Laboratory Procedure Manual

Analyte: **DEET Metabolite**

Matrix: **Urine**

Method: **DEET Metabolite and Neonicotinoid Insecticides Biomarkers in Urine by Online-SPE-HPLC/+ESI MS/MS**

Method No: **6120.02**

as performed by: Contemporary Pesticides and Flame Retardants Laboratory
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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	Analyte Name
DEET_I	URXDEA	3-(diethlycarbamoyl)benzoic acid (ng/mL)
SSDEET_I	SSEMEA	3-(ethlycarbamoyl)benzoic acid (ng/mL)
SSNEON_I	SSIMID	Imidacloprid (µg/L)
	SSACET	Acetamiprid (µg/L)
	SSCLOT	Clothianidin (µg/L)
	SSTHIA	Thiacloprid (µg/L)
	SSOHIM	5-Hydroxyimidacloprid (µg/L)

1. Clinical Relevance and Summary of Test Principle

N,N-diethyl-3-methylbenzamide, 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 100% (EPA 2017).

Neonicotinoids (neonics) are a class of insecticides that are now being used to replace the toxic organophosphates and carbamates. Neonicotinoids are used for growing genetically-modified corn, soybeans, cotton, sunflowers, and canola, as well as various other genetically-modified and non-genetically-modified vegetables and fruits (Cimino et al. 2017). Approximately 90% of the corn and 50% of the soybeans planted in the USA have been treated with neonicotinoids. Neonicotinoid insecticides are also used in lawn treatment and are very effective for flea control in cats and dogs (Tomizawa 2005). The neonicotinoid family includes acetamiprid, clothianidin, imidacloprid, nitenpyram, nithiazine, thiacloprid and thiamethoxam; imidacloprid is the world's single most widely applied insecticide. Neonics are persistent in the environment and cannot be washed off easily from food (Chen 2014). Although long-term impacts of neonicotinoids on the environment are unknown, neonicotinoids have received increased scrutiny because they may adversely affect pollinators and are linked to colony collapse disorder in bees (Hladik et al. 2014) and the decline of insectivorous birds (Hallmann, 2014). Neonics have neurotoxic action on the nicotinic acetylcholine receptor. The extent of Americans' exposure to neonicotinoids is unknown but exposures may be on the rise due to the increased use of neonicotinoids.

Although the clinical relevance of this method, on rare occasions, may extend to quantifying concentrations for emergency responses, such as accidental poisonings, its design and intended use is to provide data in support of epidemiological studies. The concentrations of these pesticides biomarkers will be used to produce for the first time ever distributions and reference ranges for the U.S. population for some of these biomarkers. This information will greatly improve our understanding of the extent of Americans' current exposure to DEET and neonicotinoid insecticides, whether exposure is changing in the United States, and whether these changes may impact the general population as a whole or in specific population groups. This method does not directly test for any disease.

This method uses online solid phase extraction coupled with high performance liquid chromatography-tandem mass spectrometry (SPE-HPLC-MS/MS) for quantifying two DEET metabolites, 3-(diethylcarbamoyl)benzoic acid and 3-(ethylcarbamoyl)benzoic acid; four neonicotinoid insecticides: acetamiprid, clothianidin, imidacloprid, and thiacloprid; and two neonicotinoid metabolites: N-desmethyl-acetamiprid and 5-hydroxy-imidacloprid in 200 µL of human urine (Baker et al., 2018). Sample preparation begins with an overnight enzymatic deconjugation of the glucuronide-bound biomakers. On the second day, the eight compounds are concentrated via online SPE and then chromatographically separated from each other and from other urine biomolecules using reversed phase HPLC. The eluting compounds are ionized using Electrospray Ionization (ESI) and then selectively filtered by mass-to-charge ratios at unit resolution. Select molecular 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.

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). Hepatitis B vaccination series is usually recommended for health care and laboratory workers who are exposed to human fluids and tissues. Observe universal precautions

d. Mechanical Hazards

The risk for mechanical hazards will be minimized by following standard safety practices while performing this procedure. 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 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. Personnel should also have a working knowledge of the basics of chemistry, SPE, HPLC-MS/MS systems including troubleshooting, maintenance and operation, and a working knowledge of basic chemical separations and analytical chemistry.

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
Data stored in Starlims are backed up daily. Raw instrument data can be temporarily backed up on a CDC-approved Jump drive until transferred to the CDC network, which is also backed up at least weekly.

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 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 in cryovials packed in boxes frozen and securely packed in dry ice. To minimize the potential degradation of the specimen, special care must be taken to avoid prolonged exposure of the urine to room or refrigerator temperatures after collection. Freeze all samples until analysis. Portions of urine that remain after the analytical aliquots are withdrawn should be refrozen after 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.

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 of similar specifications but from other sources may also be used.

Table 1. Reagents and Suggested Manufacturers

Reagents	Suggested Manufacturers
Acetonitrile	Fisher Scientific
Methanol	Fisher Scientific
Formic Acid	Fluka
Bottled Water	Fisher
Potassium Phosphate Dibasic Trihydrate	MP Biomedicals

Phosphoric Acid	Sigma-Aldrich Co.
β-glucuronidase from E.coli	Sigma-Aldrich Co.
4-methylumbelliferone (UMB)	Sigma-Aldrich Co.
4-methylumbelliferone- 2,3,4,Methyl-$^{13}C_4$	Cambridge Isotopes
3-(ethylcarbamoyl)benzoic acid (ECBA)	Cerilliant
D_5-3-(ethylcarbamoyl)benzoic acid	CanSyn Chemical Corporation
3-(diethylcarbamoyl)benzoic acid (DCBA)	Cerilliant or CanSyn Chemical Corporation
D_{10}-3-(diethylcarbamoyl)benzoic acid	CanSyn Chemical Corporation
(E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine (Clothianidin)	Chemservice
D_3-Clothianidin	Sigma-Aldrich
1-[(6-Chloro-3-pyridinyl)methyl]-N-nitro-4,5-dihydro-1H-imidazol-2-amine (Imidacloprid)	SPEX Certiprep or Sigma-Aldrich
D_4-Imidacloprid	Sigma Aldrich
(E)-N1-[(6-Chloro-3-pyridyl)methyl]-N2-cyano-N1-methylacetamidine (Acetamiprid)	SPEX Certiprep or Fluka
D_5-Acetamiprid	Cerilliant
1-[(6-Chloro-3-pyridinyl)methyl]-2-(nitroamino)-4,5-dihydro-1H-imidazol-5-ol (5-hydroxy-imidacloprid)	Gift from Dr. Heiko Kafferlain, IPA, Germany
D_4-5-hydroxy imidacloprid	ClearSynth
(1E)-N'-[(6-Chloro-3-pyridinyl)methyl]-N-cyanoethanimidamide (Acetamiprid-N-Desmethyl)	Chemservice or Fluka
$^2H_3$,^{13}C,$^{15}N_2$, Acetamiprid-N-Desmethyl	CanSyn Chemical Corporation
{(2E)-3-[(6-Chloro-3-pyridinyl)methyl]-1,3-thiazolidin-2-ylidene}cyanamide (Thiacloprid)	SPEX Certiprep or Fluka
D_4-Thiacloprid	Fluka

b. Reagent Preparation

1. **Liquid chromatography mobile phases:**
   For online solid phase extraction: Mobile Phase A= 0.1% Formic Acid in aqueous solution. For example, pipette 1 mL of Formic Acid in 999 mL of HPLC-grade water and mix. Mobile Phase B= 100% MeOH. For analytical separation: Mobile Phase A = 0.1% Formic Acid in aqueous solution. Mobile phase B =100% Acetonitrile.

2. **Buffer Solution-0.2M Potassium Phosphate Buffer (pH 6.8)**
   Suggested procedure: In a 500 mL beaker of bottled water, completely dissolve 22.8 grams of potassium phosphate dibasic trihydrate. With 500 mL beaker placed on a stirrer (moderate setting) add 1 mL phosphoric acid and place a pH electrode in the solution. Continue to

add phosphoric acid to adjust pH to 6.8 ±0.1. Label solution and keep it refrigerated.

3. **Enzyme Solution-0.66 units/µL of β-glucuronidase from E.coli**
   Suggested procedure: Gently dissolve 1 bottle of β-glucuronidase from E.coli (7.8 mg solid; 12800 units/mg-approximately 100,000 units) in 150mL of 0.2 M potassium phosphate buffer pH 6.8. Transfer to labeled glass vials and keep solutions at or below -20°C. Note: The enzyme concentration is made such that 150 µL contains 100 units (i.e., modified Fishman units) of enzyme activity.

c. Standards and Quality Control Materials Preparation

1. **Native Stock Solutions (1 mg/mL and 0.1 mg/mL)**
   Suggested procedure: Individually weigh appropriately 30 mg of the target compound into a clean 60 mL glass vial. Calculate the volume of acetonitrile needed to achieve a concentration of 1 mg/mL. Add this volume gravimetrically to the 60 mL glass vial. Vortex mix until solute is fully dissolved. Sonication may be required. Repeat for all target analytes except clothianidin, 5-hydroxy-imidacloprid, and ECBA, which require a final concentration <1 mg/mL. Note: ECBA, 5-hydroxy-imidacloprid, and clothianidin will not stay in solution 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 appropriately 30 mg of a labeled target compound into a clean 60 mL glass vial. Calculate the volume of acetonitrile needed to achieve a concentration of 1 mg/mL. Add this volume gravimetrically to the 60 mL glass vial. Vortex mix until solute is fully dissolved. Repeat for all target analytes. Sonication may be required.

3. **ISTD and Native Standard Spiking Solutions**
   Suggested procedure for Native spiking solutions: Combine individual native stock solutions of target analytes for a final concentration of 0.2 ng/µL for all analytes except 3-(diethylcarbamoyl)benzoic acid (DCBA) which should be at 1.0 ng/µL. This is the highest concentration spiking solution. Using either serial dilutions or gravimetric techniques, prepare eleven 1:1 dilutions, resulting in concentrations such that a 50 µL spike into a 200 µL urine sample, results in concentrations of 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781, 0.390, 0.195, 0.097, 0.049, and 0.024 ng/mL urine for all target analytes except DCBA, which has concentrations 5 times greater due to a predicted concentration range found in field/study samples. Concentrations may vary slightly for each analyte but are updated in the quantitation method to maintain accuracy.

Suggested procedure for ISTD spiking solution: Combine individual ISTD stock solutions of isotopically labeled target analytes and 4-methylumbelliferone, such that a 100 µL spike into a 200 µL urine sample results in concentrations of 12.5, 50, 50, 25, 50, 50, 50, 12.5, and 1024 ng/mL urine, respectively.

4. Quality Control Materials

There are three types of quality control materials: Solvent blanks, spiked urine matrix pools, and proficiency testing urine pools. All urine pools are diluted 1:1 with deionized water prior to enriching with target analytes. All quality control samples are treated identical to unknown samples. Solvent blanks are made with deionized water. All other quality control pools are made from anonymous individual urine samples that are screened for the presence of endogenous compounds or interfering compounds that co-elute with the target compounds. Selected urines are pooled and mixed. The pool is separated into five pools. The first pool is spiked at a concentration at the lower end of the linear range of the method and serves as a low concentration quality control sample that is run with each batch of samples. The second pool is spiked at a concentration at the upper end of the linear range of the method and serves as a high concentration quality control sample that is run with each batch of samples. Pools three, four, and five are spiked at three different concentrations spanning the linear range of the method and serve as material for proficiency testing (PT) samples that are run bi-annually to test the laboratory's performance. Pools are spiked and mixed over night before being aliquoted into 2 mL vials for storage at or below −20°C.

5. 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 to obtain mean and standard deviation values. QC samples, at two concentration levels (i.e., low and high), are analyzed with each analytical run and serve as calibration verification.

6. Proficiency-Testing Materials (Low, Medium, and High)

Proficiency testing materials are made just like Calibration-Verification materials (section 5 above) except they are spiked at three different concentrations spanning the useable range of the method.

d. Materials

1. Chromolith Flash RP-18e precolumn (4.6 mm x 25 mm, Merck KGaA, Germany).
2. 1.5 mL silanized autosampler vials (ThermoScientific, USA)
3. Thermo Hypersil Gold aQ 150 x 4.6mm, 3um particle size
4. Inline filters (2 µm and 0.5 µm, Upchurch).
5. Pipette tips: 1 mL, 250 µL, and 20 µL.
6. 2 mL, 96 Deep-well plates or vials.

e. Equipment

1. Pipettors (Rainin)
2. Balance (Sartorius, Genius series)
3. pH meter (Mettler Toledo Seven Compact).
4. Vortex Mixer (Fisher, Genie 2).
5. Magnetic Stirrer (Corning)

f. Analytical instrumentation

1. ThermoFinnigan TSQ Vantage Triple Quadrupole Mass Spectrometer
2. Thermo Scientific™ Dionex™ UltiMate™ 3000 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.
3. Liquid Handling System

Note: Materials, equipment and analytical instruments of similar specifications but from other sources may also be used.

7. Calibration and Calibration-Verification Procedures

a. Calibration Plot

For each analytical run, a 12-point calibration curve (section 6, c, 3) is generated by plotting the relative response factors (area native/area labeled IS) versus standard concentrations with a 1/x weighting. Slope and intercept of this curve is done using a linear least square fit with Xcalibur software. The lowest point of the calibration curve is at or below the measurable detection limits and the highest point is at or above the expected range of results.

b. Calibration Verification

1. 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.

2. Analytical runs generally include 8-12 standard calibrators for each analyte and three levels of calibration-verification materials (i.e., blank, QC low, and QC high).

3. All calibration verification runs and results are documented in the Starlims database.

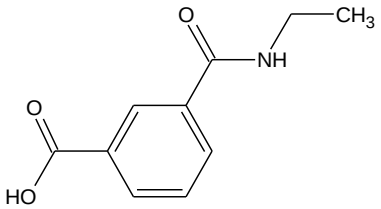
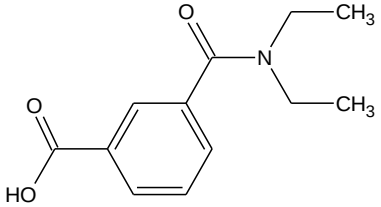
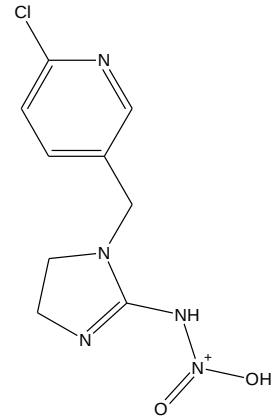
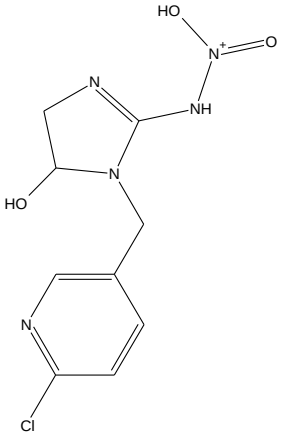
c. Proficiency testing (PT)

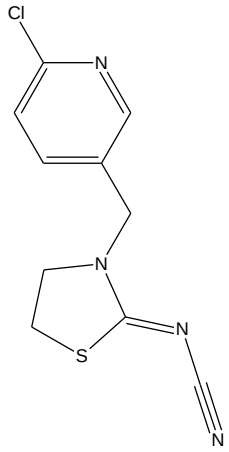
In-house proficiency testing
PT sample materials, or pools, are prepared in-house as described above. These PT samples encompass the linear range of the method and are characterized in our laboratory. The characterization data are forwarded to the division statistician (acting PT administrator) in charge of executing the PT program. The PT administrator establishes the mean and confidence limits for each analyte concentration. PT should be performed biannually. 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.

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 is not available.

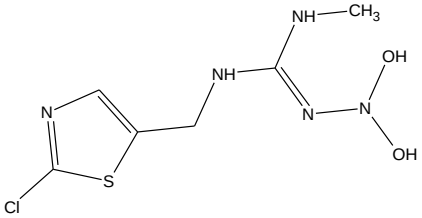
8. Analyte Nomenclature and Structures

Structure	Common Name/NHANES Code	IUPAC name
	3-(ethylcarbamoyl)benzoic acid (ECBA) (NHANES: MEA)	3-(ethylcarbamoyl)benzoic acid
	3-(diethylcarbamoyl)benzoic acid (DCBA) (NHANES: DEA)	3-(diethylcarbamoyl)benzoic acid
	Imidaclorid (NHANES: IMID)	1-[(6-Chloro-3-pyridinyl)methyl]-N-nitro-4,5-dihydro-1H-imidazol-2-amine
	5-hydroxy-imidacloprid (NHANES: OHIM)	1-[(6-Chloro-3-pyridinyl)methyl]-2-(nitroamino)-4,5-dihydro-1H-imidazol-5-ol



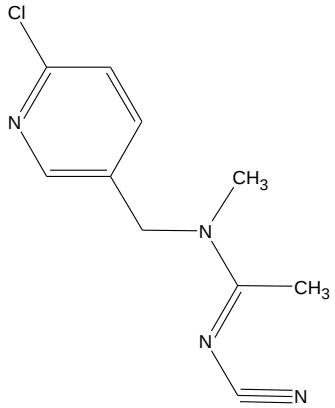
Thiacloprid
(NHANES: THIA)

{(2E)-3-[(6-Chloro-3-pyridinyl)methyl]-1,3-thiazolidin-2-ylidene}cyanamide



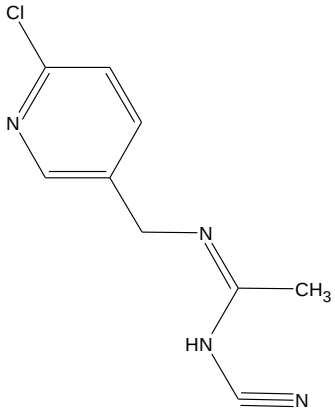
Clothianidin
(NHANES: CLOT)

(E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine



Acetamiprid
(NHANES: ACET)

(E)-N1-[(6-Chloro-3-pyridyl)methyl]-N2-cyano-N1-methylacetamidine



Acetamiprid-N-desmethyl
(NHANES: AND)

(1E)-N'-[(6-Chloro-3-pyridinyl)methyl]-N-cyanoethanimidamide

9. Operating Procedures; Calculations; Interpretation of Results

a. Preliminaries

(1) A batch normally consists of twelve calibrators, a water rinse, one reagent blank without labeled internal standard (ISTD), one reagent blank with ISTD, four quality control samples (i.e., two low concentration and two high concentration - levels spread randomly throughout the sample's positions), and up to 76 urine samples.
(2) Urine samples and quality control (QC) materials are thawed at room temperature.
(3) Thawed urine samples are vortex mixed prior to sample preparation.

b. Sample preparation

(1) To 96 deep-well plate wells, aliquot: 200 µL of urine sample, 100 µL internal standard spiking solution, 50 µL of calibrator solution (to calibrator samples only) and 300 µL of enzyme buffer solution.
(2) Incubate at ~37°C for approximately 17 hours.
(3) Remove samples from incubation, add 100 uL 10:1 Formic Acid, briefly mix with liquid handler, cap wells with silicon pre-slit mats, load 96-well plate into the SPE-HPLC/MS system for analysis. The autosampler temperature should be set to 10˚C throughout the analysis.

c. Instrumental Analysis

(1) Instrument Performance (Chromatography and Sensitivity Check)

Before an analytical run is started on the SPE-HPLC-MS/MS system, an instrument check sample (e.g., a urine- or deionized water-based pool with a concentration near the lowest standard) is injected and analyzed to confirm acceptable chromatographic peak shape, chromatographic resolution and detector sensitivity before an analytical run is started.

(2) On-line SPE-HPLC/MS analysis

The fully automated analysis is performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 x2 Dual HPLC System coupled to a ThermoFinnigan TSQ Vantage triple quadrupole mass spectrometer, equipped with a HESI (Heated Electrospray ionization (ESI)) interface. The HPLC system and the mass spectrometer are both controlled by XCalibur® software.

The online SPE-HPLC system includes:

-WPS-3000TRS temperature controlled autosampler,
-DGP-3600RS pump, a dual pump system which supports 2 x 3 eluents via proportioning valves.
-The UltiMate 3000 TCC-3000RS Rapid Separation Thermostatted Column Compartment outfitted with a six-port switching valve.

A Chromolith Flash RP-18e monolithic column (25 x 4.6 mm), manufactured by EMD Chemicals (Gibbstown, NJ), is used for on-line SPE cleanup column. Its monolithic structure allows for high flow rates at low pressure. A Thermo (San Jose, CA) Hypersil Gold aQ (150 x 4.6 mm, 3µ), is used for the analytical separation column.

The system flow paths are depicted in Figure 1. This online SPE method involves a three-step process shown in Table 2:

In the first step, 500 µL are drawn from the 96-well plate (or sample vial) and transferred onto the SPE cartridge. The Chromolith SPE cartridge is washed for 0.5 minutes with 5% methanol at 1 mL per minute and then an additional 2 minutes at 2 mL/min. While the sample is washed on the SPE cartridge, the other pump equilibrates the analytical column. The analytical column and SPE cartridge temperatures remain at 40°C throughout the run. Switching the six-port valve initiates step two.

In step two, the SPE pump is temporarily stopped and the analytical pump solvent is directed in-line with the SPE cartridge, with an opposite flow direction, and then through the analytical column. A 25% acetonitrile solvent strength elutes the analytes from the SPE cartridge and onto the analytical column. After allowing 1.1 minutes to backflush the analytes from the SPE cartridge onto the analytical column, the six-port valve is switched again, which is the beginning of step three.

In step three, the analytical pump no longer pumps eluents through the SPE cartridge, but rather, bypasses it, via the six-port valve, allowing the flow to go only through the analytical column. At the same time, the analytical pump starts a gradient using acetonitrile, causing the analytes to elute from the analytical column. Also at the same time, the SPE cartridge is washed with the other pump independently using 100% methanol for about 3.5 minutes and then equilibrates with a solution of 5:95 methanol:water to prepare for the next injection.

Table 2. Valve positions, flow rates and solvent percentages.

Valve			SPE Pump (Binary Pump #1)			Analytical Pump (Binary Pump #2)		
Time [min]	Step	Valve Pos.	Time [min]	Methanol [%]	Flow Rate [mL/min]	Time [min]	Acetonitrile [%]	Flow Rate [mL/min
0.0	1	1	0.0	5	1.0	0.0	25.0	1.0
			0.4	5	1.0			
			0.5	5	2.0			
						4.0	25.0	1.0
2.5	2	2	2.50	5	2.0			
			2.60	5	0			
3.6	3	1						
			5.1	5	0			
			5.2	100	3.0			
						8.0	50.0	1.0
						8.1	25.0	1.0
			8.5	100	3.0			
			8.6	5	1.0			
						11.0	25.0	1.0
11.0			11.0	5	1.0	11.0	25.0	1.0

Figure 1. On-line dual-pump switching scheme with flow paths and mobile phase gradient profiles (Kuklenyik 2013).

(3) Multiple Reaction Monitoring (MRM) Setup for DEET and metabolites.

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 at ~1.5 mTorr. The collision energy is specifically set for each ion (Table 3).

Table 3. Mass ion transitions and collision energies.

Analyte	Ion Type	Parent Ion	Product Ion
ECBA	Quantitation Ion	194	149
	Confirmation Ion	194	121
	ISTD	199	149
DCBA	Quantitation Ion	222	121
	Confirmation Ion	222	149
	ISTD	232	149
Acetamiprid	Quantitation Ion	223	126
	Confirmation Ion	223	90
	ISTD	226	126
N-desmethyl-acetamiprid	Quantitation Ion	209	126
	Confirmation Ion	209	90
	ISTD	215	126
Clothianidin	Quantitation Ion	250	132
	Confirmation Ion	250	113
	ISTD	253	172
Imidacloprid	Quantitation Ion	256	209
	Confirmation Ion	256	175
	ISTD	260	213
5-hydroxy-imidacloprid	Quantitation Ion	272	225
	Confirmation Ion	272	191
	ISTD	276	229
Thiacloprid	Quantitation Ion	253	126
	Confirmation Ion	253	90
	ISTD	257	126

ISTD = Isotopically-labeled internal standard

d. Calculations

The concentration of individual target analytes in each sample is calculated using the calibration curve equation derived from a linear regression 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

Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided below. **The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.**

a. Accuracy

The accuracy of this method was determined by recovery analysis 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 level 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]. Method recoveries are shown in Figure 4. Acceptable recovery should be 85-115% except at 3*LOD where it can be 80-120%. Any deviations from these ranges required approval from the Director.

Table 4. Accuracy of the method at various concentrations

Analyte	Accuracy of Concentration measurements	
	Concentration (ng/mL)	Mean Recovery (%)
ECBA	10 25 40	94.6
DCBA	50 125 200	102.2
Acetamiprid	10 25 40	105.0
N-desmethyl-acetamiprid	10 25 40	99.6
Clothianidin	10 25 40	98.6
Imidacloprid	10 25 40	97.4
5-hydroxy-imidacloprid	10 25 40	111.3
Thiacloprid	10 25 40	102.4

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. Total precision is shown in Table 5. Acceptable RSD should not exceed 15%. Any deviations from these ranges required approval from the Director.

Table 5. Total precision of the method at different concentrations.

Analyte	Average Concentration (ng/mL)	RSD(%)
ECBA	3.8 17.7	5.9 6.0
DCBA	9.3 79.9	5.3 5.2
Acetamiprid	3.8 15.2	3.8 5.1
N-desmethyl-acetamiprid	4.1 15.7	7.4 6.0
Clothianidin	7.0 17.2	5.4 5.0
Imidacloprid	3.6 14.6	4.1 4.2
5-hydroxy-imidacloprid	4.3 17.8	8.8 8.4
Thiacloprid	4.0 16.0	7.0 7.0

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). LODs are calculated as 3S0, where S0 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 concentration (Taylor 1987). The detection limits vary based on the current operating precision and the cleanliness of the analytical system. The method detection limits for each compound can also be set by the concentration of the lowest detected calibration standard. For many analytes, the concentration of S1 is given as the lowest detected standard. The reported LOD can be higher than these values if the team lead feels this is necessary, but the value cannot be below these values. Current LOD`s are shown in Table 6 and may vary over time.

Table 6. Method Limits of detection (LODs).

Analyte	LOD (ng/mL)
ECBA	0.2
DCBA	0.2
Acetamiprid	0.3
N-desmethyl-acetamiprid	0.2
Clothianidin	0.2
Imidacloprid	0.4
5-hydroxy-imidacloprid	0.4
Thiacloprid	0.03

d. Analytical Specificity

This method requires that the analytes: 1) must form a positive molecular ion with electrospray ionization; 2) produce the same two collision-induced dissociation product ions at a defined ratio; and 3) co-elute with the corresponding isotope labeled internal standard analog. The quantitation and confirmation ions for each analyte are listed in Table 3. Potential interfering substances were evaluated during method development using approximately 50 convenience urine samples collected in the bathrooms with IRB protocol 3994 approval.

e. Stability (Freeze-Thaw, Bench Top, 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 measurements were made initially and then n=3 measurements were made after three freeze-thaw cycles. 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 measurements were made initially and n=3 measurements were made after materials were stored at room temperature for one day. All stability results were within ± 15% of the nominal concentrations. 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.

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 measured 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 reportable ranges are from LOD to approximately 50 ng/mL, except for DCBA (LOD to approximately 250 ng/mL).

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 R2 that exceeds 0.98" (DLS Policy and Procedures Manual July 2016).

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.
Reference ranges for 3-(diethylcarbamoyl)benzoic acid (DCBA) can be found at:
https://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Volume1_Mar2018.pdf

11. QC Procedures

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

i. 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.

ii. 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.

iii. 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.

iv. 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.

v. The area count ratio of 4-UMB and 4-UMB (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.

vi. Unknown samples, for which all of the analyte's concentrations 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.

vii. 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.

viii. 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 result is beyond the characterization mean $\pm$ $4S_i$
   1. 3S Rule – Run result is outside a $3S_i$ limit
   2. 2S Rule – Both run results are outside the same $2S_i$ limit

   3. 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean

4. 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 results 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 $\pm$ $4S_m$
   1. 3S Rule – Run mean is outside a $3S_m$ limit
   2. 2S Rule – Both run means are outside the same $2S_m$ limit
   3. 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:
   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 Run QC samples fail to meet division specified criteria the run is deemed out of control and the run must be repeated until Run QC samples pass run criteria. No data are reported for an analyte from a run deemed out of statistical control for that particular analyte.

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. Under such circumstances, the calculated concentration for the target analyte is 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 US population (Calafat et al. 2016; CDC 2018).

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 preferably at ≤−20°C 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. If storage system fails, urine samples can be temporarily stored in the refrigerator for a maximum of 24 hours.

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 Team Lead and finally by the laboratory supervisor. The supervisor provides feedback to the Team Lead and/or his/her designee and requests confirmation of the data as needed.

b. The Quality Control officer reviews each analytical run and identifies the quality control samples within 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 memorandum to the Branch Chief, and a letter from the Division Director to the person(s) who requested the analyses reporting the analytical results.

e. These 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.

g. Final hard copies of correspondence are maintained in the office of the Branch Chief and/or his/her designee and with the quality control officer.

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. This spreadsheet system does not include NHANES samples, for which specific procedures exist.

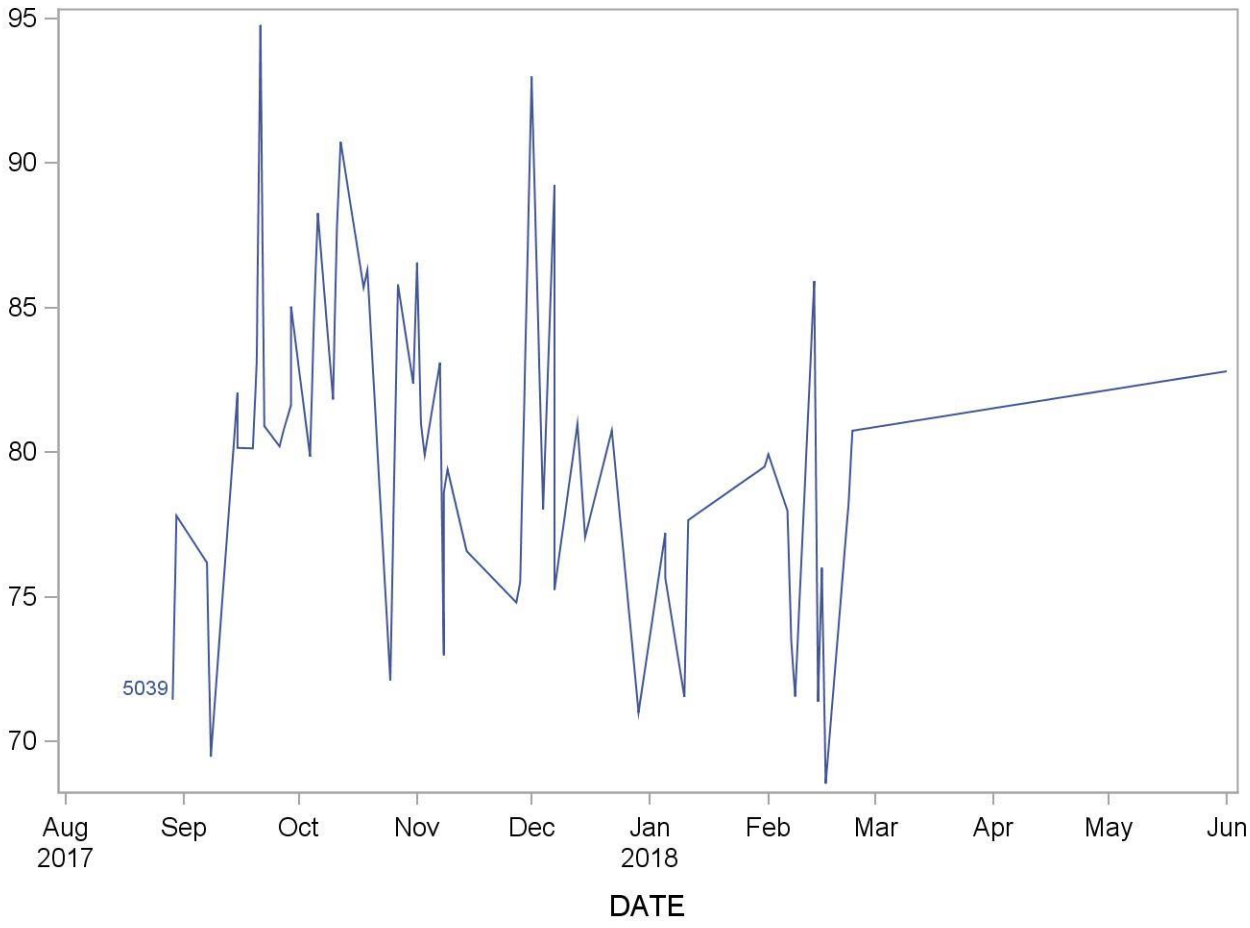
20. Summary Statistics and QC Graph

See following page.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

2015-2016 Summary Statistics and QC Chart for DEET acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5039	59	29AUG17	01JUN18	79.83963	5.77331	7.2



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