



Proposal for a semi-quantitative method for the determination of volatile compounds in cocoa liquors

Proposición de un método semi-cuantitativo para la determinación compuestos volátiles en licores de cacao

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ABSTRACT

Keywords:

Volatile compounds,
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cocoa liquor.

Seventy-four volatile compounds were identified and quantified from cocoa liquors of the ICS 95 and TCS 01 varieties produced in the department of Santander, Colombia. The compounds were extracted using the solid phase microextraction with head space (SPME-HS) technique, and identified by gas chromatography coupled to mass spectrometer (GC-MS) by comparing the mass spectra of each compound in the Wiley 275L library of mass spectra and the Kovats retention index (KI). A semi-quantitative method was proposed that included toluene as an internal standard to normalize the degree of recovery between samples and a response factor for each family, calculated using a compound characteristic for each functional group. The results associated with response factors for each family: alcohols, acids, aldehydes, ketones, esters and pyrazines (2.19, 1.02, 2.84, 0.38, 6.38, 0.88 respectively) were different between families, however, there was no difference between compounds within the same family. The implemented method obtained a LOD and LOQ of 0.024 µg/kg and 0.037 µg/kg respectively and an accuracy expressed as percentage recovery (of characteristic compounds per family) of 96% on average. According to the precision of the method, the results show that the concentrations have an average coefficient of variation (%CV) of 7.38% assuring repeatability and good precision. Finally, for the analyzed and quantified samples, it was found that the compounds with higher concentration were acetic acid (42.633 mg/kg), 2-phenylethyl acetate (29.44 mg/kg), 2,3-butanediol (345.39 mg/kg), 2-phenylethanol (12.595mg/kg) and 2,3.5.6-tetramethylpyrazine (8.601 mg/kg).

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RESUMEN**Palabras clave:**

Compuestos volátiles,
Factor de respuesta,
SPME,
Índices de Kovats,
GC-MS,
licor de cacao.

Se identificaron y cuantificaron 74 compuestos volátiles a partir de licores de cacao de las variedades ICS 95 Y TCS 01 producidas en el departamento de Santander, Colombia. Los compuestos fueron extraídos usando la técnica de micro extracción en fase sólida con espacio de cabeza (SPME-HS), e identificados por medio de cromatografía de gases acoplado a espectrómetro de masas (GC-MS) mediante la comparación de los espectros de masas de cada compuesto en la biblioteca Wiley 275L de espectros de masa y la relación de índices de retención de Kovats (IK). Se propuso un método semi-cuantitativo que incluyó al tolueno como estándar interno para normalizar el grado de recuperación entre muestras y un factor de respuesta para cada familia, calculado por medio de un compuesto característico de ese grupo funcional. Los resultados asociados con los factores de respuesta para cada familia o grupo de compuestos como alcoholes, ácidos, aldehídos, cetonas, ésteres y pirazinas (2,19, 1,02, 2,84, 0,38, 6,38, 0,88 respectivamente) fueron diferentes entre familias, sin embargo, no hubo diferencia entre los compuestos de la misma familia. El método implementado obtuvo un LOD y LOQ de 0,024 $\mu\text{g}/\text{kg}$ y 0,037 $\mu\text{g}/\text{kg}$ respectivamente y una exactitud expresada en porcentaje de recuperación (de los compuestos característicos por familia) en promedio de 96%. Según la precisión del método, los resultados muestran que las concentraciones tienen un coeficiente de variación (%CV) promedio de 7,38% aseverando repetibilidad y buena precisión. Finalmente, para las muestras analizadas y cuantificadas, se encontró que los compuestos con mayor concentración fueron el ácido acético (42,633 mg/kg), acetato de 2-feniletilo (29,44 mg/kg), 2,3-butanodiol (345,39 mg/kg), 2-feniletanol (12,595mg/kg) y 2,3,5,6-tetrametilpirazina (8,601 mg/kg).

Introduction

The cocoa produced worldwide is classified into fine and flavour cocoa and ordinary or common cocoa. Only 5-10% of the cocoa produced worldwide is considered fine and aromatic [1]. Three varieties of cocoa are grown in Colombia: forastero, which corresponds to ordinary cocoa, and criollo and trinitario, which correspond to fine and aromatic cocoa. Genotype, agroclimatic conditions and processing stages have a significant impact on the aromatic quality of cocoa beans [2], [3]. This aromatic quality is directly related to the volatile aroma precursor compounds present in the different varieties [4], [5]. The greatest development of these aroma compounds is found in the processing stages (fermentation and drying) and in roasting [6], [7]. Certain volatile compounds found and identified in cocoa liquors such as 2-heptanol (floral aroma), 2-phenylethanol (sweet aroma), benzaldehyde (butter aroma), 2-phenylethylacetate (fruit aroma), acetophenone (floral aroma), 2,5-dimethylpyrazine (nutty aroma), 2-acetylpyrrole (sweet chocolate aroma), among others, favour the aromatic quality of the cocoa liquor obtained [1], [8], [9].

Although the literature is extensive on the identification

of compounds associated with cocoa aroma, few articles propose methods for their quantification. Normally, quantification is carried out by means of an internal standard using a reference compound to quantify all other compounds in the sample; however, this approach is not very accurate in quantifying as a constant response factor is assumed for all compounds in the sample. Another approach to quantification is the use of external standards, in this case a reference compound is used for each compound to be quantified (the same compound for quantification); the drawback of this approach is the cost associated with the purchase of standards. Thus, for example Rodríguez-Campos et al [7], quantified some volatile compounds present in finished chocolates with external standards (one reference compound for each quantified compound) such as benzaldehyde, 2-heptanol, 2-phenylethylacetate, 2,3-diethyl-5-methylpyrazine, among others. Additionally, they quantified the compounds for which the commercial standard was not available using the slope obtained for a standard of an analogous compound that was structurally similar, but slightly different. [10], quantified volatile compounds in cocoa powder using external standards such as benzyl acetate, ethyl octanoate, 2,3,4,6-trimethylpyrazine, acetic acid, 2-acetylpyrrole, linalool, among others,

using a reference compound for each compound to be quantified. Similarly, Rodríguez - Campos et al applied [11] quantifying volatile compounds present in cocoa liquors at different times of fermentation and drying with external standards such as 2-phenylethylacetate, propanoic acid, 2,3,5-trimethylpyrazine, acetophenone, among others. For the compounds that did not have standards, they were quantified using an analogous compound to determine the concentration in cocoa liquors. Other authors such as Ducki et al [12], carried out the quantification of volatile compounds to cocoa powder before and after conching using external standards such as 2-methylpropanal, 3-methylbutanal, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, among others. For the compounds that did not have the external standard he used an internal standard (toluene) leaving as results concentrations equivalent to grams of toluene.

According to the approaches outlined in the previous paragraphs, the quantification of volatile compounds is complex due to the amount of compounds present in each sample of cocoa liquor. Therefore, the quantification of each compound using an external standard becomes impractical and economically unfeasible. For this reason, this paper identifies and proposes a semi-quantitative method by family or functional group of the volatile aroma precursor compounds present in cocoa liquors, using a hybrid strategy (internal standard - external standard/family) which includes toluene as an internal standard to normalize the degree of recovery between samples and a response factor for each family, calculated by means of a compound characteristic of that functional group, thus facilitating the determination of the concentration (quantification) of volatile compounds present in cocoa liquors.

Materials and Methods

Cocoa liquor samples

In this study, two varieties of cocoa liquor (ICS 95 and TCS 01) were used, obtained from the research center La Suiza, Corpoica, located in Rionegro, Santander, Colombia. The cocoa beans were fermented in 15x15-inch wooden crates for 4 days, dried in the sun and roasted at 100°C for 15 minutes. Two samples were taken from each variety, one with only well-fermented beans (BF) and the other with mixed beans taken at random (AZ). The selection of the well-fermented beans was

done following the Colombian technical standard NTC 1252. Both BF and AZ beans were husked and ground to obtain the respective cocoa liquor.

Extraction of volatile compounds from cocoa liquor

For each sample, 2g of cocoa liquor, 25 µL of toluene and 20 µL of the reference compound (concentration shown in Table 1), were added to a 10 ml vial (PTFE/Silicone septa) with magnetic stirrer, where the volatiles were extracted using the solid phase microextraction technique (SPME) with a polydimethylsiloxane fiber (DVB-CAR-PDMS 50/30µm, Supelco) [7], [12]. The sample was conditioned for 15 min and 60°C and then the extraction of volatiles was performed by introducing the fiber for 40 min and 60°C.

Table I. Representative Volatile Compounds

Reference Compound	Functional group (family)	Concentration (mg/kg)
Toluene	Standard	4200
Acetic Acid	Acid	2070
Acetophenone	Ketone	3040
Ethyl acetate	Ester	2070
Phenylacetaldehyde	Aldehyde	9300
2,3,5-trimethylpyrazine	Pyrazine	2500
Linalol	Alcohol	2680

Separation and Identification of Volatile Compounds

Fiber desorption was performed in a gas chromatograph (GC 7890A) coupled to a quadrupole mass detector (HP 5972) with electronic impact ionization system at 70 eV and source temperature of 230°C. For the separation, a non-polar 5%-phenylpolymethylsiloxane (HP-5) capillary column of 30m x 0.25mm x 0.25µm d.i. was used. The oven temperature and heating ramp for the column was set at 30°C-10min, then increased to 3°C/min-60°C-10min, then increased to 10°C/min-150°C and finally increased to 4°C/min-200°C. The carrier gas used was nitrogen at 1ml/min. The temperature of the injector was 265°C with injection in split mode.

The compounds were identified on the basis of two criteria:

By comparing the mass spectra of each compound in the Wiley 275L mass spec library and with the NIST MS 2.3 tool.

The calculation of the Kovats retention Index (KI) by means of equation 1 for each of the compounds and the

respective comparison with the NIST SRD69 library to verify the identification of the compound.

$$KI = 100n + 100 \left[\frac{t_{Rx} - t_{Rn}}{t_{RN} - t_{Rn}} \right] \quad (1)$$

Equation 1. Calculation of KI

Where:

KI: Retention rate of interest compound *x*.

n: Number of carbon atoms of the eluting *n*-alkane before the compound of interest *x*.

t_{Rx} : Retention time of interest compound *x*.

t_{Rn} y t_{RN} : Retention times of *n*-alkanes eluting before and after compound *x*.

Quantification of volatile compounds

To perform a more precise quantification, a semi-quantitative method involving an internal standard (toluene) and a response factor for each family of compounds was proposed (Table 1). For each group of compounds (pyrazines, acids, esters, aldehydes, ketones, and pyrroles), their respective response factor was determined using equation 2. Three identical samples were prepared for each analysis and the results averaged.

$$FR = \frac{A_{EI} \times C_x}{A_x \times C_{EI}} \quad (2)$$

Equation 2. Response Factor Calculation

Where:

C_x : Concentration of compound of interest *x*.

A_x : Area of compound of interest *x*.

C_{EI} : Concentration of the internal standard

A_{EI} : Area of the internal standard.

FR: response factor.

ρ : density of the cocoa liquor.

Equation 3. Calculation of analyte concentration

Once the response factors for each family were obtained, the quantification of all the volatile compounds present in the sample was carried out by means of equation 3.

$$C_x(\text{mg/kg}) = FR \times \frac{A_x \times C_{EI}}{A_{EI} \times \rho} \quad (3)$$

Method validation

In order to evaluate the analytical method, the following figures of merit were determined for the internal standard using toluene:

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined for the internal standard, preparing toluene solutions in ethanol, making dilutions until the minimum detectable signal was obtained.

The limit of detection (LOD) was determined with 95 % confidence as the concentration in mg/kg that provides a signal (LOD) in the detector that is significantly different from the 'target' or 'background noise' signal, according to the formula in Equation 4:

$$LOD = Y_B + 3S_B \quad (4)$$

Equation 4. Calculation of the detection limit

Where:

Y_B : Minimum detectable concentration of the analyte

S_B : Standard deviation of the measurements made to determine Y_B at a 95% confidence level.

The limit of quantification (LOQ) is defined as:

$$LOQ = Y_B + 10S_B \quad (5)$$

Equation 5. Calculation of the limit of quantification

Intermediate accuracy

Intermediate accuracy was evaluated in terms of the coefficient of variation (CV). For the coefficient of variation the acceptance criterion $CV < 10\%$ was taken into account. This value represents the relationship between mean size and variability and it is important that it is less than 10% to check for repeatability in data collection.

$$CV = \frac{\bar{s}}{\bar{x}} * 100 \quad (6)$$

Equation 6. Coefficient of Variation Calculation

Where:

\bar{S} : Average of the standard deviations of the concentrations of 3 repetitions for each analyte.

\bar{X} : Average of the concentrations of 3 replicates for each analyte.

Accuracy

The accuracy of the method was calculated on the basis of percentage recovery (%R), in which the sample of cocoa liquor was enriched with each of the compounds used as reference for each family with their respective concentrations shown in Table 1 (all experiments were done in triplicate and their response was averaged). The calculation was made as follows:

$$\%R = \frac{C_{ME} - C_A}{C_T} \times 100 \quad (7)$$

Equation 7. Calculation of the recovery percentage

Where:

C_{ME} : Concentration of the measured analyte in the fortified sample

C_A : Concentration of the analyte in the sample without fortification

C_T : Total concentration of the analyte added to the sample

Results and Discussion

Identification and quantification of volatile compounds

Seventy-four volatile compounds were identified and quantified in cocoa liquors from the department of Santander. Table 3 shows the identification and quantification by functional group of each sample of cocoa liquor obtained. Identification results similar to those obtained here were reported by [3], [6], [7], [10], [12], [13], among others, showing that most of the identified compounds are pleasant and associated with desired sensory attributes. Thus, for example, the presence of benzaldehyde, phenylacetaldehyde, 2-phenyl-2-butenal, 2,3 butanediol, 2-heptanol, linalool, 2-phenyl-ethanol, 3-hydroxy-2-butanone, 2-heptanone, 2-nonanone, 3-methyl-butanol acetate is beneficial, ethyl acetate, ethyl octanoate, 2,3,5,6-tetramethyl pyrazine, 1-(1h-pyrrol-2-yl) ethanone for the sensory characteristics of cocoa, providing mostly cocoa, sweet, nutty and fruity sensory notes. Looking at table

3, it was found that esters and alcohols constituted the families with the highest number of aroma compounds found in the 4 cocoa liquors and these represent 23% and 16% respectively of the total number. On the other hand, other compounds were found that have not been reported in cocoa matrices such as 2-ethylbutanoic acid, 3-methyl-2-cyclohexanone, epoxy linalol (6-ethenyl-2,2,6-trimethyloxan-3-ol), gamma-terpinene (1-methyl-4-(1-methyl ethyl)- 1,4-cyclohexadiene), 4-carvomethenol (4-methyl-1-(1-methylethyl)-3-cyclohexenol), 1-methylpropyl acetate (sectile acetate), isobutyl isobutyrate (2-methylpropanoate), cis-citral (3,7-dimethyl-2,6-octadienal), trans-citral (3,7-dimethyl-2,6-octadienal).

With respect to the family of acids, 6 acids were identified and are shown in table 3. It is observed that the concentrations of the acids in the random samples are much higher than the well-fermented samples, showing a reduction when comparing the BF samples to the AZ samples (TCS 33% and ICS 14% reduction), this is due to the selection of only well-fermented beans which denote a good fermentation process. Analyzing the behavior of acetic acid (a compound with a higher concentration; associated with perceptions of vinegar) the reduction in the TCS01 and ICS95 cocoa liquor samples was 34.7% and 12.2% respectively. The concentration of acetic acid in the TCS 01AZ sample is higher in all samples which could then lead to the hypothesis that TCS 01AZ would have undesirable attributes associated with vinegar, sour and astringent notes [13] - these characteristics are associated with under-fermented cocoa beans. However, a sensory analysis would be required to support the hypothesis made in the previous paragraph and to determine the threshold concentration at which undesirable attributes are perceived. Likewise, this work identified the presence of an unreported compound, 2-ethylbutanoic acid, which has odor-active characteristics associated with rancid, acid.

Eleven alcohols were identified, of which 2-phenylethanol and β -linalool have been found in various cocoa matrices [3], [10]. For this family of compounds, the BF samples increased the concentrations of 2,3-butanediol, 2-nonanol, and 2-phenylethanol (the first two odorless; the last one associated with sweet, floral) [6], [11] while the concentrations of 3-methylbutanol (butter flavor), and 2-heptanol (floral) were reduced. Two stereoisomers of the compound 2,3-butanediol were found, showing a

considerable increase when only BF grains are selected; in the sample TCS01BF, this compound increases approximately 18 times its concentration with respect to TCS01AZ. Similar to the other stereoisomer in the TCS01BF sample where the increase is 3 times the concentration in TCS01AZ. In the ICS 95 samples there is an increase for the BF samples (ICS95BF increase 1.2 times over ICS95AZ), however, it is not as significant as in the TCS01 variety. Compounds such as 2-ethylhexanol, 2,6-octadien-1-ol-3,7-dimethyl and phenylmethanol (associated with attributes such as sweet and floral) [10], were only identified in TCS 01 BF; their presence is possibly associated with an increase in the number of well-fermented beans and with the genetics of TCS 01. In this sense, TCS 01 is an acriolated trinitarian clone with a theobromine/caffeine ratio of 4; while ICS 95 is a normal trinitarian with a ratio of 8.

For aldehydes and ketones, 12 and 5 compounds were identified respectively. As shown in table 3, most of the aldehydes increase their concentration in the BF samples with respect to the AZ samples (TCS 77% and for ICS 7% increase), this is due to the increase of well-fermented grains that give as effect an increase in concentrations of desired volatile compounds. benzaldehyde, phenylacetaldehyde and 5-methyl-2-phenyl-2-hexenal are the aldehydes with the highest concentration found in the samples and widely reported in the literature on cocoa matrices [6], [7]; they represent 65% (TCS 01) and 56% (ICS 95) of the total increase in aldehyde concentration between the BF and AZ samples. Aldehydes not reported and identified were 3,7-dimethyl-2,6-octadienal (cis-citral) and 3,7-dimethyl-2,6-octadienal (trans-citral) with lemon citrus (PubChem) flavours. The 2-Phenyl-2-butenal, 4-methyl-2-phenyl-2-pentenal and 5-methyl-2-phenyl-2-hexenal offering cocoa aromas increase their concentration by 30% (TCS01) and 14% (ICS95) when only well-fermented beans are selected. These compounds are representative of the cocoa fruit because it is a constituent of the characteristic cocoa aroma. On the other hand, the most commonly reported ketones in cocoa matrices are 3-hydroxy-2-butanone (butter aroma), acetophenone (strong sweet aroma) and 2-heptanone [3], as well as in table 3 are the ketone compounds with the highest concentration. However, this functional group is found in higher concentration in the AZ samples than in the BF samples (43% and 21% reduction in TCS 01 BF and ICS 95 BF respectively). 3-methyl-2-cyclohexanone was identified which has not been found in other reports in

cocoa matrices obtaining a sweet, nutty aroma. Seventeen esters were identified among which 3-methylbutyl acetate, ethyl benzoate, ethyl octanoate, isoamyl benzoate, and ethyl 2-phenyl acetate have been reported in investigations associated with the identification of volatile cocoa compounds and pleasant fruit and candy flavors [7], [3]. These compounds show a reduction in BF samples of 30% (TCS01BF) and 17% (ICS95BF) with respect to AZ samples. Unlike the two compounds with higher concentrations present in the samples such as 2-phenylethyl acetate and ethyl acetate with sensory properties to sweet, fruity [6], [3]. which increase their concentration significantly in the TCS01BF sample by 8 and 3.2 times and in the ICS95BF sample by 1.2 and 1.1 times. Isobutyl isobutyrate has not been reported in other investigations, but in this work it was identified only in the TCS 01 BF variety and was one of the main compounds with higher concentration and odor-active fruit aroma (PubChem). The compounds 3-methylbutyl acetate, 2-methylbutyl acetate and 3-methyl-2-butyl acetate with fruit aroma are reduced by 47% (TCS01BF) and 21% (ICS95BF) in the BF samples due to the elimination of 2-methylbutanoic and 3-methylbutanoic acids which are the precursors of these compounds in the fermentation and drying stages [10], compounds such as ethyl 3-phenyl-2-propenoate associated with sweet and cinnamon aromas and benzyl acetate with floral and fruit aromas [10], are only found in the TCS 01 variety and increase their concentration by 2.3 and 1.6 times when working with BF grains.

Table III. Volatile compounds identified and quantified in cocoa liquors from ICS-95 and TCS-01 materials

N°	Compound	Retention time (min)	Kovats retention index ^b	TCS 01 BF		TCS 01 AZAR		ICS 95 BF		ICS 95 AZAR	
				Concentration ^a	%CV	Concentration	%CV	Concentration	%CV	concentration	%CV
Acids	1 Acetic acid	2.86	602	64.881±1.602	2.469	99.277±6.109	6.153	42.583±3.923	9.212	48.501±1.730	3.566
	2 2-methylpropanoic acid	7.98	758	N/D	-	2.622±0.176	6.712	2.918±0.258	8.841	3.410±0.141	4.134
	3 3-methylbutanoic acid	15.25	869	6.786±0.505	7.441	7.868±0.087	1.105	8.634±0.132	1.529	11.010±0.424	3.851
	4 2-methylbutanoic acid	15.86	878	5.895±0.467	7.921	6.94±0.084	1.210	4.65±0.365	7.849	5.542±0.268	4.835
	5 Octanoic acid	36.49	1180	0.186±0.01	5.376	0.237±0.006	2.531	0.052±0.001	1.923	0.070±0.002	2.857
	6 2-ethylbutanoic acid	37.39		0.668±0.018	2.694	0.138±0.004	2.898	0.227±0.015	6.607	0.173±0.006	3.468
Alcohols	7 3-methylbutanol	6.14	730	0.982±0.042	4.276	1.584±0.104	6.565	1.181±0.008	0.677	1.425±0.022	1.543
	8 2,3-butanediol	8.78	782	345.390±14.933	4.324	18.968±1.735	9.146	8.786±0.775	8.820	7.344±0.525	7.148
	9 2,3-butanediol	9.54	793	39.615±2.380	6.008	12.755±1.25	9.800	8.657±0.788	9.102	7.856±0.179	2.278
	10 2-heptanol	17.86	902	0.57±0.034	5.964	1.592±0.127	7.977	1.204±0.091	7.558	2.177±0.007	0.321
	11 2-ethylhexanol	29.05	1039	0.976±0.065	6.659	N/D	-	N/D	-	N/D	-
	12 Phenylmethanol	29.34	1042	1.168±0.092	7.876	N/D	-	N/D	-	N/D	-
	13 1-phenylethanol	31.89	1072	N/D	-	0.236±0.022	9.322	N/D	-	N/D	-
	14 β-linalool (3,7-dimethyl-1,6-dien-3-ol)	33.99	1100	1.301±0.056	4.304	1.388±0.07	5.043	1.537±0.068	4.424	1.319±0.049	3.714
	15 2-nonanol	34.07	1101	0.415±0.039	9.397	0.376±0.035	9.308	0.323±0.013	4.024	0.257±0.006	2.334
	16 2-phenylethanol	34.38	1113	13.71±0.23	1.677	5.605±0.099	1.766	12.5±0.26	2.080	9.166±0.310	3.382
	17 2,6-octadien-1-ol-3,7-dimethyl	37.54		0.238±0.001	0.420	N/D	-	N/D	-	N/D	-
Aldehydes	18 3-methylbutanal	3.02	639	3.498±0.166	4.745	1.422±0.038	2.672	4.203±0.302	7.185	4.179±0.162	3.876
	19 2-methylbutanal	3.25	645	2.603±0.102	3.918	1.261±0.096	7.613	2.361±0.0394	1.538	2.334±0.073	3.127
	20 Pentanal	10.00		N/D	-	N/D	-	0.932±0.019	2.038	1.472±0.104	7.065
	21 Benzaldehyde	21.60	945	10.083±0.473	4.691	3.827±0.252	6.584	4.413±0.049	1.110	4.291±0.158	3.682
	22 Phenylacetaldehyde	30.05	1051	3.24±0.211	6.512	1.762±0.153	8.683	4.199±0.168	4.009	3.304±0.050	1.513
	23 Nonanal	34.14	1102	0.941±0.011	1.168	0.494±0.048	9.716	0.556±0.007	1.258	0.597±0.030	5.025
	24 Decanaldehyde	37.07	1200	0.594±0.009	1.515	0.443±0.001	0.225	0.364±0.028	7.777	0.264±0.001	0.378
	25 3,7-dimethyl-2,6-octadienal (Cis-Citral)	37.81	1221	0.282±0.017	6.028	0.526±0.045	8.555	N/D	-	N/D	-
	26 3,7-dimethyl-2,6-octadienal (Trans-Citral)	38.39	1240	0.400±0.009	2.250	0.818±0.072	8.801	N/D	-	N/D	-
	27 2-Phenyl-2-butenal	38.43	1270	1.137±0.053	4.661	0.192±0.006	3.125	0.439±0.027	6.150	0.381±0.003	0.787
Ketones	28 4-methyl-2-phenyl-2-pentenal	40.31	1383	0.8±0.017	2.125	0.318±0.015	4.716	N/D	-	N/D	-
	29 5-methyl-2-phenyl-2-hexenal	42.45	1483	5.72±0.056	0.978	5.404±0.505	9.344	3.074±0.023	0.748	2.706±0.036	1.330
	30 3-hydroxy-2-butanone	4.84	707	0.844±0.053	6.279	4.476±0.158	3.529	2.211±0.175	7.914	2.415±0.126	5.217
	31 2-heptanone	17.03	890	0.056±0.001	1.785	0.177±0.006	3.389	0.186±0.017	9.139	0.448±0.011	2.455
	32 3-methyl-2-cyclohexenone	31.16		0.292±0.002	0.684	0.106±0.005	4.716	0.228±0.007	3.070	0.398±0.009	2.261
	33 1-Phenylethanone	31.94	1075	N/D	-	0.427±0.02	4.683	0.611±0.001	0.163	0.727±0.028	3.851
	34 2-nonanone	33.67	1095	1.999±0.046	2.301	0.363±0.029	7.988	0.442±0.032	7.239	0.624±0.005	0.801
	35 2-pentyl acetate	14.17	859	0.802±0.014	1.745	0.788±0.032	4.060	2.148±0.171	7.960	2.849±0.114	4.001
Esters	36 3-methylbutyl acetate	16.32	880	3.78±0.108	3.105	3.53±0.118	3.342	2.645±0.195	7.372	2.967±0.202	6.808
	37 2-methylbutyl acetate	16.39	882	N/D	-	1.239±0.073	5.891	1.001±0.040	3.996	1.422±0.086	6.044
	38 3-methyl-2-butyl acetate	17.46		N/D	-	4.656±0.247	5.304	1.215±0.074	6.092	2.093±0.034	1.624
	39 Ethyl acetate	19.91		104.367±4.287	4.108	12.952±0.822	6.346	8.374±0.22	2.627	7.011±0.277	3.950
	40 1-methylpropyl acetate	30.75		0.057±0.003	5.263	0.938±0.081	8.635	0.42±0.03	7.142	0.593±0.003	0.505
	41 Isobutyl-isobutyrate (2-methylpropyl-2-methylpropanoate)	31.90		37.94±0.48	1.265	N/D	-	N/D	-	N/D	-
	42 2,3-butanediol diacetate	32.31	1080	1.066±0.072	6.754	1.257±0.099	7.875	0.97±0.037	3.814	1.413±0.013	0.920
	43 2,3-butanediol diacetate	33.10		1.142±0.034	2.977	1.052±0.028	2.661	0.344±0.029	8.430	0.488±0.011	2.254
	44 Benzyl acetate	36.03	1165	1.943±0.019	0.977	1.203±0.085	7.065	N/D	-	N/D	-
	45 Ethyl benzoate	36.18	1170	N/D	-	0.975±0.08	8.205	1.751±0.059	3.369	2.339±0.014	0.598
	46 Ethyl octanoate	36.88	1196	2.578±0.087	3.374	3.756±0.112	2.982	4.411±0.151	3.423	6.889±0.293	4.253
	47 Ethyl-2-phenylethanoate	37.89	1224	1.921±0.082	4.268	4.074±0.183	4.491	2.155±0.005	0.232	2.827±0.025	0.884
	48 2-phenylethyl acetate	38.12	1231	32.435±0.798	2.460	10.084±0.594	3.907	29.336±2.633	8.975	27.065±1.338	4.943
	49 Ethyl decanoate	40.59	1395	N/D	-	N/D	-	1.582±0.111	7.016	1.869±0.023	1.230
50 Isoamylbenzoate	40.63	1430	3.037±0.012	0.395	3.232±0.033	1.021	2.32±0.196	8.448	2.812±0.047	1.671	
51 Ethyl-3-phenyl-2-propenoate	42.04	1462	0.972±0.010	1.028	0.432±0.015	3.472	N/D	-	N/D	-	
Pyrazines	52 2-methyl-pyrazine	12.14	826	0.435±0.007	1.609	0.267±0.019	7.116	N/D	-	N/D	-
	53 2,5-dimethylpyrazine	18.61	913	0.657±0.03	4.566	0.22±0.002	0.909	0.423±0.015	3.546	0.366±0.005	1.366
	54 2,3-dimethylpyrazine	18.93	919	0.399±0.009	2.255	0.062±0.005	8.064	0.141±0.012	8.510	0.077±0.001	1.298
	55 2-ethyl-6-methylpyrazine	25.32	992	0.402±0.014	3.482	N/D	-	N/D	-	N/D	-
	56 2,3,5-trimethylpyrazine	25.60	1005	2.358±0.008	0.339	0.997±0.006	0.601	1.24±0.01	0.806	0.975±0.023	2.358
	57 2-ethyl-6-methylpyrazine	27.04	1016	1.075±0.015	1.395	N/D	-	0.265±0.019	7.169	0.281±0.017	6.049
	58 2-ethyl-2,5-dimethylpyrazine	32.83	1083	0.14±0.01	7.142	N/D	-	0.057±0.004	7.017	0.053±0.004	7.547

Monoterpenes	59	2,3,5,6-tetramethylpyrazine	33.32	1090	30.826±0.348	1.128	7.53±0.227	3.014	8.709±0.269	3.088	7.390±0.371	5.020
	60	2-ethyl-3,5,6-trimethylpyrazine	35.96	1163	0.611±0.037	6.055	N/D	-	N/D	-	N/D	-
	61	2,3,5-trimethyl-6-propylpyrazine	36.64		0.093±0.003	3.225	N/D	-	N/D	-	N/D	-
	62	β -pinene(6,6-Dimethyl-2-methylene bicyclo [3.1.1] heptane)	22.63	963	0.56±0.039	6.964	0.435±0.003	0.689	N/D	-	N/D	-
	63	β -myrcene (1,6-Octadiene-7-methyl-3-methylene)	24.48	983	0.562±0.041	7.295	0.418±0.002	0.478	0.584±0.035	5.993	0.525±0.008	1.523
	64	α -limonene (1-methyl-4- (1-methyl-ethyl) -cyclohexene)	28.02	1028	13.933±1.103	7.916	9.861±0.539	5.465	6.808±0.343	5.038	6.237±0.388	6.220
Others	65	γ -Terpinene (1-methyl-4- (1-methyl-ethyl) -1,4-cyclohexadiene)	31.49	1064	3.848±0.319	8.290	1.679±0.142	8.457	1.787±0.084	4.700	1.058±0.022	2.079
	66	4-carvomenthenol (4-methyl-1- (1-methylethyl) -3-cyclohexenol)	36.32	1177	0.661±0.015	2.269	0.161±0.008	4.968	N/D	-	N/D	-
	67	2-acetyl-furan	9.67	802	N/D	-	N/D	-	0.336±0.024	7.142	0.488±0.015	3.073
	68	2 (3H) -dihydro furanone	18.50	910	N/D	-	0.12±0.009	7.500	0.26±0.022	8.461	0.244±0.015	6.147
	69	1,2,3-trioxane-2,4,6-trimethyl	20.38		2.852±0.117	4.102	5.01±0.361	7.205	1.829±0.105	5.740	2.443±0.133	5.444
	70	Benzonitrile	23.66	979	0.498±0.003	0.602	0.456±0.025	5.482	N/D	-	N/D	-
	71	1- (1h-pyrrol-2-yl) ethanone (2-acetilpirrol)	32.06	1076	0.885±0.047	5.310	0.366±0.014	3.825	0.54±0.053	9.814	0.543±0.034	6.261
	72	6-methyl-3,5-dihydroxy-2,3-dihydro-4h-pyran-4-one	35.40	1149	1.419±0.107	7.540	1.418±0.053	3.737	1.156±0.087	7.525	1.006±0.010	0.994
	73	6-ethenyl-2,2,6-trimethylloxan-3-o	36.30	1173	0.811±0.022	2.712	0.3±0.008	2.666	0.510±0.018	3.529	0.389±0.007	1.799
	74	2-furancarboxaldehyde	12.71	835	1.234±0.044	3.565	0.476±0.023	4.831	N/D	-	N/D	-

^aConcentrations in mg/kg, ^bCompared with literature, N/D Not determined

Ten pyrazines were identified in the four samples of processed cocoa liquor, among which 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine and 2,3,4,6-tetramethylpyrazine are widely found in roasted cocoa liquor offering aromas of nuts, roasted and dried fruits [6], [5]. Table 3 shows that there is an increase in the concentration of these compounds when BF grains are chosen. In the TCS 01BF sample, the concentration is increased by 4 times compared to the AZ sample, and the ICS95BF sample is increased by 1.2 times. In the same way, [5], they show that for grains with better indications of fermentation, the concentrations of pyrazines increase due to the increase in compounds that develop in the miallard reaction in the roasting stage. In the TCS 01 variety, this type of compound stands out due to the characteristics of tri-cryolated cocoa, which is why a greater quantity of pyrazine compounds is developed, as opposed to the ICS 95 variety, which only develops 6 of the 10 compounds found. For this reason it would be expected that this genotype has a greater richness in compounds with characteristics of fine and aroma.

Some monoterpenes were identified in the samples as beta-pinene, beta-myrcene, alpha-limonene, gamma-Terpene and 4-carvomentenol, with floral and herbal sensory characteristics (PubChem, MSDS, Kadow). Like pyrazines, these compounds increase their concentration when BF grains are chosen (56% TCS01BF and

18% ICS95BF). Compounds such as beta-pinene and 4-carvomentenol only develop in the TCS01 variety, in concentrations of 0.560 mg/kg and 0.661 mg/kg increasing 4 and 1.2 times their concentration respectively, while α -limonene obtained a concentration of 13.933 mg/kg, being the monoterpene with the highest concentration. Other families of compounds identified are furanes, oxanes, pyrroles and pyrones. However, few compounds from these functional groups are found in the samples and, moreover, in low concentrations. For example, the 2-furancarboxaldehyde that offers aroma to candy, almonds [10], Pubchem, and obtained a concentration of 1.234 mg/kg increasing 2.5 times its concentration with respect to the AZ sample and the benzonitrile that offers aromas to almonds [10], Pubchem, which maintained its concentration between BF and AZ grains; these two were only found in the variety TCS 01. The 2-acetylpyrrole that offers aromas of chocolate, hazelnut 6-methyl-3,5-dihydroxy-2,3-dihydro-4h-pyran-4-one which is odorless but has the highest concentration of the other compounds which is 1.419 mg/kg in the variety TCS 01-BF. B. and 1,2,3-trioxane-2,4,6-trimethyl with higher concentrations in the random samples as 5.01 mg/kg in TCS 01-AZ and 2.443 mg/kg ICS 95-AZ.

Method validation

LOD & LOQ

A minimum detected concentration of 0.0186 µg/kg was obtained and equations 1 and 2 were used to calculate the LOD and LOQ, which are 0.024 µg/kg and 0.037 µg/kg, respectively, which may offer high sensitivity. Similar limits were found in other investigations using the SPME solid phase micro extraction technique.

Intermediate accuracy

Table 3 shows each of the coefficients of variation (%CV) associated with the determination of concentrations for each of the identified compounds. The results reveal that in none of the cases did the CV value exceed 10%. This result is an indication that the standardized method achieves reliable results, guaranteeing repeatability and consequently good precision. Other authors who have also worked with extraction of volatile compounds by means of SPME from other matrices such as coffee and wines, cheeses, obtaining average %CV values less than 10% for alcohols, ketones, aldehydes, esters and acids similar to those reported in this work.

Accuracy

Table 4 shows the recovery percentages for each of the families of compounds worked on. The percentages obtained range from 86%-94%, with the lowest recovery for acidic compounds and the highest for pyrazines.

Table IV. Percentage of recovery for each of the representative compounds of each family

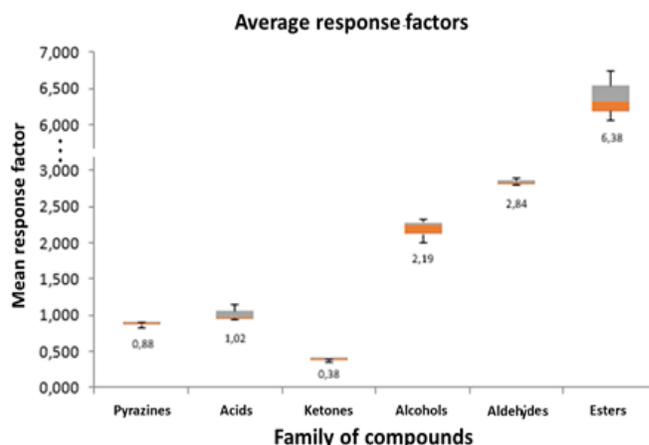
Compound family	Recovery rate (%R)
Acid	86.11
Alcohols	90.74
Aldehydes	91.60
Ketones	91.51
Esters	92.38
Pyrazines	93.40

These percentages show a high recovery capacity of the compost to achieve an accurate quantification [10].

Response factors

Figure 1 shows the mean values of the response factors,

the standard deviation and the coefficients of variation for each of the families of compounds. It can be seen that for the groups of compounds such as pyrazines, esters, ketones, pyrroles and aldehydes the percentages of coefficient of variation were less than 6%. The response factor of the acids slightly exceeded the limit value of 10% CV; this behaviour is associated with the elution difficulties presented by the chromatographic peak for the acid that was selected as the reference compound. Due to this, it is observed that there is repeatability in the determination of the response factors.



Composite Family	Average	DS	% CV
Pyrazines	0.88	0.04	4.61
Esters	6.38	0.35	5.465
Acids	1.02	0.11	11.17
Ketones	0.38	0.02	4.456
Alcohols	2.19	0.17	7.974
Pirroles	1.1	0.02	1.787
Aldehydes	2.84	0.05	1.614
Monoterpenes	ND'	ND'	ND'
Others	ND'	ND'	ND'

*ND= not determined. For the quantification we worked with a response factor equal to 1, following the internal standard approach without family approximation.

Figure 1. Response factors by compound family

Conclusion

Semi-quantitative methods have proven to be a valuable tool for the quantification of volatile compounds. The hybrid technique presented in this paper shows good levels of reliability and reproducibility for the quantification of volatile compounds in cocoa matrices.

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