

# Cotinine Analytical Workshop Report: Consideration of Analytical Methods for Determining Cotinine In Human Body Fluids as a Measure of Passive Exposure to Tobacco Smoke\*

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A two-day technical workshop was convened November 10-11, 1986, to discuss analytical approaches for determining trace amounts of cotinine in human body fluids resulting from passive exposure to environmental tobacco smoke (ETS). The workshop, jointly sponsored by the U.S. Environmental Protection Agency and Centers for Disease Control, was attended by scientists with expertise in cotinine analytical methodology and/or conduct of human monitoring studies related to ETS. The workshop format included technical presentations, separate panel discussions on chromatography and immunoassay analytical approaches, and group discussions related to the quality assurance/quality control aspects of future monitoring programs. This report presents a consensus of opinion on general issues before the workshop panel participants and also a detailed comparison of several analytical approaches being used by the various represented laboratories. The salient features of the chromatography and immunoassay analytical methods are discussed separately.

## Introduction

Environmental tobacco smoke (ETS) has increasingly become a health concern since a series of epidemiological studies between 1981 and 1986 (1-6)

\*Chairperson: J. Lewtas, US EPA, Research Triangle Park, NC; Session chairpersons: F. Sperto, CDC, Atlanta, GA, R. Watts, US EPA, Research Triangle Park, NC; invited speaker/panel participants included: Neal Benowitz and Peyton Jacob, San Francisco General Medical Center; Colin Feyerabend, New Cross Hospital, London, England; Nancy Haley, American Health Foundation; George Knight, Foundation for Blood Research; Richard Kornfeld, Battelle Columbus Laboratories; John Langone, Baylor College of Medicine; Peter McElroy, Rosewell Park Memorial Institute; M. A. H. Russell, Maudsley Hospital, London, England; Karl Verebey, New York State Division of Substance Abuse Services; and Helen Van Vunakis, Brandeis University.

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reported an association between tobacco smoke exposure and increased risk of human lung cancer. Humble and co-workers (?) recently confirmed the health risk conclusions of earlier researchers and reported that people who never smoked and were married to smokers had about a 2-fold increased risk of lung cancer.

Methods for determining the degree of exposure of individuals has received much attention in recent years, and various biological markers have been studied as surrogate analytes for determining exposures. A general consensus is that the nicotine metabolite, cotinine, has the prerequisites of specificity, retention time in the body, and detectable concentration levels that make it the analyte of choice for quantifying exposures. In recent years a number of procedures have been reported for determining cotinine in human body fluids. The majority of these procedures use either a chromatographic technique or some form of immunoassay analysis.

This paper is a report from the two-day Cotinine Analytical Workshop, which was attended by invited health scientists and analytical chemists recognized for their expertise in studies of population exposure

to ETS and/or analytical methodology related to these studies. The workshop was jointly sponsored by the U.S. Environmental Protection Agency (EPA) and Centers for Disease Control (CDC) and was attended by 32 scientists who shared their expertise in immunoassay or chromatography methods for cotinine and provided guidance for developing and establishing related programs for determining passive exposures to tobacco smoke. The meeting objective was to compare the various analytical approaches to cotinine analysis and to make recommendations regarding the general aspects of establishing and conducting monitoring programs. Discussions included quality assurance/quality control (QA/QC) programs to support cotinine monitoring studies and also the possibility of conducting a future interlaboratory methods comparison study. The diverse analytical approaches represented by chromatography and immunoassay methods for cotinine were separately discussed and reported by respective work groups. The purpose of this communication is to summarize discussions from the immunoassay and chromatography work groups relevant to the aforementioned topics and to convey the workshop general consensus on other joint issues including QA/QC aspects of ETS studies.

## Chromatography Group Report

The workshop participants with expertise in developing and applying chromatography methods for determining cotinine in biological fluids met in a one-day session. The goal of this session was to develop a group consensus on several key issues including *a*) general method considerations and approaches, *b*) QA/QC programs to support cotinine monitoring studies, and *c*) considerations related to conducting an interlaboratory methods comparison study. The following is a summary of the chromatography group discussions and a draft of their recommendations related to topics *a* and *c*. The QA/QC recommendations are contained in a separate section.

## General Method Considerations

**Sample Type.** The body fluids discussed for monitoring tobacco smoke exposure included blood serum, saliva, and urine. Group consensus was that all three are generally acceptable; however, the choice of a body fluid to analyze should be predicated on the goals of the specific monitoring program. For studies that require a quantitative assessment of exposure, blood was recommended by the group as the fluid of choice (8). Saliva was also considered acceptable, and good correlations were reported between saliva and blood for results from the same subject (9). Sample collection considerations, however, resulted in the selection of blood as the sample medium of first choice. Analysis of either blood or saliva for cotinine permits an estimate of the degree of exposure to tobacco smoke in persons passively exposed at home or in the work place. While

cotinine determination in urine was also recommended for estimating exposure, it was generally felt that estimation based on urinary cotinine excretion would be less reliable than estimation based on plasma or salivary levels. Cotinine excretion is variable across and within individuals depending on renal function, urine flow rate, and urine pH (10). Urine results may be expressed as micrograms of cotinine per milligram of creatinine in order to correct, in part, for the variable dilution effects. This correction or normalization, however, introduces additional variability since this requires another analytical determination (and opportunity for experimental error), and creatinine excretion rates for individuals are also variable. Horstmann (11) reported creatinine excretion rate for 56 subjects to be  $1.11 \pm 0.68$  g/day (mean  $\pm$  SD). Hoffman and Brunnehan (12) also found 13 nonsmokers on a controlled diet to have creatinine values of  $1.65 \pm 0.5$  g per 24 hr urine (mean  $\pm$  SD). The coefficients of variation between subjects for these two studies were 61 and 30%, respectively.

**Sample Collection and Handling.** Chromatography procedures for cotinine generally require analysis of a 1 mL sample with an additional 1 mL volume needed for reanalysis. A total sample volume of 2.5 to 3.0 mL was therefore recommended. Glass and/or polypropylene sample tubes with screw cap closures were recommended. The polypropylene tubes were preferred to avoid breakage during shipment. Minimum size sample tubes were suggested to reduce volume losses from freeze drying during long-term storage.

Blood should be centrifuged at the field site and the serum samples frozen prior to shipment to the laboratory. Urine should be frozen soon after collection to prevent bacterial degradation of the sample. Saliva may be collected by expectoration into a sample tube; however, an alternative saliva collection procedure that uses highly adsorbent dental rolls is recommended (13). The subject is asked to place a dental roll in the mouth for approximately 15 min. The sample is then placed in a tube and frozen prior to shipment to the laboratory. The thawed sample is regenerated at the laboratory by placing the dental roll in a glass syringe and compressing with a glass plunger. The resultant clear liquid may then be aliquoted for analysis.

Shipment in a frozen condition with dry ice was recommended for all three sample types to prevent bacterial degradation of the sample matrix. Loss or degradation of the cotinine analyte was not considered to be a problem since participants had found this compound to be stable.

Upon receipt at the laboratory, samples should be placed in a freezer (approximately  $-20^{\circ}\text{C}$ ) until analyzed. Samples that will be held in excess of one year should be stored at  $-80^{\circ}\text{C}$ . No cotinine degradation problems were reported for frozen samples. Precautions were recommended, however, to prevent concentration errors resulting from freeze-drying of samples stored over one year in a frost-free freezer.

## Analytical Method Considerations

The group consensus was that the analytical method should permit the determination of nicotine and cotinine in a single analysis and should allow a clear separation and distinction between these and other analytes that may be present. The method should be sufficiently sensitive to give good definition of passive exposure and thereby yield analytical results which will show a distinction, for example, between a child or other nonsmoker that is exposed in the home and one that is not. Tables 1 and 2 list the range of detection limits for both chromatography and immunoassay methods.

The importance of this sensitivity consideration was supported by the 1981 report of Hirayama (1) and the 1987 report of Humble et al. (7) which showed an increased risk of lung cancer for a spouse exposed to a smoker in the home. Russell reported that cotinine levels in children's saliva averaged  $0.44 \pm 0.68$  ng/mL where no parents are smokers,  $1.31 \pm 1.21$  ng/mL where only the father smoked,  $1.95 \pm 1.71$  where only the mother smoked, and  $3.38 \pm 2.45$  ng/mL where both parents are smokers (13). This study used an analytical method with a detection and quantification limit of 0.1 ng/mL, which permitted classification of the lowest exposures into exposure distributions differing by only 0.1 ng/mL. Over 30% of the children from nonsmoking homes had cotinine concentrations below the 0.1 ng/mL detection limit. In the groups where one or more parents smoked, the cotinines were significantly ( $p < 0.01$ ) elevated, and 50% of the children of the lowest exposed group had less than 1 ng/mL (when only the father smoked). Table 1 shows that several available chromatography methods have detection limits ranging from 0.1 to 0.2 ng/mL while the most sensitive immunoassay method in Table 2 reports a 0.3 ng/mL detection limit.

The question of analyte volatility losses during analysis was discussed, and it was generally agreed that if nicotine were included as an analyte, precautions would need to be taken to prevent loss during concentration steps. Acidification to convert nicotine to a salt form prevents losses during concentration.

Cotinine primary standards are used in the free base form by some analysts; however, a salt form was preferred by meeting participants, since the free base form is hygroscopic and difficult to maintain at a well-defined purity. A perchlorate salt of cotinine was recommended for preparation of 1 mg/mL stock solutions in 0.01 N HCl (8). This standard solution could be frozen and kept indefinitely. The group consensus was that a salt form of cotinine should be made available as a primary standard.

Chemical analysis is usually accomplished by gas chromatography with nitrogen/phosphorus thermionic detection (GC-NPD) or GC-mass spectrometry (GC-MS) using either electron impact ionization or

chemical ionization. Packed columns for GC were successfully used; however, fused silica capillary columns containing a methyl silicone or methyl phenyl silicone liquid phase were recommended (see Table 1 HRGC references).

A high-performance liquid chromatography (HPLC) method using a  $C_{18}$  reversed phase column with paired ion chromatography and UV detection (at 257 nm) was also reported by McElroy where the HPLC method of Machacek and Jiang (14) was modified for analyzing urine samples at passive exposure levels. Further improvement in HPLC sensitivity and detection limit is required before application to the more limited sample volumes generally available for blood or saliva. HPLC was considered a very promising approach due to the highly efficient columns now available and the stability and reproducibility of response commonly obtainable by UV detection.

The final quantitation of residues in all methods was accomplished with internal standards and standard curves developed from fortified blank samples. It was recommended that standard curves be prepared daily or with each batch of samples. A variety of internal standards were used ranging from deuterated cotinine and nicotine for GC-MS to chemically similar compounds for other GC or LC detectors.

Table 1 lists the chromatography methods (14-17) presented at the workshop and summarizes the salient features of each. Information for this table was derived from questionnaire responses submitted by each author/participant.

## Chromatography Group Recommendations

The chromatography group recommended that an interlaboratory methods comparison study be conducted prior to any large-scale monitoring efforts aimed at determining population exposure to tobacco smoke. Specific suggestions and recommendations relating to method comparison studies were as follows:

- Separate studies should be conducted for passive exposure levels and active smoker levels.
- Statisticians should be used in planning study samples.
- Blood, urine, and saliva should be included in each study.
- Immunoassay and chromatography methods should be included in each method comparison study.
- Samples should be fluids from exposed individuals and also from fortified blanks in order to look for bias from chromatography or immunoassay methods through measurement of artifacts or metabolites related to nicotine/cotinine.
- The study coordinator should supply standard reference material(s) to each participating laboratory.

**Table 1. Summary of passive exposure chromatography methods.**

	Machacek and Jiang (14)	Jacob, et al. (15)	Verebey et al. (16)	Feyerabend et al. (17)	Kornfeld (personal communication)
Sample type	Urine	Blood, plasma, urine	Serum	All biological fluids	Urine
Vol. analyzed, mL	6	1	0.5	1	5
Concentration step	N2 evaporated to dryness	N2 evaporated to dryness	None	N2 evaporated to dryness	N2 evaporated solvent exchange
Extraction method	SPE column Chloroform elution	Solvent extraction	Solvent extraction	Solvent extraction	Solvent extraction
Isolation step	Acid/base partition	Acid/base partition	Acid/base partition	None: plasma and saliva back extract urine	None
Determination	HPLC reverse-phase Paired ion chromatography	HRGC-NPD  HRGC-MS	HRGC-NPD	GC-NPD	HRGC-MS
Quantitation	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve
Linear range	0-500 ng	0-4000 ng/mL	40-400 ng/mL	0-15,000 ng/mL	1-500 ng/mL
Detection limit	<1 ng/mL	0.2 ng/mL	5 ng/mL	0.1 ng/mL	0.13 ng/mL
Quantification limit	1 ng/mL	0.5-5 ng/mL	5 ng/mL	0.1 ng/mL	0.25 ng/mL
CV, %	13	6.8 (3.0 ng/mL) 12 (1.0 ng/mL) 13 (0.5 ng/mL)	5.6	7.7	6
% recovery	90	107	90	90	85

## Immunoassay Group Report

Participants in the workshop with expertise in the development and use of immunoassays for detecting cotinine in biological fluids met independently of the chromatography group to discuss and make recommendations regarding methodology and applications of immunoassay in monitoring passive as well as active exposure to tobacco smoke, QA/QC programs, and interlaboratory methods comparison. The following discussion presents an overview of the available immunoassay techniques for cotinine analysis, their applications with advantages and disadvantages, and the views and recommendations of the immunoassay panel members. There is notable agreement between this group and the chromatography group on most common issues outside the technical aspects specific to each methodology.

## General Method Considerations

**Introduction.** The first radioimmunoassay (RIA) for cotinine was reported in 1973 (18,19). Antisera were raised in rabbits and goats immunized with a covalent conjugate prepared by linking cotinine 4'-carboxylic acid to immunogenic carrier proteins, such as bovine serum albumin and keyhole limpet hemocyanin. The radioactive tracer was prepared by labeling a tyramine derivative of cotinine 4'-carboxylic acid with  $^{125}\text{I}$ ; since then, [ $^3\text{H}$ ]cotinine has been prepared enzymatically (19) from [ $^3\text{H}$ ]nicotine and is now widely used. Another approach uses cotinine derivatized at the 1-position in the pyridine ring for preparing the immunogen and as a precursor of an  $^{125}\text{I}$ -labeled tracer (20). The original assay has been used to measure cotinine levels in physiological fluids, e.g., urine, blood, saliva, amniotic fluid, and spinal fluid

**Table 2. Summary of immunoassay methods.**

	Langone et al. (18) Langone and Van Vunakis (19)	Haley et al. (22)	Knight et al. (29)	Bjercke et al. (27,28)
Sample type and volume analyzed, mL	Urine (0.02-0.05) Serum (up to 0.5) Saliva (0.02)	Urine, plasma, saliva (0.005-0.025)	Urine (0.01) Serum (0.1)	Urine, serum, saliva (0.1 in RIA; 0.01 in microtiter plate assays)
Assay type	RIA ( $^{125}\text{I}$ , $^3\text{H}$ )	RIA ( $^3\text{H}$ )	RIA ( $^{125}\text{I}$ )	RIA ( $^{125}\text{I}$ , $^3\text{H}$ ), ELISA, <sup>a</sup> FIA
Quantitation	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve
Detection limit, ng/mL	2	0.37	0.3	0.5-1.5
Quantitation limit, ng/mL	2	1	1	0.5-1.5
CV, %	6-10	10	10-15	9-14

<sup>a</sup>Enzyme-labeled protein A cannot be used to assay concentrated serum.

(18,19,21-23) of active smokers and serum, urine, and saliva (24-26), of passive smokers.

More recently, monoclonal antibodies specific for cotinine have been prepared and used to develop fluid phase RIAs with the  $^{125}\text{I}$ - and  $^3\text{H}$ -labeled tracers as well as enzyme-linked immunosorbent assays (ELISA) and a fluorescence immunoassay (FIA) in a microtiter plate format (27,28). These assays also have been used to measure cotinine levels in fluids of active (27,28) and passive smokers (Langone et al., unpublished results).

**Test Samples and Standards.** Because the original RIAs and the monoclonal antibody-based nonisotopic assays have been developed for analysis of unextracted physiological fluids, careful attention must be paid to possible nonspecific inhibition of antigen-antibody binding resulting from effects of pH or high concentrations of salts or urea, e.g., in urine. In this regard the immunoassay group agreed and strongly recommended that pooled standard samples of serum, saliva, and urine containing known amounts of added or endogenous cotinine should be made available through an agency such as the National Institute of Standards and Technology. There was general agreement that GC/MS would be the best method to establish the cotinine concentration for purposes of methods comparison and that levels should cover the range from cotinine-free through concentrations found in passive and active smokers. Essentially cotinine-free samples might be collected from a population that would represent a group with minimal exposure to tobacco smoke (e.g., Mormons in Utah). The suggestion also was made that low-level or essentially cotinine-free fluids might be treated (e.g., by absorption with XAD-2 resin or charcoal) to remove possible traces of cotinine. However, because absorption could remove other constituents that might affect the assays, it was not considered to be a firm recommendation.

Although it was suggested that urine may be the fluid of choice for RIA analysis, there was no strong consensus for priority over serum or saliva. In this regard, one participant pointed out the advantage that salivary cotinine levels determined by RIA are independent of saliva flow (Van Vunakis and Regas, unpublished results). The monoclonal antibody assays also have been used to detect cotinine in saliva and urine of passively exposed children (Langone et al., unpublished observations), and these investigators tended to favor the use of saliva. In addition to the use of dental rolls as discussed by the chromatography group, one member of the immunoassay group suggested that subjects chew a piece of Teflon tape to stimulate the flow of saliva that is then collected in a glass vial. It was pointed out that Teflon will not contaminate the sample. Regardless of which fluid is tested, it was recommended that samples be centrifuged (e.g., 2000g for 10-20 min or 10000g for 1-2 min) to sediment particulate matter before analysis. Immunoassay group participants concurred with the

sample handling recommendations given in the Chromatography Group Report.

## Comparison of the Assays

The original RIA and variations of it are used by the immunoassay group participants. Therefore, the discussion focused on this method and the monoclonal antibody based assays, the salient features of which are summarized in Table 2.

**Reagents.** The same immunogen was used to produce the rabbit, goat, or sheep antisera and the monoclonal antibodies. However, it was emphasized that cotinine 4'-carboxylic acid (and the  $^{125}\text{I}$ -labeled derivative) is a mixture of stereoisomers giving rise to a heterogeneous population of polyclonal antibodies recognizing both natural (-)-cotinine and the (+)-enantiomer. Also, conventional antisera contain a population of antibodies that bind specifically to the linkage group that joins cotinine to the immunogenic carrier protein and to the tyramine group in the  $^{125}\text{I}$ -labeled derivative. The practical consequences are a relatively shallow standard inhibition curve and the failure to achieve 100% inhibition of immune binding with (-)-cotinine. Although these problems are circumvented by using (-)- $^3\text{H}$  cotinine, this assay is somewhat less sensitive, owing to the lower specific activity and counting efficiency achieved with tritium. Also, disposing of large volumes of radioactive scintillation fluid is a major concern.

Two approaches have been used with some success to improve the quality of the  $^{125}\text{I}$ -RIA with rabbit antisera. They involve removing antibody group antibodies by absorption with a nicotine-hemocyanin conjugate (29) and preparing an  $^{125}\text{I}$ -labeled cotinine derivative with a bridging group different from that present in the immunogen (30). In contrast, monoclonal antibodies to cotinine were produced in a way that avoided the problems inherent in the use of polyclonal antisera (27,28). Although the immunogen contained a mixture of isomers, the hybridomas were screened using (-)- $^3\text{H}$  cotinine to optimize chances of detecting antibodies that preferentially recognize the naturally occurring isomer, but not the bridging group in the immunogen. Furthermore, it was pointed out that monoclonal antibodies are preferred standard reagents for immunoassay because they are continuously available and are homogeneous in terms of binding affinity and specificity.

The specificity of any newly produced antiserum must be fully characterized one time with a battery of compounds that would include at least cotinine, nicotine, and metabolites such as nicotine N'-oxide, nor-nicotine, and *trans*-3'-hydroxycotinine. This recommendation holds even for new antisera prepared with a proven immunogen, since the response of individual immunized animals cannot be predicted. However, all agreed that when the properties of the antiserum had been established, it was unnecessary for each laboratory that received that antiserum to

complete a thorough reexamination of specificity, although it would be good laboratory practice to routinely compare the cotinine and nicotine inhibition curves.

Immunoassay methods have often reported using standard cotinine as the free base. However, because cotinine is hygroscopic and difficult to weigh accurately, all participants agreed that a nonhygroscopic salt of cotinine, such as the perchlorate or fumarate, would be the preferred standard.

**Assay Performance.** In the original RIA, antibody-bound and free-labeled cotinine were separated by the double-antibody method in which a heterologous antibody directed against the species of anticotinine was used to precipitate antigen-antibody complexes (18). Other techniques can be used including precipitation with ammonium sulfate or polyethylene glycol (29). Although the latter methods are faster and less expensive, there was some concern expressed that background radioactivity precipitated by ammonium sulfate, when normal serum is used in place of anti-cotinine, can exceed 10% of the added amount of cotinine tracer.

In contrast to the conventional fluid phase RIAs, the monoclonal antibody-based assays are carried out in a solid-phase system in which a cotinine-polylysine conjugate is passively adsorbed to the surface of 96-well plastic microtiter plates (27,28). Immobilized cotinine and fluid phase cotinine in the test sample compete for monoclonal anticotinine, which is detected with a variety of enzyme-labeled antiimmunoglobulin reagents including the bacterial product, protein A. Assay sensitivity can be enhanced by using a sandwich procedure in which rabbit anti-mouse immunoglobulin is added before (or along with) labeled protein A. It was emphasized that protein A reagents cannot be used to detect low levels of serum cotinine, because host IgG will bind nonspecifically to the microtiter wells giving high background binding of the enzyme-labeled protein A tracer.

Compared to times when rabbit antisera were used, assays with monoclonal antibodies were more sensitive, the standard curves were steeper, and the antigen-antibody reaction was completely inhibited by (-)-cotinine, even when the  $^{125}\text{I}$ -labeled tyramine derivative was used in RIA (28). There was good agreement between the levels of cotinine found in saliva and serum of smokers determined by conventional RIA, the monoclonal antibody ELISA and GC (27,28). It was pointed out that high quality rabbit antisera also can be used in the solid phase nonisotopic immunoassays with titers that can be 100- to 1000-fold higher than in RIA (27,28).

**Specificity and Sensitivity.** Both polyclonal and monoclonal antibodies are specific for cotinine (18,19,27). Approximately 50 to 100 compounds that have been tested in the immunoassays including several nicotine metabolites, related tobacco alkaloids, and other compounds that retain structural features of either or both ring systems found in nicotine or

cotinine fail to inhibit the antigen-antibody reactions at levels that would interfere in the assays.

One participant emphasized that literally thousands of serum and urine samples from both active smokers and nonsmokers had been analyzed over a period of several years and that few, if any, false positives had been reported. Although the subjects studied are mainly from the U.S. and England, these data support the view that diet or other factors such as prescription or other drugs do not interfere in the assays and are consistent with high specificity of anticotinine. It was pointed out that differences in diet or drug use must be considered when other populations are studied, or at least be aware that interference in the assays might arise from factors which have not appeared to date.

The immunoassays generally can detect cotinine down to the ng/mL level or less (Table 2), although it was emphasized that sensitivity can be affected by the need to dilute samples (e.g., urine) that may give spurious results when higher concentrations are tested. This point was discussed at some length with the participants in agreement that a sensitivity for cotinine of 0.1 ng/mL of physiological fluids could not generally be achieved with confidence using the available immunoassays. In this regard, it was pointed out that differences in sensitivity limits between chromatography and immunoassay likely reflect fundamental differences in methodology and are not strictly comparable. GC methods, for example, might extract and analyze a considerably larger portion of sample than would be analyzed by immunoassay.

**Analytical Results.** There was general agreement that cotinine concentration should be expressed as nanogram per milliliter. However, urine values also should be given as nanogram per milligram creatinine, as this ratio is used conventionally in the medical literature to account for differences in urine volume. Because low levels of creatinine in infants relative to adults may result in misleading values that fall into the range reported for active smokers, the need to include primary data for urine was stressed. Furthermore, experience has shown that urinary cotinine levels determined by conventional RIA generally are 30 to 50% higher than values obtained for the same samples by GC. Discussion centered on the possibility that the higher RIA values may reflect cross-reactivity of anti-cotinine with *trans*-3'-hydroxycotinine, which recently has been reported to be a major nicotine metabolite found in smokers' urine at levels up to three times higher than cotinine (31).

Since this meeting, synthetic *trans*-3'-hydroxycotinine (supplied by Dr. Peyton Jacob, San Francisco General Medical Center) has been shown to cross-react only 1 to 2% compared to cotinine in the monoclonal antibody based ELISA; one participant stated that he found only about 5% cross-reactivity with his rabbit antiserum in RIA. This degree of cross-reaction would not account for the discrepancy

Table 3. Summary comparison of chromatography and RIA methods.

	Chromatography methods	RIA methods
Sample type	Blood, saliva, urine	Blood, saliva, urine
Vol. analyzed, mL	0.5-6	0.005-0.5
Extraction and concentration	Yes	No
Quantification	Internal standard Calibration curve	Internal standard Calibration curve
Detection limit, ng/mL	0.1-5	0.3-2
Quantification limit, ng/mL	0.1-5	0.5-2
CV, %	5.6-13	6-15

in the urine values, and it was agreed that further research was needed to clarify the basis for the differences.

### Considerations in Selecting an Analytical Technique

Table 3 shows some comparisons for RIA and chromatography methods. Apparent differences are in sample volumes used, sample work-up requirements, and limits of detection. RIA methods use less than 10% of the sample volumes required for chromatography methods, and this is a major reason that RIA detection limits are not as low as those for chromatography methods. Because RIA methods do not require sample manipulations such as extraction and concentration, they are faster, simpler, and presumably less expensive. Chromatography procedures not only have the advantage of increased sensitivities, but also are more specific and can provide quantification of both nicotine and cotinine in a single analysis. Workshop participants agreed that the choice between these two approaches would depend on the goals of a particular study. Both approaches have been found to be 100% effective in discriminating smokers from nonsmokers (32). This particular goal would favor the use of an RIA method. At least one participant suggested that the more sensitive chromatography methods are recommended to characterize ETS exposures for plasma or saliva concentrations when levels are less than 1 ng/mL.

A compilation of literature values for cotinine concentrations in body fluids of nonsmokers before and after documented ETS exposures is shown in Table 4. This comparison indicates a similarity between plasma and saliva concentrations, while urine values are often a factor of two or more higher. This is a primary reason that urine is often the fluid of choice when RIA methods are used in passive smoking studies.

### Quality Assurance for Laboratories Assaying Cotinine

Participants in the cotinine workshop discussed the need for developing a quality assurance (QA) program for monitoring performance of laboratories

assaying cotinine for the purpose of assessing exposure to ETS. When assuming many subjects, such a QA program would be essential to ensure that the conclusions reached are based on reliable data. A one-time exercise where the ability of laboratories to measure cotinine levels found in both active smoking and for passive exposure to ETS was considered as an alternative possibility. This suggestion was prompted by the realization that although published data on cotinine levels found in body fluids for active smokers show reasonable agreement, levels of cotinine reported for subjects exposed to ETS show considerable variation. Such differences might not be unexpected when measuring the low levels of cotinine found in ETS exposure, given that the detection limits for existing analytical methods approximate these cotinine levels.

To evaluate the between-laboratory variation in cotinine analyses, an international study was initiated by the *Forschungsgesellschaft Rauchen und Gesundheit mBH* in Hamburg (32). Eleven laboratories experienced in measuring nicotine and cotinine by RIA and/or GC participated. Serum and urine specimens from eight nonsmokers and eight smokers, and from two nonexposed nonsmokers spiked with nicotine and cotinine were distributed on dry ice to each laboratory. Results were returned and analyzed by method and by laboratory. Recoveries on both the urine and serum specimens spiked with cotinine corresponding to levels found in smokers ranged from 79 to 119%, with the exception of one laboratory with a 20% recovery (the data from this laboratory were excluded from further analysis). The interlaboratory coefficient of variation on these same samples was excellent (9-13%). The coefficient of variation on samples from smokers was fairly large, however, ranging from 18 to 45% for serum and 21 to 59% for urine. Further, cotinine levels reported for urine were about 60% higher than from those using RIA as compared to GC, suggesting a possible interfering substance in the immunoassay system. Cotinine levels reported for nonsmokers were extremely variable, and a number of laboratories could not detect cotinine in serum from exposed nonsmokers. In addition, cotinine values reported by some laboratories bore no relationship to estimated ETS exposure, or they were so high as to be unrealistic. In spite of this variability, all laboratories were able to discriminate smokers from nonsmokers with 100% effectiveness.

**Table 4. Mean or median concentrations of cotinine in nonsmokers before and after exposure to environmental tobacco smoke.**

Study reference	Plasma cotinine, ng/mL		Saliva cotinine, ng/mL		Urine cotinine, ng/mL	
	Before	After	Before	After	Before	After
(33)	0.82	2.04	0.73	2.48	1.55	7.71
(34)	1.1	7.3	1.5	8.0	4.8	12.9
(9)	0.8	1.8-2.5	0.7	2.2-2.8	1.5	6.5-9.4
(13)			0.4	1.3-3.4		
(35)	0.9-1.7	2.6-3.3	1.0-1.7	1.4-2.5	14	21-55
(36)					8.5	25.2
(25)						2.8-29.6
(37)			1.3-1.7	2.4-5.6		

The cotinine results reported for ETS exposure should be viewed with caution, however. A number of the participants at this conference workshop, who also were in the study, indicated that the volumes provided were insufficient for repeat analysis using GC or an assay was used which had not been optimized for measuring passive levels of cotinine. A further limitation of the study was that recovery of spiked cotinine was only assessed for smoking levels. Finally, immunoassays based on monoclonal antibodies were not included, nor were HPLC methods evaluated.

This interlaboratory study indicates the need for further information on the reliability of data provided by laboratories for study subjects exposed to ETS. A quality assurance program could provide such information, as well as an ongoing assessment of quality and a mechanism for improving performance.

## QA Recommendations

**Interlaboratory QA.** The need for an interlaboratory quality assurance program was endorsed by most of the session participants, with some concern being expressed that the number of samples evaluated be kept to reasonable limits to minimize unnecessary assays. It was recommended that such a program should be administered by a QA coordinator laboratory. The coordinating laboratory would be responsible for monitoring the performance of participating laboratories and for providing specified samples as standards and/or controls. This laboratory should have in-house expertise or have access to laboratories having expertise in both immunoassay and high resolution gas chromatography/mass spectrometry (HRGC-MS).

Suggested objectives of the QA coordinator laboratory include:

- To provide an objective measure of the precision and accuracy of analytical methods used routinely by laboratories assaying cotinine.
- To identify preferred method(s) for measuring cotinine.
- To assess the reliability of results provided by different laboratories.

- To provide a mechanism for improving performance through knowledge of the performance of others.
- To serve as a resource center for communication and exchange of information among participants.

Recommended mechanisms for accomplishing the foregoing objectives are as follows:

**Interlaboratory Quality Assurance Studies: Quality Assurance Samples.** The coordinator laboratory should periodically conduct a blind or check sample study consisting of authentic biological fluids (serum, urine, or saliva) with actual or spiked levels of cotinine. Samples should be selected to represent cotinine levels typical of those found in passive and active smoking. Authentic biological samples with actual levels of cotinine are strongly recommended because only they will contain nicotine metabolites or other substances that may interfere in assays. In addition, blank samples spiked with known levels of cotinine should be distributed to evaluate recovery. Finally, samples with high levels of cotinine should be diluted with negative specimens to check for linearity. Samples should ideally have target values assigned by the QA coordinating laboratory through use of reference methods. Data returned by participants would be analyzed and reports containing results and a critique distributed.

**Field Study Samples.** The QC coordinating laboratory may assist organizations carrying out field studies in assessing the performance of the study laboratory on actual study subjects. The workshop considered that this could be accomplished by submission at intervals of blind duplicates: duplicates of the same study subject submitted at intervals to assess precision; split samples: sample is split with one portion being sent for analysis to the study laboratory and one portion to the QC coordinating laboratory for comparison purposes; blanks: samples that are considered free of analyte to serve as a check on environmental contamination.

**Ancillary Activities of the QA Coordinating Laboratory: Primary Reference Standard(s).** A strong consensus was reached that a well-characterized, pure, primary reference standard be made available. This material should be aliquoted into quantities sufficient to allow any laboratory to use

the standard for assay calibration. Handling and storage information should also be provided along with suggested methods for preparing secondary standards. It was generally agreed that cotinine should be in the form of a salt, since cotinine freebase is hygroscopic and, therefore, likely to vary in composition dependent on handling conditions. The perchlorate salt was suggested as one possibility (see chromatography group report). It was further recommended that the standard be supplied in solution to preclude errors due to dilution. The GC group made the suggestion that the National Institute of Standards and Technology might be the appropriate agency to prepare such a standard. The QC coordinating laboratory could then distribute the standard.

**Biological Reference Samples.** The suggestion was made that, in addition to providing a primary reference standard, the QA coordinating laboratory make available authentic biological samples from actual smokers and subjects exposed to ETS. Cotinine concentrations would be established by the QA coordinating laboratory using a reference method(s) and declared on each reference sample. Such samples would be important because the cotinine would be present in the matrix (urine, serum, saliva) actually used by analysts, thus allowing evaluation of possible matrix interference. In addition, such specimens would contain nicotine, nicotine metabolites other than cotinine, and other substances which might interfere in the assay.

The GC group also felt that blank samples, i.e., those essentially free of cotinine would be desirable. Suggested sources were bovine serum or human samples with very low exposure to ETS. Pooled specimens might be necessary because obtaining sufficient volume of biological reference samples could prove difficult. Samples would therefore be provided in restricted quantities only to allow laboratories to periodically evaluate their own method.

**Reference Method.** Workshop participants also discussed a reference method for establishing cotinine levels in biological samples. The consensus of the group was that GC/MS would be the ultimate reference method because of its extreme specificity. However, in the group discussion, the GC group pointed out that although the method is highly specific, it ultimately is no better than the reliability of the extraction and evaporation methods chosen to prepare samples for analysis. A further concern was that differences in assigned values may result from differences attributable to the detection method (chemical ionization or electron impact). Consequently, it appears unlikely that a gold standard will be available and acceptance of a reference method will depend ultimately on judgment of its reliability. Representatives of the National Institute of Standards and Technology indicated that their practice is to evaluate a variety of independent methods, and if sufficient agreement is reached, a certified value is

provided, albeit with the understanding that confidence limits are somewhat uncertain. In the absence of agreement between various methods, NIST provides a consensus value(s) for informational purposes.

In the event that GC/MS is adopted as a reference method, the implication for immunoassayists is that their performance would be judged against this standard. Judging immunoassay results against GC/MS is not without precedent, since other immunoassays, such as those for steroids, are already compared to this method.

## Postscript

A cotinine spiked, freeze-dried human urine reference material is being prepared by the National Institute of Standards and Technology (formerly the National Bureau of Standards). Three lots with different cotinine concentration levels are being prepared: a) an unspiked blank level (< 1 ppb), b) an approximately 50 ppb low level, and c) an approximately 500 ppb high level. This material (EPA/NIST Reference Material 8444) is planned for issue during the first quarter of 1989. The material may be ordered from: Office of Standard Reference Materials, Building 222 Room B-311, National Institute of Standards and Technology, Gaithersburg, MD 20899. Telephone 301-975-6776. Technical information may be obtained from Dr. Lane Sander, Organic Analytical Research Division, Center for Analytical Chemistry, NIST, Gaithersburg, MD 20899. Telephone 301-975-3117.

A cotinine perchlorate salt reference material is also being planned for development by NIST. A date has not been determined, however, for release of this standard.

The research described in this report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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