UNIVERSIDADE DE LISBOA
INSTITUTO SUPERIOR TÉCNICO

UNIVERSITÉ DE PAU ET DES PAYS DE L'ADOUR

ECOLE DOCTORALE DES SCIENCES EXACTES ET DE LEUR APPLICATIONS

Advanced analytical strategies in inorganic and isotopic analysis for the authentication of food products in Portugal

Inês Alexandra Terra Viana Ribeiro Coelho

Supervisors: Doctor Olivier F. X. Donard
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Thesis approved in public session to obtain the PhD Degree in Chemistry

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2022

Resumo

A origem geográfica dos géneros alimentícios é determinante para a sua qualidade final, sendo a sua correta identificação um fator chave para garantir a segurança ao longo da cadeia alimentar. Em Portugal, como no resto do mundo, existem muitos produtos como o vinho, o queijo e o azeite, aos quais o consumidor reconhece valor acrescentado por serem produzidos e classificados como Denominação de Origem Protegida (PDO). A fraude relacionada com a origem geográfica dos géneros alimentícios, é uma questão que tem vindo a ganhar importância ao longo dos anos. Os seus impactos podem ser sentidos a nível económico e de saúde pública.

Informações precisas sobre a origem dos produtos alimentícios comercializados são de extrema importância por razões econômicas e de saúde. Embora essa informação seja obrigatória na rotulagem, a documentação nem sempre é confiável, pois é suscetível a falsificação. Como tal, a composição química dos géneros alimentícios pode ser a resposta para prevenir tais práticas fraudulentas. É essencial ter ferramentas analíticas que possibilitem a detecção de tais práticas fraudulentas com uma margem mínima de incerteza.

Os dois principais objetivos deste trabalho são (1) estudar a presença e conteúdo de nutrientes e contaminantes em diferentes matrizes alimentares, com base em uma abordagem multielementar, e (2) avaliar a aplicação de estratégias analíticas avançadas em análises inorgânicas e isotópicas para a autenticação geográfica dos produtos alimentares portugueses.

Este trabalho combinou diferentes estratégias analíticas para a autenticação de dois produtos alimentares portugueses, contribuindo para a sua caracterização. Apresentou uma nova abordagem analítica baseada em isótopos estáveis tradicionais e não tradicionais e na determinação multielementar. Demonstrou que a origem geográfica pode ser rastreada através da análise multielementar e isotópica usando ferramentas estatísticas quimiométricas para processamento de dados. Os métodos propostos requerem extensas bases de dados de suporte para que os resultados possam ser aplicados recorrentemente em estudos de autenticidade de rotina. Os resultados apresentados poderão, no futuro, integrar uma base de dados de autenticidade e uma Tabela de Composição de Alimentos para peras Rocha, vinho do Douro e vinho do Porto.

Além de combater a fraude alimentar, a caracterização química de marcadores de autenticidade oferece aos produtores a oportunidade de acrescentar valor aos seus produtos autênticos. Estes podem, no futuro, vir a ser promovidos como produtos autênticos, ostentando nos seus rótulos um logótipo de autenticidade de assinatura química, complementando os logótipos de indicações geográficas da União Europeia já existentes.

Palavras chave: macro elementos, elementos vestigiais, análise isotópica, autenticidade, alimentos.

Abstract

In recent years, consumers have shown a growing awareness and concern regarding the quality of the food they eat. There are different factors contributing to this increase in consumer's interest such as recent outbreaks of foodborne diseases, a concern for animal welfare, environmental concerns, etc.

The geographical origin of foodstuffs is crucial to its final quality, being its correct identification a key factor to ensure safety along the food chain. Moreover, in Portugal, as in the rest of the world, there are many products such as wine, cheese and olive oil, to which the consumer recognizes added value for being produced and classified as Protected Designation of Origin. The recent classification of the Mediterranean diet as Intangible Cultural Heritage of Humanity by UNESCO is another factor that reinforces the importance of the origin of food products.

Fraud related to the geographical origin of foodstuffs, is an issue that has been gaining importance over the years. Its impacts can be felt at economical and public health levels.

Accurate information regarding the origin of commercialized food commodities is of uttermost importance for economic and health reasons. Although such information is mandatory in labelling, documentation is not always trustworthy as it is susceptible to falsification. As such, the chemical composition of foodstuffs may provide the answer to prevent such fraudulent practices. It is essential to have analytical tools that enable the detection of such fraudulent practices with a minimal margin of uncertainty.

The two main objectives of this work are (1) to study the presence and content of nutrients and contaminants in different food matrices, based on a multielement approach, and (2) to evaluate the application of advanced analytical strategies in inorganic and isotopic analysis for the geographical authentication of Portuguese food products.

This work proposes combining different analytical strategies for the authentication of two Portuguese food products, contributing to their characterisation. It presented a new analytical approach based on traditional and non-traditional stable isotopes and multielement determination. It demonstrated that the geographical origin could be traced through multielement and isotopic analysis using chemometric statistical tools for data processing. The proposed methods require an extensive database of results to be helpful in routine authenticity testing schemes. The results presented may, in the future, integrate both an authenticity database and a Food Composition Databank for Rocha pears, Douro wine and Port wine.

In addition to battling food fraud, the chemical characterization of authenticity tracers provides the opportunity for producers to build added value to their authentic products. These may, in the future, be promoted as authentic products by sustaining a chemical signature authenticity logo, complementing the already existing EU geographical indication logos.

Key words: major elements, trace elements, isotopic analysis, authenticity, food.

Résumé

Au cours des dernières années, les consommateurs ont montré une prise de conscience et une préoccupation croissantes concernant la qualité des aliments qu'ils consomment. Différents facteurs contribuent à cette augmentation de l'intérêt des consommateurs, tels que les récentes épidémies de maladies d'origine alimentaire, le souci du bien-être animal, les préoccupations environnementales, etc.

L'origine géographique des denrées alimentaires est cruciale pour leur qualité finale, étant son identification correcte un facteur clé pour assurer la sécurité tout au long de la chaîne alimentaire. De plus, au Portugal, comme dans le reste du monde, il existe de nombreux produits tels que le vin, le fromage et l'huile d'olive, auxquels le consommateur reconnaît une valeur ajoutée pour être produits et classés en Appellation d'Origine Protégée. Le récent classement du régime méditerranéen au patrimoine culturel immatériel de l'humanité par l'UNESCO est un autre facteur qui renforce l'importance de l'origine des produits alimentaires.

La fraude liée à l'origine géographique des denrées alimentaires est un problème qui a gagné en importance au fil des années. Ses impacts se font sentir au niveau économique et de la santé publique.

Des informations précises sur l'origine des produits alimentaires commercialisés sont de la plus haute importance pour des raisons économiques et sanitaires. Bien que ces informations soient obligatoires dans l'étiquetage, la documentation n'est pas toujours fiable car elle est susceptible d'être falsifiée. A ce titre, la composition chimique des denrées alimentaires peut apporter la réponse pour prévenir de telles pratiques frauduleuses. Il est essentiel de disposer d'outils d'analyse permettant de détecter ces pratiques frauduleuses avec une marge d'incertitude minimale.

Les deux principaux objectifs de ce travail sont (1) d'étudier la présence et la teneur en nutriments et contaminants dans différentes matrices alimentaires, sur la base d'une approche multiélémentaire, et (2) d'évaluer l'application de stratégies analytiques avancées en analyse inorganique et isotopique pour l'authentification géographique des produits alimentaires portugais.

En résumé, ce travail propose de combiner différentes stratégies analytiques pour l'authentification de deux produits alimentaires portugais, contribuant à leur caractérisation. Il a présenté une nouvelle approche analytique basée sur les isotopes stables traditionnels et non traditionnels et la détermination multiélémentaire. Il a démontré que l'origine géographique pouvait être retracée grâce à une analyse multiélémentaire et isotopique à l'aide d'outils statistiques chimiométriques pour le traitement des données. Les méthodes proposées nécessitent une vaste base de données de résultats pour être utiles dans les schémas de test d'authenticité de routine. Les résultats présentés pourraient, à l'avenir, intégrer à la fois une base de données d'authenticité et une FCDB pour les poires Rocha, le vin du Douro et le vin de Porto.

En plus de lutter contre la fraude alimentaire, la caractérisation chimique des traceurs d'authenticité offre l'opportunité aux producteurs d'apporter une valeur ajoutée à leurs produits authentiques. Ceux-ci pourraient, à l'avenir, être promus en tant que produits authentiques en conservant un logo d'authenticité de la signature chimique, en complément des logos d'indication géographique déjà existants de l'UE.

Mots clés: éléments majeurs, oligo-éléments, analyse isotopique, authenticité, produits alimentaires

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“Logic will get you from A to B. Imagination will take you everywhere. “

(Albert Einstein).

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Acronyms

ANOVA - Analysis of Variance

APN – National Association of Rocha Pear Producers

CRM – Certified reference material

DRV – Daily recommended value

EC – European Commission

EFSA – European Food Safety Authority

EU – European Union

FCDB – Food Composition Database

FIT-PTS - Food analysis using Isotopic Techniques-Proficiency Testing Scheme

HCA - Hierarchical Cluster Analysis

ICP-MS – Inductively coupled plasma mass spectrometry

ICP-OES - Inductively coupled plasma optical emission spectrometry

INSA – National Institute of Health Doutor Ricardo Jorge

IRMS – Isotope ratio mass spectrometry

IVDP - Port and Douro Wines Institute

JSI – Josef Stefan Institute

LD – Limit of detection

LDA – Linear discriminant analysis

LQ – Limit of quantification

MC-ICP-MS – Multi-collector inductively coupled plasma mass spectrometry m/z – mass to charge ratio

NIR – Near-infrared

OIV - International Organisation of Vine and Wine

PC – Principal component

PCA - Principal component analysis

PDO - Protected Designation of Origin

PGI - Protected Geographical Indication

PT – Proficiency testing

QCS – Quality control standards

RSD - Relative standard deviation

SD – Standard deviation

TIMS - Thermal ionization mass spectrometry

UNESCO - United Nations Educational, Scientific and Cultural Organization

UPPA – University of Pau

VIM - International Vocabulary for Metrology

V-SMOW – Vienna Standard Mean Ocean Water

WHO - World Health Organization

1 Scope

Accurate information regarding the origin of commercialized food commodities is of uttermost importance for economic and health reasons. Although such information is mandatory in labelling, documentation is not always trustworthy as it is susceptible to falsification. As such, the chemical composition of foodstuffs may provide the answer to preventing such fraudulent practices. This study aims to evaluate the application of advanced analytical strategies in inorganic analysis for the authentication of two Portuguese food products.

The scope of this study was limited to two foodstuffs, Rocha Pear and Wine, and four analytical techniques, inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES), multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) and isotope ratio mass spectrometry (IRMS).

For Rocha pears, the sampling plan encompassed evaluating and comparing two geographical areas of production in Portugal. The first is located in the western part of the country and has a Protected Designation of Origin (PDO), and the second is in the region of Castelo Branco. In each region, we collected pears from five farms. Within each farm, five trees were selected, and three pears were harvested from each tree and analysed as a pooled sample. The total number of collected pears was 150, and the total number of analysed pools was 50. The analytical determinations performed were multielement (Al, As, B, Be, Ca, Cd, Cu, Co, Cr, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Se, Sn, Sr, Tl, Zn) and isotopic (Sr) analysis.

For wines and to assure their authenticity, samples were provided by the Instituto dos Vinhos do Douro e do Porto. A total of 30 wines, 15 Port wines and 15 Douro wines, were analysed for multielement (Ag, Al, As, B, Be, Ca, Cd, Cu, Co, Cr, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, Se, Sn, Sr, Tl, Zn) and isotopic (B, O, Pb, Sr) determination.

The data produced were analysed using chemometric statistical tools, namely Principal Component Analysis (PCA), Hierarchiqual Cluster Analysis (HCA) and Linear Discriminant Analysis (LDA)

2 Objectives/scientific questions

This thesis concerns the following scientific questions:

What is the contribution of the mineral and isotopic profile to food authenticity?

How can isotope ratios contribute to the valorization of Portuguese food products?

Is it possible to discriminate Rocha Pear produced within the region of Protected Designation of origin from Rocha Pears produced elsewhere?

How does the winemaking process influence the inorganic and isotopic composition of wines from the Douro Demarcated Region?

What are the discriminant parameters regarding the geographical origin of wines from the Douro Demarcated Region?

The main objectives are:

To study the presence and content of chemical contaminants, nutrients and isotopic profile of Portuguese Food products

To evaluate the application of advanced analytical strategies in inorganic and isotopic analysis for food provenance authentication

To evaluate the influence of the geographical area of production and production methods on the multielement and isotopic composition of wines

3 Introduction

This chapter was partially adapted from the following papers:

Coelho, I., Castanheira, I., Bordado, J. M., Donard, O., & Silva, J. A. L. (2017). Recent developments and trends in the application of strontium and its isotopes in biological related fields. TrAC Trends in Analytical Chemistry, 90, 45–61. https://doi.org/10.1016/j.trac.2017.02.005

Coelho, I., Matos, A. S., Teixeira, R., Nascimento, A., Bordado, J., Donard, O., & Castanheira, I. (2019). Combining multielement analysis and chemometrics to trace the geographical origin of Rocha pear. Journal of Food Composition and Analysis, 77, 1–8. https://doi.org/10.1016/j.jfca.2018.12.005

Coelho, I., Matos, A. S., Epova, E., Barre, J., Cellier, R., Castanheira, I., Bordado, J., Donard, O., (2022). Multi-element and multi-isotopic profiles of Port and Douro wines as tracers for authenticity. (Submitted to Food Chemistry)

3.1 Food Fraud

Food fraud is at least as ancient as the Roman Empire, where the massive adulteration of wine with additives like honey or Pb made scientists question its healthiness (Bush, 2002). Still, it was not until 1820 when the German chemist Fredrick Accum published his book entitled “A Treatise on Adulterations of Food and Culinary Poisons”, that the issue became of interest to the scientific community. Ever since the topic has been of concern for humans.

In recent years, public awareness regarding food fraud has increased following several food scandals. Incidents like the melamine scandal, where milk and infant formula adulterated with melamine reached consumers in China in 2008, or the horsemeat scandal in 2013 in the United Kingdom made headlines worldwide. It alerted consumers and authorities to the susceptibility of products along the food chain (Morin & Lees, 2018). The demand for safety, quality assurance, and authenticity of food products are greater than ever. The information presented on the label must coincide with the product inside the package, and analytical strategies can contribute to such a goal.

Fraudulent activities within the European Union’s (EU) agri-food industry increase annually. The number of requests for administrative assistance from member countries concerning suspicion of fraud has almost doubled between 2016 and 2019, and estimated losses are around 8 to 12 billion euros per year (European Commission, 2020a, 2022d). The European Commission (EC) defines food fraud as “any suspected intentional action by businesses or individuals to deceive purchasers and gain undue advantage from there, in violation of the rules referred to in Article 1(2) of Regulation (EU) 2017/625 (the agri-food chain legislation)” (European Commission, 2022c).

According to the EU, there are seven types of food fraud: concealment, substitution; dilution; unapproved enhancement; mislabelling, grey market, and counterfeit (European Commission, 2022d). The first four fall under the category of adulteration (Morin & Lees, 2018). Mislabelling, defined as placing a false claim on packaging for economic gain (European Commission, 2022d), will be present in practically all food fraud cases considering that intentionally deceiving purchasers is involved in the definition of food fraud. Grey market deals with the sale of excess unreported products, namely in sectors where quotes limit production. Finally, counterfeit products are products that do not respect Intellectual Property Rights, such as portraying the label or package format of another product (European Commission, 2022d).

Generally, food fraud has an economic motivation. However, food fraud and food safety are interconnected issues since the former may lead to adverse health effects, making it a public health problem. The mislabelling of geographical origin induces a false sense of safety in the consumer since normative regulations in the supposed country of production may not be complied with, for example, in what concerns the use of pesticides. The adulteration of ingredients can lead to food allergies, toxicity or other undesirable health effects.

Although not being the primary target of food fraud, in resume, health problems may occur as a side effect. Battling food fraud promotes food safety and human health at the same time.

3.2 Food authenticity

The concepts of food fraud and food authenticity are deeply connected. The term authenticity refers to the correspondence between the food product and its description or labelling. An authentic food product is “a food product where there is a match between the actual food product characteristics and the corresponding food product claims; when the food product is what the claim says that it is” (Morin & Lees, 2018). In that sense, no product subject to fraudulent practices can be authentic.

The goal of food authenticity testing is to detect food fraud. In recent years, numerous severe foodborne diseases have motivated the scientific community to work more intensively on food authenticity (Drivelos & Georgiou, 2012). However, not all types of fraud are vulnerable to analytical tests. F authenticity testing can be framed under different scenarios in response to diverse kinds of food fraud. In fact, it may relate to aspects such as the identification of the production system (biological or conventional agriculture) or of ingredient adulteration or the traceability of the geographical origin of food.

Examples of the term authenticity application to the different aspects of food fraud are easily found in the literature. The concept has been used to refer to the production of organic milk (Chung, Park, Yoon, Yang, & Kim, 2014) and plants (Laursen et al., 2013); genetically modified foods (Lüthy, 1999); ingredient adulteration (Fu, Zhang, Zhou, & Liu, 2020; Gliszczyńska-Świgło & Chmielewski, 2017); processing technology (Krajnc et al., 2021); or geographical origin (Camin, Bontempo, Perini, & Piasentier, 2016; Kokkinofta et al., 2017). In the present work, the term food authenticity will refer to the geographical origin of foodstuffs.

Within Europe, the geographical origin is considered a quality criterion for food, and food ingredients, enjoying increased importance as a result of legislative protection of regional foods (Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016; Gonzalvez, Armenta, & de la Guardia, 2009). Also, consumers’ interest in the geographical origin of the food they consume has grown over the last years due to the concern of serious foodborne diseases (Drivelos & Georgiou, 2012). To protect and promote the geographical origin of European products, the EU has created two quality logos (Figure 1): PDO and Protected Geographical Indication (PGI) (European Commission, 2022a).

Figure 1. EU geographical indication logos (European Commission, 2022a)

Through these logos, consumers can easily recognize traditional quality products and assure their authenticity in terms of regional origin. Such goods are often promoted by producers as premium quality products and are commercialized at higher values, making them a desirable object for fraud (Kelly, Heaton, & Hoogewerff, 2005).

A search on the web of knowledge using the words food, authenticity and origin shows that the interest of scientists falls on different food products. Over the last year, we can find publications dealing with the geographical origin of a multitude of food matrices like olive oil, bivalves, truffles, cheese, cocoa, lettuce, asparagus, tea, wine, milk, meat, fish, rice, kimchi, vinegar, coffee, wheat, spices and peach.

The focus of the present work will be on two very well-known Portuguese food commodities with PDO: Rocha pear and wine (Port and Douro). It will, for the first time, trace the geographical authenticity of Portuguese food comodities resorting to analytical approaches that combine heavy and light isotopes, as well as multielement analysis.

3.3 Rocha Pear

A healthy diet includes consuming fruits and vegetables since they are rich in vitamins, phytochemicals, fibres and minerals (Slavin & Lloyd, 2012). The World Health Organization (WHO) recommends the daily consumption of 400g of fruits and vegetables based on solid evidence suggesting positive health effects on cardiovascular diseases, type 2 diabetes and obesity (FAO/WHO, 2004). The daily recommended values (DRV) of minerals, which play an essential role in human health, were published by the European Food Safety Authority (EFSA) (EFSA, 2017). The importance of minerals has been recently reviewed, stating that even trace elements without an established DRV, like B and Co, are important in human nutrition (Gharibzahedi & Jafari, 2017).

Recently, with the new concepts of carbon and water footprints, and glycemic index, even fruits and vegetables are the object of study to guarantee a bridge between ecology, agriculture and sustainable nutrition. Adding to the known benefits of consuming fruit in general, pears are considered a good source of fibre and K with low Na content and a low glycemic index (Barda, 2011; Foster-Powell, Holt, & Brand-Miller, 2002). Compared to other cultures, pears present low environmental impact since their carbon and water footprints are among the smallest amongst fruits (Downs & Fanzo, 2015; Yan et al., 2016). In addition, growing native varieties also contributes to reducing the environmental footprint of production since these are more adapted to the climate and soils and are more resistant to local plagues and diseases.

Figure 2. Production area of Rocha pear with Protected Designation of Origin, adapted from (Direcção Geral do Território, 2013)

Rocha pear (*Pyrus communis* L. var. Rocha) is a traditional Portuguese variety native to the Sintra municipality that represents about 95% of the national pear production. Easily recognizable due to the typical russeting around the stalk, Rocha pears contain high phenolic content and excellent consumer acceptance due to their organoleptic properties (Salta et al., 2010). Rocha Pears produced in the west region of Portugal may be classified with PDO (Figure 2). Certified products are commercialized under the designation of “Pera Rocha do Oeste” (COMMISSION REGULATION (EC) No 492/2003 of 18 March 2003, 2003).

According to the National Association of Rocha Pear Producers (APN), Portugal produces on average 173.000 tons of Rocha pears per year, out of which 60% is exported (Associação Nacional de Produtores de Pera Rocha, 2022). Exportations have increased over the last years and expanded worldwide. South America, the United States of America, Saudi Arabia, China and South Africa represent emerging markets. For Portuguese producers, Rocha pear is a favourite variety as it presents good resistance to handling and transportation and high shelf life, maintaining its properties during storage with reduced waste (Associação Nacional de Produtores de Pera Rocha, 2022).

Although there is only one classified PDO region, limited to the west part of Portugal, the production also takes place in other parts of the country, mainly in the area of Fundão.

The selection of this traditional Portuguese product relates not only to its high economic value but also to the recognized health and nutritional benefits and reduced environmental impact of this culture due to its low carbon and water footprints (Downs & Fanzo, 2015; Yan et al., 2016).

Over the last years, studies regarding the authenticity of fruits bearing the PDO or PGI designations have been sporadic. Aguzzoni et al. (2020) used the $^{87}Sr/^{86}Sr$ isotope ratio to classify PDO apples from Italy. Although considering that the isotope ratio was a good marker for studies of food traceability, the authors concluded that results would improve by combining this approach with other chemical parameters. In 2018, a study showed that the geographical origin of prickly pear fruits (*Opuntia ficus-indica* L. Miller) significantly infuences its multielement profile (Mottese et al., 2018). In the same year, using near-infrared (NIR) spectroscopy, authors were able to distinguish between PDO hazelnuts and hazelnuts coming from different regions (Biancolillo et al., 2018). Previous works can be found on PDO cherries from Spain and Greece (Ganopoulos, Argiriou, & Tsaftaris, 2011; Matos-Reyes, Simonot, López-Salazar, Cervera, & de la Guardia, 2013), PGI clementines of Calabria (Benabdelkamel et al., 2012), and PGI lemons from Italy (Amenta, Fabroni, Costa, & Rapisarda, 2016). Working with fruits Perez et al., (2006) succeeded in distinguishing strawberries and blueberries from different provenances using multielement and isotopic analysis. Still, they could not classify pears according to their country of origin using multielement and isotopic information (Perez et al., 2006).

The soil type and the environmental conditions under which plants are cultivated determine the plant's mineral and trace elements profile. Thus, trace element content and isotope ratios have been proposed to assure the geographical origin of food samples (Coelho, Castanheira, Bordado, Donard, & Silva, 2017; Gonzalvez et al., 2009). Nonetheless, work on this topic for pears is scarce. We will focus on the determination of twenty-four elements, using ICP-MS and ICP-OES to study the authenticity of pears.

In addition, the mineral profile of foods analysed under a quality control framework can be used to update food composition databases (FCDB). FCDBs are used for various purposes, ranging from clinical practice, research, public health/education, food industry to planning and policy and nutrition monitoring and surveillance (Ene-Obong et al., 2019). Online FCBDs contain nutrient values generated by laboratories and scrutinized by compilers under a validated quality system and supported by robust sampling plans.

Food composition platforms are crucial for various activities like public health, nutrition, trade, agriculture, government policy, and shaping consumer choices (Egan, Fragodt, Raats, Hodgkins, & Lumbers, 2007). The reputation of FCDBs stands on the use of highly rigorous procedures from analysis to compilation. Thus these FCBDs are solid foundations for developing rational consumer choice and education (Bhagwat, Patterson, & Holden, 2009; Castanheira, Saraiva, Rego, & Ollilainen, 2016).

Despite pears being well documented in several online FCDBs (EuroFIR, 2018), as far as we know, no information is available in any FCDB for Rocha Pear. In this study, we analyze the mineral and isotopic contents of Rocha pears to promote the linkage between the nutritional profile and geographical origin, filling the gap of absent values in FCDBs.

3.4 Port and Douro Wines

Port wine is very famous and appreciated worldwide (Instituto da Vinha e do Vinho, 2021), even appearing in some best selling novels like Agatha Cristie’s Hercule Poirot. It is produced in the Douro Demarcated Region in the northeast of Portugal. This region, created in 1756, was the first denomination of origin to define not only the wine-producing area but also regulations regarding producing methodologies and trade rules (L. Pereira et al., 2018).

In 2021, Port wine accounted for 65% of the wine production in the region and 36.4% of national wine exports (Instituto da Vinha e do Vinho, 2021; Instituto dos Vinhos do Douro e do

Porto, 2021), clearly illustrating its importance in the global wine market. The main export destinations within the EU are France, Netherlands and Belgium, while outside the EU, the United Kingdom, United States of America and Canada are the primary consumers. Asian countries like South Korea and China represent growing export markets (Instituto da Vinha e do Vinho, 2021).

Its particular wine making process with the addition of grape spirit mid fermentation, combined with the unique edapho-climatic characteristics of the region, leads to a large diversity of Port wines with a natural residual sweetness and an alcoholic content between 19% and 22% (v/v). In addition, the blending of different batches (harvest year, vine varieties or location within the region) also contributes to the diversity of Port wines. Ageing can be in the bottle, which is the case of Ruby and vintage Port, or in wood, like Tawny Port. However, all Port wines stage a minimum of two years in wood, as is the case of vintage Port.

Within the same area and region, we can also find the production of Douro dry wines. These are table wines with PDO that can age in wood or stainless steel containers and will be referred to as Douro wines from this point forward.

Although being very different products, mainly due to differences in the vinification method, both wines share their geographical origin, history, agricultural practices and grapevine varieties. In other words, these wines share their terroir, defined as an interactive cultivated ecosystem in a given place that includes climate, soil and the vine (van Leeuwen, 2010).

Studying wine is essential not only for its economic value but also because of the health effects associated with its consumption (Tariba, 2011). In the 1960s, the phenomenon known as the French paradox launched the debate around the benefits of wine consumption. Since then, several epidemiological studies have evidenced its potential protective cardiovascular effects (Burr, 1995).

Mostly known for contributing to the prevention of coronary heart disease when consumed in moderation (Castaldo et al., 2019), wine can, in addition, contribute to mineral intake but also exposure to toxic elements. Additionally, while some elements positively influence the organoleptic properties of wines, others may contribute to undesirable effects such as haze formation or changes in taste and aroma (Tariba, 2011).

The geographical origin assumes great importance in the wine industry, as the wine’s characteristics, including its chemical composition, highly depend on the terroir. Mislabelling compromises the authenticity of wines and can present itself in the form of misrepresentation of varieties and geographical origin (Kamiloglu, 2019).

The high value of origin wines supports the rising number of studies dedicated to the provenance of this beverage over the last years (Coelho et al., 2017; Kamiloglu, 2019; Kelly et al., 2005). Analytical strategies recur to multielement fingerprinting (Gonzálvez, Llorens, Cervera, Armenta, & de la Guardia, 2009; Rodrigues et al., 2011), stable isotopes (Catarino et al., 2019; Durante et al., 2015; Horacek et al., 2021; Ogrinc, Košir, Kocjančič, & Kidrič, 2001) or more frequently a combination of two or more strategies (Epova et al., 2019; Teixeira et al., 2021), as tools for assessing the geographical origin of wines. Among the most common isotopic systems proposed to trace the geographical origin of wines are $\delta^{18}O$ and $^{87}Sr/^{86}Sr$.

The EU adopted the determination of $\delta^{18}O$ using IRMS as the official method for assessing the authenticity of wines, including adulteration and conformity with the origin indicated in the label (European Commission, 2008). The EU wine databank was created in 1991 to compile and store isotopic data from wines produced across the EU. This information is not available to the general public, but the Member States can request it if suspicious cases arise (European Union, 2022).

The first application of Sr isotope ratios for food authentication was published in 1993 using wine samples (Horn, Schaaf, Holbach, H�lzl, & Eschnauer, 1993). Even nowadays, wine is still one of the most studied food commodities in this field (Coelho et al., 2017). This probably relates to this market's strict regulation and the high commercial value achieved by wines from certain PDOs.

In February 2022 alone, 10000 bottles of wine were apprehended in Italy for fraudulently presenting the logos from Italian geographical indications on their labels. Another 112 000 bottles of alcoholic beverages were mislabelled as “Made in Italy” (European Commission, 2022b). In Portugal over a thousand bottles were apprehended in 2017 for mislabelling as Barca Velha or Pera Manca, two famous Portuguese wines produced in the Douro and Alentejo Demarcated Regions, respectively (Autoridade de Segurança Alimentar e Económica, 2017).

3.5 State of the art

Often, mislabelled foods are not identifiable by appearance, smell or taste. It is up to the scientific community to provide analytical tools that allow the detection of fraudulent products.

As a result of the legislative protection of regional products, regulatory authorities are asking for analytical techniques to support such law enforcement (Danezis et al., 2016). Many studies have been published over the last few years using different markers (e.g. phenolic profile, volatile organic compounds, elemental profile, stable isotopes fingerprinting, DNA fingerprinting, organic acids). However, no harmonized approach has been established so far.

Regarding geographical origin, the focus of research needs to be on parameters that depend mainly on natural sources like soil, climate, location, and topography; rather than those influenced by anthropogenic sources.

Plants absorb elements mainly through their roots. Therefore, elements and their isotopic ratios in plants depend on the soil and climate conditions in which they grow. In this way, major elements, trace elements and stable isotopes may provide an excellent response to the scientific challenge of tracing the geographical origin of food products.

Elemental concentrations have been widely used to develop methods for authentication of foodstuffs. On the other hand, isotope ratios provide a chemical fingerprint that allows for a very precise tracing of the geographical origin of foodstuffs. The advantages of combining elemental concentrations and isotope ratios or using them separately will be a matter of interest in this research work.

Although some work can be found in Portuguese foods regarding geographical provenance, it is still a much-unexplored field. Non-exhausting literature research shows that existing studies on Portuguese food products focus primarily on wines (Almeida & Vasconcelos, 2003; Catarino et al., 2019; Almeida & Vasconcelos, 1999; Rocha, Pinto, Almeida, & Fernandes, 2019; Rodrigues et al., 2011; Teixeira et al., 2021), but also on sugarcane honey (Silva, Freitas, Nunes, & Câmara, 2021; Silva, Silva, Perestrelo, Nunes, & Câmara, 2018), meat products from the Iberian black pig (Krajnc et al., 2021) and olive oil (Albuquerque, Costa, & Oliveira, 2019).

3.5.1 Multielement analysis as a tool for food authentication

Different approaches have been suggested to address the issue of food authentication, (Danezis et al., 2016). Minerals and trace elements are naturally present in soils. Still, they may also be incorporated by anthropogenic activities, like agricultural practices (application of pesticides, fertilizers or fungicides), post-harvest processing or packaging. Some elements may also derive from atmospheric contamination (Epova et al., 2020; Pohl, 2007).

When elements are dissolved in the soil solution, they are absorbed by roots during water uptake and distributed throughout the plant along with water. Several factors affect the availability of nutrients and their uptake by plants, namely pH, redox potential, biological activity, cation-exchange capacity, and clay contents (Fageria, Baligar, & Clark, 2002). Still, the content of selected minerals and trace elements in plants reflects the soil and the environmental growing conditions. Thus, trace element content has been proposed to assure the geographical origin of food samples (Gonzalvez et al., 2009).

There are several examples of multielement profiling using ICP-MS and ICP-OES in food authenticity studies for varied matrices, namely wines (Rodrigues et al., 2011; Tanabe, Nelson, Boulton, Ebeler, & Hopfer, 2020), onions (Furia, Naccarato, Sindona, Stabile, & Tagarelli, 2011), almonds (von Wuthenau, Segelke, Müller, Behlok, & Fischer, 2022), or honey (Drivelos, Danezis, Halagarda, Popek, & Georgiou, 2021), to name a few.

However, in the majority of cases, the best results are obtained by combining multielement analysis with other approaches, in particular stable isotope analysis. This was clearly evidenced in the case of mangos, where combining multielement determination with stable isotopes provided a more accurate classification compared to the use of each technique individually (Muñoz-Redondo et al., 2021). Recent articles focus mostly on the combination of more than one analytical technique.

3.5.2 Isotopic analysis as a tool for food authentication (Sr, Pb, B, O)

Isotopes were first discovered by J.J. Thompson in 1913 when he used parallel electric and magnetic fields to separate two isotopes of neon on a photographic plate. Scientists followed his work leading to the discovery of stable isotopes for several elements (Wieser, Schwieters, & Douthitt, 2012).

It is commonly assumed that overall, isotopic abundances are constant in nature, and in that sense, isotopes represent a signature of the elements. However, variations can occur between different compartments of the environment due to isotope fractionation processes. These affect the relative abundance of isotopes either by radioactive decay or by fractionation due to physical and chemical reactions.

Stable isotopes do not decay into other elements, in contrast to radioactive isotopes. The radioactive decay of isotopes leads to extremely low differences in isotopic compositions; thus, few techniques can correctly perform isotope ratio measurements. Developments in the analytical instruments have provided high precision and accuracy measurement, which are a fundamental requirement for the widespread use of isotope ratios in different applications (Oulhote, Le Bot, Deguen, & Glorennec, 2011).

Following is presented an overview of the four elements whose isotopes were used in the present study: B, O, Sr and Pb.

3.5.2.1 Boron

Boron has two stable isotopes, ^{10}B and ^{11}B, with relative average Earth abundances of 19.7% and 80.3%, respectively (Meija et al., 2016).

The element B does not occur free in nature. Instead, it combines with O and other elements to form boric acid (H_3BO_3), or inorganic borate salts called borates (Bernard, Harrass, & Manning, 2010).

In humans, so far, no specific biochemical function has been discovered for B. However, studies have indicated that this element is a beneficial bioactive food component that promotes health and well-being. In particular, B has been associated with positive biochemical and functional responses related to bone growth and maintenance, brain function, and possibly cancer risk reduction (Nielsen, 2014).

On the other hand, in plants, B is recognized as an essential micronutrient required for growth, flowering, pollination, and seed formation (Nielsen & Eckhert, 2019; Pereira et al., 2021). It is absorbed from the soil solution by roots primarily in the form of boric acid (H_3BO_3) in acid solutions and in the form of borate anion [$B(OH)_4^-$] in alkaline solutions (Sun, Gou, Dong, Xu, & Cao, 2019). H_3BO_3 is preferentially enriched in the heavy isotope (^{11}B), while $B(OH)_4^-$ is enriched in the light isotope (^{10}B) (Sun et al., 2019). As such, the pH of the soil strongly affects the isotopic absorption of B by influencing the chemical equilibrium between H_3BO_3 and $B(OH)_4^-$ in the rhizosphere (Pereira et al., 2021). Soil moisture, temperature, salinity, organic matter, and precipitation have also been referred to as influencing factors in the equilibrium between the two chemical forms (Shireen et al., 2018). In addition to these factors, the use of fertilizers also has the potential to change the pH of the soil and promote the fractionation of B absorbed by plants.

Boron fractionation in plants has already been the object of study, and its isotopes have been previously applied to the study of wine authenticity. However, few works have been published so far. Coetzee et al. (2011) used hydroponic culture to demonstrate that the ^{11}B/^{10}B ratio in grapevine leaves depended on the variety/rootstock combination, although it also reflected the growth medium. This suggested that B suffers fractionation during root absorption or through biological processes within the plant. Another study, also using hydroponic culture, observed B fractionation in different parts of the plant, in corn, wheat, and broccoli (Marentes, Vanderpool, & Shelp, 1997). More recently, a work combining isotope ratios and elemental concentrations showed that B isotopes, together with Sr and Pb, provided significant discrimination between Lambrusco PDO wines (Lancellotti et al., 2021). However, unlike Sr and Pb isotopes, the fractionation of B isotopes is dependent on environmental pH. As such, it is possible that the changes occurring in wine´s pH during winemaking processes lead to fractionation of B isotopes, changing further its isotopic signature.

3.5.2.2 Oxygen

Oxygen has three stable isotopes, ^{16}O, ^{17}O and ^{18}O, with relative average Earth abundances of 99.76%, 0.038%, and 0.205%, respectively (Meija et al., 2016).
The absolute abundance of the heavier isotope is very small. Thus, the isotopic composition of O is conventionally reported in delta notation relative to an established certified reference material (CRM) (Coplen, 1994). Because of the typically minor isotopic differences, reporting is made by multiplying the delta value by a factor of 1000, thus expressing the difference per mill (%) (Brand, Douthitt, et al., 2014).

The relative abundance of O isotopes is influenced by the hydrological cycle (Figure 3). Isotope fractionation of O in water occurs due to the effects of evaporation and precipitation. In evaporation, the water evaporated from the ocean is richer in ^{16}O. The δ^{18}O values of the vapour are more negative than those of the ocean water that originated them (Rodrigues et al., 2013). As a consequence, the ocean is high in ^{18}O. In precipitation, the concentration of the heavy isotope (^{18}O) increases gradually with the increase of temperature, meaning that water in higher latitudes is depleted in ^{18}O as it precipitates in the lower, warmer latitudes (Jäger & Hunziker, 1979; Piana, 2010). Thus, the cold, polar regions are depleted in ^{18}O, but they are high in ^{16}O (Piana, 2010). Ice in poles is highly depleted in ^{18}O, with δ^{18}O values reported below -13‰ (Porter & Opel, 2020). The amount of ice in the poles also influences the δ^{18}O values since as glaciers melt in warmer climates, the ^{16}O trapped inside is released into the ocean, decreasing its δ^{18}O value (Piana, 2010). Higher altitudes and increased distance from the sea also contribute to the depletion of ^{18}O in precipitation (Jäger & Hunziker, 1979).

The isotope ratio $^{18}O/^{16}O$ exhibits relatively large variations in natural waters. Water enriched or depleted in ^{18}O, with a heterogeneous distribution around the globe, will eventually be absorbed by plants. It is important to note that, since evaporation and precipitation are highly correlated with temperature, climate changes can significantly impact the distribution of O isotopes around the globe (Piana, 2010).

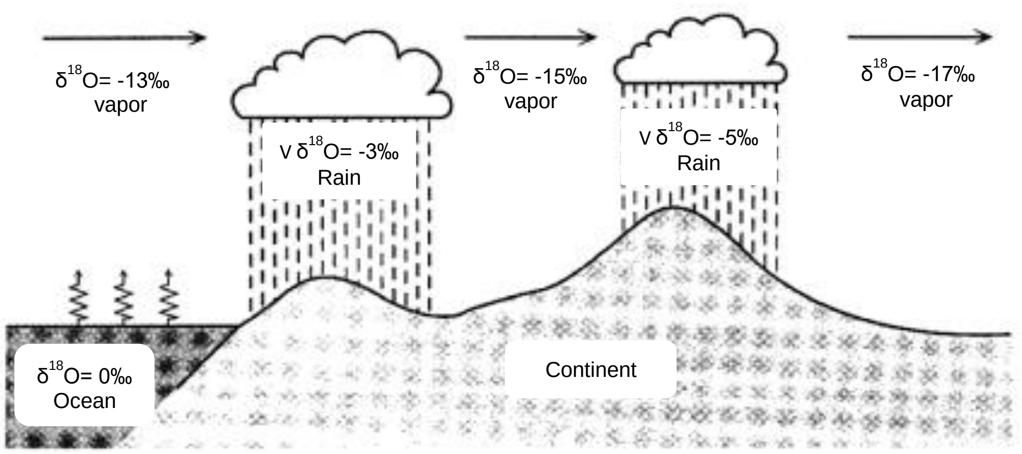

Figure 3. Oxygen isotopes and the water cycle. Adapted from (Jäger & Hunziker, 1979)

There is no evidence of fractionation of isotopes during water uptake by plant roots. Therefore the isotopic composition of water in roots and stems reflects the isotopic composition of water available to the plant (Rodrigues et al., 2013). Oxygen, $\delta^{18}O$, is used to trace the geographical origin and detect the watering of wines since the $\delta^{18}O$ value from tap or spring water is lower than $\delta^{18}O$ in wine water that originates from plants (Horacek et al., 2021; Santesteban, Miranda, Barbarin, & Royo, 2015). In fact, its value is not affected by anthropogenic factors, such as pollution or biological fractionation within the plant. Instead, it alters with the physical processes of the hydrological cycle, as previously explained (Roberts, Zanchetta, & Jones, 2010). A study combining the $\delta^{18}O$ values from 4000 wine samples with several climatic and geographical descriptors showed that in wine, this parameter is influenced by climate and location, mainly latitude, precipitation, distance from the ocean and temperature (Camin et al., 2015).

The determination of $\delta^{18}O$ by IRMS is the official method for assessing the authenticity of wines within the EU (European Commission, 2008). The European wine databank, established in 1991, compiles the isotopic composition of wines collected from across the EU so that competent authorities in the Member States can request information when disputes or court cases arise (European Commission, 2020b). However, this information is accessible only to authorised users.

3.5.2.3 Strontium

Strontium is a chemical element without biological functions in humans. In fact, only two biological roles are known, both as a biomineral constituent. However, Sr and Ca (a significant biometal) present chemical similarities, hence, promoting the absorption of the former by living organisms.

Strontium is a very reactive metal that quickly oxidizes, forming an oxide, and therefore it does not occur free in nature (Nielsen, 2004). Instead, it can be found (as Sr^{2+}) in most rocks and soils with varying concentrations as a function of the rock type, being that basalts show higher Sr content. At the same time, in sandstone and low-calcium granite, its presence is less noticeable (Höllriegl & München, 2011).

Strontium has four stable isotopes, ^{88}Sr, ^{87}Sr, ^{86}Sr and ^{84}Sr, with relative average Earth abundances of 82.58%, 7.00%, 9.86% and 0.56%, respectively (Meija et al., 2016).

One of Strontium's stable isotopes, ^{87}Sr, is radiogenic, meaning that its relative abundance will increase over time due to the radioactive decay of ^{87}Rb. Radiogenic isotopes are at the basis of various bioinorganic applications. In particular, the isotope ratio $^{87}Sr/^{86}Sr$ has been used over the past 50 years. The first application was published in 1972 (Faure & Powell, 1972), for geochronology or as a geochemical tracer for past geological processes, such as the chemical differentiation of the Earth (Charlier et al., 2012; Kleine, 2011).

Current $^{87}Sr/^{86}Sr$ values of geochemical reservoirs and rocks can be found between 0.702 for the depleted mantle (low Rb/Sr) and above 0.943 for the old continental crust (Rosner, 2010).

Weathering is responsible for releasing Sr from rocks into soils and subsequently to plants and animals and entering the oceans primarily by river transport of sediments (Bentley, 2006). Radioactive decay in rocks of different ages leads to different isotope ratios in the labile fraction of the soils, which plants later absorb. Hence, the Sr isotopic signature is a fingerprint representative of the soil on which plants were cultivated.

A study using rice and hot peppers showed that the contribution of the bedrock and the silicate fraction of the soil to bioavailable Sr is insignificant. In contrast, the composition of the water and the exchangeable fraction of the soil have a more significant influence on bioavailable Sr (Song, Ryu, Shin, & Lee, 2014). On the other hand, Song et al. (2015) analysed fruits, leaves, rocks and soils to show that Sr isotope ratios of bedrocks were maintained in soils and plants. In 2013 a study on Lambrusco wines reported a good match between the Sr isotope ratios monitored in the bio-available fraction of soils and their respective grape juice values (Durante et al., 2013). A few years later, and although the Sr isotopic signature between soils and Lambrusco wines was in agreement, it was demonstrated that the year of production did not influence the isotopic composition of Sr (Durante et al., 2015). The same conclusions were obtained by Marchionni et al. (2013). The authors concluded that the isotopic fingerprint of wines is mainly determined by the bioavailable fraction of the soils on which the vineyards are settled.

$^{87}Sr/^{86}Sr$ is also constant across the food web since apparently it is not influenced by external factors (e.g. pollution, climate) or fractionation after absorption by living organisms (Bentley, 2006; Capo, Stewart, & Chadwick, 1998). Such has been demonstrated by matching fish scales and otoliths to the surrounding water (Pouilly, Point, Sondag, Henry, & Santos, 2014) or by studying Sr isotope ratios from the soil across two trophic levels (soil-plant-butterfly) (Flockhart, Kyser, Chipley, Miller, & Norris, 2015).

Other studies have demonstrated that the Sr isotope ratio present in the wine is not affected by the wine-making process thus reflecting the soil in which the plant has grown (Coelho et al., 2017; Durante et al., 2015). This stability was demonstrated along the production line, starting from the bioavailable fraction of soils, passing to branches, musts and finally to wine, making $^{87}Sr/^{86}Sr$ an excellent biological marker of the terroir (Cellier, Bérail, et al., 2021; Durante et al., 2013; Marchionni et al., 2016).

Currently, little is known about other natural processes responsible for Sr isotope fractionation (Shalev et al., 2013). Some authors refer that high temperature and precipitation rates significantly favour higher Sr isotope fractionation (Charlier et al., 2012; Shalev et al., 2013). In this way, due to the low temperatures at which they occur, it is not expected significant fractionation of Sr isotopes motivated by biological processes, whether involved in plant or animal metabolism (Bentley, 2006; Flockhart et al., 2015).

Once absorbed by plants, the $^{87}Sr/^{86}Sr$ value is kept across the trophic levels (Figure 4), unlike the organic components of foodstuffs that can be influenced by agronomical conditions (Drivelos & Georgiou, 2012) or by technological factors (e.g. extraction systems, processing filtrations, storage conditions) (Ranalli, Malfatti, Lucera, Contento, & Sotiriou, 2005; Ranalli, Cabras, Iannucci, & Contento, 2001). In addition, Sr presents chemical similarities with Ca (an important element for all organisms), hence promoting the uptake of the former by plants and animals (Bentley, 2006), regardless of its biological relevancy. Therefore, $^{87}Sr/^{86}Sr$ in biological samples, independently of the trophic level, can be used as tracers for geographical provenance since they reflect the original sources of Sr.

Figure 4. Conservative isotope ratio throughout the food chain (Coelho et al., 2017).

3.5.2.4 Lead

Lead is an environmental contaminant that occurs naturally but mainly from anthropogenic activities like mining and smelting, and battery manufacturing (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2010a). Its use caused extensive environmental contamination, human exposure and significant public health problems in many parts of the world (World Health Organization, 2021a). It has become one of the most serious environmental threats to human health. In Europe, by the 1980s, Pb emissions decreased significantly when leaded gasoline was forbidden. However, the end of leaded petrol worldwide happened only in 2021 when the last country, Algeria, finally stopped providing it (UN Environment Programme, 2021).

According to the EFSA, human exposure to Pb occurs mainly via food and water, although it can also happen via air, dust and soil. It is particularly harmful to young children since they tend to absorb it more than adults do (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2010b; World Health Organization, 2021b). It is distributed to the brain, liver and kidneys, and, over time, it is stored in teeth and bones. Due to its long half-life in the body, the chronic toxicity of Pb is of most concern when considering the potential risk to human health. The principal possible critical adverse effects of Pb identified were developmental neurotoxicity in young children and cardiovascular effects and nephrotoxicity in adults (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2010b). There is no level of exposure to Pb that is known to be without harmful effects (World Health Organization, 2021b).

Pb has four stable isotopes, ^{204}Pb, ^{206}Pb, ^{207}Pb and ^{208}Pb, with relative average Earth abundances of 1.4%, 24.1%, 22.1% and 52.4%, respectively (Meija et al., 2016).

Similarly to ^{87}Sr, three of its isotopes, ^{206}Pb, ^{207}Pb and ^{208}Pb, are radiogenic isotopes and are the end products of the radioactive decay of ^{238}U, ^{235}U and ^{232}Th, respectively (Gulson, 2008). The difference to ^{87}Sr is that the variations in the relative abundances of Pb isotopes are a consequence of three independent decay chains and the abundances of the three radiogenic Pb isotopes co-vary strongly and depend on when the ore was formed (Cheng & Hu, 2010a).

The isotopic compositions of different types of Pb-containing minerals are a consequence of the interaction between several processes, including radioactive decay of U and Th to Pb, the relative proportion of U–Th–Pb in the system, and mixing of Pb from different sources (Cheng & Hu, 2010b). Estimates of the emissions of individual Pb sources indicate that the atmosphere is the initial primary recipient and that anthropogenic sources of Pb are major contributors compared to natural sources. Studies dealing with Pb isotopes offer a convenient approach for studying and tracing the sources of Pb pollution in different environmental compartments (Komárek, Ettler, Chrastný, & Mihaljevič, 2008).

Although to a lesser extent than Sr or O, Pb isotopes have been used to study the geographical provenance of wines for some time (Epova et al., 2020; Almeida & S.Vasconcelos, 1999; Teixeira et al., 2021). The isotopic signature of the Pb is different according to the source of the contamination. In soils, the isotopic composition of Pb reflects the mixing of these sources (Komárek et al., 2008). Similarly to other heavy metals, Pb does not fractionate during uptake by plants (Lancellotti, Sighinolfi, Marchetti, & Tassi, 2020). However, studies have shown a discrepancy between soil and wine’s Pb isotope ratios suggesting that Pb in vine and wine is not originated primarily from the soil but from other sources such as atmospheric deposition (Epova et al., 2020; Kristensen, Taylor, & Evans, 2016; Lancellotti et al., 2020). While Sr isotope ratios are geogenic tracers, Pb isotopes are indicators of environmental pollution.

3.6 Mass spectrometry techniques

Whether for health, economic, legal or transparency reasons, or even just for consumers preference, it is fundamental to have reliable analytical tools for proving the authenticity of food products. The analytical methods shall establish a confident and proven correlation between the analytical parameters of commercialized products and their geographical region of origin.

The development of new advanced analytical techniques over the last ten years made it possible to determine the geographic origin of most foodstuffs by analysing their elemental and isotopic compositions (Drivelos & Georgiou, 2012).

Developments in the analytical instruments have provided high precision and accuracy measurement, which are a fundamental requirement for the widespread use of isotope ratios in different applications (Oulhote et al., 2011). Over the past decade, with the development of new advanced analytical techniques, it is now possible to successfully retrieve elemental and isotopic compositions of any given food sample and determine the geographic origin successfully (Drivelos & Georgiou, 2012). In addition to elemental determinations, multi-isotope or single-isotope ratios can provide unique and representative fingerprints, thus making it possible to discriminate the origin of food samples.

Mass spectrometry is a technique used to separate molecules, atoms or isotopes in samples based on their mass to charge ratio (m/z). Coupling mass spectrometers to different sample introduction systems, ion sources and detectors allows for a broad field of applications.

Three techniques stand out regarding the analysis of elements and stable isotopes: ICP-MS, MC-ICP-MS and IRMS.

3.6.1 Inductively coupled plasma mass spectrometry (ICP-MS)

In 1980 Houk et al., published the first work on ICP-MS. The authors describe for the first time the equipment combining an inductively coupled plasma and a mass spectrometer to determine trace elements and isotopes in solutions.

The first commercial ICP-MS was made available only three years later. It was launched in 1983 by PerkinElmer and showed significant improvement in the limits of detection (LD) and speed for elemental analysis (Hunt, 2008). Progressively other companies produced their equipment, and the ICP-MS quickly established itself as a desired tool for determining trace, minor and major elements in almost every analytical field (AgilentTechnologies, 2005; Cubadda, 2007).

Briefly, ICP-MS uses a peristaltic pump to ensure that a continuous flow of sample is introduced to the nebulizer of the equipment. The sample forms an aerosol in the nebuliser and heads to the spray chamber. Here the larger droplets are removed while, the smaller ones move forward to the torch where the argon plasma is formed. In the plasma, the temperature is around 7500 K, meaning that aerosol droplets are rapidly dried, decomposed, vaporized and atomized, and then ionized by removing one electron from each atom (AgilentTechnologies, 2005).

The ions go through the interface, passing by two cones, the sample and the skimmer. Once they reach the vacuum chamber, ions are directed to the mass spectrometer by a lens system. Finally, at the mass spectrometer, ions are sequentially filtered according to their m/z. The separated ions reach the detector, typically an electron multiplier, which measures them in counts per second. Figure 5 shows the schematic representation of an ICP-MS instrument.

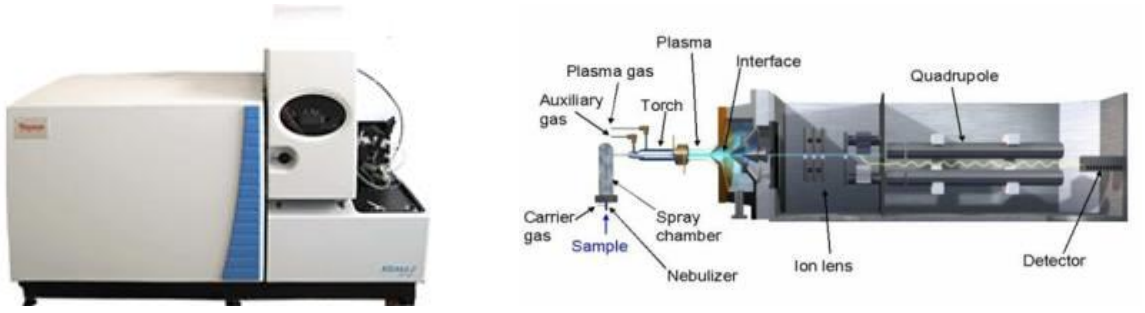

Figure 5. Thermo X Series II and schematics of a quadrupole ICP-MS (UC Davis, 2020).

Although ICP-MS offers the possibility of direct analysis of solids by coupling a laser ablation system to the equipment, the more common systems require the use of liquid samples. As such, food samples need a sample preparation step to ensure the destruction of the organic matter, thus avoiding the clogging of cones or plasma instability. Depending on the food matrix and the goal of the analysis, this step may resort to different strategies, like open or closed vessel digestion. Sample preparation is a crucial step of the analysis to obtain accurate and reliable results. The sample pre-treatment protocol must be suited to the intended analytes and the matrix. It must assure that no contamination or analyte loss occurs. Typically, ICP-MS applications for foodstuffs resort to inorganic acids for matrix digestion.

The success of the ICP-MS is due to its many advantages, namely high sample throughput, high sensitivity, multi-element capability and wide elemental coverage, wide linear dynamic range and providing some isotopic information. In addition, ICP-MS can be coupled to chromatographic techniques acting as a powerful detector in hyphenated methods that allow the determination of element species (AgilentTechnologies, 2005; Cubadda, 2007).

This technique provides high sensitivity for most elements in the periodic table and rapid throughput of samples. It allows a simultaneous quantitative determination of trace elements with low DLs at the ultra-trace level and good accuracy and precision. The main disadvantages of the technique are its high acquisition and running costs, demand for highly qualified staff, limited total dissolved solids tolerance and potential interferences due to limited mass resolution.

3.6.2 Isotope ratios mass spectrometry (IRMS)

Since its development in the 1940s, IRMS (Figure 6) has been the technique used to study the isotopic composition of a limited number of elements, namely, $^{13}C/^{12}C$, $^{2}H/^{1}H$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$ (Brand, Douthitt, et al., 2014). These are often referred to as “traditional” or “light” isotopes. However, the terminology “light isotopes” associated with IRMS is misleading. It does not account for the element B (or Li, for that matter), which is also an element of interest in the present study. When commercial equipment became available during the 1960s and 1970s, new applications were developed, and research in this field grew considerably (Brand, Douthitt, et al., 2014).

IRMS requires the introduction of gaseous samples. Solid and liquid samples need to be vaporized using a sample preparation system. Results are obtained by comparing gaseous samples to a reference gas with a well-defined isotopic composition. Then results are normalized to the suitable standards and expressed by using the isotope delta notation (Brand, Douthitt, et al., 2014).

This comparison technique has the advantage of compensating instrumental effects such as drift or mass discrimination, which may change over time and vary from instrument to instrument (Brand, Douthitt, et al., 2014).

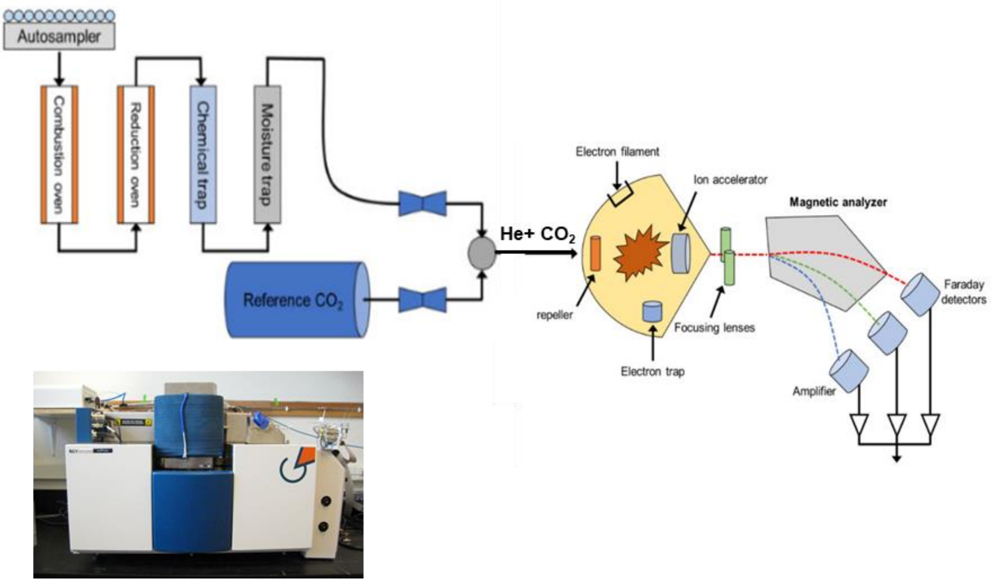

Figure 6. GV instruments isotope ratio mass spectrometer and schematics of an equipment. Adapted from (Won, Yun, Lee, & Shin, 2021).

3.6.3 Multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS)

MC-ICP-MS is used to detect changes in the relative abundances of “non-traditional” isotopes, like Sr or Pb.

Although isotopic abundances are considered constant in nature, small variations, known as isotopic fractionation, can occur due to physical and chemical reactions or because of radioactive decay. The results are extremely low differences in isotopic composition; hence, few techniques can correctly perform isotope ratio measurements. High precision measurement is a fundamental requirement.

The development of the MC-ICP-MS brought several advantages over other available techniques at the time, like thermal ionization mass spectrometry (TIMS), namely high ionization yield, versatile sample introduction system and no time-dependent mass fractionation (Krupp et al., 2001). MC-ICP-MS has become an advanced technique for measuring non-traditional isotopic compositions at high precision (Epov et al., 2010). This state of the art technique allows a high precision isotope ratio determination with an excellent limit of quantification (LQ).

MC-ICP-MS instruments (Figure 7) combine an ICP argon plasma source, where positively charged ions are created, with a complex electrostatic zoom lens system that focuses the ion beam and directs it to the magnetic field. Since isotopes of a given element present different masses, due to the different number of neutrons, they will be separated based on their m/z and directed to the respective detector (Wieser et al., 2012).

The position of the detectors is not fixed and may be arranged for each isotopic system. In that way, several detectors can be placed with micrometre precision to collect the ion current of the isotopes of interest simultaneously. This fine precision is essential to measure isotope ratios suffering from spectral interferences accurately. In such cases, the interfering ion beam must be resolved from the target ions by high mass resolution. Once the beam reaches the detector, the ions are converted into voltages. Isotope ratios are obtained by comparing voltages from the different detectors (Wieser et al., 2012).

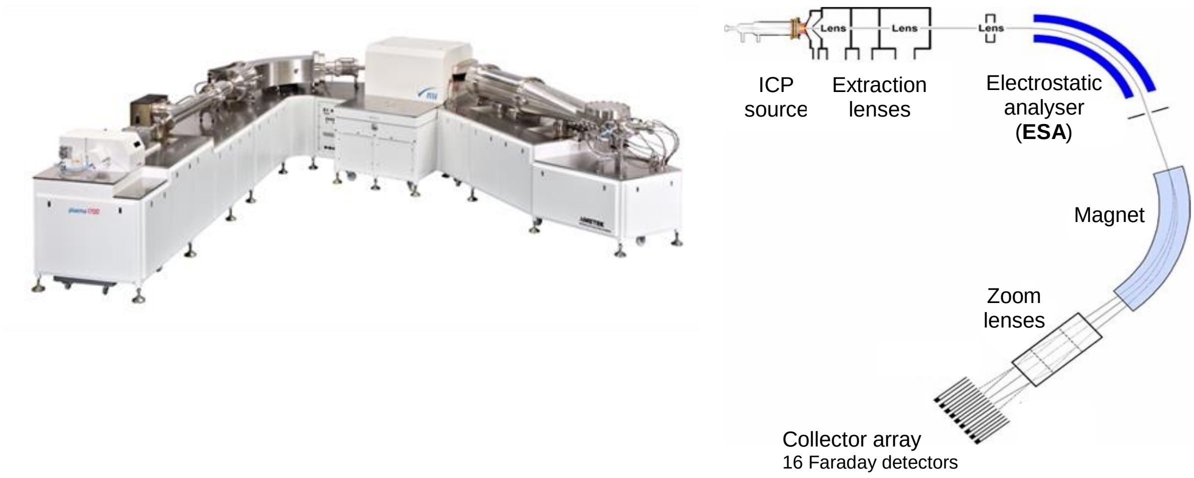

Figure 7. Nu Plasma 1700 and schematics of an MC-ICP-MS instrument. Adapted from (Yang, 2009)

MC-ICP-MS presents high ionization yield and versatile sample introduction systems well established in ICP spectrometry. It is an advanced technique for measuring non-traditional isotopic compositions at high precision, namely Sr and, more recently, Pb. Also, since isotopic determinations occur simultaneously, there is no time-dependent mass fractionation (Krupp et al., 2001).

3.7 Chemometrics

Chemometrics was established at the beginning of the 1970s and is “the chemical discipline that uses mathematical, statistical, and other methods employing formal logic to design or select optimal measurement procedures and experiments and provide maximum relevant chemical information by analysing chemical data” (Héberger, 2008).

When dealing with multielement and isotopic analysis, data treatment is as necessary as the analytical techniques employed in the isotopic determinations. The large number of variables involved originate massive datasets of values. Multivariate data analysis is essential to extract relevant information from such datasets (Drivelos & Georgiou, 2012). Appropriate modelling of the isotopic and elemental data using multivariate statistics is necessary for identifying isotopic and elemental fingerprints. The chemometrics approach uses multivariate methods, i.e., all variables are considered simultaneously (Héberger, 2008), thus providing a crucial tool for data interpretation in food authentication.

Several statistical tools have already been described in the literature (Gonzalvez et al., 2009; Kelly et al., 2005; Oulhote et al., 2011). A short description of the most relevant ones for the present work is provided below.

Although being a univariate test, Analysis of Variance (ANOVA) is a pretty helpful starting point in data analysis. It provides a statistical test to compare the means of several groups, identifying significant differences between them. ANOVA is functional in comparing two, three, or more means and has been used to compare elemental profiles of foods of different origins (Drivelos & Georgiou, 2012).

Principal Component Analysis (PCA) is an unsupervised exploratory technique that reduces the dimensionality of a dataset while preserving as much ‘variability’ (i.e. statistical information) as possible (Jolliffe & Cadima, 2016). PCA transforms the original measured variables into new uncorrelated variables called principal components (PCs). The data dimensionality reduction allows their visualization while retaining the information present in the original data as much as possible (Drivelos & Georgiou, 2012).

Hierarchical Cluster Analysis (HCA) is another frequently used unsupervised statistical technique. It assigns samples into groups, or clusters, according to their similarities. The procedure of hierarchical clustering involves the construction of a hierarchy of treelike structures (Roessner, Nahid, Chapman, Hunter, & Bellgard, 2011).

Contrary to the previously presented techniques, Linear Discriminant Analysis (LDA) is a predictive supervised pattern-recognition method (Drivelos & Georgiou, 2012). It is generally used to classify patterns between two classes, but it can be extended to more (Vaibhaw, Sarraf, & Pattnaik, 2020). It is based on the determination of linear discriminant functions, which maximize the ratio of between-class variance and minimize the within-class variance (Drivelos & Georgiou, 2012). The final step in LDA is often cross-validation to verify how accurately the predictive model performs. The cross-validation uses samples of known origin as blind samples to validate the model (Oulhote et al., 2011).

4 Material and methods

This chapter was partially adapted from the following paper:

Coelho, I., Matos, A. S., Teixeira, R., Nascimento, A., Bordado, J., Donard, O., & Castanheira, I. (2019). Combining multielement analysis and chemometrics to trace the geographical origin of Rocha pear. Journal of Food Composition and Analysis, 77, 1–8. https://doi.org/10.1016/j.jfca.2018.12.005

Coelho, I., Matos, A. S., Epova, E., Barre, J., Cellier, R., Castanheira, I., Bordado, J., Donard, O., (2022). Multi-element and multi-isotopic profiles of Port and Douro wines as tracers for authenticity. (Submitted to Food Chemistry)

This chapter presents the methodology followed for the multielement and isotopic characterization of pears and wines. Figure 8 is a schematic representation of the procedure followed for each matrix.

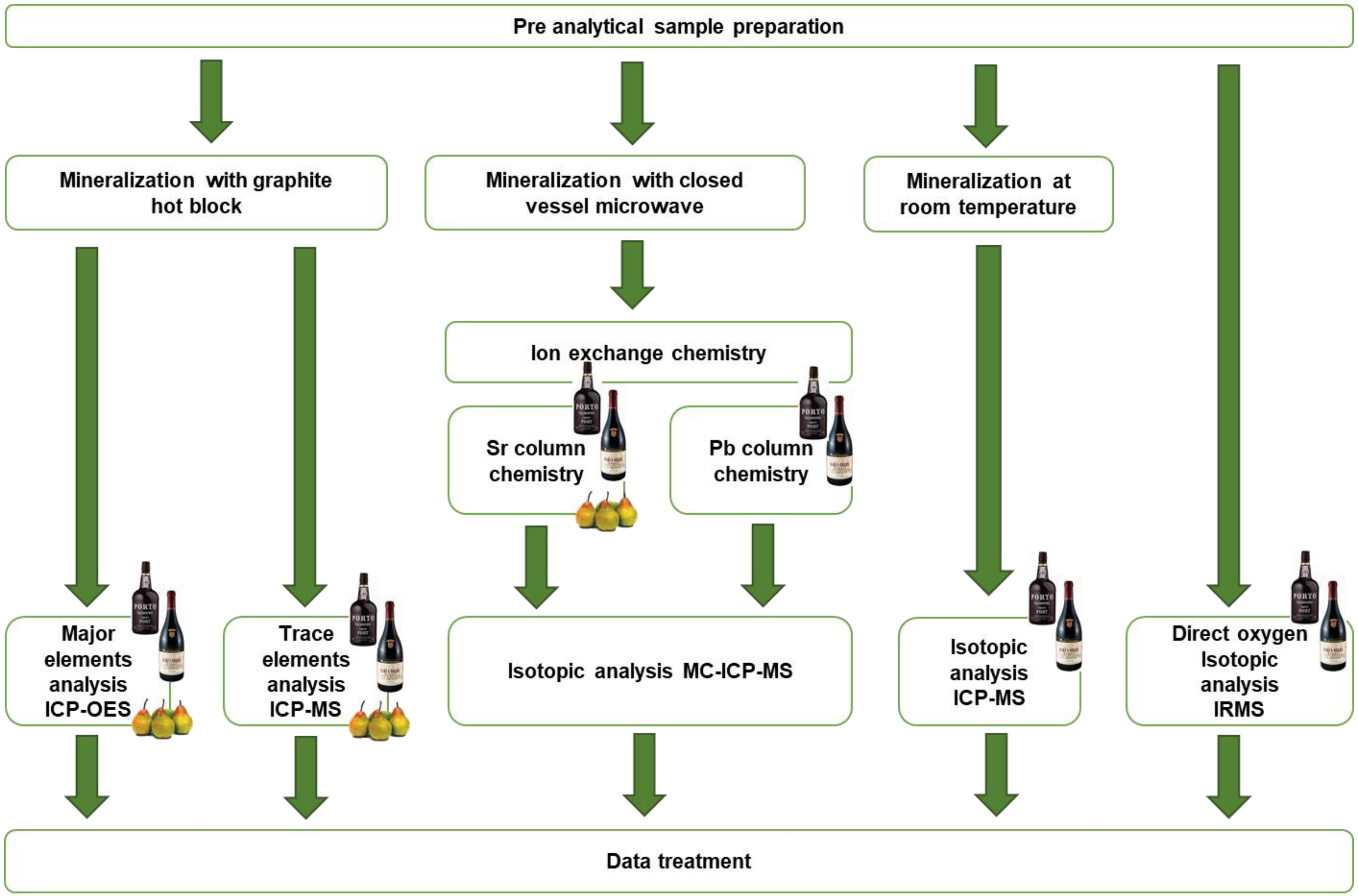

Figure 8. Schematics of the analytical approach followed for pear and wine samples.

4.1 Pre-analytical sample preparation

4.1.1 Pears

Samples were obtained directly from producers and collected according to the previously described sampling plan to ensure the geographical origin (Coelho et al., 2019).

One hundred fifty samples of Rocha pears were collected in August of 2016 from 10 different farms: 5 located in the PDO region, and the remaining five originating from the area of Fundão, a non-PDO production area (Figure 9). Five trees were selected from each farm, and within each tree, three pears were harvested and analysed as a pooled sample. This sampling scheme led to 25 pools from each region, each containing three pears from the same tree.

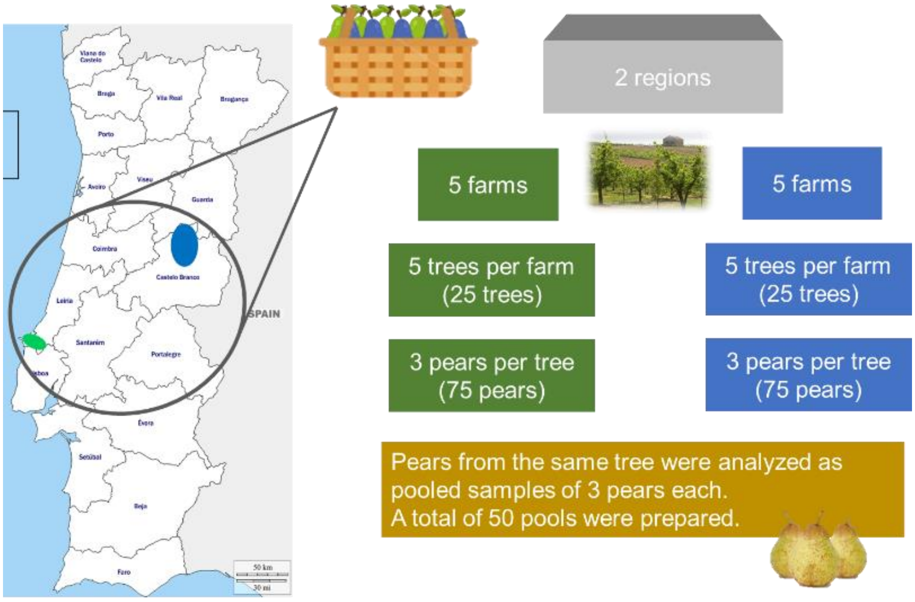

Figure 9. Sampling plan for Rocha pear. The area with PDO production is green, and in blue is the non-PDO production region.

The preparation of samples for storage is presented in Figure 10. Samples arrived at the laboratory in groups of 3 pears identified by the farm and tree of origin. For each group of samples, the procedure was identical. Samples were washed with deionized water, seeds were removed, and pears were cut into smaller pieces and placed in plastic containers. Each container represented only one pool. Each pool required two containers to fit the whole sample available.

In addition to the 25 pools, a study was conducted to evaluate the effect of peeling samples on the elemental composition of pears. Consequently, six samples were used to prepare separate pools containing peeled and unpeeled pears. In these cases, samples were cut in quarters. Two opposed quarters remained unpeeled and formed one pool. The peel was removed from the other two quarters, which originated another pool. Peels were discarded.

Afterwards, pooled samples were frozen below -18 ºC before being lyophilized for a minimum of 48 hours (Heto PowerDry PL9000/HSC500, Thermo Fisher Scientific, Waltham, USA). Moisture content was determined for all samples in duplicates by measuring the mass before and after lyophilization.

Dehydrated samples were then homogenized into a fine powder using a grinder (GRINDOMIX GM 200, Retsch, Haan, Germany), equipped with titanium blades. Samples were vacuum-sealed in polyethylene bags and kept at room temperature until analysis.

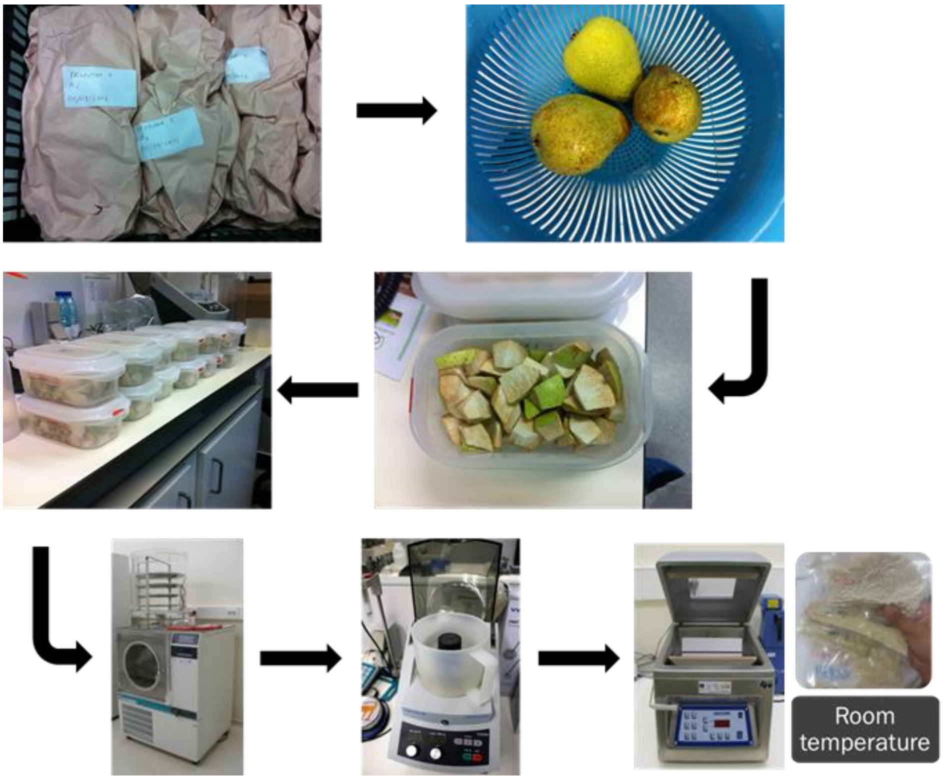

Figure 10. Preparation of pear samples for storage.

4.1.2 Wines

In this study, we analysed 30 wines originating from the Douro Demarcated Region, half of which was Port wines and the other half Douro wines. To assure the authenticity and traceability of samples, these were provided by the Port and Douro Wines Institute (IVDP), which is the public entity responsible for controlling the use of the Douro geographic indication worldwide (Instituto dos Vinhos do Douro e do Porto, 2021). Samples were numbered from 1 to 15, preceded by the letter P for Port wines and the letter D for Douro wines**.** Table 1 provides information regarding sample ID, year of production (when available since Port wines may be blended), colour, soil type and coordinates of the producer.

Table 1. Description of wine samples used in the study (n=30).

Sample ID	Type	Designation	Colour	Year	Storage	Soil	Coordinates Datum 73, Hayford-Gauss, IGOE	Observations
P1	Fortified wine	Port wine	White	n.a.	wood/stainless steel	Schist	231342;464624	
P2	Fortified wine	Port wine	Red	n.a.	wood	Schist	231342;464624	
P3	Fortified wine	Port wine	Red	2014	stainless steel	Schist	232850;476195	Laboratory sample.
P4	Fortified wine	Port wine	Red	2016	wood	Schist	247237;466080	Vintage candidate or LBV.
P5	Fortified wine	Port wine	Red	2015	wood	Schist	305964;452098	Vintage candidate or LBV.
P6	Fortified wine	Port wine	Red	n.a.	wood	Schist	227893;463437	Reserve Ruby. Bottled in 2014.
P7	Fortified wine	Port wine	Red	n.a.	wood/stainless steel	Schist	230672;465651	
P8	Fortified wine	Port wine	Red	2014	----	Schist	228104;465223	
P9	Fortified wine	Port wine	Red	2012	wood	Schist	248715;468220	Vintage- Bottled in 2014.
P10	Fortified wine	Port wine	White	> 30 years	wood	Schist	233033;477985	
P11	Fortified wine	Port wine	Red	2015	wood	Schist	261791;466042	
P12	Fortified wine	Port wine	White	n.a.	wood	Schist	232731;463230	
P13	Fortified wine	Port wine	Red	n.a.	----	Schist	232731;463230	
P14	Fortified wine	Port wine	Red	n.a.	----	Schist	237382;464948	
P15	Fortified wine	Port wine	Red	2015	----	Schist	241365;465408	
D1	Wine	Douro wine	Red	2014	stainless steel	Transition/Schist	271046;449772	
D2	Wine	Douro wine	Red	2015	stainless steel	Schist	224934;465770	
D3	Wine	Douro wine	Red	2014	wood	Schist/Transition	258438;493243	
D4	Wine	Douro wine	Red	2014	stainless steel	Schist	232076;465889	
D5	Wine	Douro wine	White	2015	stainless steel	Schist	252719;462181	
D6	Wine	Douro wine	Red	2013	wood	Schist	238181;464719	
D7	Wine	Douro wine	White	2014	stainless steel	Schist	242666;477951	
D8	Wine	Douro wine	White	2015	stainless steel	Schist	228378;464791	
D9	Wine	Douro wine	White	2015	stainless steel	Schist	231490;475602	
D10	Wine	Douro wine	Red	2013	wood	Schist	261155;467833	
D11	Wine	Douro wine	Red	2012	wood	Schist	282542;454993	
D12	Wine	Douro wine	Red	2013	stainless steel	Schist	282542;454993	
D13	Wine	Douro wine	Red	2014	stainless steel	Schist	312006;455129	
D14	Wine	Douro wine	Red	2013	wood	Schist	272041;456631	
D15	Wine	Douro wine	Red	2012	wood	Schist/Transition	275508;484130	

4.2 Reagents and chemical standards

All reagents used in the analyses were of high analytical grade. Ultrapure water, resistivity 18 M cm, was obtained using a Milli-Q plus Millipore System. Concentrated nitric acid, HNO_3 (65%) (Merck, Darmstadt, Germany), was purified in an acid distillation system (Milestone SubPUR, Shelton, USA) before usage. Purity was verified by comparing each batch of laboratory-purified acid against a commercial ultrapure HNO_3 solution (Merck, Darmstadt, Germany).

A 2% (v/v) solution of HNO_3 was used to prepare working standard solutions, dilute samples and prepare blanks. The same solution and a 4% (v/v) solution of HNO_3 were used to wash up the sample introduction systems of ICP-MS and ICP-OES, respectively, between samples.

Working multi-element standard solutions were prepared from mono-element high-purity ICP stock standards (SPC science, Montreal, Canada). Working multi-element standard solutions of the same elements were independently prepared from multi-element solution XVI (Merck, Darmstadt, Germany) and Mono-element ICP standards (PlasmaCAL, SCP Science, Montreal, Canada) and used for quality control. Internal standard correction for ICP-MS was made by Y and In (1000 mg/L; Merck, Darmstadt, Germany).

For isotopic analysis, NIST SRM 987 (Strontium carbonate, NIST, Gaithersburg, MD, United States), NIST SRM 981 (Common lead isotopic standard, NIST, Gaithersburg, MD, United States), NIST SRM 997 (Thallium isotopic standard, NIST, Gaithersburg, MD, United States) and NIST SRM 951a (Boric acid isotopic standard, NIST, Gaithersburg, MD, United States) were used.

4.3 Apparatus

Elemental concentrations were determined by ICP-MS (Thermo X series II, Thermo Fisher Scientific, Waltham, USA) and ICP-OES (Thermo iCAP 6000 series, Thermo Fisher Scientific, Waltham, USA).

A high-resolution MC-ICP-MS Plasma 1700 (Nu Instruments Ltd., Wrexham, UK) and a MC-ICP-MS Plasma 1 (Nu Instruments Ltd., Wrexham, UK) were used for the isotopic analysis of Sr and Pb, respectively.

The isotopic analysis of B was performed on an ICP-MS instrument PlasmaQuant Elite (Analytik Jena, Jena, Germany).

The isotopic analysis of O was done on a MultiFlow Bio preparation system (IsoPrime, Manchester, UK) connected to a continuous flow isotope ratio mass spectrometer (IRMS; GV instruments, Manchester, UK)

Depending on the succeeding analytical determination, sample preparation was performed using a closed vessel microwave oven (ETHOS 1 Series, Milestone, Shelton, USA), a graphite digestion block (DigiPREP, SCP Science, Montreal, Canada) and an ultrasonic water bath (Elmasonic S 60 H, Elma, Singen, Germany).

4.4 Quality control

The analytical work was carried out in three different laboratories established across Europe, namely at the National Institute of Health Doutor Ricardo Jorge (INSA) in Portugal, at the University of Pau (UPPA) in France and the Josef Stefan Institute (JSI) in Slovenia.

All work was carried out in the framework of NP EN ISO / IEC 17025 standard under rigorous metrological control (ISO/IEC, 2005).

Assessment of analytical methods and laboratory performance was based on the use of standard analytical methods, replicates, blanks, CRMs, in house reference materials, spiked samples, participation in proficiency testing (PT) schemes and estimation of the measurement uncertainty associated with analytical data (Coelho, Gueifão, Matos, Roe, & Castanheira, 2013).

The quality assurance program followed for each determination was executed in compliance with the internal guidelines established at each executing facility.

In some cases, the terminology followed by the laboratories was not always harmonized. This emphasized the need to define the concepts applied. For clarification and harmonization purposes between the terminologies used at each laboratory, the International Vocabulary for Metrology (VIM) was adopted, in particular in what relates to the concepts transcripted below (International Organization of Legal Metrology, 2007):

Precision - closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions.

Repeatability condition of measurement - Condition of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time.

Repeatability - measurement precision under a set of repeatability conditions of measurement.

Intermediate precision condition of measurement - condition of measurement, out of a set of conditions that includes the same measurement procedure, same location, and replicate measurements on the same or similar objects over an extended period of time, but may include other conditions involving changes.

Intermediate precision - measurement precision under a set of intermediate precision conditions of measurement.

Reproducibility condition of measurement - condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects.

Reproducibility - measurement precision under reproducibility conditions of measurement.

Measurement accuracy - closeness of agreement between a measured quantity value and a true quantity value of a measurand.

More detailed information on quality control is provided under each method’s chapter.

4.6 Multielement analysis

Sample contamination is of uttermost importance in trace and ultra-trace analysis. Analyses were performed in clean room facilities, and all labware was made of plastic (PFA or PTFE) to minimize the possibility of contamination.

4.6.1 Sample digestion – Pears

A total of 24 elements were determined by ICP-MS (Al, As, B, Be, Cd, Co, Cr, Cu, Li, Mn, Mo, Ni, Pb, Se, Sn, Sr, Tl, Zn) and by ICP-OES (Ca, Fe, K, Mg, Na and P). The digestion method was optimized by comparing closed vessel microwave digestion (ETHOS 1 Series, Milestone) with a graphite digestion block (DigiPREP, SCP Science, Montreal, Canada). Different parameters were tested, such as sample intake, the addition of acid and hydrogen peroxide, H_2O_2, and digestion temperature and duration. The method of choice was the graphite digestion block equipped with programmable power control. It provides the destruction of organic matter with adequate accuracy and precision and is less time consuming than closed vessel microwave digestion.

As such, 700 mg of lyophilized sample were weighted directly into the digestion tubes followed by the addition of 7 mL of concentrated HNO_3 and left overnight. On the next morning, samples were placed in the graphite digestion block with the following program:

- step (1) 5 min up to 45 ºC
- step (2) 25 min up to 95 ºC
- step (3) 120 min at 95 ºC
- step (4) cooling down.

After cooling to room temperature, the volume was made up to 25 mL with deionized water. All samples were analyzed in triplicate. Digested samples were kept at 5 ± 3 ºC until further analysis.

4.6.2 Sample digestion – Wines

A total of 26 elements were determined by ICP-MS (Ag, Al, As, B, Be, Cd, Co, Cr, Cu, Li, Mn, Mo, Ni, Pb, Rb, Se, Sn, Sr, Tl, Zn) and by ICP-OES (Ca, Fe, K, Mg, Na and P).

Before analysis, samples were mineralized. As such, 5 mL of each wine sample was evaporated to half and left to react overnight with 5 mL of HNO_3. Afterwards, samples underwent the same 4-step program presented for pear samples (chapter 4.5.1) using the graphite hot block. The final volume was made up to 25 mL with ultra-pure water. Mineralized samples were kept at 5 ± 3 ºC until further analysis.

4.6.3 Quality control

The quality assurance scheme was designed following the NP EN ISO / IEC 17025 standard and was similar for both matrices under study (ISO/IEC, 2005).

A new calibration curve was prepared daily with a minimum of five standards in different concentrations. Two independent Quality Control Standards (QCS) were used between each batch of five samples to monitor instrumental drift during each working session. One QCS had the same concentration as the first point of the calibration curve, while the other had an intermediate concentration of the working range. The acceptance criteria for the QCS was a relative error of less than10% compared to the theoretical concentration.

Samples were analysed in triplicate, and repeatability was evaluated as RSD%. In pears, the lowest repeatability was observed for Se and Sn, both consistently below 7.4%. The highest values were found in Cr, Mo and Ni, below 14%. In wines, repeatability was below 10% for all elements.

Each digestion cycle contained at least one blank to monitor for potential contaminations and one spiked sample or CRM to check accuracy.

In pears, spiked samples and the reference material - Carrot NCS ZC73031 from China National Analysis Centre - were used to check accuracy. In wines, only spiked samples were available.

The laboratory also participated regularly in PT schemes launched by two accredited providers (FAPAS® and BIPEA) with satisfactory results.

The LQ was first calculated as 10 times the SD of 10 to 20 digestion blanks series. The first point of the calibration curve was equal to or above that value and was considered the LQ. Quantification was accepted with a tolerance of 10% above or below the working range. A calculation was applied to the LQ using the average sample weight and the appropriate dilution to reflect the matrix.

4.7 Isotopic analysis

4.7.1 Boron

The B isotopic analysis was performed on a PlasmaQuant Elite following the protocol of Cellier, (2020). The mineralization of the samples for the isotopic analysis of B was carried out according to the protocol:

1. Add 5 mL of HNO_3 to 5 mL of wine
2. Leave to react for at least 8 hours
3. Add 2 mL of H_2O_2
4. Leave to react for at least 3 hours at room temperature.

Boron isotopes were measured by sample calibrator bracketing, using NIST SRM 951 (0‰ by definition) at 100 µg.L^{-1}, accounting for mass discrimination through time. Samples and calibration solutions were measured using the same method.

Repeatability was verified by analysing samples in triplicates. The maximum RSD of triplicates was 0.19%. The accuracy of δ^{11}B measurements was confirmed by measuring an in house reference material, "Champagne", at 10.1‰ (n=12) (Cellier, 2020).

The δ^{11}B values, expressed in the *δ*-notation, were reported relative to the NIST SRM 951a boric acid, according to Equation (1) (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014):

$$\delta^{11}\mathrm{B}\ (‰) = \left(\frac{\mathrm{R}(^{11}\mathrm{B}/^{10}\mathrm{B})_{sample}}{\mathrm{R}(^{11}\mathrm{B}/^{10}\mathrm{B})_{Standard}} - 1\right) \times 1000 \tag{1}$$

The delta values are multiplied by 1000 and expressed in units “per mil” (‰).

4.7.2 Oxygen

The $^{18}O/^{16}O$ ratios were determined directly in the wine after equilibration with reference gas CO_2/He (5% CO_2) at 40°C for 6 hours. Measurements were performed on a MultiFlow Bio preparation system (IsoPrime, Manchester, UK) connected to a continuous flow IRMS (GV Instruments, Manchester, UK). Analyses were normalised against laboratory reference standard seawater with the value of $\delta^{18}O = +0.36 \pm 0.04‰$, snow with the value of $\delta^{18}O = -19.73 \pm 0.02‰$ and external laboratory reference material ERM with the value of $\delta^{18}O = +4.83 \pm 0.29‰$. Samples were analysed in triplicate. A laboratory reference material, “Sweet wine” with $\delta^{18}O = -0.73 \pm 0.29‰$, was used for accuracy control. The combined uncertainty for $\delta^{18}O$ determination in wine was ± 0.2‰, while the expanded uncertainty was ±0.4‰.

Data quality control charts were systematically recorded throughout the study period. To ensure the validity and comparability of the isotope results, the laboratory regularly participates in the “Food analysis using Isotopic Techniques-Proficiency Testing Scheme” (FIT-PTS), organized by EUROFINS. This PT scheme happens three times per year and includes wine.

The stable isotope ratios of O are expressed in the *δ*-notation according to Equation (2) (Brand, Coplen, et al., 2014):

$$\delta^{18}\mathrm{O}\ (‰) = \left(\frac{\mathrm{R}(^{18}\mathrm{O}/^{16}\mathrm{O})_{sample}}{\mathrm{R}(^{18}\mathrm{O}/^{16}\mathrm{O})_{Standard}} - 1\right) \times 1000 \qquad (2)$$

The delta values are multiplied by 1000 and expressed in units “per mil” (‰). The $\delta^{18}O$ was reported relative to the V-SMOW standard (Vienna Standard Mean Ocean Water) (Brand, Coplen, et al., 2014).

4.7.4 Strontium

Strontium isotopic analysis was performed on the Nu-Plasma-1700 multi-collector as previously described (Cellier, Berail, et al., 2021; Epova et al., 2019).

In Sr isotopic analysis, it is critical to isolate Sr from the remaining matrix, paying particular attention to Rb, an important interferent. The use of columns with specific resins, Sr-selective resin Eichron®, is well suited for such a purpose.

Samples digested using the graphite digestion block showed a light yellowish colour, indicating incomplete destruction of the inorganic matter. Although this procedure is sufficient for ICP-MS and ICP-OES determination, it leads to saturation of the Sr-selective resin Eichron®.

For Sr isotopic analysis, pear and wine samples had to be mineralized using closed vessel microwave digestion (ETHOS 1 Series, Milestone). Each digestion cycle contains 12 vessels distributed by one sample in triplicate, seven single samples, one CRM or spiked sample, and one blank.

For pears, the mineralization protocol was as follows:

1. Weigh 1g of lyophilized sample into a PTFE vessel and leave overnight to react with 7 mL of HNO_3;
2. Add 1 mL of H_2O_2;
3. Place in the microwave according to the following program: (1) 10 min up to 180 ºC, (2) 5 min at 180 ºC, (3) 12 min up to 210 ºC, (4) 5 min at 210 ºC and (5) cooling down.

For wines, the mineralization protocol was as follows:

1. Evaporate 5 mL of wine to half;
2. Add 5 mL of HNO_3 and leave overnight;
3. Add 1 mL of H_2O_2 followed by microwave digestion according to the previous program.

After mineralization the digestates were concentrated by evaporation to assure a minimum of 100µg of Sr / 10 mL, as required by the MC-ICP-MS. The protocol, common to both matrices, was based on (Epova et al., 2019) as follows:

1. Transfer the samples to PFA tubes that fit the digiprep (savilex tubes).
2. Evaporate to dryness, at 80ºC, for approximately 15 hours or until being dry.
3. Add 4 mL of 3M HNO_3 and close the tubes.
4. Place in the ultrasound bath for 5-10 min.
5. Place in the digiprep for 5-10 min, at 85 °C.

6. Place in the ultrasound bath for 5-10 min.
7. Check if samples are fully dissolved. If not, repeat steps 4, 5 and 6.
8. Load to the column with Sr-Spec Eichrom® Sr-selective resin.

The procedure for the extraction of Sr was as follows:

1. Weigh between 140 to 160 mg of Sr-Spec Eichrom® Sr-selective resin to a 10 mL tube and add 4 mL of HNO_3 3M;
2. Place a filter on the column;
3. Place a tube for waste below the column;
4. Transfer the Sr-Spec Eichrom® Sr-selective resin to the column;
5. Resin Washing - rinse the column with 10 mL of HNO_3 3M (2x5 mL) followed by 20 mL H_2O (4x5 mL), Discard;
6. Resin conditioning - add 2 mL of HNO_3 3M, Discard;
7. Sample load - Add the sample (4 mL);
8. Matrix removal - rinse the column with 8 mL of HNO_3 3M (2x4 mL), Discard;
9. Place the tube for the collection below the column;
10. Sample elution - add 10 mL of H_2O (2x5 mL);
11. Collect the solution for analysis;
12. Acidify sample – add 0.2 mL of concentrated HNO_3.

The levels of Sr rand Rb were monitored at two critical points of the process using ICP-MS: one after mineralization and the other after purification. Recovery rates for Sr after purification were accepted between 70% and 100%. The maximum ratio of Rb/Sr after purification was 0.0045. In each purification cycle, at least one sample was analysed in triplicate, and one blank and NIST SRM 987 (pure SrCO3, NIST, USA) were also monitored throughout the whole process. Isotopic $^{87}Sr/^{86}Sr$ results for NIST SRM 987, under intermediate precision conditions, were at the value of 0.71027 ± 0.00002 (2SD, n=6). Instrumental and procedural blanks were permanently controlled for the traces of Rb.

NIST SRM 987 was used for daily optimization of the MC-ICP-MS as well as bracketing standard for measurement. The value of SRM 987 applied for data processing was 0.710255 according to Waight et al. (2002) and was measured with the precision of 55 ppm (2RSD, n=15).

4.7.5 Lead

Pb isotopic analysis was performed on the Nu-Plasma 1 MC-ICP-MS connected to a nebulizer DSN-100 (Nu Instruments, Wrexham, UK) under dry plasma conditions, as described (Epova et al., 2020). Briefly, before analysis, Pb in the samples was purified using Dowex 1-X8 resin following the mineralisation of wine.

SRM 981 (Common Lead Isotopic Standard, NIST, USA) was used as a bracketing standard. Data were corrected for the isobaric interferent ^{204}Hg by monitoring ^{202}Hg and using the proper correction equation. SRM 997 (Thallium Isotopic Standard, NIST, USA) was used as an internal standard to correct for instrumental mass bias. The accuracy was confirmed by measuring an in house reference material, "Champagne", in triplicate, at ^{208}Pb/^{204}Pb = 38.244 ± 0.043 (2SD, n=3), ^{207}Pb/^{204}Pb = 15.637 ± 0.014 (2SD, n=3) and ^{206}Pb/^{204}Pb = 18.234 ± 0.01 (2SD, n=3) (Cellier, 2020).

4.8 Statistical analysis

Descriptive statistical data were reported as mean, maximum and minimum values. For pears, this data considered five trees per farm, quoted on a dry weight basis, and for wines, 15 samples for each type (Port or Douro).

Values between the LD and the LQ were treated similarly to values above the LQ. Concentrations below the LD were considered as LD/$\sqrt{2}$ (Verbovše, 2011).

Tests were performed using either Statistica® 8.0 or 12 software (Statsoft Ibérica, Lisboa, Portugal) for pears and wines, respectively. Statistical significance was established at a p-value < 0.05 for all analyses but also at a p-value < 0.01 for Pearson coefficient significance. The first step consisted of testing data for normality using the Kolmogorov-Smirnov test. For parameters presenting a normal distribution, the analysis continued with One-way ANOVA to evaluate possible significant differences and Pearson correlation coefficient to establish relations between parameters. For the remaining Mann Whitney and Spearman correlation were performed.

Two unsupervised pattern recognition techniques, PCA and HCA, were used to identify groups of samples based on the measured variables and the relationship between these variables and the observed differences among groups (Brereton, 2003; Yücel & Sultanoğlu, 2013). PCA studies the relationships between variables with the aim of reducing the original number of variables while defining a new set of ("latent") variables (PCs) that explains the maximum percentage of the variation in the data. Varimax rotation was used for PCA to maximize the total sum of squares of the loadings among each axis. Although several rotational strategies can be found in the literature, varimax is the most disseminated one, available in most commercial factor analysis software.

On the other hand, HCA identifies inside a set of samples a grouping pattern by minimizing the homogeneity within-group (cluster) and maximizing the heterogeneity between groups based on distances. The strategy used was agglomerative, meaning that initially, there are as many clusters as samples, which are grouped according to their similarities. To find similarities, HCA was performed considering the Euclidean distance, and as a clustering method, Ward's method was applied. For both studies, PCA and HCA, data were auto-scaled to zero mean and unit variance due to vast differences in data dimensionality. For pears, LDA was used to build a predictive model for classifying samples according to their geographical origin.

5 Results and discussion

This chapter was partially adapted from the following paper:

Coelho, I., Matos, A. S., Teixeira, R., Nascimento, A., Bordado, J., Donard, O., & Castanheira, I. (2019). Combining multielement analysis and chemometrics to trace the geographical origin of Rocha pear. Journal of Food Composition and Analysis, 77, 1–8. https://doi.org/10.1016/j.jfca.2018.12.005 (published)

Coelho, I., Matos, A. S., Epova, E., Barre, J., Cellier, R., Castanheira, I., Bordado, J., Donard, O., (2022). Multi-element and multi-isotopic profiles of Port and Douro wines as tracers for authenticity. (Submitted to Food Chemistry)

5.1 Analysis of Rocha Pears

5.1.1 Multielement analysis

5.1.1.1 Effect of peeling

Rocha pear can be consumed with or without the peel. The peel acts as a physical barrier to protect the fruit, but in pears, it also has nutritional value as it concentrates some bioactive compounds and minerals (Li et al., 2014; Michailidis et al., 2021). On the other hand, peel tends to accumulate pesticides which represent a risk for human health (Han et al., 2015).

A comparative study was made to determine whether pear samples should be analysed with or without peel using five different samples, identified from S1 to S5. Two-quarters of each pear were peeled while the other two remained unpeeled. Individual results of samples are presented in Table A1 from Appendix 1.

The Paired Data t-Test was used to compare results from peeled and unpeeled samples. This bilateral test is used to compare the mean of differences between two measurements taken from the same sample, as is in the present case.

In terms of nutrients and contaminants in peeled and unpeeled Rocha pears, some considerations may be taken from the obtained results. However, regarding the effects on human health, no conclusions are to be taken from the present data, as such a study requires a fit for purpose sampling plan and risk-benefit analysis, which is not within the scope of the present work.

Most of the elements did not display any significant differences between peeled and unpeeled samples. However, four elements did present significant differences according to the Paired Data t-Test, considering a significant level of 5% ($|T_0| < T_{(0.025;\ 5)}$). These were Al, Cu, K and Pb, as shown in Figure 11.

Cooper and potassium were significantly higher in peeled samples. These results align with a previous study that found lower Cu and K values in unpeeled Nashi pears and Gala apples (Michailidis et al., 2021). The same authors also reported higher levels of Ca and Mn in unpeeled pears and apples. In the present study, these two elements also presented higher concentrations in unpeeled samples. However, these differences were not deemed significant by the Paired Data t-Test.

Some of the most relevant differences found between peeled and unpeeled samples were found for the elements Al and Pb. The levels of Pb were above the LQ only in peeled samples. The opposite behaviour was observed for Al, quantifiable only in unpeeled samples. Aluminium is the third most abundant element on the planet. Natural processes such as rock weathering cause the release of Al into soils. Its availability to plants depends mainly on the soil's pH. When available, it is absorbed through the roots and transported to other plant parts (Rahman & Upadhyaya, 2021). On the other hand, although Pb also occurs naturally, its presence is highly influenced by human activity (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2010b). As such, the levels of Pb may be conditioned by environmental contamination.

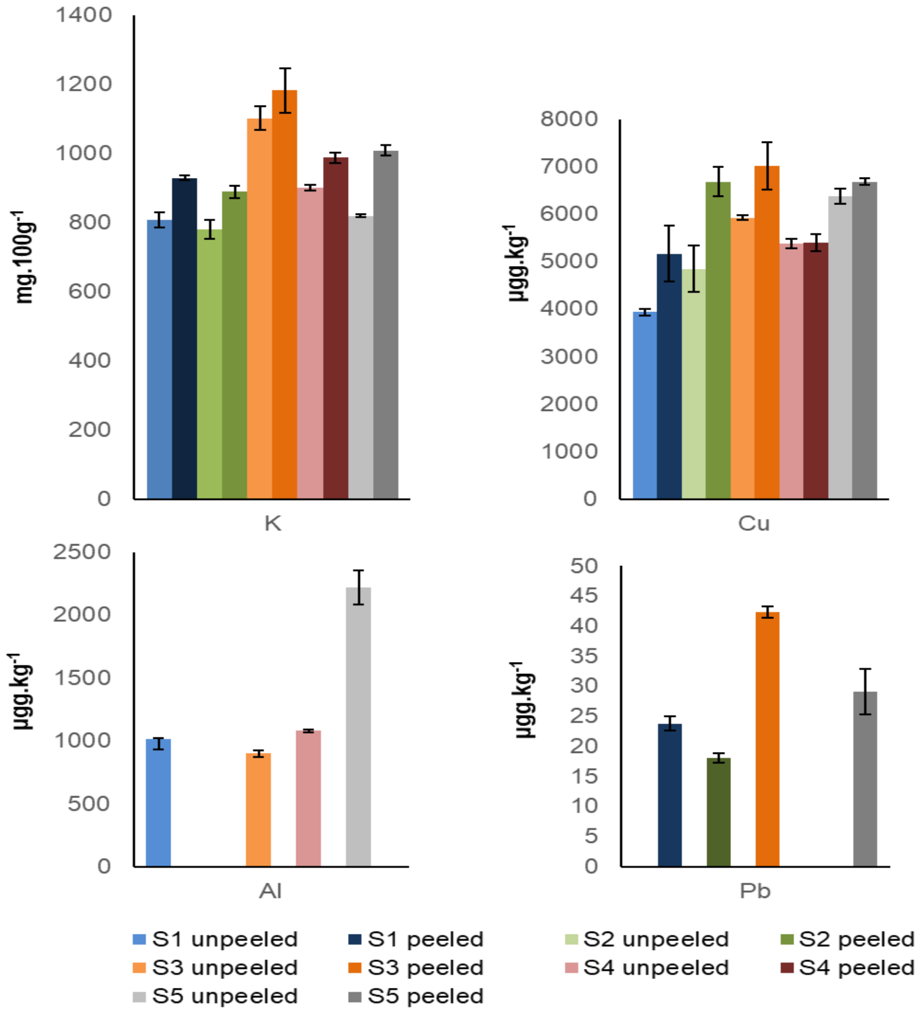

Figure 11. Comparison between the levels of K, Ca, P, Cu, Mn, Al, Ni and Pb in peeled and unpeeled pear samples (n=5).

Keeping in mind that the aim of this work is to evaluate the contribution of the multielement profile of Rocha pears to trace its authenticity, the choice was to proceed with the samples that provided quantifiable Al instead of Pb. As such, the study continued using unpeeled samples.

5.1.1.2 Sample homogeneity

After lyophilisation, pear samples were stored in vacuum-sealed bags at room temperature. When opened for analysis, samples had formed a compact mass with a rubbery texture. Since, from this point on, samples were complicated to grind, it was necessary to conduct a study to assess the homogeneity of samples without grinding.

One sample was randomly selected, identified as S6, and used for the repeatability study. Ten independent replicates were weighed from this sample by cutting approximately 0.7g of sample with a ceramic knife. Elements determined by ICP-MS were evaluated, and the results above the LQ are presented in Table 2. The RSD was below 10% for all elements analysed. Therefore, the results followed the quality criteria established and within-sample homogeneity was assured by cutting the sample with a ceramic knife. All further studies on the pear samples were performed in triplicate, ensuring repeatability of measurements and without grinding lyophilized samples.

Table 2. Homogeneity study of lyophilized pears (n=10). Results are expressed in µg.kg^{-1} of dry weight.

	S6 (n=10)		
	Mean	**SD**	**RSD %**
Li	26	0.75	2.8
B	12618	219	1.7
Al	2394	225	9.4
Cr	322	29	9.0
Mn	2336	103	4.4
Co	123	2.7	2.2
Ni	701	23	3.2
Cu	5955	301	5.1
Zn	13504	1275	9.4
Se	88	8.6	9.7
Sr	790	38	4.8
Mo	50	2.8	5.6
Cd	7	0.21	2.9
Sn	9	0.67	7.4

5.1.1.3 Multielement characterization

The concentration of 24 elements was determined by ICP-MS and ICP-OES in 50 pooled samples of Rocha Pear.

Table 3 shows the results obtained for the analytical performance of the method. Whenever CRM was not available, fortified samples were used to check for accuracy. Quality control was within the criteria established by the laboratory and in accordance with the USDA Data Quality Evaluation System and EuroFIR guidelines, assuring that the data are suitable to integrate FCDBs in the future (Bhagwat et al., 2009; Castanheira et al., 2016).

Table 3. Method performance for multielemental determination in pear samples. Elements showing m/z were determined by ICP-MS. Elements showing emission lines were determined by ICP-OES.

Element	Units	m/z	emission lines (nm)	Working range	LQ	LD	Repeatability* (RSD %)	Recovery (%)	MRC Carrot NCS ZC73031 (n=8)	
					(in pears)				Certified value	Found value
Al	µg.kg^{-1}	27	---	25 - 250	810	243	11	98-100	460000	47970±571
As		75	---	0.25 - 2.5	8.1	2.4	9.2	89-114	110±20	99±3
B		11	---	50 - 500	1620	486	9.9	90-106	18100±1100	19577±1187
Be		9	---	0.10 - 2.5	3.2	1.0	8.8	94-104	6.5±1.5	4.7±2.6
Cd		111	---	0.10 - 2.5	3.2	1.0	9.8	88-102	34±4	31±1
Co		59	---	0.25 - 2.5	8.1	2.6	10	110-117	66±7	62±5
Cr		52	---	0.50 – 5.0	16	4.9	14	104-112	1040±130	932±120
Cu		65	---	0.50 – 5.0	16	4.9	10	100-107	4100±410	4097±206
Li		7	---	0.10 - 2.5	3.2	1.0	9.8	108-113	160±20	166±22
Mn		55	---	0.50 – 5.0	16	4.9	12	92-115	12100±2210	12905±1300
Mo		95	---	0.50 – 5.0	16	4.9	14	86-110	100±10	113±2
Ni		60	---	0.50 – 5.0	16	4.9	14	106-112	670±100	530±65
Pb		208	---	0.50 – 5.0	16	4.9	10	83-99	430±70	376±15
Se		77	---	0.50 – 5.0	16	4.9	7.4	87-110	31±10	34±3
Sn		118	---	0.25 - 2.5	8.0	2.6	7.4	111-120	22	15.4±0.4
Rb		85	---	0.50 – 5.0	16	4.9	9.9	---	6900±500	6916±701
Sr		88	---	0.50 – 5.0	16	4.9	13	101-116	22000±2200	21348±1278
Tl		205	---	0.10 - 2.5	3.2	1.0	12	87-98	10.7±2.1	8.8±0.5
Zn		66	---	5.0 – 50	160	48	10	93-103	11200±500	10329±706
Ca	mg.(100g)$^{-1}$	---	184.006	0.2-2.0	6.4	2.1	8.9	---	255±10	229±13
Fe		---	259.940	0.005-0.005	0.16	0.053	12	---	14.8±1.5	11±0.2
K		---	769.896	0.25 - 2.5	8.0	2.7	10	---	1080±40	1138±31
Mg		---	279.553	0.1-1	3.2	1.1	8.8	---	91±3	89±4
Na		---	589.592	0.2 – 2.0	6.4	2.1	9	---	650±30	596±50
P		---	178.284	0.2-2.0	6.4	2.1	9.9	---	230±20	219±9.2

*maximum RSD% between triplicates

The multielement content of minerals and trace elements, expressed on a dry weight basis, is presented in Tables 4 and 5 for the PDO region and Fundão, respectively. Results are presented by farm and expressed by the mean, minimum and maximum value found for each farm. Individual sample results are shown in Appendix 2, Tables A2 and A3.

From the 24 elements studied, Be, Se, Sn, and Pb showed values below the LQ systematically and therefore were not included in Tables 4 and 5 or any statistical analysis performed. Data had to be converted to a wet weight basis for comparison purposes with previous works. Considering that the moisture content of pear samples varied between 81 and 87%, calculations were made according to Equation (3):

$$WW = DW \times [(100 - \%moisture)/100] \qquad (3)$$

where WW = concentration in wet weight basis and DW = concentration in dry weight basis

The content of Cr and Mo was higher than the values reported in the Danish FCDB (Fødevareinstituttet, 2017) but in agreement with the ones found by Nardi et al. (2009). The values found in our study suggest that analysed contaminants such as Ni, As, Cd, Sn and Pb in pears do not present a risk for consumers. Tl, a toxic element with evidence of carcinogenicity (Cvjetko, Cvjetko, & Pavlica, 2010), is not a common element in fruits but can be present in food due to endogenous sources or from pesticides (although forbidden in the EU). Samples from the area of Fundão, mainly farm 5, presented higher Tl content than the PDO region. Even so, these values are below the toxicity level, which is located around 1.5 mg.kg^{-1} (WHO, 1996). Cu and Zn present similar levels, ranging from 0.29 to 1.2 mg.kg^{-1} and 0.42 to 2.7 mg.kg^{-1}, respectively, thus suggesting this fruit may be a good contributor to the recommended daily intake (RDI) of these elements. On the other hand, for Ca, the values obtained are low but in line with previous studies (Fødevareinstituttet, 2017; Perez et al., 2006; USDA, 2017).

Table 4. Mineral composition of pears by farm, from the PDO region, expressed by dry weight.

Farm[1] (n=5)		Li	B	Al	Cr	Mn	Co	Ni	Cu	Zn	As	Sr	Mo	Cd	Tl	Fe	Mg	Ca	Na	P	K
		µg.kg^{-1}														mg.(100g)$^{-1}$					
PDO 1	mean	10	13217	2254	209	1703	48	310	3260	4910	25	579	100	5.7	< LQ	0.66	35	36	34	59	841
	max	12	21903	2704	329	2315	74	500	4820	6355	38	736	138	9.8	4.4	0.81	44	39	45	71	915
	min	5.8	9786	1628	120	1474	13	225	1778	3293	15	353	45	< LQ	< LQ	0.57	27	33	28	45	754
PDO 2	mean	18	9369	2003	227	2488	85	466	4748	14219	15	679	70	9.8	< LQ	0.80	51	54	17	70	810
	max	20	10706	2150	356	3218	117	649	5511	17127	20	918	108	11	3.5	0.96	58	74	22	82	886
	min	16	8250	1742	126	2070	55	336	3655	10958	10	547	58	8.1	< LQ	0.71	46	40	14	57	727
PDO 3	mean	44	12226	1497	227	1747	108	850	5255	6904	12	535	57	18	7.7	0.76	29	34	15	65	681
	max	109	15213	1856	347	1960	136	1362	6747	8360	16	762	61	23	11	0.82	36	46	21	71	761
	min	10	9865	1104	147	1649	82	406	3906	4973	8.2	382	45	11	< LQ	0.59	25	30	8.8	55	596
PDO 4	mean	28	9687	2402	217	3167	30	311	4543	4851	< LQ	1131	80	8.9	27	0.71	37	49	14	56	939
	max	38	10873	3272	333	3946	34	339	6132	5767	< LQ	1651	89	13	48	0.85	45	61	22	66	1098
	min	19	7566	1550	141	2333	22	270	3698	3952	< LQ	611	69	7.2	13	0.62	32	42	9.4	47	860
PDO 5	mean	11	15144	1288	240	2096	65	326	4966	6112	< LQ	583	70	7.8	3.5	0.54	24	38	16	63	730
	max	16	18848	1674	463	2748	96	445	7045	8071	< LQ	768	103	10	6.3	0.65	26	48	27	79	799
	min	9.5	12199	884	134	1315	34	265	3834	5066	< LQ	320	39	5.0	< LQ	0.47	22	23	9.1	49	597

[1] five trees per farm, each tree represented by a pool of three pears analyzed in triplicate.

Table 5. Mineral composition of pears by farm from the region of Fundão, expressed by dry weight.

Farm[1] (n=5)		Li	B	Al	Cr	Mn	Co	Ni	Cu	Zn	As	Sr	Mo	Cd	Tl	Fe	Mg	Ca	Na	P	K
		µg.kg^{-1}														mg.(100g)$^{-1}$					
Fundão 1	mean	25	7897	895	208	1803	35	169	3647	3442	< LQ	953	62	4.1	28	0.59	31	23	< LD	47	735
	max	36	9776	1064	322	2269	55	210	4113	3786	11	1201	69	5.9	49	0.71	38	27	< LD	50	798
	min	18	6138	< LQ	137	1373	23	134	3231	2609	< LQ	699	56	< LQ	18	0.45	27	22	< LD	45	697
Fundão 2	mean	12	13901	1018	216	1640	25	212	3995	4825	< LQ	1709	95	16	33	0.58	45	47	< LD	61	744
	max	18	16191	1557	347	2238	30	293	5016	6120	< LQ	2318	126	20	37	0.70	53	71	< LD	68	786
	min	6.5	11616	< LQ	140	1339	18	144	2981	3306	< LQ	1303	79	13	28	0.50	39	31	< LD	53	706
Fundão 3	mean	55	17996	1238	150	7042	123	318	3876	5890	18	820	101	10	17	0.65	32	29	< LD	53	852
	max	88	25529	1640	176	11289	266	414	4331	6698	30	1121	170	13	25	0.86	39	35	< LD	63	988
	min	24	10432	925	136	5189	35	237	3078	4876	10	526	60	7.5	7.3	0.56	26	24	< LD	46	692
Fundão 4	mean	4.9	8253	958	144	2060	43	87	3806	4163	< LQ	2333	42	3.4	9.7	0.66	36	27	< LD	58	905
	max	7.6	12297	1241	162	2600	57	108	4854	4515	< LQ	2981	55	11	15	0.75	46	40	< LD	61	984
	min	< LQ	5629	< LQ	127	1596	14	64	3396	3853	< LQ	2048	22	< LQ	3.8	0.49	29	21	< LD	50	779
Fundão 5	mean	34	20559	979	147	2721	29	200	5679	5280	16	743	124	11	73	0.54	36	28	< LD	67	1109
	max	59	29623	111	177	3554	41	292	7542	6411	34	898	156	16	114	0.78	45	31	< LQ	81	1256
	min	22	14529	< LQ	137	1931	20	139	4709	3749	< LQ	622	95	8.0	43	0.42	27	26	< LD	58	890

[1] five trees per farm, each tree represented by a pool of three pears analyzed in triplicate.

Potassium is the mineral present at higher concentrations, followed by P. These results are similar to the data provided by FCDBs, where pears are richer in these two minerals than in others (Fødevareinstituttet, 2017; USDA, 2017). Na content varies according to the geographical origin of pears. The PDO region is located near the seashore leading to samples with high Na content, ranging from 14 to 72 mg.kg^{-1}, when compared to previous studies which report Na values between 5 and 35 mg.kg^{-1} (Fødevareinstituttet, 2017; Perez et al., 2006; USDA, 2017). On the other hand, the levels of Na in all samples from the Fundão region, an interior area near the mountains, were not quantifiable. Similar results were reported for cocoa beans from Indonesia, where the concentration of Na in samples varied according to the proximity of cultures to the sea (Febrianto & Zhu, 2022). Besides Na, ANOVA identified significant differences between the two regions in the following elements: Al, Ni, Zn, Sr, Tl, Fe and Ca. For the remaining elements, neither ANOVA nor Mann-Whitney identified significant differences. This line of research was further developed resorting to chemometric tools.

5.1.1.4 Correlation analysis

Correlation analysis was performed to identify possible relationships between the content of the elements using the 50 available samples. Two elements, Cr and Mn, did not present a normal distribution. Therefore, a non-parametric test, Spearman correlation, was used. Cr did not show any significant correlations, while Mn evidenced a significant positive correlation with Li, Zn and K. For the remaining variables, a parametric test was used, as presented in Table 6. Among these elements, As was the element with the least correlations, showing two significant positive correlations with Mo and Na ($p < 0.05$) and one significant negative correlation with Sr.

On the other hand, Zn and Ni showed 10 and 9 correlations, respectively, mainly positive with $p < 0.01$. Several elements, such as Li, B, Cu, Mo, Cd, Mg, Ca and P, showed only significant positive correlations, whereas the remaining elements evidenced both positive and negative significant correlations. The three strongest correlations were found between Al-Na (0.71), Cu-P (0.67), and Mg-Ca (0.62). Further conclusions regarding the correlations between elements require more advanced statistical tools such as PCA.

Table 6. Correlation matrix for element concentrations in pears from PDO and Fundão regions (figures in **bold** indicate higher correlations between two elements considering a p-value < 0.05).

	Li	B	Al	Co	Ni	Cu	Zn	As	Sr	Mo	Cd	Tl	Fe	Mg	Ca	Na	P	K
Li	1.00																	
B	0.23	1.00																
Al	-0.03	-0.08	1.00															
Co	**0.34**	0.28	0.15	1.00														
Ni	**0.50**	0.03	0.23	**0.48**	1.00													
Cu	0.16	**0.36**	0.09	0.23	0.21	1.00												
Zn	0.03	-0.04	**0.38**	**0.40**	**0.38**	**0.41**	1.00											
As	0.16	0.15	0.22	0.11	0.18	-0.15	0.19	1.00										
Sr	-0.19	-0.27	-0.25	-0.27	**-0.41**	-0.26	**-0.30**	**-0.46**	1.00									
Mo	0.09	**0.45**	-0.01	-0.25	-0.11	-0.03	-0.12	**0.41**	-0.23	1.00								
Cd	**0.31**	**0.30**	-0.01	0.27	**0.56**	**0.38**	0.22	-0.08	-0.21	0.14	1.00							
Tl	0.24	**0.28**	**-0.29**	**-0.31**	-0.26	0.10	-0.26	0.09	0.10	**0.39**	0.06	1.00						
Fe	0.08	-0.19	0.26	**0.32**	**0.43**	0.01	**0.38**	0.27	0.01	-0.15	0.12	**-0.32**	1.00					
Mg	-0.06	-0.17	0.21	-0.07	-0.02	-0.02	**0.47**	0.02	**0.33**	0.21	0.11	0.10	**0.39**	1.00				
Ca	-0.01	-0.09	**0.51**	0.01	**0.28**	0.11	**0.50**	-0.08	0.13	0.09	0.26	-0.16	**0.28**	**0.62**	1.00			
Na	-0.14	-0.08	**0.71**	0.12	**0.39**	-0.06	**0.29**	**0.40**	**-0.42**	0.02	-0.02	**-0.44**	0.27	0.04	**0.35**	1.00		
P	0.02	**0.30**	0.20	0.17	0.27	**0.67**	**0.57**	0.10	-0.08	0.18	**0.33**	0.05	0.24	**0.40**	**0.41**	0.25	1.00	
K	0.11	0.21	0.00	**-0.32**	**-0.29**	0.16	-0.07	0.25	0.17	**0.54**	-0.21	**0.50**	0.05	**0.35**	-0.00	-0.11	**0.29**	1.00

5.1.1.5 Principal component analysis

PCA was carried out to identify relationships between samples and mineral content according to their geographical provenance. The data considered for this statistical treatment was obtained by measuring the concentration of twenty elements, in triplicate, in 50 samples of pears from two different regions, PDO and Fundão.

Data was pre-treated by matrix standardization taking into account the vast differences in data scaling, as can be seen in Tables 4 and 5. Varimax rotation was chosen from the available rotational strategies in PCA.

The analysis identified six PCs, with eigenvalues above 1, which explained 74.7% of total data variation. The first component presented an eigenvalue of 4.26, representing 21.3% of the total variance. The second PC with an eigenvalue of 2.98 explained 14.9% of the total variance. The following four PCs had eigenvalues of 2.57, 2.03, 1.65 and 1.45, respectively, corresponding to 12.9%, 10.1%, 8.3% and 7.2% of the total variance.

Table 7 shows the loadings of the first six PCs. Elements were considered strongly related to a PC whenever the respective loading, in absolute value, was equal to or above 0.65. As such, the elements B, Cr, Fe and Zn did not show a strong influence on any PC. According to this criterion, the first PC is strongly associated with Mg and Ca. The second PC is defined by Mo, K and Tl, while the dominant elements in PC3 are Mn, Co and Li. According to Table 6, the elements within these PCs show strong positive correlations between them ($p < 0.01$). PC4 is strongly associated with four elements, Na, Al, As, and Sr. Sr is the only element that presents a significant negative correlation ($p < 0.01$) with As and Na, supported by the negative loading (0.65) in PC4. Particular attention should be paid to this element when interpreting the PC's Biplot. Like PC1, the fifth PC incorporates only two elements, Cu and P, with one of the strongest correlations (0.67), as described in chapter 5.1.1.4. Ni, Cr and Cd provided the highest loadings in PC6.

Table 7. The loadings of the first six rotated PCs (n=50, figures in **bold** indicate the dominant loadings > 0.6 in each PC).

Element	PC1	PC2	PC3	PC4	PC5	PC6
Li	-0.05	0.20	**0.66**	-0.11	0.00	0.46
B	-0.32	0.47	0.30	0.00	0.45	0.13
Al	0.34	-0.11	0.01	**0.69**	0.10	-0.03
Cr	0.18	0.08	-0.20	0.14	-0.32	**0.63**
Mn	0.02	0.16	**0.89**	-0.06	-0.02	-0.08
Co	-0.03	-0.38	**0.73**	0.19	0.26	0.20
Ni	0.09	-0.22	0.25	0.32	0.16	**0.77**
Cu	-0.03	0.01	0.07	-0.05	**0.91**	0.06
Zn	0.48	-0.23	0.19	0.38	0.52	0.06
As	-0.05	0.42	0.29	**0.67**	-0.12	-0.05
Sr	0.45	-0.09	-0.12	**-0.65**	-0.25	-0.28
Mo	0.01	**0.87**	-0.03	0.16	0.02	0.11
Cd	0.04	0.02	0.08	-0.13	0.45	**0.70**
Tl	-0.08	**0.70**	0.02	-0.40	0.07	-0.03
Fe	0.56	-0.20	0.29	0.30	-0.07	0.18
Mg	**0.89**	0.20	-0.00	-0.04	0.08	-0.04
Ca	**0.75**	-0.03	-0.14	0.18	0.18	0.27
Na	0.14	-0.12	-0.19	**0.87**	0.01	0.13
P	0.40	0.17	-0.06	0.17	**0.76**	0.11
K	0.29	**0.75**	0.09	-0.04	0.08	-0.34

Figure 12 illustrates the scores and loading plots for the first four PCs. Samples are identified by coloured letters and numbers according to their geographical provenance. Samples with a high score (| Score| > 1) in a specific PC show a strong association with that PC. By analysing the scores of each sample in the PCs, it is possible to find resemblances in the mineral content of farms. All trees from farm P2 present a high score in PC1, meaning that this location is richer in Mg and Ca when compared to other farms under study. Likewise, in the PC2, farm F5 has a higher content of Mo, Tl and K, as shown in Figure 12a and 12e. Although from the same region, Fundão, farms F2 and F3 present opposite scores in PC3 (Figure 12b). The five trees from F3, with positive scores, present the highest levels of Li, Mn and Co, whereas F2 presents the lowest mean concentration of Mn and Co, as can be confirmed in Tables 4 and 5. In PC4, all significant scores from Fundão are negative, whereas, in PDO, they are positive. This can be explained since Na presents the highest loading in PC4, and its value in Fundão samples is always below the LQ, while in PDO, it is always quantifiable (Tables 4 and 5). Still, within PC4, farms F2 and F4 stand out due to their absolute high scores indicating high Sr content but lower Al, As and Na concentration. Farm P1 is also differentiated in this PC by the high concentration of Al, As, and Na, as can be seen in Figure 12c and Table 7. PC5, rich in Cu and P, does not highlight any particular farm where these elements show a pattern (Table 7).

Finally, the analysis of PC6 evidences one farm per region, P3 and F4, that show significant scores although with opposite signs. Samples collected from farm P3 are richer in Ni and Cd, whereas samples from farm F4 present the lowest mean values for the same minerals.

PCA was not able to identify relationships between all samples and their geographical origin. However, with the exception of P4, P5 and F1, the first six PCs identified relationships between the five trees on each farm.

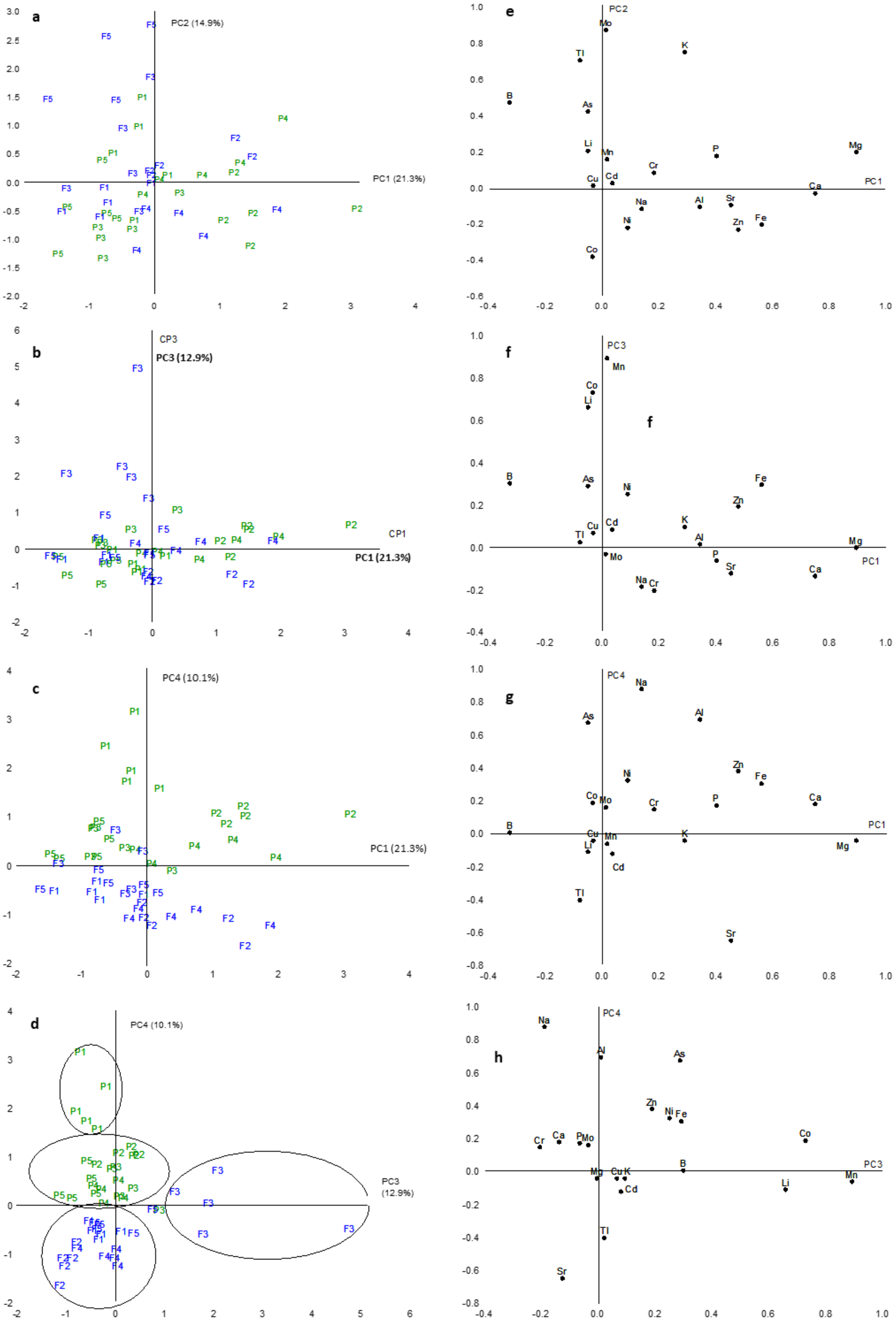

Figure 12. Score (a, b, c and d) and loading (e, f, g and h) plots for pear samples. “**P**” represents PDO region and “**F**” represents the region of Fundão. Numbers 1 to 5 identify farms.

5.1.1.6 Hierarchical cluster analysis

The results presented so far, although valuable for the elemental characterization of Rocha Pears, were not able to fully distinguish between the two geographical provenances under analysis. Thus, other statistical strategies need to be pursued. HCA was the statistical method chosen.

The obtained results for auto-scaled data are presented in the form of a dendrogram in Figure 13. Unlike in PCA, in this representation, it is evident the existence of only two clusters distinguished by their geographical origin. Elements without strong influence in any PC (B, Cr, Fe and Zn) were not considered for HCA.

Cluster 1, represented by blue letters in Figure 13, includes all samples coming from the region of Fundão. The second cluster contains all the pears from the PDO region and is identified by green letters. Samples were matched to their production area with 100% accuracy. In fact, a deeper analysis also shows a good matching of trees to their corresponding farms.

Looking at Table 6, we can verify that the five predominant elements in HCA present significant correlations. Two groups of elements can be identified within which the correlations are positive. The first encompasses two elements, Sr and Tl, and the second Al, Ni and Na. On the other hand, the correlations between group elements when significant are negative: Al-Tl (-0.29), Ni-Sr (-0.41), Ni-Tl (-0.31), Na-Sr (-0.42) and Na-Tl (-0.44). This shows that while cluster 1 is richer in the elements of the first group (Sr and Tl), cluster 2 has higher values for the remaining elements.

While PCA was not able to provide a total separation between geographical provenances, HCA entirely separated both regions. Nonetheless, the two exploratory techniques showed that it is possible to distinguish between geographical origins based on multielement data. Thus, the next step consisted in building a predictive model and the correspondent validation.

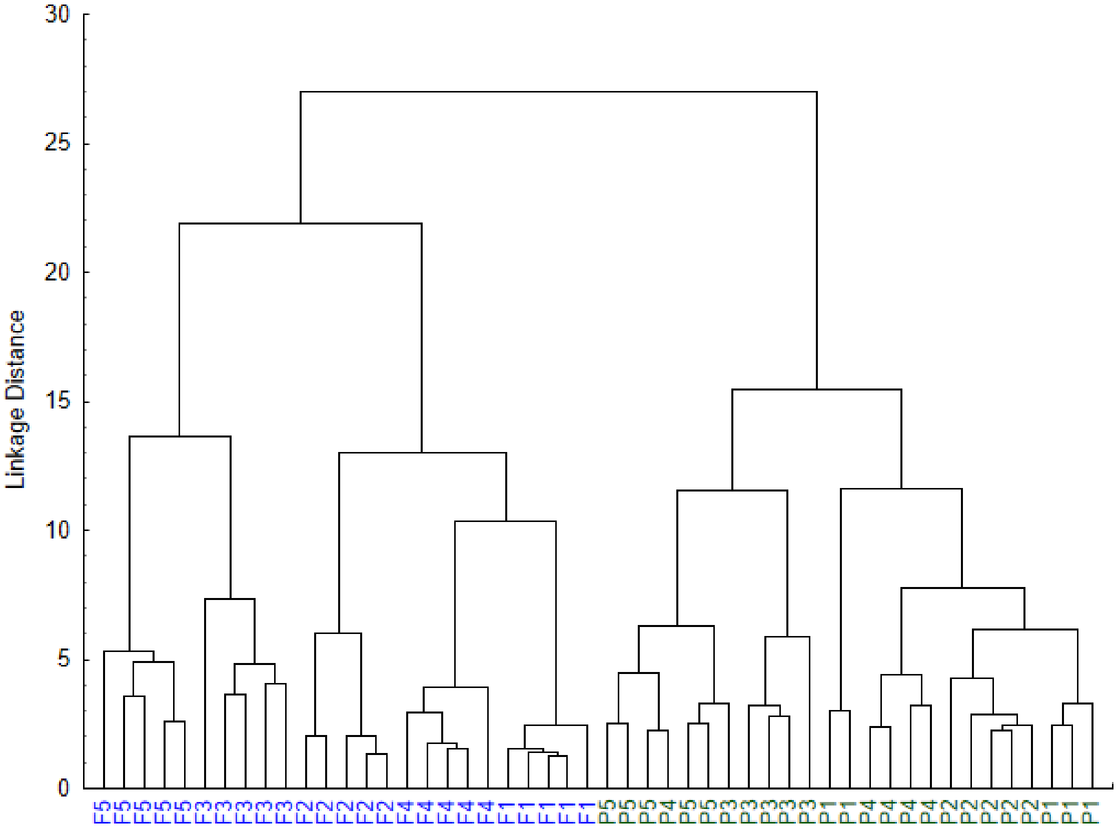

Figure 13. Dendrogram obtained by HCA considering samples of pears (n=50) and 16 variables. “P” represents PDO region and “F” represents the region of Fundão. Numbers 1 to 5 identify farms.

5.1.1.7 Linear discriminant analysis

A predictive model using LDA was built and validated to classify pear samples according to their origin. This approach differs from PCA and HCA since the predictive model is a supervised pattern recognition technique based on pre-defined groups (classes). Cross-validation of the model is essential in this technique. Based on the results from the PCA, four elements (B, Cr, Fe and Zn) were not included in the LDA. Mn was also removed due to a lack of normality, and the model was built considering 15 minerals. Samples were divided into two sets: training and cross-validation. The training set included two-thirds of the samples (n=34) and the cross-validation set had the remaining one third (n=16) (StatSoft, 2007). Figure 14 represents the distribution of samples, from both sets, based on the first two discriminant functions of LDA.

Samples selected for the cross-validation were classified with 100% accuracy according to the place of origin.

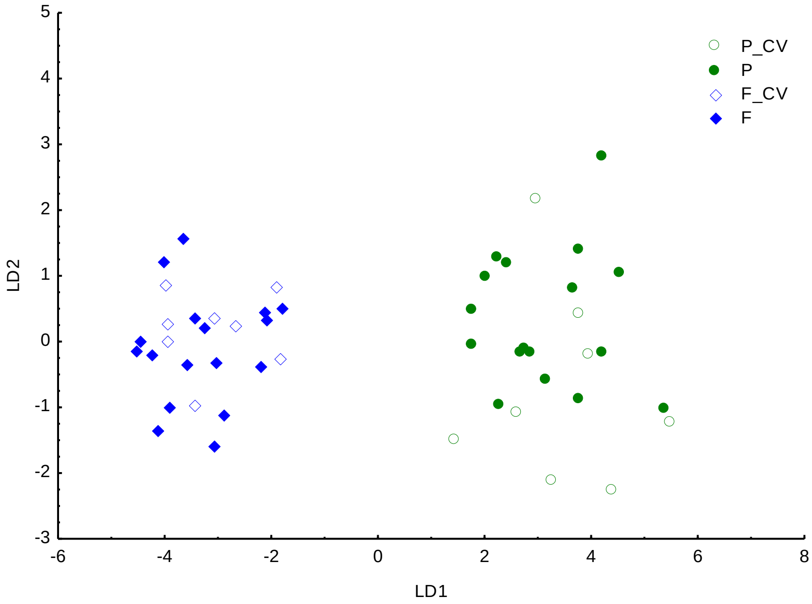

Figure 14. Scatter plot of the first two discriminant functions of LDA, representing the distribution of training and cross-validation pear samples according to the place of origin. PDO region is represented by “P”, and the region of Fundão is represented by “F”. “P_CV” and “F_CV” identifies the cross-validation set.

5.1.2 Isotopic analysis

The $^{87}Sr/^{86}Sr$ isotope ratios were measured in 50 pooled samples using MC-ICP-MS.

The isotopic analysis of Sr in Rocha pears was done for the first time. Consequently, it was necessary to validate the procedure, assuring that the method effectively isolated Sr from the remaining matrix components, particularly the interferent Rb, while obtaining reasonable recovery rates. As such, the elution profiles from Sr extraction were carefully examined, and an intercomparison exercise was promoted with an independent laboratory. Following the validation, the work carried on with the evaluation of $^{87}Sr/^{86}Sr$ in pear samples.

5.1.2.1 Method validation

Elution profile

The Sr content in pear samples ranged from 320 µg.kg^{-1} to 2980 µg.kg^{-1}. Due to this large variation, the extraction procedure was validated in three levels of concentration: low ([Sr] = 300 µg.kg^{-1}), medium ([Sr] = 600 µg.kg^{-1}) and high ([Sr] = 1200 µg.kg^{-1}). First, samples were mineralized using closed vessel microwave digestion. For the extraction of Sr, the procedure described in chapter 4.6.3 was followed. To evaluate the elution profile of Sr and Rb, the sample was loaded to the column, and the following addition of reagents to the column (HNO_3 for sample removal followed by H_2O for sample elution) was divided into smaller volumes of 5,2,4,1 or 0.5 mL. Each fraction was collected and measured in the ICP-MS after completing the volume to 5 mL with ultrapure water. As such, the levels of Rb and Sr were measured on 32 tubes, and elution profiles were designed, as presented in Figure 15. To evaluate the recovery of Sr, measurements were made using the same three samples but following the normal digestion and extraction procedures. The recovery rates of Sr for the low, medium and high levels were 70%, 84% and 88%, respectively.

The elution profiles showed that Rb is eluted with the first 3 or 4 mL of HNO_3 3M used for matrix removal. Rb was successfully removed to a residual level that does not interfere with Sr during MC-ICP-MS analysis. On the other hand, Sr only eluted during the sample collection phase through the addition of 10 mL of H_2O. Sr post-column recoveries were considered acceptable for the three levels of concentration.

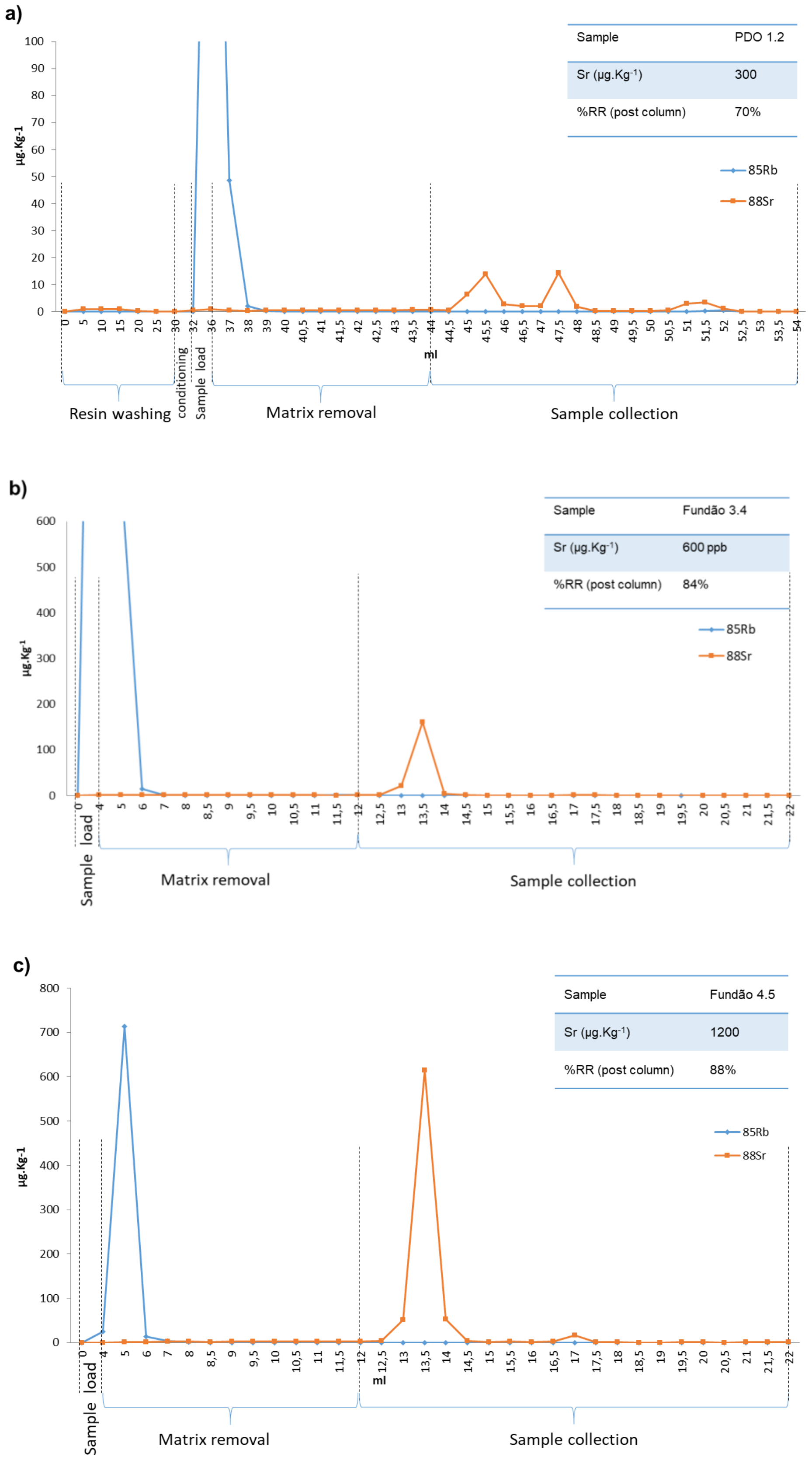

Figure 15. Elution Profiles of pear samples, using Sr-selective resin Eichron® with (a) low, (b) medium and (c) high concentrations of Sr.

Intercomparison exercise

An intercomparison exercise was performed between the UPPA and the JSI to validate further the results. The extracts of six samples (S1 to S6) were analysed in parallel by both laboratories, using MC-ICP-MS. The results obtained are presented in Table 8. The Paired Data t-Test was used to compare results from both laboratories.

Table 8. Intercomparison exercise, between two laboratories, for the determination of $^{87}Sr/^{86}Sr$.

	$^{87}Sr/^{86}Sr$		
	UPPA	JSI	Error (diference)
S1	0.71299	0.71298	0.00001
S2	0.71446	0.71448	-0.00002
S3	0.71344	0.71338	0.00006
S4	0.71243	0.71232	0.00011
S5	0.71307	0.71286	0.00021
S6	0.71202	0.71208	-0.00006
		mean	0.00005
		SD	0.00010
		T_0	1.29
		$T_{(0.025;\ 5)}$	2.57

T-Student statistics showed that there are no significant differences between the results obtained at UPPA and the ones obtained at JSI, considering a significance level of $\alpha = 5\%$ ($|T_0| < T_{(0.025;\ 5)}$).

The method was considered fit for purpose, and the work carried on with the determination of $^{87}Sr/^{86}Sr$ isotope ratios in all pear samples.

5.1.2.2 Results

The Sr isotope ratios were determined in 50 pears from the regions of Fundão and the PDO region.

The PDO region is primarily based on Meso-Cenozoic sedimentary rocks. In Fundão, the geology is characterized by Palaeozoic magmatism intruded in Proterozoic and Palaeozoic (Eggenkamp & Marques, 2010). Figure 16 shows the geological map for both regions.

In Fundão, the exact location of the fruit farms is represented by numbers 1 to 5. Unfortunately, the precise coordinates of farms for the PDO region were not registered during harvest. In this case, Figure 16 presents the whole sampling area, represented by a black line, without identifying the farms. Two different geological settings are recognised for the farms in Fundão. While farms 1, 2, 3 and 5 are grown on biotite granites with calcic plagioclase. Farm 4 is the only one in the region of Fundão planted on different geology, namely Granites and orthogneisses. Within the PDO region, three substrata can be observed in the geological map: Limestone, marlstone, sandstone; Sandstone, Limestone, marlstone and dolomites; and sand, gravel, silt and clay.

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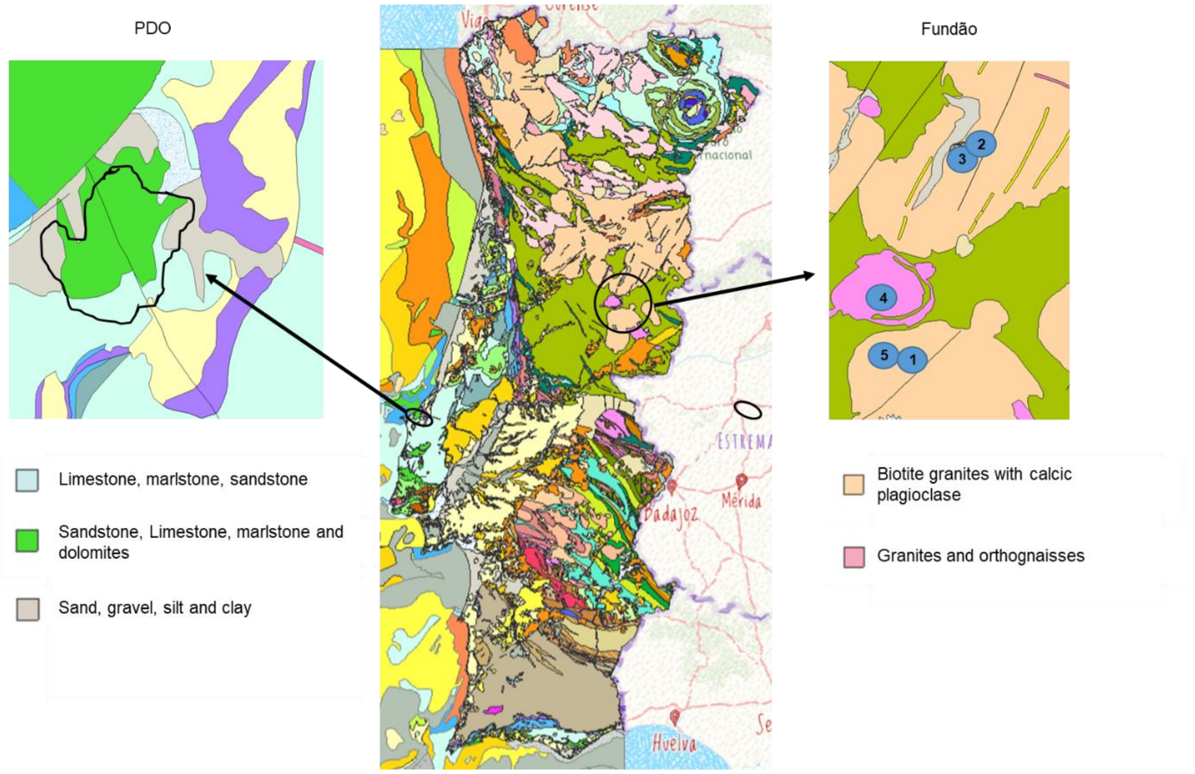

Figure 16. Geological map of the sampling areas of pears. Adapted from (LNEG, 2021).

One Way ANOVA was applied to the data. Significant differences were identified between the two regions (p-value= 0.0001), with a confidence level of 95%. Also, significant differences were identified between producers within each region, as illustrated in Figure 13 by the letters a, b, c and d, placed above the columns.

The individual analysis of producers shows that the PDO region presents a narrower profile compared to Fundão. The $^{87}Sr/^{86}Sr$ in the samples from the PDO region were in the range of 0.70715-0.71446 (n=25). For the region of Fundão, the $^{87}Sr/^{86}Sr$ ratio varied between 0.70890 and 0.72877 (n=25). The isotopic signature of Sr, dependent on the initial Rb/Sr ratio and the age of the rocks, is higher in granites (0.710-0.750) than in limestone (0.706-0.709) (Faure & Powell, 1972). Weathering releases Sr into the soil, and subsequently, it will be absorbed by plants. Higher $^{87}Sr/^{86}Sr$ values in Fundão are consistent with the underlying geology of both regions.

Producer PDO4 presents significant differences from all other PDO producers, regarding the value of $^{87}Sr/^{86}Sr$, except for PDO1 (Figure 17). There are no significant differences among the remaining four producers within the PDO region. Considering the lower mean $^{87}Sr/^{86}Sr$ value in PDO4 combined with the highest SD from PDO farms, one possible explanation could be that this farm encompasses different soil types. Another reason may be that PDO4 is closest to the sea hypothetically. It has been reported that the influence of seawater Sr ($^{87}Sr/^{86}Sr$ = 0.70918) in the plant-available reservoir is noticeable near the seashore (Bentley, 2006). This proximity to the ocean associated with the limestone geological background explains the lower Sr isotopic signature for the PDO region compared to Fundão. However, as far as we know, no data for Sr isotopes ratios are available for the region, which adding to the absence of the exact location coordinates for the producers, obstructs further conclusions.

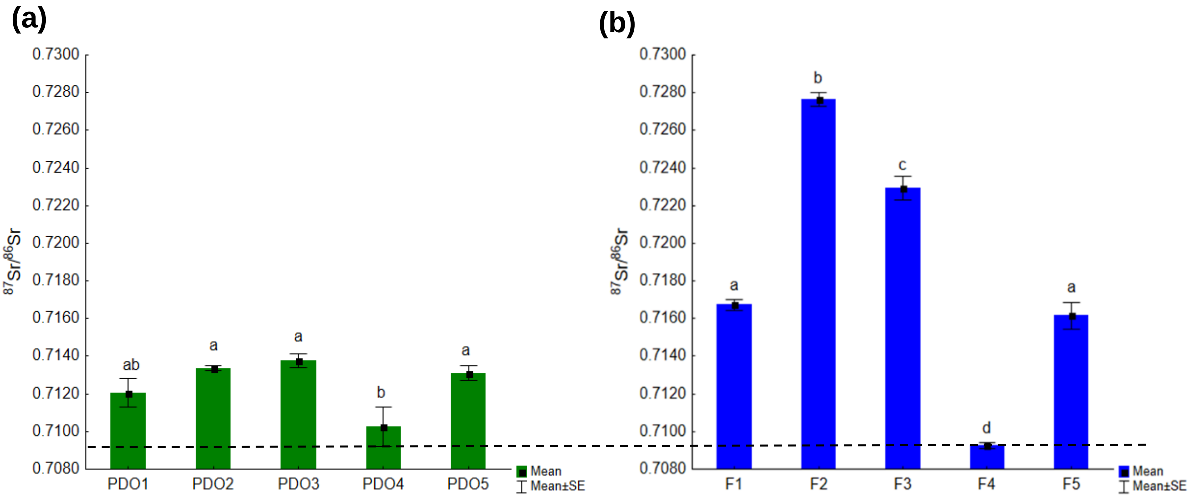

Figure 17. Characterization of the $^{87}Sr/^{86}Sr$ isotope ratio in Rocha pears (n=50). (**a**) represents the PDO region and (**b**) the region of Fundão. Producers are identified by numbers from 1 to 5. (----) modern seawater Sr isotope values $^{87}Sr/^{86}Sr$ = 0.70918 (Jones, 2001).

In Fundão, producers F1 and F5 presented similar results. These two farms are closely located, as demonstrated in Figure 16. F2 and F3 are also found near each other. Although ANOVA identified significant differences between their results, the two are the highest for all samples. On the other hand, producer F4 showed the lowest values for $^{87}Sr/^{86}Sr$. In fact, the geological setting in this farm is distinct from the remaining 4 (Figure 16). On average, pears from F4 are even lower than the values obtained for all producers from the PDO region and very similar.

While four producers from Fundão showed higher values than those found in the PDO region, producer F4 presented the lowest result from all analysed samples. Although the two regions presented distinct values of $^{87}Sr/^{86}Sr$, it is not clear that this parameter would adequately attest to the authenticity of pears produced in the PDO region. A suggestion would be to combine $^{87}Sr/^{86}Sr$ isotope ratios with other isotopic systems, namely O. Unfortunately, it is not possible to pursue this line of research in the present work as pear samples have been lyophilized for storage purposes.

5.1.3 Final remarks

In the present study, 50 Rocha pear samples from two Portuguese regions were analysed. The content of 24 minerals was determined by ICP-MS and ICP-OES, contributing to the characterization of this traditional product. The analytical methods, with high sensitivity and low LQ, were suitable for the purpose and are recommended methods for the analysis of inorganic components even at low values.

After the pre-analytical sample preparation, a study was successfully conducted to evaluate sample homogeneity. The compliance of the replicates with the established RSD% criteria indicated that samples were sufficiently homogeneous and that no further sample pre-treatment, e.g. homogenization, was required to continue the study.

The comparison study between peeled and unpeeled pears, using five samples, supported the decision to carry on using unpeeled samples as the significant differences found were between Al, Cu, K and Pb.

The obtained results showed that pear samples are richer in K and P than in other minerals, regardless of the geographical origin. Samples from the region of Fundão showed a higher content of Tl, Sr and Mn, while PDO samples are richer in Zn, Al, Ni and Na.

The exploratory analysis, based on the unsupervised models PCA and HCA, identified relationships between samples and their geographical provenance. In PCA, the first six PCs explained 74.7% of total data variation. Two distinct clusters were obtained with HCA, fully separating the 25 samples from each region under study. LDA confirmed that it is possible to trace the authenticity of pear samples to the correspondent origin based on the mineral profile.

Samples used for cross-validation of the predictive model were matched to its origin with 100% accuracy.

The method applied for the extraction of Sr prior to its isotopic analysis by MC-ICP-MS was used for the first time to pears. Therefore validation of the extraction method was required. It was demonstrated that the technique effectively removed Rb from the matrix, and recoveries of Sr were acceptable for three concentration levels. In addition, the reproducibility, evaluated by comparing the obtained results of six samples with an external laboratory, provided satisfactory results since no significant differences were found between the two laboratories. As such, the extraction method is considered suitable after validation and the analysis of samples from both regions carried on.

The $^{87}Sr/^{86}Sr$ ratio presented differences between the two regions under study. Values acquired for pears produced in the PDO region were lower than those obtained for the region of Fundão. The exception was producer 4 in Fundão, whose results were closer to the seawater value, even though this region is not located near the seashore. These pears are cultivated in granite and orthogneiss bedrocks, while the remaining four producers from the region share the same geological formation. At this point, it is not possible to clarify the low values found in F4.

In conclusion, the chemometric techniques used allowed the differentiation of samples according to their geographical origin based on their mineral profile, contributing to their authenticity and traceability as well as to a deeper knowledge of the mineral content of Rocha pear. The methodology was successfully applied in the detection of pears cultivated far from the ocean shore. The developed method ensured the precision, accuracy, sensitivity, and specificity needed for the unequivocal identification of Rocha Pear.

This study produced robust data, in conformity with quality criteria, fit to integrate FCDBs and fill an existing gap of absent values for Rocha pears. Furthermore, the data could be used by farmers and agroindustry to support the fundaments of discrimination against PDO Rocha pear from similar pear ripened in other regions. The method can be extended in the future to identify the geographic origin of other fruits and vegetables from a specific region.

5.2 Analysis of Wines

5.2.1 Multielement and isotopic characterization

Port and Douro wines are produced in the Douro Demarcated Region, located in the northeast of Portugal. Agricultural practices are common to both types of wine, as are climate and soil. The difference between the two types of wine lies in the winemaking process due to the addition of grape spirit to Port wine during the fermentation stage.

Although the geology in Portugal is quite diverse throughout the country, this region shows a relatively homogeneous geological substratum originating from old metamorphic rocks that date from the Upper Proterozoic-Cambrian Era; over 541 million years ago (Figure 18). Wines in the region are produced in Quintas (farms), and the same wine may contain grapes from different vineyards within the same farm. All samples in the present study come from vineyards grown in schist soils within the Douro Valley Valley, as defined in Figure 18.

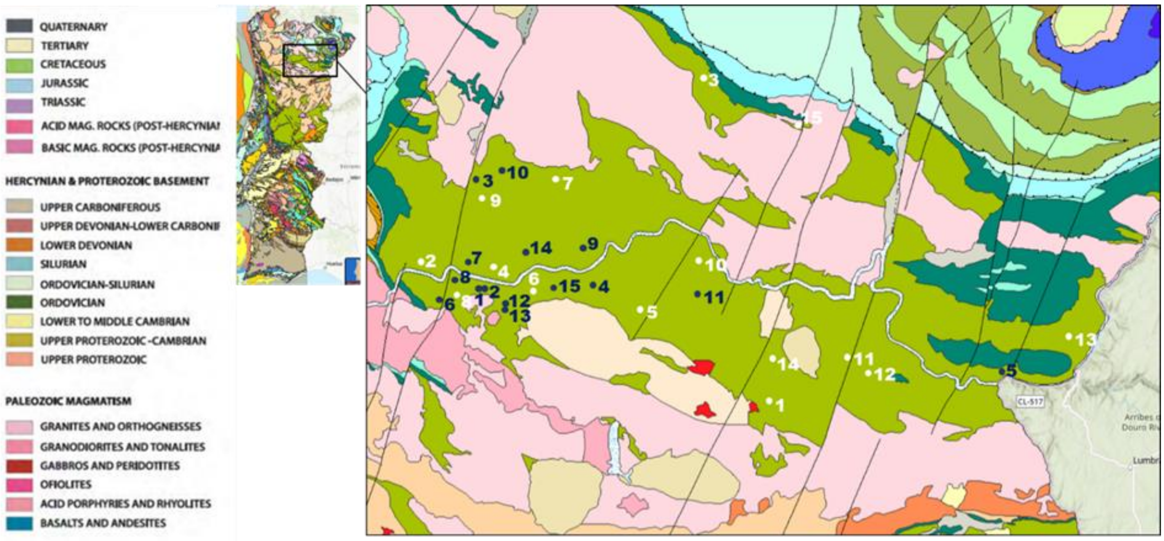

Figure 18. Geological map of Portugal highlighting the Douro Demarcated Region with the location of the selected *Quintas*. Blue numbers represent samples of Port wine (n=15). White numbers represent samples of Douro wine (n=15). Adapted from (LNEG, 2021)

Thirty wines were analysed for multielement and isotopic composition.

Table 9 shows the results obtained for the analytical performance of the method. Since there was no CRM available at the time, accuracy was evaluated using spiked samples with recoveries ranging between 83% (Ni) and 118% (K). Repeatability for each element is presented as the highest obtained RSD% between triplicates. Quality control was within the criteria established by the laboratory.

Table 9. Method performance for multielemental determination in wine samples. Elements showing m/z were determined by ICP-MS. Elements showing emission lines were determined by ICP-OES.

Element	Units	m/z	emission lines (nm)	Working range	LQ	LD	Repeatability* (RSD %)	Recovery (%)
					(in wine)			
Ag	µg.kg^{-1}	107	---	0.50 – 5.0	2.3	0.76	2.6	---
Al		27	---	25 - 250	113	37	9.1	101-115
As		75	---	0.25 - 2.5	1.1	0.36	8.9	100-117
B		11	---	50 - 500	225	74	9.1	104-113
Be		9	---	0.25 - 2.5	1.1	0.36	3.4	93-117
Cd		111	---	0.25 - 2.5	1.1	0.36	2.0	87-112
Co		59	---	0.25 - 2.5	1.1	0.36	7.2	94-115
Cr		52	---	0.50 – 5.0	2.3	0.76	8.8	103-117
Cu		65	---	0.50 – 5.0	2.3	0.76	8.0	90-112
Li		7	---	0.25 - 2.5	1.1	0.36	8.9	90-117
Mn		55	---	0.50 – 5.0	2.3	0.76	7.6	94-112
Mo		95	---	0.50 – 5.0	2.3	0.76	9.3	86-113
Ni		60	---	0.50 – 5.0	2.3	0.76	9.7	83-114
Pb		208	---	0.50 – 5.0	2.3	0.76	8.6	88-116
Se		77	---	0.50 – 5.0	2.3	0.76	9.3	97-115
Sn		118	---	0.25 - 2.5	1.1	0.36	7.7	102-111
Rb		85	---	0.50 – 5.0	2.3	0.76	5.8	---
Sr		88	---	0.50 – 5.0	2.3	0.76	4.9	85-116
Tl		205	---	0.25 - 2.5	1.1	0.36	1.9	85-107
Zn		66	---	5.0 – 50	23	7.4	6.5	90-104
Ca	mg.(100g)$^{-1}$	---	184.006	0.2-2.0	0.9	0.33	7.8	99-102
Fe		---	259.940	0.005-0.005	0.025	0.023	8.6	94-108
K		---	769.896	0.25 - 2.5	1.1	0.36	4.3	90-118
Mg		---	279.553	0.1-1	0.45	0.17	3.5	92-113
Na		---	589.592	0.2 – 2.0	0.9	0.33	3.5	91-104
P		---	178.284	0.2-2.0	0.9	0.33	2.8	97-101

*maximum RSD% between triplicates

Table 10 shows the results obtained according to the type of wine (Port vs Douro). The minimum, median and maximum values of each element are presented. Median was chosen over mean since it better represents the variability of results within each type of wine due to a large number of independent samples within each group (n=15). Elements were divided into major and minor elements depending on their median concentration being higher or lower than 500 µg.L^{-1}. Major elements are expressed in mg.L^{-1} and minor elements in µg.L^{-1}. Individual sample results are presented in Appendix 3 Tables A4, A5 and A6.

On average, the RSD% for major elements, among all samples of the same type of wine, was 33% and similar for both types of wine. The percentage was two times higher for minor elements, reaching 66%. However, considering the high number of independent samples (30), this should not be a matter of concern.

Among the major elements, the most abundant one was K ranging between 399 mg.L^{-1} and 1458 mg.L^{-1}. This result was also observed in previous studies (Geană, Sandru, Stanciu, & Ionete, 2017; Hopfer, Nelson, Collins, Heymann, & Ebeler, 2015). The order of abundance of the remaining major elements was identical for both types of wine, as follows: P>Mg>Ca>Na>B>Mn ≈ Rb >Sr>Fe. These results are in good agreement with the existing literature, except for Mn in other Portuguese wines reported with lower values than ours (Catarino et al., 2018; Serapinas et al., 2008). The presence of Mn in wines may come from agricultural practices due to the use of fertilizers and pesticides (Catarino et al., 2018).

Regarding the minor elements, Zn and Al were the most abundant ones ranging from 106 µg.L^{-1} to 586 µg.L^{-1} and 75 µg.L^{-1} to 1043 µg.L^{-1}, respectively. The median values of Al are in agreement with those previously reported for the region (170 µg.L^{-1} and 270.17 µg.L^{-1}). At the same time, for Zn, our results are lower than the reported values (1000 µg.L^{-1} and 430 µg.L^{-1}) (Almeida & Vasconcelos, 2003). The least abundant were Ag, Cd and Tl, with concentrations below the LQ in all samples and removed from further data analysis.

Unlike major elements, the order of abundance for minor elements is not the same for Port and Douro wines. In fact, while Port wine exhibits Cu (12 µg.L^{-1} to 332 µg.L^{-1}) and Cr (31 µg.L^{-1} to 52 µg.L^{-1}) as the third and fourth most abundant minor elements; in Douro wines, these positions are occupied by Li and Ni.

While some elements analysed are essential for humans (Ca, Cu, Fe, K, Mg, Mn, Mo, P, Se, and Zn) and have nutritional recommendations established, others are predominantly toxic or depend on the dose or the chemical species present (As, Cd, Co, Cr, Na, Ni, Pb, and Sn). Some also affect the quality of the wine (Al, Cu, Fe, Ni, and Zn) (Tariba, 2011). Several entities and countries have established maximum limits for elements. All analysed samples comply with the maximum acceptable limits set by the International Organization of Vine and Wine (OIV) and presented in Table 10 (International Organisation of Vine and Wine, 2015). Therefore, these samples do not pose a risk to human health regarding these toxic elements.

Table 10. Characterization of Port and Douro wines in terms of their **a)** elemental composition and **b)** Isotopic profile.

*Limit of quantification (LQ); $^{1)}$maximum limits established by the OIV at the time of harvest (International Organisation of Vine and Wine, 2015); ^{2} for liqueur wines produced from unfermented or slightly fermented grape must; ^{3} for wine made, starting from the 2007 harvest year; n.a. – not applicable

a)			Port wine			Douro wine			OIV limit^{1}
		P10 (n=1)	(n=14)			(n=15)			
			min	median	max	min	median	max	
Major elements mg.L^{-1}	B	2.4	2.7	4.4	6.4	2.5	5.3	8.2	14
	Ca	128	26	52	72	45	63	76	n.a.
	Fe	12	0.13	0.57	3.4	0.57	0.95	1.7	n.a.
	K	954	534	872	1345	399	1090	1458	n.a.
	Mg	118	66	92	147	60	106	123	n.a.
	Mn	3.3	0.50	1.6	3.3	0.93	1.8	3.0	n.a.
	Na	17	2.6	6.4	14	6.3	11	32	80
	P	97	117	162	210	94	189	240	n.a.
	Rb	1.8	0.93	1.3	2.6	0.87	2.2	4.4	n.a.
	Sr	1.6	0.75	1.2	1.7	0.47	1.2	1.9	n.a.
Minor elements µg.L^{-1}	Ag	< 2.3*	< 2.3*	< 2.3*	< 2.3*	< 2.3*	< 2.3*	< 2.3*	n.a.
	Al	2697	< 113*	164	1043	124	260	911	n.a.
	As	40	1.5	2.7	14	< 1.1*	2.5	5.6	200
	Be	< 1.1*	< 1.1*	< 1.1*	1.5	< 1.1*	< 1.1*	2.7	n.a.
	Cd	< 1.1*	< 1.1*	< 1.1*	< 1.1*	< 1.1*	< 1.1*	< 1.1*	10
	Co	14	3.0	7.2	10	2.0	4.6	10	n.a.
	Cr	53	31	37	52	10	17	32	n.a.
	Cu	1716	12	91	332	4.2	31	158	2000^{2}/1000
	Li	63	14	23	184	9.0	48	244	n.a.
	Mo	4.9	< 2.3*	< 2.3*	3.7	< 2.3*	2.5	14	n.a.
	Ni	54	9.1	22	44	14	26	57	n.a.
	Pb	120	< 2.3*	4.1	19	2.8	8.5	21	150 ^{3}
	Se	6.2	< 2.3*	< 2.3*	4.8	< 2.3*	< 2.3*	2.8	n.a.
	Sn	5.7	< 1.1*	< 1.1*	4.7	< 1.1*	< 1.1*	3.6	n.a.
	Tl	< 1.1*	< 1.1*	< 1.1*	< 1.1*	< 1.1*	< 1.1*	< 1.1*	n.a.
	Zn	1675	197	330	586	106	258	435	5000

b)			Port wine			Douro wine		
		P10 (n=1)	(n=14)			(n=15)		
			min	median	max	min	median	max
Isotopic Ratio	$^{208}Pb/^{204}Pb$	38.306	37.609	38.029	38.289	37.728	38.147	38.560
	$^{207}Pb/^{204}Pb$	15.633	15.587	15.618	15.641	15.606	15.632	15.653
	$^{206}Pb/^{204}Pb$	18.226	17.647	18.062	18.314	17.854	18.141	18.672
	$^{208}Pb/^{206}Pb$	2.102	2.085	2.108	2.131	2.044	2.104	2.113
	$^{206}Pb/^{207}Pb$	1.166	1.132	1.157	1.172	1.144	1.160	1.193
	$^{87}Sr/^{86}Sr$	0.71712	0.71652	0.71985	0.72160	0.71683	0.71969	0.72290
	$\delta^{18}O$ ‰	2.67	4.23	5.38	7.27	0.25	5.24	6.91
	$\delta^{11}B$ ‰	27.2	3.6	16.0	24.9	10.7	20.3	33.5

The sample P10 (Port wine) presents considerably higher concentrations of the following elements Al, As, Cu, Zn, and Pb, among all analysed wines. This sample is a white Port wine from a small family production, aged in wood barrels and stored for over 30 years. For this reason, it is presented in a separate column, and it was removed from all statistical analyses. The high concentration of Pb in this sample is in agreement with Pb values reported in the literature for older fortified wines (International Organisation of Vine and Wine, 2020). According to the International Organisation of Vine and Wine (2020), environmental sources of Pb contamination in wines are factories and Pb in gasoline. The former is not a reasonable possibility since the region is not industrialized. However, leaded petrol was abolished in Portugal in 1999 and was still used at the time of production. To look further into this possibility, it is necessary to analyse the Pb isotope ratios. Although sample P10 is over 30 years old, its Pb isotopic signature is similar to the remaining samples. In fact, its value for $^{206}Pb/^{207}Pb$ is the second-highest among Port wines and the sixth of all samples, and it is reported that, in Europe, the atmospheric $^{206}Pb/^{207}Pb$ value has increased since the forbidding of leaded gasoline (Epova et al., 2020; Komárek et al., 2008). The values of Pb isotope ratios in Port and Douro wines, including P10, are consistent with previous results for the region ($15.6244 \leq {}^{207}Pb/^{204}Pb \leq 15.6699$, n=5) (Teixeira et al., 2021). Compared to other European wines, namely French wines, where values for $^{206}Pb/^{207}Pb$ ranged from 1.1518 to 1.1914 (n=43), our results are consistent although showing higher dispersion (Epova et al., 2020). Six samples presented values below the minimum French result, and one sample was above. Figure 19 compares the Pb isotope ratios in Port and Douro wines and Bordeaux wines. Sample B15 was removed for being an outlier according to the Grubbs test. The overlapping of the two regression lines (Port and Bordeaux) is indisputable and clearly illustrates a similar profile for Pb isotopes in Portugal and France.

Therefore, a non-environmental source of contamination is at the origin of the higher levels of Pb found in this sample. Sample P10 also presents the highest levels of Cu and As among all samples, suggesting that Pb may be coming from vine sprays used at the time (lead arsenate, lead in copper sulphate). Another possible source is the metal material used at the time, like pipes and tubing, that came in contact with the wine during the winemaking process (International Organisation of Vine and Wine, 2020). The Pb isotope ratios are not distinctive of the year of production in the present samples.

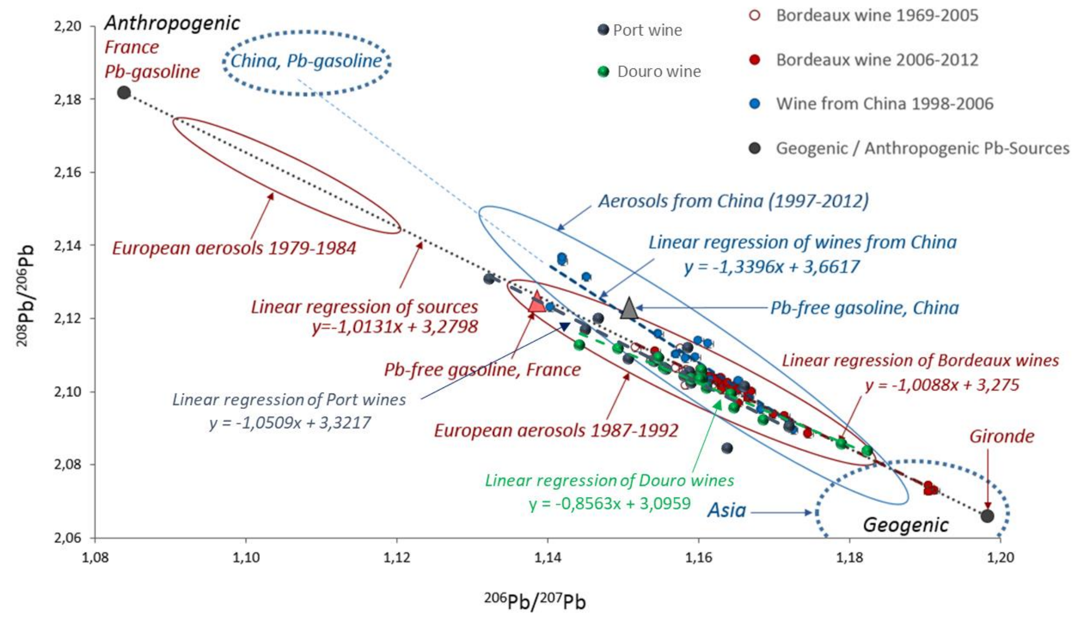

Figure 19. ^{208}Pb/^{206}Pb and ^{207}Pb/^{206}Pb ratios in Port, Douro and Bordeaux wines compared with the predominant anthropogenic and geogenic Pb sources. Adapted from (Epova et al., 2020).

The isotopic profile of all 30 wine samples was analysed for four isotopic systems (Table 10b). Light isotopes, B and O, are presented in delta notation, while Sr and Pb show results as ratios of radiogenic and non-radiogenic isotopes.

The value of δ^{18}O ranged between +0.25‰ and +7.27‰. The fact that all results are positive depicts an enrichment in ^{18}O. The value of δ^{18}O is highly dependent on the climate and location, mainly latitude, temperature and precipitation (Camin et al., 2015). Its value in wine reflects alterations in climate conditions during the harvesting period (Roßmann et al., 1999). The Douro Demarcated Region is located between latitudes 45º53” N and 45º68” N, extending more longitudinally than in latitude. During the years under study (2012 to 2016), the amount of annual precipitation across the region varied amply between its three sub-regions, from 493mm in Douro Superior to 730 mm in Cima Corgo (IPMA, 2022). However, during the harvesting period, typically between September and October, the average total precipitation was similar for the three sub-regions, ranging from 138 mm in Douro Superior to 182 mm in Cima Corgo.

On the other hand, the mean annual temperature of about 14.1 °C is relatively constant across the region, with an average of 17.2 °C reached during the harvesting period (IPMA, 2022). The value δ^{18}O obtained for all wines is positive (+0.25‰ to +7.27‰), and there are no apparent differences between wines produced within the region, which is coherent with the fact that the major influencing conditions are similar throughout the sampling area.

Our results are higher than the values in the literature compared to other southern European countries like Italy (Lancellotti et al., 2021). However, as far as we know, although Portugal reports $\delta^{18}O$ values for the European Wine DataBank, there are no published data available.

(Ogrinc et al., 2001) reports $\delta^{18}O$ values in Slovenian wines between -8.24 ‰ and +4.31 ‰. In another latest work, average $\delta^{18}O$ values in wines from Austria, Slovenia, Romania, Montenegro and Argentina were as listed: -1.3‰, +1.4‰, +3.5‰, +8.0‰ and +2.8‰, respectively (Horacek et al., 2021). All samples, except one from Montenegro ($\delta^{18}O$ = +8.0‰), were below our maximum value of +7.27‰, obtained in a sample of Douro wine. $\delta^{18}O$ values in wine in Portugal are expected to be enriched in ^{18}O since as latitude, altitude and distance from the ocean increase; the values $\delta^{18}O$ in rainwater become lower (Santesteban et al., 2015). Therefore, $\delta^{18}O$ values are lower in countries in central and northern Europe compared to those in the south. In addition, hot temperatures and a dry climate, like those typical in the Douro region during the harvesting season, contribute to higher evaporation of water from vines with further enrichment in the isotope ^{18}O (Santesteban et al., 2015).

Values for $^{87}Sr/^{86}Sr$ ranged between 0.71652 and 0.72290. These results are higher than values reported for Bordeaux wines (0.70829 – 0.71020, n=43) or Lambrusco wines (0.70864 - 0.70911, n=40) (Epova et al., 2019; Lancellotti et al., 2021). They are also higher compared to three other Portuguese PDO regions, namely Dão (0.713-0.715, n=4), Óbidos (0.7080-0.7095, n=4) and Palmela (0.7075-0.709, n=4) (Catarino et al., 2016). However, when compared to other wines from the Douro Demarcated Region, our results fall within the previously reported range (Figure 20). Soils from the region have been analysed for $^{87}Sr/^{86}Sr$, to study for wine authenticity, and are reported to have values ranging from 0.708 to 0.780, while the values for the bedrock are between 0.72794 and 0.77001 (Catarino et al., 2019; Teixeira et al., 2021). Several authors have observed depletion in $^{87}Sr/^{86}Sr$ from bulk soils to wine, probably related to the texture of soils or weathering processes (Marchionni et al., 2016; Teixeira et al., 2021). Douro vineyards are cultivated mainly in soils originating from schistous rocks. These are metamorphic rocks from the Neoproterozoic Era. Together, a high initial concentration of Rb in bedrocks and the age of this geological support favour such relatively higher values of the ratio $^{87}Sr/^{86}Sr$. As far as we know, other than the Douro Demarcated Region, such high values have only been reported for Romanian wines from the Vrancea region (Geană et al., 2017).

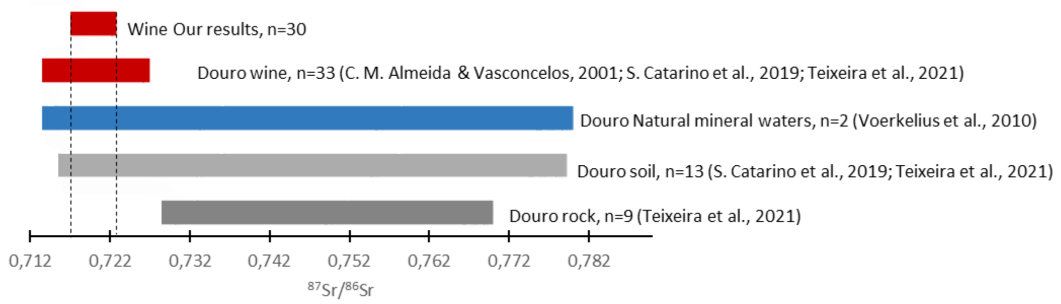

Figure 20. Range of values of $^{87}Sr/^{86}Sr$ for wines, soil, rocks and water from Portugal, as reported in literature. References: (Almeida & Vasconcelos, 2001) (Teixeira et al., 2021) (Catarino et al., 2019) (Voerkelius et al., 2010) (Ribeiro, Azevedo, Santos, Medina, & Costa, 2014)

The $\delta^{11}B$ shows a large dispersion of results within both groups, with the maximum value for a Douro wine (33.5‰) being almost 10-fold higher than the lowest for a Port wine (3.60‰). Samples under study come from a limited geographical area with the same geological formation. Such indicates a high variability of this parameter, possibly linked to anthropogenic sources like the use of fertilizers. Another hypothesis to study further is the role of the vine variety in the fractionation of B after root absorption (Coetzee et al., 2011). In the present work, only two wines are monovarietal; thus, it is not possible to formulate any assumptions on this matter. Some works are available for comparison in the literature regarding $\delta^{11}B$. Our results show a higher dispersion than Lambrusco wines, where $\delta^{11}B$ varied between 8.49‰ and 19.94‰ (n=40) (Lancellotti et al., 2021). The present results are the first ever-obtained results that point to the necessity of a more profound study, especially for elucidating the factors behind the high variability of $\delta^{11}B$.

5.2.2 Statistical analysis

Since all studied wines come from the homogeneous broad swath of land of pre-Cambrian schist and granite along the valley of the Douro River (Figure 18), the geological signature of these wines is expected to be relatively similar. However, while this is true for most elements, Port and Douro wines presented differences in their inorganic composition for some elements and isotopes, suggesting an anthropogenic input. Statistical analysis was used to clarify some of the results obtained.

5.2.2.1 One-way analysis of variance

ANOVA was applied to study further such variations and evaluate significant differences between the two types of wine.

Significant differences were identified in seven elements; Ca, Na, Rb, Zn, Cu, Cr and Se and four isotope ratios; $^{206}Pb/^{204}Pb$, $^{207}Pb/^{204}Pb$, $^{206}Pb/^{207}Pb$ and $\delta^{11}B$; with p-values of 0.002, 0.002, 0.001, 0.031, 0.017, 0.000, 0.000, 0.042, 0.044, 0.045 and 0.031, respectively.

Figure 21 illustrates the boxplots for elements with significant differences and the isotopic systems under study. Minerals and trace elements may come from natural or anthropogenic sources. In the particular case of this study, all vineyards are grown in the same geological substratum and climate. Thus the soil and climate are not expected to be responsible for these differences. Agricultural practices are also common to both types of wine. Instead, differences between Port and Douro wines likely result from the winemaking process or storage conditions.

The levels of Cu and Cr were significantly higher in Port wines than in Douro wines. Similar results had already been observed for Cu but not for Cr between fortified and table wines of the region (Almeida & Vasconcelos, 2003). Nonetheless, results for both types of wine were in accordance with the literature (Catarino et al., 2018; Hopfer et al., 2015; Serapinas et al., 2008). Copper is present in wine because of agricultural practices, namely the use of pesticides, the winemaking process or oenological practices (Tariba, 2011). It comes from the application of fungicide in the form of copper sulphate (Cu_2SO_4) or from additions used during winemaking to control unwanted sulfidic aromas. But the levels of Cu have been reported to decrease during wine fermentation due to the formation of insoluble copper sulphides (Bica, Sánchez, & Todolí, 2020; Tariba, 2011). The early stopping of the fermentation in Port wine could explain the higher levels of Cu.

Regarding the concentrations of Cr, Almeida & Vasconcelos, (2003), also observed the contamination of fortified wines with Cr coming from oak barrels and the vinification system. On the other hand, the use of chromium oxides for pigmentation of glass bottles contributes to an increase in Cr during storage (Cabrera-Vique, Teissedre, Cabanis, & Cabanis, 1997). According to Médina & Sudraud, (1980), the contamination of Cr is higher in glass bottles than in stainless steel tanks. This is consistent with Douro wines presenting lower levels of Cr than Port wines. The three highest concentrations of Cr were found in sample P10 and samples P13 and P14, both bottled in 2014, while the lowest five are in Douro wines stored in stainless steel tanks (D9, D7, D8, D5). Since this was not the primary goal of the present study, a tailor-made sampling plan is required to further elaborate on the origin of Cr in fortified wines.

The levels of Se were between the LD and the LQ (Table 10 a) in little over half of the results. They were considered in the statistical analysis similarly to quantifiable values (Verbovše, 2011). Considering the Adequate Intake (AI) established by EFSA of 70 µg of Se per day, no health effects, either positive or negative, are expected at this level (EFSA NDA Panel, 2014). However, the analytical technique provided enough sensitivity to detect significant differences between Port and Douro wines in this element (p-value ≈ 0.000).

In the case of Zn, although there were significant differences in the level of this element, the range of values obtained for Douro wines is broader and covers most results for Port samples.

The levels of Ca, Na and Rb, are higher in Douro wines and significantly different from Port wines. The explanation for this fact is in the dilution factor introduced by the addition of grape spirit to Port wines. Grape spirit is added to Port wines during fermentation, in the proportion of 1L of grape spirit to 4L of must, diluting the samples and consequently lowering the elemental content. Other elements (B, Fe, K, Mg, Mn, P, Al, Li, Ni, Pb) exhibited the same behaviour but without statistically significant differences.

ANOVA also identified significant differences between the two types of wine regarding some of the Pb isotope ratios and δ^{11}B. However, a visual inspection of the boxplots of δ^{11}B and ^{207}Pb/^{207}Pb, in Figure 21 shows large overlapping values for both types of wine.

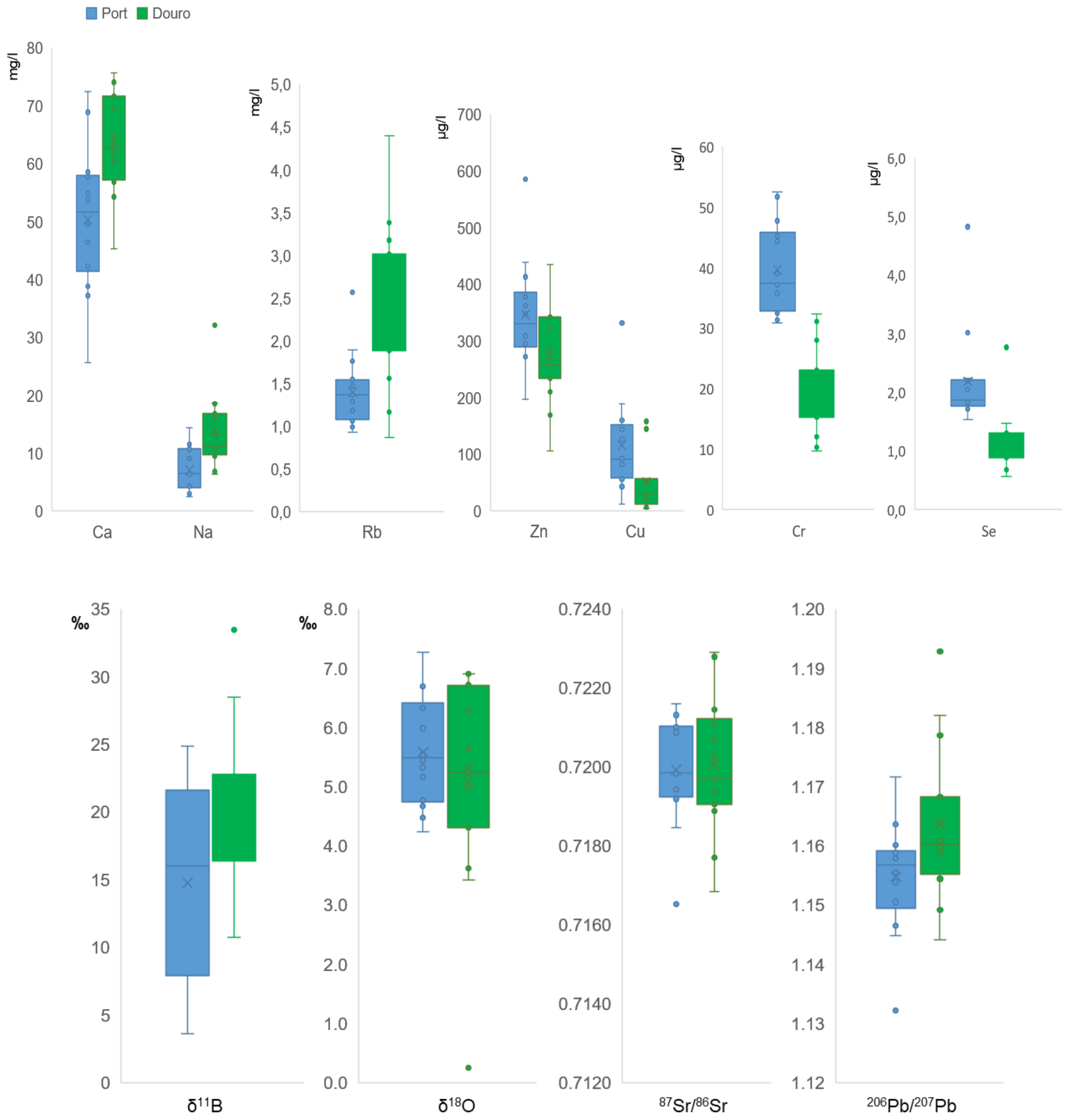

Figure 21. Boxplots for the concentration of the inorganic elements and isotope ratios evidencing significant differences between Port and Douro wines, at $p < 0.05$.

The remaining elements (Li, Be, B, Al, Mn, Co, Ni, As, Sr, Mo, Ag, Cd, Sn, Tl, Pb, Fe, Mg, P, and K) and isotopic systems ($\delta^{18}O$, $^{87}Sr/^{86}Sr$, $^{208}Pb/^{204}Pb$ and $^{208}Pb/^{206}Pb$) did not present any significant differences between the two types of wines, suggesting they may be characteristic of the region and not influenced by anthropogenic sources. These parameters may provide good tracers for provenance traceability.

The values of $^{87}Sr/^{86}Sr$ and $\delta^{18}O$ are referred to in the scientific literature as good geographical markers for wine (Epova et al., 2019; Horacek et al., 2021; Lancellotti et al., 2021). Although a synergistic approach, combining different data is usually more effective, both these parameters may serve as an excellent first indicator of the geographical origin of wines from the Douro Demarcated Region.

The elemental composition of wines is highly influenced by the geochemistry of the soils, and the majority of the elements under study showed a concentration range unrelated to the type of wine. However, some elements showed a variation in their content deriving from the wine production method. This is the case for Ca, Na and Rb due to dilution effects and for Cu because of the incomplete fermentation in Port wines. In the case of Cr, multiple possible causes such as the spirit added, contamination during storage or the interruption of the fermentation require further studies for clarification. Two isotopic analyses, $^{87}Sr/^{86}Sr$ and $\delta^{18}O$, are promising tracers of geographical authenticity for the Douro Demarcated Region since none presented statistically significant differences between the type of wine and values for both were distinctive from the literature. Multivariate statistics was applied to the data to elaborate further on the role of these elements/isotopes in the characterization of Port and Douro wines.

5.2.2.2 Correlation analysis

The aim of the correlation analysis is to identify possible relationships between the 32 variables, elements and isotopes amongst the 29 wine samples. The correlation matrix is provided in Table 11. According to the Kolmogorov-Smirnov test, all variables except Li follow a normal distribution within each type of wine. Spearman´s correlation was used for Li, while for the remaining variables, a parametric test was applied.

All variables showed at least one correlation. This was the case of Mn and $\delta^{18}O$, each with one single significant positive correlation with Mg (0.43) and Al (0.40), respectively. Both Mo and Sn presented significant correlations with only two other variables, Mo-P (0.41), Mo-Pb (0.44), Sn-Pb (0.40) and Sn-Cu (0.64). The three strongest correlations, excluding intra Pb isotope correlations, were Ni-Mg (0.70), Li-Mg (0.68) and Al-Na (0.65). Cr, Mg and Na presented the highest number of significant correlations with other variables, ten in total, followed by Li and Rb, each with nine correlations.

Table 11. Correlation matrix between variables in wines (figures in **bold** indicate significant correlations between two variables considering a p-value < 0.05)

	^{87}Sr/^{86}Sr	δ^{18}O	^{206}Pb/^{207}Pb	^{208}Pb/^{204}Pb	^{207}Pb/^{204}Pb	^{206}Pb/^{204}Pb	^{208}Pb/^{206}Pb	^{207}Pb/^{206}Pb	δ^{11}B	Li	Be	B	Al	Cr	Mn	Co	Ni	Cu	Zn	As	Se	Rb	Sr	Mo	Sn	Pb	Fe	Mg	Ca	Na	P	K
^{87}Sr/^{86}Sr	1.000	-0.272	0.096	-0.136	-0.045	0.094	-0.153	-0.096	0.282	**0.407**	0.053	0.185	0.133	0.079	0.057	-0.130	0.134	0.186	-0.277	0.332	0.225	**0.444**	-0.024	0.260	0.118	0.033	0.076	0.083	0.176	0.262	0.147	**0.567**
δ^{18}O		1.000	0.138	0.129	0.003	0.130	-0.108	-0.138	-0.257	0.025	-0.037	0.002	-0.364	0.011	-0.049	-0.210	-0.156	-0.001	-0.062	-0.269	-0.032	-0.131	-0.003	-0.079	-0.011	-0.104	-0.095	-0.010	-0.049	-0.266	0.224	-0.153
^{206}Pb/^{207}Pb			1.000	**0.834**	**0.820**	**0.994**	**-0.947**	-1.000	0.270	0.059	0.218	-0.010	0.181	**-0.388**	-0.164	-0.259	0.082	-0.277	**-0.386**	-0.155	-0.221	0.077	-0.115	0.016	-0.276	-0.076	-0.090	-0.056	0.230	0.325	-0.058	0.151
^{208}Pb/^{204}Pb				1.000	**0.867**	**0.852**	**-0.660**	**-0.834**	0.114	-0.235	0.187	-0.125	0.182	**-0.461**	-0.237	-0.242	-0.205	**-0.381**	-0.083	**-0.421**	-0.289	-0.207	-0.092	-0.038	**-0.434**	-0.074	-0.015	-0.210	0.097	0.193	-0.224	0.026
^{207}Pb/^{204}Pb					1.000	**0.845**	**-0.701**	**-0.820**	0.227	-0.106	0.013	-0.014	0.181	**-0.455**	-0.278	-0.226	0.002	-0.288	-0.093	-0.280	-0.297	-0.091	-0.200	0.035	-0.301	0.134	0.109	-0.109	-0.007	0.313	-0.184	0.102
^{206}Pb/^{204}Pb						1.000	**-0.932**	**-0.994**	0.275	0.024	0.195	-0.057	0.156	**-0.392**	-0.203	-0.259	0.048	-0.281	-0.345	-0.180	-0.210	0.039	-0.138	0.000	-0.300	-0.086	-0.114	-0.086	0.171	0.310	-0.103	0.129
^{208}Pb/^{206}Pb							1.000	**0.947**	-0.291	-0.115	-0.214	-0.048	-0.201	0.297	0.110	0.208	-0.194	0.183	**0.392**	0.057	0.131	-0.162	0.116	-0.013	0.242	0.046	0.157	0.039	-0.268	-0.309	0.057	-0.148
^{207}Pb/^{206}Pb								1.000	-0.270	-0.059	-0.218	0.010	-0.181	**0.388**	0.164	0.259	-0.082	0.277	**0.386**	0.155	0.221	-0.077	0.115	-0.016	0.276	0.076	0.090	0.056	-0.230	-0.325	0.058	-0.151
δ^{11}B									1.000	0.347	0.205	-0.078	-0.028	-0.279	0.166	-0.269	-0.027	-0.119	**-0.383**	0.018	-0.269	**0.400**	0.266	0.022	-0.112	0.163	0.051	0.142	0.167	0.202	-0.007	0.343
Li										1.000	0.038	**0.583**	-0.094	0.120	0.125	0.084	**0.565**	0.355	-0.286	**0.465**	-0.026	**0.583**	**0.378**	0.252	0.332	0.119	0.229	**0.684**	-0.050	0.199	**0.509**	**0.582**
Be											1.000	-0.148	**0.389**	-0.288	**0.373**	0.110	-0.178	-0.242	-0.061	-0.092	-0.138	0.145	0.231	0.052	-0.044	0.223	0.019	-0.023	0.229	**0.391**	0.209	0.230
B												1.000	-0.187	0.060	0.048	0.048	**0.428**	0.000	-0.075	0.149	-0.059	**0.393**	**0.425**	0.175	-0.021	-0.088	0.209	**0.492**	0.169	0.050	**0.376**	**0.535**
Al													1.000	-0.336	0.101	0.063	0.179	0.104	0.010	0.319	-0.104	0.045	**-0.389**	0.261	0.276	**0.549**	**0.447**	-0.111	0.247	**0.655**	-0.098	-0.079
Cr														1.000	-0.143	0.281	0.131	**0.569**	0.209	0.290	**0.807**	-0.312	0.160	-0.076	0.256	-0.333	-0.280	0.043	**-0.393**	**-0.529**	0.069	0.033
Mn															1.000	0.358	0.270	-0.034	-0.082	0.310	-0.032	0.229	0.257	-0.310	-0.075	0.181	0.261	**0.531**	0.342	0.220	0.156	-0.012
Co																1.000	**0.448**	**0.382**	**0.407**	0.271	**0.401**	-0.066	0.119	-0.357	0.297	0.209	0.155	**0.429**	**-0.380**	0.058	-0.182	-0.199
Ni																	1.000	**0.370**	0.021	**0.626**	0.092	0.320	-0.027	0.053	**0.392**	0.312	0.256	**0.699**	-0.024	**0.391**	0.153	0.096
Cu																		1.000	0.098	**0.440**	**0.528**	-0.097	-0.012	0.109	**0.627**	0.244	0.111	0.251	-0.328	-0.168	0.051	0.028
Zn																			1.000	-0.219	0.264	**-0.450**	-0.079	-0.030	-0.066	0.233	0.065	-0.161	**-0.538**	-0.133	-0.350	-0.286
As																				1.000	0.305	0.167	-0.154	0.019	**0.426**	0.067	0.230	**0.506**	-0.010	0.309	0.179	-0.018
Se																					1.000	-0.321	0.056	-0.275	0.207	-0.250	-0.157	0.009	-0.333	**-0.391**	-0.180	-0.053
Rb																						1.000	0.323	0.133	0.135	0.211	0.238	**0.423**	**0.390**	0.284	**0.491**	**0.597**
Sr																							1.000	-0.023	-0.134	-0.171	0.083	**0.438**	0.121	-0.217	0.306	**0.602**
Mo																								1.000	**0.409**	0.312	0.127	-0.054	0.035	0.282	**0.404**	**0.436**
Sn																									1.000	**0.488**	0.343	0.253	-0.236	0.183	0.292	0.082
Pb																										1.000	**0.541**	0.171	-0.037	**0.537**	0.099	0.071
Fe																											1.000	0.303	0.010	**0.518**	0.049	0.156
Mg																												1.000	-0.020	0.179	**0.432**	0.295
Ca																													1.000	0.307	0.163	0.215
Na																														1.000	0.021	0.132
P																															1.000	**0.549**
K																																1.000

5.2.2.3 Principal Component Analysis

The PCA revealed six PCs explaining 85% of the total variance. Only 17 of the initial 35 variables influenced at least one PC, considering a factor loading ≥ 0.6, and remained during the construction of the final model. The obtained loadings can be consulted in Table 12. Out of the eleven parameters identified by ANOVA with significant differences between the types of wine, only seven remained in the factorial analysis. Such indicates that the values of these seven parameters (Cr, Cu, Rb, Se, $^{206}Pb/^{204}Pb$, $^{207}Pb/^{204}Pb$ and $\delta^{11}B$) are more influenced by the winemaking process or the storage conditions than by the edafo-climatic characteristics of the production area. All other parameters should be evaluated as potential tracers of geographical authenticity. In particular, variables with no significant differences between the types of wine and no significance in the PCA like $\delta^{18}O$, $^{87}Sr/^{86}Sr$, B, Be, Co, Mo, Mn, P and Sn. These are mainly dependent on climate, soil and agricultural practices that are the common characteristics of Port and Douro wines.

Table 12. Loadings for Douro and Port wines (n = 29, in **bold** Loadings > 0.6).

	PC1	PC2	PC3	PC4	PC5	PC6
$^{87}Sr/^{86}Sr$	0.088	0.011	0.045	-0.017	0.112	**0.942**
Li	-0.239	**0.604**	-0.111	0.043	-0.016	**0.658**
Cr	0.318	0.157	-0.154	0.106	**0.822**	-0.070
Ni	0.007	**0.867**	0.165	0.152	0.020	0.061
As	0.382	**0.773**	-0.017	-0.294	0.207	0.101
Se	0.157	-0.054	-0.076	0.044	**0.787**	0.051
Rb	0.008	0.302	0.009	0.243	-0.409	**0.714**
Sr	0.086	0.171	-0.070	**0.931**	0.034	0.017
Pb	-0.033	0.083	**0.840**	0.048	-0.240	-0.002
Fe	0.059	0.021	**0.860**	0.005	0.062	0.098
Mg	0.298	**0.790**	0.050	0.424	-0.023	0.082
K	-0.091	-0.038	0.082	**0.684**	0.008	**0.648**
Al	-0.260	-0.016	**0.628**	-0.521	-0.157	-0.159
Cu	0.136	0.154	**0.623**	-0.099	**0.610**	0.031
$^{206}Pb/^{204}Pb$	**-0.954**	0.001	-0.090	-0.091	-0.121	0.152
$^{208}Pb/^{204}Pb$	**-0.909**	-0.210	0.028	0.017	-0.134	-0.220
$^{207}Pb/^{204}Pb$	**-0.942**	-0.115	0.076	-0.047	-0.130	0.0505
Expl.Var	3.155	2.577	2.327	1.994	2.028	2.389
Prp.Totl	0.186	0.152	0.137	0.117	0.119	0.141

The bi-plot between the factors PC3 and PC4 shows the formation of two groups of samples. The first on the upper quadrants identifies samples with higher levels of Sr and K (samples D2, D4, D6, D10, D14 and P13), and the other on the lower quadrant groups the samples with the lowest levels of these two elements (samples D5, D7, D8, D9 and P1). However, the explanation for this grouping is not the winemaking process (Port or Douro) since both groups are mainly composed of Douro wines, and each includes one Port wine.

Looking at the PC plots in Figure 22, it becomes evident that the factor PC5 provides the best separation between Port and Douro wine. Three elements have a stronger influence here, Cr, Se and Cu. The three evidenced significant differences between the type of wine with ANOVA and a positive, strong correlation between all of them, Cr-Se (0.580), Cr-Cu (0.569) and Cu-Se (0.528). Douro wines have a lower content of Cr, Se and Cu compared to Port wines and these differences permit their differentiation.

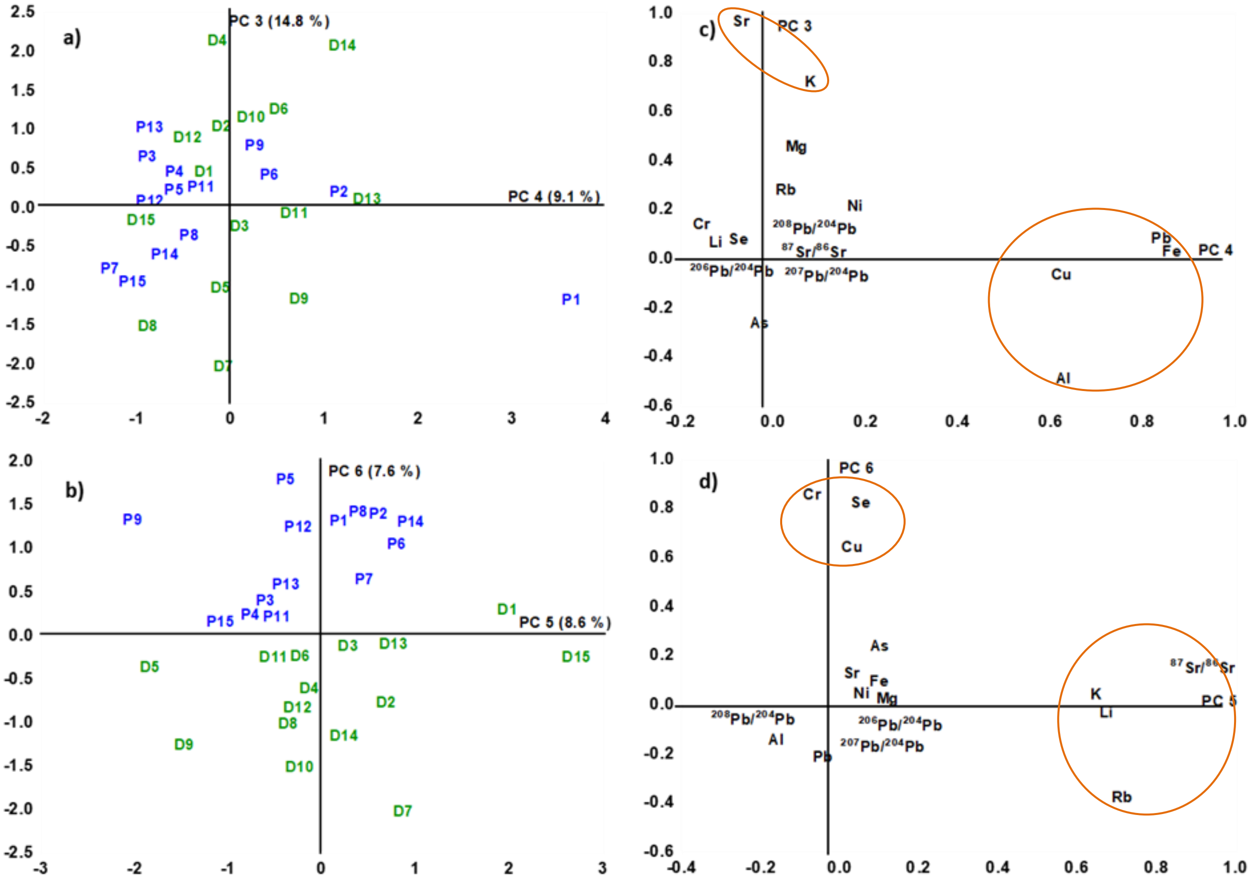

Figure 22. PCA plots representing scores (a, b) and loadings (c, d) for wine samples. The letters “**P**” and “**D**” represent Port and Douro wines, respectively. Numbers from 1 to 15 identify the samples

In sum, PCA identified common characteristics, based on the levels of Sr and K, to a group of samples that includes Port and Douro wines (PC3 vs PC4). Still, it also provided the complete differentiation between Port and Douro wines based on the levels of Cr, Cu and Se (PC5 vs PC6).

5.2.2.4 Hierarchical Cluster Analysis

To reduce background noise, HCA used only the 17 variables highlighted in the PCA (Table 12).

HCA showed the formation of four clusters (Figure 23). The separation between the types of wines is evident for two clusters in this statistical analysis. The first cluster contains only Douro wines, nine in total, and eleven Port wines form the third cluster. These last ones are characterized by higher levels of Cr, Se and lower Rb and Na when compared to the global mean. On the other hand, Douro wines in the first cluster show values closer to the mean for all variables with no evident distinctive values.

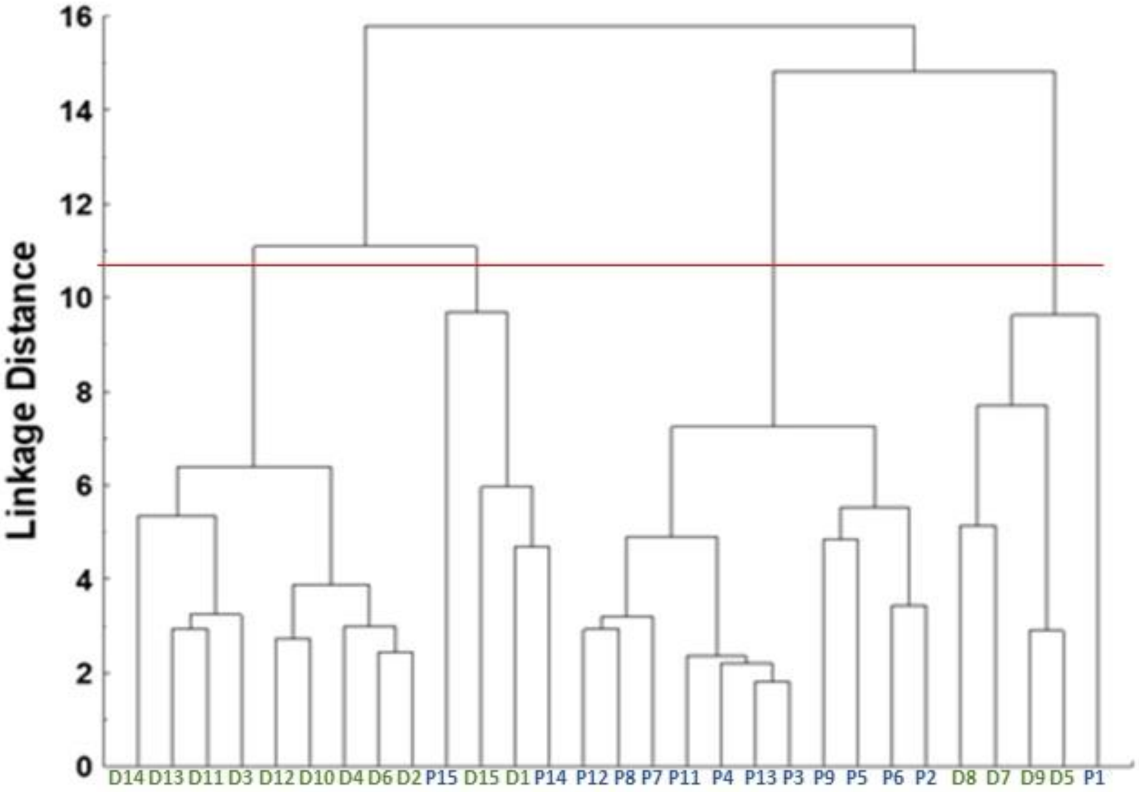

Figure 23. Dendrogram obtained by HCA considering wine samples (n=29). The letters "**P**" and "**D**" represent Port and Douro wines, respectively. Numbers from 1 to 15 identify the samples.

The second cluster is a mixture of Port and Douro wines, containing two of each namely P14, P15, D1 and D15. On average, these wines have the highest concentrations of Li, Rb and As, and the lowest of Pb, which explains the grouping of this cluster.

Four Port wines and one Douro wine form the fourth and last cluster. The common factor is that all five are white wines. In fact, the only white wine understudy not classified in this cluster is P12. The analysis assumed that this sample presented more similarities with other Ports than with white wines and placed it in the third cluster. The wines in the fourth cluster show the highest levels of Al and Na and the lowest of Cr, Sr and K. Cr and Na presented significant differences between Port and Douro wines in ANOVA (Figure 21). As such, the predominance of Douro wines in this cluster explains the levels of these two elements.

On the other hand, Al, Sr and K did not show significant differences between the two types of wine. White wines have been reported to have higher Al and lower Sr than red wines (Gajek, Pawlaczyk, & Szynkowska-Jozwik, 2021; Karaś, Zioła-Frankowska, & Frankowski, 2020). Unlike red wines, the fermentation process in white wines does not occur in contact with skin and stalk, which can explain the lower levels of K. Combining this analysis with the interpretation of the PCA, we verify that the plot PC3 (Al) vs PC4 (Sr, K) (Figure 22 a) provides a grouping of these wines, coherent with the HCA. In addition, Pearson´s test showed a negative correlation between Al-Sr (-0.389) and a positive one between Sr-K (0.602). Due to the reduced number of white wine samples under study, these are preliminary results and should be further investigated.

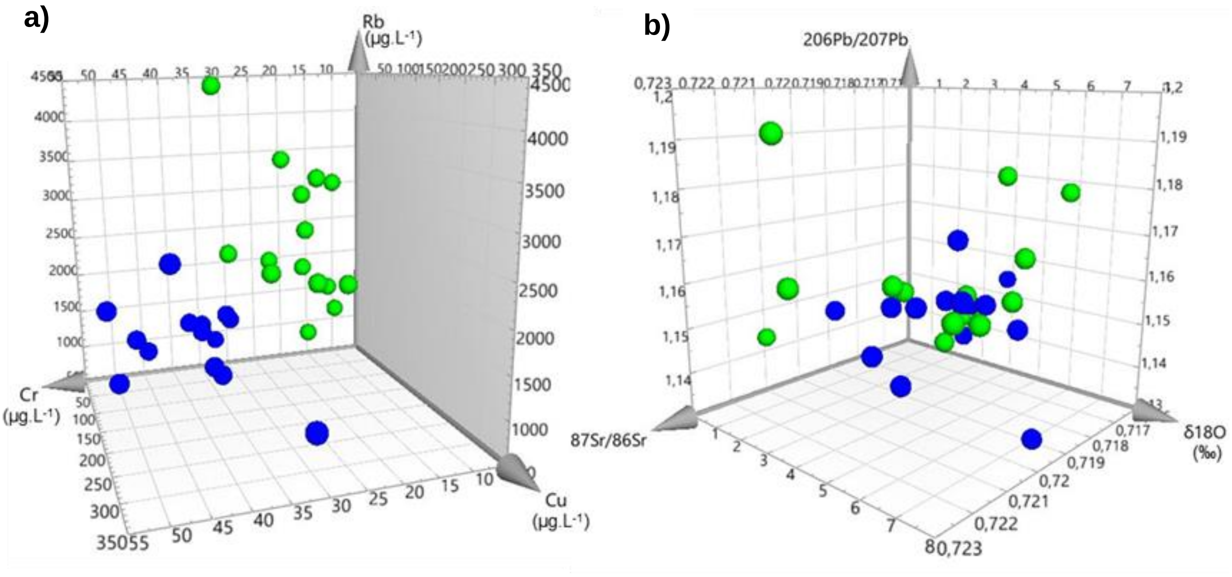

Figure 24. A 3-D plot of (a) discriminant parameters process-oriented by type of wine and (b) non-discriminant isotope ratios.

Based on the results from the statistical analysis, two 3-D Plots (Figure 24) were constructed to represent the interaction between parameters and the type of wine. Figure 24 (a) illustrates the characterization of wines using parameters influenced by the winemaking process or storage (Cr, Cu, Rb). The distinction between Port and Douro wines is unequivocal as there are no overlapping samples. On the other hand, parameters mainly influenced by the climate (like $\delta^{18}O$), atmospheric contamination or underlying geology ($^{87}Sr/^{86}Sr$) display a range of values common to both types of wines (Figure 24 (b)). This plot represents a characteristic pattern of the region. The obtained results could integrate a reference database for the Douro Demarcated Region.

5.2.3 Final remarks

The Douro Demarcated Region produces Port and Douro wines. These wines share the same climate conditions, geological formation and agricultural practices. The main difference between the two wines is the addition of grape spirit at mid fermentation to Port wine, producing a fortified wine. All vineyards in this study are grown in soils from schists. The present study aimed to evaluate the inorganic content of these wines and assess the elements and isotopes dependent exclusively on the production area and those in

The analytical methods provided enough sensitivity to detect the inorganic content in the wine samples. The analytical determinations revealed that all analysed samples comply with OIV maximum limits and that K, P and Mg are the most abundant elements, in decreasing order. On the other hand, Cd, Tl and Ag were below the LQ in all samples.

As a result of ANOVA, seven elements (Ca, Na, Rb, Zn, Cu, Cr and Se) and four isotopic systems (^{206}Pb/^{204}Pb, ^{207}Pb/^{204}Pb, ^{206}Pb/^{207}Pb and δ^{11}B) showed significant differences between the two types of wine, suggesting a greater influence of the winemaking process or storage on these parameters rather than soil or climate. Douro wines are richer in Ca, Na and Rb due to dilution effects coming from the addition of grape spirit to Port wine. The interruption of the fermentation leads to higher Cu values in Port wines. As for Cr, further studies are required to clarify the causes behind its increased levels in Port wine.

Three elements (Cu, Cr and Se) provided a good distinction between Port and Douro wines observed in the PCA. HCA also the samples were separated, the formation of a cluster composed only of white wines showed these wines have lower levels of Sr and K but are enriched in Al. However, more samples are required to support these assumptions, and further studies should be conducted.

Results obtained for both ^{87}Sr/^{86}Sr and δ^{18}O values are distinct from the scientific literature, and the type of wine did not condition either of these variables. The addition of grape spirit to Port wines, which may come from areas outside the Douro Demarcated Region and even other countries, did not induce statistically significant differences between Port and Douro wines, nor did storage conditions. Such indicates that both parameters are specific to the region and are promising natural tracers for the geographical origin of the Douro Demarcated Region, as the winemaking process or storage conditions did not influence either of them.

This work provides comprehensive and solid analytical data to integrate an authenticity database for wines from the Douro Demarcated Region.

6 Conclusions

The scope of this thesis was the characterization and evaluation of the mineral and isotopic profile of two Portuguese food products, Rocha pear and wine, for food authenticity purposes. The strategy for both matrices differed. As for pears, there were no published results available; the plan was to characterize and compare pear samples produced in the PDO region with those produced in another area. In wines, the strategy followed comprised the characterization of two types of wine produced within the same demarcated region but following different winemaking processes. This allowed us to evaluate the influence of the wine-making process on the inorganic and isotopic composition of wines from the Douro Demarcated Region and evaluate the discriminant parameters regarding the geographical origin of wines from this region.

The multielement and Sr isotopic profile of Rocha pears was characterized for the first time to evaluate its contribution to the study of pears authenticity. Samples from two regions were collected and analysed in 50 pools. Regardless of the geographical origin, pear samples are richer in K and P than in other minerals. The levels of inorganic contaminants were low in all samples and did not pose a risk to human health.

Chemometric tools, applied to the multielement data, differentiated between Rocha pears and classified them with 100% accuracy according to their production area. Pears from the PDO region distinguished themselves by having higher Al, Na, Ni, and Zn levels. Na was quantifiable only in PDO pears due to the proximity to the seashore. The Sr isotope ratio, $^{87}Sr/^{86}Sr$, was evaluated and, except for one farm from Fundão, results varied according to the underlying geology. Granites from Fundão were associated with higher $^{87}Sr/^{86}Sr$ values in pears, while samples from the PDO region, characterized by limestone and sandstone, presented lower Sr isotope ratios.

In the future, combining isotopic and multielement information may help assess the authenticity of Rocha pears, particularly when compared to other coastal areas of production.

Multielement and isotopic analyses were used to identify discriminant parameters regarding the geographical origin of wines from the Douro Demarcated Region. A comparative study using chemometrics was performed on the data obtained for 15 Port wines and 15 Douro wines.

Three elements were essential to distinguish between the two types of wines studied. Cr, Cu, and Se levels were significantly higher in Port wines, leading to a clear separation between Port and Douro wines, as exemplified in the PCA and the 3-D plots. Considering that the climate, soil, and agricultural practices are typical to both types of wine, these three parameters are primarily influenced by variable factors, namely the winemaking process or storage conditions. On the other hand, two isotopic systems, $^{87}Sr/^{86}Sr$ and $\delta^{18}O$ presented a similar profile for all wines. The similarities of the two parameters in both types of wines, deriving fundamentally from the characteristics of the soil and climate, associated with a comparison with the scientific literature, indicated that both are region-specific and can serve as valuable tools in the combat against wine fraud regarding geographical provenance in the Douro Demarcated Region. Combining the information from two isotopic systems, namely Sr (reflecting the soil) and O (reflecting climate), seems promising in detecting wine fraud. This work provides the foundations for developing an authenticity testing scheme for the Douro Demarcate Region.

The study of the Pb isotopic signature of Port and Douro wines provided valuable insight into atmospheric contamination across Europe. The comparison of the obtained Pb isotopic ratios against previously published results for Bordeaux wines showed a very good alignment between the two indicating a common source of Pb contamination.

This work contributed to the characterization of two Portuguese food products. It demonstrated that the geographical origin could be traced through multielement and isotopic analysis. The proposed methods require an extensive database of results to be helpful in routine authenticity testing schemes. Because the results presented were obtained under rigorous quality control criteria, they may, in the future, integrate both an authenticity database and a FCDB for Rocha pears, Douro wine and Port wine.

In addition to battling food fraud, the chemical characterization of authenticity tracers provides the opportunity for producers to build added value to their authentic products. These may, in the future, be promoted as authentic products by sustaining a chemical signature authenticity logo, complementing the already existing EU geographical indication logos.

7 Future perspectives

Following the obtained results, several questions arise that do not fall within the scope of the present thesis but should be a matter of research in the future, namely:

- In pears, expand the sampling plan to include other coastal areas and inner areas of the PDO region, and evaluate the impact on the Na and $^{87}Sr/^{86}Sr$ content, as these parameters were the main discriminant factors between the two regions.
- Foresee the determination of the $\delta^{18}O$ value in future analyses of pears because the results of this parameter in wine were promising.
- Conduct a study at the various stages of the wine production process, including the influence of bottle ageing, to clarify the causes behind the differences encountered between Port and Douro wines, particularly for Cr. Grape spirit, should also be analysed to discard this option as a contamination source.
- Build a national databank compiling isotopic information of $\delta^{18}O$ and $^{87}Sr/^{86}Sr$ from authentic wines produced in the different demarcated regions within the country. Such a database would facilitate the authentication of samples in the future.
- Optimize the column extraction process for Sr and Pb isotopes. This process is tedious and time-consuming. Developing a more automated process would be very beneficial and increase sample throughput.
- Implement a sampling plan aimed at evaluating differences between white and red wines. Multivariate analysis showed common characteristics between analysed white wines, regardless of the type of wine. However, to obtain proper conclusions, it is necessary to adequate the sampling plan for such a purpose.

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Appendix 1 – Elemental concentrations in peeled and unpeeled pear samples

Table A1.Elemental concentrations in peeled and unpeeled Rocha Pears. Samples are identified from S1 to S5.

	Al	As	B	Cd	Cr	Co	Cu	Li
	µg.kg^{-1} (dry weight)							
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
S1 unpeeled	1013 ± 14	12.2 ± 0.5	10432 ± 155	8.5 ± 0.3	176 ± 11	47 ± 1	3937 ± 76	88 ± 1
S1 peeled	< LQ	13 ± 1	13852 ± 238	10 ± 1	130 ± 15	70 ± 1	5168 ± 592	89 ± 5
S2 unpeeled	< LQ	< LQ	12297 ± 682	11 ± 1	162 ± 3	57 ± 5	4854 ± 490	4.0 ± 0.2
S2 peeled	< LQ	< LQ	14430 ± 368	14 ± 1	125 ± 6	69 ± 3	6683 ± 308	5.2 ± 0.3
S3 unpeeled	902 ± 26	13.7 ± 0.3	14938 ± 214	10.1 ± 0.2	137 ± 2	38 ± 1	5931 ± 53	22 ± 1
S3 peeled	< LQ	13 ± 2	13509 ± 1314	13 ± 1	124 ± 10	40 ± 3	7024 ± 496	25 ± 2
S4 unpeeled	1081 ± 11	< LQ	13137 ± 360	8.2 ± 0.2	109 ± 5	67 ± 1	5384 ± 98	6.0 ± 0.1
S4 peeled	< LQ	< LQ	12673 ± 319	7.1 ± 0.2	126 ± 5	63 ± 3	5397 ± 183	6.0 ± 0.2
S5 unpeeled	2217 ± 135	< LQ	10760 ± 269	14 ± 1	124 ± 1	145 ± 4	6381 ± 155	6.0 ± 0.4
S5 peeled	< LQ	< LQ	10424 ± 99	15.5 ± 0.4	135 ± 2	147 ± 2	6690 ± 69	3.7 ± 0.1

Table A1. Elemental concentrations in peeled and unpeeled Rocha Pears. Samples are identified from S1 to S5. (Continuation)

	Mn	Mo	Ni	Pb	Sr	Tl	Zn
	$\mu g.kg^{-1}$ (dry weight)						
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
S1 unpeeled	6319 ± 48	84 ± 1	269 ± 9	< LQ	1121 ± 17	7.3 ± 0.2	4876 ± 63
S1 peeled	4305 ± 370	75 ± 13	379 ± 35	24 ± 1	865 ± 40	7.4 ± 0.1	5851 ± 427
S2 unpeeled	2284 ± 30	22 ± 1	106 ± 17	< LQ	2048 ± 90	3.8 ± 0.2	4515 ± 319
S2 peeled	2190 ± 90	24 ± 1	175 ± 5	18 ± 0.8	2315 ± 36	5.6 ± 0.2	5010 ± 180
S3 unpeeled	1931 ± 57	111 ± 6	154 ± 11	< LQ	758 ± 40	42.7 ± 1.2	5016 ± 244
S3 peeled	1630 ± 123	119 ± 9	208 ± 10	42 ± 1	1099 ± 51	54.3 ± 3.4	5486 ± 514
S4 unpeeled	2274 ± 42	< LQ	364 ± 15	< LQ	323 ± 11	3.3 ± 0.1	6731 ± 178
S4 peeled	1933 ± 20	31.8 ± 0.3	367 ± 3	< LQ	330 ± 5	3.5 ± 0.1	5719 ± 291
S5 unpeeled	4811 ± 143	83 ± 1	982 ± 3	< LQ	1195 ± 2	11.8 ± 0.2	6352 ± 277
S5 peeled	2348 ± 258	76 ± 9	1339 ± 93	29 ± 4	1402 ± 160	10.5 ± 0.1	5550 ± 86

Table A1. Elemental concentrations in peeled and unpeeled Rocha Pears. Samples are identified from S1 to S5. (Continuation)

	Ca	Fe	K	Mg	Na	P
	mg.100g^{-1} (dry weight)					
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
S1 unpeeled	35.5 ± 0.7	0.6 ± 0.01	807 ± 23	27.3 ± 0.7	1.4 ± 0.1	46 ± 1
S1 peeled	27 ± 1	0.66 ± 0.05	929 ± 6	35 ± 0.3	< LQ	53 ± 4
S2 unpeeled	20.9 ± 0.7	0.75 ± 0.03	779 ± 27	28.6 ± 0.9	< LQ	56 ± 5
S2 peeled	19.6 ± 0.8	0.84 ± 0.02	888 ± 19	36 ± 2	< LQ	77 ± 3
S3 unpeeled	30 ± 1	0.45 ± 0.01	1101 ± 34	37 ± 1	1.7 ± 0.1	60 ± 1
S3 peeled	31 ± 2	0.53 ± 0.03	1182 ± 64	40 ± 2	1.4 ± 0.2	73 ± 5
S4 unpeeled	40.3 ± 0.8	0.69 ± 0.01	900 ± 8	41.6 ± 0.1	6.1 ± 0.1	60 ± 2
S4 peeled	35.6 ± 0.9	0.65 ± 0.02	988 ± 15	40.1 ± 0.7	6.5 ± 0.1	68 ± 2
S5 unpeeled	25.2 ± 0.9	0.92 ± 0.09	819 ± 4	36 ± 0.4	< LQ	66 ± 1
S5 peeled	18.8 ± 0.3	0.75 ± 0.01	1008 ± 14	28.9 ± 0.2	< LQ	64 ± 1

Appendix 2 – Elemental concentrations and isotope ratios in pear samples

Table A2.Elemental concentrations, Sr isotope ratios and moisture content in Rocha Pears from the PDO region.

Sample ID	Moisture %	Al	As	B	Be	Cd	Cr	Co	Cu	Li
		µg.kg^{-1} (dry weight)								
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
PDO 1.1	85	2704 ± 154	17 ± 1.1	21903 ± 737	<LD	5.2 ± 0.1	329 ± 28	69.5 ± 2.2	4820 ± 178	11.7 ± 0.5
PDO 1.2	85	2231 ± 176	36.8 ± 1	11471 ± 151	<LD	4.4 ± 0.2	319 ± 21	38.7 ± 1	1778 ± 35	9.0 ± 0.2
PDO 1.3	84	2274 ± 50	16.3 ± 0.7	10060 ± 266	<LD	6.2 ± 0.2	120 ± 5	44.4 ± 0.6	3530 ± 27	10.2 ± 0.3
PDO 1.4	84	2434 ± 62	15.1 ± 0.8	12864 ± 347	<LD	9.8 ± 0.5	132 ± 10	74.2 ± 1.7	4235 ± 93	12.4 ± 0.6
PDO 1.5	84	1628 ± 59	37.8 ± 1.1	9786 ± 176	<LD	<LQ	143 ± 9	13.5 ± 0.2	1936 ± 18	5.8 ± 0.2
PDO 2.1	87	1742 ± 36	9.8 ± 0.2	10706 ± 241	<LD	8.1 ± 0.3	345 ± 10	67 ± 1	3655 ± 54	18 ± 0.9
PDO 2.2	87	1937 ± 37	15.6 ± 0.2	10342 ± 643	<LD	10.9 ± 0.3	356 ± 25	117 ± 6.9	5360 ± 9	19.8 ± 1.9
PDO 2.3	87	2106 ± 28	15 ± 0.6	8250 ± 121	<LD	10.9 ± 0.2	164 ± 14	80.3 ± 0.5	5511 ± 82	20.3 ± 0.4
PDO 2.4	87	2150 ± 26	13.4 ± 0.5	9243 ± 187	<LD	9.1 ± 0.2	126 ± 6	105.2 ± 2.6	5095 ± 110	15.8 ± 0.4
PDO 2.5	86	2080 ± 83	19.6 ± 0.2	8304 ± 204	<LD	10.3 ± 0.3	145 ± 17	55.4 ± 1.2	4118 ± 50	17.8 ± 0.2
PDO 3.1	83	1259 ± 121	10.3 ± 0.5	15213 ± 1511	<LD	19.7 ± 0.9	347 ± 7	103.1 ± 2.9	5070 ± 149	109.3 ± 7
PDO 3.2	84	1104 ± 65	13.9 ± 0.8	12136 ± 584	<LD	14.3 ± 0.3	331 ± 5	135.5 ± 7.3	3906 ± 88	30 ± 3
PDO 3.3	87	1639 ± 78	15.6 ± 0.2	13950 ± 464	<LD	11.4 ± 0.3	154 ± 8	82.2 ± 1.1	6747 ± 45	9.7 ± 0.2
PDO 3.4	83	1630 ± 97	8.2 ± 0.2	9964 ± 264	<LD	20.4 ± 0.4	147 ± 8	85.3 ± 1.8	5709 ± 110	58.5 ± 1.7
PDO 3.5	84	1856 ± 105	10.4 ± 0.1	9865 ± 210	<LD	22.7 ± 0.2	159 ± 10	134.2 ± 2.4	4842 ± 59	11.4 ± 0.2
PDO 4.1	85	3272 ± 232	<LQ	10873 ± 437	<LD	7.4 ± 0.4	307 ± 12	22.2 ± 0.9	3781 ± 106	37.8 ± 2.1
PDO 4.2	85	2231 ± 122	<LQ	10650 ± 46	<LD	7.2 ± 0.2	333 ± 16	33.2 ± 0.6	3698 ± 218	37.9 ± 1.6
PDO 4.3	83	2495 ± 204	<LQ	9733 ± 929	<LD	9.8 ± 0.8	156 ± 19	33.6 ± 3.2	6132 ± 638	21.9 ± 2
PDO 4.4	85	2460 ± 133	<LQ	9611 ± 302	<LD	12.6 ± 0.3	147 ± 11	27.7 ± 0.7	5405 ± 134	24.2 ± 0.9
PDO 4.5	84	1550 ± 27	<LQ	7566 ± 427	<LD	7.8 ± 0.3	141 ± 6	31.2 ± 1.6	3701 ± 164	18.9 ± 1.2
PDO 5.1	84	1125 ± 11	<LQ	15280 ± 691	<LD	10.1 ± 0.4	463 ± 42	34.4 ± 1.3	3934 ± 149	9.9 ± 0.4
PDO 5.2	84	884 ± 84	<LQ	12199 ± 191	<LD	9.9 ± 0.2	316 ± 7	64.1 ± 0.8	4914 ± 92	9.5 ± 0.6
PDO 5.3	85	1674 ± 71	<LQ	13318 ± 595	<LD	5.1 ± 0.4	152 ± 6	96.0 ± 0.0	7045 ± 179	15.7 ± 0.1
PDO 5.4	85	1639 ± 40	<LQ	18848 ± 368	<LD	5 ± 0.2	135 ± 19	61.0 ± 1.4	5102 ± 57	10.9 ± 0.3
PDO 5.5	84	1118 ± 106	<LQ	16073 ± 33	<LD	9.1 ± 0.9	134 ± 7	67.8 ± 0.6	3834 ± 96	10.1 ± 0.2

Table A2. Elemental concentrations, Sr isotope ratios and moisture content in Rocha Pears from the PDO region. (Continuation)

Sample ID	Mn	Mo	Ni	Pb	Se	Sn	Sr	Tl	Zn
	µg.kg^{-1} (dry weight)								
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
PDO 1.1	1651 ± 164	135 ± 7	225 ± 24	<LQ	<LQ	13 ± 2	730 ± 28	<LQ	5978 ± 163
PDO 1.2	1518 ± 78	138 ± 2	279 ± 12	<LD	<LQ	<LQ	353 ± 20	<LQ	3293 ± 83
PDO 1.3	2315 ± 97	82 ± 4	307 ± 9	<LQ	<LQ	<LQ	576 ± 23	4.4 ± 0.1	5488 ± 72
PDO 1.4	1559 ± 24	45 ± 2	241 ± 9	<LD	<LQ	<LQ	736 ± 11	<LQ	6355 ± 392
PDO 1.5	1474 ± 26	102 ± 5	500 ± 29	<LD	<LQ	<LQ	500 ± 18	<LQ	3434 ± 49
PDO 2.1	2306 ± 30	108 ± 8	396 ± 22	<LD	<LQ	<LQ	615 ± 16	<LQ	10958 ± 284
PDO 2.2	3218 ± 157	62 ± 2	649 ± 22	<LQ	<LQ	<LQ	918 ± 45	<LQ	17127 ± 249
PDO 2.3	2709 ± 80	61 ± 1	408 ± 24	<LQ	<LQ	<LQ	547 ± 15	<LQ	14642 ± 120
PDO 2.4	2139 ± 54	58 ± 3	539 ± 20	24.4 ± 0.9	<LQ	<LQ	686 ± 16	<LQ	14808 ± 127
PDO 2.5	2070 ± 62	60 ± 5	336 ± 5	24.1 ± 1.1	<LQ	<LQ	630 ± 14	<LQ	13561 ± 122
PDO 3.1	1787 ± 21	58 ± 4	1362 ± 38	<LD	<LQ	<LQ	762 ± 7	10.8 ± 1.1	6228 ± 299
PDO 3.2	1669 ± 158	61 ± 3	852 ± 21	<LD	<LQ	<LQ	651 ± 73	7.6 ± 0.2	4973 ± 59
PDO 3.3	1960 ± 32	45 ± 2	406 ± 25	16.7 ± 1.3	18 ± 1	<LQ	382 ± 11	<LQ	8360 ± 40
PDO 3.4	1668 ± 16	61 ± 1	911 ± 10	n.d.	<LQ	<LQ	450 ± 6	10.6 ± 0.5	8215 ± 406
PDO 3.5	1649 ± 22	61 ± 1	721 ± 4	19.9 ± 0.8	<LQ	<LQ	430 ± 14	7.5 ± 0.0	6747 ± 109
PDO 4.1	3936 ± 145	73 ± 1	326 ± 7	<LQ	<LQ	<LQ	1396 ± 61	23.9 ± 1.0	3952 ± 103
PDO 4.2	3946 ± 231	89 ± 6	322 ± 15	<LQ	<LD	<LQ	1651 ± 113	48.4 ± 1.2	4839 ± 53
PDO 4.3	2909 ± 280	69 ± 7	270 ± 20	19.3 ± 0.3	<LD	<LQ	611 ± 48	22.6 ± 2.0	5767 ± 514
PDO 4.4	2333 ± 56	88 ± 3	299 ± 12	22.8 ± 1.6	<LQ	<LQ	1267 ± 27	12.5 ± 0.4	5153 ± 177
PDO 4.5	2709 ± 128	81 ± 4	339 ± 17	<LQ	<LD	<LQ	729 ± 24	26 ± 1.4	4545 ± 201
PDO 5.1	2464 ± 281	103 ± 1	445 ± 29	<LD	<LQ	<LQ	768 ± 101	6.3 ± 0.3	5069 ± 163
PDO 5.2	1682 ± 44	66 ± 2	293 ± 25	<LD	<LQ	<LQ	320 ± 11	4.9 ± 0.2	5327 ± 187
PDO 5.3	2748 ± 15	83 ± 12	295 ± 40	<LQ	<LQ	<LQ	737 ± 99	<LQ	8071 ± 350
PDO 5.4	2270 ± 49	58 ± 6	333 ± 14	17.8 ± 1	<LQ	<LQ	655 ± 11	<LQ	7030 ± 86
PDO 5.5	1315 ± 88	39 ± 1	265 ± 23	18.6 ± 1.6	17 ± 1	<LQ	437 ± 26	<LQ	5066 ± 194

Table A2. Elemental concentrations, Sr isotope ratios and moisture content in Rocha Pears from the PDO region. (Continuation)

Sample ID	Ca	Fe	K	Mg	Na	P	$^{86}Sr/^{87}Sr$
	mg.(100g)$^{-1}$ (dry weight)						
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
PDO 1.1	39 ± 1	0.61 ± 0.02	830 ± 51	34.0 ± 2.0	71.2 ± 4.7	71.2 ± 4.7	0.70977 ± 0.00003
PDO 1.2	33 ± 33	0.70 ± 0.02	915 ± 46	35.9 ± 1.8	56.6 ± 2.3	56.6 ± 2.3	0.71257 ±0.00002
PDO 1.3	36 ± 36	0.60 ± 0.01	885 ± 11	43.7 ± 1.1	60.7 ± 0.7	60.7 ± 0.7	0.71283 ± 0.00003
PDO 1.4	39 ± 39	0.57 ± 0.02	754 ± 16	34.7 ± 0.9	61.6 ± 1.0	61.6 ± 1.0	n.d.
PDO 1.5	33 ± 33	0.81 ± 0.02	819 ± 7	26.8 ± 0.2	44.9 ± 0.2	44.9 ± 0.2	0.71210
PDO 2.1	52 ± 52	0.72 ± 0.02	812 ± 26	46.5 ± 0.3	65.4 ± 0.3	65.4 ± 0.3	0.71291
PDO 2.2	74 ± 74	0.96 ± 0.02	886 ± 26	57.9 ± 1.7	82.2 ± 3.7	82.2 ± 3.7	0.71344
PDO 2.3	40 ± 40	0.82 ± 0.03	884 ± 9	51.8 ± 0.6	77.1 ± 0.7	77.1 ± 0.7	0.71373
PDO 2.4	55 ± 55	0.81 ± 0.01	727 ± 22	48.8 ± 1.6	67.7 ± 2.3	67.7 ± 2.3	0.71331
PDO 2.5	50 ± 50	0.71 ± 0.04	741 ± 25	47.3 ± 1.5	57.3 ± 2.1	57.3 ± 2.1	0.71335
PDO 3.1	46 ± 46	0.81 ± 0.02	761 ± 10	35.9 ± 0.7	71.5 ± 0.9	71.5 ± 0.9	0.71439 ± 0.00003
PDO 3.2	36 ± 36	0.82 ± 0.03	637 ± 34	31.0 ± 1.9	55.1 ± 3.9	55.1 ± 3.9	0.71446
PDO 3.3	30 ± 30	0.76 ± 0.01	708 ± 13	25.1 ± 0.1	71.2 ± 0.6	71.2 ± 0.6	0.71344
PDO 3.4	30 ± 30	0.59 ± 0.02	703 ± 30	26.4 ± 1.1	67.6 ± 2.6	67.6 ± 2.6	0.71404
PDO 3.5	30 ± 30	0.80 ± 0.04	596 ± 14	25.3 ± 0.3	61.0 ± 1.4	61.0 ± 1.4	0.71234
PDO 4.1	61 ± 61	0.72 ± 0.03	913 ± 16	38.3 ± 0.6	50.1 ± 1.6	50.1 ± 1.6	0.70959 ± 0.000001
PDO 4.2	52 ± 52	0.85 ± 0.00	1098 ± 40	44.8 ± 1.9	66.4 ± 4.3	66.4 ± 4.3	0.70932 ± 0.00001
PDO 4.3	42 ± 42	0.62 ± 0.01	860 ± 6	31.9 ± 0.5	51.9 ± 0.5	51.9 ± 0.5	0.71199
PDO 4.4	46 ± 46	0.73 ± 0.02	916 ± 21	37.1 ± 1.0	66.1 ± 1.3	66.1 ± 1.3	0.70715 ± 0.00003
PDO 4.5	43 ± 43	0.64 ± 0.03	911 ± 37	35.1 ± 1.6	47.0 ± 3.1	47.0 ± 3.1	0.71318
PDO 5.1	42 ± 42	0.49 ± 0.03	799 ± 80	23.3 ± 1.3	49.4 ± 2.4	49.4 ± 2.4	0.71307
PDO 5.2	23 ± 23	0.56 ± 0.02	740 ± 22	22.7 ± 0.3	66.9 ± 2.2	66.9 ± 2.2	0.71202
PDO 5.3	48 ± 48	0.47 ± 0.04	724 ± 15	26.1 ± 2.3	78.8 ± 0.2	78.8 ± 0.2	0.71433
PDO 5.4	45 ± 45	0.55 ± 0.01	791 ± 1	25.2 ± 0.9	69.1 ± 0.7	69.1 ± 0.7	0.71251
PDO 5.5	30 ± 30	0.65 ± 0.06	597 ± 54	21.6 ± 1.7	49.6 ± 4.9	49.6 ± 4.9	0.71358

n.d. – not determined. Repeatability for $^{86}Sr/^{87}Sr$ in pears was estimated on independent triplicates and evaluated as RSD (RSDmax = 119 ppm).

Table A3.Elemental concentrations, Sr isotope ratios and moisture content in Rocha Pears from the region of Fundão.

Sample ID	Moisture %	Al	As	B	Be	Cd	Cr	Co	Cu	Li
		µg.kg^{-1} (dry weight)								
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Fundão 1.1	83	918 ± 8	10.8 ± 0.2	6138 ± 73	10.6 ± 0.2	< LQ	297 ± 7	28.4 ± 0.2	3231 ± 29	26.3 ± 0.4
Fundão 1.2	83	1064 ± 41	< LQ	6180 ± 170	17.6 ± 0.7	< LQ	322 ± 13	36.5 ± 0.9	3334 ± 99	26.3 ± 1.1
Fundão 1.3	83	856 ± 56	< LQ	9085 ± 56	7.9 ± 0.2	5.0 ± 0.1	137 ± 7	23.3 ± 0.5	4051 ± 29	17.9 ± 0.2
Fundão 1.4	83	< LQ	< LQ	8307 ± 157	11.6 ± 0.3	4.2 ± 0.1	142 ± 4	31.1 ± 0.6	3507 ± 55	20.1 ± 0.4
Fundão 1.5	83	856 ± 84	< LQ	9776 ± 454	27.3 ± 0.9	5.9 ± 0.4	141 ± 7	54.7 ± 2.8	4113 ± 146	36.2 ± 1.8
Fundão 2.1	84	< LQ	< LQ	11616 ± 195	<LD	15.5 ± 0.4	347 ± 38	19.7 ± 0.1	3540 ± 32	6.5 ± 0.1
Fundão 2.2	85	< LQ	< LQ	12279 ± 338	< LQ	12.6 ± 0.5	255 ± 12	18.4 ± 0.7	2981 ± 67	14.3 ± 0.5
Fundão 2.3	87	1118 ± 104	< LQ	14316 ± 556	< LQ	19.7 ± 0.7	140 ± 4	30.4 ± 1.1	4534 ± 124	13.8 ± 0.8
Fundão 2.4	84	956 ± 7	< LQ	15103 ± 1347	< LQ	16 ± 1.1	178 ± 11	25.2 ± 1.3	5016 ± 428	9.3 ± 0.8
Fundão 2.5	85	1557 ± 68	< LQ	16191 ± 282	< LQ	13.5 ± 0.6	162 ± 10	29.6 ± 0.3	3905 ± 56	17.6 ± 0.4
Fundão 3.1	84	1013 ± 80	12.2 ± 1.1	10432 ± 238	< LQ	8.5 ± 0.6	176 ± 15	46.8 ± 1.1	3937 ± 382	88.1 ± 3.4
Fundão 3.2	83	925 ± 60	30.4 ± 0.9	19451 ± 510	< LQ	7.5 ± 0.2	148 ± 7	120.7 ± 3.8	4174 ± 76	39.4 ± 1.7
Fundão 3.3	84	1640 ± 95	10.4 ± 0.2	21271 ± 305	4.8 ± 0.3	11.3 ± 0.4	138 ± 14	265.6 ± 4	4331 ± 83	79.2 ± 1.6
Fundão 3.4	83	1509 ± 159	12 ± 0.3	25529 ± 418	3.8 ± 0.1	10.5 ± 0.3	154 ± 22	146.8 ± 3	3859 ± 37	23.8 ± 0.7
Fundão 3.5	83	1105 ± 68	23.3 ± 0.1	13298 ± 137	< LQ	12.7 ± 0.3	136 ± 4	35.4 ± 0.7	3078 ± 35	43.1 ± 1
Fundão 4.1	83	< LQ	< LQ	12297 ± 368	< LQ	10.9 ± 0.7	162 ± 6	57.2 ± 2.6	4854 ± 308	4.0 ± 0.3
Fundão 4.2	81	< LQ	< LQ	7048 ± 245	4.1 ± 0.3	< LQ	136 ± 8	56.9 ± 2.8	3624 ± 45	7.6 ± 0.7
Fundão 4.3	81	1052 ± 107	< LQ	8198 ± 437	< LQ	< LQ	156 ± 15	14.3 ± 0.7	3644 ± 192	4.0 ± 0.3
Fundão 4.4	81	1241 ± 124	< LQ	5629 ± 73	3.4 ± 0.1	<LD	140 ± 13	52.2 ± 0.8	3513 ± 36	5.9 ± 0.2
Fundão 4.5	81	1068 ± 83	< LQ	8093 ± 36	1.8 ± 0.2	<LD	127 ± 8	33.2 ± 0.2	3396 ± 51	<LQ
Fundão 5.1	83	902 ± 87	13.7 ± 1.1	14938 ± 1314	<LD	10.1 ± 1	137 ± 10	37.6 ± 3.4	5931 ± 496	22.2 ± 1.6
Fundão 5.2	84	977 ± 42	15.6 ± 0.9	14529 ± 514	< LQ	9.2 ± 0.3	143 ± 10	22.7 ± 0.7	4821 ± 177	27.8 ± 1.4
Fundão 5.3	85	< LQ	9.7 ± 0.9	29623 ± 1421	4.2 ± 0.3	16.2 ± 0.5	177 ± 19	40.6 ± 2.2	7542 ± 331	33.6 ± 1.3
Fundão 5.4	83	1119 ± 29	< LQ	25378 ± 178	3.7 ± 0.3	9.9 ± 0.1	141 ± 12	19.9 ± 0.4	4709 ± 22	30 ± 0
Fundão 5.5	81	1113 ± 2	33.8 ± 1.9	18326 ± 532	5.2 ± 0.1	8.0 ± 0.2	137 ± 4	26.4 ± 1.3	5394 ± 154	59 ± 1.6

Table A3. Elemental concentrations, Sr isotope ratios and moisture content in Rocha Pears from the region of Fundão. (Continuation)

Sample ID	Mn	Mo	Ni	Pb	Se	Sn	Sr	Tl	Zn
	µg.kg^{-1} (dry weight)								
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Fundão 1.1	2007 ± 46	59 ± 3	195 ± 4	< LD	< LD	< LD	1201 ± 25	49 ± 1	3677 ± 45
Fundão 1.2	1456 ± 48	69 ± 3	162 ± 11	< LD	< LD	< LD	859 ± 33	24 ± 1	2609 ± 64
Fundão 1.3	2269 ± 29	56 ± 1	134 ± 6	19 ± 1	< LD	< LQ	699 ± 3	18 ± 0	3563 ± 272
Fundão 1.4	1909 ± 38	66 ± 1	142 ± 12	20 ± 1	< LD	< LD	960 ± 21	27 ± 0	3572 ± 61
Fundão 1.5	1373 ± 57	59 ± 3	210 ± 16	19 ± 0	< LD	< LD	1047 ± 51	21 ± 1	3786 ± 203
Fundão 2.1	1680 ± 207	80 ± 9	260 ± 17	< LD	< LD	< LD	1303 ± 165	32 ± 0	3658 ± 49
Fundão 2.2	1339 ± 2	88 ± 1	144 ± 6	< LD	< LD	< LD	1601 ± 5	33 ± 1	3306 ± 80
Fundão 2.3	1489 ± 31	100 ± 5	188 ± 15	< LQ	< LD	< LQ	2318 ± 57	37 ± 2	5531 ± 172
Fundão 2.4	2238 ± 132	126 ± 8	293 ± 1	< LQ	< LD	< LD	1980 ± 180	28 ± 2	6120 ± 99
Fundão 2.5	1456 ± 3	79 ± 3	178 ± 10	< LD	< LQ	< LQ	1341 ± 13	37 ± 0	5510 ± 478
Fundão 3.1	6319 ± 262	84 ± 12	269 ± 35	< LQ	< LD	< LQ	1121 ± 40	7 ± 0	4876 ± 427
Fundão 3.2	5189 ± 243	104 ± 5	237 ± 7	< LQ	< LD	< LD	526 ± 37	15 ± 0	5223 ± 152
Fundão 3.3	11289 ± 426	60 ± 4	414 ± 21	< LD	< LQ	< LQ	939 ± 35	25 ± 1	6532 ± 46
Fundão 3.4	6474 ± 349	86 ± 1	361 ± 7	< LQ	< LD	< LQ	797 ± 24	20 ± 1	6119 ± 602
Fundão 3.5	5936 ± 61	170 ± 7	310 ± 2	< LD	< LQ	< LQ	719 ± 23	19 ± 0	6698 ± 74
Fundão 4.1	2284 ± 90	22 ± 1	106 ± 5	< LD	< LD	< LD	2048 ± 36	4 ± 0	4515 ± 180
Fundão 4.2	1787 ± 80	54 ± 4	84 ± 3	< LD	< LQ	< LD	2203 ± 250	15 ± 1	4197 ± 105
Fundão 4.3	1596 ± 63	44 ± 1	71 ± 4	< LD	< LQ	< LD	2106 ± 81	11 ± 1	4082 ± 190
Fundão 4.4	2035 ± 12	38 ± 3	64 ± 1	< LD	< LQ	< LD	2326 ± 22	11 ± 0	3853 ± 87
Fundão 4.5	2600 ± 63	55 ± 3	108 ± 15	25 ± 1	< LQ	< LD	2981 ± 13	8 ± 1	4166 ± 421
Fundão 5.1	1931 ± 123	111 ± 9	154 ± 10	< LD	< LQ	< LQ	758 ± 51	43 ± 3	5016 ± 514
Fundão 5.2	2175 ± 95	151 ± 7	176 ± 2	< LD	< LQ	< LQ	790 ± 9	100 ± 1	5241 ± 162
Fundão 5.3	3554 ± 163	156 ± 10	292 ± 29	< LQ	< LQ	< LD	649 ± 16	47 ± 2	5986 ± 203
Fundão 5.4	2456 ± 4	106 ± 6	139 ± 3	< LQ	< LQ	< LD	622 ± 6	59 ± 0	3749 ± 89
Fundão 5.5	3490 ± 364	95 ± 10	238 ± 19	65 ± 7	< LQ	< LD	898 ± 82	114 ± 3	6411 ± 467

Table A3. Elemental concentrations, Sr isotope ratios and moisture content in Rocha Pears from the region of Fundão. (Continuation)

Sample ID	Ca	Fe	K	Mg	Na	P	^{86}Sr/^{87}Sr
	mg.(100g)$^{-1}$ (dry weight)						
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Fundão 1.1	26.8 ± 0.4	0.66 ± 0.01	697 ± 8	37.5 ± 0.5	< LD	49.5 ± 0.4	0.71756
Fundão 1.2	21.9 ± 0.7	0.58 ± 0.01	798 ± 37	28.7 ± 0.6	< LD	45.0 ± 0.9	0.71626
Fundão 1.3	21.6 ± 0.4	0.45 ± 0.00	729 ± 3	26.7 ± 0	< LD	46.3 ± 0.2	0.71635
Fundão 1.4	24.2 ± 0.4	0.54 ± 0.03	720 ± 3	35.3 ± 0.3	< LD	44.6 ± 0.5	0.71674
Fundão 1.5	21.9 ± 1	0.71 ± 0.04	732 ± 17	27.3 ± 1.3	< LD	47.1 ± 1.9	n.d.
Fundão 2.1	31.3 ± 0.3	0.63 ± 0.01	706 ± 15	38.3 ± 1	< LD	52.6 ± 0.4	0.72610
Fundão 2.2	38.2 ± 0.6	0.55 ± 0.01	723 ± 13	39.4 ± 0.5	< LD	54.4 ± 0.5	0.72819
Fundão 2.3	70.8 ± 2.2	0.5 ± 0.02	762 ± 30	49.8 ± 1.6	< LD	68.1 ± 2.6	0.72877± 0.00004
Fundão 2.4	56.6 ± 1.9	0.7 ± 0.09	786 ± 13	49.8 ± 1.5	< LD	68.3 ± 1.6	0.72705
Fundão 2.5	37.6 ± 1.6	0.5 ± 0.01	741 ± 11	39.9 ± 0.2	< LD	60.6 ± 1.5	0.72727
Fundão 3.1	35.5 ± 1.1	0.6 ± 0.05	807 ± 6	29.1 ± 0.3	< LD	46.2 ± 3.0	n.d.
Fundão 3.2	23.6 ± 1	0.86 ± 0.03	985 ± 23	30.9 ± 0.4	< LD	63.2 ± 1.1	0.721373
Fundão 3.3	30.9 ± 0.8	0.65 ± 0.02	787 ± 32	35.6 ± 1.3	< LD	52.7 ± 1.3	0.72294 ± 0.00005
Fundão 3.4	25.3 ± 0.8	0.56 ± 0.03	692 ± 24	26.5 ± 0.7	< LD	49.1 ± 2.0	0.72443 ± 0.00003
Fundão 3.5	31.3 ± 0.4	0.59 ± 0.00	988 ± 13	38.9 ± 0.1	< LD	52.6 ± 1.2	0.722986
Fundão 4.1	20.9 ± 0.8	0.75 ± 0.02	779 ± 19	28.6 ± 1.5	< LD	55.7 ± 3.4	0.708895
Fundão 4.2	24.5 ± 0.5	0.61 ± 0.04	904 ± 9	37.3 ± 0.4	< LD	60.6 ± 3.2	0.709044
Fundão 4.3	24.7 ± 0.7	0.49 ± 0.01	959 ± 57	29.3 ± 1.1	< LD	59.5 ± 2.8	0.709099
Fundão 4.4	27.4 ± 0.5	0.73 ± 0.01	899 ± 7	38.4 ± 0.1	< LD	50.3 ± 1.1	0.709428
Fundão 4.5	39.8 ± 1.4	0.80 ± 0.00	984 ± 4	46.4 ± 0.2	< LD	61.3 ± 4.0	0.709874
Fundão 5.1	30.5 ± 2.1	0.45 ± 0.03	1101 ± 64	37.3 ± 2.4	< LD	60.4 ± 5.2	0.71415 ± 0.00002
Fundão 5.2	29.8 ± 0.3	0.55 ± 0.01	1162 ± 26	40.3 ± 0.9	< LD	67.8 ± 1.2	0.71528 ± 0.00009
Fundão 5.3	28.6 ± 1.2	0.78 ± 0.04	1257 ± 49	45.5 ± 1.3	< LD	80.7 ± 3.7	0.71623
Fundão 5.4	26.4 ± 0.2	0.42 ± 0.02	890 ± 3	26.6 ± 0.8	< LD	58.4 ± 1.2	0.71686 ± 0.00007
Fundão 5.5	26.5 ± 0.5	0.52 ± 0.01	1063 ± 67	30.2 ± 0.4	< LD	69.8 ± 0.8	0.71828

n.d. – not determined. Repeatability for ^{86}Sr/^{87}Sr in pears was estimated on independent triplicates and evaluated as RSD (RSDmax = 119 ppm).

Appendix 3 – Elemental concentrations and isotope ratios in wine samples

Table A4.Elemental concentrations in Port wines.

Sample ID	Ag	Al	As	B	Be	Cd	Co	Cr	Cu	Li
	$\mu g.L^{-1}$									
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
P1	<LD	1043 ± 16	5.8 ± 0.1	3442 ± 11	<LD	<LD	8.7 ± 0.2	30.8 ± 1.4	332 ± 2	24.9 ± 0.4
P2	<LD	272 ± 21	3.0 ± 0.2	3647 ± 76	<LD	<LD	4.7 ± 0.1	37.7 ± 0.6	160 ± 10	22.3 ± 0.6
P3	<LD	148 ± 2	2.3 ± 0.1	4406 ± 140	<LD	<LD	8.6 ± 0.2	35.7 ± 2.6	43 ± 3	13.8 ± 0.5
P4	<LD	<LQ	1.52 ± 0.03	5144 ± 126	<LQ	<LD	8.0 ± 0.2	37.2 ± 5.4	90 ± 1	25.8 ± 0.7
P5	<LD	149 ± 5	2.4 ± 0.1	4819 ± 77	<LQ	<LD	7.4 ± 0.3	33 ± 4.9	86 ± 16	15.5 ± 0.1
P6	<LD	298 ± 3	3.1 ± 0.1	4423 ± 119	<LQ	<LD	7.3 ± 0.2	52.4 ± 7.4	126 ± 5	27.4 ± 0.9
P7	<LD	<LQ	1.6 ± 0.1	4394 ± 44	<LQ	<LD	3.03 ± 0.03	32.5 ± 4.5	11.6 ± 0.1	15.4 ± 0.3
P8	<LD	276 ± 3	3.4 ± 0.1	5593 ± 105	<LQ	<LD	4.00 ± 0.01	47.7 ± 6	93 ± 3	15.7 ± 0.3
P9	<LD	179 ± 3	3.90 ± 0.01	4197 ± 34	<LQ	<LD	10.1 ± 0.1	51.7 ± 4	149 ± 62	19 ± 0.5
P10	<LD	2697 ± 49	39.7 ± 1.1	2431 ± 58	<LQ	<LQ	14 ± 0.5	52.7 ± 6.6	1716 ± 54	63.4 ± 1.5
P11	<LD	183 ± 5	2.1 ± 0.1	3402 ± 104	<LD	<LD	7.1 ± 0.3	31.3 ± 4.5	56 ± 2	22.3 ± 0.8
P12	<LD	264 ± 6	2.1 ± 0.1	2686 ± 77	<LQ	<LD	3.2 ± 0.1	35.9 ± 4.9	144 ± 118	27.1 ± 1.2
P13	<LD	138 ± 1	1.64 ± 0.04	4960 ± 136	<LD	<LD	3.99 ± 0.04	39 ± 5.7	82 ± 1	22.9 ± 0.6
P14	<LD	128 ± 1	7.8 ± 0.5	3116 ± 16	<LD	<LD	9.2 ± 0.2	44.4 ± 7	189 ± 1	183.8 ± 1.6
P15	<LD	<LQ	13.6 ± 0.2	6424 ± 236	<LQ	<LD	6.8 ± 0.2	45.2 ± 4	58 ± 3	66.6 ± 2.3

Table A4. Elemental concentrations in Port wines. (Continuation)

Sample ID	Mn	Mo	Ni	Pb	Rb	Se	Sn	Sr	Tl	Zn
	$\mu g.L^{-1}$									
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
P1	2192 ± 24	2.8 ± 0.2	26.3 ± 0.2	19.4 ± 1.5	1115 ± 30	<LQ	3 ± 0.1	861 ± 5	<LD	586 ± 9
P2	1745 ± 104	3.30 ± 0.02	16.7 ± 0.5	10.6 ± 0.2	1181 ± 17	3.02 ± 0.04	4 ± 0.1	1227 ± 60	<LD	311 ± 7
P3	3296 ± 58	<LQ	22.9 ± 2.2	<LQ	1286 ± 3	<LQ	<LD	1450 ± 37	<LD	309 ± 7
P4	1239 ± 26	<LQ	20.9 ± 0.4	3.1 ± 0.2	1364 ± 10	<LQ	<LQ	1216 ± 33	<LD	348 ± 9
P5	1031 ± 35	<LQ	21.5 ± 0.4	3.84 ± 0.03	1544 ± 4	4.8 ± 4.6	<LQ	1308 ± 18	<LD	362 ± 4
P6	1510 ± 57	4 ± 0	31 ± 1.8	13.8 ± 0.2	1891 ± 8	<LQ	3.1 ± 0.1	1204 ± 35	<LD	439 ± 15
P7	500 ± 22	<LQ	9.1 ± 0.1	<LQ	926 ± 8	<LQ	<LQ	747 ± 21	<LD	295 ± 3
P8	1399 ± 27	2.9 ± 0.1	26.9 ± 2.6	4.31 ± 0.03	1368 ± 20	<LQ	<LQ	914 ± 6	<LD	353 ± 5
P9	1717 ± 77	<LQ	41.4 ± 4.2	5.2 ± 0.1	1076 ± 11	<LQ	1.53 ± 0.03	1421 ± 58	<LD	309 ± 4
P10	3322 ± 94	4.9 ± 0.4	53.5 ± 1.1	119.9 ± 2.7	1761 ± 27	6.2 ± 0.2	5.7 ± 0.1	1549 ± 35	<LD	1675 ± 41
P11	1462 ± 31	<LQ	18.7 ± 0.6	4.5 ± 0.2	1362 ± 114	<LQ	<LQ	1199 ± 28	<LD	413 ± 11
P12	1288 ± 49	<LQ	11.6 ± 0.3	<LQ	989 ± 11	<LQ	<LD	1314 ± 51	<LD	272 ± 3
P13	1641 ± 14	2.28 ± 0.04	13.6 ± 0.2	<LQ	1471 ± 14	<LQ	<LQ	1720 ± 17	<LD	276 ± 4
P14	2452 ± 74	<LQ	42 ± 1.4	6.5 ± 0.1	2567 ± 17	2.2 ± 0.6	4.7 ± 0.2	1169 ± 30	<LQ	197 ± 2
P15	2040 ± 94	2.2 ± 0.1	44.2 ± 1.8	<LQ	1060 ± 12	<LQ	<LQ	1189 ± 52	<LD	378 ± 11

Table A4. Elemental concentrations in Port wines. (Continuation)

Sample ID	Ca	Fe	K	Mg	Na	P
	mg.L^{-1}					
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
P1	25.7 ± 0.3	3.45 ± 0.03	847 ± 3	93 ± 1	11.6 ± 0.0	146 ± 2
P2	58.5 ± 1.6	2.13 ± 0.06	1058 ± 35	92 ± 3	11.0 ± 0.3	160 ± 6
P3	72.5 ± 5.6	0.44 ± 0.04	866 ± 27	107 ± 5	4.6 ± 0.1	163 ± 6
P4	49.6 ± 1.5	0.56 ± 0.02	872 ± 26	90 ± 3	3.0 ± 0.1	176 ± 6
P5	53.7 ± 0.9	0.59 ± 0.01	704 ± 3	92 ± 2	4.3 ± 0.1	145 ± 2
P6	57.7 ± 1.2	0.58 ± 0.01	1345 ± 25	90 ± 2	14.5 ± 0.3	189 ± 4
P7	42.3 ± 0.4	0.26 ± 0.00	935 ± 25	66 ± 2	2.6 ± 0.1	117 ± 2
P8	54.8 ± 0.5	0.55 ± 0.01	1059 ± 26	91 ± 2	4.70 ± 0.05	210 ± 5
P9	37.2 ± 0.6	1.60 ± 0.03	653 ± 13	108 ± 3	7.1 ± 0.2	119 ± 2
P10	127.6 ± 1.1	11.6 ± 0.0	954 ± 24	118 ± 3	16.7 ± 0.2	97 ± 2
P11	38.8 ± 1.0	1.06 ± 0.03	864 ± 20	93 ± 4	6.5 ± 0.2	161 ± 5
P12	55.1 ± 1.2	0.13 ± 0.01	955 ± 39	82 ± 4	6.4 ± 0.1	150 ± 6
P13	68.9 ± 0.7	0.31 ± 0.00	981 ± 26	90 ± 2	3.3 ± 0.1	163 ± 5
P14	46.5 ± 0.7	0.19 ± 0.01	873 ± 11	120 ± 1	9.1 ± 0.2	198 ± 1
P15	43 ± 0.8	0.65 ± 0.02	534 ± 9	147 ± 6	10.7 ± 0.1	189 ± 8

Table A5.Elemental concentrations in Douro wines.

Sample ID	Ag	Al	As	B	Be	Cd	Co	Cr	Cu	Li
	µg.L^{-1}									
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
D1	<LD	395 ± 7	5.5 ± 0.2	5704 ± 548	<LQ	<LD	6.7 ± 0.1	32.3 ± 2.3	31 ± 1	121.6 ± 3.7
D2	<LD	148 ± 16	1.73 ± 0.02	6344 ± 134	<LQ	<LD	5.2 ± 0.05	17.3 ± 0.8	31 ± 1	49.7 ± 1.4
D3	<LD	433 ± 13	4.1 ± 0.1	5245 ± 69	<LD	<LD	8.1 ± 0.1	15.7 ± 0.8	149 ± 7	47.8 ± 0.6
D4	<LD	124 ± 2	1.26 ± 0.05	3721 ± 135	<LQ	<LD	3.51 ± 0.04	16.7 ± 0.1	21 ± 1	19.4 ± 0.6
D5	<LD	857 ± 8	2.46 ± 0.04	3928 ± 77	<LQ	<LD	6.1 ± 0.1	15.2 ± 0.3	11 ± 1	13 ± 0.5
D6	<LD	260 ± 14	2.1 ± 0.1	6026 ± 93	<LQ	<LD	6.8 ± 0.1	31.1 ± 0.7	52 ± 1	30.6 ± 0.3
D7	<LD	639 ± 5	3.5 ± 0.1	2465 ± 46	<LQ	<LD	4.1 ± 0.1	10.3 ± 0.5	6.2 ± 0.4	10.4 ± 0.1
D8	<LD	176 ± 1	<LQ	2798 ± 34	<LQ	<LD	2.03 ± 0.01	12 ± 0.2	21.7 ± 0.2	9.0 ± 0.2
D9	<LD	911 ± 13	1.09 ± 0.01	3451 ± 31	<LQ	<LD	5.6 ± 0.1	9.7 ± 0.7	4.3 ± 0.4	15.1 ± 0.1
D10	<LD	140 ± 1	1.7 ± 0.1	4534 ± 64	<LD	<LD	3.79 ± 0.05	17.7 ± 0.9	51.5 ± 0.0	58.0 ± 0.7
D11	<LD	470 ± 7	5.6 ± 0.1	4220 ± 31	<LD	<LD	3.6 ± 0.1	28 ± 1.6	145 ± 2	59.1 ± 0.7
D12	<LD	257 ± 9	4.3 ± 0.2	6378 ± 81	<LQ	<LD	4.6 ± 0.2	23 ± 0.3	26 ± 2	30.7 ± 1.0
D13	<LD	295 ± 5	3.1 ± 0.1	7303 ± 83	<LQ	<LD	3.17 ± 0.03	21.2 ± 1.1	158 ± 3	82.6 ± 1.1
D14	<LD	185 ± 2	2.1 ± 0.1	8234 ± 333	<LD	<LD	10.3 ± 0.1	15.9 ± 0.8	56 ± 1	87.6 ± 1.2
D15	<LD	172 ± 17	4.6 ± 0.4	7181 ± 20	<LQ	<LD	2.4 ± 0.1	20.0 ± 0.0	12 ± 1	8.4 ± 1.8

Table A5. Elemental concentrations in Douro wines. (Continuation)

Sample ID	Mn	Mo	Ni	Pb	Rb	Se	Sn	Sr	Tl	Zn
	µg.L^{-1}									
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
D1	1793 ± 18	2.27 ± 0.04	40.3 ± 1.0	2.8 ± 0.1	4400 ± 71	2.8 ± 0.2	1.35 ± 0.03	1479 ± 23	<LQ	258 ± 8
D2	2662 ± 56	<LQ	21.4 ± 1.1	3.59 ± 0.02	2922 ± 3	<LQ	<LD	1554 ± 24	<LD	321 ± 1
D3	1672 ± 71	2.5 ± 0.3	37.5 ± 0.8	4.6 ± 0.1	1989 ± 6	<LQ	1.4 ± 0.1	1046 ± 47	<LQ	210 ± 2
D4	1744 ± 39	2.30 ± 0.03	14 ± 0.8	6.5 ± 0.1	1882 ± 53	<LQ	<LQ	1878 ± 52	<LD	285 ± 4
D5	2968 ± 59	<LQ	28.6 ± 0.7	8.7 ± 0.3	870 ± 6	<LQ	<LD	966 ± 12	<LD	349 ± 3
D6	2461 ± 48	<LQ	26.2 ± 0.9	9.9 ± 0.1	2248 ± 11	<LQ	<LQ	1437 ± 37	<LQ	257 ± 3
D7	2525 ± 59	<LQ	14.2 ± 0.5	8.5 ± 0.1	3016 ± 67	<LQ	<LQ	624 ± 19	<LQ	235 ± 3
D8	1446 ± 32	2.48 ± 0.03	25.2 ± 0.9	3.24 ± 0.03	1561 ± 13	<LQ	<LQ	470 ± 12	<LD	290 ± 4
D9	926 ± 11	5.3 ± 0.1	14.5 ± 0.6	16.2 ± 0.4	1166 ± 20	<LQ	1.2 ± 0.1	744 ± 8	<LD	343 ± 6
D10	1808 ± 43	5.3 ± 2.2	24.6 ± 2.9	15.5 ± 0.3	2477 ± 9	<LQ	<LQ	1502 ± 33	<LD	343 ± 2
D11	1631 ± 8	13.9 ± 0.2	29.5 ± 0.3	11.5 ± 0.2	2213 ± 4	<LQ	1.37 ± 0.03	1193 ± 7	<LQ	258 ± 14
D12	2177 ± 74	3.3 ± 0.3	24.3 ± 1.0	3.2 ± 0.01	2032 ± 9	<LQ	2.8 ± 0.02	1593 ± 50	<LD	169 ± 3
D13	1631 ± 33	2.8 ± 0.3	30.2 ± 0.7	19.9 ± 0.4	2074 ± 2	<LQ	3.6 ± 0.1	1061 ± 25	<LD	233 ± 2
D14	1900 ± 13	2.26 ± 0.05	57.2 ± 1.3	21.2 ± 0.2	3176 ± 16	<LQ	<LQ	1777 ± 26	<LQ	435 ± 4
D15	1251 ± 40	3.2 ± 0.3	29 ± 0.5	2.98 ± 0.05	3385 ± 17	<LQ	<LQ	1235 ± 31	<LQ	106 ± 4

Table A5. Elemental concentrations in Douro wines. (Continuation)

Sample ID	Ca	Fe	K	Mg	Na	P
	mg.L^{-1}					
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
D1	62.7 ± 0.8	1.23 ± 0.02	1310.1 ± 24.7	120.5 ± 2.5	13.1 ± 0.1	196 ± 3
D2	56.8 ± 0.3	1.64 ± 0.07	1160.2 ± 49.6	102.3 ± 1.7	6.83 ± 0.05	189 ± 3
D3	60.3 ± 1.3	0.62 ± 0.06	1044.8 ± 5.6	119.9 ± 2.3	16.8 ± 0.2	167 ± 2
D4	61.6 ± 0.4	0.82 ± 0.02	1450.2 ± 32	106.2 ± 1.9	11.1 ± 0.1	215 ± 3
D5	74.6 ± 0.4	0.61 ± 0.01	398.9 ± 6.8	92.4 ± 1.5	18.5 ± 0.1	94 ± 1
D6	65.4 ± 1.0	1.45 ± 0.03	1184.3 ± 13.1	113.4 ± 1.4	10.8 ± 0.1	240 ± 2
D7	75.6 ± 1.4	0.93 ± 0.04	541.6 ± 12.3	82.8 ± 2.9	16.7 ± 0.3	152 ± 4
D8	69.4 ± 0.1	0.57 ± 0.01	541.1 ± 1.5	59.9 ± 0.9	9.43 ± 0.05	139 ± 1
D9	45.3 ± 0.7	1.01 ± 0.01	547.1 ± 4.4	65.3 ± 0.8	32.1 ± 0.4	173 ± 2
D10	54.3 ± 1.1	0.57 ± 0.01	1089.8 ± 13.7	119.5 ± 0.5	6.3 ± 0.1	211 ± 2
D11	62.4 ± 0.9	1.15 ± 0.02	930.5 ± 12.9	110.9 ± 2.2	10.6 ± 0.2	209 ± 4
D12	71.7 ± 2.4	0.77 ± 0.03	1173.2 ± 30.8	107.4 ± 3.3	9.7 ± 0.3	213 ± 6
D13	74.1 ± 1.1	1.66 ± 0.04	1081.8 ± 12.9	104.3 ± 1.3	11.2 ± 0.2	198 ± 3
D14	57.2 ± 0.5	1.34 ± 0.01	1306.8 ± 23.3	122.7 ± 2.6	16.0 ± 0.0	124 ± 3
D15	64.7 ± 1.5	0.95 ± 0.02	1457.9 ± 6.9	100.7 ± 0.8	18.7 ± 0.5	178 ± 1

Table A6.Isotope ratios in wines.

Sample ID	^{208}Pb/^{204}Pb	^{207}Pb/^{204}Pb	^{206}Pb/^{204}Pb	^{208}Pb/^{206}Pb	^{206}Pb/^{207}Pb	^{86}Sr/^{87}Sr	^{11}B/^{10}B	$\delta^{11/}$B (‰)	δ^{18}O (‰)
P1	38.05636	15.62609	18.03855	2.10977	1.15430	0.720871	4.1306	21.5	2.67
P2	37.83922	15.60938	17.87127	2.11734	1.14486	0.721311	4.1127	17.1	5.32
P3	38.10160	15.61648	18.09345	2.10585	1.15859	0.719826	4.0592	3.8	5.16
P4	37.94996	15.61284	17.89988	2.12025	1.14646	0.718450	4.0914	11.8	4.47
P5	37.99714	15.61668	18.02066	2.10861	1.15384	0.719184	4.0645	5.1	6.70
P6	37.87163	15.60661	17.95564	2.10922	1.15048	0.721605	4.0859	10.4	4.77
P7	38.20760	15.64110	18.14584	2.10553	1.16013	0.721006	4.1279	20.8	5.44
P8	38.08857	15.63129	18.11390	2.10275	1.15878	0.720977	4.0794	8.8	4.67
P9	38.02545	15.62197	18.05145	2.10645	1.15551	0.716520	4.1167	18.1	4.23
P10	38.30648	15.63346	18.22645	2.10172	1.16584	0.717119	4.1536	27.2	6.32
P11	38.23020	15.62376	18.09747	2.11244	1.15828	0.719431	4.1039	14.9	5.98
P12	38.28870	15.63033	18.31413	2.09069	1.17166	0.719878	4.1319	21.8	5.53
P13	38.03327	15.60844	18.07270	2.10449	1.15784	0.719487	4.1400	23.8	5.57
P14	37.89678	15.61937	18.17557	2.08501	1.16359	0.721063	4.1442	24.9	6.76
P15	37.60890	15.58701	17.64734	2.13120	1.13212	0.719268	4.0583	3.6	7.27
D1	38.12882	15.62195	18.19232	2.09588	1.16444	0.722788	4.1315	21.7	3.62
D2	38.07888	15.61951	18.10001	2.10378	1.15874	0.720690	4.1100	16.4	6.91
D3	38.20701	15.63190	18.19710	2.09968	1.16403	0.721226	4.0870	10.7	4.98
D4	38.22281	15.64092	18.14732	2.10627	1.16019	0.719688	4.1358	22.8	5.64
D5	38.55951	15.65223	18.50225	2.08416	1.18201	0.717693	4.1269	20.6	5.07
D6	38.16106	15.63490	18.14057	2.10374	1.16020	0.719430	4.1163	18.0	6.71
D7	37.72768	15.60563	17.85428	2.11310	1.14403	0.721094	4.1327	22.0	0.25
D8	38.22242	15.63271	18.26427	2.09271	1.16826	0.719053	4.1098	16.3	6.72
D9	38.47000	15.64658	18.44284	2.08603	1.17864	0.716828	4.0917	11.9	6.28
D10	37.88843	15.60989	17.93892	2.11218	1.14913	0.719694	4.1440	24.8	5.02
D11	38.01596	15.61971	18.04438	2.10683	1.15519	0.718885	4.1237	19.8	5.35
D12	38.00764	15.60738	18.01795	2.10946	1.15441	0.719389	4.1198	18.8	5.24
D13	38.12234	15.62747	18.14145	2.10140	1.16084	0.721455	4.1258	20.3	6.90
D14	38.14671	15.63395	18.13357	2.10371	1.15986	0.720256	4.1790	33.5	4.30
D15	38.16102	15.65301	18.67194	2.04384	1.19278	0.722899	4.1588	28.5	3.41

Repeatability was estimated on independent triplicates and evaluated as RSD. The maximum RSD obtained were as follows: 578 ppm (^{208}Pb/^{204}Pb), 436 ppm (^{207}Pb/^{204}Pb), 316 ppm (^{206}Pb/^{204}Pb), 270 ppm (^{208}Pb/^{206}Pb), 124 ppm (^{206}Pb/^{207}Pb), 48 ppm (^{86}Sr/^{87}Sr), 0.19% (^{11}B/^{10}B). Expanded uncertainty for δ^{18}O (‰) ±0.4‰.

Appendix 4 – Scientific contribution

International publications

Coelho, I., Castanheira, I., Bordado, J. M., Donard, O., & Silva, J. A. L. (2017). Recent developments and trends in the application of strontium and its isotopes in biological related fields. *TrAC Trends in Analytical Chemistry*, *90*, 45–61. https://doi.org/10.1016/j.trac.2017.02.005

Coelho, I., Matos, A. S., Teixeira, R., Nascimento, A., Bordado, J., Donard, O., & Castanheira, I. (2019). Combining multielement analysis and chemometrics to trace the geographical origin of Rocha pear. *Journal of Food Composition and Analysis*, *77*, 1–8. https://doi.org/10.1016/j.jfca.2018.12.005

Coelho, I., Matos, A. S., Epova, E., Barre, J., Cellier, R., Castanheira, I., Bordado, J., Donard, O., (2022). Multi-element and multi-isotopic profiles of Port and Douro wines as tracers for authenticity. (Submitted to Food Chemistry)

National publications

Coelho, I.; Ventura, M.; Bordado, J.; Castanheira, I., (2017) "Análise de B, Cu, Zn, As, Cd e Pb em vinhos portugueses". INSA - Boletim Epidemiológico de Observações, 9 (20), 37-40. http://hdl.handle.net/10400.18/4880.

Scientific meetings*

Coelho, I., Epova E., Barre, J., Castanheira I., Donard, O., (2022) Mineral and isotopic characterization of Wines from the Douro Demarcated Region. NUTRITION 2022 LIVE ONLINE, 14-16 June, American Society Nutrition, Online Event. (Poster presentation)

Donard, O., Cellier, R., Coelho, I., Berail, S., Barre, J., Epova, E., Milcent, S., Ors, P., (2020). Combined non-traditional isotopes for quality, authenticity and high geographical resolution assessments of wines. 5th IMEKOFOODS, September, Czech Republic. (Oral presentation)

Coelho, I., Matos, A., Nascimento A., Bordado, J., Donard, O., Epova E., Barre, J., Berail S., Castanheira I., (2020). Building a database for provenance studies of origin wines from the Douro Valley. 5th IMEKOFOODS, September, Czech Republic. (Poster presentation)

Donard, O., Berail, S., Epova, E., Barre, J., Cellier, R., Ors, P., Vacchina, V., Seby, F., Medina, B., Coelho, I., Castanheira, I., (2020) High Resolution Geographical Provenance Determination of Wines With Combined Non – Traditional Isotopic Signatures. 42nd Congress of Vine and Wine, 15-19 July, Genève, Switzerland. (Oral presentation)

Coelho, I., Matos, A., Jesus S., Nascimento A., Bordado, J., Donard, O., Epova E., Berail S., Castanheira I., (2019). Multielement fingerprinting, isotope ratios and chemometrics as tools to trace the geographical origin of wine. 13th IFDC, 14-18 October, Lisbon, Portugal. (poster presentation)

Coelho, I., Matos, A., Nascimento A., Bordado, J., Donard, O., Epova E., Berail S., Castanheira I., (2019). Tracing the geographical origin of food products with multielement fingerprinting, isotope ratios and chemometrics: the case of Rocha pear. European Winter Conference on Plasma Spectrochemistry, 3-8 February, Pau, France (poster presentation)

Coelho, I., Gueifão, S., Ventura, M., Castanheira, I., (2018). Contaminantes alimentares de origem ambiental: percurso, exposição e monitorização Seminário: Agricultura inteligente para o equilíbrio ambiental, económico e social ANIPLA, 27 November, Coimbra, Portugal. (Oral presentation)

Coelho, I., Matos, A., Teixeira, R., Nascimento, A., Bordado, J., Donard, O., Castanheira I., (2018). Análise multielementar e quimiometriana autenticidade de alimentos: Pera Rocha. Encontros com ciência no DAN, 28 September, Lisbon, Portugal. (Oral presentation)

Coelho, I., Matos, A., Teixeira, R., Nascimento, A., Bordado, J., Donard, O., Castanheira I., (2018). Tracing the geographical origin of PDO products with multielement fingerprinting and chemometrics: The case of Rocha pear. 1st International Meeting on Innovation& Development in the Food Sector, 5 June, Viseu, Portugal (Oral presentation)

Coelho, I., Rego, A., Teixeira, R., Nascimento, A., Bordado, J., Santos, R., Castanheira, I., (2017). ´Rocha' pear antioxidant and mineral characterization. 12th International Food Data Conference (IFDC), 11-13 October, Buenos Aires, Argentina. (Oral presentation)

Castanheira, I., Coelho, I., Teixeira, R., Ventura, M., (2017). Development of a digestion method to assist multi-element determination in Rocha pear by ICP-MS. 3rd IMEKOFOODS, 1 – 4 October, Thessaloniki, Greece. (poster presentation)

*the presenting author is underlined.

Appendix 5 - *Curriculum Vitae*

Inês Alexandra Terra Viana Ribeiro Coelho

Inês Ribeiro Coelho completed the degree in Engenharia Agronómica in 2004 by the Universidade de Lisboa Instituto Superior de Agronomia. Is finishing the PhD program in Chemistry in co-tutelle between the University of Lisbon - Instituto Superior Técnico and the University of Pau under the theme "Advanced analytical strategies in inorganic and isotopic analysis for the authentication of food products in Portugal". Is Head of the Laboratory of Reference Materials of the National Institute of Health since 2018. Is Quality Manager of the Laboratory of Reference Materials since 2015. Published 39 articles in journals. Published 1 book section(s). Organized 3 scientific events. Participates and/or participated in 15 national and/or international projects. Currently, she is PI of the project ILiFOOD, ref EXPL/CTA-AMB/0977/2021. Is the Portuguese deputy for Metrofood-PP since 2019, as well as the Workpackage leader and Task leader in Metrofood-PP. Works mainly in the area(s) of Bio-inorganic chemistry, Chemical speciation of arsenic in foodstuffs, Assessment of trace element composition of foodstuffs, Development of Reference Materials and in-house quality control materials and Food authenticity.

PERSONAL INFORMATION

Inês Alexandra Terra Viana Ribeiro Coelho

📍 Instituto Nacional de Saúde Dr Ricardo Jorge. Av. Padre Cruz, 1649-016 Lisboa, Portugal

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✉ ines.coelho@insa.min-saude.pt

Sex Female | Date of birth 19/06/1979 | Nationality Portuguese

POSITION

Head of the Laboratory of Reference Materials

National Institute of Health Dr Ricardo Jorge, Department of Food Safety and Nutrition

WORK EXPERIENCE

11/2018–Present **Head of the Laboratory of Reference Materials**

National Institute of Health Dr Ricardo Jorge, Department of Food Safety and Nutrition, Lisbon (Portugal)

01/2015–Present **Quality Manager of the Laboratory of Reference Materials**

National Institute of Health Dr Ricardo Jorge, Department of Food Safety and Nutrition, Lisbon (Portugal)

07/2006–Present **Senior Laboratory Technician**

National Institute of Health Dr Ricardo Jorge, Department of Food Safety and Nutrition, Lisbon (Portugal)

Production of reference materials. Analysis of foodstuffs using liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS) techniques.

01/2010–01/2015

Quality Manager Substitute of the Laboratory of Reference Materials

National Institute of Health Dr Ricardo Jorge, Department of Food Safety and Nutrition, Lisbon (Portugal)

03/2008–05/2008

Trainee in chemical speciation (HPLC-ICP-MS)

University of Cincinnati, Department of Chemistry, Cincinnati (United States)
Chemical speciation using HPLC-ICP-MS techniques

11/2007–12/2007

Trainee in chemical speciation (HPLC-ICP-MS)

Complutense University, Department of chemistry, Madrid (Spain)
Chemical speciation using HPLC-ICP-MS techniques

09/2004–05/2005

Professional Trainee

Instituto da Vinha e do Vinho, Lisbon (Portugal)
Ampelographic characterization of portuguese wine varieties

EDUCATION AND TRAINING

10/2005–07/2006

Post-graduation

Instituto Nacional de Administração Pública, Lisboa (Portugal)

Advanced studies in public management.

09/1998–07/2004

Agronomic Engineer

Instituto Superior de Agronomia, Universidade Técnica de Lisboa

Ongoing

PhD student in Chemistry

Instituto Superior Técnico, University of Lisbon. University of Pau and Pays de l'Adour.

PERSONAL SKILLS

Organisational / managerial skills

- leadership (currently responsible for a team of 7 people)
- Good laboratory organisational skills

Job-related skills

- good command of quality management processes (currently responsible for quality management system)

Digital skills

- good command of office suite (word processor, spread sheet, presentation software)
- basic command of access

ADDITIONAL INFORMATION

Projects

2022/01/01 - 2024/12/31	Edible insects: From a sustainable food production to a food safety concern. PTDC/CTA-AMB/0730/2021
2022/01/01 - 2023/06/30	Lithium in food: The impact of lithium mining. – Principal Investigator EXPL/CTA-AMB/0977/2021
2021 - 2023	Alternative protein sources in the European diets – integrating health risk-benefit and sustainability GP/EFSA/ENCO/2020/03 - GA 2
2018/09/19 – 31/05/2022	METROFOOD-RI Preparatory Phase Project - Deputy for the Portuguese Node; Work package leader; Task leader info:eu-repo/grantAgreement/EC/H2020/871083/EU
2018/09/19 - 2022/09/18	Exploring the potential of bioactive nutrients for the prevention of Alzheimer's disease and cognitive aging through in vitro assays, in vivo verification, and in silico quantification of health benefits (NewFood4Thought) PTDC/SAU-NUT/30455/2017
2018/05/01 – 2021/04/30	SEAFOODfortomorrow - Nutritious, safe and sustainable seafood for consumers of tomorrow
2017/12/01 - 2020/06/30	MISAGE - Mitigation strategies of acrylamide and advanced glycation end-products in bread. LISBOA-01-0145-FEDER-024172
2017/01/01 - 2017/12/31	Progressing towards the construction of METROFOOD-RI (PRO-METROFOOD) info:eu-repo/grantAgreement/EC/H2020/739568/EU
2012/02/01 – 2016	Total Diet Study Exposure info:eu-repo/grantAgreement/EC/FP7/289108/EU
2012 – 2015	ORQUE SUDOE - Observatoire de Recherche sur la Qualité de l'Environnement du Grand Sud-Ouest Européen SOE3/P2/F591
2012 – 2015	Liracork – Ligantes Inócuos para Rolhas e Aglomerados de Cortiça QREN – projecto nº: 2009/005012
2012-2014	VALCAP - Valorização da Casca do Arroz Português ValCap 23290
2010/02/01 - 2013/12/31	Benefícios e riscos associados ao consumo de produtos da pesca: Uma análise de benefício-risco baseada na abundância e bioacessibilidade de n-3 PUFA e Selénio, Mercúrio e Arsénio em produtos crus e cozinhados (GOODFISH) PTDC/SAU-ESA/103825/2008
2010/05/01 - 2013/10/31	HERA - Avaliação de risco ambiental de um ambiente estuarino contaminado: um estudo de caso PTDC/SAU-ESA/100107/2008
2004 – 2008	LABORATÓRIO DE MATERIAIS DE REFERÊNCIA PARA A SEGURANÇA ALIMENTAR (LMARSA) POR-3.1.015/DRELVTde Economia

Publications

Refereed articles

1. A. Rego, I. Coelho, C. Motta, C. Cardoso, A. Gomes-Bispo, C. Afonso, J.A. M. Prates, N.M. Bandarra, J.A.L. Silva, I. Castanheira "Seasonal variation of chub mackerel (*Scomber colias*) selenium and vitamin B12 content and its potential role in human health". (2022). Journal of Food Composition and Analysis. 109 https://doi.org/10.1016/j.jfca.2022.104502
2. David M. Ribeiro, Cristina M. Alfaia, José M. Pestana, Daniela F, P. Carvalho, Mónica Costa, Cátia F. Martins, José P. C. Lemos, Miguel Mourato, Sandra Gueifão, Inês Delgado, Patrícia Carvalho, Diogo Coelho, Inês Coelho, João P. B. Freire, André M. Almeida, José A. M Prates "Influence of Feeding

Weaned Piglets with Laminaria digitata on the Quality and Nutritional Value of Meat” (2022) Foods. https://doi.org/10.3390/foods11071024

3. Mónica M. Costa; José M. Pestana; Diogo Osório; Cristina M. Alfaia; Cátia Falcão Martins; Miguel Pedro Mourato; Sandra Gueifão; Andreia Rego; Inês Coelho; Diogo Coelho, et al. “Effect of Dietary Laminaria digitata with Carbohydrases on Broiler Production Performance and Meat Quality, Lipid Profile, and Mineral Composition” (2022). Animals DOI: 10.3390/ani12081007. https://www.mdpi.com/2076-2615/12/8/1007
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Contaminantes alimentares de origem ambiental: percurso, exposição e monitorização. 2018. Seminário: Agricultura inteligente para o equilíbrio ambiental, económico e social

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Book chapter

Morgado, Cátia; Bernardo, Patrícia; Henriques, Inês; Jesus, Susana; Rego, Andreia; Delgado, Inês; Coelho, Inês; et al. "Effect of Rocha pear peel extracts added to wheat and rye bread formulations on acrylamide reduction and sensory quality maintenance". Springer International Publishing, 2019. 10.1007/978-3-030-30938-1_15

Event organisation

8º Simpósio para a promoção de uma alimentação saudável e segura - SPASS 2021, subordinado ao tema da "Alimentação vegetariana: desafios e recomendações" (2021/11/22 - 2021/11/22) - Symposium (Member of the Organising Committee)

13th International Food Data Conference (2019/10/15 - 2019/10/18) - Conference (Member of the Organising Committee)

3º Simpósio Nacional "Promoção de uma Alimentação Saudável e Segura - SPASS 2016", subordinado ao tema "Contaminantes de Preocupação Emergente: Desafios Laboratoriais" (2016/11/14 - 2016/11/14) - Symposium (Member of the Organising Committee)