



Article de périodique (Journal article)

"Degradation of (-)-Epicatechin and Procyanidin B2 in Aqueous and Lipidic Model Systems. First Evidence of "Chemical" Flavan-3-ol Oligomers in Processed Cocoa."

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Abstract

Despite the key role of flavan-3-ols in many foods, very little is yet known concerning the modification of their chemical structures through food processes. Degradation of model media containing (-)-epicatechin and procyanidin B2, either separately or together, was monitored by RP-HPLC-DAD-ESI(-)-MS/MS. Medium composition (aqueous or lipidic) and temperature (60 and 90 °C) were studied. In aqueous medium at 60 °C, (-)-epicatechin was mainly epimerized to (-)-catechin, but it was also oxidized to "chemical" dimers, a "chemical" trimer, and dehydrodi(epi)catechin A. Unlike oxidation, epimerization was enhanced at 90 °C. In lipidic medium, epimerization proved slow but degradation was faster. Procyanidin B2 likewise proved able to epimerize, especially at 90 °C and in aqueous medium. At high temperature only, the interflavan linkage was cleaved, yielding the same compounds as those found in the monomer-containing model medium. Oxidation to procyanidin A2 was also evidenced. With little epimerization and slow oxidation even at 90 °C, procyanidin B2 proved more stable in lipidic medium. [...]

Référence bibliographique

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Degradation of (–)-Epicatechin and Procyanidin B2 in Aqueous and Lipidic Model Systems. First Evidence of “Chemical” Flavan-3-ol Oligomers in Processed Cocoa

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ABSTRACT: Despite the key role of flavan-3-ols in many foods, very little is yet known concerning the modification of their chemical structures through food processes. Degradation of model media containing (–)-epicatechin and procyanidin B2, either separately or together, was monitored by RP-HPLC-DAD-ESI(–)-MS/MS. Medium composition (aqueous or lipidic) and temperature (60 and 90 °C) were studied. In aqueous medium at 60 °C, (–)-epicatechin was mainly epimerized to (–)-catechin, but it was also oxidized to “chemical” dimers, a “chemical” trimer, and dehydrodi(epi)catechin A. Unlike oxidation, epimerization was enhanced at 90 °C. In lipidic medium, epimerization proved slow but degradation was faster. Procyanidin B2 likewise proved able to epimerize, especially at 90 °C and in aqueous medium. At high temperature only, the interflavan linkage was cleaved, yielding the same compounds as those found in the monomer-containing model medium. Oxidation to procyanidin A2 was also evidenced. With little epimerization and slow oxidation even at 90 °C, procyanidin B2 proved more stable in lipidic medium. Synergy was also observed: in the presence of the monomer, the dimer degradation rate increased 2-fold at 60 °C. This work states for the first time the presence of newly formed flavan-3-ol oligomers in processed cocoa.

KEYWORDS: polyphenol, flavan-3-ol, lipidic medium, cocoa, chocolate, conching

■ INTRODUCTION

Many raw materials used in the food industry, such as cocoa,^{1–3} hops,^{4–6} grapes,⁷ and sorghum,^{8,9} contain between 0.5 and 10% polyphenols. Among these, flavan-3-ols are often the major form. Catechins and procyanidins have been extensively investigated for their impact on reduction power,^{10–15} their health properties,^{16,17} their astringency and bitterness,^{18–20} and their role in colloidal instability.^{5,21,22} In cocoa, bitterness is also brought by diketopiperazines²³ and the two main alkaloids caffeine and theobromine.^{24,25} The caffeine content differs between varieties with amounts from 0.05% in Forastero to 1% in Criollo. Forastero beans usually have a high theobromine/caffeine ratio (>9), Criollo a much lower ratio (<3), and Trinitario in the mid range.²⁶

Food processes can greatly affect the polyphenol content and profile of raw materials.^{27–34} Flavan-3-ols are subject to epimerization, oxidation, polymerization, and depolymerization.^{5,35–38} The most detailed flavan-3-ol degradation mechanisms have been published by Guyot et al.,^{39,40} Contreras-Dominguez et al.,⁴¹ and Poupard⁴² for apple and apple juice media. These authors have shown how oxidation can induce dimerization and trimerization, forming new conjugated compounds. Our group⁵ has confirmed that oxidative reactions occurring during beer storage convert colorless dehydrodicatichin B to dehydrodicatichin A dimers, responsible for the yellow-brown color found in aged lager beers. Extensive polymerization appears much less efficient than condensations between A and B cycles, further rigidified by complex intramolecular bridges. Adducts involving epicatechin and Maillard-reactive carbonyls were also evidenced by Totlani and Peterson.⁴³

Some polyphenol transformations have been evidenced during cocoa bean fermentation,^{44,45} roasting,^{18,46} and cocoa powder

alkalization.⁴⁷ In chocolate, HPLC analyses on a chiral column have enabled Cooper² to identify (–)-catechin as the second major polyphenol issued from epimerization of native cocoa (–)-epicatechin. Indirect data such as the evolution of the reduction power during chocolate conching confirm that various chemical modifications occur from cocoa to chocolate, leading sometimes to higher antioxidant activity.⁴⁸

The physicochemical environment of polyphenols is strongly modified from cocoa beans to chocolate. Native polyphenols are initially enclosed in the pigmentary cells of the beans (aqueous environment in the vacuoles). After fermentation, drying will reduce the moisture content from about 60 to 7–8%. Grinding of roasted cocoa beans and conching will put polyphenols into a lipidic environment constituted of cocoa butter and lecithin. The aim of the present work was to compare the degradation rates of flavan-3-ol monomer and dimer, including both separately and together in aqueous and lipidic model media heated at 60 or 90 °C. Although present in only the very first steps of cocoa processing, water gives the enormous advantage of avoiding long polyphenol extraction steps before analysis. In both media, the main degradation products of native flavan-3-ols were characterized by RP-HPLC-DAD-ESI(–)HRMS/MS. Some of them were further evidenced in processed cocoa.

■ MATERIALS AND METHODS

Chemicals. Acetonitrile (99.99%), diethyl ether (99.9%), ethanol (97%), ethyl acetate (97%), acetone (97%), and methanol (99.9%) were

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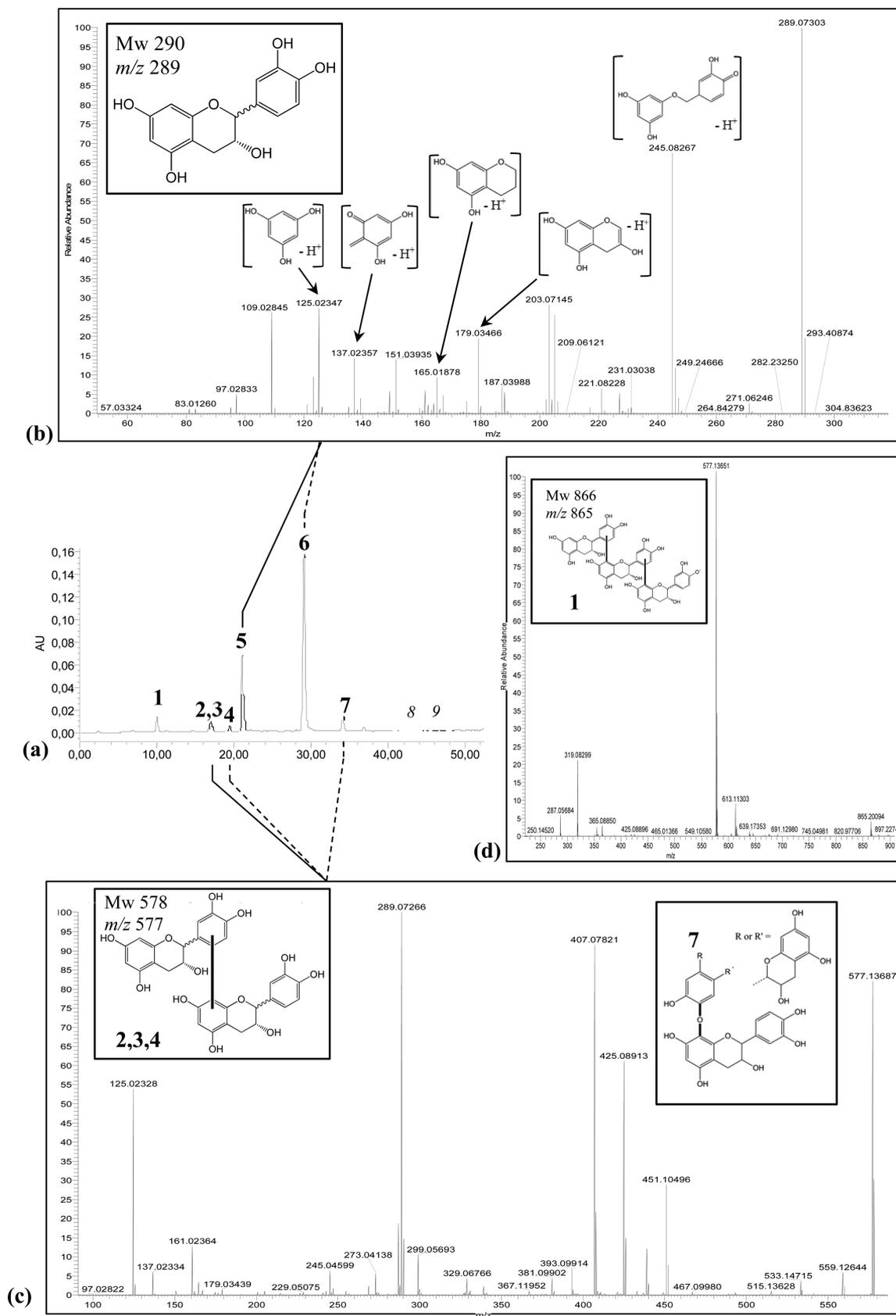


Figure 1. (a) RP-HPLC-UV chromatogram (280 nm) of (-)-epicatechin heated at 60 °C for 12 h in aqueous media. ESI(-)HRMS/MS spectra at (b) m/z 289 \pm 0.1 (compounds 5 and 6), (c) 577 \pm 0.1 (compounds 2, 3, 4 and 7), and (d) m/z 865 \pm 0.1. The bold lines in the molecule indicate that the precise position of the chemical bonds is not known. The dotted lines indicate that these compounds gave an HRMS/MS spectrum very similar to that here displayed.

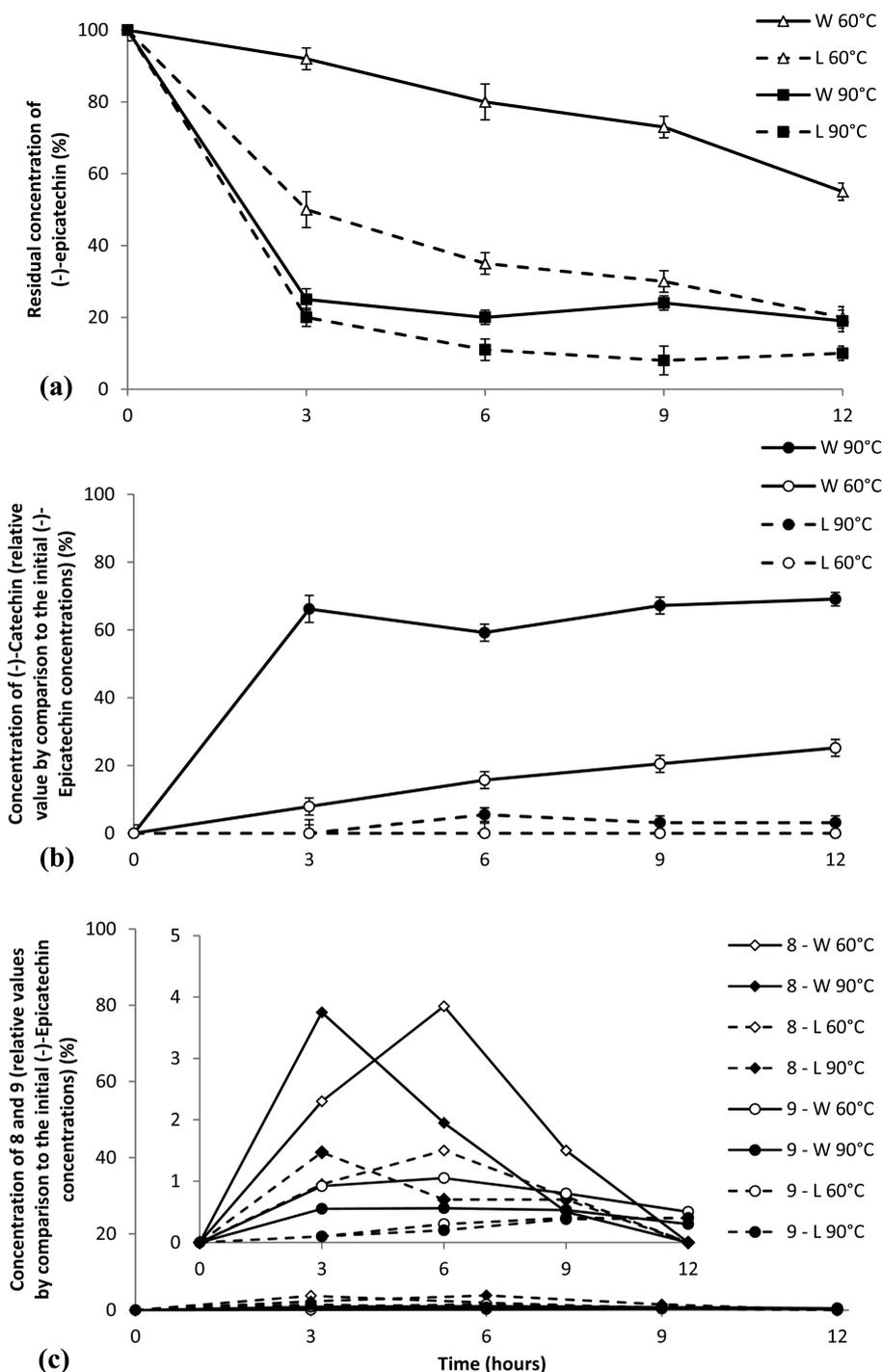


Figure 2. Evolution of the (-)-epicatechin model medium at 60 and 90 °C: (a) degradation of (-)-epicatechin; (b) synthesis of (-)-catechin; (c) synthesis of compounds 8 and 9. Quantitation is in epicatechin equivalents by ESI(-)-MS/MS. Standard deviation was <math><5\%</math>.

supplied by VWR (Leuven, Belgium). Formic acid (99%) was obtained from Acros Organic (Geel, Belgium). (-)-Epicatechin (98%), (+)-catechin (98%), caffeine, and theobromine (>99%) were supplied by Sigma-Aldrich (Bornem, Belgium). (-)-Epicatechin-4 β -8-(-)-epicatechin (B2, 90%), (-)-epicatechin-2O-7,4 β -8-(-)-epicatechin (A2, 99%), and kaempferol (>90%) were obtained from Extrasynthese (Genay, France). (-)-Epicatechin-4 β -8-(-)-epicatechin-4 β -8-(-)-epicatechin (C1, 99%) and (-)-epicatechin-4 β -8-(+)-catechin (B1, 99%) were supplied by PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Aqueous solutions were made with Milli-Q (Millipore, Bedford, MA, USA) water (resistance = 18 m Ω). Cocoa butter, sugar, and lecithin were obtained from Belcolade (Puratos Group, Erembodegem, Belgium).

Cocoa and Chocolate Samples. Ecuadorian National cocoa beans (before and after roasting for 30 min at 150 °C) and derived chocolate (72% cacao, conching step of 12 h at 75 °C) were provided by the chocolate factory Pierre Marcolini (Brussels, Belgium).

Preparation of Model Media. Aqueous model media were obtained by diluting (-)-epicatechin or B2 at a concentration of 1000 mg/L in ultrapure water (stock solution of 10000 mg/L prepared in methanol). Lipidic model media contained sugar (59.7% w/w), cocoa butter (39.8% w/w), and lecithin (0.5% w/w, addition after 6 h) and (-)-epicatechin or B2 (1000 mg/L). All media were treated in duplicates at 60 or 90 °C, and samples were taken at 0, 3, 6, 9, and 12 h.

Extraction of Flavan-3-ols from Lipidic Model Systems. This method has been adapted from the one developed in our laboratory for

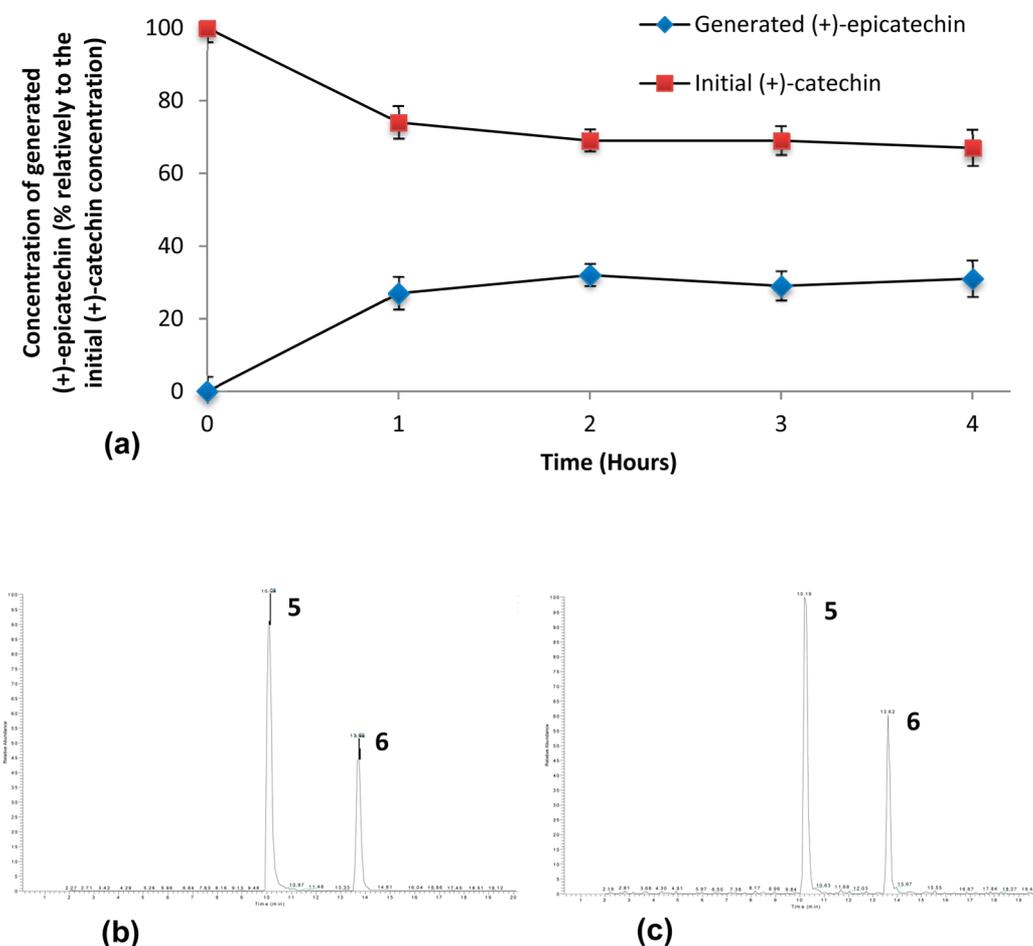


Figure 3. (a) Evolution of the (+)-catechin model medium at 90 °C. (b, c) RP-HPLC-MS chromatograms obtained after 12 h at 90 °C, from the (+)-catechin or (-)-epicatechin aqueous model media, respectively.

the analysis of flavan-3-ols in chocolate.¹⁴ All extraction steps have been done in duplicate.

Lipid Removal. Lipidic model systems (500 mg) were introduced into a centrifugal vial. By successive 10 min extractions, lipids were removed with 3 × 1.5 mL diethyl ether at room temperature under gentle stirring. At the end of each step, the sample was centrifuged for 10 min at 2500g, and the supernatant was eliminated. After the last step, the powder was dried under vacuum to get rid of residual solvent.

Flavan-3-ol Extraction. Defatted samples were extracted three times with 3 mL of acetone/water/acetic acid (70:28:2, v/v/v), each time for 1 h under shaking at room temperature. After each extraction, the suspension was centrifuged for 10 min at 2500g, and the supernatant was collected. The combined supernatants were concentrated by rotary evaporation to obtain ~3 mL of extract, and residual particles were removed by filtration.

Solid-Phase Extraction Purification Step. To purify the flavan-3-ol extract, a 360 mg C18 Sep-Pak cartridge (Waters, Millipore) was preconditioned with 10 mL of methanol and 20 mL of water. The extract was loaded on the cartridge, and sugars were removed with 5 mL of water. Flavan-3-ols were finally eluted with 5 mL of acetone/water/acetic acid (70:28:2, v/v/v). The eluates were concentrated by rotary evaporation (<40 °C) and freeze-dried. The extract was dissolved in methanol and stored under nitrogen at -80 °C in the dark until used.

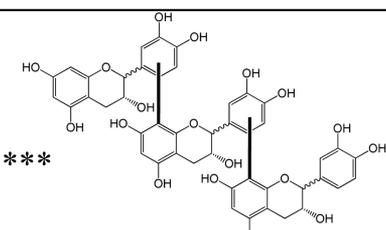
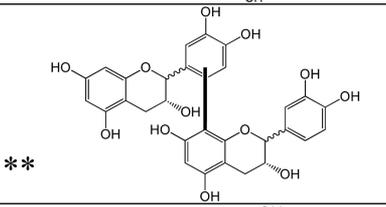
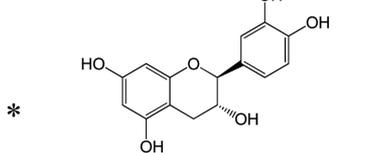
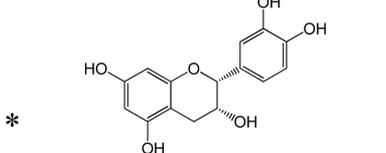
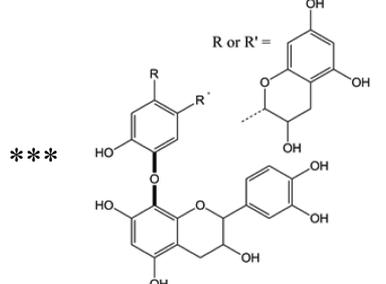
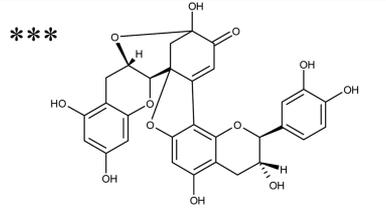
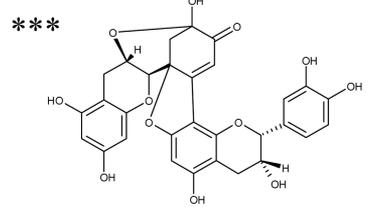
Extraction of Flavan-3-ols from Cocoa and Chocolate. The method was close to the one used here above for model systems. Seven grams of cocoa beans or chocolate was defatted by diethyl ether (3 × 50 mL) at room temperature under gentle stirring. After centrifugation, samples were dried under vacuum. Defatted samples spiked with 500 μL of kaempferol at 10000 mg/L in methanol (used as internal standard; 714 mg/kg if calculated in beans) were extracted with

3 × 50 mL of acetone/water/acetic acid (70:28:2, v/v/v) and purified on a 10 g C18 Sep-Pak cartridge (Waters, Millipore) preconditioned with 200 mL of methanol and 300 mL of water. The samples were finally eluted with 50 mL of acetone/water/acetic acid (70:28:2, v/v/v), concentrated by rotary evaporation/ and freeze-dried.

RP-HPLC-DAD-ESI(-)MS/MS Quantitation of Flavan-3-ols.

Quantitations were performed on a C18 Kinetex column (150 × 2.1 mm, 2.6 μm) (Phenomenex, Torrance, CA, USA) using a linear gradient from A (water containing 1% acetonitrile and 2% formic acid) to B (acetonitrile containing 2% formic acid). Gradient elution was as follows: from 97% A to 91% in 5 min, from 91 to 85% in 25 min, from 85 to 64% in 35 min, from 64 to 10% in 10 min, and isocratic for 20 min at a flow rate of 200 μL/min. Five microliters of the sample was injected in duplicates onto the column kept at 20 °C. A SpectraSystem equipped with an AS3000 autosampler and a P4000 quaternary pump was used. The system was controlled with Xcalibur software version 1.2 (Thermo Fisher Scientific, Austin, TX, USA). Flavan-3-ols were monitored from 200 to 800 nm with a UV6000LP diode array detector. Mass spectra were acquired using an LCQ ion trap mass spectrometer equipped with an ESI source (ThermoFisher). Collision-induced dissociation spectra were recorded at a relative collision energy of 30, 35, and 40%, respectively, for singly charged $[M - H]^{-1}$ ions of monomers (m/z 289), dimers (m/z 577, 575, and 573), and trimers (m/z 865). The ESI inlet conditions were as follows: source voltage, 4.9 kV; capillary voltage, -4 V; capillary temperature, 200 °C; and sheath gas, 39 psi. For ESI(-)MS/MS semiquantitation in cocoa beans and chocolate (kaempferol used as internal standard), a relative recovery factor = 1 was applied for all compounds. Flavan-3-ol monomers were quantitated according to the calibration curve of (-)-epicatechin (0, 10, 25, 50, 100 mg/L, $R^2 = 0.99648$), procyanidins B2 and B5 with the one of B2

Table 1. Polyphenols Detected in the (-)-Epicatechin Model Media^a

Peak n°, name and formula	Rt [min] (relative retention time)	[M-H] ⁻¹	MS/MS ions m/z (%)	Suspected structure
1 Chemical trimer (C ₄₅ H ₃₈ O ₁₈)	10.0 (0.34)	865	577.1 (100) 319.1 (23) 613.1 (11) 287.1 (8)	 ***
2,3,4 Chemical dimer (C ₃₀ H ₂₆ O ₁₂)	16.8 19.3 (0.57) (0.65)	577	393.2 (100) 559.1 (94) 425.1 (57) 439.1 (50)	 **
5 (-)-Catechin (C ₁₅ H ₁₄ O ₆)	21.2 (0.72)	289	245.1 (100), 205.1 (34), 179.1 (12)	 *
6 (-)-Epicatechin (C ₁₅ H ₁₄ O ₆)	29.6 (1.00)	289	245.1 (100), 205.1 (34), 179.1 (12)	 *
7 Chemical dimer (C ₃₀ H ₂₆ O ₁₂)	34.0 (1.15)	577	393.2 (100) 559.1 (94) 425.1 (57) 439.1 (50)	 ***
8 Dehydrodiepicatechin A (epicatechin-catechin) (C ₃₀ H ₂₄ O ₁₂)	53.1 (1.79)	575	449.1 (100), 423.0 (75), 539.2 (28), 289.1 (22), 407.1 (20), 557.1 (13)	 ***
9 Dehydrodiepicatechin A (epicatechin-epicatechin) (C ₃₀ H ₂₄ O ₁₂)	58.0 (1.95)	575	449.1 (100), 423.0 (75), 539.2 (28), 289.1 (22), 407.1 (20), 557.1 (13)	 ***

^aRt = retention time. * Comparison with purified commercial standards (retention time and ESI(-)-HRMS/MS spectra). ** From refs 38 and 42 and ESI(-)-HRMS/MS. *** From refs 21 and 42 and ESI(-)-HRMS/MS.

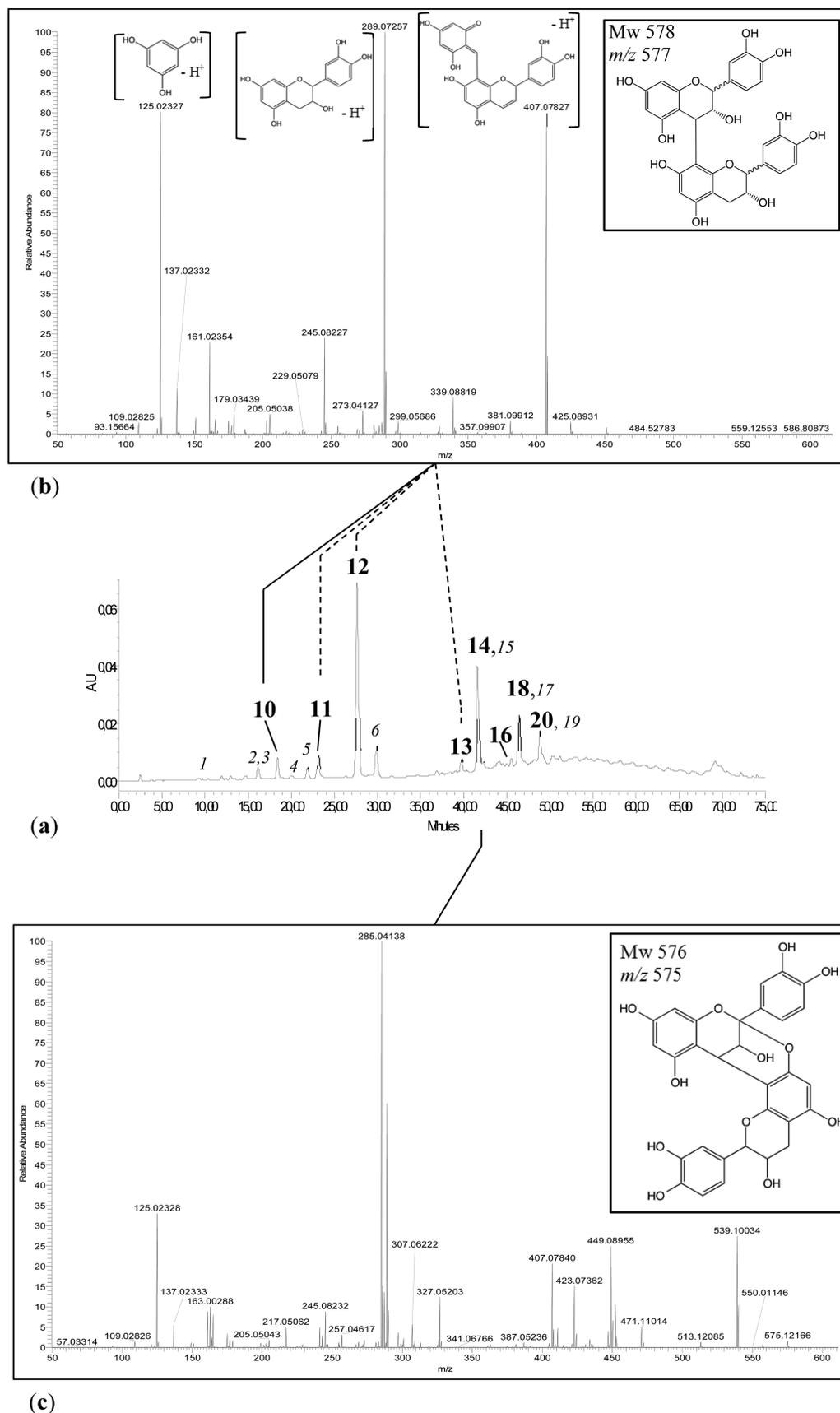


Figure 4. (a) RP-HPLC-UV chromatogram (280 nm) of procyanidin B2 heated at 60 °C for 12 h in aqueous media. ESI(-)HRMS/MS spectra at (b) m/z 577 \pm 0.1 (peaks 10–13) and (c) 575 \pm 0.1 (peak 14). The dotted lines indicate that these compounds gave an HRMS/MS spectrum very similar to that here displayed.

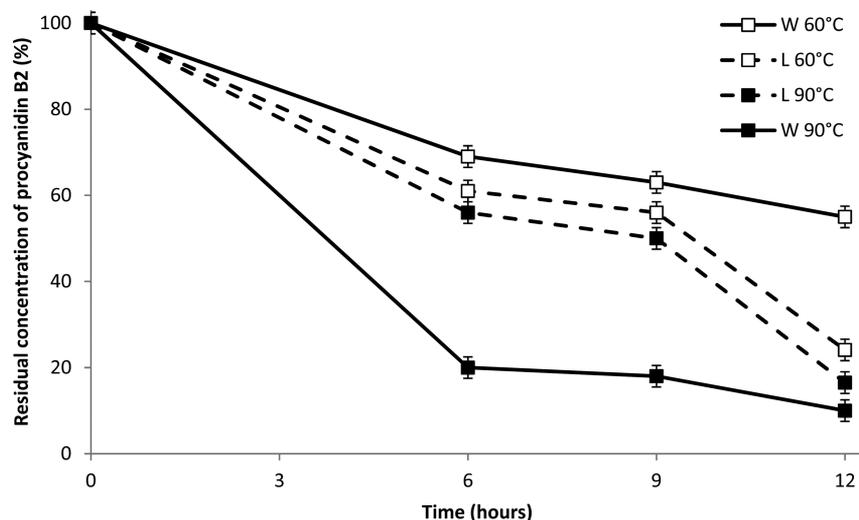


Figure 5. Evolution at 60 and 90 °C of the procyanidin B2 model medium. Quantitation was in B2 equivalents by ESI(–)-MS/MS. Standard deviation was <5%.

(0, 10, 25, 50, 100 mg/L, $R^2 = 0.99831$), and C1 with itself (0, 10, 25, 50, 100 mg/L, $R^2 = 0.99873$). No extraction was applied on aqueous model systems. For the lipidic model systems, a theoretical recovery factor of 100% was also used (relative values by comparison to the initial concentrations).

HRMS/MS Identification. High-resolution MS/MS spectra were obtained by connecting the same column with the same elution program to an Exactive system composed of Accela LC coupled to the Orbitrap mass spectrometer and controlled with Xcalibur software version 2.0.7 (Thermo Fisher Scientific). The ESI inlet conditions were as follows: sheet gas flow rate, 70; auxiliary gas flow rate, 5; sweep gas flow rate, 0; spray voltage, 3.50 kV; capillary temperature, 320 °C; S-lens level, 80%; heater temperature, 50 °C.

RESULTS AND DISCUSSION

The stability of (–)-epicatechin and procyanidin B2 was monitored by investigating model media after 0, 3, 6, 9, and 12 h at 60 and 90 °C. After a first series of experiments conducted in water (W; extraction steps here not required before HPLC separation), lipidic media (L) were also investigated. As for typical Belgian consumer chocolates,⁴⁷ the lipidic medium was composed of 59.7% (w/w) saccharose and 39.8% (w/w) cocoa butter spiked at midtime with lecithin (0.5%). Lecithin is often added in the last part of the conching process chocolate (heat treatment of 6–18 h at 60–90 °C) to improve rheological properties (0.1% of lecithin provides a similar effect as 1% cocoa butter).

Monitoring (–)-Epicatechin Degradation in Aqueous Medium. As depicted in Figure 1a, eight new peaks were detected in the aqueous (–)-epicatechin medium heated for 12 h at 60 °C (six at 280 nm and two at 410 nm). Most of them were already apparent after 3 h, especially when the temperature was increased to 90 °C (73% loss of (–)-epicatechin after 3 h at 90 °C versus only 11% loss at 60 °C; solid lines in Figure 2a).

The epimerization of (–)-epicatechin (peak 6) to (–)-catechin (peak 5, Figure 1a,b) accounted for 25% of the degradation of the monomer at 60 °C and even reached 70% at 90 °C (Figure 2b). Of course, catechin can also epimerize to epicatechin, but as depicted in Figure 3, because of lesser steric hindrance, catechin epimerization is less favored ((+)-catechin used in this experiment). Thus, whatever the initial epimer used, an equilibrium between epicatechin and catechin is reached before 3 h at 90 °C, with the same final ratio close to 70:30 in

favor of catechin (Figure 3). At 60 °C, on the other hand, (–)-epicatechin remained the major monomer in epicatechin-spiked medium after 12 h (Figure 1a).

Although not found in the 280 nm UV chromatogram (peaks 8 and 9 in Figure 1a), two yellow-orange compounds clearly appeared at 410 nm during (–)-epicatechin degradation, explaining the observed color of the medium (Figure 2c). Dehydrodi(epi)catechins A, previously described in a model medium²¹ and in 5-day-stored beer⁵ as arising through (+)-catechin degradation, eluted at the corresponding retention times (structures detailed in Table 1).

Three “chemically derived” dimers similar to the dehydrodi(epi)catechin B4 described by Guyot et al.²¹ and Sun and Miller³⁸ in model apple media were evidenced at 280 nm (peaks 2, 3, and 4, Figure 1a). They are here referred to as “chemical dimers” because contrary to native flavan-3-ols, the interflavan linkage is always created between aromatic rings. The formulas of these chemical dimers were confirmed by high-resolution mass spectrometry ($C_{30}H_{26}O_{12}$ experimental $m/z = 577.13687$; theoretical $m/z 577.13460$; $\delta 3.93$). In all three ESI(–)-HRMS/MS spectra (Figure 1c), the characteristic m/z peak = 393.09914 ($C_{21}H_{12}O_{16}$, not found for the natural procyanidin B2, Figure 4b) was evidenced, allowing further differentiation between “natural” (C4–C8 or C4–C6 linkages) and “chemical” (C2′–C8 or C6′–C8) dimers in complex media. Due to different possible structures that cannot be distinguished by HRMS/MS, the bold lines drawn in Table 1 and Figure 1 do not specify the precise position of the chemical bonds. Also worth stressing is the presence of compound 7, which elutes much later than compounds 2, 3, and 4, but with exactly the same mass spectrum (Figure 1c). We suspect it could be another chemical dimer, dehydrodi(epi)catechin B (Table 1), previously described by Guyot et al.²¹ and Poupard⁴² (same theoretical $m/z 577.13460$). All four compounds together account for <6%.

A trimer (peak 1; $C_{45}H_{38}O_{18}$, experimental $m/z 865.20094$; theoretical $m/z 865.19854$; $\delta 2.77$) was also detected at 280 nm and suspected to have arisen through addition of a “chemical dimer” to an oxidized monomer (Figure 1d). The commercial C1 trimer eluted 13.3 min after compound 1 by RP-HPLC (RT = 23.3 and 10 min, respectively). Only trimers containing (+)- or (–)-catechin units could elute before catechin.⁴⁹ Therefore, we

Table 2. Characterization of the Degradation Products in the Procyanidin B2 Model Media

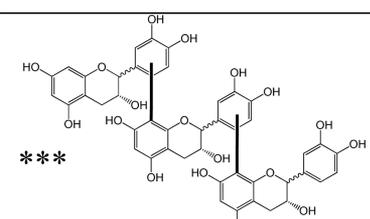
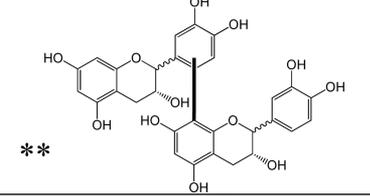
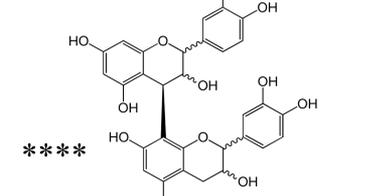
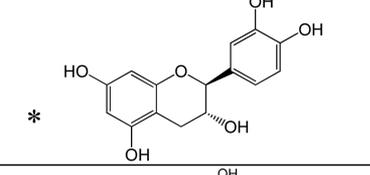
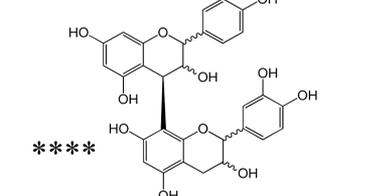
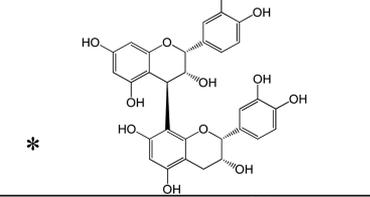
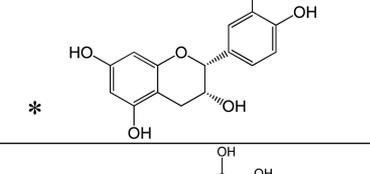
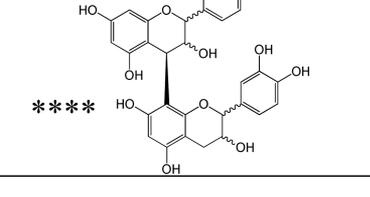
Peak n ^o , name and formula	Rt (min) (relative retention time)	[M-H] ⁻¹	MS/MS ions m/z (%)	Structure
1 Chemical trimer (C ₄₅ H ₃₈ O ₁₈)	10.0 (0.34)	865	577.1 (100) 319.1 (23) 613.1 (11) 287.1 (8)	 ***
2,3,4 Chemical dimer (C ₃₀ H ₂₆ O ₁₂)	16.8 19.3 (0.57) (0.65)	577	393.2 (100) 559.1(94) 425.1 (57) 439.1 (50)	 **
10 B2 epimer (C ₃₀ H ₂₆ O ₁₂)	18.5 (0.62)	577	425.1 (100), 451.1 (65), 407.2 (47), 289.1 (28)	 ****
5 (-)-Catechin (C ₁₅ H ₁₄ O ₆)	21,2 (0.72)	289	245.1 (100), 205.1 (34), 179.1 (12)	 *
11 B2 epimer (C ₃₀ H ₂₆ O ₁₂)	23.3 (0.78)	577	425.1 (100), 451.1 (65), 407.2 (47), 289.1 (28)	 ****
12 Procyanidin B2 (C ₃₀ H ₂₆ O ₁₂)	27.8 (0.94)	577	425.1 (100), 451.1 (65), 407.2 (47), 289.1 (28)	 *
6 (-)-Epicatechin (C ₁₅ H ₁₄ O ₆)	29.6 (1.00)	289	245.1 (100), 205.1 (34), 179.1 (12)	 *
13 B2 epimer (C ₃₀ H ₂₆ O ₁₂)	39.8 (1.34)	577	425.1 (100), 451.1 (65), 407.2 (47), 289.1 (28)	 ****

Table 2. continued

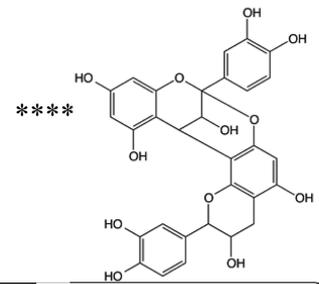
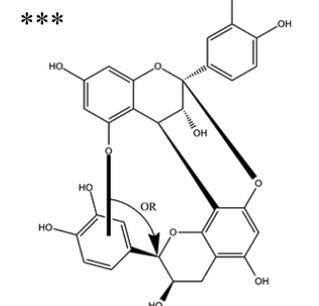
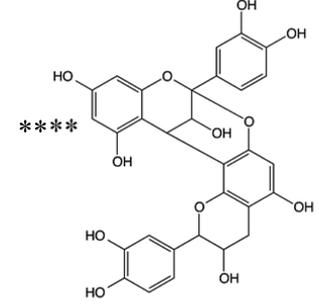
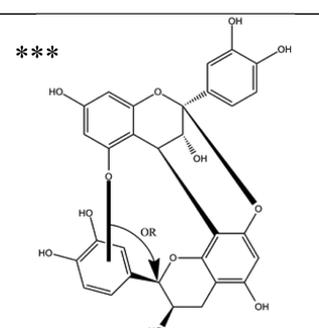
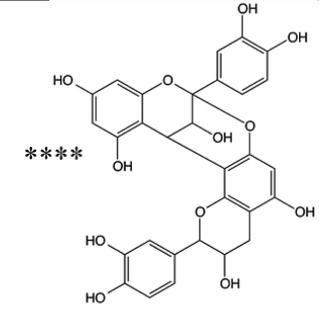
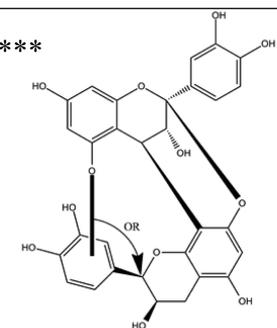
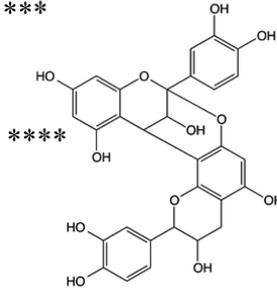
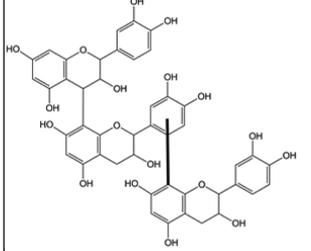
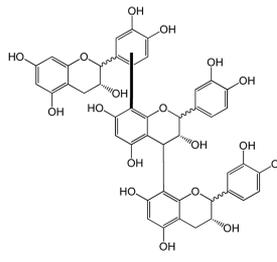
Peak n ^o , name and formula	Rt (min) (relative retention time)	[M-H] ⁻¹	MS/MS ions m/z (%)	Structure
14 A2 (C ₃₀ H ₂₄ O ₁₂)	41.9 (1.42)	575	449.1 (100), 539.2 (47), 423.1 (42), 289.1 (30), 407.2 (15), 557.1 (14)	**** 
15 Oxidized A2 epimer (C ₃₀ H ₂₂ O ₁₂)	42.3 (1.43)	573	285.1 (100), 125.1 (75), 555.1 (40), 161.1 (31) 423.1 (23)	*** 
16 A2 Epimer (C ₃₀ H ₂₄ O ₁₂)	45.5 (1.54)	575	449.1 (100), 539.2 (47), 423.1 (42), 289.1 (30), 407.2 (15), 557.1 (14)	**** 
17 Oxidized A2 epimer (C ₃₀ H ₂₂ O ₁₂)	46.7 (1.58)	573	285.1 (100), 125.1 (75), 555.1 (40), 161.1 (31) 423.1 (23)	*** 
18 A2 epimer (C ₃₀ H ₂₄ O ₁₂)	46.2 (1.56)	575	449.1 (100), 423.0 (75), 539.2 (28), 289.1 (22), 407.1 (20), 557.1 (13)	**** 

Table 2. continued

Peak n ^o , name and formula	Rt (min) (relative retention time)	[M-H] ⁻¹	MS/MS ions m/z (%)	Structure
19 Oxidized A2 epimer (C ₃₀ H ₂₂ O ₁₂)	46.7 (1.58)	573	285.1 (100), 125.1 (75), 555.1 (40), 161.1 (31) 423.1 (23)	*** 
20 A2 epimer (C ₃₀ H ₂₄ O ₁₂)	48.7 (1.56)	575	449.1 (100), 423.0 (75), 539.2 (28), 289.1 (22), 407.1 (20), 557.1 (13)	*** **** 
21^a Chemical trimer (C ₄₅ H ₃₈ O ₁₈)	28.1 (0.95)	865	864.2 (100) 865.2 (50) 846.2 (30) 820.3 (20)	 OR 

^aTrimer only observed in the medium containing both (-)-epicatechin and procyanidin B2 * Comparison with purified commercial standards (retention time and ESI(-)-HRMS/MS spectra). ** From refs 38 and 42 and ESI(-)-HRMS/MS. *** From refs 21 and 42 and ESI(-)-HRMS/MS. **** Similarities with the ESI(-)-HRMS/MS of a commercial epimer.

suspect that peak 1 could be a chemical trimer containing one to three units of (-)-catechin.

Monitoring Procyanidin B2 Degradation in Aqueous Medium. Figures 4 and 5 show that procyanidin B2 (peak 12) was also degraded in aqueous medium, especially at 90 °C, and yielded a large number of derived compounds (Table 2). Again, epimerization was found to occur, leading to three peaks (10, 11, and 13) in addition to B2 (12), all with an ESI(-)-HRMS/MS spectrum identical to that of the native dimer (epimers commercially unavailable) (Figure 4b). Those epimers are (-)-epicatechin-4 β -8-(-)-catechin, (-)-catechin-4 β -8-(-)-epicatechin,

and (-)-catechin-4 β -8-(-)-catechin. In B2 equivalents, none of them exceeded 5% of the initial procyanidin B2 concentration. Compound 10 could be suspected to coelute with commercially available procyanidin B1 [(-)-epicatechin-4 β -8-(+)-catechin]. Our gradient elution applied on the C18 column was able to differentiate the natural procyanidin B1 from the suspected (-)-epicatechin-4 β -8-(-)-catechin epimer, called here “B1-like” (elution of compound 10 0.6 min after B1; data not shown). It was nonetheless impossible in our model medium to create B1 that includes a (+)-catechin moiety in its structure.

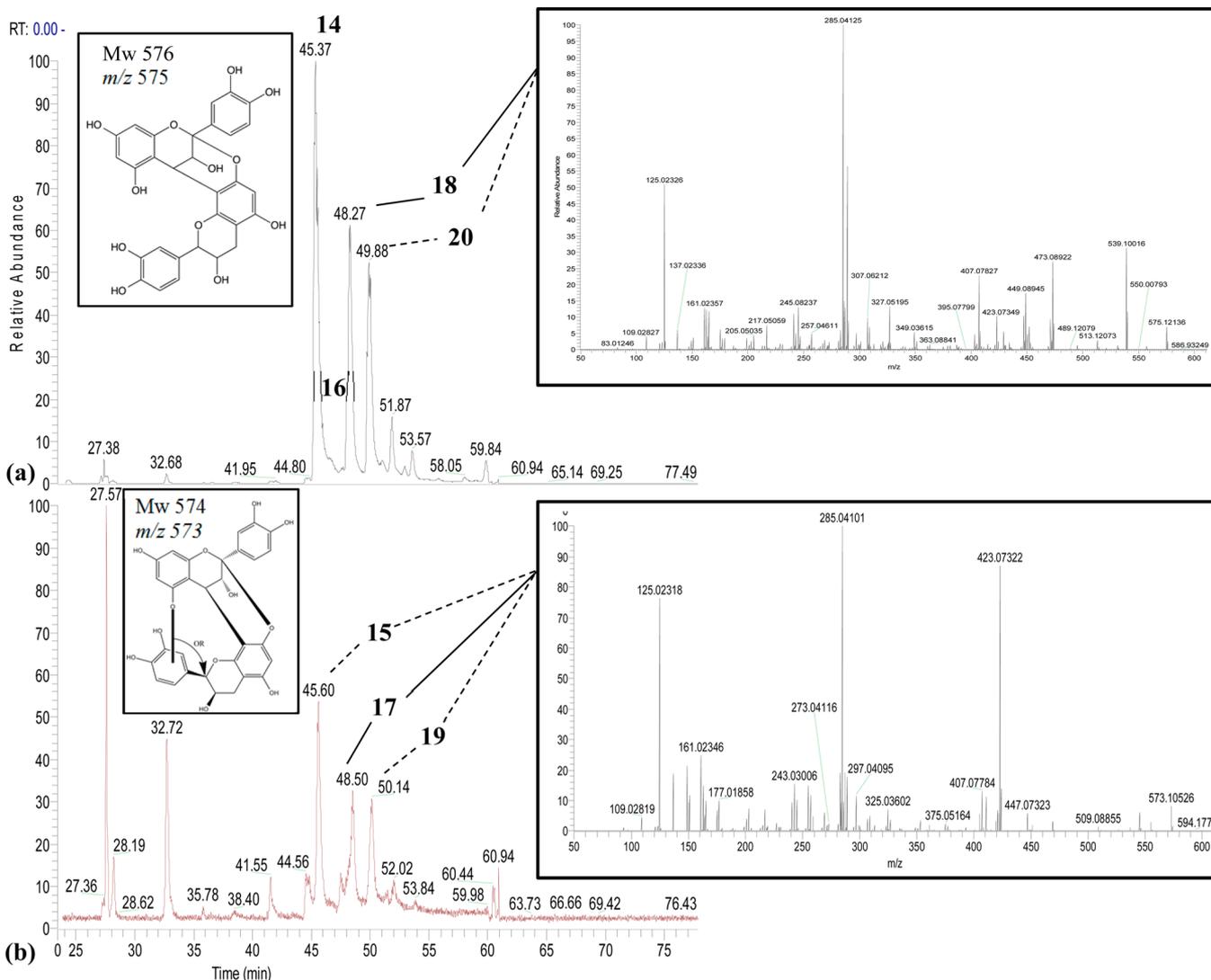


Figure 6. RP-HPLC-ESI(-)-HRMS/MS chromatogram of B2 heated at 90 °C for 12 h in aqueous media: (a) m/z 575 \pm 0.1; (b) m/z 573 \pm 0.1. The bold lines in the molecule indicate that the precise position of the chemical bonds is not known. The dotted lines indicate that these compounds gave an HRMS/MS spectrum very similar to that here displayed.

Depolymerization and further epimerization also generated some peaks [all discussed here above in relation to Figure 1]: (–)-epicatechin (peak 6), (–)-catechin (peak 5), and dehydrodiepicatechins A (peaks 8 and 9, resolved only at 410 nm). The aqueous B2-containing media were, as expected, slightly yellow after degradation at 60 °C and red-brown at 90 °C. Only 50 mg/L (–)-epicatechin and (–)-catechin were observed after 12 h at 90 °C, accounting for <5% of the initial procyanidin B2 (1000 mg/L).

No trimer (**1**) was detectable on the UV chromatogram (Figure 4a). Yet traces were found by selecting m/z 865 at the mass detector (fragmentation similar to that of compound **1** in the epicatechin model system). These results are in line with those previously published by Vidal et al.⁷

Procyanidin A2 (compound **14**, Figure 4a,c) was also produced from B2 (detectable only at 90 °C). Poupard et al.⁴² also evidenced A2 in apple juice media. Three analogues (**16**, **18**, and **20**) with the same mass spectrum (Figure 6a) were found in lower amounts. They are suspected to be three A2 epimers ($C_{30}H_{24}O_{12}$: experimental m/z for **18** 575.12136; theoretical m/z 575.11950; δ 3.23). These compounds can undergo further

oxidation leading to compounds **15**, **17**, and **19** ($C_{30}H_{22}O_{12}$: experimental m/z for **17** 573.10526; theoretical m/z 573.10385; δ 2.46 ppm; Figure 6b). The position of the third interunit linkage must still be determined.

Comparison between Aqueous and Lipidic Media.

(–)-Epicatechin degradation at 60 °C proved much faster in lipidic medium (dotted lines in Figure 2a, 11% loss after 3 h in water versus 50% in cocoa butter). This contrasted with the epimerization pathway, an important degradation pathway in aqueous medium, which proved to be much slower in lipidic medium (<5% after 12 h at 90 °C, Figure 2b). Epimerization of epicatechin involves the breaking of the C-ring, leading to a radical intermediate. The higher efficiency of epimerization in the aqueous medium is most probably explained by the higher relative stability of this intermediate in an aqueous environment.

In contrast to (–)-catechin, dehydrodi(epi)catechins A were formed more quickly in lipidic medium and obviously much more quickly at higher temperature (Figure 2c). Yet none of the studied modalities allowed generating more than the equivalent of 4% of the epicatechin initially present. The “chemical” dimers (**2**, **3**, and **4**), intermediate compounds involved in the reaction

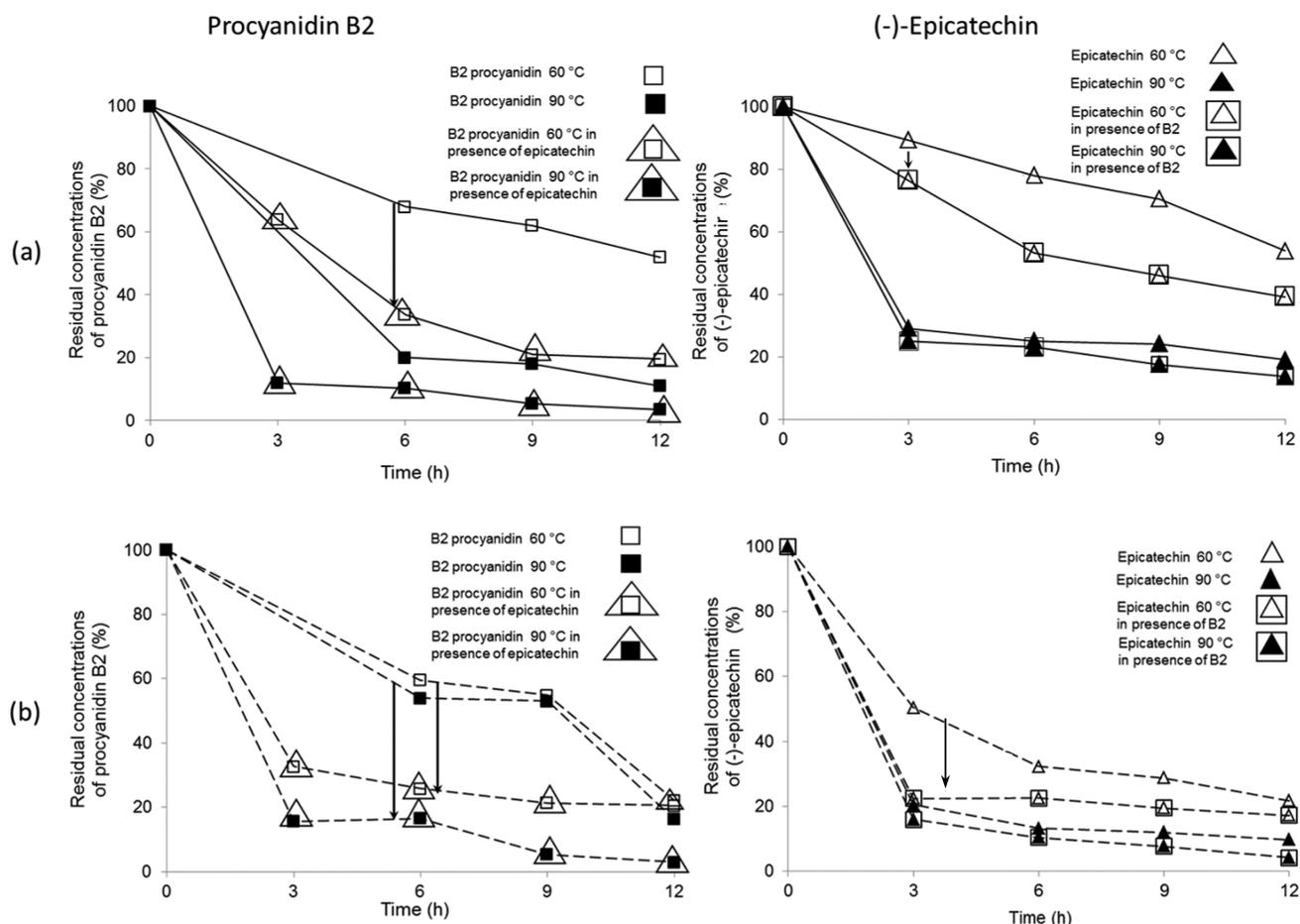


Figure 7. Comparison of the evolution at 60 and 90 °C of (-)-epicatechin and procyanidin B2 in (a) aqueous and (b) lipidic media, pure or mixed together. Quantitation was by ESI(-)-MS/MS. Standard deviation was <5%.

forming the dehydrodi(epi)catechins A, were found in both media, but in lower amounts in the lipidic environment.

Dimer B2 also behaved differently in the fatty environment. At 90 °C, B2 was degraded more slowly in the lipidic medium, although the two systems showed similar residual concentrations after 12 h (Figure 5). At 60 °C on the other hand, as previously seen for the monomer, the procyanidin B2 concentration decreased more rapidly in lipidic medium than in water (78% degradation versus 42%, after 12 h at 60 °C) (Figure 5).

Synergy between Monomers and Dimers. To determine whether a monomer might influence the degradation of the dimer or vice versa, aqueous and lipidic model media containing (-)-epicatechin and procyanidin B2 together (1000 mg/L each) were analyzed.

As depicted in Figure 7, the presence of the monomer was found to strongly accelerate degradation of the dimer, in both aqueous (Figure 7a) and lipidic (Figure 7b) media (e.g., 34% residual procyanidin B2 for the aqueous medium after 6 h at 60 °C in the presence of monomer versus 68% in its absence).

Likewise, the degradation rate of (-)-epicatechin was influenced, at least at 60 °C, by the presence of procyanidin B2. After 3 h, the residual concentrations of (-)-epicatechin were significantly lower in the presence of the dimer (76% in water and 22% in lipidic medium) than in its absence (89% in water and 50% in lipidic medium, coefficient of variation below 5% for our data). Epimerization, in contrast, was 10% less efficient than in the media containing only the monomer. At 90 °C, the (-)-epicatechin

degradation rate was not significantly influenced by the presence of procyanidin B2.

As depicted in Figure 8a,b, in addition to the previously described “chemical” trimer 1, a new trimer (21) emerged in this mixed medium, with a retention time of 28.1 min (HRMS/MS confirmation of $C_{45}H_{38}O_{18}$: experimental m/z 865.20094; theoretical m/z 865.19854; δ 2.77). Most probably, this was due to polymerization of an oxidized monomer with the natural dimer (both at 60 and at 90 °C). Not present for the natural C1 trimer (Figure 8c), the m/z 319 fragment was found for both chemical trimers. Further investigations are required to reveal their accurate structure.

Occurrence of Native Flavan-3-ol Degradation Products in Roasted Cocoa and Chocolate.

As depicted in Figure 9, unroasted Ecuadorian cocoa beans (Figure 9a) contained (-)-epicatechin (1523 mg/kg, 6) and very little catechin (77 mg/kg, 5), procyanidins B2 (561 mg/kg, 12), B5 (397 mg/kg), and C1 (309 mg/kg). As expected, high temperature during roasting (30 min at 150 °C) significantly decreased the amount of natural monomer (-51% epicatechin), dimers (-50% B2 and -57% B5), and trimer (-81% C1). On the other hand, degradation through conching (12 h at 75 °C) was moderate except for the monomer (-53% epicatechin, -12% dimer B2, -8% dimer B5, and -9% trimer C1, taking into account the 72% cocoa content in the resulting chocolate). Of course, due to the presence of various higher oligomers in cocoa (not detectable in our RP-HPLC chromatograms), part of the “natural” monomers and

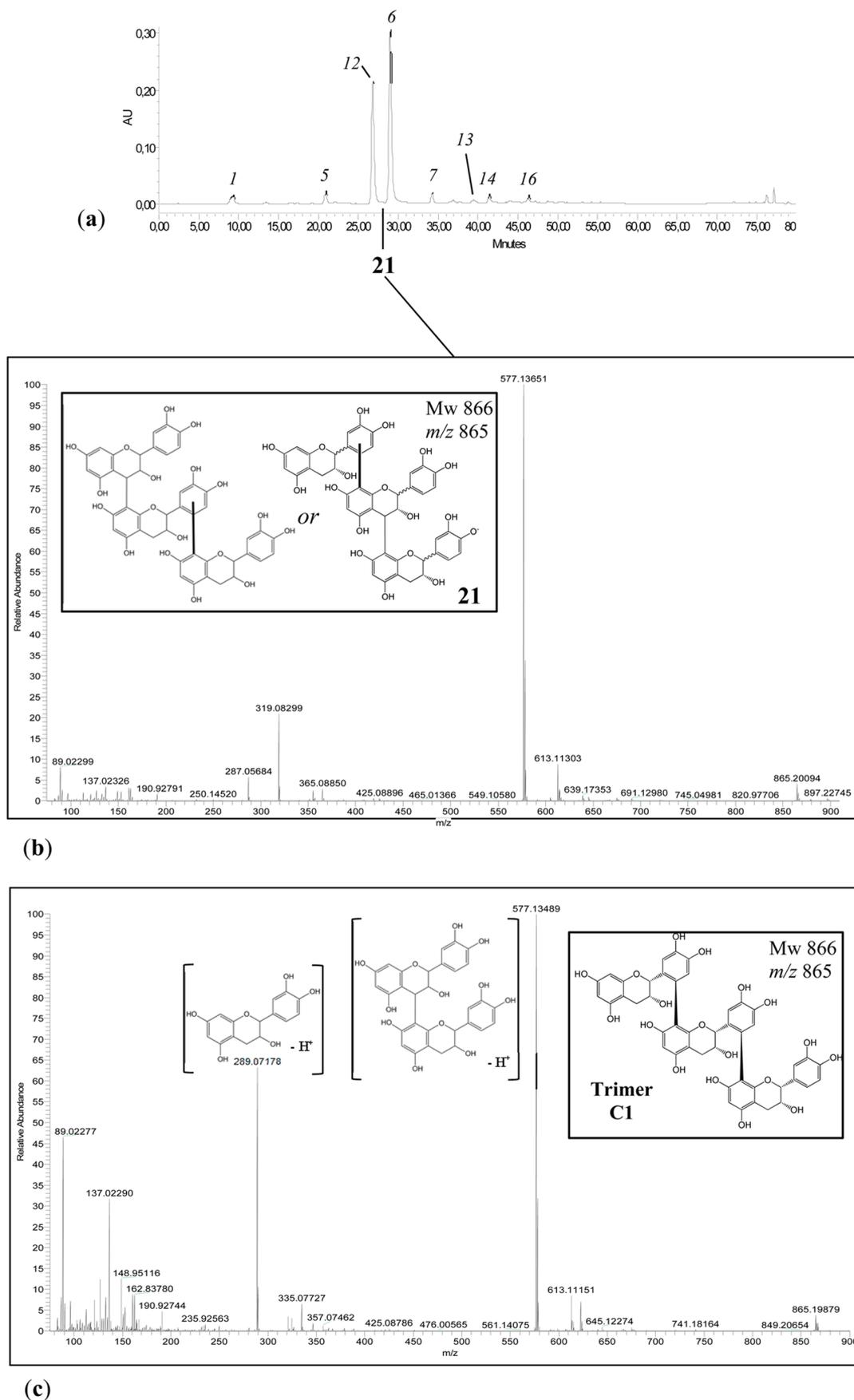


Figure 8. (a) RP-HPLC-UV chromatogram (280 nm) of B2 heated at 60 °C for 12 h in aqueous media in the presence of (–)-epicatechin and ESI(–)HRMS/MS spectra of (b) compound 21 and (c) the natural procyanidin C1.

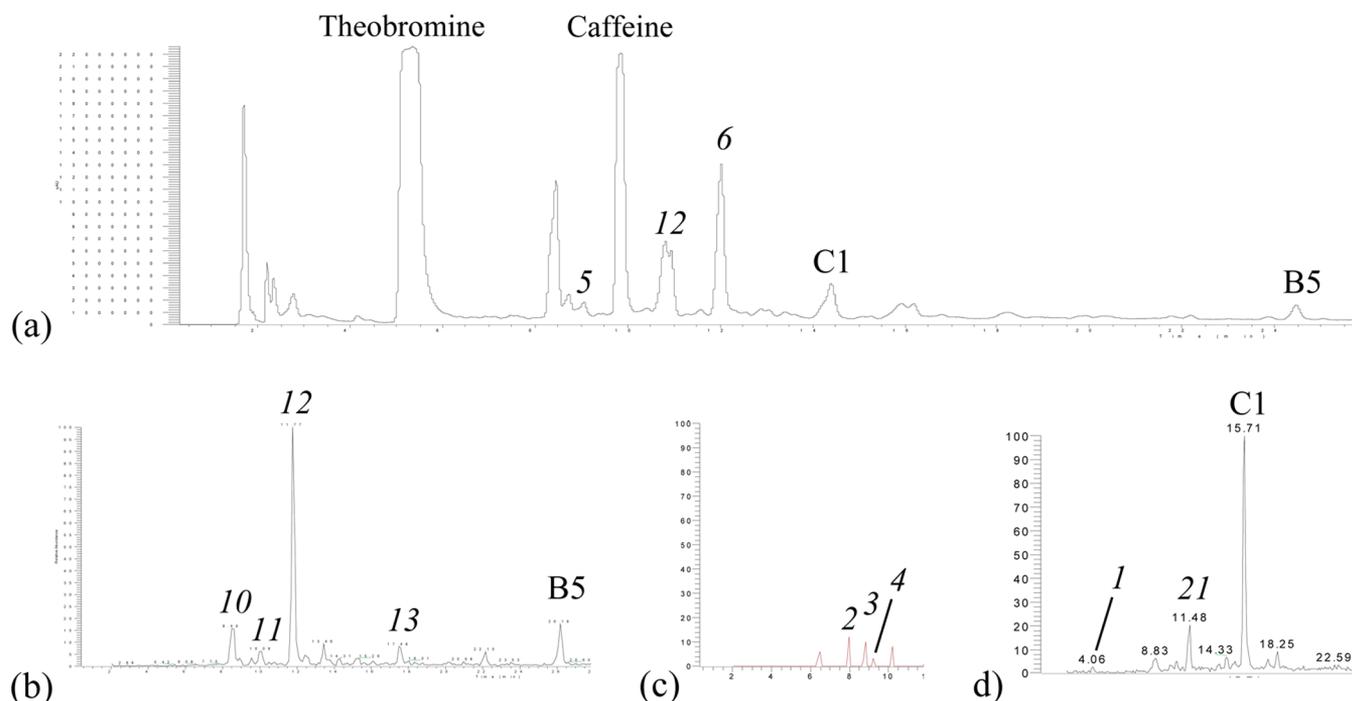


Figure 9. (a) RP-HPLC-UV(280 nm) chromatogram of the polyphenolic extract issued from unroasted cocoa beans. (b–d) RP-HPLC-MS/MS chromatograms of the extract issued from the corresponding roasted cocoa beans at (b) m/z 577, (c) m/z 577 \rightarrow 393, and (d) m/z 865.

dimers found in roasted cocoa beans could also be issued from depolymerization.

Most of the degradation products evidenced in our model media have been found in the roasted cocoa beans (Figure 9b,c) as well as in chocolate. Epimerization of (–)-epicatechin significantly occurred through roasting, as shown by the epicatechin/catechin ratios of 19.8 and 5.2 before and after roasting, respectively. B2 epimers (peaks 10, 11, and 13) were also formed (observed only by MS/MS on m/z 577, Figure 9b). Epimerization was revealed to be less efficient during chocolate conching (the epicatechin/catechin ratio remained close to 5 in the derived chocolate).

Compounds 2, 3, and 4, all three suspected to be “chemical” newly formed dimers, were absent in unroasted beans but were evidenced after heat treatment (Figure 9c; selection of the specific fragment 393 issued from m/z 577). In the same way, 1 and 21 suspected to be “chemical” trimers were detectable after roasting (Figure 9d). To our best knowledge, this is the first work that states the presence of chemical oligomers in processed cocoa.

The “chemical” dimers and oligomers produced during cocoa processing still require deeper investigations (detailed structure, antioxidant capacity, bioavailability, etc.). The further step of this research will be to optimize the roasting and conching temperature programs to maximize the content of bioavailable antioxidant polyphenols.

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Notes

The authors declare no competing financial interest.

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