PhD in Chemical Sciences and Technologies
Cycle XXXVI
Scientific Disciplinary Sector: CHIM/01

ELEMENTAL METABOLOMICS
New Analytical Tools for Food Valorization and Authentication

PhD Student — Andrea Mara

Coordinator — Prof. Carla Cannas

Supervisor — Prof. Gavino Sanna

Academic Year 2022– 2023

Thesis defense: May 2024 Session

SUMMARY

FOOD AUTHENTICATION AND VALORIZATION BY ELEMENTAL METABOLOMICS

1. OMICS SCIENCES IN FOOD ANALYSIS

In an interconnected world, globalization has created both opportunities and challenges for food producers (Steffen et al., 2015). The network has facilitated the international exchange of food supplies, giving consumers greater choice and access to food products throughout the year, while providing producers more opportunities and business (Kearney, 2010). However, the distribution of food resources remains a critical issue (Kearney, 2010). Similarly, differences in food safety regulations make it difficult to monitor product safety and have high quality standards (Godfray et al., 2010). Finally, the high production and waste of food resources have made the system unsustainable (Hoekstra & Wiedmann, 2014). Based on this evidence, sustainability and food quality are the two most important aspects and challenges for the future of the food industry (Godfray et al., 2010; Hu et al., 2019; Nosratabadi et al., 2019).

Consumer preferences suggest that food should be sustainable and derived from a circular economy with a low environmental impact (Lewandowski, 2016). Simultaneously, the food produced should be of high quality and nutritional value, while also being safe and free of toxic substances (Godfray et al., 2010; Siró et al., 2008). Additionally,it must be traceable and authentic to safeguard local productions and consumers from food frauds (Kendall et al., 2019).

Challenges in this framework involve researching in new feeding or cultivation methods and recovery of production waste and by products (Lambin & Meyfroidt, 2011; Naylor et al., 2000; Tuck et al., 2012). On the other hand, new analytical methods are required to monitor food quality, safety and authenticity (Danezis et al., 2016; Hu et al., 2019).

Among the possible approaches, Foodomics is one of the most promising disciplines because it combines omics analysis with food science (Valdés et al., 2022). By using omics sciences (e.g. genomics (Kumar et al., 2022), transcriptomics (Balkir et al., 2021), lipidomics (Mahrous et al., 2023), proteomics (Afzaal et al., 2022), and metabolomics (Li et al., 2021)) integrated with advanced statistical

methodologies, food can be investigated in terms of quality, safety, traceability, and processing (Valdés et al., 2022). Lipids, proteins, and metabolites in foods are related to the health status and genetics of the animals or plants from which they originate. Similarly, nutrition, cultivation and environment also have a strong bearing on the omics profile of a food (Herráiz-Gil et al., 2023). On the other hand, the composition of food is also related to all trasformation and operations prior to sale to consumers (e.g., cooking, processing, storage, or packaging) (S. Ali et al., 2023). The correlation between all these aspects and the food composition allows for an overall authentication, ascertaining quality and safety (Balkir et al., 2021), tracing origin (Chien et al., 2023), and enhancing the presence of metabolites of nutraceutical interest (Ortea, 2022).

Mass spectrometry-based analytical techniques are widely used in omic sciences and foodomics (Zaikin & Borisov, 2021). These techniques have a wide range of applications depending on the ionization source, such us electron ionization (EI), electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and ion analyzer, such us Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS), orbital ion trap Orbitrap (LTQ, Orbitrap), combination of quadrupole and time-of-flight mass spectrometer mass analyzers (QTOF), and ion mobility mass spectrometry (MS-IM) (Zaikin & Borisov, 2021). These techniques can be combined with other separation techniques such as gas gaschromatrography (GC/MS) (Aspromonte et al., 2023; Esteki et al., 2020) and liquid chromatrography (LC/MS) (Muguruma et al., 2022; Perini & Bontempo, 2022).

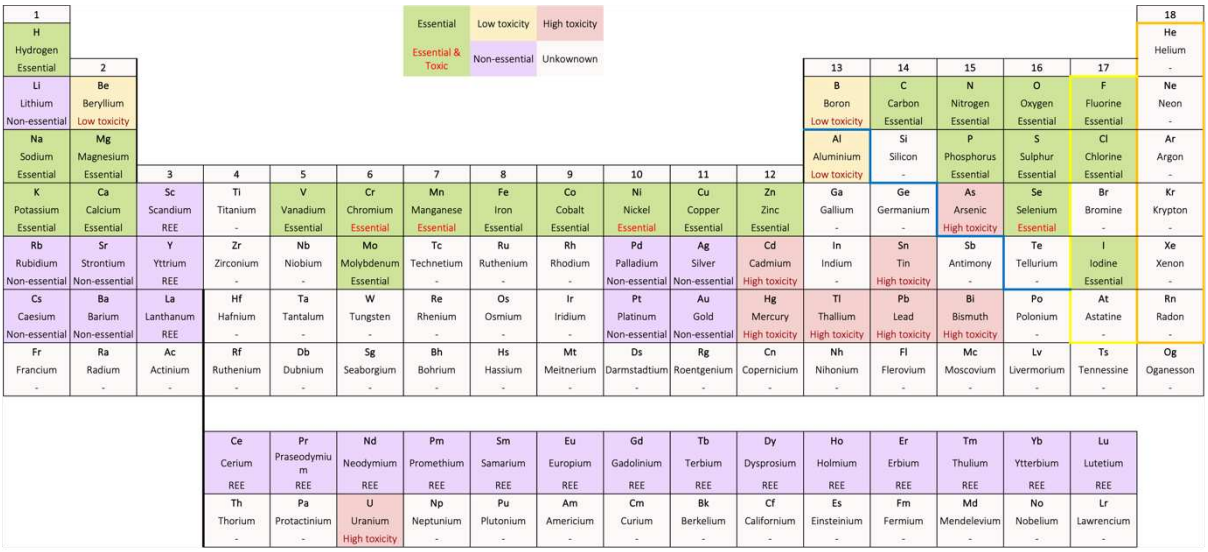
All these analytical methods can be perfomed to investigate food composition, from a quantitative or qualitative point of view. Basically, an analysis can results in a targeted or untargated screening, respectevely (Creydt & Fischer, 2020). The general workflow involves a first untargated approach to evaluate the compounds or markers that can discriminate food depending on the factor of interest (e.g. quality, safety, processing, geografical origin etc.) (S. Ali et al., 2023). Finally, the identified markers are determined using a targeted or quantitative approach (Fanelli et al., 2021). The foodomics approach generates a large amount of data that necessitates the use of advanced statistical tools based on multivariate analysis and machine learning (González-Domínguez et al., 2022). These tools

enable the analysis of data by considering all variables simultaneously, which is essential for understanding complex relationships between variables and identifying any hidden patterns or structures in the data (González-Domínguez et al., 2022).

2. ELEMENTAL METABOLOMICS

Metabolomics is one of the most investigated foodomics sciences. Metabolites can serve as biomarkers to monitor the physiological or pathological state of organisms, as therapeutic targets for drug development, and for food authentication (Chen et al., 2023). They include amino acids, proteins, monosaccharides, carbohydrates, nucleic acids, enzymes, lipids, and ionic species like metals and trace elements. Lipidomics and proteomics focus on the composition of lipids and proteins (Afzaal et al., 2022; Mahrous et al., 2023), respectively, while elemental metabolomics studies elements (Zhang et al., 2017).

Elemental metabolomics involves determining total and speciated elements, their distribution, and monitoring changes as a function of internal or external factors. Ionomics and metallomics also involve elemental analysis, but elemental metabolomics provides a more comprehensive view (Zhang et al., 2017). Approximately 96% of the human body is composed of four elements: carbon, hydrogen, oxygen, and nitrogen. These elements are the major components of all living organisms and are not useful for diagnostic purposes. The remaining 3.25% of elements are known as macroelements, which include calcium, chlorine, potassium, magnesium, sodium, phosphorus, and sulfur (Aversa et al., 2016). These elements are crucial for metabolic processes and are therefore of significant nutritional and diagnostic interest. The remaining 0.75% consists of trace or ultratrace elements, including both essential or nutraceutical elements and elements of toxicological interest (Versieck & McCall, 1985) (Figure A).



1																	18
H Hydrogen Essential	2											13	14	15	16	17	He Helium -
Li Lithium Non-essential	Be Beryllium Low toxicity											B Boron Low toxicity	C Carbon Essential	N Nitrogen Essential	O Oxygen Essential	F Fluorine Essential	Ne Neon -
Na Sodium Essential	Mg Magnesium Essential	3	4	5	6	7	8	9	10	11	12	Al Aluminium Low toxicity	Si Silicon -	P Phosphorus Essential	S Sulphur Essential	Cl Chlorine Essential	Ar Argon -
K Potassium Essential	Ca Calcium Essential	Sc Scandium REE	Ti Titanium -	V Vanadium Essential	Cr Chromium Essential	Mn Manganese Essential	Fe Iron Essential	Co Cobalt Essential	Ni Nickel Essential	Cu Copper Essential	Zn Zinc Essential	Ga Gallium -	Ge Germanium -	As Arsenic High toxicity	Se Selenium Essential	Br Bromine -	Kr Krypton -
Rb Rubidium Non-essential	Sr Strontium Non-essential	Y Yttrium REE	Zr Zirconium -	Nb Niobium -	Mo Molybdenum Essential	Tc Technetium -	Ru Ruthenium -	Rh Rhodium -	Pd Palladium Non-essential	Ag Silver Non-essential	Cd Cadmium High toxicity	In Indium -	Sn Tin High toxicity	Sb Antimony -	Te Tellurium -	I Iodine Essential	Xe Xenon -
Cs Caesium Non-essential	Ba Barium Non-essential	La Lanthanum REE	Hf Hafnium -	Ta Tantalum -	W Tungsten -	Re Rhenium -	Os Osmium -	Ir Iridium -	Pt Platinum Non-essential	Au Gold Non-essential	Hg Mercury High toxicity	Tl Thallium High toxicity	Pb Lead High toxicity	Bi Bismuth High toxicity	Po Polonium -	At Astatine -	Rn Radon -
Fr Francium -	Ra Radium -	Ac Actinium -	Rf Ruthenium -	Db Dubnium -	Sg Seaborgium -	Bh Bohrium -	Hs Hassium -	Mt Meitnerium -	Ds Darmstadtium -	Rg Roentgenium -	Cn Copernicium -	Nh Nihonium -	Fl Flerovium -	Mc Moscovium -	Lv Livermorium -	Ts Tennessine -	Og Oganesson -

Ce Cerium REE	Pr Praseodymium REE	Nd Neodymium REE	Pm Promethium REE	Sm Samarium REE	Eu Europium REE	Gd Gadolinium REE	Tb Terbium REE	Dy Dysprosium REE	Ho Holmium REE	Er Erbium REE	Tm Thulium REE	Yb Ytterbium REE	Lu Lutetium REE
Th Thorium -	Pa Protactinium -	U Uranium High toxicity	Np Neptunium -	Pu Plutonium -	Am Americium -	Cm Curium -	Bk Berkelium -	Cf Californium -	Es Einsteinium -	Fm Fermium -	Md Mendelevium -	No Nobelium -	Lr Lawrencium -

Figure A. Periodic table with essential and toxic trace elements.

Trace elements are vital to organisms in small amounts, but can be toxic at concentrations even slightly above those where they show efficacy in metabolic cycles (Versieck & McCall, 1985). Even a slight excess or deficiency can alter physiological processes and cause health problems. Therefore, maintaining an adequate intake of trace elements through a balanced diet is essential for overall health and well-being. These elements play several roles in biological processes (Jomova et al., 2022). For instance, iron is a crucial component of hemoglobin, the protein responsible for transporting oxygen throughout the body (Mégier et al., 2022). Zinc acts as a cofactor for several enzymes involved in metabolism, immune function, and DNA synthesis (Kitala et al., 2023). Copper contributes to connective tissue formation and nervous system function (Liu & Miao, 2022). Selenium is a vital component of antioxidant enzymes and is essential for thyroid function and immune response (Ye et al., 2022). Iodine is essential for synthesizing thyroid hormones that regulate metabolism and growth (Opazo et al., 2022). Manganese acts as a cofactor for enzymes involved in bone formation and antioxidant defense (Wong et al., 2022). Fluorine strengthens tooth enamel and prevents tooth decay (Everett, 2011).

Toxic elements, sometimes incorrectly referred to as “heavy metals”, have no physiological role in organisms and are harmful to them even at low concentrations (Jaishankar et al., 2014). The most common toxic elements are lead (Vallverdú-Coll et al., 2019), mercury (Kim et al., 2016), cadmium

(Charkiewicz et al., 2023), and arsenic (Ozturk et al., 2022). Their impact on plants and animals is dependent on various factors, such as the amount, duration of exposure, and phisyology (Chai & Schachtman, 2022; de Almeida Ribeiro Carvalho et al., 2022; Nag et al., 2022; Yang et al., 2022). The toxicity of many elements can be different depending on their chemical form. For instance, chromium(III) is an essential nutrient involved in glucose metabolism, while chromium(VI) is highly toxic and carcinogenic (De Almeida Rodrigues et al., 2022). Again, inorganic arsenic compounds are more toxic than their organic species (Leermakers et al., 2006). Furthermore, methylmercury is the most toxic form of mercury and is of particular concern due to its bioaccumulation in the food chain (Li et al., 2024). For these reasons, the measurement of the total concentration of a toxic element does not provide reliable information on its actual level of toxicity. In such cases, elemental speciation, i.e. the assessment of the concentrations of the different chemical forms of the toxic present in a given matrix, is the most informative determination. Ingestion and inhalation are the primary routes of intake for toxic elements (Frazzoli et al., 2007; Salim et al., 2023; Yang et al., 2022). Efforts to mitigate their damage to human health and the environment include the adoption of environmental and food regulations and occupational safety measures. Beyond trace elements and toxic elements, there are minority elements that do not have well-established physiological functions, such as rubidium, strontium, some precious metals and the rare earth elements. Their levels show great variability, often related with the environment, making them useful for geographic markers and valuable for food traceability (Danezis & Georgiou, 2022; Magdas et al., 2020).

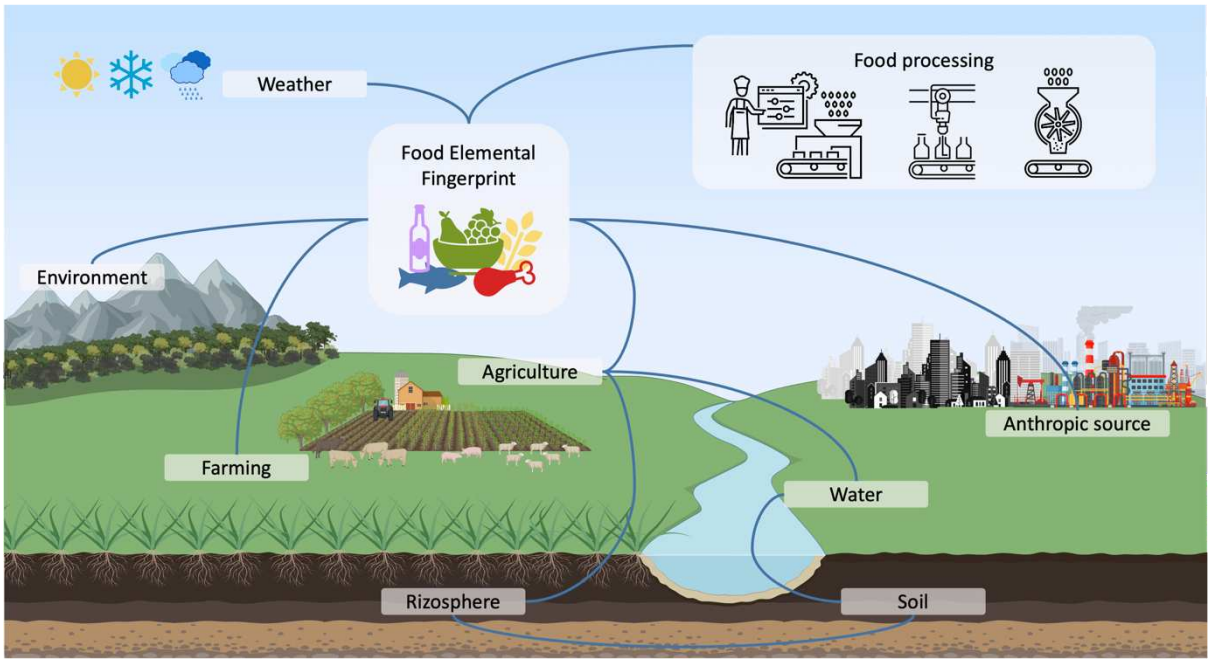


Figure B. Elemental flow from the abiotic to the biotic sphere and factors influencing the elemental fingerprint of foods.

The elemental composition of foods can be influenced by both internal and external factors of the organism (Figure B). Interactions between the abiotic and biotic compartments regulate the flow of elements (Manahan, 2022). The translocation of elements between the lithosphere and hydrosphere are determined by both environmental and climatic conditions (H. Ali et al., 2019). The environment has an impact on the translocation of elements in flora and fauna and, ultimately, on their level of bioavailability (Gupta et al., 2019). The characteristics of the rhizosphere influence the uptake of elements by the plant root system, while their concentration in plant and animal tissues , and thus elemental fingerprints, depends on the relevant physiological characteristics (Antoniadis et al., 2017). Elements in foods are influenced not only by environmental phenomena but also by cultivation and breeding practices (Zhang et al., 2017). The elemental fingerprint of plant foods will vary depending on irrigation techniques and the nature (extensive or intensive) of farming (Christophe et al., 2021; Spanu et al., 2020). In addition, food processing techniques affect the quality and safety of the final product. Based on these considerations, elemental metabolomics has the potential for elemental authentication and valorization (Danezis & Georgiou, 2022; Mazarakioti et al., 2022; Zhang et al., 2017).

3. ELEMENTAL ANALYSIS BY INDUCTIVE COUPLED PLASMA MASS SPECTROMETRY

Measuring the elemental fingerprint of a food requires a versatile technique to analyze many elements simultaneously with the highest accuracy. Amonge the possible analytical methods, inductive coupled plasma mass spectrometry (ICP-MS) ensures the highest performance (Schlemmer et al., 2019). In summary, ICP-MS operates by converting samples into ions in a high-temperature plasma environment, followed by mass separation and detection. The process begins with introducing the sample, typically converted into a thin aerosol or vapor, into the inductively coupled plasma (ICP). In this plasma, which is characterized by temperatures exceeding 10,000 Kelvin, the sample undergoes complete atomization and ionization. This results in the formation of ions that represent the elemental constituents of the sample. The ions then pass through the interface and are directed into the mass spectrometer, where they undergo mass separation based on their mass-to-charge ratio (m/z). Only ions with a specific mass can pass through the mass analyzer and reach the detector, where they generate electrical signals proportional to their abundance. This allows for precise quantification of the elemental composition of the sample. ICP-MS has exceptional sensitivity, capable of detecting elements at ultra-low concentrations, often in the parts per trillion (ppt) range. ICP-MS offers a wide dynamic range, covering concentrations from traces to higher levels, making it suitable for various applications. It is also capable of analyzing multiple elements simultaneously within a single sample, thereby improving efficiency and productivity (Schlemmer et al., 2019). The primary challenges in ICP-MS are isobaric interferences resulting from the creation of polyatomic ions and overlap between the masses of certain isotopes. These interferences can be addressed by utilizing a quadrupole as a collision and reaction cell (CRC) and the KED (kinetic energy discrimination) and DRC (dynamic reaction chamber) analysis modes (Lum & Sze-Yin Leung, 2016). In the former, an inert gas such as helium is used to collide with polyatomic ions and enable discrimination based on kinetic energy. In the second case, a reactive gas such as O_2 is used to react with the analyte (shift-mass) or interfering ion (on-mass) (Yamada, 2015). Although these techniques are well-established, matrix effects in complex

samples may require choosing the appropriate calibration strategy and optimizing sample preparation (Núñez Burcio & Lucci, 2016).

4. SAMPLE PREPARATION FOR FOOD ELEMENTAL ANALYSIS

Sample preparation is a crucial phase in any analytical method. For ICP-MS analysis, it is common to analyze acidic aqueous solutions obtained through ashing or digestion (Schlemmer et al., 2019). The first involves treating samples in muffle furnaces capable of reaching temperatures high enough to completely decompose the organic matrix of the samples. This can be conducted either dry or wet with oxidizing reagents and chemically inert crucibles. On the other hand, the digestionof the sample can be efficiently performed using strongly oxidizing mixtures in microwave-based systems (Flores, 2014). This treatment is carried out in closed vessels, reaching in this way temperatures higher than the boiling points of the oxidizing mixtures. This significantly reduces preparation time and minimizes contamination and the loss of volatile elements. Microwave closed vessel technology is commonly used to prepare food samples for elemental analysis. However, its performance depends on the number of vessels per run, and different matrices cannot be digested simultaneously. In addition, vessel closing and opening operations can be complicate and harmful (Flores, 2014).

A new technology with great potential is the Single Reaction Chamber (SRC). SRC technology differs from traditional microwave digestion because it uses a vessel-into-vessel approach (Nóbrega et al., 2012) (Figure C).

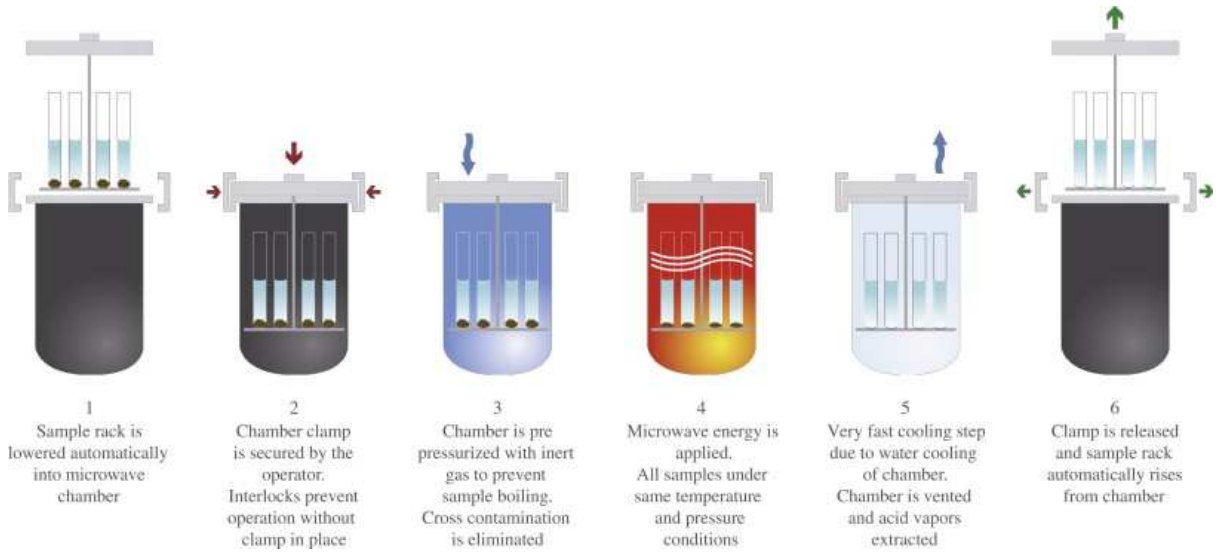


Figure C. Single Reaction Chamber operating sequence **(**Nóbrega et al., 2012**)**.

The vessels containing samples and oxidizing mixtures are not hermetically closed but pressurized with an inert gas (Ar or N_2) inside a larger vessels. The rack containing the sample vessels is immersed in an acidic aqueous solution, ensuring greater heating and temperature uniformity. Additionally, SRC technology simplifies operations and improves operator safety. This technology offers superior performance compared to traditional methods in all aspects. It allows higher temperatures and pressures, simultaneous digestion of different matrices, and, based on matrix reactivity, can treat large amounts of samples with lower volumes of oxidants (Muller et al., 2016). All of these features enable high quality digestion with low residual organic carbon and low acidity. Both parameters are critical because they play a role in the matrix effect in the subsequent ICP-MS analysis. High levels of residual acid and organic carbon reduce the nebulization efficiency of the sample by increasing the viscosity and surface tension of the solution. This can ultimately decrease the amount of sample in the plasma and, consequently, worsen the sensitivity of the analytical method (Nóbrega et al., 2012).

Therefore, the use of SRC technology can help meet the requirements of blank and green analytical chemistry . Achieving high-quality digestions with low residual acidity and organic carbon, using small amounts of oxidants, reduces analysis costs and saves reagents. In addition, higher nebulization efficiency and a lower dilution factor due to lower residual acidity increase the sensitivity of the method making it suitable for the ultra-trace analysis (Muller et al., 2017).

REFERENCES

Afzaal, M., Saeed, F., Hussain, M., Shahid, F., Siddeeg, A., & Al-Farga, A. (2022). Proteomics as a promising biomarker in food authentication, quality and safety: A review. Food Science & Nutrition, 10(7), 2333–2346. https://doi.org/10.1002/fsn3.2842

Ali, H., Khan, E., & Ilahi, I. (2019). Environmental Chemistry and Ecotoxicology of Hazardous Heavy Metals: Environmental Persistence, Toxicity, and Bioaccumulation. Journal of Chemistry, 2019, 1–14. https://doi.org/10.1155/2019/6730305

Ali, S., Rezende, V. T., Ullah, S., De Paiva, E. L., Tonin, F. G., Abdullah, Corassin, C. H., & Oliveira, C. A. F. D. (2023). Food processing and challenges in the food production and quality: The foodomics approach. *Food Bioscience*, *56*, 103217. https://doi.org/10.1016/j.fbio.2023.103217

Antoniadis, V., Levizou, E., Shaheen, S. M., Ok, Y. S., Sebastian, A., Baum, C., Prasad, M. N. V., Wenzel, W. W., & Rinklebe, J. (2017). Trace elements in the soil-plant interface: Phytoavailability, translocation, and phytoremediation–A review. *Earth-Science Reviews*, *171*, 621–645. https://doi.org/10.1016/j.earscirev.2017.06.005

Aspromonte, J., Mascrez, S., Eggermont, D., & Purcaro, G. (2023). Solid-phase microextraction coupled to comprehensive multidimensional gas chromatography for food analysis. *Analytical and Bioanalytical Chemistry*. https://doi.org/10.1007/s00216-023-05048-0

Aversa, R., Petrescu, V., Apicella, A., & Petrescu, I. T. (2016). The Basic Elements of Life's. *American Journal of Engineering and Applied Sciences*, *9*(4), 1189–1197. https://doi.org/10.3844/ajeassp.2016.1189.1197

Balkir, P., Kemahlioglu, K., & Yucel, U. (2021). Foodomics: A new approach in food quality and safety. *Trends in Food Science & Technology*, *108*, 49–57. https://doi.org/10.1016/j.tifs.2020.11.028

Chai, Y. N., & Schachtman, D. P. (2022). Root exudates impact plant performance under abiotic stress. *Trends in Plant Science*, *27*(1), 80–91. https://doi.org/10.1016/j.tplants.2021.08.003

Charkiewicz, A. E., Omeljaniuk, W. J., Nowak, K., Garley, M., & Nikliński, J. (2023). Cadmium Toxicity and Health Effects—A Brief Summary. *Molecules*, *28*(18), 6620. https://doi.org/10.3390/molecules28186620

Chen, D., Zhao, S., Li, L., & Li, L. (2023). Controlling pre-analytical process in human serum/plasma metabolomics. *TrAC Trends in Analytical Chemistry*, *169*, 117364. https://doi.org/10.1016/j.trac.2023.117364

Chien, H., Zheng, Y., Wang, W., Kuo, C., Hsu, Y., & Lai, C. (2023). Determination of adulteration, geographical origins, and species of food by mass spectrometry. *Mass Spectrometry Reviews*, *42*(6), 2273–2323. https://doi.org/10.1002/mas.21780

Christophe, O. S., Grelet, C., Bertozzi, C., Veselko, D., Lecomte, C., Höeckels, P., Werner, A., Auer, F.-J., Gengler, N., Dehareng, F., & Soyeurt, H. (2021). Multiple breeds and countries' predictions of mineral contents in milk from milk mid-infrared spectrometry. *Foods*, *10*(9). Scopus. https://doi.org/10.3390/foods10092235

Creydt, M., & Fischer, M. (2020). Food authentication in real life: How to link nontargeted approaches with routine analytics? *ELECTROPHORESIS*, *41*(20), 1665–1679. https://doi.org/10.1002/elps.202000030

Danezis, G. P., & Georgiou, C. A. (2022). Elemental metabolomics: Food elemental assessment could reveal geographical origin. *Current Opinion in Food Science*, *44*, 100812. https://doi.org/10.1016/j.cofs.2022.100812

Danezis, G. P., Tsagkaris, A. S., Camin, F., Brusic, V., & Georgiou, C. A. (2016). Food authentication: Techniques, trends & emerging approaches. *TrAC - Trends in Analytical Chemistry*, *85*, 123–132. Scopus. https://doi.org/10.1016/j.trac.2016.02.026

de Almeida Ribeiro Carvalho, M., Botero, W. G., & de Oliveira, L. C. (2022). Natural and anthropogenic sources of potentially toxic elements to aquatic environment: A systematic literature review. *Environmental Science and Pollution Research*, *29*(34), 51318–51338. Scopus. https://doi.org/10.1007/s11356-022-20980-x

De Almeida Rodrigues, P., Ferrari, R. G., Kato, L. S., Hauser-Davis, R. A., & Conte-Junior, C. A. (2022). A Systematic Review on Metal Dynamics and Marine Toxicity Risk Assessment Using Crustaceans as Bioindicators. *Biological Trace Element Research*, *200*(2), 881–903. https://doi.org/10.1007/s12011-021-02685-3

Esteki, M., Shahsavari, Z., & Simal-Gandara, J. (2020). Gas Chromatographic Fingerprinting Coupled to Chemometrics for Food Authentication. *Food Reviews International*, *36*(4), 384–427. https://doi.org/10.1080/87559129.2019.1649691

Everett, E. T. (2011). Fluoride's Effects on the Formation of Teeth and Bones, and the Influence of Genetics. *Journal of Dental Research*, *90*(5), 552–560. https://doi.org/10.1177/0022034510384626

Fanelli, V., Mascio, I., Miazzi, M. M., Savoia, M. A., De Giovanni, C., & Montemurro, C. (2021). Molecular Approaches to Agri-Food Traceability and Authentication: An Updated Review. *Foods*, *10*(7), 1644. https://doi.org/10.3390/foods10071644

Flores, É. M. M. (2014). *Microwave-assisted sample preparation for trace element determination*. Elsevier.

Frazzoli, C., D'amato, M., Caroli, S., & Záray, G. (2007). Arsenic and Other Potentially Toxic Trace Elements in Rice. In *The Determination of Chemical Elements in Food* (pp. 383–400). https://doi.org/10.1002/9780470141007.ch12

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M., & Toulmin, C. (2010). Food Security: The Challenge of Feeding 9 Billion People. *Science*, *327*(5967), 812–818. https://doi.org/10.1126/science.1185383

González-Domínguez, R., Sayago, A., & Fernández-Recamales, Á. (2022). An Overview on the Application of Chemometrics Tools in Food Authenticity and Traceability. *Foods*, *11*(23), 3940. https://doi.org/10.3390/foods11233940

Gupta, N., Yadav, K. K., Kumar, V., Kumar, S., Chadd, R. P., & Kumar, A. (2019). Trace elements in soil-vegetables interface: Translocation, bioaccumulation, toxicity and amelioration - A review. *Science of The Total Environment*, *651*, 2927–2942. https://doi.org/10.1016/j.scitotenv.2018.10.047

Herráiz-Gil, S., Del Carmen De Arriba, M., Escámez, M. J., & León, C. (2023). Multi-omic data integration in food science and analysis. *Current Opinion in Food Science*, *52*, 101049. https://doi.org/10.1016/j.cofs.2023.101049

Hoekstra, A. Y., & Wiedmann, T. O. (2014). Humanity's unsustainable environmental footprint. *Science*, *344*(6188), 1114–1117. https://doi.org/10.1126/science.1248365

Hu, K., Liu, J., Li, B., Liu, L., Gharibzahedi, S. M. T., Su, Y., Jiang, Y., Tan, J., Wang, Y., & Guo, Y. (2019). Global research trends in food safety in agriculture and industry from 1991 to 2018: A data-driven analysis. *Trends in Food Science & Technology*, *85*, 262–276. https://doi.org/10.1016/j.tifs.2019.01.011

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary Toxicology*, *7*(2), 60–72. Scopus. https://doi.org/10.2478/intox-2014-0009

Jomova, K., Makova, M., Alomar, S. Y., Alwasel, S. H., Nepovimova, E., Kuca, K., Rhodes, C. J., & Valko, M. (2022). Essential metals in health and disease. *Chemico-Biological Interactions*, *367*, 110173. https://doi.org/10.1016/j.cbi.2022.110173

Kearney, J. (2010). Food consumption trends and drivers. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1554), 2793–2807. https://doi.org/10.1098/rstb.2010.0149

Kendall, H., Clark, B., Rhymer, C., Kuznesof, S., Hajslova, J., Tomaniova, M., Brereton, P., & Frewer, L. (2019). A systematic review of consumer perceptions of food fraud and authenticity: A European perspective. *Trends in Food Science & Technology*, *94*, 79–90. https://doi.org/10.1016/j.tifs.2019.10.005

Kim, K.-H., Kabir, E., & Jahan, S. A. (2016). A review on the distribution of Hg in the environment and its human health impacts. *Journal of Hazardous Materials*, *306*, 376–385. https://doi.org/10.1016/j.jhazmat.2015.11.031

Kitala, K., Tanski, D., Godlewski, J., Krajewska-Włodarczyk, M., Gromadziński, L., & Majewski, M. (2023). Copper and Zinc Particles as Regulators of Cardiovascular System Function—A Review. *Nutrients*, *15*(13), 3040. https://doi.org/10.3390/nu15133040

Kumar, P., Rani, A., Singh, S., & Kumar, A. (2022). Recent advances on DNA and omics-based technology in Food testing and authentication: A review. *Journal of Food Safety*, *42*(4), e12986. https://doi.org/10.1111/jfs.12986

Lambin, E. F., & Meyfroidt, P. (2011). Global land use change, economic globalization, and the looming land scarcity. *Proceedings of the National Academy of Sciences*, *108*(9), 3465–3472. https://doi.org/10.1073/pnas.1100480108

Leermakers, M., Baeyens, W., De Gieter, M., Smedts, B., Meert, C., De Bisschop, H. C., Morabito, R., & Quevauviller, Ph. (2006). Toxic arsenic compounds in environmental samples: Speciation and validation. *TrAC Trends in Analytical Chemistry*, *25*(1), 1–10. https://doi.org/10.1016/j.trac.2005.06.004

Lewandowski, M. (2016). Designing the Business Models for Circular Economy—Towards the Conceptual Framework. *Sustainability*, *8*(1), 43. https://doi.org/10.3390/su8010043

Li, S., Li, Z., Wu, M., Zhou, Y., Tang, W., & Zhong, H. (2024). Mercury transformations in algae, plants, and animals: The occurrence, mechanisms, and gaps. *Science of The Total Environment*, *911*, 168690. https://doi.org/10.1016/j.scitotenv.2023.168690

Li, S., Tian, Y., Jiang, P., Lin, Y., Liu, X., & Yang, H. (2021). Recent advances in the application of metabolomics for food safety control and food quality analyses. *Critical Reviews in Food Science and Nutrition*, *61*(9), 1448–1469. https://doi.org/10.1080/10408398.2020.1761287

Liu, Y., & Miao, J. (2022). An Emerging Role of Defective Copper Metabolism in Heart Disease. *Nutrients*, *14*(3), 700. https://doi.org/10.3390/nu14030700

Lum, T.-S., & Sze-Yin Leung, K. (2016). Strategies to overcome spectral interference in ICP-MS detection. *Journal of Analytical Atomic Spectrometry*, *31*(5), 1078–1088. https://doi.org/10.1039/C5JA00497G

Magdas, D. A., Marincas, O., Cristea, G., Feher, I., & Vedeanu, N. (2020). REEs – a possible tool for geographical origin assessment? *Environmental Chemistry*, *17*(2), 148. https://doi.org/10.1071/EN19163

Mahrous, E., Chen, R., Zhao, C., & Farag, M. A. (2023). Lipidomics in food quality and authentication: A comprehensive review of novel trends and applications using chromatographic and spectroscopic techniques. *Critical Reviews in Food Science and Nutrition*, 1–24. https://doi.org/10.1080/10408398.2023.2207659

Manahan, S. E. (2022). *Environmental Chemistry* (11th ed.). CRC Press. https://doi.org/10.1201/9781003096238

Mazarakioti, E. C., Zotos, A., Thomatou, A.-A., Kontogeorgos, A., Patakas, A., & Ladavos, A. (2022). Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), a Useful Tool in Authenticity of Agricultural Products' and Foods' Origin. *Foods*, *11*(22), 3705. https://doi.org/10.3390/foods11223705

Mégier, C., Peoc'h, K., Puy, V., & Cordier, A.-G. (2022). Iron Metabolism in Normal and Pathological Pregnancies and Fetal Consequences. *Metabolites*, *12*(2), 129. https://doi.org/10.3390/metabo12020129

Muguruma, Y., Nunome, M., & Inoue, K. (2022). A Review on the Foodomics Based on Liquid Chromatography Mass Spectrometry. *Chemical and Pharmaceutical Bulletin*, *70*(1), 12–18. https://doi.org/10.1248/cpb.c21-00765

Muller, E. I., Muller, C. C., Souza, J. P., Muller, A. L. H., Enders, M. S. P., Doneda, M., Frohlich, A. C., Iop, G. D., & Anschau, K. F. (2017). Green microwave-assisted wet digestion method of carbohydrate-rich foods with hydrogen peroxide using single reaction chamber and further elemental determination using ICP-OES and ICP-MS. *Microchemical Journal*, *134*, 257–261. https://doi.org/10.1016/j.microc.2017.06.012

Muller, E. I., Souza, J. P., Muller, C. C., Muller, A. L. H., Mello, P. A., & Bizzi, C. A. (2016). Microwave-assisted wet digestion with H2O2 at high temperature and pressure using single reaction chamber for elemental determination in milk powder by ICP-OES and ICP-MS. *Talanta*, *156–157*, 232–238. https://doi.org/10.1016/j.talanta.2016.05.019

Nag, R., O'Rourke, S. M., & Cummins, E. (2022). Risk factors and assessment strategies for the evaluation of human or environmental risk from metal(loid)s – A focus on Ireland. *Science of The Total Environment*, *802*, 149839. https://doi.org/10.1016/j.scitotenv.2021.149839

Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C. M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., & Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature*, *405*(6790), 1017–1024. https://doi.org/10.1038/35016500

Nóbrega, J. A., Pirola, C., Fialho, L. L., Rota, G., De Campos Jordão, C. E. K. M. A., & Pollo, F. (2012). Microwave-assisted digestion of organic samples: How simple can it become? *Talanta*, *98*, 272–276. https://doi.org/10.1016/j.talanta.2012.06.079

Nosratabadi, S., Mosavi, A., Shamshirband, S., Kazimieras Zavadskas, E., Rakotonirainy, A., & Chau, K. W. (2019). Sustainable Business Models: A Review. *Sustainability*, *11*(6), 1663. https://doi.org/10.3390/su11061663

Núñez Burcio, O., & Lucci, P. (Eds.). (2016). *New trends in sample preparation techniques for food analysis*. Nova Publishers.

Opazo, Ma. C., Coronado-Arrázola, I., Vallejos, O. P., Moreno-Reyes, R., Fardella, C., Mosso, L., Kalergis, A. M., Bueno, S. M., & Riedel, C. A. (2022). The impact of the micronutrient iodine in health and diseases. *Critical Reviews in Food Science and Nutrition*, *62*(6), 1466–1479. https://doi.org/10.1080/10408398.2020.1843398

Ortea, I. (2022). Foodomics in health: Advanced techniques for studying the bioactive role of foods. *TrAC Trends in Analytical Chemistry*, *150*, 116589. https://doi.org/10.1016/j.trac.2022.116589

Ozturk, M., Metin, M., Altay, V., Bhat, R. A., Ejaz, M., Gul, A., Unal, B. T., Hasanuzzaman, M., Nibir, L., Nahar, K., Bukhari, A., Dervash, M. A., & Kawano, T. (2022). Arsenic and Human Health: Genotoxicity, Epigenomic Effects, and Cancer Signaling. *Biological Trace Element Research*, *200*(3), 988–1001. https://doi.org/10.1007/s12011-021-02719-w

Perini, M., & Bontempo, L. (2022). Liquid Chromatography coupled to Isotope Ratio Mass Spectrometry (LC-IRMS): A review. *TrAC Trends in Analytical Chemistry*, *147*, 116515. https://doi.org/10.1016/j.trac.2021.116515

Salim, S. A., Sarraf Ov, N., Dana, Z., Hashami, Z., Afrah, A., Sadeghi, E., & Bashiry, M. (2023). A comprehensive image of environmental toxic heavy metals in red meat: A global systematic review and meta-analysis and risk assessment study. *Science of The Total Environment*, *889*, 164100. https://doi.org/10.1016/j.scitotenv.2023.164100

Schlemmer, G., Balcaen, L., Todolí, J. L., & Hinds, M. W. (2019). *Elemental analysis: An introduction to modern spectrometric techniques*. De Gruyter. https://doi.org/10.1515/9783110501087-201

Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance—A review. *Appetite*, *51*(3), 456–467. https://doi.org/10.1016/j.appet.2008.05.060

Spanu, A., Valente, M., Langasco, I., Leardi, R., Orlandoni, A. M., Ciulu, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2020). Effect of the irrigation method and genotype on the bioaccumulation of toxic and trace elements in rice. *Science of The Total Environment*, *748*, 142484. https://doi.org/10.1016/j.scitotenv.2020.142484

Steffen, W., Broadgate, W., Deutsch, L., Gaffney, O., & Ludwig, C. (2015). The trajectory of the Anthropocene: The Great Acceleration. *The Anthropocene Review*, *2*(1), 81–98. https://doi.org/10.1177/2053019614564785

Tuck, C. O., Pérez, E., Horváth, I. T., Sheldon, R. A., & Poliakoff, M. (2012). Valorization of Biomass: Deriving More Value from Waste. *Science*, *337*(6095), 695–699. https://doi.org/10.1126/science.1218930

Valdés, A., Álvarez-Rivera, G., Socas-Rodríguez, B., Herrero, M., Ibáñez, E., & Cifuentes, A. (2022). Foodomics: Analytical Opportunities and Challenges. *Analytical Chemistry*, *94*(1), 366–381. https://doi.org/10.1021/acs.analchem.1c04678

Vallverdú-Coll, N., Mateo, R., Mougeot, F., & Ortiz-Santaliestra, M. E. (2019). Immunotoxic effects of lead on birds. *Science of The Total Environment*, *689*, 505–515. https://doi.org/10.1016/j.scitotenv.2019.06.251

Versieck, J., & McCall, J. T. (1985). Trace Elements in Human Body Fluids and Tissues. *CRC Critical Reviews in Clinical Laboratory Sciences*, *22*(2), 97–184. https://doi.org/10.3109/10408368509165788

Wong, M. M. H., Chan, K. Y., & Lo, K. (2022). Manganese Exposure and Metabolic Syndrome: A Systematic Review and Meta-Analysis. *Nutrients*, *14*(4), 825. https://doi.org/10.3390/nu14040825

Yamada, N. (2015). Kinetic energy discrimination in collision/reaction cell ICP-MS: Theoretical review of principles and limitations. *Spectrochimica Acta Part B: Atomic Spectroscopy*, *110*, 31–44. https://doi.org/10.1016/j.sab.2015.05.008

Yang, Z., Yang, F., Liu, J.-L., Wu, H.-T., Yang, H., Shi, Y., Liu, J., Zhang, Y.-F., Luo, Y.-R., & Chen, K.-M. (2022). Heavy metal transporters: Functional mechanisms, regulation, and application in phytoremediation. *Science of The Total Environment*, *809*, 151099. https://doi.org/10.1016/j.scitotenv.2021.151099

Ye, R., Huang, J., Wang, Z., Chen, Y., & Dong, Y. (2022). The Role and Mechanism of Essential Selenoproteins for Homeostasis. *Antioxidants*, *11*(5), 973. https://doi.org/10.3390/antiox11050973

Zaikin, V. G., & Borisov, R. S. (2021). Mass Spectrometry as a Crucial Analytical Basis for Omics Sciences. *Journal of Analytical Chemistry*, *76*(14), 1567–1587. https://doi.org/10.1134/S1061934821140094

Zhang, P., Georgiou, C. A., & Brusic, V. (2017). Elemental metabolomics. *Briefings in Bioinformatics*, bbw131. https://doi.org/10.1093/bib/bbw131

AIM OF THE THESIS

Elemental metabolomics is a field of research with a huge potential. Many studies in the literature have used this approach to authenticate and valorize food products. However, a common issue encountered in these studies is the low number of samples compared to the number of factors under investigation. In addition, all the parts of the analytical methods used are often suboptimized. The objectives of this doctoral project of research were to develop, to optimize, and to validate analytical methods for studying three food matrices of great importance to the economy of the Sardinia region (Italy): honey, dairy sheep products, and rice.

1. All analytical methods used in this project were specifically developed and validated for each food matrix. A special attention was given to optimize the sample preparation, which was accomplished using the SRC technology.

2. Honey and beehive products are considered high-value foods due to their nutraceutical and nutritional properties. For these reasons, they are highly susceptible to counterfeiting and adulteration. The composition of these products is closely related to the environment and the floral sources from which bees forage for pollens. Therefore, they are often used also as indicators of environmental pollution. The project initially evaluated the influence of botanical origin on the elemental signature of the most characteristic unifloral honeys produced in Sardinia, including asphodel, eucalyptus, strawberry tree, and thistle honey. In the second phase of the project, the sampling was extended to both multifloral and unifloral honeys produced in Spain. Honeys of common botanical origin were then analyzed to verify and compare the influence of botanical and geographical origin on the elemental fingerprint.

3. The dairy industry is a leading economic sector in Sardinia, Italy. Livestock farming is culturally important and emblematic of the region's population. However, the industry's lack of innovation and industrial backwardness have led to problems related to product diversification and valorization. Two renowned products are the PDO Romano cheese and the PDO Sardinian cheese, both made from sheep's milk, but with different cheese-making processes. In collaboration with the Regional

Agriculture Agency AGRIS, we analyzed samples from all over the island. The samples included both types of cheese produced in different periods of the year. The aim was to evaluate their nutritional and safety properties. At the same time, the influence of the cheese-making process on the elemental signature was evaluated, also taking into account seasonal variations.

4. Rice is a staple food for the world's population due to its high nutritional value and widespread consumption. However, it is important to follow strict safety standards when consuming rice, as it has a tendency to accumulate toxic elements such as As and Cd. This is partly due to the traditional method of continuous flooding irrigation, which creates favorable conditions for the translocation and accumulation of these metals in rice grains. Intermittent methods, such as periodic saturation and sprinkling, can save significant amounts of water compared to continuous flooding irrigation and reduce the translocation of toxic elements. This research group has been investigating the use of these methods to produce safe and environmentally friendly rice for years. As part of this project, a method for analyzing arsenic speciation using HPLC-ICP-MS has been developed, since the toxicity of arsenic depends on its chemical species. In fact, the toxicity of organic arsenic species is lower than that of inorganic species, the latter being regulated in terms of concentration. The project aimed to evaluate arsenic speciation based on irrigation techniques and rice genotype/subspecies, in addition to developing and validating methods.

In conclusion, the influence of botanical and geographical origin on the elemental fingerprint of honey has been evaluated. Furthermore, the analysis of dairy sheep products allowed to evaluate the impact of processing methods and seasonality, which is strongly correlated with the health status of the animals. Finally, the study of rice assesses the impact of agricultural practices on the translocation of elements. The following chapters provide a comprehensive overview of the topics briefly mentioned in this section. These chapters are derived from articles published by our research group and are based on this Ph.D. dissertation project.

Published on Molecules, 2022, https://doi.org/10.3390/molecules27062009

MULTIELEMENTAL ANALYSIS AS TOOL TO ASCERTAIN THE SAFETY AND THE ORIGIN OF BEEHIVE PRODUCTS. DEVELOPMENT, VALIDATION, AND APPLICATION OF AN ICP-MS METHOD ON FOUR UNIFLORAL HONEYS PRODUCED IN SARDINIA, ITALY

1.1. INTRODUCTION

Honey is an ancient natural and functional food used frm centuries in the traditional medicine of many cultures. Beyond monosaccharides such as glucose and fructose, which constitute it up to 80% w/w, honey is a very complex matrix containing many bioactive compounds such as proteins, amino acids and enzymes, organic acids, polyphenols, flavonoids, vitamins, and inorganic elements (Bogdanov et al., 2008). This combination makes honey an outstanding functional food with many health-promoting activities and antimicrobial, antiviral, antifungal, anticancer, and antidiabetic properties (Cianciosi et al., 2018; Da Silva et al., 2016; Machado De-Melo et al., 2018; Rahman et al., 2017; Solayman et al., 2016).

Sardinia, Italy, is the second-largest island in the Mediterranean Sea. Its extension and distance from the European and African continental shelves characterize the endemic flora and fauna. Furthermore, due to the paucity of population and industries, the poor anthropogenic pressure is considered by consumers such an intrinsic guarantee of the quality of agri-food products, albeit sometimes this is not enough supported by scientific data. For these reasons, Sardinian honey is a typical product universally appreciated and recognized for its quality and for their peculiar organoleptic features. Beekeeping is a significant sector of the regional agriculture, since Sardinia produces more than the 11% of the Italian honey and Italy is the fourth largest producer in the European Union (Istituto di Servizi per il Mercato Agricolo Alimentare (Ismea), 2019). Besides the multiflora ones, the production of Sardinian honey is mainly focused towards four unifloral varieties: asphodel (*Asphodelus spp*), eucalyptus (*Eucalyptus spp*), strawberry tree (*Arbutus unedo L.*), and thistle (*Galactites tomentosa*) (Floris et al., 2007, 2021).

Given the importance of the sector for the economy of Sardinia, the traceability of their unifloral honeys should be ascertained with analytical methods aimed to food authentication. Among the possible methods, those based on elemental metabolomics [10] provided great achievements in both botanical (Danezis & Georgiou, 2022; Voyslavov et al., 2021) and geographical classification (Danezis & Georgiou, 2022; Drivelos & Georgiou, 2012). The elemental metabolomics approach involves the determination of trace elements to achieve an elemental signature (Zhang et al., 2017). Hence, although they are not directly related with most of the therapeutical and nutraceutical properties of honey (Afrin et al., 2020; Cornara et al., 2017; Denisow & Denisow-Pietrzyk, 2016; Viuda-Martos et al., 2008), trace elements are nevertheless of great scientific interest.

First, the health-promoting properties of honey must be coupled with the highest level of food safety, and consequently the concentration of toxic elements should be negligible. Then, honey is considered a valuable bioindicator of environmental pollution (Goretti et al., 2020; Kastrati et al., 2021; Rashed et al., 2009; Satta et al., 2012) because of its chemical composition strictly related to the environmental quality of the area next to the beehive (Bargańska et al., 2016; Ćirić et al., 2021). In fact, the concentration of some potentially toxic elements has been found higher in hive matrices from industrial and urban areas with respect to what measured in uncontaminated areas (Fodor & Molnar, 1993; Perna et al., 2021; Satta et al., 2012; Tuzen et al., 2007; Yayinie & Atlabachew, 2022). For these reasons, the concern for the potential presence of toxic elements in honey moved the European Union to set maximum levels for Hg (0.01 mg kg^{-1}) and for Pb (0.1 mg kg^{-1}) (European Community, 2015, 2018). Last, the elemental signature of honey has been frequently used for its authentication and traceability because foraged plants tend to accumulate specific elements in the nectar, allowing the classification according to their botanical origin (Bogdanov et al., 2007; Drivelos et al., 2021). In addition, soil composition affects element availability in honey, allowing in this case their georeferentiation (Drivelos et al., 2021; Quinto et al., 2016).

Despite trace elements were determined in honeys from many countries, such as Bulgaria (Voyslavov et al., 2021), Kosovo (Kastrati et al., 2021), Ethiopia (Yayinie & Atlabachew, 2022), Turkey (Tuzen et al., 2007), Italy (Perna et al., 2021; Pisani et al., 2008; Quinto et al., 2016; Satta et al., 2012), Poland and Greece (Drivelos et al., 2021), Hungary (Czipa et al., 2015) and New Zealand (Grainger et al., 2021), there are few literature contributions reporting the amounts of trace elements in unifloral honeys produced in Sardinia. Literature is also poor of studies reporting the elemental composition of the honeys of asphodel, eucalyptus, strawberry tree and thistle produced out of Sardinia. Among them, eucalyptus honey was the most investigated were reported in the literature. In contrast, the elemental composition of strawberry tree honey has been reported only for those coming from Croatia (Tariba Lovaković et al., 2018), whereas, at the best of our knowledge, no literature report dealt with a trace elements characterization of both asphodel and thistle honeys.

On an analytical viewpoint, microwave-assisted digestion (Astolfi et al., 2020; Pohl et al., 2017) and inductively coupled plasma spectrometry (ICP-MS) (Caroli, 1999; Pohl et al., 2017) were the preferred techniques for sample pre-treatment and for elemental analysis, respectively. Among the former ones, microwave-assisted digestion ensures high efficiency and performances (Leme et al., 2014; Muller et al., 2017; Oliveira et al., 2019; Sadowska et al., 2021). On the other hand, ICP-MS allows to achieve great results in trace and ultratrace analysis and to perform a reliable investigation for food authentication and traceability (Drivelos & Georgiou, 2012; Pohl et al., 2017). Regardless of the tool chosen for the elemental analysis, the data obtained should be elaborated using a multivariate approach, essential for classification purpose. Principal Component Analysis (PCA) (Di Bella et al., 2021), Cluster Analysis (CA) (Quinto et al., 2016), Discriminant analysis (DA) (Grainger et al., 2021), Partial Least Squares (PLS) (Drivelos et al., 2021) and Self-Organizing Maps (SOMs) (Voyslavov et al., 2021), provided great results in exploratory analysis and honeys classification, respectively.

This research group is active frm decades in the assessment and the validation of original analytical methods applied to beehive matrices to ascertain their quality (Spano et al., 2008, 2006; Salis et al., 2021; Sanna et al., 2000; Ciulu et al., 2011; Spano et al., 2009; Ciulu et al., 2015, 2013) as well as the

origin (Ciulu et al., 2018, 2020). Hence, the principal aim of this research is to ascertain the elemental signature of the most renowned unifloral honeys of Sardinia as a reliable tool to ensure their healthiness and guarantees on the origin. Therefore, an original ICP-MS method able to simultaneously measure the total amount of 23 elements of potential health concern (i.e., Ag, As, Ba, Be, Bi, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sb, Sn, Sr, Te, Tl, V, and Zn) has been developed and thoroughly validated. Finally, the proposed method was applied to a very large sampling of honeys from asphodel, eucalyptus, strawberry tree, and thistle.

1.2. MATERIALS AND METHODS

1.2.1. HONEY SAMPLES

The honeys were mainly gathered in the last year directly from local beekeepers and, in a minor amount, purchased in Sardinian markets. The collection, summarized in Figure 1.1, consists of 133 samples of the four most renowned unifloral varieties of honey: asphodel (n = 33), eucalyptus (n = 30), strawberry tree (n = 31) and thistle (n = 39), respectively. Samples were stored in the dark at 4°C until the analysis.

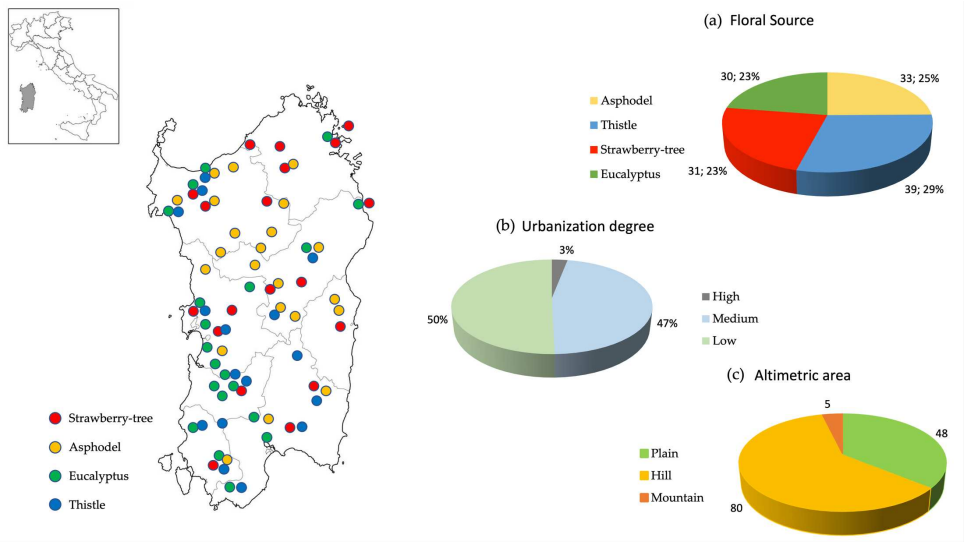


Figure 1.1. Information about Sardinian honey collection (n = 133). a) Floral source; b) Urbanization degree; c) Altimetric area.

The floral origin of each sample, primarily based on information directly provided by beekeepers, has been confirmed by the melissopalynological analysis, which provided data within the relevant ranges measured by Floris et al. [8]. Briefly, Sardinia is mainly hilly, therefore, 60% (n = 80) of the honeys are from the hills, 36% (n = 48) from the plains and only 4% (n = 5) from the mountains. The production areas are divided into rural areas 50% (low urbanization degree, n = 67), slightly urbanized areas 45% (medium urbanization degree, n = 62) and urbanized 3% (high urbanization degree, n = 4) (Istituto Nazionale di Statistica (Istat), 2022). The odd distribution of the samples in the region, as

reported in Figure 1.1, is representative of the different production areas and the distribution of the botanical sources.

1.2.2. REAGENTS AND STANDARD SOLUTIONS

In all the analytical phases, type I water (resistivity > 18 MW cm^{-1}), produced by means of a MilliQ plus System (Millipore, Vimodrone, Italy) was used. Nitric acid (67-69% w/w, NORMATON® for ultra-trace analysis) and hydrogen peroxide (30% w/w, NORMATON® for ultra-trace metal analysis) were from VWR (Milan, Italy). The multi-element standard Periodic Table mix 1 for ICP (TraceCert®, 33 elements, 10 mg dm^{-3}) and the internal standard solution (3% HNO_3 v/v aqueous solution containing 1000 mg dm^{-3} of Rh) were from Sigma-Aldrich (St. Louis, USA), while the elemental standards of Hg (10 mg dm^{-3}), Mo (10 mg dm^{-3}), Sb (1000 mg dm^{-3}) and Sn (100 mg dm^{-3}) were from Carlo Erba (Milan, Italy). NexION KED Setup Solution (1% HCl v/v aqueous solution containing Co 10 mg dm^{-3} and Ce 1 mg dm^{-3}) and NexION Setup Solution (1% HNO_3 v/v aqueous solution containing 1 µg dm^{-3} each of Be, Ce, Fe, In, Li, Mg, Pb and U) were from Perkin Elmer (Milano, Italy). A standard solution of NaOH 0.5 mol dm^{-3}, the potassium dichromate, the ammonium iron (II) sulphate and the sulfuric acid (96% v/v) used for the titrations were from Sigma-Aldrich.

1.2.3. INSTRUMENTATION

The multielement analysis has been performed by a NexION 300X ICP-MS spectrometer, equipped with a S10 autosampler, a glass concentric nebulizer, a glass cyclonic spray chamber, and a kinetic energy discrimination (KED) collision cell, all produced by Perkin Elmer (Milan, Italy). Samples were mineralized by means of a microwave single reaction chamber (SCR) system (ultraWAVE™, Milestone, Sorisole, Italy) equipped with fifteen polytetrafluoroethylene (PTFE) vessels (volume: 15 cm^3 each). An Ultraturrax mixer model T18 (IKA, Staufen, Germany) was used to homogenize honey samples before analysis. The residual acidity and the dissolved organic carbon (DOC) in digested sample were determined by acid–base titration procedure and by the Walkley-Black method (Food and Agriculture organization of the United Nations (FAO), 2020) using a Thermo Scientific Orion 950 titrator, whereas the total organic carbon (TOC) was determined by means of a CHN analyzer Leco 628. Nylon filters (pore diameter: 0.22 µm), polypropylene (PP) metal-free tubes, and polyethylene (PE) flasks were from VWR (Milan, Italy).

1.2.4. ICP-MS METHOD ASSESSMENT, QUALITY CONTROL AND ASSURANCE

The minimization of the dissolved organic carbon (DOC) in digested honey samples is an essential prerequisite to make a reliable ICP-MS analysis. Failing in this, high residual amounts of organic substances from a partial oxidation of the saccharides contained in honey could cause the formation of molecular ions in the plasma that may interfere with the determination of very important elements such as ^{52}Cr, ^{63}Cu and ^{75}As. Beyond this specific issue, the need to avoid the interference of molecular ions of any origin on the determination of the chosen isotopes of the analytes suggested to operate in kinetic energy discrimination (KED) mode for 15 elements out of 23. Therefore, the He flow has been carefully optimized for each element, to find the best compromise between the minimization of the polyatomic ion interferences and the maximization of the instrumental signal (Langasco et al., 2022; Mara et al., 2021; Spanu, Langasco, et al., 2020; Spanu, Valente, et al., 2020).

As a result, ^{75}As, ^{138}Ba, ^{111}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{57}Fe, ^{55}Mn, ^{60}Ni, ^{121}Sb, ^{120}Sn, ^{88}Sr, ^{120}Te, ^{51}V and ^{66}Zn were analyzed in KED mode using a He flow rate between 3 $cm^3\ min^{-1}$ and 4 $cm^3\ min^{-1}$, while ^{107}Ag, ^{9}Be, ^{209}Bi, ^{7}Li, ^{202}Hg, ^{98}Mo, ^{208}Pb and ^{205}Tl were analyzed in normal mode. The optimized instrumental parameters and the elemental settings used for each analyte are reported in Table A2.

The matrix effect was determined comparing the slopes of the calibration function obtained in the absence (2% HNO_3 v/v aqueous solutions) or presence (digested honey samples spiked with known amounts of analyte) of the matrix (De Oliveira et al., 2017). Data obtained accounted for a substantial absence of any matrix effect for all the analytes considered in the whole calibration range, hence quantification has been accomplished by means of external calibration. The samples, blank-diluted when necessary to lead analyte concentrations within the relevant calibration range, were analyzed in duplicate. Data obtained as results of a triplicate ICP-MS measurement were blank-corrected. To compensate any signal instability, a solution of Rh (10 mg dm^{-3}) has been used as internal standard, while the reliability of the measurements was ensured analyzing one blank and a standard solution containing each analyte (50 mg dm^{-3}) every 10 samples. Memory effects between consecutive samples were eliminated interposing a washing cycle of 60 seconds with a 2% HNO_3 v/v aqueous solution.

1.2.5. OPTIMIZATION OF THE COMPOSITION OF THE ACIDIC/OXIDIZING MIXTURE

After some preliminary evaluations, the digestion program (pression, temperature and time) and water volume of the oxidizing mixture (4 cm^3) were kept constant while the factors considered in the design were the sample amount (0.5 - 1.0 g) and the ratio between the volumes of HNO_3 and H_2O_2 (0.5/3 cm^3 – 2/1.5 cm^3). As a result, a two-levels 2^2 full factorial design was applied to improve the composition of the oxidizing mixture and minimize the dissolved organic carbon in digested sample.

To estimate the experimental error and validate the regression model, a duplicate of the central point was added to the factorial design. The responses of the design were the residual acidity in the digested sample and the efficiency of organic matter decomposition (Bizzi et al., 2017) (EOMD%), which was calculated with the equation 1. The experimental matrix obtained with the two-levels full factorial design is reported in Table 1.1, as well as the experimental plan.

$$EOMD\ \% = [(TOC\text{-}DOC)/TOC]\ \% \quad (1)$$

where TOC is the total organic carbon (mg kg^{-1}) and DOC is the dissolved organic carbon (mg kg^{-1})

Table 1.1. Two-level, 2^2 full factorial experimental design with center point.

Exp	Sample amount (g)	Ratio HNO_3 / H_2O_2	X_1 [a]	X_2 [a]	Residual acidity (mol dm^{-3})	EOMD%
1	0.50	0.17	-1	-1	0.39	99.7
2	1.00	0.17	+1	-1	0.06	87.2
3	0.50	1.33	-1	+1	1.41	97.7
4	1.00	1.33	+1	+1	0.16	99.1
5	0.75	0.56	0	0	0.48	95.8
6	0.75	0.56	0	0	0.39	96.4

[a] X_1 and X_2 represent the sample amount and the ratio HNO_3/H_2O_2, respectively.

The results obtained with the full factorial design are reported in Table 1.1. Multilinear regression (MLR) provided the coefficients of the equation 2 for both the responses, residual acidity (Y_1) and EOMD% (Y_2) respectively, which are reported in Table 1.2.

$$Y_n = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 \quad (2)$$

where b_0 is a constant, b_1 and b_2 are the coefficients of the main effects of the factors X_1 and X_2, whereas b_{12} is the coefficient of their interaction.

Table 1.2. Values of MLR coefficients and their significance levels for both the design's responses.

	Residual Acidity		EOMD%	
coefficient	**coefficient value**	**significance [a]**	**coefficient value**	**significance [a]**
b_0	0.49	***	95.9	***
b_1	-0.41	**	-2.8	*
b_2	0.27	*	2.5	*
b_{12}	-0.25	*	3.5	**

[a] Blank, not statistically significant, *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$.

As reported in Table 1.2 all the coefficients were significant for both the responses, including the coefficient of the interactions (b_{12}) especially for EOMD%. From the experiments at the central point, the experimental error can be estimated, and the standard deviation for Y_1 and Y_2 were 0.06 and 0.42, respectively. The predicted values for the residual acidity and for EOMD% at the center point were 0.5 ± 0.1 and 96 ± 1. Since the difference values between the experimental and predicted values are not significant (t-test, $\alpha = 0.05$), the model can be accepted.

The effects of the sample amount and the ratio between the oxidizing compounds on the residual acidity and the EOMD% of the digested samples are shown in the contour plots in Figure 1.2.

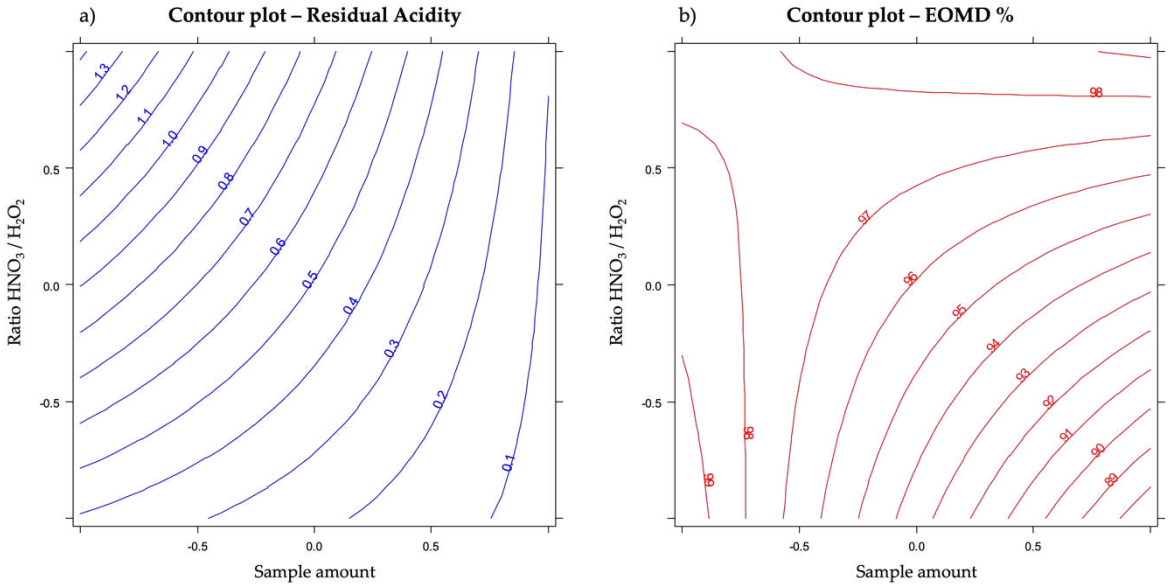


Figure 1.2. Contour plots for the effects on the design's responses. a) Residual acidity; b) EOMD%.

From Figure 1.2 it is possible to understand the interaction between the two variables:

Y_1. Residual Acidity: the effect of the ratio HNO_3/H_2O_2 (X_2) on the residual acidity is present only at the lower sample amount (X_1) where its increase leads to higher acidity, while at higher amount var. X_2 has no effect; on the other hand, at higher ratios (X_2) an increase of the sample amount (X_1) leads to a higher decrease of the response, greater than the decrease occurring at lower ratios (X_2).

Y_2. EOMD%: the effect of the ratio HNO_3/H_2O_2 (X_2) on the EOMD is present only at the higher sample amount (X_1) where its increase leads to higher response, while at the lower sample amount var. X_2 has no effect; on the contrary, at lower ratios (X_2) an increase of the sample amount (X_1) leads to a decrease of the response, while at higher ratios the sample amount has no effect.

It is evident that a good compromise between the two responses provides a sample amount of ca. 0.7 g of honey and a ratio HNO_3/H_2O_2 less than 0.5. Therefore, in order to minimize the consumption of nitric acid for the benefit of the greenest hydrogen peroxide, the acceptable composition of the acidic/oxidizing mixture is 0.5 cm^3 HNO_3, 3 cm^3 of H_2O_2 and 4 cm^3 of H_2O.

1.2.6. STATISTICAL ANALYSIS

A two-tail t-test at $\alpha = 0.05$ was used in ascertaining the existence of matrix effect as well as in the trueness evaluation. Principal Components Analysis (PCA), Linear Discrimination Analysis (LDA) and Multilinear Regression (MLR) were performed by means of the R-based software Chemometric Agile Tool (CAT) developed by the Italian group of Chemometrics (Leardi et al., 2024).

1.3. RESULTS AND DISCUSSION

1.3.1. SAMPLE PRE-TREATMENT

To avoid the matrix effect and maximize the signal-to-noise ratio, a great attention was paid to the optimization of the sample pre-treatment step (Leme et al., 2014; Muller et al., 2017; Oliveira et al., 2019; Sadowska et al., 2021). Although the sample amounts and the instrumental conditions depend on the specific features of the microwave system, according to the recent method proposed by Astolfi et al. (Astolfi et al., 2020), HNO_3 and H_2O_2 were chosen as acidic/oxidizing mixture due their proven capability to minimize the formation of the interfering polyatomic ions in the plasma and their efficiency in organic matter decomposition. According to those reported by Muller et al. (Muller et al., 2017), a simply 2^2 full factorial design was applied to improve the digestion efficiency and the composition of the oxidizing mixture. The optimization allowed the reduction of the HNO_3 volume required for sample digestion, increasing the amount of the greenest hydrogen peroxide and the mass of sample digested, which is an important achievement for trace elements analysis. These results were also achieved thanks to the versatile performance of the ultraWAVE digestion system. The optimized method is reported in Table 1.3.

Table 1.3. ultraWAVE SRC microwave digestion operational program and conditions.

Step		Time (min)	Temperature (°C)	
1st	Heating	25	240	Initial pressure: 40 bar Final temperature: < 40°C Pressure release rate: 8 bar/min Rack : 15 positions Vessel : volume 15 cm^3, PTFE Sample amount: 0.7 g of honey Reagents: 0.5 cm^3 HNO_3, 3 cm^3 of H_2O_2 and 4 cm^3 H_2O
2nd	Holding	10	240	
3rd	Cooling	ca. 30	< 40	

First, honey sample was heated until 40°C and then homogenized by an Ultraturrax. Next, ca. 0.7 g of honey, exactly weighted on an analytical balance (± 0.0001 g uncertainty), was treated with 0.5 cm3 of HNO_3, 3 cm^3 of H_2O_2 and 4 cm^3 of type I water inside a PTFE vessel of the SCR mineralization system. After the digestion, samples were diluted to the final volume of 15 cm^3 and filtered through a 0.22 μm nylon filter. Finally, they were stored at 4°C in the dark until the analysis. The typical amount of

residual acidity found in digested samples was 0.2 mol dm^{-3}, whereas the efficiency of organic matter decomposition (EOMD%) is generally higher than 94%.

1.3.2. VALIDATION OF THE ICP-MS METHOD

The validation of the ICP-MS method proposed was accomplished in terms of the limit of detection (LoD), limit of quantification (LoQ), linearity, precision, and trueness. Table 1.4 reports the validation parameters of the ICP-MS method for the determination of the total amount of 23 trace elements in honey.

Table 1.4. Validation parameters of the ICP-MS method aimed to the determination of 23 trace elements in unifloral honeys.

Element	LoD[a] ($\mu g\ kg^{-1}$)	LoQ[a] ($\mu g\ kg^{-1}$)	Calibration Range[b] ($\mu g\ dm^{-3}$)	Repeatability[c] CV (%)	Intermediate precision[d] CV (%)	Trueness Recovery (% ± sd)
Ag	5	17	0.1 - 50	4	8	106 ± 5
As	2	7	0.1 - 50	4	6	92 ± 1
Ba	20	70	1 - 250	1	3	90 ± 20
Be	0.4	1.3	0.02 - 50	4	13	103 ± 1
Bi	0.1	0.3	0.005 - 50	4	12	85 ± 7
Cd	0.3	1.0	0.01 - 50	4	8	117 ± 1
Co	0.3	1.0	0.01 - 50	8	9	99 ± 1
Cr	7	23	0.1 - 50	4	5	97 ± 1
Cu	20	70	1 - 100	4	11	107 ± 4
Fe	30	100	1 - 100	4	17	105 ± 15
Hg	6	20	0.1 - 50	4	13	130 ± 10
Li	2	7	0.1 - 500	4	14	96 ± 1
Mn	8	27	0.1 - 500	3	3	107 ± 4
Mo	0.7	2.3	0.04 - 50	4	8	94 ± 1
Ni	3	10	0.1 - 100	6	18	95 ± 2
Pb	3	10	0.1 - 250	4	6	92 ± 1
Sb	0.7	2.3	0.04 - 50	4	9	115 ± 1
Sn	2.1	6.9	0.1 - 50	5	5	103 ± 2
Sr	3	10	0.1 - 100	3	7	103 ± 1
Te	1.2	3.9	0.04 - 50	4	21	108 ± 3
Tl	0.04	0.13	0.005 - 50	7	5	96 ± 1
V	0.2	0.7	0.01 - 50	4	6	94 ± 2
Zn	40	130	1 - 500	12	16	101 ± 1

[a] The LoD and LoQ values were measured according to (Currie, 1999); [b] instrumental calibration range; [c] evaluated on the sample replicates in the same analytical session (n = 3); [d] evaluated on the sample replicates within one month (n = 3)

The LoDs and LoQs of the method were calculated according to Currie (Currie, 1999) on 30 measurements of method blanks obtained in different analytical sessions. LoDs were generally below 10 mg dm^{-3} for all the elements except for Ba, Cu, Fe and Zn, which were between 10 mg dm^{-3} and 40 mg dm^{-3}. Because of the huge variability of the analyte concentrations, great attention has been paid on the instrument calibration phase. Although the linearity of the ICP-MS could be extended over several orders of magnitude of concentration, in this case this parameter was explored for each element only within the relevant operative range of concentration. For each analyte, the calibration function was the result of a linear regression of six standard solutions, three for each extreme point of the calibration range. To evaluate the experimental error and verify the linearity of the calibration function, three different solutions at an analyte concentration equal to the central point of the calibration range were analyzed. For each analyte, the difference between the experimental and predicted values was not significant (t-test, $\alpha = 0.05$). Hence, keeping into consideration the random distribution of the residuals around the mean value as well as the very high coefficient of determination, R^2 (always above 0.999), the linearity of the calibration function was successfully ascertained.

Precision, measured as coefficient of variation (CV%), was assessed in terms of repeatability and intermediate precision. For this purpose, a batch of honey samples was replicated in the same and in different analytical sessions. Table 1.4 reports the CV%s calculated at the analyte's average concentration. For elements which concentration in honey was below the LoD, precision was measured spiking the samples with standard solutions of analyte. Repeatability exhibited CV% between 1% (Ba) and 12% (Zn), while intermediate precision ranged between 3% (Ba and Mn) and 21% (Te). All precision parameters were in the range of CV% defined by the Horwitz's theory (Horwitz, 1982), hence the overall precision level of the method was acceptable.

Due to the lack of certified reference material (CRM) for the determination of trace elements in honey, trueness was evaluated by recovery tests. Hence, three aliquots of each sample were spiked with increasing amounts of a standard solution containing all analytes. All aliquots were then

undergone to the whole analytical procedure described in Section 1.3.1. Furthermore, an additional aliquot for each honey sample was analyzed without any spiking. Recoveries ranged between 85% (Bi) and 130% (Hg). Quantitative recoveries (t-test, $\alpha = 0.05$) were observed for most of the elements, except for As, Li, Mo, Pb and V (underestimation bias) and for Be, Cd, Sb (overestimation bias). Nevertheless, also the recoveries obtained in these cases were acceptable according to the AOAC guidelines (Association of Official Agricultural Chemists (AOAC), 2016). Summarizing, the proposed method has been fully and successfully validated and the parameters here considered are comparable or better than those reported in previous works (Astolfi et al., 2020; Di Bella et al., 2021; Drivelos et al., 2021; Grainger et al., 2021; Pohl et al., 2017).

1.3.3. HONEY ANALYSIS

The Table 1.5 summarizes, in terms of both mean value and range, the amount of toxic and trace elements belonging to the four unifloral Sardinian honeys.

The variability of the elemental concentrations measured was very high, not only between samples belonging to different floral origin, but also within samples of the same botanical nature. Except for Mn, which concentration was always over its LoD, the amount of other elements such as Ba, Co, Cu, Fe, Ni, Sn, Sr, V and Zn ranged between values below the relevant LoDs and few mg kg^{-1} (i.e., Co, Ni, Sn, Sr, and V) or mg kg^{-1} (i.e., Ba, Mn, Fe, Cu and Zn). On the other hand, the concentrations of both Ag and Te have never been found above the relative LoQs, while the amount of Be was always below its LoD. For what concerns the most fearsome toxic elements, like As, Cd, Pb, Hg, Sb and Tl, their concentrations were almost always below a few tens (As, Hg and Pb) or few units (Cd, Sb and Tl) of mg kg^{-1}, confirming an overall high level of food safety for unifloral honeys coming from Sardinia.

The results suggest for a possible discrimination of unifloral honeys based on their botanical origin, whereas no significant differences were observed between samples from areas with different degree of pollution or urbanization.

Table 1.5. Mean and range concentrations (in mg kg^{-1}) of 23 among toxic and trace elements in unifloral honey samples of asphodel, eucalyptus, strawberry tree, and thistle.

Element	Asphodel (n = 33)		Eucalyptus (n = 30)		Strawberry tree (n = 31)		Thistle (n = 39)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Ag	*< 5*	*< 5* - < 17	*< 5*	*< 5* - *< 5*	*< 5*	*< 5* - < 17	*< 5*	*< 5* - *< 5*
As	< 3	*< 2* - 9	< 6	*< 2* - 19	*< 2*	*< 2* - < 7	< 4	*< 2* - < 7
Ba	180	*< 20* - 1600	340	80 - 690	550	< 70 - 2600	300	< 70 - 1800
Be	*< 0.4*	*< 0.4* - *< 0.4*	*< 0.4*	*< 0.4* - *< 0.4*	*< 0.4*	*< 0.4* - *< 0.4*	*< 0.4*	*< 0.4* - *< 0.4*
Bi	< 0.2	*< 0.1* - 1.6	< 0.2	*< 0.1* - 1.2	< 0.2	*< 0.1* - 0.8	< 0.6	*< 0.1* - 10.7
Cd	< 0.7	*< 0.3* - 2.5	1.7	*< 0.3* - 9.2	*< 0.3*	*< 0.3* - < 1	1.4	*< 0.3* - 5.2
Co	2.5	< 1 - 10.2	7.6	< 1 - 109	1.8	*< 0.3* - 9.7	5.1	< 1 - 15.5
Cr	< 11	*< 7* - 24	< 17	*< 7* - 26	< 15	*< 7* - 24	< 8	*< 7* - 24
Cu	90	*< 20* - 250	170	< 70 - 630	90	*< 20* - 250	180	< 70 - 1030
Fe	160	< 100 - 660	570	< 100 - 1600	180	< 100 - 630	340	120 - 820
Hg	*< 6*	*< 6* - *< 6*	*< 6*	*< 6* - *< 6*	*< 6*	*< 6* - < 10	*< 6*	*< 6* - < 20
Li	*< 2*	*< 2* - 14	11	*< 2* - 30	280	*< 2* - 8500	< 5	*< 2* - 22
Mn	190	40 - 770	2600	140 - 5100	330	< 27 - 4900	330	40 - 3300
Mo	< 1.8	*< 0.7* - 3.6	< 2.3	*< 0.7* - 3.9	1.8	*< 0.7* - 3.8	< 2.1	*< 0.7* - 3.7
Ni	19	*< 3* - 170	22	*< 3* - 122	12	*< 3* - 33	24	< 10 - 220
Pb	23	*< 3* - 400	16	*< 3* - 95	< 10	*< 3* - 90	< 9	*< 3* - 30
Sb	< 0.8	*< 0.7* - < 2.3	< 1.3	*< 0.7* - 4.7	< 0.9	*< 0.7* - < 2.3	< 0.8	*< 0.7* - 2.8
Sn	44	*< 2.1* - 210	30	< 6.9 - 110	43	*< 2.1* - 200	46	< 7.1 - 240
Sr	38	*< 3* - 174	180	20 - 290	140	22 - 350	98	18 - 420
Te	< 1.5	*< 1.2* - 6.8	*< 1.2*	*< 1.2* - *< 1.2*	*< 1.2*	*< 1.2* - *< 1.2*	*< 1.2*	*< 1.2* - < 3.9
Tl	< 0.13	*< 0.04* - 0.4	0.3	*< 0.04* - 2.2	< 0.13	*< 0.04* - 1.4	0.18	*< 0.04* - 1.3
V	< 0.4	*< 0.2* - < 0.7	4.1	< 0.7 - 12.8	< 0.6	*< 0.2* - 1.8	1.3	< 0.7 - 5.6
Zn	550	< 130 - 1400	660	330 - 1400	400	*< 40* - 1200	800	300 - 2000
Total (mg kg^{-1})	1.30	0.56 -2.70	2.20	0.74 -6.60	2.10	0.76 -10.30	4.70	1.00 -8.40

Each sample has been analyzed twice. In *italics*, data below the LoD; in underlined, data below the LoQ.

To verify a possible discrimination among Sardinian and foreign honeys, a meta-analysis has been conducted. Since no previous literature's contribution deals with the assessment of the elemental signature of asphodel (*Asphodel spp*) and thistle (*Galactites tomentosa*) honeys, it was not possible to make a comparison fr these two unifloral varieties. On the other hand, a comparison could be made between eucalyptus (*Eucalyptus spp*) and strawberry tree (*Arbutus unedo L*) honeys. Table 1.6 shows the results of the amounts of toxic and trace elements reported for unifloral eucalyptus honeys produced in different countries.

Table 1.6. Mean concentration and range[a] (both in mg kg^{-1}) of toxic and trace elements in unifloral Eucalyptus honeys from different geographical origins.

Element	Tunisia (n = 3) (Di Bella, 2021)	Argentina (n = 1) (Conti, 2014)	Italy (n = 1) (Forte, 2001)	Unknown (n =1) (Caroli, 2000)	Sardinia (n = 29) (This work)
Ag					*< 5; < 5 - < 5*
As	19.08	<10	5.99	3.33	< 6; *< 2* - 19
Ba					340*;* 80 - 690
Be		<10			*< 0.4; < 0.4 - <*
Bi					< 0.2*; < 0.1* -
Cd	<0.01	<10	0.592	0.70	1.7*; < 0.3* - 9.2
Co		10			7.6; < 1 - 109
Cr	130	<10	1.50	2.73	< 17; *< 7* - 26
Cu	800	120	219	140	170; < 70 - 630
Fe	7100	3380	1008	914	570; < 100 -
Hg			< 0.75		*< 6; < 6 - < 6*
Li					11; *< 2* - 30
Mn	1250	8840	1009	1976	2600; 140 -
Mo					< 2.3; *< 0.7* -
Ni	220	50	11.3	8.04	22; *< 3* - 122
Pb	250	10	5.00	141	16; *< 3* - 95
Se	130	10	5.60		
Sb	100				< 1.3; *< 0.7* -
Sn				7.85	30; < 6.9 - 110
Sr					180; 20 - 290
Ti	610				
Te					*< 1.2; < 1.2 - <*
Tl		<10			0.3; *< 0.04* - 2.2
U		<10			
V	50	<10		3.36	4.1; < 0.7 - 12.8
Zn	2060	550	791	414	660; 330 - 1400

[a] data are presented in the "average; range" format. n = number of honey samples analyzed. In *italics*, data below the LoD; in underlined, data below the LoQ.

The determination of As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn was performed in all studies, and the amounts of these elements in honeys from different geographical origin normally span over one or two orders of magnitude. The different concentrations of the most abundant elements (Cr, Cu, Fe, Ni, Pb and Zn, significantly higher in the samples from Tunisia, or the very high concentration of Mn in the Argentinian honey) let to envisage the feasibility of a discrimination of these honeys according to the geographical origin. However, due to the paucity of the samples analysed in literature studies (Caroli et al., 2000; Conti et al., 2014; Di Bella et al., 2021; Forte et al., 2001), no definitive conclusion could be obtained by this comparison.

The comparison of the dataset obtained from the determination of 23 elements in nine samples of strawberry-tree from Croatia (Tariba Lovaković et al., 2018) was more reliable. In this case, 17 out of 23 elements were measured in both Countries. Meaningful differences in the average concentration of many trace elements (like Co, Cu, Mo, Ni, Pb, Sb, Sn and V) appeared to support the possibility to achieve a differentiation according to the different geographical origin. The average distributions reported in Figure 1.3 highlights the differences between Croatian and Sardinian strawberry tree honeys.

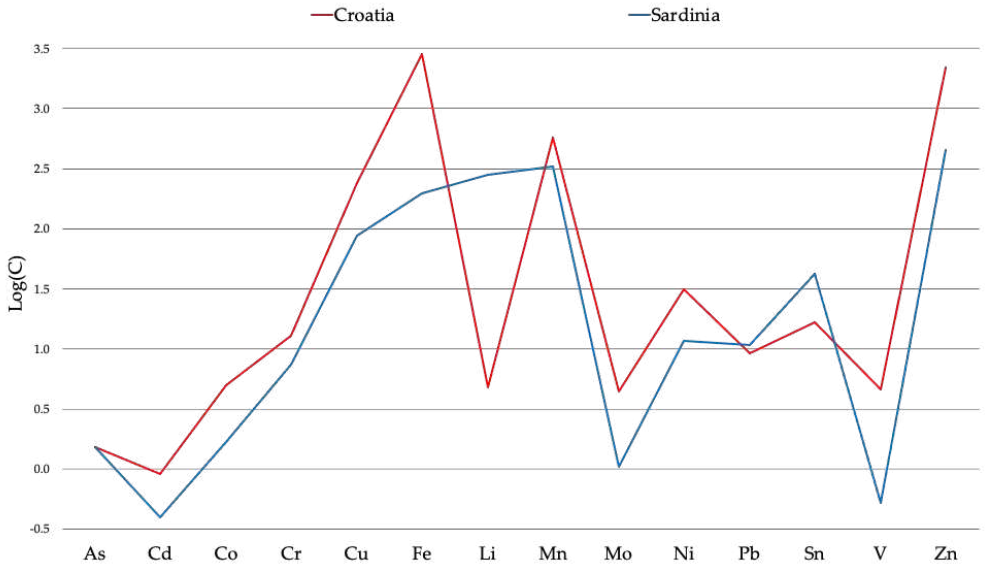


Figure 1.3. Average distributions of strawberry tree honeys from Croatia and Sardinia. Elemental concentrations are in mg kg^{-1}.

1.3.4. CHEMOMETRIC ANALYSIS

In order to evaluate the suitability of trace elements to ascertain the origin of Sardinian honeys, principal component analysis (PCA) and linear discriminant analysis (LDA) were performed for exploratory and classification purposes, respectively.

The original dataset, constituted by 133 samples and 18 variables (Ag, Be, Hg, Sb and Te were removed because almost never quantified), were randomly divided into a training (85 samples) and a validation (48 samples) set for internal validation.

The logarithmic transformation (log10) was used to reduce the skewness of the probability density distribution present in the original data. However, the information embodied in the loadings has changed and so it could lead to dangerous misunderstandings (Oliveri et al., 2019).

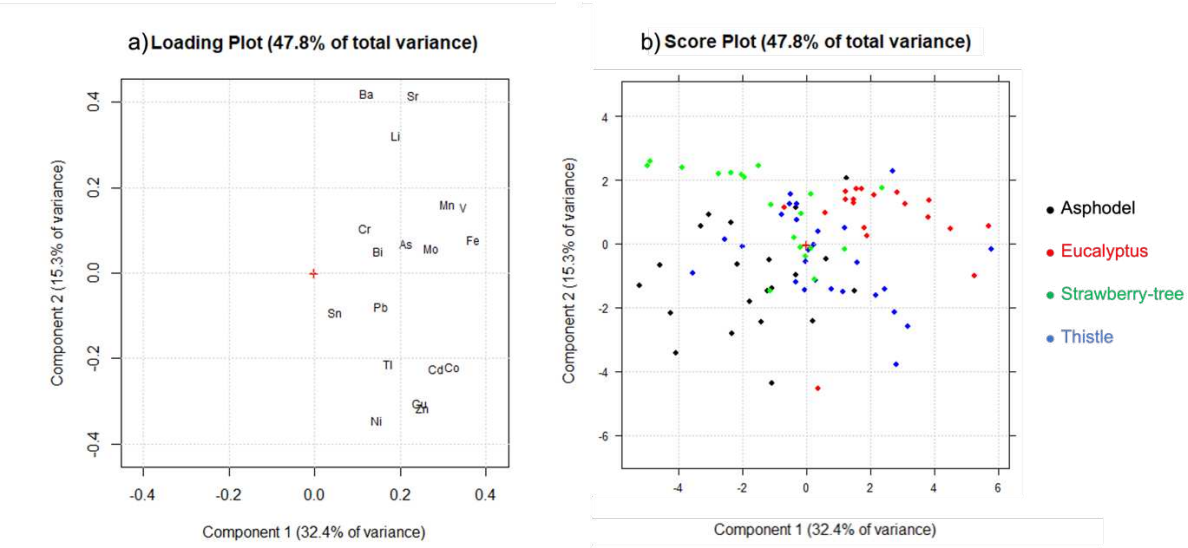


Figure 1.4. PCA performed on 85 unifloral honey samples and 18 trace elements; a) Loading Plot; b) Score Plot, objects colored according to the different botanical origin of the samples.

From the loading plot (Figure 1.4a), PC1 explained mainly the global concentration of the trace elements characterized by positive loadings on PC1 (Fe, V, Mo, Mn). On the other hand, PC2 explained the contrast between the two clusters of correlated trace elements: Li, Sr, Ba, at positive loadings, and Co, Cd, Cu, Zn, Ni at negative loadings. Couples of elements like Mn and V, Cd and Co and, mainly, Cu and Zn, were strongly correlated among them. Finally, Sn, Cr, Pb and Bi were the less significant variables, as suggested by their overall low expression in both PC1 and PC2. Looking at the score plot

in the plane PC1-PC2 (Figure 1.4b), with the objects colored according to the different botanical origin, it was noticed that the four classes were generally characterized by a specific location on the plane: asphodel honey samples (1) can be found at negative scores of both PC1 and PC2; eucalyptus samples (2) at positive scores of both PC1 and PC2; strawberry-tree samples (3) at negative score of PC1 and positive scores of PC2 and, finally, thistle samples (4) can be found at positive scores of PC1 and negative scores of PC2. The information on the within-class variability of the four classes was smoothed by the logarithmic transformation, as well as the correlations between the variables. The high variability can be confirmed from the width of the ranges reported in Table 1.5. Furthermore, an analysis of the correlations was performed before and after the logarithmic transformation (Table A1) to ensure a correct interpretation of the variables. The coefficients highlight a strong correlation between Fe – Mn – V and Cu – Ni – Zn, confirmed by the loading plot, in contrast the correlations between Ba – Sr – Li and Cd – Co were emphasized by the data pre-treatment.

For classification purposes, linear discriminant analysis (LDA) was used. This method is based on the description of data by means of probability density distributions, under two hypotheses: i) probability distributions are multivariate normal within all the classes; ii) dispersion and correlation structures are the same within all the classes. Fortunately, the method is quite robust against slight deviations from these hypotheses (Oliveri et al., 2021).

In this case, after the pre-processing it is possible to use LDA because the classes are similar in size and orientation and therefore it is possible to assume that they have a similar variance and covariance matrix.

Table 1.7. Results of the LDA, % of correct classified samples in CV and prediction.

% Correctly classified	Asphodel	Eucalyptus	Strawberry tree	Thistle	Total
Cross validation	61.9	84.2	78.9	76.9	75.5
Prediction [a]	91.7	81.5	75.0	100.0	87.1

Internal validation

Table 1.8. Confusion Matrix in prediction.

	Asphodel	Eucalyptus	Strawberry tree	Thistle
Asphodel	11	0	0	1
Eucalyptus	1	9	0	1
Strawberry tree	1	0	9	2
Thistle	0	0	0	13

The results obtained in cross validation and prediction are reported in Table 1.7. The botanical origin with the lowest percentage of correct prediction was asphodel (61.9%), whose samples were mainly confused with thistle (n = 3) and strawberry tree (n = 5), while the best results are obtained for eucalyptus (84.2%). A permutation test was performed to compare the performance obtained with the correct assignment with a series of random permutation of the classes (in this case, 100 permutations were calculated). Figure A3 shows how the model works, since in no event the classification results were obtained randomly. In prediction, a correct classification percentage of 87.1% was achieved, quite in agreement with the results obtained in CV. By observing the confusion matrix (Table 1.8), it is possible to look at the samples characterized by the wrong assignment highlighting that asphodel and thistle are relatively less accurate but still acceptable: strawberry tree twice erroneously classified as thistle and once as asphodel; eucalyptus erroneously re-classified as both thistle and asphodel; a sample of asphodel wrongly classified as a thistle.

The level of discrimination achieved in this contribution is less than those obtained by this research group using chemometric approaches based on untargeted physical and chemical data (Spano et al., 2006) or FT-ATR spectroscopic data (Salis et al., 2021).

Several factors could affect the ascertainment of the botanical origin. First, Sardinian unifloral honeys are typically underrepresented from a melissopalynological point of view since the pollen spectrum is often largely overlapping due to both accompanying and rare pollens (Floris et al., 2007). This highlights a partly common botanical origin, especially for spring productions of asphodel and thistle and secondly also for summer and autumn production of eucalyptus and strawberry tree, respectively. Finally, a last possible factor, could be related to the geographical origin of some honeys,

especially for those coming from the mining areas (central and southwestern Sardinia) where the natural characteristics of soil composition may have influenced the concentration of some elements.

In addition, a second LDA was unsuccessfully used in the attempt to achieve a geographical classification of Sardinian samples belonging to the same floral origin (data not reported). Probably the reason for this failure is related both to the difficulty to localize the exact position of the hives (Floris et al., 2007) and to the extreme geochemical and pedological variability of the soil of Sardinia (Aru et al., 1991).

Summarizing, the elemental signature provided rather robust descriptors for the botanical discrimination of Sardinian unifloral honeys, whereas the intra-regional geographical discrimination was currently compromised and should be investigated by acquiring additional data. Hence, efforts will be paid to carefully solve these issues. Moreover, additional samples of both eucalyptus and strawberry tree honeys produced outside Sardinia will be gathered in order to achieve a reliable geographical discrimination.

1.4. CONCLUSIONS

For the first time, the concentration of 23 trace elements have been measured in a very large sampling of the most renowned unifloral honeys from Sardinia, Italy, using an original and validated ICP-MS method. Special attention has been paid to the development of the acid microwave digestion procedure, as well as the optimization of instrumental parameters, to improve the efficiency in the organic matter decomposition and minimize the polyatomic interferences, respectively. Among the most abundant elements (Ba, Mn, Fe, Cu and Zn), only Mn was measured in all the samples, whereas the others ranged from the relevant LoDs to few mg kg^{-1}. Toxic elements have been almost always below the amounts of potential health concern, confirming hence a very good level of food safety for Sardinian honeys. Since no elemental signature was reported in literature for asphodel and thistle honey, the meta-analysis was carried out only on eucalyptus and strawberry tree honeys and it highlighted the possibility of a geographical discrimination thanks to their elemental signature, mainly for strawberry tree honeys from Croatia and Sardinia. Finally, through the elemental signature of the four unifloral honeys here considered, a good classification based on the botanical origin was accomplished by means of linear discrimination analysis.

1. REFERENCES

Afrin, S., Haneefa, S. M., Fernandez-Cabezudo, M. J., Giampieri, F., al-Ramadi, B. K., & Battino, M. (2020). Therapeutic and preventive properties of honey and its bioactive compounds in cancer: An evidence-based review. *Nutrition Research Reviews*, *33*(1), 50–76. https://doi.org/10.1017/S0954422419000192

Aru, A., Baldaccini, P., & Vacca, A. (1991). *Carta dei suoli della Sardegna*. http://www.sardegnaportalesuolo.it/cartografia/carte-dei-suoli/carta-dei-suoli-della-sardegna-scala-1250000.html

Association of Official Agricultural Chemists (AOAC). (2016). *Guidelines for Standard Method Performance Requirements*. https://www.aoac.org/wp-content/uploads/2019/08/app_f.pdf

Astolfi, M. L., Conti, M. E., Marconi, E., Massimi, L., & Canepari, S. (2020). Effectiveness of Different Sample Treatments for the Elemental Characterization of Bees and Beehive Products. *Molecules*, *25*(18), 4263. https://doi.org/10.3390/molecules25184263

Bargańska, Ż., Ślebioda, M., & Namieśnik, J. (2016). Honey bees and their products: Bioindicators of environmental contamination. *Critical Reviews in Environmental Science and Technology*, *46*(3), 235–248. https://doi.org/10.1080/10643389.2015.1078220

Bizzi, C. A., Pedrotti, M. F., Silva, J. S., Barin, J. S., Nóbrega, J. A., & Flores, E. M. M. (2017). Microwave-assisted digestion methods: Towards greener approaches for plasma-based analytical techniques. *Journal of Analytical Atomic Spectrometry*, *32*(8), 1448–1466. https://doi.org/10.1039/C7JA00108H

Bogdanov, S., Haldimann, M., Luginbühl, W., & Gallmann, P. (2007). Minerals in honey: Environmental, geographical and botanical aspects. *Journal of Apicultural Research*, *46*(4), 269–275. https://doi.org/10.1080/00218839.2007.11101407

Bogdanov, S., Jurendic, T., Sieber, R., & Gallmann, P. (2008). Honey for Nutrition and Health: A Review. *Journal of the American College of Nutrition*, *27*(6), 677–689. https://doi.org/10.1080/07315724.2008.10719745

Caroli, S. (1999). Determination of essential and potentially toxic trace elements in honey by inductively coupled plasma-based techniques. *Talanta*, *50*(2), 327–336. https://doi.org/10.1016/S0039-9140(99)00025-9

Caroli, S., Forte, G., Alessandrelli, M., Cresti, R., Spagnoli, M., D'Ilio, S., Pauwels, J., & Kramer, G. N. (2000). A pilot study for the production of a certified reference material for trace elements in honey. *Microchemical Journal*, *67*(1–3), 227–233. https://doi.org/10.1016/S0026-265X(00)00066-7

Cianciosi, D., Forbes-Hernández, T., Afrin, S., Gasparrini, M., Reboredo-Rodriguez, P., Manna, P., Zhang, J., Bravo Lamas, L., Martínez Flórez, S., Agudo Toyos, P., Quiles, J., Giampieri, F., & Battino, M. (2018). Phenolic Compounds in Honey and Their Associated Health Benefits: A Review. *Molecules*, *23*(9), 2322. https://doi.org/10.3390/molecules23092322

Ćirić, J., Spirić, D., Baltić, T., Lazić, I. B., Trbović, D., Parunović, N., Petronijević, R., & Đorđević, V. (2021). Honey Bees and Their Products as Indicators of Environmental Element Deposition. *Biological Trace Element Research*, *199*(6), 2312–2319. https://doi.org/10.1007/s12011-020-02321-6

Ciulu, M., Floris, I., Nurchi, V. M., Panzanelli, A., Pilo, M. I., Spano, N., & Sanna, G. (2013). HPLC determination of pantothenic acid in royal jelly. *Analytical Methods*, *5*(23), 6682. https://doi.org/10.1039/c3ay41284a

Ciulu, M., Floris, I., Nurchi, V. M., Panzanelli, A., Pilo, M. I., Spano, N., & Sanna, G. (2015). A Possible Freshness Marker for Royal Jelly: Formation of 5-Hydroxymethyl-2-furaldehyde as a Function of Storage Temperature and Time. *Journal of Agricultural and Food Chemistry*, *63*(16), 4190–4195. https://doi.org/10.1021/acs.jafc.5b00873

Ciulu, M., Oertel, E., Serra, R., Farre, R., Spano, N., Caredda, M., Malfatti, L., & Sanna, G. (2020). Classification of Unifloral Honeys from SARDINIA (Italy) by ATR-FTIR Spectroscopy and Random Forest. *Molecules*, *26*(1), 88. https://doi.org/10.3390/molecules26010088

Ciulu, M., Serra, R., Caredda, M., Salis, S., Floris, I., Pilo, M. I., Spano, N., Panzanelli, A., & Sanna, G. (2018). Chemometric treatment of simple physical and chemical data for the discrimination of unifloral honeys. *Talanta*, *190*, 382–390. https://doi.org/10.1016/j.talanta.2018.08.025

Ciulu, M., Solinas, S., Floris, I., Panzanelli, A., Pilo, M. I., Piu, P. C., Spano, N., & Sanna, G. (2011). RP-HPLC determination of water-soluble vitamins in honey. *Talanta*, *83*(3), 924–929. https://doi.org/10.1016/j.talanta.2010.10.059

Conti, M. E., Finoia, M. G., Fontana, L., Mele, G., Botrè, F., & Iavicoli, I. (2014). Characterization of Argentine honeys on the basis of their mineral content and some typical quality parameters. *Chemistry Central Journal*, *8*(1), 44. https://doi.org/10.1186/1752-153X-8-44

Cornara, L., Biagi, M., Xiao, J., & Burlando, B. (2017). Therapeutic Properties of Bioactive Compounds from Different Honeybee Products. *Frontiers in Pharmacology*, *8*, 412. https://doi.org/10.3389/fphar.2017.00412

Currie, L. A. (1999). Detection and quantification limits: Origins and historical overview1Adapted from the Proceedings of the 1996 Joint Statistical Meetings (American Statistical Association, 1997). Original title: "Foundations and future of detection and quantification limits". Contribution of the National Institute of Standards and Technology; not subject to copyright.1. *Analytica Chimica Acta*, *391*(2), 127–134. https://doi.org/10.1016/S0003-2670(99)00105-1

Czipa, N., Andrási, D., & Kovács, B. (2015). Determination of essential and toxic elements in Hungarian honeys. *Food Chemistry*, *175*, 536–542. https://doi.org/10.1016/j.foodchem.2014.12.018

Da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry*, *196*, 309–323. https://doi.org/10.1016/j.foodchem.2015.09.051

Danezis, G. P., & Georgiou, C. A. (2022). Elemental metabolomics: Food elemental assessment could reveal geographical origin. *Current Opinion in Food Science*, *44*, 100812. https://doi.org/10.1016/j.cofs.2022.100812

De Oliveira, F. A., De Abreu, A. T., De Oliveira Nascimento, N., Froes-Silva, R. E. S., Antonini, Y., Nalini, H. A., & De Lena, J. C. (2017). Evaluation of matrix effect on the determination of rare earth elements and As, Bi, Cd, Pb, Se and In in honey and pollen of native Brazilian bees (Tetragonisca angustula – Jataí) by Q-ICP-MS. *Talanta*, *162*, 488–494. https://doi.org/10.1016/j.talanta.2016.10.058

Denisow, B., & Denisow-Pietrzyk, M. (2016). Biological and therapeutic properties of bee pollen: A review. *Journal of the Science of Food and Agriculture*, *96*(13), 4303–4309. https://doi.org/10.1002/jsfa.7729

Di Bella, G., Potortì, A. G., Beltifa, A., Ben Mansour, H., Nava, V., & Lo Turco, V. (2021). Discrimination of Tunisian Honey by Mineral and Trace Element Chemometrics Profiling. *Foods*, *10*(4), 724. https://doi.org/10.3390/foods10040724

Drivelos, S. A., Danezis, G. P., Halagarda, M., Popek, S., & Georgiou, C. A. (2021). Geographical origin and botanical type honey authentication through elemental metabolomics via chemometrics. *Food Chemistry*, *338*, 127936. https://doi.org/10.1016/j.foodchem.2020.127936

Drivelos, S. A., & Georgiou, C. A. (2012). Multi-element and multi-isotope-ratio analysis to determine the geographical origin of foods in the European Union. *TrAC Trends in Analytical Chemistry*, *40*, 38–51. https://doi.org/10.1016/j.trac.2012.08.003

European Community. (2015). *Commission Regulation (EU) 2015/1005 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs.* https://eur-lex.europa.eu/eli/reg/2015/1005/oj

European Community. (2018). *Commission Regulation (EU) 2018/73 of 16 January 2018 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for mercury compounds in or on certain prod-ucts.* https://eur-lex.europa.eu/eli/reg/2018/73/oj

Floris, I., Pusceddu, M., & Satta, A. (2021). The Sardinian Bitter Honey: From Ancient Healing Use to Recent Findings. *Antioxidants*, *10*(4), 506. https://doi.org/10.3390/antiox10040506

Floris, I., Satta, A., & Ruiu, L. (2007). Honeys of Sardinia (Italy). *Journal of Apicultural Research*, *46*(3), 198–209. https://doi.org/10.1080/00218839.2007.11101393

Fodor, P., & Molnar, E. (1993). Honey as an environmental indicator: Effect of sample preparation on trace element determination by ICP-AES. *Mikrochimica Acta*, *112*(1–4), 113–118. https://doi.org/10.1007/BF01243327

Food and Agriculture organization of the United Nations (FAO). (2020). *Standard operating procedure for soil organic carbon. Walkley-Black method: Titration and colorimetric method*. https://www.fao.org/publications/card/en/c/CA7471EN/

Forte, G., D'Ilio, S., & Caroli, S. (2001). Honey as a Candidate Reference Material for Trace Elements. *Journal of AOAC INTERNATIONAL*, *84*(6), 1972–1975. https://doi.org/10.1093/jaoac/84.6.1972

Goretti, E., Pallottini, M., Rossi, R., La Porta, G., Gardi, T., Cenci Goga, B. T., Elia, A. C., Galletti, M., Moroni, B., Petroselli, C., Selvaggi, R., & Cappelletti, D. (2020). Heavy metal bioaccumulation in honey bee matrix, an indicator to assess the contamination level in terrestrial environments. *Environmental Pollution*, *256*, 113388. https://doi.org/10.1016/j.envpol.2019.113388

Grainger, M. N. C., Klaus, H., Hewitt, N., & French, A. D. (2021). Investigation of inorganic elemental content of honey from regions of North Island, New Zealand. *Food Chemistry*, *361*, 130110. https://doi.org/10.1016/j.foodchem.2021.130110

Horwitz, W. (1982). Evaluation of Analytical Methods Used for Regulation of Foods and Drugs. *Analytical Chemistry*, *54*(1), 67A-76A. https://doi.org/10.1021/ac00238a765

Istituto di Servizi per il Mercato Agricolo Alimentare (Ismea). (2019). *Il settore apistico: Analisi di mercato*. https://www.ismeamercati.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/9685

Istituto Nazionale di Statistica (Istat). (2022). *Principali statistiche geografiche sui comuni*. https://www.istat.it/it/archivio/156224

Kastrati, G., Paçarizi, M., Sopaj, F., Tašev, K., Stafilov, T., & Mustafa, M. K. (2021). Investigation of Concentration and Distribution of Elements in Three Environmental Compartments in the Region of Mitrovica, Kosovo: Soil, Honey and Bee Pollen. *International Journal of Environmental Research and Public Health*, *18*(5), 2269. https://doi.org/10.3390/ijerph18052269

Langasco, I., Barracu, F., Deroma, M. A., López-Sánchez, J. F., Mara, A., Meloni, P., Pilo, M. I., Estrugo, À. S., Sanna, G., Spano, N., & Spanu, A. (2022). Assessment and validation of ICP-MS and IC-ICP-MS methods for the determination of total, extracted and speciated arsenic. Application to samples from a soil-rice system at varying the irrigation method. *Journal of Environmental Management*, *302*, 114105. https://doi.org/10.1016/j.jenvman.2021.114105

Leardi, R., Melzi, C., & Polotti, G. (2024). *CAT (Chemometric Agile Software)*. http://gruppochemiometria.it/index.php/software

Leme, A. B. P., Bianchi, S. R., Carneiro, R. L., & Nogueira, A. R. A. (2014). Optimization of Sample Preparation in the Determination of Minerals and Trace Elements in Honey by ICP-MS. *Food Analytical Methods*, *7*(5), 1009–1015. https://doi.org/10.1007/s12161-013-9706-5

Machado De-Melo, A. A., Almeida-Muradian, L. B. D., Sancho, M. T., & Pascual-Maté, A. (2018). Composition and properties of *Apis mellifera* honey: A review. *Journal of Apicultural Research*, *57*(1), 5–37. https://doi.org/10.1080/00218839.2017.1338444

Mara, A., Langasco, I., Deidda, S., Caredda, M., Meloni, P., Deroma, M., Pilo, M. I., Spano, N., & Sanna, G. (2021). ICP-MS Determination of 23 Elements of Potential Health Concern in Liquids of e-Cigarettes. Method Development, Validation, and Application to 37 Real Samples. *Molecules*, *26*(21), 6680. https://doi.org/10.3390/molecules26216680

Muller, E. I., Muller, C. C., Souza, J. P., Muller, A. L. H., Enders, M. S. P., Doneda, M., Frohlich, A. C., Iop, G. D., & Anschau, K. F. (2017). Green microwave-assisted wet digestion method of carbohydrate-rich foods with hydrogen peroxide using single reaction chamber and further elemental determination using ICP-OES and ICP-MS. *Microchemical Journal*, *134*, 257–261. https://doi.org/10.1016/j.microc.2017.06.012

Oliveira, S. S., Alves, C. N., Boa Morte, E. S., De Freitas Santos Júnior, A., Araujo, R. G. O., & Santos, D. C. M. B. (2019). Determination of essential and potentially toxic elements and their estimation of bioaccessibility in honeys. *Microchemical Journal*, *151*, 104221. https://doi.org/10.1016/j.microc.2019.104221

Oliveri, P., Malegori, C., Mustorgi, E., & Casale, M. (2021). Qualitative pattern recognition in chemistry: Theoretical background and practical guidelines. *Microchemical Journal*, *162*, 105725. https://doi.org/10.1016/j.microc.2020.105725

Oliveri, P., Malegori, C., Simonetti, R., & Casale, M. (2019). The impact of signal pre-processing on the final interpretation of analytical outcomes – A tutorial. *Analytica Chimica Acta*, *1058*, 9–17. https://doi.org/10.1016/j.aca.2018.10.055

Perna, A. M., Grassi, G., Gambacorta, E., & Simonetti, A. (2021). Minerals content in Basilicata region (southern Italy) honeys from areas with different anthropic impact. *International Journal of Food Science & Technology*, *56*(9), 4465–4472. https://doi.org/10.1111/ijfs.15112

Pisani, A., Protano, G., & Riccobono, F. (2008). Minor and trace elements in different honey types produced in Siena County (Italy). *Food Chemistry*, *107*(4), 1553–1560. https://doi.org/10.1016/j.foodchem.2007.09.029

Pohl, P., Bielawska-Pohl, A., Dzimitrowicz, A., Jamroz, P., Welna, M., Lesniewicz, A., & Szymczycha-Madeja, A. (2017). Recent achievements in element analysis of bee honeys by atomic and mass spectrometry methods. *TrAC Trends in Analytical Chemistry*, *93*, 67–77. https://doi.org/10.1016/j.trac.2017.05.009

Quinto, M., Miedico, O., Spadaccino, G., Paglia, G., Mangiacotti, M., Li, D., Centonze, D., & Chiaravalle, A. E. (2016). Characterization, chemometric evaluation, and human health-related aspects of essential and toxic elements in Italian honey samples by inductively coupled plasma mass spectrometry. *Environmental Science and Pollution Research*, *23*(24), 25374–25384. https://doi.org/10.1007/s11356-016-7662-5

Rahman, M. M., Alam, Md. N., Fatima, N., Shahjalal, H. Md., Gan, S. H., & Khalil, Md. I. (2017). Chemical composition and biological properties of aromatic compounds in honey: An overview. *Journal of Food Biochemistry*, *41*(6), e12405. https://doi.org/10.1111/jfbc.12405

Rashed, M. N., El-Haty, M. T. A., & Mohamed, S. M. (2009). Bee honey as environmental indicator for pollution with heavy metals. *Toxicological & Environmental Chemistry*, *91*(3), 389–403. https://doi.org/10.1080/02772240802294870

Sadowska, M., Hyk, W., Ruszczyńska, A., Roszak, A., Mycka, A., & Krasnodębska-Ostręga, B. (2021). Statistical evaluation of the effect of sample preparation procedure on the results of determinations of selected elements in environmental samples. Honey bees as a case study. *Chemosphere*, *279*, 130572. https://doi.org/10.1016/j.chemosphere.2021.130572

Salis, S., Spano, N., Ciulu, M., Floris, I., Pilo, M. I., & Sanna, G. (2021). Electrochemical Determination of the "Furanic Index" in Honey. *Molecules*, *26*(14), 4115. https://doi.org/10.3390/molecules26144115

Sanna, G., Pilo, M. I., Piu, P. C., Tapparo, A., & Seeber, R. (2000). Determination of heavy metals in honey by anodic stripping voltammetry at microelectrodes. *Analytica Chimica Acta*, *415*(1–2), 165–173. https://doi.org/10.1016/S0003-2670(00)00864-3

Satta, A., Verdinelli, M., Ruiu, L., Buffa, F., Salis, S., Sassu, A., & Floris, I. (2012). Combination of beehive matrices analysis and ant biodiversity to study heavy metal pollution impact in a post-mining area (Sardinia, Italy). *Environmental Science and Pollution Research*, *19*(9), 3977–3988. https://doi.org/10.1007/s11356-012-0921-1

Solayman, Md., Islam, Md. A., Paul, S., Ali, Y., Khalil, Md. I., Alam, N., & Gan, S. H. (2016). Physicochemical Properties, Minerals, Trace Elements, and Heavy Metals in Honey of Different Origins: A Comprehensive Review. *Comprehensive Reviews in Food Science and Food Safety*, *15*(1), 219–233. https://doi.org/10.1111/1541-4337.12182

Spano, N., Casula, L., Panzanelli, A., Pilo, M., Piu, P., Scanu, R., Tapparo, A., & Sanna, G. (2006). An RP-HPLC determination of 5-hydroxymethylfurfural in honeyThe case of strawberry tree honey. *Talanta*, *68*(4), 1390–1395. https://doi.org/10.1016/j.talanta.2005.08.003

Spano, N., Ciulu, M., Floris, I., Panzanelli, A., Pilo, M. I., Piu, P. C., Scanu, R., & Sanna, G. (2008). Chemical characterization of a traditional honey-based Sardinian product: Abbamele. *Food Chemistry*, *108*(1), 81–85. https://doi.org/10.1016/j.foodchem.2007.10.046

Spano, N., Piras, I., Ciulu, M., Floris, I., Panzanelli, A., Pilo, M. I., Piu, P. C., & Sanna, G. (2009). Reversed-Phase Liquid Chromatographic Profile of Free Amino Acids in Strawberry-tree (Arbutus unedo L.) Honey. *Journal of AOAC INTERNATIONAL*, *92*(4), S73–S84. https://doi.org/10.1093/jaoac/92.4.S73

Spanu, A., Langasco, I., Valente, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2020). Tuning of the Amount of Se in Rice (Oryza sativa) Grain by Varying the Nature of the Irrigation Method: Development

of an ICP-MS Analytical Protocol, Validation and Application to 26 Different Rice Genotypes. *Molecules*, *25*(8), 1861. https://doi.org/10.3390/molecules25081861

Spanu, A., Valente, M., Langasco, I., Leardi, R., Orlandoni, A. M., Ciulu, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2020). Effect of the irrigation method and genotype on the bioaccumulation of toxic and trace elements in rice. *Science of The Total Environment*, *748*, 142484. https://doi.org/10.1016/j.scitotenv.2020.142484

Tariba Lovaković, B., Lazarus, M., Brčić Karačonji, I., Jurica, K., Živković Semren, T., Lušić, D., Brajenović, N., Pelaić, Z., & Pizent, A. (2018). Multi-elemental composition and antioxidant properties of strawberry tree (Arbutus unedo L.) honey from the coastal region of Croatia: Risk-benefit analysis. *Journal of Trace Elements in Medicine and Biology*, *45*, 85–92. https://doi.org/10.1016/j.jtemb.2017.09.022

Tuzen, M., Silici, S., Mendil, D., & Soylak, M. (2007). Trace element levels in honeys from different regions of Turkey. *Food Chemistry*, *103*(2), 325–330. https://doi.org/10.1016/j.foodchem.2006.07.053

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., & Pérez-Álvarez, J. A. (2008). Functional Properties of Honey, Propolis, and Royal Jelly. *Journal of Food Science*, *73*(9). https://doi.org/10.1111/j.1750-3841.2008.00966.x

Voyslavov, T., Mladenova, E., & Balkanska, R. (2021). A New Approach for Determination of the Botanical Origin of Monofloral Bee Honey, Combining Mineral Content, Physicochemical Parameters, and Self-Organizing Maps. *Molecules*, *26*(23), 7219. https://doi.org/10.3390/molecules26237219

Yayinie, M., & Atlabachew, M. (2022). Multi-element Analysis of Honey from Amhara Region-Ethiopia for Quality, Bioindicator of Environmental Pollution, and Geographical Origin Discrimination. *Biological Trace Element Research*, *200*(12), 5283–5297. https://doi.org/10.1007/s12011-021-03088-0

Zhang, P., Georgiou, C. A., & Brusic, V. (2017). Elemental metabolomics. *Briefings in Bioinformatics*, bbw131. https://doi.org/10.1093/bib/bbw131

Published on Foods, 2024, https://doi.org/10.3390/foods13020243

ELEMENTAL FINGERPRINTING COMBINED WITH MACHINE LEARNING TECHNIQUES AS A POWERFUL TOOL FOR GEOGRAPHICAL DISCRIMINATION OF HONEYS FROM NEARBY REGIONS

2.1. INTRODUCTION

Honey is a natural sweet food produced by bees (*Apis mellifera*) with numerous nutraceutical and therapeutical properties (Afrin et al., 2020; Almasaudi, 2021; Alvarez-Suarez et al., 2010; Cornara et al., 2017; Da Silva et al., 2016; Martinello & Mutinelli, 2021; Ranneh et al., 2021). Several factors, including botanical origin, environmental conditions, and bee species, impact the composition of honey and play a key role in determining its sensorial and health-promoting properties (Da Silva et al., 2016). Owing to its unique qualities, honey is particularly vulnerable to unauthorized food manipulation. Adulteration and mislabeling are the most prevalent forms of honey fraud (Jurica et al., 2021). According to European (EU) legislation (Thrasyvoulou et al., 2018), honey labeling must indicate EU or non-EU origins, whereas botanical and local geographical origins are optional (Mădaş et al., 2020). Nevertheless, because these factors strongly affect the economic value and price of honey, they are frequently falsified. This type of counterfeiting has a remarkable economic impact, especially on small beekeepers who tend to produce rare or unifloral honey (European Commission, 2023). For these reasons, there is considerable scientific interest in developing innovative analytical methods to verify the honey authenticity (Tsagkaris et al., 2021).

Honeys' botanical origin is generally ascertained through melissopalynological analysis. However, this method is ineffective if the honey has been filtered. On the other hand, there are no standard methods for determining geographical origin. Current methods involve advanced analytical tools coupled with chemometrics (X.-H. Zhang et al., 2023). Physicochemical (Ciulu et al., 2018; Demir Kanbur et al., 2021; Fernández-Estellé et al., 2023; Solayman et al., 2016), elemental (Pohl et al., 2017), isotopic (Drivelos & Georgiou, 2012), chromatographic and hyphenated mass spectrometry methods (Hernanz et al., 2023; Wei et al., 2023), NMR (Lemus Ringele et al., 2022; Schievano et al., 2023), DNA-

based (Mohamadzade Namin et al., 2022; Q. Wu et al., 2024), electronic sensing (Masoomi et al., 2024; romano et al., 2016) and spectroscopic (Caredda et al., 2023; Ciulu et al., 2020; Egido et al., 2024; Suhandy et al., 2023; Suhandy & Yulia, 2021) techniques are nowadays the most used for predicting botanical and geographical origin or detecting adulterants (Wang et al., 2021). Usually, botanical issue is investigated using chromatographic techniques and physicochemical analyses (Ciulu et al., 2018; García-Seval et al., 2022; Tsagkaris et al., 2021). However, while vibrational spectroscopy is commonly used to detect adulterated honeys (Tsagkaris et al., 2021; Wang et al., 2021), it is also highly effective in predicting the authenticity of almost all types of honey. Furthermore, the use of portable instrumentation enhances the versatility of this approach (Escuredo et al., 2021; Guelpa et al., 2017). Finally, elemental, and isotopic methods are prevalently used for geographical origin discrimination (Drivelos et al., 2021; Drivelos & Georgiou, 2012), but they are also useful in determining botanical origin (Drivelos et al., 2021; Mara et al., 2022). Elements in honey reflect the soil composition (Kastrati et al., 2023), flora (Voyslavov et al., 2021), and anthropogenic activities (Satta et al., 2012). The translocation of elements is influenced by season (Bilandžić et al., 2019), bee species (J. Wu et al., 2020), and, in general, by environmental conditions (P. Zhang et al., 2017). Furthermore, honey reflects the urbanization grade (Girolametti et al., 2023; Grainger et al., 2021; Kastrati et al., 2023). All these factors determine the elemental fingerprint of honey, allowing its authentication in terms of both geographical and botanical origins (Danezis & Georgiou, 2022).

The elemental composition of honey has been characterized worldwide. Recent studies investigated honeys from Argentine (Pellerano et al., 2012), Bulgaria (Atanassova et al., 2016; Pavlin et al., 2023; Voyslavov et al., 2021), China (Liu et al., 2021; F. Wu et al., 2021; J. Wu et al., 2020; Zhu et al., 2020), Croatia (Bilandžić et al., 2017, 2019; Lazarus et al., 2021; Pavlin et al., 2023), Denmark (Grainger et al., 2023), Egypt (Karabagias et al., 2017), Eritrea (Czipa et al., 2023), Ethiopia (Yayinie & Atlabachew, 2022), France (Magdas et al., 2021), Germany (Grainger et al., 2023), Greece (Drivelos et al., 2021; Karabagias et al., 2017; Louppis et al., 2017), Hungary (Bodó et al., 2021; Czipa et al., 2015), Israel (Bommuraj et al., 2019), Italy (Conti et al., 2022; Di Bella et al., 2015; Drivelos et al., 2021; Giglio

et al., 2017; Girolametti et al., 2023; Gulino et al., 2023; Mara et al., 2022; Perna et al., 2021; Quinto et al., 2016; Scivicco et al., 2022; Squadrone, Brizio, Stella, Mantia, et al., 2020; Squadrone, Brizio, Stella, Pederiva, et al., 2020), Kazakhstan (Squadrone, Brizio, Stella, Mantia, et al., 2020), Kosovo (Đogo Mračević et al., 2020), Montenegro (Vukašinović-Pešić et al., 2020), Morocco (Karabagias et al., 2017; Pavlin et al., 2023), New Zealand (Grainger et al., 2021, 2023), Poland (Drivelos et al., 2021), Romania (Drivelos et al., 2021; Magdas et al., 2021), Serbia (Đogo Mračević et al., 2020), Slovenia (Pavlin et al., 2023), Spain (Álvarez-Ayuso & Abad-Valle, 2017; Díaz et al., 2019; Karabagias et al., 2017; Rodríguez-Flores et al., 2016; Serra Bonvehi et al., 2019), Thailand (Kastrati et al., 2023), Tunisia (Di Bella et al., 2015), and Turkey (Demir Kanbur et al., 2021; Pavlin et al., 2023). Typically, data management and elaboration involve multivariate analysis and machine learning techniques, including Principal Component Analysis (PCA) (Mara et al., 2022; Yayinie & Atlabachew, 2022), Cluster Analysis (CA) (Drivelos et al., 2021; Kastrati et al., 2023), Linear Discriminant Analysis (LDA) (Karabagias et al., 2017; Yayinie & Atlabachew, 2022), Partial Least Squares regression (PLS)(Drivelos et al., 2021; F. Wu et al., 2021), Decision Tree Analysis (DTA) (Grainger et al., 2023), Self-Organizing Maps (SOMs) (Voyslavov et al., 2021), and Soft Independent Modelling of Class Analogy (SIMCA) (Magdas et al., 2021). Few papers in the literature report cases where elemental fingerprinting discriminated honeys according to their botanical and geographical origin (Bilandžić et al., 2019; Drivelos et al., 2021; Magdas et al., 2021; Pavlin et al., 2023). Research often focuses on authenticating honeys from various origins based on botanical sources or regions with unique climates, soils, or levels of urbanization. European legislation requires differentiation between EU and non-EU honeys, which is usually feasible due to the numerous environmental factors that affect their elemental fingerprints. On the other hand, discriminating honey from nearby areas is a more challenging task.

The main goal of this study is to distinguish unifloral and multifloral honeys from two nearby regions. In this context, Sardinia (Italy) and Spain have been chosen as a case study. Geologically, the Corsica-Sardinian microplate separated from the Iberian Peninsula during the Miocene. Therefore, the soil composition of Sardinia is more similar to that of southeastern Spain than Italy (Gattacceca et al., 2007). In addition, the two regions have similar climatic conditions.

A comprehensive selection of multifloral and unifloral honeys from common botanical species, such as rosemary and eucalyptus, was considered for this purpose. The physicochemical characterization of eucalyptus and rosemary honeys from Spain (Gomez et al., 1993; Perezarquillue et al., 1994) and Italy (*Persano Oddo, L; Sabatini, AG; Accorti, M; Colombo, R; Marcazzan, GL; Piana, L; Piazza, MG; Pulcini, P. I Mieli Uniflorali Italiani. Nuove Schede Di Caratterizzazione*, 2000a; *Persano Oddo, L; Sabatini, AG; Accorti, M; Colombo, R; Marcazzan, GL; Piana, L; Piazza, MG; Pulcini, P. I Mieli Uniflorali Italiani. Nuove Schede Di Caratterizzazione*, 2000b) have been reported in literature. Possible differences among the eucalyptus and rosemary honeys are related to their diastase activity and acidity parameters. The diastase activity of Italian eucalyptus honey samples is higher than that of Spanish ones, whereas the acidity parameters of Spanish eucalyptus honey samples are higher than those of Italian ones. Additionally, Spanish rosemary honey samples exhibit higher diastase activity than Italian ones. Furthermore, some typical unifloral honeys from Sardinia, such as strawberry tree, asphodel, and thistle honey (Floris et al., 2007), were analyzed. Strawberry tree honey is famous worldwide for its unique “bitter” taste (Cabras et al., 1999) and for their healing properties (Afrin et al., 2017). The chemical composition of strawberry tree honey confirms its botanical origin due to the presence of high concentrations (several hundred mg kg^{-1}) of homogentisic acid (Cabras et al., 1999; Scanu et al., 2005), a compound not found in other unifloral honeys. The botanical origin of asphodel honey is also guaranteed by the peculiar presence of methyl syringate in it (Tuberoso et al., 2009). Among all the Sardinian unifloral honeys considered in this study, the thistle honey is characterized by the lowest values of pH, electrical conductivity and color (Ciulu et al., 2018).

The honeys elemental fingerprints were determined using a validated Inductively Coupled Plasma Mass Spectrometry (ICP-MS) method (Mara et al., 2022). Four major elements (Na, Mg, K, Ca), twenty-three among trace and toxic elements (Ag, As, Ba, Be, Bi, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sb, Sn, Sr, Te, Tl, V, Zn) and fourteen lanthanides (La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu) were analyzed. The data were processed using PCA for multivariate data visualization, whereas LDA and Random Forest were used for honey classification.

2.2. MATERIALS AND METHODS

2.2.1. HONEY SAMPLES

The study analyzed honeys from two areas in the Western Mediterranean: Spain (SPA) and Sardinia (Italy, ITA). The geographical and botanical origins of the honey are shown in Figure 2.1.

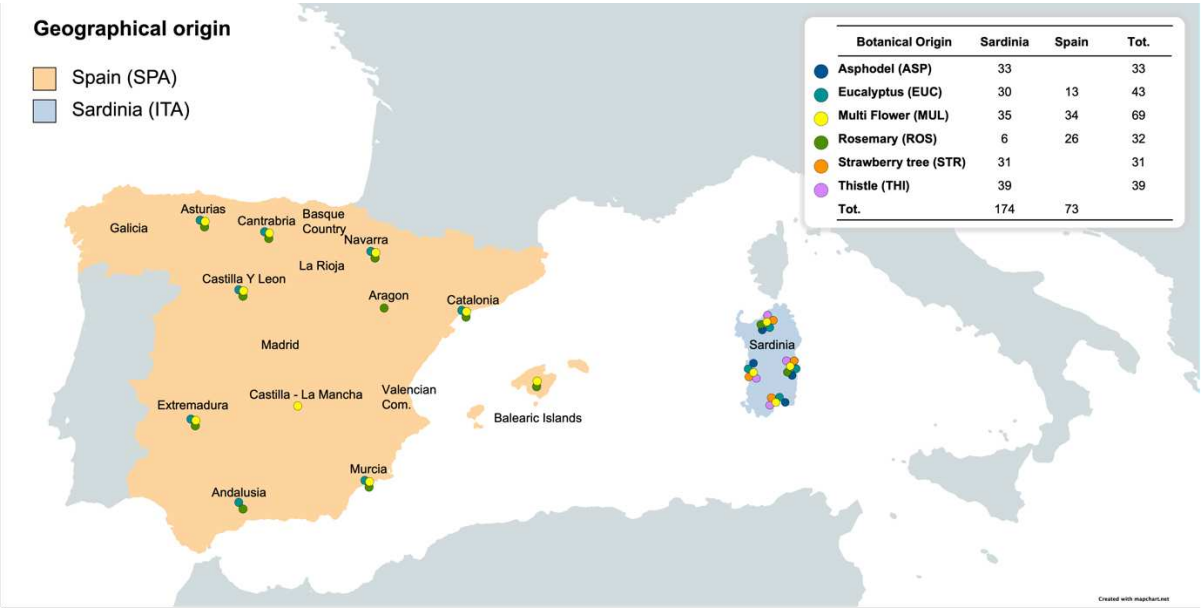


Figure 2.1. Geographical and botanical origins of honey samples.

In total, 247 honey samples from Spain (SPA = 73) and Sardinia (ITA = 174) were examined. The sample set consisted of both multifloral and unifloral honeys. Among the common honeys from the two regions, multifloral (MUL, SPA = 34, ITA = 35), eucalyptus (EUC, SPA = 13, ITA = 30), and rosemary (ROS, SPA = 26, ITA = 6) were included. Additionally, three characteristic unifloral Sardinian honeys, asphodel (ASP, ITA = 33), strawberry tree (STR, ITA = 31), and thistle (THI, ITA = 39), were considered for the geographical attribution. The botanical origin of the samples was determined by melissopalynological analysis. The collection was recorded between 2020 and 2022, reflecting the flowering and seasonality of the botanical sources. In general, eucalyptus, thistle and multifloral honeys were produced in spring, summer and fall, respectively. Rosemary and strawberry tree honeys were collected in fall and winter, while asphodel honeys were produced in winter and spring. Sardinian honeys were gathered throughout the island (Figure 2.1), whereas the Spanish honeys were from

Andalusia, Aragon, Asturias, Cantabria, Castilla la Mancha, Castilla-Leon, Catalonia, Extremadura, Balearic Islands, Navarre, and Basque Country (Figure 2.1). Previous studies [81] have reported details on the chemical-physical and sensory characteristics of the honeys, such as color, pH, moisture, taste, and botanical markers. Honeys were stored in the dark at 4 °C until analysis.

2.2.2. INSTRUMENTATION AND REAGENTS

The elemental analysis was performed using a NexION 300X ICP-MS spectrometer from Perkin Elmer (Milan, Italy). The spectrometer was equipped with an S10 autosampler, glass concentric nebulizer, glass cyclonic spray chamber, and kinetic energy discrimination (KED) collision cell. Microwave acid digestion was performed using an ultraWAVE™ from Milestone (Sorisole, Italy) equipped with a single reaction chamber (SCR) system, a fifteen-position rotor, and polytetrafluoroethylene (PTFE) vessels (15 cm^3). Dry ashing was performed using a Controller P320 muffle from Nabertherm (Lilienthal, Germany). Samples were homogenized before analysis using an Ultraturrax mixer model T18 (Staufen, Germany). Nylon filters (pore diameter, 0.22 µm), syringes, metal-free polypropylene tubes (15 and 50 cm^3), and porcelain crucibles (150 cm^3) were supplied by VWR (Milan, Italy).

A MilliQ Plus System (Millipore, Vimodrone, Italy) was used to produce type I water (resistivity >18 $M\Omega\ cm^{-1}$). Nitric acid (67–69% w/w, NORMATON® for ultra-trace analysis) and hydrogen peroxide (30% w/w, NORMATON® for ultra-trace metal analysis) were supplied by VWR (Milan, Italy). Periodic table mix 1 for ICP (TraceCert®, 33 elements, 10 mg dm^{-3} in 10% HNO_3), periodic table mix 3 for ICP (TraceCert®, 16 elements, 10 mg dm^{-3} in 5% HNO_3), Rh solution (1000 mg dm^{-3} in 3% HNO_3), apple leaves NIST SRM® 1515 and mussel tissue BCR 668 were from Sigma-Aldrich (St. Louis, USA). Single standard solutions of Na, Mg, K, Ca, Hg, Mo, Sb, and Sn (100-1000 mg dm^{-3} in 2-5% HNO_3) were obtained from Carlo Erba (Milan, Italy).

2.2.3. SAMPLE PREPARATION

Sample preparation involved the use of microwave acid digestion and dry ashing techniques. Microwave acid digestion was utilized to determine macroelements, trace elements, and toxic elements, while dry ashing was employed for lanthanide analysis (Gulino et al., 2023). Microwave acid digestion, performed according to a previously described method (Mara et al., 2022), was optimized by a 2^2 full factorial experimental design. In this manner, the residual carbon and acidity levels were minimized, preventing the need for unnecessary dilutions and reducing the matrix effect. Initially, the samples were homogenized at 40 °C. Then, approximately 0.700 g of honey was weighed in 15 cm^3 PTFE vessels and treated with 0.5 cm^3 of HNO_3, 3 cm^3 of H_2O_2, and 4 cm^3 of type I water. After digestion at 240 °C, samples were collected, diluted to 15 cm^3, and filtered before analysis. Dry ashing was performed weighing about 5.0 g of honey in porcelain crucibles (150 cm^3). After ashing at 600 °C, samples were treated with 10 cm^3 of 5% HNO_3 aqueous solution, diluted to 15 cm^3, and filtered before analysis. Table B1 reports the operational conditions of both methods.

2.2.4. ELEMENTAL ANALYSIS

A previously developed and validated procedure (Mara et al., 2022) was used on a NexION 300X ICP-MS (Perkin Elmer) to perform elemental analysis. Here, detailed information regarding instrumental parameters, elemental settings, method assessment, performance, quality control, and validation is reported. The literature method for the analysis of trace and toxic elements (i.e., Ag, As, Ba, Be, Bi, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sb, Sn, Sr, Te, Tl, V, Zn) in honey (Mara et al., 2022) was implemented to analyze macro elements (i.e., Na, Mg, K, Ca) and lanthanides (i.e., La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Ho, Er, Tm, Yb, Lu). Tables B2 and Table B3 report the instrumental conditions and elemental settings for the analysis of macro elements and lanthanides, respectively. Trueness was evaluated by analyzing two certified reference materials, apple leaves NIST SRM® 1515 and mussel tissue BCR 668. The results are presented in Table B4.

2.2.5. STATISTICAL ANALYSIS

Data analysis was conducted using the statistical freeware software R (v. 4.3.1) run in the free integrated development environment R-Studio (v. 2023.3.1), GraphPad Prism (v. 9.1.0 221), and Chemometric Agile Tool (CA) (Leardi et al., 2021). For data visualization, PCA was performed removing all elements that were rarely quantified. Compositional data analysis (CoDa) was applied as a data pre-treatment before PCA to improve the interpretability using centered log-ratio transformation (Templ & Templ, 2021). To remove missing values (i.e., those below the limit of detection or quantification), the dl23 method (two-thirds of the limit of detection) was used (Templ & Templ, 2021). To classify honeys according to botanical origin, geographical origin, and their combination, LDA was performed. A RF machine-learning algorithm was also used (R package randomForest, (Liaw & Wiener, 2001)). Data for each factor (i.e. geographical, botanical origin, and their combination) were extracted and divided into train and test sets. The train set included an equal number of samples from each group, equivalent to half of the least populated group. The remaining data constituted the test set. The classification algorithms were applied by removing chemical elements with missing (i.e., below detection or quantification limits) or repeated values, iterating 100 times both train and test sampling to increase the statistical significance. Accuracy was measured by averaging over the accuracies obtained in the 100 replicas. In particular, RF analysis was performed using 1000 trees and setting the mtry parameter equal to the square root of the number of predictors (Liaw & Wiener, 2001). To find the smallest set of chemical elements required to efficiently discriminate between groups, the importance for the classification of variables was first measured using the mean decrease in the Gini index (Liaw & Wiener, 2001; C. Zhang & Ma, 2012). Predictors with the lower Gini index were removed and the analysis was iterated with the remaining predictors. The smallest set of chemical elements was identified as the one preceding a visible reduction in the overall classification accuracy. Statistical significance was set at $p < 0.05$.

2.3. RESULTS

2.3.1. ELEMENTAL FINGERPRINTS

Data relative to the elemental composition of honeys from Spain and Sardinia (Italy) are reported in Table B5. For each type of honey, the minimum, average, and maximum concentrations are reported. K was generally the most abundant macroelement in all honeys, followed by Ca, Na, and Mg. The most abundant trace elements were Zn, Cu, Mn, Sr, Ba, Fe, and Ni. The remaining trace elements were present at lower concentrations or below the limit of quantification (LoQ). Li was only quantified in EUC and STR. As, Cd, Pb, Sn, and Tl were rarely quantified, while Be, Bi, Hg, Sb, and Te were always below the relevant LoQs. Finally, the concentrations of lanthanides (range between μg kg^{-1} and ng kg^{-1}) followed the pattern predicted by the Oddo-Harkins rule, which holds that the elements with even atomic numbers are more abundant than those with immediately adjacent atomic numbers. Notably, Eu deviated from the expected trend in all Spanish honeys.

2.3.2. PRINCIPAL COMPONENT ANALYSIS

Before conducting the PCA, the data underwent a centered log-ratio transformation. This pre-treatment enhances the interpretability of the PCA outcomes by emphasizing sample percentage compositions. For comparison, Figure B1 shows the PCA performed with standardization. The comparison indicates that the centered log-ratio transformation increases the variance of PC2. Thus, scores and loadings were more scattered.

Figure 2.2 shows the loading and score plots of the PCA, with the first two components accounting for 42.7% and 11.6% of the total variance, respectively (Scree plot, Figure B2).

From the loading plot (Figure 2.2A), positive PC1 values indicate a greater percentage of macro and trace elements, whereas negative values indicate a higher percentage of lanthanides. On the other hand, PC2 distinguishes by negative values the alkaline and alkaline earth elements (Na, Mg, K, Ca, Ba, Sr), and by positive values the transition metals (Cu, Ni, Mn, Zn). Notably, Fe shows a higher correlation

with the cluster formed by alkaline and alkaline-earth elements than with that formed by transition trace elements.

Looking at the score plots (Figures 2.2B-H), objects are colored to highlight honeys according to geographical (Figure 2.2B) and botanical origins (Figure 2.2C), common botanical origins to both geographical areas (Figures 2.2D-G), and uncommon origins (Figure 2.2H). Overall, samples exhibit differentiation based on geographical origin along PC1 (Figure 2.2B), whereas the discrimination based on botanical origin is less evident (Figure 2.2C). However, when considering the unifloral honeys that are common in Sardinia and Spain (Figures 2.2D-G), the distinction between the two regions is less evident. Only eucalyptus honeys were separated (Figure 2.2F). Finally, as expected, the groups formed by samples from uncommon origins were the most differentiated ones (Figure 2.2H).

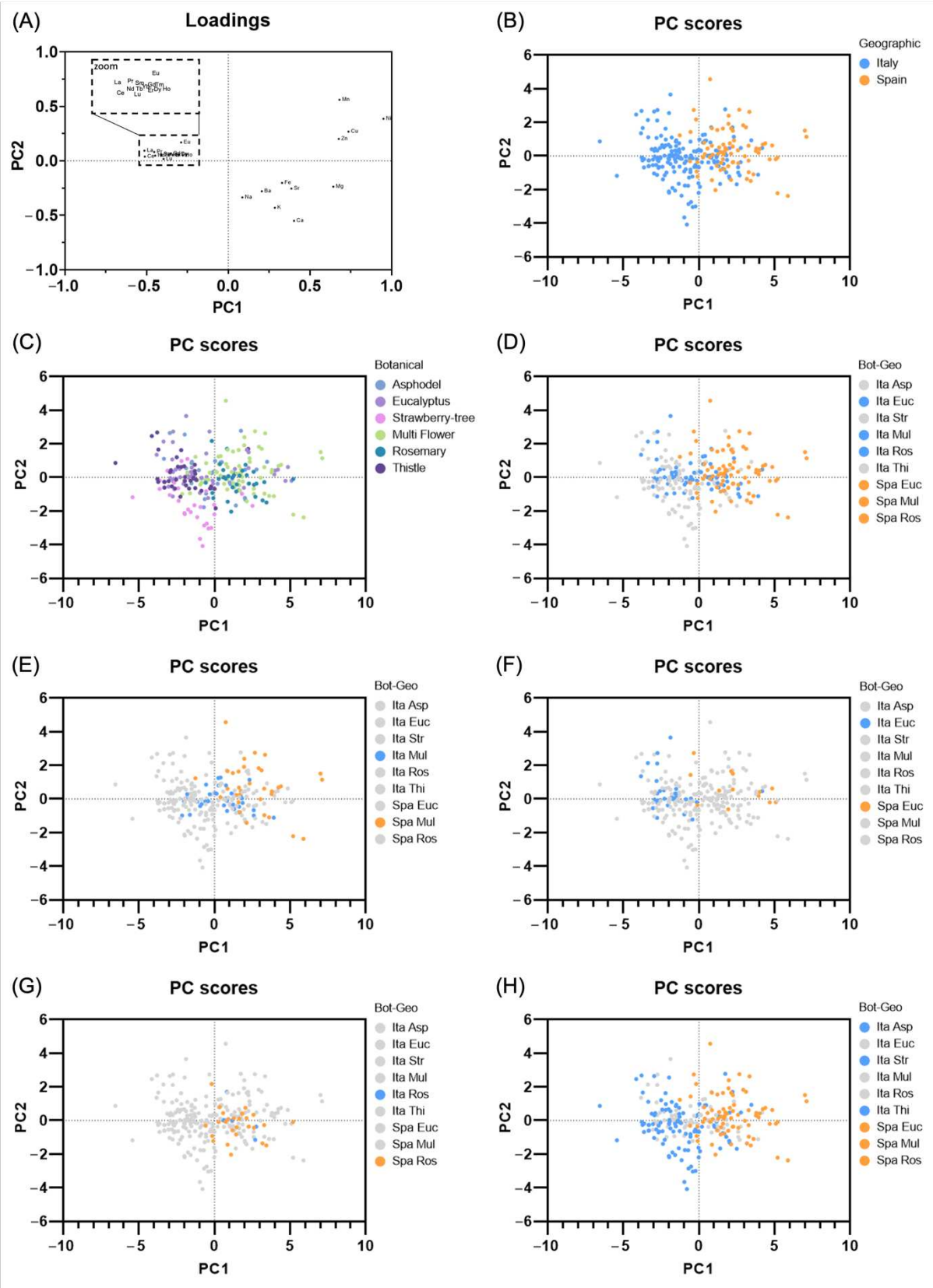


Figure 2.2. PCA analysis. A) loading plot; B) score plot, object colored according to geographical origin; C) score plot, object colored according to botanical origin; D) score plot, object colored according to common botanical origin; E) score plot, object colored according to multi flower honey; F) score plot, object colored according to eucalyptus honey; G) score plot, object colored according to rosemary honey; H) score plot, object colored according to uncommon origins.

2.3.3. CLASSIFICATION BY LDA AND RF

Honeys were classified according to their geographical and botanical origins. For this purpose, LDA and RF were used and compared. Table 2.1 reports the results obtained.

Table 2.1. Linear Discrimination Analysis (LDA) and Random Forest (RF) for honey classification according to their origins. Results are expressed as percentage accuracy (± standard deviation).

Geographical Origin	**LDA**		**Random Forest**	
	Train	**Test**	**Train**	**Test**
ITA	91 ± 5	78 ± 6	91 ± 3	88 ± 4
SPA	92 ± 4	79 ± 8	92 ± 3	91 ± 5
Botanical Origin	**LDA**		**Random Forest**	
	Train	**Test**	**Train**	**Train**
ASP	91 ± 6	83 ± 9	77 ± 9	79 ± 9
EUC	84 ± 8	63 ± 9	80 ± 10	81 ± 9
MUL	80 ± 10	50 ± 10	45 ± 10	47 ± 7
ROS	74 ± 9	50 ± 15	70 ± 10	70 ± 10
STR	90 ± 5	70 ± 10	78 ± 8	80 ± 10
THI	76 ± 9	60 ± 10	76 ± 9	78 ± 9
Geographical and Botanical	**LDA**		**Random Forest**	
Origins	**Train**	**Test**	**Train**	**Test**
ITA ASP	90 ± 7	82 ± 9	75 ± 10	78 ± 9
ITA EUC	89 ± 6	80 ± 10	83 ± 7	84 ± 9
ITA MUL	80 ± 7	60 ± 10	65 ± 10	69 ± 9
ITA ROS	98 ± 7	50 ± 30	20 ± 30	30 ± 20
ITA STR	89 ± 6	70 ± 10	70 ± 10	70 ± 10
ITA THI	74 ± 9	60 ± 10	69 ± 8	70 ± 10
SPA EUC	80 ± 15	40 ± 20	40 ± 30	50 ± 20
SPA MUL	70 ± 10	30± 10	50 ± 10	50 ± 10
SPA ROS	75 ± 10	50 ± 20	74 ± 9	80 ± 10

Both LDA and RF perform well in classifying botanical origin across different categories. Notably, both algorithms accurately predicted the asphodel and strawberry-tree honeys during training and testing. Conversely, multifloral and rosemary honeys were more difficult to classify. In both training and testing sets, these categories show lower accuracy scores than others. The two algorithms demonstrate excellent performance in classifying samples from Sardinia (Italy) and Spain based on their geographical origin. However, when considering both geographical and botanical origins, the accuracy of LDA and RF tends to decrease. Overall, the algorithms were better at predicting geographical origin than botanical origin, while predicting both origins at the same time posed a greater challenge. LDA and RF demonstrated accuracy and competitiveness across various categories

and origins. Nevertheless, RF showed more stable performance and better agreement between training and testing.

Additionally, the algorithm allows to directly assess which elements were the most important for the models. The classification accuracy was evaluated by iterating the calculations while varying the number of predictors (Figure 2.3). The reported results indicate that Na, K, Ca, Mn, Sr, Ce, Eu, and Lu are the most significant elements for classifying honeys based on their botanical origin. The elements Na, Mg, Mn, Sr, Zn, Ce, Nd, Eu, and Tb are the most effective predictors for classifying geographical origin. Na, K, Mn, Sr, Ce, Eu, and Lu are the most relevant elements for both origin classifications.

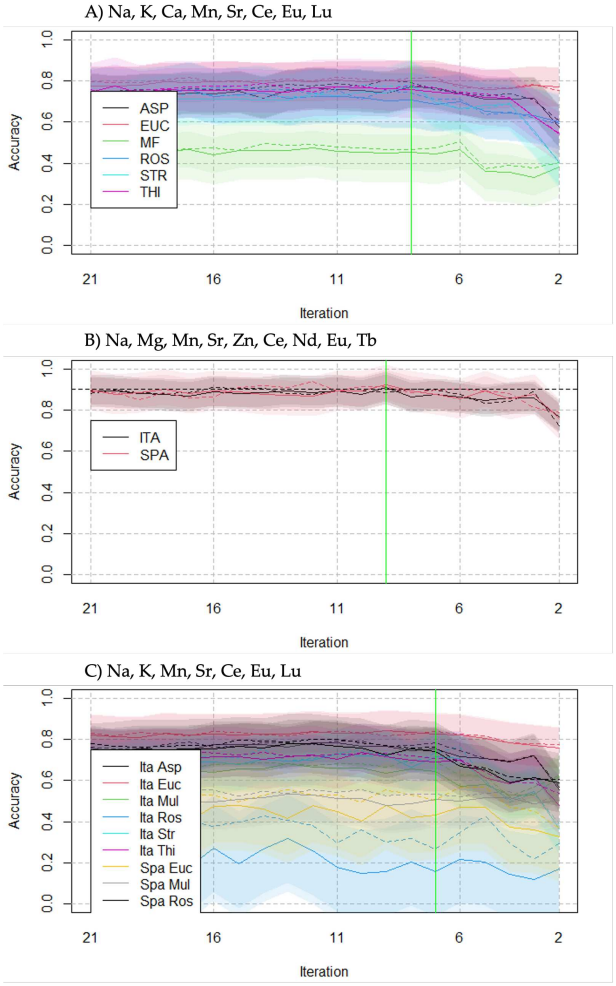


Figure 2.3. Accuracy of RF algorithm at varying of iterations, which indicates the number of predictors (chemical elements) used for classification purposes. Continuous line: accuracy in training. Dashed lines: accuracy in testing. Shades: mean ± standard deviation. Green line: accuracy drop. A) RF classification of honeys based on botanical origin; B) RF classification based on geographical origin; C) RF classification based on both geographical and botanical origin. All values are given in Table B6, Table B7, and Table B8.

2.4. DISCUSSION

Elemental analysis allows to evaluate the content of toxic and nutritional elements. The levels of harmful elements are similar to or frequently lower than those found in other Spanish (Álvarez-Ayuso & Abad-Valle, 2017; Díaz et al., 2019) and Italian (Girolametti et al., 2023; Quinto et al., 2016; Scivicco et al., 2022) honeys analyzed in previous studies. On the other hand, honeys have a relatively high content of minerals such as Na, Mg, K, Ca, Zn, Mn, and Cu. However, assuming a daily honey intake of 20 g, elements of nutritional interest do not cover daily requirements, while the toxic elements do not pose any health risk.

Elemental fingerprints were tested for classifying honeys according to geographical and botanical origins. PCA was performed using two different data pre-treatment, centered log-ratio transformation (Figure 2.2) and standardization (Figure B1). As previously reported (Templ & Templ, 2021), the centered log-ratio transformation improves the data interpretability by distributing a part of the variance explained by the first component into the other components. Consequently, loadings and scores in the first two components are more dispersed (Figure 2.2), allowing a more comprehensive differentiation of honey categories. The PCA results show that botanical information predominates over geographical information (compare Figure 2.2B with Figure 2.2C). Except for rosemary honeys (for which Sardinia is underrepresented), multifloral honeys tend to overlap in the graph (Figure 2.2E), while eucalyptus honeys are more distinguished (Figure 2.2D). It is hypothesized that this difference is attributable to the origin of Spanish eucalyptus honeys. These are produced mainly in the northern regions, which are geographically more different from Sardinia. Furthermore, the results of the PCA analysis indicate that geographical discrimination is facilitated, as expected, when considering different botanical origins (compare Figure 2.2D with Figure 2.2H). Finally, the score plots allow also to compare honey elemental compositions. Broadly, the percentage of lanthanides is lower in Spanish samples compared to Sardinian unifloral varieties. On the other hand, macroelements and trace elements are relatively less abundant in unifloral honey than in multifloral ones (Figure 2.2).

Regarding honey authentication, the RF algorithm performs better than LDA in classifying honey according to their origins. Overall, the classification by geographical origin is the most accurate (90%, predictors: Na, Mg, Mn, Sr, Zn, Ce, Nd, Eu, Tb). The accuracy tends to decrease when classifying honey by botanical origin (73%, predictors: Na, K, Ca, Mn, Sr, Ce, Eu, Lu) and when combining geographical and botanical origins (65%, predictors: Na, K, Mn, Sr, Ce, Eu, Lu). Based on these results, it is possible that geographical origin may have a greater influence than botanical origin on the elemental fingerprint. However, predicting the botanical origin is challenging, and combining the two factors reduces the classification model's accuracy.

To our knowledge, this is the first case where RF combined with elemental fingerprinting has been used to distinguish the origin of honey. Thus, the accuracy of the models cannot be easily assessed if compared with data presented in the literature. Few studies have investigated the authentication of honey in terms of both geographical and botanical origin (Bilandžić et al., 2019; Drivelos et al., 2021; Magdas et al., 2021; Pavlin et al., 2023). Often the data were not processed with classification algorithms (Bilandžić et al., 2019; Pavlin et al., 2023). Drivelos et al. achieved excellent results in geographical classification (Drivelos et al., 2021). On the other hand, Magdas et al. achieved also excellent results, but their classification models were obtained using isotopic markers in addition to elements (Magdas et al., 2021).

In general, as expected the models obtained indicate that unifloral varieties are more accurately classified than multifloral honey. Regarding elemental predictors, Na, Mn, Sr, Ce, and Eu are common to all origin classifications, while Mg, Zn, Nd, and Tb are useful for geographical classification. On the other hand, K, Ca, and Lu are relevant for botanical origin. These findings can be compared and are in rather good agreement with those of Pavlin et al. (Pavlin et al., 2023), who analyzed different varieties of unifloral and multifloral honeys from Slovenia, Croatia, Bulgaria, Turkey, and Morocco (Pavlin et al., 2023). They reported Mn, K, and Ca as botanical markers, whereas Na, Mg, and Fe as geographical ones. However, the labeling of elements as markers for the determination of a specific origin may vary depending on honey origin or variety.

Regarding lanthanides, they have primarily been reported as indicators of geographical origin (Drivelos & Georgiou, 2012; Magdas et al., 2021). In this study, Ce, Nd, Eu, and Tb are significant for geographical classification, while Ce, Eu, and Lu are significant for botanical classification. Previously, Squadrone et al. (Squadrone, Brizio, Stella, Pederiva, et al., 2020) reported that lanthanides can help in discriminating unifloral and multifloral honey using the ratio of light and heavy rare earths (LREE/HREE), while Gulino et al. (Gulino et al., 2023) reported that the fractionation of heavy lanthanides partially helps in geographical classification. The results of this research suggest that Ce and Eu are among the most important lanthanides in all models. Eu exhibits anomalous behavior in Spanish honey, not observed in those from Sardinia. Gulino et al. (Gulino et al., 2023) suggested that these anomalies could be attributed to Ba interferences during the analyses. Although it may be possible, any potential bias would affect all samples and should be highly correlated with Ba. However, the level of correlation between these parameters is low ($r = 0.16$), so this hypothesis can be ruled out. Based on the results obtained, Eu may be considered a reliable marker in all classification models.

2.5. CONCLUSIONS

In conclusion, the analysis of macroelements, trace elements, toxic elements, and lanthanides allows for the assessment of their potential use as markers for the botanical and geographical classification of honeys. Specifically, the investigation compared honey originating from the same botanical source but produced in neighbors' geographic locations for climate, flora, and geology. The accuracy of honey classification based on geography is reliable when comparing honey of different botanical origins but tends to decrease when comparing the same botanical varieties. As expected, multifloral honeys are more difficult to classify in terms of both botanical and geographical origin.

This case study confirmed the usefulness of elemental fingerprinting and suggested its potential use to effectively discriminate honeys from similar regions. One possible application could be to discriminate European honeys from those produced in neighboring non-EU countries or those that share similar Mediterranean floral resources. As a future perspective, honey from these regions will be studied.

2. REFERENCES

Afrin, S., Forbes-Hernandez, T., Gasparrini, M., Bompadre, S., Quiles, J., Sanna, G., Spano, N., Giampieri, F., & Battino, M. (2017). Strawberry-Tree Honey Induces Growth Inhibition of Human Colon Cancer Cells and Increases ROS Generation: A Comparison with Manuka Honey. *International Journal of Molecular Sciences*, *18*(3), 613. https://doi.org/10.3390/ijms18030613

Afrin, S., Haneefa, S. M., Fernandez-Cabezudo, M. J., Giampieri, F., al-Ramadi, B. K., & Battino, M. (2020). Therapeutic and preventive properties of honey and its bioactive compounds in cancer: An evidence-based review. *Nutrition Research Reviews*, *33*(1), 50–76. https://doi.org/10.1017/S0954422419000192

Almasaudi, S. (2021). The antibacterial activities of honey. *Saudi Journal of Biological Sciences*, *28*(4), 2188–2196. https://doi.org/10.1016/j.sjbs.2020.10.017

Álvarez-Ayuso, E., & Abad-Valle, P. (2017). Trace element levels in an area impacted by old mining operations and their relationship with beehive products. *Science of The Total Environment*, *599–600*, 671–678. https://doi.org/10.1016/j.scitotenv.2017.05.030

Alvarez-Suarez, J. M., Tulipani, S., Romandini, S., Bertoli, E., & Battino, M. (2010). Contribution of honey in nutrition and human health: A review. *Mediterranean Journal of Nutrition and Metabolism*, *3*(1), 15–23. https://doi.org/10.1007/s12349-009-0051-6

Atanassova, J., Pavlova, D., Lazarova, M., & Yurukova, L. (2016). Characteristics of Honey from Serpentine Area in the Eastern Rhodopes Mt., Bulgaria. *Biological Trace Element Research*, *173*(1), 247–258. https://doi.org/10.1007/s12011-015-0616-9

Bilandžić, N., Sedak, M., Đokić, M., Bošković, A. G., Florijančić, T., Bošković, I., Kovačić, M., Puškadija, Z., & Hruškar, M. (2019). Element content in ten Croatian honey types from different geographical regions during three seasons. *Journal of Food Composition and Analysis*, *84*, 103305. https://doi.org/10.1016/j.jfca.2019.103305

Bilandžić, N., Tlak Gajger, I., Kosanović, M., Čalopek, B., Sedak, M., Solomun Kolanović, B., Varenina, I., Luburić, Đ. B., Varga, I., & Đokić, M. (2017). Essential and toxic element concentrations in monofloral honeys from southern Croatia. *Food Chemistry*, *234*, 245–253. https://doi.org/10.1016/j.foodchem.2017.04.180

Bodó, A., Radványi, L., Kőszegi, T., Csepregi, R., Nagy, D. U., Farkas, Á., & Kocsis, M. (2021). Quality Evaluation of Light- and Dark-Colored Hungarian Honeys, Focusing on Botanical Origin, Antioxidant Capacity and Mineral Content. *Molecules*, *26*(9), 2825. https://doi.org/10.3390/molecules26092825

Bommuraj, V., Chen, Y., Klein, H., Sperling, R., Barel, S., & Shimshoni, J. A. (2019). Pesticide and trace element residues in honey and beeswax combs from Israel in association with human risk assessment and honey adulteration. *Food Chemistry*, *299*, 125123. https://doi.org/10.1016/j.foodchem.2019.125123

Cabras, P., Angioni, A., Tuberoso, C., Floris, I., Reniero, F., Guillou, C., & Ghelli, S. (1999). Homogentisic Acid: A Phenolic Acid as a Marker of Strawberry-Tree (Arbutus unedo) Honey. *Journal of Agricultural and Food Chemistry*, *47*(10), 4064–4067. https://doi.org/10.1021/jf990141o

Caredda, M., Mara, A., Ciulu, M., Floris, I., Pilo, M. I., Spano, N., & Sanna, G. (2023). Use of genetic algorithms in the wavelength selection of FT-MIR spectra to classify unifloral honeys from Sardinia. *Food Control*, *146*, 109559. https://doi.org/10.1016/j.foodcont.2022.109559

Ciulu, M., Oertel, E., Serra, R., Farre, R., Spano, N., Caredda, M., Malfatti, L., & Sanna, G. (2020). Classification of Unifloral Honeys from SARDINIA (Italy) by ATR-FTIR Spectroscopy and Random Forest. *Molecules*, *26*(1), 88. https://doi.org/10.3390/molecules26010088

Ciulu, M., Serra, R., Caredda, M., Salis, S., Floris, I., Pilo, M. I., Spano, N., Panzanelli, A., & Sanna, G. (2018). Chemometric treatment of simple physical and chemical data for the discrimination of unifloral honeys. *Talanta*, *190*, 382–390. https://doi.org/10.1016/j.talanta.2018.08.025

Conti, M. E., Astolfi, M. L., Mele, G., Ristorini, M., Vitiello, G., Massimi, L., Canepari, S., & Finoia, M. G. (2022). Performance of bees and beehive products as indicators of elemental tracers of atmospheric pollution in sites of the Rome province (Italy). *Ecological Indicators*, *140*, 109061. https://doi.org/10.1016/j.ecolind.2022.109061

Cornara, L., Biagi, M., Xiao, J., & Burlando, B. (2017). Therapeutic Properties of Bioactive Compounds from Different Honeybee Products. *Frontiers in Pharmacology*, *8*, 412. https://doi.org/10.3389/fphar.2017.00412

Czipa, N., Andrási, D., & Kovács, B. (2015). Determination of essential and toxic elements in Hungarian honeys. *Food Chemistry*, *175*, 536–542. https://doi.org/10.1016/j.foodchem.2014.12.018

Czipa, N., Kovács, B., Alexa, L., & Gebreyesus, M. (2023). Determination of Trace, Micro and Macro Elemental Concentration of Eritrean Honeys. *Biological Trace Element Research*. https://doi.org/10.1007/s12011-023-03821-x

Da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry*, *196*, 309–323. https://doi.org/10.1016/j.foodchem.2015.09.051

Danezis, G. P., & Georgiou, C. A. (2022). Elemental metabolomics: Food elemental assessment could reveal geographical origin. *Current Opinion in Food Science*, *44*, 100812. https://doi.org/10.1016/j.cofs.2022.100812

Demir Kanbur, E., Yuksek, T., Atamov, V., & Ozcelik, A. E. (2021). A comparison of the physicochemical properties of chestnut and highland honey: The case of Senoz Valley in the Rize province of Turkey. *Food Chemistry*, *345*, 128864. https://doi.org/10.1016/j.foodchem.2020.128864

Di Bella, G., Lo Turco, V., Potortì, A. G., Bua, G. D., Fede, M. R., & Dugo, G. (2015). Geographical discrimination of Italian honey by multi-element analysis with a chemometric approach. *Journal of Food Composition and Analysis*, *44*, 25–35. https://doi.org/10.1016/j.jfca.2015.05.003

Díaz, S., Paz, S., Rubio, C., Gutiérrez, Á. J., González-Weller, D., Revert, C., Bentabol, A., & Hardisson, A. (2019). Toxic Metals and Trace Elements in Artisanal Honeys from the Canary Islands. *Biological Trace Element Research*, *190*(1), 242–250. https://doi.org/10.1007/s12011-018-1538-0

Đogo Mračević, S., Krstić, M., Lolić, A., & Ražić, S. (2020). Comparative study of the chemical composition and biological potential of honey from different regions of Serbia. *Microchemical Journal*, *152*, 104420. https://doi.org/10.1016/j.microc.2019.104420

Drivelos, S. A., Danezis, G. P., Halagarda, M., Popek, S., & Georgiou, C. A. (2021). Geographical origin and botanical type honey authentication through elemental metabolomics via chemometrics. *Food Chemistry*, *338*, 127936. https://doi.org/10.1016/j.foodchem.2020.127936

Drivelos, S. A., & Georgiou, C. A. (2012). Multi-element and multi-isotope-ratio analysis to determine the geographical origin of foods in the European Union. *TrAC Trends in Analytical Chemistry*, *40*, 38–51. https://doi.org/10.1016/j.trac.2012.08.003

Egido, C., Saurina, J., Sentellas, S., & Núñez, O. (2024). Honey fraud detection based on sugar syrup adulterations by HPLC-UV fingerprinting and chemometrics. *Food Chemistry*, *436*, 137758. https://doi.org/10.1016/j.foodchem.2023.137758

Escuredo, O., Rodríguez-Flores, M. S., Meno, L., & Seijo, M. C. (2021). Prediction of Physicochemical Properties in Honeys with Portable Near-Infrared (microNIR) Spectroscopy Combined with Multivariate Data Processing. *Foods*, *10*(2), 317. https://doi.org/10.3390/foods10020317

European Commission. (2023). *EU coordinated action "From the Hives" (Honey 2021-2022)*. https://food.ec.europa.eu/safety/eu-agri-food-fraud-network/eu-coordinated-actions/honey-2021-2022_en#qapdf

Fernández-Estellé, M., Hernández-González, V., Saurina, J., Núñez, O., & Sentellas, S. (2023). Characterization and Classification of Spanish Honeydew and Blossom Honeys Based on Their Antioxidant Capacity. *Antioxidants*, *12*(2), 495. https://doi.org/10.3390/antiox12020495

Floris, I., Satta, A., & Ruiu, L. (2007). Honeys of Sardinia (Italy). *Journal of Apicultural Research*, *46*(3), 198–209. https://doi.org/10.1080/00218839.2007.11101393

García-Seval, V., Martínez-Alfaro, C., Saurina, J., Núñez, O., & Sentellas, S. (2022). Characterization, Classification and Authentication of Spanish Blossom and Honeydew Honeys by Non-Targeted HPLC-UV and Off-Line SPE HPLC-UV Polyphenolic Fingerprinting Strategies. *Foods*, *11*(15), 2345. https://doi.org/10.3390/foods11152345

Gattacceca, J., Deino, A., Rizzo, R., Jones, D. S., Henry, B., Beaudoin, B., & Vadeboin, F. (2007). Miocene rotation of Sardinia: New paleomagnetic and geochronological constraints and geodynamic implications. *Earth and Planetary Science Letters*, *258*(3–4), 359–377. https://doi.org/10.1016/j.epsl.2007.02.003

Giglio, A., Ammendola, A., Battistella, S., Naccarato, A., Pallavicini, A., Simeon, E., Tagarelli, A., & Giulianini, P. G. (2017). Apis mellifera ligustica, Spinola 1806 as bioindicator for detecting environmental contamination: A preliminary study of heavy metal pollution in Trieste, Italy. *Environmental Science and Pollution Research*, *24*(1), 659–665. https://doi.org/10.1007/s11356-016-7862-z

Girolametti, F., Illuminati, S., Annibaldi, A., Ajdini, B., Fanelli, M., & Truzzi, C. (2023). Mercury in honey from the Marche region (central Italy). Risk assessment from human consumption and its use as bioindicator of environmental pollution. *Heliyon*, *9*(10), e20502. https://doi.org/10.1016/j.heliyon.2023.e20502

Gomez, M. E. M., Hernandez, E. G., Gomez, J. Y. M., & Marin, J. L. M. (1993). Physicochemical analysis of Spanish commercial *Eucalyptus* honeys. *Journal of Apicultural Research*, *32*(3–4), 121–126. https://doi.org/10.1080/00218839.1993.11101296

Grainger, M. N. C., Klaus, H., Hewitt, N., & French, A. D. (2021). Investigation of inorganic elemental content of honey from regions of North Island, New Zealand. *Food Chemistry*, *361*, 130110. https://doi.org/10.1016/j.foodchem.2021.130110

Grainger, M. N. C., Klaus, H., Hewitt, N., Gan, H., & French, A. D. (2023). Graphical Discrimination of New Zealand Honey from International Honey Using Elemental Analysis. *Biological Trace Element Research*. https://doi.org/10.1007/s12011-023-03680-6

Guelpa, A., Marini, F., Du Plessis, A., Slabbert, R., & Manley, M. (2017). Verification of authenticity and fraud detection in South African honey using NIR spectroscopy. *Food Control*, *73*, 1388–1396. https://doi.org/10.1016/j.foodcont.2016.11.002

Gulino, F., Calà, E., Cozzani, C., Vaccari, L., Oddone, M., & Aceto, M. (2023). On the Traceability of Honey by Means of Lanthanide Distribution. *Foods*, *12*(9), 1803. https://doi.org/10.3390/foods12091803

Hernanz, D., Jara-Palacios, M. J., Santos, J. L., Gómez Pajuelo, A., Heredia, F. J., & Terrab, A. (2023). The profile of phenolic compounds by HPLC-MS in Spanish oak (Quercus) honeydew honey and their relationships with color and antioxidant activity. *LWT*, *180*, 114724. https://doi.org/10.1016/j.lwt.2023.114724

Jurica, K., Brčić Karačonji, I., Lasić, D., Bursać Kovačević, D., & Putnik, P. (2021). Unauthorized Food Manipulation as a Criminal Offense: Food Authenticity, Legal Frameworks, Analytical Tools and Cases. *Foods*, *10*(11), 2570. https://doi.org/10.3390/foods10112570

Karabagias, I. K., Louppis, A. P., Karabournioti, S., Kontakos, S., Papastephanou, C., & Kontominas, M. G. (2017). Characterization and geographical discrimination of commercial Citrus spp. Honeys produced in different Mediterranean countries based on minerals, volatile compounds and physicochemical parameters, using chemometrics. *Food Chemistry*, *217*, 445–455. https://doi.org/10.1016/j.foodchem.2016.08.124

Kastrati, G., Sopaj, F., Tašev, K., Stafilov, T., Šajn, R., & Paçarizi, M. (2023). Analysis of chemical elements in honey samples in the territory of Kosovo. *Journal of Food Composition and Analysis*, *124*, 105505. https://doi.org/10.1016/j.jfca.2023.105505

Lazarus, M., Tariba Lovaković, B., Orct, T., Sekovanić, A., Bilandžić, N., Đokić, M., Solomun Kolanović, B., Varenina, I., Jurič, A., Denžić Lugomer, M., & Bubalo, D. (2021). Difference in pesticides, trace metal(loid)s and drug residues between certified organic and conventional honeys from Croatia. *Chemosphere*, *266*, 128954. https://doi.org/10.1016/j.chemosphere.2020.128954

Leardi, R., Melzi, C., & Polotti, G. (2021, September 14). *CAT (Chemometric Agile Tool Software)*. http://gruppochemiometria.it/index.php/software

Lemus Ringele, G. B., Beteinakis, S., Papachristodoulou, A., Axiotis, E., Mikros, E., & Halabalaki, M. (2022). NMR Metabolite Profiling in the Quality and Authentication Assessment of Greek Honey—Exploitation of STOCSY for Markers Identification. *Foods*, *11*(18), 2853. https://doi.org/10.3390/foods11182853

Liaw, A., & Wiener, M. (2001). Classification and Regression by RandomForest. *R News*, *2*.

Liu, T., Ming, K., Wang, W., Qiao, N., Qiu, S., Yi, S., Huang, X., & Luo, L. (2021). Discrimination of honey and syrup-based adulteration by mineral element chemometrics profiling. *Food Chemistry*, *343*, 128455. https://doi.org/10.1016/j.foodchem.2020.128455

Louppis, A. P., Karabagias, I. K., Kontakos, S., Kontominas, M. G., & Papastephanou, C. (2017). Botanical discrimination of Greek unifloral honeys based on mineral content in combination with physicochemical parameter analysis, using a validated chemometric approach. *Microchemical Journal*, *135*, 180–189. https://doi.org/10.1016/j.microc.2017.09.004

Mădaş, M. N., Mărghitaş, L. A., Dezmirean, D. S., Bobiş, O., Abbas, O., Danthine, S., Francis, F., Haubruge, E., & Nguyen, B. K. (2020). Labeling Regulations and Quality Control of Honey Origin: A Review. *Food Reviews International*, *36*(3), 215–240. https://doi.org/10.1080/87559129.2019.1636063

Magdas, D. A., Guyon, F., Puscas, R., Vigouroux, A., Gaillard, L., Dehelean, A., Feher, I., & Cristea, G. (2021). Applications of emerging stable isotopes and elemental markers for geographical and varietal recognition of Romanian and French honeys. *Food Chemistry*, *334*, 127599. https://doi.org/10.1016/j.foodchem.2020.127599

Mara, A., Deidda, S., Caredda, M., Ciulu, M., Deroma, M., Farinini, E., Floris, I., Langasco, I., Leardi, R., Pilo, M. I., Spano, N., & Sanna, G. (2022). Multi-Elemental Analysis as a Tool to Ascertain the Safety and the Origin of Beehive Products: Development, Validation, and Application of an ICP-MS Method on Four Unifloral Honeys Produced in Sardinia, Italy. *Molecules*, *27*(6), 2009. https://doi.org/10.3390/molecules27062009

Martinello, M., & Mutinelli, F. (2021). Antioxidant Activity in Bee Products: A Review. *Antioxidants*, *10*(1), 71. https://doi.org/10.3390/antiox10010071

Masoomi, S., Sharifi, H., & Hemmateenejad, B. (2024). A paper-based optical tongue for characterization of iranian honey: Identification of geographical/botanical origins and adulteration detection. *Food Control*, *155*, 110052. https://doi.org/10.1016/j.foodcont.2023.110052

Mohamadzade Namin, S., Yeasmin, F., Choi, H. W., & Jung, C. (2022). DNA-Based Method for Traceability and Authentication of Apis cerana and A. dorsata Honey (Hymenoptera: Apidae), Using the NADH dehydrogenase 2 Gene. *Foods*, *11*(7), 928. https://doi.org/10.3390/foods11070928

Pavlin, A., Kočar, D., Imperl, J., Kolar, M., Marolt, G., & Petrova, P. (2023). Honey Origin Authentication via Mineral Profiling Combined with Chemometric Approaches. *Foods*, *12*(15), 2826. https://doi.org/10.3390/foods12152826

Pellerano, R. G., Uñates, M. A., Cantarelli, M. A., Camiña, J. M., & Marchevsky, E. J. (2012). Analysis of trace elements in multifloral Argentine honeys and their classification according to provenance. *Food Chemistry*, *134*(1), 578–582. https://doi.org/10.1016/j.foodchem.2012.02.125

Perezarquillue, C., Conchello, P., Arino, A., Juan, T., & Herrera, A. (1994). Quality evaluation of Spanish rosemary (Rosmarinus officinalis) honey. *Food Chemistry*, *51*(2), 207–210. https://doi.org/10.1016/0308-8146(94)90258-5

Perna, A. M., Grassi, G., Gambacorta, E., & Simonetti, A. (2021). Minerals content in Basilicata region (southern Italy) honeys from areas with different anthropic impact. *International Journal of Food Science & Technology*, *56*(9), 4465–4472. https://doi.org/10.1111/ijfs.15112

Persano Oddo, L; Sabatini, AG; Accorti, M; Colombo, R; Marcazzan, GL; Piana, L; Piazza, MG; Pulcini, P. I mieli uniflorali italiani. Nuove schede di caratterizzazione. (2000a). http://profilomieli.albomiele.it/html/4_mieli_eucalipto.html

Persano Oddo, L; Sabatini, AG; Accorti, M; Colombo, R; Marcazzan, GL; Piana, L; Piazza, MG; Pulcini, P. I mieli uniflorali italiani. Nuove schede di caratterizzazione. (2000b). http://profilomieli.albomiele.it/html/4_mieli_rosmarino.html

Pohl, P., Bielawska-Pohl, A., Dzimitrowicz, A., Jamroz, P., Welna, M., Lesniewicz, A., & Szymczycha-Madeja, A. (2017). Recent achievements in element analysis of bee honeys by atomic and mass spectrometry methods. *TrAC Trends in Analytical Chemistry*, *93*, 67–77. https://doi.org/10.1016/j.trac.2017.05.009

Quinto, M., Miedico, O., Spadaccino, G., Paglia, G., Mangiacotti, M., Li, D., Centonze, D., & Chiaravalle, A. E. (2016). Characterization, chemometric evaluation, and human health-related aspects of essential and toxic

elements in Italian honey samples by inductively coupled plasma mass spectrometry. *Environmental Science and Pollution Research*, *23*(24), 25374–25384. https://doi.org/10.1007/s11356-016-7662-5

Ranneh, Y., Akim, A. M., Hamid, H. Ab., Khazaai, H., Fadel, A., Zakaria, Z. A., Albujja, M., & Bakar, M. F. A. (2021). Honey and its nutritional and anti-inflammatory value. *BMC Complementary Medicine and Therapies*, *21*(1), 30. https://doi.org/10.1186/s12906-020-03170-5

Rodríguez-Flores, S., Escuredo, O., & Seijo, M. C. (2016). Characterization and antioxidant capacity of sweet chestnut honey produced in North-West Spain. *Journal of Apicultural Science*, *60*(1), 19–30. https://doi.org/10.1515/jas-2016-0002

romano, andrea, Cuenca Quicazan, M., Makhoul, S., Biasioli, F., Martinello, L., Fugatti, A., & Scampicchio, M. (2016). Comparison of e-Noses: The case study of honey. *Italian Journal of Food Science*, *28*(2), 326–337. https://doi.org/10.14674/1120-1770/ijfs.v325

Satta, A., Verdinelli, M., Ruiu, L., Buffa, F., Salis, S., Sassu, A., & Floris, I. (2012). Combination of beehive matrices analysis and ant biodiversity to study heavy metal pollution impact in a post-mining area (Sardinia, Italy). *Environmental Science and Pollution Research*, *19*(9), 3977–3988. https://doi.org/10.1007/s11356-012-0921-1

Scanu, R., Spano, N., Panzanelli, A., Pilo, M. I., Piu, P. C., Sanna, G., & Tapparo, A. (2005). Direct chromatographic methods for the rapid determination of homogentisic acid in strawberry tree (Arbutus unedo L.) honey. *Journal of Chromatography A*, *1090*(1–2), 76–80. https://doi.org/10.1016/j.chroma.2005.06.092

Schievano, E., Piana, L., & Tessari, M. (2023). Automatic NMR-based protocol for assessment of honey authenticity. *Food Chemistry*, *420*, 136094. https://doi.org/10.1016/j.foodchem.2023.136094

Scivicco, M., Squillante, J., Velotto, S., Esposito, F., Cirillo, T., & Severino, L. (2022). Dietary exposure to heavy metals through polyfloral honey from Campania region (Italy). *Journal of Food Composition and Analysis*, *114*, 104748. https://doi.org/10.1016/j.jfca.2022.104748

Serra Bonvehi, J., Ventura Coll, F., & Orantes Bermejo, J. F. (2019). Characterization of avocado honey (Persea americana Mill.) produced in Southern Spain. *Food Chemistry*, *287*, 214–221. https://doi.org/10.1016/j.foodchem.2019.02.068

Solayman, Md., Islam, Md. A., Paul, S., Ali, Y., Khalil, Md. I., Alam, N., & Gan, S. H. (2016). Physicochemical Properties, Minerals, Trace Elements, and Heavy Metals in Honey of Different Origins: A Comprehensive Review. *Comprehensive Reviews in Food Science and Food Safety*, *15*(1), 219–233. https://doi.org/10.1111/1541-4337.12182

Squadrone, S., Brizio, P., Stella, C., Mantia, M., Pederiva, S., Brusa, F., Mogliotti, P., Garrone, A., & Abete, M. C. (2020). Trace elements and rare earth elements in honeys from the Balkans, Kazakhstan, Italy, South America, and Tanzania. *Environmental Science and Pollution Research*, *27*(11), 12646–12657. https://doi.org/10.1007/s11356-020-07792-7

Squadrone, S., Brizio, P., Stella, C., Pederiva, S., Brusa, F., Mogliotti, P., Garrone, A., & Abete, M. C. (2020). Trace and rare earth elements in monofloral and multifloral honeys from Northwestern Italy; A first attempt of characterization by a multi-elemental profile. *Journal of Trace Elements in Medicine and Biology*, *61*, 126556. https://doi.org/10.1016/j.jtemb.2020.126556

Suhandy, D., Al Riza, D. F., Yulia, M., & Kusumiyati, K. (2023). Non-Targeted Detection and Quantification of Food Adulteration of High-Quality Stingless Bee Honey (SBH) via a Portable LED-Based Fluorescence Spectroscopy. *Foods*, *12*(16), 3067. https://doi.org/10.3390/foods12163067

Suhandy, D., & Yulia, M. (2021). The Use of UV Spectroscopy and SIMCA for the Authentication of Indonesian Honeys According to Botanical, Entomological and Geographical Origins. *Molecules*, *26*(4), 915. https://doi.org/10.3390/molecules26040915

Templ, M., & Templ, B. (2021). Statistical Analysis of Chemical Element Compositions in Food Science: Problems and Possibilities. *Molecules*, *26*(19), 5752. https://doi.org/10.3390/molecules26195752

Thrasyvoulou, A., Tananaki, C., Goras, G., Karazafiris, E., Dimou, M., Liolios, V., Kanelis, D., & Gounari, S. (2018). Legislation of honey criteria and standards. *Journal of Apicultural Research*, *57*(1), 88–96. https://doi.org/10.1080/00218839.2017.1411181

Tsagkaris, A. S., Koulis, G. A., Danezis, G. P., Martakos, I., Dasenaki, M., Georgiou, C. A., & Thomaidis, N. S. (2021). Honey authenticity: Analytical techniques, state of the art and challenges. *RSC Advances*, *11*(19), 11273–11294. https://doi.org/10.1039/D1RA00069A

Tuberoso, C. I. G., Bifulco, E., JerkoviĆ, I., Caboni, P., Cabras, P., & Floris, I. (2009). Methyl Syringate: A Chemical Marker of Asphodel (Asphodelus microcarpus Salzm. et Viv.) Monofloral Honey. *Journal of Agricultural and Food Chemistry*, *57*(9), 3895–3900. https://doi.org/10.1021/jf803991j

Voyslavov, T., Mladenova, E., & Balkanska, R. (2021). A New Approach for Determination of the Botanical Origin of Monofloral Bee Honey, Combining Mineral Content, Physicochemical Parameters, and Self-Organizing Maps. *Molecules*, *26*(23), 7219. https://doi.org/10.3390/molecules26237219

Vukašinović-Pešić, V., Blagojević, N., Brašanac-Vukanović, S., Savić, A., & Pešić, V. (2020). Using Chemometric Analyses for Tracing the Regional Origin of Multifloral Honeys of Montenegro. *Foods*, *9*(2), 210. https://doi.org/10.3390/foods9020210

Wang, Z., Ren, P., Wu, Y., & He, Q. (2021). Recent advances in analytical techniques for the detection of adulteration and authenticity of bee products – A review. *Food Additives & Contaminants: Part A*, *38*(4), 533–549. https://doi.org/10.1080/19440049.2020.1871081

Wei, Q., Sun, J., Guo, J., Li, X., Zhang, X., & Xiao, F. (2023). Authentication of chaste honey adulterated with high fructose corn syrup by HS-SPME-GC-MS coupled with chemometrics. *LWT*, *176*, 114509. https://doi.org/10.1016/j.lwt.2023.114509

Wu, F., Zhao, H., Sun, J., Guo, J., Wu, L., Xue, X., & Cao, W. (2021). ICP-MS-based ionomics method for discriminating the geographical origin of honey of Apis cerana Fabricius. *Food Chemistry*, *354*, 129568. https://doi.org/10.1016/j.foodchem.2021.129568

Wu, J., Duan, Y., Gao, Z., Yang, X., Zhao, D., Gao, J., Han, W., Li, G., & Wang, S. (2020). Quality comparison of multifloral honeys produced by Apis cerana cerana, Apis dorsata and Lepidotrigona flavibasis. *LWT*, *134*, 110225. https://doi.org/10.1016/j.lwt.2020.110225

Wu, Q., Xu, J., Yao, L., Chen, Q., Yao, B., Zhang, Y., & Chen, W. (2024). Accuracy and stability enhanced honey authenticity visual tracing method via false positive-eradicating PCR assisted nucleic acid-capturing lateral flow strip. *Food Chemistry*, *435*, 137587. https://doi.org/10.1016/j.foodchem.2023.137587

Yayinie, M., & Atlabachew, M. (2022). Multi-element Analysis of Honey from Amhara Region-Ethiopia for Quality, Bioindicator of Environmental Pollution, and Geographical Origin Discrimination. *Biological Trace Element Research*, *200*(12), 5283–5297. https://doi.org/10.1007/s12011-021-03088-0

Zhang, C., & Ma, Y. (Eds.). (2012). *Ensemble Machine Learning*. Springer.

Zhang, P., Georgiou, C. A., & Brusic, V. (2017). Elemental metabolomics. *Briefings in Bioinformatics*, bbw131. https://doi.org/10.1093/bib/bbw131

Zhang, X.-H., Gu, H.-W., Liu, R.-J., Qing, X.-D., & Nie, J.-F. (2023). A comprehensive review of the current trends and recent advancements on the authenticity of honey. *Food Chemistry: X*, *19*, 100850. https://doi.org/10.1016/j.fochx.2023.100850

Zhu, M., Zhao, H., Wang, Q., Wu, F., & Cao, W. (2020). A Novel Chinese Honey from Amorpha fruticosa L.: Nutritional Composition and Antioxidant Capacity In Vitro. *Molecules*, *25*(21), 5211. https://doi.org/10.3390/molecules25215211

Submitted on Molecules, 2024, https://doi.org/10.3390/moleculesXXXXXXXX

ELEMENTAL FINGERPRINTING OF PECORINO ROMANO AND PECORINO SARDO PDO: CHARACTERIZATION, AUTHENTICATION AND NUTRITIONAL VALUE

3.1. INTRODUCTION

Dairy products and milk are among the most valuable foods due to their high nutritional value. Milk is the primary food of mammals at birth and contains high amounts of sugars, proteins, fats, minerals and vitamins (Pereira, 2014). Dairy products are derived from the milk of major and minor ruminant species, such as cows, buffaloes, sheep, and goats. Besides their nutritional properties, dairy products are important for the economy and traditions of many countries. Among them, Italy is one of the most recognized in the world to produce protected designation of origin (PDO) products. The most widely renowned dairy products include Parmigiano Reggiano, Grana Padano, and Pecorino Romano.

Pecorino Romano (PR) is a sheep cheese primarily produced in Sardinia, an Italian region where the sheep dairy industry is economically relevant (Pulina et al., 2018). In fact, two other PDO sheep's milk cheeses are produced here: Pecorino Sardo PDO (PS) and Fiore Sardo PDO. The production of PDO cheeses from sheep's milk is the main source of income for the livestock industry on the island. Semi-extensive farming is the primary method for rearing milk sheep in Sardinia. This results in cheeses that exhibit unique sensorial properties due to the distinguishing features of pastures and climate.

Despite its relevance to the industry, the economic model is fragile due to its dependence on price fluctuations of PR. This often results in farms failing to cover production costs during times of price drops (Pulina et al., 2018). For these reasons, recent studies have aimed to enhance economic performance and sustainability of the supply chain (Vagnoni et al., 2017), develop new marketing strategies, improve farm technologies (Pulina et al., 2021), and diversify the production (Camanzi et al., 2018; Lai et al., 2023). This latter strategy appears to be the most promising in reducing the reliance of milk costs on PR. Another key method for enhancing product value is to capitalize on consumer awareness of dairy quality and nutritional properties (Kraus, 2015; Sajdakowska et al., 2020). For

instance, vitamins and minerals are of significant interest because of their association with various health benefits (de la Fuente & Juárez, 2015; Kraus, 2015).

Elements such as Na, Mg, K, Ca, Fe, Cu, Zn and Se are essential for supporting the immune response, cellular processes and antioxidant defences (Cannas et al., 2020; Islam et al., 2023). These elements can be obtained naturally through a balanced and tailored diet, which prevents health complications caused by deficiency or excess. On the other hand, toxic elements such as As, Cd, Hg and Pb pose health risks to humans at any concentration (Jaishankar et al., 2014). Anthropogenic activities often lead to pollution by toxic elements, which contaminate food through water, soil, and air (de Almeida Ribeiro Carvalho et al., 2022). To ensure food safety, regulations and safety measures have been implemented to monitor and limit the presence of toxic elements in food. For instance, the European community has established maximum levels of toxic elements in food (European Commission, 2006, 2011). However, most of these contaminants in dairy products currently have no regulations. The limit for Pb in milk and dairy products is 0.020 mg kg^{-1} (European Commission, 2006). Previous studies have investigated the dietary intake of toxic metals from milk and its derivatives (Crupi et al., 2022; Giri et al., 2021; Koch et al., 2022).

In addition to nutritional and safety aspect, the elemental composition of foods (Zhang et al., 2018) can provide valuable information for authentication (G. P. Danezis et al., 2016), traceability (Aceto, 2016), and origin assessment of dairy products (Drivelos & Georgiou, 2012). The concentration of elements in foods can be affected by various factors, including climate and translocation from soil, water, and air (Zhang et al., 2018). Research has shown that elemental fingerprints of dairy products can be used to discriminate their geographical origin (de Andrade et al., 2022; Magdas et al., 2019; Tedesco et al., 2021), verify their PDO authenticity (G. P. Danezis et al., 2016, 2020; Di Donato et al., 2021; Nečemer et al., 2016; Santarcangelo et al., 2022), identify breeding methods (Rodríguez-Bermúdez et al., 2018), assess production processes (de Oliveira Filho et al., 2022), and trace the production chain (Aceto et al., 2017). Accurate sampling is necessary to achieve these goals and encompass all variables that can influence the elemental fingerprint. This includes seasonality,

product processing steps, soil characteristics, pollution sources, and class variability (G. P. Danezis & Georgiou, 2022).

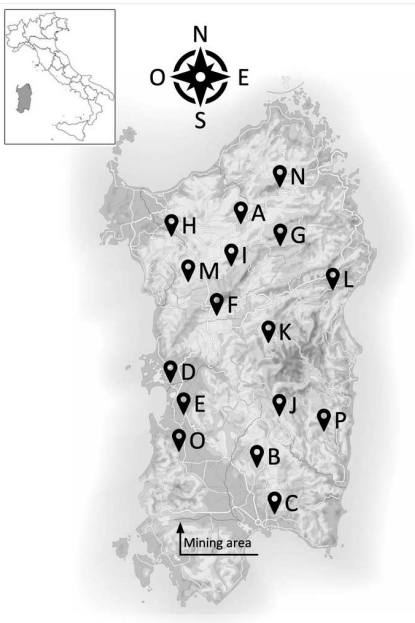
For several decades, this research group has concentrated on the valorization valorization (Caredda et al., 2016; Idda et al., 2016, 2018; Pulinas et al., 2017; Urgeghe et al., 2012), quality protection quality (Dedola et al., 2020; Piga et al., 2009, 2010, 2013), classification (Caredda et al., 2017), and food safety (Guiso et al., 2022; Spano et al., 2023; Zazzu et al., 2019) of dairy products from Sardinia. Therefore, the potential of elemental analysis in food valorization and authentication was evaluated in this study by measuring the elemental fingerprint of two Sardinian PDO sheep cheeses. The concentrations of 31 elements in 200 samples of PR and PS were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) and inductively coupled plasma-mass spectrometry (ICP-MS). The main objective was to evaluate nutritional properties and food safety to enhance products and record data for food authentication studies, e.g. for geographical discrimination. In addition, the effects of cheesemaking and seasonality on elemental fingerprints were evaluated.

3.2. MATERIALS AND METHODS

3.2.1. SAMPLES

A total of 200 samples of Pecorino cheese produced in Sardinia in 2021 were obtained from 16 dairy farms that collected milk from livestock farming in surrounding areas. Two PDO sheep cheeses were considered: PR (n=103) and PS (n=97). The production technologies adhere closely to the specifications outlined in their respective consortium regulations (Romano, 2023; Sardo, 2023). Pasteurized whole milk is curdled using cultures of milk enzymes from the milk's place of origin. To produce PR, the milk is coagulated at 38-40 °C and the curd is cooked at 45-48 °C. The resulting 20-35 kg wheels mature for 5-18 months. On the other hand, PS is produced by coagulating the milk at 35-39 °C, cooking the curd at 43 °C and ripening the 1.7-4.0 kg wheels for 2-6 months. The salting process can occur through both dry and wet methods. For PR dry salting, the side and plate of the cheese receive about 2800 g of NaCl distributed thrice over a maximum span of 50 days. Alternatively, the wheels are immersed in a dynamic brine (at 12°C and a NaCl concentration of 20-22%) for 20 days. Similarly, PS can also be salted dry or wet, but generally, it is kept in brine (24% of NaCl) for 10 hours kg^{-1} of cheese at a temperature of 10-12 °C.

In this study, cheeses were produced by each dairy in three periods of the year: winter, 37%; spring, 35%; and summer, 28%. Thus, samples differed in seasoning and cheesemaking. Additionally, the cheeses varied in maturation (PR: 5-18 months, PS: 2-6 months). The wheels were first divided according to ISO 707:2008 (Subramanian & Rodriguez-Saona, 2010). Then, aliquots obtained were aggregated, homogenized, and stored at a temperature between -18 °C and -24 °C until analysis. Figure 3.1 shows the selected information on Pecorino cheese samples.



Dairy Factory	Pecorino Romano			Pecorino Sardo			Total
	Winter	Spring	Summer	Winter	Spring	Summer	
A	2	2	3				7
B	3	3	3				9
C				6	6	4	16
D	3	3	3	6	5	2	22
E	3	3	3				9
F	3	3	3				9
G	2	2	3				7
H	2	3	3				8
I	3	3	3				9
J				6	6	4	16
K	3	3	3				9
L	2	4	3				9
M	3	3	3	6	4	2	21
N	3	3	3	6	4	2	21
O				6	4	2	12
P				6	6	4	16
Total	32	35	36	42	35	20	200

Figure 3.1. Description of pecorino sampling in terms of dairies, samples, and period of production.

3.2.2. INSTRUMENTATION AND REAGENTS

Elemental analysis was performed on a NexION350X spectrometer equipped with an S10 autosampler, a glass concentric nebulizer, a glass cyclonic spray chamber, and a kinetic energy discrimination (KED) collision cell, all from Perkin Elmer (Milan, Italy). The most abundant elements were determined using an OPTIMA 7300 DV spectrometer (Perkin Elmer, Waltham, MA, USA) equipped with a GemTip Cross-Flow II nebulizer (Perkin Elmer) and an autosampler (SC-2 DX, Elemental Scientific Inc., Omaha, NE, USA). To determine macro elements (Ca, K, Mg, Na, P, and S) cheese samples were previously dried in a drying oven (Memmert, Schwabach, Germany) and then calcined in a muffle furnace (Gelman Instrument, Opera, Italy). To detect trace elements (Zn, Fe, Mn, Cu, Se, Rb, Sr, Al, B, Co, Ni, Cr, V, Li, and Ag) and toxic elements (As, Cd, Hg, Pb, Sn, Sb, Tl, Te, Bi, and U) samples were digested using an ultraWAVE™ microwave single reaction chamber (SCR) system (Milestone, Sorisole, Italy) equipped with a rotor at 15 positions and 15 cm^3 polytetrafluoroethylene (PTFE) vessels. In all procedures, type I water (resistivity > 18 MΩ cm^{-1}) was produced using a MilliQ Plus System (Millipore, Milan, Italy). Hydrochloric acid (37% w/w), Nitric acid (67-69% w/w, NORMATON® for ultra-trace analysis), hydrogen peroxide (30% w/w, NORMATON® for ultra-trace

metal analysis), syringes (HENKE-JECT®, 20 cm^3), syringe filters (25 mm diameter, 0.22 μm pores, nylon), paper filters (ashless, Grade 40, Whatman®) and metal-free falcon tubes (15 cm^3 and 50 cm^3) were purchased from VWR (Milan, Italy). Periodic table mix 1 (TraceCERT®, 33 elements, 10 mg dm^{-3} in 10% HNO_3), periodic table mix 2 (TraceCERT®, 17 elements, 10 mg dm^{-3} in 5% HCl), periodic table mix 3 (TraceCERT®, 16 elements, 10 mg dm^{-3} in 5% HNO_3), and certified skimmed milk powder ERM®-BD151 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Single standard solutions of Sc, Y, Ge, Rh, Ir, Mo, Sb, Sn, Hg, and U (100-1000 mg dm^{-3} in 2-5% HNO_3) were obtained from LabKings (Hilversum, Netherlands).

3.2.3. SAMPLE PREPARATION

Sample preparation for macro elements analysis was performed as previously described (Lai et al., 2023). Sample digestion for trace and toxic elements was made using an ultraWAVE™ microwave single reaction chamber (SRC) system (Milestone, Sorisole). With respect to conventional microwave instruments, SRC technology reaches higher temperatures and pressures and can manage higher amounts of samples using lower amounts of reagents (Mara et al., 2022). In accordance with previous studies (Astolfi et al., 2020; Mara et al., 2022), nitric acid and hydrogen peroxide were used as oxidizing agents in this study. Approximately 0.450 g of the sample (exactly weighted on the analytical balance) was treated with 1 cm^3 of HNO_3 (67-69%), 2 cm^3 of H_2O_2 (30%), and 4 cm^3 of ultrapure H_2O.

The digestion program is listed in Table 3.1. After cooling, the samples were collected, diluted to 15 cm^3 using ultrapure H_2O, and filtered using a syringe filter. The final residual acidity determined by titration with 0.1 mol dm^{-3} sodium hydroxide was 2.5 ± 0.2%. To ensure the quality of the analytical data, each digestion batch included a blank and a sample of certified reference material (CRM), ERM-BD151. The same CRM was used to assess the efficiency of microwave acid digestion in terms of matrix effect and trueness.

Table 3.1. Cheese digestion conditions using an ultraWAVE™ SRC system (Milestone).

Step		Time (min)	Temperature (°C)
1	Heating	25	240
2	Holding	10	240
3	Cooling	ca. 30	< 40

Initial pressure: 4 MPa; Release pressure rate: 0.8 MPa min^{-1}; Rotor: 15 positions; Vessels: 15 cm^3 (PTFE), Method: 0. 450 g cheese + 1 cm^3 HNO_3 (67-69%) + 2 cm^3 H_2O_2 (30%) + 4 cm^3 H_2O

3.2.4. ELEMENTAL ANALYSIS, VALIDATION, QUALITY CONTROL AND ASSURANCE

Macro elements (Ca, K, Mg, Na, P, and S) were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES), whereas trace and toxic elements were analyzed using inductively coupled plasma-mass spectrometry (ICP-MS). The instrumental parameters used for the analysis are reported in Table C1 (ICP-OES) and Table C2 (ICP-MS). Further details regarding the ICP-OES method have been previously reported (Lai et al., 2023), whereas the ICP-MS method was fully developed and validated in this study. For each PDO cheese, three samples were randomly selected and analyzed using the semi-quantitative TotalQuant® method (Syngistix software v 2.3). This preliminary analysis allowed the assessment of the elements that were always within the instrumental detection limit. Excluding these, the elements of interest for a possible ICP-MS quantification were Ag, Al, As, B, Bi, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Ni, Pb, Rb, Sb, Se, Sn, Sr, Te, Tl, U, V, and Zn. Subsequently, the possible presence of polyatomic interferences in the real matrix was ascertained, and for each element, the most suitable analysis mode (STD mode or KED mode) was determined. Validation was accomplished in terms of limits of detection and quantification, precision, and trueness. The validation parameters are listed in Table C3. The limits of detection (LoD) and quantification (LoQ) were calculated according to Currie (Currie, 1999). Method repeatability ($CV\%_{r)}$ was assessed by analyzing samples in triplicate within the same analytical session, whereas intermediate precision ($CV\%_{IP}$) was calculated using data obtained from different analytical sessions. Finally, trueness was evaluated by analyzing certified milk CRM ERM BD-151 and spiking tests. In the last case, for each analyte, samples were spiked three times at increasing concentration levels. The trueness measured by analyzing the CRM (Table C4) was between 89 ± 5% (P) and 114 ± 5% (Na) for the macro elements (ICP-OES method), whereas that measured for the trace and toxic elements (ICP-MS method) ranged from 92 ± 5% (Cd) to 110 ± 5%

(Se). Furthermore, the recoveries measured by spiking tests ranged from 86 ± 1% (Ag) to 149± 7% (As). The recovery results (Table C3) show that the determination of more than 80% of the elements was bias-free (criteria: t-test, p = 95%). Moderate underestimation was observed for Ag and Bi, and slight overestimation for Sr and V. However, the observed bias are acceptable according to the AOAC guidelines (Association of Official Agricultural Chemists (AOAC), 1998). Finally, for Na and As, a meaningful overestimation bias was observed.

Quantification was performed by external calibration using single- and multi-element standard solutions in 2.5% HNO_3. The calibration was performed according to the expected analyte concentrations. Additionally, measurements were performed in triplicate and the data were blank-corrected. To account for signal fluctuations and matrix effects, Rh (50 µg dm^{-3}) and Ir (1 µg dm^{-3}) were used as internal standards. A 60-second wash with a 2% aqueous solution of HNO_3 was introduced between consecutive samples to prevent memory effects.

3.2.5. STATISTICAL ANALYSIS

Data analysis was performed using R-Studio (v. 4.3.1) and Chemometric Agile Tool (CAT) (Leardi et al., 2021). The Shapiro-Wilk test was used to confirm the normal distribution of the data. ANOVA and MANOVA tests were used to compare groups, and Tukey's HSD test was used as a post-hoc test. Principal Component Analysis (PCA) was performed for data visualization. Linear Discriminant Analysis (LDA) was used to discriminate samples from different categories. Statistical significance was set at a < 0.05.

3.3. RESULTS AND DISCUSSION

3.3.1. ELEMENTAL COMPOSITION OF PECORINO ROMANO PDO AND PECORINO SARDO PDO

The elemental analysis of PR and PS was conducted using ICP-OES to determine the concentrations of macroelements (Ca, K, Mg, Na, P, and S), and ICP-MS to measure the amounts of trace elements (Zn, Fe, Mn, Cu, Se, Rb, Sr, Al, B, Co, Ni, Cr, V, Li, and Ag) and toxic elements (As, Cd, Hg, Pb, Sn, Sb, Tl, Te, Bi, and U). The results are presented in Table 3.2 and expressed in kg of dry matter in the cheese.

Table 3.2. Elemental analysis of Pecorino Sardo PDO and Pecorino Romano PDO.

Element		Pecorino Sardo PDO			Pecorino Romano PDO		
		min	**mean ± sd**	**max**	**min**	**mean ± sd**	**max**
Macro (mg kg^{-1})	Ca	10000	14000 ± 1000	16000	10000	14000 ± 1000	16000
	K	1000	1300 ± 200	1600	700	1000 ± 100	1300
	Mg	600	700 ± 50	800	500	600 ± 40	800
	Na	5000	8000 ± 1000	11000	17000	25000 ± 5000	38000
	P	8000	9000 ± 500	10000	7000	9000 ± 700	10000
	S	500	700 ± 100	1000	500	1000 ± 200	1400
Trace elements (μg kg^{-1})	Zn	37000	56000 ± 9000	78000	18000	47000 ± 7500	60000
	Fe	2600	7000 ± 3000	14300	2400	6000 ± 950	8500
	Mn	560	850 ± 100	1250	310	800 ± 100	1150
	Cu	600	1200 ± 500	2100	500	1000 ± 350	1800
	Se	210	340 ± 90	530	220	400 ± 100	580
	Rb	1000	1700 ± 500	2600	600	1600 ± 500	2600
	Sr	7800	13400 ± 2500	17900	4400	14300 ± 2500	20500
	Al	200	6000 ± 3000	13900	2500	6000 ± 2000	11300
	B	< 54	2000 ± 2000	7100	< 54	8000 ± 8000	30000
	Co	0.9	4 ± 1	9	1.3	4 ± 1	6
	Ni	< 10	30 ± 10	60	< 10	27 ± 5	40
	Cr	< 3.1	40 ± 20	95	< 3.1	20 ± 10	50
	V	5	10 ± 5	21	7	15 ± 5	24
	Li	< 55	< 55	< 55	< 55	< 55	< 55
	Ag	< 1.6	5 ± 5	8	< 1.6	5 ± 5	10
Toxic elements (μg kg^{-1})	As	< 3.3	6 ± 1	8.4	5.7	8 ± 1	10.8
	Cd	0.5	1 ± 0.5	1.7	0.5	1.2 ± 0.5	1.9
	Hg	< 30	< 30	< 30	< 30	< 30	< 30
	Pb	< 3.4	20 ± 10	40	< 3.4	20 ± 10	45
	Sn	< 2.4	20 ± 10	54	< 2.4	10 ± 10	32
	Sb	< 3.6	12 ± 5	16	< 3.6	10 ± 5	18
	Tl	< 0.5	1.9 ± 0.5	2.3	< 0.5	< 0.5	< 0.5
	Te	< 1.2	130 ± 50	220	< 1.2	9 ± 5	15
	Bi	< 0.5	< 0.5	< 0.5	< 0.5	2 ± 1	2.8
	U	< 0.19	1 ± 1	5.2	< 0.19	2 ± 1	7.1

Both cheeses had comparable levels of Ca, Mg, K and P in terms of macroelements, with an order of abundance that reflected their concentration in the original milk: Ca > P > K > S ⩾ Mg. However, due to the distinct salting process employed, the Na concentration in PR was higher than that in PS. Typically, the NaCl concentration in PR ranges from 3% to 7%, whereas in PS it rarely exceeds 2%. Even in terms of trace elements, both cheeses have a similar elemental content. Consistent with the initial milk composition, the trace elements found in highest abundance were Zn, Fe, and Cu. Both PR and PS contained similar amounts of Se, Rb, Sr, and Al. Other trace elements, such as Co, Ni, Cr, V, Li, and Ag, were present in both cheeses at levels near or below the limit of quantification.

Regarding toxic elements, both cheeses contained low levels of As, Cd and Pb. Hg was never detected. Other toxic elements, such as Sn, Sb, Te, Tl, Bi, and U, were generally either not quantified or present at very low levels. It is worth noting that the level of Te in PS was significantly higher than that in PR. However, the European Food Safety Agency (EFSA) is currently investigating the potential toxicity of Te (Kowalczyk et al., 2022).

Finally, a semi-quantitative analysis of rare earth elements (REEs) was performed (data not shown). The REEs were seldom detected above the instrumental detection limit, with a few exceptions for the LREEs. Further investigations will be conducted to optimize the limits of quantification of the analytical method and enable the determination of markers for traceability of the production chain (Aceto et al., 2017).

To the best of our knowledge, the determination of trace elements in PR and/or PS has rarely been accomplished. Previous literature has mainly focused on quantifying macro elements (Coni et al., 1999; Di Donato et al., 2021; Manuelian et al., 2017), with only occasional attention given to trace elements such as Zn, Fe, Se, Cu (Manuelian et al., 2017), Ba (Di Donato et al., 2021), and Al, Ba, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Pt, Sr, and Zn (Coni et al., 1999). The data obtained in this study are in good agreement. Table C5 enables a comparison of the elemental compositions of PS and PR as measured in this study and in the literature.

3.3.2. DIFFERENTIATION DUE TO CHEESE-MAKING PROCESS TECHNOLOGY

Elemental fingerprinting has been reported in the literature as a method for authenticating cheeses (G. Danezis et al., 2019; G. P. Danezis et al., 2020; de Andrade et al., 2022; Di Donato et al., 2021; Magdas et al., 2019; Nečemer et al., 2016). This technique is commonly used to discriminate cheeses made from milk of different animal origins (G. Danezis et al., 2019; G. P. Danezis et al., 2020; Nečemer et al., 2016), from different geographical areas (de Andrade et al., 2022; Di Donato et al., 2021), or from significantly different cheese-making processes, such as final moisture content and salting (G. P. Danezis et al., 2020; Magdas et al., 2019). Samples were collected from various dairies in Sardinia (Italy) and varied in cheese-making technologies and production period (seasonality).

Principal Component Analysis (PCA) was used for data visualization. The data was cleaned by removing any elements that were not quantified in at least 90% of the samples or were not significant for the analysis. Additionally, Na was excluded as a variable to eliminate the influence of the salting process. Outliers were identified and removed using T^2 and Q statistics after performing a preliminary PCA with $p > 0.05$. The results of the PCA are presented in Figure 3.2.

PC1 and PC2 accounted for 24.5% and 19.7% of the total variance, respectively. Figure 3.2a shows that the most abundant trace elements, such as Zn and Cu, are characterized by positive values of PC1, while macro elements, including Ca, Mg, P, and K, are characterized by positive PC2 values. Looking at the score plot (Figure 3.2b), positive PC1 values tended to occur in the PS cluster (red samples), which was associated with a higher concentration of trace elements, while the PR cluster (black samples) tended to have negative PC1 values. The differentiation between the two clusters was accentuated upon observing PC3, which explained 12.1% of the variance (3D score plot, Figure 3.2c). This evidence suggests that the elemental fingerprint may be used to discriminate between the two types of cheese.

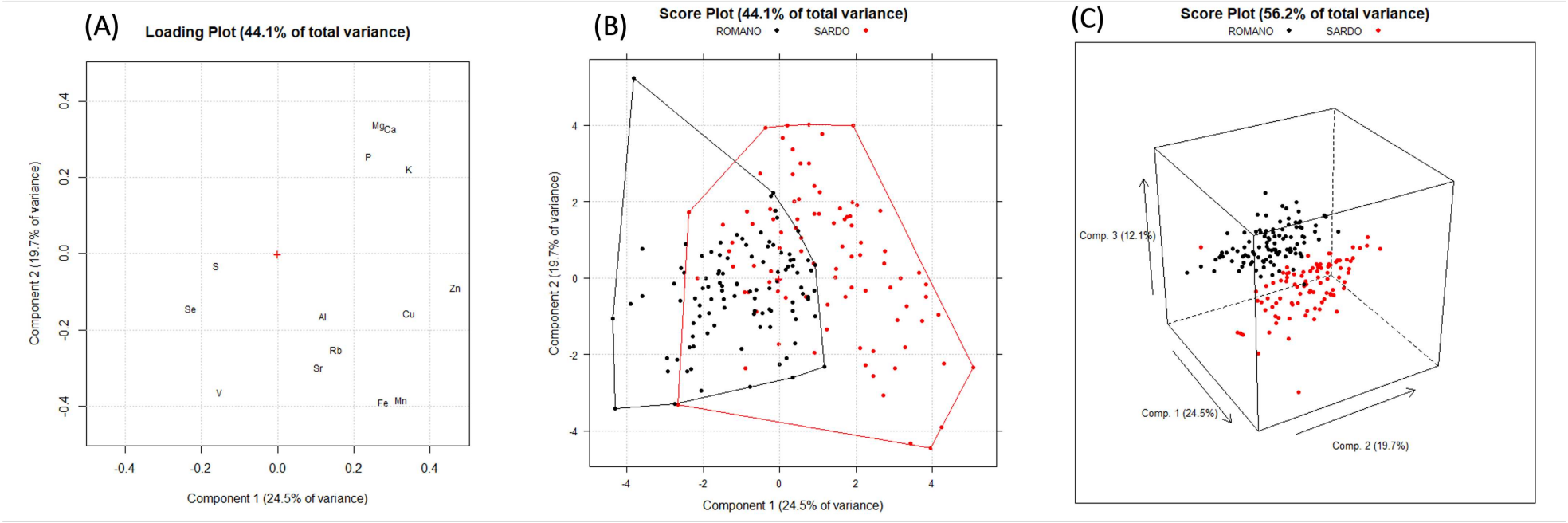


Figure 3.2. PCA analysis was performed on 196 pecorino samples and 14 elements: (a) loading plot; (b) score plot; (c) 3D score plot. Object colored according to cheese type.

Linear Discriminant Analysis (LDA) was used for classification. The MANOVA test showed a significant difference between the two groups (F (15, 180), Wilks = 0.175, approx. F = 56.78, $p < 0.001$). Prior to LDA, the dataset was randomized and split into a training set (n=140) and a test set (n=55). The results obtained from cross-validation and prediction are reported in Table 3.3.

Table 3.3. Results of the LDA performed for the discrimination based on cheese type. Confusion matrix and accuracy in cross-validation (training) and prediction (testing).

Confusion matrix

Training	Romano	Sardo	Testing	Romano	Sardo
Romano	67	3	Romano	32	0
Sardo	1	69	Sardo	2	21

Accuracy

Romano	Sardo	Total	Romano	Sardo	Total
95.7%	98.6%	97.1%	100%	91.3%	95.7%

The levels of discrimination achieved in cross-validation (97.1%) and prediction (95.7%) were highly accurate. The elemental fingerprint can discriminate between PR and PS using macro-elements (i.e. Ca, K, Mg, P, and S) and trace elements (i.e. Zn, Fe, Mn, Cu, Se, Rb, Sr, Al, Co, and V). These findings were consistent when LDA was used to analyze data from samples produced by three farms that yielded both PR and PS during the same period. The statistical significance of the data was reduced due to the smaller sample size (n=62). However, the Principal Component Analysis (PCA) in Figure C1 showed that the samples were distinguishable, and the LDA successfully classified them with an accuracy of 98.1% in cross-validation.

Among the literature reviewed, the study by Di Donato et al (Di Donato et al., 2021) was the most comparable to the present study as it adopted a similar approach for authenticating Italian Pecorino cheese. However, it is important to note that the samples in their study were geographically diverse, collected from three different regions of Italy. In contrast, the differences found in our study were solely attributed to the cheese production method used.

The study confirmed the impact of cheese-making technology on the elemental fingerprint. The differences in the chemical equilibrium of milk's various elements, as well as the chemical form (ionic soluble or colloidal bound to milk proteins and fats), and the variations in cheese-making techniques (coagulation, wheying, and salting) could explain the distinct elemental fingerprints of the two cheeses analyzed in this

research. During cheese production, the main stages that lead to significant variance in the concentration of elements found in the cheese are salting and coagulation.

Salting triggers osmotic phenomena, resulting in fluctuations in the levels of unbound minerals, which can cause a loss of water and cationic elements such as Na, K, Al, Cd, Co, and Rb. On the other hand, the elements bound to caseins and fats, such as Ca, P, Fe, Mg, Mn, Ni, Pt, and Zn, become concentrated.

During coagulation, there is a non-uniform distribution of elements between the curd and whey. Na and K are soluble elements and tend to be distributed in the aqueous phase (whey). On the other hand, Ca, Mg and P are associated in different proportions with the colloidal suspension of casein micelles and are more concentrated in the curd during cheese production (Recio et al., 2009). Several studies have shown that the soluble phase of sheep's milk may contain different percentages of Ca, Mg and P, with deviations from the total content ranging from 20-25%, 35-64% and 35-40%, respectively (M.A et al., 1997; O et al., 1994; Polychroniadou & Vafopoulou, 1985). Currently, there is limited information available on the distribution of trace elements during sheep milk cheese production. However, data indicate that Zn and Mn are primarily distributed in the curd, accounting for about 90%, while Fe and Cu account for about 70% (M.A et al., 1997). Fluctuations in pH, temperature, and milk storage conditions affect the equilibrium between soluble and colloidal forms. Generally, a decrease in pH and temperature shifts the balance towards soluble ionic forms, while an increase in pH and temperature favors solubilization. This results in an increase in the retention of certain elements in the curd during cheese production.

Based on these considerations, the various conditions of acidification (which are more intense in PR compared to PS), curd breaking (which is more extensive in PR compared to PS), cooking (with curd cooking at 45°C in PR and 43°C in PS), whey removal, heating, curd cooling, and salting may have induced differences in the balance between curd and whey. As a result, the different cheesemaking technologies favor the retention of certain elements, especially Zn, Fe, Cu, and Mn in PS compared to PR (see Figure 3.2). Milder acidification and breaking of the curd may have resulted in less demineralization during the production of PS. This could have led to greater retention of trace elements that are partially bound to casein micelles, such as Zn, Mg, Fe, and Cu, as previously observed. Additionally, rapid lactic fermentation followed by effective whey removal promotes curd demineralization (Martín-Hernández et al., 1992).

3.3.3. EFFECT OF SEASONALITY

In Sardinian sheep farming births are synchronized and the lactation period starts in November and ends in June - July. The chemical composition of sheep's milk changes during this period, depending on the diet, lactation stage, and climate (Pulina et al., 2021). The concentration of minerals and major classes of compounds can be affected by the lactation stage (Timlin et al., 2021). Therefore, we evaluated the effect of seasonality on the elemental composition of PR and PS cheeses using PCA and ANOVA.

The PCA analysis in Figure C2 shows a distinct trend for Pecorino Romano PDO cheese. The loading plot indicates that PC1 describes the correlation between trace element concentrations and seasonality. Negative values indicate elements that are more abundant in PR produced in summer (V, Al, Rb, and Fe), while positive values indicate elements that are more abundant in PR produced in winter (Zn and Cu). The ANOVA results confirmed that seasonality had an impact on 12 out of 15 elements (see Figure C3). Winter-produced cheeses had the highest concentrations of Zn, Cu, K, and Mn, while spring-produced cheeses had the highest concentrations of Ca, Mg, P, and S. Summer-produced cheeses had the highest concentrations of Rb, Fe, Al, and V. The concentrations of Se, Na, and Sr were not affected by seasonal variations.

In contrast, the impact of seasonality on Pecorino Sardo PDO is relatively minor. Although the PCA did not reveal any clear trends (see Figure C4), the ANOVA indicates a significant effect of seasonality on 8 out of 15 elements (Zn, Ca, K, P, S, Cu, Rb, and V), as shown in Figure C5. Notably, the trends for these elements were like those observed for PR, with higher concentrations of Ca, P, and Mg found in PS during the spring season. Additionally, the concentration of Cu was highest in winter cheese.

As expected, both types of pecorinos exhibit similar seasonal variations in their composition, reflecting the composition of sheep's milk. Ca is closely linked with P in casein micelles, which provide the structure and stability of the micelles. Colloidal calcium phosphate links the casein submicelles together, occupying 6% of the micellar structure. Therefore, there is a positive correlation between Ca, P, and casein content in ruminant milk (Holt, 2011). Both cheeses (PS and PR) exhibit the highest concentrations of Ca, Mg, P, and S during the spring season when the amount of casein in the ewe's milk reaches its daily maximum level (Pulina et al., 2021). S is not directly involved in the stabilization of micelles, but it is present in proteins, specifically whey proteins (cysteine and cystine amino acids). Therefore, the higher concentration of sulfur in spring cheeses could be linked to the protein concentration found in the milk of spring sheep. Despite this, the cheese still contains only small amounts of whey proteins. When examining the alkaline elements, no evident trends were found. Na cannot be evaluated due to the salting process. K remained constant in winter and spring but decreased significantly in summer. Rb in PR increased from winter to summer, but this trend was not observed in PS. This difference was likely due to different production methods. Regarding trace elements, there were two opposite trends observed. At the start of the lactation period, the concentrations of Zn, Mn, and Cu were highest. However, towards the end of the lactation period, the concentrations of Fe and V tended to increase.

The elemental composition of sheep's milk during lactation is subject to seasonal variations, which are influenced by various factors such as the lactation stage, nutritional status of the animal, as well as environmental and genetic factors (Fox & McSweeney, 2009; Recio et al., 2009). The mineral content in milk is weakly affected by ruminant feeding because the maternal skeleton tends to demineralize during periods when dietary mineral intake does not meet the mineral requirements of the newborn, thus compensating for the deficit (Woodrow et al., 2006). Skeletal demineralization typically occurs during periods of high

mineral demand, such as early lactation and colostrum production (Liesegang et al., 2000). The influence of the lactation stage on the mineral composition of milk is not well-documented. In bovine milk, Ca, P, Mg, and Na levels tend to increase towards the end of the lactation period (Gulati et al., 2018). This is likely due to increased permeability of the mammary epithelium as lactation progresses (Hettinga, 2019). Finally, the mineral content of milk can also be influenced by the animal's health status and genetic type. The concentration of most minerals in milk decreases when mastitis is present in the mammary gland, except for sodium and chloride ions, which increase instead (Frédéric, 2005).

3.3.4. NUTRITIONAL AND SAFETY ASPECTS

Milk and dairy products are considered highly nutritious. Mineral content is an important factor in determining food value, according to consumer preferences. Cheese is a well-known source of minerals, especially Ca, P, and Mg. Casein peptides in milk or cheese prevent the precipitation of calcium in the intestine, making it easily bioavailable (Ebringer et al., 2008). Although the etiology of osteoporosis is complex, adequate calcium intake during childhood and adolescence is important for developing high peak bone mass. Maximizing bone mass early in life is considered a crucial preventive factor against osteoporosis (Theobald, 2005). This study's results indicate that Pecorino Romano PDO and Pecorino Sardo PDO are sources of several nutritional elements. Figure 3.3 displays the daily mineral intake for both cheeses across genders. The data were calculated based on the Dietary Recommended Intakes (DRI) published by the US Department of Health and Human Services, National Institutes of Health (U.S. Department of Health & Human Services. National Institutes of Health, 2019).

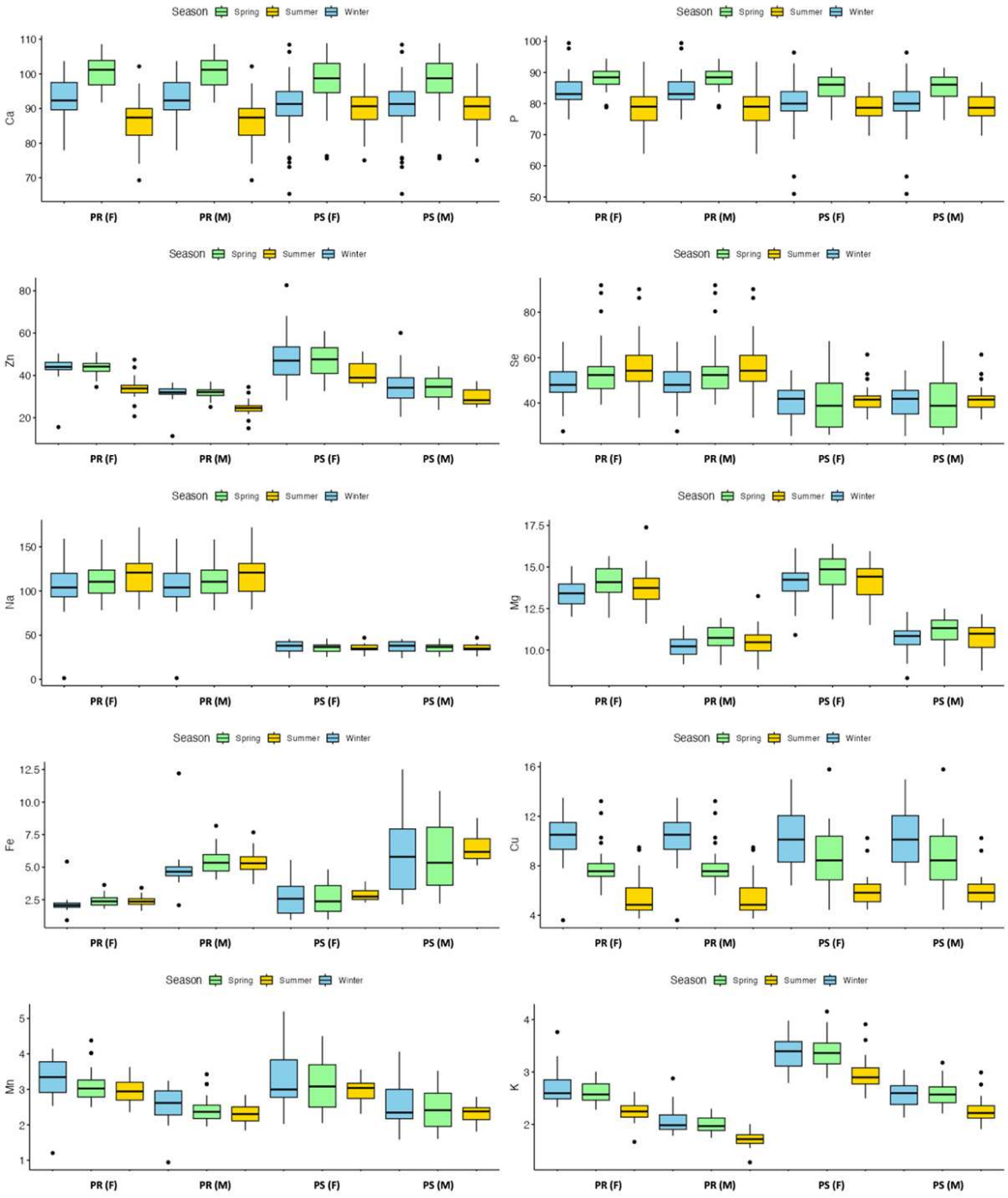


Figure 3.3. Mineral daily intakes for adult males and females (17-70 years old) for the consumption of 100g of Pecorino Romano PDO and Pecorino Sardo PDO**.**

Figure 3.3 shows that the daily consumption of both cheeses meets the Recommended Dietary Allowances (RDAs) and Adequate Intakes (AIs) for many elements (daily portion of 100g). According to European guidelines (European Union, 1990, 2006), both cheeses are rich in Ca, P, Zn, and Se (DRI > 30%). Additionally, PR and PS cheeses are potential sources of Mg (females, DRI > 15%) and Cu (DRI > 15%). The Cu content in cheeses may vary seasonally, as shown in Figures C3-S5. Therefore, it may be possible to produce cheeses with high Cu content during the winter season. These findings are significant as they allow dairies and protection Consortia to implement nutritional labeling in accordance with European regulations (Commission, 2012). As for toxic elements such as As, Cd, Hg, Pb, and Sn, both PR and PS cheeses have a high level of food safety. Additionally, the concentrations of Tl, Bi, and U were frequently below their limits of quantification. Moreover, the cheeses analyzed in this study were obtained from various locations (see Figure 3.1), indicating the high level of food safety in Sardinian sheep's milk production.

3.4. CONCLUSIONS

Pecorino Romano PDO and Pecorino Sardo PDO are two of the most popular and appreciated cheeses. However, there is a lack of knowledge regarding their elemental composition and trace element content. To address this research gap, this study analyzed 31 elements (macro, trace, and toxic) in a comprehensive sample of cheeses using ICP-based methods. The analysis provided valuable insights into the nutritional properties of both pecorino cheeses. They are rich in essential minerals such as Ca, P, Zn and Se, and potentially a source of Cu and Mg, depending on the season of production of the cheese and the gender daily requirement intake (DRI). These findings emphasize the nutritional and safety aspects of both cheeses and suggest that Consortia should implement nutritional labelling.

Moreover, the data allowed for an assessment of the impact of cheesemaking and seasonality on the elemental composition. Linear discriminant analysis demonstrated that the elemental fingerprint effectively discriminates dairy products based on their production method, while excluding other variables such as geographical and animal origin. Even though the two cheeses are not in competition, discriminate cheese using the elemental fingerprinting could be a useful methodology for detecting food fraud involving other cheaper pecorinos.

Future perspectives include the use of the recorded data for further studies on food authentication, e.g. for discrimination based on geographical origin. In this context, the ICP-MS method needs to be optimized for the analysis of other markers such as rare earth elements. Additionally, the methodology could be applied to detect fraudulent activities, such as mixing lower value cheeses with grated PDO cheeses. Finally, additional food technology studies will be conducted to monitor the distribution of elements along the cheese-making process.

3. REFERENCES

Aceto, M. (2016). The Use of ICP-MS in Food Traceability. In *Advances in Food Traceability Techniques and Technologies: Improving Quality Throughout the Food Chain*. https://doi.org/10.1016/B978-0-08-100310-7.00008-9

Aceto, M., Musso, D., Calà, E., Arieri, F., & Oddone, M. (2017). Role of Lanthanides in the Traceability of the Milk Production Chain. *Journal of Agricultural and Food Chemistry*, *65*(20), 4200–4208. https://doi.org/10.1021/acs.jafc.7b00916

Association of Official Agricultural Chemists (AOAC). (1998). Peer-Verified Methods Program—Policies and procedures. *AOAC International*.

Astolfi, M. L., Conti, M. E., Marconi, E., Massimi, L., & Canepari, S. (2020). Effectiveness of Different Sample Treatments for the Elemental Characterization of Bees and Beehive Products. *Molecules*, *25*(18), 4263. https://doi.org/10.3390/molecules25184263

Camanzi, L., Arba, E., Rota, C., Zanasi, C., & Malorgio, G. (2018). A structural equation modeling analysis of relational governance and economic performance in agri-food supply chains: Evidence from the dairy sheep industry in Sardinia (Italy). *Agricultural and Food Economics*, *6*(1). https://doi.org/10.1186/s40100-018-0099-z

Cannas, D., Loi, E., Serra, M., Firinu, D., Valera, P., & Zavattari, P. (2020). *Relevance of essential trace elements in nutrition and drinking water for human health and autoimmune disease risk*. https://doi.org/10.3390/nu12072074

Caredda, M., Addis, M., Ibba, I., Leardi, R., Scintu, M. F., Piredda, G., & Sanna, G. (2016). Prediction of fatty acid content in sheep milk by Mid-Infrared spectrometry with a selection of wavelengths by Genetic Algorithms. *LWT*, *65*, 503–510. Scopus. https://doi.org/10.1016/j.lwt.2015.08.048

Caredda, M., Addis, M., Ibba, I., Leardi, R., Scintu, M. F., Piredda, G., & Sanna, G. (2017). Building of prediction models by using Mid-Infrared spectroscopy and fatty acid profile to discriminate the geographical origin of sheep milk. *LWT*, *75*, 131–136. Scopus. https://doi.org/10.1016/j.lwt.2016.08.053

Commission, E. (2012). *European Union Regulation (EC) No. 432/2012 16 May 2012 on the compilation of a list of permitted health claims on food, other than those referring to the reduction of disease risks and to the development and health of children*. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:136:0001:0040:en:PDF

Coni, E., Bocca, B., & Caroli, S. (1999). *Minor and trace element content of two typical Italian sheep dairy products*. https://doi.org/10.1017/S0022029999003775

Crupi, R., Lo Turco, V., Gugliandolo, E., Nava, V., Potortì, A. G., Cuzzocrea, S., Di Bella, G., & Licata, P. (2022). Mineral Composition in Delactosed Dairy Products: Quality and Safety Status. *Foods*, *11*(2). https://doi.org/10.3390/foods11020139

Currie, L. A. (1999). Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC Recommendations 1995). *Analytica Chimica Acta*, *391*(2), 105–126. https://doi.org/10.1016/S0003-2670(99)00104-X

Danezis, G. P., & Georgiou, C. A. (2022). Elemental metabolomics: Food elemental assessment could reveal geographical origin. *Current Opinion in Food Science*, *44*. https://doi.org/10.1016/j.cofs.2022.100812

Danezis, G. P., Pappas, A. C., Tsiplakou, E., Pappa, E. C., Zacharioudaki, M., Tsagkaris, A. S., Papachristidis, C. A., Sotirakoglou, K., Zervas, G., & Georgiou, C. A. (2020). Authentication of Greek Protected Designation of Origin cheeses through elemental metabolomics. *International Dairy Journal*, *104*. https://doi.org/10.1016/j.idairyj.2019.104599

Danezis, G. P., Tsagkaris, A. S., Camin, F., Brusic, V., & Georgiou, C. A. (2016). Food authentication: Techniques, trends & emerging approaches. *TrAC - Trends in Analytical Chemistry*, *85*, 123–132. https://doi.org/10.1016/j.trac.2016.02.026

Danezis, G., Theodorou, C., Massouras, T., Zoidis, E., Hadjigeorgiou, I., & Georgiou, C. A. (2019). Greek graviera cheese assessment through elemental metabolomics—Implications for authentication, safety and nutrition. *Molecules*, *24*(4). https://doi.org/10.3390/molecules24040670

de Almeida Ribeiro Carvalho, M., Botero, W. G., & de Oliveira, L. C. (2022). *Natural and anthropogenic sources of potentially toxic elements to aquatic environment: A systematic literature review*. https://doi.org/10.1007/s11356-022-20980-x

de Andrade, B. M., Margalho, L. P., Batista, D. B., Lucena, I. O., Kamimura, B. A., Balthazar, C. F., Brexó, R. P., Pia, A. K. R., Costa, R. A. S., Cruz, A. G., Granato, D., Sant'Ana, A. S., Luna, A. S., & de Gois, J. S. (2022). Chemometric classification of Brazilian artisanal cheeses from different regions according to major and trace elements by ICP-OES. *Journal of Food Composition and Analysis*, *109*. https://doi.org/10.1016/j.jfca.2022.104519

de la Fuente, M. A., & Juárez, M. (2015). Milk and dairy products. In *Handbook of Mineral Elements in Food* (pp. 645–668). wiley. https://doi.org/10.1002/9781118654316.ch28

de Oliveira Filho, E. F., Miranda, M., Ferreiro, T., Herrero-Latorre, C., Castro Soares, P., & López-Alonso, M. (2022). Concentrations of Essential Trace and Toxic Elements Associated with Production and Manufacturing Processes in Galician Cheese. *Molecules*, *27*(15). https://doi.org/10.3390/molecules27154938

Dedola, A. S., Piras, L., Addis, M., Pirisi, A., Piredda, G., Mara, A., & Sanna, G. (2020). New analytical tools for unmasking frauds in raw milk-based dairy products: Assessment, validation and application to fiore sardo PDO cheese of a RP-HPLC method for the evaluation of the α-l-fucosidase activity. *Separations*, *7*(3), 1–11. https://doi.org/10.3390/separations7030040

Di Donato, F., Foschi, M., Vlad, N., Biancolillo, A., Rossi, L., & D'archivio, A. A. (2021). Multi-elemental composition data handled by chemometrics for the discrimination of high-value italian pecorino cheeses. *Molecules*, *26*(22). https://doi.org/10.3390/molecules26226875

Drivelos, S. A., & Georgiou, C. A. (2012). Multi-element and multi-isotope-ratio analysis to determine the geographical origin of foods in the European Union. *TrAC - Trends in Analytical Chemistry*, *40*, 38–51. https://doi.org/10.1016/j.trac.2012.08.003

Ebringer, L., Ferencik, M., & Krajcovic, J. (2008). Beneficial Health Effects of Milk and Fermented Dairy Products. *Folia Microbiologica*, *53*, 378–394.

European Commission. (2006). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, *L364*, 5–24. Scopus.

European Commission. (2011). Commission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. *Off. J. Eur. Union*, *L6*, 3–6. Scopus.

European Union. (1990). *Council Directive 90/496/EEC of 24 September 1990 on nutrition labelling rules of foodstuffs.* http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri= CELEX:31990L0496&from=EN.

European Union. (2006). *Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods.*

Fox, P., & McSweeney, P. (2009). Advanced Dairy Chemistry. In *Advanced Dairy Chemistry* (Vol. 1). https://doi.org/10.1007/978-0-387-84865-5

Frédéric, G. (2005). The minerals of milk. *Reprod. Nutr. Dev.*, *45*(4), 473–483. https://doi.org/10.1051/rnd:2005030

Giri, A., Bharti, V. K., Kalia, S., Kumar, B., & Chaurasia, O. P. (2021). Health Risk Assessment of Heavy Metals Through Cow Milk Consumption in Trans-Himalayan High-Altitude Region. *Biological Trace Element Research*, *199*(12), 4572–4581. https://doi.org/10.1007/s12011-021-02593-6

Guiso, M. F., Battacone, G., Canu, L., Deroma, M., Langasco, I., Sanna, G., Tsiplakou, E., Pulina, G., & Nudda, A. (2022). Essential and Toxic Mineral Content and Fatty Acid Profile of Colostrum in Dairy Sheep. *Animals*, *12*(20). https://doi.org/10.3390/ani12202730

Gulati, A., Galvin, N., Lewis, E., Hennessy, D., O'Donovan, M., McManus, J. J., Fenelon, M. A., & Guinee, T. P. (2018). Outdoor grazing of dairy cows on pasture versus indoor feeding on total mixed ration: Effects on gross composition and mineral content of milk during lactation. *Journal of Dairy Science*, *101*(3), 2710–2723. https://doi.org/10.3168/jds.2017-13338

Hettinga, K. (2019). *Lactose in the dairy production chain* (pp. 231–266). https://doi.org/10.1016/B978-0-12-811720-0.00006-4

Holt, C. (2011). Milk Salts | Interaction with Caseins. In J. W. Fuquay (Ed.), *Encyclopedia of Dairy Sciences (Second Edition)* (Second Edition, pp. 917–924). Academic Press. https://doi.org/10.1016/B978-0-12-374407-4.00356-3

Idda, I., Spano, N., Addis, M., Galistu, G., Ibba, I., Nurchi, V. M., Pilo, M. I., Scintu, M. F., Piredda, G., & Sanna, G. (2018). *Optimization of a newly established gas-chromatographic method for determining lactose and galactose traces: Application to Pecorino Romano cheese*. https://doi.org/10.1016/j.jfca.2018.09.006

Idda, I., Spano, N., Ciulu, M., Nurchi, V. M., Panzanelli, A., Pilo, M. I., & Sanna, G. (2016). *Gas chromatography analysis of major free mono- and disaccharides in milk: Method assessment, validation, and application to real samples*. https://doi.org/10.1002/jssc.201600583

Islam, M. R., Akash, S., Jony, M. H., alam, M. N., Nowrin, F. T., Rahman, M. M., Rauf, A., & Thiruvengadam, M. (2023). *Exploring the potential function of trace elements in human health: A therapeutic perspective*. https://doi.org/10.1007/s11010-022-04638-3

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). *Toxicity, mechanism and health effects of some heavy metals*. https://doi.org/10.2478/intox-2014-0009

Koch, W., Czop, M., Iłowiecka, K., Nawrocka, A., & Wiącek, D. (2022). Dietary Intake of Toxic Heavy Metals with Major Groups of Food Products—Results of Analytical Determinations. *Nutrients*, *14*(8). https://doi.org/10.3390/nu14081626

Kowalczyk, E., Givelet, L., Amlund, H., Sloth, J. J., & Hansen, M. (2022). *Risk assessment of rare earth elements, antimony, barium, boron, lithium, tellurium, thallium and vanadium in teas*. https://doi.org/10.2903/j.efsa.2022.e200410

Kraus, A. (2015). Development of functional food with the participation of the consumer. Motivators for consumption of functional products. *International Journal of Consumer Studies*, *39*(1), 2–11. https://doi.org/10.1111/ijcs.12144

Lai, G., Caboni, P., Piras, C., Pes, M., Sitzia, M., Addis, M., Pirisi, A., & Scano, P. (2023). Development and Chemico-Physical Characterization of Ovine Milk-Based Ingredients for Infant Formulae. *Applied Sciences (Switzerland)*, *13*(1). https://doi.org/10.3390/app13010653

Leardi, R., Melzi, C., & Polotti, G. (2021, September 14). *CAT (Chemometric Agile Tool Software)*. http://gruppochemiometria.it/index.php/software

Liesegang, A., Eicher, R., Sassi, M.-L., Risteli, J., Kraenzlin, M., Riond, J.-L., & Wanner, M. (2000). Biochemical Markers of Bone Formation and Resorption Around Parturition and During Lactation in Dairy Cows with High and Low Standard Milk Yields1,2. *Journal of Dairy Science*, *83*(8), 1773–1781. https://doi.org/10.3168/jds.S0022-0302(00)75048-X

M.A, de la F., A, O., & M, J. (1997). Distribution of calcium, magnesium, phosphorus, zinc, manganese, copper and iron between the soluble and colloïdal phases of ewe's and goat 's milk. *Lait*, *77*(4), 515–520. https://doi.org/10.1051/lait:1997437

Magdas, D. A., Feher, I., Cristea, G., Voica, C., Tabaran, A., Mihaiu, M., Cordea, D. V., Bâlteanu, V. A., & Dan, S. D. (2019). Geographical origin and species differentiation of Transylvanian cheese. Comparative study of isotopic and elemental profiling vs. DNA results. *Food Chemistry*, *277*, 307–313. https://doi.org/10.1016/j.foodchem.2018.10.103

Manuelian, C. L., Currò, S., Penasa, M., Cassandro, M., & De Marchi, M. (2017). Characterization of major and trace minerals, fatty acid composition, and cholesterol content of Protected Designation of Origin cheeses. *Journal of Dairy Science*, *100*(5), 3384–3395. https://doi.org/10.3168/jds.2016-12059

Mara, A., Deidda, S., Caredda, M., Ciulu, M., Deroma, M., Farinini, E., Floris, I., Langasco, I., Leardi, R., Pilo, M. I., Spano, N., & Sanna, G. (2022). Multi-Elemental Analysis as a Tool to Ascertain the Safety and the Origin of Beehive Products: Development, Validation, and Application of an ICP-MS Method on Four Unifloral Honeys Produced in Sardinia, Italy. *Molecules*, *27*(6). https://doi.org/10.3390/molecules27062009

Martín-Hernández, M. C., Amigo, L., Martín-Álvarez, P. J., & Juárez, M. (1992). *Differentiation of milks and cheeses according to species based on the mineral content*. https://doi.org/10.1007/BF01185480

Nečemer, M., Potočnik, D., & Ogrinc, N. (2016). Discrimination between Slovenian cow, goat and sheep milk and cheese according to geographical origin using a combination of elemental content and stable isotope data. *Journal of Food Composition and Analysis*, *52*, 16–23. https://doi.org/10.1016/j.jfca.2016.07.002

O, P., F, R., & M, R. (1994). Evolution of physico-chemical characteristics and renneting properties of ewe's milk collected in the 'Roquefort area'. *Lait*, *74*(6), 425–442. https://doi.org/10.1051/lait:1994635

Pereira, P. C. (2014). Milk nutritional composition and its role in human health. *Nutrition*, *30*(6), 619–627. https://doi.org/10.1016/j.nut.2013.10.011

Piga, C., Urgeghe, P. P., Piredda, G., Scintu, M. F., Di Salvo, R., & Sanna, G. (2013). *Thermal inactivation and variability of γ-glutamyltransferase and α-l-fucosidase enzymatic activity in sheep milk*. https://doi.org/10.1016/j.lwt.2013.05.018

Piga, C., Urgeghe, P. P., Piredda, G., Scintu, M. F., & Sanna, G. (2009). *Assessment and validation of methods for the determination of γ-glutamyltransferase activity in sheep milk*. https://doi.org/10.1016/j.foodchem.2009.01.041

Piga, C., Urgeghe, P. P., Piredda, G., Scintu, M. F., & Sanna, G. (2010). *Analytical methods for the evaluation of α-l-fucosidase activity in sheep milk*. https://doi.org/10.1007/s12161-008-9069-5

Polychroniadou, A., & Vafopoulou, A. (1985). Variations of Major Mineral Constituents of Ewe Milk During Lactation. *Journal of Dairy Science*, *68*(1), 147–150. https://doi.org/10.3168/jds.S0022-0302(85)80808-0

Pulina, G., Atzori, A. S., Dimauro, C., Ibba, I., Gaias, G. F., Correddu, F., & Nudda, A. (2021). The milk fingerprint of Sardinian dairy sheep: Quality and yield of milk used for Pecorino Romano P.D.O. cheese production on population-based 5-year survey. *Italian Journal of Animal Science*, *20*(1), 171–180. https://doi.org/10.1080/1828051X.2021.1875896

Pulina, G., Milán, M. J., Lavín, M. P., Theodoridis, A., Morin, E., Capote, J., Thomas, D. L., Francesconi, A. H. D., & Caja, G. (2018). Invited review: Current production trends, farm structures, and economics of the dairy sheep and goat sectors. *Journal of Dairy Science*, *101*(8), 6715–6729. https://doi.org/10.3168/jds.2017-14015

Pulinas, L., Spanu, C., Idda, I., Ibba, I., Nieddu, G., Virdis, S., Scarano, C., Piras, F., Spano, N., Sanna, G., & De Santis, E. P. L. (2017). *Production of farmstead lactose-free pecorino di osilo and ricotta cheeses from sheep's milk*. https://doi.org/10.4081/ijfs.2017.6353

Recio, I., de la Fuente, M. A., Juárez, M., & Ramos, M. (2009). Bioactive Components in Sheep Milk. In *Bioactive Components in Milk and Dairy Products* (pp. 83–104). John Wiley & Sons, Ltd. https://doi.org/10.1002/9780813821504.ch4

Rodríguez-Bermúdez, R., López-Alonso, M., Miranda, M., Fouz, R., Orjales, I., & Herrero-Latorre, C. (2018). Chemometric authentication of the organic status of milk on the basis of trace element content. *Food Chemistry*, *240*, 686–693. https://doi.org/10.1016/j.foodchem.2017.08.011

Romano, C. per la T. del F. P. (2023). *Disciplinare Pecorino Romano DOP*. https://www.pecorinoromano.com/pecorino-romano/disciplinare-e-norme

Sajdakowska, M., Gębski, J., Guzek, D., Gutkowska, K., & Żakowska-Biemans, S. (2020). Dairy Products Quality from a Consumer Point of View: Study among Polish Adults. *Nutrients*, *12*(5). https://doi.org/10.3390/nu12051503

Santarcangelo, C., Baldi, A., Ciampaglia, R., Dacrema, M., Di Minno, A., Pizzamiglio, V., Tenore, G. C., & Daglia, M. (2022). Long-Aged Parmigiano Reggiano PDO: Trace Element Determination Targeted to Health. *Foods*, *11*(2). https://doi.org/10.3390/foods11020172

Sardo, C. per la T. del F. P. (2023). *Disciplinare Pecorino Sardo DOP*. https://www.politicheagricole.it/flex/files/9/b/b/D.2baf7e91401d2f673d3e/Disciplinare_pecorino_sardo.pdf

Spano, N., Bortolu, S., Addis, M., Langasco, I., Mara, A., Pilo, M. I., Sanna, G., & Urgeghe, P. P. (2023). *An Analytical Protocol for the Differentiation and the Potentiometric Determination of Fluorine-Containing Fractions in Bovine Milk*. https://doi.org/10.3390/molecules28031349

Subramanian, A., & Rodriguez-Saona, L. (2010). Chemical and Instrumental Approaches to Cheese Analysis. In 20610176, *Adv. Food Nutr. Res.* (Vol. 59, pp. 167–213). Academic Press Inc.; Scopus. https://doi.org/10.1016/S1043-4526(10)59005-3

Tedesco, R., Villoslada Hidalgo, M. D. C., Vardè, M., Kehrwald, N. M., Barbante, C., & Cozzi, G. (2021). Trace and rare earth elements determination in milk whey from the Veneto region, Italy. *Food Control*, *121*. https://doi.org/10.1016/j.foodcont.2020.107595

Theobald, H. (2005). Dietary calcium and health. *Nutrition Bulletin*, *30*, 237–277. https://doi.org/10.1111/j.1467-3010.2005.00514.x

Timlin, M., Tobin, J. T., Brodkorb, A., Murphy, E. G., Dillon, P., Hennessy, D., O'donovan, M., Pierce, K. M., & O'callaghan, T. F. (2021). The impact of seasonality in pasture-based production systems on milk composition and functionality. *Foods*, *10*(3). Scopus. https://doi.org/10.3390/foods10030607

Urgeghe, P. P., Piga, C., Addis, M., Di Salvo, R., Piredda, G., Scintu, M. F., Wolf, I. V., & Sanna, G. (2012). *SPME/GC-MS Characterization of the Volatile Fraction of an Italian PDO Sheep Cheese to Prevalent Lypolitic Ripening: The Case of Fiore Sardo*. https://doi.org/10.1007/s12161-011-9302-5

U.S. Department of Health & Human Services. National Institutes of Health. (2019). *Nutrient Recommendations and Databases*. https://www.ncbi.nlm.nih.gov/books/NBK545442/table/appJ_tab3/?report=objectonly

Vagnoni, E., Franca, A., Porqueddu, C., & Duce, P. (2017). Environmental profile of Sardinian sheep milk cheese supply chain: A comparison between two contrasting dairy systems. *Journal of Cleaner Production*, *165*, 1078–1089. https://doi.org/10.1016/j.jclepro.2017.07.115

Woodrow, J., Sharpe, C., Fudge, N., Hoff, A., Gagel, R., & Kovacs, C. S. (2006). Calcitonin Plays a Critical Role in Regulating Skeletal Mineral Metabolism during Lactation. *Endocrinology*, *147*, 4010–4021. https://doi.org/10.1210/en.2005-1616

Zazzu, C., Addis, M., Caredda, M., Scintu, M. F., Piredda, G., & Sanna, G. (2019). *Biogenic amines in traditional fiore sardo pdo sheep cheese: Assessment, validation and application of an rp-hplc-dad-uv method*. https://doi.org/10.3390/separations6010011

Zhang, P., Georgiou, C. A., & Brusic, V. (2018). Elemental metabolomics. *Briefings in Bioinformatics*, *19*(3), 524–536. https://doi.org/10.1093/bib/bbw131

Published on Journal of Environmental Management, 2022, https://doi.org/10.1016/j.jenvman.2021.114105

ASSESSMENT AND VALIDATION OF ICP-MS AND IC-ICP-MS METHODS FOR THE DETERMINATION OF TOTAL, EXTRACTED AND SPECIATED ARSENIC

4.1. INTRODUCTION

Arsenic is an element largely released into the environment by both anthropogenic and natural sources. Its toxicity depends in an utmost way on the nature of its chemical species. Among all, the most toxic form of As is the inorganic one (iAs) (Abernathy et al., 1999), a non-threshold, class 1 carcinogen, absorbed and bonded to plasmatic proteins, and accumulated in the liver and kidneys (Meharg et al., 2009; Narukawa & Chiba, 2010). The principal chemical species of iAs are arsenite, As(III), and arsenate, As(V), where the former is generally considered much more toxic (*Arsenic*, 1977; Huang et al., 2010) and carcinogenic (Martinez et al., 2011) than the latter. On the other hand, organoarsenical compounds have been considered less toxic than the inorganic ones. Arsenobetaine, arsenocholine and arsenosugars are virtually non-toxic ('Opinion of the Scientific Panel on Contaminants in the Food Chain [CONTAM] Related to Arsenic as Undesirable Substance in Animal Feed.', n.d.) while it has assumed that species like monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) may be cancer promoters (Brown et al., 1997, p. 19). Diet is the main source of arsenic intake for humans (Tao & Michael Bolger, 1999; World Health Organization, 2003). Bioaccumulation of As species in rice represents a real health concern for over three billion of people (S. Islam et al., 2016). The attempt to set a "safe" daily intake level for such a toxic element turned out to be not an easy task. The amount of the provisional tolerable weekly intake (PTWI) of 15 μg kg^{-1} bw, set by FAO in 1988 (Food and Drug Administration (FDA) & World Health Organization, 1988) has been later withdrawn in 2010 (Food and Drug Administration (FDA) & World Health Organization, 2010). In addition, the European Food Safety Authority (EFSA) stressed the need to produce reliable speciation data for different foods to better evaluate the health risk associated with dietary exposure to As (Scientific Opinion on Arsenic in Food, 2009).

Rice is the staple food for over half of the world's population, since it contributes up to 15% of the human daily protein intake (International Rice Research Institute, 2021), and it provides more than 20% of the calories consumed by humans worldwide (International Rice Research Institute, 2021; B. D. Smith, 1995).

Although almost the 98% of rice is produced in Asia, Africa and South America (Food and Drug Administration (FDA), 2019), intensive but small productions can be found in Europe and in the United States (Frazzoli et al., 2007; Zavala & Duxbury, 2008). Rice obtained from the major producing Countries is almost completely used by them for internal consumption (International Rice Research Institute, 2013). Such a largely consumed food should have the highest level of food safety, but, unfortunately, rice is able to bioaccumulate large quantities of toxic elements like As (Agency for Toxic Substances and Disease Registry (ATSDR), 2007; Meharg et al., 2009). Bioaccumulation of As in rice grain has both natural (S. Islam et al., 2016; Meharg & Rahman, 2003; Williams et al., 2006) and anthropogenic (X.-Y. Liao et al., 2005; J. F. Ma et al., 2008; Zhu et al., 2008) origin. Elevated As amounts in rice grain may be found also when both soils and irrigation waters are unpolluted. This happens because the constant anaerobiosis of the paddy field shifts the redox equilibrium of the As chemical species towards the most mobile and bioavailable form, As(III), which represents the 90% of the total As found in the pore water (S. Das et al., 2016). Here, the arsenite anion is readily absorbed by roots of rice and efficiently conveyed to the grain by specific aquaporins (J. F. Ma et al., 2008). This mechanism of transport is so efficient that, when the irrigation waters are severely polluted by As, the total amount of As in rice reaches concentrations even up to 2 mg kg^{-1} (M. R. Islam et al., 2004), being As(III) the principal form of iAs found in kernels (S. Islam et al., 2016).

World Health Organization (WHO) and the European Commission (EC) have set advisory levels of iAs in white rice grain at 0.2 mg kg^{-1} and in brown rice at 0.4 mg kg^{-1} ('Codex Alimentarius Commission, 37th Session', 2014; European Community, 2015). China is currently the most restrictive country in fixing limits posed to regulate the level of iAs in rice (0.15 mg kg^{-1}) (Hojsak et al., 2015), Brazil posed a limit of 0.30 mg kg^{-1} of total As in rice grain (Ciminelli et al., 2017), whereas a total As concentration below 1 mg kg^{-1} is still considered "safe" in Bangladesh (H. K. Das et al., 2004). In the United States of America, the issue is still debated (Food and Drug Administration (FDA), 2016). Again, adverse health effects on children and babies caused by As contained in rice-based infant food have led both EC and US Food and Drug Administration (FDA) to pose a limit of 0.2 mg kg^{-1} of iAs (European Community, 2015; Food and Drug Administration (FDA), 2016). Furthermore, the EC has discouraged parents from giving children younger than 36 months rice milk as a milk substitute (European Community, 2015).

Beyond the preliminary evaluation of the concentration of the total As in rice grain (Costa et al., 2016; Frazzoli et al., 2007; M. R. Islam et al., 2004; Jackson & Punshon, 2015; Mandal & Suzuki, 2002; Meharg & Rahman, 2003; Nardi et al., 2009; Williams et al., 2006), it is evident that only a reliable speciation of the main As chemical forms in kernels could allow a careful risk assessment for consumers (Cullen & Reimer, 1989; Foà et al., 1984; S. Islam et al., 2016; Juhasz et al., 2006; Sarwar et al., 2021). A speciation method usually consists of two steps: extraction, the most critical, and analysis. The ideal extraction of As species from a sample should contemporarily ensure: i) a short extraction time ii) quantitative recoveries of As species; iii) no interconversion among the analytes. Unfortunately, the real situation for rice is rather different. Redox interchanges between As(III) and As(V) species have been sometimes observed (Huang et al., 2010; Liang et al., 2010; Narukawa et al., 2014; Williams et al., 2005). The extreme difficulty to not perturb the redox equilibria between As(III) and As(V) in the extraction step has led some research groups to quantify them only as iAs (Llorente-Mirandes et al., 2012; Williams et al., 2005). In addition, low recoveries of As species have been observed on real samples (Heitkemper et al., 2001; Williams et al., 2005), whereas higher recoveries were sometimes obtained only using drastic conditions (Perkin Elmer, 2021b; E. Smith et al., 2008). Usually, pure water (Heitkemper et al., 2001; Liang et al., 2010; L. Ma et al., 2016; Narukawa et al., 2014; Perkin Elmer, 2021a; Zavala & Duxbury, 2008), methanol (Heitkemper et al., 2001), water/methanol solutions (Heitkemper et al., 2001; Liang et al., 2010), nitric acid (Batista et al., 2011; Huang et al., 2010; Liang et al., 2010; Llorente-Mirandes et al., 2012; Narukawa et al., 2014; Perkin Elmer, 2021b) or trifluoroacetic acid (Heitkemper et al., 2001; Liang et al., 2010; Meharg et al., 2009; E. Smith et al., 2008; Williams et al., 2005; Zavala & Duxbury, 2008) aqueous solutions, were used as extracting solvents. The extraction step was frequently heath-assisted (Batista et al., 2011; Heitkemper et al., 2001; Huang et al., 2010; Liang et al., 2010; L. Ma et al., 2016; Narukawa et al., 2014; Perkin Elmer, 2021b, 2021a; E. Smith et al., 2008; Williams et al., 2005) or, unusually, microwave- (Heitkemper et al., 2001; Llorente-Mirandes et al., 2012; Narukawa & Chiba, 2010) ultrasound-assisted (Heitkemper et al., 2001; Narukawa & Chiba, 2010; Zavala & Duxbury, 2008). Again, it was sometimes accomplished by Accelerated Soxhlet Extraction (ASE) (Heitkemper et al., 2001; Narukawa & Chiba, 2010) or, even more rarely, performed also at room temperature (Narukawa & Chiba, 2010). Finally, the conflictual results obtained by different research groups (Heitkemper et al., 2001; Williams

et al., 2005) leave still open the debate on the optimization of the extraction technique of As species by rice flour. The extraction of the As species from soil is also a crucial step, since pH and Eh affect the equilibrium between the chemical forms of As, and micro-organisms can promote methylation/demethylation of As species (Kumarathilaka et al., 2018). Both the key extraction solvent systems and extraction techniques are summarized in several authoritative reviews (Gong, 2002; Leermakers et al., 2006). Among others, mixtures of aqueous acid and organic solvents (Chappell, 1995; Giacomino et al., 2010; Leermakers et al., 2006) and phosphoric acid aqueous solutions in the presence/absence of reducing species (Garcia-Manyes, 2002; Gong, 2002; Leermakers et al., 2006; Ruiz-Chancho et al., 2005) were used as extracting solvents, whereas the microwave-assisted extraction was a technique regularly used (Garcia-Manyes, 2002; Giacomino et al., 2010; Gong, 2002; Leermakers et al., 2006; Ruiz-Chancho et al., 2005, 2007).

Among all instrumental methods addressed to the determination of the total amount of As in rice grain and in soils, undoubtedly the Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) is the preferred instrumentation, due to its sensitivity, its wide dynamic range and its accuracy (Al Rmalli et al., 2005; Costa et al., 2016; H. K. Das et al., 2004; Frazzoli et al., 2007; Jackson & Punshon, 2015; Jayasekera & Freitas, 2005; Lin et al., 2020; Mandal & Suzuki, 2002; Meharg & Rahman, 2003; Nardi et al., 2009; Phuong et al., 1999; Williams et al., 2006). On the other hand, only liquid chromatography coupled with ICP-MS spectrometry can accomplish the quantification of the most representative As species in these matrices in a reliable way (Ackerman et al., 2005; Heitkemper et al., 2001; Huang et al., 2010; Juhasz et al., 2006; Kato et al., 2019; Kohlmeyer et al., 2003; Liang et al., 2010; Llorente-Mirandes et al., 2012; Narukawa et al., 2014; Narukawa & Chiba, 2010; Perkin Elmer, 2021b, 2021a; Ruiz-Chancho et al., 2005, 2007; E. Smith et al., 2008; Williams et al., 2005; Zavala & Duxbury, 2008). In these cases, the As compounds extracted from rice and/or soils in the previous step can be separated by means of High-Performance Liquid Chromatography (HPLC) (Narukawa et al., 2014; Narukawa & Chiba, 2010; Perkin Elmer, 2021b, 2021a; Zavala & Duxbury, 2008) or Ionic Chromatography (IC) (Ackerman et al., 2005; Batista et al., 2011; Huang et al., 2010; Kato et al., 2019; Kohlmeyer et al., 2003; Liang et al., 2010; L. Ma et al., 2016; Zavala et al., 2008), whereas the ICP-MS represents a very suitable detector for revealing and quantifying the As contained in each species separated in the chromatographic run.

This research group is active for years in the optimization of irrigation methods, alternative to the traditional one (Spanu et al., 2004, 2009, 2012, 2021; Spanu, Langasco, et al., 2020; Spanu, Valente, et al., 2020) (i.e. the continuous flooding, CF, worldwide used for rice cultivation). These methods allowed to obtain huge water savings (up to 70% the amounts normally required by CF irrigation) (Spanu et al., 2009) without any loss in the yield of rice grain (Spanu et al., 2004). In addition, an outstanding and unprecedented decrease of 98% (average on 53 different rice genotypes) (Spanu et al., 2012, 2021; Spanu, Valente, et al., 2020) of the total As concentration in the edible fraction has been measured irrigating rice with sprinkler irrigation (SP), optimized for rice cultivation by some researchers of this group (Spanu et al., 2004, 2009). Despite of the demonstrated major role played by the nature of the irrigation methods in the bioaccumulation mechanisms of toxic elements (Ashraf et al., 2018; Duxbury & Panaullah, 2007; Hu, Huang, et al., 2013; Hu, Li, et al., 2013; L. Liao et al., 2013; Spanu et al., 2018; Sun et al., 2014; Yang et al., 2009) and oligoelements (Da Silva et al., 2020; Orasen et al., 2019; Spanu, Langasco, et al., 2020; Y. Xu et al., 2019) in rice grain, at the best of our knowledge no attention has been paid until now towards the effects caused by intermittent irrigation methods on the amounts and on the distribution of the principal chemical species of As in kernels.

Based also on the expertise of members of this research group on the assessment of speciation methods of As in foods (Llorente-Mirandes et al., 2012), the principal goal of this contribution is to develop and completely validate IC-ICP-MS procedures aimed to extract and quantify the most representative As species contained in rice flours and in soils. To ensure the highest reliability of the whole analytical procedure, also the ICP-MS methods aimed to the determination of the total amount of As (or the total amount of extracted As) in soils and rice have been optimized and validated. Beyond the analyses of a lot of certified reference materials of both rice flour and soil, all the procedures have been tested also on samples of a real soil-rice system at varying of the irrigation methods. Hence, an *Aleramo* rice genotype was cultivated in the same site and irrigated with the same water using three different irrigation methods like CF, SP and periodical saturation of the soil (SA). Soils used for these cultivations and rice grains obtained in this way have been analysed with all the proposed methods.

4.2. MATERIALS AND METHODS

4.2.1. SAMPLES AND CULTIVATION CONDITIONS

Rice samples used for testing the proposed method were cultivated in the "Santa Lucia" experimental farm of the University of Sassari, Sardinia, Italy (39°59'N, 8°40'E; 15 m AMSL). The soil is a *Pantofluvic Eutric Fluvisol Loamic*, according to the World Reference Base for soil resources (Food Agriculture Organization (FAO), 2014). Irrigation water was from Lake Omodeo (i.e. the largest artificial basin in Sardinia). An *Aleramo* rice genotype was cultivated in three adjoining plots of soil: the first plot was irrigated by CF, whereas the second and the third ones were irrigated by SA and SP, respectively. An extensive description of all the irrigation methods used has been reported elsewhere (Spanu et al., 2012, 2018, 2021; Spanu, Langasco, et al., 2020; Spanu, Valente, et al., 2020). Briefly, where CF irrigation was used, soil is completely flooded for almost the whole vegetation cycle of rice, whereas this does not happen using SA and SP methods. Soil irrigated by SA is cyclically saturated whenever its upper layer is dry, while soil irrigated by SP was irrigated each 2–5 days with the exact water amount aimed to compensate any evaporative and transpiration losses by the soil-plant system. Using SP irrigation, the soil is never saturated, and its redox potential is held constantly positive (i.e. roughly between +100 and + 130 mV vs. Saturated Calomel Electrode, SCE), whereas the redox potential of soil irrigated by CF is constantly below −200 mV vs. SCE. Finally, large oscillations of redox potential (i.e. roughly between −100 mV and +350 mV vs. SCE) were observed along a SA irrigation cycle. The experimental design was a randomized block with four replications, and the surface of each sub-plot was typically of 10 m2. Further details of cultivation conditions are also summarized in the paragraph 1 of the Appendix D. For each sub-plot, 100 g of paddy rice was dried at 32 °C, mechanically husked and bleached, and then mineralized for the determination of total As (by ICP-MS), or grounded and extracted with a proper solvent system for the determination of the total extracted As (by ICP-MS) and As species (by IC-ICP-MS). Finally, soil sampling has been accomplished by means of conventional quartering/mixing techniques, hence the laboratory sample was carefully dried and stored at 40 °C in a thermostatic vacuum dryer and sieved immediately before mineralization (or extraction).

4.2.2. REAGENTS AND STANDARDS

Only type-I ultrapure water (resistivity >18 MΩ cm^{-1} and total organic carbon<30 µg dm^{-3}) was used throughout all the phases of the analytical procedures. Ultrapure water was prepared with doubly deionized water obtained from a Milli-Q® IQ 7003 system (Millipore, Vimodrone, Italy).

Nitric acid (69%, Hiperpur), sulphuric acid (93–98%, for trace metal analysis), L (+)-ascorbic acid (>99%, ACS) and aqueous ammonia (30%, ACS) were from Panreac AppliChem, Castellar del Vallès, Spain, 30% hydrogen peroxide (30%, ULTREX II), was from JTBaker, Rodano, Italy, dihydrogen ammonium phosphate (>99.5%, BioUltra) was purchased by Merck, Darmstadt, Germany. Further details on reagents and standard used have been reported in the paragraph 2 of the Appendix D.

4.2.3. INSTRUMENTS AND APPARATUS

The speciation of As(III), As(V), DMA and MMA in rice grains and soils has been performed by means of an IC-ICP-MS instrument, constituted by i) an Agilent 1200 Series Gradient HPLC system (Agilent Technologies, Santa Clara, CA, USA); ii) a PRP-X100 Anion Exchange HPLC Column (Hamilton Company, Reno, NE, USA) and iii) an Agilent 7500ce ICP-MS (Agilent Technologies, Santa Clara, CA, USA). The outlet of the IC column was connected by means of a polyether ether ketone (PEEK) capillary to the ICP-MS nebulizer. The Agilent 7500ce ICP-MS has been also used for the determination of the total amount of As and the total extracted As in rice samples as well as in soils. The trace elements content in paddy soils, where the rice samples have been cultivated, were determined by a NexION 300X ICP-MS spectrometer (PerkinElmer, Milan, Italy), equipped with a nebulization system composed of a glass concentric nebulizer, a glass cyclonic spray chamber, a S10 autosampler and a kinetic energy discrimination (KED) collision cell. Further details on instruments and apparatus used for sample pre-treatment are reported in the paragraph 3 of the Appendix D.

4.2.4. ANALYTICAL METHODS

Literature methods have been used to measure the Eh potential and all the parameters of interest for soils (Gazzetta Ufficiale, 1999; 'Redox Potential', 2006; Spanu, Valente, et al., 2020) and irrigation waters (Birke et al., 2010). Mineralization of rice grains and soils has been performed according to methods already proposed

by this research group (Spanu, Langasco, et al., 2020). Conversely, both original or significantly improved methods previously developed by researchers of this group (Llorente-Mirandes et al., 2012; Ruiz-Chancho et al., 2005, 2007; Spanu, Valente, et al., 2020) were extensively reported in Section 4.3.

4.2.5. STATISTICAL ANALYSIS

A two-tailed *t*-test (p = 95%) was used in ascertaining the existence of matrix effect as well as in the trueness evaluation.

4.3. RESULTS AND DISCUSSION

4.3.1. METHODS ASSESSMENT

4.3.1.1. Extraction of As species

Without any doubt, this is the most critical phase of the whole analytical procedure of speciation. Indeed, a wrong choice of the extraction solvent as well as of the extraction conditions can alter the equilibrium conditions among As chemical species present in the sample, setting the conditions to obtain analytical artefacts. For this reason, and to keep the extraction conditions under control as far as possible, all these procedures have been developed working only on Certified Reference Material (CRM).

4.3.1.1.1. Extraction of As species from soils

The assessment of this method is extensively described in the paragraph 4 of the Appendix D. 0.1 g of the soil sample was weighed on an analytical balance (accuracy ±0.0001 g), transferred in a 20 cm^3 polypropylene centrifugation tube and then treated with 15 cm^3 of Ar-deoxygenated aqueous solution containing 1 mol dm^{-3} of phosphoric acid and 0.5 mol dm^{-3} of L (+)-ascorbic acid. Next, the sample was thoroughly stirred for 16 h at room temperature by means of a rotating mixer for tubes working at 30 rpm (i.e. 0.4×*g*). The suspension was centrifuged at 4600×*g* for 30 min, the solution was first separated from the solid, then filtered through a 0.22 µm nylon filter. Finally, the vials containing the extracts were kept tightly closed at 4 °C under a constant Ar atmosphere no longer than 24 h until the ICP-MS determination of the total amount of As extracted as well as the IC-ICP-MS determination of As(III), As(V), DMA and MMA were performed.

4.3.1.1.2. Extraction of As species from flours of rice grains

The development of this method is thoroughly reported in the paragraph 5 of the Appendix D. 0.25 g of rice flour was weighed on an analytical balance in a PTFE vessel for microwave digestion and then treated with 10 cm^3 of 0.2% nitric acid (w/v). Figure D1 in the Appendix D shows the time-temperature diagram describing the extraction cycle. After extraction, the mixture was first allowed to reach the room temperature and then transferred into a polypropylene centrifuge tube and centrifuged at 1800×*g* for 12 min. After that,

the supernatant solution was filtered through a 0.22 µm nylon filter and preserved. Until the analysis. Also in this case, the vials containing the extracts were kept tightly closed at 4 °C under a constant Ar atmosphere for no more than 24 h until the determination of the total amount of As extracted and the determination of the concentrations of As(III), As(V), DMA and MMA.

4.3.1.2. ICP-MS and IC-ICP-MS methods

All the measurements reported in this paper have been accomplished by means of the IC-ICP-MS and ICP-MS Agilent instrumentation, with the only exception of the trace elements determination in soils, that has been performed with the ICP-MS PerkinElmer NexION 300X.

4.3.1.2.1. ICP-MS determinations in rice grains and in soils

The ICP-MS methods proposed here were mainly devoted to the measurement of i) the total amount of As, and ii) the total amount of extracted As. In addition to As, fourteen selected elements were measured to properly describe the pedochemical nature of the soils used in these experiments. The assessment of these methods is described in detail in paragraph 6 of the Appendix D. Special attention was given to the elimination of any interferences by molecular ions on the $^{75}As^{+}$ signal. This goal has been accomplished by a careful optimization, for each matrix and for each instrument used, of the He flow used working in KED mode, as shown in Figure D2 in the Appendix D. In addition, Table D2 reports the instrumental parameters and the elemental settings used for the concomitant determination of all toxic and trace elements in soils used in the cultivation of *Aleramo* rice genotype at varying of the irrigation method.

4.3.1.2.2. IC-ICP-MS determination of the chemical species of As

4.3.1.2.2.1. IC conditions

The separation among As(III), DMA, MMA and As(V) has been performed using a PRP-X100 column coupled with an aqueous solution 20 mmol dm^{-3} in sodium dihydrogen phosphate (pH = 5.8). Further details on the optimization of the IC conditions are reported in the paragraph 7 of the Appendix D. Table D3 summarizes the optimized ion chromatography conditions used for the separation of the As species.

4.3.1.2.2.2. ICP-MS conditions

Table D4 reports the optimized instrumental conditions used for the ICP-MS detection of As in samples. The concomitant application of the methods described in Table D3 and Table D4 provides the separation of As(III), As(V), DMA and MMA at the baseline level. The whole chromatographic run lasts less than 10 min. Figure 4.1 shows the IC-ICP-MS chromatogram of a standard mixture containing 15 µg dm^{-3} each of the analytes.

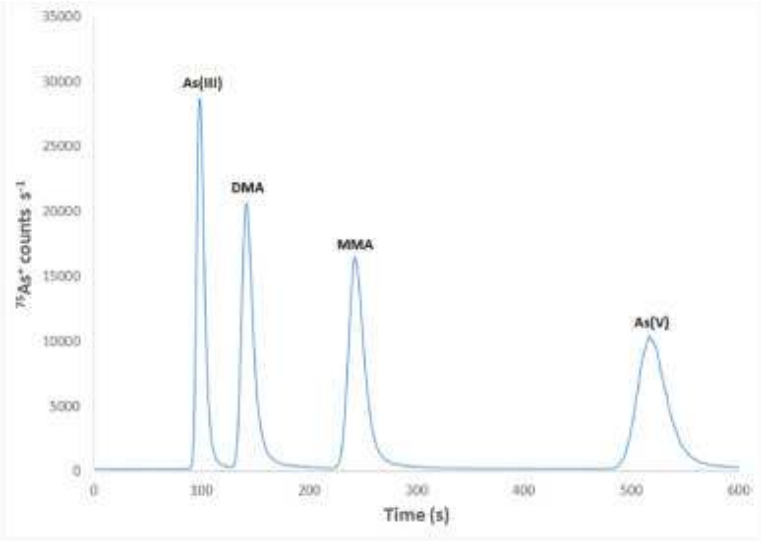


Figure 4.1. IC-ICP-MS chromatogram of a standard mixture containing 15 µg dm^{-3} each of As(III), DMA, MMA and As(V).

4.3.1.2.3. Quality assurance and quality control in the ICP-MS based methods

All samples were blank-diluted, when necessary to lead the analyte concentration within the relevant calibration range. A solution of Rh (10 μg dm^{-3} for measurements performed with the NexION 300X instrument, 20 μg dm^{-3} for measurements performed with the Agilent 7500ce instrument) was used as an internal standard to compensate for any signal instability, while a washing cycle of at least 80 s was interposed between two consecutive samples (160 s between groups of real samples obtained by different irrigation methods) to eliminate any potential memory effect. All reported data have been blank-corrected. In order to constantly monitor the overall level of method accuracy, one blank every five samples was performed, while a standard solution containing 10 μg dm^{-3} in As species (or a standard solution containing 10 μg dm^{-3} of As, Cd, Hg, Mo, Pb, Sb, Se and Tl; 100 μg dm^{-3} of Cr, Cu, Mn, Ni and Zn, and 500 μg dm^{-3} of Al and Fe, for the determination of elements in paddy soils) was processed every ten samples. Lastly, a mineralized solution of CRM (typically, one aliquot of ERM-BC211 for rice and one 1:10 diluted aliquot of CRM 025–050 for soil samples) was processed every fifteen samples. No significant statistical difference (criteria: two-tailed *t*-test, p = 0.95) was found between the trends of the regression lines obtained by both external calibration and multiple standard additions on a CRM sample. For this reason, quantification was always performed using external calibration. Each sample was analysed at least three times and each analytical data is the average of four replicated ICP-MS (or IC-ICP-MS) measurements.

4.3.2. VALIDATION

Validation of proposed methods has been accomplished in terms of limit of detection, LoD; limit of quantification, LoQ; linearity, precision, and trueness. Table 4.1 and Table 4.2 report the features describing the performances of the methods here considered.

Table 4.1. Validation parameters for the ICP-MS determination of the total amount of As in rice flour and in soils.

Matrix	**LoD (μg kg^{-1})[a]**	**LoQ (μg kg^{-1})**	**Linearity concentration range (μg kg^{-1}) Y = (a ± s_a)X + (b ± s_b)**
Rice grain	0.18	0.59	0.6–360
Soil	5	16	a = 700; s_a = 10; b = 140; s_b = 50; R^2 = 0.9992
Matrix (C_{As} ± s,)	**Repeatability[b], CV (%)**	**Intermediate Precision[c], CV(%)**	**Trueness, Recovery (%±sd)**
NCSZC 73008 rice flour (102 ± 8 μg kg^{-1})	1.7	3.7	105 ± 4
NCSZC 11007 rice flour (110 ± 20 μg kg^{-1})	1.3	1.5	105 ± 6
NIST 1568a rice flour (290 ± 30 μg kg^{-1})	3.4	2.6	98 ± 2
IRMM 804 rice flour (49 ± 4 μg μg kg^{-1})	1.2	2.3	99 ± 1
CRM SS-1 soil (20.7 ± 1 mg kg^{-1})	1.6	2.5	95 ± 3

[a] The LoD value is measured according to (Currie, 1999). [b] Evaluated by analyzing the CRM five times within the same analytical session. [c] Evaluated by analyzing the CRM five times in five different analytical sessions within one month. [d] Standard deviation.

Table 4.2. Validation parameters for the IC-ICP-MS determination of As(III), As(V), DMA and MMA in rice flour and in soils.

As species	Rice flour		Soil		Linearity concentration range ($\mu g\ kg^{-1}$) $Y = (a \pm s_a)X + (b \pm s_b)$
	LoD ($\mu g\ kg^{-1}$)[a]	LoQ ($\mu g\ kg^{-1}$)	LoD ($\mu g\ kg^{-1}$)a	LoQ ($\mu g\ kg^{-1}$)	
As(III)	0.4	1.3	1.5	5	1.3–400 a = 3400; s_a = 100; b = 130; s_b = 50; R^2 = 0.9995
As(V)	0.28	0.92	1	3.5	0.9–400 a = 2920; s_a = 30; b = 240; s_b = 90; R^2 = 0.9998
DMA	0.2	0.65	0.75	2.5	0.7–400 a = 3400; s_a = 90; b = 600; s_b = 300; R^2 = 0.9995
MMA	0.28	0.92	1	3.5	0.8–400 a = 1750; s_a = 150; b = 220; s_b = 90; R^2 = 0.9998

Rice flour

As species	Repeatability[b], CV (%)	Intermediate Precision[c], CV (%)	Trueness Recovery (% ± sd)
As(III)	1.5	4	100.0 ± 0.1[e] 98±5[f]
As(V)	1.5	5.5	100.0 ± 0.1[e] 103±6[f]
DMA	0.3	2	99.8 ± 0.2[g]
MMA	1.7	3.5	89±4[f]

Soil

As species	Repeatability[b], CV (%)	Intermediate Precision[c], CV (%)	Trueness Recovery (% ± sd)
As(III)	1.3	2.7	93±6[h]
As(V)	1.1	3.5	98±7[h]
DMA	1.7[i]	3.4[i]	104±2[i]
MMA	2.1[i]	3.9[i]	94±2[i]

[a] The LoD values are measured according to (Currie, 1999). [b] Evaluated by analyzing the CRM ERM-BC211 rice flour (or the CRM 025–050 soil) five times within the same analytical session. [c] Evaluated by analyzing the CRM ERM-BC211 rice flour (or the CRM 025–050 soil) five times in five different analytical sessions within one month. [d] Standard deviation. [e] Data, measured analyzing the CRM ERM-BC211 rice flour, is referred to the concentration of iAs. [f] Recovery measured by multiple spikes of analyte. [g] Recovery measured analyzing the CRM ERM-BC211 rice flour. [h] Recovery measured analyzing the CRM 025–050 soil. [i] Measured on samples of CRM 025–050 soil fortified with known amounts of analyte.

The proposed methods are characterized by a very low LoD, measured according to (Currie, 1999), for all considered As species and matrices. The values measured for the IC-ICP-MS method are at least one order of magnitude lower than those previously reported in literature (Heitkemper et al., 2001; L. Ma et al., 2016; Narukawa & Chiba, 2010; Perkin Elmer, 2021b; Williams et al., 2005). In addition, an excellent linearity within the experimental range of concentration (i.e. roughly three orders of magnitude from LoQ) is observed, although it can easily be extended up to the mg kg^{-1} level without any significant worsening of the R^2 coefficient of determination. Furthermore, the casual dispersion of the residuals of the regression plot around zero supports in both cases the linearity of the method.

The availability of many certified reference materials (mainly, rice flours) exhibiting a wide range of total As concentration (between 49 ± 4 μg kg^{-1} for IRMM 804 and 290 ± 30 μg kg^{-1} for NIST 1568a for rice flours, between 20.7 ± 1 mg kg^{-1} for CRM SS-1 and 339 ± 17 mg kg^{-1} for CRM 025–050 soils) has allowed to measure accuracy of the method along a large interval of analyte concentration. Precision was evaluated in terms of both repeatability and intermediate precision. As far as the repeatability is concerned, the CV on the determination of the total amount of As ranged from 1.2% (IRMM 804) to 3.4% (NIST 1568a), whereas the uncertainty for the intermediate precision measurements is only slightly higher (i.e. between 1.5% for NCSZC 11007 and 3.7% for NCSZC 73008). Good precision data were from also by repeated analyses of the CRM SS-1 soil: the CV measured are always less than 3%. Even the precision performances for As speciation are very good, both in rice flour (CRM ERM-BC211) and in the soil (CRM 025–050): in the former case, the repeatability ranged from 0.3% (DMA) to 1.7% (MMA), while intermediate CV precision is between 2.0% (DMA) to 5.5% (As (V)), whereas, in the latter, precision data are always below 4%. Overall, precision data are comparable, and in some cases better, than those reported in previous studies for some of these species (Frazzoli et al., 2007; Llorente-Mirandes et al., 2012). All precision parameters were found acceptable according to Horwitz's theory (Horwitz, 1982).

Trueness for the ICP-MS determination of the As total amount was measured by means of repeated analyses of four rice flour CRMs and a CRM SS-1 of soil. A quantitative recovery (criteria: two-tailed *t*-test, p = 0.95) was observed for NCSZC 11007, NIST 1568a and IRMM 804 certified rice flours, whereas slight bias was ascertained for NCSZC 73008 rice flour (overestimation) and for CRM SS-1 soil (underestimation).

Nevertheless, in both cases the systematic errors were found to be acceptable for the level of As concentration, according to the guidelines reported in the Peer Verified Methods by the AOAC international (Association of Official Agricultural Chemists (AOAC), 2016). Conversely, the CRMs certificating As species in rice flour as well as in soils are quite scarce. In particular, the CRM ERM-BC211 rice flour certificates only iAs and DMA, while the CRM 025–050 certificates the amount of As(III) and As(V) in soil. Hence, recovery tests have been used to evaluate the trueness for remaining analytes. Quantitative recoveries (criteria: two-tailed *t*-test, p = 0.95) were found in certificated rice flour and soil for As(III), As(V) and DMA, while the amount of MMA was underestimated for both matrices. Also in this case, the bias was acceptable according to AOAC guidelines (Association of Official Agricultural Chemists (AOAC), 2016).

4.3.3. APPLICATION OF ICP-MS AND IC-ICP-MS METHODS FOR THE CHARACTERIZATION OF SAMPLES FROM A SOIL-RICE SYSTEM

In addition to analyses on CRM we also performed the methods on grown samples from a soil-rice plant system. Three adjoining fields have been used to cultivate the same rice genotype (variety *Aleramo*, belonging to the Japonica rice subspecies). As evidenced by data reported in Table 4.3, the three soils considered have very similar pedological, hydrological and physical-chemical properties. In each of these fields, the *Aleramo* rice was irrigated with the same water, but using three different irrigation methods: continuous flooding (CF), periodical saturation (SA) and sprinkler irrigation (SP), respectively.

Table 4.3. Pedological, hydrological and physical-chemical parameters of the soils (layer depth 0–20 cm, particle size: <0.2 mm for the elemental analysis, < 2 mm for the other measurements) and of the irrigation water used for producing rice samples. CF, continuous flooding irrigation, SA, saturation irrigation, SP, sprinkler irrigation.

Parameters	CF	SA	SP	Irrigation water
Texture	Clay loam	Clay loam	Clay loam	
pH	7.9	7.7	7.4	7.6
Eh[a] (mV vs SCE)	−210	−110[b]; 355[c]	115[d]	[e]
Carbonates (% as $CaCO_3$)	<0.01	0.01	0.02	0.005[f]
Total nitrogen (%)	0.04	0.06	0.12	[e]
Organic carbon (%)	1	1.2	1.4	[e]
Assimilable P (mg kg^{-1} as P_2O_5)	104	141	97	<0.5 mg dm−3
Exchangeable K (mg kg^{-1} as K_2O)	225	231	216	2.3 mg dm−3
Field capacity (%, v/v)	[e]	[e]	38.9	[e]
Permanent wilting point (%, v/v)	[e]	[e]	20.8	[e]
Al (mg kg^{-1})	22,000 ± 3000	20,000 ± 1000	36,000 ± 1000	15 ± 2 μg dm^{-3}[g]
As (mg kg^{-1})	4.4 ± 0.2	4.5 ± 0.1	3.99 ± 0.09	3.0 ± 0.4 μg dm^{-3}[g]
Cd (mg kg^{-1})	0.184 ± 0.007	0.126 ± 0.003	0.098 ± 0.003	0.08 ± 0.02 μg dm^{-3}[g]
Cr (mg kg^{-1})	55 ± 4	45 ± 5	80 ± 2	0.17 ± 0.06 μg dm^{-3}[g]
Cu (mg kg^{-1})	16 ± 2	10 ± 2	16.0 ± 0.5	1.7 ± 0.3 μg dm^{-3}[g]
Fe (mg kg^{-1})	18,000 ± 2000	18,000 ± 600	27,000 ± 1000	24 ± 3 μg dm^{-3}[g]
Hg (mg kg^{-1})	0.021 ± 0.002	0.018 ± 0.002	0.011 ± 0.001	0.030 ± 0.007 μg dm^{-3}[g]
Mn (mg kg^{-1})	38 ± 2	40 ± 5	105 ± 2	22 ± 3 μg dm^{-3}[g]
Mo (mg kg^{-1})	0.30 ± 0.02	0.85 ± 0.07	0.22 ± 0.01	2.2 ± 0.4 μg dm^{-3}[g]
Ni (mg kg^{-1})	13 ± 1	20 ± 2	35 ± 2	1.3 ± 0.3 μg dm^{-3}[g]
Pb (mg kg^{-1})	7.4 ± 0.3	6.9 ± 0.3	8.7 ± 0.4	0.041 ± 0.009 μg dm^{-3}[g]
Sb (mg kg^{-1})	0.25 ± 0.02	0.205 ± 0.004	0.120 ± 0.005	0.24 ± 0.05 μg dm^{-3}[g]
Se (mg kg^{-1})	1.81 ± 0.02	1.30 ± 0.06	1.2 ± 0.1	0.017 ± 0.009 μg dm^{-3}[g]
Tl (mg kg^{-1})	0.070 ± 0.001	0.054 ± 0.002	0.061 ± 0.002	0.005 ± 0.002 μg dm^{-3}[g]
Zn (mg kg^{-1})	67 ± 3	50.0 ± 0.9	60 ± 2	1.7 ± 0.5 μg dm^{-3}[g]

Soils and irrigation waters have been analyzed in quadruplicate. If otherwise not reported, all parameters were evaluated according to (Gazzetta Ufficiale, 1999). [a] Evaluated according to (Redox Potential, 2006). [b] Measured 5 h after saturation. [c] Measured just before saturation. [d] Measured halfway through each sprinkler cycle. [e] Parameter not pertinent or not measured. [f] Expressed as HCO_3^- ion. [g] Measured according to (Birke, 2010).

4.3.3.1. Characterization of soils and irrigation waters

Table 4.3 summarizes the chemical and hydrological soil and irrigation water characterization. The surface horizons of the experimental soils have a sandy-clayey texture, a sub-alkaline pH, a very low quantity of carbonates and bicarbonates, a quantity of organic carbon and total nitrogen lower than 1.5% and 0.15%, respectively, while the quantities of assimilable phosphorus and exchangeable potassium were sufficient for the needs of rice cultivation. Although the quantities of almost all elements in all experimental soils fell within the typical range observed worldwide for uncontaminated soils (Adriano, 2001; Alloway, 2013), significant differences were observed between the composition of the trace elements found in the three soils considered. In particular, the slow leaching of Al, Cr, Fe, Mn, Ni, and Pb observed in soils irrigated from years by means CF is responsible for lower concentration here measured in comparison to soils irrigated by SA and, mainly, by SP. This difference has been emphasized by the lack of any deep ploughing, seldom practiced in paddy fields, which has prevented the soil from regenerating from the elements leached over the years. Moreover, the total quantity of As present in the three soils is comparable, although the concentration measured in SP soil is slightly less than that measured in CF and SA soils. Finally, the concentration of trace elements measured in irrigation water spanned over five orders of magnitude, (i.e. from about a few ng dm^{-3} to one hundred µg dm^{-3}), always well within the range typical for unpolluted inland waters (European Community, 1998).

4.3.3.2. As speciation in soils

Table 4.4 summarizes the speciation data measured in the soils as a function of the nature of the irrigation method. Data reported in Table 4.4 reveal that the amounts of total measured As and of total extracted As in the three soils are statistically not different (criteria: two tails *t*-test, p = 0.95). As(III) is the only species available in CF soil, and it is the most abundant chemical form in SA soil, whereas As(V) is the 25% of the total amount of extracted As. These data are in good agreement with literature (Khanam et al., 2020; X. Y. Xu et al., 2008). In particular, Xu et al. (X. Y. Xu et al., 2008) showed that arsenic was mainly present like As(III) in CF soil, whereas also As(V) was present in aerobic soils. Hence, passing from the CF irrigation method to intermittent methods of irrigation, the Eh of the soil increases and the equilibria between As(III) and As(V) is

progressively shifted towards the oxidized form. In SP soil, As(V) is the only As species found. Also the amount of inorganic arsenic (iAs) measured is constant in all soils. Interestingly, the concentration of DMA and MMA are always below than the relevant LoDs in all soils considered, and this is consistent with the observations of many research groups (Huang et al., 2010; Huang & Matzner, 2006, 2007; Takamatsu et al., 1982). Furthermore, it is also interesting to observe that the column recovery measured in CF soil is slightly lower (i.e. 96%) than that measured in both SA and SP soils. Although the values of the relevant standard deviations prevent to substantiate statistically differences among these recoveries, it could be supposed that further not extracted As forms, different from the searched analytes, are present in CF soil (Huang et al., 2010).

Table 4.4. Concentrations (in mg kg^{-1}) of As species present in the soil irrigated with CF, SA and SP methods (n = 3).

Parameters	Irrigation methods		
	CF	**SA**	**SP**
Total As	4.8 ± 0.2	4.7 ± 0.3	4.5 ± 0.3
Total extracted As	4.6 ± 0.4	4.5 ± 0.5	4.4 ± 0.3
As(III)	4.4 ± 0.2	3.4 ± 0.4	<0.0015[a]
As(V)	<0.0010[a]	1.10 ± 0.06	4.4 ± 0.3
iAs	4.4 ± 0.2	4.5 ± 0.4	4.4 ± 0.3
DMA	<0.00075[a]	<0.00075[a]	<0.00075[a]
MMA	<0.0010[a]	<0.0010[a]	<0.0010[a]
Sum of As species	4.4 ± 0.2	4.5 ± 0.4	4.4 ± 0.3
Extraction efficiency (%)	97±9[b]	96±7[b]	97±8[b]
Column recovery (%)	96±5[b]	100±4[b]	100±3[b]

[a] Value below LoD. [b] Ratios calculated using unrounded terms of the division.

4.3.3.3. Total and speciated arsenic in rice

Table 4.5 reports the As speciation data and the amounts of total As measured in the *Aleramo* rice sample irrigated by CF, SA and SP, respectively, whereas Figure 4.2 shows a typical IC-ICP-MS chromatogram of the *Aleramo* rice genotype irrigated by SP.

Table 4.5. Concentration (in µg kg^{-1}) of the total As and As species in the *Aleramo* rice grain irrigated by CF, SA and SP. (n = 4).

Irrigation Method	Total As	Total extracted As	As(III)	As(V)	iAs
CF	160 ± 10	160 ± 2	55 ± 3	<0.28[a]	55 ± 3
SA	70 ± 8	72 ± 4	66 ± 5	<0.28[a]	66 ± 5
SP	6 ± 2	6 ± 3	1.6 ± 0.7	4.4 ± 0.3	6.0 ± 0.8
Irrigation Method	**DMA**	**MMA**	**Sum of As species**	**Extraction efficiency (%)[b]**	**Column recovery (%)[b]**
CF	104 ± 5	<0.28[a]	159 ± 6	100 ± 4	99 ± 2
SA	<0.20[a]	<0.28[a]	66 ± 5	102 ± 6	92 ± 7
SP	<0.20[a]	<0.28[a]	6.0 ± 0.8	101 ± 5	99 ± 4

[a] Value below LoD. [b] Ratios calculated using unrounded terms of the division.

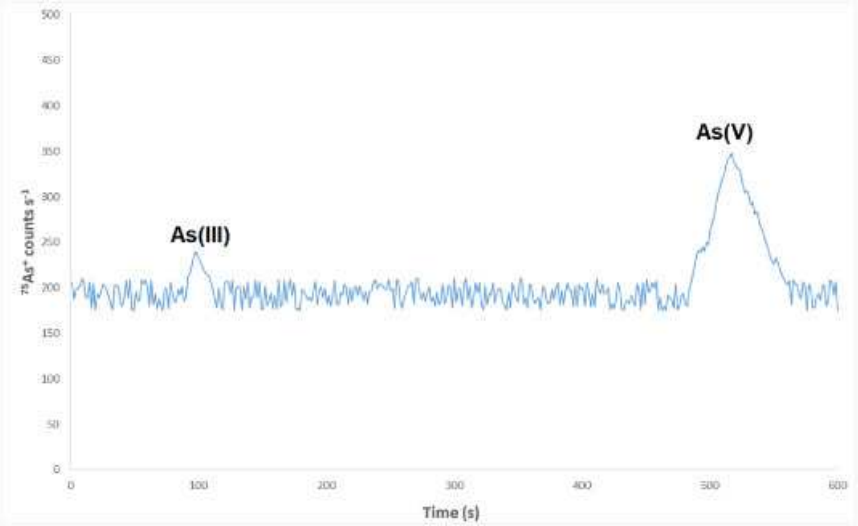


Figure 4.2. IC-ICP-MS chromatogram of the *Aleramo* rice genotype irrigated by SP.

Although the concentrations of total As measured this time in the *Aleramo* rice genotype are quite higher than that observed in the same site in previous agricultural years (Spanu et al., 2012; Spanu, Valente, et al., 2020), reduction of As concentration in kernels passing from CF to intermittent irrigation methods agrees with literature data (Spanu et al., 2012; Spanu, Valente, et al., 2020). In particular, the decrease of the As concentration in rice grain is higher than 50% passing by rice irrigated with CF to that irrigated by SA, but it reaches more than 95% in rice grains irrigated by SP. As expected, As(III) is abundant in CF rice, accounting for almost one third of the total amount of extracted As, but the dominant species in this genotype is DMA, which represents the remaining part of the extracted As. Indeed, no other As species are detectable in this case. The prevalent presence of both As(III) and DMA in rice irrigated by CF has been largely observed in literature (Batista et al., 2011; Heitkemper et al., 2001; Huang et al., 2010; Liang et al., 2010; Llorente-Mirandes et al., 2012; L. Ma et al., 2016; Meharg et al., 2009; Narukawa & Chiba, 2010; Williams et al., 2005; Zavala et al., 2008), but it is quite infrequent that DMA prevails in a so large extent with respect to As(III). A similar behavior has been first observed, mainly in rice produced in the United States, by Zavala et al. (Zavala et al., 2008). Interestingly, the concentration of methylated species of As in all soils used to cultivate the *Aleramo* rice genotype was always below the relevant LoDs (i.e. 0.28 μg kg^{-1} for MMA and 0.20 μg kg^{-1} for DMA), and this fact strengthens the hypotheses made by Zavala concerning the capability, also for the rice plant, to promote the biomethylation of inorganic species of As to afford DMA. On the other hand, it is evident that, since this experimental evidence is based on the behavior of only one rice genotype, it is currently impossible to draw any conclusion of general meaning from this data. As a final remark, this *Aleramo* rice genotype irrigated by CF should be categorized, according to Zavala's results, in the range of DMA-type rice, which is very common in the United States, but seldom found in Europe and China. However, the huge diversity among the thousands of different rice genotypes may be the reason to justify this behavior. Passing to the rice produced by intermittent irrigations, it is noteworthy to observe that As(III) is the only species found in SA-irrigated rice. These results allow to envisage that the changes introduced replacing the CF irrigation with the SA irrigation can stop the suggested biosynthesis of DMA in rice plant. Furthermore, the domain, among As species, of As(V) in SP-irrigated rice reflects what already observed in the soil irrigated by SP. Hence, not only the replacement of CF irrigation with SP irrigation is able to lower up to 96% the As

bioaccumulation in rice grain, but also the chemical form of As found in SP rice is less toxic than the As(III), largely found in rice irrigated by CF. Finally no organic As species are detectable in rice irrigated with intermittent methods, and this supports the fact that the CF irrigation may be an essential prerequisite to form DMA in the grain of rice. Finally, it is helpful to remember that intermittent methods of irrigation allow to greatly reduce the amount of water consumed for rice cultivation with respect the traditional continuous flooding method (i.e. up to −30% using the periodical saturation, up to −70% using sprinkler irrigation).

4.4. CONCLUSIONS

Despite the huge interest of the scientific community on the development and application of analytical protocols aimed to the speciation of As chemical forms in the soil-rice systems, literature is still lacking simple and reliable methods able to couple sensitivity and accuracy in both matrices. Hence, a complete analytical protocol of ICP-MS and IC-ICP-MS methods aimed to quantify the total amount of As, the total extracted As, and the main chemical species of As (i.e. As(III), As(V), DMA and MMA) in real samples from a soil-rice system has been developed and validated in this contribution. A particular attention has been devoted to carefully establish the procedure for the extraction of As by soils and by rice flour by microwave-assisted procedures. Analyses of CRMs and recoveries on fortified samples have allowed to verify that both the extraction methods do not alter the chemical equilibria among the analytes, mainly those between As(III) and As(V) species, also ensuring very high recoveries of extracted As. The IC separation among As(III), As(V), DMA and MMA, which has ensured a complete separation at the baseline level of the four analytes, has been accomplished in less than 10 min. All methods have been successfully validated in terms of LoD, LoQ, linearity, precision, and trueness. These methods could hence represent a valuable tool for evaluating all potentially interesting As species (i.e. the total amount, the total extracted amount, and the amounts of As(III), As(V), DMA and MMA) in soils as well as in rice. It should be applied in routine analysis, as required in food control laboratories according to the ISO/IEC 17025:2005 standard.

4. References

Abernathy, C. O., Liu, Y. P., Longfellow, D., Aposhian, H. V., Beck, B., Fowler, B., Goyer, R., Menzer, R., Rossman, T., Thompson, C., & Waalkes, M. (1999). Arsenic: Health effects, mechanisms of actions, and research issues. *Environmental Health Perspectives*, *107*(7), 593–597. https://doi.org/10.1289/ehp.99107593

Ackerman, A. H., Creed, P. A., Parks, A. N., Fricke, M. W., Schwegel, C. A., Creed, J. T., Heitkemper, D. T., & Vela, N. P. (2005). Comparison of a Chemical and Enzymatic Extraction of Arsenic from Rice and an Assessment of the Arsenic Absorption from Contaminated Water by Cooked Rice. *Environmental Science & Technology*, *39*(14), 5241–5246. https://doi.org/10.1021/es048150n

Adriano, D. C. (2001). *Trace Elements in Terrestrial Environments*. Springer New York. https://doi.org/10.1007/978-0-387-21510-5

Agency for Toxic Substances and Disease Registry (ATSDR). (2007). *Toxicological profile for Arsenica*. https://www.atsdr.cdc.gov/toxprofiles/tp2.pdf

Al Rmalli, S. W., Haris, P. I., Harrington, C. F., & Ayub, M. (2005). A survey of arsenic in foodstuffs on sale in the United Kingdom and imported from Bangladesh. *Science of The Total Environment*, *337*(1–3), 23–30. https://doi.org/10.1016/j.scitotenv.2004.06.008

Alloway, B. J. (Ed.). (2013). *Heavy Metals in Soils: Trace Metals and Metalloids in Soils and their Bioavailability* (Vol. 22). Springer Netherlands. https://doi.org/10.1007/978-94-007-4470-7

Arsenic: Medical and Biological Effects of Environmental Pollutants (p. 9003). (1977). National Academies Press. https://doi.org/10.17226/9003

Ashraf, U., Hussain, S., Akbar, N., Anjum, S. A., Hassan, W., & Tang, X. (2018). Water management regimes alter Pb uptake and translocation in fragrant rice. *Ecotoxicology and Environmental Safety*, *149*, 128–134. https://doi.org/10.1016/j.ecoenv.2017.11.033

Association of Official Agricultural Chemists (AOAC). (2016). *Guidelines for Standard Method Performance Requirements*. https://www.aoac.org/wp-content/uploads/2019/08/app_f.pdf

Batista, B. L., Souza, J. M. O., De Souza, S. S., & Barbosa, F. (2011). Speciation of arsenic in rice and estimation of daily intake of different arsenic species by Brazilians through rice consumption. *Journal of Hazardous Materials*, *191*(1–3), 342–348. https://doi.org/10.1016/j.jhazmat.2011.04.087

Birke, M., Reimann, C., Demetriades, A., Rauch, U., Lorenz, H., Harazim, B., & Glatte, W. (2010). Determination of major and trace elements in European bottled mineral water—Analytical methods. *Journal of Geochemical Exploration*, *107*(3), 217–226. https://doi.org/10.1016/j.gexplo.2010.05.005

Brown, J. L., Kitchin, K. T., & George, M. (1997). Dimethylarsinic acid treatment alters six different rat biochemical parameters: Relevance to arsenic carcinogenesis. *Teratogenesis, Carcinogenesis, and Mutagenesis*, *17*(2), 71–84. https://doi.org/10.1002/(SICI)1520-6866(1997)17:2<71::AID-TCM3>3.0.CO;2-B

Chappell, J. (1995). Speciation of arsenic in a contaminated soil by solvent extraction. *Talanta*, *42*(3), 323–329. https://doi.org/10.1016/0039-9140(95)01395-R

Ciminelli, V. S. T., Gasparon, M., Ng, J. C., Silva, G. C., & Caldeira, C. L. (2017). Dietary arsenic exposure in Brazil: The contribution of rice and beans. *Chemosphere*, *168*, 996–1003. https://doi.org/10.1016/j.chemosphere.2016.10.111

Codex Alimentarius Commission, 37th Session. (2014). *Codex Alimentarius Commission, 37Th Session*, 14–18. Scopus.

Costa, B. E. S., Coelho, L. M., Araújo, C. S. T., Rezende, H. C., & Coelho, N. M. M. (2016). Analytical Strategies for the Determination of Arsenic in Rice. *Journal of Chemistry*, *2016*, 1–11. https://doi.org/10.1155/2016/1427154

Cullen, W. R., & Reimer, K. J. (1989). Arsenic speciation in the environment. *Chemical Reviews*, *89*(4), 713–764. https://doi.org/10.1021/cr00094a002

Currie, L. A. (1999). Detection and quantification limits: Origins and historical overview1Adapted from the Proceedings of the 1996 Joint Statistical Meetings (American Statistical Association, 1997). Original title: “Foundations and future of detection and quantification limits”. Contribution of the National Institute of Standards and Technology; not subject to copyright.1. *Analytica Chimica Acta*, *391*(2), 127–134. https://doi.org/10.1016/S0003-2670(99)00105-1

Da Silva, J. T., Paniz, F. P., Sanchez, F. E. S., Pedron, T., Torres, D. P., Da Rocha Concenço, F. I. G., Barbat Parfitt, J. M., & Batista, B. L. (2020). Selected soil water tensions at phenological phases and mineral content of trace elements in

rice grains – mitigating arsenic by water management. *Agricultural Water Management*, *228*, 105884. https://doi.org/10.1016/j.agwat.2019.105884

Das, H. K., Mitra, A. K., Sengupta, P. K., Hossain, A., Islam, F., & Rabbani, G. H. (2004). Arsenic concentrations in rice, vegetables, and fish in Bangladesh: A preliminary study. *Environment International*, *30*(3), 383–387. https://doi.org/10.1016/j.envint.2003.09.005

Das, S., Chou, M.-L., Jean, J.-S., Liu, C.-C., & Yang, H.-J. (2016). Water management impacts on arsenic behavior and rhizosphere bacterial communities and activities in a rice agro-ecosystem. *Science of The Total Environment*, *542*, 642–652. https://doi.org/10.1016/j.scitotenv.2015.10.122

Duxbury, J. M., & Panaullah, G. (2007). Remediation of Arsenic for Agriculture Sustainability, Food Security and Health in Bangladesh. *Remediation of Arsenic for Agriculture Sustainability, Food Security and Health in Bangladesh*. Scopus.

European Community. (1998). *Council Directive No 98/83/EC on the quality of water intended for human consumption*. https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:31998L0083&from=IT

European Community. (2015). *Commission Regulation (EU) 2015/1005 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs.* https://eur-lex.europa.eu/eli/reg/2015/1005/oj

Foà, V., Colombi, A., Maroni, M., Buratti, M., & Calzaferri, G. (1984). The speciation of the chemical forms of arsenic in the biological monitoring of exposure to inorganic arsenic. *Science of The Total Environment*, *34*(3), 241–259. https://doi.org/10.1016/0048-9697(84)90066-4

Food Agriculture Organization (FAO). (2014). *World reference base for soil resources*. https://www.fao.org/3/i3794en/I3794en.pdf

Food and Drug Administration (FDA). (2016). *Supporting document for action level for inorganic arsenic in rice cereals for infants*. https://www.fda.gov/media/97121/download

Food and Drug Administration (FDA). (2019). *FAOStat Data*. http://www.fao.org/faostat/en/#data/QC

Food and Drug Administration (FDA), & World Health Organization. (1988). *Expert Committee on Food Additives. Evaluation of certain food additives and contaminants: 33th report*. http://www.fao.org/tempref/codex/Reports/Reports_2014/REP14_CFe.pdf

Food and Drug Administration (FDA), & World Health Organization. (2010). *Expert Committee on Food Additives. Evaluation of certain food additives and contaminants: 72th report*. http://apps.who.int/iris/bitstream/handle/10665/44514/WHO_TRS_959_eng.pdf?sequence=1

Frazzoli, C., D'amato, M., Caroli, S., & Záray, G. (2007). Arsenic and Other Potentially Toxic Trace Elements in Rice. In *The Determination of Chemical Elements in Food* (pp. 383–400). https://doi.org/10.1002/9780470141007.ch12

Garcia-Manyes, S. (2002). Arsenic speciation in contaminated soils. *Talanta*, *58*(1), 97–109. https://doi.org/10.1016/S0039-9140(02)00259-X

Gazzetta Ufficiale. (1999). *Suppl. Ordinario n. 185. Metodi Ufficiali di Analisi Chimica del Suolo*. https://www.gazzettaufficiale.it/eli/gu/1999/10/21/248/so/185/sg/pdf

Giacomino, A., Malandrino, M., Abollino, O., Velayutham, M., Chinnathangavel, T., & Mentasti, E. (2010). An approach for arsenic in a contaminated soil: Speciation, fractionation, extraction and effluent decontamination. *Environmental Pollution*, *158*(2), 416–423. https://doi.org/10.1016/j.envpol.2009.08.010

Gong, Z. (2002). Arsenic speciation analysis. *Talanta*, *58*(1), 77–96. https://doi.org/10.1016/S0039-9140(02)00258-8

Heitkemper, D. T., Vela, N. P., Stewart, K. R., & Westphal, C. S. (2001). Determination of total and speciated arsenic in rice by ion chromatography and inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry*, *16*(4), 299–306. https://doi.org/10.1039/b007241i

Hojsak, I., Braegger, C., Bronsky, J., Campoy, C., Colomb, V., Decsi, T., Domellöf, M., Fewtrell, M., Mis, N. F., Mihatsch, W., Molgaard, C., Van Goudoever, J., & ESPGHAN Committee on Nutrition. (2015). Arsenic in Rice: A Cause for Concern. *Journal of Pediatric Gastroenterology and Nutrition*, *60*(1), 142–145. https://doi.org/10.1097/MPG.0000000000000502

Horwitz, W. (1982). Evaluation of Analytical Methods Used for Regulation of Foods and Drugs. *Analytical Chemistry*, *54*(1), 67A-76A. https://doi.org/10.1021/ac00238a765

Hu, P., Huang, J., Ouyang, Y., Wu, L., Song, J., Wang, S., Li, Z., Han, C., Zhou, L., Huang, Y., Luo, Y., & Christie, P. (2013). Water management affects arsenic and cadmium accumulation in different rice cultivars. *Environmental Geochemistry and Health*, *35*(6), 767–778. https://doi.org/10.1007/s10653-013-9533-z

Hu, P., Li, Z., Yuan, C., Ouyang, Y., Zhou, L., Huang, J., Huang, Y., Luo, Y., Christie, P., & Wu, L. (2013). Effect of water management on cadmium and arsenic accumulation by rice (Oryza sativa L.) with different metal accumulation capacities. *Journal of Soils and Sediments*, *13*(5), 916–924. https://doi.org/10.1007/s11368-013-0658-6

Huang, J.-H., Ilgen, G., & Fecher, P. (2010). Quantitative chemical extraction for arsenic speciation in rice grains. *Journal of Analytical Atomic Spectrometry*, *25*(6), 800. https://doi.org/10.1039/c002306j

Huang, J.-H., & Matzner, E. (2006). Dynamics of organic and inorganic arsenic in the solution phase of an acidic fen in Germany. *Geochimica et Cosmochimica Acta*, *70*(8), 2023–2033. https://doi.org/10.1016/j.gca.2006.01.021

Huang, J.-H., & Matzner, E. (2007). Mobile arsenic species in unpolluted and polluted soils. *Science of The Total Environment*, *377*(2–3), 308–318. https://doi.org/10.1016/j.scitotenv.2007.01.059

International Rice Research Institute. (2013). *Rice Almanac*. http://books.irri.org/9789712203008_content.pdf

International Rice Research Institute. (2021). *The importance of rice*. http://www.knowledgebank.irri.org/ericeproduction/Importance_of_Rice.htm

Islam, M. R., . M. J., & . S. I. (2004). Assessment of Arsenic in the Water-Soil-Plant Systems in Gangetic Floodplains of Bangladesh. *Asian Journal of Plant Sciences*, *3*(4), 489–493. https://doi.org/10.3923/ajps.2004.489.493

Islam, S., Rahman, M. M., Islam, M. R., & Naidu, R. (2016). Arsenic accumulation in rice: Consequences of rice genotypes and management practices to reduce human health risk. *Environment International*, *96*, 139–155. https://doi.org/10.1016/j.envint.2016.09.006

Jackson, B. P., & Punshon, T. (2015). Recent Advances in the Measurement of Arsenic, Cadmium, and Mercury in Rice and Other Foods. *Current Environmental Health Reports*, *2*(1), 15–24. https://doi.org/10.1007/s40572-014-0035-7

Jayasekera, R., & Freitas, M. C. (2005). Concentration Levels of Major and Trace Elements in Rice from Sri Lanka as Determined by the k₀ Standardization Method. *Biological Trace Element Research*, *103*(1), 083–096. https://doi.org/10.1385/BTER:103:1:083

Juhasz, A. L., Smith, E., Weber, J., Rees, M., Rofe, A., Kuchel, T., Sansom, L., & Naidu, R. (2006). *In Vivo* Assessment of Arsenic Bioavailability in Rice and Its Significance for Human Health Risk Assessment. *Environmental Health Perspectives*, *114*(12), 1826–1831. https://doi.org/10.1289/ehp.9322

Kato, L. S., De Nadai Fernandes, E. A., Raab, A., Bacchi, M. A., & Feldmann, J. (2019). Arsenic and cadmium contents in Brazilian rice from different origins can vary more than two orders of magnitude. *Food Chemistry*, *286*, 644–650. https://doi.org/10.1016/j.foodchem.2019.02.043

Khanam, R., Kumar, A., Nayak, A. K., Shahid, Md., Tripathi, R., Vijayakumar, S., Bhaduri, D., Kumar, U., Mohanty, S., Panneerselvam, P., Chatterjee, D., Satapathy, B. S., & Pathak, H. (2020). Metal(loid)s (As, Hg, Se, Pb and Cd) in paddy soil: Bioavailability and potential risk to human health. *Science of The Total Environment*, *699*, 134330. https://doi.org/10.1016/j.scitotenv.2019.134330

Kohlmeyer, U., Jantzen, E., Kuballa, J., & Jakubik, S. (2003). Benefits of high resolution IC?ICP?MS for the routine analysis of inorganic and organic arsenic species in food products of marine and terrestrial origin. *Analytical and Bioanalytical Chemistry*, *377*(1), 6–13. https://doi.org/10.1007/s00216-003-2064-1

Kumarathilaka, P., Seneweera, S., Meharg, A., & Bundschuh, J. (2018). Arsenic speciation dynamics in paddy rice soil-water environment: Sources, physico-chemical, and biological factors - A review. *Water Research*, *140*, 403–414. https://doi.org/10.1016/j.watres.2018.04.034

Leermakers, M., Baeyens, W., De Gieter, M., Smedts, B., Meert, C., De Bisschop, H. C., Morabito, R., & Quevauviller, Ph. (2006). Toxic arsenic compounds in environmental samples: Speciation and validation. *TrAC Trends in Analytical Chemistry*, *25*(1), 1–10. https://doi.org/10.1016/j.trac.2005.06.004

Liang, F., Li, Y., Zhang, G., Tan, M., Lin, J., Liu, W., Li, Y., & Lu, W. (2010). Total and speciated arsenic levels in rice from China. *Food Additives & Contaminants: Part A*, *27*(6), 810–816. https://doi.org/10.1080/19440041003636661

Liao, L., Xu, J., Peng, S., Qiao, Z., & Gao, X. (2013). Uptake and Bioaccumulation of Heavy Metals in Rice Plants as Affect by Water Saving Irrigation. *Advance Journal of Food Science and Technology*, *5*(9), 1244–1248. https://doi.org/10.19026/ajfst.5.3090

Liao, X.-Y., Chen, T.-B., Xie, H., & Liu, Y.-R. (2005). Soil As contamination and its risk assessment in areas near the industrial districts of Chenzhou City, Southern China. *Environment International*, *31*(6), 791–798. https://doi.org/10.1016/j.envint.2005.05.030

Lin, H.-T., Wong, S.-S., & Li, G.-C. (2020). Heavy metal content of rice and shellfish in Taiwan. *Journal of Food and Drug Analysis*, *12*(2). https://doi.org/10.38212/2224-6614.2649

Llorente-Mirandes, T., Calderón, J., López-Sánchez, J. F., Centrich, F., & Rubio, R. (2012). A fully validated method for the determination of arsenic species in rice and infant cereal products. *Pure and Applied Chemistry*, *84*(2), 225–238. https://doi.org/10.1351/PAC-CON-11-09-30

Ma, J. F., Yamaji, N., Mitani, N., Xu, X.-Y., Su, Y.-H., McGrath, S. P., & Zhao, F.-J. (2008). Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proceedings of the National Academy of Sciences*, *105*(29), 9931–9935. https://doi.org/10.1073/pnas.0802361105

Ma, L., Wang, L., Tang, J., & Yang, Z. (2016). Optimization of arsenic extraction in rice samples by Plackett–Burman design and response surface methodology. *Food Chemistry*, *204*, 283–288. https://doi.org/10.1016/j.foodchem.2016.02.126

Mandal, B. K., & Suzuki, K. T. (2002). Arsenic round the world: A review. *Talanta*, *58*(1), 201–235. https://doi.org/10.1016/S0039-9140(02)00268-0

Martinez, V. D., Vucic, E. A., Becker-Santos, D. D., Gil, L., & Lam, W. L. (2011). Arsenic Exposure and the Induction of Human Cancers. *Journal of Toxicology*, *2011*, 1–13. https://doi.org/10.1155/2011/431287

Meharg, A. A., & Rahman, Md. M. (2003). Arsenic Contamination of Bangladesh Paddy Field Soils: Implications for Rice Contribution to Arsenic Consumption. *Environmental Science & Technology*, *37*(2), 229–234. https://doi.org/10.1021/es0259842

Meharg, A. A., Williams, P. N., Adomako, E., Lawgali, Y. Y., Deacon, C., Villada, A., Cambell, R. C. J., Sun, G., Zhu, Y.-G., Feldmann, J., Raab, A., Zhao, F.-J., Islam, R., Hossain, S., & Yanai, J. (2009). Geographical Variation in Total and Inorganic Arsenic Content of Polished (White) Rice. *Environmental Science & Technology*, *43*(5), 1612–1617. https://doi.org/10.1021/es802612a

Nardi, E. P., Evangelista, F. S., Tormen, L., Saint´Pierre, T. D., Curtius, A. J., Souza, S. S. D., & Barbosa, F. (2009). The use of inductively coupled plasma mass spectrometry (ICP-MS) for the determination of toxic and essential elements in different types of food samples. *Food Chemistry*, *112*(3), 727–732. https://doi.org/10.1016/j.foodchem.2008.06.010

Narukawa, T., & Chiba, K. (2010). Heat-Assisted Aqueous Extraction of Rice Flour for Arsenic Speciation Analysis. *Journal of Agricultural and Food Chemistry*, *58*(14), 8183–8188. https://doi.org/10.1021/jf101317n

Narukawa, T., Suzuki, T., Inagaki, K., & Hioki, A. (2014). Extraction techniques for arsenic species in rice flour and their speciation by HPLC–ICP-MS. *Talanta*, *130*, 213–220. https://doi.org/10.1016/j.talanta.2014.07.001

Opinion of the Scientific Panel on contaminants in the food chain [CONTAM] related to Arsenic as undesirable substance in animal feed. (n.d.). *EFSA Journal*, *EFSA Journal*. https://doi.org/10.2903/j.efsa.2005.180

Orasen, G., De Nisi, P., Lucchini, G., Abruzzese, A., Pesenti, M., Maghrebi, M., Kumar, A., Nocito, F. F., Baldoni, E., Morgutti, S., Negrini, N., Valè, G., & Sacchi, G. A. (2019). Continuous Flooding or Alternate Wetting and Drying Differently Affect the Accumulation of Health-Promoting Phytochemicals and Minerals in Rice Brown Grain. *Agronomy*, *9*(10), 628. https://doi.org/10.3390/agronomy9100628

Perkin Elmer. (2021a). *Arsenic speciation analysis in Brown rice by HPLC/ICP-MS using the NexION 300D/350D. Perkin elmer HPLC-ICP-MS application note*. https://www.perkinelmer.com/lab-solutions/resources/docs/nexion300d-arsenicspeciationinbrownrice.pdf

Perkin Elmer. (2021b). *Arsenic speciation analysis in white rice by HPLC/ICP-MS using the NexION 300D/350D. Perkin elmer HPLC-ICP-MS application note*. https://www.perkinelmer.com/lab-solutions/resources/docs/nexion300d-arsenicspeciationinwhiterice.pdf

Phuong, T. D., Kokot, S., Chuong, P. V., & Tong Khiem, D. (1999). Elemental content of Vietnamese ricePart 1. Sampling, analysis and comparison with previous studies. *The Analyst*, *124*(4), 553–560. https://doi.org/10.1039/a808796b

Redox Potential. (2006). In M. Pansu & J. Gautheyrou, *Handbook of Soil Analysis* (pp. 581–591). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-31211-6_16

Ruiz-Chancho, M. J., López-Sánchez, J. F., & Rubio, R. (2007). Analytical speciation as a tool to assess arsenic behaviour in soils polluted by mining. *Analytical and Bioanalytical Chemistry*, *387*(2), 627–635. https://doi.org/10.1007/s00216-006-0939-7

Ruiz-Chancho, M. J., Sabé, R., López-Sánchez, J. F., Rubio, R., & Thomas, P. (2005). New Approaches to the Extraction of Arsenic Species from Soils. *Microchimica Acta*, *151*(3–4), 241–248. https://doi.org/10.1007/s00604-005-0405-9

Sarwar, T., Khan, S., Muhammad, S., & Amin, S. (2021). Arsenic speciation, mechanisms, and factors affecting rice uptake and potential human health risk: A systematic review. *Environmental Technology & Innovation*, *22*, 101392. https://doi.org/10.1016/j.eti.2021.101392

Scientific Opinion on Arsenic in Food. (2009). *EFSA Journal*, *10*. https://doi.org/10.2903/j.efsa.2009.1351

Smith, B. D. (1995). *The emergence of agriculture*. Scientific American Library : Distributed by W.H. Freeman.

Smith, E., Juhasz, A. L., Weber, J., & Naidu, R. (2008). Arsenic uptake and speciation in rice plants grown under greenhouse conditions with arsenic contaminated irrigation water. *Science of The Total Environment*, *392*(2–3), 277–283. https://doi.org/10.1016/j.scitotenv.2007.11.023

Spanu, A., Daga, L., Orlandoni, A. M., & Sanna, G. (2012). The role of irrigation techniques in arsenic bioaccumulation in rice (Oryza sativa L.). *Environmental Science and Technology*, *46*(15), 8333–8340. Scopus. https://doi.org/10.1021/es300636d

Spanu, A., Langasco, I., Serra, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2021). Sprinkler irrigation in the production of safe rice by soils heavily polluted by arsenic and cadmium. *Chemosphere*, *277*, 130351. https://doi.org/10.1016/j.chemosphere.2021.130351

Spanu, A., Langasco, I., Valente, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2020). Tuning of the Amount of Se in Rice (Oryza sativa) Grain by Varying the Nature of the Irrigation Method: Development of an ICP-MS Analytical Protocol, Validation and Application to 26 Different Rice Genotypes. *Molecules*, *25*(8), 1861. https://doi.org/10.3390/molecules25081861

Spanu, A., Murtas, A., & Ballone, F. (2009). Water Use and Crop Coefficients in Sprinkler Irrigated Rice. *Italian Journal of Agronomy*, *4*(2), 47. https://doi.org/10.4081/ija.2009.2.47

Spanu, A., Murtas, A., Ledda, L., & Ballone, F. (2004). Innovative agronomic techniques for rice cultivation. *Conference Proceedings of Challenger and Opportunities for Sustainable Rice-Based Production Systems*, 207–216. Scopus.

Spanu, A., Valente, M., Langasco, I., Barracu, F., Orlandoni, A. M., & Sanna, G. (2018). Sprinkler irrigation is effective in reducing cadmium concentration in rice (Oryza sativa L.) grain: A new twist on an old tale? *Science of The Total Environment*, *628–629*, 1567–1581. https://doi.org/10.1016/j.scitotenv.2018.02.157

Spanu, A., Valente, M., Langasco, I., Leardi, R., Orlandoni, A. M., Ciulu, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2020). Effect of the irrigation method and genotype on the bioaccumulation of toxic and trace elements in rice. *Science of The Total Environment*, *748*, 142484. https://doi.org/10.1016/j.scitotenv.2020.142484

Sun, L., Zheng, M., Liu, H., Peng, S., Huang, J., Cui, K., & Nie, L. (2014). Water Management Practices Affect Arsenic and Cadmium Accumulation in Rice Grains. *The Scientific World Journal*, *2014*, 1–6. https://doi.org/10.1155/2014/596438

Takamatsu, T., Aoki, H., & Yoshida, T. (1982). DETERMINATION OF ARSENATE, ARSENITE, MONOMETHYLARSONATE, AND DIMETHYLARSINATE IN SOIL POLLUTED WITH ARSENIC: *Soil Science*, *133*(4), 239–246. https://doi.org/10.1097/00010694-198204000-00007

Tao, S. S.-H., & Michael Bolger, P. (1999). Dietary arsenic intakes in the United States: FDA Total Diet Study, September 1991-December 1996. *Food Additives and Contaminants*, *16*(11), 465–472. https://doi.org/10.1080/026520399283759

Williams, P. N., Islam, M. R., Adomako, E. E., Raab, A., Hossain, S. A., Zhu, Y. G., Feldmann, J., & Meharg, A. A. (2006). Increase in Rice Grain Arsenic for Regions of Bangladesh Irrigating Paddies with Elevated Arsenic in Groundwaters. *Environmental Science & Technology*, *40*(16), 4903–4908. https://doi.org/10.1021/es060222i

Williams, P. N., Price, A. H., Raab, A., Hossain, S. A., Feldmann, J., & Meharg, A. A. (2005). Variation in Arsenic Speciation and Concentration in Paddy Rice Related to Dietary Exposure. *Environmental Science & Technology*, *39*(15), 5531–5540. https://doi.org/10.1021/es0502324

World Health Organization. (2003). *Arsenic, Drinking-Water and Health Risks Substitution in Arsenic Mitigation: A Discussion Paper*. https://www.who.int/water_sanitation_health/dwq/WSH03.06fulltext.pdf

Xu, X. Y., McGrath, S. P., Meharg, A. A., & Zhao, F. J. (2008). Growing Rice Aerobically Markedly Decreases Arsenic Accumulation. *Environmental Science & Technology*, *42*(15), 5574–5579. https://doi.org/10.1021/es800324u

Xu, Y., Gu, D., Li, K., Zhang, W., Zhang, H., Wang, Z., & Yang, J. (2019). Response of Grain Quality to Alternate Wetting and Moderate Soil Drying Irrigation in Rice. *Crop Science*, *59*(3), 1261–1272. https://doi.org/10.2135/cropsci2018.11.0700

Yang, J., Huang, D., Duan, H., Tan, G., & Zhang, J. (2009). Alternate wetting and moderate soil drying increases grain yield and reduces cadmium accumulation in rice grains. *Journal of the Science of Food and Agriculture*, *89*(10), 1728–1736. https://doi.org/10.1002/jsfa.3648

Zavala, Y. J., & Duxbury, J. M. (2008). Arsenic in Rice: I. Estimating Normal Levels of Total Arsenic in Rice Grain. *Environmental Science & Technology*, *42*(10), 3856–3860. https://doi.org/10.1021/es702747y

Zavala, Y. J., Gerads, R., Gürleyük, H., & Duxbury, J. M. (2008). Arsenic in Rice: II. Arsenic Speciation in USA Grain and Implications for Human Health. *Environmental Science & Technology*, *42*(10), 3861–3866. https://doi.org/10.1021/es702748q

Zhu, Y.-G., Sun, G.-X., Lei, M., Teng, M., Liu, Y.-X., Chen, N.-C., Wang, L.-H., Carey, A. M., Deacon, C., Raab, A., Meharg, A. A., & Williams, P. N. (2008). High Percentage Inorganic Arsenic Content of Mining Impacted and Nonimpacted Chinese Rice. *Environmental Science & Technology*, *42*(13), 5008–5013. https://doi.org/10.1021/es8001103

Published on Journal of Environmental Management, 2022, https://doi.org/10.1016/j.jenvman.2022.115984

INFLUENCE OF IRRIGATION METHODS ON ARSENIC SPECIATION IN RICE GRAIN

5.1. INTRODUCTION

Rice (*Oryza sativa* L.) is a herbaceous plant of the *Poaceae* family. The high availability of rice and its high nutritional value make it a staple food for more than 3.5 billion people (Consultative Group for International Agricultural Research, n.d.). Rice supplies 19% of the world human energy and, in many countries, the average consumption per capita is over 100 kg year^{-1} (Food and Drug Administration (FDA), 2019). Moreover, Asia consumes 90% of the world production ('International Year of Rice, Economics and the International Year of Rice', 2004) and, for more than half a billion low-income people, rice represents the main source of caloric intake (Consultative Group for International Agricultural Research, n.d.; International Rice Research Institute, 2013). Therefore, any potential health risk associated with its consumption should be assessed with the highest priority and care. On this respect, one of the main reasons for concern about any rice-based food is its possible contamination with toxic elements. While rice pollution by certain heavy metals such as Pb, Cd, and Hg is generally of anthropogenic origin (Fangmin et al., 2006; Tang et al., 2020), bioaccumulation of metalloids like As is very often of natural origin (Mandal & Suzuki, 2002). For this reason, and since As bioaccumulation in rice is more efficient than in other cereals (Schoof et al., 1999; Williams et al., 2007), this pollution should be considered of even greater concern. For instance, in several Asian countries such as Bangladesh, China, India, and Taiwan the frequent As contamination of groundwater from wells used to irrigate fields caused a dangerous alarming increase in the As concentration in rice grains produced in these areas (Mandal & Suzuki, 2002; Meharg & Rahman, 2003).

Arsenic is a broadly distributed element in the Earth crust and may exist in many inorganic and organic forms, mostly in +3 and + 5 oxidation states (Mandal & Suzuki, 2002). Nearly all As compounds are toxic for humans: inorganic As species (iAs) are generally more toxic than organic ones and, among them, trivalent species are more toxic than pentavalent ones (Mandal & Suzuki, 2002). Overall, iAs species are a non-threshold class I carcinogens to humans. Since the toxicity of As species increases with their solubility in water, the health risk represented by the most common forms of iAs is high (Rumble, 2021) (i.e., arsenite

and arsenate ions, all soluble in water). Among the possible factors that increase the spread of As from aquifer sediments into groundwater, the reductive dissolution of ferric oxyhydroxide containing As, which derives from base-metal sulfide oxidation, is the most significant geochemical trigger (Nickson et al., 1998). Therefore, As(III) is often the main redox form present in groundwater from wells that, in southeastern Asia, were frequently used to irrigate rice fields (Korte & Fernando, 1991). In particular, As contamination in groundwater reached the highest level of danger to humans in Bangladesh, where As concentrations were found to be above 4 mg dm^{-3} (Nickson et al., 1998; Shankar et al., 2014). Similarly to what happens in groundwater, As(III) represents the most abundant chemical form also in paddy soils, which in turn are characterized by a very low redox potential due to their continuous flooding (CF) conditions.

From groundwater and soils, As is then transferred to rice plants. Roots easily absorb As(III) through the Lsi1 and Lsi2 silicon protein transporters contained in aquaporins, i.e., the channel proteins contained in root cells, thereby exploiting their close chemical similarity with the Si (Bakhat et al., 2017). Although the Lsi transporters are not significantly involved in the translocation of As(III) from the roots to the epigean parts of the rice plant, As(III) is very efficiently conveyed towards the grains by the phloem (Awasthi et al., 2017), and this happens also in the absence of any water and/or soils pollution (Meharg et al., 2009; Spanu et al., 2012). Consequently, it is not surprising that the limit of 0.20 mg kg^{-1} for iAs in polished rice established both by FAO-WHO ('Codex Alimentarius Commission, 37th Session', 2014) and European Community (EC) (European Community, 2015) is often exceeded. Furthermore, As-polluted environments can lead to producing rice where the As concentration is above the 1 mg kg^{-1} level (Islam et al., 2004), which results in a significant arsenicosis risk for more than 150 million people living in the south of Asia, where rice is the staple food (Consultative Group for International Agricultural Research, n.d.).Therefore, the determination of the main chemical forms of As in rice grain is of utmost importance to evaluate its level of pollution and, consequently, the health risk of the consumer.

Literature data (Batista et al., 2011; Heitkemper et al., 2001; Hu et al., 2015; Huang et al., 2012; Liang et al., 2010; Llorente-Mirandes et al., 2012; Meharg et al., 2009; Williams et al., 2005; Zavala et al., 2008; Zavala & Duxbury, 2008) reveal the marked influence of the geographical origin (in terms of As concentration measured both in the soil and in the irrigation water) and the nature of the genotype on the As speciation equilibria in rice grains. In CF-irrigated rice, As(III) and dimethylarsinic acid (DMA) are normally the most abundant species, As(V) is a minority species, whereas the amount of monomethylarsonic acid (MMA) is usually low or negligible (Batista et al., 2011; Heitkemper et al., 2001; Hu et al., 2015; Huang et al., 2012; R.-Y. Li et al., 2009; Liang et al., 2010; Llorente-Mirandes et al., 2012; Meharg et al., 2009; Narukawa & Chiba, 2010; Williams et al., 2005; Zavala et al., 2008). Despite the importance in reducing the overall amount of As in rice grains demonstrated by the adoption of intermittent irrigation methods (Arao et al., 2009; Duxbury & Panaullah, 2007; Hu et al., 2015; R.-Y. Li et al., 2009; Sarkar et al., 2012; Somenahally et al., 2011a; Spanu et al., 2012; Suriyagoda et al., 2018),only a few studies paid attention to their effects on the As speciation in rice grains (Arao et al., 2009; Hu et al., 2015; R.-Y. Li et al., 2009; Somenahally et al., 2011a).

For years our research group has studied the effect of intermittent irrigation methods on the bioaccumulation of elements of health concern in rice grains (Spanu et al., 2012, 2018a; Spanu, Langasco, et al., 2020, 2020; Spanu et al., 2021). The replacement of CF irrigation with sprinkler irrigation (SP) revealed the simultaneous minimization of As and Cd in grains of twenty-six different rice genotypes (Spanu, Valente, et al., 2020). In particular, an unprecedented 98% reduction of the total As concentration (average value in grains of 53 different rice genotypes) was achieved using SP in place of CF irrigation (Spanu et al., 2012; Spanu, Valente, et al., 2020). Moreover, the SP-irrigated rice cultivated in soil polluted by several tens of mg kg^{-1} of As and Cd showed a total concentration of the two toxic elements well below the strictest limits set by the FAO-WHO and the EC (Spanu et al., 2021) (i.e. 200 µg kg^{-1} for both iAs and Cd). In addition, periodic soil saturation (SA) also results in a significant reduction in As concentration in grains compared to the concentrations measured in rice irrigated by CF. Both intermittent irrigation methods resulted in non statistically significant loss of grain yield, achieving up to 70% of water savings with respect to the amounts normally required by CF irrigation (Spanu et al., 2004, 2009a).

To the best of our knowledge, no studies have been conducted on the impact of intermittent irrigation methods on the determination of the amounts of the most important As species in rice grains. Therefore, the main purpose of this contribution is to evaluate the differences in concentrations of As(III), As(V), DMA, and MMA in the kernels of twenty-six different rice genotypes grown in an open field with the same soil/water system but irrigated by conventional (CF) or intermittent (SP or SA) methods. Analyses were performed by a comprehensive set of IC-ICP-MS and ICP-MS methods, previously developed and validated by this group (Langasco et al., 2022).

5.2. MATERIAL AND METHODS

5.2.1. CULTIVATION AND RICE GENOTYPES

All rice genotypes were cultivated in three adjoining soils (one for each irrigation methodchosen for the study) inside the "Santa Lucia" experimental farm of the University of Sassari, Sardinia, Italy (39°59' N, 8°40' E; 15 m AMSL). According to the World Reference Base for soil resources, the soils are classified as *Pantofluvic Eutric Fluvisol Loamic* (Food Agriculture Organization (FAO), 2014). All soils were clayey with good water retention capacity, and those devoted to CFor SA irrigation were used as paddy fields for the last 35 years. During this period, none of the soils used in the experiments was treated with pesticides containing organoarsenic species, as evidenced by the negligible quantities of DMA and MMA found in it (Langasco et al., 2022). Irrigation water was from Lake Omodeo (40°08 N, 8°55' E; 118 m AMSL). The site has a Mediterranean climate (Spanu, Valente, et al., 2020). Further details on the site were reported in previous contributions (Langasco et al., 2022; Spanu et al., 2018a; Spanu, Langasco, et al., 2020; Spanu, Valente, et al., 2020). The experimental design was a randomized block with 4 replications for each genotype. Each plot was divided into sub-plots of 10 m^2 each. The seedbed was prepared first by plowing to a 20 cm depth and then by secondary tillage with a field cultivator. Sowing was performed on dry soil using a seed drill. Fertilization and herbicide treatments were accomplished according to previous contributions (Spanu et al., 2018a; Spanu, Valente, et al., 2020). On the basis of the ripening level of each rice genotype, harvesting was performed between the last days of September and the first days of October using a small plot combine harvester. Yields of rice grain ranged between 8.2 and 13 t ha^{-1}, depending on the rice genotype and irrigation method, whilst the average yields for all genotypes irrigated by the three methods are always 11 ± 3 t ha^{-1}. Table E1 of the Appendix E provides all yields for both each rice genotype and irrigation method. Twenty-six genotypes were cultivated in this study, six of them belonging to the Indica subspecies (i.e., *Apollo, Oceano, Salvo, Sprint, Thaibonnet,* and *Urano*) and twenty belonging to the Japonica subspecies (i.e., *Aleramo, Antares, Balilla, Brio, Carnaroli, Carnise, Cerere, CRV 04, CRV 108, CRV 114, CRV 390, Galileo, Gloria, Luxor, Musa, Opale, Orione, Ronaldo, Selenio* and *Virgo*). Their denominations are those reported in the Italian

Register of Rice Varieties (Servizio Informativo Agrario Nazionale, 2018), with the only exceptions of the four CRV genotypes, which are currently under registration.

5.2.2. IRRIGATION METHODS

CF is the traditional irrigation method, used worldwide for cultivating rice. A laser-leveled paddy field was first surrounded by embankments, aimed to contain a 10 cm blanket of water on average, and then divided into subplots. CF ensures constant reducing conditions in the soil-water-rice system. The subplots were flooded from seeding to approximately two weeks before harvest. Contrary to CF, in SA the soil was never flooded, but it was cyclically saturated. Leveling of soil was needed, but perimetral embankments were unnecessary. One time a week, water was provided until reaching the soil water storage capacity. The field was saturated through the periodic flooding of 10 cm-depth furrows, dug every 25–30 m. Between two consecutive irrigations, the redox potential of soil swung between reductive conditions (at least −100 mV vs. saturated calomel electrode, SCE) and strongly oxidating conditions (even +400 mV vs. SCE). Lastly, in SP the soil was never flooded or saturated, and its typical redox potential usually ranged between +100 and + 200 mV vs. SCE. Water was sprayed into the air using low-flow sprinkler heads and dropped onto the soil as rainfall. In each irrigation cycle, water was provided until soil reached its field capacity in amount proper to compensate the losses of the soil-plant system due to both evaporation and transpiration. At variance, in both CF and SP methods the rice plant is under drought stress, which could not be guaranteed by SA irrigation, due to the constant time intervals between successive saturations. Abundant details on these irrigation methods were provided elsewhere (Spanu et al., 2004, 2009a, 2016, 2018a, 2018a; Spanu, Valente, et al., 2020, p. 20, 2020, 2020; Spanu et al., 2021).

5.2.3. INSTRUMENTATION

5.2.3.1. Total amount of As

Rice samples were mineralized using an Ethos Easy Lab Station microwave oven (Milestone, Sorisole, Italy). The determination of the total amount of As in rice grains was performed using a NexION 300X ICP-MS spectrometer (PerkinElmer, Milan, Italy), equipped with a glass nebulization/spray chamber system, an S10 autosampler, and a KED collision cell.

5.2.3.2. As speciation

The extraction of As from rice grains was accomplished using an Ethos Touch Control microwave digestion system (Milestone, Sorisole, Italy). The speciation of As(III), As(V), DMA and MMA were performed by means of ion chromatography - inductively coupled plasma – mass spectrometry instrument (IC-ICP-MS), constituted of i) an Agilent 1200 Series Gradient HPLC system (Agilent Technologies, Santa Clara, CA, USA); ii) a PRP-X100 Anion Exchange HPLC Column (Hamilton Company, Reno, NE, USA); and iii) an Agilent 7500ce ICP-MS (Agilent Technologies, Santa Clara, CA, USA).

Additional instrumentation and the list of chemicals and standards used in this study have been reported in the Appendix E..

5.2.4. *SAMPLING AND ANALYTICAL METHODS*

Sampling and analysis of irrigation water, paddy soils, and rice grains were extensively described in a previous contribution of this research group (Langasco et al., 2022). Rice analysis steps are briefly summarized below. For each subplot, a sample of 100 g of paddy rice was collected by a repeated quartering of the whole kernels here obtained; it was dried at 32 °C, husked, bleached, mineralized with HNO_3 67% and H_2O_2 30% (for the determination of the total amount of As) or ground and extracted with an aqueous solution containing 0.2 mol dm^{-3} of HNO_3 (for the determination of the As(III), As(V), DMA and MMA species). Finally, samples were analyzed by ICP-MS or IC-ICP-MS methods. Analytical and instrumental settings used during all ICP-MS based measurements, as well as the validation parameters and the quality assurance and quality control procedures, were thoroughly reported elsewhere (Langasco et al., 2022).

5.2.5. *STATISTICAL ANALYSIS AND CHEMOMETRICS*

A two-tail *t*-test (p = 95%) was used for the comparison of paired data. The multivariate analysis of variance (MANOVA) with the Hoteling's test was performed with RStudio (version 2022.02.3 + 492). Principal Component Analysis (PCA) was performed on rice data using the R-based software CAT (Chemometric Agile Tool) (Leardi et al., 2021).

5.3. RESULTS AND DISCUSSION

5.3.1. AS SPECIES IN RICE GRAINS

The total amount of As and its most representative chemical forms in rice grains (i.e., As(III), As(V), DMA and MMA) were measured for each rice genotype irrigated by CF, SA, or SP. The total amount of As was measured for both mineralized and extracted samples; As speciation was accomplished only on the latter. Table 5.1 summarizes the results of the determination of the twenty-six rice genotypes (average concentration and range) and the analytical performance of the method (extraction efficiency and column recovery) for each irrigation method. Data for each sample are reported in Tables E2–E4.

Table 5.1. Average and range concentration (in μg kg^{-1}) of As species in twenty-six genotypes of rice grains irrigated by CF, SA, and SP irrigation, n = 4.

Parameter	Continuous flooding (CF)		Saturation (SA)		Sprinkler (SP)	
	Average[d]	Range	Average[d]	Range	Average[d]	Range
Total As	154	109 - 197	52	31 - 84	5.5	1.4 - 18
Total extracted As	147	103 - 190	52	31 - 83	5	1.2 - 16
As(III)	70	46 - 120	50	31 - 76	0.6	< 0.04 - 5.4
As(V)	<1	<0.2 - 19	<0.2	<0.2 - <0.2	4.2	1.2 - 10.6
iAs	70	46 - 120	50	31 - 76	4.8	1.2 - 14.9
DMA	70	18 - 109	<0.3	<0.3 - <0.3	<0.3	<0.3 - <0.3
MMA	<0.16	<0.16 - <0.16	<0.16	<0.16 - <0.16	<0.16	<0.16 - <0.16
Sum of speciated As [a]	141	98 - 190	50	31 - 76	4.9	1.2 - 14.9
Extraction efficiency [b] (%)	94.7	80 - 106	98.7	82 - 112	93.8	75 - 110
Column recovery [c] (%)	96.5	82 - 110	96.9	85 - 101	95.6	80 - 101

Data preceded by the < sign are below the relevant limit of detection, LoD. [a] Sum of speciated As = As(III)+As(V)+DMA+MMA; [b] extraction efficiency = (Total extracted As)/(Total As) ratio; [c] column recovery = (Sum of speciated As)/(Total extracted As) ratio. [d] no significant differences were observed between average and median. Average data, the sum of speciated As, the extraction efficiency and the column recovery were calculated on unrounded data. For the sake of clarity, data are here presented without the relevant standard deviation, which is reported for all samples in the Tables E2-E4 in the Appendix E.

The concentrations of total As in kernels measured in this experiment were in good agreement with those measured by this research group in previous agricultural years (Spanu et al., 2012, 2016; Spanu, Valente, et al., 2020). The average As extraction efficiency from rice grains (95.7%, Table 5.1) and the column recoveries of the total As extracted (96.3%, Table 5.1) agreed with the performances of the method recently published by this group (Langasco et al., 2022).

The results of the As speciation confirmed the influence of the irrigation method on the concentration of the analytes considered. For a simple and comprehensive data visualization, a Principal Component Analysis (PCA) was performed on the dataset constituted of 78 rice samples (26 for each irrigation method; CF, SA, and SP) and 5 variables (i.e., the total amount of As and the percent amounts of As(III), of As(V), of DMA, and of other As species, respectively, where the last variable is the difference between total extracted As and the sum of the As species measured in the IC-ICP-MS analysis). Since MMA was always found below its LoD for any irrigation method used, it cannot be considered for statistical analysis. Figure 5.1 reports the loading plot and the score plot from the PCA.

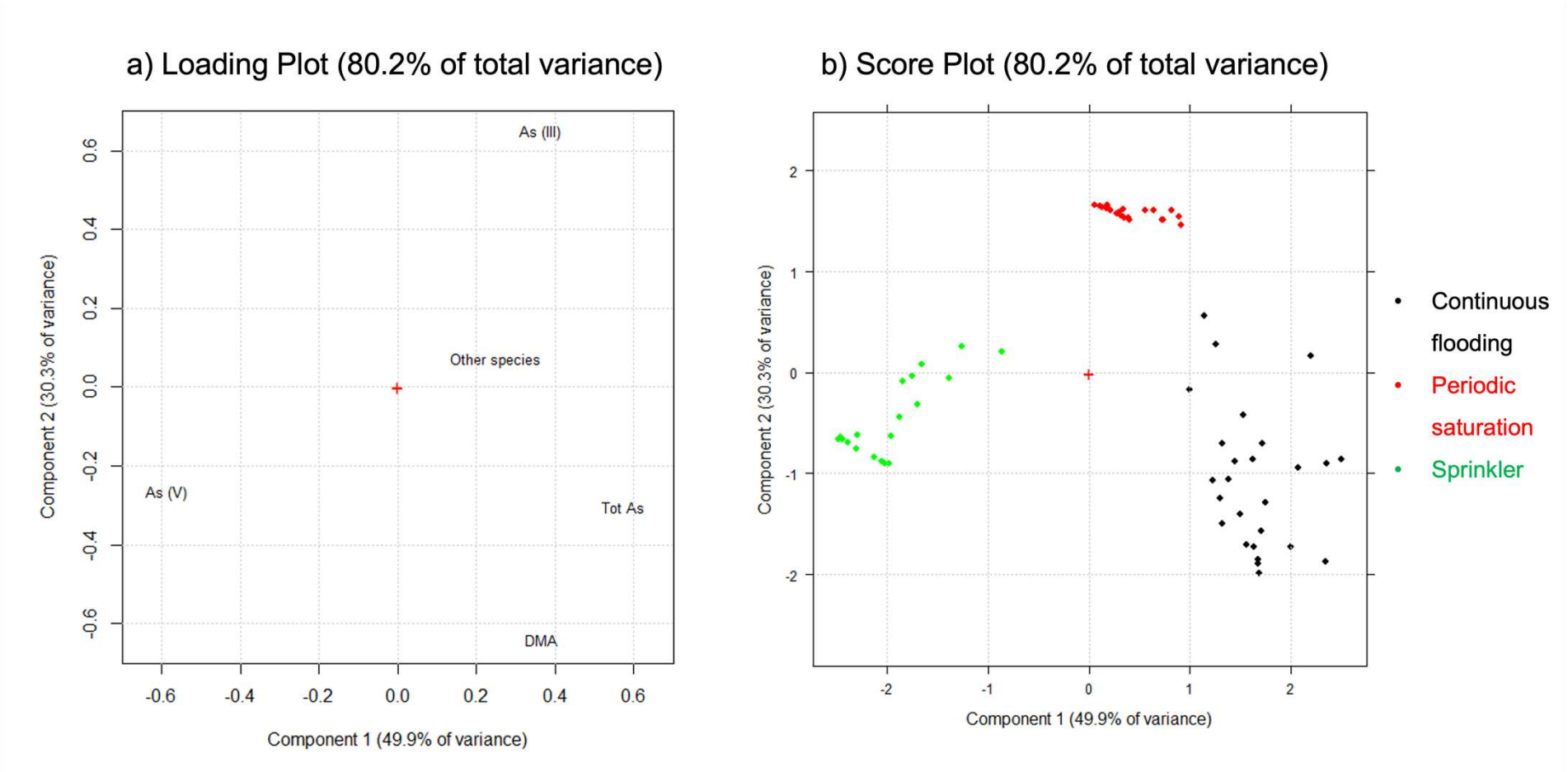


Figure 5.1. PCA performed on 78 rice samples (26 for each irrigation method; CF, SA and SP) and 5 variables (i.e., % of As(III), % of As(V), % of DMA, % of unknown species (difference between total extracted As and the sum of the As species measured in the IC-ICP-MS analysis), and total extracted As). Loading plot (a) and score plot (b), samples colored according to the irrigation method.

After autoscaling, PC1 and PC2 accounted for 49.9% and 30.3% of the total variance, respectively. The loading plot (Figure 5.1a) reveals an inverse correlation between each of the three As species quantified. Hence, As(V) is characterized by negative values of both PC1 and PC2, As(III) is represented by positive values of both PC1 and PC2, whereas DMA is described by positive amounts of PC1 and negative values of PC2. In addition, the total amount of extracted As is quite close to the DMA in the PC1-PC2 plan, while the sum of the other As species is essentially not described by the first two components.

On the other hand, the score plot (Figure 5.1b) exhibits a clear differentiation of the three irrigation methods along with the PC1-PC2 plan: highly positive values of PC1 are always representative of rice genotypes irrigated by CF, SA-irrigated rice genotypes are identified by slightly positive amounts of PC1 and highly positive amounts of PC2; by contrast, highly negative amounts of PC1 are typical of SP-irrigated rice. Finally, the comparative view of the two PCA outputs provides comprehensive evidence that rice irrigated by CF is mainly described by high amounts of total As and DMA, whereas As(III) is the only representative species to describe rice irrigated by SA, as well as As(V) is for the SP-irrigated rice.

5.3.1.1. As speciation in rice grain irrigated by CF

A closer look at the concentrations of the As species in rice irrigated by CF reveals that in half of the genotypes studied the As(III)/DMA ratio is > 1, whereas in the remaining 50% the ratio is ≤ 1. In both cases, the average amounts of As(III) and DMA are 70 μg kg^{-1}. This disagrees with the conclusions of Zavala et al. (Zavala et al., 2008) who, through the meta-analysis of a wide literature dataset and the speciation analysis of 24 different rice samples from the United States of America (USA), postulated the existence of two different types of rice, depending on the prevalence of iAs or DMA in it. The so-called “DMA-type rice”, in which the concentration of DMA is much more abundant than iAs, is quite common in the USA, whereas the “iAs-type rice”, normally found in Europe and Asia, is characterized by an amount of DMA largely higher than iAs. According to that study, marked differences among both slopes and correlation coefficients of the relationships among the concentrations of iAs (or DMA) as a function of the total concentration of As in rice (see Figure 5 in (Zavala et al., 2008)) were observed. Data in this study show that the overall trend,

summarized in Table 5.1 and Figure E1, is not representative of either DMA-type rice or, as expected by the Zavala's conclusions, of iAs-type rice.

In particular, Figure E1 reveals that, for the rice genotypes irrigated by CF, the experimental points representing the amounts of total extracted As vs DMA (blue dots) or iAs (red dots) are significantly overlapped and the relevant regression lines show a quite low value of the correlation coefficients R^2.

On the contrary, the average speciation data reported in this study agree rather well with those obtained for 389 samples of polished rice produced in Brazil (i.e., from 58 μg kg^{-1} to 62 μg kg^{-1} for As(III) and DMA, respectively) (Barnet et al., 2021) and to those measured in more than 100 samples of dehusked rice produced in Spain (i.e., from 71 μg kg^{-1} to 55 μg kg^{-1} for As(III) and DMA, respectively) (Signes-Pastor et al., 2016). As far as regards Italian productions of rice, Šlejkovec et al. (Šlejkovec et al., 2021) reported that the average amounts of iAs and DMA in 24 samples of polished rice were 87 μg kg^{-1} and 31 μg kg^{-1}, respectively. On the other hand, for Tenni et al. (Tenni et al., 2017), the average amount of iAs in 168 samples of polished rice was 102 μg kg^{-1}. Furthermore, in a subset of 61 samples of rice grains from the latter study, also As(III), As(V), and DMA were measured, obtaining average concentrations of 91 μg kg^{-1}, 8 μg kg^{-1}, and 55 μg kg^{-1}, respectively (Tenni et al., 2017). In this study, As(V) was quantified only in the *Orione* and in the *Sprint* rice genotypes, at concentrations of 19 ± 1 μg kg^{-1} and 13 ± 1 μg kg^{-1}, respectively. Hence, the average amount of iAs found in this study is not only significantly less than those previously measured in Italian rice irrigated by CF (Šlejkovec et al., 2021; Tenni et al., 2017; Williams et al., 2005), but it also ensures an excellent level of food safety. In the worst of the cases, the amount of iAs in it reaches the concentration of 120 ± 10 μg kg^{-1}, i.e., only the 60% of the maximum concentration allowed by both EC and FAO-WHO for polished rice (European Community, 2015; Food and Drug Administration (FDA), 2014) and, for twenty-four out of twenty-six genotypes (see Table E2), is below the limit of 0.1 mg kg^{-1} fixed by the EU for foods intended for infants and young children (European Community, 2015).

5.3.1.2. As speciation in rice kernels irrigated through intermittent methods

5.3.1.2.1. Further remarks on SP and SA methods of irrigation

While the definition of the CF irrigation method is unambiguous, this is not the case for intermittent irrigation methods in that the lack of sufficient details in their description prevents a reliable comparison of the results obtained by using them. The same irrigation method was sometimes called by two different names or, even worse, the same name was used to identify different irrigation methods (Spanu, Valente, et al., 2020). Although the most important characteristics of SA and SP irrigations have been summarized in Section 5.2, the discussion of the data obtained from this experiment requires the knowledge of additional details about these methods. While SP and SA belong to the family of the alternate watering and drying methods of irrigation (AWD) (Ishfaq et al., 2020), the different nature of water management, the redox potential of the soil, and the stress level of the plants make them very different from each other.

The primary purpose of SP irrigation is to restore the amount of water lost through transpiration of the rice plant and by evaporation from the soil in the short period between two consecutive irrigations (normally this time is 3–5 days, at the latitude of this experiment) (Spanu et al., 2012). As a consequence the amount of water supplied in every irrigation cycle is not constant, but it is dependent on both the phenological phases of the rice plant and weather conditions (Spanu et al., 2009b). At the end of each irrigation, the soil was brought to the field capacity. This means that the wider soil pores are occupied by air, and the narrower pores are occupied by water. Because irrigation cycles are so close, the rice plant is never under drought stress (Spanu et al., 2018b). This is confirmed by the high yields of rice grains, which do not differ statistically from those measured in CF irrigation (Spanu et al., 2012, 2018b) (Table E1), and by the constancy of the redox potential in the soil, ca. 300 mV vs. SCE higher than the values normally measured in CF soils (Spanu et al., 2012, 2016, 2018b; Spanu, Valente, et al., 2020).

On the other hand, the main target of SA irrigation is to saturate all soil pores with water, thereby reaching the so-called “water storage capacity” (Spanu et al., 2018b), i.e., the stage just before the appearance of the first pools of water in the topsoil. Thus, the soil will be no longer irrigated until its surface layer becomes dry, and this usually happens - at the latitude of this experiment - after one week of the last saturation. This

means that the dry period between two successive SA irrigations is constant (Spanu et al., 2016; Spanu, Valente, et al., 2020) and, as a function of the phenological stages of the plant and weather conditions, drought stress can sometimes arise in rice plants in the last days of the dry period (Spanu et al., 2018b).

Finding similarities between the AWD methods reported in the literature and those used in this study is a very difficult task. For this reason, it was deemed appropriate to indicate at least two methods, i.e., alternate wetting and moderate soil drying (Yang & Zhang, 2010) and alternate wetting and severe soil drying (Yang et al., 2009), which are closest to the SP and the SA, respectively. The comparison between literature methods (Yang et al., 2009; Yang & Zhang, 2010) and SP and SA, respectively, gave somewhat similar results, primarily about effects on the bioaccumulation of toxic elements in grains and on the level of drought stress on rice but, unfortunately, they do not report any result as far as the As speciation in rice.

5.3.1.2.2. As speciation in rice grains irrigated by SA and SP

Replacing CF irrigation with SA irrigation reduces the quantity of As(III) in rice grains from 30% to 40%, depending on the genotype. However, the most remarkable change induced by SA irrigation is the almost total disappearance of DMA, whose concentration is always below the LoD (i.e., 0.3 μg kg^{-1}). Since also As(V) is always below its LoD, As(III) is the only species quantifiable in rice irrigated by SA.

On the other hand, in comparison to that measured in CF the adoption of SP irrigation produces an exceptional reduction in the amount of As(III) in the grain (average reduction: 99.1%). As(V) is the main species present in the SP-irrigated rice grains, at concentrations ranging from 1.2 μg kg^{-1} to 10.6 μg kg^{-1} (average concentration: 4.2 μg kg^{-1}). Again, the quantity of DMA is always less than its LoD. Only two open-field studies carried out in Extremadura, Spain, reported the use of SP irrigation in the cultivation of a *Gladio* rice genotype (Alvarenga et al., 2022; Moreno-Jiménez et al., 2014). By switching from CF to SP irrigation, both showed a significant reduction in the total concentration of As (from −57% to −84%), of the iAs (from −50% to – 82%), and – mainly - of the organic As (from −60% to – 91%) in the grain. On the other hand, several details indicate that SP is probably not the intermittent irrigation method used in these studies, but another that is much closer to the SA (Spanu et al., 2016, 2018b; Spanu, Valente, et al., 2020). In particular, the excessive amount of water used (from 57% to 67% of the amount needed for the CF irrigation (Alvarenga et

al., 2022), the very low rice yields (only one-third of the average production of rice obtained in Extremadura in the two decades prior the trial (Moreno-Jiménez et al., 2014)) and, especially, the large increase in the concentration of Cd in rice grain produced using the intermittent irrigation (Alvarenga et al., 2022; Moreno-Jiménez et al., 2014) fueled these doubts. Beyond the differences in intermittent methods, also those related to cultivation techniques (i.e., pot cultivation (Arao et al., 2009; Hu et al., 2015; R. Y. Li et al., 2009; Xu et al., 2008) in place of open field cultivation here used) make it difficult to compare the results obtained in this experiment with those of the literature. In two pot experiments, Li et al. (R. Y. Li et al., 2009) and Xu et al. (Xu et al., 2008) used an *Oochikara* rice genotype irrigated by continuous flooding or "aerobic" irrigation. In the latter case, the complete disappearance of DMA and the reduction in the amount of iAs (between 67.6% (Xu et al., 2008) and 87.9% (R. Y. Li et al., 2009) of the corresponding amounts measured in CF rice) were observed. It should be noted that, despite of the two studies conducted by the same research group and using the same cultivation technique, the reported results differed widely. This suggests that the experimental conditions might lack in reproducibility. Nevertheless, these data fully support the findings observed in this study about DMA. Also, the reduction of As(III) levels measured by them by replacing the CF method with the aerobic method is within the range found in this research (i.e. 28.6% (SA) to 93.1% (SP) of the amounts measured in rice irrigated by CF, average value of twenty-six rice genotypes). Hu et al. (Hu et al., 2015) carried out speciation on Brazilian upland rice grown in a pot using polluted soil both with As (22 mg kg^{-1}) and Cd (2 mg kg^{-1}) and irrigated with four different methods (i.e., aerobic, flooding, intermittent or intermittent-aerobic methods). Also in these cases, the adoption of intermittent irrigation methods caused a dramatic decrease in DMA, and a significant decrease in iAs with respect to the relevant concentrations measured in rice irrigated by the flooding method (−72% and −96% for DMA, and −12% and −47% for iAs, using the intermittent or the intermittent-aerobic method, respectively). Unfortunately, data on aerobic irrigation are lacking since rice irrigated with this method has not reached the heading phase. This is probably due to the very high sensitivity of this genotype (i.e., the *IAPAQ-9*) to As and/or Cd pollution, since the literature reports (Spanu et al., 2021) that a different rice genotype (i.e., the *Carnise* one), irrigated with SP in soil containing 50 mg kg^{-1} each of As and Cd, has supplied edible rice, containing a quantity of As well below the limit established by the FAO-WHO and the EC (European Community, 2015; Food and Drug

Administration (FDA), 2014). Other research groups (Arao et al., 2009; Somenahally et al., 2011a) did not use aerobic irrigation methods throughout the vegetative cycle of the rice plant. A 45% reduction in the quantities of As(III) and DMA in grains of a field-grown *Cocodrie* rice genotype was observed by Somenahally et al. (Somenahally et al., 2011a) when the flooding irrigation was replaced with intermittent flooding. Finally, Arao et al. (Arao et al., 2009) made a comparison between the amounts of iAs and DMA measured in a *Koshihikari* rice genotype grown in a pot using two different soils and seven different irrigation methods. (i.e., continuous flooding and six different methods of intermittent flooding, each with a different length and frequency of flooding). Reductions of 75% for iAs and more than 98% for DMA were observed with respect to relevant amounts found in rice irrigated with CF when using the shortest flooding periods and, mainly, when no flooding was applied during the heading period.

5.3.1.2.3. Behavior of DMA in rice grains by varying the nature of the irrigation method

While the opposite behavior of As(III) and As(V) in the variation of the irrigation method can be rationalized in terms of their different bioavailability in the soil-root system, the substantial disappearance of DMA in rice grains irrigated by intermittent methods such as SA or SP merits additional comment. First, the data reported here are consistent with recent preliminary evidence from this research group (Langasco et al., 2022). Moreover, intermittent irrigation methods used in rice cultivation instead of CF irrigation can significantly change the microbial community present in the soil. In particular, microbial biomethylation of iAs seems to be possible only under anaerobic soil conditions, typically provided by CF irrigation (Somenahally et al., 2011b).

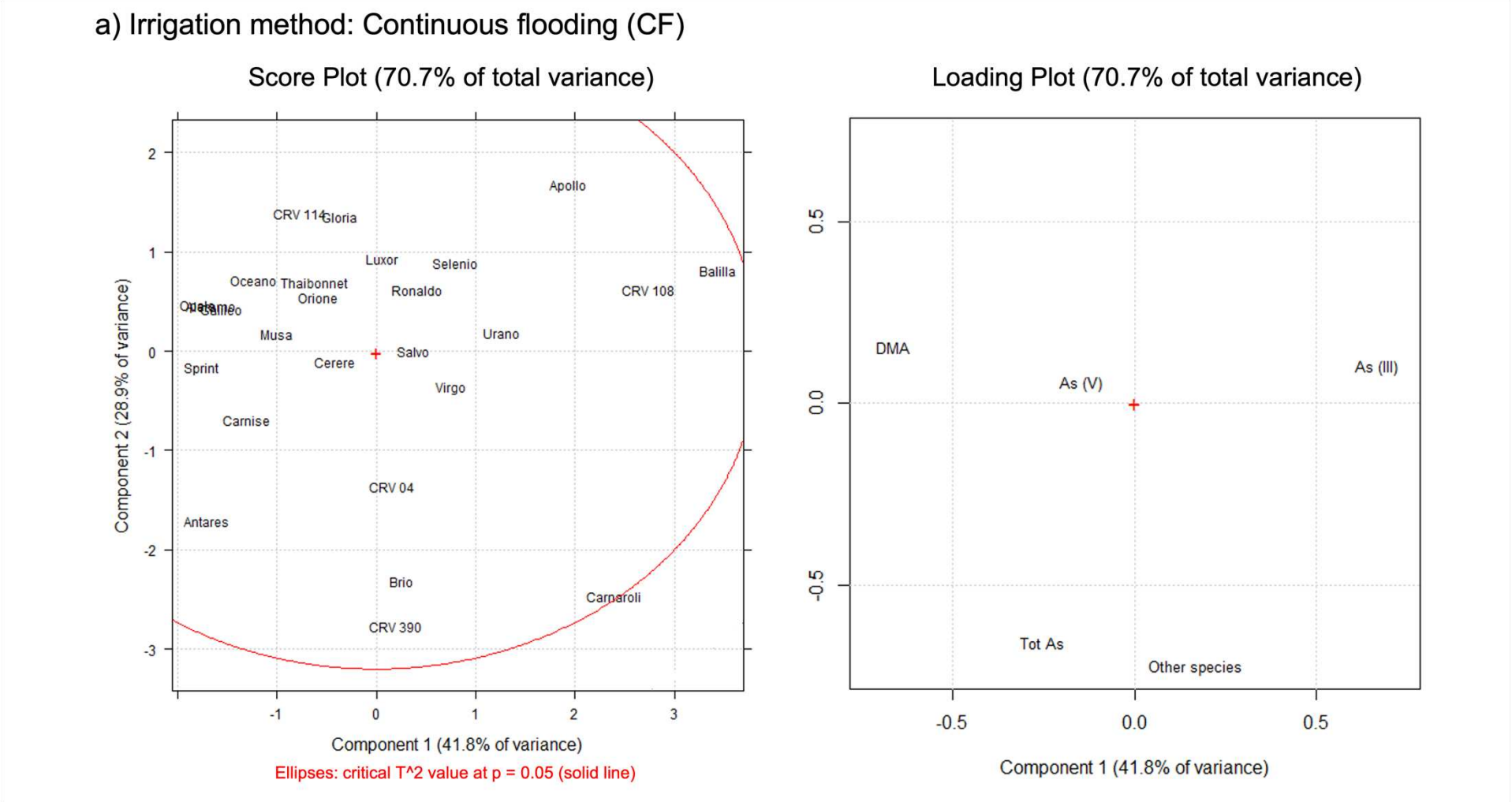
In this study, only As(III) was quantified in CF soils (Langasco et al., 2022) while the increase in redox potential caused by SA irrigation favored the oxidation of 25% of the total amount of As to As(V) (Langasco et al., 2022).These data are consistent with those reported in the literature for irrigation methods more closely related to SA (Khanam et al., 2020; Xu et al., 2008), while the constant positive redox potential measured in SP soil was consistent with the presence of only As(V) (Langasco et al., 2022). On the other hand, and even if DMA was one of the two most abundant species found in rice grains irrigated by CF, organoarsenic species such as DMA and MMA were always below their LOD (i.e., 0.75 μg kg^{-1} and 1 μg kg^{-1}, respectively) in

all soils used in this experiment (Langasco et al., 2022). This result agree with literature findings (Huang et al., 2010; Huang & Matzner, 2006, 2007; Lomax et al., 2012; Takamatsu et al., 1982), and it is also consistent with the known lack of any organoarsenic treatment of these soils. The apparent discrepancy can be explained in two ways: i) the rice plant was able to biomethylate As(III) to form the DMA that was found in rice grains or ii) the very small amount of DMA present in the soil was very efficiently transferred to the grain. Literature results primarily support this latter option (Lomax et al., 2012). Compared to iAs, DMA is absorbed more slowly through rice roots (Lomax et al., 2012) but is transferred with extraordinary efficiency to the grain via xylem and phloem (Carey et al., 2010; Khanam et al., 2020; R.-Y. Li et al., 2009; Ma et al., 2007; Raab et al., 2007). Furthermore, the transport pathway of DMA between roots and grains is influenced by rice genotype (Ma et al., 2007), and this may explain the broad range of DMA concentrations measured among the twenty-six rice types considered here (Table E2).

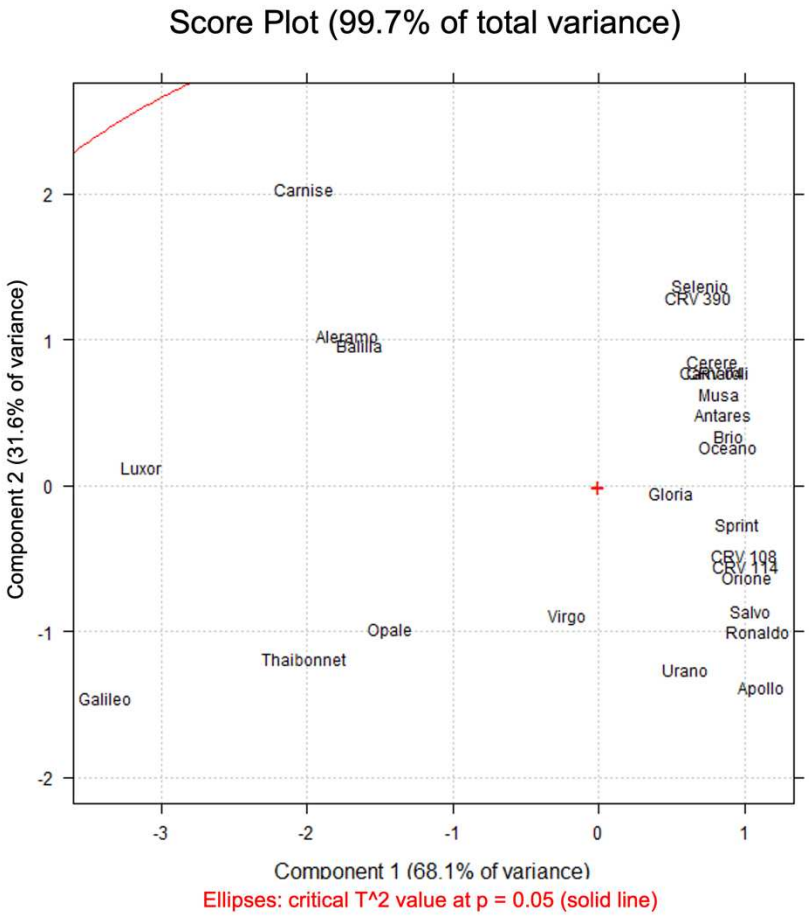
5.3.2. INFLUENCE OF THE RICE GENOTYPES AND ITS SUBSPECIES ON THE AS SPECIATION AS A FUNCTION OF THE NATURE OF THE IRRIGATION METHOD

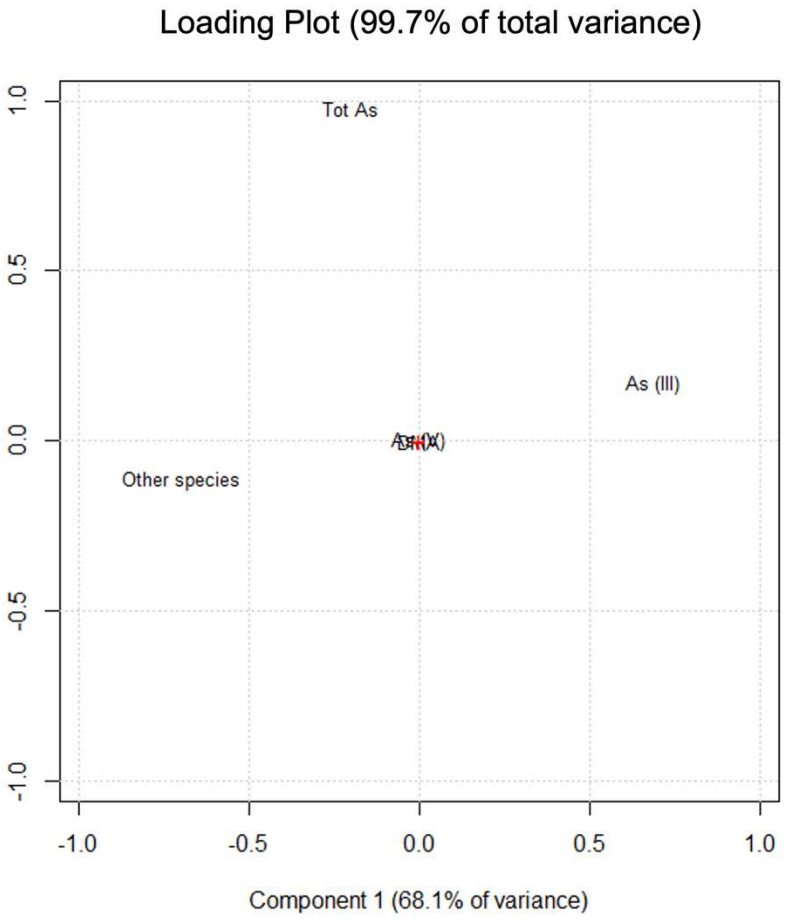
Although the irrigation method is the main factor influencing the total amount of As and its speciation in rice grains, rice genotypes also play a well-known role here (Batista et al., 2011; Liang et al., 2010). To highlight the differences and correlations between the twenty-six genotypes analyzed in this research, three separate PCAs were performed, one for each dataset obtained from each irrigation method. Figure 5.2 shows the resulting plots.

Figure 5.2. Score and loading plots of the PCA performed on the samples of each irrigation method. CF irrigation, (a), SA irrigation, (b), and SP irrigation, (c).

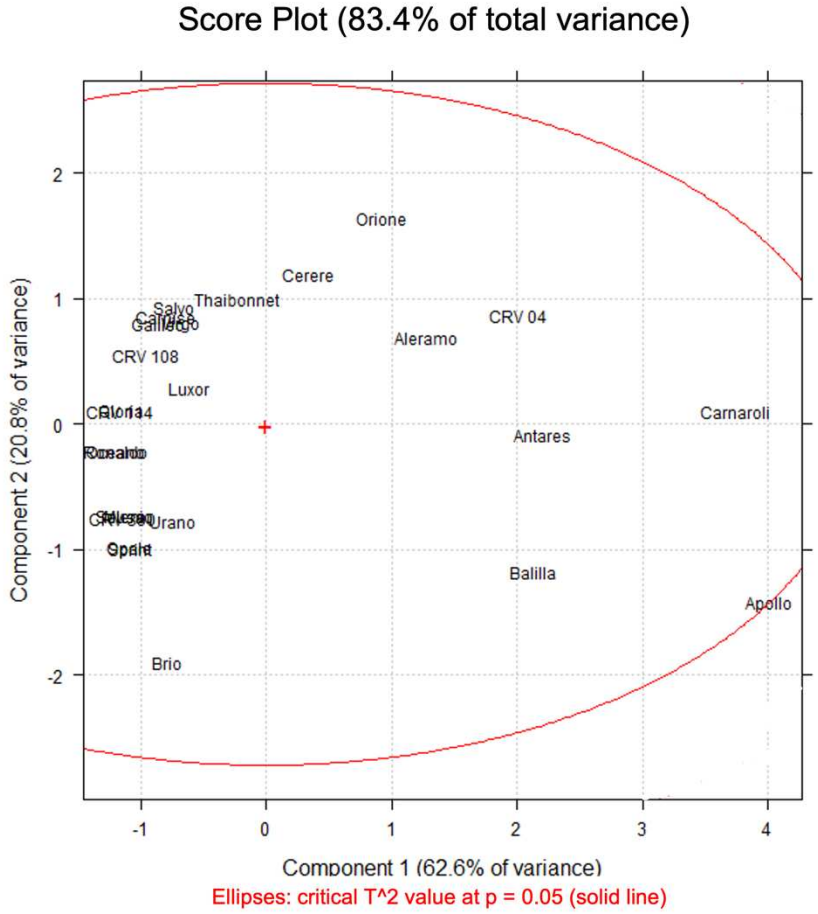


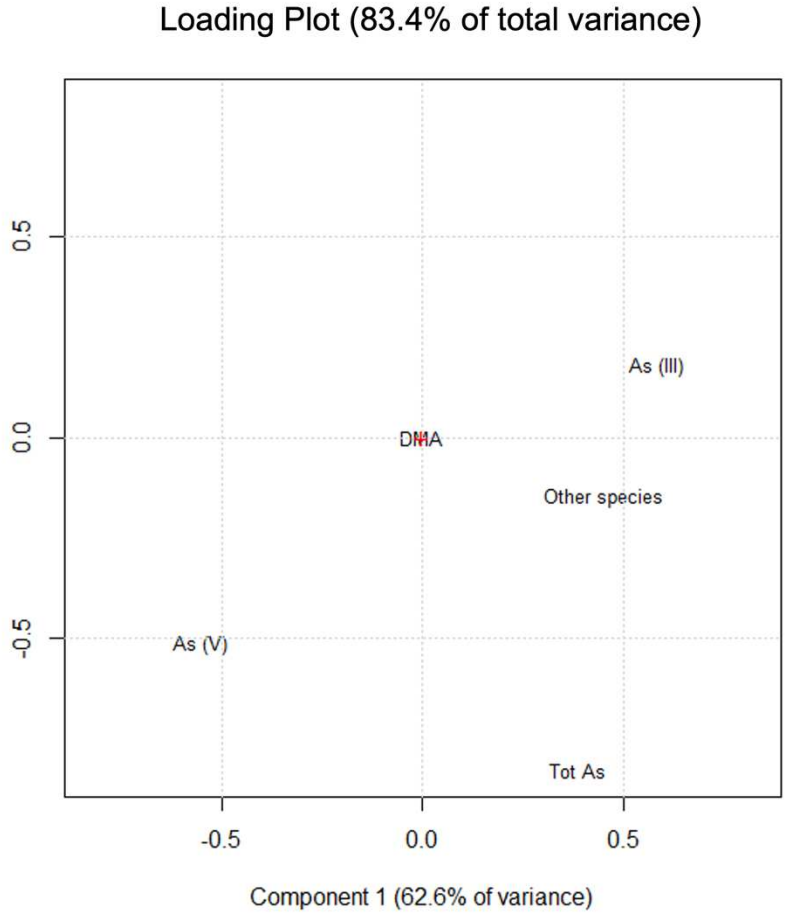
b) Irrigation method: Periodic saturation (SA)





c) Irrigation method: Sprinkler (SP)





From Figure 5.2, it is possible to understand the influence of the rice genotype on the speciation of As at varying the irrigation method. In general, no outliers were detected for each group (criteria: T^2 test, p = 99.9%). The total variance explained by PCA decreases from SA (Figure 5.2c and d, 99.7% of the total variance) to CF-PCA (Figure 5.2a and b, 70.7% of the total variance). This suggests that the genotype effect tends to decrease in the order CF > SP > SA, and this is consistent with the PCA reported in Figure 5.1, where the dispersion of each class tends to decrease in a similar way.

In particular, from the loading plot of CF irrigation (Figure 5.2b), the most significant variables are the total As, the As(III), and the DMA, while the As(V) is not described by the first two components. As(III) and DMA are inversely correlated with each other, and both orthogonal to total As. The score plot in Figure 5.2a shows that genotypes with negative values of PC1 have a higher amount of DMA, whereas positive values of PC1 were found for those characterized by high amounts of As(III). Furthermore, the total amount of As tends to increase going from positive to negative values of PC2. For example, Apollo is the genotype with the lowest amount of total As (109 ± 8 μg kg^{-1}), Carnaroli is the one with the highest amount of both As(III) (120 ± 10 μg kg^{-1}) and total As (197 ± 3 μg kg^{-1}), and Oceano is that with the lowest amount of As(III) (46 ± 4 μg kg^{-1}). Data for each genotype are reported in Table E2.

Figure 5.2c and Figure 5.2d show the PCA for SA-irrigated rice genotypes. The loading plot reveals that only three variables (i.e., total As, As(III), and other species) are significant in that As(V) and DMA are always below their LoD. From the score plot (Figure 5.2c), most genotypes are described for PC1 positive values (large amounts of As(III)), while those described with negative PC1 values show the highest quantities of unknown As species. On the other hand, positive amounts of PC2 are descriptive of genotypes showing the higher concentrations of total As. Compared to that observed in Figure 5.2a, the score plot of Figure 5.2c reveals a higher level of genotype grouping, and this gives visual evidence of their low influence on As speciation in comparison to that observed for the CF. In general, SA irrigation leads to an average 26% reduction of the concentration of total As compared to the amount measured in CF-irrigated rice. However, for five out of twenty-six genotypes, the concentration of

As(III) increases with respect that measured in CF, reaching a +18% for the *Aleramo* genotype. Data for each genotype are reported in Table E3.

Finally, Figure 5.2e and f report the plots of the PCA performed on SP-irrigated rice. In the loading plot (Figure 5.2f), positive PC1 values mostly describe As(III) and other species, while the negative values for PC2 mainly represent the total As (positive values for PC1) and As(V) (negative values for PC1). Here, As(III) and As(V) are in inverse correlation. The negative PC1 values frequently shown for many genotypes in the score plot (Figure 5.2e) reflect the abundance of As(III), which is below the LoD for seventeen out of twenty-six genotypes. As(V) is the main species in SP irrigated rice, and its concentration increases when PC2 decreases. Therefore, *Carnise*, *Salvo,* and *Galileo* exhibit the lowest amount of total As, which is always in the As(V) chemical form. Contrary to that observed with CF and SA irrigation, *Apollo* is now the genotype with the highest amount of total As (18 ± 4 μg kg^{-1}). This points out that the effect of each irrigation method in this study is specific to each genotype. Data for each genotype are reported in Table E4.

In addition to the genotype effect, also the influence of the rice subspecies on As speciation was investigated. To assess the differences among Japonica and Indica subspecies, a MANOVA analysis was performed. The results are reported in Table 5.2.

Table 5.2. MANOVA results for the analysis of the influence of the As speciation between Japonica and Indica subspecies at varying the irrigation method.

Irrigation method	Hoteling's trace value	F	Hypothesis df	Residual df	p
Continuous flooding (CF)	0.321	1.684	4	21	0.191
Periodical saturation (SA)	0.392	4.507	2	23	0.022
Sprinkler (SP)	0.031	0.230	3	22	0.875

df = degree of freedom

Data presented in Table 5.2 show that the subspecies do not influence the speciation of As in rice depending on the nature of the irrigation method. This agrees with other data previously reported (Spanu et al., 2012, 2016; Spanu, Valente, et al., 2020).

As a concluding remark, the speciation of As in rice grains is highly dependent on the nature of the irrigation method, significantly depends upon the nature of the rice genotype, while it is independent by the nature of the subspecies Indica or Japonica.

5.4. CONCLUSIONS

Twenty-six rice genotypes belonging to the subspecies Indica or Japonica were cultivated in the same open field under the same agronomic conditions, but using three different irrigation methods (i.e., CF, SA and SP, respectively). The data obtained have demonstrated that the nature of the irrigation method has a great influence on the As speciation of rice grain. While As(III) and DMA dominate the CF rice roughly in equal amounts, the adoption of intermittent methods of irrigation consistently reduces the total amount of As, compared to what found in rice CF (from −65% (SA) to −96% (SP)), leading also the DMA concentration below its LoD. As(III) is the only species quantified in SA rice, while As(V) is the one largely prevalent in SP rice. The PCA performed on speciation data clearly differentiates the three irrigation methods. Within each of them, the differences among the rice genotypes are evident. The effect on As speciation in grains attributed to the irrigation method clearly prevails over that attributable to the nature of rice genotype. In addition, belonging to the subspecies Indica or Japonica does not cause any statistically significant difference in the speciation of As in rice by varying the irrigation method. The enhanced level of food security that can be achieved by irrigating rice with SP rather than CF is coupled with other significant benefits. Among others, the huge water savings (up to 70% of the amount necessary for CF) and the absence of any change in soil composition or any genetic engineering approach are worth mentioning. These features make this approach highly eco-friendly. Since there is interest in explaining changes in the translocation mechanisms of the As chemical species as a function of the irrigation methods, speciation studies in different parts of the rice plant will be planned as a key objective of forthcoming research.

5. REFERENCES

Alvarenga, P., Fernández-Rodríguez, D., Abades, D. P., Rato-Nunes, J. M., Albarrán, Á., & López-Piñeiro, A. (2022). Combined use of olive mill waste compost and sprinkler irrigation to decrease the risk of As and Cd accumulation in rice grain. *Science of the Total Environment*, *835*. Scopus. https://doi.org/10.1016/j.scitotenv.2022.155488

Arao, T., Kawasaki, A., Baba, K., Mori, S., & Matsumoto, S. (2009). Effects of Water Management on Cadmium and Arsenic Accumulation and Dimethylarsinic Acid Concentrations in Japanese Rice. *Environmental Science & Technology*, *43*(24), 9361–9367. https://doi.org/10.1021/es9022738

Awasthi, S., Chauhan, R., Srivastava, S., & Tripathi, R. D. (2017). The Journey of Arsenic from Soil to Grain in Rice. *Frontiers in Plant Science*, *8*, 1007. https://doi.org/10.3389/fpls.2017.01007

Bakhat, H. F., Zia, Z., Fahad, S., Abbas, S., Hammad, H. M., Shahzad, A. N., Abbas, F., Alharby, H., & Shahid, M. (2017). Arsenic uptake, accumulation and toxicity in rice plants: Possible remedies for its detoxification: A review. *Environmental Science and Pollution Research*, *24*(10), 9142–9158. Scopus. https://doi.org/10.1007/s11356-017-8462-2

Barnet, L. S., Pozebon, D., Dressler, V. L., & Cioato, D. (2021). Method validation for As speciation in rice using LC-ICP-MS and the inorganic arsenic limit for Brazilian rice. *Journal of Food Composition and Analysis*, *99*. Scopus. https://doi.org/10.1016/j.jfca.2021.103849

Batista, B. L., Souza, J. M. O., De Souza, S. S., & Barbosa, F. (2011). Speciation of arsenic in rice and estimation of daily intake of different arsenic species by Brazilians through rice consumption. *Journal of Hazardous Materials*, *191*(1–3), 342–348. https://doi.org/10.1016/j.jhazmat.2011.04.087

Carey, A.-M., Scheckel, K. G., Lombi, E., Newville, M., Choi, Y., Norton, G. J., Charnock, J. M., Feldmann, J., Price, A. H., & Meharg, A. A. (2010). Grain unloading of arsenic species in rice. *Plant Physiology*, *152*(1), 309–319. Scopus. https://doi.org/10.1104/pp.109.146126

Codex Alimentarius Commission, 37th Session. (2014). *Codex Alimentarius Commission, 37Th Session*, 14–18. Scopus.

Consultative Group for International Agricultural Research. (n.d.). *Research Program on Rice. Ricepedia.* https://ricepedia.org.

Duxbury, J. M., & Panaullah, G. (2007). Remediation of Arsenic for Agriculture Sustainability, Food Security and Health in Bangladesh. *Remediation of Arsenic for Agriculture Sustainability, Food Security and Health in Bangladesh*. Scopus.

European Community. (2015). *Regulation 2015/1006 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of inorganic arsenic in foodstuffs*. http://www.eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:JOL_2015_161_R_0006

Fangmin, C., Ningchun, Z., Haiming, X., Yi, L., Wenfang, Z., Zhiwei, Z., & Mingxue, C. (2006). Cadmium and lead contamination in japonica rice grains and its variation among the different locations in southeast China. *Science of the Total Environment*, *359*(1–3), 156–166. Scopus. https://doi.org/10.1016/j.scitotenv.2005.05.005

Food Agriculture Organization (FAO). (2014). *World reference base for soil resources*. https://www.fao.org/3/i3794en/I3794en.pdf

Food and Drug Administration (FDA). (2014). *Codex Alimentarius Commission, 37th Session*. http://www.fao.org/tempref/codex/Reports/Reports_2014/REP14_CFe.pdf

Food and Drug Administration (FDA). (2019). *FAOStat Data*. http://www.fao.org/faostat/en/#data/QC

Heitkemper, D. T., Vela, N. P., Stewart, K. R., & Westphal, C. S. (2001). Determination of total and speciated arsenic in rice by ion chromatography and inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry*, *16*(4), 299–306. Scopus. https://doi.org/10.1039/b007241i

Hu, P., Ouyang, Y., Wu, L., Shen, L., Luo, Y., & Christie, P. (2015). Effects of water management on arsenic and cadmium speciation and accumulation in an upland rice cultivar. *Journal of Environmental Sciences (China)*, *27*(C), 225–231. Scopus. https://doi.org/10.1016/j.jes.2014.05.048

Huang, J.-H., Fecher, P., Ilgen, G., Hu, K.-N., & Yang, J. (2012). Speciation of arsenite and arsenate in rice grain—Verification of nitric acid based extraction method and mass sample survey. *Food Chemistry*, *130*(2), 453–459. Scopus. https://doi.org/10.1016/j.foodchem.2011.07.059

Huang, J.-H., Ilgen, G., & Fecher, P. (2010). Quantitative chemical extraction for arsenic speciation in rice grains. *Journal of Analytical Atomic Spectrometry*, *25*(6), 800. https://doi.org/10.1039/c002306j

Huang, J.-H., & Matzner, E. (2006). Dynamics of organic and inorganic arsenic in the solution phase of an acidic fen in Germany. *Geochimica et Cosmochimica Acta*, *70*(8), 2023–2033. https://doi.org/10.1016/j.gca.2006.01.021

Huang, J.-H., & Matzner, E. (2007). Mobile arsenic species in unpolluted and polluted soils. *Science of The Total Environment*, *377*(2–3), 308–318. https://doi.org/10.1016/j.scitotenv.2007.01.059

International Rice Research Institute. (2013). *Rice Almanac*. http://books.irri.org/9789712203008_content.pdf

International Year of Rice, Economics and the International Year of Rice. (2004). *International Year of Rice, Economics and the International Year of Rice*. Scopus.

Ishfaq, M., Farooq, M., Zulfiqar, U., Hussain, S., Akbar, N., Nawaz, A., & Anjum, S. A. (2020). Alternate wetting and drying: A water-saving and ecofriendly rice production system. *Agricultural Water Management*, *241*. Scopus. https://doi.org/10.1016/j.agwat.2020.106363

Islam, M. R., . M. J., & . S. I. (2004). Assessment of Arsenic in the Water-Soil-Plant Systems in Gangetic Floodplains of Bangladesh. *Asian Journal of Plant Sciences*, *3*(4), 489–493. https://doi.org/10.3923/ajps.2004.489.493

Khanam, R., Kumar, A., Nayak, A. K., Shahid, Md., Tripathi, R., Vijayakumar, S., Bhaduri, D., Kumar, U., Mohanty, S., Panneerselvam, P., Chatterjee, D., Satapathy, B. S., & Pathak, H. (2020). Metal(loid)s (As, Hg, Se, Pb and Cd) in paddy soil: Bioavailability and potential risk to human health. *Science of The Total Environment*, *699*, 134330. https://doi.org/10.1016/j.scitotenv.2019.134330

Korte, N. E., & Fernando, Q. (1991). A review of arsenic (III) in groundwater. *Critical Reviews in Environmental Control*, *21*(1), 1–39. https://doi.org/10.1080/10643389109388408

Langasco, I., Barracu, F., Deroma, M. A., López-Sánchez, J. F., Mara, A., Meloni, P., Pilo, M. I., Estrugo, À. S., Sanna, G., Spano, N., & Spanu, A. (2022). Assessment and validation of ICP-MS and IC-ICP-MS methods for the determination of total, extracted and speciated arsenic. Application to samples from a soil-rice system at varying the irrigation method. *Journal of Environmental Management*, *302*, 114105. https://doi.org/10.1016/j.jenvman.2021.114105

Leardi, R., Melzi, C., & Polotti, G. (2021, September 14). *CAT (Chemometric Agile Tool Software)*. http://gruppochemiometria.it/index.php/software

Li, R. Y., Stroud, J. L., Ma, J. F., Mcgrath, S. P., & Zhao, F. J. (2009). Mitigation of arsenic accumulation in rice with water management and silicon fertilization. *Environmental Science and Technology*, *43*(10), 3778–3783. Scopus. https://doi.org/10.1021/es803643v

Li, R.-Y., Ago, Y., Liu, W.-J., Mitani, N., Feldmann, J., McGrath, S. P., Ma, J. F., & Zhao, F.-J. (2009). The Rice Aquaporin Lsi1 Mediates Uptake of Methylated Arsenic Species. *Plant Physiology*, *150*(4), 2071–2080. https://doi.org/10.1104/pp.109.140350

Liang, F., Li, Y., Zhang, G., Tan, M., Lin, J., Liu, W., Li, Y., & Lu, W. (2010). Total and speciated arsenic levels in rice from China. *Food Additives & Contaminants: Part A*, *27*(6), 810–816. https://doi.org/10.1080/19440041003636661

Llorente-Mirandes, T., Calderón, J., López-Sánchez, J. F., Centrich, F., & Rubio, R. (2012). A fully validated method for the determination of arsenic species in rice and infant cereal products. *Pure and Applied Chemistry*, *84*(2), 225–238. https://doi.org/10.1351/PAC-CON-11-09-30

Lomax, C., Liu, W.-J., Wu, L., Xue, K., Xiong, J., Zhou, J., McGrath, S. P., Meharg, A. A., Miller, A. J., & Zhao, F.-J. (2012). Methylated arsenic species in plants originate from soil microorganisms. *New Phytologist*, *193*(3), 665–672. Scopus. https://doi.org/10.1111/j.1469-8137.2011.03956.x

Ma, J. F., Yamaji, N., Tamai, K., & Mitani, N. (2007). Genotypic Difference in Silicon Uptake and Expression of Silicon Transporter Genes in Rice. *Plant Physiology*, *145*(3), 919–924. https://doi.org/10.1104/pp.107.107599

Mandal, B. K., & Suzuki, K. T. (2002). Arsenic round the world: A review. *Talanta*, *58*(1), 201–235. https://doi.org/10.1016/S0039-9140(02)00268-0

Meharg, A. A., & Rahman, Md. M. (2003). Arsenic Contamination of Bangladesh Paddy Field Soils: Implications for Rice Contribution to Arsenic Consumption. *Environmental Science & Technology*, *37*(2), 229–234. https://doi.org/10.1021/es0259842

Meharg, A. A., Williams, P. N., Adomako, E., Lawgali, Y. Y., Deacon, C., Villada, A., Cambell, R. C. J., Sun, G., Zhu, Y.-G., Feldmann, J., Raab, A., Zhao, F.-J., Islam, R., Hossain, S., & Yanai, J. (2009). Geographical Variation in Total and Inorganic Arsenic Content of Polished (White) Rice. *Environmental Science & Technology*, *43*(5), 1612–1617. https://doi.org/10.1021/es802612a

Moreno-Jiménez, E., Meharg, A. A., Smolders, E., Manzano, R., Becerra, D., Sánchez-Llerena, J., Albarrán, T., & López-Piñero, A. (2014). Sprinkler irrigation of rice fields reduces grain arsenic but enhances cadmium. *Science of the Total Environment*, *485–486*(1), 468–473. Scopus. https://doi.org/10.1016/j.scitotenv.2014.03.106

Narukawa, T., & Chiba, K. (2010). Heat-Assisted Aqueous Extraction of Rice Flour for Arsenic Speciation Analysis. *Journal of Agricultural and Food Chemistry*, *58*(14), 8183–8188. https://doi.org/10.1021/jf101317n

Nickson, R., McArthur, J., Burgess, W., Ahmed, K. M., Ravenscroft, P., & Rahmanñ, M. (1998). Arsenic poisoning of Bangladesh groundwater. *Nature*, *395*(6700), 338–338. https://doi.org/10.1038/26387

Raab, A., Williams, P. N., Meharg, A., & Feldmann, J. (2007). Uptake and translocation of inorganic and methylated arsenic species by plants. *Environmental Chemistry*, *4*(3), 197–203. Scopus. https://doi.org/10.1071/EN06079

Rumble, J. R. (Ed.). (2021). *CRC handbook of chemistry and physics* (102nd edition 2021-2022). CRC Press.

Sarkar, S., Basu, B., Kundu, C. K., & Patra, P. K. (2012). Deficit irrigation: An option to mitigate arsenic load of rice grain in West Bengal, India. *Agriculture, Ecosystems and Environment*, *146*(1), 147–152. Scopus. https://doi.org/10.1016/j.agee.2011.10.008

Schoof, R. A., Yost, L. J., Eickhoff, J., Crecelius, E. A., Cragin, D. W., Meacher, D. M., & Menzel, D. B. (1999). A market basket survey of inorganic arsenic in food. *Food and Chemical Toxicology*, *37*(8), 839–846. Scopus. https://doi.org/10.1016/S0278-6915(99)00073-3

Servizio Informativo Agrario Nazionale. (2018). *Registro della varietà di specie agrarie*. https://www.sian.it/downloadpub/zfadlx010?id=8044

Shankar, S., Shanker, U., & Shikha. (2014). Arsenic Contamination of Groundwater: A Review of Sources, Prevalence, Health Risks, and Strategies for Mitigation. *The Scientific World Journal*, *2014*, 1–18. https://doi.org/10.1155/2014/304524

Signes-Pastor, A. J., Carey, M., Carbonell-Barrachina, A. A., Moreno-Jiménez, E., Green, A. J., & Meharg, A. A. (2016). Geographical variation in inorganic arsenic in paddy field samples and commercial rice from the Iberian Peninsula. *Food Chemistry*, *202*, 356–363. Scopus. https://doi.org/10.1016/j.foodchem.2016.01.117

Šlejkovec, Z., Gorše, L., Grobler, A., Jagodic, M., & Falnoga, I. (2021). Arsenic speciation and elemental composition of rice samples from the Slovenian market. *Food Chemistry*, *342*. Scopus. https://doi.org/10.1016/j.foodchem.2020.128348

Somenahally, A. C., Hollister, E. B., Yan, W., Gentry, T. J., & Loeppert, R. H. (2011a). Water Management Impacts on Arsenic Speciation and Iron-Reducing Bacteria in Contrasting Rice-Rhizosphere Compartments. *Environmental Science & Technology*, *45*(19), 8328–8335. https://doi.org/10.1021/es2012403

Somenahally, A. C., Hollister, E. B., Yan, W., Gentry, T. J., & Loeppert, R. H. (2011b). Water management impacts on arsenic speciation and iron-reducing bacteria in contrasting rice-rhizosphere compartments. *Environmental Science and Technology*, *45*(19), 8328–8335. Scopus. https://doi.org/10.1021/es2012403

Spanu, A., Daga, L., Orlandoni, A. M., & Sanna, G. (2012). The role of irrigation techniques in arsenic bioaccumulation in rice (Oryza sativa L.). *Environmental Science and Technology*, *46*(15), 8333–8340. Scopus. https://doi.org/10.1021/es300636d

Spanu, A., Langasco, I., Serra, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2021). Sprinkler irrigation in the production of safe rice by soils heavily polluted by arsenic and cadmium. *Chemosphere*, *277*, 130351. https://doi.org/10.1016/j.chemosphere.2021.130351

Spanu, A., Langasco, I., Valente, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2020). Tuning of the Amount of Se in Rice (Oryza sativa) Grain by Varying the Nature of the Irrigation Method: Development of an ICP-MS Analytical Protocol, Validation and Application to 26 Different Rice Genotypes. *Molecules*, *25*(8), 1861. https://doi.org/10.3390/molecules25081861

Spanu, A., Murtas, A., & Ballone, F. (2009a). Swater use and crop coefficients in sprinkler irrigated rice. *Italian Journal of Agronomy*, *4*(2), 47–58. Scopus. https://doi.org/10.4081/ija.2009.2.47

Spanu, A., Murtas, A., & Ballone, F. (2009b). Water Use and Crop Coefficients in Sprinkler Irrigated Rice. *Italian Journal of Agronomy*, *4*(2), 47. https://doi.org/10.4081/ija.2009.2.47

Spanu, A., Murtas, A., Ledda, L., & Ballone, F. (2004). Innovative agronomic techniques for rice cultivation. *Conference Proceedings of Challenger and Opportunities for Sustainable Rice-Based Production Systems*, 207–216. Scopus.

Spanu, A., Valente, M., Langasco, I., Barracu, F., Orlandoni, A. M., & Sanna, G. (2018a). Sprinkler irrigation is effective in reducing cadmium concentration in rice (Oryza sativa L.) grain: A new twist on an old tale? *Science of the Total Environment*, *628–629*, 1567–1581. Scopus. https://doi.org/10.1016/j.scitotenv.2018.02.157

Spanu, A., Valente, M., Langasco, I., Barracu, F., Orlandoni, A. M., & Sanna, G. (2018b). Sprinkler irrigation is effective in reducing cadmium concentration in rice (Oryza sativa L.) grain: A new twist on an old tale? *Science of The Total Environment*, *628–629*, 1567–1581. https://doi.org/10.1016/j.scitotenv.2018.02.157

Spanu, A., Valente, M., Langasco, I., Leardi, R., Orlandoni, A. M., Ciulu, M., Deroma, M. A., Spano, N., Barracu, F., Pilo, M. I., & Sanna, G. (2020). Effect of the irrigation method and genotype on the bioaccumulation of toxic and trace elements in rice. *Science of The Total Environment*, *748*, 142484. https://doi.org/10.1016/j.scitotenv.2020.142484

Spanu, A., Valente, M., Leardi, R., Ciulu, M., Orlandoni, A. M., Barracu, F., & Sanna, G. (2016). Influence of the nature of the irrigation methods on the phenomena of bioaccumulation of eight health-threatening elements in rice grains. *IMEKOFOODS Conf.: Promot. Object. Meas. Food Qual. Saf.*, 262–269. Scopus. https://www.scopus.com/inward/record.uri?eid=2-s2.0-85041358584&partnerID=40&md5=a757c75a39e153c1beede8a8340052d3

Suriyagoda, L. D. B., Dittert, K., & Lambers, H. (2018). Arsenic in Rice Soils and Potential Agronomic Mitigation Strategies to Reduce Arsenic Bioavailability: A Review. *Pedosphere*, *28*(3), 363–382. https://doi.org/10.1016/S1002-0160(18)60026-8

Takamatsu, T., Aoki, H., & Yoshida, T. (1982). Determination of arsenate, arsenite, monomethylarsonate, and dimethylarsinate in soil polluted with arsenic. *Soil Science*, *133*(4), 239–246. Scopus. https://doi.org/10.1097/00010694-198204000-00007

Tang, Z., Fan, F., Deng, S., & Wang, D. (2020). Mercury in rice paddy fields and how does some agricultural activities affect the translocation and transformation of mercury—A critical review. *Ecotoxicology and Environmental Safety*, *202*. Scopus. https://doi.org/10.1016/j.ecoenv.2020.110950

Tenni, D., Martin, M., Barberis, E., Beone, G. M., Miniotti, E., Sodano, M., Zanzo, E., Fontanella, M. C., & Romani, M. (2017). Total As and As Speciation in Italian Rice as Related to Producing Areas and Paddy Soils Properties. *Journal of Agricultural and Food Chemistry*, *65*(17), 3443–3452. Scopus. https://doi.org/10.1021/acs.jafc.7b00694

Williams, P. N., Price, A. H., Raab, A., Hossain, S. A., Feldmann, J., & Meharg, A. A. (2005). Variation in Arsenic Speciation and Concentration in Paddy Rice Related to Dietary Exposure. *Environmental Science & Technology*, *39*(15), 5531–5540. https://doi.org/10.1021/es0502324

Williams, P. N., Villada, A., Deacon, C., Raab, A., Figuerola, J., Green, A. J., Feldmann, J., & Meharg, A. A. (2007). Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain levels compared to wheat and barley. *Environmental Science and Technology*, *41*(19), 6854–6859. Scopus. https://doi.org/10.1021/es070627i

Xu, X. Y., McGrath, S. P., Meharg, A. A., & Zhao, F. J. (2008). Growing Rice Aerobically Markedly Decreases Arsenic Accumulation. *Environmental Science & Technology*, *42*(15), 5574–5579. https://doi.org/10.1021/es800324u

Yang, J., Huang, D., Duan, H., Tan, G., & Zhang, J. (2009). Alternate wetting and moderate soil drying increases grain yield and reduces cadmium accumulation in rice grains. *Journal of the Science of Food and Agriculture*, *89*(10), 1728–1736. https://doi.org/10.1002/jsfa.3648

Yang, J., & Zhang, J. (2010). Crop management techniques to enhance harvest index in rice. *Journal of Experimental Botany*, *61*(12), 3177–3189. Scopus. https://doi.org/10.1093/jxb/erq112

Zavala, Y. J., & Duxbury, J. M. (2008). Arsenic in Rice: I. Estimating Normal Levels of Total Arsenic in Rice Grain. *Environmental Science & Technology*, *42*(10), 3856–3860. https://doi.org/10.1021/es702747y

Zavala, Y. J., Gerads, R., Gürleyük, H., & Duxbury, J. M. (2008). Arsenic in Rice: II. Arsenic Speciation in USA Grain and Implications for Human Health. *Environmental Science & Technology*, *42*(10), 3861–3866. https://doi.org/10.1021/es702748q

CONCLUSIONS AND PERSPECTIVES

The objective of this thesis is to use elemental metabolomics to authenticate and add value to food products. Specific results have been achieved in developing analytical methods and using elemental fingerprints of honey, dairy sheep products, and rice for quality assessment:

1. **Sample preparation:** Inductively coupled plasma mass spectrometry (ICP-MS) was used for elemental analysis and microwave acid digestion was performed using a single reaction chamber (SRC) technology for sample preparation. The use of SRC technology has enabled the development of efficient methods that meet the requirements of blank and green analytical chemistry. The honey digestion method was optimized using an experimental design, resulting in a significantly lower amount of oxidizing agents than previously reported in the literature. The optimized oxidizing mixture contained less nitric acid than hydrogen peroxide, leading to less acidic digested solutions that require less dilution and resulting in higher sensitivity of the analytical method. However, it is important to note that the use of mixtures with high amounts of hydrogen peroxide should not be considered an absolute advantage. While hydrogen peroxide is a considered a green reagent, it is more expensive and difficult to purify than nitric acid. The amount of hydrogen peroxide should be carefully regulated to ensure the regeneration of nitric acid in the solution, taking into account the overall oxidizing power of the mixture. For these reasons, the future aim is to use a more comprehensive experimental design that considers factors and responses beyond those in the preliminary stage. In addition, this approach will be extended to more food matrices working, where possible, on certified reference materials. The ultimate goal is to develop a library of green and blank SRC methods for food mineralization that will be propaedeutic for elemental analysis.

2. **Elemental analysis:** Each method developed for every matrix was fully validated. Compared to more complex matrices, such as soils or alloys, ICP-MS analysis of food is considered relatively simple. However, to ensure the accurate analysis of all elements, for each matrix it is necessary to tune all instrumental parameters for maximize sensitivity and remove any possible interferences. To minimize the matrix effect resulting from differences in composition between samples and standards, it is important to reproduce the relative abundance of elements in the calibration standards. Although these well-known practices are essential for the development and validation of analytical methods, literature shows that they are often overlooked. The HPLC-ICP-MS method for analyzing speciated arsenic was developed based on the aforementioned considerations. Sample preparation involved two phases: microwave extraction for the analysis of inorganic species (As(III), As(V)) and organic species (DMA, MMA), and microwave digestion for total arsenic analysis. Extraction is the most delicate step because it requires meantaining equilibrium between the species. The excellent validation parameters achieved for this method ensure that all criteria have been reached.

3. **Honey**: This study examinated the elemental fingerprints of multifloral and unifloral honeys from Sardinia (Italy) and Spain to determine the influence of their geographical and botanical origins. Elemental analysis was used to evalate the nutritional and toxic element contents. The samples analyzed showed low levels of toxic elements and relatively high levels of elements of nutritional interest, such as potassium, magnesium, zinc, copper, and manganese. However, honey cannot be considered a primary source of minerals, and its daily consumption alone is insufficient to meet daily requirements. The results of this study suggest that the elemental fingerprint is primarily influenced by geographical rather than botanical origin. Elemental markers include both high-concentration and ultra-trace elements, such as lanthanides. Because of the determination of those elements requires incineration as a sample preparation technique, two separate analytical runs are necessary to fully analyze the most important elements for obtaining the elemental fingerprint of honeys. This approach is highly effective in differentiating between honey samples based on their geographical origin rather than their botanical origin. This is also true when the samples come from neighboring regions, and accuracy increases when discriminating honey samples of different botanical origins. Other techniques, such as vibrational spectroscopy or chromatography, can accurately authenticate honey samples by allowing the determination of organic markers. In the future perspectives, the approach will be expanded to include other factors such as anthropogenic impact and adulteration. Additionally, it is important to investigate other botanical and geographical origins. Finally, the investigation will consider expanding to include honeys from other botanical or geographical origins.

4. **Dairy sheep products**: The analysis of sheep‘s dairy products has allowed to the achievement of various diversification and protection strategies for two cheeses with Protected Designation of Origin (PDO) produced in Sardinia, (Italy). The Pecorino Romano PDO and Pecorino Sardo PDO were taken into account for this purpose. Both cheeses were made from sheep milk using different cheesemaking processes. This results in a different fractionation of elements in the curd, and therefore, a different elemental composition in the final products. Both cheeses use Sardinian sea salt for salting. However, the Pecorino Romano PDO has a higher sodium chloride content than the Pecorino Sardo PDO due to different salting methods used. Therefore, coagulation is the most significant step in cheesemaking. Seasonal variations in the concentration of elements in milk are reflected in cheese composition. These variations are correlated with lactation period. The results suggest that the nutritional value of cheeses varies depending on the season and the amount of oligoelements present. Therefore, it may be possible to update the nutritional labels of these products to reflect the seasonal changes of specific oligoelements. Further investigation will be conducted on the parameters that may affect the fractionation of elements during cheesemaking. These results indicate the potential application of elemental metabolomics for the authenticating of counterfeit or adulterated products. Moreover, understanding the distribution of toxic and nutritional elements could lead to the regulation of the maximum levels of the former in milk. Additionally, the recovery of some cheesemaking byproducts as mineral additives can be considered.

5. **Rice**: This study evaluated the impact of various irrigation methods on arsenic speciation. The total concentration of arsenic was significantly reduced by 50-70% and 90-100% with the use of intermittent irrigation techniques, namely periodic saturation and sprinkler irrigation, respectively. Additionally, these methods influenced the distribution of As chemical forms in both soil and rice grains. In the soil, only the inorganic species As(III) and As(V) were detected. Rice produced under continuous flooding contains both As(III) and DMA species in roughly equal amounts. The plant efficiently translocates As(III) and synthesizes organic DMA species. However, when using periodic saturation, the plant only translocates As(III), whereas the DMA species is virtually absent. In contrast, in sprinkler irrigation, As(V) is the predominant species, As(III) is present in low amounts, and organic As species are not detected. The results indicate that while both intermittent techniques reduce total arsenic, only sprinkler irrigation minimizes As(III), the most toxic chemical species of arsenic. Additionally, the irrigation methods have a greater effect on the bioaccumulation of total and speciated arsenic in rice grain compared to the rice genotypes and subspecies. In conclusion, sprinkler irrigation is the most effective technique for reducing the toxicity caused by As species in rice grain. It also saves large amounts of water without any significant loss in yield, making it an environmentally friendly method for producing high-quality and safe rice. The data obtained suggest that irrigation techniques could be an interesting instrument for modulating the translocation of non-toxic trace elements in rice. For authentication purposes, further studies will evaluate the translocation of other elements, such as lanthanides. Additionally, to comprehensively assess the eco-compatibility of the irrigation method, greenhouse gas emissions will be determined through continuous flooding, saturation, and sprinkler irrigations.

The elemental metabolomics approach has been used in all studies to evaluate the factors that can influence the elemental fingerprint and authenticate food, ensuring its quality and safety. Future research should aim to improve analytical methods, from sample preparation to elemental analysis. Although ICP-MS is a well-established technique, its routine use for quality control is limited by its high cost and the need for advanced skills. Finally, further studies will be conducted to evaluate all the factors that influence the flow of elements between the environment and organisms, as well as the impact of human activities on element translocation. These findings are essential for uniformly regulating the content of toxicological elements and establishing minimum quality criteria for nutritional elements. When combined with other disciplines of foodomics, this approach can significantly contribute to the advancement of the current food industry, which aims for sustainability and quality.

APPENDIX

APPENDIX A

Figure A1. Scree plot of the PCA performed of the training set (85 samples, 18 variables).

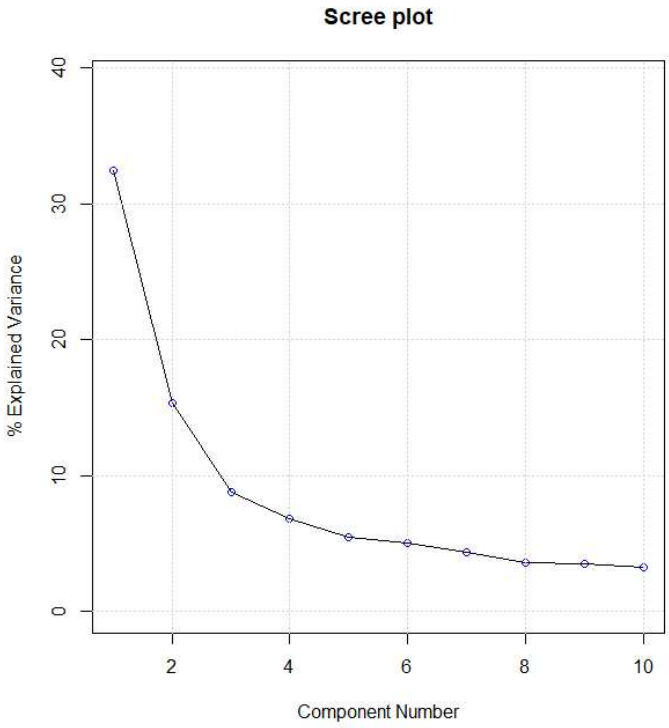


Figure A2. Influence plot of the PCA performed of the training set (85 samples, 18 variables).

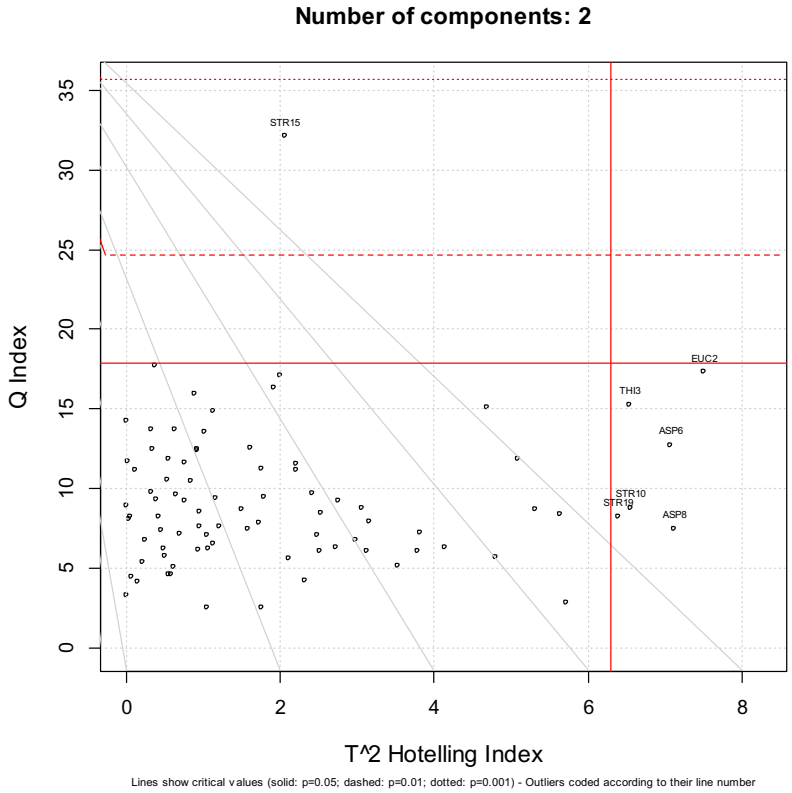


Figure A3. Permutation test of the LDA in calibration.

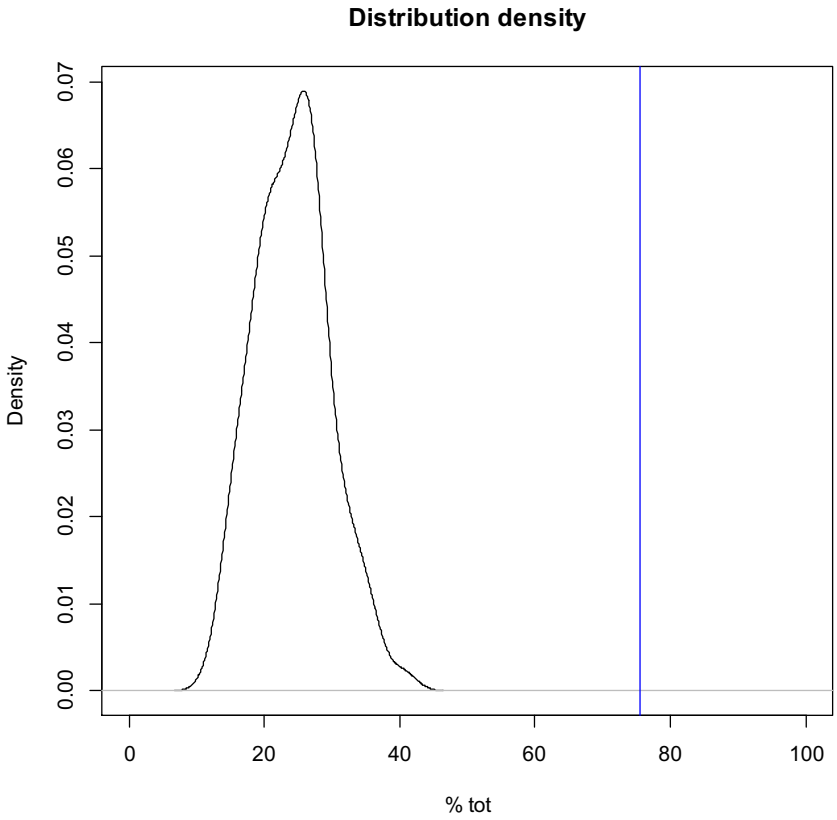


Table A1. Correlation's coefficients of the variables, before (blue) and after (red) the logarithmic transformation.

	As	*Ba*	*Bi*	*Cd*	*Co*	*Cr*	*Cu*	*Fe*	*Li*	*Mn*	*Mo*	*Ni*	*Pb*	*Sn*	*Sr*	*Tl*	*V*	*Zn*
As	1.00	0.08	-0.05	0.26	0.02	-0.04	0.10	0.40	-0.04	0.34	0.29	-0.06	-0.01	0.01	0.29	0.08	0.40	0.16
Ba	0.22	1.00	-0.03	-0.05	-0.02	-0.04	-0.10	0.06	-0.04	0.05	0.07	-0.11	-0.09	0.11	0.37	0.13	0.10	-0.17
Bi	0.19	0.09	1.00	-0.03	0.05	-0.01	0.00	0.00	0.00	-0.01	0.01	-0.01	-0.04	-0.04	0.06	-0.04	-0.02	-0.01
Cd	0.44	-0.08	0.19	1.00	0.13	-0.01	0.30	0.33	-0.02	0.24	0.25	0.06	0.03	-0.04	0.13	0.37	0.33	0.56
Co	0.36	0.09	0.33	0.63	1.00	0.22	0.35	0.27	-0.03	0.27	0.19	0.19	-0.03	-0.08	0.18	0.25	0.34	0.30
Cr	0.02	0.03	0.19	0.18	0.24	1.00	0.00	0.17	-0.01	0.13	0.18	0.16	-0.02	0.04	0.12	0.02	0.17	0.04
Cu	0.23	0.01	0.21	0.52	0.68	0.01	1.00	0.37	0.05	0.21	0.23	0.75	-0.05	-0.05	0.04	0.25	0.22	0.62
Fe	0.49	0.32	0.43	0.48	0.62	0.24	0.60	1.00	0.07	0.72	0.51	0.15	-0.08	0.01	0.56	0.34	0.88	0.28
Li	0.24	0.39	0.23	0.13	0.13	0.13	0.16	0.49	1.00	-0.03	0.10	-0.01	-0.01	-0.08	0.05	0.01	0.02	-0.02
Mn	0.43	0.35	0.26	0.35	0.52	0.14	0.49	0.75	0.51	1.00	0.28	0.03	-0.04	-0.15	0.59	0.15	0.78	0.09
Mo	0.30	0.23	0.22	0.36	0.40	0.23	0.42	0.56	0.21	0.39	1.00	0.04	-0.07	0.11	0.28	0.19	0.49	0.25
Ni	0.05	-0.13	0.22	0.35	0.52	0.23	0.59	0.37	0.00	0.22	0.25	1.00	0.00	-0.01	-0.11	0.12	0.03	0.35
Pb	0.23	0.10	-0.06	0.23	0.14	0.01	0.16	0.20	0.02	0.14	0.10	0.17	1.00	0.23	-0.11	0.11	-0.03	0.05
Sn	0.14	0.12	-0.01	-0.01	0.00	-0.05	0.02	0.06	-0.11	-0.10	0.14	0.12	0.25	1.00	0.04	0.07	-0.09	0.11
Sr	0.35	0.72	0.28	0.11	0.26	0.17	0.15	0.56	0.61	0.55	0.29	-0.06	0.13	-0.03	1.00	0.08	0.59	-0.01
Tl	0.21	-0.01	0.19	0.44	0.47	0.13	0.27	0.23	0.01	0.08	0.13	0.23	0.23	0.07	0.03	1.00	0.37	0.33
V	0.47	0.29	0.31	0.47	0.60	0.30	0.47	0.84	0.48	0.76	0.56	0.19	0.21	-0.03	0.54	0.20	1.00	0.18
Zn	0.24	-0.17	0.25	0.60	0.62	0.16	0.61	0.45	0.12	0.32	0.34	0.46	0.09	0.07	0.06	0.41	0.41	1.00

Table A2. Instrumental parameters and elemental settings used for the ICP-MS determination of 23 trace elements in unifloral honeys.

ICP-MS NexION 300X Perkin Elmer settings			
RF power generator	1300	KED mode cell entrance	-8.0
Ar plasma flow (dm^3	18.0	KED mode cell exit voltage (V)	-25.0
Ar auxiliary flow (dm^3	1.20	Resolution (Da)	0.7
Ar nebulizer flow (dm^3	0.91	Scan mode	Peak hopping
Nebulizer	Meinhardt®, glass	Detector mode	Dual
Spray chamber	Cyclonic, glass	Dwell time (ms)	50
Skimmer and	Nickel	Number of points per peak	3
Sampling depth (mm)	0	Acquisition time (s)	6
Deflector voltage (V)	-8.00	Acquisition dead time (ns)	35
Analog stage voltage	-1750	KED gas	Helium 99.999%
Pulse stage voltage	+1350	Masses of optimization	^{7}Li, ^{115}In and ^{205}Tl

Quantification ion	Interfering ions	Analysing mode	He flow rate	Correction equation
$^{107}Ag^{+}$ (51.84)	$^{91}Y^{16}O^{+}$, $^{91}Zr^{16}O^{+}$	Normal	-	
$^{75}As^{+}$ (100)	$^{40}Ar^{35}Cl^{+}$; $^{59}Co^{16}O^{+}$; $^{39}K^{36}Ar^{+}$; $^{63}Cu^{12}C^{+}$;	KED	3.0	
$^{138}Ba^{+}$ (71.7)	$^{40}Ar_2^{58}Ni^{+}$; $^{138}La^{+}$; $^{122}Sn^{16}O^{+}$;	KED	4.0	-0.000901x^{139}La -
$^{9}Be^{+}$ (100)	none	Normal	-	
$^{209}Bi^{+}$ (100)	none	Normal	-	
$^{111}Cd^{+}$ (12.80)	$^{95}Mo^{16}O^{+}$; $^{97}Mo^{14}N^{+}$; $^{79}Br^{16}O_2^{+}$;	KED	4.0	
$^{59}Co^{+}$ (100)	$^{24}Mg^{35}Cl^{+}$; $^{40}Ar^{18}O^{1}H^{+}$; $^{27}Al^{16}O_2^{+}$;	KED	3.5	
$^{52}Cr^{+}$ (83.79)	$^{40}Ar^{12}C^{+}$; $^{36}Ar^{16}O^{+}$; $^{1}H^{35}Cl^{16}O^{+}$;	KED	3.0	
$^{63}Cu^{+}$ (69.17)	$^{40}Ar^{23}Na^{+}$; $^{31}P^{16}O_2^{+}$; $^{47}Ti^{16}O^{+}$; $^{28}Si^{35}Cl^{+}$;	KED	4.0	
$^{57}Fe^{+}$ (2.12)	$^{40}Ar^{16}O^{1}H^{+}$; $^{40}Ca^{16}O^{1}H^{+}$; $^{40}K^{16}O^{1}H^{+}$	KED	3.0	
$^{7}Li^{+}$ (92.50)	none	Normal	-	
$^{202}Hg^{+}$ (22.86)	$^{186}W^{16}O^{+}$	Normal	-	
$^{55}Mn^{+}$ (100)	$^{40}Ar^{14}N^{1}H^{+}$; $^{37}Cl^{18}O^{+}$; $^{39}K^{16}O^{+}$	KED	3.0	
$^{98}Mo^{+}$ (24.13)	$^{98}Ru^{+}$; $^{81}Br^{17}O^{+}$; $^{40}K_2^{18}O^{+}$; $^{58}Ni^{40}Ar^{+}$;	Normal	-	-0.10961 x ^{101}Ru
$^{60}Ni^{+}$ (26.22)	$^{44}Ca^{16}O^{+}$; $^{43}Ca^{16}O^{1}H^{+}$; $^{23}Na^{37}Cl^{+}$;	KED	3.5	
$^{208}Pb^{+}$ (52.40)	none	Normal	-	
$^{121}Sb^{+}$ (57.21)	$^{107}Ag^{14}N^{+}$; $^{109}Ag^{12}C^{+}$; $^{105}Pd^{16}O^{+}$;	KED	3.5	
$^{120}Sn^{+}$ (32.58)	$^{39}K^{81}Br^{+}$; $^{80}Se^{40}Ar^{+}$; $^{104}Pd^{16}O^{+}$;	KED	3.5	
$^{88}Sr^{+}$ (82.58)	$^{56}FeO_2^{+}$; $^{87}Rb^{1}H^{+}$; $^{48}Ti^{40}Ar^{+}$	KED	3	
$^{130}Te^{+}$ (34.08)	$^{95}MoO_2^{+}$; $^{114}Cd^{16}O^{+}$; $^{90}Zr^{40}Ar^{+}$	KED	4	-0.0094 x ^{137} Ba – 0.1543 x
$^{205}Tl^{+}$ (70.26)	$^{189}Os^{16}O^{+}$	Normal	-	
$^{51}V^{+}$ (99.75)	$^{35}Cl^{16}O^{+}$	KED	3.0	
$^{66}Zn^{+}$ (27.90)	$^{50}Ti^{16}O^{+}$; $^{65}Cu^{1}H^{+}$; $^{26}Mg^{40}Ar^{+}$; $^{31}P^{35}Cl^{+}$;	KED	3.0	

APPENDIX B

Figure B1. Principal Component Analysis (PCA) performed using autoscaling as data pre-treatment. (A) Loading plot; (B) Score plot.

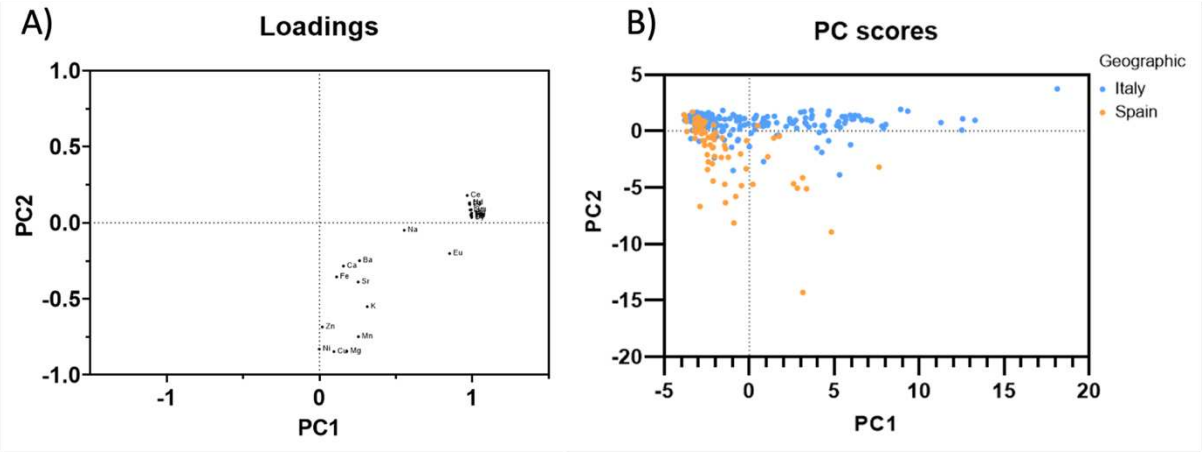


Figure B2. Proportion of variance (PCA using centered log-ratio transformation).

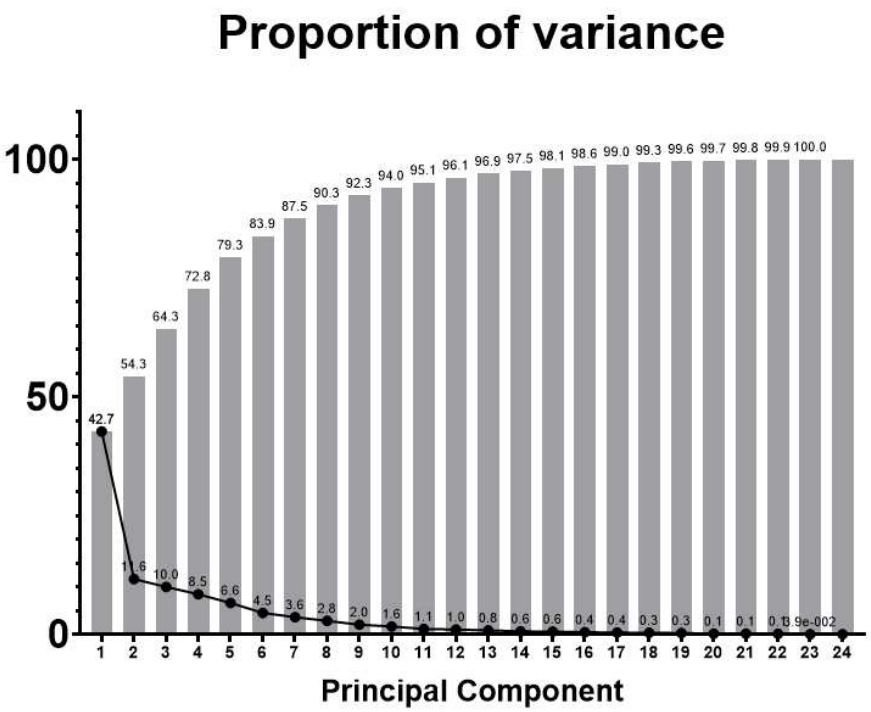


Table B1. Operational conditions of microwave acid digestion and dry ashing for honey elemental analysis.

Microwave acid digestion (ultraWAVE Milestone)			
Step		Time (min)	Temperature (°C)
1st	Heating	25	240
2nd	Holding	10	240
3rd	Cooling	ca. 30	< 40

Dry ashing (Controller P320 Nabertherm)			
Step		Time (min)	Temperature (°C)
1st	Heating	10	105
2nd	Heating	30	150
3rd	Holding	30	150
4th	Heating	30	200
5th	Holding	30	200
6th	Heating	360	600
7th	Holding	360	600

Table B2. Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) instrumental conditions for honey elemental analysis.

ICP-MS NexION 300X Perkin Elmer settings			
RF power generator (W)	1300	KED mode cell entrance voltage (V)	-8.0
Ar plasma flow (dm^3 min^{-1})	18.0	KED mode cell exit voltage (V)	-25.0
Ar auxiliary flow (dm^3 min^{-1})	1.20	Resolution (Da)	0.7
Ar nebulizer flow (dm^3 min^{-1})	0.91	Scan mode	Peak hopping
Nebulizer	Meinhardt®, glass	Detector mode	Dual
Spray chamber	Cyclonic, glass	Dwell time (ms)	50
Skimmer and sampling cones	Nickel	Number of points per peak	3
Sampling depth (mm)	0	Acquisition time (s)	6
Deflector voltage (V)	-8.00	Acquisition dead time (ns)	35
Analog stage voltage (V)	-1750	KED gas	Helium 99.999%
Pulse stage voltage (V)	1350	Masses of optimization	^{7}Li, ^{115}In and ^{205}Tl

Table B3. Elemental settings and validation parameters for the ICP-MS determination of macroelements and lanthanides in honey.

Macroelements

Element	Mode	Calibration Range (mg dm^{-3})	R^2	LoD (mg kg^{-1})	LoQ (mg kg^{-1})	$CV\%_r$	$CV\%_{IP}$
^{23}Na	KED	0.05 - 5	0.9998	0.10	0.30	6	8
^{24}Mg	KED	0.01 – 10	0.9996	0.26	0.80	2	5
^{39}K	KED	0.1 – 100	0.9998	0.70	0.20	2	6
^{43}Ca	KED	0.05 - 50	0.9997	0.16	0.50	2	5

He flow rate = 3.6 cm^3 min^{-1}

Lanthanides

Element	Mode	Calibration Range (μg dm^{-3})	R^2	LoD (μg kg^{-1})	LoQ (μg kg^{-1})	$CV\%_r$	$CV\%_{IP}$
^{139}La	KED	0.001 - 10	0.9999	0.015	0.050	15	21
^{140}Ce	KED	0.001 - 10	0.9998	0.024	0.079	15	18
^{141}Pr	KED	0.001 - 10	0.9999	0.006	0.020	15	18
^{142}Nd	KED	0.001 - 10	0.9993	0.018	0.059	16	19
^{152}Sm	KED	0.001 - 10	0.9997	0.003	0.010	11	16
^{153}Eu	KED	0.001 - 10	0.9998	0.003	0.010	15	16
^{158}Gd	KED	0.001 - 10	0.9999	0.003	0.010	6	15
^{159}Tb	KED	0.001 - 10	0.9996	0.001	0.003	7	13
^{164}Dy	KED	0.001 - 10	0.9999	0.003	0.010	8	12
^{165}Ho	KED	0.001 - 10	0.9999	0.003	0.010	12	16
^{166}Er	KED	0.001 - 10	0.9996	0.003	0.010	8	18
^{169}Tm	KED	0.001 - 10	0.9999	0.001	0.003	3	16
^{174}Yb	KED	0.001 - 10	0.9999	0.001	0.004	10	17
^{175}Lu	KED	0.001 - 10	0.9998	0.001	0.002	5	18

He flow rate = 4.6 cm^3 min^{-1}

Macroelements and lanthanides were determined after microwave acid digestion and dry ashing, respectively.

Detailed information about the instrumental parameters and elemental settings for trace and toxic elements analysis, method assessment, performance, quality control, and validation can be found in Chapter 1.

Table B4. Trueness evaluation for the ICP-MS determination of macroelements and lanthanides by means analysis of certified reference materials (NIST SRM 1515 apple leaves and BCR 668 mussel tissue).

NIST SRM 1515 apple leaves

Element	Certified value (mg kg^{-1})	Experimental value (mg kg^{-1}) (n=3)	Recovery %*
Na	24.4 ± 2.1	23 ± 3	105 ± 15
Mg	2710 ± 120	2600 ± 200	104 ± 7
K	16080 ± 210	15300 ± 900	105 ± 6
Ca	15250 ± 100	15700 ± 900	97 ± 6

BCR 668 mussel tissue

Element	Certified value (μg kg^{-1})	Experimental value (μg kg^{-1}) (n=3)	Recovery %*
La	80 ± 6	75 ± 4	94 ± 8
Ce	89 ± 7	84 ± 9	90 ± 10
Pr	12.3 ± 1.1	11.9 ± 1.5	100 ± 10
Nd	54 ± 4	54 ± 9	100 ± 30
Sm	11.2 ± 0.8	11.9 ± 0.9	110 ± 10
Eu	2.79 ± 0.16	2.6 ± 0.5	90 ± 20
Gd	13.0 ± 0.6	14 ± 1	110 ± 10
Tb	1.62 ± 0.12	1.73 ± 0.08	107 ± 9
Dy	8.9 ± 0.6	9.7 ± 0.3	109 ± 8
Ho	1.8 ± 0.6	1.9 ± 0.8	105 ± 20
Er	4.5 ± 0.5	4.3 ± 0.2	100 ± 10
Tm	0.48 ± 0.08	0.5 ± 0.03	110± 20
Yb	2.8 ± 0.5	2.8 ± 0.2	100 ± 20
Lu	0.389 ± 0.024	0.44 ± 0.04	110 ± 10

* Recoveries has been calculated using unrounded experimental concentrations

Table B5. Concentration of macroelements, trace elements, toxic elements and lanthanides in honeys from Sardinia and Spain (min; mean ± sd; max).

Macroelements(mg kg^{-1})

Elements	SPA ROS (n = 26)	SPA MUL (n = 34)	SPA EUC (n = 13)	ITA THI (n = 39)	ITA STR (n = 31)	ITA ROS (n = 6)	ITA MUL (n = 35)	ITA EUC (n = 30)	ITA ASP (n = 33)
Na	3; 20 ± 10; 35	4; 20 ± 10; 48	13; 40 ± 10; 56	11; 50 ± 20; 99	3; 20 ± 20; 68	6.1; 8 ± 3; 12.9	10; 60 ± 40; 150	20; 110 ± 60; 240	1; 16 ± 8; 37
Mg	2; 12 ± 6; 29	3; 30 ± 20; 85	7; 40 ± 15; 62	1; 9 ± 4; 19	2; 12 ± 8; 32	3.5; 5 ± 1.5; 6.6	4; 12 ± 7; 27	2; 13 ± 5; 23	2; 7 ± 4; 16
K	60; 300 ± 150; 650	< LoD; 600 ± 400; 1700	200; 1000 ± 400; 1600	90; 600 ± 300; 1270	200; 1500 ± 800; 3000	110; 190 ± 90; 330	140; 550 ± 300; 1080	70; 500 ± 200; 880	10; 200 ± 90; 400
Ca	4; 30 ± 10; 51	8; 50 ± 25; 97	11; 80 ± 30; 120	4; 30 ± 10; 59	10; 70 ± 40; 150	11; 20 ± 20; 52	10; 35 ± 15; 70	10; 70 ± 30; 130	2; 14 ± 8; 33

Lanthanides (ng kg^{-1})

Elements	SPA ROS (n = 26)	SPA MUL (n = 34)	SPA EUC (n = 13)	ITA THI (n = 39)	ITA STR (n = 31)	ITA ROS (n = 6)	ITA MUL (n = 35)	ITA EUC (n = 30)	ITA ASP (n = 33)
La	200; 1100 ± 300; 1800	800; 1600 ± 800; 3500	900; 2100 ± 900; 4100	1200; 5000 ± 2000; 9400	800; 300 ± 2000; 7900	360; 360 ± 10; 370	400; 1300 ± 800; 3300	1000; 7000 ± 3000; 14000	200; 1100 ± 500; 2000
Ce	300; 1400 ± 400; 2300	900; 1600 ± 500; 3100	1300; 3000 ± 1500; 6100	2000; 8000 ± 4000; 18000	2000; 5000 ± 3000; 15000	400; 800 ± 200; 900	400; 2000 ± 1000; 4600	1000; 12000 ± 6000; 23000	300; 1400 ± 600; 2700
Pr	100; 300 ± 100; 400	200; 400 ± 200; 1000	200; 600 ± 300; 1200	300; 1300 ± 600; 2600	200; 900 ± 600; 2500	94; 95 ± 1; 97	100; 300 ± 200; 800	200; 1900 ± 900; 3900	100; 300 ± 100; 600
Nd	400; 1000 ± 300; 1700	600; 1500 ± 900; 3700	900; 2500 ± 1000; 5100	1300; 5500 ± 3000; 11400	900; 4000 ± 3000; 10800	300; 500 ± 200; 700	300; 1400 ± 800; 3100	1000; 8000 ± 4000; 15000	300; 1100 ± 500; 2200
Sm	100; 200 ± 100; 300	100; 300 ± 200; 1000	200; 600 ± 300; 1200	300; 1100 ± 600; 2400	200; 800 ± 600; 2300	100; 100 ± 100; 200	100; 400 ± 300; 1200	100; 1600 ± 700; 3000	100; 200 ± 100; 500
Eu	20; 100 ± 30; 140	100; 300 ± 200; 600	100; 300 ± 300; 800	100; 600 ± 300; 1200	100; 400 ± 300; 1000	100; 100 ± 100; 200	100; 500 ± 400; 1500	100; 700 ± 300; 1100	< LoD; 100 ± 100; 200
Gd	< LoD; 200 ± 10; 210	100; 200 ± 100; 700	100; 500 ± 200; 900	200; 900 ± 400; 1800	200; 700 ± 500; 1800	100; 100 ± 100; 200	100; 400 ± 200; 800	100; 1300 ± 600; 2600	< LoD; 200 ± 100; 400
Tb	10; 20 ± 10; 21	20; 40 ± 20; 90	< LoD; 100 ± 2; 110	< LoD; 100 ± 100; 200	< LoD; 100 ± 100; 200	23.6; 23.9 ± 0.3; 24.2	< LoD; 100 ± 100; 200	< LoD; 200 ± 100; 300	10; 30 ± 10; 50
Dy	30; 110 ± 30; 150	100; 200 ± 100; 500	100; 300 ± 200; 600	100; 600 ± 300; 1200	100; 400 ± 300; 1100	50; 90 ± 30; 130	< LoD; 300 ± 100; 600	< LoD; 800 ± 300; 1500	< LoD; 100 ± 100; 200
Ho	10; 20 ± 10; 30	10; 30 ± 20; 80	20; 50 ± 30; 100	< LoD; 100 ± 100; 200	< LoD; 100 ± 100; 200	19.2; 19.5 ± 0.2; 19.8	20; 40 ± 20; 90	< LoD; 100 ± 100; 200	10; 20 ± 10; 30
Er	10; 50 ± 10; 70	< LoD; 100 ± 100; 200	< LoD; 100 ± 100; 200	100; 200 ± 100; 500	< LoD; 200 ± 100; 400	29; 29 ± 1; 29	< LoD; 100 ± 100; 200	< LoD; 300 ± 100; 600	10; 50 ± 20; 100
Tm	6; 7 ± 10; 9	< LoD; 10 ± 10; 30	10; 20 ± 10; 30	10; 30 ± 20; 60	10; 20 ± 20; 60	3.6; 3.6 ± 0.1; 3.7	< LoD; 10 ± 1; 11	10; 50 ± 20; 80	4; 7 ± 3; 14
Yb	10; 40 ± 10; 60	< LoD; 100 ± 10; 200	< LoD; 100 ± 100; 200	< LoD; 200 ± 100; 400	< LoD; 100 ± 100; 400	24.6; 24.9 ± 0.3; 25.2	< LoD; 100 ± 100; 200	< LoD; 300 ± 100; 500	10; 40 ± 20; 70
Lu	5; 5 ± 1; 7	< LoD; 10 ± 10; 20	< LoD; 10 ± 10; 30	10; 30 ± 20; 60	10; 20 ± 20; 60	4.3; 4.4 ± 0.1; 4.4	< LoD; 10 ± 10; 20	< LoD; 40 ± 20; 80	3; 7 ± 3; 12

Trace and toxic elements (mg kg^{-1})

Elements	SPA ROS (n = 26)	SPA MUL (n = 34)	SPA EUC (n = 13)	ITA THI (n = 39)	ITA STR (n = 31)	ITA ROS (n = 6)	ITA MUL (n = 35)	ITA EUC (n = 30)	ITA ASP (n = 33)
Co	1.2; 3 ± 2; 7	2; 11 ± 9; 34	6; 14 ± 6; 24	1.4; 5 ± 3; 13.2	1.1; 1.8 ± 0.1; 3	4; 6 ± 3; 9.1	1.1; 3 ± 1; 7	2; 4 ± 1; 6.7	1.2; 3 ± 1; 6.4
Cr	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
Cu	170; 220 ± 80; 490	100; 400 ± 300; 1400	100; 700 ± 400; 1400	70; 130 ± 10; 240	80; 110 ± 10; 190	110; 150 ± 10; 200	90; 180 ± 70; 340	80; 140 ± 10; 220	70; 120 ± 10; 180
Fe	< LoD; 1500 ± 300; 2100	< LoD; 2400 ± 1000; 3600	< LoD; 2800 ± 900; 4400	120; 300 ± 200; 730	110; 190 ± 10; 360	< LoD	590; 800 ± 300; 1420	100; 500 ± 200; 840	100; 180 ± 10; 320
Mn	50; 300 ± 200; 710	< LoD; 3000 ± 3000; 12000	900; 4000 ± 2000; 7200	40; 200 ± 100; 460	40; 200 ± 100; 470	40; 110 ± 80; 240	70; 300 ± 100; 730	100; 3000 ± 1500; 5100	40; 120 ± 70; 330
Mo	< LoD	< LoD	4.6; 5.4 ± 0.1; 6.6	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
Ni	18; 30 ± 10; 46	20; 70 ± 60; 280	20; 200 ± 100; 480	11; 22 ± 1; 44	10; 16 ± 1; 28	16; 25 ± 10; 40	11; 20 ± 10; 52	11; 18 ± 1; 34	13; 22 ± 1; 34
Sr	50; 120 ± 60; 270	60; 150 ± 60; 270	70; 210 ± 60; 270	18; 80 ± 20; 128	20; 130 ± 50; 240	37; 60 ± 20; 77	20; 80 ± 30; 170	20; 180 ± 60; 290	10; 30 ± 10; 56
V	3; 12 ± 5; 18	< LoD	2.3; 7 ± 3; 10.6	0.8; 1.2 ± 0.1; 1.9	0.7; 0.8 ± 0.1; 1	< LoD	1.1; 1.6 ± 0.1; 2.6	1; 4 ± 1; 7.3	< LoD
Zn	560; 900 ± 200; 1370	400; 1100 ± 400; 2100	700; 1800 ± 700; 2800	300; 700 ± 300; 1480	260; 600 ± 200; 1180	420; 600 ± 200; 850	400; 800 ± 400; 1900	330; 600 ± 150; 1000	250; 500 ± 200; 1000
Ag	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
Ba	118; 138 ± 1; 151	100; 200 ± 100; 500	170; 500 ± 200; 720	80; 200 ± 100; 520	100; 500 ± 400; 1300	< LoD	110; 150 ± 10; 270	80; 300 ± 100; 540	70; 190 ± 90; 340
Li	< LoD	< LoD	< LoD	< LoD	7.3; 12.1 ± 0.1; 16.7	< LoD	< LoD	7; 12 ± 1; 20	< LoD
As	< LoD	< LoD	8; 9 ± 1; 11	< LoD	< LoD	< LoD	< LoD	7.5; 8.8 ± 0.1; 10.3	< LoD
Be	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
Bi	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
Cd	< LoD	1.3; 1.9 ± 0.1; 3.1	1; 1.9 ± 0.1; 2.8	1.1; 1.6 ± 0.1; 2.6	< LoD	< LoD	1.2; 1.9 ± 0.1; 3.5	1; 1.9 ± 0.1; 3.4	< LoD
Hg	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
Pb	< LoD	< LoD	< LoD	10.2; 12.7 ± 0.1; 15.1	< LoD	< LoD	< LoD	14; 19 ± 1; 27	13.1; 13.4 ± 0.1; 13.6
Sb	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
Sn	9.1; 12.8 ± 0.1; 21	7; 15 ± 1; 29	10; 14 ± 1; 19	10; 40 ± 30; 120	10; 40 ± 40; 140	19; 24 ± 1; 27	8; 17 ± 8; 29	7; 30 ± 20; 88	11; 40 ± 30; 104
Te	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
Tl	< LoD	1.6; 4 ± 2; 7.1	1; 1.7 ± 0.8; 3.1	< LoD; 0.20 ± 0.01; 0.21	0.13; 0.1 ± 0.1; 0.15	< LoD	< LoD	< LoD	< LoD; 0.20 ± 0.01; 0.21

SPA, Spain, ITA, Sardinia, Italy; ROS, rosemary; MUL, multifloral; EUC, eucalyptus; THI, thistle; STR, strawberry tree; ASP, asphodel.

Table B6. Random forest (RF) accuracy table for each set of predictors tested: botanical origin.

		ASP		EUC		MUL		ROS		STR		THI	
Index	**Predictors**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**
21	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu	73±9	70±10	78±10	80±9	40±10	47±7	70±10	70±10	70±10	70±10	74±9	80±10
20	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu	72±10	75±10	77±10	79±8	45±10	47±9	70±10	70±10	70±10	70±10	77±9	70±10
19	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Gd, Tb, Dy, Er, Tm, Yb, Lu	73±10	80±10	80±10	79±10	40±10	48±8	70±10	75±10	70±10	70±10	74±8	75±9
18	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Gd, Tb, Dy, Tm, Yb, Lu	73±10	75±10	79±9	80±8	50±10	46±8	70±10	80±10	70±10	70±10	76±8	80±10
17	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Gd, Tb, Dy, Tm, Lu	74±10	80±10	79±8	82±8	50±10	47±8	80±10	75±10	71±9	70±10	76±8	77±9
16	Na, Mg, K, Ca, Mn, Sr, Zn, Ce, Pr, Nd, Eu, Gd, Tb, Dy, Tm, Lu	74±9	80±10	80±10	79±9	40±10	47±8	70±10	80±10	71±9	70±10	75±8	80±10
15	Na, Mg, K, Ca, Mn, Sr, Zn, Ce, Pr, Nd, Eu, Tb, Dy, Tm, Lu	75±10	75±10	80±9	80±8	50±10	46±8	73±9	80±10	70±10	70±10	76±9	80±10
14	Na, K, Ca, Mn, Sr, Zn, Ce, Pr, Nd, Eu, Tb, Dy, Tm, Lu	72±8	80±10	79±9	80±10	50±10	49±7	74±9	80±10	70±10	70±10	75±8	80±10
13	Na, K, Ca, Mn, Sr, Zn, Ce, Pr, Nd, Eu, Tb, Dy, Lu	75±10	78±9	80±10	81±9	50±10	49±7	70±10	80±10	70±10	70±10	75±9	80±10
12	Na, K, Ca, Mn, Sr, Zn, Ce, Nd, Eu, Tb, Dy, Lu	77±9	80±10	80±10	80±9	50±10	49±8	73±9	80±10	74±9	70±10	76±8	75±10
11	Na, K, Ca, Mn, Sr, Zn, Ce, Nd, Eu, Tb, Lu	76±9	80±10	80±9	81±9	50±10	48±8	72±8	75±10	73±9	80±10	77±9	80±10
10	Na, K, Ca, Mn, Sr, Ce, Nd, Eu, Tb, Lu	75±10	78±9	80±9	81±8	45±10	47±9	72±9	70±10	74±9	80±10	77±8	80±10
9	Na, K, Ca, Mn, Sr, Ce, Eu, Tb, Lu	74±10	80±10	80±10	80±9	45±10	47±8	70±10	75±10	70±10	80±10	76±9	80±10
8	Na, K, Ca, Mn, Sr, Ce, Eu, Lu	77±9	79±9	80±10	81±9	45±10	47±7	70±10	70±10	78±8	80±10	76±9	78±9
7	K, Ca, Mn, Sr, Ce, Eu, Lu	77±9	80±10	80±8	82±9	40±10	47±8	70±10	70±10	69±9	70±10	75±9	77±9
6	K, Mn, Sr, Ce, Eu, Lu	74±8	74±10	78±9	77±9	50±10	50±8	70±10	70±10	70±10	70±10	74±9	75±10
5	K, Mn, Sr, Ce, Lu	71±9	70±10	76±9	77±8	40±10	37±8	65±10	60±10	70±10	70±10	72±9	70±10
4	K, Mn, Sr, Lu	71±9	73±10	80±10	80±10	35±10	39±9	60±10	65±10	69±9	70±10	71±9	70±10
3	Mn, Sr, Lu	72±10	71±10	78±9	78±8	30±10	38±8	60±10	60±10	60±10	50±10	60±10	60±10
2	Mn, Lu	58±10	60±10	80±10	76±10	40±10	40±9	60±10	60±10	40±10	40±10	50±10	55±10

Values in tables B6-B8 represent the percentage of accuracy in classifying the data in the test and the training datasets. The values have been rounded according to the relevant standard deviation.

Table B7. RF accuracy table for each set of predictors tested: geographical and botanical origin.

		ITA ASP		ITA EUC		ITA MUL		ITA ROS		ITA STR		ITA THI		SPA EUC		SPA MUL		SPA ROS	
Index	**Predictors**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**	**Training**	**Test**
21	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu	80±10	80±10	82±8	83±9	60±10	65±10	20±30	40±20	70±10	70±10	70±10	70±10	45±25	50±20	50±10	50±10	73±8	80±10
20	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Er, Tm, Yb, Lu	70±10	75±10	81±9	80±10	66±9	60±10	30±35	35±20	70±10	70±10	70±9	72±9	40±30	50±15	50±10	50±10	74±9	80±10
19	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Gd, Tb, Dy, Er, Tm, Yb, Lu	75±10	75±101	81±9	80±10	60±10	70±10	30±30	40±20	70±10	70±10	70±10	70±10	50±30	50±20	50±10	50±10	75±10	80±10
18	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Gd, Tb, Dy, Tm, Yb, Lu	75±9	78±9	83±7	80±10	65±10	70±10	30±33	40±20	70±10	70±10	71±9	72±9	50±25	50±15	50±10	50±10	75±8	80±10
17	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Tb, Dy, Tm, Yb, Lu	75±9	80±10	82±8	82±9	65±10	70±10	20±30	40±20	70±10	70±10	70±10	70±10	40±25	50±20	50±10	55±10	75±8	80±10
16	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Tb, Dy, Tm, Lu	77±9	80±10	82±8	84±8	60±10	70±10	30±30	40±20	70±10	70±10	72±8	70±10	50±20	50±10	50±10	55±10	75±10	80±10
15	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Tb, Tm, Lu	78±9	77±9	82±8	83±9	65±10	70±10	20±30	40±20	70±10	70±10	70±10	70±10	50±20	50±15	50±10	50±10	77±9	80±10
14	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Tb, Lu	80±10	78±9	83±9	80±10	70±10	70±10	30±30	40±20	70±10	75±10	70±9	74±9	50±20	50±15	50±15	55±10	76±9	80±10
13	Na, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Eu, Tb, Lu	80±10	80±10	83±8	80±10	70±10	70±10	30±35	40±20	70±10	70±10	71±9	70±10	40±20	60±20	50±10	50±10	78±8	80±10
12	Na, K, Ca, Mn, Sr, Zn, Ce,Pr, Nd, Eu, Tb, Lu	78±9	80±10	84±8	80±10	70±10	70±10	30±30	40±20	70±10	70±10	73±9	70±10	50±20	50±20	50±10	60±10	78±9	80±10
11	Na, K, Ca, Mn, Sr, Zn, Ce, Nd, Eu, Tb, Lu	78±9	79±8	83±8	84±8	70±10	70±10	20±30	30±20	70±10	70±10	70±10	70±10	45±20	50±20	50±10	50±10	77±9	80±10
10	Na, K, Ca, Mn, Sr, Ce, Nd, Eu, Tb, Lu	80±10	79±8	83±8	84±9	70±10	70±10	15±30	40±20	70±10	75±10	74±9	73±9	40±20	50±20	50±10	60±10	76±9	80±10
9	Na, K, Ca, Mn, Sr, Ce, Nd, Eu, Lu	77±8	80±10	84±7	80±10	60±10	66±9	20±30	30±20	70±10	70±10	72±9	70±10	50±20	60±10	50±10	50±10	73±9	80±10
8	Na, K, Mn, Sr, Ce, Nd, Eu, Lu	75±10	77±9	83±8	82±9	65±10	70±10	20±30	30±20	70±10	70±10	70±10	72±9	40±20	50±15	50±10	50±10	75±10	80±10
7	Na, K, Mn, Sr, Ce, Eu, Lu	75±10	78±9	83±7	84±9	65±10	69±9	20±30	30±20	70±10	70±10	69±8	70±10	40±30	50±20	50±10	50±10	74±9	80±10
6	Na, K, Mn, Sr, Ce, Lu	70±10	70±10	80±10	80±10	60±10	60±10	20±30	35±30	70±10	80±10	70±10	70±10	50±20	60±15	50±10	50±10	70±10	70±10
5	Na, Mn, Sr, Ce, Lu	71±9	70±10	80±7	82±9	60±10	60±10	20±30	40±25	60±10	60±10	60±10	60±10	50±25	60±20	50±10	50±10	65±10	70±15
4	Mn, Sr, Ce, Lu	70±10	70±10	78±9	80±10	50±10	50±10	10±20	30±25	50±10	50±10	60±10	60±10	40±20	50±20	50±10	55±10	60±10	60±10
3	Mn, Sr, Lu	70±10	70±10	80±10	80±10	50±10	60±10	10±20	20±20	55±10	50±10	60±10	60±10	40±20	45±15	50±10	50±10	60±10	60±10
2	Mn, Lu	55±10	60±10	80±10	80±10	60±10	60±10	20±30	30±20	40±10	40±10	50±10	50±10	30±20	40±20	50±10	50±10	60±10	60±10

Table B8. RF accuracy table for each set of predictors tested: geographical origin.

		ITA		SPA	
Index	**Predictors**	**Training**	**Test**	**Training**	**Test**
21	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu	89±5	88±5	90±3	89±6
20	Na, Mg, K, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Er, Tm, Yb, Lu	89±4	90±3	88±3	89±7
19	Na, Mg, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Er, Tm, Yb, Lu	88±4	88±4	89±4	85±8
18	Na, Mg, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Er, Tm, Lu	88±5	88±4	90±3	89±5
17	Na, Mg, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Tm, Lu	87±4	89±3	88±3	86±8
16	Na, Mg, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Tm, Lu	89±2	91±4	89±4	86±8
15	Na, Mg, Ca, Mn, Sr, Zn, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Lu	88±5	91±3	89±4	91±5
14	Na, Mg, Ca, Mn, Sr, Zn, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Lu	88±5	90±3	88±4	90±10
13	Na, Mg, Ca, Mn, Sr, Zn, Ce, Pr, Nd, Eu, Gd, Tb, Lu	89±5	88±5	87±5	91±5
12	Na, Mg, Mn, Sr, Zn, Ce, Pr, Nd, Eu, Gd, Tb, Lu	89±5	88±3	87±4	94±5
11	Na, Mg, Mn, Sr, Zn, Ce, Nd, Eu, Gd, Tb, Lu	89±4	89±5	89±5	89±5
10	Na, Mg, Mn, Sr, Zn, Ce, Nd, Eu, Gd, Tb	88±3	89±5	89±5	91±4
9	Na, Mg, Mn, Sr, Zn, Ce, Nd, Eu, Tb	91±3	88±4	92±3	91±5
8	Na, Mg, Mn, Sr, Zn, Ce, Nd, Tb	86±5	90±3	89±5	89±9
7	Mg, Mn, Sr, Zn, Ce, Nd, Tb	88±5	90±3	88±4	90±5
6	Mn, Sr, Zn, Ce, Nd, Tb	86±5	88±5	86±3	90±5
5	Mn, Zn, Ce, Nd, Tb	84±4	84±4	89±3	89±5
4	Mn, Zn, Ce, Tb	86±6	84±4	85±3	88±6
3	Mn, Zn, Ce	86±4	89±3	88±4	81±6
2	Mn, Zn	77±8	73±7	76±7	80±10

APPENDIX C

Table C1. Instrumental conditions of the ICP-OES OPTIMA 7300 DV, Perkin Elmer.

ICP-OES OPTIMA 7300 DV, Perkin Elmer	
RF power generator (W)	1300
Ar plasma flow (dm^3 min^{-1})	15.0
Ar auxiliary flow (dm^3 min^{-1})	0.20
Ar nebulizer flow (dm^3 min^{-1})	0.80
Nebulizer	GemTip Cross-Flow II

Table C2. Instrumental conditions of the ICP-MS NexION 350X, Perkin Elmer.

ICP-MS NexION 350X, Perkin Elmer			
RF power generator (W)	1400	KED cell entrance voltage (V)	-8
Ar plasma flow (dm^3 min^{-1})	18.0	KED cell exit voltage (V)	-38
Ar auxiliary flow (dm^3 min^{-1})	1.40	Resolution (Da)	0.7
Ar nebulizer flow (dm^3 min^{-1})	0.90	Scan mode	Peak hopping
KED He flow (cm^3 min^{-1})	4.60	Detector mode	Dual
Nebulizer	Meinhardt glass	Dwell time (ms)	50
Spray chamber	Cyclonic glass	Number of points per peak	3
Skimmer and sampling cones	Nickel	Acquisition time (s)	6
Deflector voltage (V)	-10	Acquisition dead time (ns)	35
Analog stage voltage (V)	-2350	KED gas	Helium, 99.999%
Pulse stage voltage (V)	1800	Masses of optimization	^{7}Li,^{115}In and ^{208}Pb

Gas nebulizer optimization: $^{141}Ce^{16}O^{+}/^{141}Ce^{+} < 0.03$ (NexION Setup Solution).

KED he-flow optimization: $^{35}Cl^{16}O^{+}/^{59}Co^{+} < 0.005$ (NexION KED Solution).

Table C3. Analysis of the CRM ERM BD-151 (skimmed milk powder).

Macro elements [a]	Certified value (g kg^{-1})	Experimental value (g kg^{-1}, n=3)	Trueness %
Ca	13.9 ± 0.7	14.7 ± 0.6	106 ± 4
K	17.0 ± 0.8	17.7 ± 0.5	104 ± 3
Mg	1.26 ± 0.07	1.31 ± 0.04	104 ± 3
Na	4.19 ± 0.23	4.8 ± 0.2	114 ± 5
P	11.0 ± 0.6	9.8 ± 0.6	89 ± 5
Trace elements [b]	**Certified value (mg kg^{-1})**	**Experimental value (mg kg^{-1}, n=3)**	**Trueness %**
Cd	0.106 ± 0.013	0.098 ± 0.005	92 ± 5
Cu	5.00 ± 0.23	4.7 ± 0.2	94 ± 4
Fe	53 ± 4	49 ± 3	92 ± 6
Mn	0.29 ± 0.03	0.28 ± 0.01	97 ± 4
Pb	0.207 ± 0.014	0.215 ± 0.005	104 ± 2
Se	0.19 ± 0.04	0.21 ± 0.01	110 ± 5
Zn	44.9 ± 2.3	44 ± 2	98 ± 5

a) ICP-OES; b) ICP-MS

Table C4. Validation parameters of the ICP-MS method for the elemental analysis of Pecorino cheeses.

Element	Mode	Calibration Range (mg dm^{-3})	R^2	LoD (mg kg^{-1})	LoQ (mg kg^{-1})	$CV\%_r$	$CV\%_{IP}$	Recovery %
^{107}Ag	STD	0.1 - 50	0.99996	0.5	1.6	11%	21%	86 ± 1
^{27}Al	KED	0.5 - 200	0.99999	35	115	16%	24%	105 ± 1
^{75}As	KED	0.1 - 200	0.99996	1.0	3.3	7%	24%	149 ± 7
^{11}B	STD	0.5 - 200	0.99995	16	54	6%	13%	111 ± 4
^{209}Bi	STD	0.05 - 50	1.00000	0.1	0.5	9%	21%	88 ± 2
^{111}Cd	KED	0.05 - 100	1.00000	0.03	0.10	7%	17%	89 ± 1
^{59}Co	KED	0.1 - 200	1.00000	0.02	0.08	4%	15%	102 ± 1
^{52}Cr	KED	0.1 - 200	0.99997	0.9	3.1	9%	12%	102 ± 1
^{63}Cu	KED	0.1 - 500	1.00000	60	200	6%	16%	101 ± 2
^{57}Fe	KED	0.1 - 500	0.99996	29	90	9%	19%	112 ± 1
^{202}Hg	STD	0.1 - 50	0.99987	9	30	13%	18%	88 ± 6
^{7}Li	STD	0.1 - 200	0.99995	17	55	4%	12%	106 ± 5
^{55}Mn	KED	0.1 - 500	1.00000	0.4	1.2	5%	23%	103 ± 1
^{60}Ni	KED	0.1 - 200	0.99998	3.20	10.0	8%	17%	96 ± 2
^{208}Pb	STD	0.05 - 100	0.99996	1.0	3.4	8%	14%	95 ± 1
^{85}Rb	STD	0.1 - 500	0.99999	0.2	0.7	5%	14%	101 ± 1
^{121}Sb	KED	0.1 - 50	1.00000	1.1	3.6	8%	15%	99 ± 1
^{82}Se	KED	0.1 - 500	0.99999	2.3	7.6	9%	19%	147 ± 3
^{118}Sn	KED	0.1 - 50	1.00000	0.7	2.4	5%	14%	97 ± 2
^{88}Sr	STD	0.1 - 500	0.99999	0.8	2.6	4%	15%	109 ± 2
^{130}Te	STD	0.1 - 50	0.99999	0.4	1.2	4%	17%	107 ± 5
^{105}Tl	STD	0.05 - 50	0.99998	0.1	0.5	4%	18%	83 ± 1
^{238}U	STD	0.05 - 50	0.99998	0.06	0.19	9%	18%	101 ± 1
^{51}V	KED	0.1 - 200	0.99998	0.2	0.6	3%	18%	109 ± 2
^{66}Zn	KED	0.1 - 500	0.99997	90	300	6%	18%	115 ± 4

$CV\%_r$, Variation coefficient (repeatability); $CV\%_{IP}$, Variation coefficient (intermediate precision)

Table C5. Average elemental composition of Pecorino cheeses measured in this study and from literature data.

	Elements	Pecorino Romano PDO			Pecorino Sardo PDO		Other Italian Pecorino[a]	
		Coni, 1999, n = 7	**DiDonato, 2021, n = 17**	**This study, n = 103**	**DiDonato, 2021, n = 20**	**This study, n = 97**	**Manuelian, 2017, n = 10**	**DiDonato, 2021, n = 16**
Macro (mg kg^{-1})	Ca		13000	14000 ± 1000	13000	14000 ± 1000	7280	12000
	K		1100	1000 ± 100	1500	1300 ± 200	1430	1700
	Mg	221	730	600 ± 40	700	700 ± 50	330	650
	Na		21000	25000 ± 5000	10000	8000 ± 1000	7820	11000
	P		9000	9000 ± 700	8000	9000 ± 500	4300	8000
	S			1000 ± 200		700 ± 100	1300	
Trace and toxic (µg kg^{-1})	Ag			5 ± 5		5 ± 5		
	Al	2250		6000 ± 2000		6000 ± 3000		
	As			8 ± 1		6 ± 1		
	Ba	1730	3500		2700			1200
	B			8000 ± 8000		2000 ± 2000		
	Bi			2 ± 1		< 0.5		
	Cd	25		1.2 ± 0.5		1 ± 0.5		
	Co	26		4 ± 1		4 ± 1		
	Cr	40		20 ± 10		40 ± 20		
	Cu	550		1000 ± 350		1200 ± 500	600	
	Hg			< 30		< 30		
	Fe	2470	3800	6000 ± 950	2500	7000 ± 3000	3380	2400
	Li			< 55		< 55		
	Mn	270		800 ± 100		850 ± 100		
	Ni	460		27 ± 5		30 ± 10		
	Pb	19		20 ± 10		20 ± 10		
	Pt	132						
	Rb			1600 ± 500		1700 ± 500		
	Sb			10 ± 5		12 ± 5		
	Se			400 ± 100		340 ± 90	780	
	Sn			10 ± 10		20 ± 10		
	Sr	1880		14300 ± 2500		13400 ± 2500		
	Te			9 ± 5		130 ± 50		
	Tl			< 0.5		1.9 ± 0.5		
	U			2 ± 1		1 ± 1		
	V			15 ± 5		10 ± 5		
	Zn	18800	49000	47000 ± 7500	48000	56000 ± 9000	21750	40000

a) no-PDO cheeses

Figure C1. PCA analysis performed on Pecorino Sardo and Pecorino Romano produced by 3 farms in the same period: (a) loading plot; (b) score plot.

Figure C1a

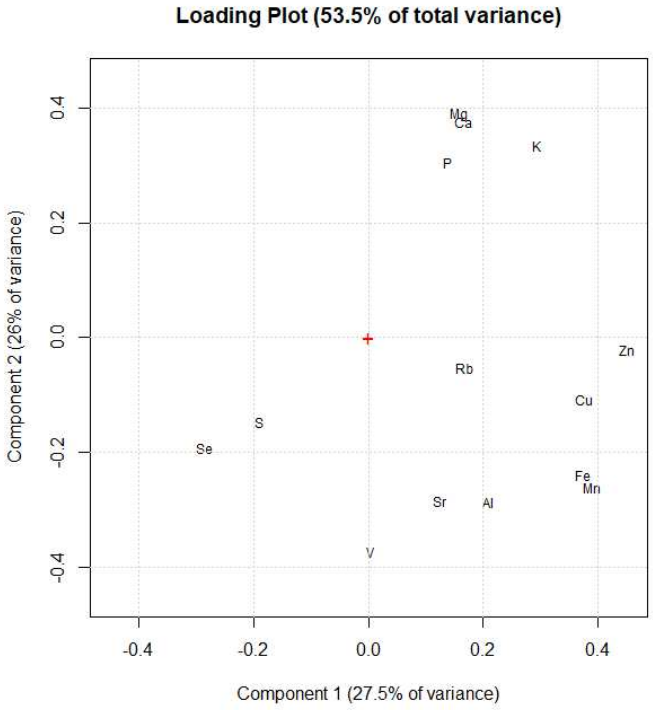


Figure C1b

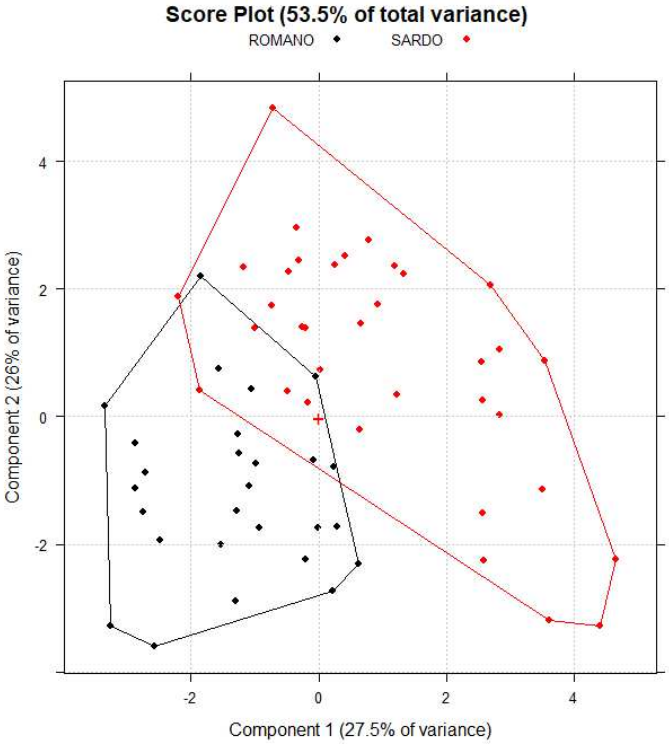


Figure C2. PCA analysis performed on Pecorino Romano samples and 14 elements: (a) loading plot; (b) score plot. Object colored according to seasonality**.**

Figure C2a

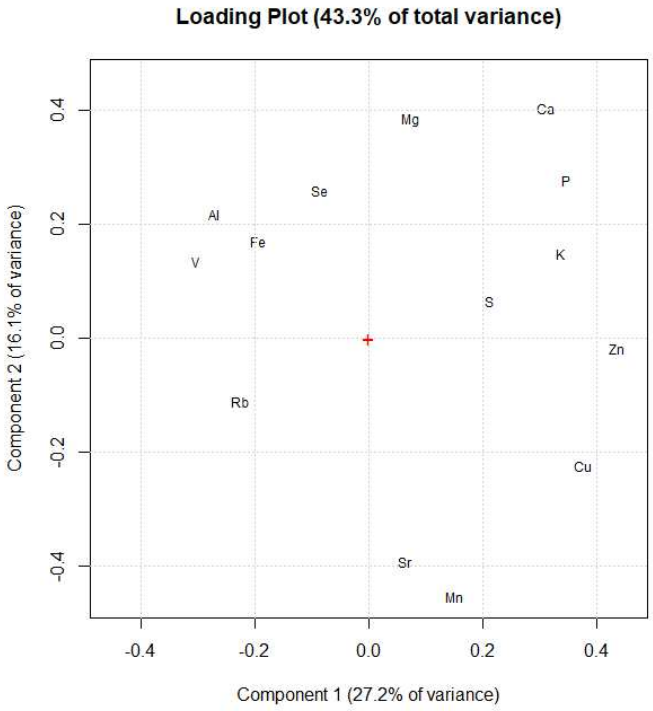


Figure C2b

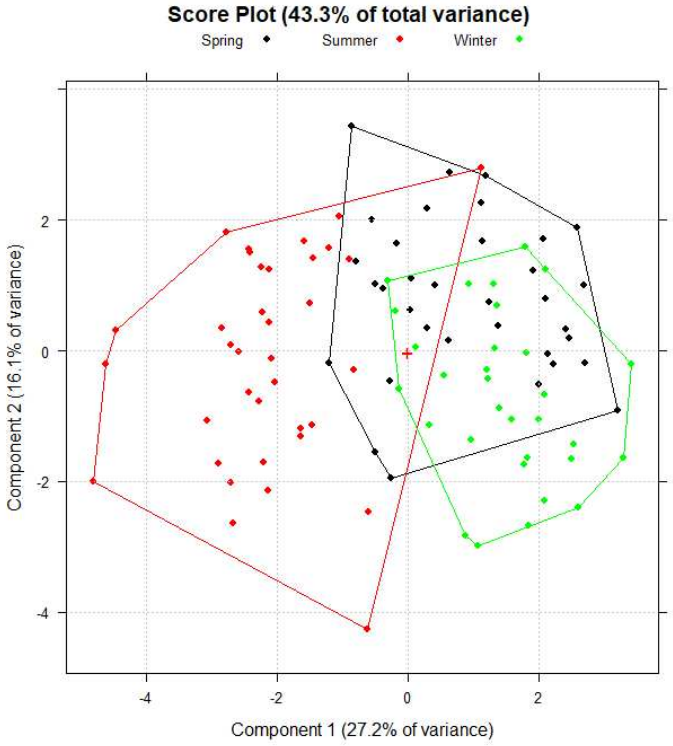


Figure C3. ANOVA analysis of macro and trace elements in Pecorino Romano PDO as a function of the seasonality.

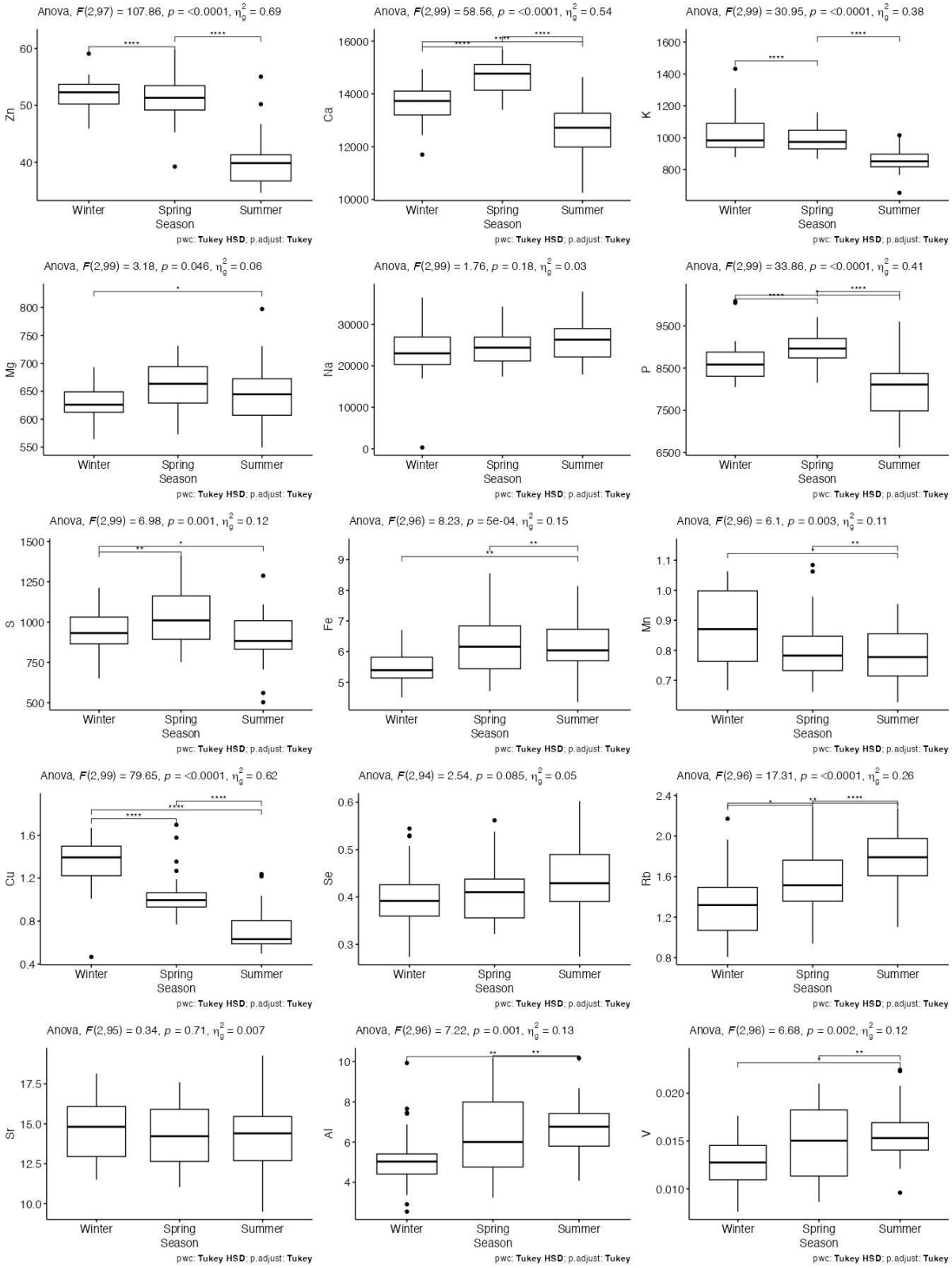


Figure C4. PCA analysis performed on Pecorino Sardo samples and 14 elements: (a) loading plot; (b) score plot. Object colored according to seasonality.

Figure C4a

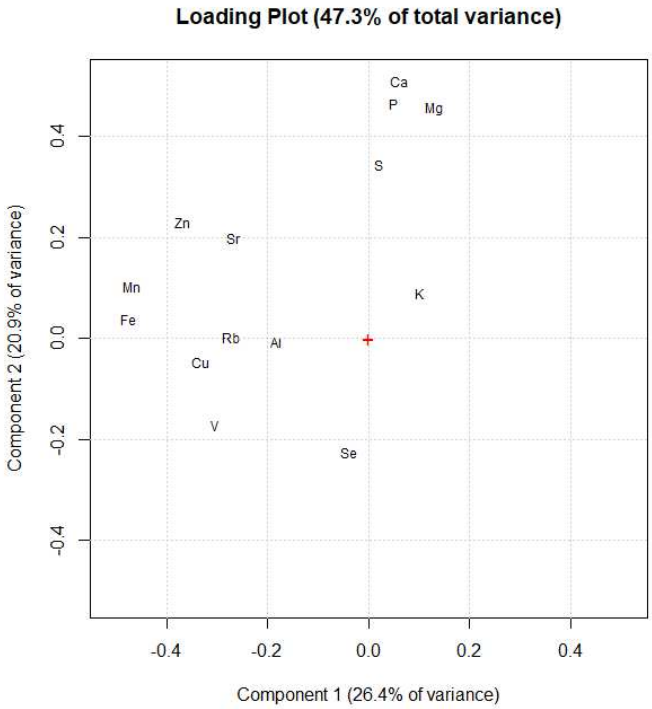


Figure C4b

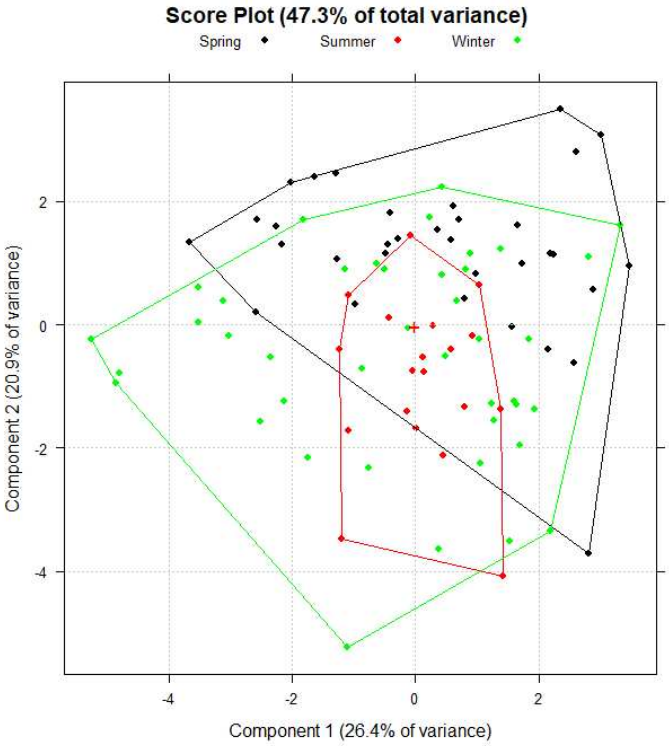
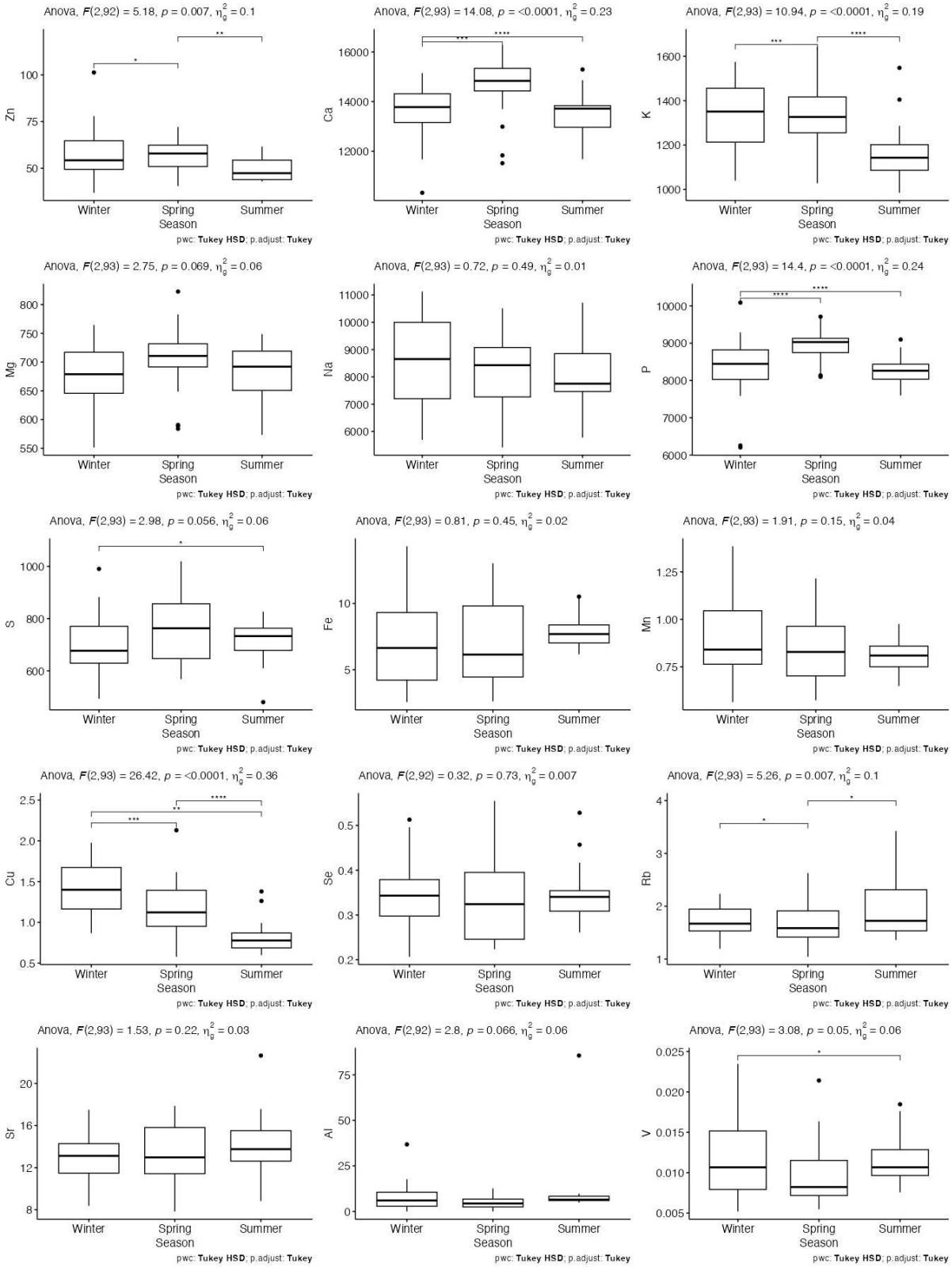


Figure C5. ANOVA analysis of macro and trace elements in Pecorino Sardo as a function of the seasonality.



APPENDIX D

D1) Further details on cultivation conditions of an Aleramo rice genotype at varying of the irrigation methods

Sowing was accomplished in four sub-plots (surface of each sub-plot was 10 m^2) for each irrigation method. The preparation of the seed bed was accomplished by chisel plowing to a depth of 20 cm, followed by a secondary tillage with a field cultivator. A preliminary leveling of the field was performed when either CF or SA irrigation methods have been used, while no leveling was required when SP irrigation was used. Sowing was always performed on dry soil using a seed drill. Seeds were placed at a depth of 3 cm with 14 cm of inter-row distance, with 500 viable seeds m^{-2}. The main hydrological parameters and the majority composition of the soils, as well as of the irrigation waters, have been accomplished by means of literature methods. Of contrast, an original method here described has been used to measure the amount of fifteen elements of potential health concern in the paddy soils irrigated by CF, SA, and SP. Fertilization and treatments against weeds are reported in Table D1. Harvesting was performed between last days of September and first days of October, depending on the irrigation method. For each sub-plot, yield was measured using a small plot combine harvester.

Table D1. Fertilization and treatments against weed performed on fields irrigated by continuous flooding (CF), saturation (SA), and sprinkler (SP) methods, respectively.

CF and SA	**Treatments**
Pre-sowing fertilization	N_2, 63 kg ha^{-1}; P_2O_5, 92 kg ha^{-1}; K_2O, 50 kg ha^{-1}
Pre-emergent weed treatment	*Pendimethalin®,* 1,320 g ha^{-1}
Post-emergent weed treatment	*MCPA®,* 376 g ha^{-1} and *Propanil®,* 3,640 g ha^{-1} or *Cinosulfuron®,* 80 g ha^{-1}
Coverage fertilization	Three treatments, between 36 kg ha^{-1} of N_2 and 45 kg ha^{-1} of N_2 each, according needs of the cultivation.
SP	**Treatments**
Pre-sowing fertilization	N_2, 63 kg ha^{-1}; P_2O_5, 92 kg ha^{-1}; K_2O, 50 kg ha^{-1}
Pre-emergent weed treatment	*Pendimethalin®,* 1,650 g ha^{-1}
Post-emergent weed treatment	*MCPA®,* between 470 g ha^{-1} and 790 g ha^{-1}, as a function of severity of the weed infestation.
Coverage fertilization	Three treatments, 30 kg ha^{-1} of N_2 each

MCPA: 4-chloro-o-tolyloxyacetic acid; (4-chloro-2-methylphenoxy) acetic acid.

2) Further details on reagents and standard used in the study

Inorganic As stock standard solutions in water (1000 mg dm^{-3}) were purchased by Fluka (Rodano, Italy) for As(III), and by Merck (Darmstadt, Germany) for As(V), respectively, whereas stock standard aqueous solutions (1000 mg dm^{-3}) of organic species of As were prepared dissolving proper amounts of cacodylic acid (>99.0%) from Sigma-Aldrich, St. Louis, USA for DMA and disodium methyl arsonate hexahydrate (98%, Supelco, St. Louis, Missouri, USA) for MMA. All stock solutions were stored at 4°C, while the working solutions were daily prepared from them by a proper dilution of the stock solution. NCSZ 11007 and NCSZ 73008 certified rice flours were produced by the China National Analysis Centre (Beijing, China), whereas NIST 1568a rice flour was from National Institute of Standards and Technology (Gaithersburg, USA), and IRMM 804 and ERM-BC211 rice flours were from the European Commission's Joint Research Centre – Institute for the Reference Materials and Measurements (Geel, Belgium). SS-1 EnviroMAT contaminated soil was produced by SCP Science (Baie D'Urfé, Canada), while CRM 025-050 sandy loam soil was produced by the Resource Technology Corporation (Laramie, USA). The ICP-MS Setup Solution (a 1% (v/v) aqueous solution of nitric acid containing 1 µg dm^{-3} each of Be, Ce, Fe, In, Li, Mg, Pb and U), the KED Setup Solution (a 1% (v/v) aqueous solution of nitric acid containing 10 µg dm^{-3} of Co and 1 µg dm^{-3} of Ce) and the internal standard solution containing 10 µg dm^{-3} of Rh in a 1% (v/v) aqueous solution of nitric acid was all from Perkin Elmer (Milan, Italy). A commercial solution ICP-MS Stock Tuning Solution (a 2% (v/v) aqueous solution of nitric acid containing 10 µg dm^{-3} each of Li, Y, Ce, Tl and Co) and the internal standard solution containing 20 µg dm^{-3} of Be, Rh, and Tl in a 1% (v/v) aqueous solution of nitric acid was from Agilent Technologies (Santa Clara, CA, USA).

3) Further details on instruments and apparatus used in the study

A microwave digestion system Milestone Ethos Touch Control (FKV-Milestone, Torre Boldone, Italy) was used to mineralize samples. An electronic millivoltmeter model 210A (Thermo Scientific Orion, Chelmsford, USA) connected to a Pt electrode (model 805/SPG/12) and a calomel electrode (model 303/SCG/12), or to a combined pH electrode (model 411/CGG/12), all from Amel Instruments (Milan, Italy), were used for Eh and pH measurements, whereas an agate mortar pestle grinder model SFM-8 (TOB New Energy Technology, Xiamen, China) was used to grind and homogenize samples. In addition, a centrifuge model Rotonda 460RS (Hettich, Tuttlingen, Germany) was used for separating the solid fraction by the aqueous extracts, while a rotating mixer for tubes model MRH-04 (SBS, Barcelona, Spain) was used for continuous shaking the centrifugation tubes during the extraction process.

4) Assessment of method of extraction of As species from soils

In this case, both SS-1 EnviroMAT contaminated soil and CRM 025-050 sandy loam were used as certified samples (certified total As of 20.7±1 mg $kg^{-1}$and 339±17.3 mg kg^{-1}, respectively). Hence, these CRMs certificate their total As amount spanning over more than one order of magnitude. Unfortunately, only CRM 025-050 certificates the inorganic species of As, hence it is possible to obtain trueness information for both CRMs only as far as the total amount of As extracted is concerned. To not use extraction conditions significantly different from those typical for soils, attention was primarily addressed to extraction methods working at room temperature. In this case, high recoveries of As in the extracts from soils are frequently observed when acidic aqueous solutions were used [SM1, SM2]. Among others, phosphoric acid was found to be the most performing extracting agent, and this is likely since the As bound to the various mineral phases is displaced by phosphate ions through a ligand exchange mechanism [SM1]. Also sulphuric acid, hydrochloric acid and hydrofluoric acid provided good recoveries in the extraction process of As from soils. Nevertheless, the presence of halogenic acids was, for different reasons (i.e. spectral interference of $^{40}Ar^{35}Cl^{+}$ molecular ion on the monoisotopic $^{75}As^{+}$, or chemical aggression by HF on glass parts of the ICP-MS instrument), undesirable in the following phases of analysis. Hence, a preliminary comparative evaluation of the extraction capabilities at room temperature of three extraction systems (i.e. an aqueous solution containing: i) 1 mol dm^{-3} of phosphoric acid or ii) 1 mol dm^{-3} of sulphuric acid or iii) 1 mol dm^{-3} of phosphoric acid and 0.5 mol dm^{-3} of

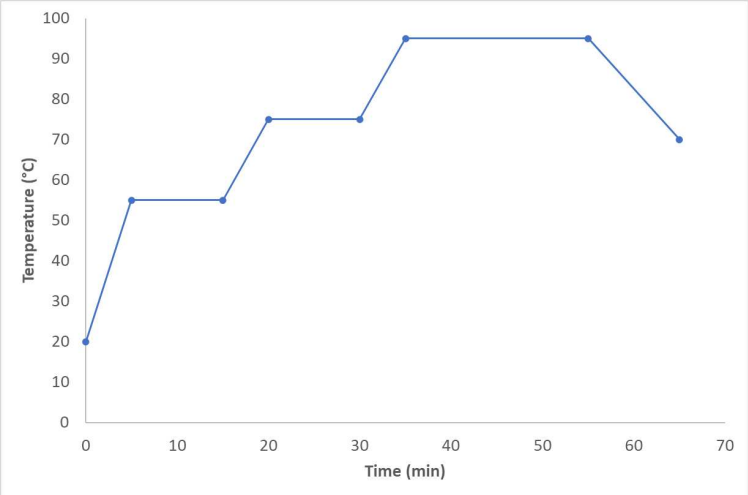
L(+)-ascorbic acid) was carried out on both CRMs of soil. Results obtained demonstrated that extraction solutions i) and iii) provided the best recoveries in As, whereas only the extraction solution iii) minimized the transformation of As(III) in As(V) species, due to the strong antioxidant action of the L(+)-ascorbic acid. Hence, the solution iii) have been chosen to develop the extraction method.

5) Assessment of method of extraction of As species from flours of rice grain

Also in this case, different CRMs of rice flour were used to assess this extraction method. Five CRMs were used for this task: NCSZ 11007, NCSZ 73008, NIST 1568a, IRMM 804 and ERM-BC211. The first four rice flours certificate only the total amount of As, in a range between 49±4 μg kg^{-1} (IRMM 804) and 290±30 μg kg^{-1} (NIST 1568a). On the contrary, the ERM-BC211, beyond the total amount of As (i.e. 260±13 μg kg^{-1}), also certificates the concentrations of iAs and DMA (124±11 μg kg^{-1} and 119±13 μg kg^{-1}, respectively). The exam of the literature demonstrated that each solvent and each condition of extraction exhibited several pros and cons. As a general remark, the extraction with acid solutions usually provided best recoveries of As than that observed for neutral solutions (water and water/methanol mixtures). Among acid extracting solutions, diluted nitric acid or trifluoroacetic acid were those most frequently used. However, literature provides contradictory results in recoveries of extracted As when solutions of trifluoroacetic acid were used. Hence, based on our expertise, nitric acid has been used in the extraction of As species in the research here reported. In a previous paper, aimed to quantify the total iAs rather than both chemical forms of inorganic As, an aqueous solution containing 0.2% (w/v) of nitric acid and 1% (v/v) of hydrogen peroxide was used as As extracting agent. In this case, however, the aim is to quantify both As(III) and As(V) inorganic species, hence extraction of As was attempted by means of an aqueous solution containing 0.2% (w/v) of the only nitric acid. Literature agrees in excluding the possibility of an oxidation of As(III) to As(V) species when the extraction has been performed with a very diluted solution of nitric acid, but Huang et al. (Huang et al., 2010) observe that a reduction of As(V) to As(III) is possible when these solutions are used for extracting this element from rice samples. In that case, it was suggested that thiolate groups released from a slow acidic hydrolysis of rice may be able to reduce As(V) when the concentration of nitric acid is below 0.28 mol dm^{-3} (i.e. the 1.76%, w/v). On the other hand, it is possible that the adoption of a further diluted solution of nitric acid could greatly slow down the hydrolysis of rice towards thiolates, and the use of a fast and efficient extraction

system might be able to further minimize the course of the As(V) reduction. Repeated recovery tests made on As(V)-spiked CRM samples of rice flour confirmed this hypothesis: a microwave-assisted extraction, performed with an aqueous solution of a 0.2% nitric acid (w/v) on a As(V)-spiked sample of CRM rice flour and lasted 65 minutes, provided recoveries always > 96% on the speciated As(V) (whose concentration is below its LoD in the unspiked CRM). Extraction was always accomplished by means a microwave oven. Figure D1 reports the time-temperature conditions of extraction used.

Figure D1. Time – temperature diagram describing the conditions of microwave-assisted extraction of As from rice flour using a 0.2% (w/v) solution of nitric acid.



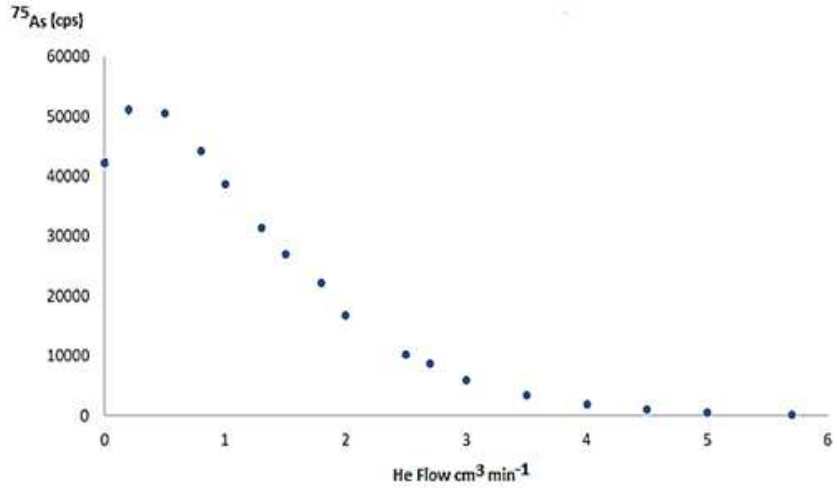
It is interesting to observe that the highest temperature reached by the solution is the same of the method described by Huang et al. (Huang et al., 2010), but it was kept only for 20 minutes (i.e. less than 25% of the time used by Huang et al. (Huang et al., 2010)).

6) Assessment of ICP-MS methods of elemental determination in rice grain and soils

Before any reliable As speciation measurement, researchers should be at least aware of i) the total amount of As in the matrix considered, and ii) the total amount of extracted As. Hence, attention must be paid also to correctly develop an accurate ICP-MS method aimed to carefully measure these quantities. ^{75}As is the only isotope available in nature, and this could be a problem if other interfering species with a similar m/z ratio are already present in the matrix or, more probably, are formed in the plasma phase. Chlorine-containing matrices easily form in the plasma the molecular ion $^{40}Ar^{35}Cl^{+}$, which strongly overestimates the counts of the $^{75}As^{+}$ ion. A mathematical approach to minimize this interference is to estimate the number of counts of the ions of m/z = 77 (i.e. $^{40}Ar^{37}Cl^{+}$ and ^{77}Se). In this way it is possible to evaluate the net counts attributable to $^{75}As^{+}$ using correction equations able to calculate the counts of the $^{40}Ar^{35}Cl^{+}$ based on those of $^{40}Ar^{35}Cl^{+}$. However, the most accurate way to overcome this drawback is to operate with an ICP-MS instrument equipped with a collision/reaction cell. In this case, the use of a proper gas (like as He or methane) can remove the interference caused by $^{40}Ar^{35}Cl^{+}$ through a collision process (using an inert gas like He) or a reaction process (using oxygen or methane) [SM2]. Obviously, the flux of the colliding/reacting gas should be carefully optimized: whereas an insufficient flow does not completely eliminate the interference, an excessive flow

reduces also the signal of the analyte, increasing hence the instrumental LoD of the analyte. Figure D2 explicitly shows the behaviour of the signal at m/z ratio of 75 amu from a solution containing 10 μg dm^{-3} of As as a function of the increasing flow of He. The sharp linear decreasing trend (left side of the diagram) is indicative of the collisional suppression of the signal due to $^{40}Ar^{35}Cl^{+}$ interfering ion, while the second linear trend (right side of the diagram) is related to the gradual suppression also of the signal of $^{75}As^{+}$ due to very high flows of He. The abscissa of the intersection of the regression lines from both trends provides the optimized flux of He.

Figure D2. Behaviour of the signal (in cps) at m/z ratio of 75 amu from a solution containing 10 μg dm^{-3} of As as a function of the increasing flow of He (in cm^3 min^{-1}). ICP-MS instrument: Agilent 7500ce.



For the Agilent 7500ce the optimized flow is 2.7 cm^3 min^{-1} while for the Perkin Elmer Nexion 300X the relevant analytical flow is 4.0 cm^3 min^{-1}. Obviously, the optimized He fluxes depend only on the ICP-MS instrument settings, irrespectively that the ICP is (or not) coupled with a chromatography system. Table D2 reports the instrumental conditions used for the ICP-MS determinations of 15 among toxic elements and trace elements in the soils where the real samples of rice were grown.

Table D2. ICP-MS parameters and elemental settings for the determination of fifteen among toxic and trace elements in paddy soil.

Instrument: Perkin Elmer NexION 300X			
RF power generator (W)	1300	**KED gas**	Helium, 99.999%
Ar plasma flow (dm^3 min^{-1})	17.995	**KED gas flow (cm^3 min^{-1})**	4.0
Ar auxiliary flow (dm^3 min^{-1})	1.203	**Masses of optimization**	^{7}Li, ^{89}Y and ^{205}Tl
Ar nebulizer flow (dm^3 min^{-1})	0.991	**Dwell time (ms)**	50
Nebulizer	Meinhard®, glass	**Number of points per peak**	3
Spray chamber	Cyclonic, glass	**Acquisition time (s)**	3
Skimmer and sampling cones	Nickel	**Quantification mass**	^{75}As
Sampling depth (mm)	0	**Quantification**	External calibration

Element	**Quantification isotopic ion (% elemental abundance)**	**Interferents**	**Analysing mode**	**He flow (cm^{3}min^{-1})**	**Correction equation**
Al	^{27}Al+ (100)	$^{11}B^{16}O^+$; $^{13}C^{14}N^+$; $^{11}Be^{16}O^+$; $^{26}Mg^1H^+$; $^{12}C^{15}N^+$; $^{54}Cr^{2+}$; $^{54}Fe^{2+}$	KED	3.5	
As	$^{75}As^+$ (100)	$^{40}Ar^{35}Cl^+$; $^{59}Co^{16}O^+$; $^{39}K^{36}Ar^+$; $^{63}Cu^{12}C^+$; $^{40}Ca^{35}Cl^+$; $^{58}Ni^{16}O^1H^+$	KED	4.0	
Cd	$^{111}Cd^+$ (12.80)	$^{95}Mo^{16}O^+$; $^{97}Mo^{14}N^+$; $^{79}Br^{16}O_2^+$; $^{94}Zr^{16}O^1H^+$; $^{71}Ga^{40}Ar^+$	Normal		
Cr	$^{52}Cr^+$ (83.79)	$^{40}Ar^{12}C^+$; $^{36}Ar^{16}O^+$; $^{1}H^{35}Cl^{16}O^+$; $^{104}Pd^{2+}$; $^{51}V^1H^+$; $^{40}Ca^{12}C^+$; $^{38}Ar^{14}N^+$	KED	3.0	
Cu	$^{63}Cu^+$ (69.17)	$^{40}Ar^{23}Na^+$; $^{31}P^{16}O_2^+$; $^{47}Ti^{16}O^+$; $^{28}Si^{35}Cl^+$; $^{51}V^{12}C^+$	Normal		
Fe	$^{57}Fe^+$ (2.12)	$^{40}Ar^{16}O^1H^+$; $^{40}Ca^{16}O^1H^+$; $^{40}K^{16}O^1H^+$	KED	3.0	
Hg	$^{202}Hg^+$ (22.86)	$^{86}W^{16}O^+$	Normal		
Mn	$^{55}Mn^+$ (100)	$^{40}Ar^{14}N^1H^+$; $^{37}Cl^{18}O^+$; $^{39}K^{16}O^+$	Normal		
Mo	$^{98}Mo^+$ (24.13)	$^{98}Ru^+$; $^{81}Br^{17}O^+$; $^{40}K_2{}^{18}O^+$; $^{58}Ni^{40}Ar^+$; $^{63}Cu^{35}Cl^+$	Normal		-0.10961x^{101}Ru
Ni	$^{60}Ni^+$ (26.22)	$^{44}Ca^{16}O^+$; $^{43}Ca^{16}O^1H^+$; $^{23}Na^{37}Cl^+$; $^{25}Mg^{35}Cl^+$; $^{28}Si^{16}O_2^+$	Normal		
Pb	$^{208}Pb^+$ (52.40)		Normal		
Sb	$^{121}Sb^+$ (57.21)	$^{107}Ag^{14}N^+$; $^{109}Ag^{12}C^+$; $^{105}Pd^{16}O^+$; $^{81}Br^{40}Ar^+$; $^{120}Sn^1H^+$	Normal		
Se	$^{82}Se^+$ (8.73)	$^{82}Kr^+$; $^{81}Br^1H^+$; $^{66}Zn^{16}O^+$; $^{68}Zn^{14}N^+$; $^{164}Dy^{2+}$; $^{65}Cu^{16}O^1H^+$	KED	3.5	-0.00783x^{83}Kr
Tl	$^{205}Tl^+$ (70.26)	$^{189}Os^{16}O^+$	Normal		
Zn	$^{66}Zn^+$ (27.90)	$^{50}Ti^{16}O^+$; $^{34}S^{16}O_2^+$; $^{132}Ba^{2+}$; $^{50}Cr^{16}O^+$; $^{65}Cu^1H^+$; $^{26}Mg^{40}Ar^+$; $^{31}P^{35}Cl^+$; $^{52}Cr^{14}N^+$	Normal		

Within the elements kept into consideration in this study, three of them (i.e. ^{27}Al, ^{75}As, ^{55}Mn) are monoisotopic but, for the remaining twelve elements, the candidate isotope for quantification should ensure both the highest abundance as well as the absence of any severe interferences. Although ^{57}Fe is a minority isotope with respect to ^{56}Fe, it does not suffer the very hard molecular interference by $^{40}Ar^{16}O^+$ which affects the signal at m/z = 56. However, also working on ^{57}Fe isotope, it has been chosen to operate with the kinetic energy discrimination (KED) mode, due to the demonstrated presence of a spectral interference, likely ascribable to $^{1}H^{40}Ar^{16}O^+$. The choice of the best analytical isotope for Cr is critical, because its four stable

isotopes are all potentially prone to be interfered by molecular ions. Hence, ^{52}Cr has been chosen only because of its highest abundance (83.79%) The possible, but unlikely, interferences of $^{38}Ar^{14}N^{+}$, $^{1}H^{35}Cl^{16}O^{+}$ and, mainly, the known and severe interference given by $^{40}Ar^{12}C^{+}$ have, in this case, required the use of the KED mode of analysis in the determination of $^{52}Cr^{+}$. For each of these elements, ^{66}Zn, ^{121}Sb, ^{202}Hg, and ^{205}Tl are the most abundant natural isotopes. None of them are interfered isobarically (elemental ion on elemental ion), but many of them might undergo interferences by oxide ions, that however have not been observed in these cases. On the other hand, the interference by polyatomic ions was ascertained for five of the fifteen elements: beyond ^{57}Fe and ^{52}Cr, this happens also for ^{27}Al, ^{75}As and ^{82}Se. In these last cases, the minimization of spectral interference was accomplished using optimized fluxes of He for each element (between 3.0 and 4.0 cm^3 min^{-1}). The remaining elements, for which no meaningful spectral interference was substantiated, were analysed in normal mode. A correction equation was used for considering the isobaric interferences of $^{98}Ru^{+}$ on $^{98}Mo^{+}$ and of $^{82}Kr^{+}$ on $^{82}Se^{+}$, respectively.

7) Optimization of the IC conditions for the separation of the As species

The first step of the assessment of the chromatographic separation is the choice of both stationary and mobile phases. As far as the As speciation is concerned, the ion chromatography approach is largely preferred to the RP-HPLC one. Columns like AS16, IC-Pak Anion HR, Ion Pac AS7 & AG7, and – mainly – Hamilton PRP-X100 have been used for this purpose. In particular, the PRP-X100 column provided an excellent separation at the baseline level of the main As chemical species in a chromatographic run typically in less than 10 minutes, and for this reason it has been chosen to assess this method. The mobile phase most frequently used with the PRP-X100 column is an aqueous solution of ammonium dihydrogen phosphate, normally used in concentration in the range between units of mmol dm^{-3} and a few tens of mmol dm^{-3}, and at pH values between 5.5 and 6.5. The optimization of these parameters, performed in terms of peaks resolution and length of the chromatographic run, provided a concentration of ammonium dihydrogen phosphate of 20 mmol dm^{-3} and a pH value of 5.8.

Table D3. IC conditions for the chromatographic separation of As(III), As(V), DMA and MMA in extracts of rice flour and soils.

Instrument: Agilent 1200 Series Gradient HPLC system	
Precolumn	Hamilton PRP X-100 (20 mm x 2.0 mm, 10 m)
Stationary phase	Anionic exchange Hamilton PRP X-100 (250 mm x 4.1 mm, 10 m)
Mobile phase	$NH_4H_2PO_4$ 20 mmol dm^{-3}, pH=5.8 (adjusted with NH_3 25%)
Elution mode	Isocratic
Temperature	20°C
Injection volume (cm^3)	0.100
Flow rate ($cm^3 \cdot min^{-1}$)	1.5
Pressure (MPa)	13 (130 bar)

8) Optimized ICP-MS conditions for the detection of the As species

Table D4. ICP-MS parameters for the determination of As species in rice and in soils.

Instrument: Agilent 7500ce			
RF power generator (W)	1550	**KED gas flow (cm^3 min^{-1})**	2.7
Ar plasma flow (dm^3 min^{-1})	18.00	**Masses of optimization**	^{7}Li, ^{89}Y and ^{205}Tl
Ar auxiliary flow (dm^3 min^{-1})	0.94	**Dwell time (s)**	2.0
Ar nebulizer flow (dm^3 min^{-1})	0.85	**Number of points per peak**	3
Ar make-up flow (dm^3 min^{-1})	0.32	**Acquisition time (s)**	3
Nebulizer	Bergener Ari Mist HP type, glass	**QP/OctP Bias difference (V)**	3
Spray chamber	Scott-type, quartz	**Quantification mass**	^{75}As
Skimmer and sampling cones	Nickel	**Quantification**	Ext. calibration
Sampling depth (mm)	8.0	**As signal processing**	Peak area (counts)
KED gas	He, 99.999%		

References of the Appendix D

SM1 Tokunaga, S.; Hakuta, T. Acid Washing and Stabilization of an Artificial Arsenic-Contaminated Soil. *Chemosphere* **2002**, *46*, 31–38. https://doi.org/10.1016/s0045-6535(01)00094-7.

SM2 Hsieh, M.-W.; Liu, C.-L.; Chen, J.-H.; Jiang, S.-J. Speciation Analysis of Arsenic and Selenium Compounds by CE-Dynamic Reaction Cell-ICP-MS. *Electrophoresis*, **2010**, *31*, 2272–2278. https://doi.org/10.1002/elps.200900632

APPENDIX E

1) Miscellaneous

An electronic millivoltmeter model 210A (Thermo Scientific Orion, Chelmsford, USA) connected to 1) a model 805/SPG/12 Pt electrode and a model 303/SCG/12 calomel electrode or 2) a model GG/12 combined pH electrode, all by Amel Instruments (Milan, Italy), was used for the measurements of both redox potential and pH. Samples were homogenized and ground employing an agate mortar pestle grinder model SFM-8 (TOB New Energy Technology, Xiamen, China). A Rotonda centrifuge model 460RS (Hettich, Tuttlingen, Germany) was used for separating the solid fraction from the aqueous extracts, whereas a rotating mixer for tubes model MRH-04 (SBS, Barcelona, Spain) was used for continuously shaking the centrifugation tubes during the extraction process.

2) Chemicals and standards

Type-I ultrapure water (resistivity > 18 MΩ cm-1, total organic carbon < 30 µg dm-3), always used in this study, was prepared using a Milli-Q® IQ 7003 system (Millipore, Vimodrone, Italy). Nitric acid (69%, Hiperpur) and aqueous ammonia (30%, ACS) were from Panreac AppliChem, Castellar del Vallès, Spain, hydrogen peroxide (30%, ULTREX II) was from JTBaker, Rodano, Italy, dihydrogen ammonium phosphate (>99.5%, BioUltra) was from Merck, Darmstadt, Germany. As(III) and As(V) stock solutions in water (1000 mg dm-3) were purchased by Fluka (Rodano, Italy) and by Merck (Darmstadt, Germany), respectively. Stock aqueous solutions of organic species of As (1000 mg dm-3) were prepared to weigh the needed amount of the pure compounds (>99.0%, Sigma-Aldrich, St. Louis, USA) or disodium methyl arsonate hexahydrate (98%, Supelco, St. Louis, Missouri, USA, for DMA and MMA, respectively), and dissolving them in water. All stock solutions were stored at 4°C, while the working solutions were daily prepared by a proper dilution of the relevant stock solutions. NCSZ 11007 and ERM-BC211 certified rice flours were produced by the China National Analysis Centre (Beijing, China) and the European Commission's Joint Research Centre – Institute for the Reference Materials and Measurements (Geel, Belgium), respectively. Finally, the ICP-MS Stock Tuning Solution and the internal standard solution (a 20 µg dm−3 of Be, Rh, and Tl in a 1% (v/v) aqueous solution of nitric acid) were from Agilent Technologies (Santa Clara, CA, USA), while the ICP-MS Setup Solution, the KED Setup Solution

and the internal standard solution (a 10 µg dm−3 of Rh in a 1% (v/v) aqueous solution of nitric acid) were all from Perkin Elmer (Milan, Italy).

Table E1. Rice yields (t ha-1 ± SD), 13% moisture, n = 4, for twenty-six genotypes irrigated by continuous flooding (CF), saturation (SA) and sprinkler (SP).

Rice Genotype	CF	SA	SP
Aleramo	10.7 ± 0.8^{aa}	11.4 ± 0.6^{aa}	10.9 ± 0.1^{aa}
Antares	9.4 ± 0.6^{cb}	11.4 ± 0.4^{ac}	10.8 ± 0.6^{ba}
Apollo	10.8 ± 0.3^{aa}	11.2 ± 0.4^{aa}	10.9 ± 0.5^{aa}
Balilla	12.9 ± 0.6^{aa}	12.5 ± 0.7^{aa}	12.4 ± 0.7^{aa}
Brio	11.8 ± 0.4^{aa}	12.3 ± 0.8^{aa}	12.4 ± 0.5^{aa}
Carnaroli	10.6 ± 0.7^{aa}	10.2 ± 0.3^{aa}	9.7 ± 0.9^{aa}
Carnise	10.1 ± 0.7^{ab}	10.0 ± 0.3^{ba}	8.9 ± 0.6^{bb}
Cerere	9.4 ± 0.5^{bc}	10.5 ± 0.4^{ca}	10.9 ± 0.5^{ab}
CRV-04	11.4 ± 0.6^{ab}	10.9 ± 0.4^{aa}	10.6 ± 0.2^{ba}
CRV-108	10.8 ± 0.4^{ba}	10.1 ± 0.4^{ab}	10.5 ± 0.6^{aa}
CVR-114	11.2 ± 0.4^{aa}	11.5 ± 0.4^{aa}	10.9 ± 0.6^{aa}
CVR-390	11.7 ± 0.4^{aa}	11.6 ± 0.8^{aa}	11.9 ± 0.7^{aa}
Galileo	10.7 ± 0.6^{aa}	10.1 ± 0.3^{aa}	10.8 ± 0.6^{aa}
Gloria	11 ± 1^{aa}	10.5 ± 0.3^{aa}	9.8 ± 0.7^{aa}
Luxor	10.2 ± 0.8^{ca}	13 ± 1^{dc}	10.9 ± 0.1^{ad}
Musa	11.8 ± 0.7^{aa}	11.4 ± 0.6^{aa}	12 ± 1^{aa}
Oceano	9.6 ± 0.7^{aa}	9.6 ± 0.4^{aa}	9.9 ± 0.4^{aa}
Opale	10.2 ± 0.5^{ab}	10.1 ± 0.3^{ca}	11.0 ± 0.2^{bc}
Orione	10.3 ± 0.6^{aa}	10.1 ± 0.4^{aa}	10.8 ± 0.5^{aa}
Ronaldo	11.4 ± 0.1^{bc}	12.0 ± 0.3^{ab}	12.1 ± 0.3^{ca}
Salvo	10.1 ± 0.4^{aa}	10 ± 1^{aa}	10.4 ± 0.3^{aa}
Selenio	12.6 ± 0.5^{bc}	11.5 ± 0.4^{ab}	10.8 ± 0.5^{ca}
Sprint	11.8 ± 0.2^{ca}	11.1 ± 0.3^{cc}	11.9 ± 0.5^{ac}
Thaibonnet	10.7 ± 0.5^{ba}	11.5 ± 0.1^{aa}	12 ± 1^{ab}
Urano	8.2 ± 0.7^{bb}	9.6 ± 0.4^{ab}	10.1 ± 0.8^{ba}
Virgo	12.2 ± 0.5^{ab}	11.6 ± 0.2^{aa}	12.4 ± 0.5^{ba}
Average	11 ± 3^{aa}	11 ± 3^{aa}	11 ± 3^{aa}

SD, standard deviation, Indica rice genotypes are reported in *italic character*. Results for each yield marked with the same letter are not significantly different (two-tail t-test, $p > 0.05$).

Table E2. Concentrations (in µg kg^{-1}) of As species in twenty-six rice genotypes irrigated by CF, n = 4.

Genotype	Total As	Total extracted As	As(III)	As(V)	iAs	DMA	MMA	Sum of speciated As [a]	Extraction efficiency [b] (%)	Column recovery [c] (%)
Aleramo	160±20	160±2	55.4±0.3	<0.2	55.4±0.3	104.3±0.3	<0.16	160±4	100	100
Antares	192±7	179.1±0.2	54±1	<0.2	54±1	109±6	<0.16	163±6	93	91
Apollo	109±8	103±5	70±20	<0.2	70±20	34±1	<0.16	100±20	94	97
Balilla	140±10	121±1	115±2	<0.2	115±2	18±1	<0.16	133±3	86	110
Brio	190±40	183.9±0.3	87±4	<0.2	87±4	73±3	<0.16	160±6	97	87
Carnaroli	197±3	168±2	120±10	<0.2	120±10	27.5±0.2	<0.16	150±10	85	89
Carnise	192±7	180±20	72.2±0.2	<0.2	72.2±0.2	104±3	<0.16	176±3	94	98
Cerere	180±20	190±4	95±7	<0.2	95±7	95±9	<0.16	190±10	106	100
CRV 04	160±20	159±2	70±10	<0.2	70±10	70±10	<0.16	140±20	99	88
CRV 108	110±10	105.3±0.1	74±6	<0.2	74±6	24±3	<0.16	98±9	96	93
CRV 114	124±3	124±5	50±2	<0.2	50±2	74±1	<0.16	124±3	100	100
CRV 390	180±20	170±2	71±1	<0.2	71±1	68.7±0.5	<0.16	140±2	94	82
Galileo	150±10	139±5	46±7	<0.2	46±7	90±10	<0.16	136±10	93	98
Gloria	121±5	128±3	56±1	<0.2	56±1	71±2	<0.16	127±2	106	99
Luxor	140±10	131±2	67.1±0.3	<0.2	67.1±0.3	65±7	<0.16	132±1	94	101
Musa	170±30	159±6	68±1	<0.2	68±1	91±1	<0.16	159±2	94	100
Oceano	150±20	119.2±0.3	46±4	<0.2	46±4	74.4±0.9	<0.16	120±6	79	101
Opale	160±20	150±20	50±2	<0.2	50±2	100±20	<0.16	150±20	94	100
Orione	160±20	164±5	79.7±0.3	19±1	99±1	70±20	<0.16	170±20	102	104
Ronaldo	140±10	142±3	75±4	<0.2	75±4	64±2	<0.16	139±6	101	98
Salvo	130±20	110±10	51.1±0.4	<0.2	51.1±0.4	50±20	<0.16	100±20	85	91
Selenio	140±10	137±5	80±1	<0.2	80±1	56.7±0.6	<0.16	137±2	98	100
Sprint	170±10	167±2	55±2	13±1	68±2	94±3	<0.16	162±4	98	97
Thaibonnet	150±20	144±3	64.3±0.1	<0.2	64.3±0.1	80±10	<0.16	140±10	96	97
Urano	130±10	135.2±0.6	77±3	<0.2	77±3	49.2±0.1	<0.16	126±4	104	93
Virgo	160±10	150±7	84±2	<0.2	84±2	58.4±0.3	<0.16	142±3	94	95

In *Italics*, rice genotypes belonging to Indica subspecies. Data preceded by the < sign are below the relevant limit of detection, LoD. [a] Sum of speciated As = As(III)+As(V)+DMA+MMA, data rounded according to the standard deviations; [b] extraction efficiency = (Total extracted As)/(Total As) ratio; [c] column recovery = (Sum of speciated As)/(Total extracted As) ratio.

Table E3. Concentrations (in µg kg^{-1}) of As species in twenty-six rice genotypes irrigated by SA, n = 4.

Genotype	Total As	Total extracted As	As(III)	As(V)	iAs	DMA	MMA	Sum of speciated As [a]	Extraction efficiency [b] (%)	Column recovery [c] (%)
Aleramo	70±8	71.5±0.2	65.6±0.1	<0.2	65.6±0.1	<0.3	<0.16	65.6±0.1	102	92
Antares	56±7	59±4	59±1	<0.2	59±1	<0.3	<0.16	59±1	105	100
Apollo	31±3	30.7±0.5	30.7±0.4	<0.2	30.7±0.4	<0.3	<0.16	30.7±0.4	99	100
Balilla	69±3	67.4±0.1	62±3	<0.2	62±3	<0.3	<0.16	62±3	98	92
Brio	54±4	58.4±0.3	59±4	<0.2	59±4	<0.3	<0.16	59±4	108	101
Carnaroli	60±10	58±8	58±1	<0.2	58±1	<0.3	<0.16	58±1	97	100
Carnise	84±9	83±7	76±4	<0.2	76±4	<0.3	<0.16	76±4	99	92
Cerere	61±7	59±3	59±4	<0.2	59±4	<0.3	<0.16	59±4	97	100
CRV 04	60±4	58±5	58±2	<0.2	58±2	<0.3	<0.16	58±2	97	100
CRV 108	43±6	42.1±0.2	42.1±0.4	<0.2	42.1±0.4	<0.3	<0.16	42.1±0.4	98	100
CRV 114	42±2	42±3	42±1	<0.2	42±1	<0.3	<0.16	42±1	100	100
CRV 390	67±4	57±1	57.0±0.6	<0.2	57.0±0.6	<0.3	<0.16	57.0±0.6	85	100
Galileo	42±9	47±2	40±5	<0.2	40±5	<0.3	<0.16	40±5	112	85
Gloria	50±10	41±7	40±2	<0.2	40±2	<0.3	<0.16	40±2	82	98
Luxor	62±9	64.4±0.3	56±3	<0.2	56±3	<0.3	<0.16	56±3	104	87
Musa	58±7	58.0±0.2	58±4	<0.2	58±4	<0.3	<0.16	58±4	100	100
Oceano	53±2	54.3±0.3	54±1	<0.2	54±1	<0.3	<0.16	54±1	102	99
Opale	53±1	58±1	53±2	<0.2	53±2	<0.3	<0.16	53±2	109	91
Orione	41±6	41±3	41±9	<0.2	41±9	<0.3	<0.16	41±9	100	100
Ronaldo	36±5	39.1±0.2	39.5±0.9	<0.2	39.5±0.9	<0.3	<0.16	39.5±0.9	109	101
Salvo	38±6	36±2	36±3	<0.2	36±3	<0.3	<0.16	36±3	95	100
Selenio	68±7	67.2±0.6	67±2	<0.2	67±2	<0.3	<0.16	67±2	99	100
Sprint	46±3	41±2	41±5	<0.2	41±5	<0.3	<0.16	41±5	89	100
Thaibonnet	42±4	41±3	36.6±0.8	<0.2	36.6±0.8	<0.3	<0.16	36.6±0.8	98	89
Urano	34±5	33±1	32±8	<0.2	32±8	<0.3	<0.16	32±8	97	97
Virgo	41±5	39±2	37±2	<0.2	37±2	<0.3	<0.16	37±2	95	95

In *Italics*, rice genotypes belonging to Indica subspecies. Data preceded by the < sign are below the relevant limit of detection, LoD. [a] Sum of speciated As = As(III)+As(V)+DMA+MMA, data rounded according to the standard deviations; [b] extraction efficiency = (Total extracted As)/(Total As) ratio; [c] column recovery = (Sum of speciated As)/(Total extracted As) ratio.

Table E4. Concentrations (in µg kg^{-1}) of As species in twenty-six rice genotypes irrigated by SP, n = 4.

Genotype	Total As	Total extracted As	As(III)	As(V)	iAs	DMA	MMA	Sum of speciated As [a]	Extraction efficiency [b] (%)	Column recovery [c] (%)
Aleramo	6±2	6.08±0.01	1.6±0.1	4.4±0.1	6.0±0.1	<0.3	<0.16	6.0±0.1	101	99
Antares	7±2	6.9±0.2	1.1±0.4	4.8±0.7	5.9±0.7	<0.3	<0.16	5.9±0.7	99	86
Apollo	18±4	16.0±0.2	5.4±0.2	9.5±0.3	14.9±0.3	<0.3	<0.16	14.9±0.3	89	93
Balilla	14±3	14.0±0.1	3.3±0.2	10.6±0.9	13.9±0.2	<0.3	<0.16	13.9±0.2	100	99
Brio	11±2	10.8±0.5	<0.04	10.6±0.1	10.7±0.1	<0.3	<0.16	10.7±0.1	98	99
Carnaroli	8±1	7.5±0.3	1.7±0.1	4.4±0.3	6.1±0.3	<0.3	<0.16	6.1±0.3	94	89
Carnise	1.5±0.6	1.47±0.02	<0.04	1.3±0.1	1.3±0.1	<0.3	<0.16	1.3±0.1	98	88
Cerere	2.4±0.5	2.3±0.3	0.4±0.1	1.8±0.1	2.2±0.1	<0.3	<0.16	2.2±0.1	96	96
CRV 04	7±2	6.1±0.3	2.0±0.2	4±1	6±1	<0.3	<0.16	6±1	87	98
CRV 108	2.1±0.6	1.9±0.02	<0.04	1.7±0.2	1.7±0.2	<0.3	<0.16	1.7±0.2	90	89
CRV 114	3±1	2.8±0.2	<0.04	2.8±0.1	2.8±0.1	<0.3	<0.16	2.8±0.1	93	100
CRV 390	6.0±0.8	5.7±0.3	<0.04	5.6±0.1	5.6±0.1	<0.3	<0.16	5.6±0.1	95	98
Galileo	1.5±0.6	1.4±0.2	<0.04	1.4±0.3	1.4±0.3	<0.3	<0.16	1.4±0.3	93	100
Gloria	3±1	2.8±0.4	<0.04	2.7±0.3	2.7±0.3	<0.3	<0.16	2.7±0.3	93	96
Luxor	1.4±0.1	1.6±0.3	<0.04	1.4±0.3	1.4±0.3	<0.3	<0.16	1.4±0.3	114	88
Musa	6.0±0.8	5.4±0.1	<0.04	5.3±0.1	5.3±0.1	<0.3	<0.16	5.3±0.1	90	98
Oceano	4±1	3.7±0.4	<0.04	3.68±0.02	3.68±0.02	<0.3	<0.16	3.68±0.02	92	99
Opale	7.0±0.2	6.6±0.9	<0.04	6.5±0.1	6.5±0.1	<0.3	<0.16	6.5±0.1	94	98
Orione	2.1±0.6	2.0±0.3	0.5±0.1	1.5±0.2	2.0±0.2	<0.3	<0.16	2.0±0.2	95	100
Ronaldo	4.0±0.1	3.8±0.2	<0.04	3.7±0.1	3.71±0.01	<0.3	<0.16	3.71±0.01	95	98
Salvo	1.4±0.5	1.22±0.02	<0.04	1.2±0.6	1.2±0.6	<0.3	<0.16	1.2±0.6	87	98
Selenio	6±2	5.5±0.1	<0.04	5.3±0.2	5.3±0.2	<0.3	<0.16	5.3±0.2	92	96
Sprint	7±2	6.6±0.2	<0.04	6.6±0.1	6.6±0.1	<0.3	<0.16	6.6±0.1	94	100
Thaibonnet	2.0±0.8	1.8±0.1	0.2±0.1	1.6±0.2	1.8±0.2	<0.3	<0.16	1.8±0.2	90	100
Urano	6±1	6.6±0.5	<0.04	6.1±0.3	6.1±0.3	<0.3	<0.16	6.1±0.3	110	92
Virgo	2.0±0.8	1.5±0.2	<0.04	1.2±0.1	1.2±0.1	<0.3	<0.16	1.2±0.1	75	80

In *Italics*, rice genotypes belonging to Indica subspecies. Data preceded by the < sign are below the relevant limit of detection, LoD. [a] Sum of speciated As = As(III)+As(V)+DMA+MMA, data rounded according to the standard deviations; [b] extraction efficiency = (Total extracted As)/(Total As) ratio; [c] column recovery = (Sum of speciated As)/(Total extracted As) ratio.

Figure E1. Relationships between the amounts of the total extracted As and DMA (blue dots) or iAs (red dots) in twenty-six rice genotypes irrigated by CF.