Scientific Research Publishing

American Journal of Analytical Chemistry, 2021, 12, 87-95
https://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

Use of the $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$ Isotopic Ratios of Theobromine and Caffeine in the Characterization of Geographic Origin

Didier Diomandé[1,2*], Adjon Kouassi[3], Jacques Sagne Akpa[1], Ingrid Antheaume[2], Maël Leroux[2], Illa Tea[2], Gérald S. Remaud[2]

[1]Laboratoire de Constitution et Réaction de la Matière (LCRM), UFR SSMT, Université Félix Houphouët Boigny, Abidjan, Côte d'Ivoire
[2]Elucidation of Biosynthesis by Isotopic Spectrometry (EBSI) Group, CEISAM, University of Nantes-CNRS UMR6230, Nantes, France
[3]Laboratory of Environmental Sciences and Technology (LSTE), Jean Lorougnon Guédé University, Daloa, Côte d'Ivoire
Email: *dggbe@yahoo.fr

How to cite this paper: Diomandé, D., Kouassi, A., Akpa, J.S., Antheaume, I., Leroux, M., Tea, I. and Remaud, G.S. (2021) Use of the $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$ Isotopic Ratios of Theobromine and Caffeine in the Characterization of Geographic Origin. *American Journal of Analytical Chemistry*, **12**, 87-95.
https://doi.org/10.4236/ajac.2021.124007

Received: February 13, 2021
Accepted: April 12, 2021
Published: April 15, 2021

Abstract

The aim of this work is to characterize the geographical origin of cocoa beans and coffee beans. This study aims to contribute to the traceability of raw materials in order to fight against falsification. For this purpose, we based our work on the measurement of isotope ratios in ^{13}C, ^{15}N and ^{18}O. The multi-element isotope ratios have been evaluated as a means to distinguish fermented cocoa beans of different geographic and varietal origins. The isotopic ratios of ^{13}C, ^{15}N and ^{18}O were measured in theobromine obtained from samples of fermented cocoa beans. Twenty-two (22) samples of different geographical origins covering the four mainland cocoa producing areas were analyzed on the one hand and on the other hand, 16 caffeine samples from various origins were also analyzed. The treatment of the values resulting from these isotopic analyzes by statistical methods, namely the principal component analysis (PCA) makes it possible to visualize the discriminations between the different origins. The most discriminating variables identified as responsible for the geographic and varietal differences were the $\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$ values of cocoa beans and certain extracts and tissues. We have shown that the isotope ratios are correlated with the altitude and precipitation conditions encountered in the different cocoa growing regions.

Keywords

Cocoa Beans, Traceability, Isotopic Analyzes, Theobromine, Caffeine

1. Introduction

The cocoa tree (*Theobroma cacao* L.) is a tropical perennial plant belonging to the Sterculiaceae family. The cultivation of cocoa dates long before the arrival of the Spaniards in Central America. It is difficult to pinpoint the origin of the cocoa tree. However, the most probable hypothesis locates the cocoa tree in the Amazon basin [1] [2] [3] [4]. Three main genetic groups of cocoa named Criollo, Forastero and Trinitario have been established on the basis of their morphological characteristics and geographic origins [5]-[11]. Although *Theobroma cacao* is native to America, nearly 70% of the world's harvest is currently produced in West Africa. Cocoa beans are mainly used in the preparation of chocolate. Before being exported, cocoa beans undergo fermentation following a drying process. These two steps are generally conducted as traditional indigenous processes (Ho, Zhao & Fleet, 2014), the details of which depend on the country of origin, but which influence the taste and flavor of cocoa products. The characteristics of the geographical origin of fermented cocoa beans could thus be linked both to the fermentation and drying procedure and to the varieties of cocoa, giving a range of economic values to the product, hence the need for adequate methods. of traceability. The processed cocoa bean is composed mainly of triacylglycerides (cocoa butter, 45% - 54%) and protein (11.5%) and with sufficient levels of xanthine theobromine alkaloids (1.2% - 1.8%) and caffeine (0.25%) so that these are easily detected and quantified. The chemical composition of cocoa beans is related not only to the variety but also to the quality of fermentation and drying. Thus, most publications in the field of cocoa traceability have mainly focused on analyzing the chemical composition of processed cocoa beans (Crews, 2002; Oracz, Zyzelewicz, & Nebesny, 2013) by several methods, including infrared and NMR spectroscopy the chemical composition depends on the type of bean, the variety, the quality of fermentation and drying. Thus, most publications in the field of cocoa traceability have mainly focused on analyzing the chemical composition of processed cocoa beans (Crews, 2002; Oracz, Zyzelewicz, & Nebesny, 2013) by several methods, including infrared and NMR spectroscopy (Caligiani, Acquotti, Cirlini, & Palla, 2010; Caligiani, Palla, Acquotti, Marseglia and Palla, 2014), HPLC (Oracz *et al.*, 2013) and mass spectrometry (Crews, 2002; Oracz and al., 2013). These different studies have shown that it is possible to differentiate the 3 varieties of cocoa (Forastero, Criollo and Trinitario) and to distinguish 3 cultivation zones in Côte d'Ivoire (Aboisso, Daloa and Divo), in particular on the basis of the caffeine and theobromine content. Recently, 1H NMR spectra of hydroalcoholic extracts from commercial cocoa beans have been used to characterize fermented cocoa beans according to variety and geographic origin (Caligiani *et al.*, 2014), although a discrimination satisfactory was only possible after the complete assignment of the ^{1}H NMR spectra. In 2015, Diomande *et al.* used for the first time the Nitrogen and Carbon isotopic signature to characterize the geographic origin of cocoa bean of various origins. Although the results obtained were

satisfactory, the measurement of the $^{18}O/^{16}O$ isotopic ratios had not been made. This work aims to characterize the origin of the cocoa bean from the isotopic ratios (in $^{18}O/^{16}O$, in $^{15}N/^{14}N$ and $^{13}C/^{12}C$) of theobromine taken as a probe molecule. A principal component analysis taking into account these three parameters makes it possible to highlight the characterization of the geographical origin. This same method will also be applied to caffeine with a view to the traceability of the various natural substances containing this molecule.

2. Materials and Methods

2.1. Samples

Theobromine comes from cocoa beans from the 2008 to 2010 harvest periods from 17 different geographical origins and one of commercial origin, giving a total of 24 samples (Table 1), were supplied by CIRAD or obtained directly from producers in Ivory Coast.

Table 1. Isotopic composition in ^{13}C, ^{15}N and ^{18}O of theobromine of various origins.

Origines	^{13}C (‰)	^{15}N (‰)	$^{18}O_{smow}$ (‰)
Synthesis (1)	−38.73	−2.14	23.63
Duékoué (CI) (2)	−28.20	2.91	3.61
Jamaïque (3)	−25.68	1.90	4.65
Madagascar (4)	−26.90	2.17	4.84
Colombie (5)	−25.40	4.07	−1.94
Equateur (6)	−26.80	3.10	5.68
Vietnam (7)	−27.70	0.93	3.21
Rep. Dominicaine (8)	−28.20	2.49	0.42
Pérou (9)	−27.20	2.77	−0.23
Costa Rica (10)	−26.50	0.09	4.42
PNG (11)	−27.70	1.91	3.54
Indonésie (12)	−27.01	−0.24	0.45
Malaisie (13)	−28.00	0.75	4.53
Brésil (14)	−27.50	3.38	4.49
Azaguié (CI) (15)	−26.62	4.53	3.30
Iles Salomon (16)	−28.85	1.61	−0.77
Trinidad (17)	−27.60	2.06	4.24
Gabon (18)	−26.97	2.73	−0.71
Guatemala (19)	−27.40	3.55	0.72
Kouibly (CI) (20)	−28.60	3.13	4.51
San Pedro (CI) (21)	−27.80	4.89	3.02
Divo (CI) (22)	−28.50	2.12	2.05

CI: Ivory Coast.

Caffeine has been extracted from tea, mate, Guarana powder, Redbull © cola nut drink, and coffee purchased from the store.

2.2. Chemical Products

Sulfuric acid, chloroform, soda and cyclohexane were purchased from VWR Prolabo, ammonia solution (25% v/v) from Merck, propan-2-ol from Fluka and ethanol (99.9%) of Docks Des Alcools (France).

2.3. Extraction of Theobromine

For each sample, we applied the theobromine extraction method of extracting cocoa butter from the cotyledons before the theobromine extraction step. 10 g of cotyledons obtained from the cocoa beans are finely ground and the powder obtained is extracted hot (90˚C) at reflux, for 2 hours, with cyclohexane (3 × 100 mL) of cocoa butter. After cooling and filtration, the residue constitutes lean cocoa. The filtrate is evaporated and gives cocoa butter (45% to 50%). The lean cocoa is then used for the extraction of theobromine. This is done in two stages. First we have a solid-liquid extraction followed by a liquid-liquid extraction. In the solid-liquid extraction, 5 g of the lean cocoa are heated at reflux (110˚C) in a solution of sulfuric acid (3 × 150 mL at 4N) for 1 hour. After filtration, a violet-colored solution is obtained and the residue constitutes the proteins. This solution is then based on an ammonia solution (28%) until a brown color is obtained. In the liquid-liquid extraction, the brown basified solution is extracted several times with a mixture of chloroform/Isopropanol in the proportions (3/1), using a separating funnel. The organic phases are combined and concentrated on a rotary evaporator to remove the solvent. The residue obtained is washed with ethanol to remove impurities. It is then dried in an oven to evaporate the traces of ethanol. Theobromine is obtained with a yield of 0.4% to 2%.

2.4. Caffeine Extraction

A mass of 100 g of coffee is weighed into a 1 L flask. A 500 mL solution of a 0.1 mol/L aqueous sodium hydroxide solution is added. Then using a reflux assembly, the whole is brought to the boil for a period of 2 hours. After heating, filtration is carried out hot. The filtrate is extracted several times with chloroform. The organic phases are combined and then evaporated. The residue obtained is recrystallized from ethanol to obtain caffeine with a yield of 1% to 2%.

2.5. Isotope Analyzes

2.5.1. $\delta^{13}C$ and $\delta^{15}N$ Isotope Analysis

The overall isotope compositions, $\delta^{13}C$ and $\delta^{15}N$, were determined using a Delta-V Advantage isotope ratio mass spectrometer (http://www.thermo.com) coupled to an NA2100 elemental analyzer (irm EA/MS). The compound (generally 0.8 to 1.0 mg) was encapsulated in a tin capsule and burnt in an EA as described. The isotopic composition of the resulting gases, CO_2 and N_2, was determined by reference to a GA working standard standardized against an internationally cali-

brated reference material (IAEA CH_6 and IAEA CH_7 for carbon isotope ratio and IAEA N1 for nitrogen isotope ratio, International Atomic Energy Agency, Austria). The analytical performance was verified by inserting laboratory standards which is glutamic acid (GA) ($\delta^{13}C$ = 27.30‰, (0.45 as correction factor); $\delta^{15}N$ = 4.85‰, (0.14 as a correction factor)) between samples to check stability and allow drift correction to be made if necessary.

2.5.2. $\delta^{18}O$ Isotope Analysis

After a period of development on commercial theobromine standards. The analyzes were calibrated against the usual working standard which is NBS120c, *i.e.* a phosphorite-type rock whose δ^{18}OSMOW is calibrated at 21.7‰ and which also serves to control drift over time. Series of measurements on the spectrometer. For each sample, we made 3 capsules. Rather than keeping the distribution of standards 2 by 2 for every 10 samples, we preferred to group them together and pass them 4 by 4. The reported results represent the mean and the standard deviation over the 3 capsules. In about 10% of cases we removed the result of the first capsule which could have a memory effect. The results are expressed in δ^{18}OSMOW (‰).

2.6. Statistical Method

For the statistical processing of the data, we used the R software. It is both a statistical software and a programming language. It works in the form of a command interpreter. It has a very large library of statistical functions, all the more extensive since it is possible to integrate new ones by the system of "packages", compiled external modules. The R software also offers a wide range of graphics functions. It is possible to use R in interactive mode without ever having to program. We therefore obtained Figures 1-4 from the statistical processing of the results of Table 1 and Table 2.

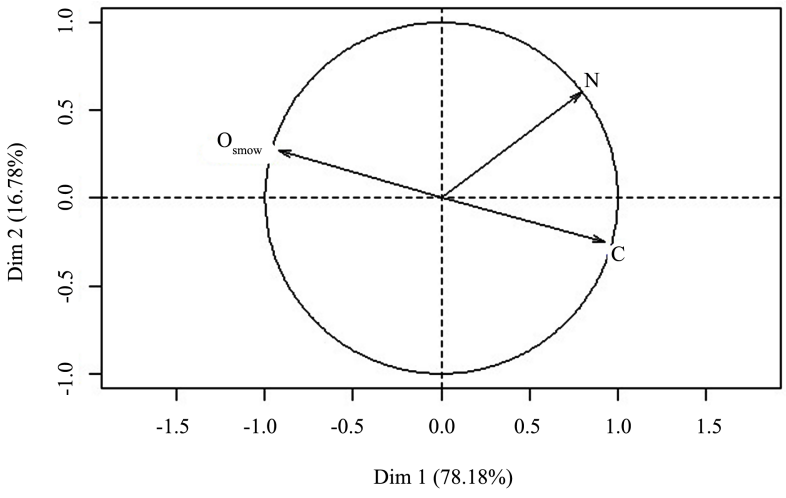

Figure 1. Representation of the characterization vectors ($\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$) used for performing the principal component analysis (PCA) of cocoa samples with a total of 94.96% discrimination.

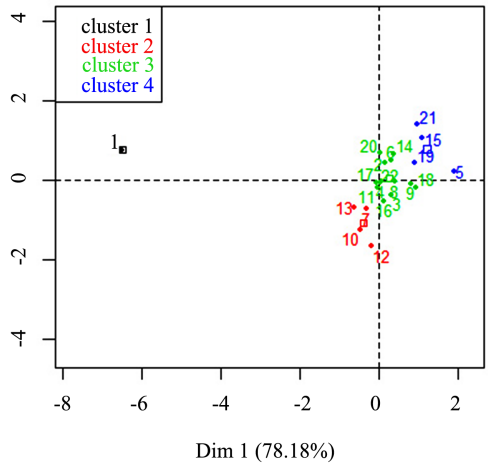

Figure 2. Representation of the PCA to obtain information on cocoa samples and highlighting the presence of 4 clusters (the different figures represent the number of samples in Table 1).

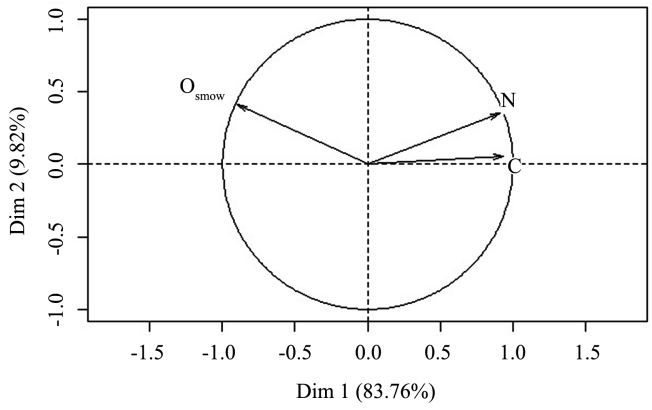

Figure 3. Representation of the characterization vectors (δ^{15}N, δ^{13}C and δ^{18}O) used to perform the principal component analysis of samples containing caffeine with a total of 93.58% discrimination.

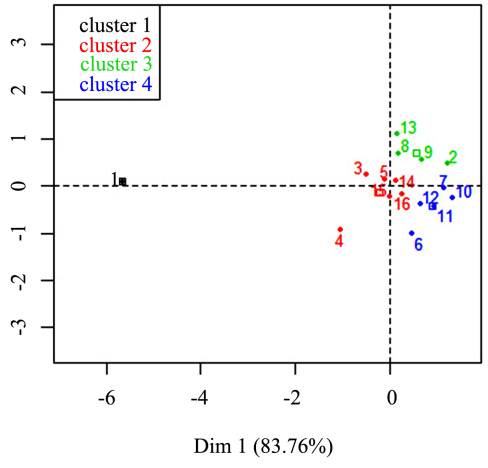

Figure 4. Representation of PCA allowing to obtain information on samples containing caffeine and showing the presence of 4 clusters (the different figures represent the number of samples in Table 2).

Table 2. Isotopic composition in ^{13}C, ^{15}N and ^{18}O of caffeine of various origins.

Origins of caffeine	$d^{13}C$ (‰)	$d^{15}N$ (‰)	$d^{18}O_{smow}$ (‰)
Synthesis (1)	−34.55	−23.64	19.02
Cola nuts (2)	−24.97	5.01	−1.49
China black tea (3)	−28.14	0.30	3.95
Chine green tea (4)	−31.57	1.5	1.99
Guarana powder (5)	−28.41	4.77	3.28
Maté (Argentine) (6)	−27.67	−1.33	−6.40
Redbull© (7)	−25.97	4.77	−3.35
Café du Brésil (8)	−26.07	1.39	2.96
Robusta coffee from Cameroon (9)	−25.65	3.42	0.81
Arabica coffee from Haïti (10)	−26.47	7.44	−4.26
Arabica coffee from Costa Rica (11)	−26.11	0.67	−5.42
Arabica coffee from Colombie (12)	−26.97	1.75	−3.53
Arabica coffee from Kenya (13)	−25.79	2.72	5.23
Arabica coffee from Congo (14)	−27.54	3.14	1.40
Arabica coffee from Ethiopia (15)	−28.14	1.72	0.18
Robusta coffee from Ivory Coast (16)	−27.49	2.05	−0.73

3. Results and Discussions

The results obtained are shown in Table 1 and Table 2. The isotopic signature values vary from −38.73‰ to −25.40‰ for $\delta^{13}C$, from −2.14‰ to 4.89‰ for $\delta^{15}N$ and from −1.94‰ to 23.63‰ for $\delta^{18}O$ in the theobromine molecule (Table 1). We also observe a variation of the isotope signature from −34.55‰ to −25.65‰ for $\delta^{13}C$, from −23.64‰ to 7.44‰ for $\delta^{15}N$ and from −6.40‰ to 19.02‰ for $\delta^{18}O$ in the caffeine molecule (Table 2). Waters of mountainous origin have an isotopic signature $\delta^{18}O$ that varies around −5.21 [12]. The values ($\delta^{18}O$ = −6.40‰) for the Maté plant from Argentina and ($\delta^{18}O$ = −5.42‰) for Arabica coffee from Costa Rica (Table 2) suggest that these two plantations are of mountainous origin. The isotopic signatures $\delta^{15}N$ of theobromine are almost all positive except for two values (Table 1). This general ^{15}N enrichment could be explained by a convergence in cultivation techniques. We also observe almost the same trends in the caffeine samples. However, a strong depletion of ^{15}N in synthetic caffeine should be noted (Table 2).

The results obtained were the subject of a statistical treatment which was shown in Figures 1-4.

We have four (4) groups (clusters) that emerge from the principal component analysis (PCA). We observe a discrimination of 78.18% and 16.78% respectively on the first and second principal components. The $\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$ isotope ratios of commercial theobromine are different from those obtained from naturally occurring theobromine (Figure 2). The same result is obtained with caffe-

ine as a probe molecule (**Figure 4**). The environment of the plant can affect isotope ratios and may be responsible for the differences observed. These results can also be explained by the cultivation techniques (use of fertilizers) which influence the isotopic ratio $\delta^{15}N$, and also the influence of carbon dioxide (CO_2) on $\delta^{13}C$ from cocoa and coffee plantations located near large metropolitan areas. explain these results. Note also the rainfall which has an influence on the oxygen isotopic deviation ($\delta^{18}O$). The samples of theobromins of natural origin can be distinguished into three (3) groups. The discrimination within the groups corresponding to clusters 2 and 4 is very pronounced while cluster 3 is located between the two groups. This difference is related on the one hand to the isotopic ratios of ^{18}O and ^{13}C. With regard to the isotopic content of $\delta^{15}N$, we have a strong similarity, this can be explained by a convergence of agricultural technique. To some extent, the observed differences in isotopic values can be explained on the basis of the elevation and precipitation parameters. However, regarding cocoa, an aspect still to be dealt with more closely is the fermentation process, which could also introduce isotopic fractionation, due to different rates of enzymatic reactions during fermentation processes which impact on alkaloids [13]. The different treatments of coffee beans, as well as those of other matrices which contain caffeine, can have an influence on the discrimination observed at the level of isotopic signatures.

4. Conclusion

This study has shown that the isotopic signatures of nitrogen, carbon and oxygen obtained from theobromine obtained from cocoa beans of various geographical origins can be exploited to characterize their geographical origins. These same isotopic signatures of nitrogen, carbon and oxygen have also been obtained from caffeine from different plants. The results obtained demonstrate the characterization within the cocoa samples in four (4) groups. The nitrogen, carbon and oxygen isotopic signature of the synthetic theobromine sample is very different from that of natural origin. Within the cocoa samples, the isotopic signatures of nitrogen, carbon and oxygen obtained from theobromine show 3 groups (**Figure 2**). We get the same results using caffeine as molecules in the matrixes that contain caffeine from the same isotope ratios. Analysis of the $^{2}H/^{1}H$ isotope ratio could provide further information on geographic origins. The use of quantitative ^{13}C NMR on theobromine and caffeine respectively could allow traceability of cocoa and coffee. Cocoa butter, which represents nearly 50% of the cocoa bean by mass, could also be the subject of an isotopic study. We can also apply site-specific ^{13}C NMR to cocoa butter in order to characterise the geographical origin of cocoa. Near infrared spectroscopy (NIRS) applied to theobromine and caffeine and to cocoa butter could also contribute to the geographical origin [14].

Acknowledgements

D. Diomande thanks the University of Nantes and the Région des Pays de Loire

for funding his Ph.D. The authors thank also CIRAD for purchasing cocoa samples. We are grateful to our colleague Richard Robins for assistance with the preparation of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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