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Article

Integrated Chemometric Approach to Optimize Sample Preparation for Detecting Metabolic Changes Provoked by Abiotic Stress in *Coffea arabica* L. Leaf Fingerprints

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The effects of water-deficit stress on irrigated and unirrigated field plants of *Coffea arabica* L. genotype IAPAR 59 were investigated. Plant extracts were obtained following an ethanol-dichloromethane-hexane statistical mixture design. Proton nuclear magnetic resonance (^{1}H NMR) fingerprints of the extracts were discriminated using factor analysis (FA) and hierarchical clustering techniques. Extracts from the 1:1:1 ternary mixture presented the largest discriminations compared with those from the pure solvents or their 1:1 binary mixtures. Metabolites resulting from fermentation processes and nutritional deficiencies as well as senescence and abscission precursors such as lactate, arginine and methionine were prevalent in unirrigated plants that can provoke expressive decreases in bean productivity as well as premature plant aging. Amino acids that control regulatory, physiological processes and soil salinization have higher concentrations in the irrigated plants. The NMR assignments of eighteen substances observed here were confirmed by electrospray ionization mass spectrometry.

Keywords: *Coffea arabica*, environmental stress, water-deficit stress, metabolite classes

Introduction

Climate changes carry important implications for almost every aspect of life on Earth and can result in limiting factors for agriculture production, such as those caused by drought, salinity, cold or heat stress, oxidative stress and heavy metals.[1,2] Some environmental conditions can affect the availability of water and hydric necessities of several agricultural sectors that can cause large changes in ecosystems that depend on water for development.[3] With increasing droughts and heat waves in some places, it is possible that the necessity of irrigation increases in the future or even in the development of genetically modified organisms with water-resistant genes.[4-7] In 2080, climate changes are expected to cause negative impacts on the productivity of *Coffea arabica* L. as well as on the regions where it normally grows.[8] Changes in coffee production capacities will have economic and food-safety implications, not only at the regional level but also at the world level. Current analysis on irrigation technologies to improve food availability generally focus on production yields but does not bring information or understanding about molecular changes occurring in plants.[9-13]

Metabolite concentrations and diversities present in vegetal material provide integrated information about cellular function at the molecular level with which it is possible to determine cell or tissue phenotype responses to environmental and even genetic alterations besides predicting organism behavior for possible improvements.[14-16] The group of metabolites (i.e., metabolome) of a cell or vegetal tissue has a rapid response to any environmental alteration, although, owing to the enormous diversity existing in organisms,

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changes at the molecular level have been considered to be quite unique for each kind of plant increasing the difficulty of generalizing biomarkers as a function of specific stresses.[17-21] As cellular metabolism is integrated several metabolites participate in different biochemical pathways leading to metabolic data of very high complexity.[22,23] The association of numerous biochemical pathways, chemical concentration changes and high complexity of metabolic groups increases the difficulty of a global analysis of cell metabolome.[24]

Knowledge of metabolomic variations is fundamental for Metabolic Engineering.[25,26] The development of strategies for altering any biosynthetic mechanism to increase or diminish metabolite levels needs to be preceded by elucidation of the molecular alterations caused by stress factors. Based on knowledge of biosynthetic mechanisms it is possible to understand probable metabolic effects on organisms and evaluate plant viability leading to a development strategy at the gene level of the vegetal material. Studies have shown that water deficiency leads to production loss with lower yields and inferior attributes affecting coffee quality with direct economic impacts.[27-29] But few studies have been published concerning chemical profiles for plants subjected to water-deficit stress, the profile and levels of bioactive amines in sorghum,[30] phenolic compounds in cherry tomatoes[31] and phenolic compositions in grapes[32] being recent examples.

The extraction of groups of metabolites, which represent the whole of the vegetal matrix, has required evermore complex chemical analysis, requiring the association of many sample preparation techniques with different analytical methods. Considering extraction to be the probable limiting step of this analysis, our group has been focusing on metabolite extractions from different plant matrices using statistical mixture designs.[33-39]

The objective of the research reported here is to develop an analytical fingerprinting procedure to study metabolite abundance changes in *Coffea arabica* L. samples suffering water-deficit stress. An integrated chemometrics approach involving statistical mixture design and factor analysis applied to proton nuclear magnetic resonance (^{1}H NMR) data combined with confirmatory mass spectrometric evidence clarifies the metabolic adaptation process owing to this environmental stress. The mixture design is used to determine the solvent mixtures most appropriate for this procedure.

Experimental

Plants

The coffee field plant experiment was established at the Instituto Agronômico do Paraná (IAPAR), Londrina (−23°18'37" S, 51°09'46" W, 585 m altitude), Paraná, Brazil. *Coffea arabica* L. genotype IAPAR 59 seedlings were planted in the field at the beginning of 2010, in a 2.5 × 0.5 m arrangement. Plants were grown under two water regimes, drip irrigation and water field conditions (unirrigated ones). The irrigation system had the intensity of 3.5 L h^{-1} in each dripper and was triggered when necessary. Drippers were distributed linearly every half meter of the trunk of the coffee trees. Leaves from four plants of each treatment were collected in October 2012, when plants were about 120 cm high. Leaves were collected in the middle layer (40-80 cm), when that layer had the higher leaf area compared to the lower and superior ones, and the light interception was slightly lower than in the superior.[40] Leaf drying occurred with air circulation at room temperature. The leaves were distributed in trays and turned over every 24 h to homogenize the drying. The drying process was carried out for 15 days. After complete drying, the leaves were ground in an IKA A11 mill, sieved and packed in a vacuum and stored in an ultra-freezer at −65 °C.

Extraction

The extracts were prepared using ethanol (e), dichloromethane (d) and hexane (h) solvents in different proportions according to a statistical mixture design with three components as given in Table 1. The organic solvents used were of analytical grade being ethanol (Impex, São Paulo, Brazil), dichloromethane (Alphatec, São Paulo, Brazil) and hexane (Anidrol, São Paulo, Brazil). Extractions were randomly done. The solvents were selected based on Snyder's solvent selectivity triangle.[41] Table 2 presents the acidity (α), basicity (β), dipolarity (π) and polarity (P), which are relative measures of the strength and type of molecular interaction of the solute with the solvents for the 7 points of the statistical mixture design. The crude extracts were prepared from 2.0 g of the dried leaves with 60 mL of extractive solvent. The extraction process was carried

Table 1. Statistical mixture design for ethanol (e), dichloromethane (d) and hexane (h) solvents

Extract notation	Solvent		
	Ethanol	Dichloromethane	Hexane
e	1	0	0
d	0	1	0
h	0	0	1
ed	0.50	0.50	0
eh	0.50	0	0.50
dh	0	0.50	0.50
edh	0.333	0.333	0.333

out by shaking in an ACB Labor shaker-incubator with an agitation speed of 150 rpm and temperature of 15 °C. After 24 h, the extract was filtered and separated from the leaves. This procedure was repeated three more times. The total solvent volume (240 mL) for each point of the blending design was evaporated in a rotary evaporator followed by forced air circulation. The dried extracts were lyophilized.

Table 2. Chemical properties of the pure solvents and their mixtures (linear combination) according to the statistical mixture design: acidity (α); dipolarity (π); basicity (β) and polarity (P)

Statistical mixture design	Chemical property			
	α	π	β	P
e	0.39	0.25	0.36	4.30
d	0.27	0.73	0	3.10
h	0.10	0.06	0.03	0.10
ed	0.33	0.49	0.18	3.70
eh	0.25	0.16	0.20	2.20
dh	0.19	0.40	0.02	1.60
edh	0.25	0.35	0.13	2.50

^{1}H NMR measurements

For NMR analysis, 0.05 g of extract was dissolved in 0.5 mL of dimethyl sulfoxide-d_6 (DMSO-d_6, Sigma-Aldrich, St. Louis, USA) with tetramethylsilane (TMS) as internal reference. The ^{1}H NMR experiments were carried out at 25 °C on a Bruker Model Avance III spectrometer operating at 400 MHz equipped with a 5 mm broadband inverse (BBI) multinuclear probe. Spectra were acquired with 32 scans, an acquisition time of 3 min and 4 s with 1 s of waiting time. Signal suppression was accomplished removing chemical shifts owing to residual water using the water suppression sequence, zgpr (Bruker standard sequence). The 90° pulse calibration were conducted based on each sample before data acquisition. The data were processed using the v.3.5.7 TopSpin software.[42]

Mass spectrometric measurements (ESI-MS)

An aliquot of 3.0 mg of crude extract from the ternary mixture extract (edh) of each the irrigated and unirrigated plant leaves were dissolved in 1.0 mL of liquid chromatography-mass spectrometry (LC-MS) Fluka analytical methanol (St. Louis, USA). This solution was diluted with 0.01% formic acid from Vetec (Rio de Janeiro, Brazil) to obtain a 1×10^{-6} mg mL^{-1} extract solution. This was injected directly into the source of the Bruker mass spectrometer equipped with electrospray ionization (ESI) and a quadrupole analyzer. The analysis was carried out in both the positive and negative modes to determine the different fragments of compounds in the extracts. Data were acquired in the scan mode covering the 80 to 1000 Da *m/z* range. The ionization source conditions were: 3 kV capillary voltage, 150 °C source temperature, 80 L h^{-1} conical gas flow, 800 L h^{-1} desolvation gas flow and 350 °C desolvation temperature. Nitrogen nebulizer gas was 99% pure. The data were processed using the v.4.1 MassLynx software.[43]

Statistical analysis of the ^{1}H NMR data

Analysis of the NMR spectrometric data was made using the Statistica 7.0 software.[44] Origin Pro 8[45] was used to graph the loadings. The ^{1}H NMR spectra were referenced to the TMS signal (0.0 ppm). The working interval of spectrometric data was 0.00-10.0 ppm. The chemical shift region owing to deuterated solvent (2.39-2.59 ppm) was removed from the data matrix for the factor analysis. Normalization to unit area preprocessing was carried out, but there were no significant changes in the factor scores and loadings, so the original data with no scaling were chosen for analysis.

Results and Discussion

The sum of all the mixture design extracts can be considered to be a digitized global metabolomic fingerprint that carries a maximum amount of possible information for the three solvents. Figures 1a and 1b show the average ^{1}H NMR spectra obtained from the different solvent mixtures in Table 1 for the irrigated and unirrigated leaves, respectively. Figure 1c shows the subtraction result of these two averaged spectra, exhibiting a set of metabolite signals that present differences in relative intensities owing to water-deficit stress. As can be seen in Figure 1c, the larger variations occur in the 4.0-0.5 ppm range. The peak at 1.20 ppm is characteristic of pigments,[46-49] that correspond to the most characteristic diverse class of metabolites in the extracts of the irrigated plants. Generally, under increasing water availability, the leaf chlorophyll content increases,[50] allowing higher photosynthetic rates,[51] which supports the differentiated peaks for pigments in our study.

The peak at 1.24 ppm is assigned to lipids,[52-54] the most characteristic diverse class of metabolites found for extracts of the unirrigated plants. Proteins and lipids are the principal components of thylakoid membranes, which are dynamic systems in which the lateral mobility of their principal constituents plays a key role in physiological processes, including electron transport, regulation of light-harvesting, membrane biogenesis and turnover and repair of proteins.[55] Plants need to compensate changes in thylakoid

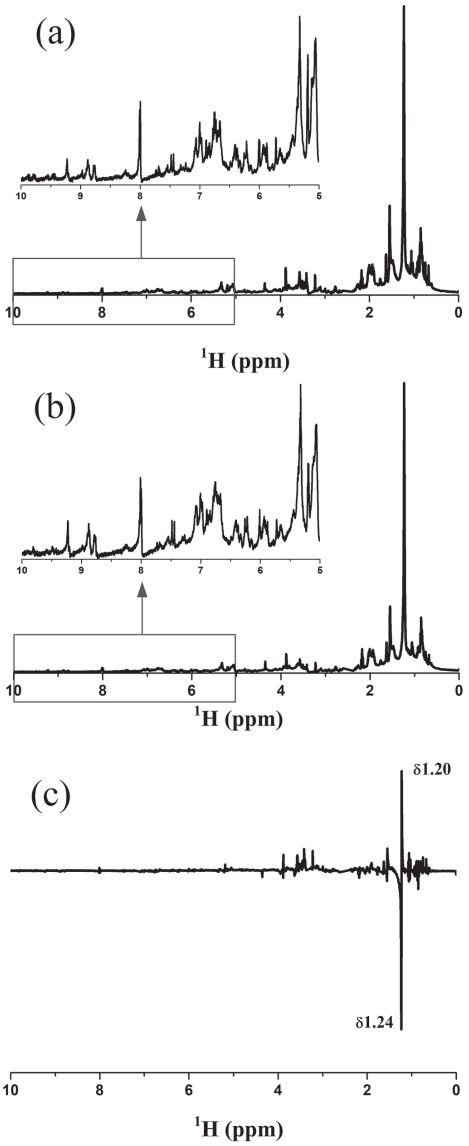

Figure 1. Spectrometric ^{1}H NMR averages for the three-component solvent mixture design: (a) averaged spectrum of irrigated plant extracts; (b) averaged spectrum of unirrigated plant extracts; (c) subtraction result of averaged NMR spectra for irrigated and unirrigated plants ((a) – (b)).

membrane fluidity to prevent the negative impacts of environment.[56] Lipids are interchanged among membrane-bound organelles.[57] Common strategies to maintain adequate membrane fluidity involve the incorporation of polyunsaturated fatty acids, the conformation of double bonds, the length of fatty acyl chains and the presence of sterols.[58] Our results indicate that the characteristic lipid presence could be related to membrane maintenance under the drought stress conditions.

Factor analysis was applied to the spectrometric data to obtain more information about metabolite differences in coffee leaves from irrigated and unirrigated plants. The NMR spectral data from all the mixture design extracts were digitized into a matrix with 14 rows and 8178 columns, where the rows are the samples (seven for the irrigated and seven for the unirrigated leaf extracts) and columns are the spectral intensities The first three factors explain 96.07% of the total data variance.

Factor analysis results in a two-dimensional projection of this multivariate data conserving 92.57% of the spectral information. It is then possible to identify differences between the two sets of leaf extracts in an objective way. Figure 2 contains a graph of scores for the first two factors. The distribution of the extract points along both factors correspond to the polarities of the mixture design extraction blends. Hexane and its mixtures of lowest polarity occupy the upper left side of the graph with gradual changes owing to polarity increases until the ethanol mixtures in the lower right corner. Dipole moments of polar solvents interacting with relatively strong intermolecular forces and hydrogen bonding preferentially extract polar and hydrophilic compounds. The nonpolar solvents tend to extract low polarity and hydrophobic compounds.[40]

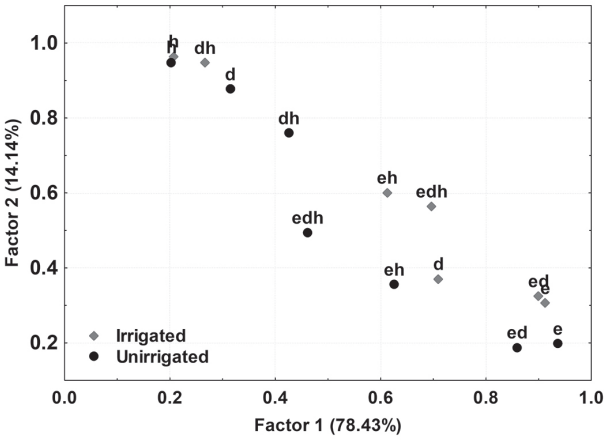

Figure 2. Two-dimensional score graph of the ^{1}H NMR spectral data of the irrigated and unirrigated leaf extracts from the mixture design solvents.

Figure 3a shows a graph of the loadings for the first factor. As can be seen a very high positive loading occurs for the 1.21 ppm indicating a greater abundance of pigments in samples with high factor 1 scores, extraction mixtures containing ethanol relative to those without ethanol. Furthermore the 1.24 ppm peak is somewhat negative indicating lower lipid abundances in the ethanolic mixtures. On the other hand, the second factor (Figure 3b) loadings have a positive peak for the 1.24 ppm lipid signal and slightly negative one for the 1.21 ppm pigment peak. As the non-ethanolic extracts contain higher score values on this factor they have higher lipid abundances compared to the ethanolic extracts. So, the relative abundances of pigments and lipids in these extracts are negatively correlated.

Figure 4 contains the ^{1}H NMR spectra for irrigated leaf extracted with pure hexane and pure ethanol. Four separate

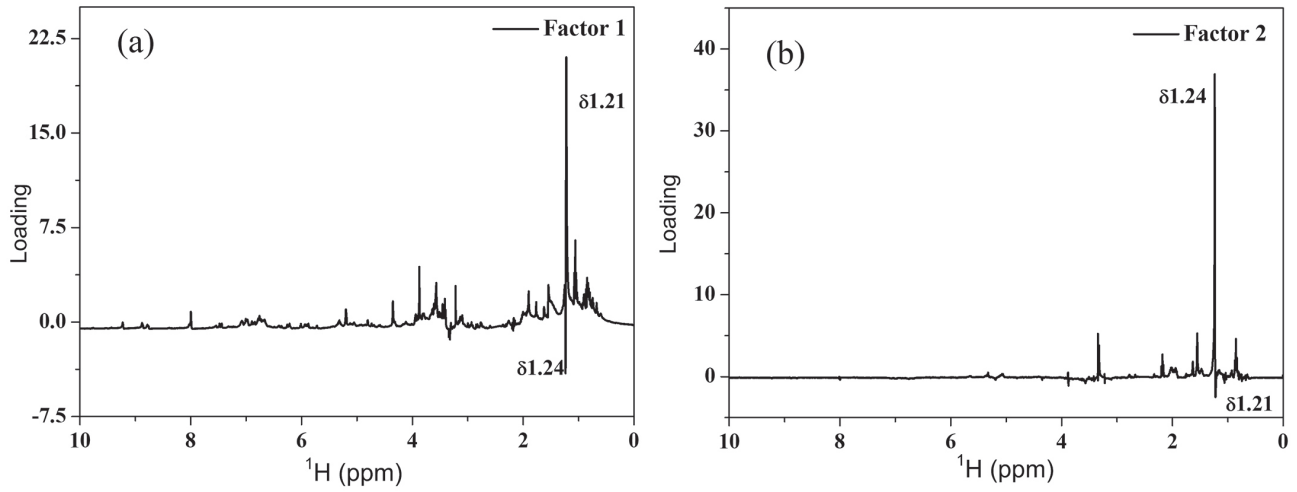

Figure 3. Loading graph for leaf extracts of *Coffea arabica* genotype IAPAR 59 cultivated with and without irrigation for (a) factor 1 and (b) factor 2.

regions are shown to facilitate visualization and discussion (10-6.5, 6.5-4.0, 4.0-2.0 and 2.0-0.1 ppm).

In Figure 4 one sees that the hexane extract does not provide useful chemical information between 10.0-6.5 ppm as might be expected since it does not tend to extract nonpolar metabolites. However, the spectra obtained from the pure ethanol extract had many peaks in this range and also at higher field, such as those of trigonelline, at 8.75,

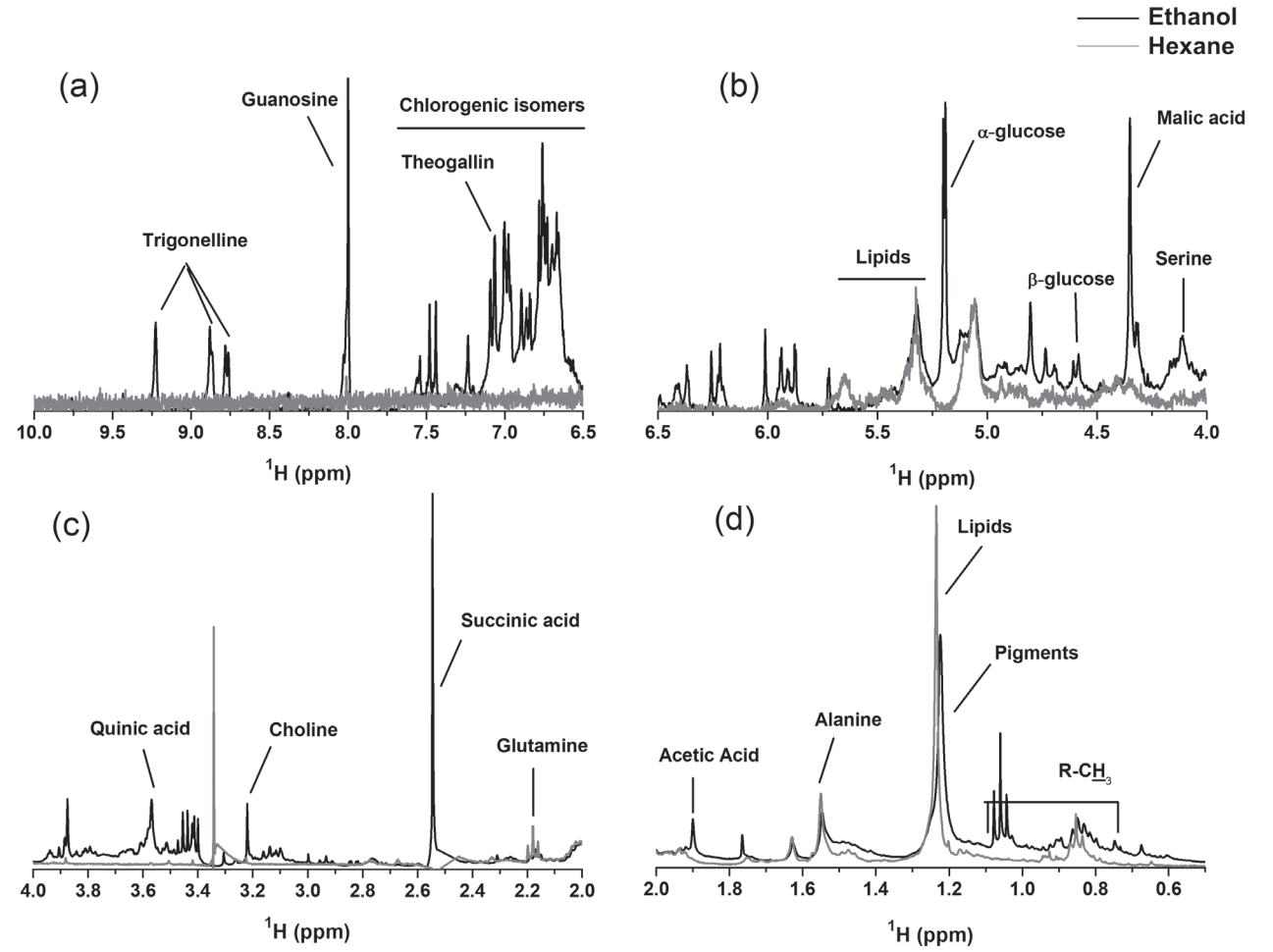

Figure 4. ^{1}H NMR spectra of pure ethanol and pure hexane extracts of *Coffea arabica* leaves cultivated with irrigation (a) 10-6.5; (b) 6.5-4.0; (c) 4.0-2.0; (d) 2.0-0.1 ppm.

8.87 and 9.22 ppm,[59,60] guanosine at 8.00 ppm,[54,61] the region for the chlorogenic acids at 7.8-6.1 ppm,[62] theogallin at 7.08 ppm,[63] α-glucose at 5.20 ppm,[60,64] β-glucose at 4.61 ppm,[60,64] malic acid at 4.33 ppm,[64] serine at 4.08 ppm,[65] quinic acid at 3.56 ppm,[63] choline at 3.20 ppm,[59,60] succinic acid at 2.54 ppm,[52,64] glutamine at 2.16 ppm,[60,64] acetic acid at 1.90 ppm,[52,59] alanine at 1.52 ppm,[59,60,64] pigment methyl groups at 1.21 ppm[46-49] and valine, leucine and isoleucine at 1.08-0.70 ppm.[54,60,61]

In Figure 4, on the other hand, the hexane extract exhibits strong peaks at 5.65 and 5.3 ppm characteristic of the –CH=CH–R structure in fatty acid chains.[66] One intense peak for the hexane extract at 1.24 ppm, characteristic of the lipid chemical structure $-(CH_2)_n$ is also found.[52-54] Other peaks at 3.2 and 5.1 ppm are characteristic of the hydroxyl proton in fatty acid.[66]

Figure 5 shows a three-dimensional projection of scores involving the first three factors. The third factor, despite its small explained variance (3.50%), contains information concerning the important metabolites that discriminate irrigated and unirrigated plants. The extracts from irrigated points are found at lower third factor scores than the ones from unirrigated points.

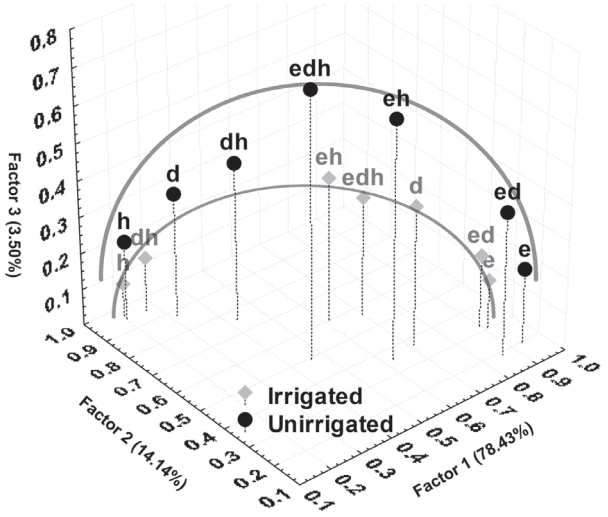

Figure 5. Three-dimensional graph of the scores of the first three factors from the ^{1}H NMR spectra of mixture design extracts of irrigated and unirrigated plants.

The factor loadings for the ^{1}H NMR spectra of the irrigated and unirrigated extracts can be divided into three distinct regions: the aromatic region (10.0-5.5 ppm), sugar region (5.5-3.0 ppm) and amino acid/aliphatic compound one (3.0-0.0 ppm).[52,53,64] Figure 6 contains graphs of the third factor loadings, with peaks in the sugar and amino acid/aliphatic compound regions associated with the discrimination between the irrigated and unirrigated plants. The score values indicate that negative peaks correspond to the irrigated extracts whereas positive ones indicate metabolite peaks predominant in the unirrigated extracts. The peaks at 1.34 ppm characteristic of lactate,[67] at 1.65 ppm of arginine[60] and at 3.88 ppm of methionine[61] are more intense in the leaf extracts of the unirrigated plants, while those at 1.30, 3.20, 3.34 and 3.86 ppm assigned for threonine,[65] choline,[52] proline[60] and serine,[65] respectively, are higher in irrigated leaf extracts, indicating the higher

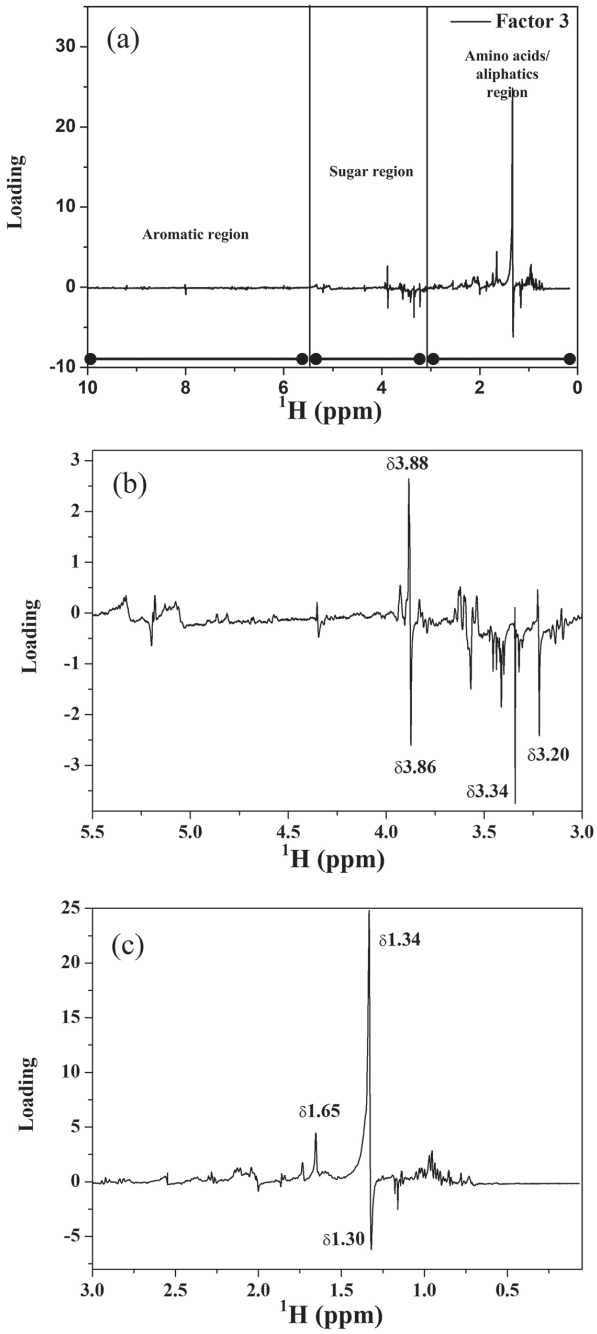

Figure 6. Loading graphs of the third factor loadings for irrigated and unirrigated leaf extracts of *Coffea arabica* L. genotype IAPAR 59: (a) 10.0-0.0 ppm; (b) sugar region, 5.5-3.0 ppm; (c) amino acid/aliphatic compound region, 3.0-0.0 ppm.

abundances of these metabolites in the two typologies of samples.

In Figure 5 one can see that the best solvent discriminator of the mixture design is the ternary mixture as the third factor distance between irrigated and unirrigated leaf extracts is greater. The extract separation shown by the factor analysis is confirmed by the hierarchical analysis dendrogram presented in Figure 7 for the triplicate edh mixture. The dendrogram shows that irrigated and unirrigated extracts form two groups. Table 3 shows the *m/z* ratios for the mass spectrometric results in the positive and negative modes for the ethanol/dichloromethane/hexane ternary extracts of both the irrigated and unirrigated leaf extracts. The presence of characteristic masses provides confirmatory evidence of the metabolites indicated by the NMR chemical shifts. For the chlorogenic acids, ($[M - H]^-$, *m/z*) values of 367, 353 and 337 are characteristic of the chlorogenic isomeric derivatives of feruloylquinic (FQA), caffeoylquinic (CQA) and coumaroylquinic acids (pCoQA).

The higher presence of lactate in the unirrigated leaf extracts could be related to the gas exchange conditions of these plants by physiological routes in the plant. Insufficient water conditions for the plant maintenance can provoke stomatal closure.[68] The stomates are responsible for gas exchange and stomatal transpiration.[69] On closure the photosynthesis rate is reduced so the plant does not lose water by transpiration.[70,71] At low O_2 levels respiration does not occur by aerobic processes and anaerobic behavior begins to occur in pyruvate metabolic ways.[72] Lactate is one of the products of fermentation processes.[73] Its accumulation indicates hypoxic stress in plants[74] as the higher concentrations alter the pH of cytosol (cytosolic acidosis) and can lead to cellular death.[75]

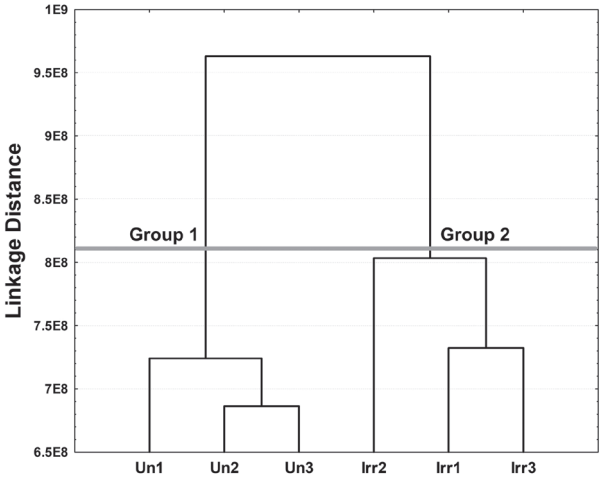

Figure 7. Hierarchical dendrogram of the ^{1}H NMR spectra of the irrigated and unirrigated plant extracts of the 1:1:1 ethanol-dichloromethane-hexane mixture.

The increase in relative abundance of arginine in plants is indicative of a phosphorus deficiency in the metabolism,[76] being still a precursor of metabolites that are responses to biotic and abiotic stresses such as the polyamines.[77] Another

Table 3. Characteristic ESI-MS *m/z* ratios for protonated molecules and their corresponding characteristic NMR chemical shifts for ternary mixture extracts (ethanol/dichloromethane/hexane) of irrigated and unirrigated *Coffea arabica* leaves for the metabolites

Metabolite	*m/z*	Protonated molecule	δ / ppm
Lactate	89	$[M - H]^-$	1.34, 4.22
Alanine	90	$[M + H]^+$	1.52, 3.81
Choline	105	$[M + H]^+$	3.20, 4.10
Serine	106	$[M + H]^+$	3.88, 4.08
Proline	116	$[M + H]^+$	2.01, 2.32, 3.34, 8.78
Succinic acid	117	$[M - H]^-$	2.56
Threonine	118	$[M - H]^-$	1.30, 4.02
Valine	118	$[M + H]^+$	1.08-0.70, 2.27, 3.63
Glutamine	128	$[M - H]^-$	2.16
Leucine/isoleucine	132	$[M + H]^+$	1.08-0.70
Malic acid	133	$[M - H]^-$	2.66, 2.75, 4.33
Trigonelline	138	$[M + H]^+$	8.75, 8.87, 9.22
Methionine	148	$[M - H]^-$	2.15, 2.18, 2.66, 3.88
Arginine	175	$[M + H]^+$	1.65, 1.90, 3.23, 3.74, 7.23
α-Glucose/β-glucose	181	$[M + H]^+$	5.20/4.61
Quinic acid	193	$[M + H]^+$	1.90, 1.92, 2.03, 2.04, 3.56, 4.05
Guanosine	284	$[M + H]^+$	8.00
Theogallin	345	$[M + H]^+$	2.02, 2.15, 2.19, 3.85, 4.22, 5.44, 7.08

possible metabolite present in the extracts of unirrigated *Coffea arabica* leaves is methionine that is a precursor of ethylene in plants.[78] Methionine has higher abundances in the unirrigated plants. The metabolites of the synthetic route of ethylene are related to stress factors.[79] In the leaves, ethylene exercises functions in senescence and abscission processes.[80]

The spectral evidence and loading values indicate that amino acids have high abundance values in irrigated plants. A large part of physiological processes are regulated by amino acids.[71,81] Proline has been investigated to verify that its accumulation is related to water availability in leaves of rice plants, higher levels of water leading to higher proline levels.[82] Choline has numerous functional properties inherent to vegetation,[83] having its bioavailability involved also in water stress situations[84] and saline stress situations.[85] Irrigated areas are sensitive to salinization processes in soils, but series of physical and chemical processes must be aligned for these phenomena to occur, such as water composition and culture care.[86] Antagonistic salinization effects can occur with the growth and development of plants annihilating the synergic effect of water disponibility.[87,88]

Conclusions

The metabolite changes found provide an indication of the changes in regulatory metabolites in *Coffea arabica* L. leaves suffering water-deficit stress. Definitive studies using a larger number of diverse representative samples is needed to confirm the results above for water-deficit stress. A key point in the proposed analytical procedure is the use of the ethanol-hexane-dichloromethane ternary mixture. Ternary mixtures are rarely used in extraction procedures and its necessary application here only became evident through the integrated statistical mixture design and multivariate analysis methodology. Indeed, mixture design results are not only very useful for discriminating classes but also for assigning NMR signals to metabolite classes.

Acknowledgments

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