http://dx.doi.org/10.5935/0103-5053.20140172

J. Braz. Chem. Soc., Vol. 25, No. 11, 1929-1938, 2014.
Printed in Brazil - ©2014 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

Spectroscopic and Chromatographic Fingerprint Analysis of Composition Variations in *Coffea arabica* Leaves Subject to Different Light Conditions and Plant Phenophases

Fernanda Delaroza,[a] Miroslava Rakocevic,[b] Galileu Bernardes Malta,[a] Roy Edward Bruns[c] and Ieda Spacino Scarminio*[,a]

[a]Laboratório de Quimiometria em Ciências Naturais, Departamento de Química, Universidade Estadual de Londrina, CP 6001, 86051-990 Londrina-PR, Brazil

[b]Instituto Agronômico do Paraná (IAPAR), CP 481, 86047-902 Londrina-PR, Brazil

[c]Instituto de Química, Universidade Estadual de Campinas, CP 6154, 13083-970 Campinas-SP, Brazil

Impressões digitais de folhas de *Coffea arabica* expostas à luz solar e autossombreadas colhidas na mesma planta, foram usadas para determinar as variações das concentrações dos metabólitos devido a diferentes ambientes de luz e fases fenológicas. Os rendimentos dos extratos nos solventes etanol, acetona, hexano e diclorometano, bem como as suas misturas são apresentados. Os maiores rendimentos para todas as folhas autossombreadas e expostas ao sol são obtidos com misturas binárias etanol-acetona. Análise de componentes principais (PCA) dos espectros de infravermelho com transformada de Fourier (FTIR) dos extratos indicam diferenças espectrais entre 2962-2828, 1759-1543 e inferiores a 1543 cm^{-1} que podem ser atribuídos a maiores concentrações de ésteres de ácidos graxos ou grupo éster em triglicerídeos, cafeína, ácidos clorogênicos e carboidratos, que são mais predominantes em folhas na fase de floração. Os espectros dos picos de cromatografia líquida de alta eficiência com detector UV de arranjo de diodos (HPLC-UV-DAD) dos extratos mostraram que folhas expostas ao sol apresentam absorções mais fortes para a cafeína, ácido clorogênico e teobromina. Experimentos confirmatórios para determinar a concentração de cafeína realizados com curvas de calibração UV, mostram que a cafeína de folhas expostas ao sol são cerca de duas vezes maior que as folhas autossombreadas na fase de floração. O conhecimento da quantidade de cafeína em folhas de *Coffea arabica* é de importância ecológica, já que plantas expostas ao sol parecem mais estressadas do que as autossombreadas para esta espécie. As concentrações lipídicas em folhas autossombreadas são quase o dobro daquelas expostas ao sol.

Fingerprints of self-shaded and sunlight-exposed leaves of the same *Coffea arabica* plant were obtained to determine metabolic concentration changes owing to different light environments and phenological stages. Leaf extract yields of the ethanol, acetone, dichloromethane and hexane solvents, as well as their statistical design mixtures, are reported. Highest yields are obtained with binary 1:1 ethanol-acetone mixtures for all sun-exposed and self-shaded leaves. Principal component analysis (PCA) of Fourier transform infrared (FTIR) spectra of leaf extracts indicate spectral differences between 2962-2828, 1759-1543 and below 1543 cm^{-1} that can be attributed to higher concentrations of fatty acid esters or the ester group in triglycerides, caffeine, chlorogenic acids and carbohydrates that are more prevalent in leaves of flowering plants. High-performance liquid chromatography with UV diode array detector (HPLC-UV-DAD) spectra of the chromatographic peaks for the extracts showed that sun-exposed samples contain stronger absorptions for caffeine, chlorogenic acid and theobromine. Confirmatory experiments carried out with reference UV calibration curves determined caffeine contents for sun-exposed leaves that are about double those for self-shaded leaves of flowering plants. Knowledge of leaf caffeine content in *Coffea arabica* is of ecological importance since sun-exposed conditions seem more stressful than self-shading ones for this species. Lipid concentrations in self-shaded leaves are almost double those that were sun-exposed.

Keywords: fingerprint, *Coffea arabica* leaves, mixture design, principal component analysis, HPLC-UV-DAD, light exposure

*e-mail: ieda@uel.br

Introduction

Within the biological system framework, the functional analysis of metabolomes has had an intense level of activities in the last decade. Chromatographic and spectroscopic fingerprinting have been increasingly used to provide information for the study of vegetal material. Among these applications, chemical fingerprinting has proved especially useful for diverse applications, such as quality control,[1] taxonomic classifications[2] and disease diagnostics.[3] Fingerprint techniques[4,5] have also become very powerful approaches to simultaneously analyze several metabolites and identify differences among them. They are described by a variety of analytical methods that can identify and approximately quantify a group of metabolites associated with specific pathways. The advantage of the use of these profiles is that metabolite variations are observed principally by total spectroscopic or chromatographic pattern changes, without previous knowledge of the identities of the investigated compounds. Recently, our group has shown that statistical mixture designs permit the development of rigorous but economical procedures for the development of fingerprint profiles of the extracted metabolites of plant material.[4-7]

Coffee Arabica (*Coffea arabica* L.) has its primary center of diversity in the southwestern Ethiopia highlands, the Boma Plateau of Sudan and Mount Marsabit of Kenya.[8] In its natural habitats, *Coffea arabica* is a perennial woody shrub shaded by taller trees. Its external form is characterized by a dimorphic growth, which consists of vertical trunk (orthotropic), and horizontal (plagiotropic) branches (Roux's architectural model).[9] Plant architecture is directly or indirectly related to multiple plant functions, like light interception, photosynthesis and transpiration.[10]

Coffee crops may be produced under shade or in monoculture.[11-14] Shade-grown coffee is planted among taller trees or under a shading canopy of natural forest. In modern and intensive planting systems, *Coffea arabica* is most often grown in monoculture, as done in parts of Brazil,[15] because those systems produce the highest berry yields.

In plants, especially in medicinal and alimentary ones, environmental conditions induce the physiological reactions and adaptations closely related to the biochemical changes observed in their metabolism. In coffee plants, these changes may be important for a final product – coffee bean quality. Many biosynthetic processes of secondary metabolites and purine alkaloid production by *C. arabica* cells are enhanced by light irradiance.[16] Environmental stress conditions are expected to enhance accumulation of qualitative defensive substances such as alkaloids, glycosides, and others. Agroforestry systems[12,17-21] decrease the incident radiation for coffee plants, change the phenological development of coffee and can reflect positively on beverage quality.[11] Since coffee is one of the most important worldwide trade commodities, it is essential to know how light intensity and plant density can affect leaf biochemistry and metabolome, and subsequently, the grain quality and metabolome.

The caffeine alkaloid is the most studied metabolite in coffee, mainly due to its pharmacological activity.[22] It is produced in young leaves and accumulates in mature ones.[23] It is considered toxic to both insects and fungi. The 'chemical defense theory' proposes that caffeine in young leaves, fruits and flower buds serves to protect soft tissues from predators such as insect larvae and beetles.[24] In *Coffea arabica*, caffeine content in leaves changes with mineral nutrition and omission of K induced the greatest increase of caffeine contents in leaves, while the omission of both P and K showed lowest contents.[25] Mazzafera *et al.*[26] dismissed the allelopathic or anti-herbivory role of caffeine in coffee leaves, discussing the role of caffeine as a nitrogen-storage compound and its involvement in resistance against diseases.

The metabolomic fingerprint approach permits separating the dynamics of any biotic, abiotic or genetic plant perturbation for accurate assessment. In this sense, it is assumed that the impact of any genetic, physiological or environmental factor could be diagnosed by chemical profiling of vegetal material. Our hypothesis is that *Coffea arabica* leaves are grown under stressful conditions when exposed to intense light in upper layers of monocultures, because it is a species of deeper forest layers. To demonstrate this hypothesis, metabolomic fingerprints of self-shaded and sunlight-exposed leaves of the same plant were obtained. A statistical mixture design[27] was performed to develop spectroscopic and chromatographic fingerprints to compare metabolite compositions of the *C. arabica* leaves grown under different arrangements and sunlight conditions (self-shaded and sun-exposed leaves). Principal component analysis (PCA) was used to analyze the fingerprints as well as to compare the performances of fingerprints for the discrimination of self-shading and sunlight-exposed *C. arabica* leaves.

Experimental

Coffea arabica leaf samples

The *Coffea arabica* plants, cultivar IAPAR 59, were cultivated in the experimental area of the Agronomic Institute of Paraná, Londrina (23°18' S, 51°17' W),

Paraná, Brazil. The coffee trees were planted in 1995 and trimmed twice (2000 and 2008) close to the ground. Plants were cultivated in two distinct spatial distributions: a rectangular arrangement (3.0 m × 0.41 m) allowing 1.25 m^2 for the development of each plant (8,000 plants ha^{-1}), and a square one (0.84 × 0.84 m) with 0.71 m^2 for each plant (14,000 plants ha^{-1}). Leaves were harvested from two canopy layers: upper (leaves directly exposed to the sun) and lower (self-shaded leaves). Leaves were collected from plagiotropic branches by taking care that they were of about the same age. Plants were observed in two phenophases: fruit ripening in April 2010 and end of flowering in October 2011.

Reagents

High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from VETEC Química Fina (Rio de Janeiro, Brazil). Mobile phase mixture preparations were made using water prepared with the Millipore Milli-Q purification system. Hexane, dichloromethane, acetone and ethanol were also purchased from VETEC and were of analytical grade.

Extract preparation

The collected ripe berries were dried in sunlight on a concrete floor during two weeks. The grains of green coffee were crushed in a mill (Laboratory Mill 3600) with liquid nitrogen and then were passed through a 0.71 mm (ABNT #25) sieve, packaged in sealing Selovac 2B and then stored in a freezer. Each extract was prepared by weighing 2.5 g of dried and crushed *Coffea arabica* L. leaves and adding 60 mL of the solvent mixtures according to a simplex-centroid design of four components consisting of ethanol (e), acetone (a), dichloromethane (d) and hexane (h) pure solvents, six 1:1 binary mixtures, four ternary mixtures in equal proportions and one quaternary 1:1:1:1 mixture (Table 1). Each mixture was placed in an ultrasonic bath (Unique, model USC 1400) for 2 hours. The bath water was kept cold with the addition of ice cubes. The extract was filtered to separate the solution from the coffee leaves. This procedure was repeated eleven more times, so the total volume of solvent mixture added to the leaves was 720 mL. This solvent was evaporated in a rotary evaporator and then kept under forced ventilation until reaching constant weight.

Analysis by Fourier transform infrared spectroscopy (FTIR)

A Thermo Scientific Nicolet iS10 FTIR spectrometer equipped with a Ge crystal attenuated total reflectance (ATR) accessory was used for analysis. The spectra of the extracts were recorded in the 4000-675 cm^{-1} region, with 4 cm^{-1} resolution and 32 scans. Principal component analysis (PCA) was performed on the entire infrared spectra using the Statistica 6.0 software (Statistica for Windows 6.0, Statsoft, Tulsa, OK, USA, 1999). Before performing PCA the data matrix was auto-scaled, with each data vector having a zero mean and unit variance.

Table 1. Simplex centroid design extraction mixtures

Extract	Proportion			
	Ethanol	Acetone	Dichloromethane	Hexane
1	1	0	0	0
2	0	1	0	0
3	0	0	1	0
4	0	0	0	1
5	0.5	0.5	0	0
6	0.5	0	0.5	0
7	0.5	0	0	0.5
8	0	0.5	0.5	0
9	0	0.5	0	0.5
10	0	0	0.5	0.5
11	0.333	0.333	0.333	0
12	0.333	0.333	0	0.333
13	0.333	0	0.333	0.333
14	0	0.333	0.333	0.333
15	0.25	0.25	0.25	0.25

Sample preparation for HPLC analysis

Each sample was prepared by weighing 0.1 mg of concentrated extract and re-dissolving it in 1.00 mL of methanol. This was placed in an ultrasonic bath (Unique, model Ultracleaner 1400) for 15 min, and later filtered through a 0.20 µm CHROMAFIL® XTRA PTFE-20/25 membrane filter (Macherey-Nagel GmbH & Co. KG, Germany). Five hundred microliters of the mobile phase were added to 100 µL of this extract. A 20 µL aliquot of this diluted solution was injected into the HPLC. The chromatographic conditions were: Kinetex C18 column, 2.6 µm Hilic 100 Å, with dimensions of 150 mm × 4.6 mm (Phenomenex), Kinetex Hilic guard column 4.6 mm (Phenomenex) and 0.5 mL min^{-1} mobile phase flow rate. HPLC analysis was conducted on an SPD-M10AV Finnigan Surveyor 61607 liquid chromatograph equipped with a Finnigan Surveyor PDA Plus diode array detector and four Thermo-Electron Corporation pumps. The gradient program used was as follows ACN: H_2O (15:85 v/v),

0-0.44 min; ACN:H_2O (35:65 v/v), 0.45-2.67 min; ACN:H_2O (90:10 v/v), 2.68-3.33 min; ACN:H_2O (15:85 v/v), 3.34-15 min. Elution was monitored at 263, 274 and 325 nm. Satisfactory separation was achieved at 274 nm. The data were processed using ChromQuest 4.2 software.

Caffeine and lipid analysis

For caffeine extraction,[28] 2.0 g of dried and crushed leaves of *C. arabica* L. with 4 mL of 98% sulfuric acid were heated in a water bath at 80 ± 2 °C for 15 minutes. Then, 50 mL of water were added and kept at a boil for 15 minutes. After this, the sample was filtered while hot and washed with 10 mL of hot water acidified with 50 µL of 98% sulfuric acid. This washing procedure was repeated twice more. The filtrate was transferred to a separation funnel with 30 mL of chloroform and extracted three times. The decanted portion was filtered with filter paper wetted with chloroform. The chloroform was evaporated in a rotary evaporator at 50 ± 2 °C. The extract was kept in the kiln until complete evaporation of the chloroform. Extracted caffeine was diluted in 10 mL of Milli-Q water and a 30 µL aliquot of this solution was added to 2970 µL of Milli-Q water. For caffeine quantification, standard stock solution was prepared adding 0.10 g of caffeine (Sigma Hidrus, PA) to 100 mL of Milli-Q water and then diluted to concentrations of 0.0060, 0.0080, 0.0100, 0.0200, 0.0300, 0.0400 mg mL^{-1}. Caffeine extractions were performed in triplicate and the analytical calibration curve was obtained at 274 nm in UV-Vis spectrophotometer.

For lipid extraction,[28] 5.00 g of dried and crushed leaves of *C. arabica* L were weighed and placed in a kiln for four hours at 105 ± 2 °C. The samples were subjected to Soxhlet extraction with a mixture of ether and petroleum ether (1:1) for 6 hours at 55 ± 2 °C. The solution was concentrated on a rotary evaporator and subjected to forced ventilation until reaching constant mass. The lipid mass to sample percentages were determined in duplicate.

Results and Discussion

The effects of solvent composition on the yields of crude extracts of *Coffea arabica* leaves are shown in Figure 1. For

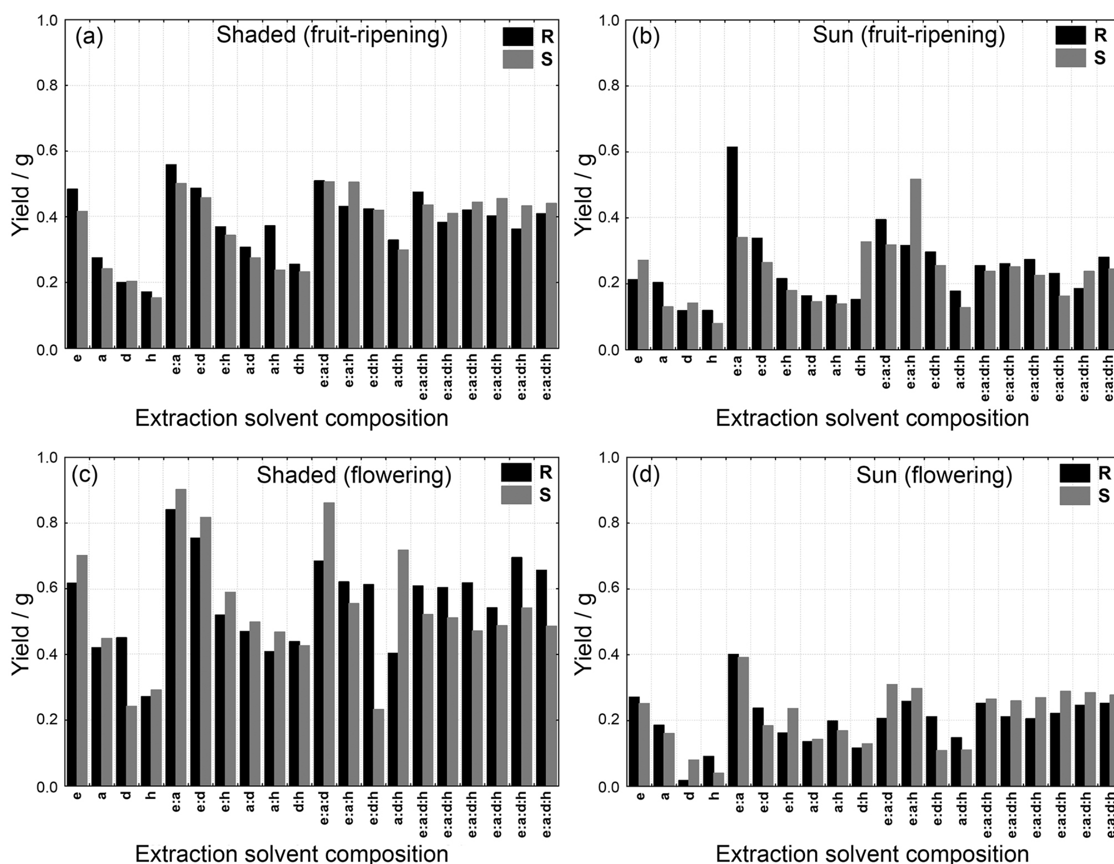

Figure 1. Crude extract yields for different proportions of ethanol, acetone, hexane, and dichloromethane solvents for: (a) self-shaded and (b) sun-exposed samples of the fruit-ripening harvest and (c) self-shaded and (d) sun-exposed samples of the flowering harvest, for the square (S) and rectangular (R) arrangements.

both the fruit-ripening and flowering harvests, regardless of the plant arrangement, yields are higher for extracts of the self-shaded leaves. The differences were especially pronounced in the second harvest with leaves from the more mature plants. The shaded leaves in the flowering harvest had much larger yields than in the fruit-ripening one.

The yield variations with solvent composition are similar for both plant densities and both light conditions of each phenophase. Local relative maximum yields in all the bar graphs in Figure 1 are found for pure ethanol, the binary ethanol-acetone and ethanol-dichloromethane mixtures, the ternary ethanol-acetone-dichloromethane mixtures, and the quartenary mixture involving all the solvents.

The similar profiles of the yield values (Figure 1) can be better understood by determining response surfaces for the crude extract yield as a function of the extraction solvent composition. The models for these surfaces were validated by performing ANOVA and testing for lack of fit. The quadratic models for the yields of the fruit-ripening (fr) and flowering phases (fp) and for the rectangular arrangement of the sun-exposed (se) and self-shaded (ss) leaves, showed no significant lack of fit at the 95% confidence level (Table 2). The simplified equations, containing only statistically significant binary coefficients, for the predicted yields are:

$$\hat{y}_{Rss(fr)} = \underset{(\pm0.04)}{0.49e} + \underset{(\pm0.04)}{0.29a} + \underset{(\pm0.04)}{0.20d} + \underset{(\pm0.15)}{0.18h} + \underset{(\pm0.15)}{0.61ea} + \underset{(0.15\pm)}{0.57ed} + \underset{(\pm0.15)}{0.45ah} \quad (1)$$

$$\hat{y}_{Rss(fp)} = \underset{(\pm0.04)}{0.62e} + \underset{(\pm0.04)}{0.43a} + \underset{(\pm0.04)}{0.46d} + \underset{(\pm0.04)}{0.27h} + \underset{(\pm0.17)}{1.19ea} + \underset{(\pm0.17)}{0.82ed} \quad (2)$$

$$\hat{y}_{Rse(fr)} = \underset{(\pm0.04)}{0.21e} + \underset{(\pm0.04)}{0.20a} + \underset{(\pm0.04)}{0.12d} + \underset{(\pm0.04)}{0.12h} + \underset{(\pm0.19)}{1.66ea} + \underset{(\pm0.19)}{0.72ed} \quad (3)$$

$$\hat{y}_{Rse(fp)} = \underset{(\pm0.03)}{0.28e} + \underset{(\pm0.03)}{0.20a} + \underset{(\pm0.03)}{0.02d} + \underset{(\pm0.03)}{0.09h} + \underset{(\pm0.11)}{0.56ea} + \underset{(\pm0.11)}{0.31ed} + \underset{(\pm0.11)}{0.27dh} \quad (4)$$

where e, a, d and h represent the ethanol, acetone, dichloromethane and hexane proportions, respectively. Standard error estimates are given in parentheses below the corresponding model coefficients. In all these equations the linear coefficients for ethanol are larger than those of the other pure solvents. As shown in Figure 1, of all the pure solvents, ethanol extracts the highest yield. All the models contain significant binary coefficients for the ethanol-acetone and ethanol-dichloromethane solvents. These positive coefficients indicate the existence of synergic interactions between these solvents in the mixtures resulting in higher yields. The synergic acetone-hexane coefficient is only significant in equation 1 and the dichlorometane-hexane one only in equation 4.

The special cubic model for the yields of the first and second harvests for the square arrangements for sun-exposed and self-shaded leaves, showed no significant lack of fit at the 95% confidence level. The simplified prediction equations for the yields are:

Table 2. ANOVA regression and lack of fit significance level probabilities

			Significance Level	
Harvest	Arrangement	Exposure	Regression	Lack of fit
1	R	ss	0.000	0.633
1	R	se	0.003	0.163
1	S	ss	0.000	0.089
1	S	se	0.011	0.054
2	R	ss	0.000	0.878
2	R	se	0.000	0.122
2	S	ss	0.000	0.072
2	S	se	0.000	0.010

$$\hat{y}_{Sss(fr)} = \underset{(\pm0.02)}{0.41e} + \underset{(\pm0.02)}{0.24a} + \underset{(\pm0.02)}{0.20d} + \underset{(\pm0.02)}{0.15h} + \underset{(\pm0.10)}{0.75ea} + \underset{(\pm0.09)}{0.68ed} + \underset{(\pm0.10)}{0.29eh} + \underset{(\pm0.09)}{0.28ad} + \underset{(\pm0.09)}{0.28dh} + \underset{(\pm0.67)}{2.34eah} \quad (5)$$

$$\hat{y}_{Sss(fp)} = \underset{(\pm0.03)}{0.70e} + \underset{(\pm0.03)}{0.45a} + \underset{(\pm0.03)}{0.24d} + \underset{(\pm0.03)}{0.29h} + \underset{(\pm0.17)}{1.34ea} + \underset{(\pm0.17)}{1.42ed} + \underset{(\pm0.17)}{0.65ad} + \underset{(\pm0.17)}{0.42ah} + \underset{(\pm0.17)}{0.67dh} - \underset{(\pm1.03)}{5.17eah} - \underset{(\pm1.03)}{12.96edh} + \underset{(\pm1.03)}{4.63adh} \quad (6)$$

$$\hat{y}_{Sse(fr)} = \underset{(\pm0.05)}{0.28e} + \underset{(\pm0.05)}{0.13a} + \underset{(\pm0.05)}{0.14d} + \underset{(\pm0.23)}{0.57ea} + \underset{(\pm0.25)}{0.93dh} + \underset{(\pm1.48)}{5.59eah} - \underset{(\pm1.58)}{4.96adh} \quad (7)$$

$$\hat{y}_{Sse(fp)} = \underset{(\pm0.03)}{0.26e} + \underset{(\pm0.03)}{0.17a} + \underset{(\pm0.03)}{0.09d} + \underset{(\pm0.03)}{0.05h} + \underset{(\pm0.09)}{0.74ea} + \underset{(\pm0.08)}{0.33eh} + \underset{(\pm0.08)}{0.23ah} + \underset{(\pm0.50)}{2.18ead} \quad (8)$$

As found for the models obtained for the rectangular arrangement, the blending coefficients of ethanol are the highest of all the linear coefficients in these equations. In this arrangement (equations 5-8), all models contain significant binary and ternary coefficients.

All models contain significant positive binary coefficients for ethanol and acetone. The two models for the self-shaded leaves have significant binary coefficients for ethanol and dichloromethane although the interaction of these solvents is not significant for the sun-exposed leaves. The square arrangement models do have a higher number of significant binary coefficients and even some ternary ones. Most of them are positive indicating synergic mixture effects among the solvents on extraction.

The contour plots containing the maximum predicted yields of equations 1-8 are given in Figure 2. The models for the yields of the fruit-ripening and flowering harvests for both plant arrangements for sun-exposed and self-shaded conditions predict that higher yields can be achieved with equal volume or ethanol-rich ethanol-acetone binary mixtures.

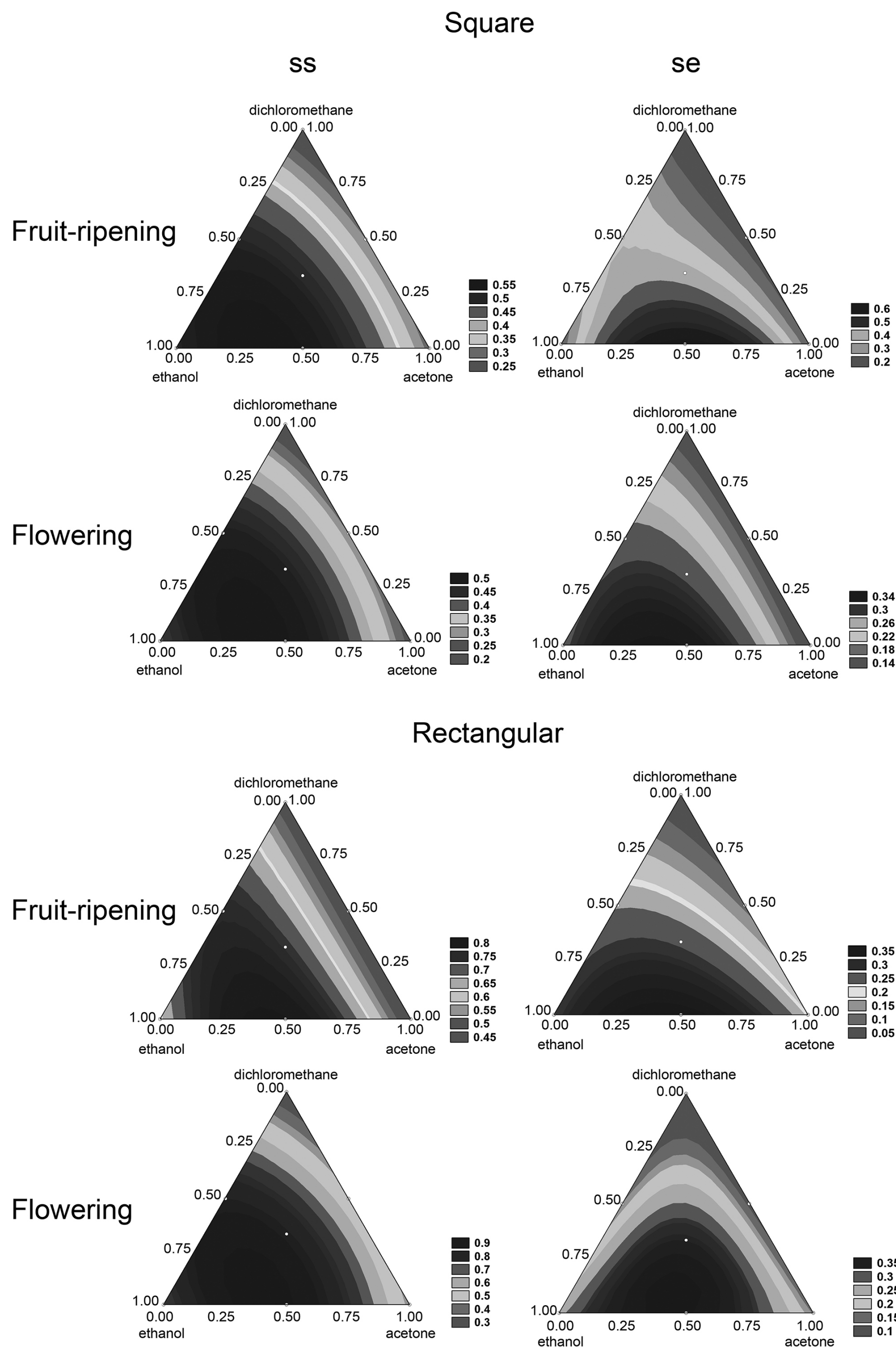

Figure 2. Mixture response surfaces for self-shaded (ss) and sun-exposed (se) fruit-ripening harvest samples, for the square and rectangular arrangements of both harvests.

PCA was applied to FTIR spectra to search for the fingerprint as well as to explore the spectroscopic differences of chemical compositions for different *Coffea arabica* leaf light conditions and plant phenophases. The spectral data were arranged in a 152 × 1725 matrix, corresponding to spectra of 15 different proportions of solvent with a pentuplicate at the central point, for leaves harvested in the rectangular and square arrangements under sun-exposed and self-shaded conditions.

Figure 3a shows the scores plot for PC1 against PC3, which accounts for 93.2% of total variance. Although PC3 explains a small percentage of variance, this plot shows a relatively clear separation between leaves in the fruit-ripening and flowering phases. A greater portion of leaf samples from the fruit-ripening phase are located at positive PC3 scores, while those from the flowering period are located at its negative values. Figure 3b shows the loading plot for PC1 and PC3 for the spectral range

studied. Three main regions contributed to the separation of leaves from the two coffee phenophases, 2962-2828, 1759-1543 and below 1543 cm^{-1}, including the fingerprint region. The 1744 cm^{-1} band can be attributed to the C=O vibration associated with fatty acid esters or the ester group in triglycerides whereas the bands at 1658 and 1704 cm^{-1} are associated with caffeine absorption. Since these bands have negative loadings (Figure 3b) one can conclude that the leaves in the flowering phase are richer in these compounds than in those of the fruit-ripening phase. Spectral regions around 2920-2850 cm^{-1} are attributed to CH_2 asymmetrical stretching of methyl groups and C–H symmetrical stretching of methyl groups. The bands at 1739 and 1660 cm^{-1} are consistent with the C=O stretching of polysaccharides and C=C stretching band of lipids and fatty acids, respectively.[29] The region below 1543 cm^{-1}, including the classical fingerprint region, has negative loadings and can be associated with chlorogenic acids and carbohydrates that appear to be more prevalent in leaves from flowering plants. Therefore, these results suggest that this spectral separation between leaves from the two phenophases is mainly due to polysaccharides, fatty acids, caffeine and proteins. Best separation was obtained in the plot of PC2 against PC3 not shown here. For the fruit-ripening phase, the ethanol: hexane (1:1 v/v) mixture discriminated the self-shaded leaves from those directly exposed to the sun, whereas for plants in flowering leaf discrimination was not clear with this extractor. For the other statistical design mixtures, no separation of self-shaded and sun-exposed leaves could be found.

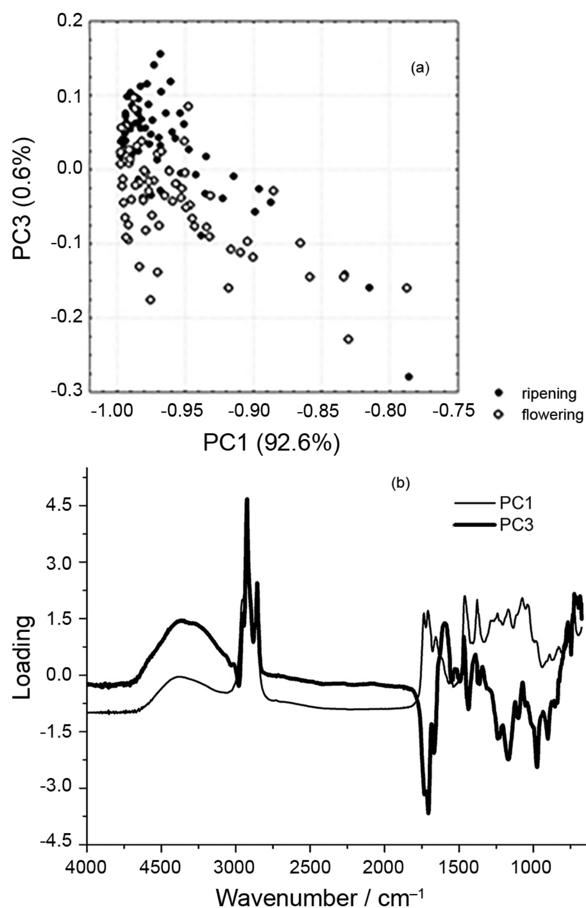

Figure 3. (a) PC1 against PC3 score plot and (b) PC1 against PC3 loading plot of the FTIR spectra obtained from the simplex centroid design mixtures obtained for the square and rectangular arrangements of both harvests.

Figure 4 contains a three dimensional PC graph of the UV-Vis spectra between 200 and 611 nm of the leaf extracts containing 94.7% of the total data variance. The data matrix contained 140 rows and 601 columns. Four main groups can be seen, and the group in the center, well separated from the other three, is indicated within a circle. This group contains points representing spectra of only self-shaded leaf extracts, obtained with dichloromethane, 1:1 ethanol-hexane, 1:1 hexane-dichloromethane and 1:1:1 ethanol-dichloromethane-hexane solutions.

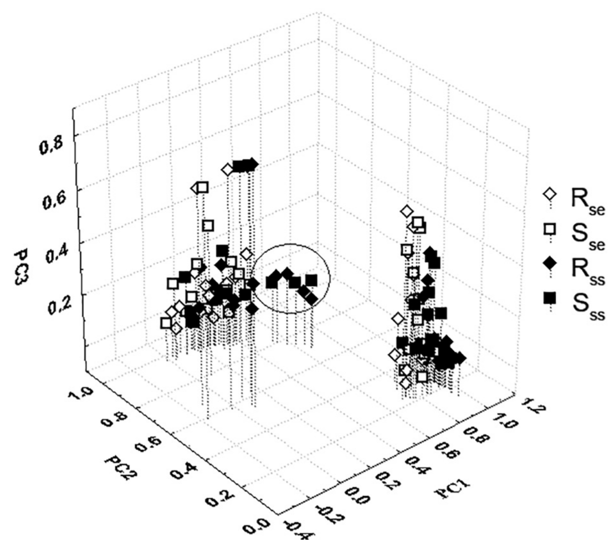

Figure 4. Three dimensional PC1 *vs.* PC2 *vs.* PC3 score plot containing 94.7% of the variance of the FTIR spectral data obtained from the simplex centroid design mixtures for the square (S) and rectangular (R) arrangements and fruit-ripening and flowering harvests of sun-exposed (se) and self-shaded (ss) samples.

Figure 5 shows the diode array detector (DAD) spectra of the chromatographic peaks for the extract prepared in the 1:1 ethanol-hexane mixture, obtained for self-shaded and sun-exposed samples of the square and rectangular arrangements. Sun-exposed samples contain stronger absorptions for caffeine, chlorogenic acid and theobromine, which indicate higher concentrations in these leaves than in those that were self-shaded. The leaf caffeine content in *Coffea arabica* leads to an ecological interpretation for which sun-exposed conditions seem more stressful than the

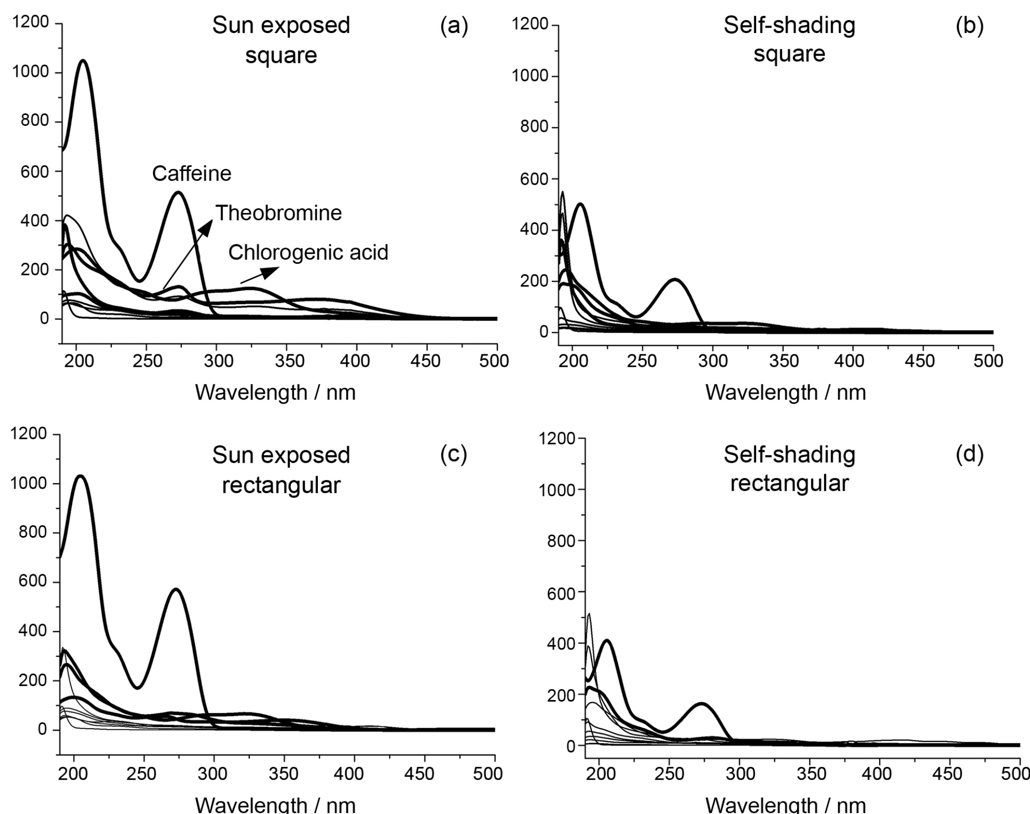

Figure 5. HPLC-UV-DAD spectra of the chromatographic peaks for the extract prepared in ethanol-hexane mixtures obtained for sun exposed and self-shaded samples of the square (S) and rectangular (R) arrangements.

self-shaded ones originating from lower forest layers for this species. This contrasts with higher caffeine contents in *Ilex paraguarienses* yerba mate leaves grown in the shade compared with those exposed to sunlight, where shade conditions are more stressful.[30] Yerba mate originates from forests with dominant *Araucaria angustifolia* where, in its natural habitat, it exists in a first high layer under a dominant species.

The difference between the arrangements is much less pronounced than the effect of direct sun exposure or not. Absorbance spectra between 190 to 210 nm[31] are attributed to sugars and lipids, so these results are in agreement with those obtained from the IR spectra.

Figure 6 contains bar graphs of the caffeine abundances estimated from the peak heights at 274 nm of the DAD spectra of the caffeine chromatographic peak. Caffeine abundances are normally larger for all extracts of sun-exposed leaves compared to those for the self-shaded leaves. This is true for both plant arrangements and harvests. A paired *t*-test was performed on all 68 differences between DAD caffeine peak heights of sun-exposed and self-shaded leaf extracts. The higher caffeine abundances in the sun-exposed leaves are highly significant, well above the 99% confidence level. Separate *t*-tests of the differences were also performed on leaf extracts of each harvest. These differences for the flowering harvest were significant, well-above the 99% confidence level, whereas those from the fruit-ripening stage were only significant, below that level but above the 95% level.

Triplicate confirmatory experiments were carried out directly on the self-shaded and sun-exposed leaf materials determining the percentage abundances of caffeine from the UV calibration curves and lipids from the gravimetric results of the rectangular and square arrangements of both fruit-ripening and flowering plants. These results are presented in Table 3 along with moisture and ash contents. As can be seen the caffeine contents for the sun-exposed leaves of both arrangements for the flowering plants are about double the values of the self-shaded leaves. The differences are much smaller for the leaves of the plants in the fruit-ripening phase indicating an interaction effect between light exposure and harvest period as found above for the DAD caffeine abundance estimates. In an ecological interpretation, caffeine is considered a protective secondary metabolite,[24] and flowering plants also emit many new leaves, much more than fruit-ripening plants. A newly formed leaf area is responsible for carbon assimilation needed for both fruit development and plant structure growth and accumulates more protective metabolites, whereas during fruit-ripening the leaf area is responsible

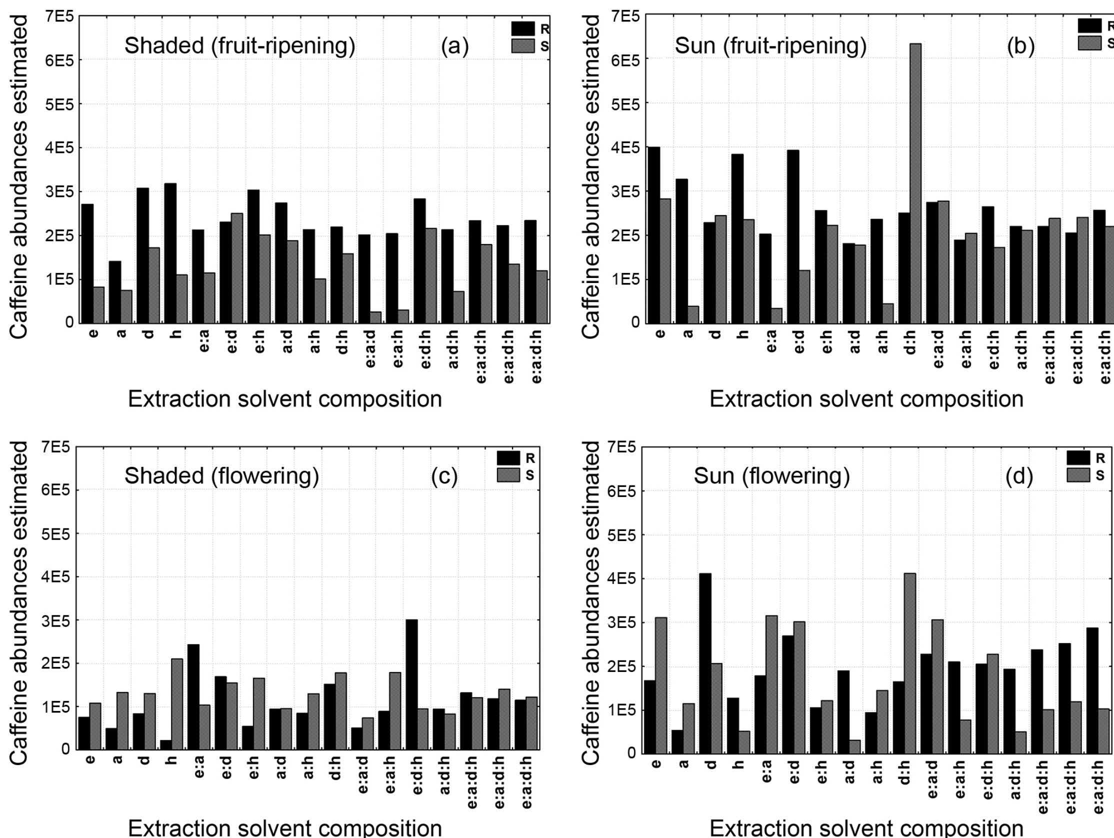

Figure 6. Caffeine abundance estimates from DAD spectral peak heights for extracts with different proportions of ethanol, acetone, hexane and dichloromethane solvents for: (a) self-shaded and (b) sun-exposed samples of the fruit-ripening harvest, and (c) self-shaded and (d) sun-exposed samples of the flowering harvest, for the square (S) and rectangular (R) arrangements.

Table 3. Moisture, total ash, caffeine and lipid percentage amounts determined in triplicate for leaves harvested in the fruit-ripening and flowering plants

Arrangement	Moisture	Total ash	Caffeine	Lipids
	Fruit-ripening harvest			
Rss	8.25	8.43	0.50	3.18
Rse	9.26	7.78	0.52	1.92
Sss	9.74	8.25	0.65	3.49
Sse	11.64	7.56	0.71	1.91
	Flowering harvest			
Rss	8.34	8.51	0.35	3.19
Rse	8.80	7.99	0.83	1.96
Sss	7.75	7.96	0.43	3.51
Sse	8.80	7.07	0.74	2.02

only for final seed formation. Paired *t*-tests confirm the caffeine differences between sun-exposed and self-shaded leaves at close to the 90% confidence level.

Lipid content is much higher in the self-shaded leaves of both plant arrangements and phenophases indicating the efficient lipid protective role of secondary metabolism products in the shade. There are no significant lipid differences for type of arrangement or plant phenophase.

Conclusions

The metabolic fingerprint approach permitted the chemical profiling of vegetal material. Solvent composition was found to be very important in determining the extraction yield. FTIR, HPLC-UV-DAD and classical chemical analysis show that caffeine, theobromine and chlorogenic acid contents in sun-exposed *Coffea arabica* leaves are about double those found in self-shaded leaves, indicating that sun-exposed conditions are more stressful than self-shaded ones from lower forest layers for this species. This contrasts with higher caffeine contents in *Ilex paraguarienses* yerba mate leaves grown in the shade compared with those exposed to sunlight.[29] Leaves from the flowering phase are more sensitive to light exposure than those of the fruit-ripening phase. Lipid concentrations in *Coffea arabica* self-shaded leaves are about double those exposed to direct sunlight, indicating the efficient lipid protective role of secondary metabolism products in the shade.

Acknowledgements

This work was support by CNPq, Fundação Araucária and Consórcio Brasileiro de Pesquisa e Desenvolvimento de Cafés, Brazilian granting agencies.

References

1. Xu, X.; Jiang, J.; Liang, Y.; Yi, L.; Cheng, J.; *Anal. Methods* **2010**, *2*, 2002.
2. Choi, Y. H.; Sertic, S.; Kim, H. K.; Wilson, E. G., Michopoulos, F.; Lefeber, A. W. M.; Erkelens, C.; Prat Kricun, S. D.; Verpoorte, R.; *J. Agric. Food Chem*. **2005**, *53*, 1237.
3. Ellis, D. I.; Dunn, W. B.; Griffin, J. L.; Allwood, J. W.; Goodacre, R.; *Pharmacogenomics* **2007**, *8*, 1243.
4. Moreira, I.; Scarminio, I. S.; *Talanta* **2013**, *107*, 416.
5. Soares D. X.; Scarminio I. S.; *Anal. Chim. Acta* **2011**, *702*, 288.
6. Soares, P. K.; Scarminio, I. S.; *Phytochem. Anal.* **2008**, *19*, 78.
7. Soares, P. K.; Bruns, R. E.; Scarminio, I. S.; *Anal. Chim. Acta* **2012**, *736*, 36.
8. Anthony, F.; Combes, M. C.; Astorga, C.; Bertrand, B.; Graziosi, G.; Lashermes, P. *Theor. Appl. Genet*. **2002**, *104*, 94.
9. Hallé, F.; Oldeman, R. A. A.; Tomlinson, P. B.; *Tropical Trees and Forests: an Architectural Analysis*, 1st ed.; Springer-Verlag: Berlin, Germany, 1978, pp. 441.
10. Sinoquet, H.; Stephan, J.; Sonohat, G.; Lauri, P. E.; Monney, P.; *Plant, Cell Environ*. **2001**, *24*, 395.
11. Beer, J.; Muschler, R.; Kass, D.; Somarriba, E.; *Agrofor. Syst*. **1998**, *38*, 139.
12. López-Gómes, A. M.; Willians-Linera, G.; Manson, R. H.; *Agric. Ecosyst. Environ*. **2008**, *124*, 160.
13. Muschler, R. G; *Agrofor. Syst*. **2001**, *51*, 131.
14. Perfecto, I.; Vandermeer, J.; Mas, A.; Pinto, L. S.; *Ecol. Econ*. **2005**, *54*, 435.
15. Clay, J. C.; *World Agriculture and the Environment: a Commodity-by-Commodity Guide to Impacts and Practices*, 1st ed.; Island Press: Washington, USA, 2004, ch. 3.
16. Kurata, H.; Matsumura, S.; Furusaki, S.; *Plant Sci*. **1997**, *123*, 197.
17. Bosselmann, A. S.; Dons, K.; Oberthur, T.; Smith Olsen, C.; Ræbild, A.; Usma, H.; *Agric. Ecosyst. Environ*. **2009**, *129*, 253.
18. Souza, H. N.; Goede, R. G. M. ; Brussaard, L.; Cardoso, I. M.; Duarte, E. M. G.; Fernandes, R. B. A.; Gomes, L. C.; Pulleman, M. M.; *Agric. Ecosyst. Environ*. **2012**, *146*, 179.
19. Rice, R. A.; *Agric. Ecosyst. Environ*. **2008**, *128*, 212.
20. Babbar, L. I.; Zak, D. R.; *Agric. Ecosyst. Environ.* **1994**, *48*, 107.
21. Lin, B. B.; *Agric. Forest Meteorol*. **2007**, *144*, 85.
22. Viani, R. In *Physiologically Active Substances in Coffee*; Clarke, R. J; Macrae, R., eds; New York: Elsevier, 1988, ch. 1.
23. Ashihara, H.; Sano, H.; Crozier, A.; *Phytochemistry* **2008**, *69*, 841.
24. Ashihara, H.; Crozien, A.; *Trends Plant Sci*. **2001**, *6*, 407.
25. Mazzafera, P.; *Bragantia* **1999**, *58*, 387.
26. Mazzafera, P.; Yamaoka-Yano, D. M.; Vitória, A. P.; *Rev. Bras. Fisiol. Veg*. **1996**, *8*, 67.
27. Barros, B. B; Bruns, R. E.; Scarminio, I. S.; *Statistical Design - Chemometrics*; Elsevier: Amsterdam, 2006, ch. 7.
28. Zenebon, O.; Pascuet, N. S.; Tiglea, P.; *Métodos Físico-Químicos para Análise de Alimentos*, 1a ed.; Instituto Adolfo Lutz: São Paulo, 2008, ch. 4.
29. Wang, N.; Fu, Y.; Lim, L.-T.; *J. Agric. Food Chem*. **2011**, *59*, 3220.
30. Mazzafera, P.; *Braz. J. Plant Physiol*. **1994**, *6*, 149.
31. Veloso, A. C. A.; Rodrigues, L. R.; Dias, L. G.; Peres, A. M. In *UV Spectrophotometry Method for Dietary Sugars*; Preedy, V. R., ed.; RSC Publishing: Cambridge, UK, 2012, ch. 14.

Submitted: May 12, 2014
Published online: July 29, 2014

FAPESP has sponsored the publication of this article.