

Fate of Anthocyanins through Cocoa Fermentation. Emergence of New Polyphenolic Dimers

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ABSTRACT: Fresh, ripe cocoa beans from Cameroon (German cocoa/Amelonado group and ICS 40/Trinitario group) were subjected to fermentation-like incubations in acetic acid, lactic acid, or both and to natural fermentation. Two naturally fermented samples from Cuba (UF 654/Trinitario group and C 411/Criollo group) were also investigated. Both cyanidin-3-galactoside and cyanidin-3-arabinoside (found as major anthocyanins in colored beans only) were drastically degraded through fermentation, especially in small beans and in the presence of acetic acid. On the other hand, emergence of a cyanidin-rhamnose isomer was evidenced, even in Criollo beans. In addition to the recently described structures F1 and F2 [$m/z = 575$ in ESI(-)], three additional polyphenolic structures [F3, F4, and F5; $m/z = 557$ in ESI(+)] were found after fermentation, the two former ones resulting from epicatechin oxidation. Synthesis of F5 requires an interclass reaction between cyani(di)n and epicatechin, which explains its absence in fermented Criollo beans.

KEYWORDS: cocoa fermentation, anthocyanin, epicatechin, Criollo, cyanidin-rhamnoside

INTRODUCTION

After flavan-3-ols, anthocyanidins are the most represented flavonoids in cocoa.^{1–3} Mainly present as glycosides of cyanidin (galactoside and arabinoside), these water-soluble pigments give their characteristic purple-red color to non-Criollo unfermented cocoa beans.⁴ This color is due to major, specific delocalization of π -electrons through the aromatic structure (chromophore with eight conjugated double bonds).⁵ The pigments accumulate in vacuoles of pigmentary cells, located at the periphery of bean cotyledons. In freshly harvested seeds, cyanidin-3-galactoside and cyanidin-3-arabinoside levels can reach 100–3500 and 400–4500 mg/kg, respectively.^{6,7}

Proper cocoa fermentation is a key step in achieving high-quality cocoa.^{1,8–11} The well-defined succession of microorganisms through time (4–7 days) allows production of ethanol by yeasts and its oxidation by acetic acid bacteria.^{12,13} These two exothermic reactions increase the temperature in the fermentation heap or boxes up to 50 °C. Heat, together with alcohol and acid penetration into the bean, leads to death of the embryo and triggers a cascade of biological reactions, including physical and chemical changes that allow enzymes and substrates to meet.^{11,14} As natural fermentations remain hard to control, some authors have used fermentation-like incubations in acids to better assess the evolution of cocoa components through fermentation.^{15,16} A recent study by Eyamo et al. has confirmed that cocoa flavan-3-ols evolve in a similar way through natural fermentations and through incubations in acetic and/or lactic acid.¹⁷

In the presence of acids, the vacuolar lipidic structure changes in cotyledon cells, allowing further intracellular enzymatic reactions. For example, glycosidases cleave the sugar moiety from cyanidin-3-galactoside and cyanidin-3-arabinoside to release free cyanidin.¹⁸ As shown in fermentation index assays, this

cleavage leads to loss of the original bean color, due to a shift in absorbance between the glycosylated form of cyanidin (530 nm) and its aglycon (460 nm).^{19,20} Niemenak et al., however, observed in some clones an increase in total anthocyanins through fermentation.⁷

Released cyanidin is a highly reactive, positively charged compound. As described by Sun et al., it can easily be degraded to protocatechuic acid and phloroglucinaldehyde.¹¹ It can also act as an electrophile toward other cocoa constituents. In wine, for instance, anthocyanidins react with endogenous epicatechin to form uncharged pyrano molecules, which can be further dehydrated to pyranoanthocyanins or react with a carbocation issued from dimer cleavage to form flavanol–anthocyanin structures.^{21–23}

Very recently, Eyamo et al. evidenced the emergence through cocoa fermentation (natural or simulated in acid) of two newly identified compounds [named F1 and F2, $m/z = 575$ in ESI(-)-MS/MS].¹⁷ They suspected that these compounds were issued from reactions between polyphenols of different families, including anthocyanidins. The aim of the present work was to assess the fate of cocoa anthocyan(id)ins through model and natural fermentations, in both colored beans (Forastero and Trinitario) and white Criollo beans. An attempt was made to identify F1 and F2, along with additional fermentation-derived molecules evidenced here, by comparison with model reaction media containing (+)-epicatechin (the major flavan-3-ol in cocoa) and major cocoa cyanidin glycosides.

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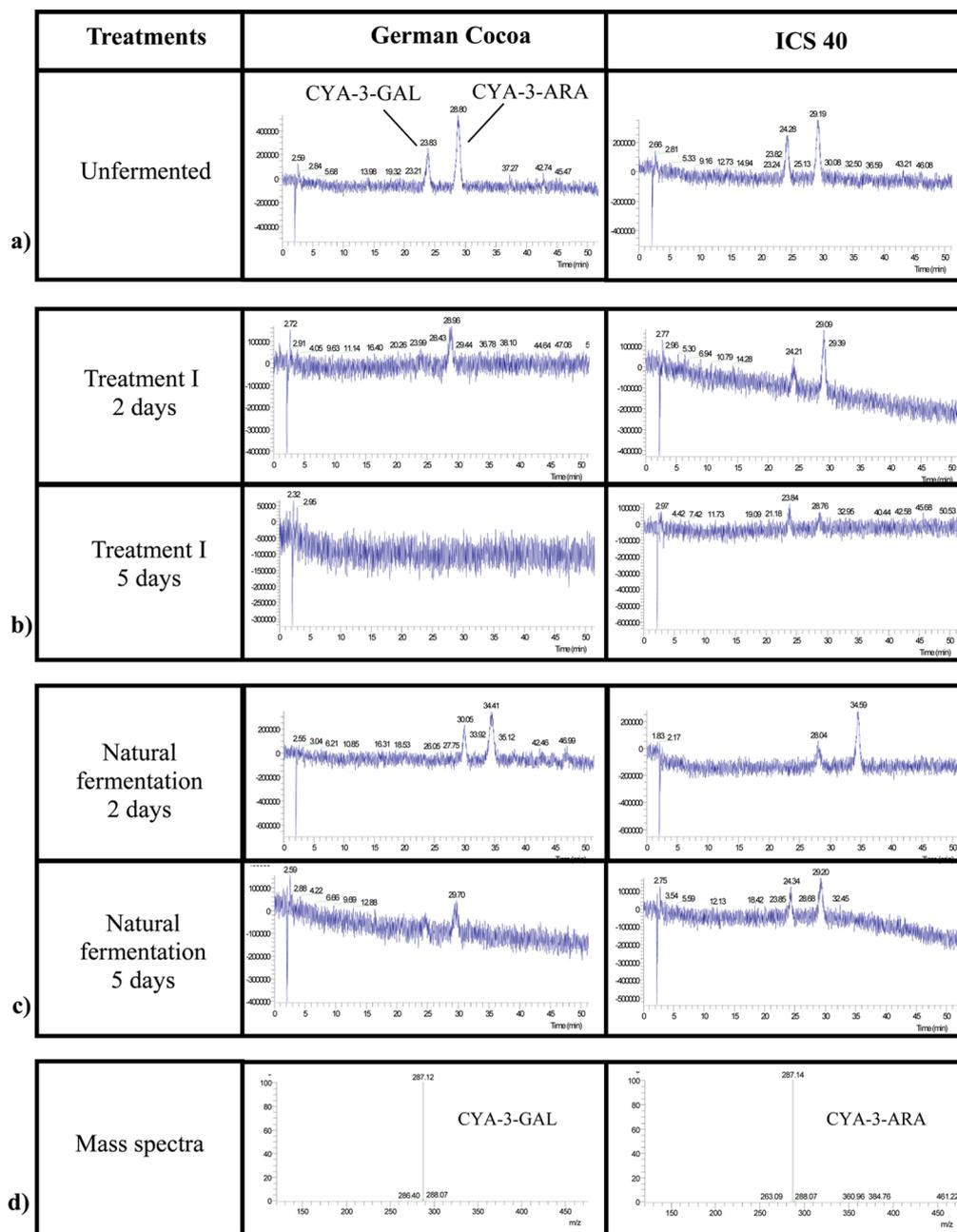


Figure 1. RP-HPLC–UV chromatograms (530 nm) of CYA-3-GAL and CYA-3-ARA in (a) unfermented and (b, c) fermented GC and ICS 40 beans; (b) fermentation-like incubation in acetic acid; (c) natural fermentation on days 2 and 5. (d) Experimental mass spectrum of the two compounds.

MATERIALS AND METHODS

Chemicals. Acetic acid (99%) was supplied by Fluka. Acetonitrile (99.9%) and methanol (99.9%) were supplied by VWR (Leuven, Belgium). Formic acid (99%) was obtained from Acros Organic (Geel, Belgium). Lactic acid (98%), (–)-epicatechin (98%), (+)-catechin (98%), kaempferol (97%), protocatechuic acid (99%), and phloroglucinaldehyde (99%) were supplied by Sigma-Aldrich (Bornem, Belgium). Cyanidin chloride (99%), cyanidin 3-arabinoside chloride (99%), and cyanidin 3-galactoside chloride were supplied by PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Aqueous solutions were made with Milli-Q water (Millipore, Bedford, MA) (resistance = 18 mΩ).

Cocoa Samples. German cocoa pods (the name refers to old Amelonado clones introduced in Cameroon by Germans at the beginning of the 20th century) were obtained from an old plantation near

Ngoumou, Cameroon. ICS 40 pods (Trinitario group) came from the SODECAO cocoa station in Nkoemvone, Cameroon. Both cultivars are recognized in the country for their high productivity. Damaged pods were discarded and fermentation-like incubations were carried out directly after harvest. Unfermented and naturally fermented Criollo C 411 and Trinitario UF 654 beans were from Cuba (Baracoa region).

Natural Fermentations and Fermentation-like Incubations. Natural fermentations were conducted in Cameroon and Cuba for 5 days. About 50 kg of cocoa beans of each clone were fermented separately in wooden boxes covered with banana leaves. The beans were stirred manually, morning and evening, every 2 days and then sun-dried.

For the fermentation-like incubations done in Cameroon, three different treatments were applied to both Cameroon cocoa clones, according to the method of Rohsius,¹⁶ with slight modifications.

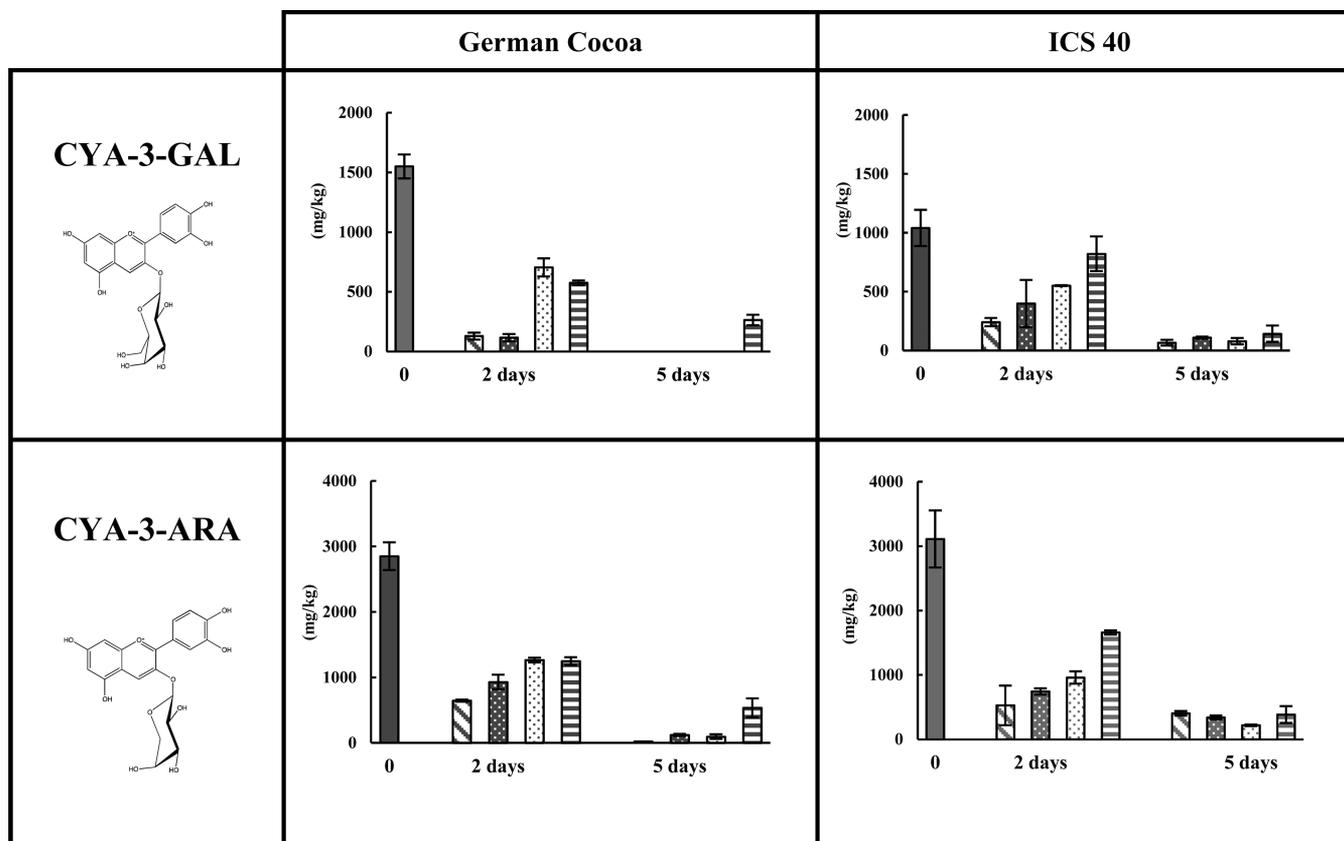


Figure 2. CYA-3-GAL and CYA-3-ARA contents (mg/kg) of German cocoa and ICS 40 beans after 0, 2, and 5 days of natural fermentation or fermentation-like incubation: solid gray = initial content, diagonal stripes = treatment I, white dots on a dark background = treatment II, dark dots on a white background = treatment III, and horizontal stripes = natural fermentation.

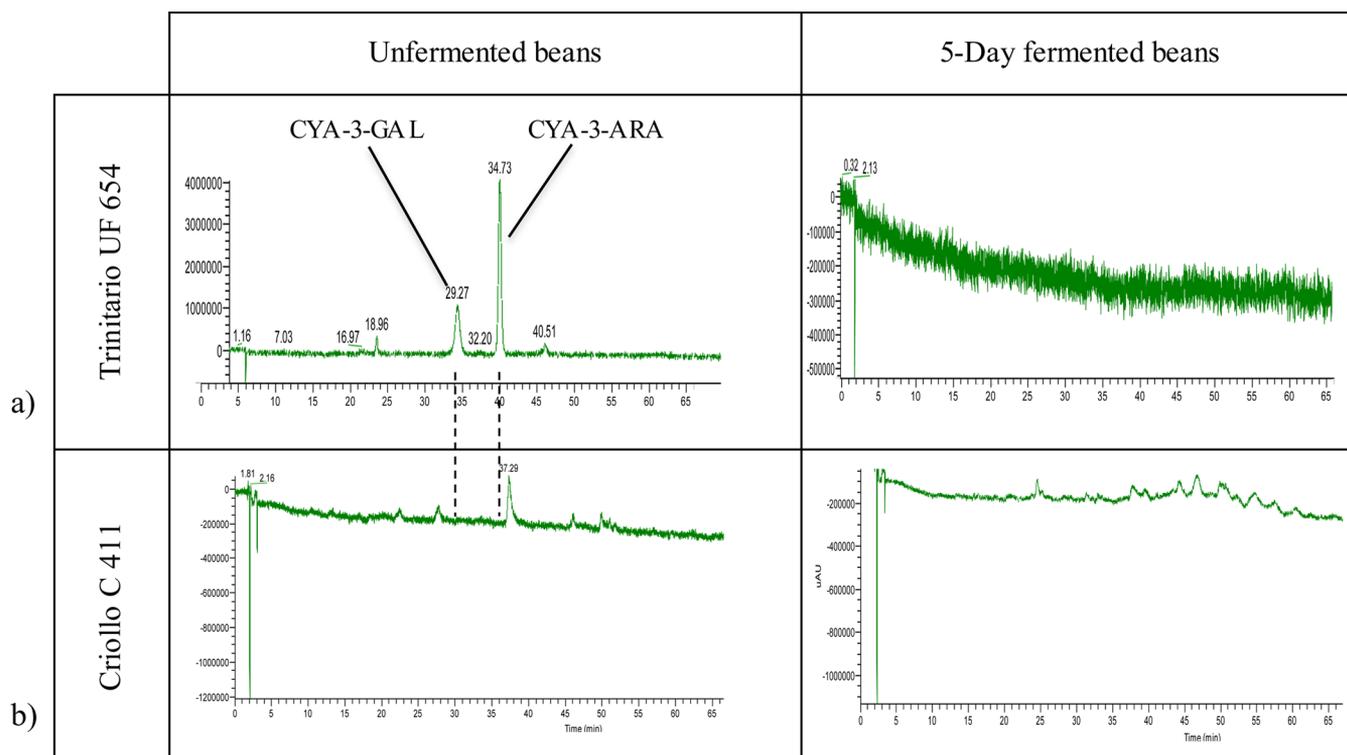


Figure 3. RP-HPLC–UV chromatograms (530 nm) of CYA-3-GAL and CYA-3-ARA in unfermented and 5-day naturally fermented (a) Trinitario UF 654 and (b) Criollo C 411 cocoa beans.

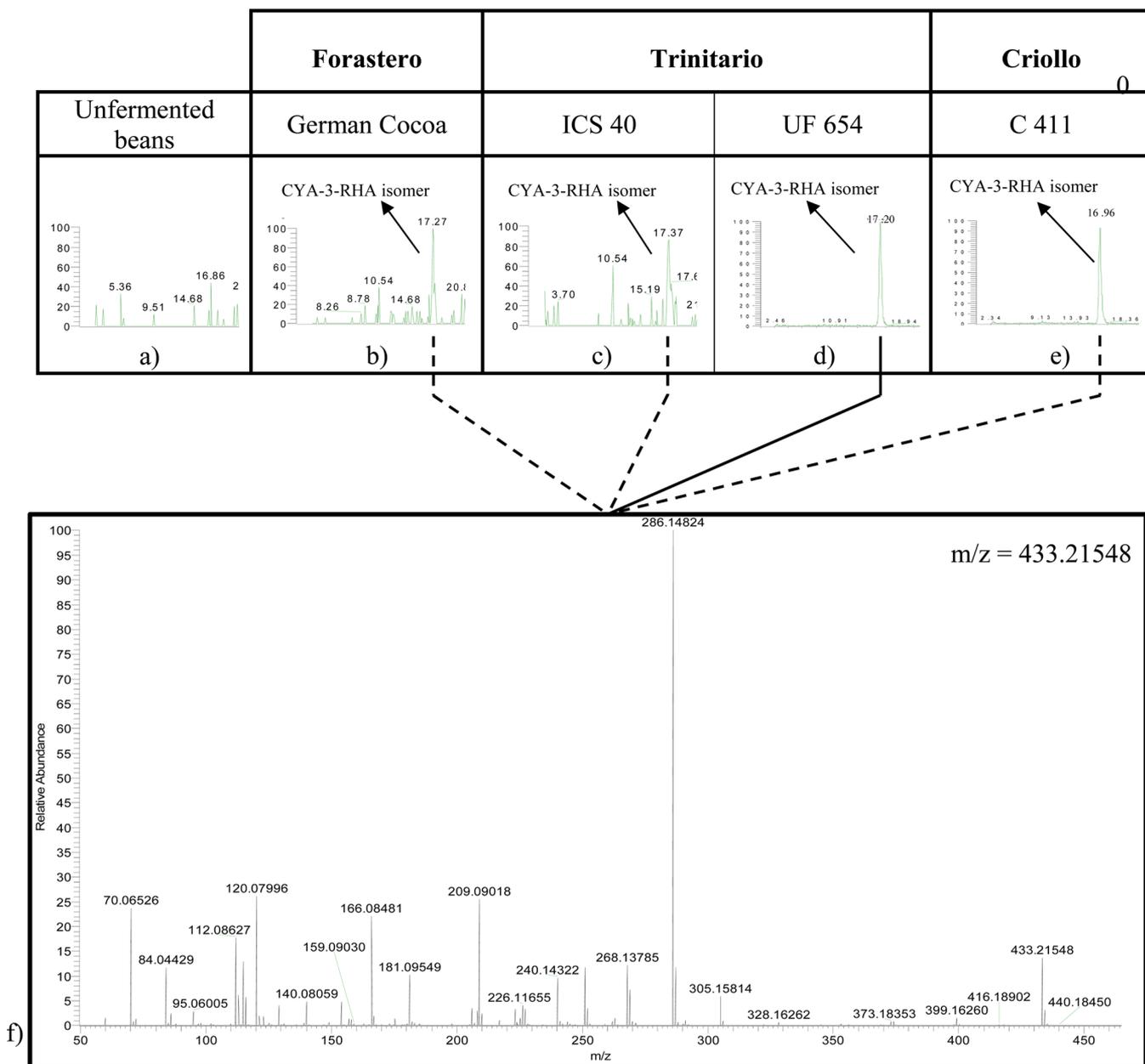


Figure 4. RP-HPLC–ESI(+)-MS/MS ($m/z = 433$) chromatograms of extracts issued from (a) unfermented GC, (b) 5-day naturally fermented GC, (c) 5-day naturally fermented ICS 40, (d) 5-day naturally fermented Trinitario UF 654, and (e) 5-day naturally fermented Criollo C 411 beans. (f) Experimental MS/MS spectrum (The dotted lines indicate that these compounds gave a MS/MS spectrum very similar to that displayed here).

Each treatment, mimicking similar acidic conditions as the ones achieved in natural fermentation, was applied four times, and the resulting dried beans were mixed together. In each experiment, one cocoa pod was washed twice for 15 min in sodium hypochlorite solution (30 mg/L) and then rinsed three times with distilled water. After natural drying under a laminar flow hood, the pod was sprayed with ethanol, flamed, and opened. Twenty-five beans were put into a sterilized glass bottle containing 150 mL of incubation medium [100 mmol/L acetic acid (treatment I), 100 mmol/L lactic acid (treatment II), or both acids (treatment III) at 100 mmol/L each, adjusted to pH 4 with 1 mmol/L NaOH].¹⁷ After 2 days of incubation, the beans were transferred under the hood to a second sterilized glass bottle containing the same volume of 200 mmol/L acetic acid (pH 5) and incubated for 3 days more. All the fermentation-like incubations were done away from light. The temperature was controlled throughout the experiments by incubating the bottles in a 30–50 °C water bath. After incubation, all the cocoa beans were sun-dried between 8 a.m. and 5 p.m. for 7 days with stirring

every 3 h (final water content: 6.9%). The dried beans were stored in black plastic bags at room temperature (25–28 °C) until analysis.

Polyphenol Degradation Model Media. Aqueous model media were prepared by diluting (–)-epicatechin and/or cyanidin, cyanidin-3-galactoside, or cyanidin-3-arabinoside at 1000 mg/L (each) in ultra-pure water (stock solutions of 10 000 mg/L prepared in methanol) before heat treatment at 50 °C for 24 h. After filtration (0.22 μm), the samples were kept at –80 °C before injection.

Extraction of Flavan-3-ols and Anthocyanins from Cocoa Beans. This method was adapted from that developed in our laboratory²⁴ for the analysis of flavan-3-ols in chocolate. All extraction steps were done in duplicate. Chocolate or cocoa beans (7 g) were defatted with 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 10 000 mg/L in methanol (used as internal standard; 714 mg/kg if reported to bean weight) were extracted with 3 × 50 mL of acetone:water:acetic acid

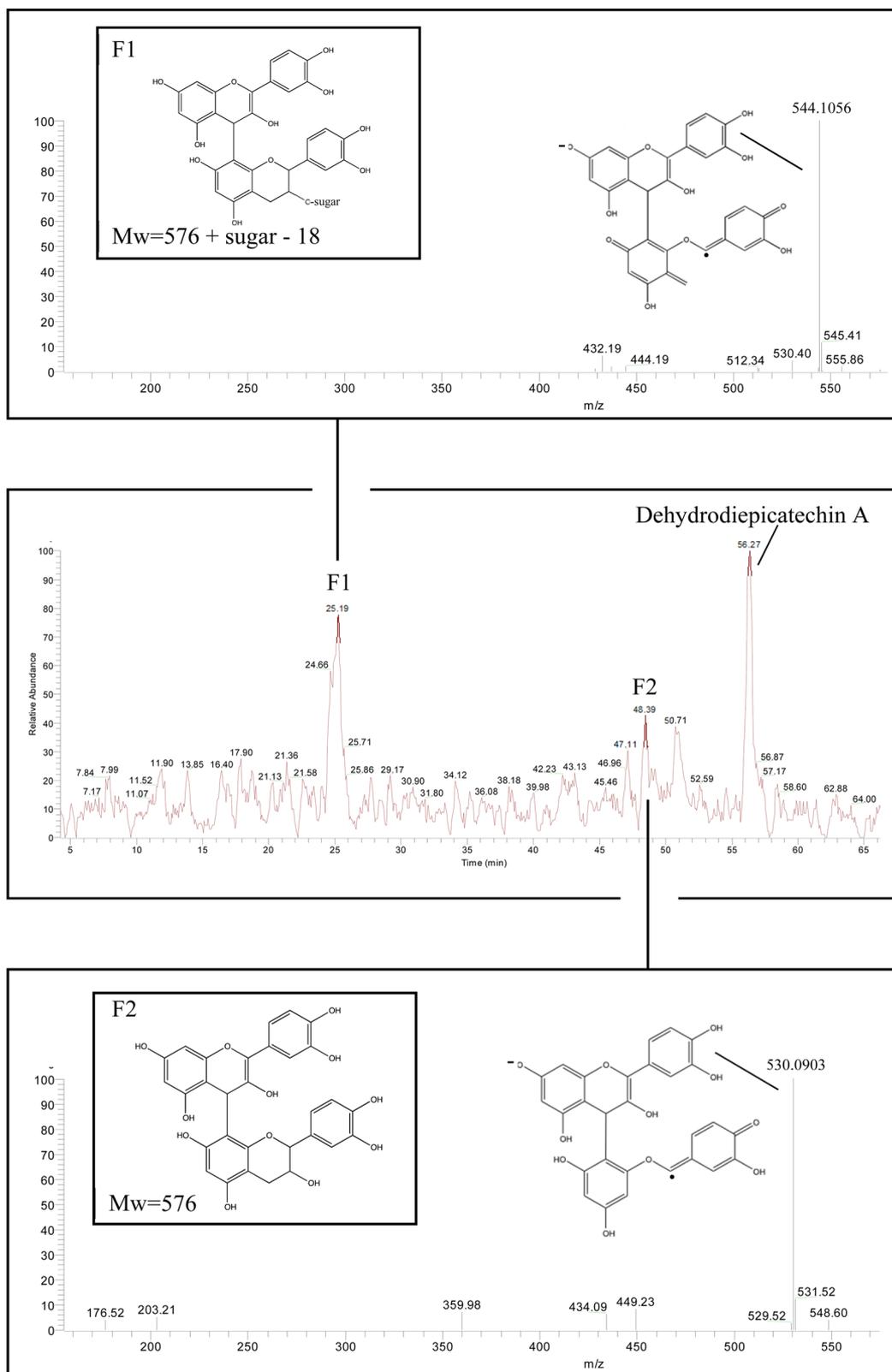


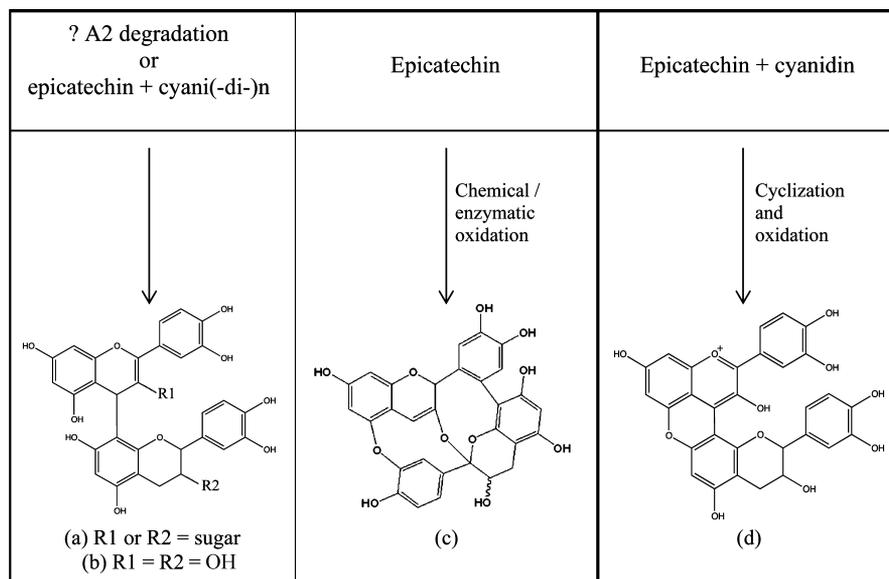
Figure 5. RP-HPLC–ESI(–)-MS/MS ($m/z = 575$) chromatogram of extracts issued from naturally fermented GC cocoa beans, with the corresponding suspected structures and mass spectra of compounds F1 and F2.

(70:28:2, v/v/v). After each extraction step, the extract was kept under nitrogen and then the three extracts were mixed together. The mixture was concentrated by rotary evaporation and freeze-dried.

RP-HPLC-DAD–ESI-MS/MS Semi-Quantitation. Quantitation was done by connecting a C18 Prevail column (150 × 2.1 mm, 2,7 μm)

(Grace, Deerfield, IL) to a SpectraSystem equipped with an AS3000 autosampler and a P4000 quaternary pump. A linear gradient from A (water containing 1% acetonitrile and 2% formic acid) to B (acetonitrile containing 2% formic acid) was used 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

Table 1. Hypothetical Structures of (a) F1, (b) F2, (c) F3 and F4, and (d) F5 and Suspected Precursors



64% to 10% in 10 min, and isocratic for 20 min at a flow rate of 200 $\mu\text{L}/\text{min}$. Five microliters of sample was injected onto the column kept at 20 $^{\circ}\text{C}$. The system was controlled with Xcalibur software version 1.2 (Thermo Fisher). Compounds were monitored from 200 to 800 nm with a UV6000LP diode array detector. Mass spectra were acquired with an LCQ ion trap mass spectrometer equipped with an ESI source (Thermo Fisher). Collision-induced dissociation spectra were recorded at a relative collision energy of 30 or 35 for singly charged $[\text{M} - \text{H}]^{-}$ ions of monomers (m/z 289) or dimers (m/z 577, 575), respectively, and at 42% for $[\text{M}]^{+}$ ions of cyanidin (m/z 287), cyanidin-3-galactoside (m/z 449), cyanidin-3-arabinoside (m/z 419), and F3, F4, and F5 (m/z 557). The ESI inlet conditions were as follows: source voltage, 4.9 kV; capillary voltage, -4 V; capillary temperature, 200 $^{\circ}\text{C}$; and sheath gas, 39 psi. Semiquantitation of anthocyanins was done with the calibration curves of cyanidin-3-arabinoside and cyanidin-3-galactoside, while cyanidin was used for itself.

HRMS/MS Analyses. 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, Austin, TX).

RESULTS AND DISCUSSION

Cyanidin-3-arabinoside and Cyanidin-3-galactoside in Forastero and Trinitario Cocoa Beans before and after Fermentation-like Incubations or Natural Fermentations. In agreement with the literature, both cyanidin-3-galactoside [CYA-GAL; m/z = 449 in ESI(+), major fragment at m/z = 287, t_{R} = 23.6 min] and cyanidin-3-arabinoside [CYA-ARA; m/z = 419 in ESI(+), major fragment at m/z = 287, t_{R} = 28.8 min] were detected at 530 nm in dried unfermented German cocoa (GC) and ICS 40 cocoa beans (Figure 1a). Unlike its glycosides, free cyanidin [m/z = 287 in ESI(+), t_{R} = 46.6 min, monitoring at 460 nm] was absent in both unfermented clones. As depicted in Figure 2, the initial CYA-3-ARA content was 2–3 times as high as the CYA-3-GAL content (2850 versus 1550 mg/kg and 3112 versus 1041 mg/kg in dried GC and ICS 40 unfermented beans, respectively).

After 2 days of fermentation-like incubation, both cyanidin glycosides already showed a sharp decrease (Figures 1b and 2). As observed previously for flavan-3-ol degradation,⁵ acetic acid alone (treatment I) most drastically affected the CYA-3-GAL

and CYA-3-ARA contents (up to -91% and -77% for GC, up to -77% and -83% for ICS 40, respectively). In GC beans, treatment III (fermentation-like incubation with lactic acid only) led to a residual anthocyanin content on day 2 very similar to that found in the naturally fermented sample (Figure 2). In natural box fermentations (Figures 1c and 2), acid bioavailability is of course limited by microorganism growth. In fermentation-like incubations, we can assume that the lower pK_{a} of lactic acid (3.85 at 25 $^{\circ}\text{C}$, resulting in a higher proportion of charged lactate molecules) reduces the rate at which it can migrate into cotyledon cells, as compared to acetic acid (pK_{a} = 4.75). In the bigger ICS 40 beans, into which acids penetrate less readily, cyanin loss on day 2 was more drastic in all fermentation-like incubations than in natural fermentation. After 5 days (Figures 1a,b and 2), CYA-3-GAL was undetectable in incubated GC beans whatever the treatment, whereas residual CYA-3-ARA was still found (0.7–18.8% recovered). In 5-day-fermented ICS 40 beans, recovery was in the range 7.6–13.6% for the native CYA-3-GAL and 7.0–13.0% for CYA-3-ARA.

In most cases, the greatest loss occurred during the first 2 days of fermentation, being less pronounced between days 3 and 5. Yet, most probably for kinetic reasons of acid penetration into a bigger bean, the opposite was observed for CYA-3-GAL in naturally fermented ICS 40 beans. As depicted in Figure 3a for Trinitario beans from Cuba (UF 654), both cyanins could even be totally degraded in well-fermented beans.

Anthocyanins in Criollo Beans. In unfermented colorless Criollo cocoa beans (e.g., C 411 from Cuba), as expected, CYA-3-GAL and CYA-3-ARA were not detected at 530 nm before fermentation (Figure 3b). On the other hand, traces of a compound—still to be identified—eluting at 37 min (before cyanidin, which elutes at 42 min) were found at the same wavelength, in this sample only.

A very interesting result was the first evidence in fermented beans [ESI(+) mode] of a molecule eluting at 17 min, with an experimental m/z of 433.215 48 (corresponding to $\text{C}_{21}\text{H}_{21}\text{O}_{10}^{+}$ with a theoretical mass of 433.214 92, δ = 1.29 ppm). The MS/MS mass spectrum was very close to those of CYA-3-GAL and CYA-3-ARA (Figure 4f), but with a major fragment at

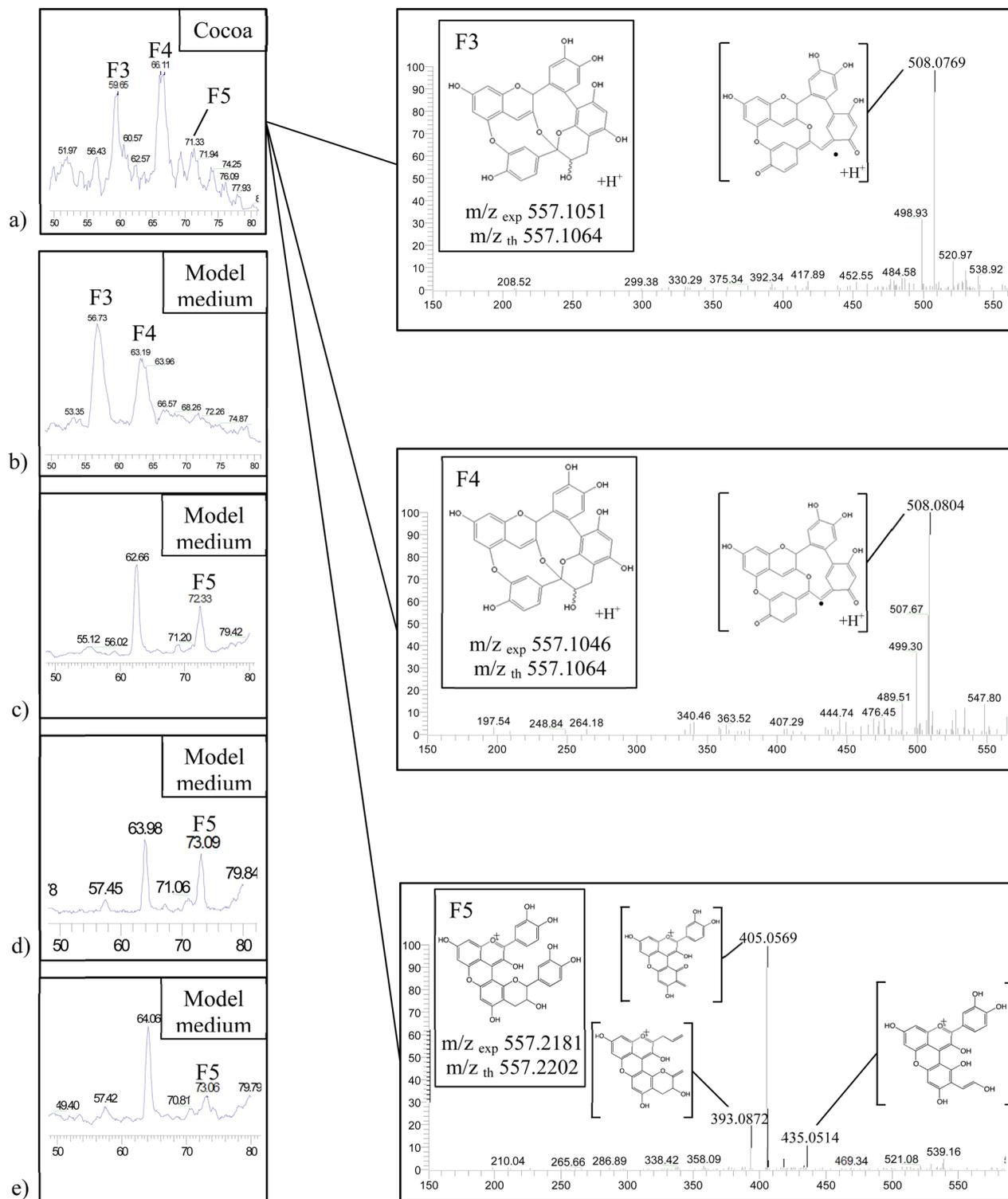


Figure 6. RP-HPLC–ESI(+)-MS/MS ($m/z = 557$) chromatograms of (a) an extract of naturally fermented ICS 40 cocoa beans, (b) (–)-epicatechin (1000 mg/kg) thermal degradation medium (at 50 °C for 24 h), and medium supplied with (–)-epicatechin (1000 mg/kg) and (c) cyanidin (1000 mg/kg), (d) cyanidin-galactoside (1000 mg/kg), or (e) cyanidin-araboside (1000 mg/kg) after thermal degradation at 50 °C for 24 h. Experimental mass spectra corresponding to compounds F3, F4, and F5, in the cocoa extract, on the right.

m/z 286 instead of 287. This compound was found in all cocoa samples, even Criollo, after fermentation (Figure 4a–e). The $m/z = 433$ [ESI(+)] corresponds to a rhamnose isomer ($M_w = 164$) linked to cyanidin ($M_w = 287$), after loss of a water molecule ($M_w = 18$). It is referred to here as F0, by analogy to F1 and F2, recently evidenced in fermented beans only.

Identification Attempt of Five Fermentation-Derived Polyphenols by Comparison with Cyanin/Epicatechin-Containing Aqueous Model Media. Figure 5 depicts the RP-HPLC–ESI(–)-MS/MS ($m/z = 575$) of an extract issued from GC cocoa beans and the mass spectra corresponding to compounds F1 and F2. HRMS analysis of this extract by

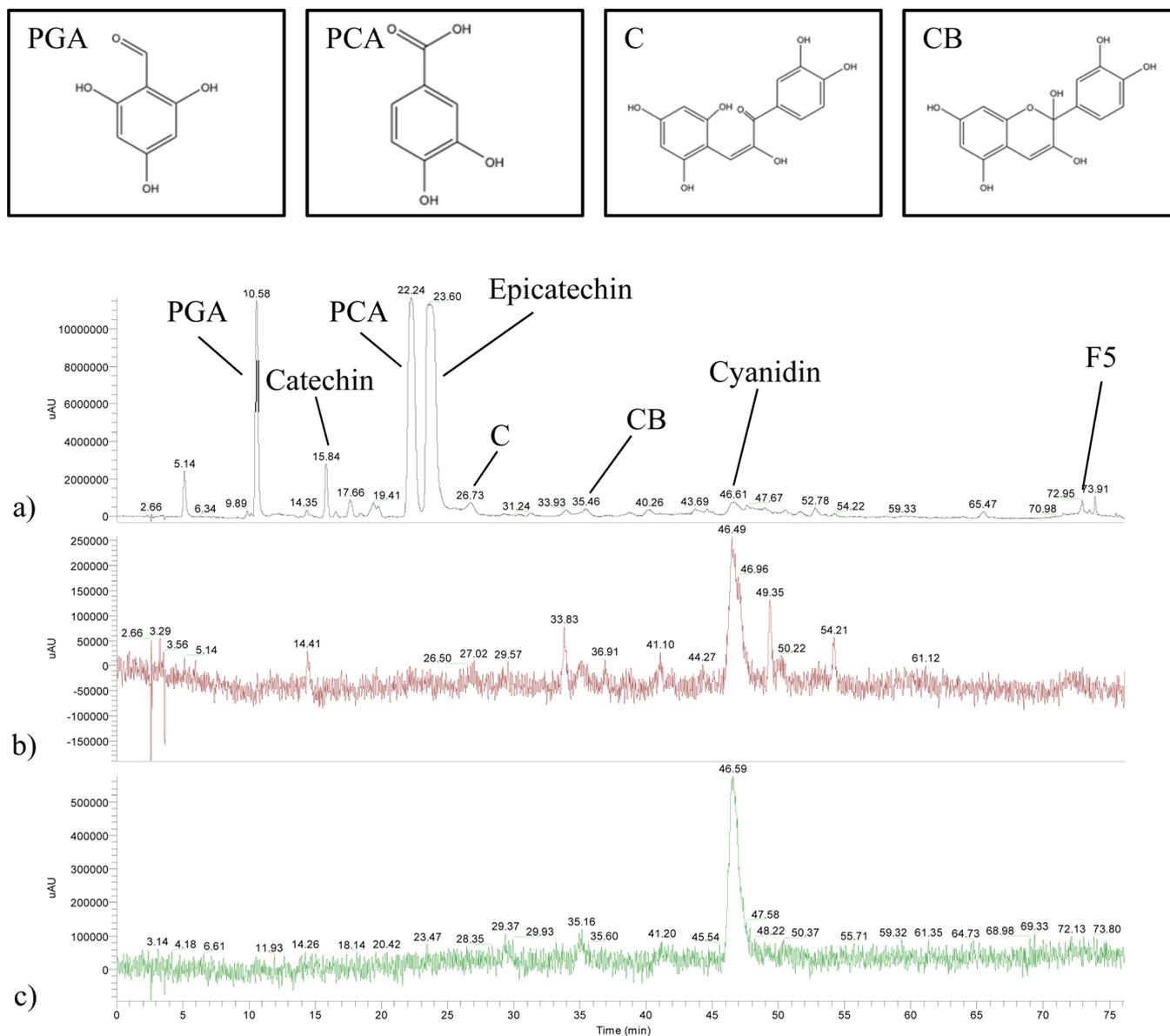


Figure 7. RP-HPLC–UV chromatograms at (a) 280 nm, (b) 460 nm, and (c) 530 nm of medium supplied with cyanidin and (–)-epicatechin (1000 mg/kg each) and subjected to thermal degradation. PGA = phloroglucinaldehyde. PCA = protocatechuic acid. C = chalcone. CB = carbinol base.

ESI(–) confirmed the elemental composition $C_{30}H_{24}O_{12}$ [theoretical mass in ESI(–) = 575.1294] for F1 (experimental mass of 575.1309, $\delta = 1.98$ ppm) and F2 (experimental mass of 575.1195, $\delta = 1.72$ ppm). The main fragment of F1 was found at m/z 544.1056 ($C_{29}H_{20}O_{11}$, theoretical mass of 544.1087, $\delta = 5.69$ ppm), while for F2, m/z 530.0903 was measured ($C_{28}H_{19}O_{11}$, theoretical mass of 530.0932, $\delta = 5.47$ ppm). F2 could arise through procyanidin A2 degradation or through nucleophilic attack of an epicatechin on the C4 position of the heterocycle (either a cyanidin or an activated flavan-3-ol issued from depolymerization) (Table 1a,b). Unfortunately, we have not yet achieved the synthesis of these structures in model media (see the last paragraph below containing the cyanidin/epicatechin model medium). Compound F1, eluting much sooner at $t_R = 25.2$ min, could be a glycoside of F2, as drawn in Table 1.

Figure 6 shows that besides F1 ($t_R = 25.2$ min) and F2 ($t_R = 48.4$ min), three additional compounds were evidenced by RP-HPLC–ESI(+)-MS/MS in fermented cocoa beans at

$m/z = 557$ (F3, F4, and F5; $t_R = 56.7$, 63.2, and 72.3 min, respectively).

Found only after fermentation (Figure 6a), compounds F3 and F4 were also suspected of being native polyphenol degradation products. HRMS gave two very close experimental masses: 557.105 12 for F3 and 557.104 65 for F4 ($C_{30}H_{21}O_{11}$, theoretical mass = 557.106 39, $\delta = 2.28$ and 3.12 ppm for F3 and F4, respectively). The two compounds also exhibited very close MS/MS spectra, with a major fragment at $m/z = 508$ [$C_{29}H_{16}O_9$, theoretical mass of 508.079 43, experimental masses of 508.076 91 and of 508.080 43 ($\delta = 4.95$ and 1.97 ppm for F3 and F4 fragments, respectively)]. The hypothetical structures drawn in Table 1c indicate that F3 and F4 could arise through oxidation of epicatechin (the major polyphenol in cocoa), in keeping with the recent finding that newly formed chemical dimers can emerge in a heated model medium containing epicatechin.²⁵ As $m/z = 557$ [ESI(+)] was not previously monitored in such model media,²⁵ the experiment was here reconducted in a 1000 mg/kg (–)-epicatechin model medium heated at 50 °C

for 24 h. These new results confirm that if oxidation continues, two new interflavane linkages (C5–O–C2' and C3–O–C2) are created to generate two MW = 556 epimers detected at m/z 557 in the MS/MS ESI(+) mode, sharing the same structure except for the stereochemistry of C3 in the lower unit. As depicted in Figure 6a,b, F3 and F4 are present both in the ICS 40 and GC cocoa bean extracts and in (–)-epicatechin model medium.

Lastly, the post-fermentation metabolite F5 (Figure 6a) proved to arise through interclass polyphenol reactions [anthocyani(-di)n + flavan-3-ol (Table 1)]. HRMS–ESI(+) confirmed the cationic $C_{30}H_{21}O_{11}$ structure (experimental mass 557.218 12, theoretical mass 557.220 21, δ = 3.75). The mass spectrum of F5 exhibited three major fragments, at m/z = 405.056 93 ($C_{22}H_{13}O_8$, theoretical mass 405.058 58, δ = 4.07 ppm), 393.087 18 ($C_{22}H_{17}O_7$, theoretical mass 393.086 34, δ = 2.14 ppm), and 435.051 42 ($C_{23}H_{15}O_9$, theoretical mass 435.053 26, δ = 4.22 ppm). F5 was found in Trinitario ICS 40 (Figure 6a) and Amelonado GC cocoa beans but not in Criollo C 411 beans, suggesting that a cyani(di)n moiety was required for its synthesis.

To mimic what might happen in cotyledon cells after entry of acetic or lactic acid, aqueous model media containing (–)-epicatechin and cyanidin, CYA-3-GAL, or CYA-3-ARA were heated at 50 °C for 24 h (pH 4). Only 28% residual cyanidin, 68% residual CYA-3-GAL, and 73% residual CYA-3-ARA were found after treatment [ESI(+), m/z = 287; t_R = 46.6, 28.1, and 39.4 min, respectively]. As expected and as depicted in Figure 7 (RP-HPLC with detection at 280, 460, and 530 nm), known degradation products of cyanidin²¹ and (–)-epicatechin²⁵ were detected in the media. Their structures were confirmed by RP-HPLC–ESI(–)-MS/MS and co-injections for all commercially available compounds. Among them, phloroglucinaldehyde [PGA, m/z = 153 in ESI(–), t_R = 9.56 min] and protocatechuic acid [PCA, m/z = 153 in ESI(–), t_R = 20.8 min] were issued from cyanidin, while very little (–)-catechin (t_R = 15.8 min) arose through (–)-epicatechin epimerization. Two other compounds derived from cyanidin or its glycosides were detected at m/z = 303: the chalcone (C) and the carbinol base (CB) at t_R = 26.7 and 35.5 min, respectively.^{26,27} Their shorter elution time was in keeping with the additional oxygen atom providing increased polarity. Compared to previous results obtained on model media containing flavan-3-ols only,²⁵ it was obvious that the presence of cyanidin modified the behavior of (–)-epicatechin [epimerization was less pronounced; no chemical dimers (m/z = 577) were detected]. The most interesting result in these model media was obviously evidence of the presence of F5 obtained by ESI(+)-MS/MS at m/z = 557 (Figure 6c–e). Compared to F5 found in the cocoa extracts, the same major fragments, at m/z = 405.057 28 ($C_{22}H_{13}O_8$, δ = 3.21 ppm), 393.084 81 ($C_{22}H_{17}O_7$, δ = 3.90 ppm), and 435.050 96 ($C_{23}H_{15}O_9$, δ = 5.28 ppm) were found, corresponding to three different flavan-3-ol heterocycle openings. In all fragments, the interflavane linkage (C5–O–C7) seemed to be preserved (multiple surrounding aromatic cycles providing enhanced stability). F5 synthesis could be linked to the emergence of F2 (nucleophilic attack of epicatechin on the C4 position of cyanidin, followed by cyclization and oxidation), but our experimental conditions did not enlighten the relation between them. Complementary investigations are also required to confirm the structures of F1 and F2. Moreover, a large series of fermented Criollo beans should now be analyzed to confirm that F5 could be used as an authenticating marker after postharvest treatments. Additional studies are also needed to

understand how an isomer of cyanidin-rhamnose can emerge in these Criollo beans.

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Notes

The authors declare no competing financial interest.

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