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Screening Antioxidants Using LC-MS: Case Study with Cocoa

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Oxidative stress enhances pathological processes contributing to cancer, cardiovascular disease, and neurodegenerative diseases, and dietary antioxidants may counteract these deleterious processes. Because rapid methods to evaluate and compare food products for antioxidant benefits are needed, a new assay based on liquid chromatography−mass spectrometry (LC-MS) was developed for the identification and quantitative analysis of antioxidants in complex natural product samples such as food extracts. This assay is based on the comparison of electrospray LC-MS profiles of sample extracts before and after treatment with reactive oxygen species such as hydrogen peroxide or 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). Using this assay, methanolic extracts of cocoa powder were analyzed, and procyanidins were found to be the most potent antioxidant species. These species were identified using LC-MS, LC-MS/MS, accurate mass measurement, and comparison with reference standards. Furthermore, LC-MS was used to determine the levels of these species in cocoa samples. Catechin and epicatechin were the most abundant antioxidants followed by their dimers and trimers. The most potent antioxidants in cocoa were trimers and dimers of catechin and epicatechin, such as procyanidin B2, followed by catechin and epicatechin. This new LC-MS assay facilitates the rapid identification and then the determination of the relative antioxidant activities of individual antioxidant species in complex natural product samples and food products such as cocoa.

KEYWORDS: LC-MS/MS; antioxidant; catechin; epicatechin; procyanidin; cocoa

INTRODUCTION

Oxidative stress is caused by an imbalance between the effects of pro-oxidants such as reactive oxygen species and defense mechanisms such as antioxidants in tissues, with the balance being shifted in favor of the pro-oxidants (*1*). As a result, excessive oxidative damage can occur to biological molecules including DNA, lipids, and proteins that might contribute to the development of cancer, cardiovascular disease, or neurodegenerative diseases (*2*, *3*). One of the most abundant reactive oxygen species formed in tissue is hydrogen peroxide (*4*). Although hydrogen peroxide has low reactivity compared to some other reactive oxygen species such as superoxide and hydroxyl radicals, it can cross biological membranes and then contribute significantly to pathological processes through the generation of hydroxyl radicals via Fenton chemistry (reactions requiring transition metal ions such as iron) (*5*). Cellular defenses against reactive oxygen species include enzymes such as catalase, which degrades hydrogen peroxide into water and oxygen, and antioxidants such as tocopherol and ascorbic acid, which neutralize reactive oxygen species. Most of these antioxidants in cells and tissues are supplied by the diet (*6*).

Measurements of antioxidant levels in biological samples such as food extracts, urine, or serum are usually carried out using assays based either on hydrogen atom transfer reactions or on electron transfer reactions (*7*). The most popular hydrogen atom transfer assays include the oxygen radical absorbance capacity assay (ORAC), the total radical trapping antioxidant parameter assay, and crocin bleaching assays. Electron transfer reaction-based assays include the total phenols assay using the Folin−Ciocalteu reagent, the Trolox equivalence antioxidant (TEAC) capacity assay, the ferric ion reducing antioxidant power assay (FRAP), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay. Although these assays enable the determination of the total antioxidant capacity of a sample or, in some cases, the antioxidant capacity of only the most potent antioxidants present, they do not provide information regarding the identities, chemical structures, or relative amounts of specific antioxidant compounds. To address the need for identification and quantitative analysis of specific antioxidants in biomedical specimens and foods, we developed a new antioxidant assay based on liquid chromatography−mass spectrometry (LC-MS) and LC−tandem mass spectrometry (LC-MS/MS).

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This approach utilizes the selectivity of HPLC separation and high-resolution tandem mass spectrometric analysis for the characterization, identification, and then quantitative analysis of antioxidant species in complex samples.

As a first application, the LC-MS antioxidant screening assay was applied to the analysis of antioxidants in cocoa. Commonly called cacao or cocoa, *Theobroma cacao* L. (Sterculiaceae) is a native species of Mesoamerica, has been used in traditional medicine as a diuretic, cardiotonic, and antiseptic (*8*), and is currently consumed in food products as cocoa powder or chocolate prepared from cocoa beans. Antioxidants in cocoa such as catechins and procyanidins have been reported to have beneficial effects as chemoprevention agents (*9*), and the levels of these antioxidants in cocoa can be higher than in black tea, green tea, or red wine (*10*). Screening cocoa using LC-MS facilitated the identification of the antioxidant constituents that function as free radical scavengers as well as the determination of the levels of individual compounds, which is information not available from previous antioxidant assays.

MATERIALS AND METHODS

Chemicals. (−)-Epicatechin, (+)-catechin, (−)-epigallocatechin, procyanidins B1 and B2, quercetin-3-*O*-*β*-D-glucopyranoside, (−)-gallocatechin, (−)-epigallocatechin gallate, (−)-gallocatechin gallate, (−)-epicatechin gallate, and DPPH were purchased from Sigma-Aldrich (St. Louis, MO). Genistein was purchased from Indofine Chemical (Somerville, NJ). HPLC-grade methanol, acetonitrile, hexanes (mixture of isomers), certified hydrogen peroxide 50%, and nylon syringe filters (0.2 μm pore size) were purchased from Fisher (Fair Lawn, NJ). Formic acid was purchased from EMD Chemicals (San Diego, CA). Purified water was prepared by using a Millipore Milli-Q purification system (Millipore, Billerica, MA). All other chemicals and solvents were of ACS reagent grade, unless stated otherwise.

Preparation of Cocoa Powder. The cocoa powder of *T. cacao* L. used in this study was provided by the Hershey Co. (Hershey, PA). Cocoa powder (1 g) was defatted with a mixture of hexanes. The defatted powder (100 mg) was extracted three times with methanol, and the methanol extracts were combined, sonicated for 15 min, and centrifuged for 4 min at 4 °C. The supernatants were filtered (0.2 μm) and evaporated to dryness under a stream of nitrogen. The yield of cocoa extract was 14.8%.

Hydrogen Peroxide Oxidation Assay. Oxidation of the cocoa extract was carried out using hydrogen peroxide according to the method of Zhu et al. (*11*) with modifications. Briefly, 100 μL of cocoa extract (0.25 mg/mL) was treated with 100 μL of hydrogen peroxide (50%; 7.35 M final concentration) at 25 °C. Aliquots were removed at 0, 3, 6, 12, and 24 h and placed in an ice bath to stop the oxidation reaction. As a control, a duplicate sample of cocoa extract was treated identically except that 100 μL of water was substituted for the hydrogen peroxide solution. A 100 μL aliquot of each the reaction mixture was diluted with 100 μL of water containing genistein (final concentration = 2.5 μg/mL) as an internal standard, and 10 μL was injected onto the LC-MS or LC-MS/MS to profile the hydrogen peroxide reactivity of each constituent. The cocoa extract was analyzed using LC-MS to make certain that no genistein was present.

In addition to the cocoa extract, a mixture of botanical antioxidants including catechin, epicatechin, procyanidin B2, epigallocatechin, and epigallocatechin gallate (10 μg/mL each) was treated with hydrogen peroxide and analyzed as described above. As a control, the stability of each catechin with water substituted for hydrogen peroxide solution was investigated at 25 °C for 72 h. The percentage of each remaining compound in each sample was determined as the percentage ratio between the (compound peak area)/(internal standard peak area) at 0, 3, 6, 12, and 24 h divided by the (compound peak area)/(internal standard peak area) at 0 h. Finally, the extent of oxidation of each compound was calculated by subtracting the percentage of remaining compound at each time point from 100%, and the average extent of oxidation (percent) was calculated using triplicate measurements of three different samples.

DPPH Oxidation Assay. Reactions of DPPH with cocoa powder extracts were carried out as follows. A 10 μL aliquot of cocoa extract (0.25−4.82 mg/mL) in methanol/water (20:80; v/v) was vortex mixed with 90 μL of 2.25 mM (0.9 mg/mL) DPPH in methanol. After 60 s, the reaction solution was diluted with 100 μL of water (to lower the organic solvent concentration prior to reversed phase HPLC) and then analyzed immediately using LC-MS. Control samples were treated identically except for the substitution of blank solvent for either the cocoa powder extract or the DPPH solution.

LC-MS Screening for Oxidation by Hydrogen Peroxide. LC-MS and LC-MS/MS analyses of samples from the hydrogen peroxide reactions were carried out using a Shimadzu (Columbia, MD) LC-10A HPLC system and Leap (Carrboro, NC) HTS PAL autosampler interfaced to an Applied Biosystems (Foster City, CA) API 4000 triple-quadruple mass spectrometer. Botanical antioxidants were separated on a 150 mm × 3 mm i.d. X-Terra C_{18} analytical column (Waters, Milford, MA) using a linear gradient from 10 to 60% acetonitrile/methanol (1:1; v/v) in water (containing 0.1% formic acid) at a flow rate of 0.2 mL/min. The injection volume was 10 μL. Negative ion electrospray tandem mass spectrometric analysis for quantitative analysis of each botanical antioxidant was carried out at unit resolution using collision-induced dissociation and selected reaction monitoring (SRM). The ion source temperature was 350 °C, the ion spray voltage was −4200 V, and the declustering potential was varied from −85 to −105 V. Nitrogen was used as the collision gas at −20 to −35 eV, and the dwell time was 1 s/ion. During SRM, catechin, epicatechin, procyanidin B1, procyanidin B2, epigallocatechin, quercetin-3-*O*-*β*-D-glucopyranoside, and epigallocatechin gallate were measured by recording the signals for the transitions of the deprotonated molecules of *m*/*z* 289, 289, 577, 577, 305, 463, and 457 to the most abundant fragment ions of *m*/*z* 245, 245, 407, 289, 299, and 169, respectively. The SRM transition from *m*/*z* 269 to 133 was monitored for the internal standard genistein. The limits of detection (LOD), defined as a signal-to-noise of 3:1, were 50 ng/mL for catechin, epicatechin, epigallocatechin, and epigallocatechin gallate and 75 ng/mL for procyanidins B1 and B2 and quercetin-3-*O*-*β*-D-glucopyranoside. The internal standard genistein was stable during sample handling and analysis. Data were acquired and analyzed using Analyst software version 1.2 from Applied Biosystems.

LC-MS Screening for Oxidation by DPPH. Samples from the DPPH reactions were analyzed using a Shimadzu LC-20AD HPLC system interfaced to a high-resolution Shimadzu ion trap time-of-flight hybrid mass spectrometer. Both negative ion and positive ion mass spectra were acquired during each analysis over the range *m*/*z* 125−950 using polarity switching using Shimadzu Solutions software version 3.0. The ion source temperature (CDL and heat block) was 200 °C. The injection volume, HPLC column, and flow rate were identical to those used for the analysis of the hydrogen peroxide oxidation products. However, the mobile phase consisted of a 60 min linear gradient from 10 to 37% acetonitrile/methanol (1:1; v/v) in water at flow rate of 0.2 mL/min over 60 min.

High-Resolution LC-MS Analyses. For the characterization and identification of antioxidants in cocoa, positive ion electrospray mass spectra and tandem mass spectra were obtained at a resolving power of 20000 using a Thermo Finnigan (San Jose, CA) LTQ Fourier transform ion cyclotron resonance mass spectrometer equipped with a Michrom (Auburn, CA) microcapillary HPLC system and a 150 mm × 2.1 mm i.d., 3 μm, BioSuite C_{18} column. The HPLC mobile phase consisted of a 50 min linear gradient from 95:4.9:0.1 to 40:59.9:0.1 (v/v/v) water/acetonitrile/0.1% formic acid at a flow rate of 200 μL/min. Acetonitrile was used in place of methanol/acetonitrile during these high-performance LC-MS analyses to improve the separation of catechins. Product ion tandem mass spectra were obtained using collision-induced dissociation with the collision gas at 22 eV and a collision energy of 35 eV.

RESULTS AND DISCUSSION

Catechin and Cocoa Oxidation by Hydrogen Peroxide. The time course of the oxidation of a mixture of procyanidin and catechins by hydrogen peroxide was investigated to validate the hypothesis that LC-MS screening may be used to compare the relative antioxidant properties of compounds in mixtures. Because cocoa is known to contain procyanidins and catechins (*12*), these natural products were used to test this assay instead of synthetic

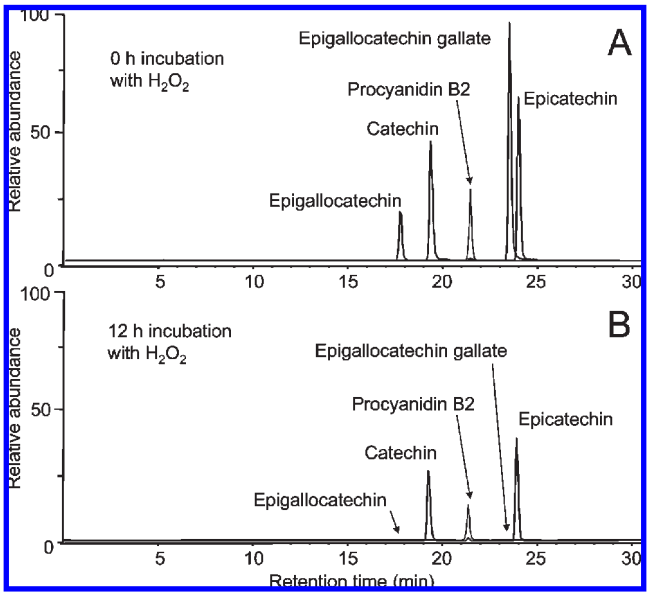

Figure 1. Negative ion LC-MS total ion chromatograms showing the HPLC separation and mass spectrometric detection of the deprotonated molecules of procyanidins and catechins in a standard mixture at (**A**) 0 h and (**B**) 12 h after the addition of hydrogen peroxide. The decrease in peak height of each antioxidant is the result of oxidative degradation.

antioxidants such as butylated hydroxytoluene or natural antioxidants that do not occur in cocoa. Negative ion electrospray LC-MS analyses of the botanical antioxidant test mixture at 0 and 12 h after the addition of hydrogen peroxide are shown in **Figure 1**. The HPLC retention times for catechin, epicatechin, epigallocatechin, epigallocatechin gallate, and procyanidin B2 were 19.3, 23.9, 17.8, 23.8, and 21.3 min, respectively. The reduction in peak heights of the deprotonated molecules of the antioxidants in the chromatogram 12 h after treatment with hydrogen peroxide indicates that the concentrations of the antioxidants decreased due to oxidation.

To verify that the concentrations of these antioxidants did not decrease due to other factors during the incubation with hydrogen peroxide, a control incubation in which water was added in place of hydrogen peroxide was carried out. LC-MS analyses of aliquots of the incubation mixture obtained at 0, 3, 6, 12, and 24 h indicated that catechin, epicatechin, epigallocatechin, epigallocatechin gallate, and procyanidin B2 showed 0% oxidation at 25 °C for up to 24 h (data not shown). Therefore, these compounds did not degrade during the assay due to factors other than oxidation by hydrogen peroxide.

The graph in **Figure 2** shows how the relative amounts (based on LC-MS detection) of each antioxidant in the mixture of standards decreased over time during treatment with hydrogen peroxide. Because the rate of reaction of each antioxidant in the presence of hydrogen peroxide was not the same, this suggests that this LC-MS assay may be used to determine the relative antioxidant activity of each species. In this mixture, the oligomeric procyanidins were the most reactive, and the monomeric catechin and epicatechin were the least reactive antioxidants. As indicated by **Figure 2**, the order of reactivity of the antioxidant standards with hydrogen peroxide was epigallocatechin > procyanidin B2 > procyanidin B1 > catechin ≈ epicatechin.

Next, incubation with hydrogen peroxide and LC-MS analysis were used to investigate the antioxidants in a cocoa powder extract. On the basis of inspection of the LC-MS data, eight chromatographic peaks were observed, corresponding to

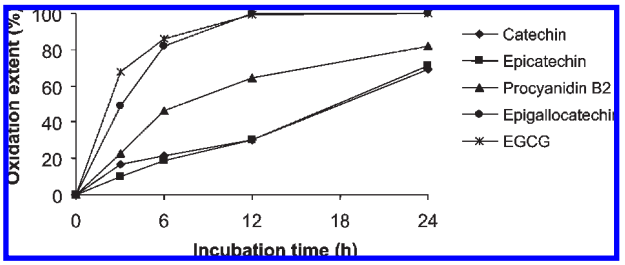

Figure 2. Time course of catechin and procyanidin oxidation by hydrogen peroxide in a mixture of standards. Each data point represents the mean of three experiments measured using LC-MS/MS.

compounds in the cocoa powder extract that decreased over time due to oxidation by hydrogen peroxide. Two of these antioxidants, detected as their deprotonated molecules of *m*/*z* 739 and 867, were initially unknown. The identities of the other six antioxidants in the cocoa extract were determined on the basis of comparison with authentic standards including the demonstration of coelution during LC-MS, identical tandem mass spectra, and identical elemental compositions as indicated by high-resolution accurate mass measurement. The antioxidants identified in this manner were catechin ($[M - H]^-$ detected at *m*/*z* 289), epicatechin ($[M - H]^-$ of *m*/*z* 289), procyanidin B1 ($[M - H]^-$ of *m*/*z* 577), procyanidin B2 ($[M - H]^-$ of *m*/*z* 577), epigallocatechin ($[M - H]^-$ of *m*/*z* 305), and quercetin-3-*O*-*β*-D-glucopyranoside ($[M - H]^-$ of *m*/*z* 463). **Figure 3** shows a comparison of the LC-MS/MS detection of these six antioxidants in cocoa powder with a mixture of botanical antioxidant standards.

The detection of abundant catechin and epicatechin in cocoa (**Figure 3A**) is consistent with reports in the literature that these compounds are the most abundant antioxidants in chocolate (*13*). In addition, the flavonoid glucoside quercetin-3-*O*-*β*-D-glucopyranoside has been reported previously in cacao liquor (*14*). The shoulder at 22.6 min on the HPLC peak for quercetin-3-*O*-*β*-D-glucopyranoside (eluting at 23.0 min) in both the cocoa extract and the standard suggests the formation in solution of the conformational isomer, quercetin-3-*O*-*β*-D-glucofuranoside (**Figure 3B**). Procyanidin oligomers (**Figure 3**) have also been reported in cocoa (*15*), and epigallocatechin has been identified in *T. cacao* (*16*) and chocolate (*17*). However, no epigallocatechin gallate or epicatechin-3-*O*-gallate was found in these samples of cocoa powder.

To characterize the unknown cocoa antioxidant eluting at 21.8 min (**Figure 4**), high-resolution positive ion electrospray MS and MS/MS analyses were carried out. Positive ion electrospray was used instead of negative ion mode for the structural characterization and identification of larger phenolic antioxidants such as procyanidins, because structurally significant fragment ions have been reported in the literature for these compounds (*15*, *19*). Using high-resolution accurate mass measurement, the protonated molecule of this antioxidant was observed at *m*/*z* 739.1860 corresponding to a compound with an elemental composition of $C_{36}H_{34}O_{17}$, which is consistent with a procyanidin dimer. Next, the high-resolution product ion tandem mass spectrum of this protonated molecule was obtained, and three abundant fragment ions were observed at *m*/*z* 721, 577, and 425 corresponding to $C_{36}H_{33}O_{16}$ (loss of water), $C_{30}H_{25}O_{12}$ ($[MH - 162]^+$). and $C_{22}H_{17}O_9$ ($[MH - 314]^+$), respectively (**Figure 5**). The structure of this antioxidant is consistent with that of 3T-*O*-*β*-D-galactopyranosyl-*ent*-epicatechin-(2α→7,4α→8)-epicatechin reported by Porter et al. (*18*) and Natsume et al. (*15*) in cacao beans and cocoa liquor, respectively. The ion of *m*/*z* 577 corresponds to loss of dehydrated galactose, and the ion of *m*/*z* 425 was probably formed by both loss of galactose and retro-Diels−Alder

fragmentation as is typical of flavan-3-ols (*19*). Because no standard of this compound was available, the structure of the unknown antioxidant of *m*/*z* 739 eluting at 21.8 min could not be confirmed but is proposed to be 3T-*O*-*β*-D-galactopyranosyl-*ent*-epicatechin-(2α→7,4α→8)-epicatechin.

High-resolution positive ion electrospray mass spectrometric analysis of the antioxidant eluting at 14.5 min in **Figure 4** indicated an elemental composition of $C_{45}H_{38}O_{18}$. The high-resolution product ion tandem mass spectrum of the protonated molecule of *m*/*z* 867 is shown in **Figure 6** and is consistent with a trimeric procyanidin. Three abundant fragment ions were observed at *m*/*z* 715, 577, and 425. The product ion of *m*/*z* 715 corresponds to the retro-Diels−Alder fragmentation of the C-ring of a terminal monomeric subunit, $[MH - 152]^+$. Elimination of an entire terminal monomeric subunit, $[MH - 290]^+$, probably was responsible for the formation of the ion of *m*/*z* 577, and an additional retro-Diels−Alder fragmentation of the C-ring of one of the remaining monomeric subunits, $[MH - 290 - 152]^+$, is consistent with the formation of the ion of *m*/*z* 425. Another fragment ion was detected at *m*/*z* 289, which probably represents cleavage between a terminal monomeric unit and the other two subunits with the charge remaining on the monomer. Proposed structures for these ions are shown in **Figure 6** and are supported by high-resolution accurate mass measurements. The proposed fragmentation patterns of this antioxidant are consistent with the data reported by Natsume et al. (*15*) and Pati et al. (*19*) for a trimeric type B procyanidin (C4→C8) in cocoa, which has been named procyanidin C1 by Natsume et al. (*15*).

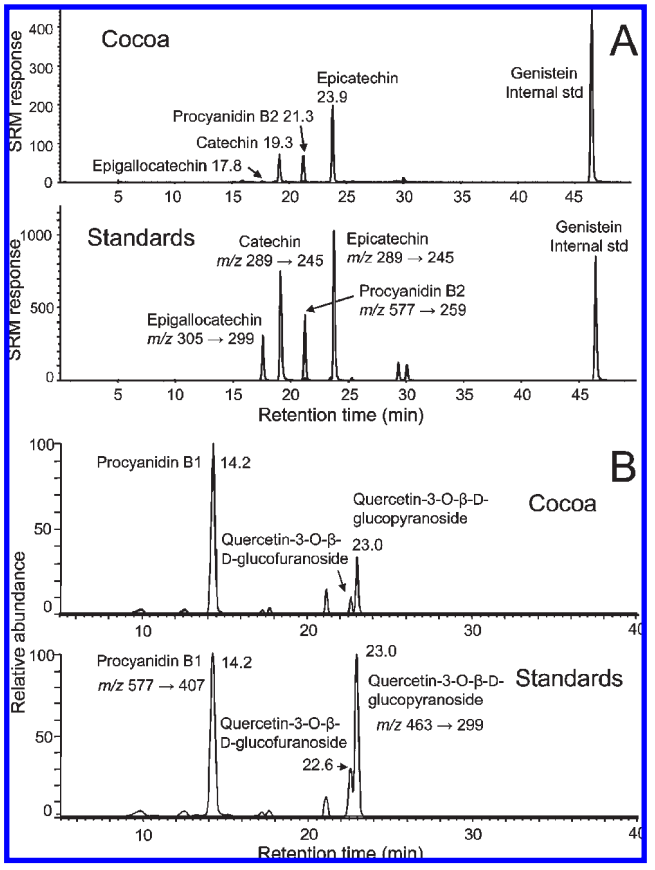

Figure 3. Negative ion electrospray LC-MS/MS selected reaction monitoring (SRM) detection of antioxidants in a methanol extract of cocoa powder and the corresponding standards: (**A**) LC-MS/MS SRM chromatograms for epigallocatechin (*m*/*z* 305 → 299), catechin and epicatechin (*m*/*z* 289 → 245), procyanidin B2 (*m*/*z* 577 → 259), and standards (bottom trace); (**B**) LC-MS/MS SRM chromatograms for procyanidin B1 (*m*/*z* 577 → 407), quercetin-3-*O*-*β*-D-glucofuranoside and quercetin-3-*O*-*β*-D-glucopyranoside (*m*/*z* 463 → 299), and standards (bottom trace).

Next, the LC-MS antioxidant assay was used to determine the relative reactivities of each major antioxidant in the cocoa powder extract. These data are summarized in **Figure 7** and indicate that the most reactive antioxidant was epigallocatechin followed by procyanidin C1 and then procyanidins B2 and B1. The least reactive antioxidants were 3T-*O*-*β*-D-galactopyranosyl-*ent*-epicatechin-(2α→7,4α→8)-epicatechin, catechin, epicatechin, and, last, quercetin-3-*O*-*β*-D-glucopyranoside. This order of reactivity toward hydrogen peroxide for the catechins and procyanidins was also observed in the standard mixture of antioxidants (see **Figure 2**).

To confirm the compatibility of the LC-MS antioxidant assay with oxidizing agents other than hydrogen peroxide, the cocoa extract was reacted with the standard free radical oxidizing agent DPPH. When excess DPPH was reacted with the cocoa extract, LC-MS analysis of the characteristic purple solution showed no detectable antioxidants such as catechins or procyanidins. However, when cocoa antioxidants were present in excess, the purple color of the DPPH radical disappeared upon mixing, resulting in a yellow solution. Then, LC-MS analysis of the reaction mixtures

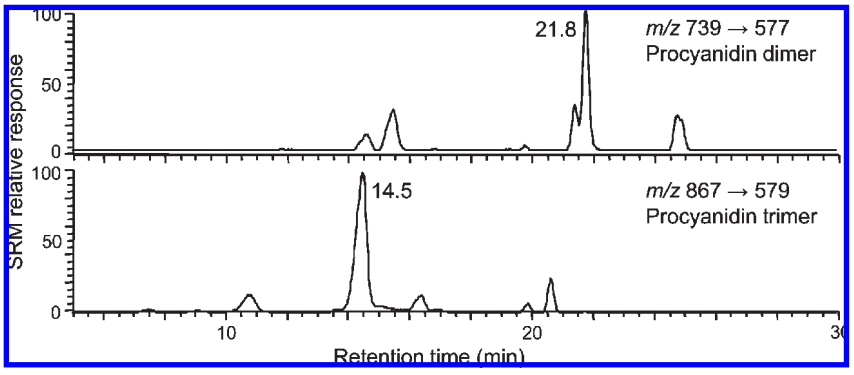

Figure 4. Positive ion electrospray LC-MS/MS detection of a procyanidin dimer antioxidant of *m*/*z* 739 at a retention time of 21.8 min and a procyanidin trimer of *m*/*z* 867 eluting at 14.5 min in a cocoa powder extract.

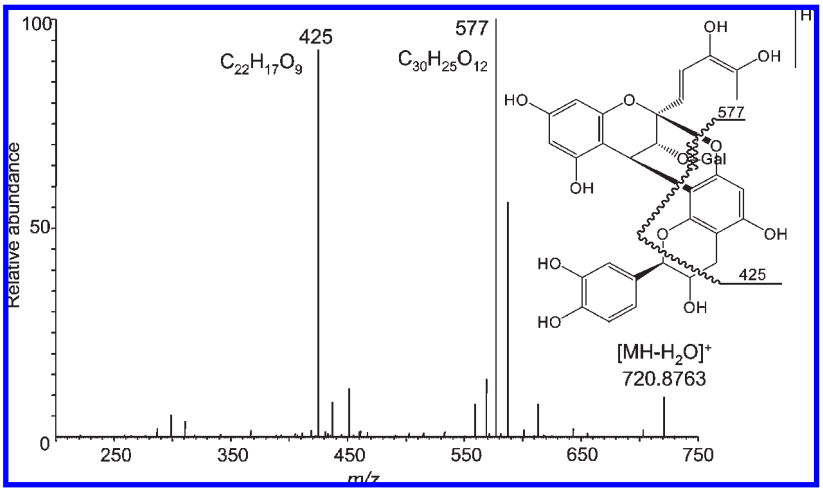

Figure 5. Positive ion electrospray high-resolution product ion tandem mass spectrum of *m/z* 739.1860. The base peak of *m/z* 577 was formed by retro-Diels–Alder fragmentation as indicated.

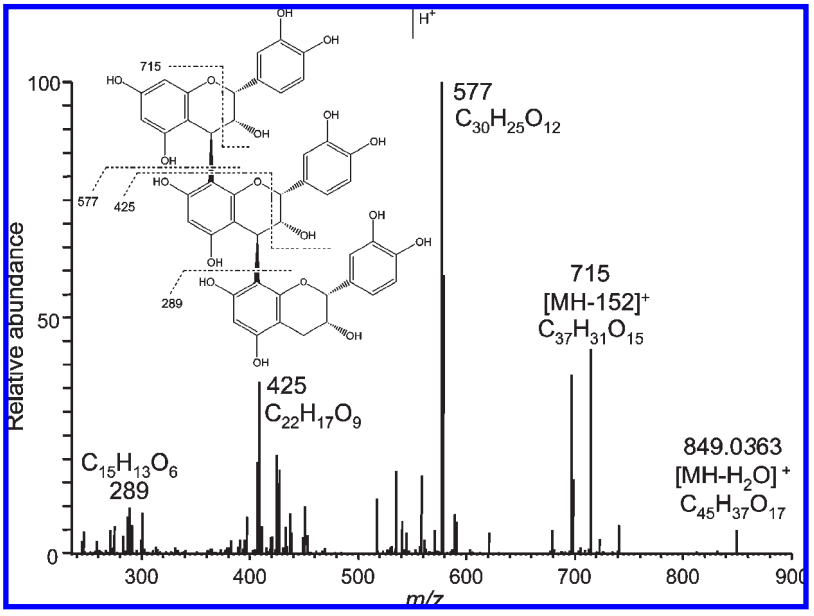

Figure 6. Positive ion electrospray high-resolution product ion tandem mass spectrum of the protonated procyanidin trimer of *m/z* 867.2110 detected at a retention time of 14.5 min during LC-MS/MS analysis of a cocoa extract (see chromatogram in **Figure 4**). The fragment ion of *m/z* 715 was probably formed by retro-Diels–Alder fragmentation of the C-ring of a monomeric subunit (loss of 152). Elimination of a terminal monomeric subunit resulted in formation of the ion of *m/z* 577, loss of an additional group of 152 units from the C-ring of a remaining monomeric subunit resulted in the ion of *m/z* 425, and loss of two monomeric subunits formed the monomer ion of *m/z* 285.

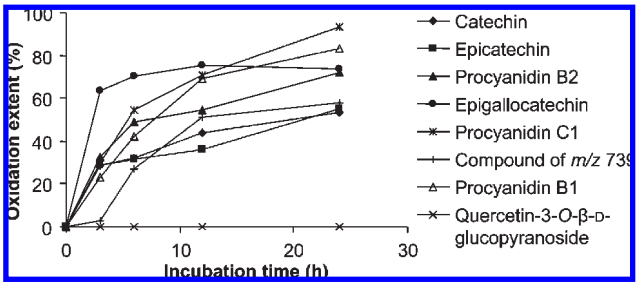

Figure 7. Time course of antioxidant degradation by hydrogen peroxide in a cocoa powder extract. Each data point represents the mean of three experiments measured using LC-MS-MS.

(see **Figure 8**) indicated that the most reactive cocoa antioxidants such as the procyanidins were eliminated first through reaction with DPPH before the less reactive antioxidants such as catechin and epicatechin. These results show that although the time course of oxidation of a sample by excess hydrogen peroxide may be followed easily using LC-MS, fast-reacting oxidants such as DPPH need to be assayed using excess antioxidants instead. By using a range of antioxidant concentrations in the DPPH reactions, the most reactive antioxidants can be observed to be depleted first by using LC-MS (**Figure 8**).

Both the hydrogen peroxide and the DPPH reaction data indicate that catechin and epicatechin are less reactive antioxidants than their dimers and trimers. Such differences in antioxidant reactivity have been observed previously, resulting in the categorization of antioxidants as "fast-acting" or "retarding" (*20*–*22*). Fast-acting antioxidants are able to inhibit oxidation by oxidants such as hydrogen peroxide or DPPH. Examples of fast-acting antioxidants in cocoa are epigallocatechin, procyanidin B1, procyanidin B2, and procyanidin C1. Retarding antioxidants are able to slow the rate of oxidation by hydrogen peroxide but not neutralize it completely. Examples of retarding antioxidants from

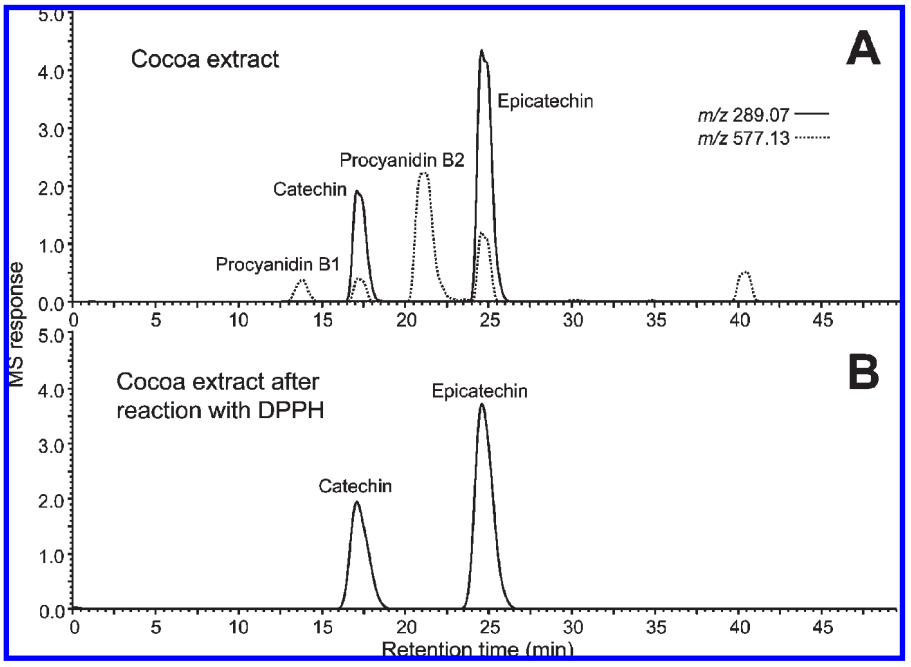

Figure 8. Computer-reconstructed negative ion electrospray mass chromatograms of cocoa extracts showing the detection of the $[M - H]^-$ signals of catechin (retention time of 17.1 min) and epicatechin (24.6 min) at *m/z* 289 and procyanidins B1 and B2 (retention times of 13.8 and 21.2 min, respectively) at *m/z* 577: (**A**) control incubation without DPPH showing the detection of both catechins and procyanidins; (**B**) incubation of excess cocoa extract (4.82 mg/mL) with DPPH showing the detection of the less reactive catechin and epicatechin but no procyanidins. The more reactive antioxidants such as procyanidin B1 and procyanidin B2 were completely neutralized by DPPH and were not detected.

this study are catechin, epicatechin, and 3T-*O*-*β*-D-galactopyranosyl-*ent*-epicatechin-(2α→7,4α→8)-epicatechin.

Oxidation of epigallocatechin gallate and epigallocatechin by hydrogen peroxide has been studied by Zhu et al. (*11*), who suggested that the A-ring was the site most susceptible to oxidation. Valic et al. (*23*) reported that the B-ring of epigallocatechin gallate was responsible for antioxidant activity, whereas Nanjo et al. (*24*) and Wan et al. (*25*) suggested that the gallate ester moieties of epigallocatechin gallate and epigallocatechin were involved in antioxidant reactions. Regardless of which site on an antioxidant is most reactive toward an oxidant such as hydrogen peroxide, the LC-MS assay reported here may be used to determine the relative reactivity of each antioxidant present in a mixture.

The use of hydrogen peroxide or DPPH treatment and LC-MS screening for antioxidants in cocoa powder indicated that the major antioxidant constituents phenolic compounds including flavon-3-ols (catechin and epicatechin), dimeric procyanidins (procyanidin B1, procyanidin B2; 3T-*O*-*β*-D-galactopyranosyl-*ent*-epicatechin-(2α→7,4α→8)-epicatechin), and trimeric proanthocyanidins (procyanidin C1). By using LC-MS and LC-MS-MS with high-resolution accurate mass measurement, the antioxidants in cocoa could be identified rapidly by comparison to standards. When no standards were available, compounds could still be characterized and compared to antioxidant compounds in cocoa that have been described in the literature. The use of high-resolution tandem mass spectrometry with HPLC separation provided much more structural information than would have been possible using other systems such as HPLC with UV absorbance detection or electrochemical detection. Conventional methods for identifying antioxidants in complex mixtures imply time-consuming assay-guided fractionation procedures, followed by the spectroscopic identification of the purified active compounds. The much more rapid approach described here may be used to screen for antioxidants in extracts of foods, dietary supplements, or other complex natural product mixtures and to compare their reactivities toward oxidizing agents such as hydrogen peroxide. As shown by the experiments using DPPH, this approach is compatible with other oxidizing agents and is not limited to hydrogen peroxide.

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