

ORIGINAL ARTICLE

The effect of fermentation and roasting on free amino acids profile in Criollo cocoa (*Theobroma cacao* L.) grown in Venezuela

Efeitos da fermentação e torrefação no perfil de aminoácidos livres do cacau Criollo (Theobroma cacao L.) cultivado na Venezuela

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Abstract

The development of cocoa flavor and aroma is a complex phenomenon that depends on how the fermentation and roasting processes are carried out. During fermentation, the formation of some compounds so-called "aroma and flavor precursors" takes place, which are finally expressed during the roasting stage. Therefore, the evaluation of aroma precursors such as the amino acids formed during fermentation is crucial in order to determine the quality of the cocoa. In this context, we developed and validated a method for the study of these compounds in cocoa samples. The amino acids were quantitatively converted into their trimethylsilyl derivatives before their determination by gas chromatography with mass spectrometry detection. The results were verified performing precision and accuracy studies. The inter and intra assay coefficients of variation (C.V, n = 5) were lower than 4.7% and 4%, respectively. The analytical recoveries (95% to 108% with C.V < 4.2, n = 5) demonstrated the high performance of the extraction procedure. The method was successfully applied to the analysis of the amino acids in 110 samples of Venezuelan Criollo cocoa during the three days of fermentation and roasting (110 °C for 25 min). All samples had an appreciable content of free amino acids ranging between 3.87 and 5.97 g/kg in the absence of fermentation. We observed degradation of the acidic amino acids during the first day of fermentation, while the rest of amino acids increased progressively during the fermentation process with a predominance of the hydrophobic ones, mainly leucine, phenylalanine, valine, alanine and isoleucine. Additionally, during the roasting stage a fraction of the amino acids, especially the hydrophobic ones, was partially degraded through Maillard reaction to form the compounds associated with the cocoa aroma and flavor.

Keywords: Gas chromatography; Mass spectrometry; Microwave; BSTFA; Aroma precursors; Trimethylsilyl derivatives; Hydrophobic amino acids.



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Resumo

O desenvolvimento do sabor e do aroma do cacau é um fenômeno complexo que depende de como os processos de fermentação e torrefação são realizados. Durante a fermentação, ocorre a formação de alguns compostos chamados "precursores de aroma e sabor", que são finalmente expressos durante a fase de torrefação. Portanto, a avaliação de precursores de aroma, como os aminoácidos formados durante a fermentação, é crucial para determinar neste contexto como o método foi desenvolvido e validado para o estudo desses compostos em amostras de cacau, sendo os aminoácidos convertidos quantitativamente em seus derivados de trimetilsilil antes de serem determinados por cromatografia gasosa com detecção por espectrometria de massa. Os resultados foram verificados por meio de estudos de precisão e acurácia. Os coeficientes de variação inter e intraensaio (CV; n = 5) foram inferiores a 4,7% e 4%, respectivamente. As recuperações analíticas (95% to 108% com CV < 4,2; n = 5) demonstraram alto desempenho do procedimento de extração. O método foi aplicado com sucesso na análise dos aminoácidos em 110 amostras de cacau venezuelano Criollo durante os três dias de fermentação e torrefação (110 °C por 25 min). Todas as amostras estudadas apresentaram um conteúdo apreciável de aminoácidos livres variando entre 3,87 e 5,97 g/kg na ausência de fermentação. A degradação dos aminoácidos ácidos foi observada durante o primeiro dia de fermentação, enquanto os demais aminoácidos aumentaram progressivamente durante o processo de fermentação com predomínio dos hidrofóbicos, principalmente leucina, fenilalanina, valina, alanina e isoleucina. Além disso, durante a fase de torrefação, uma fração dos aminoácidos, especialmente os hidrofóbicos, foi parcialmente degradada pela reação de Maillard para formar os compostos associados ao aroma e ao sabor do cacau.

Palavras-chave: Cromatografia gasosa; Espectrometria de massa; Micro-ondas; BSTFA; Precursores de aromas; Derivados de trimetilsililo; Aminoácidos hidrofóbicos.

1 Introduction

Cocoa was first cultivated in Central and South America. Currently, there are three widely recognized varieties of cocoa: Forastero, Criollo and Trinitario (Afoakwa et al., 2015; Parra et al., 2002). Moreover, according to the International Cocoa Organization (2017), another category is known as fine aroma cocoa, a classification that describes a cocoa of exquisite aroma and flavor, which represents around 8% of the cocoa production in the world. Countries such as Colombia, Ecuador, Venezuela and Peru produce about 76% of the world's fine aroma cocoa. On the other hand, Venezuela is the only country in the world where Porcelana Criollo cocoa is produced and harvested. Porcelana is a variety of Criollo cocoa recognized worldwide for its organoleptic properties (Portillo et al., 2005) and its completely different aroma, taste and texture (Portillo et al., 2007, 2009). The favorable soil and climate conditions of Venezuela may give Porcelana an intrinsic quality seal. However, it is necessary to optimize its production and commercial value. On the other hand, the chocolate flavor demand is the factor that propels the production and consumption of cocoa. For this reason, the whole procedure implied in the development of cocoa flavor and aroma has been studied profusely. The development of cocoa flavor and aroma is a complex phenomenon that depends on how the fermentation and roasting processes are carried out (Lima et al., 2011; Afoakwa et al., 2008; Alvarado et al., 2014). Fresh almonds must be fermented to develop the chemical bases that give the cocoa and floral flavor after roasting. During fermentation, physical, chemical and biochemical changes occur within the almond leading to the formation of the so-called "aroma and flavor precursors" that are finally expressed during the roasting stage (Voigt et al., 2016, 2018; Jinap et al., 2010; Afoakwa et al., 2013). In cases of very short or long fermentation times, the flavor precursors do not develop to reach a good level (Afoakwa et al., 2015; Voigt et al., 2018). Additionally, the different varieties of cocoa presents also distinct fermentation times. Therefore, one of the critical factors to obtain a good quality product is the use of technological tools, highlighting among others the use of reliable genetic material in terms of its origin and quality, as well as an appropriate post-harvest management (Kongor et al., 2016).

During the fermentation process, the mucilaginous pulp that surrounds the grains breaks and causes the death of the cotyledon (Afoakwa et al., 2008). This stage triggers the biochemical transformation inside the grains, with the combined activity of enzymes, endoprotease aspartate and carboxypeptidase, leading to the reduction of bitterness and astringency and the development of taste precursors such as free amino acids, peptides and sugars (Rohan, 1964; Rohan & Stewart, 1967; Brito et al., 2000; Afoakwa & Paterson, 2010; Janek, et al., 2016). Unfermented cocoa beans have an astringent and unpleasant taste and must be fermented, dried and roasted to obtain the characteristic chocolate flavor (Rohan, 1964; Bracco et al., 1969; Voigt & Biehl, 1995).

Other studies have focused on analyzing the behavior of various physical and chemical parameters during the fermentation, drying and roasting processes on some types of Venezuelan cocoas. Thus, the influence of factors such as type of fermenting, frequency of removal, endurance of the cocoa pod and fermentation time have been evaluated (Portillo et al., 2005, 2007; Graziani de Fariñas et al., 2003) as well as the drying methodology (Ortiz de Bertorelli et al., 2004) and the mixing of different types of grains, that also affects the fermentation process and the final quality of the product (Graziani de Fariñas et al., 2002; Lemus et al., 2002). Regarding the fruits, morphological (grain size and percentage of husk) (Álvarez et al., 2002, 2007) and chemical characteristics (moisture content, crude protein, crude fat, ashes, crude fiber, total sugars, reducing sugars, purines and polyphenols) have been studied. The influence of the fermentation, drying and roasting processes over the aromatic quality of Criollo-type cocoa has been analyzed by some authors (Portillo et al., 2009), while other works show the effect of edaphoclimatic conditions on certain quality parameters of Merideño Criollo cocoa (Zambrano et al., 2010). However, the available information regarding the behavior of amino acids during the postharvest stages is scarce (Rohan & Stewart, 1967; Puziah et al., 1998; Kirchhoff et al., 1989a, 1989b; Rohsius et al., 2006; Mulono, 2017; Mulono et al., 2017). For this reason, this work aimed to optimize and validate an analytical methodology using gas chromatography (GC) with mass spectrometry (MS) detection for the evaluation of amino acids. GC is a sensitive, selective and very accessible technique to many routine analysis laboratories. Nevertheless, it is restricted to the analysis of volatile compounds (Barquero, 2006). Amino acids are polar compounds and their volatility needs to be increased by a silylation reaction with BSTFA, in order to obtain volatile and thermally stable derivatives before a GC analysis (Schummer et al., 2009). In general, silylation reactions occur at temperatures ranging between 75 and 100 °C with long reaction times (more than 30 minutes) (Villas-Bôas et al., 2011). In this sense, recent studies have proved that microwave irradiation can accelerate the process (Kíngston & Jassie, 1988; Tierney & Lidström, 2005). Thus, we used microwave irradiation in order to reduce the derivatization reaction time.

The developed method was applied to the analysis of the amino acid profile in Criollo cocoa samples from Venezuela taken during the fermentation and roasting stages. The obtained information aims to improve and strengthen the production of the country in this important area, prioritizing the current trend of producing a fine cocoa with its characteristic aroma and flavor.

2 Material and methods

2.1 Instrumentation

A GC-MS (VARIAN 3800 with ion trap mass spectrometer VARIAN Saturn 2000) was used for the determination of the analytical parameters and samples analysis. The GC was equipped with an automatic autosampler (VARIAN 8200) for 48 vials with 2.0 and 0.1 mL.

Analytes were separated on a 30 m × 0.25 mm i.d. × 0.25 µm film thickness HP5 (methyl phenyl polysiloxane) capillary column.

2.2 Reagents, solvents and solutions

All reagents were analytical grade and all solvents were HPLC grade. Acetonitrile (ACN) was purchased from J. T Baker (Phillisbrug, NJ, USA). Water was purified in a Milli-Q SP TOC system (Water, Millipore, Milliford, MA, USA). Air (99.999%). Nitrogen (99.999%) and helium (99.999%) were supplied by AGA (Venezuela).

Standards of the amino acids present in cocoa were purchased from Sigma (St. Louis, MO, USA). Aqueous stock solutions of 17 amino acids were prepared by accurately weighing 0.05 g of each compound and by dissolving it in 5 mL of Mili-Q water in order to obtain a final concentration of 10.000 $\mu\text{g mL}^{-1}$, except for tryptophan, which was dissolved with 0.1 M hydrochloric acid. Stock solutions were stored at 4 °C. Working solutions were weekly prepared by dilution of the stock solutions in Mili-Q water.

N, O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA) was purchased from Sigma and used as derivatization reagent.

2.3 Samples

The varieties of the Criollo cocoa samples analyzed were Guasare, Zea and San Juan, from San Juan de Lagunillas' genetic bank (INIA-Mérida, Venezuela), and Porcelana, from the Chama's Local Station (CORPOZULIA, Venezuela).

The fermentation and roasting procedures were as follows: once cocoa was harvested, seeds were fermented for 3 days; the fermenting mass was turned over every 24 h. This process was performed in wooden boxes (60 cm³), in triplicate. We collected daily samples for analysis. Once the fermentation time was finished, the dryness process lasted 6 days.

Next, a fraction of the samples was grinded, sieved (≤ 42 mesh) and defatted following the Soxhlet extraction method (International Office of Cocoa, Chocolate and Sugar Confectionary, 1996). Then, they were stored at -20 °C until analysis. The rest of the dried almonds were toasted at 125 °C for 25 minutes and subsequently ground and sieved.

All samples were supplied in hermetically sealed plastic containers and were stored at -20 °C until analysis.

2.4 Amino acids extraction and derivatization

First, for the extraction of the amino acids from the cocoa sample, 0.8000 g of defatted cocoa powder were weighed. Then, the sonication-assisted extraction was performed in 15 mL of purified water at 70 °C for 15 minutes. The extract was allowed to cool to room temperature and centrifuged at 4000 rpm. Supernatant was transferred to a 25 mL volumetric flask and filtered using 0.22 μm membranes (support[®]-220 Membrane, Waters, USA).

Next, derivatization reaction was performed as follows: 250 μL of the extract were evaporated to dryness on a water bath (80 °C) under a pure and dry nitrogen steam. The residue was redissolved in a hermetically closed vial with 50 μL of acetonitrile and 100 μL of BSTFA. The mixture was vortex-stirred for 20 seconds and exposed to microwave radiation in a domestic microwave oven (MS-0745V, LG Intelowave) for 1 min at 630 W. Finally, 1 μL was injected into the chromatographic system for the analysis (Schummer et al., 2009; Kingston & Jassie, 1988; Tierney & Lidström, 2005).

2.5 GC-MS analysis

For the amino acids derivatives analysis, the injection port was operated in splitless mode, with the solenoid valve opened for 0.7 min and an injection temperature of 280 °C. The MS was operated in the electron impact (EI) mode (70 eV). The transfer line, manifold and ion trap temperatures were maintained at 230 °C, 40 °C, and 280 °C, respectively. The column temperature program was as follows: started at 80 °C

for 2 min, increased at $8\text{ }^{\circ}\text{C min}^{-1}$ to $155\text{ }^{\circ}\text{C}$, then increased at $11\text{ }^{\circ}\text{C min}^{-1}$ to $300\text{ }^{\circ}\text{C}$ and hold for 3 min, with a total run < 29 min. Helium was employed as carrier gas with a flow rate of 1.0 mL min^{-1} .

Derivatives detection was carried out in the selected ion monitoring mode (SIM) and analytes identification was performed with the mass spectral libraries NIST and WILEY. Quantitative ions for each analyte were set as follows: m/z 102 for glycine, 106 for alanine, 142 for proline, 144 for valine, 158 for leucine and isoleucine, 156 for lysine, 176 for methionine, 202 for tryptophan, 204 for serine, 218 for threonine, tyrosine and phenylalanine, 232 for aspartic acid and 246 for glutamic acid.

3 Results and discussion

3.1 Method validation

The proposed method was validated by performing accuracy, precision, linearity and matrix effect studies.

Precision study results, expressed as the coefficient of variation (CV), were $\text{CVs} < 4.67\%$ ($n = 5$) for the analyses performed during the same day, and $\text{CVs} < 4.67\%$ ($n = 5$) for the analyses carried out between days. These values indicated that the precision of the method and stability of the CG-MS system used were satisfactory.

Cocoa samples enriched with different concentration levels of the amino acids were analyzed to perform recovery studies and estimate the accuracy of the method. For all cases, recovery values were between 95 and 108% with CVs lower than 4.2% ($n = 5$). These results confirmed the high performance of the extraction procedures (solid-liquid) and of the derivatization reaction.

To evaluate the linearity of the method, calibration curves were constructed using standard solutions of the amino acids and enriched cocoa samples with the same concentration levels, according to the standard addition method. Values of the linear regression coefficient were $r > 0.9990$ for all cases, which showed a good linearity within the range of concentrations studied.

3.2 Cocoa samples

We analyzed cocoa samples collected before the fermentation procedure, during the three days of fermentation and after roasting.

Figure 1 shows the variation of total free amino acids content as a function of the fermentation and roasting time.

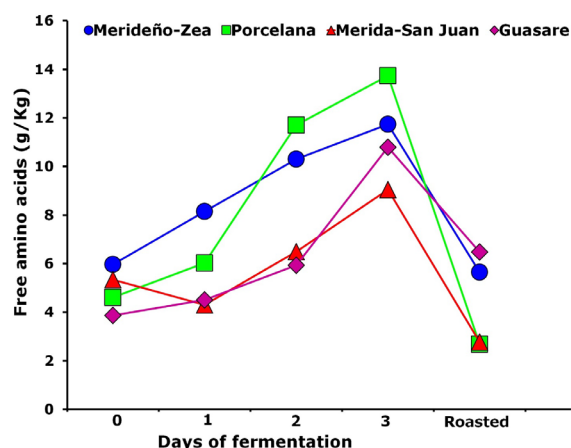


Figure 1. Total free amino acids content during the processes of fermentation and roasting for the different varieties of Venezuelan Criollo cocoa.

A progressive increase of amino acids is observed as fermentation progresses, with a maximum on the third day and a significant decrease during roasting. Values for the third day ranged between 9.05 and 13.73 mg kg⁻¹ with a maximum value for Porcelana Criollo cocoa (14.0 mg kg⁻¹). It is noteworthy that all the studied samples had a significant amount of free amino acids in a range between 3.87 and 5.97 g kg⁻¹ before the fermentation process (fermentation time: tf = 0). However, free amino acids level is substantially higher in fermented cocoa beans than in unfermented ones (Kirchhoff et al., 1989a, 1989b; Rohsius et al., 2006; Biehl et al., 1985). This trend has been previously reported by several researchers who studied Forastero and Trinitario cocoas in different countries (Rohan & Stewart, 1967; Puziah et al., 1998; Kirchhoff et al., 1989a, 1989b; Rohsius et al., 2006), and confirms that amino acids are also enzymatically released from proteins during fermentation (Biehl et al., 1985).

On the other hand, Figure 2 shows the free amino acids profile for each variety once fermentation is completed.

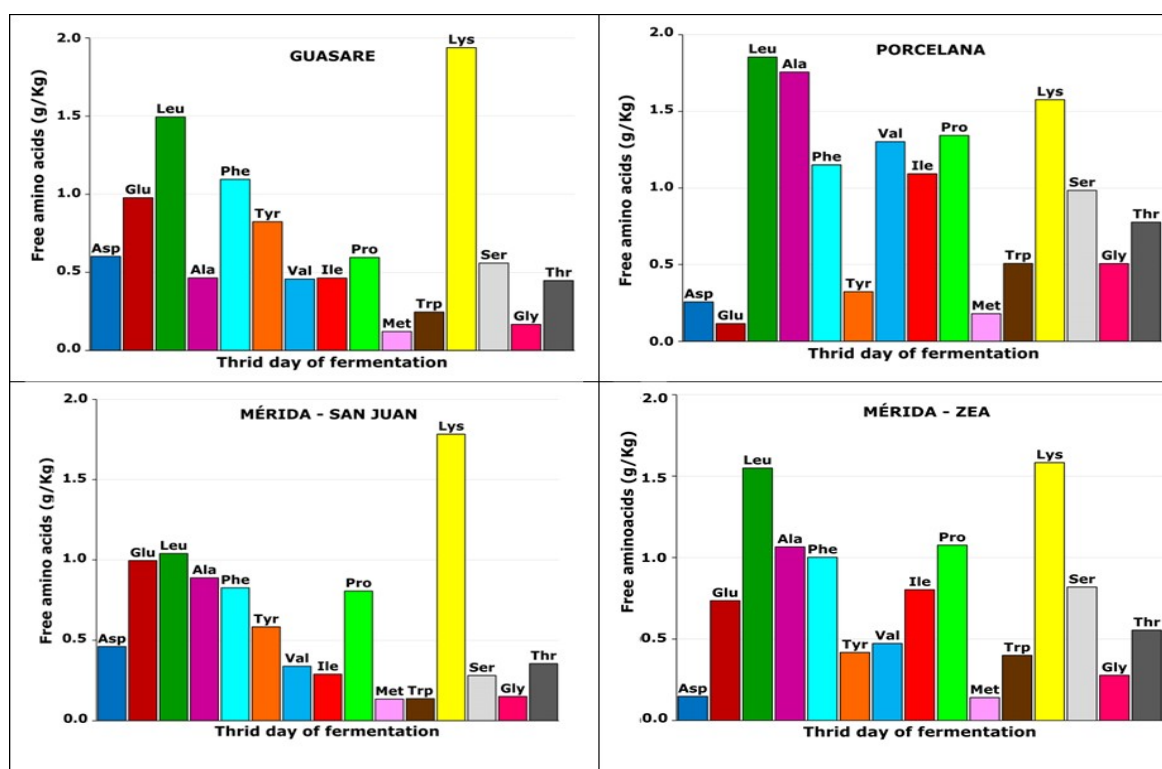


Figure 2. Amino acids content after three days of fermentation for venezuelan Criollo cocoas.

After the fermentation process the predominant hydrophobic amino acids were leucine (11% to 14%), phenylalanine (8% to 10%), valine (4% to 10%), alanine (4% to 13%) and isoleucine (3% to 8%) in all cocoa varieties, obtaining maximum values for the Porcelana one (14%, 8%, 10%, 13% and 8%). These amino acids are associated with the formation of the cocoa aroma, due to its greater capacity to react with sugars in the roasting stage. A similar behavior was reported by Rohan & Stewart (1966), Puziah et al. (1998), and Kirchhoff et al. (1989a, 1989b) in their foreign cocoas studies, observing hydrophobic amino acids increase at the end of fermentation, specially leucine, phenylalanine, alanine and valine.

Moreover, Figure 3 illustrated the total content of acidic, hydrophobic and other amino acids during the fermentation and roasting processes.

In all cases, the greater content increase during fermentation occurs for hydrophobic amino acids, which several researchers (Voigt & Biehl, 1995; Kirchoff et al., 1989a, 1989b) have associated to the proteolytic activity of certain enzymes of cocoa beans proteins. The enzymes involved in this process are aspartic endoproteinase (optimal pH 3.5) and carboxypeptidase (optimal pH 5.8). First, one acts on proteins by degrading them to oligopeptides with hydrophobic carboxyl-terminal amino acid residues, while the second one acts on the oligopeptides carboxyl-terminal ends, thereby releasing hydrophobic amino acids (Jinap et al., 2010; Lagunes Gálvez et al., 2007; Voigt et al., 1994). The trend observed in these figures has been previously described for other cocoa varieties (Rohan & Stewart, 1967; Puziah et al., 1998; Kirchoff et al., 1989a, 1989b; Rohsius et al., 2006) although the individual amounts obtained in this work differ from the reported ones, both for unfermented and fermented beans, due to their genetic origin. However, the influence of other parameters has to be taken into account.

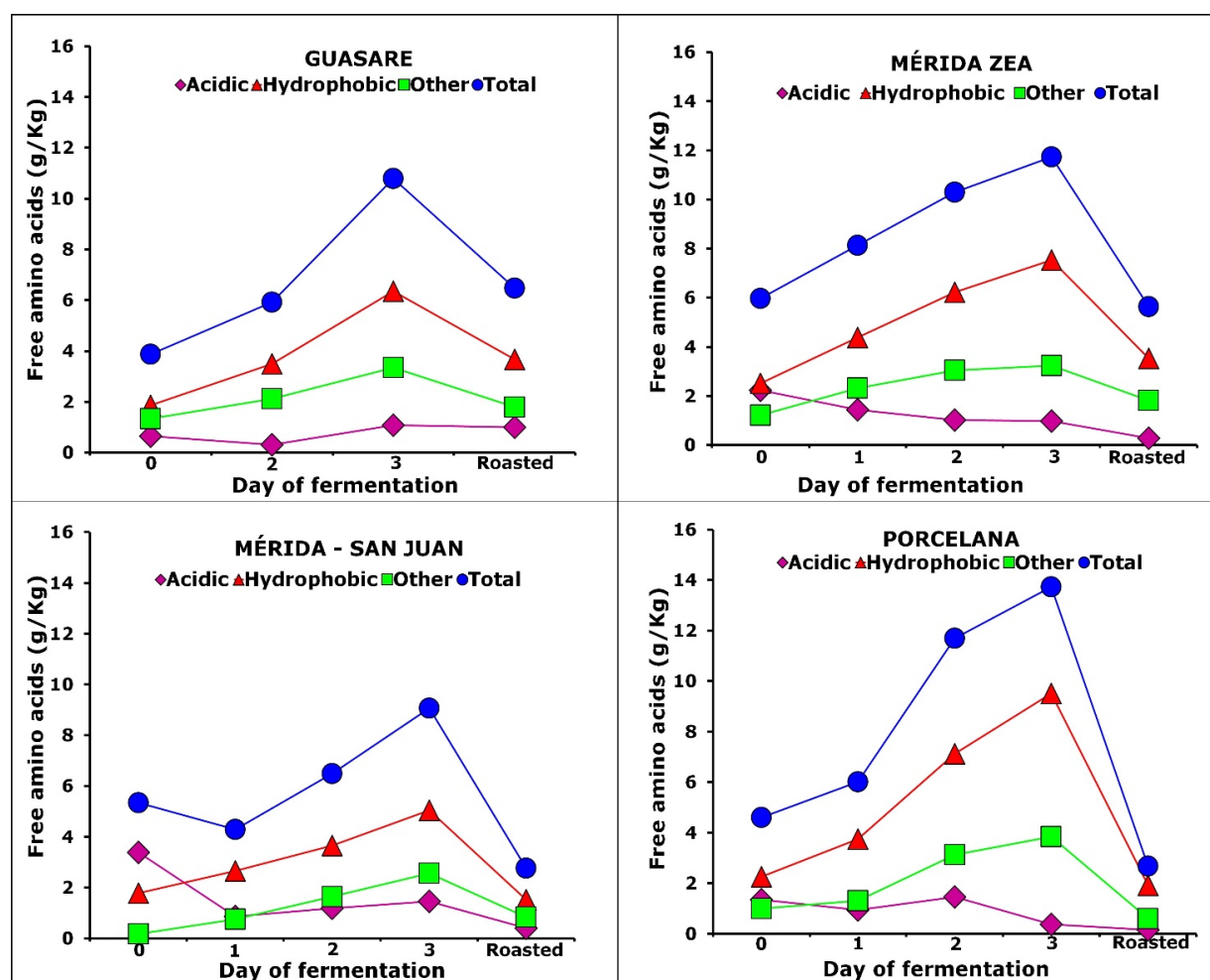


Figure 3. Free acidic, hydrophobic and other amino acids content during the fermentation and roasting processes for the different varieties of Venezuelan Criollo cocoa.

For instance, Rohan (1964), reported that the amount of free amino acids and oligopeptides that are formed and accumulated in the seeds during fermentation are not constant since they depend on the pH during the proteolysis stage. For example, carboxypeptidase, which is responsible for the release of hydrophobic amino acids, decreases its action at low temperatures and high pH (Puziah et al., 1998). In this sense, some parameters of the fermentation stage, such as the amount of grain that is fermented and the number of removals carried out, have a direct influence on the temperature and pH of the process. Removal of the grains allows their aeration, which leads to a regulation of both the acidity of the product and the speed of the

fermentation process (Portillo et al., 2005; Ortiz de Bertorelli et al., 2009a,b). Excessive aeration leads to low temperatures and, consequently, to decreased metabolic rates as well as a high concentration of acetic acid. On the other hand, Portillo et al. (2005), pointed out that the delay in the shattering and the opening of the cocoa pods leads to an increase in temperature during fermentation, which may favor the processes of proteolysis and consequently the release of hydrophobic amino acids.

In addition, Figure 3 shows a decrease in the acidic amino acids content (glutamic acid and aspartic acid) during the first days of fermentation. Similar results were reported by Puziah et al. (1998), who noticed a decrease in glutamic and aspartic acid content during the first two days of fermentation. According to Kirchhoff et al. (1989a, 1989b), it is not possible to guarantee a variation in the content of the rest of amino acids but the acid ones, since their degradation can be compensated by its proteolytic formation, especially in the case of hydrophobic amino acids.

The content and type of amino acids that predominates in the almond allow the differentiation between fermented and unfermented cocoas. In this work, unfermented cocoa beans presented small amounts of free amino acids (between 2.33 and 5.96 g/kg) with a high content of acidic amino acids (between 17% and 63%). On the other hand, well-fermented cocoa beans have a higher content of free amino acids (9.05 to 13.73 g/kg), with a predominance of hydrophobic amino acids (56% to 70%) and a drastic decrease of more than 70% of the acidic amino acids at the end of the fermentation process. Thus, the ratio between hydrophobic:acidic:other amino acids was between 45:36:19 and 49:29:21 for the unfermented almonds and between 63:8:29 and 70:3:27 for the fermented ones (Figure 4). This trend was reported by Puziah et al. (1998) and Kirchhoff et al. (1989a, 1989b) for cocoa from Malaysia and Ghana, in which the unfermented beans presented small amounts of free amino acids (2.25-6.24 g/kg) while the well-fermented, dry and fat-free beans presented amounts of free amino acids between 8 and 14 g/kg. In that case, the ratio between hydrophobic:acidic:other amino acids ranged from 30:18:52 for unfermented beans to 46:6:48 in fermented ones for Malaysian cocoa, and from 33:30:37 to 58:16:26 for the cocoa from Ghana.

Besides, Figure 4 depicts significant differences between Merideño San Juan and Zea varieties, which are genetically equivalent cocoas but grown in different areas. These differences can be attributed to the edaphoclimatic conditions in which the crop and harvest were developed. Zambrano et al. (2010) studied some parameters affecting the flavor and aroma of these cocoas and reported variations in the content of fat, total sugars, pyrazines, caffeine and theobromine, which indicates that the edaphoclimatic conditions led to changes in the chemical behavior of the precursors of the aroma and flavor. Moreover, other researchers have also reported that factors such as climatic conditions during the fruit formation, degree of maturity of the berries, nutritional status of the mother plant and genetic material significantly influence the amino acid profile during fermentation and drying processes (Puziah et al., 1998; Rohsius et al., 2006).

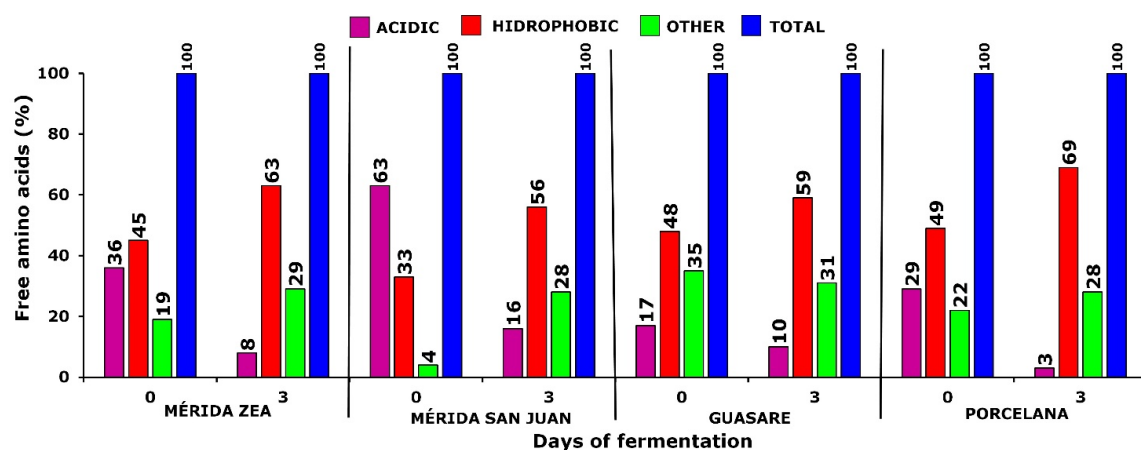


Figure 4. Total, hydrophobic, acid and other free amino acids content (%) for the unfermented and fermented Criollo cocoa varieties.

The roasting stage is an essential step for the development of the chocolate. In this step, Maillard reactions are crucial for the free amino acids degradation that forms the compounds associated with flavor and aroma from the precursors obtained during fermentation and drying. In accordance with it, Figure 3 shows the decrease of free amino acids during roasting. Hydrophobic amino acids showed the greatest reduction, particularly in Porcelana variety. This performance is associated with a significant increase in pyrazines, which have hydrophobic amino acids as potential precursors (Portillo et al., 2009; Serra Bonvehí & Ventura Coll, 2002). Therefore, pyrazine levels can be used to evaluate the efficiency of the roasting process due to its pronounced increase during this stage and its influence on the final aroma of the cocoa.

Finally, the free amino acids profile during the last day of fermentation and during the roasting stage was compared with the pyrazine content obtained by Brunetto et al. (2009) for the same samples. As it is shown in Figure 5, the obtained results are consistent with the premises discussed above.

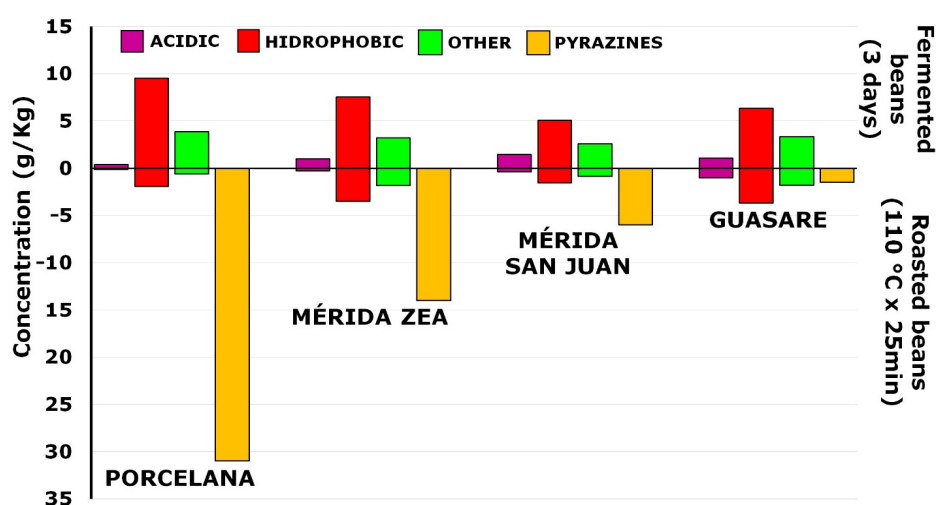


Figure 5. Effect of roasting process on the content of free amino acids and pyrazines in the samples of Criollo cocoa.

To sum up, the obtained results indicate that amino acids are released in limited and inconstant amounts due to the great influence of the fermentation pH and temperature. In addition, fermented and unfermented cocoas can be differentiated by the amount of free amino acids and the hydrophobic and acidic amino acids ratio present in the almond.

4 Conclusions

We developed a GC-MS method to perform the selective evaluation of the cocoa samples profile. The results obtained when analyzing different validation parameters indicated a good accuracy and stability of the chromatographic system used as well as the effectiveness of the extraction procedures. The method was successfully applied for the analysis of cocoa samples. Overall, we noticed an increase in the content of free amino acids during the fermentation stage, particularly of the hydrophobic ones that had a significant influence on the pyrazines formation during the roasting stage. Moreover, we also observed that the fermented and unfermented cocoas can be differentiated by the amount of free amino acids and the proportion between the hydrophobic and acidic ones.

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