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RESEARCH

Meat Authentication Based on Animal Species and Other Quality Meat Attributes (Protected Geographical Indication, Organic Production, and Halal and Kosher Products) by HPLC–UV Fingerprinting and Chemometrics

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Abstract
A simple and economic high-performance liquid chromatography with UV–vis detection (HPLC–UV) metabolomic fingerprinting methodology was developed and applied after a water extraction procedure to obtain sample chemical descriptors suitable for meat authentication by chemometrics. Three hundred meat samples involving different species (lamb, beef, pork, rabbit, quail, chicken, turkey, and duck) as well as different non-genetic attributes (protected geographical indications, organic production, and Halal and Kosher meats) were analyzed, and the obtained HPLC–UV fingerprints subjected to PCA and PLS-DA for classification and authentication. Excellent PLS-DA discrimination and classification performance was accomplished for calibration and cross-validation, with sensitivity and specificity values higher than 100% and 99.3%, respectively, and classification errors below 0.4%, when meat species were considered. The prediction capability when employing a classification decision tree consisting on consecutive dual PLS-DA models built using a hierarchical model builder was of 100% accuracy when 48 meat samples were subjected to the model as unknown samples. Multiclass PLS-DA classification performances when addressing meat geographical origin, organic productions and Halal and Kosher products were also very acceptable, with overall sensitivity and specificity values higher than 91.2%, and classification errors below 6.9%. Finally, fraudulent meat adulteration cases involving PGI, organic and Halal and Kosher adulterated meats were evaluated by partial least squares (PLS) regression, allowing the detection and quantitation of adulteration levels within the range from 15 to 85% with prediction errors below 6.6%, demonstrating the suitability of the proposed methodology to assess meat authenticity.

Keywords HPLC–UV fingerprinting · Meat authentication · Chemometrics · Meat geographical origin · Meat production practices · Classification decision tree

Introduction

The quality and integrity of food is one of the aspects that most concerns society today, being willing to pay higher prices for foods with specific characteristics such as the region where the food has been produced, the use of sustainable production practices, foods enriched with bioactive substances, etc. However, this situation has led to an increase in fraudulent practices that seek to illicit financial enrichment at the expense of consumers by taking advantage of their predisposition to such products considered to be of higher quality. Food fraud refers to the deliberate production, marketing, and distribution of counterfeit or adulterated foods for profit. These fraudulent practices may include the incorrect labelling (false information included on the product

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packaging or label, such as untrue nutritional statements, products labelled as organic without being so, additives not declared), the food dilution or substitution with lower-value components to minimize production costs without reflecting this information on the label, or the addition of unknown or undeclared substances (sometimes to conceal such fraud), among other practices. The great danger of fraudulent practices in food, apart from the economic deception, is that they can alter its safety, which can lead to health problems for consumers (addition of potentially toxic substances, possibility of allergy episodes, etc.). For this reason, food fraud is prohibited throughout the world, and the competent bodies must guarantee its rapid detection. To achieve this, it is necessary to develop feasible, simple, and fit-for-purpose analytical methodologies able to assess food authenticity.

Meat and meat products are worldwide consumed as one of the main dietary sources of protein, contributing to 40% of the total global protein consumption, leading to an increase in demand for these products with the population growth, and generating significant challenges in the food sector (Henchion et al. 2014). Incidents related to species substitution, adulteration, and fraud-labelling on meat and meat-derived products are common to generate illegal financial benefits, requiring new analytical approaches to assess their authenticity, quality, and safety (Cavin et al. 2018; Hrbek et al. 2020; Candoğan et al. 2021; Khalil et al. 2021). Since the huge food safety and security scandal of the detection of horsemeat in ready-meal food products marketed as 100% beef in Europe in 2013 (Kerschke Risch 2017), the number of fraudulent practices based on animal species substitution is increasing. This kind of fraud is even more relevant in countries with cultural/religious practices prohibiting the consumption of meat products from some specific animal species such as on Muslim countries regarding Halal products, where the consumption of pork, for example, is not allowed, or the case of Kosher products in Jewish communities (Hossain et al. 2021; Mortas et al. 2022). Despite the number of analytical methodologies developed to address this kind of authenticity issues, the identification of the animal species present in meat-derived products is easily solved by genetic detection tools based on DNA determination such as the use of real-time polymerase chain reaction (PCR) among other techniques (Ali et al. 2012; Khalil et al. 2021; Prachugsorn et al. 2022; Ramachandran et al. 2022; Rohman et al. 2024).

However, several meat authentication issues not involving different meat species cannot be solved by means of genetic detection tools. These cases also involved cultural/religious practices, for instance, in Halal or Kosher products where the way in which animals permitted for consumption are slaughtered, or how the meat products are processed, is perfectly established (Hossain et al. 2021). Other examples are related to the geographical origin of meat products, where the region where the animal has been raised becomes important, especially in those meat products with protected geographical indication (PGI) seals. For example, only in Spain, on 2022, 20 PGI raw meat products, including beef, pork, lamb, goatling, ox, and chicken, are registered (2023). Meat sustainable production practices that consider aspects such as organic production or animal welfare are also examples where fraudulent practices will not be detected by genetic methodologies. As previously mentioned, due to the fact that society is increasingly aware of good practices in meat production, and is willing to pay more for specific attributes that result in supposedly higher quality products at least from an organoleptic point of view, not so much as a nutritional one, the number of fraudulent cases based on the examples previously mentioned is increasing. In these cases, metabolomic analytical methodologies are emerging as the best options to address these authentication issues.

The metabolome is the complete set of small molecules called metabolites (such as metabolic intermediates, hormones and other signaling molecules, and secondary metabolites) that can be found in any biological sample, including food samples from animal or plant origin. Thus, all animal metabolites that may be present in a meat product will not only depend on the genetics of the animal but also on the phenotype, that is, all those external factors that have affected the animal at some point or another. Therefore, any environmental factor (geographical origin), feeding (organic production), animal care practices (animal welfare), or how the animal has been slaughtered (Halal or Kosher products) will affect the animal metabolome and, consequently, the metabolites that can be detected in its meat or derivatives. As a consequence, metabolomic studies will be very useful to assess meat authentication and to fight against fraudulent practices that cannot be easily solved by genetics (Cubero-Leon et al. 2014; García-Cañas and Simó 2019; Selamat et al. 2021; Zhang et al. 2021). Two main analytical strategies are normally employed in metabolomic studies: targeted (profiling) and non-targeted (fingerprinting) strategies. In targeted (profiling) metabolomic analysis, specific compounds or families of compounds, perfectly identified, are monitored by means of specifically designed methodologies providing high precision, selectivity, and reliability (Selamat et al. 2021). Despite some advantages of targeted methodologies such as low detection limits, simple data interpretation, and quantitative analysis, these approaches have several limitations such as the requirement of purified standards of the targeted compounds for correct identification, confirmation, and quantitation. In contrast, non-targeted (fingerprinting) metabolomics focus on monitoring all detectable analytes in one sample without requiring knowledge of which metabolites are detected before the gathering of the data (Selamat et al. 2021). Thus, non-targeted approaches are more high-throughput and comprehensive and may allow, if necessary,

the discovery of new compounds. However, the interpretation of data can be difficult, and normally will require the use of chemometric analysis to reduce the comprehensive data sets into smaller collections of controllable variables, especially when addressing classification and authentication issues.

In both cases, but specially in non-targeted approaches, the use of chromatographic separation techniques, mainly liquid chromatography (LC), in combination with high-resolution mass spectrometry (LC-HRMS) are among the most powerful techniques to address meat authentication issues by metabolomics (Man et al. 2021; Windarsih et al. 2022b, 2024c, b). For example, LC-HRMS metabolomics and chemometrics has been recently proposed for the analysis of dog meat adulteration in beef meatballs for halal authentication studies (Windarsih et al. 2024b). In another work, a mass spectrometry-based untargeted metabolomics approach was described for the discrimination among beef of different origins including geographical origins as well as feeding regimes (Man et al. 2021). Despite the great advantages of HRMS in metabolomic studies due to its high-resolution and accurate mass measurements, these techniques are quite expensive and require of specialized users, being not affordable in worldwide control laboratories specially in developing countries.

More affordable, simple, and less-expensive non-targeted methodologies based on spectroscopic (UV–vis, near-infrared, or Fourier transform infrared, among others) or even liquid chromatography with spectroscopic detection are also employed in food (including meat products) authentication taking advantage of fingerprinting metabolomic approaches, that is, recording as much instrumental chemical responses as possible of the analyzed samples without having to know the identity of the chemical compounds responsible for those responses (Candoğan et al. 2021; Hossain et al. 2021; Haider et al. 2024; Putri et al. 2024). These fingerprints are then proposed as sample chemical descriptors to address sample discrimination/classification by chemometrics, allowing fit-for-purpose methodologies to solve authentication issues.

Within this line, the present contribution aims to develop a simple high-performance liquid chromatography with ultraviolet detection (HPLC–UV) fingerprinting method to assess meat authenticity after a simple sample extraction procedure. This work does not aim to propose a fully validated method, an aspect that will be carried out later, but rather to evaluate the applicability of HPLC–UV fingerprints to tentatively assess meat authenticity as a proof-of-concept. The obtained HPLC–UV fingerprints were then employed as meat chemical descriptors for principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) chemometric methodologies in classification and authentication studies based on animal species, geographical origin, organic production, and Halal and Kosher products. Finally, several meat fraud cases based on the adulteration of organic, PGI, Halal, or Kosher meats were studied, and the adulteration levels detected and quantified by partial least squares (PLS) regression.

Materials and Methods

Materials

Methanol (Chromosolv™ for HPLC, ≥ 99.9%), acetonitrile (Supergradient ACS quality for UHPLC), and ethanol (absolute) were obtained from Panreac AppliChem (Barcelona, Spain). Formic acid (≥ 96%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water (Milli-Q quality) was purified using a Milli-Q Reference A + system from Merck (Darmstadt, Germany), and filtered through 0.22-µm nylon filters.

Instrumentation

An HPLC instrument (Agilent 1100 Series, Waldbronn, Germany) equipped with a binary pump (G1312A model), an automatic sample injector (WPALS G1367A model), a diode-array UV–visible detector (G1315B model), and a PC with the Agilent Chemstation for LC software was employed. Chromatographic separation for meat fingerprinting was accomplished using a Phenomenex Kinetex® C18 (100 × 4.6 mm I.D., 2.6 µm partially porous particle size) fused-core column (Torrance, CA, USA) under a universal gradient elution program using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) as the mobile phase components. The flow-rate employed was of 0.4 mL min^{-1}. Briefly, the gradient elution program used was as follows: from 0 to 1 min at 3% of solvent B (initial conditions); then a universal lineal gradient from 1 to 20 min up to 95% solvent B; the elution was then kept at 95% solvent B from 20 to 22 min; then back to initial conditions at 3% solvent B in 0.1 min (20–22.1 min), and finally these initial conditions were kept until minute 25 for column equilibration. UV–visible acquisition was performed from 190 to 400 nm, and HPLC–UV fingerprints at 280 nm were selected as meat chemical descriptors for authentication purposes. A meat aqueous extract injection volume of 5 µL was used.

Meat Samples

Meat samples belonging to eight different animal species and, in some cases, involving different geographical production regions, production practices (organic vs. non-organic), or religious production practices (Halal and Kosher meats) were obtained from local markets and supermarkets in Spain.

To evaluate the discrimination capability of the proposed HPLC–UV fingerprinting methodology based on the animal species, a total of 160 meat samples were analyzed (20 different samples for each animal species), including mammals such as rabbit, beef, pork, and lamb, and birds such as chicken, turkey, quail, and duck.

To evaluate meat geographical production region authenticity, lamb samples belonging to two different production regions in Spain (20 different samples for each sample class) were employed. Samples involved lamb produced in Catalonia and lamb produced in Aragon with a PGI stamp.

To evaluate meat production system authenticity, chicken and beef samples were employed (20 different samples for each sample class). In the first case, non-organic chicken meat samples and organic chicken meat samples were used, all of them produced in Catalonia (Spain). In the second case, non-organic beef meat samples, and organic beef meat samples coming from two different producer companies, all of them produced in Catalonia (Spain), were analyzed.

To evaluate meat production systems including religious practices, such as the way of animal slaughtering, lamb and beef samples were employed (20 different samples for each sample class). In the case of lamb, Halal and non-Halal lamb samples were analyzed, while for beef samples, beef, Halal beef, and Kosher beef samples were used.

For the optimization of the extraction solvent conditions, five meat samples (rabbit, beef, pork, lamb, and chicken) were used.

Sample Treatment

Meat samples were first processed by eliminating the fatty parts and grinding them using a meat blended-processor from Moulinex (Alençon, France). Only meat muscle parts were employed. Then, 1 g of meat was weighed in a 50-mL PTFE centrifuge tube (Serviquimia, Barcelona, Spain) and mixed with 10 mL of water by vigorously shaking with the help of a VibraMix Vortex (OVAN, Barcelona, Spain) for 1 min. Ultrasound-assisted extraction (UAE) was then performed for 15 min in a 5510 Branson ultrasonic bath (Barcelona, Spain). The samples were then centrifuged (5 min, $4000 \times g$) with a Rotina 420 Centrifuge (Hettich, Tuttingen, Germany). The obtained aqueous meat extracts were then filtered into 2-mL HPLC vials using 0.45-μm nylon syringe filters (FILTER-LAB, Barcelona, Spain), and kept at 4 °C until analysis.

For the optimization of the extraction solvent conditions, several extraction solvents with different polarity were employed: Milli-Q water (W), methanol (M), ethanol (E), and acetonitrile (A). Additionally, 1:1 (v/v) mixtures of these solvents were also tested: water–methanol (WM), water–ethanol (WE), water-acetonitrile (WA), methanol-ethanol (ME), methanol–acetonitrile (MA), and ethanol-acetonitrile (EA). All these extraction experiments were performed by triplicate.

Data Analysis

Meat Classification Studies

Aqueous meat extract samples were randomly analyzed with the proposed HPLC–UV method to minimize the influence of instrumental drift on the results. A quality control (QC) sample was prepared mixing 50 μL of each extract to evaluate the repeatability and robustness of the proposed methodology. For that purpose, the QC was analyzed after each ten aqueous meat extract samples. All the registered HPLC–UV chromatograms were then exported to a spreadsheet using Unichrom software from New Analytical Systems (Minsk, Belarus), obtaining for each sample an HPLC–UV fingerprint consisting of 3750 absorbance signal values at 280 nm as a function of chromatographic retention time. For chemometric analysis, only the chromatographic retention segment from 7 to 17 min was employed, thus obtaining for each sample an HPLC–UV fingerprint consisting of 1500 absorbance signal values at 280 nm. The SOLO autoscaling preprocessing was applied to all fingerprints prior to chemometric analysis to ensure that all variables were equally weighted. This autoscaling consisted on a mean-centering followed by division of each column (variable) by the standard deviation of that column. No other data preprocessing correction was employed.

Different data matrices were then built from the non-targeted HPLC–UV fingerprints, and they were submitted to a PCA, used as an exploratory method to evaluate the distribution of the meat samples and the QC samples behavior, and, to PLS-DA, employed as a supervised sample classificatory method according to meat species, geographical origin, or production (organic vs. non-organic; Halal vs. non-Halal; Kosher vs. non-Kosher) system. SOLO 8.6 chemometrics software from Eigenvector Research (Manson, WA, USA) was used to carry out these analyses. In PCA, the X-data matrix was built with the absorbance values recorded at 280 nm at a specific time over the chromatogram segment from 7 to 17 min for each sample. The same X-data matrix was employed for the PLS-DA analyses (without the QCs) together with a Y-data matrix defining each sample class (species, geographical origin, or production system). The number of LVs used to build PLS-DA models was established by the first relevant minimum of the cross-validation (CV) error from Venetian blind. When necessary, the ellipses delimitation areas in the score plots of both analyses were drawn manually to help with the visualization of the different sample clusters.

PLS-DA classification performance was evaluated by means of cross-validation sensitivities, specificities, and

accuracies (classification errors) (Riedl et al. 2015). Sensitivity describes the capability of the model to detect true positives, and it is calculated as TP/(TP + FN) in percentage, where TP (true positive) is the number of positive samples correctly assigned to the corresponding class and FN (false negative) is the number of positive samples incorrectly assigned as not belonging to the class. Specificity describes the capability of the model to detect true negatives, and it is calculated as TN/(TN + FP) in percentage, where TN (true negative) is the number of negative samples correctly assigned (i.e., not belonging to the studied class) and FG (false positive) is the number of negative samples incorrectly assigned to the studied class. Finally, the accuracy is calculated as the classification error as (TP + TN)/TS in percentage, where TS is the total number of samples.

When necessary, paired PLS-DA models were also built and validated using 70% of the samples (randomly selected) as a calibration set, and the remaining 30% of the samples being employed as the prediction (unknown) set.

Besides, to improve the classification performance, a classification decision tree consisting of consecutive dual PLS-DA models built using hierarchical model builder (HMB) was employed. For that purpose, the first 30% of the samples of each sample group were randomly selected to form a group of prediction samples (in total, 48 meat samples for the meat species authentication study), and the other 112 samples were then used to build the corresponding PLS-DA calibration models. First, a non-supervised PCA model was performed with the calibration set to detect (and remove) outliers. Then, in a first step, a multiclass PLS-DA model with these 112 aqueous meat extract samples employed for calibration was built to select the sample class that was better discriminated based on cross-validation sensitivity and specificity performance (if more than two sample groups provided similar performance, then the sample class was randomly selected). Then, a paired PLS-DA model of the selected sample class (i.e., sample class 1) against all the other samples was performed (first rule node of the classification tree), by employing the 112 samples of the calibration set, and the 48 "unknown samples" for prediction. Then, prediction samples belonging to sample class 1 correctly assigned to its group and prediction samples belonging to the other sample groups incorrectly assigned to sample class 1 were removed from the prediction set, while prediction samples belonging to sample class 1 incorrectly assigned as belonging to the other sample group were kept. Then, the second rule node of the classification tree is performed, also removing all the calibration samples belonging to sample class 1. A new multiclass PLS-DA model was then built to select the next sample group that was better discriminated (for instance, sample class 2), and then a paired PLS-DA model to sample class 2 versus all the other remaining samples was performed (second rule node of the classification tree), and the same process is performed until all the classification tree rule nodes are built. The classification decision tree performance was then evaluated by means of sensitivity, specificity, and accuracy (classification error) for calibration, cross-validation, and prediction, respectively.

Detection and Quantitation of Meat Adulteration

The potential of the proposed HPLC–UV fingerprints to detect and quantify meat adulteration by PLS was evaluated by means of six adulteration cases based on blended-meat adulterations from different geographical production regions or different production systems (including organic vs. non-organic, Halal vs. non-Halal and Kosher vs. non-Kosher meats). For that purpose, PLS calibration was performed at adulteration levels of 0 (pure meat), 20, 40, 60, 80, and 100% (pure adulterant meat). PLS validation was performed at adulteration levels of 15, 25, 50, 75, and 85%. Each adulteration percentage in calibration and validation was done by quintuplicate. In addition, an additional 50% adulteration level was employed as QC extract to control the reproducibility and robustness of the proposed methodology. The PLS X-data matrix consisted of the HPLC–UV fingerprints at 280 nm within the chromatographic segment from 7 to 17 min for each meat-blended sample and QC, while the Y-data matrix defined the adulteration percentages. The number of LVs in the PLS models was selected by Venetian blinds cross-validation.

Results and Discussion

Optimization of Sample Extraction Solvent

As commented in the introduction section, the aim of the present contribution is to evaluate the potential of HPLC–UV fingerprints obtained after a simple metabolite meat extraction, working in a non-targeted metabolomic approach without the requirement of identifying the identity of the extracted chemical compounds, to address meat authentication issues. With this aim, first, different pure solvents (Milli-Q water, methanol, ethanol, and acetonitrile) were employed for meat metabolite extraction, following the procedure described in materials and methods section, and the obtained extracts analyzed with the proposed HPLC–UV methodology. For this optimization, five meat samples (rabbit, beef, pork, lamb, and chicken) were tested, and all the extractions were performed in triplicate. As an example, Fig. S1 (supplementary information) shows the non-targeted HPLC–UV chromatogram at 280 nm of a pork sample extracted with pure Milli-Q water. In general, the HPLC–UV fingerprints obtained for the five meat samples under study were characterized for the presence of some

quite polar compounds, with high-signal intensities, eluting close to the chromatographic dead volume, and a group of less polar compounds, with lower signal intensities, and eluting within the range from 7 to 17 min. It should be mention that no important differences were observed for the first group of polar compounds within the five meat samples analyzed, while more notable difference in both compound profiling and signal distribution was observed for the second group of compounds. Thus, to evaluate the extraction capacity of the studied solvents and conditions, the area of the chromatogram signal registered at 280 nm in the chromatographic range from 7 to 17 min was employed (this parameter will be referred as the "total metabolite signal (TMS)" from now). Fig. S2 (supplementary information) compares the obtained TMS values for the different pure solvents employed on rabbit, beef, pork, lamb, and chicken meat samples. As can be seen, without any doubt, water is the pure solvent that most metabolites extract under the evaluated extraction conditions, with a significance difference, followed by methanol. Regarding the other two solvents, ethanol and acetonitrile, the metabolite extraction is clearly higher for acetonitrile in the case of beef, while no significant differences were observed for the other analyzed meats. Besides, important differences were also observed on the TMS parameter depending on the meat sample under study, with lamb showing the highest metabolite extraction, followed by beef, pork, chicken, and rabbit when, for example, water was employed.

Even though it seems clear that water is the solvent that can extract most metabolites under the studied conditions, extraction was also valuated by employing 1:1 (v/v) solvent mixtures, to evaluate if their mixed properties helps in the solvent extraction capacity. Thus, six 1:1 (v/v) solvent combinations were employed, i.e., water:methanol (WM), water:ethanol (WE), water:acetonitrile (WA), methanol:ethanol (ME), methanol:acetonitrile (WA), and ethanol:acetonitrile (EA), and the comparison of the obtained TMS values on the five meats under study is shown in Fig. S3 (supplementary information). As observed, solvent mixtures with water are the ones with the highest extraction capacity, being the mixture of water with methanol the one providing the highest TMS value, followed by the mixtures of water with ethanol and acetonitrile, respectively. In any case, none of the solvent mixtures evaluated provided higher TMS values than those obtained when using pure Milli-Q water (Fig. S2); thus, water was selected as the optimal extraction solvent for the present work.

HPLC–UV Fingerprints of Meat Samples

As previously commented in the introduction section, chromatographic metabolomic fingerprinting studies focus on recording the instrumental signal (absorbance, in this case) as a function of chromatographic retention time, with no previous knowledge of the components present in the samples, and monitoring as much instrumental response signals as possible, to be able to assess sample discrimination based on different sample attributes. The obtained HPLC–UV fingerprints can then be employed as potential meat chemical descriptors to address its characterization and classification.

Thus, once the extracting solvent was selected, 1 g of each meat sample was extracted with water following the extraction procedure described in the sample treatment section. Chromatographic fingerprints of the aqueous meat extracts were obtained by reversed-phase separation using a porous-shell C18 column and 0.1% aqueous formic acid and acetonitrile as mobile phase components under universal gradient elution conditions. All the samples were analyzed randomly, and injecting a QC solution every ten samples. A total of 160 meat samples involving eight meat species (mammals: pork, beef, lamb, and rabbit; birds: chicken, turkey, duck, and quail) were analyzed. Figure 1 shows, for illustration, the obtained HPLC–UV fingerprints, within the 7- to 17-min range, for each meat sample group under study. Important differences can be observed on the obtained peak signal profiling regarding both the number of peaks as well as the peak intensities depending on the meat species, especially on the region from 9 to 10 min and from 12.5 to 15 min, where the most intense peaks are appearing. Besides, other compounds with lower peak signals can also be observed through the chromatographic region from 10 to 12.5 min that may also be important discriminant chemical descriptors when addressing sample classification. Therefore, the differences observed in the obtained HPLC–UV fingerprints (in both metabolite signal profiles and intensities), and the fact that the obtained fingerprints seem to be reproducible for all the samples within their specific sample class (meat species), suggested that this HPLC–UV fingerprinting data could be proposed as sample chemical descriptors to study the classification of meat species by chemometrics.

Exploratory Non-supervised PCA

Once the HPLC–UV fingerprints of the 160 meat samples and QCs were obtained, the characterization and classification of the analyzed meat samples was assessed by chemometrics. First, an exploratory non-supervised PCA study was conducted. The main objective when employing PCA was to evaluate the reproducibility and robustness of the proposed methodology by studying the behavior of all the QCs analyzed through the sample sequence, as well to see if there is also an initial sample discrimination among the different sample groups (meat species) under study. Therefore, an X-data matrix with the corresponding HPLC–UV fingerprints (280 nm, segment from 7 to

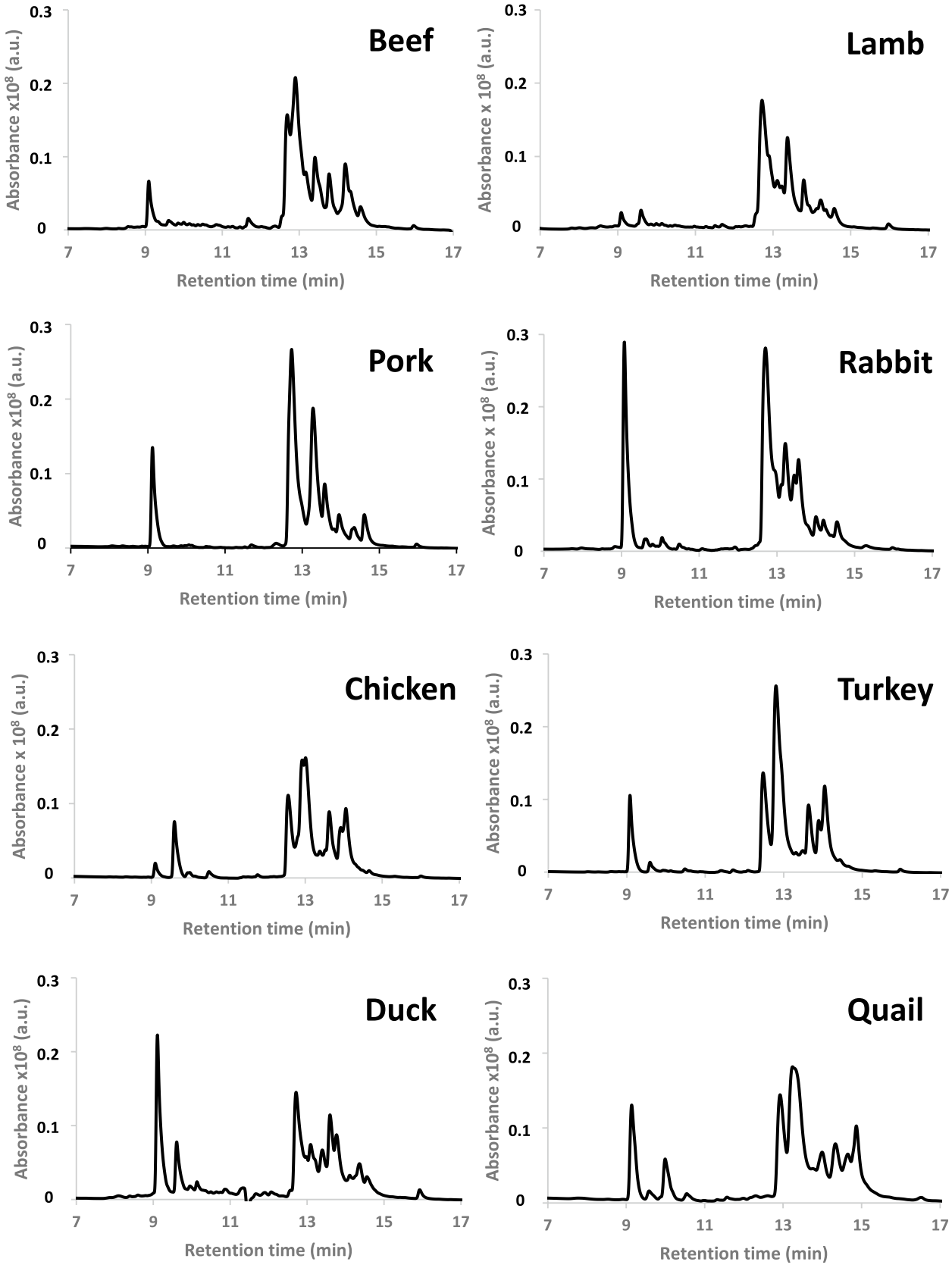

Fig. 1 HPLC–UV fingerprints (at 280 nm, segment from 7 to 17 min) for one selected sample of each meat specie under study

17 min) for the 160 meat samples and QCs analyzed was built and submitted to PCA. This data matrix has dimension of (180 × 1500) being defined by Samples + QCs × absorbance values (as a function of retention time). The obtained PCA model was built by employing eight PCs (describing 87.68% of the total sample variance). For illustration, Fig. S4 (supplementary information) shows the obtained PCA score plot of PC1 vs. PC2. As can be seen, QCs appeared clustered close to the center area of the plot, showing that no instrumental drifts affected the obtained results and, consequently, validating the robustness and reproducibility of the proposed HPLC–UV fingerprinting strategy. This behavior with the corresponding QCs was also observed in all the PCA studies performed in this work prior to classification studies, demonstrating the long-term stability of the HPLC–UV fingerprints over extended periods. Besides, meat samples also tend to be clustered according with the animal species, even though some of them appeared overlapped (when only two PCs are plotted). Briefly, beef and quail samples are the two sample groups completely discriminated from the others, exhibiting in both cases positive PC1 values, and being differentiated among them by PC2, with positive and negative PC2 values for quail and beef, respectively. Chicken and turkey samples appeared overlapped on the left-top area of the plot (with negative PC1 and positive PC2 values). All the other sample groups tend to be located at the bottom of the plot (with negative PC2 values), with some overlapping and a certain discrimination through PC1. In any case, it should be considered that sample discrimination is accomplished with the eight PCs obtained in the PCA model, and cannot be visualized when only two PCs are depicted in 2D plots.

Meat Classification Based on Animal Species by PLS-DA

After verifying the HPLC–UV method robustness and reproducibility by the PCA behavior of QCs, a supervised PLS-DA study was conducted employing the HPLC–UV fingerprints previously obtained to classify the analyzed samples according to the animal species. In this study, QCs were not considered. Thus, a X-data matrix with a dimension of (160 × 1500) being defined by Samples × absorbance values (as a function of retention time) was employed, while the Y-data matrix defined the sample category (animal species). After subjecting the data matrix to PLS-DA, a model employing seven LVs was built (explaining 83.59% of the sample variance), and the obtained score plots of LV1 vs. LV2 and of LV1 vs. LV2 vs. LV3 are shown in Fig. 2. As can be seen, a good discrimination capacity was accomplished. Samples tend to be grouped according to the animal species, and when plotting the first three LVs, almost all the sample groups are visually discriminated, with the exception of chicken and turkey samples. However, as previously commented, sample discrimination is accomplished with the seven LVs defined in the PLS-DA model, so to better evaluate the PLS-DA classification performance of the proposed methodology, sensitivity, specificity, and accuracy values were determined. Table 1 shows the PLS-DA cross-validated multiclass prediction performance for the set of meat samples when using seven LVs. Figures of merit demonstrate the good performance of the proposed methodology, with overall sensitivities, specificities, and accuracies of 100% for calibration, with the exception of a 0.36% classification error (99.64% accuracy) for quail

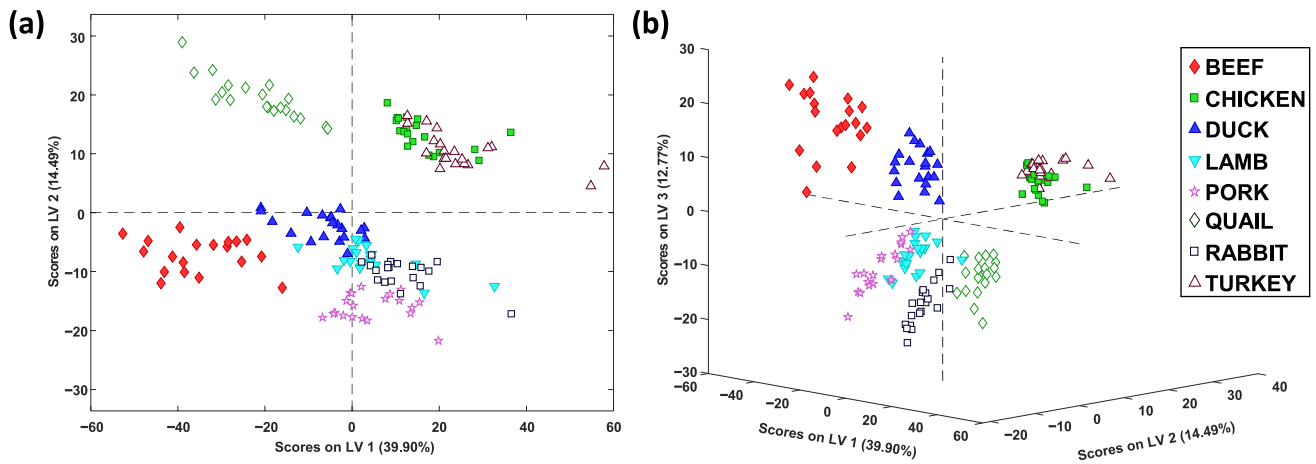

Fig. 2 Partial least squares-discriminant analysis (PLS-DA) score plots of **a** LV1 vs. LV2 and **b** LV1 vs. LV2 vs. LV3 when employing HPLC–UV fingerprints (280 nm, segment from 10 to 17 min) as sample chemical descriptors of the analyzed meat samples. The PLS-DA model was built by employing 7 LVs

Table 1 PLS-DA multiclass predictions by cross-validation for the set of meat samples based on animal species

Sample class variety	Sensitivity (%)		Specificity (%)		Accuracy (classification error, %)	
	Calibration	Cross-validation	Calibration	Cross-validation	Calibration	Cross-validation
Beef	100	100	100	100	0	0
Chicken	100	100	100	99.3	0	0.36
Duck	100	100	100	100	0	0
Lamb	100	100	100	100	0	0
Pork	100	100	100	100	0	0
Quail	100	99.3	100	99.3	0.36	0.36
Rabbit	100	100	100	100	0	0
Turkey	100	100	100	100	0	0

samples. Regarding cross-validation parameters, again sensitivity, specificity, and accuracy values of 100% are obtained for most of the cases, with the exception of chicken samples, showing a specificity of 99.3% and an accuracy with a classification error of 0.36%, and quail samples, showing both sensitivity and specificity values of 99.3%, and again an accuracy of 99.64% (classification error of 0.36%).

The obtained data demonstrate the suitability of the proposed HPLC–UV fingerprints (at 280 nm, chromatographic segment from 10 to 17 min), obtained after a simple sample extraction with pure water, as meat chemical descriptors to assess their classification and authenticity based on the animal species.

PLS-DA models to study the classification of mammal and bird meat animal species, independently, were also evaluated following the same procedure. In both cases, a X-data matrix with a dimension of (80 × 1500) being defined by Samples × absorbance values (as a function of retention time) were employed, while the Y-data matrix defined the sample category (animal species). For illustration, Fig. S5 (supplementary information) shows the obtained 3D-PLS-DA score-plots of LV1 vs. LV2 vs. LV3 for the classification of (a) mammal meat and (b) bird meat animal species, respectively. In addition, Table S1 and S2 (supplementary information) summarize the PLS-DA performance data when mammal meat and bird meat animal species are studied, respectively. As can be seen, in both cases, the discrimination capability of the proposed HPLC–UV fingerprints is very acceptable. In the case of mammal meat animal species, sensitivity, specificity, and accuracy values of 100% were observed for all sample groups (Table S1). The same trend is observed for most of the sample classes in the case of bird meat animal species, with the exception of chicken that depicted a calibration specificity of 95.0% and a cross-validation classification error of 2.5%, and of turkey, showing both sensitivity and specificity cross-validation values of 98.3%, and both calibration and cross-validation classification errors of 0.8%. In any case, these are also very acceptable PLS-DA performance values.

Finally, although the multiclass PLS-DA model employed for the classification of the eight meat animal species showed a very good performance, the prediction capability of the model was evaluated by means of a classification decision tree consisting of consecutive dual PLS-DA models built using hierarchical model builder. Thus, 30% of the samples were randomly selected for each sample class (48 samples in total) and used as unknown samples for prediction, while the other 112 samples were employed to build the different dual PLS-DA models for each rule node. For illustration, Fig. 3 shows the flow-chart of the designed classification tree employed with the seven rule nodes involved, together with the matrix dimensions and the LVs used for the paired PLS-DA calibrations: (1) beef vs. others; (2) duck vs. others (excluding beef samples); (3) pork vs. others (excluding beef and duck samples); (4) chicken vs. others (excluding beef, duck, and pork samples); (5) lamb vs. others (excluding beef, duck, pork, and chicken samples); (6) quail vs. others (excluding beef, duck, pork, chicken, and lamb samples); and (7) rabbit vs. turkey (excluding beef, duck, pork, chicken, and quail samples). Table 2 shows the obtained classification decision tree confusion matrix for prediction, and the accuracy for each meat species under study.

As shown in Table 2, the prediction capability of the proposed classification decision tree was excellent, with a 100% accuracy in the prediction of the 48 "unknown" samples within their corresponding sample group, clearly improving the performance obtained when a conventional multiclass PLS-DA model was employed.

Although a simple HPLC–UV fingerprinting strategy is proposed, the accomplished meat specie discrimination is similar or even better than the one described in the literature with more complex and expensive methodologies. For example, Windarsih et al. (2024c) demonstrated that non-targeted LC-HRMS metabolomics in combination with chemometrics was able to perfectly discriminate between

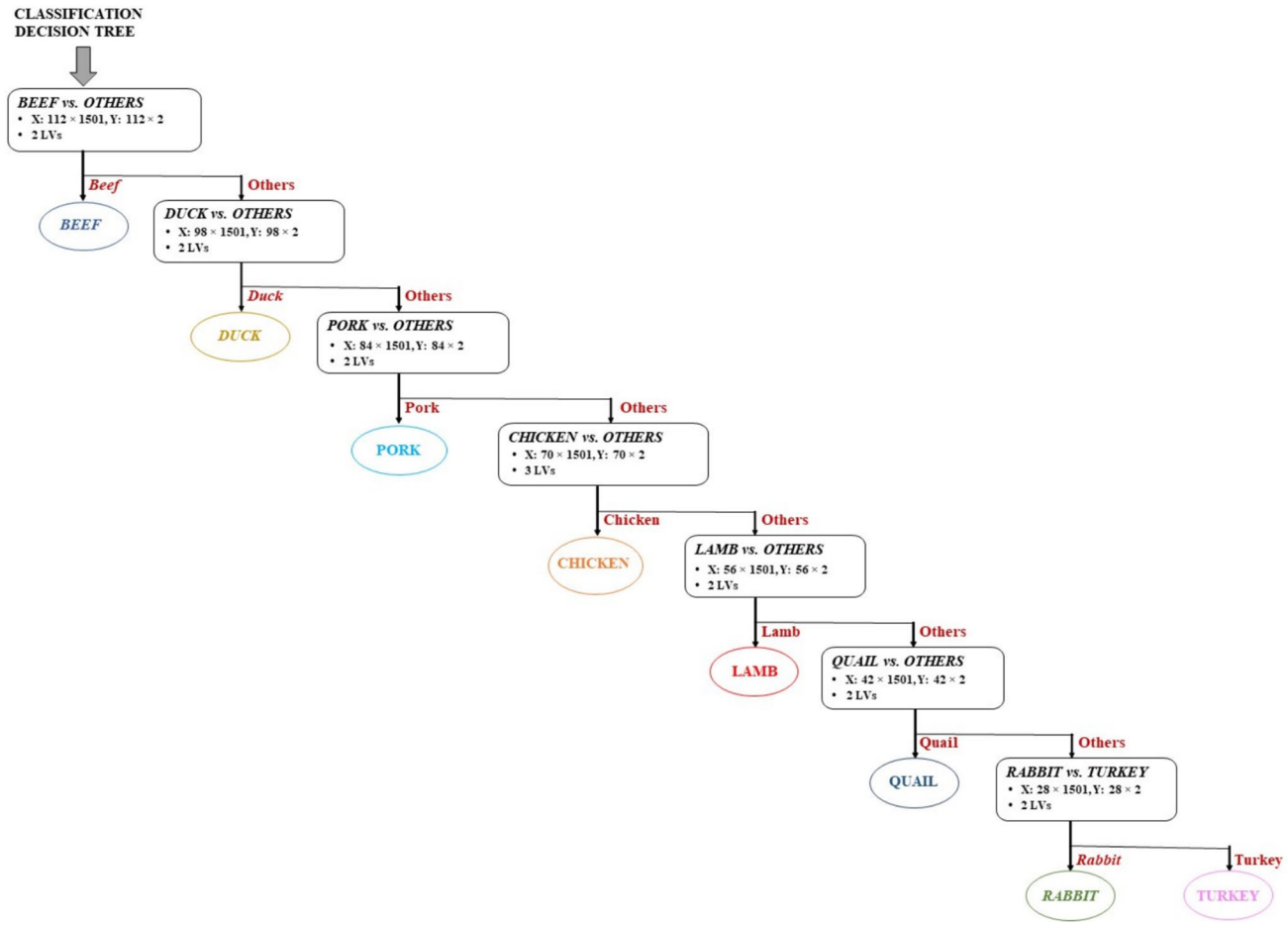

Fig. 3 Flowchart of the classification decision tree constructed using PLS-DA models as the rule nodes. The dimensions of the data matrices and LVs employed to build the paired PLS-DA calibration models are indicated

Table 2 Prediction confusion matrix for animal meat species when using a classification decision tree based on dual PLS-DA models

	Beef	Chicken	Duck	Lamb	Pork	Quail	Rabbit	Turkey	Accuracy (%)
Beef	6	0	0	0	0	0	0	0	100
Chicken	0	6	0	0	0	0	0	0	100
Duck	0	0	6	0	0	0	0	0	100
Lamb	0	0	0	6	0	0	0	0	100
Pork	0	0	0	0	6	0	0	0	100
Quail	0	0	0	0	0	6	0	0	100
Rabbit	0	0	0	0	0	0	6	0	100
Turkey	0	0	0	0	0	0	0	6	100

beef, chicken, and wild boar meats. Obviously, the use of HRMS allow the identification of metabolites, being one of the disadvantages of our proposed methodology, but for meat specie authentication purposes this is not required, and more simple, non-expensive, and affordable techniques such as HPLC–UV fingerprinting can be employed, as demonstrated.

Meat Classification Based on Different Meat Attributes (Production Systems, Geographical Origin, Halal, or Kosher meats) by PLS-DA

As previously demonstrated, the obtained HPLC–UV fingerprints within the chromatographic segment from 7 to 17 min resulted on excellent sample chemical descriptors to

accomplish meat discrimination and authentication based on the animal species. However, nowadays, this authentication issue can also be solved by employing genetic-based methodologies. In contrast, as commented in the introduction section, other meat authentication issues based on some meat attributes highly considered nowadays by society are not easily detected by genetics. This is the case, for instance, when the geographical production region of meat needs to be assessed, especially in the case of meat products with protected geographical indications (PGIs), or the case of different meat production systems, such as those based on organic-production practices, or meats produced under religious production systems such as Halal or Kosher products. The potential of the obtained HPLC–UV fingerprints to assess these meat authenticity issues was also evaluated by employing chicken, lamb, and beef meats, based on sample availability.

Thus, chicken samples were employed to address meat organic production authenticity issues. For that purpose, 20 conventional and 20 organic produced chicken samples (all of them from Catalonia, Spain) were analyzed with the proposed methodology, and the obtained HPLC–UV fingerprints (segment from 7 to 17 min) submitted to PLS-DA analysis. Important differences on the metabolite signals detected in the obtained fingerprints (Fig S6, supplementary information) can be observed, especially regarding the intensity of some of them in the organic-produced chickens. Figure 4a shows the obtained PLS-DA score plot of LV1 vs. LVs (model built with two LVs), depicting a perfect discrimination of both sample groups based on LV1, and attaining sensitivity, specificity, and accuracy values of 100%.

In the case of lamb meats, three sample classes were employed: (i) lamb meat produced in Catalonia (Spain), (ii) lamb meat with PGI produced in Aragon (Spain), and (iii) Halal lamb meat produced in Catalonia (Spain). Twenty samples per group were analyzed with the proposed methodology, and the obtained HPLC–UV fingerprints (segment from 7 to 17 min) subjected to PLS-DA analysis to address both geographical production region and Halal produced meat issues, simultaneously. Fig. S7 (supplementary information) depicts some differences in the detected metabolite signals of the corresponding chromatographic fingerprints that will help on the chemometric discrimination of the analyzed samples. The PLS-DA score plot (building the model with four LVs) of LV1 vs. LV2 is depicted in Fig. 4b. Excellent discrimination was accomplished for the three sample groups, with LV1 allowing to discriminate conventional lamb (negative LV1 values) against the other two sample groups, and LV2 allowing to discriminate between Halal lamb samples (negative LV2 values) against the other two sample groups. Sensitivity, specificity and accuracy values of 100% were also obtained for the three sample groups.

Finally, beef meat samples belonging to five sample classes were also analyzed (all of them produced in Catalonia (Spain)): (i) beef meat, two different organic beef meats (grown from different organic producers) labeled as (ii) organic beef 1 and (iii) organic beef 2, (iv) Halal beef meat, and (v) Kosher beef meat. Examples of the obtained HPLC–UV fingerprints (segment from 7 to 17 min) are provided in Fig. S8 (supplementary information), showing differences in the detected metabolites extracted with water. PLS-DA was then employed to address discrimination of organic, Halal, and Kosher production systems, simultaneously, and the obtained score plot (model built with three LVs) of LV1 vs. LV2 vs. LV3 is depicted in Fig. 4c. PLS-DA multiclass prediction values by cross-validation are summarized in Table 3. As can be seen in the 3D PLS-DA plot, a very acceptable sample discrimination among the five beef sample groups under study was obtained. Calibration and cross-validation sensitivity values higher than 95.0% were achieved, while specificity values higher than 95.0% and 91.2% were obtained for calibration and cross-validation, respectively. Accuracy worsened in comparison to the previous addressed examples, although classification errors lower than 6.25% and 6.9% were observed for calibration and cross-validation, respectively, which are quite acceptable considering the sample types under study. In any case, when addressing each beef meat attribute under study separately, i.e., organic production and halal/kosher production, results improved considerably, as can be seen in Fig. S9 (supplementary information) depicting the corresponding PLS-DA plots when studying beef samples against organic produced beef (Fig. S6a) and beef samples against Halal and Kosher beef (Fig. S6b). In the case of the beef organic production, sensitivity, specificity, and accuracy values of 100% were obtained, with the exception of cross-validation specificity (> 95.0%) and accuracy (> 97.5%). However, when addressing Halal and Kosher beef production, sensitivity, specificity, and accuracy values of 100% were observed for all the sample groups.

Overall, the obtained results demonstrate that the proposed HPLC–UV fingerprinting strategy by obtaining metabolite instrumental responses (without the requirement of compound identification) after a simple meat water sonication extraction provide excellent sample chemical descriptors for chemometrics to address meat authenticity issues based on different meat attributes such as geographical region, organic, and Halal/Kosher production systems.

As commented in the introduction section, the main objective of the present contribution was to evaluate the applicability of simple HPLC–UV fingerprints as chemical descriptors to assess several meat authentication issues that cannot be easily solved my beans of genetic tests. However, to ensure the method performance for future validation, an independent group of meat samples will be employed for prediction purposes. In any case, the prediction capability accomplished when using the PLS-DA model based on

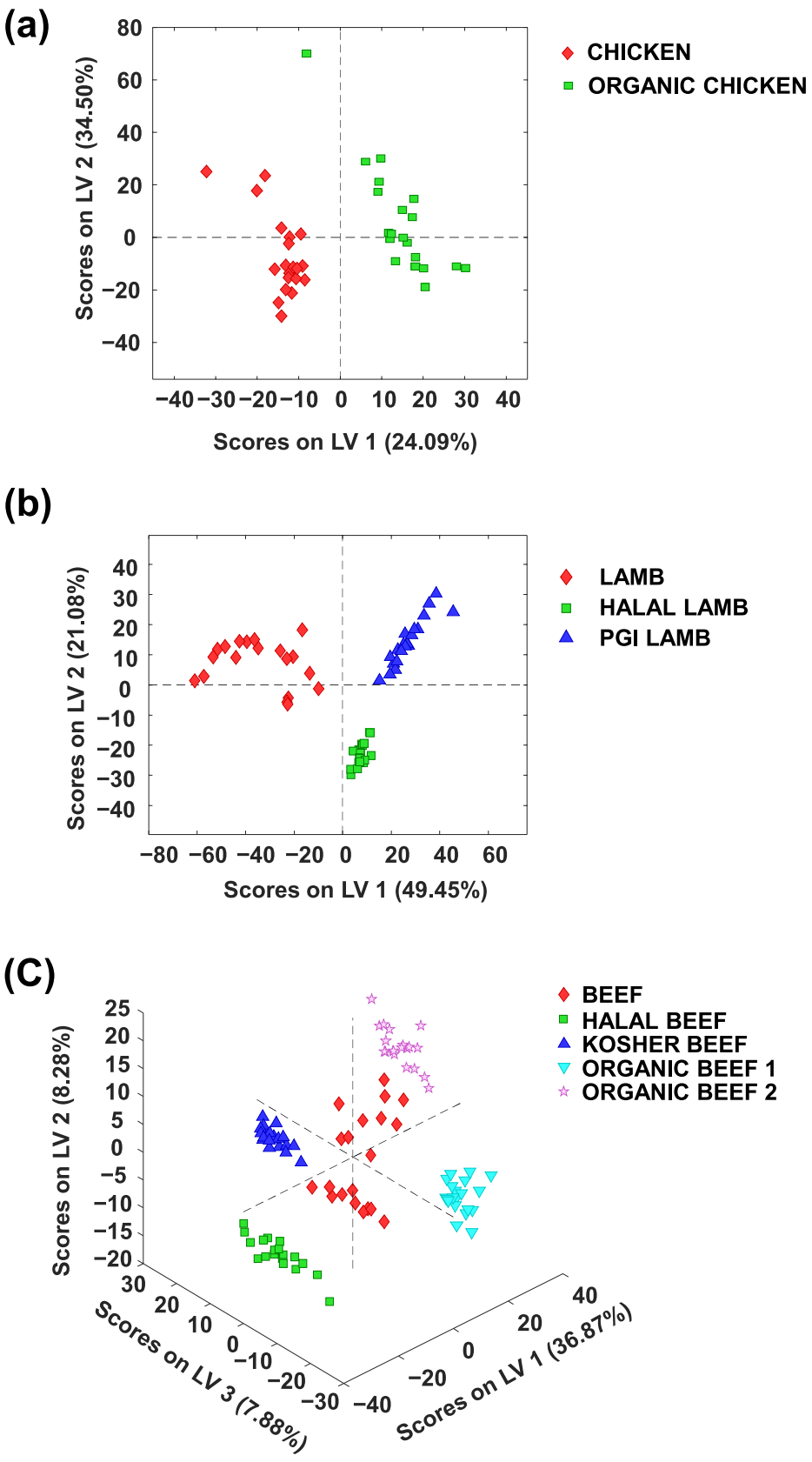

Fig. 4 Partial least squares-discriminant analysis (PLS-DA) score plots when employing HPLC–UV fingerprints to address classification of meat samples based on different meat production attributes. **a** Chicken samples, **b** lamb samples, and **c** beef samples

Table 3 PLS-DA multiclass predictions by cross-validation for the set of beef meat samples

Sample class variety	Sensitivity (%)		Specificity (%)		Accuracy (classification error, %)	
	Calibration	Cross-validation	Calibration	Cross-validation	Calibration	Cross-validation
Beef	95.0	95.0	95.0	91.2	5	6.9
Halal beef	100	100	100	100	0	0.36
Kosher beef	100	100	100	100	0	0
Organic beef 1	100	100	98.8	97.5	6.25	1.3
Organic beef 2	100	95.0	100	100	0	2.5

HCA was excellent, showing that important discriminants features are present on the obtained HPLC–UV fingerprints. Identification of these discriminant chemicals will also be performed in future studies by means of LC–MS and LC-HRMS methodologies.

Detection and Quantitation of Meat Adulteration Frauds by PLS Regression

The potential of the proposed HPLC–UV fingerprints to detect and quantify meat adulterations based on the meat attributes previously described by PLS regression was also evaluated. For that purpose, six adulteration cases were studied: (i) PGI lamb (produced in Aragon, Spain) adulterated with lamb (produced in Catalonia, Spain), (ii) organic chicken adulterated with non-organic chicken, (iii) organic beef adulterated with non-organic beef, (iv) Halal lamb adulterated with non-Halal lamb, (v) Halal beef adulterated with non-Halal beef, and (vi) Kosher beef adulterated with non-Kosher beef. For PLS regression, two sets of adulterated blended meat samples were prepared for training and validation/prediction. Meat blended samples were prepared by pooling different meat samples belonging to the same sample group. The training set included the adulteration levels of 0 (pure studied meat), 20, 40, 60, 80, and 100% (pure meat considered as the adulterant). The set for validation/prediction consisted on adulteration levels of 15, 25, 50, 75, and 85%. All the adulteration levels were prepared in quintuplicate. An additional 50% adulteration level was prepared for each case under study to be employed as QC to assess and control the robustness and reproducibility of the PLS predictions. All the adulterated samples of each case under study were analyzed randomly and the QC was injected every ten samples injections. For each adulteration case studied, first the obtained HPLC–UV fingerprints (segment from 7 to 17 min) were subjected to PCA to evaluate the behavior of the QCs and to see the distribution of the different adulteration levels in the PC1 vs. PC2 plot. In all cases, QCs appeared clustered together, demonstrating the good robustness and reproducibility of the proposed methodology, and samples tend to be distributed through PC1 based on the adulteration content with pure samples located at opposite ends of PC1. Then PLS regression was performed for each adulteration case studied, and the obtained Y predicted 1 vs. Y measured 1 plots are shown in Fig. 5. PLS performances accomplished with the six meat adulteration cases are summarized in Table 4.

As can be seen, overall, very acceptable performances were observed for de detection and quantitation of meat frauds based on blended adulterations with different meat production systems. Good calibration linearity was obtained in all the adulteration cases under study, with R^2 values in the range 0.928–0.998 for the calibration scatter plots of predicted vs. measured adulteration levels.

PLS calibration, cross-validation, and prediction errors within the ranges of 1.7–9.0%, 3.9–10.6%, and 2.4–6.6%, respectively, were obtained. These figures of merit demonstrate the potential of the obtained HPLC–UV fingerprints (segment from 7 to 17 min) as good sample chemical descriptors not only for classification purposes, as previously described, but also to detect and quantify meat adulteration frauds that cannot easily be detected by genetics such as the meat geographical production region or meat production practices (organic, or Halal and Kosher products).

It must be highlighted that these chemical descriptors are obtained after a very simple meat sample extraction procedure based on sonication with water, and the corresponding analysis with a simple HPLC–UV strategy, methodologies affordable in most food quality laboratories, without the requirement of more expensive instrumentation, such as LC–MS or LC-HRMS, nor the necessity of identifying or targeting specific metabolites thanks to the fingerprinting approach, resulting in an easy-to-use and economical methodology. Obviously, the proposed methodology will lack the potential of compound identification that can be affordable with LC–MS or LC-HRMS methods (Man et al. 2021; Windarsih et al. 2022b, a, 2023, 2024a). Another handicap of the proposed methodology that will need to be evaluated in the future is the detection capability on meat adulteration, which at the moment cannot exceed those of LC-HRMS methodologies. Although this factor will not be important in some of the meat attributes under study if an illicit profit is intended (relatively high adulteration levels can be expected, for example, in the case of geographical

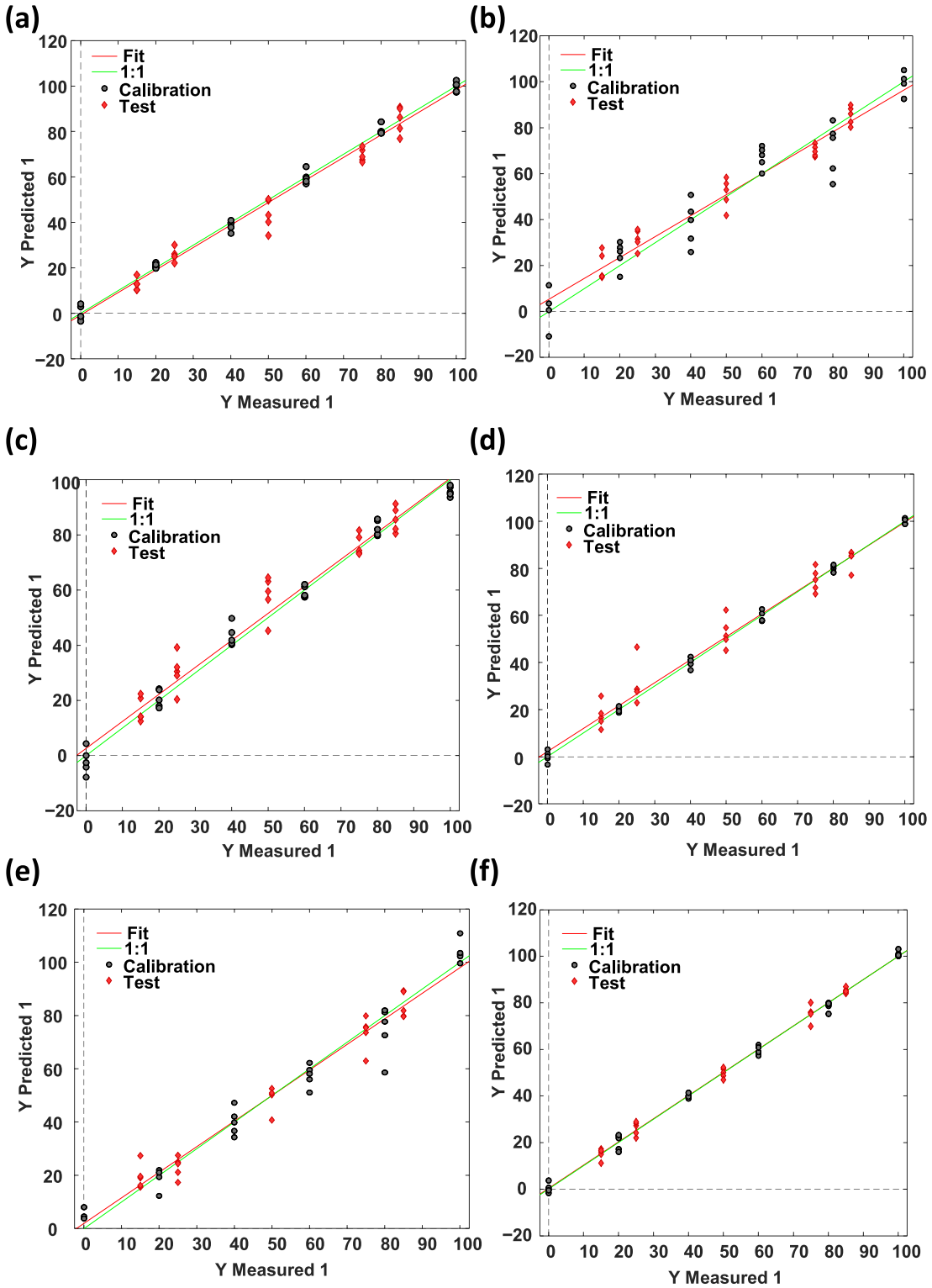

Fig. 5 Partial least squares (PLS) regression results depicting the scatter plots of predicted vs. measured percentages when evaluating different meat adulteration cases. **a** PGI lamb adulterated with conventional lamb, **b** organic chicken adulterated with non-organic chicken, **c** organic beef adulterated with non-organic beef, **d** Halal lamb adulterated with non-Halal lamb, **e** Halal beef adulterated with non-Halal beef, and **f** Kosher beef adulterated with non-Kosher beef

Table 4 Evaluation of meat adulterations by PLS using HPLC–UV fingerprints as chemical descriptors

Adulteration case[a]	LVs	Linearity (R^2)	Calibration error (%)	Cross-validation error (%)	Prediction error (%)
PGI lamb	4	0.996	2.2	4.1	5.7
Organic chicken	2	0.928	9.0	10.0	6.1
Organic beef	3	0.987	3.9	10.6	6.6
Halal lamb	6	0.998	1.7	5.8	6.4
Halal beef	2	0.966	6.2	7.9	5.2
Kosher beef	5	0.997	2.0	3.9	2.4

indications or organic production), in other situations, such as the case of Halal or Kosher products, the capability to detect low adulteration levels will be mandatory, and at least levels lower than 5% will need to be detected. In any case, and as a proof-of-concept, we demonstrated that at least we can detect and quantify meat adulteration levels down to 15%, which can be a first approach for a simple and affordable screening method in food control laboratories.

Conclusions

The results obtained in this work demonstrate that a relatively simple technique widely implemented in the vast majority of laboratories, such as liquid chromatography with ultraviolet detection, used for non-targeted metabolomic analysis, allows to obtain HPLC–UV fingerprints that are very good chemical descriptors in meat authentication and in the detection of meat fraud. In addition, different (relatively polar) solvents were evaluated for the non-targeted extraction of metabolites in meat, with the best results being observed when simply using water as the extraction solvent, which allows us to propose a simple, inexpensive sample treatment method that generates little waste. The study of the HPLC–UV fingerprints of the aqueous extracts obtained from the different meats analyzed allowed us to detect that most of the metabolites of interest elute in the chromatographic segment from minute 7 to minute 17. These differences were based on both the metabolite profile and the relative abundances of the obtained signals, as well as between the different differentiating attributes of the samples analyzed such as the animal species, the geographical origin of the samples, and the organic or non-organic production of the animals, as well as Halal or Kosher products. Thus, HPLC–UV fingerprints (chromatographic segment from 7 to 17 min) were proposed as the final meat chemical descriptors for meat authentication by chemometrics.

As a proof of concept, despite the fact that genetic detection methodologies are able to detect meat fraudulent practices involving different animal species, the proposed methodology was evaluated for the classification and authentication of eight meat group samples (involving mammals and birds) by PLS-DA. Results demonstrated a very good sample discrimination capability of the obtained fingerprints (when simultaneously considering the eight groups of samples), with sensitivity and specificity values of 100% and higher than 99.3% for calibration and cross-validation, respectively. The observed accuracy was also very good, with classification errors below 0.36% for both calibration and cross-validation. In order to evaluate the prediction capability of the proposed methodology for the authentication of meat species, a classification decision tree consisting of consecutive dual PLS-DA models built using a hierarchical model builder was applied by using only 70% of the samples (randomly selected) for the calibration models, and submitting the other 30% of the samples (48 meat samples) as unknown samples for prediction. Excellent results were observed, with a 100% accuracy on the prediction capability of the proposed methodology.

It must be highlighted the high-capability of the proposed methodology to address meat authentication issues involving meat attributes that cannot be solved by genetic methodologies, such as the meat geographical origins, the animal organic production systems, or cultural/religious aspects such as Halal or Kosher meats. PLS-DA discrimination was perfect when addressing organic chicken versus non-organic chicken, and lamb vs. halal lamb and PGI lamb. In the case of beef samples, involving five sample groups (beef, two organic beefs, Halal beef, and Kosher beef), results were also very acceptable, with overall calibration and cross-validation values higher than 95.0% and 91.2% for sensitivity and specificity, respectively, and with classification errors below 6.9%. In any case, when addressing independently beef vs. organic beefs, or beef vs. Halal/Kosher beefs, the PLS-DA discrimination performance improved.

Finally, the obtained fingerprints were also very appropriate to easily detect and quantify meat adulteration levels by PLS regression. As a proof of concept, six adulteration cases involving adulterations on PGI, organic, Halal, and Kosher meats were evaluated, with good PLS linearities, and with calibration, cross-validation, and prediction errors below 9%, 10%, and 6.6%, respectively.

Thus, HPLC–UV fingerprinting analysis of meat samples after water extraction working in a non-targeted metabolomic approach (without the requirement of metabolite identification) resulted in a simple, feasible, fit-for-purpose, and non-expensive methodology to assess meat authentication issues based on meat fraudulent practices involving species, geographical origin, and organic, Halal, or Kosher production systems. Future studies will be performed to ensure full method validation by evaluating the PLS-DA prediction performance employing an independent meat data set, as well as to establish the detection capability of the proposed methodology specially on Halal/Kosher authentication issues. Liquid chromatography coupled with mass spectrometry (both low- and high-resolution mass spectrometry) will also be employed for the characterization and identification of the most discriminant metabolites aiming to propose future meat biomarkers for authenticity purposes.

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Data Availability Data is provided within the manuscript or supplementary information files. Other data will be made available by authors under request.

Declarations

Competing Interests The authors declare no competing interests.

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