Dimethylsulfoxide, HPLC grade (Burdick & Jackson Laboratories, Muskegan, MI)
- 23) Water, HPLC grade (Aqua Solutions, Jasper, GA)
- 24) Ammonium Hydroxide (28-30%, Fisher Scientific Co., Fairlawn, NJ)
- 25) Ammonium Acetate, HPLC grade (Sigma, St. Louis, MO)
- 26) Acetic acid, glacial, reagent grade (Sigma, St. Louis, MO)
- 27) 4-methylumbelliferyl β -D-glucuronide hydrate (Sigma, St. Louis, MO)
- 28) 4-methylumbelliferyl sulfate (Sigma, St. Louis, MO)
- 29) β -Glucuronidase from *Helix pomatia* (Sigma, St. Louis, MO)
- 30) Enterolactone (Sigma, St. Louis, MO)
- 31) Enterodiol (Sigma, St. Louis, MO)
- 32) Equol (Sigma, St. Louis, MO)
- 33) Genistein (Indofine Chemical Company, Somerville, NJ)
- 34) Daidzein (Indofine Chemical Company, Somerville, NJ)
- 35) o-Desmethylangolensin (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 36) $^2\text{H}_8$ -Enterodiol, (Dr. Kristiina Wähälä, University of Helsinki, Finland)
- 37) $^2\text{H}_6$ -Enterolactone, (Dr. Kristiina Wähälä, University of Helsinki, Finland)
- 38) $^2\text{H}_4$ -Genistein, (Dr. Kristiina Wähälä, University of Helsinki, Finland)
- 39) $^2\text{H}_3$ -Daidzein, (Dr. Kristiina Wähälä, University of Helsinki, Finland)
- 40) $^2\text{H}_4$ -Equol, (Dr. Kristiina Wähälä, University of Helsinki, Finland)
- 41) $^2\text{H}_5$ -o-Desmethylangolensin, (Dr. Kristiina Wähälä, University of Helsinki, Finland)
- 42) $^{13}\text{C}_3$ -Enterodiol, (Dr. Nigel Botting, University of St. Andrews, Scotland)

- 43) $^{13}\text{C}_3$ -Enterolactone, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 44) $^{13}\text{C}_3$ -Genistein, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 45) $^{13}\text{C}_3$ -Daidzein, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 46) $^{13}\text{C}_3$ -Equol, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 47) $^{13}\text{C}_3$ -O-Desmethylangolensin, (Dr. Nigel Botting, University of St. Andrews, Scotland)

e. Instrumentation

- 1) Agilent 1200 HPLC system (Agilent, Santa Claire, CA)
 - a) Model G1379B-Degasser
 - b) Model G1312A-Binary pump
 - c) Model G1367B-High Performance Autosampler
 - d) Model G1316A-Thermostatted Column Compartment
- 2) Sciex API 4000 triple quadrupole mass spectrometer, turboionspray as ion source (TIS) in ESI mode, with Analyst 1.4.2 software (Applied Biosystems, Foster City, CA)
- 3) Parker-Balston model LCMS-5001NTNA Tri-Gas Generator that supplies curtain, exhaust, and source gases to the instrument in addition to nitrogen gas for the collision cell (Peak Scientific Instruments, Chicago, IL)
- 4) Gilson 215 automated solid phase extraction system (Gilson, Inc., Middleton WI)
- 5) Speedvac SPD2010-220 (Thermo Savant, Bellefonte, PA)
- 6) Economy incubator, model 3EM (Precision, Winchester, VA)
- 7) Eppendorf pipette (100-1000 μL , Brinkmann Instruments, Inc, Westbury, NY)
- 8) Eppendorf pipette (10-100 μL , Brinkmann Instruments, Inc, Westbury, NY)
- 9) Eppendorf pipette (2-20 μL , Brinkmann Instruments, Inc, Westbury, NY)
- 10) Eppendorf Repeater Plus pipettor, (Brinkmann Instruments, Inc, Westbury, NY)
- 11) Vortexer (VWR, Suwanee, GA)
- 12) Magnetic stirrer (Fisher Scientific Co., Fairlawn, NJ)
- 13) Pinnacle model 530 pH meter (Corning Inc., Corning, NY)
- 14) Mettler Toledo analytical balance, model AG104 (Mettler Instrument Corp., Hightstown, NJ)
- 15) Harvard syringe pump (Harvard apparatus, Inc, Holliston Massachusetts)

7. Calibration and Calibration Verification Procedures

Results of in-house SPE recovery independent of the internal standard showed a mean (\pm SD) recovery of 88.0% \pm 5.7% for equol, 105.2% \pm 5.5% for daidzein, 105.3% \pm 7.9% for O-DMA, 100.5% \pm 1.7% for genistein, 99.7% \pm 6.4% for enterolactone, and 102.8% \pm 1.8% for enterodiol for phytoestrogens added externally to urine at levels equivalent to the 3rd through 8th calibrators.

The calibration process is internal, based on the area ratios of each of the individual six phytoestrogens in each standard with the internal standard. A nine-point linear curve, not

forced through zero, is generated using 1/x weighting and calculated on the basis of the single analysis of nine different standard concentrations. The concentrations are calculated from the regression equation based on the area ratios between analyte and internal standard.

Calibration standards are not carried through the sample preparation procedure.

At the beginning of each run, nine mixed calibrators (S1-S9) are prepared using the mixed working analytical standard solutions and the mixed working internal standard solution prepared as described in section 6.b. of this document. 50 μ L of mixed working internal standard solution is added to each mixed working analytical standard solution. The contents are vortexed thoroughly, transferred to a 12x75mm glass culture tube, and the solvent is then evaporated. The contents of the tube are then redissolved in 100 μ L of reconstitution solution, vortexed, and are then ready for analysis.

At the end of each run, the calibrators are reanalyzed as unknowns. The measured concentrations of these calibrators must agree within 15% of their set values.

International reference materials are not available for urine phytoestrogens. External quality assessment programs don't exist either. Proficiency testing is conducted in-house at least twice a year.

a. Calibration of Instrument (Sciex API 4000)

The calibration of the instrument is scheduled on a semi-annual basis, as part of the preventive maintenance, and is performed by the service engineer from Applied Biosystems. If necessary, the analyst can recalibrate using the calibration standards described below and by following the instructions contained in the operator's manual.

- 1) The tuning and mass calibration of the first and third quadrupoles of the API 4000 is performed using a solution of polypropylene glycol (PPG) by infusion and running the instrument in either Manual Tuning mode or using Automatic Mass Calibration.
- 2) The PPG calibration solution kit contains PPG standard 2000 vials for positive ion mode calibration and PPG standard 3000 for negative ion mode calibration.
- 3) The PPG calibration standards are infused using a Harvard syringe pump, typically using a 1 mL syringe (diameter of 4.61 mm) at a flow rate of 10 μ L/min, in manual tuning using Q1 and Q3 scans.

b. Instructions for Calibration of Instrument (Sciex API 4000)

Follow the API 4000 operators' manual for instructions on calibration with PPG's (API 4000 Operator Course, and API 4000 L/MS/MS Hardware manual, Applied Biosystems).

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A typical run (written in this document in the order in which they are injected into the LC/MS/MS) consists of one injection containing mobile phase only, injection of the blank, 9 calibrators, 3 front QC's (low, medium, and high), 73 patient samples, 3 back QC's (low, medium, and high), reinjection of all nine calibrators, all followed by the shutdown method.

a. Sample Preparation

- 1) Enzymatic Deconjugation (Quality control samples and unknown samples)
 - a) Remove all necessary QC's and unknown samples from the freezer (-80°C), as well as internal standard solution and deconjugation internal standard solution (prepared

- as described in section 6.c of this document), and allow them to reach ambient temperature.
- b) Set up and label one 16x100 mm screw-top glass culture tube per sample.
 - c) Add 50- μ L of internal standard solution to each glass tube.
 - d) Add 10- μ L of deconjugation internal standard solution to each glass tube.
 - e) Add 500- μ L of QC or unknown urine sample to the appropriate glass tube according to the label.
 - f) Allow the mixture to sit at room temperature while preparing the β -glucuronidase solution (described in section 6.a. of this document).
 - g) Add 500 μ L of HPLC-grade water to each sample.
 - h) Add 500 μ L of the β -glucuronidase solution to each sample.
 - i) Cap each sample and gently rock (not vigorously and do not vortex as this may cause deactivation of the enzyme), making sure that all contents are washed from the tube wall.
 - j) Incubate overnight (at least 17 hours) at $37^{\circ} \pm 2^{\circ}\text{C}$.
- 2) Automated Solid Phase Extraction using Gilson 215 SPE system (Quality control samples and unknown samples)
- a) Preparation the of the Gilson 215 SPE System
 1. Rinse and refill 700 mL methanol solvent bottle and 700 mL water solvent bottle.
 2. Refill reservoir with SPE Reservoir Solution (prepared as described in section 6.a. of this document).
 3. After filling reservoir, purge the syringes and lines: under the "Applications" tab on the left side of the screen, double-click on the appropriate application for running samples for phytoestrogens (see SOP for Sample Preparation in the Phytoestrogens QA book for specific method names and information). Once it opens, click on the Manual Control tab in the window (if this prompts you to initialize the system, click yes) and click purge. Let the syringes and lines purge for at least five minutes.
 - b) Loading the Gilson 215 SPE System
 1. Load as many Oasis HLB flangeless cartridges as needed in the mobile cartridge holders.
 2. Load as many 12x75mm glass culture tubes (labeled appropriately) as needed in the collection racks, beginning in position 1 (top left position of the rack on the left).
 3. Vortex each sample for 2 seconds and remove the caps.
 4. Load all samples on the sample racks, beginning in position 1 (top left position of the rack on the left). Only place samples in the first 40 slots of each sample rack (the last two rows of each sample rack should remain empty). There are two sample racks, for a total of 80 possible samples.
 - c) Running the Gilson 215 SPE System
 1. After purging the syringes and lines and loading the system, open the appropriate application for running samples for phytoestrogens (see SOP for Sample Preparation in the Phytoestrogens QA book for specific method information) if it is not already open. Note: the application is set up to run 80

samples, so if running less, simply highlight and delete the samples that are not needed.

2. To begin the run, click the green “play” button.

The Gilson 215 SPE system processes 4 samples simultaneously. All steps of the method, such as cartridge conditioning, sample loading, cartridge washing, sample elution, and collection, are performed on one row before moving to the next row. After starting the run, this process is performed automatically until all designated samples have been completed.

d) Description of the SPE Method

1. Rinse Needle: inside volume – 2 mL of reservoir solution
outside volume – 3 mL of reservoir solution
2. Condition Cartridge: 2 mL of methanol
3. Condition Cartridge: 2 mL of water
4. Dispense: 1 mL of water to each sample
5. Mix: syringe draws up 2.2 mL of sample and dispenses (2 cycles)
6. Load Cartridge: contents of the sample vial (2.4 mL) are loaded onto the cartridge
7. Wash Cartridge: 1 mL of water
8. Wash Cartridge: 2 mL of reservoir solution
9. Elution: 3 mL of methanol
10. Rinse Needle: inside volume – 1 mL of water
outside volume – 2 mL of water

3) Sample Preparation for LC/MS/MS Analysis

- a) 2 mL HPLC vials are placed in the autosampler rack according to the number of samples to be run (1 vial per sample); place a 300 μ L glass insert in each vial. Include an extra HPLC vial (no insert) for use as a wash vial.
- b) One set of the nine mixed working analytical standard solutions (preparation is described in section 6.b. of this document) are removed from the freezer (-80°C) and allowed to come to room temperature.
- c) 50 μ L of mixed working internal standard solution is then added to each working analytical standard solution. The contents are vortexed thoroughly and transferred to a labeled 12x75mm glass culture tube.
- d) The 9 calibrators (S1-S9) and the QCs and unknown samples obtained from SPE are placed in a Thermo Savant SPD2010-220 SpeedVac for approximately 2.5 hours at 55°C to allow the solvent to evaporate.
- e) 100 μ L of reconstitution solution (prepared as described in section 6.a. of this document) is added to each sample that is vortexed thoroughly.
- f) The contents of each glass tube are then transferred to the inserts within the HPLC vials and each vial is capped. Each vial must be visually checked to make sure there is no air bubble in the bottom of the glass insert. If an air bubble exists, simply vortex the vial for a few seconds or tap the bottom of the vial to remove bubble. The wash vial must also be filled with 100% methanol. The sample set is then ready for analysis.

b. Instrument Preparation

1) HPLC Preparation

- a) Fill all solvent bottles: aqueous HPLC mobile phase (line A1) should be made fresh daily; organic HPLC mobile phase (line B1) should be refilled daily; HPLC-grade water (line A2) and HPLC-grade acetonitrile (line B2) should be checked daily and refilled as needed. Check the waste bottle to ensure that it will not overflow during the run.
- b) Phenomenex C18 (ODS) guard column, 4mm L x 2.0mm ID, should be replaced as needed, but no less than every 3 runs (assuming a full set of samples is being run each time).
- c) Before each run, review the chromatographic spectra of the previous runs' calibrators to ensure that the ThermoElectron Betasil C18 analytical column (50x3mm, 5µm) is in suitable condition (i.e. no double peaking, peak trailing, broad peaks, etc.). Replace the analytical column as needed.

2) Mass Spectrometer Preparation

- a) Check the interface and turbo ionspray probe before each run to make sure the needle height, probe height, and probe width settings are correct.
- b) Clean the interface as needed by removing the interface housing (caution, if the instrument is in ready mode the housing will be very hot) and curtain plate, followed by wiping the curtain plate and orifice plate with water and then methanol.

3) Run Samples on the LC/MS/MS

- a) Open Analyst software using the icon on the desktop of the instrument PC.
- b) Select the project (such as "Phytoestrogens 2008" for all samples ran in the year 2008) at the top of the screen.
- c) Double-click "Hardware Configuration" on the left side of the screen and make sure that the LCMS LC 1200 profile is activated (it will have a green check mark beside it). If it is not, click the activate profile button.
- d) Build the batch file

A quick and easy way to build a batch (especially for NHANES runs) is to open an existing batch and change the necessary information (i.e. Sample Name and ID, Data file name, Comments, etc.). Otherwise, use the following procedure.

1. Double-click "Build Acquisition Batch"
2. Specify a Set Name
3. Click Add Set, then Add Samples
4. Specify the Sample Name and number of samples to be run and click okay
5. Using the pull down tabs, select a quantitation method as well as an acquisition method (see SOP for Instrument Preparation in the Phytoestrogens QA book for specific method names and information) and check the multiple methods box if planning on utilizing more than one method in the same batch (see Table 3 for gradient information, Table 5 for example MS parameters).

6. Input all necessary information for each sample, including Sample Name, Sample ID, Data File (the typical data file is named (mmddy)), Comments, rack code, rack position, plate code, plate position, and if running more than one method in the batch, select a method for each sample. For any non-research run where data will be reported, e.g. NHANES runs, make sure that all calibrators, QC's, and patient samples are run with the same acquisition method. A blank should be run at the end of each sample set using the "Shutdown_Phytoestrogens" (see Table 4 for gradient information) acquisition method. The purpose of the shutdown method is to flush any residual components from the analytical column and prepare it for potential long term storage.
 7. Click the Locations tab at the top of the window and choose which type of autosampler is being used.
 8. Double-click inside the appropriate plate box (plate 1 or plate 2) and begin numbering each vial according to the order in which it is to be injected by left-clicking inside each circle (you can switch between the plates by using the Autosampler View/Plate View button). When selected, each sample should turn red and the number inside the circle denotes the order that the samples will be run. To inject a sample more than one time, push the control key on the keyboard and click the sample that is to be repeated (it will turn green, and the number will disappear). To verify the order of each sample injection, click the sample tab and look at the Sample Vial column. It is a good idea at this point to verify that the order of the samples in Analyst matches the order of the samples in the autosampler tray.
 9. Click the Quantitation tab at the top of the window and select a type for each sample (i.e. unknown, standard, QC, blank, etc.). For each calibrator (standard), enter a value for the concentration of each analyte in units of ng/mL. Also, specify a dilution factor if necessary (default value is 1).
 10. Once the batch file is finished, be sure and save it. The typical filename for each batch is "Phyto mmddy".
- e) Submit the batch by clicking the Submit tab at the top of the batch window, verify that all sample information is correct, and click the submit button.
 - f) Open the Queue window by clicking the View Queue button.
 - g) Click on the Equilibrate icon.
 - h) Select the appropriate sample acquisition method and enter an equilibration time of 60 minutes (recommended, but can be less if need be).
 - i) Make sure the line coming from the analytical column of the HPLC is attached to the interface housing of the mass spectrometer.
 - j) Click Okay to begin equilibrating the column.
 - k) After the system has equilibrated for 60 minutes, make sure the system is in Ready mode and click the Start Sample button to begin sample acquisition.

c. Quantification and Data Review

- 1) Using Analyst software, generate a Results file from the raw data.

- a) Double-click "Quantitation Wizard" shown on the left side of the screen.
 - b) Select the data file corresponding to the run in which you are generating results for.
 - c) Highlight which samples are to be included in the Results file and click the right arrow button. This will move them to the box on the right for Selected Samples.
 - d) Click Next
 - e) Using the pull-down tab, select Summary. Also select None under Default Query
 - f) Click Next
 - g) Using the pull-down tab, select the quantitation method that corresponds to the sample acquisition method used for the run.
 - h) Click Finish
 - i) Analyst will generate a results file based on the data selected and will automatically select the desired peak and perform the integration according to the parameters of the quantitation method.
 - j) Click File, then Save As to save the Results file. It is typically given the same name as the corresponding Data file ("mmddy").
- 2) Data Review
- a) For each sample, there are a total of 19 mass transitions monitored by the mass spectrometer, thus a total of 19 peaks generated per injection (6 analytes – 2 mass transitions per analyte, 6 internal standard transitions, and 1 deconjugation internal standard transition).
 - b) The integration should be verified by clicking the Peak Review button.
 - c) Right-click in the Peak Review window, choose Options and select the desired number of peaks per row/column, how to view each peak (i.e. 100% of the peak, 5X the baseline, etc.), and select the box to zoom to a 2 minute time window.
 - d) To perform manual integration, click Manual Integration Mode.
 - e) After checking all analyte and internal standard peaks for all calibrators, QC's, and patient samples, close the Peak Review window.
 - f) Click the Calibration button and, using the pull-down tab, select 1/x weighting for each calibration curve (if it was not originally designated when the quantitation method was generated).
 - g) Use the following criteria for selecting points to include/exclude from the calibration curves:
 1. Equol Q1 : S1 - S9
 2. Daidzein Q1: S1 – S7
 3. Daidzein Q2: S6 – S9
 4. O-DMA Q1: S1 – S7
 5. O-DMA Q2: S6 - S9
 6. Genistein Q1: S1 – S7
 7. Genistein Q2: S6 – S9
 8. Enterolactone Q1: S1 – S7
 9. Enterolactone Q2: S6 – S9

10. Enterodiol Q1: S1 – S9

- h) Close the Calibration window and save the Results data.
- 3) Importing into the Access database
- a) With a Results file open in Analyst, right-click and select “Full”. This shows a full layout of all data (all transitions for each sample) for a given run, not just for one analyte. NOTE: This is necessary because if only one transition is visible, e.g. Equol Q1, then only data for that analyte will be exported, not the full data set.
 - b) Click File, then Export
 - c) Using the “Save in” pull-down tab, select the location in which to save the exported results, e.g. *Q:\VTN\Nutrition Lab\Phytoestrogens\Data files for import*.
 - d) Using the “Save as type” pull-down tab, select Text Files (*.txt)
 - e) Name the file to be exported the same as the Results file (“mmddy”).
 - f) Click “All Columns” to export all Results data (including hidden columns), or select “Visible Columns” to export only the Results data that is visible in the Results file.
 - g) Click Save
 - h) The text file is then imported into the Access database for statistical analysis.

d. System Maintenance

- 1) Gilson 215 SPE – Preventative maintenance is performed annually by the service engineer.
- 2) Agilent 1200 HPLC – Preventative Maintenance is performed annually by the service engineer.
- 3) Sciex API 4000 Mass Spectrometer – Preventative maintenance and tuning and calibration of the instrument is performed semi-annually by the service engineer.

e. CDC Modifications

This method is based on the procedure described by Valentin-Blasini et al⁸.

9. Reportable Range of Results

The reportable range of results for each of the six phytoestrogens is as follows:

Equol:	0.3 – 100 ng/mL
Daidzein:	0.3 – 1,600 ng/mL
O-DMA:	0.2 – 300 ng/mL
Genistein:	0.3 – 730 ng/mL
Enterolactone:	0.3 – 3,300 ng/mL
Enterodiol:	0.3 – 320 ng/mL

Samples with concentrations exceeding the highest calibrator are diluted, re-extracted, and reanalyzed so that the measured value is within the range of the calibration.

10. Quality Control (QC) Procedures

a. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Inorganic Toxicology and Nutrition Branch. These specimens are prepared at two levels so as to emulate the patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

b. Bench Quality Controls

Bench QC specimens are prepared from three urine pools that represent low, medium and high levels of phytoestrogens. Samples from these pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The results from the pools are checked after each run. The system is declared "in control" if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits, apply the rules below and reject if any condition is met - the run is then declared "out of control":

1. 1_{3s} Any of the three QC results are outside the 3s limit.
2. 2_{2s} Two of the three QC results in the run are outside the 2s limit (same side of mean).
3. R_{4s} Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean.
4. 10_x Ten sequential QC results (across pools and across runs) are on the same side of the mean.

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in the ACCESS database. For runs that are not imported into ACCESS (exception, research-type runs), QC results are stored electronically in the analyte-specific folder on Q:\ITN\Nutrition Lab\Data handling\QC results in Excel. A hardcopy of the QC results from each run is also kept by the analyst.

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

1. Check to make sure that the hardware is functioning properly. Make sure the Mass spectrometer calibrations are proper. Run PPG's in Q1 Scan to check the instrument calibration.
2. Run standards in Q1 Scan to see if molecular ion is detected.
3. Check the proper gas flow for curtain, exhaust, and source from the nitrogen generator.

4. Check the autosampler for proper sample injections.
5. Look for sample preparation errors, i.e., if the analyst forgot to add internal standard, specimen, etc.
6. Check the calibrations of the pipettes.
7. If the steps outlined above do not result in correction of the “out of control” values for QC materials, consult the supervisor for other appropriate corrective actions.
8. Do not report analytical results for runs not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

The most common causes of imprecision are intermittently inaccurate micropipettors and pipetting errors.

Buffers should be made fresh monthly and pH should be checked.

Occasionally, the concentration of phytoestrogens in urine is much higher than the highest standard in the calibration curves, so 500- μ L of urine may be too much to use. In this case, a smaller aliquot of urine can be used as described earlier.

13. Reference Ranges (Normal Values)

Refer to Table 6.

14. Critical Call Results (“Panic Values”)

There is currently insufficient data 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 the refrigerator to expedite thawing prior to aliquotting. Samples should be allowed to warm to and be maintained at room temperature during preparation and testing.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

There are no acceptable alternative methods for the analysis of phytoestrogens in the Nutrition laboratory. If the analytical system fails, we recommend that the specimens or prepared samples be stored at -80°C until the analytical system is restored to functionality.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Test results are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically on a periodic basis to the Westat ISIS computer and then are transferred to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

The Microsoft Access database is used to keep records and track specimens for NHANES 1999+. If urine analyses are used for smaller, non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab on the DLS LAN.

We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual urine from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

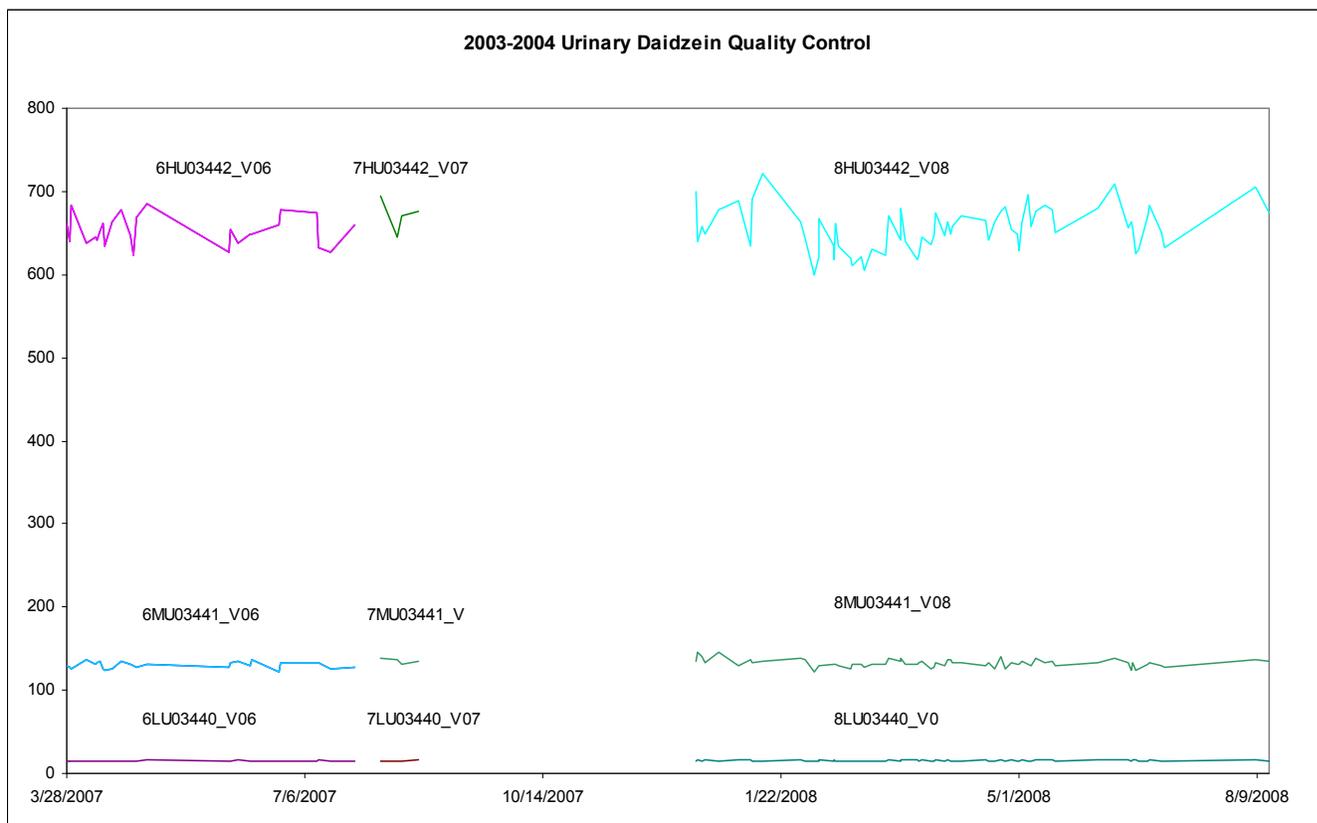
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -80°C. The specimen ID is read off of the vial by a barcode reader attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

19, Summary Statistics and QC Graphs

a. Daidzein

Summary Statistics for Urinary Daidzein by Lot

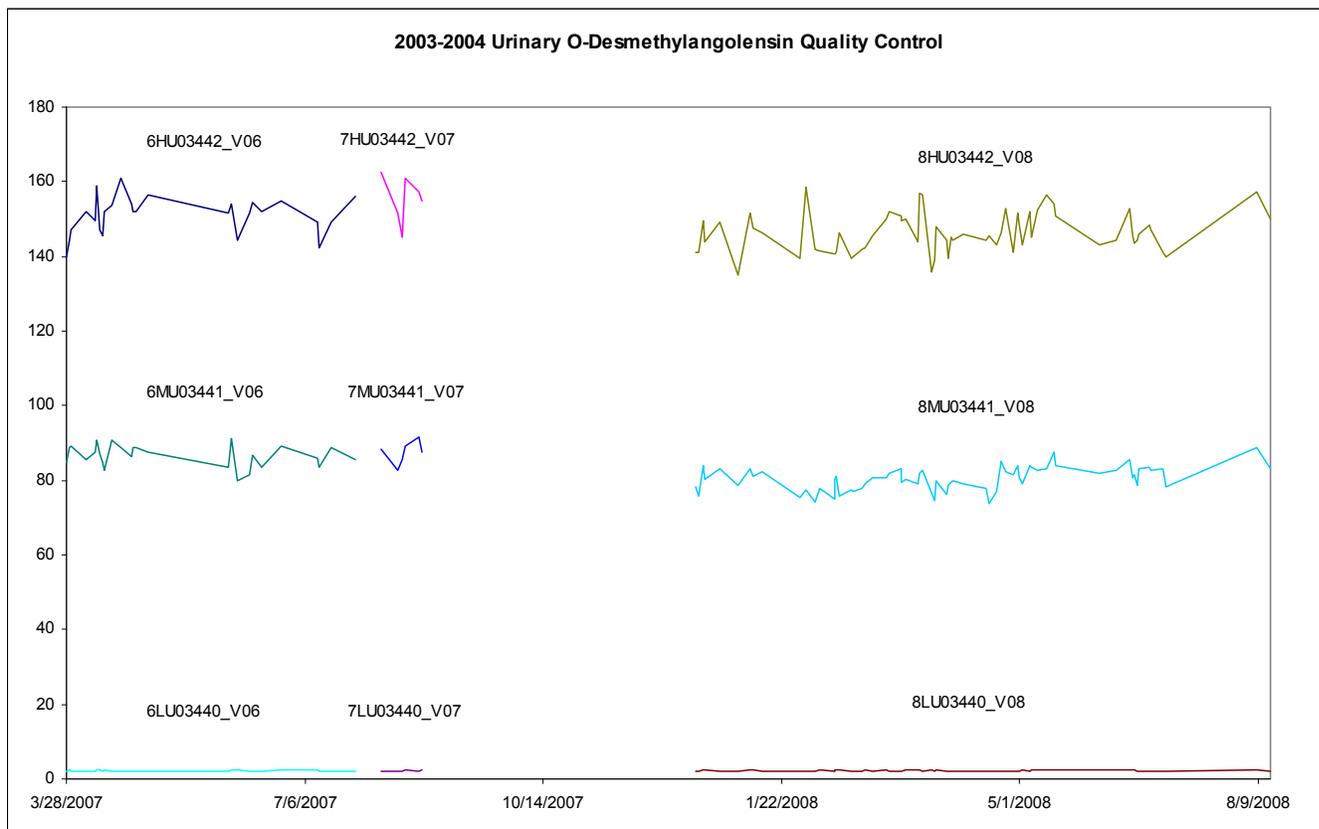
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
6LU03440_V06	26	3/28/2007	7/27/2007	15.000	0.456	3.0
6MU03441_V06	26	3/28/2007	7/27/2007	130.212	3.948	3.0
6HU03442_V06	26	3/28/2007	7/27/2007	652.615	18.004	2.8
7LU03440_V07	4	8/7/2007	8/23/2007	15.263	0.446	2.9
7MU03441_V07	4	8/7/2007	8/23/2007	135.250	2.986	2.2
7HU03442_V07	4	8/7/2007	8/23/2007	672.000	20.240	3.0
8LU03440_V08	67	12/17/2007	8/14/2008	15.488	0.555	3.6
8MU03441_V08	67	12/17/2007	8/14/2008	132.352	4.656	3.5
8HU03442_V08	67	12/17/2007	8/14/2008	654.771	26.277	4.0



b *o*-Desmethylangolensin (O-DMA)

Summary Statistics for Urinary Desmethylangolensin by Lot

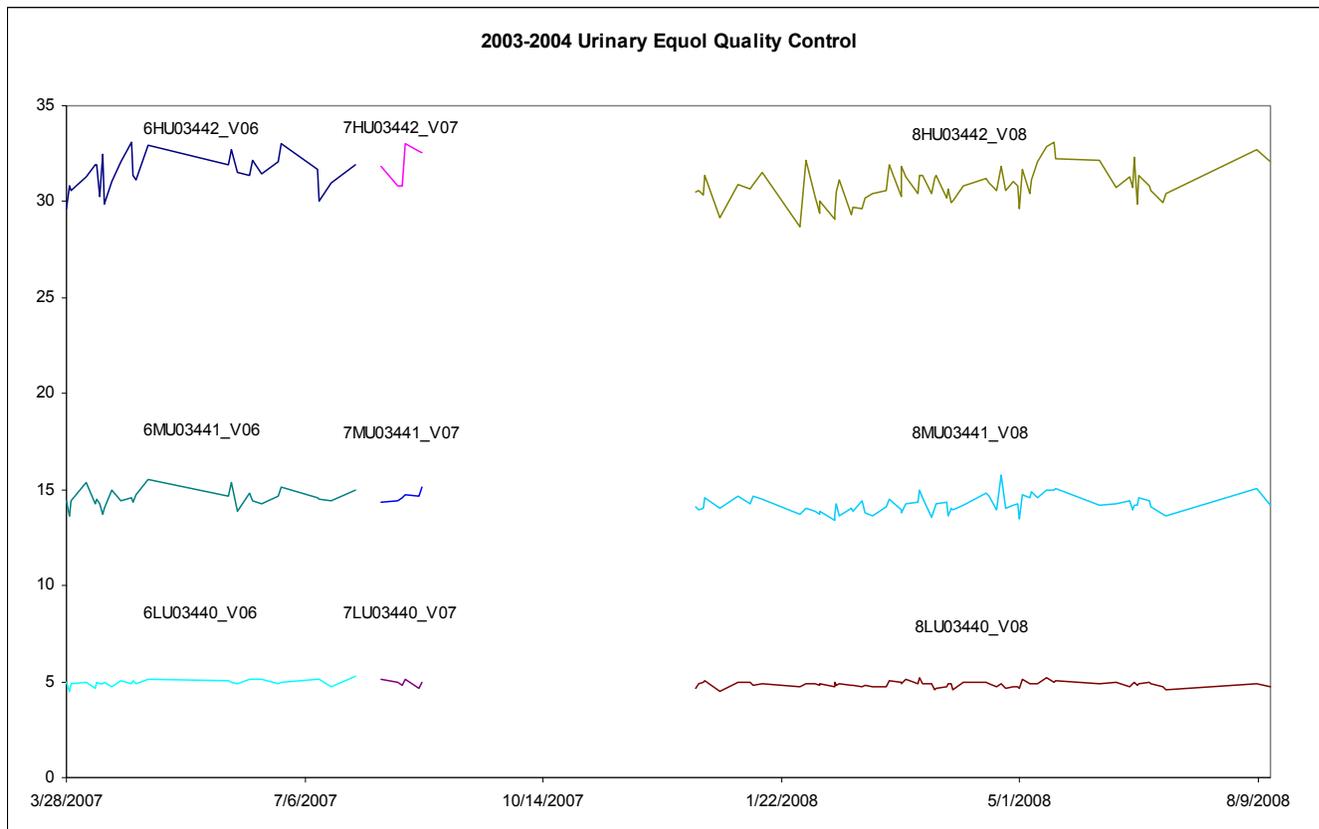
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
6LU03440_V06	26	3/28/2007	7/27/2007	2.210	0.072	3.3
6MU03441_V06	26	3/28/2007	7/27/2007	86.535	2.923	3.4
6HU03442_V06	26	3/28/2007	7/27/2007	150.962	5.010	3.3
7LU03440_V07	6	8/7/2007	8/24/2007	2.233	0.061	2.7
7MU03441_V07	6	8/7/2007	8/24/2007	87.458	3.127	3.6
7HU03442_V07	6	8/7/2007	8/24/2007	155.417	6.476	4.2
8LU03440_V08	66	12/17/2007	8/14/2008	2.188	0.086	3.9
8MU03441_V08	66	12/17/2007	8/14/2008	80.465	3.180	4.0
8HU03442_V08	66	12/17/2007	8/14/2008	146.189	5.393	3.7



c. Equol

Summary Statistics for Urinary Equol by Lot

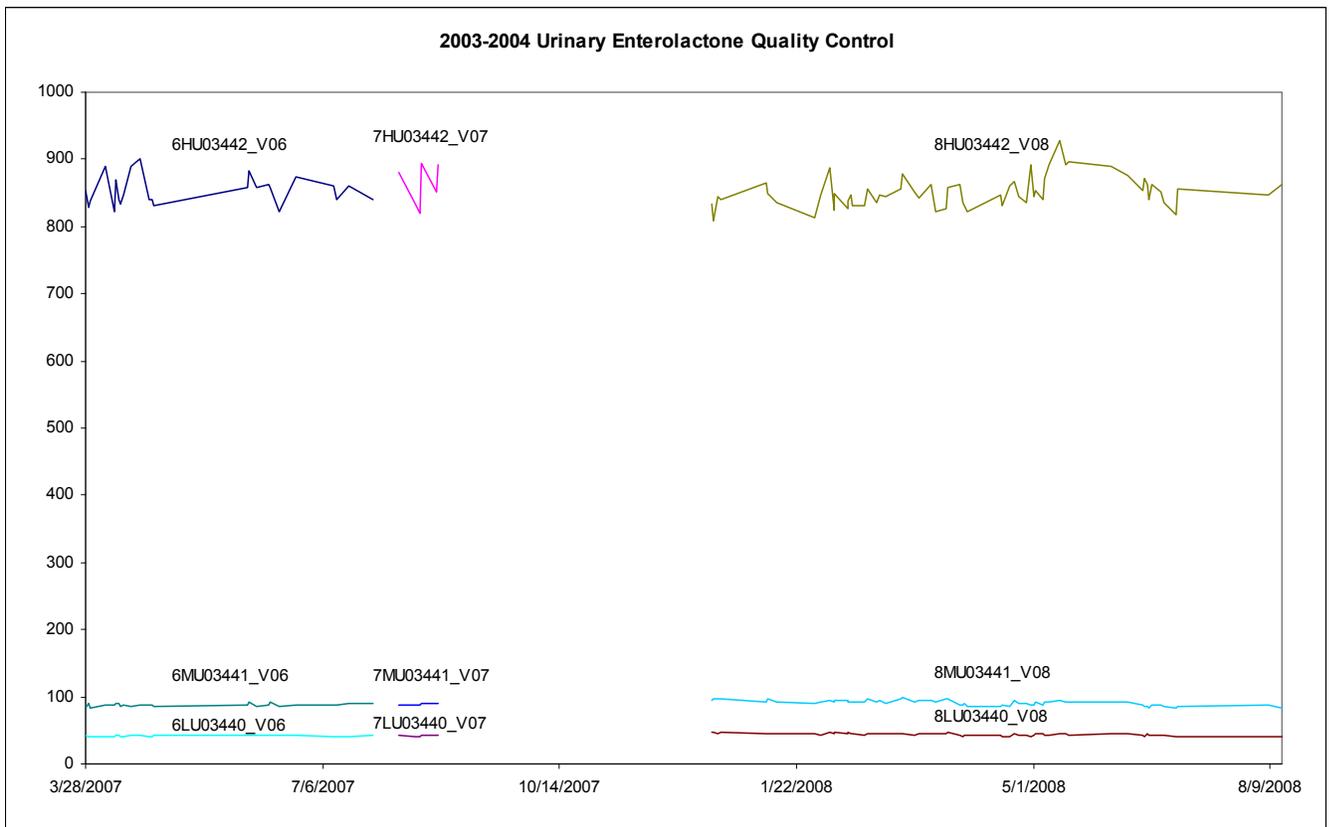
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
6LU03440_V06	27	3/28/2007	7/27/2007	4.952	0.168	3.4
6MU03441_V06	27	3/28/2007	7/27/2007	14.565	0.469	3.2
6HU03442_V06	27	3/28/2007	7/27/2007	31.535	0.934	3.0
7LU03440_V07	6	8/7/2007	8/24/2007	4.942	0.188	3.8
7MU03441_V07	6	8/7/2007	8/24/2007	14.650	0.283	1.9
7HU03442_V07	6	8/7/2007	8/24/2007	31.950	0.948	3.0
8LU03440_V08	67	12/17/2007	8/14/2008	4.849	0.146	3.0
8MU03441_V08	67	12/17/2007	8/14/2008	14.227	0.453	3.2
8HU03442_V08	67	12/17/2007	8/14/2008	30.823	0.944	3.1



d. Enterodiol

Summary Statistics for Urinary Enterodiol by Lot

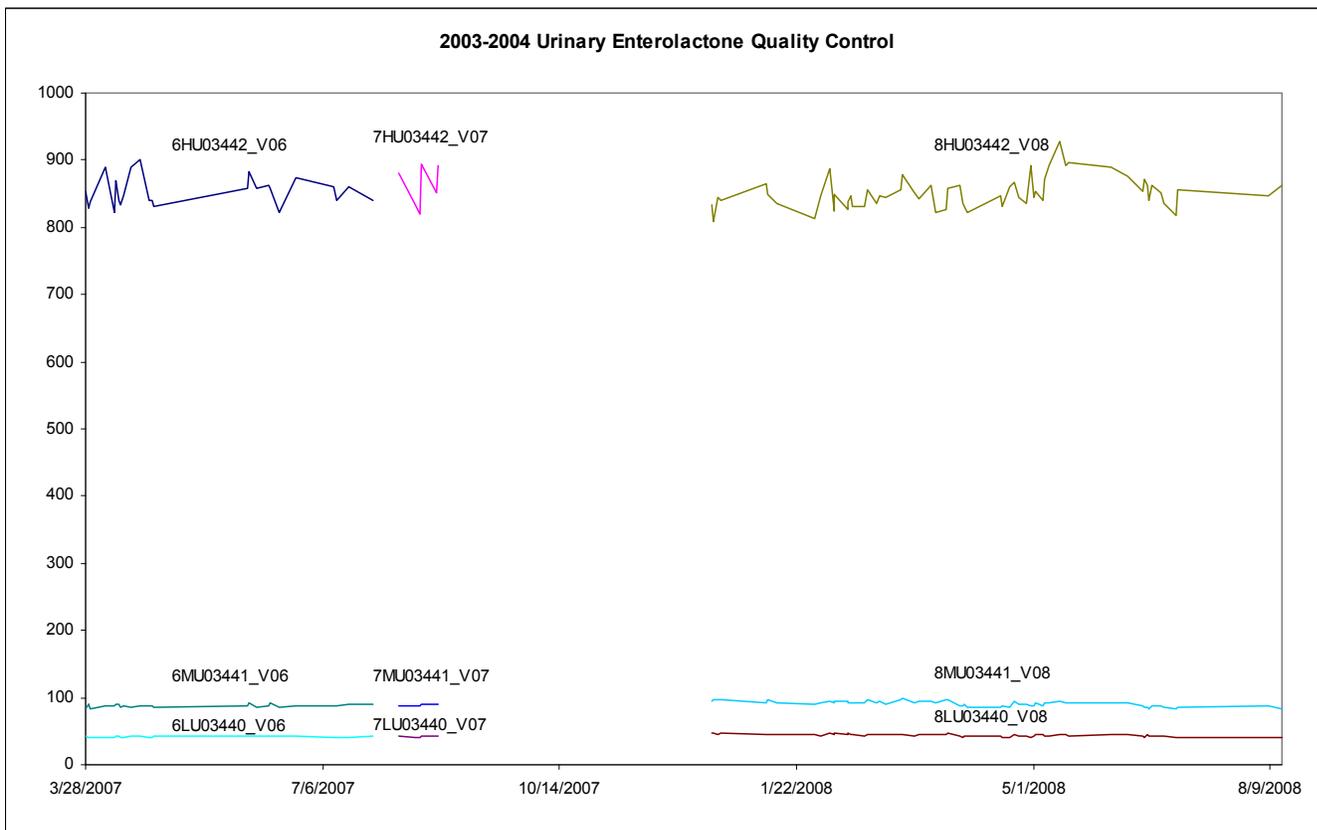
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
6LU03440_V06	25	3/28/2007	7/27/2007	17.974	0.509	2.8
6MU03441_V06	25	3/28/2007	7/27/2007	53.836	1.223	2.3
6HU03442_V06	25	3/28/2007	7/27/2007	61.602	1.781	2.9
7LU03440_V07	6	8/7/2007	8/24/2007	18.225	0.698	3.8
7MU03441_V07	6	8/7/2007	8/24/2007	53.983	0.642	1.2
7HU03442_V07	6	8/7/2007	8/24/2007	62.208	1.933	3.1
8LU03440_V08	67	12/17/2007	8/14/2008	18.088	0.517	2.9
8MU03441_V08	67	12/17/2007	8/14/2008	55.248	1.519	2.7
8HU03442_V08	67	12/17/2007	8/14/2008	61.755	1.759	2.8



e. Enterolactone

Summary Statistics for Urinary Enterolactone by Lot

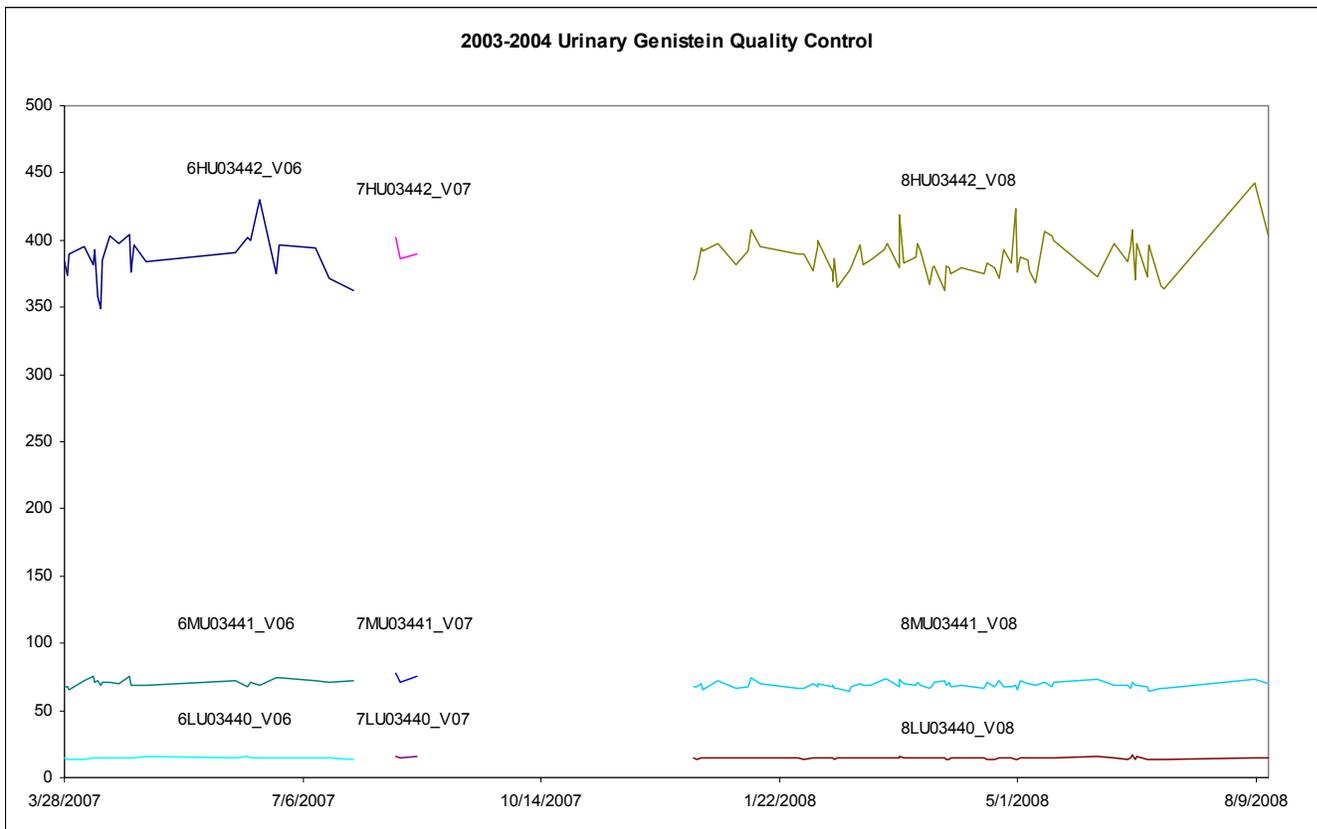
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
6LU03440_V06	25	3/28/2007	7/27/2007	41.842	1.095	2.6
6MU03441_V06	25	3/28/2007	7/27/2007	87.852	2.277	2.6
6HU03442_V06	25	3/28/2007	7/27/2007	853.520	21.667	2.5
7LU03440_V07	6	8/7/2007	8/24/2007	42.025	1.162	2.8
7MU03441_V07	6	8/7/2007	8/24/2007	88.892	1.463	1.6
7HU03442_V07	6	8/7/2007	8/24/2007	861.667	31.830	3.7
8LU03440_V08	61	12/17/2007	8/14/2008	43.966	1.936	4.4
8MU03441_V08	61	12/17/2007	8/14/2008	91.324	3.769	4.1
8HU03442_V08	61	12/17/2007	8/14/2008	851.072	23.032	2.7



f. Genistein

Summary Statistics for Urinary Genistein by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
6LU03440_V06	24	3/28/2007	7/27/2007	14.556	0.545	3.7
6MU03441_V06	24	3/28/2007	7/27/2007	70.538	2.553	3.6
6HU03442_V06	24	3/28/2007	7/27/2007	387.229	17.117	4.4
7LU03440_V07	3	8/14/2007	8/23/2007	15.283	0.475	3.1
7MU03441_V07	3	8/14/2007	8/23/2007	74.617	3.347	4.5
7HU03442_V07	3	8/14/2007	8/23/2007	392.833	8.129	2.1
8LU03440_V08	67	12/17/2007	8/14/2008	14.482	0.605	4.2
8MU03441_V08	67	12/17/2007	8/14/2008	68.947	2.267	3.3
8HU03442_V08	67	12/17/2007	8/14/2008	386.682	14.789	3.8



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Table 1. Preparation of mixed working standard solutions for analytes and internal standards *

Analyte	Analytical Standards									Internal Standard	
	S1	S2	S3	S4	S5	S6	S7	S8	S9	² H	¹³ C
Equol	0.3	1	4	6	8	14	18	60	100	96	48
Daidzein	1	10	40	55	70	100	125	1000	1600	75	35
O-DMA	0.4	1	3	4	5	14	20	110	300	5	5
Genistein	0.4	2	15	22	27	55	75	325	730	25	25
Enterolactone	2	10	150	250	315	550	750	2100	3300	96	34
Enterodiol	0.3	2	25	30	34	55	75	175	320	27	27

Table 2. Target values for quality control specimens

Analyte	Concentration (ng/mL)		
	Low	Medium	High
Equol	10	20	40
Daidzein	15	70	1000
O-DMA	5	25	200
Genistein	5	30	500
Enterolactone	25	300	2500
Enterodiol	5	35	200

Table 3. Solvent gradient for phytoestrogens LC-MS/MS sample acquisition method

Solvent	Time (min)						
	0	2	4	5	7	7.5	9.0
B: ACN:MeOH (1:1)	35%	35%	50%	55%	95%	35%	35%
A: 10 mM ammonium acetate, pH 6.5	65%	65%	50%	45%	5%	65%	65%

Table 4. Solvent gradient for phytoestrogens LC-MS/MS shutdown method

Solvent	Time (min)			
	0	10	11	20
B: ACN	20%	20%	95%	95%
A: Water	80%	80%	5%	5%

* Table specifies amount (ng) in 100 µL Spike of Mixed Working Analytical Standard Solutions and 50 µL Spike of deuterated and carbon-13 labeled Mixed Working Internal Standard Solutions

Table 5. Mass spectrometric parameters

The values below are an example of mass spectrometric parameters based on previous acquisition methods. These values are only provided as an example; actual values used should be determined by optimizing the instrument as suggested in the API 4000 instrument manual.

Analyte	Signal	MS/MS transition (m/z)		Dwell Time (ms)	MS/MS potentials (V)			
		Molecular Ion	Product Ion		DP	EP	CE	CXP
Equol	Q1	241.1	119.1	50	-100	-10	-30	-10
	Q2	241.1	135.0	50	-40	-8	-30	-10
	² H IS	245.0	123.0	50	-70	-10	-24	-5
	¹³ C IS	243.9	123.1	50	-90	-10	-18	-7
Daidzein	Q1	253.1	223.1	50	-110	-10	-47	-15
	Q2	253.1	117.1	50	-110	-10	-47	-15
	² H IS	256.0	226.0	50	-110	-10	-47	-15
	¹³ C IS	255.9	134.0	50	-95	-10	-54	-7
O-DMA	Q1	257.1	108.1	50	-25	-6	-40	-5
	Q2	257.1	148.0	50	-25	-6	-40	-5
	² H IS	261.0	111.0	50	-70	-10	-33	-7
	¹³ C IS	260.0	136.8	50	-50	-10	-32	-7
Genistein	Q1	269.1	133.0	50	-80	-10	-45	-13
	Q2	269.1	224.1	50	-80	-10	-40	-13
	² H IS	273.0	137.0	50	-70	-10	-47	-7
	¹³ C IS	271.8	63.0	50	-100	-10	-62	-9
Enterolactone	Q1	297.3	107.1	50	-50	-11	-45	-7
	Q2	297.3	93.0	50	-50	-11	-45	-7
	² H IS	303.0	110.0	50	-70	-10	-60	-15
	¹³ C IS	299.9	108.3	50	-75	-10	-36	-9
Enterodiol	Q1	301.3	253.2	50	-10	-9	-35	-6
	Q2	301.3	241.1	50	-10	-9	-35	-6
	² H IS	309.0	261.0	50	-70	-10	-32	-7
	¹³ C IS	304.0	255.7	50	-75	-10	-32	-5
Umbelliferone		174.7	133.0	50	-46	-3	-35	-20

Table 6. Urinary phytoestrogen levels from the U.S. population

Phytoestrogen	Geometric Mean	Urinary concentration (µg/L)						Sample Size
		10 th	25 th	50 th	75 th	90 th	95 th	
Daidzein [†]	75.1	11.1	24.4	69.7	229	538	1310	2554
Enterodiol [†]	26.6	2.94	11.8	34.0	78.7	165	266	2527
Enterolactone [†]	239	25.4	107	315	726	1970	2790	2548
Equol [†]	8.37	< LOD	< LOD	8.02	17.2	35.0	53.5	2182
Genistein [†]	24.4	1.91	8.67	27.0	93.6	284	562	2557
Matairesinol ^{‡§}	6.6		3.2	8.6	18			199
O-DMA [†]	4.34	< LOD	.750	4.88	22.7	100	217	2295

[†] Representative sample of the U.S. population aged 6 years and older from the National Health and Nutrition Examination Survey, 1999-2000 (10)

[‡] Non-representative multiethnic subset of adults aged 20 to 58 from the Third National Health and Nutrition Examination Survey.

[§] Geometric mean expressed in µg/g creatinine.