



Laboratory Procedure Manual

Analyte: **N,N-diethyl-3-methylbenzamide, N,N-diethyl-3-hydroxymethylbenzamide, and 3-diethyl-carbamoyl benzoic acid**

Matrix: **Urine**

Method: **Online-SPE-HPLC/+APCI MS/MS**

Method No: **6111.02**

as performed by: Pesticide Laboratory
Organic Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

contact:
Liza Valentín-Blasini, PhD
Phone: 770-488-7902
Email: lvb5@cdc.gov

James L. Pirkle, M.D., Ph.D.
Director, Division of Laboratory Sciences

Important Information for Users

The Centers for Disease Control and Prevention (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_E	URXDEE	N,N-diethyl- <i>meta</i> -toluamide (DEET) (ug/L)
	URXDHD	N,N-Desethyl hydroxy benzamide DEET (ug/L)
	URXDEA	DEET acid (ug/L)

1. Clinical Relevance and Summary of Test Principle

N, N-diethyl-3-methylbenzamide, commonly known as DEET, is the principle 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 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 repellent products with concentrations ranging from 10 to 100% in a variety of liquids, lotions, gels, sprays, sticks and impregnated materials and more than 30 million packages of DEET-containing products are sold annually. Approximately 230 products containing DEET are registered with the Environmental Protection Agency (EPA) by about 70 different companies ([EPA 2012](#)).

The method design and intended use is to provide data in support of epidemiological studies. It 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 DEET and two of its metabolites, N,N-diethyl-3-hydroxymethylbenzamide (DHMB) and 3-diethyl-carbamoyl benzoic acid (DCBA), in 100 μ L of urine (Kuklenyik et al. 2013). Sample preparation begins with an over-night enzymatic deconjugation of the glucuronide-bound metabolites. On the second day, the three compounds being measured are concentrated via online SPE and then chromatographically separated from each other and from other urine biomolecules using reversed phase HPLC. The eluting molecular ions are converted to gas phase ions using Atmospheric Pressure Chemical Ionization (APCI) and then selectively filtered by mass-to-charge ratios at unit resolution. Select molecular ions are then fragmented with chemical induced dissociation 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.

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

Note: Material Safety Data Sheets (MSDS) 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 MSDS prior to using chemicals.

b. **Radioactive Hazards**

There are no radioactive hazards to report with this method.

c. **Microbiological Hazards**

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. Laboratory personnel handling human fluids and tissues are required to take the "Bloodborne Pathogens Training" course offered at CDC to insure proper compliance with CDC safe workplace requirements.

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. Contact with the heated surfaces of the mass spectrometer should be avoided.

e. **Protective Equipment**

Standard personal protective equipment 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. Operators are required to read the laboratory standard operating procedures manual. Formal training is not necessary; however, an experienced user should train all of the operators.

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 diluted bleach solution). To insure proper compliance with CDC requirements, laboratory personnel are required to take annual hazardous waste disposal courses.

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 frequently. Raw instrument data are temporarily backed up (e.g., on a CDC-approved Jump drive) until transferred to the CDC network which is also backed up on a routine basis.

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. Preferably, at least five milliliters of urine is collected, and can be stored frozen in polypropylene vials or specimen cups. 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 0.1-mL if 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

Table 1. Reagents and Suggested Manufacturers

Reagents	Suggested Manufacturers
Acetonitrile	Fisher Scientific
Methanol	Fisher Scientific
Glacial Acetic Acid	JT Baker
Bottled Water	JT Baker
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
N,N-diethyl-m-toluamide (DEET)	Sigma-Aldrich Co.
d ₁₀ - N,N-diethyl-m-toluamide	CanSyn Chemical Corporation
N,N-diethyl-m-hydroxymethylbenzamide (DHMB)	CanSyn Chemical Corporation
d ₁₀ - N,N-diethyl-m-hydroxymethylbenzamide	CanSyn Chemical Corporation
3-diethyl-carbamoyl benzoic acid (DCBA)	CanSyn Chemical Corporation
d ₁₀ -3-diethyl-carbamoyl benzoic acid	CanSyn Chemical Corporation

b. Reagent Preparation

1. **Liquid chromatography mobile phases:**

For online solid phase extraction: Mobile Phase A= 0.1% Acetic Acid in aqueous solution. For example, pipette 1 mL of Acetic Acid in 999 mL of HPLC-grade water and mix. Mobile Phase B= 100% MeOH. For analytical separation: Mobile Phase A = 0.1% Acetic 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 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 \pm 0.1. Label solution and keep it refrigerated.

3. **Enzyme Solution-0.66 units/ μ L 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 in a -20°C freezer. Note: The enzyme concentration is made such that 150 μ L contains 100 units (i.e., modified Fishman units) of enzyme activity resulting in 1 unit of enzyme activity per 1 μ L of urine.

c. Standards and Quality Control Materials Preparation

1. **Native Stock Solutions (1 mg/mL)**

Suggested procedure: For N, N-diethyl-3-methylbenzamide (DEET), N, N-diethyl-3-hydroxymethylbenzamide (DHMB), 3-diethyl-carbamoyl benzoic acid (DCBA) and 4-methylumbelliferone (UMB), individually weigh appropriately 30 mg of compound into a clean 60mL glass vial. Calculate the volume of solvent (i.e., acetonitrile) needed to achieve an exact concentration of 1 mg/mL. Add this volume gravimetrically to the 60 mL glass vial. Vortex mix until solute is fully dissolved in solvent. Repeat for all native analytes. Store stock solutions in a -20 °C freezer.

2. **Internal Standard Stock Solutions (1mg/mL)**

Suggested procedure: For D₁₀-N, N-diethyl-3-methylbenzamide (DEET_L), D₁₀-N, N-diethyl-3-hydroxymethylbenzamide (DHMB_L), D₁₀ 3-diethyl-carbamoyl benzoic acid (DCBA_L), and 4-methylumbelliferone- 2, 3, 4, Methyl-¹³C₄ (UMB_L), individually weigh appropriately 30 mg of one compound into a clean 60mL glass tube. Calculate the volume of solvent (i.e., H₂O, except methanol for UMB_L) 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 in solvent. Repeat for each isotopically-labeled compound. These are the individual ISTD stock solutions.

3. **ISTD and Native Standard Spiking Solutions**

Suggested procedure for preparing native standard spiking solution: Combine individual native stock solutions of DEET, DHMB, and DCBA for final concentrations of 0.5, 0.5, 0.5 and 5.0 µg/mL, respectively. This is the highest concentration spiking solution. Prepare nine 1:1 dilutions, resulting in concentrations such that a 10 µL spike into 100 µL urine sample results in concentrations of 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781, 0.390, 0.195, and 0.097 ng/mL urine for DEET, and DHMB, respectively. Note: DCBA has concentrations 10 times greater due to a higher instrument limit of detection and a predicted concentration range found in field samples. Concentrations may vary slightly for each analyte but are updated in the quantitation method to maintain accuracy.

Suggested procedure for preparing the ISTD spiking solution: Combine individual stock solutions of DEET_L, DHMB_L, DCBA_L, UMB_L and UMB such that a 30 µL spike into 100 µL of urine results in a concentration of approximately 10, 10, 10, 600, and 600 ng/mL urine, for DEET_L, DHMB_L, DCBA_L, UMB_L, and UMB, respectively.

4. **Quality Control Materials**

There are four types of quality control materials: Solvent blanks, urine matrix blank pools, spiked urine matrix pools, and proficiency testing urine pools. All quality control samples are treated as study 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 six pools. The first pool is not spiked with any target analytes and is used as urine matrix blank material. The second 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 (QC) sample. The third pool is spiked at a concentration at the upper end of the linear range of the method and serves as a high concentration QC sample. Pools four, five and six 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 two through six 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 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 three concentration levels (i.e., blank, 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 (see section 4 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. Phenomenex-Prodigy 5 μ Phenyl-3, 4.6 x 100mm (Torrance, CA).
4. Inline filters (2 μm and 0.5 μm , Upchurch).
5. Pipette tips: 1 mL, 250 μL , 100 μL , 50 μL , 20 μL , and 10 μL sizes.

e. Equipment

1. Pipettors (Rainin)
2. Balance (Sartorius, Genius series)
3. pH meter (Corning pH/ion analyzer 455, Corning, New York).
4. Vortex Mixer (Fisher, Genie 2).
5. Magnetic Stirrer (Corning)

f. Analytical instrumentation

- a. ThermoFinnigan TSQ Vantage Triple Quadrupole Mass Spectrometer
- b. Agilent 1200 series High Pressure Liquid Chromatography system (degasser, quaternary pump, binary pump, autosampler, peltier cooling device, column oven compartment).

7. Calibration and Calibration-Verification Procedures

Calibration Verification

1. Calibration verification is not required by the manufacturer(s). However, it 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. 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.
3. Analytical runs generally include 10 standard calibrators and three levels of calibration-verification materials (i.e., QC blank, QC low, and QC high). Therefore, the conditions above are met with the calibration procedures for this method. Therefore, no additional calibration verification is required by CLIA.
4. All calibration verification runs and results are documented in the Starlims database.

b. Proficiency testing

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 a 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.

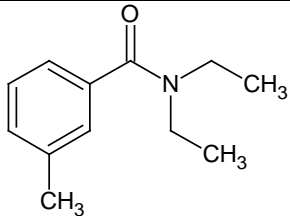
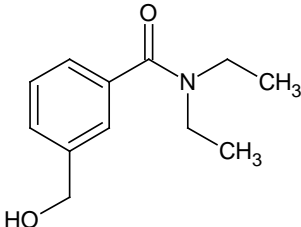
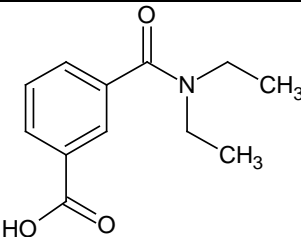
Proficiency testing should be performed biannually. When proficiency testing is required, the laboratory supervisor or his/her designee will notify the PT administrator who will randomly select

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

At this time, external proficiency testing for this method is not available.

8. Analyte Nomenclature and Structures

Structure	Common Name	IUPAC name
	N,N-diethyl-m-toluamide (DEET)	<i>N,N</i> -diethyl-3-methylbenzamide
	N,N-diethyl-m-formylbenzamide (DHMB)	<i>N,N</i> -diethyl-3-(hydroxymethyl)benzamide
	m-toluic-acid (DCBA)	3-diethyl-carbamoyl benzoic acid

9. Operating Procedures; Calculations; Interpretation of Results

a. Preliminaries

- (1) A batch normally consists of one reagent blank, one matrix blank not spiked with labeled internal standard, one matrix blank spiked with labeled internal standard, four quality control samples (i.e., two low concentration and two high concentration levels), ten calibrators, and up to 70 urine samples needing quantitative analysis.
- (2) Urine samples and quality control (QC) materials are thawed at room temperature.
- (3) Thawed urine sample vials are vortex mixed prior to sample preparation.

b. Sample preparation

- (1) To labeled 1.5 mL silanized autosampler vials, aliquot: 100 μ L of urine sample, 30 μ L internal standard spiking solution, 10 μ L of calibrator solution (to calibrator samples only) and 150 μ L of enzyme buffer solution.
- (2) Cap vials and incubate at 37°C for approximately 17 hours.
- (3) Remove samples from incubation, briefly vortex mix, load samples 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 (i.e., a urine 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 two-pump Agilent 1200 series HPLC system coupled to a ThermoFinnigan TSQ Vantage triple quadrupole mass spectrometer, equipped with an APCI interface. The HPLC system and the mass spectrometer are both controlled by XCalibur® software.

The online SPE-HPLC system includes: two Agilent 1200 series HPLC pumps (one functions as the analytical pump and the other functions as the SPE pump), a temperature-controlled auto sampler, a Peltier cooling module, and a temperature-controlled column compartment outfitted with a six-port switching valve. The system flow paths are depicted in figure 1.

A Chromolith Flash RP-18e monolithic column (25 x 4.6 mm) is used for on-line SPE cleanup column. Its monolithic structure allows for high flow rates at low pressure. A Prodigy Phenyl (100 x 4.6 mm, 5 μ), is used for the analytical separation column.

The exact system is shown in figure 1. This online SPE method involves a three step process.

In the first step, 100 μ L is drawn from the sample vial and transferred onto the SPE cartridge. The Chromolith SPE cartridge is washed for 2.5 min with 10% methanol at 4 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 35°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 30% acetonitrile solvent strength elutes the analytes from the SPE cartridge and onto the analytical column. After allowing one minute 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 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 4 minutes and then equilibrates with a solution of 10:90 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	1	1	0	10	1.00	0	30.00	1.00
1.00			10	1.00				
1.10			10	4.00				
3.50	2	2	3.50	10	4.00	3.00	30.00	1.00
			3.60	50	0			
4.50	3	1	4.60	50	0	8.00	50.00	1.00
			5.00	100	4.00			
			10.00	100	4.00			
			10.10	10	1.00			
			12.00	10	1.00			
					10.00	100.00	1.00	
					10.50	30.00	1.00	
12.00						12.00	30.00	1.00

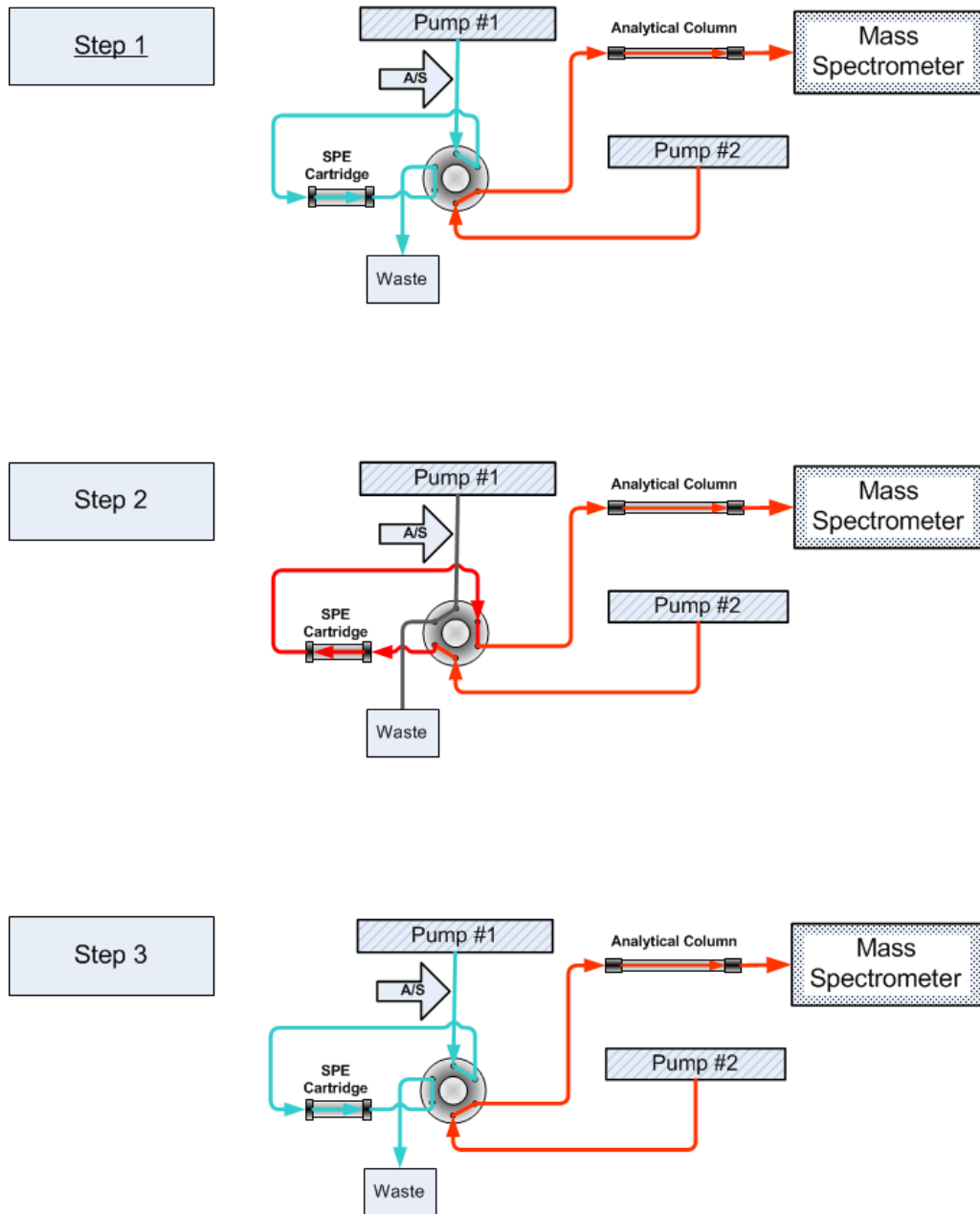


Figure 1 On-line dual-pump switching scheme with flow paths and mobile phase gradient profiles.

(3) Multiple Reaction Monitoring 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 for target analytes.

Analyte (NHANES ID)		Parent Ion	Product Ion	Collision Energy [V]
DEET	Quantitation Ion	192.139	119.00	16
	Confirmation Ion	192.139	91.055	29
	DEET_L1	202.202	119.050	16
	DEET_L2	202.202	91.055	29
DHMB	Quantitation Ion	208.134	135.045	18
	Confirmation Ion	208.134	89.039	35
		218.197	135.045	18
	DHMB_L2	218.197	89.039	35
DCBA	Quantitation Ion	222.113	149.024	19
	Confirmation Ion	222.113	121.029	27
	DCBA_L1	232.176	149.024	19
	DCBA_L2	232.176	121.029	38

DEET = *N,N*-diethyl-3-methylbenzamide

DHMB = *N,N*-diethyl-3-(hydroxymethyl) benzamide

DCBA = 3-diethyl-carbamoyl benzoic acid

_L1 = Isotopically-labeled analogue for internal standard

_L2 = Isotopically-labeled analogue for internal standard (alternate)

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. Normally, a calibration curve is run with each analytical run and used by the Xcalibur® data analysis software to quantify concentrations of all unknowns, QC, and blank samples.

10. Reportable Range of Results

The reportable range of results for each analyte is determined by the linear range of the standard calibration curve and the method limit of detection (LOD).

a. Linearity Limits

The linearity of the standard curve is determined from its R^2 value. Standard curves with R^2 values greater than 0.985 are considered acceptable. The upper limit of linearity is determined by the highest concentration standard in the standard curve and the lower limit of linearity is determined by the concentration of the lowest standard in the standard curve. Urine samples whose concentrations exceed the highest standard's concentration must be reanalyzed using a smaller urine volume.

b. Method Limit of Detection (LOD)

The lower limit of the reportable range is determined by the method LOD (Table 4). However, if the concentration of the method LOD is lower than the concentration of the lowest standard in the standard curve, the concentration of the lowest standard in the analytical run is used as the LOD.

Table 4. Method Limits of detection (LODs).

Analyte	LOD (ng/mL)
DEET	0.083
DHMB	0.089
DCBA	0.475

c. Accuracy

The accuracy of the method for each analyte is listed in table 5 below.

Table 5. Accuracy of the method at various concentrations

Analyte	Accuracy of Concentration measurements	
	Concentration (ng/mL)	Bias (%)
DEET	2.8	2.7
	45	1.0
DHMB	2.8	-5.0
	49	0.60
DCBA	29	6.3
	504	2.3

d. Precision

The precision of the method is determined by calculating the average coefficient of variation (CV) of repeated measurements ($n = 30$) of the QC materials over a 5-week period.

Table 6. Precision of the method at different concentrations.

Analyte	Concentration (ng/mL)	CV (%)
DEET	2	11
	15	7
	25	7
DHMB	2	8
	15	6
	25	6
DCBA	10	14
	50	9
	225	9

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

- 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 dispensed in the autosampler vial.
- 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 ($0.05\% \times$ 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 concentration in an unknown sample is above the concentration of the highest calibration standard (with diluted samples this test is based on the response ratio), 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

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. Currently, reference range values are only available for DEET (CDC 2009). Reference range values for the DEET metabolites measured in this method (i.e., N, N-diethyl-3-hydroxymethylbenzamide (DHMB), and 3-diethyl-carbamoyl benzoic acid (DCBA) are currently not available.

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 $\leq -20^\circ\text{C}$ in sealed autosampler vials 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

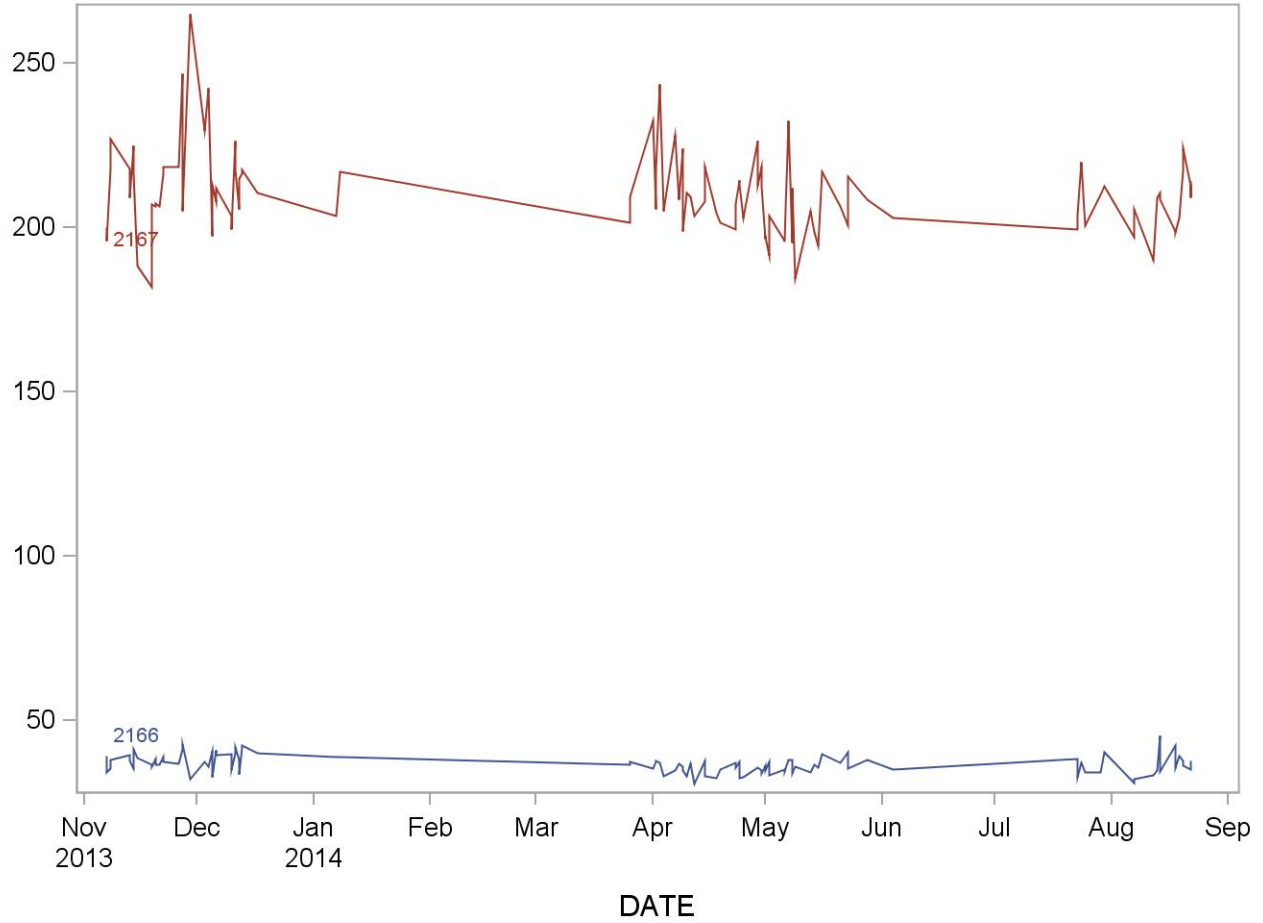
An Excel spreadsheet with information for receiving/transferring specimens is kept in an electronic form on the laboratory's shared workspace. In this form, samples are logged in when received. This spreadsheet also includes information regarding sample storage location, relevant inventory file(s), and 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 Graphs

See following pages.

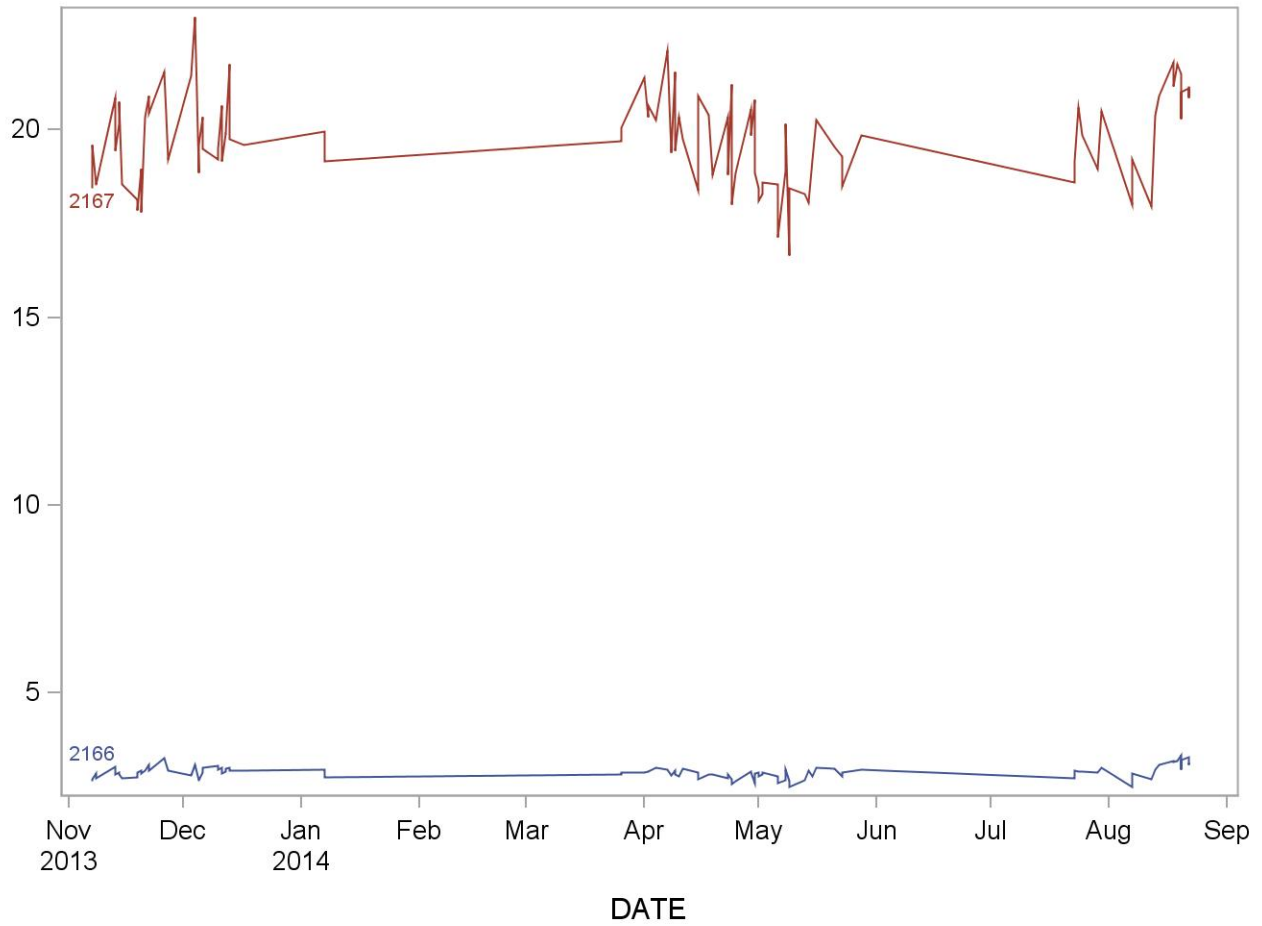
2007-2008 Summary Statistics and QC Chart for DEET acid (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2167	103	07NOV13	22AUG14	210.1942	12.93635	6.2
2166	103	07NOV13	22AUG14	36.68689	2.81348	7.7



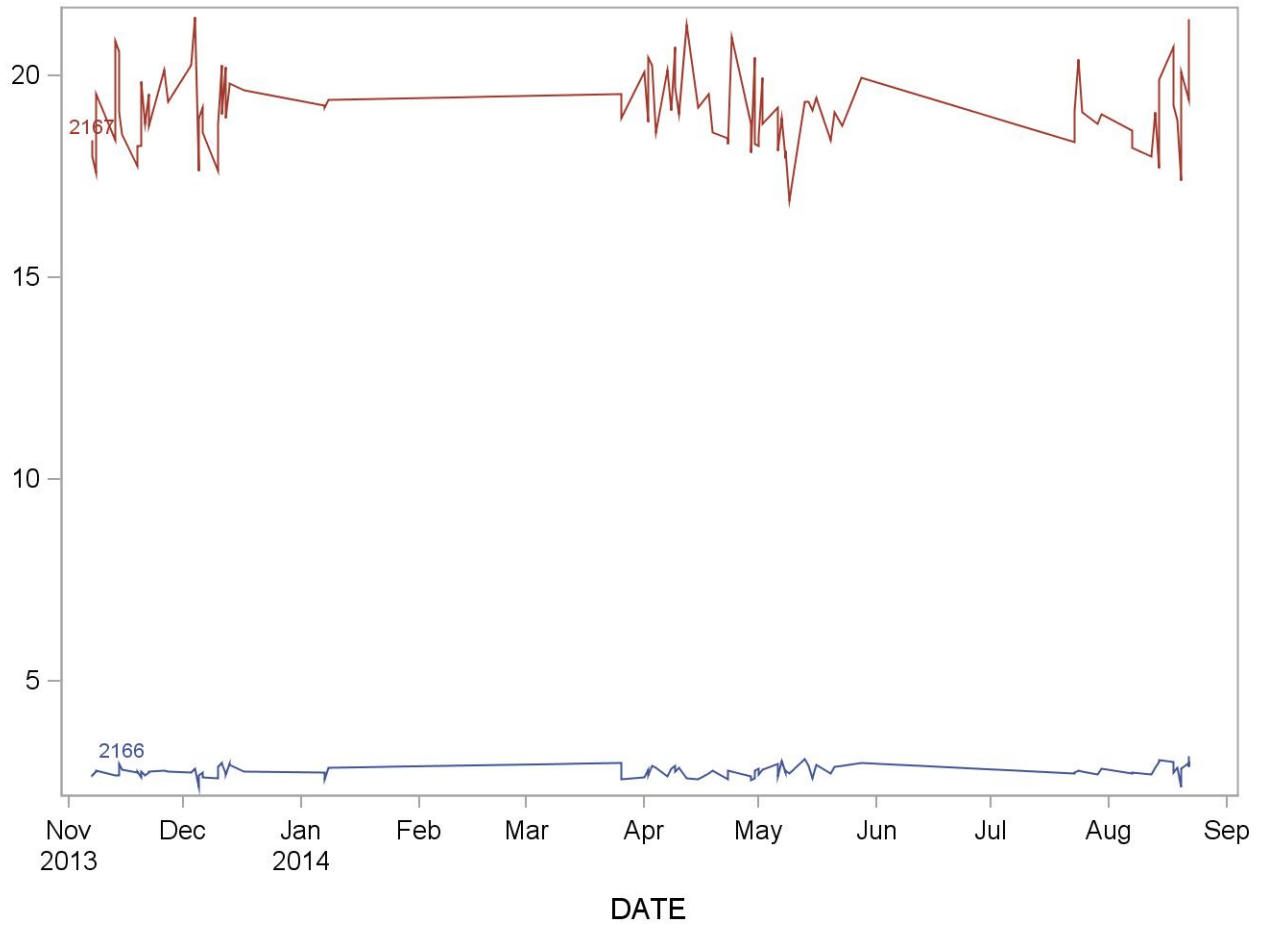
2007-2008 Summary Statistics and QC Chart for N,N-Desethyl hydroxy benzamide DEET (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2167	98	07NOV13	22AUG14	19.72551	1.20541	6.1
2166	98	07NOV13	22AUG14	2.88893	0.16499	5.7



2007-2008 Summary Statistics and QC Chart for N,N-diethyl-meta-toluamide (DEET) (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2167	98	07NOV13	22AUG14	19.16939	0.93309	4.9
2166	98	07NOV13	22AUG14	2.77061	0.13927	5.0



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.

References

Caudill SP, Schleicher RL, and Pirkle JL. "Multi-rule quality control for the age-related eye disease study". *Statist. Med.* 2008. 27:4094-4106.

CDC. Fourth National Report on Human Exposure to Environmental Chemicals. [\[http://www.cdc.gov/exposurereport/pdf/FourthReport.pdf\]](http://www.cdc.gov/exposurereport/pdf/FourthReport.pdf). 2009. Atlanta, GA, Centers for Disease Control and Prevention; National Center for Environmental Health; Division of Laboratory Sciences.

EPA (2012) Pesticides: Topical & Chemical Fact Sheets. The Insect Repellent DEET. US Environment Protection Agency, Washington, DC (available online <http://www.epa.gov/opp00001/factsheets/chemicals/deet.htm>, last accessed August 11, 2013).

Kuklenyik P, Baker SE, Bishop AM, Morales-A P, Calafat AM. "On-line Solid Phase Extraction-High Performance Liquid Chromatography-Isotope Dilution-Tandem Mass Spectrometry Approach to Quantify N,N-Diethyl-m-Toluamide and Metabolites in Urine". *Anal. Chim. Acta* 2013. 787: 267–273.