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ORIGINAL ARTICLE

Environmental stress evaluation of *Coffea arabica* L. leaves from spectrophotometric fingerprints by PCA and OSC–PLS–DA

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Abstract The effects of hydric stress and sunlight access conditions on metabolic compounds in coffee leaves were investigated utilizing statistical mixture design extractor solvents. PCA and OSC–PLS–DA chemometric methods were used to analyze UV–visible spectra of irrigated and non-irrigated *Coffea arabica L.* leaves from low (< 40 cm, self-shaded) and higher (> 80 cm, light exposed) strata. The first latent variable of the OSC–PLS–DA score plot perfectly discriminated extracts of 34 calibration and 14 validation samples of irrigated and non-irrigated leaves. Higher spectral signals observed at the 410, 505, 535, 607 and 665 nm wavelengths are attributed to conjugate double bond pigments, mainly pheophytin a, indicating that non-irrigated conditions are more stressful than irrigated ones for this species. No significant difference was found for leaf sample extracts with varying light access conditions.

1. Introduction

Coffee is one of the most popular beverages in the world (Wang and Lim, 2012) and an important raw material in international trade (Barbin et al., 2014). The coffee plant is a woody perennial evergreen dicotyledon that belongs to the Rubiaceae family (Davis et al., 2006 and Lima et al., 2013). Among the several identified species of the genus *Coffea*, *Coffea arabica* L. has significant importance for high quality and of the principal cultivars, the 'Catuaí Vermelho IAC 99' has high adaptability to different regions and growth conditions (Fazuoli et al., 2007).

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Environmental conditions to which plants are exposed can impact metabolite synthesis through induction of physiological responses and adaptations related to biochemical changes observed in their metabolisms. In coffee plants, these changes may be important for coffee bean quality (Delaroza et al., 2014 and Simões et al., 2004). For plants, any external factor that exerts disadvantageous influence and induces changes and responses may be considered stressful (Gobbo-Neto and Lopes, 2007).

The major environmental stress affecting coffee production in most producing countries is hydric stress. This stress is defined as any water content of a tissue/cell that is below the highest content displayed in its higher hydration state. This affects all the plant's vital processes, causing cellular damage and secondary effects such as oxidative stress (DaMatta and Ramalho, 2006). Unlike the considerable quantity of research on the relationship between hydric stress and coffee (Carr, 2001; Custódio et al., 2012; Maestri and Barros, 1977 and Villa Nova et al., 2002), few studies have reported changes in metabolic contents of other coffee plant parts such as leaves (Campa et al., 2012; Chaves et al., 2004 and Mondolot et al., 2006).

By being naturally present in leaves, the photosynthetic pigments, chlorophylls, carotenoids and their derivatives, can be analyzed and used as stress indicators. Plant stress is caused by a variety of factors, generally decreasing the chlorophyll concentrations and apparently increasing those of carotenoids and other pigments (Carter and Knapp, 2001 and Schoefs, 2002). Their molecules are extracted and used as natural colorants and antioxidants (Schoefs, 2002). Emphasizing that the interest in natural antioxidants found in plants has increased due to the worldwide increase in using plant extracts for scavenging free radicals to ensure high quality and/or stability of lipids and lipid-containing products (Loranty et al., 2010).

Some of the most modern approaches to simultaneously analyze several metabolites are fingerprint techniques (Dunn and Ellis, 2005; Esslinger et al., 2014; Grauwet et al., 2014; Longobardi et al., 2013; Oliveira and Catharino, 2015; Ren et al., 2011 and Rossi et al., 2011). They are fast with high information content and analyze samples as a whole permitting separation of the dynamics of any biotic, abiotic or genetic plant perturbation for accurate assessment. The advantage of using these profiles is that metabolic variations are mainly observed by spectroscopic and chromatographic pattern changes without previous knowledge of the identities of the investigated compounds (Dunn and Ellis, 2005). Ultraviolet–visible spectroscopy (UV–Vis), as a screening tool, is capable of discriminating patterns of various origins due to the large proportion of metabolites which absorb ultraviolet light, including substances that contain one or more double bonds as well as substances with unpaired electrons (Giovannetti, 2012 and Valladão et al., 2009).

Recently, our group has shown that statistical mixture designs permit the development of rigorous and economical procedures for the development of fingerprint profiles of extracted metabolites of plant material (Delaroza and Scarminio, 2008; Garcia et al., 2009, 2010; Moreira and Scarminio, 2013; Moreira et al., 2014 and Souza et al., 2009). Allied with chemometric approaches containing mathematical and statistical methods for treating instrumental data they increase the understanding of chemical information and related quality parameters, permitting product quality evaluation leading to more efficient laboratory practices or automated quality control systems (Barros Neto et al., 2006 and Brereton, 2013).

According to the suggestions of Campa et al. (2012) coffee leaves have the potential for health benefit, emphasizing the necessity of more research in this field. When grown under environmental stress conditions, coffee leaves are susceptible to metabolic changes especially in chlorophyll derivatives and carotenoids (Carter and Knapp, 2001). The aim of this study was to investigate variations occurring for extracted metabolites as well as photosynthetic pigments in coffee leaves under plant hydric stress and different light conditions by evaluating spectrophotometric fingerprints obtained with simplex–centroid mixture design and Principal Component Analysis (PCA). Also, the feasibility of using UV–Vis spectral fingerprinting of these extracts for Orthogonal Signal Correction and Partial Least Squares-Discrimination Analysis (OSC–PLS–DA) was investigated to discriminate the coffee leaves of plants cultivated under irrigated, non-irrigated, self-shaded and sun-exposed conditions.

2. Materials and methods

2.1. Plant materials

Coffea arabica leaves, from Catuaí IAC 99 genotype, were collected from irrigated and non-irrigated plants cultivated for 3 years at the Paraná Agronomic Institute (IAPAR, Londrina) in October 2012. In order to check plant architectural influence and light irradiation effects, the harvest was stratified into an inferior layer (40 cm, self-shaded) and a superior one (> 80 cm, high light exposed).

Drying was carried out with circulating air at ambient temperature. The leaves were distributed into trays and every 24 h turned over to allow complete drying. This drying process lasted 15–20 days depending on the number of leaves in each tray. When completely dry, the leaves were crushed in a domestic blender, passed through a plastic sieve, packed in plastic bags, subjected to vacuum and stored in a freezer/cooler.

2.2. Extract preparation

The crude extracts were prepared using mixtures of (e) ethanol, (d) dichloromethane and (h) hexane whose proportions were varied according to the simplex-centroid mixture design, presented in Table 1. For plant extraction, all organic solvents were of analytical grade with ethanol obtained from Impex (São Paulo, Brazil), dichloromethane from Alphatec (São Paulo, Brazil) and hexane from Anidrol (São Paulo, Brazil). Extraction consisted of three pure solvents, three (1:1) binary mixtures and one (1:1:1) ternary and three axial ternary mixtures, (4:1:1), (1:4:1) and (1:1:4) as in Table 1. These mixtures were prepared in random order including five replicates at the (1:1:1) center point mixture. Solvent selection was based on diversity considering Snyder's solvent selectivity triangle (Snyder et al., 1993).

Table 1 Mixture design solvent proportions used for extraction given in mL.

Extract notation	Solvents		
	Ethanol	Dichloromethane	Hexane
e	60	0	0
d	0	60	0
h	0	0	60
ed	30	30	0
eh	30	0	30
dh	30	0	00
Edh	40	10	10
eDh	10	40	10
edH	10	10	40
edh 1	20	20	20
edh 2	20	20	20
edh 3	20	20	20
edh 4	20	20	20
edh 5	20	20	20

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Each crude extract was prepared adding 60 mL of extractor solvent to 2.0 g of dried and crushed *C. arabica* L. leaves. After 24 h the extract was filtered to separate the solution from the coffee leaves. This procedure was repeated three more times, for a total of four repetitions. Thus the total volume of solvent mixture added to the leaves was 240 mL for each point of centroid-simplex design. These solvents were evaporated in a rotary evaporator and kept under forced ventilation after which the extracts were lyophilized.

2.3. Spectrophotometric fingerprint measurements

UV–Vis spectral fingerprinting was carried out with 3.0 mg of each crude extract solubilized in 1 ml of respective extractor solvent (Table 1). An aliquot of 100 μL of this solution was diluted in 1900 μL of solvent extractor. The absorptions were performed in a 1 cm quartz cuvette, with a Thermo Scientific® Evolution 60S model UV–Vis spectrophotometer, coupled to Vision Lite software and monitored in the 200–800 nm range with a resolution of 1 nm.

2.4. Data treatment

Pre-processing treatments, PCA and OSC–PLS–DA model calculations, cross validation and predictions were performed using Matlab R2007a (Mathworks Inc. Natick, MA, USA) functions included in PLS Toolbox 5.8.1 (Eigenvector Research Inc., Wenatchee, WA, USA).

Every spectral fingerprint of each leaf crude extract (see Table 1) was monitored in the 200–800 nm region. However the low and high spectral wavelengths contained no information and the 255–720 nm working range was selected. Preliminary spectra exploration applied PCA to a 56×466 data matrix assembled with each row corresponding to one of the crude extracts (28 for each irrigation mode) and each column representing the spectra data at a given wavelength (Jolliffe, 1986). PCA reduces the dimensionality of a large number of interrelated variables, while retaining, as much as possible, the variations in the data set (Jolliffe, 1986). This method is commonly employed for information data analysis due to its compression capacity as a correlation function among many variables (Sabin et al., 2004). This results in a few components retaining most of the variation of the original data (Dominguez-Vidal et al., 2016). Then, the PCA scores are examined for similarities and differences among samples while variable importance information is contained in the PC loadings (Almeida et al., 2013). Data were mean-centered prior to applying PCA.

PLS–DA is a supervised method based on searching an optimal set of latent variable data for classification purposes (Barker and Rayens, 2003). It is developed from algorithms for Partial Least Squares (PLS) regression employing a set of predictor variables **X** and defined categories, **y**. It has the advantage of being suitable when the number of objects is fewer than the number of variables (Pizarro et al., 2013). The orthogonal signal correction (OSC) (Wold et al., 1998) algorithm was utilized to eliminate unnecessary information from the model. The main benefit of using the OSC-filter in PLS–DA, i.e. OSC–PLS–DA, compared with only PLS–DA is its ability to separate predictive from non-predictive (orthogonal) variations (Bylesjö et al., 2006).

OSC–PLS–DA was applied to the data matrix with dimensions of a 48 (crudes extract) × 466 (wavelengths) data matrix assembled so that each row corresponded to PCA relevant crude extracts. The crude extracts were randomly divided into calibration and validation subsets with the **y** variables associated with the two different classes (irrigated and non-irrigated leaf crude extracts). Data were also mean-centered before PLS analysis. Subsequently, Variables Importance in Projection (VIPs) scores (Bylesjö et al., 2006 and Wold et al., 2001) were calculated for selection of the more relevant variables which really have an effect on the sample separation. The importance of each variable is reflected by its VIP score, which is a weighted sum of squares of the PLS-weights, calculated from the amount of **y**-variance of each PLS component (Wold et al., 2001).

3. Results and discussion

Being one variable reduction procedure, PCA is the first chemometric stage in data processing and is usually applied for exploratory analysis. A reduced number of new variables called principal components account for most of variance in the observed variables (Koley et al., 2011). The PCA model utilized to explore the solvent effects on the extraction procedure was evaluated using the mixture design given in Table 1. Spectral fingerprint data were initially subjected to PCA after mean centering. The score plot of the two first principal components, which accounts for 97.85% of total variance, is shown in Fig. 1a. A small group in the positive PC_2 and negative PC_1 quadrant contains the pure ethanol extracts. Highly polar and hydrophilic compounds are more easily extracted by ethanol and they cause this group formation (Matos, 2009). Positive PC_2 loadings in Fig. 1b are characterized by the band at 272 nm which corresponds to caffeine or its derivatives. In order to confirm its presence, caffeine was extracted from *C. arabica* leaves by an established methodology (Zenebon et al., 2008). The comparison of its UV spectrum with an analytical caffeine standard and a crude extract spectrum (Table 1) in the 255–320 nm range proves its metabolic presence, as shown in Fig. 2.

A second group of extracts obtained with hexane is located in the negative PC_1 and PC_2 quadrant and is characterized by bands at 410 and 665 nm, that according to its PC_2 loadings (Fig. 1b), are very similar to carotenoid bands. Non-polar solvents, such as hexane that easily extract low polarity compounds and some low hydrophilic polar compounds from plants, form this second group (Matos, 2009). A third group containing the remainder of the crude extracts was clustered owing to their intermediate solvent polarities (Palamareva and Palamarev, 1989) (between ethanol and hexane) and their spectral similarities. Since this group contains the most similar spectral fingerprints, it was decided to apply OSC–PLS–DA only to these data (named the **X** matrix).

A PLS–DA model was determined for the 255–720 nm spectral range (UV–Vis spectral fingerprints of crude extracts of *C. arabica* leaves) by applying the OSC method to remove information from the UV–Visible data which is not necessary for fitting the **y** variables (Niazi et al., 2015). The **y** variables were associated with two different classes (irrigated leaf crude extracts categorized in class 0 and non-irrigated ones in class 1). The spectral fingerprints of the **X** matrix were

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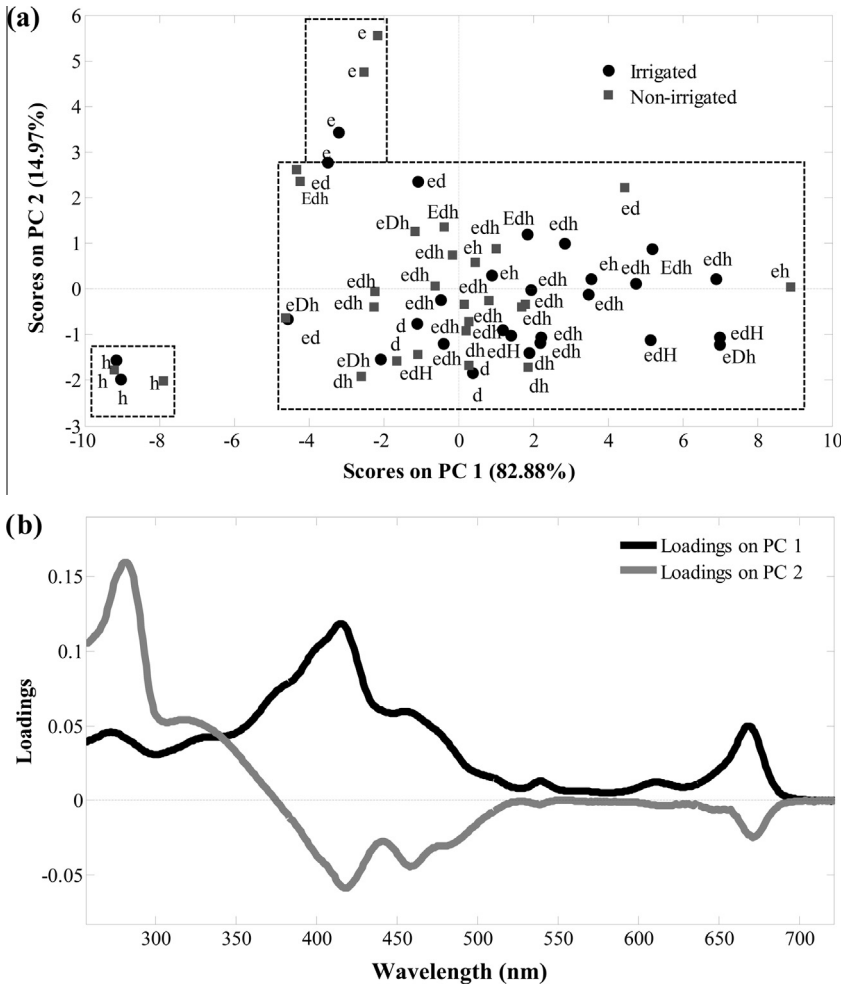

Figure 1 (a) PC_1 vs. PC_2 score plot of the fingerprint data (56 crude extracts × 466 wavelength) and (b) corresponding loading plot.

mean-centered and randomly divided into subsets: the training subset composed of 34 crude extracts to determine the calibration rule, and the test subset, containing 14 crude extracts to validate the model. The dimensionality of the model was determined by random subset cross-validation, based on the lowest RMSECV value.

Two latent variables were needed to develop a classification model showing high correlation with *R* of 0.91 between the real and predicted classes of the training subset extracts. In addition, the 86.33% of explained variance in **y** corresponds to 91.28% of variance in the **X** matrix, with RMSEVC equal to 0.37 and a mean error of 0.15 for the training set (RMSEC). The model obtained was able to discriminate between the two classes, as can be seen in the OSC–PLS–DA score plot, Fig. 3a. The first latent variable (LV_1 explains 88.74% of **y** variance and 24.82% of the variance of matrix **X**) discriminates the irrigated and non-irrigated crude extracts. Positive scores on LV_1 are related to non-irrigated samples and its negative values to irrigated ones. The discriminant analysis was based on the spectral fingerprint profile of the crude extracts obtained from the mixture design solvents in Table 1.

The loading plot of latent variables, presented in Fig. 3b, shows the UV–Vis bands which contribute to the differentiation between classes. By analyzing the loading plot of the latent variables, it is noted that the irrigated samples have higher concentrations of metabolites corresponding to the 410, 505, 535, 607 and 665 nm bands (negative weights) which are very similar to the absorbances of conjugated double bond pigment systems, mainly pheophytin a (Milenković et al., 2012). The VIP scores evaluate the importance of the variables and provide a useful tool in interpreting the importance of each variable. Fig. 3c shows the VIP scores obtained for the OSC–PLS–DA model with the mean centered data, where values greater than 1 indicate the important spectral bands for discrimination. In these spectral fingerprints of *C. arabica* leaf extracts the band at 410 nm can be selected for optimal discrimination.

Pheophytin a is the simplest derivative of chlorophyll a, with a Mg atom dechelated from its porphyrin ring, naturally present in plants as a breakdown product (Hsu et al., 2013 and Milenković et al., 2012). In the UV–Vis region it is characterized by a band at 410 nm (blue region), denominated as the Soret band, and a band at 665 nm (red region), called the Q band. Between the Soret and Q bands exist minor bands at 505, 535 and 606 nm. These bands are characteristics of the porphyrin ring and occur by electron promotion from a π bonding orbital to a π^* anti-bonding one (Milenković et al., 2012). Hence the OSC–PLS–DA discrimination between the

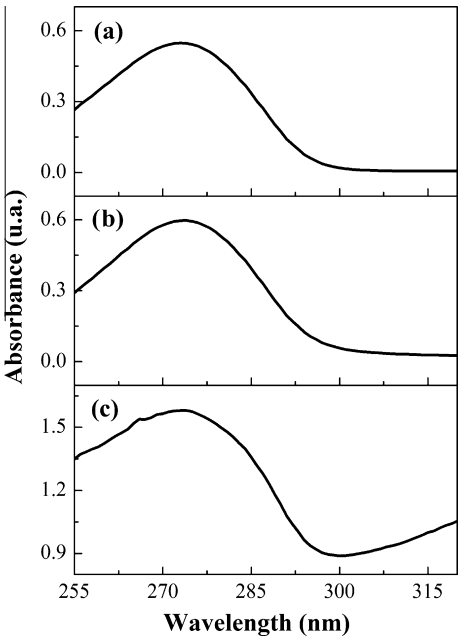

Figure 2 Comparison between caffeine spectra: (a) of the analytical standard; (b) isolated from *Coffea arabica* leaves; (c) in crude extract obtained from the ethanol and dichloromethane binary mixture.

two groups seems to be due to higher pheophytin concentrations in the irrigated crude extracts.

The results suggest that the discrimination can be attributed to variations of photosynthetic pigment contents of chlorophyll derivatives (Suzuki and Shioi, 2003). Under stressful conditions, such as hydric stress, changes and responses are induced at all organism levels. The observed variation can be explained by the important role of water in photosynthesis and cellular respiration. Irrigated leaves have more water availability to be oxidized by photons to produce species used in carbon dioxide reduction, requiring greater amounts of photosynthetic pigments to absorb light. Non-irrigated leaves decrease the degree of the stomata opening to maintain hydric equilibrium, increasing the entrance resistance of carbon dioxide and consequently limiting photosynthesis in chloroplasts and reducing photosynthetic pigment amounts (DaMatta and Ramalho, 2006).

The OSC–PLS–DA model was applied to classify the 14 crude extracts contained in the validation subset (7 from each class). The prediction performance can be observed in Fig. 4. The spectral fingerprints of irrigated samples are above the threshold value of 0.4339 whereas the non-irrigated points are below, showing a 100% correct classification according to the irrigation system. Owing to its simplicity, versatility, speed, cost-effectiveness, among others (Mohammadzadeh kakhki et al., 2013) these results confirm the usefulness of UV–Vis spectroscopy allied with chemometric methods in analytical chemistry. Furthermore, the use of spectral fingerprinting in the UV–Vis region associated with the application of OSC–PLS–DA models appears promising for classifying and predicting hydric stress of coffee leaves.

In order to verify whether the differences between irrigated and non-irrigated layers are significant, a paired *t*-test was

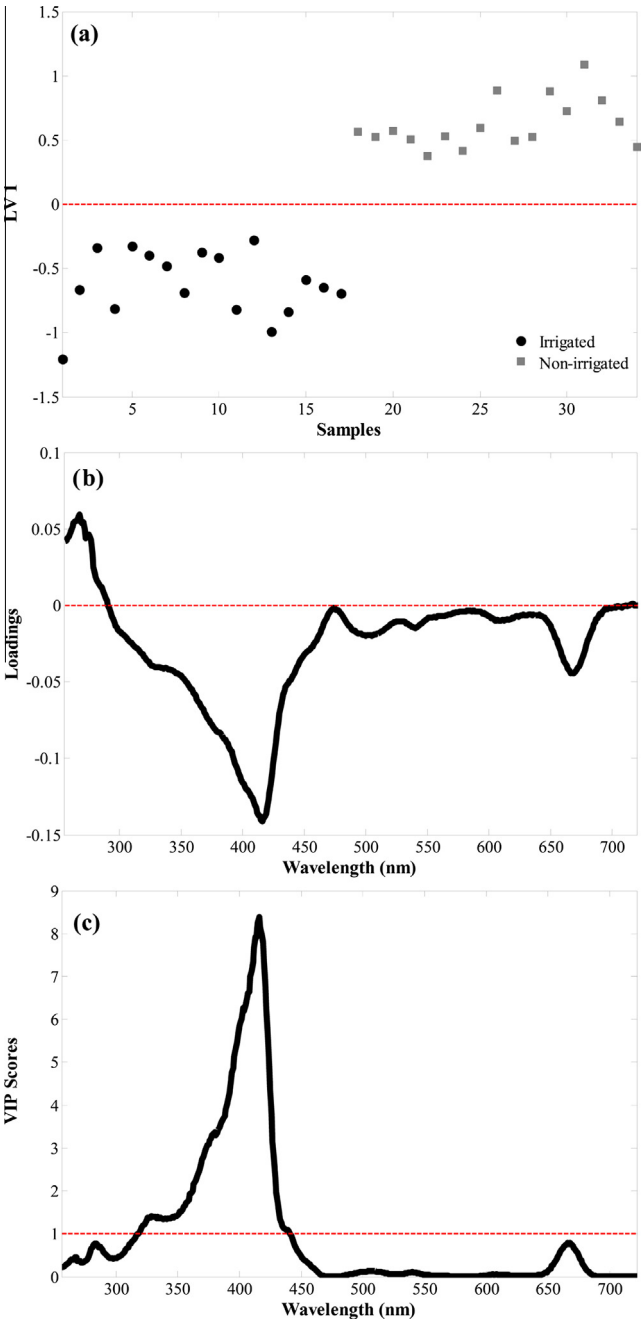

Figure 3 OSC–PLS–DA results of spectral fingerprint analysis of crude extracts of *Coffea arabica* leaves from irrigated and non-irrigated leaves analyzed in the 250–720 nm region for simplex centroid mixture design extracts: (a) LV_1 score and (b) loading plots (wavelength); (c) variable importance (VIP) score plot.

applied to the absorption intensity value at 410 nm for pheophytin a for all crude extracts from the **X** matrix. First, the data were tested for leaves stratified into the inferior layer (40 cm, self-shaded) and superior layer (> 80 cm, high light exposed) of irrigated sample extracts. The results did not show significant differences at the 95% confidence level between the light irradiation levels. This was based on calculated t value of 0.28 that is much lower than the 2.20 critical value. The same occurred for non-irrigated leaves, with a calculated *t* value of

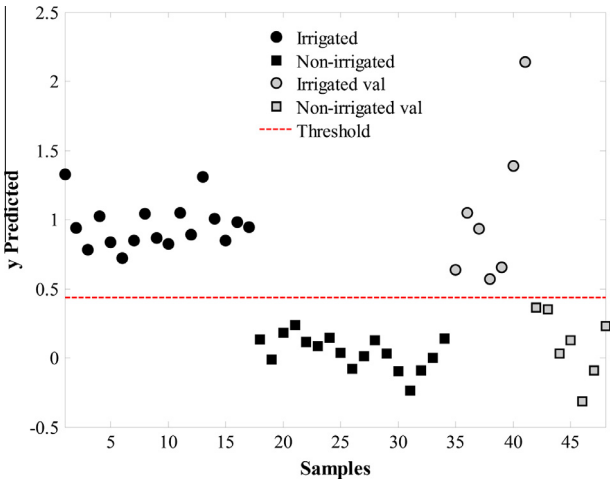

Figure 4 Results of the OSC–PLS–DA model of the UV–Visible spectral fingerprinting of irrigated and non-irrigated *Coffea arabica* leaves for the calibration and validation sets.

1.26. These results reinforce that the response changes observed for photosynthetic pigments are caused by hydric stress.

4. Conclusions

The metabolic fingerprint approach allied with PCA and OSC–PLS–DA permitted chemical profiling of vegetal material and discrimination of different irrigation conditions based on metabolic changes. Statistical mixture design was found to be very important for obtaining the working data set eliminating spectral discrepancies presented in crude extracts obtained with pure solvents. The UV–Vis analysis shows that photosynthetic pigments, demonstrated as pheophytin, in irrigated *C. arabica* leaves are higher than in non-irrigated leaves, indicating that non-irrigated conditions are more stressful than irrigated ones for this species. On the other hand no significant difference was found for these leaf samples owing to varying light conditions.

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