Simultaneous Determination of Theobromine, (+)-Catechin, Caffeine, and (–)-Epicatechin in Standard Reference Material Baking Chocolate 2384, Cocoa, Cocoa Beans, and Cocoa Butter

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Abstract

sA reverse-phase liquid chromatography analysis is used to access the quantity of theobromine, (+)-catechin, caffeine, and (–)-epicatechin in Standard Reference Material 2384 Baking Chocolate, cocoa, cocoa beans, and cocoa butter using water or a portion of the mobile phase as the extract. The procedure requires minimal sample preparation. Theobromine, (+)-catechin, caffeine, and (–)-epicatechin are detected by UV absorption at 273 nm after separation using a 0.3% acetic acid–methanol gradient (volume fractions) and quantified using external standards. The limit of detection for theobromine, (+)-catechin, caffeine, and (–)-epicatechin averages 0.08, 0.06, 0.06, and 0.06 µg/mL, respectively. The method when applied to Standard Reference Material 2384 Baking Chocolate; baking chocolate reference material yields results that compare to two different, separate procedures. Theobromine ranges from 26000 mg/kg in cocoa to 140 mg/kg in cocoa butter; (+)-catechin from 1800 mg/kg in cocoa to below detection limits of < 32 mg/kg in cocoa butter; caffeine from 2400 mg/kg in cocoa to 400 mg/kg in cocoa butter, and (–)-epicatechin from 3200 mg/kg in cocoa to BDL, < 27 mg/kg, in cocoa butter. The mean recoveries from cocoa are 102.4 ± 0.6% for theobromine, 100.0 ± 0.6 for (+)-catechin, 96.2 ± 2.1 for caffeine, and 106.2 ± 1.7 for (–)-epicatechin.

Introduction

Reverse-phase high-performance liquid chromatography (RP-HPLC) has been used for the determination of theobromine and caffeine in baking chocolate (1,2), cocoa (1,3-8), cocoa beans (8-11), and cocoa butter (12). Other separate methods are used for analyzing (+)-catechin and (–)-epicatechin in baking chocolate, cocoa, and cocoa beans (13–16). Most procedures do not allow the separation and quantitation of theobromine, (+)-catechin, caffeine, and (–)-epicatechin present in the same sample.

Preparation of these samples for the elimination of the sample matrix includes extraction in methanol (16), defatting with petroleum ether or hexane prior to further sample treatment (1–11,13,14) and, in some cases, solid-phase extraction (7,8,11,14–16). Although amperometric detection has been used (7), most procedures employ UV detection for the determination of theobromine and caffeine (2–6,8–11) and (+)-catechin and (–)-epicatechin (13–16) in cocoa, cocoa beans, and cocoa butter. UV (1,2) as well as mass selective detection (13) has been used to monitor the extract of baking chocolate.

Two procedures were found which extract cocoa using only water with no further sample preparation (5,17). One procedure quantifies only theobromine and caffeine of the water extract of cocoa (5), while the other, although capable of determining theobromine, (+)-catechin, caffeine, and (–)-epicatechin in a concentrated water extract of cocoa before and after treatment with polyvinylpyrrolidone, lacks validation (17). No validated procedure exists for the simultaneous determination of theobromine, (+)-catechin, caffeine, and (–)-epicatechin in baking chocolate, cocoa, cocoa beans, and cocoa butter. Because all of these compounds have some solubility in water, it was decided to use water or the aqueous portion of the mobile phase as the extractant in order to minimize the use of organic solvents. The use of more costly and unsafe solvents such as petroleum ether or hexane, which can cause loss of theobromine and caffeine, for fat removal (3) was eliminated. Sample preparation consists of simple dilution of the sample in water or mobile phase, warming the extract to melt the sample in the case of solid samples and filtering the extract prior to analyses; no tedious sample pretreatments are used. No solid-phase extraction is used for sample preparation as is done in other procedures where loss of the analyte can occur (7,8,14,15). UV detection, which is more commonplace in many laboratories, is employed for the analyses of these four compounds. The method described here has the capability of quantitatively determining theobromine, (+)-catechin, caffeine, and (–)-epicatechin from the water or mobile phase extract of Standard Reference Material 2384 Baking Chocolate, cocoa, cocoa beans, and cocoa butter.

Experimental

Samples

Standard Reference Material 2384 Baking Chocolate was purchased from the National Institute of Standards & Technology (NIST) (Gaithersburg, MD) and stored in a freezer at –12°C until

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assayed. Cocoa powder (10–12% cocoa butter fat), fermented cocoa beans, and cocoa butter were obtained from Blommer Chocolate Company (Chicago, IL) and stored under ambient conditions in clear glass jars. Hershey's cocoa was purchased from a local supermarket in the United States in January 2006 and left in its original container under ambient conditions.

Reagents

Methanol (MeOH) was liquid chromatography grade (Burdick & Jackson, Muskegon, MI) and was used in the HPLC mobile phase. Water was first subjected to reverse osmosis then passed through a Nanopure system (Barnstead/Thermolyne, Dubuque, IA), which consisted of an organic removal cartridge, two anion/cation mixed bed cartridges, a carbon and mixed bed cartridge and a 0.20-µm filter and was used as an extracting solution. Glacial acetic acid (HAc) (Fisher Scientific Company L.L.C., Suwanee, GA) was used to prepare the 0.3% HAc solution (volume fraction) by diluting 3 mL in 1 L of water and was used as a portion of the mobile phase. A second extract was prepared by mixing 0.3% HAc with MeOH (85:15, volume fractions).

Theobromine, theophylline, (+)-catechin, caffeine, and (–)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. The stock standard concentrations were chosen so as not to exceed their solubility in water. The stock standard of theobromine was prepared at 300 µg/mL; theophylline 160 µg/mL, (+)-catechin 130 µg/mL; caffeine 200 µg/mL, and (–)-epicatechin 130 µg/mL in water or 85:15, 0.3% HAc–MeOH (volume fractions). The ranges of the working standards were 0.5–72 µg/mL for theobromine, 0.5–4 µg/mL for (+)-catechin, 0.8–6 µg/mL for caffeine, and 0.5–16 µg/mL for (–)-epicatechin. The standards were analyzed before and after the sample extracts. The stock solutions and working standards were stored a 5°C when not in use.

Table I. HPLC Parameters for the Analysis of Standard Reference Material 2384 Baking Chocolate, Cocoa, Cocoa Beans, and Cocoa Butter

Parameter		Conditions	
Mobile phase	Time (min)	0.3% HAc	%MeOH
Gradient (volume fractions)	Initial	85	15
	10.0	85	15
	11.0	75	25
	18.0	75	25
	18.1	70	30
	25.0	70	30
	25.1	0	100
	30.0	0	100
	30.1	100	0
	35.0	100	0
Flow rate	0.50 mL/min		
Injection volume	5 µL		
Column	Phenomenex Gemini C18, 5 µm, 150 × 2.0 mm i.d. with Gemini C18, 2 mm i.d. guard cartridge		
Column temperature	Ambient		
Autosampler temperature	10°C		
Detector	UV at 273 nm		
Runtime	35 min		

Sample preparation

Standard Reference Material 2384 Baking Chocolate (0.020 ± 0.001 g), cocoa (0.004 ± 0.001 g), cocoa beans (0.020 ± 0.001 g) ground in a coffee mill to about 40 mesh (0.420 mm), and cocoa butter (0.020 ± 0.001 g) were weighed into 16 × 100 mm test tubes and extracted using 5.0 mL of water or 85:15, 0.3% HAc–MeOH (volume fractions) by vortexing 15 min using a VWR VX-2500 Multi-Tube Vortexer (Henery Troemner LLC, Thorofare, NJ). The extract was heated to 60 ± 2°C for 10 min in a water bath to melt cocoa butter and baking chocolate samples prior to extraction. After extraction, the extract was filtered using a 0.45-µm pore size polyvinylidene fluoride Whatman Autovial (Whatman, Clifton, NJ) into a glass autosampler vial and sealed with a screw cap containing a septum.

HPLC

The HPLC system consisted of a 680 gradient controller, two 515 pumps, a 717 plus autosampler, and a 2487 absorbance detector (Waters, Milford, MA) (Table I). The absorbance detector wavelength was set at 273 nm using different sensitivity setting (absorbance units full scale, AUFS) throughout and within a run to avoid saturation of the detector or non-detection of the analyte.

An equilibration delay was not used prior to the next injection. The mobile phase was degassed by sparging with helium. Data were acquired and results calculated in mg/kg using EZChrom Elite version 3.0.0 (Scientific Software, Pleasanton, CA).

Table II. Evaluation of Other Extractants for Cocoa (mg/kg, n = 6, mean ± σ)*

Extractant†	Theobromine	(+)-Catechin	Caffeine	(–)-Epicatechin
MeOH–1% HAc‡	19512 ± 546	681 ± 44	1841 ± 55	2037 ± 69
1% NH_4OH	19650 ± 357	< 118§	1880 ± 44	< 86§
1% HAc	19478 ± 959	693 ± 36	1904 ± 95	2055 ± 117

* σ = standard deviation.
† Volume fractions.
‡ MeOH–1% HAc ratio = 80:20
§ Based on 0.004 g sample in 5 mL, 0.02 AUFS, S/N ≥ 2.

Method validation procedures

Quantitation was determined by injecting 5 µL of each sample extract into the HPLC and the height of the peak observed at the retention time corresponding to each authentic standard was recorded. This value was used to calculate the amount in mg/kg of the analyte in the samples using the linear equation obtained from the standard curve, the extract volume and sample weight. Precision was obtained by analyzing each sample at least three times. Recoveries and y-intercept values from standard addition experiments were

determined in duplicate by spiking pure standards at one-half, one, and two times the amounts originally present in the samples to the extract solutions prior to sample analysis. For recovery, the mass found by analysis was compared to the known mass added to the extract and expressed as a percentage. The y-intercept values indicate the concentration of the sample when no additional standard solution was added to the sample extract. All statistical and mathematical calculations were done using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA).

Results and Discussion

Mobile phases and extractants

Most mobile phases used for the analysis of theobromine, (+)-catechin, caffeine, and (–)-epicatechin in baking chocolate in cocoa and cocoa beans use predominately water with MeOH (8–31% volume fraction MeOH) and a low level of HAc (1–5% volume fraction) or phosphate buffer at a pH of 3.5 under isocratic conditions (1,3–7,10,11,14,15). Acetonitrile and water (20% volume fraction acetonitrile) have been used in an isocratic mode for cocoa and cocoa beans (8) as well as a 10% volume fraction acetonitrile in water, pH adjusted to 2.5 with HAc, for baking chocolate (2). Gradients consisting of 2.5% volume fraction HAc and 30% volume fraction acetonitrile in water (17), 20mM phosphate buffer at pH 6.9 and acetonitrile (9) and water with 0.05% trifluoroacetic acid and acetonitrile with 0.05% trifluoroacetic acid (volume fractions) have been used (13). No gradient of 0.3% HAc (volume fraction) and MeOH as described in Table I has been used for the determination of theobromine, (+)-catechin, caffeine, and (–)-epicatechin in Standard Reference Material 2384 Baking Chocolate, cocoa, cocoa beans, and cocoa butter.

Initially, water was used as the mobile phase on reversed-phase octadecylsilane (C18) columns using column temperatures as high as 55°C. Caffeine was strongly retained (> 60 min) on the columns using water as the mobile phase, but the main drawback of the C18 columns using water as the mobile phase was phase collapse resulting in poor or no resolution after only about 10 injections (18). Ethanol was not evaluated as a mobile phase component because other workers experienced difficulties with reproducibility only after 10 injections of the MeOH extract of cocoa, which had been fractionated on a Sephadex LH-20 column using an isocratic mobile phase of water–ethanol–HAc (87:8:5 volume fractions) (16).

The gradient described in Table I was chosen because five compounds found in cocoa of can be separated in a single run. Figure 1 is a chromatogram of a composite standard containing the five analytes evaluated in this work. Theophylline (Theop) was not detected in any of the samples analyzed as determined by comparison of retention times with authentic standards and spiking of the sample extract with solutions of authentic standards.

The 100% MeOH column wash (Table I) would be optional depending on the cleanliness of the sample extract, but may be included to prevent phase collapse. The last segment of 100% of 0.3% HAc may also be an option, but helped better resolve earlier

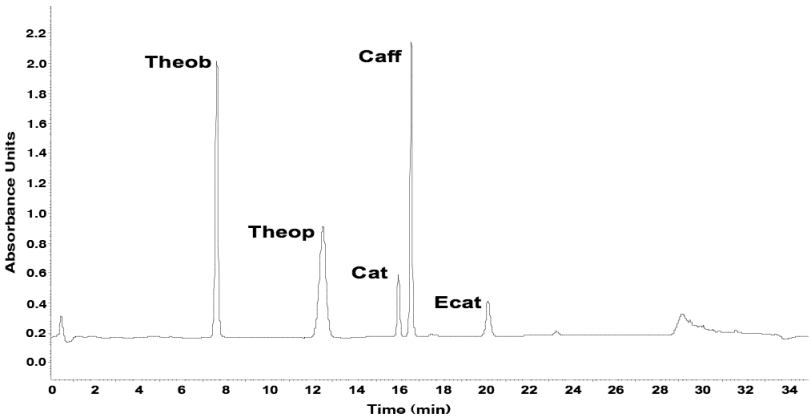

Figure 1. Chromatogram theobromine (Theob), theophylline (Theop), catechin (Cat), caffeine (Caff), and epicatechin (Ecat) standards in 85:15, 0.3% HAc–MeOH (volume fractions). Conditions: Gemini C18 column (2.0 × 150 mm, 5 µm particle size); mobile phase gradient of 0.3% acetic acid and MeOH at 0.50 mL/min; injection volume of 5 µL; autosampler temperature of 10°C; absorbance detection at 273 nm and 0.04 absorbance units full scale (AUFS); concentration of all compounds is 10.0 µg/mL.

Table III. Instrument Precision (n = 12), Linearity of Response, Limit of Detection, and Limit of Quantitation*

Compound	% Relative standard deviation†	Concentration range (µg/mL)‡	r^2§	LOD (µg/mL)**	LOQ (µg/mL)††
Water					
Theobromine	2.74	1.00–15.84	0.9986	0.08	0.26
Theophylline	2.47	1.00–16.32	0.9996	0.07	0.24
(+)-Catechin	4.01	0.50–10.10	0.9976	0.06	0.19
Caffeine	2.61	1.00–15.50	0.9995	0.06	0.19
(–)-Epicatechin	3.89	0.50–11.70	0.9976	0.06	0.19
Initial mobile phase‡‡					
Theobromine	3.38	1.00–17.60	0.9996	0.11	0.37
Theophylline	1.91	1.00–16.96	0.9990	0.06	0.21
(+)-Catechin	1.80	0.50–10.16	0.9998	0.03	0.09
Caffeine	3.86	1.00–15.68	0.9999	0.12	0.40
(–)-Epicatechin	2.60	0.50–13.68	0.9999	0.04	0.13

* Absorbance units full scale (AUFS) = 0.100 for all compounds except (+)-catechin and (–)-epicatechin where AUFS = 0.008.
† % Relative standard deviation = height of lowest standard standard deviation/height of lowest standard average × 100.
‡ Five levels.
§ r^2 = Linear correlation coefficient.
** LOD = limit of detection, three times the standard deviation of the lowest standard analyzed as a sample.
†† LOQ = limit of quantitation, ten times the standard deviation of the lowest standard analyzed as a sample.
‡‡ Initial mobile phase is 85:15, 0.3% HAc–MeOH (volume fractions).

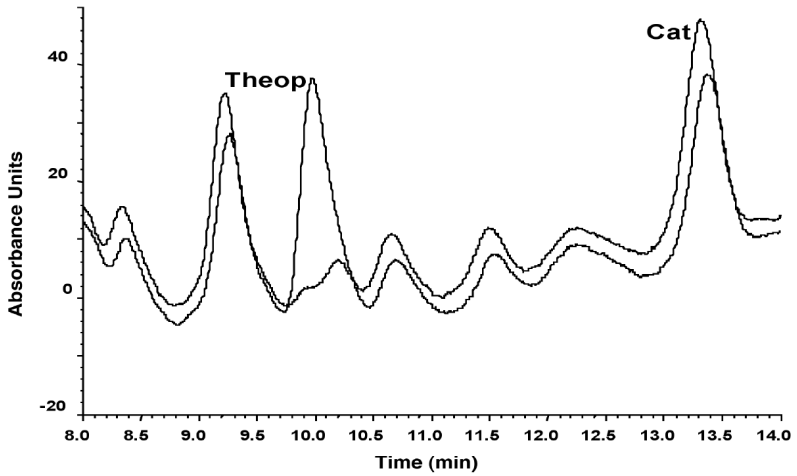

Figure 2. Overlaid chromatograms of the extract of Standard Reference Material 2384 Baking Chocolate (dark trace, extract contains 50 mg/kg theophylline). Conditions: Gemini C18 column (2.0 × 150 mm, 5-µm particle size); mobile phase gradient of 0.3% acetic acid and MeOH at 0.50 mL/min; injection volume of 5 µL; autosampler temperature of 10°C; absorbance detection at 273 nm and 0.02 absorbance units full scale (AUFS); 1 g sample extracted in 25 mL 85:15, 0.3% HAc–MeOH (volume fractions); theophylline (Theop), (+)-catechin (Cat).

Table IV. Overall Precision, Extract Stability, Recovery, and Standard Addition from Cocoa

Compound	Initially (n = 6) Mean	%RSD†	r^2‡	After Two Days (n = 18) Mean	%RSD	r^2	Standard concentration range (µg/mL)*
Overall precision (mg/kg, water)							
Theobromine	19217	2.91	0.9998	18817	3.21	0.9998	6.7-46.8
(+)-Catechin	785	2.77	0.9615	1321	37.39	0.6764	0.5–1.8
Caffeine	1830	2.90	0.9998	1757	3.93	0.9996	0.6-4.5
(–)-Epicatechin	2096	3.41	0.9939	3519	37.88	0.6983	1.0–5.5
Overall precision (mg/kg, initial mobile phase)§							
Theobromine	18917	3.43	0.9998	18902	3.33	0.9994	7.3–43.7
(+)-Catechin	685	5.92	0.9987	723	3.86	0.9972	0.2–1.0
Caffeine	1864	2.62	0.9996	1875	3.31	0.9990	0.7–4.5
(–)-Epicatechin	2037	3.13	0.9993	2044	3.30	0.9995	1.0–6.2

Compound	% Recovery, mean ± σ (n = 2)**	mg/kg, mean ± σ Y-Intercept (n = 2)
Recovery and standard addition (water)		
Theobromine	104.6 ± 1.3	20764 ± 387
(+)-Catechin	102.1 ± 2.0	718 ± 8
Caffeine	94.7 ± 4.1	1670 ± 18
(–)-Epicatechin	94.5 ± 1.3	1977 ± 49
Recovery and standard addition (initial mobile phase)		
Theobromine	102.4 ± 0.6	19037 ± 214
(+)-Catechin	100.0 ± 0.6	676 ± 16
Caffeine	96.2 ± 2.1	1990 ± 5
(–)-Epicatechin	106.2 ± 1.7	2006 ± 16

* Five levels.
† % RSD = % relative standard deviation = height standard deviation/height mean × 100.
‡ r^2 = standard linear correlation coefficient.
§ Initial mobile phase is 85:15, 0.3% HAc–MeOH (volume fractions).
** σ = standard deviation.

eluting compounds such as theobromine.

Prior to using water and the initial composition of the mobile phase as extractants, three other solutions were evaluated to extract cocoa. These are listed in Table II. The results using 80:20, MeOH–15HAc (volume fractions) and 1%HAc (volume fraction) are the same for all analytes (within experimental error) as those using water and 85:15, 0.3% HAc– MeOH (volume fractions) in Table IV. The extract of cocoa using 1% NH_4OH (volume fraction) gives similar results for theobromine and caffeine, but (+)-catechin and (–)-epicatechin were found to be below detection limits. It is doubtful that (+)-catechin and (–)-epicatechin are not extracted in 1% NH_4OH because water was found to extract these compounds. (+)-Catechin and (–)-epicatechin probably under go a reaction under alkaline conditions. Dutching (alkalinization) of cocoa powder is suspect to reduce (–)-epicatechin (19). Other workers have used NH_4OH in chloroform to extract theobromine, theophylline, and caffeine from cocoa and cocoa beans, but did not report values for (+)-catechin and (–)-epicatechin (10,20,21). Because (+)-catechin and (–)-epicatechin were of interest, 1% NH_4OH was not used as an extractant. The other two extractants, 80:20, MeOH–1% HAc (volume fractions) and 1% HAc (volume fraction), were not further evaluated because it was decided to thoroughly evaluate water and use less than 80% MeOH to extract the samples.

Precision, linearity, and recovery

Table III lists the instrument precision, linearity of response, limit of detection (LOD), and limit of quantitation (LOQ) of the standards used in this work in water and the initial conditions of the mobile phase gradient (initial mobile phase, 85:15, 0.3% HAc–MeOH, volume fractions). The percent relative standard deviations (%RSD) were less than 4% using the initial mobile phase. (+)-Catechin and (-)-epicatechin in water have higher % RSD values and lower correlation coefficients than those standards prepared in the initial mobile phase. This may be due to the stability of (+)-catechin and (–)-epicatechin in water. This will be discussed later.

The linear correlation coefficients (r^2) for the standards over the specified concentration ranges at five levels for both extractants are greater then 0.99. The LOQ values are below the concentration of the standards used to analyze the samples.

Table IV gives the overall precision, extract stability (both initially and after two days),

recovery, and standard addition using the two different extractants. Water and the initial mobile phase were used as the extract for cocoa and medium for the standards prepared in the same range. The same standards and sample extracts were used to determine precision over two days. The standards and sample extracts were stored in vials in the autosampler at 10°C.

Initially, the mean precision results for theobromine, (+)-catechin, caffeine, and (–)-epicatechin using water or the initial mobile phase as the extract for cocoa were equivalent, relative standard deviations (%RSD) were in the same range and standard linear correlation coefficients (r^2) were greater than 0.99 except for (+)-catechin in water whose r^2 value was around 0.96. After two days, the mean values of (+)-catechin and (–)-epicatechin increased about 68% due to the degradation (loss in height response) of (+)-catechin and (–)-epicatechin standards in water. This loss in response resulted in a high and unacceptable %RSD for the samples extracted in water. As can be seen in Table IV, the standard concentrations are no longer linear with height response in water after two days. This did not occur with theobromine and caffeine standards in water. Even though some workers have used ascorbic acid to reduce the degradation of catechins (22), no effort was made to stabilize the (+)-catechin and (–)-epicatechin standards in water. Loss of the height response for (+)-catechin and (–)-epicatechin in the water extract of cocoa after two days was approximately 5%. The less loss of response of the extract versus the loss of the standard in water is presumably due to the natural antioxidants, tocopherols, found in cocoa (23,24).

The standards and extracts in the initial mobile phase showed no deterioration after two days, the %RSD and r^2 values being similar after two days (Table IV). Standards and cocoa extracts were stable for at least one week in the autosampler at 10°C when in the presence of the initial mobile phase. For this reason the initial mobile phase is preferred over water as the medium for standards of (+)-catechin and (–)-epicatechin.

Recovery experiments were done by adding authentic standard solutions to the sample extracts in water and the initial mobile phase. Table IV shows the percent recovery of theobromine, (+)-catechin, caffeine, and (–)-epicatechin to be acceptable, ~100% using either extractant indicating accuracy and that the procedure quantitates the four compounds very well.

Standard addition yielded the y-intercept value where no analyte was added and should be equivalent to the amount determined by external standard. Overall, the amounts found by standard addition (n = 2) are in the same range as those found by external standard (n = 6), showing little interferences with the compounds analyzed in the cocoa matrix (Table IV).

Table V. Comparison of Results with the National Institute of Standards & Technology (NIST) Standard Reference Material 2384, Baking Chocolate (n = 6, mg/kg mean ± σ), Percent Recovery (n = 2, percent mean ± σ), and Standard Addition from NIST Baking Cocoa (n = 2, mg/kg mean ± σ)

	Theobromine	(+)-Catechin	Caffeine	(–)-Epicatechin	Theophylline
Results by this procedure (water extract)*					
Mean	12532	222	1117	1463	< 11†
σ‡	1166	16	40	47	
% RSD§	9.30	7.22	3.56	3.18	
Results by this procedure (initial mobile phase extract)*					
Mean	11973	195	1195	1294	< 11†
σ‡	102	3	14	19	
% RSD	0.85	1.51	1.13	1.44	
*Results by this procedure** (defatted, diluted with initial mobile phase extract)*					
Mean	11579	219	1041	1197	< 4†
σ‡	111	1	9	20	
% RSD	0.95	0.53	0.82	1.71	
Certified results of NIST					
Mean	11600	245	1060	1220	151
σ‡	1100	51	50	240	3
% RSD	9.48	20.82	4.72	19.67	1.96
Recovery and standard addition from NIST Baking Chocolate (water extract)					
% Recovery	99.6 ± 1.2	104.3 ± 0.9	102.1 ± 2.5	101.0 ± 0.4	
Standard addition	11659 ± 122	211 ± 1	1126 ± 2	1517 ± 15	
Recovery and standard addition from NIST Baking Chocolate (initial mobile phase extract)††					
% Recovery	103.7 ± 3.0	98.9 ± 0.7	100.9 ± 3.1	98.0 ± 0.8	
Standard addition	12530 ± 22	226 ± 1	1214 ± 6	1293 ± 21	

* 0.020 ± 0.001 g in 5 mL heated to 60°C.
† Based on S/N ≥ 2 at 0.002 AUFS.
‡ σ = Standard deviation.
§ % RSD = Percent relative standard deviation = standard deviation/average × 100.
** 0.200 ± 0.001 g defatted four times with 10 mL hexane, final dilution in 50 mL initial mobile phase.
†† Initial mobile phase is 85:15, 0.3% HAc–MeOH (volume fractions).

Comparison of results with NIST baking chocolate

Table V compares the results for theobromine, (+)-catechin, caffeine, (–)-epicatechin and theophylline in a baking chocolate Standard Reference Material (NIST Standard Reference Material 2384) obtained by the procedure described in the "Experimental" section with those stated by NIST (24) using two different extractants and after defatting the baking chocolate sample prior to dilution with the initial mobile phase. The results are comparable to the NIST certified results and those found by other workers for theobromine and caffeine in baking chocolate (1). No theophylline was detected in the baking chocolate reference material using the procedure described here. Even though a value for theophylline is stated in the certificate of analysis, the analysts clearly state in their manuscript that theophylline is not a certified value, but did find theophylline in the standard reference material baking chocolate (2). Theobromine, caffeine, and theophylline results obtained by NIST were determined on a 1 g sample defatted with hexane four times, dried in a stream of nitrogen and reconstituted in water. The calibration standards were also prepared in water. The extract was analyzed using

absorbance detection at 274 nm and quantified using an internal standard of β-hydroxyethyltheophylline after eluting theobromine, caffeine, and theophylline using an isocratic mobile phase of 10% acetonitrile and 90% water (volume fractions) adjusted to pH 2.5 with HAc (2). β-Hydroxyethyltheophylline as well as theophylline have been used as internal standards for the determination of theobromine and caffeine in *Theobroma* seeds and cocoa (9,25). The procedure described here uses a 0.02 g sample weight extracted in 5 mL of water or initial mobile phase and a 0.20 g sample weight diluted in 50 mL of initial mobile phase after defatting the baking chocolate four times with 10 mL of hexane. The results were calculated based on external standard and a 0.3% acetic acid–methanol gradient (volume fractions) (Table 1) was used to elute theobromine, (+)-catechin, caffeine, (–)-epicatechin. Theophylline was found to be below detection limits (< 11 mg/kg), but an amount of 151 mg/kg, in the range of fermented cocoa beans (10), should have given a response around 12 min (see Figure 1) since (+)-catechin, even though its response at the same concentration is less than theophylline (Figure 1), at 195 mg/kg was detected (Table V). Even upon defatting the sample with hexane four times, theophylline was below detection limits (< 4 mg/kg). Figure 2 shows overlaid chromatograms of spiked (dark trace, contains 50 mg/kg theophylline) and unspiked extracts of a 1 g sample of NIST baking chocolate extracted in 25 mL of the mobile phase, a 50-fold increase in sample weight to extract volume. As seen in Figure 2, the unspiked extract is below 50 mg/kg and not in the range of 151 mg/kg as stated in the certificate of analysis by NIST (24). Other workers have also reported not detecting theophylline in *Theobroma* seeds and dark and milk chocolates where theophylline was detected in some samples, but not in others (9,26).

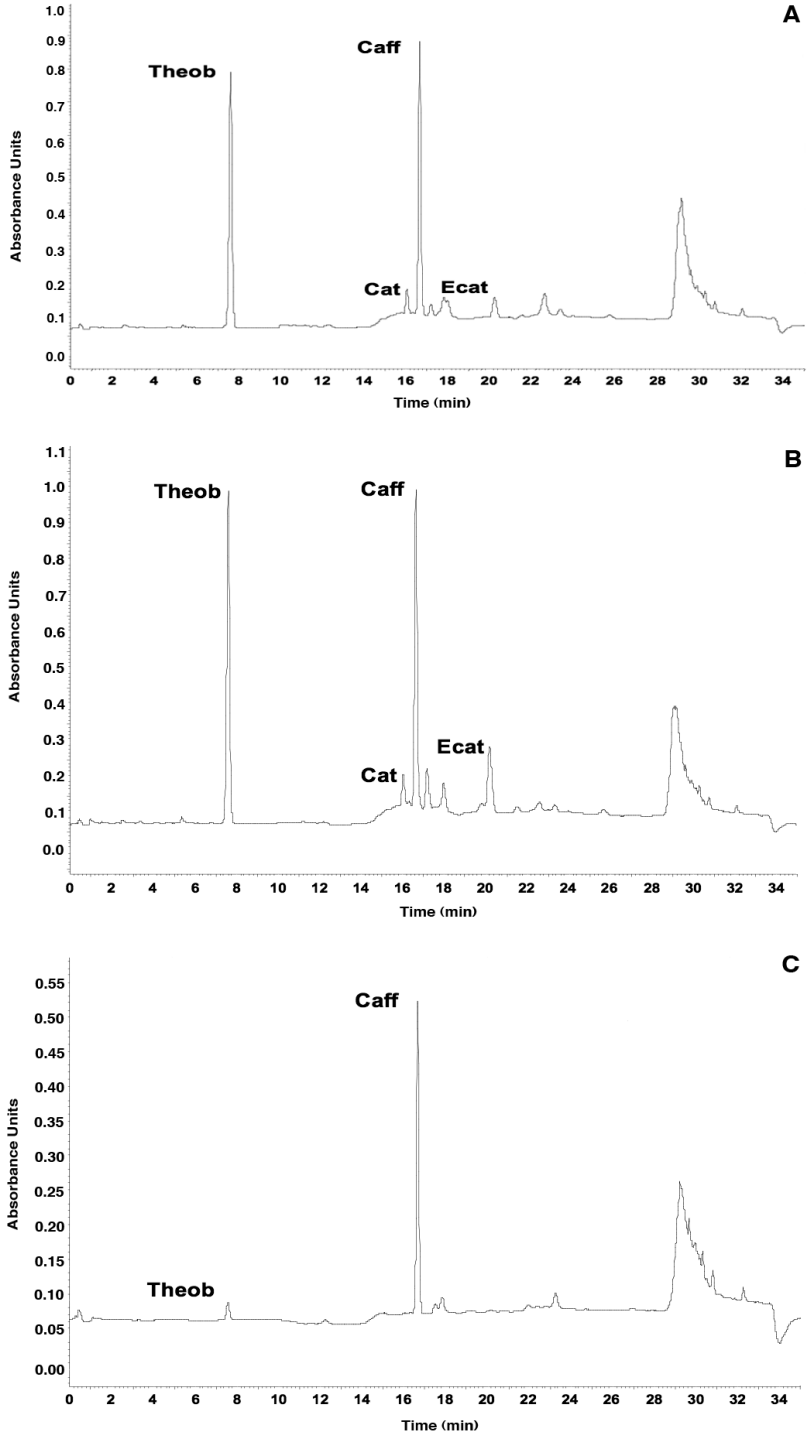

Figure 3. Chromatograms of Hershey's cocoa extract (0.004 g in 5 mL) (A), cocoa bean extract (0.020 g in 5 mL) (B), and cocoa butter (0.020 g in 5 mL) extract (C) extracted in 85:15, 0.3% HAc–MeOH (volume fraction). Conditions: Gemini C18 column (2.0 × 150 mm, 5 µm particle size); mobile phase gradient of 0.3% acetic acid and MeOH at 0.50 mL/min; injection volume of 5 µL; autosampler temperature of 10°C; absorbance detection at 273 nm; 0.30 absorbance units full scale (AUFS) initially then 0.03 AUFS after 10 min; theobromine (Theob), (+)-catechin (Cat), caffeine (Caff) and (–)-epicatechin (Ecat).

It is not understood why (+)-catechin and (–)-epicatechin were not detected using the NIST procedure for the determination of theobromine, theophylline and caffeine since essentially the same wavelength (274 nm used by NIST, 273 nm used in this procedure) was applied to monitor the eluent. It may be that the detector sensitivity setting was too coarse for the detection of (+)-catechin and (–)-epicatechin or that the isocratic mobile phase used by NIST was too weak to elute (+)-catechin and (–)-epicatechin from the column.

The NIST results for (+)-catechin and (–)-epicatechin (quantified using an internal standard of trytophan methyl ester hydrochloride) were done using mass selective detection after removing the fat from the sample with three extractions of hexane, drying under nitrogen, extracting two times in MeOH, combining the MeOH extracts and final dilution in water (13). The simpler procedure described here is as effective for quantifying (+)-catechin and (–)-

Table VI. Results for Other Cocoa Samples, Cocoa Beans, and Cocoa Butter (mg/kg, mean ± σ, n = 3)*

Sample	Theobromine	(+)-Catechin	Caffeine	(–)-Epicatechin
Water as extract				
Cocoa 1	22882 ± 259	932 ± 31	1875 ± 21	2607 ± 28
Cocoa 2	20483 ± 311	765 ± 37	1615 ± 12	1931 ± 22
Cocoa 3	20494 ± 127	302 ± 23	1521 ± 24	1616 ± 27
Cocoa 4	18614 ± 95	929 ± 28	1637 ± 24	2177 ± 25
Cocoa 5	21472 ± 211	1835 ± 162	1636 ± 23	3272 ± 74
Hershey's Cocoa	26321 ± 501	1122 ± 49	2431 ± 52	1922 ± 42
Cocoa Beans	8909 ± 406	434 ± 15	827 ± 24	1909 ± 136
Cocoa Butter	122 ± 13	< 32†	413 ± 2	< 27†
Initial mobile phase as extract‡				
Cocoa 1	22447 ± 545	974 ± 26	1958 ± 56	2538 ± 60
Cocoa 2	19292 ± 770	841 ± 33	1719 ± 65	1807 ± 66
Cocoa 3	20179 ± 232	329 ± 4	1669 ± 62	1536 ± 6
Cocoa 4	19125 ± 931	735 ± 31	1802 ± 77	2214 ± 141
Cocoa 5	22084 ± 356	1668 ± 36	1756 ± 35	3520 ± 49
Hershey's Cocoa	24600 ± 904	1026 ± 46	2343 ± 130	1560 ± 71
Cocoa Beans	8623 ± 222	388 ± 72	908 ± 5	1846 ± 167
Cocoa Butter	139 ± 32	< 32†	416 ± 0.4	< 27†

* σ = standard deviation.
† Based on S/N ≥2 at 0.002 absorbance units full scale of a 0.02g in 5 mL.
‡ Initial mobile phase is 85:15, 0.3% HAc–MeOH (volume fractions).

epicatechin in the baking chocolate matrix.

Table V shows the percent recovery of theobromine, (+)-catechin, caffeine, and (–)-epicatechin to be acceptable, varying from 98.9 to 104.3% using either extractant. The amounts found by standard addition compare with those obtained by external standard, indicating no interferences with the analytes in the baking chocolate sample matrix.

The overall results obtained here (excluding the defatted sample) are higher than those using the two, separate NIST procedures except for possibly (+)-catechin. This may be due to the additional sample preparation steps used in the NIST procedures which can result in the loss of the analyte or change in the internal standard concentration which does not compensate for analyte loss. Lower results could also be caused by the compounds (including the internal standards) having varying solubilities in hexane, water and MeOH.

Application to other cocoas, cocoa beans, and cocoa butter

Table VI gives the results for six different cocoas, cocoa beans, and cocoa butter extracted in water and the initial portion of the mobile phase. The results for theobromine and caffeine in cocoa using both extractants are within the range of the amounts found by other analysts who defatted the cocoa using either petroleum ether or hexane (4–7). The ratios of theobromine to caffeine vary from 10.5:1 to 13.0:1 for these cocoas containing 10–12% cocoa butter fat. Kreiser and others found ratios of theobromine to caffeine to vary from 23:1 to as low as 2.5:1 (3,4). The theobromine and caffeine results for cocoa beans (8–11) and cocoa butter (12) are also in the range reported by other workers who analyzed defatted (petroleum ether) samples. There is little difference between the results for theobromine and caffeine using the two different extractants even though it has been reported that water extracts slightly more theobromine and caffeine from cocoa samples that were extracted in water without defatting (5). This reported difference may be due to the loss of theobromine and caffeine in the defatting solvent, hexane (3).

The amount of (+)-catechin and (–)-epicatechin in cocoa and cocoa beans are in the sample range as those of Subagio (16) who extracted the samples in MeOH with no defatting and Kim (15) who determined (–)-epicatechin on defatted samples after compensating for a estimated 50% butter fat content (27).

Cocoa beans contain all four compounds analyzed, but at overall lower levels relative to cocoa (Figures 3A and 3B). This is probably due to the higher cocoa butter fat content in cocoa beans which would be insoluble in water and the initial mobile phase. Cocoa butter contains more caffeine than theobromine and at much lower levels than cocoa and cocoa beans. (+)-Catechin and (–)-epicatechin are not detected in cocoa butter under these chromatographic conditions (Figure 3C).

Conclusions

The method developed for the determination of theobromine, (+)-catechin, caffeine, and (–)-epicatechin has been shown to be reliable and simple. The procedure can be applied for the assay of these compounds in Standard Reference Material 2384 Baking Chocolate, cocoa, cocoa beans and cocoa butter. Due to the instability of (+)-catechin and (–)-epicatechin, water may not be the choice as the medium for standards for (+)-catechin and (–)-epicatechin. The procedure can be used to replace separate techniques for the determination of theobromine and caffeine and (+)-catechin and (–)-epicatechin in materials containing extracts from *Theobroma cacao*.

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