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MULTIELEMENT PROFILING BY ICP-MS IN FOOD TRACEABILITY

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Esame finale anno 2017

To my

Mother

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List of abbreviations

AAS	Atomic absorption spectroscopy
AES	Atomic Emission spectroscopy
AMS	Accelerator mass spectrometry
amu	Atomic mass unit
ANNs	Artificial neural networks
AOC	Appellation d'Origine Contrôlée
CCDs	Charge coupled devices
CE	Central Europe
CE	Capillary electrophoresis
CRC	Collision/Reaction cells
EC	European Commission
ED	Energy discrimination
EFSA	European Food Safety Authority
ETAAS	Electrothermal Atomic Absorption Spectrometry
EU	European Union
FAAS	Flame atomic absorption spectroscopy
FAO	Food and Agriculture Organization
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
FTICR	Fourier transform ion cyclotron resonance
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GFAAS	Graphite furnace atomic absorption spectroscopy
HCA	Hierarchical Cluster Analysis
HEPA	High Efficiency Particulate Air filter
HPLC	High performance liquid chromatography
ICP	Inductively coupled plasma
ICP-AES	Inductively coupled plasma Atomic emission spectroscopy
ICP-FTICR-MS	Inductively Coupled Plasma-Fourier transform ion cyclotron resonance-mass spectrometers

ICP-MS	Inductively coupled plasma Mass spectrometry
ICP-OES	Inductively coupled plasma Optical emission spectrometry
ICP-QQQ	Inductively coupled plasma Triple Quadrupole
ICP-SFMS	Inductively Coupled Plasma Sector field mass spectrometry
INAA	Instrumental neutron-activation analysis
IR	Infrared spectroscopy
IRMS	Isotope ratio mass spectrometry
IS	Internal Standard
ISO	International Organization for Standardization
KNN	K nearest neighbor method
LA-ICP-MS	Laser Ablation-Inductively coupled plasma mass spectrometry
LDA	Linear discriminant analysis
LIMS	Laser Ionization Mass Spectrometry
LODs	Limit of Detection
LOQs	Limit of Quantification
m/z	mass-to-charge ratio
MC-ICP-MS	Multicollector-Inductively Coupled Plasma Mass Spectrometer
N	Number of observations
NI	Northern Italy
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance spectroscopy
OES	Optical Emission Spectrometry
ORS3	Octopole Reaction System
PCA	Principal component Analysis
PCR	Polymerase chain reaction technique
PCs	Principal components
PDO	Protected Designation of Origin
PFA	Perfluoroalkoxy
PGI	Protected Geographical Indication
PLC	Programmable Logic Controller
PLS	Partial least squares
PTFE	Polytetrafluoroethylene
PTR-MS	Proton transfer reaction mass spectrometry
Q1	First Quadrupole

Q2	Second Quadrupole
QA	Quality assurance
QC	Quality control
QCs	Quality control standards
R^2	Coefficient of determination
RASFF	Rapid Alert System for Food and Feed
REEs	Rare Earth Elements
RF	Radio-frequency
RFID	Radio-frequency identification
RSD	Relative standard deviations
SD	Standard deviation
SI	Southern Italy
SIMCA	Soft independent modeling of class analogy
SIMS	Secondary ion mass spectrometry
SRM	Standard Reference Materials
SSMS	Spike ion source mass spectrometry
TOF	Time-of-flight
TSG	Traditional Specialities Guaranteed
UN	United Nations
UNI	Italian Standards Institute
XRF	X-ray fluorescence spectrometry

List of symbols

µg	Microgram
Al	Aluminium
Ar	Argon
As	Arsenic
Ba	Barium
Bi	Bismuth
C	Carbon
Ca	Calcium
Cd	Cadmium
Ce	Cerium
Cl	Chlorine
Co	Cobalt
CO_2	Carbon Dioxide
Cr	Chromium
Cs	Caesium
Cu	Copper
Dy	Dysprosium
Er	Erbium
Eu	Europium
Fe	Iron
g	Gram
Gd	Gadolinium
Ge	Germanium
H_2	Hydrogen
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
He	Helium
Hg	Mercury
HNO_3	Nitric acid
Ho	Holmium
I	Iodine

In	Indium
K	Potassium
Kg	Kilogram
La	Lanthanum
Li	Lithium
Lu	Lutetium
Mg	Magnesium
mg	milligram
Mn	Manganese
Mo	Molybdenum
Na	Sodium
Nd	Neodymium
ng	Nanogram
NH_3	Ammonia
Ni	Nickel
nm	nanometer
O_2	Oxygen
P	Phosphorus
Pb	Lead
Pm	Promethium
Pr	Praseodymium
Rb	Rubidium
S	Sulfur
Sc	Scandium
Se	Selenium
Sm	Samarium
Sn	Tin
Sr	Strontium
Tb	Terbium
Th	Thorium
Ti	Titanium
Tl	Thalium
Tm	Thulium
U	Uranium

V	Vanadium
Y	Yttrium
Yb	Ytterbium
Zn	Zinc
σ	Standard deviation

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Summary

Fingerprinting techniques based on elemental composition and multivariate statistical analysis of compositional data (Di Salvo, Fadda, Sanguinetti, Naes, & Del Caro, 2014; Ku et al., 2010; Maietti et al., 2012; Preys, Vigneau, Mazerolles, Cheynier, & Bertrand, 2007) can be used for the identification and classification of a specific agricultural product according to its geographical provenance (Coetzee et al., 2005). The analytical approach assumes that the elemental composition of an agricultural product such as for example, wine (Baxter, Crews, Dennis, Goodall, & Anderson, 1997; Jakubowski, Brandt, Stuewer, Eschnauer, & Gortges, 1999; Thiel, Geisler, Blechschmidt, & Danzer, 2004), coffee, tea, olive oil, and fruit juice (Ogrinc, N, Košir, IJ, Spangenberg, JE, Kidrič, 2003) will reflect the composition of the soil on which they are cultivated, the practices to which they are subjected and to the local environmental conditions thanks to biogeochemical cycling.

The geographically/geologically sensitive parameters such as trace element composition are of significant relevance in order to characterize and subsequently identify the origin of a given food product.

In recent years, many serious disease appeared related to foodstuff, so providing the motivation for the scientific community to work more intensively in this area (Drivelos & Georgiou, 2012). Moreover, consumers have renewed their interest in food strongly identified with a place of origin. The reason for this increasing interest varies from the global trend for organic products to the concern about animal welfare and environmental friendly methods of production (Luykx & van Ruth, 2008). Thus the consumers demand food products of high quality and one of the basic parameters is the knowledge of the geographical origin (Drivelos & Georgiou, 2012).

In this framework, proof of provenance has become an important issue, in accordance with national legislation and international standards. based on EU has been supporting the potential of differentiating quality products on a regional basis (Luykx & van Ruth, 2008). The EC regulations N. 509/2006, 510/2006, 1898/2006, protect consumers through a system of effective and impartial control that defines the safeguard of the Protected Designation of Origin (PDO), the Protected Geographical Indication (PGI) and the Traditional Specialties Guaranteed (TSG). The typical foodstuffs with PDO are wines, cheeses, olive oils, honey, beers, meats and potatoes (Sacco et al., 2009). These

indications are particularly important in Europe, where there has been a long tradition of associating certain food products with particular regions (Luykx & van Ruth, 2008).

Consequently, the determination and measurement of multi-element concentrations (Benincasa, Lewis, Perri, Sindona, & Tagarelli, 2007; Benincasa, Lewis, Sindona, & Tagarelli, 2008; Brescia, Monfreda, Buccolieri, & Carrino, 2005) in selected regional products may provide unique compositional fingerprint for characterizing their geographical origin (Kelly, Heaton, & Hoogewerff, 2005).

The aim of this PhD thesis have been firstly to set up a high level analytical facility for elemental analysis based on a triple quadrupole ICP-MS and subsequently to test and apply this approach to a selected set of food products. In this framework the chosen food has been milk from different farms and regions. The samples analyzed were characterized in term of trace and ultra-trace elements. The results were validated through proper quality control practices based upon the use of certified reference materials, while the determined elemental array of each cow milk sample was tested through chemometric analysis (in particular multivariate tools were chosen) to investigate the potential for associating milk inorganic fingerprinting to a given geographical origin on a regional basis. Milk is known as the most complete food in human diet, because it provides all the necessary macronutrients (proteins, lipids and carbohydrates) and all the essential micronutrients (elements, vitamins and enzymes) (Ataro, McCrindle, Botha, McCrindle, & Ndibewu, 2008); on the other hand, it is one of the foodstuffs more often and historically exposed to adulteration (OJEC, 1990), such as for example by water addition, reducing its nutritional value and causing additional health problems.

In the field of food quality control, the monitoring of trace element contents in this kind of food is very important because of their nutritional and toxicological relevance in human health. For instance, Cr and Mn are essential elements but may become toxic at higher concentrations, while Pb and Cd are always toxic and their health effect can be cumulative (Ataro et al., 2008; Onianwa, Adetola, Iwegbue, Ojo, & Tella, 1999; Rivero Martino, Fernàndez Sànchez, & Sanz Medel, 2000). This is because they are readily transferred through the food chain and are known to serve any essential biological function (Z. P. Liu, 2003).

Moreover, recent improvements in routine analytical technique, the use of REE fertilization in agriculture, at least in East Asian agriculture, and the importance of these

elements as indicators in physiological processes and reactions have contributed to an increasing interest (Tyler, 2004).

Furthermore, the characteristics of cow milk are highly depending on the geographical region and farming practices in which the cows are grown. Thus the origin of a milk product is an important factor, affecting its quality (Sola-Larrañaga & Navarro-Blasco, 2009).

Mass spectrometry techniques, and in particular Inductively Coupled Plasma Mass Spectrometry (ICP-MS), is a powerful tool for the quali-quantitative determination of a range of metal and non-metal elements at low and ultra-low concentration levels, in chemical matrices (Abdrabo, Grindlay, Gras, & Mora, 2015; Dressler et al., 2012; Husáková et al., 2011; Leme, Bianchi, Carneiro, & Nogueira, 2014; Mamone, Picariello, Caira, Addeo, & Ferranti, 2009; Mir-Marqués, Domingo, Cervera, & De La Guardia, 2015; Mohd-Taufek et al., 2016; Muller et al., 2013; Wang et al., 2013).

In this thesis, a new Agilent 8800 Triple Quadrupole ICP-MS was set into operation, its management and utilization were optimized and applied for the determination of essential, trace and Rare Earth elements in cow milk samples from different origin (Amr, 2012).

The results were analyzed chemometrically using multivariate techniques with the aim of classifying cow milk samples according to different origin.

Chemometric methods coupled with Inductively coupled plasma mass spectrometry (ICP-MS) have proved to be an effective way to characterize food products from different geographical origin, providing a fingerprint of the element patterns in the samples (Benincasa et al., 2008; Sola-Larrañaga & Navarro-Blasco, 2009).

Introduction

Chapter 1 The Food traceability problem

1.1 Food Traceability: tracking and tracing

Food traceability is an important issue in food safety and quality control, with impacts on food security[1], its quantity and overall availability (Gonzalvez, Armenta, & de la Guardia, 2009).

Producing safe and high quality food is a prerequisite to ensure consumer health and successful domestic and international trade, and is critical to the sustainable development of national agricultural resources. Systems to trace food or feed products through specified stages of production, processing and distribution play a key role in assuring food safety (IAEA, 2011).

Traceability is the ability to trace and follow a food, feed, food-producing animal or substance going to be processed into a food, through all stages of production, processing and distribution, according to the Regulation EC N°178/2002 (European Parliament, 2002). Thus traceability can be defined as the history of a product in terms of the direct properties of that product and/or properties that are associated with that product once it has been subject to particular value-adding processes using associated production means and in associated environmental conditions (Regattieri, Gamberi, & Manzini, 2007).

It is important to distinguish between two different processes involved in the **traceability** of a product, such as the tracking and the tracing. The **tracking** is to follow the product path **forwards** from the starting point to wherever it currently is. The **tracing** is to follow the completed path **backwards** from its current point to where it began.

The two processes are obviously strongly interconnected, as shown in Figure 1.1.

According to the traceability system, the information concerning relationships at the origin/source may be used upstream in the supply chain (e.g., in the ordering process to define the requirements of an ordered product), or downstream (e.g., in delivery processes to specify the characteristics of products) (Regattieri et al., 2007).

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[1] Food security is a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an Food safety is a scientific discipline describing handling, preparation, and storage of food in ways that prevent foodborne illness. Codex Alimentarius, 1963, WHO and FAO.

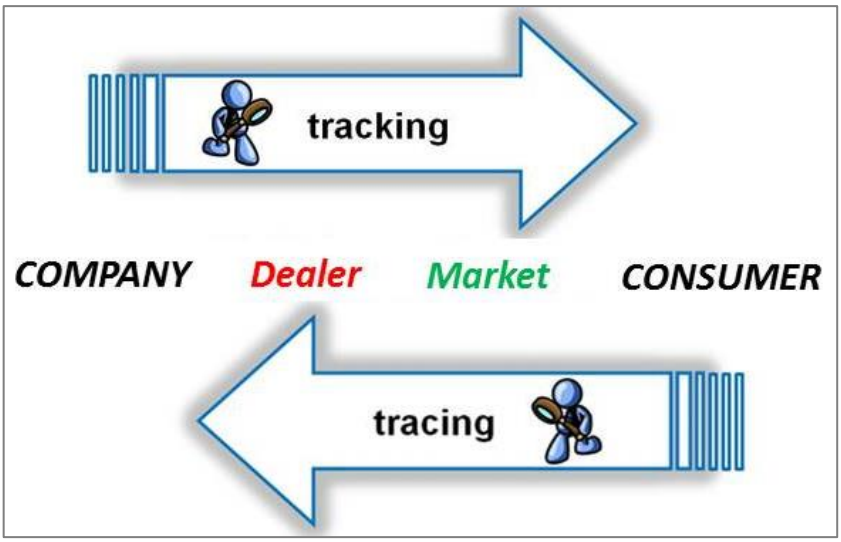

Figure 1.1 Diagram of the traceability system as the sum of the both tracking and tracing processes.

In order to ensure the safety of food, it is necessary to consider all aspects of the **food production chain** as a continuum from and including primary production and the production of animal feed up to and including sale or supply of food to the consumer because each element may have a potential impact on food safety.

In particular, product safety is the fundamental factor in the food sector that is making traceability relevant as recent studies on food safety show that about seven million people a year are affected by food borne illness (Sarig et al., 2003).

Only with an efficient tracing system is possible to have a prospective product recall and effective research into what caused the problems.

These concepts were reinforced by the EU through ‘‘The European White Paper on Food Safety” (European Commission, 2000) and by the FAO in ‘‘The Bangkok Declaration and Strategy on Aquaculture Development” (Naca/Fao, 2000).

Unfortunately, there is currently no general legal requirement for the establishment of traceability systems in food chains. The only mandatory traceability system currently enforced throughout a complete food chain enables beef on sale within the EU to be traced back to where it originated.

Therefore, in a context where traceability is basically voluntary, a small number of pioneer companies are developing their own systems, but they lack standards, are very differentiated, and are producing different economic results.

1.2 Food Traceability: Legal and Regulatory Aspects

The scientific and technical issues connected to food and feed safety are becoming increasingly important and complex.

In 1985 a UN General Assembly resolution gave rise to the Guidelines for consumer protection, which were published in 1986 (United Nation, 1986). These guidelines identify food as one of three priority areas of essential concern to consumer health. The Codex Alimentarius (FAO, 2006) evolved from these UN guidelines as the latter were selected as the reference point for the FAO Codex Alimentarius guidelines regarding food. While this codex also deals with quality issues, it reflects an emphasis on ensuring that consumers receive products that are safe and do not pose a threat to health.

The United States Department of Agriculture published ‘‘Traceability for Food Marketing and Food Safety: What’s the Next Step” (2002). The paper set out the case for voluntary traceability within the food industry, and then argues that government should ensure that the private sector meets performance targets for food safety, but there are no prescriptions and only suggestions on how this goal should be achieved.

The US National Institute of Standards and Technology (NIST) of the US Commerce Department developed the NIST Policy on Traceability, which presents the definition of measurement traceability used by NIST, and clarifies the roles of NIST and others in achieving results using their measure of traceability. Thus, the primary role of NIST is to assist its customers in establishing the measurement system of traceability and to assess the claims of traceability made by others, but in this case too, no proposals are formulated (Regattieri et al., 2007).

In 2002 EU General Food Law Regulation stated that a broad non descriptive traceability requirement would be introduced from 1st January 2005 (European Parliament, 2002).

The Regulation (EC) N° 178/2002 (European Parliament, 2002) lays down the general principles and requirements of food law and laying down procedures in terms of food safety.

In particular, the Regulation (EC) N° 178/2002 provides the basis for the assurance of a high level of protection of human health and consumers' interest in relation to food, taking into account in particular the diversity in the supply of food including traditional products, whilst ensuring the effective functioning of the internal market. It is applying to all stages of production, processing and distribution of food and feed.

In addition to a proposed EU regulation, several countries have introduced their own traceability regulations. For example, in Italy the Italian Standards Institute (UNI) has enacted specific legislative measures. Two specific standards have been issued: UNI 10939 ‘‘Traceability system in agricultural food chain – General principles for design and development” in April 2001, and UNI 11020 ‘‘Traceability system in agro-food industries – Principles and requirements for development” in December 2002 (Italian Standards Institute, 2001, 2002).

Other legislative acts have been introduced in several European countries, such as France, Spain and Greece, but they primarily relate to quality issues rather than food safety.

1.2.1 EFSA: European Food Safety Authority

https://www.efsa.europa.eu/

The Regulation (EC) N° 178/2002 institutes the European Food Safety Authority (EFSA). The agency was set up in 2002 following a series of food crises in the late 1990s to be a source of scientific advice and communication on risks associated with the food chain.

The establishment of EFSA reinforces the system of scientific and technical support which is no longer able to respond to increasing demands on it (European Union, 2002). In the European system of food safety, the risk assessment and management are two separate processes.

EFSA, as responsible for risk assessment, develop scientific opinions and expert advice in order to provide a solid foundation linked to legislative and policy making in Europe and to allow the European Commission, the European Parliament and the EU Member States to make timely and effective decisions in risk management.

The contact point between the Commission, EFSA, EEA and at national level in member countries, exchanging information in a clear and structured way by means of an online system, is the Rapid Alert System for Food and Feed (RASFF).

1.2.2 RASFF: Rapid Alert System for Food and Feed

http://ec.europa.eu/food/safety/rasff_en

The EU has one of the highest food safety standards in the world, largely thanks to the solid set of EU legislation in place, which ensures that food is safe for consumers. A key tool to ensure the cross-border follow of information to swiftly react when risks to public health are detected in the food chain is RASFF, the Rapid Alert System for Food and Feed (European Union, 2016). The legal basis of the RASFF is Regulation (EC) No 178/2002.

Created in 1979, RASFF enables information to be shared efficiently between its members (EU-28 national food safety authorities, Commission, EFSA, ESA, Norway, Liechtenstein, Iceland and Switzerland) and provides a round-the-clock service to ensure that urgent notifications are sent, received and responded to collectively and efficiently. Thanks to RASFF, many food safety risks had been averted before they could have been harmful to European consumers.

Whenever a member of the network has any information relating to the existence of a serious direct or indirect risk to human health deriving from food or feed, this information is immediately notified to the Commission under the RASFF. The Commission immediately transmits this information to the members of the network.

The scheme of the RASFF's work is reported in Figure 1.2.

Figure 1.2 The scheme of the shared information in the RASFF's work system.

1.3 Food Traceability: European Protected Food Names

The European Union Protected Food Names Schemes came into force in 1992 (2081/92, 1992) and offers an independent inspection and labelling system for the protection of food names on a geographical basis, comparable to the French system 'Appellation d'Origine Contrôlée (AOC)' used for wine (Kelly et al., 2005).

Agricultural products produced in the European Union (EU) reflect the rich diversity of different traditions and regions in Europe. To help protect and promote products with particular characteristics linked to their geographical origin as well as traditional products, the EU created quality logos, named "Protected Designation of Origin", "Protected Geographical Indication" and "Traditional Speciality Guaranteed". Product designations fall into two categories: those linked to a territory and those relating to a particular production method. Geographical indications and designations of origin are names identifying a product as originating in a given territory and testifying to a link between a given quality, reputation or characteristic of the product and its geographical origin (Drivelos & Georgiou, 2012).

The EC regulations No. 510/2006, 509/2006, 1898/2006, protect consumers through a system of effective and impartial control that defines the safeguard of the Protected Designation of Origin (PDO), the Protected Geographical Indication (PGI) and the Traditional Specialities Guaranteed (TSG).

1.3.1 Protected Designation of Origin (PDO)

PDO is the term used to describe foodstuffs, with a strong regional identity, that are produced, processed and prepared in a specific geographical area using prescribed techniques that may be unique to that region.

Well-known PDO products are Bordeaux PDO (France, wine), Cava PDO (Spain, wine), Manouri PDO (Greece, cheese), Tiroler Bergkäse PDO (Austria, cheese), Prés-salés du Mont-Saint-Michel PDO (France, fresh meat product) or Pistacchio verde di Bronte PDO (Italy, fruit) (European Commission (EC), 2007; The Council of the European Union, 2006b).

1.3.2 Protected Geographical Indication (PGI)

PGI covers agricultural products and foodstuffs closely linked to the geographical area. At least one of the stages of production, processing or preparation takes place in the area.

Typical products with recognized PGI are Sorrento Lemons PGI (Italy, fruit), Liliputas PGI (Lithuania, cheese), Gofio canario PGI (Spain, cereals product), Walbecker Spargel PGI (Germany, vegetable), České pivo PGI (Czech Republic, beer), Lammefjordskartofler PGI (Denmark, vegetable) or Primorska PGI (Slovenia, wine) (European Commission (EC), 2007; The Council of the European Union, 2006b).

1.3.3 Traditional Specialities Guaranteed (TSG)

A TSG does not refer to a specific geographical origin, but define traditional character, either in terms of production techniques or composition. Famous examples are Mozzarella cheese TSG (Italy, cheese), Kriek TSG (Belgium, beer), Hollandse maatjesharing TSG (Netherlands, fish product), File Elena TSG (Bulgaria, meat product) or Prekmurska gibanica TSG (Slovenia, cake) (European Commission (EC), 2007; The Council of the European Union, 2006a).

Two of these logos, such as the Protected Designation of Origin (PDO) and the Protected Geographical Indication (PGI), have a specific link to the region where the product comes from, while the third one, the Traditional Speciality Guaranteed (TSG), logo highlights a traditional production process.

Figure **1.3** shows food products with protected signature name in Europe in the past few years.

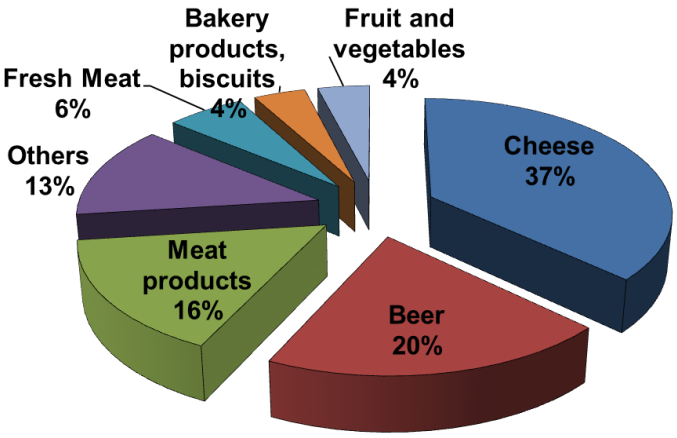

Figure 1.3 European food products with registered names (PDO, PGI and TSG) (Drivelos & Georgiou, 2012)

Manufacturers of Protected foods usually charge a premium for their produce due to increased production costs and consequently economic incentives exist to replace genuine articles with inferior ones for financial gain.

The typical foodstuffs with PDO are wines, cheeses, olive oils, honey, beers, meats and potatoes (Sacco et al., 2009).

These indications are particularly important in Europe, where there has been a long tradition of associating certain food products with particular regions (Luykx & van Ruth, 2008).

1.4 Food Traceability: Technical tools

A product traceability system requires the identification of all the physical entities and locations from which the product originates, that is to say, where it is processed, packaged, and stocked, and so this includes every agent in the supply chain (Regattieri et al., 2007).

In practice, different technical solutions can be used in a traceability system:

1. alphanumerical code;
2. bar code;
3. radio-frequency identification (RFID).

Alphanumerical codes are a sequence of numbers and letters of various sizes placed on labels, see Figure 1.4, which in turn are placed on the product or on its packaging. In the

food sector the latter of these two practices is the most suitable. The risk of data integrity corruption is very high.

Figure 1.4 An example of alphanumerical codes divided in company prefix and product cod number.

Today, alphanumerical codes are not frequently used because bar codes (see Figure 1.5) offer several significant advantages. The automation, the high speed, the great precision (it is a practically an error-free system) guaranteed by a bar code structure allows simpler, cheaper, and precise traceability systems.

Figure 1.5 An example of bar codes.

In addition to bar code technology, also a radio frequency based identification system (RFID) is available. RFID is an identification tool using wireless microchips to create tags that do not need any physical contact or any particular alignment with the reader. The reading phase is very fast and fully automated.

RFID tags (see Figure 1.6) are very small (a few millimeters reading distance) and they have no compatibility problem with foods.

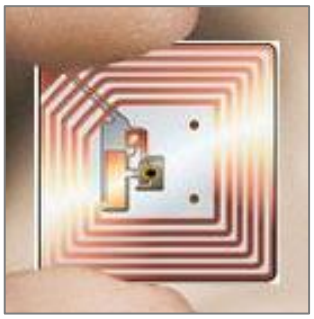

Figure 1.6 An example of microchips using in RFID.

Thus, the best technical instruments to use for a product traceability system are bar codes and RFID systems. In particular, RFID presents very favorable properties for the food sector, but the tag cost remains a problem.

Final choice must consider the degree of compatibility with the product and the production process, the degree of automation supported by the supply chain analyzed, and in general knowledge along the supply-production chain.

Such technical tools are typically used in traceability systems based on a continuous "paper-trail" and effective labelling. All stakeholders involved in the production process from raw materials, semi-finished products, accessories etc., must be traceable by means of a management that identifies the label with a code characterizing all steps of the supply chain. Food products are very critical, and their traceability system must be particularly complete.

1.5 Food Traceability: Analytical tools

The traceability of the supply chain in food industry/market is the result of internal traceability processes to each operator in the chain, linked by efficient communication flows. Applying food traceability techniques can reduce food losses by minimizing recalls of food consignments if the production region can be identified based upon a scientific methodology.

Proof of provenance has become an important issue in the context of food safety, food quality and consumer protection in accordance with national legislation and international standards and guidelines. Provenance means to identify and ensure the origin of a commodity and thereby the region where it was produced.

Therefore, an independent and universally applicable analytical strategy to verify the declared country of origin of food can be an invaluable tool to enable regulatory

authorities to trace contaminated foods back to their source. However, analytical techniques enabling the provenance of food to be determined provide an independent means of verifying "paper" traceability systems and also help **to prove product authenticity**, **to combat fraudulent practices** and **to control adulteration**, which are important issues for economic, religious or cultural reasons.

In this context, the development of new and increasingly sophisticated techniques for determining the geographical origin of food products is highly desirable for consumers, agricultural farmers, retailers and administrative authorities (Luykx & van Ruth, 2008; Reid, O'Donnell, & Downey, 2006).

Literature on analytical methods for determining the geographical origin of food products have been increasing in number in the last years. The initial focus was on processed agricultural products such as wine (Baxter et al., 1997; Jakubowski et al., 1999; Thiel et al., 2004), honey, teas, olive oil, and orange juice (Ogrinc, N, Košir, IJ, Spangenberg, JE, Kidrič, 2003), while later studies examined fresh products such as potatoes, Welsh onions, pistachios, and garlic, chiefly because world-wide trade in fresh agricultural products has increased year by year and the law now enforces labelling of their geographical origin. Various techniques have been studied based on organic constituents, mineral contents or composition, light- or heavy-element isotope ratios, or combinations thereof.

These techniques, when used in conjunction with food safety surveillance programmes, provide independent verification of food traceability systems, thereby helping to protect human health and facilitate international trade worldwide. Chemometric processing of the data provided by analytical procedures and instruments which have the ability to determine more than one component at a time in a sample are of enormous support to establish links to the food origin.

Chapter 2 Analytical approach for food traceability

2.1 Markers of provenance

Fingerprinting techniques based on the determination of the specific markers of provenance is the most promising analytical method for traceability of food products (Coetzee et al., 2005; Drivelos & Georgiou, 2012; Fortunato et al., 2004b; Galgano, Favati, Caruso, Scarpa, & Palma, 2008).

It is well known that the normal range of organic compounds in foods varies with fertilization, climatic conditions in the year of cultivation, history of fields and variety or species as well as geographical location and soil characteristics, so it is sometimes difficult to determine the authenticity of a material from its organic components, so there is an ever increasing demand for more effective techniques in food control. On the other side, the content of selected minerals and trace elements in foods clearly reflects the soil type and the environmental growing conditions. In this framework a better approach has be found to include a wide range of inorganic species including macro, trace and ultra-trace elements, such as Rare Earth elements, whose content altogether has been proposed to ensure the geographical origin of food samples (Gonzalvez et al., 2009).

Consequently, the determination and the measurement of a multi-elemental array in a given food (Benincasa et al., 2007, 2008; Brescia et al., 2005) in selected regional products may represent a unique fingerprint for characterizing its geographical origin.

In addition, the content of hydrogen and oxygen and the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios are indicators of climatic characteristics, which depend on local agricultural practices and animal diets (Kelly et al., 2005).

The most common elements used as markers of origin are subdivided in four macro-groups:

- Macro- and Trace elements (Drivelos & Georgiou, 2012; Voica, Dehelean, Iordache, & Geana, 2012; Voica, Dehelean, & Kovacs, 2012);
- Rare earth elements (Bentlin, dos Santos, Flores, & Pozebon, 2012; Bettinelli, HM., Spezia, S., Baffi, C., Beone, G.M., Rocchetta, R., Nassisi, 2005; Capron, Smeyers-Verbeke, & Massart, 2007; Jakubowski et al., 1999; Joebstl, Bandoniene, Meisel, & Chatzistathis, 2010; Oddone, Aceto, Baldizzone, Musso,

& Osella, 2009; Santos, Nardini, Cunha, Barbosa, & De Almeida Teixeira, 2014; Thiel et al., 2004);

- Multi-isotope ratio;
  - ✓ Heavy Isotope Ratio – Strontium (Durante et al., 2013, 2015; Silvestri et al., 2013);
  - ✓ Light Isotope Ratio - (C, H, O, N) (Longobardi, Casiello, Sacco, Tedone, & Sacco, 2011; Zhao et al., 2014);

In recent years, the use of multi-element composition for characterizing food products has grown significantly, owing to both the legal interest in protecting PDO products and to the improvement in available technical instrumentation. On the other side, the study on the multi-isotope ratio seems to have reached a steady state (Drivelos & Georgiou, 2012; Gonzalvez et al., 2009)**.**

2.2 The influence of geological origin of soil on elements content

The rationale between chemical fingerprinting in a given food and its traceability is found within the food chain linking the local production to water, soil and the resulting agricultural product supposedly homogeneous at a reasonably regional scale.

For example, magmatic and sedimentary rocks which contain highly distinct amounts of macro, trace and ultra-trace elements, affect at a comparably distinct extent the composition of soil and water as a result of weathering and biogeochemistry.

During weathering, elements may undergo dissolution from the parent rock by rain/snow water, redox and complexation reactions according to the properties of each element, leading to final composition highly related to the overall processing described, including eventually further interactions with bioorganic derivatives (Kabata-Pendias, 2011; Merian, Anke, Ihnat, & Stoeppler, 2004).

The bioavailability of the elements in the soil is influenced by many factors, such as its pH, drainage status, organic matter, cation- and anion-exchange capacity and the plant species.

Consequently, the geological origin of soil significantly affects also most of the elements up taken by plants and therefore of the final agricultural products.

Based on the study of Anke et al., and reported by Merian et al., the concentrations of the four indicator plants (wheat, rye, field and meadow red clover) from 13 different soils, varied for many elements.

The concentration of light, heavy and non-metals obtained from the study, are summarized in Table 2.1.

Table 2.1. Influence of geological origin of soil on macro-, trace- and ultra-trace element contents of indicator plants (Anke, Dorn, Schäfer, & Müller, 2003; Merian et al., 2004)

Geological Origin	**Relative number (%)***											
	P	**Ca**	**Cr**	**Fe**	**Zn**	**Al**	**Cd**	**Cu**	**Ni**	**U**	**As**	**Se**
Alluvial river-side soils	93	85	74	83	76	71	71	74	79	71	70	65
Moor, peat	89	94	87	89	89	73	52	52	47	50	70	80
Loess	89	89	74	87	67	78	60	86	71	72	66	100
Diluvial sands	89	83	79	93	86	78	70	70	71	46	59	49
Boulder clay	93	89	77	71	66	73	86	70	71	64	56	49
Keuper weathering soils	85	97	74	76	57	54	67	85	65	52	46	40
Muschelkalk weathering soils	94	100	79	78	64	71	69	93	64	74	50	50
Bunter weathering soils	96	91	79	66	66	68	75	80	60	55	49	36
Rotliegende weathering soils	100	96	100	100	91	100	90	100	100	75	87	75
Phyllite weathering soils	96	84	86	83	100	93	82	93	94	67	38	37
Granite weathering soils	94	83	96	89	92	90	100	82	69	100	58	37
Gneiss weathering soils	94	89	77	88	82	83	79	93	83	50	100	47
Slate weathering soils	100	83	81	73	89	89	81	94	84	64	44	38

*Content is expressed as number in % respect to Soils with highest concentration=100

Trace element composition varies in different soils in the range of 15% for phosphorus, 17% for calcium, by 25 to 35% for chromium and iron and 43% for zinc, in respect to the soils with highest element concentration, such as Loess for Se, Muschelkalk weathering soils for Ca, Rotliegende weathering soils for P, Cr, Fe, Al, Cu and Ni, Phyllite weathering soils for Zn, Granite weathering soils for Cd and U, Gneiss weathering soils for As and Slate weathering soils for P (Anke et al., 2003; Merian et

al., 2004). The geological origin of soil also alters aluminum, cadmium, copper and nickel by 45 to 55% and the uranium, arsenic and selenium by 54 to 64% in the indicator plants used in this study (Table 2.2).

Table 2.2. List of the indicator plants used in the study

Indicator Plats	**Plant species**
Wheat	*Triticum sativum*
Rye	*Secale cereale*
Red Clover (field)	*Trifolium pretense sativum*
Red Clover (meadow)	*Trifolium pretense spontaneum*

The green plants were harvested when the rye was in blossom, the wheat shooting, the field red clover in bud and the meadow red clover in blossom.

Based on the results of the study summarized in Table 2.1, plant grown on the weathering soils of the Rotliegende accumulated the highest, and those on Keuper weathering soils the lowest concentrations.

2.3 Macro- and Trace elements

The essential elements for the nutrition of animals and man include macro-elements such as P, Na, K, Ca, Mg, S, Cl and trace-elements such as Fe, Cu, Zn, Mn, Mo, Ni, I, Se. Furthermore, ultra-trace elements (Li, Rb, Cs, Sr, Ba, Cd, Hg, Al, Tl, Ti, Sn, Pb, V, As, Bi, Cr, W, U, Co) are important since some of them, are or may become toxic at a given concentration threshold.

2.3.1 Uptake, distribution and analytical method

The transfer of inorganic components from soil to plants and into the food chain of animals and man have been a topic of intensive research since the beginning of modern environmental and agricultural chemistry, as well as of biological and nutrition sciences, owing to their influence on animal and human health (Merian et al., 2004).

The transfer of all metals and non-metals from plants to animals and man via the terrestrial food chain is the basis for animal and human nutrition.

The macro-, trace- and ultra-trace elemental content significantly differs among both soil typologies and geographic regions. In most cases, the highest levels of the inorganic

components in the plants are concentrated into the leaves. The elements transfer from the stem to the leaf, which depends on the type of plant and its age, influences the concentration of the inorganic components in the whole plant.

The concentrations of elements in vegetable foodstuffs range from <1 µg kg^{-1} dry weight for uranium in sugar, to 127 g kg^{-1} dry matter for potassium in lettuce as reported by Anke et al. (Table 2.3).

Table 2.3. Elemental composition of several vegetable foodstuffs (mg kg-1 dry matter) (Anke et al., 2003; Merian et al., 2004)

Element	Sugar	Wheat flour	Lentil	Apple	Potato	Asparagus	Lettuce	Mushroom
Ca	31	264	401	488	288	2556	15329	1104
Mg	2	341	1191	433	1349	1869	2496	366
P	18	1187	4401	657	2627	5143	11949	2460
K	61	1777	12269	11388	27576	42934	127438	1297
Na	67	95	78	113	153	436	1732	46214
Fe	5.3	16	8.3	13	35	116	208	270
Mn	0.24	9.9	13	4.3	6.2	24	34	5.1
Ni	0.140	0.173	2.142	0.188	0.975	9.183	4.767	1.575
Zn	0.76	10	48	4.2	18	94	94	–
Cu	0.31	2.1	6.3	2.9	3.9	5.8	11	7.8
Mo	0.023	0.156	4174	0.037	0.537	0.602	0.665	0.390
I	0.002	0.021	0.029	0.031	0.028	0.101	0.150	0.634
Se	<0.002	0.084	0.521	0.022	0.027	0.334	0.025	0.476
As	0.010	0.054	0.250	0.046	0.024	0.223	0.122	0.342
Li	0.199	0.905	0.748	1.449	1.592	2.217	4.502	5.788
Rb	0.11	0.76	6.02	5.02	4.084	68.0	21.8	0.57
Sr	0.17	1.6	1.9	2.5	1.9	12	58	12
Ba	3.9	0.9	5.4	1.5	1.5	2.8	11.8	11.4
Cd	0.005	0.038	0.058	0.019	0.124	0.083	0.547	0.040
Hg	0.002	0.006	0.020	0.011	0.034	0.050	0.045	0.263
Al	4.4	4.1	18	12	30	66	269	148
Ti	0.071	0.115	0.285	0.277	0.442	2.181	3.968	4.288
V	0.008	0.018	0.041	0.021	0.019	0.097	0.377	0.625
Cr	0.145	0.113	0.358	0.202	0.333	0.948	1.260	1.052
U	0.001	0.0015	0.002	0.002	0.003	0.053	0.039	0.105

Sugar contains the lowest amounts of macro, trace and ultra-trace elements, owing to the efficient sequential purification steps by fractionated crystallization during its industrial processing, as well as wheat flour which is relatively poor in essential and toxic elements.

Vegetables which are especially calcium-rich include lettuce (15 g kg^{-1} dry matter) and asparagus (2.6 g kg^{-1} dry matter). Both species store also relatively high amounts of

magnesium (2.5 and 1.9 g kg^{-1} dry matter, respectively), phosphorus (12 and 5.1 g kg^{-1}) and potassium (127 and 43 g kg^{-1}).

Additionally, elemental content of the foodstuff are also influenced by the farming system as well as by the processing of the raw materials for food production. Conventionally, working farms use fertilizers, herbicides, fungicides, insecticides, growth promoters and other pesticides, whereas organic farmers use only dung, compost and organic waste of the agricultural production as fertilizers and do not apply any pesticides. Under these conditions, elemental composition of conventionally and ecologically produced foodstuffs contains different element- and food-specific amounts of macro, trace and ultra-trace elements.

As reported by scientific literature, animal foodstuffs from green production contain lower concentrations of macro, trace and ultra-trace elements (Anke et al., 2003; Merian et al., 2004; Rohrig, Anke, Drobner, Jaritz, & Holzinger, 1998).

Thus, the concentrations of the inorganic components in food products are both foodstuff- and element-specific.

Consequently, the determination and the measurement of their elemental composition (Benincasa et al., 2007, 2008; Brescia et al., 2005) in selected regional products may provide unique "markers" for characterize geographical origin (Kelly et al., 2005).

Mass spectrometry techniques, and in particular the Inductively Coupled Plasma Mass Spectrometry (ICP-MS), is a powerful tool for the quantitative determination of a range of metal and non-metal elements at low and ultra-low concentration levels, in many sample types (Abdrabo et al., 2015; Dressler et al., 2012; Husáková et al., 2011; Leme et al., 2014; Mamone et al., 2009; Mir-Marqués et al., 2015; Mohd-Taufek et al., 2016; Muller et al., 2013; Wang et al., 2013).

2.4 Rare earth elements

Rare earth elements (REE) include the yttrium (Y), lanthanum (La) and the 14 lanthanides, such as cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb), and lutetium (Lu) (Tyler, 2004) as shown in Figure 2.1.

One of them, the promethium, is unstable and does not occur in nature.

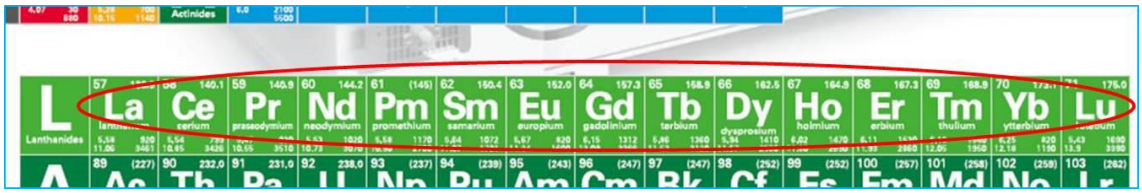

Figure 2.1 Lanthanides group in the periodic table

REEs are frequently subdivided into three groups: light (lanthanum to europium), middle (samarium to holmium) and heavy REEs (gadolinium to lutetium) (Tyler, 2004; Wen, Yuan, Shan, Li, & Zhang, 2001).

Lanthanide series is used in industries and technologies involving metallurgy, illumination, glass, ceramics, magnets, petroleum, electronics, medical imaging, and nuclear energy.

The term "rare earth elements" is misleading because these elements are not rare but they are found in abundance in the Earth's crust. REE concentrations in soil vary according to material properties, history and weathering state of soil, its content of organic matter and clay minerals.

The total concentrations of REEs in Earth's crust and in some soils are listed in Table 2.4.

Table 2.4. Mean concentrations of lanthanides in the Earth's crust and some selected soils (Tyler, 2004)

Elements	**Symbol**	**Atomic mass**	**Earth's crust [a] (mg Kg^{-1})**	**Japan [b] (mg Kg-1)**	**China [c] (mg Kg^{-1})**
Lanthanum	La	139	30	18	44
Cerium	Ce	140	60	40	86
Praseodymium	Pr	141	8.2	4.5	-
Neodymium	Nd	144	28	18	36
Samarium	Sm	150	6.0	3.7	8.4
Europium	Eu	152	1.2	1.0	-
Gadolinium	Gd	157	5.4	3.7	-
Terbium	Tb	159	0.9	0.6	-
Dysprosium	Dy	162.5	3.0	3.3	-
Holmium	Ho	165	1.2	0.7	-
Erbium	Er	167	2.8	2.0	-
Thulium	Tm	169	0.5	0.3	-
Ytterbium	Yb	173	3.0	2.0	-
Lutetium	Lu	175	0.5	0.3	-

[a](Demayo, 1986)
[b](Yoshida, Muramatsu, Tagami, & Uchida, 1998)
[c](Ran & Liu, 1999)

The abundances of cerium (average concentration in the Earth's crust 60 mg kg^{-1}), lanthanum (average 30 mg kg^{-1}), and neodymium (average 28 mg kg^{-1}) are similar to those of copper (average 55 mg kg^{-1}), lead, tin, and cobalt (Demayo, 1986; Hedrick, 2000; Merian et al., 2004). The less abundant lanthanides, lutetium and thulium, are actually more abundant in the Earth's crust than cadmium and selenium (Tyler, 2004).

REE's occur in all plants, but no lanthanide elements are known to be nutritionally essential in animals or humans nor considered toxic; however, many of these elements can compete with calcium in a number of calcium-mediated biological processes. In humans, lanthanides tend to accumulate in liver and bones, while occupational exposure may increase concentrations in specific organs (Gerhardsson, Wester, Nordberg, & Brune, 1984; Merian et al., 2004).

2.4.1 Uptake, distribution and analytical method

The uptake and contents of REEs in plants differ considerably between plant species, even under natural conditions without supplementation (Wyttenbach, Furrer, Schleppi, & Tobler, 1998). Soil properties and the specific differences between species hardly affect the uptake of REEs by plants.

Normally their uptake occurs exclusively by roots. As with most trace elements, the distribution of REEs in plants varied among different plant parts, and the following order was generally observed: root > leaf > stem > grain as shown in Figure 2.2.

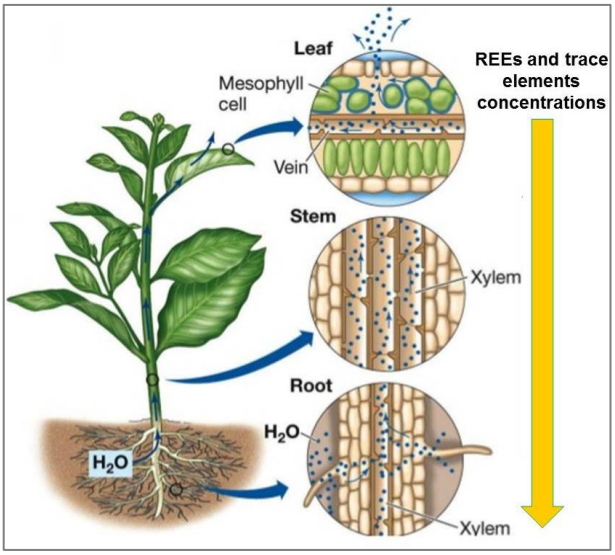

Figure 2.2 Distribution of REEs in different parts of the plants in different parts of plants follows the order: root>leaf>stem>grain.

The fact that REE concentrations in roots is higher than that in other parts proved that REEs can be accumulated and retained by roots, so that only small portions of REEs reached other parts of plants.

The concentrations of REEs in root, leaf and stem increase remarkably when REE fertilizers were applied (Redling, 2006; Tyler, 2004). In recent years, the utilization of REEs has been widely applied not only in industry but also in agriculture and forestry (Wen et al., 2001).

Mixtures of REEs in fertilizers are nowadays widely used in Chinese agriculture to improve crop nutrition (Xu, Zhu, Wang, & Witkamp, 2002) and has been in common use for about 20 years (Pang, Li, & Peng, 2002). Mainly, there are two kinds of fertilizers in commerce:

1. *Nongle* (translated into English meaning happy farmer), which consists of a complex of soluble chloride forms of rare earth compound, was the first fertilizer produced for testing the efficacy of rare earths in field experiments (Guo, 1985);
2. *Changle* (meaning happiness forever), which contains about 38% rare earth oxides, it also included a number of elements essential to plant growth (Brown, Rathjen, Grahahm, & Tribe, 1990; Guo, 1986).

Rare earth-products used today basically comprise rare earth nitrates, rare earth chlorides, compound rare earth fertilizers with multiple trace elements, compound fertilizers of rare earth and ammonium bicarbonate (Xiong, 1995) and rare earth compounds mixed with amino-acids (MAR) (Pang et al., 2002). However, rare earth nitrates found in *Changle* still constitute the main preparation used in plant production (Redling, 2006; Wen et al., 2001).

These fertilizers are reported to contain 25-28% lanthanum oxide (La_2O_3), 49-51% cerium dioxide (CeO_2), 5-6% praseodymium oxide (Pr_6O_{11}), 15-17% neodymium oxide (Nd_2O_3) and less than 1% other rare earths (Xiong, 1995).

Application of REE fertilizer does not change the distribution patterns of REEs in plants. With the widespread application of fertilizer containing REEs in China, the research interests have also been accelerated.

Recent improvements in more routine analytical technique, the use of REEs as fertilizers, at least in East Asian agriculture, and the importance of these elements as

indicators in physiological processes and reactions have contributed to an increased interest in these elements previously less considered in environmental sciences (Tyler, 2004). Therefore, considering that REEs fertilizers are used to promote the growth of vegetables, more research is required on the toxicological effects of the presence of REEs in vegetables at these concentrations (Wen et al., 2001).

Generally, the concentration of REE in food is very low, ng L^{-1} (Ming & Bing, 1998), and only the most sensitive analytical techniques can be used to determine these elements at such concentration levels.

ICP-MS is one of the most widely used techniques for the determination of REE in different sample types, because of its ability to carry out rapid multi-element detection at low and ultra-low concentration levels at highly performing levels (Bettinelli, HM., Spezia, S., Baffi, C., Beone, G.M., Rocchetta, R., Nassisi, 2005; Coetzee et al., 2005; Galgano et al., 2008; Joebstl et al., 2010; Spalla, JS, Baffi, C, Barbante, C, Turretta, C, Cozzi, G, Beone, GM, Bettinelli, 2009; Thiel et al., 2004).

Hirano & Suzuki summarized the detection limits of REE in four analytical methods (Table 2.5).

Table 2.5. Detection limits of REE in four analytical instrumental techniques (Hirano & Suzuki, 1996)

Elements	**Detection limit (μg L^{-1})[1]**			
	ICP-AES[2]	**ICP-MS[3]**	**FAAS[4]**	**GFAAS[5]**
La	0.1	0.002	2000	24
Ce	0.4	0.004	-	-
Pr	10	0.003	4000	80
Nd	0.3	0.007	2000	200
Sm	30	1.5	600	-
Eu	0.06	0.007	40	0.2
Gd	0.4	0.009	4000	80
Tb	0.1	0.002	2000	100
Dy	4	0.007	200	3.4
Ho	3	0.002	100	1.8
Er	1	0.005	100	9
Tm	0.2	0.002	40	0.2
Yb	0.02	0.005	20	0.1
Lu	0.1	0.002	2000	80

[1] (Date & Hutchison, 1987) and (Kawaguchi & Nakahara, 1994)
[2] Inductively coupled plasma atomic emission spectroscopy
[3] Inductively coupled plasma mass spectrometry
[4] Flame atomic absorption spectroscopy
[5] Graphite furnace atomic absorption spectroscopy

Data in Table 2.5, shows that the most sensitive method for analysis of lanthanides is Inductively coupled plasma mass spectrometry (ICP-MS) where detection limits for the series of ions range from 0.002 to 0.009 μg L^{-1}.

Recent improvements in ICP-MS technique, such as the use of collision/reaction cell technology coupled with a triple quadrupole system (Amr, 2012), allow to carry out REEs determination at ultra-low concentration levels (ng L^{-1}).

2.5 Multi-isotope ratio

Over the years, isotopic analysis of different elements present in various products (such as the 'light' elements H, C, N, O and S and/or 'heavy' elements, such as Sr and Pb), has been used in provenance studies.

Many natural phenomena, defined as physico-chemical effects, can lead to isotope fractionation (measurable changes in the ratio of the 'heavy' to 'light' isotope of a given element). For example, evaporation and condensation, crystallization and melting, absorption and desorption, diffusion and thermos-diffusion may affect the isotopic partitioning of a given element based on the different rate as a function of the a heavier/lighter isotopic mass (Kelly et al., 2005).

Table 2.6 reports the influence of the natural abundance of stable isotope in food provenance determinations.

Table 2.6. The provenance informations provide by the natural abundance of stable isotope ratio

Isotope ratio	Fractionation	Provenance information
$^{2}H/^{1}H$	Evaporation, condensation, precipitation	Geographical
$^{13}C/^{12}C$	C3 and C4 plants	Diet
$^{15}N/^{14}N$	Agricultural practice, marine and terrestrial plants	Diet
$^{18}O/^{16}O$	Evaporation, condensation, precipitation	Geographical
$^{87}Sr/^{86}Sr$	Age of the rock and Rb/Sr ratio	Underlynig geology, Geographical

On a first approximation natural abundance measurements will provide information on plant species or diet (carbon and nitrogen isotope ratios as a function of the protein content), and geographical origin (hydrogen, oxygen, and strontium isotope ratios).

2.5.1 Light Isotope Ratio - (C, H, O, N)

Each plant has its own unique pattern of naturally occurring stable isotopes of carbon (^{12}C, ^{13}C), nitrogen (^{14}N, ^{15}N), hydrogen (^{1}H, ^{2}H) and oxygen (^{16}O, ^{18}O), whose distribution has been influenced by a number of physical and/or biochemical properties and geo-climatic conditions (Ogrinc, N, Košir, IJ, Spangenberg, JE, Kidrič, 2003).

Many natural phenomena, such as evaporation and condensation, crystallization and melting, absorption and desorption, diffusion and thermo-diffusion can lead to a change in isotope distribution (Kelly et al., 2005). The effect of these conditions on the final isotopic composition of a molecule is known as isotopic fractionation (Reid et al., 2006).

Consequently, isotope distribution in agricultural products depends on their botanic and geographical origin. For instance, the measurement of the stable isotope ratio of hydrogen and oxygen as related to water cycling is applied to the determination of the geographical origin because of their dependence from latitude. The oxygen isotopic composition in fact mainly reflects that of local groundwater affected by precipitation and meltwater through percolation and runoff (Craig, 1961; Longobardi et al., 2011).

On the other side, local agricultural practices and animal diet affect $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratio, respectively (Kelly et al., 2005). The carbon isotopic composition of plant materials strongly depends on the carbon fixation process, such as the C_3 or C_4 cycle (Smith & Epstein, 1971).

In particular, C_3 plants use the Calvin photosynthetic pathway[2] to assimilate CO_2. During this process the plants discriminate against ^{13}C and therefore possess relatively lower $^{13}C/^{12}C$ ratios than C_4 plants that utilize the more energy efficient Hatch–Slack pathway[3].

Since, C_3 plants predominate at higher latitudes and C_4 plants are more common in warmer climates at lower latitudes (such as the tropics), there is a gradient of decreasing $^{13}C/^{12}C$ in plant material from the equator to the poles, which can be provided information about the geographical provenance.

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[2]The Calvin cycle or C_3 cycle is one of three metabolic pathways for carbon fixation in photosynthesis and it is a series of biochemical redox reactions that take place in the stroma of chloroplast in photosynthetic organisms.

[3]C_4 carbon fixation or the Hatch-Slack pathway is a process in some plants. It is the first step in extracting carbon from carbon dioxide to be able to use it in sugar and other biomolecules. It is one of three known processes for carbon fixation.

Nitrogen isotopic composition instead mainly depends on soil nutrients, affected by the natural biogeochemical cycle of this element as well as by the use of fertilizers (Kohl, Shearer, & Commoner, 1973; Meints, Shearer, Kohl, & Kurtz, 1975).

In this framework, the adulteration of several food products, such as honey (Padovan, De Jong, Rodrigues, & Marchini, 2003; Tosun, 2013), juice (Simpkins, Patel, Harrison, & Goldberg, 2000), olive oil (Angerosa, Camera, Cumitini, Gleixner, & Reniero, 1997), and wine (Rossmann et al., 1999) can be detected by differences in the carbon, nitrogen and/ or oxygen isotopic distribution which are known to varies latitudinally according to the above specifications.

Furthermore, the geographical origin of agricultural products such as cereal crops (Kaoru Ariyama & Yasui, 2006; Goitom Asfaha et al., 2011; Kelly et al., 2002), wine (Baxter et al., 1997) and olive oil (Camin, Larcher, Nicolini, et al., 2010; Camin, Larcher, Perini, et al., 2010) and animal products such as meat (Schmidt et al., 2005) and dairy products (Ritz et al., 2005; Rossmann et al., 2000; Sacco et al., 2009), have been traced by using natural variations of their isotopic compositions.

A prerequisite of this analytical method for food traceability is the availability of a database including all the analytical data obtained from different geographical areas. In this case, considering that the isotopic variations might have been affected by climatic change and seasonal variations, it's necessary to update the databank periodically.

2.5.2 Heavy Isotope Ratio - Strontium

Strontium is an alkaline earth metal with an average concentration of 370 mg kg^{-1} in the 16 km-thick Earth's crust (Merian et al., 2004). It occurs as four stable isotopic forms with atomic masses respectively of 84, 86, 87, and 88 amu. The latter isotope, ^{88}Sr, with a relative abundance of 82.58%, is the most abundant (Rosman & Taylor, 1998).

The relative natural abundances of the all strontium isotope are listed in Table 2.7.

Table 2.7. The relative natural abundances of the strontium isotopes

Symbol	Mass Number	Isotopic composition (%)
Sr	84	0.56
	86	9.86
	87	7.00
	88	82.58

Among these isotopes, ^{87}Sr is radiogenic, deriving, in part, from the natural β-decay of rubidium-87 (^{87}Rb), with a half-life of $4.88 \cdot 10^{+10}$ years, and its concentration in the minerals depends on the age of the rock and on the Rb/Sr ratio. Thus, the amount of ^{87}Sr in geologic materials is controlled by the age and the amount of ^{87}Rb in the sample. A stable isotope of Sr, ^{86}Sr, has been designated as the reference isotope in the system. Hence, it is the $^{87}Sr/^{86}Sr$ isotope ratio which is used as an isotopic tracer and an important parameter in geochronology (Hurst, Davis, & Elseewi, 1991).

Usually, older acidic rocks (e.g. silica-rich granites) have high $^{87}Sr/^{86}Sr$ isotope ratio (typically > 0.710) due to the increasing Rb/Sr ratios over time, while younger basic rocks (e.g. basaltic and carbonate rocks) have relatively lower values typically between 0.702 and 0.705 (Barbaste, Robinson, Guilfoyle, Medina, & Lobinski, 2002; Kelly et al., 2005; Montgomery, Evans, & Wildman, 2006).

The $^{87}Sr/^{86}Sr$ isotope ratio was demonstrated not to change during biological processes and reflects in plants, the environmental of growth: bedrock, soil and soil water. In the soil–vegetation–atmosphere system, Sr is inherited from the parent rock and stored in primary and authigenic minerals, soil solution, cation exchange sites, and in vegetation, including foliage, wood and roots (Stewart, Capo, & Chadwick, 1998).

In this way, the strontium isotopic ratio was stored in the plants and in the animals that eat them, entering in the food chain and providing an important geographical tracer for several type of food products.

Compared to the lighter isotopes, the fractionation for strontium isotope, due to biological processes, whether involved in plant or animal metabolism, is insignificant as compared to the original isotopic ratios because the nuclides have high mass compared to the mass differences of the isotopes (Stewart et al., 1998).

In this case, stable isotopic ratios depend essentially upon the origin of the rocks and can be used for identification of the source of a material or characterization of its provenance.

Several studies have demonstrated that $^{87}Sr/^{86}Sr$ has been successfully applied to different types of food matrices (Baroni et al., 2011; Fortunato et al., 2004a; Rosner, 2010), such as ginger (Choi, Lee, Lee, & Han, 2008), asparagus (Swoboda et al., 2008), butter (Rossmann et al., 2000), cereals (Goitom Asfaha et al., 2011) and beverages such as mineral water (Voerkelius et al., 2010) and wine (Almeida & Vasconcelos, 2004; Durante et al., 2013, 2015; Marchionni et al., 2016).

Thus, $^{87}Sr/^{86}Sr$ isotope ratio may represent a unique and highly efficient geographical tracer for several type of food products.

The use of strontium isotope ratios in the food traceability provides some advantages respect to light isotopes determination. $^{87}Sr/^{86}Sr$ isotope ratio can discriminate between different micro-regions within the same climate; additional Sr isotope ratio analysis may provide an additional level of geographical resolution provided that different lithologies exist with that region (Kelly et al., 2005) .

2.6 Analytical techniques

Various techniques, based on organic compounds, mineral contents or composition and light- or heavy-element isotope ratios, have been studied in order to determine the possible markers of geographical provenance of the food products.

The analytical approaches, which have been successfully applied to food authentication, are classified into four groups:

- mass spectrometry techniques;
- spectroscopic techniques;
- separation techniques;
- other techniques.

All analytical techniques are summarized in Table 2.8.

Table 2.8. Summary of the current analytical techniques applied to food traceability, abbreviations in brackets (Luykx & van Ruth, 2008).

Principal techniques	Core Technique
Mass spectrometry techniques	Isotope ratio mass spectrometry (IRMS)
	Inductively coupled plasma mass spectrometry (ICP-MS)
	Proton transfer reaction mass spectrometry (PTR-MS)
	Gas chromatography mass spectrometry (GC-MS)
Spectroscopic techniques	Nuclear magnetic resonance spectroscopy (NMR)
	Infrared spectroscopy (IR, FTIR)
	Fluorescence spectroscopy
	Atomic spectroscopy (AAS, AES)
Separation techniques	High performance liquid chromatography (HPLC)
	Gas chromatography (GC)
	Capillary electrophoresis (CE)
Others techniques	Sensor technology
	DNA technology (PCR)

These analytical techniques are applied both to organic and inorganic components in order to shed light on specific markers for food traceability.

The analytical techniques, see Figure 2.3, employed to determine the elemental profile in food products, are subdivided according to the kind of analysis (trace-element and multi-isotope ratio).

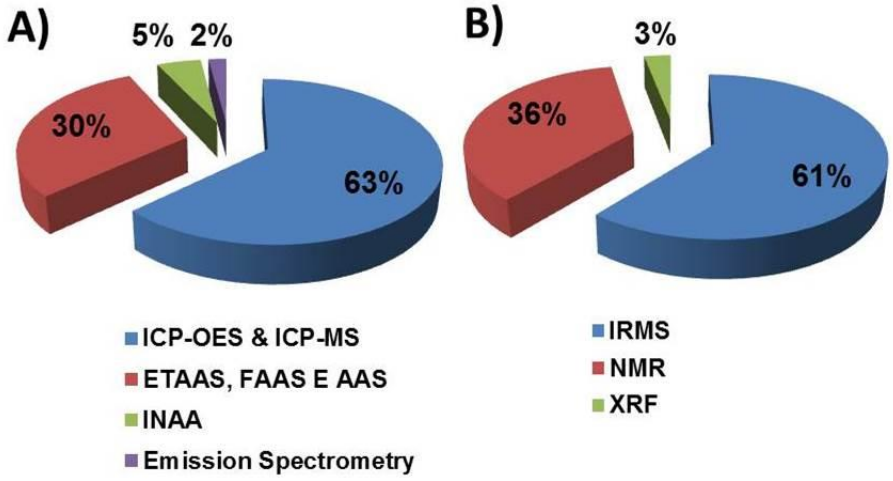

Figure 2.3 Distribution of analytical techniques used to determine: A) trace-elements and B) multi-isotope ratio in food products (Gonzalvez et al., 2009).

The group A) comprises plasma spectrometry such as Inductively coupled plasma - optical emission spectrometry (ICP-OES) and ICP-MS, atomic spectrometry such as Electrothermal Atomic Absorption Spectrometry (ETAAS), Flame atomic absorption spectroscopy (FAAS) and Atomic absorption spectroscopy (AAS) and instrumental neutron-activation analysis (INNA), whereas the group B) includes the isotope-ratio mass spectrometry (IRMS), the nuclear magnetic resonance (NMR) and X-ray fluorescence spectrometry (XRF).

The techniques of choice to obtain the elemental fingerprint of food products are those with multi-element detection capability, such as ICP-MS and ICP-OES, and the isotope-ratio mass spectrometry (IRMS).

Elemental analysis has been performed using a number of techniques, including flame and graphite furnace atomic absorption spectrometry, ICP-MS, ICP-optical emission spectrometry (ICP–OES), also known as ICP-atomic emission spectrometry (ICP-AES), and ICP-MS/MS.

ICP-MS, ICP-OES and ICP-MS/MS are highly sensitive, have wide linear calibration ranges and facilitate simultaneous multi-element quantification (Agilent Technologies Inc., 2005) .

2.6.1 Inductively Coupled Plasma (ICP)

Since its introduction over five decades ago, the inductively coupled plasma source (ICP) has exhibited a large number of special attributes. Some of the most important characteristics of the Argon ICP are outlined in Table 2.9.

Table 2.9. Characteristics of the Ar ICP*

The axial channel
High gas temperature (4500-8000 K) and electron temperature (8000-10000K)
Long residence time of the sample aerosol inside of the plasma (2-3 ms)
Vaporization and atomization in a nearly chemically inert environment
Molecular species are either absent or present at very low levels
Robustness of the plasma

*(Montaser, 1998)

These unique properties mainly are responsible for the success of the Ar ICP as an optimum vaporization, atomization, excitation and ionization source for simultaneous multi-element analysis.

2.6.1.1 Plasma Formation

Basically, the plasma is formed in a stream of Argon gas (usually 8-20 L/min) flowing through an assembly of three concentric quartz tubes known as the plasma torch as shown in Figure 2.4.

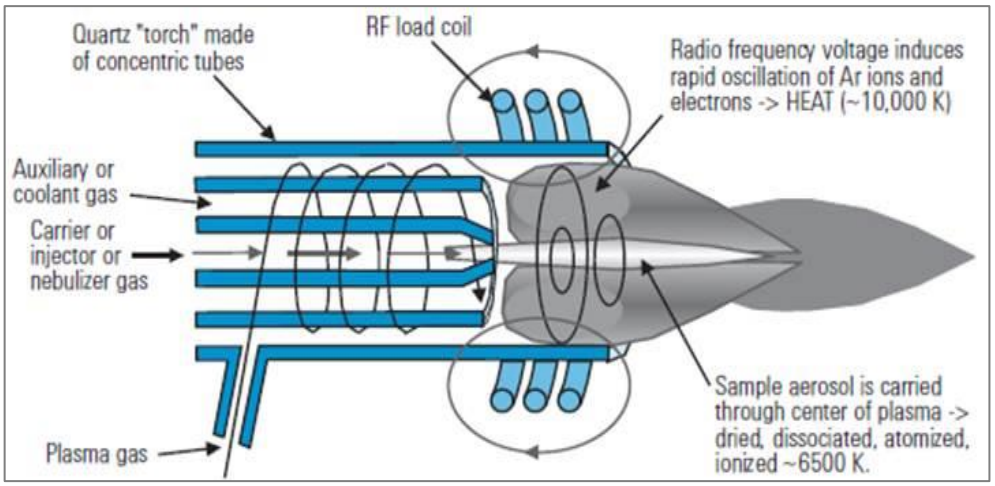

Figure 2.4 Schematic drawing of an Argon ICP torch (Agilent Technologies Inc., 2005)

The torch is surrounded at the top by an induction coil, also called the load coil, connected to a free-running or crystal-controlled radiofrequency (RF) generator. The induction coil is made from copper and is cooled, either by water or the argon gas. The generator can operate at frequencies ranging from 4 to 50 MHz.

The magnetic field generated by the RF current through the load coil induces a current in the Argon gas stream. Plasma is formed almost instantaneously when the Argon gas is seeded with energetic electrons. These electrons are produced either by a high-voltage Tesla discharge, or a solid state piezoelectric transducer.

Stable, self-sustaining plasma is maintained as long as the magnetic field strength is sufficiently high and the gas flows in a symmetrical pattern.

Importantly, the largest current flow occurs on the periphery of the plasma, which gives the ICP a distinctive annular configuration that sets it apart from most other discharges.

This doughnut-type structure ensures the efficient introduction of sample aerosol into the central channel of the plasma, thus resulting in the efficient desolvation, vaporization, atomization, excitation, and ionization of the sample.

Figure 2.5, shows a schematic representation of processes in an ICP plasma source.

The Argon ICP is capable of exciting-ionizing a wide range of elements, particularly metals, and it therefore allows simultaneous multi-element determination.

The inductively coupled plasma source (ICP) systems are currently available in two varieties, such as the ICP-OES and ICP-MS. Basically, the sample introduction system and plasma of both the instruments look similar.

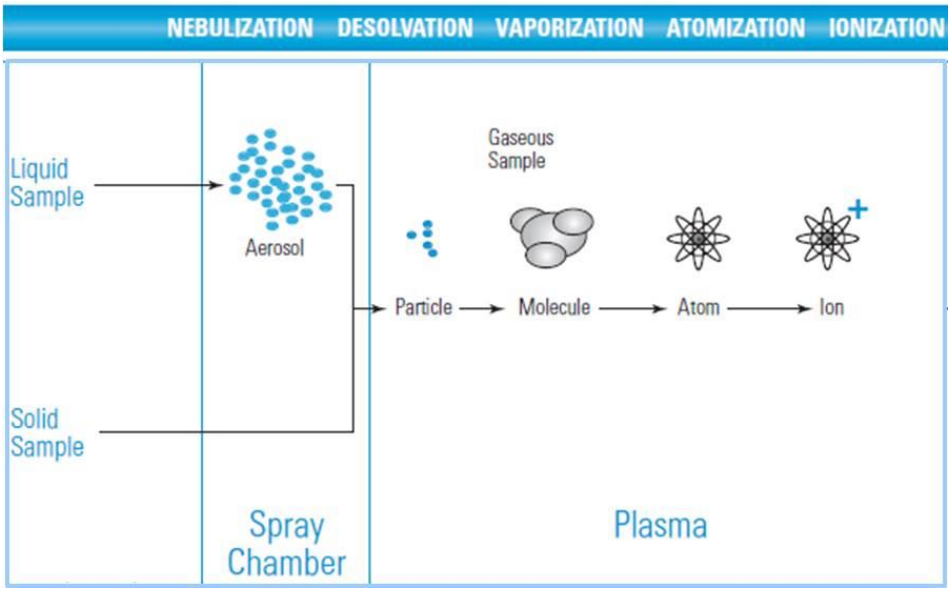

Figure 2.5 Schematic representation of processes in ICP plasma source (Agilent Technologies Inc., 2005)

2.6.2 Inductively coupled plasma optical emission spectrometry (ICP-OES)

Inductively coupled plasma optical emission spectrometry (ICP-OES) is used for single or multi-elemental analysis at concentrations of parts-per billion based on the wavelength of light emitted.

As reported in the previous section, the ICP-OES uses extremely high temperature Argon plasma to excite atoms to the point where they emit their characteristic wavelengths of light (emission lines).

In ICP-OES the optical spectrum with a typical range of 165 to 800 nm is viewed and measured, either sequentially and simultaneously.

The simultaneous ICP-OES can be faster for large numbers of elements, but it is more expensive than sequential ICP-OES.

Cost is greatly dependent on the number of elements and the concentrations required. More recently, several ICP-OES spectrometers have been able to reach to 120 nm, thus enabling the determination of Cl at the primary wavelength of 134.664 nm with sub-ppm detection limits (Ammerman et al., 2013).

Figure 2.6, shows an optical diagram of an ICP-OES instrument.

One or two transfer lenses are then used to focus the emitted light on a diffraction grating where it is separated into its component wavelengths in the optical spectrometer.

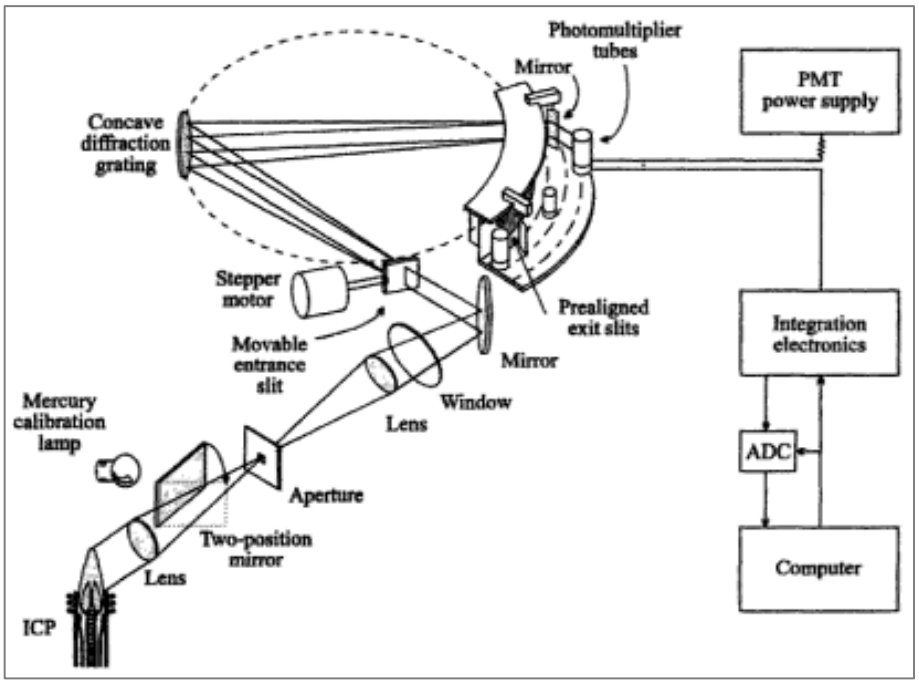

Figure 2.6 Optical diagram of an ICP-OES instrument (Montaser, 1998)

Within the optical chamber, after the light is separated into its different wavelengths, the light intensity is measured with a photomultiplier tube or tubes physically positioned to "view" the specific wavelength for each element line involved, or, in more modern units, the separated wavelength fall upon an array of semiconductor photodetectors such as charge coupled devices (CCDs). Using these detector arrays, the intensities of all wavelengths (within the system's range) can be measured simultaneously, allowing the instrument to analyze every element to which the unit is sensitive all at once. Thus, samples can be analyzed very quickly.

The intensity of the emitted light (emission intensity) is directly proportional to the concentration of the elements in the sample. By measuring the emitted light by known and varying concentrations of an element, by performing a calibration, the concentration of an unknown sample can be determined by comparison.

2.6.3 Inductively coupled plasma mass spectrometry (ICP-MS)

The inductively coupled plasma source (ICP), which is currently the most commonly used plasma ion source in inorganic mass spectrometry.

Mass spectrometry is one of the most important analytical techniques used today in inorganic analysis especially at trace and ultra-trace level, for surface and isotope analysis, as well as for the structural analysis of organic and bioorganic compounds, due

to its extremely high sensitivity, low detection limits and the possibility of analyzing very small sample volumes (Johanna Sabine Becker, 2007).

In general, MS is applied to elucidate the composition of a sample by generating a mass spectrum representing the masses of the sample components (Luykx & van Ruth, 2008). All types of mass spectrometric systems for analysis of organic and inorganic compounds use the same basic principle.

A general setup of mass spectrometer is given in Figure 2.7.

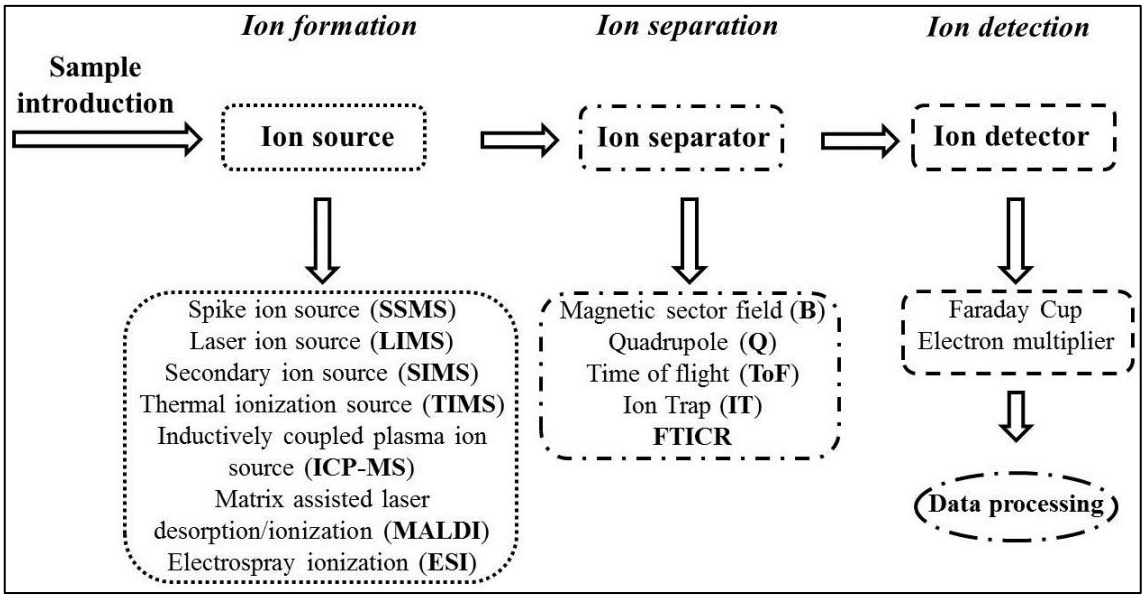

Figure 2.7 General diagram of a mass spectrometer for analysis of organic and inorganic compounds (Johanna Sabine Becker, 2007)

The mass spectrometer encompasses four fundamental processes: the introduction of the sample in the ion source, the subsequent generation of ions from the compounds in the sample, the separation of these ions by their mass-to-charge ratio in the mass separator and the detection of ions in the ion detector (J. Becker & Dietze, 2000). The sample introduction system differs according to the type of the sample (gases, liquids or solids). Liquids are introduced by nebulization of the solution and the solids by evaporation or laser ablation or often directly inserted into the ion source (SSMS, LIMS).

The major advantage of mass spectrometry techniques is the possibility to determine the isotope ratio and abundance of isotopes in all type of samples with high precision in order to investigate the fine isotope variation in various fields, such as quality assurance, waste control and traceability of food products.

Nowadays, this prospect is of great importance because of the many requests from industry and research for sensitive and accurate multi-element analytical analysis.
In the last few decades, the advanced research in elemental, surface and isotope analysis recognizes the inorganic mass spectrometry techniques, such as ICP-MS, laser ablation ICP-MS (LA-ICP-MS), secondary ion mass spectrometry (SIMS) and accelerator mass spectrometry (AMS) (Kutschera, 2005), as the most important and sensitive analytical techniques at the trace and ultra-trace concentration level.

The ICP-MS technique is presently indispensable in environmental, geological, clinical, nuclear, and semiconductor analysis laboratories. In particular, the Inductively coupled plasma mass spectrometry (ICP-MS), is a powerful tool for the quantitative determination of a range of metal and non-metal elements at low and ultra-low concentration levels, in many sample types (Abdrabo et al., 2015; Dressler et al., 2012; Husáková et al., 2011; Leme et al., 2014; Mamone et al., 2009; Mir-Marqués et al., 2015; Mohd-Taufek et al., 2016; Muller et al., 2013; Wang et al., 2013).
In recent years, the ICP-MS technique, used for food analytical traceability, has been successfully applied to food products such as onions (K Ariyama et al., 2007; Kaoru Ariyama & Yasui, 2006), nuts (Gómez-Ariza, Arias-Borrego, & García-Barrera, 2006), beverages (Coetzee et al., 2005; García-Ruiz, Moldovan, Fortunato, Wunderli, & García Alonso, 2007; Magdas, Dehelean, & Puscas, 2012; Pilgrim, Watling, & Grice, 2010) and oils (Benincasa et al., 2012, 2007; Castillo, Jiménez, & Ebdon, 1999; Jimenez, Velarte, & Castillo, 2003; Llorent-Martínez, Ortega-Barrales, Fernández-De Córdova, Domínguez-Vidal, & Ruiz-Medina, 2011), in order to determine their geographical origin.
Inductively coupled plasma mass spectrometry (ICP-MS) was first demonstrated about 39 years ago and has been commercially available for just over 33 years.
Koppenaal et al. proposed the history of ICP-MS in terms of development periods in the community, as shown in Table 2.10.
It is seen that much of the development activity in ICP-MS has been directed at the elimination of interferences.
Today, the ICP mass spectrometers with different instrumental arrangements are produced by several companies worldwide.

Table 2.10. Proposed development periods of ICP-MS*

Period	Development
1978–1983	Initial ICP-MS evolution, quadrupoles, demonstration, adoption
1983–1988	Quadrupole ICP-MS reigns
1988–1993	Sector-field, high-resolution ICP-MS arrives
1993–1998	Miscellaneous analyzers in vogue (ion trap, TOF, ICR, MC)
1998–present	Collision/reaction cells rage

*(Koppenaal, Eiden, & Barinaga, 2004)

An overview of commercial ICP mass spectrometers from different companies (quadrupole based ICP-MS, triple quadrupole also, with and without collision/reaction cell, sector field ICP-MS with single ion collector (ICP-SFMS) and multiple ion collector ICP-MS (MC-ICP-MS), time-of-flight (ToF), ICP-ion-trap-MS and non-commercial ICP Fourier transform ion cyclotron resonance (FTICR) mass spectrometers is given in Figure 2.8.

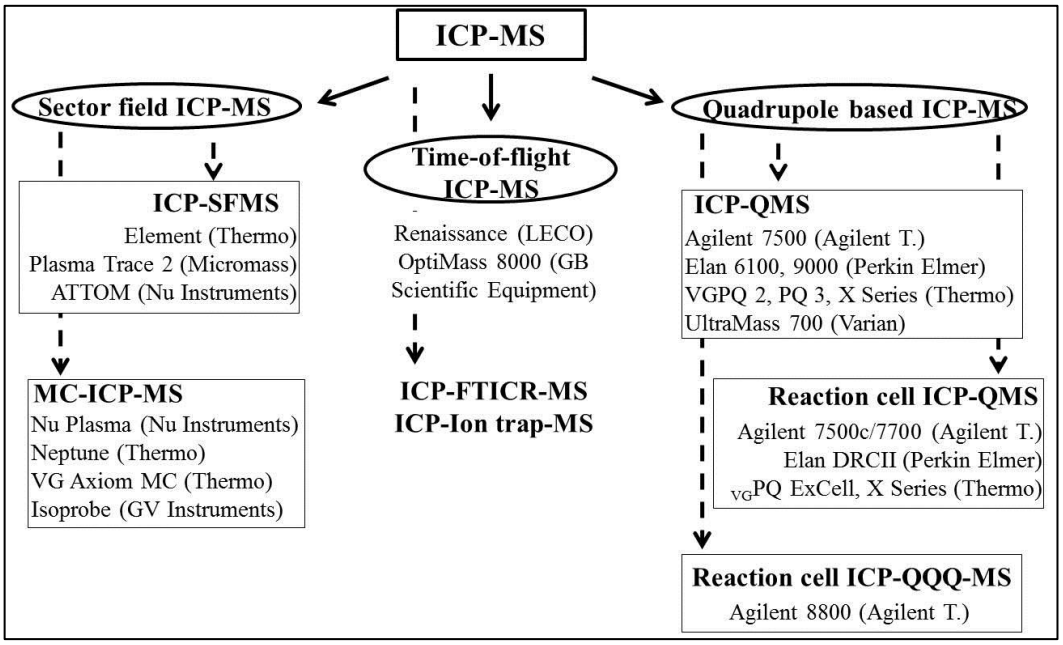

Figure 2.8 Summary of ICP-MS instrumentations from different companies

The Inductively coupled plasma mass spectrometry[4] (ICP-MS) instrument consists of several distinct parts (Agilent Technologies Inc., 2005):

[4] Hyphenated ICP-MS: An ICP-MS may be coupled with techniques such as high performance LC, GC, or capillary electrophoresis in order to have complementary information on the investigational compound based on specific elements present in the molecule (Ammerman et al., 2013).

- ✓ Sample introduction;
- ✓ Ion generation in the ICP;
- ✓ Plasma/vacuum interface;
- ✓ Ion focusing;
- ✓ Ion separation and measurement.

Sample introduction: The sample is typically introduced into the Inductively Coupled Plasma (ICP) as an aerosol, produced by passing the liquid sample through a simple pneumatic nebulizer. Larger aerosol droplets are removed from the gas stream by a spray chamber, and the remaining smaller droplets are swept into the central channel of the argon plasma.

Ion generation in the ICP: The sample aerosol is passed into the plasma, which is generated in a stream of argon (Ar) contained in a quartz torch. More details have been already reported in the previous section.

A schematic of an inductively coupled plasma ion source including the quartz plasma torch and induction load coil together with sampler and skimmer as part of the interface region of an ICP mass spectrometer is shown in Figure 2.9.

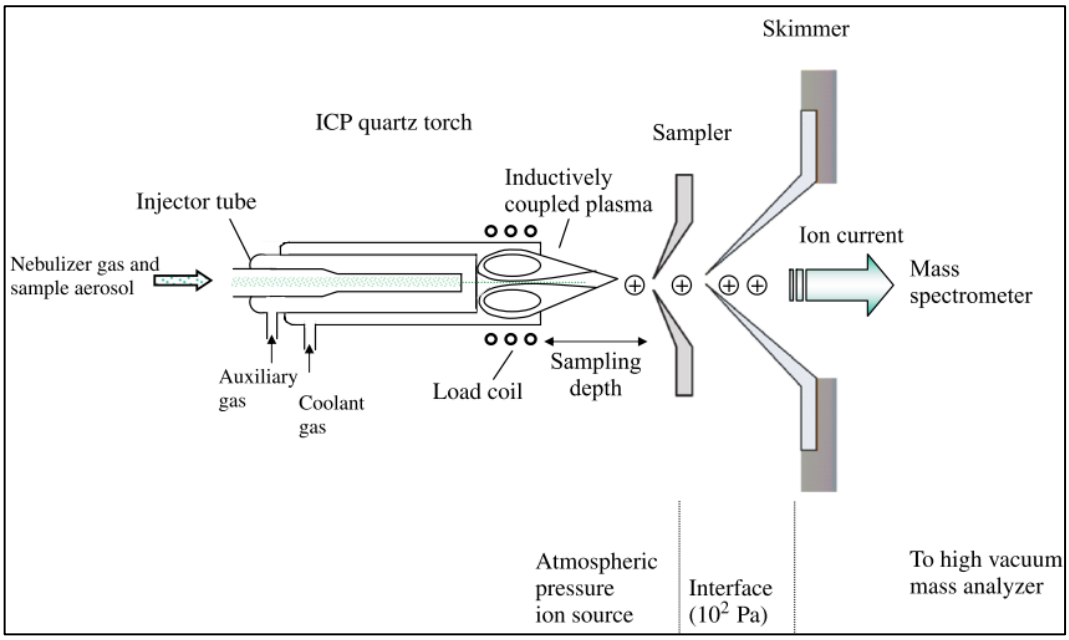

Figure 2.9 Schematic of inductively coupled plasma source in an ICP-MS instrument (J S Becker, 2007)

Interface: The positively charged ions that are produced in the plasma are extracted into the vacuum system, via a pair of interface cones, namely "sampler" and "skimmer". The cones are essentially metal plates with central orifices through which the ions pass. Small orifices are used, typically 1mm diameter or less, to maintain the high vacuum in the mass spectrometer region (see Figure 2.9).

Ion focusing: Electrostatic lenses keep the ions focused in a compact "ion beam" as they pass through the vacuum system to the final chamber, where the mass spectrometer (MS) and detector are housed. The ion lenses perform a second, essential, function of separating the ions from the photons and residual neutral material.

Mass spectrometer: Three different types of mass analyzers have been used with ICP-MS; these are quadrupole, magnetic sector, and time-of-flight analyzers.
A quadrupole mass analyzer is the most commonly used filter in mass spectrometers. This filter consists of four rods (approximately 1 cm in diameter and 15-20 cm long) that are arranged in perpendicular pairs. Alternating AC and DC voltages are applied to opposite pairs of the rods, allowing ions with a single mass-to-charge ratio (m/z) to cross the between-rods path to the detector at a specific point in time (Amr, 2012).
For a given voltage setting, only one m/z is stable and the quadrupole scans rapidly across the mass range (2-260 amu), passing each mass of interest sequentially to the electron multiplier (EM) detector.

Ion detection: The detector, so-called "electron multiplier" device, detects each ion as it exits the quadrupole. When a positive ion arrives at the detector, it is deflected onto the first dynode, which is held at a high negative voltage. The impact of the ion releases several free electrons from the dynode surface, which are repelled from the high negative voltage at the front and strike the next dynode. Each electron which strikes the second dynode releases several electrons from that surface and so on down the many stages of the detector, hence the name "electron multiplier". By the time the electron cascade reaches the final dynode, the multiplication factor has built up a pulse large enough to be measured reliably as an ion "count"(Agilent Technologies Inc., 2005).
The intensities for individual isotopes are converted into concentrations via calibration and a dedicated software (Mohd-Taufek et al., 2016).

2.6.3.1 Spectral Interferences in ICP-MS

While quadrupole-based ICP-MS is an immensely powerful multi-element analytical technique, it does suffer from some well-documented spectral interferences.

The main sources of spectral interferences in ICP-MS are:

- ✓ Direct overlap from a different element with an isotope at the same nominal mass (isobars), known as an isobaric interference, e.g. 87Rb overlap on 87Sr;
- ✓ Overlap from a polyatomic ion formed from the combination of species derived from the plasma gas (typically Argon), solution (water, organic), matrix (acids, buffers, salts, etc.) and plasma entrained atmospheric gas (O_2, N_2, etc.) sources, e.g. $^{40}Ar^{16}O$ overlap on ^{56}Fe;
- ✓ Doubly-charged species resulting from ions created by the loss of two electrons instead of just one. Because the quadrupole separates ions based on m/z (mass over charge ratio), a doubly-charged ion (M^{2+}) will appear at mass M/2. An example of a doubly-charged interference would be the $^{136}Ba^{2+}$ overlap on $^{68}Zn^{+}$.

Isobaric interferences can be avoid choosing another (interference free) isotope of the element of interest, for example ^{114}Cd is interfered with by the presence of ^{114}Sn, so ^{111}Cd, which has no isobaric overlap, can be used.

Generally, if a high plasma temperature is maintained, most potential polyatomic interferences will be reduced, often to levels where, in practice, they become negligible. The level of polyatomic interferences can be monitored using the production of refractory oxide ions of specific elements. Cerium (Ce) is an element commonly used for this purpose as it forms a strong oxide bond and therefore has one of the highest oxide formation rates. The CeO/Ce ratio is often referred to as a measure of plasma robustness in ICP-MS. Most ICP-MS systems operate at CeO/Ce ratios of 2-3%, whereas a well-designed ICP-MS can achieve a ratio of 0.3 - 0.5% CeO/Ce, about 5-10 times lower.

2.6.3.2 Collision/reaction cell technology

Collision/Reaction cells (CRC's) are a means to remove spectral interferences in ICP-MS and have been incorporated into instruments since the late 1990's. They have become so powerful and popular that most ICP-MS sold since the early 2000's are

equipped with a CRC. There are different configurations of CRC but fundamentally the device consists of RF multipole ion guides, which is enclosed in a cell that can be pressurized with a gas and is located after the main ion lenses (Agilent Technologies Inc., 2005; Koppenaal et al., 2004).

The gas interacts with the ion beam to remove polyatomic interferences in one of two ways:

- ✓ Reaction Mode, the gas reacts with an interference to convert it to a different species. A reactive gas e.g. H_2, O_2, NH_3, etc. is added to the cell to react with the interference, either converting it to a different species or neutralizing it (converting it to an un-charged atom or molecule);
- ✓ Collision Mode, the gas collides with the polyatomic interference, causing it to lose energy. Since polyatomic species are large, they undergo more collisions than the analytes, and so lose more energy. The lower energy interference is then separated from the higher energy analyte by energy discrimination (ED).

The use of ICP-MS system configured with collision/reaction cell technology has been demonstrated to be an efficient way to eliminate polyatomic interferences (Amr, 2012; Avula, Wang, Duzgoren-Aydin, & Khan, 2011; Döker & Uslu, 2014; Feldmann, Jakubowski, & Stuewer, 1999; Kawabata, Kishi, & Thomas, 2003; Luykx & van Ruth, 2008).

As an example we report the scheme of a single quadrupole Agilent 7500c equipped with a collision cell (Agilent's version of the CRC is the Octopole Reaction System (ORS^3)) for interferences correction as shown in Figure 2.10.

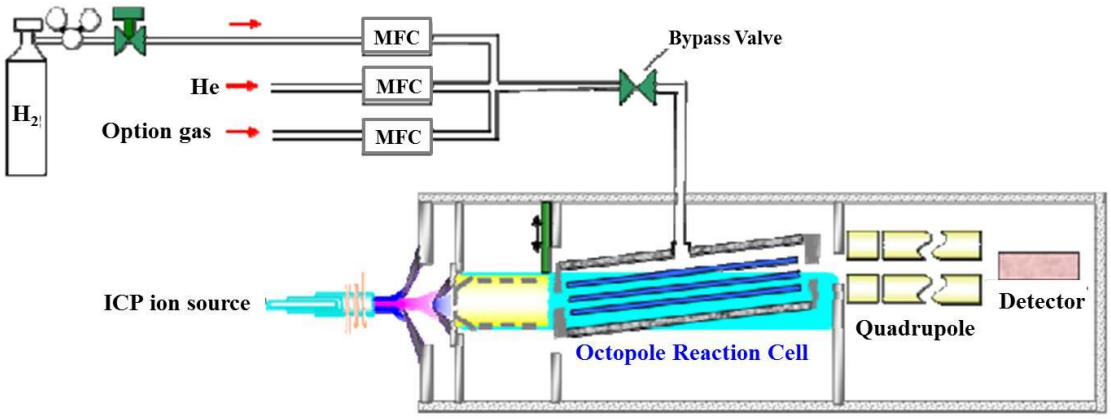

Figure 2.10 Schematic diagram of the single quadrupole mass spectrometer (Agilent 7500c)

Figure 2.11, shows the schematic diagram of a new Triple Quadrupole mass spectrometer (Agilent 8800) developed by Agilent Technologies (Mizobuchi, Kuwabara, Takakashi, Shikamori, & Sugiyama, 2012; Tyler & Olsson, 2006; Vanhaecke, 2015) which has been set up and used within this PhD thesis.

Compared to conventional quadrupole ICP-MS (or ICP-QMS), the ICP-QQQ features an additional quadrupole mass filter (Q1), situated in front of the Octopole Reaction System (ORS^3) cell and quadrupole mass filter (now called Q2).

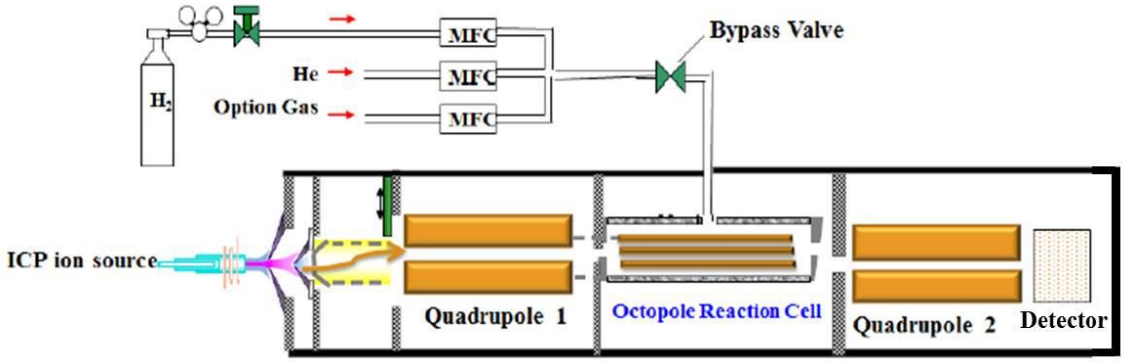

Figure 2.11 Schematic diagram of the triple quadrupole mass spectrometer (Agilent ICP-QQQ 8800)

The first quadrupole (Q1) selects only the m/z of the ion of interest to enter the ORS^3, avoiding the entering of possible interfering ion, while the second quadrupole (Q2) acts as a filter, selecting only the ion product after reaction with a reaction gas in the cell (MS/MS mode) and sending to the detector. In this case, the polyatomic interferences are eliminated successfully.

Details on the instrument characteristics and set up will be given in the following chapters concerning the work carried out within this PhD thesis.

2.7 Milk

In the human diet, milk is known as the most complete food because it provides all the macronutrients (proteins, lipids and carbohydrates) and all the essential micronutrients (elements, vitamins and enzymes), as reported in Table 2.11.

Human milk is particularly important in the case of early childhood because it is the only source of nutrients during the first months of an infant's life (Ataro et al., 2008).

This fact has an outstanding importance for some elements such as Se and Zn which are not stored by the fetus.

Table 2.11. Milk composition of some nutrients in 1 L whole milk, and their main health effects

Milk component	Concentration in 1 L whole milk*		Health effects
Fat	33	g/L	Energy rich
Saturated fatty acid	19	g/L	Increase HDL, small dense LDL, and total cholesterol. Inhibition of bacteria, virus
Oleic acid	8	g/L	Prevent CHD, gives stable membranes
Lauric acid	0.8	g/L	Antiviral and antibacterial
Myrisitc acid	3	g/L	Increase LDL and HDL
Palmitic acid	8	g/L	Increase LDL and HDL
Linoleic acid	1.,2	g/L	Omega-6 fatty acid
Alpha linolenic acid	0.75	g/L	Omega-3 fatty acid
Protein	32	g/L	Essential amino acids, bioactive proteins, peptides. Enhanced bioavailability
Lactose	53	g/L	Lactosylation products
Calcium	1.1	g/L	Bones, teeth, blood pressure, weight control
Magnesium	100	mg/L	For elderly, asthma treatment
Zinc	4	mg/L	Immune function. Gene expression
Selenium	37	µg/L	Cancer, allergy, CHD
Vitamin E	0.6	mg/L	Antioxidant
Vitamin A	280	µg/L	Vision, cell differentiation
Folate	50	µg/L	DNA synthesis, cell division, amino acid metabolism
Riboflavin	1.83	mg/L	Prevent a riboflavinosis
Vitamin B12	4.4	µg/L	Key role in folate metabolism

*data from USDA Food Composition Data

In fact, in the "formula" milk, essential elements have been usually added in order to satisfy nutritional requirements (Rivero Martino et al., 2000).

In cow milk, the mineral fraction, which is a small fraction of milk (about 8-9 g L^{-1}), contains cations (calcium, magnesium, sodium and potassium) and anions (inorganic phosphate, citrate and chloride). Concentration ranges of the different minerals in cow milk are indicated in Table 2.12.

This composition is considered as relatively constant, but slight variations can be observed in some cases.

Table 2.12. Mineral composition of cow milk*

Mineral	**Concentration ($mg \cdot kg^{-1}$)**
Calcium	1043–1283
Magnesium	97–146
Inorganic phosphate	1805–2185
Total phosphore	930–992
Citrate	1323–2079
Sodium	391–644
Potassium	1212–1681
Chloride	772–1207

*(Gaucheron, 2005)

For example, the calcium and phosphate contents are higher in milks rich in proteins, or the milk from Normandy cows has a higher mineral content than the milk from Friesian, Red Pied and Holstein cows (Gaucheron, 2005).

A good understanding of the properties of milk minerals is very important because of their nutritional and toxicological relevance in human health.

Milk and milk products, being an important source of beneficial nutrients for humans, are one of the most important food consumed around the world, (Inam, 2000; Oreste et al., 2016); on the other hand, milk is one of the earliest foodstuffs expose to adulteration (OJEC, 1990), such as water addition, reducing its nutritional value and causing additional health problems. For quality control, the investigation of the trace element contents in this kind of food is very important because of their nutritional and toxicological relevance in human health. For instance, Cr and Mn are essential elements but may become toxic at higher concentrations, while Pb and Cd are toxic and can be cumulative (Ataro et al., 2008; Onianwa et al., 1999; Rivero Martino et al., 2000). This is because they are readily transferred through food chains and are known to serve any essential biological function (Z. P. Liu, 2003).

Furthermore, the characteristics of the cow milk are highly depending on the geographical region and farming practices in which the cows are grown. Thus the origin of a milk product is an important factor, affecting its quality (Sola-Larrañaga & Navarro-Blasco, 2009).

Thus, it is important to evaluate safety and quality of milk products using their elemental composition as a quality parameter in order to assess the good value of the product (Kelly et al., 2005).

2.8 Aim of the thesis

Food product authenticity and traceability are major concerns not only for consumers, but also for agricultural farmers, retailers and administrative authorities. Food product provenance is of particular importance for consumer protection; meanwhile, geographic origin is another essential factor for evaluating the quality of agro-product, not because of changes in nutritional values but in terms of consumers deception, selling cheap foreign products as high-price regional agro-product (Zhao et al., 2014).

In this context, the food traceability is an important issue in food safety and quality control (Gonzalvez et al., 2009). New regulations and in particular the European regulation 178/2002 (European Parliament, 2002), applicable on January 1st 2005, imposed the requirement for the traceability of agro-products (Kelly et al., 2005).

At present, several trace and ultra-trace elements have been used successfully for the identification of the authenticity of some food products, such as wine, cheese, olive oils, honey, vegetables like onion, tomatoes, and others (Benincasa et al., 2007; Bettinelli, HM., Spezia, S., Baffi, C., Beone, G.M., Rocchetta, R., Nassisi, 2005; Jakubowski et al., 1999; Joebstl et al., 2010; Oddone et al., 2009; Pillonel et al., 2003; Spalla, JS, Baffi, C, Barbante, C, Turretta, C, Cozzi, G, Beone, GM, Bettinelli, 2009).

In this framework, the development of new and increasingly sophisticated techniques for determining the geographical origin of food products is highly desirable for consumers, agricultural farmers and administrative authorities (Reid et al., 2006).

On this basis, the aim of this PhD thesis have been firstly to set up a high level analytical facility for elemental analysis based on a Triple Quadrupole ICP-MS and subsequently to test and apply this approach to a selected set of food products in order to determine their elemental profile in order to assess the feasibility of geographical traceability through a chemometric approach.

The first part of this PhD has been therefore devoted to the installation, set-up and optimization of the ICP-MS Lab, including the mineralization system and sample handling in clean-lab conditions (Andreozzi et al., 2013).

In particular, my work has been focused on the:

- choice and installation of the Clean Room facility (ISO 6 class, see Section 3.4.2);
- choice, installation, set-up and optimization of the Agilent 8800 Triple Quadrupole ICP-MS (see Section 3.4.3);
- choice, installation, and set-up of the mineralization system for sample treatment using standard reference materials (NIST 1849a) (see Section 3.4.1);
- set-up and optimization of a clean lab suitable for sample treatment prior trace analysis by means of ICP-MS (see Section 3.4.1).

In order to respect all clean-lab conditions, reagents, chemicals and all required accessories for clean room and chemical lab were accurately evaluated, chosen and finally acquired.
The set-up and optimization of the ICP-MS Lab also included the validation of the analytical methods for elemental analysis at low and ultra-low level based on Linearity, Accuracy, Precision, Limit of detection, Limit of quantification, Repeatability and Reproducibility determinations (see Section 3.5).
These activities took about two years for reaching optimal working conditions.

The second part of my PhD activity has been focused on the thorough investigation of relevant elemental profiles in cow milk samples from various regions.
50 milk samples from different farms in Northern and Southern Italy and from Central Europe were characterized in term of trace and ultra-trace elements.
All samples were digested prior to ICP-MS, with a microwave digestion system according to the method developed to the scope and validated by means of suitable standard reference material (Milk Powder, NIST1849a).
The obtained composition data was firstly subjected to basic statistical analysis for data screening and compared to literature data. Subsequently data was tested for statistically significant differences among the three regions from which milk was studied.
Finally, the results were evaluated chemometrically using multivariate techniques with the aim of classifying cow milk samples according to different origin.
According to the results obtained, chemometric methods, such as Principal component Analysis (PCA) and Hierarchical Cluster Analysis (HCAA), coupled with Inductively

coupled plasma mass spectrometry (ICP-MS) have proved to be an extremely powerful tool to characterize and classify food products from different geographical origin, providing a fingerprint of the element patterns in the samples (Benincasa et al., 2008; Sola-Larrañaga & Navarro-Blasco, 2009).

Material and Methods

Chapter 3 Materials and Methods

3.1 Sample collection

A total of 50 samples of raw cow milk were collected from farms in Italy and in Europe, as reported in Table 3.1.

Table 3.1. Geographical origin of cow milk samples.

Standard number	Origin	Region
1-20	Camugnano (Bo)	Emilia Romagna - Northern Italy
21-34	Salerno (Sa)	Campania - Southern Italy
35-42	France	Europe
43-44	Germany	Europe
45-46	Slovenia	Europe
47-50	Hungary	Europe

Thirty-four Italian samples were supplied by two different farms in Northern and Southern Italy, namely Emilia Romagna (sample ID 1-20) and Campania (sample ID 21-34) regions, respectively.

The Italian samples were obtained between February and June 2015 on a weekly basis. The sixteen European samples were supplied by different farms in Central-Europe (sample ID 35-50) and the sampling was between February and July 2014 on a weekly basis.

Factory milk samples consisted of France (8 samples), Germany (2 samples), Slovenia (2 samples) and Hungary (4 samples).

Figure 3.1, shows the sampling sites of raw cow milk samples from selected regions of the Europe.

Samples of fresh raw milk (0.1 L) were taken from the bulk tanks at the farms and poured into 0.1 L polypropylene boxes prior to freezing at -20°C until analysis.

Figure 3.1 European raw cow milk sampling sites.

3.2 Sample treatment

All samples were digested with a microwave digestion system, Speedwave Four model (Berghof, Germany), equipped with temperature and pressure control for twelve digestion vessels completely made of TFM^{TM}-PTFE[5] was used in all experiments (maximum operating temperature 260°C; maximum operating pressure 40 bar).

The samples were digested in closed vessels, according to the following procedure inspired to the milk digestion method reported in the Application report of Speedwave Four V.8.0 of Berghof Products + Instruments GmbH (Berghof Products + Instruments GmbH, 2011). The digestion method used in this work was developed by myself in order to assess the best procedure for the digestions of both the milk powder, used as Standard Reference Materials (SRM) (NIST 1849a, Milk powder), and the fresh milk samples. Thus, the digestion procedure was improved using 7 mL of HNO_3 (69%) and 2 ml of H_2O_2 (30%) as acid mixture and increasing the microwaves power in percent from the 70 to 80% for the first step of the microwave heating program reported in Table .

[5] TFM^{TM}-PTFE has proven to be the material of choice for digestion vessels and is the only material used by Berghof. It is a perfluorated plastic with perfluoroalkoxy side chain (<1 % by weight).

5 ml of each milk sample were introduced directly in the Teflon digestion vessel and an acid mixture containing 7 mL of HNO_3 (69%) (Fluka, TraceSELECT®) and 2 mL of H_2O_2 (30%) (Carlo Erba, Ultrapure grade) was added.

The mixture was shaken carefully and after 10 minutes for reacting, the vessel was closed. The acid digestion was carried out using the microwave heating program shown in Table 3.2.

Table 3.2. Microwave heating program for cow milk digestion.

Step	Temperature (°C)	Ranging Time (min)	Time (min)	Power (%)
1	145	2	5	80
2	170	5	10	80
3	190	2	15	90
4	50	1	10	0

All samples were digested in triplicate and for each cycle of mineralization nine fresh milk samples and one blank sample (7 mL of HNO_3 and 2 mL of H_2O_2) were processed. The obtained acid solution was clear and the complete digestion was checked by running the analysis of a Standard Reference Material.

After the digestion, the acid mixture was left to cool to room temperature (about 20 minutes). After checking the clearly of the resulting solution, it was quantitatively transferred into plastic vials and made up to a final volume of 50 mL with a 2% HNO_3 and 1% HCl solution. Figure 3.2 shows the comparison between the milk samples a) before and b) after the microwave digestion.

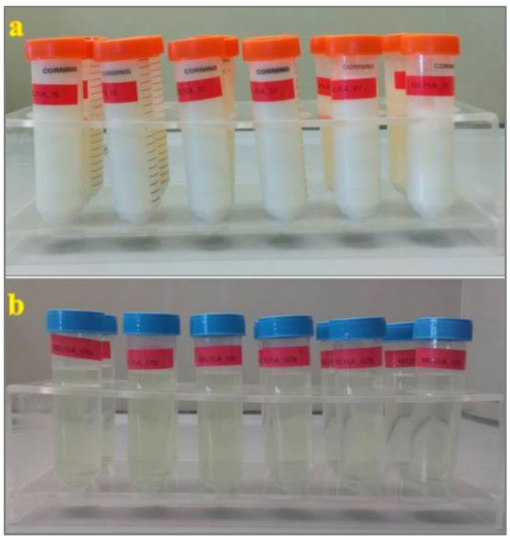

Figure 3.2 Cow milk samples a) before and b) after the mineralization process.

It is found that the resulting digested solutions are clear without any organic matrix residues. In addition, the final solution was diluted 5 and 100 times with a 2% HNO_3 and 1% HCl solution for the ICP-MS analysis. The first dilution (5 times) was carried out for the analysis of trace and toxic elements, whereas, the second dilution (100 times) was made for the macro-elements analysis.

The whole samples preparation procedure was performed in a clean laboratory dedicated to trace level analysis thanks to use of HEPA absolute filters that clean the air entering in the room in order to minimize the possible contamination (see further on in this chapter for details).

After each digestion cycle, the vessels were cleaned by means of the microwave digestion system using nine milliliters of HNO_3 and one milliliter of HCl as cleaning solution applying the heating program reported in Table 3.3.

Table 3.3. Microwave heating program for vessels cleaning.

Step	Temperature (°C)	Ranging Time (min)	Time (min)	Power (%)
1	170	5	5	80
2	200	1	15	90
3	50	1	10	0

After cooling, the vessels were washed three times with deionized water and dried in the oven at 100°C overnight.

3.3 Chemicals

All the reagents used in the experiment were analytical-reagent grade. TraceSELECT® grade 69% HNO_3 and 37% HCl, and ultra-pure grade 30-32% H_2O_2 were acquired from Sigma-Aldrich (St. Louis, MO, USA) and Carlo Erba Reagents (Milan, Italy), respectively.

High purity de-ionized water (resistivity 18.2mΩ cm^{-1}) was obtained from a Milli-Q Advantage A10 water purification system (Millipore, Bedford, MA, USA).

Two multi-element stock standard solutions containing respectively 43 (10 mg/L each in 3% HNO_3, P/N: IV-ICPMS-71A) and 16 elements (100 mg/L each in 7% HNO_3, P/N: CCS-4) supplied by Inorganic Ventures (Christiansburg, VA, USA) were used for calibration and spike recovery studies. The multi-element standards include 43 elements, such as Ag, Al, As, B, Ba, Be, Ca, Cd, Ce, Co, Cr3, Cs, Cu, Dy, Er, Eu, Fe,

Ga, Gd, Ho, K, La, Lu, Mg, Mn, Na, Nd, Ni, P, Pb, Pr, Rb, S, Se, Sm, Sr, Th, Tl, Tm, U, V, Yb, and Zn, and 16 elements, such as Al, As, Ba, Be, Bi, Ca, Cs, Ga, In, K, Li, Mg, Na, Rb, Se, and Sr.

A single-element standard solution containing 10 mg/L of Mercury (Hg) in 10% HNO_3 (P/N: MSHGN-10PPM, Inorganic Ventures, Christiansburg, VA, USA) was used for the calibration of the mercury in order to prevent the memory effect specific of this element. The memory effect, characteristic of Mercury, is caused by the contact of mercury with the materials that comprise the sample introduction system.

In order to overcome this problem, a series of blank solutions (n=5) was analyzed in sequence after the standard solutions used for the calibration of the mercury during the ICP-MS analysis. After the fifth sample, the signal (cps) for the mercury was controlled and compared with blank solution in order to verify the cleaning of the sample introduction line.

A solution containing ^{6}Li, Sc, Ge, Y, In, Tb and Bi in 5% HNO_3 (10 mg/L of each) provided by Agilent Technologies, was used for internal standardization. The internal standard (IS), after convenient dilution, was added in line using a peristaltic pump and a T piece, during the ICP-MS analysis in order to compensate for any variation in the intensity of the target element signal. The IS was used both for the calibration standards and the milk samples, to correct for analytical steps such as pipetting, auto-sampler injection, instrument fluctuations, and to some extent, matrix effects. To be successful, the IS and the target element should be similar in mass, and ideally have similar ionization potentials, and thus undergo similar enhancement and suppression effects. Elements that are similar in mass will succumb to similar effects of instrument drift because these effects are mass-dependent on the ICP-MS system (Ammerman et al., 2013).

Standards and samples were prepared daily with the acidified aqueous solution (2% HNO_3/1% HCl) used as carrier solution during the ICP-MS analysis.

All solutions, prepared in a clean laboratory in order to remove the possibility of contaminant, were stored in polypropylene containers.

Glassware was avoided in favor of plastic ware in order to prevent contamination from glass surface retention/release of elements introducing analytical artefacts into the samples/standards. All containers and plastic wares were kept closed tightly or covered when not in use to avoid the possibility of contamination.

Blanks Preparation

The acidified aqueous solution used as both the carrier solution during the ICP-MS analysis and the blanks solutions was prepared by adding 0.04 L of HNO_3 (69%) to 0.02 L of HCl (37%) and the solution was made to volume (2 L) using high purity de-ionized water (resistivity 18.2 mΩ cm^{-1}). A series of 10 blank solutions was prepared, transferring the carrier solution into ten vials used for ICP-MS analysis.

3.4 The instrumental facility

With the increasing demand for ultra-trace element determinations and quality assurance and quality control (QA/QC) in the studies of life sciences, environment and new materials the need for controlled laboratory environment is ever growing (Sloof, 2003).

In order to prevent and reduce the natural contamination of the samples, it's necessary to use appropriate precautions, working in specific laboratory where prepare and analyze the samples, to avoid the possible contamination due to the atmospheric fallout and researchers.

Within the "Polo Tecnologico" initiative in the Emilia Romagna Region, and refers to the Traceability lab, high level instrumental facility for trace element/isotopic analysis to provide information on the origin of foods, has been implemented in ENEA C.R. Brasimone.

The facility includes:

- a Clean Laboratory for sample treatment and preparation for the ICP-MS analysis;
- a Clean Room, (ISO 14644-1 Clean room Standard ISO 6 Class), with controlled pressure, temperature and humidity, suitable for the ICP-MS analysis;
- a Triple quadrupole inductively coupled plasma mass spectrometer (QQQ-ICP-MS) for trace and ultra-trace analysis.

3.4.1 Clean Laboratory

The facility includes a clean working lab for sample handling and mineralization by a microwave digestion system prior to ICP-MS analysis.

The Clean Laboratory has been set up with a system of absolute filters, HEPA H14 class, that makes the environment "clean" and suitable for trace analysis, in order to minimize contamination from the external environment, according to EN 1822:2009. This system provides a positive pressure condition in the room by means of a stainless steel system equipped with HEPA filter, developed and build by ENEA C.R. Brasimone. Figure 3.3 shows the inside (a, b) and outside (c) view of the system of absolute filters in the clean laboratory. The aspiration system was turned on until 30 minutes before entering in the laboratory, in order to remove the possible environmental contaminant.

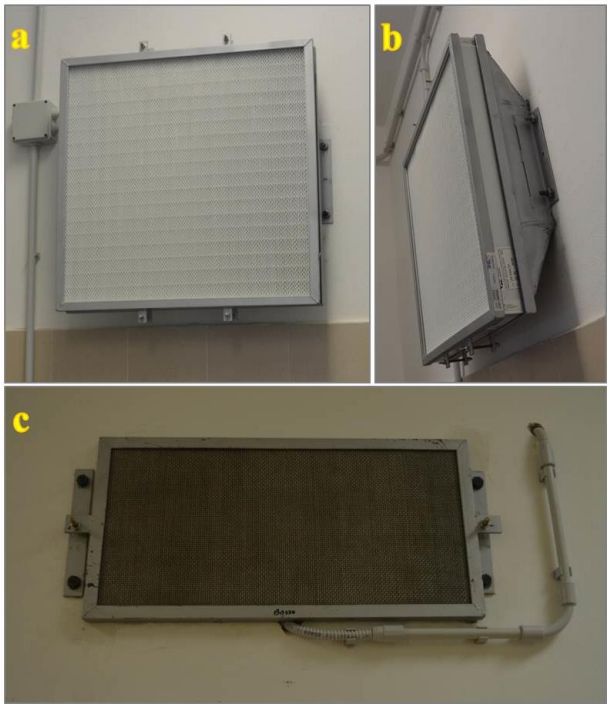

Figure 3.3 Clean Laboratory equipment: system of absolute filters. a), b) inside and c) outside view of system of filters across the lab wall.

The sample treatment was carried out by means of the instrumentations located in the Clean Laboratory, such as:

- a microwave digestion system, Speedwave Four model (Berghof, Germany), for sample digestion (see Figure 3.4). The system was equipped with both temperature and pressure control on all 12 vessels. The temperature monitoring system determines the temperature inside the pressure vessels by measuring the direct infrared radiation emitted by the sample, in each vessel. The pressure monitoring system controls the pressure in the vessels by using polarized light to measure any change in the photoelastic behavior of a glass ring in the vessel lid;

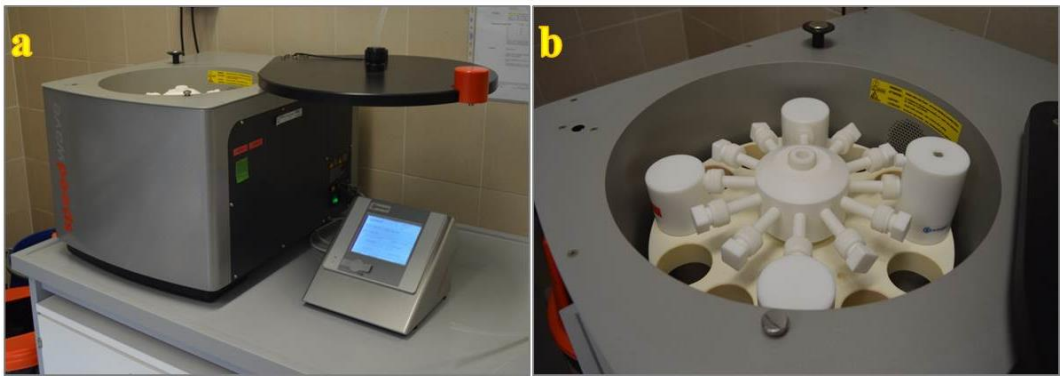

Figure 3.4 Clean Laboratory equipment: microwave digestion system. a) the microwave oven and the separate control unit and b) the opened oven with the rotor and the vessels.

- a fume hood for high heat and acid treatment such as (Hydrofluoric acid) (UNI EN 14175-7, 2012) (see Figure 3.5). The fume cupboards was made in selected materials, such as polypropylene and makrolon, that ensure suitability against chemical erosion and thermal deformation at the temperature of use;

Figure 3.5 Clean Laboratory equipment: fume hood for acid treatment.

- laboratory glassware in PTFE or PFA, being inert coating and high chemical resistant material and suitable for ultra-trace elements analysis.

Figure 3.6 Clean Laboratory equipment: laboratory glassware in PTFE or PFA.

3.4.2 Clean Room

All the measurements were carried out by means of a ICP-MS installed in a dedicated Clean Room ISO Class 6, (ISO 14644-1 Clean room), with controlled pressure, temperature and humidity.

The level of cleanliness in a laboratory is defined by a given maximum permissible number of particles of a particular size, per unit volume of air. Several standards of classification are currently in use worldwide, including the US Federal Standard 209E and the new ISO 14644 series developed by the International Organization for Standardization in Switzerland.

Table 3.4 shows airborne particulate cleanliness classes and maximum number of permissible particles for defined particle sizes according to the new ISO 14644 guidelines. The airborne particulate cleanliness, according to ISO 14644, is designated by a classification number (N).

The clean room, within the Traceability lab, satisfies all standard request for food trace analysis, with a maximum concentration limits (particles/m^3) of 1 x 10^6 for particles equal to and larger than 0.1 µm for ISO Class 6.

The clean room is structured in three main environments separated from each other: technical compartment, dressing room (ISO Class 8) and clean room (ISO Class 6) as shown in Figure 3.7.

Table 3.4. Selected airborne particulate cleanliness classes for clean rooms and clean zones

ISO Class Number, N	Maximum concentration limits (particles/m^3 of air) for particles equal to and larger than the considered sizes show below					
	≥0.1 µm	≥0.2 µm	≥0.3 µm	≥0.5 µm	≥1 µm	≥5 µm
ISO Class 4	1.0×10^4	2370	1020	352	83	
ISO Class 5	1.0×10^5	23700	10200	3520	832	29
ISO Class 6	1.0×10^6	237000	102000	35200	8320	293
ISO Class 7				352000	83200	2930
ISO Class 8				3520000	832000	29300

(International Organization for Standardization ISO 14644-1, 2015)

Each compartment is separated from other by a door with "interlock" system, so that it's impossible open two doors at the same time, in order to avoid the environmental contamination (see Figure 3.8a).

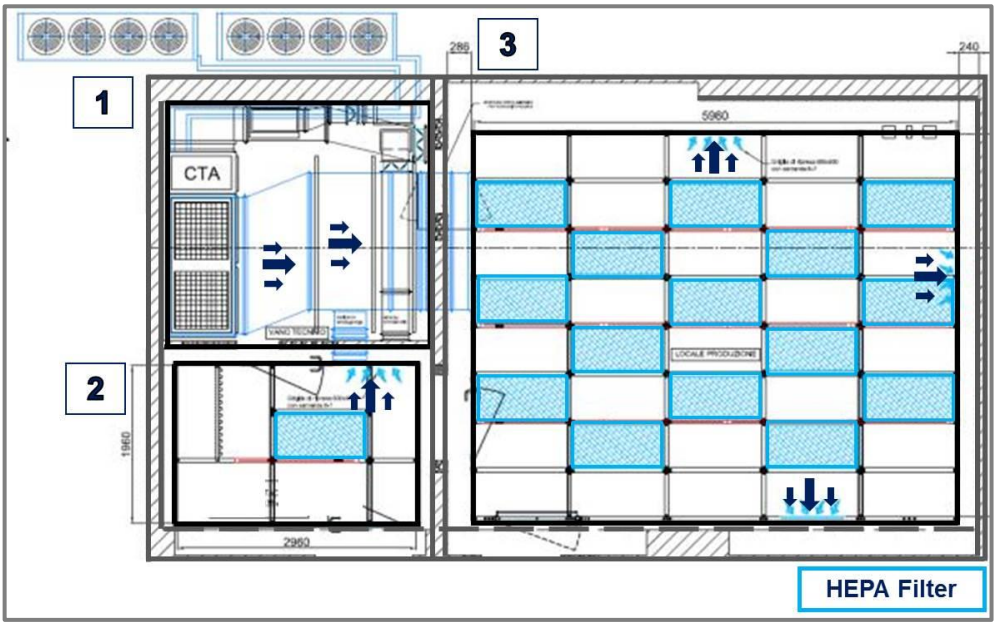

Figure 3.7 Scheme of the compartments of the clean room facility: 1) technical compartment; 2) dressing room; 3) clean room.

Near each door, a manometer in cmH_2O (from -0.1 to 8 cmH_2O) with a red fluid, measures the pressure within the local, as shown in Figure 3.8b.

Figure 3.8 The interlock system a) and the pressure control system b) that measures the pressure between the dressing room and outside.

The reference pressure values set for the clean room facility are:

- pressure between the dressing room and outside: 0.08 cmH_2O (7.85 Pa);
- pressure between the dressing room and the clean room ranged from 0.12 to 0.14 cm H_2O (12-14 Pa);
- pressure between clean room and outside ranged from 0.22 cm H_2O (22 Pa).

The parameters set for the clean room facility are pressure, temperature and humidity. Table 3.5 reports the operation condition of the clean room. This parameters are controlled by a PLC (Programmable Logic Controller) located in the technical compartment.

Table 3.5. Operation parameters of the Clean Room

Air flow (m^3/h)	**Humidity (%)**	**Temperature (°C)**
9400	≤ 50	20.8 - 21.5

In order to prevent the environmental contamination and obtain high pure solutions, the Milli-Q Advantage A10 water purification system (Millipore, Bedford, MA, USA) has been installed in the clean room (ISO Class 6), as shown in Figure 3.9.

Figure 3.9 The Milli-Q Advantage A10 water purification system located in the Clean Room in ENEA Brasimone Research Center.

The ICP-MS spectrometer has been installed in the clean room, as shown in Figure 3.10.

Figure 3.10 The inductively coupled plasma mass spectrometer (ICP-MS 8800 Agilent T.) installed in the Clean Room (ISO Class 6) in ENEA Brasimone Research Center.

Before entering in the Clean Room, all personnel needs to wear all required accessories left in the dressing room (see Figure 3.11).

After dressing, the personnel can access the clean area for sample processing and analysis.

Figure 3.11 The operating personnel, dressed with all required accessories, before entering in the Clean Room (ISO Class 6) in ENEA Brasimone Research Center.

All required accessories and procedures (hair cap, lab coats, masks, gloves and foot covers wearing) to minimize the release of particles generated by the operators has been applied and used in the clean room (see Figure 3.12).

Figure 3.12 The researcher at work in the Clean Room (ISO Class 6) in ENEA Brasimone Research Center.

The disposable clean room overalls are in TYVEC, a Teflon based fabric with very low particle emission, good chemical resistance, good flexibility and low cost.

3.4.3 The inductively coupled plasma mass spectrometer (ICP-MS)

A Triple quadrupole inductively coupled plasma mass spectrometer (QQQ-ICP-MS, 8800 model, Agilent Technologies, Santa Clara, CA, USA), equipped with two quadrupoles, one (Q1) before and one (Q2) after the Octopole Reaction System (ORS^3), was installed in the clean room facility, see Figure 3.13.

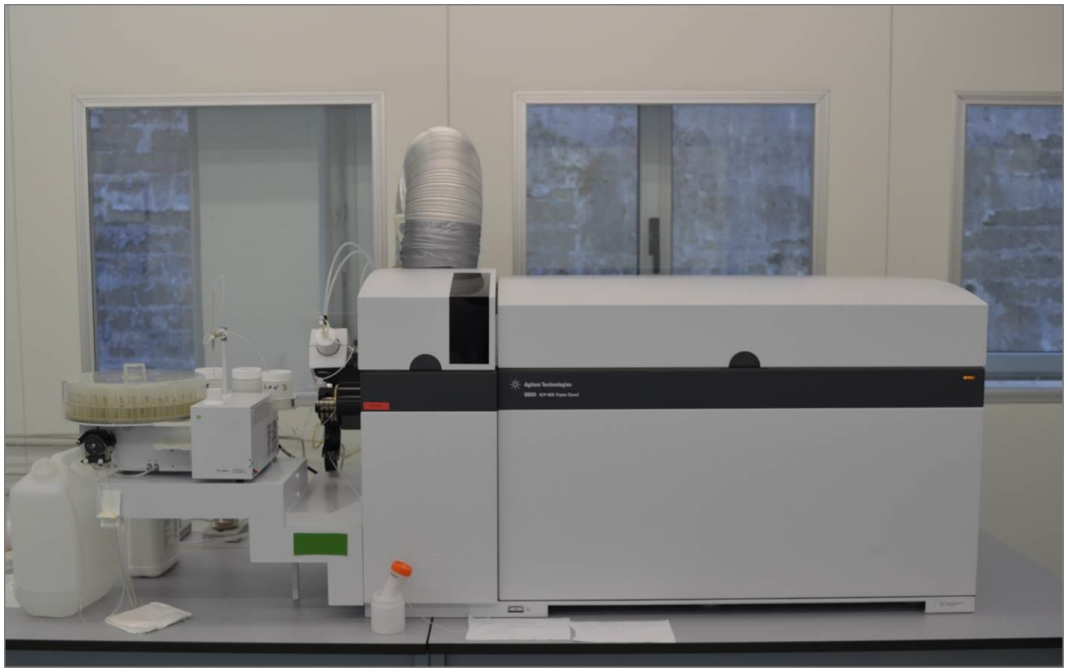

Figure 3.13 The triple quadrupole inductively coupled plasma mass spectrometer (QQQ-ICP-MS, 8800 model, Agilent Technologies) installed in the Clean Room facility.

The ICP-MS 8800 triple quadrupole operates in tandem MS (MS/MS) configuration: Q1 operates as a mass filter, allowing only the target analyte mass to enter the cell, and rejecting all other masses. This ensures that the reaction processes in the ORS^3 are precisely controlled and so accurate measurements can be performed even in complex, high-matrix sample. The second quadrupole (Q2) then selects only the ion of interest emerging from the cell, and rejects the ions at all other mass-to-charge ratios (Vanhaecke, 2015).

The scheme of MS/MS configuration is reported in Figure 3.14.

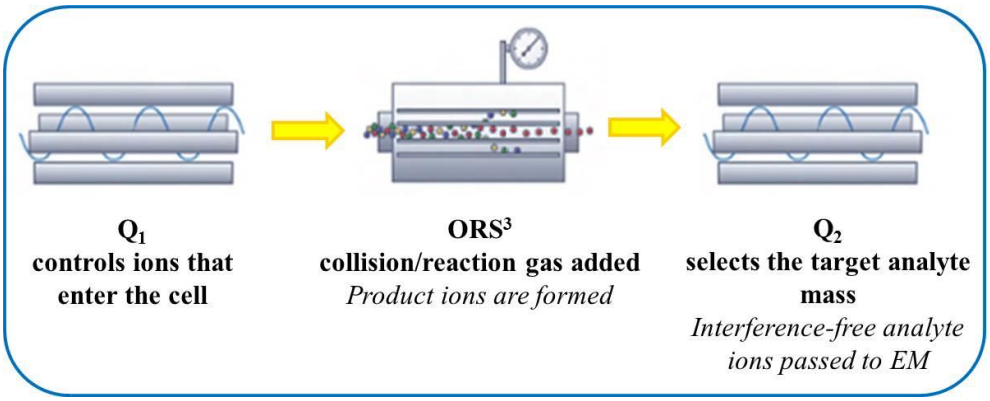

Figure 3.14 Scheme of tandem MS/MS configuration in the QQQ-ICP-MS 8800 model from Agilent Technologies

The octopole reaction system includes an octopole ion guide inside a pressurized reaction cell and can be operated in both collision and reaction modes for the removal of polyatomic spectral interferences.

The schematic of the ORS with the octopole ion guide in evidence is reported in Figure 3.15.

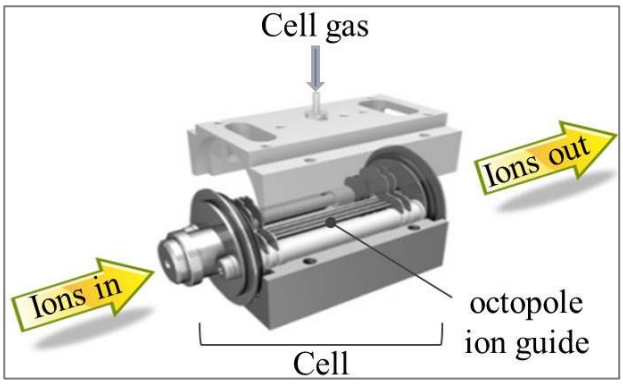

Figure 3.15 Schematic of Octopole Reaction System (ORS3) in the QQQ-ICP-MS, 8800 model from Agilent Technologies

All gases were supplied by Air Liquide (Milan, Italy) as ultrapure Alphagaz grade (99.999%).

The supply gas system (see Figure 3.16) was installed in the clean room, and it can accommodate up to five lines of gases, such as Argon, essential for plasma torch plus Helium, Hydrogen and Oxygen, used in the collision/reaction cell, according to their applications. In order to optimize the instrument capability, the ICP-MS was coupled with an integrated autosampler (Agilent I-AS model) capable of handling multiple sample vials, as shown in Figure 3.17.

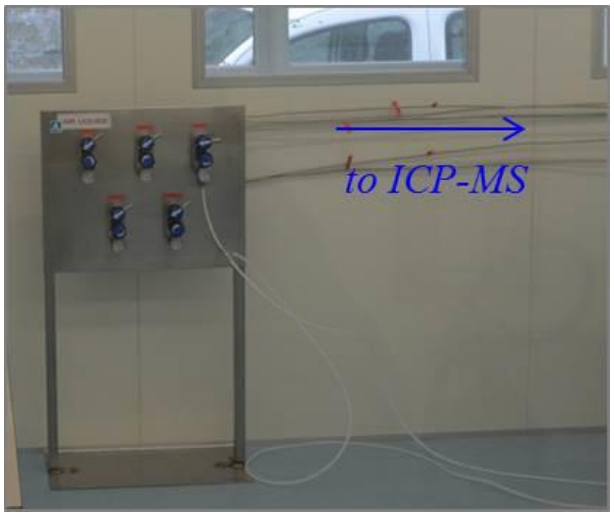

Figure 3.16 The supply gases system installed in the Clean Room for gases delivery to ICP-MS.

The sample tray contains slots for 89 sample vials with a volume up to 6 ml.

Figure 3.17 The Integrated Autosampler coupled with the ICP-MS installed in the Clean Room.

The instrument configuration includes Nichel interface cones, standard ion lens and sample introduction system consisting of a MicroMist glass concentric nebulizer (400 µL min^{-1}), a quartz Scott double-pass spray chamber cooled by a Peltier thermoelectric module down to 2°C to reduce the water vapor present in the sample aerosol, and a quartz shielded torch with 2.5 mm injector.

The tuning was performed by monitoring the signal in counts per second (CPS) of ^{7}Li, ^{89}Y, and ^{205}Tl contained in the tuning solution (1 µg/L of Li, Mg, Y, Ce, Tl, Co) supplied by Agilent Technologies (Santa Clara, CA, USA). A Nitric/Hydrochloric acid solution at 2% and 1% concentration (v/v), respectively was used as a carrier solution.
The instrument operating conditions as determined within the optimization work carried out within this thesis, are summarized in Table 3.6.

Table 3.6. Operating conditions of ICP-MS triple quadrupole.

QQQ-ICP-MS, 8800	
Cones	Nichel
Nebulizer	MicroMist
Plasma Torch	Quartz, 2.5 mm
Plasma gas	15 L/min
RF power	1550 W
Sample depth	8.0 mm
Carrier gas flow rate	0.8 L/min
Dilution gas flow rate	0.35 L/min
Total flow	1.15 L/min

3.5 Validation parameters and quality control

Accuracy. In order to validate the procedure for the preparation of cow milk samples, a Standard Reference Materials (SRM) (NIST 1849a, Milk powder) supplied by National Institute of Standards and Technology (Gaithersburg, MD, USA) was analyzed together with the different samples using the same experimental approach. The SRM (~ 700 mg weighing) and the samples were digested and analyzed as previously explained in Section 3.2.
Each cycle of digestion was made up of a reagent blank (i.e. clean vessel + acids) and three replicates for each sample. The use of the SRM allowed to determine the efficiency of the recovery for each of the certified element following the mineralization procedure applied prior to ICP-Ms analysis, as well as to assess the overall laboratory performance by determining systematically the bias between the certified and the experimental data on it.

In Figure 3.18, the content of certified elements in NIST 1849a is reported (available at https://www-s.nist.gov/srmors/certificates/archive/1849.pdf, visited on March 22nd 2017).

Table 2. Certified Mass Fraction Values for Elements in SRM 1849a

	Mass Fraction (mg/kg)	Coverage Factor, k
Calcium (Ca)[a,b,c]	5253 ± 51	2.00
Copper (Cu)[a,b,c]	19.78 ± 0.26	2.00
Chromium (Cr)[a,b,c,d]	1.072 ± 0.032	2.00
Iodine (I)[b,d,e]	1.29 ± 0.11	2.00
Iron (Fe)[a,b,c]	175.6 ± 2.9	2.00
Magnesium (Mg)[a,b,c]	1648 ± 36	2.00
Manganese (Mn)[a,b,e]	49.59 ± 0.97	2.00
Molybdenum (Mo)[a,b,c,d]	1.707 ± 0.040	2.00
Phosphorus (P)[a,b,c]	3990 ± 140	2.00
Potassium (K)[a,b,c]	9220 ± 110	2.00
Selenium (Se)[b,c,d]	0.812 ± 0.029	2.00
Sodium (Na)[a,b,c]	4265 ± 83	2.00
Zinc (Zn)[a,b,c]	151.0 ± 5.6	2.00

[a] NIST ICP-OES
[b] Collaborating laboratories
[c] Manufacturer
[d] NIST ICP-MS
[e] NIST INAA

Figure 3.18 Certified Mass Fraction Values for Elements in Reference Standard Material 1849a from NIST.

Spike recovery studies. In order to evaluate the accuracy of the proposed method, spike recovery studies were performed for all 40 elements chosen in the analytical procedure herein optimized. The Standard Reference Materials (NIST 1849a, Milk powder) was spiked with an appropriate volume of multi-element standard solution with 3 standard additions: Standard addition 1, 250 µL to a final concentration of 10 µg/L for trace elements, Standard addition 2, 250 µL to a final concentration of 0.5 µg/L for Mercury and Standard addition 3, 250 µL to a final concentration of 0.5 ng/L for Rare Earth elements. The samples were spiked before their microwave digestion in order to assess the recovery for the whole process. The microwave digestion was carried out using the developed method described above.

Limit of Detection and Limit of Quantification. As required in a traceable and reliable analytical procedure, Limit of Detection (LODs) and Limit of Quantification (LOQs) for 40 elements at low concentration level in milk were determined.

LODs and LOQs were estimated respectively as three and ten times of the standard deviation (σ) of 10 consecutive measurements of the reagent blanks, which were prepared with the same procedure as the real milk samples, according to EURACHEM recommendation (O. Magnusson, 2014).

Internal Standard (IS). The internal standard mix (stock concentration 200 µg/L) was used both for the calibration standards and the milk samples, to correct the matrix effects. Automatic addition of internal standard was carried out by adding, using a T-piece, the internal standard solution into the sample line using the fitted-in peristaltic pump. The pump tubing used for both the sample and internal standard solutions determined the degree of dilution of both solutions for accurate matrix compensation, see Figure 3.19.

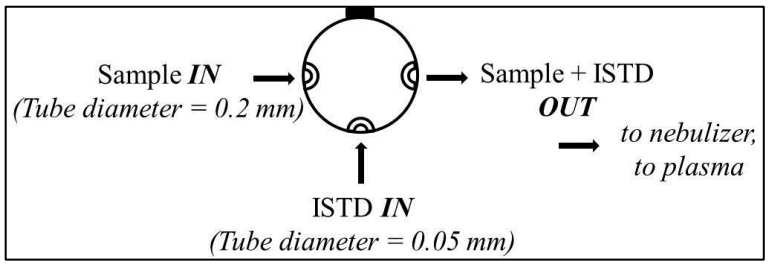

Figure 3.19 Schematic representation of Sample/ISTD introduction system on the ICP-MS 8800 triple quadrupole.

The recovery values for the internal standard, included in the range 70-120% (see the results and discussion section for details), ensure a long term stability during the ICP-MS analysis.

Quality Control Standards (QCs). Quality control standards (QCs) were added in the batch of analysis, every 10 samples, in order to control the accuracy of the analysis.

External Calibration. Quantitative analysis of the elements in digested SRM and milk samples, were based on calibration curves obtained at different concentration levels: 1, 5, 10, 50, 100, 200, 300, 400 µg/L for the elements ^{9}Be, ^{11}B, ^{31}P, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{78}Se, ^{85}Rb, ^{88}Sr, ^{111}Cd, ^{133}Cs, ^{137}Ba, ^{205}Tl, ^{208}Pb, ^{232}Th, ^{238}U; 0.5, 0.7, 0.8, 1, 2, 3, 4 mg/L for macro elements ^{23}Na, ^{24}Mg, ^{39}K, ^{40}Ca; 0.10, 0.25, 0.50, 1.00 µg/L for ^{202}Hg and 0.25, 0.50, 1, 2.5, 5, 7.5, 10 ng/L for ultra-trace elements ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm, ^{157}Gd, ^{163}Dy, ^{165}Ho, ^{166}Er, ^{169}Tm, ^{175}Lu.

The calibration solutions were prepared using a programmable pipette (Gilson). Calibration ranges were modified according to the expected elements concentration ranges in digested milk samples.

Repeatability and Reproducibility. The repeatability and reproducibility of the proposed procedure were also evaluated. The intra-day repeatability was studied by digestion of one milk sample for five subsequent times in one day and the obtained solutions were analyzed by means of ICP-MS with five replicated measurements each. Moreover, the inter-day reproducibility was studied by digestion of one milk sample three times in three consecutive days and with three replicated measurements by ICP-MS.

Multivariate data processing. In order to classify the samples according to their geographical origin, chemometric treatment of data based on multivariate techniques was applied. Multivariate methods for data reduction applied included factor and cluster analysis with the aim of pointing out the association between element arrays and source region of the milk samples analyzed . Details on the statistical methods adopted are reported further on in this thesis

All the statistical and multivariate analyses were carried out using the software STATISTICA 7.0.61.0. Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA) on the complete data set

Chapter 4 Multivariate statistical tools

4.1 Chemometric techniques

Chemometric analysis of the data provided by analytical instruments, such as ICP-MS, which have the ability to determine more than one component at a time in a sample, can be used to establish relations to the food provenance.

The set of concentrations of the components, that have a satisfactory discriminatory power, provides a characteristic pattern or 'fingerprint' relating to the geographical origin of the sample (Luykx & van Ruth, 2008).

Thus, chemometric techniques provide the ability to detect these patterns, and is really useful when the number of components necessary to differentiate samples from different geographical origins increases.

Multivariate data are classified into target groups by chemometric pattern recognition methods (Table 4.1) (Kaoru Ariyama & Yasui, 2006).

Table 4.1. Typical chemometric techniques used to determine geographic origin*

Pattern recognition method		
Unsupervised (conducting exploratory analysis and classification using only explanatory variable data)	**Supervised** (conducting analysis and classification by selecting and weighting explanatory variables to classify the target groups)	
	Non-parametric method (does not hypothesize statistical distribution; applicable with only little sample data)	**Parametric method** (hypothesizes statistical distribution; provides considerable statistical information)
Principal Component Analysis (PCA), Cluster Analysis (CA)	K-Nearest Neighbors (KNN), Artificial Neural Networks (ANN)	Linear Discriminant Analysis (LDA), Soft Independent Modeling of Class Analogy (SIMCA)

*(Kaoru Ariyama & Yasui, 2006)

The unsupervised pattern recognition methods, such as Principal component analysis (PCA) and Cluster analysis (CA), are used to simply recognize differences among obtained data. On the other hand, the supervised classification (discriminant) techniques, such as Linear discriminant analysis (LDA), Partial least squares (PLS), K

nearest neighbor (KNN) method, Soft independent modeling of class analogy (SIMCA) and Artificial neural networks (ANNs), are used to establish a technique for determining geographic origin because these pattern recognition methods give clearer discrimination results.

4.1.1 Principal Component Analysis (PCA)

PCA (Jackson, 1991) is a technique that, by the reduction of the data dimensionality, allows their visualization, while retaining as much as possible of the information present in the original data (Berrueta, Alonso-Salces, & Héberger, 2007).

So, PCA transforms the original measured variables into new uncorrelated variables called principal components (PCs). Each PC is a linear combination of the original measured variables. This technique affords a group of orthogonal axes that represent the directions of greatest variance in the data. The first PC (PC1) accounts for the maximum of the total variance, the second (PC2) is uncorrelated with the first and accounts for the maximum of the residual variance, and so on, until the total variance is accounted for (Drivelos & Georgiou, 2012).

The values that represent the samples in the space defined by the principal components are the component scores. The scores can be used as input to other multivariate techniques, instead of the original measured variables (Guimet, Ferré, & Boqué, 2005; Tewari & Irudayaraj, 2005). Generally, the result of performing PCA is represented in a plot of scores of PCs.

Figure 4.1, shows an example of the plot of scores of PC2 versus PC1 for the data of a case study on the chromatographic columns behavior (Brereton, 2003).

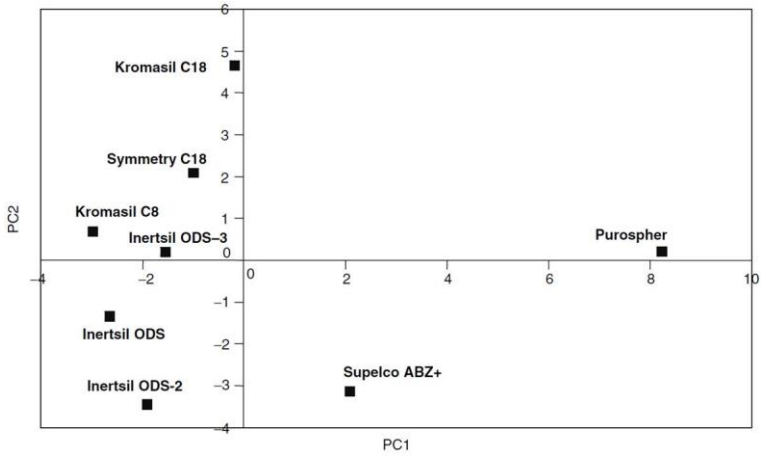

Figure 4.1 Plot of scores of PC2 versus PC1 after standardization for case study 2 (Brereton, 2003)

Principal components loadings plot shows the grouping of the columns and suggests that the three Inertsil columns behave very similarly whereas other two (Kromasil C18 and Supelco ABZ+) behave in a diametrically opposite manner.

Nowadays, PCA is the most widely used multivariate analysis technique in science and engineering (Lavine, 2006).

The efficacy of this procedure has been demonstrated in several studies including determination of geographical origin of several food products such as artichoke (Di Salvo et al., 2014), tea (Ku et al., 2010), ciders (García-Ruiz et al., 2007), fruits (Benabdelkamel et al., 2012; Hidalgo, Fechner, Marchevsky, & Pellerano, 2016; Maietti et al., 2012; Santos et al., 2014), cheeses (Moreno-Rojas, Sánchez-Segarra, Cámara-Martos, & Amaro-López, 2010; Pillonel et al., 2003), olive oils (Longobardi et al., 2012), wines (Geana et al., 2013; Gonzálvez, Llorens, Cervera, Armenta, & de la Guardia, 2009; Thiel et al., 2004), hazelnuts (Oddone et al., 2009) and honey (Pellerano, Uñates, Cantarelli, Camiña, & Marchevsky, 2012).

4.1.2 Hierarchical Cluster Analysis (HCA)

Another unsupervised pattern recognition technique used for preliminary evaluation of the information contents in the data matrices is the Hierarchical cluster analysis (HCA) (Jackson, 1991).

In cluster analysis, samples are grouped on the basis of similarities without taking into account the information about the class membership (Möller, Frese, & Bro, 2005).

HCA groups samples according to a similarity metric, which can be distance, correlation or some combination of both. This technique is based on the idea that the similarity is inversely related to the distance between samples. So, HCA calculates the distances (or correlation) between all samples using a defined metric such as Euclidean distance, Manhattan distance, etc. A pre-treatment of the data is required in order to avoid the effect of different scales of the variables. (Berrueta et al., 2007)

The results of HCA are presented in the form of a dendrogram, as reported in the example in Figure 4.2.

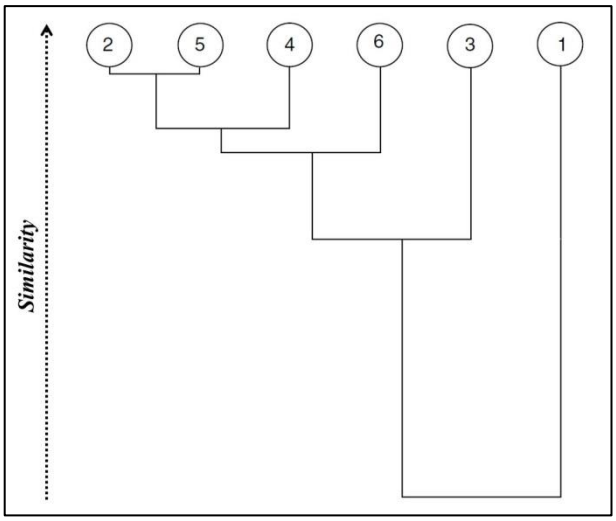

Figure 4.2 Dendrogram for cluster analysis example (Brereton, 2003).

The objects are organized in a row, according to their similarities: the vertical axis represents the similarity measure at which each successive object joins a group. In this case, the objects from two to six appear to form a single group, while object 1 is very different from the others.

Published reviews have shown the value of this pattern recognition technique in the determination of geographical origin of specific food such as rice (Lagad, Singh, & Rai, 2017), coffee (Pohl, Stelmach, Welna, & Szymczycha-Madeja, 2013), onions (K Ariyama et al., 2007) and cereals (Li, Dong, Luo, Xian, & Fu, 2015), and beverages such as wine (Bentlin et al., 2012; Frías, Conde, Rodríguez-Bencomo, García-Montelongo, & Pérez-Trujillo, 2003; Galgano et al., 2008; Haswell & Walmsley, 1998) and tea (Fernández, Pablos, Martín, & González, 2002).

Experimental Section and Discussion

Chapter 5 Development and validation of the analytical method

5.1 Development and validation of analytical method for multi-element analysis

5.1.1 Interferences

The interferences occurring in ICP-MS can be divided into two sources, polyatomic ions and isobaric overlap. These interferences can significantly affect the determination of multi-elements at trace and ultra-trace level (Amr, 2012).

Polyatomic ions

Polyatomic ions result from the short-lived combination of two or more atomic species, e.g., ArO^+. The polyatomic or molecular spectral interference is the most common type of interferences and it is associated with either the plasma and nebulizer gas used, matrix components in the solvent or other elements in the sample (Thomas, 2002b).

Isobaric overlaps

An isobaric overlap exists where two elements have isotopes of essentially the same mass that cannot be resolved by the conventional quadrupole (IUPAC, 1991). Most of the elements in the periodic table have at least one (e.g., Co), two (e.g., Sm), or even three (e.g., Sn) isotopes that are free from isobaric overlap. No isobaric peak interference is observed for those isotopes below m/z = 36. The severity of this type of interference is dependent to some extent on the sample matrix and relative proportions of the elements concerned (Amr, 2012).

5.1.2 Optimization of the ICPMS method

In order to check the performance of the ICP-MS, a multi-element standard solution from Inorganic Ventures was evaluated as calibration standard. The standard solution contains different elements including heavy metals, trace and macro elements.

The optimized acquisition parameters of ICP-MS triple quadrupole developed for the multi-element analysis are reported in Table 5.1.

Table 5.1. Acquisition parameters of ICP-MS triple quadrupole.

QQQ-ICP-MS, 8800	
Replicates	3
Sweeps/replicate	90
Peak Pattern	3 Points
Isotopes *(no gas-mode)*	^{9}Be, ^{11}B, ^{202}Hg, ^{232}Th, ^{238}U
Isotopes *(helium mode)*	^{23}Na, ^{24}Mg, ^{31}P, ^{39}K, ^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{111}Cd, ^{133}Cs, ^{137}Ba, ^{205}Tl, ^{208}Pb
Isotopes *(hydrogen mode)*	^{40}Ca, ^{56}Fe, ^{78}Se
Integration time (s)	0.30 - 3
Collision/Reaction cell parameters	
He cell gas flow rate	4.5 mL/min
He stabilization time (s)	15
H_2 cell gas flow rate	7.0 mL/min
H_2 stabilization time (s)	15

The instrumental method was developed modifying the collision/reaction gases (He and H_2) in the ORS^3 for each element, depending on the interference type, i.e. isobaric or polyatomic.

The ORS^3 was used in no gas-mode for ^{9}Be, ^{11}B, ^{202}Hg, ^{232}Th, ^{238}U, in Helium mode (He) for ^{23}Na, ^{24}Mg, ^{31}P, ^{39}K, ^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{111}Cd, ^{133}Cs, ^{137}Ba, ^{205}Tl, ^{208}Pb, and in hydrogen mode (H_2) for ^{40}Ca, ^{56}Fe and ^{78}Se.

After the first quadrupole (Q1), the ions are extracted under vacuum into a collision/reaction cell that is positioned before the analyzer quadrupole (Q2).

A collision/reaction gas, such as helium or hydrogen, is then bled into the cell, which consists of a multipole (such as an octapole), operated in the radio frequency (RF)-only mode. The RF-only field does not separate the masses like a traditional quadrupole, but instead has the effect of focusing the ions, which then collide and react with molecules of the collision/reaction gas. By a number of different ion-molecule collision and reaction mechanisms, polyatomic interfering ions like ^{40}Ar, ^{40}Ar^{16}O, and ^{40}Ar^{40}Ar^{+}, will

either be converted to harmless noninterfering species, or the analyte will be converted to another ion which is not interfered with (Thomas, 2002a).

Helium mode

Collision mode (using **Helium** gas in the cell) was used successfully to remove the polyatomic interferences for ^{23}Na, ^{24}Mg, ^{31}P, ^{39}K, ^{51}V, ^{52}Cr,^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{111}Cd, ^{133}Cs, ^{137}Ba, ^{205}Tl, and ^{208}Pb.

The molecules of helium collide with the large polyatomic ions formed in the plasma enabling them to be separated in the second quadrupole mass analyzer (Q2). In this way, the polyatomic interferences are converted to harmless noninterfering species (Feldmann et al., 1999).

Hydrogen mode

As reaction gas, H_2 was selected for the removal of the polyatomic interferences associated with use of argon necessary in the plasma torch operations. In particular, the **Hydrogen** was used as reaction gas for ^{40}Ca, ^{56}Fe, and ^{78}Se, to reduce polyatomic argon ion species ($^{40}Ar^+$, $^{40}Ar^{16}O^+$, $^{40}Ar^{40}Ar^+$), which are a significant source of severe spectroscopic interferences in ICP-MS (Avula et al., 2011).

Reaction chemistry of H_2 is well known, and the mainly three different processes are reported in Table 5.2.

Table 5.2. Reaction chemistry of Hydrogen

Chemical process	Reaction
Hydrogen atom transfer	$Ar^+ + H_2 \rightarrow ArH^+ + H$
Proton transfer	$ArH^+ + H_2 \rightarrow H_3^+ + Ar$
Charge transfer	$Ar^+ + H_2 \rightarrow H_2^+ + Ar$

The probability of the different reactions can be expressed in terms of gas phase ion-molecule reaction rate constants, which are high for all the three processes above (in the order of 10^{-9} cm^3 s^{-1}), making these reaction processes extremely selective (Feldmann et al., 1999; Ikezoe, Matsuoka, Takebe, & Viggiano, 1987).

The reduction of three typical interferences by polyatomic argon ions (Ar^+ at m/z= 40, ArO^+ at m/z= 56 and Ar_2^+ at m/z= 80) as a function of varying H_2 gas flow rates from measurement of carrier acid solution, was carried out and shown in Figure 5.1.

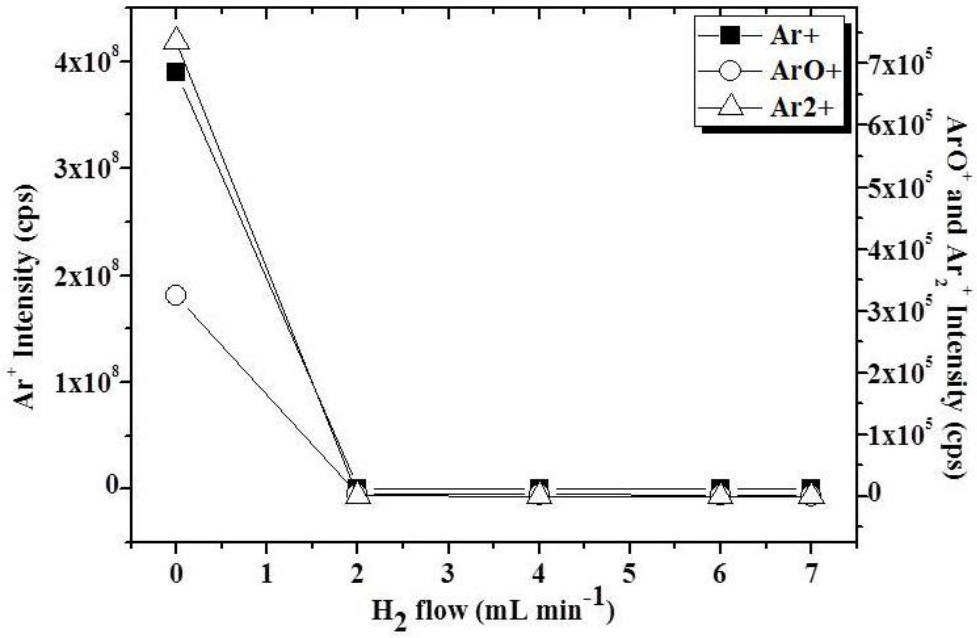

Figure 5.1 Intensities of Ar^+, ArO^+ and Ar_2^+ ions as a function of the hydrogen gas flow into the collision cell.

H_2 gas flow rates ranged from 0 to 7 mL min^{-1}. The intensities of Argon ions species were measured in cps and ranged from $3x10^5$ cps for ArO^+ ions to $4x10^8$ for Ar^+ ions.
The intensities in cps decrease considerably until a constant value is reached at flow rate of about 7 mL min^{-1}.
The results demonstrate that the use of hydrogen as reaction gas can be an effective tool to eliminate the polyatomic argon species interferences.

5.1.3 Validation of the Method

Linearity

Instrumental response vs calibration standards was found to be strongly linear over the whole concentration range investigated for all analyzed elements with correlation coefficient (R^2) in the range from 0.9997 for ^{24}Mg to 1.0000 for ^{23}Na, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{78}Se, ^{202}Hg, and ^{232}Th.
All standards and samples were injected in triplicate.
All calibration data, including gas mode, calibration range and correlation coefficient for the analysis of 27 elements, are reported in Table 5.3.

Table 5.3. Calibration data for the analysis of 27 elements.

Element	Isotope	Mode	Calibration range	Unit	R^2
Be	9	No gas	1-400	µg/L	0.9999
B	11	No gas	1-400	µg/L	0.9999
Na	23	He	0.5-4	mg/L	1.0000
Mg	24	He	0.5-4	mg/L	0.9997
P	31	He	1-400	µg/L	0.9999
K	39	He	0.5-4	mg/L	0.9998
Ca	40	H_2	0.5-4	mg/L	0.9998
V	51	He	1-400	µg/L	1.0000
Cr	52	He	1-400	µg/L	0.9999
Mn	55	He	1-400	µg/L	1.0000
Fe	56	H_2	1-400	µg/L	1.0000
Co	59	He	1-400	µg/L	1.0000
Ni	60	He	1-400	µg/L	0.9999
Cu	63	He	1-400	µg/L	1.0000
Zn	66	He	1-400	µg/L	1.0000
As	75	He	1-400	µg/L	0.9999
Se	78	H_2	1-400	µg/L	1.0000
Rb	85	He	1-400	µg/L	0.9998
Sr	88	He	1-400	µg/L	0.9999
Cd	111	He	1-400	µg/L	0.9998
Cs	133	He	1-400	µg/L	0.9999
Ba	137	He	1-400	µg/L	0.9999
Hg	202	No gas	0.10-1.00	µg/L	1.0000
Tl	205	He	1-400	µg/L	0.9999
Pb	208	He	1-400	µg/L	0.9999
Th	232	No gas	1-400	µg/L	1.0000
U	238	No gas	1-400	µg/L	0.9999

Accuracy

In order to check the accuracy of the method, the certified reference material (Milk powder NIST1849a) was spiked with two different standard additions (10 µg/L and 0.5 µg/L of multi-element standard for trace elements and Hg, respectively). The standard addition method was used because the certified elements in the standard reference materials did not fit all the 40 elements chosen and analyzed in this work. In this case for elements not covered by certification, accuracy was tested not by the bias evaluation but adopting an experimental approach based on the internal standard spiking (RIFF). The samples were therefore spiked prior to microwave digestion in order to assess the recovery for the whole process.

Table 5.4 lists the recoveries for the 27 analyzed elements determined with three measurements of concentration for each element, together with the relative standard deviation (both in percent).

Table 5.4. Recoveries (in percent) and relative standard deviation (in percent) for NIST1849a, in order to check the accuracy of the microwave digestion and ICP-MS method.

Element	Isotope	Recovery (%)	RSD (%)
Be[a]	9	81.4	1.5
B[a]	11	107.6	2.8
Na[b]	23	107.3	3.5
Mg[b]	24	96.3	3.1
P[b]	31	98.8	3.1
K[b]	39	100.1	3.3
Ca[b]	40	109.7	1.6
V[a]	51	91.6	1.8
Cr[b]	52	93.6	1.9
Mn[b]	55	90.0	1.2
Fe[b]	56	96.2	0.2
Co[a]	59	87.9	2.0
Ni[a]	60	91.9	2.0
Cu[b]	63	88.3	0.6
Zn[b]	66	83.4	0.5
As[a]	75	96.7	1.6
Se[b]	78	99.8	2.1
Rb[a]	85	98.8	0.4
Sr[a]	88	97.3	0.7
Cd[a]	111	82.8	0.4
Cs[a]	133	92.3	0.6
Ba[a]	137	97.5	0.9
Hg[c]	202	97.9	0.8
Tl[a]	205	87.1	0.1
Pb[a]	208	88.2	0.5
Th[a]	232	103.9	1.6
U[a]	238	104.1	1.9

[a] Standard addition of 10 µg/L for trace elements
[b] Elements contained in SRM NIST1849a with Certified Mass Fraction in mg/kg
[c] Standard addition of 0.5 µg/L for Hg

The agreement between measured and certified values is satisfactory and confirmed that the method is accurate for quantitative analysis of multi-elements in milk samples. The accuracy values ranged from 81.4 % for Be to 109.7 % for Ca. The best results were obtained for P (98.8%), K (100.1%), Se (99.8%), Rb (98.8%), Sr (97.3%) and Th (103.9%).

Precision

The precision of the method is defined as Relative Standard Deviation (RSD) calculated using the standard deviation divided by the mean of replicated samples see Table). The RSD for the analyzed samples ranged between 0.1% for Tl to 3.5 % for Na, thus presenting satisfactory values were found.

Limits of detection (LODs) and Limits of quantification (LOQs)

Limits of detection (LODs) and Limits of quantification (LOQs) were estimated for 27 elements. Results for the multi-element analysis are reported in Table 5.5.

Table 5.5. Limit of detection and Limit of quantification data for 27 elements.

Element	**Isotope**	**LOD ($\mu g\ L^{-1}$)**	**LOQ ($\mu g\ L^{-1}$)**
Be	9	0.033	0.110
B	11	0.714	2.379
Na	23	0.318	1.062
Mg	24	0.058	0.195
P	31	1.045	3.482
K	39	0.501	1.669
Ca	40	1.212	4.039
V	51	0.009	0.030
Cr	52	0.008	0.026
Mn	55	0.006	0.019
Fe	56	0.638	2.128
Co	59	0.003	0.009
Ni	60	0.013	0.043
Cu	63	0.012	0.042
Zn	66	0.145	0.482
As	75	0.456	1.521
Se	78	0.333	1.111
Rb	85	0.028	0.094
Sr	88	0.029	0.098
Cd	111	0.002	0.008
Cs	133	0.022	0.074
Ba	137	0.034	0.112
Hg	202	0.001	0.002
Tl	205	0.016	0.055
Pb	208	0.003	0.009
Th	232	0.016	0.055
U	238	0.003	0.009

Limits of detection (LODs) and Limits of quantification (LOQs) were estimated for all the elements, as 3σ and 10σ of 10 consecutive measurements of the reagent blanks.

The lowest LOD values found is 0.001 µg/L for Hg, whereas the highest LOD is 1.212 µg/L for Ca.

Repeatability

Additionally, five replicated measurements with 3 injections for each measurement on the spiked SRM were performed within 1 day to confirm the accuracy of the method and to check the repeatability. The relative standard deviations (RSD) of the five replicate samples for each element are presented in Table 5.6.

Table 5.6. Repeatability and relative standard deviation (in percent) for the analyzed elements in spiked SRM NIST1849a (5 measurements per day with 3 injection for measurement).

Element	Unit	Mean Concentration	RSD (%)
Be[a]	µg/L	8.11	9.61
B[a]	µg/L	11.8	10.1
Na[b]	mg/Kg	4537	2.40
Mg[b]	mg/Kg	1577	2.32
P[b]	mg/Kg	3894	1.96
K[b]	mg/Kg	9228	2.44
Ca[b]	mg/Kg	5716	1.25
V[a]	µg/L	8.88	8.53
Cr[b]	mg/Kg	0.98	3.23
Mn[b]	mg/Kg	43.4	2.53
Fe[b]	mg/Kg	163	2.96
Co[a]	µg/L	8.51	8.47
Ni[a]	µg/L	8.35	8.34
Cu[b]	mg/Kg	17.0	2.43
Zn[b]	mg/Kg	122	2.65
As[a]	µg/L	8.56	7.99
Se[b]	mg/Kg	0.74	5.75
Rb[a]	µg/L	15.2	8.28
Sr[a]	µg/L	12.9	8.40
Cd[a]	µg/L	7.60	8.11
Cs[a]	µg/L	8.71	8.54
Ba[a]	µg/L	9.15	8.34
Hg[c]	µg/L	0.54	4.06
Tl[a]	µg/L	8.39	8.49
Pb[a]	µg/L	8.50	10.2
Th[a]	µg/L	9.46	10.3
U[a]	µg/L	9.44	10.4

[a] Standard addition of 10 µg/L for trace elements
[b] Elements contained in SRM NIST1849a with Certified Mass Fraction in mg/kg
[c] Standard addition of 0.5 µg/L for Hg

Satisfactory values for the RSD ranging from 1.25 % for Calcium to 10.4 % for Uranium were found for the analyzed samples.

Reproducibility

Reproducibility was also checked with samples analyzed in three different days with 3 replicates for 3 injections of spiked SRM, and RSD for each element was calculated (Table 5.7).

Table 5.7. Reproducibility and relative standard deviation (in percent) for the analyzed elements in spiked SRM NIST1849a (3 replicates x 3 days).

Element	Unit	Day 1	RSD (%)	Day 2	RSD (%)	Day 3	RSD (%)
Be[a]	µg/L	8.69	1.14	8.25	2.15	8.14	1.48
B[a]	µg/L	12.51	0.55	11.13	1.70	10.76	2.83
Na[b]	mg/Kg	4508	2.9	3356	3.0	4574	3.5
Mg[b]	mg/Kg	1560	2.5	1326	3.1	1587	3.1
P[b]	mg/Kg	3845	4.6	3406	1.8	3942	3.1
K[b]	mg/Kg	9125	3.5	7602	2.4	9230	3.3
Ca[b]	mg/Kg	5634	0.8	5209	0.4	5763	1.6
V[a]	µg/L	9.55	0.94	9.44	1.25	9.16	1.76
Cr[b]	mg/Kg	0.97	1.14	0.85	2.79	1.00	1.87
Mn[b]	mg/Kg	43.45	1.45	38.33	2.67	44.61	1.19
Fe[b]	mg/Kg	164.33	0.18	141.49	0.28	168.88	0.23
Co[a]	µg/L	9.17	1.42	9.13	0.89	8.79	2.02
Ni[a]	µg/L	8.92	1.24	16.88	0.50	9.19	1.96
Cu[b]	mg/Kg	17.10	0.48	15.00	2.38	17.46	0.56
Zn[b]	mg/Kg	122.71	0.69	110.34	2.50	125.94	0.52
As[a]	µg/L	9.25	0.91	9.99	1.88	9.67	1.62
Se[b]	mg/Kg	0.74	1.55	0.68	4.71	0.81	2.12
Rb[a]	µg/L	9.19	1.04	10.13	1.05	9.88	0.39
Sr[a]	µg/L	9.27	0.50	10.09	0.98	9.73	0.69
Cd[a]	µg/L	8.21	0.58	8.48	0.84	8.28	0.41
Cs[a]	µg/L	9.46	1.31	9.51	0.61	9.23	0.59
Ba[a]	µg/L	9.92	0.67	10.12	0.32	9.75	0.94
Hg[c]	µg/L	0.52	0.80	0.49	0.70	0.49	0.80
Tl[a]	µg/L	9.11	0.91	8.96	0.12	8.71	0.11
Pb[a]	µg/L	9.55	0.91	9.95	0.78	8.82	0.54
Th[a]	µg/L	10.18	1.17	10.62	0.27	10.39	1.59
U[a]	µg/L	10.19	1.12	10.63	0.82	10.41	1.95

[a] Standard addition of 10 µg/L for trace elements
[b] Elements contained in SRM NIST1849a with Certified Mass Fraction in mg/kg
[c] Standard addition of 0.5 µg/L for Hg

The RSD values ranged from 0.11 % for Thallium (Day 3) to 4.71 % for Selenium (Day 2) for the analyzed samples.

5.2 Development and validation of analytical method for REE analysis

When ICP-MS is used for REEs analysis, it is necessary to consider and apply further precautions. In fact, in this case another type of polyatomic interferences is produced by elements in the sample combining with H, ^{16}O, or ^{16}OH, (either from water or air) to form molecular hydride (H), oxide (^{16}O), and hydroxide (^{16}OH) ions, which occur at 1, 16, and 17 mass unit higher than its mass (Vaughan & Horlick, 1986). These interferences are typically produced in the cooler zones of the plasma, immediately before the interface region. They are usually more serious when rare earth elements are present in the sample, because many of them readily form molecular species (particularly oxides), which create spectral overlap problems on other elements in the same group (Thomas, 2002b). To overcome this problem an instrumental method was further developed using a suitable reaction gas, in this case O2, in the ORS^3. These types of interferences are associated either with the plasma and nebulizer gas used, or matrix components in the solvent or other elements in the sample. The optimized acquisition parameters of ICP-MS triple quadrupole developed for the REE element analysis are reported in Table 5.8.

Table 5.8. Acquisition parameters of ICP-MS triple quadrupole.

QQQ-ICP-MS, 8800	
Replicates	3
Sweeps/replicate	90
Peak Pattern	3 Points
Isotopes *(no gas-mode)*	^{153}Eu, ^{172}Yb
Isotopes *(oxygen mode)*	^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm, ^{157}Gd, ^{163}Dy, ^{165}Ho, ^{166}Er, ^{169}Tm, ^{175}Lu
Integration time (s)	8
Collision/Reaction cell parameters	
O_2 cell gas flow rate	0.3 mL/min (30% of full scale)
O_2 Stabilization Time (s)	20

The ORS^3 was used in no gas-mode for ^{153}Eu and ^{172}Yb, and in Oxygen mode (O_2) for the others, in order to remove the spectral interferences.

Oxygen mode

Oxygen was used as the reaction gas in the cell, for the following lanthanides: ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm, ^{157}Gd, ^{163}Dy, ^{165}Ho, ^{166}Er, ^{169}Tm and ^{175}Lu.

The major interferences in this case are polyatomic species, e.g. hydride (MH^+) and/or oxide (MO^+) ions, derived from other REE elements. These interfering ions create spectral overlap problems on other elements in the same group.

REE elements (with the exception of ^{153}Eu and ^{172}Yb) readily react with oxygen to form oxide ions MO^+ (*m/z* M+16) that can be detected at the product (oxide) ion mass with the MS/MS mass-shift method.

In the MS/MS mass-shift method, the first quadrupole (Q1) operates as a mass filter set to the appropriate REE precursor ion mass. Q1 rejects all other masses, thereby removing the existing interfering ions and preventing them from overlapping the new analyte product ions.

Then, the ions pass through the ORS^3, where they react with the oxygen as reaction gas, providing ions oxide as product ion mass. Q2 selects this product ions at a mass value shifted of + 16, thus avoiding the overlap interferences.

This way, REE analysis was performed using the ICPMS spectrometer in MS/MS "mass-shift" mode using O_2 as reaction gas in the cell. The ICPMS-QQQ was used in No Gas-Mode only for the ^{153}Eu and ^{172}Yb, which do not react with oxygen (Sugiyama, 2012; Sugiyama & Woods, 2012).

5.2.1 Validation of the Method

Linearity

The instrumental response for REE analysis in the concentration range investigated proved to be highly linear for all the analyzed elements with correlation coefficients (R^2) in the range from 0.9997 for ^{157}Gd to 1.0000 for ^{139}La and ^{175}Lu. All standards and samples were injected in triplicate.

All calibration data, including gas mode, calibration range and correlation coefficient for the analysis of 13 elements, are reported in Table 5.9.

Table 5.9. Calibration data for the analysis of 13 elements.

Element	Isotope	Mode	Calibration range	Unit	R^2
Eu	153	No gas	0.25-10	ng/L	0.9999
La	139	O_2	0.25-10	ng/L	1.0000
Ce	140	O_2	0.25-10	ng/L	0.9999
Pr	141	O_2	0.25-10	ng/L	0.9999
Nd	146	O_2	0.25-10	ng/L	0.9999
Sm	147	O_2	0.25-10	ng/L	0.9999
Yb	172	No gas	0.25-10	ng/L	0.9999
Gd	157	O_2	0.25-10	ng/L	0.9997
Dy	163	O_2	0.25-10	ng/L	0.9999
Ho	165	O_2	0.25-10	ng/L	0.9999
Er	166	O_2	0.25-10	ng/L	0.9999
Tm	169	O_2	0.25-10	ng/L	0.9999
Lu	175	O_2	0.25-10	ng/L	1.0000

Accuracy

In order to check the accuracy of the method, the certified reference material (NIST1849a) was spiked with the standard additions (0.5 ng/L of multi-element standard for rare earth elements).

The standard addition method was used because the standard reference materials for the determination of all these elements were not available. The samples were spiked before their microwave digestion in order to assess the recovery for the whole process.

The recoveries for the 13 analyzed elements, determined with three measurements of concentration for each element, together with the relative standard deviation, are listed in Table 5.10.

The agreement between measured and certified values is satisfactory and confirmed that the method is accurate for quantitative analysis of rare earth elements in milk samples. The obtained recoveries ranged from 94.0 % for Lu to 100.4 % for Yb, which suggests that no significant elemental losses occurred during the digestion process.

The best results were obtained for Yb (100.4%), Nd (99.3%), Dy (98.0%), and La (97.7%).

Precision

The precision of the method is defined as Relative Standard Deviation (RSD) calculated using standard deviation divided by the mean of replicated samples. Satisfactory values

for the RSD ranging between 0.5% for Pr to 4.7 % for Lu were found for the analyzed samples.

Table 5.10. Recoveries (in percentage) and relative standard deviation (in percent) for NIST1849a, in order to check the accuracy of the microwave digestion and ICP-MS method.

Element	Isotope	[a] Recovery (%)	RSD (%)
Eu	153	97.5	1.8
La	139	97.7	1.5
Ce	140	96.9	3.7
Pr	141	96.4	0.5
Nd	146	99.3	4.6
Sm	147	95.1	3.9
Yb	172	100.4	1.7
Gd	157	95.9	2.1
Dy	163	98.0	2.1
Ho	165	96.0	0.6
Er	166	97.5	1.6
Tm	169	95.2	3.3
Lu	175	94.0	4.7

[a] Standard addition of 0.5 ng/L for REE elements

Limits of detection (LODs) and Limits of quantification (LOQs)

Limits of detection (LODs) and Limits of quantification (LOQs) were estimated for 13 elements. Results for the rare earth elements analysis are reported in Table 5.11.

Table 5.11. Limit of detection and Limit of quantification data for 13 elements.

Element	Isotope	LOD (ng L^{-1})	LOQ (ng L^{-1})
Eu	153	0.0004	0.0015
La	139	0.0424	0.1414
Ce	140	0.0556	0.1854
Pr	141	0.0158	0.0527
Nd	146	0.0293	0.0976
Sm	147	0.0056	0.0188
Yb	172	0.0032	0.0105
Gd	157	0.0025	0.0085
Dy	163	0.0283	0.0944
Ho	165	0.0510	0.1699
Er	166	0.0091	0.0303
Tm	169	0.0105	0.0349
Lu	175	0.0443	0.1475

Limits of detection (LODs) and Limits of quantification (LOQs) were estimated for all elements, as 3σ and 10σ of 10 consecutive measurements of the reagent blanks.

The lowest LOD values found is 0.0004 ng/L for Eu, whereas the highest LOD is 0.0556 ng/L for Ce.

Repeatability

Additionally, five replicated measurements with 3 injections for measurement on the spiked SRM were performed within 1 day to confirm the accuracy of the method and to check the repeatability. The relative standard deviations (RSD) of the five replicate samples for each element are presented in Table 5.12.

Table 5.12. Repeatability and relative standard deviation (in percent) for the analyzed elements in spiked SRM NIST1849a (5 measurements per day with 3 injection for measurement).

Element[a]	**Unit**	**Mean Concentration**	**RSD (%)**
Eu	ng/L	0.44	9.35
La	ng/L	0.41	11.3
Ce	ng/L	0.40	11.6
Pr	ng/L	0.43	9.73
Nd	ng/L	0.43	7.93
Sm	ng/L	0.42	10.0
Yb	ng/L	0.44	8.72
Gd	ng/L	0.42	7.88
Dy	ng/L	0.43	9.08
Ho	ng/L	0.42	9.66
Er	ng/L	0.43	7.88
Tm	ng/L	0.42	10.4
Lu	ng/L	0.43	11.5

[a] Standard addition of 0.5 ng/L

Satisfactory values for the RSD ranging from 7.88 % for Gadolinium to 11.6 % for Cerium were found for the analyzed samples.

Reproducibility

Reproducibility was also checked with samples analyzed in three different days with 3 replicates for 3 injections of spiked SRM, and RSD for each element was calculated (Table 5.13).

Table 5.13. Reproducibility and relative standard deviation (in percent) for the analyzed elements in spiked SRM NIST1849a (3 replicates x 3 days).

Element[a]	**Unit**	**Day 1**	**RSD (%)**	**Day 2**	**RSD (%)**	**Day 3**	**RSD (%)**
Eu	ng/L	0.48	2.02	0.49	1.80	0.48	1.10
La	ng/L	0.47	1.51	0.49	1.52	0.46	1.61
Ce	ng/L	0.44	2.61	0.48	3.68	0.46	3.06
Pr	ng/L	0.48	0.34	0.48	0.54	0.46	1.88
Nd	ng/L	0.47	1.18	0.50	4.61	0.46	2.20
Sm	ng/L	0.46	4.48	0.48	3.86	0.46	1.59
Yb	ng/L	0.47	1.35	0.50	1.70	0.47	3.84
Gd	ng/L	0.46	2.47	0.48	2.10	0.45	2.40
Dy	ng/L	0.46	4.50	0.49	2.09	0.45	2.75
Ho	ng/L	0.47	0.07	0.48	0.61	0.46	2.12
Er	ng/L	0.46	3.03	0.49	1.59	0.47	4.18
Tm	ng/L	0.48	1.04	0.48	3.29	0.47	3.55
Lu	ng/L	0.50	2.15	0.47	4.68	0.47	1.99

[a] Standard addition of 0.5 ng/L

The RSD values ranged from 0.07 % for Holmium (Day 1) to 4.68 % for Lutetium (Day 2) for the analyzed samples.

Chapter 6 Elemental profile of cow milk samples

6.1 Cow milk samples multi-element profile

The elemental profile of 50 raw cow milk samples obtained from different farms in Italy and Europe was investigated. All samples were digested with a microwave digestion system according to the method developed based on the reference standard material and described in detail in the previous chapters. The updated mineralization method was optimized and applied allowing the determination of 27 elements including 22 essential and 5 toxic elements.

6.1.1 Multi elements content in cow milk samples from different regions

The multi-element screening was performed on the Italian and European raw cow milk samples against 27 elements (22 essential and 5 toxic).

All concentration values obtained are in the limit of LODs and LOQs parameters.

The results for multi-elements contents determined in raw cow milk samples, according to their geographical origin, are listed in Table 6.1.

The data are expressed as mean values and SD for the samples originated in the same place following a three replicate per sample approach.

Nine elements, i.e. Be, As, Se, Cd, Cs, Hg, Tl, Th and U, presented concentration levels below their LOQs in all the cow milk samples studied in this work.

All the concentrations data were evaluated to ascertain the occurrence of significant differences between regions. The Student's t-test was used to this scope.

The results show no significant differences in the concentrations of B, Ca, V, Cr, Mn, Cu, and Pb between Northern and Southern Italy at the 95% confidence level. However, the other 11 elements show significant differences ($p < 0.05$) in their concentration values, which may derive from the different geographical origin.

Comparing the concentrations of the elements in Northern Italy and Central Europe, significant differences ($p < 0.05$) in the concentrations of B, Na, Mg, K, V, Cr, Cu and Rb are observed.

Table 6.1. Multi-element contents of raw cow milk samples from different regions in Italy and Europe

Element	Northern-Italy		Southern-Italy		Central Europe	
	Mean concentration (mg L^{-1})	**σ (mg L^{-1})**	**Mean concentration (mg L^{-1})**	**σ (mg L^{-1})**	**Mean concentration (mg L^{-1})**	**σ (mg L^{-1})**
Be	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
B	0.24	0.03	0.26	0.02	0.17	0.02
Na	452	23	295	32	368	30
Mg	106.9	3.5	87.7	3.3	100.0	5.5
P	904	35	979	68	940	51
K	1565	49	1668	128	1648	70
Ca	1220	93	1181	74	1215	78
V	0.0024	0.0004	0.0025	0.0002	0.0017	0.0002
Cr	0.003	0.001	0.01	0.01	0.003	0.001
Mn	0.020	0.005	0.020	0.006	0.022	0.003
Fe	0.3	0.3	1.1	1.1	0.31	0.08
Co	0.0007	0.0002	0.0009	0.0002	0.0007	0.0002
Ni	0.04	0.08	0.2	0.2	<LOQ	<LOQ
Cu	0.029	0.004	0.028	0.006	0.039	0.006
Zn	3.3	0.2	2.8	0.3	3.2	0.2
As	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Se	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Rb	0.62	0.05	0.44	0.09	1.8	1.1
Sr	0.33	0.03	0.63	0.07	0.3	0.1
Cd	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Cs	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Ba	0.048	0.005	0.13	0.02	0.07	0.06
Hg	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Tl	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Pb	0.002	0.003	0.003	0.002	0.0011	0.0006
Th	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
U	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Moreover, significant differences ($p < 0.05$) between milk samples from Southern Italy and Central Europe were found for B, Na, Mg, V, Fe, Co, Ni, Cu, Zn, Rb, Sr, Ba and Pb, while the concentration of nutrient elements, i.e. P, Ca, but also Mn, is substantially comparable ($p > 0.05$) in all the milk samples independently from the geographical region, and in agreement with the main nutritional properties of this food product.

Figure 6.1, shows the box plots of the concentration distribution of the elements in each region of provenance.

The box plots display the distribution of the parameters for each region, i.e. Northern Italy (NI), Southern Italy (SI) and Central Europe (CE).

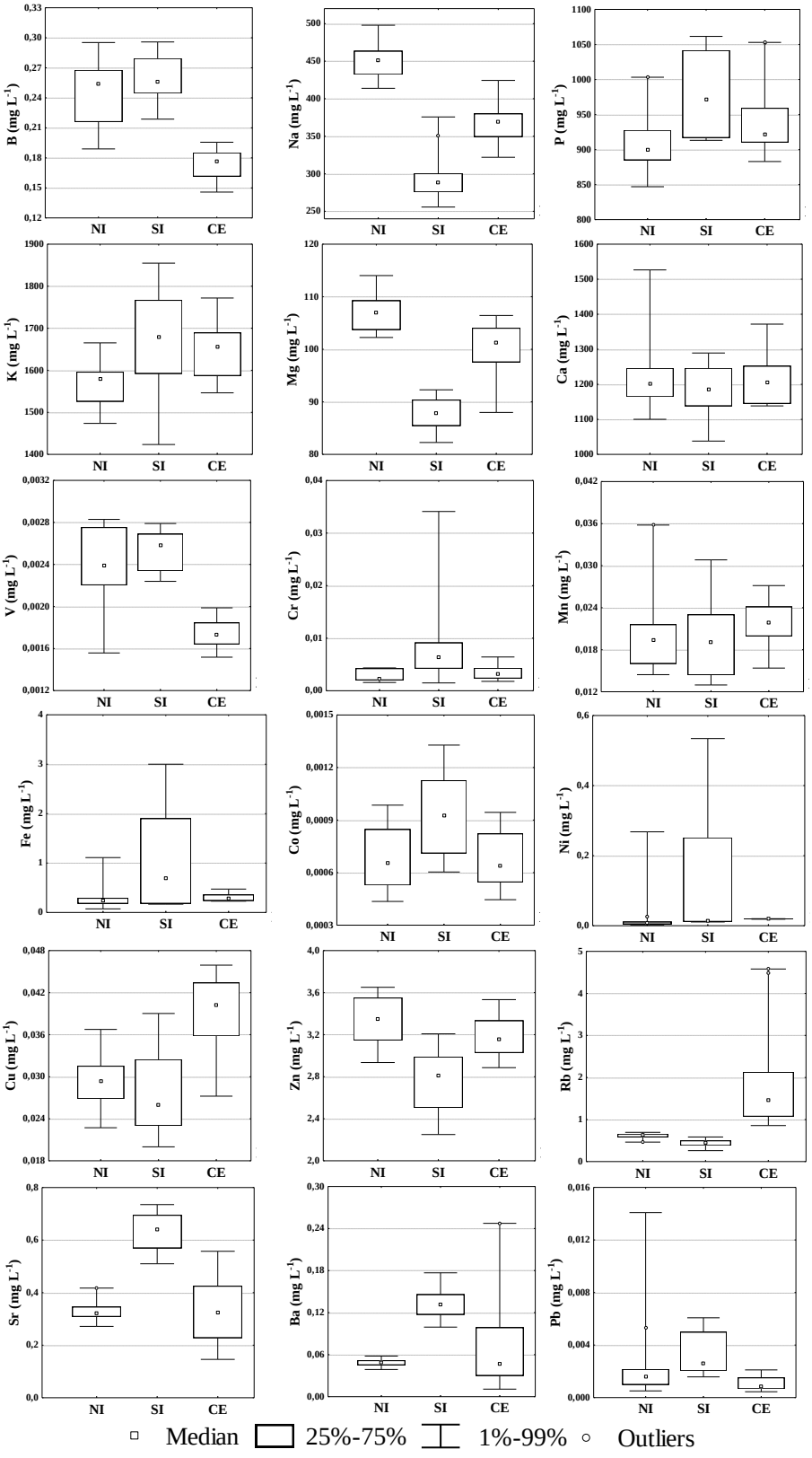

Figure 6.1. 2D box plots of 50 cow milk samples based on multi-element content according to their geographical origin. (NI= Northern Italy, SI= Southern Italy, CE= Central Europe). The square in the box indicates the median level, whiskers fence the upper and lower quartiles, and ‘‘o’’ labels the outliers.

Essential and Trace Elements content

The most abundant element in all raw cow milk samples is Potassium (K), followed by Calcium (Ca), Phosphorus (P), Sodium (Na) and Magnesium (Mg) for both Italian and European milk samples. In general, milk is considered a good source of these major elements, and especially of Ca and P that are indicators of proteins rich milk (Gaucheron, 2005).

The concentration of Ca, Mn, P, K and Co was similar in all cow milk samples studied. Essential elements such as Na and Mg were higher in NI samples as compared to SI and CE samples. Trace elements B and V were similar in both Northern and Southern Italy cow milk samples whereas Fe, Ni, Sr and Ba were higher in SI samples as compared to NI ones. The content in Cu and Rb was higher in CE samples than in the other two types of milk samples but Zn was lower in SI samples with respect to NI and CE samples.

The results are in line with the available literature concentration ranges for these elements (Ataro et al., 2008; Benincasa et al., 2008; Coni, Bocca, Ianni, & Caroli, 1995; Khan et al., 2014; Rivero Martino et al., 2000; Tunick, Van Hekken, Paul, Ingham, & Karreman, 2016; Zain, Behkami, Bakirdere, & Koki, 2016).

Table 6.2, shows the literature data in mg L^{-1} for raw cow milk samples from other countries compared to the average values obtained in the present work.

The comparison of results obtained in this study with literature data shows that Mn, Cu, Zn and Fe were lower, while Na, Mg, P, K, Ca and Ba were comparable or slightly lower than values reported by (Khan et al., 2014; Tunick et al., 2016; Zain et al., 2016).

The content in Rb, Sr, Cr and V was lower than (Ataro et al., 2008; Benincasa et al., 2008; Khan et al., 2014), while Co was comparable with that obtained by (Benincasa et al., 2008).

Toxic Trace Elements content

The concentration levels of potentially toxic trace elements, such as As, Cd, Pb, Hg, Cr, are very low or even below the LOQ values in all cow milk samples. This indicates the absence of contamination and therefore of any toxicological risks in the studied farms.

The content of As, Cd and Hg was below the their LOQ values in all the cow milk samples analyzed. The concentration of Cr and Pb was similar in NI and CE samples whereas it was higher in milk samples from SI.

Table 6.2. Literature data (mg L-1) for raw cow milk samples from other countries compared to Northern and Southern Italy and Central Europe

Element	Northern Italy[1]	Southern Italy[1]	Central Europe[1]	Italy[2]	South Africa[3]	USA[4]	South Korea[5]	New Zealand[6]	Belgium[6]	Iran[6]	Australia[6]	Malaysia[6]	Turkey[6]	USA[6]	Canada[6]
B	0.24	0.26	0.17	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Na	452	295	368	NR	NR	660	NR	511.0	385.6	441.8	478.6	450.0	430.6	437.7	434.1
Mg	106.9	87.7	100.0	NR	NR	120	NR	99.2	105.8	105.6	99.4	123.7	108.6	96.8	93.1
P	904	979	940	775	NR	1110	NR	NR	NR	NR	NR	NR	NR	NR	NR
K	1534	1668	1648	1190	NR	1550	NR	1573.4	1493.2	1598.3	1598.7	1690.5	1501.6	1583.9	1624.6
Ca	1220	1181	1215	1220	NR	1340	NR	1178.0	1162.3	1048.0	1133.3	1160.4	1105.2	1207.8	1103.3
V	0.0024	0.0025	0.0017	0.003	0.02-0.04	NR	0.006	NR	NR	NR	NR	NR	NR	NR	NR
Cr	0.003	0.01	0.003	0.0094	0.2-0.4	NR	0.364	NR	NR	NR	NR	NR	NR	NR	NR
Mn	0.019	0.020	0.022	0.032	0.1-0.3	0.03	0.133	0.080	NR	0.079	0.081	0.08	0.084	NR	NR
Fe	0.3	1.1	0.31	0.325	NR	0.39	NR	5.300	2.76	1.59	3.75	3.65	1.3	2.6	3.24
Co	0.0007	0.0009	0.0007	0.0014	NR	NR	0.006	NR	NR	NR	NR	NR	NR	NR	NR
Ni	0.04	0.2	<LOQ	NR	NR	NR	0.153	NR	NR	NR	NR	NR	NR	NR	NR
Cu	0.028	0.028	0.039	NR	NR	NR	0.383	0.850	1.21	0.77	0.73	0.87	0.87	0.81	0.93
Zn	3.2	2.8	3.2	3.81	NR	3.87	4.754	4.410	4.76	3.25	4.10	5.05	3.68	4.13	3.51
Rb	0.59	0.44	1.8	2.09	NR	NR	2.330	NR	NR	NR	NR	NR	NR	NR	NR
Sr	0.34	0.63	0.3	0.698	1.9-3.0	NR	0.517	NR	NR	NR	NR	NR	NR	NR	NR
Ba	0.046	0.14	0.07	0.226	NR	NR	0.163	0.110	0.046	0.05	0.071	0.09	0.07	0.06	0.095
Pb	0.002	0.003	0.0011	NR	0.010	NR	0.00335	NR	NR	NR	NR	NR	NR	NR	NR

[1]Present work; [2](Benincasa et al., 2008); [3](Ataro et al., 2008); [4](Tunick et al., 2016); [5](Khan et al., 2014); [6](Zain et al., 2016).

The content of Cr ranged from 0.003 mg/L for NI and CE cow milk samples to 0.010 mg/L for SI cow milk samples.

The concentration of Pb in milk samples ranged from 0.001 mg/L for CE cow milk samples to 0.003 mg/L for SI cow milk samples.

(Ataro et al., 2008) and (Coni et al., 1995) reported Pb levels sensibly higher than those obtained in this study, while (Rivero Martino et al., 2000) recorded 0.0009 mg/L Pb in cow's milk whey by using a double focusing ICP-MS.

The Codex Alimentarius Commission (Codex Alimentarius Commission, 2003) establishes a limit for Pb in milk (0.02 mg/L). The mean concentration of Pb in the milk samples studied was smaller than the established Codex standard.

6.2 Cow milk samples REE profile

The elemental profile of 50 cow milk samples obtained from different farms in Italy and Europe has been investigated. All samples were digested with a microwave digestion system according to the method developed based on the reference standard material. The new ICP-MS method was developed and the study was performed on 13 elements.

As previously reported for multi-elements determination, the European cow milk samples were evaluated in a big macro-area named Central Europe area.

6.2.1 REE fingerprint in cow milk samples from different regions

The REEs screening based on 13 elements was carried out on the whole sample range of the Italian and European raw cow milk samples of this study. They are presented separately at this stage, because, as previously described, they require different instrumental conditions for optimal determination.

All values of concentration obtained are above the LODs and LOQs parameters.

The results for REE contents determined in cow milk samples, according to their geographical origin, are reported in Table 6.3.

The data are expressed as means values and SD for the samples originated from the same place, each sample being analyzed in triplicate.

Similarly to trace elements, REE data collected were subjected to Student's t-test between couples of regional data, in order to assess the occurrence of distinct patterns between regions.

Table 6.3. REE fingerprint of cow milk samples from different regions

Element	Northern-Italy		Southern-Italy		Central Europe	
	Mean concentration (ng L^{-1})	**σ (ng L^{-1})**	**Mean concentration (ng L^{-1})**	**σ (ng L^{-1})**	**Mean concentration (ng L^{-1})**	**σ (ng L^{-1})**
Eu	2.1	0.9	3.6	0.5	14.7	8.9
La	4.2	1.2	2.8	0.8	17.8	6.7
Ce	7.7	2.5	4.8	0.9	44	15
Pr	1.5	0.8	0.8	0.2	4.3	1.3
Nd	3.1	1.1	2.5	0.9	13.5	5.9
Sm	1.3	0.8	0.7	0.2	3.0	1.4
Yb	1.0	0.8	0.3	0.2	2.0	0.9
Gd	1.4	0.8	0.6	0.2	3.9	1.3
Dy	1.3	0.8	0.5	0.2	2.2	1.1
Ho	1.0	0.8	0.2	0.2	0.8	0.4
Er	1.0	0.8	0.3	0.2	3.1	1.1
Tm	0.9	0.8	0.2	0.2	1.0	0.5
Lu	1.8	1.0	0.6	0.2	7.0	1.4

REE elements show significant differences ($p < 0.05$) between Northern and Southern Italy throughout the whole elemental range considered.

Concerning REEs in Italy and Central Europe, significant differences ($p < 0.05$) in the concentrations of Eu, La, Ce, Pr, Nd, Sm, Gd, Er and Lu are observed between milk samples from Northern Italy and Central Europe, whereas all the 13 REEs analyzed from Southern Italy are significantly different from Central Europe ($p < 0.05$) values.

Figure 6.2, shows the REEs distribution of the mean concentrations of milk samples from different origins.

REE distributions in diagram a) have a similar trend based on mean concentrations of milk samples obtained from different geographical origins, with a minor exception of lanthanides from Ho to Lu.

Moreover, diagrams b), c) and d) show that the REE distribution patterns are highly reproducible within the locality.

The results demonstrate that cow milk samples from Europe presented a higher content in rare earth elements and significantly different than all other samples.

This observation may derive from the type of animal feed used in the two Italian farms, being typical small family-conducted farms, where cow feeding (and water) is always very local and fresh. On the other hand, the European farms which provided the samples herein analyzed used higher proportions of prepackaged food not always of local origin, because of climate and large numbers of cows.

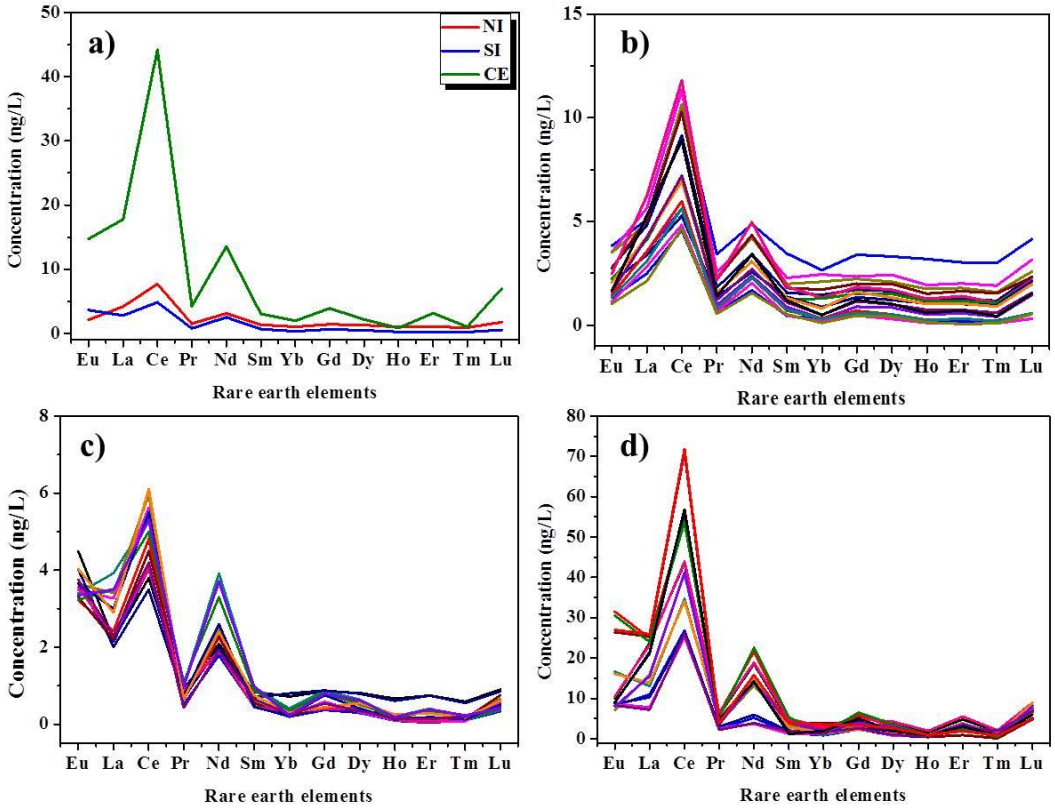

Figure 6.2 REE patterns of cow milk samples from different locations in Italy and Europe. a) Mean concentrations based on each provenance; b), c), and d) all milk samples from NI= Northern Italy, SI= Southern Italy, and CE= Central Europe, respectively.

Though the higher content of REEs in CE milk could be associated to local biogeochemistry, we speculate this might be the result of REE's addition in industrial cow feed. In fact, though this is presently just hypothetical, this fact could be reasonably real as the practice of REE addition is widely used worldwide, and especially in China, where REEs are successfully used in agriculture as fertilizer in plant production and as feed additives in animal nutrition (Redling, 2006).

In China, the improvements of the growth due to dietary supplementation of rare earths at low concentrations are described for nearly all categories of farming animals, including beef cattle, sheep, pigs, rabbits, ducks, chickens and fish (M. Liu, 2005; Shen, Zhang, & Wang, 1991; Yang, Zhang, Cheng, Zhang, & Zhu, 2005).

Hence, application of rare earths to animal diets was reported in terms of increasing body weight by up to 29 % as reported in Table 6.4.

However, prior to their commercial utilization not only the effectiveness but also the safety of rare earth application was assessed in China.

Table 6.4. Effects of rare earth elements on livestock, poultry and fishery in China (Redling, 2006; Xiong, 1995).

Animal species	**Physiological-biochemical indices and results of application**
Pigs	6 - 29 % increased body weight gain increased feed utilization efficiency of 10 %
Sheep	increased wool clip of 8 % 6 - 13 % increased body weight gain
Egg-laying Poultry	increased survival rate of 5 - 10 % increased laying rate of 8 %
Duck	6 - 10 % increased body weight gain increased laying rate of 9 %
Fish (Grass Carp, Silver Carp, Carp)	3.4 - 3.7 % increased body weight gain increased survival rate of 5 %
Long Hair Rabbit	increased body weight gain 7 - 10 % increased hair yield of 7 - 9 %
Domestic Silkworm	increased digestible protein increased cocoon production per 10000 larvae of 9 % increased total cocoon weight of 3 % grade of raw silk raised

From the literature it is known that the metabolism and toxicity of REE highly depends on the route of administration as well as on the chemical form administered. In general, the systemic absorption of lanthanides administered as soluble salts increases in the order per oral<subcutaneous<intramuscular<intraperitoneally<inhalation<intravenously (Evans, 1990; Schwabe et al., 2012). Concerning the use of REE as a new feed additive in order to enhance performance in animal husbandry, oral application is of great relevance. Several studies reported that only very small amounts of orally applied REE are absorbed in the gastrointestinal tract (Damment & Pennick, 2007; G. Magnusson, 1963). Absorption of ionic REE takes place the small intestine, predominantly in the ileum (Kostial, Kargacin, & Landeka, 1989). (Arvela, 1979) reported that in general only 0.05% of an orally administered dose is absorbed from the gastrointestinal tract.

Similar results were obtained in human absorption study by (Pennick, Dennis, & Damment, 2006), reporting that only 0.00127% ± 0.00080% (range 0.00015% - 0.00224%) of orally applied lanthanum carbonate was absorbed from the human gastrointestinal tract.

Rare earth feed additives were tested on two million animals and the results showed that rare earths were no toxic to either humans or animals (Rosewell, 1995).

At present, since the European legislation has not prohibited the purchase of REEs as feed additives so far, REEs are experimentally used and were proved to determine positive effects on animal growth especially in pigs and poultry (M. L. He, Ranz, & Rambeck, 2001; Schwabe et al., 2012).

Feeding experiments conducted on poultry (M. He, Wehr, & Rambeck., 2006) and rats (M. He, Wang, Xu, Chen, & Rambeck, 2003) proved that rare earths can also improve body weight gain of high performance animals that are kept under optimized housing and feeding conditions.

Though rare earths were shown to enhance animal performance under both Chinese and Western conditions, the underlying mechanism has not been clarified yet. (Ou, Guo, & Wang, 2000), for example, suggested four possible mechanisms to explain growth promoting effects of rare earths in animals, including enhanced enzyme activity, improved protein metabolism, suppression of bacterial growth and promoted secretion of digestive fluids in the stomach. Furthermore, effects on hormone activity as well as on cell proliferation were considered as possible explanation for performance enhancing effects of rare earths (M. He et al., 2003, 2006).

The Regulation (EC) No. 1831/2003 governs the placing of feed additives on the market as well as their use in animal nutrition. According to this regulation, feed additives can only be marketed if they have no dangerous effects on human and animal health or on the environment (European Commission (EC), 2003).

At present, rare earth containing feed additives may be purchased from the Swiss company *Zehentmayer*, as Lancer® for feedstuff companies (Redling, 2006). Lancer (lanthanum and cerium in their citrate forms) is still not authorized in the European Union. Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety of Lancer as a feed additive for weaned piglets.

After submission of additional information related to the safety of the additive, the FEEDAP Panel concluded that the additive Lancer is safe for weaned piglets when used at the maximum recommended dose of 250 mg/kg complete feed.

However, the absence of studies of long-term toxicity, carcinogenicity, reproductive toxicity and developmental toxicity of Lancer, and the absence of residue data in edible tissues, the FEEDAP Panel cannot conclude on the safety of Lancer for the consumer (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2016). Hence, the use of REE as feed additives in Europe is still under evaluation.

Figure 6.3, shows the box plots of the concentration distribution of the Rare Earth Elements in each region of provenance.

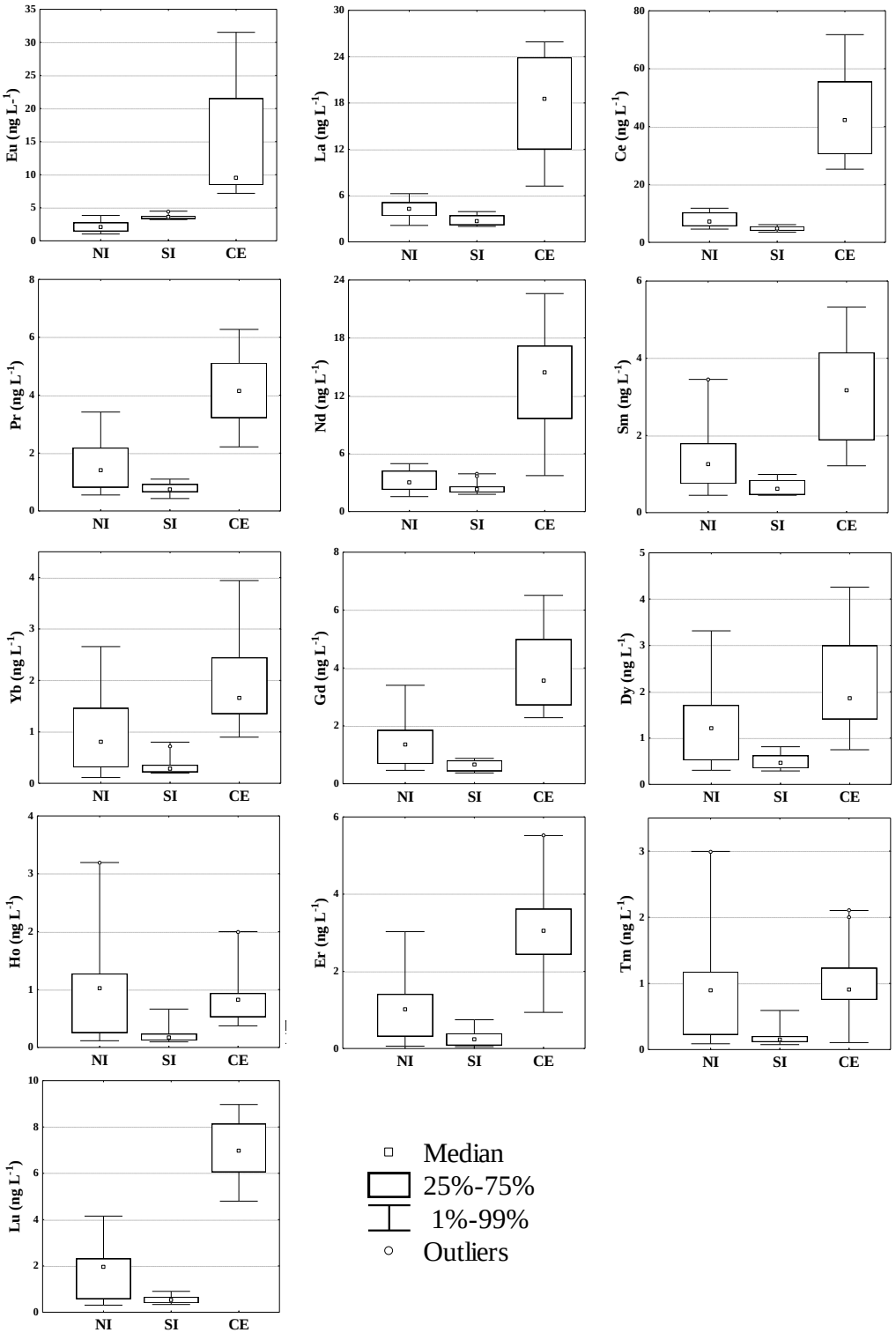

Figure 6.3 2D box plots of 50 cow milk samples based on REEs content according to their geographical origin. (NI= Northern Italy, SI= Southern Italy, CE= Central Europe). The square in the box indicating the median level, whiskers fences the upper and lower quartiles, and ‘‘o’’ labels the outliers.

The box plots display the distribution of the parameters for each group, such as Northern Italy (NI), Southern Italy (SI) and Central Europe (CE).

The most abundant lanthanides are Cerium (Ce), Lanthanum (La), Europium (Eu) and Neodymium (Nd) for both Italian and European cow milk samples.

The concentrations of Eu, La, Ce, Pr, Nd, Sm, Yb, Gd, Dy, Er and Lu were high in European milk samples with respect to Italian samples.

The content of Ho and Tm was similar in NI and CE milk samples with respect to SI samples, whereas the concentrations of lanthanides from Yb to Lu in Southern Italy milk samples are lower than NI and CE milk samples.

Chapter 7 Chemometrics

The results from ICP-MS analysis were subjected to chemometric analysis in order to assess the existence of elemental profiles associated with each of the macro areas of milk provenance as herein studied, namely Northern Italy (NI), Southern Italy (SI) and Central Europe (CE).

In particular, following a basic statistic evaluation, data were analyzed using multivariate modelling.

In this work, the technique used was based on cluster analysis and Principal Component Analysis as described in Chapter 4 of this thesis, two of the most widely used chemometric methods for data reduction owing to their efficiency in establishing data groupings/association based on shared properties between the matrix of observed data.

7.1 Descriptive statistic

Descriptive statistics of all data for essential, trace and rare earth elements analyzed in milk samples are presented in Table 7.1.

The Table reports the number of observations (N), mean and median values and standard deviation, against 33 elements in 50 milk samples.

The number of observations (N) for the most of the elements is more than the 50% of total observations (N = 50).

Only Cs and U present a low number of observations, equal to 24 and 13, respectively. For this reason, these elements were removed from the data matrix used in the following chemometric models because they could significantly lower the reliability of the statistical results.

Table 7.1. Descriptive statistics of all data in milk samples

Element	Valid N*	Mean	Median	Minimum	Maximum	Std. Dev.
Essential and trace elements (mg L^{-1})						
B	48	0.23	0.23	0.15	0.30	0.04
Na	50	381	375	256	498	71
Mg	50	99.3	102.6	82.3	114.0	8.9
P	44	931	917	847	1061	55
K	50	1620	1596	1423	1855	94
Ca	50	1207	1201	1038	1527	84
V	36	0.0023	0.0023	0.0015	0.0028	0.0004
Cr	36	0.006	0.004	0.001	0.034	0.007
Mn	50	0.021	0.020	0.013	0.036	0.005
Fe	44	0.5	0.3	0.1	3.0	0.7
Co	43	0.0008	0.0007	0.0004	0.0013	0.0002
Ni	36	0.082	0.012	0.002	0.534	0.142
Cu	50	0.032	0.031	0.020	0.046	0.007
Zn	50	3.1	3.2	2.2	3.7	0.3
Rb	50	1.0	0.6	0.3	4.6	0.9
Sr	50	0.4	0.3	0.1	0.7	0.2
Cs	24	0.008	0.007	0.004	0.031	0.006
Ba	44	0.07	0.05	0.01	0.25	0.05
Pb	48	0.0022	0.0017	0.0004	0.0141	0.0023
U	13	0.0007	0.0006	0.0005	0.0014	0.0002
Rare Earth Elements (ng L^{-1})						
Eu	47	6.9	3.6	1.0	31.5	7.7
La	47	8.4	4.3	2.0	25.9	7.9
Ce	47	19	7.2	3.5	72	20
Pr	47	2.2	1.6	0.4	6.3	1.7
Nd	47	6.5	3.4	1.5	22.6	6.2
Sm	47	1.7	1.2	0.4	5.3	1.4
Yb	47	1.1	0.9	0.1	3.9	1.0
Gd	47	2.0	1.5	0.4	6.5	1.7
Dy	47	1.4	1.0	0.3	4.3	1.0
Ho	47	0.7	0.6	0.1	3.2	0.6
Er	47	1.5	1.0	0.0	5.5	1.4
Tm	47	0.74	0.60	0.07	3.00	0.66
Lu	47	3.2	2.1	0.3	9.0	3.0

*N= number of observations

7.2 Principal component analysis (PCA)

Principal component analysis (PCA) is a powerful visualization tool providing a way to reduce the dimensionality of data and allowing the elimination of redundant information, in order to explore the presence of groups, outliers or trends in data (Benincasa et al., 2008). PCA reduces a large number of variables in the original matrix into a smaller set of components by investigating the correlation between variables. These components, the PCs, give linear combinations of variables which account for more of the variance than any other combination (Vandeginste et al., 1998). In the present work, PCA was applied to a matrix of 31 analytical parameters for 50 raw milk

samples to assess the distribution pattern of essential, trace and rare earth elements concentrations according to their geographical origin.

The Principal Components and their eigenvalues together with the cumulative variance (%) are reported in Table 7.2.

Table 7.2. Eigenvalues and relative cumulative variance in percent

PCs	Eigenvalues	Cumulative variance %
1	12.6	37.1
2	5.7	53.9
3	3.0	62.8
4	2.4	69.7
5	1.6	74.5
6	1.5	78.9
7	1.2	82.5
8	1.1	85.8

According to the eigenvalue criterion, only the PCs with eigenvalues greater than one are considered important (Pellerano et al., 2012). On the basis of eigenvalues > 1, PCA identified eight principal components explaining up to 85.8 % of the total variance, which indicates that the first eight PCs explain the 85.83% of the information obtained from the multi-elemental analysis.

The first two PCs explain over 53.9% of the total variance of the system, with the first principal component explaining 37.1% of total variability, and the second one 16.8%, while the third one explains the 8.8% of total variance.

Figure 6.4, shows the scree plot of the data of PCA based on essential, trace and REEs content.

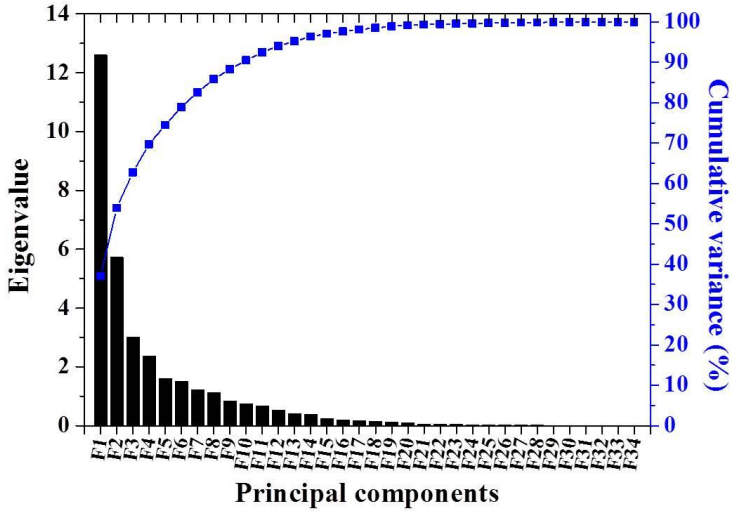

Figure 7.1. PCA scree plot of all data obtained from 50 cow milk samples based on essential, trace and REEs content.

Table 7.3, shows the factor loadings for each variable on the principal components. Each number represents the correlation between the variable and the PCs. The absolute value of the loading in a component (between 0 and 1) describes the importance of the contribution of the component, so the farther the variable from the origin, the higher its contribution in the statistic model generated by PCA.

The first PC is mainly correlated (loading >0.60) with fifteen trace and REE elements (B, Cu, Rb, Eu, La, Ce, Pr, Nd, Sm, Yb, Gd, Dy, Er, Tm and Lu), all of which present at very low concentrations in milk (<0.3 mg L^{-1} for B and <45 ng L^{-1} for REEs). Sodium, Magnesium, Nickel, Strontium, Barium and Zinc are the dominating variables in the second principal component and the Manganese in the third one. As previously observed with the use of boxplots, the variability of these elements contents could be an outcome of geographical variability.

Table 7.3. First eight principal component-loading* matrix obtained from the data matrix

Loadings	PC-1	PC-2	PC-3	PC-4	PC-5	PC-6	PC-7	PC-8
B	**0.72**	-0.07	0.23	0.10	0.38	-0.17	-0.07	-0.25
Na	-0.26	**-0.77**	0.26	-0.03	0.24	0.33	-0.05	-0.03
Mg	-0.35	**-0.76**	0.23	-0.09	-0.02	0.26	-0.20	0.12
P	-0.04	*0.44*	0.36	**-0.67**	0.21	-0.04	-0.04	-0.09
K	-0.04	*0.49*	0.13	**-0.64**	0.20	0.05	-0.13	0.21
Ca	-0.19	-0.08	0.39	*-0.59*	0.18	0.35	0.03	*-0.41*
V	*0.48*	0.10	0.23	0.36	0.32	0.09	0.23	0.22
Cr	0.35	0.11	-0.27	-0.22	0.05	-0.22	*0.51*	-0.23
Mn	-0.37	0.38	**0.71**	0.27	0.06	0.15	0.09	-0.06
Fe	0.39	*0.56*	*0.48*	*0.40*	-0.23	0.09	-0.09	-0.11
Co	0.37	*0.49*	0.40	0.06	-0.04	-0.13	-0.04	-0.33
Ni	0.17	**0.62**	*0.51*	0.36	-0.28	0.08	-0.08	-0.06
Cu	**-0.63**	0.08	-0.08	-0.32	-0.39	-0.12	0.05	-0.33
Zn	-0.38	**-0.73**	0.03	-0.18	-0.07	0.14	0.20	-0.06
Rb	**-0.67**	0.30	-0.34	0.13	0.26	0.31	-0.13	0.20
Sr	*0.60*	**0.62**	0.10	-0.29	0.25	-0.07	-0.01	0.19
Ba	0.08	**0.62**	-0.16	-0.02	*0.55*	0.07	0.04	0.07
Pb	0.36	-0.06	-0.22	-0.10	0.00	-0.19	*0.56*	0.09
Eu	**-0.71**	0.39	-0.37	0.27	0.20	0.01	-0.06	-0.08
La	**-0.89**	0.28	-0.19	0.04	0.06	-0.10	-0.06	-0.18
Ce	**-0.87**	0.28	-0.23	0.01	0.00	-0.08	-0.14	-0.21
Pr	**-0.96**	0.19	0.03	0.01	0.04	0.02	0.05	0.01
Nd	**-0.89**	0.32	-0.18	0.09	0.11	-0.03	0.01	-0.08
Sm	**-0.90**	0.09	0.01	0.21	0.11	-0.22	0.08	0.04
Yb	**-0.83**	-0.05	0.15	0.15	0.08	-0.30	0.03	-0.17
Gd	**-0.95**	0.17	0.01	-0.02	0.15	-0.08	-0.05	0.05
Dy	**-0.87**	-0.03	0.23	0.12	0.19	-0.22	0.15	0.07
Ho	*-0.56*	-0.36	*0.58*	0.07	0.18	-0.16	0.26	0.12
Er	**-0.91**	0.06	0.18	-0.15	-0.19	0.01	0.11	0.13
Tm	**-0.62**	-0.32	*0.55*	-0.06	-0.03	-0.14	0.17	0.24
Lu	**-0.91**	0.11	-0.01	-0.08	-0.27	0.08	0.02	0.12

* loadings > 0.60 are reported in bold; loadings in the range 0.40-0.60 are reported in cursive.

Figure 6.5 presents the graphic distribution of the samples according to the first two principal components scores.

The scatterplot of PC1 and PC2 component scores of the milk shows that the first two components clearly distinguish among the three regions of provenance. The three clusters obtained represent respectively the Northern Italy (NI, in red), the southern Italy (SI, in blue) and the Central Europe samples (CE, in green).

The obtained grouping emphasizes the high potential of the selected elements as geographical tracers.

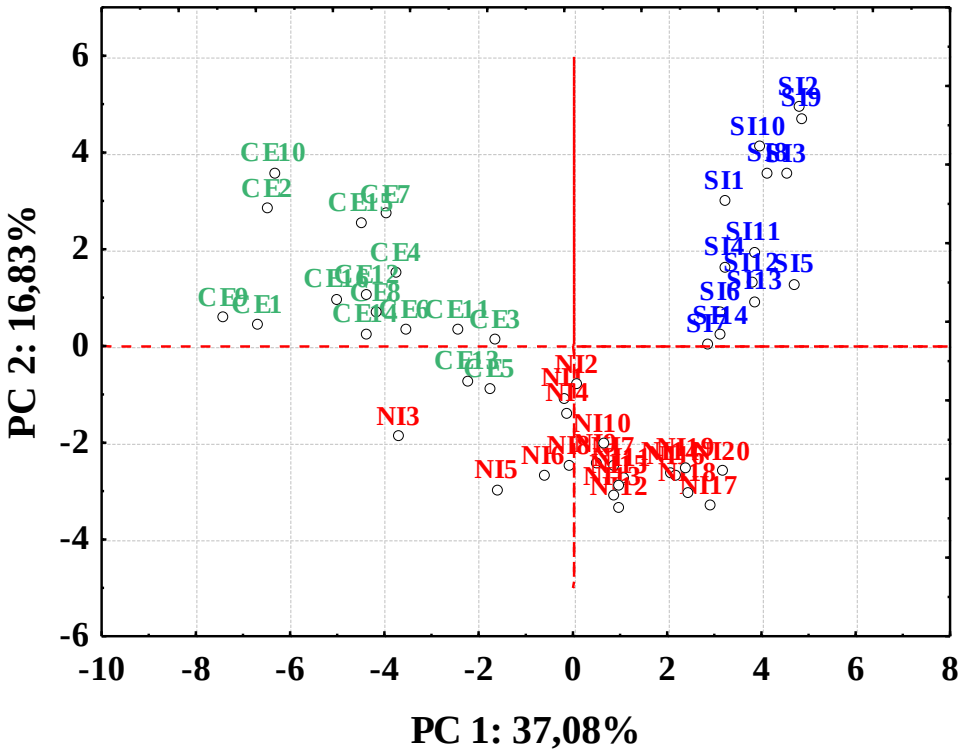

Figure 7.2. Scatterplot of the PC1 and PC2 component scores of milk samples showing differentiation according to regions. Green for Central Europe (CE) samples, red for Northern Italy (NI), and blue for Southern Italy (SI).

Figure 6.6 represents the biplot for all raw milk samples against 31 elements analyzed in this research.

Three sets of clusters are observed in the Figure and reported in black such as Northern Italy samples (NI), Sothern Italy samples (SI) and Central European samples (CE), including their characterizing elements (31 elements in red).

The cluster in the negative side of PC1 and PC2 is cluster 3 (CE samples) while cluster 1 (NI samples) and cluster 2 (SI samples) are in the positive side of PC2 and PC1 and in the positive side of PC1 and negative side of PC2, respectively.

Cluster 1 consists of milk samples which are loaded by Na, Mg and Zn, whereas milk samples in cluster 2 are loaded by Pb, B, Cr, V, Co, Fe, Sr, Ni, Ba, P and K, while cluster 3 contains milk samples loaded by Mn, Cu, Rb, Eu, La, Ce, Nd, Pr, Gd, Lu, Sm, Er, Dy and Yb. Some milk samples, such as CE5, CE13 and NI3 are borderline and are loaded by Ca, Ho and Tm.

These parameters form the discriminating factors for separation of samples in three clusters, according to their geographical origin.

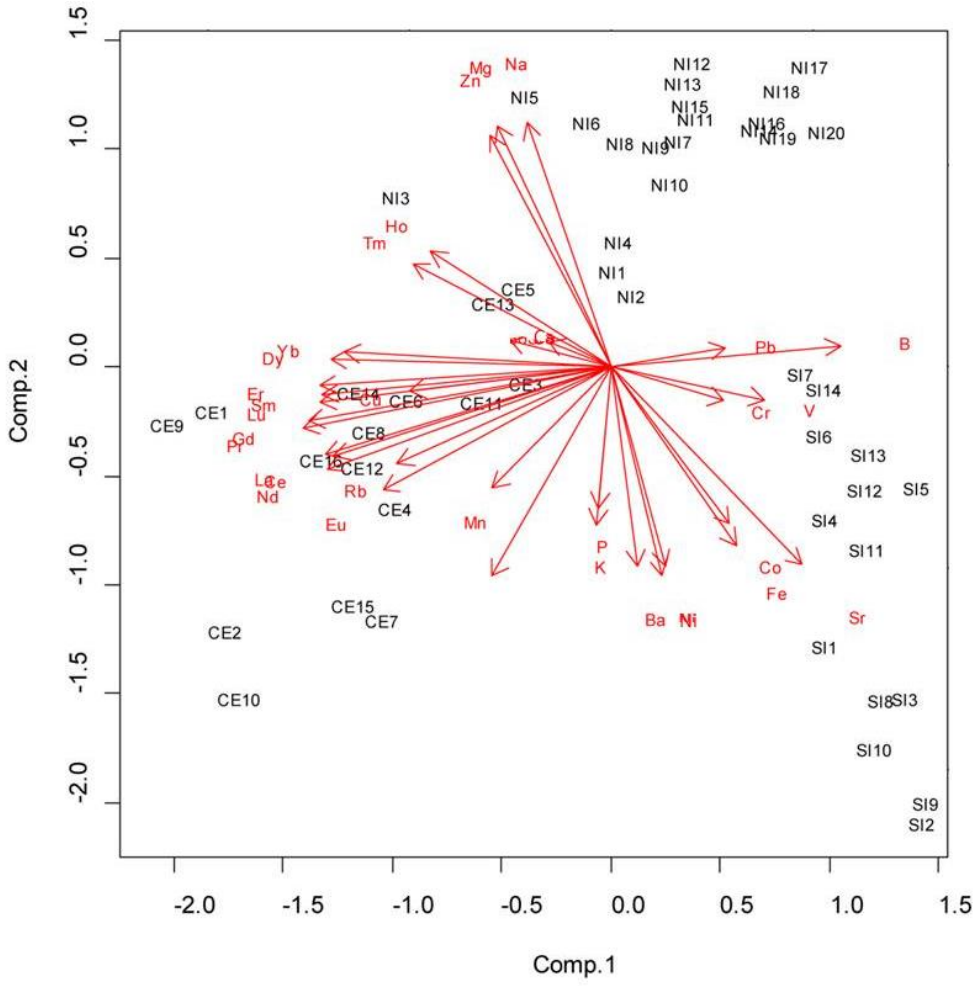

Figure 7.3. Biplot of all cow milk samples from Italy and Europe (in black, NI= Northern Italy, SI= Southern Italy, CE= Central Europe) including their characterizing elements (in red).

The results obtained by PCA show a natural grouping of the samples in three different groups, according to their geographical origin.

7.3 Hierarchical Cluster Analysis (HCA)

Hierarchical Cluster analysis (HCA) based on Euclidean distances and Ward's method supplied a first image about the structure of the data and was used to find similarity of the milk samples using 50 variables against 31 elements as input data.

The results of the HCA are reported as a dendrogram in Figure 6.7 and show the presence of milk clusters.

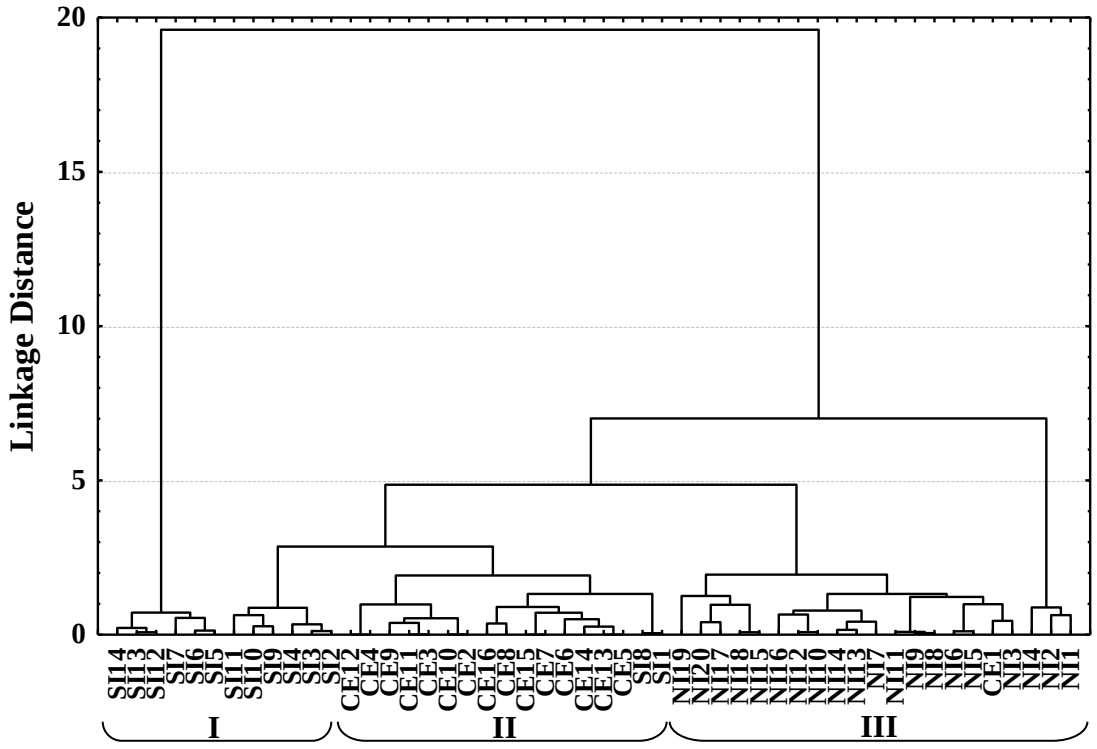

Figure 7.4. Dendrogram of Hierarchical Cluster Analysis to visualize the structure of the data set of 50 milk samples using 31 elements

Three clusters can be identified at a linkage distance of 20. A total of 15 of the 16 samples from Central Europe, together with two samples from Southern Italy, are grouped in the second cluster, while the first cluster is composed of 12 of the 14 samples from Southern Italy. Finally, milks from Northern Italy are principally grouped in the third cluster together with one sample from Central Europe. The results obtained by HCA confirm a separation of milk samples according to their geographical origin.

CONCLUSIONS

A high level analytical facility for elemental analysis based on a Clean Room (class ISO 6) that satisfies all standards requested for ultra-trace analysis and an 8800 Triple Quadrupole ICP-MS was set up and optimized, enabling multi-elemental analysis at low and ultra-low concentration level in various type of samples.

Fast and accurate method for preparation of raw cow milk samples by means of microwave digestion system was optimized and validated using a certified Standard Reference Material (NIST 1849a).

Analytical methods for the analysis of trace and ultra-trace elements and Rare Earth Elements through the high performance ICP-MS available at the ENEA-Brasimone facility was developed and validated.

Solution of isobaric interferences was achieved optimizing gas mixtures in the reaction cell leading to the following results:

a) no gas-mode for ^{9}Be, ^{11}B, ^{202}Hg, ^{232}Th, ^{238}U;

b) in Helium mode (He) for ^{23}Na, ^{24}Mg, ^{31}P, ^{39}K, ^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{111}Cd, ^{133}Cs, ^{137}Ba, ^{205}Tl, ^{208}Pb;

c) in Hydrogen mode (H_2) for ^{40}Ca, ^{56}Fe and ^{78}Se and in O_2 mode for ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm, ^{157}Gd, ^{163}Dy, ^{165}Ho, ^{166}Er, ^{169}Tm and ^{175}Lu.

The validated method presented satisfactory linearity, LOD, LOQ, accuracy, repeatability and reproducibility for a total of 40 elements, namely Be, B, Na, Mg, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Cd, Cs, Ba, Hg, Tl, Pb, Th, U, Eu, La, Ce, Pr, Nd, Sm, Yb, Gd, Dy, Ho, Er, Tm and Lu.

In the final part of the thesis the facility optimized as explained above was explored for its application in food traceability studies. It was then used for the determination of the element content in raw cow milk samples obtained from different Italian and European farms.

A total of 50 samples of milk from 3 regions widely apart from one another (Northern Italy, Southern Italy end Central Europe) against 40 elements were analyzed and the results obtained showed that multi-elemental profiles provide an extremely efficient tool for discriminating milks from different geographical origin.

The most abundant element in all raw cow milk samples is Potassium (K), followed by Calcium (Ca), Phosphorus (P), Sodium (Na) and Magnesium (Mg) for both Italian and

European milk samples. In general, milk is considered a good source of these major elements, especially Ca and P that are indicators of proteins rich milk.
Nine elements, i.e. Be, As, Se, Cd, Cs, Hg, Tl, Th and U, show concentration levels below their LOQs in all the cow milk samples analyzed in this work.
The concentration levels of potentially toxic trace elements were also evaluated and the results show that the concentration of As, Cd, Pb, Hg and Cr are very low or even below the LOQ values in all cow milk samples. This indicates the absence of contamination and therefore of any toxicological risks in the studied farms.

Regarding the trace elements content, the results show statistically significant differences in the concentrations of Na, Mg, P, K, Fe, Co, Ni, Zn, Rb, Sr and Ba between Northern and Southern Italy at the 95% confidence level.
Comparing the concentrations of the elements in Northern Italy and Central Europe, significant differences ($p < 0.05$) in the concentrations of B, Na, Mg, K, V, Cr, Cu and Rb are observed. Moreover, significant differences ($p < 0.05$) between milk samples from Southern Italy and Central Europe is found for B, Na, Mg, V, Fe, Co, Ni, Cu, Zn, Rb, Sr, Ba and Pb, while the concentration of nutrient elements, i.e. P, Ca, but also Mn, is substantially comparable ($p > 0.05$) in all the milk samples independently from the geographical region, and in agreement with the main nutritional properties of this food product.

Concerning REEs profiling, significant differences ($p < 0.05$) in the concentrations of Eu, La, Ce, Pr, Nd, Sm, Gd, Er and Lu are observed between milk samples from Northern Italy and Central Europe, whereas all the 13 REEs analyzed from Southern Italy are significantly different from Central Europe ($p < 0.05$) values.
The results of the REE screening show significant differences ($p < 0.05$) between Northern and Southern Italy throughout the whole elemental range considered.
The results demonstrate that cow milk samples from Europe presented a higher content in rare earth elements and significantly different than all other samples.
This observation may arise from the type of animal feed used in the two Italian farms, being typical small family-conducted farms, where cow feeding (and water) is always very local and fresh. On the other hand, the European farms, which provided the samples herein analyzed, used higher proportions of prepackaged feed not always of local origin, because of climate, large numbers of cows and market strategies. Though

the higher content of REEs in CE milk could be associated to local biogeochemistry, we cannot exclude this might be the result of REE's addition in industrial cow feed, as it is a widely used worldwide practice, especially in China, where REEs are successfully used in Chinese agriculture as fertilizer in plant production and as feed additives in animal nutrition (Redling, 2006).

In order to investigate the milk fingerprints from different Italian (Emilia-Romagna and Campania) and European (Central Europe) regions and to provide a basis for geographical traceability evaluation, 31 elements, of 40 total analytes, among which macro-elements (Na, Mg, P, K and Ca), trace-elements (B, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Ba and Pb) and ultra-trace elements (Eu, La, Ce, Pr, Nd, Sm, Yb, Gd, Dy, Ho, Er, Tm and Lu), were analyzed .

The PCA results show that the first eight PCs account for 85.83% of the total variance, which indicates that the first eight PCs explain the 85.83% of the information obtained from the multi-elemental analysis. Three clusters are obtained which represent the Northern Italy (cluster 1), the Southern Italy (cluster 2) and the Central Europe (cluster 3) samples. Each cluster is populated by a number of characterizing elements which form the discriminating factors for the separation of samples in three clusters, according to their geographical origin. Hence, the obtained results show that the cluster 1 consists of milk samples which are populated by Na, Mg and Zn, whereas milk samples in cluster 2 consist of by Pb, B, Cr, V, Co, Fe, Sr, Ni, Ba, P and K, while cluster 3 contains milk samples populated by Mn, Cu, Rb, Eu, La, Ce, Nd, Pr, Gd, Lu, Sm, Er, Dy and Yb. Moreover, the results obtained by HCA confirm a separation of milk samples in three clusters, according to their geographical origin.

According to the results obtained, multivariate techniques were proved to efficiently distinguish and classify raw cow milk on the basis of trace and ultra-trace elemental profile.

Correlations between studied variables were highlighted and recognized, by application of PCA and HCA. The obtained grouping emphasizes the high potential of the selected elements as geographical markers. The great capability of the analytical facility set up in food traceability investigations was demonstrated.

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