Laboratory Procedure Manual

Analyte: **Phthalate Monoesters**

Matrix: **Urine**

Method: **HPLC–MS/MS**

Method No.:

Revised:

as performed by: *Toxicology Branch*
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.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2001–2002 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
Lab24_b	URXMBP	Mono-*n*-butyl phthalate (ng/mL)
	URXMC1	Mono-(3-carboxypropyl) phthalate
	URXMCP	Mono-cyclohexyl phthalate (ng/mL)
	URXMEP	Mono-ethyl phthalate (ng/mL)
	URXMHH	Mono-(2-ethyl-5-hydroxyhexyl) phthalate
	URXMHP	Mono-(2-ethyl)-hexyl phthalate (ng/mL)
	URXMIB	Mono-isobutyl pthalate
	URXMNM	Mono-*n*-methyl phthalate (ng/mL)
	URXMNP	Mono-isononyl phthalate (ng/mL)
	URXMOH	Mono-(2-ethyl-5-oxohexyl)
	URXMOP	Mono-*n*-octyl phthalate (ng/mL)
	URXMZP	Mono-benzyl phthalate (ng/mL)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A. Test Principle

The test principle utilizes high-pressure liquid chromatography tandem mass spectrometry (HPLC-MS/MS) for the quantitative detection of monomethylphthalate, monoethylphthalate, monobutylphthalate, monocyclohexylphthalate, monoethylhexylphthalate, monooctylphthalate, monobenzylphthalate, and monoisononylphthalate in human urine. Human urine samples are processed using enzymatic deconjugation of the glucuronides followed by solid-phase extraction. The eluate is concentrated, and the phthalate metabolites are chromatographically resolved by reversed-phase HPLC, detected by APCI- tandem mass spectrometry (MS/MS), and quantified by isotope dilution. Assay precision is improved by incorporating 13C4-labeled internal standards for each of the eight analytes, as well as a conjugated internal standard and it's 13C4-labeled internal standard to monitor deconjugation efficiency. This selective method allows for rapid detection of seven metabolites of commonly used dialkyl phthalates in human urine with limits of detection in the low parts per billion (ppb) range.

B. Clinical Relevance

The dialkyl or alkyl aryl esters of 1,2-benzenedicarboxylic acid, commonly called phthalates, have a myriad of commercial uses and are considered ubiquitous environmental contaminants. Globally, over 18 billion pounds of phthalates are used each year, primarily as additives to polyvinyl chloride (PVC) plastics, as industrial solvents, and as components of many consumer products. Humans are potentially exposed to many products containing phthalates, and specific sub-populations, such as medical patients undergoing transfusions, dialysis or apheresis are potentially more heavily exposed. Measurement of an internal dose, or biomarker of exposure, is a key aspect of assessing exposure. Phthalates are lipophilic compounds but are rapidly metabolized in humans and therefore do not appear to bio-accumulate. In humans and animals, phthalates are metabolized to their respective monoesters (alkyl or aryl esters of 1,2-benzenedicarboxylic acid, commonly called phthalate monoesters) and further oxidative products, which are excreted through the urine and feces. In animal studies, several phthalates and/or their monoester metabolites act as potent reproductive and developmental toxicants.

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 in the laboratory, and on a CD-ROM in the Chamblee Library. Personnel must review the MSDS prior to using chemicals.

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

C. Exercise caution in the handling of biological samples.

Personnel should use appropriate protection to minimize possible. Observe Universal Precautions; wear safety glasses, protective gloves and labcoat during all steps of this method because of both infectious and chemical related hazards. Use of the following is imperative when performing this method:

- Laboratory coats;
- Safety glasses; and
- 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 analysis utilizes a Waters Alliance 2690 HPLC coupled with a Finnigan TSQ 7000 equipped with an APCI interface. Data acquisition and analysis are controlled by the Finnigan Xcaliber data system. The software selects the appropriate peak based on parent/daughter ion combination and chromatographic retention time and subsequently integrates the peak area. All data are exported from Xcaliber 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 CDR and Jazz disks. The Access data base is located on the Local Area Network (LAN) and is automatically backed up nightly to tape by the EHLS LAN support staff. Documentation for data system maintenance is contained in copies of data records.

B. Quality Control Data, Charts

Once the data are loaded into the database by the analyst, the data are exported as a text file. This file is used by a SAS program (Statistical Analysis Systems software SAS Institute, Cary, NC) to generate QC charts for all analytes in the sample.

C. Final Reports

Final reports are generated using a SAS program. Data are transmitted to the Branch Chief via an inter-office memorandum. After branch review, the final report is reviewed by the EHLS 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 Toxicology Branch under the miscellaneous case number assigned by the Nutritional Biochemistry Branch. Data from the case are also maintained in the notebook of the analyst.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; 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) Pediatric urine collection bag (pre-screened)
(3) Labels
(4) 5 mL plastic cryovial (pre-screened)

B. Urine collection, storage and handling

(1) Urine specimens for phthalate analysis should be fresh or frozen. Urine specimens should be collected by using either a pre-screened urine collection cup or a pre-screened 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 to prevent desiccation of the sample.

(4) Specimens may be stored frozen at –20°C to –70°C for one year 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 collection and 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 at no more than 4°C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen below –20°C. Samples thawed and refrozen several times are not compromised.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable

6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), MATERIALS; EQUIPMENT AND INSTRUMENTATION

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

A. Reagent Preparation

(1) Mobile phase A: (0.1% acetic acid in water). To make 1L, 1.0ml of acetic acid is added to 999 ml of HPLC-grade water. This solution is stored at room temperature and discarded after three days.

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

(3) (3)Basic Buffer for Solid-Phase Extraction: To make 1L, 5.0 mL of concentrated ammonium hydroxide solution is added to 1L of 50:50 acetonitrile and reagent water. This buffer is stored at room temperature and discarded after one week.

(4) (4)Acidic Buffer for Solid Phase Extraction: To make 1L, 20.0 g of NaH_2PO_4 and 10.0 mL H_3PO_4 is added to 1L of reagent water. This buffer is stored at room temperature and discarded after one month.

*Acetonitrile and HPLC grade water are purchased from Tedia
*Phosphoric Acid (85%) is purchased from Caledon
*Monosodium phosphate monohydrate (ultrapure bioreagent) is purchased from JT Baker
*Ammonium hydroxide (30%) is purchased from JT Baker

B. Analytical Standards

(1) Source: Phthalate monoester native and labeled standards were purchased from:
(a) Cambridge Isotope Laboratories
50 Frontage Road
Andover, MA 01810-5413

(b) AccuStandard Inc.
New Haven, CT

(2) Standards preparation

(a) Individual native standards (phthalate monoester metabolites) are purchased from Cambridge Isotope Laboratories. The stock solutions are prepared by accurately transferring approximately 5 mg of material to a 10 mL Pyrex beaker (methanol rinsed). The phthalate monoester is then dissolved in acetonitrile and quantitatively transferred to a 50 mL

volumetric flask. This stock solution is stored at –20°C in a Teflon-capped glass bottle (methanol rinsed) until use.

(b) Internal standards (13C4-labeled phthalate monoesters and 13C4-labeled 4-methyl umbelliferon) were purchased from Cambridge Isotope Laboratories. 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 is confirmed empirically by tandem mass spectral analysis and contained less than 1% of the native compound.

(c) 4-Methyl umbelliferone glucuronide standard was purchased from Sigma. A 25 mL volume of stock standard solution was prepared by transferring approximately 10 ng 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 was stored at –20°C in a Teflon-capped glass bottle (methanol rinsed) until use. The 4-methyl umbelliferone glucuronide spiking solution was prepared by adding 2.5 mL of the stock standard into 25 mL of HPLC-grade water to make a final concentration of 40 ppm.

(d) Eleven unique working standards with all eight analytes and 4-methyl umbelliferone were prepared in water from the stock solutions of native and the 13C4-labeled internal standards to cover the linear range of the assay for each analyte (approx. 1–1000 ppb). The linear range for mEP was extended to 2500 ppb due to the relatively high levels of this analyte found in human urine.

Table 1. Concentrations of final working standard solutions.

Analyte	Std* 0ng/.2mL	Std* 1ng/.2mL	Std* 2ng/.2mL	Std* 3ng/.2mL	Std*4ng/.2mL	Std* 5ng/.2mL	Std* 6ug/.2mL	Std* 7ng/.2mL	Std* 8ng/.2mL	Std* 9ng/.2mL	Std* 10ng/.2mL	ISTDConcng/.2mL
mMP	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	320.0	800.0	2500	50
mEP	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	320.0	800.0	2500	50
mBP	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	160.0	400.0	-	20
mCHP	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	160.0	400.0	-	20
mBzP	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	160.0	400.0	-	20
mEHP	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	260.0	400.0	-	12
mOP	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	320.0	800.0	-	30
mNP	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	320.0	800.0	-	20
mDP	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	320.0	800.0	-	25
4-Me Umb**	5.0	10.0	15.0	20.0	25.0	30.0	35.0	40.0	50.0	75.0	-	50

* Calibration curves were made with the unit ng/200 µL, in order to get the final analyte concentration in urine sample in ppb.

** 4-MeUmb: 4-methyl umbelliferon

(3) Storage and Stability

(a) All standards should be kept in amber Boston Round Bottles with Teflon-lined screw caps. Working standards are kept at refrigerator temperature and remade as needed from stock. Stock solutions are kept in the freezer at 0°C (± 20°C). Stock standards should be remade, at least, on a yearly basis.

(b) Proficiency Testing Standards. Aliquots of each stock standard were added to 1 L of filtered urine pools. The volume of each standard is varied, as in the table below, to produce proficiency testing standards of 4 different concentrations. The spiked pools were mixed overnight and were aliquoted into prescreened vials and frozen until needed. These

standards were characterized by at least 20 repeat determinations to characterize the mean and standard deviation for evaluation.

Table 2. Target analyte concentrations.

Analyte	Target Analyte Concentrations (ng/mL)			
	PT1	PT2	PT3	PT4
mMP	5	200	640	2400
mEP	5	200	640	2400
mBP	2	40	150	400
mCHP	2	40	150	400
mBzP	2	40	150	400
mEHP	2	40	150	400
mOP	5	80	300	700
mNP	5	80	300	700
mDP	5	80	300	700

C. Materials

(1) Varian Nexus solid phase extraction cartridges (Varian); 3 mL/60 mg and 12 mL/200 mg.
(2) Disposable valve liners for Visiprep vacuum manifold.
(3) Disposable borosilicate glass tubes (16 x 100 mmol, Corning), with Teflon-lined screw caps.
(4) Disposable borosilicate glass tubes (16 x 125 mmol, Corning), without screw caps.
(5) Keystone Betasil phenyl column (5 um, 50 mm X 2 mm).
(6) Inline filters (2 um and 0.5 um, Upchurch).
(7) 5 mm phenyl guard column (Keystone).
(8) Pipette tips: 5ml, 1 mL, 100 µL, 50 µ20 µL and 10 µL sizes.
(9) Glass HPLC autosampler vial inserts, vials, and caps (caps with PTFE/Silicone).

D. Equipment

(1) Renin Repipettor
(2) Visiprep vacuum manifold equipped with single use Teflon flow lines (Supelco).
(3) Balance (TR-203 Series Denver Instrument Company).
(4) Sonicating Waterbath (Branson 5210).
(5) Renin Reference Pipettes.
(6) Fisher Isotemp Incubator (300 Series Model 350D).
(7) Vortexer (Fisher, Genie 2).
(8) Magnetic Stirrer (Corning).
(9) Rainen Reference Pipettes.

E. Instrumentation

(1) Waters Alliance 2690 HPLC.
(2) TSQ 7000 Mass Spectrophotometer equipped with APCI 2 interface (Finnigan).
(3) TurboVap LV Evaporator (Zymark).

(4) pH Meter (Corning pH/Ion Analyzer 455)

7. PROCEDURE OPERATING INSTRUCTIONS

A. Procedure preliminaries

(1) The vacuum manifold is prepared for extraction (a typical run consists of: 1 Reagent Blank, Quality Control Low (QCL) and Quality Control High (QCH) per 13 samples and a set of 10 standards at the front of the front).

(2) The samples and QC materials are allowed to thaw at room temperature.

(3) The samples are mixed well by vortexing.

(4) The glucuronidase enzyme solution (mix fresh just prior to addition to sample) is prepared as follows.

(5) For a single run; 6.25 ml of 1 mmol, pH 6.5 ammonium acetate buffer is transferred accurately into a rinsed beaker to pipette 250 µL into each sample to be analyzed.

(6) 125µL of glucuronidase (5 µL to be added to each sample) is pipetted into the beaker.

(7) The solution is swirled to mix.

B. Sample preparation for solid phase extraction

(1) 1 mL of unknown urine sample, 1 mL HPLC Grade Reagent Water (for Reagent Blank), and 1 mL of QC High or QC Low are transferred into properly labeled borosilicate glass test tubes (16 x 100 mm, Corning).

(2) 50 µL 4-Methylumbelliferone glucuronide is pipetted into each tube.

(3) 100 µL Internal Standard (labeled mixture) spiking solution is pipetted into each tube.

(4) 255 µL of glucuronidase/ ammonium acetate solution is added into each tube.

(5) The tubes are sealed Teflon-lined screw caps and mixed gently and incubated at 37°C for 90 minutes.

C. Solid phase extraction procedure

(1) Varian Nexus solid phase (3 mL/60 mg) extraction cartridges (Varian Corp) are equilibrated with 1.0 mL acetonitrile followed by 2.0 mL basic buffer.

(2) The deconjugated urine samples are diluted with 1.0 mL of basic buffer solution and mixed by vortexing for 5 seconds.

(3) The samples are poured into the properly equilibrated solid phase cartridges.

(4) The elute is collected in tubes inside the vacuum manifold.

(5) The residual analytes are eluted from the cartridge by adding 1.0 mL of basic buffer to the original borosilicate glass tube and pouring the contents onto the cartridge.

(6) The breakthrough eluant is collected in the same tube in the vacuum manifold as the previous eluant.

(7) The combined eluant is acidified by adding 3.0 mL of acidic buffer and mixed by vortexing.

(8) The original small cartridge is discarded.

(9) The second Varian cartridge (10 mL/60 mg) is equilibrated by washing with acetonitrile (1.0 mL) and acidic buffer (3.0 mL).

(10) The previously acidified sample (step 7) is further purified by retaining the analytes in the second SPE cartridge and washing with acidic buffer (3.0 mL).

(11) The residual salts are removed with the addition of water (9 mL) and the SPE cartridges are dried for 5 seconds by vacuum-induced air flow.

(12) The retained analytes are eluted from the SPE cartridge by adding 2 mL of acetonitrile followed by 2 mL of ethyl acetate.

(13) The eluant is evaporated to dryness under a stream of dry nitrogen (UHP grade) in a Turbovap evaporator at 55°C.

(14) The analytes containing residue is resuspended in 200 µL of water and transferred to a glass HPLC autosampler vial insert, caped and analyzed by HPLC–MS/MS. (Prepared samples may be stored at 4°C for up to two weeks prior to analysis.)

D. Instrumental Analysis

(1) Waters HPLC/TSQ7000 Operating Conditions:

A Waters Alliance 2690 HPLC coupled with a Finnigan TSQ 7000, equipped with APCI 2(Atmospheric Pressure Chemical Ionization) interface is used for analysis. The system is controlled by Xcaliber Software and the chromatographic resolution is accomplished using a Keystone Betasil phenyl column and a solvent gradient from 100% Mobile phase A to 100% Mobile phase B at 0.6 mL per minute. After reaching 100% Mobile phase B and remaining at that mobile phase composition for 0.2 minutes, the column is subsequently equilibrated with 100% Mobile phase A for 0.5 minutes at the flow rate of 0.8 ml/min. Each sample (25 µL) is injected using the Waters 2690 autosampler, configured with syringe washes between injections to minimize carryover. Inline filters are used to remove particulate materials from the injected samples before reaching the column. A 5 mm phenyl guard column is used with the analytical column to extend the useful life span of the analytical column. The total run cycle time for the assay is 12.0 minutes.

Waters Alliance 2690 Operation Conditions

Injector parameters:

Injection volume: 25 µL

Pump Settings:

Solvent A: 0.1% Acetic acid in water

Solvent B: 0.1% Acetic acid in acetonitrile

*Solvent C: Acetonitrile

*Solvent D: HPLC grade water

Table 3. Waters Alliance 2690 Operation Conditions.

Time (min)	Flow (mL/min)	A%	B%	C%	D%
0.0	0.8	100.0	0.0	0.0	0.0
0.1	0.6	100.0	0.0	0.0	0.0
1.0	0.6	85.0	15.0	0.0	0.0
10.0	0.6	55.0	45.0	0.0	0.0
11.0	0.6	0.0	100.0	0.0	0.0
11.2	0.6	0.0	100.0	0.0	0.0
11.5	0.8	100.0	0.0	0.0	0.0
12.0	0.8	100.0	0.0	0.0	0.0

*C and D are used for syringe washes and seal washes.

Table 4. Single Reaction Monitoring (SRM) Setup for Phthalate Monoesters and 4-MeUmb

SRM Analysis Of Phthalate Monoesters				
Analyte	**Parent (Mass)**	**Daughter (Mass)**	**C_{off}(V) (Collision Offset)**	**RT Window (min)**
mMP	179	107	15.0	1.0-5.5
$^{13}C_4$-mMP	181	110	15.0	1.0-5.5
mEP	193	77	21.5	1.0-5.5
$^{13}C_4$-mEP	197	79	21.5	1.0-5.5
mBP	221	77	22.0	5.5-8.8
$^{13}C_4$-mBP	225	79	22.0	5.5-8.8
mCHP	247	77	24.5	5.5-8.8
$^{13}C_4$-mCHP	251	79	24.5	5.5-8.8
mBzP	255	183	14.2	5.5-8.8
$^{13}C_4$-mBzP	259	186	14.2	5.5-8.8
mEHP	277	134	22.0	8.8-11.5
$^{13}C_4$-mEHP	281	137	22.0	8.8-11.5
mOP	277	125	22.0	8.8-11.5
$^{13}C_4$-mOP	281	127	22.0	8.8-11.5
mNP	291	247	16.5	8.8-11.5
$^{13}C_4$-mNP	295	250	16.6	8.8-11.5
4-MeUmb	175	119	33.0	1.0-5.5
$^{13}C_4$-4-MeUmb	179	121	33.0	1.0-5.5

Negative ion atmospheric pressure chemical ionization (APCI) is used to form negatively charged analyte ions with the following settings: nitrogen sheath gas (40 psi), API vaporizer, temperature (500°C). Heated capillary temperature (280°C), corona discharge needle charge (9 uA), and electron multiplier (1800 V). During an analysis, the instrument is set in single reaction monitoring mode so that parent and daughter 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. Daughter ions are formed in the collision cell using argon at 2.0 mTorr. The collision offset is set specific for each daughter ion.

(2) Limit of Detection (LOD)

The limit of detection (LOD) for the analysis of phthalate monoesters in 1 mL of urine ranges from 0.5 to 2 ppb. The analytical limit of detection for each of the eight analytes is calculated as 3 So where So is the value of the standard deviation as the concentration approaches zero. The So is determined by analyzing quintuplicates of the lowest five standards and plotting the standard deviation versus the known standard concentration; *y* intercept of the best-fit line of this plot was used as So.

Table 5. Limit of detection for phthalate monoesters in human urine.

Analyte LOD ($3S_o$)	(ppb)
mMP	0.70
mEP	1.21
mBP	0.94
mCHP	0.93
mBzP	0.47
MEHP	0.86
mOP	0.77
mNP	0.79

8. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration

Before mass spectral analysis of unknowns, a known standard is injected to confirm acceptable chromatographic resolution and mass spectral sensitivity. Once the instrument yields acceptable performance, a full set of 11 standards followed by the unknowns, QC samples and the blank 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 Xcaliber 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.) The test samples with values below the lowest standard are reported as non detectable; the samples with values exceeding the highest standard are diluted and reprocessed.

B. Proficiency Testing (PT)

(1) Because no standard reference materials exist for urinary analysis of phthalate monoesters 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 were prepared in-house as described in the standard preparation section. These standards encompass the entire linear range of the method and were characterized in our laboratory. This data was forwarded to an DLS QOA officer who is in charge of executing the PT program.

(2) Proficiency testing should be performed a minimum of once every 6 months. The PT administrator will randomly select five of the PT materials for analysis. 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).

(3) All proficiency results shall be appropriately documented.

9. CALCULATIONS; INTERPRETATION OF RESULTS

A. Calculations

The concentration of the individual analytes in each sample is calculated using the calibration curve derived from 11 known standard mixtures.

Area ratio of analyte/internal standard = concentration ratio of analyte/Internal standard

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

B. Interpretation of Results

The phthalate monoester values obtained using this method of analysis are individual markers of phthalate exposure only. Future human exposure assessment studies should help reveal the potential role of phthalates in causing an increased risk of cancer and reproductive dysfunction. In addition, diisononyl phthalate is a mixture of phthalate isomers. In this method only one isomer is used as a standard and may underestimate exposure.

10. REPORTABLE RANGE OF RESULTS

The range of concentrations reported for analytes in an unknown specimen are determined by the extent of exposure experienced by the donor, the time lapse since that exposure occurred, and the half-life of the analyte. The value can range from nondetectable to low parts per million. If a sample has levels higher than the calibration range, it is diluted so the level is in this range. The final result is then calculated by adjusting for the dilution.

11. QUALITY CONTROL (QC) PROCEDURES

A. Source of Quality Control Materials

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 in spanned over one month prior to analysis of unknown samples.)

12. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

A. System is declared “Out of Control”

The system is declared "out-of-control" if any of the following events occur:

(1) Mean Chart: QC Rules

(a) A single run value for QC Low or QC High falls outside upper or lower 3 Standard deviation limit (13s violation).

(b) Both QC Low or QC High in a single run fall outside the upper or lower 2 Standard deviation limit (22s if on same side of mean; 44s if on opposite sides) .

(c) Sequential pooled so that QCH and QCL on same chart: If either QC is outside 2 Std limit and is preceded by 9 QC results on the same side of mean (10x sequential violation).

(2) Range Chart:

(a) Not applicable since specimens are not analyzed in duplicates.

(b) Remedial Action

While there are possibly a number of alternatives to be checked during the "out of control" period, the following five are probably the most expedient:

- Determine if a clerical error has occurred, i.e. were the data simply transcribed wrong.

- Has a sample mixup occurred; i.e. has a patient sample been substituted for a quality control sample.
- Has an instrumentation error occurred, this can take two forms, either mechanical (bad column, bad injection, etc.) or electrical (data system, attenuation, etc.).
- Determine if a change in the concentration of standards has occurred.
- Review the sample sequence in the analytical run to ensure that "carryover" from a concentrated sample did not contaminate the "out of control" QC sample.

If none of the previously mentioned alternatives prove viable for resolving the "out of control" situation, then the set of unknowns accompanying the QC is re-extracted.

13. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The procedure is very labor intensive and requires very 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.

14. REFERENCE RANGES (NORMAL VALUES)

The data from NHANES 1999+ will be used to describe levels of phthalate exposure. This reference range is considered preliminary until 3 years of NHANES are complete.

Table 6. Distributions of creatinine-adjusted phthalate monoester levels in urine, 1999+ (ppb).

mBzP	1031	27.88	14.62	0.34–1053.13	8.47	80.39
mCHP	1031	0.30	0.05	0.00–28.00	0.00	1.00
mEHP	1031	5.31	2.80	0.00–538.39	1.34	12.89
mEP	1025	396.56	134.79	5.69–19201.37	60.67	1493.89
mNP	1031	1.61	0.00	0.00–132.96	0.00	8.42
mOP	1031	0.54	0.00	0.00–23.79	0.00	2.43

15. 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.

16. SPECIMEN STORAGE AND HANDLING

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. Cleaned-up extracts of specimens can be stored (≤–20°C) in sealed ampules for an extended period of time.

17. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

The current analytical method utilizes a Waters Alliance 2690 HPLC coupled with a Finnigan TSQ 7000 equipped with an APCI interface for the final analysis. An alternative analytical approach would be the use of HP 1100 HPLC coupled with API 3000 (PE Nelson) equipped with a heated nebulizer using mass spectral detection (LC MS/MS).

18. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

A. The 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.

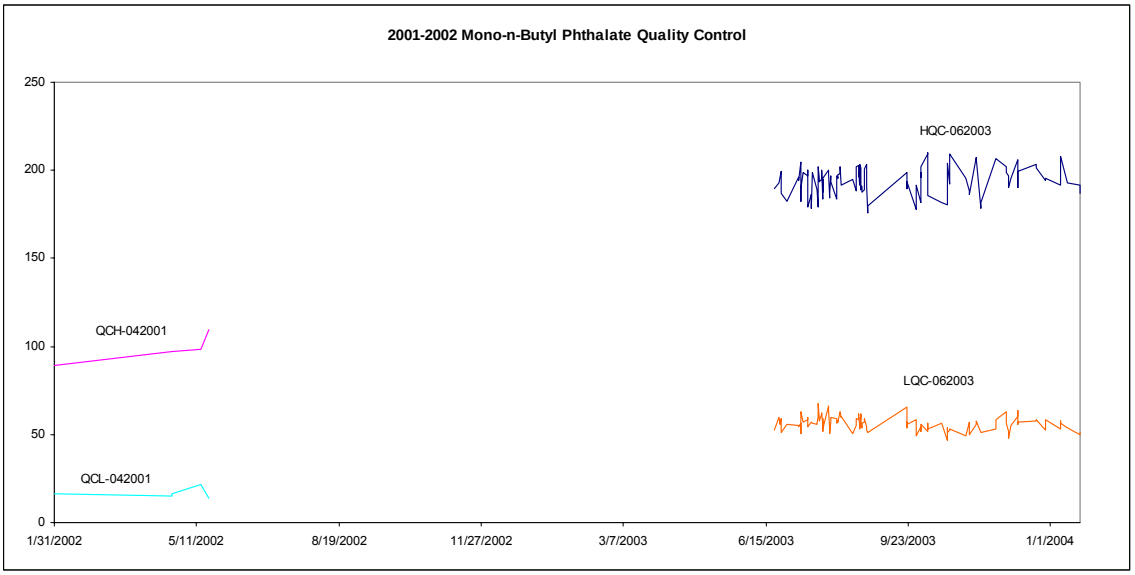
19. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

There are two types of specimen accountability forms maintained. One type accounts for specimen transfer from one branch of the division to another branch [(section, e.g. Nutritional Biochemistry Branch to the Toxicology Branch)] via the Specimen Information System--Specimen Shipping List, where signature and date of receipt of specimens are recorded. The other type accounts for sample transfer within the Contemporary Pesticide Laboratory among laboratorians via the Interlaboratory Sample Transfer Sheet Log. Here again signature and date are required.

20. SUMMARY STATISTICS AND QC GRAPHS

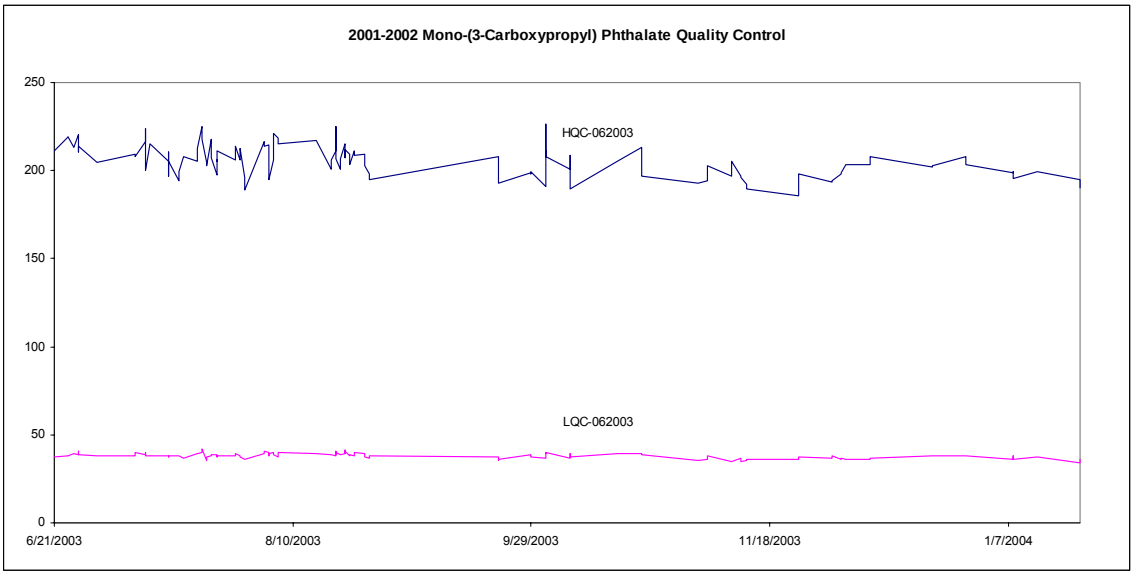
A. Mono-*n*-butyl phthalate

Summary Statistics for Mono-*n*-Butyl Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
QCL-042001	5	1/31/2002	5/20/2002	16.700	2.875	17.2
QCH-042001	5	1/31/2002	5/20/2002	98.422	7.435	7.6
LQC-062003	120	6/21/2003	1/22/2004	56.213	3.958	7.0
HQC-062003	120	6/21/2003	1/22/2004	194.197	7.843	4.0



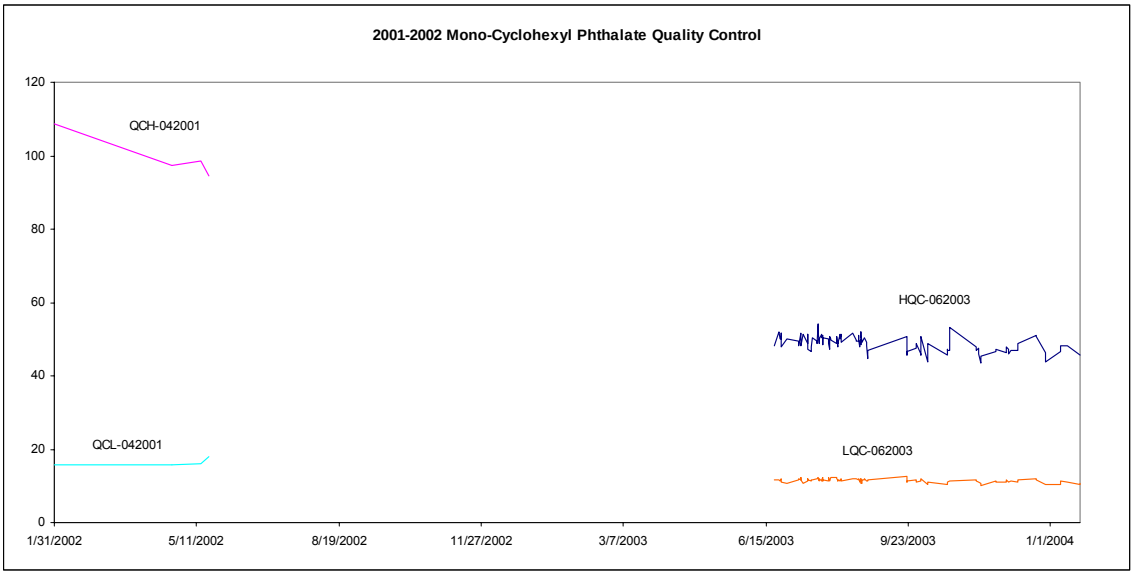
B. Mono-(3-carboxypropyl) phthalate

Summary Statistics for Mono-(3-Carboxypropyl) Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
LQC-062003	116	6/21/2003	1/22/2004	38.0919	1.5732	4.1
HQC-062003	116	6/21/2003	1/22/2004	205.3908	8.7346	4.3



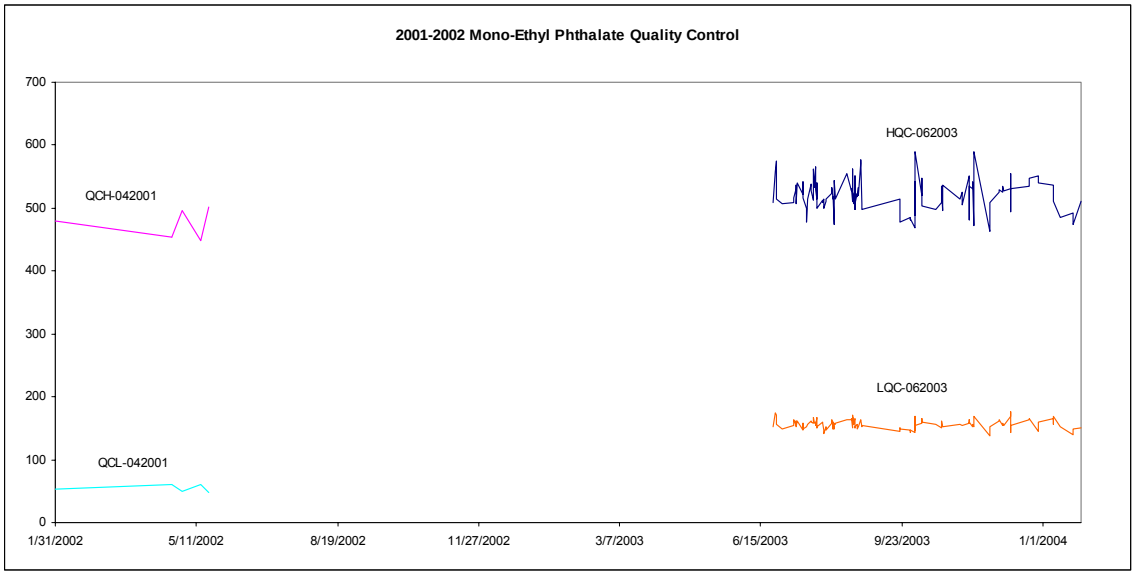
C. Mono-cyclohexyl phthalate

Summary Statistics for Mono-Cyclohexyl Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
QCL-042001	4	1/31/2002	5/20/2002	16.435	1.036	6.3
QCH-042001	4	1/31/2002	5/20/2002	99.778	6.157	6.2
LQC-062003	115	6/21/2003	1/22/2004	11.444	0.509	4.4
HQC-062003	115	6/21/2003	1/22/2004	48.760	2.115	4.3



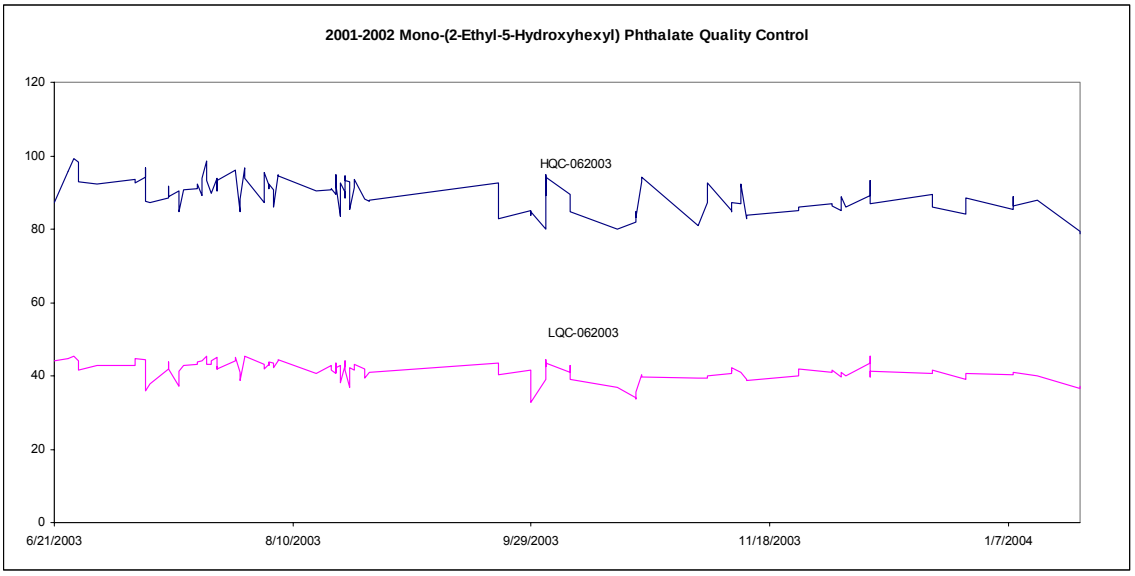
D. Mono-ethyl phthalate

Summary Statistics for Mono-Ethyl Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
QCL-042001	5	1/31/2002	5/20/2002	54.608	5.784	10.6
QCH-042001	5	1/31/2002	5/20/2002	476.262	24.490	5.1
LQC-062003	125	6/24/2003	1/28/2004	156.303	7.259	4.6
HQC-062003	125	6/24/2003	1/28/2004	521.409	24.766	4.7



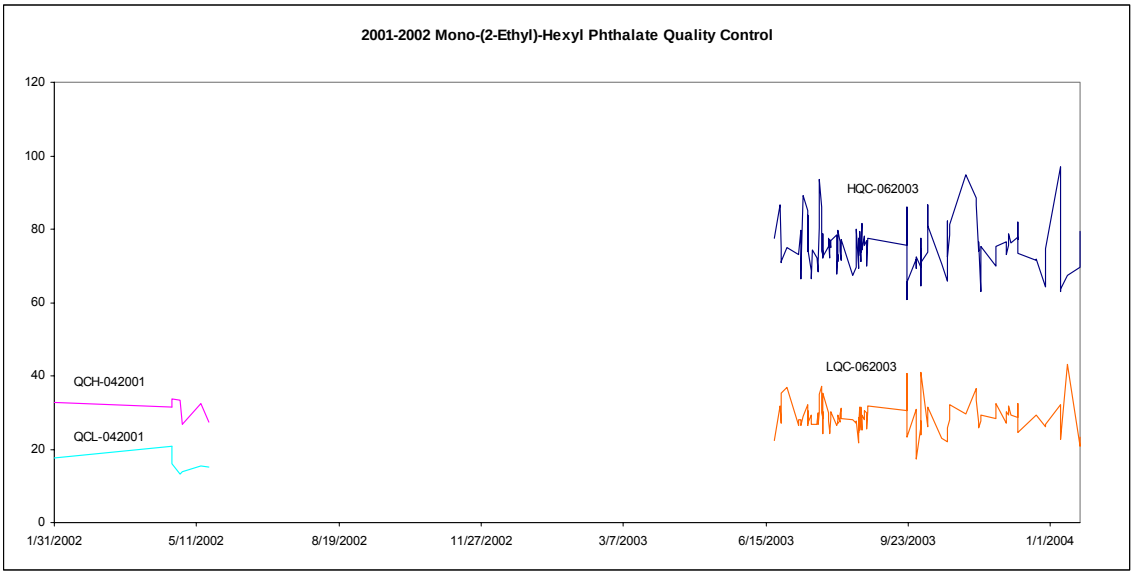
E. Mono-(2-ethyl-5-hydroxyhexyl) phthalate

Summary Statistics for Mono-(2-Ethyl-5-Hydroxyhexyl) Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
LQC-062003	125	6/21/2003	1/22/2004	41.525	2.488	6.0
HQC-062003	124	6/21/2003	1/22/2004	89.488	4.334	4.8



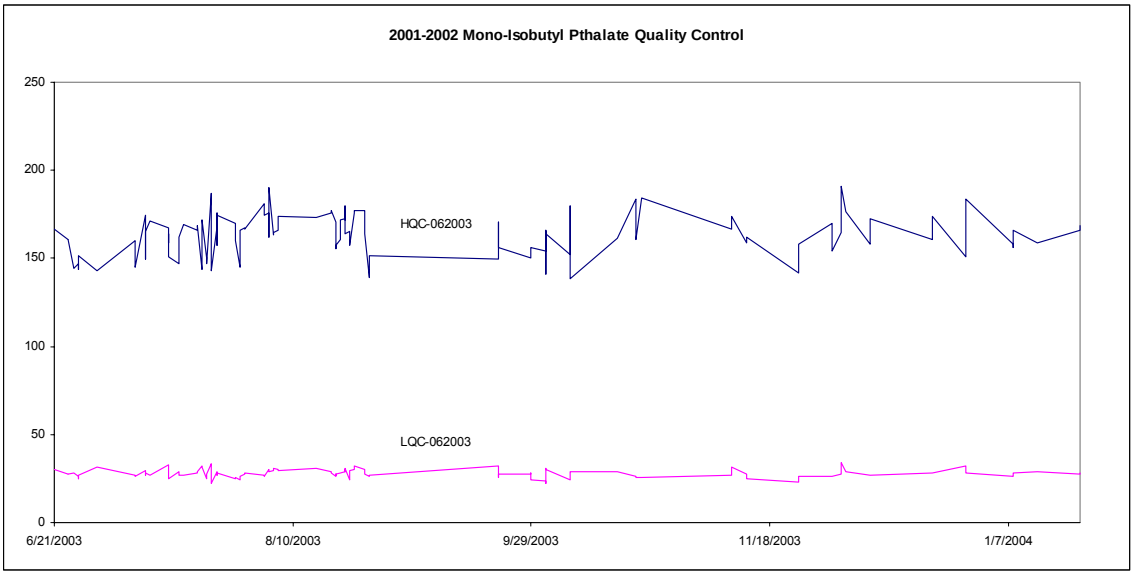
F. Mono-(2-ethyl)-hexyl phthalate

Summary Statistics for Mono-(2-Ethyl)-Hexyl Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
QCL-042001	7	1/31/2002	5/20/2002	16.021	2.529	15.8
QCH-042001	7	1/31/2002	5/20/2002	31.156	2.881	9.2
LQC-062003	119	6/21/2003	1/22/2004	28.623	3.991	13.9
HQC-062003	119	6/21/2003	1/22/2004	75.039	6.426	8.6



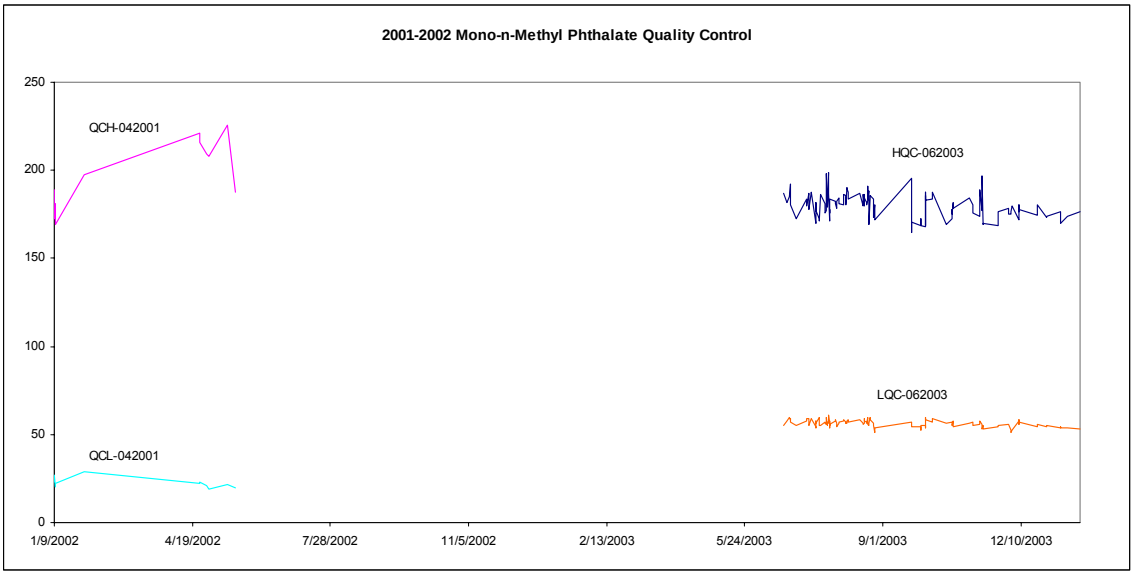
G. Mono-isobutyl pthalate

Summary Statistics for Mono-Isobutyl Pthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
LQC-062003	112	6/21/2003	1/22/2004	27.8174	2.3100	8.3
HQC-062003	112	6/21/2003	1/22/2004	163.3958	11.6890	7.2



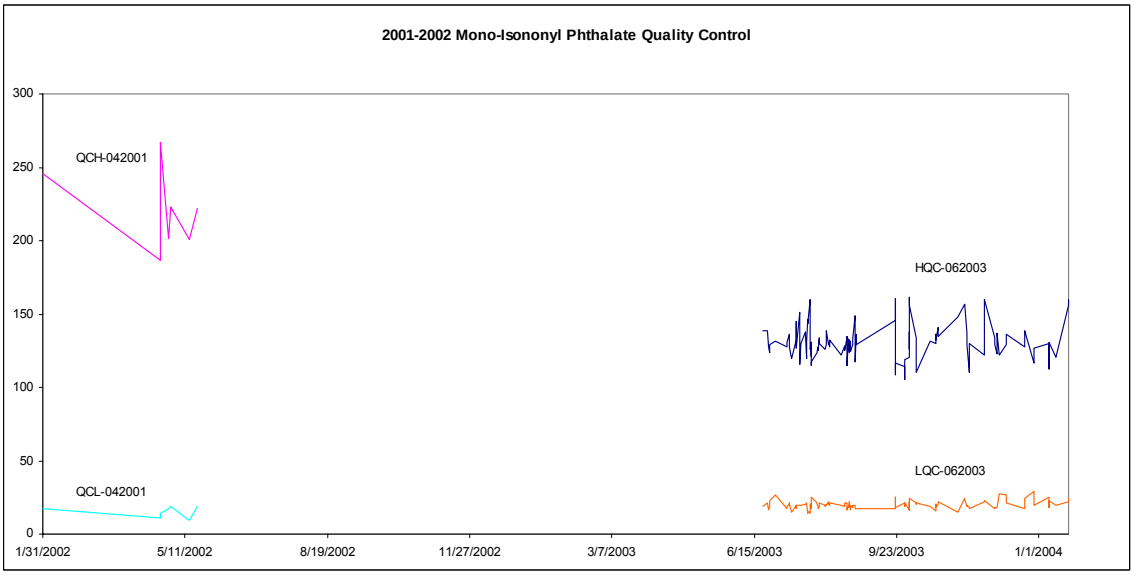
H. Mono-*n*-methyl phthalate

Summary Statistics for Mono-*n*-Methyl Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
QCL-042001	11	1/9/2002	5/20/2002	22.532	2.920	13.0
QCH-042001	11	1/9/2002	5/20/2002	197.931	19.553	9.9
LQC-062003	120	6/21/2003	1/22/2004	56.357	1.914	3.4
HQC-062003	120	6/21/2003	1/22/2004	179.925	6.911	3.8



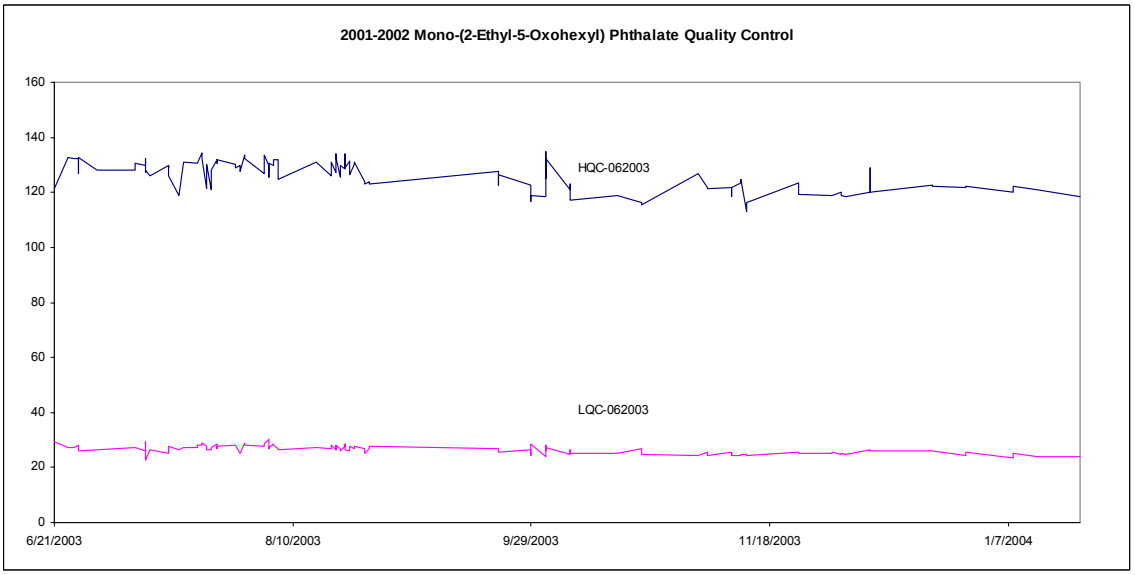
I. Mono-isononyl phthalate

Summary Statistics for Mono-Isononyl Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
QCL-042001	7	1/31/2002	5/20/2002	15.339	3.826	24.9
QCH-042001	7	1/31/2002	5/20/2002	220.963	27.984	12.7
LQC-062003	119	6/21/2003	1/22/2004	19.933	2.688	13.5
HQC-062003	118	6/21/2003	1/22/2004	131.061	11.471	8.8



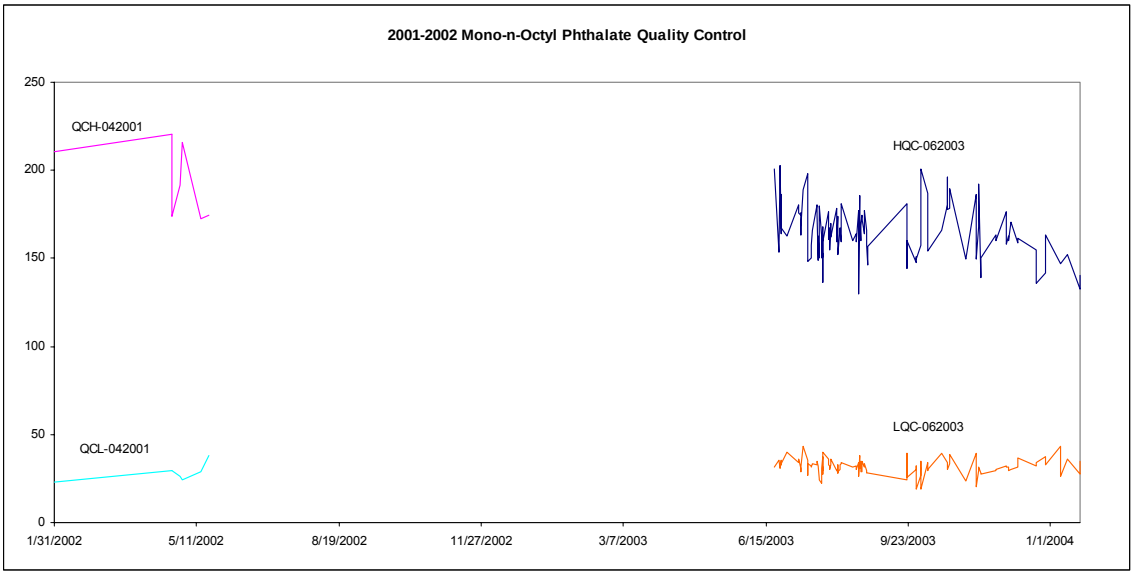
J. Mono-(2-ethyl-5-oxohexyl) phthalate

Summary Statistics for Mono-(2-Ethyl-5-Oxohexyl) Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
LQC-062003	119	6/21/2003	1/22/2004	26.5019	1.4702	5.5
HQC-062003	118	6/21/2003	1/22/2004	126.0372	5.2629	4.2



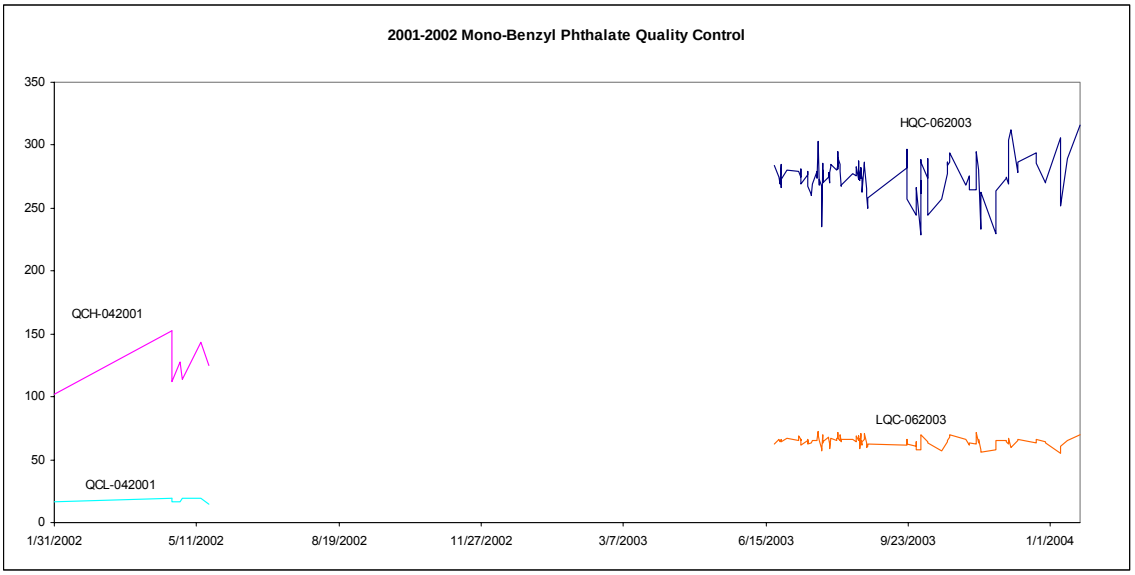
K. Mono-*n*-octyl phthalate

Summary Statistics for Mono-*n*-Octyl Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
QCL-042001	7	1/31/2002	5/20/2002	28.496	4.928	17.3
QCH-042001	7	1/31/2002	5/20/2002	194.191	21.203	10.9
LQC-062003	119	6/21/2003	1/22/2004	31.505	4.329	13.7
HQC-062003	118	6/21/2003	1/22/2004	164.878	15.499	9.4



L. Mono-benzyl phthalate

Summary Statistics for Mono-Benzyl Phthalate by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
QCL-042001	7	1/31/2002	5/20/2002	17.466	1.722	9.9
QCH-042001	7	1/31/2002	5/20/2002	125.294	17.842	14.2
LQC-062003	118	6/21/2003	1/22/2004	64.629	3.597	5.6
HQC-062003	118	6/21/2003	1/22/2004	275.183	14.867	5.4



21. GLOSSARY

A. mMP: Monomethyl phthalate
B. mEP: Monoethyl phthalate
C. mBP: Monobutyl phthalate
D. mCHP: Monocyclohexyl phthalate
E. mBzP: Monobenzyl phthalate
F. mEHP: Mono-2-ethylhexyl phthalate
G. mOP: Mono-*n*-octyl phthalate
H. mNP: Mono-3,5,5-trimethyl-1-hexyl phthalate

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