Laboratory Procedure Manual

Analyte: **Bisphenol A, 4-tert-octylphenol, ortho-phenylphenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, benzophenone-3, and triclosan.**

Matrix: **Urine**

Method: **On line SPE-HPLC-MS/MS**

Method No:

Revised: **May 25, 2005**

as performed by:

Organic Analytical Toxicology Branch
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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.

Environmental Phenols (bisphenol A (BPA) and alkylphenols (APs)) in urine
NHANES 2003-2004
Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Lab Number	Analyte	SAS Label
EPH_D	URXBPH	Urinary Bisphenol A (ng/mL)
	URXBP3	Urinary Benzophenone-3 (ng/mL)
	URX4TO	Urinary 4-tert-octylphenol (ng/mL)
	URXTRS	Urinary Triclosan (ng/mL)

1. Clinical Relevance and Summary of Test Principle

BPA and APs have been previously measured in biological matrixes by using gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled with different detection techniques. To achieve enhanced sensitivity and selectivity, the phenols have been derivatized to alkyl or acyl derivatives before GC-mass spectrometry (GC/MS) analysis.[23-31] We have developed a sensitive method for measuring BPA, 4-tert-octylphenol (tOP), benzophenone-3 (BP-3), and five chlorophenols [2,4-dichlorophenol(24-DCP), 2,5-dichlorophenol (25-DCP), 2,45,-trichlorophenol (245-TCP), 2,4,6-trichlorophenol (246-TCP) and triclosan]. The method uses solid phase extraction (SPE) coupled on-line to HPLC and tandem mass spectrometry (MS/MS). With the use of isotopically labeled internal standards, the detection limits in 100 µL of urine are 0.1-2 nanograms per milliliter (ng/mL), sufficient for measuring urinary levels of phenols in non-occupationally exposed subjects.

2. Safety Precautions

A. Reagent Toxicity or Carcinogenicity

Some of the reagents used are toxic. Special care should be taken to avoid inhalation or dermal exposure to the reagents necessary to carry out the procedure.

Radioactive Hazards

None.

B. Microbiological Hazards

The possibility of being exposed to various microbiological hazards exists. Appropriate measures should be taken to avoid any direct contact with the specimens (i.e., utilize gloves, chemical or biological hoods, etc.). A Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues. Laboratory personnel handling human fluids and tissues is required to attend the “Bloodborne Pathogens Training” course offered at CDC to insure proper compliance with CDC safe work place requirements.

C. Mechanical Hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should avoid any direct contact with the electronics of the mass spectrometer, unless all power to the instrument is off. Generally, only qualified technicians should perform the electronic maintenance and repair of the mass spectrometer. Contact with the heated surfaces of the mass spectrometer should be avoided; also, care must be taken to avoid puncture wounds from the corona discharge needle when removing the atmospheric pressure chemical ionization (APCI) interface.

D. Protective Equipment

Standard safety protective equipment should be utilized when performing this procedure. This includes lab coat, safety glasses, durable gloves, and a chemical fume hood.

E. Training

Training and experience in the use of a triple quadrupole mass spectrometer and the automated SPE extractor should be obtained by anyone using this procedure. Operators are required to read the operation manuals or laboratory SOP. Formal training is not necessary; however, an experienced user should train all of the operators.

F. Personal Hygiene

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

G. Disposal of Wastes

Solvents and reagents are disposed of in an appropriate container clearly marked for waste products and temporarily stored in a flame resistant cabinet. Containers, glassware, etc., that come in direct contact with the specimen were either autoclaved or decontaminated with 10% bleach. Contaminated analytical glassware is treated with bleach, washed and reused; disposable labware is autoclaved prior to disposal. To insure proper compliance with CDC requirements, laboratory personnel are required to attend annual hazardous waste disposal courses.

3. Computerization; Data-System Management

A. Software and Knowledge Requirements

All samples are queued for analysis in a database created using Microsoft Access. Mass spectrometry data are collected and stored using the Analyst software (Applied Biosystems, Ontario, Canada). During sample preparation and analysis, samples are identified by their Sample Name and Sample ID. The Sample Name is a number that is unique to each sample during the sample preparation and the mass spectrometry measurement. The Sample ID is used to identify each specimen. In case of repeated measurements, the sample can have more then one Sample Name, but only one Sample ID in the database. The Sample ID links the laboratory information with the demographic data recorded by the sample takers. All raw mass spectral data are archived for future reference. All raw data files are analyzed using the Analyst/Quantitation Wizard program, which allows manual peak selection and area integration. These raw data (peak area, peak height, retention time, analyte name, MRM name) are exported to the Access database used for storage and retrieval. This Access database is stored on the DLS-PC Network (Q:\Phtalates\Phenol) as well as in several archive locations. Statistical analysis of

the data, programming, and reporting are performed using the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). Knowledge and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

B. Sample Information

Sample Names and Sample IDs, sample volume and project number are entered into the Access database before sample preparation. If possible, for unknown samples, the sample IDs are read in by a barcode reader directly from the sample vials. Sample IDs for QCs are entered manually. The Sample Log Sheet containing Sample Names and Sample IDs is printed from the Access database and is used to record information during the sample preparation. After MS data collection and peak integration, the data are exported into a space delimited text file which is then imported into the Access database.

C. Data Maintenance

All sample and analytical data are checked after being entered into the database for transcription errors and overall validity. The database is routinely (at least once weekly) backed up onto a computer hard drive and onto a network magnetic tape. Data from completed studies are saved on CD-R/RW, and on the hard drive. Additionally, final reported data are saved as paper copy as an official government record.

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

A. Sample Collection

Urine specimens are collected from subjects in standard urine collection cups. Samples should be refrigerated as soon as possible. The specimen should be transferred to specimen vials within 4 hours of collection. A minimum of five milliliters of urine is collected, and can be stored frozen in borosilicate glass or polypropylene vials or specimen cups. Teflon coated stoppers are used to plug vial and the vial is sealed with an aluminum seal. Crimped caps with rubber stoppers should not be used because of their tOP content. The specimen are then labeled and frozen immediately to 20 °C, and stored on dry ice for shipping. Special care must be taken in packing to protect bottles from breakage during shipment. All samples should be stored at, at least, -20 °C until analysis.

B. Sample Handling

Samples are thawed, sonicated, aliquoted, and the residual specimen is again stored at 20 °C until needed.

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

A. Reagents and Sources

Methanol (MeOH), formic acid, and water, purchased from Tedia (Fairfield, OH) were analytical or HPLC grade. Bisphenol A (BPA), 4-tert-octylphenol (tOP), ortho-phenylphenol (OPP), triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol), 2,4-dichlorophenol (2,4-DCP), 2,5-diclorophenol (2,5-DCP), 2,4,5-trichlorophenol (2,4,5-TCP), 2,4,6-trichlorophenol (2,4,6-TCP), ammonium acetate, 4-methylumbelliferyl glucuronide and 4-methylumbelliferyl sulfate were purchased from Sigma Aldrich Laboratories, Inc. (St. Louis, MO). β-glucuronidase (E.Coli) and arysulfatase were purchased from Roche (Penzberg, Germany). Benzophenone-3 (BP-3, 2-hydroxy-4-methoxybenzophenone, Eusolex 4360) was kindly provided by EMD Chemicals Inc. (Hawthorne, NY). $^{13}C_{12}$-BPA, $^{13}C_6$-OPP, $^{13}C_6$-triclosan, $^{13}C_6$-2,4-DCP, $^{13}C_6$-2,5-DCP, $^{13}C_6$-2,4,5-TCP, and $^{13}C_6$-2,4,6-TCP were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). D_4-tOP was from Hayashi Pure Chemical Ind., Co. Ltd. (Japan).

B. Working Solutions

1. **0.5 M Ammonium acetate buffer, pH 6.5**
   Weigh 38.54 g of ammonium acetate into a 500 mL beaker. Add 400 mL HPLC-grade H_2O and mix vigorously until all ammonium acetate is dissolved. Add glacial acetic acid drop wise until pH 6.5. Transfer the solution into a 1000 mL volumetric flask, and fill to volume with HPLC-grade H_2O. Prepare monthly and store at 10 °C or below.

2. **0.1 M formic acid solution**
   Dilute 3930 µl of formic acid (96%, density 1.22) to 1 liter with HPLC grade water. Prepare monthly and store at 10 °C or below.

3. **β-glucuronidase (E.Coli)+arysulfatase/buffer solution**

   Prepare daily for each run. Add 70 µL of β-glucuronidase (E.Coli) and 210 µL of arysulfatase to 3.5 mL of ammonium acetate buffer (pH 6.5) solution. Mix gently to prevent denaturation of the enzyme. 50 µL of this enzyme/buffer solution will be used for incubation of each sample.

4. **HPLC Mobile Phase**
   HPLC grade water is used as mobile phase A (aqueous); HPLC grade MeOH is used as mobile phase B (organic) for both SPE and HPLC pumps.

5. **Synthetic Urine**
   Prepare monthly and store in the refrigerator. The mixing procedure is shown as follows,

a. 500 mL Deionized water

b. 3.8 g Potassium Chloride

c. 8.5 g Sodium Chloride

d. 24.5 g Urea

e. 1.03 g Citric Acid

f. 0.34 g Ascorbic Acid

g. 1.18 g Potassium Phosphate

h. 1.4 g Creatinine

i. 0.64 g Sodium Hydroxide (add slowly)

j. 0.47 g Sodium Bicarbonate

k. 0.28 mL. Sulfuric Acid

l. Fill up to 1 L with deionized water

C. Standards Preparation

1. Stock Solutions and Analytical Standard Solutions

   Initial stock solutions were prepared by dissolving measured amounts of phenols in methanol. Serial dilutions of these stock solutions were made in methanol to create ten mixed standard stock solutions containing all 9 analytes. 100-μl aliquot of this mixed stock solution to 100 μL urine will result in the desired concentration range.

2. Internal Standard Solution

   Initial stock solutions for all isotope labeled compounds were prepared by dissolving measured amounts of the solid compounds in MeOH. The internal standard working solution was prepared by diluting the stock solutions in MeOH, so that a 50-μL aliquot in 100 μL urine resulted in an appropriate concentration level for each compound. The native and the isotope labeled standard solutions were then dispensed into small vials. The native standard solution was stored at -20 °C, while the isotope labeled standard solution was stored in the refrigerator (2-5 °C).

3. Deconjugation Internal Standard Solution

   4-methylumbelliferyl sulfate, 4-methylumbelliferyl glucuronide, and $^{13}C_4$-4-methylumbelliferone are used as deconjugation standards to monitor the extent

of the enzymatic reaction. The deconjuation solution is prepared by dissolving 240 μg of 4-methylumbelliferyl glucuronide, 200 μg of 4-methylumbelliferyl sulfate, and 200 μg of $^{13}C_4$-4-methylumbelliferone in 100 mL of MeOH.

4. MS Instrument Operational Check Standard

   Prepare 100 mL of native compounds at a concentration level of Standard 5. Dispense into 1.5 mL autosampler vials, and store at -20 ^{o}C. This solution is used to check the sensitivity of the mass spectrometer before starting each day's run.

5. Proficiency Testing (PT) Standard

   Aliquots of each standard stock II solution are added to 100 mL filtered urine pools. The volume of each standard varies to produce PT standards of 3 different concentrations. The spiked pools are mixed overnight, aliquoted into vials, and frozen until needed. The PT standards are characterized by at least 20 repeat measurements to determine the mean concentration and standard deviation for evaluation.

D. Materials

1. HPLC conical glass autosampler vials (1.5 mL, SUN-Sri)

2. Tip ejector variable volume micropipettes (Wheaton, Millville, NJ), and disposable pipette tips (Rainin Instruments Co., Woburn, MA).

3. LiChrosphere RP-18 ADS cartridge (25-4mm, Merck GaA, Germany), and cartridge holder.

4. Chromolith Performance PR-18e (100-4.6mm, Merck GaA, Germany) HPLC column.

5. Assorted glassware.

E. Equipment

1. Agilent 1100 HPLC system (Agilent Tech., Wilmington, DE), which includes 2 binary pumps, 1 autosampler, and 1 column compartment with a 10-port switching valve.

2. High pressure mixing Tee.

3. Applied Biosystems API 4000 mass spectrometer (Applied Biosystems, Forster City, CA).

4. Sartorius Genius Series ME models electronic analytical & semi- microbalances (Sartorius AG, Goettingen, Germany).

5. Sartorius top-loading balance (Sartorius AG, Goettingen, Germany).

6. pH meter (Corning pH/ion analyzer 455, Corning, New York).

7. Allegra 6 Centrifuge (Beckman Coulter)

8. Fisher Isotemp Incubator (300 Series Model 350D).

9. Vortexer (Fisher, Genie 2).

10. Magnetic Stirrer (Corning).

F. Instrumentation

1. On line SPE-HPLC-MS/MS Configuration

The on-line SPE-HPLC-MS/MS system was constructed from several Agilent 1100 modules coupled to a triple quadrupole API 4000 mass spectrometer equipped with an APCI interface. The on-line SPE-HPLC system consisted of two binary pumps with degassers, an autosampler with a 900-µL injection loop, a high pressure mixing Tee, and one column compartment with a ten-port switching valve. The mass spectrometer and HPLC modules were programmed and controlled using the Analyst 1.4 software (Applied Biosystems, Ontario, Canada). The SPE column was a LiChrosphere RP-18 ADS (25 $\times$ 4 mm, 25 µm particle size, 60 Å pore size, Merck KGaa, Germany), and the HPLC columns were two Chromolith™ Performance RP-18 (100 $\times$ 4.6 mm; Merck KGaA).

The procedure for extracting the phenols from the urine involved concurrent SPE and HPLC-MS/MS cycles (Table 2, Figure 1). While the autosampler and Pump 1 were used for the SPE of one sample, the ten-port switching valve, Pump 2 and the mass spectrometer were used to acquire data from the previous sample. The HPLC-MS/MS acquisition method was built in 'no sync' mode (i.e., all devices were programmed to start at the same time). The different combinations of autosampler valve and switching valve positions, and the timing of the gradient of the two binary pumps divided the concurrent regeneration and equilibration of the SPE column for the clean-up of the next sample, and the collection of the HPLC-MS/MS data into six periods (Table 1).

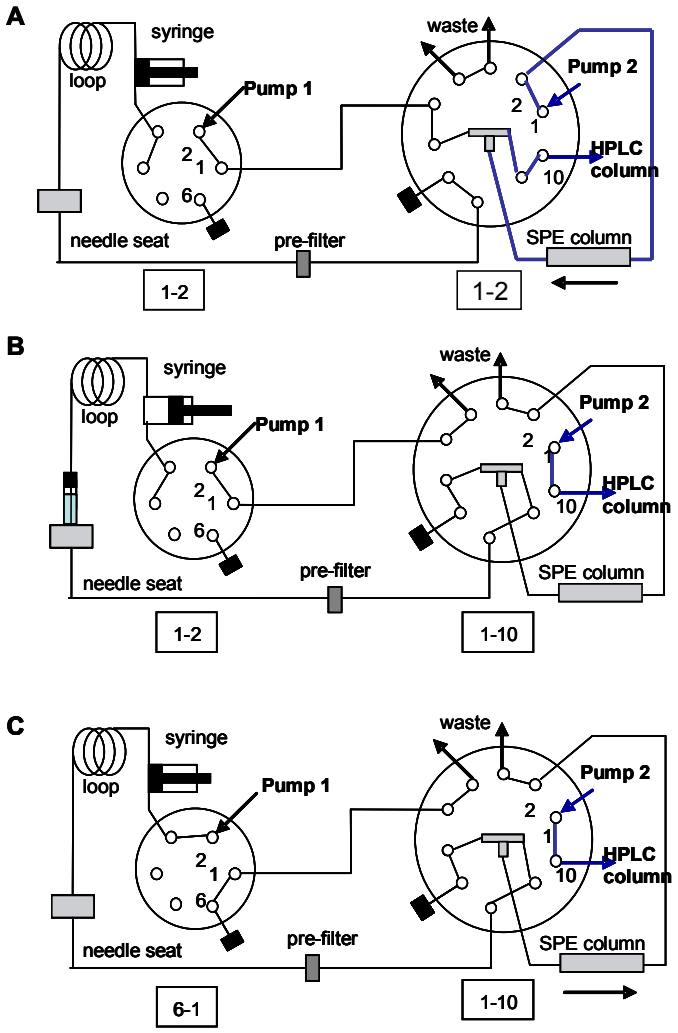
Table 1. Concurrent SPE clean-up and HPLC-MS/MS analysis time line

Period			1	2	3	4	5	6
Time (min)		0	0.1-2	2 - 5	5 - 9	9 - 15.5	15.5-18.8	18.8 - 21
SPE of Sample N+1		Start	Analyte Transfer and dilution	Rege-nerate SPE column	Equi-librate SPE column	Sample loading	SPE column Wash	Stop Pump 1
	Autosampler valve	1-2	1-2	6-1	6-1	1-2	6-1	1-2
	Pump 1 mL/min	0	0.25	1.0	1.0	1.0	1.0	0
	Pump 1 MeOH%		20%	100%	20%	20%	20%	20%
HPLC of Sample N			Analyte Transfer	HPLC separation and MS/MS acquisition				Equi-librate Pump 2
	Ten-port valve	10-1	1-2	10-1				10-1
	Pump 2 mL/min	0.5	0.5	0.75 HPLC gradient elution				0.75
	Pump 2 MeOH%	50%	50%	Time (min)	2.1	10	17	18.5	50%
				MeOH %	50	65	100	100	

First, the analytes from the previously injected sample that had been retained by the SPE column were eluted using 50% MeOH: 50% water at 0.5 mL/min provided by pump 2. Through a mixing Tee, the 0.5 mL/min SPE elute was diluted with 20% MeOH: 80% water (0.25 mL/min) provided by pump 1, and then, the analytes were transferred to the HPLC column (Figure 1A, Table 2). At 2 minutes, the collection of the HPLC-MS/MS data started while the SPE column was regenerated and equilibrated with 100% MeOH (1 mL/min for 3 minutes) and 20% MeOH:80% water (1 mL/min for 4 minutes), respectively. The injection (1 mL of sample containing 100 µL urine) was programmed as two sequential "400 µL sample draw" and "400 µL eject into the needle seat" commands in Analyst 1.4. Tube connections inside the autosampler were modified in-house to connect the needle seat directly to the SPE column. In this way, the execution of the "eject into the needle seat" command resulted in loading of the sample directly onto the SPE column by the autosampler syringe (Figure 1B). A needle rinse, performed by lowering the needle into a vial containing MeOH, was included before the second ejection. After the sample loading was complete, the SPE

column was washed for 3 minutes while unbound urine components were carried to waste by a flow (1 mL/min) of 20% MeOH:80% water (Figure 1C, Table 2). The collection of HPLC-MS/MS data lasted 16.6 min, after which the HPLC pump was equilibrated for 2.2 minutes for the next elution cycle while the flow through the SPE column was brought to a complete stop (Table 2).

Figure 1. Tubing set-up for the autosampler and ten-port valves with configurations for 3 selected periods of Table 2: A) Analyte transfer and dilution (Period 1), B) Sample loading (Period 4) and C) SPE column wash (Period 5).



G. Mass Spectrometry

The API 4000 mass spectrometer is used in negative ion APCI mode. Laboratory-grade air is used for both auxillary gas and nebulizing gas. The mass spectrometer parameters are included in the acquisition method 2005-0511. The negative fragment ions used for quantification and the retention time for the analytes are listed in Table 2.

Table 2. Analyte retention time (RT) and precursor ions to product ion transitions monitored for quantitation (and confirmation) of native compounds and corresponding internal standards**.**

Analyte	RT (min)	Precursor Ion to Product Ion (m/z)	
		Native Analyte	Internal Standard
Bisphenol A	13.6	227 to 133 (212)	239 to 139
4-t-Octyl phenol	19.3	205 to 133	209 to 137
Triclosan	19.1	161 to 125 (287 to 142)	167 to 131 (299 to 148)
Benzophenone-3	17.8	227 to 183 (211)	*
ortho-phenylphenol	15.1	169 to 115 (141)	175 to 121
2,4-dichlorophenol	14.8	161 to 125 (163 to 125)	167 to 131
2,5-dichlorophenol	14.2	161 to 125 (163 to 125)	167 to 131
2,4,5-trichlorophenol	17.4	195 to 159 (197 to 161)	201 to 165
2.4,6-trichlorophenol	16.9	195 to 159 (197 to 161)	201 to 165

*We used $^{13}C_{12}$-BPA as the internal standard for BP-3

7. Calibration and Calibration-Verification Procedures

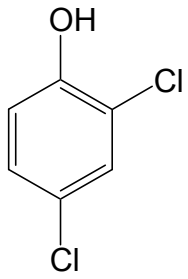
A. Mass Spectrometer

The Sciex API 4000 mass spectrometer is calibrated and tuned using a polypropylene glycol (PPG) solution according to the instructions contained in the API 4000 operator's manual.

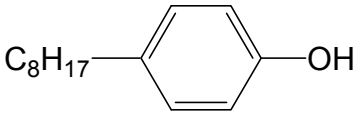
B. Calibration Curve

Calibration curves are calculated daily from the area ratios ([analyte peak area]/ [internal standard peak area]) from freshly analyzed standards and linear regression analysis where each concentration is weighed by 1/ [measured concentration]. Acceptable calibration curves must have correlation coefficients greater than 0.98. Samples with values exceeding the highest calibration point are reanalyzed using less urine diluted with water.

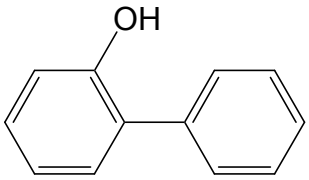
Analytes nomenclature and structures



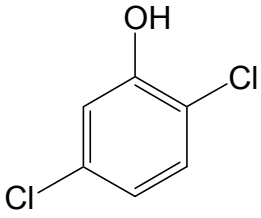
2,4-diclorophenol (24-DCP)



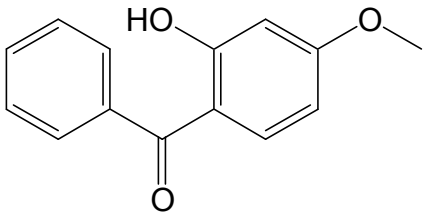
t-octylphenol (t-OP)



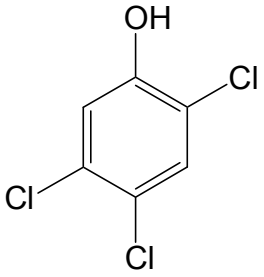
o-phenyl-phenol (o-PP)



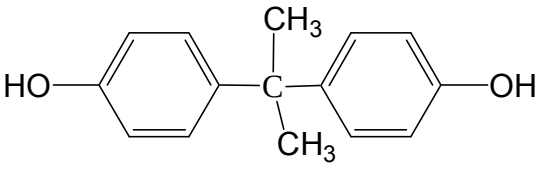
2,5-dichlorophenol (25-DCP)



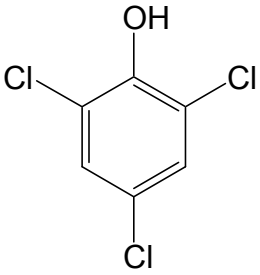
bezophenone-3 (BP-3)



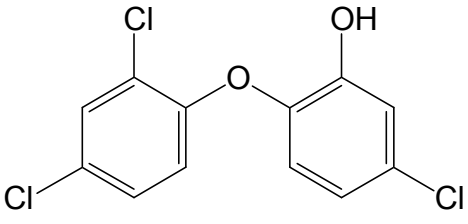
2,4,5-trichlorophenol (245-TCP)



bisphenol A (BPA)



2,4,6-trichlrophenol (26-TCP)



triclosan

1. Calibration Verification

   a. 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.

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

   c. All calibration verification runs and results shall be appropriately documented.

   d. 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.

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

2. Proficiency Testing

   a. Three pools of PT samples, which encompass the entire linear range of the method, are prepared in-house as described in the standard preparation section. Characterization of PT materials requires at least 20 separate determinations. Once the PT pools are characterized, the mean concentration and standard deviation of the PT materials are forwarded to a DLS representative responsible for executing the PT program. These PT samples are blind-coded by the PT administrator and returned to the laboratory staff for storage. When proficiency testing is required, the laboratory supervisor or his/her designee will notify the PT administrator, and the PT administrator will provide the blinded reference numbers for the 5 PT samples to be analyzed.

   b. Proficiency testing should be performed a minimum of once per 6 months. The PT administrator will randomly select five PT materials for analysis. Following analysis, the results will be forwarded directly to the PT administrator for comparison with the values previously characterized. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the PT administrator. The PT administrator will notify the laboratory staff of the PT results (i.e. pass/fail).

   c. All proficiency results shall be appropriately documented.

8. Operating Procedures; Calculations; Interpretation of Results

A. Sample preparation

1. Remove urine samples from the freezer (-20 °C) and let them thaw.
2. Included max 50 unknown samples, 2 QC blanks, and 2 QCL and 2 QCH for each batch. Label the autosampler vials. Print out Sample Log Sheet with Sample Name.
3. Add 50 µL of internal standard mix to the autosampler vials.
4. Add 10 µL of 4-methylumbelliferyl sulfate, 4-methylumbelliferyl glucuronide, and $^{13}C_4$-4-methylumbelliferone mix solution.
5. Add 100 µL of QC or urine sample. For the reagent blank (QCB), add 100 µL of HPLC grade water instead.
6. Prepare enzyme/buffer solution. Add 70 µL of β-glucuronidase (E.coli) and 210 µL of arylsulfatase to 3.5 mL of ammonium acetate buffer (0.5 M, pH-6.5). Gently mix the enzyme. *Do not shake or enzyme will be inactivated.*
7. Add 50 µL of buffer/enzyme solution to each sample.
8. Gently mix the sample manually, incubate for 4 hours at 37 °C.
9. After incubation, add 790 µL of 0.1M formic acid to each sample.
10. Vortex for 10 seconds, and centrifuge. Keep the samples at 10 °C in the refrigerator until the SPE-HPLC-MS/MS run.

B. Analysis

1. Check out the LC/MS interface
   a. If the instrument is in ready mode, wait until the interface cools down. When the interface is cold enough, take out the capillary. Rinse the capillary with MeOH; sonicate it in MeOH for 20 min if necessary. Once a week, take off the interface house, and wipe the skimmer plate.
   b. Open the rough pump cabinet, check for oil leaks and strange noise. Report anything unusual.
2. Check out the LC system
   a. Refill the mobile phase for both HPLC and SPE pumps directly from the original 4 L brown reagent bottles (water and MeOH) so that solvent contamination from a third container would be eliminated.

b. Change the two pre-filters, which are located before SPE and HPLC columns, for each batch of samples.

c. Click on the Equilibrate icon, select the current method and enter equilibration time (about 10 min). Run the instrument check sample by opening the batch file named Instrument_test.dab, change the date in sample name field. Make sure the proper Acquisition Method and Vial Position is entered, and then submits the batch. The file should be saved into the Instrument_test.wiff file. Open the chromatogram and compare the intensities and peak shape from those obtained one day and one week before. If peaks appear distorted (long tail, after-peak, too broad etc.) check with the lab supervisor or his/her designee.

d. Building the batch files
From Excel, open up the text file containing the batch table created by Access. This file should not require any editing. Save the edited table into the text file named import.txt into the batch directory (overwrite). Remember to CLOSE THE FILE IN EXCEL!! Go to the analyst and import the import.txt file (Sample pull down, go to gray header and click RMB, then Import From/File, select Agilent 1100G1313 autosampler).

Check and make sure that the proper Acquisition Method and Quantitation Method are entered.

Always submit a dummy sample first with the vial position of the first real sample. Then submit the batch file with the vial position of each sample shifting by 1. For example, if the sample is in position N, then in the batch file, the vial position for this sample should be N+1. Remember to put an empty vial right next to the last sample on the autosampler tray, so when the MS acquire data for the last sample, the autosampler could withdraw the sample from this empty vial, otherwise the system will stop because of the error message of missing vial. The reasons for building the sequence file this way have been explained in “Instrumentation/On line SPE-HPLC-MS/MS Configuration.”

C. Processing data

1. Quantification

2. All raw data files are analyzed using the Quantitation Wizard application in Analyst, which allows both automatic and manual peak selection, and area integration. All information, including sample name, sample ID and calculated concentration are exported into a tab delimited text file with the name YYYY-MMDD.txt.

3. Importing Data into the database

4. The YYYY-MMDD.txt tab-delimited file is read into the Access database. No prior editing is required.

5. Statistical Analysis and Interpretation of Data

6. Data are exported from the Access database to a fixed ASCII text file and imported into SAS. For statistical analysis the concentrations calculated by Analyst will be used. However, standard curve generation, QC analysis, blank analysis, limit of detection determination, unknown calculations, and data distribution programs have been created and may be executed in SAS when this information is needed.

D. Replacement and periodic maintenance of key components

1. API 4000 Sciex Mass Spectrometer

   a. Maintenance of the API interface, L1x lenses and the analyzer assembly requires venting the system; maintenance of the API ion probe does not. After venting, the system will usually require around 4-6 hours to reestablish the high vacuum.

   b. When a partial blockage of the vacuum is suspected, the orifice is probed with a syringe-cleaning wire.

   c. Cleaning the spray shield and the entrance end of the heated capillary, described in the Sciex API 4000 Hardware Manual (p. 100), is performed weekly. First, wash with a solution of water: methanol (1:1), second with 100 % methanol, then wipe it using flake free paper wipes.

   d. The pump oil is changed approximately every six months as part of the periodic maintenance.

2. Regeneration of HPLC columns

   HPLC columns are regenerated with a proteinase preparation weekly to remove any protein that might bind to the column. The procedure for the column regeneration is as follows:

   a. Wash the columns with 100 %ACN at a flow rate of 1 ml/min for 10 min.

   b. Wash the columns with 100 % H_2O at a flow rate of 1 ml/min for 10 min.

   c. Saturate the columns with proteinase at a flow rate of 1ml/min for 10 min. Proteinase could be recycled.

   d. Seal the columns with the caps, and put them in a sealed plastic bag.

   e. Incubate the columns @ 75 °C in a water bath for at least 30 min.

   f. Wash the columns with H_2O at a flow rate of 1 ml/min for 10 min.

g. Wash the columns with ACN at a flow rate of 1 ml/min for 10 min.

h. Check the column by running the instrument check sample.

3. Agilent 1100 on line SPE-HPLC

a. Additional maintenance of the on line SPE-HPLC is only necessary if a decrease in the system performance (low sensitivity, low resolution, and/or low S/N ratio) is detected.

b. The HPLC columns need to be replaced if the analyte resolution begins to fail. Since there are two HPLC columns used simultaneously, we suggest replacing one HPLC column at a time before deciding whether to replace either one or both columns.

c. If the analyte peaks start to tail, problem may be with HPLC or SPE column. Check each one individually for peak shape and replace whichever necessary.

d. If high pressure error messages are observed, systematically check the purge valve frit, the pre-filter, analytical column frit, HPLC lines, needle seat, or injector components to find out the source of the plug and replace the part with a new one.

e. Reestablishment of performance and calibration. Each time the system is down for cleaning or maintenance, a MS operational check standard is analyzed to assess the system performance. For the mass spectrometer rerun of the system may or may not be necessary. If the instrument does not pass this test, then the instrument is retuned using the polypropylene glycol as described previously.

9. Reportable Range of Results

The linear range of the standard calibration curves and the method limit of detection (LOD) determine the reportable range of results. The reportable range must be within the range of the calibration curves. However, samples with analytical data values exceeding the highest reportable limit may be diluted, re-extracted, and reanalyzed so that the measured value will be within the range of the calibration.

A. Linearity Limits

The high linearity limit is determined by the highest standard analyzed in the method. Unknown urine samples whose concentrations exceed this limit 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. **Table 3** summarizes the linear range for each analyte in urine.

Table 3. Calibration curve parameters

Analyte	Linear range (ng/mL) LOD - Highest Standard
Bisphenol A	0.36-100
4-tert-octylphenol	0.17-100
Triclosan	2.27-1000
Benzophenone-3	0.34-200
2,4-dichlorophenol	0.17-100
2,5-dichlorophenol	0.12-1000
2,4,5-trichlorophenol	0.10-100
2,4,6-trichlorophenol	0.17-100
ortho-phenylphenol	0.10-100

B. Analytical Sensitivity

The limits of detection (LOD) are defined for each analyte by repetitive analysis of low level standards by the calculation of the standard deviation at zero concentration (S_o). The formal limit of detection is defined as $3S_o$.

C. Accuracy

The accuracy is calculated from repeated analyses of synthetic urine spiked with standards 3, 5 and 7. We use the isotope-dilution technique with isotope-labeled phenols, which allows for automatic recovery correction for each sample and improves the method precision and accuracy.

Table 4. Spiked recoveries (%) of the standards

Analyte	Standard 3	Standard 5	Standard 7
Bisphenol A	113	102	98
4-tert-octylphenol	129	104	102
Triclosan	106	97	108
Benzophenone-3	99	107	96
2,4-dichlorophenol	102	101	96
2,5-dichlorophenol	132	122	107
2,4,5-trichlorophenol	102	98	96
2,4,6-trichlorophenol	115	95	94
ortho-phenylphenol	113	108	112

D. Precision

The precision of this method is reflected in the variance of two quality control (QC) pools over time. The coefficient of variation (CV) of repeated measurements of these QC pools is used to estimate the method precision. The QC low concentration ranges from 2 to 50 ng/mL; for the QC high, the concentration ranges from 10 to 250 ng/mL. Table 5 lists the CV % for QCL and QCH for each analytes.

Table 5. Urinary phenol levels in the QC pools

Analyte	QC High		QC Low	
	Mean	CV%	Mean	CV%
Bisphenol A	20.4	12.1	3.16	18.6
4-tert-octylphenol	18.47	18.4	5.04	24.4
Triclosan	237.5	14.4	40.8	20.9
Benzophenone-3	46.2	16.2	18.5	16.2
2,4-dichlorophenol	21.3	12.3	1.72	23.9
2,5-dichlorophenol	241.0	10.9	30.5	11.5
2,4,5-trichlorophenol	16.3	7.5	2.14	8.6
2,4,6-trichlorophenol	27.9	11.1	5.9	14.9
ortho-phenylphenol	7.4	10.5	1.5	17.2

E. Analytical Specificity

The method that requires that the analytes 1) coelute with the corresponding isotope labeled internal standard analog; 2) elute at a specific retention time; 3) have precursor ions with specific mass/charge ratios; and 4) have two specific product ions formed from the precursor ion with specific mass/charge ratios. The quantitation and confirmation ions for each analyte are listed in Table 3.

F. Deconjugation Optimization

Accurate quantification of phenol metabolites urinary levels assumes complete hydrolysis of the conjugated forms. Therefore, deconjugation was optimized to identify the time required for enzyme-mediated deconjugation. A time course experiment was conducted with urine containing conjugated bisphenol A. This experiment revealed that the incubation of a sample with β-glucuronidase/sulfatase for a minimum of 4 hours resulted in quantitative deconjugation. Overnight deconjugation (12-16 hours) ensured completion of hydrolysis, and didn't induce artifactual changes to the levels of the analytes.

10. QC Procedure

A. QC Materials
Quality control (QC) materials are prepared in bulk from urine pools collected from several anonymous donors. These QC samples are analyzed along with unknown samples to monitor for accuracy and precision throughout the analytical run.

B. QC Pools
The QC pools were mixed uniformly, and divided into two subpools. The subpools were enriched with phenols as needed to afford low concentration (QCL, ~ 2-50 ng/mL) and high concentration (QCH, 10-250 ng/mL) subpools. The pools were dispensed into sample vials and frozen at –20 °C until needed.

C. Characterization of QC Materials
The QC pools were characterized to define the mean and the 95% and 99% control limits of phenols concentrations from 60 QCL and 60 QCH runs over 3 weeks. In each run, one pair of QCL and one pair of QCH materials were analyzed and averaged. Using the pair average value from the 60 runs, we calculated the mean, and upper and lower $99^{\%}$ and $95^{\%}$ control limits.

D. Use of Quality Control Samples

Each analytical run consists of 56 samples: 2 QCL, 2 QCH, 2 reagent blanks, and 50 unknowns. The concentrations of the two QCH and the two QCL are averaged to obtain one measurement of QCH and QCL for each batch.

E. Final evaluation of Quality Control Results

Standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control. When using <u>2 QC pool levels per run, the rules are:</u>

<u>For 1 QC result per pool</u>

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. Check for any irregularities (i.e., low calibration curve regression, change in slope or intercept, high blank concentration, low internal standard sensitivity, etc). If the source of failure is easily identifiable, for instance, 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

Occasionally, the concentration of the analytes in urine is much higher than the highest standard in the calibration curves, and 100 µL of urine may be too much to use. This is evident by the low recovery of the isotope-labeled standard after the SPE extraction. In this case, a smaller aliquot of urine can be used to successfully extract the analytes. Most likely, the LOD is not higher in this case because of the concentrated nature of the urine.

The procedure requires expensive instrumentation.

13. Reference Ranges (Normal Values)

The results from the National Health and Nutrition Examination Survey 1999-2000 (NHANES 1999-2000) will be used reference range to describe levels of phenol exposure among the general US population (Table 6):

Table 6. Urinary concentrations (in ng/mL) of selected phenols from NHANES 99-00 [32]

Analytes	geometric mean	10th	25th	50th	75th	90th	95th	Sample Size
2,4-dichlorophenol	1.11 (.883-1.4)	<LOD	<LOD	0.75 (.6-1)	2.9 (1.8-4.7)	11 (6.4-17)	22 (17-31)	1990
2,5-dichlorophenol	6.01 (4.22-8.57)	<LOD	1.4 (.71-2.1)	6.5 (4.6-9.9)	37.8 (23-52)	144 (88-240)	440 (240-700)	1989
2,4,5-trichlorophenol	*	<LOD	<LOD	<LOD	1.4 (1-2.4)	5.4 (2.5-15)	16 (4.3-39)	1998
2,4,6-trichlorophenol	2.85 (2.58-3.15)	<LOD	1.2 (<LOD-1.2)	2.45 (2.3-2.7)	4.8 (4-6.62)	14.8 (8.8-21)	25 (17-37)	1989
o-phenyl phenol	0.494 (.412-.593)	<LOD	<LOD	0.49 (0.3-0.59)	0.85 (.65-1.1)	1.46 (1.1-1.8)	2 (1.6-2.5)	1991
Bisphenol A*	1.33	0.22	0.58	1.28	2.46	4.10	518	394
4-t-O-Phenol	NA	NA	NA	NA	NA	NA	NA	NA
Triclosan	NA	NA	NA	NA	NA	NA	NA	NA
BP-3	NA	NA	NA	NA	NA	NA	NA	NA

* Data are from a population of 394 adults.[33]

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

Insufficient data exist to correlate urinary phenol values with serious health effects. Therefore, critical call values have not been established.

15. Specimen Storage and Handling During Testing

Urine samples may be stored overnight in the refrigerator to expedite thawing prior to aliquoting the sample. The urine extracts are stored in autosampler vials at –20°C or below after analysis. Stability studies suggest that the extracts remain stable at room temperature for up one week.

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

Validated SPE/derivatization-GC/MS analysis protocols are available on site if necessary.

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

Once the validity of the data has been established by the QC/QA system outlined above and has been verified by a DLS statistician, one hardcopy and one electronic copy (ASCII format) of the data will be generated. This data, a cover letter, and a table of method specifications and reference range values will be routed through the appropriate channels for approval (i.e. supervisor, Branch chief, Division director).

Once approved at the Division level, the data will be sent to the contact person who requested the analyses.

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

Standard record keeping systems (i.e. notebooks, sample logs, data files, creatinine logs, demographic logs) should be employed to keep track of all specimens. Specimens will only be transferred or referred to CLIA certified laboratories. 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.

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

19. Summary Statistics and QC Graphs

A. Triclosan

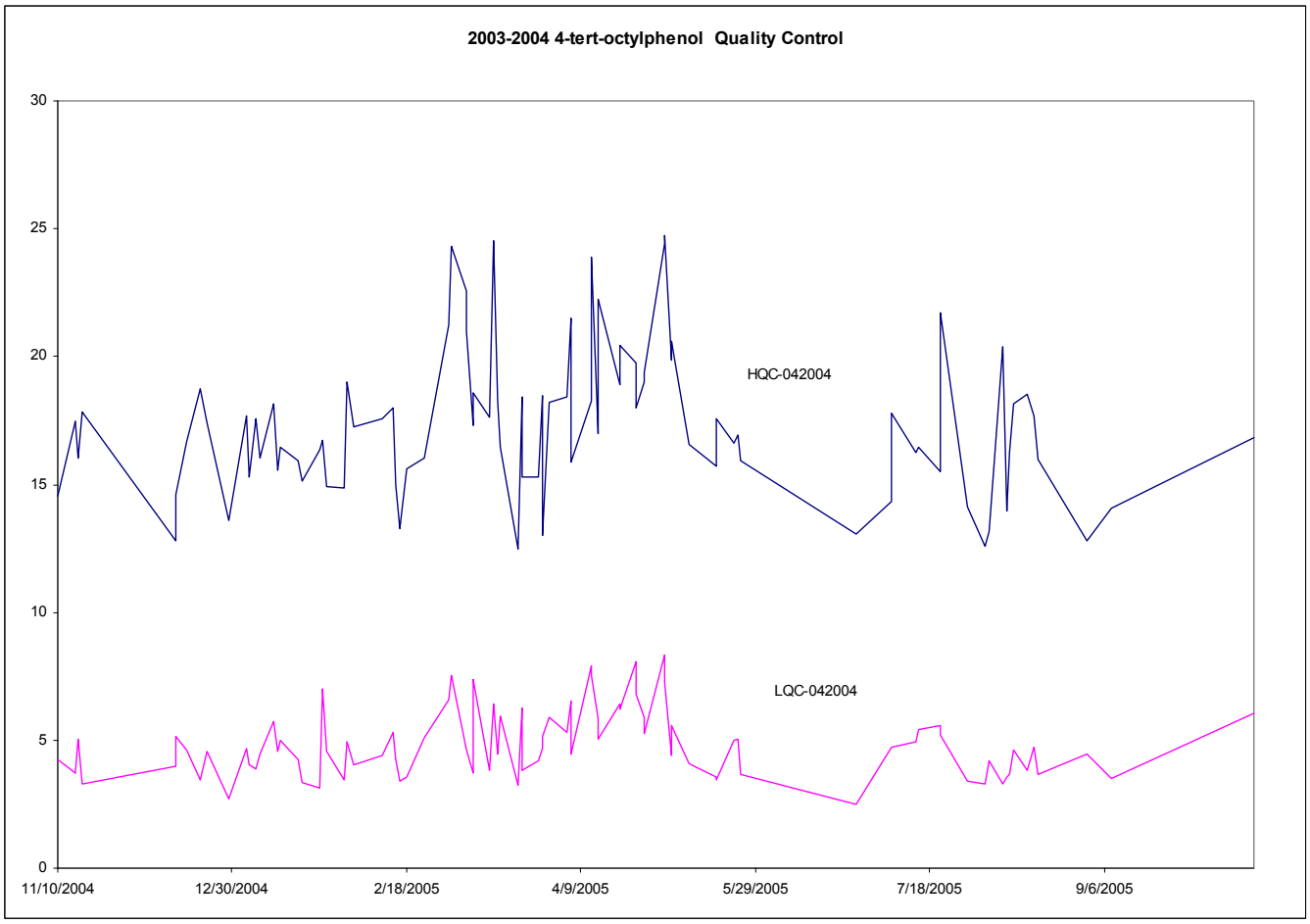
Summary Statistics for Triclosan by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	102	11/10/2004	10/19/2005	39.840	5.777	14.5
HQC-042004	102	11/10/2004	10/19/2005	231.525	24.668	10.7

B. 4-tert-octylphenol

Summary Statistics for 4-tert-octylphenol by Lot

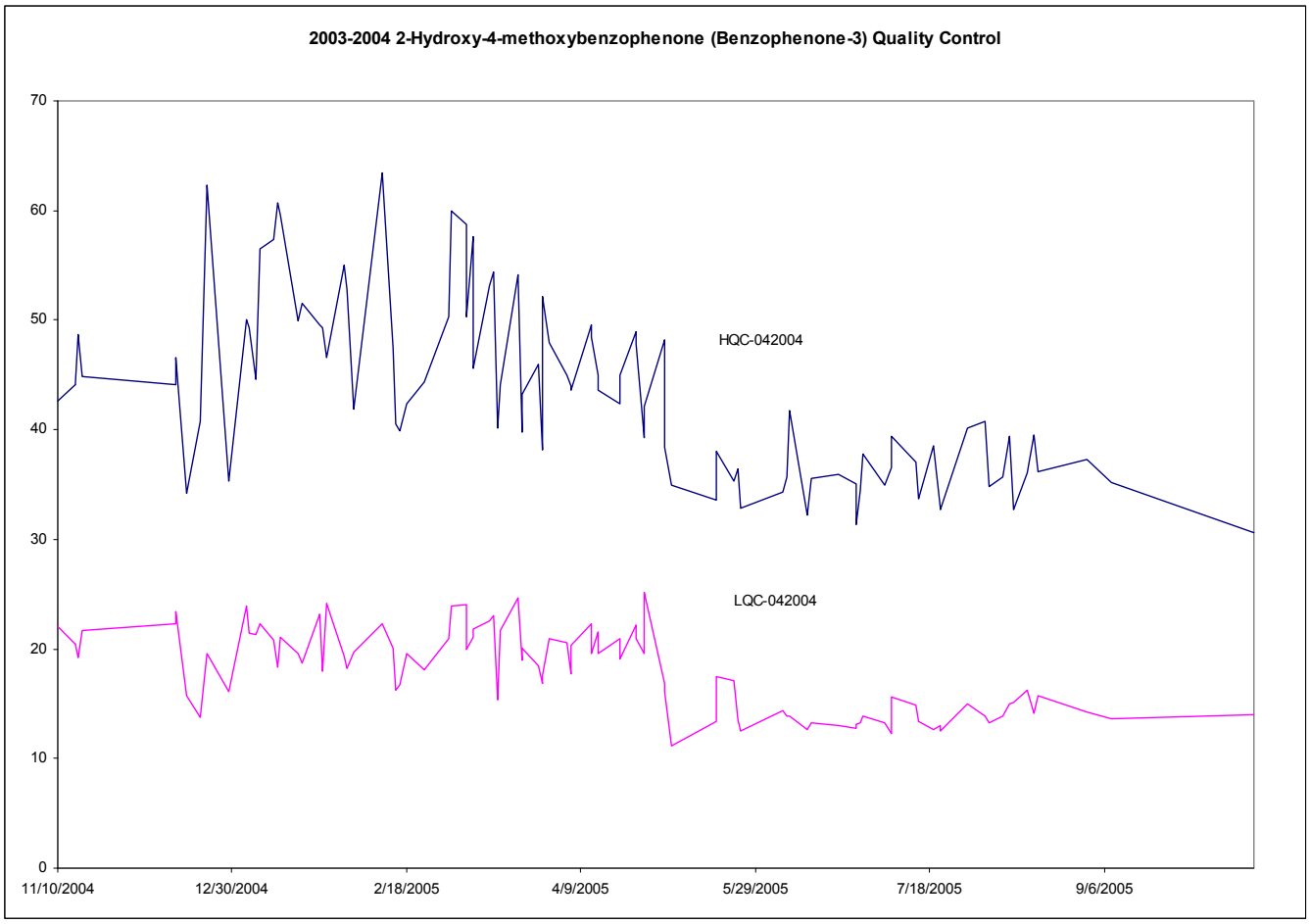
Lot	N	Start Date	End Date	Mean	Standard	Coefficient of Variation
LQC-042004	91	11/10/2004	10/19/2005	4.83	1.29	26.7
HQC-042004	91	11/10/2004	10/19/2005	17.33	2.85	16.4



C. 2-Hydroxy-4-methoxybenzophenone (Benzophenone-3)

Summary Statistics for 2-Hydroxy-4-methoxybenzophenone (Benzophenone-3) by Lot

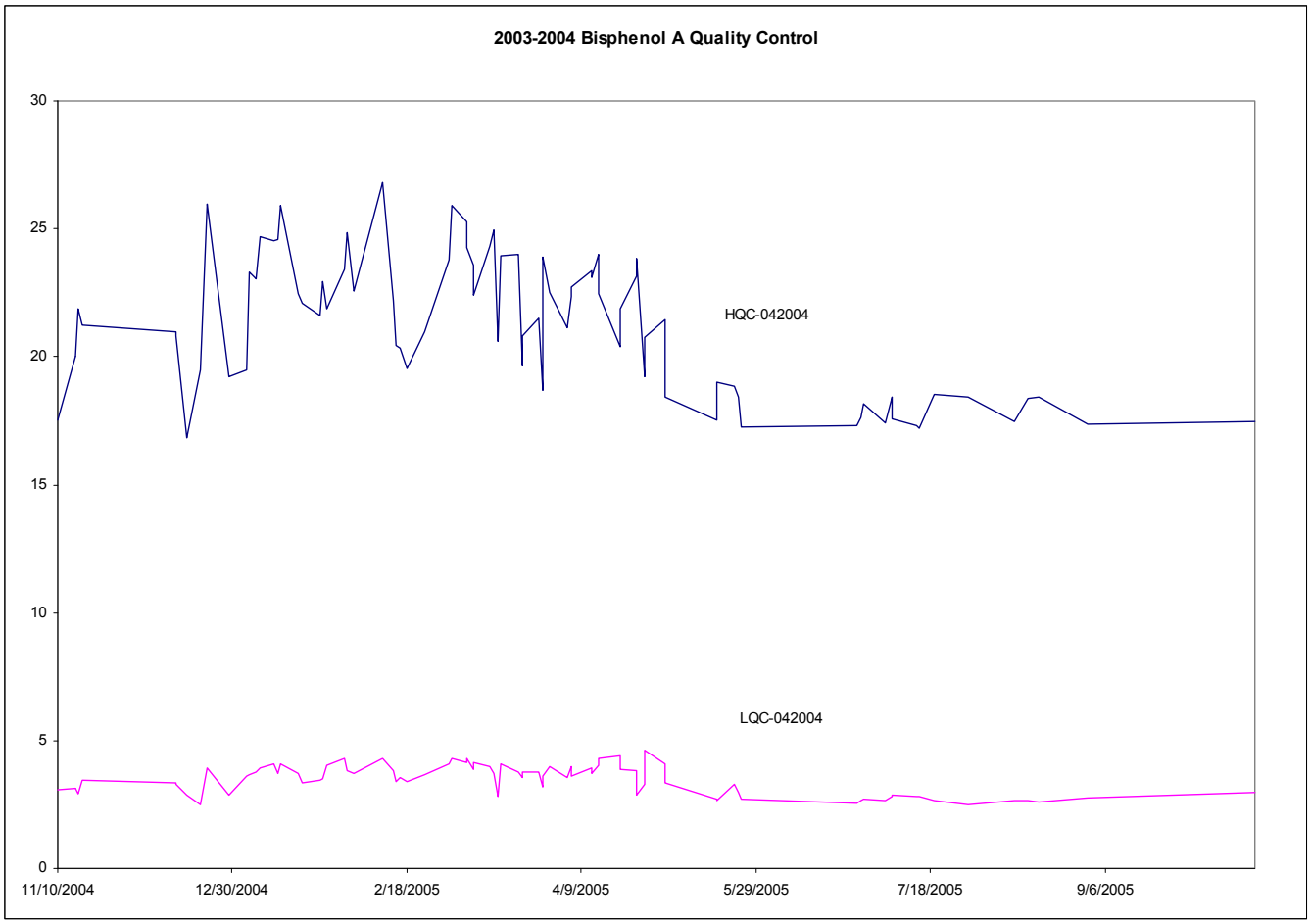
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	99	11/10/2004	10/19/2005	17.898	3.707	20.7
HQC-042004	99	11/10/2004	10/19/2005	43.361	7.976	18.4



D. Bisphenol A

Summary Statistics for Bisphenol A by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	83	11/10/2004	10/19/2005	3.468	0.564	16.3
HQC-042004	83	11/10/2004	10/19/2005	21.180	2.667	12.6



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