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Quantification of the Predominant Monomeric Catechins in Baking Chocolate Standard Reference Material by LC/APCI-MS

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Catechins are polyphenolic plant compounds (flavonoids) that may offer significant health benefits to humans. These benefits stem largely from their anticarcinogenic, antioxidant, and antimutagenic properties. Recent epidemiological studies suggest that the consumption of flavonoid-containing foods is associated with reduced risk of cardiovascular disease. Chocolate is a natural cocoa bean-based product that reportedly contains high levels of monomeric, oligomeric, and polymeric catechins. We have applied solid−liquid extraction and liquid chromatography coupled with atmospheric pressure chemical ionization−mass spectrometry to the identification and determination of the predominant monomeric catechins, (+)-catechin and (−)-epicatechin, in a baking chocolate Standard Reference Material (NIST Standard Reference Material 2384). (+)-Catechin and (−)-epicatechin are detected and quantified in chocolate extracts on the basis of selected-ion monitoring of their protonated $[M + H]^+$ molecular ions. Tryptophan methyl ester is used as an internal standard. The developed method has the capacity to accurately quantify as little as 0.1 μg/mL (0.01 mg of catechin/g of chocolate) of either catechin in chocolate extracts, and the method has additionally been used to certify (+)-catechin and (−)-epicatechin levels in the baking chocolate Standard Reference Material. This is the first reported use of liquid chromatography/mass spectrometry for the quantitative determination of monomeric catechins in chocolate and the only report certifying monomeric catechin levels in a food-based Standard Reference Material.

KEYWORDS: Catechin; chocolate; epicatechin; liquid chromatography; mass spectrometry; standard reference material

INTRODUCTION

Catechins (flavan-3-ols) are polyphenolic plant compounds that may offer potential benefits to human health. These benefits have been identified through in vitro and in vivo investigations that indicate that certain catechins possess strong antioxidant, anticarcinogenic, and antimutagenic properties (*1*−*3*). The highest levels of catechins are found in beverages and foods of plant origin such as tea (*4*−*6*), wine (*7*), fruit (*8*), and cocoa beans (*Theobroma cacao* L.) (*3*). Recently, attention has focused on the possible health benefits of catechin intake via the consumption of cocoa bean-based products such as cocoa and chocolate. There is growing evidence that chocolate consumption has positive effects on platelet function, low-density lipoprotein oxidation, and plasma total antioxidant capacity that would tend to reduce the risk of cardiovascular disease (*9*−*13*). The predominant monomeric catechins in chocolate are (+)-catechin (C) and (−)-epicatechin (EC); however, quantitative data regarding the distribution of these compounds in chocolate are inconsistent (*3*, *14*). For example, selected studies on dark chocolate show variabilities (%RSD) >100% for composite C/EC levels (micrograms per gram) (*3*). This inconsistency arises from both the lack of standardized analysis procedures and the large variety of nonstandardized chocolate products that have been analyzed and reported in the literature. A food-based Standard Reference Material (SRM) containing certified levels of catechins, and especially of C and EC, did not previously exist, and this lack of a catechin reference material has also contributed to the significant interlaboratory variability and disagreement regarding the distribution of catechins in both cocoa and chocolate (*3*). Because of the lack of standardized analysis procedures (i.e., reference methods) and standardized chocolate products (i.e., reference materials), it is difficult to objectively and accurately evaluate the dietary intake and resulting bioavailability of C and EC from chocolate. Further, the commercial use of C and/or EC in concentrated preparations (nutraceuticals) or in the form of supplemented foods (functional foods) could be more easily controlled with better quality assurance through the use of an appropriate SRM containing certified levels of C and EC. It is for this reason that values for catechins were assigned in SRM 2384. SRM 2384 (*15*) is one in a series of food-matrix reference materials available from the National Institute of Standards and Technology (NIST).

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SRM 2384 is intended for use as a primary control material for assigning values to in-house control materials and for validation of analytical methods that measure relevant nutrients for which product labeling is required by the Nutrition Labeling and Education Act of 1990 (*16*), as well as for additional analytes of interest such as catechins, caffeine, theobromine, and theophylline.

The most widely used approaches for the determination of monomeric catechins in chocolate have focused on the use of liquid chromatography with ultraviolet absorbance detection (LC/UV) (*17*−*19*), LC with fluorescence detection (LC/FD) (*20*), and LC with electrochemical detection (LC/ED) (*17*). To our knowledge, no quantitative determination of monomeric catechins in chocolate by LC coupled with mass spectrometry (LC/MS) has been reported; however, two reports describing the qualitative identification of catechins in chocolate via LC/MS analyses have recently been published (*21*, *22*), and both groups of authors assisted in value assignment of the SRM, as discussed herein. The present report describes the quantitative extraction of C and EC from a baking chocolate SRM (NIST SRM 2384) and the subsequent separation and determination of C and EC by LC coupled with atmospheric pressure chemical ionization−mass spectrometry (LC/APCI-MS). C and EC are simultaneously detected and confirmed in defatted chocolate extracts via selected-ion monitoring (SIM) of their protonated molecular ions ($[M + H]^+$, *m*/*z* 291) and their retro-Diels−Alder (RDA) fragmentation products ($[M + H - H_2O]^+$, *m*/*z* 139) (*6*), respectively. Tryptophan methyl ester (TME) was used as an internal standard compound during the extraction and analysis procedures. The resulting peak area ratios (catechin monomer/TME) were used in conjunction with calibration peak area ratios and mass ratios to determine the level (milligrams per gram) of catechin monomer in samples of baking chocolate. Further, C and EC are assigned certified values in the baking chocolate reference material based upon values determined with this method, in combination with values determined from interlaboratory measurements utilizing other analytical techniques, such as LC/UV, LC/FD, and LC/MS (nonpublished electrospray method).

Materials. Catechin primary standards [(−)-epigallocatechin (EGC), (−)-gallocatechin (GC), (+)-catechin hydrate (C), (−)-catechin gallate (CG), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin (EC), (−)-gallocatechin gallate (GCG), (−)-epicatechin gallate (ECG)], tryptophan methyl ester hydrochloride (TME), and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co. (St. Louis, MO). Procyanidin B2 was purchased from Indofine Chemical Co. (Somerville, NJ). The identity and analytical purity of all catechins, procyanidin B2, and TME were confirmed by LC and MS analysis before initial use. Specifically, the purities of C (99%, lot no. 106H0990) and EC (>97%, lot no. 085H2616), as listed on the certificates of analysis, were confirmed by both full-scan LC/MS and LC/UV absorbance analysis. HPLC-grade methanol, acetonitrile, and hexane were purchased from J.T. Baker (Phillipsburg, NJ). The baking chocolate was NIST Standard Reference Material 2384. Nylon syringe filters (0.45 μm pore size) were obtained from Alltech Associates (Deerfield, IL). Purified water (18 MΩ), prepared by using a Millipore Milli-Q purification system, was used to prepare all samples and standards. All other chemical reagents and solvents were ACS reagent grade, unless stated otherwise.

Catechin Standard Mixture. A catechin standard mixture consisting of 50 μg/mL of each of the eight catechin primary standards was prepared in 70/30 water/methanol (volume fractions) and used for methods development.

C and EC Calibration Standards. *Note: All steps were performed gravimetrically.* C and EC calibration standards were prepared as mixed standards in TME. Briefly, a stock (∼2 L) of TME internal standard solution (approximately 1 μg/mL) was prepared in 70/30 water/methanol (volume fractions). Four calibration mixtures (containing four different catechin mass ratios) were prepared by weighing known amounts (milligrams) of the catechin powders into 500-mL volumetric flasks. Because the C primary standard exists in the form of a hydrate, the weighed amount was corrected for the hydrate contribution. The contents of each flask were then diluted to volume with a known mass (grams) of the 1 μg/mL TME internal standard solution. The calibration mixtures were analyzed in duplicate by the LC/MS method, and the average peak area ratios and mass ratios were subjected to a linear least-squares fitting procedure to generate independent C and EC calibration curves.

Sample Preparation. *Note: All steps were performed gravimetrically.* One sample from eight different bars of baking chocolate was prepared and analyzed as follows: approximately 100 mg of 1000 μg/mL TME (prepared in water, not 70/30 water/methanol) was weighed (using an Eppendorf pipet) into a 50-mL plastic centrifuge tube containing approximately 1 g of chocolate. The chocolate with the internal standard was gently melted over a water bath (45 ± 2 °C) for approximately 30 min. After melting, the chocolate was stirred with a spatula to homogeneity, 40 mL of hexane was added (using a graduated cylinder) to the tube, and the chocolate was stirred again. The tube was sonicated for 15 min and then centrifuged at 3500*g* (4 min). The hexane layer containing the extracted lipids was removed, a fresh portion (40 mL) of hexane was added to the tube, and the lipid extraction was repeated. The entire lipid extraction procedure was repeated a total of three times. After the third extraction, the defatted chocolate was dried under a gentle N_2 stream for approximately 10 min. At this point, the dried chocolate was carefully weighed to determine the defatted chocolate mass. The chocolate was mixed by stirring with a spatula, and approximately 250 mg was transferred into a clean 15-mL plastic centrifuge tube. Approximately 5 mL of extraction solvent (pure methanol) was added (using a graduated cylinder) into the tube, and the tube was sonicated for 15 min (50 ± 2 °C). The tube was centrifuged at 3500*g* (4 min), and the supernatant was filtered (0.45 μm pore filter) directly into a clean 15-mL centrifuge tube. Another 5-mL portion of methanol was added into the tube of defatted chocolate, the entire extraction process was repeated once more, and the methanolic extracts were combined. A 500-μL aliquot of the methanol extract was combined with 1000 μL of water in a 2-mL plastic microcentrifuge tube. The tube was vortex-mixed, and a 300-μL aliquot of the extract was transferred into a sample vial. Each chocolate extract was analyzed once by the LC/MS method. The chocolate extracts were analyzed between alternate single injections of the calibration standards.

Methods. LC/MS analyses were performed on an Agilent 1100 series LC system coupled to a single-quadrupole Agilent 1100 series mass-selective detector with an APCI source. The LC system was outfitted with a binary pump, a variable-wavelength UV absorbance detector, a room-temperature autosampler, and an in-line mobile-phase vacuum degasser. Samples were analyzed using a Zorbax Eclipse XDB C_{18} reversed-phase analytical column (4.6 mm × 250 mm, 4 μm particle size). In all instances, the column temperature was thermostated at 30 °C, the UV absorbance detection wavelength was 280 nm, the

Table 1. LC Elution Conditions for the LC/APCI-MS Method

time (min)	eluent A (%)[a]	eluent B (%)[b]
0	88	12
25	79	21
26	0	100
29	0	100
30	88	12
40	88	12

[a] 0.05% (volume fraction) TFA in water. [b] 0.05% (volume fraction) TFA in acetonitrile.

Table 2. Analytical Method and Extraction Solvent Used by Collaborating Laboratories To Determine Catechins in SRM 2384 Baking Chocolate

analytical method	extraction solvent[a]
LC/UV (1)	70/29.5/0.5
LC/UV (2)	70/30/0.25
LC/FD	70/29.5/0.5
LC/ESI-MS	70/29.8/0.2

[a] Volume fractions of acetone/water/acetic acid.

flow rate was 750 μL/min, and the injection volume was 10 μL. The LC elution conditions are described in **Table 1**. Full-scan and SIM mode mass spectra of C, EC, and TME were obtained and optimized via positive-ion APCI. Automated tuning and mass calibration were performed using the APCI tuning solution (proprietary) provided with the instrument. The following instrument parameters were used for the analysis of C, EC, and TME in full-scan mode: corona needle, 10 μA; capillary voltage, +4000 V; scan range, 100−400 *m*/*z*; s/scan cycle, 0.93; threshold, 100; step size, 0.15; gain, 3; fragmentor voltage, 60 V; drying gas flow rate (N_2), 3.5 L/min; drying gas temperature, 50 °C; vaporizer temperature, 500 °C; nebulizer pressure, 414 kPa (60 psi). Instrument parameters used in SIM mode, except for the scan range and s/scan cycle, were identical to the parameters used in scan mode. The relevant SIM ions were *m*/*z* 291 for C and EC and *m*/*z* 219 for TME.

Analyses by Collaborating Laboratories. Four collaborating laboratories also measured catechins in SRM 2384 using their usual analytical methods (**Table 2**). All laboratories used either a published procyanidin extraction solvent (*20*, *23*) consisting of 70/29.5/0.5 acetone/water/acetic acid (volume fractions) or a slight modification of this solvent (**Table 2**). The laboratories were asked to make single measurements in samples taken from each of four bars of chocolate; one laboratory chose to make duplicate measurements. Following extraction, two laboratories used LC (reversed-phase mode) with absorbance detection (LC/UV-1 and LC/UV-2), one laboratory used LC (normal-phase mode) with fluorescence detection (LC/FD), and one laboratory used LC (reversed-phase mode) with electrospray ionization−mass spectrometry detection (LC/ESI-MS).

Two of the collaborating laboratories measured "total procyanidins" (the sum of C, EC, and the dimer through the decamer of the procyanidin oligomers) in four samples using LC and either absorbance or mass spectrometric detection. The laboratory that used absorbance detection also provided an analytically determined value for total monomers (the sum of C and EC), and this value was combined with the mathematically summed monomer values to generate the certified value for total catechin monomers.

Value Assignment for Catechins in SRM 2384 Baking Chocolate. Assigned values were derived from the combination of results provided by NIST and collaborating laboratories. The mean for each laboratory was determined from the values reported, and a grand mean was calculated for the collaborating laboratories. For C and EC, for which NIST also made measurements, this grand mean was averaged with the NIST mean to obtain the certified value. [A NIST certified value (*24*) is a value for which NIST has the highest confidence in its accuracy, in that all known or suspected sources of bias have been investigated or accounted for by NIST.] Each certified concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in **Table 2** and NIST, i.e., (mean concentration value from all outside laboratories + mean concentration value from NIST/2). The uncertainty in the certified values, calculated using the Type B on Bias method (*25*) based on guidelines described in the ISO *Guide to the Expression of Uncertainty in Measurement*, is expressed as an expanded uncertainty, *U*. The expanded uncertainty is calculated as $U = ku_c$, where u_c (unexpanded uncertainty) 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 (multiplier), *k*, is determined from the Student's *t* distribution corresponding to the appropriate associated degrees of freedom and 95% confidence for each analyte. Further details concerning the calculation of the certified concentration values and their associated uncertainties can be found in the original article by Levenson et al. (*25*).

RESULTS AND DISCUSSION

The detection and quantitation of C and EC in chocolate extracts by LC/APCI-MS were based on slight modifications of our previously published LC/APCI-MS method for the qualitative identification of catechins in tea extracts (*6*). The LC flow rate was reduced from 1000 to 750 μL/min to enhance detection sensitivity, the gradient portion of the chromatographic separation was amended by removal of two unnecessary linear gradients, and the column re-equilibration time was extended by an additional 5 min to increase retention time reproducibility. The ion detection parameters of the mass analyzer were modified to enable detection of the internal standard compound, TME ($[M + H]^+$, *m*/*z* 219).

Optimization of Catechin Extraction Conditions. Catechins are relatively stable compounds in their native environments, but they can readily epimerize, oxidize, or otherwise degrade during extraction procedures. Six different solvent systems were tested for the quantitative extraction of monomeric catechins from chocolate: (1) deionized water, (2) methanol, (3) ethyl acetate, (4) 50/50 methanol/1 mol/L HCl (volume fractions), (5) 50/50 water/ethanol (volume fractions), and (6) 70/29.5/0.5 acetone/water/acetic acid (volume fractions). Chocolate samples were melted/defatted as described under Sample Preparation and extracted with a single 5-mL portion of each extraction solvent. A catechin standard mixture consisting of 50 μg/mL of each of the eight catechin primary standards was used to help identify the presence or absence of the analytes in the resulting extracts. On the basis of LC retention times and continuously recorded mass spectra, only C and EC could be positively identified in the extracts. The best results, in terms of the largest total ion signals, were observed with the pure methanol, methanol/HCl, and deionized water extraction solvents. Both the water/ethanol and acetone/water/acetic acid extracts resulted in broad catechin peaks, while the ethyl acetate extract contained no identifiable catechins. Among the methanol, methanol/HCl, and deionized water extracts, the methanol extract resulted in cleaner selected-ion chromatograms and was thus chosen as the standard extraction solvent.

A single chocolate sample was melted/defatted and exhaustively extracted (four times) with methanol in order to estimate the efficiency of the extraction procedure. The four extracts were analyzed for C, EC, and TME, and the efficiency of each extraction was calculated by ratioing the analyte area counts for a single extraction (or combined extractions) to the analyte area counts for all four extractions summed together. The calculated efficiencies (98% ± 4% for C, 98% ± 2% for EC, and 94% ± 5% for TME) of the methanolic extractions were deemed adequate after two successive extractions. The third and fourth extractions steps did not contribute significantly to the calculated extraction efficiencies. Double methanolic extractions were adopted as a standard procedure for extraction of monomeric catechins from chocolate.

The extraction recoveries of C, EC, and TME from hexane and from chocolate were determined in separate experiments. For the hexane experiment, known concentrations (100 μg/mL) of the catechins and TME were added to 40 mL of hexane, and analyte recoveries before and after sonication were determined on the basis of analyte responses in an externally prepared standard. The recoveries of C, EC, and TME from hexane before and after sonication were all 100%, indicating that the analytes were not extracted into hexane. In a similar manner, the extraction recoveries of C, EC, and TME spiked into chocolate samples and carried throughout the lipid removal (defatting) and double methanol extraction steps (see Sample Preparation) were determined. However, the chocolate sample size was reduced to 0.25 g, and the entire quantity of chocolate was extracted and analyzed to calculate recoveries. Briefly, six chocolate samples were weighed out, and three of the samples were spiked with a known mass of a standard solution containing known masses of C, EC, and TME. The remaining three chocolate samples were left unspiked (controls). Each chocolate sample was prepared and analyzed (three injections each), and the resulting area responses for the analytes in the spiked sample were subtracted from the area responses for the analytes in the unspiked (control) sample to calculate the absolute area responses for the spiked catechins and TME. A known mass of the standard solution containing known masses of C, EC, and TME was also carried throughout the lipid removal and double methanol extraction steps to mimic the dilution of the chocolate samples. The extraction recoveries were calculated by utilizing the resulting area/mass response ratios for the spiked analytes in conjunction with the area/mass response ratios for the analytes in the standard solution. The calculated recoveries were 90% ± 3% for C, 98% ± 6% for EC, and 89% ± 9% for TME. Based upon the extraction results, there was no evidence of significant epimerization, oxidation, or degradation of the catechins during the extraction or analysis procedures.

Confirmation of Catechins. The identities of C and EC in chocolate were confirmed on the basis of the alignment of the chromatographic retention times of the catechin peaks with the retention times from reconstructed ion chromatograms by matching the mass spectra of the catechin peaks in chocolate with the mass spectra obtained from analysis of authentic catechin primary standards and by simultaneous selected-ion monitoring of the protonated molecular ions and the catechin-specific retro-Diels−Alder (RDA) fragmentation product ions (*6*, *26*, *27*). The LC peaks for C and EC matched perfectly with the ions extracted on the *m*/*z* 291 ion channel during full-scan LC/MS analysis of chocolate extracts (data not shown). An unknown catechin-like compound eluting between C and EC was also observed on the *m*/*z* 291 ion channel and was identified as procyanidin B2 (see below). A typical mass spectrum

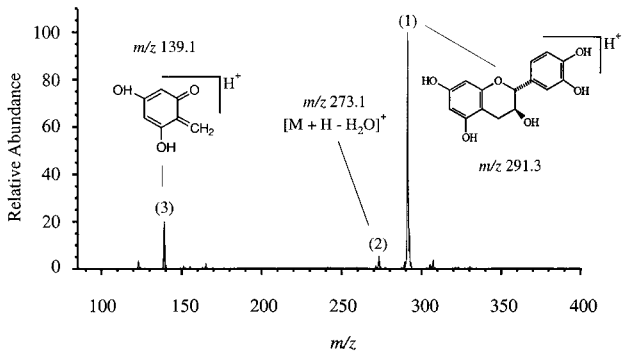

Figure 1. Background-corrected mass spectrum of a C standard (10 μg/mL). Peak identification: (1) protonated molecular ion $[M + H]^+$ of C; (2) loss of water from the protonated molecular ion; (3) catechin-specific retro-Diels–Alder (RDA) fragment ion. The spectrum was collected using the full-scan mode LC/MS conditions described in Methods.

obtained from the LC/MS analysis of a C standard (**Figure 1**) exhibits a strong protonated molecular ion at *m*/*z* 291 and two characteristic fragment ions at *m*/*z* 273 and 139. These fragment ions represent the loss of a molecule of water from the molecular ion and the formation of the catechin-specific RDA fragmentation ion, respectively. This fragmentation pathway has previously been shown to be consistent for unmodified and nongallated catechin compounds (*6*). Because EC is an epimer of C, the mass spectrum for EC was identical to that for C. The mass spectra for C and EC in the chocolate extracts were identical. The catechin-specific RDA fragment ion forms due to fragmentation of the nonvariable portion of the catechin ring structure and can serve as a fingerprint ion for the presence of catechins in complex matrices, such as chocolate. Simultaneous monitoring of the protonated catechin molecular ions (*m*/*z* 291) and the RDA fragment ions (*m*/*z* 139) by LC/MS analysis of chocolate extracts and of catechin standards is illustrated in **Figure 2**. C and EC in the chocolate extracts and standards are readily confirmed on the basis of the presence of the RDA fingerprint ions. The peak that eluted between C and EC in the chocolate extracts (**Figure 2A**,**B**) also exhibited the RDA fingerprint ion, which suggested that the peak was due to a catechin-like component. Full-scan LC/MS analysis of this component produced characteristic ions at *m*/*z* 579 (base peak), 427, 291, 247, and 139. The base peak at *m*/*z* 579 suggested that this component could be a type of catechin dimer (2 × 290 g/mol = 580 g/mol), possibly corresponding to the protonated molecular ion of procyanidin B2, an EC dimer (578 g/mol). Additionally, procyanidin B2 is a known component of cocoa beans and cocoa products (*14*). Full-scan LC/MS analysis of an authentic sample of procyanidin B2 resulted in a fragmentation pattern that included the following ions: *m*/*z* 579 (base peak), 427, 409, 291, 247, and 139. Five of the six observed ions were identical to the ions produced by full-scan analysis of the peak in the chocolate extracts. Further, the LC retention time of the procyanidin B2 standard was identical to the LC retention time of the component in the chocolate extracts. On the basis of these data, peak 2 in **Figure 2** was identified as procyanidin B2.

Figures of Merit. A typical selected-ion chromatogram of a chocolate extract showing elution of C, EC, procyanidin B2, and the internal standard, TME, is shown in **Figure 3**. Estimates of the method's linear dynamic range, limit of detection (LOD), and limit of quantitation (LOQ) for C and EC were assessed by analyzing a set of 10 serially prepared catechin standards. Results from duplicate injections of each standard were plotted and analyzed by unweighted linear least-squares regression

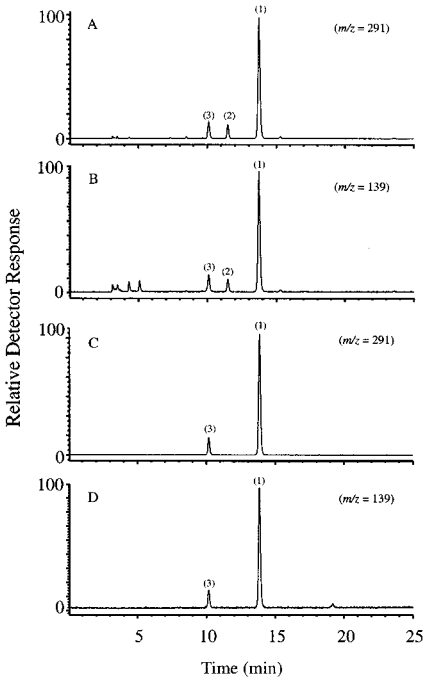

Figure 2. Comparison of the selected-ion chromatograms for the protonated catechin molecular ion channel (*m*/*z* 291) versus the retro-Diels–Alder (RDA) fragment ion channel (*m*/*z* 139). (A) Protonated catechin molecular ion channel for chocolate extract; (B) RDA fragment ion channel for chocolate extract; (C) protonated catechin molecular ion channel for C and EC in a standard mixture; (D) RDA fragment ion channel for C and EC in a standard mixture. Peak identification: (1) EC; (2) procyanidin B2; (3) C. Profiles were collected using the LC/MS conditions described in Methods.

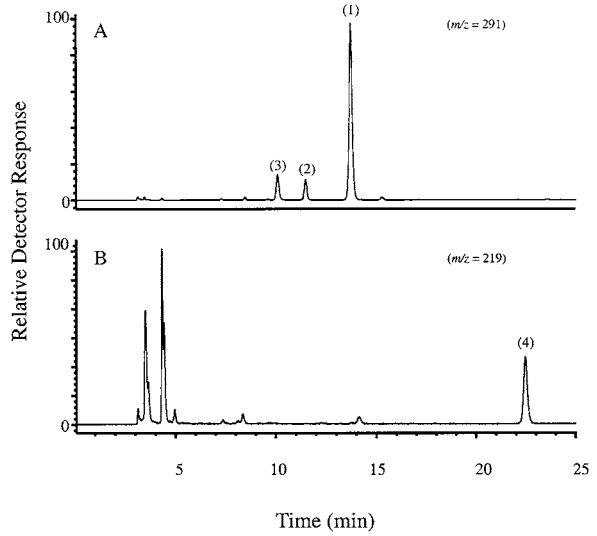

Figure 3. Typical selected-ion chromatograms resulting from the extraction and LC/MS analysis of SRM 2384 baking chocolate. (A) Protonated catechin molecular ion channel (*m*/*z* 291); (B) protonated TME molecular ion channel (*m*/*z* 219). Peak identification: (1) EC; (2) procyanidin B2; (3) C; (4) TME. Analysis conditions are described in Methods.

analysis. A plot of the area response ratio (catechin monomer/TME) versus concentration ratio (catechin monomer/TME) resulted in calibration equations of $y = 0.6365x - 0.0062$ ($r^2 = 0.999$) for C and $y = 0.7432x - 0.0075$ ($r^2 = 0.999$) for EC, respectively. The calculated r^2 values and visual inspection of each plot indicated that excellent response linearity from 0.2 to 100 μg/mL was achieved for both C and EC. On the basis of the calibration equation and the minimum detectable catechin ion signals, the estimated LOD and LOQ for both C and EC were 0.01 and 0.1 μg/mL (S/N $\geq$ 3), respectively.

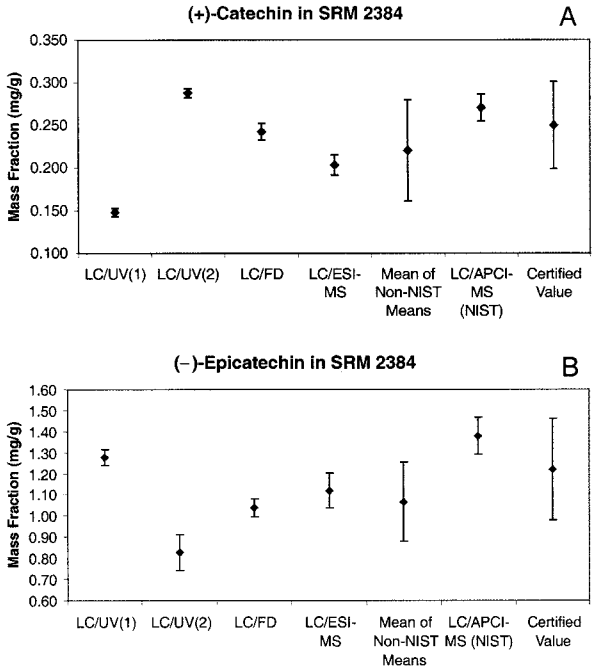

Figure 4. Interlaboratory determinations of C and EC in SRM 2384 baking chocolate. (A) Measurement results for C; (B) measurement results for EC. Error bars associated with individual laboratories' data and with the mean of non-NIST means represent one standard deviation. The error bars on the certified values represent the 95% confidence interval.

Interlaboratory Comparison and Certification of C and EC in Chocolate. The LC/MS method was applied to the determination of C and EC in baking chocolate (NIST SRM 2384). Samples were also sent to several other laboratories that had proven expertise in the measurement of catechins. These laboratories utilized a variety of analytical methods (**Table 2**) to determine C and EC in the SRM, and the results of their measurements in comparison to the NIST measurements are presented graphically in **Figure 4**. **Figure 4A** presents the results for C, and **Figure 4B** presents the results for EC. The mean values for C were 0.22 mg/g with an RSD of 26.9% for the non-NIST determinations and 0.27 mg/g with an RSD of 5.8% for the NIST determination. The mean values for EC were 1.07 mg/g with an RSD of 17.6% for the non-NIST determinations and 1.38 mg/g with an RSD of 6.3% for the NIST determination. In general, the measurement repeatability of the methods was good, with ranges of 1.8−5.8% for C and 3.1−10.2% for EC. The NIST LC/MS determinations were on the high end of the measurement spread for both C and EC, but no obvious bias was evident. The variability in the catechin values determined via the NIST LC/MS method was not inordinantly high. However, it is probable that the current variability could have been further reduced through the use of stable isotopically labeled catechin internal standard compounds combined with an isotope-dilution LC/MS quantification scheme. At this time, appropriately labeled catechin compounds are not commercially or academically available. The NIST catechin values, along with the catechin values determined at the other laboratories, were subsequently combined to certify the levels of monomeric C and EC in the SRM, and these results are summarized in **Table 3**.

The nutritional and clinical use of catechins and other phytochemicals for the improvement of human health will

Table 3. Certified Concentration Values for Catechins Monomers in SRM 2384 Baking Chocolate

analyte	mass fraction (g/kg)
C	0.245 ± 0.051
EC	1.22 ± 0.24
total catechin monomers	1.49 ± 0.22

require SRMs with certified component levels for use in quality assessment. Mass spectrometry has the capacity to both accurately quantify and unambiguously identify these phytochemicals in complex matrices. The reported LC/APCI-MS method has been applied to the specific determination and certification of the predominant monomeric catechins (C and EC) in a baking chocolate SRM. NIST SRM 2384 is the first food-based reference material with certified levels of C and EC, and as such, it should be immediately useful to nutritionists, clinicians, and food scientists involved in catechin-related bioavailability studies and value assignment in functional foods and dietary supplements.

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