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Free and Conjugated Biogenic Amines in Green and Roasted Coffee Beans

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This work reports the composition of arabica and robusta coffees in terms of biogenic amines. The analyses were conducted before and after acid hydrolysis with the aim of the determining both free and conjugated forms in green and roasted coffee beans. The amines (putrescine, cadaverine, serotonin, tyramine, spermidine, and spermine) were determined by reverse-phase high-performance liquid chromatography (RP-HPLC) after derivatization with dansyl chloride. Multivariate analyses were applied in order to evaluate the possible use of these amines as chemical descriptors for arabica and robusta coffees. It was found that putrescine, the main biogenic amine present in the green beans, could be used in the discrimination of the referred species. There is also some evidence that these compounds can be used for discrimination between green coffees subjected to different postharvest processes and that tyramine can be considered a chemical marker for Angolan robustas. The variations in biogenic amine levels after roasts is also discussed, but the statistical significance for species discrimination is reduced.

KEYWORDS: Coffee; biogenic amines; free; conjugated; discriminant analysis; geographical origin; processing method

INTRODUCTION

The *Coffea* family includes many species and varieties. In commercial terms, the two most important species are *Coffea arabica* L and *Coffea canephora* var. *robusta*, the former, being the oldest-known species with numerous varieties (which will be referred to as arabica), and the latter usually called robusta. Green arabica coffees are usually traded as Brazilian, milds (common name for wet-processed arabicas), and with a variety of other names when referring to dry-processed arabicas. Robusta coffees are usually only dry-processed and commercialized as such (*1*). Arabica coffees are generally preferred to robusta for their flavor, despite being less resistant to diseases. Consequently, robusta accounts only for around 25% of world coffee production, despite its cheaper price (*2*).

These differences in price and flavor make it of utmost importance to guarantee coffee authenticity, especially after roast (*3*), mainly checking for adulterations of arabica coffees with beans of the robusta varieties, and a number of different approaches and techniques have been used to assess this authenticity. Many groups of compounds have been used to differentiate coffee species, namely, chlorogenic acids (*4*, *5*), amino acids (*6*, *7*), and lipid and volatile fractions (*8*−*10*). Nevertheless, and despite the huge amount of work in the area, no specific marker has yet been found (*11*), with the exception of the diterpene 16-*O*-methylcafestol, only found in robusta beans, which is currently considered the best marker for the presence of small amounts of robusta beans in arabica coffee (*8*).

Biogenic amines are known to be present in a wide range of food products including fish, meat, dairy products, vegetables, fruit, nuts, chocolate, wine, and beer (*12*), being either natural endogenous constituents or formed during food processing by the action of decarboxylase-positive microorganisms (*13*). References on the biogenic amine contents of coffee are very scarce. Amorim et al. (*14*) reported free polyamines contents of arabica coffee for the first time, aiming to find correlations between their levels and beverage quality. More recently, preliminary studies on free biogenic amines levels in some arabica and robusta coffee samples were carried out in our laboratory, gathering some evidence that these compounds could be used as chemical markers for the two coffee species (*15*). A recent work by Cirilo et al. (*16*) complemented these observations, although only in relation to arabica samples.

The biogenic amines in raw vegetables are usually present both as free bases and conjugated with other molecules, like phenolic acid and proteins, with levels depending on variety, ripening stage, and storage conditions (*17*). On the basis of these observations and following our works on the discrimination of coffee varieties (*7*, *9*, *18*), the aims of the present work were

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Table 1. Free and Conjugated Biogenic Amines in Arabica and Robusta Green Coffee[a]

	free					acid-soluble conjugates					acid-insoluble conjugates				
	arabica		robusta			arabica		robusta			arabica		robusta		
biogenic amine	mean	SD	mean	SD	*t*-test[b]	mean	SD	mean	SD	*t*-test[b]	mean	SD	mean	SD	*t*-test[b]
putrescine	47.9	11.4	11.1	3.6	***	3.7	2.8	3.2	2.4	ns	2.9	0.8	0.6	0.3	***
cadaverine	0.2	0.2	0.4	0.2	*	0.3	0.2	0.4	0.3	ns	0.1	0.1	0.1	0.1	ns
serotonin	2.5	0.7	2.1	0.8	ns	0.8	0.6	1.2	0.9	ns	nd		nd		ns
tyramine	0.2	0.1	3.1	4.6	*	0.2	0.2	2.4	3.2	*	0.4	0.3	0.9	0.8	ns
spermidine	9.0	3.0	8.3	2.8	ns	3.5	2.7	2.6	3.0	ns	0.5	0.2	0.4	0.2	ns
spermine	5.5	3.3	6.0	3.8	ns	2.0	2.6	3.6	3.4	ns	5.8	1.7	5.2	1.7	ns

[a] Given as milligrams per kilogram dry weight. nd, not detectable; SD, standard deviation. [b] ns, not significant ($p > 0.05$); *, significant differences at $p \leq 0.05$; **, significant differences at $p < 0.01$; ***, significant differences at $p < 0.001$.

(i) to determine the composition of arabica and robusta species in terms of free and conjugated biogenic amines and (ii) to study the usefulness of these compounds as discriminators for both green and roasted coffee beans when compared with other chemical descriptors, or even as markers for the discrimination of the postharvest processing method applied to coffee beans (either wet or dry process).

MATERIALS AND METHODS

Reagents. Amine hydrochlorides (putrescine, cadaverine, serotonin, tyramine, spermidine, and spermine), internal standard (1,7-diaminoheptane), and dansyl chloride were all obtained from Sigma−Aldrich (Steinheim, Germany). All other reagents were analytical- or HPLC-grade, mostly from Merck (Darmstadt, Germany).

Coffee Samples. A local broker and industrial coffee roaster supplied all coffee samples and was able to confirm their botanical and geographical origin, as well as the general type of postharvest processing method (dry/wet process). No further details were known on the samples' historical background except that they were all from the same harvesting year. The samples were refrigerated until analysis. Each sample's moisture content was determined in order to express the results on a dry weight (dw) basis. Samples were labeled with a first letter indicating the coffee species (A = arabica, R = robusta) and a second letter indicating the postharvest processing treatment (D = dry, W = wet). These two letters were followed by a hyphen and another letter for geographical origin (listed below), and finally one numeral indicating the sample number within each country.

A total of 30 coffee samples (19 robustas and 11 arabicas) from distinct geographical origins were analyzed, being representative of the coffees generally consumed in Portugal, usually for espresso blends. Regarding robusta coffee, 16 samples from Africa [I, Ivory Coast (6); A, Angola (5), U, Uganda (3); C, Cameroon (2)], and three from Asia [V, Vietnam (2); D, India (1)] were used. Among the arabicas, 5 samples were from Central America [R, Costa Rica (2); G, Guatemala (1); M, Mexico (1); H, Honduras (1)] and 6 were from South America [B, Brazil (5); L, Colombia (1)].

Sample Preparation: Biogenic Amines Extraction. The biogenic amines extraction was based on published literature (*19*, *20*) with small modifications. Briefly, 500 mg of ground coffee was macerated with 8.0 mL of 5% (w/v) trichloroacetic acid (TCA) and left overnight at 4 °C. After centrifugation, the supernatant and residue were kept for further treatments. Free biogenic amines were determined directly from the supernatant. Conjugated amines were extracted by hydrolyzing an aliquot of the supernatant with 12 M HCl (1:1 v/v) for 18 h at 110 °C, on a Reacti-therm heating module (Pierce). This last fraction contained the free polyamines as well as the acid-soluble ones liberated by hydrolysis. The residue, containing the acid-insoluble conjugates, was washed two times with 5% TCA, neutralized with 1.0 M NaOH to the initial volume (±8.5 mL), and stored overnight at 4 °C. An aliquot of the resuspended residue was then hydrolyzed as described above. The hydrolyzed suspensions were filtered (0.45 μm), taken to dryness under a nitrogen stream (50 °C), and resuspended in 1.0 mL of 5% TCA.

Cleanup. A 1.0 mL portion of sample extracts was subjected to an ion-pair cleanup with bis(2-ethylhexyl) phosphate (BEHPA) as described by Casal et al. (*15*). The polyamines were initially extracted with 0.1 M BEHPA in chloroform (2 mL) after adjustment to pH 7.4 with 0.2 M phosphate buffer and then back-extracted with 0.1 M HCl (2 mL).

Derivatization Procedure. The amine aqueous extracts were dansylated at controlled pH (11.2) with 1.0 mL of 7.5 mg/mL dansyl chloride in acetone, in the presence of 1.0 mL of saturated sodium carbonate, for 12 min at 60 °C, according to the method previously developed (*15*). The excess of dansyl chloride was converted to dansylproline and the derivatives were extracted with toluene. The toluene phase was collected and dried under nitrogen, and the residues were solubilized in acetonitrile.

HPLC Analysis. The liquid chromatograph consisted of a Jasco integrated system (Japan) equipped with two model PU-980 pumps, a AS-950 automated injector, a MD-910 multiwavelength diode-array detector (DAD), and a FP-920 fluorometric detector (excitation 252 nm, emission 500 nm). A reversed-phase Tracer-Excel 120 ODS-A (250 × 4 mm i.d., 5 μm) column (Teknokroma, Spain), operating at 40 °C, with a gradient of 0.05 M phosphoric acid and methanol/acetonitrile, as reported by Hornero-Mendez and Garrido-Fernandes (*21*), was used.

Quantification was based on the internal standard method with 1,7-diaminoheptane. The DAD response at 254 nm was used for serotonin quantification while all other amines were quantified by fluorescence signal response.

Statistical Data Treatment. Univariate statistics (means, standard deviations, and extreme and quartile values) were calculated for general data inspection and description. Cluster analysis, following Ward's method with Euclidean distances, was carried out for all individual coffees analyzed. After samples were divided into three groups (robusta dry-processed, arabica wet-processed, and arabica dry-processed), the following analyses were also carried out: (i) MANOVA applied to the three coffee groups for determination of differences between them with all groups and amines taken simultaneously; (ii) Hotelling's T^2 tests following significant MANOVAs for determination of significantly different groups, applied to all different pairs of groups; (iii) *t*-tests for univariate analysis of amines for which between-group differences were significant; and (iv) canonical variate analysis (standard discriminant analysis) to determine the main amines responsible for observed between-groups differences, and also to enable the construction of graphs for visualization of the magnitude of differences. All statistical methods were standard (*22*) and applied as implemented in the Statistica for Windows statistical package (StatSoft, Tulsa, OK).

RESULTS AND DISCUSSION

The extraction procedure used throughout this work allows division of the biogenic amines into three groups—free, acid-soluble conjugated, and acid-insoluble conjugated (*23*)—although without allowing further divisions by the chemical nature of the conjugations involved. The composition of robusta and arabica green coffee samples [milligrams per kilogram on a dry weight basis (dw)], in terms of these three fractions, is presented in **Table 1**, together with an evaluation of the

Table 2. Free Biogenic Amines in Arabica and Robusta Roasted Coffee[a]

	free				
	arabica		robusta		
biogenic amine	mean	SD	mean	SD	*t*-test[b]
putrescine	1.0	0.9	1.3	1.2	ns
cadaverine	nd		nd		
serotonin	2.3	1.2	3.3	0.8	*
tyramine	nd		nd		
spermidine	0.1	0.1	0.1	0.1	ns
spermine	1.2	0.6	1.8	0.8	ns

[a] Given as milligrams per kilogram dry weight. nd, not detectable; SD, standard deviation. [b] ns, not significant differences; *, significant differences ($p < 0.05$).

significance of observed differences between the two varieties according to ANOVA tests.

Generally, putrescine was the most abundant amine in both species, followed by spermidine, spermine, and serotonin. Cadaverine and tyramine were generally present in low amounts. The results for the free biogenic amines in the arabica samples are in accordance with those reported in our last publication on the subject (*15*) and also with Amorim et al. (*14*) but are somewhat higher than the ones reported by Cirilo et al. (*16*), with the exception of serotonin, which was also found by these researchers to be the main amine present in the samples included in their work. If it is true that the different analytical methods used may be the source of some discrepancy, it is more likely that the differences in the reported results may be due to the fact that different arabica varieties were considered in the different works.

All biogenic amine contents were higher in the free form. In this fraction the differences between the two species were highly significant in what concerns putrescine, with higher values for the arabica samples. Differences were also significant for free cadaverine and tyramine. The acid-soluble conjugated forms were quite similar between the two species, with the three main amines presenting similar levels, again followed by serotonin. In what concerns the acid-insoluble conjugated forms, the levels were also quite similar between the two species, spermine being the main amine present. It is not clear whether the higher putrescine content in the arabica samples can be considered natural or simply formed in higher amounts during storage. The analysis of fresh green beans can probably clarify this situation.

The levels of tyramine were significantly different between robusta from Angola (Amboim and Ambriz) and from other countries. Also, robustas from countries other than Angola were still found to be statistically different from the arabicas ($p < 0.001$). These results are in line with published observations on coffee samples from Angola, where a tyramine precursor, tyrosine, in the form caffeoyltyrosine, was classified as a probable important marker for Angolan robustas (*24*, *25*).

The biogenic amines were also analyzed in the same coffee samples after they were submitted to a standard dark roasting procedure (160−220 °C, 14 min) generally used in Portugal. These samples presented roast losses of ∼11% for the arabica samples and ∼13% for the robusta ones (on a dry weight basis). All the major amines were still detectable after roast, as reported in **Table 2**, but in very low amounts and mainly as free forms. Since they are degraded with heat, the remaining amounts can vary with the roasting degree. Serotonin was the amine most resistant to the effects of this roasting procedure, and it was observed for some samples in higher amounts after roast than in the green beans, when expressed on a dry basis and without adjustment to the roast loss occurred during roast. Differences between species in what concerns serotonin contents were also evident, with higher amounts being detected for the robusta samples. The presence of free serotonin in roasted coffee and the consequent possibility of finding high levels of this amine in the brew can be interesting from the point of view of its physiological action in humans, since it is known to be an important neurological mediator.

The results obtained were further analyzed by considering two groups according to the method of processing applied to the green beans, dry or wet. Free putrescine values were significantly higher ($p < 0.001$) in the wet-processed arabicas (58.6 ± 5.9 mg/kg dw) when compared to the dry-processed ones (38.9 ± 4.7 mg/kg dw) but only before roasting. Tyramine levels were also higher in the wet-processed samples but with lower statistical significance ($p < 0.01$). The acid-soluble conjugated spermidine was higher in the wet-processed samples ($p < 0.05$) as it was true for putrescine in the form of acid-insoluble conjugates ($p < 0.05$). In the so-called wet process, a postharvest treatment used frequently in arabica coffees, a fermentation step is performed in order to remove the remaining adherent mucilage after pulping (*26*). This step may explain the higher biogenic amine levels found in the wet-processed samples, due to the possible action of naturally present microorganisms or coffee enzymes.

To use the biogenic amines as chemical descriptors for the two coffee species, we have further analyzed our results by employing multiple statistical techniques. The free fraction, the one showing higher statistical differences between species, was chosen for this purpose. Cluster analysis was carried out in order to search for natural groupings based on the studied biogenic amines, with the corresponding dendrogram for the green coffees being shown in **Figure 1**.

It is immediately obvious that there is a sharp distinction between robusta and arabica green coffees, forming two distinct clusters, which means that the biogenic amines provide a clear distinction between these two coffee species. On the basis of the same cluster analysis, a subdivision of the arabica cluster in a wet-process cluster and a dry-process cluster is also evident. Also, inside the robusta cluster, the samples from Angola are included in a distinct cluster, probably due to their tyramine content, as discussed before. To determine the most important biogenic amines for discrimination between the three groups considered, a discriminate analysis, by the standard method, was carried out.

Since there are three groups defined, canonical variate analysis develops only two canonical variates, with a relative importance given by the corresponding eigenvalues. **Figure 2** shows the plot of the two canonical variates. The first variate, with an eigenvalue equal to 24.9, represents the main structure (97% of the total variation in the data values) and explains the separation between robusta and arabica species. This separation is a direct consequence of the higher levels of putrescine in the arabica samples. The second variate, with an eigenvalue of 0.7 (3% of the total variation) shows that the second data structure is only relative to the discrimination between samples of the arabica species subjected to different postharvest treatments, bearing no apparent important relationship to the robusta samples. Wet-processed arabicas are distinguished by higher levels of putrescine and spermine and lower levels of spermidine. These differences due to processing method, which are visible in **Figure 2**, are, however, only significant at the $p \cong 0.09$ level, as determined by Hotelling's T^2 test (results not shown), which is a consequence of the existence of a sample

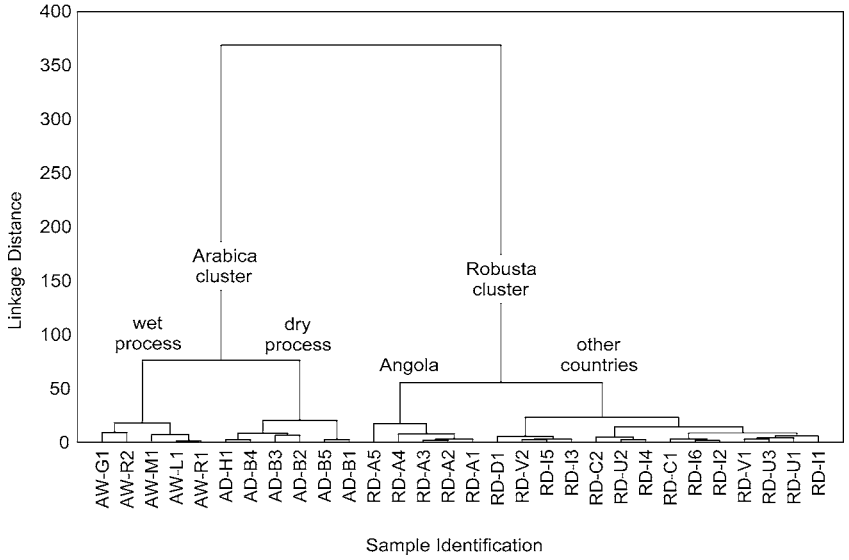

Figure 1. Dendrogram expressing the result of cluster analysis on the free biogenic amines content of green coffee samples.

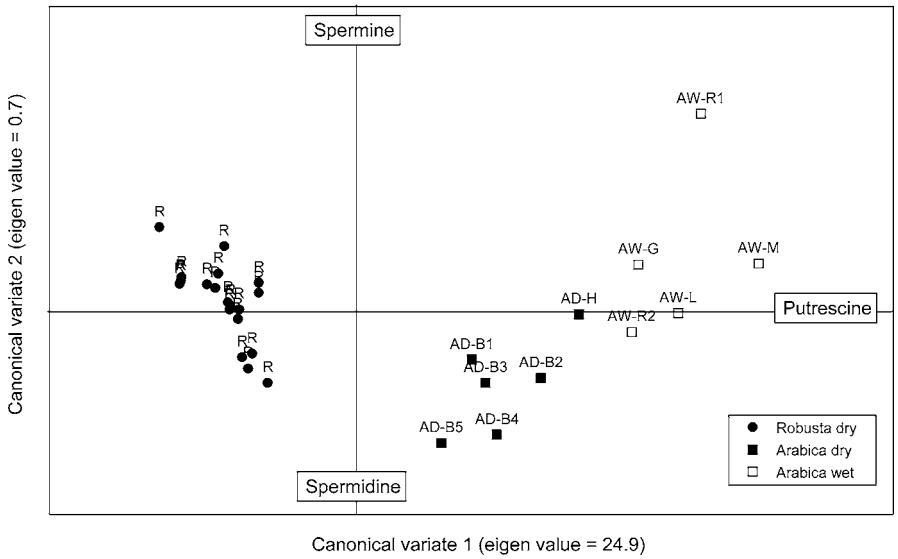

Figure 2. Canonical variate 1 versus 2 of free biogenic amines data.

classified as wet-processed but very similar to the samples classified as dry-processed. However, it must be kept in mind that these observations must be viewed with caution due to the reduced number of samples used.

As a general conclusion, one can say that putrescine shows a high potential as a coffee species discriminator. The analysis of putrescine, by means of a rapid enzymatic test, may constitute an interesting, fast chemical method for discrimination between arabica and robusta samples when compared with other wet chemical analyses published in the literature. Simultaneously, it can also provide some contribution to the assessment of the processing method performed on the beans, although being less effective than the determination of the amino acid profile, as previously reported (*7*). Further studies on the nature of the conjugations may also provide some enlightening aspects of coffee beans for these purposes. It must be realized, however, that the present investigation is mainly a way of obtaining tentative information about differences between processing methods. To be able to be more peremptory, it is important to increase the number of the samples under study, including different harvesting years and storage conditions, if definitive conclusions on the significance of the differences caused by the two postharvest processes are intended. With the data collected during this work, and since the biogenic amines were found to be greatly reduced by the roasting procedure, it is concluded that these compounds cannot be used with the same purpose in roasted beans.

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