Laboratory Procedure Manual

Analyte: **Phthalate Metabolites**

Matrix: **Urine**

Method: **HPLC/ESI-MS/MS**

Method No: **12-OD**

Revised: **May 03, 2006**

as performed by:

Organic Analytical Toxicology Branch
Division of Laboratory Sciences
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Division of Laboratory Sciences

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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.

Phthalate Metabolites in Urine
NHANES 2003-2004

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released variables follows:

Data File Name	Variable Name	SAS Label
l24ph_c	URXECP	Mono-2-ethyl-5-carboxypentyl phthalate
	URXMBP	Mono-n-butyl phthalate (ng/mL)
	URXMC1	Mono-(3-carboxypropyl) phthalate (ng/mL)
	URXMCP	Mono-cyclohexyl Phthalate (ng/mL)
	URXMEP	Mono-ethyl phthalate (ng/mL)
	URXMHH	Mono-(2-ethyl-5-hydroxyhexyl) (ng/mL)
	URXMHP	Mono-(2-ethyl)-hexyl phthalate (ng/mL)
	URXMIB	Mono-isobutyl pthalate (ng/mL)
	URXMNM	Mono-n-methyl phthalate (ng/mL)
	URXMNP	Mono-isononyl phthalate (ng/mL)
	URXMOH	Mono-(2-ethyl-5-oxohexyl) (ng/mL)
	URXMOP	Mono-n-octyl phthalate (ng/mL)
	URXMZP	Mono-benzyl phthalate (ng/mL)

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NHANES 2003-2004

1. Clinical Relevance and Summary of Test Principle

a. Test Principles

The test principle utilizes high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) for the quantitative detection in urine of the following phthalate metabolites: monomethyl phthalate (mMP), monoethyl phthalate (mEP), monobutyl phthalate (mBP), mono-isobutyl phthalate (miBP), mono (3-carboxypropyl) phthalate (mCPP), monocyclohexyl phthalate (mCHP), mono (2-ethylhexyl) phthalate (mEHP), monooctyl phthalate (mOP), monobenzyl phthalate (mBzP), monoisononyl phthalate (mNP), mono(2-ethyl-5-oxohexyl) phthalate (mEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (mEHHP), and mono(2-ethyl-5-carboxypentyl) phthalate (mECPP) [1]. Urine samples are processed using enzymatic deconjugation of the glucuronidated phthalate monoesters followed by on-line solid phase extraction (SPE) coupled with reversed phase HPLC-ESI-MS/MS. Assay precision is improved by incorporating isotopically-labeled internal standards for each of the phthalate metabolites. In addition, 4-methyl umbelliferone glucuronide is used to monitor deconjugation efficiency. This selective method allows for rapid detection of up to fifteen monoester metabolites of commonly used phthalate diesters in human urine with limits of detection in the low parts per billion (ppb) range.

b. Clinical Relevance

Phthalates, are a group of industrial chemicals widely used in consumer products and as solvents, additives, and plasticizers [2]. Humans are potentially exposed to many products containing phthalates. Phthalates are rapidly metabolized in humans to their respective monoesters, which depending on the phthalate can be further metabolized to their oxidative products. Monoesters and the oxidative metabolites of phthalates may be glucuronidated, and these conjugates excreted in the urine and feces [3-7]. Several phthalates are carcinogenic in animal models [6,8,9]. Some phthalates and their monoester metabolites can cause reproductive and developmental toxicities in animals [10-13], but little is known about the effects of phthalate exposure on humans. Information on the concentration of phthalates in people is essential to understand the human exposure to phthalates. Measurement of an internal dose, or biomarker of exposure, is a key aspect of assessing exposure [14].

2. Safety Precautions

a. 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.

(3) Use solvents only in well-ventilated areas.

Note: The Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure are maintained on the PC network (www.ilpi.com/msds/index.html), as a hard copy found in the laboratory and on a CD-ROM in the Chamblee Library. Laboratory personnel must review the MSDS prior to using chemicals.

Care should be exercised in the handling of all chemical standards.

Exercise caution in the handling of biological samples.

Laboratory personnel should use appropriate protection to minimize possible exposure, and should observe Universal Precautions, such as wearing safety glasses, protective gloves and lab coat during all steps of this method because of both infectious and chemical related hazards. Use of the following is imperative when performing this method: (1) laboratory coats; (2) safety glasses; and (3) protective gloves.

The Hepatitis B vaccination series is strongly recommended for all testing personnel. Any residual sample material should be appropriately discarded and prepared for autoclaving after analysis is completed. All disposal laboratory supplies, and laboratory glassware, used in this procedure must also be placed in an autoclave bag for disposal or decontamination and cleaning for re-use.

3. Computerization; Data-System Management

a. Data Collection and Processing

The analytical procedure for the detection of the phthalate metabolites utilizes a ThermoFinnigan Surveyor liquid chromatograph coupled with a ThermoFinnigan TSQ Quantum mass spectrometer equipped with an electrospray ionization (ESI) interface. Data acquisition and analysis are controlled by the ThermoFinnigan Xcalibur software. The software selects the appropriate peak based on the precursor/product ion combination and chromatographic retention time and subsequently integrates the peak area. All data are exported from the Xcalibur Quan software as an Excel spreadsheet report and imported into a relational database (Microsoft Access, Redmond, WA) using an automated, custom - written Visual Basic module. Further manipulation of the data, including QC evaluation, reagent blank subtraction, and statistical analyses, are performed using SAS statistical software (SAS Institute, Cary, NC). After any additional calculations or corrections by the analyst are completed and the reviewing supervisor approves the final values for release, a hard copy of the final data (SAS output) is made. Raw files are regularly backed up onto a CDR. The Access database is located on the Local

Area Network (LAN) and is automatically backed up nightly to a tape by the DLS LAN support staff. Documentation for data system maintenance is contained in copies of data records.

b. **Quality Control Data, Charts**

The data, loaded into the database by the analyst, are exported as a text file. This file is used by a SAS program to generate QC charts for all analytes monitored.

c. **Final Reports**

Final reports are generated using a SAS program, and are reviewed by the DLS statistician before being transmitted to the Branch Chief via an inter-office memorandum. Then, the report is finally reviewed by the DLS Division Director. From the inter-office memorandum, a report is generated to the individual(s) requesting the analysis. Hard copies of data and correspondences are maintained in the office of the Branch Chief of the Organic Analytical Toxicology Branch under the miscellaneous project/case number assigned by the DLS specimen-receiving personnel. Data from the case are also maintained in the notebook of the analyst, and by the laboratory supervisor and/or his/her designee.

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

a. **Materials needed for urine collection and storage**

(1) Urine collection cup (150-250 mL plastic, sterile, pre-screened for phthalate metabolites) with cap.
(2) Labels
(3) 5 mL plastic cryovial (pre-screened)
(4) Cryovials (pre-screened)
(5) Cotton wool (pre-screened)
(6) Other sampling collection materials (pre-screened)

b. **Urine collection, storage and handling**

(1) Preferably, urine specimens for phthalate analysis should be collected by using a pre-screened urine sampling collection device (e.g., cup, pediatric collection bag).
(2) A minimum sample volume of 3 mL is required for the assay.
(3) Specimens may be stored in a 5 mL plastic cryovials as long as the vials are tightly sealed.
(4) Specimens may be stored frozen at temperatures below -20 °C for one to two years prior to analysis.
(5) Specimens should arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.

(6) Specimen handling conditions are outlined in the Division protocol for urine handling (copies are available in the laboratory). In the protocol, collection, transport, and special equipment required are discussed. In general, urine specimens should be transported and stored cold (dry ice, ice or blue ice can be used). Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen below -20 °C. The integrity of samples thawed and refrozen several times is not compromised.

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

Not applicable for this procedure.

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

Note: Class A glassware such as pipettes and volumetric flasks are used unless otherwise stated.

a. Reagent Preparation

Mobile phase A (0.1% acetic acid in water). To make 1L, 1.0 mL of acetic acid is added to 999 mL HPLC grade water. This solution is stored at room temperature and kept for a maximum of three days.

Mobile phase B (0.1% acetic acid in acetonitrile). To make 1L, 1.0 mL of acetic acid is added to 999 mL HPLC grade acetonitrile. This solution is stored at room temperature and discarded after one week.

* Acetonitrile, methanol, and HPLC grade water are purchased from Tedia (Fairfield, OH),
* Formic acid (FA, 98% min, GR) is purchased from EM Science (Gibbstown, NJ).
* Acetic acid (glacial) is purchased from Sigma Aldrich Laboratories, Inc (St. Louis, MO)
* Ammonium hydroxide (30%) is purchased from JT Baker (Phillipsburg, NJ).
* β-Glucuronidase (*Escherichia coli*-K12) is purchased from Roche Biomedical (Mannheim, Germany).

b. Analytical Standards

(1) Source

Phthalate metabolite native and labeled standards were obtained from:

(a) Cambridge Isotope Laboratories Inc
50 Frontage Road

Andover, MA 01810-5413

(b) Los Alamos National Laboratory
Los Alamos, NM

(c) Professor Jurgen Angerer
Germany

(d) $^{13}C_4$-4-methyl umbelliferone was purchased from Cambridge Isotope Laboratories Inc. 4-methyl umbelliferone glucuronide was purchased from Sigma Chemicals (Cat. M5664)

(2) Standards Preparation

(a) Individual native standards (phthalate metabolites). The stock solutions are prepared by accurately transferring approximately 5 mg of material to a tared 50 mL volumetric flask. The phthalate metabolite is then dissolved in acetonitrile. This stock solution is stored at -20 °C in a Teflon-capped amber glass bottle (methanol rinsed) until use.

(b) Internal standards (isototopically-labeled phthalate metabolites and $^{13}C_4$-4-methyl umbelliferone). These internal standards are prepared similarly to the native standards and stored sealed at -20 °C until use, except for the working solution (4 °C). The isotopic purity of each internal standard, confirmed empirically by tandem mass spectral analysis, was determined to contain less than 1% of the native compound.

(c) 4-methyl umbelliferone glucuronide standard. A 25 mL volume of stock standard solution is prepared by transferring approximately 10 mg of the 4-methyl umbelliferone glucuronide standard to a flask (methanol rinsed) and then adding 2.5 mL of acetonitrile and 22.5 mL of HPLC grade water for a concentration of 400 ppm. This stock solution is stored at -20 °C in a Teflon-capped glass bottle (methanol rinsed) until use. An intermediate 4-methyl umbelliferone glucuronide stock solution (40 ppm) is prepared by adding 2.5 mL of the stock standard into 25 mL of HPLC grade water. The 4-methyl umbelliferone glucuronide pre-spiking solution is prepared by adding 2.0 mL of the intermediate stock standard into 100 mL of HPLC grade water to make a final concentration of 0.8 ppm. The spiking solution of 0.16 ppm 4-methyl umbelliferone glucuronide is prepared by transfering 5 mL of the intermediate stock solution into a 25 mL volumetric flask and diluting with water.

(d) Eleven unique working standards with all analytes and 4-methyl umbelliferone are prepared in 1:9 acetonitrile: water from the stock solutions of native and the isotopically-labeled internal standards to cover the linear range of the assay for each analyte. The linear range for mEP was extended to 3750 ppb due to the relatively high urinary levels of this analyte found in humans.

(3) Storage and Stability

All standards are kept in amber Boston round bottles with Teflon-lined screw caps. Working standards are kept in the refrigerator (4 °C) and remade as needed from the stock solutions. Stock standard mixtures are kept in the freezer at 0 °C (+/- 2 °C). Stock standard mixtures are remade, as necessary.

(4) Proficiency Testing Standards

Aliquots of each stock standard were added to 1L filtered urine pools. The volume of each standard varied to produce 3 concentrations of proficiency testing (PT) standards. The spiked pools were mixed overnight and were aliquoted into prescreened vials and frozen until needed. The PT standards were characterized by at least 20 repeat determinations to characterize the mean and standard deviation for evaluation.

(5) Materials

(a) Chromolith Flash RP-18e precolumn (4.6 mm x 25 mm, Merck KGaA, Germany).
(b) 1.5 mL autosampler vials (Agilent, USA) and caps (caps with PTFE/Silicone).
(c) ThermoFinnigan-Keystone Betasil phenyl column (3 µm, 150 mm × 2.1 mm).
(d) Inline filters (2 µm and 0.5 µm, Upchurch).
(e) Pipette tips: 5 ml, 1 mL, 100 µL, 50 µL, 20 µL and 10 µL sizes.

(6) Equipment

(a) Repipettors (Rainin, Woburn, MA and Eppendorf. New York) and Reference Pipettes (Rainin, Woburn, MA)
(b) Balance (TR-203 Series Denver Instrument Company, Denver, Co)
(c) Balance (Sartorius, Genius series, Cleveland, Oh)
(d) Sonicating waterbath (Branson 5210, Danbury Conn).
(e) Fisher Isotemp Incubator (300 Series Model 350D).
(f) Vortexer (Fisher, Genie 2).
(g) Magnetic Stirrer (Corning, Corning New York).
(h) ThermoFinnigan Surveyor autosampler (Waltham, Mass)
(i) 10 port switching valve (Rheodyne, Deerfield, Il)

(7) Analytical instrumentation

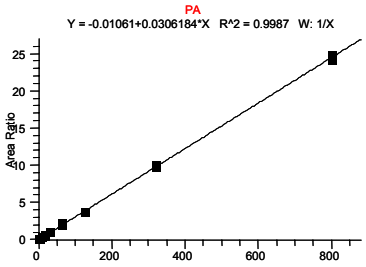
(a) ThermoFinnigan Surveyor High Pressure Liquid Chromatograph system (Waltham, Mass)
(b) ThermoFinnigan Quantum Triple Quadrupole Mass spectrometer (Waltham, Mass)

7. Calibration and Calibration-Verification Procedures

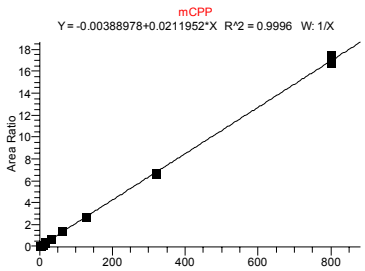
a. Calibration

Before mass spectral analysis of unknown samples, a known standard is injected to confirm acceptable chromatographic resolution and mass spectral sensitivity. If the instrument yields acceptable performance, a full set of 11 standards followed by the unknowns, QC samples and blanks are analyzed. The analysis is completed by re-injecting the same eleven standards. The duplicate standards are used to draw a daily calibration curve for each analyte (known concentration versus analyte/internal standard area ratio). Each point in the calibration curve is weighted (1/x), with correlation coefficients typically > 0.99. The minimal contributions of the isotope to the native ion and native to the isotope ion are corrected by the Xcalibur Quan software for all reported data. The calibration curve is used by the Xcalibur data analysis software for all unknowns, QC samples and blanks analyzed on that day.

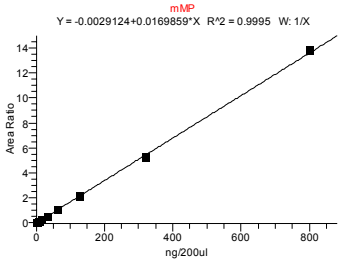
b. Example Calibration Curves



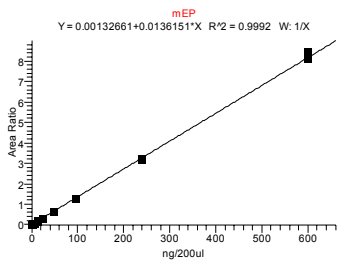
Calibration curve for PA



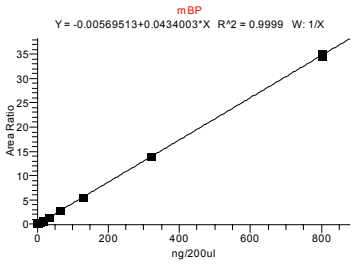
Calibration curve for mCPP



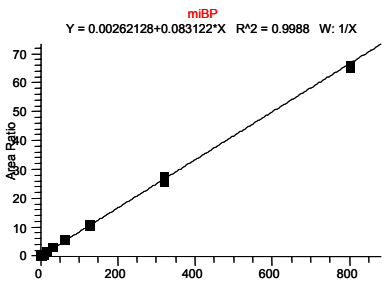
Calibration curve for mMP

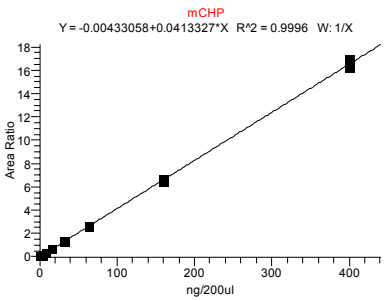


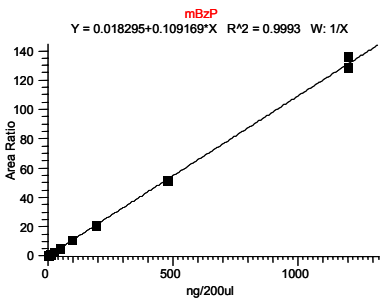
Calibration curve for mEP



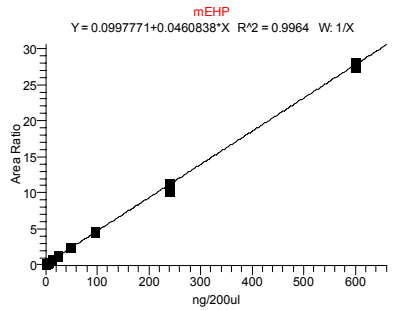
Calibration curve for mBP



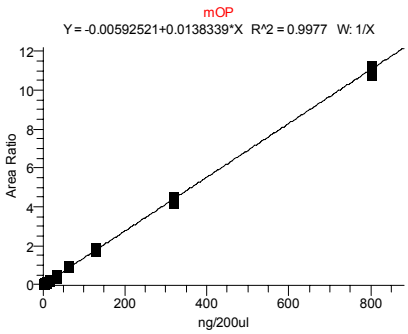




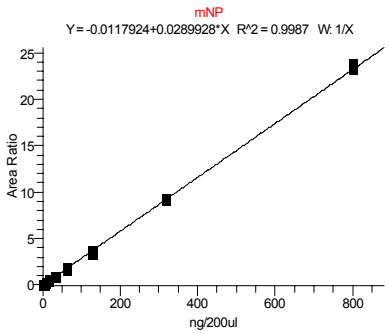
Calibration curve for miBP



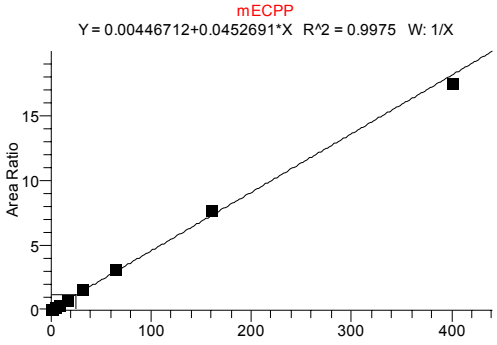
Calibration curve for mCHP



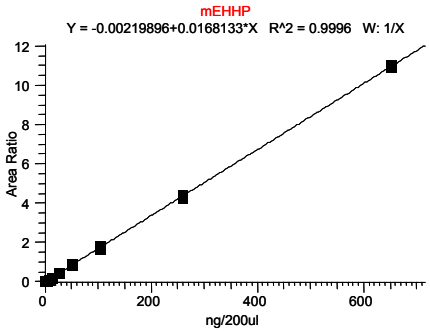
Calibration curve for mBzP



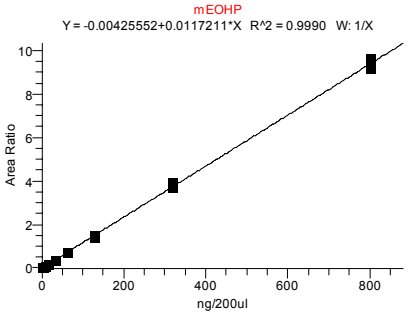
Calibration curve for mEHP



Calibration Curve for mOP

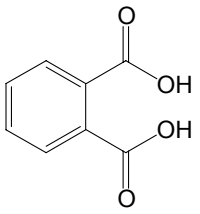


Calibration curve for mNP

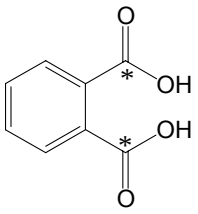


Calibration curve for mECPP **Calibration curve for mEHHP** **Calibration curve for MEOHP**

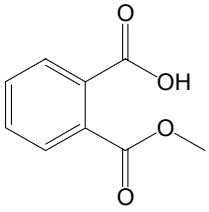
c. Analyte Nomenclature and Structures



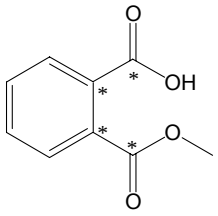
PA
phthalic acid



PA*
phthalic acid-$^{13}C_2$



mMP
monomethyl phthalate



mMP*
monomethyl phthalate-$^{13}C_4$

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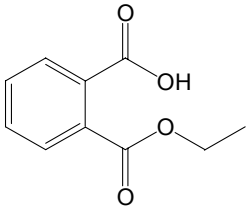
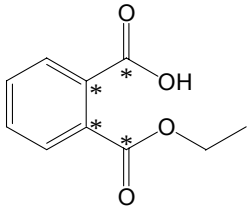
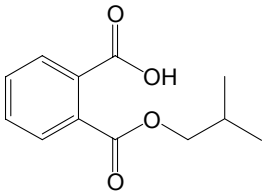


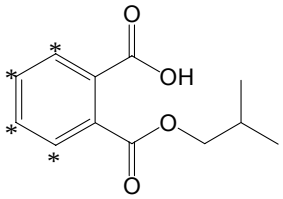
mEP
monoethyl phthalate



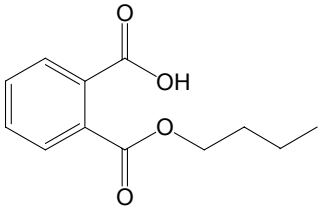
mEP*
monoethyl phthalate-$^{13}C_4$



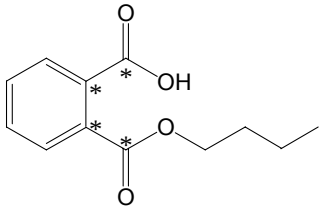
miBP
mono(2-methylpropyl) phthalate
mono-isobutyl phthalate



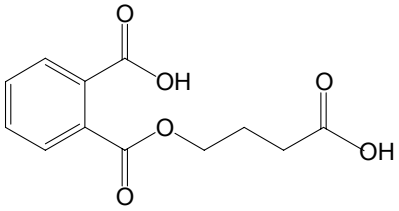
miBP*
mono(2-methylpropyl) phthalate-D_4
mono-isobutyl phthalate-D_4



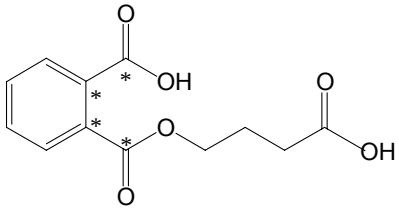
mBP
mono-n-butyl phthalate



mBP*
mono-n-butyl phthalate-$^{13}C_4$



mCPP
mono(3-carboxypropyl) phthalate



mCPP*
mono(3-carboxypropyl) phthalate-$^{13}C_4$

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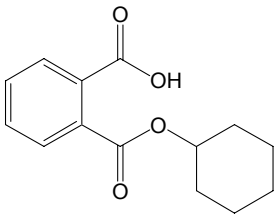
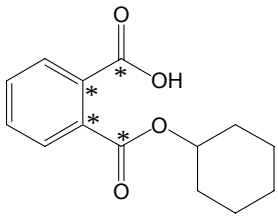
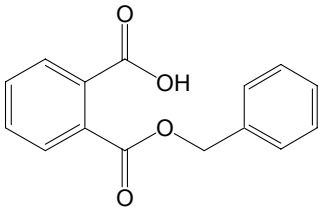


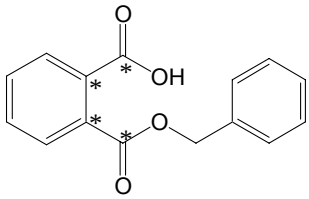
mCHP
monocyclohexyl phthalate



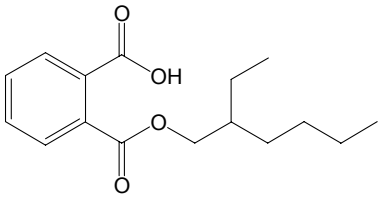
mCHP*
monocyclohexyl phthalate –$^{13}C_4$



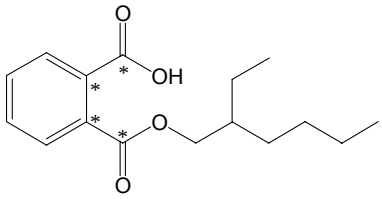
mBzP
monobenzyl phthalate



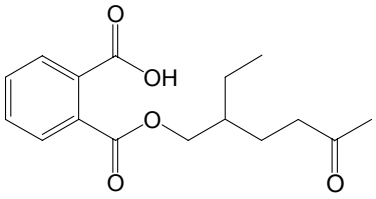
mBzP*
monobenzyl phthalate-$^{13}C_4$



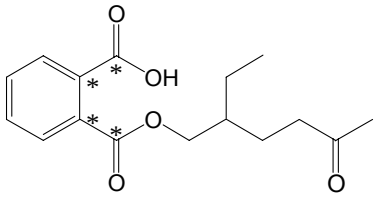
mEHP
mono (2-ethylhexyl) phthalate



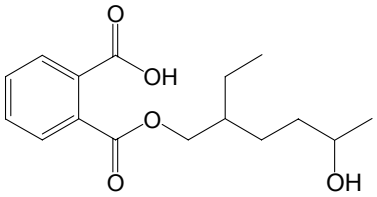
mEHP*
mono (2-ethylhexyl) phthalate-$^{13}C_4$



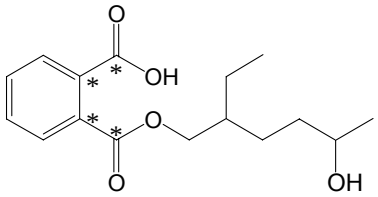
mEOHP
mono (2-ethyl-5-oxohexyl) phthalate



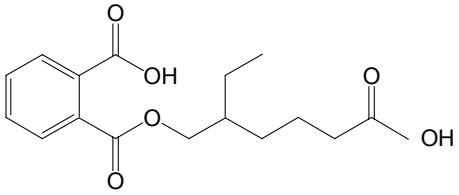
mEOHP*
mono (2-ethyl-5-oxohexyl) phthalate-$^{13}C_4$



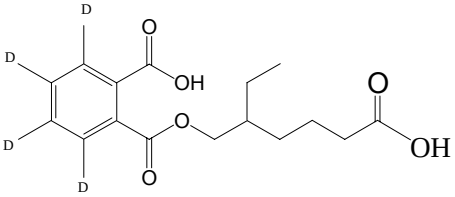
mEHHP
mono (2-ethyl-5-hydroxyhexyl) phthalate



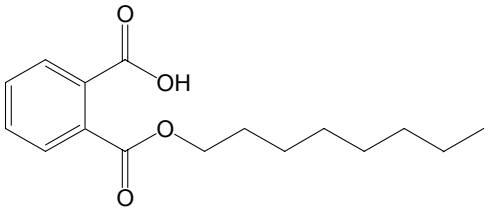
mEHHP*
mono (2-ethyl-5-hydroxyhexyl) phthalate-$^{13}C_4$



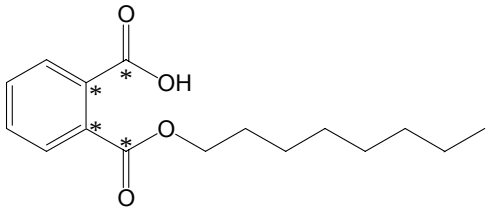
MECPP
Mono (2-ethyl-5-carboxypentyl) phthalate



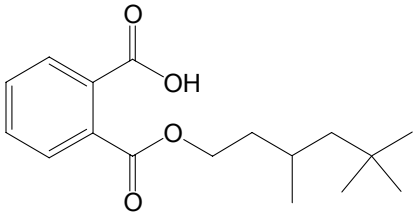
mECPP*
D_4-Mono (2-ethyl-5-carboxypentyl) phthalate



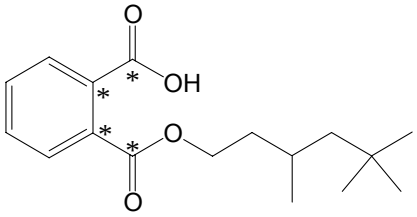
mOP
monooctyl phthalate



mOP*
monooctyl phthalate-$^{13}C_4$



mNP
mono (3, 5, 5-trimethyl-1-hexyl) phthalate
(mono-isononyl pthalate)



mNP*
mono (3, 5, 5-trimethyl-1-hexyl) phthalate-$^{13}C_4$
(mono-isononyl pthalate $^{13}C_4$)

d. **Calibration Verification**

(1)Calibration verification is not required by the manufacturer. 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) Calibration verification must be performed at least once every 6 months.

(3) All calibration verification runs and results shall be appropriately documented.

(4) 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 a low, mid, and high value, and is performed at least once every six months.

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

e. Proficiency Testing (PT)

Because no standard reference materials exist for measuring phthalate monoesters in urine and there is no recognized PT program or other laboratories routinely performing this procedure, DLS performs a rigorous in-house proficiency testing process. PT samples are prepared in-house as described in the standard preparation section. These PT samples encompass the entire linear range of the method and are characterized in our laboratory. The characterization data are forwarded to a DLS officer (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 a minimum of once every 6 months. The PT administrator 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 our laboratory of its PT status (i.e. pass/fail).

All proficiency results shall be appropriately documented.

8. Operating Procedures; Calculations; Interpretation of Results

a. Preliminaries

(1) The on-line solid phase extraction batch typically consists of: 4 Reagent Blanks, 2 Quality Control Low (QCL) and 2 Quality Control High (QCH) materials, and 40 samples.
(2) The samples and QC materials are allowed to thaw at room temperature in a sonicating water bath.
(3) The samples are mixed well by vortexing.
(4) The glucuronidase solution (mix fresh just prior to addition to sample) is prepared as follows.

(a) For a run of 50 samples; 1.5 mL of 1 M, pH 6.5, ammonium acetate buffer is transferred accurately into an autosampler vial.
(b) 30µL of β-glucuronidase is pipetted into the autosampler vial containing the ammonium acetate buffer.
(c) The solution is swirled to mix gently.

b. Sample preparation

(1) 100 µL of unknown urine sample, 100 µL mL HPLC Grade water (for the reagent blank), or 100 µL mL of QCH or QCL are transferred into properly labeled autosampler vial (1.5 mL).
(2) The vial is caped with Teflon-lined screw cap.
(3) The vial is placed in the sample tray in the sample preparation autosampler.
(4) The autosampler tray is set at 37°C for incubation of samples.
(5) 25 µL of 0.16 ppm 4-Methylumbelliferone glucuronide spiking solution, 100 µL Internal Standard (labeled mixture) spiking solution and 25 µL of β-glucuronidase/ammonium acetate solution are automatically added into each vial and mixed.
(6) After 90 min incubation, the enzyme activity is stopped by automatically adding 50 µL of glacial acetic acid and 200 µL of 5% acetonitrile in water.
(7) The autosampler tray temperature is set to 0^{o}C after preparation of the whole set.
(8) The autosampler tray is moved to the HPLC/MS analytical system for analysis.

c. Instrumental Analysis

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

The analysis is performed using a ThermoFinnigan LC pump, ThermoFinnigan Surveyor liquid chromatograph coupled with a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer, equipped with an ESI (Electrospray Ionization) interface [1]. All three systems and the ten port Reodyne switching valve are controlled by the Xcalibur Software. With the LC pump in the sample loading position, 500 µL of the pre-treated deconjugated urine sample is injected using the Surveyor autosampler. The sample is loaded for 1 min onto a Chromolith Flash RP-18e SPE column and rinsed using 0.1% acetic acid in water: 0.1% acetic acid in acetonitrile at 1.5 mL per min. The Reodyne valve is automatically switched to its alternate position, reversing the flow and allowing the analytes to be transferred from the SPE column on to HPLC column. The chromatographic resolution is accomplished using a 3 µm, 150 mm × 2.1 mm Thermofinnigan Keystone Betasil phenyl column and a solvent gradient from 77% Mobile phase A to 100% Mobile phase B at 0.35 mL per minute. After reaching 100% mobile phase B and remaining at that mobile phase composition, the column is equilibrated with 100% mobile phase A for 2 minutes (Table 1). Each sample (500 µL) is injected using the liquid chromatograph autosampler, configured with syringe washes between injections to minimize carryover. Inline filters are used to remove particulate

materials from the injected samples and extend the lifetime of the analytical column [1].

Table 1. On-line SPE and HPLC solvent gradient programs

	Agilent SPE pump			Agilent valve	Surveyor HPLC pump			Quantum valve
Time (min)	A (%)	B (%)	Flow (mL/min)		A (%)	B (%)	Flow (mL/min)	
0	90	10	1.50	SPE/Waste	77	33	2.00	HPLC/Waste
1	90	10	1.50	SPE/Waste	77	33	2.00	HPLC/Waste
1.1	100	0	0.10	SPE/HPLC	77	33	0.35	HPLC/Waste
3	100	0	0.10	SPE/HPLC			0.35	HPLC/Waste
5	100	100	0.50	SPE/Waste			0.35	HPLC/Waste
7	0	100	0.50	SPE/Waste			0.35	HPLC-MS/MS
9	0	100	0.50	SPE/Waste			0.35	HPLC-MS/MS
12	0	100	0.50	SPE/Waste	65	45	0.35	HPLC-MS/MS
13	0	100	0.50	SPE/Waste			0.35	HPLC-MS/MS
15	0	10	0.25	SPE/Waste	40	20	0.35	HPLC-MS/MS
19	90	10	0.25	SPE/Waste	20	80	0.35	HPLC-MS/MS
19.1	90	10	0.25	SPE/Waste	0	100	0.35	HPLC-MS/MS
21	90	10	0.25	SPE/Waste	0	100	0.35	HPLC/Waste

Electrospray ionization (ESI) in negative ion mode was used to transfer the negatively charged analyte ions into the gas phase. The source collision induced dissociation voltage was set to 10 V to break down acetate clusters.

During an analysis, the instrument was set in the multiple reactions monitoring mode so that parent and daughter ion combinations specific to the eluting analyte could be monitored. Reproducible chromatography allowed for the use of different data acquisition windows for different analyte groups. Daughter ions are formed in the collision cell using argon at ~1.5 mTorr. The collision offset is specifically set for each ion (Table 2).

(2) Multiple Reaction Monitoring (MRM) Setup for Phthalate Metabolites and 4-MeUmb

Table 2. Phthalate metabolites and their native and labeled precursor and product ion transitions, collision energies, and retention times.

	retention time (min)	Precursor/ Product ion	Collision energy (V)
MCPP	7.74	251/103	10
$^{13}C_4$-MCPP		255/103	

MMP	8.24	179/77	20
$^{13}C_4$-MMP		183/79	
MEP	9.65	193/77	22
$^{13}C_4$-MEP		197/79	
MECPP	11.88	307/159	17
D_4-MECPP		311/159	
MiBP	13.49	221/77	26
D_4-MiBP		225/81	
MBP	13.76	221/77	26
$^{13}C_4$-MBP		225/79	
MEOHP	14.67	291/121	26
$^{13}C_4$-MEOHP		295/124	
MEHHP	14.74	293/121	27
$^{13}C_4$-MEHHP		297/124	
MCHP	15.10	247/77	27
$^{13}C_4$-MCHP		251/79	
MBzP	15.34	255/183	16
$^{13}C_4$-MBzP		259/186	
MEHP	19.16	277/134	19
$^{13}C_4$-MEHP		281/137	
MOP	19.56	277/125	22
$^{13}C_4$-MOP		281/127	
MNP	19.64	291/247	17
$^{13}C_4$-MNP		295/250	

d. Calculations

The concentration of the individual analytes in each sample is calculated using the calibration curve derived from the known standard mixtures.

Area ratio of analyte/internal standard is proportional to the concentration ratio of analyte/Internal standard.

The final amounts of phthalate monoesters in urine are adjusted for creatinine.

e. Interpretation of Results

Several phthalates are carcinogenic in animal models [6,8,9]. Some phthalates and their monoester metabolites can cause reproductive and developmental toxicities in animals [10-13], but little is known about the effects of phthalate exposure on humans. More research is needed to determine whether exposure to phthalates can result in an increased risk of cancer and reproductive dysfunction in humans. The concentrations of phthalate monoesters obtained using this analytical method can be used to estimate recent exposure to phthalate diesters.

Di-isononyl phthalate (DINP) is a mixture of several phthalate isomers. With this method, only the monoester metabolite of one of the isomers can be measured. Therefore, exposure to DINP may be underestimated.

9. Reportable Range of Results

The linear range of the standard calibration curves and the method LOD determine the reportable range of results. The reportable range must be within the range of the calibration curves.

a. Linearity Limits

The calibration curve is linear for all analytes (R^2>0.98) over three orders of magnitude. The limit on the linearity is determined by the highest standard analyzed in the method. Unknown urine samples whose concentrations exceed this range must be reanalyzed using a smaller aliquot. The low end of the linear range is limited by the method LOD. Samples whose concentrations are below the method LOD are reported as non-detectable.

b. Limit of Detection (LOD)

The formal limit of detection (LOD) for each analyte was calculated as $3S_0$, where S_0 is the standard deviation value as the concentration approaches zero [15]. S_0 was determined from the replicate analysis of low-level standards (Table 3). The functional LOD is equal to the formal LOD unless the lowest point in the calibration curve is higher, then the functional LOD is defined as the lowest standard concentration used in the calibration curve.

Table 3. Limits of detection (LODs) of the method.

Analyte	LOD(ng/mL)
PA	0.42
MCPP	0.16
MMP	1
MEP	0.40
MiBP	0.26
MBP	0.40
MEHHP	0.32
MECPP	0.25
MEOHP	0.45
MCHP	0.20
MBzP	0.11
MEHP	0.90
MOP	1.0
MNP	1.0

c. Accuracy

The method accuracy was assessed through 5 replicate analyses of urine spiked at three different concentrations (8-16 ng/mL, 25-64 ng/mL, and 50-192 ng/mL) and expressed as a percentage of the expected value (Table 4).

Table 4. Accuracy of the method at three different concentrations.

Analyte	Conc. (ng/mL)	accuracy %	CV (%)	Conc. (ng/mL)	accuracy %	CV (%)	Conc. (ng/mL)	accuracy %	CV (%)
PA	8.0	88.5	2.3	16.0	90.5	1.7	128.0	95.4	3.5
MCPP	8.0	96.4	2.5	16.0	92.8	3.4	128.0	97.8	3.2
MMP	8.0	103.3	2.9	16.0	100.6	3.6	128.0	99.4	0.9
MEP	8.0	100.9	5.2	16.0	94.1	2.9	128.0	97.8	0.5
MiBP	8.0	103.8	3.7	5.0	99.4	3.8	128.0	100.4	2.3
MBP	8.0	95.2	4.2	16.0	107.2	1.4	128.0	107.5	2.3
MEHHP	8.0	93.6	2.8	16.0	108.6	1.1	128.0	98.4	1.3
MECPP	2.0	94.2	3.8	16.0	97.1	0.5	50.0	92.5	3.7
MEOHP	8.0	98.9	4.2	16.0	99.8	1.4	128.0	96.3	1.5
MCHP	4.0	97.8	4.7	8.0	102.5	4.4	64.0	101.5	2.4
MBzP	12.0	96.2	4.9	24.0	100.6	2.7	96.0	95.6	3.8
MEHP	6.0	94.5	6.7	12.0	103.8	4.1	48.0	100.2	4.3
MOP	8.0	95.8	5.1	16.0	97.8	3.9	128.0	97.8	4.2
MNP	8.0	94.4	3.4	16.0	92.8	3.4	128.0	97.2	3.7

d. Precision

The precision of the method is assessed by calculating the average coefficient of variation (CV) of repeated measurements of the QC materials over at least a 5-week period. This value reflects both the intraday and interday variability of the assay (Table 5).

Table 5. Urinary QC concentrations (ng/mL) and %CV

Analyte	Mean QCL	%CV QCL	Mean QCH	%CV QCH
mBP	25.7	7.4	143	7
mBzP	28.9	6.4	213	6.3
mCHP	7.8	6.9	43.7	7.1
mCPP	5.3	10.9	16	12.3
mECPP	22.3	6.1	88.2	6.2
mEHHP	14.2	8.4	86.5	6.7
mEHP	12.3	7.6	69.9	10.5
mEOHP	6.7	9.6	45.2	6.6
mEP	89.2	5.1	394	6
mMP	37.8	15.4	218	8.6
mNP	12.1	6.5	73.4	11.7
mOP	8.8	7.5	28.4	14.3
miBP	25.7	8.6	134	5.8

10. QC Procedures

Quality control (QC) materials are prepared from urine pools collected from several anonymous donors. Preliminary human quantification is used to set target ranges for baseline levels (QC Low – 5-20 ppb), and higher levels (QC High – 30-500 ppb). The human urine pool is spiked with additional phthalate monoester analytes as needed. The urine is then thoroughly mixed and dispensed into pre-rinsed glass vials. The vials are tightly sealed with Teflon-lined closures and stored at -40 °C until used. The QC pools are characterized to determine the mean concentration and the 95th and 99th confidence

intervals for both means and variance. QC characterization involved at least 100 discrete measurements spanned over one month 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. When using 2 QC pool levels per run, the rules are:

For 1 QC result per pool

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
   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.

For 2 or more QC results per pool

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).

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

If the QC systems or the calibrations failed to meet acceptable criteria, all operations are suspended until the source or cause of failure is identified and corrected. If the source of

failure is easily identifiable, for instance, failure of the mass spectrometer or a pipetting error, the problem is immediately corrected. Otherwise, fresh reagents are prepared and the mass spectrometer is cleaned. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration standards (in the case of calibration failure) are reanalyzed. After calibration or quality control has been reestablished, analytical runs may be resumed.

12. Limitations of Method; Interfering Substances and Conditions

The procedure requires expensive instrumentation.

Sources of imprecision in the procedure may be intermittently imprecise pipetting and/or phthalate contamination in extraction materials and contaminated solvents.

Any contact with plastics during specimen acquisition, storage, or sample analysis can result in interference.

13. Reference Ranges (Normal Values)

The results from the National Health and Nutrition Examination Survey (NHANES) 1999-2002 (Table 6) will be used as the reference range to describe levels of phthalate exposure among the general US population.

Table 6. Creatinine adjusted phthalate metabolite concentrations in urine (μg/g creatinine) from NHANES 1999-2000 and 2001-2002.

Metabolite	Survey years	N	Geometric mean	Median	75th percentile	90th percentile	95th percentile
mMP	01-02	2772	1.08	1.33	2.62	5.0	7.97
mEP	99-00	2536	163	141	360	898	1950
	01-02	2772	167	147	388	975	1860
mBP	99-00	2541	22.4	21.9	38.9	68.3	97.5
	01-02	2772	17.8	17.4	30.4	52.4	81.3
miBP	01-02	2772	2.53	2.44	4.50	8.02	12.0
mBzP	99-00	2541	14.0	13.3	25.1	50.1	77.4
	01-02	2772	14.1	13.5	26.6	55.1	90.4
mCPP	01-02	2772	2.57	2.45	4.07	7.25	11.4
mCHP	99-00	2541	*	<LOD	<LOD	<LOD	3.0
	01-02	2782	*	<LOD	<LOD	0.588	0.854
mEHP	99-00	2541	3.12	3.08	5.88	10.8	18.5
	01-02	2772	3.99	3.89	7.94	18.2	32.8

mEHHP	01-02	2772	18.8	16.6	32.3	70.8	147
mEOHP	01-02	2772	12.6	11.2	21.3	45.1	87.5
mNP	99-00 01-02	2541 2772	*	< LOD < LOD	< LOD < LOD	< LOD < LOD	4.29 <LOD
mOP	99-00 01-02	2541 2772	*	< LOD < LOD	< LOD < LOD	2.40 < LOD	3.51 < LOD

14. Critical-Call Results ("Panic" Values)

Reported urine levels of some phthalate monoesters can approach the low ppm range. The phthalate monoester values obtained using this method of analysis are investigational markers of phthalate exposure only; therefore critical values have not been determined.

15. Specimen Storage and Handling During Testing

Specimens are stored under lock and key in the laboratory, at freezer conditions (≤ -20 °C) prior to analysis and at refrigerator temperatures during analysis. Frozen samples are allowed to thaw slowly at room temperature prior to the initiation of the procedure.

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

The current analytical method utilizes a ThermoFinnigan Surveyor liquid chromatograph coupled with a ThermoFinnigan TSQ Quantum mass spectrometer. An alternative analytical approach would be using a Waters Alliance 2690 liquid chromatograph coupled with a Finnigan TSQ 7000 equipped with an ESI interface. The solid phase extraction can also be done manually [16,17] or automated [18]. If the test system fails, sample extracts can be stored (≤ -20 °C) in sealed 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 (≤ 5 °C).

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

a. Data from analytical runs of unknowns are initially reviewed by the laboratory supervisor.

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. If the quality control data are acceptable the laboratory supervisor generates a memorandum to the Branch Chief reporting the results.

d. These data are then sent to the person(s) that made the initial request.

e. All data (chromatograms, etc.,) are stored in electronic format in the laboratory.

f. Final hard copies of correspondence are maintained in the office of the Branch Chief and with the quality control officer.

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

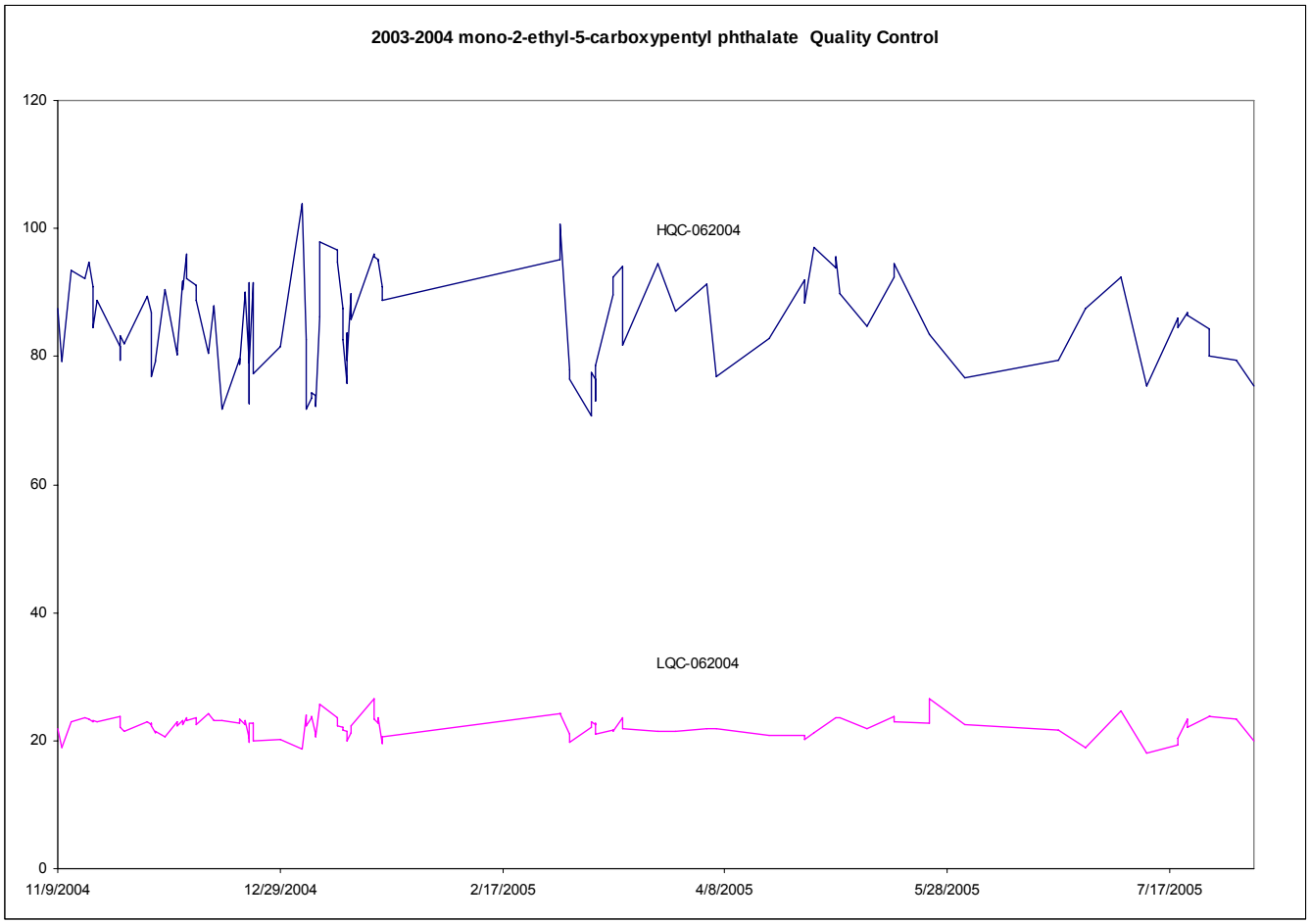
One spreadsheet form (CLIA Specimen Tracking Records) with information for receiving/transferring specimens is kept in the laboratory. In this form, the samples received are logged in when received and when stored/transferred after analysis. For NHANES samples, the person receiving the specimens signs and dates the shipping manifests. The shipping manifests for NHANES and other samples are kept in a binder in the Laboratory.

19. Summary Statistics and QC Graphs

A. Mono-2-ethyl-5-carboxypentyl phthalate

Summary Statistics for mono-2-ethyl-5-carboxypentyl phthalate by Lot

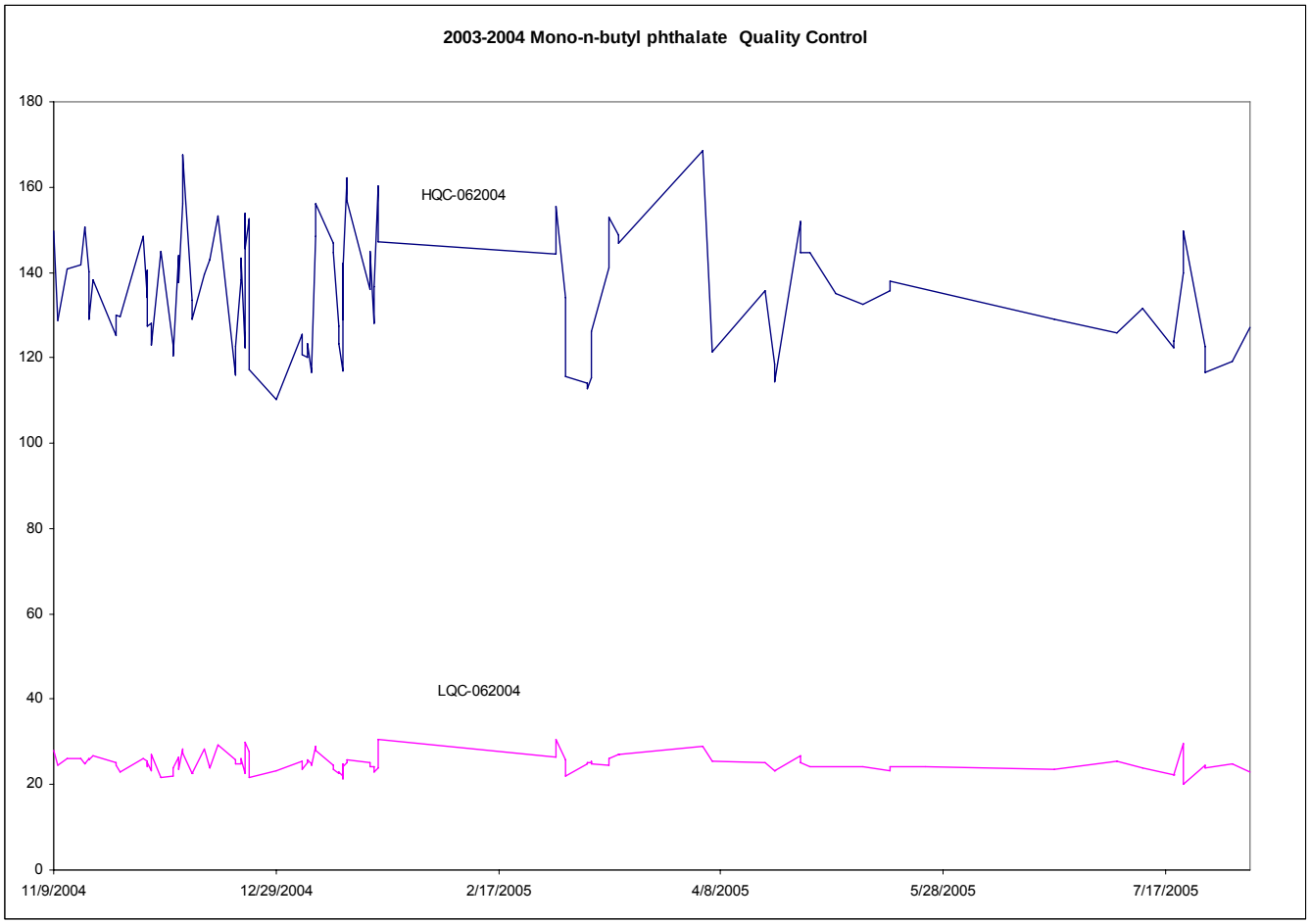
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	109	11/9/2004	8/5/2005	22.26	1.58	7.1
HQC-062004	108	11/9/2004	8/5/2005	85.38	7.54	8.8



B. Mono-n-butyl phthalate

Summary Statistics for Mono-n-butyl phthalate by Lot

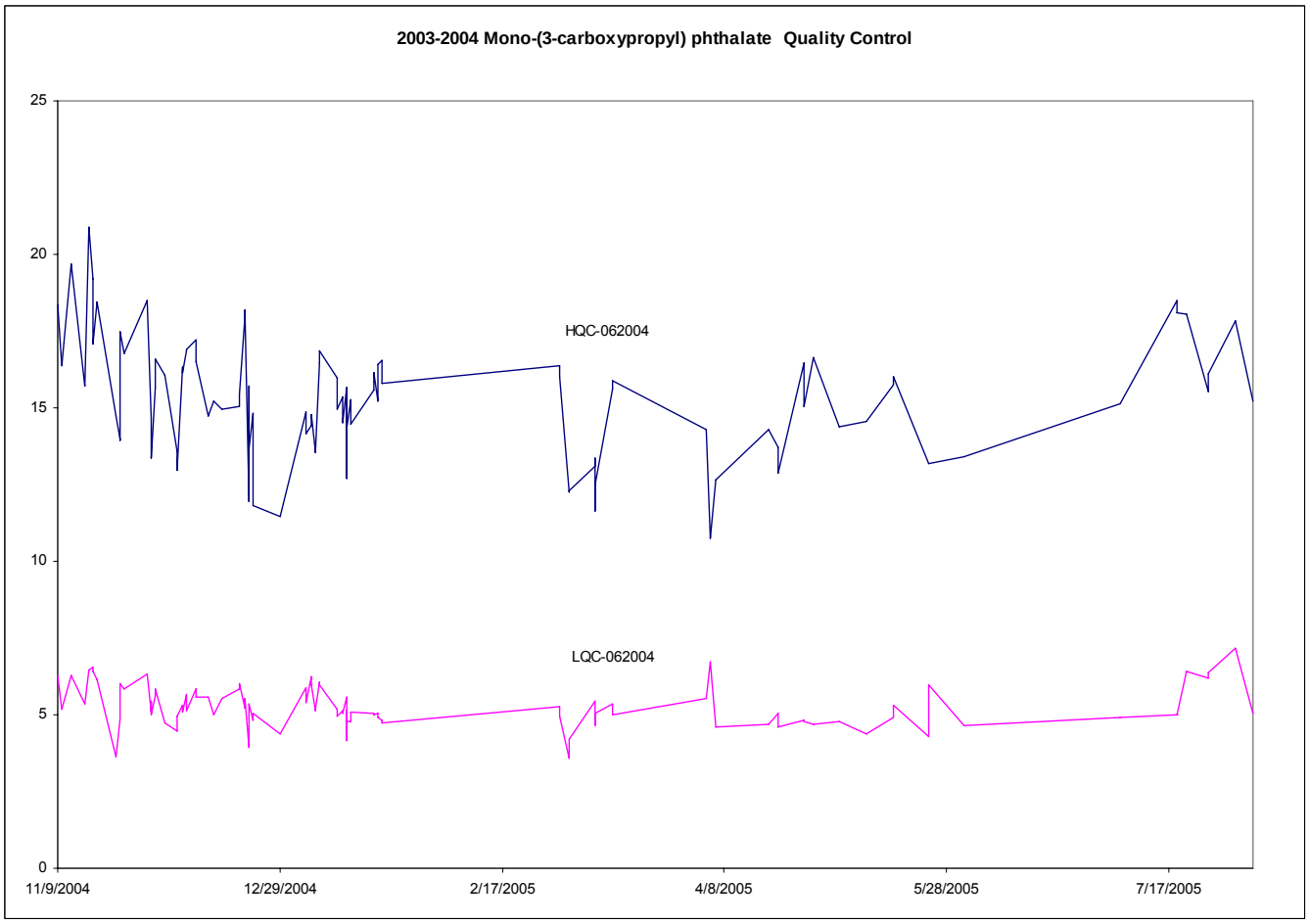
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	104	11/9/2004	8/5/2005	25.01	2.11	8.4
HQC-062004	103	11/9/2004	8/5/2005	134.70	13.49	10.0



C. Mono-(3-carboxypropyl) phthalate

Summary Statistics for Mono-(3-carboxypropyl) phthalate by Lot

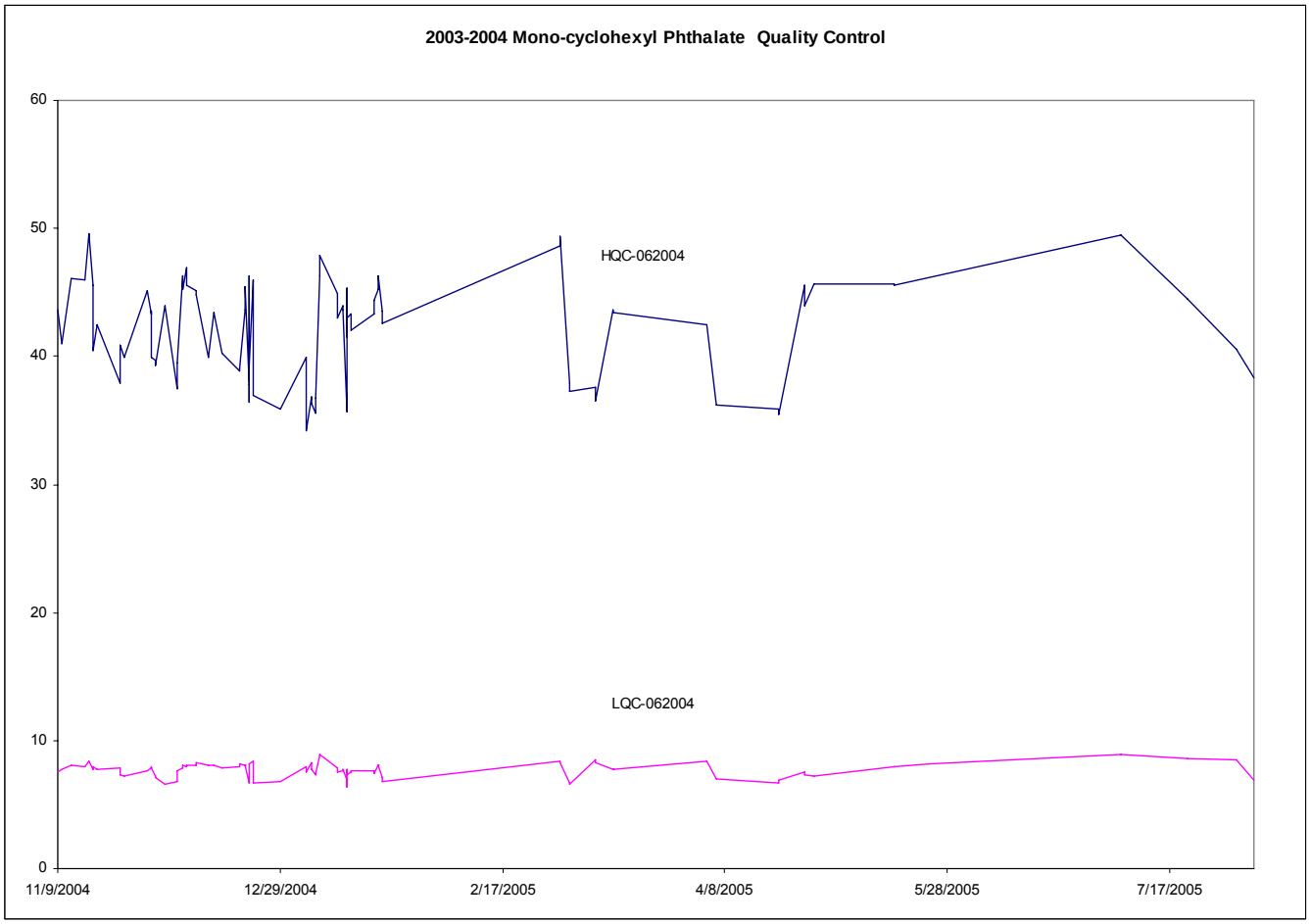
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	101	11/9/2004	8/5/2005	5.25	0.67	12.8
HQC-062004	100	11/9/2004	8/5/2005	15.27	1.93	12.6



D. Mono-cyclohexyl Phthalate

Summary Statistics for Mono-cyclohexyl Phthalate by Lot

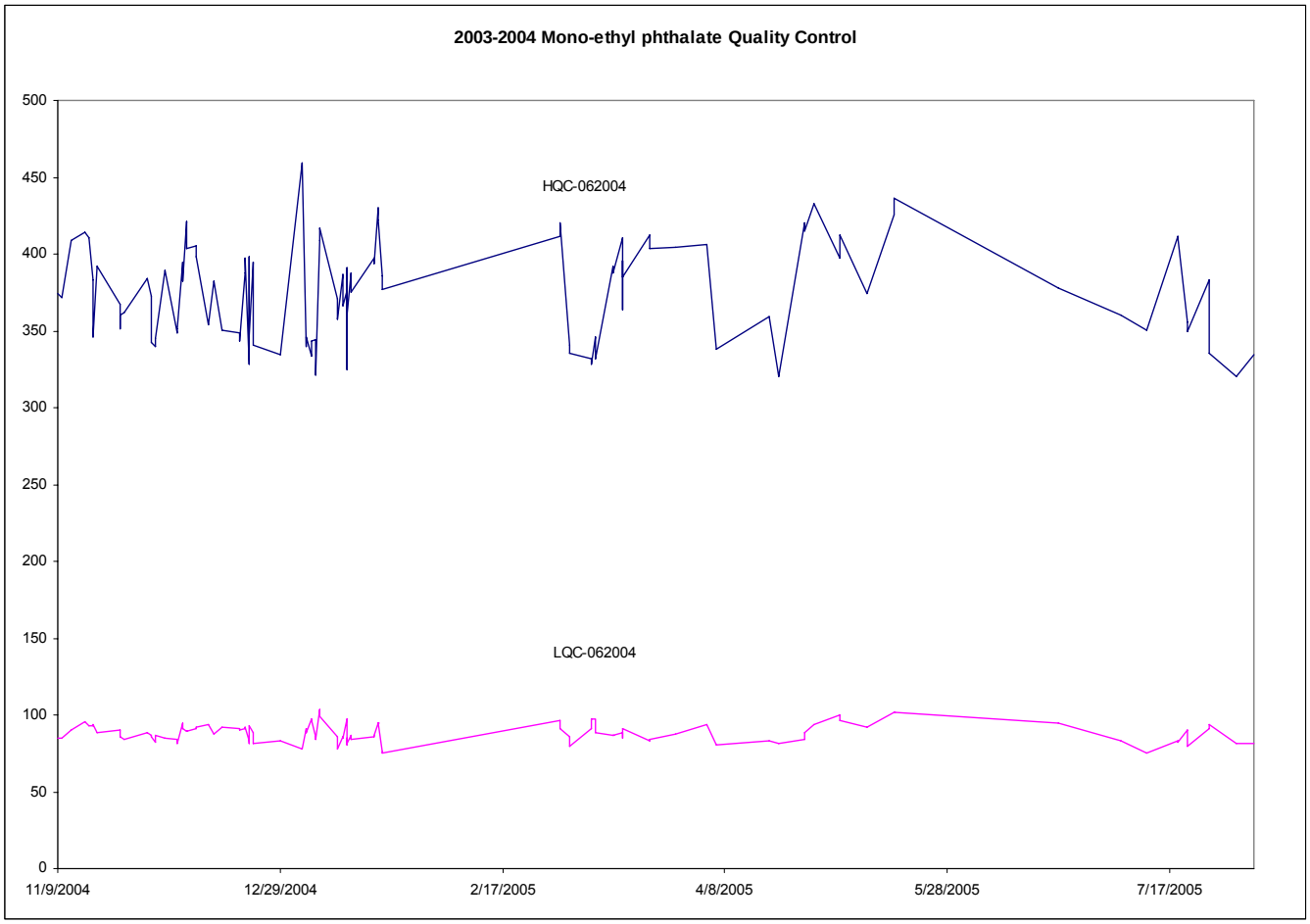
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	88	11/9/2004	8/5/2005	7.69	0.58	7.5
HQC-062004	87	11/9/2004	8/5/2005	42.00	3.88	9.2



E. Mono-ethyl phthalate

Summary Statistics for Mono-ethyl phthalate by Lot

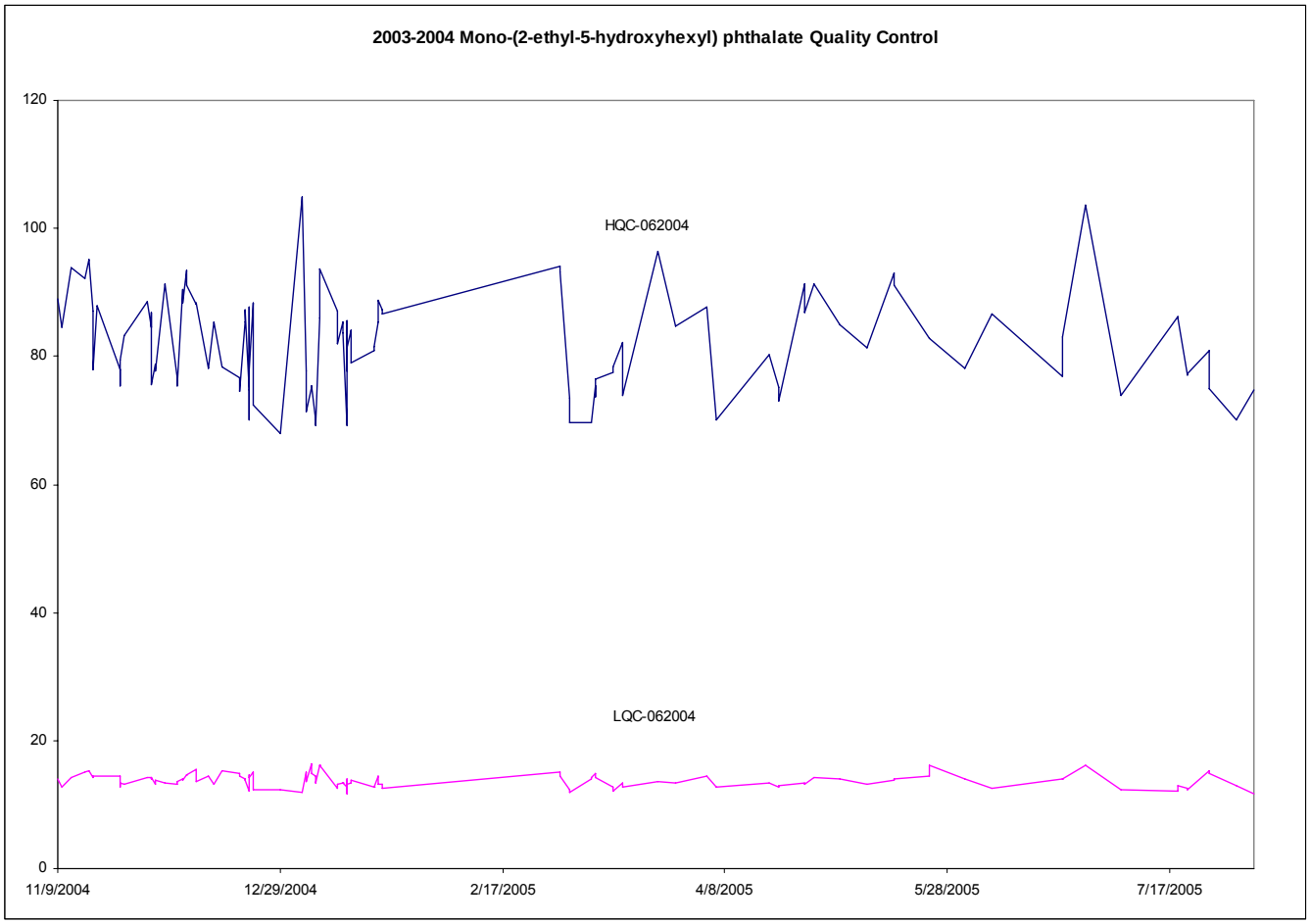
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	109	11/9/2004	8/5/2005	88.32	5.94	6.7
HQC-062004	109	11/9/2004	8/5/2005	374.48	31.70	8.5



F. Mono-(2-ethyl-5-hydroxyhexyl)

Summary Statistics for Mono-(2-ethyl-5-hydroxyhexyl) phthalate by Lot

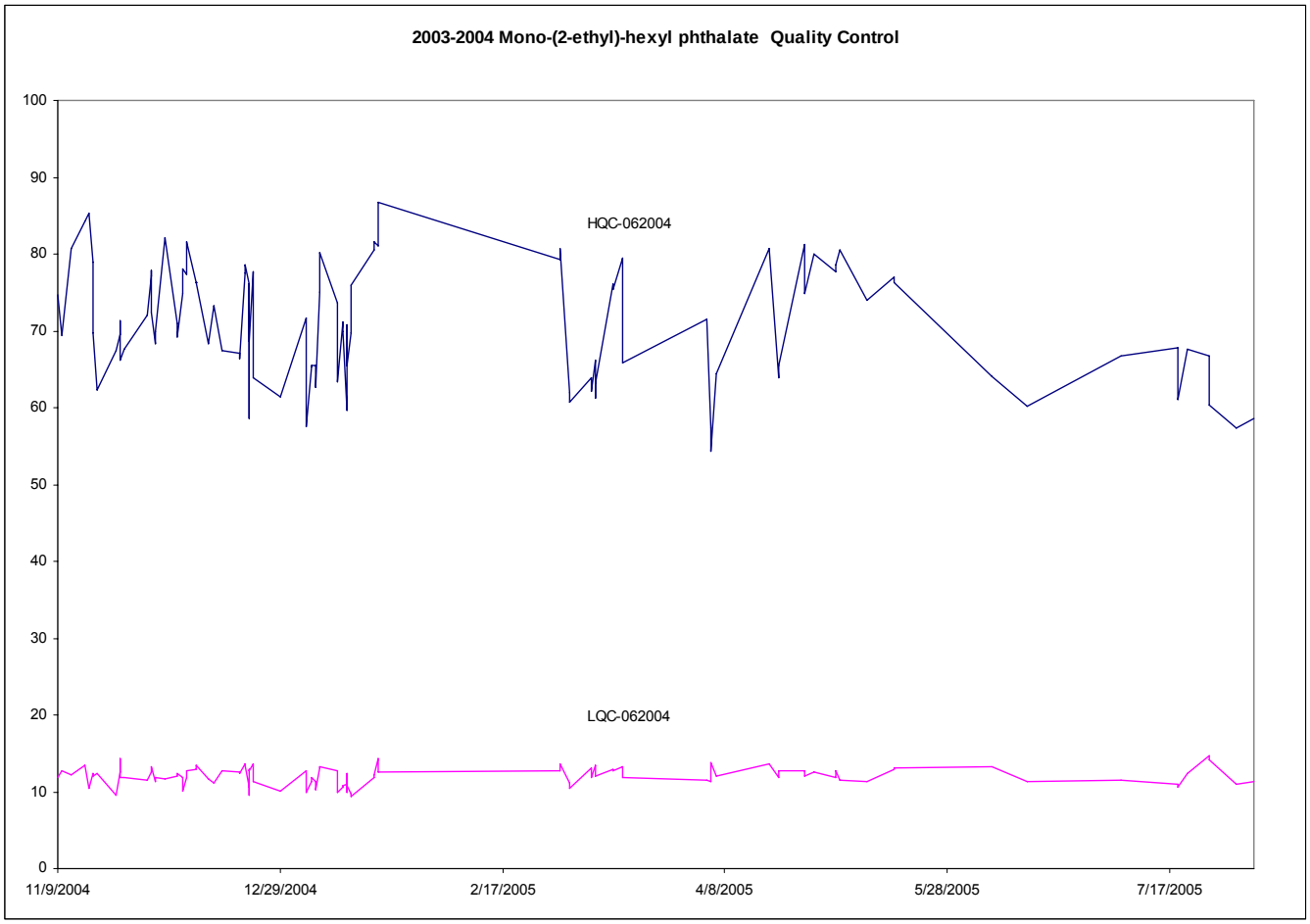
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	111	11/9/2004	8/5/2005	13.73	1.08	7.9
HQC-062004	110	11/9/2004	8/5/2005	81.93	7.77	9.5



G. Mono-(2-ethyl)-hexyl phthalate

Summary Statistics for Mono-(2-ethyl)-hexyl phthalate by Lot

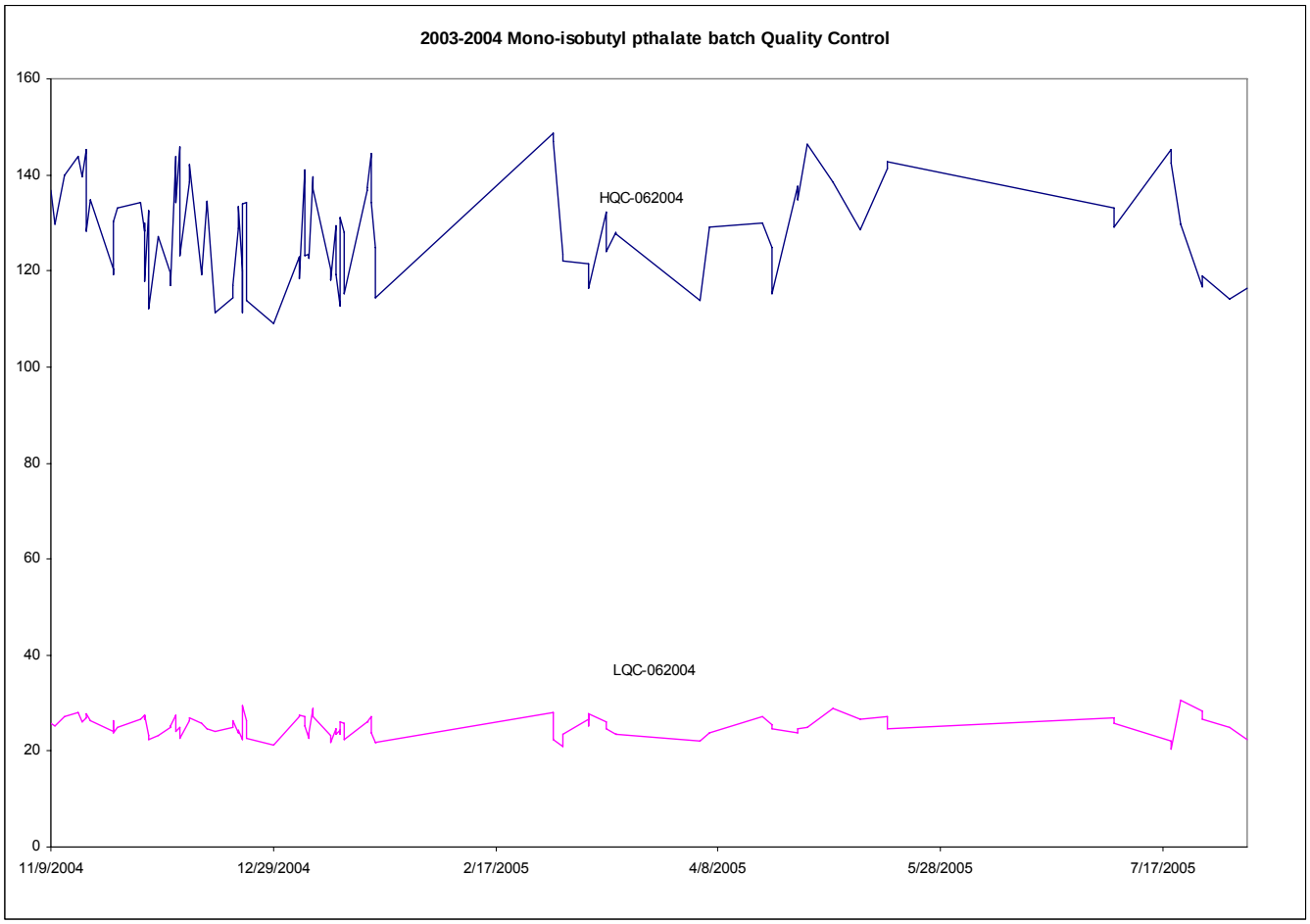
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	105	11/9/2004	8/5/2005	12.07	1.17	9.7
HQC-062004	105	11/9/2004	8/5/2005	70.69	7.52	10.6



H. Mono-isobutyl pthalate

Summary Statistics for Mono-isobutyl pthalate by Lot

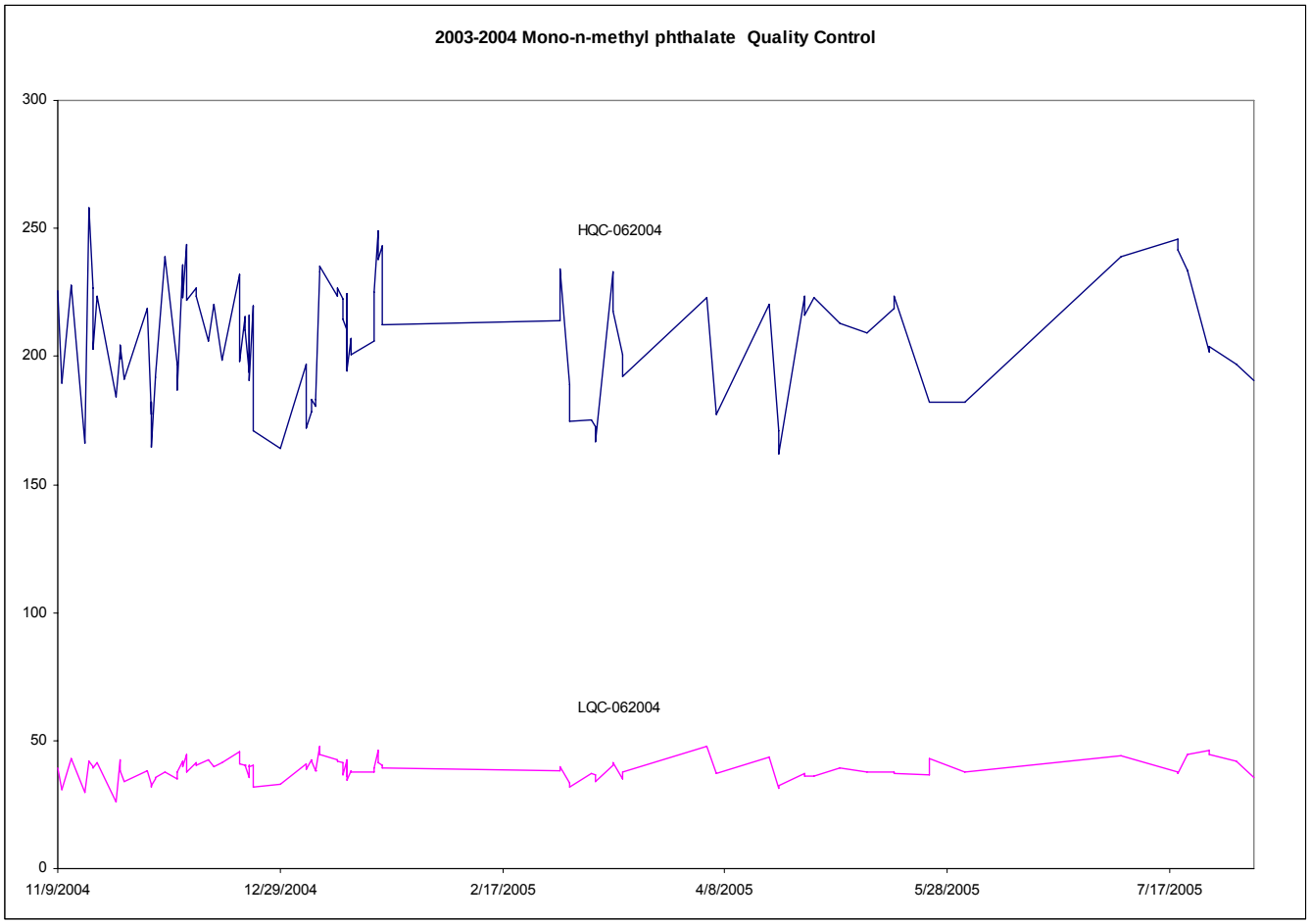
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	97	11/9/2004	8/5/2005	25.13	2.09	8.3
HQC-062004	97	11/9/2004	8/5/2005	128.16	10.21	8.0



I. Mono-n-methyl phthalate

Summary Statistics for Mono-n-methyl phthalate by Lot

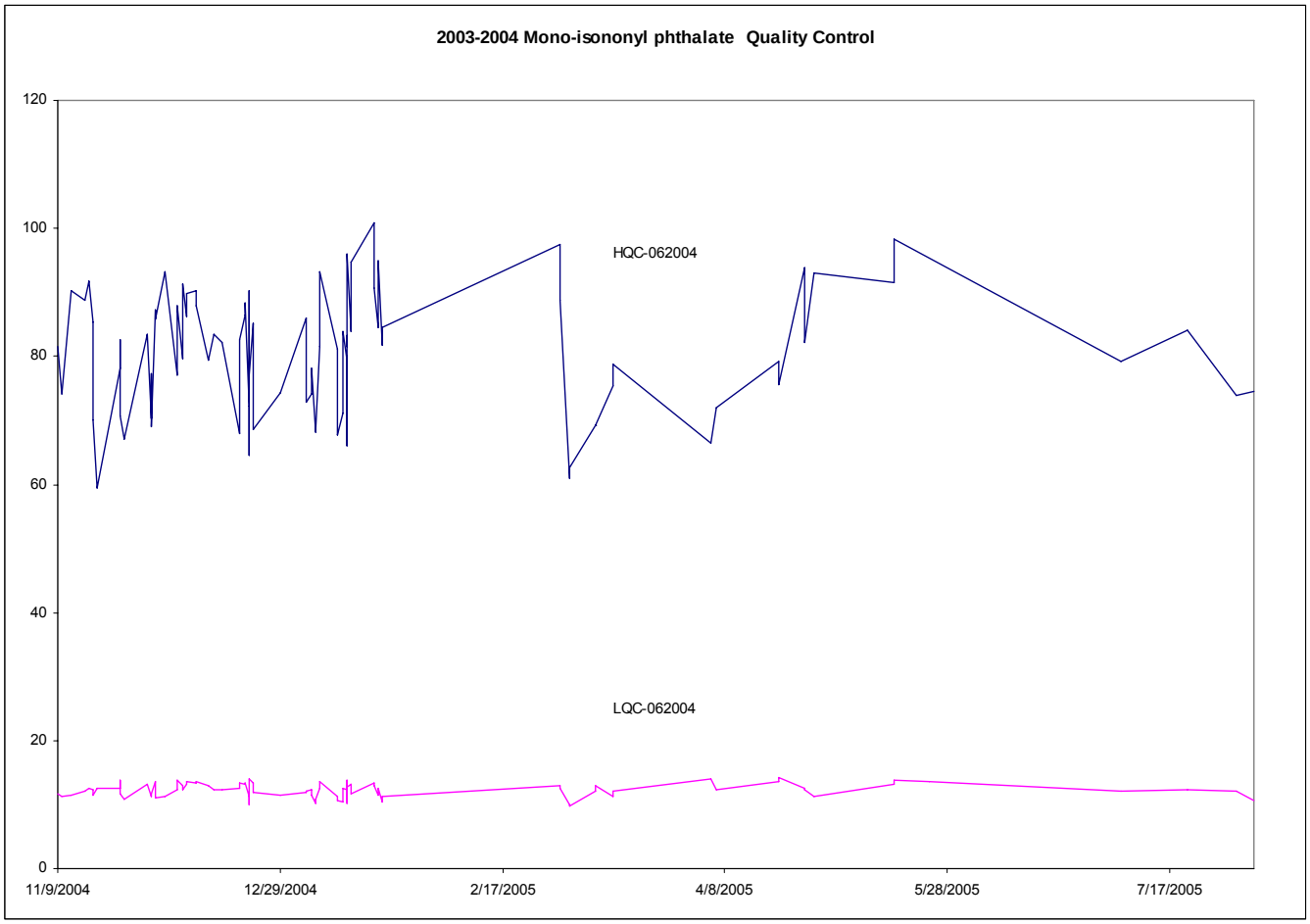
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	101	11/9/2004	8/5/2005	38.78	4.05	10.4
HQC-062004	100	11/9/2004	8/5/2005	206.87	22.67	11.0



J. Mono-isononyl phthalate

Summary Statistics for Mono-isononyl phthalate by Lot

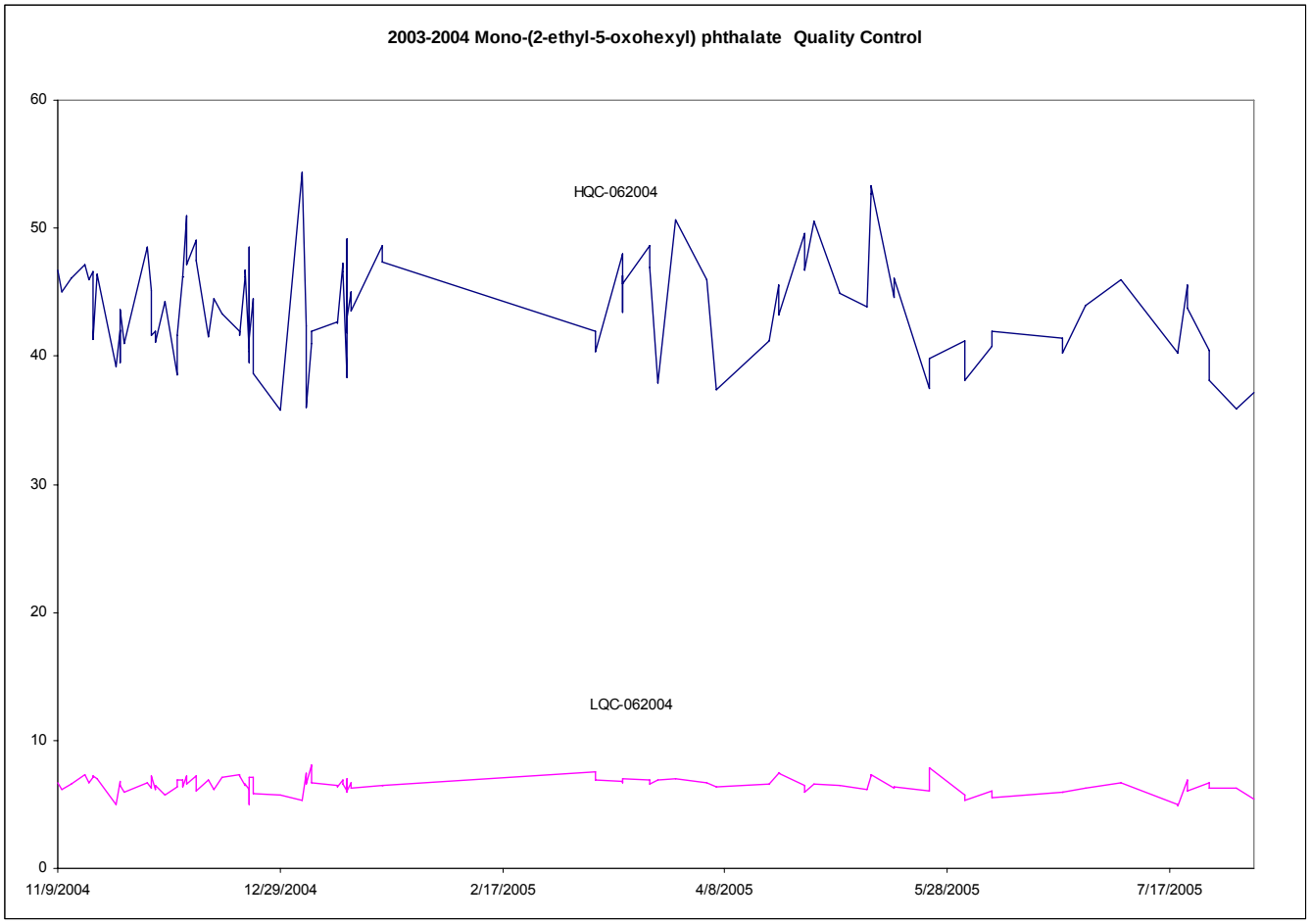
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	88	11/9/2004	8/5/2005	12.24	1.09	8.9
HQC-062004	87	11/9/2004	8/5/2005	80.62	9.55	11.8



K. Mono-(2-ethyl-5-oxohexyl)

Summary Statistics for Mono-(2-ethyl-5-oxohexyl) phthalate by Lot

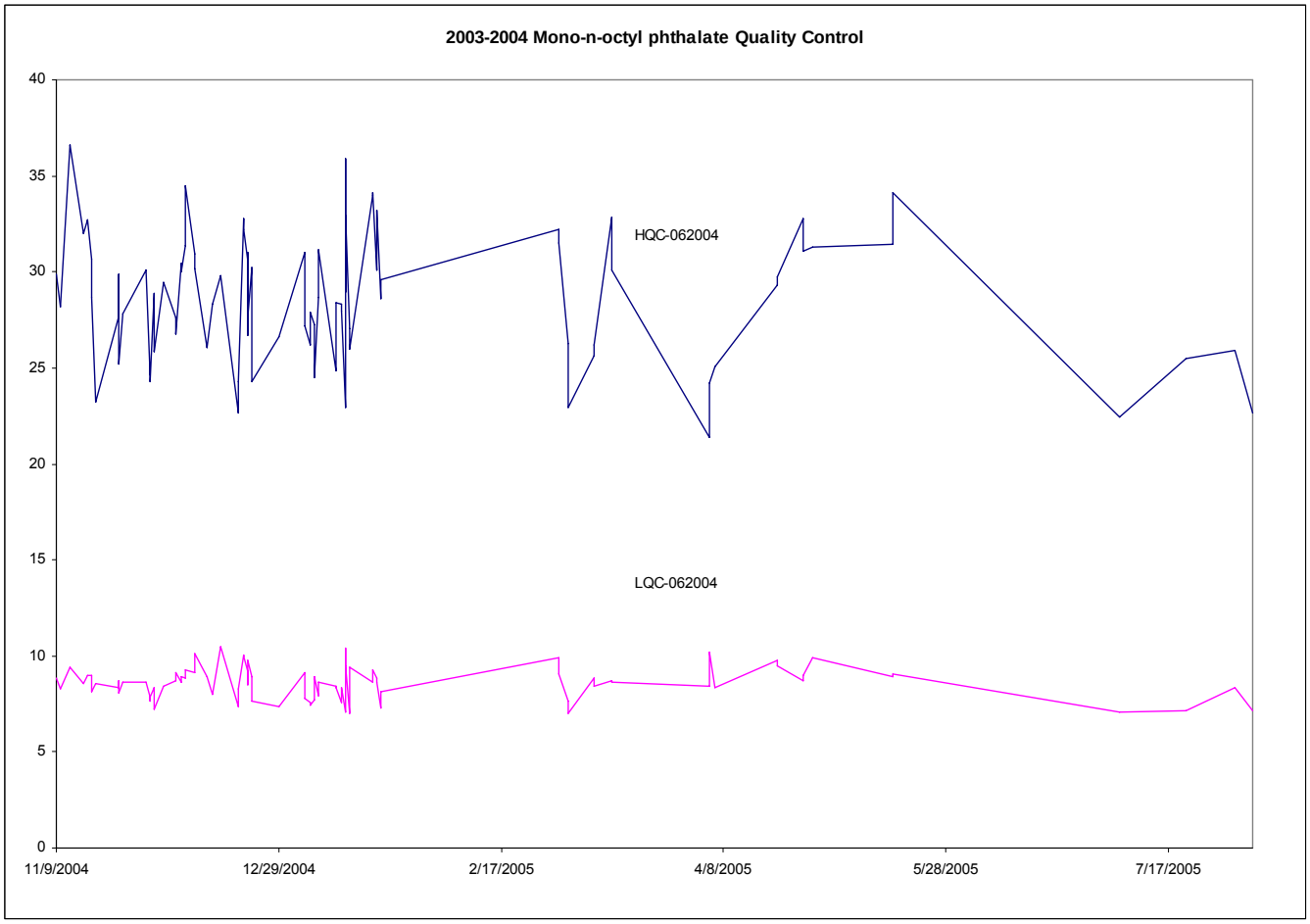
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	102	11/9/2004	8/5/2005	6.52	0.63	9.6
HQC-062004	102	11/9/2004	8/5/2005	43.72	3.95	9.0



L. Mono-n-octyl phthalate

Summary Statistics for Mono-n-octyl phthalate by Lot

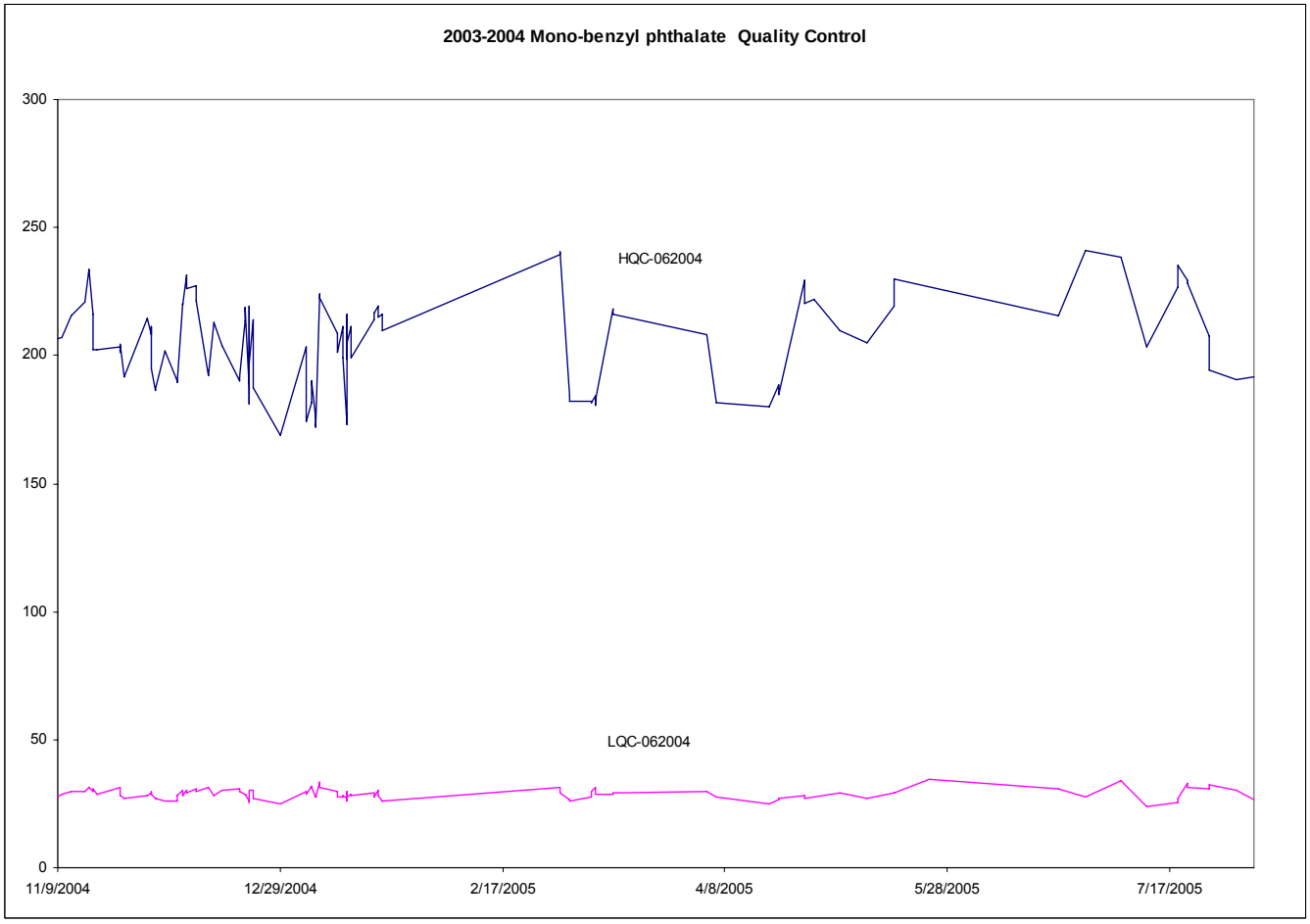
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	88	11/9/2004	8/5/2005	8.60	0.84	9.8
HQC-062004	88	11/9/2004	8/5/2005	28.54	3.38	11.9



M. Mono-benzyl phthalate

Summary Statistics for Mono-benzyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation
LQC-062004	103	11/9/2004	8/5/2005	28.81	2.03



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