

# Quantitative Measurement of the Chemical Composition of Fatty Acid of Cocoa Butter and the Isotopic Content of Glycerol Contained in Cocoa Butter by the NMR $^{13}\text{C}$ from the INEPT Sequence and Characterization of the Geographical Origin of the Cocoa

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## Abstract

The determination of the chemical composition of cocoa butter and the isotopic contents of the carbons (C-2, C-1 and C-3) of the glycerol contained in the cocoa butter were measured by the NMR- $^{13}\text{C}$  from the INEPT sequence. This technique has already been applied to triglycerides of animal origin. Thus, cocoa butter is made up of nearly 66% saturated fatty acid composed mainly of stearic acid and palmitic acid. Monounsaturated fatty acids represented by oleic acid have an average content of 31% and polyunsaturated fatty acids represent less than 4%. Likewise, the isotopic contents of the glycerol of the triglycerides of cocoa butter in  $^{13}\text{C}$  of the carbons C-2, C-1 and C-3 were measured directly and values vary from  $-11\text{‰}$  to  $-19\text{‰}$  for the C-2 and from  $-39\text{‰}$  to  $-41\text{‰}$  for all C-1 and C-3 because of the symmetry of glycerol.

## Keywords

Chemical Composition, Cocoa Butter, INEPT, Isotopic Content

## 1. Introduction

Lipids are produced by plants and animals, and they constitute the fat of living

things [1]. Lipids are used in cosmetics, pharmaceuticals and human food. Lipids are constituents of cell membranes, inter and intracellular messengers and an energy reserve for the human body [2] [3]. The form of lipids present in the body is that of fatty acid triglycerides. Fatty acids can be divided into three classes: saturated, monounsaturated and polyunsaturated. Unsaturated fatty acids, in particular oleic acid and linoleic acid, play an essential nutritional role. They have a preventive action against cardiovascular disease by lowering cholesterol levels, which is not the case for certain saturated fatty acids [4]. The chemical composition in fatty acids is linked to the botanical or animal origin or to the processes used to obtain it, but also to the geographical origin [5]. Today's society is demanding on the origin of the products consumed. Thus each actor, from the product to the consumer, wants the origin of the animal or vegetable fats used to be guaranteed, whether in a raw or processed product, in order to control its quality. It is therefore in this sense that this work is part of the determination of the fatty acid composition but also of the determination of the  $^{13}\text{C}$  isotopic signature of the glycerol contained in cocoa butter by the  $^{13}\text{C}$ -INEPT method. This technique has already made it possible to analyze triacylglycerols in olive oil and also to quantify several fatty acids, triglycerols in egg yolks [6].

## 2. Materials and Methods

### 2.1. Preparation of the Sample

We worked on 41 samples from 21 producing countries. These samples were provided by the International Agricultural Research Center for Development (CIRAD) and those from Côte d'Ivoire were obtained from farmers in different producing regions. Cocoa butter is extracted from the cocoa beans separated from the shells. 10 g of cotyledons are finely ground and the powder obtained is extracted hot ( $90^\circ$ ) at reflux, for 2 hours, with ( $3 \times 100$  mL) of cyclohexane (Prolabo). Other solvents such as hexane, acetone can also be used for the extraction of cocoa butter. After cooling and filtration, the residue constitutes lean cocoa. The filtrate is evaporated and gives cocoa butter (45% to 50%). After separation on a vacuum pump to remove traces of cyclohexane, a mass of 403.2 mg of cocoa butter is dissolved in 630 mg of  $\text{CDCl}_3$  (Eurisotop). The solution is well homogenized and then introduced into a 5 mm diameter NMR tube.

### 2.2. Isotopic $^{13}\text{C}$ NMR Acquisition Parameters

Carbon has two isotopes,  $^{12}\text{C}$ , which has an isotopic abundance of about 98.9%, and  $^{13}\text{C}$ , which is only 1.1%. In the  $^{13}\text{C}$  NMR study, the spectrum obtained gives low intensities, which does not allow spectrum analysis. In order to obtain a spectrum favorable to the analysis, polarization transfers from hydrogen to carbon-13 are carried out, among the polarization transfer techniques we use INEPT (Insensitive Nuclei Enhanced by Polarization Transfer). The INEPT or Insensitive Core Reinforced by Polarization Transfer provides a spectrum favorable to analysis by proceeding by polarization transfer from  $^1\text{H}$  to  $^{13}\text{C}$ . In

INEPT, the variation of the pulse angle depends on the chemical shift and the  $J_{C-H}$  decoupling constant, hence the presence of signal distortion caused by different couplings present. The principle can be broken down as follows, we have the  $^1H$  excitation which firstly allows the polarization to be transferred from  $^1H$  to  $^{13}C$  and secondly to detect information on the  $^{13}C$  nucleus. The start of this sequence is a spin echo that inverts the population of a subpopulation of protons resulting in carbon selectivity. This results in a gain in intensity of the  $^{13}C$  signals. The linear combinations of these different spectra then make it possible to separate the  $CH$ ,  $CH_2$  and  $CH_3$  sub-spectra. However, this assumes that all the groups have identical (or in any case very close)  $J_{C-H}$  coupling constants, which is rarely verified.

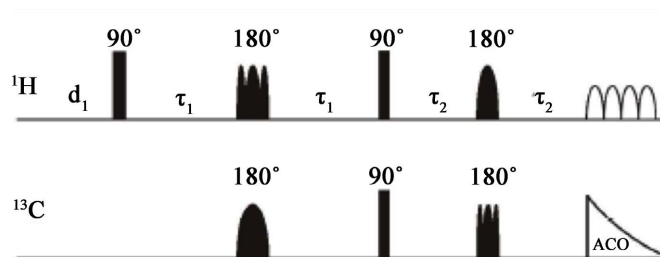
The  $^{13}C$ -INEPT method has already been discussed in the CEISAM “Chemistry and Interdisciplinarity, Synthesis, Analysis, Modeling” laboratory in the EBSI team under these fundamental and application aspects [7]. The application with the resulting cocoa butter was carried out in collaboration with the NMR specialists of the EBSI team. The  $^{13}C$  NMR experiments were carried out on the 500 MHz Bruker Avance III spectrometer equipped with a  $^{13}C/^1H$  dual cryo probe with  $^2H$  lock. All the experiments were carried out at a temperature of 293 K and without rotating the tubes.

The  $^{13}C$  NMR isotopic analysis of the glycerol of the triglycerides of cocoa butter was also carried out using a  $^{13}C$  acquisition sequence, decoupled  $^1H$  consisting of a recovery time followed by the application of an excitation pulse of which the rocking angle is equal to  $90^\circ$  and decoupling  $^1H$  during the collection of the FID only (zsig sequence). This sequence will serve as a reference to calibrate the results obtained with the  $^{13}C$ -INEPT sequence. The parameters used for this measurement are as follows:

- Signal acquisition time (AQ): 1 s and  $T_{1_{max}} = 3.6$  s
- Time between two passages (D1): 42 s ( $TR = D1 + AQ = 43$  s  $> 10 T_{1_{max}}$ )
- Number of passages (NS): 72 with a S/N ratio  $\geq 500$

The duration of the isotopic analysis of the glycerol of the triglycerides of cocoa butter being relatively long (55 min per spectrum), a polarization transfer method making it possible to increase the sensitivity and therefore to reduce the analysis time for the same S/B was used (12 min per spectrum). The transfer method that was chosen is a refocused INEPT (Insensitive Nuclei Enhancement by Polarization Transfer) in which the  $180^\circ$  inversion and refocusing pulses are adiabatic pulses [8] (Figure 1). The parameters of this sequence and more particularly the delays of the two spin echoes were optimized so as to obtain the same  $\delta^{13}C$  (‰) as those obtained by the zsig sequence on the 2 sites relating to the glycerol of the triglycerides of cocoa butter. The optimized parameters for this refocused INEPT-sequence are as follows:

- Signal acquisition time (AQ): 1 s and  $T_{1_{max}} = 2.8$  s
- Time between two passages (D1): 24 s ( $TR = D1 + AQ = 25$  s  $\geq 8 T_{1_{max}}$ )
- Times  $t_1 = 2.7$  msec and  $t_2 = 1.405$  msec – Number of passages (NS): 24 with



**Figure 1.**  $^{13}\text{C}$ -INEPT-refocused sequence in which the  $180^\circ$  inversion and refocus are adiabatic pulses.

a  $S/N$  ratio  $\geq 800$ .

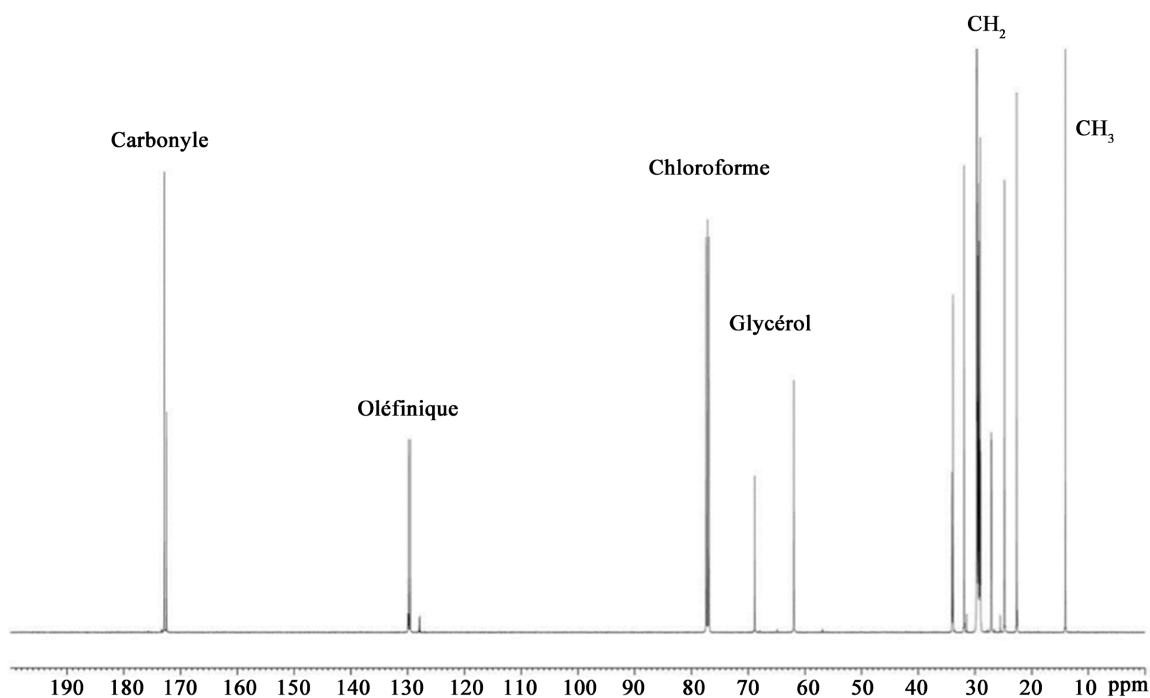
For the two sequences, 5 spectra were carried out. However, all the spectra carried out on the cocoa butter samples were obtained with the refocused INEPT-sequence, only on one of the samples of the series the zsig sequence was operated in order to ensure that we always obtained the same results. The acquired  $^{13}\text{C}$  NMR spectra were subsequently processed with a decreasing exponential type apodization function using a line broadening factor (lb) of 1.5 Hz. The baseline correction was made by part with a polynomial of degree 5. The area of the different lines present on the NMR- $^{13}\text{C}$  spectra was determined using the Perch software (University of Kuopio, Finland). The different components of the lines corresponding to the carbon sites relating to glycerol and to the fatty acids of the triglycerides of cocoa butter were deconvoluted by Lorentzian lines whose chemical shifts, the width at mid-height and the amplitude of the peaks were adjusted. In the case of the measurements obtained with the zsig sequence our results are perfectly validated, in the case of the INEPT-refocused sequence, by the sequence itself the areas of the signals are not only proportional to the number of  $^{13}\text{C}$  nuclei [9]. Thus, an average corrective factor established on the results of the 9 samples measured with the zsig sequence was applied to the areas obtained with the INEPT-refocused sequence.

### 3. Results and Discussion

#### 3.1. Application to Glycerol and Fatty Acids of the Triglycerides of Cocoa Butter

There are two types of information that can be obtained from the  $^{13}\text{C}$ -NMR spectra acquired with the zsig and refocused INEPT sequences: on the one hand, information on the  $\delta^{13}\text{C}$  (‰) isotopic deviations of the two observable sites due to the symmetry of the glycerol (C-1, C-3 and C-2) and on the other hand information on the fatty acid composition of triglycerides in cocoa butter. Indeed, the NMR- $^{13}\text{C}$  spectrum of cocoa butter is divided into 4 regions (**Figure 2**):

- The carbonyl region from 165 to 180 ppm
- The region of olefinic carbons from 120 to 140 ppm
- The glycerol region from 55 to 74 ppm
- The region of aliphatic carbons from 10 to 40 ppm



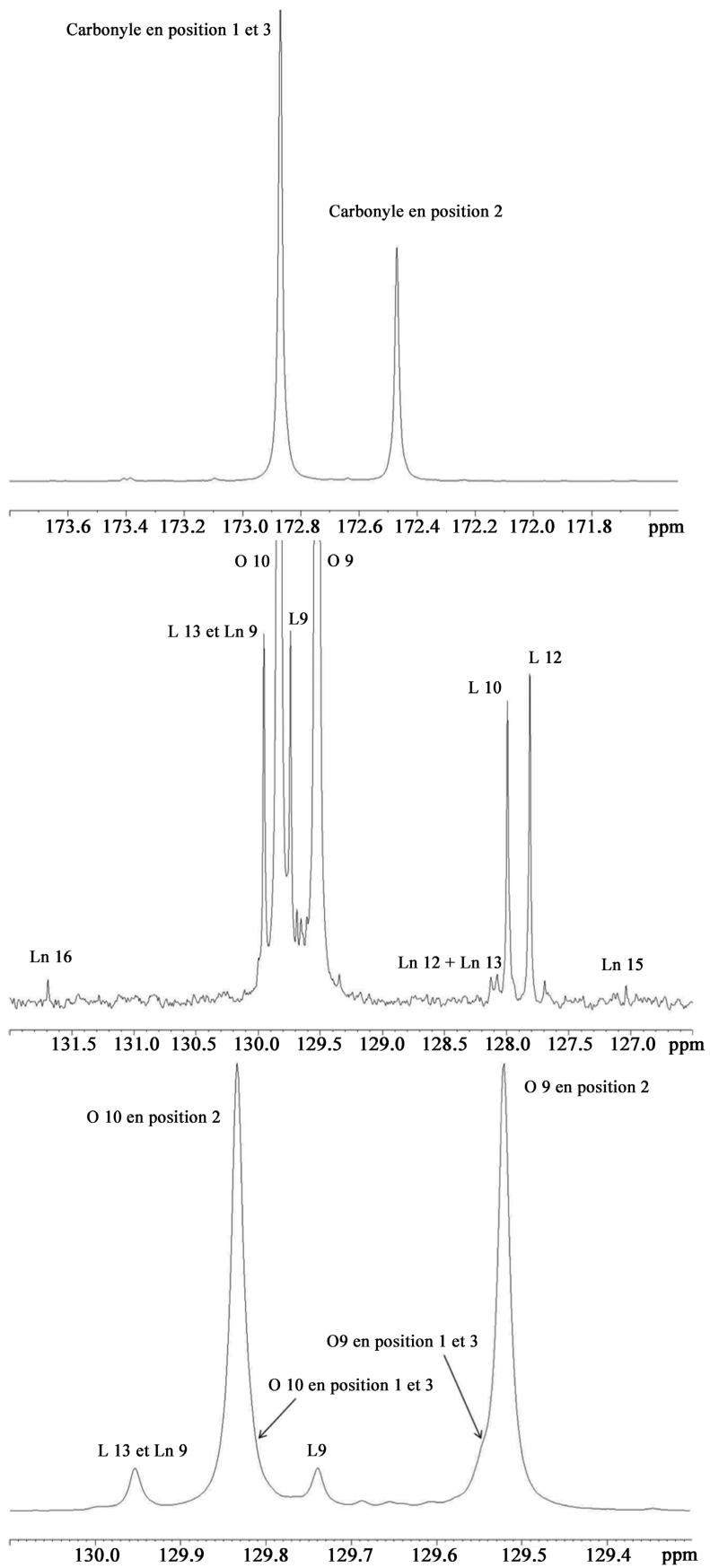
**Figure 2.** <sup>13</sup>C NMR spectrum (zgig sequence) of cocoa butter in CDCl<sub>3</sub> on the 500 MHz spectrometer.

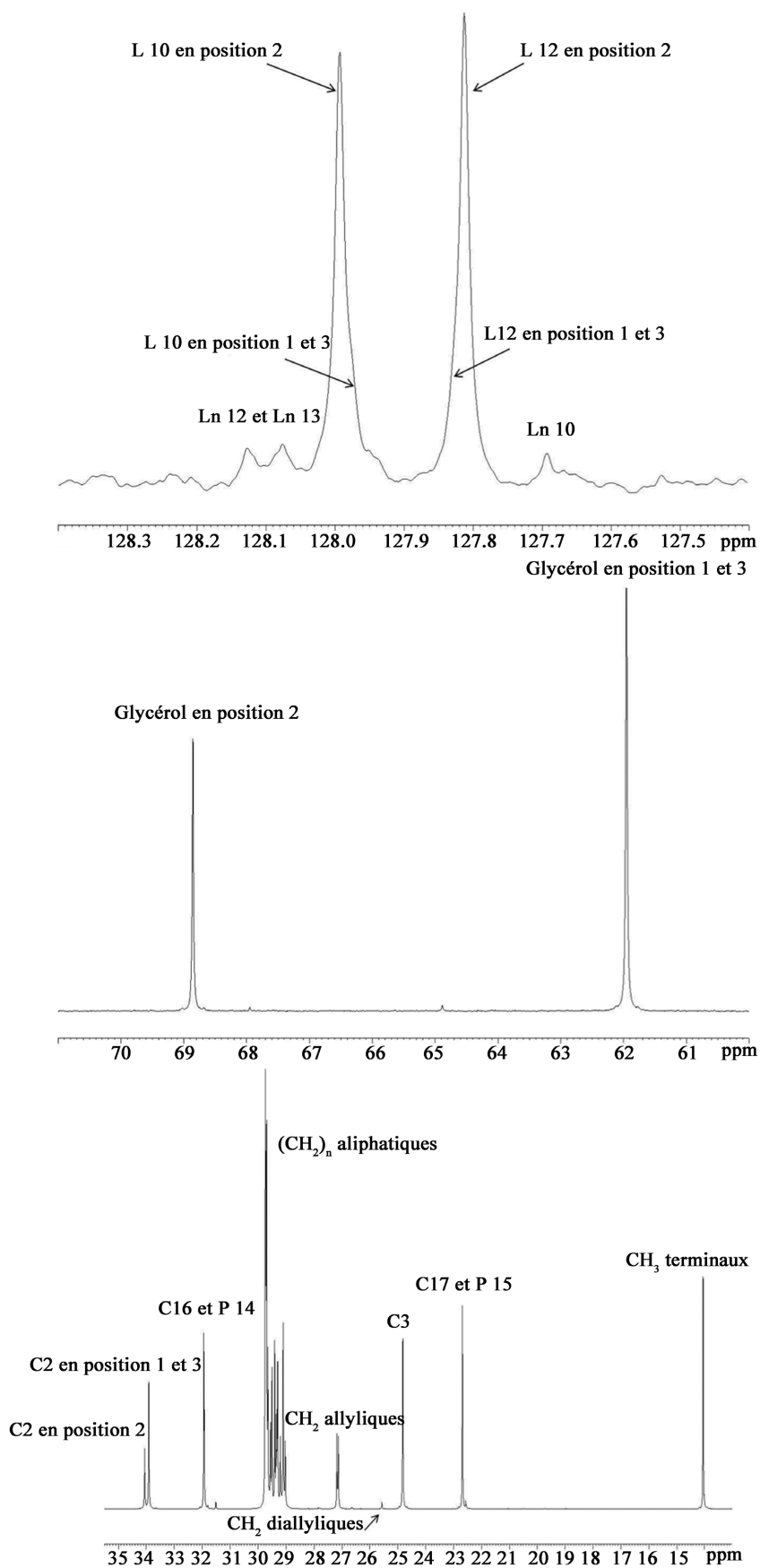
Each of these regions can provide us with information of a different nature and/or validate the results obtained on other areas of the spectrum, a complete allocation of each of the regions was therefore carried out according to the literature (**Figure 3**). The number following the name of the esterified fatty acid chain corresponds to the carbon number according to the classical nomenclature used to describe fatty acids. As a reminder, the general chemical formula of triglycerides is shown in **Figure 4**. The carbon chains R1, R2 and R3 are fatty acids. The three fatty acids are not necessarily the same. The position on the glycerol backbone of the fatty acid chains is designated by the numbering position 1, 2 and 3. In triglycerides, the fatty acid chains can have 4 to 32 carbon atoms, but those of 16 and 18 atoms are the most common lengths. Triglycerides can be saturated, monounsaturated, or polyunsaturated. The fatty acids mainly contained in cocoa butter are: oleic, palmitic and stearic acid.

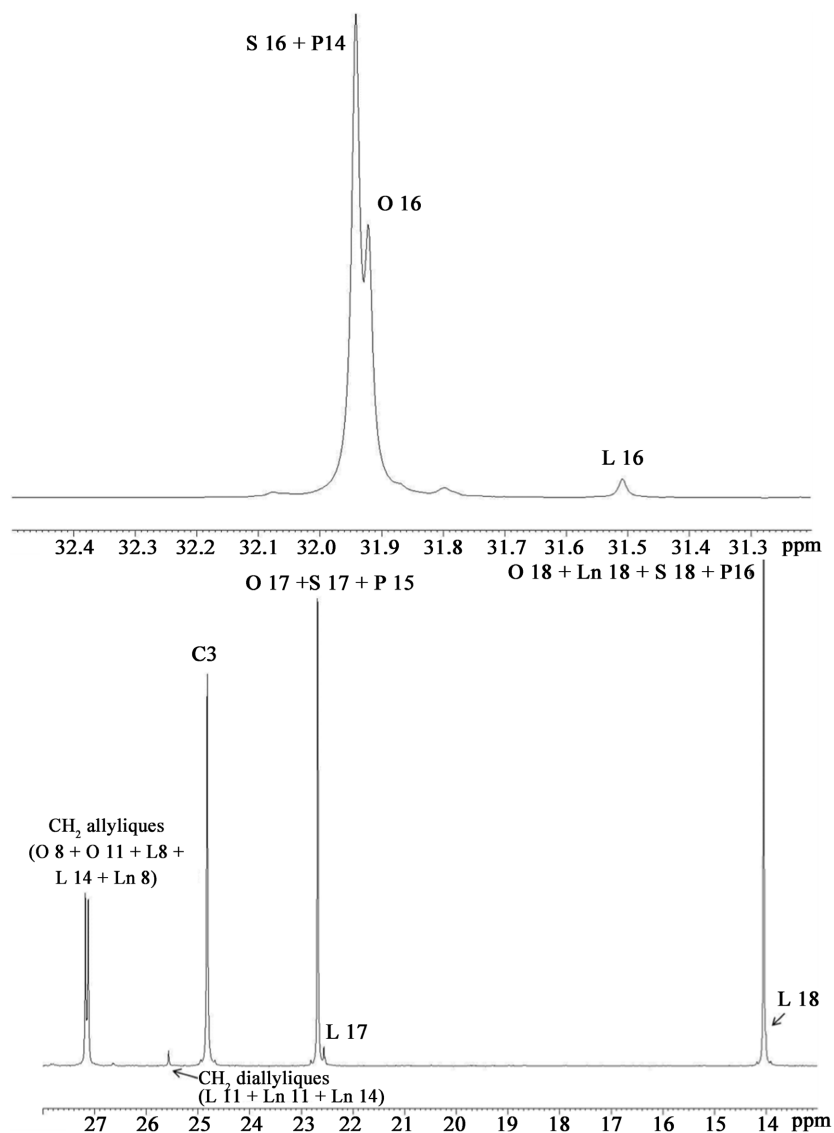
In the case of the measurements obtained with the zgig sequence our results are perfectly validated, in the case of the refocused INEPT-sequence, by the sequence itself the areas of the signals are not only proportional to the number of <sup>13</sup>C nuclei [9]. Thus, an average corrective factor established on the results of the 9 samples measured with the zgig sequence was applied to the areas obtained with the refocused INEPT-sequence. The chemical compositions obtained from the cocoa butters, as well as the isotopic deviations determined on the glycerol contained in the cocoa butters are given in **Table 1**.

### 3.2. Statistical Treatment of the Data Obtained

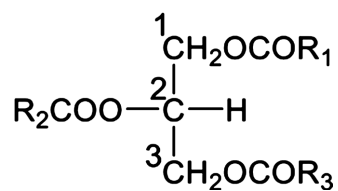
Analysis of these results shows that the chemical fatty acid composition of cocoa







**Figure 3.** Assignment of the different areas of the  $^{13}\text{C}$  NMR spectrum of cocoa butter. The signals corresponding to the chains of fatty acids esterified at position 1, 3 or 2 of glycerol are indicated as follows: L (Linoleyl), Ln (Linolenyl), O (Oleyl), P (Palmityl) and S (Stearyl), the position of the esterification of the fatty acid chain on glycerol is indicated.



**Figure 4.** General chemical formula of triglycerides.

butter is predominantly made up of saturated fatty acids, followed by the monounsaturated ones. Thus, we have nearly 63% to 68% of saturated fatty acids composed mainly of stearic acid and palmitic acid, while monounsaturated fatty acids (oleic acid) represent 30% to 32%. Polyunsaturated acids represent less



**Table 1.** Chemical composition of fatty acids in cocoa butter and the  $\delta^{13}\text{C}$ -2 and  $\delta^{13}\text{C}$ -1-3 isotopic signatures of the glycerol contained in cocoa butter.

Origines	% du linoléique à partir du C16		% des oléiques 1-3 = O10		% des oléiques 2 = O10		% des linoléiques 1-3 = L10	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Abengourou (21)	2.46	0.03	9.44	0.35	90.69	0.32	14.53	1.00
Aboisso (21)	2.58	0.03	6.61	0.57	93.28	0.52	8.03	1.10
Agboville (21)	2.44	0.02	5.56	0.12	94.23	0.11	10.32	0.96
Alepé (21)	2.62	0.02	10.39	0.77	89.82	0.70	10.34	1.18
Azaguié (21)	2.37	0.01	7.55	0.48	92.42	0.44	11.65	0.89
Biankouma (21)	2.40	0.01	6.93	0.37	92.99	0.34	11.24	1.38
Bolivie (1)	2.12	0.01	8.59	1.35	91.47	1.24	16.72	0.84
Brésil (2)	3.23	0.01	11.52	1.55	88.79	1.41	12.80	1.27
Cameroun (3)	2.23	0.01	16.18	0.10	84.54	0.09	23.23	2.20
Colombie (4)	3.29	0.03	8.16	0.20	91.86	0.19	11.70	0.18
Costa Rica (5)	2.88	0.02	9.62	0.93	90.52	0.85	11.82	1.11
Dabou (21)	2.82	0.03	4.27	0.28	95.41	0.26	8.73	0.46
Daloa (21)	2.96	0.03	9.93	0.43	90.24	0.39	12.50	0.63
Danané (21)	2.53	0.03	6.33	0.26	93.53	0.24	8.50	1.04
Dimbokro (21)	2.46	0.02	7.49	2.40	92.48	2.19	14.68	1.32
Divo (21)	2.44	0.01	6.48	0.27	93.39	0.25	11.23	2.34
Douroubé (21)	2.60	0.01	10.41	0.41	89.81	0.37	13.45	1.53
Duékoué (21)	2.57	0.02	6.37	0.36	93.50	0.33	12.61	1.34
Equateur (6)	2.40	0.02	9.04	0.38	91.06	0.35	10.36	0.44
Gabon (7)	3.08	0.02	12.55	0.76	87.86	0.70	13.01	1.39
Ghana (8)	2.66	0.01	11.31	1.60	88.99	1.46	13.72	1.56
Guatemala (9)	2.17	0.03	15.12	1.39	85.51	1.27	23.03	1.16
Gueyo (21)	2.63	0.02	6.51	1.56	93.37	1.42	8.49	1.66
Guiglo (21)	2.61	0.03	11.66	1.41	88.66	1.29	11.38	1.46
Indonésie (10)	2.57	0.04	9.06	1.27	91.05	1.16	13.84	1.25
Jamaïque (11)	3.15	0.03	15.27	1.17	85.38	1.07	14.14	0.88
Kouibly (21)	2.57	0.03	5.83	0.21	93.99	0.19	11.28	2.05
Madagascar (12)	2.07	0.02	7.70	0.27	92.28	0.25	16.78	1.42
Malaisie (13)	2.65	0.02	9.11	1.71	90.99	1.56	10.59	1.07
Man (21)	2.93	0.03	9.33	0.35	90.79	0.32	10.97	1.18
Martinique (14)	2.66	0.02	6.31	0.25	93.55	0.23	13.83	1.60

**Continued**

Oumé (21)	2.59	0.03	7.58	0.37	92.39	0.34	15.41	1.20
Pérou (15)	2.53	0.03	6.39	0.27	93.48	0.25	14.57	3.93
PNG (16)	2.31	0.01	3.97	0.25	95.68	0.23	1218	1.14
REP. Dominicaine (17)	3.13	0.02	16.73	0.56	84.04	0.51	19.39	0.64
San Pedro (21)	2.70	0.01	9.47	0.26	90.67	0.23	50.39	0.29
Soubré (21)	2.50	0.03	8.02	0.27	91.99	0.25	13.19	0.83
Trinidad (18)	2.68	0.03	4.63	0.30	95.08	0.28	12.59	1.57
Venezuela (19)	2.73	0.03	13.20	0.15	87.26	0.14	20.75	1.16
Vietnam (20)	2.27	0.02	6.54	0.13	93.34	0.12	9.49	1.20
Yakro (21)	1.99	0.02	10.43	0.22	89.79	0.20	17.61	4.18

Origines	% des linoléiques 2 = L10		% Insaturés (O + L + Ln)		% des polyinsaturés		% linoléique Ln16	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Abengourou	95.22	1.18	33.91	0.02	2.67	0.03	0.15	0.03
Aboisso	102.93	1.30	34.45	0.03	2.88	0.02	0.15	0.03
Agboville	100.21	1.14	33.37	0.03	2.78	0.18	0.14	0.03
Alepé	100.18	1.40	33.47	0.02	2.83	0.02	0.15	0.02
Azagué	98.64	1.05	33.48	0.01	2.58	0.03	0.15	0.02
Biankouma	99.12	1.64	33.83	0.03	2.63	0.01	0.14	0.03
Bolivie	92.62	0.99	34.58	0.02	2.47	0.02	0.17	0.03
Brésil	97.27	1.50	35.48	0.01	3.49	0.02	0.18	0.03
Cameroun	84.91	2.60	33.15	0.01	2.52	0.03	0.12	0.02
Colombie	98.58	0.22	34.09	0.01	3.51	0.03	0.18	0.02
Costa Rica	98.43	1.31	35.29	0.01	3.15	0.04	0.20	0.03
Dabou	102.10	0.54	33.56	0.02	3.05	0.01	0.17	0.02
Daloa	97.62	0.75	35.25	0.03	3.14	0.02	0.18	0.02
Danané	102.36	1.23	33.77	0.02	2.99	0.34	0.14	0.02
Dimbokro	95.04	1.57	33.01	0.02	2.62	0.02	0.12	0.02
Divo	99.14	2.78	33.57	0.02	2.68	0.03	0.14	0.02
Douroubé	96.50	1.81	33.52	0.03	2.75	0.01	0.12	0.02
Duékoué	97.50	1.59	33.50	0.03	2.86	0.12	0.18	0.03
Equateur	100.16	0.52	33.96	0.02	2.71	0.02	0.17	0.02
Gabon	97.02	1.64	36.14	0.03	3.32	0.03	0.16	0.04
Ghana	96.18	1.85	33.75	0.01	2.91	0.03	0.15	0.01

**Continued**

Guatemala	85.15	1.37	33.30	001	2.69	0.05	0.08	002
Gueyo	102.37	1.96	34.11	0.02	2.82	0.04	0.18	003
Guiglo	98.95	1.73	33.90	0.07	2.77	0.03	0.14	004
Indonésie	96.04	1.48	34.85	0.01	2.88	0.02	0.19	001
Jamaïque	95.68	1.04	36.06	0.01	3.42	003	0.18	003
Kouibly	99.08	2.43	33.85	0.02	2.81	0.03	0.14	001
Madagascar	92.56	1.68	34.15	0.01	2.43	0.03	0.19	003
Malaisie	99.88	1.26	34.00	0.02	2.95	0.01	0.20	001
Man (CI)	99.45	1.40	34.81	0.03	3.13	0.05	0.17	002
Martinique	96.05	1.90	34.33	0.02	2.89	0.04	0.18	002
Oumé	94.18	1.42	33.48	0.04	2.78	0.02	0.16	003
Pérou	95.17	4.65	33.04	0.02	2.75	0.02	0.14	000
PNG	98.00	1.35	33.77	0.65	2.61	0.04	0.17	004
REP. Dominicaine	89.46	0.76	36.45	0.02	3.40	0.02	0.18	003
San Pedro	52.73	0.97	34.15	0.03	2.90	0.04	0.17	002
Soubré	96.80	0.98	33.26	0.02	2.78	0.04	0.18	002
Trinidad	97.53	1.87	33.58	0.02	2.98	0.02	0.17	003
Venezuela	87.85	1.38	34.72	0.03	3.05	0.04	0.21	001
Vietnam	101.19	1.42	33.82	0.02	2.61	0.04	0.16	003
Yakro	91.57	4.95	32.51	0.03	2.32	0.01	0.12	002

Origines	% monoinsaturés		% des saturés (P+S)		C-2		C-1 et C-3	
	Mean	Standard deviation	Mean	Standard deviation	$\delta$ i mean (‰)	Standard deviation	$\delta$ i mean (‰)	Standard deviation
Abengourou	31.24	0.03	66.10	0.02	-14.62	1.35	-39.55	0.67
Aboisso	31.57	0.03	65.54	0.03	-13.97	0.35	-39.81	0.17
Agboville	30.59	0.20	66.66	0.03	-15.52	0.31	-40.18	0.15
Alepé	30.64	0.04	66.56	0.02	-16.65	0.80	-38.85	0.40
Azaguilé	30.90	0.02	66.55	0.01	-13.07	0.84	-38.67	0.42
Biankouma	31.20	0.03	66.18	0.03	-16.36	0.66	-39.19	0.33
Bolivie	32.11	0.03	65.40	0.02	-16.29	0.73	-40.45	0.36
Brésil	31.99	0.04	64.47	0.02	-14.32	1.02	-39.05	0.51
Cameroun	30.63	0.02	66.89	0.01	-18.19	0.41	-39.23	0.20
Colombie	30.58	0.03	65.91	0.01	-11.14	0.57	-39.59	0.28
Costa Rica	32.14	0.04	64.66	0.01	-12.76	0.70	-39.80	0.35
Dabou	30.51	0.03	66.46	0.02	-14.49	0.49	-40.81	0.25

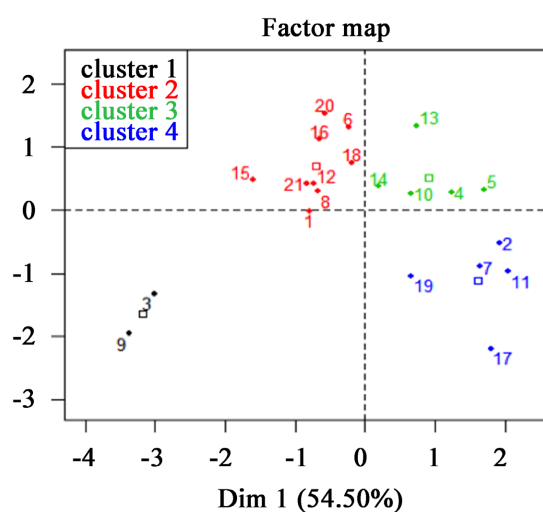
**Continued**

Daloa	32.11	0.01	64.70	0.03	-15.70	0.89	-40.07	0.44
Danané	30.78	0.34	66.25	0.02	-15.25	0.69	-39.64	0.35
Dimbokro	30.39	0.01	67.04	0.02	-16.34	1.16	-39.18	0.58
Divo	30.88	0.02	66.46	0.02	-14.13	0.73	-39.91	0.36
Douroubé	30.77	0.03	66.51	0.03	-14.84	1.06	-40.41	0.53
Duékoué	30.65	0.11	66.53	0.03	-16.85	1.31	-38.09	0.66
Equateur	31.25	0.02	66.05	0.02	-15.39	1.00	-38.68	0.50
Gabon	32.82	0.03	63.78	0.03	-17.92	0.69	-38.44	0.35
Ghana	30.83	0.03	66.27	0.01	-14.16	0.88	-40.05	0.44
Guatemala	30.62	0.05	66.73	0.01	-15.77	0.75	-39.54	0.38
Gueyo	31.29	0.04	65.89	0.02	-15.46	1.08	-39.55	0.54
Guiglo	31.13	0.09	66.11	0.07	-17.22	1.38	-39.36	0.69
Indonésie	31.97	0.02	65.12	0.01	-15.05	0.22	-39.32	0.11
Jamaïque	32.64	0.03	63.87	0.01	-13.56	1.22	-37.87	0.61
Kouibly	31.05	0.04	66.16	0.02	-16.72	0.95	-39.77	0.47
Madagascar	31.72	0.03	65.85	0.01	-13.66	0.34	-38.38	0.17
Malaisie	31.05	0.03	66.01	0.02	-15.11	0.50	-39.59	0.25
Man (CI)	31.68	0.03	65.17	0.03	-13.97	0.58	-39.72	0.29
Martinique	31.43	0.04	65.67	0.02	-14.04	0.71	-38.80	0.36
Oumé	30.70	0.05	6655	005	-14.74	0.15	-38.03	0.08
Pérou	30.29	0.03	6700	002	-13.16	0.80	-39.47	0.40
PNG	31.16	0.63	6625	067	-11.59	0.82	-40.55	0.41
REP. Dominicaine	33.05	0.02	6346	002	-15.98	1.24	-40.66	0.62
San Pedro	31.25	0.02	6585	003	-18.24	0.54	-384.0	0.27
Soubré	30.48	0.03	6677	002	-14.55	0.64	-39.99	0.32
Trinidad	30.60	0.02	6644	002	-14.28	1.20	-40.75	0.60
Venezuela	31.67	0.04	6526	003	-14.79	0.35	-39.10	0.18
Vietnam	31.21	0.05	6620	002	-13.99	0.61	-39.83	0.30
Yakro	30.19	0.03	6756	003	-14.99	0.84	-39.53	0.42

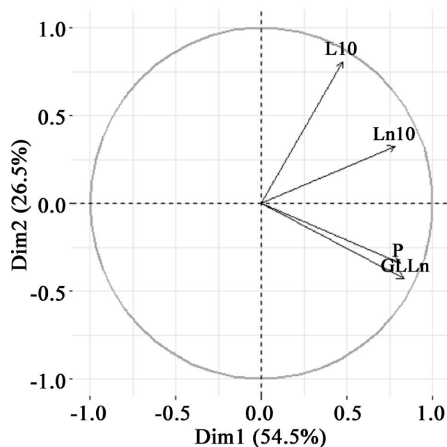
than 4%. These results are consistent with those obtained by Anklam and Lipp (1998) [10].

Statistical processing was performed using R software [11]. The objective of this statistical treatment is to highlight the variations in the chemical composition of discriminating fatty acids on the one hand and the variations in the  $\delta^{13}\text{C}$  isotopic signature of glycerol on the other. Data from unsupervised univariate and multivariate analyzes will be used as appropriate. The unsupervised analysis

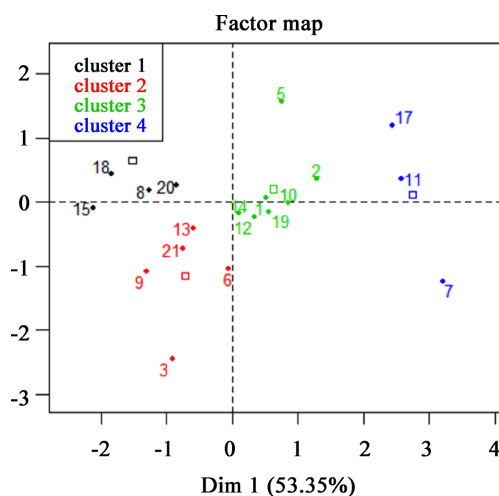
allows to have a global view of all the data while the supervised analysis aims to bring out a reduced number of discriminating parameters while allowing the distinction between two or more groups. Principal component analysis was performed in order to materialize the discrimination between the samples from the 21 countries. So the samples from the Ivory Coast were all concentrated in one value and then the difference between these different values was insignificant. The numbers assigned to the countries in the tables represent are used in the ACP. Thus, the principal component analysis of **Figure 5** makes it possible to highlight four (4) groups (clusters) with 81% discrimination. The parameters at the origin of this discrimination are the chemical compositions of fatty acid linoleic (L10), linolenic (Ln10), palmitic (P) and unsaturated fatty acid (Oleic (O), Linoleic and Linolenic Ln) (**Figure 6**). Also the PCA in **Figure 7** also highlighted (4) groups with 77.4% discrimination. The parameters responsible for this discrimination are the  $\delta^{13}\text{C}$  isotopic signatures of glycerol ( $\delta^{13}\text{C}$ -2,  $\delta^{13}\text{C}$ -1-3) and the chemical compositions of saturated (Palmitic and Stearic) and monounsaturated fatty acids (**Figure 8**). Thus the chemical composition of fatty acids is linked to their biosynthesis in plants [12]. As a result, the varieties of cocoa trees can influence the discrimination observed on the different PCA. In fact, linoleic fatty acid is synthesized in abundance by gradual desaturation of oleic acid over a short period during the development of seeds or leaves [13]. The  $^{13}\text{C}$  isotopic deviation from glycerol is also a very important parameter in discriminating geographic origin [14] [15]. Atmospheric  $\text{CO}_2$  is the main source of carbon used by plants during photosynthesis. The isotopic distribution of  $\text{CO}_2$  is homogeneous in the Earth's atmosphere, however it can be incorporated in two different ways by plants. So we have plants C3 and C4. In C3 plants, atmospheric  $\text{CO}_2$  first diffuses through the epidermis and stomata to the interior of the plant by a reversible process in which the fractionation is about 4‰. Then carboxylation by the enzyme RuBP further discriminates the heavy isotope, with a fractionation of



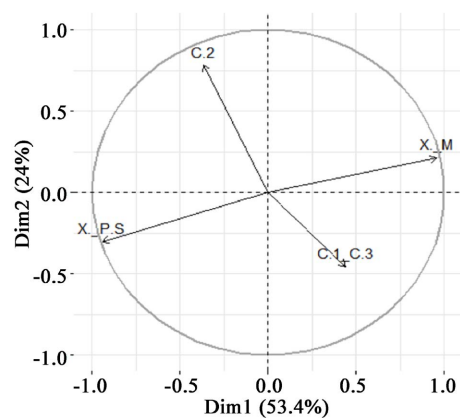
**Figure 5.** Main component analysis from the chemical composition of linoleic fatty acids (L10), linolenic (Ln10), palmitic (P) and unsaturated (oleic (O), linoleic (L), linolenic (Ln)).



**Figure 6.** The parameters used for carrying out the main component analysis: the chemical composition of the linoleic fatty acids (L10), linolenic (Ln10), palmitic (P) and unsaturated acid (oleic (O), linoleic (L), Linolenic (Ln)).



**Figure 7.** Main component analysis from the chemical composition of saturated fatty acids (palmitic, stearic: xp.s), monounsaturated (XM)  $\beta$  cocoa butter and isotopic signatures  $\delta^{13}\text{C}$ -2 and  $\delta^{13}\text{C}$ -1-3 of glycerol.



**Figure 8.** The parameters used for the realization of the main component analysis: the chemical composition of saturated fatty acids (palmitic, stearic: xp.s), monounsaturated (XM) of cocoa butter and isotopic signatures  $\delta^{13}\text{C}$ -2 and  $\delta^{13}\text{C}$ -1-3 glycerol.

about 29‰ [16]. If carbon uptake were limited only by the carboxylation process, fractionation would be 29‰ and C3 plants would have an isotopic deviation of -37‰. At the other extreme, if carbon uptake were limited only by diffusion processes, fractionation would be 4‰ and plants would have an isotopic signature of -12‰. In reality, the total fractionation is between the fractionation due to diffusion and that due to carboxylation and the C3 plants have a between -22‰ and -30‰ [17] [18].

The isotopic composition in C4 plants differs substantially from that of C3 plants because the first carboxylating enzyme is much less discriminating for the heavy isotope [16]. After entering the plant by the same diffusion processes as for C3 plants, the CO<sub>2</sub> is taken over by the PEP-carboxylase enzyme. This reaction is equivalent to a 5‰ - 6‰ fractionation. The products of this reaction are then taken over by the enzyme RuBP, which slightly discriminates against the heavy isotope of carbon [16]. Thus, the sudden fractionations during the diffusion and the action of the enzymes PEP-carboxylase and RuBP determine the isotopic composition of the C4 plants, which have a δ<sup>3</sup>C between -10‰ and -14‰ [19].

Therefore, isotopic fractionation at the carbon level is linked to the nature of the plant.

#### 4. Conclusion and Perspectives

This study made it possible to determine the chemical composition of cocoa butter in fatty acids. Thus cocoa butter is mainly composed of saturated fatty acids and then come the monounsaturated ones. We have around 63% to 68% saturated fatty acids composed mainly of stearic acid and palmitic acid, while monounsaturated fatty acids (oleic acid) represent 30% to 32%. Polyunsaturated acids represent less than 4%. We were also able to measure the δ<sup>3</sup>C isotopic signature of the glycerol contained in cocoa butter. The statistical treatment of these different results made it possible to highlight a discrimination within the samples. However, a study of the different varieties of cocoa could better make it possible to bring out the most discriminating parameters while eliminating the varietal factor. Also taking into account seasonal parameters (precipitation, sunshine, atmospheric conditions, etc.) could allow a more in-depth study with a view to characterizing the geographical origin of the cocoa bean.

#### Conflicts of Interest

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

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