Effect of the Number of Flavanol Units on the Antioxidant Activity of Procyanidin Fractions Isolated from Chocolate

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Of three different solvents (acetone, ethanol, and methanol) mixed with water and acetic acid, the acetone/water/acetic acid mixture (70:28:2, v/v) proved to be best for extracting dark-chocolate procyanidins. High-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC−MS-ESI) was further used to identify oligomers found in the extract. After HPLC fraction collection, the reduction power of flavanoid fractions was measured in the AAPH [2,2′-azobis-(2-amidinopropane)dihydrochloride] assay, where oxidation of linoleic acid is induced in an aqueous dispersion. Even expressed in relative monomeric efficiency units, the oxidation-inhibiting power of polymerized oligomers is much stronger than that of monomers. A comparison with 10 usual antioxidants indicated that oligomers with three or more (epi)catechin units are by far the most efficient.

INTRODUCTION

Polyphenols, widely distributed in nature, are found in many common foods (fruits, vegetables, etc.) and beverages (wine, tea, etc.). Chocolate containing cocoa is a rich source of flavonoids, particularly flavan-3-ols, mainly (−)-epicatechin, (+)-catechin, traces of (+)-gallocatechin, (−)-epigallocatechin, and epicatechin 3-*O*-gallate (*1*), and procyanidins (*2−4*). Small quantities of quercetin, quercetin glycosides, naringenin, luteolin, apigenin, and phenolic compounds such as clovamide, deoxy-clovamide, and caffeic, ferulic, gallic, and *p*-coumaric acids have also been found in cocoa products (*5*, *6*).

Procyanidins, mostly constituted by a variable number of flavan-3,4-diol units [mainly (−)-epicatechin] linked by 4 → 6 or 4 → 8 bonds, may be present in chocolate in a mixture consisting of dimers, trimers, tetramers, and polymers of up to 10 units (*7−9*). Monomers contribute most to the total procyanidin content of chocolate (*10*). Chocolate contains larger amounts of procyanidins per unit weight (3.8−4.9 mg/g) than foods such as red wine (close to 0.2 g/L), cranberry juice (0.1 g/L), and apple (0.5−1 mg/g). Few differences have been noted in the procyanidin concentrations of chocolate samples that originated from the same geographical region (*3*).

Attention has recently focused on the polyphenols of chocolate and cocoa because of their antioxidant properties (*2*, *11−17*) and other physiological functions such as their antimutagenic and immunomodulatory activities (*18−21*). The antioxidant properties of simple polyphenols, i.e., flavanols, flavonols, flavanones, and flavones, have been studied extensively. Flavonoids are known to scavenge reactive oxygen species (ROS): the superoxide, hydroxyl, and peroxyl radicals

(*22*). Their antioxidant effect is commonly attributed to the presence of a catechol group in the B-ring, capable of both trapping radicals and chelating metals.

Among flavonoid monomers isolated from cocoa liquor, clovamide appears to inhibit linoleic acid autoxidation most efficiently. It is followed by (−)-epicatechin, (+)-catechin, quercetin, quercetin 3-glucoside, quercetin 3-arabinoside, and dideoxyclovamide (*23*). Phenolic compounds of cocoa such as caffeic, *p*-coumaric, and ferulic acids also appear to be good antioxidants (*24*, *25*).

Catechins with galloyl groups [e.g., epigallocatechingallate (EGCG), gallocatechingallate (GCG), epigallocatechin (EGC), and gallocatechin (GC)] also exhibit a great ability to scavenge superoxide anion radicals, singlet oxygen, AAP• [2,2′-azobis-(2-amidinopropane)dihydrochloride], and DPP• (1,1-diphenyl-2-picrylhydrazyl) radicals (*26*), ABTS⁻• [2,2′-azobis(2-amidinopropane)hydrochloride] radicals (*27*), and nitro-induced peroxynitrite radicals (*28*). They can inhibit plasma oxidation by the cupric ion (*29*), oxidation of oil in a water emulsion (*30*), and oxidation of corn oil triglycerides and soy lecithin liposomes (*31*). Whatever the galloyl compound, replacement of (+)-catechin with (−)-epicatechin decreases the scavenging activity (*26*).

It is recognized that procyanidins also have a strong antioxidant capacity. Ariga *et al*. (*32*) tested several oligomeric flavonoids (dimers B1 and B3) from azuki beans and found that the ability to scavenge the azo-generated peroxyl radical is proportional to the degree of polymerization. Saint-Cricq de Gaulejac *et al*. (*33*) used an enzymatic method (hypoxanthine HPX/xanthineoxidase XOD) to study the effect of wine procyanidins on superoxide anion radicals. They found oligomeric procyanidins (dimers B1−B8) to be efficient free radical scavengers even at low concentrations in wines. For dimers, de

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Freitas *et al.* (*34*) measured the following increasing antioxidant effect: B3 ∼ B4 < B7 ∼ B6 < B1 ∼ B2 < B8. According to Teissedre *et al.* (*35*), catechin, epicatechin, B2 and B8 dimers, and C1 trimer are the best antioxidants in wines. In apple, trimers and tetramers proved to be more effective scavengers than the B2 dimer and the epicatechin monomer (*36*).

Scant and conflicting information is available regarding the impact of the degree of polymerization of cocoa procyanidins on their antioxidant activity. According to Arteel and Sies (*37*) and Bearden *et al.* (*38*), long-chain procyanidins are better scavengers than short-chain procyanidins. More recently, Os-akabe *et al.* (*39*) showed oligomeric procyanidins are better antioxidants than (+)-catechin in an MeO-AMVN-mediated [i.e., 2,2-azobis(4-methoxy-2,4-dimethylvaleronitrile)-mediated] LDL oxidation assay, while (+)-catechin was most efficient in the Cu-mediated LDL oxidation test. According to Lotito *et al.* (*40*), monomers, dimers, and trimers appear to be better antioxidants in the aqueous phase while higher-molecular weight procyanidins seem to be more efficient in the lipid phase. Recently, Steinberg *et al.* (*41*) reported that, expressed per monomeric unit, the ability of different cocoa procyanidins to inhibit LDL oxidation is similar.

In this paper, collections of chocolate oligomers from monomer P1 to decamer P10 were gathered to determine their antioxidant power by a method recently proposed by Liégeois *et al.* (*42*). As advised in the review by Wollgast *et al.* (*1*), removal of chocolate lipids followed by a solvent extraction was necessary for successfully extracting, identifying (HPLC−MS-ESI), and quantifying (HPLC−UV diode array) chocolate procyanidins. To increase the recovery of higher-order oligomers, different solvent extraction methods have first been compared.

EXPERIMENTAL PROCEDURES

Materials. Dark chocolate (consisting of sugar, cocoa butter, cocoa mass, vanillin as aroma, and lecithin as an emulsifier) was supplied by Belcolade (Puratos Group).

Chemicals. Acetone (99.9%), (−)-epicatechin, (+)-catechin, theobromine, caffeine, linoleic acid (99%), and AAPH were from Sigma-Aldrich (Bornem, Belgium). Methanol (99.9%) and dichloromethane (99.9%) were purchased from Romil (Cambridge, U.K.). Acetic acid (99.8%) was from Acros (Geel, Belgium) and diethyl ether (99.5%) from Fluka (Buchs, Switzerland), and absolute ethanol (99.8%) and Tween 20 were from Merck (Darmstadt, Germany). Aqueous solutions were made with Milli-Q (Millipore, Bedford, MA) double-distilled water (resistance = 18 mΩ/cm²).

Extraction of the Procyanidins. *Lipid Removal.* Dark chocolate (70 g) was reduced to a powder with a mixer and introduced into a Soxhlet filtration cartridge (Schleicher & Schüll). By means of the Soxhlet extractor (Waterkeyn), lipids were removed for 24 h with diethyl ether (375 mL); 50 g of defatted chocolate was finally obtained. This method (symbolized in this paper as D^S for diethyl ether and Soxhlet) was compared with the procedure proposed by Hammerstone *et al.* (*7*) (100 g of dark chocolate extracted three times with 450 mL of hexane, symbolized in this paper as H^L for hexane and liquid−solid extraction).

Procyanidin Extraction. Defatted chocolate (10 g) was extracted three times with 50 mL of solvent (3 × 1 h, 25 °C to avoid any thermal degradation of procyanidins). Three organic solvents frequently used for procyanidin extraction (either acetone, ethanol, or methanol) were mixed with water and acetic acid in different proportions (**Table 1**, A, E, or M for organic solvent proportion and H for acid percentage). After each extraction, the suspension was centrifuged for 10 min at 3000*g*, and the supernatant was collected. After filtration to remove residual particles, the combined supernatants were concentrated by rotary evaporation under partial vacuum (40 °C) to obtain ∼50 mL of extract.

Table 1. Abbreviations Used To Describe How Lipids Are Removed and Polyphenols Further Extracted

abbreviation	compound used for lipid removal[a]	mixture for polyphenol liquid−solid extraction
H^{L}A$^{70H0.5}$	hexane^L	acetone/water/acetic acid (70:29.5:0.5, v/v)
D^{S}A$^{70H0.5}$	diethyl ether^S	acetone/water/acetic acid (70:29.5:0.5, v/v)
D^{S}A^{100}, D^{S}M^{100}, or D^{S}E^{100}	diethyl ether^S	acetone, methanol, or ethanol (100%)
D^{S}S^{70}, D^{S}M^{70}, or D^{S}E^{70}	diethyl ether^S	acetone, methanol, or ethanol/water (70:30, v/v)
D^{S}A^{70H2}, D^{S}M^{70H2}, or D^{S}E^{70H2}	diethyl ether^S	acetone, methanol, or ethanol/water/acetic acid (70:28:2, v/v)
D^{S}A^{50}, D^{S}M^{50}, or D^{S}E^{50}	diethyl ether^S	acetone, methanol, or ethanol/water (50:50, v/v)
D^{S}A^{30}, D^{S}M^{30}, or D^{S}E^{30}	diethyl ether^S	acetone, methanol, or ethanol/water (30:70, v/v)

[a] L and S refer to liquid−solid and Soxhlet extraction, respectively, for lipid removal.

Solid-Phase Extraction of Procyanidin Extracts. The 5 g C18 Sep-Pack cartridge (Waters, Millipore) was preconditioned with methanol, and then with deionized water. Approximately 50 mL of procyanidin mixture was loaded on the cartridge, and sugars were removed with 100 mL of deionized water. Procyanidins were then eluted with 15 mL of methanol, ethanol, or acetone (100%). For A^{50}, E^{50}, M^{50} and A^{50}, E^{50}, M^{30} (high water contents), a mixture (70:30 v/v) of the organic solvent with water was used for elution. The eluates were concentrated by rotary evaporation under partial vacuum (40 °C, ∼50 mL of extract) and freeze-dried.

HPLC−MS Analyses. *High-Performance Liquid Chromatography Analysis of Procyanidins.* A SpectraSystem (Finnigan Mat, San Jose, CA) equipped with an SCM degasser, an AS3000 autosampler, a P4000 quaternary pump, and a diode array detector UV6000LP was used. The system was controlled with Xcalibur version 1.2 (Finnigan Mat). Procyanidins were separated on a Phenomenex 5 μm normal-phase Luna silica column, 250 mm × 4.6 mm (inside diameter) (Bester) at 25 °C. Separations were carried out at a flow rate of 1 mL/min with a linear gradient from A (dichloromethane) to B (methanol) and a constant 4% level of C (acetic acid and water, 1:1, v/v). Gradient elution was as follows: from 14 to 28% B from 0 to 30 min, from 28 to 50% B from 30 to 60 min, from 50 to 86% B from 60 to 65 min, isocratic from 65 to 70 min. Ten milligrams of procyanidin extract was diluted in 1 mL of methanol before injection with the 20 μL Rheodyne loop.

Mass Spectrometry Analysis. MS analyses were carried out using an LCQ Duo (Finnigan Mat) multipole mass spectrometer equipped with an ESI interface. The negative ion mode with a source voltage of 4.5 kV, a capillary voltage of −39 kV, a capillary temperature of 225 °C, and a shear gas (N$_2$) of 50 arb (arbitrary units) was selected for procyanidin analysis. For alkaloids (caffeine and theobromine), the positive ion mode was preferred with a source voltage of 4.5 kV, a capillary voltage of 12 kV, a capillary temperature of 220 °C, and a shear gas (N$_2$) of 50 arb. Only one-tenth of the HPLC flow was directed to the ESI interface of the mass analyzer. Data were collected on a computer (Xcalibur) using the selective ion monitoring (SIM) mode.

Collection of Oligomeric Procyanidins. Fractions of the D^{S}A^{70H2} extract were collected using the HPLC chromatograph described in the HPLC analysis section. Twenty microliters of each sample (Rheodyne loop) was injected seven times into the HPLC system. Fractions of each oligomer were obtained by mixing the adequate vials collected every minute by the automatic collector (Pharmacia). To avoid any polyphenol oxidation, these fractions were flushed with argon for 1 min and stored at −20 °C.

Antioxidant Assay. *AAPH Method.* The reduction power of flavonoid fractions was measured by a method developed in our laboratory

Table 2. Influence of the Lipid Removal Process on the Concentration of Oligomeric Procyanidins ($\lambda = 280$ nm)

procyanidin [b]	concentration in the extract (ppm or mg/L)[a]	
	H^{L}A$^{70H0.5}$ [c]	D^{S}A$^{70H0.5}$ [c]
P1	380	358
P2	177	181
P3	175	172
P4	107	116
P5	67	72
P6	46	46

[a] Concentration measured in epicatechin equivalents in the 10 mg/mL methanol extract. For three replicates, average variation coefficients for P1–P6 were 1.64, 1.98, 1.32, 3.94, 3.12, and 4.23%, respectively. [b] P1–P6 are monomeric to hexameric procyanidins, respectively. [c] D^S represents Soxhlet extraction with diethyl ether. H^L represents liquid–liquid extraction with hexane. A$^{70H0.5}$ represents extraction with 70% acetone and 0.5% acetic acid (H) in water.

Table 3. Concentration in Epicatechin Equivalents (ppm or mg/L in the 10 mg/mL Methanol Extract) of Oligomeric Procyanidins from Different Extracts ($\lambda = 280$ nm)[a]

	D^{S}A$^{70H0.5}$	D^{S}A^{100}	D^{S}A^{70}	D^{S}A^{70H2}	D^{S}A^{50}	D^{S}A^{30}
P1	358	87	331	364	294	217
P2	181	37	171	188	169	122
P3	172	22	166	173	155	118
P4	116	13	112	123	95	74
P5	72	T	69	76	54	46
P6	46	T	45	47	41	29

	D^{S}M^{100}	D^{S}M^{70}	D^{S}M^{70H2}	D^{S}M^{50}	D^{S}M^{30}
P1	358	290	121	297	285
P2	186	168	70	157	163
P3	130	131	72	124	117
P4	91	116	66	106	88
P5	47	60	71	59	50
P6	25	40	36	44	T

	D^{S}E^{100}	D^{S}E^{70}	D^{S}E^{70H2}	D^{S}E^{50}	D^{S}E^{30}
P1	21	220	240	112	39
P2	11	96	97	72	37
P3	14	84	116	61	33
P4	7	54	83	54	22
P5	T	34	47	33	13
P6	T	23	34	26	7

[a] P1–P6 are monomeric to hexameric procyanidins, respectively. T represents a trace, as the signal:noise ratio is too small. D^S represents Soxhlet extraction with diethyl ether. A^X, M^X, and E^X represent the percentage of acetone, methanol, and ethanol in water, respectively. H^Y represents the percentage of acetic acid (H) in water. For three replicates, average variation coefficients for P1–P6 were 1.64, 1.98, 1.32, 3.94, 3.12, and 4.23%, respectively.

by Liégeois *et al.* (*42*). The oxidation of linoleic acid was induced by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) in an aqueous dispersion in the absence or presence of antioxidant. The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. A Shimadzu (Antwerp, Belgium) UV−visible 240 spectrophotometer equipped with an automatic sample positioner allowed analysis of six samples per minute. In all cases, the measurements were run in duplicate against the buffer and compared with a separate AAPH-free control to check for any spontaneous oxidation.

RESULTS AND DISCUSSION

Choice of the Best Extraction Procedure for Isolating Chocolate Procyanidin Fractions. Because high-molecular weight procyanidins are commercially unavailable, dark choco-
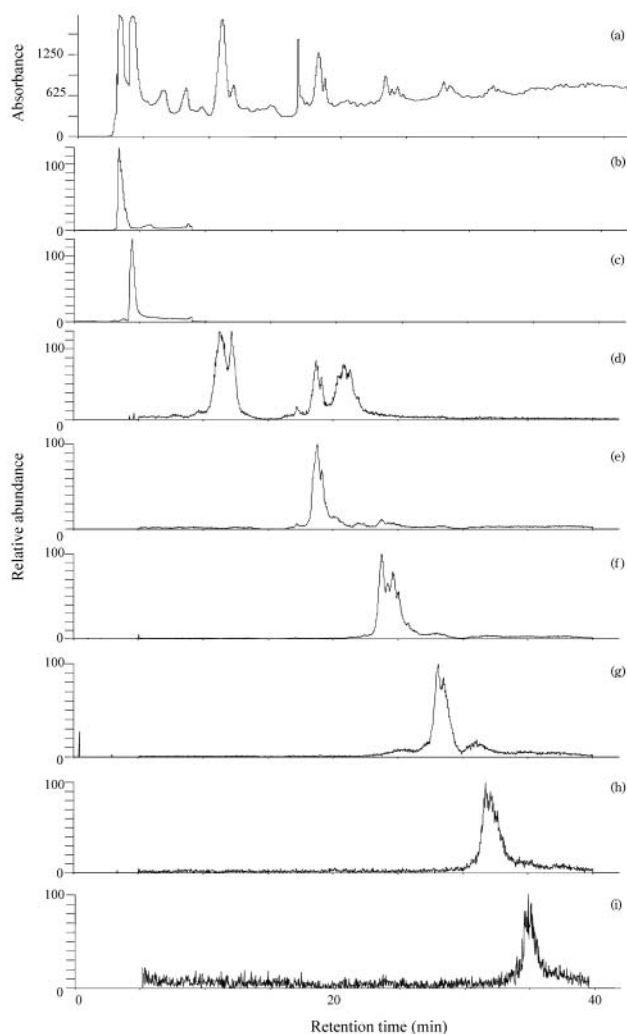
Figure 1. Comparison between HPLC−UV and SIM HPLC−MS chromatograms of the D^{S}A^{70H2} extract. (a) HPLC−UV ($\lambda = 280$ nm) and SIM HPLC−MS chromatograms for (b) caffeine (M + H) = 195, (c) theobromine (M + H) = 181, (d) monomers (M − H) = 289, (e) dimers (M − H) = 577, (f) trimers (M − H) = 865, (g) tetramers (M − H) = 1153, (h) pentamers (M − H) = 1441, and (i) heptamers (M − H) = 1729.

late was chosen here as a raw material from which to purify P1−P10 fractions. According to Hammerstone *et al.* (*7*), liquid extraction of defatted chocolate with an acetone/water/acetic acid mixture (70:29.5:0.5, v/v) yields good extracts for HPLC analyses, yet to increase the recovery of higher-order oligomers, the choice of the solvent mixture was further optimized in the study presented here.

According to Adamson *et al.* (*2*), complete removal of lipid is necessary to achieve maximum-efficiency extraction of chocolate procyanidins. Two processes of lipid elimination were compared: liquid−solid extraction with hexane (Hammerstone's method) and Soxhlet extraction with diethyl ether. As depicted in **Table 2**, for the same subsequent polyphenol extraction (see below), apparent concentrations of P2, P4, and P5 oligomeric procyanidins [(−)-epicatechin equivalents] proved to be slightly higher (2−8%) after lipid removal with diethyl ether in the Soxhlet extractor (D^{S}A$^{70H0.5}$ extract) than after hexane−solid extraction (H^{L}A$^{70H0.5}$ extract). The former, requiring 10 min of preparation (vs 1 day for the hexane−solid extraction), was therefore chosen for all subsequent experiments (P1 is not very important since it is commercially available).

Procyanidin Fractions Isolated from Chocolate

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Table 4. Antioxidant Activity (T_{inh} and R_{pinh}/R_o) of P1–P10 Fractions Obtained from the D^SA^{70H2} Chocolate Extract

procyanidin	$T_{inh}{}^a$ for 0.1 ppm (min)	RME[b]	$R_{pinh}/R_o{}^c$
P1	10.1	1.0	0.57
P2	16.4	1.6	0.62
P3	31.9	3.2	0.61
P4	34.8	3.5	0.62
P5	49.3	4.9	0.61
P6	58.2	5.8	0.61
P7	77.2	7.7	0.53
P8	80.3	8.0	0.57
P9	107.1	10.7	0.53
P10	121.4	12.1	0.52

[a] T_{inh} is the mean of the inhibition time of two AAPH assays for each of two HPLC collections; variation coefficients are always <2%. [b] RME is the relative monomeric efficiency. [c] R_{pinh}/R_o is the ratio of the propagation rate (maximum oxidation rate) in the presence of antioxidants to the initial propagation rate in their absence (mean of two AAPH assays).

$$y = 0{,}0456x^2 + 0{,}7383x$$
$$R^2 = 0{,}9887$$
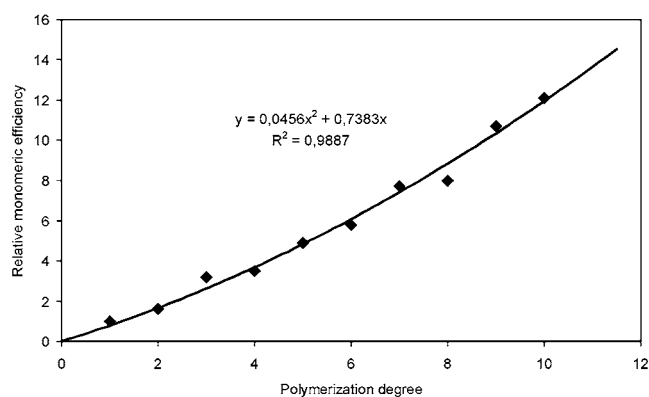
Figure 2. Relative monomeric efficiency vs the degree of polymerization of procyanidins (P1–P10).

Among the 16 solvent mixtures that were tested, a slightly modified version (2% acetic acid instead of 0.5%) of that proposed by Hammerstone *et al.* (*7*) emerged as the best for recovering P1–P6 oligomeric procyanidins (D^SA^{70H2} in **Table 3**). With acetone and ethanol, all procyanidin yields were higher when 30% water and 2% acetic acid were added (D^SE^{70H2} and D^SA^{70H2}). When pure acetone or ethanol was used (D^SA^{100} and $D^SE^{100}$ extracts), the procyanidin yields were much lower, especially for higher-order oligomers. In this case, only monomers to tetramers were detected at the UV detector ($\lambda = 280$ nm). On the other hand, in terms of total extraction efficiency, a significant decrease in the procyanidin yield was measured if the percentage of water in the acetone or ethanol exceeded 30% (e.g., D^SE^{70}, P1 = 220 ppm; D^SE^{30}, P1 = 39 ppm). Of the three pure solvents that were tested, methanol (the most polar one) was clearly the best procyanidin extractor, ethanol being by far the worst. The results for methanol may seem surprising, since the best P1–P3 yields were obtained with pure methanol (D^SM^{100}, sufficient polarity suspected).

As is the case for most matrixes described in the literature (*43–45*), the amount of procyanidin detected in our chocolate extract decreased from P1 to P6, whichever solvent was used. It is difficult to conclude, however, that this is also the case in chocolate, because recovery factor artifacts might occur. Moreover, the (−)-epicatechin absorption coefficient (measured at a λ of 280 nm and converted to ppm^{-1} cm^{-1}) was used here to quantify each fraction. In using a single apparent absorption coefficient, we assumed that a given weight of procyanidin absorbs the same amount of light whatever its degree of polymerization. We were able to confirm this assumption only for our four major fractions (P1–P4), by weighing them with a Mettler (Greifensee, Switzerland) UM3 microbalance after freeze-drying (see below for fraction collection) and resolubilizing them in methanol for absorption measurement.

Identification of Procyanidins by SIM HPLC−MS-ESI. To confirm procyanidin identification, chocolate extract D^SA^{70H2} was analyzed by HPLC−MS in the single-ion monitoring mode (SIM). A comparison of HPLC−UV and SIM HPLC−MS chromatograms (**Figure 1**) enabled us to confirm peak attribution for P1−P7. For P1 (M = 290, **Figure 1d**), the presence of two peaks at 10.6 and 11.2 min was explained by the presence of both (−)-epicatechin and (+)-catechin in chocolate (*2−4*). For dimers, trimers, or higher-order oligomers, many more possibilities must be considered (variation of the monomer structure and C4−C8 or C4−C6 possible linkages). For M − 1 = 289 (**Figure 1d**), the peak coeluting with the procyanidin dimers (retention time of 21 min) was suspected to be the monomer glycoside decomposed by electrospray ionization.

The presence in our chocolate extract of two alkaloids, caffeine and theobromine, was also confirmed by mass spec-
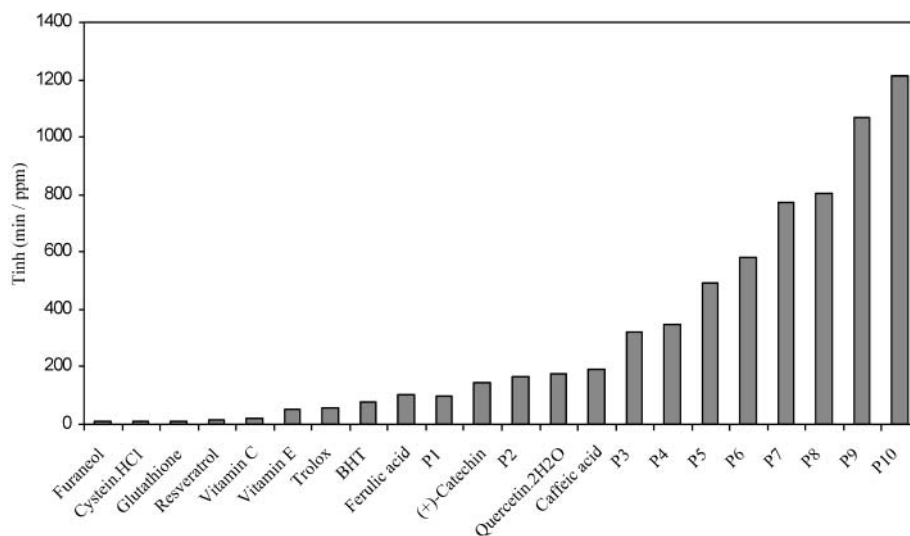
Figure 3. Antioxidant efficiency of chocolate procyanidin fractions in comparison with those of various commercial antioxidants.
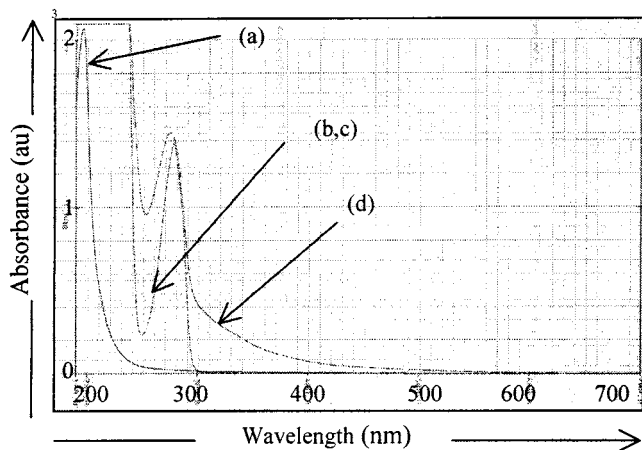
Figure 4. Absorption spectra (190 and 700 nm) of pure methanol (a), 0.1 g/L (+)-catechin (b), 0.1 g/L (−)-epicatechin (c), and 0.1 g/L D^SA^{70H2} chocolate extract (d).

trometry (M + 1 = 180 and 195, respectively, panels b and c of **Figure 1**).

Antioxidant Activity of the Procyanidin Fractions. Collection of pure fractions of P1−P10 was undertaken with the D^SA^{70H2} chocolate extract. The AAPH antioxidant activity assay (*42*) was then applied to each of them. Even when expressed with respect to the same weight (relative monomeric efficiency), the antioxidant activity of procyanidin oligomers was found to increase significantly with the degree of polymerization (see **Table 4**). As depicted in **Figure 2**, the relationship clearly was not linear.

In **Figure 3** are classified the antioxidant efficiencies of procyanidins and other reference compounds (*42*). Compared to Furaneol, cystein, glutathione, vitamin C, vitamin E, Trolox, BHT, and ferulic acid, oligomeric procyanidins exhibited the best ability to scavenge the radical species produced in the assay. Not surprisingly, the P1 fraction, which was a mixture of (+)-catechin and (−)-epicatechin, appears in the figure close to commercial (+)-catechin. Although better than (+)-catechin, quercetin and caffeic acid appeared to be much less efficient than the P3−P10 fractions.

Table 4 also gives the ratio of R_{pinh} (maximum oxidation rate in the presence of antioxidant) to R_o (initial propagation rate in the absence of antioxidant) for each fraction. As previously described by Liégeois *et al.* (*42*), an R_{pinh}/R_o ratio of ∼1 indicates that oxidation starts after the inhibition time at the same rate as in the absence of antioxidant (case of most commercial antioxidants, except polyphenols). A ratio of ∼0.5−0.6 was measured here for all oligomeric procyanidins, confirming their prolonged efficiency.

Antioxidant Activity of Chocolate Extract According to Its Procyanidin Composition. Taking into account the amounts of P1−P6 procyanidins in the fractions (P7−P10 unquantifiable), we calculated the antioxidant activity of our D^SA^{70H2} chocolate extract and compared the value to the experimental value (T_{inh} = 58.8 min/ppm). It appeared that procyanidins P1−P6 could account for ∼40% of the total antioxidant activity of the extract. Theobromine and caffeine, also present in the extract (150 and 75 ppm, respectively), did not delay oxidation in the AAPH assay (zero inhibition time). We must conclude that part of our extract that remains unidentified (88%, considering that P1−P6 = 9710 ppm in the D^SA^{70H2} extract) contributes greatly to the antioxidant activity. These results are not very surprising, since our protocol most probably also extracts melanoidins (*46*) and perhaps higher-order tannins,

as indicated by the brown color and the absorbance at wavelengths above 300 nm (**Figure 4**). To better characterize this unidentified fraction, dialysis with a cutoff of 3500 was applied. The retentate obtained in this way could account for 33% of the weight of the extract, and the AAPH assay applied to this retentate confirmed the presence of potent antioxidants (T_{inh} = 51.1 min/ppm) contributing up to 28% of the antioxidant activity of the extract. The residual antioxidant activity (∼32%) might be due to lower-molecular weight melanoidins and tannins (MW < 3500) or to synergetic effects between compounds (*47*).

CONCLUSIONS

In agreement with those of Arteel and Sies (*37*), Bearden *et al.* (*38*), and Lotito *et al.* (*40*), our results clearly confirm that long-chain procyanidins are by far the best to inhibit lipid oxidation induced by radicals in an aqueous medium. More pure fractions are now needed to assess how the structure (monomers, type of linkages, etc.) could modify the antioxidant activity of long-chain oligomers. Before an assessment of their real impact on human health is carried out, the bioavailability of procyanidins should also be investigated in the immediate future.

ACKNOWLEDGMENT

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