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Non-Targeted Ultra-High Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UHPLC-HRMS) Fingerprints for the Chemometric Characterization and Classification of Turmeric and Curry Samples

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Abstract: In this work, non-targeted ultra-high performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS) fingerprints obtained by C18 reversed-phase chromatography were proposed as sample chemical descriptors for the characterization and classification of turmeric and curry samples. A total of 21 turmeric and 9 curry commercially available samples were analyzed in triplicate after extraction with dimethyl sulfoxide (DMSO). The results demonstrated the feasibility of non-targeted UHPLC-HRMS fingerprints for sample classification, showing very good classification capabilities by partial least squares regression-discriminant analysis (PLS-DA), with 100% classification rates being obtained by PLS-DA when randomly selected samples were processed as "unknown" ones. Besides, turmeric curcuma species (*Curcuma longa* vs. *Curcuma zedoaria*) and turmeric *Curcuma longa* varieties (*Madras*, *Erodes*, and *Alleppey*) discrimination was also observed by PLS-DA when using the proposed fingerprints as chemical descriptors. As a conclusion, non-targeted UHPLC-HRMS fingerprinting is a suitable methodology for the characterization, classification, and authentication of turmeric and curry samples, without the requirement of using commercially available standards for quantification nor the necessity of metabolite identification.

Keywords: non-targeted UHPLC-HRMS analysis; fingerprinting chemical descriptors; turmeric characterization; principal component analysis; partial least squares regression-discriminant analysis

1. Introduction

Turmeric (*Curcuma longa)* is a rhizomatous herbaceous perennial plant related to the ginger family (*Zingiberaceae*) [1], originally from India, although nowadays it is cultivated around the world, especially in Southeast Asia, China, and Latin America [2]. The interest in this plant-derived curry spice for both society and the scientific community has increased because of its recognized medicinal properties, attributed to the presence of curcumin and its derivatives [3–11]. Curcumin, (1,7-bis(4-

hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also known as diferuloylmethane, is the main natural bioactive compound found in turmeric as well as in other *Curcuma* species. In addition, other polyphenolic-related bioactive substances such as phenolic acids and flavonoids are also abundant in this kind of products. Turmeric is also a very appreciated culinary spice, being an important component of Asian curries, to which it gives the yellow color, as well as in mustard and sauces in the West [12]. Besides, it is used to add flavor and color to rice, pasta, meat and vegetable dishes, and salads.

Few critical reviews addressing the characterization and determination of curcumin and curcumin derivatives in turmeric samples have been reported [1,2,13,14]. Analytical methodologies involved spectroscopy such as ultraviolet [15], fluorescence [16], infrared [17,18], and nuclear magnetic resonance (NMR) [19], as well as separation techniques such as capillary electrophoresis (CE) [20–22], liquid chromatography (LC) [23–26], and gas chromatography (GC) [27,28].

The use of mass spectrometry (MS) techniques, using either low- or high-resolution mass analyzers, is frequently proposed for the analysis of turmeric samples. Among them, liquid chromatography coupled with mass spectrometry (LC-MS) [27,29] or high-resolution mass spectrometry (LC-HRMS) [30–33] are among the most common techniques to address the characterization, determination, and identification of curcuminoids and other bioactive substances in turmeric samples. For instance, ultra-high performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS) was proposed for the identification and characterization of curcuminoids and related metabolites in turmeric samples [31], while UHPLC-HRMS in a Q-Orbitrap mass analyzer was applied to the characterization of the phenolic and antioxidant profiles of several culinary herbs and spices, including turmeric [30].

Nowadays, the use of non-targeted fingerprinting ("metabolomic") approaches is gaining relevance to address food characterization and classification for authentication purposes [34]. In contrast to targeted strategies where selected known chemicals are monitored (requiring the use of commercially available standards for analyte identification and/or quantitation), non-targeted fingerprinting methods are based on the analysis of complex instrumental responses without assuming any previous knowledge of food components. For example, in the case of LC-HRMS methodologies, the fingerprinting information consists of the peak intensity values recorded as a function of *m/z* (from 100 to 1500 Da) and retention times [35]. Besides, the identification of components that may be relevant for product authentication is not mandatory. A qualitative strategy based on the chemometric comparison between the samples under study and/or with authentic reference samples is normally exploited, clearly simplifying the method, since confirmation strategies are not required.

This work aims to evaluate the applicability of non-targeted UHPLC-HRMS fingerprinting using a Q-Exactive Orbitrap mass analyzer for the characterization and classification of commercial turmeric and curry samples. For that purpose, a total of 30 samples (21 turmeric and 9 curry samples) were analyzed in triplicate based on a simple sample extraction procedure and subsequent non-targeted reversed-phase UHPLC-HRMS using a C18 column under gradient elution with water and acetonitrile (both acidified with 0.1% formic acid) as mobile phase components. The suitability of the obtained UHPLC-HRMS fingerprints as sample chemical descriptors to address turmeric and curry classification was evaluated with multivariate chemometric methods such as principal component analysis (PCA) and partial least squares regression-discriminant analysis (PLS-DA).

2. Materials and Methods

2.1. Chemicals and Solutions

All the chemicals, reagents, and standards employed in this work were of analytical grade unless otherwise indicated. LC-MS Chromasolv® quality water, methanol, and acetonitrile, formic acid (98-100% purity), and dimethyl sulfoxide (DMSO) were provided by Sigma-Aldrich (Steinheim, Germany). Curcumin (98% purity) was also obtained from Sigma-Aldrich, while

demethoxycurcumin (>95% purity) and bisdemethoxycurcumin (>95% purity) were purchased from Biopurify Chemicals Ltd. (Chengdu, Sichuan, China).

2.2. UHPLC-HRMS Instrumentation

Non-targeted UHPLC-HRMS (metabolomic) fingerprints were obtained in an Accela UHPLC system coupled to a Q-Exactive Orbitrap HRMS instrument, both of them from Thermo Fisher Scientific (San Jose, CA, USA), by using a heated electrospray ionization (HESI II) source in negative ionization mode. Nitrogen (purity higher than 99.98%) was used for the HESI-II sheath gas, ion-sweep gas, and auxiliary gas, at flow rates of 60, 0, and 10 a.u. (arbitrary units), respectively. In addition, capillary and S-Lens RF voltages were set at −2.5 kV and 50 V, respectively, and heater and capillary temperatures at 350 °C and 320 °C, respectively.

A full scan HRMS mode from 100 to 1500 *m/z* using a resolution of 70,000 full width at half maximum (FWHM) at *m/z* 200 was employed for UHPLC-HRMS fingerprinting. An automatic gain control (AGC) value (number of ions to fill the C-trap) and a maximum injection time (IT) of 2.5×10^5 and 200 ms, respectively, were used. The Q-Exactive Orbitrap HRMS system was tuned and calibrated every 3 days to ensure a working mass accuracy error lower than 5 ppm by using a calibration solution commercially available from Thermo Fisher Scientific for that purpose.

The analytical column consisted of a porous-shell Ascentis® Express C18 reversed-phase (150 × 2.1 mm I.D., 2.7 µm partially porous particle size) from Supelco (Bellefonte, PA, USA). Water and acetonitrile (both acidified with 0.1% formic acid) were the mobile phase components. The gradient elution program is summarized in Table 1. A mobile phase flow rate of 300 µL/min was used. Separation was performed at room temperature, and the volume injection was 10 µL in full-loop mode.

Instrument control and data acquisition and processing were performed using Xcalibur v3.1 software (Thermo Fisher Scientific).

Table 1. Chromatographic elution program for the generation of liquid chromatography-high-resolution mass spectrometry (LC-HRMS) fingerprints.

Time (min)	Acetonitrile (%)	Elution Mode
0	10	Initial conditions
1	10	Isocratic step
20	95	Linear gradient
23	95	Isocratic step
24	10	Linear gradient
30	10	Isocratic step for column re-equilibration

2.3. Samples and Sample Treatment

A total of 21 turmeric and 9 curry commercially available samples were purchased at local markets in Barcelona (Spain). Table 2 summarizes sample characteristics and commercial brands. Samples were analysed in triplicate, thus obtaining 63 turmeric and 27 curry sample extracts.

Table 2. Characteristics of the analyzed turmeric and curry samples.

Sample	Commercial Brand	Number of Samples	Number of Extracts	Compositional Characteristics
Turmeric	Hacendado	5	15	Curcuma longa (Erode)
	MG	1	3	Curcuma longa (Alleppey)
	Burriac	1	3	Curcuma longa
	Carmencita	1	3	Curcuma longa (Erode)
	Ducros	1	3	Curcuma longa
	Artemis Bio	1	3	Curcuma longa
	Natco	1	3	Curcuma longa
	Pelotari	1	3	Unknown

Dani	2	6	Curcuma zedoaria	
Especies	1	3	Curcuma longa (Alleppey)	
Ocena	1	3	Curcuma longa (Madras)	
Tata Sampann	1	3	Unknown	
Herbalist	1	3	Curcuma longa (Madras)	
Street market	1	3	Curcuma longa (Madras)	
Biospirit	1	3	Curcuma longa	
NAAI	1	3	Unknown	
Curry	Hacendado	2	6	White pepper, coriander, ginger, cardamom, clove, cinnamon, anise, mustard
	Carrefour	1	3	Pepper, coriander, ginger, cumin, fenugreek, laurel, fennel, mustard
	Species Kania	1	3	Pepper, coriander, cumin, fenugreek, parsley, chili, garlic, fennel
	Condis	1	3	Pepper, coriander, fennel, cumin, cayenne, garlic, anise
	Burriac	1	3	White pepper, coriander, ginger, cardamom, clove, cinnamon, anise, mace
	Eroski	1	3	Coriander, cardamom, ginger, fenugreek, anise, garlic, clove, mustard
	Ducros	1	3	Pepper, coriander, cumin, ginger, laurel, anise, garlic, clove, cinnamon, mace
	Street market	1	3	Unknown

Sample extracts were obtained following previously described procedures with some modifications [24]. Turmeric or curry samples (10 mg) were suspended in 5 mL of DMSO and extracted by sonication (Branson 5510 bath, Branson Ultrasonics, Danbury, CT, USA) at room temperature during 15 min. Next, a centrifugation step at 3500× *g* for 15 min (Rotanta 460 RS centrifuge, Hettich, Tuttlingen, Germany) was performed. The resulting extracts were then filtered through 0.45-μm nylon filters (Whatman, Clifton, NJ, USA), and 2 mL were transferred into amber injection glass vials. Extracts were stored at −18 °C until analysis.

A quality control (QC) extract solution was prepared by mixing 50 μL of each sample extract. The QC solution was used to evaluate the method reproducibility, to ensure the robustness of the obtained chemometric data, and to detect signal tendencies attributed to the sample sequence analysis. With this aim, the QC solution and a blank of acetonitrile were injected every 10 samples. All the sample extracts were analyzed randomly.

2.4. Data Analysis

Data matrices for non-targeted analysis were obtained using R software (R Foundation, Vienna, Austria). For that purpose, the obtained UHPLC-HRMS raw data was processed with the MSConvert free-software (ProteoWizard Software Foundation and Project, available at www.proteowizard.sourceforge.net/tools.shtml), obtaining Excel files with all the chemical features detected and registered by means of the peak signal intensities as a function of *m/z* values and retention times. For data simplification, a threshold peak filter of 10^5 (absolute intensity) was applied. Common features from different samples were further grouped on the same columns under a tolerance filter of 2 ppm in the *m/z* domain and 0.25 min in the time domain. A mass defect filter was finally applied to exclude those features with more than 0.2 mass units in the corresponding whole number, and the *m/z* working range was 100 to 1500.

Eigenvector Stand-alone Chemometric Software (SOLO) v8.6 was employed for PCA and PLS-DA chemometric calculations [36]. The theoretical background of these chemometric methods have been described in detail elsewhere [37]. The X-data matrices for both PCA and PLS-DA calculations consisted of the UHPLC-HRMS (Orbitrap) fingerprints (peak intensities as a function of retention time and *m/z* value from 100 to 1500) obtained in the negative ionization mode. With the aim of providing similar weights to all the samples, a normalization pretreatment regarding the overall peak signal was applied in all the cases. The Y-data matrix in the PLS-DA models consisted of the membership of each sample to its corresponding class. The structure of maps of both samples and

variables was investigated from the scatter plots of scores and loadings of principal components (PCs) in PCA, and of latent variables (LVs) in PLS-DA, thus providing information regarding sample correlations and dependences. The first significant minimum point of a cross-validation (CV) error using a Venetian blind approach was considered to establish the most appropriate number of LVs for each PLS-DA model evaluated. PLS-DA chemometric models were validated for sample classification by using only 70% of the samples of each group randomly selected as the calibration set to build the model, and the remaining 30% of samples of each group as the "unknown" prediction set.

3. Results and Discussion

3.1. Non-Targeted UHPLC-HRMS Fingerprinting

As previously mentioned, this work aimed to evaluate the applicability of non-targeted UHPLC-HRMS "metabolomic" fingerprints as chemical descriptors to address the characterization and classification of turmeric and curry samples. Thus, 21 turmeric samples of different curcuma varieties and 9 curry samples were analyzed in triplicate. As a non-supervised fingerprinting approach was considered, a generic and simple sample treatment using DMSO as extractant solvent was applied prior to the chromatographic separation to extract as many chemical descriptors as possible. In the case of spice-based products such as turmeric and curry samples, their fingerprint will depend not only on the spice variety genotype but also on the product phenotype (food attributes dependent on external conditions such as ambient conditions, agricultural practices, and food-processing procedures among others). Therefore, UHPLC-HRMS "metabolomic" fingerprints are expected to provide good chemical features to address sample classification. Polyphenolic and curcuminoid compounds are among the bioactive substances most frequently found in turmeric and curry samples, and they are usually determined by LC-MS with electrospray in negative ionization mode [14,38].

As an example, Figure 1 shows the 3D UHPLC-HRMS fingerprint of a curry sample. For simplification, only signal intensity of features within retention times (RT) 0–20 min and *m/z* 100–1000 (which are the richer segments) are shown. As can be seen, features providing higher intensities appeared at *m/z* values mainly lower than 500 Da, which correspond to small molecules such as polyphenolic and phenolic acids as well as curcumin and curcuminoids. Besides, most of these bioactive substances are relatively polar presenting RT below 15 min when using C18 reversed-phase chromatography.

Figure 2 compares the UHPLC-HRMS total ion chromatograms, the extracted ion chromatograms for curcumin (*m/z* 367.1187), and the HRMS spectra at RT 13.53 (curcumin retention time confirmed using a commercially available standard) of two turmeric samples of different curcuma species (*Curcuma longa* and *Curcuma zedoaria*), and a curry sample. As can be seen, important differences in both chromatographic profiles (Figure 2a,b) and MS signal intensities (Figure 2c) are observed. Although these differences are enhanced between turmeric and curry samples, especially at retention times ranging from 10 to 17 min, where mainly curcuminoids are eluting, noticeable differences are also observed within the different turmeric samples according to their species. For example, the turmeric *curcuma longa* sample shows a fingerprint with higher signal intensities than turmeric *curcuma zedoaria*, as well as richer profiles at higher retention times. Because of these differences, non-targeted UHPLC-HRMS fingerprints are expected to be good sample chemical descriptors for classification purposes using chemometrics.
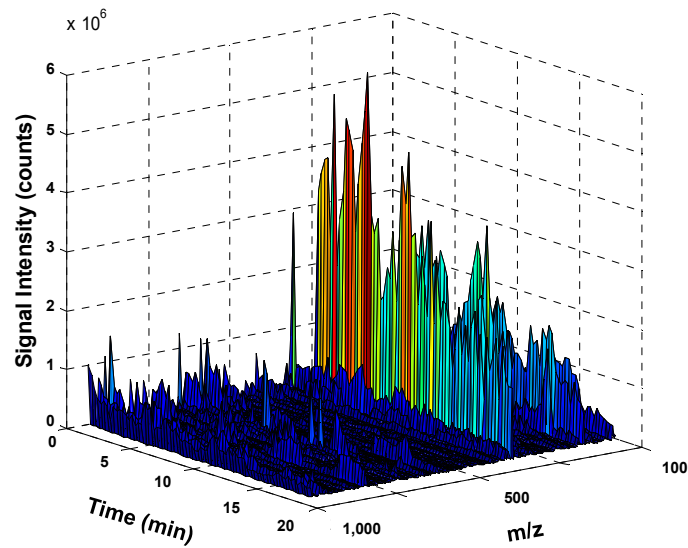
Figure 1. 3D ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) fingerprint of a curry sample. For simplification, only the time range from 0–20 min and *m/z* range from 100 to 1000 is depicted.
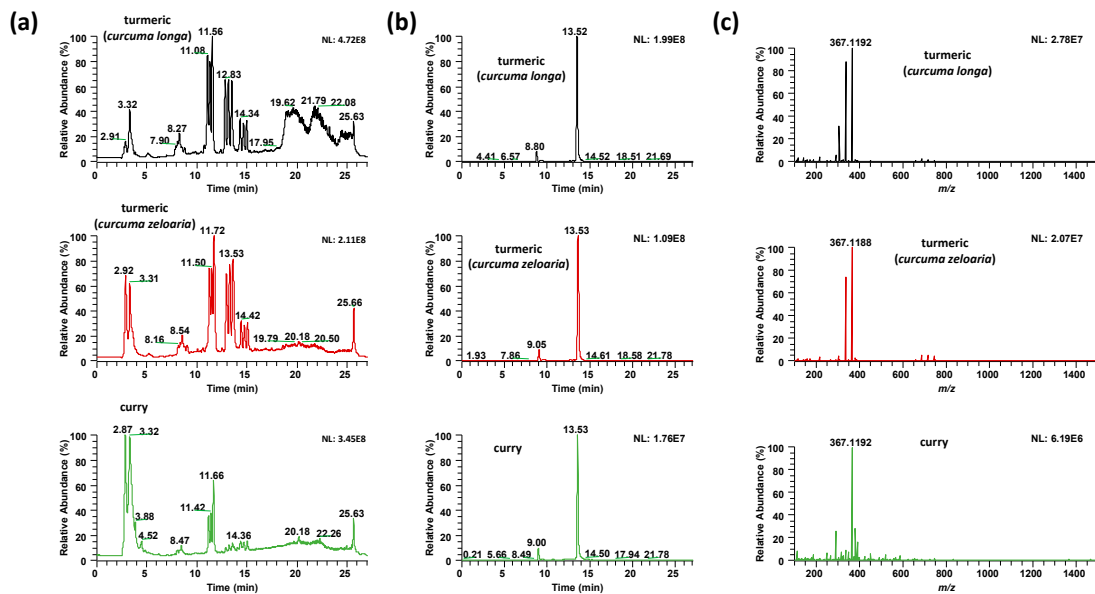

Figure 2. (**a**) Ultra high-performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) total ion chromatogram (negative ionization mode), (**b**) UHPLC-HRMS extracted ion chromatogram for curcumin (accurate mass at *m/z* 367.1187), and (**c**) HRMS spectrum at RT 13.53 (corresponding to elution of curcumin) for a *Curcuma longa* turmeric sample (Biospirit brand), a *Curcuma zedoaria* turmeric sample (Dani brand), and a curry sample (Hacendado brand).

Important differences were also observed in the MS signals as depicted in the HRMS spectra at RT 13.53 min, shown in Figure 2c. Differences are not only based on the signal intensity, but also in the presence of different ions due to different chemical coelutions depending on the sample. All these differences will help with obtaining discriminant sample chemical descriptors to address sample characterization and classification by chemometrics.

3.2. PCA Study

First, the reproducibility of the proposed UHPLC-HRMS fingerprinting method as well as the robustness of the chemometric results were evaluated by studying the behavior of the QCs by PCA. Raw UHPLC-HRMS "metabolomic" fingerprints, consisting of peak signal intensities as a function of both chromatographic retention times and HRMS *m/z* values, contained a great amount of features, including a lot of background noise signals common in all the analyzed samples. Thus, noise was mainly avoided by applying a threshold peak filter of 10^5 (absolute signal intensity). These filtered data were then processed with R software to obtain the final data matrix containing the non-targeted UHPLC-HRMS fingerprints of both QCs and samples to be subsequently processed by PCA.

Figure 3a shows the obtained PCA score plot of PC1 vs. PC2. As can be seen, although QCs are replicates of the same solution injected throughout the analysis sequence, they are not correctly grouped in the PCA score plot, revealing a lack of reproducibility of the proposed UHPLC-HRMS fingerprinting procedure, which hinders the chemometric evaluation of the samples. As shown, QCs follow a trend within the score plot probably due to losses in the H-ESI ionization efficiency. Nevertheless, as the QC is the same solution, all the obtained non-targeted UHPLC-HRMS fingerprints can be corrected using the QC signals as the reference. To perform this correction, each sample fingerprinting was divided by the equivalent one in the closest QC injected in the sequence, while each QC signal was divided by itself (resulting in fingerprinting variables defined by the value 1). The PCA score plot of PC1 vs. PC2 when using corrected fingerprints is depicted in Figure 3b. Although a perfect discrimination of both groups of samples is not accomplished, a general trend is observed through PC1 with turmeric samples exhibiting positive PC1 values being located at the right of the plot, while curry samples tend to be located at the left of the plot with negative PC1 values.

PCA sample discrimination slightly improves when using a higher number of PCs, as can be seen in the PCA score plot of PC1 vs. PC3 vs. PC4 depicted in Figure 3c. Turmeric samples exhibited positive PC1 and PC4 values, while curry samples showed negative ones.
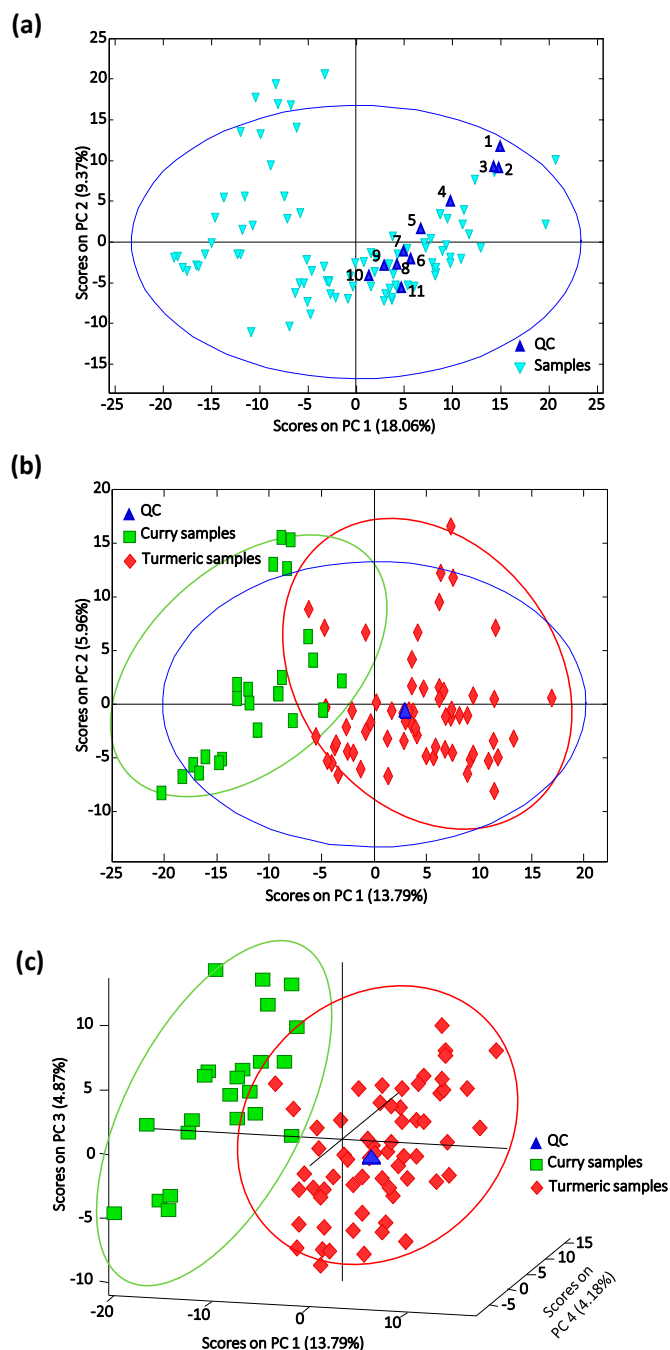
(a)

(b)

(c)

Figure 3. Study of turmeric and curry samples by principal component analysis (PCA). (**a**) Score plot of PC1 vs. PC2 when using non-normalized UHPLC-HRMS fingerprints as chemical descriptors. (**b**) PCA score plot of PC1 vs. PC2 using normalized data to build the model. (**c**) PCA score plot of PC1 vs. PC3 and PC4 when using corrected UHPLC-HRMS fingerprints.

3.3. Sample Classification by PLS-DA

PLS-DA was also applied to evaluate the feasibility of non-targeted UHPLC-HRMS corrected fingerprints as potential chemical descriptors for turmeric and curry classification. The X-data matrix was that used in the PCA, while the Y-data matrix defined the membership of each analyzed sample. Four LVs were required to build the PLS-DA model, as deduced by the first significant minimum error in CV from a Venetian blind approach. The obtained PLS-DA score plots of (a) LV1 vs. LV2 and

(b) LV1 vs. LV2 vs. LV4 for turmeric and curry sample classification is depicted in Figure 4. Sample discrimination improved in comparison with the results previously obtained by PCA, as expected. LV1 is responsible for the discrimination between turmeric and curry samples. As can be seen in Figure 4a, curry samples tend to exhibit higher LV1 values in comparison with turmeric samples, which are located to the left of the plot. Again, when using the sample information explained by higher LVs, such as LV4, excellent classification and separation of both groups of samples are accomplished (Figure 4b).
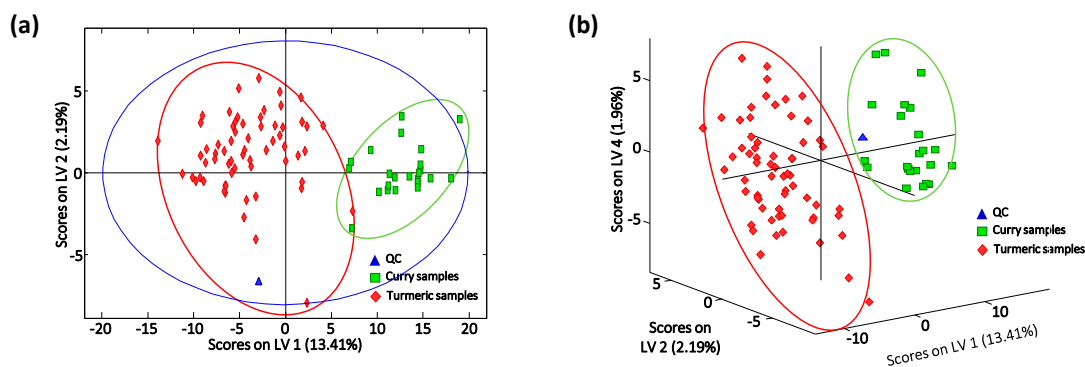

Figure 4. Partial least square regression-discriminant analysis (PLS-DA) score plots of (**a**) LV1 vs. LV2 and (**b**) LV1 vs. LV2 vs. LV4 by non-targeted UHPLC-HRMS using selected fingerprint features as sample chemical descriptors for the classification of the analyzed samples. Four latent variables (LVs) were required to build the models.

The assignation of "unknown" turmeric samples into their corresponding classes, regarding their curcuma species (*Curcuma longa* vs. *Curcuma zedoaria*), as well as among different *Curcuma longa* varieties (*Alleppey, Madras,* and *Erode*) was also studied by PLS-DA. Figure 5 shows the obtained PLS-DA score plot of LV1 vs. LV2. As can be seen, excellent discrimination was accomplished between turmeric *Curcuma longa* vs. *Curcuma zedoaria* species (Figure 5a), being perfectly differentiated by LV1. *Curcuma zedoaria* samples tend to be located at the bottom-right area of the PLS-DA plot, while *Curcuma longa* turmeric samples are distributed throughout the plot but mainly exhibiting negative LV1 values. Excellent sample discrimination was also achieved when comparing different *Curcuma longa* varieties, as can be observed in Figure 5b. Both LV1 and LV2 are playing an important role in the sample distribution. *Erode* samples are located in the center of the plot, and the other samples are distributed in different areas, with *Alleppey* samples appearing in the top-right area, *Madras* samples in the bottom-right area, and the *curcuma zedoaria* turmeric samples in the bottom-left part of the plot. The sample labeled only as *Curcuma longa* is located in the top-left area of the plot, while the unknown curcuma variety sample overlaps with those of *Curcuma longa Erode*, but is also very close to *Curcuma zedoaria* samples. Obviously, a higher number of samples will be required to perform a deeper study in order to perfectly identify this sample, although this was not the aim of the present work.
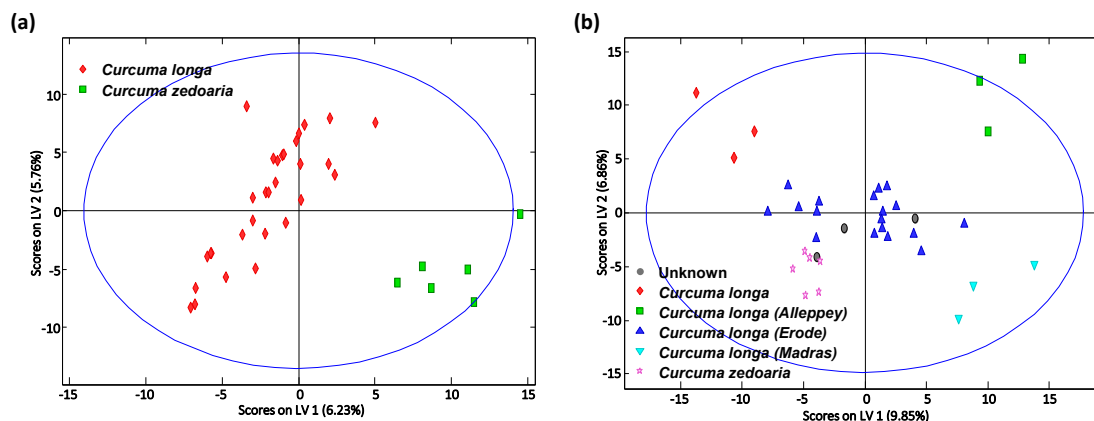
Figure 5. PLS-DA score plots of LV1 vs LV2 when using corrected non-targeted UHPLC-HRMS fingerprints as sample chemical descriptors for (**a**) classification of *curcuma longa* vs. *curcuma zedoaria* turmeric samples, and (**b**) classification of turmeric *curcuma longa* varieties. A total of four LVs were used to build the model.

Finally, the applicability of the proposed fingerprinting methodology to assign turmeric and curry samples to predefined classes was also studied by evaluating the classification rate of the corresponding PLS-DA model. This evaluation was carried out by randomly selecting 70% of the samples of each group as the calibration set to build the PLS-DA model, and using the remaining 30% as the "unknown" set for prediction/validation purposes. The classification plot obtained by PLS-DA by using four LVs to build the model is depicted in Figure 6, showing excellent results. As can be seen, all the samples used as "unknown" samples were correctly classified within the corresponding group.
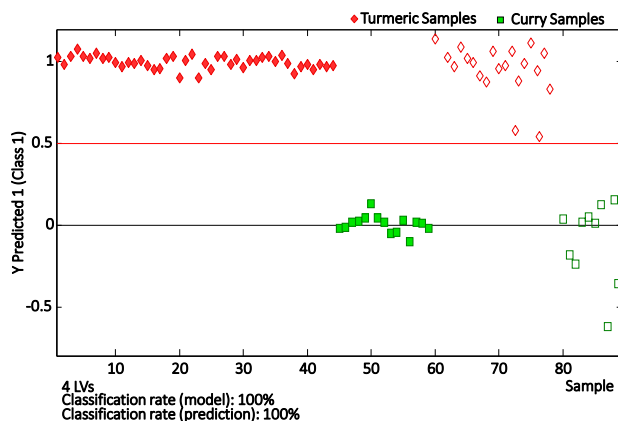

Figure 6. Class prediction plot for turmeric vs. curry samples. Filled and empty symbols correspond to calibration and validation sets, respectively. Four LVs were used to generate the model.

All the results obtained by PLS-DA demonstrated the applicability and good performance of non-targeted UHPLC-HRMS fingerprints as sample chemical descriptors for the characterization, classification, and authentication of curry and turmeric samples.

4. Conclusions

Non-targeted C18 reversed-phase UHPLC-HRMS fingerprints have been proposed as sample chemical descriptors to achieve turmeric and curry characterization, classification, and authentication by chemometrics. Both PCA and PLS-DA chemometric methods showed good discrimination

capacity with the analyzed turmeric and curry samples. Besides, PLS-DA models showed 100% classification rates in both calibration and prediction steps.

The characterization and classification of turmeric samples regarding the different turmeric curcuma species (*curcuma longa* vs. *curcuma zedoaria*) and between different curcuma longa varieties (*Madras*, *Erode*, and *Alleppey*) was successfully accomplished, despite the low number of turmeric samples available, by using non-targeted UHPLC-HRMS fingerprints as chemical descriptors and the PLS-DA chemometric method.

The results obtained in this work show that non-targeted UHPLC-HRMS fingerprints can be proposed as excellent sample chemical descriptors for the characterization, classification, and authentication of turmeric and curry samples. The method is advantageous in comparison with targeted approaches as the availability of chemical standards for sample quantitation is not required, and the identification of metabolites is not mandatory.

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