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GC-MS Detection of Chiral Markers in Cocoa Beans of Different Quality and Geographic Origin

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ABSTRACT Fermented cocoa beans (*Theobroma cacao* L., Sterculiaceae) from different countries of origin (Ecuador, Ghana, Trinidad) and cocoa beans roasted under defined conditions (industrial roasting; 150–220°C for 20 min, dry roasting in conventional oven) were analyzed for their contents of certain chiral hydroxy acids, catechins, and amino acids. Cocoa beans are fermented, dried, and industrially transformed by roasting for the production of chocolate, cocoa powders, and other cocoa-related products. Fermentation and roasting conditions influence the contents of chiral compounds such as hydroxy acids, amino acids, and polyphenols, depending on technological procedures as well as some technical parameters. The aim of this work was to check if the content and nature of the named chiral compounds present both in fermented and roasted cocoa beans could be related to the traditional parameters used to classify the variety of seeds and the degree of fermentation. The extent of racemization of amino acids in fermented cocoa beans was low while it slowly increased during roasting, depending on the temperature applied. L-lactic acid was always higher than the D-form while citric acid was generally the most abundant hydroxy acid detected in beans. A correlation was found between polyphenol content and degree of fermentation, while epimerization of (−)-epicatechin to (+)-catechin was observed during roasting. On the whole, results showed that several chiral compounds could be considered as good quality markers for cocoa seeds and cocoa-related products of different quality and geographic origin. *Chirality 19:329–334, 2007.*

KEY WORDS: D,L-amino acids; hydroxy acids; (−)-epicatechin; (+)-catechin; cocoa; chiral capillary column

INTRODUCTION

Food constituents are natural substances and, in principle, are expected to be optically pure. Therefore, the detection of the presence of the other enantiomer may be significant for assessing the quality of a food product.[1] Chiral food components may racemize as a consequence of technological processes, such as severe heating, irradiation, or treatment at extreme pH. Some chiral compounds are related to microbial contamination (namely, D-amino acids from bacterial peptidoglycans) or by action of microbial enzymes. Thus, the screening of the optical purity of food components has become a very interesting issue during the past few years.[2] The most studied chiral compounds in food are flavor compounds[3] and D-amino acids.[4] Now, chiral analysis is frequently used for foods and beverages authentication with respect to source (natural/synthetic), geographic origin, processing/aging treatments, and mechanisms of formation (chemical/enzymatic).

Cocoa beans represent the seed of the tropical cocoa tree. Different subspecies of *Theobroma cacao* L. (Sterculiaceae) are commonly employed: "Criollo," "Forastero," and "Trinitario" (the last is a cross breed among both).

Many of the constituents of cocoa beans, such as amino acids, hydroxy acids, and polyphenols such as (−)-epicatechin and (+)-catechin, are chiral. Moreover, cocoa beans used for chocolate manufacture are subjected to microbial fermentation and roasting that could change the enantiomeric distribution of these chiral compounds.

Cocoa bean fermentation is the first stage in chocolate production. Microbial fermentation is a spontaneous (natural) but controlled phenomenon, operated by wild yeast (*Kloeckera* and *Saccharomyces* spp.) and by bacteria of genera like *Lactobacillus*, *Bacillus*, *Pediococcus*, *Acetobacter*, and *Gluconobacter*, causing acetic acid and alcoholic/lactic acid fermentations. During and after fermentation, internal

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autolytic enzymic reaction proceeds, principally resulting in proteolysis and breakdown of polysaccharides and formation of flavor precursors.

For the manufacturer of chocolate or cocoa powders the degree of fermentation of the beans is a major quality criterion. Fully fermented cocoa beans have a brown color. It is shown that nonfermented (slaty color) or partly fermented (violet color) beans result in a lack of cocoa flavor in the end product. The slaty beans have a very acid and astringent flavor profile, whereas the violet beans cause a bitter and harsh flavor.[5] The degree of fermentation is also affected by climatic conditions as well as by the harvesting period of the year. The color of the fermented beans is related to their initial polyphenol content and enzymatic browning catalyzed by polyphenol oxidase.[6]

The fermentation of beans is generally carried out by a traditional process.[7] As previously reported, a wide range of yeasts and bacteria produce a range of metabolic end products (alcohols and organic acids). The organic acids diffuse into the beans, kill the microorganisms, and give rise to flavor and color, together with protein precursors of the cocoa aroma.[8] It is known that bacterial fermentations could release D-amino acids in the product.[9]

It is also known that organic acids such as lactic acid could be produced in both enantiomeric forms, depending on the fermenting microrganism.[10] So it becomes interesting to study the enantiomeric composition of these substances in cocoa beans, to obtain information on microrganisms involved in fermentation.

Another important step of chocolate production is the roasting process; flavor is partially formed during this step. The main flavor precursors are developed during fermentation and drying of cocoa beans. The aroma precursors in cocoa beans, which include free amino acids, low-molecular weight peptides, and reducing sugars, develop into cocoa-specific aroma through Maillard reactions during roasting.[11]

The heat treatments during roasting could lead to racemization of chiral compounds such as amino acids and polyphenols.[12] Pätzold and Brückner[13] recently suggested a Streacker-related mechanism in a model study based on the experimental roasting of fructose-L-phenylalanine (Amadori compound), showing the release of D-amino acids. In the same recent study, only the free D-amino acids were considered as chiral markers in roasted cocoa.

So the aim of this study was (i) to determine if chiral compounds and racemization could be considered markers of fermentation and roasting processes and (ii) if contents of amino acids and phenols could also be related to the geographical origin of beans. The analyses of chiral compounds were mainly carried out using capillary GC-MS with chiral stationary phases.

MATERIALS AND METHODS

Cocoa Samples

Fermented cocoa beans, roasted cocoa beans (nibs), liquor, and chocolate were provided by Streglio (Turin, Italy) from the different varieties Forastero and Trinitario and the following geographical origin: Ecuador, Ghana, Trinidad. Fermented cocoa bean samples were from different periods (February, May, July) and were divided in three categories: fully fermented (brown color); partly fermented (violet color); unfermented (slaty color).

Chemicals

Standard amino acids and lactic acid were purchased from Sigma; succinic acid, glutaric acid, malic acid, citric acid, Amberlite 200, Amberlite IRA-958, (−)-epicatechin, and (+)-catechin were purchased from Fluka Chemie, Buchs, Switzerland.

Sample Preparation

Determination of bound and free D,L-amino acids. For bound amino acids 0.2 g of finely ground cocoa samples were hydrolyzed overnight in 2 ml hydrochloric acid (6 N) at 100°C; free amino acids were recovered by extracting 1 g of cocoa samples with 5 ml hydrochloric acid (6 N) for 10 min at 20°C. In both cases the residue was filtered, evaporated to dryness, added with 1 ml of standard internal solution (*N*-leucine 500 ppm), and dissolved in 20 ml of distilled water. The solution was poured into a column filled with 3 ml cation exchange resin (Amberlite 200, Fluka Chemie, Buchs, Switzerland) previously regenerated with 2 N HCl (10 ml), and washed with distilled water. Amino acids were then recovered by the resin with 10 ml of 2 N NH_3, which was then evaporated to dryness. The residue was dissolved in 2.0 ml 1 N HCl in 2-propanol and kept in a screw-capped tube at 90°C for 1 h. The sample was evaporated, dissolved in 2.0 ml of dichloromethane, and treated with 0.5 ml of trifluoroacetic anhydride in a screw-capped tube at 50°C for 1 h. The tube was cooled, was opened, and the solvent was evaporated. The residue was then dissolved in 0.5 ml of dry dichloromethane and injected (1 μl) in GC/MS. D,L amino acids have been resolved and quantified using a chiral capillary column (Chirasil-L-Val −30 m × 0.25 mm I.D. × 0.25 μm film thickness-Chrompack).

GC-MS analysis was performed on a HP-5890 Series2 gas chromatograph coupled to an HP-5971 mass selective detector (Hewlett-Packard, Palo Alto, CA).

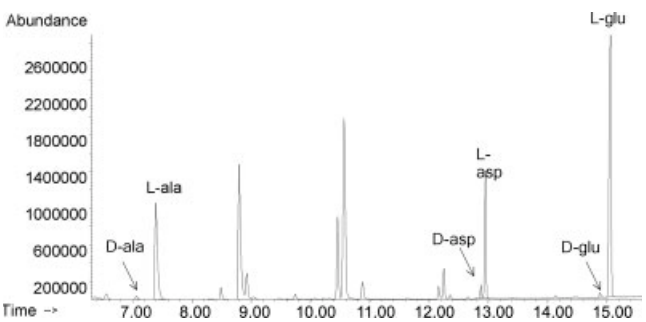

Fig. 1. Resolution of D,L-amino acids on Chirasil-L-Val capillary columns on a nibs sample.

TABLE 1. Percentage of racemization (D/D + L) of free amino acids in cocoa beans

	% D-ala	% D-asp	% D-glu
Ecuador beans (Forastero)	2.72	0.94	0.95
Ghana beans (Forastero)	1.81	1.29	0.45
Trinidad beans (Trinitario)	4.07	0.88	0.26

SD%, 3%.

GC conditions were as follows: oven temperature increased from 50 to 180°C, at 10°C/min, after an initial hold at 50°C for 3 min; injector temperature: 250°C; detector temperature: 250°C; carrier: helium.

MS conditions were as follows: ion source temperature: 180°C; electron impact: 70 eV; acquisition mode:

Scan (*m/z* 40–400).
SIM (*m/z*: 140, 168, 126, 166, 182, 184, 198, 91, 203, 171, 180).

Thermal treatments of cocoa beans for amino acid racemization. Forastero variety cocoa beans from Ghana were heated at temperatures from 150 to 220°C in a laboratory oven for 20 min. Samples were then ground, hydrolyzed, and derivatized as previously described for cocoa bean samples.

Determination of α-hydroxy acids. One gram of ground cocoa sample was added to 8.5 ml of distillate water, 1.5 ml of methanol, 1 ml of standard solution (Glutaric acid 1000 ppm in water). The solution was heated, kept at the boiling point for 10 min, cooled at room temperature, and filtered.

Four milliliter of the filtered solution was poured into a column filled with 2-ml anion exchange resin (Amberlite IRA-958 previously regenerated with 8 ml of NaOH 1.5 N, and washed with distilled water to reach neutral pH). Resin was then washed with 5 ml of distilled water and 20 ml of methanol, to eliminate sugars and polyols. Organic acids were then recovered by the resin with 4 ml of 4 N HCl in methanol; the solution was then heated at 40°C for 45 min to obtain complete esterification of organic acids.

TABLE 2. Percentage of racemization (D/D + L) of protein amino acids in cocoa beans and roasted derivatives

	% D-ala	% D-asp	% D-glu
Ecuador beans (fermented)	0.48	1.39	0.73
Ghana beans (fermented)	0.67	1.51	0.72
Trinidad beans (fermented)	0.50	1.47	0.85
Ecuador nibs (roasted)	2.42	8.54	1.15
Ghana nibs (roasted)	3.57	5.97	0.95
Trinidad nibs (roasted)	4.05	7.13	1.15
Ecuador liquor	2.20	6.20	0.84
Ghana liquor	3.40	6.41	0.97
Trinidad liquor	3.79	6.36	1.11

SD%, 3%.

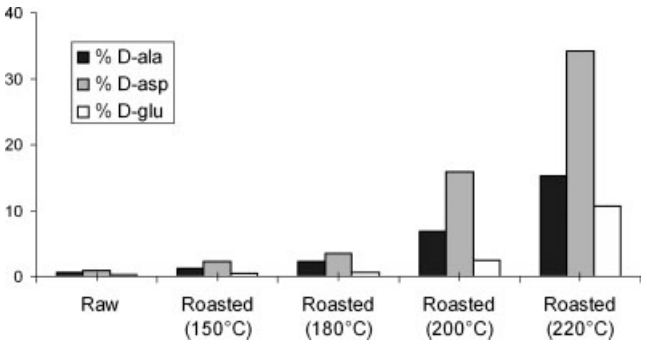

Fig. 2. D/D + L ratios of amino acids detected at different roasting temperatures on Ghana cocoa bean samples.

GC-MS was performed on an Agilent Technologies 6890 N GC coupled to an Agilent Technologies 5973 MSD. Analyses were carried out split using Chirasil-DEX capillary columns (30 m × 0.25 mm I.D. × 0.25 μm film thickness-Chrompack).

GC conditions were as follows: Oven temperature increased from 60 to 180°C, at 10°C/min after an initial hold at 60°C for 3 min; Injector and detector temperature: 230°C.

MS conditions were as follows: Ion source temperature: 240°C; electron impact: 70 eV; acquisition mode: scan (*m/z* 40–400).

Determination of (−)-epicatechin and (+)-catechin. One gram of ground cocoa beans was hot-extracted with 10 ml of CH_3OH for 10 min, added with 1 ml of β-phenylglucoside standard solution (500 ppm in methanol), filtered, and taken to dryness. The residue was dissolved in 1 ml of dry dimethylformamide and polyphenols were transformed into trimethylsililethers by addition of 0.3 ml of trimethylchlorosilane and 0.6 ml of hexamethyldisilazane, heating for few minutes at 60°C.

GC-MS analysis was performed on an Agilent Technologies 6890 N gas-chromatograph coupled to an Agilent Technologies 5973 mass selective detector.

Analyses were carried out split using DB5 capillary columns (30 m × 0.25 mm I.D. × 0.25 μm film thickness).

GC conditions were as follows: Oven temperature increased from 60 to 280°C, at 20°C/min after an initial hold at 60°C for 3 min; injector and detector temperature: 280°C.

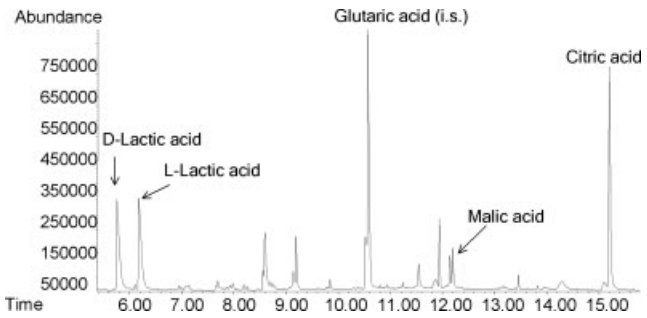

Fig. 3. Determination of α-hydroxy acids as methyl esters by GC-MS, on Chirasil DEX capillary columns in a sample of Ecuador cocoa beans.

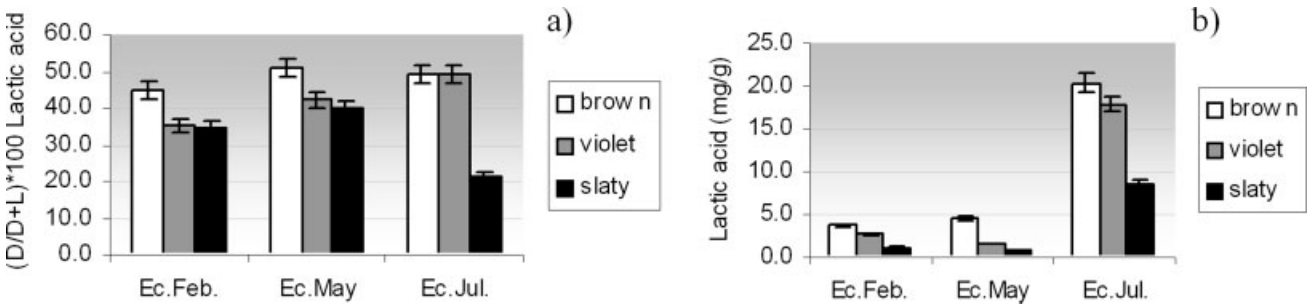

Fig. 4. (a) D/D + L ratio for lactic acid and (b) total amount of lactic acid (mg/g) in Ecuador beans of different fermentation level and of different lots.

MS conditions were as follows: Ion source temperature: 240°C; electron impact: 70 eV; acquisition mode: full scan (m/z 40–550).

RESULTS AND DISCUSSION

Amino Acids

First, the results obtained in this study provided some information about the presence in cocoa of both free and bound D-amino acids. Another recent work[13] confirmed the formation of D-amino acids in roasted cocoa, but the results are restricted to the presence of free D-amino acids. Moreover, the quantities of D-amino acids (conventionally expressed as relative quantities of D-amino acids) recovered in our study are in agreement with those reported in literature.

Amino acid enantiomers were separated on a Chirasil-L-Val capillary column; analyses were performed on fermented cocoa beans from Ecuador (Forastero), Ghana (Forastero), and Trinidad (Trinitario) and on a mixed derived product (nibs and liquor). In Figure 1a chromatogram shows a typical GC-MS separation of the protein D,L-amino acids (nibs sample).

Initially, investigation was made on free amino acids, representing less than 1% of the total amino acid content of the fermented beans, to determinate the level of racemization due to bacterial fermentation. Results reported in Table 1 showed that the D-amino acids present in the samples are D-ala (prevalent), D-asp, and D-glu, characteristic of the bacterial peptidoglycans. The level of racemization (D/D + L) of alanine and aspartic acid is less than 5%, indicating that fermentation is mainly due to yeasts rather than bacteria, as yeasts do not induce racemization. D-ala was recovered as prevalent free D-amino acids by Pätzold and Brückner.[13]

Investigation was then extended to the racemization of protein amino acids of the beans (nibs, liquor), to verify the levels of racemization due to the roasting treatments. Results reported in Table 2 showed that only aspartic acid racemizes significantly but not more than 10%; the level of the other D-amino acids is low, thus indicating that temperatures during roasting were lower than 200°C.

A simulation of the roasting process was made by heating the beans at 150, 180, 200, and 220°C (dry heating, presence of O_2) for 20 min, to check the effect of heating on the racemization level of amino acids. Results are shown in Figure 2; test was done on beans of the Ghana (Forastero) variety. As expected, there is a correlation between temperature and level of racemization that significantly increases after heating over 180°C; thus the percentage of D-amino acid could be considered a good chiral marker of the roasting process only if temperatures applied are higher than 180°C.

α-Hydroxy Acids

Hydroxy acids were recovered from samples by aqueous methanol, separated with anion exchanger, esterified with acid methanol, and analyzed by GC-MS on a Chirasil-Dex capillary column, that allows resolution of the enantiomers; analysis were carried out on fermented cocoa beans of Ecuador, Ghana, and Trinidad varieties supplied in different seasons (February, May, and July 2004) and on a series of cocoa products (nibs, liquor, and chocolate) derived from mixed beans of the February 2004 lot. Figure 3 shows a typical GC-MS separation of organic acid (Ecuador bean sample).

Citric acid, L-malic acid, and a mixture of D- and L-lactic acid were the main α-hydroxy acids present in cocoa beans; the D/L ratio for lactic acid depends on the fermentation process.

Results showed that citric and lactic acids are predominant in all the samples. Both D- and L-lactic acid isomers were recovered in all the samples in about equivalent amounts, thus indicating that lactic acid is rather produced

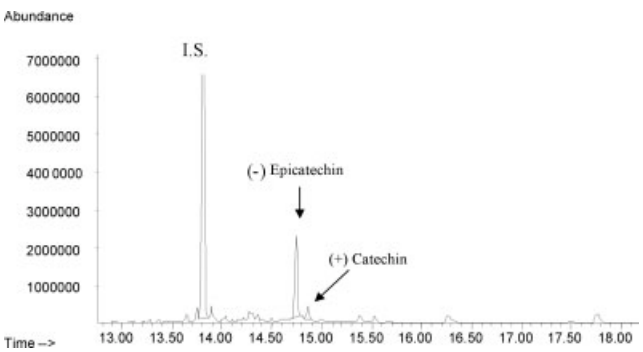

Fig. 5. GC-MS determination of (+)-catechin end (–)-epicatechin as trimethylsilil derivatives in Ecuador cocoa beans of a slaty color.

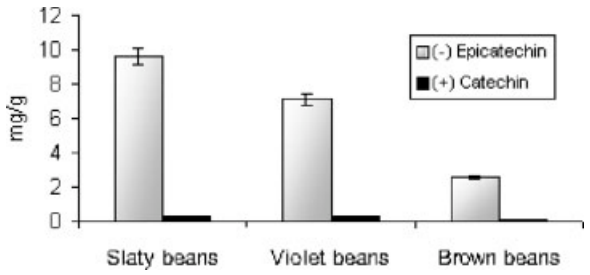

Fig. 6. Amounts of (−)-epicatechin and (+)-catechin in Ecuador beans with a different level of fermentation.

by sugar fermentation than from malolactic fermentation as the last should mainly produce L-lactic acid.

The trends of the lactic acid content and D/(D + L) values for Ecuador cocoa beans of different season and fermentation level (brown, violet, slaty) are reported in the Figures 4a and 4b.

There are no great variations of D/D + L ratio for lactic acid in analyzed samples, while lower amounts of the D-lactic acid isomer were always detected in the less fermented beans (slaty color). The trend of the D/(D + L) ratio seems not to be influenced by the absolute quantity of lactic acid that is much higher for July lot samples (Fig. 4b); this could indicate that the microorganisms involved in fermentation are quite similar, but their fermentation potential depends on the harvesting period.

The behavior of the other lots of bean samples (Ghana and Trinidad) are very similar to those recorded for the Ecuador beans.

Polyphenols

The total content of polyphenols was determined with the Folin reagent and was lower for more fermented beans (brown color). The same trend was found for (−)-epicatechin, determined by GC-MS. (+)-Catechin was also detected as isomerization product of (−)-epicatechin.

The determination of the epimers (+)-catechin and (−)-epicatechin was performed on an achiral capillary column (DB-5), after extraction from beans and derivatization. Analyses were carried out on fermented cocoa bean varieties from Ecuador, Ghana, and Trinidad and on the series of derived products (nibs and liquor). In Figure 5 a typical chromatogram shows the GC-MS separation of (+)-catechin and (−)-epicatechin on a DB-5 capillary column.

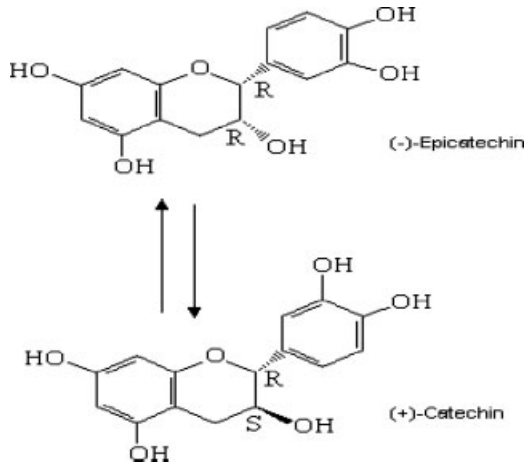

Fig. 7. Thermal equilibration of (+)-catechin and (−)-epicatechin.

TABLE 3. Quantity of (−)-epicatechin and (+)-catechin in cocoa beans and related products

	(−)-Epicatechin (mg/g)	(+)-Catechin (mg/g)
Ecuador beans[a]	5.71	0.23
Ecuador nibs	2.11	1.3
Ecuador liquor	3.83	1.92
Ghana beans[a]	4.05	0.15
Ghana nibs	0.87	0.41
Ghana liquor	0.93	0.52
Trinidad beans[a]	1.24	0.08
Trinidad nibs	0.55	0.28
Trinidad liquor	0.65	0.38
Chocolate[b]	1.35	0.67

SD%, 4% for (−)-epicatechin and 6% for (+)-catechin.
[a]Beans are a blend of brown, violet, and slaty beans.
[b]Chocolate was obtained from a blend of Ecuador, Ghana, and Trinidad liquor.

Absolute quantities of (−)-epicatechin and (+)-catechin were determined in differently fermented beans of the Ecuador varieties (Fig. 6).

These results are in agreement with literature data[14]: during fermentation polyphenols diffuse with cell liquids from their storage sites and are subjected to oxidation, polymerization, and binding with protein; so the content of catechins and soluble polyphenols is lowered during fermentation.

The amount of (+)-catechin increases significantly during the roasting process as a consequence of the thermal isomerization of (−)-epicatechin reported in Figure 7.

The effect of roasting on the catechins content was studied on several series of cocoa beans product (nibs, liquor, and chocolate). The quantity of (−)-epicatechin decreased significantly during heating, while catechin values increase, as reported in Table 3.

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