Green and Roasted Arabica Coffees Differentiated by Ripeness, Process and Cup Quality
via Electrospray Ionization Mass Spectrometry Fingerprinting

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A habilidade da técnica de espectrometria de massas com infusão direta e ionização por eletronebulização (IES-EM), nos modos de íons positivos e negativos, foi avaliada na diferenciação de cafés Arábica verdes e torrados e com diferentes estágios de amadurecimento (verde, maduro e semi-úmido) e cafés classificados por prova de xícara. No modo negativo, a análise dos cafés verdes mostrou que os íons correspondentes aos ácidos graxos e ácidos clorogênicos desprotonados são os mais importantes para a discriminação da maturidade. No modo positivo, a maturidade é diferenciada através de íons correspondentes a cafeína, ácidos clorogênicos protonados e adutos de K+ de ácidos graxos. Na diferenciação da pós-colheita, em ambos os modos de ionização, são mais importantes os íons correspondentes aos ácidos graxos, ácidos clorogênicos, açúcares e ácidos carboxílicos formados da fermentação. Cafés Arábica torrados também são discriminados com eficiência. No modo negativo, são importantes os íons correspondentes aos ácidos clorogênicos e ácidos orgânicos de cadeia curta, derivados de açúcares. No modo positivo, a discriminação é realizada por íons de baixa m/z tais como piridina e alquil piridinas protonadas, formadas através da degradação da trigonelina. Ambos os IES(+)-EM e IES(-)-EM são capazes de discriminar diferentes cafés Arábica torrados classificados por prova de xícara e os íons que permitem esta diferenciação são os mesmos descritos para a maturidade e processos pós-colheita.

Keywords: Arabica coffee, ripeness, post-harvest, cup quality, ESI-MS fingerprinting

Introduction

Coffee is the most popular beverage in the world and a commodity of extreme importance to developing countries. There are several species in the genus Coffea (Rubiaceae), but Coffea arabica and Coffea canephora are the two most commercialized around the world, which are commonly known as Arabica and Robusta coffees, respectively.

A considerable amount of research has been undertaken on the chemical composition and flavors in green and
roasted coffees, which helps to understand botanical, agricultural and process influences on coffee quality and also to tentatively assure origin authentication and avoid adulteration. Most studies on differentiation deals with Arabica and Robusta green and roasted coffees in relation to their major chemical composition such as metal, lipids focused on sterolic and alcohol diterpenes, alkaloids, chlorogenic acids and sugar profiles. Chemometric discrimination using principal component analysis (PCA) has been successfully used as an important tool to differentiate roasting processes, geographical origin of green and roasted coffees and other aspects of coffee process. To search for faster methods for coffee discrimination based on fingerprinting analysis, NIR and NMR studies were also applied.

A new and potential technique is direct infusion electrospray ionization mass spectrometry (ESI-MS). ESI-MS employs little sample preparation and gives immediate compositional information for ESI-ionizable compounds. It has been very efficient for fingerprinting complex mixtures such as that of soybean extracts, tea, spices, vegetable oil and more recently, it has been applied to investigate the differences between Arabica and Robusta defective green coffees.

We report herein the ESI-MS fingerprinting of the methanolic extracts of Arabica coffees in the positive and negative ion modes, ESI(+)-MS and ESI(-)-MS, respectively. The ESI-MS data were handled by principal component analysis (PCA) to differentiate both green and roasted Arabica coffees with different stages of ripeness (green or immature, cherry or ripe and overripe), post-harvesting process (dry, wet and semi-wet) and also coffees with different cup qualities. Cup qualities is a Brazilian classification method for Arabica coffees based on the brewing method of steeping, that generates a classification list, from the best to the worst, using the following denominations: strictly soft, soft, barely soft, hard, riosh, rio and rio zona. For the analysis of coffees with different cup qualities, discriminant analysis was applied on PCA data, since PCA alone was unable to perform such discrimination.

**Experimental**

**Sample description**

**Ripeness and post-harvest process samples**

Five samples of Brazilian Arabica coffee from the 2006 crop were directly obtained from the Fazenda São José, São José do Rio Preto, RJ, Brazil. The coffee plants of the 2.5 ha area chosen for the experiment had 20 years of production and the harvest was made by strip-picking with the cherries being collected on a sheet. The harvested cherries were then sorted and cleaned. The green (immature) and overripe (overripe) cherries were transferred to dry on a sun terrace. The harvested ripe coffee cherries were split in three parts, one part being dry processed in the same way than green and overripe cherries (ripe or dry), and the other two parts were semi-wet and wet processed. In the semi-wet process, the pulp was machine removed and the resulting cherry was transferred to dry on a sun terrace (semi-wet). In the wet process the pulp was also machine removed from the cherries, after which the cherries were fermented in tanks with water for 16 h. After this, the coffee cherries were thoroughly washed and transferred to dry on a sun terrace (wet). All samples were dried for 5 days on the sun terrace, with temperatures ranging from 35 to 40 °C and were finally dried in a hot air wood burner until reaching ± 11% moisture (hot air temperature of 50 °C and coffee temperature of 40 °C).

Coffee samples were roasted in a convection oven at 155 °C for 15 min and stored in sealed plastic bags in a freezer at −8 °C until used. Prior to each analysis, the coffee was brought to ambient temperature and ground in a Wiley mill (Tecnal Te-650, Brazil) for 60 s.

**Cup quality samples**

Five samples with medium roast, considered to be typical of different cup quality, identified as soft, hard, riosh, rio and rio zona, were kindly supplied by ABIC (Associação Brasileira das Indústrias do Café).

**Extraction**

Ground coffee samples (1 g) were extracted by reflux with 4 mL of methanol for 2 h (condenser at 5 ± 2 °C). After that time, samples were filtered in a qualitative Whatman filter paper. Samples were kept in a freezer at −8 °C until used. For each sample, three different extractions were made, identified in the figures by the number following the name of the sample.

**Mass spectrometry**

Mass spectra were acquired by using a quadrupole/time-of-flight (Q-ToF) mass spectrometer (Micromass, Manchester, U.K.). General conditions were as follows: source temperature of 100 °C, capillary voltage of 2.1 kV, and cone voltage of 15 to 30 V. Prior to the ESI-MS analysis, 1 μL of an aqueous solution of 0.1% ammonium hydroxide (v/v) or formic acid was added to 1.00 mL of each sample and the mixture vigorously stirred for 15 s. Sample introduction was performed by using a syringe pump (Harvard Apparatus, Pump 11) at a flow rate of
10.0 μL min⁻¹ and pumped through an uncoated fused silica capillary. Each analysis required about 60 s. Mass spectra were acquired by scanning over the 50-1000 m/z range. ESI tandem mass spectra were obtained by selection of a specific ion by Q1, by using a unitary m/z window, which was then submitted to collision-induced dissociation (CID) with argon in the hexapole collision chamber at energies of 15-25 eV. The product ion MS analysis was accomplished with the orthogonal TOF (time-of-flight) analyzer.

**ESI-MS data handling and statistical treatment**

All mass spectra were accumulated over 60 s, centered, aligned, and handled using MassLynx 3.5 software (Waters, Manchester, U.K.). The abundance readings, for each mass spectrum, were summed into integral m/z ion readings and normalized to maximum abundance value using an in-house built program. To discard noise, only the ions with a relative abundance higher than 5% were included in the final data matrix. The remaining m/z ion values were aligned and compiled to generate a final matrix where each line was a sample and each column a variable (m/z ratios and relative intensities of detected ions). Multivariate analyses by PCA and Partial Least Squares (PLS) were performed using the software Unscrambler, version 9.1. PLS was performed, with autoscaled data, to select the most important variables able to discriminate between different groups. The regression coefficients were calculated and, since the ones with the highest positive or negative values are the most important for the model, the variables related to them were selected for running the PCA with autoscaled data. Data to determine cup quality was first submitted to PCA and the first eight principal components were calculated and submitted to discriminant analysis using the software Statistica, version 7.0.

**Results and Discussion**

**ESI-MS fingerprints of green and roasted Arabica coffees**

Green and roasted coffees were ground and refluxed with methanol. The samples were not defatted, differently to other reported procedures, to investigate the real contribution of each class of compounds present in coffee. The contents of lipids have been shown to be an important group in coffee differentiation.³,¹⁶

Figure 1 shows the ESI-MS fingerprints both in the negative and positive ion modes of two typical samples of green and roasted Arabica coffees. Due to the complex chemical nature of coffee,²⁻⁶ both ionization modes contribute with important ions to characterize coffee composition.

In general, ESI(-)-MS of green coffees showed [M–H]⁻ ions of chlorogenic acids as the most abundant compounds. Frequently, the most intense ion is of m/z 353, which can

![Graphs showing ESI-MS fingerprints of methanol extracts of coffees: (A) crude in the negative ion mode, (B) crude in the positive ion mode, (C) roasted in the negative ion mode and (D) roasted in the positive ion mode.](image-url)
be associated with caffeoylquinic acid isomers (3, 4 and 5-CQA). Other characteristic ions are those of m/z 529, 515, 367 due to caffeoylferuloyl (CFQA), dicaffeoyl (CDQA) and feruoylquinic (FQA) acids, respectively, besides their fragment ions of m/z 191, 179, 173 and 163 (for example) as extensively discussed by Clifford and co-workers.17-19 Chlorogenic acids are well-known constituents of coffee

Coffee cherries turn red when ripe due to the replacement of chlorophyll in the pericarp by red flavonoid pigments. The color of cherry is a good marker of maturation and coffee cherries can be classified into three maturation classes: green or immature, mature or ripe and overripe. Maturation clearly favors the development of high quality flavors in coffee brew. The main volatile precursors identified in coffees are trigonelline, aminoacids, sugars, chlorogenic acids, lipids and carotenoids.25

Green Arabica coffees (immature, ripe and overripe) are differentiated via ESI(-)-MS by ions related to deprotonated fatty acids (m/z 255, 279, 283 from palmitic, linoleic acid and stearic acids) and to the monomers, dimers and trimers of chlorogenic acids (m/z 353, 557 and 695).

Another relevant group in ripeness discrimination is observed between m/z 500 and 600. Two of these ions, those of m/z 535 and 511, show in their ESI-MS/MS the fragment ions of m/z 353, 191, 179 and 173, suggesting the presence of caffeoylquinic acid. The difference of the molecular mass from 353 Da and the presence of ions of m/z 279 and 255 with their CO₂ loss fragments observed in the ESI-MS/MS suggest the presence of 2-methylbut-2-enoic acid (tiglic acid, C₆H₁₀O₂) and 2-butenoic acid (crotonic acid, C₅H₈O₂) acids esterifying caffeoyl quinic moieties. Similar compounds have been described in studies of phenylpropane metabolism of tomato (Solanaceae) cotyledons and in chrysanthemum (Asteraceae) phytochemical screening.26,27

The concentration of chlorogenic acid oligomers are known to decrease along ripeness, which reflects in the better quality of the beverage obtained with ripe cherry beans. The variability of lipids concentration was studied by Jham et al.,28 who showed a pronounced decrease of diacylglycerols in coffees beans from unripe to ripe, the same happening with fatty acids. These previous results justify the relevance of these ions to discriminate between coffee ripeness.

From a matrix of 9 samples and 23 variables, the ions selected as being the most important for the separation in ESI (-)-MS are those of m/z 191, 192, 255, 279, 353, 511, 535, 557 and 695. In this plot, PC1 explains 55% and PC2 44% of the total data variation, summing up to 99% in total. Although the separation among the different ripeness samples has been achieved with the use of only PC1 or only PC2, a better separation is obtained with the two PCs. The immature samples are positively correlated with ions of m/z 511 and 535, the ripe samples are positively correlated with ions of m/z 191 and 192 but negatively correlated with that of m/z 353 and the overripe samples are positively correlated with ions of m/z 695 but negatively correlated with those of m/z 255 and 279.

In the ESI(+) -MS of green coffees, the main ions responsible for maturity differentiation are relative to

Ripeness

Coffee cherries turn red when ripe due to the replacement of chlorophyll in the pericarp by red flavonoid pigments.
protonated caffeine (m/z 195) and chlorogenic acids. Caffeine concentration decreases along ripeness but not so much as chlorogenic acids, as it has been pointed out by Clifford and Kazi. K⁺ adducts of fatty acids such as linoleic acid (m/z 319) are also representative. Another important ion, commonly observed in all spectra, is that of m/z 104. By ESI-MS/MS, it could be associated with malonic acid by the loss of CH₃CO₂H (m/z 60), as observed for the deprotonated species of m/z 103. Malonic acid was found in roasted coffees but in minor amounts and, one supposition is that it could be generated in the rearrangement of malonylglycosides. O- and C-glycosylflavonoids have been found to be acylated by malonic, succinic and malic acids, as pointed out by Harborne in his extensive study of anthocyanins and related phenolics. These compounds could give rise to malonic acid during fragmentation.

From a matrix of 9 samples and 24 variables, the ions selected as being the most important for this separation are those of m/z 104, 132, 133, 195, 221, 355, 394, 407, 419 and 747. In this plot, PC1 explains 75% and PC2 20% of the total data variation, summing up to 95% in total. The separation among the immature, ripe and overripe has been achieved with the use of only PC1 and the variable with more influence in PC1 is that of m/z 104, which is positively correlated with the immature samples.

Although carbohydrate contents show very significant alterations during coffee maturity, ions related to reduced and non-reduced sugars were not between the 10 first variables used to discriminate ripeness in this work. Only minor carboxylic acids such as oxaloacetic acid of m/z 133 are seen probably related to its presence as an intermediate in the citric acid cycle.

Figure 2 shows the PCA scores and loadings bi-plot obtained with selected ESI-MS, (a) negative ion mode, (b) positive ion mode, data of green coffees with different ripeness.

The different ripeness stages of roasted Arabica coffee were well discriminated by ESI-MS. In the negative ion mode, contributions due to ions of higher m/z from chlorogenic acids are the most important for discrimination. Due to roasting, low m/z ions are observed in the spectra and short-chain organic acids ions such as glycolic (m/z 75), oxalic (m/z 89) and mesaconic acids (m/z 129 and 128) together with ferulic, cinnamic and caffeic acids and phenols as catechol (m/z 109) are also observed.

From a matrix of 9 samples and 48 variables, the ions selected as being the most important for ESI(-)-MS separation are those of m/z 109, 128, 133, 337, 353, 354, 373 and 527. In this plot, PC1 explains 66% and PC2 25% of the total data variation, summing up to 91% in total. Although the separation among the different ripeness samples has been achieved with the use of only PC1, a better separation is obtained with the two PCs. The immature samples are negatively correlated with all variables, whereas the ripe and overripe samples are positively correlated with all. PC2 discriminates between ripe and overripe samples, with ions of m/z 109, 128 and 337 are positively correlated with ripe samples and ions of m/z 133, 337, 353, 354 and 527 positively correlated with overripe samples.

In the ESI(+)–MS, discrimination of roasted coffees are mainly performed by low m/z ions such as protonated pyridine (m/z 80) and alkylpiridines (m/z 94 and 108 due to methylpyridinium and 1,4-dimethylpyridinium, respectively), formed via trigonelline degradation. Because N-alkylpyridinium ions are charged species, they are readily detected from solution. Carboxylic acids formed on the roasting process such as octanoic and decanoic acids (m/z 145 and m/z 173) and also the phenolics benzoic, coumaric and cinnamic acids [m/z 123, 165 and 187 in the [M + K]⁺ form, respectively] are also representative.

In the ESI(+)–MS, from a matrix of 9 samples and 418 variables the ions selected as being the most important for this separation are those of m/z 80, 94, 108, 122, 144, 154, 165, 173, 176, 187 and 352. In this plot, PC1 explains 49% and PC2 28% of the total data variation, summing up to 77% in total. The separation between the overripe and the
other samples has been achieved in PC1, with the variables of m/z 144, 165 and 176 being positively correlated with the overripe samples. The separation between ripe and immature was obtained in PC2 and the variables positively correlated with the immature samples were ions at m/z 80, 187 and 352, while the variables positively correlated with the ripe samples are those of m/z 94, 108, 122, 154 and 173.

Figure 3 shows the PCA scores and loadings bi-plot obtained with selected ESI-MS, (a) negative ion mode, (b) positive ion mode, data of roasted coffees with different ripeness.

Post-Harvest process

Dry and wet methods were employed to process green cherry coffees. In the dry method, coffee beans were dried as a whole in the cherry state, with pulp and mucilage. In the wet method, coffee beans were pulped (removal of fruit skin) or pulped and demucilated (removal of mucilaginous mesocarp under fermentation). Dry process is slow, leading to the translocation of chemical constituents from the pulp to the inner bean and also to chemical transformations that depend to ambient conditions whereas in the wet method, fermentation occurs in water at controlled temperatures, giving rise to reduced levels of undesirable flavors. The wet method has been associated with better quality coffees. Greatest amounts of reduced sugars were found in cherry dry processed beans followed by pulped coffees and, at last, lower levels were found for demucilated wet beans.31 No significant differences were seen by the same authors for soluble solids.

The ESI-MS of green coffees in the positive and negative ion modes are found to be effective to discriminate between post-harvesting processes. In both ionization modes, ions from fatty acids such as palmitic, oleic and linoleic acids are characterized via ESI-MS/MS analysis and found to be representative on differentiation, along with chlorogenic acids.

In the ESI(−)-MS, from a matrix of 9 samples and 25 variables, the ions selected as being the most important for this separation are those of m/z 75, 192, 264, 279, 281, 341, 353, 354, 367 and 695. It can be seen that 99% of total data variation is explained in this plot, PC1 explaining 69% and PC2 30%. The separation among the differently processed samples has been achieved in PC1, with PC2 discriminating the wet samples from the others. The variables of m/z 192, 279, 281 and 341 are positively correlated with the dry samples, the variables of 353, 354 and 695 with the wet samples and variables of 75, 264 and 367 with the semi-wet samples. Most of these ions are also present in the previous ESI(−)-MS ripeness analyses already discussed.

In the ESI(+)–MS for green coffees, ions are also seen from sugars such as saccharose of m/z 383 [saccharose + Na]+, m/z 219 [glucose + K]+ and K+ adducts of carboxylic acids generated in the fermentation process along the wet treatment.

From a matrix of 9 samples and 32 variables, the ions selected as being the most important to differentiate between dry and wet methods via ESI(+)–MS are those of m/z 104, 133, 175, 176, 219, 220, 221, 231, 266 and 383. In this plot, PC1 explains 56% and PC2 42% of the total data variation, summing up to 98% in total. The separation among the differently processed samples has been achieved in PC1, with PC2 discriminating the dry samples from the others. The variables of m/z 176, 221, 231 and 383 are positively correlated with the dry samples, the variable of m/z 175 with the wet samples and variables of m/z 220 and 266 with the semi-wet samples.

Important even mass ions for discrimination are detected via ESI (+)-MS of green coffees accompanied by their [M + H]+ counterpartners. This hypothesis was investigated in the generation of malonic acid and for other relevant ions such as those of m/z 220 and 221. ESI-MS/MS shows a fragment ion of m/z 148, typical of a pentose glycoside.
moiety accompanied by loss of CH$_3$CO$_2$H, suggesting the presence of a monoacetylated pentose. Butanoyl and butenoylsucroses have already been identified by Weckerle et al.$^{32}$ in green Arabica coffees.

Figure 4 shows the PCA scores and loadings bi-plot obtained with selected ESI-MS, (a) negative ion mode, (b) positive ion mode, data of green coffee obtained with different post-harvest process.

In roasted coffees, from a matrix of 9 samples and 65 variables the ions selected as being the most important for the separation in ESI (-)-MS are those of m/z 117, 133, 191, 249, 350, 365, 373 and 383. In this plot, PC1 explains 47% and PC2 42% of the total data variation, summing up to 89% in total. The separation among the dry samples, the other samples has been achieved in PC1, with the variables of m/z 91, 200, 210, 486, 758, 796 and 820 being the most important in this separation. The separation among wet and the other samples was obtained in PC2 and the variables with the stronger influence are those of m/z 139, 200, 210, 429, 430, 486 and 758. Ions of m/z 200 and 210 could not be associated to any constituent but they seem to bear a flavonoidic moiety due to the loss of water and fragment ions from an aromatic nucleus. The low intensity of the ions in the m/z 400-500 range makes any structural proposal difficult.

Figure 5 shows the PCA scores and loadings bi-plot obtained with selected ESI-MS, (a) negative ion mode, (b) positive ion mode, of roasted coffee obtained with different post harvest processes.

In roasted coffees, from a matrix of 9 samples and 65 variables the ions selected as being the most important for this separation are those of m/z 94, 139, 200, 210, 429, 430, 486, 758, 796 and 820. In this plot, PC1 explains 51% and PC2 34% of the total data variation, summing up to 95% in total. The separation among the dry, and the other samples has been achieved in PC1, with the variables of m/z 91, 200, 210, 486, 758, 796 and 820 being the most important in this separation. The separation among wet and the other samples was obtained in PC2 and the variables with the stronger influence are those of m/z 139, 200, 210, 429, 430, 486 and 758. Ions of m/z 200 and 210 could not be associated to any constituent but they seem to bear a flavonoidic moiety due to the loss of water and fragment ions from an aromatic nucleus. The low intensity of the ions in the m/z 400-500 range makes any structural proposal difficult.

In Brazil, coffees are graded through a unique classification by using type and cup evaluations. Type is related to the appearance and number of defects of the beans, whereas in cup test sensory evaluations such as flavor
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and aroma are more relevant. In this test, trained “cuppers” describe their sensations so as to classify coffees as: strictly soft, soft, barely soft, hard, rioysh, rio and rio zona, going from the best to the worst coffee. The beverages for the Brazilian cup test are made by pouring boiling water onto the ground roasted coffees and describing the flavor and smell for some minutes. Following the official Brazilian coffee classification beverage,33 the description of these coffees are: strictly soft, that has a very smooth flavor, slightly sweet and low acidity; soft, also with a smooth flavor and slightly sweetness; barely soft, that is similar but with a slight astrignency; hard, with an astringent flavor; rough taste that lacks sweetness; rioysh, with a slight taste of iodoform; rio, that has a strong unpleasant taste reminding iodoform and rio zona, with an intolerable taste and smell.34 These differences were tentatively associated to the presence of defects occasioned by excess of fermentation, skin oxidation, immature and broken beans, for example. Franca and co-workers16 suggested that these defects were mostly related to fermentation processes and could be monitored by sugar and chlorogenic acids contents.

The matrices obtained for these analyses had 15 samples and 12 variables for the negative ion mode and 15 variables for the positive ion mode. PCA was initially used but provided no clustering. So, discriminant analysis was performed on PCA scores and Figure 6 shows the plots obtained for ESI(-)-MS and ESI(+)-MS data.

Again, both ESI-MS in the positive and negative ion modes are able to differentiate cup quality Arabica roasted coffees. In the ESI(-)-MS, the ions (most deprotonated molecules) used to perform discrimination are the same ones as previously discussed: m/z 75 (glycolic acid), 133 (malic acid), 176/175 (dimethoxycinnamic acid), 191 (quinic acid), 255 (palmitic acid), 257, 279 (linoleic acid), 341 (saccharose), 353 (cafeoylquinic acid), 367 (feruloylquinic acid) and 515 (dicafeoylquinic acid).

In the ESI-(+)-MS, the major ions detected are either protonated molecules or K⁺ adducts are, for the protonated molecules, those of m/z 83, 104 (malonic acid), 138 (trigonelline), 139 (p-hydroxybenzoic acid), 151 (pentose) and 195 (caffeine), and for the K⁺ adducts those of 175, 176 (trigonelline), 233 (caffeine), 317 (linolenic acid), 319 (linolenic acid), 381 (saccharose), 382 and 393 (cafeoylquinic acid).

Attempts to correlate the Brazilian cup quality classification to chemical composition of coffees pointed to major differences on chlorogenic acids, trigonelline and caffeine contents.16 By ESI-MS discrimination, it is clear that lipids and sugars imparted a very important contribution to discriminate between Brazilian cup quality coffees.

It must be emphasized, however, that this is a preliminary work, which, in the future, should be increased, with samples from other crops, allowing for further development on the conclusions on the chemical compounds responsible for coffee ripeness and processing methods.

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