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Selective Liquid CO_2 Extraction of Purine Alkaloids in Different *Ilex paraguariensis* Progenies Grown under Environmental Influences

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In this investigation, liquid carbon dioxide at 20 °C and 150 bar was used for the selective extraction of caffeine and theobromine from dry leaves of mate. A comparison between the chromatograms from CO_2 extraction and traditional solvent extraction supports the selectivity of carbon dioxide for these purine alkaloids. The advantages of selective liquid CO_2 extraction in terms of speed and resolution of UV/HPLC is also evidenced. A randomized block design of experiments was proposed to investigate the influence of 16 progenies of *Ilex paraguariensis* grown in 3 diverse sites on the contents of caffeine and theobromine in liquors of mate leaves obtained by extraction with compressed CO_2. A significant effect of both these factors on the parameters investigated was observed by involving the *F* distribution in the statistical analysis. A cluster analysis based on the experimental uncertainties in the contents of these two methylxanthines has identified from four to six different groups of mate progenies.

KEYWORDS: Compressed; carbon dioxide; extraction; progeny; *Ilex paraguariensis*; mate

INTRODUCTION

Dry leaves of mate (*Ilex paraguariensis* St Hil) are extensively marketed because a well appreciated non-alcoholic beverage, quite similar in taste and color to the green tea manufactured from the shoots of the *Camellia sinensis* bush, is produced from their extracts. Nowadays, 96% of all of Brazil's production of dry leaves of mate, which represents approximately 210 thousands tons per year, (*1*) has this destination (*2*). However, in spite of the popularity of this aqueous infusion, an increase in the international trade of dry mate leaves depends on development of new products derived from mate liquors, where was identified the occurrence of a large number of organic chemical compounds with therapeutic properties in humans (*3*−*8*). A great contribution in this direction is the production of selective natural extracts from *Ilex paraguariensis* plants that present a particular genetic ability to synthesize an interesting clinical compound in preference. In this framework, selective extraction and identification of these compounds are essential technological aspects to be investigated.

Although the number of only volatile constituents in mate extracts is approximately 250 (*9*), many of the overall chemical species with special interest for pharmaceutical purposes often investigated (*10*, *11*) may be classified in merely three groups, namely, phenols, saponins, and purine alkaloids. Phenolic compounds act as antioxidants (*5*) and inhibitors of diabetic complications (*7*) as well inhibitors of carcinoma cells proliferation (*6*), whereas to saponins and alkaloids are credited anti-oedematogenic activity on induced oedemas (*12*) and stimulant effects on the central nervous, muscle, and circulatory systems in humans (*13*).

Supercritical and near-critical fluid extractions are closely related. In this way, many of the physical properties of solvents are similar. These types of operations are well-known methods applied in the laboratory scale or in industrial plants to fractionate natural matter (*14*) in order to have high added value products such as food additives (*15*, *16*), pharmaceuticals compounds (*17*), pesticide, and insecticide principles (*18*), or for the removal of pollutants from a solid matrix (*19*, *20*). Abundant information is available in the literature regarding the many advantages of using compressed and liquefied gases as solvents instead of conventional organic solvents on extraction (*14*, *21*−*24*). The first great benefit is the elimination of the expensive postprocessing operations for solvent removal from

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Table 1. Growth Conditions of Mate (*Ilex paraguariensis*) Bushes Involved in the Experiments (*25*)

	first site	second site	third site
city	Ivaí	Rio Azul	Guarapuava
S latitude	25°01′	25°44′	25°26′
W longitude	50°48′	50°46′	51°15′
altitude, m	600	950	1100
rain, mm	1500–1600	1400–1500	1500–1600
average temp, °C	17–18	16–17	16–17
soil type	red latosol dystrophic	haplic nitosol dystrophic	humic cambisol dystrophic
climate type	maritime temperate climate, Cfb (humid all year with warmest month lower than 22 °C and at least 4 months higher than 10°C)		

extracts in which organic solvents obviously have a detrimental effect. In fact, this is a result of the low latent heat of evaporation of several common supercritical fluids as well as the nontoxic nature of some of them, such as H_2O or CO_2. The use of liquid CO_2 as solvent for extraction also occurs at low temperatures and consequently helps to preserve thermally degradable products. Moreover, liquid CO_2 extraction consumes small amounts of solvent when compared with traditional extraction because of the possible continuous recuperation in a closed loop system. Higher solubilities and mass transfer rates also make compressed gases more attractive than the conventional solvent extraction. Despite all these above-mentioned advantages, an important characteristic of liquid carbon dioxide extraction for obtaining a specific compound in solid samples with multisoluble constituents is the selectivity. It is relevant to remember that conventional solvent extraction produces nonselective extracts that also lead to an arduous process of separation and screening in the chromatographic columns and chromatograms.

In this investigation, liquid carbon dioxide was involved for the selective extraction of caffeine and theobromine from dry leaves of mate. A comparison between typical mate liquors obtained with an aqueous ethanol solution and liquid carbon dioxide extraction was carried out in order to evidence this expected selectivity. The influence of CO_2 selectivity for these purine alkaloids on speed and resolution of the analytical procedure by isocratic HPLC in combination with spectrophotometry was examined. A total of 48 experimental extraction runs with liquid carbon dioxide at 20 °C and 150 bar were carried out to investigate the effect of 16 progenies of *Ilex paraguariensis* grown in 3 different sites of cultivations on the individual content of both of the methylxanthines mentioned above. A cluster analysis based on the experimental uncertainties in the measurements of caffeine and theobromine was also performed.

EXPERIMENTAL PROCEDURES

Materials. Sixteen progenies of mate with major production of leaves were selected from a genetic improvement experiment performed by the Brazilian Agricultural Research Corporation (*25*) in Paraná, Brazil. In particular, this program of plant selection involved 175 first generation half-sib progenies randomly planted in a linear sequence and with plant spacing of approximately 2 m. A set of six parallel lines with these characteristics spaced by 3 m in both directions, without a systematic arrangement of progenies in the rows, represents a rectangular area of cultivation where the sampling of mate leaves was carried out. A number of 8 to 10 of these clumps were distributed to each of the three different sites, where the plants were grown under the conditions defined in **Table 1** (*25*). The leaves of mate that were approximately 4 years old were manually plucked from all the 16 selected progenies in three clumps of each of the sites described in **Table 1**. Although harvesting is possible in the four seasons, the leaves used in this investigation were plucked in the winter, from June to August. Discolored, nibbled, crushed, soiled, or dried out leaves were discarded. All of the samples obtained to account for the effect of different progenies and cultivation sites on purine alkaloids were dived in hot water at 100 °C for 10 s to kill off enzymatic reaction. An oven with air circulation and control of temperature was applied to dehydrate the leaves at 45 °C, which were later manually reduced in size and passed over a single 0.35 mm (42 mesh Tyler) sieve shaken on a mechanical shaker.

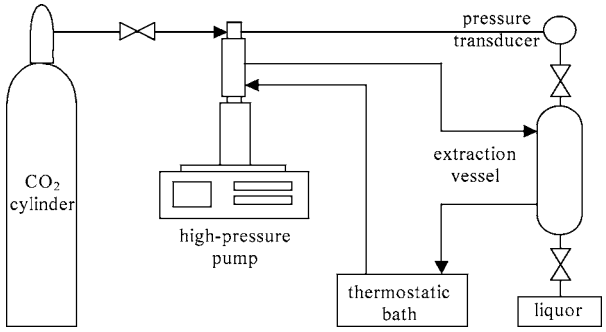

Figure 1. Schematic of the apparatus used for extraction with compressed carbon dioxide.

Extraction Procedures and Analysis for Caffeine and Theobromine. The experimental apparatus for extraction with compressed carbon dioxide is schematically shown in Figure 1. In essence, the process of extraction occurs by adding approximately 20 g of dry and milled particles of mate (Tyler 42) in a sealed extraction vessel, where carbon dioxide is pumped at a constant flow rate of 1 mL/min for 1 h. Although all of the extractions were carried out at 150 bar and 20 °C (902 kg/m^3) in semi-batch mode, several preliminary tests were performed by combining pressure and temperature in order to obtain the best condition in which the solvent selectively extracts the purine alkaloids. The individual contents of methylxanthines were determined by isocratic high performance liquid chromatography. The HPLC apparatus (Model CG 480-C) was equipped with an UV−visible spectrophotometer (Model Jasco UV-970-975) monitoring extracts obtained with liquid CO_2 at 280 nm. A 5 μm C_{18} packing column (Supelco, 250 mm × 4.6 μm) maintained at 30 °C was used. The isocratic solvent was prepared by diluting 40% of methanol and 0.5% of glacial acetic acid in water. The flow rate was kept at 1.0 mL/min. The reference samples of caffeine and theobromine were obtained from commercial sources (Synth). Triplicates of all of the analyses were carried out. The experimental results are expressed in milligrams of either caffeine or theobromine per gram of extract.

A single procedure of extraction with a conventional solvent was also carried out in order to compare this extract with the results obtained from liquid carbon dioxide extraction in terms of selectivity. In particular, these extracts were prepared by the maceration of 1.5 g of dried leaves for 24 h with a 100 mL (5 × 20 mL) portion of 70% v/v ethanol/water solution. After treating the bulked extracts with Carrez Reagent to eliminate colloidal material (*10*), mate liquors were filtered with a 0.45 μm nylon filter. Although the aqueous ethanol liquors were also examined by HPLC with a C_{18} column and the eluates monitored by UV−visible spectrophotometry, slightly different equipment and procedures were applied to avoid overlapping chromatographic peaks and to reduce the retention times of components that are more readily retained by the column. In particular, chromatography was performed by gradient HPLC on Shimadzu equipment (Model SCL-10A) consisting of an SIL-10AF injector, an LC-10AT pump, an FCV-10AL mixer, a DGU-14A degasser, and an injector valve. The column was also maintained at 30 °C using the CTO-10AS oven integrated to the HPLC machine. The solvent system consisted of A, acidulated water with a 0.3% acetic acid, and B, methanol. Solvents were run at a flow rate of 1.0 mL min^{-1} using the following linear gradients: 15−20% B in 20 min; 20−85% B in 5 min; and 85% B in 5 min (*26*). Caffeine and

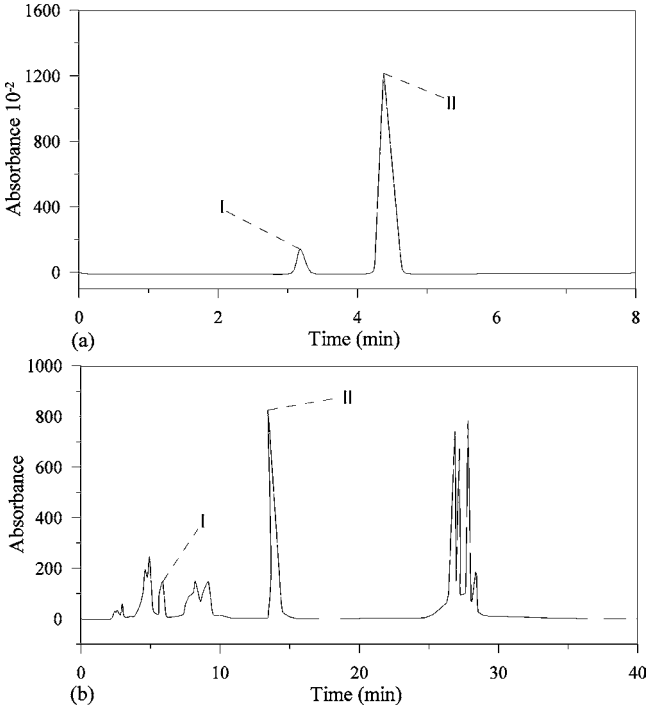

Figure 2. Comparison between a typical chromatogram by involving extraction with supercritical CO_2 at 20 °C and 150 bar (902 kg/m^3) followed by isocratic HPLC at 280 nm (a) and with a 70% v/v ethanol solution followed by gradient HPLC at 265 nm (b). Peaks I and II indicate theobromine and caffeine, respectively

theobromine detection was monitored at 265 nm by using an SPD-10A UV detector. All samples were run in triplicate.

RESULTS AND DISCUSSION

Liquid Carbon Dioxide Extraction. A typical chromatogram for the mate extracts obtained by using liquid carbon dioxide is presented in **Figure 2a**, where selectivity of CO_2 for caffeine and theobromine is promptly observed. However, the large number of compounds shown in **Figure 2b** evidence the nonselective extraction of purine alkaloids by involving the conventional aqueous ethanol solution. Because the identities of the peaks located between approximately 25 and 35 min on the right side of the chromatogram were not necessary, gradient elution has been achieved in order to maximize the speed of HPLC. Despite this procedure, individual time analysis was increased from about 6 to 35 min by replacing isocratic HPLC analysis and CO_2 extraction to gradient HPLC and aqueous ethanol extraction. In addition, a higher resolution is observed on the left side of the chromatogram, where only the two areas representing the content of caffeine and theobromine are accurately integrated because they are separated by different retention times. Improvements in the speed and resolution of HPLC by applying the selective extraction with liquid carbon dioxide is still more evident when the overall number of analyses involved in the current investigation is considered. Triplicate analyses for all of the 48 extracts with CO_2 result in 144 chromatograms to be monitored for approximately 15 h ($6 \times 144 = 864$ min), whereas up to 84 h ($35 \times 144 = 5040$ min) would be necessary to examine the mate liquors from conventional extraction with organic solvents. The higher selectivity of pressurized CO_2 for caffeine and the significant decrease in the total extraction time without significant differences in the qualities of the extracts are findings verified in the literature (*27*).

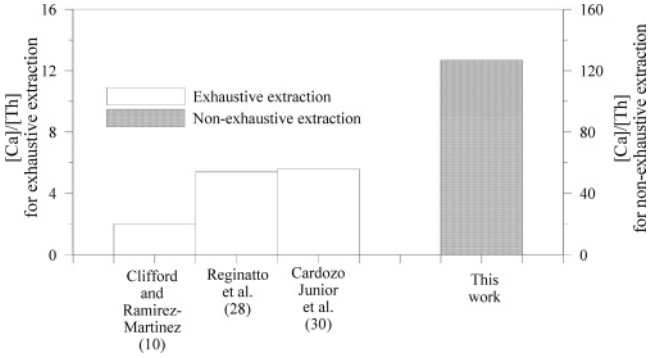

Figure 3. Comparison between the ratio of caffeine and theobromine for exhaustive and non-exhaustive extraction.

Because exhaustive extraction was not currently carried out, any comparison between the absolute contents of individual purine alkaloids from this investigation and analogous data reported in the literature for the often performed exhaustive extraction with either CO_2 or organic solvents (*10*, *28*−*30*) is not useful to corroborate the present results. However, a comparison between the ratio of caffeine and theobromine concentration at an exhaustive and non-exhaustive condition of extraction is important to confirm the much higher selectivity of carbon dioxide for caffeine when compared with theobromine, which is a finding previously reported by Saldaña et al. (*11*). **Figure 3** reports the increase of the ratio between caffeine and theobromine by applying the non-exhaustive compressed carbon dioxide extraction. This behavior is attributed to a higher removal rate of caffeine than theobromine in the early stages of extraction, where the operation is solubility dependent rather than controlled by diffusion, as evidenced by Saldaña et al. (*11*). According to the literature, caffeine is more soluble than theobromine because of the difference between the chemical structures of these methylxanthines. A detailed explanation based on this premise is presented in the literature (*11*, *31*).

Experimental results for supercritical CO_2 extraction of purine alkaloids from mate leaves available in the literature are almost exclusively obtained at exhaustive circumstances. Moreover, the operating conditions from which they were obtained are usually very different. Because the effect of temperature and pressure on extraction is significant (*11*, *32*), comparisons among these results lead to discrepancies. A rare exception that may be used as a reference to this investigation is the study proposed by Esmelindro et al. (*33*), which was performed at 30 °C and 175 bar. Although the contents of caffeine and theobromine presented by Esmelindro et al. (*33*) were obtained after 5 h of extraction against only 1 h applied in this work, a good agreement is noticed. For instance, the mean concentration of caffeine by considering the 48 averaged experimental results presented in **Figure 4** is approximately 41.5 ± 18.4 mg g^{-1} extract (137 ± 62 mg kg^{-1} dry mate), whereas the value obtained by Esmelindro et al. (*33*) is 123.5 ± 137.2 mg g^{-1} extract (949 ± 547 mg kg^{-1} dry mate). The concentration of theobromine, which is 0.327 ± 0.189 mg g^{-1} extract (1.12 ± 0.79 mg kg^{-1} dry mate), in this work is also in excellent accordance with the value of 1.09 ± 1.96 mg g^{-1} extract (6.57 ± 6.41 mg kg^{-1} dry mate) reported by Esmelindro et al. (*33*). Except for the retrograde behavior of caffeine solubility (a decrease in solubility with increase in temperature) at pressures lower than 190 bar (*11*), all of the operating conditions adopted by Esmelindro et al. (*33*) contribute to an increase of extraction (*11*), which explains the slightly higher contents of both of the purine alkaloids when compared with the results obtained in this investigation. The contents of caffeine and theobromine in

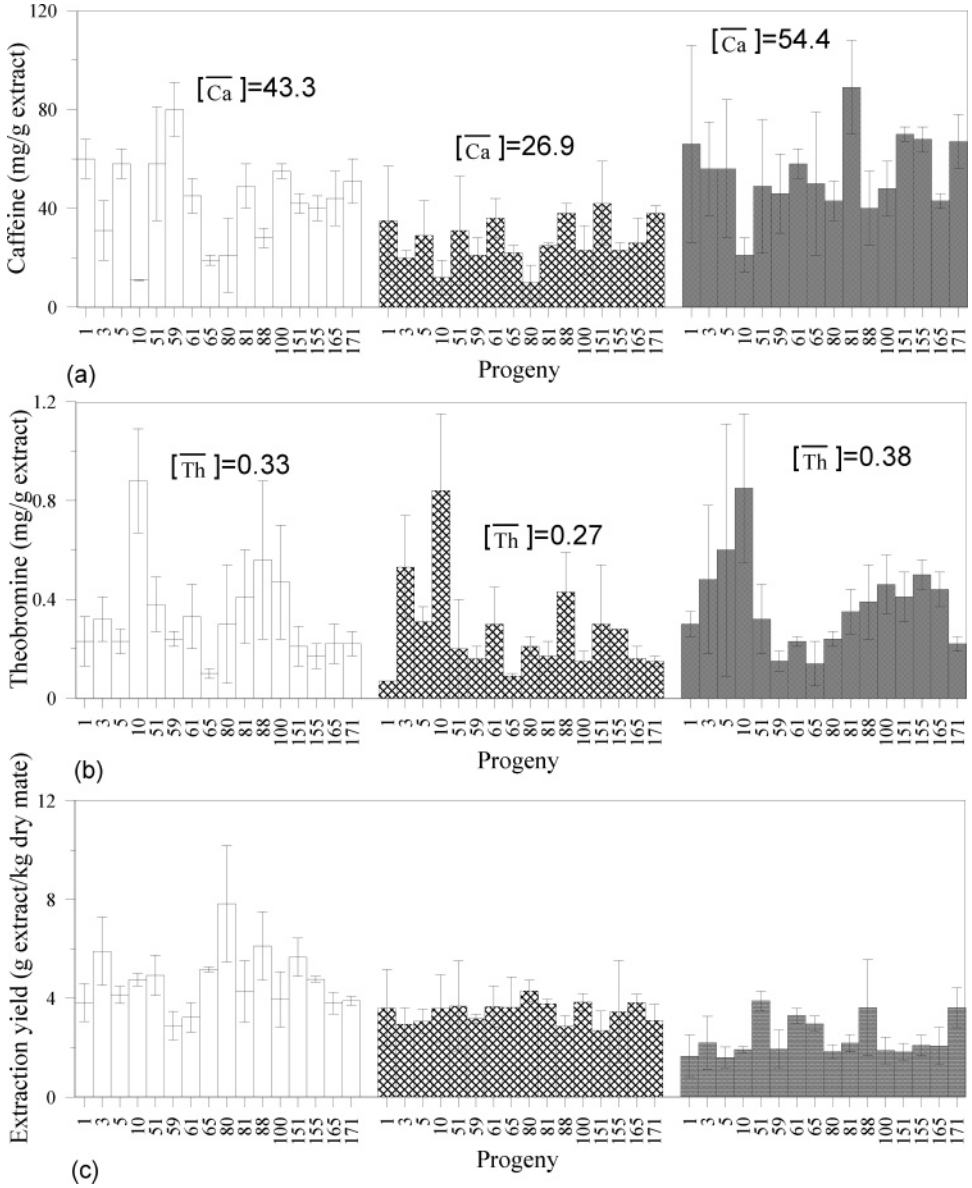

Figure 4. Contents of caffeine (a), theobromine (b), and extraction yield (c) in sixteen progenies grown in the first (white bar), second (diagonal cross bar), and third (dark bar) site. [Ca] and [Th] are the mean contents of caffeine and theobromine in each different site, respectively.

mg per kg of dry leaves of mate were obtained by multiplying the extraction yield in g of extract per kg of dry mate, reported in **Figure 4c**, times the results shown in **Figure 4a** and **b**, respectively.

An additional result that is useful for checking the consistency of the current data may be calculated from a curve of cumulative amount of both caffeine and theobromine in mate liquors, which was obtained by Saldaña et al. (*11*) by pumping supercritical carbon dioxide into a sealed vessel at 70 °C and 225 bar up to exhaustive extraction. The contents of caffeine and theobromine found by Saldaña et al. (*11*) in the first hour of extraction were approximately 533 and 29 mg kg^{-1} dry mate, respectively. In spite of the different conditions of temperature, pressure, and samples of mate leaves between these systems, caffeine contents are in the same order of magnitude, while theobromine is about 1 order of magnitude higher than those found in this investigation.

The possible reasons for the high selectivity of carbon dioxide for purine alkaloids are an important issue to be investigated. In the first hour of extraction, when the operation is governed almost exclusively by solubility (*11*), extraction is influenced by polarity effects and by CO_2 density (*34*). According to Johansen and Brunner (*34*), an increase in solubility with density is observed for xanthines. Because the purine alkaloids have similar polarities, and our conditions of temperature and pressure lead to a high density of carbon dioxide (902 kg/m^3), selective extraction of caffeine and theobromine is expected. In summary, the higher selectivity of carbon dioxide for purine alkaloids is based on an increase of the intermolecular interactions between these compounds, which is attributed to the effect of the similar polarities of methylxanthines and the high density of solvent (902 kg/m^3).

Effect of Different Progenies and Sites of Cultivation on the Contents of the Investigated Purine Alkaloids. On the basis of the advantage of applying a selective procedure of extraction in terms of speed and resolution of HPLC, a total of 48 extracts were obtained with compressed carbon dioxide to investigate the influence of both the progeny and site of cultivation on the contents of caffeine and theobromine. **Figure 4** reports the concentrations of the individual purine alkaloids obtained at all of the conditions involved in the randomized complete block design of experiments. The random order in

Table 2. Analysis of Variance for the Randomized Complete Block Design Experiment

parameter	source of variation	degrees of freedom	sum of squares	mean square	F_0	$F(\alpha = 0.5)$	effect
contents of caffeine	progeny	15	5632	376	2.71	2.01	significant
	site of cultivation	2	6094	3047	21.9	3.32	significant
	error	30	4160	139			
	total	47	15886				
contents of theobromine	progeny	15	1.290	0.086	8.43	2.01	significant
	site of cultivation	2	0.094	0.047	4.61	3.32	significant
	error	30	0.306	0.0102			
	total	47	1.690				

which the progenies are distributed within each site of cultivation and the presence of all of the different progenies in each block (site of cultivation) define the experimental design currently applied. An interesting procedure for statistical analysis involving randomized blocks is well described by Montgomery (*35*). This approach requires calculating the total sum of squares (SS_t) as well as the sum of squares for progenies (SS_p) and sites of cultivation (SS_s) as follows:

$$SS_t = \frac{N[\sum_{i=1}^{a}\sum_{j=1}^{b}(y_{ij})^2] - (\sum_{i=1}^{a}\sum_{j=1}^{b}y_{ij})^2}{N} \quad (1)$$

$$SS_p = \frac{\sum_{i=1}^{a}(\sum_{j=1}^{b}y_{ij})^2}{b} - \frac{(\sum_{i=1}^{a}\sum_{j=1}^{b}y_{ij})^2}{N} \quad (2)$$

$$SS_s = \frac{\sum_{j=1}^{b}(\sum_{i=1}^{a}y_{ij})^2}{a} - \frac{(\sum_{i=1}^{a}\sum_{j=1}^{b}y_{ij})^2}{N} \quad (3)$$

where a is the number of progenies, b is the number of cultivation sites, N is the total number of experimental runs, and y_{ij} is the concentration of either caffeine or theobromine. The error sum of squares (SS_e), which is another parameter required in this analysis of variance, is just the total sum of squares minus the sum of squares for progenies and sites of cultivation.

The importance of the investigated factors in regard to the contents of either caffeine or theobromine is verified by a comparison between the critical value of F distribution and the statistics F_0 for progenies and sites of cultivation given by eqs 4 and 5, respectively. The hypothesis of a significant effect of a factor is rejected if F_0 is lower than F.

$$F_0^p = \frac{SS_p/(a-1)}{SS_e/[(a-1)(b-1)]} \quad (4)$$

$$F_0^s = \frac{SS_s/(b-1)}{SS_e/[(a-1)(b-1)]} \quad (5)$$

In the current investigation, the entire analysis of variance is summarized in **Table 2**. According to the results shown in the last column, both the genotype and the site of cultivation affect the contents of caffeine and theobromine with a probability of 95%. These data also indicate that the influence of progeny on the content of theobromine is more significant than that on caffeine because the difference between F_0 and F for this factor is much greater in the first case. An analogous procedure for the site of cultivation implies that caffeine is more affected than theobromine by this second independent variable. Despite these conclusions, the specific nature of the differences between the content of either caffeine or theobromine involving the different progenies and sites of cultivation is not easily discovered, and a cluster analysis is required.

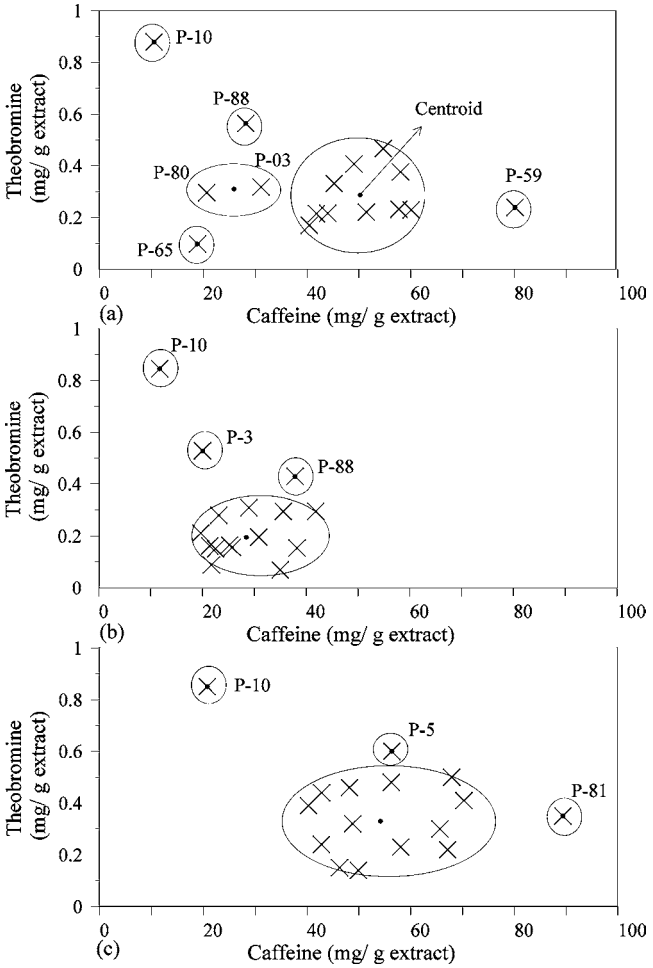

Figure 5. Cluster of progenies in the first (a), second (b), and third (c) sites of cultivation.

A simplified cluster algorithm based on the experimental uncertainties in the measurements of caffeine and theobromine is proposed. The first step toward this analysis is to determine a weighted mean of experimental standard deviation for both caffeine and theobromine. However, as three replicates were always considered, two identical arithmetic and weighted mean errors approximately equal to 10.8 mg g^{-1} extract and 0.12 mg g^{-1} extract were obtained by involving the values represented by all of the error bars in **Figure 4a** and **b**, respectively. The algorithm assumes that a single progeny initially constitutes a cluster. A new test member is admitted to a particular cluster only if the difference between the content of caffeine, as well as of theobromine, involving at least one progeny of that cluster and the test member is lower than the combined standard deviation for these variables. **Figure 5** reports the results obtained by applying this procedure in the different sites of cultivation, where 4 to 6 groups involving one or more members without significant differences in terms of caffeine and theo-

Table 3. Summary of Cluster Analysis Involving All of the Sites of Cultivation

first site			second site			third site		
cluster no./ no. of members	centroids (mg g^{-1}) [Ca]	[Th]	cluster no./ no. of members	centroids (mg g^{-1}) [Ca]	[Th]	cluster no./ no. of members	centroids (mg g^{-1}) [Ca]	[Th]
1/1	10.5	0.88	7/1	11.7	0.84	11/1	20.8	0.85
2/1	18.8	0.10	8/1	20.0	0.53	12/13	54.2	0.33
3/2	25.9	0.31	9/13	28.5	0.20	13/1	56.4	0.60
4/1	28.2	0.56	10/1	37.9	0.43	14/1	89.4	0.35
5/10	50.3	0.29						
6/1	80.1	0.24						

bromine were identified. **Table 3** presents the average contents of caffeine and theobromine as well as the number of members in the different clusters. An inverse correlation between the content of caffeine and theobromine is noticed in the different sites because when the amount of the first compound increases, the concentration of the second chemical specie is reduced. In fact, this behavior was already evidenced in the literature by involving the same progenies of mate but with traditional solvent extraction (*30*). This almost linear dependence is credited to the biosynthesis of purine alkaloids, which states that caffeine is produced via xanthosine by involving theobromine as an intermediate species (*36*, *37*).

It is interesting to observe that among the number of identified clusters, approximately 67−75% of them are constituted of a single progeny. The number of progenies that present an exclusive behavior in terms of caffeine and theobromine production in the individual sites varies from 3 to 4. These results indicate that despite the reduced number of progenies investigated, the probability of finding a plant with a particular genetic ability to produce methylxanthines in a genetic improvement program carried out in a single region of cultivation is in the range of approximately 19% (3/16) to 25% (4/16). Moreover, this genetic variability of progenies is increased to approximately 44% (7/16) by accounting for the different sites of cultivation investigated here and draws attention to the important role of environmental factors in the genetic transmission of the character. This variability allows one to obtain extracts that present extremely high caffeine with low theobromine (P-59 and P-81), moderate caffeine and low theobromine (P-5, Clusters 5 and 12), and a combination of low contents of both alkaloids (Clusters 3 and 9, and P-3, P-65, and P-88). Another relevant result that emerges from this analysis is that genotype P-10 is a valuable, unique, and constant source of genes for obtaining liquors that combine low caffeine with high theobromine.

The influence of environmental conditions on the contents of purine alkaloids is also promptly observed when their average values at the three different sites are compared. These data, which are clearly evidenced in **Figure 4**, show that the highest values of caffeine and theobromine were found in the third site, whereas the intermediate and lowest amounts of both purine alkaloids were extracted from the plants cultivated in the first and second sites, respectively. Because all of the plants were grown under similar meteorological conditions (see **Table 1**), these discrepancies are mainly attributed to natural soil properties or slight differences in fertilization practices. A more detailed explanation concerning the influence of this factor is presented in a study proposed by Cardozo Junior et al. (*30*), where an identical quantitative effect was noticed involving the same progenies and sites of cultivation but with traditional extraction using sulfuric acid, hot water, and chloroform partition. In fact, this is strong evidence of the reliability of the selective extraction with compressed carbon dioxide followed by isocratic HPLC analysis for the high resolution and rapid quantification of methylxanthines in mate liquors.

Conclusions. Selective extraction of caffeine and theobromine from mate leaves by applying compressed carbon dioxide at 20 °C and 150 bar was performed. A comparison between extracts from liquid CO_2 and conventional solvent extraction reveals the benefits of selective extraction in terms of speed and resolution of HPLC. Despite the use of gradient HPLC to speed up elution during the analysis of extracts obtained with aqueous ethanol solution, a reduction of approximately 83% in time analysis was noticed by involving liquors from liquid CO_2 extraction analyzed by using isocratic HPLC. A randomized block design of experiments was proposed to investigate the influence of 16 progenies of *Ilex paraguariensis* grown in 3 diverse sites on the contents of caffeine and theobromine in extracts obtained with liquid carbon dioxide. A significant effect of both these factors on the parameters investigated was evidenced by involving the statistical test of *F* distribution for a randomized block design of experiments. A cluster analysis based on the experimental uncertainties in the contents of these two methylxanthines has identified 4 to 6 groups of mate progenies with similar characteristics in terms of caffeine and theobromine production. Individual site analysis indicates a probability of 19−25% of finding a plant with a particular genetic ability regarding the production of methylxanthines, whereas the overall set of data suggests a genetic variability of 44% and highlights the importance of environmental factors in the genetic transmission of the character. Analogous findings available in the literature corroborate the reliability of the results obtained in this investigation.

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