Laboratory Procedure Manual

Analytes:	**3-diethyl-carbamoyl benzoic acid, 3-ethyl-carbamoyl benzoic acid, acetamiprid-*N*-desmethyl, hydroxyimidacloprid isomers, flupyradifurone, and sulfoxaflor**
Matrix:	**Urine**
Method:	**DEET and Neonicotinoid Insecticides Biomarkers in Urine by Automated SPE-HPLC/+ESI MS/MS**
Method No:	**6124**

as performed by: Contemporary Pesticides and Flame Retardants Laboratory
Organic Analytical Toxicology Branch
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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:

Data File Name	Variable Name	SAS Label
DEET_J	URXDEA	3-(diethylcarbamoyl)benzoic acid (ug/L)
	URXMEA	3-(ethylcarbamoyl) benzoic acid (ug/L)

1. Clinical Relevance and Summary of Test Principle

N,N-Diethyl-m-toluamide, commonly known as DEET, is the principal ingredient in many personal insect repellents worldwide and is highly effective against a broad spectrum of insect pests, including potential disease vectors such as mosquitoes, biting flies and ticks (including many ticks that may carry Lyme disease). DEET was first developed and patented in 1946 by the U.S. Army for use by military personnel and later registered for general public use in 1975. Every year, approximately one-third of the U.S. population uses DEET-containing insect repellents, including more than 500 products registered with the Environmental Protection Agency in a variety of liquids, lotions, gels, sprays, sticks and impregnated materials with DEET concentrations ranging from 5 to 99% (EPA 2020 and 2022a).

Neonicotinoids are a class of insecticides chemically similar to nicotine. They are widely used in both urban and agricultural settings around the world. Neonicotinoids have been viewed as ideal replacements for more toxic pesticides, like methylcarbamates and organophosphates because of their low mammalian toxicity and their perceived limited potential to affect the environment and human health (Casida, 2018). Neonicotinoid insecticides are ubiquitous in food and the environment because of wide use (Chen et al., 2020). Available toxicological data from animal studies indicate possible genotoxicity, cytotoxicity, impaired immune function, and reduced growth and reproductive success at low concentrations (Thompson et al., 2020). Limited data from ecological or cross-sectional epidemiological studies have identified acute and chronic health effects ranging from acute respiratory, cardiovascular, and neurological symptoms to oxidative genetic damage and birth defects. Because of heavy use of neonicotinoids and potential for cumulative chronic exposure, further study to fully understand their potential risks to humans is of interest (Thompson et al., 2020).

We developed a method to measure concentrations of two urinary biomarkers of DEET: 3-diethyl-carbamoyl benzoic acid and 3-ethyl carbamoyl benzoic acid, two urinary biomarkers of neonicotinoids: acetamiprid-N-desmethyl and hydroxyimidacloprid isomers, and two neonicotinoid-like pesticides: flupyradifurone and sulfoxaflor, which share the same mode of action as neonicotinoids.

This method replaces a previously described method (Baker et al., 2019) incorporating changes in solid phase extraction (SPE) and mass spectrometry (MS). The analytical approach begins with an overnight enzymatic deconjugation of 200 µL urine, followed by extraction of the target compounds with a SPE weak cation exchange (WCX) sorbent on a 96-well plate platform, concentration to dryness with nitrogen, and reconstitution with acidified deionized water. The target compounds are separated using reversed phase high performance liquid chromatography (HPLC), ionized and de-solvated using a heated electrospray ionization (HESI) probe, and selectively filtered by mass-to-charge ratios at unit resolution via MS. Select target ions are then fragmented with collision induced dissociation (CID) and the resulting product ions are filtered at unit resolution before detection via an electron multiplier.

This method is designed to provide data in support of epidemiological studies and on specific occasions, may extend to quantifying concentrations for emergency responses, such as accidental poisonings. This information will continue to improve

our understanding of the extent of Americans' exposure to DEET, neonicotinoids, and neonicotinoid-like insecticides, whether exposure is changing in the United States, and whether these changes may affect the general population as a whole or in specific population groups. This method does not directly test for any disease.

2. Safety Precautions

a. Reagent hazards, Toxicity or Carcinogenicity
Several organic solvents are used in the method, precautions should be taken to: (1) Avoid contact with eyes and skin, (2) avoid use in the vicinity of an open flame, and (3) use solvents only in well-ventilated areas. Care should be exercised in handling of all solvent/solutions/chemicals.

β-Glucuronidase is a known sensitizer. Prolonged or repeated exposure to this compound may cause allergic reactions in certain sensitive individuals.

Note: Safety Data Sheets (SDS) for the chemicals and solvents used in this procedure can be found at http://www.ilpi.com/msds/index.html. Laboratory personnel must review the SDS prior to using chemicals.

b. Radioactive Hazards
There are no radioactive hazards to report with this method.

c. Microbiological Hazards
Although urine is generally regarded as less infectious than serum, the possibility of exposure to various microbiological hazards exists. Take appropriate measures to avoid contact with the specimen (see "Protective equipment" below). Observe universal precautions

d. Mechanical Hazards
Avoid direct contact with electronic components of all laboratory equipment and instrumentation. Only qualified technicians should perform electronic maintenance and repairs.

e. Protective Equipment
Standard personal protective equipment (PPE) should be utilized when performing this procedure. This includes lab coat, safety glasses, and nitrile/latex gloves.

f. Training
Personnel performing this method must have a basic understanding of analytical chemistry principles, chemical separation techniques, and competency to operate, maintain, troubleshoot, and correct various SPE prep stations, automated pipettors, mass spectrometer, and HPLC instrument problems that arise from daily operations.

g. Personal Hygiene
Care should be taken in handling any biological specimen. Routine use of gloves and proper hand washing should be practiced. No food or drink is allowed in laboratory areas.

h. Disposal of Wastes

All solvents, chemicals, and reagents must be disposed of according to CDC's guidelines. All disposable laboratory items that come in direct contact with biological specimens must be autoclaved before transport to land fields. All reusable laboratory items that come in direct contact with biological specimens must be decontaminated appropriately (for example, using a five percent bleach solution).

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

A working knowledge of Xcalibur™, the software controlling the HPLC-MS/MS system is required. In addition, a basic understanding of the Division-approved database called STARLIMS is required. Personnel performing this method must be able to create a run, create and export a sequence, and import the instrument data into STARLIMS.

b. Sample Information

Sample information related to the analysis of a given sample is tracked with a CDC-generated ID number. This number is used as a reference number to track the location and status of any sample.

c. Data Maintenance

The folder named Xcalibur™ on the instrument computer, which contains all MS and LC methods and procedures, along with all raw data, is backed up every 30 minutes to the Z: drive, which is connected to the Isolated Secure Laboratory Instruments Environment (ISLE) network. After processing data offline (i.e., integrating chromatographic peaks), ".RAW", ".RST", ".XQN", Excel Long reports, and STARLIMS-importable files (i.e., files that are created with a macro to make the Long Report file importable to STARLIMS database) are manually backed up on a CDC network drive. Lastly, the final mass spec Result data (i.e., the STARLIMSReady file) is imported into the STARLIMS database which is backed according to the policies laid out in the Division's Policy and Procedures Manual Continuity and Recovery section.

4. Procedures for Collecting, Storing, and Handling Specimens; Criteria for Specimen Rejection

a. Sample Collecting, Handling, and Storing

Urine can be collected in standard urine collection cups. Samples should be refrigerated as soon as possible and transferred to specimen vials preferably within 4 hours of collection. Specimen handling conditions are outlined in the Division of Laboratory Sciences (DLS) protocol for urine collection and handling (copies are available in the laboratory and in the DLS intranet). In the protocol, collection, transport, and special equipment required are discussed. In general, urine specimens should be shipped overnight in cryovials packed in boxes frozen and securely packed in dry ice. To minimize the potential degradation of the specimen, avoid prolonged exposure of the urine to room or refrigerator temperatures after collection. Freeze all

samples until analysis at or below -20 °C. Remaining urine should be refrozen after aliquots are withdrawn for analysis.

b. Sample Rejection

Reject specimens that have leaked, are broken or otherwise appear to be compromised or tampered with. Also, generally reject samples with volumes less than 0.2-mL because they cannot be reliably processed for this method.

5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

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

a. Reagent Sources

Solvents and other reagents (but not standards) of similar specifications but from other sources may also be used (Table 1).

Table 1. Reagents and Suggested Manufacturers

Reagents (common names)	CAS#	Suggested Manufacturers
Acetonitrile	75-05-8	Fisher Scientific
Methanol	67-56-1	Fisher Scientific
Formic Acid	64-18-6	Honeywell-Fluka
Acetic Acid	64-19-7	Sigma-Aldrich
Ammonium Formate	540-69-2	Sigma-Aldrich
Ammonium Acetate	631-61-8	Sigma-Aldrich
β-glucuronidase E. coli K-12 liquid *	9001-45-0	Roche
N'-[(6-chloropyridin-3-yl)methyl]-N-cyanoethanimidamide (Acetamiprid-N-desmethyl)	190604-92-3	ChemServ or Sigma Aldrich
Acetamiprid-N-desmethyl $^{2}H_3$,^{13}C,$^{15}N_2$	NA	CanSyn Chemical Corporation
3-diethyl-carbamoyl benzoic acid (DCBA)	72236-23-8	Cerilliant or CanSyn Chemical Corporation
D_{10}-3-diethyl-carbamoyl benzoic acid	NA	CanSyn Chemical Corporation
3-ethyl carbamoyl benzoic acid (ECBA)	126926-33-8	Cerilliant
D_5-3-ethyl carbamoyl benzoic acid	NA	CanSyn Chemical Corporation
3-[(6-chloropyridin-3-yl)methyl-(2,2-difluoroethyl)amino]-2H-furan-5-one (Flupyradifurone)	951659-40-8	HPC Standards Inc. or Sigma-Aldrich or Toronto Research Chemicals
Flupyradifurone D5	NA	Toronto Research Chemicals
(NE)-N-[1-[(6-chloropyridin-3-yl)methyl]-5-hydroxyimidazolidin-2-ylidene]nitramide (5-hydroxy-imidacloprid)	155802-61-2	EPP or IPA (Germany) or Cambridge Isotopes Laboratories
5-hydroxy imidacloprid-^{13}C, $^{15}N_2$	NA	Cambridge Isotopes Laboratories

4-Methylumbelliferyl β-D-glucuronide hydrate (UMB_glucuronide)	881005-91-0	Sigma-Aldrich
4-methylumbelliferone (UMB)	90-33-5	Sigma-Aldrich
4-methylumbelliferone- 2,3,4, Methyl-$^{13}C_4$	NA	Cambridge Isotopes Laboratories
[methyl-oxo-[1-[6-(trifluoromethyl)pyridin-3-yl]ethyl]-λ6-sulfanylidene]cyanamide (Sulfoxaflor)	946578-00-3	LGC/Dr. Erhrenstorfer or HPC Standards
Sulfoxaflor-$^{2}H_3$,$^{13}C_2$,$^{15}N_2$	NA	Cambridge Isotopes Laboratories

NA = not available or not applicable
* = approximately 140 international units per mg protein at 37 degrees C

b. Reagent Preparation

1. **1M ammonium acetate (pH 6.5) |Enzyme Buffer|**
   Suggested procedure: in a clean beaker, dissolve 7.7g of ammonium acetate (77g/mol) in 80mL of HPLC grade water. Add stir bar to mix thoroughly. Add acetic acid incrementally, mixing thoroughly, until pH is stable at 6.5. Record volume of acid used. Transfer volume to a 100 mL graduated cylinder. Add remaining HPLC grade water to reach final volume of 100mL. Mix thoroughly and transfer to storage container.

2. **β-glucuronidase from E. coli K-12 |Enzyme-Buffer Working Solution|**
   Suggested procedure: Add 1mL of liquid enzyme solution to 20mL of 1M Ammonium Acetate Solution pH 6.5. Gently mix. Aliquot 50µL for each 0.2 mL urine sample. If more enzyme solution volume is required, maintain reagent ratios.

3. **1M ammonium formate (pH 3.75) |Sample Equilibration Solution|**
   Suggested procedure: in a clean beaker, dissolve 16 g of ammonium formate (64 g/mol) in 200 mL of HPLC grade water. Add stir bar to mix thoroughly. Measure pH. Add formic acid incrementally, mixing thoroughly until pH is stable at 3.75. Record volume of acid used. Transfer volume to 250 mL graduated cylinder. Add remaining volume of HPLC grade water to reach 250mL. Mix thoroughly and transfer to storage container.

4. **10mM ammonium formate (pH 3.75) |SPE Equilibration Solution|**
   Suggested procedure: in a clean beaker, dissolve 0.32 g of ammonium formate (64 g/mol) in 400 mL of HPLC grade water. Add stir bar to mix thoroughly. Measure pH. Add 100% formic acid incrementally, mixing thoroughly until pH is stable at 3.75. Record volume of acid used. Transfer volume to 500 mL graduated cylinder. Add remaining volume of HPLC grade water to reach 500mL. Mix thoroughly and transfer to storage container.

5. **100mM ammonium formate (pH 3.75) |for making SPE Elution Solution|** Suggested procedure: in a clean beaker, dissolve 0.64 g of ammonium formate (64g/mol) in 80 mL of HPLC grade water. Add stir bar to mix thoroughly. Measure pH. Add 100% formic acid incrementally, mixing thoroughly until pH is stable at 3.75. Record volume of acid used. Transfer volume to a 100 mL graduated cylinder.

Add remaining volume of HPLC water to reach 100mL. Mix thoroughly and transfer to storage container.

6. **15:85 methanol/10mM ammonium formate pH 3.75 |SPE Wash Solution|**
   Suggested procedure: Combine 85 mL of 10mM ammonium formate (pH 3.75) to 15mL of methanol. Mix thoroughly and transfer to storage container.

7. **90:10 methanol/100mM ammonium formate pH 3.75 |SPE Elution Solution|**
   Suggested procedure: Using a 10 mL graduated cylinder, fill to 10 mL with 100mM ammonium formate (pH 3.75) and transfer volume into a 100 mL graduated cylinder. Add methanol to bring total volume to 100 mL. Mix thoroughly and transfer to storage container.

8. **0.02% formic acid |Sample Reconstitution Solution|**
   Suggested procedure: Using a 200 µL pipette, dispense 200 µL of formic acid into 1L (i.e., 999.8 mL in a graduated cylinder) of HPLC grade water. Mix thoroughly and transfer to a storage container.

9. **Liquid Chromatography Mobile Phase**
   Suggested procedure: Mobile Phase A = 0.02% Formic Acid – Add 200 µL formic acid to 999.8 mL HPLC grade water. Mix thoroughly. Mobile phase B =100% Acetonitrile.

c. Standards and Quality Control Materials Preparation

1. **Native Stock Solutions (1 mg/mL and 0.1 mg/mL)**
   Suggested procedure: Individually weigh approximately 10-30 mg of the target compound into a clean 40 mL glass vial. Record weight. Calculate the volume of acetonitrile needed to achieve a concentration of 1 mg/mL. Add this volume gravimetrically to the 40 mL glass vial. Vortex mix until solute is fully dissolved and goes into solution. Sonication may be required. Repeat for all target analytes except 5-hydroxyimidacloprid, and ECBA, which will not remain in solution at 1 mg/mL at room temperature. Recommend making 0.1 mg/mL solutions for these two analytes. Note: 5-hydroxy-imidacloprid, and ECBA are insoluble in acetonitrile at 1 mg/mL and are thus prepared at a lower concentration.

2. **Internal Standard Stock Solutions (1 mg/mL and 0.1 mg/mL)**
   Suggested procedure: Individually weigh approximately 10-30 mg of an isotopically labeled compound into a clean 40 mL glass vial. Record weight. Calculate the volume of acetonitrile needed to achieve a concentration of 1 mg/mL. Add this volume gravimetrically to the 40 mL glass vial. Vortex mix until solute is fully dissolved and goes into solution. Repeat for all labeled internal standard materials. Sonication may be required. Repeat for all isotopically labeled internal standard materials except labeled 3-ethyl-carbamoyl benzoic acid and labeled 5-hydroxy-imidacloprid, which require a final concentration <1 mg/mL. Recommend

making 0.1 mg/mL solutions for these two labeled compounds to avoid compounds coming out of solution at room temperature.

3. Native Standard Calibrator Solutions

Suggested procedure for Native spiking solutions: Combine individual native stock solutions of target analytes for a final concentration of 10 µg/L for all analytes except 3-ethyl carbamoyl benzoic acid (ECBA) which should be 100 µg/L and 3-diethyl carbamoyl benzoic acid (DCBA) which should be 200 µg/L and two sulfoxaflor isomers (Sulfoxaflor 1 and 2) which should be 10 µg/L each. Blowdown acetonitrile to dryness with gentle nitrogen stream. Reconstitute with HPLC grade water. This is the highest concentration (i.e., level) calibrator solution. Using either serial dilutions or gravimetric techniques, prepare 11 one to one dilutions with HPLC grade water for a total of 12 calibrators. In addition, prepare one calibrator solution with a concentration halfway between that of the S10 and S11 calibrator solutions and another calibrator solution halfway between the S11 and S12 calibrator solution concentrations. This can be done as a dilution of the stock "cocktail" or by a gravimetric technique. Note: the addition of the two additional calibrator levels at the upper portion of the calibration curve improve the accuracy of the regression curves for DCBA and flupyradifurone, which have instrument responses that are slightly quadratic. Final concentrations may vary slightly for each analyte, but the actual concentrations are updated in the quantitation method to maintain accuracy.

4. ISTD Spiking Solution

Suggested procedure for ISTD spiking solution: Combine individual ISTD stock solutions of DCBA_L, flupyradifurone_L, 5-hydroxyimidacloprid_L, ECBA_L, acetamiprid-N-desmethyl_L, sulfoxaflor_L, UMB_L ('_L' designates isotopically labeled compound), for final concentrations of 3 µg/L for all except sulfoxaflor_L which should be 6 µg/L. Include UMB_L and UMB_glucuronide for final concentrations of 30 µg/L to be used as deconjugation standards to monitor the extent of the enzymatic reaction.

5. Quality Control Materials

This method uses three types of quality control samples including Solvent (Water) Blank samples, Run Quality Control (QC) samples, and Proficiency testing (PT) samples. Solvent blank and Run QC samples are included in every analytical run. Solvent blanks are made of HPLC grade water. Run QC samples are made by spiking urine pools at predetermined concentrations. The urine pools are made by combining urine samples from anonymous individual donors and spiking predetermined amounts of target analytes to reach desired concentrations. PT samples are made like QC samples but have three concentration levels (Low, Medium, and High) spread throughout the linear range of the method. All quality control samples are treated identical to unknown samples.

6. Calibration-Verification Materials

CLIA defines testing calibration materials as "a solution which has a known amount of analyte weighed in or has a value determined by

repetitive testing using a reference or definitive test method." According to this definition, our quality control (QC) materials qualify as calibration verification materials. QC pools are made from urine samples that were anonymously donated, pooled, mixed and spiked with known amounts of the target analytes. Each pool should be characterized with at least 20 analytical runs (on 20 different days) to obtain mean and standard deviation values. QC samples, at two concentrations (i.e., low and high), are analyzed with each analytical run and serve as calibration verification.

d. Materials

1. Microtube (Simport) Polypropylene 2mL sample vials and lids
2. Phenomenex 2 mL, 96 deep well plates, conical, polypropylene
3. Phenomenex 96-well plate silicone injection mats
4. BIOTAGE ISOLUTE+ filter + 96-well plate, 25µm/0.2µm
5. BIOTAGE EVOLUTE EXPRESS WCX 30mg 96-well plate
6. BIOTAGE 300 mL Reservoirs, polypropylene
7. IDEX Health and Science A-100X Frit SS 2µm 0.94 X 0.65
8. Accucore aQ C18 endcapped 150 X 3mm, 2.6µm analytical column
9. Universal Uniguard Holder
10. Accucore aQ guard column 10x3.0mm 2.6µm Defender

e. Equipment

1. Aqua Solutions 2121BL Water Purification System
2. Balance (Sartorius, Genius series)
3. pH meter (Mettler Toledo Seven Compact).
4. Vortex Mixer (Fisher, Genie 2).
5. Magnetic Stirrer (Corning)
6. Integra VIAFLO 96
7. Biotage Extrahera Liquid Handler/SPE Prep System
8. Ultravap Mistral blowdown evaporator (Porvair Sciences)

f. Analytical instrumentation

1. Thermo Scientific™ Dionex™ Ultimate™ U3000 x2 Dual HPLC System including: DGP-3600RS Dual Ternary Rapid Separation Pump, SRD-3600 Integrated Solvent and Degasser Rack, WPS-3000TRS Autosampler, TCC-3000RS Column Thermostat Compartment equipped with one 2p-6p valve
2. Thermo Scientific™ Altis Triple Quadrupole Mass Spectrometer

7. Calibration and Calibration-Verification Procedures

a. Calibration Plot

For each analytical run, a calibration curve is generated by plotting the relative response factors (area native/area labeled IS) versus the standard concentrations with a 1/x weighting. The slope and intercept of each analyte's curve is determined using a linear least squares regression (except for analytes DBCA and flupyradifurone, which are quadratic) with Xcalibur™ software. The lowest concentration of the calibration curve is at or below the method limit of detection and the highest point is at or above the expected range of results.

b. Calibration Verification

1. Calibration verification should be performed after any substantive changes in the method or instrumentation (e.g., new internal standard, change in instrumentation), which may lead to changes in instrument response, have occurred.

2. Calibration verification must be performed at least once every 6 months.

3. According to the updated CLIA regulations from 2003 (http://www.cms.hhs.gov/CLIA/downloads/6065bk.pdf), the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes low, mid, and high calibration-verification materials, and is performed at least once every six months.

4. For this method, analytical runs generally include 8-14 standard calibrators for each analyte and three levels of calibration-verification materials (i.e., QC blank, QC low, and QC high).

5. All calibration verification data are documented in the STARLIMS database.

6. All of the conditions above are met with the calibration procedures for this method. Therefore, no additional calibration verification is required by CLIA.

c. Proficiency Testing (PT)

Proficiency testing should be performed a minimum of once every 6 months. Because no formal PT testing program exists for most of the target analytes of this method, an in-house program is used. This in-house program currently includes pools prepared in-house but could also include independently prepared materials whose preparation was contracted out to an external laboratory. Where applicable, NIST matrix-based certified reference materials may be included as PT materials.

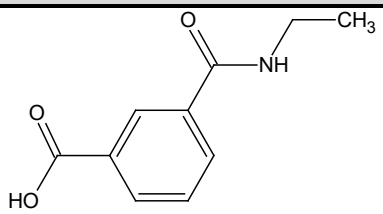
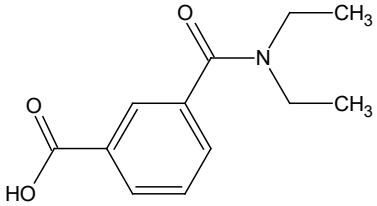
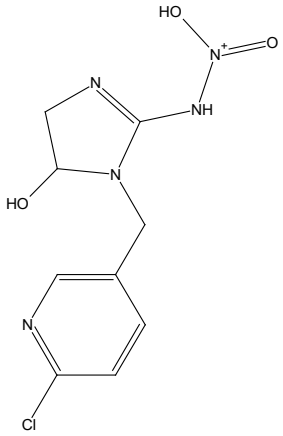
In-house proficiency testing

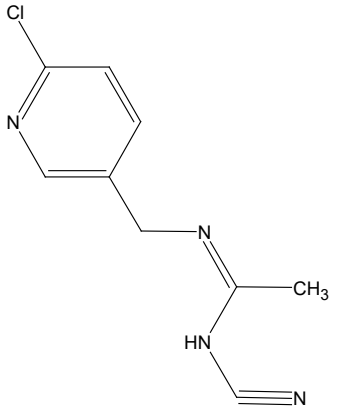
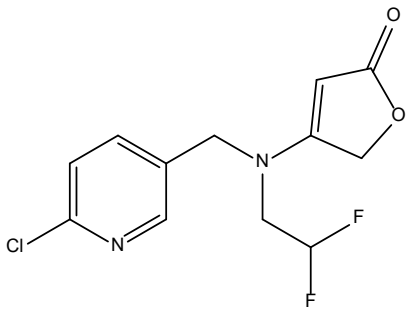
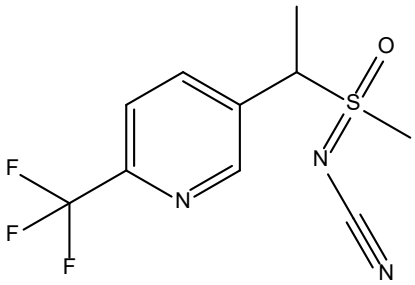
PT sample materials, or pools, are prepared in-house as described in section 6.c (Standards and Quality Control Materials Preparation). These PT samples encompass the measurable concentration ranges of the method and are characterized in our laboratory. The characterization data are forwarded to the division statistician (acting PT administrator) in charge of executing the PT

program. The PT administrator establishes the mean and confidence limits for each analyte concentration. A PT challenge should be performed every six months. When PT is required, the laboratory supervisor or his/her designee will notify the PT administrator who will randomly select five PT materials for analysis, at least one from each concentration level. The PT samples are treated as unknown samples and the analytical results are forwarded directly to the PT administrator for interpretation. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the administrator. The PT administrator will notify the laboratory of its PT status (i.e., pass/fail). All proficiency test results must be appropriately documented.

External proficiency testing
External proficiency testing for the analytes included in this method is not available.

8. Analyte Nomenclature and Structures

Structure	Common Name/NHANES Code	IUPAC name
	3-ethyl carbamoyl benzoic acid (NHANES: MEA)	3-ethyl carbamoyl benzoic acid (ECBA)
	m-toluic-acid (NHANES: DEA)	3-diethyl-carbamoyl benzoic acid (DCBA)
	5-hydroxy-imidacloprid (NHANES: OHIM)	1-[(6-Chloro-3-pyridinyl)methyl]-2-(nitroamino)-4,5-dihydro-1H-imidazol-5-ol

	Acetamiprid-N-desmethyl (NHANES: NDAC)	(1E)-N'-[(6-Chloro-3-pyridinyl) methyl]-N-cyanoethanimidamide
	Flupyradifurone (NHANES: FLUP)	3-[(6-chloropyridin-3-yl) methyl-(2,2-difluoroethyl) amino]-2H-furan-5-one
	Sulfoxaflor (NHANES: SLF1, SLF2)	[methyl-oxo-[1-[6-(trifluoromethyl)pyridin-3-yl]ethyl]-λ6-sμLfanylidene]cyanamide

9. Operating Procedures; Calculations; Interpretation of Results

a. Preliminaries

(1) A batch normally consists of 14 calibrators (number of calibrators used varies among different analytes), one solvent blank without labeled internal standard (ISTD), one solvent blank with ISTD, four quality control samples (i.e., two low and two high concentrations, which are spread randomly throughout the batch), and up to 76 urine samples.

(2) Urine samples and quality control (QC) materials are thawed at room temperature before sample preparations.

(3) Thawed urine and quality control samples are vortex-mixed prior to sample preparation.

b. Sample preparation

(1) Day 1 General Overview: the first day of sample preparation consists of aliquoting urine samples, quality control samples, calibrators, internal standard, and enzyme solution into a 96-well plate using an Integra Viaflo 96 automated pipettor.

(2) Day 2 General Overview: a Biotage Extrahera Automated Sample Preparation System performs three major steps: filters the Day1 samples, performs solid phase extraction (SPE), and finally reconstitutes wells with 0.02% formic acid after an evaporation step using a Porvair Ultravap Mistral Evaporator. The 96-well plate is then loaded into a LC autosampler for analysis on a triple quadrupole mass spectrometer.

(3) More specific steps: On Day 1 remove standard calibrators, quality control materials and unknown urine samples from the storage freezer and allow them to come to room temperature. When samples are at room temperature, vortex samples, then use an Integra VIAFLO 96 (program name = 6124) to pipette 200 µL of urine sample, 200 µL of quality control samples, 200 µL internal standard spiking solution (exclude water-only blank sample), 200 µL of calibrator solutions and 50 µL of enzyme buffer solution into predetermined positions in a 96 deep-well plate.

Integra VIAFLO 96 Program named “6124” steps are as follows:

Step1= MOVE X,Z (-81.4, 161.3)
Step2=ASPIRATE 200 µL speed 6
Step3=MOVE Z(86mm)
Step4=ASPIRATE 200 µL, speed6
Step5=MOVE X,Z(-80.4,192.4)
Step6=MOVE X,Z(79.4, 92.7)
Step7=DISPENSE 200 µL speed6
Step8=MOVE Z (112.7mm)
Step9=DISPENSE 200 µL speed 7
Step10=MOVE X,Z (78.9, 192.4)
Step11=MOVE X,Z (-93.6, 192.4)

Integra VIAFLO 96 Program named “6124_Enzyme” steps are as follows:

Step1= MOVE X,Z (-81.4, 161.3)
Step2=ASPIRATE 50 µL speed 6
Step3=MOVE Z(81mm)
Step4=ASPIRATE 50 µL , speed6
Step5=MOVE X,Z(-80.4,192.4)
Step6=MOVE X,Z(79.4, 92.7)
Step7=DISPENSE 50 µL Speed6
Step8=MOVE Z (112.7mm)
Step9=DISPENSE 50 µL speed 7
Step10=MOVE X,Z (78.9, 192.4)
Step11=MOVE X,Z (-93.6, 192.4)

(4) To accommodate spiking from 10 mL sample vials into each 2mL well of the 96-well plate using the Integra VIAFLO 96, custom-made 24-position plates were made (i.e., fabricated at CDC with a 3D printer) that allow all 96 wells to be pipetted into from four custom-made 24-position plates with standard 96-well plate outer dimensions. Each plate is color coded (i.e., Green, Yellow, Pink, and Blue). Each of the positions in each of the four unique 24-position plates corresponds to a different set of 24 of 96 positions in a standard 96-well plate such that all 96 positions can be aliquoted into from the four 24-position plates. After each aliquot from one 24-position plate, the user inserts the next 24-position plate to accommodate the next 24 aliquots. Etc. The GREEN 24-position plate is used to spike rows A, C, E, and G of odd numbered columns 1, 3, 5, 7, 9, and 11. The YELLOW 24-position plate is used to spike the same rows (i.e., A, C, E, and G) but to even numbered columns 2, 4, 6, 8, 10, and 12. The PINK 24-position plate is used to spike rows B, D, F, and H of odd numbered columns 1, 3, 5, 7, 9, and 11. The Blue 24-position plate is used to spike rows B, D, F, and H into even numbered columns 2, 4, 6, 8, 10, and 12. Four corresponding color-coded custom-made tip loading guides are placed over the tip rack when loading fresh tips to ensure accurate placement of tips for each of the four 24-position plates before loading tips onto the Integra ViaFlow096 head. Each sample is vortex-mixed, scanned into an Excel spreadsheet, and placed in its defined position of the 24-position plate before aliquoting. The Excel spreadsheet automatically keeps track of each scanned ID, the position in the 24-position plate, and the position it will be aliquoted into in the 96-well plate. From this spreadsheet an instrument sequence (i.e., a csv file) is created that is transferred to the mass spectrometer computer (via CDCs Isolated Secure Laboratory Environment (ISLE) network) and used to create the final sequence (i.e., ".sld" file) in Xcalibur™. After all samples, calibrators, QC materials, and reagents have been aliquoted into the 96-well plate it is gently vortex-mixed, covered with a mat or aluminum foil, and incubated at 37°C overnight.

On Day 2, there are four major steps to complete the sample preparation:

Step 1: Remove the plate from the incubator and transfer to the Biotage Extrahera. Run Program "Neo Isolute filter+". This program pipettes 1000 µL of 1M ammonium acetate pH 3.75 into each well, mixes with multiple aspirate/dispense cycles, transfers the contents to an ISOLUTE Filter+, and pushes the contents through the filter with positive pressure and collects the contents in a clean 96-well plate. This filter step removes solid particles larger than 0.2 um. The settings for this program are below.

Settings for "Neo Isolute Filter+" program:

Sample Plate/Rack: 2mL Sample Plate, 96
Extraction Media: Isolute Filter+
Pretreatment: On
Collection: On

<u>Sample tab:</u>

Sample Type: Neo Enzymes

Starting Sample Volume in plate/rack (µL): 500

Reuse Tips for pretreatment mix and sample load: Yes

Sample pipette tip type: 1000 µL Biotage tip

Pretreatment tab
Number of Steps: 1
Solvent: 1M Ammonium Formate pH 3.75
Volume (µL): 1000
Mix Number of Times: 0
Mix volume (µL): 800
Wait Time (min): 0
Pause After Last Step: No
Dispose Solvent Tips After Each Step: No

Collection tab
Sample Volume (µL): 1500
Premix: Yes
Number of Times: 5
Pressure (bar): 5
Positive Pressure Time (s): 360
Collect in Position: A

Step 2: Run Biotage Extrahera program, "Neo SPE". This program performs solid phase extraction. Load reagents into 100 mL solvent trays (i.e., HPLC grade water, 100% Methanol, 10 mM ammonium formate, 15% Methanol in 10 mM ammonium formate, and 90% Methanol in 10 mM ammonium formate). Below are the settings for program "Neo SPE":

Program "Neo SPE" Settings

Sample plate/rack: 2 mL Sample Plate, 96
Extraction media: Evolute WCX Express 96-Well Plate
Pretreatment: Off
Conditioning: On
Equilibration: On
Load: On
Wash: On
Elution: On

Sample
Sample Type: Neo Enzymes
Starting Sample Volume in plate/rack (µL): 1500
Reuse Tips for pretreatment mix and sample load: No
Sample pipette tip type: 1000 µL Biotage tip

Conditioning tab
Number of Steps: 1
Pressure (bar): 3.0
Solvent: Methanol
Volume (µL): 700
Collect in Position: D (waste)

Use Advance Pressure Settings: Yes
Number of Steps: 2
Pressure (bar): 3
Positive Pressure Time (s): 60
Pressure (bar): 5
Positive Pressure Time (s): 60
Repeat (Number of Times): 1
Pause After This Step: No
Dispose Solvent Tips After Each Step: No

Equilibration
Number of Steps: 1
Pressure (bar): 3.0
Solvent: 10mM Ammonium Formate pH 3.75
Volume (µL): 700
Collect in Position: D (waste)
Positive Pressure time (s): 60
Repeat (Number of Times): 1
Pause After This Step: No
Dispose Solvent Tips After Each Step: No

Load
Sample Volume (µL): 1500
Pressure (bar): 3.0
Use Advance Pressure Settings: Yes
Number of Steps: 3
Pressure (bar): 1
Positive Pressure Time (s): 90
Pressure (bar): 3
Positive Pressure Time (s): 60
Pressure (bar): 5
Positive Pressure Time (s): 60
Rinsing: No
Premix: Yes
Number of Times: 2
Pause After Each Load: No
Collect in Position: D (waste)
Tip Conditioning: No

Wash
Number of Steps: 1
Pressure (bar): 0
Solvent: 15% Methanol
Volume (µL): 700
Collect in Position: D (waste)
Use Advance Pressure Settings: Yes
Number of Steps: 3
Pressure (bar): 1
Positive Pressure Time (s): 60
Pressure (bar): 3
Positive Pressure Time (s): 60
Pressure (bar): 5

Positive Pressure Time (s): 60
Repeat (Number of Times): 1
Pause After This Step: No
Plate Dry After Last Wash: No
Plate Dry Time (s): 0
Dispose Solvent Tips After Each Step: No

Elution
Number of Steps: 1
Pressure (bar): 0
Solvent: 90% Methanol
Volume (µL): 700
Collect in Position: A
Use Advance Pressure Settings: Yes
Number of Steps: 3
Pressure (bar): 1
Positive Pressure Time (s): 60
Pressure (bar): 3
Positive Pressure Time (s): 60
Pressure (bar): 4
Positive Pressure Time (s): 60
Repeat (Number of Times): 1
Pause After This Step: No
Plate Dry After Last Wash: No
Plate Dry Time (s): 0
Dispose Solvent Tips After Each Step: No

Step 3: Transfer the Elution 96-well plate from the Biotage Extrahera and place it in the Ultravap Mistral evaporator. Run program, “Method 6124 Dryness”. Step three is used to evaporate solvents in the wells down to approximately 25-45µL.

Table 2. Mistral Program Settings

	Start (mm)	End (mm)	Minutes	°C	L/min
Stage 1	30	30	9	40	70
Stage 2	30	31	9	40	70
Stage 3	31	33	9	40	75
Stage 4	33	36	9	40	75
Stage 5	36	39	9	40	80

Step 4: Transfer the 96-well plate from the evaporator back to the Biotage Extrahera, load sufficient volume of 0.02% formic acid in a 100 mL solvent tray, and run program, “Neo Reconstitute”. This final program pipettes 350 µL of 0.02% formic acid into each well of the 96-well plate to reconstitute the sample extract volume to make it compatible with the LC mobile phase and analytical column characteristics. The 96-well plate is then removed from the Biotage Extrahera and a sealing mat (with slits) is applied. After

the plate is vortex mixed it is placed in the LC/MS autosampler for injection.

c. Instrumental analysis

(1) Instrument Performance (Chromatography and Sensitivity Check)

Before an analytical run is started on the HPLC-MS/MS system, an instrument check sample (e.g., an HPLC grade water-based pool with a concentration near the lowest standard) is injected and results are reviewed to confirm acceptable chromatographic peak shape, detector sensitivity, chromatographic resolution and retention times before an analytical run is started. If retention times have shifted outside the acquisition windows (for example, when a new analytical column has been installed), mass spectrometer acquisition segment times are updated in the acquisition method accordingly to accommodate the altered retention times of the affected analytes.

(2) HPLC-MS/MS analysis

The automated HPLC-MS/MS analysis is performed using:

Thermo Scientific™ Dionex™ Ultimate™ 3000U system including:
DGP-3600RS Dual Pump HPLC
WPS-3000RS/TRS Temperature Controlled Autosampler
TCC-3000RS Rapid Separation Thermostatted Column Compartment

Coupled to:

Thermo Scientific™ Altis Triple Quadrupole Mass Spectrometer equipped with a HESI (Heated Electrospray ionization) interface

The HPLC system and the mass spectrometer are both controlled by Xcalibur™ software.

LC Columns used:

A Thermo Scientific™ (San Jose, CA) Accucore aQ C18 endcapped 150 X 3mm, 2.6µm column is used for the analytical separation along with a matching guard column.

Table 3. HPLC separation gradient elution

Time (min)	Flow Rate (mL/min)	%A (0.02% formic acid)	%C (100% acetonitrile)
0.0	0.7	91	9.0
8.5	0.7	76.3	23.7
8.6	0.7	10	90.0
9.6	0.7	10	90.0

9.7	0.7	91	9.0
15.0	0.7	91	9.0

Mass Spectrometer Ion Source Properties:
Ion Source Type: HESI
Spray Voltage Positive: 2500 (V)
Spray Voltage Negative: 2000 (V)
Sheath Gas (Arb): 55
Aux Gas (Arb): 7
Sweep Gas (Arb): 2
Ion transfer Tube Temp (°C): 375
Vaporizer Temp (°C): 400

(3) **Multiple Reaction Monitoring (MRM) Setup for DEET and Neonicotinoids biomarkers.**

During an analysis, the instrument is set in the multiple reaction monitoring mode so that precursor and product ion combinations, specific to the eluting analyte, can be monitored. Reproducible chromatography allows for the use of different data acquisition windows for different analyte groups. Product ions are formed from the precursor ions in the collision cell using argon setting at 1.5 mTorr. The collision energy is set for each ion (Table 4).

Table 4. Mass ion transitions, collision energies, and dwell times for target analytes.

Analyte	Ion Type	Precursor Ion	Product Ion	Collision Energy [V]	Dwell Time (ms)
DCBA	Q	222	149	27	25
	C	222	121	12	25
	ISTD	232	149	19	25
ECBA	Q	194	149	15	25
	C	194	121	23	25
	ISTD	199	149	17	25
Acetamiprid-N-desmethyl	Q	209	126	17	25
	C	209	90	31	25
	ISTD	215	126	17	25
5-hydroxyimidacloprid	Q	272	225	16	25
	C	272	191	19	25
	ISTD	275	229	17	25
UMB	Native	177	105	17	75
	ISTD	181	123	17	75
Flupyradifurone	Q	289	126	20	25
	C	289	90	38	25
	ISTD	294	126	20	25
Sulfoxaflor*	Q	276	213	15	100
	C	276	212	15	100
	ISTD	283	220	15	100

Q = Quantitation ion
C = Confirmation ions

ISTD = Isotopically-labeled internal standard
S-Lens set to “Calibrated RF Lens values”
*SLF is in negative mode, all others in positive mode

d. Calculations

The concentration of individual target analytes in each sample is calculated using the calibration curve equation derived from a regression analysis of the response ratios (area counts of the native / area counts of the ISTD) versus the known concentrations of standard calibrators. A calibration curve is included with each analytical run and used by the Xcalibur™ data analysis software to quantify concentrations of all target analytes in unknowns, QCs, and blank samples.

10. Method Performance Documentation

a. Accuracy

The accuracy of this method was determined after spiking two biological materials with target analytes at zero concentration and three additional levels, i.e., low, medium and high. Spiked concentrations were spread throughout the linear range. Three replicates were spiked per concentration resulting in a total of 12 samples per quality control material per analytical run. The 12 spiked samples for each quality control material were prepared according to the method on two separate days and analyzed in two separate analytical runs on two separate days. Recovery of the spiked analytes was calculated as [(final concentration - initial concentration)/spiked concentration]. Acceptable recovery should be 85-115% except at 3*LOD where it can be 80-120%. Any deviations from these ranges require approval from the CLIA Director.

Table 5. Accuracy

Analyte	Accuracy of Concentration measurements	
	Concentration (µg/L)	Mean Recovery (%)
DCBA	12.5 25 50	94.1
ECBA	12.5 25 50	94.4
Acetamiprid-N-desmethyl	2 4 8	99.8
5-hydroxy-imidacloprid	2 4 8	99.7
Flupyradifurone	2 4 8	86.5
Sulfoxaflor 1	2 4	94.3

	8	
Sulfoxaflor 2	2 4 8	88.8

b. Precision

Precision was determined by calculating the relative standard deviation (RSD) of repeat measurements (N=20) of quality control materials at two concentrations, performed in duplicates during 10 different analytical runs on 10 different days. Within-run, between-run and total precision were calculated from these data. Acceptable RSD should not exceed 15%. Any deviations from these ranges require approval from the CLIA Director.

Table 6. Precision

Analyte	Average Concentration (µg/L)	RSD(%)
DCBA	2.7	7.3
	55.9	7.2
ECBA	2.3	6.2
	27.7	7.1
Acetamiprid-N-desmethyl	2.6	6.5
	7.5	4.4
5-hydroxyimidacloprid	2.5	6.4
	6.2	5.9
Flupyradifurone	2.2	5.5
	5.4	5.0
Sulfoxaflor 1	2.2	5.6
	5.9	4.2
Sulfoxaflor 2	2.2	4.0
	5.7	4.1

c. Analytical Sensitivity (Method Limit of Detection)

Analytical sensitivity is the lowest analyte concentration that can be measured with acceptable accuracy and precision and is expressed as the limit of detection (LOD). An LOD is not acceptable below the lowest viable calibrator. LODs are calculated as $3S_0$, where S_0 is the estimated standard deviation (SD) at zero concentration and is determined by linear regression analysis of the absolute standard deviation (SD) at concentrations near the LOD versus calibrator concentration (Taylor 1987). The detection limits vary based on the current

operating precision and the cleanliness of the analytical system. The reported LOD can be higher than these values if the program lead feels this is necessary, but the value cannot be below these values. LODs may vary over time.

Table 7. Analyte Detection Limits

Analyte	LOD (µg/L)
DCBA	0.10
ECBA	0.10
Acetamiprid-N-desmethyl	0.05
5-hydroxyimidacloprid	0.10
Flupyradifurone	0.05
Sulfoxaflor 1	0.05
Sulfoxaflor 2	0.05

d. Analytical Specificity

This method requires that the analytes: 1) must form a positive $(M+H)^+$ ion with electrospray ionization (except sulfoxaflor which must form a negative $(M-H)^-$ ion); 2) produce two collision-induced dissociation product ions (i.e., quantitation ion and confirmation ion). The confirmation ion is used above pre-determined concentrations for confirmation, for each analyte, within pre-determined acceptable ion ratio ranges; and 3) co-elute with (or relative to) the corresponding isotope labeled internal standard analog. The quantitation and confirmation ions for each analyte are listed in Table 4. Potential interfering substances were evaluated during method development using approximately 50 convenience urine samples collected anonymously (STAR ID 0900f3eb81c6ff5e).

e. Stability (Freeze-Thaw, Bench Top, Processed Sample, and Long-term)

Freeze-thaw stability was determined by comparing analyte concentrations before and after three subsequent freeze-thaw cycles, for two quality control materials. For each quality control material, n=3 replicate measurements were made after three freeze-thaw cycles and compared to the characterized mean of each control material.

Bench-top stability was assessed by comparing analyte concentrations in two quality control materials before and after materials were stored at room temperature for one day. For each pool, n=3 replicate measurements were made

initially and n=3 measurements were made after materials were stored at room temperature for one day.

Processed Sample stability was assessed by comparing analyte concentrations in two quality control materials before and after materials had been extracted and left at room temperature for a day. For each pool, n=3 measurements were made initially and n=3 measurements were made after prepared samples were stored at room temperature for one day.

For each stability test, characterized values were used for "initial measurements" in subsequent calculations. Long term stability of the analytes should be assessed by analyzing two quality control samples after freezer storage for an extended time. This method has not been active long enough for this assessment.

Table 8. Stability results expressed as percent relative standard deviations of the concentrations of the materials used for the test; n=3 for all measurements

Analyte	Material 1			Material 2		
	Three freeze-thaw cycles	Bench-top stability	Processed sample stability	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
DCBA	-11.5	-10.8	-5.9	-7.0	-7.4	-4.5
ECBA	-5.3	-6.4	-2.8	-6.6	-0.9	-1.7
Acetamiprid-N-desmethyl	-3.3	-6.7	-5.9	-4.2	-3.1	-4.3
5-hydroxyimidacloprid	-1.3	-2.0	-3.7	2.8	-1.1	0.0
Flupyradifurone	-5.2	-2.3	7.7	1.7	0.2	11.0
Sulfoxaflor 1	-3.8	-5.5	-2.3	-0.9	-3.2	-2.7
Sulfoxaflor 2	-8.5	-10.0	-6.8	-6.2	-5.3	-4.8

f. Reportable Range of Results

The reportable range of results is set by the method limit of detection at the low end and by the highest concentration standard at the high end. Samples with analyte values exceeding the highest standard may be diluted, re-extracted, and reanalyzed so that the measured value will be within the acceptable range and the dilution factor used to calculate the correct concentration. The LODs (in µg/L) are as follows: 0.05 for Flupyradifurone, Acetamiprid-N-desmethyl, and Sulfoxaflor 1 and 2; 0.1 for DCBA, ECBA, and 5-hydroxyimidacloprid. The highest concentration standard for each analyte is 10 µg/L, except for DCBA which is 200 µg/L and ECBA which is 100 ug/L.

g. Linearity Limits

"In the method validation, linearity of the measurement range (e.g., from lowest to highest calibrator) should be verified by visual examination of a residual

regression plot (assuring no curvilinear shape of residuals) and by an R^2 that exceeds 0.98" (DLS Policies and Procedures Manual, January 2023).

h. Reference Range (normal range)

National Health and Nutrition Examination Survey (NHANES) data are used to describe exposure to chemicals (or their precursors) among the general U.S. population.
The reference ranges for 3-(diethylcarbamoyl)benzoic acid, 3-(ethylcarbamoyl)benzoic acid, 5-hydroxy imidacloprid, and Acetamiprid N-desmethyl can be found at: https://www.cdc.gov/exposurereport/

11. QC Procedures

a. QC procedures for Individual samples (i.e., standards, unknown samples, and quality control (QC) materials)

(1) The relative retention time (RRT) of standards, unknowns, and QCs should be within a specified range. If the RRT falls outside the range, check the RT(s) of the peaks of analyte and IS to make sure the program picked the correct peak for integration.

(2) The area counts of IS for each analyte should be within a defined range. Low IS area counts could indicate ion suppression from sample matrix, or a spiking error. For example, high IS counts could indicate a double spike. Depending on the findings, the sample may need to be reanalyzed.

(3) The calculated concentration of the reagent blank should be less than three times the method LOD. Values exceeding this level could indicate a potential contamination in the reagents used for sample preparation and (or) mobile phases. Samples failing this test should be reanalyzed.

(4) In the absence of interfering compounds, the ratio of the calculated concentration of the quantitation ion divided by the calculated concentration of the confirmation ion, for a given analyte, should follow the same general ratio.

(5) The area count ratio of UMB and UMB_L (IS) for the unknown samples should be greater than a pre-determined value. This area ratio is used to monitor the activity of the enzyme used for deconjugation in each sample.

(6) Unknown samples, for which concentrations of the analytes fall below the LOD, may be re-analyzed to confirm that urine was successfully aspirated from the vial (or well) and fully injected by the autosampler.

(7) When sample (A+1) run after a sample (A) which contained a high concentration of any given analyte (e.g., ~ ppm levels), sample (A+1) might have to be repeated to eliminate the possibility of

carryover. If the calculated carryover amount (0.05 % x concentration of sample A) is greater than 30% of the calculated concentration of sample (A+1), sample (A+1) may need to be reanalyzed.

(8) If a given analyte's response ratio in an unknown sample is above the response ratio of the highest calibration standard, the sample needs to be re-analyzed with a smaller amount of urine or a dilution.

b. Analytical batch quality control procedures

QC pools are characterized to determine the mean and the 95th and 99th control limits. QC characterization should include at least 20 discrete measurements spanning over at least 20 days prior to analysis of unknown samples. Standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control (Caudill et al., 2008).

When using 2 QC pool levels (1QCL and 1 QCH) per run, the rules are:

(1) If both QC run results are within $2S_i$ limits, then accept the run.
(2) If 1 of the 2 QC run results is outside a $2S_i$ limit - reject run if:
- Extreme Outlier – Run results is beyond the characterization mean $\pm$ $4S_i$
- 3S Rule – Run result is outside a $3S_i$ limit
- 2S Rule – Both run results are outside the same $2S_i$ limit
- 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
- R 4S Rule – Two consecutive standardized run results differ by more than $4S_i$ (standardized results are used because different pools have different means). Since runs have single measurements per pool for 2 pools, comparison of results for the R 4S rule will be with the previous result within run or the last result of the previous run.

When using 2 QCs per QC pool levels (2QCL and 2 QCH) per run, the rules are:

(1) If both QC run means are within $2S_m$ limits and individual resµLts are within $2S_i$ limits, then accept the run.
(2) If 1 of the 2 QC run means is outside a $2S_m$ limit - reject run if:
- Extreme Outlier – Run mean is beyond the characterization mean $\pm$ $4S_m$
- 3S Rule– Run mean is outside a $3S_m$ limit
- 2S Rule – Both run means are outside the same $2S_m$ limit
- 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

(3) If one of the 4 QC individual results is outside a $2S_i$ limit - reject run if:

(4) R 4S Rule – Within-run ranges for all pools in the same run exceed $4S_w$ (i.e., 95% range limit). Since runs have multiple measurements per pool for 2 pools, the R 4S rule is applied within runs only.

Abbreviations:

S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).
S_m = Standard deviation of the run means (the limits are shown on the chart).
S_w = Within-run standard deviation (the limits are not shown on the chart).

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

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until the source or cause of failure is identified and corrected. Check for any irregularities (e.g., low calibration curve regression coefficient, change in calibration curve slope or intercept, high reagent blank concentration, low internal standard sensitivity). If the source of failure is easily identifiable, for example, a pipetting error, correct the problem immediately. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, re-analyze several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure). After re-establishing calibration or QC, resume analytical runs. All CLIA methods conducted in the Organic Analytical Toxicology Branch follow the Division-wide guidelines for Quality Improvements, Corrective Actions and Preventive Actions (QI/CAPA) described in STARLIMS document SG-017.

13. Limitations of Method; Interfering Substances and Conditions

On occasions, interfering substances may co-elute with target analytes, biasing the measured amount beyond acceptable values. If repeat analysis still results in an interference the concentration for the target analyte is set to not reportable.

14. Reference Ranges (Normal Values)

The results from the National Health and Nutrition Examination Survey (NHANES) can be used as reference ranges for the general U.S. population (https://www.cdc.gov/exposurereport/)

15. Critical-Call Results (“Panic” Values)

There are currently no critical-call values established for these target analytes.

16. Specimen Storage and Handling during Testing

Specimens are stored frozen in the laboratory prior to analysis. Frozen samples are thawed at room temperature prior to the initiation of the procedure.

17. Alternate Methods for Performing Test and Storing Specimens if Test System Fails

If the test system fails, prepared samples can be stored frozen in sealed 96-well plates (or autosampler vials, if being used) for an extended period of time until the analytical system is restored. Otherwise, samples can be re-extracted.

There are currently no alternate methods for measuring the target analytes.

18. Test-Result Reporting System; Protocol for Reporting Critical Calls (if Applicable)

a. The data from analytical runs of unknowns are initially reviewed by analyst, then by the program lead and finally by the laboratory supervisor. The supervisor provides feedback to the program lead and/or his/her designee and requests confirmation of the data as needed.

b. The quality control officer reviews each analytical run and determines whether the analytical run is performed under acceptable control conditions.

c. One of the Division statisticians' reviews and approves the quality control charts pertinent to the results being reported.

d. If the quality control data are acceptable, the laboratory supervisor or his/her designee generates a letter from the CLIA Laboratory Director to the person(s) who requested the analyses reporting the analytical results.

e. The final data are then sent (generally electronically by e-mail) to the person(s) who made the initial request.

f. All data (chromatograms, etc.,) are stored in electronic format.

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

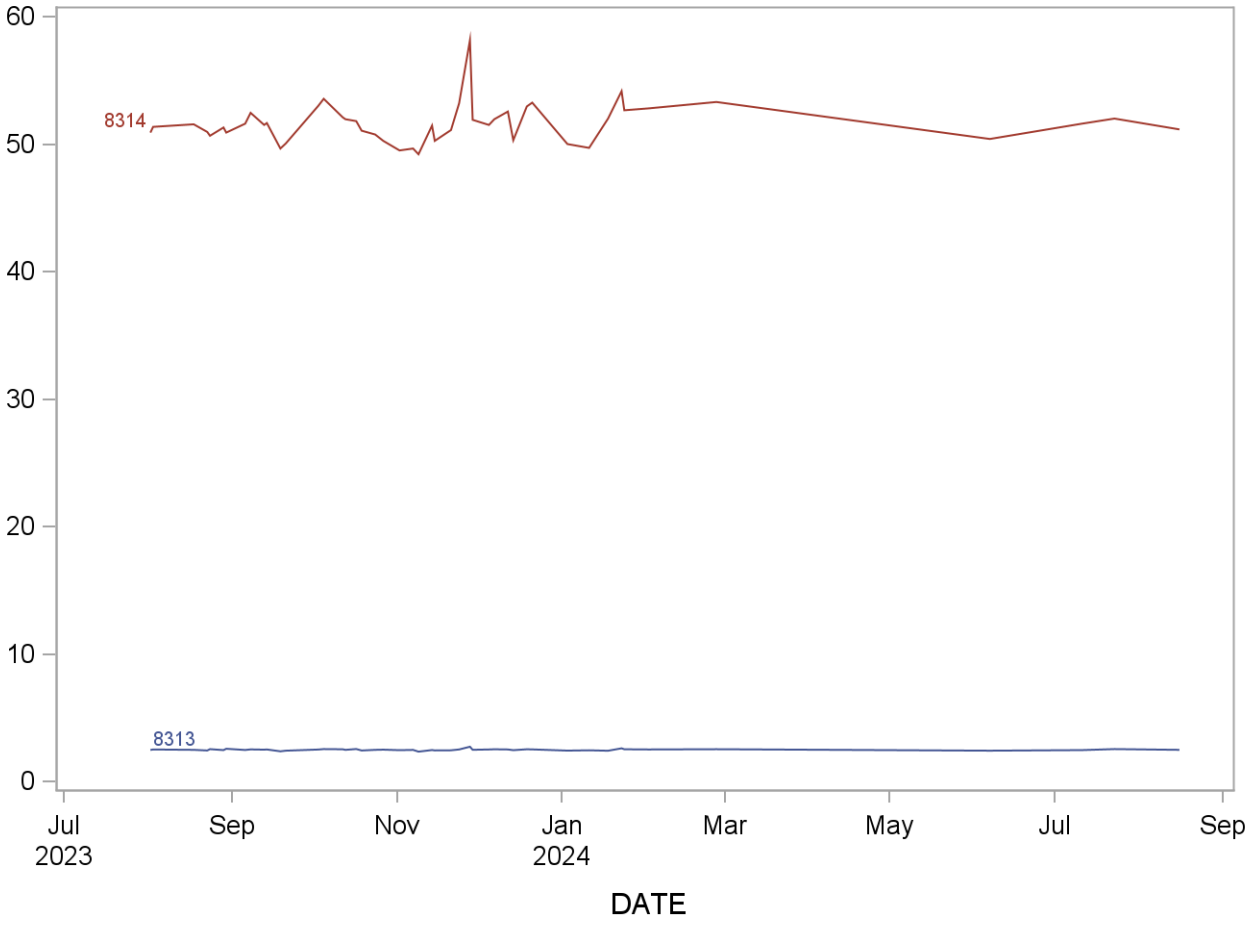
An Excel spreadsheet with information for receiving/transferring specimens is kept in an electronic form on the laboratory's shared workspace. In this form, samples are logged in when received. This spreadsheet also includes information regarding sample storage location, relevant inventory file(s), and if any samples are transferred out of the possession of the laboratory. Transfer of specimens is facilitated through the DLS Sample Logistics Laboratory.

20. Summary Statistics and QC Chart

Please see following pages.

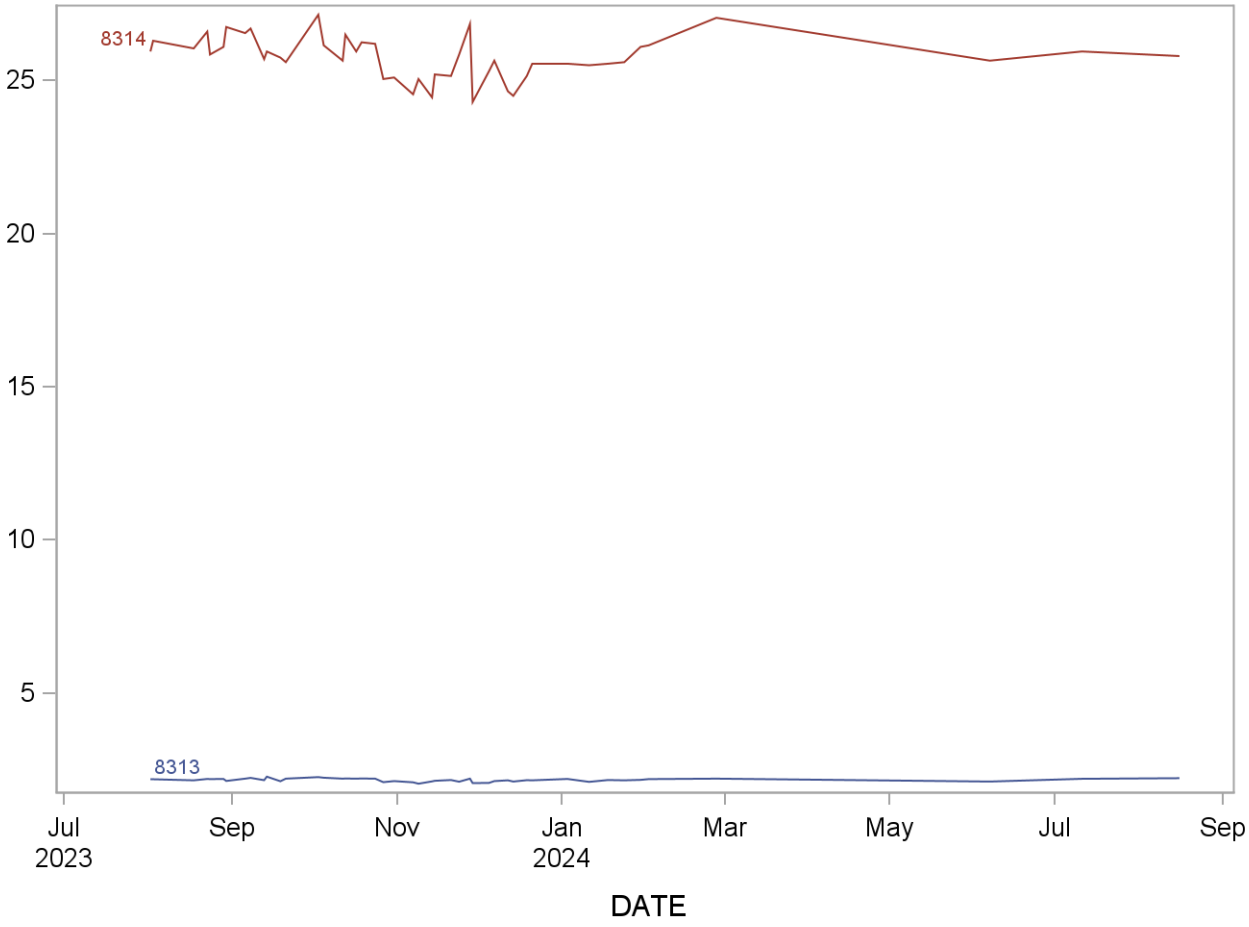
2017-2018 Summary Statistics and QC Chart
URXDEA (DEET acid (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
8314	47	02AUG23	16AUG24	51.60851	1.52783	3.0
8313	47	02AUG23	16AUG24	2.49287	0.06159	2.5



2017-2018 Summary Statistics and QC Chart
URX3MEA (3-(Ethylcarbamoyl) benzoic acid)

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
8314	46	02AUG23	16AUG24	25.75978	0.68586	2.7
8313	46	02AUG23	16AUG24	2.15326	0.05290	2.5



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