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Multi-analytical approach for the characterization and geographical authentication of black pepper: Volatile analysis, antioxidant activity, and spectroscopic profiling

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ABSTRACT

In this study, a selection of black peppercorns was analyzed to investigate their chemical and functional properties. The samples included peppercorns from the Kampot region in Cambodia (protected geographical indication, PGI; one of which is also certified organic), from Madagascar, and some commercially available products purchased from Italian supermarkets. For comparative purposes, a sample of green peppercorns was also included. Volatile compounds were analyzed by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC–MS) and explored using principal component analysis (PCA). The results highlighted clear differences in aroma profiles among the samples, with specific terpenoid compounds (such as sabinene, 3-carene, β-caryophyllene, and limonene) emerging as key contributors to sample differentiation. Antioxidant activity, evaluated through the DPPH radical scavenging assay, showed significant variability across varieties, with Madagascar pepper exhibiting the highest antioxidant capacity, indicating a higher content of bioactive constituents. Fourier transform infrared (FTIR) spectroscopy, applied as a rapid and non-destructive technique on individual peppercorns, coupled with partial least squares discriminant analysis (PLS-DA), enabled an effective classification of samples according to their geographical origin. The optimized FTIR–PLS-DA model achieved high classification accuracy in both cross-validation and external prediction, demonstrating its robustness for origin authentication. Overall, the results confirm that the integration of complementary analytical techniques provides a characterization of black peppercorns, allowing simultaneous evaluation of aromatic composition, functional quality, and geographical authenticity. This integrated workflow represents a practical and effective strategy for quality control and traceability of high-value spices.

1. Introduction

Black pepper (*Piper nigrum L.*) is one of over 700 species in the Piperaceae family, widely used as a spice worldwide (Parmar et al., 1997). The three most common forms: green, white, and black pepper, derive from the same fruit at different processing stages. Green pepper is produced from unripe berries via freeze-drying or sulfur dioxide treatment to retain a bright green hue, while black pepper is obtained by sun-drying mature green berries for several days, during which polyphenol oxidase reacts with phenolic compounds to darken the pericarp [1]. Famous as "black gold" and the "king of spices," black pepper is prized for its unique flavor and pungency [2], and is mainly harvested in tropical regions [3,4]. Beyond culinary use, it holds significant economic value and has traditional medicinal applications for muscle pain, rheumatism, fever, headaches, digestive, and cardiac ailments, owing to pharmacological activities such as anti-inflammatory, analgesic, anticancer, and antioxidant effects [5,6]. Phytochemical studies have isolated numerous bioactive compounds—alkaloids/amides, propenylphenols, lignans, neolignans, terpenes, steroids, kavapyrones, piperolides, chalcones, dihydrochalcones, flavones and flavanones [7]—and its essential oil exhibits remarkable antibacterial, antifungal and antioxidant properties, with applications in pharmaceuticals, cosmetics and especially the food industry [8]. Among spices, black pepper is preeminent, representing one of the most commonly traded spices [4]. Black pepper's quality hinges on colour, sheen, pungency, and aroma, primarily due to piperine ((2*E*,4*E*)-5-(2*H*-1,3-Benzodioxol-5-yl)-1-(piperidin-1-yl)penta-2,4-dien-1-one) for pungency and essential oils for flavor [9].

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Volatile profiling of black pepper has revealed a composition dominated by terpenes, with compounds such as caryophyllene, 3-carene, limonene, β-pinene, and copaene being particularly abundant [10,11] (Izcara et al., 2022; Lee et al., 2020). Seasonal and anatomical variations influence essential oil yield and antioxidant activity (Feitosa et al., 2024). Additionally, black pepper contains several bioactive compounds, including piperine and related alkaloids, which have demonstrated antitumor properties [12–14].

Food authentication and characterization, especially for high-value spices like black pepper, are critical due to the prevalence of economically motivated adulteration. FT-IR and NIR spectroscopy coupled with chemometrics (PCA, GA-SVM, PLS-DA) have effectively detected adulteration by papaya seeds, chili, and spent pepper material, achieving 100% discrimination of pure pepper and > 96% accuracy on unknown samples (Wilde et al., 2019). Diffuse reflectance mid-IR (DRIFTS) offers rapid, non-destructive screening and, when combined with chemometric models, yields reliable geographical origin and authenticity verification, supporting quality control across import/export, market surveillance, and incident investigation [15].

The present study aims to characterize black pepper samples from different geographical regions using spectroscopic techniques, gas chromatography analysis of the aromatic profile, antioxidant activity, and chemometric modeling. The spectroscopic characterization was carried out using FTIR spectroscopy, while the volatile composition was analyzed through headspace solid-phase microextraction (HS-SPME) followed by gas chromatography coupled with mass spectrometry (GC–MS). The collected data were processed using chemometric methods, including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), to identify specific patterns related to the geographical origin of the samples. The antioxidant activity of hydroalcoholic extracts of black pepper was assessed by measuring the IC50 value using the DPPH assay. Both commercial-quality samples and high-quality samples, such as Kampot black pepper from Cambodia and pepper from Madagascar, were used. Additionally, a green pepper sample was analyzed for comparison. Although the individual analytical techniques employed in this study are well established, the novelty of the present work lies in their integrated and coordinated application within a unified analytical workflow to address the multi-dimensional characterization and geographical authentication of black peppercorns. By combining volatile profiling, infrared spectroscopic fingerprinting, and chemometric modeling, this approach enables the extraction of complementary chemical information from a complex food matrix and provides a practical analytical framework for authenticity assessment and traceability purposes.

2. Materials and methods

2.1. Samples

Six lots of peppercorn samples were collected for analysis. Among these, two are commercially available products purchased from local supermarkets, two are from the Kampot region in Cambodia (they are PGI, one is also organic), and one lot originates from Madagascar. For comparison, a green peppercorn sample was also included in the analysis. Details regarding sample preparation are provided in the relevant sections.

2.2. HS-SPME-GC–MS analysis

The SPME fiber used in this work was coated with polydimethylsiloxane (PDMS), 100 μm thickness (Supelco, Bellefonte, PA, USA). For headspace sampling, peppercorns were ground using a ceramic laboratory mortar to obtain a homogeneous sample. A 200 mg portion of ground pepper was placed into a 4 mL glass vial sealed with a Mininert cap for SPME. The vial was then placed in an oil bath maintained at 40 °C under constant magnetic stirring. HS-SPME extraction was performed by exposing the PDMS fiber to the headspace of the sample for 5 min. After extraction, the fiber was retracted into the needle, removed from the vial, and inserted into the GC injection port for thermal desorption at 280 °C for 5 min. Five replicates were performed for each pepper type. After each analysis, the fiber was conditioned in the GC injection port at 280 °C for 15 min, followed by a blank run to verify the cleanliness of the sorbent.

All analyses were carried out on a Trace 1300 ISQ LT GC–MS system (Thermo Fisher Scientific, Waltham, MA, USA). The GC apparatus was equipped with a split/splitless injector fitted with a dedicated SPME liner. All injections were performed in splitless mode. A J&W HP-5MS capillary column (5% phenyl–polymethylsiloxane, 30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA, USA) was used. Helium 5.5 IP was employed as the carrier gas at a constant flow rate of 1.1 mL/min. The oven temperature program was as follows: initial temperature 40 °C held for 5 min, ramped to 100 °C at 25 °C/min, to 150 °C at 12 °C/min, to 165 °C at 15 °C/min, to 200 °C at 5 °C/min, and finally to 270 °C at 125 °C/min, held for 2 min. Compounds were identified by comparing their mass spectra with those in the NIST 14 library (NIST14. In *Mass Spectral Database*; NIST—National Institute of Standards and Technology: Gaithersburg, MD, USA, 2014) and by calculating their linear retention indices using a C7–C40 n-alkane mixture injected under the same chromatographic conditions.

2.3. DPPH analysis and IC_{50} determination

The antioxidant activity of hydroalcoholic extracts from different *Piper nigrum* L. varieties was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, with IC50 values determined for each variety to enable comparative evaluation. DPPH is found to be a stable free radical and, therefore, used as a reagent in the analysis of antioxidant activity [16]. This method is based on the ability of the antioxidant to reduce the free radical (DPPH•) by decreasing its concentration. This decrease causes the solution to turn from purple to pale yellow, depending on the concentration of the antioxidant present. The change in absorbance, related to the reduction in DPPH, is evaluated by spectrophotometric analysis, with readings at the absorption maximum of 516 nm. From this analysis, different IC_{50} values, defined as the concentration of pepper extract capable of reducing the absorbance of the DPPH solution by 50%, were subsequently calculated. Both commercial samples and fine varieties of pepper, such as Kampot PGI black pepper, were used for this study [17–19].

To proceed with the assay, a stock solution of DPPH was prepared at a concentration of 0.5 mg·mL^{-1} and used for assay execution. Extracts were prepared by grinding peppercorns with a ceramic mortar. The resulting powder was sieved with a 1 mm sieve, and 250 mg of powder was weighed and placed in a 20 mL volumetric flask. 10 mL of extractant mixture, H_20:EtOH (60:40, *v*/v), was then added. This was subjected to magnetic stirring inside a thermostatic circulating water bath at 60 °C for 45 min while protecting the sample from exposure to light. The samples were then centrifuged for 5 min at 1100 rpm, filtered using a 0.22 μm nylon membrane, and stored at −20 °C for 24 h.

To proceed with the assay, a stock solution of DPPH at 0.5 mg·mL^{-1} in methanol was prepared and allowed to stabilize at −20 °C for 48 h before use. A working solution at 0.025 mg·mL^{-1} was then prepared and used for the assay. For each extract, five dilutions were prepared. For each, 4.35 mL of DPPH working solution was taken, and methanol was added to a volume of 5 mL (t_0). The reacted solution, on the other hand, was obtained by adding 150 μL of the extract to 4.35 mL of DPPH working solution and brought to volume in 5 mL volumetric flask with methanol (t_1). Both solutions (t_0 and t_1), before being analyzed in the UV–Vis spectrophotometer (Onda UV-30 Scan UV, Onda, Carpi, MO, Italy), were left to react for one hour in the dark. All measurements were performed in duplicate, as preliminary tests confirmed good repeatability of the absorbance values.

The percentage of decrease in absorbance ($A_{DPPH\%}$) was calculated

according to the equation:

$$A_{DPPH\%} = \frac{t_0 - t_1}{t_0} \times 100 \quad (1)$$

where t_0 is the absorbance value of the working solution of DPPH at time zero (without the addition of the extract solution), while t_1 is the absorbance value recorded after one hour of reaction of the DPPH solution in the presence of the extract.

For each sample, five dilutions were prepared, and from the dose-response curve equations, the IC_{50} values (defined as the concentration of extract required to achieve a 50% reduction in absorbance) and their standard deviations were estimated.

2.4. FTIR analysis

FTIR analysis was conducted using an attenuated total reflectance Fourier-transform infrared (ATR FTIR) spectrometer PerkinElmer Spectrum Two™ (PerkinElmer, Waltham, MA, USA). The spectrometer was equipped with a deuterated triglycine sulfate (DTGS) detector and a PerkinElmer Universal Attenuated Total Reflectance (uATR) accessory fitted with a single-bounce diamond crystal. The background spectrum was collected by exposing the diamond crystal to air after cleaning it with methanol. Each scan was acquired in the spectral range of 4000–500 cm^{-1}. For each variety, around 100 spectra were collected on individual pepper grains, resulting in a total of 602 acquisitions. After each scan, the ATR crystal was cleaned with absorbent paper soaked in methanol before acquiring the next spectrum of the peppercorns.

2.5. Reagents

To perform the DPPH assay, the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich (St. Louis, MO, USA); methanol (≥99.0%, HPLC grade) provided by Carlo Erba Reagenti (Milano, Italy) and Milli-Q water (Millipore, Bedford, MA, USA) were used for the preparation of solutions and samples. To calculate the retention index of molecules analyzed by HS-SPME-GC–MS were used Retention Index Standard aliphatic C7–C40 hydrocarbons dissolved in hexane from Sigma-Aldrich, Saint Louis, MO, USA.

2.6. Chemometric modeling and validation

The data were analyzed using different approaches depending on their nature and the number of available samples. GC–MS data, characterized by a more limited number of observations, were explored using Principal Component Analysis (PCA), while FTIR data, which included a larger dataset, were subjected to classification through Partial Least Squares Discriminant Analysis (PLS-DA).

Exploratory data analysis (EDA) enables the identification of patterns, grouping tendencies, and outliers [20]. Among other methods, Principal Component Analysis (PCA) is one of the most widely used for summarizing high-dimensional data [21–26] (Pearson, 1901;. Mathematically, PCA decomposes the original data matrix $\boldsymbol{X}$ ($N \times M$) into a bilinear form:

$$\boldsymbol{X} = \boldsymbol{T}\boldsymbol{P}^T + \boldsymbol{E} \quad (2)$$

Here, $\boldsymbol{T}$ ($N \times F$) is the scores matrix containing the projections of the samples onto the new reduced subspace, $\boldsymbol{P}$ ($M \times F$) is the loadings matrix that defines the principal component directions, and $\boldsymbol{E}$ ($N \times M$) contains the residuals, or the part of the data not captured by the model. Usually, the number of components F is much smaller than the number of original variables M, allowing for effective data compression with minimal information loss.

Although PCA is an effective tool for uncovering structure in complex datasets and exploring potential groupings or trends, it is an unsupervised method and does not utilize prior information about sample classes. To achieve this, supervised classification techniques, like Partial Least Squares Discriminant Analysis (PLS-DA), are required. PLS-DA is a classifier that extends the traditional PLS regression framework to handle categorical response variables [27]. Briefly, PLS-DA extracts latent variables that maximize the covariance between predictors $\boldsymbol{X}$ and a categorical response matrix $\boldsymbol{Y}$ can be obtained in different ways; in this study, the approach suggested by Perez et al. [28] has been used.

In this work, all the model parameters, including data preprocessing and the optimal number of latent variables, are defined through a 7-fold cross-validation procedure applied to the training set. Since PLS-DA was applied to FTIR spectral data, several preprocessing strategies were tested, including bare mean-centering, Standard Normal Variate (SNV) [29] and derivatives [30]. The model's predictive capability was then evaluated on an independent external test set.

3. Results

The analytical workflow of this study integrates complementary approaches aimed at providing a characterization of black peppercorn samples. The antioxidant activity assessment, performed through the DPPH assay, pertains to the functional quality attributes of the samples, providing information on their bioactive potential. In parallel, volatile profiling was carried out using HS-SPME-GC–MS to evaluate the aromatic composition of the peppercorns. Data were explored by PCA, to visualize underlying patterns among samples, and to highlight potential compositional markers. Finally, FTIR spectral data were subjected to PLS-DA to classify samples according to the geographical origin. This integrated multi-analytical approach allowed the joint investigation of functional, compositional, and origin-related attributes of black peppercorns; a detailed description of the outcome of the analysis can be found in the following paragraphs. The results are discussed following the integrated analytical workflow adopted in this study and functional assessments provide complementary perspectives on the chemical characterization and geographical differentiation of black peppercorns.

3.1. Volatilome composition

The volatile fraction of the peppercorn samples was analyzed using HS-SPME coupled with GC–MS, as detailed in Section 2.2. The resulting chromatographic profiles revealed a general similarity among the samples, though with subtle yet meaningful differences in the relative abundance of specific compounds. A total of 44 volatile compounds were identified, all belonging to the terpenoid family, which includes terpene hydrocarbons as well as oxygenated and aromatic derivatives [10,31] [10].

Of these, 24 compounds (entries #1 to #24) were classified as monoterpenoids. These dominated the volatile profile in all the samples, accounting for approximately 80% in Commercial 1, Commercial 2, and Madagascar black pepper; around 70% in Kampot and Kampot IGP; and 62% in Green Pepper. Notably, six of the seven major compounds (i.e. those exceeding 5% relative abundance in at least one of the five different sample) were monoterpenes: α-pinene (#2), sabinene (#4), β-pinene (#5), β-myrcene (#6), 3-carene (#8), and limonene (#12).

Among the sesquiterpenoids, β-caryophyllene (#31) was the only compound consistently present at high levels across all samples. Its average relative abundance was approximately 12% in Commercial 1, Commercial 2, and Madagascar peppercorns, and around 21% in Kampot, Kampot IGP, and Green Pepper, peaking at 25.25% in the latter.

Each sample exhibited distinctive features. Commercial 2 was marked by a high sabinene (#4) content (16.30%), while Commercial 1 was dominated by 3-carene (#8) at 17.40%. Madagascar black pepper stood out for its elevated limonene (#12) level (16.82%). Kampot and Kampot IGP shared highly similar profiles, both rich in 3-carene (#8) and β-caryophyllene (#31), with the latter reaching 20.70% and 18.04%, respectively. Green pepper was characterized by its exceptionally high β-caryophyllene content (25.25%) and a substantial

amount of sabinene (14.44%).

In comparative terms, β-caryophyllene (#31) emerged as the most representative compound across all samples, particularly in Green Pepper. 3-Carene (#8) was prominent in Kampot and Commercial 1 but less so in Green Pepper. Sabinene (#4) was notably abundant only in Commercial 2 and Green Pepper, while nearly absent elsewhere. Both β-pinene (#5) and α-pinene (#2) were moderately present in all samples, with the highest concentrations in Madagascar pepper. Limonene (#12) was especially prominent in Commercial 1 and Madagascar, whereas β-myrcene (#6) remained consistently low, with the lowest level observed in Green Pepper.

While the relative abundances of the major volatile compounds provide a general overview of the aroma profiles, they are not necessarily sufficient to discriminate between pepper varieties. Although such compounds tend to be present across all samples, their variations may not fully capture the complexity required for accurate classification. In order to properly assess their discriminative potential through supervised modeling, a larger number of samples would have been required. Given the limited dataset available for GC–MS analysis, only exploratory analysis through Principal Component Analysis (PCA) was conducted to investigate underlying patterns and grouping tendencies. Classification was instead applied to the FTIR dataset, which included a sufficient number of observations to build and validate a supervised model.

3.2. Exploratory analysis of aroma profiles

Principal Component Analysis (PCA) was applied to the matrix containing the relative area percentages of volatile compounds across all pepper samples. Even at this exploratory stage, a clear separation among different pepper varieties was observed. Two types of data preprocessing (autoscaling and mean-centering) were tested, and the PCA scores plot (Fig. 1) generated with mean-centering highlighted the best distinct clustering of sample groups, indicating that the aroma profiles varied significantly between them (See Table 1.)

Kampot and Kampot IGP peppers present negative PC1 and positive PC2 values, revealing similar profiles between them and clearly different from the other groups. Madagascar and Commercial 2 peppers showed negative values for both PCs, suggesting a similarity in their volatile profiles. Eventually, Commercial 1 fall at positive values of PC1 and negative of PC2, whereas green pepper present positive scores on both PCs, both indicating a unique volatile profile. In general, very clear grouping tendencies can be recognized. In particular, Kampot samples fall all at negative PC1 and positive PC2, whereas Madagascar and commercial samples present all negative PC2 scores.

The loadings plot (Fig. 2) identified the variables (volatile compounds) that contribute the most to the grouping tendencies of the samples.

Sabinene is one of the main contributors to the positive scores of PC1, and is therefore associated to Commercial 1 and green pepper, whereas 3-Carene dominates negative scores of PC1 and is relevant in Kampot IGP, Kampot, Madagascar, and Commercial 2.

Looking at PC2, it is possible to conclude that β-Caryophyllene (at positive PC2) could be a relevant marker for Kampot, Kampot IGP, and green pepper samples, while limonene, showing negative values onPC2, is associable to Commercial 1 samples.

The identification of these markers is particularly interesting given their known biological activity. Indeed, Sabinene contributes to spiciness and it has antifungal and anti-inflammatory properties [32], limonene shows anti-inflammatory, antioxidant, antitumor, gastroprotective, and immunomodulatory properties [33,34], β-Caryophyllene, has anti-inflammatory, antioxidant, neuroprotective, and analgesic properties [35].

3.3. Determination of IC_{50} and antioxidant activity evaluation

The antioxidant activity of extracts of different pepper varieties was evaluated by the DPPH assay, followed by determination of the IC_{50} value. The latter, as already described in section 2.3, represents the concentration of the extract capable of inhibiting the oxidative capacity of the DPPH radical by 50%.

Table 2 shows the IC_{50} values for each pepper variety analyzed.

IC_{50} values are expressed in terms of dry pepper concentration,

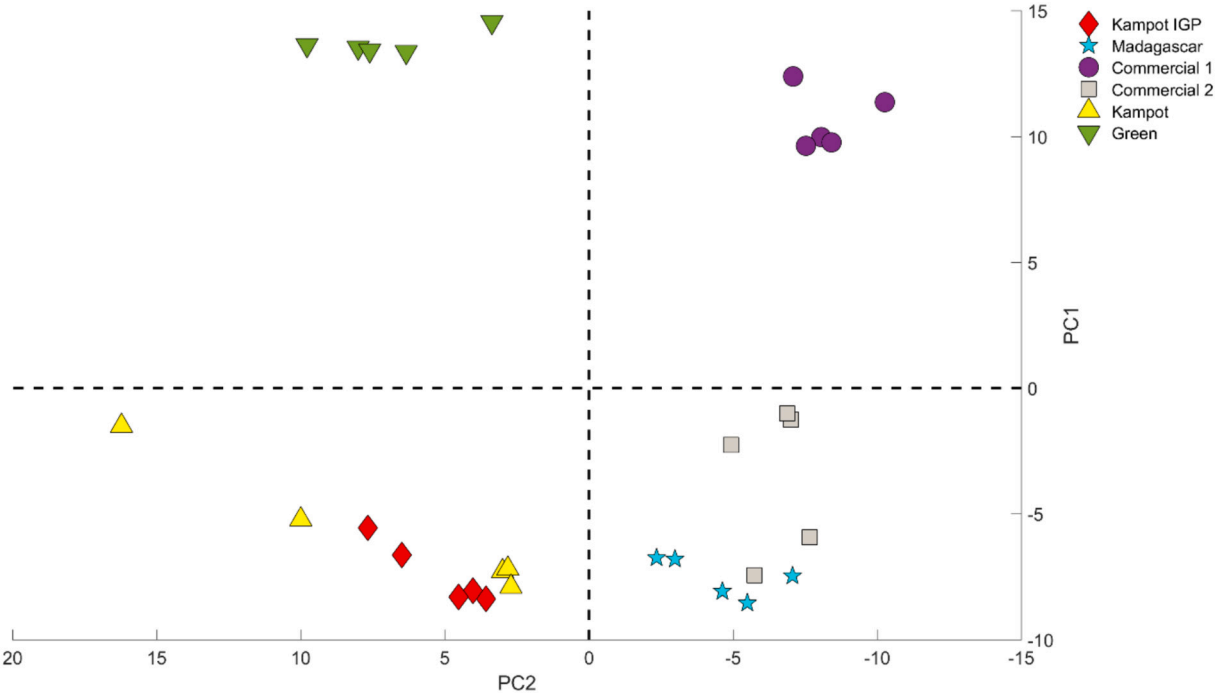

Fig. 1. PCA Scores plot on volatile compounds. Legend: Red diamonds: Kampot IGP; Cyan stars: Madagascar; Purple dots: Commercial 1; Gray squares: Commercial 2; Yellow triangles: Kampot; Green dawn-ward triangles: Green pepper. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Volatile profiles of peppercorn. For each compound: peak number (#), retention time (RT, min), assigned structure, experimental and literature retention indices, and mean relative peak area (%) ± standard error (SE), calculated from five GC–MS runs on independent amounts of the same sample. Relative areas are expressed as percentages of the total area of the 44 identified compounds. Values are reported for: Commercial 1, Commercial 2, Madagascar, Kampot IGP, Kampot, black peppercorns and Green Pepper.

			Retention Index		Commercial 1	Commercial 2	Madgascar	Kampot IGP	Kampot	Green Pepper
#	RT min	Compound	[1]Exp	[2]Lit	Mean A% ± se	Mean A% ± se	Mean A% ± se	Mean A% ± se	Mean A% ± se	Mean A% ± se
1	7.68	α-Thujene	930	929 ± 2	1.45 ± 0.23	4.65 ± 0.29	0.25 ± 0.03	0.31 ± 0.04	0.33 ± 0.05	3.16 ± 0.21
2	7.79	α-Pinene	938	937 ± 3	9.08 ± 0.14	8.42 ± 0.23	12.51 ± 0.30	6.57 ± 0.22	7.25 ± 0.52	6.41 ± 0.13
3	7.98	Camphene	954	952 ± 2	0.54 ± 0.03	0.39 ± 0.03	1.26 ± 0.11	0.27 ± 0.02	0.32 ± 0.06	0.19 ± 0.02
4	8.29	Sabinene	976	974 ± 2	7.08 ± 0.94	16.30 ± 0.37	1.10 ± 0.27	0.55 ± 0.04	0.64 ± 0.11	14.44 ± 0.53
5	8.38	β-Pinene	979	979 ± 2	12.40 ± 0.07	11.41 ± 0.54	14.27 ± 0.20	9.28 ± 0.25	10.11 ± 0.68	6.77 ± 0.14
6	8.46	β-Myrcene	992	991 ± 2	6.01 ± 0.40	4.56 ± 0.08	6.68 ± 0.35	5.35 ± 0.34	6.04 ± 0.89	2.48 ± 0.13
7	8.69	α-Phellandrene	1007	1005 ± 2	2.22 ± 0.48	2.21 ± 0.13	2.49 ± 0.40	2.20 ± 0.26	2.60 ± 0.22	2.68 ± 0.22
8	8.72	3-Carene	1014	1011 ± 2	17.40 ± 0.78	7.34 ± 0.74	16.38 ± 0.30	20.49 ± 0.49	19.05 ± 0.52	8.32 ± 0.12
9	8.79	α-Terpinene	1021	1017 ± 2	0.35 ± 0.03	0.37 ± 0.05	0.28 ± 0.04	0.21 ± 0.02	0.26 ± 0.04	0.26 ± 0.02
10	8.83	o-Cymene	1024	1022 ± 2	0.35 ± 0.01	0.022 ± 0.001	0.08 ± 0.01	0.29 ± 0.01	0.23 ± 0.01	0.002 ± 0.001
11	8.88	p-Cymene	1029	1025 ± 2	3.22 ± 0.35	0.62 ± 0.10	1.13 ± 0.11	3.27 ± 0.18	2.72 ± 0.33	0.28 ± 0.01
12	8.97	Limonene	1033	1030 ± 2	16.91 ± 0.28	16.09 ± 0.72	16.82 ± 0.29	13.64 ± 0.51	12.35 ± 0.77	10.23 ± 0.25
13	9.02	β-Phellandrene	1041	1031 ± 2	0.97 ± 0.10	2.85 ± 0.56	2.19 ± 0.14	1.12 ± 0.45	2.75 ± 0.30	3.36 ± 0.08
14	9.10	trans-β-Ocimene	1048	1049 ± 2	0.22 ± 0.04	0.70 ± 0.09	0.49 ± 0.05	0.18 ± 0.01	0.20 ± 0.03	0.41 ± 0.03
15	9.26	γ-Terpinene	1063	1060 ± 3	0.47 ± 0.01	0.69 ± 0.04	0.50 ± 0.04	0.45 ± 0.03	0.54 ± 0.07	0.59 ± 0.04
16	9.40	Sabinene hydrate	1075	1077 ± 9	0.09 ± 0.02	0.34 ± 0.04	0.018 ± 0.001	0.012 ± 0.002	0.008 ± 0.001	0.232 ± 0.004
17	9.52	p-Mentha- 2.4(8)-diene	1086	1086 ± 3	0.92 ± 0.10	0.24 ± 0.04	0.95 ± 0.05	1.12 ± 0.07	1.23 ± 0.13	0.24 ± 0.01
18	9.57	p-Mentha-1.4(8)-diene	1090	1088 ± 2	1.80 ± 0.16	0.92 ± 0.06	2.19 ± 0.09	2.09 ± 0.12	2.43 ± 0.23	0.78 ± 0.03
19	9.61	1-methyl-4-(1-methylethenyl)-benzene	1094	1090 ± 2	0.04 ± 0.01	0.014 ± 0.002	0.030 ± 0.001	0.07 ± 0.01	0.041 ± 0.004	0.004 ± 0.001
20	9.68	Linalool	1100	1099 ± 2	0.20 ± 0.02	0.16 ± 0.02	0.29 ± 0.02	0.84 ± 0.03	0.73 ± 0.05	0.358 ± 0.005
21	9.75	cis-β-Terpineol	1106	1144 ± 1	0.06 ± 0.01	0.17 ± 0.02	0.009 ± 0.001	0.011 ± 0.001	0.015 ± 0.003	0.16 ± 0.01
22	10.30	Camphor	1156	1145 ± 2	0.043 ± 0.004	0.009 ± 0.001	0.060 ± 0.003	0.003 ± 0.001	0.004 ± 0.001	0.006 ± 0.001
23	10.65	Terpinen-4-ol	1188	1182 ± 0	0.07 ± 0.02	0.20 ± 0.02	0.018 ± 0.001	0.022 ± 0.002	0.020 ± 0.001	0.42 ± 0.01
24	10.79	α-Terpineol	1201	1190 ± 3	0.042 ± 0.005	0.036 ± 0.003	0.09 ± 0.01	0.028 ± 0.002	0.024 ± 0.002	0.060 ± 0.005
25	12.31	δ-Elemene	1344	1338 ± 2	1.43 ± 0.02	1.12 ± 0.12	2.38 ± 0.12	1.20 ± 0.08	1.16 ± 0.19	3.90 ± 0.10
26	12.43	α-Cubebene	1355	1351 ± 2	0.22 ± 0.02	0.21 ± 0.02	0.08 ± 0.01	0.27 ± 0.02	0.13 ± 0.02	0.046 ± 0.004
27	12.71	Isoledene	1383	1375 ± 2	0.09 ± 0.01	0.09 ± 0.01	0.15 ± 0.01	0.052 ± 0.003	0.03 ± 0.01	0.056 ± 0.003
28	12.75	α-Copaene	1386	1376 ± 2	2.66 ± 0.20	2.34 ± 0.22	0.09 ± 0.02	0.35 ± 0.02	0.18 ± 0.03	0.11 ± 0.01
29	12.87	β-Elemene	1398	1391 ± 2	0.66 ± 0.05	0.85 ± 0.04	0.80 ± 0.06	2.03 ± 0.14	1.12 ± 0.19	1.12 ± 0.04
30	13.10	α-Gurjunene	1404	1409 ± 2	0.09 ± 0.01	0.21 ± 0.03	0.09 ± 0.01	0.45 ± 0.02	0.22 ± 0.05	0.16 ± 0.01
31	13.30	β-Caryophyllene	1419	1419 ± 3	10.34 ± 0.58	12.41 ± 0.62	11.91 ± 0.78	18.04 ± 0.88	20.70 ± 2.57	25.25 ± 0.98
32	13.37	α-Guaiene	1444	1439 ± 2	0.12 ± 0.01	0.24 ± 0.02	0.58 ± 0.05	1.28 ± 0.08	1.12 ± 0.19	0.047 ± 0.004
33	13.45	(E)-β-Famesene	1451	1457 ± 2	0.07 ± 0.01	0.15 ± 0.02	0.027 ± 0.002	0.18 ± 0.02	0.10 ± 0.02	0.63 ± 0.03
34	13.55	Cadina-3.5-diene	1461	1458 ± n/a	0.04 ± 0.01	0.04 ± 0.01	0.011 ± 0.001	0.008 ± 0.001	0.003 ± 0.001	0.017 ± 0.003

(*continued on next page*)

Table 1 (*continued*)

#	RT min	Compound	Retention Index [1]Exp	[2]Lit	Commercial 1 Mean A% ± se	Commercial 2 Mean A% ± se	Madgascar Mean A% ± se	Kampot IGP Mean A% ± se	Kampot Mean A% ± se	Green Pepper Mean A% ± se
35	13.65	α-Caryophyllene	1470	1454 ± 3	0.88 ± 0.06	1.25 ± 0.11	1.29 ± 0.10	2.39 ± 0.15	2.38 ± 0.37	2.36 ± 0.10
36	13.92	Germacrene D	1494	1481 ± 3	0.16 ± 0.04	0.14 ± 0.01	1.12 ± 0.11	0.09 ± 0.01	0.08 ± 0.01	0.72 ± 0.02
37	13.98	(Z.*E*)-α-Farnesene	1500	1491 ± 3	0.010 ± 0.004	0.07 ± 0.03	n.d.	0.024 ± 0.004	0.02 ± 0.01	0.16 ± 0.01
38	14.03	δ-Guaiene	1504	1505 ± 3	0.25 ± 0.01	0.58 ± 0.06	0.72 ± 0.07	2.46 ± 0.16	1.41 ± 0.27	0.54 ± 0.03
39	14.10	α-Selinene	1510	1494 ± 3	0.22 ± 0.02	0.40 ± 0.05	0.45 ± 0.04	1.63 ± 0.12	0.88 ± 0.18	0.16 ± 0.01
40	14.13	β-Bisabolene	1512	1509 ± 3	0.35 ± 0.07	0.55 ± 0.06	0.04 ± 0.01	0.77 ± 0.05	0.39 ± 0.07	1.18 ± 0.07
41	14.29	δ-Cadinene	1525	1524 ± 2	0.35 ± 0.04	0.33 ± 0.03	0.028 ± 0.004	0.032 ± 0.005	0.015 ± 0.002	0.027 ± 0.003
42	14.38	α-Panasinsene	1533	1527 ± 8	0.010 ± 0.001	0.08 ± 0.01	0.023 ± 0.004	0.15 ± 0.01	0.08 ± 0.02	0.09 ± 0.01
43	14.88	Germacrene B	1573	1557 ± 3	0.02 ± 0.01	0.10 ± 0.02	0.039 ± 0.003	0.027 ± 0.002	0.02 ± 0.01	1.41 ± 0.08
44	15.18	Caryophylene oxide	1598	1581 ± 2	0.069 ± 0.003	0.12 ± 0.03	0.06 ± 0.01	0.19 ± 0.03	0.10 ± 0.02	0.18 ± 0.01

n.d.: not detected.
n/a: not available.
[1] Exp: experimental retention indices calculated using a C_7-C_{40} linear alkane mixture.
[2] Lit.: semistandard non polar retention indices reported in the NIST14 mass spectra database.

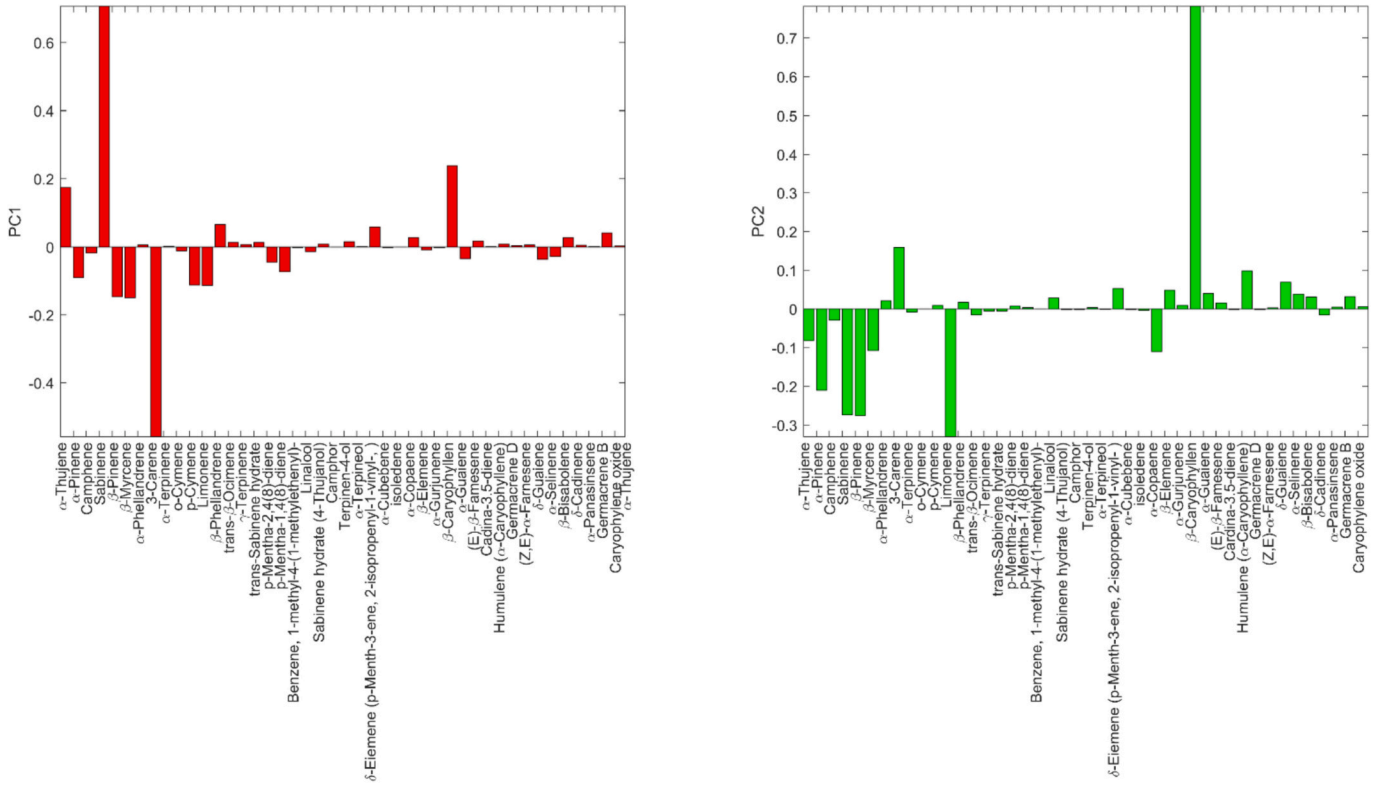

Fig. 2. PCA on volatile compounds. Loading plots associated to PC1 (on the left) and PC2 (on the right).

reflecting the antioxidant potential of the extract on a dry weight, without interference from external processing-related factors.

Since a lower IC_{50} value indicates higher antioxidant activity (since a lower concentration is required to achieve 50% inhibition of oxidative capacity), a higher IC_{50} value indicates lower inhibitory efficacy. Based on the data obtained, Madagascar pepper showed the lowest IC_{50} value (7.94 ± 0.04 mg·mL^{-1}), thus being the variety with the highest efficacy in inhibitory activity among those tested. On the contrary, Kampot pepper showed the highest IC_{50} value (16.32 ± 1.94 mg·mL^{-1}), resulting to be the variety with lower antioxidant efficacy than the others tested. The other pepper varieties analyzed were found to have intermediate IC_{50} values. The results obtained suggest that factors such as processing process, geographical origin (including soil, climate, cultivation methods), post-harvest processing techniques and storage

Table 2
IC_{50} values (mg·mL-1) obtained for each pepper analyzed.

Pepper variety	IC_{50} (mg·mL^{-1})
Madagascar	7.94 ± 0.04
Commercial 1	9.71 ± 0.69
Kampot PGI	10.23 ± 0.49
Commercial 2	12.11 ± 1.89
Kampot	16.32 ± 1.94

conditions significantly influence the biological activity of pepper by making some varieties more concentrated in bioactive compounds, responsible for antioxidant activity, than others. In particular, Madagascar pepper, with the highest antioxidant efficacy, can be considered richer in bioactive compounds than other varieties.

It should be noted that, due to the limited number of independent samples available for each class, a formal statistical comparison of IC_{50} values was not performed. Accordingly, the reported IC_{50} values are intended as descriptive indicators of antioxidant activity rather than as statistically comparable parameters, and their inclusion serves as a complementary quality-related assessment within the broader multi-analytical framework of the study.

An interesting future perspective would be the investigation of total phenolic content and its possible correlation with the antioxidant activity expressed as IC_{50} values. While such information could further support the interpretation of the results, this analysis was not included in the present study and will be addressed in future investigations.

3.4. FTIR spectra and classification modeling

As anticipated, the PLS-DA was used to classify samples according to six classes: Kampot PGI, Madagascar, Commercial 1, Commercial 2, Kampot, and Green.

Fig. 3 displays, in the upper panel, all raw ATR-FTIR spectra recorded for the analyzed peppercorn samples. In the spectral region between 3700 and 3000 cm^{-1}, a broad and weak absorption band is observed around 3300 cm^{-1}, indicative of O—H stretching vibrations typically associated with alcohols or carboxylic acids. Between 3000 and 2800 cm^{-1}, two intense absorption peaks appear in the range of 2950 to 2850 cm^{-1}, corresponding to aliphatic C—H stretching vibrations, suggesting the presence of aliphatic hydrocarbon chains commonly found in terpenes and lipids. In the region around 1700–1650 cm^{-1}, a prominent peak near 1700 cm^{-1} is characteristic of the C═O stretching of carbonyl groups, such as those present in piperine, the primary compound responsible for the pungency of black pepper. The region between 1600 and 1400 cm^{-1} exhibits bands that are typically associated with aromatic ring vibrations, likely due to the presence of piperine and other aromatic constituents of the essential oils. Finally, the intense bands observed in the 1300–900 cm^{-1} region are attributed to C–O–C stretching vibrations of polysaccharides and starches, which are abundant in black pepper.

The lower panel of the figure shows the average spectra for each geographical group. Differences can be observed in both the relative intensity and subtle shifts in band positions, particularly in the 1700–1600 cm^{-1} region (C═O stretching of carbonyl groups) and the 1300–900 cm^{-1} region (C–O–C vibrations), which may reflect compositional variations related to the samples' geographical origin. Although these differences are nuanced, they suggest that the spectral features may be informative for distinguishing the different categories of pepper. This observation is consistent with the classification results obtained through PLS-DA modeling discuss below.

To optimize the classification performance of the PLS-DA model based on FTIR spectral data, various preprocessing strategies were tested on the training set. The evaluated pretreatments included Standard Normal Variate (SNV), first derivative (D1), second derivative (D2), and the combined approaches SNV + D1 and SNV + D2. For each preprocessing method, a PLS-DA model was constructed using 7-fold cross-validation on the training data, and classification error rates were recorded (Table 3).

Among the tested options, the combination of SNV followed by first derivative (SNV + D1) produced the best performance in cross-validation (91.0% average correct classification in cross validation). The application of the model for the classification of the external test set yielded to a global classification error of 95.0%, corresponding to the confusion matrix and the correct classification rates by class shown in Table 4.

These findings underscore the critical role of preprocessing in spectroscopic classification and support the selection of SNV + D1 as the most suitable pretreatment for FTIR-based geographical authentication of black pepper.

Fig. 4 presents the projection of samples onto the space defined by the first three canonical variates (CV1, CV2, and CV3) extracted from the PLS-DA model applied to preprocessed ATR-FTIR spectra. Fig. 4A shows the projection of the samples onto the space defined by CV1 and CV2. It is evident that both Kampot sample classes, regardless of their PGI status, cluster at negative values of CV1, whereas all other samples fall at positive values of this component. The remaining samples are more

Table 3
PLS-DA: Cross validated (CV) average correct classification rates (%) and number of selected latent variables (LVs) for the calibration models built on data preprocessed by different pretreatments.

Preprocessing	LVs	Average Correct Classification Rate (%CV)
Mean-centering	12	81.5
SNV	12	89.3
D1	12	90.5
D2	12	90.5
SNV + D1	11	91.0
SNV + D2	12	90.0

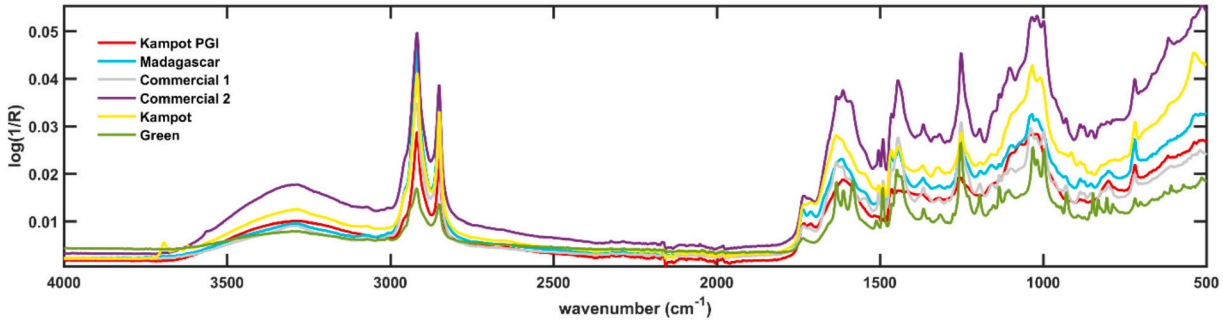

Fig. 3. FTIR spectra collected on individual peppercorns. Average spectra collected on the different classes of peppercorns. Legend: Red: Kampot PGI; Cyan: Madagascar Gray: Commercial 1; Purple: Commercial 2; Yellow: Kampot; Green:Green Pepper. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
PLS-DA: Confusion matrix and Correct Classification rates in prediction on the external test set.

	Kampot PGI	Madagascar	Commerc.1	Commerc. 2	Kampot	Green	Correct Classification Rate (%)
Predicted Kampot PGI	63	1	0	0	0	0	98.4
Predicted Madagascar	0	56	4	1	0	0	91.8
Predicted Commercial 1	0	0	69	0	0	1	98.6
Predicted Commercial 2	0	3	5	52	0	2	83.9
Predicted Kampot	0	0	2	0	66	0	97.0
Predicted Green	0	0	8	0	0	69	89.6

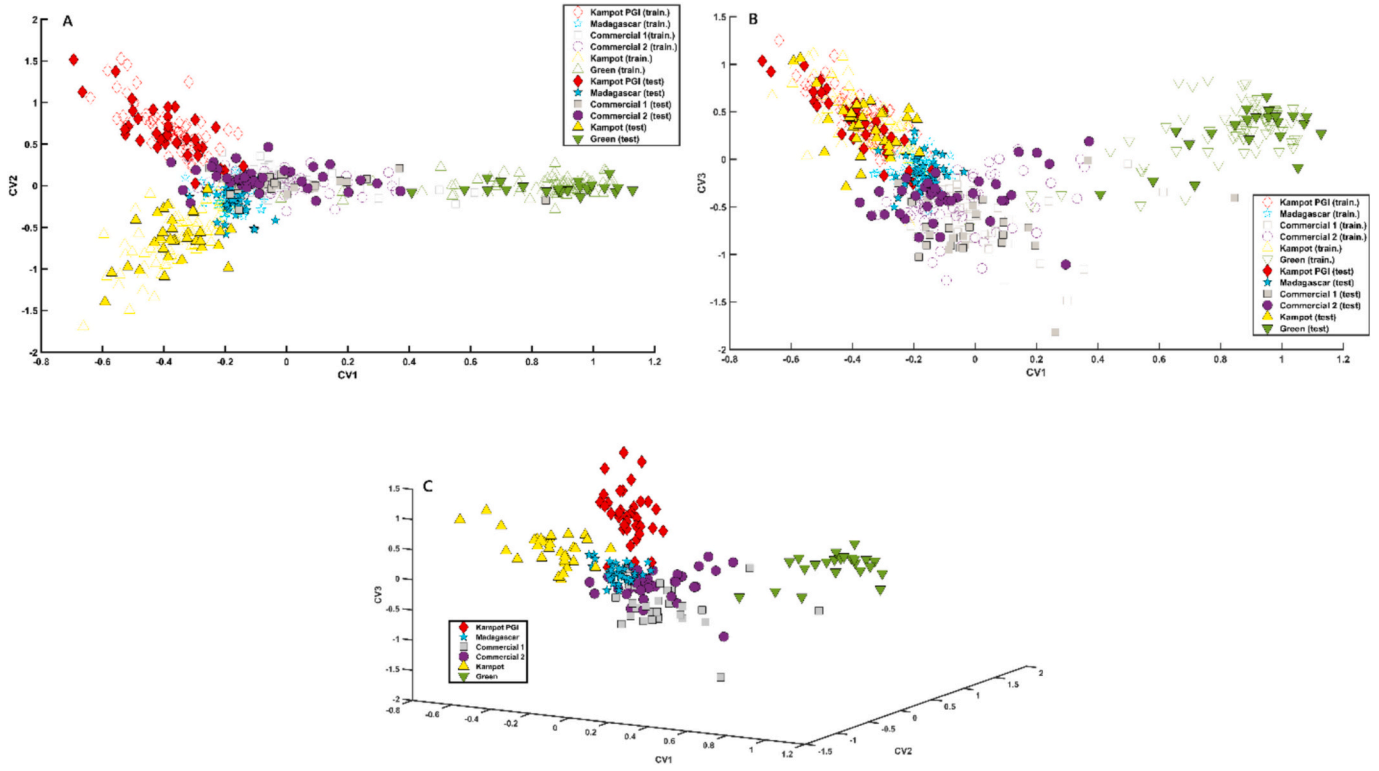

Fig. 4. PLS-DA: Projection of samples onto the space spanned by A) the first two canonical variates (CV1 and CV2); B) the first and the third canonical variates (CV1 and CV3); C) all the available canonical variates. Legend: Red diamonds: Kampot IGP; Cyan stars: Madagascar; Purple dots: Commercial 1; Gray squares: Commercial 2; Yellow triangles: Kampot; Green dawn-ward triangles: Green pepper. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

widely distributed along CV1, with the green pepper samples positioned at the most extreme positive end. Fig. 4B displays the projection onto the CV1–CV3 plane. In this case, a strong overlap is observed between the two Kampot classes (both showing positive CV1 values), as well as among the commercial samples and those from Madagascar, which cluster around the origin of the axes. As expected, green pepper forms a clearly distinct group, located far from the others at negative values of CV1.

In the three-dimensional space, a clear clustering of samples according to their class membership is observed, confirming the model's capability to capture compositional differences relevant for classification. The 3D plot is consistent with the trends already observed in the CV1–CV2 and CV1–CV3 projections. All commercial samples tend to cluster around the origin and/or at positive values of CV1, close to Madagascar objects, while Cambodian samples show negative CV1 scores. Green pepper samples are still quite well distinguishable from all other sample classes.

The relatively low overlap between classes, combined with compact intra-class grouping (especially for high-quality single-origin samples) demonstrates the effectiveness of the PLS-DA model in capturing geographic signatures from the FTIR spectral data. These findings reinforce the utility of vibrational spectroscopy, in combination with supervised multivariate analysis, for the robust classification and authentication of black pepper according to origin.

4. Conclusions

In the literature, the chemical composition of black pepper (*Piper nigrum* L.) shows considerable variability, mainly related to geographical origin, degree of ripeness of the berries, drying conditions, and storage methods. Moisture content, for instance, generally ranges from approximately 2% to 11%, depending on whether the sample is completely dried or only partially stabilized [36]. Lower values (<5%) are typically found in samples subjected to prolonged drying or stored under controlled conditions, whereas higher values (up to ~11%) may reflect incomplete drying or moisture absorption from the environment. The protein content of black pepper usually ranges between 10% and 13% on a dry weight basis. This protein fraction mainly derives from the seed storage proteins and contributes to the nutritional value of the spice, as well as to certain functional properties during extraction or thermal processing. Regarding the lipid fraction, several studies have reported a total fat content ranging between 6% and 10%. This fraction includes both non-volatile lipids (fixed oils) and a smaller portion of volatile compounds, such as terpenes and sesquiterpenes, which

constitute the essential oil responsible for the characteristic aroma of pepper. It is also known that the essential oil content can vary from 0.4% to 7% on a dry weight basis, depending on the cultivar and growing conditions [37]. In addition to proteins, lipids, and water, black pepper contains approximately 2–3% ash, indicative of its mineral content (K, Ca, Fe, Mg, Mn, Zn), as well as a significant amount of crude fiber (7–9%) and carbohydrates (60–70%), which represent the major portion of the plant matrix. These components, together with characteristic alkaloids such as piperine, contribute to defining the nutritional and bioactive profile of black pepper.

This study demonstrates the effectiveness of a multi-analytical approach combining volatile profiling (GC–MS), antioxidant activity assessment (DPPH assay), and vibrational spectroscopy (FTIR) for the characterization and geographical authentication of black pepper. Aroma profiles revealed distinctive compositional patterns among the samples, with PCA highlighting class-specific volatile markers such as sabinene, 3-carene, β-caryophyllene, and limonene—several of which are known to have significant biological activities. The antioxidant activity, evaluated through the DPPH assay, showed marked differences among the varieties, with Madagascar pepper exhibiting the highest radical scavenging capacity, suggesting a higher content of bioactive compounds.

Infrared spectroscopy, coupled with PLS-DA proved to be a powerful tool for classifying peppercorns according to their geographical origin. Among the preprocessing strategies tested, the combination of Standard Normal Variate and first derivative (SNV + D1) provided the best classification performance. The final PLS-DA model achieved high classification accuracy both in cross-validation and in external prediction (a global classification error of 95.0%), confirming the model's robustness and generalizability. While several studies have investigated either the volatile composition or the antioxidant activity of black pepper, these aspects are typically examined in isolation. The present work introduces an integrated, multi-analytical workflow that combines volatile profiling, antioxidant capacity assessment, and infrared spectroscopic classification to provide a characterization of black peppercorns from different geographical origins. This holistic approach enables simultaneous evaluation of functional, chemical, and authenticity-related features within a single analytical framework. Overall, the findings support the use of rapid, cost-effective, and non-destructive analytical methods in combination with chemometrics for quality control and authentication purposes in the spice supply chain. The integrated workflow proposed here may contribute to the development of traceability and certification systems for high-value agricultural products such as black pepper.

CRediT authorship contribution statement

Alessandra Biancolillo: Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Michela Rossi:** Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Samantha Reale:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis. **Claudia Scappaticci:** Writing – original draft, Formal analysis, Data curation. **Martina Foschi:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Angelo Antonio D'Archivio:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

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.

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Data availability

Data will be made available on request.

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