Laboratory Procedure Manual

Analyte:	**Cotinine**
Matrix:	**Serum**
Method:	**ID HPLC-APCI MS/MS**
Method No.:	
Revised:	
as performed by:	*Organic Analytical Toxicants Branch* *Division of Laboratory Sciences* *National Center for Environmental Health, CDC*
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Important Information for Users

The Centers for Disease Control and Prevention (CDC) periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

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

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
I06_b	LBXCOT	Cotinine (ng/mL)
	LBDCOTSI	Cotinine (nmol/L)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

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.

Clinical Relevance. Cotinine is a major metabolite of nicotine that may be used as a marker for both active smoking, and as an index to Environmental Tobacco Smoke (ETS) exposure, or "passive smoking". Cotinine is generally preferred over nicotine for such assessments because of its substantially longer half-life. The half-life of cotinine in plasma has been estimated to be about 15–20 hrs (1-3); by contrast, the half-life of nicotine is only 0.5–3 hrs (3-5). Cotinine may 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 higher (3–8×) in urine than in serum; however, for studies requiring a quantitative assessment of exposure, plasma or serum is regarded as the fluid of choice (6). Therefore, serum was chosen for NHANES cotinine analyses.

Assay Principle. Serum cotinine is measured by an isotope dilution - high performance liquid chromatography / atmospheric pressure chemical ionization tandem mass spectrometry (ID HPLC-APCI MS/MS). Briefly, the serum sample is spiked with methyl-D3 cotinine as an internal standard, and following an equilibration period, the sample is applied to a basified solid phase extraction column. Cotinine is extracted off the column with 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, along with additional ions for the internal standard, external standard, and for confirmation. Cotinine concentrations are derived from the ratio of native to labeled cotinine in the sample by comparisons to a standard curve.

Special precautions. Because of the nature of these analyses, all analysts in this study must be non-smokers.

NOTE: this same method may be used without change for salivary cotinine assays by substituting salivary cotinine QC pools. All other aspects are identical to serum analyses.

2. SAFETY PRECAUTIONS

Eye protection and suitable protective clothing must be worn during the extraction and processing of samples by this method.

A. Biological hazards. This assay involves human serum samples. Universal Precautions must be followed. Analysts working directly with the specimens must use proper technique and avoid any direct contact with the sample. Lab coats, gloves, and protective eyewear (as required) should be worn while handling the specimens, and all sample aliquoting must be performed in a biological safety cabinet. The Hepatitis B vaccination series is recommended for all analysts working with intact serum samples.

B. Chemical hazards. Reagents and solvents are used in this study include those listed below. MSDSs for these chemicals are readily accessible through the Division LAN CD-ROM system; hardcopies are filed in the supervisor's office.

(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 be irritating to the eyes, nose and throat, and skin or eye contact with the liquid should be avoided. Flush copiously with water if any contact should occur. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator, or in a chemical fume hood.

(2) Methanol - this is a flammable solvent, and may form explosive vapors. The vapor may be irritating to the eyes, nose and throat; liquid methanol is poisonous and may be absorbed through the skin. Any exposed skin areas should be immediately flushed with water.

(3) Toluene - this is a flammable liquid, and also may form explosive vapors. Remember that the vapor is heavier than air and may travel some distance to an ignition source. Toluene forms an irritating vapor. As a liquid it is a skin irritant, and may be absorbed through the skin. Large volumes of toluene should be handled with gloves in a chemical fume hood.

(4) 3,3',5,5'-Tetramethylbenzidine - The substrate reagent used in the EIA screening contains this chemical. It is toxic if inhaled, touched or swallowed. Sensitization of the skin may occur after contact. Wash the contacted area well with water. If feeling unwell, contact a doctor or seek medical advice.

(5) Sulfuric acid, 2 M - a corrosive oxidizing acid used as the stopping reagent in the EIA screening. Avoid contact with skin. Wash areas of contact with water.

NOTICE: POTENTIAL EXPLOSION HAZARD IN THIS ASSAY

C. Special hazard. Particular care must be exercised when a high percentage of methanol is used as the mobile phase during analyses involving the API III heated nebulizer. It is possible for explosive mixtures of methanol vapor and air to accumulate in the ionization chamber, and if arcing should occur, this mixture can ignite. This has already happened on several occasions during these analyses. Although inlet source modifications by the manufacturer have been installed on this instrument that should significantly reduce the possibility of an explosion, the following precautions must be observed:

(1) Never change between heated nebulizer and IonSpray state files while the electronics are on and mobile phase is flowing, or within 1–2 minutes after flow has been stopped (danger of arcing).

(2) Be very careful when opening and closing the orifice shutter. If the plate passes close to the corona discharge needle, arcing may occur. Electronics-1 should be OFF when the shutter is opened or closed.

(3) Make certain that the sample pump is on whenever the system is in operation and solvent is flowing.

(4) Be careful at all times when working in the region between the heated nebulizer inlet and the HPLC. If an explosion should occur, the entire heated nebulizer inlet could be ejected with sufficient force to cause a potentially serious injury if it were to strike a person.

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 ID number or an analytical result. Nevertheless, manual checks and proofreading of information are essential parts of the protocol.

The entire database for this study consists of several files. The NHANES database is an RBase file that contains the demographic information, the link between the HANES sample ID (a seven-digit number) and the Cotinine LC/MS Laboratory working ID which is a seven character alphanumeric label, and the final, validated cotinine result. This master file is maintained by the HANES Laboratory in the Nutritional Biochemistry Branch. The sample Cleanup file is a simple custom database that records sample preparation information for each run such as ID, date of analysis, analyst, sample volume, special notes, etc. Finally, analytical results are maintained in a SAS data file that contains the merged data from both the sample cleanup and LC/MS results files. The latter two databases are maintained by the Cotinine LC/MS Laboratory in the Air Toxicants Branch.

B. The sample Cleanup file is maintained on an O:\APPS directory on a Network drive. This file is automatically copied by a DOS batch file to an L:\LINK directory on a separate Network drive each time the processing program is run (generally daily). A printout of each run of samples is made and

manually checked for validity immediately following entry. A simple record locking procedure is used to restrict any further changes in the Cleanup files after the records have been transferred and merged with the analytical results data.

The SAS data file (NH4COT.DAT) is the main results file maintained by this laboratory. This file, and associated files maintained for standards results, and for "extra" (non-HANES data) results, are automatically copied using the same DOS batch file as above to an L:\LINK directory on a Network drive, each time the processing program is run. Raw instrument data files are archived onto two hard drives or two Jaz drive cartridges on the Macintosh network as described in Section 8 below. The supervisor should be contacted for emergency assistance with these files or with the Macintosh LocalTalk network; the EHLS LAN manager should be contacted for assistance with any EHLS network problems.

C. Documentation for system maintenance, and any malfunction reports, are contained in the "Sample Log" files maintained on the Macintosh, and in printed form in the associated notebook; in the processing documentation file ; and in the electronic system log files maintained by the archiving software.

Both commercial and custom programs are used in support of this method. All serum cotinine custom programs are written in either Delphi Version 2.0 (Windows), or in Think Pascal 4.0 (Macintosh). Commented source code for these programs is maintained on the appropriate system (PC or Mac) in room 2424, and in printed form in the "HANES Cotinine Programs" folder. All source files contain revision history headers. SAS processing files are maintained on a laboratory PC.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; 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.

C. This assay involves both an EIA pre-screening analysis that requires 0.2 mL of serum, and the LC/MS analysis with nominal requirements of 1.0 mL of serum for samples with "low" cotinine concentrations in the passive exposure range, and 0.05 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, 1.5–2 mL of serum is needed for this assay. The optimum sample size is 3 mL or greater to provide sufficient volume for a repeat analysis if indicated.

D. No anticoagulants, special preservatives, or unusual sterility procedures are required for sample processing. For NHANES samples, blood is collected using standard Vacutainer equipment, and the serum is stored in plastic cryogenic, screw-cap vials.

E. Both the experience of this laboratory over the past 8 years, and literature reports confirm that serum cotinine is stable when the samples are stored frozen at low temperatures (6), and both normal and accelerated stability studies of pure cotinine conducted at CDC indicated that the compound is relatively stable under a variety of conditions.

F. Specimen handling and transport is handled by the HANES program according to standard protocols. In general, blood should be processed as soon as possible after clotting, and the sample maintained in the frozen state during shipment and subsequent storage. The HANES serum cotinine samples at CDC are maintained in low-temperature freezers at –70°C.

G. At this time, there is no evidence that atypical specimen characteristics such as hemolysis or lipemia influence the LC/MS analysis of serum cotinine. However, unusual sample characteristics are recorded in the sample Cleanup file, and this information is maintained in the database files for tracking purposes.

H. Although an attempt is made to analyze all samples received whenever possible, a low cotinine sample with a low sample volume (< 0.5 mL) when received for LC/MS analysis may not generate reliable results. However, the results from such samples in the HANES study are maintained in the laboratory database.

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

Not applicable for this procedure.

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

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

A. Preparation of Reagents

Cleanup reagents (#1 and #2) are prepared fresh each Friday for the following week's analyses. The diluent is Burdick & Jackson HPLC grade water. The ammonium acetate stock used to prepare the HPLC mobile phase (#3) is prepared fresh each Monday.

(1) 5 N Potassium hydroxide. Fisher P-250, 85–90% reagent, FW = 56.11; stored at room temperature in an overhead cabinet in bay #3, room 2415. To prepare 100 mL, 0.5 mol or 28.06 g will be needed. Using the percentage purity listed on the bottle, calculate the needed weight by dividing 28.06 by the (decimal) purity. For example, if the reagent is 85%, then the needed weight of KOH is 28.06 / 0.85 = 33.01 grams. Weigh out the indicated amount of KOH, dissolve in 50–60 mL of Burdick & Jackson HPLC grade water, transfer to a 100-mL volumetric flask and q.s. Transfer to a polypropylene bottle, label, date and initial.

(2) 13 mM Ammonium acetate. Aldrich #37,233-1 (99.999%), FW = 77.08. This reagent is used with methanol to form the HPLC mobile phase. A fresh stock of 1500 mL is prepared each Monday. Weigh out 1.5 g of the crystalline material and add it to 1500 mL of Burdick & Jackson HPLC water. Adjust the pH of this solution to 5.0 with JT Baker glacial acetic acid (about 1.5 mL). Store in a labeled, glass stoppered reagent bottle in the refrigerator.

(3) HPLC mobile phase. Methanol / 13 mM ammonium acetate, 29:71 by volume. The HPLC mobile phase is made up daily or as needed. Using 250 mL and 500 mL graduated cylinders, place 204 mL of Burdick & Jackson methanol in a clean LC solvent flask and add 500 mL of the 13 mM ammonium acetate solution. Residual mobile phase is stored in the LC flask with a Teflon stopper in the refrigerator.

B. Liquid/Liquid Extraction Columns

Extube liquid/liquid extraction columns with Chem Elut packing (Varian #1219-8002, 1mL capacity) are generally prepared one week prior to use by the following method:

(1) 0.5 N Potassium hydroxide. Dilute the 5 N KOH stock solution (described above) 1:10 with Burdick & Jackson HPLC grade water to make 0.5 N KOH solution.

(2) Set up the Chem Elut columns in groups of 24 in a Varian Cerex SPE Processor rack. Apply 2 mL of 0.5 N KOH to each column and allow to drain though. Let the columns sit for 3 minutes.

(3) Add 4 mL of methylene chloride (Burdick & Jackson, "GC2", capillary GC-GC/MS grade) to each column and elute under nitrogen gas pressure. Repeat with a second 4-mL aliquot of methylene chloride.

(4) Maintain the columns under nitrogen gas pressure for 15 minutes to facilitate drying.

(5) Place the columns in a 50°C oven and allow to dry for 2 days. If the columns are not going to be used immediately place them in an airtight container until needed.

(6) Record the date of preparation, the lot number of the columns, the initials of the analyst, and the quantity prepared in the Chem Elut column preparation record for future reference.

C. Standards

One complete set of cotinine calibration standards is prepared at one time for use over a period of several years. These standards are prepared as described in detail below. The standards are analyzed over a period of two weeks to confirm their suitability, and then flame-sealed in washed and silanized ampules and stored at –20°C. The cotinine-D3 internal standard is prepared at the same time or as needed and aliquoted, sealed and then stored at 5°C. A total of 14 standards were prepared at first ranging in concentration from 0 to 50 ng/mL. However, at present only the first 12 standards are used, ranging from 0 to 25 ng/mL.

The external standard is 2-hydroxy-6-methyl-pyridine. It is prepared as needed in toluene.

Cotinine. Aldrich 28,471-8 [486-56-6]. Labeled as (-)-cotinine, 98%, FW = 176.22, BP = 250 deg @ 150 mm, MP = 40–42o, * D20 = –16o (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 = 158–160o.
Toluene. Burdick & Jackson #347-4.

Isopropanol. Burdick & Jackson #323-4

Original Stocks: Stock C0: 255.1 mg of (98%) cotinine is dissolved in toluene; q.s. to 100 mL. Final concentration = 2500*g/mL. Stock D0: cotinine-D3 is dissolved in isopropanol (5–10 mL) to make a nominal final concentration of 2500*g/mL.

Working Stocks: Stock A: dilute stock C0 1:100 with toluene, 2 mL of C0 q.s. 200 mL with toluene; 25 *g/mL. Stock B: dilute stock A 1:10 with toluene, 10 mL of A, q.s. to 100 mL with toluene; 2.5 *g/mL. Stock C: dilute stock B 1:10 with toluene, 10 mL of B, q.s. to 100 mL; 0.25 *g/mL. Stock D: dilute stock C 1:10 with toluene, 10 mL of C, q.s. to 100 mL; 0.025*g/mL. Stock DA: dilute stock D0 1:100 with isopropanol, 2 mL of D0, q.s. to 200 mL; 25 *g/mL.

ng/20 L	ng/mL	Final	Total ng Volume	Stock	mL	ISTD Vol DA	ISTD ng/20 µL
50.000	2500	100	250000	A	10	1	5
35.000	1750	100	175000	A	7	1	5
25.000	1250	200	250000	A	10	2	5
17.500	875	200	175000	A	7	2	5
10.000	500	200	100000	A	4	2	5

5.000	250	200	50000	B	20	2	5
2.500	125	200	25000	B	10	2	5
1.000	50	200	10000	B	4	2	5
0.500	25	200	5000	C	20	2	5
0.250	12.5	200	2500	C	10	2	5
0.100	5	200	1000	C	4	2	5
0.050	2.5	200	500	D	20	2	5
0.025	1.25	200	250	D	10	2	5
0.000	0	200	0	–	0	2	5

ISTD Spiking Solution – 20 mL of stock solution DA, q.s. to 1000 mL with HPLC water (final isopropanol concentration = 2%). 500,000 ng in 1000 mL = 500 ng/mL. 10 *L = 5 ng.

All standards are sealed in silanized ampules (preferably amber colored ampules). The ampules are stored in labeled boxes in the walk-in freezer. Calibration standards are prepared with ca. 3 mL in 5 mL ampules. The residual stock solutions are sealed in larger ampules as well and stored at –60°C for use in future standards preparations.

The ISTD spiking solution is also sealed in ampules (the ampules are rinsed with MeOH and then water – not silanized) in aliquots of about 3 mL per 5 mL ampule. These are then stored in the refrigerator at about 5°C.

The External Standard (ESTD) Stock solution is prepared as needed by dissolving 0.05 g of 2-hydroxy-6-methyl-pyridine in 200 mL toluene; 250 ng/*L. (The solid is dissolved with the aid of a sonicator). The External Standard Working solution is prepared by taking 400 *L of the Stock solution and q.s. to 200 mL with toluene; 500 pg/*L (10 ng/20 *L). This ESTD working solution is used as the final diluent prior to injecting the samples into the LC/MS.

D. Controls

There are four QC serum pools that need to be prepared for the HANES study: pools series #80x000, series #90x000, and two BQ pools. The first two are bench QC pools for runs containing low or high cotinine samples, respectively. The latter two are the corresponding blind QC pools that are inserted in all of the HANES runs. All QC serum pools are prepared in the Cotinine LC/MS Laboratory as described below.

All QC pools are prepared from two pools of serum: a low cotinine stock pool and a high cotinine stock pool. The target concentrations for the four pools are:

series #80x000 (Bench Lo)	ca. 2 ng/mL
Blind Lo	ca. 200 pg/mL
Series #90x000 (Bench Hi)	ca. 220 ng/mL
Blind Hi	ca. 179 ng/mL

Based on preliminary analyses of the two stocks, a small amount of the High stock pool is added to a large amount of the Low stock pool to make the two low QC pools. The High-QC pools are the result of combining a large amount of the High stock pool with a small amount of the Low stock pool to attain the appropriate concentrations. The resulting pools are stirred overnight at 4°C in the cold room. The

following day the pools are further mixed at room temperature for about 5 hours, then continuously stirred while being dispensed into 2-mL cryovials The bench QC vials are appropriately labeled but the blind QC vials remain unlabeled and are placed in labeled racks. A Micromedic dispenser is used for dispensing. The pools are dispensed in order from low to high concentration with rinsing of the Micromedic dispenser in between pools.

Random samples from all pools are removed for homogeneity testing, and the remaining samples are stored at –70°C. Residual bulk volumes of both pools are also labeled and stored at –70°C.

E. Major Instrumentation and Other Equipment

HPLC Hewlett-Packard model 1090L, Series II, equipped with the binary DR5 solvent delivery system, variable volume injector, and an autosampler (CDC # 75068).

Mass Spectrometer PE-Sciex API III Triple Quadrupole mass spectrometer with heated nebulizer interface (CDC # 70605).

Data System Consists of three Macintosh computers: IIci, IIfx, and Server G3, interfaced to the mass spectrometer and interconnected via a LocalTalk network (CDC # 70610, 70611, and 106268).

Vacuum Evaporator Savant Automatic Environmental SpeedVac System AES2010 (CDC # 106160).

Orbital Shaker Eberbach shaker (CDC # 16032).

SPE Processor (Two) Varian Cerex SPE Processors.

Microtiter Plate Reader Anthos Labtec Instruments model Anthos htII, (CDC # 94814).

Automated Pipetting Station Hamilton Micro Lab 2200, (CDC # 87052).

Automatic Plate Washer Denley Instruments\Wellwash 4 mK2.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Curve

The calibration curve for this assay is based on the analysis of the standards set described above in Section 6.C. A set of 12 standards contained in silanized 2-mL glass vials and ranging from 0–25 ng/mL are assayed under standard conditions a total of two times with each day's samples. The standards are analyzed in order from 0–25 ng/mL, and then repeated in reverse order (from 25–0 ng/mL). These data are used to establish calibration curves as described below in Section 8.D.

B. Verification

(1) Initial. The accuracy of cotinine measurements based on standards SN was evaluated by the analysis of aqueous standards prepared gravimetrically from a primary standard of cotinine perchlorate (purity > 99 mol%) obtained from the National Institute of Standards and Technology (NIST), 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 procedure.

(2) Daily. The results from the two sets of standards assayed each day are evaluated immediately, and the results are verified before any samples are assayed. The twenty-four data files are processed with the Sciex quantitation software (MacQuan and Method-3) as described below in Section 8.C.(3). The "HANES Check" program is then run on these data. This program (1) reformats and organizes the integrated area counts for all three ions for each standard, (2) calculates the quantitation ion ratios, (3) back-calculates a cotinine concentration for each

standard using a 5-point moving regression as described in Section 8.D., (4) determines the mean internal standard area counts, and (5) checks the quantitation ratios measured for the "zero" standard. The results of this evaluation, including a system-generated interpretation of the operational status of the assay, are displayed on screen, and sent to the printer for archiving. Acceptable results at this point are:

(a) Standard calculated value = nominal concentration ± 10%*
(b) Mean area counts for m/z 180*80 * 182,655
(c) "zero" standard ratio < 0.0219

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

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Samples are pre-screened using an EIA procedure in this laboratory. The samples are classified as being either above or below a nominal cut-off of 25 ng/mL based on the screening results, and then incorporated into either "low" or "high" runs as indicated by following a computerized sorting algorithm. Each run consists of a prepared rack of 50 samples of the same type (either "low" or "high"). At the time 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 position of the sample in the run (e.g. 025). These working ID numbers are linked to the HANES IDs in the NHANES database in the Nutritional Biochemistry Branch. The first run series in the NHANES cotinine analysis has been designated AA001, with each subsequent run incremented by one. Each run is set up in the following format: samples #001 and 050 = water blanks, #002 and 049 are bench QC, and samples #003 - 048 are unknowns (including blind QC).

A. EIA Pre-Screening Procedure

The cotinine pre-screening procedure is a competitive EIA for the qualitative and semi-quantitative determination of cotinine in serum, urine, and saliva. The assay relies on the competition between free cotinine in the sample and cotinine bound to enzyme, for antibody fixed on a polystyrene 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 done in this lab uses a commercially available cotinine EIA kit intended for confirmation of active smoking, rather than passive exposure to ETS (cotinine concentration greater than 20–25 ng/mL). In order to use the EIA test as a screening tool for mass spectrometry confirmation of cotinine concentration, we had to make the following modifications to the original EIA kit instructions:

- The sample size is increased from 10 *L to 50 *L to enhance sensitivity.
- New standards are prepared from National Institutes of Standards and Technology (NIST) primary standard cotinine perchlorate ranging from 0.0 to 50 ng/mL in order to lower the detection range and permit improved quantitation.
- All specimens are assayed undiluted, in duplicate.

(1) EIA Materials

(a) STC Diagnostics cotinine EIA kits, 480-test size. The kit contains the following reagents, in quantities adequate for 5 plates: (NOTE: All necessary reagents are prepared in working form by the manufacturer. No modification is made to the assay protocol for reagents.

(b) Anticotinine coated microtiter plates, antibody immobilized on polystyrene plates, supplied in dry form.

(c) Enzyme conjugate, horseradish peroxidase labeled with cotinine and diluted in a protein matrix with protein stabilizers.

(d) Substrate reagent, one bottle containing a solution of 3,3',5,5' tetramethylbenzidine.

(e) Stopping reagent, one bottle of 2 N sulfuric acid.

(f) Bovine serum albumin (BSA), Sigma Chemical Co. catalog # A-7906. This is diluted with deionized water to make a solution of 8 g/dL BSA which is used as a dilution matrix for standards and elevated-concentration specimens. (Dissolve 80 g of purified BSA with 500 mL deionized water in a 1-L volumetric flask by gently stirring on a magnetic stirrer. Dilute to 1 L volume with deionized water, and mix again to ensure solution homogeneity. Store at 4–8°C for 1 month.)

(2) EIA Standards Preparation

(a) 50,000 ng/mL Cotinine Stock EIA Standard. Dissolve 5 mg cotinine perchlorate in 8 g/dL BSA q.s. to 100 mL

(b) 100 ng/mL Cotinine Spiking Stock EIA Solution. Dilute 1 mL of 50,000 ng/mL cotinine stock EIA standard with 8 g/dL BSA; q.s. to 500 mL.

(c) 0–50 ng/mL Working Cotinine EIA Standards. Using the Micromedic Digiflex dilutor and reagent dispenser, prepare working standards as needed by diluting the 100 ng/mL EIA standard with 8 g/dL BSA according to the scheme in the following table.

Working Standard Concentration (ng/mL)	**Volume 100 ng/mL Standard (mL)**	**Volume 8 g/dL BSA Diluent (mL)**	**Final Volume (mL)**
0.0	0.0	200.0	200
0.6	2.0	198.0	200
1.5	4.0	196.0	200
4.0	10.0	190.0	200
10.0	20.0	180.0	200
13.0	32.0	168.0	200
20.0	48.0	152.0	200
36.0	80.0	120.0	200
42.0	100.0	100.0	200

The resulting nine EIA standard solutions are then placed in 2-mL cryovials in 0.5-mL aliquots and frozen at –70°C.

(3) EIA QC Pools

Quality control materials are prepared from serum processed from whole blood collected with no anticoagulant. The blood is collected from normal humans whose previous smoking/nonsmoking status has been verified. Four pools with different levels of cotinine are prepared: a low pool consisting of serum from nonsmokers, a high pool consisting of serum from smokers, and two intermediate pools prepared by blending serum from smokers and nonsmokers to desired cotinine concentration levels. The various pools are well mixed and analyzed for final cotinine concentrations. Aliquots of 0.5 mL are dispensed in 2-mL cryovials and stored at –70°C.

The system is declared "out-of-control" if a single run mean for one or more pools falls outside the upper or lower 2.57 SD limit.

(4) EIA Procedure, Operating Instructions and Calculations

EIA is a "batch" method (i.e., all specimens, standards, and QC pools are treated to the same processes, such as extraction, simultaneously). During an average day, 105 specimens are analyzed in duplicate, with replicate analyses of four levels of bench QC pools.

(a) Procedure

(i) Thaw specimens and QC materials at room temperature. Mix well using a vortex mixer.

(ii) Using the Hamilton Micro Lab, add 50 *L of standard, control, or sample to the wells of the microtiter plate as shown in the Plate Layout below. Specimens will be analyzed in duplicate.

(iii) Add 100 *L enzyme conjugate to all wells, start the stopwatch when the conjugate is added to the first well.

(iv) Incubate the plate for 35 minutes at room temperature in the dark using a box to cover the plate on the bench top.

(v) Wash the plate six times with deionized water using the microtiter plate washer.

(vi) Add 100 *L of substrate to all wells and incubate 30 minutes at room temperature in the dark as above.

(vii) Add 100 *L stopping reagent to all wells.

(viii) Using the ANTHOS microtiter plate reader, measure the absorbance of the well solution at 450 nm within 30 minutes after the reaction stops. Consult the ANTHOS microtiter plate reader manual for operating instructions.

(b) Calculations

Data from the absorbance readings on the ANTHOS microtiter plate reader are communicated to the computer. Unknown sample concentrations are determined by using the Biolise software and a four-parameter (log/linear) logistics fit standard curve. Refer to the Biolise software manual for further information regarding software features and operations.

(5) Remedial Action for EIA Procedure

If the system should be declared "out of control," take the following remedial action(s):

(a) Check the kit expiration date; only use viable kits.

(b) Make sure standards, controls, and any sample dilutions are fresh. Using old preparations can lead to variable results.

(c) Verify that the plate washer is working properly. Uneven or incomplete washing will lead to false results. Make sure all areas of the plate receive an equal number of washes. Different numbers of washes will give different OD readings.

(d) Check tips on the multichannel pipette. Make sure each tip is dispensing the same volume of reagent.

(e) Ensure that all reagents are at room temperature; cold reagents will give false results.

(f) The most crucial step in EIA is the color development step. 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 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 so that curves are shaped so that the lowest standard when read at 450 nm (before the addition of acid) gives an absorbance of approx. 1.0 OD value. This assures a standard curve with good differentiation along all points of the curve.

Samples are not repeated due to volume restrictions and this assay is only used to characterize samples into high and low runs for LC/MS analysis.

After EIA screening analysis, the specimens are re-racked for LC/MS confirmatory analysis according to their concentration (i.e., those <25 ng/mL in "low" runs, and those *25 ng/mL in "high" runs). Runsheets for the new runs are provided to the LC/MS supervisor, and specimens are stored at –70°C until analysis. After analysis, they are retained at –70°C until all survey data for cotinine are released, in case repeat analyses are required.

B. LC/MS Sample Preparation

(1) Remove the designated rack of samples from the freezer and allow to thaw at room temperature. 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 and allowed to thaw overnight.

(2) Enter the sample information for the run into the Cotinine Sample Prep Program. Start the program (double click on the Cotinine Prep icon), choose 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:

Field	Entry
QC Name:	choose the appropriate QC (e.g. 801)
Run # :	enter the current designation (e.g. AA001)
Samples:	enter the number of samples, normally = 50
ISTD:	enter the ampule number of the current ISTD
Date:	defaults to the current date; can be changed if necessary.
Sample Vol.:	set to 1.0 for LOW runs; set to 0.05 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 appropriate comments here

After filling in the required fields, press "OK" to enter the samples into the Cleanup database. The data can now 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. Change any values as necessary.

(3) Label 3 sets of 13×100 mm silanized culture tubes using pre-printed labels. Check the current settings of the M-25 microman, P-200 pipetman and P-1000 pipetman pipettes and confirm that they are set to the proper volumes (normally 10 *L, 50 *L and 1 mL, respectively). Remove the current ISTD stock solution from the refrigerator, warm to room temperature, mix well. Take one set of labeled, silanized culture tubes, add 10 *L of the ISTD solution directly to the bottom of each tube in the set using the Gilson microman pipette (# A-86-11087) reserved for this purpose.

(4) Check the HANES ID number on the 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. Mix well (vortex) and remove:

LOW runs = 1.00 mL of sample (P-1000 pipette)
HIGH runs = 0.05 mL of sample (P-200 pipette)

using a fresh tip for each sample, and add to the sample tube containing ISTD. If there is insufficient sample volume in a low run, remove a smaller aliquot as necessary (e.g. 0.5 mL) and RECORD 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.

(5) If less than 1 mL of sample was taken for analysis, dilute to volume with Burdick & Jackson HPLC water. For example, in High runs, add 0.95 mL of water to each tube (except for samples #001 and 050, the water blanks). For samples #001 and 050 add 1.0 mL Burdick & Jackson HPLC water to the sample tubes.

(6) 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.

(7) Remove a set of prepared Chem Elut columns from the 50°C oven or airtight container, label the columns and set them up on a column rack with the second set of the labeled, silanized 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. Let sit on column for 3 minutes.

(8) 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. 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 let elute by gravity into the same culture tubes.

(9) Place a set of sodium sulfate columns (1.1–1.2 g EM SC-0760-E Na2SO4 in 10-mL polypropylene columns; packed by Analytichem) in a column rack, and pre-rinse with 2–3 mL of methylene chloride. Place the methylene chloride extract from step #8 above on the washed sodium sulfate column and collect the eluant in the third set of clean, labeled 13×100-mm silanized glass culture tubes. Do not attempt to force out the residual liquid from the column. If any aqueous material is eluted from the column it will generally be visible as white droplets in the methylene chloride. In that case, carefully transfer the methylene chloride layer (avoiding the droplets) to another clean, labeled 13×100-mm tube before drying the sample.

(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, add more time.

(11) Add 200 *L of methylene chloride to each sample using a Hamilton multipipette. Swirl gently to insure that the bottom region of the tube is well rinsed.

(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 Plexiglas enclosure (prevents dust particles from contaminating the vials).

Note: 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.

C. LC/MS/MS Analysis

(1) Remove cotinine standards (1-12) and ammonium acetate mobile phase from the refrigerator and allow to warm up to room temperature. On Mondays, make up 1.5 L of fresh stock as described in Section 6.A.(2).

(2) Record the following:

- gas cylinder pressures
- He pump temperature and pressure range
- Electronics 2 on time (if not on during recycle)

Note: Electronics 2 must warm-up for one hour before MS operation if not left on during recycle. Failure to do so will allow calibration drift during early runs.

(3) N2 off temperature, pressure and time.

Note: the temperatures and pressures of the MS are stored in the "Vacuum log" Excel file on the Macintosh G3 Power PC desktop. All other values are stored in the "Sample log " MacWrite file on the Macintosh G3 Power PC desktop.

(4) Open the curtain gas valve. The Autogate system should automatically open the gate valve and turn on the Auxiliary and Nebulizer gas flows to their preset values. Record the curtain gas flow, the Auxiliary gas flow and the Nebulizer gas flow in the Sample Log.

Optimum settings for serum cotinine:

Curtain gas:	1.2 L/min	Neb flow:	0.6 L/min
Neb pressure:	80 psi	Auxiliary flow:	1.3 L/min

(5) Wait at least 5 minutes after opening the Autogate system to record the MS temperature and pressure ("N2 on") in the Vacuum Log.

(6) Turn on Electronics 1.

"NON00X" is used for all other runs.

Each Batch file contains:

- Method file
- Log of the samples being run
- Output folder/file in which data is collected

A typical "Method file" for cotinine analysis might be named D3 120699 or D3 +OH-Me-Pyr 120699. "D3" designates the # of deuteriums substituted for hydrogens present in the Internal Standard. "120699" designates the date the method file was most recently updated. "OH-Me-Pyr" designates the External standard (if used).

Since each Method file contains:

- state file
- Q1 and Q2 calibration files
- parent / daughter ions being scanned
- Dwell time
- Data acquisition interval (Delay/Acquire)

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

A typical "State File" within the method file for cotinine analysis might be named:

Example: COT-ANIL 12-06-99 .

"COT-ANIL" designates the compounds used to establish the state file parameters. (Cotinine , Acetanilide and Benzanilide). "12-06-99" is the date the state file was established.

An example of a state file:

DI	5.00	L7	0.0
ISV	6000	R2	5.0
IN	650.0	M3	100.0
OR	35.0	RE3	129.3
RO	29.5	DM3	0.11
M1	200.0	RX	–5.0
RE1	116.9	R3	–1.0
DM1	0.12	L9	–250.0
R1	27.0	FP	–250.0
		MU	–4000.0

A typical Q1 calibration file and Q2 calibration file within the method file for cotinine analysis might be named:

Examples: Q1 12/06/99 COT-ANIL CAL
Q3 12/06/99 COT-ANIL CAL

"Q1" and "Q3" designate the quadrupole. "12/06/99" is the date of calibration. "COT-ANIL" designates the compounds used for calibration (Cotinine, Acetanilide and Benzanilide)

(7) Turn on the HPLC, load method 3 and open the nitrogen and helium gas valves. Place the pre-mixed mobile phase in reservoir A. Allow to degas for 10 minutes.

HPLC method #3:

SDS-CONFIG A = 1 , B = 1

Flow = 1.1
%B = 0
MAXPRESS = 400
MIN PRESS = 0
SLOW DOWN = 2
STOP TIME = 2.6
POST TIME = 0
E1 = 0 E2 = 0 E3 = 0 E4 = 0
INJVOLUM = - INJECTOR PROGRAM
SPEED: DRAW 2 ; EJECT 1 ; MIX 2
HOLD: DRAW 0 sec; EJECT 0 sec
1 DRAW 5 *L From Sample 1
2 DRAW 0.1 *L From Air
3 DRAW 0.1 *L From Sample +51
4 INJECT
5 WAIT 2.0
6 Injector Valve to Bypass
7 Wait 0.1 min.
8 Injector valve to Mainpass
END INJ PROG(0.0*L in Syringe)
AT 1.9 %B __0__ Flow____
AT 1.91 %B 100 Flow____
AT 2.1 %B 100 Flow____
AT 2.11 %B__0__Flow____

(8) Switch on the HN temp. Start HPLC flow at 1.1 mL/min. after HN temperature reaches 100°C. Confirm that the HN temp reaches the set point of 525°C.

(9) On Mondays, do Q1 and Q3 scans of the mobile phase from 10 to 220 amu in 0.1 amu steps with a dwell time of 2 ms collecting 10 scans in MCA mode. Do this twice, and make a hardcopy of the second set of scans and retain in the "Buffer Scans" folder.

(10) In RAD, load batch "SN000X" (located in the RAD Files Folder). Change the File Name and the Sample Name to the current run number, close the Batch window and activate the start button on the Control Panel. The Ar will switch on. After about 10 minutes, record the MS temp. and pressure and the initial CGT (collision gas thickness) value in the Vacuum Log.

(11) Set up the run on the HPLC: press the Start button, assign the proper start and stop vial numbers and then press "Enter". Record the HPLC HP and LP (high pressure/ low pressure) values at start up.

(12) Prepare the current set of 12 calibration curve standards and analyze twice – first in the forward direction (standards#1-12), and then in the reverse direction (standards #12-1)

(13) On Mondays, the tune and mass assignments are validated with the anilide solutions and a (5ng/20*L) cotinine standard using flow-injection analysis, and other maintenance analyses may be performed as indicated.

(14) On other days, the first two sets of cotinine standards are processed using MacQuan on the IIfx and the results are screened using the "HANES Check" program on the G3 power PC. If "OK", then the current set of samples is reconstituted by adding 20*L of the ESTD working solution to each vial, which is then capped, vortexed and placed in the autosampler rack. Prepare and load a

new batch file for the current set of samples (normally, a HANES run will consist of 50 samples) and analyze. To avoid loss of data, the samples should be monitored during the automated analysis, and the run should be aborted immediately if a problem should arise. At the completion of a HANES run the External standard recovery should be immediately reviewed in MacQuan and recovery falling below 50% of the mean external standard recovery should be reinjected and overlaid in data file. Record any anomalies in individual sample behavior or handling in a note in the "Sample log" file on the G3 Power PC desk top.

Sciex Shutdown:

(15) Turn off Electronics 1. Change mobile phase to 100% B (MeOH). Remove the buffer reservoir (A) from the LC, stopper and store in refrigerator. Replace reservoir A with !00% MeOH. Switch flow to 100%A (MeOH) at 1.1 mL/min. for 10 minutes to remove all buffer from HPLC column and lines.

(16) Turn off HN temp. When temperature is <100°C, turn off the HPLC pump. When the HN temp has dropped to about 60°C, close the curtain gas valve. The autogate system should automatically turn off the nebulizer flow, auxiliary flow, curtain gas flow and close the gate valve. Manually check the gate valve to assure that it is closed securely. The gate valve may be operated manually, if necessary, by turning the "Autogate Defeat switch located on the front control panel to the "on"(up) position.

(17) Turn off the LC, close the nitrogen and helium gas cylinder valves (second stage only), and close the helium sparge valve located on the back of the LC.

(18) On Mondays-Thursday set the Recycle timer to 10 hrs, on Fridays set it to 60 hrs. Then press the recycle button.

D. Data processing

(1) Data files for cotinine standards are collected using the name "SN000X", where "000X"= #0001-#9999. HANES serum cotinine data files are collected using the name "AA00X", where "00X" = #001-#999. Cotinine data on saliva samples are named "SA00X", where "00X’ = #001-#999. "NON00X"(00X = 001-#999) runs may be used for method evaluation, diagnostic runs, validation runs or other purposes as designated in the sample log. Special studies (non-HANES) may be assigned names containing two letters and three numbers, such as "BA00X, where "00X" = #001-#999. Other examples may be "CA00X", "DA00X", "EA00X", etc...

(2) After each batch of samples has been analyzed, copy the data files from the Macintosh IIci to the desk top file folder on the Macintosh IIfx for processing. Retain a copy on the Macintosh IIci. This copy is transferred to an external Jaz drive disc for permanent storage (copy one). The data file transferred to the Macintosh IIfx is permanently saved on the G3 Power PC internal Jaz drive (copy two) following processing of data.

(3) Processing: Samples are processed using MacQuan and "D3 Method"(D3 cotinine as internal standard, no external standard) or "D3+ EXT Method"(D3 cotinine as the internal standard with 2-OH-6-CH_3-pyridine as the external standard).

The method parameters are as follows:

Noise Threshold	6.0
Quant Threshold	3.0
Minimum Width	2
Multiplet Width	0
Baseline Width	200
R.T. Window	8.0

(4) Process all data files using "D3 Method" or "D3+EXT Method". Visually check all of the integrations and redraw the baselines as necessary.

(5) Prepare an AQ file in TeachText and, using the clipboard, transfer the information (ion, sample ID, time, RT, area, etc) from each ion to the file.

(6) For each sample set, a preliminary assessment of the cotinine data is made using the "MultiQuant" program. This program calculates cotinine values for each sample using the data in the AQ file. A hardcopy of the results is printed out and is kept together with the chromatograms printouts (see below). From these results the values of the Bench QC's and blanks can immediately be determined.

(7) Following integration of samples a hardcopy of each integrated peak is printed. The printout gives a visual representation of the integrated Quantitation ion (177/80), Internal Standard (180/80), Confirmation ion (177/98), and External Standard (110/92) printed on a single page for each individual sample. The first set of standards run each week should also be printed for hardcopy preservation.

(8) Data may be deleted from the computers after being permanently stored on two separate Jaz drive cartridges. There should be at least 2 copies of each HANES data file maintained on separate drives (disks) at all times.

(9) Using a Mac formatted 1.44 Mb floppy disk, transfer a copy of all of the *AQ files from the Mac G-3 Server to the laboratory PC used for SAS data processing. This is accomplished by first copying the AQ files from the Mac G-3 Server to the floppy disk then inserting the floppy disk into drive 'A' of the processing computer, opening the AQ File Process program, then clicking on the "transfer files" button. The files will transfer all the *AQ files from the floppy disk to the "Current" directory on the hard drive.

(10) Using the features in the AQ File Process program, select files from the "Current" directory (Samples or Standards), select the target SAS data file (NH4COT.DAT for samples and NH4STDS.DAT for standards) and process them. As part of this processing the integrity of the data files will be checked, the MS sample data files will be merged on a sample by sample basis with the information in the sample cleanup files, and the resulting data will be appended to the SAS data file for subsequent analysis. Similar processing is performed for standards but without cleanup information merging.

E. Calculations

The calibration of this analysis is not completely linear over the > 3 orders covered by the standard curve. Therefore, the results are calculated from the quantitation ratio (m/z 177*80 / m/z 180*80) of the sample compared to the standard curve by using a 5-point moving regression. The same basic algorithm for this calculation has been incorporated into "HANES Check", "MultiQuant" and the SAS programs used for final data processing. In each case, the sample ratio is compared with the individual standard ratios, and that standard concentration whose mean ratio has the minimum difference from the sample ratio is taken as the midpoint of a 5-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 both smokers and non-smokers, thus a rather large range of serum cotinine values may be expected, from less than 0.1 ng/mL to greater than 1000 ng/mL. These assays are performed on pre-screened samples that have been divided into two groups based on the preliminary EIA results, with a nominal cutoff of 25 ng/mL. Any sample from a "Low" run with a nominal cotinine concentration greater than 25 ng/mL – or from a "High" run with a calculated concentration greater than

500 ng/mL – must be diluted and reanalyzed. In addition, any sample classified as "High" with an LC/MS value less than 25 ng/mL should be reanalyzed without dilution in a subsequent run.

10. QUALITY CONTROL (QC) PROCEDURES

There are four human serum pools that are used in this study. Their preparation was described previously in Section 6.C. In addition, two water blanks are included in each analytical run of 50 samples. All cotinine data are reviewed immediately when processed by using the "MultiQuant" program. Obvious problems and/or invalid QC results are used to identify potential repeat runs at that point. In addition, prior to release of a set of data, all samples are subjected to a final evaluation according to the following criteria:

A. All QC results (Blanks, Bench and Blind QC) are confirmed once more for the mean and range values using EHLSQC (a SAS QC program). For the run to be accepted it must meet the following criteria based on the characterization data – these data may be updated periodically as indicated:

   Blanks – Reject if any blank in the run is >0.5 ng/mL.

   Bench & Blind QC – Reject if any pool is outside of 3-σ limits (mean or range), or if any two pools are outside of 2-σ limits in the same direction.

B. The relative retention time (RRT) is defined as the retention time for the quantitation ion divided by the retention time for the ISTD. A mean RRT is calculated for all of the Bench QC's that are run during a particular study. If the RRT of a sample does not fall within 3-σ of that mean then the chromatogram must be inspected for.

C. Confirmation ratios are calculated from the ratio of the confirmation ion (m/z 177* 98) divided by the quantitation ion (m/z 177*80). Because these values are not fundamental and may be influenced by several factors such as small variations in Q2 fragmentation conditions, they can vary somewhat over time; therefore, the individual values are examined in comparison to the mean obtained for samples in that run. 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. Samples are selected for further evaluation that have a cotinine concentration of >0.5 ng/mL and a confirmation ratio greater than 3-σ from the mean.

D. Concentrations are checked to make certain that the values are within the range of the method; in general, that means the actual measured value (prior to correction for dilution) must be no greater than 25 ng/mL. Samples with (uncorrected) cotinine values greater than 25 ng/mL are selected for repeat analysis at a greater dilution.

E. 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. Any sample with an estimated W_Recov of less than 20% is repeated if sufficient residual sample is available. However, low W_Recov alone is not grounds for rejection of a sample.

F. The mean instrumental recovery (I_Recov) of each sample is estimated 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. Any sample with an estimated I_Recov of less than 25% is reinjected if sufficient residual sample is available. However, low I_Recov alone is not grounds for rejection of a sample.

G. All non-empty note fields in the raw data file are printed out and examined for limiting and excluding factors affecting individual samples. Also, the hardcopies are examined once more for normal ion chromatograms and for other indications of possible problems.

   Based on the above criteria, a set of samples is generated containing the (lab) ID numbers and raw cotinine concentrations in ng/mL, for all samples. This list is edited as necessary to replace either individual values, or entire runs, with repeat analysis values where indicated.

These values are then adjusted in a two step process. First, the data are sorted by concentration and a "check value" is generated for each sample by multiplying the cotinine concentration times the sample volume in mL taken for the assay. Then, the samples are corrected by subtracting the mean blank value from each result (both corrected and check value). Samples with a check value less than the LOD (defined a 3 * SD of the blanks) that were based on a volume of 1 mL are changed to –999 (below LOD), while values with a check value less than the LOD that were based on sample volumes less than 1 mL are changed to –997 (QNS). In addition, samples with unconfirmed cotinine values are marked as –998 at this time. The latter would include, for example, samples with deviant confirmation ratios that could not be confirmed on repeat analysis.

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

A. Calibration. System calibration and general readiness is assessed on a daily basis from a review of the instruments operating conditions (temperature, pressure, etc) and from the results of the "HANES Check" evaluation of the first two sets of standards. In the latter case, potential problems are noted – such as low sensitivity as indicated by the mean ISTD ion counts, or high background levels of cotinine in the blank – by the program and possible corrective actions are suggested. Following corrective actions, the system is re-evaluated with standards as before and HANES Check is run again until acceptable results are obtained.

B. Quality Control. If two or more quality control sample values from either the bench or blind QC samples are outside of 95% limits in the same direction, or if any values are outside of 99% limits, then the following steps are taken:

(1) The run is flagged in the processing file.
(2) An attempt is made to identify reasons for the apparent problem and these are recorded. If indicated, further HANES sample processing is halted until the problem can be corrected.
(3) The run is repeated at some later date. Note that this is NOT always possible with Low sample runs due to limited residual volumes. Repeats of High runs are not a problem in this regard.

In addition to QC evaluations, each individual sample is also checked for the proper relative retention time for the quantitation ion (relative to that for the internal standard), the absence of interfering peaks, adequate recovery, and for the correct confirmation ratio. Samples that appear to be questionable according to any of these criteria are individually repeated.

Any questionable sample identified by either QC or individual sample evaluation that cannot be repeated due to inadequate residual volume is flagged in the database.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Other nicotine metabolites such as trans-hydroxycotinine, and physiological substances such as caffeine have been reported to interfere with immunoassay and/or chromatographic assays of cotinine in some studies. However, no known interferants have yet been reported in mass spectrometric methods. 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 pemoline may interfere if present. 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/ mz 177*80 for that sample.

13. REFERENCE RANGES (NORMAL VALUES)

The NHANES population includes both smokers and non-smokers, thus a rather large range of serum cotinine values may be expected. Several investigators have estimated urinary cotinine cut-off values likely to distinguish between smokers and non-smokers, but relatively few comparisons of blood levels have been reported. Jarvis et al. (7) estimated a cut-off value of 13.7 ng/mL for plasma cotinine levels as measured by gas chromatography, a value approximately 3.6-fold lower than the urine estimate in the same population. Benkirane et al. (8) estimated cut-offs of 17–50 ng/mL by an ELISA method, and 28–70 ng/mL by RIA, whereas Van Vanukis (9) reported a serum cotinine cut-off of 8–20 ng/mL by RIA. In general, serum cotinine concentrations greater than 10–20 ng/mL are probably indicative of a currently active smoker.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable for this procedure.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Samples are stored frozen at –70°C until they are analyzed. The rack of samples is removed from the freezer and allowed to thaw overnight in a refrigerator. The samples are brought to room temperature on the morning of the analysis, and the vials are vortexed briefly immediately prior to sampling. All handling of intact serum samples is carried out in a biological safety cabinet. The residual samples are replaced in the racks and refrozen.

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

Generally, if a problem with the method exists, samples are held in the freezer until it can be resolved. If necessary, extracted samples ready for analysis can be stored at –70°C for at least one week before they are assayed. In principle, these samples could also be analyzed by capillary gas chromatography / mass spectrometry; however, samples with relatively low cotinine levels (ca. *2–5ng/mL) require the use of high-resolution mass spectrometers to attain sufficient sensitivity for the assay, and the daily throughput of 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. Data are cross-checked for accuracy by the assigned analyst and the supervisor. The supervisor releases final, validated results for the NHANES samples which are transferred electronically to the HANES RBase file (maintained by the HANES Laboratory, Nutritional Biochemistry Branch) for release to NCHS. Critical call reporting is not applicable for this method.

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

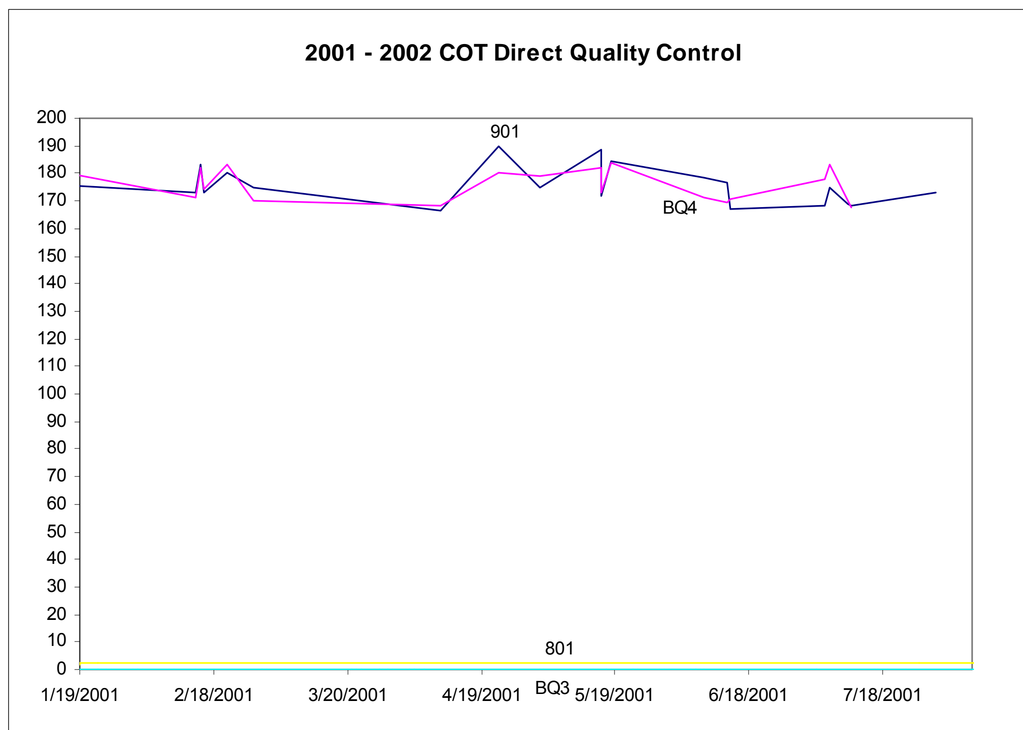
Residual sample from these assays is maintained in the same freezer that is used to hold samples waiting to be processed. Periodically, racks of completed samples are transferred to dense-pack boxes which are labeled with the contents and transferred for archive storage in a –70°C freezer in building 105. A sample tracking system documents the status and location of completed samples in the "HANESARK" database, which is maintained on the laboratory network PC. This file contains the laboratory ID, HANES ID #, current

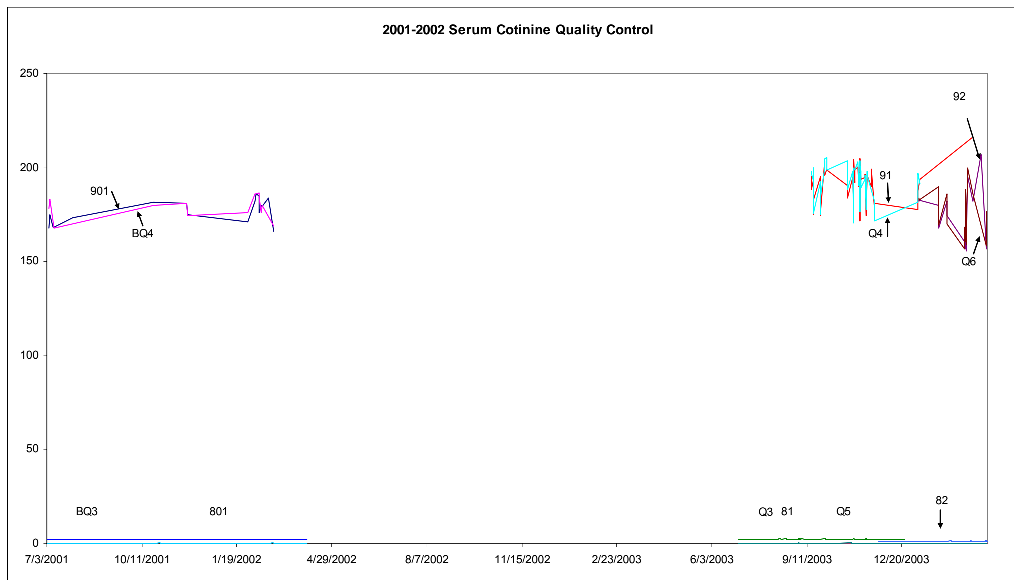
freezer location, and date of archiving (if applicable) of each sample that has been transferred to the Cotinine LC/MS laboratory for analysis.

19. Summary Statistics and QC Graphs

Summary Statistics for Serum Cotinine by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
BQ3	31	7/3/2001	4/3/2002	0.2566	0.0151	5.9
801	35	7/3/2001	4/3/2002	2.2627	0.0780	3.4
901	17	7/5/2001	2/27/2002	176.4600	6.4342	3.6
BQ4	14	7/5/2001	2/27/2002	179.1527	6.0026	3.4
Q3	232	7/1/2003	3/18/2004	0.2418	0.0135	5.6
81	240	7/1/2003	12/23/2003	2.2734	0.1013	4.5
91	50	9/16/2003	3/3/2004	190.3421	9.7912	5.1
Q4	47	9/16/2003	1/8/2004	191.0827	9.5166	5.0
Q5	124	9/23/2003	3/19/2004	0.1800	0.0118	6.6
82	122	11/25/2003	3/19/2004	1.2524	0.0736	5.9
92	20	1/7/2004	3/18/2004	175.9811	14.0430	8.0
Q6	19	1/7/2004	3/18/2004	176.8943	12.6266	7.1





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