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Application of ^{1}H NMR for the characterisation of cocoa beans of different geographical origins and fermentation levels

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ABSTRACT

This study reports for the first time the use of ^{1}H NMR technique combined with chemometrics to study the metabolic profile of cocoa (*Theobroma cacao* L.) beans of different varieties, origin and fermentation levels. Results of PCA applied to cocoa bean ^{1}H NMR dataset showed that the main factor influencing the cocoa bean metabolic profile is the fermentation level. In fact well fermented brown beans form a group clearly separated from unfermented, slaty, and underfermented, violet, beans, independently of the variety or geographical origin. Considering only well fermented beans, the metabolic profile obtained by ^{1}H NMR permitted to discriminate between some classes of samples. The National cocoa of Ecuador, known as Arriba, showed the most peculiar characteristics, while the samples coming from the African region showed some similar traits. The dataset obtained, representative of all the classes of soluble compounds of cocoa, was therefore useful to characterise fermented cocoa beans as a function of their origin and fermentation level.

1. Introduction

Cocoa beans (*Theobroma cacao* L., Sterculiaceae) represent the seed of the tropical cocoa tree, growing in a limited geographical zone, of approximately 10° to the north and south of the Equator (in particular Central America, West Indian islands, South America, Africa). Three important varieties of cocoa are commonly employed: Forastero, Criollo and Trinitario (the last is a cross breed between the other two). Forastero comprises 95% of the world production of cocoa, but the highest quality of cocoa comes from the Criollo variety and from the native Forastero variety of Ecuador (known as Arriba). Fresh cocoa seeds undergo fermentation and drying process in the countries of origin, therefore local variations in cocoa plant materials, fermentation procedures (generally carried out according to traditional processes) and drying processes lead to a traded good typical of the country of origin (Ardhana & Fleet, 2003). As a result, the composition profile of the fermented cocoa beans, which is one of the most important factor influencing taste and flavour of the cocoa products, is strictly related to both the cocoa variety and its geographical origin (Roelofsen, 1958). The crucial aspect of cocoa bean processing is fermentation. Fresh cocoa beans fermentation is the first stage in cocoa processing and consists in a microbial fermentation of the pulp surrounding the beans. It is generally a spontaneous phenomenon, operated by a microbial succession of a wide range of yeast (*Kloeckera* and *Saccharomyces* spp.) and of lactic-acid and acetic-acid bacteria (*Lactobacillus*, *Bacillus*, *Pediococcus*, *Acetobacter* and *Gluconobacter*), producing a wide range of metabolic end products, in particular alcohol and organic acids. During and after fermentation, internal autolitic enzymes are activated by microbial metabolites, such as acetic acid, starting the chemical reactions (proteolysis and break-down of polysaccharides) that form the precursors of cocoa flavour (Schwan & Wheals, 2004). The colour of the fermented beans is related to initial polyphenol content and enzymatic browning catalysed by polyphenol oxidase (Misnawi Jinap, Jamilah, & Nazamid et al., 2003). Fully fermented cocoa beans have brown colour. For the manufacturer of chocolate or cocoa powders the degree of fermentation of the beans, affected by climatic condition as well as by the harvesting period of the year, is the principal quality criterion. In fact, too high contents of unfermented (slaty colour) or underfermented (violet colour) beans result in a lack of cocoa flavour in the end product. The slaty beans cause a very acid and astringent flavour profile, whereas the violet beans cause a bitter and harsh flavour (Kattenberg & Kemmink, 1993).

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Since commercial samples usually lack information on fermentation and drying practices as well as on planting material used, it would be desirable to have an efficient rapid method for the quality control of traded cocoa beans.

In previous work (Caligiani, Acquotti, Cirlini, & Palla, 2010) the full assignment of the ^{1}H NMR spectra of hydro-alcoholic extracts of a series of well-fermented cocoa beans was performed, and qualitative and quantitative information on cocoa beans composition was provided. In this work we extend this NMR approach to a larger number of samples, including unfermented and underfermented cocoa beans. A chemometric approach was applied to obtain information about possible grouping of samples and on the metabolites characterising each group of cocoa beans.

2. Materials and methods

2.1. Materials

Samples of cocoa beans of different varieties (Forastero, Trinitario and Criollo), different geographical origins (Ecuador, Ghana, Trinidad, Grenada, Cameroon, Ivory Coast) and different fermentation level (well fermented, brown colour; underfermented, violet colour; unfermented, slaty colour) were considered. The detailed information on samples is reported in Table 1. The samples were kindly provided by Italian chocolate manufacturers. From each batch, a variable number of sub-samples were obtained and analysed separately, as reported in Table 1.

D_2O, CD_3OD and 3-(trimethylsilyl)-propionate-d4 (TSP, internal standard for NMR analysis) were purchased from Sigma–Aldrich (Milan, Italy).

2.2. Sample preparation for NMR analyses

About 200 mg of fermented cocoa beans, finely ground, were extracted with 20 ml distilled water/methanol mixture (8:2 v/v), kept at the boiling point for 10 min under magnetic stirring. Extracts were cooled, filtered, taken to dryness, dissolved in 1 ml D_2O/CD_3OD (8:2 v/v) containing 0.1% of 3-(trimethylsilyl)-propionate-*d4* (TSP), filtered again and transferred in a 5 mm NMR sample tube. TSP was used for chemical shift referencing (δ = 0 ppm) and as internal standard for the quantitative analysis.

2.3. ^{1}H NMR acquisition

^{1}H NMR spectra were acquired on a VARIAN-INOVA 600 MHz spectrometer, equipped with a triple resonance inverse probe (HCN), operating at 599.729 MHz for proton. The experiments were carried out with water suppression by low power selective water signal presaturation of 1.5 s. Spectra were acquired at 308 K, with 32 K complex points, using a 45° pulse length. 128 scans were acquired with a spectral width of 9611.9 Hz, an acquisition time of 1.3 s and a relaxation delay (d1) of 3 s.

2.4. NMR spectra processing and statistical analysis

To analyse the profiles by pattern recognition techniques, FIDs were converted into Bruker formats, Fourier transformed with FT size of 64 K and 0.2 Hz line-broadening factor, phased and baseline corrected with Topspin 2.1 software. NMR spectra were subsequently transferred to Amix 3.9.7 software (Bruker) and referenced to TSP. A first integration pattern was defined choosing buckets manually on all the considered spectra in the overlapped form. Buckets were chosen as large as to compensate the little chemical shifts fluctuation in each single spectrum. A total of 39 signals were chosen in the ^{1}H NMR spectra of cocoa beans, considering both previously identified and unknown signals. The defined pattern was used for the automatic integration of all the spectra and the integrals were normalised to the total spectral area, excluding the zones of residual deuterated methanol and water signals (3.29–3.35 ppm and 4.70–4.75 ppm respectively). A second pattern was defined by manually integrating only the identified signals (corresponding to 20 substances) and the absolute amounts of the selected substances were calculated utilising TSP as internal standard. The integral tables obtained were analysed with SPSS 18.0 software package. Principal Component Analysis (PCA) and, when appropriate, Stepwise Discriminant Analysis (SDA) was

Table 1
Characteristics of the cocoa beans analysed.

Variety	Provenience	Fermentation level	Number of sub-samples
Forastero	Camerun	Well fermented (brown)	2
Forastero	Ivory Coast	Well fermented (brown)	2
Forastero	Ghana	Well fermented (brown)	2
Forastero	Ghana	Well fermented (brown)	3
Forastero	Ghana	Underfermented (violet)	2
Forastero	Ghana	Well fermented (brown)	2
Forastero	Ghana	Underfermented (violet)	2
Forastero	Ghana	Well fermented (brown)	3
Forastero	Ghana	Underfermented (violet)	3
Forastero	Ghana	Unfermented (slaty)	2
Forastero (Arriba)	Ecuador	Well fermented (brown)	2
Forastero (Arriba)	Ecuador	Well fermented (brown)	3
Forastero (Arriba)	Ecuador	Underfermented (violet)	1
Forastero (Arriba)	Ecuador	Unfermented (slaty)	2
Forastero (Arriba)	Ecuador	Well fermented (brown)	2
Forastero (Arriba)	Ecuador	Underfermented (violet)	2
Forastero (Arriba)	Ecuador	Unfermented (slaty)	2
Forastero (Arriba)	Ecuador	Well fermented (brown)	2
Forastero (Arriba)	Ecuador	Underfermented (violet)	2
Forastero (Arriba)	Ecuador	Unfermented (slaty)	2
Criollo	Grenada	Well fermented (brown)	2
Criollo	Grenada	Underfermented (violet)	2
Criollo	Grenada	Well fermented (brown)	3
Criollo	Grenada	Underfermented (violet)	2
Trinitario	Trinidad	Well fermented (brown)	3
Trinitario	Trinidad	Underfermented (violet)	2