

Development of a Gas–Liquid Chromatographic Method for the Analysis of Fatty Acid Tryptamides in Cocoa Products

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The determination of the occurrence and level of cocoa shells in cocoa products and chocolate is an important analytical issue. The recent European Union directive on cocoa and chocolate products (2000/36/EC) has not retained the former limit of a maximum amount of 5% of cocoa shells in cocoa nibs (based on fat-free dry matter), previously authorized for the elaboration of cocoa products such as cocoa mass. In the present study, we report a reliable gas–liquid chromatography procedure suitable for the determination of the occurrence of cocoa shells in cocoa products by detection of fatty acid tryptamides (FATs). The precision of the method was evaluated by analyzing nine different samples (cocoa liquors with different ranges of shells) six times (replicate repeatability). The variations of the robust coefficient of variation of the repeatability demonstrated that FAT_{C22} , FAT_{C24} , and total FATs are good markers for the detection of shells in cocoa products. The trueness of the method was evaluated by determining the FAT content in two spiked matrices (cocoa liquors and cocoa shells) at different levels (from 1 to 50 mg/100 g). A good relation was found between the results obtained and the spiking (recovery varied between 90 and 130%), and the linearity range was established between 1 and 50 mg/100 g in cocoa products. For total FAT contents of cocoa liquor containing 5% shells, the measurement uncertainty allows us to conclude that FAT is equal to 4.01 ± 0.8 mg/100 g. This validated method is perfectly suitable to determine shell contents in cocoa products using FAT_{C22} , FAT_{C24} , and total FATs as markers. The results also confirmed that cocoa shells contain FAT_{C24} and FAT_{C22} in a constant ratio of nearly 2:1.

KEYWORDS: Cocoa product; cocoa shell; fatty acid tryptamide; gas–liquid chromatography

INTRODUCTION

The determination of the occurrence and level of cocoa shells in cocoa products and chocolate is an important analytical issue. The recent European Union directive on cocoa and chocolate products (1) has not retained the former limit of a maximum amount of 5% of cocoa shells in cocoa nibs (based on fat-free dry matter), previously authorized for the elaboration of cocoa products such as cocoa mass and cocoa liquor (2). Interestingly, the Codex Alimentarius has for the time being maintained that limit. Therefore, this quality criterion of cocoa raw materials depends on national legislation.

For technological reasons and to build confidence between suppliers and users of cocoa raw materials, analytical methods to accurately measure the shell content in cocoa shells, mass, and butter are needed. Fatty acid tryptamides (FATs) are compounds that are much more abundant in cocoa shells and have, therefore, a strong potential to be used as quantitative shell markers in cocoa products.

Methodologies have been developed to measure FATs in cocoa products (3–7). The current official method, the so-called

“blue value”, is not specific and requires carbon tetrachloride, which is highly toxic. However, an accurate liquid chromatography methodology has been developed (8–12) and optimized (13). Briefly, the method consists of extracting lipids from cocoa product samples, isolating a fraction enriched in FATs by chromatography on a silica gel cartridge, and analyzing the sample by high-performance liquid chromatography (HPLC). The detection is achieved using a fluorescent detector, and FAT_{C17} (margaric acid tryptamide) is used as an internal standard (IS). This method allows accurate quantification of the major FATs, which are used for the evaluation of shells in cocoa products. Authentic standards of the main FATs are now commercially available, but the need of a fluorescent detector could be the drawback of this procedure since this type of detector is not widely accessible in laboratories dedicated to quality control.

Gas–liquid chromatography (GLC) using a flame ionization detector (FID) is widely used for lipid analysis, and in the present study, a rapid and simple GLC method useful for the determination of FATs as markers indicating the presence of shells in cocoa products was developed. Preliminary trials aimed at quantifying cocoa shells from FATs were also performed.

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MATERIAL AND METHODS

Standards and Materials. FAT compounds (C₁₇, C₂₂, C₂₃, C₂₄, and C₂₆) were obtained from Fluka Chemicals (Buchs, Switzerland). FAT_{C17} tryptamide was used as an IS at 1 mg/mL in dry chloroform. Cocoa products were obtained from Nestlé factories and Sitos (Netherlands).

Sample Preparation. Cocoa products (1 g) were homogenized in chloroform (10 mL), and IS (1 mL, 1 mg/mL) was consequently added. FATs were extracted by sequenced ultrasonic treatment (10 min), centrifugation (10 min, 2000 rpm), and filtration of the supernatant. The extraction of FATs from cocoa butter (1 g) was performed by dilution in chloroform (10 mL) in the presence of IS (1 mL, 1 mg/mL).

Solid Phase Extraction Procedure. The extract (2 mL) was purified by solid phase extraction using a silica cartridge (Waters, Switzerland) conditioned with 3 mL of hexane/ethyl acetate (9:1 v/v) according to the literature procedure (13). Elution of four fractions was done using (i) hexane (10 mL), (ii) hexane/ethyl acetate (9:1 v/v, 10 mL), and (iii) hexane/ethyl acetate (8:2 v/v, 3 mL), and finally, FATs were recovered using pure ethyl acetate (2 mL). The latest fraction was evaporated under nitrogen and diluted in pure ethyl acetate (300 µL) before GLC analysis.

GLC Analysis. Analysis of FATs was performed by GLC using a DB-5HT capillary column (15 m × 0.25 mm i.d.; film thickness, 0.10 µm; J&W, Palo Alto, CA). Split injection (5:1) and FID were achieved at 380 °C. The oven temperature programming was 250 °C, increased to 300 °C at 50 °C min⁻¹, then to 340 °C at 10 °C min⁻¹, and to 380 °C at 10 °C min⁻¹, and isothermal at 380 °C for 1 min. A carrier gas (H₂) was used in constant flow mode at 2.5 mL min⁻¹.

Statistics. Analytical data are often subject to potential outlying values. To reduce their influence in the precision characteristics calculation, robust statistics were used. Their main advantage was to be less sensitive to outliers than classical statistics and to then provide more reliable results. Q-Stat 1.1.5 software (Nestlé, Switzerland) was used for statistical analyses. The measurement uncertainty was calculated according to a standardized procedure (14–16).

RESULTS AND DISCUSSION

According to the cocoa directive 73/241/ECC (2), the shell content in cocoa nibs is limited to 2.5%, relating to the product for quality reasons. After conversion of the new cocoa directive EEC/2000/36 (1) in national law, this adjustment will be dropped. Because of these circumstances, there is, for quality purposes, a high interest in developing an analytical method to detect the shell contents in cocoa products. FATs are suitable indicators for ascertaining the shell contents in cocoa products (8–13). The main compounds are behenic (FAT_{C22}) and lignoceric (FAT_{C24}) acid tryptamides (BAT and LAT, respectively), which represent about 90% of total FATs. On the basis of the sample preparation procedure developed by Munch and Schieberle (8, 9), which was later on optimized by Janssen and Matissek (13), a rapid detection of FATs was developed by GLC (see Figure 1). The absolute quantification was achieved using FAT_{C17} as an IS according to literature procedure (13). The identifications of the FAT_{C22}, FAT_{C23}, FAT_{C24}, and FAT_{C26} were performed using pure standards while tentative identifications of FAT_{C21} and FAT_{C25} were performed by calculation of their theoretical retention indexes. We confirmed that cocoa shells contain FAT_{C24} and FAT_{C22} in a constant ratio of nearly 2:1 (see Table 1).

Validation and Measurement Uncertainty of the Developed Methodology. *Precision of the Method.* The precision (see Table 2) of the method was evaluated by analyzing nine different samples (cocoa liquors with different ranges of shells) six times (replicate repeatability). These results indicate that the more promising markers to quantify shell content in cocoa products will be the major FATs (FAT_{C22} and FAT_{C24}) and the total FAT content. FAT_{C23} and FAT_{C26} are present in concentra-

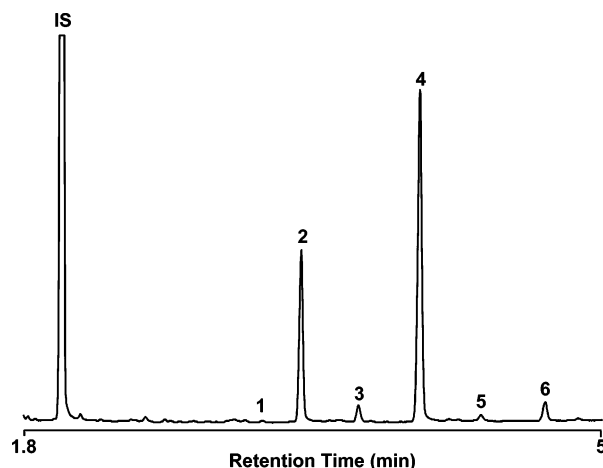


Figure 1. Partial chromatogram of FATs extracted from cocoa shells (see Materials and Methods for experimental conditions). FAT_{C17} is used as an IS for the quantification of FAT_{C22} (2), FAT_{C23} (3), FAT_{C24} (4), and FAT_{C26} (6). Compounds 1 and 5 were tentatively identified as FAT_{C21} and FAT_{C25}, respectively, based on their theoretical retention indexes.

Table 1. Comparison between Literature Data and Results Found for FAT_{C22} and FAT_{C24} Ratios

products	FAT (mg/100 g)		FAT _{C24} /FAT _{C22}	
	FAT _{C22}	FAT _{C24}	experimental	literature (13)
cocoa shells	10.30	22.83	2.22	1.79
cocoa liquor	1.16	2.20	1.90	1.97
cocoa butter	1.00	2.35	2.35	2.07

Table 2. Replicate Repeatability of the Method (Calculated Using Q-Stat 1.1.5)

FATs (mg/100 g)	n	median	SD(<i>r</i>) _{robust}	repeatability	<i>r</i> (%)	robust coefficient of variation (%)
				limit (<i>r</i>) at 95%		
FAT _{C22}	6	0.915	0.0818	0.2268	24.8	8.9
FAT _{C23}	6	0.115	0.0909	0.2519	219	79.0
FAT _{C24}	6	2.160	0.1550	0.431	19.9	7.2
FAT _{C26}	6	0.210	0.0586	0.1624	77.3	27.9
total FAT	6	3.316	0.319	0.883	26.6	9.61

tions close to the limit of quantification (LOQ = 1 mg/100 g) and do not represent valuable markers.

Trueness of the Method. The trueness of the method was evaluated by determining the FAT content in two spiked matrices (cocoa shells and cocoa liquors). The statistical results have shown a good relation between the results obtained and the spiking (recovery was between 90 and 130%). In addition, the statistical evaluation has shown that the results depend neither on the matrix nor on the FAT compound. Furthermore, these results showed that the detection limit (LOD) is between 0.01 and 0.05 mg FAT/mL solution, which means 1 and 5 mg FAT/100 g cocoa product. This will be calculated during the determination of the method linearity.

Linearity of the Method, LOD, and LOQ. The linearity of the method depends on the response of the detector vs sample concentration. The linearity was checked by using a solution containing FAT in a range of concentrations between 0.1 and 0.5 mg FAT/mL solution. The sensitivity of the method (corresponding to the slope of the curve) is 10⁶. That means that a little variation of the concentration gives a huge variation of the measured signal. The statistical evaluation of the results (residual standard deviation = 5 × 10⁻⁶) shows that the method

is linear from LOD to 0.5 mg/mL for each FAT in solution. That means that it is linear from 1 to 50 mg of each FAT per 100 g of product. The results of the method linearity have demonstrated that under the described conditions, the LOD is around 0.01 mg/mL for each FAT in solution. The LOQ is 1 mg FAT/100 g of product. However, considering the linearity range (1–50 mg/100 g product), the precision range (2–40 mg/100 g product), and the trueness range (2–40 mg/100 g product), the working range is between 2 and 40 mg FAT/100 g product.

Measurement Uncertainty of the Method. Before calculating the measurement uncertainty of the method, the sources of this uncertainty have to be identified (see **Figure 3**). The results of calculated uncertainty sources are listed in **Table 3**. The calculated uncertainty budget was plotted under a drawing form (see **Figure 2**). Then, it was rapidly seen that the response factor, the repeatability, and the recovery of the method represent the largest contributions to the measurement uncertainty of the method. The final uncertainty is expressed as an interval. To transform the deviation standard in intervals, it must be multiplied by 2 ($14-16$). Accordingly, the expression of these results would be as follows, for example, for cocoa liquor containing 5% shells:

$$\text{FAT}_{\text{C22}} = 1.14 \pm (2 \times 0.1437) \text{ mg}/100 \text{ g} \approx 1.1 \pm 0.3 \text{ mg}/100 \text{ g}$$

$$\text{FAT}_{\text{C23}} = 0.09 \pm (2 \times 0.0718) \text{ mg}/100 \text{ g} \approx 0.1 \pm 0.1 \text{ mg}/100 \text{ g}$$

$$\text{FAT}_{\text{C24}} = 2.59 \pm (2 \times 0.3535) \text{ mg}/100 \text{ g} \approx 2.6 \pm 0.7 \text{ mg}/100 \text{ g}$$

$$\text{FAT}_{\text{C26}} = 0.19 \pm (2 \times 0.0634) \text{ mg}/100 \text{ g} \approx 0.2 \pm 0.1 \text{ mg}/100 \text{ g}$$

$$\text{total FATS} = 4.01 \pm (2 \times 0.3935) \text{ mg}/100 \text{ g} \approx 4.01 \pm 0.8 \text{ mg}/100 \text{ g}$$

In conclusion, this validated method allows quantifying FATS in cocoa products. Then, to verify if this conclusion is acceptable and to find the markers of the shells in cocoa products, the following experiment was carried out.

Quantification of Cocoa Shells in Cocoa Products by GLC Analysis of FATS: Results of Preliminary Trials. During the method validation, it was demonstrated that the determination of the FATS is not dependent on the matrix. Therefore, it was decided to determine shell content in cocoa liquors. Cocoa liquor samples were prepared from cocoa beans (Ivory Coast) taken after roasting, and cocoa beans were manually peeled to avoid shell contamination. These beans were ground and homogenized to obtain cocoa nibs. The shells from the same batch of cocoa beans were taken after separation from cocoa nibs. The shells were sieved, and possible pieces of nibs were eliminated by visual control. The cocoa nibs and the shells were mixed following the scheme described in **Table 3**. The mixing was directly done in the crushing cuve, and each of the liquors was recovered in an airtight beaker. The cocoa liquors were left at about 50 °C to maintain them in the liquid state. Then, the total fat of each sample was determined using the Mojonnier method (see **Table 4**). Then, FATS were determined on these samples using the developed and validated GLC method. The samples were analyzed six times. **Figure 3** shows a perfect correlation ($R^2 = 0.9893$) between both FAT and shell contents in cocoa liquors. Even if the shell content in cocoa liquors varies from 0 to 100%, only the part of the curve between 0 and 10% was

Table 3. Uncertainty Budget for Each FAT in Cocoa Products

parameter	FAT	value	unit	uncertainty	
				standard $u(x)$	relative $u(x)/x$
repeatability of the method	BAT (FAT _{C22})	0.915	mg/100 g	0.0818	0.0894
	TAT (FAT _{C23})	0.115	product	0.0909	0.7904
	LAT (FAT _{C24})	2.16		0.1550	0.0718
	CAT (FAT _{C26})	0.210		0.0586	0.2790
recovery of the method		1.150	ratio	0.0720	0.0626
response factor	BAT (FAT _{C22})	1.000	ratio	0.0635	0.0637
	TAT (FAT _{C23})	1.000		0.0940	0.0878
	LAT (FAT _{C24})	1.000		0.1067	0.0976
	CAT (FAT _{C26})	1.000		0.2153	0.1720
concentration of IS		5000	mg/L	31.612	0.0063
volume of IS		1.000	mL	0.0035	0.0035
mass of sample		1000	mg	0.1288	0.0001
total uncertainty ^a	BAT (FAT _{C22})	0.915	mg/100 g	0.1156	0.1264
	TAT (FAT _{C23})	0.115	product	0.0917	0.7978
	LAT (FAT _{C24})	2.160		0.2946	0.1364
	CAT (FAT _{C26})	0.210		0.0701	0.3337
total FATS ^b		3.320		0.3369	0.1015

^a To determine the total uncertainty, the values of each uncertainty component were added according to the rules of error propagation. ^b The total FAT was calculated as $\sum \text{FAT}_i$, and the total FAT uncertainty was calculated as $\sqrt{\sum u(x_i)^2}$.

Table 4. Composition of Composite Samples Prepared from Cocoa Nibs and Cocoa Shells Used for the Evaluation of the Method for the Detection of Cocoa Shells in Cocoa Products

sample	content (g/100 g)		fat content (%)
	shell	nibs	
A	0.0	100.0	54.83
B	1.0	99.0	54.21
C	1.5	98.5	53.83
D	2.0	98.0	53.66
E	2.5	97.5	53.35
F	3.5	96.5	52.74
G	5.0	95.0	51.97
H	7.5	92.5	50.61
I	10.0	90.0	49.34
Z	100.0	0.0	2.65

considered because the maximum admitted avoiding technological problems is 2.5% of shells in the cocoa liquor. The statistical comparison between the results obtained and the true values determining the equations and the different coefficients of correlation has shown a good relationship. From these values, some calculations were done on a cocoa liquor containing 5% of shells (**Table 5**). The results confirmed that the best markers are the major FATS (FAT_{C22} and FAT_{C24}) and the total FATS. FAT_{C23} and FAT_{C26} could not be used as markers because their concentrations in the products are close to the LOD and the values found are not representative.

This method appears to be a suitable tool to determine and predict the shell content in cocoa liquor using FAT_{C22}, FAT_{C24}, and total FATS as markers. FAT could be analyzed by either GLC (present study) or HPLC techniques (8–13); therefore, FAT analysis could be implemented in different laboratories. Regarding the quantification of shell contents in cocoa liquors, the study confirmed that it is possible to predict % shells in cocoa liquors using FAT_{C22}, FAT_{C24}, and total FAT markers. For cocoa liquor containing about 5% shells, these markers indicated $4.2\% \pm 1.1\%$. This correlation has to be refined because it could depend on geographical origin and the crop of

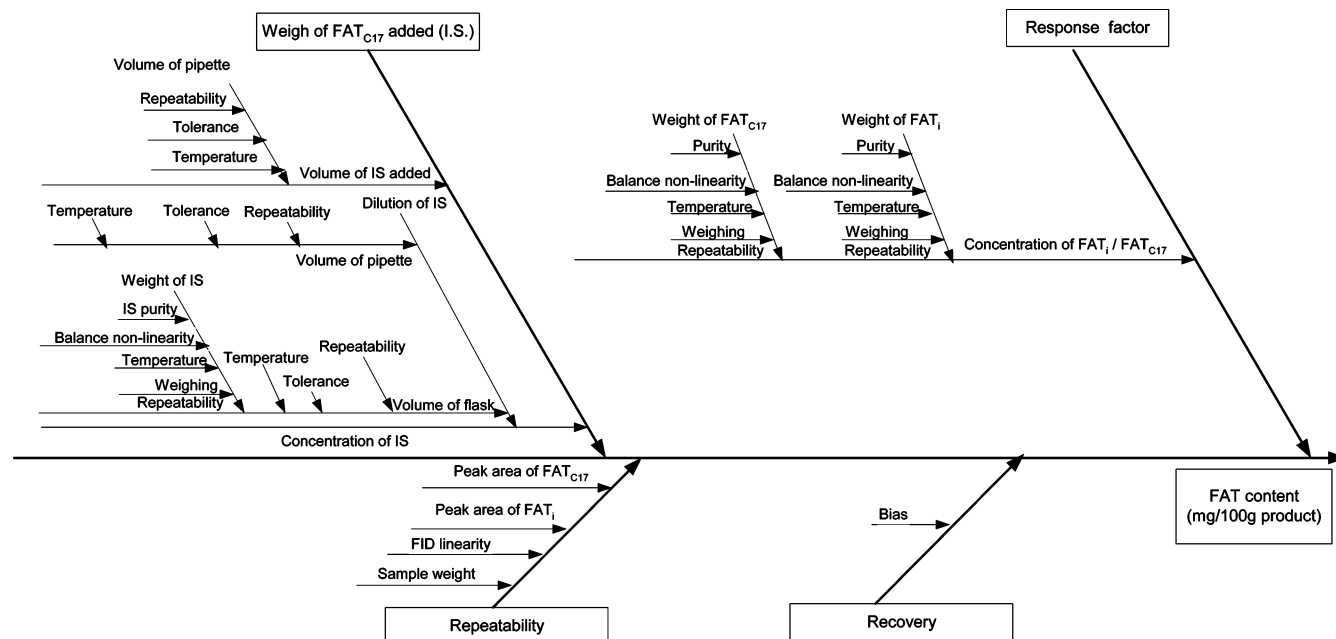


Figure 2. Sources of the uncertainty of the method.

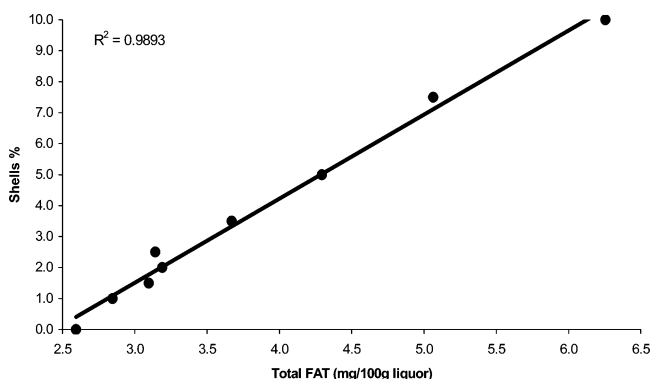


Figure 3. Correlation between FAT levels measured by the developed GLC methodology and the cocoa shell contents on spiked cocoa liquor samples.

Table 5. Equation Calculated to Predict the Shell Content in Cocoa Liquor from FAT Results and Data Obtained for Samples Containing 5% Shells

FAT	equation	% shells found
FAT _{C22}	shell content = $-4.971 + 8.408 \text{ FAT}_{C22}$	4.6 ± 1.6
FAT _{C23}	shell content = $3.61 + 0.41 \text{ FAT}_{C23}$	3.6 ± 2.2
FAT _{C24}	shell content = $-5.878 + 3.972 \text{ FAT}_{C24}$	4.4 ± 1.2
FAT _{C26}	shell content = $-0.13 + 17.73 \text{ FAT}_{C26}$	3.2 ± 1.3
total FAT	shell content = $-6.088 + 0.257 \text{ total FATs}$	4.2 ± 1.1

the cocoa beans. It should be noted that only cocoa originating from the Ivory Coast was tested; the study on the variation of these markers following the geographical origins or processing conditions will be further tested in future work.

ABBREVIATIONS USED

BAT, behenic acid tryptamide; CV, robust coefficient of variation of the repeatability; FAT, fatty acid tryptamides; FID, flame ionization detector; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; IS, internal

standard; LAT, lignoceric acid tryptamide; LOD, limit of detection; LOQ, limit of quantification.

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