Matrix effect on quantification of sugars and mannitol developed during the postharvest of cocoa: an alternative method for traceability of aroma precursors by liquid chromatography with an evaporative detector

Maritza Gil^{1,2} · Sandra Llano^{1,2,3} · Yamile Jaramillo^{1,3} · Jairo Quijano⁴ · Julian Londono-Londono⁵

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Abstract The profile of reducing sugars developed during each stage of the postharvest of cocoa beans is an important quality index; these sugars are found to be one of the main precursors of flavor and neoformed compounds. However, its quantification after extraction from different parts of the bean is a challenge due to the complexity of the matrix. The primary aim of this study was to track the formation of reducing sugars and mannitol in samples obtained from various steps of the fermentation and drying processes of cocoa beans by validating a rapid matrix-corrected chromatographic method utilizing a corona-charged aerosol detector for improved sensitivity. The analytes were

extracted from ground cocoa beans by solid phase extraction without a defatting step (20 mg raw fermented and 10 mg dried). The experimental variables influencing the effective detection were evaluated at different temperatures and signal filtering levels. Method validation studies showed an average recovery between 77.8 and 120% for fermented cocoa and between 79.6 and 117.7% for dried cocoa. A linear response was achieved for fructose, glucose, sucrose, and mannitol for a concentration range of 0.1–40 mg/L, and maltose showed linearity in the range of 0.1–70 mg/L. Regression coefficients (R) were 0.9991, 0.9993, 0.9992, 0.9995 and 0.9994, respectively. This method was successfully applied to a clone mix of cocoa from Antioquia, Colombia, to confirm the hydrolysis reaction of sucrose into glucose and fructose during fermentation and drying. A quality indicator of an efficient postharvest process in this study was determined to be a glucose percentage of 0.66% w/w and a fructose percentage of 1.46% w/w, which were higher than the values reported by other studies.

✉ Maritza Gil
maritzagil@itm.edu.co; magil@unal.edu.co;
maritzagil@itm.edu.co

Sandra Llano
llanogils@gmail.com

Yamile Jaramillo
yamile.garces@gmail.com

Jairo Quijano
jquijano@unal.edu.co

Julian Londono-Londono
jalondo@gmail.com

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Introduction

Consumption of chocolate has become a trend with considerable growth in the world, creating an increased challenge for cocoa producers. Consumers have a demand oriented toward products with differentiated profiles of aroma and flavor (International Cocoa Organization 2014) (e.g., fruity, floral, nutty, and herbal profiles, among others) (Frauendorfer and Schieberle 2006, 2008; Rodriguez-Campos et al. 2011), with a presence of bioactive

- ¹ Grupo de investigación de Ingeniería de Alimentos (GRIAL), Corporación Universitaria Lasallista, Caldas, Antioquia, Colombia
- ² Instituto Tecnológico Metropolitano, Medellín, Antioquia, Colombia
- ³ Food and Nutrition Innovation, Corporación Universitaria Lasallista, Caldas, Antioquia, Colombia
- ⁴ Universidad Nacional de Colombia, Medellín, Antioquia, Colombia
- ⁵ Sosteligroup S.A.S, Rionegro, Antioquia, Colombia

compounds, such as antioxidants (Counet et al. 2004; de Oliveira et al. 2015; Patras et al. 2014; Ioannone et al. 2015; Sarriá et al. 2015), and products that do not contain toxic compounds (e.g., acrylamide, carboxymethyl lysine, and hydroxymethylfurfural) (Niquet-Léridon and Tessier 2011).

One of the most significant challenges, both for cocoa producers and for the industry in charge of its transformation, is the quality control measures that are currently in place for this raw material during cocoa postharvest (Kongor et al. 2016), which, in most cases, are very emergent. In recent years, studies have increased focus on the importance of traceability of flavor precursors obtained during fermentation only. Examples of these precursors include reducing sugars and amino acids, which initiate their interaction during the drying of cocoa shells to form a series of compounds responsible for the aroma and flavor of the product that later will be fully developed during industrialization stages (Crafack et al. 2014; Krämer et al. 2015; Teye et al. 2015; Vázquez-Ovando et al. 2015; Kongor et al. 2016).

The implementation of robust and efficient methods is required to follow precursors such as reducing sugars and, in this way, implement the incorporation of this practice in the quality control protocols (Araujo et al. 2014). One of the greatest difficulties in using these methods for the analysis of cacao is the complexity of the matrix, which persists from the obtained raw beans that initiate fermentation until the end of the process. The matrix consists of cotyledons surrounded by a mucilaginous layer accompanied by fat and proteins macromolecules, and it is supplemented with organic acids, alkaloids, and polyphenols, among others. The matrix undergoes drastic changes from beginning to end. Similarly, the same change in matrix occurs when the raw beans obtained are well-fermented and are subjected to posterior drying. The bean is hardened at the shell surrounding the cotyledon and darkened at the beginning of the formation of compounds as a result of the Maillard reaction (Saltini et al. 2013).

The above methods of extraction are used for the elimination of interferents, such as ethereal extract, proteins, and polyphenols, among others. These methods of extraction permit the stability and recovery of different essential reducing sugars, such as glucose and fructose, complemented by other sugars present in lower amounts, such as maltose.

According to current procedures used in the extraction of sugars from cocoa beans, these methods do not differentiate between each part or process state of the bean used for the extraction (mucilage or raw or dry grain), despite the fact that these different sources have different matrix compositions that can trigger a matrix effect of greater complexity in the subsequent quantification step (Magalhães et al. 2013; Schwan 2015).

The reported methods are based mainly on the use of precipitation by centrifugation followed by filtration from an aqueous extract or are exposed to a redox reaction in the presence of dinitrosalicylic acid (DNS) prior to centrifugation. However, the latter treatment is not recommended for cocoa since it is highly colored and is specific only for reducing sugars (Lacerda Ramos et al. 2014; Sandhya et al. 2016; Bello Gil et al. 2006).

In the same way, methods reported for the analysis of sugars in cocoa do not contain information on differentiation of the treatment of the external standards used for the quantification of reducing sugars. The effect of the matrices can influence the validation of obtained results, and even though the results could be correct, they may not be accurate or precise.

The treatment of external standards used for the quantification of reducing sugars should be prepared using different matrices to keep their specificity for the sample in order to minimize the matrix effect and obtain results that are correct, accurate and precise. The cocoa bean's profile will allow for the traceability of the aroma and flavors, which are critical parameters for today's cocoa market.

Finally, the technology used for the detection of reducing sugars and their precursors in cocoa, as well as other compounds resulting from the biochemical changes that they can be subjected to, make the separation of these compounds difficult through a traditional system, such as liquid chromatography coupled with a refraction index detector (RID). These extraction methods have been used to quantify sugars not only in cocoa but also in several other food matrices. Additionally, these methods have some disadvantages, such as a lack of sensitivity depending on temperature and flow rate and an incompatibility with gradient elution (Ma et al. 2014). The RID is also quite susceptible to interference from coeluting sample components (Calull et al. 1992). Other alternative methods have been developed for the identification of sugars, such as detectors of universal response, including the evaporative light scattering detector (ELSD), which is used to determine carbohydrates, but their efficiency can be dramatically reduced with particle size and analyte concentration, limiting signals, and sensitivity (Magnusson et al. 2015).

The corona-charged aerosol detector (C-CAD) was introduced in 2004 by ESA Biosciences (Chelmsford, MA) as a quasi-universal detection system for HPLC. The operating principle of C-CAD is based on the detection of charged particles with a selected range of mobility, eliminating interferences due to physical–chemical properties of the compounds (Márquez Sillero 2014).

The aim of this work was to validate a simultaneous method for the quantification of soluble sugars (glucose, fructose, sucrose and maltose) and mannitol present in raw cocoa beans for the monitoring of aroma and flavor

precursors during certain postharvest stages. The sources of cocoa for this experiment were obtained from the harvest and during fermentation, as well as for dry fermented cocoa, by means of UHPLC-C-CAD.

Materials and methods

Chemicals

HPLC-grade acetonitrile (Merck), analytical grade fructose, glucose, sucrose, and maltose (all contents > 99%) were purchased from Sigma-Aldrich (North Harrison, USA). Mannitol was obtained from the European Pharmacopoeia Reference Standard. Ultrapure water (MilliQ) was used in all experiments.

Cocoa fruits

Fresh fruit material was provided by the Federación Nacional de Cacaoteros de Colombia – Fedecacao from the harvest of May 2017 in Urabá, Antioquia, Colombia. A mixture of Collection Castro Naranjal (CCN-51), Imperial Clone Selection (ICS-1), Fedecacao El Carmen, Santander (FEC-2), and Fedecacao Lebrija, Santander (FLE-2) clones was used in the experiments.

Fermentation and drying process

A mixture of the clones FEC-2 (7.9%), FLE-2 (3.2%), ICS-1 (3.0%), and CCN-51 (85.9%) was fermented. The fermentation process was conducted as follows: 1 day after harvest, in the region of Tulenapa (a research farm owned by Universidad de Antioquia, Urabá, Antioquia), the mixture was placed in three boxes of Oakwood (109.7 g/each box) covered with Bijao leaves and Jute sacks in order to promote anaerobic conditions for the first 48 h. Subsequently, each experimental unit was aerated with a wooden stick every 24 h for 132 h.

For the solar drying of the beans collected every day during the fermentation process, the mixture was scattered in a monolayer on a marquee and turned every 6 h during the first day until reaching a humidity between 5 and 7%.

Sampling and pretreatment

Every 24 h during the fermentation, a sample of 600 g was taken at three different heights within each box. Of each sample, 100 g was stored at -18 ± 2 °C. Samples of dry well-fermented cocoa beans (500 g) were collected daily from the marquee at 5 p.m. until reaching 132 h. The cocoa nibs were vacuum packed and refrigerated at 8 °C.

The nibs obtained during fermentation and drying were husked and ground (Hamilton Beach, Model No. 80370, USA) to reduce them to a size between 600 and 420 µm (Mesh 40). The powders were packed in airtight polypropylene containers for later analysis.

Solid-phase extraction (SPE) of sugars and mannitol

The treatment of the samples started with extraction assisted by ultrasound. First, 20 mg of the cocoa nibs collected during fermentation (moisture > 20%) or 10 mg of the samples obtained during drying (moisture < 20%) were diluted in 600 µL of MilliQ H₂O in a 2 mL microcentrifuge tube. Second, after dilution, the suspension was mixed for 5 min × 3000 rpm. Third, each microcentrifuge tube was placed in an ultrasound bath (15 min, 25 Hz, 25 °C, 99%), followed by centrifugation at 10,000 rpm for 15 min.

Solid-phase extraction (SPE) cartridges were used to clean up the samples before UHPLC-C-CAD analysis (UltiMate™ 3000 Dionex, Thermo Scientific, Sunnyvale, CA, USA). A total of 80 µL of supernatant was passed through a cartridge (Sep-Pak C18, 1 cc Vac, 50 mg, Waters, USA) previously activated with 1 mL methanol and 1 mL of MilliQ water. The extracts of sugars and mannitol were eluted with 720 µL of MilliQ water. The eluate was collected as the sample solution for chromatographic separation.

Properties of the cocoa beans in this experiment

pH

A pH measurement at the cotyledons during the fermentation process was determined according to the method reported by Bedoya (2016).

Moisture content

The moisture content ($\text{kg}_{\text{water}}/\text{kg}_{\text{dried solids}}$) was determined by the gravimetric method according to AOAC (931.04). For the determination, 2 g of milled and sieved cotyledons (M_t) were dried in a convection oven (Memmert, model UN55) at 105 ± 0.2 °C until the sample reached a constant weight (M_s). The result was reported as moisture content on a wet basis (Xbh) (Official Methods of Analysis 2012).

Preparation of standard solutions

Stock solutions of fructose, glucose, maltose, sucrose, and mannitol were prepared with MilliQ H₂O at concentrations of 1000 mg/L. Stock solutions were stable for 1 month at 3 °C in refrigeration.

Standard solution in a solvent

The working standard solutions for the calibration curve in a solvent (acetonitrile) were prepared as needed by appropriate dilution of the aqueous concentrated stock solutions. Standard solutions containing 3, 5, 20, 50, 70, 100, 120, and 150 mg/L for each of the sugars (fructose, glucose, maltose, sucrose, and mannitol) were prepared fresh daily.

Standard solution in the matrix

Standard solutions containing 0.1, 0.5, 1, 5, 10, 20, and 30 mg/L of sugars (fructose, glucose, sucrose, and mannitol) and two additional concentrations for maltose (40 and 50 mg/L) were diluted with 200 µL of the matrix reagent solution and brought to 1 mL volume with acetonitrile.

The matrix solutions were obtained from two mixtures. The first mixture consisted of 1 g from each ground sample collected every day during the fermentation, and the second mixture was obtained by combining 1 g grounded samples collected over 5 days from the beginning until the end of the drying process. Both mixtures were submitted to the aforementioned extraction. A microcentrifuge tube was changed to collect the extract liquid after eluting an additional 1 mL of MilliQ through the cartridge. This solution was used as the blank solutions for the fermentation and drying stages.

Chromatography

The chromatographic analysis was performed using an Ultra-HPLC UltiMate 3000 (Thermo Scientific™ Dionex™ Corona™ Veo™). Chromatographic separation was carried out with a Shodex Asahipak NH2 P-50 4E column (5 µm, 150 mm × 4.6 mm) at 30 °C, and 10 µL of the sample was defined as the injection volume. The mobile phase composition was acetonitrile/water 75/25 (v/v), and the flow rate was fixed at 1 mL/min for 19 min for each run.

The corona-charged aerosol detector (C-CAD) was set as follows: the nitrogen generator (Peak Scientific, USA) was selected as the nebulizing gas, a pressure of 60 ± 7 psi was selected (gas purity ≥ 99%), and a collection velocity of 10 Hz was also fixed for all analyses. Three evaporative chamber temperatures (35, 40, and 45 °C) were evaluated using a mix of standards followed by a test of the signal filtering at low, medium and high levels (0.2, 3.6, and 10 s, respectively). The chromatographic data analysis was carried out using Chromeleon 7.0 software.

Method validation

The proposed method was applied to fermented and dried samples of cocoa. All samples were analyzed following the validation data sets carried out according to the European SANTE/11975/2015 guidelines (European Commission Directorate-General for Health and Food Safety 2015). Specificity or selectivity, matrix effect, linearity, accuracy and precision, working range, limit of detection (LOD), and limit of quantification (LOQ) were evaluated.

Specificity and selectivity

Specificity was evaluated by performing a blank injection consisting of only sample diluent and injecting a “clean” sample not spiked with the mix of carbohydrates and mannitol. The absence of interferents in the diluent blank and the sample, combined with the precise and accurate recovery of the standard mix from spiked samples (see the section “Accuracy and Precision” below), was the acceptance criteria to demonstrate the specificity of the method.

Matrix effect

The matrix effect was studied through the comparison between calibration curves of the working standard prepared in solvent and in a matrix-matched diluent for both studied matrices of raw and dried cocoa.

To determine the % range of the matrix effect, the calibration curve slopes were input into Eq. 1:

$$\% \text{Matrix effect} = \frac{(\text{curve solvent slope} - \text{curve matrix slope})}{\text{curve solvent slope}} \times 100 \quad (1)$$

Linearity

Linearity of all analytes was assessed using curves generated from concentration levels of 3, 5, 20, 50, 70, 100, 120, and 150 mg/L in a solvent. In the matrix, the concentrations were 0.1, 0.5, 1, 5, 10, 20, 40, 50, and 70 mg/L.

Accuracy and precision

Accuracy was determined as the recovery of fermented and dried samples spiked at 5.0, 15.0 and 30.0 mg/L with working standards. After analyzing six replicates at each concentration, the acceptance criteria of recovery were set from 70% to 120%. The interday precision was evaluated by analyzing six spiked samples at the three concentration levels on different days by two analysts (n = 6). Repeatability was assessed for samples of the mixtures for fermented and dried cocoa matrices described in “Standard

solution in the matrix” section, and the response was expressed in terms of relative standard deviation (RSD). The acceptance criteria were set at $RSD < 20\%$ (European Commission Directorate-General for Health and Food Safety 2015).

Limit of quantification and detection, LOQ–LOD

The LOD and LOQ values were calculated from the calibration curves in the matrices of fermented and dried cocoa as 3 s/slope and 10 s/slope, respectively, where s is the standard deviation of the signal obtained from six independent measurements (International Conference on Harmonisation 2005).

Statistical analysis

Fisher’s test was used to verify interday precision. The LOQ and the difference between retention times during the study of the matrix effect were evaluated via analysis of variance (ANOVA). A comparison of means was carried out to analyze the time of the treatment using Tukey’s test ($p < 0.05$). Analysis was conducted using R statistical software (Version 0.98.1103, GNU, Affero General Public License, available in <https://cran.r-project.org>).

Results and discussion

Optimization of the chromatographic method

The separation of analytes was optimized in part by testing different ratios of the mobile phases: 80/20, 75/25, and 70/30. Of the different ratios evaluated, acetonitrile/water 75/25 (v/v) under isocratic conditions was chosen as the optimal ratio and composition, as it provided the best resolution for the sugars and mannitol (fructose: 3.05; mannitol: 2.69, glucose: 7.03; sucrose: 4.13). This ratio also provided a stable baseline with higher and narrower chromatographic peaks when compared to the other ratios, where sucrose and glucose were not baseline separated. A runtime of 19 min allowed for a reasonable period of time for the forward analysis of the complex matrix obtained during fermentation and drying of cocoa.

The dilution of the samples with acetonitrile was required to obtain a signal. Samples diluted in water had not revealed a chromatographic peak, which is different from other reports on the analysis of soluble sugars in milk samples diluted in water using a mobile phase of acetonitrile/water (70/30) and parameters for detection by C-CAD that were similar to those used in this study (Márquez-Sillero et al. 2013).

Optimization of the detection parameters

Evaporative detection based on the transfer of charge is affected by different controllable parameters, such as nebulization, temperature of the evaporation chamber, nebulizing gas pressure, signal attenuation, and extent of signal filtering (Márquez-Sillero et al. 2013). The parameters chosen to evaluate the influence of the response for the detector were evaporation chamber temperature (35, 40 and 45 °C) and extent of signal filtering (0.1, 3.6 and 10 s). Figure 1 describes differences with respect to the response of the detection from a standard mix at 5.0 mg/mL. There was no significant difference for the separation among each chromatographic peak, but the highest separation of the nearest peaks (glucose and mannitol) was at 35 °C. This is a critical parameter relevant to the study of a complex matrix, as is the case for cocoa. This temperature was chosen as the appropriate temperature for conducting the experiments. The efficiency of the process was then improved by using a mobile phase with a high content of organic solvent (Dionex 2013).

Additionally, the peaks’ sharpness was improved at the highest filter value (10 s) compared with the other evaluated levels; this result corresponded with the aim of the use of a filter, which is used to electronically reduce the noise of the baseline within the chromatographic run. Table 1 also shows an increase in the areas with the higher filter values (3.6 and 10 s). This aspect may be related to the sensitivity of the method to decrease the noise of the baseline, as illustrated in Fig. 1.

In the chromatographic profile, it should be noted that the order and the intensity of the response for each peak varied. This behavior could be due to the capability of the molecules to retain the charge and vary their size. It is well known that the equilibrium between open and closed structures for monosaccharides facilitates the charge state of the functional groups, as was observed when furanose facilitated a charge on the functional groups over the pyranose form (Badui Dergal 2006). Additionally, the same profile showed that the disaccharide sucrose, which was composed of glucose-fructose, eluted before maltose (glucose–glucose) and produced a higher peak intensity and area, independent of the concentration, for either 0.191 or 0.106 pA, respectively.

Validation of the method for the accurate quantification of sugars and polyol

Optimum chromatographic parameters were defined for the method validation and analysis of cocoa samples by the characteristics described below.

Fig. 1 Chromatograms for the analysis of sugars and sugar alcohol standards by UHPLC-CAD. Instrumental conditions: using the mobile phase acetonitrile: water 75:25 (v/v) and a flow of 1 mL/min with a Shodex Asahipak NH2 P-50 4E column at different

evaporation chamber temperatures (**A**) and low, medium and high level of the filter (**B**). The concentration of the analytes was 5.0 mg/mL. The peak designation was (1) fructose, (2) mannitol, (3) glucose, (4) maltose, and (5) sucrose

Specificity and sensitivity

Shodex Asahipak NH2 P-50 4E columns are designed for applications intending to separate monosaccharides (rhamnose, fructose, xylose, arabinose, mannose, glucose, and galactose), disaccharides (sucrose and lactose), some sweeteners, and sugar alcohols. According to Schwan (2015), cocoa beans contain monosaccharides, such as fructose and glucose, and in lower concentrations, rhamnose. Sucrose is the primary sugar for the raw cocoa that is hydrolyzed during fermentation. Polyols, such as mannitol and myo-inositol, could also be present in cocoa shells. For these reasons, all mentioned

analytes were evaluated to discard possible interferences (Schwan 2015). The chromatogram illustrates the absence of interferences during the analysis of blank and spiked samples with the standard solution of the four sugars and mannitol in both fermented and dried cocoa matrices (Fig. 1).

Matrix effect

A possible effect of the complex matrix to quantify sugars under the proposed extraction and chromatographic methods was studied for both matrices (fermented and dried cocoa). The results of the percentage matrix effect are described in Table 2.

Table 1 Response of the delta of retention time (DRT) according to temperature variation (35, 40 and 45 °C) and area for the different filter values (0.1, 3.6 and 10 s) for fructose, glucose, sucrose, maltose, and mannitol

Temperature (°C)	Delta of the retention time, DRT (min)				
	Mannitol–fructose	Glucose–mannitol	Sucrose–glucose	Maltose–sucrose	
35	1.10	0.99	3.61	2.86	
40	1.11	0.98	3.60	2.85	
45	1.13	0.94	3.58	2.83	
Filter (s)	Area				
	Fructose	Mannitol	Glucose	Sucrose	Maltose
0.2	0.20	0.27	0.16	0.31	0.20
3.6	0.27	0.29	0.23	0.33	0.20
10	0.26	0.26	0.22	0.32	0.20

Maximum DRT reached values are highlighted in bold

The established range to determine the matrix effect comprises values of $< -15\%$ and $> 15\%$. According to this range, affected sugars were sucrose in the dried cocoa matrix (18.2%) and maltose in the fermented cocoa matrix (15.8%).

Although the rest of the sugars and mannitol had not presented values within a range of matrix effects, the chromatographic peaks were displaced, and the use of a calibration curve prepared in the matrix corrected this effect and allowed the correct quantification of sugars (see Fig. 2).

There were no significant differences between the retention times of the compounds in solvent when compared to the retention times of the compounds in the fermentation matrix, but there was a displacement during the analysis of soluble sugars and polyol in the presence of the dried matrix.

Linearity, LOD, and LOQ

The linear behavior of standard solutions containing fructose, glucose, sucrose, maltose, and mannitol in solvent had regression coefficients of 0.9991, 0.9993, 0.9992, 0.9995, and 0.9994, respectively. The variation in the matrices of fermented and dried cocoa is displayed in Table 2. The LOD and LOQ values for fructose, glucose, and sucrose in dried cocoa were greater than those in fermented cocoa. These results were opposite the limit of detection and quantification results for maltose and mannitol.

Accuracy and precision

The recovery (accuracy) in both matrices of cocoa spiked with a standard mix of carbohydrates, and the polyol values at low (5 mg/L), intermediate (15 mg/L), and high (30 mg/L) concentrations levels were determined. Recoveries and the ranges of the four sugars and mannitol for fermented

cocoa (77.8–120%) and dried cocoa (79.6–117.7%) are summarized in Table 2. All recoveries fell within the established range between 70.0% and 120.0%.

Precision was determined by preparing a set of six samples spiked at the three concentration levels by the leading analyst.

Intermediate precision was evaluated by preparing a second set of six samples spiked at the three concentration levels by a second analyst on a different day. The results did not present significant differences according to Fisher's test since the tabulated F-value for a confidence level of 95% and $n = 6$ was 3.11, which was higher than all calculated F-values from the ratio of the variance between analysts and different days (Table 3) (María et al. 2001).

The repeatability of the injections ($n = 6$) kept an RSD $< 10\%$; thus, for the fermented matrix, the values were fructose 2.67%, glucose 3.76%, sucrose 2.14%, maltose 8.79%, and mannitol 1.88%. For dried cocoa, the matrix values were as follows: fructose 6.76%, glucose 6.31%, sucrose 9.81%, maltose 2.53%, and mannitol 1.18%.

Application of the UHPLC-C-CAD assay to the quantification of sugars and polyol in cocoa

A mixture of the clones FEC-2, FLE-2, ICS-1 and CCN-51 was fermented and dried by the conventional solar drying system and was analyzed according to the changes in the profile of soluble sugars and mannitol in the cotyledons. The process was performed every 24 h during fermentation, followed by solar drying until the shells reached a humidity below 7%, as shown in Fig. 3.

This monitoring was established as a method to verify the relationship between days of fermentation and the formation of reducing sugars. The result of this analysis can be considered as one of the quality indexes of postharvest because these components act as precursors in

Table 2 Linear equation, correlation coefficient, method LOD, and recovery of the developed method

	Matrix	Slope	Percent of matrix effect	Linear equation	Linear range (mg/L)	R ²	Method LOD (% w/w)	Method LOQ (% w/w)	Recovery (%)
Fructose	Solvent	0.0452		$y = 0.0452x + 0.1261$	3–150	0.9983			
	Fermented cocoa	0.0424	6.2	$y = 0.0424x - 0.0006$	0.1–40	0.9983	0.06	0.21	106.4–117.0
	Dried cocoa	0.0431	4.6	$y = 0.0431x + 0.0138$	0.1–40	0.9963	0.13	0.42	111.8–117.7
Glucose	Solvent	0.0383		$y = 0.0383x + 0.0964$	3–150	0.9986			
	Fermented cocoa	0.0424	– 10.7	$y = 0.0424x + 0.015$	0.1–40	0.9973	0.15	0.50	77.8–103.4
	Dried cocoa	0.0432	– 12.8	$y = 0.0432x + 0.0101$	0.1–40	0.9966	0.19	0.63	79.6–104.0
Sucrose	Solvent	0.0534		$y = 0.0534x + 0.1516$	3–150	0.9984			
	Fermented cocoa	0.0457	14.4	$y = 0.0457x + 0.0111$	0.1–40	0.9975	0.12	0.39	110.2–120.0
	Dried cocoa	0.0437	18.2	$y = 0.0437x + 0.0288$	0.1–40	0.9968	0.12	0.41	84.8–111.2
Maltose	Solvent	0.0373		$y = 0.0373x + 0.0444$	3–150	0.9991			
	Fermented cocoa	0.0314	15.8	$y = 0.0314x + 0.0188$	0.1–70	0.9956	0.09	0.29	102.0–118.1
	Dried cocoa	0.0347	7.0	$y = 0.0347x - 0.0396$	0.1–70	0.9976	0.04	0.13	91.0–119.7
Mannitol	Solvent	0.0515		$y = 0.0515x + 0.0888$	3–150	0.9988			
	Fermented cocoa	0.0473	8.2	$y = 0.0473x - 0.0161$	0.1–40	0.9995	0.11	0.38	84.7–106.4
	Dried cocoa	0.0491	4.7	$y = 0.0491x - 0.0146$	0.1–40	0.9976	0.06	0.18	104.6–110.9

Percent of matrix effect for fructose, glucose, sucrose, maltose, and mannitol in fermented and dried cocoa

Fig. 2 Profile chromatographic of fructose, glucose, sucrose, maltose, and mannitol detected in a solvent (a), fermented matrix (b), and dried cocoa matrix (c) by UHPLC-CAD

Table 3 Average for standard deviation of the low, intermediate, and high concentration levels for fructose, glucose, sucrose, maltose, and mannitol, measured by two analysts in different days (intermediate precision)

Fermented cocoa	Analist 1														
	Fructose			Glucose			Sucrose			Maltose			Mannitol		
	Mean (% w/w)	STD	RSD	Mean	STD	RSD	Mean	STD	RSD	Mean	STD	RSD	Mean	STD	RSD
<i>Level</i>															
Low	0.43	0.05	0.11	0.32	0.05	0.16	0.43	0.02	0.05	0.49	0.05	0.10	0.87	0.09	0.10
Intermediate	1.17	0.07	0.06	1.09	0.11	0.10	1.27	0.09	0.07	1.07	0.15	0.14	1.11	0.09	0.08
High	2.76	0.20	0.07	2.73	0.25	0.09	2.17	0.14	0.06	2.51	0.17	0.07	1.97	0.14	0.07
Average	1.45	0.10	0.08	1.38	0.13	0.12	1.29	0.09	0.06	1.36	0.12	0.10	1.31	0.11	0.09
Variance		0.01			0.02			0.01			0.02			0.01	
Fermented cocoa	Analist 2														
	Fructose			Glucose			Sucrose			Maltose			Mannitol		
	Mean (% w/w)	STD	RSD	Mean	STD	RSD	Mean	STD	RSD	Mean	STD	RSD	Mean	STD	RSD
<i>Level</i>															
Low	0.53	0.03	0.07	0.98	0.06	0.06	0.46	0.03	0.07	0.49	0.05	0.11	0.70	0.10	0.14
Intermediate	1.33	0.07	0.05	0.98	0.10	0.10	1.35	0.08	0.06	1.28	0.09	0.07	1.26	0.06	0.05
High	3.30	0.12	0.04	3.16	0.16	0.05	2.56	0.12	0.05	3.12	0.16	0.05	2.51	0.13	0.05
Average	1.72	0.08	0.05	1.71	0.11	0.07	1.46	0.08	0.06	1.63	0.10	0.08	1.49	0.10	0.08
Variance		0.01			0.01			0.01			0.01			0.01	
$F_{\text{calculate}} = VA_1/VA_2$		1.94			1.60			1.19			1.49			1.20	
Dried Cocoa	Analist 1														
	Fructose			Glucose			Sucrose			Maltose			Mannitol		
	Mean (% w/w)	STD	RSD	Mean	STD	RSD	Mean	STD	RSD	Mean	STD	RSD	Mean	STD	RSD
<i>Level</i>															
Low	1.14	0.02	0.02	0.87	0.01	0.01	0.66	0.01	0.02	0.84	0.02	0.02	1.15	0.01	0.01
Intermediate	2.26	0.03	0.01	1.70	0.04	0.02	2.11	0.02	0.01	1.33	0.01	0.01	1.94	0.04	0.02
High	3.27	0.03	0.01	2.77	0.06	0.02	2.97	0.06	0.02	2.90	0.05	0.02	2.79	0.06	0.02
Average	2.22	0.02	0.01	1.78	0.03	0.02	1.91	0.03	0.02	1.69	0.03	0.02	1.96	0.03	0.02
Variance		0.001			0.001			0.001			0.001			0.001	
Dried Cocoa	Analist 2														
	Fructose			Glucose			Sucrose			Maltose			Mannitol		
	Mean (% w/w)	STD	RSD	Mean	STD	RSD	Mean	STD	RSD	Mean	STD	RSD	Mean	STD	RSD
<i>Level</i>															
Low	1.26	0.02	0.02	0.85	0.01	0.02	0.74	0.01	0.01	0.77	0.01	0.02	1.10	0.01	0.01
Intermediate	2.35	0.02	0.01	1.68	0.02	0.01	1.99	0.01	0.00	1.64	0.01	0.01	2.10	0.03	0.01
High	3.78	0.08	0.02	2.97	0.15	0.05	3.20	0.18	0.06	3.08	0.33	0.11	3.24	0.14	0.04
Average	2.46	0.04	0.02	1.83	0.06	0.03	1.98	0.07	0.03	1.83	0.12	0.05	2.14	0.06	0.02
Variance		0.002			0.004			0.005			0.014			0.003	
$F_{\text{calculate}} = VA_1/VA_2$		0.38			0.30			0.22			0.05			0.38	

SD standard deviation, RSD relative standard deviation (coefficient of variation), VA variance

the formation of volatile compounds that are important in the following stages of industrialization.

Araujo et al. (2014) established minimum limits for the content of glucose ($> 0.085\%$ w/w) and fructose ($> 0.259\%$ w/w) in the well-fermented and dry cotyledons of interest for the development of flavor in the chocolate industry (Araujo et al. 2014), which confirmed the effectiveness of the postharvest process applied in this study (fructose: $1.71 \pm 0.238\%$ w/w and glucose: $0.66 \pm 0.19\%$ w/w).

With the purpose of monitoring the concentration of sugars mentioned as a quality control method for reaching the recommended levels, it is necessary to analyze the hydrolysis of the main disaccharides present in cocoa (sucrose and maltose) in parallel with the development of reducing sugars and possible alternative products (mannitol); this last factor was caused by heterofermentative reactions during fermentation (Schwan 2015).

The traceability of each compound was verified from the unfermented raw cotyledons at the zero moment of fermentation (0-URC). This same sample was subjected to drying, simulating the fact that the producers do not carry out the fermentation step and dry it immediately (0-UDC). Unfermented and raw cotyledons showed a greater concentration of sucrose (6.8% w/w), and this value remained constant. This result of no changes could be considered a quality indicator because it allows for the confirmation that the fermentation was not initiated or that hydrolysis of this disaccharide to produce reducing sugars did not occur as expected during the fermentation. The opposite behavior was observed as the fermentation progressed because the sucrose decreased up to 94.8% due to the hydrolysis

produced by the increase in the acidity of the medium ($\text{pH}_{0\text{h}}: 6.2 \pm 0.23$; $\text{pH}_{48\text{h}}: 4.8 \pm 0.06$). After 48 h of fermentation, the decrease in sucrose was up to 49.1%, which was probably due to the action of LAB under the micro-anaerobic conditions of this period, generating an equimolar formation of fructose and glucose (0.79% w/w), which was two key precursors of aroma and flavor (Schwan 2015).

After the aeration of beans during fermentation started and the pH continued to change ($\text{pH}_{72\text{h}}: 4.7 \pm 0.06$) as a consequence of acetic acid bacteria (AAB) and LAB, the aerobic conditions and exothermic reactions caused a gradual increase in temperature. This led to a rise in the rate of sucrose hydrolysis up to 75.5% at 72 h. However, there is evidence of uneven formation at a ratio of 2.6:1.2 for both fructose and glucose. This possibly happens due to the increased reactivity of glucose in the form of aldose in the presence of free amino acids and the high recorded temperatures during the fermentation at 72 h (42°C) and 120 h ($46.5 \pm 2.5^\circ\text{C}$) and 970 Lux $\times 100$ max (Badui Dergal 2006).

The low concentrations of mannitol present in the unfermented raw cotyledons were expected. At 48 h, the metabolism of fructose into this polyol (0.20% w/w) started as a result of the action of heterofermentative species, such as *L. fermentum* (Lefeber et al. 2011). The amount of mannitol continued to increase until 120 h (0.87% w/w) and then underwent a slight decrease at the end of fermentation. This value was an indicator of quality and can be related to the reduction of acidity when the route of formation of sugar alcohol is promoted over lactic acid

Fig. 3 Profile of soluble sugars extracted from unfermented cotyledon, during fermentation (0–120 h) and well-fermented and dried cotyledon (132 h) determined by UHPLC-CAD

formation by LAB, thereby generating fewer acidic beans (Schwan 2015).

The highest maltose concentration was present in the mucilage (max. 0.91% w/w and min. 0.21% w/w), which was approximately tenfold less than that in sucrose (max. 10.15% w/w and min. 3.47% w/w). The loss during fermentation of the maltose disaccharide present in the mucilage occurred during drainage (together with pectin), migration inside of the cotyledon and the hydrolysis of the O-glycosidic bond between the two glucose molecules (Gil et al. 2016).

Greater sensitivity was observed during sugar analysis performed on the dry cotyledons, which may be due to the handling of the sample and obtaining a more homogenous sample through the sieve. According to previous studies, sugar extraction by SPE required the defatting of cocoa, but it should be noted that for this method, this stage was modified to ensure the recovery of the analytes of interest, mainly the sugars and polyol present at low concentrations (Jinap et al. 2002).

Conclusion

The cocoa matrix has great complexity and requires different treatment that depends on the stage of the bean: raw, during fermentation, dried, or at other stages during industrialization. It should also be considered that the texture, moisture, and diverse profile of compounds at these different stages produce unique matrix effects, as demonstrated in this study. This finding implies the importance of preparing an individual calibration curve for raw and dried cocoa to correct for the displacement of retention time during chromatographic quantification using UHPLC with a corona-charged aerosol detector (C-CAD).

The validation parameters of the analytical procedure were verified with respect to the limits of detection (0.06–0.15% w/w and 0.04–0.19% w/w for each studied analyte in dried and fermented cocoa, respectively). The average recovery was between 77.8 and 120% for fermented cocoa and between 79.6 and 117.7% for dried cocoa. The regression coefficients were 0.9991 for fructose, 0.9993 for glucose, 0.9992 for sucrose, 0.9995 for maltose, and 0.9994 for mannitol. Finally, the reproducibility was tested between two analysts on different days of analysis. This was applied to standards at concentrations ranging between 0.1 and 40 mg/L for all analytes, except for maltose, which ranged from 0.1 to 70 mg/L.

The profile of sugars and mannitol in the studied mixture of crude and dried Colombian cocoa clones under the validated method allowed for the confirmation of this test as an indicator of process quality. This study followed

complete fermentation by hydrolysis of disaccharides into reducing sugars as precursors of aromatic compounds that begin to develop in the drying stage. This also implies a decrease in fructose and glucose, occurring in different proportions according to their reactivities in the Maillard reaction. Therefore, this application could be useful for the traceability of cocoa quality in the future.

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