



Laboratory Procedure Manual

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

Matrix: **Urine**

Method: **HPLC-APPI-MS/MS**

Method No: 4066.03

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.

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) |
|------------|---------|---------------------------------------|
| PHYTO_E | URXEQU | Equol (ng/mL) |
| | URXETD | Enterodiol (ng/mL) |
| | URXETL | Enterolactone (ng/mL) |
| | URXGNS | Genistein (ng/mL) |
| | URXDAZ | Daidzein (ng/mL) |
| | URXDMA | o-Desmethylangolensin (O-DMA) (ng/mL) |

1. Summary of Test Principle and Clinical Relevance

a. Clinical relevance

Phytoestrogens are plant-derived polyphenolic compounds, such as isoflavones, lignans, coumestans and stilbenes that bear structural similarities to endogenous estrogens and are capable of estrogen-receptor binding. Their endocrine activity, as well as their potential influence on other biologic pathways, has led to considerable interest in phytoestrogens from an epidemiological standpoint. The consumption of diets high in phytoestrogen-rich foods has been associated with lower rates of such hormone-dependent cancers as breast and prostate cancer, with improved bone health, with post-menopausal symptoms, and with cardiovascular disease. Whether phytoestrogens are indeed the active components responsible for these benefits, however, has come under scrutiny, and the significance of their purported health benefits has been challenged. Individual studies and meta analyses have often resulted in apparently conflicting findings, such as whether phytoestrogens do or do not significantly reduce the frequency and intensity of menopausal hot flashes. Potential toxic effects associated with phytoestrogen exposure have also been identified. 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-atmospheric pressure photoionization-tandem mass spectrometry (HPLC-APPI-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 size-exclusion filtration. Phytoestrogens are then separated from other urine components by reversed phase HPLC, detected by APPI-MS/MS, and quantified by isotope dilution. Assay precision is improved by incorporating carbon-13 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. Computerization; Data System Management

- a. Mass spectrometry data are collected and stored using the Analyst Software of the API 4000 (AB Sciex) 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 FrontEnds:

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

Step 2 – Analyst – Review run in FrontEnds:

- [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 FrontEnds database as described below.

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

- Double click the FrontEnds 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 Team leader (for Blind QC review) specifying Bench QC status of the run.

Step 5 – Team leader – Review Blind QC and other parameters and set results ready in FrontEnds:

- Double click the FrontEnds 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, that final values have been set correctly and that repeat results are as expected
- [Set Ready] – Final results will be set ready to be exported
- [Set Reviewed]
- Forward email from QA Officer to Supervisor specifying that results can be exported; include any relevant comments.

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

- Double click the FrontEnds icon on desktop, password entry required
- [Run Review] (under Batch & X-Batch) – Select assay, then desired run
- Perform final review
- [Set Reviewed]
- [Export/Report Results] (under Study Functions) – Select study, select analytes/panel, use selected panel
- [Generate Pre-Export Text File] – Review file on
- [Generate Export Text File and Set Results Exported] –FTP file to Westat
- Send Westat an email that file was transmitted
- Move transmittal file

For NHANES, data is transmitted electronically regularly 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.

Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up regularly onto a floppy disk or a CD-ROM using a CD writer.

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.

- 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.

3. 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 200 µL 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 Biomarkers Branch. 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.

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

Not applicable for this method.

5. 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 (2.5 M)**

For 500 mL, weigh 96.25 g of ammonium acetate into a 1 L beaker and dissolve in 100 mL of HPLC-grade water. While stirring, add approximately 132 mL of glacial acetic acid. Additional glacial acetic acid or NH_4OH can be added to adjust pH as needed. Transfer the solution to a 500 mL volumetric flask and fill to the mark using HPLC-grade water. Prepare every 3 months and store at 10°C or below.

(2) **β -Glucuronidase Solution**

120 units of enzyme are to be added to each urine sample. Accordingly, the β – glucuronidase powder enzyme should be prepared in HPLC-grade water at a concentration of 40 mg/mL, and allowed to dissolve (this process could take several minutes). 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.

(3) **HPLC Mobile Phase (Aqueous)**

100% HPLC-grade water. Refill as needed.

(4) **HPLC Mobile Phase (Organic)**

100% HPLC-grade methanol. Refill as needed.

(5) **Dopant Solvent**

100% HPLC-grade toluene. Refill as needed.

(6) **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 ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) |
| 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 Appendix 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 Solution

A mixed working internal standard solution containing the appropriate concentration of each compound (see Appendix 1) is prepared by pipetting the following amounts of each internal standard stock solution into a volumetric flask of appropriate size:

| | |
|------------------------------------------|----------|
| $^{13}\text{C}_3$ -Equol | 15.79 mL |
| $^{13}\text{C}_3$ -Daidzein | 19.89 mL |
| $^{13}\text{C}_3$ -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 .

5) Deconjugation Internal Standard Solution

4-methylumbelliferyl glucuronide and 4-methylumbelliferyl sulfate are used as deconjugation standards to qualitatively determine 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 below the 25th percentile for each analyte based on currently available reference data (e.g., The Second National Report on Human Exposure to Environmental Chemicals or The National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population). Urine selected for the medium pool contains levels of phytoestrogens ranging from the 25th to 75th percentile for each analyte. Urine selected for the high pool has target levels of phytoestrogens above the 75th percentile (see Appendix 2 for target quality control values; see Appendix 6 for U.S. population percentiles).

Urine (1.8 mL) is aliquoted into 2.0-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) Kinetex C18 analytical column, 50x2.1mm, 2.6µm (Phenomenex, Torrance, CA)
- 2) Krud Katcher Ultra in-line filter, 0.5µm x 0.004" ID (Phenomenex, Torrance, CA)
- 3) 12x75mm disposable glass culture tubes (Corning Glassworks, Corning, NY)
- 4) 9" Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)
- 5) Nunc 1 mL deepwell 96-well plates (Nalge Nunc International, Rochester, NY)
- 6) 96-well pre-slit silicon plate seals, 8.6mm, (Thermo-Fisher Scientific, Fair Lawn, NJ)
- 7) 96-well filter plates, Ultracell 10, regenerated cellulose, 10K MWCO (Millipore, Billerica, MA)
- 8) High Five nitrile examination gloves (High Five Products Inc., Chicago, IL)
- 9) N-Dex nitrile examination gloves (Best Manufacturing Corporation, Menlo, GA)
- 10) Blue tips (50-1000 µL) for Eppendorf pipette (Brinkmann Instruments Inc., Westbury, NY)
- 11) Yellow tips (2-200 µL) for Eppendorf pipettes (Brinkmann Instruments Inc., Westbury, NY)
- 12) Combitip plus (500 µL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)
- 13) Combitip plus (1.0 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)
- 14) Combitip plus (2.5 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)
- 15) 2.0 mL Polypropylene cryovials (Nalgene Company, Rochester, NY)
- 16) 1.5 mL micro centrifuge tubes (VWR, Suwanee, GA)
- 17) 15 mL BD Falcon Tubes (Becton Dickinson, Franklin Lakes, NJ)
- 18) 50 mL BD Falcon Tubes (Becton Dickinson, Franklin Lakes, NJ)
- 19) Various glass beakers, volumetric flasks, graduated cylinders, and bottles, class A glassware.
- 20) Methanol, HPLC grade (Tedia Company, Fairfield, OH)
- 21) Acetonitrile, HPLC grade (Tedia Company, Fairfield, OH)
- 22) Toluene, HPLC grade (Thermo-Fisher Scientific, Fair Lawn, NJ)
- 23) Ethanol, HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI)
- 24) Dimethylsulfoxide, HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI)
- 25) Water, HPLC grade (Aqua Solutions, Jasper, GA)

- 26) Ammonium Hydroxide (28-30%, Thermo-Fisher Scientific, Fair Lawn, NJ)
- 27) Ammonium Acetate, HPLC grade (Sigma, St. Louis, MO)
- 28) Acetic acid, glacial, reagent grade (Sigma, St. Louis, MO)
- 29) 4-methylumbelliferyl β -D-glucuronide hydrate (Sigma, St. Louis, MO)
- 30) 4-methylumbelliferyl sulfate (Sigma, St. Louis, MO)
- 31) β -Glucuronidase (powder), type H-1 from *Helix pomatia* (Sigma, St. Louis, MO)
- 32) Enterolactone (Sigma, St. Louis, MO)
- 33) Enterodiol (Sigma, St. Louis, MO)
- 34) Equol (Sigma, St. Louis, MO)
- 35) Genistein (Indofine Chemical Company, Somerville, NJ)
- 36) Daidzein (Indofine Chemical Company, Somerville, NJ)
- 37) O-Desmethylangolensin (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 38) $^{13}\text{C}_3$ -Enterodiol, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 39) $^{13}\text{C}_3$ -Enterolactone, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 40) $^{13}\text{C}_3$ -Genistein, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 41) $^{13}\text{C}_3$ -Daidzein, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 42) $^{13}\text{C}_3$ -Equol, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- 43) $^{13}\text{C}_3$ -O-Desmethylangolensin, (Dr. Nigel Botting, University of St. Andrews, Scotland)

e. Instrumentation

- 1) Agilent 1200 SL HPLC system (Agilent, Santa Claire, CA)
 - a) Model G4208A – Control Module
 - b) Model G1379B – Degasser
 - c) Model G1310A – Isocratic Pump
 - d) Model G1312A – Binary Pump SL
 - e) Model G1367D – High Performance Autosampler SL+
 - f) Model G1330B – Autosampler Thermostat Module
 - g) Model G1316B – Thermostatted Column Compartment SL
 - h) Model G1315C – Diode Array Detector SL
- 2) API 4000 triple quadrupole mass spectrometer, atmospheric pressure photoionization source, with Analyst 1.4.2 software (AB Sciex, Foster City, CA)
- 3) API 5000 triple quadrupole mass spectrometer, atmospheric pressure photoionization source, with Analyst 1.4.2 software (AB Sciex, Foster City, CA)
- 4) 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 (Parker Balston, Haverhill, MA)
- 5) Hamilton Microlab Starlet liquid handler (Hamilton Company, Reno, NV)
- 6) Economy incubator, model 3EM (Precision, Winchester, VA)

- 7) Eppendorf pipette (100-1000 μ L, Brinkmann Instruments, Inc, Westbury, NY)
- 8) Eppendorf pipette (10-100 μ L, Brinkmann Instruments, Inc, Westbury, NY)
- 9) Eppendorf pipette (2-20 μ L, Brinkmann Instruments, Inc, Westbury, NY)
- 10) Eppendorf Repeater Plus pipettor, (Brinkmann Instruments, Inc, Westbury, NY)
- 11) Vortexer (VWR, Suwanee, GA)
- 12) Magnetic stirrer (Thermo-Fisher Scientific, Fair Lawn, NJ)
- 13) Pinnacle model 530 pH meter (Corning Inc., Corning, NY)
- 14) Sartorius analytical balance, model ME235S (Sartorius AG, Goettingen, Germany)
- 15) Mettler Toledo analytical balance, model AG104 (Mettler Instrument Corp., Hightstown, NJ)
- 16) Harvard syringe pump (Harvard apparatus, Inc, Holliston Massachusetts)

6. Calibration and Calibration Verification Procedures

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 quadratic 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, based on the mixed working analytical standard solutions and the mixed working internal standard solution described in section 6.b. of this document, are carried through the sample preparation procedure as described in section 8.a. of this document.

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 (API 4000/5000 mass spectrometer)

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 AB Sciex. 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 mass spectrometer 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 4000/5000)

Follow the instruments operators' manual for instructions on calibration with PPG's (API 4000/5000 LC-MS/MS Hardware manual, AB Sciex).

7. Procedure Operating Instructions; Calculations; Interpretation of Results

A typical run (written here in the order in which they are injected into the LC-MS/MS) consists of 5 column equilibration injections (urine samples randomly selected from the current sample set), injection of the double blank and the blank, 9 calibrators, 3 front QC's (low, medium, and high), 79 patient samples, 3 back QC's (low, medium, and high), and lastly, reinjection of 9 calibrators.

a. Sample Preparation

Remove the following materials from their existing locations and allow them to reach ambient temperature: from the -80°C freezer, remove necessary QC's, unknown samples, one set of nine calibrators (S1-S9), mixed working internal standard solution, and deconjugation internal standard solution (prepared as described in section 6.b. of this document); from the -20°C freezer, remove β -glucuronidase enzyme; and from the 4°C refrigerator, remove the pH 5.0 ammonium acetate buffer and synthetic urine.

- 1) Enzymatic Deconjugation (place all contents into one 96-well deepwell [1 mL] plate)
 - a) Prepare β -glucuronidase solution (described in section 6.a. of this document)
 - b) Label a 96-well deepwell plate as suggested in Appendix 5 (NOTE: it is imperative that this sample layout be used when performing sample aliquotting with the Hamilton; if done manually, layout can be varied).
 - c) Calibrator Preparation
 1. Add the following to each calibrator (S1-S9 in 1.5 mL microcentrifuge tubes).
 - a. 250 μ L of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)
 - b. 50 μ L of deconjugation internal standard solution, diluted 20x (1 part solution, 19 parts water)
 - c. 100 μ L pH 5.0 (2.5M) ammonium acetate buffer
 - d. 900 μ L synthetic urine
 - e. 50 μ L of β -glucuronidase solution (IMPORTANT – add enzyme last)
 2. Thoroughly mix each calibrator.
 3. Transfer 290 μ L of each calibrator (S1-S9) to the appropriate well, as labeled on the 96-well plate.
 4. Discard remaining calibrator solution in the 1.5 mL microcentrifuge tube.
 - d) Double Blank Preparation
 1. Add the following to the well labeled “double blank” on the 96-well plate.
 - a. 10 μ L of deconjugation internal standard solution, diluted 20x (1 part solution, 19 parts water)
 - b. 20 μ L pH 5.0 (2.5M) ammonium acetate buffer
 - c. 250 μ L synthetic urine (no urine or mixed working internal standard solution present in the double blank)
 - d. 10 μ L of β -glucuronidase solution (IMPORTANT – add enzyme last)

- e) Blank Preparation
1. Add the following to the well labeled "blank" on the 96-well plate.
 - a. 50 μ L of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)
 - b. 10 μ L of deconjugation internal standard solution, diluted 20x (1 part solution, 19 parts water)
 - c. 20 μ L pH 5.0 (2.5M) ammonium acetate buffer
 - d. 200 μ L synthetic urine (no urine present in the blank)
 - e. 10 μ L of β -glucuronidase solution (IMPORTANT – add enzyme last)
- f) Urine Sample Preparation (quality control materials and unknown samples)
1. Add the following to all wells labeled "QC" and "Unknowns" on the 96-well plate.
 - a. 50 μ L of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)
 - b. 10 μ L of deconjugation internal standard solution, diluted 20x (1 part solution, 19 parts water)
 - c. 20 μ L pH 5.0 (2.5M) ammonium acetate buffer
 - d. 200 μ L urine*
 - e. 10 μ L of β -glucuronidase solution (IMPORTANT – add enzyme last)

*Part d. of Urine Sample Preparation (aliquotting of urine) can be performed manually, as described above, or can be automated whereby urine aliquoting is performed by the Hamilton Microlab Starlet. If using the Hamilton, add all contents (parts a. through e.) in the order in which it is written above, substituting only part d. with the Hamilton – all other steps (parts a., b., c., and e.) must be added manually. For directions regarding the specific Hamilton method to use for part d., see method SOP for sample preparation.

- g) Place pre-slit silicone plate mat on 96-well plate and mix gently by hand (not vigorously and do not vortex as this may cause deactivation of the enzyme), making sure that all contents are washed from the walls of each well.
- h) Incubate overnight (at least 12 hours) at $45^{\circ} \pm 2^{\circ}\text{C}$.
- 2) Filtration* (all sample types [calibrators, double blank, blank, QC's, unknowns], after incubation)

*Parts a) and b) of Filtration can be performed manually, as described below, or can be automated whereby those steps are performed using the Hamilton Microlab Starlet. For directions regarding the specific Hamilton method to use for parts a) and b), see method SOP for sample preparation.

- a) Add 100 μ L of HPLC-grade methanol to each sample and mix thoroughly.
- b) Transfer 200 μ L of each sample (all specimen types: calibrators, blanks, QC's, and unknowns) to a 96-well filter plate, such that the positions correspond to that of the sample preparation plate.

- c) Place a new, clean 96-well deepwell plate (same type as used during sample preparation) under the filter plate to serve as a collection plate, and centrifuge at 3000xg for 1 hour. **IMPORTANT** – be sure to place an additional filter plate (containing water only) and corresponding collection plate in the centrifuge at the same time to serve as a counterweight.
- d) After centrifugation, place a new, clean silicone plate mat on the 96-well deepwell plate used for collection and place in the HPLC autosampler for LC-MS/MS analysis.

b. Instrument Preparation

1) HPLC Preparation

- a) Solvent bottles should be checked daily and refilled as needed. Line A1 contains aqueous HPLC mobile phase, and line B1 contains organic HPLC mobile phase (both described in section 6a of this document). Also described in section 6a of this document is the dopant solvent, which should be checked daily and refilled as needed. The waste bottle should also be checked daily to ensure that it will not overflow during the run.
- b) Phenomenex Krud Katcher Ultra in-line filter, 0.5 μ m x 0.004" ID, should be replaced as needed.
- c) Before each run, review the chromatographic spectra of the previous runs' calibrators to ensure that the Phenomenex Kinetex C18 analytical column (50x2.1mm, 2.6 μ m) 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 settings before each run to make sure the lamp 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 APPI 2010" for all samples ran in the year 2010) at the top of the screen.
- c) Double-click "Hardware Configuration" on the left side of the screen and make sure that the appropriate profile is activated (if 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 multiple runs from a single study) is to open an existing batch and change the necessary information (i.e. sample name, 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 Appendix 3 for gradient information, Appendix 4 for example MS parameters).
 6. Input all necessary information for each sample, including sample name, data file (the typical data file is named (yyyy-mm-dd), 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. If a shutdown method is used, the blank should be injected as the sample.
 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 96-well plate.
 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 and enter a concentration of 1 for each corresponding internal standard (do this only for the calibrator internal standards). 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 yyyy-mm-dd".
- 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 all of the lines and fittings coming from the analytical column of the HPLC, to the switching valve, then to waste and to the interface housing of the mass spectrometer are connected properly and are tight.
- j) Click Okay to begin equilibrating the column.
- k) After the system has equilibrated for the required amount of time, 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 ("yyyy-mm-dd").
- 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 (e.g. 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 analyte and internal standard peaks for all injections (blanks, 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) Verify that the following information is set correctly for each curve. If it is not, click Regression, and select the appropriate Fit using the pull-down tab.
 - 1. Quantifier transition: use Quadratic Fit (e.g. EQU_Q, DAZ_Q, etc.)
 - 2. Confirmation/Qualifier transition: use Linear Fit (e.g. EQU_C, DAZ_C, etc.)
 - h) Close the Calibration window and save the Results file.
- 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. EQU_Q, 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. *\\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\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 ("yyyy-mm-dd").
 - f) Click "All Columns" to export all Results data (including hidden columns). NOTE: it is better to select "All Columns," given that the database will filter the data set according to what it needs, rather than selecting "Visible Columns" and attempting to manually select certain columns to export.
 - g) Click Save
 - h) The text file is then ready to be imported into the Access database for statistical analysis.

d. System Maintenance

- 1) Hamilton Microlab Starlet – Preventative Maintenance is performed annually by the service engineer.
- 2) Agilent 1200 HPLC – Preventative Maintenance is performed annually by the service engineer.
- 3) AB Sciex API 4000/5000 Mass Spectrometer – Preventative maintenance and tuning and calibration of the instrument is performed semi-annually by the service engineer.

e. CDC Modifications

N/A. This manuscript is a description of an original method and has been submitted for publication.

8. Reportable Range of Results

The reportable range of results for each of the six phytoestrogens is as follows:
Equal: 0.06 – 100 ng/mL

| | |
|----------------|-------------------|
| Daidzein: | 0.4 – 1,600 ng/mL |
| O-DMA: | 0.2 – 300 ng/mL |
| Genistein: | 0.2 – 730 ng/mL |
| Enterolactone: | 0.1 – 3,300 ng/mL |
| Enterodiol: | 0.04 – 320 ng/mL |

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

9. Quality Control (QC) Procedures

a. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Nutritional Biomarkers 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),

10. 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.

11. Limitations of Method; Interfering Substances and Conditions

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

Buffer should be made fresh every 3 months and pH should be verified monthly.

Occasionally, the concentration of phytoestrogens in urine is much higher than the highest standard in the calibration curves, so 200 μ L of urine may be too much to use. In this case, a smaller aliquot of urine can be used as described earlier.

This method has also undergone a series of in-house **ruggedness testing** experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. A total of five parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a test specimen with the selected parameter set at a value substantially lower and higher than that specified in this method while holding all other experimental variables constant. **The ruggedness testing findings for this method are presented in Appendix 7.** Please refer to Chapter 21 of the 2008 DLS Policies and Procedures Manual for further information on ruggedness testing.

12. Reference Ranges (Normal Values)

| Phytoestrogen | Urinary Concentration (µg/L) | | | | Sample Size |
|---------------|------------------------------|------------------|------------------|------------------|-------------|
| | Geometric mean | 10 th | 50 th | 90 th | |
| Daidzein | 61.0 | 6.75 | 59.2 | 545 | 5347 |
| Enterodiol | 31.1 | 4.35 | 36.7 | 173 | 5321 |
| Enterolactone | 249 | 23.6 | 330 | 1710 | 5342 |
| Equol | 8.77 | <LOD | 8.28 | 38.5 | 4976 |
| Genistein | 28.5 | 3.27 | 28.4 | 293 | 5351 |
| O-DMA | 4.18 | <LOD | 4.00 | 98.7 | 5065 |

13. 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.

14. 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.

15. 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 Nutritional Biomarker Branch. 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.

16. 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.

17. 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,

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.

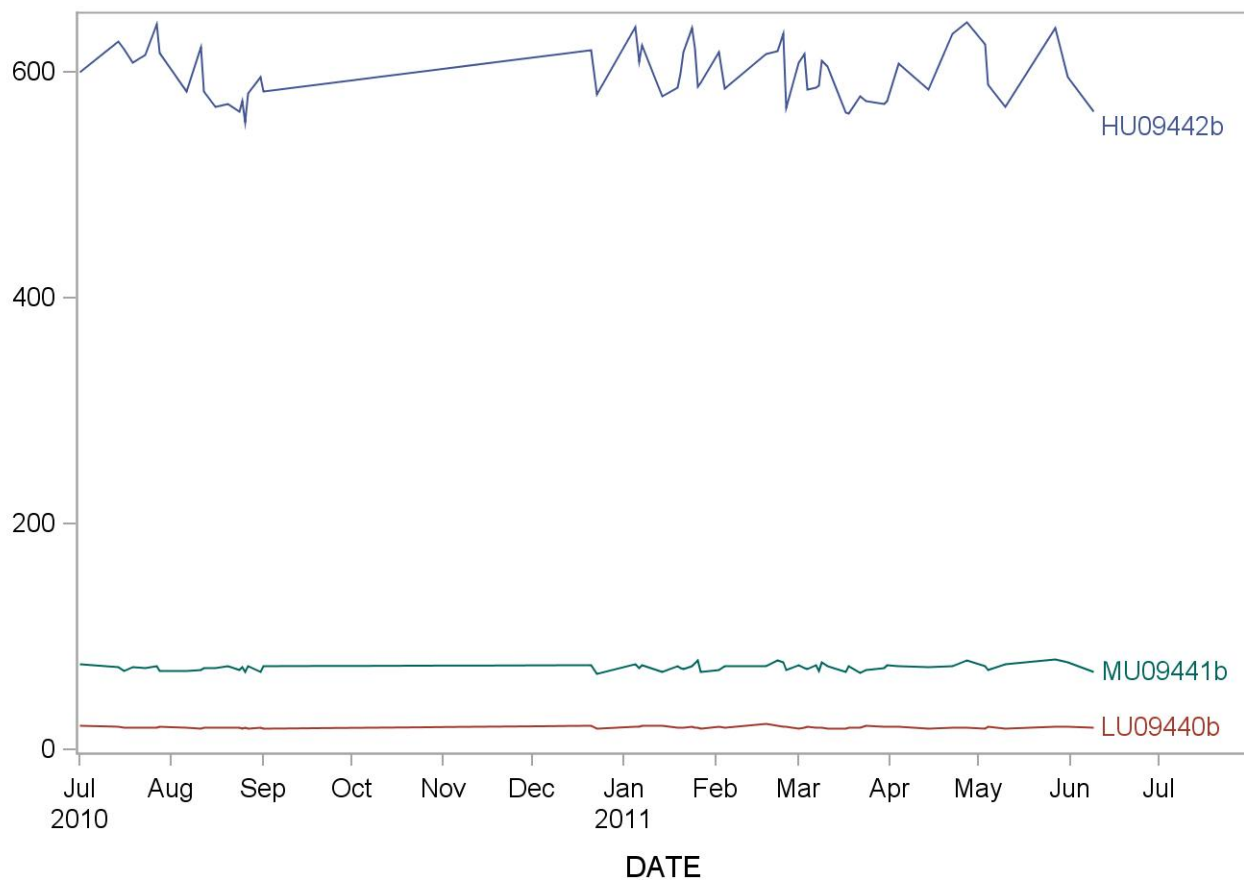
18. Summary Statistics and QC graphs

See following pages.

Summary Statistics for U-Phyto Daidzein (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|----------|----|------------|----------|--------|--------------------|--------------------------|
| HU09442b | 18 | 01JUL10 | 01SEP10 | 595.11 | 24.77 | 4.2 |
| HU09442b | 44 | 01JUL10 | 09JUN11 | 599.68 | 23.50 | 3.9 |
| LU09440b | 18 | 01JUL10 | 01SEP10 | 19.77 | 0.64 | 3.3 |
| LU09440b | 44 | 01JUL10 | 09JUN11 | 20.08 | 0.90 | 4.5 |
| MU09441b | 18 | 01JUL10 | 01SEP10 | 72.08 | 1.99 | 2.8 |
| MU09441b | 44 | 01JUL10 | 09JUN11 | 73.60 | 3.13 | 4.3 |

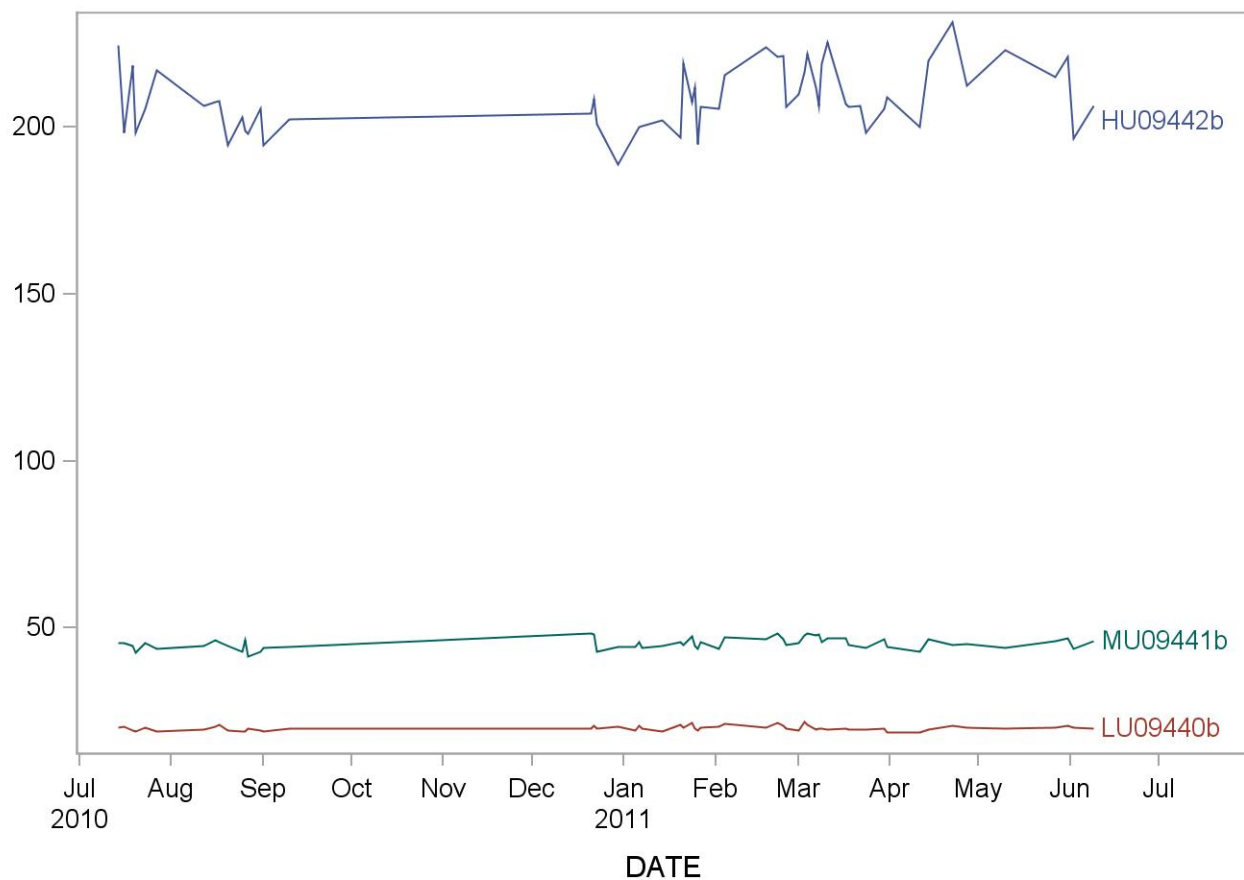
2007-2008 U-Phyto Daidzein (ng/mL) Quality Control



Summary Statistics for U-Phyto Enterodiol (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|----------|----|------------|----------|---------|--------------------|--------------------------|
| HU09442b | 16 | 14JUL10 | 10SEP10 | 205.094 | 8.659 | 4.2 |
| LU09440b | 16 | 14JUL10 | 10SEP10 | 19.584 | 0.672 | 3.4 |
| MU09441b | 16 | 14JUL10 | 10SEP10 | 44.359 | 1.431 | 3.2 |
| HU09442b | 44 | 20JUL10 | 09JUN11 | 209.004 | 9.884 | 4.7 |
| LU09440b | 44 | 20JUL10 | 09JUN11 | 19.983 | 0.761 | 3.8 |
| MU09441b | 44 | 20JUL10 | 09JUN11 | 45.564 | 1.653 | 3.6 |

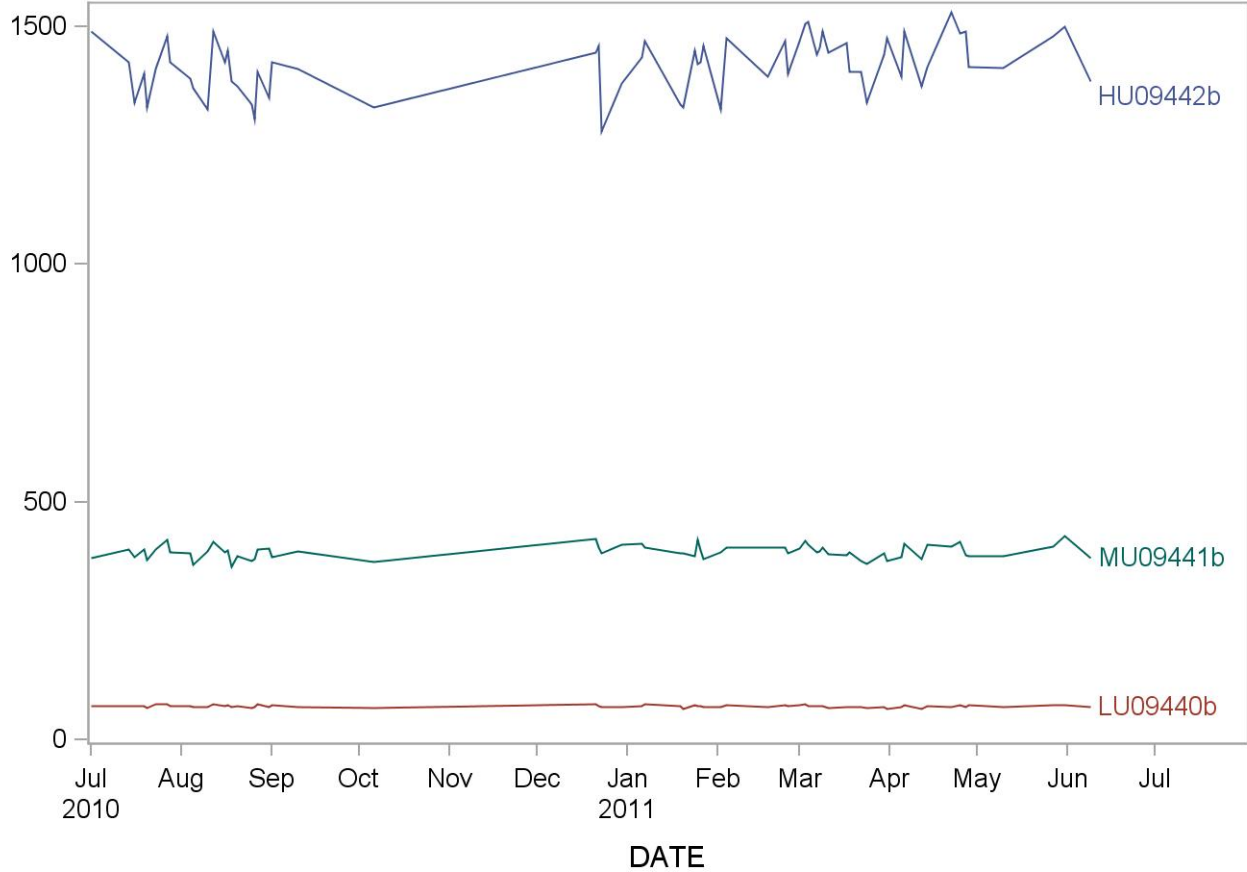
2007-2008 U-Phyto Enterodiol (ng/mL) Quality Control



Summary Statistics for U-Phyto Enterolactone (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|----------|----|------------|----------|---------|--------------------|--------------------------|
| HU09442b | 23 | 01JUL10 | 06OCT10 | 1394.35 | 53.82 | 3.9 |
| HU09442b | 44 | 01JUL10 | 09JUN11 | 1433.82 | 55.71 | 3.9 |
| LU09440b | 23 | 01JUL10 | 06OCT10 | 70.68 | 2.38 | 3.4 |
| LU09440b | 44 | 01JUL10 | 09JUN11 | 70.52 | 2.56 | 3.6 |
| MU09441b | 23 | 01JUL10 | 06OCT10 | 390.85 | 14.06 | 3.6 |
| MU09441b | 44 | 01JUL10 | 09JUN11 | 397.41 | 13.76 | 3.5 |

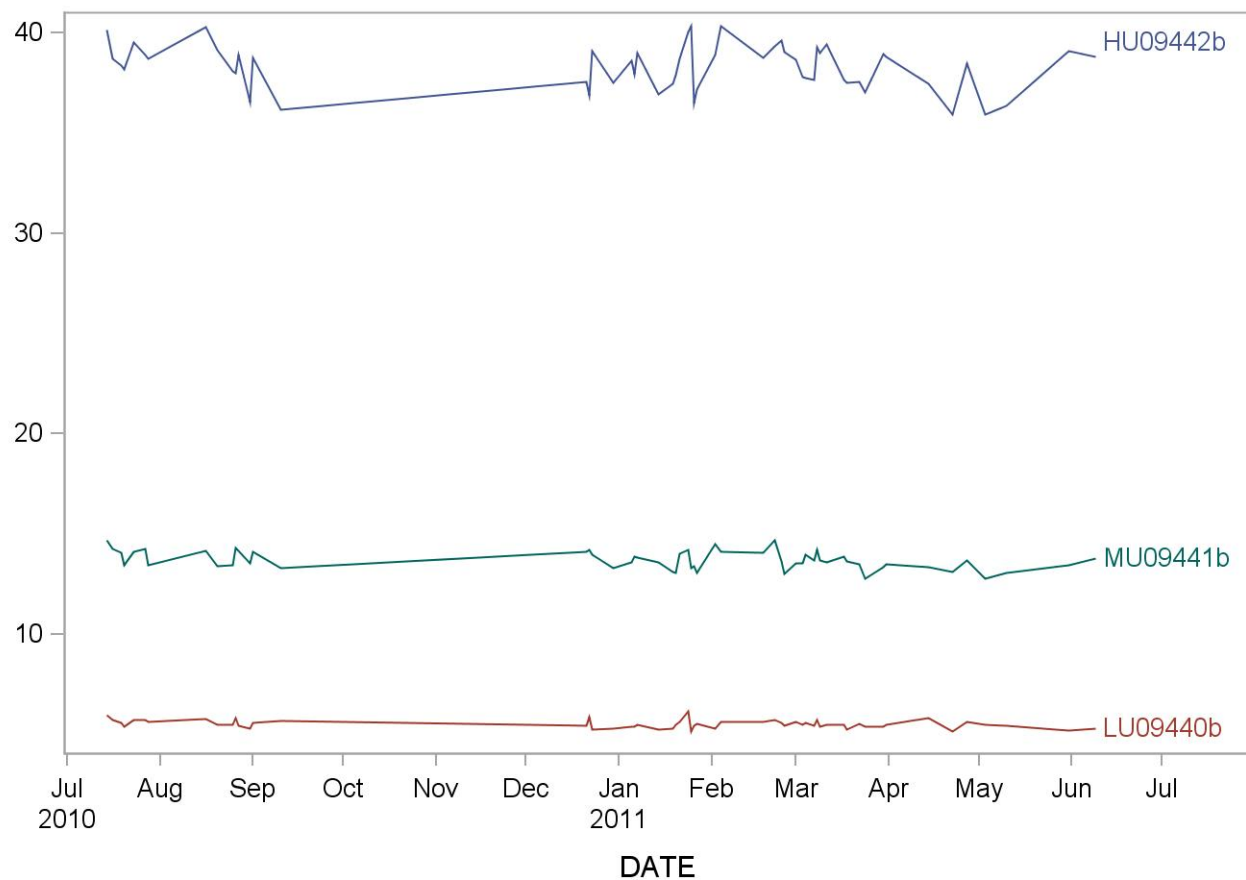
2007-2008 U-Phyto Enterolactone (ng/mL) Quality Control



Summary Statistics for U-Phyto Equol (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|----------|----|------------|----------|--------|--------------------|--------------------------|
| HU09442b | 15 | 14JUL10 | 10SEP10 | 38.567 | 1.125 | 2.9 |
| LU09440b | 15 | 14JUL10 | 10SEP10 | 5.613 | 0.178 | 3.2 |
| MU09441b | 15 | 14JUL10 | 10SEP10 | 13.910 | 0.432 | 3.1 |
| HU09442b | 43 | 20JUL10 | 09JUN11 | 38.230 | 1.107 | 2.9 |
| LU09440b | 43 | 20JUL10 | 09JUN11 | 5.469 | 0.199 | 3.6 |
| MU09441b | 43 | 20JUL10 | 09JUN11 | 13.614 | 0.438 | 3.2 |

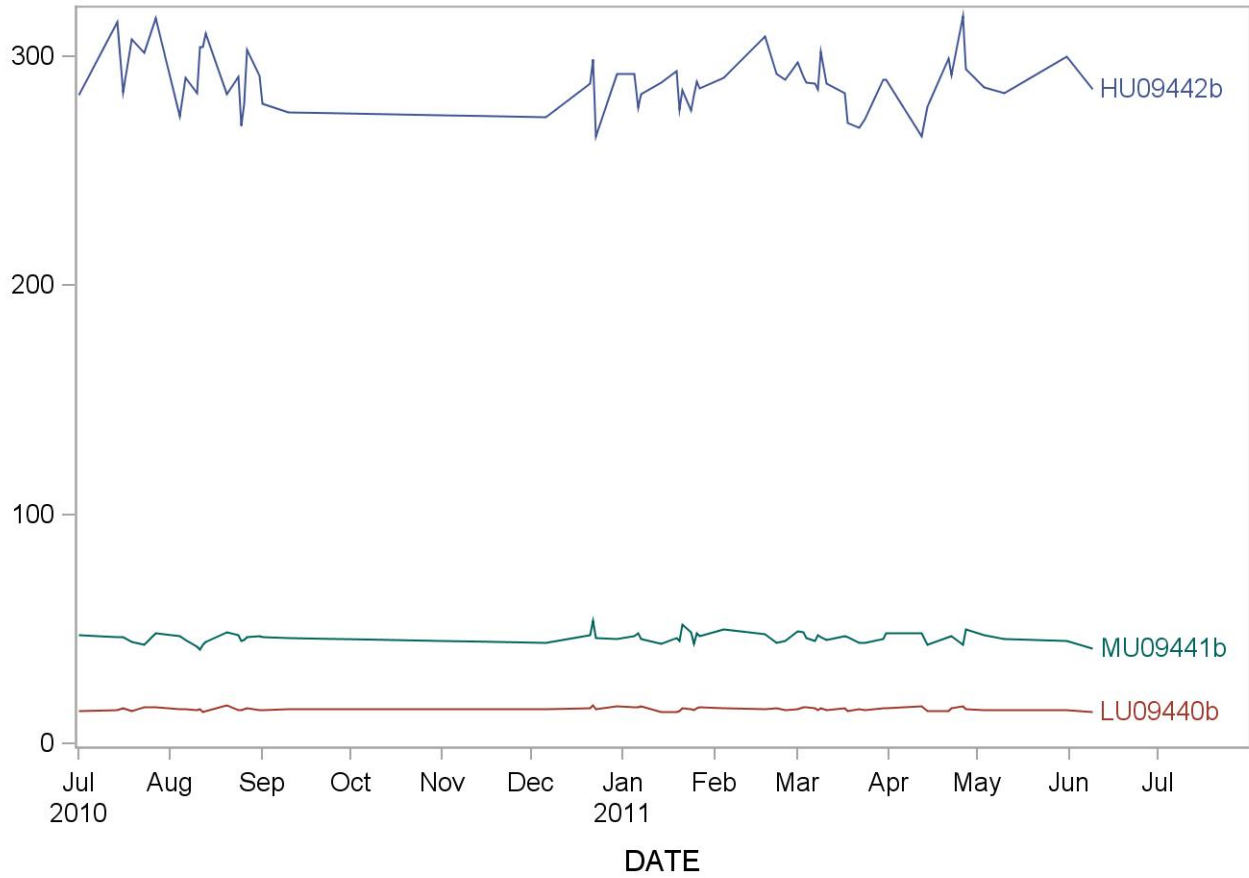
2007-2008 U-Phyto Equol (ng/mL) Quality Control



Summary Statistics for U-Phyto Genistein (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|----------|----|------------|----------|--------|--------------------|--------------------------|
| HU09442b | 21 | 01JUL10 | 06DEC10 | 291.48 | 14.53 | 5.0 |
| HU09442b | 44 | 01JUL10 | 09JUN11 | 287.25 | 10.48 | 3.6 |
| LU09440b | 21 | 01JUL10 | 06DEC10 | 15.06 | 0.63 | 4.2 |
| LU09440b | 44 | 01JUL10 | 09JUN11 | 15.24 | 0.74 | 4.9 |
| MU09441b | 21 | 01JUL10 | 06DEC10 | 45.55 | 1.98 | 4.3 |
| MU09441b | 44 | 01JUL10 | 09JUN11 | 46.65 | 2.34 | 5.0 |

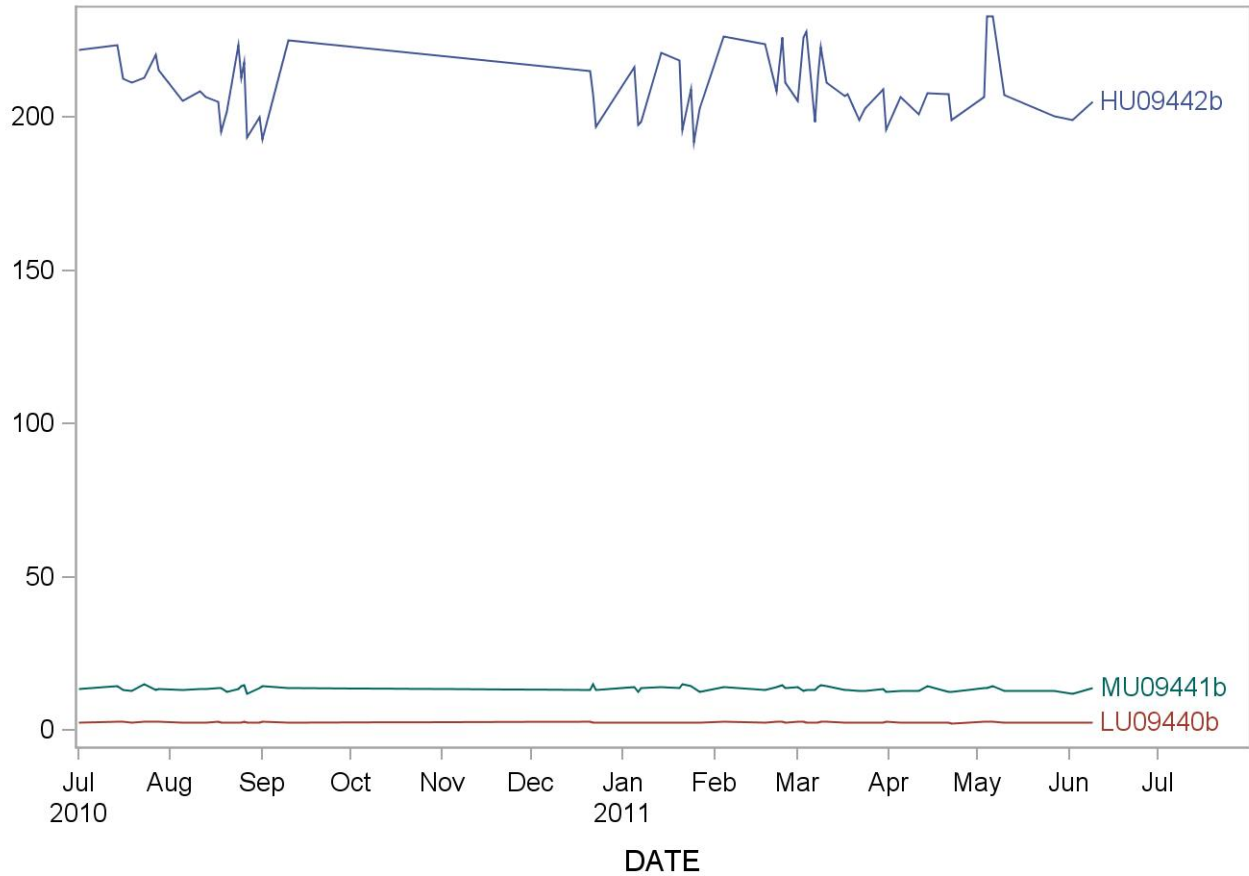
2007-2008 U-Phyto Genistein (ng/mL) Quality Control



Summary Statistics for U-Phyto o-DMA (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|----------|----|------------|----------|--------|--------------------|--------------------------|
| HU09442b | 19 | 01JUL10 | 01SEP10 | 209.61 | 9.81 | 4.7 |
| HU09442b | 45 | 01JUL10 | 09JUN11 | 209.81 | 11.19 | 5.3 |
| LU09440b | 19 | 01JUL10 | 01SEP10 | 2.69 | 0.11 | 4.2 |
| LU09440b | 45 | 01JUL10 | 09JUN11 | 2.64 | 0.13 | 4.7 |
| MU09441b | 19 | 01JUL10 | 01SEP10 | 13.67 | 0.75 | 5.5 |
| MU09441b | 45 | 01JUL10 | 09JUN11 | 13.60 | 0.76 | 5.6 |

2007-2008 U-Phyto o-DMA (ng/mL) Quality Control



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Acknowledgements

We gratefully acknowledge the contributions of Daniel Parker, Michael Rybak, Ph.D., and Christine Pfeiffer, Ph.D., who assisted in developing the methodology, performing the analyses for phytoestrogens, and preparing the manuscript for this chapter.