Laboratory Procedure Manual

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

Matrix: **Urine**

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

Method No: 4066.04

Revised:

As performed by: Bioactive Dietary Compounds Laboratory (BDCL)
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.

Phytoestrogens in Urine
NHANES 2009-2010

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_F	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)

Phytoestrogens in Urine
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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 [1-4]. Their endocrine activity, as well as their potential influence on other biologic pathways, has led to considerable interest in phytoestrogens from an epidemiological standpoint [5]. The consumption of diets high in phytoestrogen-rich foods has been associated with lower rates of such hormone-dependent cancers as breast [1,2] and prostate [3,4] cancer, with modulation of osteoporosis [6], with reduced severity of menopausal symptoms [7,8], and with lower risk for cardiovascular disease [9,10]. Whether phytoestrogens are indeed the active components responsible for these benefits, however, has come under scrutiny [5, 11], and the significance of their purported health benefits has been challenged [12]. Individual studies and meta analyses have often resulted in apparently conflicting findings, such as whether phytoestrogens do [13] or do not [14] significantly reduce the frequency and intensity of menopausal hot flushes. Potential toxic effects associated with phytoestrogen exposure have also been identified [11]. 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 [12]; glucuronidated forms also predominate in the urine [13].

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 [14].

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, sample preparation plates, 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

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.

The raw data file and respective batch file from the tandem mass spectrometer are collected using the instrument software and stored on the instrument workstation. The data file and batch file are transferred via USB flash drive to the network where the data file is processed into a results file that is also saved on the CDC network. Results are typically generated by auto-integration, but may require in some cases manual integration. The results file (including analyte and internal standard names, peak areas, retention times, sample dilution factor, data file name, acquisition time, etc) is imported into a LIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See “SOP for Computerization and Data System Management” for a step-by-step description of data transfer, review, and approval.

For NHANES, data is transmitted electronically. 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. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.

Data files from the instrument workstation are typically copied to the CDC network on a run-by-run basis. This is the responsibility of the analyst under the guidance of the team lead and/or supervisor. Further data processing is typically conducted on a networked computer and saved directly to the CDC network. Files stored on the CDC network are automatically backed up nightly by ITSO support staff.

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

We recommend that specimen donors fast prior to specimen collection, but fasting is not required.

Specimens for phytoestrogen analysis are performed on fresh or frozen urine.

3-5 mL of urine is preferable to allow for repeat analyses. A volume of 200 µL is required for each analysis.

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.

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.

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.

Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Biomarkers Branch and the electronic copy of this file is located at \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\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 0.45 µm filtered deionized water with a resistance of at least 18 MΩ/cm, and HPLC-grade solvents and reagents. Use Class A volumetric glassware in all cases. Perform all steps involving concentrated acids, bases, and organic solvents in a chemical fume hood. Though each reagent preparation specifies a total volume of reagent prepared, these directions may be scaled up or down to prepare larger or smaller quantities if desired.

(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 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 with 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 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% water. Refill as needed.

(4) HPLC Mobile Phase (Organic)

100% methanol. Refill as needed.

(5) Dopant Solvent

100% toluene. Refill as needed.

(6) Synthetic Urine

For one L, quantitatively transfer 500 mL water to a one L beaker. Using a magnetic stir bar to agitate the solution, add the following chemicals in the quantities and order specified:

- 3.8 g Potassium Chloride
- 8.5 g Sodium Chloride
- 24.5 g Urea
- 1.03 g Magnesium Sulfate ($MgSO_4.7H_2O$)
- 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.)

Once all compounds have dissolved in solution, quantitatively transfer the mixture to a one L volumetric flask. Bring the solution up to volume with water. Seal the volumetric flask and mix the contents by inversion. Transfer to a storage vessel. This solution can be stored at 4 °C for up to one year.

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). 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 µL 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 is prepared by pipetting the following amounts of each internal standard stock solution into a volumetric flask of appropriate size:

$^{13}C_3$-Equol	15.79 mL
$^{13}C_3$-Daidzein	19.89 mL
$^{13}C_3$-O-Desmethylangolensin	3.13 mL
$^{13}C_3$-Genistein	15.63 mL
$^{13}C_3$-Enterolactone	15.18 mL
$^{13}C_3$-Enterodiol	12.98 mL

The flask is then filled to volume with 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 Fourth National Report on Human Exposure to Environmental Chemicals [15], or The Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population [16]. 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 for target quality control values;.

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

Note: With some exceptions, a material listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product

listed. In the case of standards, internal standards, chemicals and reagents, the chemical and/or isotopic purity of the substituted must meet or exceed that of the listed product. In the case of the HPLC column and filter, equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures.

(1) General consumables

- Kinetex C18 analytical column, 50 x 2.1 mm, 2.6 µm (Phenomenex, Torrance, CA)
- Krud Katcher Ultra in-line filter, 0.5 µm x 0.004” ID (Phenomenex, Torrance, CA)
- 12 x 75 mm disposable glass culture tubes (Corning Glassworks, Corning, NY)
- 9” Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)
- Nunc 1 mL deepwell 96-well plates (Nalge Nunc International, Rochester, NY)
- 96-well pre-slit silicon plate seals, 8.6mm, (Thermo-Fisher Scientific, Fair Lawn, NJ)
- 96-well filter plates, Ultracell 10, regenerated cellulose, 10KD MWCO (Millipore, Billerica, MA)
- High Five nitrile examination gloves (High Five Products Inc., Chicago, IL)
- N-Dex nitrile examination gloves (Best Manufacturing Corporation, Menlo, GA)
- Blue tips (50-1000 µL) for Eppendorf pipette (Brinkmann Instruments Inc., Westbury, NY)
- Yellow tips (2-200 µL) for Eppendorf pipettes (Brinkmann Instruments Inc., Westbury, NY)
- Combitip plus (500 µL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)
- Combitip plus (1.0 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)
- Combitip plus (2.5 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)
- 2.0 mL Polypropylene cryovials (Nalgene Company, Rochester, NY)
- 1.5 mL micro centrifuge tubes (VWR, Suwanee, GA)
- 15 mL BD Falcon Tubes (Becton Dickinson, Franklin Lakes, NJ)
- 50 mL BD Falcon Tubes (Becton Dickinson, Franklin Lakes, NJ)
- Various glass beakers, volumetric flasks, graduated cylinders, and bottles, class A glassware.

(2) Chemicals and solvents

- Methanol, HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI)
- Toluene, HPLC grade (Thermo-Fisher Scientific, Fair Lawn, NJ)
- Ethanol, HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI)
- Dimethylsulfoxide, HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI)
- Water, HPLC grade (Aqua Solutions, Jasper, GA)
- Ammonium Hydroxide (28-30%, Thermo-Fisher Scientific, Fair Lawn, NJ)
- Ammounim Acetate, HPLC grade (Sigma, St. Louis, MO)
- Acetic acid, glacial, reagent grade (Sigma, St. Louis, MO)
- 4-methylumbelliferyl β-D-glucuronide hydrate (Sigma, St. Louis, MO)
- 4-methylumbelliferyl sulfate (Sigma, St. Louis, MO)
- β-Glucuronidase (powder), type H-1 from *Helix pomatia* (Sigma, St. Louis, MO)
- Enterolactone (Sigma, St. Louis, MO)
- Enterodiol (Sigma, St. Louis, MO)

- Equol (Sigma, St. Louis, MO)
- Genistein (Indofine Chemical Company, Somerville, NJ)
- Daidzein (Indofine Chemical Company, Somerville, NJ)
- O-Desmethylangolensin (Dr. Nigel Botting, University of St. Andrews, Scotland)
- $^{13}C_3$-Enterodiol, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- $^{13}C_3$-Enterolactone, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- $^{13}C_3$-Genistein, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- $^{13}C_3$-Daidzein, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- $^{13}C_3$-Equol, (Dr. Nigel Botting, University of St. Andrews, Scotland)
- $^{13}C_3$-O-Desmethylangolensin, (Dr. Nigel Botting, University of St. Andrews, Scotland)

E. Instrumentation

Note: In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of advanced laboratory instrumentation (e.g., HPLC components, tandem quadrupole mass spectrometer, automated liquid handler) equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures.

(1) Agilent 1200 SL HPLC system (Agilent Technologies, Palo Alto, CA), including:

- Model 4208A Control Module
- Model G1379B Degasser
- Model G1310A Isocratic Pump
- Model G1312A Binary pump SL
- Model G1367D High Performance Autosampler SL+
- Model G1316B Thermostatted Column Compartment SL

(2) AB Sciex API 5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA), including:

- APPI (PhotoSpray) ion source
- Parker-Balston model LCMS-5001NTNA tri-gas generator (Parker-Balston, Haverhill, MA)
- Peak model NM18L nitrogen gas generator (Peak Scientific, Billerica, MA)

(3) Hamilton Starlet 8-channel with auto-load arm (Hamilton), including:

- Two pipette tip carriers, TIP_CAR_480_A00
- Three sample vial carriers, SMP_CAR-32_A00
- One plate carrier, PLT_CAR_L5AC_A00

(4) Other laboratory instrumentation:

- Harvard syringe pump (Harvard Apparatus, Inc, Holliston, Massachusetts)
- Eppendorf pipette,100-1000µL (Brinkmann)
- Eppendorf pipette, 10-100µL (Brinkmann)
- Eppendorf pipette, 2-20µL (Brinkmann)
- Eppendorf Repeater Plus pipette (Brinkmann)
- Vortexer (VWR)
- Magnetic stirrer (Fisher Scientific)
- Economy incubator, model 3EM (Precision, Winchester, VA)

- Pinnacle model 530 pH meter (Corning Inc., Corning, NY)
- Analytical balance (AG104, Mettler Instrument Corp., Hightstown, NJ)

7. Calibration and Calibration Verification Procedures

A. Method Calibration

Ten calibrators (S0-S9) prepared in synthetic urine are added to the reaction plate and processed as regular samples. These 10 calibrators are analyzed at the beginning of each run. At the end of each run, the calibrators are re-analyzed as unknown samples. The measured concentrations of these calibrators should agree within 15% of their set values. A quadratic calibration equation with 1/x weighting is used.

Method accuracy and specificity can be accessed by amending low and medium QC pools with each of the analytes and calculating the recovery of the exogenous analyte addition. Results of in-house spiking experiment showed that the recovery was 102-112%.

Reference materials are not available for urinary phytoestrogens. Calibration verification is conducted as outlined in "**4066.04 SOP for Calibration and Calibration Verification**."

External proficiency testing programs currently do not exist for urinary phytoestrogens. An in-house proficiency testing program has been developed and is conducted at least twice a year, details of which can be found in "**4066.04 SOP for In-House Proficiency Testing**."

Results from a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied**.**

B. Instrument Calibration

(1) API 5000 Mass Spectrometer

The calibration of the mass spectrometer is scheduled on a semi-annual basis as part of a preventive maintenance program 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.

The tuning and mass calibration of the first and third quadrupoles of the API 5000 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. Please refer to the API 5000 User's Manual for additional details.

(2) Hamilton Microlab Starlet

Twice a year a Hamilton service engineer performs a preventative maintenance including volume verification at 10 µL and 1000 µL.

A volume verification of the various steps of the method can also be performed gravimetrically (e.g., using online gravimetric kit, Hamilton) by the user. Imprecision should be commensurate or exceed that obtained using manual pipettes.

8. 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, 10 calibrators, 3 front QC's (low, medium, and high), 79 patient samples, 3 back QC's (low, medium, and high), and lastly, reinjection of the 10 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 ten calibrators (S0-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

- Prepare β-glucuronidase solution (described in section 6.A. of this document)
- Label a 96-well deepwell plate

(a) Calibrator Preparation
- Add the following to each calibrator (S0-S9)
  - 250 µL of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)
  - 50 µL of deconjugation internal standard solution, diluted 20x (1 part solution, 19 parts water)
  - 100 µL pH 5.0 (2.5M) ammonium acetate buffer
  - 900 µL synthetic urine
  - 50 µL of β-glucuronidase solution (IMPORTANT – add enzyme last)
- Thoroughly mix each calibrator.
- Transfer 290 µL of each calibrator (S0-S9) to the appropriate well, as labeled on the 96-well plate.
- Discard remaining calibrator solution.

(b) Double Blank Preparation
- Add the following to the well labeled "double blank" on the 96-well plate.
  - 10 µL of deconjugation internal standard solution, diluted 20x (1 part solution, 19 parts water)
  - 20 µL pH 5.0 (2.5M) ammonium acetate buffer
  - 250 µL synthetic urine (no urine or mixed working internal standard solution present in the double blank)
  - 10 µL of β-glucuronidase solution (IMPORTANT – add enzyme last)

(c) Blank Preparation
- Add the following to the well labeled "blank" on the 96-well plate.
  - 50 µL of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)

- 10 µL of deconjugation internal standard solution, diluted 20x (1 part solution, 19 parts water)
- 20 µL pH 5.0 (2.5M) ammonium acetate buffer
- 200 µL synthetic urine (no urine present in the blank)
- 10 µL of β-glucuronidase solution (IMPORTANT – add enzyme last)

(d) Urine Sample Preparation (quality control materials and unknown samples)

- Add the following to all wells labeled "QC" and "Unknowns" on the 96-well plate.
  - 50 µL of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)
  - 10 µL of deconjugation internal standard solution, diluted 20x (1 part solution, 19 parts water)
  - 20 µL pH 5.0 (2.5M) ammonium acetate buffer
  - 200 µL urine
  - 10 µL of β-glucuronidase solution (IMPORTANT – add enzyme last)
- 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.
- Incubate overnight (at least 12 hours) at 45° ± 2°C.

(2) Filtration (all sample types [calibrators, double blank, blank, QC's, unknowns], after incubation)

- Add 100 µL of HPLC-grade methanol to each sample and mix thoroughly.
- Transfer 200 µL 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 96-well plate.
- Place a new, clean 96-well 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.
- After centrifugation, place a new, clean silicone plate mat on the 96-well plate used for collection and place in the HPLC autosampler for LC-MS/MS analysis.

B. Instrument Preparation

(1) HPLC Preparation

- 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 6.A. 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.
- Phenomenex Krud Katcher Ultra in-line filter, 0.5µm x 0.004" ID, should be replaced as needed.
- Before each run, review the chromatographic spectra of the previous runs' calibrators to ensure that the Phenomenex Kinetex C18 analytical column (50x2.1mm, 2.6µm) is in

suitable condition (i.e. no double peaking, peak trailing, broad peaks, etc.). Replace the analytical column as needed.

(2) Mass Spectrometer Preparation

- Check the interface settings before each run to make sure the lamp height, probe height, and probe width settings are correct.
- 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

- Open Analyst software using the icon on the desktop of the instrument PC.
- Select the project (such as "Phytoestrogens APPI 2012 – Rudy" for all samples ran in the year 2012) at the top of the screen.
- 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.

(a) 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.

- Double-click "Build Acquisition Batch"
- Specify a Set Name
- Click Add Set, then Add Samples
- Specify the Sample Name and number of samples to be run and click "okay"
- Using the pull down tabs, select a quantitation method as well as an acquisition method (see "SOP for Instrument Preparation" for specific method names and information) and check the multiple methods box if planning on utilizing more than one method in the same batch for gradient information, 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.
- Click the Locations tab at the top of the window and choose which type of autosampler is being used.
- 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.

- 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).
- Once the batch file is finished, be sure and save it. The typical filename for each batch is "Phyto yyyy-mm-dd".
  - 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.
  - Open the Queue window by clicking the View Queue button.
  - Click on the Equilibrate icon.
  - Select the appropriate sample acquisition method and enter an equilibration time of 60 minutes (recommended, but can be less if need be).
  - 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.
  - Click Okay to begin equilibrating the column.
  - 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) Generate a Results File.

- Double-click "Quantitation Wizard" shown on the left side of the screen.
- Select the data file corresponding to the run in which you are generating results for.
- 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.
- Click Next
- Using the pull-down tab, select Summary. Also select None under Default Query
- Click Next
- Using the pull-down tab, select the quantitation method that corresponds to the sample acquisition method used for the run.

- Click Finish
- 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.
- 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").

D. Data Review

- 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).
- The integration should be verified by clicking the Peak Review button.
- 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.
- To perform manual integration, click Manual Integration Mode.
- After checking analyte and internal standard peaks for all injections (blanks, calibrators, QC's, and patient samples), close the Peak Review window.
- 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).
- 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.
  - Quantifier transition: use Quadratic Fit (e.g. EQU_Q, DAZ_Q, etc.)
  - Confirmation/Qualifier transition: use Linear Fit (e.g. EQU_C, DAZ_C, etc.)
- Close the Calibration window and save the Results file.

E. Data Export

- 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.
- Click File, then Export
- 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*.
- Using the "Save as type" pull-down tab, select Text Files (*.txt)
- Name the file to be exported the same as the Results file ("yyyy-mm-dd").
- 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.

- Click Save
- The text file is then ready to be imported into the LIMS database for statistical analysis.

F. System Maintenance

Hamilton Microlab Starlet – Preventative Maintenance is performed annually by the service engineer.

Agilent 1200 HPLC – Preventative Maintenance is performed annually by the service engineer.

AB Sciex API 5000 Mass Spectrometer – Preventative maintenance and tuning and calibration of the instrument is performed semi-annually by the service engineer.

G. CDC Modifications

N/A. This manuscript is a description of an original method and has been published in a peer-reviewed journal [14].

9. Reportable Range of Results

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

Analyte	Reportable Range (ng/mL)
Equol	0.06 – 100
Daidzein	0.4 – 1,600
O-Desmethylangolensin	0.2 – 300
Genistein	0.2 – 730
Enterolactone	0.1 – 3,300
Enterodiol	0.04 – 320

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.

10. 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 urinary 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 using a multi-rule quality control system [17] based their characterization data, namely: the pool mean; the pooled within-run standard deviation associated with individual QC results measured in the same run (S_w); the standard deviation associated with individual QC results (S_i); and the standard deviation associated with run mean QC results (S_m). QC rules have been designed to accommodate the use of 1–3 different QC pools during a run, the use of 1–2 measurements of each pool per run, and as many instruments as needed. In the case of three QC pools per run with two QC results per pool:

(1) If all three QC run means are within 2 S_m limits and individual results are within 2 Si limits, accept the run

(2) If one of the three QC run means is outside a 2 Sm limit, reject run if:

- (a) 1 3S Rule—run mean is outside a 3 S_m limit or
- (b) 2 2S Rule—two or more of the three run means are outside the same 2 S_m limit or
- (c) 10 Xbar Rule—current and previous nine run means are on the same side of the characterization mean

(3) If one of the six QC individual results is outside a 2 Si limit, reject run if:

- (a) Outlier—one individual result is beyond the characterization mean ±4 S_i or
- (b) R 4S Rule—two or more of the within-run ranges in the same run exceed 4 S_w (i.e. 95 per cent range limit).

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 \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\Data handling\QC. 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

The following steps are provided as a general guideline for identifying possible problems resulting in “out of control” values for QC materials. The troubleshooting process should be done in consultation with the supervisor and may involve additional experiments beyond what is indicated below. Analytical results for runs not in statistical control should not be reported.

- 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.
- Run standards in Q1 Scan to see if molecular ion is detected.

- Check the proper gas flow for curtain, exhaust, and source from the nitrogen generator.
- Look for sample preparation errors, i.e., if the analyst forgot to add internal standard, specimen, etc.
- Check the calibrations of the pipettes.

12. Limitations of Method; Interfering Substances and Conditions

The most common cause of poor method performance is a pipetting error. All reagents and mobile phases should be made fresh whenever possible and verified for performance. Occasionally, the concentration of phytoestrogens in urine will exceed the highest calibrator. In this case, a smaller aliquot of urine can be used as described earlier. When using a quadratic equation for calibration, care must be taken to minimize excessive "roll-over" of the curve at higher concentrations. This phenomenon is typically indicative of too much analyte being injected. If it is observed, reducing the sample injection volume is recommended.

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.

13. Reference Ranges (Normal Values)

Urinary phytoestrogen levels from the U.S. population[1]

	Urinary Concentration (µg/L)				
Analyte	**Geometric mean**	**5th**	**50th**	**95th**	**Sample Size**
Daidzein	66.6	5.35	60.4	1,170	5,122
Enterodiol	38.6	2.48	43.9	377	5,122
Enterolactone	290	10.9	390	2,740	5,122
Equol	8.21	0.953	8.33	64.8	5,117
Genistein	29.9	2.45	26.1	523	5,122
O-DMA	4.80	<LOD	4.09	251	5,109

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 aliquoting. 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 Nutritional Biomarkers 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.

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.

[1]Representative sample of the U.S. population aged 6 years and older from the National Health and Nutrition Examination Survey, 2003-2006, as published in the National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population (CDC – 2012)

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

The LIMS database is used to keep records and track specimens for NHANES 1999+. If urinary phytoestrogens analyses are used for smaller, non-NHANES studies, records may be kept in Excel files on the network.

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

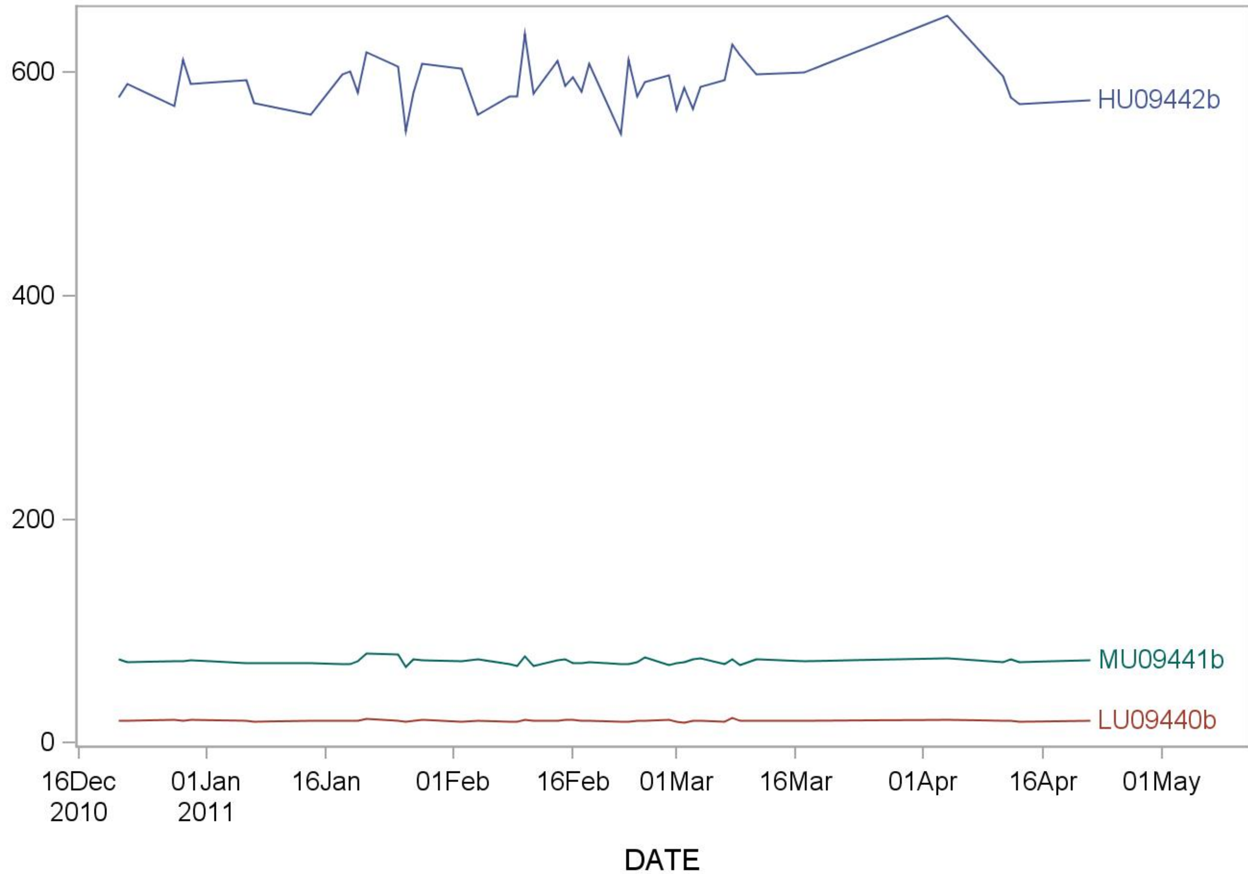
incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

19. Summary Statistics and QC Graphs

See following pages.

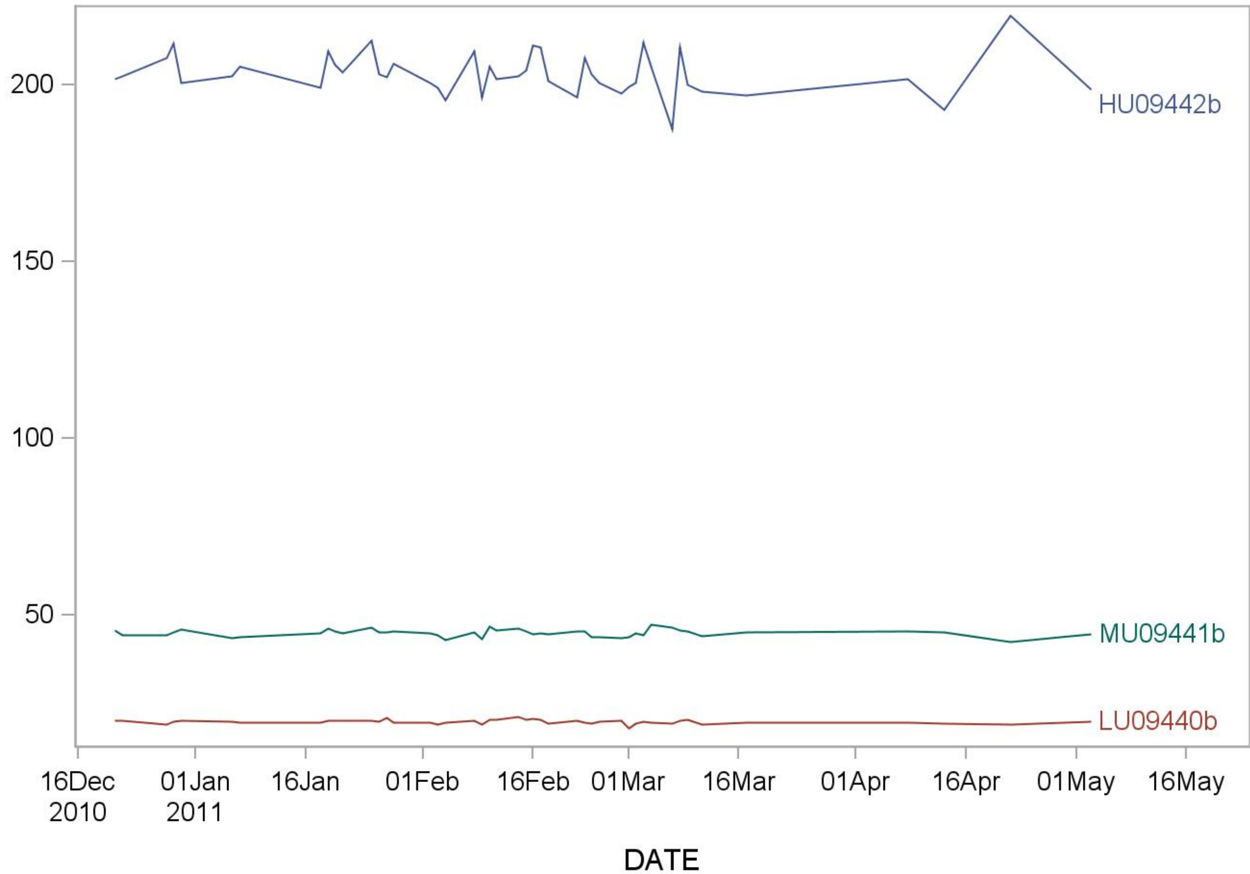
Summary Statistics for Urine Daidzein (ng/mL)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
URCZDH3	HU09442b	46	21DEC10	22APR11	590.78	20.99	3.6
URCZDL3	LU09440b	46	21DEC10	22APR11	19.94	0.73	3.7
URCZDM3	MU09441b	46	21DEC10	22APR11	73.05	2.55	3.5



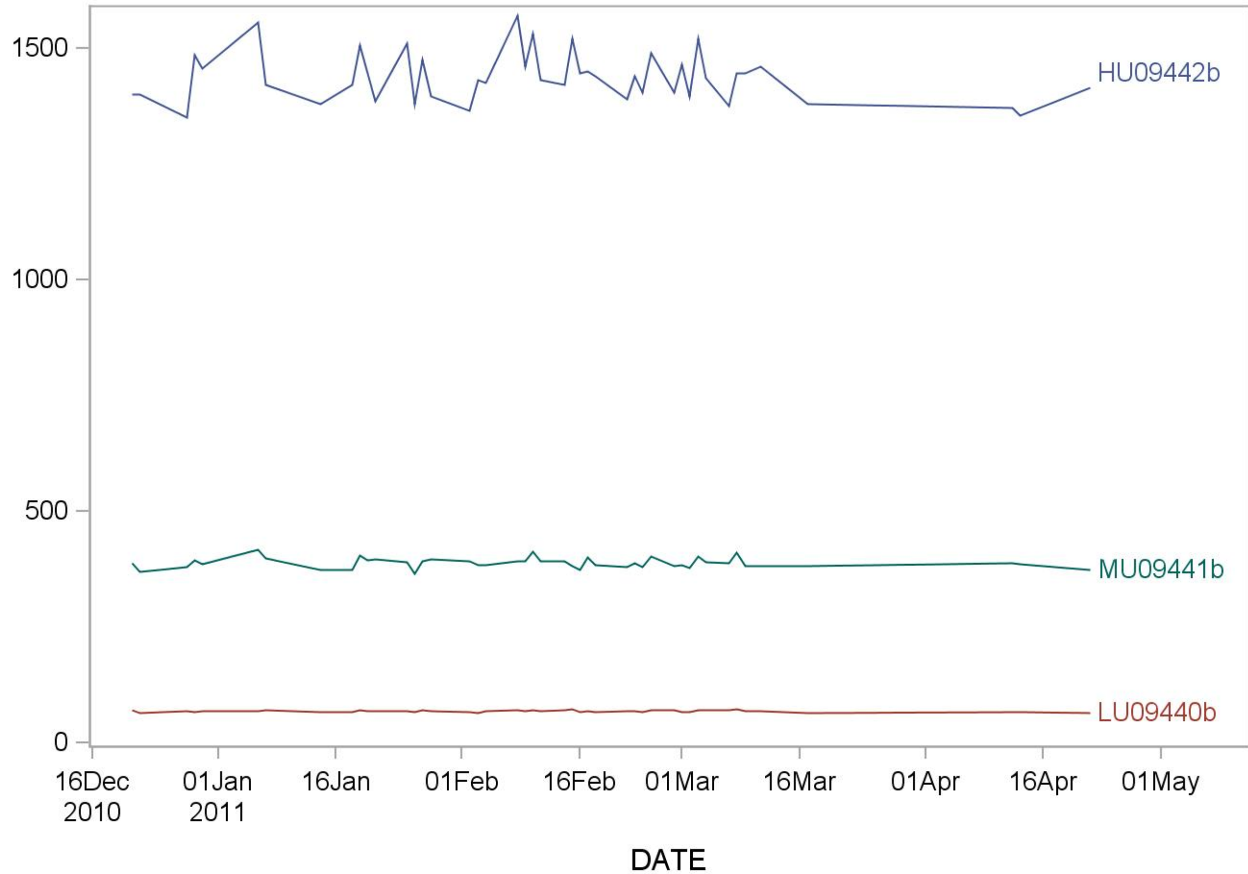
Summary Statistics for Urine Enterodiol (ng/mL)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
URCEDH3	HU09442b	45	21DEC10	03MAY11	203.022	5.895	2.9
URCEDL3	LU09440b	45	21DEC10	03MAY11	19.807	0.584	3.0
URCEDM3	MU09441b	45	21DEC10	03MAY11	44.879	1.020	2.3



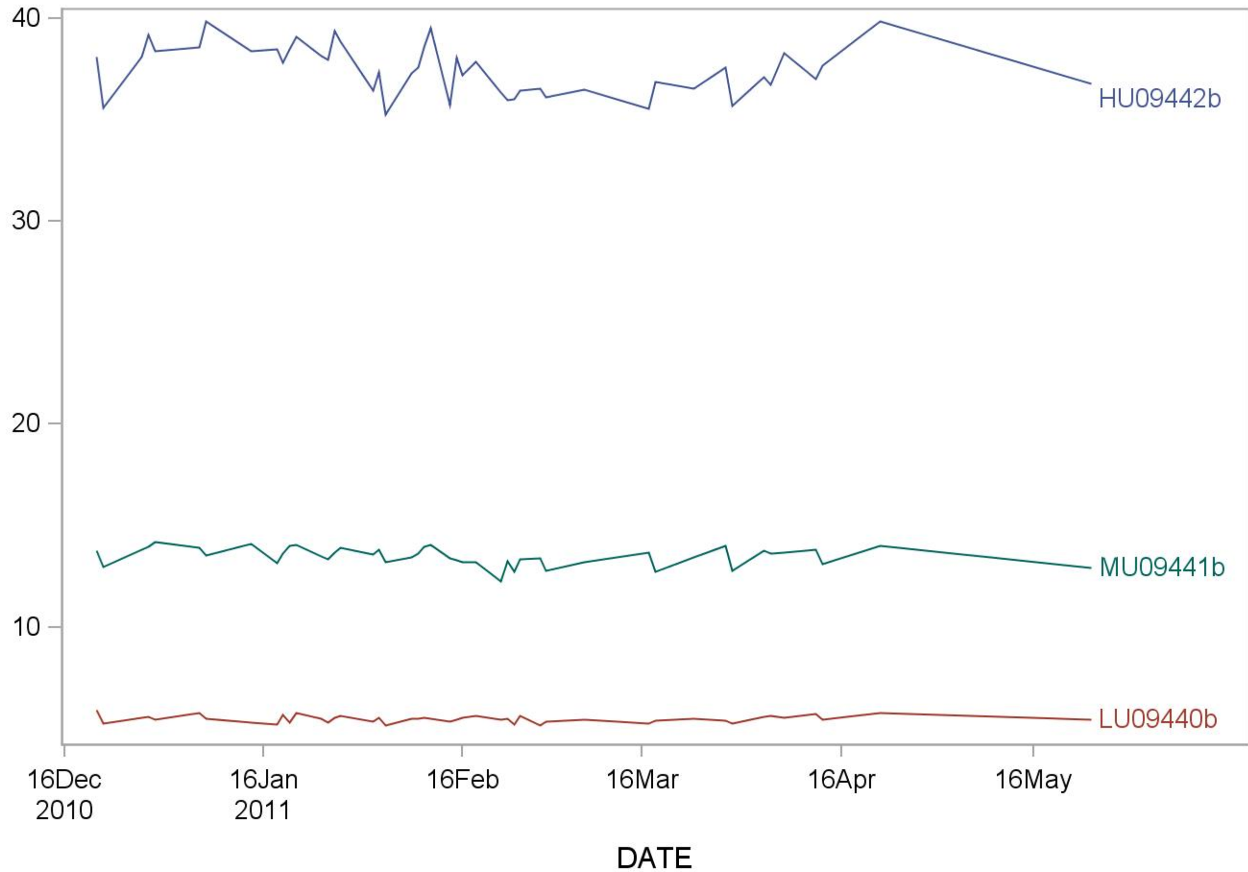
Summary Statistics for Urine Enterolactone (ng/mL)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
URCELH3	HU09442b	45	21DEC10	22APR11	1436.33	53.41	3.7
URCELL3	LU09440b	45	21DEC10	22APR11	68.58	2.10	3.1
URCELM3	MU09441b	45	21DEC10	22APR11	388.37	11.26	2.9



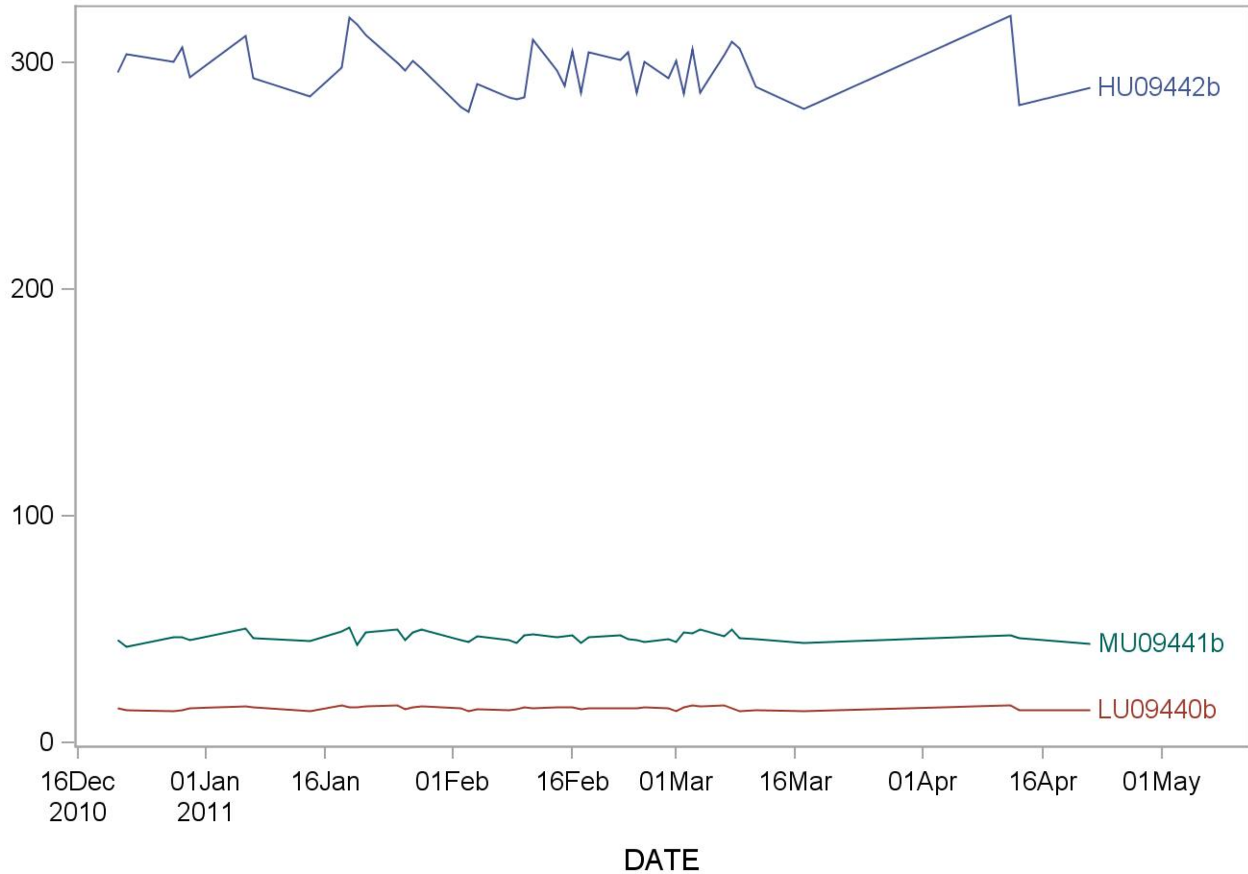
Summary Statistics for Urine Equol (ng/mL)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
URCEQH3	HU09442b	46	21DEC10	25MAY11	37.501	1.232	3.3
URCEQL3	LU09440b	46	21DEC10	25MAY11	5.474	0.173	3.2
URCEQM3	MU09441b	46	21DEC10	25MAY11	13.498	0.439	3.3



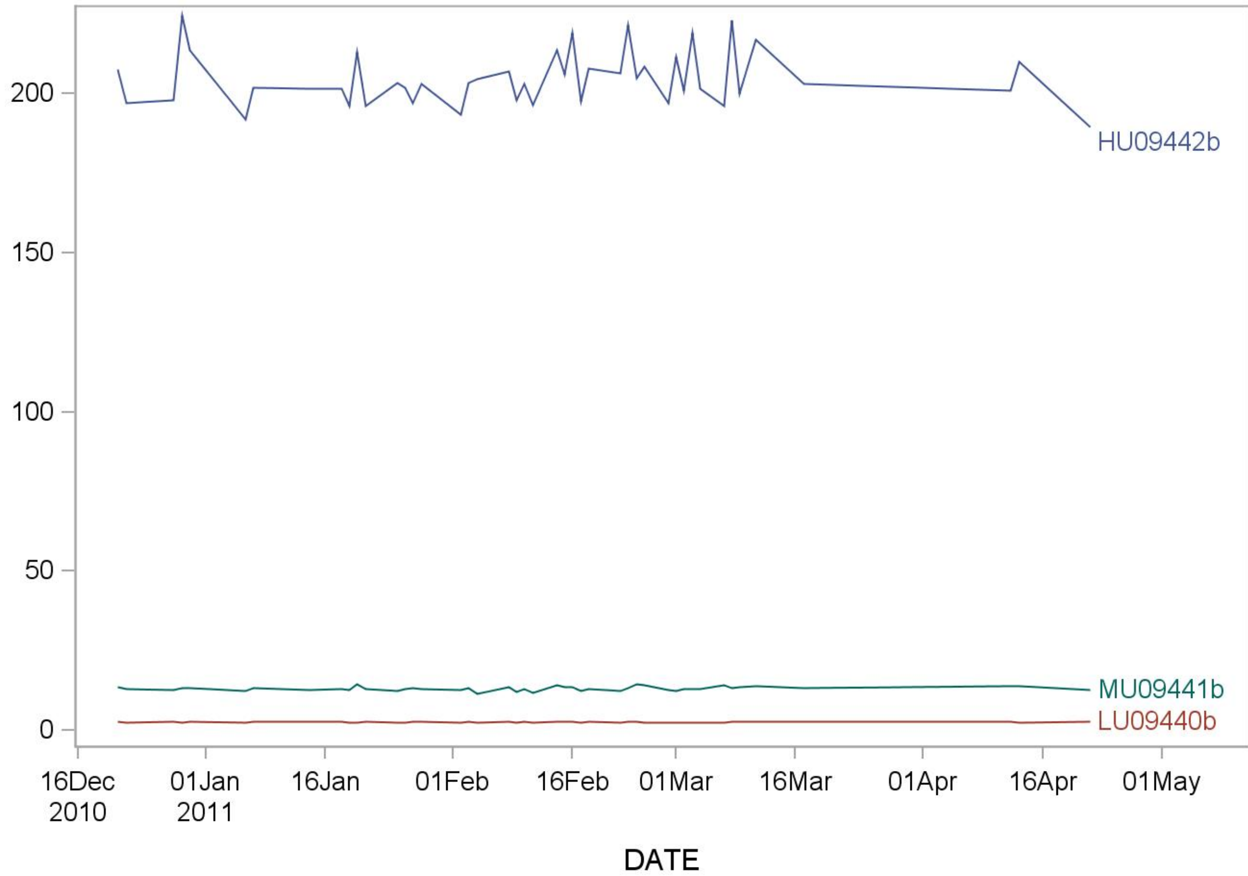
Summary Statistics for Urine Genistein (ng/mL)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
URCGNH3	HU09442b	45	21DEC10	22APR11	297.40	11.01	3.7
URCGNL3	LU09440b	45	21DEC10	22APR11	15.31	0.76	5.0
URCGNM3	MU09441b	45	21DEC10	22APR11	46.66	2.11	4.5



Summary Statistics for Urine O-DMA (ng/mL)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
URCDMH3	HU09442b	45	21DEC10	22APR11	204.64	8.46	4.1
URCDML3	LU09440b	45	21DEC10	22APR11	2.62	0.10	4.0
URCDMM3	MU09441b	45	21DEC10	22APR11	13.18	0.67	5.1



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