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Chemometric discrimination of genetically modified *Coffea arabica* cultivars using spectroscopic and chromatographic fingerprints

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ABSTRACT

Multivariate statistical design and principal component analysis (PCA) applied to RP-HPLC-DAD and FTIR spectroscopic data were performed to investigate the fingerprints of four coffee cultivars, traditional red bourbon and three genetically modified cultivars. The design and response surface results showed that extraction dependence on solvent composition of one of the genetically modified cultivars, IAPAR 59, was very similar to that found for the red bourbon standard. PCA of the FTIR spectra obtained from all the simplex centroid design mixtures indicated that the 1:1 binary ethanol–dichloromethane solution resulted in the best separation of the four cultivars. The IPR 108 cultivar has more intense vibrational bands in the 3200–3600 cm^{-1} and 1100–1600 cm^{-1} regions indicating higher acid and fat levels than those of the other cultivars. The UV absorptions close to 275 nm of the RP-HPLC-DAD spectra are correlated with the strengths of the infrared absorptions between 3400 and 3460 cm^{-1} and can be explained by varying caffeine concentrations in the four cultivars.

1. Introduction

It is well known that coffee is one of the most popular beverages consumed worldwide. It is one of the firmest pillars of the economy in several Brazilian states, and in the first week of August 2012, the daily revenue with coffee exports reached US $ 21.5 million. The two most important varieties of commercial coffee are *Coffea arabica* and *Coffea canephora* syn. Coffea robusta. Commercial coffee beverage is made from arabica or robusta beans or blends of them, but *C. arabica* is of superior quality. For this reason several papers report methods to discriminate the two species [1–6]. Although there are thousands of papers in the literature about defective and non-defective coffees [7,8] its antioxidant properties [9], the effects of drinking coffee on health [10–12] and the effect of roasting coffee beans [9], among others, few reports on the genetic variability of the Coffea genus can be found. Furthermore no published work on the discrimination of different *C. Arabica* cultivars in terms of genetic variability was encountered. Genetic variability of coffee promotes gains in productivity and desirable agronomical characteristics while also affecting the chemical composition of the product and consequently its attributes and sensory qualities [13].

The Agronomic Institute of Paraná (IAPAR), located in Londrina, Brazil, was established in 1974, and contains a gene bank of *Coffea arabica* with over a thousand hits of this kind. The database also contains a collection of 144 accessions of *C. arabica* collected in Ethiopia, region of the species' origin. This collection has been used as the basis for genetic improvements at IAPAR and resulted in many cultivars with rust resistant genes as well as some with resistance to nematodes [14]. The chemical composition of grain and, consequently, the quality and acceptability of coffee depends on the genetic factors, cropping systems, altitudes, temperatures, water demands, types and levels of fertilization, harvesting times, preparation methods, storage and roasting processes [13].

High performance liquid chromatographic (HPLC) and Fourier transform infrared spectroscopic (FTIR) studies in combination with chemometric techniques have been successfully applied for food quality assessment as well as the detection of food adulteration [15]. Fourier transform infrared spectroscopy is a method that reveals information about the functional groups present in the sample and the relative changes in their amounts.

Recently, fingerprint techniques have become one of the most powerful approaches for the quality control of foods. It describes a variety of analytical methods that can provide the identification and approximate quantification of a group of metabolites associated to specific pathways. Metabolite variations are observed principally by total spectroscopic or chromatographic pattern changes without previous knowledge of the identities of the investigated compounds. Generally, samples with similar spectroscopic or chromatographic fingerprints have similar compositions.

In the last few years, our group has shown that statistical mixture designs permit the development of rigorous but economical

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procedures for demonstrating the effects of solvent changes on the extracted metabolites of plant material [16–21]. Considering the difficulties in chemically differentiating cultivars by conventional means, this research uses a statistical mixture design [22] for four components: (1) ethanol, (2) ethyl acetate, (3) dichloromethane and (4) hexane, to find adequate extraction mixture compositions and experimental conditions for discriminating the traditional cultivar (red Bourbon) from cultivars developed by the Agronomic Institute of Paraná, IAPAR 59, IPR 101 and IPR 108. All these cultivars were produced under the same climatic conditions.

The main objective here was to apply multivariate statistical designs and models associated with liquid chromatographic and spectroscopic data to compare the fingerprints of the four coffee cultivars obtained from different extraction mixtures. As a consequence, method development can be undertaken using experimental conditions capable of differentiating genetically modified cultivars from the traditional cultivar (bourbon) that is of superior quality.

2. Materials and methods

2.1. Coffee samples

The green grains of four different cultivars, the traditional red Bourbon cultivar (used as a standard) and cultivars developed by the Instituto Agronômico do Paraná were investigated. IAPAR 59 carries the *Coffea Arabica* genes, Villa Sarchi × Hibrido de Timor (Sarchimor), IPR 101 has Catuaí × Sarchimor (with S_H2, S_H3 rust resistant genes) and IPR 108, the Iapar59 × (Catuaí × Icatu) genes. All samples were kindly provided by the Agronomic Institute of Paraná.

2.2. Reagents

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, ethyl acetate and ethanol were also purchased from VETEC and were of analytical grade.

2.3. Extract preparation

The grains of green coffees were previously immersed in liquid nitrogen to make them brittle, facilitating their crushing, and then were sieved. The extraction mixtures were chosen according to a Simplex-Centroid Design for four components, amounting to 15 mixtures. Fig. 1 shows the compositions of the experimental extraction mixtures. Each extract was prepared by weighing 10 g and adding 150 mL of one of the solvent mixtures listed in Table 1. These mixtures were placed in an ultrasonic bath (Unique, model Ultracleaner 1400) for 30 min with the bathwater being changed every 15 min to avoid heating. The extracts were filtered through filter paper to separate the solution from the coffee samples. This procedure was repeated two more times. Then an extraction solution of 15 mL was removed and stored in a capped vial under refrigeration for subsequent HPLC analysis. The remainder was evaporated in a rotary evaporator, removing all the solvents still present in the sample, until attaining constant weight. Then the yield of the crude extract was determined and used for chromatographic and spectral analysis.

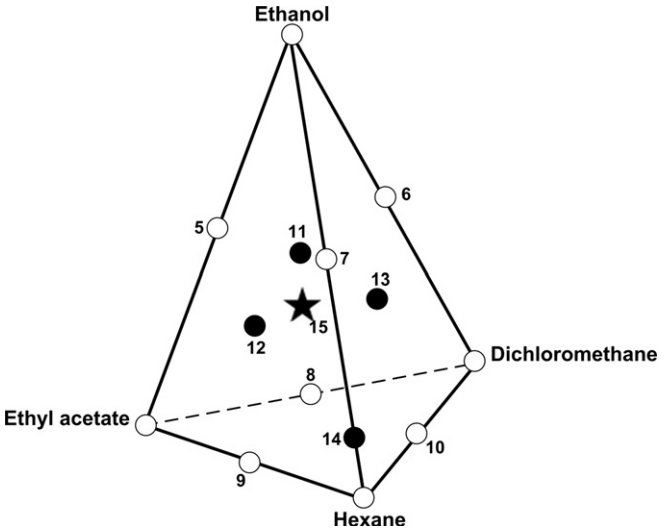

Fig. 1. The simplex centroid mixture design for the ethanol, ethyl acetate, dichloromethane and hexane solvents. Open circles represent pure solvents and binary mixtures, the darkened circles ternary mixtures, and the star a quartenary mixture. Numbers correspond to those in the extract column of Table 1.

Table 1
Extract yields (in grams) of the four coffee cultivars for the simplex centroid design mixtures.

Extract	Ethanol	Ethyl acetate	Dichloro methane	hexane	Bourbon	Iapar 59	IPR 101	IPR 108
1	1	0	0	0	1.390	1.505	1.565	1.265
2	0	1	0	0	0.820	0.870	0.920	0.835
3	0	0	1	0	1.050	1.065	1.170	1.070
4	0	0	0	1	0.820	0.850	0.945	0.830
5	0.5	0.5	0	0	1.610	1.490	1.740	1.865
6	0.5	0	0.5	0	2.010	2.170	1.710	1.510
7	0.5	0	0	0.5	1.695	1.830	1.805	1.475
8	0	0.5	0.5	0	0.870	0.915	0.960	0.830
9	0	0.5	0	0.5	0.890	0.975	1.010	0.840
10	0	0	0.5	0.5	1.030	0.940	1.125	1.240
11	0.333	0.333	0.333	0	1.275	1.465	1.650	1.310
12	0.333	0.333	0	0.333	1.255	1.270	1.280	1.185
13	0.333	0	0.333	0.333	1.305	1.290	1.365	1.240
14	0	0.333	0.333	0.333	0.970	0.940	1.090	0.930
15	0.25	0.25	0.25	0.25	1.130	1.145	1.155	1.190

2.4. Analysis by infrared spectroscopy

For Fourier transform infrared analysis (FTIR) 1.5 mg of the crude extract were weighed with 0.3 g of dry solid KBr that was then homogenized in an agate mortar with a few drops of chloroform. The spectra were recorded in the 4000–400 cm^{-1} region, with 4 cm^{-1} resolution and 90 scans, using a Shimadzu FTIR-8300 spectrophotometer. The data analysis was performed using the entire infrared spectra.

2.5. Sample preparation for HPLC analysis

A 20 μL aliquot was pipetted from each 15 mL extraction solution and added to 800 μL of mobile phase. The samples were filtered through 0.22 μm Millex Millipore paper and analyzed immediately. The chromatographic conditions were: Phenomenex C18 column, 2.6 mM Kinetex HILIC 100 A, with dimensions of 150 mm × 4.6 mm, 20 μL injection volume and 1.0 mL min^{-1} mobile phase flow rate. HPLC analysis was conducted on a Finnigan Surveyour 61607 liquid chromatograph equipped with a Finnigan Surveyour PDA Plus diode array detector. Elution was

monitored at the 210, 240 and 254 nm wavelengths. The data were processed using ChromQuest 4.2 software.

2.6. Software

Statistical models and principal components (PC) were calculated using the Statistica 6.0 software (Statistica for Windows 6.0, Statsoft, Tulsa, OK, USA, 1999).

3. Results

The compositions of the mixtures as well as the average yields of the Bourbon, Iapar 59, IPR 101 and IPR 108 cultivars are given in Table 1. Since the average response values for the binary mixtures are larger than the values for the pure components, quadratic and special cubic models were used to fit the crude extract and cultivar data. Analysis of variance (ANOVA) was used to test for model lack of fit and regression significance. The quadratic models presented lack of fit at the 95% confidence level, so consequently, special cubic models were used. Table 2 summarizes the ANOVA results, sums of squares, mean squares, F values, and the significance probabilities for all the fitted models at the 95% confidence level. Since the lack of fit mean square/pure error mean square ratios are less than the F distribution critical values at the 95% confidence level, these results validate all the models. The experimental data are well reproduced by the simplified special cubic equations, where only coefficients significant at the 95% confidence level are used. The prediction equations for the yields are

$$\hat{y}_{Bourbon} = \underset{(\pm 0.047)}{1.39e} + \underset{(\pm 0.038)}{0.82a} + \underset{(\pm 0.038)}{1.06d} + \underset{(\pm 0.038)}{0.88h} + \underset{(\pm 0.038)}{1.99ea} + \underset{(\pm 0.038)}{3.11ed} + \underset{(\pm 0.038)}{2.21eh} - \underset{(\pm 1.40)}{9.86ead} - \underset{(\pm 1.40)}{6.11eah} - \underset{(\pm 1.40)}{10.27edh} \quad (1)$$

Table 2
Analysis of variance (ANOVA) results for the reduced mixture models obtained for the four coffee cultivars.

Variation source	Sum of squares	Degrees of freedom	Mean square	Calculated F-value[a]	Probability[b]
Bourbon					
Model	3.416	9	0.379	86.99	0.000
Total error	0.087	20	0.004		
Lack of fit	0.038	5	0.008	2.364	0.090
Pure error	0.049	15	0.003		
Total	3.503	29	0.121		
IAPAR 59					
Model	4.140	9	0.460	61.84	0.000
Total error	0.149	20	0.007		
Lack of fit	0.026	5	0.005	0.644	0.670
Pure error	0.122	15	0.008		
Total	4.289	29	0.148		
IPR 101					
Model	2.739	8	0.342	53.12	0.000
Total error	0.135	21	0.006		
Lack of fit	0.055	6	0.009	1.703	0.188
Pure error	0.080	15	0.005		
Total	2.874	29	0.099		
IPR 108					
Model	2.161	6	0.360	13.51	0.000
Total error	0.613	23	0.027		
Lack of fit	0.339	8	0.042	2.320	0.076
Pure error	0.274	15	0.018		
Total	2.774	29	0.096		

[a] Lack of fit mean square/pure error mean square ratio. Corresponding critical F values at the 95% confidence level for special cubic model fits are $F_{9,20,0.05}=2.39$; $F_{8,21,0.05}=2.395$; $F_{6,23,0.05}=2.395$; $F_{5,15,0.05}=2.90$ and $F_{6,23,0.05}=2.545$.
[b] Significance probability level.

$$\hat{y}_{IAPAR59} = \underset{(\pm 0.06)}{1.50e} + \underset{(\pm 0.05)}{0.89a} + \underset{(\pm 0.05)}{1.03d} + \underset{(\pm 0.05)}{0.88h} + \underset{(\pm 0.29)}{1.16ea} + \underset{(\pm 0.29)}{3.60ed} + \underset{(\pm 0.29)}{2.53eh} - \underset{(\pm 1.40)}{5.42ead} - \underset{(\pm 1.40)}{6.16eah} - \underset{(\pm 1.40)}{14.21edh} \quad (2)$$

$$\hat{y}_{IPR101} = \underset{(\pm 0.056)}{1.56e} + \underset{(\pm 0.046)}{0.92a} + \underset{(\pm 0.046)}{1.67d} + \underset{(\pm 0.046)}{1.00h} + \underset{(\pm 0.046)}{2.05ea} + \underset{(\pm 0.25)}{1.44ed} + \underset{(\pm 0.27)}{2.53eh} - \underset{(\pm 1.71)}{10.18eah} - \underset{(\pm 1.71)}{8.26edh} \quad (3)$$

$$\hat{y}_{IPR108} = \underset{(\pm 0.047)}{1.37e} + \underset{(\pm 0.038)}{0.75a} + \underset{(\pm 0.038)}{1.16d} + \underset{(\pm 0.038)}{0.92h} + \underset{(\pm 0.038)}{3.00ea} + \underset{(\pm 0.038)}{1.23eh} - \underset{(\pm 1.40)}{8.11eah} \quad (4)$$

where e, a, d and h represent the ethanol, ethyl acetate, dichloromethane and hexane proportions, respectively. Standard error estimates are given in parentheses below the corresponding model coefficients.

Note that for all the four models, Eqs. (1)–(4), the ethanol and dichloromethane linear blending coefficients are significantly larger than the ones for ethyl acetate and hexane. Of the pure solvents, ethanol is the most effective extractor, as can be seen in Table 1. Of the six binary coefficients, Eq. (1) (bourbon) and Eq. (2) (IAPAR 59) have higher coefficients for the ethanol:dichloromethane cross terms followed by those for ethanol:hexane and ethanol:ethyl acetate. In general, positive interaction coefficients indicate synergism contributing to higher yields whereas negative coefficients indicate antagonism, as in the case of the ternary ethanol:ethyl acetate:dichloromethane interaction that results in lower yields. Eq. (3) (IPR 101) has its highest binary interaction coefficient for the ethanol:hexane pair followed by the ethanol:ethyl acetate and ethanol:dichloromethane terms with antagonistic ternary ethanol:ethyl acetate:hexane and ethanol:dichloromethane:hexane effects. Interestingly, a somewhat different pattern was found for IPR 108, where the highest binary interaction coefficient occurs for the ethanol:ethyl acetate pair and only one antagonistic ternary interaction involving ethanol: ethyl acetate: hexane was found, Eq. (4).

The contour plots for yields predicted by Eqs. (1)–(4) are given in Fig. 2. As can be seen there, the contour plots for the Bourbon and IAPAR 59 cultivars are very similar. Higher yields can be achieved with 1:1 binary mixtures of ethanol: dichloromethane. The models predict that 2.00 g and 2.17 g will be extracted by this mixture for Bourbon and IAPAR 59, respectively. These results are consistent with the experimental yield averages of 2.01 g and 2.17 g. The contour plots for the IPR 101 and IPR 108 samples in Fig. 2 fit the experimental data very well and are quite different from those for Bourbon and IAPAR 59, mostly due to the large ethanol–dichloromethane synergic interactions in Eqs. (1) and (2). This coefficient is small for the IPR 101 model and not significant at the 95% confidence level for the IPR 108 model. For IPR 101, the model predicts significantly lower yields, 1.72 g with a binary 1:1 ethanol:dichloromethane mixture and 1.75 g with an 1:1 ethanol:ethyl acetate binary mixture compared to the experimental values of 1.71 and 1.74 g, respectively. This is also true for the IPR 108 model, for which a predicted yield of 1.79 g is obtained for the 1:1 ethanol:ethyl acetate binary mixture extract corresponding to an experimental yield of 1.87 g. The 50–50% ethanol–dichloromethane mixture results in an even lower experimental yield, just 1.51 g.

The FT-IR spectra of the extracts obtained with different solvent proportions for the four cultivars were compared by principal component analysis (PCA) to investigate their similarities and/or dissimilarities. The chemometric data matrix, **X**, consisted of 60 rows and 1866 columns. Each row corresponds to a spectrum of an extract obtained using a simplex centroid mixture and each column contains the absorbance values at the different wavelengths. The results obtained from the PCA show

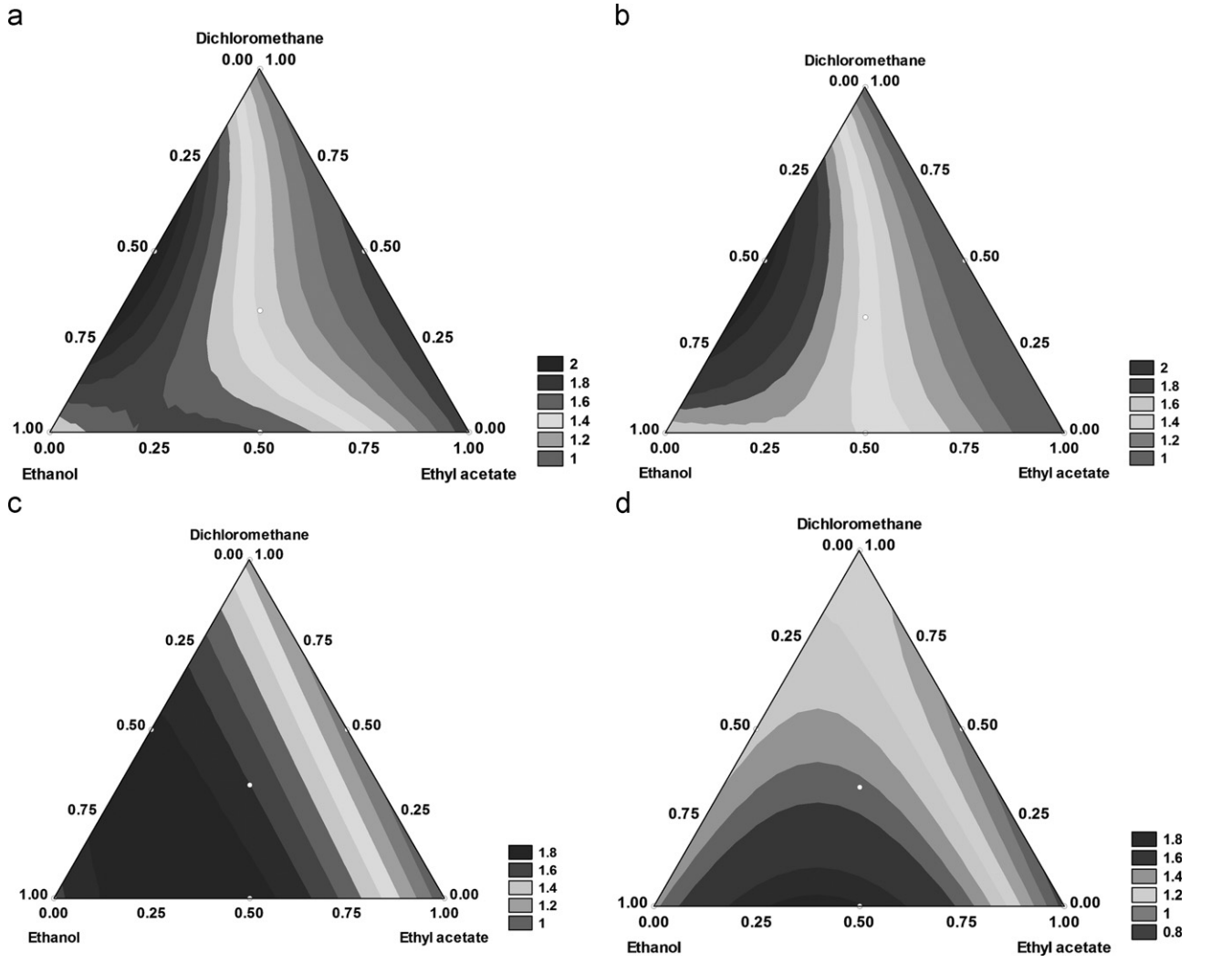

Fig. 2. Mixture response surfaces for each coffee cultivar obtained for the ethanol, dichloromethane and ethyl acetate proportions. The hexane proportion was zero for all these surfaces. (a) Boubon, (b) IAPAR 59, (c) IPR 101 and (d) IPR 108.

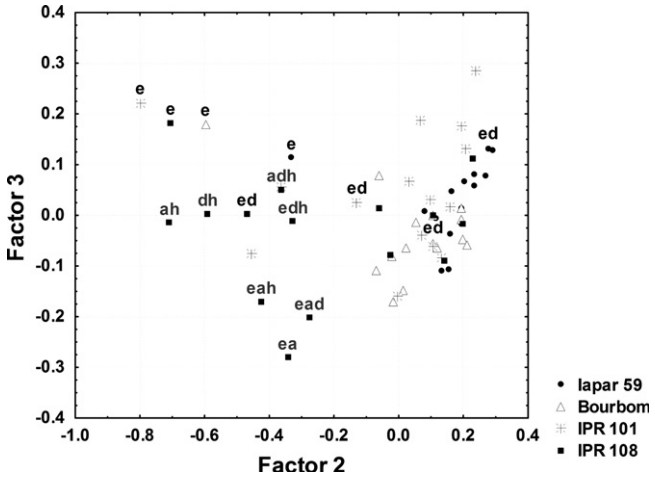

Fig. 3. Principal component score graph of the FTIR spectra of the extracts obtained from the simplex centroid design mixtures. e, d, a and h represent the ethanol, dichloromethane, ethyl acetate and hexane solvents.

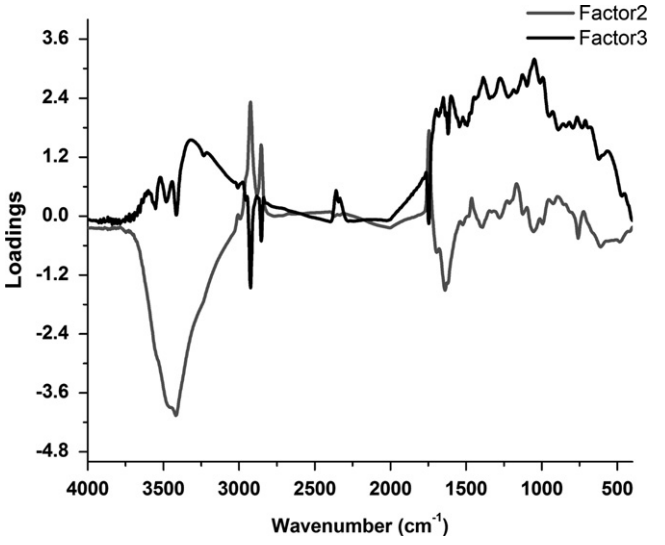

Fig. 4. Loading plots of the second and third principal components of the FTIR spectra of the extracts obtained using the simplex centroid design mixtures.

that the first component explains 89.9% of the variance, but does not contain useful information for discriminating samples of the different coffee cultivars. Although the second and third components only account for 7.9% and 1.1% of the total variance, respectively, they do contain useful information. In the score plot in Fig. 3, the samples are clearly separated along factor 2 into two

clusters, one on the right (positive side) and the other on the left (negative side). Fig. 3 shows that extracts prepared in ethanol do not discriminate the four cultivars. Of the eight experimental conditions containing hexane, for the IPR108 samples, five are located on the negative side of factor 2 (ah, dh, adh, edh, and eah), as are e, ea, and ead. This separation suggests different extraction efficiencies for the metabolites. Examination of the factor 2 loadings suggests that this separation is due to spectral domains situated around ~1650 cm^{-1} and 3432 cm^{-1} (see Fig. 4). Heterocyclic compounds containing N–H groups have N–H stretching bands in the 3500–3200 cm^{-1} region. On the other hand, caffeine molecules absorb infrared radiation at 1656 cm^{-1}. Thus, the factor 2 scores and loadings suggest that mixtures positioned on the left hand side of Fig. 3 extract higher relative amounts of nitrogen-containing compounds, such as alkaloids, than those on the right. Positive factor 3 loadings suggest that this separation was due to domains situated in the ~600–1700 cm^{-1} and 3000–3800 cm^{-1} ranges. These are large regions that can characterize different stretching vibrations. For example, the absorption of the OH stretching for hydroxyl groups is in the 3650–3200 cm^{-1} range whereas the N–H stretching vibrations show absorptions between 3500 and 3300 cm^{-1}. Absorptions between 1500 and 1300 cm^{-1} provide information on C–H bending vibrations and the symmetrical stretching vibration of benzene. The 910–650 cm^{-1} interval is an important region for determining the positions of substituted groups on benzene [23].

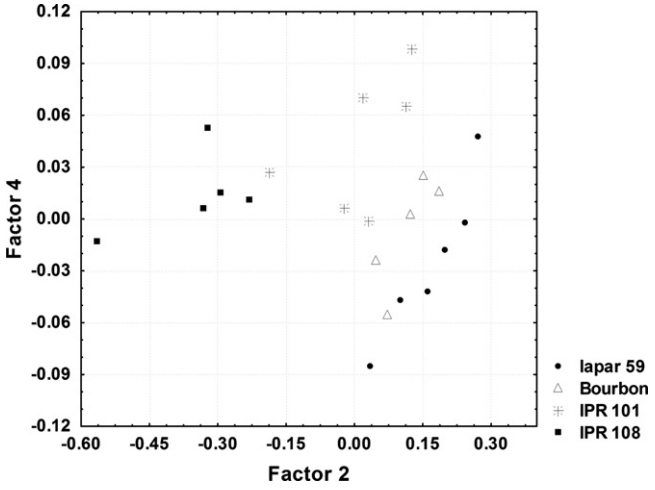

Fig. 5. Score graph of the second and fourth principal components of the FTIR spectra of extracts obtained from samples of the four coffee cultivars with a 1:1 binary mixture of ethanol and dichloromethane.

Fig. 3 also shows that extracts prepared in binary mixtures of ethanol:dichloromethane are furthest apart when compared with separations occurring for other solvent mixtures of the simplex centroid design. Since one of the intentions of this work is to establish a fingerprint in order to discriminate among the cultivars, six samples of each cultivar were prepared with this mixture. Fig. 5 shows the scores of the second and fourth principal components for which the best PCA separation of sample points was observed. As shown there, the IPR108 samples are clearly discriminated on the left of factor 2 with the IPR101 sample points located in the middle. The separation of the IPR101 samples from the Bourbon and IAPAR 59 points is diagonal, meaning that both components are important for discrimination.

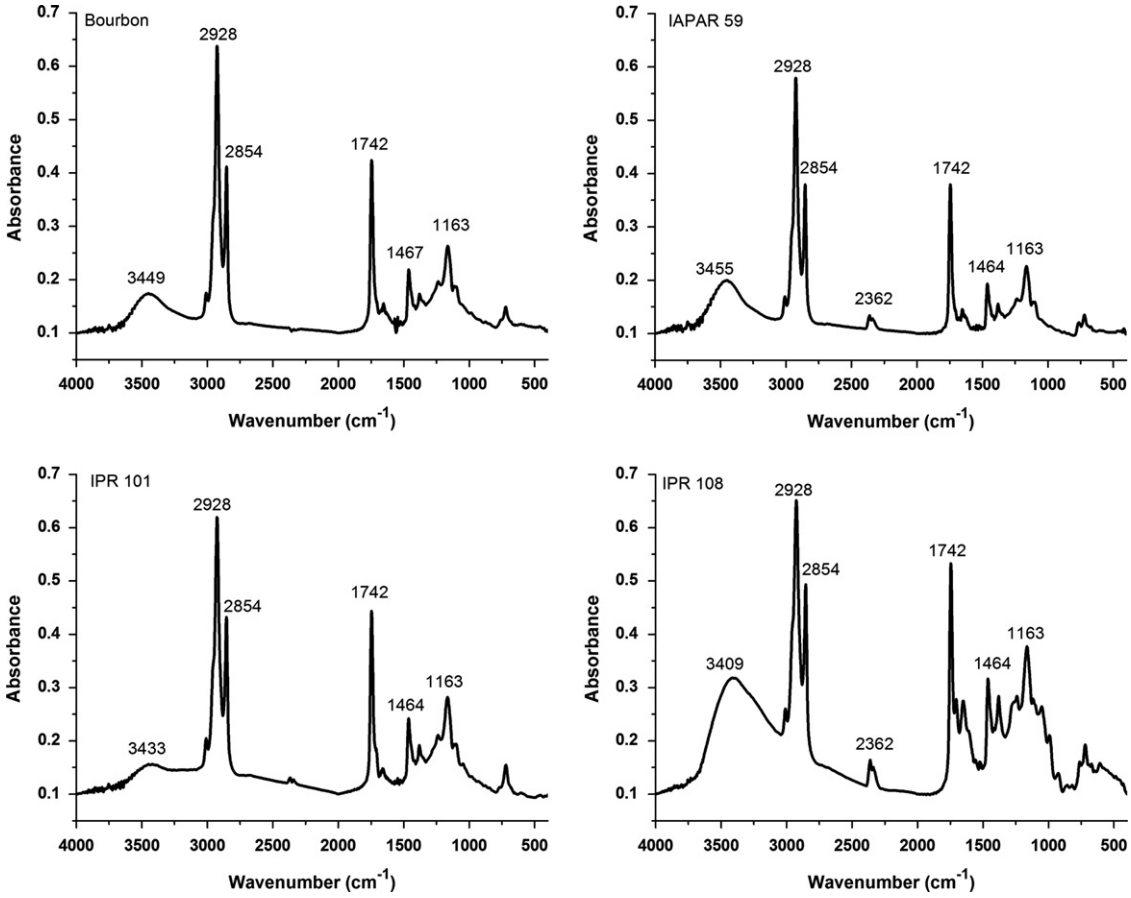

Fig. 6. FTIR spectra of sample extracts of the four coffee cultivars with a 1:1 binary mixture of ethanol and dichloromethane.

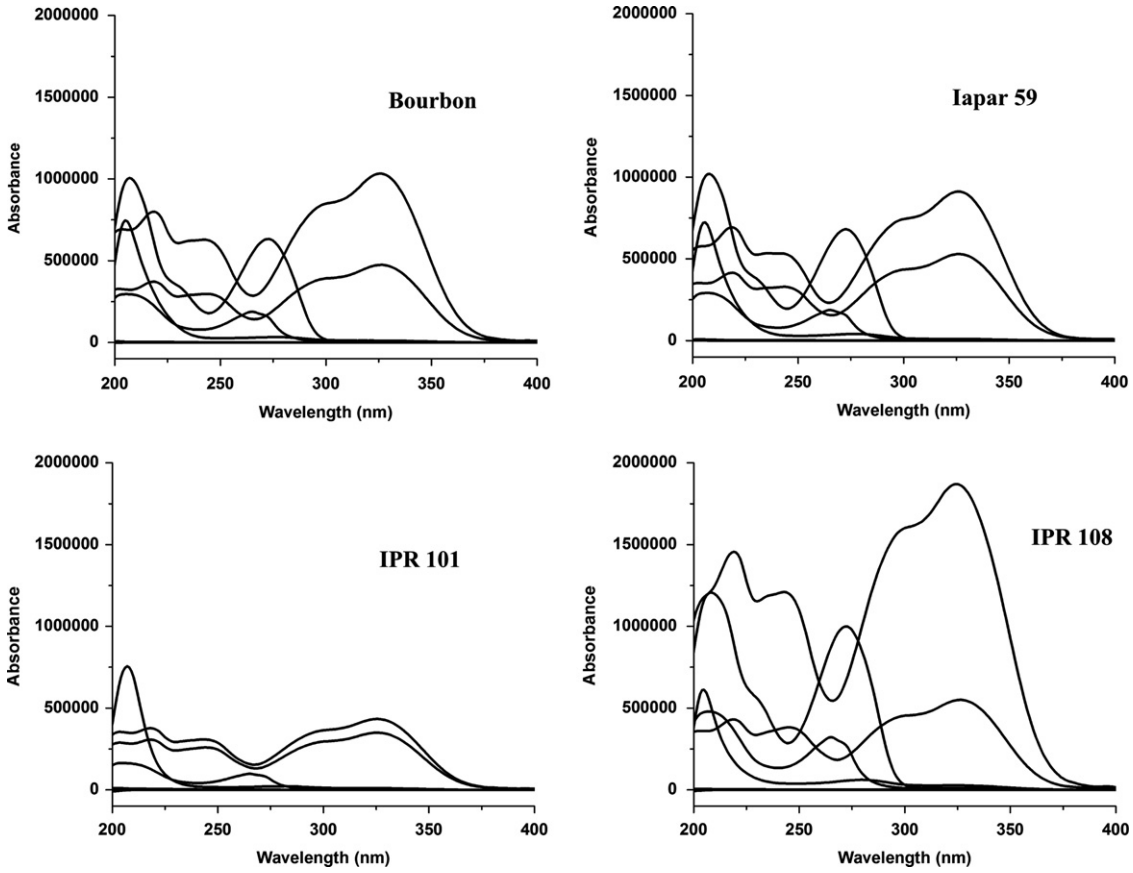

Fig. 7. RP-HPLC-DAD spectra of the various chromatographic peaks for each of the four coffee cultivars. The spectra corresponding to the different peaks are characteristic of different metabolites, such as caffeine, trigonelline and chlorogenic acids.

Also on the right of factor 2 bourbon and IAPAR 59 exhibit similar patterns.

The IPR 108 spectrum is significantly different from the others, specially the stronger absorption peaks in 3000–3600 cm^{-1} and 700–1700 cm^{-1} regions. The stronger absorption peak at 1742 cm^{-1} can be attributed to carbonyl bond stretching from lipids and fatty acids [24], whereas bands in the 1500–1250 cm^{-1} range might be attributed to acids owing to vibrational coupling between a base and a sugar. The band at $\sim$2928 cm^{-1} can be attributed to the asymmetric stretch of methylene groups and the one at $\sim$1465 cm^{-1} to the methylene scissoring vibration. Comparing the spectra of Fig. 6, one can conclude that IPR 108 has larger amounts of acids and fats than the others.

RP-HPLC-DAD mobile phase and detection wavelength conditions were investigated to obtain more information about the metabolic classes for each cultivar. The effects of nine mobile phase compositions on chromatographic separation were first investigated. Three wavelengths, 210, 240 and 254 nm, were also investigated to compare the number of eluted peaks. The largest number of peaks was observed with the 51:23:26 (v/v/v) water: MeOH:ACN mobile phase at 210 nm and these conditions were selected for obtaining the RP-HPLC-DAD data.

Fig. 7 shows the DAD spectra of the chromatographic peaks obtained from the four different cultivars of *C. Arabica*. Differences can be observed among these spectra mainly for IPR 108 and IPR 101. IPR 108 contains stronger absorptions which indicates higher concentrations of chlorogenic acids and caffeine. Moreover the IPR 101 cultivar has lower concentrations of these metabolites. Bourbon and IAPAR 59 exhibit similar patterns that are intermediate between those of IPR 108 and IPR 101. This behavior is similar to the one found for the infrared spectra. The UV absorptions close to 275 nm are correlated with the strengths of the infrared absorptions between 3400 and 3460 cm^{-1} and can be explained by varying caffeine concentrations in the four cultivars. The strong infrared absorptions at 1163, 1464 and 1742 cm^{-1} may be related to the very strong DAD band between 275 and 325 nm. These results are consistent with sensory characteristics of different arabica coffee cultivars described in Ref. [13]. There the IAPAR 59 cultivar was described as being similar to Bourbon while the IPR 101 cultivar with Catuai genes was distinguished by its less intense acidic taste.

4. Conclusions

Principal component analysis of the FTIR spectra of 15 different simplex centroid design mixtures involving ethanol, ethyl acetate, hexane and dichloromethane indicated that the 1:1 binary mixture is most adequate for discriminating and fingerprinting the red Bourbon and three genetically modified coffee cultivars. UV absorptions close to 275 nm of RP-HPLC-DAD spectra are correlated with the intensities of infrared absorptions between 3400 and 3460 cm^{-1} and can be explained by varying caffeine concentrations in the four cultivars. Quantitative analytical determinations of caffeine and other important metabolites for these cultivars are currently underway in our laboratory.

Novelty statement

FTIR spectroscopic analysis of statistical mixture design extracts was performed to investigate the fingerprints of four

coffee cultivars, traditional red Bourbon and three genetically modified cultivars. This innovative use of statistical mixture design extracts resulted in the graphical separations of the spectra of the four cultivars using either FTIR or RP-HPLC-DAD data as well as the identification of the compounds differentiating the four cultivars. This work has immediate relevance since the genetic variability of coffee promotes gains in productivity while affecting the chemical composition of the product and consequently its attributes and sensorial qualities.

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References

[1] S. Casal, M.B. Oliveira, M.R. Alves, M.A. Ferreira, J. Agric. Food Chem. 48 (2000) 3420–3424.
[2] A.G. González, F. Pablos, M.J. Martin, M. León-Camacho, M.S. Valdenebro, Food Chem. 73 (2001) 93–101.
[3] C.P. Bicchi, A.E. Binello, G.M. Pellegrino, A.C. Vanni, J. Agric. Food Chem. 45 (1995) 1549–1555.
[4] R.C. Alves, S. Casal, M.R. Alves, M.B. Oliveira, Food Chem. 114 (2009) 295–299.
[5] S. Casal, M.R. Alves, E. Mendes, M. Beatriz, P.P. Oliveira, M.A. Ferreira, J. Agric. Food Chem. 51 (2003) 6495–6501.
[6] M.J. Martin, F. Pablos, A.G. Gonzalez, Food Chem. 66 (1999) 365–370.
[7] A.P. Craig, A.S. Franca, L.S. Oliveira, Food Chem. 132 (2012) 1368–1374.
[8] A.P. Craig, A.S. Franca, L.S. Oliveira, Food Sci. technol. 47 (2012) 505–511.
[9] M.C. Nicole, M. Anese, L. Manzocco, C.R. Lerici, Food Sci. technol. 30 (1997) 292–297.
[10] A.V. Mattioli, A. Farinetti, C. Mirolo, P. Pedrazzi, G. Mattioli, Nutr. Metab. Cardiovasc. Dis. 21 (2011) 412–417.
[11] S. Gallus, A. Tavani, E. Negri, C. La Vecchia, Ann. Epidemiol. 12 (2002) 202–205.
[12] N.J. Frost-Meyer, J.V. Logomarsino, J. Funct, Foods 4 (2012) 819–830.
[13] C.S.G. Kitzberger, M.B. dos, S. Scholz, J.B.G.D. da Silva, M. de Benassi, Braz. J. Food Technol. 6 (2010) 39–48.
[14] D.S. Ito, T. Sera, G.H. Sera, L.D. Grossi1, F.S. Kanayama, Crop Breeding Appl. Biotechnol. 8 (2008) 99–103.
[15] L.E. Rodriguez-Saona, M.E. Allendorf, Annu. Rev. Food Sci. Technol. 2 (2011) 467–483.
[16] E.B.R. de Souza, R.R. da Silva, S. Afonso, I.S. Scarminio, J. Sep. Sci. 32 (2009) 4176–4185.
[17] L.M.Z. Garcia, E.P. Daiane, C.A.P. da Câmara, I.S. Scarminio, S.L. Nixdorf, J. Chromatogr. Sci. 47 (2009) 825–832.
[18] P.K. Soares, R.E. Bruns, I.S. Scarminio, J. Sep. Sci. 32 (2009) 644–652.
[19] P.K. Soares, I.S. Scarminio, Phytochem. Anal. 19 (2008) 78–85.
[20] F. Delaroza, I.S. Scarminio, J. Sep. Sci. 31 (2008) 1034–1041.
[21] L.M.Z. Garcia, R.E. Bruns, I.S. Scarminio, Chemom. Intell. Lab. Sys. 103 (2010) 1–7.
[22] B.B. Barros, R.E. Bruns, I.S. Scarminio, Statistical Design—Chemometrics, Elsevier, Amsterdam, 2006, pp. 313–340.
[23] W.J.H. Liu (Ed.), Traditional Herbal Medicine Research Methods: Identification, Analysis, Bioassay and Pharmaceutical and Clinical Study, John Wiley & Sons, 2011.
[24] M. Kansiz, P. Heraudn, B. Woodn, F. Burdenn, J. Beardalln, D. McNaughton, Phytochemistry 52 (1999) 407–417.