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.

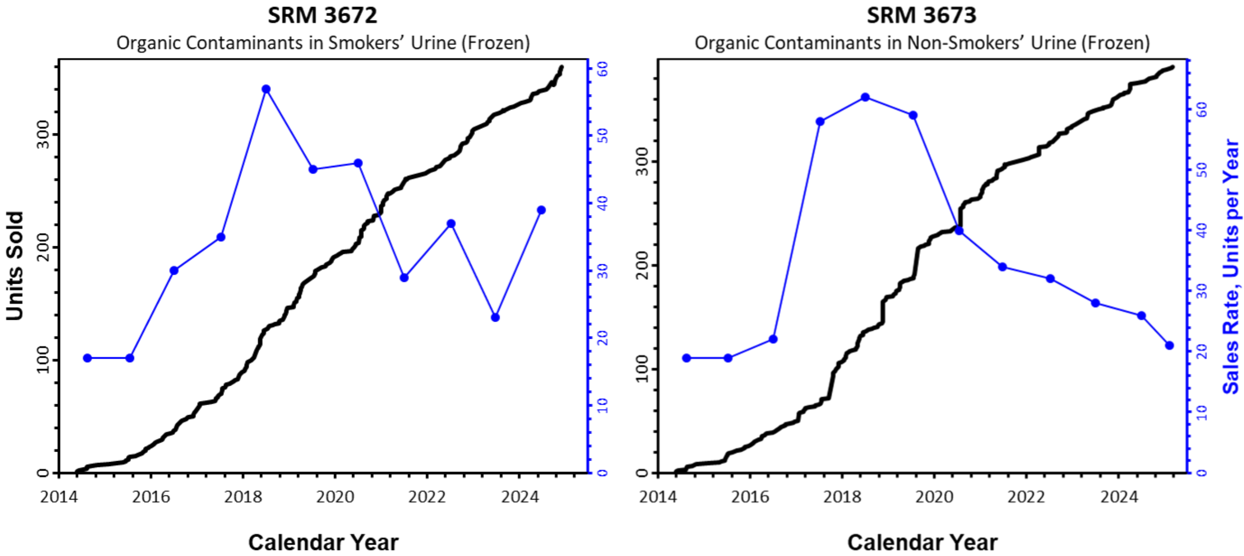


Fig. 1. Sales History of SRMs 3672 and 3673.

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.

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).

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:

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

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:

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

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:

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

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$^{\mathrm{th}}$ percentile to the 97.5$^{\mathrm{th}}$ 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:

$$\left(w_{\text{analyte}}\frac{\text{g}}{\text{g}}\right) = \left(x_{\text{analyte}}\frac{\text{g}}{\text{L}}\right) \Big/ \left(\rho_{\text{matrix}}\frac{\text{g}}{\text{mL}}\frac{1000\ \text{mL}}{\text{L}}\right)$$

$$u(w_{\text{analyte}}) = w_{\text{analyte}}\sqrt{\left(\frac{u(x_{\text{analyte}})}{x_{\text{analyte}}}\right)^2 + \left(\frac{u(\rho_{\text{matrix}})}{\rho_{\text{matrix}}}\right)^2}. \tag{4}$$

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:

$$\left(w_{\text{analyte}}\frac{\text{g}}{\text{g}}\right) = \left(c_{\text{analyte}}\frac{\text{mol}}{\text{L}}\right)\left(\frac{M_{\text{analyte}}\frac{\text{g}}{\text{mol}}}{\rho_{\text{matrix}}\frac{\text{g}}{\text{mL}}\frac{1000\ \text{mL}}{\text{L}}}\right)$$

$$u(w_{\text{analyte}}) = w_{\text{analyte}}\sqrt{\left(\frac{u(x_{\text{analyte}})}{x_{\text{analyte}}}\right)^2 + \left(\frac{u(\rho_{\text{matrix}})}{\rho_{\text{matrix}}}\right)^2 + \left(\frac{u(M_{\text{analyte}})}{M_{\text{analyte}}}\right)^2}. \tag{5}$$

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.

5.2.1.2. 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.

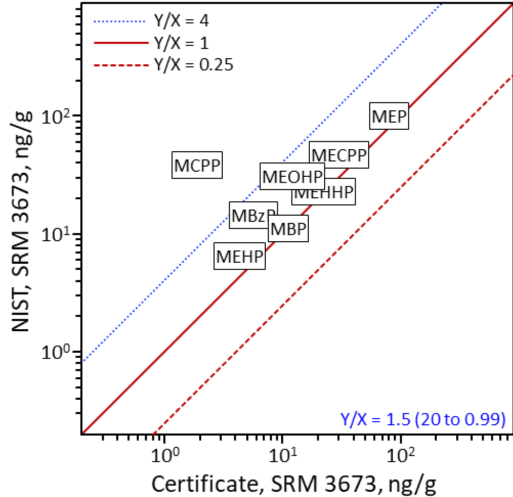


Fig. 9. SRM 3673 Phthalate-Related Results as a Function of the 2013 Non-Certified Values.

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.

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^{1}H 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

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].

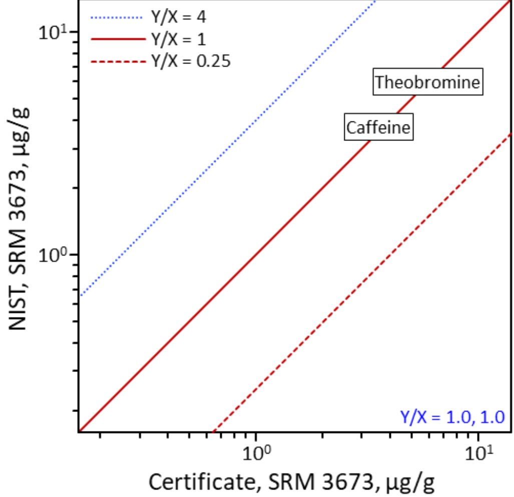


Fig. 25. Caffeine and Theobromine Results for SRM 3673 as a Function of 2013 Non-Certified Values.

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.

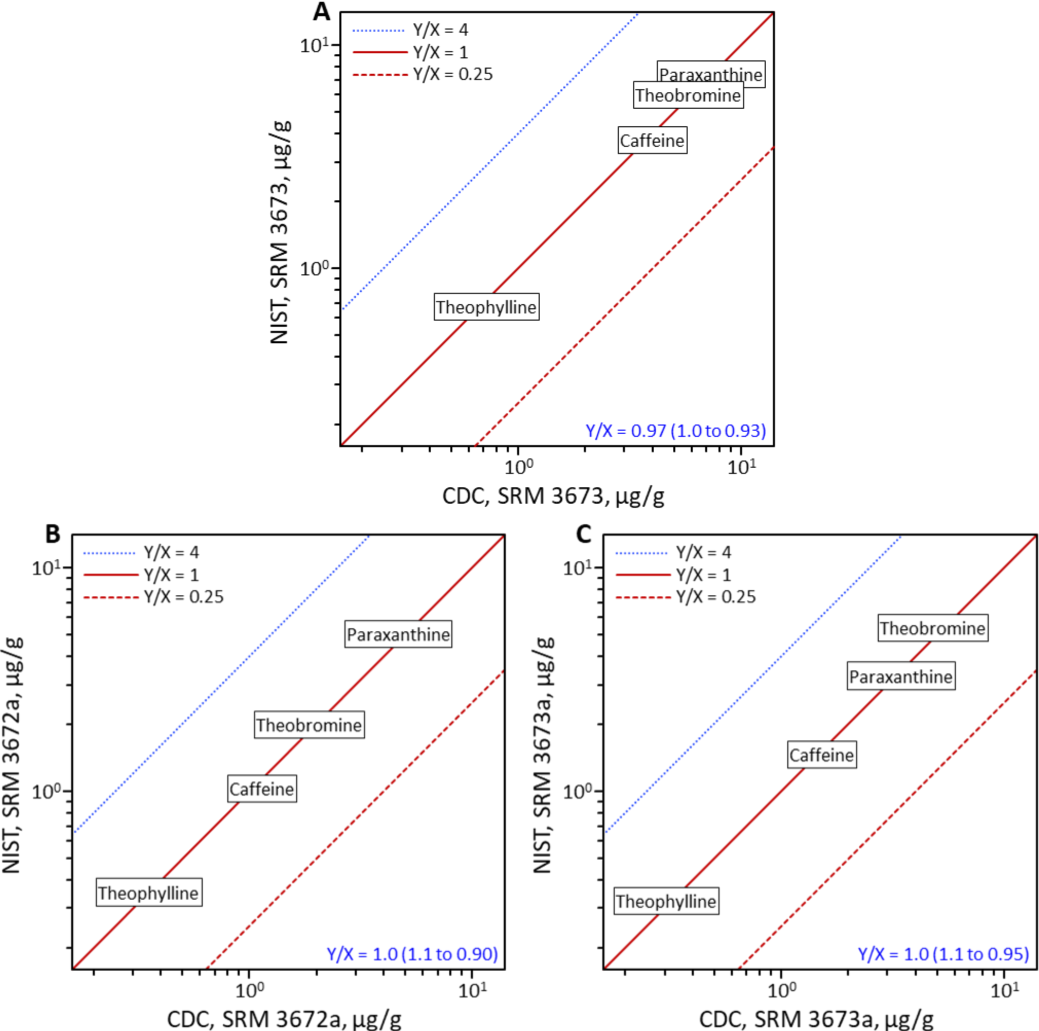


Fig. 26.NIST Caffeine-Related Analyte Results as a Function of CDC Results.

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.

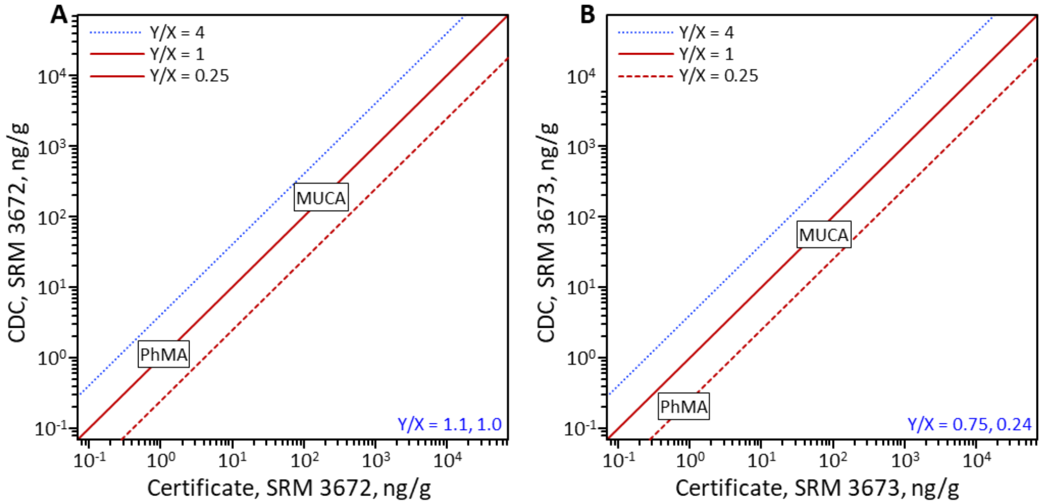


Fig. 27. Benzene and Furfural Metabolite Results as a Function of 2012 Values.

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).

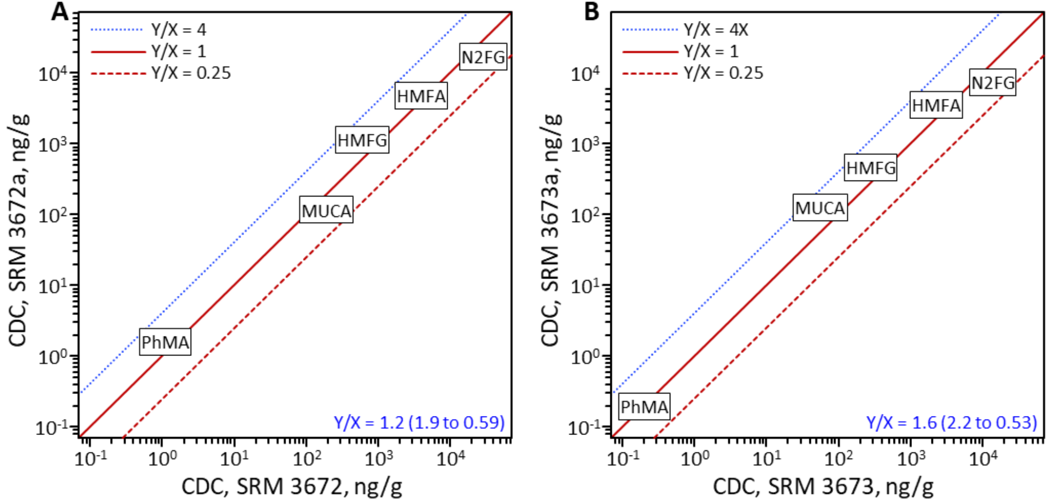


Fig. 28. Benzene and Furfural Metabolite Results for New SRMs as a Function of Original SRM Results.

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).

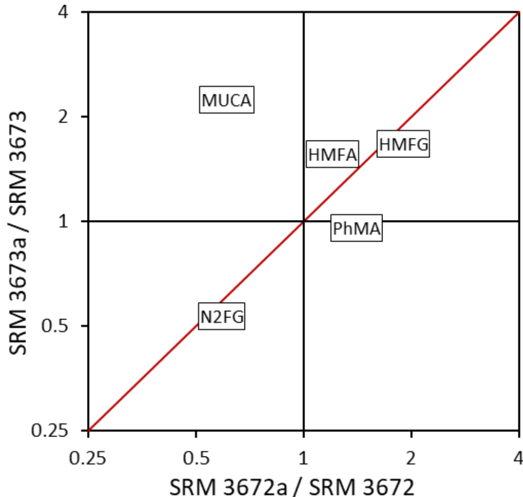


Fig. 29. Benzene and Furfural Metabolite 3673a/3673 Results as a Function of 3672a/3672 Results.

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).

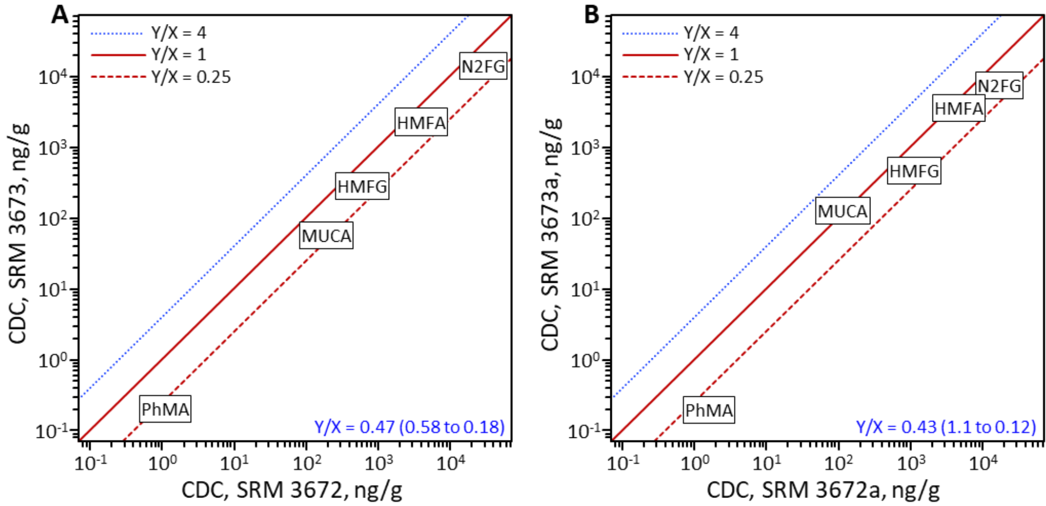


Fig. 30. Benzene and Furfural Metabolite Non-Smoker Results as a Function of Smoker Results.

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].

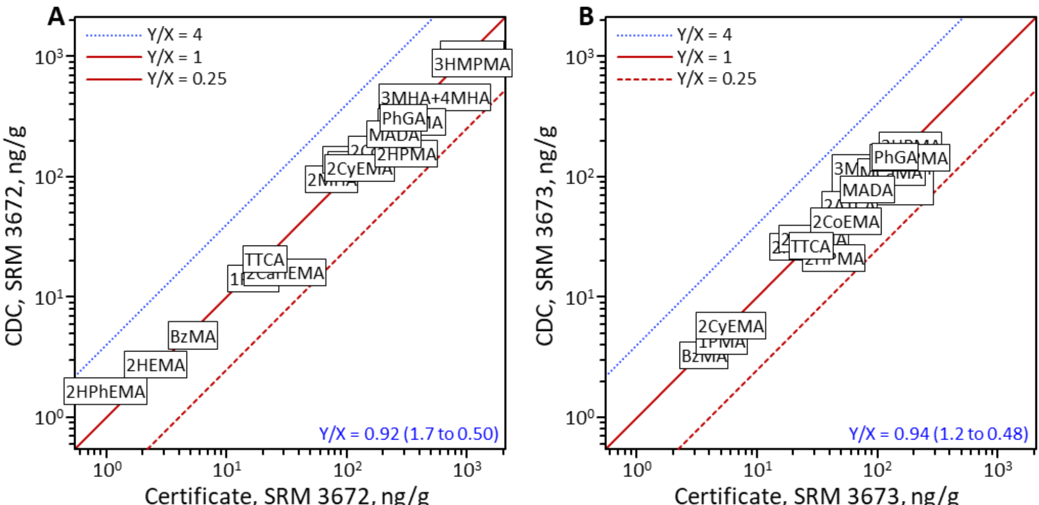


Fig. 31. Mercapturic and Related Biomarker Results as a Function of the CDC's 2012 Values.

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.

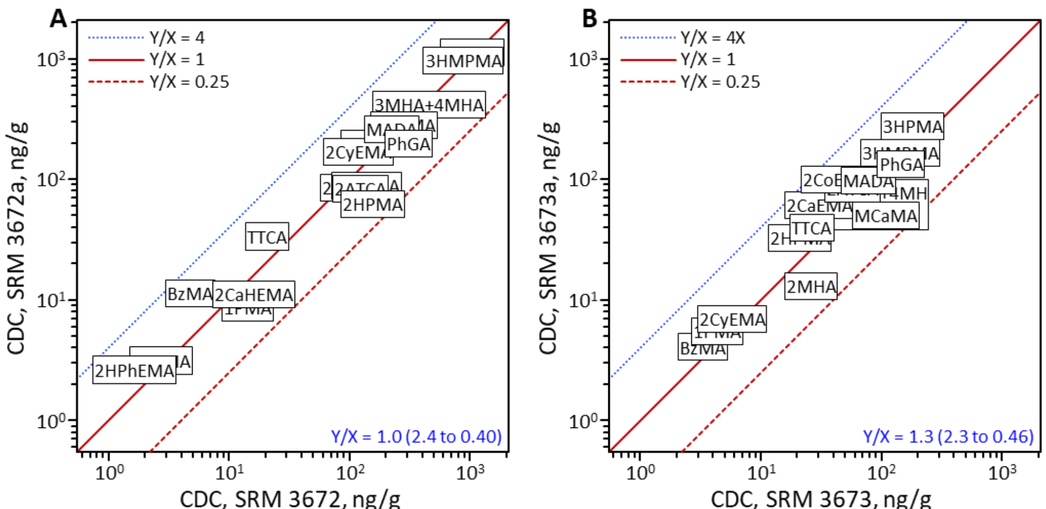


Fig. 32. Mercapturic and Related Biomarker Results for New SRMs as a Function of Original SRM Results.

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.

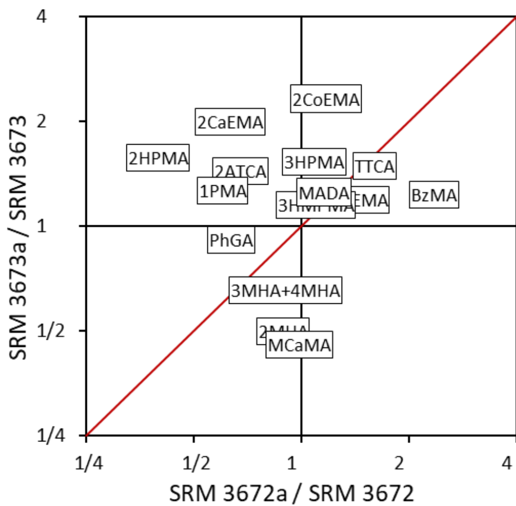


Fig. 33. Mercapturic and Related Biomarker 3673a/3673 Results as a Function of 3672a/3672 Results.

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.

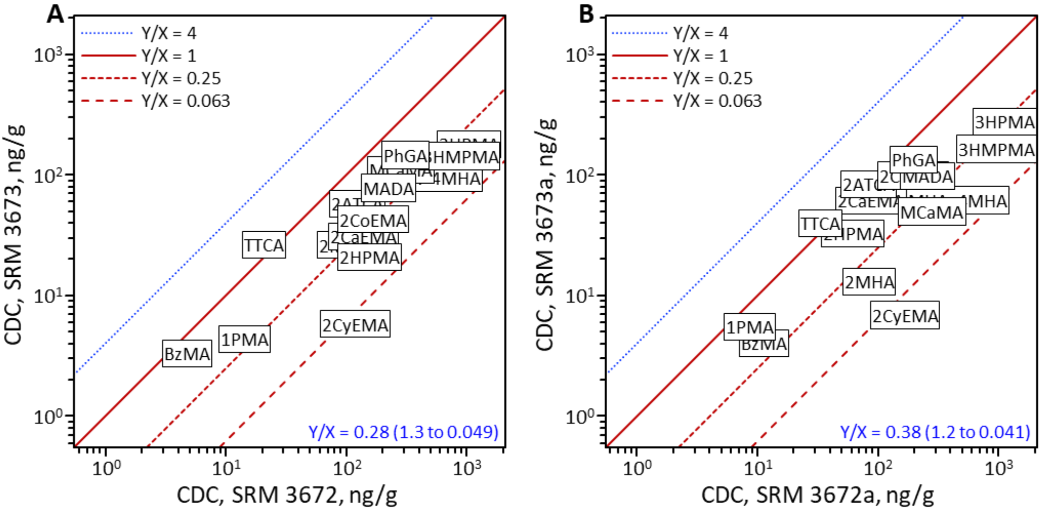


Fig. 34. Mercapturic and Related Biomarker Results Non-Smoker Results as a Function of Smoker Results.

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	

Compound	Structure
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 soluntion, 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.

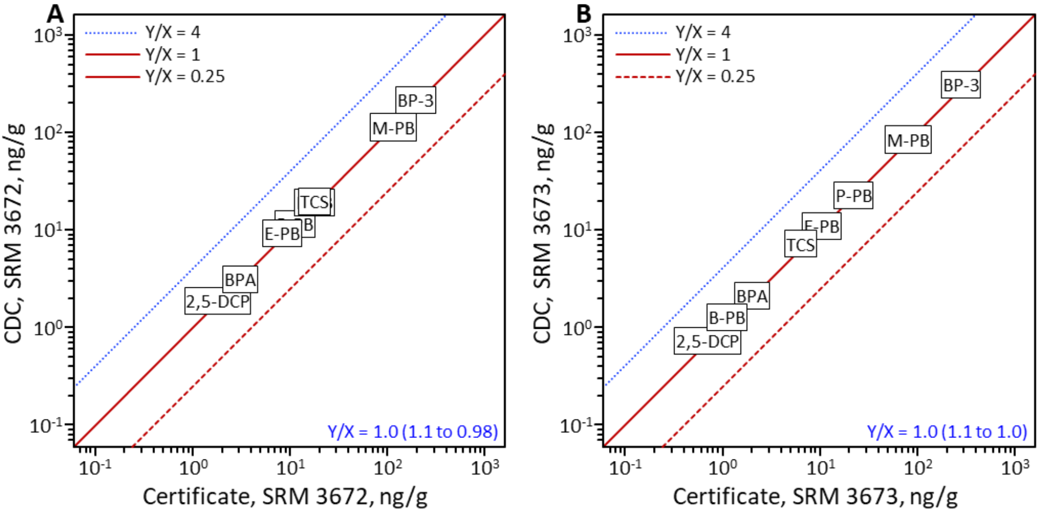


Fig. 35. Phenolic Metabolite Results as a Function of COA Values.

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.

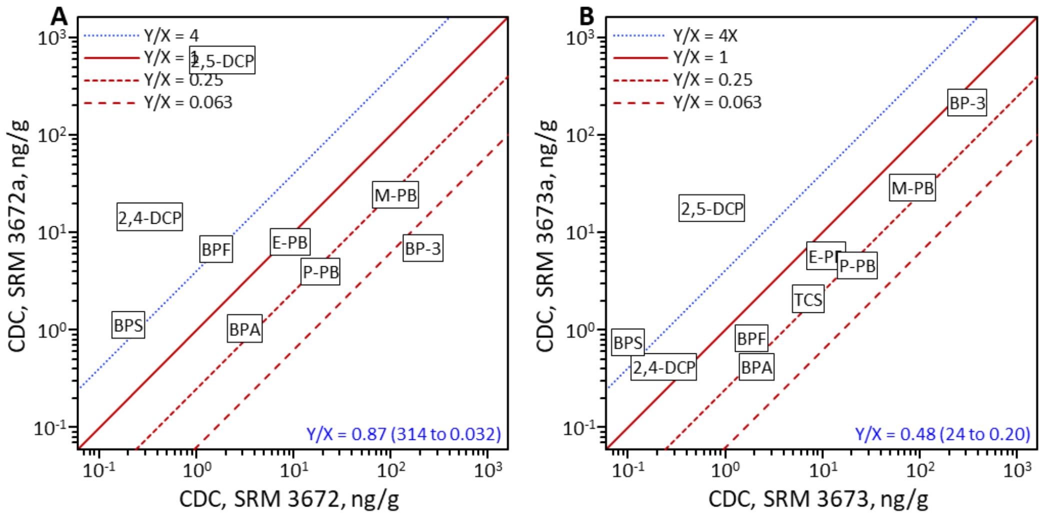


Fig. 36. Phenolic Results for New SRMs as a Function of Original SRM Results.

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.

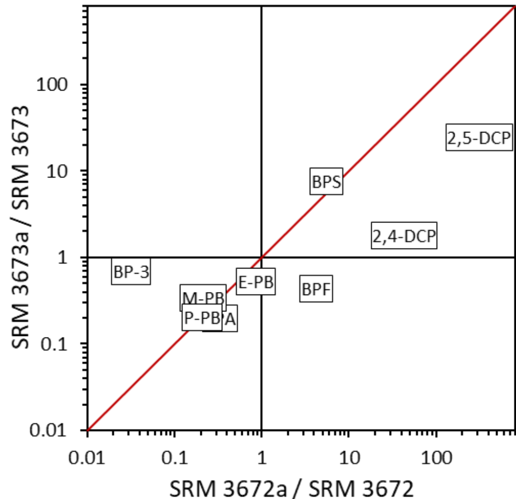


Fig. 37. Phenolic 3673a/3673 Results as a Function of 3672a/3672 Results.

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.

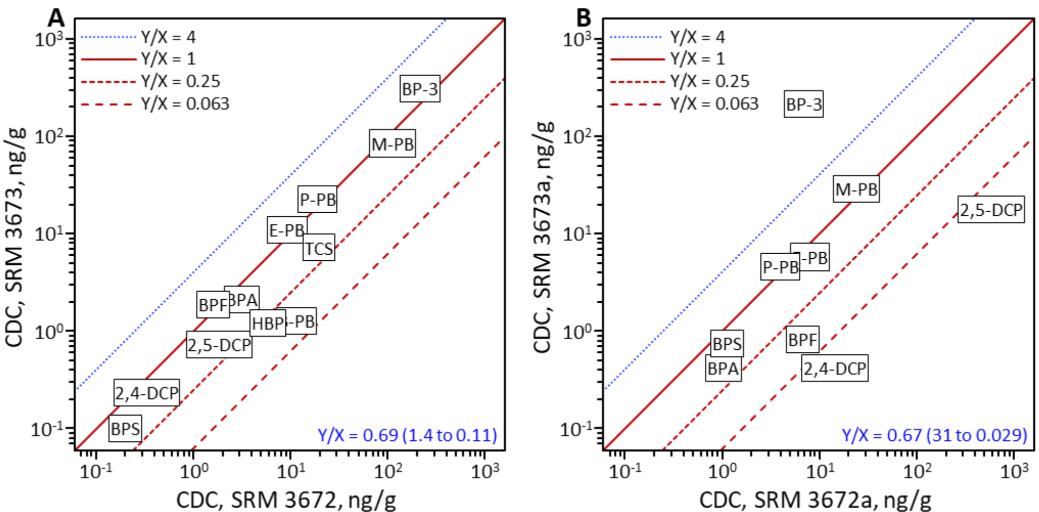


Fig. 38. Non-Smoker Phenolic Metabolite Results as a Function of Smoker Results.

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

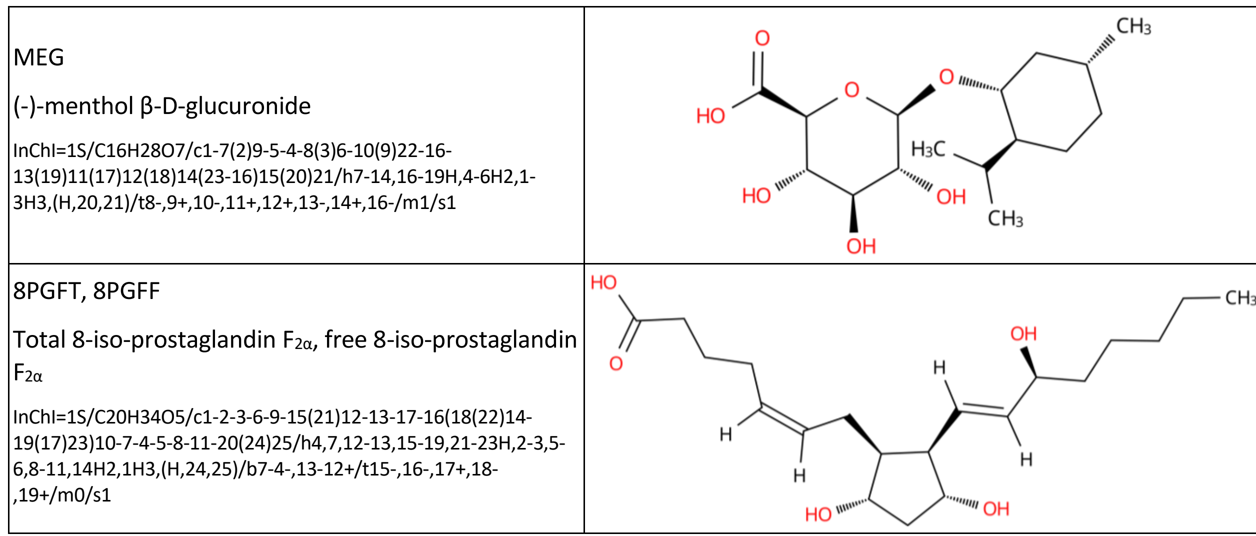
Nicotine is the primary tobacco-specific alkaloid in tobacco plants and tobacco smoke. Although not a direct cause of most diseases associated with tobacco use, nicotine addiction leads to chronic exposure to carcinogens and other bioactive compounds. The relative concentration of nicotine to its major 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	N, H N
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	N, CH_3, N
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	N, O^-, CH_3, N^+
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	O=N, N, N
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	N, CH_3, N, O

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	



Analyte	Structure
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.

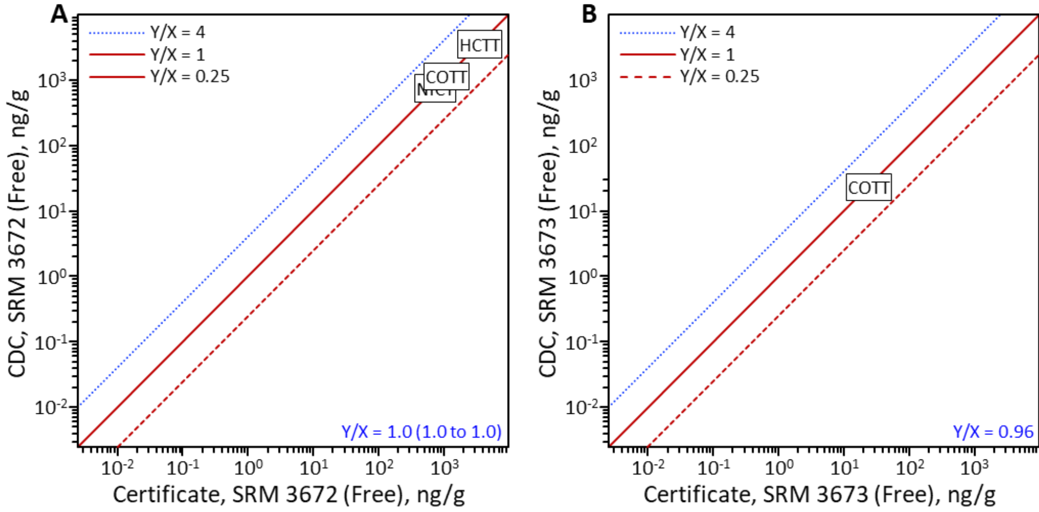


Fig. 39. Non-Smoker Nicotine and Smoking Metabolite Results as Function of COA Values.

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.

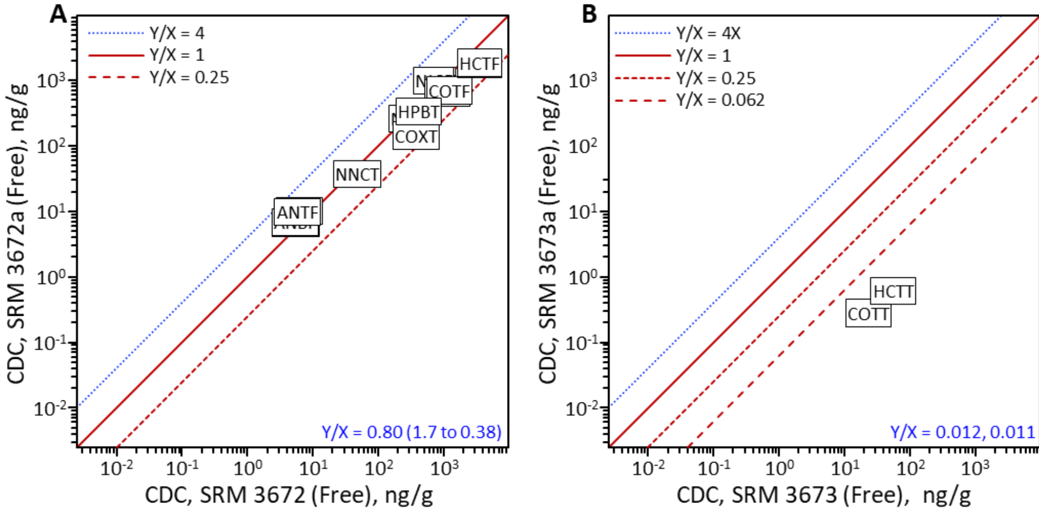


Fig. 40. Non-Smoker Nicotine and Smoking Metabolite Results for New SRMs as a Function of Original SRM Results.

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.

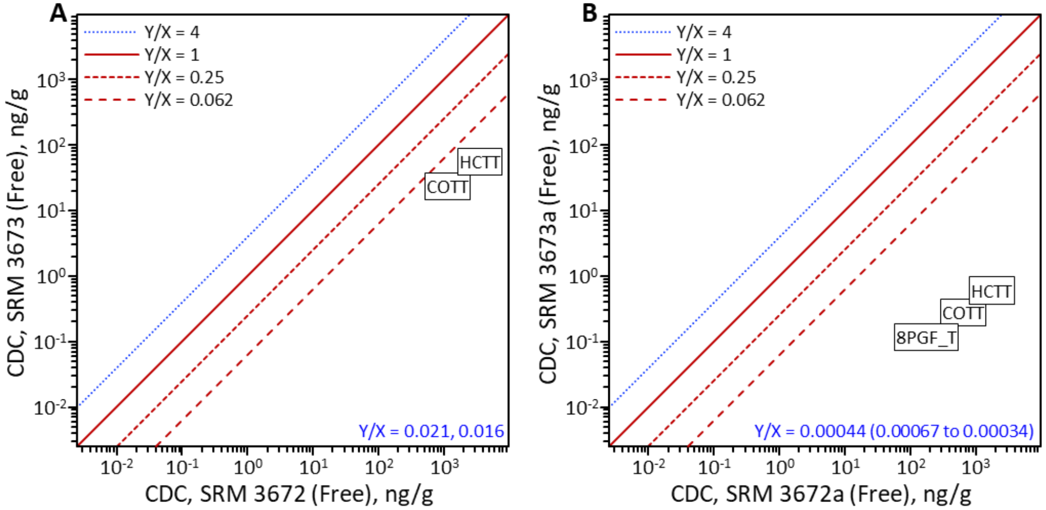


Fig. 41. Non-Smoker Nicotine and Smoking Metabolite Results as Functions of Smoker Results.

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.

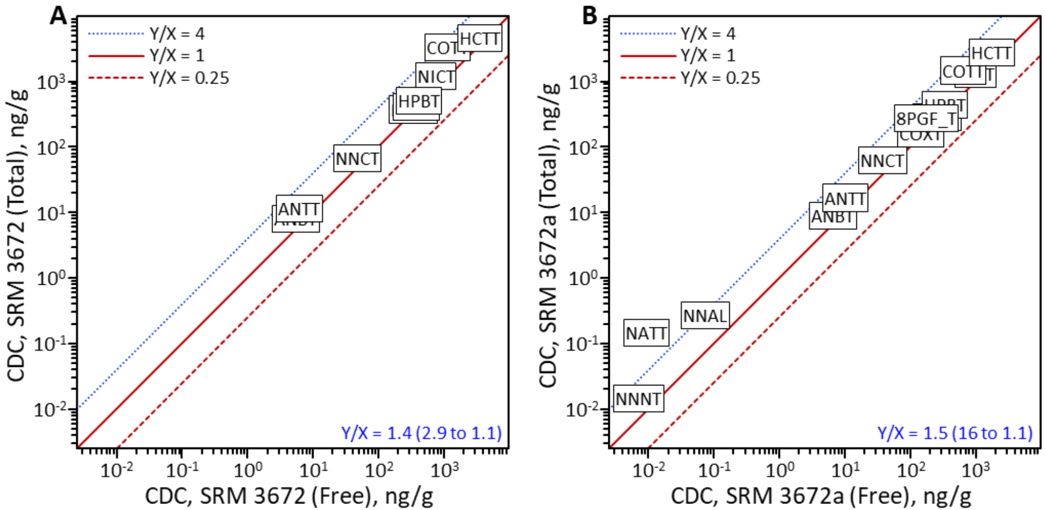


Fig. 42. Total Nicotine and Smoking Metabolite Results as Functions of Free Analyte Results.

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

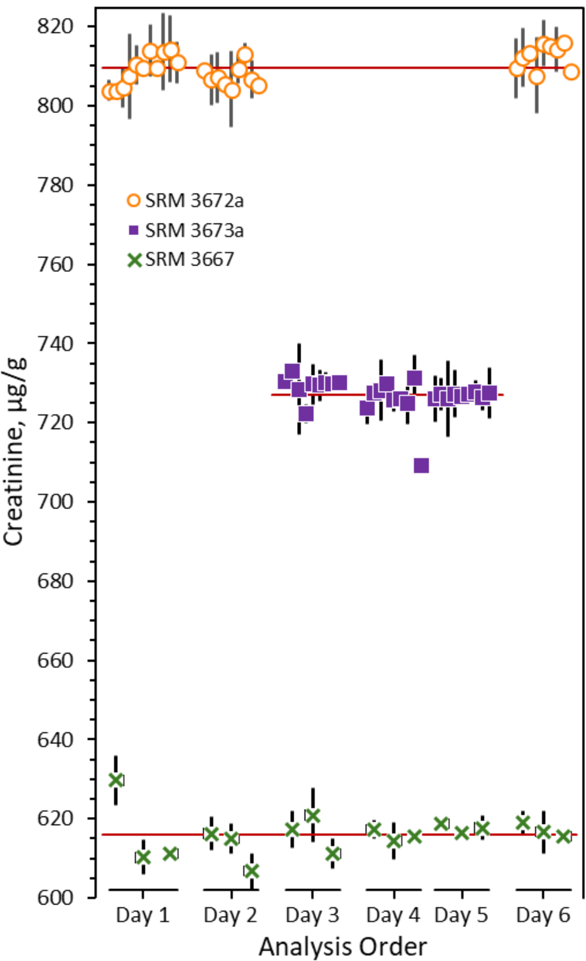


Fig. 44. Creatinine Results as a Function of Analysis Order.

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.

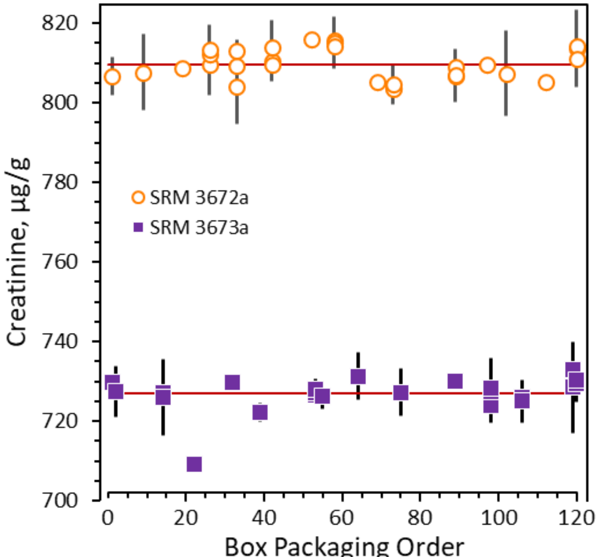


Fig. 45. Creatinine Results as a Function of Packaging Order.

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].

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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.