Withdrawn NIST Technical Series Publication

Warning Notice

The attached publication has been withdrawn (archived), and is provided solely for historical purposes. It may have been superseded by another publication (indicated below).

Withdrawn Publication	
Series/Number	NIST Special Publication 260-256
Title	Certification of Standard Reference Material®s 3672a & 3673a: Organic Contaminants in Smokers' Urine (Frozen) & Organic Contaminants in Non-Smokers' Urine (Frozen)
Publication Date(s)	May 2025
Withdrawal Date	February 25 2026
Withdrawal Note	NIST SP 260-256 is withdrawn and superseded in its entirety by NIST SP 260-256r1

Superseding Publication(s) *(if applicable)*	
The attached publication has been **superseded by** the following publication(s):	
Series/Number	NIST Special Publication 260-256r1
Title	Certification of Standard Reference Material®s 3672a & 3673a: Organic Contaminants in Smokers' Urine (Frozen) & Organic Contaminants in Non-Smokers' Urine (Frozen)
Author(s)	Ashley S.P. Boggs; Benjamin C. Blount; Julianne Cook Botelho; Christina Brosius; Carolyn Burdette; Antonia M. Calafat; Johanna Camara; Maria Morel Espinosa; June Feng; N. Alan Heckert; Jennifer C. Hoguet; Kevin Huncik; Maria Ospina; Jared Ragland; Christopher Reese; Michael Rybak; Tiffany Seyler; Liza Valentin-Blasini; Lanqing Wang; Elena Shiao Ching Wood; Baoyun Xia.
Publication Date(s)	February 25 2026
URL/DOI	https://doi.org/10.6028/NIST.SP.260-256r1

Additional Information *(if applicable)*	
Contact	
Latest revision of the attached publication	
Related Information	

NIST NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY U.S. DEPARTMENT OF COMMERCE

Date Updated: February 25, 2026

NIST Special Publication 260
NIST SP 260-256

Certification of Standard Reference Material®s 3672a & 3673a:

Organic Contaminants in Smokers' Urine (Frozen) & Organic Contaminants in Non-Smokers' Urine (Frozen)

Ashley S.P. Boggs
Carolyn Burdette
Johanna Camara
N. Alan Heckert
Jennifer C. Hoguet
Kevin Huncik
Jared Ragland
Elena Shiao Ching Wood

This publication is available free of charge from:
https://doi.org/10.6028/NIST.SP.260-256

NIST NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY
U.S. DEPARTMENT OF COMMERCE

Page Intentionally Blank

NIST Special Publication 260
NIST SP 260-256

Certification of Standard Reference Material®s 3672a & 3673a:

Organic Contaminants in Smokers' Urine (Frozen) & Organic Contaminants in Non-Smokers' Urine (Frozen)

Ashley S.P. Boggs, Carolyn Q. Burdette, Johanna Camara, Jennifer C. Hoguet, Kevin Huncik, Jared Ragland, Elena Shiao Ching Wood
Chemical Sciences Division
Material Measurement Laboratory
National Institute of Standards and Technology

N. Alan Heckert
Statistical Engineering Division
Information Technology Laboratory
National Institute of Standards and Technology

This publication is available free of charge from:
https://doi.org/10.6028/NIST.SP.260-256

May 2025

U.S. Department of Commerce
Howard Lutnick, Secretary

National Institute of Standards and Technology
Craig Burkhardt, NIST Acting Director and Under Secretary of Commerce for Standards and Technology

NIST SP 260-256
May 2025

Certain equipment, instruments, software, or materials, commercial or non-commercial, are identified in this paper in order to specify the experimental procedure adequately. Such identification does not imply recommendation or endorsement of any product or service by NIST or the CDC, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

NIST Technical Series Policies
Copyright, Use, and Licensing Statements
NIST Technical Series Publication Identifier Syntax

Publication History
Approved by the NIST Editorial Review Board on 2025-05-12

How to Cite this NIST Technical Series Publication
Boggs ASP, Burdette CQ, Camara J, Hoguet J, Huncik K, Ragland J, Wood ESC (2025) Certification of Standard Reference Material®s 3672a & 3673a: Organic Contaminants in Smokers' Urine (Frozen) & Organic Contaminants in Non-Smokers' Urine (Frozen). (National Institute of Standards and Technology, Gaithersburg, MD), NIST Special Publication (NISTSP) 260-256. https://doi.org/10.6028/NIST.SP.260-256

Author ORCID iDs
Boggs ASP:0000-0001-9765-7559
Burdette CQ;0000-0002-0843-9224
Camara J: 0000-0002-9415-8452
Heckert NA:0000-0002-8430-6757
Hoguet JC:0009-0001-9584-9166
Huncik K:0009-0004-8039-5563
Ragland J:0000-0002-8055-2432
Wood ESC:0009-0000-8537-3006

Contact Information
Please address technical questions you may have about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Project Leader responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov

Abstract

Standard Reference Material (SRM) 3672a Organic Contaminants in Smokers' Urine (Frozen) and SRM 3673a Organic Contaminants in Non-Smokers' Urine (Frozen) are intended for use in evaluating analytical methods for the determination of selected naturally-occurring smoking-related compounds and metabolites in urine. The development of SRMs 3672a and 3673a was a collaboration between the National Institute of Standards and Technology (NIST) and the Division of Laboratory Sciences, Centers for Disease Control and Prevention (CDC). This publication documents the production, analytical methods, and statistics used to characterize these products.

Keywords

Caffeine; Creatinine; Mercapturic Acids; Nicotine; Phenolics; Phthalates; Polycyclic Aromatic Hydrocarbons (PAHs); Thiocyanate; Volatile Organic Compounds (VOCs).

Table of Contents

List of Tables

List of Figures

Acknowledgements

We would like to thank our colleagues, Deepak Bhandari, Benjamin Blount, Julianne Botelho, Christina Brosius, Antonia M. Calafat, June Feng, Maria Morel-Espinosa, Christopher Reese, Michael Rybak, Tiffany Seyler, Liza Valentin-Blasini, Lanqing Wang, and Baoyun Xia of the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention for their contributions of analyte values and measurement science expertise. We also thank David Duewer for his expertise and assistance in the production and editing of this SP-260.

1. Introduction

Biomonitoring is essential for evaluating exposures that come from people's diet and surroundings and urine sampling offers a non-invasive, easily repeatable, and low collection risk method of sample collection. In addition, urine is the bodily fluid used for the biomonitoring of compounds with short biological half-lives. Many organic compounds, including certain nicotine metabolites, polycyclic aromatic hydrocarbon (PAH), phthalate, phenol, paraben, and volatile organic compound (VOC) metabolites, are monitored in urine as part of studies such as the National Health and Nutrition Examination Survey (NHANES) [1] and the Canadian Health Measures Survey (CHMS). Accurate quantitation of urinary metabolites is critical for facilitating comparability of datasets, assessing temporal trends, and developing public health initiatives associated with exposures. Other compounds of interests are monitored for clinical health. For example, urine creatinine levels are indicators of kidney function, and the measurement of other analytes is often normalized to urine creatinine levels to account for sample dilution. Therefore, accurate measurement of urine creatinine is of importance to the clinical, forensic, and toxicology communities.

In collaboration with the Centers for Disease Control and Prevention (CDC), the National Institute of Standards and Technology (NIST) introduced SRM 3672 Organic Contaminants in Smokers' Urine (Frozen) and SRM 3673 Organic Contaminants in Non-Smokers' Urine (Frozen) in 2014 [2,3,4]. These reference materials provided researchers with valuable first-of-their-kind tools for evaluating analytical methods for hydroxylated PAHs (OH-PAHs), phthalate metabolites, phenol metabolites, VOC metabolites, and other key analytes, such as creatinine and nicotine. The sales history of SRM 3672 and 3673 are displayed in Fig. 1 as a function of calendar year.

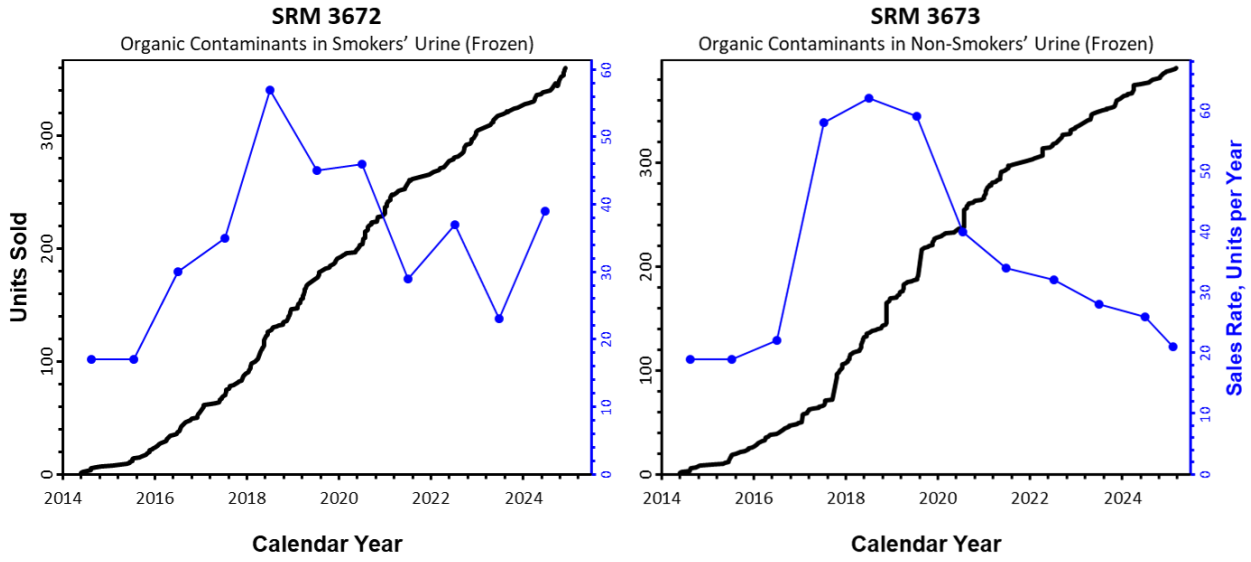


Fig. 1. Sales History of SRMs 3672 and 3673.

The thick black line in each panel depicts the cumulative distribution of sales as a function of the invoice date; it is plotted using the "Units Sold" axis at the left of each panel. The thin blue line depicts the total units sold per year; it is plotted using the "Sales Rate, Units per Year" axis to the right of each panel.

The proportions of sales to customers in the USA, Europe, Asia, and the rest of the world are displayed in Fig. 2 and Fig. 3 respectively as a whole and as a function of calendar year.

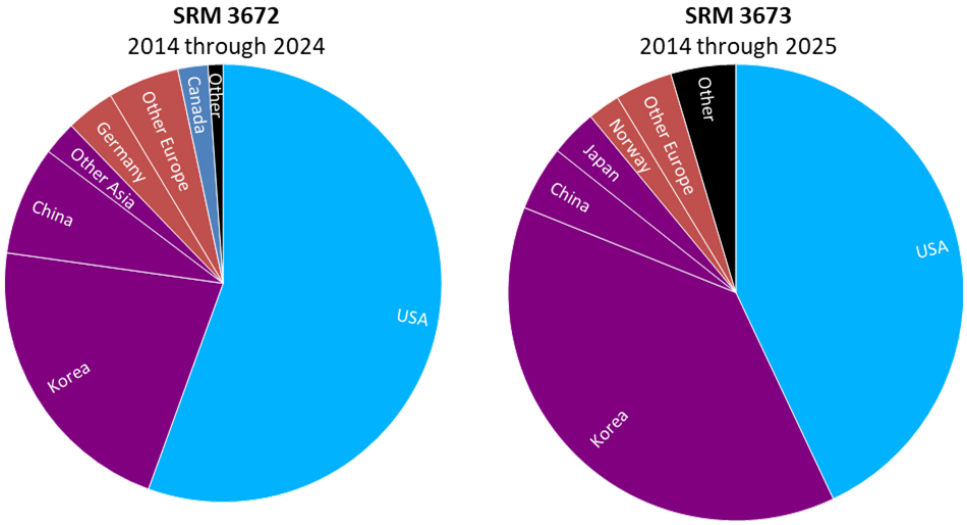


Fig. 2. Location of Customers for SRM 3672 and SRM 3673.

The pie charts display the proportion of sales to various countries or geographic regions from 2014 through October 2024. Slices are shown for individual countries only when they accounted for at least 2.5 % of the units sold. The area of the SRM 3672 pie is proportional to the number of SRM 3673 units sold.

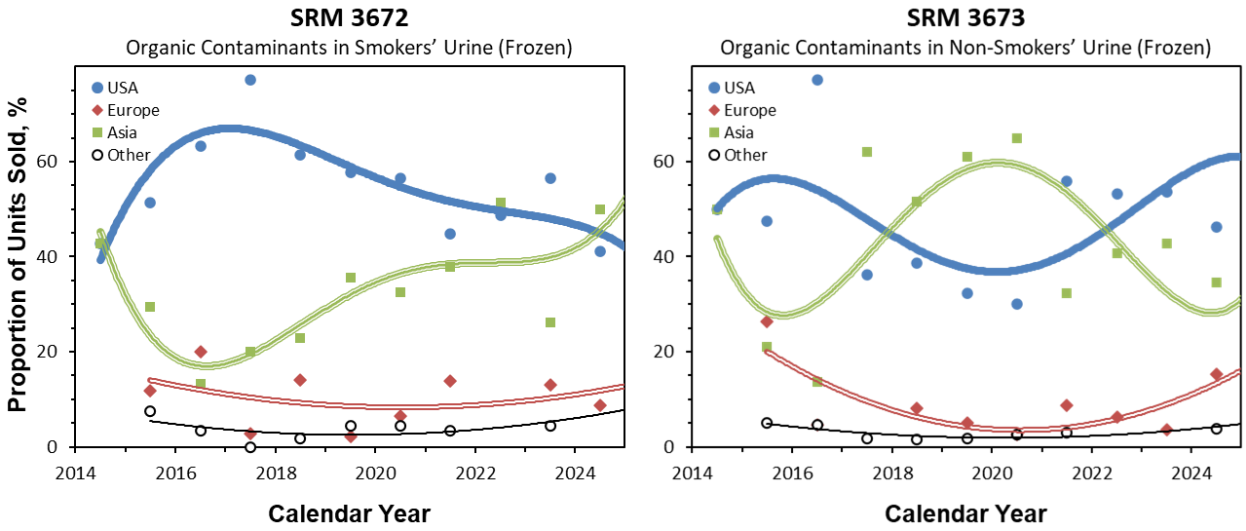


Fig. 3. Location of Customers for SRMs 3672 and 3673.

The solid circles and the thick trendlines display the proportion of sales to customers within the USA from the first sales in May of 2014 through December of 2024. Solid diamonds and the double-line trendline display the proportion of units sold to customers in Europe (including the United Kingdom) customers; solid squares and the triple-line trendline display the proportion solid to customers in Asia. The open triangles and thin single-line trendline display the proportion of units sold to customers elsewhere.

With consistent sales ranging from 20 to > 60 units per year since their release, it became evident that the SRM 3672 and SRM 3673 stocks would eventually be depleted. In 2020, NIST procured two new pools to develop the replacement materials, SRM 3672a and SRM 3673a. These materials were not amended, meaning all analytes are reflective of real-world exposures. These updated reference materials are designed to meet the evolving needs of the analytical chemistry community and ensure the continuity of high-quality standards for exposure analysis.

A unit of SRM 3672a consists of 5 vials each containing 5 mL of frozen human urine from a smokers' pool, and a unit of SRM 3673a consists of 5 vials each containing 5 mL of frozen human urine from a non-smokers' pool. The materials were analyzed at NIST for density, caffeine and metabolites, OH-PAHs, creatinine, and phthalate metabolites. The materials were also sent to collaborators at the CDC for additional measurements for caffeine and metabolites, nicotine and metabolites, OH-PAHs, phthalate metabolites, VOCs, pesticides, phenols, parabens, and other compounds from personal care products.

Metrological traceability to the International System of Units (SI) of the certified creatinine values in SRMs 3672a and 3673a is through calibration with a neat creatinine that was purity assessed by 1H-qNMR through determinations traceable to the NIST PS1 Primary Standard for qNMR (Benzoic Acid). The purity analysis characterized this material with respect to chemical components that yield creatinine entities when analyzed using the ID-LC-MS RMP.

Similarly, metrological traceability to the SI of the certified caffeine values in SRMs 3672a and 3673a is through calibration with a neat caffeine that was purity assessed by 1H-qNMR through determinations traceable to the NIST PS1 Primary Standard for qNMR (Benzoic Acid). Two higher-order measurement methods, GC-MS and LC-MS/MS, were conducted by NIST and the CDC respectively and SRM 3673 was used by both laboratories for quality assessment.

The metrological traceability of the non-certified results for the other analytes delivered by these materials is to the calibrants used in their analysis. While not extending traceability to the SI, these results are suitable for use in quality control and harmonization between methods and laboratories.

Based on experience with SRMs 3672 and 3673, the assigned analyte levels of the SRM 3672a and SRM 3673a are expected to be stable for at least five years post-production. Stability will be evaluated within one year of the expiration date, with intermittent monitoring through its use as a control whenever caffeine and metabolites, nicotine and metabolites, OH-PAHs, phthalate metabolites, VOCs, pesticides, phenols, and/or parabens in urine measurements are performed within NIST.

2. Materials and Production

Market research was conducted and a Statement of Requirements for 3000 vials of 5 mL of urine for each material was advertised on 04/23/2020. The SRM was reduced in volume from the original 10 mL after speaking with stakeholders at the CDC. Measurement methods have improved in sensitivity, thus requiring smaller volumes for analysis. Vial size of 10 mL was considered too large considering that freeze thaw cycles would invalidate the value confidence. Amber glass vials (6 mL) with aluminum crimp caps were selected for the new materials.

The required biosafety testing of the two materials differed from each other slightly due to the sources of urine used by the Contractor. The smokers' urine was sourced from a contact that conducts viral testing in the blood for all donors. Therefore, the donors for this material have been tested and found to be hepatitis B surface antigen negative, HBV NAT negative, HIV 1 and 2 antibody negative, HIV NAT negative, HCV antibody negative, syphilis negative, west nile virus negative, zika virus RNA negative, and *T. cruzi* negative.

The source for the non-smokers' urine does not test the blood of donors for biosafety. OSHA states that "The Bloodborne Pathogens Standard, 29 CFR 1910.1030, lists a number of body fluids, in addition to blood, that are reasonably likely to transmit bloodborne pathogens. OSHA provides the basis for including the body fluids listed in the June 1988 Centers for Disease Control (CDC) Guidelines (MMWR, Volume 37, Number 24, 1988), under the definition of 'other potentially infectious materials' at page 64102 in the Federal Register, Vol. 56, No. 235, published December 6, 1991. Under these guidelines, urine is not classified as a body fluid that could reasonably transmit bloodborne pathogens. In order for urine to be classified as potentially infectious, blood must be visibly present or the presence of blood reasonably anticipated due to the patient having a medical condition that would lead to blood in the urine" [5]. Therefore, we requested that all donor samples be tested for blood in the urine (to prevent potential transmission of bloodborne pathogens in the urine) and to be tested for protein content (to prevent inclusion of pregnant or ill donors). Each urine donation was tested to ensure blood content was below five cells per microliter and protein content was less than 14 mg/dL. To maintain reasonable costs, sterility testing was completed on the full-lot post filtration at 0.2 µm to test for presence of infectious bacterial organisms.

Due to unforeseeable delays due to the pandemic, the acceptance testing period was extended to 60 days post receipt of the material as opposed to the standard 30 days to accommodate reduced staff and potential laboratory closures.

2.1. Statement of Requirements

The following sections have been extracted from the Statement of Requirements "Request for human urine for SRMs 3672a and 3673a, replacements for SRM 3672, Organic Contaminants in Smokers' Urine and SRM 3673, Organic Contaminants in Non-smokers' Urine."

2.1.1. Section II. Specifications

The Contractor shall collect 15 L of human urine with the following specifications. The donors must smoke a minimum of one pack of cigarettes per day. Cigars, pipes, or other smoking methods are not prohibited, but cigarette requirements must be met. A minimum of 10 different donors is required for pooling to ensure identity of the donor is protected.

The Contractor shall collect a second lot of 15 L of human urine with the following specifications. The donors must be non-smokers who also do not vape and are not exposed to second-hand smoke or second-hand vapor. A minimum of 10 different donors is required for pooling to ensure identity of the donor is protected.

The Contractor shall test the urine units for biosafety using culture of microorganisms. The Contractor shall provide written documentation stating the negative results of all donor units utilized for standard reference materials (SRMs) 3672a and 3673a preparations. The Contractor shall test donor urine for sterility. The Contractor shall test for blood and/or protein in the urine. If the urine is not sterile or contains blood or protein, it will be excluded from the preparation and not be added to either pool.

Donors must be 18 years old or older but can be either male or female. Female donors shall not be pregnant which can be confirmed by verbal or written questionnaire. Pregnancy testing is not required.

Urine shall be pooled into the appropriate pool, filtered and homogenized. The urine in all pools shall be dispensed into amber glass vials that are capable of withstanding ultra-cold temperatures (-80 °C). Vials shall be filled with 5.1 mL of urine (with an accuracy of 0.1 mL), stoppered with a butyl rubber stopper, and sealed with an aluminum crimp cap with scoring for easy removal. NIST will supply the Contractor with labels to affix to the vials. The delivery requirement is 3,000 vials of each lot for a total of 6,000 vials.

The Contractor shall freeze the urine at -80 °C immediately after completion of bottling in packaging that shall not break upon freezing or thawing. Packages should be labeled only with the labels provided by NIST, indicating the appropriate SRM name. No personal identification or information of the donor shall be given to NIST. The Contractor shall maintain the urine at -80 °C until ready to overnight ship to NIST, frozen on dry ice. The Contractor shall ensure that the urine is NEVER thawed once frozen or the package may be rejected.

The contractor shall number the boxes containing the vials according to the order they were filled during aliquoting. The boxes shall be marked with a “start” and “end” position and a directionality of filling.

The Contractor shall provide NIST with details of the steps involved in material preparation no later than 21 calendar days after the receipt of the labels provided by NIST to ensure documentation of any deviations as well as to provide information that shall be utilized in the NIST material acquisition Report of Analysis. The Contractor shall provide NIST with the proof of protocol approval from a private Institutional Review Board (IRB).

The Contractor will work with the NIST technical point of contact (TPOC) to obtain NIST Research Protections Office (RPO) institutional approval. The Contractor shall NEVER provide the identity of the donors to NIST.

2.1.2. Section III. Deliverables and Deliverable Due Dates

The Contractor shall deliver the urine and biosafety report to NIST, using FOB Destination shipping terms no later than 21 calendar days after the receipt of the labels provided by NIST. However, due to current access restrictions, delivery shall not take place until the Contractor coordinates with the designated TPOC and Contracting Officer (CO).

Prior to shipment, the Contractor must ensure NIST's campus is open and accepting deliveries. The contractor shall contact the NIST TPOC to coordinate a delivery date prior to shipment. If the restrictions of access to the campus impacts delivery schedules, a modification may be executed by the CO to establish an extended delivery timeframe.

At the time of shipment, the contractor shall notify the NIST TPOC by e-mail, providing the date the deliverables are shipped, shipping method, tracking number, method of delivery and the anticipated delivery date it will arrive at NIST.

Overnight shipments should not be sent on Thursday/Friday/Saturday or before a Monday Federal Holiday. Overnight shipments should not be sent on the Tuesday or Wednesday prior to the Thanksgiving holiday. Overnight shipments should not be sent on the Wednesday before Christmas or New Year's holidays.

2.1.3. Section IV. Protection of Human Subjects

(a) Research involving human subjects is not permitted under this award unless expressly authorized in writing by the Contracting Officer. Such authorization will specify the details of the approved research involving human subjects and will be incorporated by reference into this contract.

(b) The Federal Policy for the Protection of Human Subjects (the "Common Rule"), adopted by the Department of Commerce at 15 CFR part 27, requires contractors to maintain appropriate policies and procedures for the protection of human subjects in research. The Common Rule defines a "human subject" as a living individual about whom an investigator conducting research obtains data through intervention or interaction with the individual, or identifiable private information. The term "research" means a systematic investigation, including research development, testing and evaluation, designed to develop or contribute to generalizable knowledge. The Common Rule also sets forth categories of research that may be considered exempt from 15 CFR part 27. These categories may be found at 15 CFR 27.101(b).

(c) In the event that human subjects research involves pregnant women, prisoners, or children, the contractor is also required to follow the guidelines set forth at 45 CFR part 46 subpart B, C and D, as appropriate, for the protection of members of a protected class.

(d) Should research involving human subjects be included in the proposal, prior to issuance of an award, the contractor shall submit the following documentation to the Contracting Officer:

(1) Documentation to verify that contractor has established a relationship with an appropriate Institutional Review Board ("cognizant IRB"). An appropriate IRB is one that is located within the United States and within the community in which the human subjects research will be conducted;

(2) Documentation to verify that the cognizant IRB possesses a valid registration with the United States Department of Health and Human Services' Office for Human Research Protections ("OHRP");

(3) Documentation to verify that contractor has a valid Federal-wide Assurance (FWA) issued by OHRP.

(e) Prior to starting any research involving human subjects, the contractor shall submit appropriate documentation to the Contracting Officer for institutional review and approval by the NIST RPO. This documentation may include:

(1) Copies of the human subjects research protocol, all questionnaires, surveys, advertisements, and informed consent forms approved by the cognizant IRB;

(2) Documentation of approval for the human subjects research protocol, questionnaires, surveys, advertisements, and informed consent forms by the cognizant IRB;

(3) Documentation of continuing IRB approval by the cognizant IRB at appropriate intervals as designated by the IRB, but not less than annually; and/or

(4) Documentation to support an exemption for the project from the Common Rule [Note: this option is not available for activities that fall under 45 CFR part 46 subpart C].

(f) In addition, if the contractor modifies a human subjects research protocol, questionnaire, survey, advertisement, or informed consent form approved by the cognizant IRB, the contractor shall submit a copy of all modified material along with documentation of approval for said modification by the cognizant IRB to the Contracting Officer for institutional review and approval by the NIST RPO. The contractor shall not implement any IRB approved-modification without written approval by the Contracting Officer.

(g) No work involving human subjects may be undertaken, conducted, or costs incurred and/or charged to the project, until the Contracting Officer approves the required appropriate documentation in writing.

2.1.4. Section V. Acceptance Testing

2.1.4.1. Acceptable Quality Level

The urine with the associated physical and chemical properties specified in Section II of this Statement of Requirements shall be suitable for use as reference materials. If deficiencies or inconsistencies between the material and the documentation (defined in Sections II and III) are

found by the NIST TPOC, or if less than the stated volume of urine is received intact the Contractor shall not have the opportunity to repair, replace, or reperform.

2.1.4.2. Monitoring Method

The NIST TPOC will verify that the materials were successfully prepared and acceptable. Acceptance will be based upon the delivery of 3,000 vials of each pooled urine material intact and unbroken. NIST requires a two-week acceptance testing period after receipt of delivery. A minimum of 10 vials will be tested by NIST to ensure suitable homogeneity (coefficient of variation of less than 10 % for analyte measurements made under repeatability conditions using NIST-approved established analytical methods) and adequate volume (all vials containing at least 5.0 mL as determined by calibrated pipette) for use as a NIST SRM. Due to the limited supply of input materials, the Contractor shall not have the opportunity to repair, replace, or re-perform in the event the deliverables fail acceptance testing, and will not be reimbursed for performance. Should the contractor wish to retrieve the vials from NIST in the event of a failure of the acceptance testing, the Contractor shall contact the NIST TPOC within 15 calendar days of notification of performance failure to arrange for the Contractor to retrieve the vials from NIST Gaithersburg. If the Contractor does not contact NIST within this 15 calendar day timeframe, the vials will be disposed of by NIST. Testing will be completed no later than 60 calendar days after receipt of the vials under the condition that the NIST laboratory is open to the NIST TPOC. If the NIST laboratory is closed due to social distancing requirements and accommodations cannot be made to conduct acceptance testing during this time, the acceptance testing will be completed within 60 calendar days of the NIST laboratory reopening.

2.2. Contractor's Protocol: NIST Urine

The following protocol for mixing and bottling both materials was provided by the Contractor.

2.2.1. Material Procurement:

- Urine is to be collected from both smokers and non-smokers at donor collection sites.
- Urine will be collected into sterile media bottles.
- Urine will be transferred from collection sites as collected and stored at our main lab until processing.

2.2.2. Material Storage:

Urine will be stored at 4 degrees C until total volume needed is achieved.

2.2.3. Processing Materials:

- 15 ml conical tubes
- 70 % ethanol

- Donor urine
- 0.2u (1L) Stericups
- Vacuum pump
- Fisherbrand 23-111-262 Urine test strips
- 20 L Carboy
- Stirring plate
- Stir bar
- Hamilton ML615-CNT Continuous aliquotter
- Lab cart
- Sterile Glass vials
- Sterile butyl stoppers
- Aluminum tear off crimp caps
- Product labels (NIST provided)
- 20 mm cap crimper
- Cryopro boxes w/25 place dividers

2.2.4. Testing:

1. Set up the following materials in a biosafety cabinet (BSC):
2. Donor urine, 15 ml conical tubes, Fisherbrand urine test strips, and 0.2u (1 L) Stericups filters.
3. Wipe down all material with 70 % ethanol before placing items inside biosafety cabinet.
4. Set up pump outside biosafety cabinet and connect vacuum line to 0.2u filter.
5. Label Stericup with Donor ID and add material from donor 1 to top of vacuum filter and allow to pass thru the filter at low pressure.
6. Once material has filtered thru, pour off a 10 ml side sample into a labeled tube for urine testing, cap side sample and bulk sample.
7. Repeat steps 5 and 6 for all non-smoker donors.
8. Take side samples for all donors and bring to lab bench for urine testing.
9. Uncap sample from donor 1, dip in a urine test strip, wait the required times and compare to bottle to confirm values fall within desired range.
   - If sample has value with fall outside desired range, remove bulk material from the biosafety cabinet and do not use for this project.
10. Repeat testing step 9 for all non-smoker donors until required volume has been met.

2.2.5. Pooling:

1. Once required volume has been met (approx. 17L) all donors will be pooled into a single 20L carboy.
2. Wipe down a large stir bar and stirring plate with 70 % ethanol and set them up in the biosafety cabinet.
3. Place the 20L carboy on top of the stirring plate and add the stir bar to the 20L carboy.
4. Turn on the stirring plate and ensure that the urine pool is mixing properly.
5. While the urine is mixing wipe down the Hamilton continuous aliquotter and set it up in the BSC beside the urine pool on the stirring plate.
6. Flush the lines of the Hamilton continuous aliquotter with 70 % ethanol.
7. After 2 hours of mixing, add the two intake lines from the continuous aliquotter into the 20L carboy.
8. Prime the continuous aliquotter and discard the initial 200mL to flush the machine.

2.2.6. Aliquoting:

1. Bring a lab cart with the following materials: Sterile glass vials, sterile butyl stoppers, and NIST labels.
2. Wipe down the outside packaging of the glass vials with 70 % ethanol and open the packaging inside the BSC. Each package contains 219 sterile glass vials on a tray.
3. Wipe down the sterile butyl stopper package and open inside the BSC.
4. One technician in the hood will remove all vials from the tray.
5. The same technician will label the glass vials and pass to a second technician who will fill the vial to 5.1 ml using the continuous aliquotter.
6. The vial will then be passed to a third technician who will place the butyl stopper into the vial.
7. The vial will then be placed back into the original tray.
8. The bottom right corner of the tray will be marked denoting the first vials filled.
9. The tray will continue to be filled from right to left in rows starting at the front and finishing at the back of the tray.
10. Once they tray is filled, a fourth technician will remove the tray from the hood.
11. This technician will have the aluminum tear away caps.
12. They will place the caps over the butyl stopper and crimp it down using a 20mm cap crimper.
13. They will then place the finished samples into a cryobox with a 25-place divider.

14. The first sample will be in the top left with the last sample in the bottom right of the box.
15. Boxes will be numbered 1-120.
16. [Identification] labels will be added to the cryobox to denote the box numbers and reference order of filling.
17. Samples will be frozen at -20 degrees C until shipment

3. Density

The Certificates of Analysis for SRMs 3672a and 3673a report measurand concentrations in units of mass fraction (g/g). The clinical community typically uses units of mass concentration (g/mL). Interconversion of these units requires knowledge of the densities of the two materials.

Determinations of urine density were made using a commercial DMA 35 digital density meter instrument.

3.1. Materials

Three vials each of SRMs 3672a and3673a were selected for analysis. The vials were chosen to representatively span their respective production lots.

Nanopure water from the MiliQ system (18.2 Mohm) was used validate the calibration of the density meter.

3.2. Analysis

Vials were inverted gently several times to homogenize the contents then emptied into a beaker for ease of access. Approximately 2 mL was collected using a plastic 5 mL luer-lock syringe, and the urine injected slowly into the oscillation loop of a DMA 35 digital density meter. The density reading was allowed to stabilize and then recorded. Sample was then returned to the beaker and measured an additional two times for a triplicate per vial. Between samples the beaker was wiped dry, and syringe dried by collecting and ejecting air repeatedly.

Temperature and density were collected for each measurement using the record button which waits for stable measurement.

Three replicate measurements of the Nanopure water were made using the DMA 35 digital density meter with the measured valued referenced against the expected density of pure water at the measured temperature. Results are listed in Table 1. All calibration measurements were within 0.0001 g/mL.

Table 1. Calibration Validation Measurements.

		Density, g/mL		
Replicate	Temperature, °C	Measured	Reference	Difference
1	21.4	0.9980	0.99790	0.0001
2	21.4	0.9979	0.99790	0.0000
3	21.5	0.9979	0.99788	0.0000

3.3. Assigned Values

The measured densities for SRMs 3672a and 3673a are listed in Table 2. Except for the three replicates for vial 2 of SRM 3672a, the measurements are self-consistent. The standard deviations across vials and replicates for both SRMs is 0.0001 g/mL.

The sample from vial 2 was slightly contaminated with rinse water from an incompletely dried beaker. The mistake was realized, and the beaker was dried for all following samples. Since there was no significant difference between the measurements for vial 1 and vial 3 of the SRM, no further measurements were deemed necessary.

The temperature during the measurement campaign remained adequately constant, only ranging between 21.8 °C and 22.4 °C. Measurement-by-measurement temperature adjustment is not required.

Table 2.Density Measurements for SRMs 3672a and 3673a.

		SRM 3672a		SRM 3673a	
Vial	Replicate	Temperature, °C	Density, g/mL	Temperature, °C	Density, g/mL
1	1	22.3	1.0050	22.3	1.0073
1	2	22.0	1.0052	22.1	1.0074
1	3	21.8	1.0053	21.9	1.0074
2	1	22.1	contaminated	22.2	1.0074
2	2	21.9	contaminated	22.1	1.0074
2	3	21.8	contaminated	21.9	1.0075
3	1	22.3	1.0051	22.4	1.0073
3	2	22.1	1.0052	22.2	1.0074
3	3	22.1	1.0052	22.1	1.0074
Number Readings:		9	6	9	9
Mean:		22.0	1.0052	22.1	1.0074
Standard Deviation:		0.2	0.0001	0.2	0.0001
Standard Uncertainty:			0.0001		0.0001

3.3.1. Metrological Traceability

The density results are metrologically traceable to the procedure defined in documentary standards ASTM D7777 and ISO 15212-1 [6,7].

4. Value Assignment Calculations

4.1. Analyte Evaluated Using a Single Method

The value and uncertainties for an analyte, *y*, evaluated using just one measurement method are calculated using the model:

$$\boldsymbol{y_i = \mu + \epsilon_i;\ i = 1, 2 \ldots, n} \quad (1)$$

where *i* indexes replication, μ is the true value, *n* represents the number of replications, and $\boldsymbol{\epsilon_i \sim N(0, \sigma^2)}$ – which is compact notation for "the measured differences from the mean value are independent and identically distributed random variates from a normal (Gaussian) distribution of mean zero and standard deviation σ."

The assigned value is the arithmetic mean of the y_i, $\bar{y}$, which is an estimate of μ. The standard uncertainty of the mean, $u(\bar{y})$, is the standard deviation of the y_i divided by the square root of the number of replications. The expanded uncertainty, $U_{95}(\bar{y})$, is estimated using the Student's *t* 0.975 confidence level for *n*-1 degrees of freedom as the coverage factor:

$$\boldsymbol{U_{95}(\bar{y}) = t_{0.975,n-1} \times u(\bar{y})}\ . \quad (2)$$

4.2. Analyte Evaluated Using Multiple Methods

The value and uncertainties for an analyte, *y*, evaluated using two or more measurement methods are calculated using the model:

$$\boldsymbol{y_{ij} = \mu + m_i + \varepsilon_{ij}} \begin{cases} \boldsymbol{i = 1, 2, \ldots, n_{mm}} \\ \boldsymbol{j = 1, 2, \ldots, n_i} \end{cases} \quad (3)$$

where *i* indexes measurement methods, *j* indexes replication within measurement method, n_{mm} represents the number of measurement methods, n_i represents the number of replications within measurement method, $m_i \sim N(0, \sigma_m^2)$, and $\epsilon_{ij} \sim N(0, \sigma_i^2)$ independently of method.

The assigned value is the DerSimonian-Laird consensus estimator of μ, $\bar{y}_{\mathrm{DL}}$ [8]. The standard uncertainty and the expanded uncertainties that approximate a 95 % confidence interval were estimated using a parametric bootstrap method [9,10]. The standard uncertainty $u(\bar{y}_{\mathrm{DL}})$, is estimated as the standard deviation of the bootstrap values. A symmetrical expanded uncertainty, $U_{95}(\bar{y}_{\mathrm{DL}})$, is estimated as the half-width of the interval from the 2.5$^{\text{th}}$ percentile to the 97.5$^{\text{th}}$ percentile of the bootstrap values.

4.3. Unit Conversions

NIST's measurements are made using gravimetric preparation and are recorded as mass fractions, w_{analyte}, expressed in units proportional to g/g. CDC's measurements are made using volumetric preparation and are recorded as mass concentration, x_{analyte}, expressed in units proportional to g/L or as amount-of-substance concentration, c_{analyte}, expressed in units proportional to mol/L.

The mass fraction and mass concentration of an analyte are related through the density of the matrix, ρ, usually expressed in units of g/mL:

$$\left(w_{\text{analyte}}\,\frac{\text{g}}{\text{g}}\right) = \left(x_{\text{analyte}}\,\frac{\text{g}}{\text{L}}\right)\Big/\left(\rho_{\text{matrix}}\,\frac{\text{g}}{\text{mL}}\,\frac{1000\ \text{mL}}{\text{L}}\right)$$
$$u\left(w_{\text{analyte}}\right) = w_{\text{analyte}}\sqrt{\left(\frac{u\left(x_{\text{analyte}}\right)}{x_{\text{analyte}}}\right)^2 + \left(\frac{u\left(\rho_{\text{matrix}}\right)}{\rho_{\text{matrix}}}\right)^2}\,. \tag{4}$$

The mass fraction and amount-of-substance concentration and are related through both the density of the matrix and the molar mass (molecular weight) of the analyte, *M*, usually expressed in units of g/mol:

$$\left(w_{\text{analyte}}\,\frac{\text{g}}{\text{g}}\right) = \left(c_{\text{analyte}}\,\frac{\text{mol}}{\text{L}}\right)\left(\frac{M_{\text{analyte}}\,\frac{\text{g}}{\text{mol}}}{\rho_{\text{matrix}}\,\frac{\text{g}}{\text{mL}}\,\frac{1000\ \text{mL}}{\text{L}}}\right)$$
$$u\left(w_{\text{analyte}}\right) = w_{\text{analyte}}\sqrt{\left(\frac{u\left(x_{\text{analyte}}\right)}{x_{\text{analyte}}}\right)^2 + \left(\frac{u\left(\rho_{\text{matrix}}\right)}{\rho_{\text{matrix}}}\right)^2 + \left(\frac{u\left(M_{\text{analyte}}\right)}{M_{\text{analyte}}}\right)^2}\,. \tag{5}$$

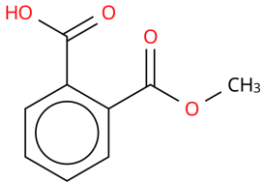
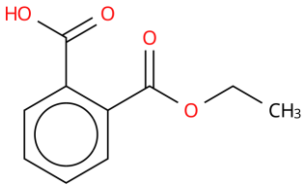
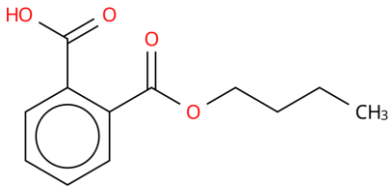
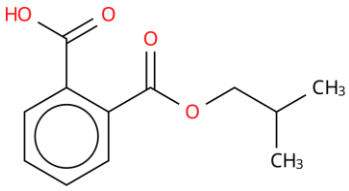
Note: The uncertainties associated with density determinations and molar masses are typically very much smaller than those associated with analyte replicate measurements but are included in the transformations to accord with metrological formalism.

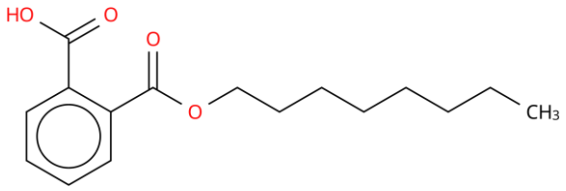
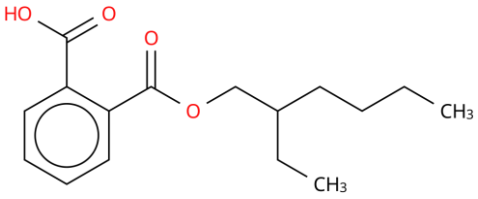
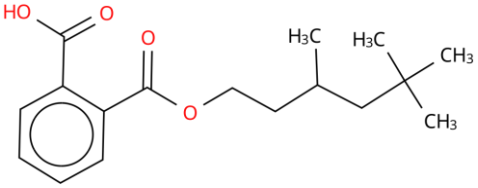
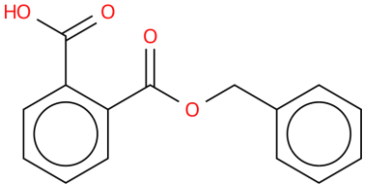
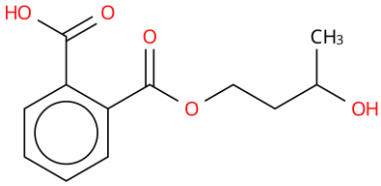
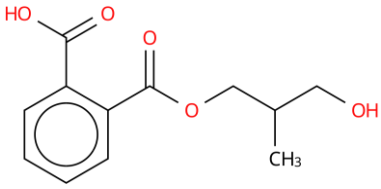
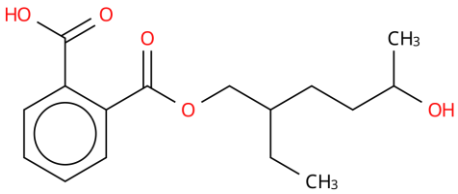
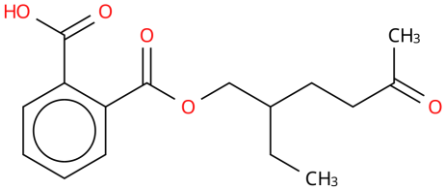
5. Phthalate, Terephthalate, and DINCH Metabolites

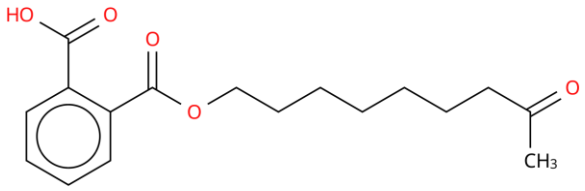
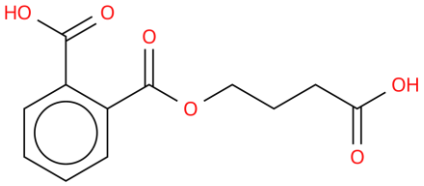
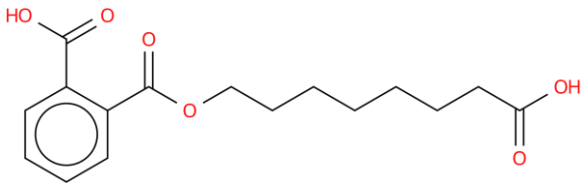
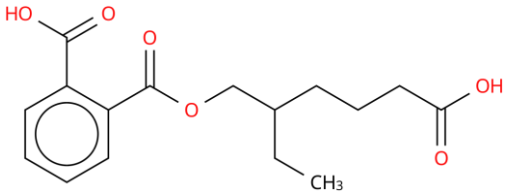
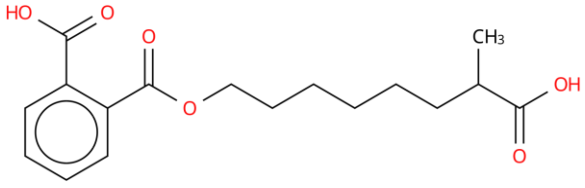
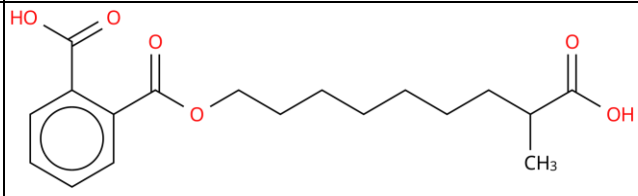
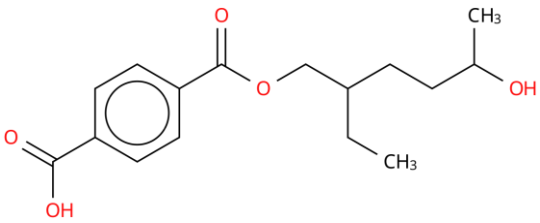
Diester phthalates, terephthalates, and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) are industrial chemicals widely used in consumer products as solvents, additives, and/or plasticizers. Some of these chemicals are known to cause carcinogenic, reproductive, and development toxicities in animals. Human exposure is through food, water, air, and use of some consumer products. After exposure, diesters are rapidly metabolized to their respective hydrolytic monoesters. Some monoesters can be further metabolized to their oxidative products before excretion in urine or feces either as free or conjugated species. These metabolites have been used as biomarkers of exposure to phthalates and related chemicals [11].

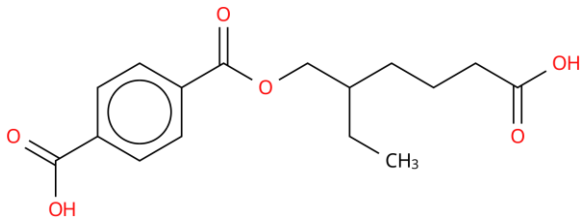
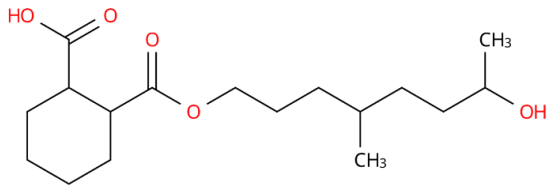
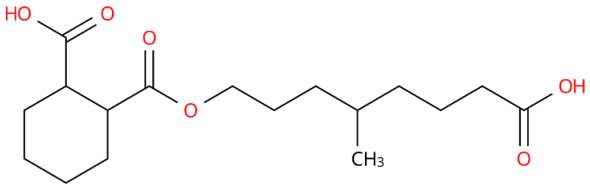
Table 3 describes the phthalate, terephthalate, and 1,2-cyclohexanedioic acid metabolites assayed by the CDC and/or NIST. The analytes are identified by their acronym, common name, International Chemical Identifier (InChI), and chemical structure. The analytes are listed in order of increasing structural complexity, with the phthalate, terephthalate, and 1,2-cyclohexanedioic acid metabolites grouped separately.

Table 3. Phthalate-Related Metabolite Structures.

Analyte	Structure
MMP mono-methyl phthalate InChI=1S/C9H8O4/c1-13-9(12)7-5-3-2-4-6(7)8(10)11/h2-5H,1H3,(H,10,11)	
MEP mono-ethyl phthalate InChI=1S/C10H10O4/c1-2-14-10(13)8-6-4-3-5-7(8)9(11)12/h3-6H,2H2,1H3,(H,11,12)	
MBP mono-n-butyl phthalate InChI=1S/C12H14O4/c1-2-3-8-16-12(15)10-7-5-4-6-9(10)11(13)14/h4-7H,2-3,8H2,1H3,(H,13,14)	
MiBP mono-isobutyl phthalate InChI=1S/C12H14O4.H3N/c1-8(2)7-16-12(15)10-6-4-3-5-9(10)11(13)14;/h3-6,8H,7H2,1-2H3,(H,13,14);1H3	

Analyte	Structure
MOP mono-octyl phthalate InChI=1S/C16H22O4/c1-2-3-4-5-6-9-12-20-16(19)14-11-8-7-10-13(14)15(17)18/h7-8,10-11H,2-6,9,12H2,1H3,(H,17,18)	
MEHP mono-2-ethylhexyl phthalate InChI=1S/C16H22O4/c1-3-5-8-12(4-2)11-20-16(19)14-10-7-6-9-13(14)15(17)18/h6-7,9-10,12H,3-5,8,11H2,1-2H3,(H,17,18)	
MiNP mono(3,5,5-trimethylhexyl) phthalate InChI=1S/C17H24O4/c1-12(11-17(2,3)4)9-10-21-16(20)14-8-6-5-7-13(14)15(18)19/h5-8,12H,9-11H2,1-4H3,(H,18,19)	
MBzP monobenzyl phthalate InChI=1S/C15H12O4/c16-14(17)12-8-4-5-9-13(12)15(18)19-10-11-6-2-1-3-7-11/h1-9H,10H2,(H,16,17)	
MHBP mono-hydroxybutyl phthalate InChI=1S/C12H14O5/c1-8(13)6-7-17-12(16)10-5-3-2-4-9(10)11(14)15/h2-5,8,13H,6-7H2,1H3,(H,14,15)	
MHiBP mono-hydroxyisobutyl phthalate InChI=1S/C12H14O5/c1-8(6-13)7-17-12(16)10-5-3-2-4-9(10)11(14)15/h2-5,8,13H,6-7H2,1H3,(H,14,15)	
MEHHP mono-2-ethyl-5-hydroxyhexyl phthalate InChI=1S/C16H22O5/c1-3-12(9-8-11(2)17)10-21-16(20)14-7-5-4-6-13(14)15(18)19/h4-7,11-12,17H,3,8-10H2,1-2H3,(H,18,19)	
MEOHP mono-2-ethyl-5-oxohexyl phthalate InChI=1S/C16H20O5/c1-3-12(9-8-11(2)17)10-21-16(20)14-7-5-4-6-13(14)15(18)19/h4-7,12H,3,8-10H2,1-2H3,(H,18,19)	

Analyte	Structure
MONP monooxononyl phthalates InChI=1S/C17H22O5/c1-13(18)9-5-3-2-4-8-12-22-17(21)15-11-7-6-10-14(15)16(19)20/h6-7,10-11H,2-5,8-9,12H2,1H3,(H,19,20)	 plus nonyl isomers
MCPP mono-3-carboxypropyl phthalate InChI=1S/C12H12O6/c13-10(14)6-3-7-18-12(17)9-5-2-1-4-8(9)11(15)16/h1-2,4-5H,3,6-7H2,(H,13,14)(H,15,16)	
MCHpP mono(7-carboxyheptyl) phthalate InChI=1S/C16H20O6/c17-14(18)10-4-2-1-3-7-11-22-16(21)13-9-6-5-8-12(13)15(19)20/h5-6,8-9H,1-4,7,10-11H2,(H,17,18)(H,19,20)	
MECPP mono-2-ethyl-5-carboxypentyl phthalate InChI=1S/C16H20O6/c1-2-11(6-5-9-14(17)18)10-22-16(21)13-8-4-3-7-12(13)15(19)20/h3-4,7-8,11H,2,5-6,9-10H2,1H3,(H,17,18)(H,19,20)	
MCOP monocarboxyisooctyl phthalate isomers InChI=1S/C17H22O6/c1-12(15(18)19)8-4-2-3-7-11-23-17(22)14-10-6-5-9-13(14)16(20)21/h5-6,9-10,12H,2-4,7-8,11H2,1H3,(H,18,19)(H,20,21)	 plus octyl isomers
MCNP mono-carboxyisononyl phthalate isomers InChI=1S/C18H24O6/c1-13(16(19)20)9-5-3-2-4-8-12-24-18(23)15-11-7-6-10-14(15)17(21)22/h6-7,10-11,13H,2-5,8-9,12H2,1H3,(H,19,20)(H,21,22)	 plus nonyl isomers
MEHHTP mono-2-ethyl-5-hydrohexyl terephthalate InChI=1S/C16H22O5/c1-3-12(5-4-11(2)17)10-21-16(20)14-8-6-13(7-9-14)15(18)19/h6-9,11-12,17H,3-5,10H2,1-2H3,(H,18,19)	

Analyte	Structure
MECPTP mono-2-ethyl-5-carboxypentyl terephthalate InChI=1S/C16H20O6/c1-2-11(4-3-5-14(17)18)10-22-16(21)13-8-6-12(7-9-13)15(19)20/h6-9,11H,2-5,10H2,1H3,(H,17,18)(H,19,20)	
MHiNCH cyclohexane-1,2-dicarboxylic acid monohydroxy isononyl ester InChI=1S/C17H30O5/c1-12(9-10-13(2)18)6-5-11-22-17(21)15-8-4-3-7-14(15)16(19)20/h12-15,18H,3-11H2,1-2H3,(H,19,20)	
MCOCH cyclohexane-1,2-dicarboxylic acid monocarboxy isooctyl ester InChI=1S/C17H28O6/c1-12(6-4-10-15(18)19)7-5-11-23-17(22)14-9-3-2-8-13(14)16(20)21/h12-14H,2-11H2,1H3,(H,18,19)(H,20,21)	

5.1. CDC Measurements

Ten vials each of SRMs 3672a and 3673a were shipped from NIST to the CDC's Division of Laboratory Sciences for assessment of phthalate, terephthalate, and DINCH metabolite concentrations. In addition to use in value-assigning these analytes, the measurements were used to assess SRM homogeneity. For both SRMs, the vials provided for assessment included the first and last vials produced and eight vials from evenly spaced intervals across the production. The vials were shipped from NIST to the CDC on dry ice and maintained in -80 °C freezers until assessment.

One vial of SRM 3673 was used for quality assessment (QA) in addition to the CDC in-house quality control (QC) materials. The QC materials were enriched with native phthalate metabolites to create low-concentration (QCL, 4–62 ng/mL depending upon the analyte) and high-concentration (QCH, 26–485 ng/mL depending upon the analyte).

5.1.1. Analysis

All sample measurements were conducted by CDC staff using the on-line solid phase extraction coupled with isotope dilution–high performance liquid chromatography–tandem mass spectrometry (SPE–HPLC–MS/MS) methods described in [11,12]]. Eighteen metabolites were assessed: MEP, MBP, MiBP, MEHP, MBzP, MHBP, MHiBP, MEHHP, MEOHP, MONP, MCPP, MECPP, MCOP, MCNP, MEHHTP, MECPTP, MHiNCH, and MCOCH. The following sections briefly describe the measurements.

5.1.1.1. Sample Preparation

A 0.1 mL aliquot of unknown and QA/QC samples and with 0.1 mL of de-ionized water was dispensed into silanized autosampler vials along with 10 calibration standards. β-glucuronidase in pH 6.5 ammonium acetate buffer was added to all samples to enzymatically deconjugate phthalate metabolites from their glucuronidated form and 4-Methyl-umbelliferyl-glucuronide as a control to monitor de-conjugation. Samples were incubated at 37 °C for a minimum of 2 hours. 200 µL of a solution containing 20 % acetic acid, 5 % acetonitrile, and 75 % water was added to each sample and held at -70 °C until analysis.

5.1.1.2. Instrumental Method

Samples were loaded onto autosampler trays for analysis. During analysis, the temperature of the autosampler tray compartment was set to 10 °C. 500 µL of each sample was loaded onto a Chromolith® HighResolution RP-18 endcapped (monolithic) guard cartridge (5 mm x 2 mm) (Merck KGaA, Germany) for preconcentration of the analytes. A gradient of 0.1 % acetic acid in water and 0.1 % acetic acid in acetonitrile was used for SPE cleanup of the sample. the analytes were transferred onto a Betasil Phenyl analytical column (3 µm, 150 mm × 2.1 mm, ThermoFisher Scientific, Bellefonte, PA, USA) which was preceded by 2 µm and 0.5 µm inline filters (Upchurch Scientific, Oak Harbor, WA, USA). The analytes were chromatographically resolved using a nonlinear solvent gradient from 77 % mobile phase A (0.1 % acetic acid in water) to 100 % mobile phase B (0.1 % acetic acid in acetonitrile) at a flow rate of 0.35 mL/min. Mass specific detection was achieved using a ThermoFisher Scientifc TSQ Altis™ Triple Quadrupole Mass Spectrometer equipped with an electrospray ionization (ESI) interface in the multiple reaction monitoring (MRM) in negative ion mode. Data acquisition and analysis were performed using Xcalibur software.

5.1.1.3. Quantitation

Matched ^{13}C or deuterated-labeled internal standards were used for analysis. Calibration curves, weighted by the reciprocal of the standard concentration of the peak area of each analyte ion divided by the peak area of its isotope-labeled standard versus standard concentration, were used to extrapolate measured values. For the analytes that represented one of many possible isomers (MCOP, MCNP, MONP, MHiNCH, and MCOCH), the whole cluster of peaks was integrated. Peak integrations were corrected manually, if necessary. The results sent to NIST were reviewed by the CDC's quality assurance officer and approved as conforming to the quality standards at the CDC.

Table 4 provides the one-vial three replicate measurements for SRM 3673. Table 5 and Table 6 provide the measurement results for SRMs 3672a and 3673a, with separate summary statistics for the ten-vial single-aliquot measurements and the one-vial three replicate measurements. These results were recorded and are listed here as amount concentrations, $x_{analyte}$. They are transformed to mass fractions, $w_{analyte}$, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3.

Table 4. CDC Results for Phthalate and DINCH Metabolites in SRM 3673, ng/mL.

Sample	MEP	MBP	MiBP	MEHP	MBzP	MHBP	MHiBP	MEHHP	MEOHP	MONP	MCPP	MECPP	MCOP	MCNP	MEHHTP	MECPTP	MHiNCH	MCOCH
Replicate-1	90.2	9.9	3.9	5.5	6.4	0.9	1.4	22.7	14.5	3.0	1.6	31.0	11.8	1.8	<0.4	0.5	<0.4	<0.4
Replicate-2	88.8	10.1	3.6	5.5	6.4	1.0	1.3	21.9	14.3	2.9	1.5	32.4	12.1	2.0	<0.4	0.5	<0.4	<0.4
Replicate-3	88.3	10.3	3.9	5.8	6.2	0.9	1.4	22.5	14.3	2.9	1.6	31.6	12.2	2.0	<0.4	0.5	<0.4	<0.4
N:	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0	3	0	0
Mean:	89.1	10.1	3.8	5.6	6.3	0.9	1.4	22.4	14.4	2.9	1.6	31.7	12.0	1.9	<0.4	0.5	<0.4	<0.4
SD:	1.0	0.2	0.2	0.2	0.1	0.1	0.1	0.4	0.1	0.1	0.1	0.7	0.2	0.1		0.0		

Table 5. CDC Results for Phthalate and DINCH Metabolites in SRM 3672a, ng/mL.

Sample	MEP	MBP	MiBP	MEHP	MBzP	MHBP	MHiBP	MEHHP	MEOHP	MONP	MCPP	MECPP	MCOP	MCNP	MEHHTP	MECPTP	MHiNCH	MCOCH
Vial-1	479	14.1	7.0	0.9	3.4	1.3	2.1	5.3	2.6	1.1	1.0	5.8	3.4	0.7	8.5	56.2	1.1	<0.5
Vial-2	498	14.8	7.4	1.1	3.3	1.1	1.9	5.3	2.5	1.1	0.9	5.9	3.4	0.6	8.5	56.2	1.0	<0.5
Vial-3	493	14.8	7.5	1.2	3.4	1.1	1.9	5.3	2.5	1.0	0.9	5.7	3.4	0.6	8.5	55.5	0.9	<0.5
Vial-4	500	14.3	7.5	1.0	3.3	1.2	1.9	5.5	2.5	1.0	0.8	5.8	3.3	0.6	8.4	56.5	1.1	<0.5
Vial-5	483	14.4	7.2	0.8	3.2	1.1	1.9	5.2	2.5	1.0	0.9	5.6	3.4	0.6	7.7	54.4	1.1	<0.5
Vial-6	478	12.0	5.9	1.0	3.1	1.0	1.8	4.8	2.3	0.9	0.9	5.1	2.9	0.6	7.8	50.1	0.9	<0.5
Vial-7	485	13.6	6.4	0.8	3.3	1.1	1.9	5.1	2.5	1.0	0.8	5.6	3.4	0.6	8.0	55.7	1.0	<0.5
Vial-8	497	14.0	7.4	1.1	3.6	1.1	2.1	5.1	2.8	1.0	1.0	5.9	3.5	0.8	9.4	60.1	1.1	0.6
Vial-9	475	13.0	6.8	1.1	3.3	1.1	1.9	5.0	2.6	1.0	0.9	5.5	3.1	0.7	8.5	54.2	0.9	<0.5
Vial-10	480	13.7	7.1	1.0	3.4	1.0	2.0	5.0	2.7	1.0	1.0	5.8	3.1	0.7	8.3	55.8	0.9	<0.5
N:	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	1
Mean:	486.8	13.9	7.0	1.0	3.3	1.1	1.9	5.2	2.6	1.0	0.9	5.7	3.3	0.7	8.4	55.5	1.0	≈0.5
SD:	9.3	0.9	0.5	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.2	0.1	0.5	2.5	0.1	
Replicate-1	487	14.3	7.2	1.1	3.4	1.1	2.0	5.4	2.6	1.0	0.9	5.9	3.2	0.8	8.5	56.9	1.1	<0.5
Replicate-2	487	14.3	7.1	1.0	3.3	1.1	1.9	5.1	2.5	1.0	0.8	5.7	3.3	0.7	8.2	54.7	1.0	<0.5
Replicate-3	473	14.9	6.9	0.8	3.3	1.2	1.9	5.1	2.5	1.0	0.9	5.6	3.1	0.6	8.3	55.8	0.9	<0.5
N:	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0
Mean:	482	14.5	7.1	1.0	3.3	1.1	1.9	5.2	2.5	1.0	0.9	5.7	3.2	0.7	8.3	55.8	1.0	<0.5
SD:	8	0.3	0.2	0.2	0.1	0.1	0.1	0.2	0.1		0.1	0.2	0.1	0.1	0.2	1.1	0.1	

Table 6. CDC Results for Phthalate and DINCH Metabolites in SRM 3673a, ng/mL.

Sample	MEP	MBP	MiBP	MEHP	MBzP	MHBP	MHiBP	MEHHP	MEOHP	MONP	MCPP	MECPP	MCOP	MCNP	MEHHTP	MECPTP	MHiNCH	MCOCH
Vial-1	20.7	6.2	4.0	<0.8	0.9	0.5	1.2	3.1	1.9	0.8	0.4	3.8	2.9	1.7	11.4	94.8	0.8	<0.5
Vial-2	20.1	6.1	4.0	<0.8	0.8	0.5	1.1	3.1	1.9	0.8	0.4	3.9	3.0	1.8	12.1	97.1	0.8	<0.5
Vial-3	20.6	6.4	4.1	<0.8	0.8	0.6	1.2	3.2	1.9	0.7	0.5	4.0	3.1	1.8	12.0	97.0	0.8	<0.5
Vial-4	20.9	6.0	4.0	0.9	0.8	0.5	1.1	3.1	1.8	0.8	0.5	3.7	3.0	1.7	12.0	92.2	0.7	<0.5
Vial-5	19.8	6.0	4.0	<0.8	0.8	0.5	1.1	3.0	1.8	0.7	<0.4	3.5	2.7	1.6	11.6	92.5	0.7	<0.5
Vial-6	20.8	6.2	4.1	<0.8	1.0	0.5	1.2	3.1	2.1	0.8	0.6	3.9	3.4	2.0	12.6	99.3	0.9	<0.5
Vial-7	20.0	5.6	4.0	0.9	1.0	0.6	1.2	3.1	2.0	0.8	0.5	3.7	3.1	1.8	11.1	93.8	0.8	<0.5
Vial-8	20.3	6.0	4.0	0.8	0.9	0.5	1.2	3.1	2.0	0.8	0.5	3.7	3.1	1.8	11.9	95.4	0.8	<0.5
Vial-9	20.6	6.6	3.9	0.8	0.9	<0.4	1.1	3.1	2.0	0.8	0.5	4.0	3.0	1.8	12.1	97.5	0.7	<0.5
Vial-10	20.5	6.1	4.1	0.9	0.9	0.5	1.2	3.0	2.0	0.8	0.5	3.7	3.0	1.8	12.1	95.6	0.7	<0.5
N:	10	10	10	5	10	9	10	10	10	10	9	10	10	10	10	10	10	0
Mean:	20.4	6.1	4.0	≈0.8	0.9	0.5	1.2	3.1	1.9	0.8	0.5	3.8	3.0	1.8	11.9	95.5	0.8	<0.5
SD:	0.4	0.3	0.1		0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.2	0.2	0.1	0.4	2.3	0.1	
Replicate-1	21.7	6.3	4.2	<0.8	0.8	0.5	1.2	3.3	1.9	0.8	0.4	3.9	2.9	1.7	11.7	95.5	0.8	<0.5
Replicate-2	21.0	6.1	4.2	<0.8	0.9	0.4	1.2	3.1	2.1	0.8	0.4	3.8	2.9	1.8	11.9	98.0	0.7	<0.5
Replicate-3	21.2	6.3	4.5	<0.8	0.9	0.5	1.1	3.3	2.0	0.7	<0.4	3.9	3.2	1.9	12.5	100.0	0.8	<0.5
N:	3	3	3	0	3	3	3	3	3	3	2	3	3	3	3	3	3	0
Mean:	21.3	6.2	4.3	<0.8	0.9	0.5	1.2	3.2	2.0	0.8	≈0.4	3.9	3.0	1.8	12.0	97.8	0.8	<0.5
SD:	0.4	0.1	0.2		0.1	0.1	0.1	0.1	0.1	0.1		0.1	0.2	0.1	0.4	2.3	0.1	

5.1.2. Homogeneity

As shown in Fig. 4, measurement repeatability (estimated from single aliquots from ten vials) is strongly related to technical precision (estimated from three aliquots from one vial). The four assessments with technical and/or repeatability relative standard deviations (RSDs) larger than a 10 % QC threshold are for analytes with mean values very close to their method limit of detection (LOD). There is no evidence for any significant between-vial heterogeneity for either SRM.

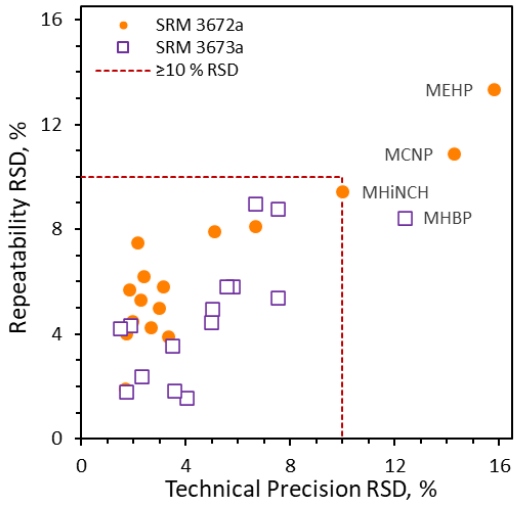


Fig. 4. Technical Precision as a Function of Repeatability for Phthalate and DINCH Analytes.

The complete sets of measurement results for most of the analytes are displayed in Fig. 5. Relatively consistent positive or negative deviations from the mean values (e.g., SRM 3672a vials 6 and 8 and SRM 3673a vial 7) suggests that much of the observed variability is related to sample preparation. There is no evidence for any production-related trend.

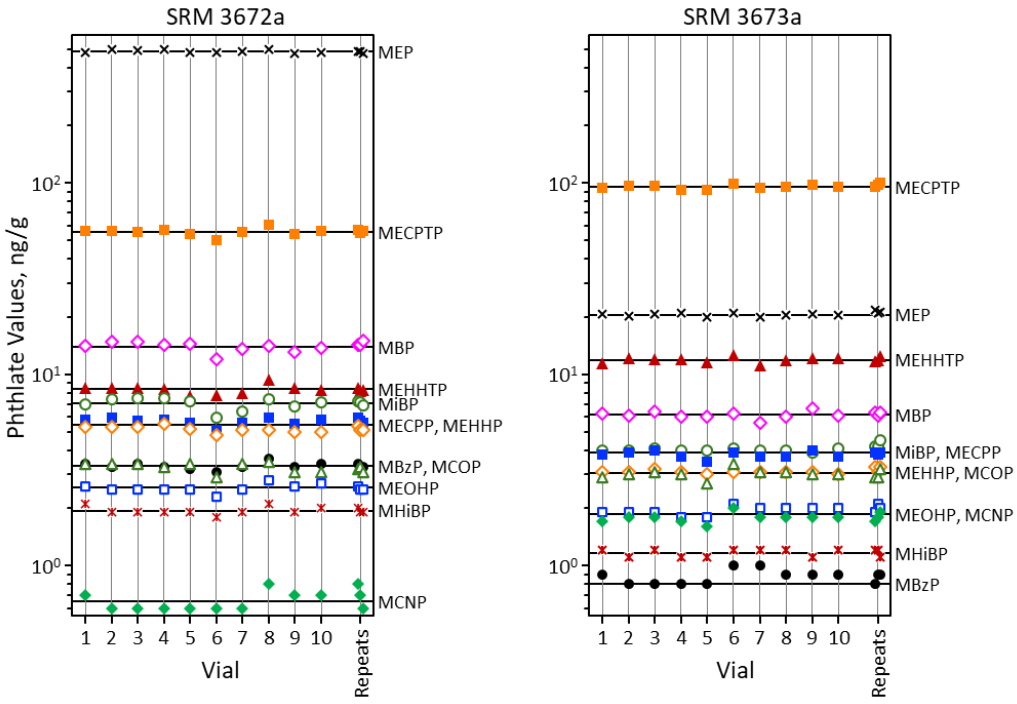


Fig. 5. Measurement Results as Functions of Vial for Phthalate-Related Analytes.

Each symbol represents one measurement result. Results for the three aliquots from one vial are displayed to the right edge of each panel. Horizontal lines denote the mean result of single aliquots from ten vials. Analyte code names are displayed at the right-end of the mean lines. Vertical lines provide visual guidance.

5.1.3. Value Assessment

To evaluate whether the measured values for SRM 3672a and 3673a are appropriate for use in value assessment, the measured values for SRM 3673 are compared to the phthalate values delivered in the SRM 3673 Certificate of Analysis (COA) [3]. The non-certified values in the certificate were determined by the CDC using the analysis methods described in [11,12].

Given that the current values are based just upon three replicate measurements and that the dispersion among these measurements represents only technical precision, Fig. 6 illustrates that there is excellent agreement between the values for the 11 phthalate metabolites common to both sets of measurement: MEP, MBP, MiBP, MEHP, MBzP, MEHHP, MEOHP, MCPP, MECPP, MCOP, MCNP. No comparison values are available for the other six metabolites assayed to be present in SRMs 3672a and/or 3673a above their LODs: MHBP, MHiBP, MONP, MEHHTP, MECPTP, and MHiNCH. However, the observed agreement for the other 11 and the use of the same analytical procedures for all metabolites suggests that all results should be at least stable over time.

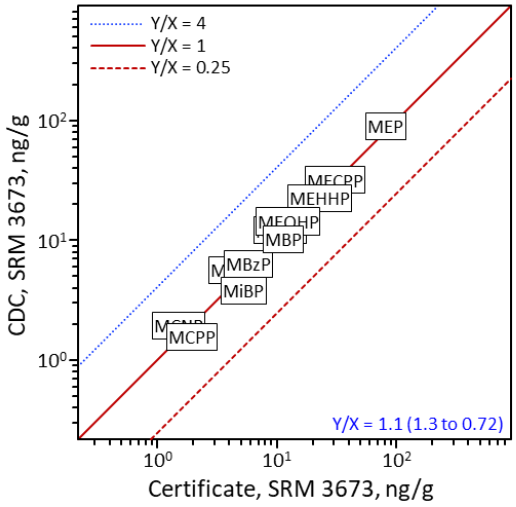


Fig. 6. CDC Phthalate-Related Results for SRM 3673 as a Function of the 2013 Non-Certified Values.

This figure compares the CDC phthalate results in SRM 3673 to the non-certified values listed in the SRM 3673 certificate of analysis (COA). Each labeled box is centered on the location {SRM 3673 COA result (X), SRM 3673 CDC current result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

5.1.4. Comparison Between SRM 3673 and SRM 3673a

As shown in Fig. 7, the concentrations of the phthalate-related metabolites in the new non-smokers' urine (SRM 3673a) are mostly about equal to about 4-fold smaller than in the original non-smokers' urine (SRM 3673). However, the concentrations of MHiBP, MCNP, and MiBP are slightly larger in SRM 3673a and the MECPTP concentration is nearly 200-fold larger. It is unknown whether these differences can be linked to changes in non-smoking exposures.

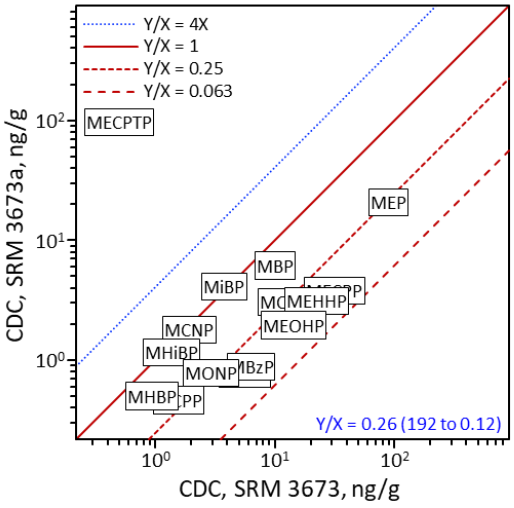


Fig. 7. SRM 3673a Phthalate-Related Results as a Function of SRM 3673 Results.

Each labeled box is centered on the location {SRM 3673 CDC current result (X), SRM 3673a CDC result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The long-dashed diagonal line denotes Y-results that are a factor of sixteen smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

5.1.5. Comparison Between Smokers' and Non-Smokers' Urine

As shown in Fig. 8, the concentrations of the phthalate-related metabolites are mostly equal to about 4-fold larger in 3672a (smokers' urine) than in 3673a (non-smokers' urine). However, the MEP concentration in SRM 3672a is about 20-fold that in 3673a and the concentrations of MCNP, MEHHTP, and MECPTP are about 2-fold larger in SRM 3673a than in 3672a. It is unknown whether these exceptional differences can be linked to smoking or are due to other exposures.

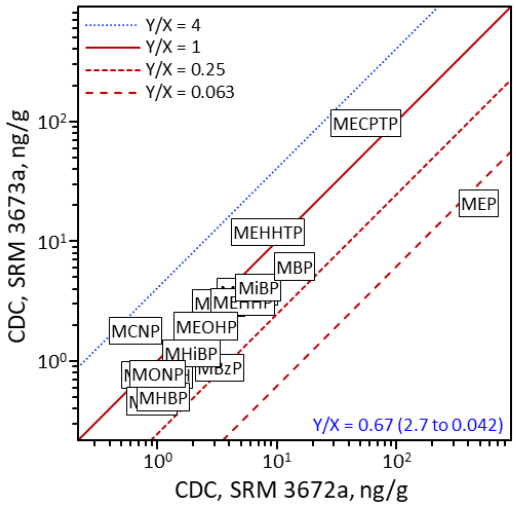


Fig. 8. SRM 3673a Phthalate and DINCH Results as a Function of SRM 3672a Results.

Each labeled box is centered on the location {SRM 3672a CDC result (X, smokers' urine), SRM 3673a CDC result (Y, non-smokers' urine)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The long-dashed diagonal line denotes Y-results that are a factor of sixteen smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

5.2. NIST Measurements

In collaboration with National Oceanic and Atmospheric Administration (NOAA) laboratories, NIST evaluated SRMs 3673, 3672a, and 3673a for thirteen phthalate metabolites using the liquid chromatography tandem mass spectrometry (LC-MS/MS) method described in [13]. All measurements were performed by NIST staff.

5.2.1. Analysis

One aliquot each of six vials of 3672a and six vials of 3673a were each analyzed. The vials of each SRM were selected based on a stratified randomized design to ensure characterization of the entire lot. Three aliquots from one vial of 3673 were used in for quality assessment. One ampoule of SRM 3060 Monoester Phthalates in Acetonitrile [14] was used to prepare stock calibration solutions.

5.2.1.1. Sample Preparation

Calibration curve preparation and gravimetric tracking of calibrants, blanks, controls and samples were conducted following NIST Technical Procedures. Three separate stock solutions were prepared by gravimetric dilution of SRM 3060 with nanopure water (18.2 Mohm). An internal standard mixture was generated from newly purchased isotopically labeled standards (Cambridge Isotope Laboratories, MA).

Samples were extracted using the NOAA approved standard operating procedure described in [13]. For each urine sample or nanopure water blank, 1 mL was gravimetrically aliquoted into an acetonitrile rinsed clean test tube and gravimetrically amended with 100 µL internal standard solution. 50 µL of 4-methylumbelliferone β-D glucuronide (4-MeUmb, 2 µg/mL) was added and vortexed. 250 µL of 1 mol/L ammonium acetate and 5 µL of β-glucuronidase (*E-coli* K12,200 units/mL, Roche, Switzerland) were added before gentle flicking to mix and incubation in a water bath at 37 °C for 90 min. Post incubation, samples were cooled to room temperature before adding 60 µL acetonitrile.

Sample cleanup was conducted using solid phase extraction with Agilent Bond Elute Nexus cartridges (3 cc, 60 mg) with the following scheme:

- condition: 1 column volume acetonitrile, 1 column volume 0.14 mol/L phosphate buffer;
- loading: at gravity speed;
- wash: 2 mL of 0.1 mol/L formic acid, 1 mL of Milli Q water;
- dry: under vacuum 30 s;
- elute: 1 mL of acetonitrile, 1 mL of ethyl acetate at gravity speed.

Eluent was dried using a Turbovap LV and reconstituted in 200 µL of Milli Q water. Samples were transferred to labeled autosampler vials containing inserts and 10 µL of 5000 ng/mL sodium dodecyl-d_{25} sulfate (SDS-d_{25}) as a recovery standard.

5.2.1.2. Instrumental Method

MMP, MEP, MBP, MiBP, MOP, MEHP, MiNP, MBzP, MEHHP, MEOHP, MCPP, MCHpP, and MECCPP concentrations were evaluated using an AB Sciex API4000 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer equipped with electrospray ionization (negative mode) attached to an Agilent 1200 Series HPLC system equipped with a binary pump and autosampler. A 5 µL injection was run on a binary gradient of acetonitrile and water both with 0.1 % acetic acid (by volume) at 350 µL/min through a Betasil Phenyl (3.0 µm, 150 mm × 2.1 mm, Thermo Scientific) column.

5.2.1.3. Quantitation

Peak areas were determined using manual integration on the Analyst (AB Sciex) software system. Calibration curves and quantitation were accomplished using NIST's Environmental Metrology Measurement Assistant (EMMA) [15]. Mass fractions of each monoester phthalate in the SRMs were calculated using linear model calibration curves without forcing the intercept through zero. Compounds were quantified using a relative response ratio to an internal standard compound that most closely matched the compound and the retention time. Final concentrations are not corrected for the purity of the neat materials.

The relative standard deviation of the peak area ratios for 4-MeUmb relative to its isotopically labeled partner were on average 3 % across all samples. This suggests that enzymatic activity of the β-glucuronidase was acceptably constant.

Table 7 provides the one-vial three replicate measurements for SRM 3673. Table 8 and Table 9 provide the measurement results for SRMs 3672a and 3673a.

Table 7. NIST Results for Phthalate-Related Metabolites in SRM 3673, ng/g.

Sample	MMP	MEP	MBP	MiBP	MOP	MEHP	MiNP	MBzP	MEHHP	MEOHP	MCPP	MCHpP	MECPP
Replicate-1		97.4	10.84			6.122		14.08	23.38	34.39	41.48	0.2255	43.72
Replicate-2		102.1	10.02			7.612		14.63	23.22	28.20	43.32	0.2427	48.92
Replicate-3		101.3	12.46			5.834		14.65	22.20	29.26	30.09	0.3522	47.68
N:		3	3			3		3	3	3	3	3	3
Mean:	<60	100.3	11.1	<3	<0.06	6.52	<6	14.45	22.93	30.6	38.3	0.273	46.8
SD:		2.5	1.2			0.95		0.32	0.64	3.3	7.2	0.069	2.7

Table 8. NIST Results for Phthalate-Related Metabolites in SRM 3672a, ng/g.

Sample	MMP	MEP	MBP	MiBP	MOP	MEHP	MiNP	MBzP	MEHHP	MEOHP	MCPP	MCHpP	MECPP
Vial-1		501.9	14.11	7.503		2.649		7.179	4.232	7.962	22.38		7.262
Vial-2		519.6	13.19	5.948		3.289		7.702	4.317	6.656	24.27		6.706
Vial-3		522.4	16.59	5.824	0.095	2.113		7.583	3.925	6.521	<11		6.849
Vial-4		500.9	15.09	6.272		1.841		7.798	4.479	7.277	24.72		7.648
Vial-5		525.9	12.66	5.817		1.607		8.129	4.353	6.878	35.57		6.361
Vial-6		521.0	12.31	7.029		1.792		7.891	4.038	7.031	21.83		6.339
N:		6	6	6		6		6	6	6	5		6
Mean:	<60	515	14.0	6.40	<0.06	2.22	<6	7.71	4.22	7.05	25.8	<0.03	6.86
SD:		11	1.6	0.71		0.64		0.32	0.21	0.52	5.6		0.52

Table 9. NIST Results for Phthalate-Related Metabolites in SRM 3673a, ng/g.

Sample	MMP	MEP	MBP	MiBP	MOP	MEHP	MiNP	MBzP	MEHHP	MEOHP	MCPP	MCHpP	MECPP
Vial-1		22.84	6.24			<1		2.012	3.029	4.069	<11		4.451
Vial-2		23.79	5.37			2.1		1.905	3.230	4.710	<11		5.341
Vial-3		22.11	6.38	4.9		1.9		2.088	3.159	5.168	<11		5.403
Vial-4		23.06	5.28			2.7		1.961	3.129	4.719	14.3		5.218
Vial-5		22.35	7.99			<1		1.953	2.855	3.840	<11		4.402
Vial-6		22.00	6.70			2.0		2.028	3.314	4.233	17.0		5.298
N:		6	6	1		4		6	6	6	2		6
Mean:	<60	22.69	6.32	<3	<0.06	≈1	<6	1.991	3.12	4.46	≈11	<0.03	5.02
SD:		0.68	0.99					0.065	0.16	0.49			0.46

Measurement values for MMP, MOP, and MiNP were at or below their reporting limits (RL, defined here as the larger of three times the standard deviation of the blank and the lowest calibrant concentration) for all three SRMs. All MCHpP values for SRMs 3672a and 3673a were below its 0.03 ng/g RL. MCPP was only observed in the highest three calibrants, leading to highly variable results. The MEHP results for SRMs 3672a and 3673a are also highly variable, with values at or near the 1 ng/g RL.

5.2.2. Value Assessment

To evaluate whether the measured values for SRM 3672a and 3673a are appropriate for use in value assessment, the measured values for SRM 3673 are compared to the non-certified phthalate values delivered in the SRM 3673 COA [3]. Given that the NIST results are based just upon three replicate measurements, Fig. 9 reveals the erratic agreement for the eight metabolites common to both sets. While the result for MBP agrees very well with the COA (NIST-to COA ratio of 0.99), the median ratio is 1.5 and the NIST result for MCPP is a factor of 20 larger than the COA.

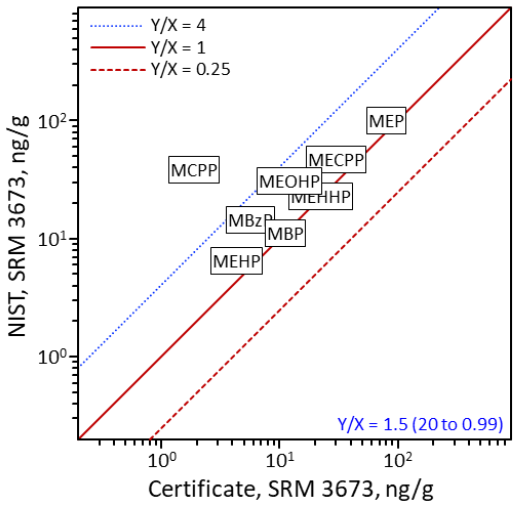


Fig. 9. SRM 3673 Phthalate-Related Results as a Function of the 2013 Non-Certified Values.

This figure compares the NIST phthalate results in SRM 3673 to the non-certified values listed in the SRM 3673 certificate of analysis (COA). Each labeled box is centered on the location {SRM 3673 COA result (X), SRM 3673 NIST current result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

5.2.3. Comparison of CDC and NIST Results

To further explore the utility of the NIST results, Fig. 10 compares the NIST results for SRMs 3673, 3672a, and 3673a to the CDC results. Given the excellent agreement between current CDC results for SRM 3673 and the non-certified values in the COA displayed in Fig. 6, the results for SRM 3673 (Panel A) closely resemble those in Fig. 9 with a median NIST/CDC ratio of 1.3. The NIST results for SRM 3672a and 3673a are also on average 1.2-fold larger than the CDC's.

NIST's MCPP results for SRMs 3673 and 3672a are both about 25-fold larger than the CDC's, suggesting that the two measurement processes identify the same metabolite. The NIST results for MBzP, MEHP, MEOHP, and MECPP are each consistently (1.3 to 2.3)-fold larger than the CDC's. The within-metabolite consistency of these ratios suggests that the two measurement processes are identifying the same metabolites but that there are quite significant calibration differences. These differences are much larger than can be explained by differences in calibrant purity or urine density.

The NIST results consistently agree closely with the CDC's only for MBP, MEHHP, MEP, and possibly MiBP.

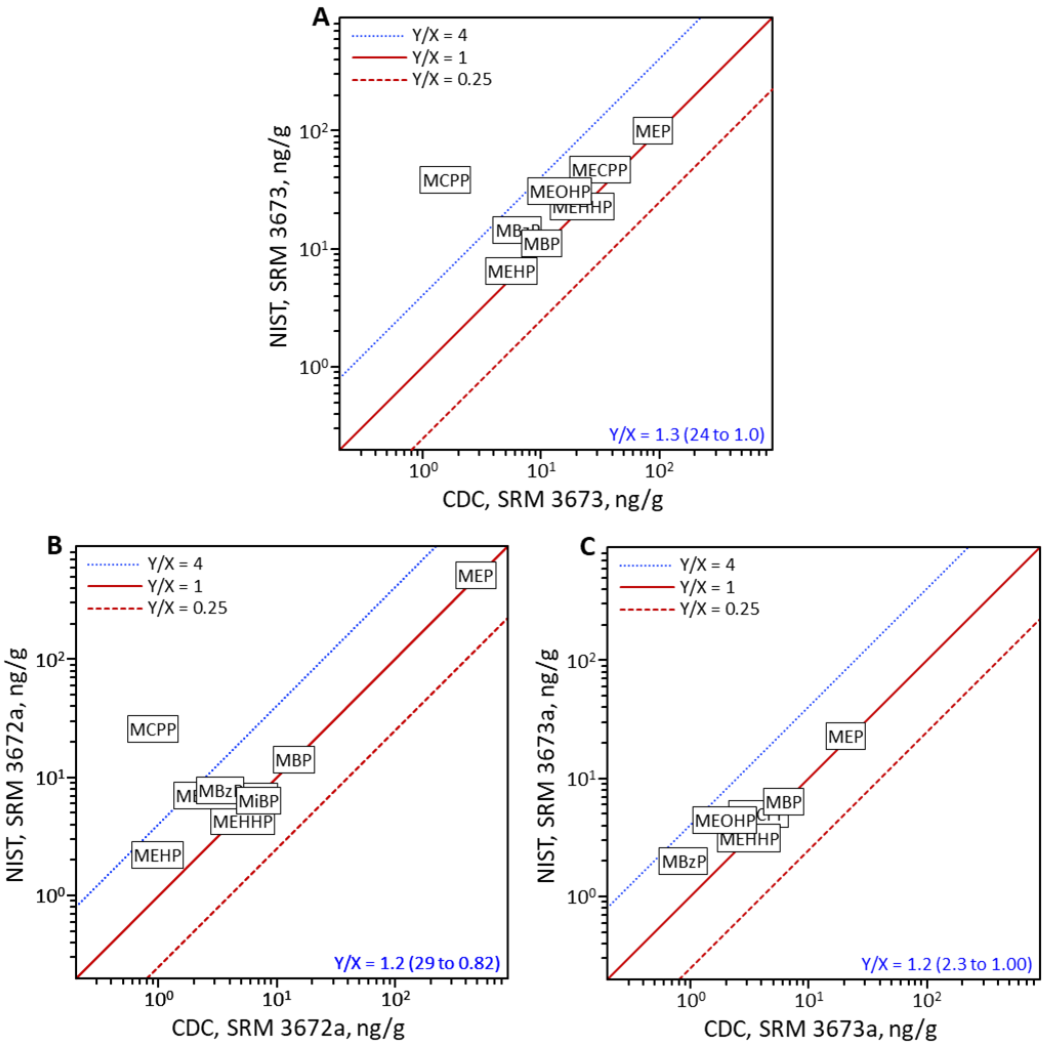


Fig. 10.NIST Phthalate-Related Analyte Results as a Function of CDC Results.

These panels compare the NIST results to the CDC results: Panel A compares results for 3673, Panel B compares results for SRM 3672a, and Panel C compares results for SRM 367a. Each labeled box within a panel is centered on the location {CDC result (X), NIST result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

5.3. Assigned Values

Table 10 lists the assigned values for phthalate and DINCH metabolites.

Table 10. Assigned Values for Phthalate and DINCH Metabolites in SRMs 3672a and 3673a, ng/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
MEP	499	16	31	2	21.6	1.1	2.2	2
MBP	13.95	0.28	0.56	2	6.11	0.14	0.32	2
MiBP	6.75	0.30	0.59	2	4.055	0.042	0.091	1
MBzP	5.5	2.2	4.3	2	1.43	0.56	1.10	2
MHBP	1.110	0.022	0.048	1				~~e~~
MHiBP	1.929	0.024	0.052	1	1.153	0.014	0.030	1
MEHHP	4.69	0.46	0.91	2	3.103	0.029	0.059	2
MEOHP	4.8	2.3	4.4	2	3.2	1.3	2.5	2
MONP	1.003	0.014	0.030	1	0.771	0.012	0.026	1
MCPP	0.896	0.020	0.043	1				~~e~~
MECPP	6.24	0.60	1.18	2	4.39	0.62	1.22	2
MCOP	3.253	0.048	0.105	1	3.001	0.047	0.101	1
MCNP				~~e~~	1.772	0.027	0.059	1
MEHHTP	8.31	0.12	0.25	1	11.84	0.11	0.25	1
MECPTP	55.27	0.61	1.32	1	95.35	0.66	1.44	1
MHiNCH	0.995	0.025	0.055	1	0.764	0.017	0.038	1

a $w_{analyte}$, mass fraction of the analyte.
b $u(w_{analyte})$, standard uncertainty associated with $w_{analyte}$.
c $U_{95}(w_{analyte})$, approximate 95 % level of confidence expanded uncertainty associated with $w_{analyte}$.
d Number of methods contributing results used to estimate $w_{analyte}$; see Section 4.
e No reliable quantitative result available.

5.3.1. Metrological Traceability

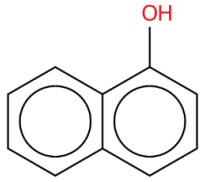
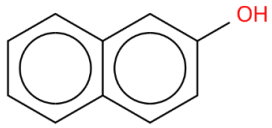
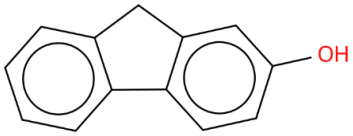
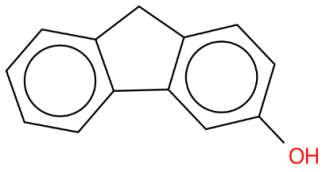
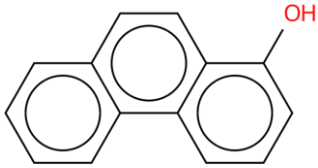
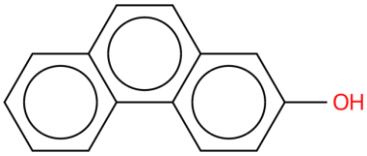
The results for MEP, MBP, MEHP, MBzP, MEOHP, and MECPP are metrologically traceable to the certified results delivered by SRM 3060 and documented in its Certificate of Analysis. The results for the other analytes are metrologically traceable to the materials used to prepare the calibration solutions.

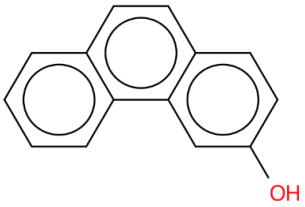
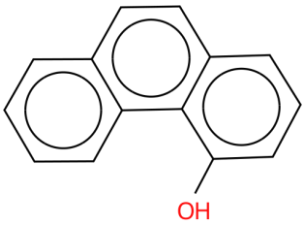
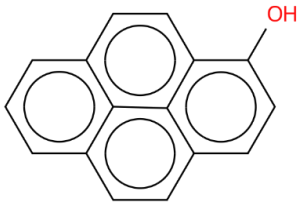
6. Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous environmental contaminants formed during incomplete combustion. Many of them are suspected human carcinogens. Occupational exposure may occur through work involving diesel fuels and coal tars including paving and roofing. Possible environmental exposures include smoking, diet, smog, and forest fires. Upon exposure, PAHs are metabolized; some of the metabolites are excreted in urine. Information on the concentration of mono-hydroxylated PAH (OH-PAH) metabolites in urine is important for understanding human exposure [16].

Table 11 describes the OH-PAHs assayed by the CDC and/or NIST. The analytes are identified by their acronym, common name, International Chemical Identifier (InChI), and chemical structure.

Table 11. Polyaromatic Mono-Hydroxylated Hydrocarbons (OH-PAHs).

Analyte	Structure
1-NAP 1-hydroxynaphtholene InChI=1S/C10H8O/c11-10-7-3-5-8-4-1-2-6-9(8)10/h1-7,11H	
2-NAP 2-hydroxynaphtholene InChI=1S/C10H8O/c11-10-6-5-8-3-1-2-4-9(8)7-10/h1-7,11H	
2-FLU 2-hydroxyfluorene InChI=1S/C13H10O/c14-11-5-6-13-10(8-11)7-9-3-1-2-4-12(9)13/h1-6,8,14H,7H2	
3-FLU 3-hydroxyfluorene InChI=1S/C13H10O/c14-11-6-5-10-7-9-3-1-2-4-12(9)13(10)8-11/h1-6,8,14H,7H2	
1-PHE 1-hydroxyphenanthrene InChI=1S/C14H10O/c15-14-7-3-6-12-11-5-2-1-4-10(11)8-9-13(12)14/h1-9,15H	
2-PHE 2-hydroxyphenanthrene InChI=1S/C14H10O/c15-12-7-8-14-11(9-12)6-5-10-3-1-2-4-13(10)14/h1-9,15H	

3-PHE 3-hydroxyphenanthrene InChI=1S/C14H10O/c15-12-8-7-11-6-5-10-3-1-2-4-13(10)14(11)9-12/h1-9,15H	
4-PHE 4-hydroxyphenanthrene InChI=1S/C14H10O/c15-13-7-3-5-11-9-8-10-4-1-2-6-12(10)14(11)13/h1-9,15H	
1-PYR 1-hydroxypyrene InChI=1S/C16H10O/c17-14-9-7-12-5-4-10-2-1-3-11-6-8-13(14)16(12)15(10)11/h1-9,17H	

6.1. CDC

The materials described in Section 5.1.1 were used for the PAH analyses: one vial each of SRMs 3672 and 3673, ten vials each of SRMs 3672a and 3673a.

6.1.1. Analysis

All sample measurements were made by CDC staff using the on-line solid phase extraction coupled with isotope dilution–high performance liquid chromatography–tandem mass spectrometry (SPE–HPLC–MS/MS) method described in [16,17]. The method involves enzymatic hydrolysis of glucuronidated/sulfated OH-PAH using β-glucuronidase/arylsulfatase. Seven analytes were assessed: 1-NAP, 2-NAP, 3-FLU, 3-FLU, 1-PHE, 2-3PHE (2-PHE combined with 3-PHE), and 1-PYR. The internal standard solution was prepared from ^{13}C-labeled analogs of each of the target OH-PAHs.

6.1.1.1. Sample Preparation

All samples, control materials, and calibrants are thawed to room temperature. 96-well plate was used for analysis. 20 µL of ascorbic acid solution (12.5 mg/mL) was added to each well, followed about the simultaneous addition of 50 µL of the internal standard solution, to account for adsorption differences. Next, 100 µL of each sample was added, followed by 50 µL of 1 mol/L sodium acetate buffer (pH 5.5) containing β-glucuronidase/arylsulfatase enzyme from *Helix pomatia* (Roman land snail, 10 mg enzyme per 1 mL buffer) to all wells and mix well. The well plate was sealed and incubated overnight (~18 h) at ~37°C.

After overnight enzymatic hydrolysis, add at least 175 µL methanol to all wells for a total volume of 395 µL to precipitate the enzyme. The solution was mixed, the well plate sealed again and centrifuged for ~15 min at 314 rad/s to 524 rad/s (3000 rpm to 5000 rpm). Transfer 200 µL of the supernatant layer to a new sample container containing 350 µL of deionized water and mix well.

6.1.1.2. Instrumental Method

An Oasis WAX online cartridge is used for online cleanup. Extract is loaded onto a Chromolith HighResolution RP-18 endcapped (5 mm × 4.6 mm) guard column followed by two jointChromolith HighResolution RP-18 endcapped (100 mm × 4.6 mm, Merck KGaA, Darmstadt, Germany) HPLC columns with a flow rate of 100 µL/min. The gradient used for separation is described in Table 12. Detection of the target analytes is made on an Sciex tandem mass spectrometer in the negative electrospray ionization mode.

Table 12. Mobile Phase Gradient for CDC OH-PAH Assessment.

Time, min	Flow Rate, µL/min	A % [a]	B % [b]
0.0	500	99.0	1.0
3.5	500	99.0	1.0
3.9	500	40.0	60.0
4.3	500	40.0	60.0
5.0	800	38.0	62.0
18.0	800	32.0	68.0
19.5	800	30.0	70.0
20.0	1000	15.0	85.0
21.0	1000	15.0	85.0
22.0	1000	10.0	90.0
24.0	1000	5.0	95.0
24.5	1000	5.0	95.0
24.6	1000	99.0	1.0
27.0	1000	99.0	1.0

a Mobile Phase A: 0.1 mm ammonium fluoride in water.
b Mobile Phase B: 0.1 mm ammonium fluoride in methanol.

The OH-PAH separation achieved with this method is illustrated in Fig. 11.

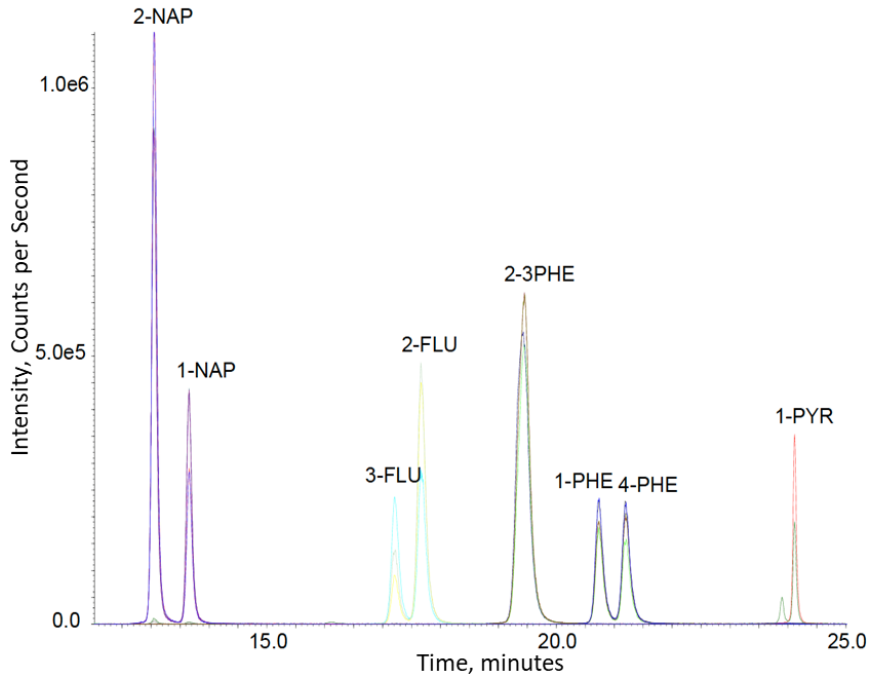


Fig. 11. Example Chromatogram of a High-Concentration Calibration Standard.

6.1.1.3. Quantitation

At least five calibration standards are analyzed with every analytical run. Calibration curves are generated by plotting the analyte area ratios (i.e., analyte area/internal standard area) against the native analyte concentrations through linear $1/x$ weighted regression, where the x is the standard concentration. Based on the ratio of analyte peak area to internal standard peak area, analyte concentrations in the unknowns are interpolated from the linear calibration models. The results sent to NIST were reviewed by the CDC's quality assurance officer and approved as conforming to the quality standards at the CDC.

Table 13 provides the one-vial three replicate measurements for SRMs 3672 and 3673. Table 14 provides the measurement results for SRMs 3672a and 3673a, with separate summary statistics for the ten-vial single-aliquot measurements and the one-vial three replicate measurements. These results were recorded and are listed here as amount concentrations, $x_{analyte}$. They are transformed to mass fractions, $w_{analyte}$, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3.

Table 13. CDC Results for OH-PAH Metabolites in SRMs 3672 and 3673, ng/mL.

	SRM 3672							SRM 3673						
Sample	1-NAP	2-NAP	2-FLU	3-FLU	1-PHE	2-3PHE [a]	1-PYR	1-NAP	2-NAP [a]	2-FLU	3-FLU	1-PHE	2-3PHE [b]	1-PYR
Replicate-1	37.8	8.89	0.727	0.429	0.121	0.205	0.198	224		0.097	0.040	0.046	0.053	
Replicate-2	38.4	9.02	0.727	0.426	0.119	0.205	0.199	217		0.093	0.041	0.045	0.051	
Replicate-3	37.4	9.11	0.716	0.433	0.121	0.200	0.200	214		0.089	0.037	0.042	0.050	
Mean:	37.87	9.01	0.7233	0.4293	0.1203	0.2033	0.1990	218.3		0.0930	0.0393	0.0443	0.0513	<0.07 [c]
SD:	0.50	0.11	0.0064	0.0035	0.0012	0.0029	0.0010	5.1		0.0040	0.0021	0.0021	0.0015	

a No reliable quantitative results available.
b 2-PHE and 3-PHE were measured together.
c The estimated concentration was at or below the method's limit of detection (LOD) for the analyte.

Table 14. CDC Results for OH-PAH Metabolites in SRMs 3672a and 3673a, ng/mL.

	SRM 3672a							SRM 3673a						
Sample	1-NAP	2-NAP	2-FLU	3-FLU	1-PHE	2-3PHE [a]	1-PYR	1-NAP	2-NAP	2-FLU	3-FLU	1-PHE	2-3PHE [a]	1-PYR
Vial-1	8.56	15.3	0.886	0.586	0.142	0.314	0.274	2.47	10.30	0.094	0.042	0.065	0.085	
Vial-2	8.69	15.2	0.893	0.587	0.148	0.321	0.272	2.32	10.00	0.092	0.040	0.058	0.080	
Vial-3	8.59	15.1	0.886	0.583	0.144	0.317	0.269	2.43	10.40	0.093	0.039	0.061	0.082	
Vial-4	8.52	15.0	0.886	0.579	0.138	0.312	0.261	2.37	10.00	0.093	0.039	0.060	0.085	
Vial-5	8.59	15.1	0.893	0.580	0.143	0.318	0.263	2.29	10.30	0.091	0.037	0.058	0.082	
Vial-6	8.63	15.1	0.893	0.588	0.143	0.314	0.266	2.34	10.40	0.093	0.038	0.063	0.083	
Vial-7	8.63	15.3	0.893	0.581	0.138	0.316	0.266	2.43	10.50	0.091	0.039	0.065	0.085	
Vial-8	8.26	14.4	0.835	0.543	0.133	0.292	0.246	2.30	10.30	0.091	0.037	0.059	0.082	
Vial-9	8.71	15.1	0.900	0.608	0.142	0.316	0.270	2.26	9.87	0.089	0.036	0.056	0.079	
Vial-10	8.63	14.9	0.893	0.581	0.145	0.318	0.266	2.37	10.70	0.094	0.041	0.060	0.085	
Mean:	8.58	15.05	0.886	0.582	0.1416	0.3138	0.2653	2.358	10.28	0.0921	0.0388	0.0605	0.0828	<0.07 [b]
SD:	0.13	0.26	0.018	0.016	0.0042	0.0081	0.0078	0.069	0.25	0.0016	0.0019	0.0030	0.0022	
Replicate-1	8.61	14.8	0.864	0.541	0.133	0.302	0.252	1.96	10.50	0.088	0.033	0.056	0.077	
Replicate-2	8.80	15.6	0.907	0.599	0.146	0.319	0.280	2.04	9.37	0.085	0.034	0.056	0.084	
Replicate-3	8.13	14.6	0.842	0.570	0.135	0.295	0.252	2.32	10.30	0.088	0.035	0.058	0.080	
Mean:	8.51	15.00	0.871	0.570	0.1380	0.305	0.261	2.11	10.06	0.0870	0.0340	0.0567	0.0803	<0.07 [b]
SD:	0.35	0.53	0.033	0.029	0.0070	0.012	0.016	0.19	0.60	0.0017	0.0010	0.0012	0.0035	

a 2-PHE and 3-PHE were measured together.
b The estimated concentration was at or below the method's limit of detection (LOD) for the analyte.

6.1.2. Homogeneity

As shown in Fig. 12, measurement repeatability (estimated from single aliquots from ten vials) is strongly related to technical precision (estimated from three aliquots from one vial). The technical and/or repeatability relative standard deviations (RSDs) for all analytes are well below the 10 % QC threshold. There is no evidence for any significant between- or within-vial heterogeneity for either SRM.

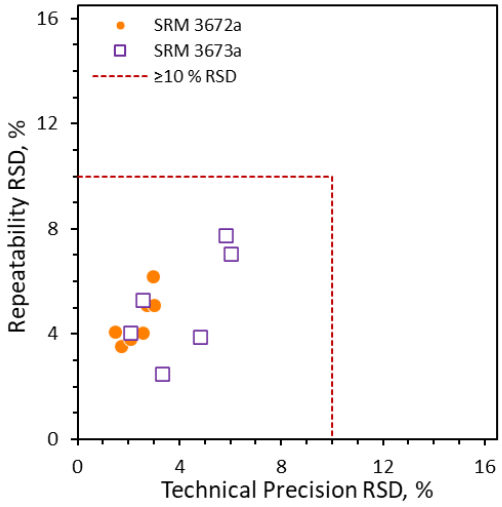


Fig. 12. Technical Precision as a Function of Repeatability for OH-PAHs.

The complete sets of measurement results are displayed in Fig. 13. Relatively consistent positive or negative deviations from the mean values (e.g., SRM 3672a vial 8 and SRM 3673a vial 1) suggests that much of the observed variability is related to sample preparation. There is no evidence for any production-related trend.

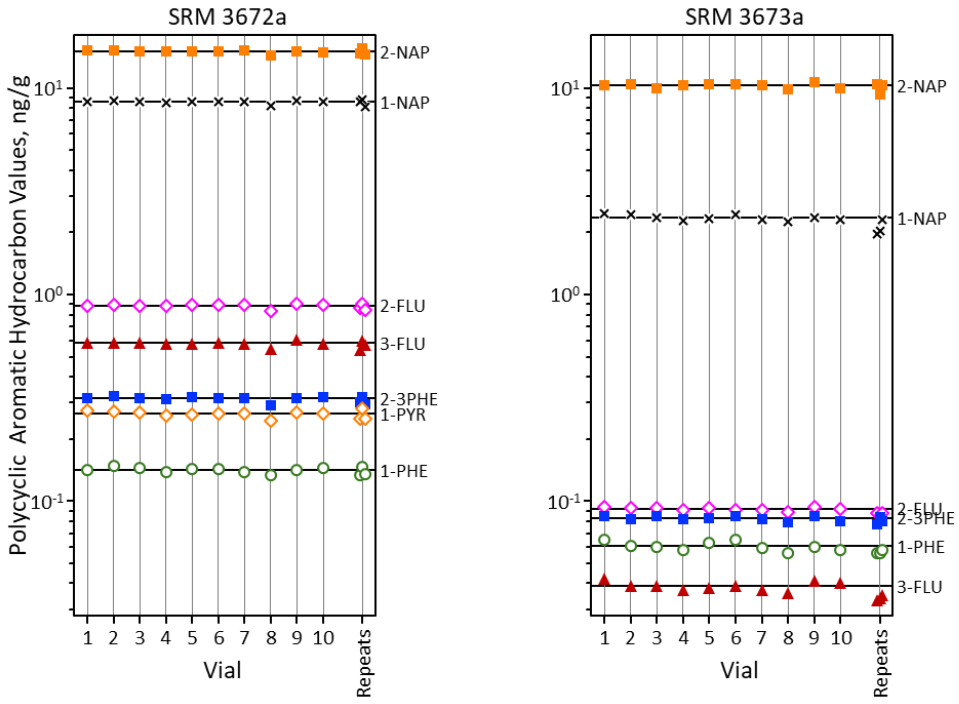


Fig. 13. Measurement Results as Functions of Vial for OH-PAHs.

Each symbol represents one measurement result. Results for the three aliquots from one vial are displayed to the right edge of each panel. Horizontal lines denote the mean result of single aliquots from ten vials. Analyte code names are displayed at the right-end of the mean lines. Vertical lines provide visual guidance.

6.1.3. Value Assessment

To evaluate whether the measured values for these metabolites in SRMs 3672a and 3673a are appropriate for use in value assessment, Fig. 14 compares the measured values for OH-PAHs in SRMs 3672 and 3673 to the non-certified values assigned in the original Certificates of Analysis (COAs) for SRMs 3672 and 3673 [4]. The values for 2-FLU in SRMs 3672 and 3673 have been removed from the current COAs for these materials [2,3] but appear to be well supported by the current measurements.

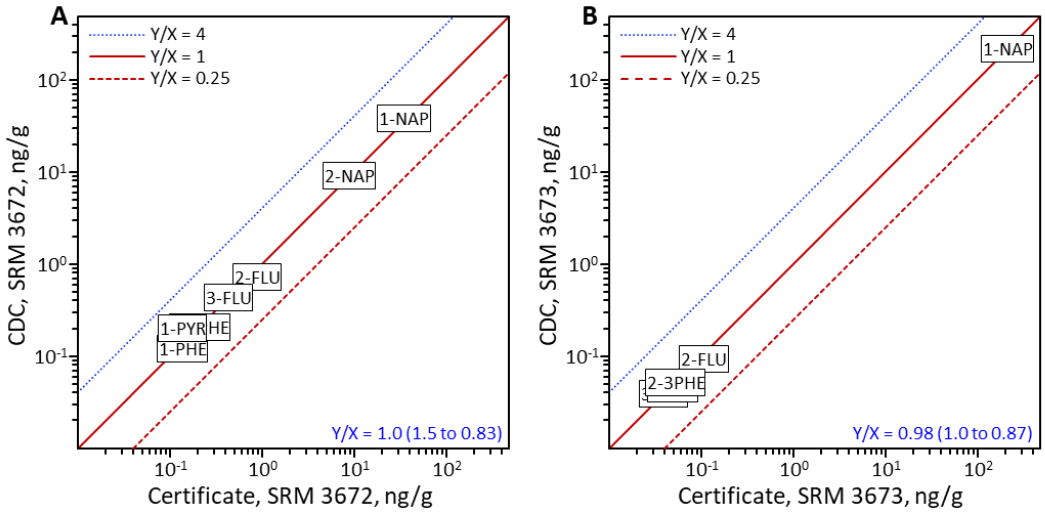


Fig. 14. OH-PAH Results for SRMs 3672 and 3673 as Functions of 2013 Non-Certified Values.

Panel A compares CDC's current results for seven mono-hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in SRM 3672 to the non-certified values listed in the current Certificate of Analysis (COA) [2] or in the original [4]; Panel B compares CDC's current results for six OH-PAHs in SRM 3673 to the non-certified values listed in [3] or [4]. Each labeled box within a panel is centered on the location {COA result (X), current CDC result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio

6.1.4. Comparisons Between the Original and the New SRMs

As shown in Fig. 15, the concentrations of the non-naphthalene OH-PAHs in the new materials (SRMs 3672a and 3673a) are about equal to the original materials (SRMs 3672 and 3673). The results for 1-NAP In the new materials are much smaller (4-fold in SRM 3672a, 100-fold in 3673a) than in the original materials. The result for 2-NAP in SRM 3672a is nearly 2-fold larger than in in 3672. In contrast to the concentrations of the other OH-PAH analytes the 1-NAP and 2-NAP concentrations in the new materials are qualitatively different than those in the original materials.

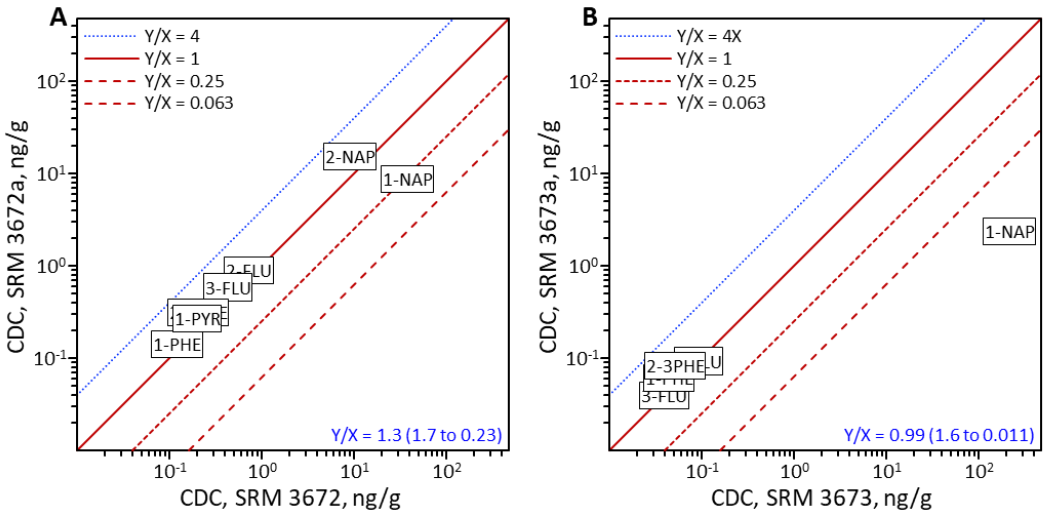


Fig. 15. OH-PAH Results for New SRMs as a Function of Original SRM Results.

Panel A compares CDC's results for the mono-hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in SRM 3672a to their current results for SRM 3672; Panel B compares CDC's results for SRM 3673a to their current results for SRM 3673. Each labeled box within a panel is centered on the location {CDC results for original SRM (X), CDC result for new SRM (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

6.1.5. Comparison Between Smokers' and Non-Smokers' Urines

As shown in Fig. 16, the concentrations of the non-naphthalene OH-PAHS in the SRM 3673 non-smokers' urine are about (2 to 10)-fold smaller than in the SRM 3672 smokers' urine while 1-NAP and 2-NAP concentrations are about 4-fold larger. On average the concentrations of all OH-PAHs in SRM 3673a are 4-fold smaller than in SRM 3672a, although the range is from (2 to 16)-fold smaller.

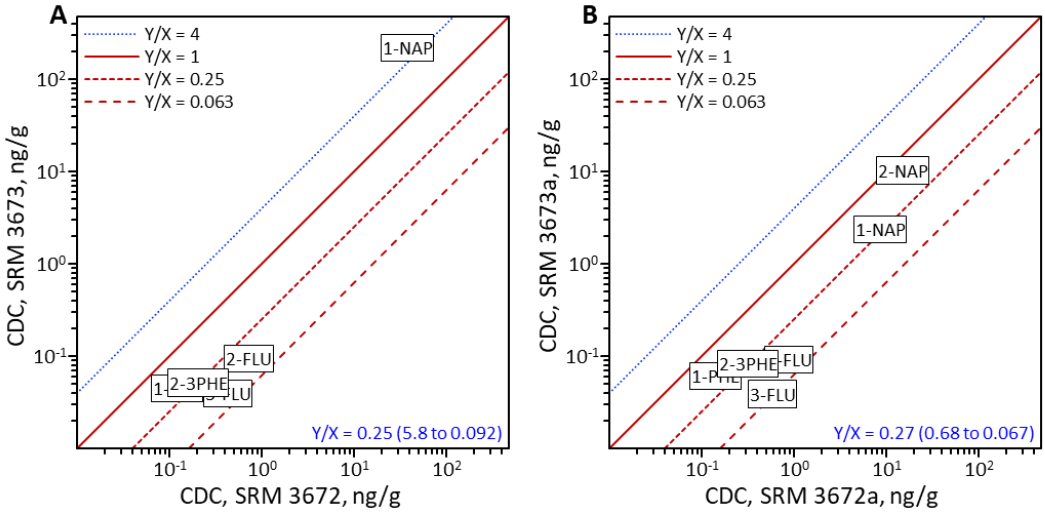


Fig. 16. OH-PAH Non-Smoker Results as a Function of Smoker Results.

Panel A compares CDC's results for the mono-hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in SRM 3672 to their results for SRM 3673; Panel B compares CDC's results for SRM 3672a to their results for SRM 3673a. Each labeled box within a panel is centered on the location {CDC results for smokers' urine (X), CDC result for non-smokers' urine (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The long-dashed diagonal line denotes Y results that are a factor of sixteen smaller than the X results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

6.2. NIST

NIST evaluated nine mono-hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in SRMs 3672, 3672a, and 3673a using the Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry (ID-LC-MS/MS) method described in [18]. This method has been described as orthogonal to method used by the CDC. Using a method inspired by [19], the measurements were also used to monitor β-glucuronidase/arylsulfatase efficiency and optimize the extraction method. All measurements were performed by NIST staff.

6.2.1. Analysis

One aliquot each of six vials of 3672a and six vials of 3673a were each analyzed. The vials of each SRM were selected based on a stratified randomized design to ensure characterization of the entire lot. Three aliquots from one vial of 3672 were used in for quality assessment. Four water blank extractions were used for quality control.

β-Glucuronidase/Arylsulfatase enzyme solution was produced by Roche Diagnostics GmbH (Mannheim, Germany). Ascorbic acid and sodium acetate were obtained through Sigma Aldrich (Burlington, MA, USA). Ammonium acetate was produced by J.T. Baker (Avator Inc., Radnor, PA, USA). All calibration and $^{13}C_4$-labeled internal standard materials were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). 4-Methylumbelliferone (MU) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Solvents were obtained through Thermo Fisher (Waltham, MA, USA); acetonitrile, ethanol, hexane, and methanol were produced by Fisher Chemical, and ethyl acetate was produced by Honeywell. MilliQ water was prepared in the lab using a MilliQ IQ 7000 ultrapure water production unit.

6.2.1.1. Calibrant Preparation

Each calibrant solution was gravimetrically diluted to a stock solution in methanol to approximately 5 µg/mL. These stock solutions were further diluted in methanol to working solutions to achieve mass fraction ratios similar to the urine samples, based on CDC target values. These working solutions were then used to make three calibrants of different levels. Calibrants were spiked with increasing amounts (55 µL to 217 µL) of MU working solution. The internal standard solution was also diluted in methanol to make a working solution.

6.2.1.2. Sample Preparation

All materials (calibrants, internal standard solutions, and urine samples) were allowed to come to room temperature for at least 1 h before preparations steps. Into 50-mL glass round bottom centrifuge tubes, aliquots of urine (2 mL to 5 mL), water (3 mL), or calibrant (0.3 mL to 2.25 mL) were gravimetrically spiked with 400 µL of the OH-PAH internal standard working solution.

For each 1 mL of sample, ~2.5 mL of acetate buffer (100 mol/L, pH 5, 3.314 g sodium acetate and 1.18 g acetic acid per 1 L of water), ~22.5 µL ascorbic acid (250 mg/mL in water), and ~50

µL of enzyme was added. The samples were vortex mixed for 5 s then incubated at 37 °C overnight (~21 h). Samples were allowed to cool to room temperature for at least 30 min.

Based on results from enzyme efficiency studies, samples were liquid-liquid extracted (LLE). To each sample, 5 mL of hexane was added and placed on a shaker for 10 min. Emulsions were broken with the addition of ethanol (1 mL to 2 mL), if needed. The top organic layer was removed with a disposable pipette and transferred to a 50-mL glass conical bottom centrifuge tube. Samples were extracted a second time with 5 mL of ethyl acetate and shaking for 3 min. Ethanol was again added to break emulsions if needed. The top organic layer was removed with a disposable pipette and combined with the first extraction volume. The extracts were dried using a TurboVap under nitrogen at 45 °C, then resuspended in 110 µL of methanol. The final samples were transferred to amber autosampler vials with a 300-µL glass inserts and stored at 20 °C until LC-MS/MS analysis.

6.2.1.3. Instrumental Method

All analyses were performed on an Agilent (Santa Clara, CA, USA) 1200 Series HPLC system equipped with a degasser, binary pump, temperature-controlled autosampler, and temperature-controlled column compartment, attached to a Sciex API 4000 LC-MS/MS using electrospray ionization in positive mode. The following parameters were the same for all compounds: autosampler tray temperature at 10 °C, curtain gas N_2 at 90 kPa (13 psi), collision gas N_2 at 62 kPa (9 psi), ion source gas 1 at 586 kPa (85 psi), ion source gas 2 at 138 kPa (20 psi), nebulizer current: at -4500 V, source temperature of 400 °C, and exit potential at -10 V. The instrument was controlled using Analyst (Sciex).

Two LC separation techniques were used.

Method "C18": The LC separation described in [18] was implemented using an Kinetex C18 (2.6 µm, 15 cm × 4 mm, Phenomenex) column with a flow rate of 0.4 mL/min and 40 °C column temperature. Mobile phase A was 0.1 % ammonium acetate in milliQ water and phase B was 0.1 % ammonium acetate in HPLC grade methanol. Initial conditions of 5 % B were held for 5 min after injection, then went to 80 % B over 15 min, and held at 80 % B for 1 min. The column returned to initial conditions for re-equilibration of 5 % B for 7 min. The total run time was 28 min. The injection volume was 5 µL or 7 µL. The target scan time was 1.5 s. The multiple reaction monitoring (MRM) mode window was 100 s. MRM transitions and timing parameters were optimized for each unlabeled compound. The achieved separation is displayed in Fig. 17.

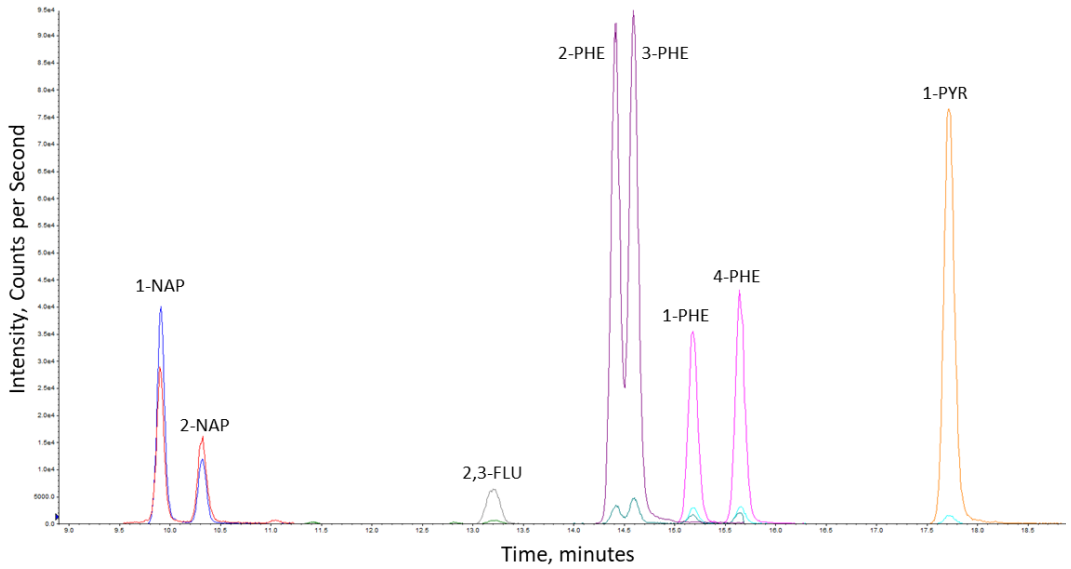


Fig. 17. Example Chromatogram of a Calibration Standard With Method "C18".

Method "F5": To resolve 2-FLU and 3-FLU, an Ascentis Express F5 (2.7 µm, 5 cm × 4.6 mm, Supelco) column was used with a flow rate of 0.8 mL/min and no column temperature control. Mobile phase A was 0.1 % ammonium acetate in milliQ water and phase B was 0.1 % ammonium acetate in HPLC grade methanol. Initial conditions of 45 % B were held for 1 min after injection, then went to 72 % B over 27 min, to 95 % B over 1 min, and held at 95 % B for 4 min. The column returned to initial conditions for re-equilibration of 45 % B for 5 min. The total run time was 38 min. The injection volume was 7 µL. Detection used the same equipment and MS parameters as above except for the scheduled MRM times. The target scan time was 1.55 s. The MRM window was 240 s. The achieved separation is displayed in Fig. 18.

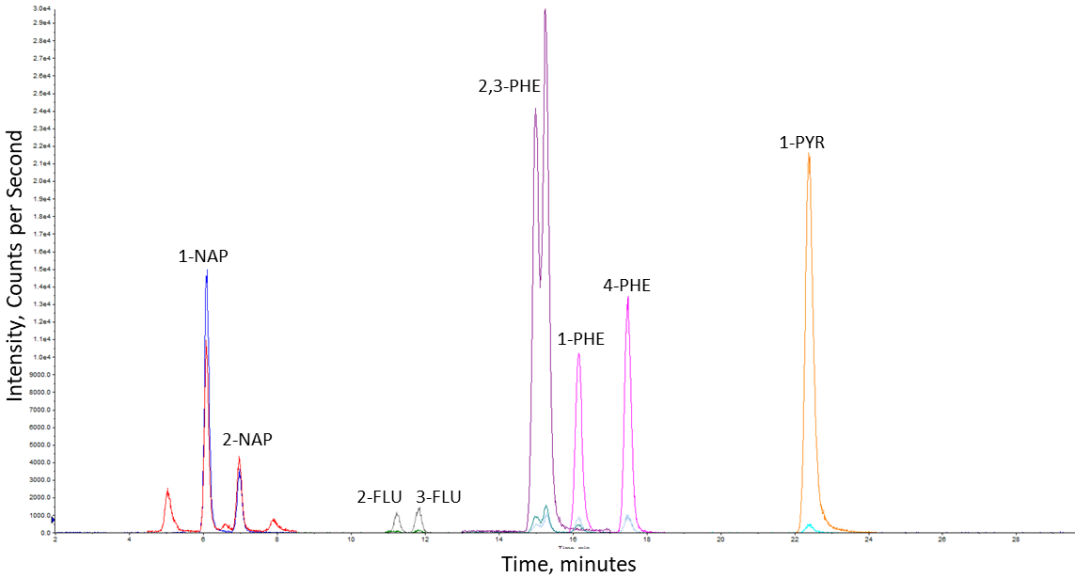


Fig. 18. Example Chromatogram of a Calibration Standard With Method "F5".

6.2.1.4. Quantitation

Six vials each of SRMs 3672a and 3673a, one vial of SRM 3672, water blanks, and seven calibrants were prepared across two days, where each day contained three preparations each of 3672a and 3673a, one or two preparations of 3672, two blank preparations, and three to four calibrant preparations. For both "C18" and "F5" analyses, all samples were analyzed in the same LC-MS/MS batch with duplicate injections in a stratified random order. Blank injections of methanol were included at the beginning and end of each sequence, as well as between the first injection and second injection sets.

Analyst software was used for peak integrations, and all peaks were checked and manually integrated if needed. NIST's Environmental Metrology Measurement Assistant (EMMA) [15]was used for data workup. By combining the data of calibrants, a linear regression was calculated using a two-parameter linear model which was used to convert the measured area ratios of analyte to mass ratios. The mass ratios were then used along with the amounts of the internal standard added to calculate analyte concentrations. Table 15 lists the measurement values and summary statistics for SRMs 3672a, 3673a, and 3672.

Table 15. NIST Results for OH-PAH Metabolites in SRMs 3672a, 3673a, and 3672, ng/g.

		SRM3672a				SRM3673a					SRM 3672			
		C18		F5		C18		F5			C18		F5	
	Vial	Inj_1	Inj_2	Inj_1	Inj_2	Inj_1	Inj_2	Inj_1	Inj_2	Extract	Inj_1	Inj_2	Inj_1	Inj_2
1-NAP	1	8.833	8.984	8.524	8.573	2.429	2.550	2.050	2.227	1	38.196	37.693	40.811	36.474
	2	9.211	9.117	8.917	8.923	2.516	2.538	2.156	1.961	2	40.956	40.593	42.021	39.090
	3	8.975	9.096	8.703	8.541	2.382	2.601	1.992	1.920	3	40.218	38.092	40.613	38.510
	4	9.114	9.080	8.696	8.782	2.637	2.654	2.327	2.015	Mean:		39.291		39.587
	5	8.979	9.205	8.881	8.989	2.471	2.645	2.266	1.916	SD:		1.450		1.979
	6	9.000	8.922	8.887	8.644	2.481	2.577	2.148	2.024					
	Mean:		9.043		8.755		2.540		2.084					
	SD:		0.114		0.163		0.088		0.138					
2-NAP	1	14.359	13.902	14.792	14.176	10.011	10.057	9.813	10.052	1	8.963	9.187	8.744	8.889
	2	14.651	14.593	14.642	14.711	9.882	10.190	9.681	9.961	2	9.372	9.270	9.167	9.198
	3	14.380	14.224	14.881	14.760	10.116	10.119	9.836	9.849	3	9.033	8.770	9.245	9.201
	4	14.432	14.135	14.755	14.947	9.997	9.823	9.962	9.722	Mean:		9.099		9.074
	5	14.591	14.277	14.820	15.093	9.952	9.949	9.701	9.874	SD:		0.220		0.206
	6	14.665	14.291	14.703	14.862	9.956	9.806	9.848	9.672					
	Mean:		14.375		14.762		9.988		9.831					
	SD:		0.229		0.221		0.119		0.121					
1-PHE	1	0.239	0.243	0.207	0.222	0.079	0.094	0.071	0.070	1	0.217	0.209	0.175	0.199
	2	0.241	0.246	0.225	0.242	0.086	0.086	0.083	0.074	2	0.223	0.220	0.229	0.250
	3	0.263	0.245	0.212	0.245	0.079	0.079	0.074	0.095	3	0.230	0.213	0.196	0.186
	4	0.256	0.258	0.253	0.241	0.082	0.087	0.089	0.094	Mean:		0.219		0.206
	5	0.242	0.246	0.257	0.228	0.087	0.086	0.100	0.080	SD:		0.007		0.028
	6	0.229	0.251	0.215	0.215	0.088	0.082	0.080	0.076					
	Mean:		0.247		0.230		0.085		0.082					
	SD:		0.009		0.017		0.005		0.010					
2-PHE	1	0.095	0.098	0.134	0.104	0.036	0.030	0.046	0.059	1	0.070	0.093	0.107	0.070
	2	0.102	0.103	0.114	0.147	0.033	0.032	0.048	0.048	2	0.089	0.084	0.096	0.094

		SRM3672a				SRM3673a					SRM 3672			
		C18		F5		C18		F5			C18		F5	
	Vial	Inj_1	Inj_2	Inj_1	Inj_2	Inj_1	Inj_2	Inj_1	Inj_2	Extract	Inj_1	Inj_2	Inj_1	Inj_2
	3	0.110	0.097	0.100	0.130	0.040	0.034	0.053	0.043	3	0.085	0.088	0.087	0.080
	4	0.110	0.117	0.135	0.132	0.040	0.034	0.041	0.032	Mean:		0.085		0.089
	5	0.112	0.109	0.117	0.103	0.038	0.037	0.036	0.040	SD:		0.008		0.013
	6	0.112	0.099	0.125	0.122	0.039	0.040	0.037	0.042					
	Mean:		0.105		0.122		0.036		0.044					
	SD:		0.007		0.015		0.003		0.008					
3-PHE	1	0.238	0.254	0.277	0.260	0.047	0.041	0.057	0.070	1	0.136	0.142	0.173	0.119
	2	0.237	0.245	0.249	0.290	0.049	0.039	0.064	0.054	2	0.132	0.143	0.140	0.153
	3	0.249	0.234	0.239	0.267	0.045	0.049	0.057	0.058	3	0.136	0.152	0.139	0.144
	4	0.267	0.248	0.292	0.262	0.052	0.047	0.052	0.045	Mean:		0.140		0.145
	5	0.247	0.261	0.252	0.256	0.048	0.048	0.046	0.051	SD:		0.007		0.018
	6	0.251	0.247	0.264	0.270	0.054	0.050	0.052	0.052					
	Mean:		0.248		0.265		0.047		0.055					
	SD:		0.010		0.016		0.004		0.007					
2&3-PHE	1	0.333	0.352	0.372	0.359	0.083	0.071	0.093	0.100	1	0.206	0.235	0.243	0.212
	2	0.339	0.347	0.351	0.392	0.082	0.071	0.097	0.087	2	0.221	0.227	0.228	0.237
	3	0.360	0.332	0.349	0.365	0.085	0.084	0.097	0.093	3	0.221	0.240	0.224	0.232
	4	0.377	0.365	0.402	0.379	0.092	0.081	0.092	0.079	Mean:		0.225		0.229
	5	0.359	0.370	0.364	0.364	0.086	0.084	0.084	0.088	SD:		0.012		0.011
	6	0.363	0.345	0.376	0.369	0.093	0.090	0.091	0.092					
	Mean:		0.354		0.370		0.084		0.091					
	SD:		0.015		0.016		0.007		0.006					
4-PHE	1	0.040	0.041	0.032	0.038	0.021	0.022	0.011	0.014	1	0.045	0.044	0.026	0.042
	2	0.039	0.042	0.032	0.031	0.022	0.017	0.011	0.013	2	0.061	0.047	0.032	0.035
	3	0.050	0.047	0.029	0.032	0.019	0.017	0.010	0.022	3	0.055	0.046	0.022	0.026
	4	0.036	0.039	0.035	0.033	0.019	0.019	0.008	0.013	Mean:		0.050		0.031
	5	0.047	0.044	0.034	0.021	0.019	0.017	0.008	0.012	SD:		0.007		0.007
	6	0.041	0.044	0.030	0.026	0.017	0.018	0.009	0.011					
	Mean:		0.043		0.031		0.019		0.012					
	SD:		0.004		0.004		0.002		0.004					
2-FLU	1	1.380	1.446	1.061	1.155			0.091	0.060	1	0.996	1.114	0.887	0.736
	2	1.251	1.380	0.998	0.995			0.076	0.075	2	1.231	1.337	0.732	0.827
	3	1.357	1.205	1.113	1.148			0.086	0.060	3	1.191	1.132	0.782	0.904
	4	1.220	1.386	1.057	1.074			0.076	0.076	Mean:		1.167		0.811
	5	1.253	1.144	0.949	1.124			0.099	0.092	SD:		0.116		0.074
	6	1.393	1.229	1.051	1.116			0.104	0.065					
	Mean:		1.304		1.070				0.080					
	SD:		0.097		0.065				0.015					
3-FLU	1	0.772	0.771	0.452	0.481	0.073	0.096			1	0.534	0.737	0.425	0.358
	2	0.857	0.919	0.604	0.533	0.095	0.070			2	0.728	0.735	0.229	0.225
	3	0.845	0.689	0.602	0.632	0.093	0.083			3	0.631	0.552	0.222	0.324
	4	0.713	0.852	0.550	0.540	0.071	0.084			Mean:		0.653		0.297
	5	0.957	0.694	0.653	0.674	0.061	0.052			SD:		0.094		0.085

		SRM3672a				SRM3673a					SRM 3672			
		C18		F5		C18		F5			C18		F5	
	Vial	Inj_1	Inj_2	Inj_1	Inj_2	Inj_1	Inj_2	Inj_1	Inj_2	Extract	Inj_1	Inj_2	Inj_1	Inj_2
	6	0.966	0.820	0.624	0.707	0.039	0.102							
	Mean:		0.821		0.588		0.077							
	SD:		0.096		0.078		0.019							
2&3-FLU	1	1.832	1.927	1.513	1.636	0.164	0.156			1	1.420	1.472	1.312	1.094
	2	1.855	1.913	1.602	1.528	0.171	0.146			2	1.460	1.563	0.961	1.053
	3	1.959	1.837	1.716	1.779	0.179	0.143			3	1.413	1.457	1.004	1.229
	4	1.770	1.926	1.607	1.614	0.148	0.160			Mean:		1.464		1.109
	5	1.906	1.818	1.602	1.798	0.160	0.144			SD:		0.054		0.136
	6	2.016	1.936	1.675	1.823	0.142	0.167							
	Mean:		1.891		1.658		0.157							
	SD:		0.070		0.102		0.012							
1-PYR	1	0.231	0.229	0.251	0.215	0.044	0.039	0.041	0.035	1	0.153	0.165	0.169	0.157
	2	0.258	0.235	0.238	0.247	0.044	0.048	0.044	0.034	2	0.174	0.175	0.164	0.178
	3	0.227	0.246	0.235	0.229	0.044	0.039	0.039	0.036	3	0.184	0.174	0.161	0.212
	4	0.235	0.234	0.225	0.230	0.049	0.033	0.050	0.036	Mean:		0.171		0.174
	5	0.242	0.245	0.246	0.222	0.042	0.047	0.038	0.026	SD:		0.011		0.020
	6	0.232	0.245	0.243	0.238	0.047	0.045	0.043	0.041					
	Mean:		0.238		0.235		0.043		0.039					
	SD:		0.009		0.011		0.005		0.006					

6.2.2. Value Assessment

To evaluate whether the measured values for SRM 3672a and 3673a are appropriate for use in value assessment, Fig. 19 compares NIST's measured values for the OH-PAHs in SRM 3672 to the non-certified values delivered in the current Certificate of Analysis (COA) [2] or in the original [4]. The current NIST results are on average about 1.1-fold larger than the 2013 values, with a range from about (1.6 to 0.8)-fold.

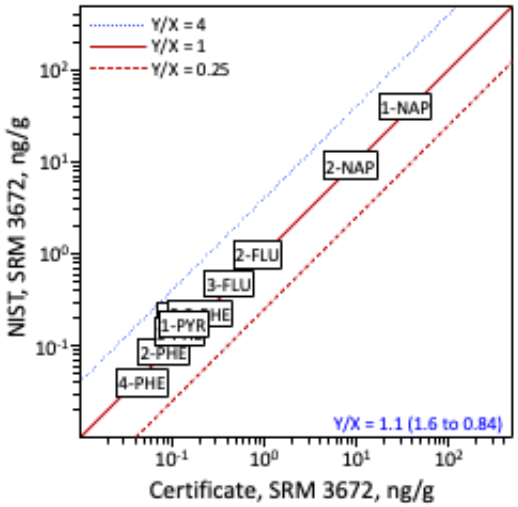


Fig. 19. NIST's OH-PAH Results for SRM 3672 as a Function of Their 2013 Non-Certified Values.

This figure compares the NIST results for ten mono-hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) analytes in SRM 3672 to the non-certified values listed in the current Certificate of Analysis (COA) [2] in the original [4]. Each labeled box is centered on the location {SRM 3672 COA result (X), SRM 3673 NIST result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

6.2.3. Comparison of CDC and NIST Results

To further explore the utility of the NIST results, Fig. 20 compares the NIST results for SRMs 3672, 3672a, and 3673a to the current CDC results. On average the NIST results are about 1.1-fold larger than the CDC's, with a range from about (1.2 to 0.9)-fold.

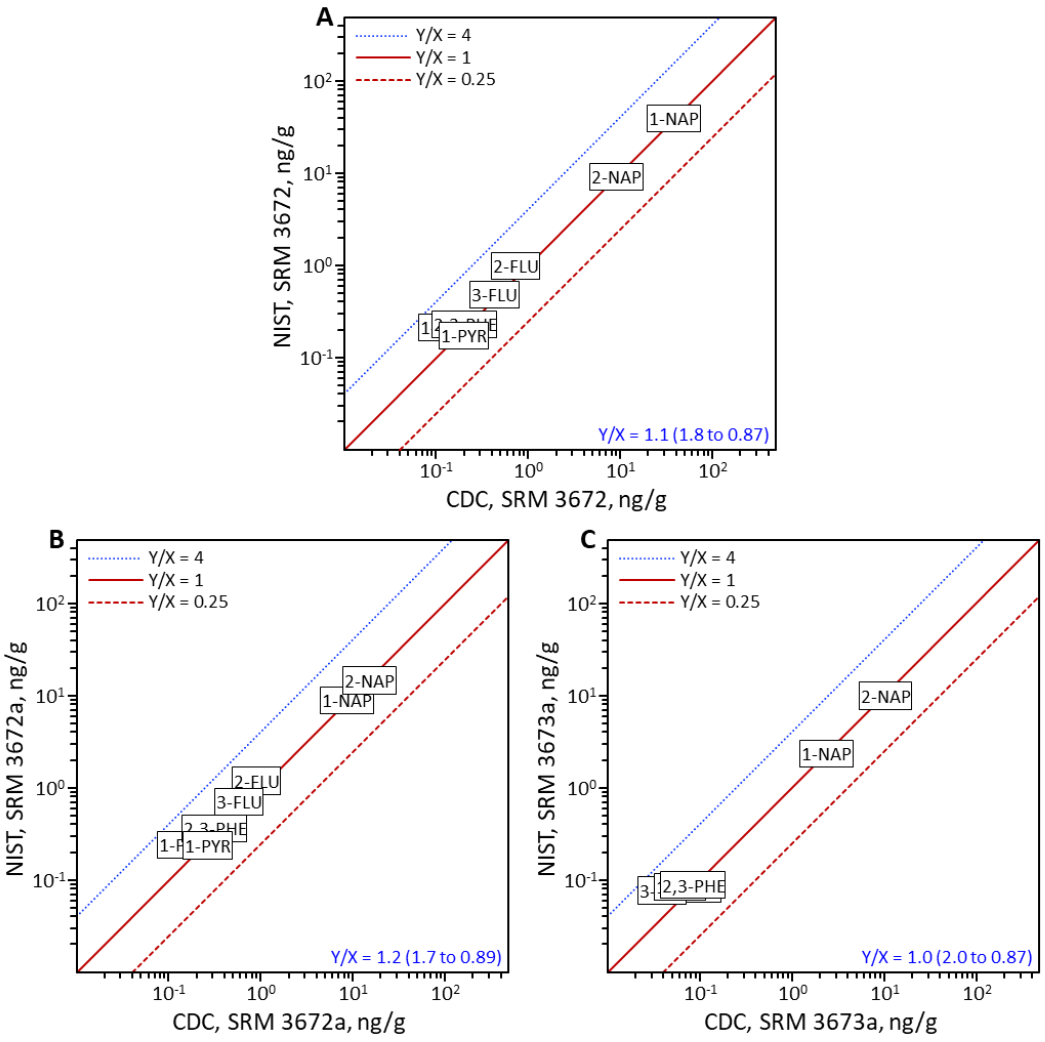


Fig. 20.NIST OH-PAH Results as a Function of CDC Results.

These panels compare the NIST results to the CDC results: Panel A compares results for 3672, Panel B compares results for SRM 3672a, and Panel C compares results for SRM 3673a. Each labeled box within a

panel is centered on the location {CDC result (X), NIST result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

6.3. Assigned Values

Table 16 lists the assigned values for the reliably determined mono-hydroxylated polyaromatic hydrocarbon (OH PAH) metabolites.

Table 16. Assigned Values for Phthalate-Related Metabolites in SRMs 3672a and 3673a, ng/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
1-NAP	8.78	0.16	0.31	3	2.30	0.14	0.28	3
2-NAP	14.70	0.17	0.33	3	9.970	0.078	0.152	3
2-FLU	0.973	0.096	0.189	2	0.0860	0.0051	0.0100	2
3-FLU	0.5760	0.0052	0.0114	1	0.03742	0.00074	0.00161	1
2&3-FLU	1.78	0.12	0.23	2	0.1567	0.0035	0.0078	1
1-PHE	0.1401	0.0014	0.0030	1	0.05918	0.00087	0.00189	1
2-PHE	0.1053	0.0021	0.0046	1	0.0361	0.0010	0.0022	1
3-PHE	0.2482	0.0028	0.0061	1	0.0474	0.0012	0.0027	1
2&3-PHE	0.345	0.020	0.039	3	0.0853	0.0030	0.0060	3
4-PHE	0.0425	0.0012	0.0026	1	0.01892	0.00054	0.00120	1
1-PYR	0.2455	0.0090	0.0177	3	0.0411	0.0024	0.0048	2

a $w_{analyte}$, mass fraction of the analyte.
b $u(w_{analyte})$, standard uncertainty associated with $w_{analyte}$.
c $U_{95}(w_{analyte})$, approximate 95 % level of confidence expanded uncertainty associated with $w_{analyte}$.
d Number of methods contributing results used to estimate $w_{analyte}$; see Section 4.

6.3.1. Metrological Traceability

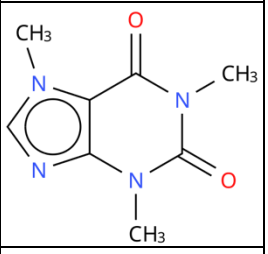
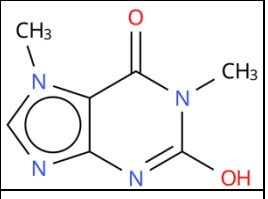
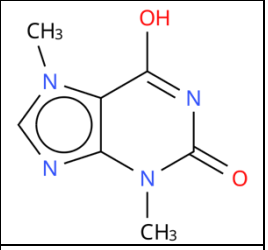
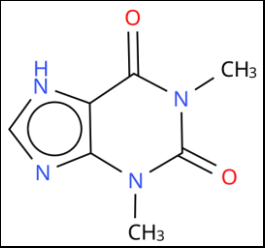
These results are metrologically traceable to the materials used to prepare the calibration solutions. None of the results are reliably traceable to the SI.

7. Caffeine-Related Analytes

Caffeine and related xanthine alkaloids are psychoactive stimulatory compounds that naturally occur in plants such as coffee beans, tea leaves, cocoa beans, and cola nuts and/or are metabolites of the naturally occurring compounds. The dietary consumption of these alkaloids comes principally from beverages (e.g., coffee, tea, cola drinks) and foods (e.g., chocolate). Caffeine consumption has been studied as a risk factor for diseases and conditions such as hypertension, cardiovascular disease, various cancers, reproduction and developmental abnormalities, and mental and behavioral disorders. Measurement of caffeine and related analytes in urine has been proposed as a means of assessing caffeine intake [20].

Table 17 describes the caffeine-related analytes assayed by the CDC and NIST. The analytes are identified by their acronym, common name, International Chemical Identifier (InChI), and chemical structure.

Table 17. Caffeine and Its Metabolites.

Analyte	Structure
Caffeine InChI=1S/C8H10N4O2/c1-10-4-9-6-5(10)7(13)12(3)8(14)11(6)2/h4H,1-3H3	
Paraxanthine InChI=1S/C7H8N4O2/c1-10-3-8-5-4(10)6(12)11(2)7(13)9-5/h3H,1-2H3,(H,9,13)	
Theobromine InChI=1S/C7H8N4O2/c1-10-3-8-5-4(10)6(12)9-7(13)11(5)2/h3H,1-2H3,(H,9,12,13)	
Theophylline InChI=1S/C7H8N4O2/c1-10-5-4(8-3-9-5)6(12)11(2)7(10)13/h3H,1-2H3,(H,8,9)	

7.1. CDC Measurements

One bottle each of SRMs 3672 and 3673 and two bottles each of 3672a, 3673a were sent overnight shipping on dry ice from NIST to the CDC. The vials were maintained in -80 °C freezers until assessment.

7.1.1. Analysis

All sample measurements were made by CDC staff using a modified version of the liquid chromatography–tandem mass spectrometry (LC–MS/MS) method described in [20]. The following sections briefly describe the measurements.

7.1.1.1. Sample Preparation

Twenty independent sample preparations (two preparations/run x ten runs) each of SRMs 3672, 3673, 3672a and 3673a were prepared and analyzed over a period of four months.

The urine samples were thawed to room temperature and mixed before use. A 50 µL aliquot of sample was transferred to a polystyrene 96-well plate and diluted tenfold with water. A 100 µL aliquot of this dilution was then transferred to a second well plate, spiked with a 20 µL aliquot of the isotopically labeled internal standard mixture, and mixed with a 20 µL aliquot of 1.2 mol/L NaOH and 80 µL of water. The sample was incubated for 30 min at room temperature. After incubation, 30 µL of 2 mol/L HCl solution and 250 µL of a 0.1 % formic acid and 10 % methanol in water solution was added to the sample, then the sample was filtered using a 0.2-µm nylon filter plate. Finally, the sample was transferred to a 96-well plate for LC-MS/MS analysis.

7.1.1.2. Instrumental Method

Analyses were performed on an Agilent 1290 series HPLC system attached to an API 6500 (Applied Biosystems, CA) using electrospray ionization (ESI) in both positive and negative modes (within-run polarity switching). LC separation was achieved using a Kinetix XB-C_{18} analytical column (2.5 µm 100 Å pore, 100 mm × 3.0 mm, Phenomenex) preceded by a KrudKatcher C18 guard filter (2.0 µm depth × 0.004" di., Phenomenex). The modified gradient program was 0 min, 0 %; 5.5 min, 10 %; 6.0 min, 40 %; 7.0 min, 40 %; 7.1 min, 0 %; and 9.0 min, 0 %. In both positive and negative ion modes, a flow rate of 800 µL/min and an injection volume of 10 µL were used. Column temperature was set to 50 °C.

For positive ion mode, the following global conditions were used: ionization voltage, 1,850 V; interface temperature, 700 °C; declustering potential, 25 V; exit potential 10 V; collision cell exit potential, 11 V. For negative ion mode, the following global conditions were used: ionization voltage, −1,850 V; interface temperature, 700 °C; declustering potential, -25 V; exit potential -10 V; collision cell exit potential, -16 V.

Table 18 lists the analyte-specific parameters used in the analysis of caffeine and the related analytes.

Table 18. CDC Analyte-Specific Parameters for Caffeine-Related Analytes

Analyte	Retention time min	Calibration range[a] µmol/L	MS/MS transition *m/z* (mode)	Collision energy V	Purpose[b]
Caffeine	6.64	0.003–50	195/138 (+) 195/110 (+) 204/144 (+)	24 32 24	Q C IS ($^{2}H_{9}$)
Paraxanthine	4.51	0.006–200	181/124 (+) 181/96 (+) 188/129 (+)	24 32 24	Q C IS ($^{13}C_{4}{}^{15}N_{3}$)
Theophylline	4.76	0.01–20	179/164 (-) 179/122 (-) 185/125 (-)	-26 -28 -28	Q C IS ($^{13}C_{4}{}^{15}N_{3}$)
Theobromine	3.36	0.004–250	181/138 (+) 181/163 (+) 187/143 (+)	24 24 24	Q C IS ($^{13}C_{4}{}^{15}N_{2}$)

a Lowest calibrator to highest calibrator. Lowest calibrator concentration = limit of detection.
b Q = Quantitation; C = Confirmation; IS = Internal Standard.

7.1.1.3. Quantitation

Results were obtained by interpolation of peak area ratios for MS/MS transitions against 11-point quadratic calibration models derived from calibrators in synthetic urine. QC limits were established by duplicate analysis of three urine QC pools over 20 independent runs. Results were reported from runs where the QC pool results satisfied the requirements of a multi-rule quality control system based on the established QC limits. The results sent to NIST were reviewed by the CDC's quality assurance officer and approved as conforming to the quality standards at the CDC.

Table 19 through Table 22 provide the ten aliquot two-injection measurement results for SRMs 3672, 3673, 3672a and 3673a. These results were recorded and are listed here as amount-of-substance concentrations, $c_{analyte}$, in units of nmol/mL. These results are transformed to mass fractions, $w_{analyte}$, using Eq. 5 with the urine densities, ρ_{matrix}, listed in Section 3.3 and the appropriate molar masses, $M_{analyte}$: (194.191 ±0.005) g/mol for caffeine and (180.164 ±0.004) g/mol for paraxanthine, theobromine, and theophylline g/mol [21,22].

Table 19. CDC Results for Caffeine-Related Analytes in SRM 3672, nmol/mL.

Caffeine				Paraxanthine				Theobromine				Theophylline			
Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD
14.9	16.0	15.45	0.78	18.4	18.3	18.35	0.07	30.2	30.3	30.25	0.07	2.33	2.32	2.325	0.007
14.3	16.6	15.45	1.63	18.4	20.5	19.45	1.48	27.1	29.4	28.25	1.63	2.19	2.41	2.300	0.156
14.8	16.5	15.65	1.20	19.4	19.6	19.50	0.14	28.6	29.8	29.20	0.85	2.42	2.38	2.400	0.028
13.7	14.8	14.25	0.78	17.3	18.6	17.95	0.92	28.8	30.7	29.75	1.34	2.05	2.22	2.135	0.120
14.2	14.9	14.55	0.49	18.6	19.6	19.10	0.71	29.6	30.8	30.20	0.85	2.17	2.29	2.230	0.085
13.8	15.4	14.60	1.13	17.7	18.7	18.20	0.71	29.8	31.6	30.70	1.27	2.12	2.34	2.230	0.156
15.3	15.6	15.45	0.21	20.3	20.2	20.25	0.07	31.0	31.1	31.05	0.07	2.36	2.43	2.395	0.049
15.6	15.4	15.50	0.14	18.4	19.3	18.85	0.64	29.3	29.8	29.55	0.35	2.20	2.38	2.290	0.127
14.4	16.3	15.35	1.34	19.0	19.5	19.25	0.35	30.1	30.7	30.40	0.42	2.41	2.16	2.285	0.177
15.6	17.1	16.35	1.06	18.6	21.1	19.85	1.77	28.8	32.4	30.60	2.55	2.27	2.37	2.320	0.071
Mean:		15.26		Mean:		19.08		Mean:		30.00		Mean:		2.291	
SD:		0.62		SD:		0.74		SD:		0.83		SD:		0.079	

Table 20. CDC Results for Caffeine-Related Analytes in SRM 3673, nmol/mL.

Caffeine				Paraxanthine				Theobromine				Theophylline			
Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD
20.1	20.6	20.35	0.35	30.5	30.8	30.65	0.21	39.2	39.3	39.25	0.07	3.95	3.94	3.945	0.007
20.6	21.5	21.05	0.64	31.6	31.6	31.60	0.00	40.4	40.4	40.40	0.00	3.89	4.10	3.995	0.148
20.7	21.9	21.30	0.85	31.0	31.7	31.35	0.49	39.8	39.4	39.60	0.28	4.11	4.01	4.060	0.071
19.6	20.1	19.85	0.35	30.9	31.9	31.40	0.71	41.0	41.3	41.15	0.21	4.08	4.09	4.085	0.007
20.0	20.1	20.05	0.07	34.0	33.7	33.85	0.21	41.7	41.5	41.60	0.14	4.07	3.98	4.025	0.064
19.7	20.0	19.85	0.21	32.0	32.5	32.25	0.35	41.8	41.2	41.50	0.42	3.93	4.26	4.095	0.233
20.0	20.2	20.10	0.14	33.5	32.6	33.05	0.64	40.6	41.0	40.80	0.28	4.03	4.22	4.125	0.134
20.9	21.1	21.00	0.14	31.9	33.2	32.55	0.92	41.3	41.2	41.25	0.07	3.85	4.14	3.995	0.205
21.0	21.4	21.20	0.28	34.3	31.6	32.95	1.91	41.8	41.8	41.80	0.00	3.68	3.55	3.615	0.092
22.2	22.5	22.35	0.21	35.9	34.2	35.05	1.20	42.4	42.6	42.50	0.14	4.30	4.14	4.220	0.113
Mean:		20.71		Mean:		32.47		Mean:		40.99		Mean:		4.016	
SD:		0.81		SD:		1.32		SD:		1.00		SD:		0.161	

Table 21. CDC Results for Caffeine-Related Analytes in SRM 3672a, nmol/mL.

Caffeine				Paraxanthine				Theobromine				Theophylline			
Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD
5.87	5.96	5.915	0.064	9.80	10.00	9.90	0.14	25.7	24.6	25.15	0.78	2.06	2.03	2.045	0.021
6.03	6.28	6.155	0.177	10.80	10.20	10.50	0.42	25.3	24.6	24.95	0.49	1.93	2.08	2.005	0.106
6.01	6.28	6.145	0.191	10.30	10.40	10.35	0.07	24.8	25.0	24.90	0.14	2.01	1.94	1.975	0.049
5.72	5.74	5.730	0.014	10.30	9.78	10.04	0.37	26.0	25.8	25.90	0.14	1.95	1.91	1.930	0.028
5.64	5.78	5.710	0.099	10.70	10.50	10.60	0.14	26.5	26.3	26.40	0.14	2.03	1.95	1.990	0.057
5.70	5.69	5.695	0.007	10.10	9.81	9.96	0.21	26.2	26.5	26.35	0.21	1.98	1.90	1.940	0.057
5.85	5.84	5.845	0.007	10.70	10.80	10.75	0.07	26.4	26.0	26.20	0.28	2.03	2.00	2.015	0.021
5.73	5.86	5.795	0.092	10.40	10.60	10.50	0.14	26.8	26.7	26.75	0.07	2.02	1.90	1.960	0.085
5.84	5.98	5.910	0.099	10.50	9.90	10.20	0.42	26.6	25.9	26.25	0.49	1.88	1.90	1.890	0.014
5.83	6.48	6.155	0.460	10.70	10.80	10.75	0.07	26.8	26.3	26.55	0.35	1.97	2.01	1.990	0.028
Mean:		5.906		Mean:		10.35		Mean:		25.94		Mean:		1.974	
SD:		0.186		SD:		0.32		SD:		0.69		SD:		0.045	

Table 22. CDC Results for Caffeine-Related Analytes in SRM 36723a, nmol/mL.

Caffeine				Paraxanthine				Theobromine				Theophylline			
Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD	Inj1	Inj2	Mean	SD
7.78	8.04	7.910	0.184	26.2	24.6	25.40	1.13	18.4	18.2	18.30	0.14	1.74	1.79	1.765	0.035
7.93	8.53	8.230	0.424	26.4	26.3	26.35	0.07	18.1	18.8	18.45	0.49	1.69	1.68	1.685	0.007
7.91	8.40	8.155	0.346	26.2	26.1	26.15	0.07	17.8	17.8	17.80	0.00	1.75	1.72	1.735	0.021
7.70	7.69	7.695	0.007	25.5	25.6	25.55	0.07	19.6	19.6	19.60	0.00	1.69	1.68	1.685	0.007
7.78	7.78	7.780	0.000	28.1	27.8	27.95	0.21	19.8	19.7	19.75	0.07	1.65	1.75	1.700	0.071
7.60	7.57	7.585	0.021	26.0	25.2	25.60	0.57	19.6	19.7	19.65	0.07	1.68	1.67	1.675	0.007
7.74	7.98	7.860	0.170	28.4	28.2	28.30	0.14	19.4	19.9	19.65	0.35	1.75	1.84	1.795	0.064
7.77	7.78	7.775	0.007	26.8	26.2	26.50	0.42	19.3	19.6	19.45	0.21	1.65	1.67	1.660	0.014
7.63	8.18	7.905	0.389	27.3	26.7	27.00	0.42	19.1	19.4	19.25	0.21	1.68	1.61	1.645	0.049
7.67	8.36	8.015	0.488	27.0	28.5	27.75	1.06	19.2	20.3	19.75	0.78	1.64	1.74	1.690	0.071
Mean:		7.891		Mean:		26.66		Mean:		19.17		Mean:		1.704	
SD:		0.200		SD:		1.05		SD:		0.71		SD:		0.047	

7.1.2. Value Assessment

To evaluate whether the measured values for SRMs 3672a and 3673a are appropriate for use in value assessment, Fig. 21 compares the measured values for caffeine and theobromine in SRMs 3672 and 3673 to the non-certified values listed in the SRM 3672 and 3673 Certificates of Analysis (COAs) [2,3]. The listed values were determined by NIST in 2011 using an analysis method like that described in [23].

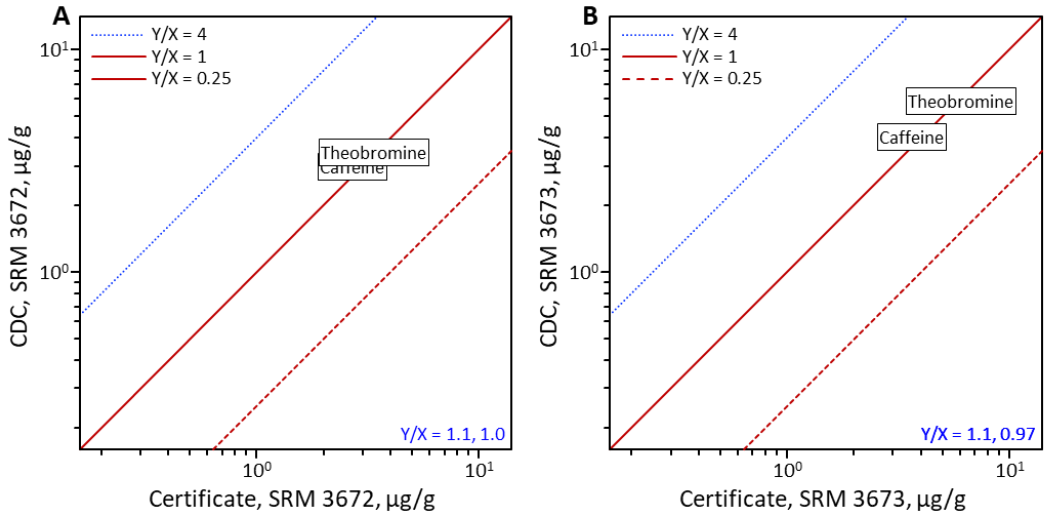


Fig. 21. Caffeine and Theobromine Results as a Function of the 2013 Non-Certified Values.

Panel A compares CDC's current results for caffeine and theobromine in SRM 3672 to the non-certified values listed in the SRM 3672 Certificate of Analysis (COA); Panel B compares CDC's current results for caffeine and theobromine in SRM 3673 to the non-certified values listed in the SRM 3673 COA. Each labeled box within a panel is centered on the location {COA value (X), CDC result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the Y/X ratios for the two analytes.

The CDC's results for caffeine and theobromine are in excellent agreement with the non-certified results given in the COAs. No comparison values are available for paraxanthine and theophylline. However, the use of the same analytical procedure for all the caffeine-related analytes suggests that the CDC's results are suitable for use in value assignment.

7.1.3. Comparisons Between the Original and the New SRMs

As shown in Fig. 22, the concentrations of the caffeine-related analytes in the new materials (SRMs 3672a and 3673a) are roughly 2-fold smaller than in the original materials (SRMs 3672 and 3673). However, only caffeine declined equally in the smoker and non-smoker donor pools.

Different concentrations of these analytes in the new materials were expected due to changes in the general public's dietary intake of foods, drinks, and supplement that contain caffeine and its related alkaloids, as well as general variation in the donor population.

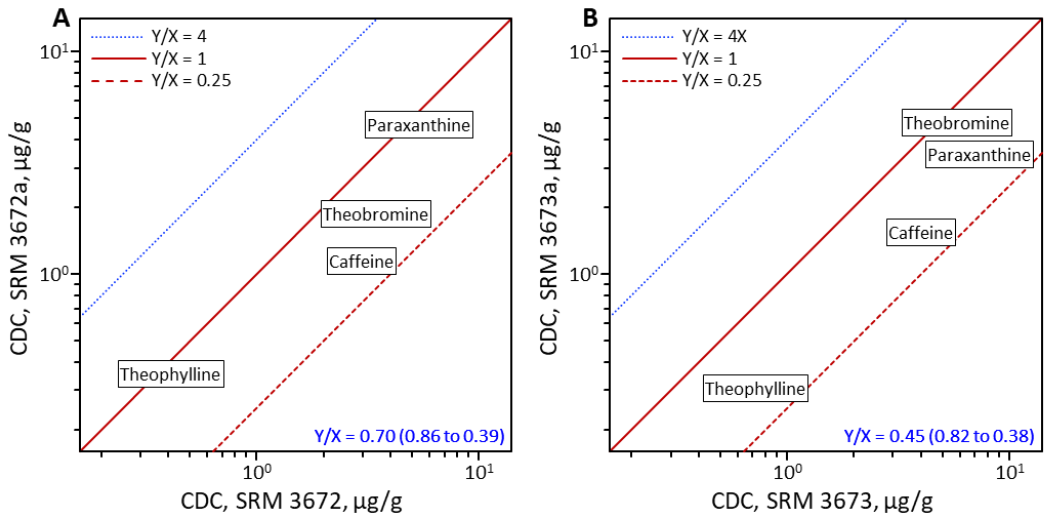


Fig. 22. Caffeine-Related Analyte Results for New SRMs as a Function of Original SRM Results.

Panel A compares CDC's results for the caffeine related analytes in SRM 3672a to their current results for SRM 3672; Panel B compares CDC's results for SRM 3673a to their current results for SRM 3673. Each labeled box within a panel is centered on the location {CDC results for original SRM (X), CDC result for new SRM (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

7.1.4. Comparison Between Smokers' and Non-Smokers' Urines

As shown in Fig. 23, the concentrations of the caffeine-related analytes in the SRM 3673 non-smokers' urine are consistently about 1.5-fold larger than in the SRM 3672 smokers' urine while there is no clear pattern to the SRM 3673a/3672a concentration ratios. This inconsistency suggests that changes in the xanthine alkaloid concentrations reflect differences in the donor populations that are not causally related to smoking.

Compared to the most recent NHANES caffeine and caffeine metabolite measurements [24], most of the analytes in both new materials are close to or between the 50th and 75th percentiles. The theobromine level in SRM 3672a is between the 25th and 50th percentiles. The materials provide a reasonable range to support NHANES measurements.

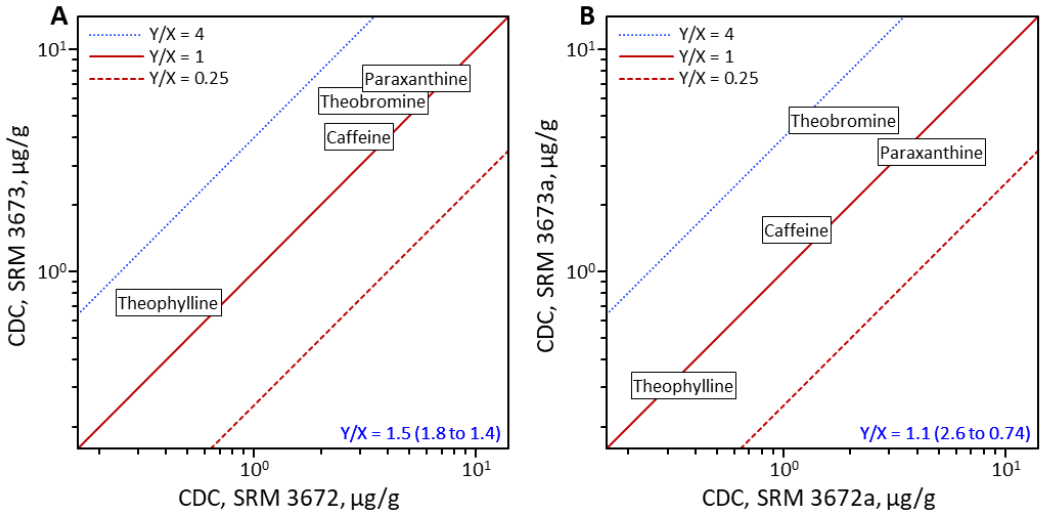


Fig. 23. Caffeine-Related Analyte Non-Smoker Results as a Function of Smoker Results.

Panel A compares CDC's results for the caffeine related analytes in SRM 3672a to their current results for SRM 3672; Panel B compares CDC's results for the caffeine related analytes in SRM 3673a to their current results for SRM 3672. Each labeled box within a panel is centered on the location {CDC results for original SRM (X), CDC result for new SRM (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

7.2. NIST Measurements

One vial of SRM 3673 and seven vials each of 3672a and 3673a were used for the assessment. The vials were maintained in -80 °C freezers.

7.2.1. Analysis

All sample measurements were made by NIST staff using a recently developed gas chromatography-mass spectrometry (GC-MS) method. The following sections describe the measurements.

7.2.1.1. Sample Preparation

Vials were thawed and brought to room temperature. Gravimetrically, 1 mL of urine was transferred to a glass centrifuge tube and 50 µL of internal standard mixture was added gravimetrically. Samples were allowed to equilibrate for 2 hours before 0.8 mL of Fisher Optima Chloroform was added. The sample was vortexed for 1 min then centrifuged at 378 rad/s (3600 rpm) for 30 min. The bottom chloroform layer was then transferred to a glass insert with feet in an amber autosampler vial for injection.

Three blanks containing only internal standards diluted in chloroform were prepared and extracted.

Calibration standards were gravimetrically prepared from neat standards diluted in Fisher Optima Acetonitrile. NIST Primary Standard Material for caffeine (PSM3) was used as the calibration standard for caffeine. The PSM3 purity had been assigned as (0.998 ±0.002) g/g against the NIST PS1 Primary Standard for qNMR (Benzoic Acid) [25] using quantitative internal standard proton nuclear magnetic resonance spectrometry ($q^1H\ NMR_{IS}$) [26]. All other calibrants and internal standards were purchased from commercial sources.

Separate calibration curves were generated for caffeine and the caffeine metabolites due to a caffeine signal detected in the metabolite standards when analyzed without the presence of caffeine.

7.2.1.2. Instrumental Method

The method used by NIST for caffeine in 2011 did not fully resolve the metabolites. A splitless injection method using a SLB-IL59 (0.2 µm, 30 m × 0.25 mm, Supelco) column with a 1 m guard column was developed following its identification by Reyes-Contreras et al. [27] as having the capability to separate the caffeine metabolites. The method was implemented using an Agilent 7000 GC/MS Triple quad with an Agilent 7693 Autosampler and 7890A GC System. The method was optimized to shorten the run-time and improve separation.

The inlet heater was set to 270 °C and a pressure of 94.5 kPa. Septum purge flow was 3 mL/min and purge flow to split vent was 25 mL/min at 0.8 min. Sample injection was 2 µL with eight wash steps with chloroform pre- and post-injection. The mass spectrometer detector transfer

line was set at 300 °C. The triple quad collision cells were not used but kept at 0.5 mL/min of He quench gas and 0.5 mL of N_2 collision gas.

Both qualifier and quantifier masses for caffeine (194, 109), paraxanthine (180, 68), theobromine (180, 109), and theophylline (180, 93, 68) were tracked using secondary ion monitoring (SIM) with three windows. Exact match internal standards were monitored using the following m/z: caffeine-$^{13}C_3$ (197), theobromine-d_6 (186), paraxanthine-d_3 (183), and theophylline-$^{13}C_2d_6$ (188).

Example SIM mode chromatograms for SRMs 3672a, 3673a, and 3673 are displayed in Fig. 24.

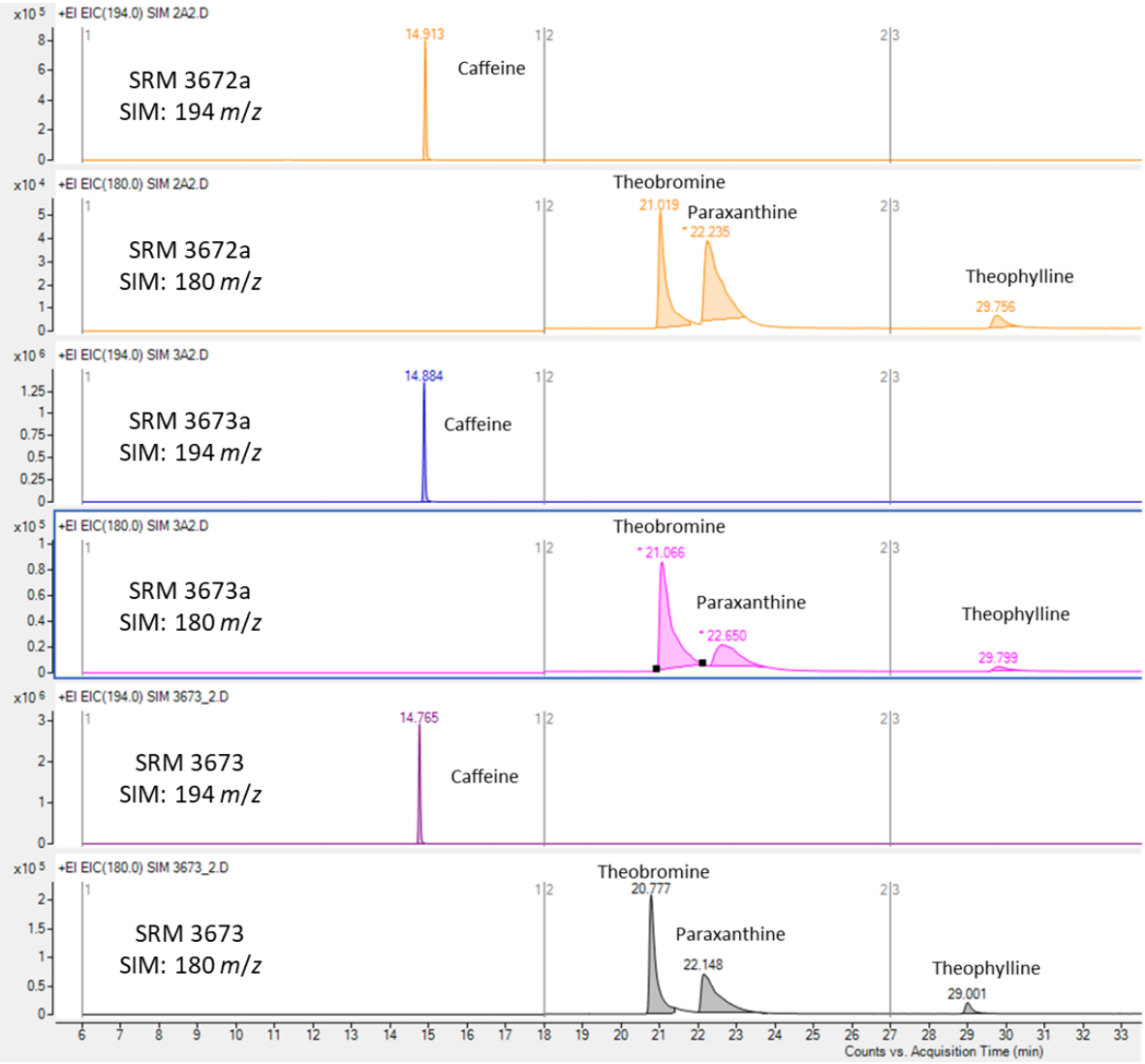


Fig. 24. SIM Chromatograms for SRMs 3672a, 3673a, and 3673.

7.2.1.3. Quantitation

Integrations were performed using MassHunter Quantitative Analysis version 10.0 (Agilent). Samples were normalized by the internal standard and mass adjusted before interpolation using linear calibration models. This calculation was done using both the quantifier mass and the qualifier mass, when available, to compare results. Mass fraction values were estimated using a bespoke spreadsheet; these values were validated against those provided by the EMMA system [15].

Table 23 lists the results for single injections of the three replicate extractions of the SRM 3673 control material. Table 24 lists the results for single injections of single extracts from seven (SRM 3672a) or six (SRM 3673a) vials of the new materials.

Table 23. NIST Results for Caffeine-Related Analytes in SRM 3673, µg/g.

	SRM 3673, µg/g			
Replicate	Caffeine	Paraxanthine	Theobromine	Theophylline
1	3.718	5.817	7.167	0.6850
2	3.752	6.190	7.543	0.6450
3	3.778	5.875	7.500	0.6900
N:	3	3	3	3
Mean:	3.749	5.961	7.403	0.6733
SD:	0.030	0.201	0.206	0.0247

Table 24. NIST Results for Caffeine-Related Analytes in SRM 3672a and 3673a, µg/g.

	SRM 3672a, µg/g				SRM 3673a, µg/g			
Vial	Caffeine	Paraxanthine	Theobromine	Theophylline	Caffeine	Paraxanthine	Theobromine	Theophylline
1	1.002	1.8310	4.890	0.3210	*a*	*a*	*a*	*a*
2	1.040	2.0330	4.924	0.3620	1.447	4.969	3.102	0.3110
3	1.032	2.0880	5.170	0.3590	1.454	5.305	3.413	0.3210
4	1.036	1.9160	4.852	0.3680	1.465	5.364	3.294	0.3250
5	1.031	1.9340	5.024	0.3510	1.455	5.482	3.189	0.3240
6	1.040	2.0650	5.188	0.3500	1.452	5.524	3.182	0.3450
7	1.031	1.9820	5.191	0.3610	1.459	5.644	3.517	0.3090
N:	7	7	7	7	6	6	6	6
Mean:	1.030	1.9784	5.034	0.3531	1.455	5.381	3.283	0.3225
SD:	0.013	0.0914	0.149	0.0155	0.006	0.235	0.157	0.0129

a) Sample precipitated during extraction. No usable extract was obtained.

7.2.2. Value Assessment

To evaluate whether the measured values for SRMs 3672a and 3673a are appropriate for use in value assessment, Fig. 25 compares the measured values for caffeine and theobromine in SRM 3673 to the non-certified values listed in the SRM 3673 Certificates of Analysis (COA) [3]. The COA values were determined by NIST in 2011 using an analysis method similar to that described in [28].

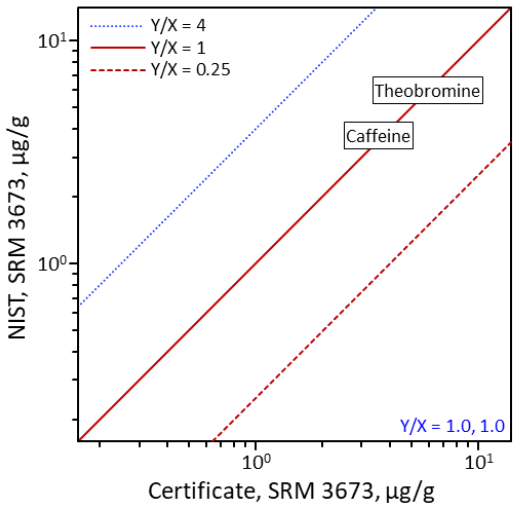


Fig. 25. Caffeine and Theobromine Results for SRM 3673 as a Function of 2013 Non-Certified Values.

This figure compares the NIST results for caffeine and theobromine in SRM 3673 to the non-certified values listed in the SRM 3673 certificate of analysis (COA). Each labeled box is centered on the location {COA value (X), NIST result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the Y/X ratios for the two analytes.

NIST's results for caffeine and theobromine are in excellent agreement with the non-certified results given in the COA. No comparison values are available for paraxanthine and theophylline.

7.2.3. Comparison of CDC and NIST Results

To further explore the utility of the NIST results, Fig. 26 compares the NIST results for SRMs 3673, 3672a, and 3673a to the CDC results. All NIST results on average are equal to the CDC results within a range of (1.1 to 0.9)-fold.

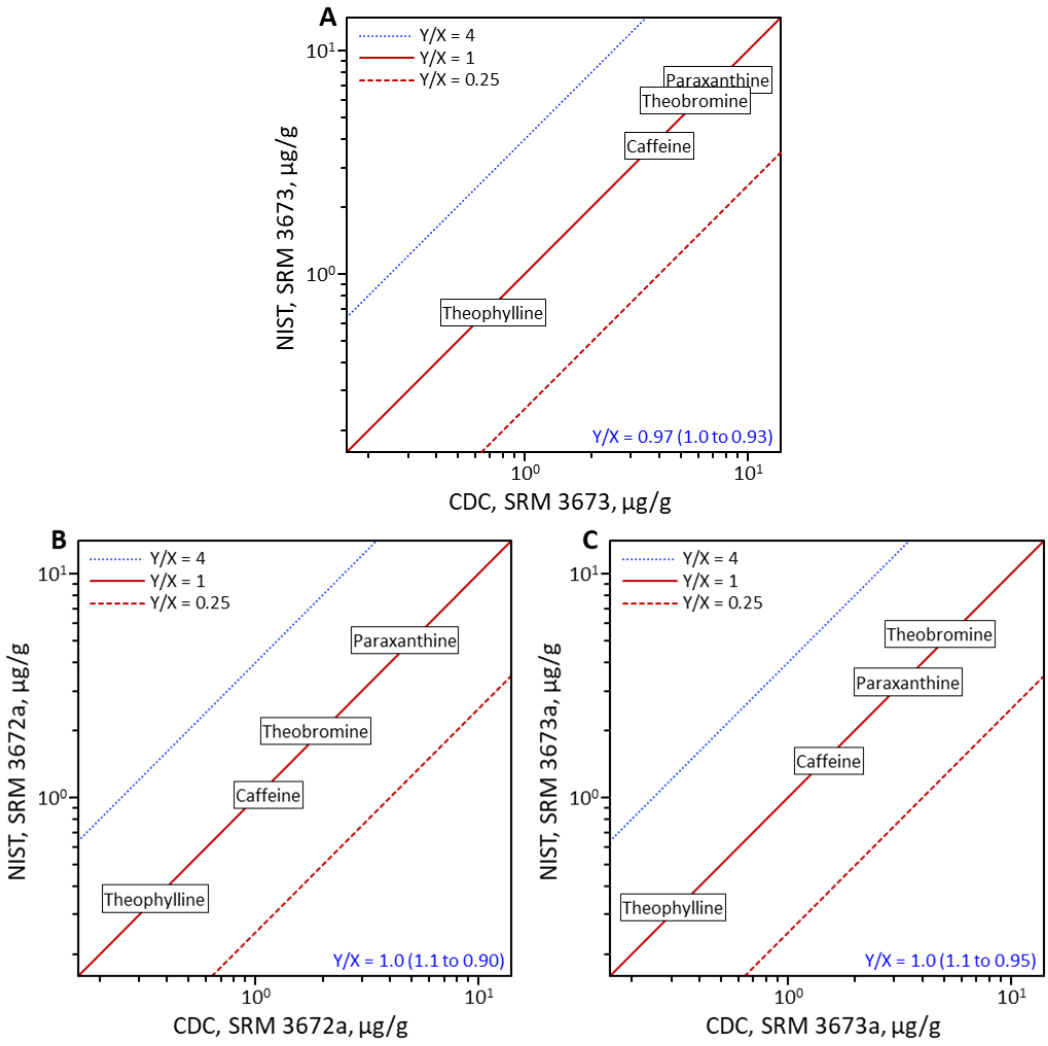


Fig. 26.NIST Caffeine-Related Analyte Results as a Function of CDC Results.

These panels compare the NIST results to the CDC results: Panel A compares results for 3673, Panel B compares results for SRM 3672a, and Panel C compares results for SRM 3673a. Each labeled box within a panel is centered on the location {CDC result (X), NIST result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

7.3. Assigned Values

Table 25 lists the assigned values for caffeine and its metabolites.

Table 25. Assigned Values for Caffeine and its Metabolites in SRMs 3672a and 3673a, µg/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
Caffeine	1.0274	0.0083	0.0164	2	1.4476	0.0092	0.0180	2
Paraxanthine	4.84	0.19	0.38	2	3.33	0.10	0.20	2
Theobromine	1.913	0.061	0.121	2	5.07	0.31	0.60	2
Theophylline	0.3538	0.0027	0.0054	2	0.3130	0.0090	0.0176	2

a $w_{analyte}$, mass fraction of the analyte.
b $u(w_{analyte})$, standard uncertainty associated with $w_{analyte}$.
c $U_{95}(w_{analyte})$, approximate 95 % level of confidence expanded uncertainty associated with $w_{analyte}$.
d Number of methods contributing results used to estimate $w_{analyte}$; see Section 4.

7.3.1. Metrological Traceability

The caffeine result is traceable to the SI through NIST PSM3 caffeine, which was value assigned using NIST PS1 Benzoic Acid [25,26] as calibrant. The values for paraxanthine, theobromine, and theophylline are metrologically traceable to the materials used to prepare the calibration solutions.

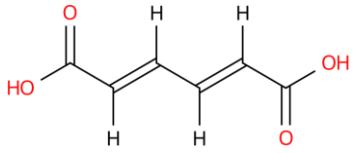
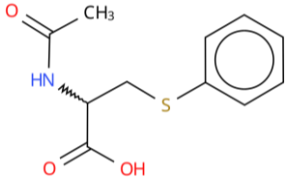
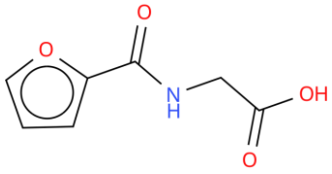
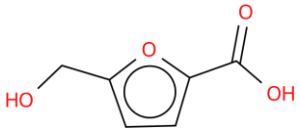
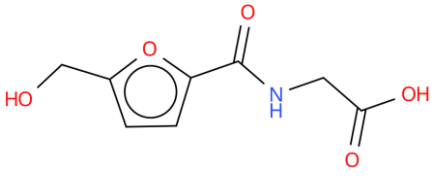
8. Volatile Organic Compounds (VOCs)

Volatile organic compounds (VOCs) are ubiquitous in the environment, originating from many different natural and anthropogenic sources. Human exposure to VOCs occurs through inhalation, ingestion, and dermal contact. Exposure to VOCs is associated with numerous health risks including respiratory, liver, blood, and hormonal toxicity as well as cancer. VOCs can be metabolized prior to urinary excretion, so VOC exposure can be assessed by measuring their metabolites in urine. The CDC monitors these metabolites through the NHANES project [1].

8.1. Benzene and Furfural Metabolites

Table 26 lists metabolites of benzene, furfural, and 5-hydroxymethylfurfural that were measured in SRMs 3672a and 3673a by the CDC using their ultra-performance liquid chromatography with electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) Method 2105.02 [29]. The metabolites are identified by their acronym, common name, International Chemical Identifier (InChI), chemical structure, and parent compound.

Table 26. Benzene and Furfural Metabolites and Their Parent Compounds.

Metabolite	Structure	Parent
MUCA *t*,*t*-Muconic acid InChI=1S/C6H6O4/c7-5(8)3-1-2-4-6(9)10/h1-4H,(H		Benzene
PhMA N-Acetyl-S-(phenyl)-L-cysteine InChI=1S/C11H13NO3S/c1-8(13)12-10(11(14)15)7-16-9-5-3-2-4-6-9/h2-6,10H,7H2,1H3,(H,12,13)(H,14,15)/t10-/m0/s1		Benzene
N2FG N-2-Furoylglycine InChI=1S/C7H7NO4/c9-6(10)4-8-7(11)5-2-1-3-12-5/h1-3H,4H2,(H,8,11)(H,9,10)		Furfural
HMFA 5-Hydroxymethyl-2-furancarboxylic acid InChI=1S/C6H6O4/c7-3-4-1-2-5(10-4)6(8)9/h1-2,7H,3H2,(H,8,9)		5-Hydroxymethylfurfural
HMFG 5-Hydroxymethyl-2-furoylglycine InChI=1S/C8H9NO5/c10-4-5-1-2-6(14-5)8(13)9-3-7(11)12/h1-2,10H,3-4H2,(H,9,13)(H,11,12)		5-Hydroxymethylfurfural

8.1.1. Analysis

Six vials of SRM 3672a, six vials of 3673a, one vial of 3672 and one vial of 3673 were sent from NIST to the CDC by overnight shipping on dry ice. Vials were stored at -80 °C until analysis.

8.1.1.1. Sample Preparation

All solutions, calibrants, control materials, and blanks used in the analysis of the SRM 3672a and 3673a materials were prepared as described in [29]. Materials in a urine matrix were diluted 1:10 in aqueous sample preparation buffer consisting of 5 mmol/L ammonium formate and 0.15 % formic acid at pH 2.89 to 2.93. External calibration solutions were prepared from nominally neat materials in the sample preparation buffer. The calibrants spanned more than a 1000-fold concentration range. High and low concentration level quality control (QC) samples were prepared from urine pools.

The 1:10 urine-matrix dilutions were achieved by adding 25 µL of an internal standard solution of isotopically labelled metabolite and 425 µL sample preparation buffer to each 50 µL sample. The diluted samples were mixed and then transferred to autosampler for analysis.

8.1.1.2. Instrumental Method

An analytical run consists of solvent blanks, blanks with internal standard, calibration standards, low level QC, high level QC and the SRM 3672, 3673, 3672a, and 3673a samples. Chromatographic separation of the analytes is achieved with a UPLC system (e.g., Waters Acquity or Shimadzu LC-40) fitted with a reversed phase pentafluorophenyl column (e.g., Waters Acquity UPLC HSS PFP) and run on a methanol and water (0.02 % formic acid by volume) gradient. A triple quadrupole mass spectrometer (e.g., Sciex 5500) on scheduled multiple reaction monitoring with an electrospray ion source is used for the detection of the urinary VOC metabolites [30].

8.1.1.3. Quantitation

A set of nine calibration solutions is analyzed twice, bracketing unknowns and QC materials in an analytical run. These calibration results are combined and used for the quantification of analytes in all samples and QC materials from that batch. Calibration curves are constructed for each analyte from the peak response ratio of standards to internal standards at the nine different concentration levels. The results sent to NIST were reviewed by the CDC's quality assurance officer and approved as conforming to the quality standards at the CDC.

Results for the low- and high-level QC materials were within expected bands. Results for SRMs 3672 and 3673 are listed in Table 27. Results for SRMs 3672a and 3673a are listed in Table 28. These results were recorded and are listed here as amount concentrations, x_{analyte}. They are transformed to mass fractions, w_{analyte}, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3.

Table 27. Results for Benzene and Furfural Metabolites in SRMs 3272 and 3673, ng/mL.

	SRM 3672					SRM 3673				
Replicate	MUCA	PhMA	N2FG	HMFA	HMFG	MUCA	PhMA	N2FG	HMFA	HMFG
1	190	1.06	28700	4090	595	56.0	0.226	13900	2170	272
2	196	1.18	29200	3860	622	56.8	0.193	13800	2270	281
3	185	1.13	29400	3870	586	56.1	0.193	14300	2370	290
N:	3	3	3	3	3	3	3	3	3	3
Mean:	190	1.12	29100	3940	601	56.3	0.204	14000	2270	281
SD:	6	0.06	361	130	19	0.4	0.019	265	100	9

Table 28. Results for Benzene and Furfural Metabolites in SRMs 3272a and 3673a, ng/mL.

	SRM 3672a					SRM 3673a				
Vial	MUCA	PhMA	N2FG	HMFA	HMFG	MUCA	PhMA	N2FG	HMFA	HMFG
1	115	1.53	16600	4900	1090	127	0.192	7400	3480	485
2	119	1.57	17800	4970	1160	123	0.186	7440	3660	446
3	118	1.68	17500	4610	1190	128	0.192	7550	3620	487
4	115	1.57	17500	4950	1080	123	0.211	7460	3270	466
5	113	1.57	16600	4550	1200	126	0.191	7480	3760	477
6	113	1.57	17300	4460	1130	126	0.195	7540	3410	444
N:	6	6	6	6	6	6	6	6	6	6
Mean:	116	1.58	17217	4740	1142	126	0.195	7478	3533	468
SD:	3	0.05	504	225	50	2	0.009	58	180	19
%RSD:	2.2	3.2	2.9	4.8	4.4	1.7	4.4	0.8	5.1	4.1

8.1.2. Value Assessment

To evaluate whether the measured values for these metabolites in SRMs 3672a and 3673a are appropriate for use in value assessment, Fig. 27 compares the measured values for the benzene metabolites MUCA and PhMA in SRMs 3672 and 3673 to the values reported by the CDC in 2012 and listed in [4].

The Certificate of Analysis (COA) for SRM 3673 [3] provides non-certified values for MUCA and PhMA; however, these results were revised in 2022 based upon the measurements listed in Table 27. While based on the original method as described in [31], the current implementation of the method [29] provides a 2-fold improvement in sensitivity which provides more reliable results for low concentration analytes. The current results for the larger concentrations of these analytes in SRM 3672 are in excellent agreement with those from the 2012 implementation.

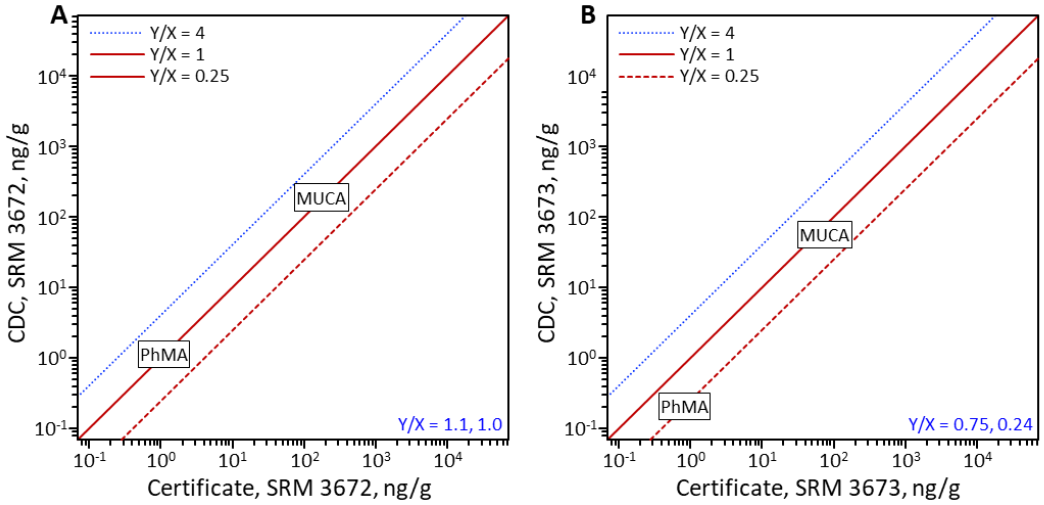


Fig. 27. Benzene and Furfural Metabolite Results as a Function of 2012 Values.

Panel A compares CDC's current results for the two benzene metabolites in SRM 3672 to the non-certified values reported by the CDC in 2012 and listed in the SRM 3672 Certificate of Analysis (COA); Panel B compares CDC's current results for the metabolites in SRM 3673 reported by the CDC in 2012 [4]. Each labeled box within a panel is centered on the location {2012 CDC result (X), current CDC result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the Y/X ratios for the two analytes.

8.1.3. Comparisons Between the Original and the New SRMs

As shown in Fig. 28, the concentrations of the benzene and furfural metabolites in the new materials (SRMs 3672a and 3673a) range from 2-fold smaller to 2-fold larger than in the original materials (SRMs 3672 and 3673).

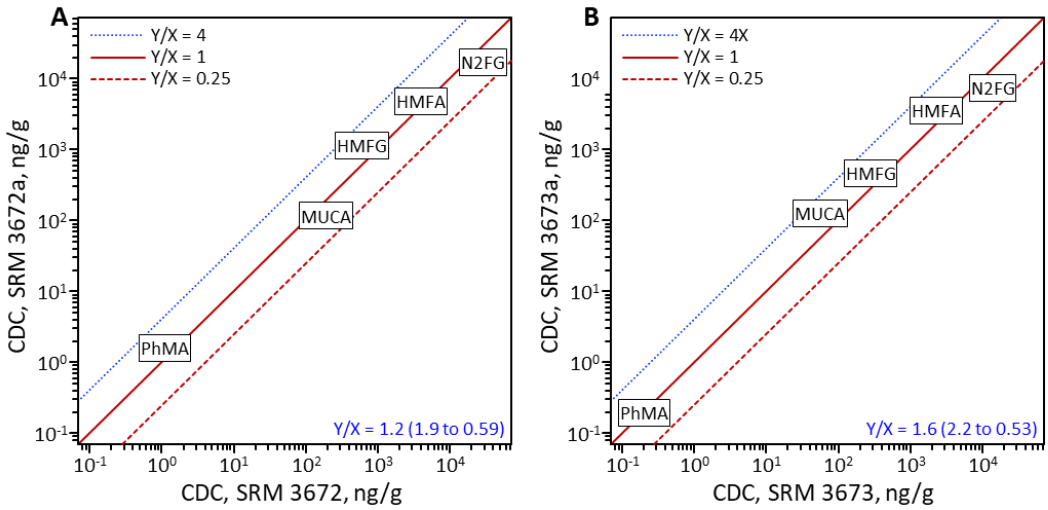


Fig. 28. Benzene and Furfural Metabolite Results for New SRMs as a Function of Original SRM Results.

Panel A compares CDC's results for the benzene and furfural metabolites in SRM 3672a to their current results for SRM 3672; Panel B compares CDC's results for SRM 3673a to their current results for SRM 3673. Each labeled box within a panel is centered on the location {CDC results for original SRM (X), CDC result for new SRM (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

As shown in Fig. 29, HMFG in the new materials is nearly 2-fold larger than that in original materials, a potential indication of an increase in the consumption of heated or cooked sugar-containing food or flavored e-cigarettes. However, N2FG in the new materials is nearly 2-fold smaller than that in the original materials, a potential indication of a decreased consumption of strongly heated foods. The parallel increase and decrease of these analytes in the smokers' and non-smokers' urines suggests that they are unlikely to be related to use of flavored e-cigarettes since the contributors to the non-smoker material were screened for vaping or exposure to second-hand vapor (see Section 2.1.1).

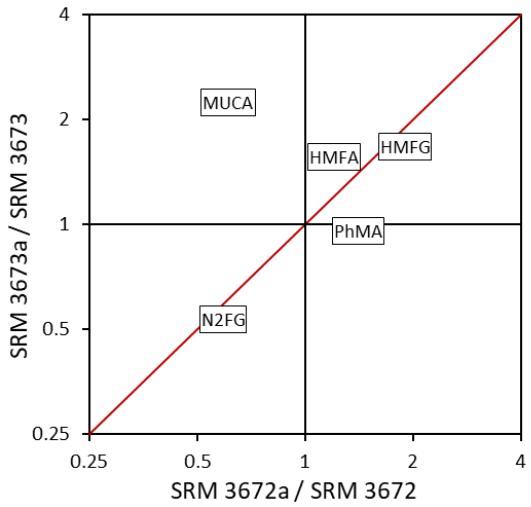


Fig. 29. Benzene and Furfural Metabolite 3673a/3673 Results as a Function of 3672a/3672 Results.

Each labeled box is centered on the location {result for SRM 3672a / result for SRM 3672 (X), result for SRM 3673a / SRM 3673 (Y)}. The diagonal line represents equality between the ratios. The horizontal and vertical lines denote unit ratios.

8.1.4. Comparison Between Smokers' and Non-Smokers' Urines

As shown in Fig. 30, the concentrations of the benzene and furfural metabolites in the SRM 3673 non-smokers' urine are consistently about 2-fold smaller than in the SRM 3672 smokers' urine. The concentrations of PhMA, HMFG, HMFA, and N2FG in SRM 3673a are likewise (2 to 8)-fold smaller than in 3672a and are consistent with the donor pool descriptions. However, the concentration of MUCA is slightly larger in the non-smoker SRM 3673a material than in SRM 3672a smokers' urine. The Statement of Requirements did not mandate that the donor pools be screened for occupational or environmental exposure to benzene (see Section 2.1.1).

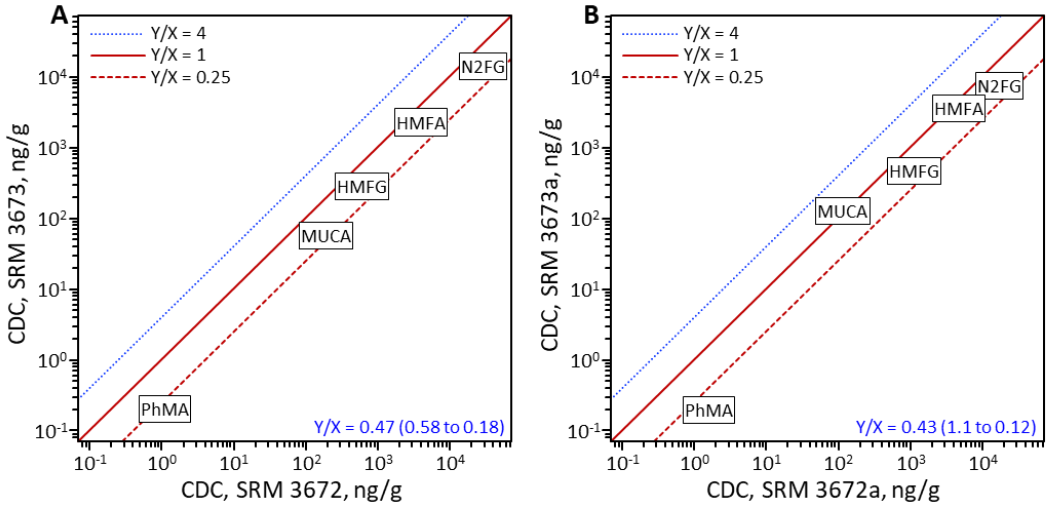


Fig. 30. Benzene and Furfural Metabolite Non-Smoker Results as a Function of Smoker Results.

Panel A compares CDC's results for the benzene and furfural metabolites in SRM 3672 to their results for SRM 3673; Panel B compares CDC's results for SRM 3672a to their results for SRM 3673a. Each labeled box within a panel is centered on the location {CDC results for smokers' urine (X), CDC result for non-smokers' urine (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

8.1.5. Assigned Values

Table 29 lists the assigned values for benzene and furfural metabolites.

Table 29. Assigned Values for Benzene and Furfural Metabolites in SRMs 3672a and 3673a, ng/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
MUCA	114.9	1.0	2.6	1	124.6	0.8	2.2	1
PhMA	1.574	0.021	0.053	1	0.1931	0.0035	0.0090	1
N2FG	17131	205	526	1	7423	24	61	1
HMFA	4716	92	235	1	3507	73	188	1
HMFG	1136	20	53	1	464.1	7.7	19.7	1

a $w_{analyte}$, mass fraction of the analyte.
b $u(w_{analyte})$, standard uncertainty associated with $w_{analyte}$.
c $U_{95}(w_{analyte})$, approximate 95 % level of confidence expanded uncertainty associated with $w_{analyte}$.
d Number of methods contributing results used to estimate $w_{analyte}$; see Section 4.

8.1.5.1. Metrological Traceability

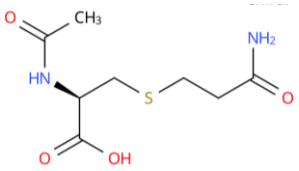
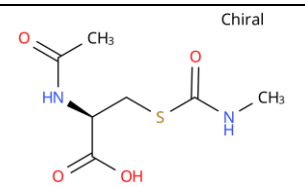
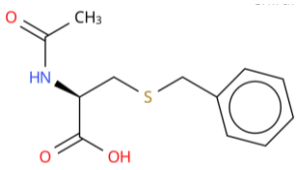
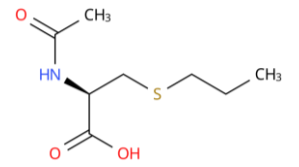
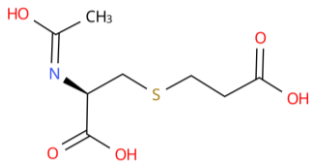
The benzene and furfural metabolite analyte results are metrologically traceable to the materials used to prepare the calibration solutions.

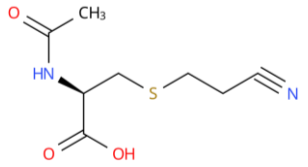
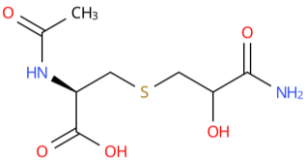
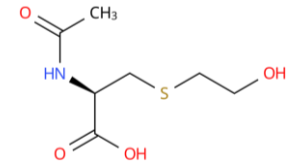
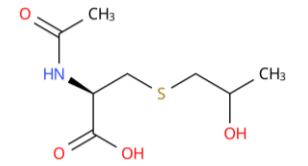
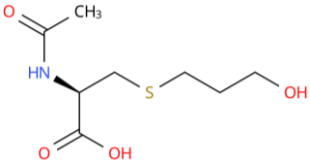
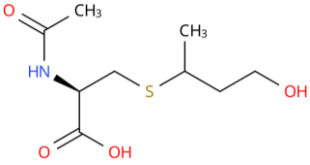
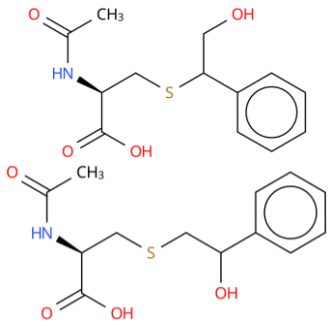
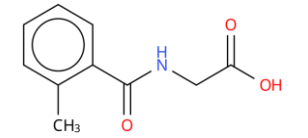
8.2. Mercapturic Acid and Related Biomarkers

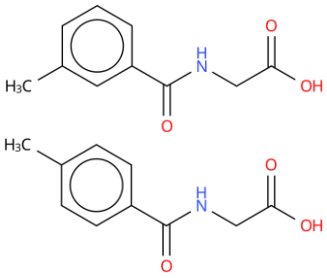
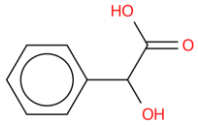
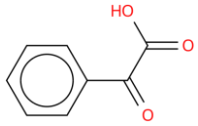
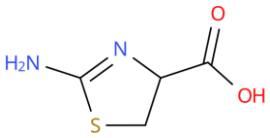
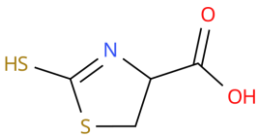
Monitoring urinary metabolites of VOCs provides complimentary data to measuring VOCs in exhaled breath or blood, and a longer time window during which biomarkers are elevated following cessation of exposure to VOCs. The non-invasive sampling of urine, longer physiological half-lives of mercapturic acids, and relatively high degree of specificity make urinary mercapturic acids useful biomarkers of exposure to VOCs. Mercapturic acids are formed primarily through the metabolism of VOCs via the glutathione pathway. VOCs and/or their metabolites can react with glutathione and undergo further metabolism to form mercapturic acids. These metabolites are then removed from the blood by the kidneys and excreted into urine.

Table 30 lists the mercapturic acid and related biomarkers of VOC exposure that were measured in SRMs 3672a and 3673a by the CDC using their ultra-performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) Method 2103 [32]. The metabolites are identified by their acronym, common name, International Chemical Identifier (InChI), chemical structure, and parent compound.

Table 30. Mercapturic and Related Biomarker and Their Parents.

Metabolite	Structure	Parent(s)
2CaEMA N-Acetyl-S-(2-carbamoylethyl)-L-cysteine InChI=1S/C8H16N2O4S/c1-5(11)10-6(8(13)14)4-15-3-2-7(9)12/h6-7,12H,2-4,9H2,1H3,(H,10,11)(H,13,14)/t6-,7?/m0/s1		Acrylamide
MCaMA N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine InChI=1S/C7H12N2O4S/c1-4(10)9-5(6(11)12)3-14-7(13)8-2/h5H,3H2,1-2H3,(H,8,13)(H,9,10)(H,11,12)/t5-/m0/s1		N,N-Dimethylformamide Methyl isocyanate
BzMA N-Acetyl-S-(benzyl)-L-cysteine InChI=1S/C12H15NO3S/c1-9(14)13-11(12(15)16)8-17-7-10-5-3-2-4-6-10/h2-6,11H,7-8H2,1H3,(H,13,14)(H,15,16)/t11-/m0/s1		Toluene Benzyl alcohol
1PMA N-Acetyl-S-(n-propyl)-L-cysteine InChI=1S/C8H15NO3S/c1-3-4-13-5-7(8(11)12)9-6(2)10/h7H,3-5H2,1-2H3,(H,9,10)(H,11,12)/t7-/m0/s1		1-Bromopropane
2CoEMA N-Acetyl-S-(2-carboxyethyl)-L-cysteine InChI=1S/C12H15NO3S/c1-9(14)13-11(12(15)16)8-17-7-10-5-3-2-4-6-10/h2-6,11H,7-8H2,1H3,(H,13,14)(H,15,16)/t11-/m0/s1		Acrolein

Metabolite	Structure	Parent(s)
2CyEMA N-Acetyl-S-(2-cyanoethyl)-L-cysteine InChI=1S/C8H12N2O3S/c1-6(11)10-7(8(12)13)5-14-4-2-3-9/h7H,2,4-5H2,1H3,(H,10,11)(H,12,13)/t7-/m0/s1		Acrylonitrile
2CaHEMA N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine InChI=1S/C8H14N2O5S/c1-4(11)10-5(8(14)15)2-16-3-6(12)7(9)13/h5-6,12H,2-3H2,1H3,(H2,9,13)(H,10,11)(H,14,15)/t5-,6?/m0/s1		Acrylamide
2HEMA N-Acetyl-S-(2-hydroxyethyl)-L-cysteine a InChI=1S/C7H13NO4S/c1-5(10)8-6(7(11)12)4-13-3-2-9/h6,9H,2-4H2,1H3,(H,8,10)(H,11,12)/t6-/m0/s1		Acrylonitrile Vinyl chloride Ethylene oxide
2HPMA N-Acetyl-S-(2-hydroxypropyl)-L-cysteine InChI=1S/C8H15NO4S/c1-5(10)3-14-4-7(8(12)13)9-6(2)11/h5,7,10H,3-4H2,1-2H3,(H,9,11)(H,12,13)/t5?,7-/m0/s1		Propylene oxide
3HPMA N-Acetyl-S-(3-hydroxypropyl)-L-cysteine InChI=1S/C8H15NO4S/c1-6(11)9-7(8(12)13)5-14-4-2-3-10/h7,10H,2-5H2,1H3,(H,9,11)(H,12,13)/t7-/m0/s1		Acrolein
3HMPMA N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine InChI=1S/C9H17NO4S/c1-6(3-4-11)15-5-8(9(13)14)10-7(2)12/h6,8,11H,3-5H2,1-2H3,(H,10,12)(H,13,14)/t6?,8-/m0/s1		Crotonaldehyde
2HPhEMA N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine N-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine InChI=1S/C13H17NO4S/c1-9(16)14-11(13(17)18)8-19-12(7-15)10-5-3-2-4-6-10/h2-6,11-12,15H,7-8H2,1H3,(H,14,16)(H,17,18)/t11-,12?/m0/s1 InChI=1S/C13H17NO4S/c1-9(15)14-11(13(17)18)7-19-8-12(16)10-5-3-2-4-6-10/h2-6,11-12,16H,7-8H2,1H3,(H,14,15)(H,17,18)/t11-,12?/m0/s1		Styrene
2MHA 2-Methylhippuric acid InChI=1S/C10H11NO3/c1-7-4-2-3-5-8(7)10(14)11-6-9(12)13/h2-5H,6H2,1H3,(H,11,14)(H,12,13)		Xylene

Metabolite	Structure	Parent(s)
3MHA+4MHA 3-Methylhippuric acid 4-Methylhippuric acid InChI=1S/C10H11NO3/c1-7-3-2-4-8(5-7)10(14)11-6-9(12)13/h2-5H,6H2,1H3,(H,11,14)(H,12,13) InChI=1S/C10H11NO3/c1-7-2-4-8(5-3-7)10(14)11-6-9(12)13/h2-5H,6H2,1H3,(H,11,14)(H,12,13)		Xylene
MADA Mandelic acid InChI=1S/C8H8O3/c9-7(8(10)11)6-4-2-1-3-5-6/h1-5,7,9H,(H,10,11)		Styrene Ethylbenzene
PhGA Phenylglyoxylic acid InChI=1S/C8H6O3/c9-7(8(10)11)6-4-2-1-3-5-6/h1-5H,(H,10,11)		Ethylbenzene Styrene
2ATCA 2-Aminothiazoline-4-carboxylic acid InChI=1S/C4H6N2O2S/c5-4-6-2(1-9-4)3(7)8/h2H,1H2,(H2,5,6)(H,7,8)		Cyanide
TTCA 2-Thioxothiazolidine-4-carboxylic acid InChI=1S/C4H5NO2S2/c6-3(7)2-1-9-4(8)5-2/h2H,1H2,(H,5,8)(H,6,7)		Carbon disulfide

8.2.1. Analysis

The SRM materials described in Section 8.1.1 were used in the analysis of these analytes.

8.2.1.1. Sample Preparation

Urine samples and QC materials were diluted 1:10 with 15 mmol/L ammonium acetate, amended with internal standard and mixed before transfer to autosampler for analysis. An analytical run consisted of a 15 mmol/L ammonium acetate blank, a 15 mmol/L ammonium acetate and internal standard blank, calibrants, low level QC, high level QC, and urine samples.

A set of nine calibration stock solutions were prepared from neat standards. Calibrants were prepared by diluting the stocks with 15 mmol/L ammonium acetate buffer. It had been previously established that for this and related analysis methods, calibrators prepared in ammonium acetate buffer produced the same results as calibrators prepared in urine [31].

8.2.1.2. Instrumental Method

A triple quadrupole mass spectrometer (e.g., AB Sciex Triple Quad 5500) with an electrospray ion source is used for the detection of urinary VOC metabolites operated under Scheduled Multiple Reaction Monitoring (MRM) mode. Chromatographic separation is achieved with a UPLC system (e.g., Waters Acquity) fitted with a reversed phase C18 column (e.g., Acquity UPLC® HSS T3) and guard column with a gradient of 15 mmol/L ammonium acetate (solvent A) and acetonitrile (solvent B). The UPLC gradient used is described in Table 31.

Table 31. UPLC Gradient Used in Analysis of Mercapturic and Related Biomarkers.

Time, min	Flow, µL/min	% Solvent A	% Solvent B
0	250	97	3
2	250	95	5
3	300	90	10
5	300	70	30
6.5	300	60	40
7	300	85	15
7.5	300	90	10
8	300	97	3
9	300	97	3

8.2.1.3. Quantitation

Unknown samples were quantified by the ratio of the analyte peak area to the internal standard peak area. Values are interpolated from the linear calibration curves. The linear calibration models were parameterized using $1/x$ weighted regression, where the x is the standard concentration.

The values assigned to the calibration stock solutions were not corrected for the purity of the neat standards. All standards and internal standards had a stated nominal purity of ≥ 98 %.

Results for the low- and high-level QC materials were within expected bands. All results sent to NIST were reviewed by the CDC's quality assurance officer and approved as conforming to the quality standards at the CDC.

Results for SRMs 3672 and 3672a are listed in Table 32. Results for SRMs 3673 and 3673a are listed in Table 33. These results were recorded and are listed here as amount concentrations, x_{analyte}. They are transformed to mass fractions, w_{analyte}, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3.

Table 32. CDC Results for Mercapturic and Related Biomarkers in SRMs 3672 and 3672a, ng/mL.

	SRM 3672					SRM 3672a							
Analyte	Prep1	Prep2	Prep3	Mean	SD	Prep1	Prep2	Prep3	Prep4	Prep5	Prep6	Mean	SD
2CaEMA	125	141	149	138	12	89.8	90.6	86.7	86.7	84.9	88.1	87.8	2.1
MCaMA	287	279	287	284.3	4.6	322	266	280	272	271	277	281	21
BzMA	5.04	4.64	4.72	4.80	0.21	10.9	11.2	11.8	11.0	11.6	11.5	11.3	0.4
1PMA	14.5	14.3	13.9	14.23	0.31	9.40	8.72	8.06	8.64	7.63	9.10	8.59	0.65
2CoEMA	172	166	160	166.0	6.0	194.0	195.0	196.0	199.0	192.0	197.0	195.5	2.4
2CyEMA	114	121	121	118.7	4.0	166	169	167	167	170	166	167.5	1.6
2CaHEMA	16.5	16.3	15.3	16.03	0.64	11.30	11.70	10.90	9.93	10.60	11.90	11.06	0.73
2HEMA	2.91	2.58	2.74	2.74	0.17	3.36	3.11	2.95	3.13	3.07	3.17	3.13	0.13
2HPMA	155	157	154	155.3	1.5	59.8	62.8	63.1	63.0	59.6	61.8	61.7	1.6
3HPMA	1050	1050	1020	1040	17	1130	1110	1140	1140	1120	1150	1132	15
3HMPMA	858	894	879	877	18	953	960	975	989	947	953	963	16
2HPhEMA	1.56	1.75	1.63	1.65	0.10	2.46	2.60	2.62	2.62	2.73	2.70	2.622	0.094
2MHA	91.5	93.5	99.1	94.7	3.9	91.7	82.3	82.9	86.3	84.5	78.3	84.3	4.5
3MHA + 4MHA	479	450	445	458	18	416	411	413	424	411	414	414.8	4.9
MADA	223	221	227	223.7	3.1	258	275	267	255	234	269	260	15
PhGA	308	311	309	309.3	1.5	201	207	200	190	194	191	197.2	6.6
2ATCA	122	130	119	123.7	5.7	91.9	81.1	84.0	78.2	100.0	67.4	83.8	11.3
TTCA	20.6	22.0	19.8	20.8	1.1	34.4	31.4	35.8	33.7	31.3	33.9	33.4	1.8

Table 33. CDC Results for Mercapturic and Related Biomarkers in SRMs 3673 and 3673a, ng/mL.

	SRM 3673					SRM 3673a							
Analyte	Prep1	Prep2	Prep3	Mean	SD	Prep1	Prep2	Prep3	Prep4	Prep5	Prep6	Mean	SD
2CaEMA	29.1	31.7	30.9	30.6	1.3	60.0	61.7	61.8	61.0	61.7	60.0	61.03	0.85
MCaMA	115	107	105	109.0	5.3	52.6	48.5	50.9	48.0	53.1	44.6	49.6	3.2
BzMA	3.52	3.43	2.88	3.28	0.35	4.04	4.20	4.08	3.83	4.08	4.02	4.04	0.12
1PMA	4.75	4.34	3.95	4.35	0.40	5.02	4.73	5.47	5.47	6.25	5.95	5.48	0.56
2CoEMA	46.0	42.5	39.1	42.5	3.5	98.3	95.7	98.7	96.7	104.0	98.9	98.7	2.9
2CyEMA	5.91	5.83	5.57	5.77	0.18	6.69	6.66	7.03	6.95	7.18	6.74	6.88	0.21
2CaHEMA				<9.4 [a]								<9.4 [a]	
2HEMA				<0.79 [a]								<0.79 [a]	
2HPMA	20.2	22.1	20.4	20.9	1.0	32.8	32.4	33.3	32.6	33.0	32.4	32.75	0.36
3HPMA	185	194	162	180	17	267	269	299	271	279	271	276	12
3HMPMA	142	147	140	143	4	164	163	168	164	164	162	164.2	2.0
2HPhEMA				<1.0 [a]								<1.0 [a]	
2MHA	28.4	25.7	24.1	26.1	2.2	13.7	12.5	11.5	14.1	12.8	13.2	12.97	0.92
3MHA + 4MHA	98.6	91.3	92.9	94.3	3.8	63.1	57.5	61.2	59.9	65.8	62.1	61.6	2.8
MADA	69.4	79.2	85.3	78.0	8.0	112.0	107.0	90.3	88.8	85.5	102.0	97.6	10.9
PhGA	152	146	139	145.7	6.5	134	133	136	132	131	132	133.0	1.8
2ATCA	66.6	56.4	50.5	57.8	8.1	90.2	82.2	91.1	75.3	89.6	70.3	83.1	8.7
TTCA	26.8	28.0	24.7	26.5	1.7	41.2	39.0	42.4	37.6	38.3	38.5	39.5	1.9

a Analyte was either not detected or the estimated concentration was at or below the method's limit of detection (LOD) for the analyte.

8.2.2. Value Assessment

To evaluate whether the measured values for these metabolites in SRMs 3672a and 3673a are appropriate for use in value assessment, Fig. 31 compares the measured values for the mercapturic acid and related biomarkers in SRMs 3672 and 3673 to the values reported by the CDC in 2012 and listed in [4].

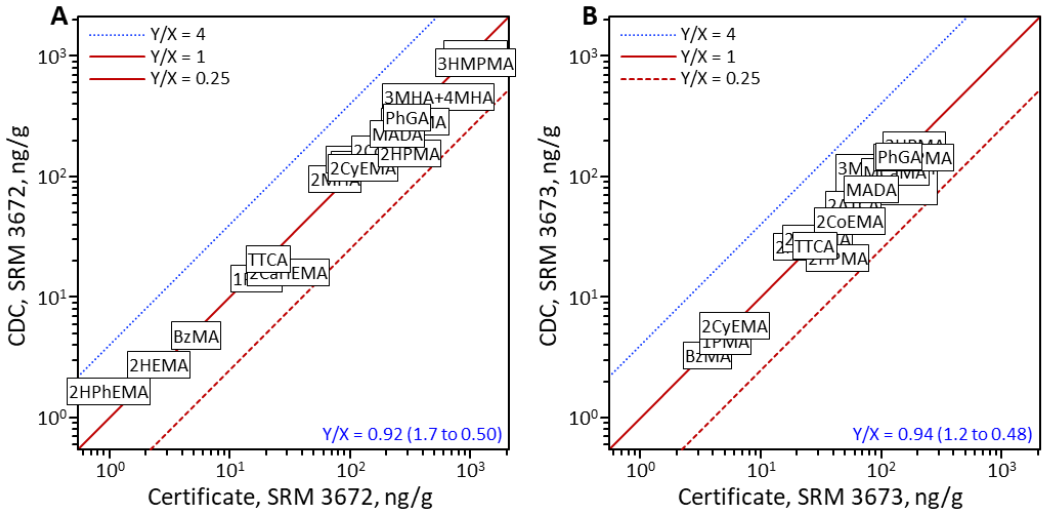


Fig. 31. Mercapturic and Related Biomarker Results as a Function of the CDC's 2012 Values.

Panel A compares CDC's current results for the 18 biomarkers in SRM 3672 to the non-certified values reported by the CDC in 2012 and listed in [4]; Panel B compares CDC's current results for the 15 biomarkers in SRM 3673 reported by the CDC in 2012 and listed in [4]. Each labeled box within a panel is centered on the location {2012 CDC result (X), current CDC result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

The Certificates of Analysis (COA) for SRM 3672 and 3673 [3] provide non-certified values for 2CaHEMA, 2HPMA, and 3HMPMA; however, these results were revised in 2022 based upon the measurements listed in Table 32 and Table 33. The CDC has updated their method for these analytes to take in account the salt in the calibration standards. The current SRM 3672 and 3673 results are smaller by about a factor of two from what was originally reported. The current method also has enhanced sensitivity, providing more reliable results for low concentration analytes. The 2012 2HPhEMA result was less than the current method's 1.0 ng/mL limit of detection (LOD).

Excluding 2CaHEMA, 2HPMA, 3HMPMA, and 2HPhEMA, the CDC's current results for the mercapturic acid and related metabolites are in good agreement with their 2012 values.

8.2.3. Comparisons Between the Original and the New SRMs

As shown in Fig. 32, the concentrations of the mercapturic acid and related biomarkers in the new materials (SRMs 3672a and 3673a) are on average equal to or slightly larger than in the original materials (SRMs 3672 and 3673), although in both of the new materials the concentrations range from 2-fold smaller to 2-fold larger than in the original materials.

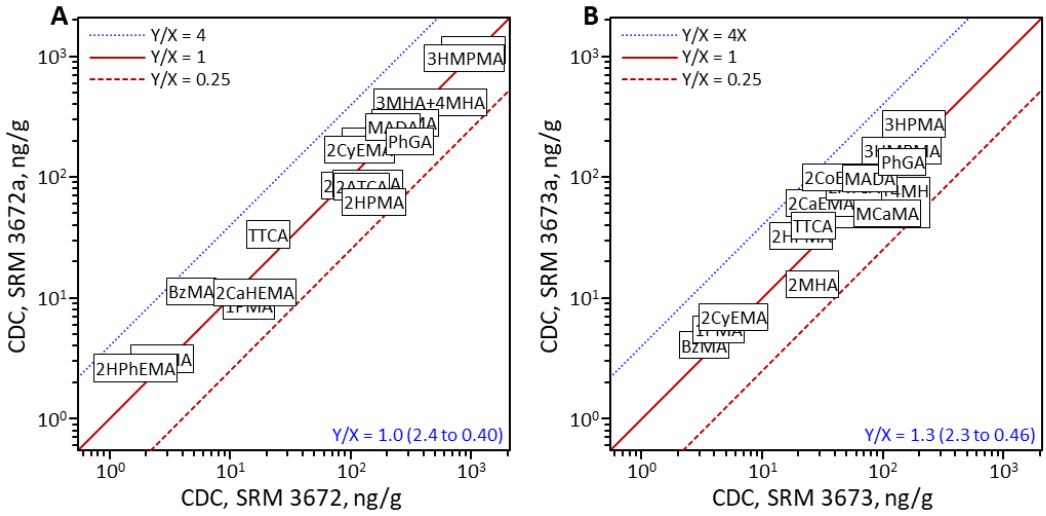


Fig. 32. Mercapturic and Related Biomarker Results for New SRMs as a Function of Original SRM Results.

Panel A compares CDC's results for the mercapturic acid and related biomarkers in SRM 3672a to their current results for SRM 3672; Panel B compares CDC's results for SRM 3673a to their current results for SRM 3673. Each labeled box within a panel is centered on the location {CDC results for original SRM (X), CDC result for new SRM (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

As shown in Fig. 33, there is very little correlation between the SRM 3672a/SRM 3672 and SRM 3673a/SRM 3673 ratios. The relative standard deviations of the SRM 3672a/SRM 3672 and SRM 3673a/SRM3763 ratios are about the same (45 % and 40 %). Given that the average biomarker levels increased more in the non-smoker material than in the smoker material, the differences between the new and the original materials may be driven by increased but variable environmental exposures.

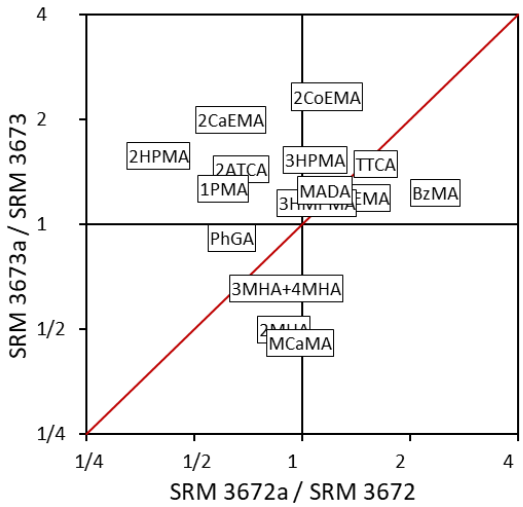


Fig. 33. Mercapturic and Related Biomarker 3673a/3673 Results as a Function of 3672a/3672 Results.

Each labeled box is centered on the location {result for SRM 2672a divided by the result for SRM 3672 (X), result for SRM 3673a divided by the result for SRM 3673 (Y)}. The diagonal line represents equality between the ratios. The horizontal and vertical lines denote unit ratios.

8.2.4. Comparison Between Smokers' and Non-Smokers' Urines

As shown in Fig. 34, the concentrations of the mercapturic acid and related biomarkers in the SRM 3673 and 3673a non-smokers' urines are consistently about 3-fold smaller than in the SRM 3672 and 3672a smokers' urines. Other than for TTCA, a marker for carbon disulfide exposure and the only biomarker that has a slightly larger concentration in the non-smokers' urines, these differences are consistent with the donor pool descriptions.

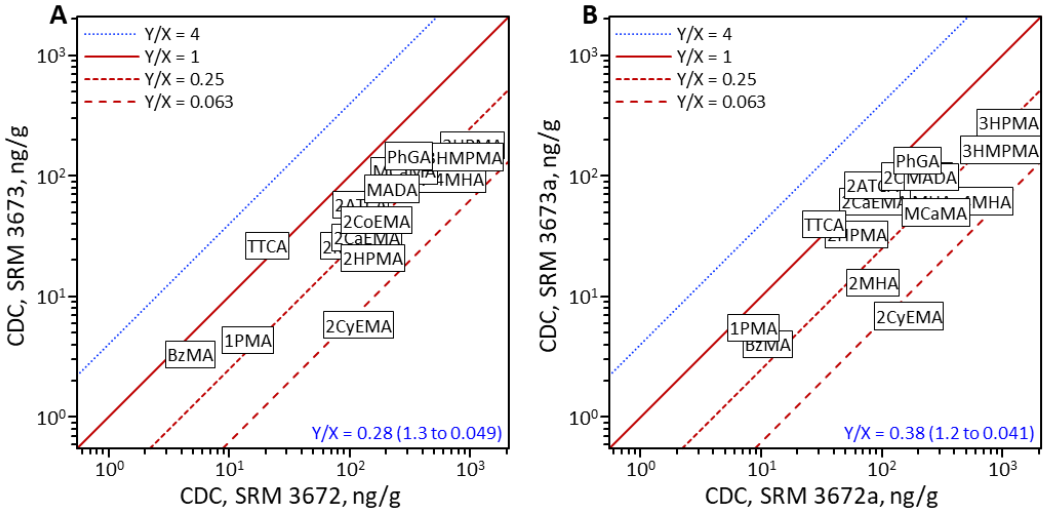


Fig. 34. Mercapturic and Related Biomarker Results Non-Smoker Results as a Function of Smoker Results.

Panel A compares CDC's results for the mercapturic and related biomarkers in SRM 3672 to their results for SRM 3673; Panel B compares CDC's results for SRM 3672a to their results for SRM 3673a. Each labeled box within a panel is centered on the location {CDC results for smokers' urine (X), CDC result for non-smokers' urine (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The long-dashed diagonal line denotes Y results that are a factor of sixteen smaller than the X results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

8.2.5. Assigned Values

Table 34 lists the assigned values for mercapturic acid and related biomarkers.

Table 34. Assigned Values for Mercapturic Acid and Related Biomarkers in SRMs 3672a and 3673a, ng/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
2CaEMA	87.36	0.87	2.22	1	60.59	0.34	0.89	1
MCaMA	279.9	8.3	21.4	1	49.3	1.3	3.4	1
BzMA	11.28	0.14	0.37	1	4.012	0.049	0.126	1
1PMA	8.55	0.27	0.68	1	5.44	0.23	0.59	1
2CoEMA	194.53	0.99	2.54	1	98.0	1.2	3.0	1
2CyEMA	166.67	0.67	1.72	1	6.83	0.09	0.22	1
2CaHEMA	11.00	0.30	0.77	1				e
2HEMA	3.116	0.055	0.141	1				e
2HPMA	61.38	0.65	1.68	1	32.51	0.14	0.37	1
3HPMA	1126.0	6.0	15.4	1	274.0	4.9	12.5	1
3HMPMA	958.0	6.5	16.7	1	163.0	0.8	2.1	1
2HPhEMA	2.609	0.038	0.098	1				e
2MHA	83.9	1.8	4.7	1	12.87	0.37	0.96	1
3MHA + 4MHA	412.8	2.0	5.1	1	61.1	1.1	2.9	1
MADA	258.4	5.9	15.2	1	96.9	4.4	11.3	1
PhGA	196.2	2.7	6.9	1	132.02	0.72	1.86	1
2ATCA	83.3	4.6	11.8	1	82.5	3.5	9.1	1
TTCA	33.25	0.72	1.84	1	39.21	0.76	1.95	1

a $w_{analyte}$, mass fraction of the analyte.
b $u(w_{analyte})$, standard uncertainty associated with $w_{analyte}$.
c $U_{95}(w_{analyte})$, approximate 95 % level of confidence expanded uncertainty associated with $w_{analyte}$.
d Number of methods contributing results used to estimate $w_{analyte}$; see Section 4.
e No reliable quantitative result available.

8.2.5.1. Metrological Traceability

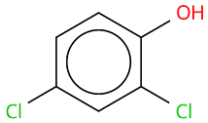
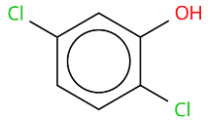
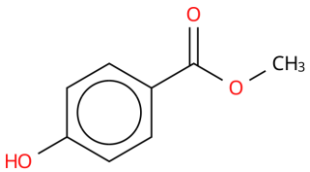
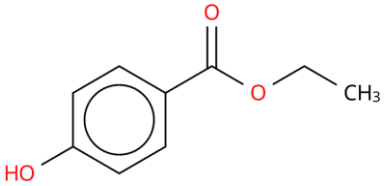
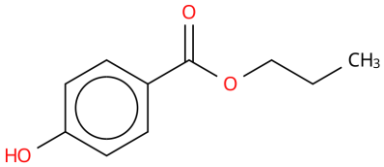
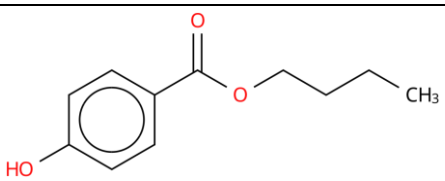
The mercapturic acid and related biomarker results are metrologically traceable to the materials used to prepare the calibration solutions.

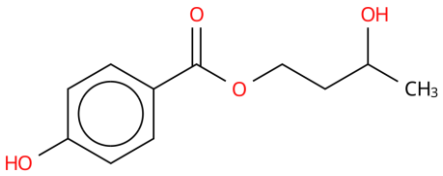
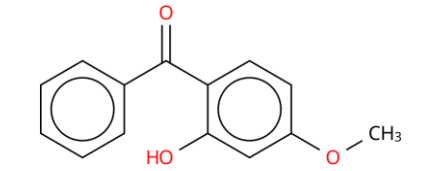
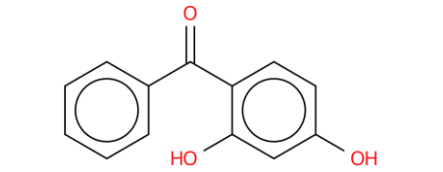
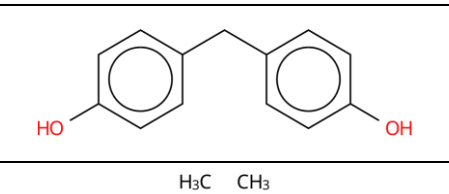
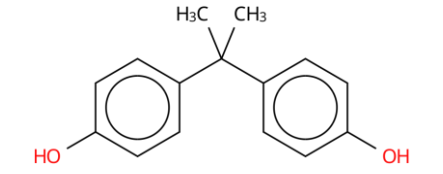
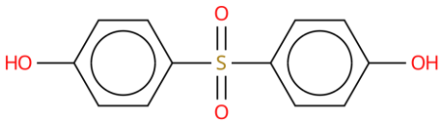
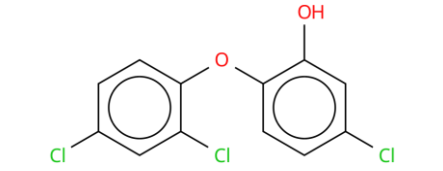
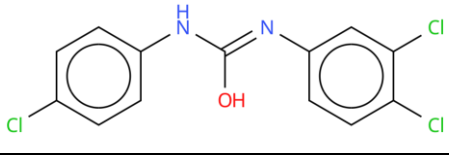
9. Phenolics

Phenols, benzophenones, triclosan, and parabens are classes of phenolic compounds that are commonly used in personal care products. Metabolites of these compounds are often measured in human urine to assess exposure to these chemicals, particularly those with known detrimental health effects. Over the past decade, some chemicals of these classes have been withdrawn from the consumer market and substitutes introduced. The CDC monitors these phenolics through measurements of their metabolites in urine through the National Health and Nutrition Examination Survey (NHANES) project [1].

Table 35 lists the phenolic metabolites that were measured in SRMs 3672a and 3673a by the CDC using their on-line solid-phase extraction liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS) method [33]. The metabolites are identified by their acronym, common name, International Chemical Identifier (InChI), and chemical structure.

Table 35. Environmental Phenolics, Names and Structures.

Analyte	Structure
2,4-DCP 2,4-Dichlorophenol InChI=1S/C6H4Cl2O/c7-4-1-2-6(9)5(8)3-4/h1-3,9H	
2,5-DCP 2,5-Dichlorophenol InChI=1S/C6H4Cl2O/c7-4-1-2-5(8)6(9)3-4/h1-3,9H	
M-PB Methyl Paraben InChI=1S/C8H8O3/c1-11-8(10)6-2-4-7(9)5-3-6/h2-5,9H,1H3	
E-PB Ethyl Paraben InChI=1S/C9H10O3/c1-2-12-9(11)7-3-5-8(10)6-4-7/h3-6,10H,2H2,1H3	
P-PB Propyl Paraben InChI=1S/C10H12O3/c1-2-7-13-10(12)8-3-5-9(11)6-4-8/h3-6,11H,2,7H2,1H3	
B-PB Butyl Paraben InChI=1S/C11H14O3/c1-2-3-8-14-11(13)9-4-6-10(12)7-5-9/h4-7,12H,2-3,8H2,1H3	

HPB 3-Hydroxy n-butyl paraben InChI=1S/C11H14O4/c1-8(12)6-7-15-11(14)9-2-4-10(13)5-3-9/h2-5,8,12-13H,6-7H2,1H3	
BP-3 Benzophenone-3 InChI=1S/C14H12O3/c1-17-11-7-8-12(13(15)9-11)14(16)10-5-3-2-4-6-10/h2-9,15H,1H3	
DHAVO Dihydroxyavobenzone InChI=1S/C13H10O3/c14-10-6-7-11(12(15)8-10)13(16)9-4-2-1-3-5-9/h1-8,14-15H	
BPF Bisphenol F InChI=1S/C13H12O2/c14-12-5-1-10(2-6-12)9-11-3-7-13(15)8-4-11/h1-8,14-15H,9H2	
BPA Bisphenol A InChI=1S/C15H16O2/c1-15(2,11-3-7-13(16)8-4-11)12-5-9-14(17)10-6-12/h3-10,16-17H,1-2H3	
BPS Bisphenol S InChI=1S/C12H10O4S/c13-9-1-5-11(6-2-9)17(15,16)12-7-3-10(14)4-8-12/h1-8,13-14H	
TCS Triclosan InChI=1S/C12H7Cl3O2/c13-7-1-3-11(9(15)5-7)17-12-4-2-8(14)6-10(12)16/h1-6,16H	
TCC Triclocarban InChI=1S/C13H9Cl3N2O/c14-8-1-3-9(4-2-8)17-13(19)18-10-5-6-11(15)12(16)7-10/h1-7H,(H2,17,18,19)	

9.1. Analysis

One vial each of SRMs 3672, 3673, 3672a, 3673a were sent from NIST to the CDC by overnight shipping on dry ice. Vials were stored at -80 °C until analysis. All sample measurements were made by CDC staff using the SPE-LC-MS/MS method described in [33].

9.1.1. Sample Preparation

Three replicates each from SRMs 3672 and 3673 and six replicates each from SRMs 3672a and 3673a were prepared. All samples were analyzed in tandem with low QC and high QC materials generated by amending pools of human urine.

Urine samples were thawed to room temperature and mixed before transferring 100 µL to an autosampler vial and mixed with 100 µL of a mixture of internal standards containing deconjugation standard solution, 100 uL of MeOH, and 100 µL of enzyme/ammonium acetate before incubation at 37 °C for 4 hours. Samples were then diluted with 600 µL of 0.1 mol/L formic acid (by volume) and centrifuged.

9.1.2. Instrumental Method

The on-line method was implemented using Agilent 1260 modules attached to a Sciex API 4000 LC-MS/MS using atmospheric pressure chemical ionization (APCI) in negative mode. The SPE column was C18 XS flash column(15 µm, 25 mm × 4.6 mm, Interchim), and the HPLC column was a Chromolith HighResolution.RP-18e column (100 mm × 4.6 mm, Merck).

9.1.3. Quantitation

Standard solutions covering the linear range of the analysis were used to generate linear calibration models. Results for the low- and high-level QC materials were within expected bands. The results sent to NIST were reviewed by the CDC's quality assurance officer and approved as conforming to the quality standards at the CDC.

Results for SRMs 3672 and 3673 are listed in Table 36. Results for SRMs 3672a and 3673a are listed in Table 37. These results were recorded and are listed here as amount concentrations, x_{analyte}. They are transformed to mass fractions, w_{analyte}, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3.

Table 36. CDC Results for Environmental Phenolics in SRMs 3672 and 3672a, ng/mL.

	SRM 3672					SRM 3672a							
Analyte	Prep1	Prep2	Prep3	Mean	SD	Prep1	Prep2	Prep3	Prep4	Prep5	Prep6	Mean	SD
2,4-DCP	0.3	0.4	0.3	0.33	0.06	14.1	14.1	15.4	14.4	14.2	14.3	14.42	0.50
2,5-DCP	1.9	1.9	1.8	1.87	0.06	576	569	631	580	580	579	586	23
M-PB	114	110	115	113.0	2.6	23.8	22.6	25.6	24.3	24.5	23.9	24.1	1.0
E-PB	9.2	9.2	9.4	9.27	0.12	8	7.7	8.7	7.8	8	7.9	8.02	0.35
P-PB	18.9	18.9	19.2	19.00	0.17	4	3.8	4.3	3.9	3.9	3.9	3.97	0.18
B-PB	11.5	11.5	11.7	11.57	0.12							<0.1 [a]	
HBP	5.8	5.7	5.9	5.80	0.10							<0.1 [a]	
BP-3	213	212	219	214.7	3.8	6.7	6.5	7.5	6.7	6.8	7	6.87	0.35
DHAVO				<0.1 [a]								<0.1 [a]	
BPF	1.5	1.6	1.7	1.60	0.10	6.7	6.4	7.3	6.4	6.7	6.9	6.73	0.34
BPA	3	3.2	3.2	3.13	0.12	1	1	1.1	1	1	1.1	1.03	0.05
BPS	0.2	0.2	0.2	0.20	0	1.1	1.1	1.2	1.1	1.1	1.1	1.12	0.04
TCS	19	20.8	19.2	19.67	0.99							<0.1 [a]	
TCC				<0.1 [a]								<0.1 [a]	

a Analyte was either not detected or the estimated concentration was at or below the method's limit of detection (LOD) for the analyte.

Table 37. CDC Results for Environmental Phenolics in SRMs 3673 and 3673a, ng/mL.

	SRM 3673					SRM 3673a							
Analyte	Prep1	Prep2	Prep3	Mean	SD	Prep1	Prep2	Prep3	Prep4	Prep5	Prep6	Mean	SD
2,4-DCP	0.3	0.2	0.2	0.23	0.06	0.4	0.4	0.4	0.5	0.4	0.4	0.42	0.04
2,5-DCP	0.8	0.7	0.7	0.73	0.06	17.9	17.8	18	18.3	17.4	17.7	17.85	0.30
M-PB	84	86.1	82.8	84.3	1.7	29.2	29	28.9	29.6	27.7	29.1	28.92	0.64
E-PB	10.9	11.1	10.7	10.90	0.20	5.8	5.8	5.7	5.9	5.6	5.7	5.75	0.10
P-PB	22.8	23.4	22.2	22.80	0.60	4.6	4.7	4.6	4.8	4.5	4.5	4.62	0.12
B-PB	1.3	1.3	1.2	1.27	0.06							<0.1 [a]	
HBP	1.2	1.2	1.2	1.20	0							<0.1 [a]	
BP-3	307	316	300	307.7	8.0	213	210	212	221	206	212	212.3	4.9
DHAVO				<0.1 [a]		0.1	0.1	0.1	0.1	0.1	0.1	0.10	0
BPF	1.9	1.8	1.9	1.87	0.06	0.9	0.8	0.9	0.8	0.7	0.8	0.82	0.08
BPA	2.2	2.1	2	2.10	0.10	0.5	0.3	0.4	0.5	0.4	0.4	0.42	0.08
BPS	0.1	0.1	0.1	0.10	0	0.7	0.7	0.8	0.8	0.7	0.8	0.75	0.05
TCS	7.8	6.9	6.8	7.17	0.55	2.2	1.8	1.9	1.9	2.9	1.8	2.08	0.43
TCC	2.5	2.5	2.3	2.43	0.12							<0.1 [a]	

a Analyte was either not detected or the estimated concentration was at or below the method's limit of detection (LOD) for the analyte.

9.2. Value Assessment

To evaluate whether the measured values for the environmental phenolic metabolites in SRMs 3672a and 3673a are appropriate for use in value assessment, Fig. 35 compares the CDC's current results for the eight phenolic metabolites that were assigned non-certified values in the SRM 3672 and 3673 Certificates of Analysis (COAs) [2,3]: 2,5-DCP, M-PB, E-PB, P-PB, B-PB, BP-3, BPA, and TCS.

The values listed in the COAs for these metabolites were assigned using both CDC and NIST measurement results. The current CDC values for these metabolites are on average equal to these composite values, within a very narrow range of (1.1 to 0.98)-fold differences.

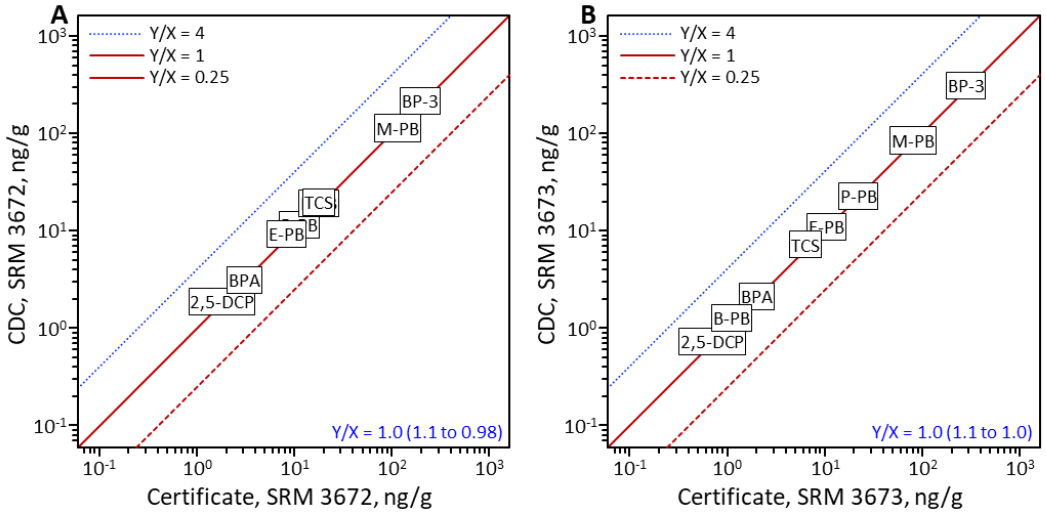


Fig. 35. Phenolic Metabolite Results as a Function of COA Values.

Panel A compares CDC's current results for eight phenolic metabolites in SRM 3672 to the non-certified values in the Certificate of Analysis (COA) [2]; Panel B compares CDC's current results for the eight analytes in SRM 3673 to the non-certified COA values in [3]. Each labeled box within a panel is centered on the location {COA result (X), current CDC result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratio.

9.2.1. Comparisons Between the Original and the New SRMs

As shown in Fig. 36, the concentrations of most of the phenolic metabolites in the new materials (SRMs 3672a and 3673a) are quite different from those in than in the original materials (SRMs 3672 and 3673). While on average the concentrations in the SRM 3672a material are about equal to those in SRM 3672, the range from 300-fold larger (2,5-DCP) to 30-fold smaller (BP-3). The differences in the non-smokers' urine are less extreme, with most of the metabolite concentrations in SRM 3673a about 4-fold smaller than in SRM 3673, but 2,5-DCP is 25-fold larger.

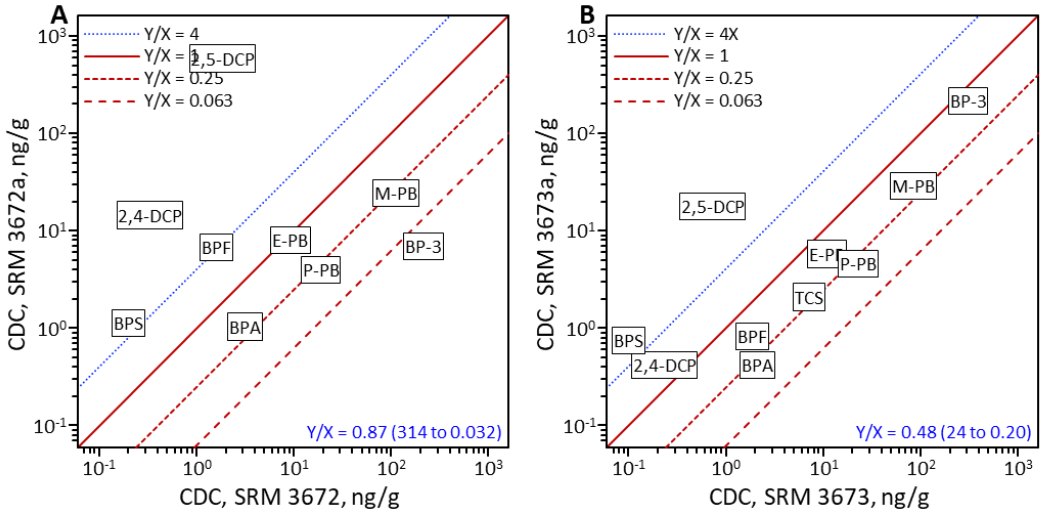


Fig. 36. Phenolic Results for New SRMs as a Function of Original SRM Results.

Panel A compares CDC's results for the phenol-related environmental metabolites in SRM 3672a to their current results for SRM 3672; Panel B compares CDC's results for SRM 3673a to their current results for SRM 3673. Each labeled box within a panel is centered on the location {CDC results for original SRM (X), CDC result for new SRM (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The long-dashed diagonal line denotes Y-results that are a factor of sixteen smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

As shown in Fig. 37, the changes in concentration between the original and new materials differ by more than four orders-of-magnitude. The changes are roughly proportional for metabolites M-PB, E-PB, P-PB, B-PB, and BFS. For BP-3, the changes are not or only weakly proportional for BP-3, BPF, 2,4-DCP, and 2,5-DCP.

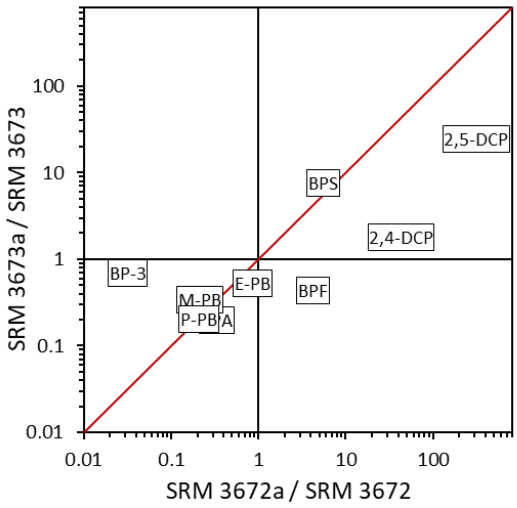


Fig. 37. Phenolic 3673a/3673 Results as a Function of 3672a/3672 Results.

Each labeled box is centered on the location {result for SRM 3672a / result for SRM 3672 (X), result for SRM 3673a / SRM 3673 (Y)}. The diagonal line represents equality between the ratios. The horizontal and vertical lines denote unit ratios.

9.2.2. Comparison Between Smokers' and Non-Smokers' Urines

As shown in Fig. 38, the concentrations of the phenolic metabolites in the original non-smokers' urine (SRM 3673) are on average 0.7-fold smaller than in the smokers' material (SRM 3672), although the range is (1.4 to 0.1)-fold. While the concentrations in the new non-smokers' material (SRM 3673a) are also on average 0.7-fold smaller than in the new smokers' material (SRM 3672a), the range is much wider: from (31 to 0.03)-fold.

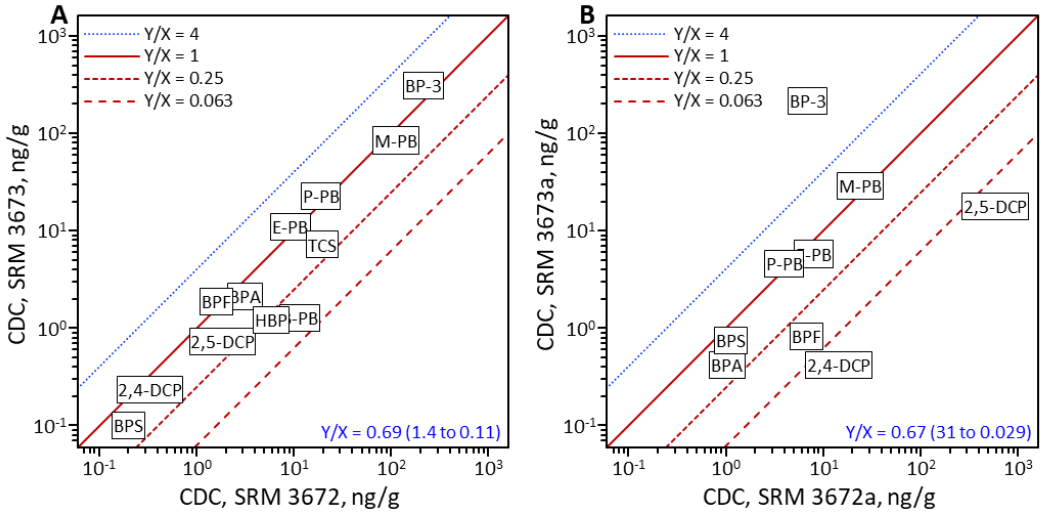


Fig. 38. Non-Smoker Phenolic Metabolite Results as a Function of Smoker Results.

Panel A compares CDC's results for the phenolic metabolites in SRM 3672 to their results for SRM 3673; Panel B compares CDC's results for SRM 3672a to their results for SRM 3673a. Each labeled box within a panel is centered on the location {CDC results for smokers' urine (X), CDC result for non-smokers' urine (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The long-dashed diagonal line denotes Y results that are a factor of sixteen smaller than the X results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

9.3. Assigned Values

Table 38 lists the assigned values for phenolic metabolites.

Table 38. Assigned Values for Phenolic Metabolites in SRMs 3672a and 3673a, ng/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
2,4-DCP	14.34	0.20	0.52	1	0.414	0.017	0.043	1
2,5-DCP	582.9	9.1	23.5	1	17.72	0.12	0.31	1
M-PB	24.00	0.40	1.03	1	28.70	0.26	0.67	1
E-PB	7.98	0.14	0.37	1	5.708	0.043	0.109	1
P-PB	3.947	0.071	0.183	1	4.583	0.047	0.122	1
BP-3	6.83	0.14	0.37	1	210.8	2.0	5.1	1
DHAVO				e	0.099267	0.000023	0.000058	1
BPF	6.70	0.14	0.35	1	0.811	0.031	0.078	1
BPA	1.028	0.021	0.054	1				e
BPS	1.111	0.017	0.043	1	0.745	0.022	0.057	1

a $w_{analyte}$, mass fraction of the analyte.
b $u(w_{analyte})$, standard uncertainty associated with $w_{analyte}$.
c $U_{95}(w_{analyte})$, approximate 95 % level of confidence expanded uncertainty associated with $w_{analyte}$.
d Number of methods contributing results used to estimate $w_{analyte}$; see Section 4.
e No reliable quantitative result available.

9.3.1. Metrological Traceability

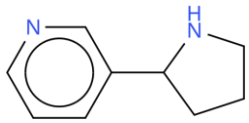
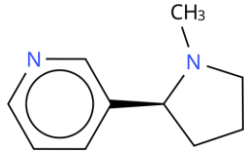
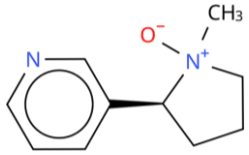
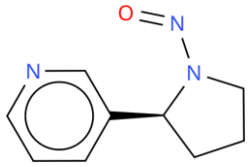
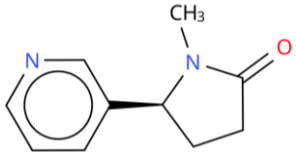
The phenol, benzophenone, and paraben results are metrologically traceable to the materials used to prepare the calibration solutions.

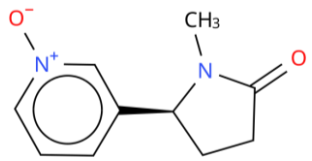
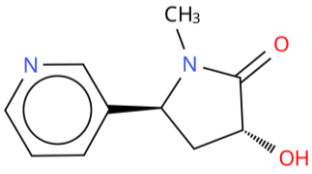
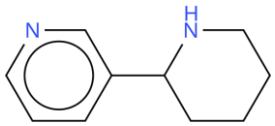
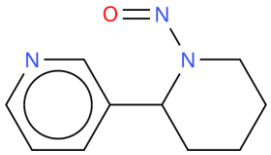
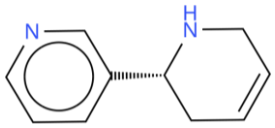
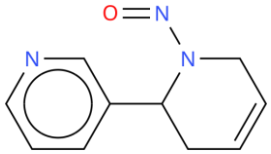
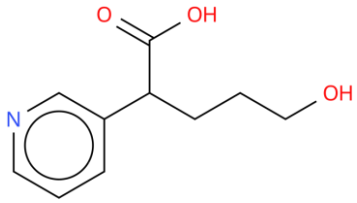
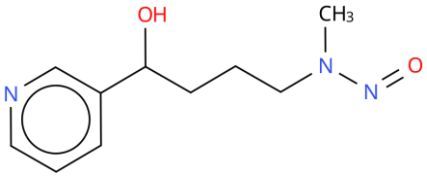
10. Nicotine and Smoking-Related Metabolites

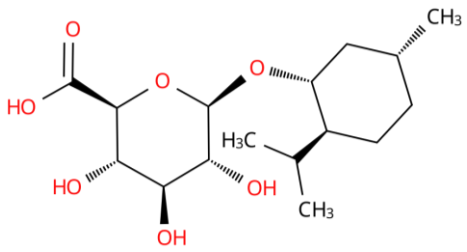
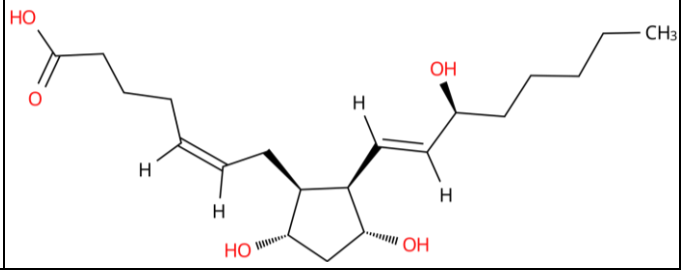
Nicotine is the primary tobacco-specific alkaloid in tobacco plants and tobacco smoke. Although not a direct cause of most diseases associated with tobacco use, nicotine addiction leads to chronic exposure to carcinogens and other bioactive compounds. The relative concentration of nicotine to its major metabolites is of interest when elucidating differences in metabolic profiles of various ethnic, age, and gender groups. Other smoking-related metabolites are used in nitrosamine-related studies and monitoring compliance of smoking cessation programs [34].

Table 39 lists smoking-related analytes that were measured in SRMs 3672, 3673, 3672a, and 3673a by the CDC using various isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) methods. These analytes are identified by their acronym, common name, International Chemical Identifier (InChI), and chemical structure. Analytes displayed with stereo-specific bonds are the most commonly occurring enantiomers.

Table 39. Nicotine and Smoking-Related Metabolites.

Analyte	Structure
NNCT, NNCF Total (R,S)-nornicotine, free (R,S)-nornicotine InChI=1S/C9H12N2/c1-3-8(7-10-5-1)9-4-2-6-11-9/h1,3,5,7,9,11H,2,4,6H2	
NICT, NICF Total (-)-nicotine, free (-)-nicotine InChI=1S/C10H14N2/c1-12-7-3-5-10(12)9-4-2-6-11-8-9/h2,4,6,8,10H,3,5,7H2,1H3/t10-/m0/s1	
NOXT, NOXF Total (1'S,2'S)-nicotine N'-oxide, free (1'S,2'S)-nicotine N'-oxide InChI=1S/C10H14N2O/c1-12(13)7-3-5-10(12)9-4-2-6-11-8-9/h2,4,6,8,10H,3,5,7H2,1H3/t10-,12?/m0/s1	
NNNT, NNNF Total *N'*-nitrosonornicotine, free *N'*-nitrosonornicotine InChI=1S/C9H11N3O/c13-11-12-6-2-4-9(12)8-3-1-5-10-7-8/h1,3,5,7,9H,2,4,6H2/t9-/m0/s1	
COTT, COTF Total (-)-cotinine, free (-)-cotinine InChI=1S/C10H12N2O/c1-12-9(4-5-10(12)13)8-3-2-6-11-7-8/h2-3,6-7,9H,4-5H2,1H3/t9-/m0/s1	

COXT, COXF Total (S)-cotinine N-oxide, free (S)-cotinine N-oxide InChI=1S/C10H12N2O2/c1-11-9(4-5-10(11)13)8-3-2-6-12(14)7-8/h2-3,6-7,9H,4-5H2,1H3/t9-/m0/s1	
HCTT, HCTF Total (-)-*trans*-3'-hydroxycotinine, free (-)-*trans*-3'-hydroxycotinine InChI=1S/C10H12N2O2/c1-12-8(5-9(13)10(12)14)7-3-2-4-11-6-7/h2-4,6,8-9,13H,5H2,1H3/t8-,9+/m0/s1	
ANBT, ANBF Total (R,S)-anabasine, free (R,S)-anabasine InChI=1S/C10H14N2/c1-2-7-12-10(5-1)9-4-3-6-11-8-9/h3-4,6,8,10,12H,1-2,5,7H2	
NABT, NABF Total *N'*-nitrosoanabasine, free *N'*-nitrosoanabasine InChI: InChI=1S/C10H13N3O/c14-12-13-7-2-1-5-10(13)9-4-3-6-11-8-9/h3-4,6,8,10H,1-2,5,7H2	
ANTT, ANTF Total (R,S)-anatabine, free (R,S)-anatabine InChI=1S/C10H12N2/c1-2-7-12-10(5-1)9-4-3-6-11-8-9/h1-4,6,8,10,12H,5,7H2	
NATT, NATF Total *N'*-nitrosoanatabine, free *N'*-nitrosoanatabine InChI= 1S/C10H11N3O/c14-12-13-7-2-1-5-10(13)9-4-3-6-11-8-9/h1-4,6,8,10H,5,7H2/t10-/m0/s1	
HPBT, HPBF Total 4-hydroxy-4-(3-pyridyl)-butanoic acid, free 4-hydroxy-4-(3-pyridyl)-butanoic acid InChI=1S/C10H13NO3/c12-6-2-4-9(10(13)14)8-3-1-5-11-7-8/h1,3,5,7,9,12H,2,4,6H2,(H,13,14)	
NNAL, NNALF 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-butanol InChI=1S/C10H15N3O2/c1-13(12-15)7-3-5-10(14)9-4-2-6-11-8-9/h2,4,6,8,10,14H,3,5,7H2,1H3	

MEG (-)-menthol β-D-glucuronide InChI=1S/C16H28O7/c1-7(2)9-5-4-8(3)6-10(9)22-16-13(19)11(17)12(18)14(23-16)15(20)21/h7-14,16-19H,4-6H2,1-3H3,(H,20,21)/t8-,9+,10-,11+,12+,13-,14+,16-/m1/s1	
8PGFT, 8PGFF Total 8-iso-prostaglandin $F_{2\alpha}$, free 8-iso-prostaglandin $F_{2\alpha}$ InChI=1S/C20H34O5/c1-2-3-6-9-15(21)12-13-17-16(18(22)14-19(17)23)10-7-4-5-8-11-20(24)25/h4,7,12-13,15-19,21-23H,2-3,5-6,8-11,14H2,1H3,(H,24,25)/b7-4-,13-12+/t15-,16-,17+,18-,19+/m0/s1	

Note: Many of these analytes are characterized with both “free” and “total” concentrations, where “free” signifies the concentration of the unconjugated form of the analyte and “total” signifies the sum of unconjugated and conjugated forms (i.e., after β-glucuronidase hydrolysis). Analyte code names that end in “F” signify analytes assayed as “free” and code names that end in “T” signify analytes assayed as “total” (except for NNAL which is the code for the total form to conform with reporting since 2006).

10.1. Analysis

One vial each of SRMs 3672 and 3673 and twelve vials each of SRMs 3672a and 3673a were sent from NIST to the CDC by overnight shipping on dry ice. Vials were stored at -80 °C until analysis. CDC Quality Control (QC) urine pools were used in conjunction with all analyses.

All results were obtained by CDC staff using five ID-LC-MS/MS methods. In all methods the new materials, SRMs 3672a and 3673a, were each characterized using one aliquot each of six vials while the original materials, SRMs 3672 and 3673, were each characterized using three replicates from one vial. Not all the methods used have sufficient sensitivity to characterize the concentrations of their target analytes in the non-smoker SRMs 3673 and 3673a materials.

All results sent to NIST were reviewed by the CDC’s quality assurance officer and approved as conforming to the quality standards at the CDC.

The following sections briefly describe the sample preparation and instrumental analysis for each of the DLS methods used. For all methods, samples were removed from the freezer and allowed to thaw completely before starting sample preparation.

10.1.1. Tobacco Specific Nitrosamines (DLS 2014)

Free and total *N’*-nitrosoanabasine (NAB), *N’*-nitrosoanatabine (NAT), *N’*-nitrosonornicotine (NNN), and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-butanol(NNAL) were assayed using the CDC’s Division of Laboratory Sciences (DLS) Method Number 2014 (DLS 2014) [35]. These tobacco-

specific nitrosamines are carcinogens formed during tobacco smoking and tobacco curing; they are important biomarkers for tobacco carcinogen uptake.

10.1.1.1. Sample Preparation

A Caliper Staccato System was used for automation of sample preparation and analysis. 50 µL of internal standard (IS) solution and 1.7 mL of urine were added to a 96 well collection plate. For total analysis 170 µL of β-glucuronidase solution type IX-A from *Escherichia coli* in 0.5N phosphate buffer (20,000 u/mL) was added and incubated with moderate shaking for 24 hours at 37 °C. For free analysis this hydrolysis step was replaced by the addition of 170 µL of HPLC-grade water.

The samples were solid-phase extracted (SPE) with 5 % ammonia in methanol after treatment with formic acid, liquid-liquid extracted (LLE) with methylene chloride after treatment with 10 mol/L sodium hydroxide and finally molecularly imprinted polymer (MIP) SPE with methylene chloride after washing with water and toluene.

10.1.1.2. Instrumental Method

Analysis was conducted on an AB Sciex 6500 triple quadrupole mass spectrometer and Shimadzu HPLC using a Gemini-NX C18 (3.0-µm, 2.0 mm × 150 mm, Phenomenex) column. Mobile phase A was 0.08 % ammonium hydroxide in water, pH 10.5. Mobile phase B was acetonitrile. The flow rate is 0.6 ml/min. The gradient from 3 % B to 30 % B over 10.51 min. A third pump was used to deliver 0.4 mL/min of acetonitrile to increase compound sensitivity.

10.1.1.3. Quantitation

Data was processed using the Indigo Ascent Automated Data Analysis and Review software (Indigo Biosystems, Indianapolis, IN). Linear calibration functions were derived using 1/*x*-weighted regression of (analyte peak area)/(IS peak area) on analyte concentration, where the *x* is the standard concentration. Sample concentrations were calculated using calibration functions derived from calibrants included in the same analytical run.

Results for the tobacco specific nitrosamines in SRMs 3672a and 3673a are listed in Table 40 and Table 41. These results were recorded and are listed here as amount concentrations, x_{analyte}. They are transformed to mass fractions, w_{analyte}, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3. SRMs 3672 and 3673 were not characterized using the DLS 2014 method as they were not previously assessed for these analytes.

Table 40. Results for Tobacco Specific Nitrosamines in SRM 3672a by DLS 2014, ng/mL.

Analyte	Form	Vial1	Vial2	Vial3	Vial4	Vial5	Vial6	Mean	SD
NNNF	Free	0.0070	0.0071	0.0072	0.0073	0.0073	0.0074	0.00722	0.00015
NABF	Free							<0.0016	
NATF	Free	0.0089	0.0091	0.0092	0.0095	0.0095	0.0101	0.00938	0.00042
NNALF	Free	0.0737	0.0742	0.0747	0.0748	0.0753	0.0754	0.07468	0.00065
NATT	Total	0.145	0.145	0.147	0.147	0.148	0.148	0.1467	0.0014
NABT	Total	0.0216	0.0221	0.0221	0.0222	0.0222	0.0231	0.02222	0.00049
NNNT	Total	0.0145	0.0146	0.0146	0.0146	0.0147	0.0149	0.01465	0.00014
NNAL	Total	0.264	0.265	0.267	0.269	0.272	0.276	0.2688	0.0045

Table 41. Results for Tobacco Specific Nitrosamines in SRM 3673a by DLS 2014, ng/mL.

Analyte	Form	Vial1	Vial2	Vial3	Vial4	Vial5	Vial6	Mean	SD
NNNF	Free							<0.0028	
NATF	Free							<0.0042	
NABF	Free							<0.0016	
NNALF	Free							<0.0006	
NNNT	Total							<0.0028	
NATT	Total							<0.0042	
NABT	Total							<0.0016	
NNAL	Total	0.001	0.001	0.001	0.001	0.001	0.001	0.001	

10.1.2. Urinary Nicotine Metabolites and Analogs (DLS 2021.02)

Free and total anabasine (ANB), anatabine (ANT), cotinine (COT), cotinine N-oxide (COX), *trans*-3'-hydroxycotinine (HCT), 4-hydroxy-4-(3-pyridyl)-butanoic acid (HPB), nicotine (NIC), nornicotine (NNC), and nicotine N'-oxide (NOX) were assayed using DLS Method Number 2021.02 (DLS 2021.02) [34]. ANB and ANT are NIC analogs that have been used in monitoring compliance of smoking cessation programs. COT and HCT are the predominant NIC metabolites in urine; because their concentrations are greater than NIC and their elimination half-lives are longer, these metabolites are generally preferred over NIC itself as exposure biomarkers. The relative concentrations of NIC and its metabolites help elucidate metabolic differences among populations.

10.1.2.1. Sample Preparation

For total analysis, a Caliper Staccato System was used for automation of sample preparation and analysis. 50 µL of IS solution and 100 µL of urine were added to a 96 well assay plate. 60 µL of β-glucuronidase solution, type H-1 from *Helix Pomatia* (Unit of 1000) was added and incubated for 12 hours at 45 °C with moderate shaking.

450 µL of cold acetone (-20 °C) was added to each sample, mixed well and incubated at -20 °C for 30 min. 180 µL of supernatant was transferred, evaporated to remove acetone, then 250 µL water was added before.instrument analysis.

For free analysis, a Hamilton STARlet System was used for automation of sample preparation and analysis. 50 µL of IS solution and 100 µL of urine were added to a 96 well assay plate. 60 µL of water was added in place of the β-glucuronidase solution and there was no incubation.

50 µL of sample was transferred to an ISOLUTE FILTER+ plate (0.2 µm) and 250 µL of water was added to each sample. 10 psi was applied for 2 min and samples were collected in a 96 well assay plate below for injection.

10.1.2.2. Instrumental Method

Analysis was conducted on an AB Sciex 6500 triple quadrupole mass spectrometer and Shimadzu HPLC using a Gemini-NX C18 (3.0-µm, 2.0 mm × 100 mm, Phenomenex) column with A-103X SS frit and A-100X SS frit (Upchurch Scientific) precolumn filters. Mobile phase A was 6.5 mmol/L ammonium acetate adjusted to pH 10.0. Mobile phase B was acetonitrile. The gradient was 0 % B to 60 % B over the course of 5.50 min.

10.1.2.3. Quantitation

Data was processed using the Indigo Ascent Automated Data Analysis and Review software (Indigo Biosystems, Indianapolis, IN). Linear calibration functions were derived using 1/*x*-weighted regression of (analyte peak area)/(IS peak area) on analyte concentration, where the *x* is the standard concentration. Sample concentrations were calculated using calibration functions derived from calibrants included in the same analytical run.

Results for NICT and its major metabolites and analogs in SRMs 3672 and 3672a are listed in Table 42. SRMs 3673 and 3673a were not characterized using the DLS 2021.02 method. These results were recorded and are listed here as amount concentrations, $x_{analyte}$. They are transformed to mass fractions, $w_{analyte}$, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3.

Table 42. Results for Nicotine Metabolites and Analogs in SRMs 3672 and 3672a by DLS 2021, ng/mL.

		SRM 3672					SRM 3672a							
Analyte	Form	Rep1	Rep2	Rep3	Mean	SD	Vial1	Vial2	Vial3	Vial4	Vial5	Vial6	Mean	SD
NNCF	Free	50.2	46.6	46.9	47.9	2.0	37.9	37.5	37.4	37.1	37.0	39.0	37.65	0.73
NICF	Free	783	725	741	750	30	994	1000	988	983	969	1030	994	21
NOXF	Free	357	331	326	338	17	261	259	261	253	254	268	259.3	5.5
COTF	Free	1200	1090	1120	1137	57	654	638	636	630	634	657	642	11
COXF	Free	395	368	375	379	14	146	142	144	142	142	144	143.3	1.6
HCTF	Free	3710	3440	3480	3540	150	1800	1780	1770	1790	1760	1820	1787	22
ANBF	Free	5.68	5.24	5.35	5.42	0.23	6.69	6.51	6.62	6.53	6.37	6.72	6.57	0.13
ANTF	Free	6.38	6.09	6.01	6.16	0.19	10.3	10.3	10.1	10.2	10.1	10.4	10.23	0.12
HPBF	Free	430	398	417	415	16	339	342	333	328	342	354	339.7	8.9
NNCT	Total	68.1	64.2	67.8	66.7	2.2	62.8	64.0	62.7	62.8	60.2	61.4	62.3	1.3
NICT	Total	1250	1140	1210	1200	56	1330	1320	1320	1310	1260	1320	1310	25
NOXT	Total	370	371	374	371.7	2.1	292	285	285	286	281	288	286.2	3.7
COTT	Total	3370	3140	3340	3280	130	1460	1470	1460	1500	1450	1430	1462	23
COXT	Total	408	400	396	401.3	6.1	159	157	158	160	155	158	157.8	1.7
HCTT	Total	4610	4400	4630	4550	130	2810	2790	2770	2730	2690	2770	2760	43
ANBT	Total	8.08	7.46	8.15	7.90	0.38	8.90	8.67	8.89	9.06	8.65	8.90	8.85	0.16
ANTT	Total	11.8	11.3	11.5	11.53	0.25	16.5	17.0	16.4	16.8	16.6	17.1	16.73	0.28
HPBT	Total	519	491	515	508	15	437	438	425	431	426	429	431.0	5.5

10.1.3. Menthol and Smoker Screening Assay (DLS 2023)

Free NIC, COT, and HCT, ANB, ANT, and menthol-glucuronide (MEG) were assayed using DLS Method Number 2023 (DLS 2023) [36]. Menthol, a major tobacco product flavor enhancer, is extensively metabolized and excreted in urine predominantly as MEG. The presence of MEG in urine is a marker for menthol exposure.

10.1.3.1. Sample Preparation

A Hamilton STARlet system was used for automation of sample preparation and solid-phase extraction (SPE). 200 µL of 4 % formic acid, 100 µL IS solution, and 100 µL of urine sample were aliquoted into a 96-well plate. An Oasis MCX 96-well SPE plate was conditioned, loaded with 400 µL of sample solution, washed the retained samples, and then eluted with 450 µL of methanol with 4 % ammonium hydroxide. Eluent was then dried under nitrogen at 40 °C and reconstituted with 400 µL of water.

10.1.3.2. Instrumental Method

Analysis was conducted on an AB Sciex 6500+ triple quadrupole mass spectrometer and a Shimadzu Nexera UHPLC using a Kinetex® EVO C18 (2.6 µm, 2.1 mm x 100 mm, Phenomenex) analytical column with a 0.2 µm frit 2.1 mm pre-column filter (Waters). Injection volume was 2 µL. Mobile phase A was 20 mmol/L ammonium acetate buffer adjusted to pH 10 (±0.15) with ammonium hydroxide. Mobile phase B was acetonitrile. The gradient was 0 % B to 60 % B over 3.3 min.

10.1.3.3. Quantitation

Data was processed using the Indigo Ascent Automated Data Analysis and Review software (Indigo Biosystems, Indianapolis, IN). Linear calibration functions were derived using $1/x$ weighted regression of (analyte peak area)/(IS peak area) on analyte concentration, where the x is the standard concentration. Sample concentrations were calculated using calibration functions derived from calibrants included in the same analytical run.

Results for the menthol and smoker screening analytes in SRMs 3672 and 3672a are listed in Table 43. Results for SRM 3673a are listed in Table 44. These results were recorded and are listed here as amount concentrations, $x_{analyte}$. They are transformed to mass fractions, $w_{analyte}$, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3. SRMs 3673 was not characterized using the DLS 2023 method.

Table 43. Results for Menthol and Smoker Screening Analytes in SRMs 3672 and 3672a by DLS 2023, ng/mL.

		SRM 3672					SRM 3672a							
Analyte	Form	Rep1	Rep2	Rep3	Mean	SD	Vial1	Vial2	Vial3	Vial4	Vial5	Vial6	Mean	SD
NICF	Free	715	698	706	706.3	8.5	970	1010	1030	956	987	951	984	31
COTF	Free	1220	1220	1210	1216.7	5.8	701	691	703	713	704	688	700.0	9.2
HCTF	Free	3600	3530	3580	3570	36	1900	1800	1940	1880	1830	1820	1862	54
ANBF	Free	5.45	5.42	5.54	5.470	0.062	6.55	7.04	6.72	7.07	6.55	6.73	6.78	0.23
ANTF	Free	5.89	5.80	5.74	5.810	0.075	9.92	9.89	9.98	9.84	9.62	9.63	9.81	0.15
MEG		4540	4400	4410	4450	78	2130	2130	2150	2020	2280	2110	2137	84

Table 44. Results for Menthol and Smoker Screening Analytes in SRM 3673a by DLS 2023, ng/mL.

		SRM 3673a							
Analyte	Form	Vial1	Vial2	Vial3	Vial4	Vial5	Vial6	Mean	SD
NICF	Free							<4.27	
COTF	Free							<1.18	
HCTF	Free							<0.2.39	
ANBF	Free							<0.77	
ANTF	Free							<0.263	
MEG		1920	2120	1950	1960	2160	2150	2040	110

10.1.4. Total and Free Cotinine (COT) and trans-3-Hydroxycotinine (HCT) (DLS 2024)

Free and total COT and HCT were assayed using DLS Method Number 2024 (DLS 2024) [37]. This method has much lower detection limits than DLS 2021.02 and is suitable for quantitatively characterizing the primary nicotine metabolites in non-smoker urine.

10.1.4.1. Sample Preparation

Total cotinine (COTT) and total *trans*-3-hydroxycotinine (HCTT) sample preparation was performed on a Hamilton STAR/Caliper Staccato System system. Free cotinine (COTF) and free *trans*-3-hydroxycotinine (HCTF) measurement was done on a Hamilton Vantage. 50 µL of IS solution and 200 µL of urine were added to a 96 well assay plate. For total analysis, 50 µL of enzyme solution containing β-glucuronidase solution, type H-1 from *Helix Pomatia* (Unit of 400) was added, and samples were incubated at 37 °C for at least 6 hours (usually overnight). For free analysis, 50 µL of water was used, and there was no incubation.

50 µL 0.2 N potassium hydroxide was added to each sample. Samples were loaded onto a 400 mg 96-well Isolute SLE+ extraction plate. Analytes were eluted by 1.8 mL (3 x 0.6 mL) of 5 % isopropanol in methylene chloride. Samples were dried under nitrogen at 40 °C and reconstituted using 0.1 mL HPLC-grade water.

10.1.4.2. Instrumental Method

Analysis was conducted on an AB Sciex 6500 triple quadrupole mass spectrometer and a Shimadzu HPLC using a Luna™ Omega C18 (1.6 µm 100 Å pore, 100 mm × 2.1 mm, Phenomenex) column. Injection volume was 5 µL. Mobile phase A was 6 mmol/L ammonium acetate. Mobile phase B was acetonitrile. The LC gradient was 0.1 % B to 98 % B over 2.61 min , hold until 4.10 min, and return to 0.1% B at 4.11 min.

10.1.4.3. Quantitation

Data was processed using the Indigo Ascent Automated Data Analysis and Review software (Indigo Biosystems, Indianapolis, IN). Linear calibration functions were derived using 1/*x*-weighted regression of (analyte peak area)/(IS peak area) on analyte concentration, where the *x* is the standard concentration. Sample concentrations were calculated using calibration functions derived from calibrants included in the same LC/MS analytical batch.

Results for the major nicotine metabolites in SRMs 3673 and 3673a are listed in Table 45. These results were recorded and are listed here as amount concentrations, x_{analyte}. They are transformed to mass fractions, w_{analyte}, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3. SRMs 3672 and 3672a were not characterized using the DLS 2024 method.

Table 45. Results for Primary Nicotine Metabolites in SRMs 3673 and 3673a by DLS 2024, ng/mL.

		SRM 3673					SRM 3673a							
Analyte	Form	Rep1	Rep2	Rep3	Mean	SD	Vial1	Vial2	Vial3	Vial4	Vial5	Vial6	Mean	SD
COTF	Free	24.2	23.0	23.0	23.40	0.69	0.271	0.274	0.270	0.271	0.292	0.285	0.2772	0.0092
HCTF	Free	56.3	55.3	56.8	56.13	0.76	0.584	0.658	0.620	0.567	0.607	0.593	0.605	0.032
COTT	Total	48.4	47.2	51.3	49.0	2.1	0.594	0.635	0.586	0.569	0.564	0.592	0.590	0.025
HCTT	Total	66.1	64.0	67.9	66.0	2.0	0.876	0.889	0.797	0.788	0.791	0.791	0.822	0.047

10.1.5. Urinary Oxidative Stress Biomarker (DLS 2025)

Free and total 8-iso-prostaglandin $F_{2\alpha}$(8PGFF and 8PGFT) were assayed using DLS Method 2025 (DLS 2025) [38].8-iso-prostaglandin $F_{2\alpha}$ is formed in vivo by non-enzymatic peroxidation of the polyunsaturated fatty acid arachidonic acid and is a marker of oxidative stress. Oxidative stress results from an imbalance between free radicals and antioxidant defenses. 8-iso-prostaglandin $F_{2\alpha}$.is present in all human urine through endogenous metabolic processes, but its levels rise following exogenous exposure to environmental toxicants such as cigarette smoke.

10.1.5.1. Sample Preparation

A Hamilton Starlet system was used for automated sample preparation and solid phase extraction. For total analysis, 40 µL of IS solution, 800 µL of HPLC water, 400 µL of sample, and 160 µL of enzyme solution (2000 units of β-glucuronidase, type IX-A from *Escherichia coli,* dissolved in 0.5 mol/L phosphate buffer at pH 6.1) were combined and incubated in a water bath overnight at 37°C. For free analysis, 160 µL HPLC-grade water was substituted for the enzyme, and the sample was not incubated.

400 µL of methanol was added to each sample tube and loaded onto a 96-well weak anion exchange SPE plate. The samples were washed with 1.8 mL methanol, evaporated to dryness under nitrogen at 37 °C, and reconstituted in 50 µL of 25 % methanol in water.

10.1.5.2. Instrumental Method

Analysis was conducted on a Sciex 6500 triple quadrupole mass spectrometer coupled with a Shimadzu Nexera X2 HPLC system using an Acquity UPLC HSS C18 (1.8 µm, 2.1 mm x 150 mm, Waters) analytical column with an Acquity UPLC C18 (1.8 µm, 2.1 mm × 5 mm) pre-column. Injection volume was 10 µL. Mobile phase A was 0.15 % formic acid in water; mobile phase B was 50:50 0.15 % formic acid in water:acetonitrile. The mobile phase gradient was 40 % B to 100 % B over 7.1 min.

10.1.5.3. Quantitation

Data was processed with Sciex MultiQuant version 3.0.3. Linear calibration functions were derived using 1/*x*-weighted regression of (analyte peak area)/(IS peak area) on analyte concentration, where the *x* is the standard concentration. Sample concentrations were calculated using calibration functions derived from calibrants included in the analytical run.

Results for 8PGFT and 8PGFF in SRMs 3672a and 3673a are listed in Table 46. These results were recorded and are listed here as amount concentrations, $x_{analyte}$. They are transformed to mass fractions, $w_{analyte}$, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3. SRMs 3672 and 3673 were not characterized using the DLS 2025 method.

Table 46. Results for 8-iso-prostaglandin $F_{2\alpha}$ in SRMs 3672a and 3673a by DLS 2025, ng/mL.

SRM	Form	Vial1	Vial2	Vial3	Vial4	Vial5	Vial6	Mean	SD
3672a	Free	0.179	0.173	0.172	0.180	0.175	0.178	0.1762	0.0033
3672a	Total	0.280	0.268	0.271	0.272	0.268	0.263	0.2703	0.0057
3673a	Free	0.121	0.115	0.115	0.116	0.121	0.120	0.1180	0.0030
3673a	Total	0.187	0.190	0.181	0.184	0.193	0.182	0.1862	0.0047

10.2. Value Assessment

To evaluate whether the measured values for these analytes in SRMs 3672a and 3673a are appropriate for use in value assessment, Fig. 39 compares the CDC's measured values for NICF, COTF, and HCTF in 3672 and 3673 to the non-certified values measured by NIST in 2013 and listed in their Certificates of Analysis (COAs)[2,3]. The current and original values are in excellent agreement.

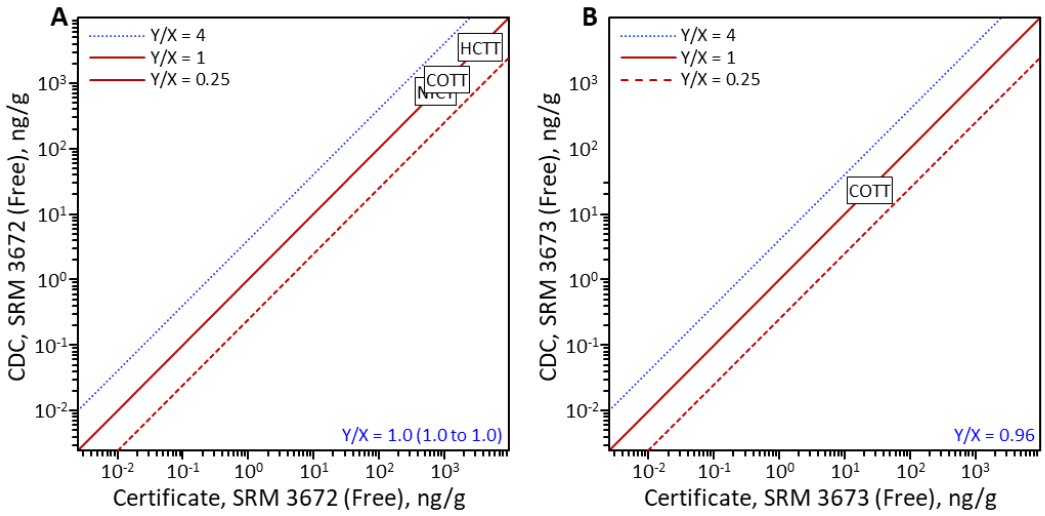


Fig. 39. Non-Smoker Nicotine and Smoking Metabolite Results as Function of COA Values.

Panel A compares CDC's results for NICF, COTF, and HCTF in SRM 3672 to the non-certified values listed in the SRM 3672 Certificate of Analysis (COA); Panel B compares CDC's result for COTT to the result listed in the SRM 3673 COA. Each labeled box within a panel is centered on the location {COA result (X), CDC result (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the Y/X ratios for the two analytes.

10.3. Comparisons Between the Original and the New SRMs

As shown in Fig. 40, the concentrations of the free values for the smoking-related analytes in the new smokers' material, SRM 3672a, are on average equal to or slightly smaller than in the original material, SRMs 3672, with a range from (1.7 to 0.4)-fold. The concentrations of the major COTF and HCTF metabolites in the new non-smokers' material, SRM 3673a, are about 90-fold smaller than in the original non-smoker material, SRM 3673.

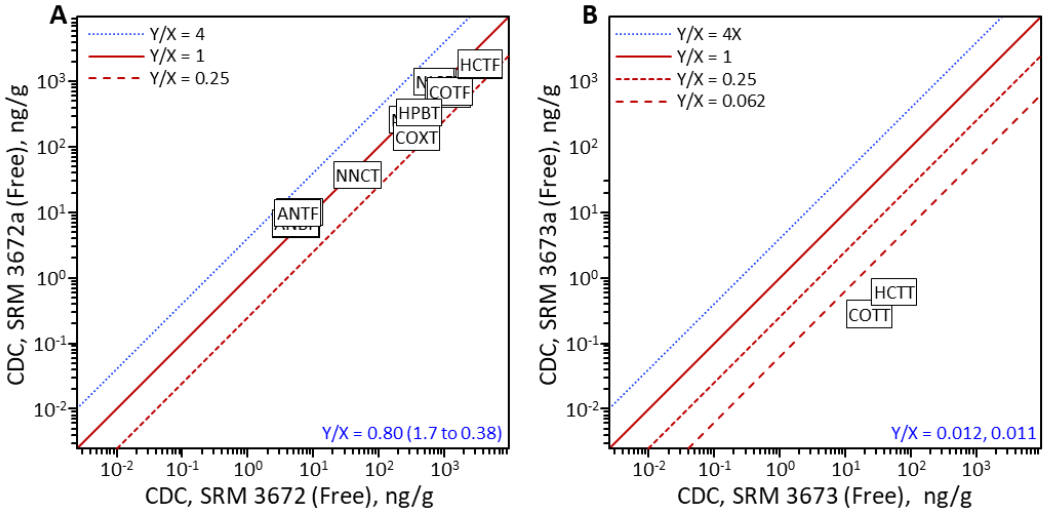


Fig. 40. Non-Smoker Nicotine and Smoking Metabolite Results for New SRMs as a Function of Original SRM Results.

Panel A compares CDC's results for the free smoking-related analytes in SRM 3672a to their results for SRM 3672; Panel B compares CDC's results for SRM 3673a to their results for SRM 3673. Each labeled box within a panel is centered on the location {CDC results for original SRM (X), CDC result for new SRM (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The long-dashed diagonal line denotes Y results that are a factor of sixteen smaller than the X results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

10.4. Comparisons Between Smokers' and Non-Smokers' Urines

As shown in Fig. 41, the concentrations of COTF and HCTF in the SRM 3673 non-smokers' urine are both about 50-fold smaller than in the SRM 3672 smokers' urine. The concentrations of COTF, HCTF, 8PGFF in SRM 3673a are more than 2000-fold smaller than in 3672a. These results are consistent with the donor pool descriptions and suggest that the donors to the new non-smoker material were considerably less exposed to second-hand smoke.

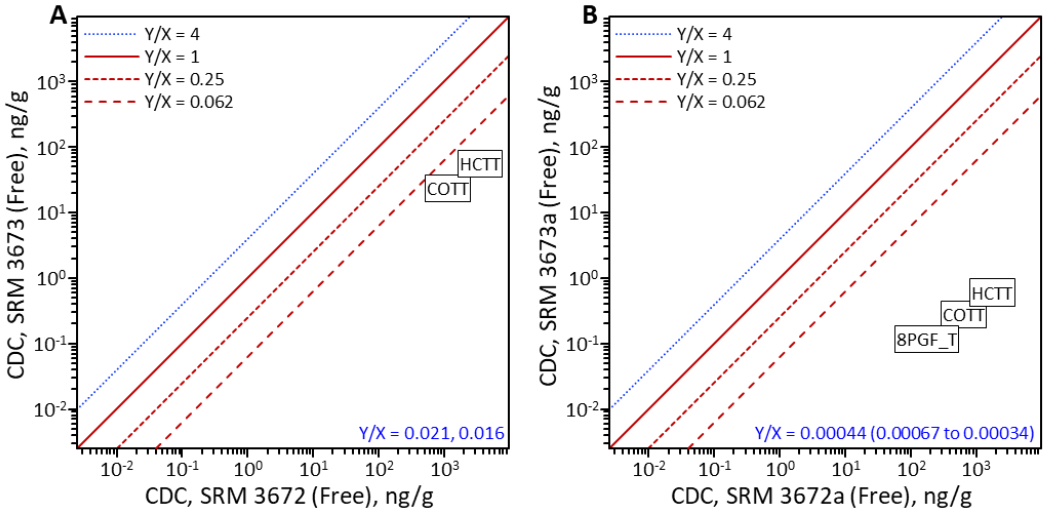


Fig. 41. Non-Smoker Nicotine and Smoking Metabolite Results as Functions of Smoker Results.

Panel A compares CDC's results for the primary nicotine metabolites in SRM 3672 to their results for SRM 3673; Panel B compares CDC's results for these metabolites and the oxidative stress biomarker 8PGFF in SRM 3672a to their results for SRM 3673a. Each labeled box within a panel is centered on the location {CDC results for smokers' urine (X), CDC result for non-smokers' urine (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The long-dashed diagonal line denotes Y results that are a factor of sixteen smaller than the X results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

10.5. Comparisons Between Free and Total Analyte Results

As shown in Fig. 42, the results for the "total" forms of the smoking-related analytes in the SRM 3672 and 3672a smoker materials are, as expected, consistently somewhat larger than their "free" forms. The average ratio is 1.5-fold with actual ratios between (3 to 1.1)-fold with the greatest difference being the 16-fold ratio for NATT in SRM 3672a.

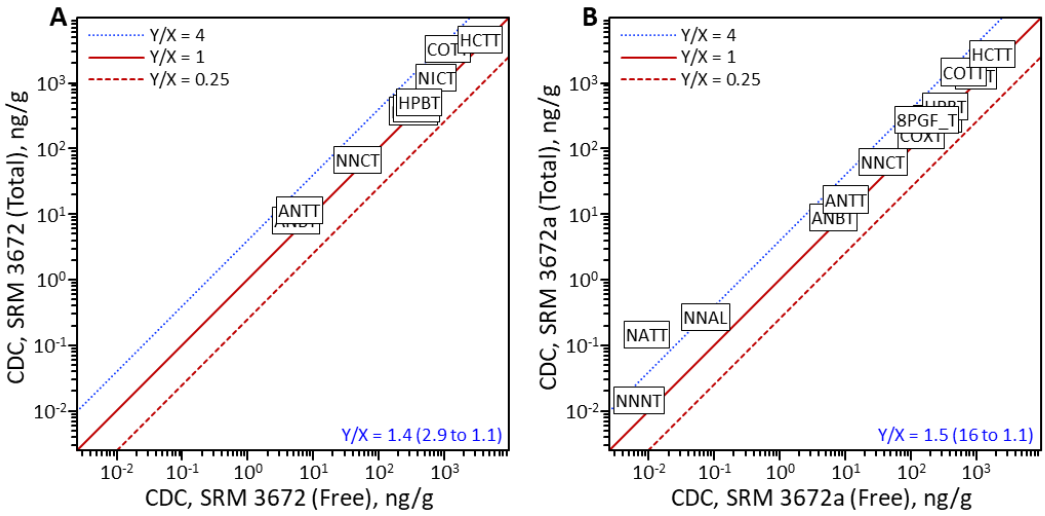


Fig. 42. Total Nicotine and Smoking Metabolite Results as Functions of Free Analyte Results.

Panel A compares CDC's "total" results for smoking-related analytes in SRM 3672 to the "free" results; Panel B compares CDC's "total" results for these analytes in SRM 3672a to the "free" results. Each labeled box within a panel is centered on the location {CDC results for smokers' urine (X), CDC result for non-smokers' urine (Y)}. The solid diagonal line denotes equality between the two sets of values. The dotted diagonal line denotes Y-results that are a factor-of-four larger than the X-results. The short-dashed diagonal line denotes Y-results that are a factor-of-four smaller than the X-results. The values at the bottom right of the chart are the median and (maximum to minimum) Y/X ratios.

10.6. Assigned Values

Table 47 lists the assigned values for nicotine and smoking-related metabolite.

Table 47. Assigned Values for Nicotine and Smoking-Related Metabolites in SRMs 3672a and 3673a, ng/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
NNCT-total	62.01	0.54	1.38	1				e
NNCF-free	37.46	0.30	0.77	1				e
NICT-total	1303	10	26	1				e
NICF-free	984	6	12	2				e
NOXT-total	284.7	1.5	3.8	1				e
NOXF-free	258.0	2.2	5.7	1				e
NNNT-total	0.01458	0.00010	0.00027	1				e
NNNF-free	0.00718	0.00011	0.00027	1				e
COTT-total	1454.4	9.4	24.2	1	0.586	0.010	0.026	1
COTF-free	667	28	56	2	0.2751	0.0037	0.0095	1
COXT-total	157.05	0.70	1.80	1				e
COXF-free	142.62	0.66	1.71	1				e
HCTT-total	2746	18	45	1	0.816	0.019	0.049	1
HCTF-free	1809	33	64	2	0.600	0.013	0.033	1
ANBT-total	8.801	0.064	0.164	1				e
ANBF-free	6.66	0.12	0.24	2				e
NABT-total	0.02211	0.00022	0.00056	1				e
ANTT-total	16.65	0.11	0.29	1				e
ANTF-free	9.97	0.21	0.41	2				e
NATT-total	0.14594	0.00056	0.00144	1				e
NATF-free	0.00934	0.00019	0.00050	1				e
HPBT-total	428.9	2.2	5.7	1				e
HPBF-free	338.0	3.6	9.3	1				e
NNAL-total	0.2675	0.0018	0.0047	1	0.001000	0.000048	0.000124	1
NNALF-free	0.07431	0.00028	0.00071	1				e
MEG	2103	26	57	1	2028	45	116	1
8PGFT-total	0.2711	0.0019	0.0041	1	0.1856	0.0021	0.0046	1
8PGFF-free	0.1784	0.0015	0.0033	1	0.11738	0.00087	0.00192	1

a $w_{analyte}$, mass fraction of the analyte.
b $u(w_{analyte})$, standard uncertainty associated with $w_{analyte}$.
c $U_{95}(w_{analyte})$, approximate 95 % level of confidence expanded uncertainty associated with $w_{analyte}$.
d Number of methods contributing results used to estimate $w_{analyte}$; see Section 4.
e No reliable quantitative result available.

10.6.1. Metrological Traceability

The nicotine and smoking-related metabolite results are metrologically traceable to the materials used to prepare the calibration solutions.

11. Thiocyanate

The thiocyanate anion (SCN^-) is a metabolite of hydrogen cyanide, a compound found in tobacco smoke. Although there are other sources of minimal thiocyanate exposure, this compound is used as a biomarker for tobacco smoke exposure. The CDC monitors thiocyanate in the urine through the NHANES project [1].

The thiocyanate mass concentrations in SRM 3672a and 3673a were determined using an ion chromatography-mass spectrometry method [39].

11.1. Materials

One vial of SRM 3672a and one vial of 3673a were sent overnight shipping on dry ice from NIST to the CDC. Vials were stored at -80 °C until analysis.

Potassium thiocyanate (Sigma-Aldrich, St. Louis, MO) was used to prepare the calibrants.

11.2. Analysis

Following the methods described in [39], urine samples were thawed to room temperature. Six replicate measurements were made for each SRM. For each replicate, 100 µL of urine was transferred to an autosampler vial and diluted with 900 µL of deionized water containing internal standard mix, then thoroughly mixed. Quality control pools having SCN^- mass concentrations of 100 ng/mL and 1100 ng/mL were analyzed at the same time as the SRM materials.

Ion chromatography used an IonPac AS 20 (2 mm × 250 mm, Dionex) analytical column with an injection volume of 24 µL. A 50 mmol/L potassium hydroxide in water eluant was used under isocratic conditions at a flow rate of 0.5 mL/min. Analyte ions were measured using a Sciex API 4000 LC-MS/MS with negative mode electrospray ionization.

Standard solutions covering the linear range of the analysis were used to generate a calibration curve for quantitation.

11.3. Results

Table 48 lists the results of the thiocyanate measurements for SRMs 3672a and 3673a. All measurements are above the method's 10 ng/mL LOD. Concentrations of thiocyanate for the smokers' and nonsmokers' materials reflect the expected order of magnitude differences. These results were reviewed by the CDC's quality assurance officer and approved as conforming to the quality standards at the CDC.

These results were recorded and are listed here as amount concentrations, $x_{analyte}$. They are transformed to mass fractions, $w_{analyte}$, using Eq. 4 with the urine densities, ρ_{matrix}, listed in Section 3.3.

Table 48. Results for Thiocyanate in SRMs 3272a and 3673a, ng/mL.

Sample	SRM 3672a	SRM 3673a
Replicate-1	4250	448
Replicate-2	4340	444
Replicate-3	4370	439
Replicate-4	4360	456
Replicate-5	4440	450
Replicate-6	4460	437
N:	6	6
Mean:	4370	446
SD:	75	7.1

11.4. Assigned Values

Table 34 lists the assigned values for thiocyanate.

Table 49. Assigned Values for Thiocyanate in SRMs 3672a and 3673a, ng/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
SCN	4348	31	79	1	442.4	2.9	7.4	1

11.4.1. Metrological Traceability

The thiocyanate results are metrologically traceable to the thiocyanate material used to prepare the calibration solutions.

12. Creatinine

Urine creatinine levels are indicators of kidney function, and the measurement of other analytes is often normalized to urine creatinine levels to account for sample dilution. Therefore, accurate measurement of urine creatinine is of importance to the clinical, forensic, and toxicology communities. The tautomeric chemical structure of creatinine is displayed in Fig. 43.

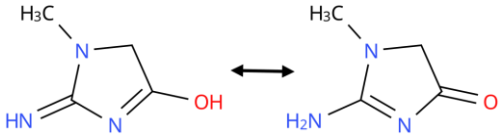


Fig. 43. Chemical Structure of Creatinine.

The creatinine concentrations of SRMs 3672a and 3673a were assigned using NIST's isotope-dilution liquid chromatography mass spectrometry (ID-LC-MS) method for determining creatinine in human urine [40,41]. This method is recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) as a reference measurement procedure (RMP) [42]. The sample preparation component of the RMP was simplified to accommodate the less complex urine sample matrix.

12.1. Materials

Measurements were made on 15 vials of SRM 3672a and 15 vials of SRM 3673a. One vial was selected from the first box (1) and one from the last box (120) of the production batch of each SRM. The remaining 13 vials were selected by random stratified sampling across the batch of each SRM, dividing boxes 2 to 119 into 13 relatively even groups.

Control measurements were made on six vials of SRM 3667 Creatinine in Frozen Human Urine [43]. Calibrants were prepared from SRM 915b [45]. The internal standard was creatinine-d_3 (Cayman Chemical). Ammonium acetate was purchased from Sigma-Aldrich. Hydrochloric acid, 2 mol/L, was purchased from Honeywell. HPLC-grade water was used in the calibration and sample preparation steps, as well as for the preparation of the LC-MS mobile phase.

Mobile phases, 0.01 mol/L HCL blank solution, and calibration solutions were prepared following the procedures detailed in [44]. Five independent calibrant stock solutions were prepared from SRM 914b several weeks before the first campaign.

12.2. Sample Preparation

Following a preliminary target level assessment, SRM 3667,3672a and 3673a samples were spiked with internal standard solution to achieve a mass ratio of 1-to-1 creatinine to creatinine-d_3. A 400 µL aliquot of internal standard solution containing 40 µg of creatinine- d_3 was added gravimetrically to a 15 mL conical centrifuge tube, then an aliquot of thawed urine estimated to contain 40 µg of creatinine was added. HPLC-grade water and 50 µL of 1 mol/L HCl solution was added so that the final HCl concentration was 0.01 mol/L in a 5 mL volume. The samples were vortex mixed and allowed to equilibrate overnight at (2 to 8) °C. Once

equilibrated, the sample tubes were vortexed, and the creatinine sample was further diluted 1-to-100 (volume fraction), where 50 µL of the sample was added into 495 µL of water.

Approximately 1 mL of each diluted sample was transferred into an HPLC vial for analysis. The remaining reconstituted sample volumes were stored at (4 to 8) °C until all measurement campaigns were successfully completed.

For each campaign, three aliquots from one vial of SRM 3667, one-to-three aliquots from five vials of SRM 3672a or 3673a, and one calibrant from each of the five calibration stock solutions were prepared on the same day.

12.3. Analysis

Seven vials of SRM 3672a and six vial of SRM 3673a were characterized using three aliquots. The remaining vials were each characterized using one aliquot. Results were obtained in six non-consecutive measurement campaigns ("Days") over the course of three weeks. Three campaigns were devoted to each SRM. Three aliquots from one vial of SRM 3667 were characterized in each of the six campaigns.

Each calibrant and sample was injected twice. Calibrants were injected before and after the sample injections. 0.01 mol/L HCl blanks were injected at the start, between calibrant and sample groupings, and end of each campaign.

All samples were characterized using the ID-LC-MS method detailed in [44]. Analyses were performed on an Agilent 1260 Infinity II Series LC system equipped with a binary pump, degasser, autosampler, and column compartment, attached to an Agilent InfinityLab Quadrupole MS. The instrument was controlled using ChemStation software (Agilent). Separation was achieved on a Luna C18(2) (5 µm, 25 cm × 4.6 mm, Phenomenex) column.

Detection was achieved using electrospray ionization in positive mode, operated in selected ion monitoring (SIM) mode to detect *m/z* 114 (creatinine) and *m/z* 117 (creatinine-d_3). The mass fraction of creatinine in the urine aliquots was determined using the average response factor internal standard approach described in [44].

12.4. Results

The response factor for every injection of every calibrant in every campaign was close to 1. The mean of the 30 mean factors was (0.9889 ±0.0065) where the "±" value is the standard deviation. The low variability (0.66 % relative standard deviation, RSD) demonstrates that the preparation of calibrants was consistent and that the calibrants were stable for the duration of their measurement campaign.

Table 50 lists creatinine results for SRM 3672a, 3673a, and 3667 in the six measurement campaigns. The results are visualized in Fig. 44.

Table 50. Results for Creatinine in SRMs 3272a, 3673a, and 3667, µg/g.

SRM 3672a

Day	Box	Aliquot	Inj_1	Inj_2	Mean	SD
	73	1	804.8	802.9	803.9	1.3
	73	2	803.9	803.3	803.6	0.4
	73	3	806.5	802.9	804.7	2.5
	102	1	811.2	803.6	807.4	5.4
	42	1	812.1	808.6	810.4	2.5
1	42	2	809.0	810.0	809.5	0.7
	42	3	811.6	816.3	814.0	3.3
	97	1	809.7	809.6	809.7	0.1
	120	1	810.2	817.1	813.7	4.9
	120	2	817.3	811.3	814.3	4.2
	120	3	812.8	809.1	811.0	2.6
	89	1	808.4	809.4	808.9	0.7
	89	2	804.3	808.8	806.6	3.2
	89	3	804.8	809.4	807.1	3.3
	69	1	805.3	805.4	805.4	0.1
2	33	1	800.8	807.5	804.2	4.7
	33	2	810.5	808.0	809.3	1.8
	33	3	812.2	814.1	813.2	1.3
	1	1	805.0	808.4	806.7	2.4
	112	1	805.2	805.4	805.3	0.1
	26	1	812.1	806.8	809.5	3.7
	26	2	809.5	814.7	812.1	3.7
	26	3	813.1	813.6	813.4	0.4
	9	1	811.0	804.3	807.7	4.7
6	58	1	817.8	813.7	815.8	2.9
	58	2	815.7	814.2	815.0	1.1
	58	3	812.2	816.2	814.2	2.8
	52	1	815.5	816.6	816.1	0.8
	19	1	808.2	809.1	808.7	0.6
				N:	29	
				Mean:	809.7	
				Standard Deviation:	3.9	

SRM 3673a

Day	Box	Aliquot	Inj_1	Inj_2	Mean	SD
	119	1	730.0	730.5	730.3	0.4
	119	2	732.7	733.0	732.9	0.2
	119	3	732.5	724.4	728.5	5.7
	39	1	723.0	721.4	722.2	1.1
	120	1	727.9	731.4	729.7	2.5
3	120	2	730.8	728.0	729.4	2.0
	120	3	729.3	731.1	730.2	1.3
	1	1	729.9	729.5	729.7	0.3
	89	1	730.2	729.9	730.1	0.2
	98	1	725.3	722.3	723.8	2.1
	98	2	728.4	726.7	727.6	1.2
	98	3	725.4	730.9	728.2	3.9
	32	1	729.3	730.4	729.9	0.8
4	106	1	724.8	726.9	725.9	1.5
	106	2	726.2	725.8	726.0	0.3
	106	3	726.8	723.1	725.0	2.6
	64	1	733.3	729.1	731.2	3.0
	22	1	708.8	709.5	709.2	0.5
	14	1	728.1	723.9	726.0	3.0
	14	2	725.7	728.6	727.2	2.1
	14	3	722.6	729.4	726.0	4.8
	75	1	725.1	729.3	727.2	3.0
5	53	1	726.0	726.9	726.5	0.6
	53	2	726.8	727.7	727.3	0.6
	53	3	726.9	728.8	727.9	1.3
	55	1	725.2	727.5	726.4	1.6
	2	1	729.6	725.1	727.4	3.2
				N:	27	
				Mean:	727.1	
				Standard Deviation:	4.3	

SRM 3667 Control

Day	Aliquot	Inj_1	Inj_2	Mean	SD
	1	631.9	627.5	629.7	3.1
1	2	611.8	608.7	610.3	2.2
	3	611.3	611.1	611.2	0.1
	1	617.8	614.8	616.3	2.1
2	2	616.3	613.7	615.0	1.8
	3	605.2	608.4	606.8	2.3
	1	615.8	619.1	617.5	2.3
3	2	623.4	618.5	621.0	3.5
	3	609.8	612.5	611.2	1.9
	1	616.5	618.2	617.4	1.2
4	2	616.1	612.8	614.5	2.3
	3	615.3	615.6	615.5	0.2
	1	618.6	619.1	618.9	0.4
5	2	616.5	616.5	616.5	0.0
	3	618.8	616.7	617.8	1.5
	1	617.9	620.0	619.0	1.5
6	2	618.6	614.8	616.7	2.7
	3	615.7	615.4	615.6	0.2
			N:	18	
			Mean:	616.1	
			Standard Deviation:	4.9	

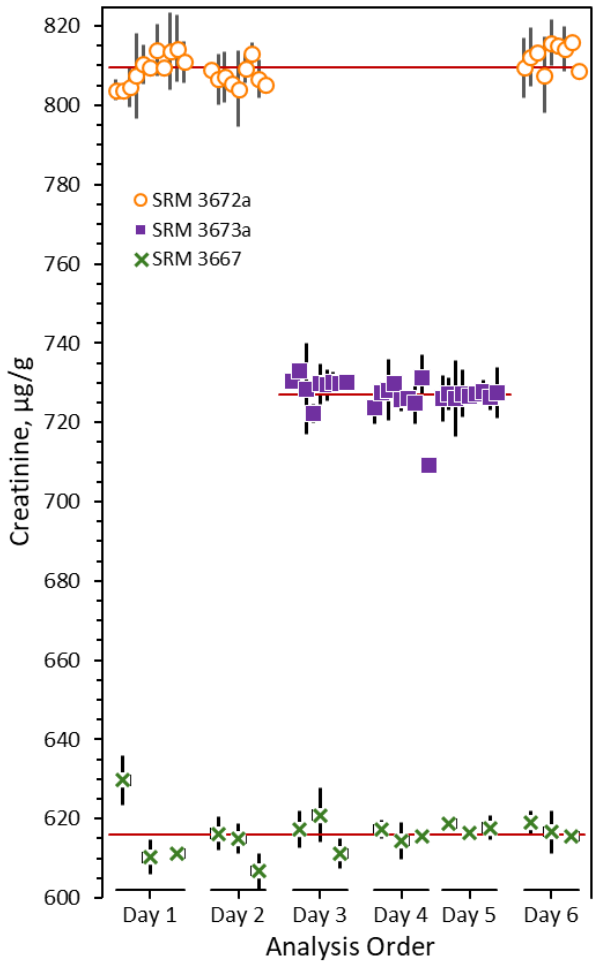


Fig. 44. Creatinine Results as a Function of Analysis Order.

Each symbol represents the mean of measurement results from two injections of a sample aliquot. Results are displayed for the SRM 3667 (×), 3673a (solid square), and 3672a (open circle) materials ordered by when the aliquots were analyzed. Error bars represent twice the standard deviation of the results for the two injections. Horizontal lines denote the means of the aliquot means.

The mean creatinine result for the 18 mean SRM 3667 control determinations was (616.1 ± 4.9) µg/g, in excellent agreement with the certified 95 % level of confidence range of (613 ± 13) µg/g. This verifies the accuracy of the current implementation of the RMP and that the creatinine measurements were in statistical control throughout the measurement campaigns.

The slightly high result of the first SRM 3667 aliquot of the Day 1 campaign is attributed to incomplete mixing. There is no known preparation, operator, or instrument cause for the slightly low result of the SRM 3673a Box 22 in the Day 4 campaign, therefore all results are considered valid. The RSDs of the SRM 3667, 3673a, and 362a results are 0.79 %, 0.59 %, and 0.48 %. These low variabilities verify the excellent within-day repeatability and day-to-day reproducibility of the RMP implementation.

There is no evidence for significant between-campaign differences for any of the three SRMs.

12.5. Homogeneity

Results for SRMs 3672a and 3673a are displayed as functions of their batch packaging order in Fig. 45. There is no evidence for any significant trend among the boxes for either SRM.

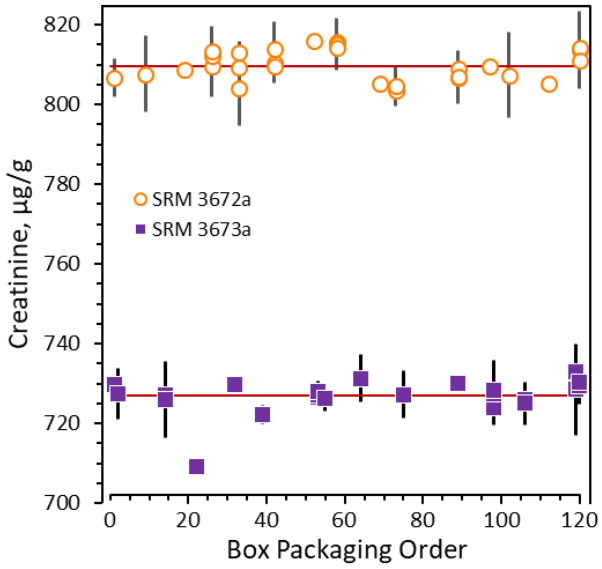


Fig. 45. Creatinine Results as a Function of Packaging Order.

Each symbol represents the mean of measurement results from two injections of a sample aliquot. Results are displayed for SRMs 3673a (solid squares) and 3672a (open circles) ordered by when the box the vials were taken from was filled during production. Error bars represent twice the standard deviation of the results for the two injections. Horizontal lines denote the means of the aliquot means.

12.6. Assigned Values

Table 51 lists the assigned values for creatinine.

Table 51. Assigned Values for Creatinine in SRMs 3672a and 3673a, µg/g.

	SRM 3672a				SRM 3673a			
Analyte	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]	w [a]	$u(w)$ [b]	$U_{95}(w)$ [c]	n_{mm} [d]
Creatinine	809.7	0.6	1.1	1	727.1	0.6	1.2	1

12.6.1. Metrological Traceability

The creatinine results are metrologically traceable to the International System of Units (SI) through calibration with SRM 914b Creatinine [45].

References

[1] National Center for Health Statistics (2023) About the National Health and Nutrition Examination Survey. https://www.cdc.gov/nchs/nhanes/about_nhanes.htm. Accessed 10/24/2024.

[2] National Institute of Standards and Technology (21 December 2022) Certificate of Analysis, Standard Reference Material 3672 Organic Contaminants in Smokers' Urine (Frozen). https://tsapps.nist.gov/srmext/certificates/3672.pdf.

[3] National Institute of Standards and Technology (3 January 2023) Certificate of Analysis, Standard Reference Material 3673 Organic Contaminants in Non-Smokers' Urine (Frozen). https://tsapps.nist.gov/srmext/certificates/3673.pdf.

[4] Schantz MM, Benner BA, Heckert NA, Sander LC, Sharpless KE, Vander Pol SS, Vasquez Y, Villegas M, Wise SA, Udeni Alwis K, Blount BC, Calafat AM, Li Z, Silva MJ, Ye X, Gaudreau E, Patterson DG Jr, Sjödin A (2015) Development of urine standard reference materials for metabolites of organic chemicals including polycyclic aromatic hydrocarbons, phthalates, phenols, parabens, and volatile organic compounds. Anal Bioanal Chem 407:2945–2954. https://doi.org/10.1007/s00216-014-8441-0.

[5] United States Department of Labor, Occupational Safety and Health Administration, Standard 1910.1030, Standard Interpretations/ The potential for the transmission of HIV from contact with urine specimens. https://www.osha.gov/laws-regs/standardinterpretations/1996-04-15. Accessed 10/24/2024.

[6] ASTM Standard D7777-13 (2018) Standard Test Method for Density, Relative Density, or API Gravity of Liquid Petroleum by Portable Digital Density Meter. ASTM International, West Conshohocken, PA. https://doi.org/10.1520/D7777-13.

[7] ISO Standard 15212-1:1998 (1998) Oscillation-type density meters. Part 1: Laboratory instruments. International Standards Organization, Geneva. https://www.iso.org/standard/28482.html.

[8] DerSimonian R, Laird N (1986) Meta-analysis in clinical trials. Control Clin Trials, 7(3):177-188. https://doi.org/10.1016/0197-2456(86)90046-2.

[9] Efron B, Tibshirani RJ (1994) An Introduction to the Bootstrap, Chapman and Hall/CRC. https://doi.org/10.1201/9780429246593.

[10] Possolo A, Hibbert D, Stohner J, Bodnar O, Meija J (2024) A brief guide to measurement uncertainty (IUPAC Technical Report). Pure Appl Chem. 96(1):113-134. https://doi.org/10.1515/pac-2022-1203.

[11] Silva MJ, Samandar E, Preau JL Jr, Reidy JA, Needham LL, Calafat AM (2007) Quantification of 22 phthalate metabolites in human urine. J Chromatogr B 860(1):106-12. https://doi.org/10.1016/j.jchromb.2007.10.023.

[12] Kato K, MJ Silva, LL Needham, AM Calafat (2005) Determination of 16 Phthalate Metabolites in Urine Using Automated Sample Preparation and On-line Preconcentration/High-Performance Liquid Chromatography/Tandem Mass Spectrometry. Anal Chem 77(9):2985-2991. https://doi.org/10.1021/ac0481248.

[13] Wenzel AG, Brock JW, Cruze L, Newman RB, Unal ER, Wolf BJ, Somerville SE, Kucklick JR (2018) Prevalence and predictors of phthalate exposure in pregnant women in Charleston, SC. Chemosphere 193:394-402. https://doi.org/10.1016/j.chemosphere.2017.11.019.

[14] National Institute of Standards and Technology (26 July 2016) Certificate of Analysis, Standard Reference Material 3060 Monoester Phthalates in Acetonitrile. https://tsapps.nist.gov/srmext/certificates/3060.pdf.

[15] Ragland J. Environmental Metrology Measurement Assistant (EMMA). https://www.nist.gov/programs-projects/environmental-metrology-measurement-assistant-emma. Accessed 11/1/2024.

[16] CDC Method 6705.02 (2013-2014) Laboratory Procedure Manual for Eight monohydroxy-polycyclic aromatic hydrocarbons: 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyfluorene, 3-hydroxyfluorene, 1-hydroxyphenanthrene, 2- & 3-hydroxyphenanthrene, 1-hydroxypyrene. Centers for Disease Control and Prevention. https://wwwn.cdc.gov/nchs/data/nhanes/2013-2014/labmethods/PAH_H_MET_Aromatic_Hydrocarbons.pdf. Accessed 11/4/2024.

[17] Wang Y, Meng L, Pittman EN, Etheredge A, Hubbard K, Trinidad DA, Kato K, Ye X, Calafat AM (2017) Quantification of urinary mono-hydroxylated metabolites of polycyclic aromatic hydrocarbons by on-line solid phase extraction-high performance liquid chromatography-tandem mass spectrometry, Anal Bioanal Chem 409(4):931-937. https://doi.org/10.1007/s00216-016-9933-x.

[18] Ramsauer B, Sterz K, Hagedorn HW, Engl J, Scherer G, McEwan M, Errington G, Shepperd J, Cheung F (2011) A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in urine of non-smokers and smokers. Anal Bioanal Chem 399:877–889. https://doi.org/10.1007/s00216-010-4355-7.

[19] Grignon C, Dupuis A, Albouy-Llaty M, Condylis M, Barrier L, Carato P, Brunet B, Migeot V, Venisse N (2017) Validation of a probe for assessing deconjugation of glucuronide and sulfate phase II metabolites assayed through LC–MS/MS in biological matrices, J Chromatogr B 1061–1062(1):72-78, https://doi.org/10.1016/j.jchromb.2017.07.013.

[20] Rybak ME, Pao CI, Pfeiffer CM (2014) Determination of urine caffeine and its metabolites by use of high-performance liquid chromatography-tandem mass spectrometry: estimating dietary caffeine exposure and metabolic phenotyping in population studies. Anal Bioanal Chem 406:771–784. https://doi.org/10.1007/s00216-013-7506-9.

[21] Juris Meija (2016-2019) IUPAC Molecular Weight Calculator, ciaaw.org [BETA release v.0.7, May 2019]. Commission on Isotopic Abundances and Atomic Weights (CIAAW), International Union of Pure and Applied Chemistry (IUPAC) Project 2015-037-2-200. https://ciaaw.shinyapps.io/calculator. Accessed 10/24/2024.

[22] Possolo A, van der Veen AMH, Meija J, Hibbert DB (2018) Interpreting and propagating the uncertainty of the standard atomic weights (IUPAC Technical Report). Pure Appl Chem 90:395-424. https://doi.org/10.1515/pac-2016-0402.

[23] Man CN, Gim L-H, Ismail S, Lajis R, Awang R. (2006) Simple, Rapid and Sensitive Method for Simultaneous Quantification of Urinary Nicotine and Cotinine Using Gas Chromatography-Mass Spectrometry. J Chromatogr B 844(2):322–327. https://doi.org/10.1016/j.jchromb.2006.07.029.

[24] Center for Disease Control and Prevention (2020) National Health and Nutrition Examination Survey: 2013-2014 Data Documentation, Codebook, and Frequencies: Caffeine & Caffeine Metabolites – Urine. https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/CAFE_H.htm. Accessed 10/24/2024.

[25] Nelson MA, Waters JF, Toman B, Lang BE, Rück A, Breitruck K, Obkircher M, Windust A, Lippa KA (2018) A New Realization of SI for Organic Chemical Measurement: NIST PS1 Primary Standard for Quantitative NMR (Benzoic Acid). Anal Chem 90(17):10510-10517. https://doi.org/10.1021/acs.analchem.8b02575.

[26] Toman B, Nelson MA, Lippa KA (2016) Chemical Purity Using Quantitative 1H Nuclear Magnetic Resonance: A Hierarchical Bayesian Approach for Traceable Calibrations. Metrologia 53(5):1193−1203. https://doi.org/10.1088/0026-1394/53/5/1193.

[27] Reyes-Contreras C, Domínguez C, Bayona JM (2012) Determination of nitrosamines and caffeine metabolites in wastewaters using gas chromatography mass spectrometry and ionic liquid stationary phases. J Chromatogr A 1261:164-70. https://doi.org/10.1016/j.chroma.2012.05.082.

[28] Man CN, Gim L-H, Ismail S, Lajis R, Awang R. (2006) Simple, Rapid and Sensitive Method for Simultaneous Quantification of Urinary Nicotine and Cotinine Using Gas Chromatography-Mass Spectrometry. J Chromatogr B 844(2):322–327. https://doi.org/10.1016/j.jchromb.2006.07.029.

[29] CDC Method 2105.02 (2019-2020) Laboratory Procedure Manual for Urinary metabolites of Benzene, Furfural, 5-Hydroxymethylfurfural, and N-Methyl-2-PYRrolidone. Centers for Disease Control and Prevention. https://wwwn.cdc.gov/nchs/data/nhanes/2019-2020/labmethods/UVOC2-K-MET-508.pdf. Accessed 10/24/2024.

[30] Bhandari D, McCarthy D, Biren C, Movassaghi C, Blount BC, De Jesús VR (2019) Development of a UPLC-ESI-MS/MS method to measure urinary metabolites of selected VOCs: Benzene, cyanide, furfural, furfuryl alcohol, 5- hydroxymethylfurfural, and N-methyl-2-PYRrolidone. J Chromatogr B 1126-1127:121746. https://doi.org/10.1016/j.jchromb.2019.121746.

[31] Alwis KU, Blount BC, Britt AS, Patel D, Ashley DL (2012) Simultaneous analysis of 28 urinary VOC metabolites using ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI/MSMS). Anal Chim Acta 750:152-160. https://doi.org/10.1016/j.aca.2012.04.009.

[32] CDC Method 2103a (2017-2018) Division of Laboratory Sciences Laboratory Procedure Manual for Volatile Organic Compounds (VOCs) Metabolites in Urine. https://wwwn.cdc.gov/nchs/data/nhanes/2017-2018/labmethods/UVOC-J-MET-508.pdf.

[33] Ye X, Kuklenyik Z, Needham LL, Calafat AM (2005) Automated On-Line Column-Switching HPLC-MS/MS Method with Peak Focusing for the Determination of Nine Environmental Phenols in Urine. Anal Chem 77(16):5407-5413. https://doi.org/10.1021/ac050390d.

[34] CDC Method 2021.02 (2013-2014) Division of Laboratory Sciences Laboratory Protocol for Nicotine, Cotinine, Trans-3'-hydroxycotinine, Nornicotine, Nicotine-N-oxide, Cotinine-N-oxide, Norcotinine, Anatabine, and Anabasine in Urine. https://wwwn.cdc.gov/nchs/data/nhanes/2013-2014/labmethods/UCOT_H_UCOTS_H_Urinary_Nicotine_Metabolites_and_Analogs.pdf. Accessed 11/6/2024.

[35] CDC Method 2014 (2013-2014) Division of Laboratory Sciences Laboratory Procedure Manual for Tobacco-Specific Nitrosamines in Urine. https://wwwn.cdc.gov/nchs/data/nhanes/2013-2014/labmethods/TSNA_H_MET.pdf. Accessed 11/6/2024.

[36] DLS 2023, Laboratory Protocol for Menthol glucuronide, Nicotine, Cotinine, Trans-3'-hydroxycotinine, Anatabine, Anabasine, β-Nicotyrine, Isonicoteine, Myosmine, Bupropion, Cytisine, Varenicline, Arecaidine, and Arecoline in Urine. Division of Laboratory Services, Center for Disease Control and Prevention. For further information, contact June Feng, czf2@cdc.gov.

[37] CDC Method 2024 (2013-2014) Division of Laboratory Sciences Laboratory Procedure Manual for Cotinine and Hydroxycotinine (Total) in Urine. https://wwwn.cdc.gov/nchs/data/nhanes/2015-2016/labmethods/UCOT_I_UCOTS_I_Urinary_Cotinine_and_Hydroxycotinine_MET.pdf. Accessed 11/6/2024.

[38] DLS 2025.01 Laboratory Protocol for Urinary 8-Isoprotane, Oxidative Stress. Division of Laboratory Sciences, Centers for Disease Control and Prevention. For further information, contact Christina Brosius, xfv5@cdc.gov.

[39] Valentín-Blasini L, Blount BC, Delinsky A (2007) Quantification of iodide and sodium-iodide symporter inhibitors in human urine using ion chromatography tandem mass spectrometry. J Chromatogr A 1155(1):40-46. https://doi.org/10.1016/j.chroma.2007.04.014.

[40] Stokes P, O'Connor G (2003) Development of a liquid chromatographY-mass spectrometry. method for the high-accuracy determination of creatinine in serum. J Chrom B 794:125-36. https://doi.org/10.1016/s1570-0232(03)00424-0.

[41] Dodder NG, Tai SS, Sniegoski LT, Zhang NF, Welch MJ (2007) Certification of creatinine in a human serum reference material by GC-MS and LC-MS. Clin Chem 53:1694-9. https://doi.org/10.1373/clinchem.2007.090027.

[42] Joint Committee for Traceability in Laboratory Medicine (JCTLM) DB Identifier C4RMP1, https://www.jctlmdb.org/#/app/search/search-results?keywords=C4RMP1&code_status=P. Accessed 10/24/2024.

[43] National Institute of Standards and Technology (24 June 2022) Certificate of Analysis, Standard Reference Material 3667 Creatinine in Frozen Human Urine. https://tsapps.nist.gov/srmext/certificates/3667.pdf.

[44] Camara J, Wood ESC, Toman B. (2023) Certification of Standard Reference Material® 967b Creatinine in Frozen Human Serum. National Institute of Standards and Technology, Gaithersburg, MD, NIST Special Publication (NISTSP) 260-239. https://doi.org/10.6028/NIST.SP.260-239.

[45] National Institute of Standards and Technology (21 November 2018) Certificate of Analysis, Standard Reference Material 914b Creatinine. https://tsapps.nist.gov/srmext/certificates/914b.pdf.

Appendix A. Abbreviations and Acronyms

APCI	atmospheric pressure chemical ionization
CDC	Centers for Disease Control and Prevention
COA	Certificate of Analysis
DINCH	1,2-cyclohexane dicarboxylic acid diisononyl ester
DLS	Division of Laboratory Sciences (CDC)
EMMA	Environmental Metrology Measurement Assistant
ESI	electrospray ionization
GC	gas chromatography
HPLC	high-performance liquid chromatography
ID	isotope dilution
IS	internal standard
InChI	International Chemical Identifier
JCTLM	Joint Committee for Traceability in Laboratory Medicine
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NHANES	National Health and Nutrition Examination Survey
NMR	nicotine metabolic ratio
NIST	National Institute of Standards and Technology
NOAA	National Oceanic and Atmospheric Administration
ORM	Office of Reference Materials (NIST)
QA	quality assessment
QC	quality control
QCH	high-concentration quality control material
QCL	low-concentration quality control material
OHRP	Office for Human Research Protections (Department of Health and Human Services)

PSM primary standard material

RMP reference measurement procedure

RSD relative standard deviation

SD standard deviation

SHS second-hand smoke

SI Internation System of Units (Système international d Unités)

SIM secondary ion monitoring

SPE solid-phase extraction

SRM Standard Reference Material, a NIST-trademarked certified reference material

UPLC ultra-performance liquid chromatography

VOC volatile organic compound

Appendix B. Analytes

Table 52. Summary of Analyte Codes and Common Names.

Code	Common	Class
1-NAP	1-hydroxynaphtholene	OH-PAH
1-PHE	1-hydroxyphenanthrene	OH-PAH
1PMA	N-Acetyl-S-(n-propyl)-L-cysteine	Mercapturic acid, etc.
1-PYR	1-hydroxypyrene	OH-PAH
2&3-PHE	2-hydroxyphenanthrene and 3-hydroxyphenanthrene	OH-PAH
2&3-FLU	2-hydroxyfluorene and 3-hydroxyfluorene	OH-PAH
2,5-DCP	2,5-Dichlorophenol	Phenolics
2ATCA	2-Aminothiazoline-4-carboxylic acid	Mercapturic acid, etc.
2CaHEMA	N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	Mercapturic acid, etc.
2CoEMA	N-Acetyl-S-(2-carboxyethyl)-L-cysteine	Mercapturic acid, etc.
2CyEMA	N-Acetyl-S-(2-cyanoethyl)-L-cysteine	Mercapturic acid, etc.
2-FLU	2-hydroxyfluorene	OH-PAH
2HEMA	N-Acetyl-S-(2-hydroxyethyl)-L-cysteine a	Mercapturic acid, etc.
2HPhEMA	N-Acetyl-S-(1-PHEnyl-2-hydroxyethyl)-L-cysteine	Mercapturic acid, etc.
2HPMA	N-Acetyl-S-(2-hydroxypropyl)-L-cysteine	Mercapturic acid, etc.
2MHA	2-Methylhippuric acid	Mercapturic acid, etc.
2-NAP	2-hydroxynaphtholene	OH-PAH
2-PHE	2-hydroxyphenanthrene	OH-PAH
2-FLU	2-hydroxyfluorene	OH-PAH
3-FLU	3-hydroxyfluorene	OH-PAH
3HPMA	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine	Mercapturic acid, etc.
3HMPMA	N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine	Mercapturic acid, etc.
3MHA + 4MHA	3-Methylhippuric acid, 4-Methylhippuric acid	Mercapturic acid, etc.
3-PHE	3-hydroxyphenanthrene	OH-PAH
4-PHE	4-hydroxyphenanthrene	OH-PAH
8PGFT	Total 8-iso-prostaglandin $F_{2\alpha}$	Nicotine, etc.
8PGFF	Free 8-iso-prostaglandin $F_{2\alpha}$	Nicotine, etc.
ANBF	Free (R,S)-anabasine	Nicotine, etc.
ANBT	Total (R,S)-anabasine	Nicotine, etc.
ANTF	Free (R,S)-anatabine	Nicotine, etc.
ANTT	Total (R,S)-anatabine	Nicotine, etc.
BP-3	Benzophenone-3	Phenolics
BPA	Bisphenol A	Phenolics
B-PB	Butyl Paraben	Phenolics
BPF	Bisphenol F	Phenolics
BPS	Bisphenol S	Phenolics
BzMA	N-Acetyl-S-(benzyl)-L-cysteine	Mercapturic acid, etc.
Caffeine	Caffeine	Caffeine etc.
COTF	Free (-)-cotinine	Nicotine, etc.
COTT	Total (-)-cotinine	Nicotine, etc.
COXF	Free (S)-cotinine N-oxide	Nicotine, etc.
COXT	Total (S)-cotinine N-oxide	Nicotine, etc.
Creatinine	Creatinine	Creatinine
DHAVO	Dihydroxyavobenzone	Phenolics
E-PB	Ethyl Paraben	Phenolics
HCTF	Free (-)-*trans*-3'-hydroxycotinine	Nicotine, etc.
HCTT	Total (-)-*trans*-3'-hydroxycotinine	Nicotine, etc.
HMFA	5-Hydroxymethyl-2-furancarboxylic acid	Benzene, etc.

Code	Common	Class
HMFG	5-Hydroxymethyl-2-furoylglycine	Benzene, etc.
HPB	3-Hydroxy n-butyl paraben	Phenolics
HPBF	Free 4-hydroxy-4-(3-pyridyl)-butanoic acid	Nicotine, etc.
HPBT	Total 4-hydroxy-4-(3-pyridyl)-butanoic acid	Nicotine, etc.
MADA	Mandelic acid	Mercapturic acid, etc.
MBP	mono-n-butyl phthalate	Phthalates, etc.
MBzP	monobenzyl phthalate	Phthalates, etc.
MCaMA	N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine	Mercapturic acid, etc.
MCHpP	mono(7-carboxyheptyl) phthalate	Phthalates, etc.
MCNP	mono-carboxyisononyl phthalate isomers	Phthalates, etc.
MCOCH	cyclohexane-1,2-dicarboxylic acid monocarboxy isooctyl ester	Phthalates, etc.
MCOP	monocarboxyisooctyl phthalate isomers	Phthalates, etc.
MCPP	mono-3-carboxypropyl phthalate	Phthalates, etc.
MECPP	mono-2-ethyl-5-carboxypentyl phthalate	Phthalates, etc.
MECPTP	mono-2-ethyl-5-carboxypentyl terephthalate	Phthalates, etc.
MEG	(1R,2S,5R)-(-)-menthol β-D-glucuronide	Nicotine, etc.
MEHHP	mono-2-ethyl-5-hydroxyhexyl phthalate	Phthalates, etc.
MEHHTP	mono-2-ethyl-5-hydrohexyl terephthalate	Phthalates, etc.
MEHP	mono-2-ethylhexyl phthalate	Phthalates, etc.
MEOHP	mono-2-ethyl-5-oxohexyl phthalate	Phthalates, etc.
MEP	mono-ethyl phthalate	Phthalates, etc.
MHBP	mono-hydroxybutyl phthalate	Phthalates, etc.
MHiBP	mono-hydroxyisobutyl phthalate	Phthalates, etc.
MHiNCH	cyclohexane-1,2-dicarboxylic acid monohydroxy isononyl ester	Phthalates, etc.
MiBP	mono-isobutyl phthalate	Phthalates, etc.
MiNP	mono(3,5,5-trimethylhexyl) phthalate	Phthalates, etc.
MMP	mono-methyl phthalate	Phthalates, etc.
MONP	monooxononyl phthalates	Phthalates, etc.
MOP	mono-octyl phthalate	Phthalates, etc.
M-PB	Methyl Paraben	Phenolics
N2FG	N-2-Furoylglycine	Benzene, etc.
NABF	Free N’-nitrosoanabasine	Nicotine, etc.
NABT	N’-nitrosoanabasine	Nicotine, etc.
NATF	Free N’-nitrosoanatabine	Nicotine, etc.
NATT	N’-nitrosoanatabine	Nicotine, etc.
NICF	(-)-Nicotine	Nicotine, etc.
NICT	Nicotine	Nicotine, etc.
NNAL	4-(Methylnitrosamino)-1-(3-Pyridyl)-1-butanol	Nicotine, etc.
NNALF	Free 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-butanol	Nicotine, etc.
NNNF	Free N’-nitrosonornicotine	Nicotine, etc.
NNNT	N’-nitrosonornicotine	Nicotine, etc.
NOXF	Free (1’S,2’S)-nicotine-N’-oxide	Nicotine, etc.
NOXT	Nicotine-1'-N-oxide	Nicotine, etc.
Paraxanthine	Paraxanthine	Caffeine etc.
PhGA	Phenylglyoxylic acid	Mercapturic acid, etc.
PhMA	N-Acetyl-S-(phenyl)-L-cysteine	Benzene, etc.
P-PB	Propyl Paraben	Phenolics
SCN^-	Thiocyanate	Thiocyanate
TCC	Triclocarban	Phenolics
Theobromine	Theobromine	Caffeine etc.
Theophylline	Theophylline	Caffeine etc.

Code	Common	Class
TCS	Triclosan	Phenolics
TTCA	2-Thioxothiazolidine-4-carboxylic acid	Mercapturic acid, etc.