

Laboratory Procedure Manual

Analyte: **Cotinine**
Matrix: **Serum**
Method: **HPLC - APCI Tandem Mass Spectrometry**
Method No.: **03-OD**
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as performed by:

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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 testing the items listed in the following table:

Data File Name	Variable name	Description
COT_D	LBXCOT	Cotinine

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1. Clinical Relevance and Summary of Test Principle

Analyte (-)-Cotinine. 1-methyl-5-(3-pyridyl)-2-pyrrolidinone; N-methyl-2-(3-pyridyl)-5-pyrrolidone. $C_{10}H_{12}N_2O$; Mol Wt 176.21; m.p. 40-42 °C.

a. Clinical Relevance

Cotinine is a major metabolite of nicotine that can be used as a marker for active smoking and as an index for environmental tobacco smoke (ETS) exposure, otherwise known as passive smoking. Cotinine generally is preferred over nicotine for such assessments because of its substantially longer half-life. The estimated half-life of cotinine in plasma is about 15-20 hours (1-3); by contrast, the half-life of nicotine is only 0.5-3 hours (3-5). Cotinine can be measured in serum, urine, or saliva—the half-life of cotinine in all three fluids is essentially the same (1). Cotinine concentrations tend to be three to eight times higher in urine than in serum; however, plasma or serum is the fluid of choice for studies requiring a quantitative assessment of exposure (6). For that reason, serum was chosen as the matrix for the National Health and Nutrition Examination Survey (NHANES) cotinine analyses.

b. Test Principle

Serum cotinine is measured by an isotope-dilution high-performance liquid chromatography /atmospheric pressure chemical ionization tandem mass spectrometric (ID HPLC-APCI MS/MS) method (7, 8). Briefly, the serum sample is spiked with methyl- D_3 cotinine as an internal standard. After an equilibration period, the sample is applied to a basified solid-phase extraction column. Cotinine is extracted by using methylene chloride; the organic extract is concentrated, and the residue is injected onto a short C18 HPLC column. The eluant from these injections is monitored by APCI-MS/MS, and the m/z 80 daughter ion from the m/z 177 quasi-molecular ion is quantitated. Additional ions for the internal standard and for confirmation are also monitored. Cotinine concentrations are derived from the ratio of native- to- labeled cotinine in the sample by comparisons to a standard curve.

Special Precaution

Because of the nature of these assays, all analysts involved in this study must be nonsmokers, and measurements must be performed in a smoke-free building environment.

Note: This same method can be used to conduct salivary cotinine assays by substituting suitable saliva QC pools. All other aspects including calibration, cleanup and analysis are identical to serum cotinine measurements (8).

2. Safety Precautions

Wear eye protection and suitable protective clothing when using this method to extract and process samples.

a. Biological Hazards

This assay involves human serum samples. Follow universal precautions. Analysts working directly with the specimens must use proper technique and avoid any direct contact with the sample. Wear a lab coat, gloves, and protective eyewear (as required) while handling the specimens, and perform all sample aliquoting in a biological safety cabinet. CDC recommends the Hepatitis B vaccination series for all analysts who work with intact serum samples.

b. Chemical Hazards

Reagents and solvents used in this study include those listed below. Material Safety Data Sheets (MSDS) for these chemicals are readily accessible through the Division of Laboratory Sciences (DLS) local area network (LAN) CD-ROM system; hard copies are filed in the supervisor's office.

If any of these chemicals come in contact with any part of the body, quickly wash the exposed area with copious quantities of water.

(1) **Methylene Chloride**

This solvent is chemically stable and relatively unreactive; it poses a relatively low hazard. It is not flammable, but the vapor can irritate the eyes, nose, throat, and skin. Avoid skin or eye contact with the liquid. If you must evaporate significant volumes of this solvent, use a properly vented Savant[®] evaporator or a chemical fume hood.

(2) **Methanol**

Methanol is a flammable solvent and may form explosive vapors. The vapors irritate the eyes, nose and throat; liquid methanol is poisonous and can be absorbed through the skin.

(3) **Toluene**

Toluene is a flammable liquid that also may form explosive vapors. These vapors are heavier than air and may travel some distance to an ignition source. The vapors can irritate the eyes, nose, and throat; liquid toluene is a skin irritant and can be absorbed through the skin. Handle large volumes of toluene with gloves in a chemical fume hood.

(4) 3,3',5,5'-Tetramethylbenzidine

The substrate reagent used in EIA screening contains this chemical. Tetramethylbenzidine is toxic if inhaled, touched, or swallowed. The skin may become sensitized after contact with the chemicals. If you become ill while working with this chemical, contact a doctor or seek medical advice immediately.

(5) Sulfuric Acid, 2M

This chemical is a corrosive oxidizing acid used as the stopping reagent in EIA screening. Avoid contact with skin.

3. Computerization; Data-System Management

- a. This procedure has been designed to incorporate computerized data handling to the maximum extent possible, both to address the relatively high throughput demands of the assay and to eliminate manual data-entry errors. At no time is it necessary (or desirable) to manually record a sample identification number (ID) or an analytical result. Nevertheless, manual checks and proofreading are essential steps of the protocol.

The entire database for this study consists of several files. The sample cleanup file is a simple, customized database that records sample preparation information such as ID, date of analysis, analyst, sample volume, internal standard and cleanup column information, and special notes for each run. Analytical results are transferred from the instruments as raw data files, and maintained in a Statistical Analysis System (SAS) file that contains the merged data from both the sample cleanup and LC/MS Results files. The TEB Laboratory in the Emergency Response and Air Toxicants Branch maintains these two databases.

- b. The sample cleanup master database is maintained on a network drive. The analyst should generate a printout of each run of samples and manually check it for validity immediately following entry. A simple record-locking procedure restricts any further changes in the cleanup file after the records have been transferred and merged with the analytical results data. The SAS[®] data file (study specific) is the main results file. Both cleanup and data files should be backed up at least weekly. In addition, archive raw instrument data files onto two hard drives and/or CD ROM as described in Section 8 below. Contact the supervisor for emergency assistance with any custom files and databases used in this method; contact the DLS LAN manager for assistance with any DLS network problems.

- c. Documentation for system maintenance and any malfunction reports are kept in the instrument "Sample Log" files maintained on the instrument data stations; a printed version should also be retained.

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

- a. There are no special requirements such as fasting or adherence to special diets for this assay.
- b. The specimen for these analyses is serum (or saliva).
- c. This assay involves both an EIA prescreening analysis that requires 0.1 mL of serum and the LC/MS analysis with nominal requirements of 0.5 mL of serum for samples with "low" cotinine concentrations in the passive exposure range, and 0.1 mL of serum for "high" cotinine samples, generally those from active smokers. Therefore, minimum requirements may vary according to the nature of the individual sample, but in general, a minimum of 1.5 - 2.0 mL of serum is needed for this assay to provide sufficient volume for a repeat analysis if indicated.
- d. Sample processing does not require anticoagulants, special preservatives, or unusual sterility procedures. Collect blood using standard, non-anticoagulated Vacutainer[®] equipment, and freeze the serum in polypropylene cryogenic, screw-cap vials. The laboratory should be contacted before samples are collected to confirm the suitability of any equipment used to collect, process or store samples intended for these analyses. Some materials can provide significant contamination sources; only equipment that has been prescreened and found to be acceptable should be used.
- e. Collection of saliva is most conveniently accomplished using a Salivette[®] or similar commercial device (8). Salivettes may be frozen directly after sample collection for subsequent transfer to the laboratory without any further processing required.
- f. Both the experiences of this laboratory over the past 10 years and earlier literature reports confirm that serum cotinine is stable when the samples are stored frozen at low temperatures (6-8). Furthermore, normal and accelerated stability studies of pure cotinine conducted at CDC indicated that the compound is relatively stable under a variety of conditions.
- g. Specimen handling and transport should be conducted according to standard protocols. Process blood within 30 to 60 minutes after clotting, and ensure that the resulting serum sample remains in the frozen state during shipment and subsequent storage. Serum cotinine samples at CDC are routinely maintained in low-temperature freezers at -70 °C.

- h. Currently, there is no evidence that atypical specimen characteristics such as hemolysis or lipemia influence the LC/MS analysis of serum cotinine. However, record unusual sample characteristics in the sample cleanup file and maintain this information in the database files for tracking purposes.
- i. Although an attempt is made to analyze all samples being submitted whenever possible, a low cotinine sample with a low sample volume (< 0.5 mL) for LC/MS analysis may not generate reportable results.

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

Not applicable for this procedure.

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

Note: Use class-A glassware, such as pipets and volumetric flasks, unless otherwise stated. The accuracy of balances, automated pipets and other measuring equipment should be confirmed and documented as needed; at least annually.

a. Reagents

The following solutions are prepared on an as-needed basis.

(1) 5.0 N Potassium Hydroxide

Fisher P-250, 85-90% reagent, FW = 56.11, stored at room temperature. To prepare 100 mL, you will need 0.5 mols or 28.06 g. Using the percentage purity listed on the bottle, calculate the required weight by dividing 28.06 by the (decimal) purity. For example, if the reagent is 85%, then the required weight of potassium hydroxide (KOH) is $28.06/0.85 = 33.01$ grams. Weigh out the indicated amount of KOH, dissolve in 50-60 mL of high purity (HPLC-grade) water, transfer to a 100-mL volumetric flask, and dilute to volume. Transfer the solution to a labeled 100-mL polypropylene bottle; then include the date and your initials on the bottle.

(2) 13 mM Ammonium Acetate

Aldrich Chemical Co. (Milwaukee WI) Materials Section # 37,233-1 (99.999%), FW = 77.08. Use this reagent with methanol to form the HPLC mobile phase.

Weigh out 1.5 g of the crystalline material and dissolve it in 1,500 mL of HPLC-grade water. Adjust the pH of this solution to 5.0 with glacial acetic acid (typically about 0.75 mL). Store in a labeled, glass-stoppered reagent bottle in the refrigerator.

(3) HPLC Mobile Phase

Methanol/13 mM ammonium acetate, 20:80 by volume. Prepare the HPLC mobile phase daily, or as needed. Using 250-mL and 500-mL graduated cylinders, place 125 mL of HPLC-grade methanol in a clean LC solvent flask, and add 500 mL of the 13-mM ammonium acetate solution. Store residual mobile phase in the LC flask with a Teflon[®] stopper in the refrigerator.

b. Liquid/Liquid-Extraction Columns

Prepare Extube liquid/liquid extraction columns with Chem Elut[®] packing (Varian# 1219-8002, 1-mL capacity) 1 week before using as follows:

0.5 N Potassium Hydroxide: Dilute the 5 N KOH stock solution (described above) 1:10 with HPLC-grade water to make 0.5 N KOH solution.

Set up the Chem Elut columns in groups of 24 in a Varian Cerex[®] Solid Phase Extraction (SPE) Processor rack. Apply 2.0 mL of 0.5 N KOH to each column and allow draining. Let the columns sit for 3 minutes.

Add 4.0 mL of methylene chloride (Burdick & Jackson, "GC²," capillary gas chromatography GC -GC/MS grade) to each column and elute under nitrogen-gas pressure. Repeat with two additional 4.0-mL aliquots of methylene chloride.

Maintain the columns under nitrogen-gas pressure for 15 minutes to facilitate drying.

Place the columns in a 50 °C oven and allow them to dry for 48 hours. If you are not going to use the columns immediately, place them in an airtight container until needed.

Record the preparation date, the column lot numbers, analyst's initials, and the quantity prepared in the Chem Elut column preparation record for future reference.

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c. Standards

Prepare one complete set of cotinine calibration standards at one time for use over a period of several years. Prepare these standards as described in detail below. Analyze the standards for 2 weeks to confirm their suitability, and then flame-seal in ampules (pre-washed with acetone, methanol and water, and then dried) and store them at -20 °C. Prepare the cotinine-D₃ internal standard at the same time or as needed and then aliquot, seal, and store it at 5 °C. A total of 12 standards are prepared, ranging from 0 to 10 ng/mL.

The external standard is 2-hydroxy-6-methyl-pyridine. Prepare it as needed in water.

Cotinine. Aldrich 28,471-8 [486-56-6]. Labeled as (-)-cotinine, 98%, FW = 176.22, BP = 250 deg @ 150 mm, MP = 40-42°, ∇ D₂₀ = -16° (C=1, EtOH)

Cotinine-D₃. Cambridge Isotopes Laboratories DLM-1819

2-Hydroxy-6-Methyl-Pyridine. Aldrich 12,874-0 [3279-76-3], 97%,
FW = 109.13, MP = 158-160 °C.

Water. Tedia WS-221, HPLC grade.

Original Stocks of Native Cotinine:

Solution A. 250.45 mg of (98%) cotinine is dissolved in water; q.s. to 100 mL. Final concentration = 2500 ug/mL.

Solution B. Dilute Solution A. 1:10 with water by taking 10 mL of Solution A and q.s. to 100 mL with water. 250 ug/mL.

Solution C. Dilute Solution B. 1:10 with water by taking 10 mL of Solution B and q.s. to 100 mL with water. 25 ug/mL.

Working Stocks of Native Cotinine:

Solution D. Dilute Solution C 1:10 with water by taking 10 mL of Solution C and q.s. to 100 mL with water. 2.5 µg/mL.

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Solution E. Dilute Solution D 1:10 with water by taking 10 mL of Solution D and q.s. to 100 mL with water. 250 ng/mL.

Solution F. Dilute Solution E 1:10 with water by taking 10 mL of Solution E and q.s. to 100 mL with water. 25 ng/mL.

Original Stocks of D₃-Cotinine:

Solution DA. Approximately 25 mg cotinine-D₃ is dissolved in water; q.s. to 100 mL. Nominal final concentration = 250 µg/mL.

Solution DB. Dilute Solution DA 1:25 with water by taking 4 mL of Solution DA and q.s. to 100 mL. 10 µg/mL.

Solution DC. Dilute Solution DB 1:10 with water by taking 10 mL of Solution DB and q.s. to 100 mL. 1000 ng/mL.

Working Solution of D₃-Cotinine:

Solution DD. dilute Solution DC 1:10 with water, 20 mL of Solution DC, q.s. to 200 mL; 100 ng/mL.

Concentrated Internal Standard Spiking Solution:

Solution DE. dilute Solution DB 1:20 with water, 10 mL of Solution DA, q.s. to 200 mL; 500 ng/mL.

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Table 1. Cotinine Calibration Standards

Ng/100 μ L	ng/mL	Final Volume	Total ng	Stock	mL	ISTD Vol DD	ISTD ng/100 μ L
10.000	100	200	20000	D	8	10	0.5
5.000	50	200	10000	D	4	10	0.5
2.500	25	200	5000	E	20	10	0.5
1.000	10	200	2000	E	8	10	0.5
0.500	5	200	1000	E	4	10	0.5
0.250	2.5	200	500	F	20	10	0.5
0.100	1.0	200	200	F	8	10	0.5
0.050	0.5	200	100	F	4	10	0.5
0.025	0.25	200	50	G	20	10	0.5
0.010	0.10	200	20	G	8	10	0.5
0.005	0.05	200	10	G	4	10	0.5
0.000	0.00	200	0	---	0	10	0.5

All standards are sealed in pre-cleaned ampules (preferably amber-colored ampules). The ampules are stored in labeled boxes in the -20 °C freezer. Calibration standards are prepared with a volume of ca. 3 mL each in 5 mL ampules.

The concentrated ISTD spiking solution is also sealed in pre-cleaned ampules in aliquots of about 1.5 mL per 2 mL ampule. These are then stored in the refrigerator at 4-5 °C. The actual ISTD spiking solution is reconstituted as needed by taking 1 mL of the concentrated ISTD spiking solution (pre-warmed to room temperature) and q.s. to 500 mL with HPLC water.

The External Standard (ESTD) Stock solution is prepared as needed by dissolving 0.05g 2-hydroxy-6-methyl-pyridine in 200 mL HPLC water to prepare a

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concentration of 250 ng/ μ L. The solid may be dissolved with the aid of a sonicator. The External Standard Working solution is prepared by taking 4 μ L of the Stock solution and q.s. to 200 mL with water; final concentration is 5 pg/ μ L (0.5 ng/100 μ L). This ESTD working solution is used as the final diluent to reconstitute the samples prior to analysis by LC/MS.

d. Quality-Control (QC) Materials

Four QC serum pools are used in the NHANES study: pool series #80x000 and series #90x000 and 2 BQx pools. The first 2 are bench QC pools for runs containing samples with low or high concentrations of cotinine, respectively. The latter 2 are the corresponding blind QC pools that are inserted in all of the NHANES runs.

Prepare all QC pools from 2 stock pools of human serum: a low-cotinine stock pool from nonsmokers with minimum exposure to ETS, and a high-cotinine stock pool from users of tobacco. The target cotinine concentrations for the four pools are:

Table 2. QC Pool Target Concentrations

QC Pools	Approximate Target Concentration
Series #80x000 (bench low)	1.5 ng/mL
Blind low	200 pg/mL
Series #90x000 (bench high)	180 ng/mL
Blind high	180 ng/mL

Stock pool concentrations are determined by preliminary analyses. Add a calculated amount of the high concentration stock pool to a large volume of the low concentration stock pool to make the two low QC pools. Make the high QC pools by combining a large amount of the high-concentration stock pool with a small volume of the low concentration stock pool as needed. Stir the resulting pools overnight at 4 °C. The next day, mix the pools at room temperature for about 5 hours, then continuously stir them while dispensing them into 2-mL cryovials. Label the bench QC vials. Do not label the blind QC vials; place them in labeled racks. Use a Micromedic Digiflex[®] pipettor (Titertek, Huntsville, AL) to

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dispense the pools in order from low to high concentration. Be sure to rinse the Digiflex well between pools.

Random samples of each pool are removed for homogeneity testing, and the remaining samples are stored at -70 °C. Label and store the residual bulk volumes of both stock pools at -70 °C.

e. Major Instrumentation and Other Equipment

HPLC. Two Shimadzu modular systems, each containing one SCL-10A system controller, two SC-10AD pumps, one DGU-14A degasser (CDC #132523 and 132460)

Injector/Autosampler/Column Oven. Two Hewlett Packard 1100 series systems, each containing one variable volume injector/autosampler and one column heater. (CDC # 143920 and 132213).

Mass Spectrometer. Two Applied Biosystems/MDS Sciex API 4000 Triple Quadrupole mass spectrometers with heated nebulizer interface, (CDC # 141278 and 141280).

Data System. Applied Biosystems International (ABI) Analyst version 1.3

Gas Generator. (Two) Peak Scientific Instruments Ltd., model NM20ZA, (CDC # 141385 and 149245)

Vacuum Evaporator. (Two) Savant Automatic Environmental SpeedVac Systems AES2010 (CDC # 106160 and 114032).

Orbital Shaker. Two Eberbach model 6010 shakers.

SPE Processor. Four Varian Cerex SPE Processors.

Dispenser. Titertek Digiflex model CX, (CDC # 132351).

Rotary Mixer. Glas-Col Rugged Rotator model 099A 4512 equipped with a test tube rack holder model RD50.

Microtiter Plate Reader. Power Wave X340, (CDC # 132501).

Automatic Plate Washer. Denley Instruments\Wellwash 4 mK2,

7. Calibration and Calibration-Verification Procedures

a. Calibration Curve for LC/MS assay

Base the calibration curve for this assay on the analysis of the standards set described above in Section 6.c. Assay set of 12 standards ranging from 0-25 ng/mL in 2-mL silicized glass vials. Analyze the standards in order from 0-25 ng/mL, then later repeat the analysis in reverse order (from 25-0 ng/mL). Use these data to establish calibration curves as described below in Section 8.d.

b. Calibration Verification

(1) Initial

The accuracy of cotinine measurements based on standards SN was evaluated by analyzing aqueous standards prepared gravimetrically from a primary standard of cotinine perchlorate (purity > 99 mol%) obtained from the National Institute of Standards and Technology (NIST) Gaithersburg, MD, and of NIST RM 8444 (cotinine in freeze-dried urine). In these analyses, the reference samples were processed as unknowns through the entire cleanup and analysis procedures. Those results have been described (7).

(2) Daily

Immediately evaluate the results from the two series of standards assayed each day, and then verify the results before assaying any samples. Process the 24 data files with the Sciex[®] quantitation software as described below in Section 8.c.3. Then run the "HANES Check" program on these data. This program (a) reformats and organizes the integrated area counts for all three ions for each standard; (b) calculates the quantitation ion ratios; (c) back-calculates a cotinine concentration for each standard using a 3-point moving regression as described in section 8.d; (d) determines the mean internal standard area counts; and (e) checks the quantitation ratios measured for the "zero" standard. The results of this evaluation which include a system-generated interpretation of the operational status of the assay, are displayed on-screen and sent to the printer. Acceptable results at this point are as follows:

- (A) Standard calculated value = nominal concentration \pm 10%*
- (B) Mean area counts for m/z 180→80 \geq 182,655
- (C) "Zero" standard ratio < 0.0219

* (30% for standards less than 0.1 ng/mL)

8. Principle Operating Procedures; Calculations; Interpretation of Results

Samples are prescreened using an enzyme immunoassay (EIA) procedure. Classify the sample as being either above or below a nominal cutoff of 20 ng/mL based on the screening results, and then incorporate them into either "low" or "high" runs by following a computerized sorting algorithm. Each run consists of a prepared rack of 25 samples of the same concentration type (either low or high). When the run is prepared, each sample or control is assigned an alphanumeric laboratory ID with a five-character prefix, and a three-digit suffix in the format XXXXX-*nnn*, where XXXXX is the run designation (e.g., AA001) and -*nnn* is the sample's position in the run (e.g., 025). These working ID numbers are linked to the original IDs in the sample database. The first run series in the NHANES cotinine analysis has been designated AA001, with each subsequent run to increment by 1. Each run is set up in the following format: sample #001 = water blank, #002 is the bench QC, and samples #003 - #025 are unknowns (including blind QCs).

a. EIA Prescreening Procedure

The cotinine prescreening assay is a competitive EIA for the qualitative and semiquantitative determination of cotinine in serum, urine, and saliva. This procedure is used solely for pre-classification of samples to enable their assignment to either "high" or "low" runs for subsequent analysis by LC/MS/MS. The assay relies on the competition between free cotinine in the sample and cotinine bound to enzyme for antibody fixed on a polystyrene microtiter plate. After excess enzyme is washed away and substrate added, the amount of free cotinine present is inversely proportional to the amount of free cotinine in the biological sample.

The EIA performed in this lab uses a commercially-available cotinine EIA kit (Cozart Bioscience Ltd.) primarily intended to confirm active smoking rather than passive exposure to ETS. Thus, to use the test as a screening tool for subsequent mass spectrometry, we established the following conditions for analysis:

- (I) The sample size is 10 μ L;
- (II) Standards are set at 0, 2.5, 5.0, 10, 25 and 50 ng/mL. The zero and high three standards are provided as part of the kit; the remaining two standards are prepared by 1:2 and 1:4 dilution of the 10 ng/mL standard.

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- (III) All specimens are assayed undiluted, in duplicate.
- (IV) The plate is washed four times with a pause/soak of approximately 30 seconds between each wash.

(1) EIA Materials

Cozart serum cotinine Microplate EIA kits, 480-test size. The kit contains the following reagents in quantities adequate for five plates: (Note: The manufacturer has prepared all necessary reagents in working form, so there is no need to modify the assay protocol for reagents.)

- (i) Anticotine-coated microtiter plates: antibody immobilized on polystyrene plates, supplied in dry form. Store at 2–8 °C
- (ii) Enzyme conjugate: buffered protein reagent with stabilizers. Store at 2-8 °C
- (iii) Substrate reagent: one bottle containing a solution of 3,3',5,5' tetramethylbenzidine
- (iv) Stopping reagent: 1 M sulfuric acid (corrosive).
- (v) Wash buffer concentrate (30x). Requires dilution with distilled water before use. Dilute vial contents to 1500 mL with distilled water.
- (vi) Standards in a protein matrix (concentration 0, 10, 25, 50 ng/mL).

(2) EIA QC Pools

Quality control materials are prepared from serum processed from whole blood collected with no anticoagulant. Collect the blood from human donors whose previous smoking/nonsmoking status has been verified. Prepare three pools with different levels of cotinine: a low pool consisting of serum from nonsmokers, a high pool consisting of serum from smokers, and an intermediate pool prepared by blending serum from smokers and nonsmokers to the desired cotinine concentration level. Mix the various pools well and analyze them to confirm the final cotinine concentrations. Dispense aliquots of 0.5 mL into 2-mL cryovials and store them at -70 °C.

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Examples of results from the long-term analysis of one set of QC pools by this screening assay are summarized in the following table:

Table 3. Serum Cotinine EIA QC Pools

Pool	Mean Cotinine (ng/mL)	SD	CV	N
E05	2.5	0.71	28.7	59
E08	8.2	1.39	17.1	59
E09	24.9	5.52	22.1	57

(3) EIA Procedure, Operating Instructions, and Calculations

This is a "batch" method (i.e., all specimens, standards, and QC pools are simultaneously treated to the same processes). During an average day, 78 specimens are analyzed in duplicate, with replicate analyses of three levels of QC and six standards per plate.

(a) Procedure

- (i) Thaw frozen specimens, standards, and QC materials at room temperature. Mix well using a Vortex mixer.
- (ii) Using a Matrix automatic pipettor, add 10 μ L of standard, control, or sample to the wells of the microtiter plate in the layout as shown in Table 4 below. Analyze specimens in duplicate.

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	1	2	3	4	5	6	7	8	9	10	11	12
A	s1	s2	s3	s4	s5	s6	s7	s8	s9	E05	E08	E09
B	s1	s2	s3	s4	s5	s6	s7	s8	s9	E05	E08	E09
C	E10	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10	p11
D	E10	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10	p11
E	p12	p13	p14	p15	p16	p17	p18	p19	p20	p21	p22	p23
F	p12	p13	p14	p15	p16	p17	p18	p19	p20	p21	p22	p23
G	p24	p25	p26	p27	p28	p29	p30	p31	p32	p33	p34	p35
H	p24	p25	p26	p27	p28	p29	p30	p31	p32	p33	p34	p35

s1-s9 = standards 0.0, 2.5, 5.0, 10, 25, and 50 ng/mL, respectively

E05, E08, and E09 are control pools

p1-p39 = unknown study samples

- (iii) Add 100 μ L of enzyme conjugate to all wells, and start the stopwatch when you add the conjugate to the first well.
- (iv) Incubate the plate for 30 minutes at room temperature in the dark, using a box to cover the plate on the bench top.
- (v) Wash the plate four times with wash buffer solution with a pause/soak step of approximately 30 sec between washes, using the microtiter plate washer.
- (vi) Add 100 μ L of substrate to all wells and incubate 30 minutes at room temperature in the dark as described above.
- (vii) Add 100 μ L of stopping reagent to all wells.
- (viii) Using the Bio-Tek Powerwave X340 plate reader, measure the absorbance of the well solution at 450 nm within 30 minutes after stopping the

reaction. Correct to the blank absorption at 605 nm. Consult the Bio-Tek plate reader manual for operating instructions.

- (b) Calculations
Data from the absorbance readings on the Bio-Tek microtiter plate reader are compiled in the attached computer. Determine unknown sample concentrations by using the KC4 Kineticcalc for Windows software and a four-parameter (log/linear) logistics fit standard curve. Refer to the *KC4® Software Manual* for further information regarding software features and operations.

(4) Remedial Action for EIA Procedure

If the analysis is declared out of control, take the following remedial action(s):

- (a) Recheck the kit expiration date; use kits which are within-date only.
- (b) Prepare fresh standards, controls, and any sample dilutions for each run and repeat the analysis.
- (c) Verify that the plate washer is working properly. Uneven or incomplete washing will lead to false results. Make sure that all areas of the plate receive an equal number of washes. Using a different numbers of washes may give different OD readings.
- (d) Check the tips on the multichannel pipet. Make sure each tip is dispensing the same volume of reagent.
- (e) Ensure that all reagents are at room temperature before use; cold reagents will give false results.
- (f) The most crucial step in EIA is color development. If the color development is inadequate (if the reaction is stopped too soon), the curve will be flat--differences between concentrations will be small, and controls will vary greatly, especially at the low end. If the color is too dark (if the reaction was stopped too late), the curve will be steep and the accuracy at the high end may be lost. Improper color development is the most frequent cause of out-of-control results. Color development should be stopped in a manner assuring that curves are shaped so that the absorbance of the lowest standard when read at 450nm (before the addition of acid) is approximately 1.0 OD. This value assures a standard curve with good differentiation along all points of the curve.

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Only use this assay to characterize samples into high and low concentration runs for LC/MS analysis. Analytical results from these assays are NOT reported by the laboratory.

After EIA screening analysis, re-rack the specimens for LC/MS/MS analysis according to their concentration (i.e., those <20 ng/mL in low runs and those ≥ 20 ng/mL in high runs) by using the "Buildrun" program. Provide the lab supervisor with run sheets for the new runs. Store specimens at -70 °C until analysis.

b. LC/MS/MS Sample Preparation

- (1) Remove the designated rack of samples from the freezer and allow thawing at room temperature. Generally the rack will contain 2 runs of 25 samples. The samples may be placed in a few inches of cool water in the sink to facilitate thawing. During the week, the next day's samples are generally placed in the refrigerator the night before to thaw.
- (2) Check each sample cap to be sure it is sealed. Place the entire rack on the Glas-Col rotary mixer for 15 minutes.
- (3) Enter the sample information for the run into the Cotinine Sample Prep Program. Start the program (double click on the Cotinine Prep icon), chose the "S" folder for serum analyses, and then click on "New Samples" sheet to create the sample set. There are ten fields of information that must be entered for each run:

QC Name - choose the appropriate QC (e.g. 801)
Run # -- enter the current designation (e.g. AA001)
Samples -- enter the number of samples, normally = 25
ISTD -- enter the ampule number of the current ISTD
Date -- defaults to the current date; can be changed if necessary
Sample Volume -- set to 0.5 for LOW runs; set to 0.02 for
HIGH runs
Diluent -- will automatically calculate to make 1.0 the total vol.;
can be manually entered if needed
Column - enter clean up date of column batch
Analyst -- initials of analyst(s); 3 characters maximum
Note - enter additional comments here

After filling in the required fields, press “ok” to enter the samples into the Cleanup database. The data can then be individually edited if needed, and notes for particular samples can be entered here. After the data have been entered, prepare and check a printout of the data. Edit any values as necessary.

- (4) Label 2 sets of 13x100 mm silicanized glass culture tubes using pre-printed labels. If the run is a high run, a third set of culture tubes must be labeled. Check the current settings of the M-1000 Microman and P-1000 Pipetman pipets and confirm that they are set to the proper volumes (normally 0.5 mL each). Remove the current ISTD spiking solution from the refrigerator, warm to room temperature, mix well. Take one set of labeled, silicanized culture tubes, add 0.5 mL of the ISTD solution directly to the bottom of each tube in the set using the Gilson M-1000 microman pipet reserved for this purpose.
- (5) Remove the sample rack from the Glas-Col rotary mixer. Check the HANES ID number on the first sample vial with the number listed on the run sheet and confirm that they match. Note any unusual aspects concerning the sample (e.g. lipemic, hemolyzed, etc) and record in the Notes field of the cleanup file. This step is done for each sample prior to removing any serum from the original vial.
- (6) If the run is a high run, the samples must be diluted before sampling using a Digiflex dispenser. The Digiflex is equipped with a 10 mL diluent syringe and a 200 μ L sample syringe. Place the diluent tubing in a flask of freshly poured HPLC water and prime 3 times. Enter the syringe volumes: diluent 2400 μ L, sample 100 μ L. Invert the sample vial once immediately before sampling. Place the dispenser tip into the sample vial and dispense into a labeled, silicanized culture tube. The resulting solution is a 1:25 dilution of the original serum. Wipe the dispenser tip with a Kay-Dry after each sample. Repeat for each sample in the run.

If the run is a low run there is no prior dilution. Invert each sample vial once immediately before sampling.

In both types of runs 0.5 mL sample (low run) or diluted sample (high run) is removed with the P-1000 Pipetman pipet using a fresh tip for each sample and added to the labeled, silicanized culture tube containing ISTD. If there is insufficient sample volume in a low run, remove a smaller aliquot as necessary (e.g. 0.3 mL) and RECORD

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THE ACTUAL VOLUME used in the sample cleanup file. Cap the tube with a 13 mm polyethylene snap cap (Precision Labs). Repeat for each sample in the run.

For tube # 001 add 0.5 mL HPLC grade water to the labeled, silicanized culture tube.

- (7) Place the entire rack of samples on the Eberbach orbital shaker and turn on (low speed). Allow the samples to equilibrate with the internal standard for at least 20 minutes, but no longer than 45 minutes.
- (8) Remove a set of prepared Chem Elut columns from the 50°C oven (allow to cool to room temperature) or airtight container and set them up on a column rack with the second set (third set if it's a high run) of the labeled, silicanized culture tubes placed below so that the eluant can be collected. Remove the samples from the shaker and pour them into their respective Chem Elut columns. Use a finger to keep track of which column is next to receiving sample in order to minimize errors. Allow to remain on the column for 3 minutes.
- (9) Using a Labindustries Repipet, add 4 mL of methylene chloride (Burdick & Jackson, "GC²", capillary GC-GC/MS grade) to each sample culture tube and mix with any residual sample. Pour this mixture into the appropriate Chem Elut column – again use a finger to keep track of which column is next. Allow the eluant to drain through the column by gravity. Add a second 4 mL aliquot of methylene chloride directly to the Chem Elut columns and elute by gravity into the same culture tubes.
- (10) Place the tubes in the Savant vacuum evaporator and take to dryness as follows:

Make sure the samples all have the same volume of methylene chloride (for balance). If necessary, add sufficient GC² methylene chloride to assure balance. Also make certain to place tubes in the Savant rotor at intervals such that rotor balance is maintained. Select Low Drying Rate and Vapornet Cryopumping On. Set drying time of 1 hour 15 min. This should be enough time to take all tubes to dryness. If not, use additional time. Samples should be taken just to dryness.
- (11) Add 200 µL of methylene chloride to each sample using a Hamilton multipipet. Vortex gently to insure that the bottom region of the tube is well rinsed.

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- (12) Carefully decant the contents of the tube into a pre-rinsed (with methylene chloride), dried, labeled autosampler vial. Allow the solvent to evaporate at room temperature under a protective bench-top plexi-glass enclosure (prevents dust particles from contaminating the vials).

Note1: make certain that any anomalies in the cleanup or in the appearance or behavior of the samples are recorded in the samples 'Note' field of the Cleanup database.

Note2: in order to reduce contamination from previous sample tubes or from spills made during the cleanup procedure, the racks that are used to hold sample tubes and vials must be washed on a regular basis. The rack-washing protocol is as follows:

HANES racks that hold the original serum vials and those that hold the final autosampler vials are washed after each use. The wire racks that hold the glass culture tubes and the polypropylene racks that hold the culture tubes and the Chem Elut columns during the extraction step are washed weekly.

All racks are soaked in a solution of 10% bleach for > 1 hour, washed with Liquinox soap, rinsed with water, and air dried.

c. LC/MS/MS Analysis

LC/MS/MS Analysis (Method Parameters)

Hardware Configuration Using Analyst version 1.3 Last updated 10-03

Configuration profile name "LCMS" or "MS"

	<u>LC</u>	<u>Col Heater</u>	<u>AutoInjector</u>
COM	5	4	3
Baud rate	9600	19200	9600
Data Bits	7	8	8
Parity	even	none	none
Stop Bits	1	1	1
Flow Control	None	hardware	hardware

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	Mass Table		
	Q1 Mass	Q3 Mass	Time
Exp – 1	177.2	98	250
SC type – MRM	177.2	80	250
Polarity – Positive	180.2	80	250
Duration – 3.00	110.0*	92*	250*
Delay – 0			

*not used for standards

Gas Settings

CAD – 10	DP - 61	Res Q1 - unit
CUR – 30	EP – 10	Res Q3 - unit
GS1 – 40	CE – 31	Pause between mass range 5.007
NC – 5	CXP - 7	
TEM – 500	IHE - on	

*HPLC***Gradient Table**

<u>Time</u>	<u>Module</u>	<u>Event</u>	<u>Parameter</u>
0.01	Controller	Start	
2.20	Pumps	%B	0.0
2.21	Pumps	%B	100.0
2.5	Pumps	%B	100.0
2.51	Pumps	%B	0.0
3.0	Controller	Stop	

Limits: Minimum Pressure 0
Maximum Pressure 6260

Injector

Injection Volume	10	Wash – enabled
D Speed	200	Wash vial # 91
E Speed	200	Wash rack #1
Needle Level	1.5	

Injection Program (Use Custom Injection Program checked)

1. DRAW def. Amount from sample, def. Speed, def. Offset
2. DRAW def. Amount from air, def. Speed, def. Offset
3. NEEDLE into Vial 81, def. Offset
4. WASH NEEDLE in default wash location, 3 times
5. INJECT

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6. WAIT 2 min
7. VALVE bypass
8. WAIT 0.10 min
9. VALVE mainpass

Column Oven

Left Temp	40
Right Temp	23
Total Tol	3.0
Ac Tol	1.0

Global Settings MS

Set the Detector current to the voltage that gives 250,000 ion counts for the ISTD.

LC/MS/MS Analysis (Weekly Tuning Procedure)

- (1) Prepare the following solutions for PPG tuning:
 - a. PPG Buffer Solution: Dissolve 15.4 mg ammonium acetate in a mixture of 49.9 mL water, 49.9 mL methanol, 0.1 mL formic acid, and 0.1 mL acetonitrile. Date and store at 4 °C. PPG Buffer Solution is good for 1 year from date of preparation.
 - b. Dilute PPG solution (2x10⁻⁶ M): Put 19.6 mL PPG Buffer Solution described above in a vial. Add 0.4 mL Standard PPG 1000/2000 solution and vortex thoroughly. Date and store at 4 °C. Dilute PPG solution is good for 6 months from date of preparation.
- (2) Fill a glass syringe with dilute PPG solution. Place this on the Harvard apparatus syringe pump and set the pump to deliver 10 µL / min.
- (3) Put the IS (IonSpray) probe in the interface for the API 4000 MS. Connect the syringe pumping PPG solution to the APCI Turbo IS probe on the MS. Change the configuration to MS only. Set the instrument to the **API Instrument** project and open the most current tuning file for Q1. Choose the Tune hot button (a capital T at the top) to prepare the

instrument for tuning. Turn on the flow to the syringe pump and start the tuning method by choosing the start hot key.

- (4) Once the instrument has run 10 scans, look to the bottom left to ensure the total ion count was sufficient (around 7×10^7) and stable. Right click on the lower right chromatogram and open that field. A series of 8 scan chromatograms will be seen on the next screen.
- (5) Ensure that the 906 peak has at least 2×10^6 counts for sensitivity. Then check that the highest two molecular weight peaks (2010 and 2242) are tuned on the middle peak and that the middle peak is the highest in the field. The valleys to these also need to be less than half height in order for the software to properly identify the middle peak as a single peak. Also verify that the peaks are not overly jagged in appearance and that the peak shape is consistent in appearance to previous tune records.
- (6) Click the calculate button at the top and on the next screen check that all the peaks are chosen for calibration. Choose calibrate. A screen will appear showing the acceptable range and average for both peak shift and width. Peak width must be between 0.6 to 0.8 with a practical target between 0.65 and 0.75. Peak shift must be less than 0.1 either direction but with a practical target of 0.05 or less. If the shift is off, it is first advisable to either update the calibration if it is fairly close or replace it if it is not – always replace if unsure. Keep rerunning the tunes and repeating this while updating calibration until the shifts fall close to the 0 line. Once the calibration is sufficient, adjust the peak widths by altering the offsets settings. To obtain wider peaks, go lower on the offsets or vice versa for narrower. When all the parameters appear to be in specifications, print out the results of each screen and place the printouts in the **Tuning Log** binder. Repeat the same procedure for Q3.
- (7) After completing the tunes, close the configuration for MS only and remove the IS probe. Clean the interface skimmer plate and the surrounding area to restore full sensitivity. Then change the two frits in front of the heater assembly, wipe off the injector needle with a cotton swab and methanol, clean the IS probe with methanol and put the APCI probe back in, clean out the syringe with methanol, and wash the buffer bottle with soap and water and replace with fresh buffer. Return the configuration to LCMS and the project to the latest current project setting. Run a set of standards up and down (see **Daily Procedure**) to

verify the instrument is running correctly. Print out the chromatograms for the standards on Mondays and store them in the binder for **Standards Weekly Printouts**.

LC/MS/MS Analysis (Daily Assay Procedure)

- (1) Remove cotinine standards (1-12) and ammonium acetate mobile phase from the refrigerator and allow warming up to room temperature.
- (2) Every day except Monday the front end of the mass spec is cleaned as described: Remove the APCI assembly from the front of the MS. Remove the skimmer plate from the orifice area. Clean the plate with soap and water. Dry it thoroughly and then rinse with methanol. Take a low lint paper towel and clean the inside cavity of the APCI with methanol on the towel making sure to wipe the needle off. Do the same with the orifice plate being careful not to allow the small orifice hole to become blocked. Replace the skimmer plate and reassemble the APCI interface making certain the shorter probe is installed in the ceramic area for APCI analysis (the longer probe is for IS only and is used to run tunes).
- (3) Check to make sure the mobile phase and Methanol LC bottles are full enough to complete the runs planned for the day. It takes approximately 80 mL of mobile phase to run 2 sets of standards or 25 samples. After topping off, purge both LC pumps to make certain there are no air bubbles in the lines and to ensure the degasser will have any effect on the pH initially.
- (4) Record the following in the daily sample log:
 - a. GS1 readings both before startup and after startup ????
 - b. 3 pressure gauges and run time clock readings on the Peak gas generator.
 - c. The HPLC pump 1 pressure readings after approximately 10 minutes run time.
 - d. The run numbers of the standards and samples for that day, the instrument analyst, and any cleaning or repair made to the instrument.

Note: This information is also stored in the file “**Sample log**” that is maintained on the instrument computer using MS Word. Hard copies of this file are kept in the maintenance log binder. Any repairs which are made to the instrument are also recorded in the front pages of this binder for quick reference.

- (5) With the line removed from the APCI, start the flow using any sample run method and allow the buffer to go to waste for approximately 10 minutes to allow the ceramic area to warm up and the backpressures to equilibrate. Failure to do so particularly on Instrument #2 will cause liquid to build up in the interface area and cause the peaks to be jagged in appearance. This has occurred only on this particular instrument.
- (6) Prepare a batch file to reflect the standards and samples to be run using the Analyst Software. Naming follows the below convention:

Hanes samples	AAXXX
Standards	SSXXXX or CCXXXX
Saliva	SAXXXX
Non Hanes Samples	BAXXX
Non-sample runs	BBXXX

A typical “**Method file**” will be named CotSpl (81-91) 03-27-03. The numbers in the bracket indicate the wash vials used. The last numbers indicate the date the method file was last edited.

Since each **Method** file contains:

- (a) state file (gas settings, detector settings, ions scanned etc) for the mass spec.
- (b) injection volume, wash injection program language, etc for the injector
- (c) gradient and flow information for the LC
- (d) column oven settings

The method file name should be updated with the current date each time a component within the file is modified.

Each data file saved contains all the information contained in the method file if access to that information is needed. To access this, go to the review peak section, pull up the sample of interest, and choose “I” for information to show the relevant method and run information.

- (7) Connect the LC line to the APCI and run a test standard to check to see that the ISTD area counts are near 250 K counts – with a goal of no greater than 300 K or less than 200 K. Adjustment can be made by raising or lowering the CEM settings under Instrument options – settings. Readings above 300 K do not constitute a run out of limit but should be avoided in order to limit the risk of approaching saturation.
- (8) Once the sensitivity is adjusted, submit the samples from the batch file followed by submitting the Wash/Shutdown batch to condition the column and shut the instrument down after the run. When submitting the samples, run no more than 13 or 14 samples using one method so that the needle wash vials are not used for too many samples which may lead to carry-over. Ten different methods are made using identical parameters except for the location of the wash and needle wash vials, and these are used both for samples and for standards.
- (9) Remove the sample tray and replace the water in the last two rows of wash vials on the right side of the tray using HPLC grade water. Prepare the current set of samples to be run by adding 100 µL of the ESTD working solution to each vial, which is then capped, vortexed and placed in the autosampler rack while carefully monitoring the correct order of the samples and recording any discrepancies in the sample log. Also report any samples which may have any other unusual physical observations.
- (10) Record the LC pump 1 pressure reading on the sample log and start the run. Make certain there is sufficient buffer and methanol to complete the run (75 mL for standards, 80 mL per set of 25 samples, 10mL shutdown) and that the LC is maintaining normal pressures. After the standards have had time to run, process standards run in each direction by adjusting the baselines and saving the results both as Analyst .pst format and as a text file with an .AQ extension (export the file as text, save as SSXXXX.aq, then choose visible tables only and all files.) Check the results using the “Standards Check” program to verify that the standards fall within specifications as outlined in Section 7.2. To do this, open the text heading file located on the desktop in the computer under the File Setup folder and change the date to reflect the correct date. Edit-copy the type and close the file. Open the AQ text file created above and paste the heading to the top and save the file. Move it to the Current folder on the computer desktop. Open the Standards Check program and choose the two AQ files and then choose run program. Put the hard copy of the results in the binder labeled “Standards Checks Printouts”.

- (11) After the sample run is complete, process the data using the software for the instrument (**Analyst**). After saving the results, make a separate text AQ file as with the standards. When copying the heading on the AQ file however, the -999 needs to be replaced with the average counts from the standards that day for the ISTD peak. Also, if more than one run is completed, the A at the top right of the heading needs to be made a B for the second run, C for the third etc. The results need to be printed out using the "Multi Quant" program and clicking on the print button at the bottom to print out the results table. Also, all the chromatograms for the samples need to be printed out. All the printouts and computer files are given to the data analyst for the group for further processing.
- (12) After running for around a quarter, a new project must be made in order to remain fewer than 650 MB file size. This is so that a backup copy can be made of the project onto CD while small enough to fit onto one CD. Two backup CD's are to be made – one stored with the analyst and one to go to the lab supervisor for safe keeping.

d. Data Processing

- (1) Collect data files for cotinine standards using the name "SN000X," where "000X" = 0001-9999. Collect NHANES serum cotinine data files using the name "AA00X," where "00X" = 001-999. Cotinine data on saliva samples are named "SA00X," where "00X" = 001-999. "NON00X"(00X=001-999) are runs for method evaluation, diagnostic runs, validation runs, or other purposes as designated in the "Sample Log." Special studies (non-NHANES) may be assigned names containing two letters and three numbers, such as "BA00X," where "00X" = 001-999. Other examples can be "CA00X," "DA00X," "EA00X," etc.
- (2) Samples are processed using Analyst and methods labeled either "Standards" (using D₃ cotinine as the internal standard, with no external standard), or "Samples" (D₃ cotinine as the internal standard and 2-Hydroxy-6-Methyl-Pyridine as the external standard).
- (3) After each batch of standards or samples has been analyzed, the data are processed using Analyst. An analysis file is created by exporting a copy of the data as a file with the same name as the data result file, but with an ".AQ" extension. A copy of the analysis file is provided to the data analyst for further processing.
- (4) After the standards have been analyzed and automatically integrated, visually check all of the peak shapes and retention times and correct any baselines as necessary. Verify that the standard runs for the day fall within QC guidelines by using the AQ file data and the "Standards Check" program. Print out a copy of the standards chromatograms on the first day of each week and store in the "Weekly Standards Printout" notebook.

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Retain a copy of the printed output from the Standards Check program and file in the corresponding notebook.

- (5) For each set of samples, again review the initial integration data and adjust any baselines as necessary. After creating the result set and AQ file, a preliminary assessment of the cotinine data is made by using the "MultiQuant" program. This program calculates an initial cotinine result for each sample in the file. A hardcopy of those results and of the chromatograms is printed.

From these results, the values for the bench QC and the blanks can be immediately evaluated for run integrity. A copy of the AQ file is then provided to the data analyst for review. In that process, the integrity of the data files is checked, and the MS data is then merged on a sample by sample basis with the information in the sample cleanup file; the resulting merged data are appended to a SAS database for further analysis. These data are then recalculated for cotinine levels, and the blanks, bench QC and blind QC are evaluated according to the criteria defined below. Standards are processed in a similar manner.

- (6) Every three months, two complete backup CDs of the Project are made which will record all of the raw data, results sets, and all relevant instrument parameters used for each sample during that quarter. The CDs also contain a backup of all AQ files and the electronic sample log for that period of time. One copy is retained by the analyst and the other copy is provided to the laboratory supervisor for storage. After these backups have been made and confirmed, project information can be deleted from the Data System hard drive to clear up space.

e. Calculations

The calibration of this analysis is not completely linear over the > 3 orders covered by the standard curve. Therefore, calculate the results from the quantitation ratio (m/z 177 \rightarrow 80 / m/z 180 \rightarrow 80) of the sample compared to the standard curve by using a 3-point moving regression. The same basic algorithm for this calculation has been incorporated into "Standards Check," "MultiQuant," and the SAS[®] programs used for final data processing. In each case, compare the sample ratio with the individual standard ratios. Then use the standard concentration whose mean ratio has the minimum difference from the sample ratio is taken as the midpoint of a 3-point curve. The regression parameters derived from that sub-region of the standard curve are then used to calculate the result.

9. Reportable Range of Results

This analysis may be applied to specimens from both smokers and nonsmokers; consequently, a rather broad range of serum cotinine values may be encountered, from less than 0.1 ng/mL to greater than 1,000 ng/mL. To accommodate this range of values, these assays are conducted using prescreened samples that have been divided into two groups based on the preliminary EIA results, with a nominal cutoff of 20 ng/mL to define the threshold for high concentration. Dilute and reanalyze any sample from a low run with a nominal cotinine concentration greater than 20 ng/mL—or from a high run with a calculated concentration greater than 500 ng/mL. In addition, any

sample classified as high with an LC/MS value less than 15 ng/mL should be reanalyzed without dilution in a subsequent run.

10. Quality Control (QC) Procedures

Four human serum pools are used in this study. Their preparation was described previously in Section 6.c. In addition, one water blank is included in each analytical run of 25 samples. Immediately review all cotinine data being processed by using the “MultiQuant” program. Obvious problems and/or invalid QC results are used to identify potential repeat runs at this point. and repeat any runs with obvious problems or invalid QC results. In addition, prior to releasing a set of data, all samples are subjected to a final evaluation according to the following criteria:

- a. Confirm all QC results (blanks, bench, and blind QCs) once more for the mean and range values using the current DLS QC rules based on the division SAS[®] QC program. These serum cotinine runs contain one sample each of the bench and blind QC pool, so for this method follow the DLS rules specified for 2 QC pools per run with 1 QC result per pool.

For the run to be accepted it must meet the following criteria based on the characterization data. The characterization data are developed initially from at least 20 analyses of the pool over a period of at least two weeks. These data may be updated periodically based on additional runs as indicated:

Blanks – Reject if any blank > 0.05 ng/mL.

If both QC run results are within 2 S_i limits, then accept the run (note: S_i = standard deviation of individual results)

If one of the two QC results is outside of a $2 S_i$ limit then reject the run if:

Extreme Outlier Rule – Run result is beyond the characterization
mean $\pm 4 * S_i$

1 3S Rule - Any run result is outside of a $3 S_i$ limit

2 2S Rule - Both run results are outside of the same $2 S_i$ limit

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

R 4S Rule – Two consecutive standardized run results differ by more than $4 * S_i$

- b. Check all relative retention times. If the retention time difference between the quantitation and ISTD ions is more than 3 seconds, then inspect the chromatogram carefully for any possible interferences. If the identity of the peak cannot be confirmed, then the sample is marked as invalid.
- c. Calculate the confirmation ratio by dividing the confirmation ion area (m/z 177→98) by the quantitation ion (m/z 177→80). The confirmation ion ratio range is determined from the mean of both bench QC and unknown samples over an extended period of time. Because of low ion counts for the confirmation ion, these evaluations are limited to samples with a calculated cotinine concentration of ≥ 0.5 ng/mL. Select those samples for further evaluation that have a cotinine concentration of > 0.5 ng/mL and a confirmation ratio greater than 3-sigma from the mean.
- d. Check concentrations to make certain that the values are within the linear range of the method; in general, that means that the actual measured value (prior to correction for dilution) must be no greater than 10 ng/mL. Select samples with (uncorrected) cotinine values greater than 10 ng/mL for repeat analysis at a greater dilution.
- e. Estimate the mean work-up recovery (W_Recov) of each sample is estimated from the raw ion counts observed for the ISTD relative to the mean observed for all of the standards (generally $n=24$) assayed that day. Reanalyze any sample with an estimated W_Recov of less than 20% if sufficient residual sample is available. However, low W_Recov alone is not grounds for rejecting a sample.
- f. Estimate the mean instrumental recovery (I_Recov) of each sample from the raw ion counts observed for the ESTD relative to the mean ISTD observed for all of the standards (generally $n = 24$) assayed that day. Reinject any sample with an estimated I_Recov of less than 20% if sufficient residual sample is available. However, low I_Recov alone is not grounds for rejecting a sample.

- g. Print out all nonempty note fields in the raw data file and examine them for limiting and excluding factors affecting individual samples. Also, examine the hard copies once more for normal ion chromatograms and for other indications of possible problems. Based on the above criteria, generate for all samples a set of samples containing the (lab) ID numbers and raw cotinine concentrations in ng/mL

Adjust these values in a two-step process. First, sort the data by concentration. Generate a “check value” for each sample by multiplying the cotinine concentration times the sample volume in mL taken for the assay. Then correct the samples by subtracting the mean blank value from each result (both the corrected and check values). Change to -999 those samples with a check value less than the limit of detection (LOD) (defined as $3 * \text{standard deviation [SD]}$ of the blanks) that were based on a volume of 1 mL. Change to -997 QNS those values with a check value less than the LOD that were based on sample volumes less than 1 mL. In addition, mark samples with unconfirmed cotinine values as -998 at this time. The latter would include, for example, samples with deviant confirmation ratios that you could not confirm on repeat analysis.

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

a. Calibration

Assess system calibration and general readiness on a daily basis by reviewing instrument operating conditions (temperature, pressure, etc.) and by reviewing the results of the "NHANES Check" evaluation of the first two sets of standards. In the latter case, make note of potential problems, such as low sensitivity as indicated by the mean ISTD ion counts, or high background levels of cotinine in the blank. Take corrective actions, if necessary, re-evaluating the system with standards as before and running the “NHANES Check” again until you obtain acceptable results.

b. Quality Control

If the run is judged to be outside of control limits based on the evaluation described above in Section 10 a., then take the following steps:

- (1) Flag the run in the processing file.
- (2) Make an attempt to identify reasons for the apparent problem and record them. If indicated, halt further sample processing until you can correct the problem.
- (3) Repeat the run at some later date. Note that this is not always possible with low sample runs because of limited residual volumes. Repeating high runs is seldom a problem. If the sample cannot be repeated due to insufficient residual sample, then the result is recorded as invalid with the appropriate code.

12. Limitations of Method; Interfering Substances and Conditions

In some studies, other nicotine metabolites (e.g., trans-hydroxycotinine) and physiological substances (e.g., caffeine) have interfered with immunoassay or chromatographic assays of cotinine. However, no known interferents have yet been reported for this mass spectrometric method. Two physiological substances with MW = 176 that might be encountered in blood are ascorbic acid and serotonin. No interference in the analysis of cotinine was noted when standards of these substances were analyzed according to our usual procedures. However, the pharmaceutical agent, pemoline, may interfere with cotinine analysis. The presence of an interfering substance in a particular sample should be indicated by a deviation in the expected confirmation ratio of m/z 177→98/ m/z 177→80 for that sample.

13. Reference Ranges (Normal Values)

Since the population includes both smokers and nonsmokers, the range of cotinine values is quite broad. The distribution of serum cotinine in the U.S. population for the period 1988 – 1991 was established from the analyses conducted as part of NHANES III, Phase 1. Those results have been published (12). Subsequent evaluations have indicated a decline in the median level of serum cotinine among nonsmokers (CDC, Second National Report).

To distinguish smokers from nonsmokers, Jarvis et al. (9) estimated a cutoff value of 13.7 ng/mL for plasma cotinine levels as measured by gas chromatography, and Benowitz (2) has suggested a cutoff of approximately 10 ng/mL. Based on NHANES evaluations, the nadir between smokers and nonsmokers is about 10-15 ng/mL (12), and either of those values might be used a suitable cutoff based on serum cotinine measurements.

14. Critical-Call Results (“Panic Values”)

Not applicable for this procedure.

15. Specimen Storage and Handling During Testing

Store samples frozen at -70 °C until you analyze them. Remove the rack of frozen samples from the freezer and allow them to thaw overnight in a refrigerator. Bring the samples to room temperature on the morning of the analysis, and mix the samples

continuously on a rotary mixer for 15 minutes before sampling. Handle all intact serum samples in a biological safety cabinet. Replace the residual samples in the racks, and re-freeze them.

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

Generally, if a problem exists with the method, store samples in the freezer until you can resolve the problem. If necessary, extracted samples that you are ready to analyze can be stored at -70°C for at least 1 week before assaying them. In principle, these samples could also be analyzed by capillary gas chromatography/mass spectrometry; however, samples with relatively low cotinine levels (approximately $\leq 2\text{-}5\text{ ng/mL}$) require the use of high-resolution mass spectrometers to attain sufficient sensitivity for the assay, and the daily throughput of such GC/MS assays would be significantly lower than that achieved with this method.

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

Analytical results are reported as ng cotinine/mL serum for each sample. The assigned analyst and the supervisor cross-check all data for accuracy. Final results that meet all QC/QA criteria are then reviewed by a DLS statistician, and formally released by the Director of DLS to the indicated recipient. Data that have successfully completed all review and validation processes may also be provided in electronic file format. Critical-call reporting is not applicable for this method.

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

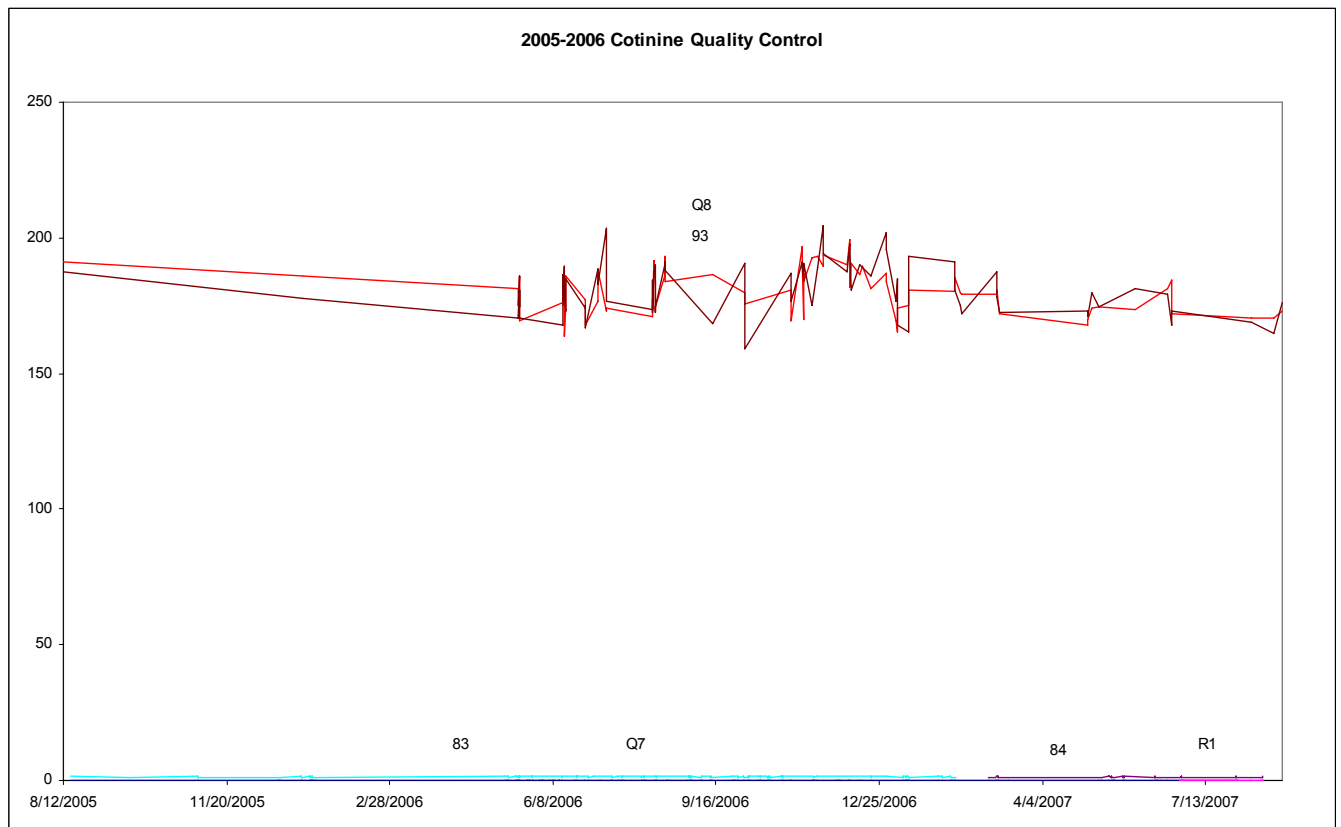
Maintain residual samples from these assays in the same freezer that is used to hold samples waiting to be processed. Periodically, transfer racks of completed samples to dense-pack boxes that are labeled for the contents and transferred for archive storage in a -70°C freezer. A sample tracking system documents the status and location of completed samples in the "NHANESARK" database, which is maintained on the laboratory network PC. This file contains the laboratory ID, NHANES ID NUMBER, current freezer location, and date of archiving (if applicable) of each sample that has been transferred to the Cotinine LC/MS laboratory for analysis. If required, the supervisor or the DLS Study Specimen Coordinator will transfer specimens according to CDC/DLS protocol.

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19. Summary Statistics and QC Graphs

Summary Statistics for Cotinine by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Q8	82	8/12/2005	8/29/2007	179.8524	7.9712	4.4
93	82	8/12/2005	8/29/2007	181.2118	9.6311	5.3
Q7	349	8/16/2005	8/2/2007	0.1971	0.0137	6.9
83	294	8/16/2005	2/9/2007	1.3348	0.0568	4.3
84	80	3/2/2007	8/17/2007	1.2530	0.0450	3.6
R1	25	6/27/2007	8/17/2007	0.1212	0.0090	7.4



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