Journal of Food Composition and Analysis 115 (2023) 104976

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

In the trail of "Maçã de Alcobaça" protected geographical indication (PGI): Multielement chemometrics as a security and anti-fraud tool to depict clones, cultivars and geographical origins and nutritional value

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ARTICLE INFO

Keywords:
Elemental fingerprints
Chemometrics
Traceability
Authenticity
"Maçã de Alcobaça" certification

ABSTRACT

Food fraud associated with the intentional mislabelling of non-Protected Geographical Indication (PGI) is a concern for consumers. "Maçã de Alcobaça" (Alcobaça apple) is one of the oldest Portuguese PGI products, characteristic of the main apple-growing regions in the country, being of utmost importance to develop traceability and authenticity tools to depict the PGI certification status of these products. Pulp multielement signatures were able to discriminate with moderate accuracy (65.7 %) different Royal Gala clones, grown within the same cultivation area. Moreover, Variable Importance in Projection Partial Least-Squares Discriminant Analysis (VIP-PLS-DA) allowed the discrimination of the Royal Gala samples from different PGI producers with 70.0 % accuracy. Apple PGI cultivars were also discriminated accurately (82.0 %). Expanding the approach to non-PGI production areas, several cultivars could be distinguished, according to their provenance with high accuracy, namely Starking (100.0 % accuracy), Granny Smith (100.0 % accuracy), Fuji (100.0 % accuracy), Royal Gala (86.7 % accuracy) and Reineta (90.3 % accuracy). The PGI fruit's microelement nutritional traits highlighted their higher nutritional value, an important trait for food fraud reduction, informing the consumer of the product authenticity, and providing insights on the nutritional value of these high-value market products.

1. Introduction

According to the European Union (EU) eAmbrosia database of agricultural products and foodstuffs, wine, and spirit drinks that are legally registered and protected across the EU, there are 3416 protected products in the EU, of which 1257 are registered as Protected Geographical Origin (PGI) (European Union, 2022). Of these, more than 28 % are fruits, vegetables, and cereals, including more than 20 apples (European Union, 2022). The "Maçã de Alcobaça" (Alcobaça apple) is a Portuguese PGI with a pomological tradition going back to the Middle Ages (XII/XIII centuries), characteristic of the main apple growing regions in the country (West coast, Alcobaça region and nearest municipalities) (Almeida et al., 2017), known by the uniqueness of its edaphoclimatic conditions. According to the European Commission implementing regulation No 2015/792 of 19 May 2015 (European Commission, 2015), the "Maçã de Alcobaça" includes apples from the cultivars Casa Nova, Golden Delicious, Red Delicious, Gala, Fuji, Granny Smith, Jonagold, Reineta and Pink produced in a geographically delimited area and characterized by elevated consistency and crispness, the high percentage of sugar and high acidity, which gives them a bittersweet taste and

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https://doi.org/10.1016/j.jfca.2022.104976
Received 2 September 2022; Received in revised form 6 October 2022; Accepted 11 October 2022
Available online 17 October 2022

intense aroma (detailed information can be found in https://www.maca.pt). The attribution of the PGI led to an inevitable economic valuation of the "Maçã de Alcobaça" products, currently around 50,000 tons per year (APMA, personal communication). These PGI products are sold at an average price of 0.75 €/kg, resulting in 37.5 M€ per year in terms of economic value, versus a market value of 0.65 €/kg when considering non-certified apples (32.5 M€/y economic value) (DGADR, 2016). This market price difference makes them also more prone to intentional food fraud, namely through substitution by non-PGI apples mislabelled as "Maçã de Alcobaça". Thus, it is highly important to develop traceability tools, that can efficiently depict the geographical location of origin of PGI products while ensuring non-PGI products are also efficiently discriminated against.

To tackle the growing issue of food fraud, the European Union (EU) published a resolution, compelling all member states to develop and adopt tools to increase food traceability and prevent mislabelling (European Commission, 2002). Several similar regulations were adopted worldwide increasing food security requirements. The increasing requirements in food security led to the implementation of geographical origin authentication methodologies, that allow consumers to unequivocally know the origin of the food products that they are consuming (Leal et al., 2015). Several techniques have been employed to trace the geographical origin of food products, including elemental analysis (Albuquerque et al., 2016; Arbuckle & Wormuth, 2014; Bennion et al., 2021; Duarte, Mamede, et al., 2022; Duarte, et al., 2022a, 2022b, 2022c; Carreiras, et al., 2022; Gonzalvez et al., 2009; Mottese et al., 2020), isotope analysis (Drivelos & Georgiou, 2012; Gonzalvez et al., 2009; Kelly et al., 2005; Varrà et al., 2019), fatty acid profiles (Fonseca et al., 2021; Mottese et al., 2018; Ricardo et al., 2021) and optical spectroscopy techniques (Ghidini et al., 2019a; 2019b; Varrà et al., 2021). All these techniques alone or combined produce large datasets that can be either analysed through classical statistical techniques or with advanced chemometric approaches (Albergamo et al., 2018; Bua et al., 2017; Costas-Rodríguez et al., 2010; Duarte, et al., 2022a; Ghidini et al. 2019a, 2019b). The environmental conditions (namely climatic and soil traits) under which plants are cultivated have the utmost influence on the multi-elemental profile of the plants. This will have inevitable impacts on the fruit composition, leaving a geographical elemental record in these products during crop season (APMA, 2014). Considering elemental profiling, total X-ray fluorescence (TXRF) spectroscopy has been pointed out as a high-throughput non--targeted analytical tool to efficiently depict the foodstuff products' elemental composition, tracing the product's geographical origin whilst simultaneously, providing accurate food nutritional values (Kelly et al., 2005; Lim et al., 2021; Rajapaksha et al., 2017). Moreover, TXRF requires minimal sample preparation, being a low-cost (reduced reagents and gas costs), fast and sensible technique for multi-elemental quantitative profiling (Feng et al., 2021).

Considering the specificity of the limited geographical distribution and agricultural practices that are explicitly stated to attain the "Maçã de Alcobaça" PGI label, the apple multi-elemental profiles appear as a potentially useful tool to assure the geographical origin of these products as was previously employed not only in apples (Bizjak Bat et al., 2016; Liu et al., 2022) and other similar commodities (Coelho et al., 2019; Gonzalvez et al., 2009). More specifically and focusing on apple products previous studies reported that elemental chemometrics based on discriminant analysis namely PLS-DA ensure good classification results when distinguishing apple products from different geographical provenances (Bizjak Bat et al., 2016; Liu et al., 2022). Thus, the present work used TXRF-based multielement signatures of "Maçã de Alcobaça" apple pulps to: 1) evaluate its fine-scale (producer-level within a limited geographical area) traceability resolution; 2) depict the PGI and non-PGI products' elemental signatures of different cultivars at a national and international scale; 3) evaluate the potential of these multielement signatures for clonal discrimination within a core collection of Royal Gala cultivars with "Maçã de Alcobaça" PGI label; 4) evaluate the nutritional microelement profile of the different cultivars from different provenances while associating these to the PGI label. To our knowledge, this is the first time where this approach is undertaken for the "Maçã de Alcobaça" PGI label at all the above-mentioned considered scales (clone, producer, regional and international level), aiming to produce chemometric models that can unequivocally depict the certification status of apple products.

2. Materials and methods

2.1. Sample collection

All labware used for elemental analysis was decontaminated in acid baths for 48 h before use. Apple samples from the different surveyed provenances were sampled at the end of the growing 2021 season (Summer 2021) and brought back to the laboratory for further processing. Samples' typology collection sites, as well as their certification status, can be consulted in Fig. 1 and Table 1 respectively. All samples were collected by the National Institute of Agriculture and Veterinary (INIAV) within their production sites (INIAV samples) or within collaborating producers' cultivation areas. Clone samples were attained from the experimental cultivation sites of INIAV and were used to evaluate the resolution of the developed chemometric models to discriminate different Gala clones grown within the same cultivation area. The IGP area is characterized by subcoral limestone and fine and clayey stoneware, and is located in a transitional area between a mountain system and the Atlantic Ocean, with temperate-oceanic microclimatic conditions resulting from the humid Atlantic air, a mean annual air temperature of 15 ºC and moderate annual rainfall (APMA, 2014). Portuguese samples from the non-PGI areas were obtained from producers in the northern part of the country, a mountain area with prevalent granite soils, lower annual air temperatures, more intense rainfall regimes and low humidity (Inácio et al., 2008). Apples from Italy were obtained from the production region of Trentino-Alto Adige/Südtirol, in the country's far north. Apples from France originated from the cultivation region Tarn-et-Garonne, located in the south of the country. These Italian and French samples were used as outgroups to evaluate the possibility of the developed chemometric approaches to differentiate Portuguese PGI from non-Portuguese non-PGI samples.

Apple pulp samples were collected using stainless steel cork cutter cylinders. Endocarp and exocarp were discarded from the collected

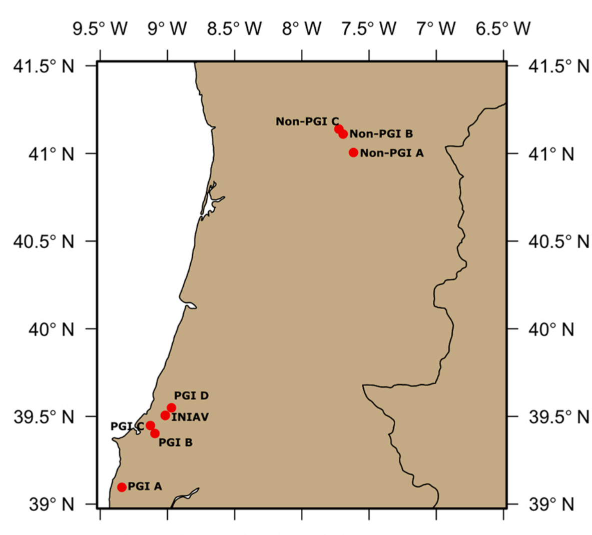

Fig. 1. Protected Geographical Origin (INIAV, PGI A, B, C and D) and non-PGI (Non-PGI A, B and C) sample collection sites in Portugal's mainland. An additional set of Royal Gala and Reineta samples was collected in Italy and France, respectively.

Table 1
Apple sample origin and sample number considering all cultivars, clones and provenances from national Protected Geographical Origin (PGI, INIAV, PGI Producers A, B C and D) and national (Non-PGI PT Producer A, B and C) and international non-PGI (Non-PGI producer from France (FR) and Italy (IT)) origins used in the present study.

	INIAV	PGI Producer A	PGI Producer B	PGI Producer C	PGI Producer D	Non-PGI PT Producer A	Non-PGI PT Producer B	Non-PGI PT Producer C	Non-PGI Producer FR	Non-PGI Producer IT
Royal Gala	10	10	10	10		10				10
Gala Clones	10 per clone									
Fuji					10			10		
Granny Smith			10					10		
Reineta	10						10		10	
Starking				10		10				

cylinder. Mesocarp samples were stored at −80 ºC and freeze-dried for 48 h at −50 ºC (Telstar laboratory freeze dryer, Cryodos-45).

2.2. Sample elemental analysis

Freeze-dried samples (10 replicates per class) were mineralized with HNO_3 in Teflon reactors, following a microwave digestion process (Multiwave GO, Anton Paar GmbH, Graz, Austria) according to the EPA 3052 method (Environmental Protection Agency (EPA), 1996). Briefly, the temperature profile is specified to permit specific reactions and incorporates reaching 180 ± 5 ºC in approximately less than 5.5 min and remaining at 180 ± 5 ºC for 9.5 min for the completion of specific reactions (Environmental Protection Agency (EPA), 1996). After cooling, an internal standard (Gallium, final concentration 1 mg/L) was added to each sample, and 5 μL of each sample was then applied to a siliconized quartz disk (Bruker Nano Analytics, Germany) and dried. Elemental concentrations (Ba, Br, Ca, Cl, Cr, Cu, Fe, K, Mn, Na, Ni, P, Pb, Pr, Rb, Ru, S, Se, Sr, Ti, V, Y and Zn) were determined by total reflection. Elemental concentrations were determined by X-ray fluorescence spectroscopy (TXRF, S2 PICOFOX, Bruker Nano GmbH, Germany) considering the fluorescence values from the individual sample application of the internal standard (Duarte, et al., 2022a, 2022b, 2022c).

Instrumental recalibration (gain correction, sensitivity analysis and multi-elemental standards) and analytical blanks were used for quality control, according to the manufacturer instructions and using Arsenic and Nickel standard discs (Bruker Nano GmbH, Germany) for calibration. Blanks with only HNO_3 were used to assess potential contaminations from the extraction process. Multi-elemental disc samples provided by the manufacturer were used to ensure the detection of a wide array of representative elements along the energetic segment surveyed (Bruker Nano GmbH, Germany). Extraction efficiency was confirmed through the analysis of International certified reference materials (ERM-CD281 Ryegrass, Table 2). The majority of the certified elements presented a high extraction efficiency (>90 %), ensuring that the applied extraction method has a good recovery and is appropriate for this type of sample (Table 2).

Table 2
Ryegrass (ERM-CD281) certified and analysed elemental values, uncertainty (mg/kg) and calculated extraction efficiency (average ± standard deviation, $N = 5$).

Element	Certified Value	Uncertainty	Measured Value	Extraction Efficiency (%)
Cr	0.73	0.22	0.67 ± 0.10	91.3 ± 5.3
Mn	4.88	0.24	3.35 ± 0.10	68.6 ± 1.9
Fe	161.0	8.0	198 ± 1	123.0 ± 0.4
Ni	0.69	0.15	0.78 ± 0.05	113.1 ± 5.8
Cu	5.98	0.27	7.10 ± 0.07	118.8 ± 1.0
Zn	71.0	4.0	73.3 ± 0.3	103.2 ± 0.4
As	6.7	0.4	7.25 ± 0.07	108.2 ± 0.9
Se	1.62	0.12	1.51 ± 0.03	93.3 ± 1.9
Rb	2.46	0.16	2.45 ± 0.05	99.6 ± 1.9
Sr	19.0	0.0	18.6 ± 0.3	97.6 ± 1.8
Cd	0.336	0.025	0.32 ± 0.02	96.6 ± 4.5
Pb	2.18	0.18	2.47 ± 0.05	113.3 ± 2.1

2.3. Chemometric data analysis

For the chemometric approach, a Partial Least-Squares Discriminant Analysis (PLS-DA) methodology was used and a variable selection method was implemented, specifically variable importance in projection (VIP) of PLS-DA. Both analyses were performed using the *DiscriMiner* package (Sanchez, 2013) in R-Studio Version 1.4.1717 (R Core Team, 2021). Cross-validation was performed using the Leave-One-Out function of the package, and the percentage of correct classification to the known geographical origin of the sample in cross-validation was used as a measure of model accuracy. For the Leave-One-Out cross-validation procedure and considering the N classes considered for each discrimination analysis, for each i^{th} case in (1,2,.,N), the data were tested (except for the i^{th} case) to build the classifier model. After this procedure, the model was applied to the i^{th} case and its classification was evaluated. This procedure was repeated N times, allowing all cases to be assigned to a classification label and the model accuracy evaluated. According to previous works (Molinaro et al., 2005) leave on out cross-validation is the most adequate for small sample size studies in comparison with resubstitution and simple split-sample estimates that lead to serious bias, being the leave-one-out cross-validation the method with the smallest bias for discriminant analysis. For each analysis, the number of components for the model was set as k-1, where k is the number of sample classes (e.g. origin, number of clones). This component number selection was confirmed through the analysis of the Receiver Operating Characteristic (ROC) Area Under the Curve (AUC) parameter, the goodness of fit value or explained variation (R^2) and the goodness of prediction or predicted variation (Q^2) values. The statistical significance of the AUC parameter was evaluated using a Wilcox test and the component selection using ROC-AUC was performed using the *MixOmics* package (Rohart et al., 2017) in R-Studio Version 1.4.1717. After ensuring a correct number of components and high AUC values, model accuracy variable components coordinates were calculated using the *DiscriMiner* package (Sanchez, 2013) in R-Studio Version 1.4.1717. The component selection was considered optimal when R^2 and Q^2 were the most similar as possible and the accuracy maximum. This was found to be optimum at k-1 components (data not shown). The VIP score is a measure of a variable's importance in the PLS-DA model, summarizing the contribution of a variable to the model (Banerjee et al., 2013). The VIP score of a variable is calculated as a weighted sum of the squared correlations between the PLS-DA components and the original variable. The weights correspond to the percentage variation explained by the PLS-DA component in the model. The number of terms in the sum depends on the number of PLS-DA components found to be significant in distinguishing the classes.

2.4. Nutritional analysis

For the Daily Recommended Value (DRV) relative percentage, the average ingestion of 2 pieces of fruit per day (160 g each) was considered, following the World Health Organization (WHO) recommendations (World Health Organization, 2014). Dietary reference values were attained from the European Food and Safety Authority (EFSA). Adequate Intake (AI) or Average Requirement (AR) values were used depending on the availability of each parameter. Average values for adult men and women (non-pregnant) were considered for each element AI or AR (European Food and Safety Authority (EFSA), 2019) (Table 3).

3. Results and discussion

3.1. "Maçã de Alcobaça" PGI label cultivars with PLS-DA

Considering the potential PGI fraud of the "Maça de Alcobaça" cultivars, the elemental, the elemental signature of each cultivar produced within the PGI area was evaluated (Fig. 2). Considering the different cultivars produced within the PGI area, the generated PLS-DA-based chemometric model showed an overall high accuracy (82.0 %) when classifying the different cultivars according to their elemental signature, with lower classification accuracies observed for the Reineta cultivar (60.0 %), which presented some similarities with Fuji and Granny Smith, leading to some misclassification with these groups (Fig. 2C). Additionally, reduced misclassification was also observed for Royal Gala (with three samples misclassified as Fuji, Granny Smith and Starking) and for Granny Smith (with only one sample classified as Fuji). Observing the elements with higher contribution to this separation among cultivars (Fig. 2B), several elements could be identified as having a significant role in the model performance (VIP > 1), with Sr and Rb being the elements with a higher importance in the projection. These were followed by several macro and micronutrients (Ca, S, Mn, Zn, Cl, Cu, K, P and Fe). Although Rb is not highly abundant in most environments, its uptake in organisms is normally regulated by other metallic and alkali elements (Campbell et al., 2011) and therefore its influence might be amplified by the also significant contribution of Ca, Mn, Zn, Cu, K and Fe. Strontium on the other hand is present in the composition of surface waters and farmed soils, mainly due to the use of agricultural lime for soil improvement and can be released from the substrate to the groundwater and surface waters, becoming bioavailable and readily taken up by plants and animals (Andreasen & Thomsen, 2021). Also, in this case, the relevance of this element might be amplified due to the well-known interaction between Sr and Ca uptake (Andreasen & Thomsen, 2021), with the latter being also one of the most important elements for the generated discrimination model. Additionally, K, P, Ca and other metallic elements uptake in orchard fruits are highly correlated presenting recognized interactions (Kuzin & Solovchenko, 2021). Thus, with exception of Rb and Sr with no known relevant physiological roles, the uptake of the remaining elements to the different apple varieties seems to be highly influenced by the variety within a certain cultivation area, and consequently, these elements appear as variables with high discriminatory power among the studied varieties.

Table 3
Average Daily Recommended Value (DRV) of the micronutrients considered, according to the European Food and Safety Authority (European Food and Safety Authority (EFSA), 2019).

Element	Daily Recommended Value (mg/day)
Ca	800
Cl	3100
Cu	1.45
Fe	6.0
Mn	3.0
Se	0.07
Na	2000
Zn	9.16

3.2. Discriminating PGI and non-PGI non-PGI (Portugal, France and Italy) geographical areas

To overcome food fraud, the development of signatures that can efficiently discriminate PGI from non-PGI apple samples is of utmost importance, to avoid intentional mislabelling of non-PGI products, aiming to increase their market value (Barendse et al., 2019). Two approaches were undertaken for this purpose: i) comparing the elemental signatures within the same cultivar between PGI and non-PGI signatures (Fig. 3) and ii) depicting the potential use of a common signature between PGI and non-PGI samples independent from the cultivar (Fig. 4).

A clear separation between Starking, Fuji and Granny Smith (Fig. 3A, D and G, respectively) pulp samples from apples produced within and outside the PGI geographical area was obtained. Royal Gala and Reineta samples from the PGI area presented some degree of overlap in the two-dimensional plot (Fig. 3J and M, respectively) with samples collected outside the PGI area both in Portugal and abroad. Nevertheless, this small projection overlap does not translate into low model accuracy values, with all models presenting overall classification accuracies above 86.7 % (Fig. 3C, F, I, L and O). Notably, the misclassifications observed for Royal Gala and Reineta samples are among samples collected outside the PGI geographical area, with the samples from PGI apples presenting 100 % classification (Fig. 3L and O). These results indicate that the pulp elemental signatures can efficiently discriminate the PGI origin of the cultivars, and thus be a potential tool to fight PGI fraud (Barendse et al., 2019). Conspicuously Rb is always the element with higher importance for the model discrimination accuracy attained (Fig. 3B, E, H, K and N). This element is more abundant in granite rocks than in other types of geological features (Zhang et al., 2021). Considering the geographical provenance of the apple samples from the non-PGI areas and when comparing their geological features, the prevalent granite soils in North Portugal and the sandy-calcareous soils from the PGI area (Inácio et al., 2008) appear to leave an elemental mark on the fruits produced, thus making Rb a key element for the models' high accuracies. Additionally, other heavy elements such as Y, Pb, and Sr without any physiological role in plants are also highlighted as key elements (VIP > 1) for the high accuracy of the chemometric models (Fig. 3B, E, H, K and N), reinforcing the geological characteristics of the cultivation areas as a major influence for the fruits elemental signature (Inácio et al., 2008). Other transitional elements such as Cu, Zn, Ni, as well as the alkali metal Ba, appeared in the list of the elements with high importance in projection (Fig. 4B, E, H, K and N). Although these elements have known physiological roles (Marschner, 1995), their bioavailability in soils depends also on the geological characteristics of the cultivation area as well as the fertilization practices of the producers, and have significant effects on the plant K, S and Mn uptake (Wyszkowski & Brodowska, 2020). Considering the different climatic and geological environments as well as the rules imposed to attain the PGI status in terms of cultivation techniques, the influence of these elements as discriminators of the apple origins becomes evident and appears to be amplified by the onsite interactions between elements either due to its geological bioavailability or by elemental fertilization techniques.

Bearing in mind, these evident elemental signatures attained at the cultivar level between PGI and non-PGI apple pulp samples, as well as the contribution of similar elements observed in the different cultivar models produced, the potential application of a similar approach was evaluated considering only the origin of the samples (within or outside the PGI geographical area) without considering the apple cultivar (Fig. 4). As abovementioned this approach aimed to evaluate the possibility of using a unique elemental signature independent to depict the PGI status of apple samples. Observing the PLS-DA biplot (Fig. 4A) two sample clusters formed by PGI and non-PGI samples are evident, with a reduced degree of overlap between the two sample groups. This clear separation is translated into a very high classification accuracy (95.0 %)

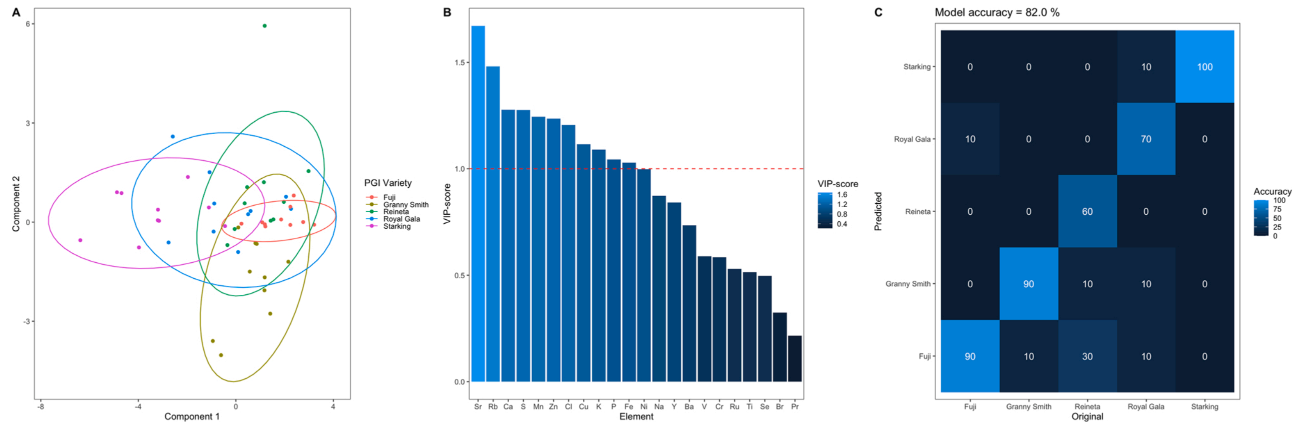

Fig. 2. Variable in Projection Partial Least Squares Discriminant Analysis (VIP-PLS-DA) biplots (A), element VIP-scores (B) and confusion matrix heatmaps (C) of the different cultivars (Fuji, Granny Smith, Reineta, Royal Gala and Starking) produced within the "Maçã de Alcobaça" Protected Geographical Indication (PGI) geographical area (N = 10 samples per class).

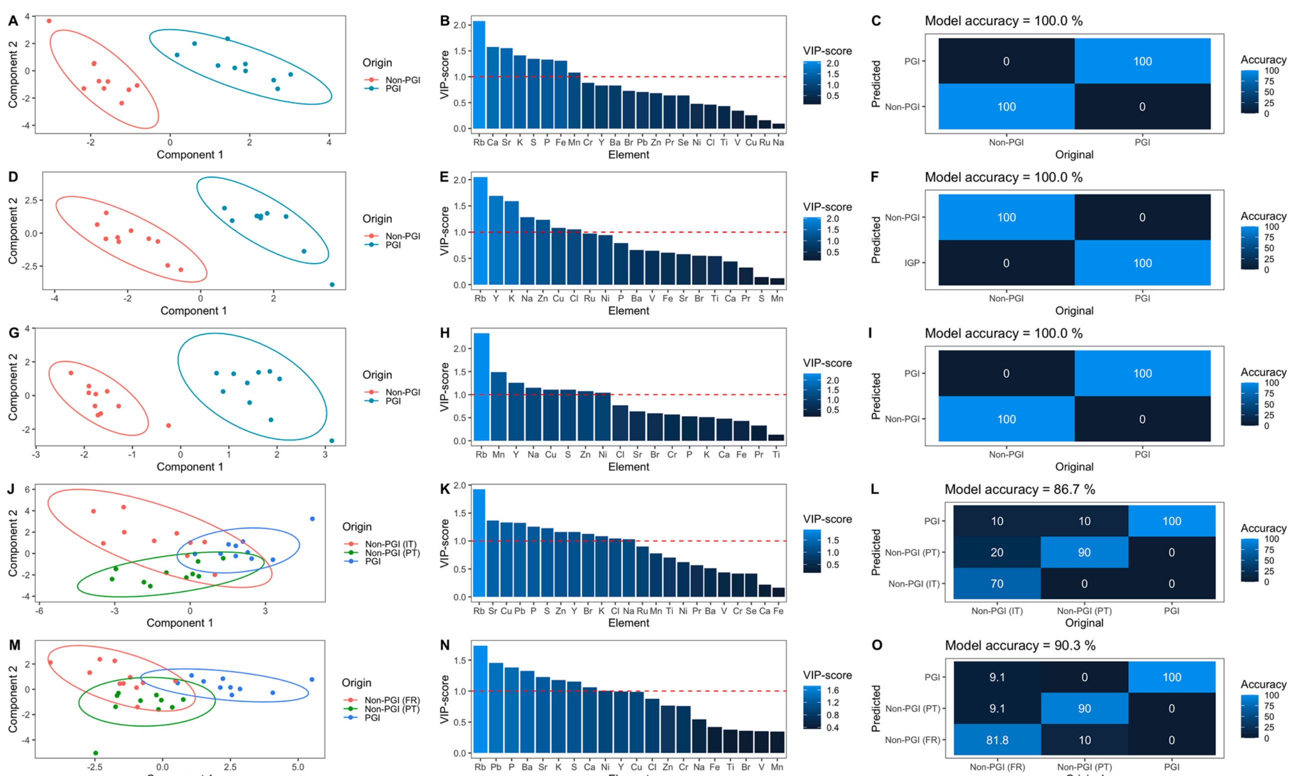

Fig. 3. Variable in Projection Partial Least Squares Discriminant Analysis (VIP-PLS-DA) biplots (A, D, G, J and M), element VIP-scores (B, E, H, K and N) and confusion matrix heatmaps (C, F, I, L and O) of the different cultivars [Starking (A-C), Granny Smith (D-F), Fuji (G-I), Royal Gala (J-L) and Reineta (M-O)] from producers within (West Portugal) and outside [North Portugal (PT), Italy (IT) and France (FR] the "Maçã de Alcobaça" Protected Geographical Indication (PGI) geographical area (N = 10 samples per class).

of the chemometric model generated with both groups being correctly classified with partial accuracies of 94.3 % and 95.8 %, respectively for PGI and non-PGI samples (Fig. 4C). When observing the elements with higher importance for the model projection, the ones which depend largely on the geological contribution (Rb, Y, Pb and Sr) are highlighted. This reinforces the geographical elemental signature as a prevalent feature and to a lower extent the different cultivar physiological traits. Nevertheless, these last potential physiological features should also be taken into account as it is possible to observe the different elements with importance in projection among cultivars, indicating a certain contribution of the cultivar physiology in the uptake and translocation of these elements to the fruit, especially when comparing element VIP scores from cultivars originating from the same cultivation area. This reinforces the abovementioned role of the agricultural soil characteristics as a key feature in shaping the elemental signature of the fruits and therefore making these elemental tracers ideal for PGI certification confirmation, independent of the apple cultivar considered and from the provenance of the non-PGI samples (either Portuguese or European cultivation areas). This also reinforces the geological and climatic features of the PGI area as not only being a mandatory requirement for attaining the "Maçã de Alcobaça" PGI certification, but also the key influence of these abiotic features as signature modulators, creating a very specific elemental mark in the apples produced within this PGI area, that can be efficiently used to confirm this PGI certification.

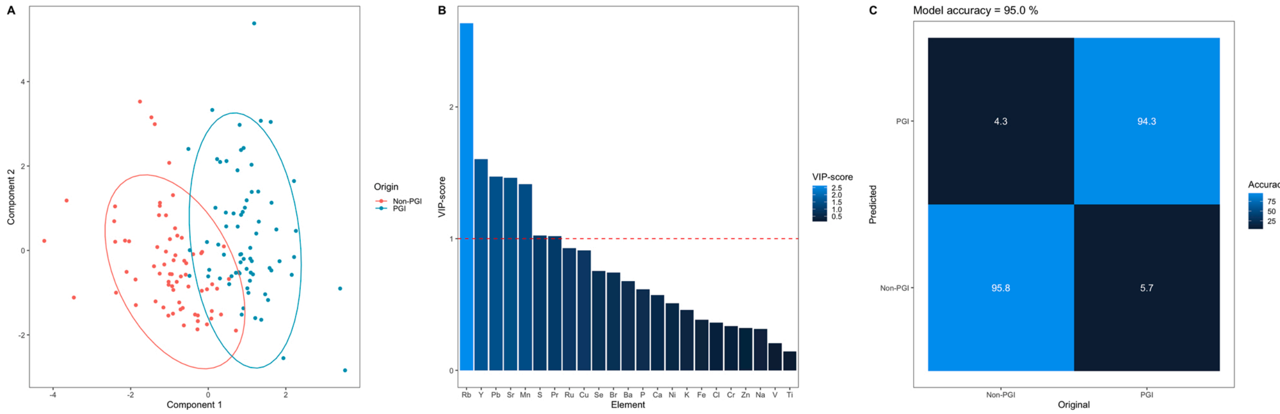

Fig. 4. Variable in Projection Partial Least Squares Discriminant Analysis (VIP-PLS-DA) biplots (A), element VIP-scores (B) and confusion matrix heatmaps (C) having as input the pulp elemental signatures of Protected Geographical Indication (PGI) (within PGI Portuguese area, N = 70 samples) and non-PGI (North Portugal, France and Italy, N = 70 samples).

3.3. *Gala cultivar clones and production areas with "Maçã de Alcobaça" PGI label*

The National Institute for Agriculture and Veterinary (INIAV) maintains an orchard core collection composed of Gala clones within the "Maçã de Alcobaça" PGI label geographical area. This allowed us to evaluate the elemental signatures of Gala clones cultivated within the same area. Analysing the chemometric classification of the Gala clones according to their multielement profile (Fig. 5A) a significant overlap among samples from different clones is evident. This overlap is more pronounced in the Galafab and Redlum clone samples and the Brookfield and Venus Fengal samples (Fig. 5A), although at a lower extent, as it is possible to observe from the low classification accuracy (Fig. 5C). Markedly, the Schniga Schnico clone has some samples that are misclassified according to the chemometric model as Schniga Schnico Red, a similar clone. These latter along with Fendeca Decarli clone samples showed the highest classification accuracy, according to the VIP-PLS-DA model generated (Fig. 5C). Although there are some misclassification issues, it should also be stated that considering common cultivation ground and the genetic proximity of the orchards (clone level, below cultivar level), the model overall accuracy resulted in 65.7 % of the samples being correctly classified, which considering the above-mentioned sample traits and the high number (7) of different clones can be considered as an interesting approach. Br, Ni, Zn, Rb and Cu were revealed to be the elements that most contributed (VIP-score > 1) to this differentiation accuracy (Fig. 2B). These slight differences observed between Gala clones, and although they are produced within the same cultivation experimental field with the same soil properties and subjected to the same climatic conditions, point to different mineral nutrition and physiological traits between clones, although this requires further investigation, in a more physiological and mineral uptake mechanistic work. When comparing the elements that were more relevant in differentiating Royal Gala samples within the "Maçã de Alcobaça" PGI cultivation area (from the INIAV core collection and 3 different producers), it could be found that only Br and Rb were common to both sets of relevant elements for the generated models (Fig. 5E). For

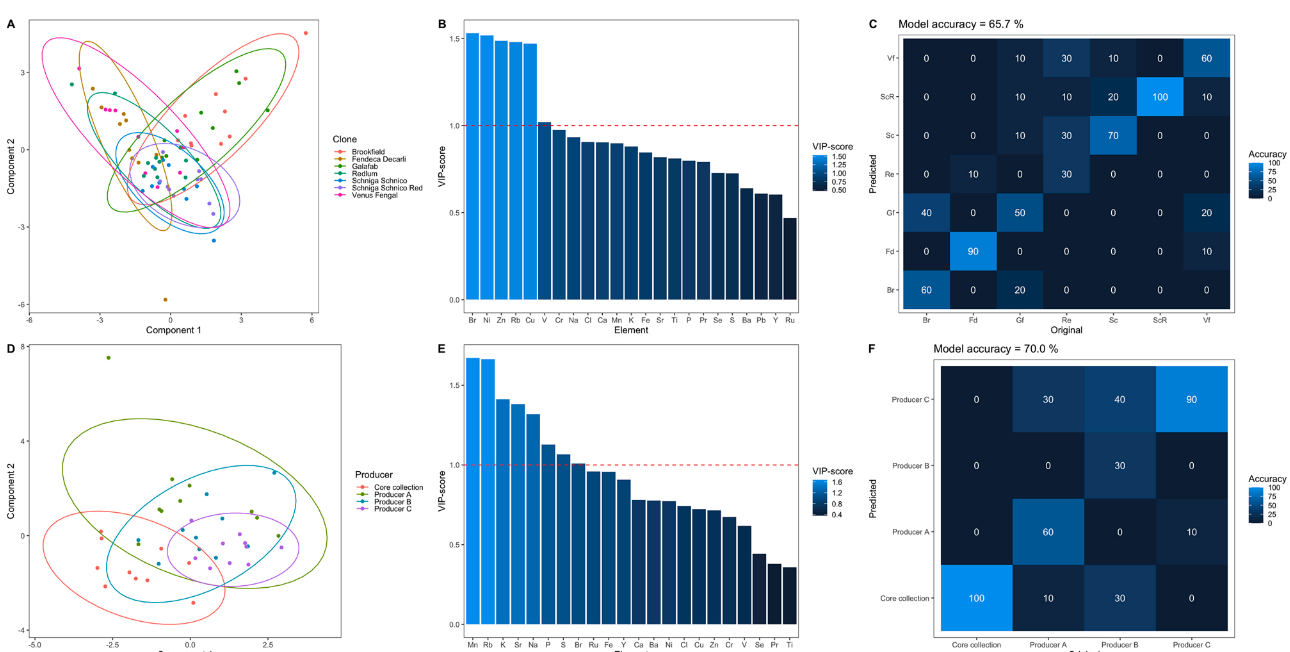

Fig. 5. Variable in Projection Partial Least Squares Discriminant Analysis (VIP-PLS-DA) biplots (A and D), element VIP-scores (B and E) and confusion matrix heatmaps (C and F) of the INIAV Gala core collection clone (A, B and C) and Gala samples from different "Maçã de Alcobaça" Protected Geographical Indication (PGI) sources (INIAV and three PGI-certified producers) (N = 10 samples per class).

this last model, besides Rb and Br also Mn, K, Sr, Na, P and S were revealed to have a high influence in discriminating the Royal Gala samples from different “Maçã de Alcobaça” PGI sources. The generated chemometric model revealed a lower degree of overlap among sample groups (Fig. 5D) and increased model accuracy (70.0 %, Fig. 5F). The Royal Gala samples from the INIAV core collection and Producer C presented a very high classification accuracy (100 % and 90 %, respectively) according to the produced model. When analysing the elements responsible (VIP > 1) for this separation accuracy it is evident that (apart from Br) are elements highly present in fertilizer formulations (Sparr, 1970; Treder et al., 2022), indicating that the different fertilizer applications either by INIAV and the producers influenced the Royal Gala apple pulp multielement signature leading to a better separation between samples from different producers.

3.4. *PGI (cultivars and clones) and non-PGI apple pulps nutritional profiles*

Beyond the generated elemental signatures, the elemental concentrations can also be used for the nutritional values of the products from different geographical origins, cultivars and clones. Considering the average EFSA’s dietary reference values (European Food and Safety Authority (EFSA), 2019) and an average daily intake of two pieces of fruit per day (assuming a mean weight per piece of 160 g), the proportion of the DRV attained from the ingestion of the different apple samples surveyed was evaluated (Fig. 6). Considering Ca, Cl, and Se, all samples presented reduced values of these elements when compared to the EFSA DRV (Fig. 6A, B and G). Observing Fe and Zn (Fig. 6D and H), it is possible to observe that there is a higher number of PGI samples that when ingested in the above-mentioned proportions comply with more than 50 % of the Fe DRV for an adult person and in a lower extent to the 50 % of the Zn DRV for an adult person. Regarding Mn, none of the apple types considered reached the 50 % DRV (Fig. 6E). Nonetheless, if the 25 % DRV is considered once again it is possible to observe that the ingestion of PGI/Core collection apples considering the abovementioned doses can contribute to reaching this nutritional threshold, whilst non-PGI apples present values far inferior to this 25 % DRV threshold. All samples were found to be rich in Cu, and their intake exceeds the 50 % DRV threshold, independently of the apple’s geographical provenance. Comparing these values with the attained for other fruit pulps (Harmankaya et al., 2012), the PGI-certified apples showed higher values than peach, orange, apricot and cherry, reinforcing their nutritional value. Again, this way, the PGI certification confirmation, not only ensures the consumer is consuming regional certified products but also of higher nutritional value in terms of essential micronutrients. Regarding the remaining elemental micronutrients, a lower degree of variation was observed between PGI and non-PGI apple pulp samples (Supplementary Fig. S1). Only to denote that, as abovementioned for the chemometric models, geological feature-related elements such as Rb and Pb showed higher concentration values in non-PGI samples.

4. Conclusions

Protected Geographical Indication (PGI) products with high socio-economic relevance and/or high market values are prone to intentional food fraud. In this regard, it is of utmost importance to develop efficient and accurate geographical traceability tools to correctly validate the origin of products at both local, national and international scales. In the present study, “Maçã de Alcobaça” PGI apple pulp samples, Portuguese PGI and non-PGI areas, as well as different PGI cultivars and

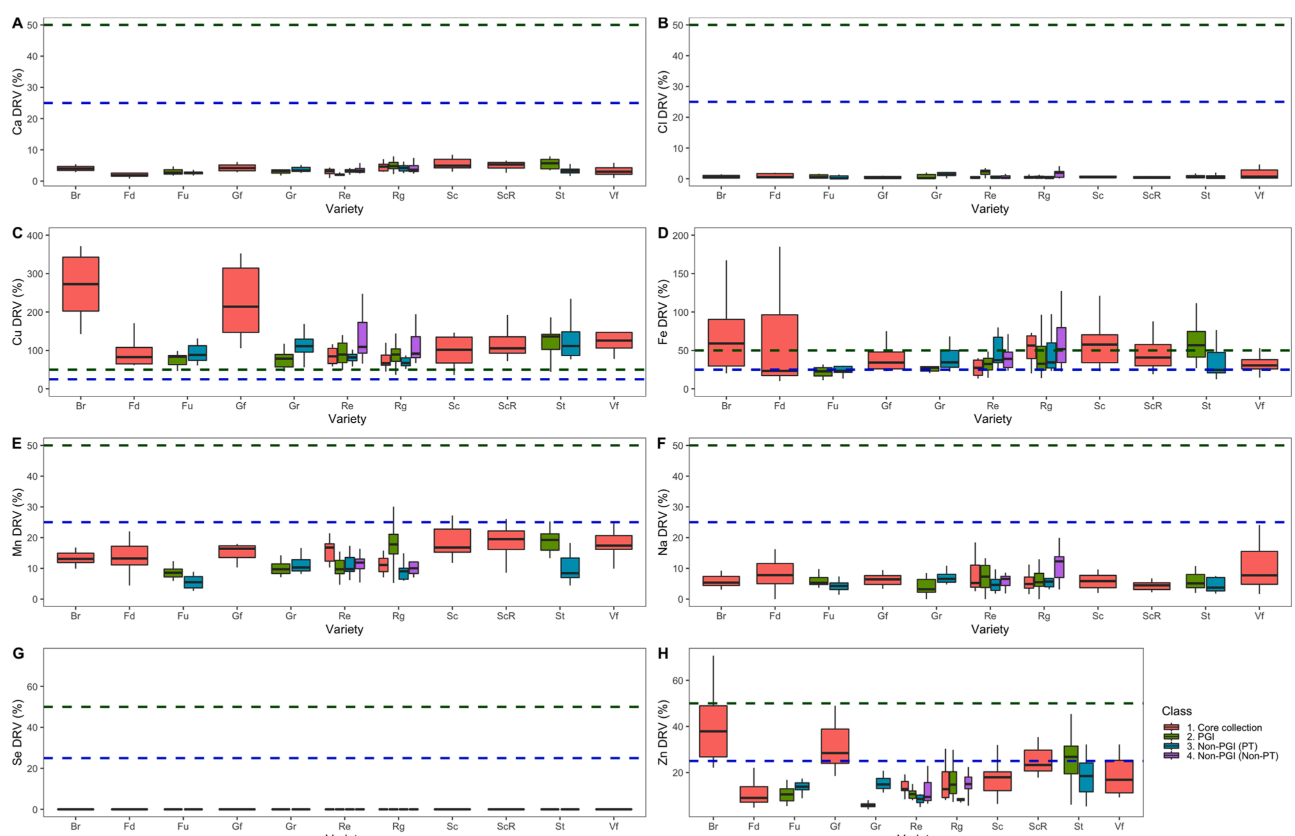

Fig. 6. Element Daily Recommended Value (DRV) percentage in the apple pulp samples attained from the INIAV core collection, Protected Geographical Indication (PGI) producers and non-PGI producers from the different Royal Gala clones (Br – Brookfield; Fd - Fendeca Decarli; Gf – Galafab; Vf - Venus Fengal; Re – Redlum;Sc - Schniga Schnico; ScR - Schniga Schnico Red) and different surveyed cultivars (Fu – Fuji, Re – Reineta; Rg – Royal Gala; St – Starking); dotted blue and green lines denote 25 % and 50 % DRV respectively.

clones, were analysed. The content of 23 elements was determined by TXRF, contributing to the characterization of this traditional product. The chemometric approaches allowed to disentangle with a high degree of accuracy PGI cultivar samples, which can be of great use when analysing second-line products such as juices. At a national scale, multielement signatures proved to be highly reliable in identifying samples from the same cultivar produced in PGI and non-PGI areas. Additionally, these models were also very efficient if samples from outside Portugal are included, allowing complete discrimination of the PGI samples versus non-PGI national or international samples. Moreover, despite the chemometric models' moderate resolution power in discriminating Royal Gala clones, they revealed a high accuracy in discriminating PGI Royal Gala fruits from certified producers at a small scale. In sum, the produced elemental signatures and chemometric methods allowed the production of a PGI signature independent of the cultivars that can discriminate with a high degree of accuracy "Maçã de Alcobaça" PGI apple pulp samples. Allied with this authentication accuracy it was also possible to evaluate the microelement nutritional traits of the traditional certified products, highlighting their higher nutritional value versus non-PGI fruits, hence potentially contributing to the reduction of food fraud, informing the consumer of the product authenticity, and providing insights on the nutritional value of these high-value market products.

CRediT authorship contribution statement

Bernardo Duarte: Resources, Conceptualization, Writing – original draft, Data curation, Project administration. **Juliana Melo:** Methodology, Investigation, Writing – review & editing. **João Carreiras:** Methodology, Investigation, Writing – review & editing. **Renato Mamede:** Methodology, Investigation, Writing – review & editing. **Andreia Figueiredo:** Writing – review & editing. **Vanessa F. Fonseca:** Writing – review & editing. **Miguel L. Sousa:** Resources, Writing – review & editing. **Anabela Silva:** Resources, Writing – review & editing, Project administration.

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.

Data Availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Fundação para a Ciência e a Tecnologia (FCT) for funding MARE (Marine and Environmental Sciences Centre, UIDB/04292/2020 and UIDP/04292/2020), BioISI (Biosystems and Integrative Sciences Institute, UIDP/04046/2020) and ARNET (Aquatic Research Network Associated Laboratory, LA/P/0069/2020). B. Duarte and V. F. Fonseca were supported by research contracts (CEECIND/00511/2017 and 2021.00244. CEECIND).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2022.104976.

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