Authentication of cocoa bean shells by near- and mid-infrared spectroscopy and inductively coupled plasma-optical emission spectroscopy

Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number: FOODCHEM-D-18-06255R1

Title: Authentication of cocoa bean shells by near-infrared and mid-infrared spectroscopy and inductive coupled plasma-optical emission spectroscopy

Article Type: Research Article (max 7,500 words)

Keywords: cocoa bean shells, food traceability, data fusion, near infrared spectroscopy, mid infrared spectroscopy, inductive coupled plasma.

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Abstract: The aim of this study was to evaluate the efficacy of a multi-analytical approach for origin authentication of cocoa beans shells (CBS). The overall chemical profiles of cocoa bean shells from different origins were collected and measured using diffuse reflectance near-infrared spectroscopy (NIRS) and attenuated total reflectance mid-infrared spectroscopy (ATR-FT-IR) for molecular composition, as well as inductive coupled plasma-optical emission spectroscopy (ICP-OES) for elemental composition. Exploratory chemometric techniques were employed to identify systematic patterns related to the geographical origin of samples based on each technique using Principal Components Analysis (PCA). A combination of the three techniques proved to be the most promising approach to establish classification models.Partial Least Squares-Discriminant Analysis model of the fused PCA scores of three independent models was used and compared with single technique models. CBS samples were better classified by the fused model. Satisfactory classification rates where obtained for Central Africa samples with accuracy of 0.84.

Highlights

- Multi-analytical approach for origin authentication of cocoa beans shells is proposed.
- Principal Component Analysis of NIR, ATR-FT-IR and ICP-OES data was discussed.
- Samples from Ecuador and central Africa were precisely classified by PLS-DA.
- Samples from São Tomé showed more features in common with the American samples than with the African samples.
- CBS samples were better classified by the fused model then by the three single analytical techniques.

Authentication of cocoa bean shells by near-infrared and mid-infrared spectroscopy and inductive coupled plasma-optical emission spectroscopy

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Abstract

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techniques were employed to identify systematic patterns related to the geographical origin of samples based on each technique using Principal Components Analysis (PCA). A combination of the three techniques proved to be the most promising approach to establish classification models. Partial Least Squares-Discriminant Analysis model of the fused PCA scores of three independent models was used and compared with single technique models. CBS samples were better classified by the fused model. Satisfactory classification rates were obtained for Central Africa samples with accuracy of 0.84.

1. Introduction

Since the 19th century cocoa has seen a continuous growth of consumption in a variety of forms, leading to an outstanding economic interest of chocolate industries for constant innovation and modernization. As many other agro-food activities, cocoa industry produces large amounts of by-products (https://www.icco.org/). Cocoa bean shells (CBS) is one of the main by-products, which represents the 12 % of weight after husking and grinding of dried cocoa seeds. CBS represents a non-negligible disposal problem and thus legislation and environmental issues are forcing industries to define process optimization and recovery/recycling strategies. Recently, bioconversion of by-products has raised the interest of scientific research and in several countries strategic vision or dedicated policies are being prepared to manage food industry wastes in the most efficient way – abandoning the "take, make and dispose" behavior and instead acting out a circular economy paradigm (Sørensen, Aru, Khakimov, Aunskjær, Engelsen, 2018). The increasing interest for byproducts has certainly an environmental basis, but an important role is played by the tendency to reduce the use of synthetic additives and replace them with natural substances in food. Research concerning new natural additives with high quality/costs ratio is increasing nowadays (Carocho, Morales, Ferreira, 2015). Moreover, the demand of new functional foods, rich in bio compounds such as polyphenols, fiber, n-3 fatty acids etc., drives interest for rich food wastes, such as seeds husks (Andrade, Gonçalvez, Maraschin, Ribeiro-do-Valle, Martínez, Ferreira, 2012; Jansman, Verstegen, Huisman, Van den Berg, 1995). Vegetal by-products are rich of nutrients, such as fiber, polyphenols, minerals and their recycling represent one of the valorization strategies. The development of CBS valorization strategies is aimed at reducing the environmental impact of the cocoa production and provides information to promote conversion of a by-product into added-value products with application in food and healthcare sectors. The definition of the chemical composition of CBS from different countries is meant to evaluate the systematic

differences due to their origin. Chemical analysis of CBS has been carried out in several research papers because of its interesting features related to flavor, phenolic compounds and nutritional values (Barbosa-Pereira, Guglielmetti, Zeppa, , 2018; Manzano, Hernández, Quijano-Avilés, Barragán, Chóez-Guaranda, Viteri, Valle, 2017; Redgwell, Trovato, Merinat, Curti, Hediger, Manez, 2003; Serra Bonvehí, and Escolá Jordà, 1998; Martín- Cabrejas, Valiente, Esteban, Mollá, Waldron, 1994;), however a complete characterization, using different methodologies to highlight similarities and differences in composition of samples from different countries has not been accomplished yet. In this work, CBS samples from different countries were analyzed with three different analytical methods. Near infrared spectroscopy (NIRS), mid infrared spectroscopy by attenuated total reflectance (ATR-FT-IR) and inductively coupled plasma-optical emission spectroscopy (ICP-OES) were used to collect a wide chemical information, both molecular and elementary. The aim of this study was to evaluate the validity of simple and rapid analytical techniques, supported by a chemometric approach, for the identification of differences due to different geographical origin of samples of CBS, with the perspective of a future application for traceability and origin authentication of CBS as food additive.

Nowadays, the exchange of food is realized in a complex and interconnected global net, and food products are often exposed to frauds, false information, contamination risk and counterfeiting. For this reason, it is extremely important to protect and valorize authentic products, including regionals specialties. Innovative, reliable strategies to individuate specific markers of origin, as well as characteristic compositional patterns that can be associated to a precise origin are needed (Mandrile, Giovannozzi, Zeppa, Rossi, 2016). Geographical origin indicators should provide an analytical response to the geographical traceability problem and support the documental certification, which is used today to guarantee food and food-additives provenience. Different techniques such as NMR and isotope ratio mass spectrometry can play a relevant role to provide origin indicators (Lee, et al., 2011). Rapid and non-destructive techniques, such as near infrared spectroscopy, are particularly interesting because of the possibility to obtain an efficient and non-biased overview of the sample chemistry (Sørensen, Khakimov, Engelsen, 2016). The chemical specificity and ease of sampling of NIR spectroscopy make it an attractive tool for rapid and comprehensive food analysis. The complex pattern of signals revealed by IR analysis, both in the near and mid infrared spectral region, is correlated to the content of the different chemical constituents, such as proteins, fatty acids, carbohydrates, alimentary fibers and phenolic compounds. Statistics and multivariate data analysis offer powerful tools to identify robust correlations between measured

77 data and geographical origin, and validated models can provide useful methods for the recognition of unknown
78 samples, with a certain probability (Peres, Barlet, Loiseau, Montet, 2007; Kelly, Heaton, Hoogewerff, 2005). In
79 this work, chemometrics was used for data analysis to calculate at first explorative, and subsequently predictive,
80 models. Principal Component Analysis for data exploration and visualization is a well-established strategy to
81 allow the extraction of useful information from numerous experimental results in food science (Munck,
82 Nørgaard, Engelsen, Bro, Andersson, 1998). Moreover, data fusion for multi-block analysis was used to improve
83 models, gaining information from several different analytical techniques (Biancolillo, Bucci, Magrì, Magrì,
84 Marini, 2014; Skov, Honoré, Hansen, Næs, Engelsen, 2014; Silvestri et. al, 2014; Zakaria, et al, 2010).
85
86 **2. Material and Methods**

87 *2.1 Samples*

88 Fermented and dried cocoa (Theobroma cacao L.) samples were selected and collected within COVALFOOD
89 project funded by European Union's Seventh Framework, involving five Italian chocolate industries. A complete
90 list of 78 samples with the associated information about supplier, provenience and variety is reported in table
91 1S.1 in supplementary information. For an easier exploration of the sample pool, charts of geographical and
92 varietal distribution are shown in figure 1S.1. All samples were imported as untreated raw materials, and the
93 geographical origin was guaranteed by the supplying industry. All samples were roasted and decorticated in
94 laboratory in a ventilated oven for 20 min at 130°C. After roasting, the fragile shell of the beans was separated
95 by mechanical rubbing and removed by hoover suction. The collected cocoa bean shells (CBS) were ground
96 using an ultra-centrifugal mill Retsch ZM 200 (RetschGmbh, Haan, Germany) and stored as dry fine powders
97 (250 µm) in a desiccator in closed containers.

98 *2.2 Near infrared spectroscopy*

99 NIR spectra of CBS were collected in the spectral range 10000 - 4000 cm^{-1} (1000 - 2500 nm) using an Antaris II
100 FT-NIR spectrometer (Thermo Fisher, Waltham, USA) in diffuse reflectance mode. The integrating sphere
101 accessorize was used to collect diffuse reflected light. CBS was analyzed without sample pretreatment; 0.1 g of
102 powder in a quartz glass vial located over the integrating sphere. 32 scans were collected per each sample with

spectral resolution of 8 cm^{-1}. A clean flat golden surface was used for background collection. Three measurement replicates were collected per sample. All samples were measured in randomized order.

2.3 Mid infrared spectroscopy

ATR-FT-IR spectra in the mid infrared region between 500 - 4000 cm^{-1} were collected using Nicolet FT-IR spectrometer (Thermo Fisher, Waltham, USA), Germanium crystal (n = 5.7) for total reflection was used which allows a maximum sample penetration of 1 µm. 64 scans were needed for a good signal to noise with 4 cm^{-1} resolution. The sample powder was pressed with a conical tip on the crystal, the pressure applied was 15 Bar. The tip and the crystal were washed with ethanol between one sample analysis and the following. Three spectra were collected for each sample, resampling at each replicate.

2.4 ICP-OES elemental composition

ICP-OES measurements were performed on an Agilent 5100 Synchronous Vertical Dual View (Agilent, Santa Clara, California, USA), equipped with an EasyFit torch (Agilent P/N G8010-60228). Samples were measured in radial mode, using a plasma flow of 12 ml/min and nebulizer flow of 0.7 ml/min, with a rinse time of 15 seconds and stabilization time of 15 seconds, in three replicates. Viewing height was set to 8 mm, and pump speed to 12. Prior to measurement, the samples were digested in an Antor Paar Multiwave GO microwave oven: 5 mg of CBS samples were placed in the oven teflon tubes, 1 ml of HNO_3 5 % v/v was added, and the tubes were sealed to manufacturer specifications. The temperature ramp was set to reach 180° in 5 min, then held constant, and the total treatment lasted 40 min. After digestion the samples were further diluted with 4 ml HNO_3 5 % v/v to obtain a clear solution, before being put in tubes and placed in the auto-sampler for the ICP analysis. All glassware, tubes and equipment were cleansed in HNO_3 5 % v/v as needed.

2.5 Data treatment

Chemometric data analysis was carried out using PLS Toolbox from Eigenvector Research, Inc. (Manson, WA) for Matlab R2015a (Mathworks, Natick, USA). Principal Components Analysis (PCA) method is a linear factorization method uniquely suited for data exploration. As an explorative tool, PCA provides visualization of multivariate data as score points in a model space (Wold, Esbensen, Geladi 1987). PCA scores plot are useful to

explore data and to find correlation between measured variables and the information of interest, such as geographical provenience of CBS, in this case. Then PLS-DA (Barker and Rayens 2003) models were calculated to compare the classification performances of the three techniques separately with the results obtained by joining the three datasets and considering all information contemporarily. Ten classes were considered: Central Africa, Ecuador, Gulf of Mexico, Indonesia, Mexico, Peru, São Tomé, Colombia, Venezuela and Brazil. All the calculated PLS-DA models were validated using leave-one group-out cross validation. The subsets of samples used as tests sets in cross validation corresponds to the country of origin. For each technique data preprocessing details are reported. Leave-one group-out cross validation was performed, using as group vector the country of origin. Sensitivity (True Positive/(True Positive+False Negative)), Specificity (True Negative/(True Negative+False Positive)), Accuracy (correctly classified samples/total samples) and Precision (True Positive/(True Positive +False Positive) were considered as model evaluation parameters for each class in cross validation to compare classification performances of different techniques.

2.5.1 NIRS data treatment

Preprocessing of NIRS data was applied to extract useful information from the dataset. Absolute absorbance variations and unwanted light scattering were removed using preprocessing of the NIRS data (Martens et al, 2003). The most effective preprocessing was chosen based on the minimum differences between replicates on the PCA scores plots relative to the distance between samples. 2^{nd} derivative (Savitzky Golay, filter width 15 and polynomial order 2) coupled with standard normal variate (SNV); normalization was useful to remove random shift of the baseline offset (Barnes, Dhanoa, Lister, 1989). In addition, the derivatives of spectra were calculated to increase sensitivity to data trends changings. Processed spectra were shown in figure 2S.1. Unwanted variability was successfully removed as demonstrated by the narrow grouping of the replicates obtained after processing shown in figure 2S.2 in supplementary information. PCA was applied to visualize data and to investigate systematic differences among samples, and variables with peculiar relevance were identified. 4LVs PLS-DA classification model was also calculated to discriminate classes of samples from different geographical areas. Same spectra preprocessing was used.

2.5.2 MIRS data treatment

Preprocessing of data was performed to suppress useless variability associated to unwanted noise. The selection criterion for data preprocessing was the maximized closeness of the scores of technical replicates on PC1, as shown in figure 3S.1 in supplementary information. Baseline correction (using asymmetric weighted least squares algorithm, with basis filter of order 2) (Peng, Peng, Jiang, Wei, Li, Tan, 2010) followed by second derivative (Savitzky Golay, filter width 15 and polynomial order 2) and mean centering was selected as optimal preprocessing. PCA model for data visualization and exploration was calculated; PLS-DA classification model using 4 LVs of the same preprocessed data was also calculated to compare MIRS classification capabilities with the other techniques.

2.5.3 ICP-OES data treatment

ICP emission spectra were evaluated for quantification using a calibration curve per element. The calibration curves were estimated using two series of standards prepared by dilution of a certified standard mix (ICP Multi-element standard solution IV, Sigma Aldrich, Germany) containing known concentration of 21 elements (Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Sr, Tl, Zn). Standard concentrations were 0, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 20, 30, 40, 60, 80 100 mg/100g of the certified standard concentration, which was 5 mg/l for all elements, out of Potassium that was 50 mg/l in the standard solution. Three emission wavelengths were monitored per each element, then the intensity revealed for only one λ was selected per each element based on the best correlation coefficient of the corresponding calibration curve and trying to avoid interferences between different elements: λ_{Al}= 237.3 nm; λ_{B}=249.7 nm; λ_{Ba}=455.4 nm; λ_{Bi}=190.2 nm; λ_{Ca}=396.8 nm; λ_{Cd}=228.8 nm; λ_{Co}=230.8 nm; λ_{Cr}=206.2 nm; λ_{Cu}=324.8 nm; λ_{Fe}=234.4 nm; λ_{K}=766.5 nm; λ_{Li}=670.8 nm; λ_{Mg}=285.2 nm; λ_{Mn}=259.4 nm; λ_{Mo}=203.8 nm; λ_{Na}=589.0 nm; λ_{Ni}=221.6 nm; λ_{Pb}=217.0 nm; λ_{Sr}=421.6 nm; λ_{Tl}=351.9 nm; λ_{Zn}=202.5 nm.

The table of results was then imported in Matlab (Mathworks, Natick, USA) and processed with the PLS Toolbox for PCA model calculation and PLS-DA classification. Autoscaling was performed on the data. Three LVs were considered for PLS-DA classification model. Cross validation was used to evaluate the classification capabilities of the model, leaving one country out at each validation step, as described for the other techniques.

2.5.4 Data fusion

The multi-block tool of PLS toolbox by Eigenvector was used to fuse the PCA scores from the three single PCA models of the different analytical techniques. A joined model exploiting mid-level data fusion was obtained (; Borràs, et al, 2014). To make the interpretation clearer, the measurement replicates were averaged, and one matrix line per each sample was maintained for the three different original datasets (NIRS, MIR-ATR and ICP). Each block was first decomposed by PCA, and the resulting scores were fused into a new dataset. The samples' scores for the most relevant PCs were considered to calculate a new fused model. Seven PCs were considered for MIRS and ICP, and six PCs were considered for NIRS. Thus, twenty initial variables were used to build the new joined PCA model. Default autoscale was applied before joining data. PLS-DA method was then performed with autoscaled data to obtain a classification model (Ballabio, Consonni, 2013). The class vector was represented by the area of origin. It was composed of 10 classes i.e. Central Africa, Colombia, Ecuador, Gulf of Mexico, Indonesia, Mexico, Peru, São Tomé, Venezuela, Brazil. Unfortunately the number of samples per each class was not balanced, due to sample availability. Five latent variables were considered for the PLS-DA model, based on the minimum average classification error in cross validation, using leave-one country-out cross validation strategy.

3. Results and Discussion

3.1 NIRS spectroscopy characterization of CBS samples

The NIRS profiles show the typical broad bands of overtones and combination bands of vibrational modes associated to the main constituents of vegetal origin materials. The assignment of the most bands of the NIR spectrum are reported in table 2S.1 in the supplementary information (Jacobsen, et. al. 2011). The mean NIR spectra of all CBS samples is shown in figure 1 a, together with the standard deviation profiles. Similar spectral shape was obtained for all samples, the same bands are present in all spectra with slight differences in mutual intensities.

Figure 1

Vibrational spectroscopy represents a rapid strategy to gather chemical information of a complex matrix, reducing costs, time and environmental impact of analysis. NIR spectra can be effectively correlated to the main

alimentary components as widely reported in literature (De Oliveira, Roque, de Maia, Stringheta, Teófilo, 2018; Dong, Sørensen, He,Engelsen, 2017; Mandrile, Fusaro, Amato, Marchis, Martra, Rossi, 2018).

The sensitivity of NIRS to the botanical variety was tested at first, since it has been previously demonstrated in literature that differences in the chemical composition of different varieties of *Theobroma Cacao L.* were present (Elwers, Zambrano, Rohsius, Lieberei, 2009). The outcome of the PCA on the NIR spectra is shown in figure 1 b. In contrast with expectations, different botanical varieties did not cause evident systematic clustering of NIR spectra. The scores of NIR spectra of *Forastero* and *Trinitario* samples were overlapped in the scores plot (figure 1 b), no separation occurred neither in the PC2/PC1 plot, nor in the later PCs (plots not shown). This can be probably attributed to the complexity of the samples' set, that introduces a lot of confusing variability. However, Arriba samples, a specific variety cultivated in Ecuador only (green squares on the scores plot in figure 1b), was specifically, even though not selectively, characterized by negative scores on PC1 and positive scores on PC2 attesting the capability of NIR spectra to catch common chemical features of Arriba samples. The loadings profiles (figure 2S.3 a) and the variance captured (figure 2S.4) hallows to define what spectral regions are involved in each relevant PC. PC1, is mainly characterized by fatty acids bands as 5670-5780 cm^{-1} (1^{st} C-H str) and 4325 cm^{-1} (1^{st} C-H str + 1^{st} C-H def CH_2), 4250 cm^{-1} (1^{st} C-H str + 1^{st} C-H def). In addition PC1 captures also some regions related to proteins such as 5170-5190 cm^{-1} (2^{nd} C=O of CONH), 5269 cm^{-1} (2^{nd} C=O of COOH), 6320 cm^{-1} (1^{st} N-H str of CONH) and 6535 cm^{-1} (1^{st} N-H str of RNH_2) and 6950 cm^{-1}. PC2, instead, shows three maxima at 4400 cm^{-1} (1^{st} O-H str + 1st C-C str, associated to starch), 4763 cm^{-1} (2^{nd} O-H def + 2^{nd} C-O str of starch) and 5000 cm^{-1} (2^{nd} O-H def + 1st C-O def of starch), this means that PC2 mostly represents the starch content into the samples. PCA highlighted a major content of fatty acids and vegetal proteins in the examined Arriba samples as shown in figure 1 c, d, whereas lower intensity in the spectral regions associable to polysaccharides, such as starch, was measured (corresponding enlarged spectral region not shown for brevity reasons).

As far as correlations between the geographical origin and NIR spectra are concerned, the information provided by the scores plot seems confused at a first look, however some interesting considerations can be underlined. Common features of all samples coming from central Africa were noticed in the scores plot (figure 2 a) when considering PC2. On average, central Africa samples (red rhombus in figure 2 a) show positive scores on PC2,

related to polysaccharides and starch bands mainly (figures 2S.3, 2S.4 can be consulted for all attributions of spectral bands to the PCs). Moreover other common features were noticed in further PCs, such as negative scores on PC3 (figure 2S.6 b) (where the main contributions are 5218 cm^{-1}, 1st O-H str of phenols, 5878 cm^{-1} 1st C-H str CH_3, 6075 cm^{-1} 1st C-H str of R-CH-CH, 7062 cm^{-1}, 2nd C-H str + 1st C-H def of aromatic compounds) and positive again on PC4 (Figure 2S.6 c) which is related mainly to carbohydrates (4790 cm^{-1} 1st O-H str + 1st O-H def ROH o sucrose and starch, 6264 cm^{-1}, 1st O-H str intramolecular H-bond of starch or glucose). Although the separation of the examined groups is not sufficient for selective discrimination, it was confirmed that the geographical origin information is captured by NIRS. As shown in figure 2 a, African samples from São Tomé (a little island in Guinea Gulf, at latitude 0°) show features in common with samples coming from America, which on average showed negative scores on PC2. The scores of São Tomé samples (light blue rhombus in figure 2 a) are mixed with Gulf of Mexico Samples, this can be attributed to similar environmental and climatic conditions of the little islands, that influences the chemical composition of Cocoa fruits, and therefore of CBS (see also figures 2S.6 a to appreciate similitudes of São Tomé with samples from the islands and coasts of Gulf of Mexico). Moreover, Ecuador samples seemed more similar to the African samples than to the American, indeed, in figure 2 a, orange circles corresponding to Ecuador samples are mixed with red rhombus corresponding to samples from Central Africa. In figure 2 b the average NIR spectra of the macro classes, Africa and America, are compared with the spectra of São Tomé and Ecuador, that show peculiar behavior in contrast with the general trend.

The Asian samples are separated from the others (blue triangles in figure 2 b), because of high values on PCs 4, 5 and 6. PC4 is characterized by a peak around 4530 cm^{-1}. This spectral region, represented in figure 2 d is assigned to ROH combination modes, so it can be hypothesized that sugars' content differs for Asian samples with respect to all the others. The most represented spectral region in PC5 (which is relevant for the clustering of Asian samples) is the side of the peak at 6300 cm^{-1}. This region, represented in figure 2 e, highlights that the bands' shape is relevant, more than its intensity in this case. PC6 is also responsible for the following spectral regions: 4466 cm^{-1} (beta-glucan), 5114 cm^{-1} (2nd C=O of esters) and 7147 cm^{-1} typical of R-OH (as already mentioned figures 2S.3, 2S.4 can be consulted for all attributions of spectral bands to the PCs).

Figure 2

The definition of rules to correlate the NIR spectra variability with the geographic area of origin based on the PCA scores plot of NIR spectra is not immediate. However, some common trends were noticed for samples from the same area, and NIR spectra demonstrated to contain useful information for geographical provenience analysis.

3.2 ATR-FT-IR spectra

Spectral profiles in the mid infrared region are shown in figure 3 a. As well as for NIRS, ATR-FT-IR spectroscopy is expected to deliver information about the chemical composition of CBS samples including most of biochemical species present in the matrix. Although absorption bands in the mid infrared region are more defined and narrower because primary vibration modes absorb in this spectral region, the visual interpretation of spectra is difficult, especially in the so-called fingerprint region, between 1750 cm^{-1} and 500 cm^{-1}. Main bands interpretation is reported in table 3S.1 in supplementary information. (Socrates, 2001; Rubio- Diaz, Rodriguez- Saona, 2010; Li-Chan, Chalmers, Griffiths, 2011). The region between 2260-2440 cm^{-1}, where CO_2 band is present, was excluded.

Figure 3

MIRS spectra provided information in agreement with NIRS investigation. Signals are more defined and spectral specificity is increased compared to NIRS, and PCA scores plots investigation resulted an effective strategy to explore spectra similarities. Similarities and differences between samples are ruled by PC1, 2 and 3. The correspondence between PCs and MIR spectral regions was evaluated analyzing figure 3S.4, where the MIR spectrum was superimposed over the histogram of the percentage of variance captured by each PC, to understand what bands drive the scores distribution on the scores plot. PC1 is mainly dominated by CH_x vibrations in the 3000-2800 cm^{-1} and 1460-1420 cm^{-1} region (samples with high intensity of signals at 2920 cm^{-1} and 1463 cm^{-1} present lower values of PC1), moreover 1730 cm^{-1} peak (C=O stretching) that showed increased intensity in Arriba samples is also represented in PC1; PC2 captures variance in 1700-1650 cm^{-1} region (high values of PC2 mean lower intensity at 1560 cm^{-1} and 1525 cm^{-1} of amide I-II and lower intensity of the 1690 cm^{-1} shoulder). Several peaks associated to carbohydrates are also relevant, for example 763 cm^{-1} related to pyranose compounds is modeled by PC5. Variety information reveals a certain grouping of Arriba sample that show high PC2 scores

and lower intensity of PC5 in Arriba samples, in agreement with NIRS results. The scores plot colored by variety information is shown in figure 3S.5.

The different geographical provenience drives a differentiation between samples and some general considerations can be extracted from the scores plot (figure 3 b,c). PC2 certainly explains interesting characteristics of Central Africa samples, that show positive scores on PC2. Samples from São Tomé showed more similarities with samples from Gulf of Mexico, Venezuela and Colombia, as attested also by NIRS data shown in the previous paragraph. This confirms that similar climatic and environmental conditions are crucial in determining the chemical composition captured by spectroscopic techniques, as previously reported in literature for cocoa samples (Marseglia, et al, 2017). African samples show higher intensity at 2954 cm^{-1} and 2870 cm^{-1} in the CH_x stretching vibrations (Figure 3 d). Moreover, PC5 and PC6 were relevant to identify features in common between Ecuadorian samples. 87% of Ecuador samples were placed to the left of the left diagonal of the PC6/PC5 plot (figure 3 c). This is due to the ratio between 1280 cm^{-1} (Amide III of β-sheet proteins) and 1320 cm^{-1} or 1440 cm^{-1} that allows to separate samples from Ecuador from other American samples, as shown in figure 3 e. Moreover low values in PC5 reflect low intensities at 673 cm^{-1} and 1600 cm^{-1} (ring breathing modes of polysaccharides) as already noticed for Arriba samples (enlarged spectral regions not shown for brevity reasons).

The ATR-FT-IR spectrum represents the sum of numerous bands of several functional groups, which are contemporarily present in more than one biochemical compound. Beyond the hypothesized interpretation, it should be stressed that an accurate understanding of what peaks and bands drive the scores distribution should by managed carefully to avoid misinterpretation. To univocally associate the relevant spectral regions to specific classes of compounds remains complicated when a whole complex matrix such as food is analyzed. However, the possibility to identify spectral features that precisely, characterize samples from the same origin is an indication that a correlation between geographical origin and vibrational spectra can be modeled.

3.3. ICP-OES elemental characterization of CBS samples

The raw ICP-OES results are shown in Table 4S.1 in supplementary information. The most abundant elements are by far Ca, Mg, K which have a concentration at least one order of magnitude higher compared to all other

311 elements. Among the secondary elements, particularly relevant were Al, Fe and Li (Barker and Rayens 2003).
312 Relevant amounts of lead were revealed in all samples (around 0.3 mg/kg), which is a high value compared with
313 the average content of lead in foods reported in 2007 by the Agency for Toxic Substances and Disease Registry
314 (Abadin H., et al. 2007). All other elements were revealed in concentration lower than 0.2 mg/kg, particularly
315 low concentrations were determined for Ni and Cr. PCA was used to identify major variance directions that can
316 be related to geographical origin. Five samples were identified as very different from the others. They were SB3,
317 SB4 from Brazil, ICAM10 from Congo, FER8 from Uganda and FER13 from Côte d'Yvoire. These samples
318 were excluded as outliers because of their very low K content. Boron, Potassium, Magnesium and Calcium are
319 responsible of the most variance captured by PC1, which resulted not to be particularly correlated to provenience
320 of samples. Aluminum, Chromium, Iron, Sodium and Nickel are particularly relevant for PC2, whereas
321 Cadmium, Cobalt and Molybdenum together with Calcium and Manganese are mostly represented in PC3, as
322 shown in figure 4 d.
323 Examining the PC2/PC3 loadings and scores plot (figure 4 a, b), high levels of Fe and Al resulted to be
324 characteristic for African continent for most of Central Africa Samples, moreover a general deficiency of Ca, K,
325 Mg, Ni, was revealed. Interestingly some similitudes of São Tomé samples with American samples were
326 captured by PC2. Precisely a relatively higher content of Fe, Al, Cu and Ni was revealed for this samples, this
327 trend makes São Tomé samples more like American than to African samples. Moreover, São Tomé samples are
328 characterized by high content of Ba with respect to others. Conversely, Ecuador samples did not show any
329 specific elemental profile.

330 **Figure 4**

331 *3.4 Data fusion to merge chemical information provided by the different analytical techniques*

332 The idea of data fusion is to merge information, provided by different analytical determinations, in one single
333 data set, to enhance the quality of the results. The obtained joined PCA model clearly shows that all the three
334 datasets provide useful information for the final model. It was noticed that the three most represented variables
335 in PC1 were one from MIR-ATR, one from ICP and one from NIRS (figure 5S.1 in supplementary information).
336 The scores plot and the loadings projected on the PC2/PC1 space are shown in figure 5. The grouping of samples

based on the geographical origin was improved by the multi analytical model. Proximity, and hence common features, were appreciated for samples from the same geographical area.

Classification models were calculated to quantify the grouping performances of the joined model compared to the three single models, based on the geographical origin. Even though interesting observations were previously discussed for the three techniques separately, and some correlation between geographical origin and the composition was defined, single technique outputs were not accurate and precise for the recognition of the geographical origin of samples in predictive classification models. In table 1 the most classification figure of merit (sensitivity, specificity, error rate, accuracy, precision) relative to PLS-DA classification models for the geographical discrimination were reported. The classification performances for the samples' classes composed of more than 5 samples were shown. Classification results were higher for the joined model compared to each of the three single models for Central Africa, Ecuador and Gulf of Mexico classes. This experimental evidence was in agreement with literature findings corroborating mid-level or high-level data fusion to increase predictive performance of classification models (Doeswijk, Smilde, Hageman, Westerhuis, Van Eeuwijk, 2011). Single techniques provide null accuracy and precision for most classes, out of Central Africa. Moreover, merging information from the three techniques, the accuracy (correctly classified samples rate) increased.

Table 1

NIRS, MIRS and ICP profiles together deliver sufficiently accurate information to capture the common features of African samples, and to distinguish them from all the others. Unfortunately, the same is not confirmed for the other classes. Low stability emerged during cross validation for Ecuador, Gulf of Mexico and Venezuela classes. Classification results for classes composed of less than 10 samples were not considered statistically valid.

4. Conclusions

Because of the low price and interesting features of CBS, such as the extraordinary similarity to cocoa powder in terms of color, taste and texture, and the potential beneficial effects on human health, research is needed to assist the valorization of this food by-product, and to prevent fraud in cocoa powder market. The present work demonstrates the existence of correlations between the geographical origin and the composition of CBS samples, even though low specificity for the single country or restricted areas emerged. Some information about what samples from the same macro-area have in common was described. The selected techniques provided significant

criteria to distinguish sample classes, such as Central Africa and Ecuador samples with adequate accuracy and precision, however it is very difficult to precisely determine what chemical species drive this separation only using vibrational spectroscopy for chemical composition analysis. Nevertheless, estimates and trends were determined. The geographical traceability of food based on chemical analysis remains complicated and always valid rules are rarely identified. The natural variability of most food materials is huge, climatic conditions and process variables represent an intrinsic limit of this field of study. However, the capability to identify leading variables, common trends and general indications using rapid and simple techniques is an encouraging result in this domain. More sensitive and accurate techniques should be used for an exhaustive investigation. Easy-to-use instrumental analysis still needs the support of heavier analytical strategies for comparison and calibration.

Acknowledgements

The present work has been supported by COVALFOOD "Valorisation of high added-value compounds from cocoa industry by-products as food ingredients and additives" project funded by European Union's Seventh Framework programme for research and innovation under the Marie Skłodowska-Curie grant agreement No 609402 - 2020 researchers: Train to Move (T2M).

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FIGURE CAPTIONS

Figure 1–a) Mean NIR spectrum of all CBS samples (green) and standard deviation limits (blue); b) Scores plot of NIRS data PCA colored in accordance with variety; c, d) Zoom of average spectrum of Arriba samples compared with the mean spectrum calculated considering all other NIR spectra.

Figure 2– a) PC2/PC1 scores plot of NIR spectra of CBS sample colored by geographical origin. b) PC4/PC5/PC6 scores plot of NIR spectra of CBS sample colored by geographical origin. c) Average NIR spectra of CBS from Africa and America as macro-classes (red and green respectively) and mean spectra of São Tomé

and Ecuador groups (light blue and orange respectively); d, e) Zoom on the spectral regions which make Asian samples different from all other CBS samples;

Figure 3– a) ATR-FT-IR average spectrum of all CBS samples (green) and standard deviation limits (blue); b) PC2 scores plot which highlight common behavior of African samples; c) PC5/PC6 scores plot that allow to highlight characteristic trend for Ecuador samples; d) MIR average spectra of CH_x stretching bands of samples different geographical origin; e) MIR average spectra of Ecuador sample compared with Americans in the spectral region where Ecuador samples show distinct characteristics with respect to American samples.

Figure 4– PCA model of ICP-OES data outputs, 2D a) loading and b) scores plots; c) Histogram of mean data for the considered macro-classes (Africa and America) and São Tomé samples that show peculiar feature with respect to others; d) Variance captured per each principal component.

Figure 5– Joined PCA model of NIRS+ICP+MIRS, a) loadings and b) scores plot on PC1 and PC2.

Table 1–Cross Validation outputs of PLS-Discriminant Analysis classification models for geographical origin discrimination: a) Joined classification model with 5 LVs, classification performances in leave-one origin-out cross validation; b) NIRS PLS-DA model with 4 LVs classification performances in leave-one origin-out cross validation; c) MIRS PLS-DA model with 4 LVs classification performances in leave-one origin-out cross validation; d) ICP-OES PLS-DA model with 3 LVs classification performances in leave-one origin-out cross validation.

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Table1

Class	Technique	N	Sensitivity (true positive ratio)	Specificity (true negative ratio)	Accuracy	Precision
Central Africa	a) **Joined**	22	0.68	0.92	0.84	0.79
	b) NIRS	19	0.68	0.86	0.81	0.00
	c) MIRS	19	0.32	0.70	0.59	0.29
	d) ICP-OES	19	0.50	0.83	0.75	0.50
Gulf of Mexico	a) **Joined**	9	0.33	0.82	0.76	0.21
	b) NIRS	9	0.00	0.87	0.75	0.00
	c) MIRS	9	0.00	0.82	0.71	0.00
	d) ICP-OES	9	0.00	0.87	0.75	0.00
São Tomé	a) **Joined**	6	0.33	0.95	0.9	0.40
	b) NIRS	6	0.00	0.91	0.86	0.00
	c) MIRS	6	0.00	0.90	0.83	0.00
	d) ICP-OES	6	0.00	0.92	0.86	0.00
Venezuela	a) **Joined**	10	0.10	0.87	0.76	0.11
	b) NIRS	12	0.00	0.89	0.74	0.00
	c) MIRS	4	0.00	0.89	0.84	0.00
	d) ICP-OES	12	0.00	0.85	0.69	0.00
Ecuador	a) **Joined**	10	0.00	0.87	0.74	0.00
	b) NIRS	10	0.00	0.85	0.72	0.00
	c) MIRS	10	0.00	0.81	0.70	0.00
	d) ICP-OES	10	0.00	0.87	0.73	0.00
Indonesia	a) **Joined**	1	0.00	0.96	0.94	0.00
	b) NIRS	1	0.00	1.00	0.99	0.00
	c) MIRS	1	0.00	1.00	0.99	0.00
	d) ICP-OES	1	0.00	0.98	0.97	0.00
Mexico	a) **Joined**	2	0.00	0.99	0.96	0.00
	b) NIRS	2	0.00	0.96	0.93	0.00
	c) MIRS	2	0.00	0.94	0.91	0.00
	d) ICP-OES	2	0.00	0.97	0.94	0.00
Peru	a) **Joined**	4	0.00	0.89	0.84	0.00
	b) NIRS	4	0.00	0.92	0.87	0.00
	c) MIRS	4	0.00	0.97	0.91	0.00
	d) ICP-OES	4	0.00	0.87	0.81	0.00
Colombia	a) **Joined**	4	0.00	0.95	0.90	0.00
	b) NIRS	4	0.00	0.92	0.87	0.00
	c) MIRS	12	0.00	0.93	0.77	0.00
	d) ICP-OES	4	0.00	0.85	0.80	0.00

Table 1: Cross Validation outputs of PLS-Discriminant Analysis classification models for geographical origin discrimination: a) Joined classification model with 5 LVs, classification performances in leave-one origin-out cross validation; b) NIRS PLS-DA model with 4 LVs classification performances in leave-one origin-out cross validation; c) MIRS PLS-DA model with 4 LVs classification performances in leave-one origin-out cross validation; d) ICP-OES PLS-DA model with 3 LVs classification performances in leave-one origin-out cross validation.

Figure 1

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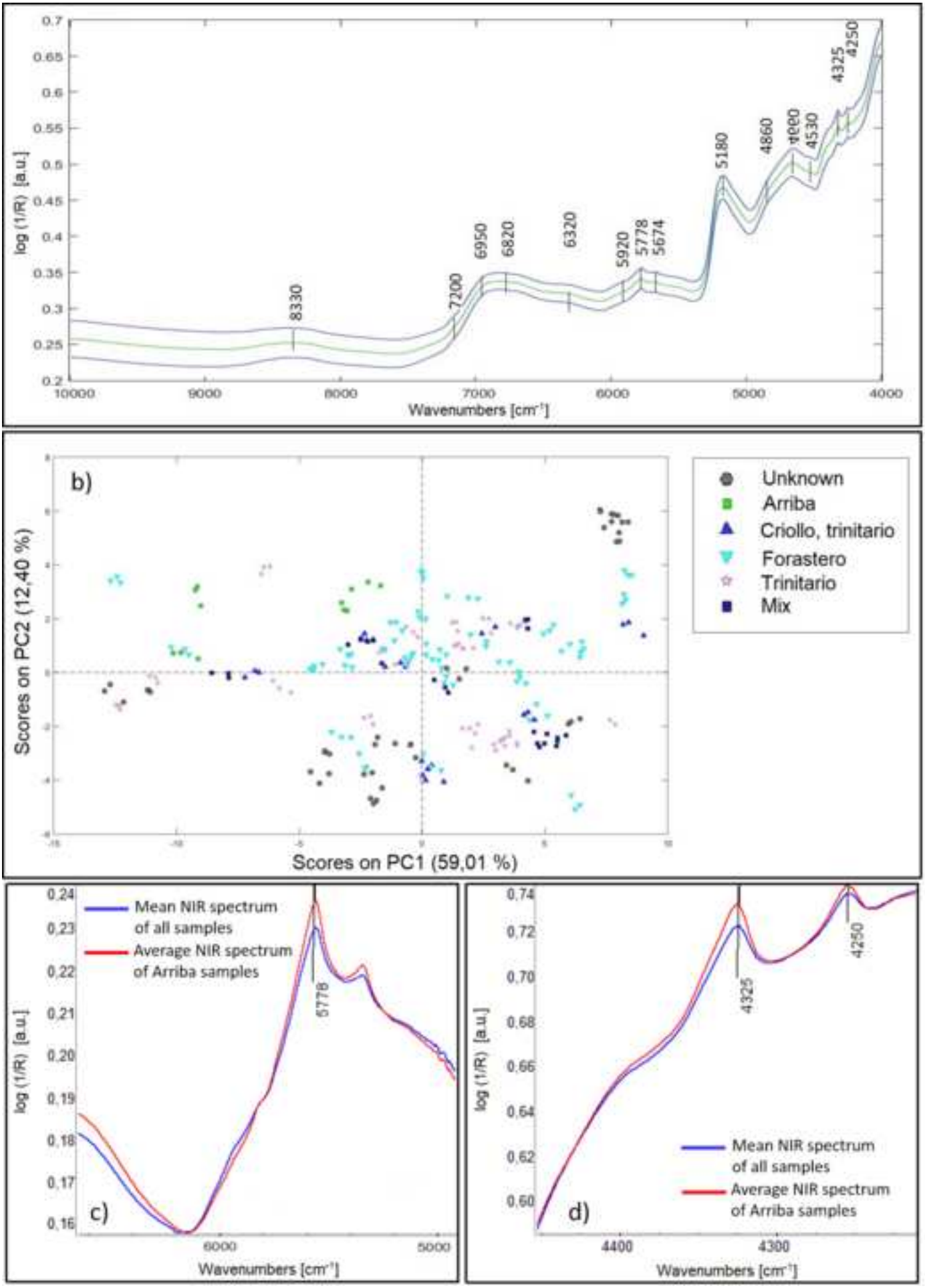

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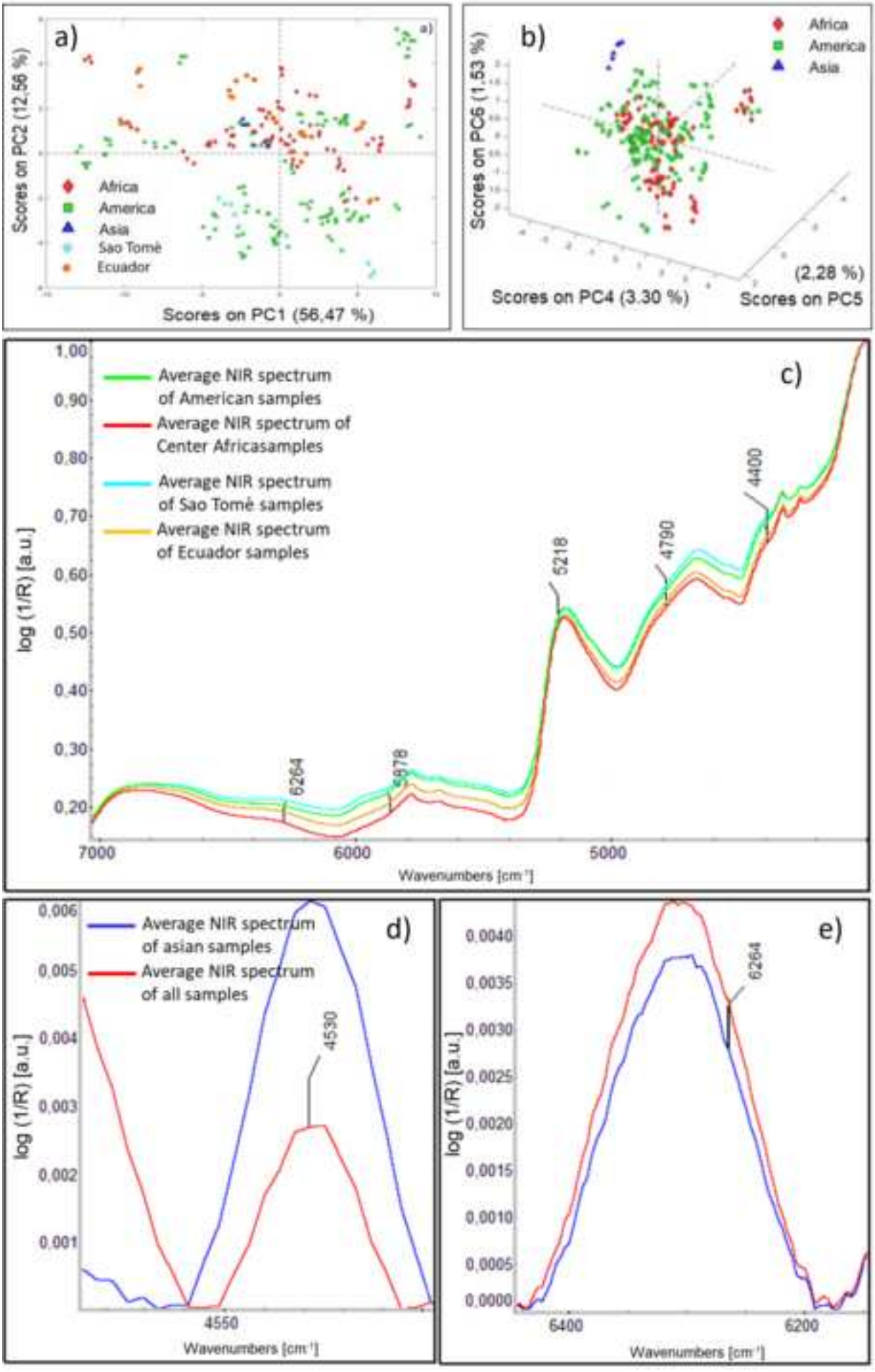

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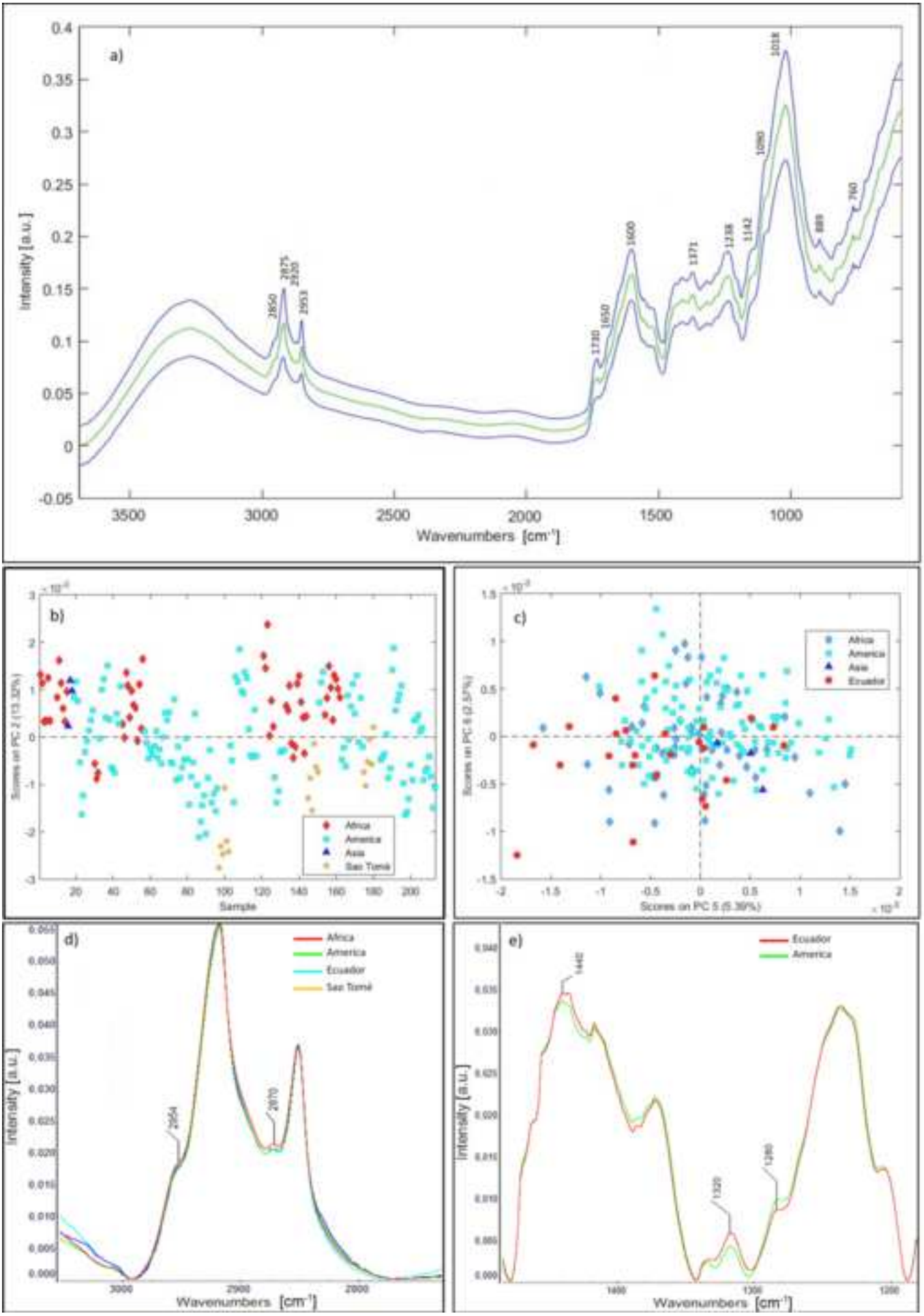

Figure 4

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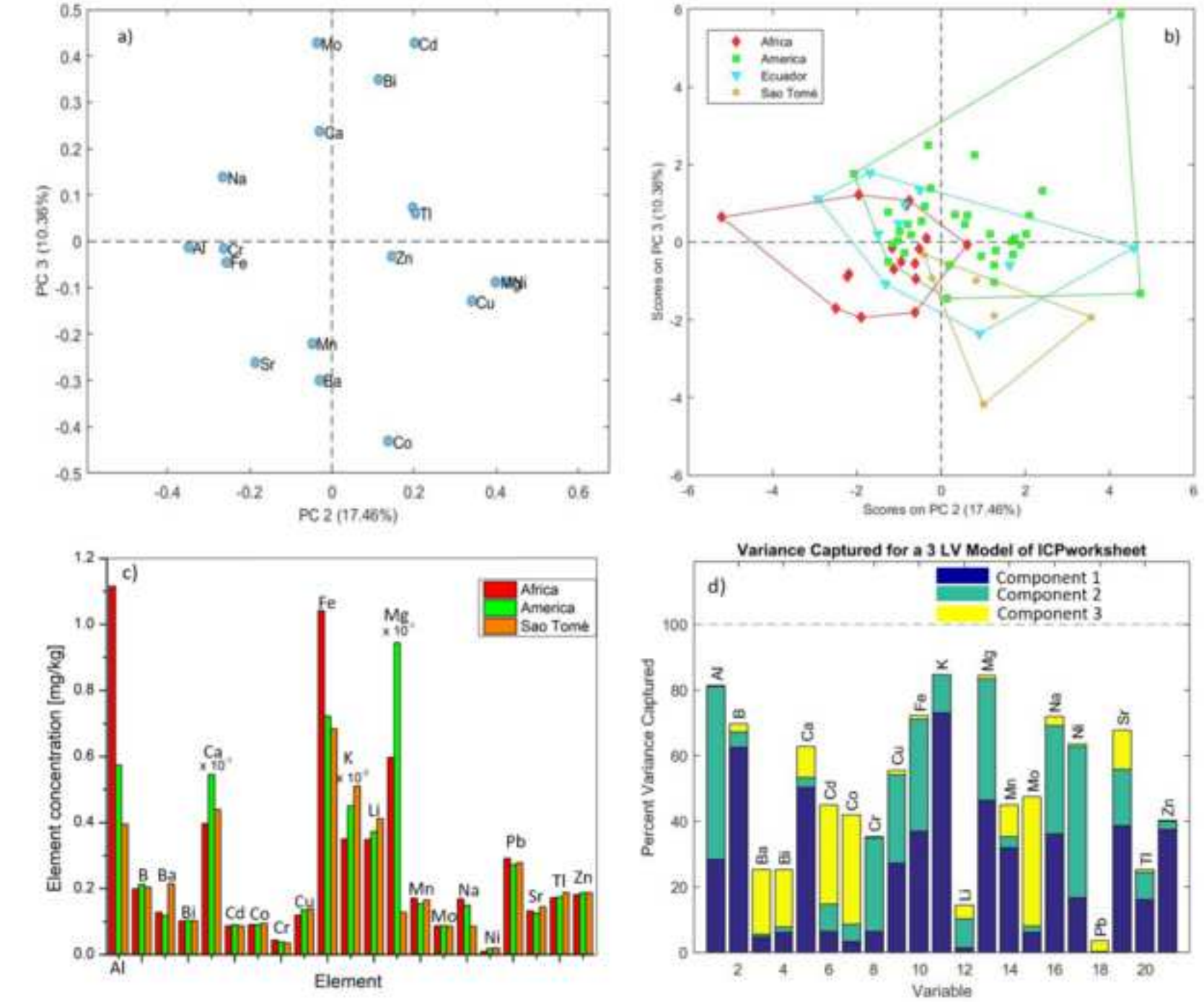

Figure 5

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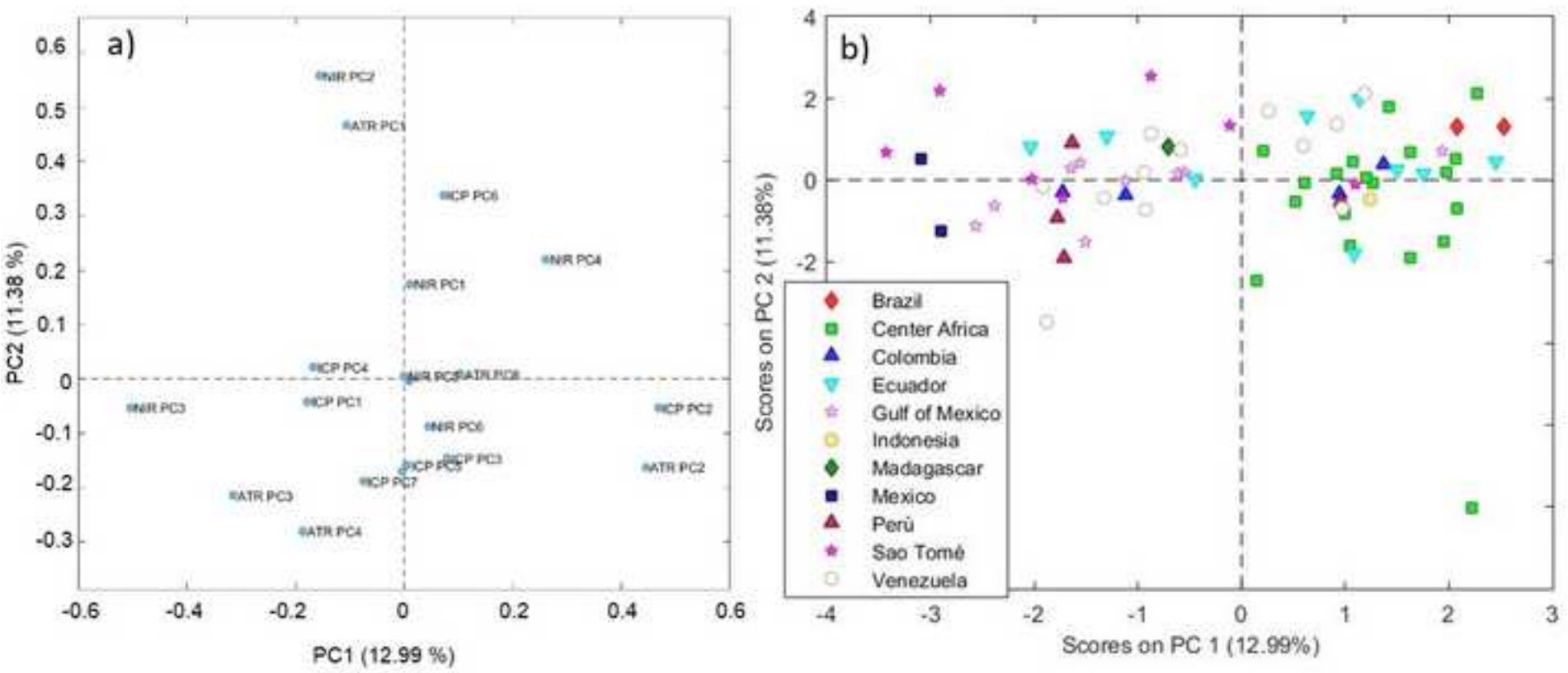

Supplementary Material

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