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Determination of Caffeine, Theobromine, and Theophylline in Standard Reference Material 2384, Baking Chocolate, Using Reversed-Phase Liquid Chromatography

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A rapid and selective isocratic reversed-phase liquid chromatographic method has been developed at the National Institute of Standards and Technology to simultaneously measure caffeine, theobromine, and theophylline in a food-matrix standard reference material (SRM) 2384, Baking Chocolate. The method uses isocratic elution with a mobile phase composition (volume fractions) of 10% acetronitrile/90% water (pH adjusted to 2.5 using acetic acid) at a flow rate of 1.5 mL/min with ultraviolet absorbance detection (274 nm). Total elution time for these analytes is less than 15 min. Concentration levels of caffeine, theobromine, and theophylline were measured in single 1-g samples taken from each of eight bars of chocolate over an eight-day period. Samples were defatted with hexane, and β-hydroxyethyltheophylline was added as the internal standard. The repeatability for the caffeine, theobromine, and theophylline measurements was 5.1, 2.3, and 1.9%, respectively. The limit of quantitation for all analytes was <100 ng/mL. The measurements from this method were used in the value-assignment of caffeine, theobromine, and theophylline in SRM 2384.

KEYWORDS: Standard reference material; caffeine; theobromine; theophylline; chocolate; liquid chromatography; reversed-phase

INTRODUCTION

Reliable analytical methods to support measurements made for compliance with nutritional and dietary supplement labeling laws are needed by laboratories in the food testing and nutrition communities. The Nutrition Labeling and Education Act (NLEA) of 1990 requires food processors to provide specific nutrition information on packages of products distributed in the United States (*1*). As a result, it has become crucial that methods and well-characterized reference materials be developed to help improve the accuracy of nutrition labeling information to assist consumers in maintaining a healthy diet and to provide traceability for food exports needed for acceptance in many foreign markets.

At needs assessment workshops held in 1997 and 1999, participants from the food industry and regulatory agencies were asked to identify priority reference materials needed to help facilitate compliance with the nutritional labeling laws. As an outcome of the workshops, the National Institute of Standards and Technology (NIST) began an effort to characterize food-based materials with values assigned for nutrients that could be used by food and nutrition measurement laboratories for analytical method assessment and quality assurance.

Since 1997, NIST has developed several food-matrix SRMs characterized for nutrient concentrations (*2*). One of the reference materials that was recently developed at NIST to address labeling accuracy is standard reference material (SRM) 2384, Baking Chocolate (*3*). This SRM is intended for use as a primary control for assigning values to in-house control materials and validation of analytical methods for the measurement of selected nutrients (fatty acids, proximates, vitamins, elements, and total dietary fiber, for which labeling is required by the NLEA, as well as for catechins, caffeine, theobromine, and theophylline). As part of the characterization process for SRM 2384, a rapid and selective isocratic reversed-phase liquid chromatographic method with ultraviolet absorbance detection was developed to simultaneously measure the concentration levels of caffeine, theobromine, and theophylline in this material. SRM 2384 is the first NIST reference material that provides concentration values for these three analytes.

Caffeine and its metabolites, known as xanthine compounds, (see **Figure 1**) are widely found in the human diet. These compounds naturally occur in food products such as tea, coffee, and cocoa beans, with theobromine and caffeine being the two most abundant xanthines in chocolate. In recent years, xanthine derivatives have received increased attention in the food and nutrition industry because they can cause various physiological effects. Caffeine is used as a central nervous system, cardiac,

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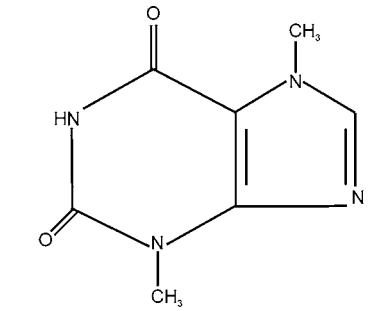
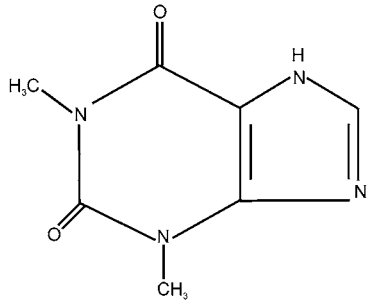


Theobromine (3,7-dimethylxanthine)



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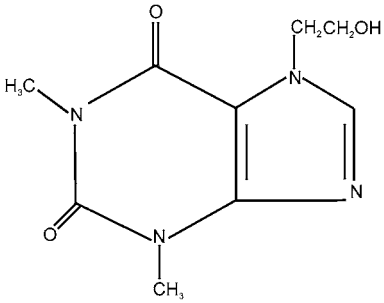
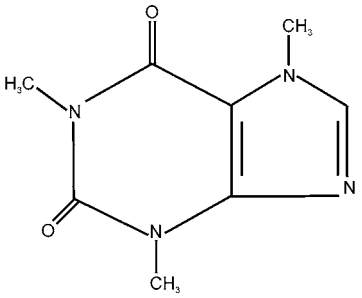


β-Hydroxyethyltheophylline (1,3-dimethyl-7[2-hydroxyethyl]-xanthine)



Caffeine (1,3,7-trimethylxanthine)

Figure 1. Chemical structures of caffeine, theobromine, theophylline, and β-hydroxyethyltheophylline.

and respiratory stimulant. Theophylline and theobromine are widely used as smooth muscle relaxants. All three of these compounds can cause diuresis (*4*). The lack of reference procedures and well-characterized food-based products makes it difficult to accurately evaluate the dietary intake and the resulting biological effects of these compounds on humans.

A limited number of analytical methods have been reported for the determination of xanthines in various food products (*5*−*12*). Some of these methods require tedious sample treatment and are not suitable for the simultaneous separation of caffeine from its potential interfering metabolites. Others may not provide accurate (adequately precise and unbiased) results for the food matrixes tested. Only a few of these methods have been applied to the analysis of food products for the determination of all three compounds in the same sample. Abuirjeie et al. used two techniques: derivative UV-spectrophotometry and liquid chromatography (LC) to simultaneously determine xanthines in selected food products (*7*). Precision of these methods was shown to be good for the selective determination of xanthine compounds over the concentration range of 5 μg/mL to 40 μg/mL. Theophylline was not determined in the cocoa- and chocolate-based food products using either method. Meyer et al. simultaneously determined caffeine, theophylline, and theobromine in different food products by LC with amperometric detection (*8*). The method provides two- to 5-fold higher detection sensitivity in comparison with UV absorbance detection. For the determination of the xanthines using this method, Soxhlet and solid-phase extraction techniques were employed to eliminate the matrix in the cocoa-based samples. Only caffeine and theobromine concentration values were provided for the cocoa-based samples analyzed. Chen et al. used ion chromatography to determine the three xanthines in foods and pharmaceutical preparations (*9*, *10*). Detection limits for all three analytes were below the sub-μg/mL level. Spiked recoveries for all three analytes (ranging from 2 mg/g to 20 mg/g) in the cocoa-based samples using anion-exchange chromatography were greater than 90%. Comparable results were provided for caffeine and theobromine using both cation- and anion-exchange techniques; theophylline was not detected in the food products analyzed by either method.

In this paper, the isocratic LC method that was developed at NIST for the value assignment of caffeine, theobromine, and theophylline in SRM 2384 Baking Chocolate is described. This method allows reliable quantitative measurements of all three xanthines in a single sample and provides a limit of quantitation for all analytes at levels $<$100 ng/mL. The measurements used to value assign these selected nutrients in the SRM are also discussed.

MATERIALS AND METHODS

Caffeine (CAS 58-08-2), theobromine (CAS 83-67-0), and theophylline (CAS 58-55-9) were obtained from Sigma (St. Louis, MO) for the study at NIST. HPLC-grade water (Baker Omnisolv, J. T. Baker, Phillipsburg, NJ) was used to prepare the calibration standards for these analytes. β-Hydroxyethyltheophylline (CAS 519-37-9; Sigma; St. Louis, MO) was used as the internal standard for this work. The concentration of each of the analytes in the calibration solutions was determined by gravimetry. Concentration ranges for the calibration solutions for caffeine were approximately 250−1000 μg/mL; approximately 100−500 μg/mL for theobromine; and approximately 50−150 μg/mL for theophylline. The internal standard concentration level was approximately 1000 μg/mL. Each solution was injected prior to sample analysis to determine retention times and detector response factors. The purities of caffeine (99.9%), theobromine (99.4%), and theophylline (99.7%) were assessed using the LC−UV conditions described below. Differential scanning calorimetry (DSC; Perkin-Elmer DSC 7, Perkin-Elmer Corporation, Norwalk, CT) was used to support these assessments.

NIST SRM 2384 Baking Chocolate. The material for SRM 2384 was obtained from a chocolate manufacturer in 1999. Thirty-five hundred 91-g (3.2-oz) bars of baking chocolate (100% cocoa beans) produced in generic molds as part of a single lot were removed from inventory. The labeled wrappers were removed from these bars. The bars of chocolate, which remained wrapped in paper-lined gold foil, were placed in zip-lock polyethylene bags in lots of five, constituting one SRM sales unit.

These bags of chocolate bars were then placed in unmarked white cardboard boxes and shipped to NIST under refrigeration. No run-order information was available for this material. The chocolate was refrigerated upon receipt. Additional details regarding the development and preparation of SRM 2384 can be found in refs 3 and 13.

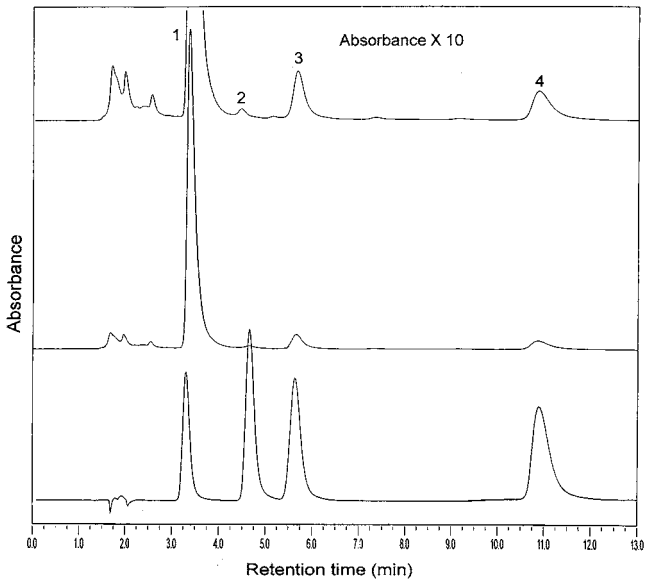


Figure 2. Lower: Chromatogram of the separation of a standard mixture of (1) theobromine, (2) theophylline, (3) internal standard β-hydroxyethyltheophylline, and (4) caffeine in water using the LC conditions described in the text. **Middle and Upper:** Chromatogram of the analysis of SRM 2384 Baking Chocolate using the LC method described in the text.

Preparation of SRM 238, Baking Chocolate for LC Analysis. Eight different bars of SRM 2384 were randomly selected and prepared using a modified AOAC Official Method 980.14 (*14*). The AOAC method was modified by using hexane (four extractions; see discussion below) instead of petroleum ether (two extractions) to defat the samples, using N_2 to remove the hexane instead of placing samples in a warm water bath to remove the solvent, and eliminating the need to quantitatively weigh the dried sample prior to transferring it into a sample vial for LC analysis. Approximately 1 g of chocolate was weighed in a 50-mL centrifuge tube and melted in a water bath at 50 °C for 15 min. A 1-mL aliquot of the internal standard solution (approximately 1000 μg/mL β-hydroxyethyltheophylline in water) was immediately added to the melted chocolate and agitated in an ultrasonicating bath (approximately 5 min) until there was a homogeneous mixture. The internal standard selected for these analyses was a sufficiently close homologue of the analyte(s) of interest, had similar extractability from the matrix, and was readily separable from the measurands by the LC method developed (see **Figure 2**). The purity (99.9%) of β-hydroxyethyltheophylline was assessed using the described LC method.

A 40-mL aliquot of hexane was added to the mixture to remove the lipids. The mixture was agitated (using an ultrasonicating bath) for 20 min and subsequently centrifuged for 10 min. The hexane layer, which contained the lipids, was then removed. This extraction procedure was repeated. On the basis of another study at NIST (*15*) in which values were assigned for fat and selected fatty acids in the Baking Chocolate SRM, it was determined that this material consisted of a mass fraction of over 50% of extractable fat. To ensure that the lipids were sufficiently extracted from the Baking Chocolate SRM and that there were no chromatographic interferences from residual fat in the sample, a total of four hexane extractions was used. After the fourth extraction, the defatted chocolate was dried using a stream of N_2.

The dried chocolate sample was reconstituted with 30 mL HPLC water and agitated in an ultrasonicating bath for approximately 15 min to ensure dissolution of the sample prior to filtering. The sample was centrifuged and the supernatant was filtered sequentially using 0.45 μm and 0.20 μm pore nylon filters. A 200-μL aliquot of the filtered sample was transferred into a sample vial for LC analysis.

LC Apparatus and Conditions. The chromatograph used in this work consisted of a ternary-pump LC solvent delivery system, a variable wavelength UV−vis absorbance detector, an autosampler, and a controller/integrator. A 5-μm analytical column (80 Å, Zorbax R_x-C_{18}; 4.6-mm × 25-cm; MAC-MOD Analytical, Inc., Chadds Ford, PA) consisting of an octadecyl stationary phase that has been chemically bonded to an inactive silica support was used. An isocratic mixture (volume fractions) of 10% acetronitrile/90% water (pH adjusted to 2.5 using 5 mL of acetic acid per liter of water to minimize peak tailing) at 1.5 mL/min with ultraviolet absorbance detection (274 nm) was used for the separation of the analytes. The sample injection volume was 15 μL. All separations were conducted at room temperature (~25 °C).

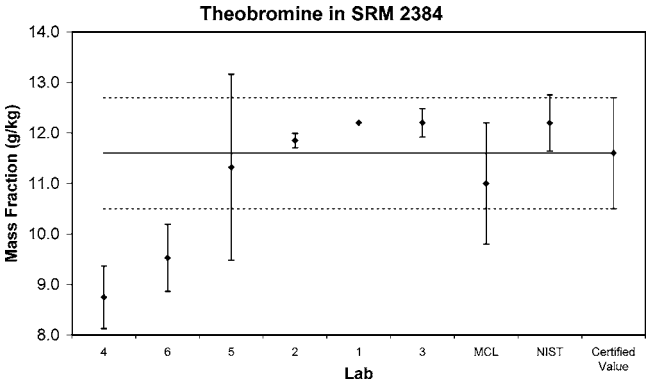


Figure 3. Comparison of the assigned value and its expanded uncertainty and the means and standard deviations for individual laboratory data for theobromine. Error bars span ± two repeatability standard deviations about the participants' means. Dashed lines represent an approximate 95% confidence level for the analytes. MCL denotes the mean of collaborating laboratories.

RESULTS AND DISCUSSION

LC Analysis of SRM 2384 Baking Chocolate. Caffeine, theobromine, and theophylline in SRM 2384 Baking Chocolate were measured simultaneously, using the reversed-phase LC method described above. The separation of a standard mixture of caffeine, theobromine, and theophylline using the method described is shown in **Figure 2**. Total elution time for these analytes is less than 15 min. The limit of quantitation for caffeine and theophylline is 50 ng/mL and 30 ng/mL for theobromine.

Figure 3 shows the simultaneous LC separation of the analytes in the SRM 2384 Baking Chocolate. All analytes are well separated from matrix interferences. **Table 1** shows the summary of the mean results from a single LC measurement of each of eight samples of SRM 2384 that were prepared and analyzed over an 8-day period using the described protocol. The uncertainty represents the standard deviation of the mean of the eight LC measurements. No significant inter-sample variability was observed; the material was found to be homogeneous. The measurement repeatability for caffeine, theobromine, and theophylline in the samples using this method was 5.1, 2.3, and 1.9%, respectively. A summary of the mean results from six collaborating laboratories (see discussion below) that helped to value assign these analytes in the baking chocolate SRM is also provided in **Table 1**.

Value Assignment of Caffeine, Theobromine, and Theophylline in SRM 2384 Baking Chocolate. Over the past several years, NIST has worked with other government agencies and the food industry to provide an increased array of SRMs with values assigned for selected nutrients. At NIST, there are three categories of assigned values—certified, reference, and information values—and seven modes for value assigning SRMs for chemical composition (*16*). Concentration values for caffeine, theobromine, and theophylline in SRM 2384 were assigned based on two of the seven modes of assignment, which involve the use of a combination of data from NIST and from interlaboratory comparison exercises. The intercomparisons have

Table 1. Summary of Data Used for Value Assignment of Theobromine, Theophylline, and Caffeine (Mass Fraction in g/kg) in SRM 2384 Baking Chocolate[a]

	theobromine			caffeine			theophylline		
laboratory	mean	SD	n	mean	SD	n	mean	SD	n
1	12.2		1	1.14		1			
2	11.85	0.07	2	1.16	0.02	2			
3	12.2	0.14	2	1		2			
4	8.75	0.31	4	1.08	0.06	4			
5	11.32	0.92	2	0.93	0.07	2			
6	9.53	0.33	8	1.11	0.04	8			
NIST	12.20	0.28	8	1.05	0.05	8	0.151	0.003	8
collaborating laboratories' mean	11.0			1.07					
standard error	0.6			0.04					
degrees of freedom	5			5					
NIST mean	12.2			1.05			0.151		
standard error	0.1			0.02			0.001		
degrees of freedom	7			7			7		
final assigned value[b]	11.6 ± 1.1			1.06 ± 0.05			0.151 ± 0.003		

[a] These data are also plotted in **Figure 4** for graphical comparison. [b] Assigned as an NIST certified value (*3*, *16*).

Table 2. Assigned Concentration Values for Theobromine, Theophylline, and Caffeine in SRM 2384 Baking Chocolate

analyte	category of SRM assignment[a]	mass fraction (g/kg)[b]
caffeine	certified	1.06 ± 0.05
theobromine	certified	11.6 ± 1.1
theophylline	reference	0.151 ± 0.003

[a] The NIST process of assigning values to its SRMs for chemical measurements identifies three categories of assigned values–certified, reference, and information values (*3*,*16*). See section on Determination and value assignment of caffeine, theobromine, and theophylline in SRM 2384 Baking Chocolate. [b] The uncertainty in the assigned values is expressed as an expanded uncertainty at the 95% level of confidence (*17*, *18*).

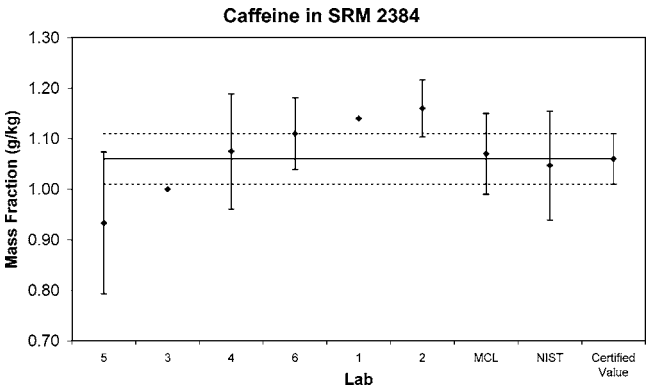


Figure 4. Comparison of the assigned value and its expanded uncertainty and the means and standard deviations for individual laboratory data for caffeine. Error bars span ± two repeatability standard deviations about the participants' means. Dashed lines represent an approximate 95% confidence level for the analytes. MCL denotes the mean of collaborating laboratories.

involved several laboratories of the National Food Processors Association's (NFPA's) Food Industry Analytical Chemists Subcommittee (FIACS) and additional collaborating laboratories. SRM 2384 was analyzed by six laboratories: Covance Laboratories (Madison, WI), General Mills, Inc. (Minneapolis, MN), Kraft Foods (Glenview, IL), M & M/Mars, Inc. (Hackettstown, NJ), Nestlé (Dublin, OH), and Ralston Purina Co. (St. Louis, MO). These laboratories participated in an interlaboratory comparison exercise where four bars of the SRM were distributed to each laboratory for analysis. Each laboratory used their usual analytical methods (sample extraction; LC with absorbance detection).

The LC method developed for this work was used to generate, in conjunction with the values provided by the collaborating laboratories, the certified concentration values (**Table 2**) for caffeine and theobromine in the Baking Chocolate SRM. An NIST-certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias in the measurements have been fully accounted for and investigated (*16*). Each certified value, expressed as a mass fraction, is the weighted mean of results provided by the laboratories mentioned above and NIST. A reference value is also provided for theophylline (**Table 1**) in the SRM. This value is derived from the mean of results obtained by NIST using the LC method described. An NIST reference value is the best estimate of the true value provided by NIST, where all known or suspected sources of bias have not been fully investigated (*16*). Reference concentration values are not certified, because they are derived from a limited number of analyses (from a single method, as in this case), the disagreement among methods is greater than expected, or the identity of components present in the measured chromatographic peak is less certain.

The uncertainty in the assigned values is expressed as an expanded uncertainty *U*, and is calculated according to the method described in the ISO *Guide to the Expression of Uncertainty in Measurement* (*17*,*18*). The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory and within-laboratory components of uncertainty. The coverage factor, *k*, is determined from the Student's *t*-distribution corresponding to the appropriate associated degrees of freedom and an approximate 95% confidence for each analyte. The relative expanded uncertainties are 5% for caffeine, 2% for theophylline, and 10% for theobromine in the SRM. Graphical data of the results in **Table 1** for caffeine and theobromine from the LC method in this report and from the six collaborating laboratories along with the assigned values are plotted in **Figure 4**. As shown from these data, the among-method variability for the theobromine measurements is greater than the among-method variability for the caffeine measurements. This observed variability may be due to the insolubility of theobromine in the samples. Well-characterized reference materials and reliable analytical methods are critical to the identification of such sources of bias.

The AOAC official method 980.14 for the determination of theobromine and caffeine in cacao products recommends that samples contain ≤0.5 g of dry, fat-free cacao solids/100 mL water to ensure the solubility of theobromine in the sample. The solubility of theobromine in water is reported to be about 0.5 g/L (*4*), for example, 1 g of theobromine dissolves in about 2000 mL of water at ambient temperature and in 150 mL of boiling water. A 1-g sample of caffeine dissolves in 46 mL of water at ambient temperature, in 5.5 mL of water at 80 °C, and in 1.5 mL of boiling water. A 1-g sample of theophylline dissolves in 120 mL of water at ambient temperature (*4*). For this study, the defatted samples of chocolate were dissolved in 30 mL of water at approximately 50 °C to ensure the solubility of the analytes being measured. Concentration data for theophylline were only provided by NIST using the LC method described. The within-method measurement repeatability for theophylline was good (1.9%).

The method described in this paper has been successfully implemented for value assignment and characterization of NIST SRM 2384 Baking Chocolate, which provides a precise and unbiased means for supporting traceability for the measurement of caffeine, theobromine, and theophylline. This method has also been used, with slight modification (less acetonitrile), for the measurement of caffeine in dietary supplement SRMs currently being developed at NIST.

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