



# Influence of acetic and lactic acids on cocoa flavan-3-ol degradation through fermentation-like incubations



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## ABSTRACT

The biochemical reactions occurring inside cocoa beans during fermentation are mostly due to penetration of lactic and acetic acids issued from microbial activities. In the present study, fresh, ripe cocoa beans were subjected to three fermentation-like incubation schemes: incubation for 2 days at pH 4 in a solution of acetic acid, lactic acid, or both, followed by a 3-days incubation at pH 5 in acetic acid solution. After each treatment applied, the fermentation index was above 1 and a cut test revealed a brown color, characteristic of well-fermented beans. As shown by RP-HPLC-ESI(−)-MS/MS analysis, the main flavan-3-ols found in German Cocoa (Amelonado group) and ICS 40 (Trinitario group) ranked as follows: epicatechin > C1 > B2 > catechin > B5 > dehydrodiepicatechin A. In both natural fermentations and fermentation-like incubations, these compounds showed a sharp decrease, this effect being strongest when acetic acid was present from the start. Lactic acid exhibited a somewhat polyphenol-protective effect. Two procyanidins with a molecular weight of 576, undetected before fermentation, were evidenced here for the first time in both incubated and naturally fermented cocoa beans.

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## 1. Introduction

Cocoa beans, the main raw material in chocolate manufacturing, are derived from the pods of cocoa trees (*Theobroma cacao* L.) (Ardhana & Fleet, 2003; Thompson, Miller, & Lopez, 2001). The world demand for cocoa increases yearly by about 4.4%, with greatest interest in high-quality fermented beans (ICCO, 2014). Fermentation releases flavor precursors for Maillard reactions occurring during roasting and conching. It also degrades polyphenols (inhibitors of Strecker aldehyde and pyrazine synthesis), thus removing some astringency (Puziah, Jinap, Sharifah, & Asbi, 1998; Camu et al., 2007). Unfermented beans are known to contain very high levels of flavan-3-ols, mainly (−)-epicatechin and its derived oligomers, accounting for up to 18% of the dry weight (Wollgast & Anklam, 2000), together with flavan-4-ones (naringenin), flavonols (quercetin), flavones (apigenin, luteolin),

benzoic acids (gallic/syringic/protocatechic/vanillic acids), cinnamic acids and derivatives (caffeic/ferulic/p-coumaric/phloretic acids, clovamide, dideoxyclovamide), and stilbens (resveratrol and *trans*-piceid) (Counet, Callemien, & Collin, 2006; Jerkovic et al., 2010). Except for the Criollo group, unfermented cocoa beans also exhibit a purple/brown color conferred by anthocyanins (mainly cyanidin-3- $\alpha$ -L-arabinoside and cyanidin-3- $\beta$ -D-galactoside). Bean color changes through fermentation, as a consequence of aglycone release. This enzymatic degradation is widely used to assess fermentation efficiency (fermentation index defined as the 460 nm–530 nm absorbance ratio), along with the cut test (visual observation of longitudinally cut beans) (Afoakwa, Kongor, Takrama, & Budu, 2013).

The residual phenolic content of roasted cocoa beans depends on both cocoa variety and post-harvest processes (*i.e.*: fermentation, drying, and roasting) (Afoakwa et al., 2013; Camu et al., 2007). Fermentation in wood boxes or under banana leaves can be divided into two stages. The first is external fermentation, which takes place directly after bean removal from the pods, thanks to mucilage

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sugars. A succession of yeasts, lactic bacteria (LAB), and acetic bacteria (AAB) results in lactic and acetic acid formation, along with pulp depectinization and liquefaction. During alcoholic fermentation, the pH, initially below 4 because of the presence of citric acid in the mucilage, slowly increases along with the temperature, thus creating ideal growth conditions for LAB and AAB. Through LAB activity, glucose is consumed for lactic acid biosynthesis, while the temperature slowly increases to 35–40 °C (Jespersen, Nielsen, Honholtand, & Jakobsen, 2005). The disappearance of mucilage allows aeration and hence AAB growth, with conversion of ethanol to acetic acid. This exothermic reaction increases the temperature to 50 °C, and the pH stabilizes around 5–5.5 (Afoakwa et al., 2013; Camu et al., 2007). After this external fermentation, lactic and acetic acids migrate into the cotyledons and lower the pH from 6.4 to 4.5, causing bean death, disintegration of cell compartments, and degradation of storage proteins and sucrose by seed-derived enzymes (Afoakwa et al., 2013; Thompson et al., 2001).

Lactic and acetic acids are essential to the production of high-quality cocoa beans. Most studies have focused on the microbial dynamics of yeasts, LAB, and AAB in spontaneous cocoa fermentations (Nielsen et al., 2007; Schwan & Wheals, 2004). Recent data show that some heterofermentative species can also simultaneously produce lactic and acetic acids after ethanolic fermentation (Lefeber, Janssens, Camu, & De Vuyst, 2010; Ouattara, Ouattara, Goualie, Kouamé, & Niameké, 2014). To date, little is known about the respective actions of lactic and acetic acid inside the beans and about any synergy between them. As natural cocoa fermentations remain hard to control, lab-scale fermentations, known as fermentation-like incubations, have been optimized by some authors (Kadow, Niemenak, Rohn, & Lieberei, 2015; Rohsius, 2007).

The aim of the present work was to use fermentation-like incubations (three different acid treatments) to assess the impact of the successive or simultaneous presence of lactic and acetic acids around the seeds on flavan-3-ol levels in cocoa beans, compared to natural fermentation. Monomers, dimers, and trimers of flavan-3-ols were quantitated by RP-HPLC-ESI(–)-MS/MS.

## 2. Materials and methods

### 2.1. Chemicals

Acetic acid (99%) was supplied by Fluka (Switzerland). Acetonitrile (99.99%), diethyl ether (99.9%), ethanol (97%), ethyl acetate (97%), acetone (97%) 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%), and (+)-catechin (98%) were supplied by Sigma–Aldrich (Bornem, Belgium). (–)-Epicatechin-4β-8-(–)-epicatechin (B2, 90%) and kaempferol (>90%) were obtained from Extrasynthese (Genay, France). (–)-Epicatechin-4β-8-(–)-epicatechin-4β-8-(–)-epicatechin (C1, 99%) and (–)-epicatechin-4β-8, 2-O-7-(–)-epicatechin (A2, 99%) were supplied by PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Aqueous solutions were made with Milli-Q water (resistance = 18.2 mΩ) (Millipore, Bedford, MA, USA).

### 2.2. Cocoa fruits

German Cocoa pods (the name refers to old Amelonado clones introduced in Cameroon by Germans at the beginning of the twentieth 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 applied directly after harvest.

### 2.3. Fermentation-like incubations and natural fermentations

As depicted in Table 1, three different treatments were applied to both cocoa clones, according to Rohsius (2007) and Kadow et al. (2015), with slight modifications. Each treatment was applied four times and the resulting dried beans were mixed together.

For each fermentation-like incubation, one cocoa pod was washed twice for 15 min in sodium hypochlorite solution (3 ml/l) and then rinsed three times in 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 incubation medium (100 mmol/L acetic acid, 100 mmol/L lactic acid, or both acids at 100 mmol/L each – adjusted to pH 4 with 1 mmol/L NaOH; Table 1). After two 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 three more days. The temperature was controlled throughout the experiments by incubating the bottles in a 30–50 °C water bath (Table 1). After incubation, all the cocoa beans were sun-dried between 8 a.m. and 5 p.m. for 7 days with stirring every three hours (final water content: 6.9%). The dried beans were stored in black plastic bags at room temperature (25–28 °C) until analysis.

Natural fermentations were conducted at the plantation for five days. About 50 kg cocoa beans of each clone were fermented separately in wooden boxes covered with banana leaves. The beans were stirred manually, morning and evening, every two days. Sun-drying was done as described above.

### 2.4. Fermentation index

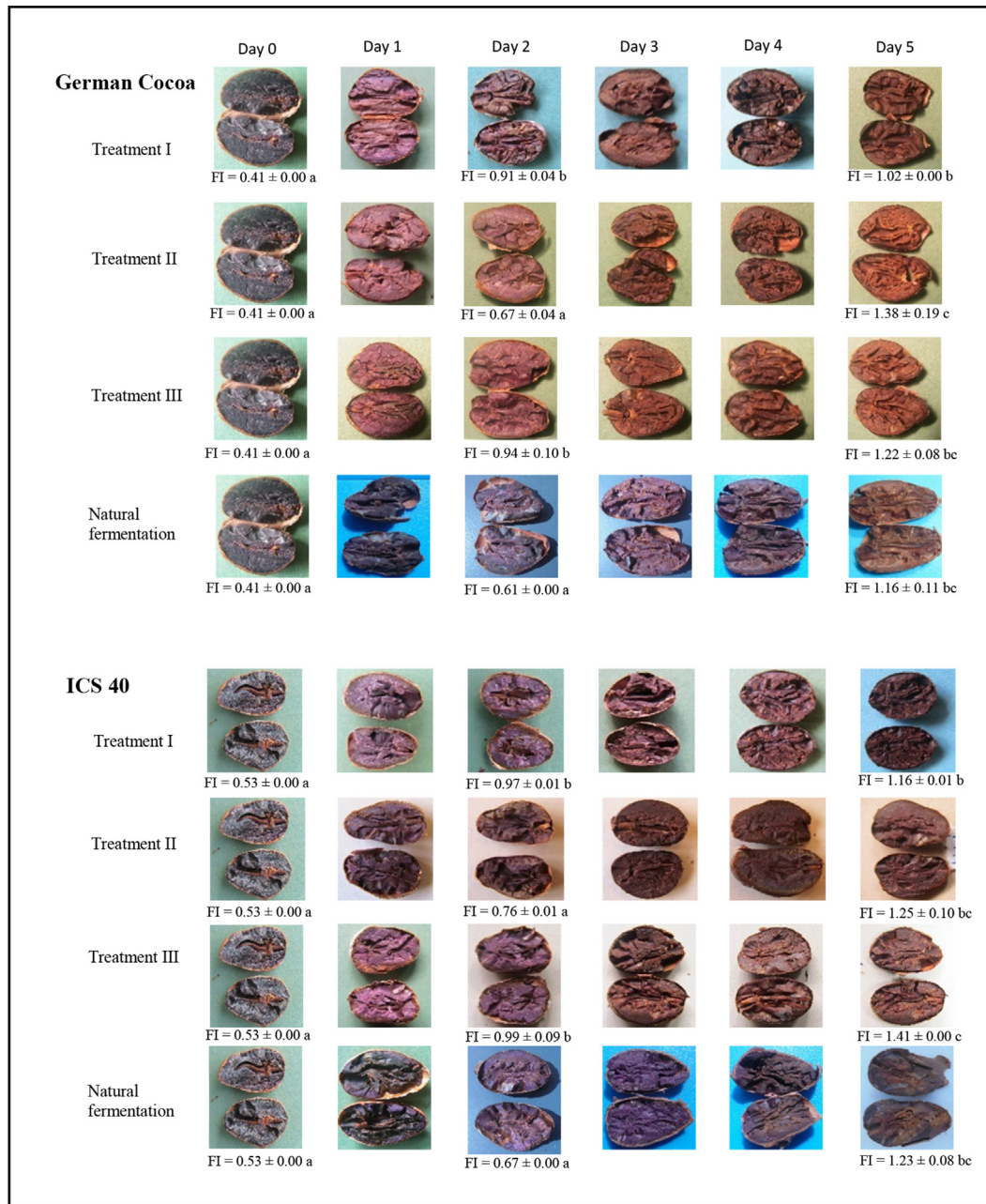
The fermentation index (FI), defined as the ratio of the absorbance measured at 460 nm (yellow oxidized polyphenols) to that measured at 530 nm (anthocyanins), was determined in duplicate, according to Misnawi, Jinap, Jamilah, and Nazamid (2003). Five hundred milligrams of ground cocoa beans were mixed in 50 ml methanol/hydrochloric acid (97:3 v/v) and then stored at 8 ± 2 °C for 18 h and filtered through Whatman n°4 (England). The filtrate was completed to 50 ml with methanol/hydrochloric acid (97:3 v/v) before absorbance determinations (Shimadzu UV-240, Japan).

### 2.5. Cut test

This test was carried out according to Shamsuddin and Dimmick (1986), with slight modifications. Ten dried cocoa beans from each incubation scheme or fermentation were cut lengthwise into two roughly equal parts. Each half was visually examined in full daylight and photographed. Fig. 1 shows the color observed in each sample for the majority of beans.

**Table 1**  
Fermentation-like incubations: experimental design.

Treatments	Days of incubation				
	Day 1	Day 2	Day 3	Day 4	Day 5
	30 °C	40 °C	45 °C	50 °C	50 °C
I	Acetic acid 100 mmol/L; pH 4		Acetic acid 200 mmol/L; pH 5		
II	Lactic acid + Acetic acid 100 mmol/L + 100 mmol/L; pH 4		Acetic acid 200 mmol/L; pH 5		
III	Lactic acid 100 mmol/L; pH 4		Acetic acid 200 mmol/L; pH 5		



**Fig. 1.** Cocoa bean color and fermentation index (FI) after different fermentation-like incubations or natural fermentation. FI values with the same letter are not significantly different according to Tukey's multiple range test at  $\alpha = 0.05$  ( $n = 2$ ).

## 2.6. Extraction of flavan-3-ols from cocoa beans

Flavan-3-ols were extracted in duplicate according to De Taeye, Kankolongo, Jerkovic, and Collin (2014). Cocoa beans (7 g) were ground and defatted with diethyl ether ( $3 \times 50$  mL) at room temperature under gentle stirring. After centrifugation, the samples were vacuum dried. Defatted samples spiked with 500  $\mu$ L kaempferol at 10 000 mg/L in methanol (used as internal standard; 714 mg/kg as calculated for raw beans) were extracted with  $3 \times 50$  mL acetone-water-acetic acid (70:28:2, v/v/v), concentrated by rotary evaporation, flushed with nitrogen, and stored at  $-80$  °C prior to freeze-drying for three days. Afterwards, the extracts were dissolved in methanol to reach 10 mg/ml.

## 2.7. RP-HPLC-DAD-ESI(-)-MS/MS quantitation of flavan-3-ols

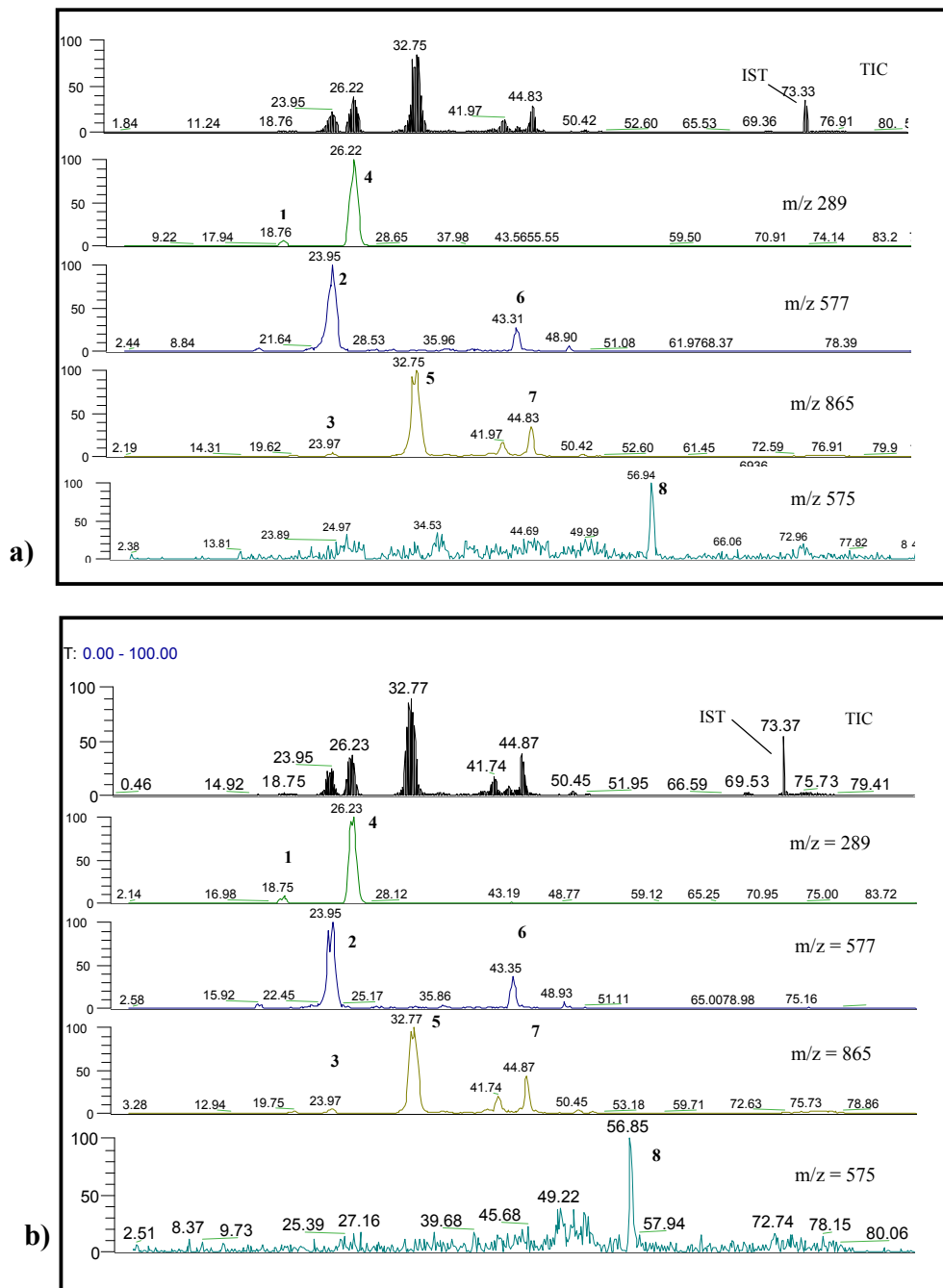
Quantitations were performed on a C18 Prevail column ( $150 \times 2.1$  mm,  $2.7 \mu$ m) (Grace, Deerfield, IL, USA) using a linear gradient from A (water containing 1% acetonitrile and 2% formic acid) to B (acetonitrile containing 2% formic acid). Gradient elution was as follows: from 97% A to 91% 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  $\mu$ L/min. Five microliters of sample were injected in duplicate onto the column kept at 20 °C. A Spectra System equipped with a P4000 quaternary pump and an AS3000 auto sampler was used. The system was controlled with the Xcalibur software version 1.2 (ThermoFisher Scientific, Austin, TX,

USA). Flavan-3-ols 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 (ThermoFisher). Collision-induced dissociation spectra were recorded at 30, 35, and 40% collision energy for singly charged  $[M-H]^{-1}$  ions of monomers ( $m/z$  289), dimers ( $m/z$  577 and 575), and trimers ( $m/z$  865), respectively. The ESI inlet conditions were as follows: source voltage, 4.9 kV; capillary voltage,  $-4$  V; capillary temperature,  $200$  °C; sheath gas, 39 psi. For ESI(-)-MS/MS quantitation in cocoa beans, kaempferol was used as internal standard (IST), and recovery factor = 1 relative to the IST was applied for all compounds. Flavan-3-ol monomers were quantitated with the calibration curve of (-)-epicatechin (0, 10, 25, 50, 100 mg/L,

$R^2 = 0.99648$ ), procyanidins B2 and B5 with that of B2 (0, 10, 25, 50, 100 mg/L,  $R^2 = 0.99831$ ),  $m/z = 575$  compounds with that of A2 (0, 10, 25, 50, 100 mg/L,  $R^2 = 0.99756$ ), and C1 with its own calibration curve (0, 10, 25, 50, 100 mg/L,  $R^2 = 0.99873$ ).

## 2.8. Statistical analysis

All data were analyzed with the SPSS software, version 17.0 (SPSS Inc., Chicago, USA). Statistical significance was assessed by variance analysis (ANOVA). The least significant difference (LSD) according to Tukey's test was used to compare and separate the means, and significance was accepted at the 5% level ( $\alpha = 0.05$ ).



**Fig. 2.** RP-HPLC-ESI(-)-MS/MS chromatograms obtained from unfermented cocoa beans (a) German Cocoa, (b) ICS 40. Each line refers to the  $m/z$  indicated on the right. Peaks are numbered as in Table 2.



**Table 2**  
Flavan-3-ols detected in German Cocoa and ICS 40 beans. Retention times and peaks refer to the unfermented German Cocoa shown in Fig. 2a.

Peak	Name	$[M-H]^{-1}$	Retention time (min)
1	Catechin	289	18.76
2	Procyanidin B2	577	23.95
3	Procyanidin C1 epimer 1	865	23.97
4	Epicatechin	289	26.22
5	Procyanidin C1	865	32.75
6	Procyanidin B5	577	43.31
7	Procyanidin C1 epimer 2	865	44.83
8	Dehydrodiepicatechin A	575	56.94
F1	A2 isomer 1 <sup>a</sup>	575	25.52
F2	A2 isomer 2 <sup>a</sup>	575	45.95

<sup>a</sup> Detected exclusively in fermented or incubated cocoa beans; retention times obtained from German Cocoa, Treatment I, day 5 in Fig. 5a.

### 3. Results and discussion

#### 3.1. Cut test and fermentation index

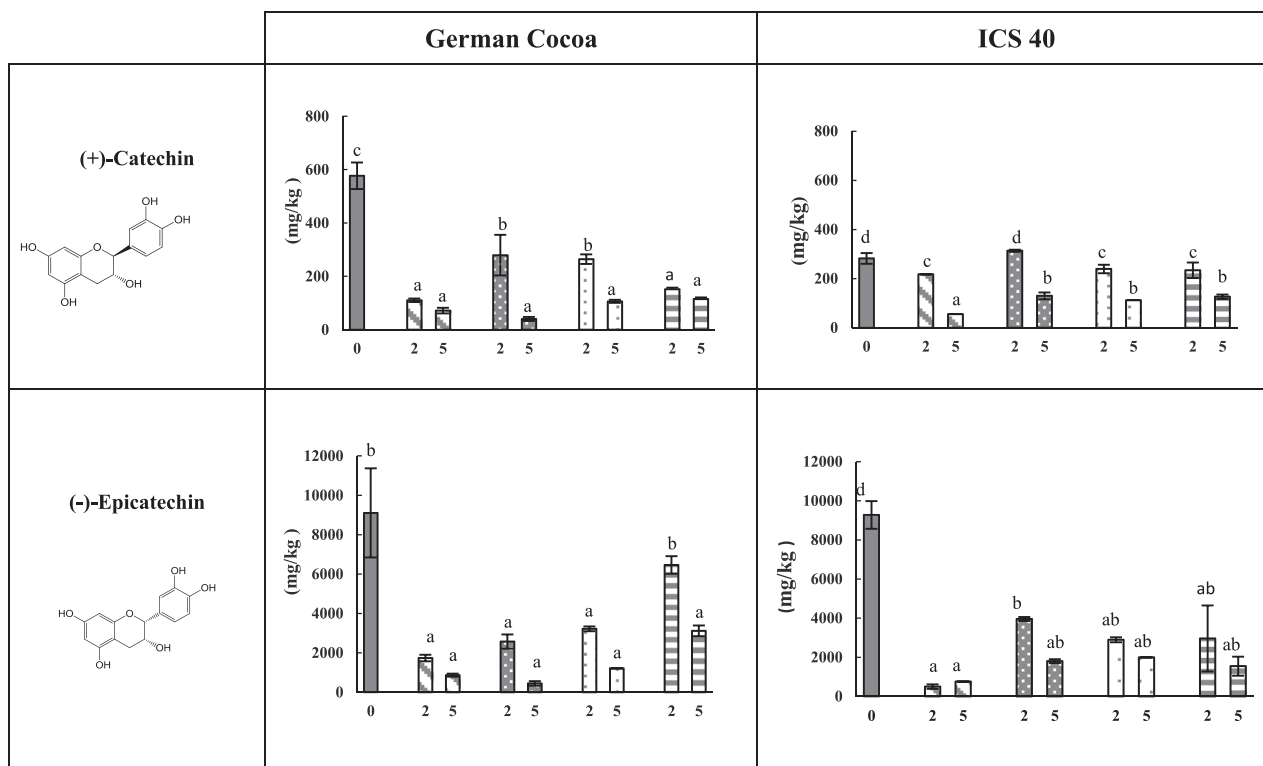
As observed in natural fermentations, bean color was found to evolve in all of our fermentation-like incubations (Fig. 1), from slaty in unfermented beans to purple on day 2 and brown on day 5 (well-fermented cocoa beans). It can thus be assumed that in our fermentation-like incubations, diffusion of both acids mimicked well the entrance of metabolites usually issued from microbial activities (Schwan & Wheals, 2004). Chemically, disappearance of the purple color can be explained by anthocyanin hydrolysis (Afoakwa, Quao, Budu, Takrama, & Saalia, 2012; Misnawi et al., 2003), while the brown color results from the oxidation of cyanidins, other free phenols, and protein-phenolic complexes (Kongor, Takrama, Budu, Mensah-Brown, & Afoakwa, 2013; Misnawi, 2008;

Nazaruddin, Seng, Hassan, & Said, 2006).

Prior to incubation (unfermented beans), the fermentation index (FI) of all samples was below 1. Values above 1 (indicative of fully fermented beans - Gourieva & Tserrevitinov, 1979) were reached after 5 days in all cases, but the time course of the color change and the final index reached depended on the treatment. Over the first two days, the presence of acetic acid alone (treatment I) or lactic acid alone (treatment III) led to a quicker FI increase than the simultaneous presence of both (treatment II; twice as many acidic molecules at the beginning). The index recorded in this last case was close to that observed after two days of natural fermentation. After 5 days, a lower FI was observed after treatment I (1.02 and 1.16 for German Cocoa and ICS 40, respectively) than after treatment II or III (final FI values above 1.22), suggesting partial inhibition of the *beta*-glucosidase activity after day 2. In treatment III, lactic acid, which first migrated into the cotyledons and allowed efficient enzymatic release of colorless aglycone from anthocyanins over the first two days (FI = 0.94–0.97), has further promoted an FI increase to 1.22–1.41 by day 5. We suspect that other chemical transformations of anthocyanins, in addition to that catalyzed by *beta*-glucosidase, occur inside the beans at low pH (e.g.: opening of the heterocycle, which also leads to loss of the red color). In the case of treatment II (same external pH but more acidic molecules able to migrate), the activity of *beta*-glucosidase was limited during the two first days (FI = 0.67–0.76 versus 0.91–0.99) but higher chemical degradations probably occurred between days 3 and 5 (FI up to 1.38).

#### 3.2. Identification and quantitation of the main flavan-3-ols

Flavan-3-ols (monomers to trimers) from unfermented, incubated, and fermented beans were extracted and analyzed by RP-



**Fig. 3.** Catechin and epicatechin contents (mg/kg) of German Cocoa and ICS 40 beans after 0, 2 and 5 days of fermentation-like incubation or natural fermentation. ■ = initial content, ▨ = Treatment I, ▩ = Treatment II, □ = Treatment III and ■ = Natural fermentation. Different letters in the same graph indicate significant differences according to Tukey's multiple range test at  $\alpha = 0.05$  (n = 2).

HPLC-ESI(-)-MS/MS for identification and quantitation.

The chromatograms obtained from unfermented cocoa beans were similar for the two clones (Fig. 2). Eight compounds were found: monomers (catechin and epicatechin) at  $m/z = 289$ , dimers (B2 and B5) at  $m/z = 577$ , trimers (C1 and two suspected epimers) at  $m/z = 865$ , and dehydrodiepicatechin A at  $m/z = 575$  (retention time = 55.9 min) (Table 2). Monomers, dimers, and C1 were assigned on the basis of retention times and mass spectra, by comparison with purified commercial standards. The latter, commercially unavailable, was identified by comparison with Guyot, Vercauteren, and Cheynier (1996) and to De Taeye et al. (2014). To our best knowledge, dehydrodiepicatechin A has never

been reported in unfermented cocoa beans. Usually issued from the chemical addition of two epicatechins after oxidation, it is often found as two epimers (the second one with terminal epicatechin → catechin; retention time = 53.6 min). Only the unepimerized form was here detected. According to their concentrations, the flavan-3-ols were ranked as follows: epicatechin > C1 > B2 > catechin > B5 > dehydrodiepicatechin A.

As depicted in Figs. 3 and 4, all the fermentation-like incubations were found to cause strong degradation of monomers (81–95% after 5 days), dimers (76–88% after 5 days), and trimers (80% after 5 days). In both cocoa clones, monomers were more affected than oligomers. In most cases, the levels decreased

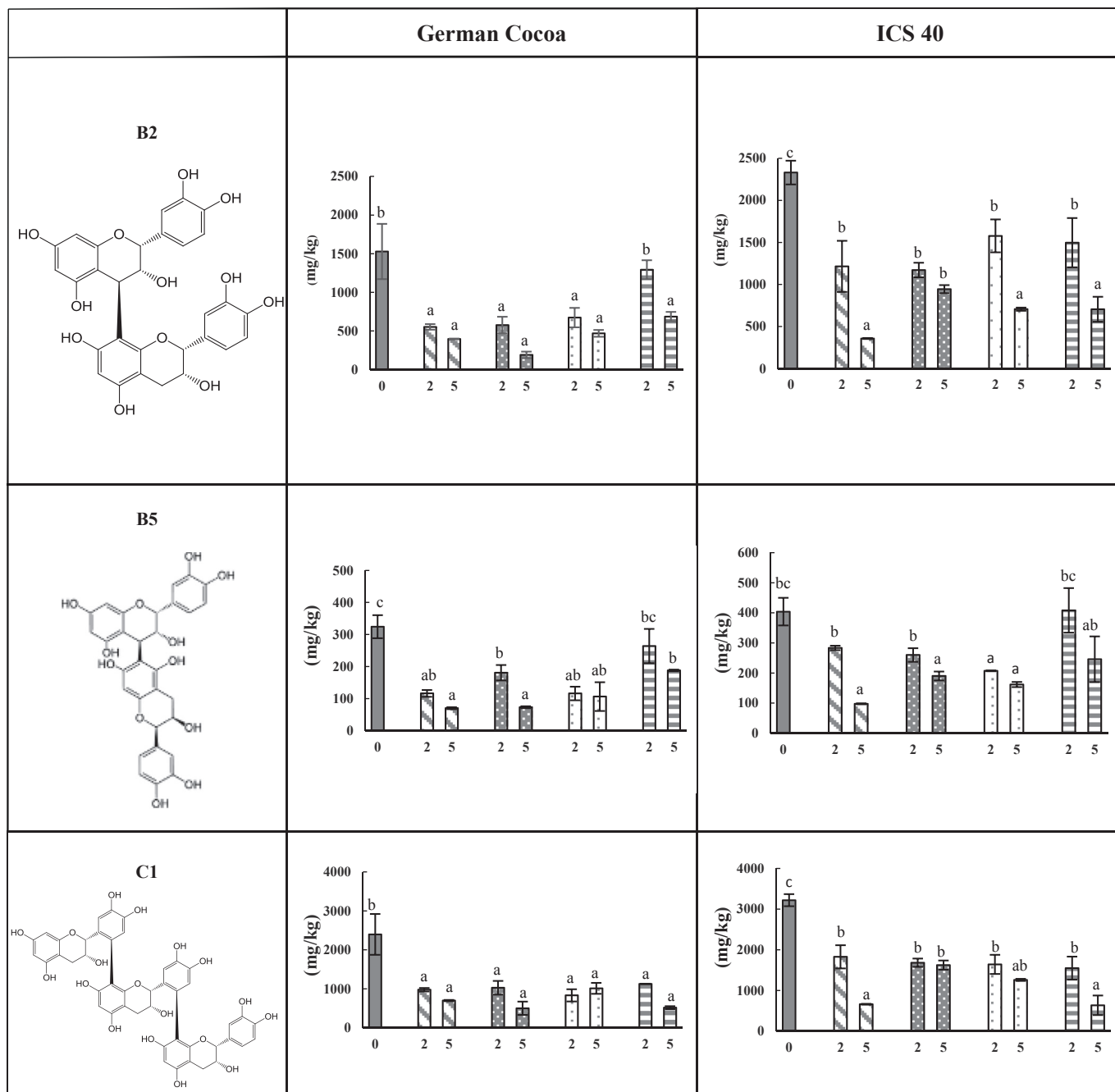
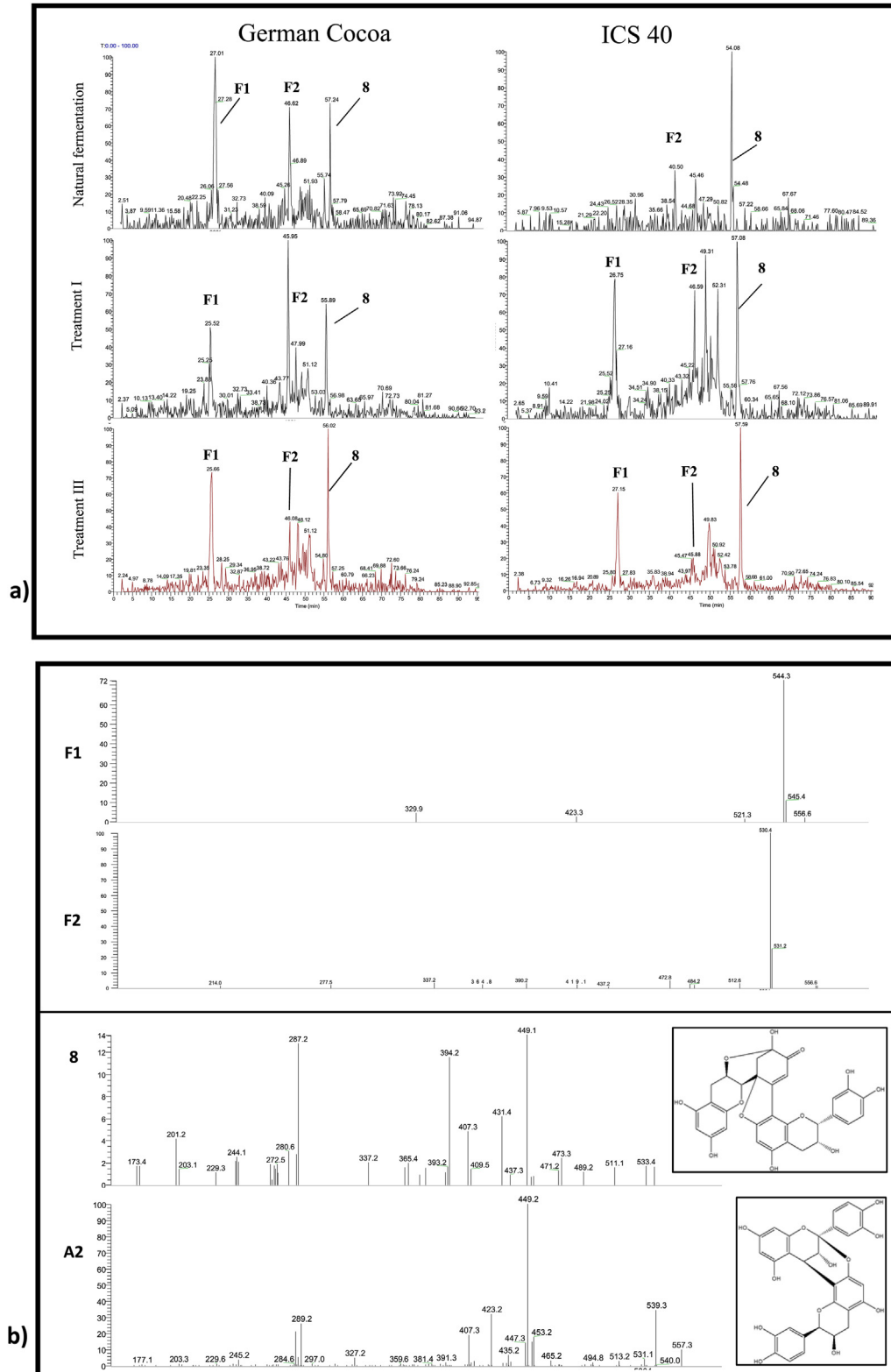


Fig. 4. Procyanidin B2, B5 and C1 contents (mg/kg) of German Cocoa and ICS 40 beans after 0, 2 and 5 days of fermentation-like incubation or natural fermentation. ■ = initial content, ▨ = Treatment I, ▩ = Treatment II, □ = Treatment III and ◻ = Natural fermentation. Different letters in the same graph indicate significant differences according to Tukey's multiple range test at  $\alpha = 0.05$  ( $n = 2$ ).

throughout the treatment, although loss was greatest over the first two days (up to 81% for epicatechin in German Cocoa after 2 days, treatment I). Degradation was often stronger for German Cocoa than for ICS 40, perhaps because the former is characterized by

smaller beans, more easily attacked by acids (e.g.: approximate weight: 1.35 g versus 1.83 g for ICS 40; size: 2.2 × 1.2 cm versus 2.6 × 1.4 cm for ICS 40). Furthermore, flavan-3-ol degradation was strongly influenced by the incubation medium. It was particularly



**Fig. 5.** a) RP-HPLC-ESI(-)-MS/MS chromatograms (m/z = 575) of German Cocoa and ICS 40 cocoa beans after 5 days of natural fermentation or treatment I and III. b) Mass spectra of the compounds identified at m/z = 575 compared with A2.

**Table 3**

F1, F2 and dehydrodiepicatechin A (8) contents (mg/kg A2 equivalents) of German Cocoa and ICS 40 beans after 0, 2 and 5 days of fermentation-like incubation or natural fermentation. Different letters on the same line indicate significant differences according to Tukey's multiple range test at  $\alpha = 0.05$  ( $n = 2$ ). n. d. means not detected.

Flavan-3-ols		Unfermented	Treatment I		Treatment II		Treatment III		Natural fermentation	
		0	2	5	2	5	2	5	2	5
German Cocoa	F1	n. d.	n. d.	62.39 ± 1.86a	n. d.	62.75 ± 1.14a	n. d.	86.89 ± 5.79a	n. d.	100.01 ± 16.98b
	F2	n. d.	n. d.	77.75 ± 0.27b	n. d.	34.89 ± 7.40a	n. d.	44.55 ± 4.59a	n. d.	49.94 ± 10.91a
	8	24.10 ± 2.37a	84.48 ± 3.97b	47.98 ± 3.61a	123.75 ± 16.75c	71.93 ± 4.04b	62.70 ± 6.40b	75.58 ± 17.68b	53.30 ± 6.52 ab	111.4 ± 12.98bc
ICS 40	F1	n. d.	45.44 ± 3.34b	48.34 ± 1.24b	31.62 ± 4.80a	29.12 ± 0.71a	n. d.	55.37 ± 2.56c	n. d.	n. d.
	F2	n. d.	n. d.	32.18 ± 4.42a	n. d.	52.53 ± 8.57b	n. d.	22.83 ± 4.83a	n. d.	32.65 ± 1.36a
	8	47.00 ± 17.60a	91.18 ± 12.37b	32.27 ± 0.97a	80.68 ± 8.23b	50.04 ± 1.87a	49.39 ± 3.77a	57.35 ± 3.43a	45.79 ± 2.93a	105.99 ± 14.32b

pronounced, especially for monomers, when the beans were incubated in acetic acid alone. On the other hand, there appeared no significant difference for monomers between treatments II and III after 2 days of incubation. This suggests that in treatment II (both acids present), the presence of lactic acid was able to inhibit the deleterious effect of acetic acid on (+)-catechin and (–)-epicatechin. In the case of the bigger, Trinitario beans, the greater effect of treatment I on monomers (Fig. 3) as compared to treatments II and III, was still measurable after 5 days.

The residual flavan-3-ol levels observed at the end of natural fermentations proved very similar to those observed after treatments II and III (20 and 45% residual catechin, 34 and 17% residual epicatechin, 45 and 30% residual B2, 58 and 61% residual B5, and 22 and 20% residual C1 for naturally fermented German Cocoa and ICS 40, respectively).

### 3.3. Investigation of two new dimers issued from fermentation

A very interesting result was the detection of two new compounds, named F1 and F2, in both incubated and naturally fermented beans. The respective retention times of these compounds, detected at  $m/z = 575$  in the negative MS mode, were 25.5 min and 45.9 min. These compounds have never been described before, although also present in naturally fermented beans (Fig. 5a). As depicted in Fig. 5b, their ESI(–)MS/MS spectra were very different from that of dehydrodiepicatechin A ( $m/z$  of the main MS/MS fragment: 544 for F1 and 530 for F2), although the three compounds share the same  $m/z$ . All three are isomers of A2, but only dehydrodiepicatechin A exhibited some fragments identical to A2 fragments (e.g.: 449).

For German Cocoa, as regards F1 and F2 production (60–100 and 30–50 mg/kg A2 equivalents of F1 and F2; Table 3), treatments II and III again emerged as the most similar to natural fermentation. As F1 remained undetected in ICS 40 beans after natural fermentation, we suspect that fermentation was in this case a bit less efficient.

In conclusion, cocoa incubation in lactic and/or acetic acid solutions revealed to well mimic natural fermentation, as judged from fermentation indexes, cut tests or flavan-3-ol quantifications. Yet, lactic acid exhibited a somewhat polyphenol-protective effect. Two new compounds named F1 and F2 were detected in both natural and incubated beans, in addition to the well identified dehydrodiepicatechin A. Further research is now needed to identify F1 and F2 and to assess how they could be used by chocolate makers as indicators of cocoa mass quality.

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