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Identification of Native Catechin Fatty Acid Esters in Green Tea (*Camellia sinensis*)

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Supporting Information

ABSTRACT: Catechins are potent antioxidants and make up the primary class of polyphenols present in tea (*Camellia sinensis*). They are especially abundant in the less-fermented green teas that have been employed in various foods to enhance shelf life stability (Senanayake, N. *J. Funct. Foods* **2013**, in press. Gramza, A.; Korczak, J. *Trends Food Sci.* **2005**, *16*, 351−358). The antioxidative activity of native (polar) catechins has proven to be useful in foods of relatively high polarity, while mixed results have been achieved in high-fat foods. However, the polarity of catechins can be attenuated by esterification with fatty acids, producing adducts that effectively partition into lipids and protect against rancidity even in high-fat foods (Cutler, S.; Fuller, E.; Rotberg, I.; Wray, C.; Troung, M.; Poss, M. International Patent WO 2013/036934 A1, March 14, 2013. Zhong, Y.; Shahidi, F. *J. Agric. Food Chem.* **2011**, *59*, 6526−6533). In this work, a search for the presence of naturally occurring lipid-conjugated catechins was undertaken in various green tea varieties. Rather than the traditional aqueous infusion, dried tea leaves were extracted with organic solvents followed by analysis for catechin adducts with both lower polarities and increased molecular weights as monitored by liquid chromatography and tandem mass spectrometry. Native catechin palmitates were identified and indirectly confirmed by synthesis and nuclear magnetic resonance as natural components of several Chinese green teas. Evidence of other fatty catechin esters was also observed.

KEYWORDS: *catechin palmitates, UPLC−MS/MS, preparative chromatography, NMR, structure determination*

INTRODUCTION

Plants exhibit a great diversity of polyphenols ranging from single-ring to multiring structures of intermediate to high polarity. Many polyphenols protect plant tissues against oxidation from the oxygen-rich atmosphere and photodamage from excessive sunlight.[6,7] Because catechins are excellent antioxidants with large far-UV extinction coefficients, these are the roles they are believed to play in plant physiology.[7] Some polyphenols exhibit even greater structural and functional variation through conjugation with other compounds, e.g., aliphatic or phenolic acids, sterols, and sugars.[8−10] These modifications are believed to facilitate autolocation into environments which they would otherwise not partition or tether them to functional locations of the plant's anatomy where they presumably serve defensive roles against oxidation, photolysis, microorganisms, and herbivory.[5]

The major polyphenols of green tea are the four catechins found in Figure 1: (−)-epicatechin (EC), (−)-epicatechin 3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin 3-gallate (EGCG). Although catechin levels vary by cultivar, environment, and harvest process, the proportions of the four aforementioned catechins are generally 5, 10, 30, and 55% of total catechins, respectively.[10,11] All catechins possess chiral centers at C2 and C3 (Figure 1). Although C2 is relatively stable, thermal lability about C3 allows substantial epimerization, thereby resulting in the epimeric pairs of each of four catechin species, e.g., epicatechin and catechin, epicatechin gallate and catechin gallate, etc.[12,13]

The polar (native) catechins have been employed in several food preparations to protect against rancidity, but their greatest efficacy is observed primarily in foods of high to intermediate polarity (e.g., well-hydrated or emulsified foods).[1,2] Although native catechins provide protection primarily in higher-polarity matrices, fatty acid-esterified catechins exhibit potent anti-rancidity activities even in fats and oils.[3,4,14] Indeed, several studies have been reported in which the catechin fatty acid esters rival synthetic antioxidants (e.g., BHA, BHT, and TBHQ) in their protection in many foods and feeds.[3]

The supply of another popular and potent natural food antioxidant, tocopherol, has been rapidly waning in recent years as prices have been volatile. Tocopherols and catechins share some interesting structural features that suggest similar functional characteristics in their food stabilizing abilities. Like catechins, the polar chromanol ring of tocopherol provides excellent antioxidant capability, but in tocopherols, this antioxidant ring is tethered to a 12-carbon phytyl chain to partition into and protect the more hydrophobic microenvironments (as evidenced by the yellow hue of many vegetable oils). It would not be surprising to find both free and lipid-conjugated catechins in *Camellia sinensis* performing similar antioxidative roles in both polar and nonpolar intracellular environments, as well. We therefore undertook an investigation in which various green tea varieties of Chinese origin were extracted with low-polarity solvents and analyzed [liquid chromatography and tandem mass spectrometry (LC−MS/MS)] for fatty acid catechin esters monitoring pseudomolecular ions calculated from the molecular weights of free catechins and various fatty acids known to be prevalent in tea. Green tea was selected for

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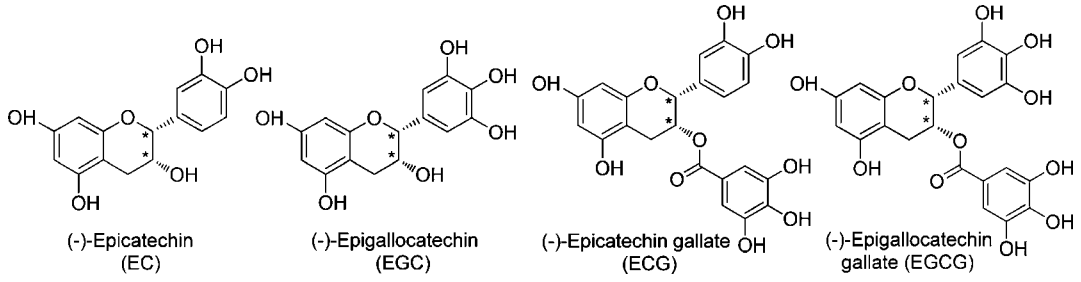

Figure 1. Chemical structures of the four prominent green tea catechins. Catechins have two chiral centers denoted by asterisks, one *ortho* and one *meta* to the benzopyran oxygen, C2 and C3, respectively, the latter being most labile to epimerization. When epimerization occurs, the corresponding epimers (without the "epi" prefix in their nomenclature) are generated; thus, equilibrium mixtures of all eight forms generally exist in extracts.

this study because catechins are more abundant than in other (more fermented) tea leaf preparations.[10,15]

This is the first report that native palmitoylated catechins have been identified in green tea.

MATERIALS AND METHODS

Reagents and Materials. Multiple dry green tea leaf samples were obtained from various sources, including a local grocery store (Lipton Green Tea); several were from the Wuxi Tea Research Institute located in Wuxi, Jiangsu province, China (itemized below). In addition, a catechin-rich green tea extract (assay, 71% catechins and 96% total polyphenols) was purchased from AVT Natural Products Ltd. (Kerala, India). Synthetic catechin palmitates were obtained from Kemin Bioscience (Ningbo, China), which were manufactured from green tea catechin extract under GMP conditions and were compliant with all commercial product specifications. Methanol, acetonitrile, ethyl acetate, and dimethylformamide (DMF) were all Optima grade and were purchased from Fisher Scientific (Pittsburgh, PA). Acetone-d_6, palmitic acid (≥98%), and myristoyl, palmitoyl, and stearoyl chlorides (98%) were from Sigma-Aldrich (St. Louis, MO). Hydromatrix inert diatomaceous earth was from Agilent. Reagent grade water (18.2 MΩ cm, charcoal-scrubbed, 0.2 μm-filtered, UV-irradiated) was Nanopure (Barnstead). When dry solvents were required, they were dried over 10% 3 Å sieves for ≥72 h prior to being used.

Reversed-Phase Ultraperformance Liquid Chromatography with Photodiode Array and Tandem Mass Spectrometric Detection (UPLC–PDA/MS/MS). Samples (0.1–10 mg) were dissolved in 1 mL of DMF, and 1–20 μL injections were analyzed by reversed-phase UPLC on a Waters Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm) at 40 °C. Mobile phases A and B were water and acetonitrile, respectively (both without formic acid), and the flow rate for all UPLC studies was 0.45 mL/min. Detection was achieved by monitoring with a photodiode array detector (PDA) from 190 to 500 nm in series with a triple-quadrupole mass spectrometer (Waters TQD) interfaced by electrospray ionization (ESI-negative) employing either selected ion recording (SIR) or multiple-reaction monitoring (MRM). Two different MRM transitions were acquired for each catechin palmitate class (except for EC palmitate, for which only a single MRM was identified). Chromatography was conducted over several weeks so retention times may vary slightly. Also note that the MS is plumbed in tandem (post-PDA), resulting in a 3–4 s delay in MS retention time relative to the PDA.

The following gradient was used with occasional slight modifications (as mentioned below) to elute the various catechin families of palmitate esters. The LC column was run isocratically for 1 min at 20% acetonitrile, and then the level of acetonitrile was increased to 64% over 1 min. Next, the level of acetonitrile was slowly increased to 68% over 29 min; finally, the level of acetonitrile was increased to 100% over 2 min and re-equilibrated.

The pseudomolecular ions ($[M - H]^-$) were calculated for the putative esters based on the calculated molecular weights of the four catechin species and are listed in Table 1. Low signal-to-noise chromatograms were smoothed as necessary by application of a standard "double-mean" protocol with a two-scan window.

Table 1. Calculated Pseudomolecular Ions ($[M - H]^-$) for Monitoring Putative Mono-Fatty Acid Catechin Esters

catechin	free catechin	myristates	palmitates	stearates	oleates
EGCG/GCG	457	667	695	723	721
ECG/CG	441	651	679	707	705
EGC/GC	305	515	543	571	569
EC/C	289	499	527	555	553

Samples (10 mg) of synthetic myristoyl, palmitoyl, and stearoyl catechins (synthesis described below) were separately dissolved in 1 mL of methanol and infused directly into the mass spectrometer interface. The acquisition parameters (e.g., ESI temperature, cone voltage, gas flows, etc.) were optimized for molecular ions (m/z) and MRMs of each of the three fatty acyl sets of catechins (catechin myristates, palmitates, and stearates).

Pressurized Liquid Extraction (PLE). Green tea leaf samples (40 g) were individually milled and blended with 20 g of Hydromatrix. Each mixture was packed into a stainless steel PLE sample column between layers of neat Hydromatrix, and the column was mounted to a PowerPrep/PLE system (Fluid Management Systems, Watertown, MA). Each sample was automatically extracted by circulating dichloromethane (DCM) at 120 °C and 1500 psi for 20 min and collected into a heated evaporation block (45 °C) where bulk solvent was removed under a stream of dry nitrogen. A 1 mL aliquot of the final volume of 40 mL was transferred to a 2 mL microfuge tube and evaporated to dryness by centrifugal evaporation (35 °C). The residue was dissolved in 1 mL of DMF, and a 20 μL injection was analyzed by UPLC–UV/MS/MS. The entire PLE sample preparation requires <2 h.

Synthesis of Catechin Fatty Acid Esters from a Catechin-Enriched Green Tea Extract. Myristoyl, palmitoyl, and stearoyl catechin esters were prepared as references against which to compare native catechin esters extracted from green tea leaves. Myristoyl catechins were prepared by first suspending 20 g of the AVT green tea extract (71% catechins, ~35 mmol) in 150 mL of dry ethyl acetate in a 250 mL Erlenmeyer flask and degassed by sonication. The suspension was transferred to a 250 mL triple-neck round-bottom flask with a 50 mL dry ethyl acetate rinse; 17.0 mL of pyridine (67 mmol) was added, and the vessel's atmosphere was replaced with argon. Next, 18.2 mL (67 mmol) of myristoyl chloride was added dropwise by buret over a 1 h period while the mixture was vigorously magnetically stirred and the temperature was maintained at 30 °C. The reaction mixture was then stirred for an additional 30 min at 30 °C. The suspension was vacuum filtered (paper), and the retentate was washed with 2 × 15 mL ethyl acetate rinses. The combined filtrates were washed with 200 mL of saturated sodium bicarbonate (gently to minimize emulsion) followed by 4 × 500 mL of water and dried over sodium sulfate, and finally, the solvent was removed by rotary evaporation (55 °C). Palmitoyl and stearoyl catechins were analogously prepared with 67 mmol of palmitoyl and stearoyl chlorides (20.3 and 22.6 mL), respectively.

Purification of Synthetic Catechin Palmitates by Preparative High-Performance Liquid Chromatography. Approximately 10 g of synthetic catechin palmitates (Kemin Bioscience) was dissolved in

DMF and pelleted at 8000 RCF (relative centrifugal force) for 10 min (<1% insolubles), and the supernatant was diluted 2-fold with DMF. This clarified and amber-colored, half-saturated sample was thus prepared to prevent the sample from crashing out of solution upon preparative HPLC injection. Ten 3.0-mL volumes were injected onto a Waters AutoPurification System with a 19 mm × 250 mm Waters SunFire C18 (5 μm) column at room temperature with the following elution gradient: isocratic at 66% B for 10 min, ramped to 89% B over 28 min with a gentle concave curve (#7 per Waters software), and ramped to 100% B over 2 min with a final 5 min hold; all steps were performed at a flow rate of 18 mL/min. Mobile phases A and B were 0.1% trifluoroacetic acid (TFA) in 20% acetonitrile (ACN) and 0.08% TFA in ACN, respectively. Separations were monitored by UV absorbance at both 250 and 275 nm and by evaporative light scattering detection (ELSD). The collection of fractions was triggered automatically using the 275 nm signal (absorption maximum of catechins and catechin palmitates) collecting 30 s slices. The duration of the postgradient column wash was 15 min with a 2-propanol/methanol/dichloromethane mixture (3:2:1) until the absorption (275 nm) returned to baseline.

The 25 fractions collected from each run were pooled with the corresponding fractions collected for all 10 runs. Aliquots were drawn from each of the 25, and the solvent was removed from each bulk fraction by a large-scale centrifugal evaporation (Genevac Rocket) at 40 °C and inspected via nuclear magnetic resonance spectroscopy (NMR). The solvent was also removed from each of the sample aliquots by a small-scale centrifugal evaporator (Labconco CentriVap) at 40 °C, taken up in 800 μL of DMF, and analyzed by UPLC–PDA/MS/MS to evaluate the purification and to positively identify the catechin esters (EGCG, EGC, ECG, and EC) by mass.

Structure Determination by One- and Two-Dimensional NMR. The actual site of each adduct's palmitoylation was determined by NMR. The samples purified as described above (5–10 mg) were each dissolved in 0.7 mL of acetone-d_6 and placed in a 5 mm NMR tube (Wilmad). Spectra were recorded with a Bruker AVIII 600 MHz spectrometer with the probe controlled at 25 °C. One-dimensional NMR spectra were first acquired for both ^{1}H and ^{13}C for each compound. Two-dimensional spectra were acquired using standard pulse sequences for homonuclear correlation spectroscopy (COSY), heteronuclear single-quantum correlation (HSQC), and heteronuclear multiple-bond correlation (HMBC) experiments. HSQC spectra played a part in the evaluation of directly bound ^{1}H–^{13}C chemical shift correlations, while long-range ^{1}H–^{13}C chemical shift correlations were determined by HMBC spectra. Resonance assignments were referenced to the residual solvent peak (2.08 ppm for ^{1}H and 29.85 ppm for ^{13}C). The specific NMR parameters are listed in Table 2.

Table 2. NMR Parameters

experiment	nucleus (f2, f1)	relaxation delay (s)	no. of scans	SW[a] (f2, f1) (Hz)	TD[b] (f2, f1)
^{1}H	^{1}H	1.0	16	12335	–
^{13}C	^{13}C	2.0	2048	36058	–
COSY	^{1}H, ^{1}H	2.0	8	5618, 5618	1024, 128
HSQC	^{1}H, ^{13}C	1.5	4	5435, 30196	512, 256
HMBC	^{1}H, ^{13}C	1.5	16	5435, 33523	1024, 128

[a]Sweep width. [b]Time domain, number of acquired data points.

Determination of Approximate Concentrations of Native Catechin Palmitates Present in Various Green Tea Varieties. Because the MRM peaks of native catechin palmitates exhibited no detectable UV absorbance at the scale analyzed, their quantitation was based on the MRM analysis of known free catechin aliquots. Chromatography of the latter provides the integrated areas of both free catechin UV absorption (275 nm) and MRM peaks, from which a free catechin-to-palmitoyl catechin conversion constant is calculated. Samples of several Chinese green tea cultivars were extracted by PLE and analyzed by UPLC–MS/MS, and their concentrations were calculated on a milligram per kilogram of dry green tea basis.

RESULTS AND DISCUSSION

Pressurized Liquid Extraction (PLE). Low-scale PLE with DCM was successfully employed early in the extraction process, while other approaches were later evaluated with the intent of extraction scale-up and to purify sufficient quantities for direct NMR structure determinations. These alternative extraction approaches were only marginally successful, and included chloroform, ethyl acetate, methylene chloride, hexane, dimethylformamide, and a variety of other solvents in which analyte stability might be questionable, e.g., methanol, ethanol, 2-propanol, *n*-propanol, and acetone. Several were evaluated in combination, e.g., chloroform and methanol (2:1), and for various periods of time and at various temperatures. While near-boiling ethyl acetate (b.p. of 71 °C) performed reasonably, PLE with 120 °C at 1500 psi produced the best yield. Therefore, elevated temperatures in aprotic, intermediate-polarity to low-polarity solvents (polarity indices from 3 to 5) appeared to produce the best yields for our purposes. Even room-temperature DMF in which the fatty acid catechin esters are most soluble proved to be an ineffective extractant. Because of these results, only data for PLE extracts in DCM are shown.

Comparison of the Chromatographic Profiles of Synthetic Myristoyl, Palmitoyl, and Stearoyl EGCG/GCG by UPLC–PDA/MS/MS. Analysis of chromatographic profiles of the synthetic mono-C14, C16, and C18 EGCG adducts reveals great similarity with variations observed in only retention time, peak width, and separation distance, as expected for isomer families in which each member differs by only a single methylene group (Figure 2). The corresponding

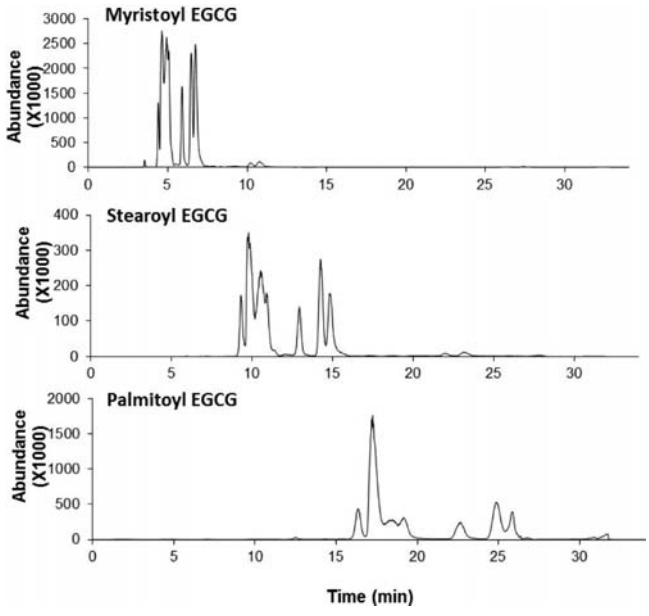

Figure 2. MRM transition profiles for each of the synthetic EGCG fatty acid esters, myristoyl EGCG (top), palmitoyl EGCG (middle), and stearoyl EGCG (bottom), following optimization. Profiles are virtually identical with uniform variations observed in only retention time, peak width, and separation, as expected for isomer families differing by only a single methylene group. The corresponding myristoyl, palmitoyl, and stearoyl esters of the non-EGCG catechins also exhibited analogous profiles within their isomeric groups, i.e., EGC/GC, ECG/CG, and EC/C (data not shown).

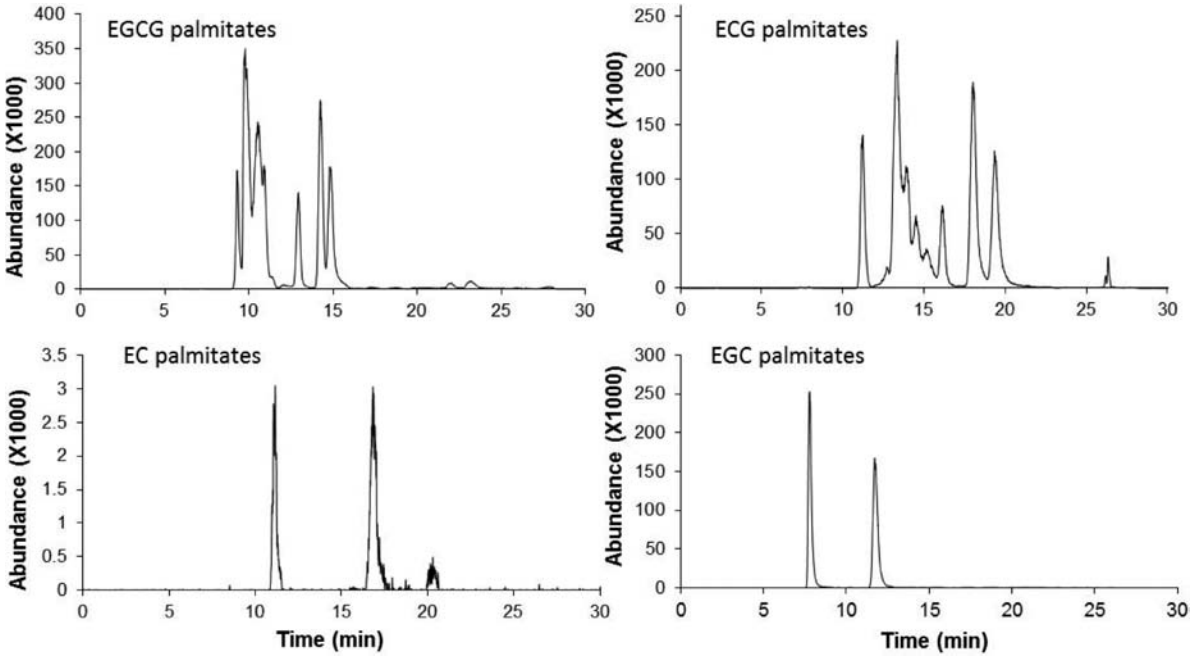

Figure 3. MRM chromatograms of synthetic EGCG, EC, ECG, and EGC palmitates. Although MRM intensities can vary substantially from analyte to analyte as a function of ionization efficiency at the MS source, the relative intensities of catechin palmitates are consistent with catechin natural abundances found in the green tea material from which they were prepared, i.e., EGCG > EGC > ECG > EC.

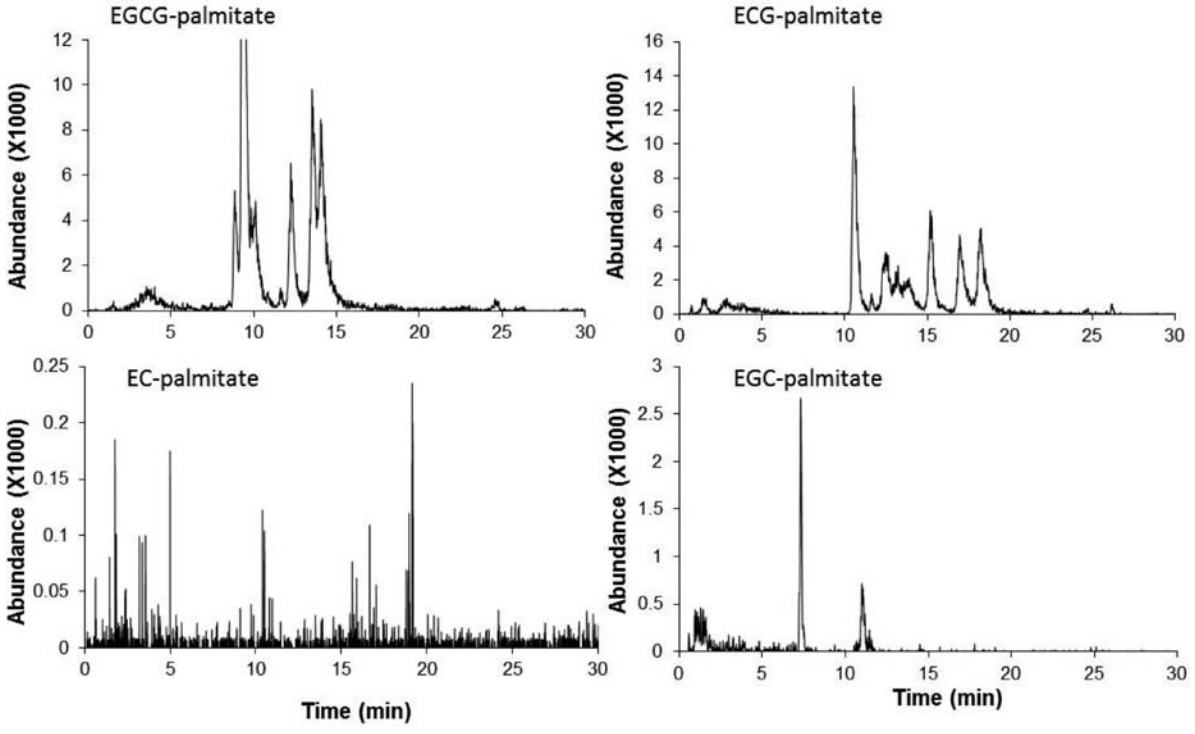

Figure 4. MRM chromatograms of catechin palmitates extracted from Lipton Green Tea, purified, and analyzed by UPLC–MS/MS. Shown are the MRM chromatograms monitoring EGCG, EC, ECG, and EGC palmitates.

myristoyl, palmitoyl, and stearoyl esters of the other three catechins also exhibited analogous profiles within their isomeric groups, i.e., EGC/GC, ECG/CG, and EC/C (data not shown).

A More Detailed UPLC–PDA/MS/MS Analysis of Synthetic Palmitoyl Catechins. Chromatograms of each of the four palmitate catechin families (from Kemin Bioscience) produced clean and positively identified (i.e., MRM) peaks (Figure 3). The first four chromatograms are the more intense of two (essentially identical) MRM transitions for each of the four catechin palmitate families. There are five, six, seven, and eight hydroxyls (acid chloride esterification sites) for C/EC, GC/EGC, CG/ECG and GCG/EGCG, respectively (see Figure 1), summing to 26 catechin esters (52 when considering both epimeric forms). Because fewer than 52 MRM peaks are

Table 3. Percentages of Palmitoyl Catechins per Dry Weight of Various Chinese Green Tea Varieties Harvested from Two Locations (Wuxi and Yixing) in China

green tea variety	catechin palmitates (mg/kg)	green tea variety	catechin palmitates (mg/kg)
Fuding DaBai Wuxi	58	Baiye No. 1 Yixing	34
Zhenong 139 Wuxi	51	Chunlan Yixing	89
Fuding Dahao Wuxi	68	Sucha 130 Yixing	19
Meizhan Wuxi	86	ZhongCha 108 Yixing	32
Fuding Dahao Yixing	27	Fuding Dahao Yixing	27
ChunBoLv Wuxi	40	Longjin 43 Yixing	26
WuNiuZao Wuxi	43	DanGui Yixing	56
Xicha No. 5 Wuxi	63	ChunBoLV Yixing	33
Zhenong 117 Wuxi	53	Zhenong 139 Yixing	61
Xicha No. 11 Wuxi	80	Zhenong 117 Yixing	57
Xicha No. 5 Yixing	57	Longjin Changye Yixing	41
Taicha 12 Yixing	81	Fuding Dabai Yixing	15
YunGuiDaYe Yixing	84	Sucha 120 Yixing	36
Soubei Yixing	55		

observed, we assume that some peaks are not individually resolved and/or not all hydroxyls are equally reactive. Both are likely correct. Peak shapes suggest envelopes of multiple unresolved peaks, while differential reactivity is suggested by the fact that EC/C and EGC/GC both possess single aliphatic hydroxyls (more reactive to esterification than phenolic hydroxyls) that would exhibit two peaks per epimer pair. From the two peaks observed for EC/C and EGC/GC, this indeed appears to be the case. ECG/CG and EGCG/GCG, however, produced more complex chromatograms of multiple peaks each. Because EGCG/GCG and EGC/GC species are known to predominate in green tea (generally ∼50 and ∼30% by mass, respectively), the UV chromatogram is dominated primarily by these contributions (data not shown). Furthermore, while MRM intensities can vary substantially from analyte to analyte as a function of ionization efficiency at the MS source, the relative intensities of catechin palmitates are consistent with catechin natural abundances found in the green tea material from which they were prepared, i.e., EGCG > EGC > ECG > EC. The observed monoisotopic pseudomolecular ions ($[M - H]^-$) were essentially identical to theoretical values for the four catechin palmitate sets (theoretical, observed): EGC/EC (543.2958, 543.53), ECG/CG (679.3118, 679.54), EC/C (527.3009, 527.47), and EGCG/GCG (695.3068, 695.52).

While the natural abundance of catechin palmitates represents only a small fraction of total green tea catechins,

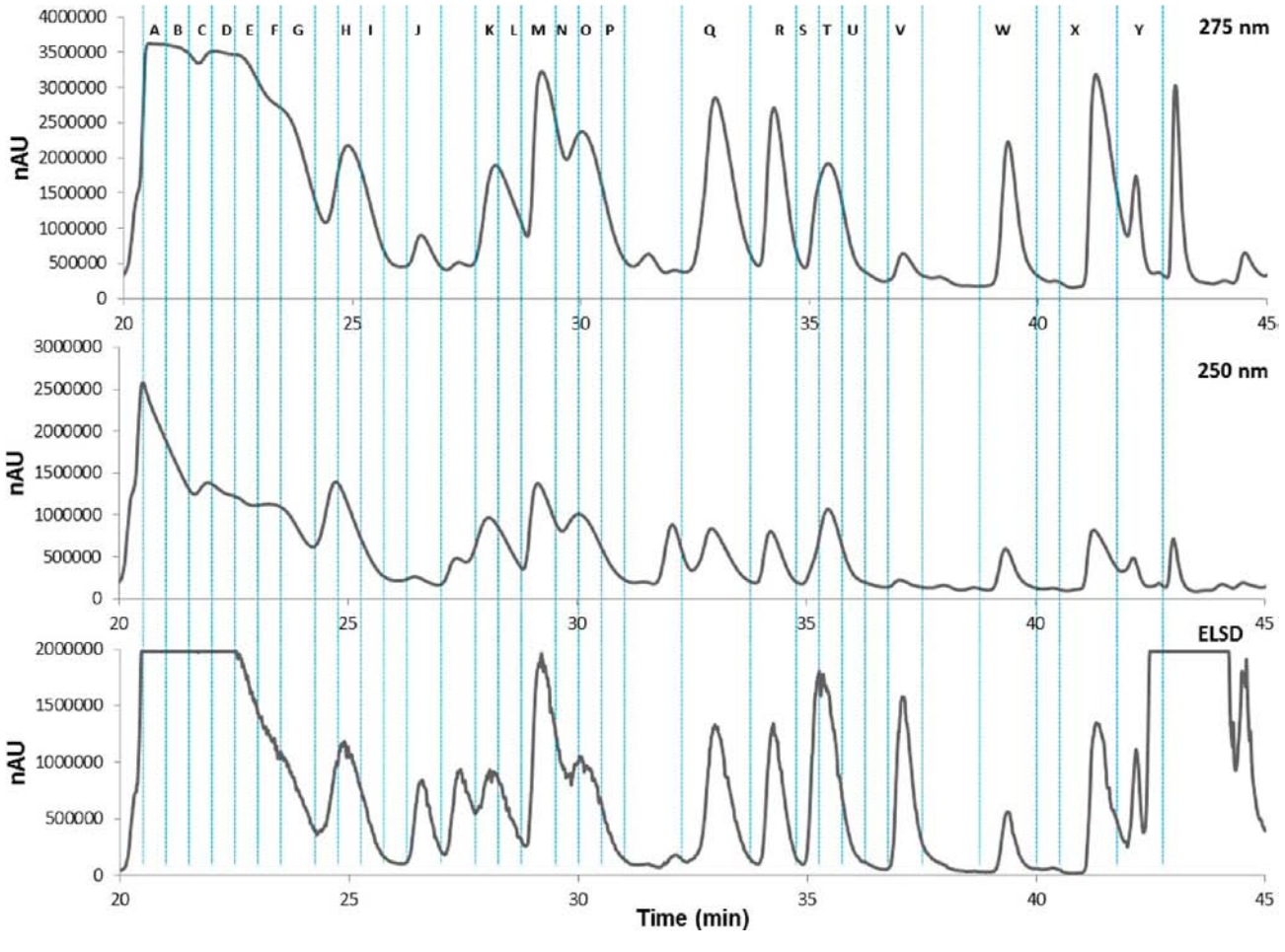

Figure 5. Chromatographic purification of individual synthetic catechin palmitates. Shown is one of 10 preparative chromatograms run in series collecting 30 s slices (vertical bands) using 275 nm peak recognition triggers. The 10 identical slices for each fraction (labeled A–Y, top chromatogram) were pooled, worked up, and analyzed by NMR. Note that fractions Q and V–Y each derive from ten 60 s slices. The top chromatogram was acquired by monitoring at 275 nm (mean absorption maximum of catechin palmitates). The middle chromatogram was monitored at 250 nm to distinguish non-catechin polyphenols (e.g., gallic and gallol palmitates eluting at ∼37 min) where the A_{250}/A_{275} absorption ratio is greater with a pronounced ELSD signal (bottom). The ELSD chromatogram also clearly records the prominent free palmitic acid peak between 42 and 44 min that is not observed at either 250 or 275 nm.

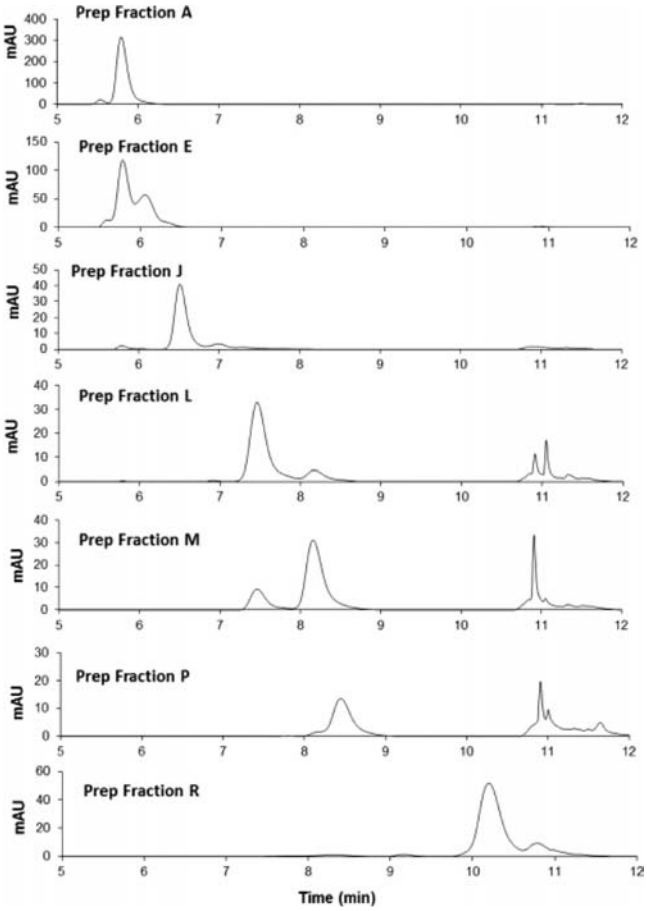

Figure 6. UPLC–PDA/MS/MS analysis of synthetic catechin palmitate preparative fractions (slice fractions from Figure 5). For the sake of simplicity, analyses of only a few of the 25 fractions are shown (275 nm); however, full mass scans were also acquired, and from these, catechin species of all major and minor peaks were easily and unambiguously identified by the pseudomolecular ions (data not shown).

we were concerned about possible contamination by synthetic materials. Every precaution was therefore taken to ensure against carryover: disposable plasticware was used whenever possible, glassware and PLE extraction cells were scrupulously washed, chromatographic columns were subjected to thorough washing and regeneration, the sample loop was cleaned 10 times with ACN before each run, and all chromatographic separations were preceded by blank DMF runs to verify flat baselines.

UPLC–MS/MS Analysis of Fatty Acid Catechin Esters Extracted from Green Tea. The MRM chromatogram of green tea extract monitoring putative myristoyl EGCG exhibited only early eluting peaks that were very weak, while signals for the other (non-EGCG palmitates) were essentially noise (data not shown). However, MRMs of palmitoyl EGCG exhibited stronger signals (Figure 4) with a profile substantially similar to that of synthetic palmitoyl EGCG shown in Figures 2 and 3. Furthermore, the profiles for both ECG and EGC palmitates are also very similar (compare the corresponding panels of Figures 3 and 4). Although the noisy profile of palmitoyl EC is mere baseline (Figure 4), this too is consistent in that EC (the least naturally abundant free catechin in green tea) simply falls below the detection limit in most green tea extracts.[2] Signals recorded for MRMs for putative stearoyl EGCG (like myristoyl catechins) were also marginal (data not shown).

The poor recovery of myristoyl and stearoyl catechins may be explained simply by the low natural abundance of these fatty acids in tea. Wang et al.[16] reported the fatty acid composition of tea seed oil as being nearly devoid of myristic and stearic acids, although the concentrations of palmitic and oleic acids were found to be the most prominent. Assuming that (1) the fatty acid profile for tea leaf is similar to that for tea seed and (2) biosynthesis of fatty acid catechin esters occurs by the likely "one-pot" system yielding product concentrations proportional to the reactant (free catechin) concentrations, one might surmise the observed results are to be expected. If said assumptions are correct, the results of Wang et al. suggest an appreciable presence of oleoyl catechins in green tea. Unfortunately, oleoyl chloride is unavailable commercially, so catechin oleoyl esters were not prepared either to optimize oleoyl EGCG MRMs or to compare their chromatographic profiles with that of extracted oleoyl EGCG. We are currently exploring ways to test this theory.

Multiple dry green tea cultivar samples were extracted by PLE; aliquots were analyzed by UPLC–MS/MS as described above, and their palmitoyl catechin concentrations were calculated (Table 3). Catechin palmitate esters were observed to vary in concentration from 0.02 to 0.09 mg/kg of dry weight of green tea leaf, which corresponds to <0.1% on a total catechin basis.

Large-Scale Purification of Multiple Synthetic Palmitoyl Catechins. The determination of several native palmitoyl catechin structures would ideally follow; however, the purification of sufficient quantities for NMR would be exceedingly difficult as the natural abundance of these compounds in green tea is very low. Moreover, catechin palmitate peaks are broad and late eluting by reversed phase (their best separatory mode), so chromatographic resolution of the 52 closely related isomers is limited. Because synthetic palmitoyl catechins exhibit the same MRM chromatographic profile and elution times as those extracted from green tea, and MRM transitions strongly confirm their identity, several

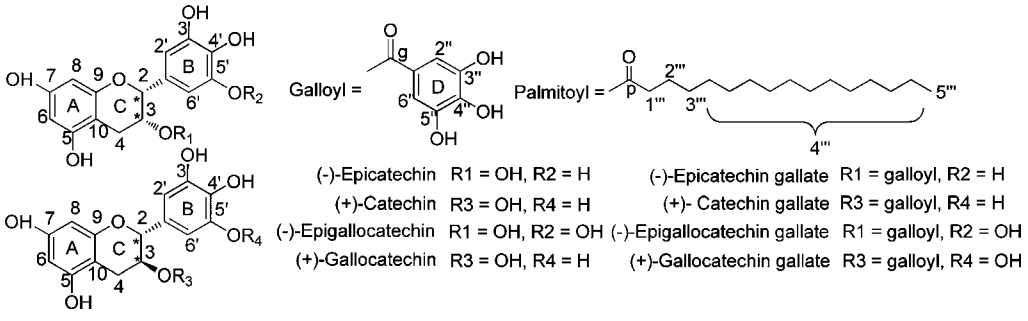

Figure 7. ^{1}H and ^{13}C chemical shift assignments of purified synthetic catechin palmitate structures.

Table 4. Selected ^{1}H Chemical Resonances of Catechins (in parts per million) and Their Shifts Due to Esterification

	EGCG	GCG	ECG	CG	EC	C	GC
			Ring A Protons (6, 8)				
catechin[1]	6.06, 6.04	6.06, 5.99	6.06, 6.04	6.07, 5.99	6.02, 5.92	6.02, 5.87	6.02, 5.88
5-*O*-palmitoyl	6.41, 6.27	6.38, 6.30	6.43, 6.28	6.27, 6.14	–	–	–
7-*O*-palmitoyl	6.31	6.32, 6.28	6.33, 6.31	6.33, 6.28	–	–	–
			Ring B Protons (2′, 5′,[a] 6′)				
catechin[1]	6.62	6.48	7.06, 6.81, 6.76	6.92, 6.79	7.05, 6.84, 6.79	6.90, 680, 6.76	6.46
3′-*O*-palmitoyl	7.00, 6.84	–	7.31, 7.25, 7.09[b]	–	–	–	–
4′-*O*-palmitoyl	6.72	6.57	7.31, 6.95, 6.87[b]	–	–	–	–
			Ring D Protons (2″, 6″)				
catechin[1]	7.03	7.05	7.03	7.05	–	–	–
3″-*O*-palmitoyl	7.37, 7.21	7.37, 7.24	–	7.37, 7.23	–	–	–
4″-*O*-palmitoyl	7.31	7.24	–	–	–	–	–
			Ring C Protons (2, 3)				
catechin[1]	5.07, 5.56	5.13, 5.40	5.13, 5.56	5.15, 5.40,	4.88, 4.21	4.56, 3.99	4.51, 3.97
3-*O*-palmitoyl	–	–	–	–	5.07, 5.41	4.99, 5.27	4.98, 5.26

[a]For ECG, CG, EC, and C. [b]Assignments interchangeable.

Table 5. Selected ^{13}C Chemical Resonances of Catechins (in parts per million) and Their Shifts Due to Esterification

	EGCG	GCG	ECG	CG	EC	C	GC
			Ring A Carbons (6, 8)				
catechin[1]	96.54, 95.87	96.40, 95.57	96.56, 95.87	96.47, 95.61	96.22, 95.75	96.19, 95.50	96.15, 95.49
5-*O*-palmitoyl	103.81, 100.94	*a*	103.41, 101.16	*a*	–	–	–
7-*O*-palmitoyl	102.03, 101.90	102.37, 102.03	102.38, 102.02	101.84, 101.68	–	–	–
			Ring A Carbons (5, 7, 9)				
catechin[1]	157.78, 157.46, 157.12[1]	158.02, 157.23, 156.24	157.81, 157.50, 157.14	158.08, 157.25, 156.32	157.61, 157.60, 157.19	157.77, 157.23, 156.95	157.74, 157.24, 156.90
5-*O*-palmitoyl	157.01, 156.24, 151.65	*a*	156.43, 156.28, 150.53	*a*	–	–	–
7-*O*-palmitoyl	156.79, 155.91, 151.21	156.79, 155.91, 151.44	157.08, 156.81, 151.22	155.85, 151.05	–	–	–
			Ring B Carbons (2′, 5′,[b] 6′)				
catechin[1]	106.81	106.20	119.25, 115.67, 114.95	119.13, 115.99, 114.30	119.41, 115.51, 115.31	120.08, 115.74, 115.27	107.25
3′-*O*-palmitoyl	113.64, 112.05	–	122.40, 118.55, 115.81[c]	–	–	–	–
4′-*O*-palmitoyl	107.07	106.20	125.67, 117.09, 115.46[c]	–	–	–	–
			Ring D Carbons (2″, 6″)				
catechin[1]	110.04	109.99	109.99	109.98	–	–	–
3″-*O*-palmitoyl	114.34, 114.17	*a*	–	116.54, 114.09	–	–	–
4″-*O*-palmitoyl	125.71, 121.58	115.47	–	–	–	–	–
			Ring C Carbons (2, 3)				
catechin[1]	78.10, 69.36	78.64, 70.36	78.11, 69.36	78.80, 70.46	79.46, 66.97	82.76, 68.38	82.80, 68.35
3-*O*-palmitoyl	–	–	–	–	77.76, 68.85	78.91, 69.93	78.76, 69.86

[a]No ^{13}C due to the low abundance of this compounds in the sample. [b]For ECG, CG, EC, and C. [c]Assignments interchangeable.

synthetic palmitoyl catechins were purified by preparative chromatography for their structures to be determined by NMR.

Ten identical preparative chromatographic runs (3.0 mL injections each) were made. The corresponding 25 slices [labeled A–Y (example chromatogram in Figure 5)] were pooled for each fraction; aliquots were removed for analysis, and then both aliquots and bulk fractions were taken to dryness as described above. Once aliquots of each fraction had been dried *in vacuo*, each was taken up in 800 μL of DMF and analyzed by UPLC–PDA/MS/MS to evaluate their purity and the masses of the components. Figure 6 shows several fractions thus analyzed. The material of most fractions was sufficiently abundant and of low complexity for direct NMR analysis without further purification.

Structures of Purified Synthetic Catechin Palmitates As Determined by One-Dimensional and Two-Dimensional (2D) NMR. There are 21 unique catechin palmitates that were positively identified in fractions that resulted from the chromatographic separation. Those most abundant species (number in parentheses) are EGCG palmitates (six) followed by GCG (five), ECG (four), and CG (three). There was only a single palmitate isomer observed for each of EC, GC, and C (at hydroxyl 3), as well as free palmitic acid. All of the novel compounds observed were fully characterized by ^{1}H, ^{13}C, and 2D NMR techniques to determine the position of the palmitoyl moiety.

In general, there are six proton signals in gallocatechin gallates (see Figure 7), namely, positions 2 (~5 ppm), 3 (~5.5 ppm), 4 (2.8–3.1 ppm), 6 and 8 (~6 ppm), 2′ and 6′ (~6.5–7 ppm), and 2″ and 6″ (~7 ppm). Catechin gallates exhibit an additional proton

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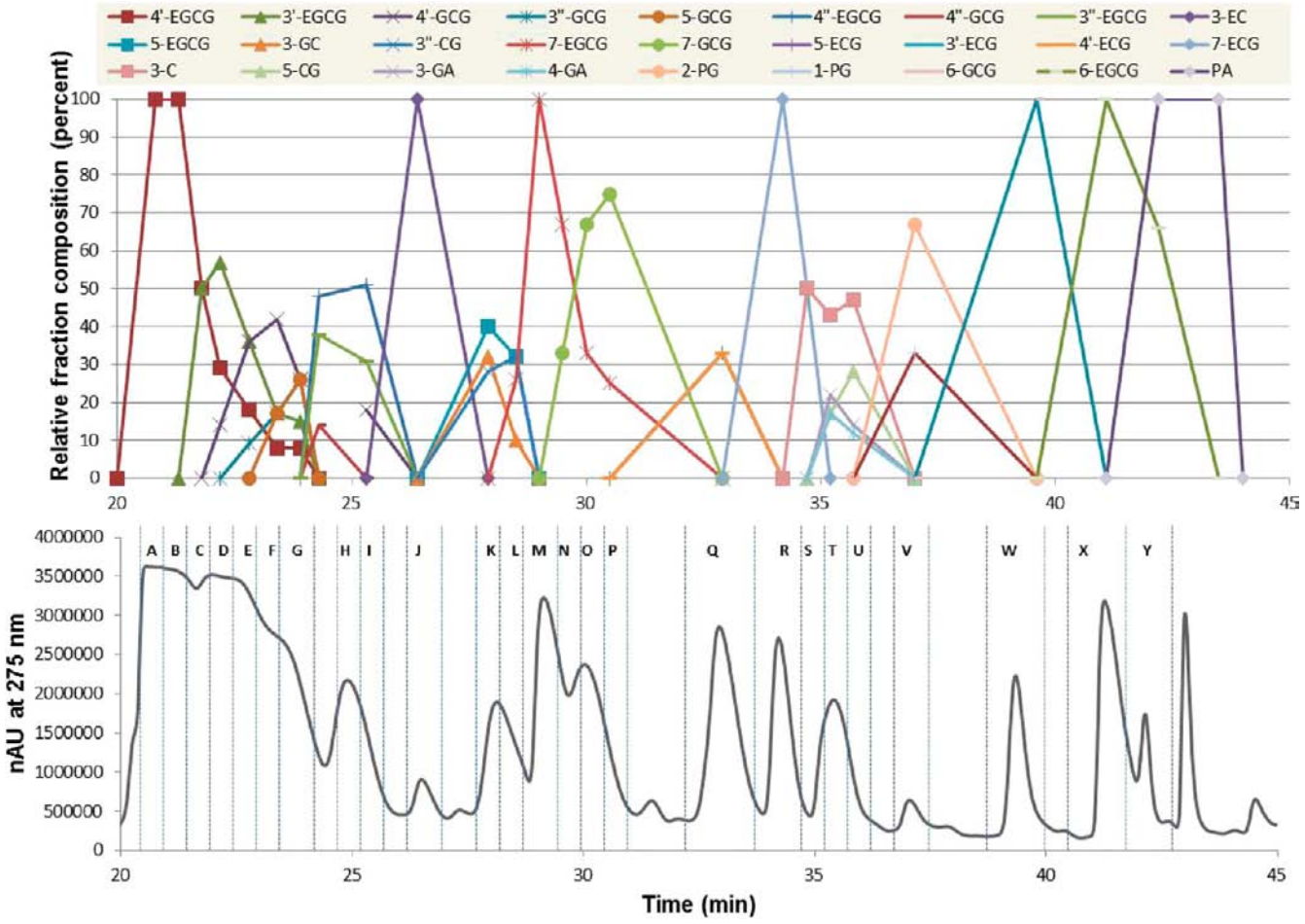

Figure 8. Composition chromatogram (top) in which each point is a fraction purified by preparative chromatography of synthetically palmitoylated catechin extract with the compound composition and the relative proportions of each, as determined by NMR. This composition chromatogram is aligned with the preparative chromatogram (bottom) from which the fractions derive (A_{275} chromatogram in Figure 5). Structure acronyms in the legend represent the catechin and its site (carbon number) of palmitate esterification according to Figure 7.

signal at position 5′ (~6.8 ppm). Epi isomers are easily recognizable in the ^{1}H spectrum because of their small coupling constant between protons 2 and 3 (~1.5 Hz),[17] which is usually not very well resolved, giving rise to a broad singlet. GCG, GC, CG, and C palmitates exhibit a quartet for proton 3 (J = 5−8 Hz, sometimes reported as a multiplet) and a doublet for position 2 (J = 5−8 Hz).[17]

Upon esterification, the chemical shift of neighboring protons and carbons in the molecule was significantly affected. Esterification of hydroxyl groups positioned on ring A of EGCG, GCG, ECG, and CG resulted in a downfield shift of protons 6 and 8 by ~0.3 ppm, as shown in Table 4. Additionally, chemical shifts of ring A protons in 5-O-substituted catechins were split apart by 0.08−0.15 ppm, as the proton at position 6 was more influenced by the ester than the distant proton 8. Carbon resonances at positions 6 and 8 shifted downfield by ~5−6 ppm upon esterification, while the carbon chemical shift of the ester position (either 5 or 7) was moved upfield by ~5 ppm (Table 5).

When ring B of EGCG or GCG was esterified at position 4′, it resulted in a shift of protons 2′ and 6′ by 0.10 ppm (Table 4). Esterification at position 3′ caused a much greater shift of these protons, specifically by 0.38 and 0.22 ppm, respectively. The carbon chemical shift of positions 2′ and 6′ changed slightly (0.26 ppm) when the ester was present at position 4′ (Table 5), in contrast with the results of esterification at the 3′-hydroxyl that caused a downfield shift of the aforementioned carbons by more than 6 ppm. ECG substituted at the 3′ or 4′ position also exhibited a downfield shift of ring B protons and carbons (positions 2′, 5′, and 6′). Esterification of the hydroxyl group at position 3 in EC, C, and GC caused a downfield shift of protons at positions 3 (by ~1.2 ppm) and 2 (by ≥0.2 ppm). Carbon 3 also shifted downfield, while the carbon at position 2 underwent upfield shifts of 1.7 (EC), 3.85 (C), and 4.04 ppm (GC). Similar to esterification of ring B, esterification of the gallate ring in EGCG, GCG, and ECG gave rise to a downfield shift of its protons and carbons (positions 2″ and 6″). Furthermore, when position 3″ was derivatized, two distinct proton resonances were observed because of the loss of symmetry in the molecule.

As expected, the palmitoyl moiety upon esterification exhibited shifts compared to palmitic acid. The most dramatic shift was experienced by proton 1‴. In catechin gallate esters, the protons at position 1‴ were shifted further downfield (2.54−2.68 ppm) than those of palmitic acid (2.28 ppm). Interestingly, esterification of catechin esters at position 3 (3-*O*-palmitoyl EC, C, and GC) resulted in upfield shifts of the 1‴ protons to 2.25−2.18 ppm.

Given the 52 possible monopalmitate isomers sharing only four different masses (one for each catechin species), baseline separation of each of the catechin palmitates was challenging, as expected. Nevertheless, with the combination of chromatographic

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Table 6. Approximate Relative Abundance of Synthetic Catechin Palmitate Structures (PA is palmitic acid)

catechin palmitate species	area counts (275 nm)	relative abundance (%)
4′-EGCG	4.76×10^6	31
3′-EGCG	2.24×10^6	14
4′-GCG	1.28×10^6	8.4
3″-GCG	5.07×10^5	3.4
G-GCG	3.98×10^5	2.6
4″-EGCG	6.87×10^5	4.5
3″-EGCG	4.93×10^5	3.2
4″-GCG	2.19×10^5	1.4
3-EC	1.00×10^5	0.66
5-EGCG	2.05×10^5	1.3
3-GC	1.40×10^5	0.92
3″-CG	1.63×10^5	1.1
7-EGCG	1.05×10^6	6.9
7-GCG	5.48×10^5	3.6
5-ECG	3.66×10^5	2.4
3′-ECG	3.66×10^5	2.4
4′-ECG	3.66×10^5	2.4
7-ECG	6.00×10^5	3.9
3-C	2.95×10^5	1.9
7-CG	6.09×10^4	0.40
3-GA	1.04×10^5	0.68
4-GA	8.42×10^4	0.55
5-CG	5.58×10^4	0.36
2-PG	1.00×10^4	0.066
1-PG	5.00×10^4	0.33
5-CG	4.30×10^4	0.28
total (%)		99.6

resolution, the collection of thinly "sliced" preparative chromatographic fractions, and the high-field (600 MHz) NMR selectivity, we were able to isolate the most abundant compounds and to determine their structures with considerable confidence. High-field NMR allowed such strong and well-resolved 2D connectivities that it was possible to acquire structural data of multiple compounds within the same fraction (Figure 6). The approximate proportions of these compounds per fraction were determined from the relative NMR signal intensities, a plot of which versus the fractions' preparative chromatographic retention times, produces a semiquantitative composition chromatogram of all the catechin palmitate structures observed in this study (Figure 8). From Table 6 and Figure 8, it is clear that 4′-*O*-palmitoyl EGCG predominates in synthetic catechin palmitates, representing approximately 30% of total catechin palmitates. Furthermore, the same adduct also appears to predominate in native green tea. This is not surprising given that (1) the oxygen of the *para* 4′-*O*-hydroxyl is more nucleophilic than the corresponding 4″-*O*-hydroxyl (*para* to its ester at C1″, deactivating and *meta*-directing) and (2) the 4′-*O*-hydroxyl is distally located on the molecule where it experiences relatively little steric obstruction. Moreover, making up approximately 50% of total green tea catechins, free EGCG is the most abundant.

From our review of the literature, this is the first report in which palmitoylated catechins have been identified in green tea. While the four synthetic catechin palmitate families of Figure 3 correspond in retention time and profile to those of native catechin palmitates extracted from green tea in Figure 4, their qualitative identity is clear. However, they exhibit substantial quantitative differences that are not surprising because one set was synthesized *in vitro* while the other was derived from the *in vivo* environment of botanical tissue. It is unknown if these catechin esters derive chemically in the cell's cytosol, but like similar (single-pot) biosynthetic processes, they are likely mediated enzymatically, perhaps with membrane association as a source of palmitate.

In conclusion, this work demonstrates the presence of palmitoylated catechins (and likely other catechin fatty acid esters) that are naturally occurring in all 28 of the green tea varieties investigated to date. Furthermore, these naturally occurring lipid-conjugated catechins have exhibited great efficacy as shelf life extenders for even fatty foods where they outperform synthetic antioxidants, and we report here that they are modeled after the natural structures of their endogenous botanical counterparts.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A list of 25 compounds identified and their respective ^{1}H and ^{13}C NMR resonance assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

EC, (−)-epicatechin; ECG, (−)-epicatechin 3-gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin 3-gallate

■ REFERENCES

(1) Senanayake, N. Green tea extract: Chemistry, antioxidant properties and food applications—A review. *J. Funct. Foods* **2013**, in press.

(2) Gramza, A.; Korczak, J. Tea constituents (*Camellia sinensis* L.) as antioxidants in lipid systems. *Trends Food Sci.* **2005**, *16*, 351−358.

(3) Cutler, S.; Fuller, E.; Rotberg, I.; Wray, C.; Troung, M.; Poss, M. Antioxidant Formulations. International Patent WO 2013/036934 A1, March 14, 2013.

(4) Zhong, Y.; Shahidi, F. Lipophilized epigallocatechin gallate (EGCG) derivatives as novel antioxidants. *J. Agric. Food Chem.* **2011**, *59*, 6526−6533.

(5) Lattanzio, V.; Lattanzio, V. M.; Cardinoli, A. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. In *Phytochemisty: Advances in Research*; Imperato, F., Ed.; Research Signpost: Kerala, India, 2006; pp 23−67.

(6) Macfarlane, A.; Macfarlane, I. *The Empire of Tea*, 1st ed.; The Overlook Press: New York, 2004.

(7) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Mechanistic studies of catechins as antioxidants against radical oxidation. *Arch. Biochem. Biophys.* **1999**, *362*, 79−86.

(8) Benavides, A.; Montoro, P.; Bassarello, C.; Piacente, S.; Pizza, C. Catechin derivatives in *Jatropha macrantha* stems: Characterization and LC/ESI/MS/MS quali-quantitative analysis. *J. Pharm. Biomed. Anal.* **2006**, *40*, 639−647.

(9) Hakala, P.; Lampi, A.; Ollilainen, V.; Werner, U.; Murkovic, M.; Wahala, K.; Karkola, S.; Piironen, V. Steryl phenolic acid esters in cereals and their milling fractions. *J. Agric. Food Chem.* **2002**, *50*, 5300−5307.

(10) Henning, S. M.; Fajardo-Lira, C.; Lee, H. W.; Youssefian, A. A.; Go, V. L. W.; Heber, D. Catechin content of 18 teas and a green tea supplement correlates with the antioxidant capacity. *Nutr. Cancer* **2003**, *45*, 226−235.

(11) Robbins, R. J. Phenolic acids in foods: An overview of analytical methodology. *J. Agric. Food Chem.* **2003**, *51*, 2866−2887.
(12) Suzuki, M.; Sano, M.; Yoshida, R.; Degawas, M.; Miyanse, T.; Meaed-Yamamoto, M. Epimerization of tea catechins and *O*-methylated derivatives of (−)-epigalocatechin-3-*O*-gallate: Relationship between epimerization and chemical structure. *J. Agric. Food Chem.* **2003**, *51*, 510−514.
(13) Velayuthan, P.; Babu, A.; Liu, D. Green tea catechins and cardiovascular health: An update. *Curr. Med. Chem.* **2008**, *15*, 1840−1850.
(14) Roleira, G. M. F.; Siquet, C.; Orru, E.; Garrido, E. M.; Garrido, J.; Milhazes, N.; Podda, G.; Paiva-Martins, F.; Reid, S.; Carvalho, R. A.; Tavares da Silva, E.; Borges, F. Lipophilic phenolic antioxidants: Correlation between antioxidant profile, partition coefficients and redox properties. *Bioorg. Med. Chem.* **2010**, *18*, 5816−5825.
(15) Murakami, I.; Nakamure, T.; Ishibashi, Y.; Shibuya, R.; Ayano, E.; Morita-Murase, Y.; Nagata, Y.; Kanazawa, H. Simultaneous determination of catechins and procyanidins in bottled tea drinks by LC/MS. *Chromatography* **2006**, *27*, 27−33.
(16) Wang, Y.; Sun, D.; Chen, H.; Qian, L.; Xu, P. Fatty acid composition and antioxidant activity of tea (*Camellia sinensis* L.) seed oil extracted by optimized supercritical carbon dioxide. *Int. J. Mol. Sci.* **2011**, *12*, 7708−7719.
(17) Davis, A. L.; Cai, Y.; Davies, A. P.; Lewis, J. R. ^{1}H and ^{13}C Assignments of Some Green Tea Polyphenols. *Magn. Reson. Chem.* **1996**, *34*, 887−890.