JOSÉ HENRIQUE DA SILVA TAVEIRA

METABOLITE PROFILE AND SENSORY QUALITY OF ARABICA GENOTYPES GROWN IN DIFFERENT ALTITUDES AND PROCESSED BY DIFFERENT POST-HARVEST METHODS

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Dr. Flávio Meira Borém

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A Deus, meu tudo.

A meus pais, José Balde e Olívia Isabel.

A meus irmãos Márcio, Denise, Elaine, Jaqueline e Vânia.

A meus sobrinhos.

DEDICO

E sempre haverá uma montanha, e nós sempre teremos que movê-la. E a cada dia surgirá uma diferente batalha, logo, teremos que saber ganhá-la ou perdê-la. O mais importante é tentar, não desistir, manter os olhos do coração no céu e a cabeça aqui na terra. Caminhando e nunca perdendo a fé, por mais difícil que seja, perceberemos que existe um sonho muito maior do que o nosso, o sonho de DEUS.

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ABSTRACT

Coffee is a product that is evaluated and commercially valued by its beverage quality, mainly in the international trading. The knowledge regarding genetic, environmental and technological parameters that produce and determine the quality is greatly important for the production of specialty coffees. In order to understand the influence of these factors on the metabolite profiles and on the final quality of the coffee, this work was performed with the following goals: i) to assess the influence of altitude, slope exposure, and processing on the metabolite and sensory profiles of different genotypes grown in *Serra da Mantiqueira*; ii) to identify a single metabolite or group of metabolites that are potential markers of coffee quality. The experimental samples were harvested during three agricultural crop seasons, with experimental design comprising 3 altitude ranges (<1000m, 1000-1200m, >1200m), two slope exposures (sunny face and shade face), two genotypes (Acaiá and Yellow Bourbon) and two processing methods (dry and wet way). The metabolite profiling was performed using the gas chromatography-quadrupole/mass spectrophotometer- GC-Q/MS and the sensory evaluation was performed according to the methodology purposed by the Specialty Coffee Association of America-SCAA. Samples harvested in different crop seasons tended to show different metabolite profiles and very similar sensory score. The processing methods were the main factors influencing the metabolite profile; however, the sensory score was similar between natural and demucilaged coffees. The altitude effects were hidden by the great variance showed by processing and genotypes, what made necessary to separate assessments. The samples harvested above 1200m of altitude tended to show the highest quality scores, regardless the processing method used, mainly the Yellow Bourbon genotypes. The slope exposure did not affect the metabolite profile of coffee seeds. The highest correlation values were observed for the samples of Yellow Bourbon and for the group of metabolites analyzed, the ones that positively correlated were: tryptophan, L-asparagine, gluconic acid, glucose, sorbitol, and mucic acid; and the ones that negatively correlated were: lactic acid, cafeic acid, myo-inositol, glycerol 1-phosphate, and L-aspartic acid.

Key words: Specialty coffees; Metabolomics; Environment; Coffee processing.

RESUMO

O café é um produto que é avaliado e comercialmente valorizado pela qualidade de sua bebida, principalmente no mercado internacional. O conhecimento dos fatores genéticos, ambientais e tecnológicos que formam e determinam a qualidade é de suma importância para a produção de cafés especiais. Buscando o entendimento da influência desses fatores no perfil metabólico e na qualidade, este trabalho foi realizado com os objetivos de: i) avaliar a influência da altitude, faces de exposição ao sol e do processamento no perfil metabólico e na qualidade da bebida de diferentes genótipos cultivados na microrregião da Serra da Mantiqueira; ii) identificar um ou mais metabólitos potenciais para marcadores da qualidade da bebida. As amostras do experimento foram coletadas durante três safras agrícolas, com delineamento composto por três faixas de altitude (<1000m, 1000-1200m, >1200m), duas faces de exposição ao sol (soalheira e contra-face), dois genótipos (Acaiá e Bourbon Amarelo) e dois métodos de processamento (via seca e via úmida). O perfil metabolômico das amostras foi realizado por CG-Q-MS a avaliação sensorial foi realizada pela metodologia da SCAA. Os métodos de processamento são os fatores que mais afetam o perfil metabólico dos grãos de café, no entanto, a qualidade sensorial em termos de notas é bastante semelhante entre os cafés descascados e os cafés naturais. O efeito da altitude é dificilmente detectável devido à grande variância apresentada pelo processamento e genótipo, tornando-se necessário sua avaliação separada. As amostras colhidas acima de 1200m, independente do processamento, sempre apresentam a tendência de terem as maiores notas sensoriais, principalmente o Bourbon Amarelo. As faces de exposição não exercem efeito significativo e nem tendencioso sobre o perfil metabolômico e nota total das amostras. Os maiores valores de correlação foram obtidos das análises das amostras de Bourbon Amarelo e para o grupo de metabólitos analisados, os que se correlacionaram positivamente foram: tryptophan, L-asparagine, ácido glucônico, glicose, sorbitol e ácido mucico; e os que se correlacionaram negativamente foram: ácido lático, ácido cafeico, glycerol 1-phosfato, mio-inositol, L-ácido aspártico.

Palavras chave: Cafés especiais; Metaboloma; Ambiente; Café-processamento.

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( ) indicated the threshold (0.2) for the selection of the most important features that influenced in the separation trend of coffee samples harvested in the three altitude ranges. Each altitude class in the dataset was pretreated by WGLS, alpha=0.001. 64

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1 INTRODUCTION

Coffee is one of the most important commercial commodities traded in the world. Nowadays, the demand for specialty coffees in the market grows in greater rates when compared to the regular ones. These coffees are characterized by high quality and a great potential for flavor after roasting, absence of any defects and relationship to origin differentiation, crop or specific genotypes. The term "coffee quality" has a peculiar meaning for each class in the coffee production chain, and from the farmer to the consumer levels it can change significantly Leroy et al., 2006.

Although Brazil is the largest coffee producer, its coffees are still worldwide known as regular, cheap, flat, and with prominent astringency. Coffees from different origins as Central America, Africa and Asia are very well known for their high quality and noticed sensory attributes. On the other hand, different origins may influence the metabolic responses to environmental conditions. According to Choi et al. (2010), coffee seeds originated from different areas have very distinct metabolite profile, mainly when they are geographically distant, located in different continents. In the same way, the species *Coffea arabica* L. and *Coffea canephora* also present great differences in their chemical composition Alonso-Salces et al. (2009) and Wei et al. (2012).

The metabolite profile of coffee seeds can significantly change because of several factors, such as genetic (FIGUEIREDO et al., 2013), environment (JOËT et al., 2010; ALONSO-SALCES et al., 2009; AVELINO et al., 2005) and post-harvest methods (JOËT et al., 2010; DUARTE; PEREIRA; FARAH, 2010; BYTOF et al., 2007; KNOPP; BYTOF; SELMAR, 2005). This way, the cup quality or sensory profile may be directly related to the metabolite profile.

Metabolomics as a comprehensive and non-target analysis covers a broad range of metabolites. It also plays a key role for describing the precursor

compounds responsible for quality. These metabolites react with proteins and amino acids releasing more than 800 new aromatic constituents Leroy et al. (2006), which provide flavor to the roasted coffee seeds. Considering the coffee chemical, it is complex to simply correlate quality to a single compound, or a specific environmental parameter, or even to a specific post-harvest technique. The differences in the metabolite involve a net of phenomena that is not easy to explain and it still demands much more studies to be understood and explained.

The metabolomics approach is also a useful tool to identify new metabolites, instead of those that are classically reported in the coffee literature, such as chlorogenic acids, caffeine, soluble sugars, fatty acids, etc. Therefore it is necessary to dig the metabolite changes and differences that are responsible to define the final coffee quality in order to provide techniques to the specialty coffee production chain. In addition, it could help to improve the strategies for coffee breeding programs.

Nowadays, it is easier to discriminate materials from different origins in the world or different species using sophisticated metabolite profiling tools, such as high performance liquid chromatography HPLC Alonso-Salces et al. (2009), gas chromatography-mass spectroscopy GC-MS (FROST et al., 2012; DU et al., 2011) nuclear resonance magnetic - NRM (WEI et al., 2012; MOCO et al., 2007). Considering the need for fast analytical methods to characterize coffee seeds produced in different environments and processed in different ways, GC-MS is a rapid, efficient and suitable analysis for metabolite profiling.

Despite many works have approached metabolomics analysis as fingerprinting to differentiate origins and coffee species, a broad study covering the relationship among environment conditions, processing methods, genetic materials, metabolite profiles and their effects on the sensory profile is still poorly found. Understanding the interaction among the environmental

phenomena, genetic materials and post-harvest treatments, may change the way we see coffee quality in Brazil.

There are several coffee producing regions in Brazil. One of the most important is the South of Minas Gerais State, where is located *Serra da Mantiqueira* micro region. This micro region is known for producing coffee showing an outstanding sensory profile and high quality. Thereby, finding marker metabolites for coffee seeds produced in the *Serra da Mantiqueira* region will favorably work on benefit of their origin denomination and also help to understand coffee quality.

Therefore, the general aim of this work is to describe the metabolite profile of coffee seeds produced in different environments and processed in different ways.

The main objectives of this work were:

To assess the influence of altitude, slope exposure, and processing on the metabolite and sensory profiles of different genotypes;

To identify one metabolite or group of metabolites that are potential markers to coffee quality.

2 LITERATURE REVIEW

2.1 Environmental conditions, genotype and coffee quality

Coffea arabica L. is native from forests in Ethiopia, Kenya, and Sudan, with average altitude between 1500-2800m and latitude between 4°N and 9°N. In these regions, the air temperature oscillation is low, thus, the impact on the environmental variation and on the climate is very light. The average of annual temperature ranges from 18 to 22 ºC and the rainfall is well distributed during the year, which varies from 1600mm to more than 2000mm. In this

environment, the *Coffea arabica* L. established as a shrub tree of a forest (SYLVAIN, 1955). In this context, it is expected that this species adapts very well in regions with similar environment, presenting good yield and high quality.

It is currently known that the most suitable geographical conditions to produce specialty coffees should have high altitude, good rainfall distribution, and soil with high fertility. Those lands are located in the equatorial zone, which have tropical climate, characterized by high temperatures during the day and low temperatures during the night. Nevertheless, it is possible to find around the world several coffee producing regions with edaphoclimatic conditions that strongly influence the final physical and sensory quality of the coffee.

Coffee quality is directly associated with the origin where the coffee trees grow. The inherent characteristics of the region, such as environmental conditions, geographic, geological, and human elements interacting with the plant's genetics provide very distinct profiles. In this context, the final quality of coffee is fully related to its origin, which can be considered a *terroir* product.

The arabica coffee is produced in many states in Brazil, including Minas Gerais, Paraná, São Paulo, Bahia and Espírito Santo. The largest state that produces coffee is Minas Gerais, which provides about 50% of the Brazilian crop every year. The spatial distribution of coffee quality in this state suggests that coffees with the better quality are produced in regions with mild temperatures and average rainfall about 1600 mm Barbosa et al. (2012).

The environmental conditions directly affects the production of the arabica coffee, mainly the photoperiod, rainfall distribution and temperature Camargo and Camargo (2001). These authors suggest that high temperatures promote stress for the coffee tree and its consequent faster growing, which will produce coffees with lower quality. This way, the temperature consists on a

climatic parameter of the production environment that can strongly affect the coffee tree physiology.

The temperature is related to altitude variation and slope exposure. It is well known that in tropical regions, for every 100 m of increase in the attitude, the average daily temperature decreases about 1 ºC Vaast et al. (2006). This way it is expected plots located in higher altitudes to have a mild climate, with lower temperatures, which can stretch the phenological cycle of the coffee tree. This could improve the maturation and quality of the fruits.

Laviola et al. (2007) concluded that altitude influences the extension of the coffee tree phenological cycle, accumulation of nutrients, and leaves/fruit competition. The authors also suggested that enzymatic reactions take place more slowly in environments with lower temperatures, as well as the photosynthetic and respiratory rates. Positive correlation was also found between the chlorogenic acids, fatty acids and the increasing altitude Avelino et al. (2005). Joët et al. (2010) did not find relationship between altitude and a broad range of analyzed metabolites, which included fatty acids, sugars, caffeine and chlorogenic acids. However, the authors clearly demonstrated the relationship between these chemical compounds and the environment temperature.

In addition to altitude, the slope exposure of the plots may promote the formation of different microclimates, which expose the coffee tree to more or less lightness during its phenological cycle. The quantity of light received by the coffee tree is responsible for changing the plant metabolism and fruits maturation, which is directly related to their final quality Bertrand et al. (2006), Geromel et al. (2006), Montavon et al. (2003), Vaast et al. (2006).

The changes of the phenological cycle according to the lightness, fruits maturation, increase of seeds size, uniformity of maturation, nutrients received and metabolized in different precursors of the quality beverage have been studied previously Geromel et al. (2008) and Moreira (2003). The quantity of

light may stimulate or retard the fruits maturation, which affects the amount of nutrients received and synthesized by the fruits, such as sucrose, chlorogenic acids, trigonelline, and others Bertrand et al. (2006) and Vaast et al. (2006). These metabolites are important when related to coffee quality, considering they are precursors of the flavor after the roasting process Farah et al. (2006), Figueiredo et al. (2013) and Geromel et al. (2008). Vaast et al. (2006) pointed that the amounts of chlorogenic acids and trigonelline are present in greater concentrations in coffee fruits exposed to higher luminosity conditions, which showed incomplete maturation, and as consequence higher bitterness and astringency in the beverage.

In addition to the environmental effects on the coffee tree metabolism, the response of each genotype to the environment plays an important role on the final quality of the beverage. Coffee genotypes respond differently when exposed to the same environmental conditions Miranda et al. (1999). Miranda et al. (1993) and Geromel et al. (2008) worked with different *Coffea arabica* L. lineages. These authors concluded that different coffee lineages also have different responses to the luminosity conditions. Vaast et al. (2006) also observed that traditional coffee cultivars and hybrids of *Coffea arabica* L. respond in different ways when exposed to the same environment conditions, regarding to yield, chemical composition and beverage quality.

On the other hand, Figueiredo et al. (2013) showed that different lineages of the same variety have completely different responses when cropped in different locations. The authors evaluated several genotypes and concluded that Bourbon had the greatest potential for quality. Ribeiro (2013) analyzed Bourbon genotype coffee seeds harvested in different levels of altitude. The author concluded that Bourbon grown above 1200 m of altitude tended to show higher levels of caffeine, trigonelline, and 3-CQA and final sensory score around 90. In the same work, Ribeiro (2013) showed that even when Acaiá genotype was

grown in higher altitudes, it tended to present lower scores and higher levels of 4-CQA than Bourbon genotypes.

2.2 Coffee processing

Besides environmental conditions, genetic materials and the farming conditions influencing the coffee quality, it is expected that flavor characteristics are also formed from several biochemical changes during the coffee processing. Studies focused on the different metabolite profiles resulting from these changes are scarce and need to be performed to clarify such differences.

There are two methods to process coffee fruits after they are harvested: the dry way and wet way Borém (2008) and Brando (2004). In the dry way process, the entire fruits are carried to be dried, with neither hulling nor pulping, which provides the natural coffee. On the other hand, in the wet way process the coffee fruit peel and part or the whole mucilage layer are removed. Depending on the used technique, the wet way may provide different types of coffees: the hulled coffee, from which the remaining mucilage is not removed; the fully-washed coffee, from which the remaining mucilage is removed by fermentation; and the demucilaged coffee, from which the mucilage is mechanically removed Borém (2008). In both processes the main objective is drying the coffee seeds quickly, by decreasing their moisture content to 11% (w.b.), ideal for benefiting and storage.

The most used method in Brazil, Ethiopia and Yemen for arabica coffees is the dry way, and the wet way is most used in the other countries Brando (2004). Despite this fact, with the technology advances, Brazil has increased the amounts of coffee seeds processed in the wet way.

The final chemical composition depends on the processing method used Bytof et al. (2004), Duarte, Pereira and Farah (2010), Joët et al. (2010 and

Knopp, Bytof and Selmar (2005), thus it is expected that coffees processed in different ways present distinct sensory profiles. In general, the coffee seeds processed in the dry way have heavier beverage body and higher sweetness than coffee seeds processed in the wet way, which present higher acidity.

Several metabolic changes take place during the post-harvest treatments, which influence the sugar levels and other relevant compounds in the coffee seeds Duarte, Pereira and Farah (2010) and Favarin et al. (2004) and Joët et al. (2010). Biochemical changes related to the seed metabolism during the processing depend on the processing method that coffee seeds were exposed, dry way or wet way Selmar et al. (2006). Bytof et al. (2004), Duarte, Pereira and Farah (2010), Joët et al. (2010) and Knopp, Bytof and Selmar (2005) concluded that the mode of processing significantly influences the chemical composition of green coffees, in which the soluble carbohydrates are strongly affected by the metabolic processes occurring during the course of wet or dry processing. In their work, Knopp, Bytof and Selmar (2005) concluded that coffees processed in the dry method present higher levels of fructose and glucose comparing to coffee seeds proceed in the wet method. Sugars present in the coffee seeds contribute to the beverage sweetness, which is one of the most desirable attributes of specialty coffees and play an important role in chemical reactions, such as Maillard and caramelization. These reactions are responsible for the color and flavor formation in the roasted coffee beans.

Bytof et al. (2004) pointed glutamic acid as a precursor of γ-amino butyric acid. This synthesis is more prominent when seeds suffer some kind of stress, which lowers glutamic acid amount in the coffee seeds processed in the dry way. In the same work, the levels of aspartic acid, asparagine and alanine were increased by dry method.

Duarte, Pereira and Farah (2010) evaluated the content of nine chlorogenic acids, caffeine, trigonelline and sucrose on *Coffea arabica* L.

cultivars and hybrids treated by wet and semi-dry post-harvesting methods. Their results showed that the wet method produced an increase in chlorogenic acids and trigonelline contents and a small loss in sucrose contents when comparing to semi-dry method.

Livramento, 2008, assessed the proteomic profile of coffee seeds processed by both methods and dried using different temperatures. The author concluded that natural coffees are more likely to lose proteins by denaturation than washed coffees. The same sensitivity of coffees processed in the dry way was observed by Taveira et al. (2012), in which the inactivation of enzymes complex were more severe for the natural coffees during the drying stage.

2.3 Metabolomics approach

Metabolomics analysis has been widely used to very efficiently differentiate coffee seeds by several groups of researchers. Alonso-Salces et al. (2009) used phenolic compounds and methylxantine contents as markers to describe the differences between *Coffea arabica* L. and *Coffea canephora*. The authors concluded that profiles of chlorogenic acids, cinnamoyl amides, cinnamoyl glycosides, free phenolic acids and methylxanthines of green coffee seeds contain adequate information for the geographical characterization of Arabica and Robusta coffees at continental, sub continental, and national levels.

Joët et al. (2010) described the effects of the environmental temperature on the final composition of the coffee seed on the final chemical composition of coffee seeds using metabolomics approach for the first time. The authors also demonstrated the influence of wet processing on the chemical composition of seeds and its interactions with the metabolic status of seeds at harvest. However, despite the very wide range of metabolites covered, it seemed that any of them explained the better flavor of high-altitude coffees.

Coffee metabolomics approach was also used to discriminate coffees from different origins, Asia, Africa, and South America Choi et al. (2010). The authors attempted to determine the coffee origin in order to efficiently control the quality of coffee, using non-target analysis and statistical algorithm (Principal Component Analysis combined with heat map). This study points that based on the feasibility of determination of plant origin, these techniques can be applied to several areas, including food, agricultural, and pharmaceutical sciences.

Following the same trend, Wei et al. (2012) used the metabolomics approach to differentiate coffee species from several origins, including Brazil, Colombia, Guatemala and Tanzania. However, this study was focused on improving the Nuclear Resonance Magnetic-NMR coupled to multivariate analysis (Principal Component Analysis- PCA and Orthogonal Partial Least Squares Discriminant Analysis- OPLS-DA) to perform their work.

Lately, metabolomics has also been used in studies focused on finding marker metabolites to work as finger prints, which could be used against forgery, for instance, *Kopi Luwak*, an exotic and high priced Indonesian coffee, which is made from coffee berries that are eaten by the Asian palm civet (*Paradoxurus hermaphroditus*). Jumhawan et al. (2013) attempted to differentiate *Kopi Luwak* of the regular commercial coffee. The authors showed the feasibility of selecting discriminant markers from metabolite profiling using Gas Chromatography Mass Spectroscopy coupled to multivariate analysis.

2.3 Sensory evaluation

Coffee quality is described by the physical characteristics of the seeds and sensory attributes of the beverage. The sensory profile has as principal

attributes aroma, uniformity, clean cup, sweetness, flavor, acidity, body, after taste, balance, and overall Lingle (2011).

The most used method for coffee quality evaluation is performed using an old fashioned but very efficient technique, the sensory analysis. This subjective technique uses the human senses (taste, smell and touch) as tools to perform the sensory evaluation. Considering the complexity of the flavor found in coffee beverage, the sensory evaluation has shown efficiency and consistency when performed by a trained panel of judges Figueiredo et al. (2013), Kathurima et al. (2009) and Ribeiro (2013).

Commonly, cuppers and traders use two methodologies to evaluate the sensory profile of coffee: Official Brazilian Classification, *"Classificação Oficial Brasileira*- COB*"* Brasil (2003), the Specialty Coffee Association of America - SCAA Lingle (2011). The traditional method in Brazil uses categories to classify the coffee beverage, which are strictly soft, soft, hard, *rio* and *rio zona* Brasil (2003). Even though it is widely used for commodity coffee evaluation, it is worth to mention that this method is not solid and does not have a standard methodology for its performing.

The SCAA method has been widely used for the evaluation of quality of specialty coffees and coffees of controlled experiments Figueiredo et al. (2013), Kathurima et al. (2009) and Ribeiro (2013). This method can be performed only by cuppers trained and qualified as "SCAA Certified Cupping Judges". Due to the excellent control and standardization of such method, it has shown suitability to evaluate coffee seeds that have huge or slight differences. It evaluates the coffee attributes aroma, uniformity, clean cup, sweetness, flavor, acidity, body, after taste, balance, and overall Lingle (2011).

It has become easier to find researchers trying to correlate coffee quality to the chemical composition of coffee seeds Bertrand et al. (2006), Bicho et al. (2012), Farah et al. (2006), Figueiredo et al. (2013), Kathurima et al. (2009),

Pimenta, Ribeiro and Carvalho (2004), Ribeiro (2013), Ribeiro, Ferreira and Salva (2011) and Vaast et al. (2006). However, it is worth to mention that consistent results are only reachable when the sensory analysis is performed following more solid criteria. In this case, the SCAA methodology should be more appropriate for the assessment of coffee quality, mainly when it is for research purpose.

3 MATERIALS AND METHODS

3.1 Experimental site and conditions

This work is part of a major project, which is titled "Identity, quality and traceability protocol to substantiate the geographical indication of coffees from *Mantiqueira*" - "*Protocolo de identidade, qualidade e rastreabilidade para embasamento da indicação geográfica dos cafés da Mantiqueira.*" Considering that "*Serra da Mantiqueira*" comprises a large area, a smaller area was selected to start the characterization studies of coffees from this region. This selection was based on the edaphoclimatic characteristics of the whole region, such as rainfall, temperature, altitude, slope, and the coffee producing area. The average value of these characteristics were then calculated for each of the 22 municipalities that the region comprises and the municipality that showed the most similar characteristics and would better represent the whole region was chosen. In this case, the municipality Carmo de Minas (-22.12º, -45.13º) was selected as a first experimental site of the pilot project, because it presented the average characteristics that represents the Serra da Mantiqueira region and also because it provided a better accessibility to information and to samples harvesting.

3.2 Experimental Factors

The experimental design is shown in Figure 1 in which during the three coffee crops (2009/10, 2010/11, 2011/12) coffee samples were harvested from plots located only in Carmo de Minas. For this experiment the six environmental combinations formed between the altitude ranges (bellow 1000m, between 1000m and 1200m, and above 1200m) and two groups of slope exposures, sunny face (NE, N, NW, W) and shade face (E, SE, S and SW) were considered. The group denominated sunny face comprised the slope exposures that would be warmer because they are exposed to longer sunlight periods, mainly in the afternoon. Conversely, the group denominated shade face should be colder due to the shorter sunlight period of exposition, mainly in the morning. For each combination, three replicate samples of coffee fruits, Acaiá and Yellow Bourbon genotypes, were collected and processed by dry way and wet way.

Acaiá genotype was chosen to constitute the experimental design mainly because it has the red fruit skin, besides an average high yield, and is one of the most cultivated varieties in Carmo de Minas. And Yellow Bourbon genotype was chosen mainly because it has yellow fruit skin, presents good consistency in the sensory score, and also shows a high potential for quality.

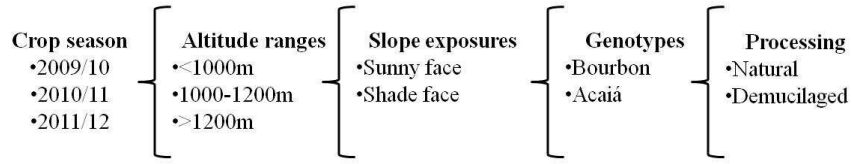

Figure 1 Experimental factors designed in a factorial mode (72 treatments comprised by 3 replicates each).

3.2.3 Harvest and Post-harvest of coffee

Three biological samples of coffee fruits were selectively harvested and manually selected when completely ripe to ensure their uniformity, integrity and high quality. The floaters were removed from the samples by washing the coffee fruits in a bucket. To ensure the sample uniformity with ripe fruits only, a new hand selection was performed. About 12 L of the whole coffee fruits were carried directly to suspended drying beds (natural coffee). To carry out the wet way method, about 20 L of coffee fruits were pulped and mechanically demucilaged. Then, the coffee seeds were also taken to the suspended drying beds.

The suspended beds were wooden frames (1m x 1m) with a net made of polyethylene yarn on the bottom. The samples were uniformly spread on the suspended beds observing the limit of 12 L for natural coffees and 8 L for the demucilaged coffee. All samples were stirred every 20 to 30 minutes during the drying under the sun, and during the night they were taken into a hangar to be protected of dew or even unexpected rain. In case of raining days, the samples were transferred to fixed bed layer dryers with air heated at 35°C in order to keep them drying and to avoid losing any sample by undesirable fermentation. The drying processes ended for all samples when they reached 11% (w.b.) moisture content, which was measured using an oven, set at 105±1°C, for 16±0,5 hours according to the method ISO 6673 (INTERNATIONAL ORGANIZATION STANDARDIZATION, 2003).

After dried, the samples were packed and stored in chambers with controlled environmental conditions set at 10 ºC and relative humidity of 60% during about 30 days. Then samples were milled, sorted and only the normal and non-defective seeds with size 16/64 to 18/64 were selected to metabolite profiling and sensory analysis. It was done to standardize the samples, to

maximize the effect of treatments and to minimize any external interferences that can increase the experimental error.

All drying and storage stages were carried out in the facilities of the *Laboratório de Processamento de Produtos Agrícolas* - LPPA of the *Universidade Federal de Lavras* - UFLA.

3.3 Metabolomic approach

3.3.1 Coffee seeds extraction

Overall, 10 mg of the lyophilized tissue powder was extracted twice in 515 µl aqueous methanol 60% (v/v) in a 2.0 ml microcentrifuge tube containing internal standards (0.18 µl of 45 mg·ml−1 adonitol and 0.37 µl of 0.59 mg·ml−1 13C6 trans-cinnamic acid). The microcentrifuge tubes were incubated in a water bath set at 70 °C for 1 min and then transferred to a dry plate set at 70 °C while they were mixed. The mixtures were cooled down to room temperature and centrifuged at 2199 rad·s−1 for 5 min at room temperature. The supernatant from the first extraction phase was transferred into a new tube. The powder remaining in the tube was mixed with 515 µl aqueous methanol 60% (v/v), mixed for 15 min using sonication, and centrifuged at room temperature. The supernatant was added to the first extraction phase, and the total extract mixture was complete. A subsample (50 µl) of each extract was transferred to a glass micro-insert, taken to the derivatization, and then analyzed using GC-Q/MS according to the methodology described by Frost et al. (2012).

3.3.2 Gas Chromatography Quadrupole Mass Spectroscopy GC-Q/MS analysis

A sub-sample (25µl) of each extract was transferred into a glass micro-insert and dried in the centrivap. Samples were methoximated with a

methoxyamine hydrochloride/pyridine solution (20 mg/ml; Sig- ma-Aldrich) containing retention index markers (pentadecane, eicosane, pentacosane, and triacontane), and silyated with N- Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma-Al- drich, St. Louis, MO). Derivitized samples were injected (1 µl per sample) into an Agilent 7890A GC in splitless mode with an inlet temperature of 250 ºC Jeong et al. (2004). Metabolites were resolved on a DB-5MS column (30 m length, 0.25 mm diameter, with a built-in 10 m DuraGuard pre-column) with a flow of 1.12 ml/min, and average velocity of 26.86 cm/sec. Thermal ramping initiated at 80^{o}C for 1 min, ramped 20^{o}C/min to 200^{o}C, then 10^{o}C/min to 310^{o}C with a 6.5 min hold at 310^{o}C. Metabolites were detected using an Agilent 5975C MS with source and quadrupole mass filter temperature setting of 230^{o}C and 150^{o}C, respectively. Mass spectra were collected in scanning ion mode (m/z 50 and 500) in ChemStation (Agilent Technologies) and deconvoluted using AnalyzerPro (SpectralWorks, Runcom, UK).

3.3.3 Metabolite identification

Putative peak identities were assigned based on the NIST08, Fiehnlib (Agilent Technologies), and in-house mass spectral libraries. The best given peak identities were provided with the confidence level for each metabolite (Table 1), and only peaks that showed confidence level above 0.7 were considered for analysis.

3.3.4 Metalab analysis

Finally, data peaks obtained from the analysis of each sample were aligned using an in-house software program (MetaLab), which was available at http://aspendb.uga.edu. In addition, it was also possible to correct possible variations in the chromatogram spectra by dividing all peaks by the internal standard peaks (adonitol and 13C6 trans-cinnamic acid).

The average spectra similarity was also obtained from the MetaLab output (Table 1). It was performed by comparing the peak spectra of each sample chromatogram with the correspondent peak spectra of all the other samples. The higher and closer to 1 the values were, more similar were the compounds aligned and assigned with the same name.

3.4 Roasting and Sensory Profiling

The roasting and sensory profiling were performed according to the protocol developed by the Specialty Coffee Association of America - SCAA Lingle (2011). Each green coffee sample (100g) was roasted using an experimental roaster, Probat TP2 (Curitiba, Brasil) within 24 hours of cupping and allowed to rest for at least 8 hours. The roast profile was light-medium roast based on reference color numbers, 65 for ground coffee and 55 for the whole seed (SCAA/Agtron Roast Color Classification). The roast was completed between 8 and 12 minutes, and samples were immediately cooled down until room temperature to avoid over roasting.

After roasted, samples were weighted according to the optimum ratio, which is 8.25 grams of coffee per 150 ml of water, as this conforms to the mid-point of the optimum balances (SCAA). Then, samples were ground in the Mahlkönig Guatemala (Hamburg, German) miller, within no more than 15 min prior cupping process. Grind particle size should be slightly coarser than typically used for paper filter drip brewing, with 70% to 75% of the particles passing through a U.S. Standard size 20 mesh sieve.

The cupping was performed according to the SCAA protocol Lingle (2011), and for each sample, five cups were assessed by 4 judges certified by SCAA. Ten attributes comprised the sensory profile, including aroma, uniformity, clean cup, sweetness, flavor, acidity, body, after taste, balance, and overall. The quality level of these attributes was analyzed, and then based on the

cupper's previous experience, samples were rated using a 0.25 numeric scale. The final score was generated by the sum of the ten sensory attributes evaluated.

As the processing methods provide coffees with very distinct sensory profiles, the evaluation rounds were performed for the natural coffee samples and demucilaged coffee samples separately. Each of these rounds contained one sample resulted from the combination between environment and genotypes.

3.5 Data Analysis

3.5.1 Pre-treatment

The data set was pretreated prior to the construction of the chemometric models. This step in the analysis was done in order to avoid outliers in large datasets and to correct possible variations in the spectra that were not related to the samples nature. It included variations in the conditions of analysis, such as environmental temperature and relative humidity, type of apparatus, worker, position of samples in the equipment and all possible perturbation. This way, prior to model calculation, Generalized Least Square Weighting-GLSW and auto scaling were used to preprocess the data. GLSW is a filtering technique used to lower and smooth signals that are known as inference Wise et al. (2006). It calculates a filter matrix based on the differences between pairs or groups of samples that should otherwise be similar.

It was also necessary to auto scale the dataset due to the differences in the nature of the analyses considering metabolite profile, which is expressed in peak intensity, and sensory profile, expressed in total score.

3.5.2 Principal Component Analysis - PCA

The multivariate PCA is an unsupervised analysis that provides a visual representation of the data, it does not require a priori knowledge of the data set and has been widely used for metabolomics analysis Choi et al. (2010),

Jumhawan et al. (2013) and Wei et al. (2012). This technique is an exploratory multivariate analysis that projects the data matrix to a lower dimensional space spanned by the eigenvector. The loading vectors corresponding to the *K* largest eigenvalues are retained to optimally capture variance of the data and to minimize the effect of random noise Jackson and Mudholkar (1979) . PCA was used to provide an explanation of the data variability.

PCA was performed using MATLAB 7.9.0 (The MathWorksTM,MA, United States) and PLS Tool- box 5.2.2 (Eigenvector Research, Inc., WA, United States).

3.5.3 Correlation analysis

Correlation analysis between the metabolites and sensory score was required in order to find the most important features that were correlated with sensory analysis. It was performed by Microsoft Office Excel 2007, data analysis toolbox.

4 RESULTS AND DISCUSSION

4.1 Metabolite profiling

GC-Q/MS was performed on aqueous extracts of raw coffee seeds in order to search the differences in their metabolite profiles, and to select potential discriminative markers related to the sensory profile. The metabolite profiling was performed in coffee samples harvested in three different crop seasons 2009/10, 2010/11, and 2011/12. Each crop season comprised samples of coffee fruits of two genotypes, Acaiá and Yellow Bourbon, harvested in three altitude ranges (<1000m, 1000-1200m, and >1200m) and two groups of slope exposures (sunny and shade faces) in each range of altitude.

Metabolomics approach is a non-target analysis that allows covering a broad range of metabolites. An average amount of 500 peak signals were found in each extract of samples, which were aligned using MetaLab. However, only 33 metabolites were picked after a very strict selection, which was based on the threshold of 70% of confidence level (Table 1), when putative peak identities were assigned. Compounds that were present at trace levels, below the limit of quantification, overlapped with other substances, or even higher in the blank runnings than in the samples extracts, were not counted for the data analysis. In this study, groups of metabolites such as amino acids, organic acids, carbohydrates, CGA's, polyalcohol and other compounds were identified in the coffee seed extracts. Despite many compounds were found in the extracts, this analysis was not capable to comprise many metabolites such as fatty acids, other isomers of chlorogenic acids, carbohydrates, and volatile compounds. The retention time, confidence level and spectra similarity of each compound found in coffee seeds extracts were shown on the Table 1.

Table 1 Metabolite profile of coffee seeds extracts performed by CG-Q/MS. RT (average Retention Time of the compounds in the GC column and the RT values were extracted from the chromatograms), Confidence (the confidence values were extracted from MetaLab output by comparing the mass spectra of the compounds to the libraries), and AVG SC (the Average Spectra Similarity values were obtained from MetaLab output and calculated by comparing the mass spectra of each compound of all samples).

Compound	RT	Confidence	AVG SC
Lactic acid	5.90	87.12	0.88
L-alanine	6.20	93.35	0.87
Oxalic acid	6.46	93.85	0.99
L-Valine	6.98	89.01	0.90
L-Isoleucine	7.53	83.50	0.88
L-proline	7.61	84.94	0.90
Nicotinic acid	7.65	89.45	0.95
L-Serine	7.95	96.56	0.99
Malic acid	8.88	95.34	0.99
L-Aspartic acid	9.15	95.06	0.92
Trigonelline	9.24	89.00	0.81
Pyroglutamic acid	9.28	94.01	0.89
Glutamine	9.97	94.64	0.98
Phenylalanine	10.16	90.45	0.96
L-asparagine	10.42	95.85	0.98
Putrescine	11.11	86.24	0.93
Glycerol 1-phosphate	11.15	87.05	0.90
Citric acid	11.70	95.26	0.98
Quinic acid	12.05	94.16	0.99
Fructose	12.18	94.27	0.95
Glucose	12.59	93.22	0.89
Sorbitol	12.70	93.48	0.96
L-tyrosine	12.88	92.70	0.93
Gluconic acid	13.31	80.43	0.86
Myo-inositol	13.39	79.27	0.98
Mucic acid	13.72	77.04	0.93
Allo-inositol	14.20	90.74	0.99
Caffeic acid	14.66	94.57	0.97
Tryptophan	15.38	89.78	0.91
Galacturonic acid	16.50	81.76	0.95
Galactinol	21.23	90.48	0.97
4-CQA	23.10	95.00	0.98
5-CQA	23.37	95.38	0.98

For the first time, this work showed the metabolite profiling approach using GC-Q/MS coupled with PCA chemometric analysis to discriminate samples of coffee samples and detect the underling metabolites that may lead to a better quality. This analysis turned out being efficient and fast, considering the time spent for running (25min/sample). Furthermore, as a non-target analysis, it provided excellent identification of several peaks in only one running.

4.2 Principal Component Analysis - PCA

The dataset was divided in classes according to the experimental factors (crops, processing methods, altitude ranges, slope exposures, and coffee genotypes) and each class was pretreated by GLSW (a=0.001). In order to observe the general trends in the dataset, PCA was performed separately for each general factor of the experiment

4.2.1 PCA of processing methods

PCA of the processing classes was performed to assess the differences between coffees processed in the dry way (natural coffee) and wet way (demucilaged coffee). The PCA score scattering plot of the processing methods was shown on the Figure 2, in which a clear differentiation between the natural and demucilaged coffees was observed. The higher variance was observed on PC1, which contributed with 30.78 of the total variance. However, there was no contribution of the total sensory score for the separation between natural and demucilaged coffees, which may suggest that there was no difference in the total scores between coffees processed by the dry and wet ways (Figure 3 A). It was worth mentioning that those coffees had distinct sensory profiles. Generally, the main differences were found in the taste, which is more fruity for the natural

coffees and floral for the demucilaged coffees. In addition, the body intensity was median to high for demucilaged coffees and high for the natural coffees.

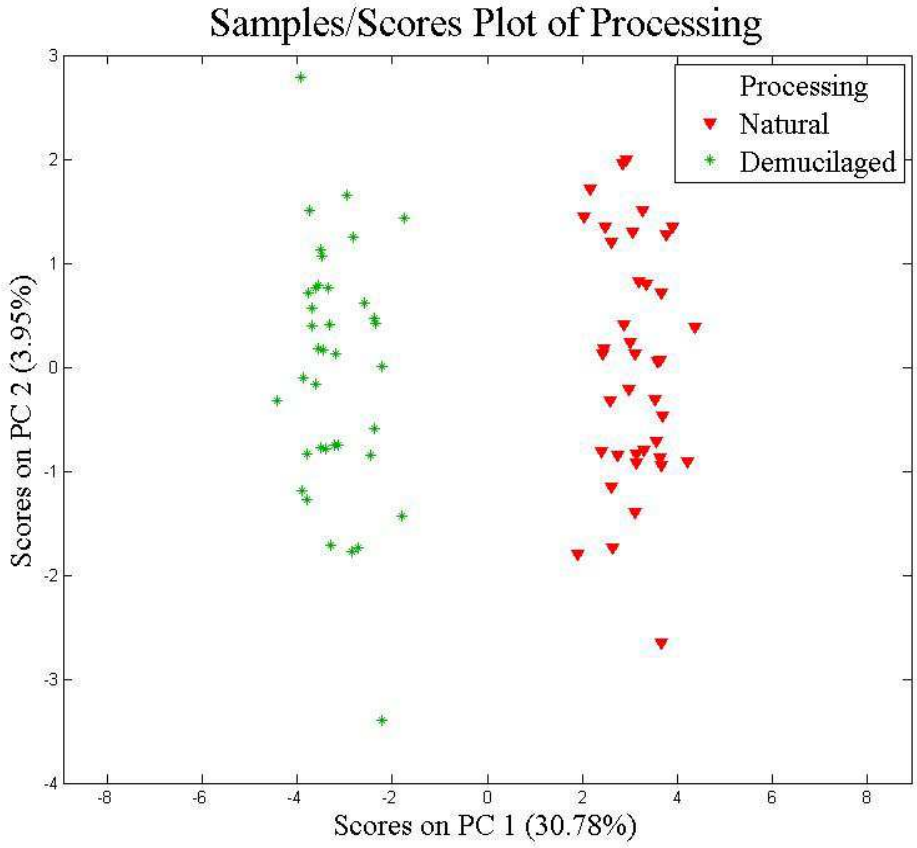

Figure 2 PCA score scattering plot of the principal component 1 (30.78% of the total variability) and principal component 2 (3.95% of the total variability) of the metabolite profile differentiating (▾) natural coffee and (✦) demucilaged coffee. Each class of the processing methods in the dataset was pretreated by WGLS, alpha=0.001.

The variable loadings were shown in Figure 3, which showed the most important features for the separation between natural and demucilaged coffees. The features that most influenced this separation were: L-valine, L-isoleucine, L-proline, pyroglutamic acid, fructose, L-tyrosine, and allo-inositol for natural

coffees; and L-aspartic acid, putrescine, mucic acid, and galactinol for demucilaged coffees (Figure 3).

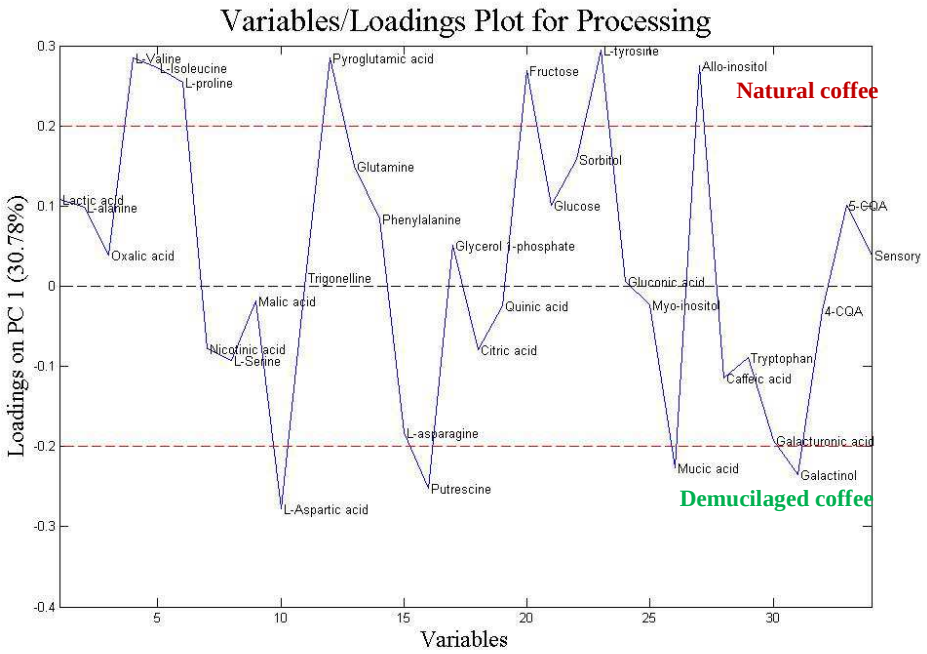

Figure 3 PC 1 metabolite loadings of the PCA of the processing methods. The positive values on the Y axis indicated the most important features for the natural coffee samples and the negative values on the Y axis indicated the most important features for the demucilaged coffee samples. The red dashed line

( ) indicated the threshold (0.2) for the selection of the most important features that influenced on the separation of the natural and the demucilaged coffees. Each class of the processing methods in the dataset was pretreated by WGLS, alpha=0.001.

Despite all those metabolites were observed separating the processing methods, any information taken at this point could lead us to wrong conclusions. In addition, the presence of the genotype and altitude variances within each processing class may also had interfered on the final results.

Whereas natural coffees and demucilaged coffees are known for showing completely different sensory profile and distinct metabolite profile, further separate analyses were required for each of these classes (natural and demucilaged coffees). These analyses were performed in order to catch detailed information about the metabolites that are more related to the sensory score and the metabolic responses of each genotype to the altitude ranges.

4.2.2 PCA altitude

The PCA score scattering plot of the principal component 1 (12.14%) and principal component 2 (5.2%) of the metabolite profile of coffee samples harvested in the three altitude ranges (<1000m, 1000-1200m, and >1200m) were shown in Figure 4.

Altitude has been popularly known for its strong influence on the final coffee quality and many studies have concentrated their efforts on finding direct and indirect parameters that could explain that Anastácio; Pereira (2008), Avelino et al. (2005), Joët et al. (2010a) and Laviola et al. (2007). However, the effect of altitude could not be clearly observed in the present study. It was probably due to the analysis performed with all parameters together, including processing, genotypes and slope exposure. As previously observed, the processing methods showed very strong influence on the separation of the coffee samples, based on their metabolite profile.

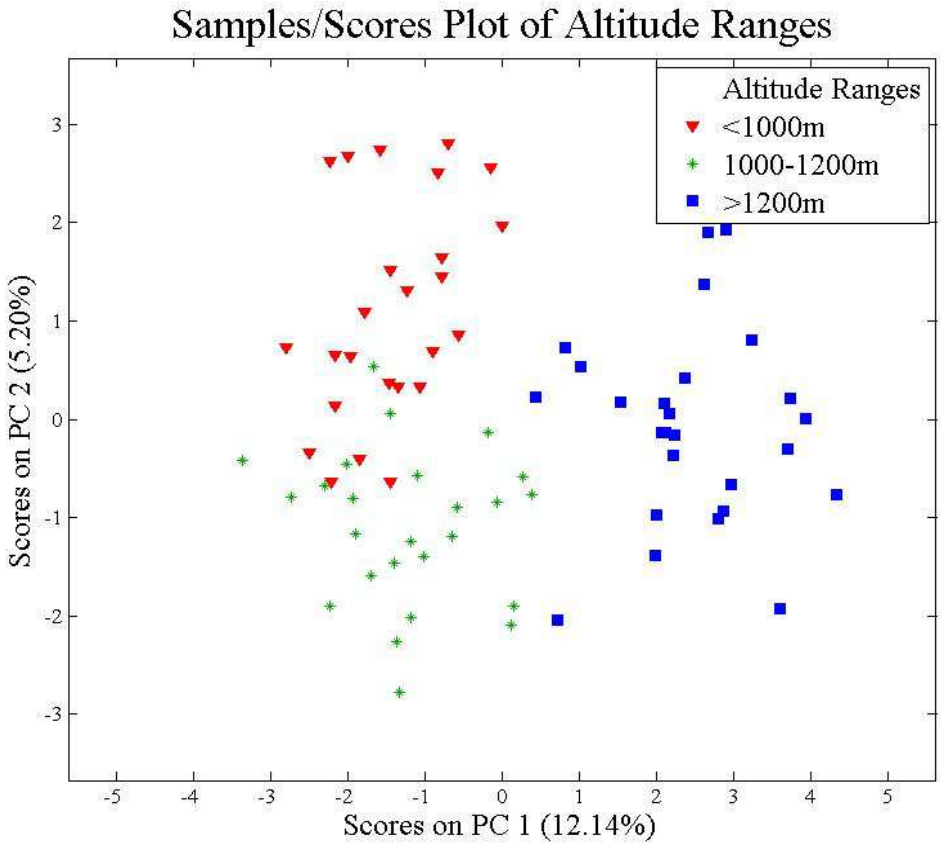

Figure 4 PCA score scattering plot of the principal component 1 (12.14% of the total variability) and principal component 2 (5.2% of the total variability) of the metabolite profile differentiating coffee seeds harvested in three different ranges of altitude (▼) <1000m, (✦) 1000-1200m, and (■) >1200m. Each class of altitude ranges in the dataset pretreated by WGLS, alpha=0.001.

Figure 5 shows the features that were responsible for the separation trend of the samples harvested in the three ranges of altitude. Although some metabolites could be considered important features, their score weights were not enough to separate the samples according to their original altitude (Figure 4).

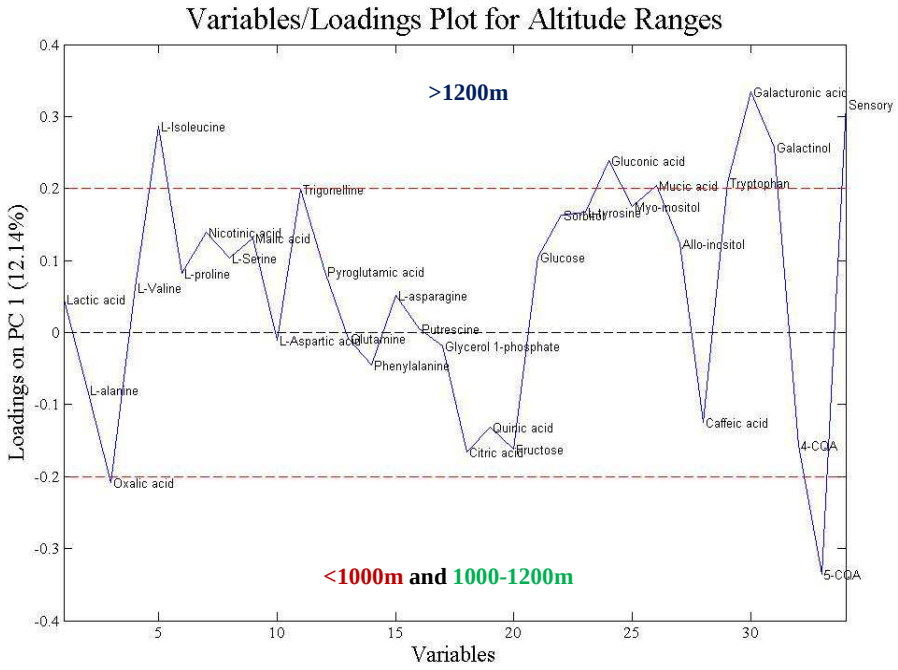

Figure 5 PC 1 metabolite loadings of the PCA of the altitude ranges. The positive values on the Y axis indicated the most important features for the coffee samples harvested above 1200m of altitude and the negative values on the Y axis indicated the most important features for the coffee samples harvested between 1000m and 1200m, and bellow 1000m. The red dashed line

( ) indicated the threshold (0.2) for the selection of the most important features that influenced in the separation trend of coffee samples harvested in the three altitude ranges. Each altitude range class in the dataset was pretreated by WGLS, alpha=0.001.

Thereby, separated analyses were performed attempting to capture detailed information about the altitude effects on the metabolite profile and final sensory scores.

4.2.3 PCA slope exposure

The Figure 6 shows the PCA score scattering plot of the principal component 1 (6.00%) and principal component 2 (5.21%) of the metabolite profiles of coffee samples harvested in two slope exposures (sunny face and shade). Although the samples were divided into two classes, any separation between the slope exposures was observed.

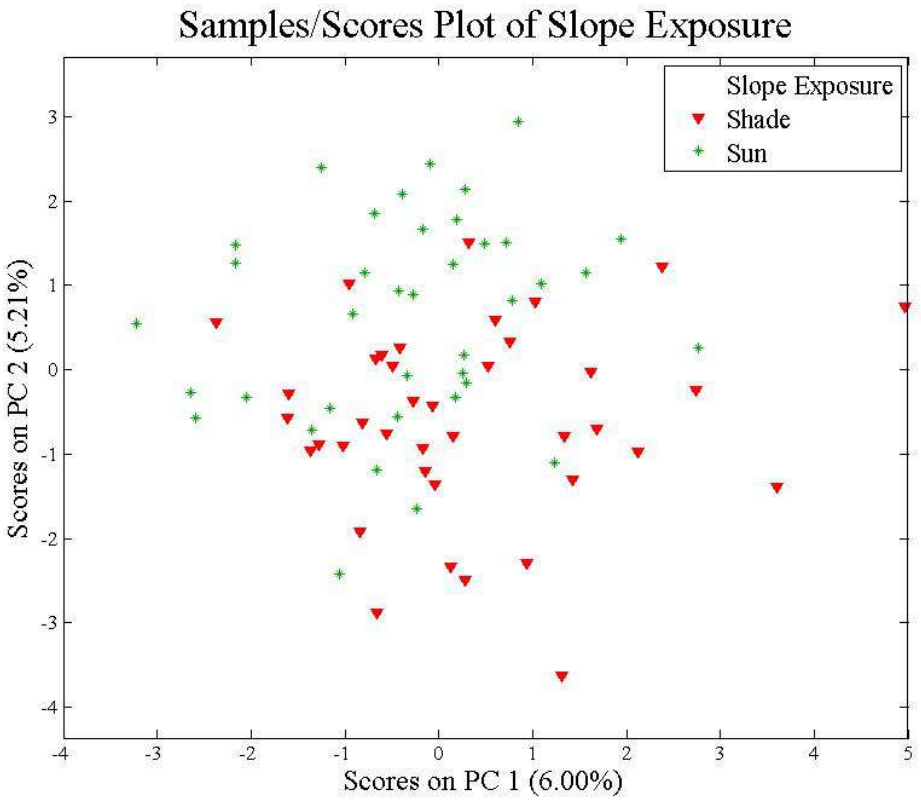

Figure 6 PCA score scattering plot of the principal component 1 (6.00% of the total variability) and principal component 2 (5.21% of the total variability) of the metabolite profile differentiating coffee seeds harvested in two different slope exposure (▾) Sunny face, (*) Shade face. Each class of the slope exposures in the dataset pretreated by WGLS, alpha=0.001.

These results did not show clear interferences of the slope exposure, neither on the metabolite profiling nor on the sensory analysis, and the variable/loadings were not required to assess the influence of the metabolites. The effects of the slope exposure might have been hidden by the way that the groups of slope exposures were setup, sunny face (NE, N, NO, and O) and shade face (E, SE, S, SO). Further studies may be required in order to find differences between slope exposures, e.g. setting different groups of faces.

It has been well known that the slope exposure affects the coffee quality in some unknown way, however these effects seemed to be very slight. There was a suggestion that east-facing plots provided coffees with better sensory characteristics in Costa Rica (AVELINO et al., 2005). Nevertheless, it is important noting that the latitude had a very clear influence on the characteristics of the slope exposures.

Conversely, the slope exposure could provide different microclimates to coffee trees. Speculations that shade face would receive less sun light and radiation, and consequently lower environmental temperatures could be easily verified by placing meteorological stations on the plots.

4.2.4 PCA genotype

The Figure 7 shows the PCA score scattering plot of the principal component 1 (15.16%) and principal component 2 (5.56%) of the metabolite profiles of coffee genotypes, Acaiá and Yellow Bourbon. A trend of separation of these genotypes was observed on Figure 7. However, a confusion zone was also observed, mixing some samples of both genotypes. In addition, the samples harvested in the altitude range between 1000-1200m may be responsible for that. This altitude range might be a transition zone, in which the samples were sometimes confused with the samples harvested bellow 1000m and sometimes with the samples harvested above 1200m. Moreover, this analysis included

natural and demucilaged coffees leading to cover the differences between the genotypes.

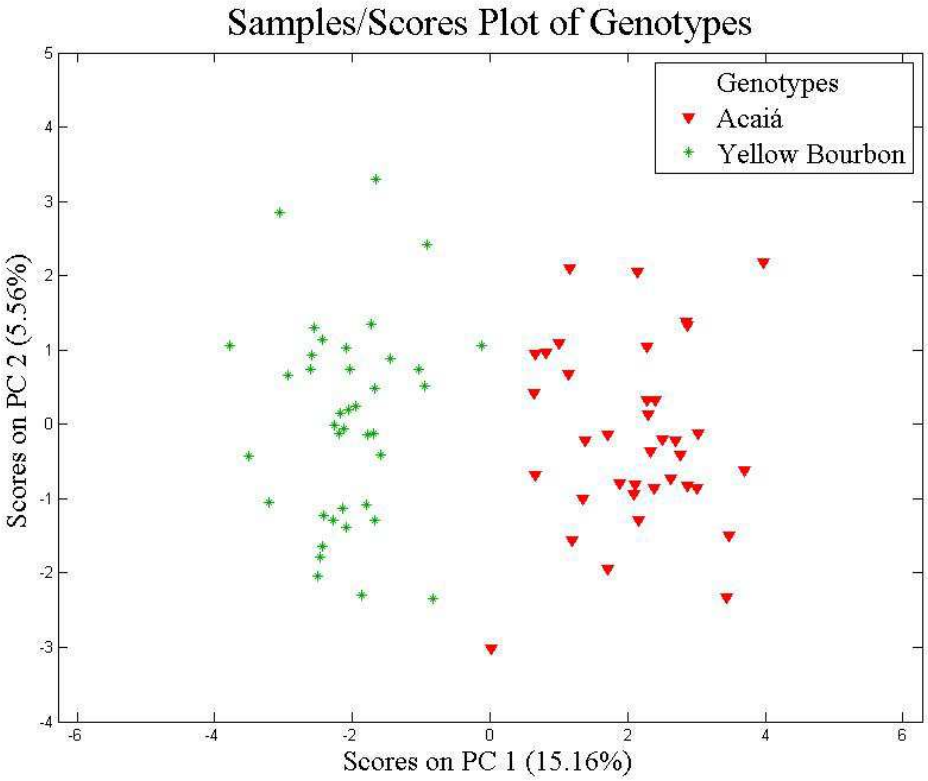

Figure 7 PCA score scattering plot of the principal component 1 (15.16% of the total variability) and principal component 2 (5.56% of the total variability) of the metabolite profile differentiating seeds of two genotypes (▾) Acaiá, (*) Yellow Bourbon. Each genotype class in the dataset was pretreated by WGLS, alpha=0.001.

Although that confusion zone was observed on Figure 7, Figure 8 shows a clear high contribution of the sensory analysis for the Yellow Bourbon separated from Acaiá. Yellow Bourbon genotype is known for its high potential for producing coffee with high cup quality Figueiredo et al. (2013) and for its excellent response to the environmental conditions where it is growing. In addition, in order to confirm that Yellow Bourbon presented a better average

quality than Acaiá genotype, the results of this research pointed to compounds that have never been related to quality. The most important features for this separation trend were glycerol 1-phosphate, myo-inositol, allo-inositol, galacturonic acid, and galactinol for the Acaiá genotype, and for the Yellow Bourbon were L-aspartic, phenylalanine, and 5-CQA. Despite those features were identified on this PCA, the slight differences on the metabolic drivers to quality may have been hidden due to the higher variances observed on the processing method, the transition range of altitude (1000-1200m).

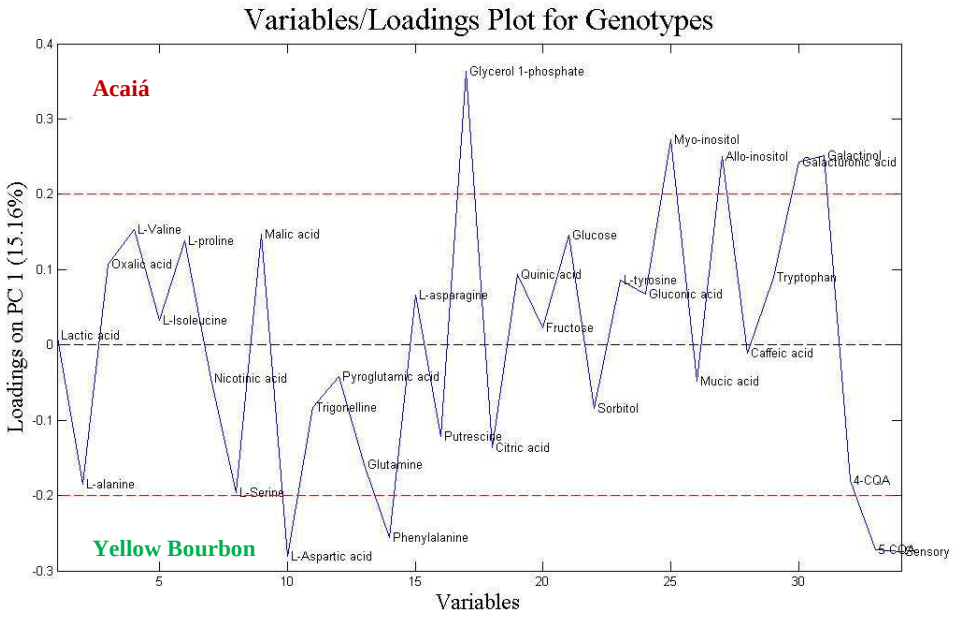

Figure 8 PC 1 metabolite loadings of the PCA of the genotypes. The positive values on the Y axis indicated the most important features for the Acaiá coffee samples harvested and the negative values on the Y axis indicated the most important features for the Yellow Bourbon coffee samples. The red dashed line

( ) indicated the threshold (0.2) for the selection of the most important features that influenced in the separation trend of the genotypes Acaiá and Yellow Bourbon coffee samples. Each genotype class in the dataset was pretreated by WGLS, alpha=0.001.

Considering all the parameters together could lead us to wrong conclusions about the metabolites that are important to characterize the genotypes. Even because this genotypes are known for showing different sensory profiles. Separate analysis was required in order to name the most important features that are responsible for the genotypes samples separation.

4.2.5 PCA and correlation analysis (processing x genotype x altitude)

The largest differences and trends for separation were observed when evaluating the PCA's for discrimination between the processing methods and between the genotypes. Higher differences were observed between the metabolite profiles of natural and demucilaged coffees, and the larger differences on the sensory score were observed on the PCA of the genotypes. The slight differences in the metabolite profile of coffee samples harvested in

different altitude ranges may have been hidden due to the larger variances in those parameters, processing and genotypes.

Separate analyses were carried out in other to capture the influence of altitude on the metabolite profile and to establish the relationship between the metabolites and the final sensory score. The dataset was divided into four sample groups, combining processing methods, genotypes and the three altitude ranges (<1000m, 1000-1200m, and >1200m).

4.2.5.1 PCA of natural coffee x Acaiá x altitude

The Figure 9 shows the PCA score scattering plot of the principal component 1 (40.23%) and the principal component 2 (29.6%) of the metabolite profiles of seeds of Acaiá coffee genotype harvested in three ranges of altitude (<1000m, 1000-1200m, and >1200m) and processed by the dry method.

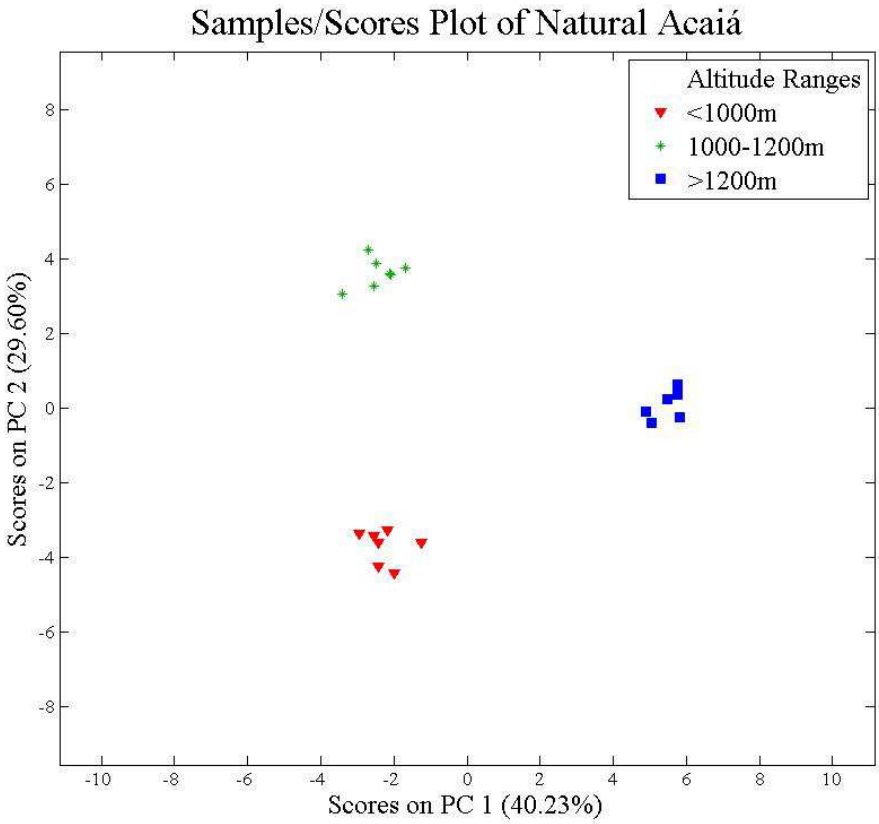

Figure 9 PCA score scattering plot of the principal component 1 (40.23% of the total variability) and principal component 2 (29.6% of the total variability) of the metabolite profile differentiating Natural Acaiá coffee seeds harvested in three different ranges of altitude (▾) <1000m, (✦) 1000-1200m, and (■) >1200m. Each class of altitude ranges in the dataset pretreated by WGLS, alpha=0.001.

The PC1 analysis showed a clear difference between the samples harvested bellow 1000m and above 1200m. However, the samples harvested in the altitude range between 1000 and 1200m were separated by PC2, which presented lower variance (29.26%). These results showed that altitude played an important role in the metabolite profile differentiation of Acaiá genotype samples processed by the dry method, mainly for those harvested above 1200m.

The Figure 10 shows the variable loadings of the principal component 1 (40.23%), which clearly distinguished the samples harvested at the most contrasting altitude range >1200m from those harvested bellow such altitude. The main metabolites of the PC1 responsible for the separation were L-serine, glutamine, L-asparagine, L-tyrosine, myo-inositol, and tryptophan for samples harvested above 1200m; and trigonelline, phenylalanine, quinic acid, mucic acid and 5-CQA for samples harvested bellow 1000m and 1000-1200m (Figure 10A). Conversely, the sensory score points to coffee samples harvested above 1200m to have higher scores than the ones harvested bellow 1200m (Figure 10A). However, the contribution was much lower for such separation comparing to the metabolites contribution below the 0.2 threshold. It may suggest that the natural Acaiá metabolic response to the altitude was larger than the sensory response.

Phenylalanine is responsible to provide the bitter taste sensation Kohl et al. (2013). This compound was the main contributor to the natural Acaiá samples harvested bellow 1000m, which might strongly contribute to the bitterness and lower total scores of coffee samples harvested at this altitude range, but bitterness is not very appreciated in the sensory analysis. Conversely, mucic acid is a sugar acid that may contribute to the acidity and also to the sweetness of coffee. However, the stability of both phenylalanine and mucic acid during the roasting process should be considered.

The samples harvested above 1200m showed higher levels of L-serine, which is a compound that has sweet taste Kawai et al. (2012). L-tyrosine and tryptophan were also present in that samples, however, they provide bitter taste. L-asparagine would be responsible for increasing the acidy taste of the coffee beverage, considering its low threshold of perception by the human sense.

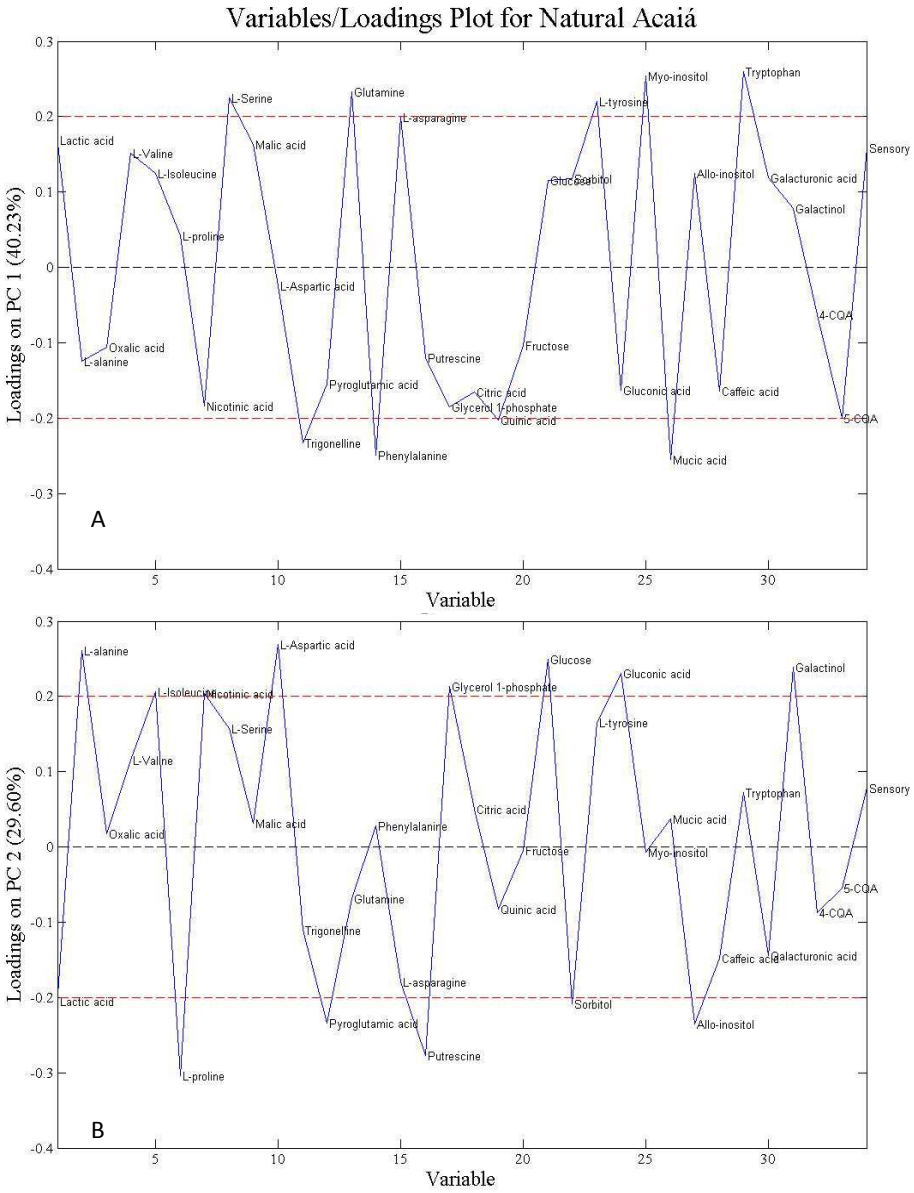

Figure 10 A) PC 1 metabolite loadings of the PCA of the Natural Acaiá coffee seeds (Figure 9). B) PC 2 metabolite loadings of the PCA of the Natural Acaiá coffee seeds (Figure 9). The red dashed line

( ) indicated the threshold (0.2) for the selection of the most important features that influenced in the separation trend of coffee samples harvested in the three altitude ranges. Each altitude class in the dataset was pretreated by WGLS, alpha=0.001.

The Figure 10B shows the PC2 metabolite loadings of the natural Acaiá coffee seeds. Despite the variance on PC2 (29.6%), information about the differences between the samples harvested bellow 1000m and 1000-1200m altitude ranges could be identified. The metabolites found differentiating the samples harvested bellow 1000m included L-proline, pyroglutamic acid, putrescine, sorbitol and allo-inositol. The metabolites found differentiating the samples harvested in the 1000-1200m altitude range were L-alanine, L-isoleucine, nicotinic acid, L-aspartic acid, glycerol 1-phosphate, glucose, gluconic acid, and galactinol.

4.2.5.2 PCA of demucilaged x Acaiá x altitude

The Figure 11 shows the PCA score scattering plot of the principal component 1 (47.37%) and principal component 2 (29.67%) of the metabolite

profile of the demucilaged Acaiá coffee seeds harvested in the three altitude ranges (<1000m, 1000-1200m, and >1200m). The demucilaged Acaiá samples harvested in these three ranges of altitude showed a clear differentiation according to those three ranges of altitude. However, the separation by PC1 was observed for the samples harvested in the 1000-1200m altitude range from the other two ranges, <1000m and >1200m. The samples harvested in the two altitude ranges, <1000m and >1200m, were separated by the PC2. Although these results showed differentiation between the samples harvested in different levels of altitude, a pattern in the metabolite profile according to the altitude increasing could not be observed. It might be due to the demucilaging processing method, which may change the final metabolite profile regardless the altitude differences.

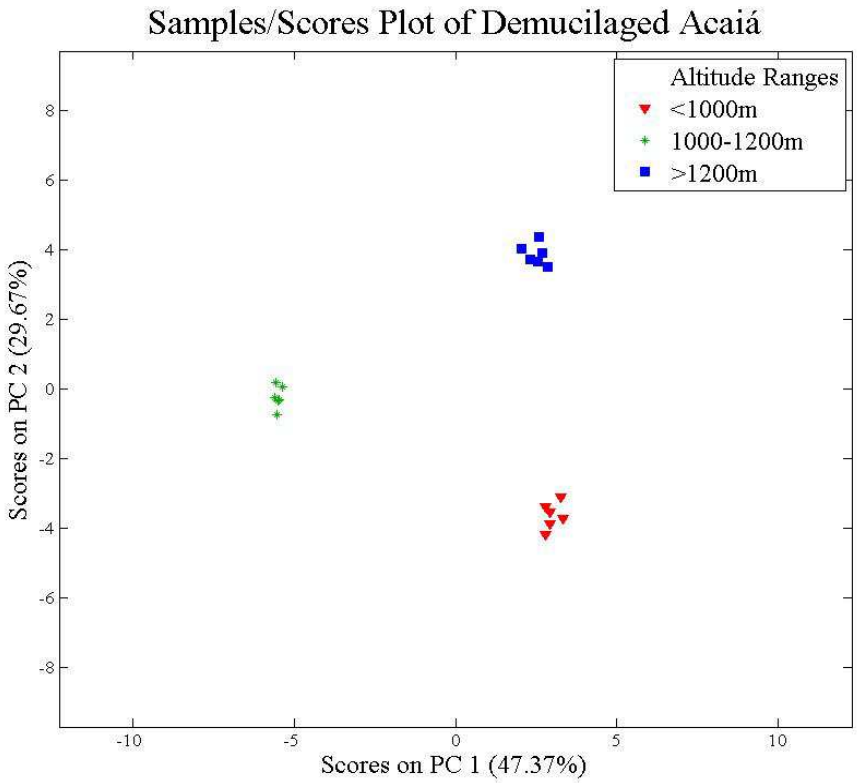

Figure 11 PCA score scattering plot of the principal component 1 (47.37% of the total variability) and principal component 2 (29.67% of the total variability) of the metabolite profile differentiating Demucilaged Acaiá coffee seeds harvested in three different ranges of altitude (▾) <1000m, (✦) 1000-1200m, and (■) >1200m. Each class of altitude ranges in the dataset pretreated by WGLS, alpha=0.001.

The Figure 12A shows the metabolite loadings of PC1 differentiating demucilaged Acaiá coffee seeds harvested in three ranges of altitude <1000m, 1000-1200m, and >1200m. The features that most influenced in the separation of the samples harvested in the 1000-1200m altitude range were oxalic acid, L-aspartic acid, phenylalanine, fructose, and galactinol. As previously observed, the samples harvested bellow 1000m and above 1200m were not separated by PC1, but by PC2.

The metabolites present in higher levels in the demucilaged Acaiá coffee samples harvested bellow 1000m were L-valine, glycerol 1-phosphate, and 5-CQA; and in the ones harvested above 1200m included L-isoleucine, L-proline, L-serine, trigonelline, putrescine, gluconic acid, myo-inositol, and galacturonic acid.

Gluconic acid and galacturonic were present in higher levels in demucilaged Acaiá coffees harvested above 1200m when comparing to the samples harvested bellow 1000m. Lactic acid and gluconic acid were also present in the demucilaged Acaiá coffees. These sugar acids may increase the acidity and the sweetness of the coffees harvested above 1200m of altitude. In addition, the acidity and sweetness are attributes appreciated when evaluating coffee quality. Coffees that present high intensity in these parameters will probably be high scored.

On the opposite side, the demucilaged Acaiá samples harvested bellow 1000m of altitude, showed higher levels of L-valine, glycerol 1-phosphate and 5-CQA, which contributes to the bitterness Akitomi et al. (2013) and Farah et al. (2006). The presence of these compounds on coffee seeds harvested bellow 1000m may explain their lower scores when compared to the samples harvested >1200m.

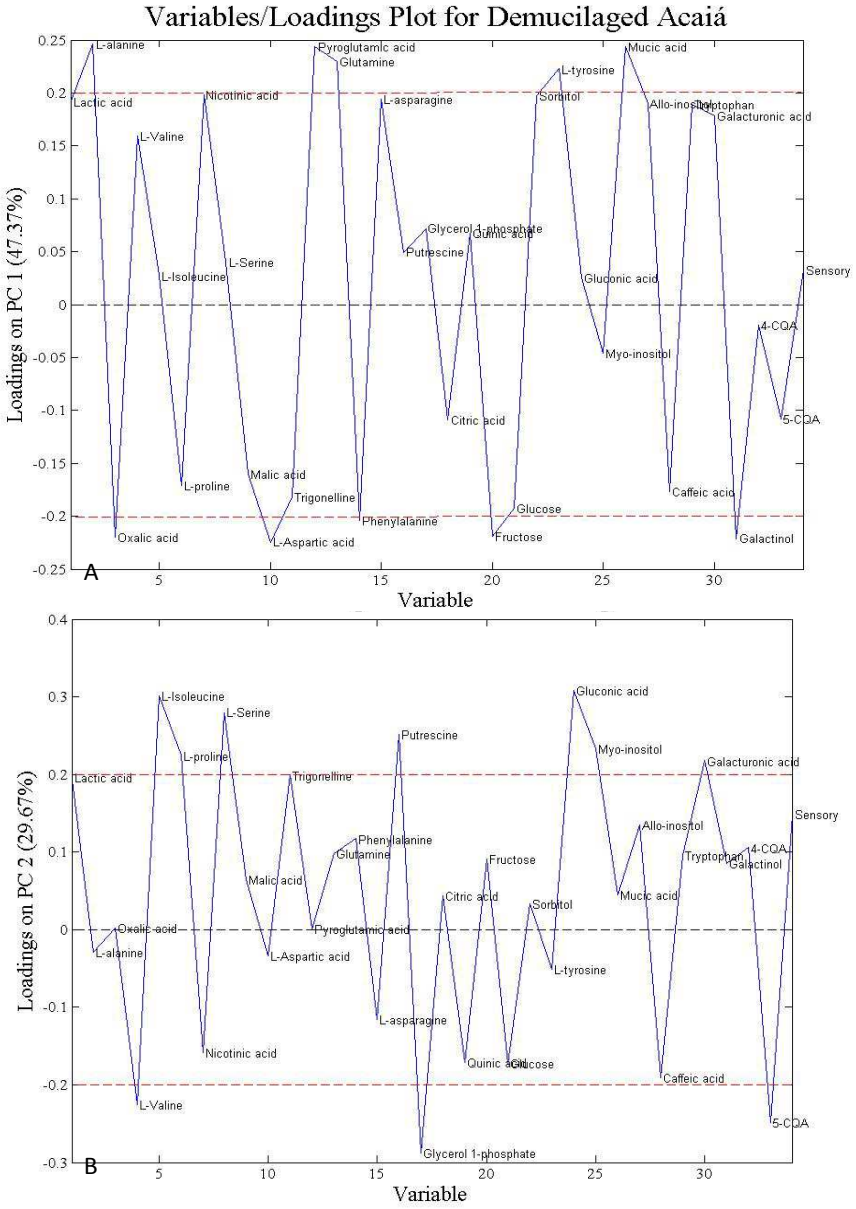

Figure 12 A) PC 1 metabolite loadings of the PCA of the demucilaged Acaiá coffee seeds (Figure 11). B) PC 2 metabolite loadings of the PCA of the Natural Acaiá coffee seeds (Figure 11). The red dashed line

( ) indicated the threshold (0.2) for the selection of the most important features that influenced in the separation trend of coffee samples harvested in the three altitude ranges. Each altitude class in the dataset was pretreated by WGLS, alpha=0.001.

4.2.5.3 PCA of natural coffee x Yellow Bourbon x altitude

The Figure 13 shows the PCA score scattering plot of the principal component 1 (51.82%) and of the principal component 2 (20.24%) of the metabolite profiles of natural Yellow Bourbon coffee seeds harvested in three ranges of altitude (<1000m, 1000-1200m, and >1200m). The natural Bourbon samples harvested in these three ranges of altitude showed clear differentiation and grouping according to the altitude range where they were harvested. However, the samples harvested in the 1000-1200m altitude range were right in the middle of the other two altitude ranges, <1000m and >1200m. This event in this PCA analysis showed that the variation in the metabolite profile may vary according to the variation in the altitude level.

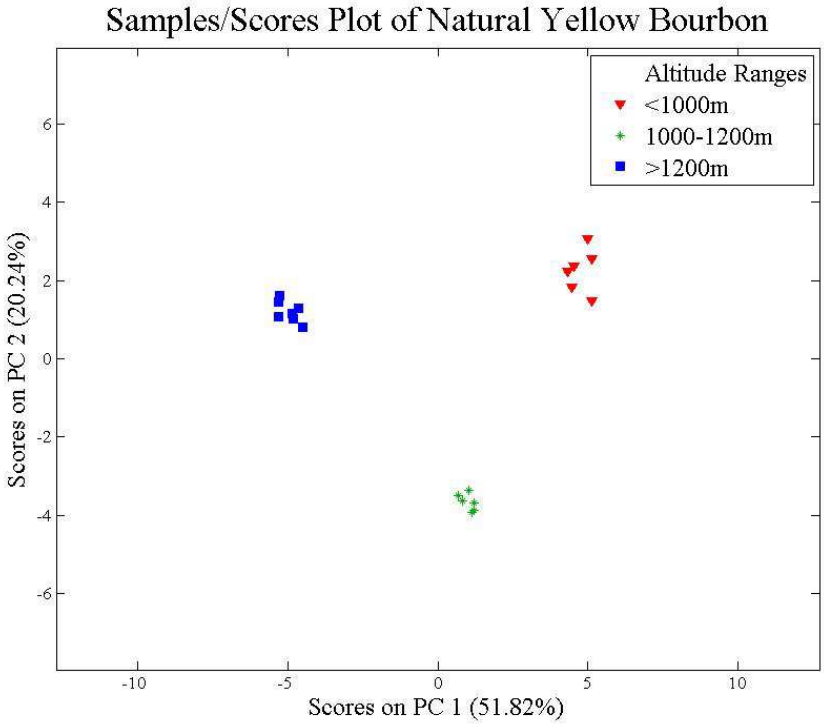

Figure 13 PCA score scattering plot of the principal component 1 (51.82% of the total variability) and principal component 2 (20.24% of the total variability) of the metabolite profile differentiating Natural Yellow Bourbon coffee seeds harvested in three different ranges of altitude (▼) <1000m, (✦) 1000-1200m, and (■) >1200m. Each class of altitude ranges in the dataset pretreated by WGLS, alpha=0.001.

The Figure 14A shows the variable loadings of the PC1 of the total variance, which clearly distinguished the samples harvested at the most contrasting ranges of altitude, <1000m and >1200m. The sensory score loading on PC1 shows that the natural Bourbon samples harvested above 1200m of altitude have higher scores than the samples harvested bellow 1000m (Figure 9B). This indicates the strong effect of the altitude on the final sensory score of the natural Yellow Bourbon samples. In addition, this result may indicate that for the group of metabolites analyzed, some of them may be related to quality.

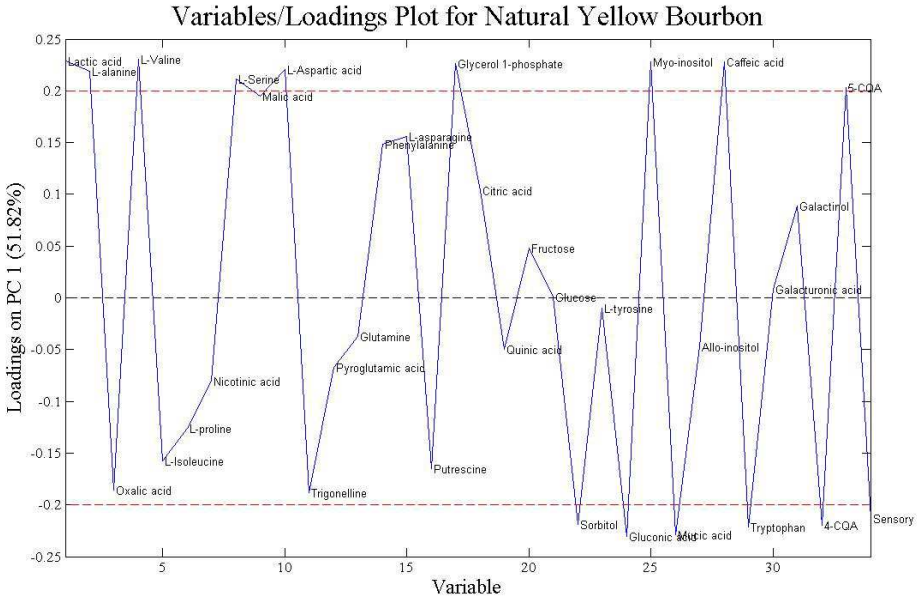

Figure 14 PC 1 metabolite loadings of the PCA of the Natural Yellow Bourbon coffee seeds (Figure 13). The positive values on the Y axis of the PC1 loadings indicated the most important features for the Natural Yellow Bourbon coffee samples harvested above 1200m of altitude and the negative values on the Y axis indicated the most important features for the Natural Yellow Bourbon coffee samples harvested bellow 1200m. The red dashed line

( ) indicated the threshold (0.2) for the selection of the most important features that influenced in the separation trend of coffee samples harvested in the three altitude ranges. Each altitude class in the dataset was pretreated by WGLS, alpha=0.001.

The features that most influenced in this differentiation were lactic acid, L-alanine, L-valine, L-serine, L-aspartic acid, glycerol 1-phosphate, myo-inosiotol, caffeic acid, and 5-CQA for natural Bourbon samples harvested bellow 1000m of altitude (Figure 14). The samples harvested above 1200m were mostly influenced by sorbitol, gluconic acid, mucic acid, tryptophan, and 4-CQA.

The higher levels of other compounds such as gluconic acid and mucic acid may increase the acidity and sweetness in a very suitable way towards quality. The high sensory score united to this group of metabolites found in the Yellow Bourbon coffee samples harvested above 1200m may suggest they are strong potential markers for good cup quality.

L-tyrosine, myo-inositol, and 5-CQA for natural Bourbon samples harvested bellow 1000m. Only myo-inositol would increase the sweetness in the

coffee samples, considering it is a sugar alcohol and it remains after the roasting Ruiz-Matute et al. (2007). Conversely, L-tyrosine and 5-CQA would increase the bitterness taste Akitomi et al. (2013), Farah et al. (2006) and Kohl et al. (2013) .

4.2.5.4 PCA demucilaged x Yellow Bourbon x altitude

The Figure 15 shows the PCA score scattering plot of the PC1 (55.44%) and PC2 (20.04%) of the metabolite profiles of demucilaged Yellow Bourbon coffee seeds harvested in three ranges of altitude (<1000m, 1000-1200m, and >1200m). The samples harvested above 1200m were different from the ones harvested bellow such altitude by PC1. Conversely, the samples harvested bellow 1000m and between 1000-1200m were separated in two different groups, however they showed much lower variance in PC2 compared to PC1. This indicated that higher altitudes play an important role for the Yellow Bourbons seeds metabolism, which allied to the processing method showed strong interference on the separation.

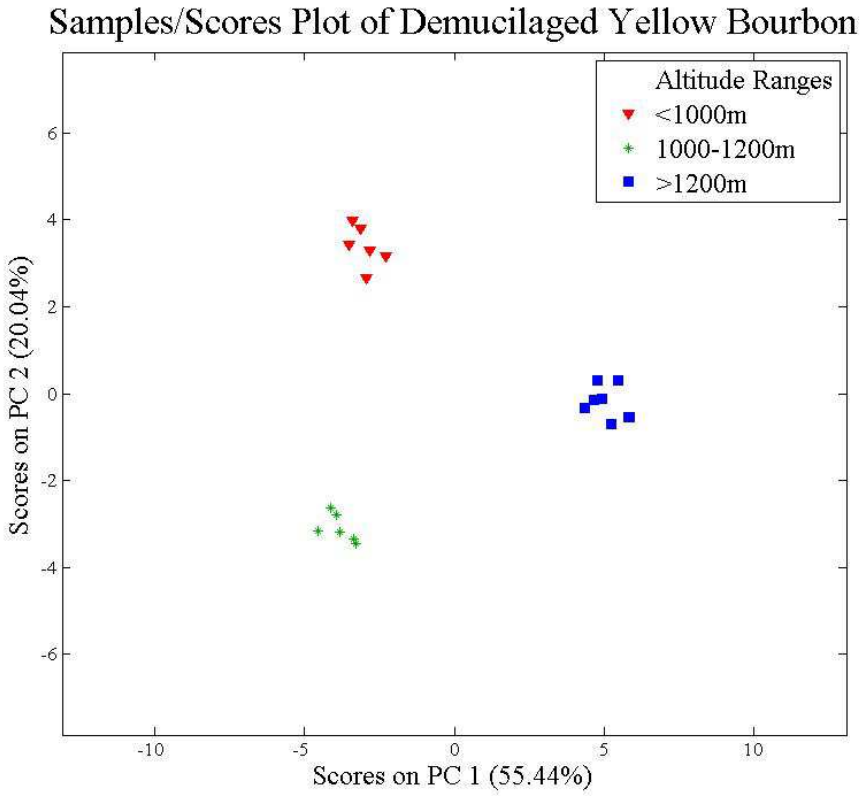

Figure 15 PCA score scattering plot of the principal component 1 (40.23% of the total variability) and principal component 2 (29.6% of the total variability) of the metabolite profile differentiating Demucilaged Yellow Bourbon coffee seeds harvested in three different ranges of altitude (▾) <1000m, (✦) 1000-1200m, and (■) >1200m. Each class of altitude ranges in the dataset pretreated by WGLS, alpha=0.001.

The Figure 16A shows the metabolite loadings of PC1 differentiating demucilaged Yellow Bourbon coffee genotype harvested in three ranges of altitude <1000m, 1000-1200m, and >1200m. The features that most influenced the separation of the samples harvested above 1200m were L-serine, L-aspartic acid, L-asparagine, fructose, glucose, and mucic acid.

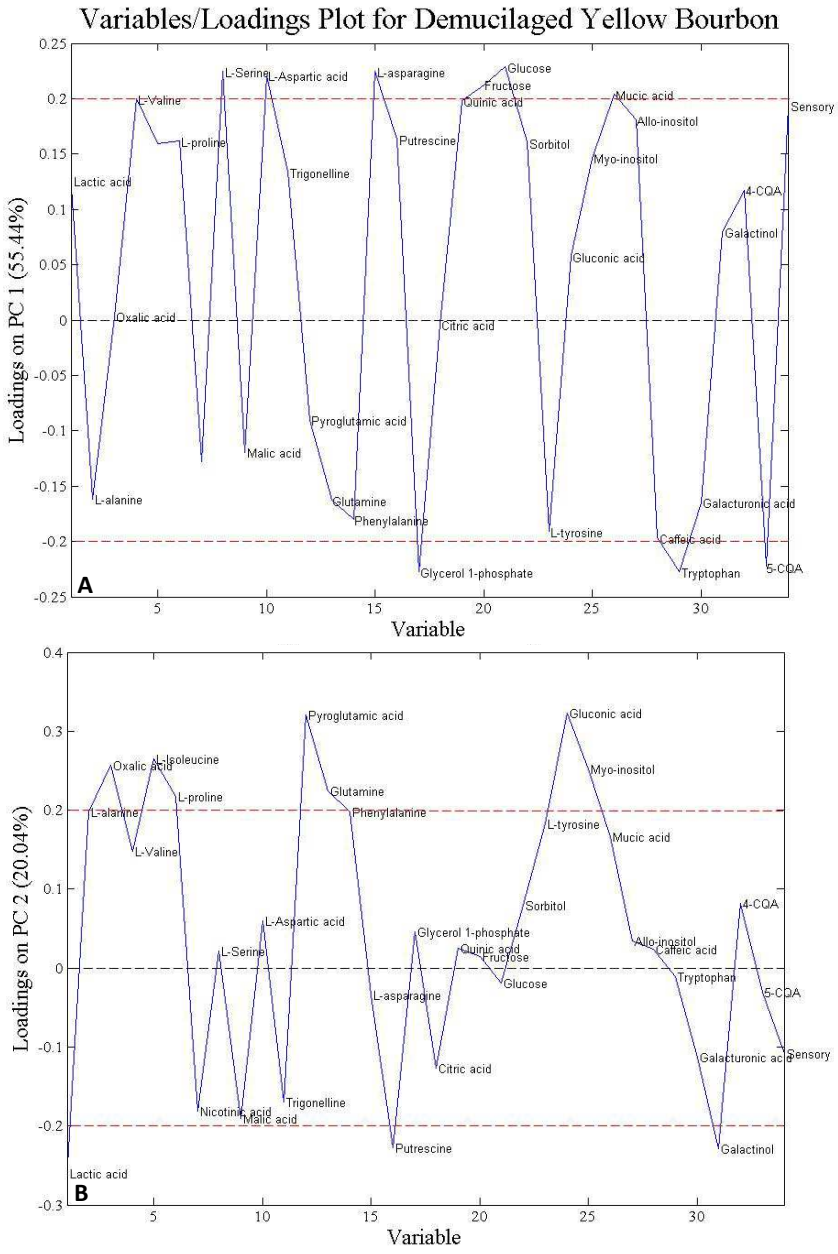

Figure 16 A) PC 1 metabolite loadings of the PCA of the Demucilaged Yellow Bourbon coffee seeds (Figure 15). B) PC 1 metabolite loadings of the PCA of the Demucilaged Yellow Bourbon coffee seeds (Figure 15).

The red dashed line (
) indicated the threshold (0.2) for the selection of the most important features that influenced in the separation trend of coffee samples harvested in the three altitude ranges. Each altitude class in the dataset was pretreated by WGLS, alpha=0.001.

The sensory score did not reached the 0.2 threshold for the separation of the samples, however it pointed to samples harvested above 1200m showing higher scores than the other ones. This could indicate a higher correlation between the compounds found for those samples and the sensory score.

Among those amino acids found in the Yellow Bourbon coffee samples, L-serine provides bitter taste, L-aspartic acid and L-asparagine provide acidy taste Kirimura et al. (1969). L-aspartic acid and L-asparagine are the major amino acids found in coffee seeds Arnold et al. (1994). In addition to contributing to increase the acidity, the threshold of detection of asparagine is very low, which means that a very small amount can be human detected Kirimura et al. (1969). In the same trend, L-aspartic acid would help to increase the acidity taste, however in much lower rates than asparagine, considering it has

much higher threshold for human detection. Their higher presence in coffee seeds would indicate higher sensory quality.

The features that most influenced on the discrimination of the samples harvested bellow 1200m (<1000m and 1000-1200m) and the ones harvested above 1200m included glycerol 1-phosphate, tryptophan, and 5-CQA.

The samples harvested bellow 1000m showed most compounds that may increase the bitterness in coffee. The compounds 5-CQA and tryptophan increases the bitter taste Akitomi et al. (2013), Farah et al. (2006) and Kirimura et al. (1969). On the other hand, glycerol 1-phosphate the sweetness as a sugar alcohol.

4.2.5.5 Correlation analysis of the metabolites and sensory score

The analysis of the four combinations showed that both genotypes positively responded to altitude variation by increasing the final sensory score. This could be observed checking on the direction of the variable loadings contribution for the separation on each PCA (Figures 10, 12, 14 and 16), where the total sensory score always points to the higher altitudes, >1200m. However, the total sensory score of the Yellow Bourbon genotypes always showed higher influence on the samples separation than on the Acaiá genotypes. It could indicate that the Yellow Bourbon genotype responded better to the altitude variation than Acaiá genotype, regardless the processing used.

A broad range of metabolites were analyzed by PCA's and the influence of the altitude variation, processing and genotype on their content were observed. However, considering many of them have never been linked to quality, the correlation analyses were performed for the following combinations: natural Acaiá harvested at <1000m, 1000-1200m, >1200m; natural Yellow Bourbon harvested at <1000m, 1000-1200m, >1200m; Acaiá harvested at <1000m, 1000-1200m, >1200m and processed in the wet way; and Yellow

Bourbon harvested at harvested at <1000m, 1000-1200m, >1200m and processed in the wet way. The TABLE 2 shows the results of the correlation analysis of all metabolites and sensory score for each combination.

The correlation coefficients might seem to be low, however it is worth noting that these analyses were carried out in green coffee beans, prior to roasting. In addition, mentioning that several reactions take place during roasting is very important. The metabolites may react among themselves disappearing after the coffee seeds are roasted, but releasing new compounds Farah et al. (2006) and Leroy et al. (2006). However, the presence of these compounds in green coffee seeds may provide a glimpse of the compounds that work as precursors of flavor, consequently determining coffee quality.

Table 2 Correlation coefficients of the analysis between the metabolite profile and the sensory score of coffees separated by processing and genotype (natural Acaiá, natural Yellow Bourbon, demucilaged Acaiá, and demucilaged Yellow Bourbon). Attributed classes according to the altitude ranges (<1000m, 1000-1200m, and >1200m) and pretreated by GLSW (a=0.001).

Natural Acaiá		Demucilaged Acaiá		Natural Yellow Bourbon		Demucilaged Yellow Bourbon	
Metabolite	**Sensory Correlation**	**Metabolite**	**Sensory Correlation**	**Metabolite**	**Sensory Correlation**	**Metabolite**	**Sensory Correlation**
Trigonelline	-0.57	5-CQA	-0.41	Lactic acid	-0.88	Glycerol 1-phosphate	-0.86
Mucic acid	-0.51	Glycerol 1-phosphate	-0.37	Caffeic acid	-0.87	Tryptophan	-0.82
Phenylalanine	-0.50	Caffeic acid	-0.36	Myo-inositol	-0.86	L-tyrosine	-0.81
Pyroglutamic acid	-0.49	Glucose	-0.33	L-Aspartic acid	-0.86	5-CQA	-0.79
Quinic acid	-0.47	L-Valine	-0.24	L-Valine	-0.84	Phenylalanine	-0.77
Putrescine	-0.46	Quinic acid	-0.19	L-alanine	-0.84	Glutamine	-0.76
Caffeic acid	-0.45	L-Aspartic acid	-0.16	Glycerol 1-phosphate	-0.83	L-alanine	-0.70
5-CQA	-0.44	Nicotinic acid	-0.12	L-Serine	-0.81	Caffeic acid	-0.69
Citric acid	-0.29	Oxalic acid	-0.11	5-CQA	-0.74	Pyroglutamic acid	-0.57
Nicotinic acid	-0.24	L-asparagine	-0.08	Malic acid	-0.71	Galacturonic acid	-0.49
Glycerol 1-phosphate	-0.23	Citric acid	0.01	L-asparagine	-0.60	Nicotinic acid	-0.30
Fructose	-0.21	Malic acid	0.01	Phenylalanine	-0.49	Malic acid	-0.30
Oxalic acid	-0.19	Galactinol	0.01	Citric acid	-0.39	Oxalic acid	-0.17
4-CQA	-0.19	Fructose	0.02	Galactinol	-0.33	Gluconic acid	0.00
Gluconic acid	-0.17	L-tyrosine	0.04	Fructose	-0.23	Citric acid	0.10
L-proline	-0.14	Phenylalanine	0.05	Galacturonic acid	0.05	Myo-inositol	0.35
L-alanine	-0.06	L-alanine	0.08	Glucose	0.09	4-CQA	0.35
Sorbitol	0.08	Pyroglutamic acid	0.12	L-tyrosine	0.11	L-Isoleucine	0.40
Allo-inositol	0.08	Sorbitol	0.14	Quinic acid	0.20	L-proline	0.41
Galacturonic acid	0.14	4-CQA	0.14	Glutamine	0.21	Galactinol	0.46
L-Aspartic acid	0.15	Trigonelline	0.18	Pyroglutamic acid	0.21	Sorbitol	0.54
Lactic acid	0.20	Mucic acid	0.19	Nicotinic acid	0.24	Trigonelline	0.60
L-asparagine	0.29	Tryptophan	0.22	Allo-inositol	0.24	Mucic acid	0.62
Galactinol	0.32	L-proline	0.23	L-proline	0.43	Allo-inositol	0.63
Malic acid	0.35	Glutamine	0.25	L-Isoleucine	0.54	L-Valine	0.63
L-Valine	0.40	Allo-inositol	0.28	Putrescine	0.56	Lactic acid	0.65
L-Isoleucine	0.41	Myo-inositol	0.30	Trigonelline	0.64	Quinic acid	0.71
Glucose	0.42	Putrescine	0.36	Oxalic acid	0.70	Fructose	0.75
Glutamine	0.44	Lactic acid	0.37	4-CQA	0.83	Putrescine	0.77
Myo-inositol	0.52	Galacturonic acid	0.39	Mucic acid	0.84	L-Aspartic acid	0.77
L-tyrosine	0.59	L-Serine	0.40	Sorbitol	0.84	L-Serine	0.80
L-Serine	0.59	L-Isoleucine	0.43	Gluconic acid	0.85	Glucose	0.84
Tryptophan	0.60	Gluconic acid	0.44	Tryptophan	0.85	L-asparagine	0.85

The higher correlation coefficients were shown for the natural and demucilaged Yellow Bourbon. On the other hand, the correlation coefficients shown by the Acaiá genotypes were very low. Thus, the Yellow Bourbon genotype can provide good information about the metabolites that are positive and negative potential markers for quality in coffee seeds.

4.2.5.5.1 Positively correlated metabolites to sensory quality

Considering a +0.8 threshold correlation, the positively correlated metabolites to the total sensory score were L-asparagine (0.85), tryptophan (0.85), gluconic acid (0.85), glucose (0.84), sorbitol (0.84), mucic acid (0.84), 4-CQA (0.83), and L-serine.

Among the positively correlated metabolites were amino acids that had previously been related to good sensory attributes; L-asparagine to the acidity; L-serine to sweetness Akitomi et al. (2013), Kirimura et al. (1969) and Solms (1969). Gluconic acid, mucic acid are sugar acids that besides increasing the sweetness of the coffee beverage may increase the acidity. Those are two of the most appreciated parameters in coffee sensory analysis.

Glucose was expected to be positively correlated, considering it is a sugar, it contributes to sweetness increasing.

4.2.5.5.2 Negatively correlated metabolites to sensory quality

Considering a -0.80 threshold correlation, the negatively correlated metabolites to the total sensory score included lactic acid (-0.88), caffeic acid (-0.87), myo-inositol (-0.86), L-aspartic acid (-0.86), glycerol 1-phosphate (-0.86), L-valine (-0.84), L-alanine (-0.84), tryptophan (-0.82), L-serine (-0.81), and L-tyrosine (-0.81).

For the first time glycerol 1-phosphate has been found as a negatively correlated compound to the total sensory score. As a polyol, it was expected to increase the sweetness to the coffee beans, which is very appreciate in the sensory analysis. However, it may had turned into a new unpleasant compound by the roasting process.

L-serine was positively correlated to the sensory quality of demucilaged Yellow Bourbon, however, it was negatively correlated to the sensory quality of natural Yellow Bourbon. Conversely, tryptophan was positively correlated to the sensory quality of natural Yellow Bourbon and negatively correlated to the sensory quality of the demucilaged Yellow Bourbon. It may have indicated that these compounds may not have strong influence on the total sensory score.

5 CONCLUSIONS

The processing method was the parameter that most influenced the differentiation of the metabolite profile, mainly L-valine, L-isoleucine, L-proline, pyroglutamic acid, fructose, L-tyrosine, and allo-inositol were observed in higher levels in the natural coffee beans; and L-aspartic acid, putrescine, mucic acid, and galactinol for demucilaged coffees were observed in higher levels in the demucilaged coffee beans;

The effects of altitude on the coffee samples was hidden by the greater variances showed by the processing and genotypes;

The slope exposure differences were not detectable using this metabolomics approach and statistical analysis;

The evaluated genotypes showed a trend to be separated by the metabolite profile and by the sensory score, which strongly contributed for the separation of Yellow Bourbon genotype compared to Acaiá genotype;

The metabolite profiles of Yellow Bourbon genotype showed a more consistent correlation to the sensory score than the metabolite profiles of Acaiá genotype.

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