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NMR based geographical characterization of roasted coffee

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ABSTRACT

The increasing attention for food quality and safety led to develop several analytical techniques suitable to address these demands. Coffee has been already demonstrated to represent a worldwide appreciated beverage and its high economical value could induce frauds or adulteration practices involving both compositional and geographical aspects. In the last years, metabolic profiling revealed to be suitable to face the quality determination of food matrices and NMR confirmed its potentiality in metabolites characterization. The present study reports the capability of NMR spectroscopy to investigate the metabolite content of roasted *Coffea arabica* samples from the three main production areas, America, Africa and Asia. OPLS-DA models performed on ^{1}H NMR data led to a clear separation of samples according to their origin: fatty acids, chlorogenic acids and lactate and finally acetate and trigonelline resulted the main compounds characterizing the American, African and Asian samples respectively. The analytical approach here presented confirmed the potentiality of the joined NMR analysis and statistical treatments in quality determination of food matrices.

1. Introduction

Food quality requirements are strongly related to the origin of products especially for those belonging to specific areas, awarded with PDO (Protected Denomination of Origin) or PGI (Protected Geographical Indication) marks. Their higher economical values are justified by unique quality characteristics, making them particularly appreciated worldwide. The increasing consumer awareness of food quality and safety required the development of new analytical techniques for authentication of highly quoted food commodities. In this contest NMR spectroscopy has already demonstrated its capability: several food matrices have been successfully investigated [1], thus enforcing the pivotal role played by this technique in quality assessment procedures.

Valuable coffee blends, endowed with specific organoleptic characteristics, are made by mixing coffee from different geographical origins, to fulfil both market and consumer requests. This procedure, increases quality and commercial value of the final product, thus enabling frauds and adulteration practices involving both compositional and geographical aspects. In this respect the possibility to verify the geographical origin of roasted coffee would be, as well as in PDO food products, very remarkable.

The specific geographic areas of Central and South America, Africa and the Middle-Southeast Asia, between "Tropic of Cancer" and "Tropic of Capricorn", constitute the so-called "coffee bean belt", where all commercially grown coffee are produced. The rich soil, moist tropical climate, high altitudes and perfect temperatures of these regions affect amiably the flavor of the beans leading to highest quality coffee. There is an increasing interest of consumers for high quality and mono-origin food products: the feasibility of this determination is not straightforward even thought for complex food matrices like tomato sauce [2] and honey [3] has been successfully performed. Nowadays the geographical origin of coffee is evaluated by the "in cup taste testers" whose subjective response is often not unanimous. Conversely, an objective analytical tool allowing the chemical composition determination and the geographical evaluation will result particularly appealing and reliable. The main problem from the chemical point of view is the complexity of the metabolic content: roasted coffee consists of different classes of chemical compounds [4], responsible for organoleptic and qualitative properties, whose different concentration is depending upon the species. Among different classes of compounds, amino acids, aromatic acids and esters, amides, sugars, proteins, lipids are usually observed, but also heterocyclic compounds derived from the Maillard reaction occurred during the roasting process. Among more than eighty described *Coffea* L. species, only two, namely *C. arabica* and *C. canephora* (known as Robusta), are worldwide cultivated, with a production of green beans larger than eight millions of tons in 2009 [5]. *C. arabica* shows higher consumer acceptance due to flagrancy, sweetness and fruity taste against less aromatic, bitter and edged taste of *Robusta* specie, thus imposing higher market prices. Balanced mixtures from different geographical origins of the same species, could further tune and modulate the final taste and organoleptic perceptions.

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Several studies have been performed on roasted coffee in the past years, focused on molecular characterization: volatile compounds formed during roasting process were analyzed by GC–MS [6], lactones derived from chlorogenic acids by LC–MS and HPLC [7,8], as well as di-terpenes by High Speed Counter Current Chromatography (HSCCC) [9] and amino acids by GC [10]. ^{1}H NMR spectroscopic studies on soluble coffee composition have been performed recently [11,12], while the evolution of significant molecules during roasting process in both Robusta and Arabica coffee species was followed by HR-MAS NMR [13]. Finally, a combined LC–MS/MS and NMR study on bitter compounds determination in roasted coffee was presented [14]. To the best of our knowledge, only very few studies on the geographical determination of roasted coffee are present in the literature and none of them was involving the NMR techniques. In one study [15], elemental analysis performed using ICPAES (inductively coupled plasma atomic emission spectroscopy) and different statistical pattern recognition methods were employed to investigate the feasibility of the method to discriminate different coffee grown sites, like south and central America, Indonesia and east Africa. The authors reported 70–86% of successful classification by using eleven elements. In another study, based on GC–TOF-MS determinations, the geographical origins of coffee from the three main producers, America, Africa and Asia [16] were investigated, analyzing and classifying authentic and non-authentic samples collections. The last work, based on analysis of trace elements by XRF (X-ray fluorescence) spectrometer, reported the successful origin classification for samples from six different production areas using only six elements [17]. These methods relayed on determination of volatile/semi-volatile compounds or trace elements and implied complex sample treatment and preparation. Other methods, like those involving mixture analysis, performed without sample treatment, could represent a valid alternative, allowing determination of chemical compounds not identifiable with the GC or MS based methods. NMR was already proposed as a good candidate for this type of analysis without any purification or derivatization process. Following our previous studies, this analytical technique could be applied to *C. arabica* roasted coffee samples to investigate the geographical origin. In several studies, it has been already demonstrated that the geographical origin is a sort of a strong marker inserted into the food product by the nature that could be detected by analyzing the metabolic content. In late 1999, the first ^{1}H NMR studies reported the coffee composition for both Arabica and Robusta species [18] followed only by the more recent papers of Ferreira [19] and Ciampa [13], where the authors essentially reconfirmed the previous assignment using ^{1}H NMR and HR-MAS NMR spectroscopy respectively. In the present work, NMR analysis combined with chemometrics were used for assessing the metabolite content of water extracts of *C. arabica* roasted coffee samples, thus simultaneously monitoring different classes of compounds to address the geographical origin determination.

2. Materials and methods

2.1. Samples

Roasted coffee samples (*C. arabica*) of certified geographical origins from three production areas (America, Africa and Asia) were bought directly from different producers. Differences in roasting conditions and powder size were present among samples.

2.2. NMR samples preparation

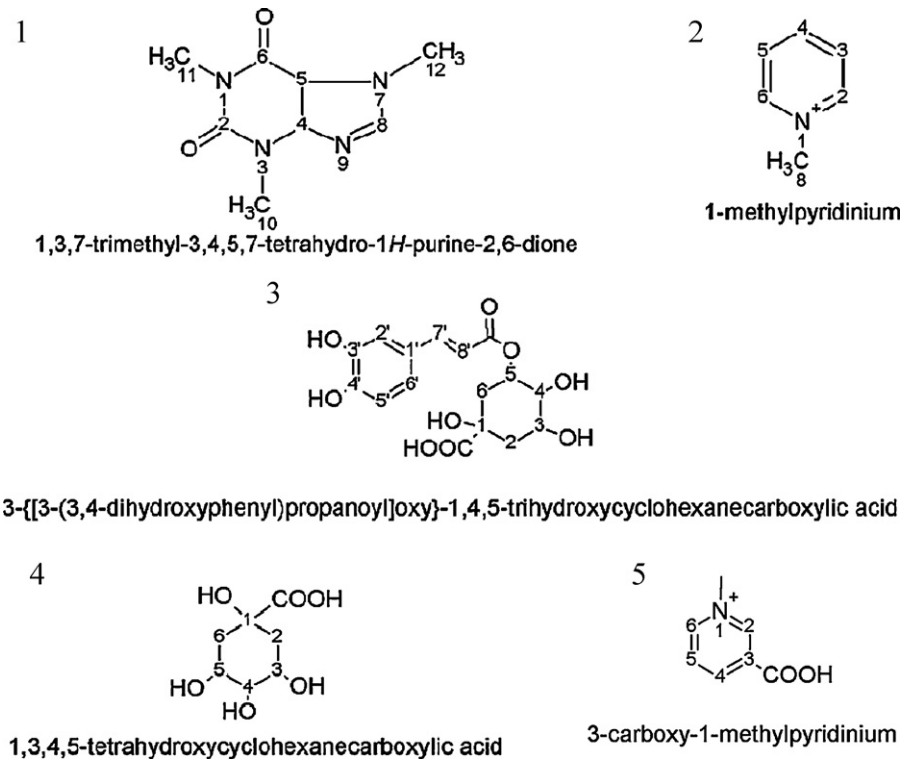
A total of 40 samples of roasted coffee were analyzed in double; the origins of samples were summarized in Table 1. 150 mg of ground roasted coffee were extracted at room temperature with 750 μL of deuterated water buffered at pH = 7. Solutions were centrifuged at 13,400 rpm for 20 min and a final volume of 400 μL were used for NMR analysis, with external TSP referencing capillary. ^{1}H NMR spectra were recorded on Bruker DMX 500 spectrometer (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany) operating at 11.7 T and equipped with a 5 mm reverse probe with z-gradient. All spectra were recorded at 300 K, with 6900 spectral width over 32 K data points. Solvent suppression was achieved by applying a water presaturation with low power radiofrequency irradiation. An exponential function with LB = 0.3 was applied before Fourier transformation; phase and baseline were adjusted with TOPSPIN software (Bruker Biospin). Spectra were aligned for bucket integration on the formic signal at 8.424 ppm. We have chosen the widely accepted binning method to conceal any peak position variation enabling more robust models. Spectra were therefore reduced to integrated regions (buckets) of equal width (0.04 ppm) from 0.15 to 10.00 ppm and buckets were normalized to the total integrals; the residual water signal between 4.850 and 4.751 ppm was set to zero constant value with ACD/NMR software (ACD Labs, version 11, Toronto, Ontario, Canada). Notwithstanding buffered solutions were used some shifts of signals were observed and for this reason some buckets needed manual adjustment in size.

Table 1
List of roasted coffee (*C. arabica*) samples analyzed.

Sample	Continent	Country
1	Africa	Cape Verde
2	Africa	Ethiopia
3	Africa	Ethiopia
4	Africa	Ethiopia
5	Africa	Ethiopia
6	Africa	Kenya
7	Africa	Kenya
8	Africa	Kenya
9	Africa	Malawi
10	Africa	Saint Helena
11	Africa	Tanzania
12	America	Brazil
13	America	Brazil
14	America	Brazil
15	America	Brazil
16	America	Brazil
17	America	Colombia
18	America	Colombia
19	America	Costa Rica
20	America	Costa Rica
21	America	El Salvador/Brazil
22	America	Galapagos
23	America	Guatemala
24	America	Hawaii
25	America	Honduras
26	America	Jamaica
27	America	Nicaragua
28	America	Nicaragua/Guatemala
29	America	Peru
30	America	Peru
31	America	Mix South America
32	Asia	India
33	Asia	India
34	Asia	India
35	Asia	Indonesia
36	Asia	Indonesia
37	Asia	Indonesia (Sumatra)
38	Asia	Indonesia (Sumatra)
39	Asia	Nepal
40	Asia	Yemen

2.3. Statistical methods

Principal Component Analysis (PCA) and Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) were



Scheme 1. Molecules IUPAC names and numbering: **1**—caffeine; **2**—N-methyl pyridine; **3**—5-O-caffeoyl-quinic acid; **4**—quinic acid; **5**—trigonelline.

performed with mean centering as data pretreatment. OPLS is a multivariate projection method that extracts linear relationships from two data blocks X and Y by removing the so-called structured noise [20,21]. When structured noise is present in a data set X, traditional projection techniques, such as PLS regression, can produce systematic variation of X having a component uncorrelated to Y. OPLS removes this structured noise by decomposing the systematic variation in the X block into two model parts: the predictive or parallel part, modeling the joint X–Y correlated variation, and the non-predictive or orthogonal part, not related to Y. OPLS can be used to perform Discriminant Analysis (DA) by introducing suitable dummy variables. The main benefit using the OPLS-DA technique is the reduced model complexity [22]. For example, in the case of N classes, the dimension of the predictive space is $N-1$ and therefore the model can be explained using only $N-1$ components. The number of latent components can be determined by cross-validation techniques, and in this study, we used 7-fold cross-validation. In addition, a permutation test on the Y block was performed to safely overcome casualty or overfitting into models and both sensitivity and specificity were calculated. When the dimension of the joint correlated space is one, useful visualization tools, such as, for example, the S-plot, can be used to highlight the role played by the variables in the model [23]. The D-optimal onion design [24] was applied to select from the candidate set both training and test sets, by using MODDE 8.0 (Umetrics, Umea, Sweden). Statistical data analysis was performed with the SIMCA-P+ 12 (Umetrics, Umea, Sweden) software.

3. Results and discussion

3.1. Determination of metabolite content of roasted coffee samples by NMR

The ^{1}H spectrum of water extracted solution of coffee showed a quite complicated spectral pattern of resonances. The chemical structures of few selected compounds are summarized in Scheme 1. The aliphatic region (Fig. 1A) was dominated by the resonances of caffeine (N-methyl singlets), acetate and trigonelline (N-methyl singlet), while the aromatic region (Fig. 1B) accounted mainly for trigonelline (aromatic moiety) and formic acid. In addition, signals due to chlorogenic acids, assigned as caffeoyl-quinic acids (CQAs), were also clearly visible. The comparison with reference molecules and the use of hetero-nuclear bidimensional experiments based on both direct (HSQC) and long range (HMBC) ^{1}H–^{13}C coupling constants, as well as homo-nuclear experiments (TOCSY) led to confirm the spin system assignments. Further inspection of the aliphatic region revealed the presence of several compounds: organic acids like lactate (1.303 ppm), acetate (1.887 ppm), citrate (2.568 and 2.712 ppm), malate (2.385 and 2.657 ppm) and the complete spin system of free quinic acid at 1.840, 1.925, 2.034, 3.517, 3.986 and 4.107 ppm were recognized. Broad signals of coffee lipids were also observed: in particular, based on previous assignments [25,26] and on our analysis of organic extracts of roasted coffee (data not shown) signals at 0.872 and 1.272 ppm could be referred to methyl and methylene protons of fatty acids chains respectively. The anomeric signal of sucrose was identified at 5.374 ppm; its intensity was varying within all samples but without any correlation with the geographical origin. This sucrose variation is most likely due to both Maillard reactions and melanoidins formation that took place during roasting processes [27], differently carried out by manufacturers for all the analyzed samples. Myo-inositol moiety was also detected (3.245, 3.501, 3.592 and 4.028 ppm). N-methyl resonances for caffeine residue were found at 3.185, 3.357 and 3.795 ppm while trigonelline was identified at 4.384 ppm. In the aromatic region, in addition to trigonelline resonances at 8.022, 8.777, 8.792 and 9.060 ppm, N-methyl pyridine resonances were identified, with appropriate signals at 7.998, 8.483, 8.732 ppm and aldehydic HMF signal at 9.380 ppm. Particular considerations must be accounted for caffeoyl-feruloyl-quinic esters, commonly named chlorogenic acids: as previously pointed out by other authors [8,28] chlorogenic acids are mainly constituted by caffeoyl-quinic acids, mainly 5-O-caffeoyl-quinic, di-caffeoylquinic acids and feruloyl

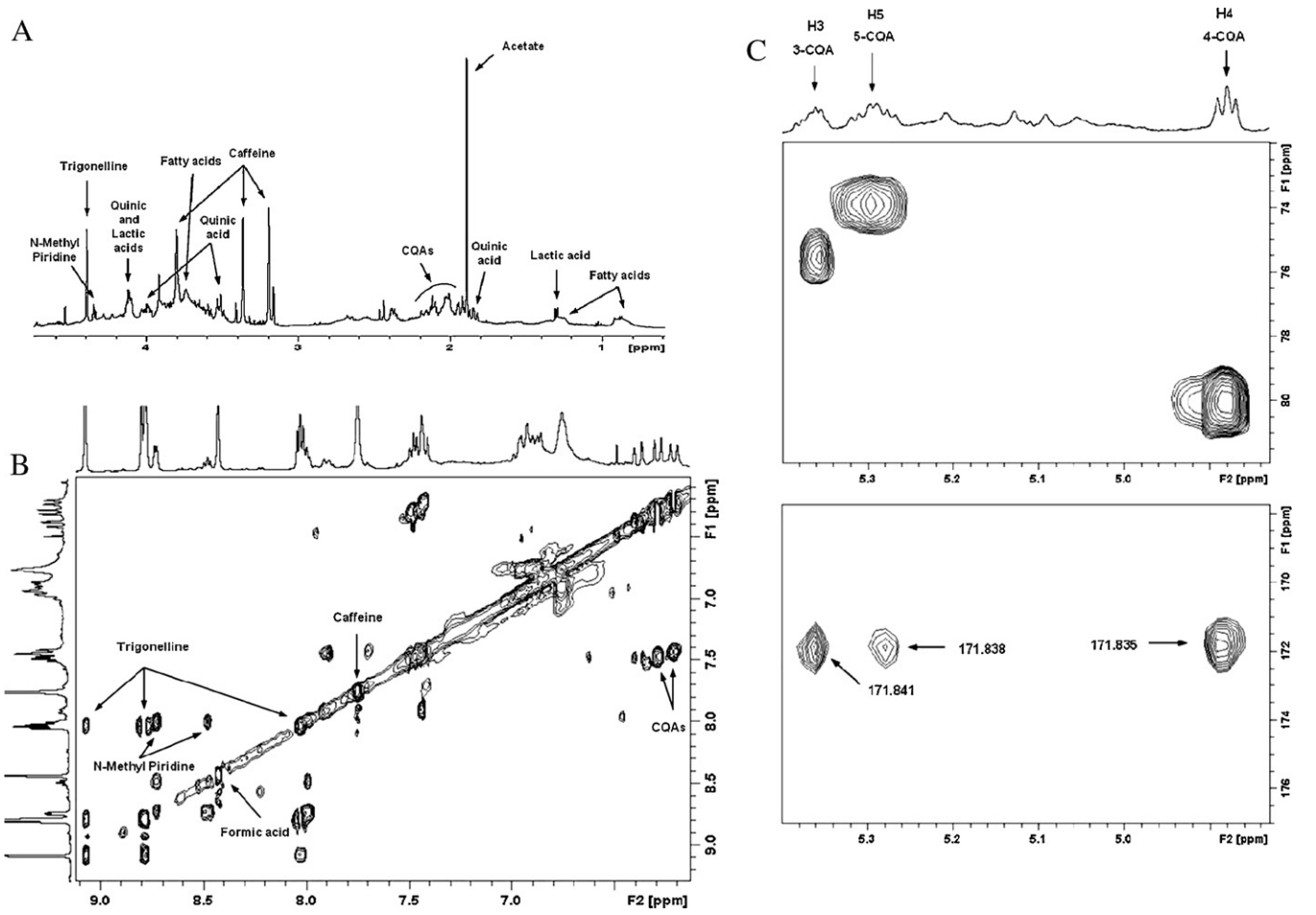


Fig. 1. (A) Aliphatic region of ^{1}H NMR spectrum of roasted coffee (*C. arabica*) from central America dissolved in water. Principal spin system assignments are indicated by arrows. (B) Expansion of TOCSY spectrum corresponding to the aromatic region with principal spin system assignments. (C) Top trace, expansion of HSQC spectrum indicating the direct ^{1}H/^{13}C correlations for 3-CQA, 4-CQA and 5-CQA; bottom trace, expansion of carboxylic region of HMBC spectrum indicating the long range correlations for 3-CQA, 4-CQA and 5-CQA. ^{13}C chemical shifts are indicated by arrows.

acids. In addition, the corresponding lactones, occurred during roasting process, could be present as well. By comparing our spectra with previously published NMR data, we did not confirmed the presence of lactones, most likely due to different roasting conditions and to our milder extraction procedures, performed at room temperature. As already pointed out [28,29], roasting process performed at high temperature enables isomerisation and degradation of chlorogenic acids. In our spectra different signals for the unsaturated moiety of caffeoyl derivatives in the region 6.0–6.5 ppm and 7.0–7.5 ppm were detected: at least two almost equally populated isomers were evaluated. The two spin systems were assigned by the use of TOCSY experiment (Fig. 1B), thus indicating 6.283 and 7.474 ppm and, 6.206 and 7.420 ppm, whose carbon resonances obtained from HSQC spectra were at 117.38 and 148.95 ppm for the first spin system while, 117.01 and 148.55 ppm for the second one. Analysis of HSQC spectra (Fig. 1C) revealed the presence of specific ^{1}H–^{13}C correlations due to chlorogenic isomers and in particular the concomitant presence of 5-CQA, 4-CQA and 3-CQA were detected: their specific cross peaks were observed at 5.287 and 74.03 ppm, 4.876 and 80.08 ppm, 5.351 and 74.79 ppm respectively. Our data was validated on the basis of recent NMR investigations of organic extracts of green coffee beans [30]. In this study the authors reported, for the first time, the presence of three different caffeoylquinic esters in mixture. Conversely to other authors [13,19], we did not observed the presence of acrylamide, pyrazines, amino acids (lysine, arginine) or catecol, most likely due to our mild extraction method and experimental conditions. On the contrary, we detected other few spin systems, still unassigned.

3.2. Statistical analysis of ^{1}H NMR data

In order to identify possible existing relations among samples based on their metabolites content, monodimensional ^{1}H NMR spectra were analyzed by PCA. This model resulted explaining 81.3% of the total variability for the first 4 PCs; the corresponding score scatter plot showed a samples separation according to the geographical origin. A supervised discriminant analysis such as OPLS-DA, was applied for a better data analysis and interpretation; this algorithm could effectively deal with large variations orthogonal to the differences of interest, allowing an enhanced separation of groups and a clearer interpretation of differences among groups. The resulting model highlighted the good separation of samples according to their geographical origin, with the overall goodness of fit (R^2Y) of 81.5% and an overall cross validation coefficient (Q^2Y) of 69.7% (Fig. 2A). To check whether this differentiation could have occurred by chance, Y scrambling validation on the corresponding PLS-DA model with $R^2Y = 81.5\%$ and $Q^2 = 39.8\%$, was performed with 200 rounds of random permutation of the Y variable (data not shown). The substantial decrease of both parameters Q^2 and R^2 (vertical axis interception point of the Q^2 and R^2 regression line at −1.62 and 0.26 respectively) enforced the statistical validity of the obtained model. The corresponding loading plot of Fig. 2B, indicated

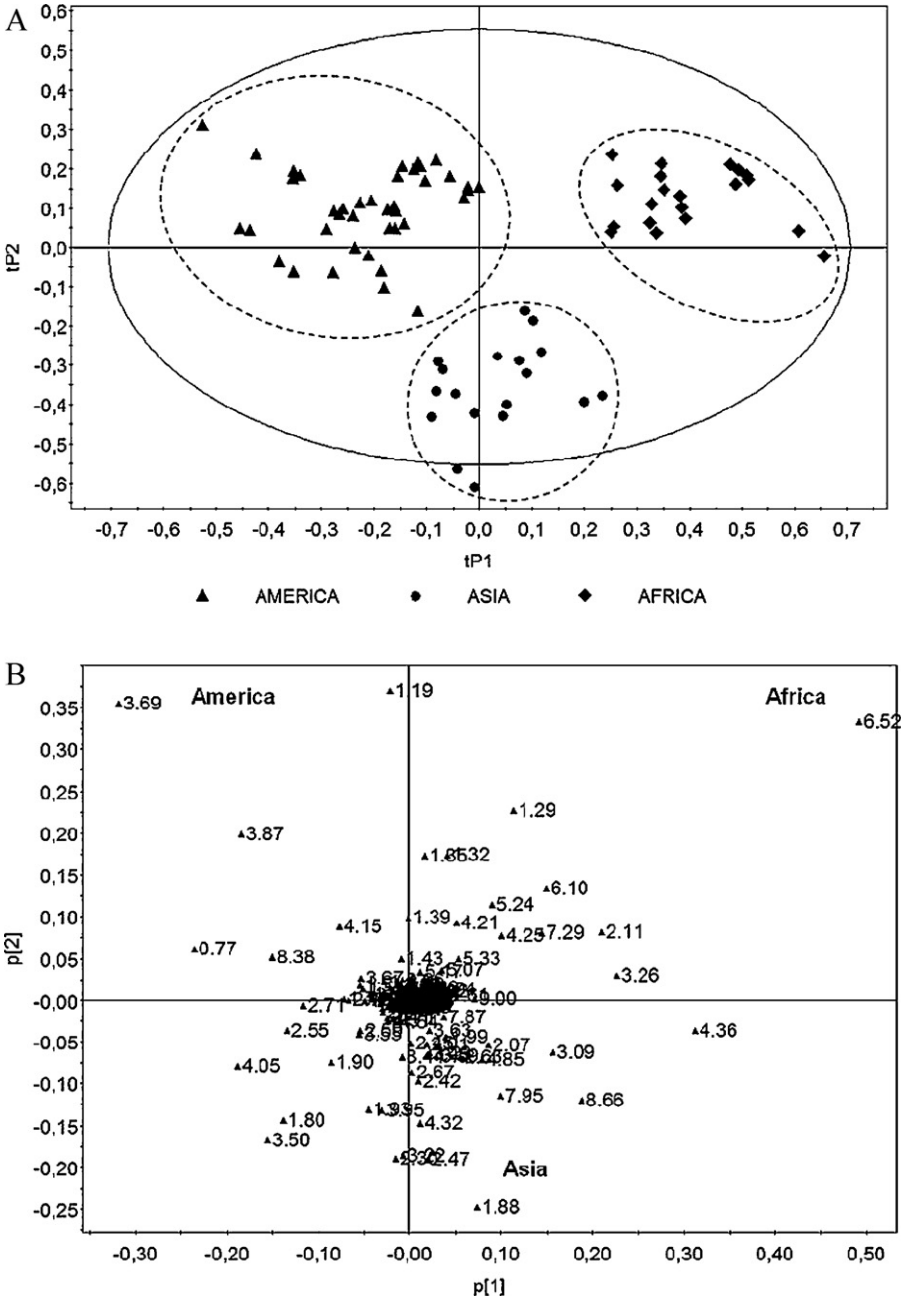


Fig. 2. (A) and (B) Score and loading plots of OPLS-DA performed by considering all roasted coffee samples: filled triangles, dots and diamonds represents American, Asian and African roasted coffee samples respectively. $R^2Y = 81.5\%$ and $Q^2 = 69.7\%$.

chlorogenic acids (buckets at 2.11, 6.52 and 6.10 ppm) and lactate (bucket at 1.29 ppm) as responsible for African differentiation, acetate (bucket at 1.88 ppm) and trigonelline (buckets at 7.95 and 8.66 ppm) for Asian samples and finally methyl and methylene protons of fatty acids chains (buckets at 0.77 and 1.19 ppm), unknown compound (bucket at 3.87 ppm) and bucket at 3.69 ppm for American. The proton resonances included into this latter bucket, needed particular consideration; they are represented by caffeine (N-methyl12), chlorogenic acids (H4) and fatty acid chains. We could safely exclude the contribution from caffeine and chlorogenic acids due to lack of other buckets belonging the same compound, not resulted as discriminant in the loading plot, thus indicating fatty acids as the main contributing compounds to bucket at 3.69 ppm, confirmed by other buckets at 0.77 and 1.19 ppm. A further model validation could be performed by applying D-Optimal onion design to select balanced training and test sets from PCA scores of the 40 candidate samples. These sets were represented by 28 and 12 samples for training and test set respectively. The training set was used to build an OPLS-DA model with three classes (American, Asian and African samples): this model resulted with the overall goodness of fit $R^2Y = 73.8\%$ and $Q^2Y = 52\%$. Misclassification table (data not shown) performed on the candidate set revealed that only 1 over 40 samples of the test set resulted "misclassified", thus confirming the model goodness.

For a better evaluation of signals affecting classes differentiation, two model classes OPLS-DA were performed. Model obtained by considering African and American samples resulted with the overall goodness of fit (R^2Y) of 86.7% and an overall cross validation coefficient (Q^2Y) of 81%. The relative contribution of bins (buckets) in clustering American and African roasted coffee samples

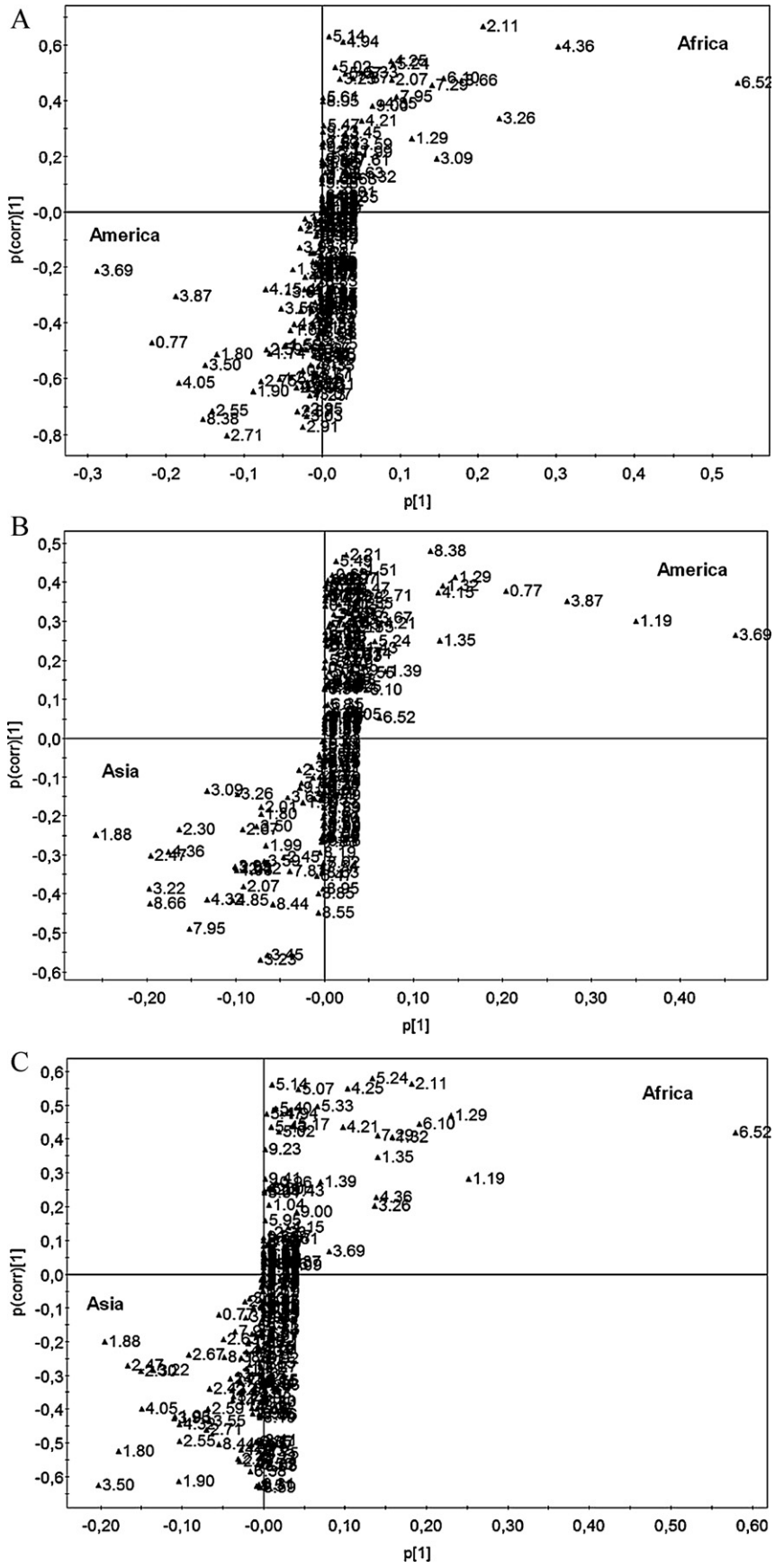


Fig. 3. (A–C) S-plots of two classes OPLS-DA models. African and American; American and Asian; African and Asian respectively.

Table 2
Sensitivity and specificity values for OPLS-DA models.

	Sensitivity %	Specificity %
Africa	90	100
America	97.5	100
Asia	88.89	100
Africa	100	100
America	97.50	100
America	90	100
Asia	94.44	100
Africa	100	100
Asia	100	100

could be easily represented with the S-plot of Fig. 3A. Chlorogenic acids (bucket at 2.11 and 6.52 ppm), trigonelline (bucket at 4.36 ppm), lactate (bucket at 1.29 ppm) and caffeine (buckets at 3.09 and 3.26 ppm) were responsible for African samples while fatty acids (bucket at 0.77 and 3.69 ppm) and unknown compounds (buckets at 3.87 ppm) were characterizing American roasted coffees.

Model obtained with American and Asian samples resulted with the overall goodness of fit (R^2Y) of 81.2% and an overall cross validation coefficient (Q^2Y) of 70.8%. In the corresponding S-plot (Fig. 3B) American roasted coffee were characterized by fatty acids (bucket at 0.77, 1.19 and 3.69 ppm) and by an unknown compound (bucket at 3.87 ppm), while Asian samples were characterized by acetate (bucket at 1.88 ppm), trigonelline (buckets at 7.95 and 8.66 ppm), citrate (bucket at 2.47) and by myo-inositol (bucket at 3.22 ppm). The last model, concerning African and Asian roasted coffee samples resulted with the overall goodness of fit (R^2Y) of 93.7% and an overall cross validation coefficient (Q^2Y) of 85.4%. From the S-plot of this latter model (Fig. 3C) African samples were characterized by chlorogenic acids (buckets at 2.11, 6.10 and 6.52 ppm), lactate (bucket at 1.29 ppm) and methylene protons of fatty acids chains (bucket at 1.19 ppm), while Asian roasted coffee samples were identified by acetate (bucket at 1.88) and quinic acid (buckets at 1.80 and 3.50 ppm). Misclassification table presented in Table 2 summarized the performances of the models with the calculated sensitivity and specificity values.

4. Conclusions

Among different analytical approaches developed to assess the geographical origin determination of foods, NMR spectroscopy has already proven to be a valid tool and the metabolite content analysis revealed to be a properly suited approach to highlight differences/similarities of samples. The main advantage of the NMR technique is based on null sample derivatization: moreover, with a single experiment, detection of several water soluble compounds with high reproducibility and limited experimental time is allowed. Within the framework of this study, the geographical discrimination among American, African and Asian *C. arabica* roasted coffee samples, was achieved by performing statistical analysis of ^{1}H NMR data. Our results indicated that American roasted coffees samples were characterized fatty acids chains, the African samples by chlorogenic acids and lactate, while the Asian ones by acetate and trigonelline.

Even if preliminary, our approach resulted well effective in geographical determination in terms of continents discrimination. Nowadays the geographical origin of coffee is not determined analytically, while NMR spectroscopy combined with chemometrics, resulted to be a powerful and objective tool to trace authenticity of roasted coffee. In our opinion, this statement is outstanding for import–export trade sectors that requires high level of traceability to ensure correct quality and geographical origin.

Our future efforts will be routed towards country discrimination by using a wider collection of certified origin samples.

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