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FOOD SAFETY IN THE AGRI-FOOD SUPPLY CHAIN

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The food you eat can be either the
healthiest and most powerful form of
medicine, or the slowest form of poison.

Ann Wigmore

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Chapter 1

FOOD SAFETY AND QUALITY

1.1. INTRODUCTION

The agri-food chain is defined as the set of steps and events that a food product goes through to get, from the production of raw materials to its arrival on the consumer's plate. Hence, every food we eat has its own supply chain, which can be intended as the story of its journey and all the processes it has undergone from producers to supermarkets, shops, restaurants and, not least, our homes. Recently, there has been a lot of talk about the transparency of supply chain[1], i.e., a fully transparent and traceable supply chain, with information available to consumers at every step. Traceability and transparency of supply chains are very important to reach new levels of sustainability, development and food safety.

The assurance of food safety and quality is one of the most widespread problems in the world today. In particular, the term 'food safety' refers to a set of practices, rules and standards designed to ensure that food is always handled and consumed in a healthy, hygienic and risk-free manner[2]. These guidelines cover how food should be handled at all stages of the production process: preparation and storage, transport and distribution to the consumers.

Food security, on the other hand, refers to programmes, regulations and instruments aimed at guaranteeing universal access to food, regardless of possible complications arising from factors such as location, social class or economic availability[3]. The right to food has been recognised as a human right since the Universal Declaration of 1948.

As we have seen, food safety therefore has mainly regulatory and sanitary connotations, whereas food security falls within the economic and social sphere. The former, therefore, has to do with

rules and standards, while the latter is closely linked to a series of much broader problems that relate to issues of poverty, conflict and the geographical configuration of different territories.

Quality and food safety work in parallel, with one determining the existence of the other. A product cannot be considered safe unless its quality is determined. Similarly, one of the factors contributing to food quality is safety. Hence, the two concepts are inextricably linked. The quality of a food product is strongly influenced by the production process throughout the supply chain. In fact, producing 'quality food' is an important objective for all manufacturing companies[4]. Companies in the food industry must create the conditions to ensure that products always meet the most stringent standards, thus, guaranteeing quality and safe food.

Food safety is closely linked to the chemical and/or biological contamination of food, where a contaminant is defined as 'any substance not intentionally added to food but present in food as a result of its manufacture, processing, preparation, treatment, packaging, transport or storage, or as a result of environmental contamination'[5]. Admittedly, the chemical contamination of agri-food products is a rather complex problem, linked both to the industrial development and to man's increasing use of xenobiotic substances (foreign to the natural environment). It follows that the chemical substances that contaminate food, which are usually present in very small quantities, (range of mg/kg or μg/kg), can have different origins, such as agronomic (pesticides, fertilisers) and zootechnical (hormones, antibiotics), practices, release phenomena from processing equipment and containers (plasticizers, bisphenols) and, not least, environmental pollution of industrial and urban origin. Chemical substances that are foreign to the composition of food, and not always harmless, are also additives, but these are not called contaminants (unintentionally added residues) because they are added to food voluntarily for various reasons, e.g. to maintain or improve its preservation, freshness, taste, texture or appearance.

The agri-food industry has a responsibility to protect the health of consumers and is therefore particularly involved in the prevention and management of risks associated with contaminants at all stages of the production process, from agricultural raw materials to the finished product.

Over the last twenty years, there have been numerous scares in the agri-food sector: baby bottles containing bisphenol A, salmon with high levels of polychlorinated biphenyls, swordfish with high levels of methylmercury, dioxin-contaminated chickens, as well as the risks posed by plant protection products on crops, making it imperative to monitor the quality and safety of food and, at the same time, to assess human exposure to chemical contaminants through the diet.

The Commission responsible for drawing up the Codex Alimentarius (a set of internationally standardised guidelines and codes of good practice that contribute to improving the safety, quality and fairness of the world food trade) established three basic principles of food safety in 1997[6], which correspond to three essential definitions, such as:

- food safety, i.e. a food is defined as safe when it cannot cause any harm to the health of the consumer when prepared and/or consumed for its intended use;
- food hygiene, i.e. the conditions and measures necessary to ensure the wholesomeness and fitness for consumption of a food at each stage of the production process;
- food suitability, i.e. a food is considered fit for human consumption for its commonly understood uses.

Today, more than ever, all the actors of the agri-food chain have a role to play: they must not "let their guard down", and it is essential to take all precautions to avoid various types of contamination, such as:

- primary contamination: which occurs in food at the production stage (raw materials) from air, water, soil and the producing animal itself;
- secondary contamination: which occurs during processing and depends on the working environment, tools used and handling;
- tertiary contamination: occurs during the marketing of the product, i.e. during transport, storage and preservation.
- quaternary contamination: which occurs when the food is consumed;

Food safety is also the lever for achieving many of the Sustainable Development Goals that make up the UN's 2030 Agenda-Goal 2, which aims to "achieve food safety, improve nutrition and promote sustainable agriculture", is interlinked with all the other goals of the 2030 Agenda, as agriculture plays an essential role in achieving relevant objectives, such as eradicating poverty, ensuring health, providing access to water, safeguarding biodiversity and energy, ensuring sustainable cities and mitigating climate change[7]. The regulatory, scientific and technological capacity of countries to ensure food safety and quality throughout the agri-food chain contributes to the spread of more sustainable models of food production and consumption, taking into account all stages of the agri-food chain, from production, harvesting and storage to processing and consumption.

1.2. REGULATORY OVERVIEW

The European Union has the highest level of food safety in the world, thanks to a solid legislative framework designed to protect consumers. The first food safety rules date back to the birth of the EU, and the food safety crisis of the 1990s highlighted the urgent need to replace what had become a hodgepodge of rules with a simple, structured approach that was also better prepared to deal with the risks of food contamination. The result was a new framework legislation called the "General Food Law", implemented between 2002 and 2005, which not only defined the principles to be applied to food safety, but also introduced the concept of traceability, whereby food and food companies (whether producers, processors or importers) ensure that every food, feed and food ingredient can be traced along the food chain, from consumer to producer[8].

The European Food Safety Authority (EFSA), located in Parma (Italy), was founded in 2002 to advise the EU institutions, mainly the European Commission, on all scientific aspects related to the production, processing and marketing of food and feed. It operates in areas such as food, genetically modified organisms, animal health and welfare and plant health[9]. In particular, EFSA's Panel on

Contaminants in the Food Chain (CONTAM) provides scientific opinions on contaminants in the food chain and undesirable substances such as unauthorised substances and residues. The members of the CONTAM Panel are scientists from all over Europe with expertise in food and feed processing in the field of chemical contaminants, assessment of dietary exposure to contaminants, human and veterinary toxicology, interpretation of toxic effects of chemical contaminants in farm animals, assessment of dose-response effects and animal nutrition[10]. EFSA provides scientific opinion to EU decision-makers in an efficient and transparent way. Once EFSA's scientific opinion has been obtained, it is essentially up to the European Commission to decide how to respond; governments and the European Parliament have given the Commission the power to act immediately in the case of imminent risks. In such cases, the Commission can, for example, impose conditions on the sale of food and feed, restrict or even prevent the sale of the food in question. These and other operational decisions are discussed with Member States in the Standing Committee on the Food Chain and Animal Health.

A key element in ensuring the rapid exchange of information between Member States in the event of a risk to human and/or animal health arising from the consumption of food and feed is the Rapid Alert System for Food and Feed (RASFF). The Rapid Alert System for Food and Feed (RASFF), first conceived in 1979 at the suggestion of the European Council, was formally established by Regulation (EC) 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety[11].

The RASFF is a 24-hour alert system for the notification of a direct or indirect risk to human or animal health or the environment arising from food or feed.

The members of the network are

- The European Commission (member and manager of the system);

- The public health authorities responsible for food safety in the Member States of the European Union;
- The European Food Safety Authority (EFSA);
- the European Free Trade Association (EFTA - Iceland, Liechtenstein, Norway, Switzerland).

Information is communicated and exchanged between network members in real time via the i-RASFF online platform, which is accessible to all network members, who can both activate and read notifications uploaded to the system by other countries. Through this rapid exchange of information, multiple risks are managed so that dangerous products are withdrawn/recalled from the market. The European Food Safety Authority provides independent scientific opinions and risk assessments that feed into EU food safety legislation; protecting human health is the aim of all EU laws and regulations in the fields of agriculture, animal husbandry and food production. A comprehensive set of EU-wide rules regulates the entire food production and processing chain within the EU, as well as imported and exported goods. The EU monitors the application and effectiveness of these laws and controls and provides the necessary training for EU and international authorities. EU food safety policy and resulting actions focus on four main areas:

- Food hygiene: food businesses, from farms to restaurants, must comply with EU food law, including when food is imported into the EU.
- Animal health: health checks and measures for pets, farm animals and wildlife to monitor and control diseases.
- Plant health: early detection and eradication of pests prevents their spread and ensures healthy seeds.
- Contaminants and residues: monitoring keeps contaminants out of food and feed. Maximum permitted levels apply to local and imported food and feed.

Food contamination can occur naturally or as a result of farming practices or production processes. Regulation (EU) 2023/915, which replaced the previous Regulation (EC) 1881/2006, came into

force in May 2023 and focuses on setting maximum levels for different contaminants in food[12]. To protect public health, maximum levels are set and regularly updated for various contaminants such as dioxins, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), nitrates, certain metals and mycotoxins in food of both animal and plant origin. Residues in food may also come from food-producing animals treated with veterinary medicines or exposed to pesticides or biocides. Foods containing unacceptable levels of contaminants cannot be marketed in the EU.

There are also regulations for materials that come into contact with food, such as materials used to transport or process food, packaging materials and kitchen and tableware. A Regulation lays down general requirements for all relevant materials and articles to ensure that they do not transfer their constituents to food in quantities that could endanger human health. Specific EU measures may also be adopted with more detailed provisions for the 17 materials and articles listed in Annex I, i.e. all materials and articles intended to come into contact with food. Regulation (EC) No 2023/2006 sets out good manufacturing practices for these materials and articles[13]. For example, for plastic materials, restrictions have been introduced on the use of bisphenol A in plastic baby bottles. In 2011, the EU consolidated its rules on plastic materials and articles intended to come into contact with food into a single document (Regulation (EU) No 10/2011) and subsequent updates[14].

EU legislation requires food additives to be authorised before they can be used in food. Once they have received this authorisation, these substances are entered into the EU Register of Authorised Food Additives contained in Regulation (EC) No 1331/2008 on food additives and subsequent amendments[15], which also defines the conditions under which these substances may be used. Feed business operators must ensure that all stages of production, processing and distribution under their control comply with EU feed hygiene rules and ensure full traceability. When feeding food-producing animals, farmers must minimise the risk of biological, chemical and physical contamination of feed, animals and animal products. A specific directive sets maximum limits for undesirable substances in feed, including heavy metals, and prohibits the dilution of contaminated

raw materials. The provisions on veterinary medicinal products and medicated feeding stuffs have been updated by Regulation (EU) 2019/6 and Regulation (EU) 2019/4 respectively[16,17].

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Chapter 2

FOOD TRACEABILITY

Traceability of agri-food products is becoming increasingly important due to consumer pressure to know not only the nutritional value of food, but also its origin and authenticity. Food traceability and authenticity are inseparable parts of food safety and quality. In recent years, we have moved from traceability intended as the monitoring of material flows, mainly consisting of documentation, to the 'scientific' traceability of food1. The difference between authenticity and traceability must be clarified.

Authenticity is based on the ability to distinguish different productions by:

- geographical area;
- botanical/animal variety;
- production technology.

Traceability, on the other hand, implies the possibility of establishing a chemical link between a food product and the area in which the raw materials for its production originate.

The diffusion of traceability systems in recent years has been driven by the need to respond to a series of "food scandals" that have had a significant impact on consumer behaviour and led to a state of deep mistrust of the agri-food production system. To see the traceability of agri-food chains in the perspective of a simple response to food emergencies caused by consumers is extremely reductive. Chain traceability is a tool whose potential is much broader and more articulated, and which can be understood in the light of the transformation process that the agri-food production system has undergone in recent years[2,3]. The changes observed in agriculture and industrial processing are mainly due to two fundamental factors: the spread of increasingly complex technologies in agriculture, and the significant increase in the food volume in industrial processes. The result is a much greater availability of food than in the past, with significant environmental impacts and increased costs throughout the supply chain. In addition, the transfer of part of the

supply chain to developing countries to overcome the seasonality of some agri-food production is becoming more frequent, but most of these countries do not have control systems and production technologies comparable to those present in developed countries.

In this context, two models can be identified: the European model, in which intensive and technologically advanced production is grafted onto a large complex of traditional production and processing techniques, and the link between the specificity of food production and the territory is very strong[4], and the non-European model, which consists mainly of large agricultural and processing enterprises with a very extensive use of modern technologies[5]. Against this background, it is not surprising that events such as the food emergencies of the 1990s (dioxin in chicken, to name but one example) were particularly disruptive and required a strong response from both the EU and individual member states. Traceability of food has been mandatory throughout the European Union since 1 January 2006 under European Regulation 178/20026; before that date, only products posing the greatest health risk to consumers, such as meat, fish and eggs, were traceable.

This legislation requires food and feed producers operating in Europe to implement a traceability system for their products. According to the Regulation, it is necessary to trace food, feed, food-producing animals and other substances intended to be part of food at all stages of production, processing and distribution of the products.

Traceability must be guaranteed by means of a universal and objective documentary identification that allows all events along the supply chain to be traced.

National policies aimed at safeguarding, protecting and developing local specialities as a synonym for quality, with a view to maximising biodiversity, are very positive. However, it is extremely complex for control authorities to guarantee the hygiene and health aspects that underpin food safety standards for consumers.

Based on these considerations, the simultaneous verification of two closely related aspects becomes essential: the characterisation of the food, to monitor and verify its ingredients, and the identification of its geographical origin, to establish the provenance of its raw materials. Product authenticity and

geographical traceability are therefore two essential aspects of food safety. For these reasons, consumers insistently demanding more and more precise information on nutritional parameters to guarantee the "quality" of food products. The authentication of a food product is the process of verifying that the product conforms to what is declared on the label and, where applicable, to what is laid down in the relevant regulations. In particular, the use of rapid, efficient and high-performance analytical methods with respect to the matrices under investigation is a valid and irreplaceable tool for the competent control authorities. Moreover, scientific innovation and the technological development of tools and methodologies can enable the rapid detection of fraud and adulteration designed to circumvent the 'legal' controls currently in force.

Traceability, on the other hand, guarantees consumers control over the origin, production and distribution of the product through the coordinated efforts of several organisations with the common goal of ensuring the integrity of the product.

Traceability standards for agri-food products aim to document the history or origin of a product or its main components, both within a single company and along the entire agri-food chain. The geographical origin of food products involves the creation of categories of typical products that can bear quality labels (PDO, PGI, etc.)[7]. This is linked to the need to diversify agri-food production to achieve a better balance between supply and demand on the market. The concepts of traceability and authenticity are very important for the protection of a typical food product, intended as identification processes that follow the food product at all stages of its production. This includes information on the origin of the raw materials, the place of production and the processing techniques used to obtain the finished product.

The scientific community has developed several methods for tracing the origin of a product. These methods are based on parameters that consider the climate, soil and possible types of fertilisers, which influence the chemical and physical composition of the product, in turn characteristic of the areas of origin.

It is possible to classify the "analytical" parameters that are indicators of geographical origin into two main categories: direct or primary indicators, which include variables that directly relate certain chemical characteristics of the foodstuff to the same characteristics measured in the territory (i.e. metals and isotopic speciation); indirect or secondary indicators linked to the compositional characteristics of the food and the transformation processes. These allow products of the same origin or protected by a designation of origin to be classified as "similar" and, thus, distinguished from all others, through a comprehensive characterisation of the matrix and the determination of many variables using various analytical techniques. In this case, the chemometric processing of analytical data, i.e. the use of mathematical classification models and their validation, are indispensable tools for the purposes described above.

In the following chapters we will often see how these chemometric tools have been applied to several food matrices to derive information and differentiate them according to geographical origin, botanical/animal variety and even production technology.

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Chapter 3

OVERVIEW OF POLLUTANTS IN THE FOOD CHAIN

In addition to the nutrients and biologically active substances needed by the human organism, food may contain a range of toxic substances that can affect its healthiness.

Food may accumulate such substances from soil, water or air contamination, compounds naturally present in the environment or synthetic products released into the ecosystem. Contamination can occur at various stages, from production to processing, from storage to transport, from preparation to consumption: in other words, 'from farm to fork'.[1]

Various natural or synthetic molecules that are potentially toxic are collectively referred to as xenobiotics. Although the term xenobiotic is usually used as a synonym for synthetic substances, it should be specified that it can also refer to natural or biological molecules in much higher concentrations than normal, or produced by certain organisms as a defence mechanism, such as toxins released by bacteria, fungi or even plants[2]. In general, xenobiotics are pollutants, drugs, industrially synthesised molecules, food additives or substances produced by cooking or the use of high temperatures.

The long persistence of a chemical compound in the environment inevitably favours its introduction into the food chain, and the chemical properties that favour its persistence in the environment are: resistance to thermal degradation; stability to electromagnetic radiation, especially sunlight, which performs the important function of photodegradation; low solubility in water, which favours the uptake of substances in soils and sediments with little dilution and dispersion; resistance to chemical degradation of an acid and/or basic nature; and resistance to degradation by microorganisms[3].

Food contaminants can be classified according to where they come from and how they come into contact with food. They are generally classified as chemical, physical and biological, but in line with recent trends it is possible to distinguish natural, environmental and contamination from one or more stages of the production chain. These include pesticides, polycyclic aromatic hydrocarbons

(PAHs), polychlorinated biphenyls (PCBs), dioxins, chlorinated compounds and toxic/potentially toxic elements.

Heat treatments commonly used in food production can also be a source of contamination. The use of high temperatures in combination with external factors can lead to the formation of toxic compounds that affect food safety and quality.

Food packaging is also a potential source of contamination due to the use of additives, stabilisers, antioxidants, plasticizers and bisphenols, although it has many benefits, including physical protection of food and extending its shelf life.

Some information on the main classes of contaminants considered in this thesis is provided in the following sections.

3.1. PESTICIDES, POLYCHLORINATED BIPHENYLS, DIOXINS and POLYCYCLIC AROMATIC HYDROCARBONS

Pesticides, Polychlorinated Biphenyls (PCBs), Dioxins, Polycyclic Aromatic Hydrocarbons (PAHs) and belong to the category of Persistent Organic Pollutants (POPs) and are characterised by a persistent effect on the environment as they are not degraded by thermal, chemical or microbial activity[5].

POPs are defined as compounds that “possess toxic properties, resist degradation, bioaccumulate, are transported across international boundaries by air, water and migratory species, and are deposited far from their point of release where they accumulate in terrestrial and aquatic ecosystems". Precisely due to these characteristics and the high chemical risk associated with persistent organic pollutants, countries that are parties to the Stockholm Convention[6] (adopted in Stockholm in 2001 under the auspices of the United Nations Environment Programme and entered into force in 2004) are committed to taking measures to protect human health and the environment by reducing and/or eliminating these types of pollutants.

The term 'pesticide' is commonly used as a synonym for phytosanitary product, but pesticide is a broader term that includes products such as biocides, which are used to control pests and disease-carrying organisms such as insects, rats and mice. Crop protection products, on the other hand, are mainly used to keep crops healthy and prevent them from being destroyed by disease and pests. They include herbicides, fungicides, insecticides, acaricides, plant growth regulators and repellents. Pesticides are mostly toxic, persistent and bioaccumulative substances that affect the physical and chemical properties of soils and are often extremely harmful not only to human health but to the entire ecosystem and all living organisms. Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005, which entered into force in September 2008, establishes harmonised Community rules on maximum residue levels (MRLs). The maximum residue limit is the highest level of a pesticide that is legally tolerated in food[7].

These MRLs are set at European level for each pesticide on the basis of its use in agriculture (quantity and frequency of application, and the growth stage of the plant during application) and two toxicological values: chronic or long-term toxicity, i.e. the acceptable daily intake (ADI) of the pesticide, and acute or short-term toxicity, i.e. the Acute Reference Dose (ARfD), that must never be exceeded, even in a single exposure.

Pesticides have different chemical and physical properties from compound to compound due to their different structures. What they all have in common is a low solubility in water, although given the high doses required for pesticide action, the amounts of pesticides found in water and surrounding environmental matrices are still significant. The most worrying category of pesticides from a human health point of view are the organochlorine pesticides, because they are very persistent in the environment and have a high capacity to accumulate in the food chain[8]. Examples of this category include dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethane (DDD), dichlorodiphenyldichloroethylene (DDE), aldrin, dieldrin, endrin and chlordane. They exert their effect through neurotoxic activity, some depolarise the nerve membrane by altering the opening of the voltage-dependent sodium channel, others act as inhibitors of geranylgeranyl pyrophosphate, an

enzyme that plays an important role in the mevalonate pathway, and others interfere with plastoquinone, which is involved in the electron transport chain in the light-dependent reactions of photosynthesis. All these mechanisms result in the death of unwanted pests or weeds. However, the highly persistent nature of pesticides means that the same neurotoxic effects also affect other living organisms, including mammals and humans.

Tests have been carried out on laboratory animals to assess specific effects on humans and possible carcinogenicity. In this context, it is interesting to note the classifications proposed by the European Council Regulation 1272/2008, IARC and EPA, according to which pesticides can be divided into two categories: probable human carcinogens (IARC Group 2B) and not classifiable as human carcinogens (IARC Group 3)[9].

When discussing food hazards due to the presence of pesticides, it is important to consider multi-residues, i.e. the co-occurrence of several residues of harmful substances in the same sample. Multiple residues in a single foodstuff may result from the presence of several active ingredients in the same pesticide, or from the use of different types of active ingredients (e.g. herbicides, fungicides or insecticides), or from contamination during food processing, uptake of persistent residues by the soil, or spread from treatments on neighbouring fields. The interaction of several active ingredients causes different effects in the body, depending on their chemical structure. Antagonistic, additive or even synergistic effects can result, causing damage that is more severe, irreversible and even unpredictable than their individual effects.

The generic term "dioxins" refers to a group of 210 polychlorinated aromatic chemical compounds divided into two families: dibenzo-p-dioxins (PCDDs, or properly "dioxins") and dibenzo-p-furans (PCDFs, or "furans"). These are chlorinated aromatic hydrocarbons, mostly of anthropogenic origin, articularly stable and persistent in the environment, toxic to humans, animals and the environment itself; dioxins and furans are two of the twelve classes of persistent organic pollutants.

There are a total of 75 dioxin congeners and 135 furan congeners, of which only 17, 7 PCDDs and 10 PCDFs are of particular toxicological concern. In common terminology, the term "dioxin" is

sometimes used as a synonym for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic congener. Dioxins are semi-volatile, thermostable, weakly polar, water-insoluble, highly fat-soluble substances that are extremely resistant to chemical and biological degradation. These chemical and physical properties make these substances easily transported by atmospheric currents and, to a lesser extent, by river and sea currents, thus, allowing them to contaminate sites distant from the emission sources. Due to their ubiquitous presence in the environment, their persistence and their liposolubility, dioxins tend to accumulate over time in living organisms, i.e. in the tissues and organs of humans and animals. In addition, the concentration of these substances can increase ('biomagnification') as they move up the food chain. Dioxins accumulate in the fatty tissues of organisms, so when contaminated grass and soil is ingested by herbivores, these substances accumulate in the fat of their meat and in the fat of the milk produced.

Polychlorinated biphenyls (PCBs) are hydrocarbons of industrial origin characterised by a biphenyl structure (two aromatic rings) to which 1 to 10 chlorine atoms are bonded. PCBs are a family of 209 synthetic, polyaromatic, lipophilic, persistent (half-life 1->10 years) chemical compounds with ubiquitous bioaccumulation in all biological matrices (soil, sediment, air, water).

The term PCB is often used to define the whole class of compounds or mixtures rather than the individual congener. Various classifications of PCBs have been proposed over the years: the most widely used is that of Ballschmiter and Zell from 1980[10], which assigns a progressive number from 1 to 209 to the individual congeners according to the lesser or greater degree of chlorination (IUPAC classification). Numerous studies conducted by the International Agency for Research on Cancer (IARC) and the US Environmental Protection Agency (EPA) on both laboratory animals and humans have highlighted the high toxicity and chemical risk associated with PCBs. Toxicity and mechanisms of action vary from congener to congener, resulting in symptoms ranging from skin rashes and liver dysfunction due to acute intoxication to the formation of cancer cells. In general, the 209 PCBs can be divided into two large subgroups: dioxin-like PCBs and non-dioxin-like PCBs. Dioxin-like PCBs include 12 so-called coplanar congeners (congeners 77, 81, 126, 169, 105, 114,

118, 123, 156, 157, 167, 189), which act according to the same biological mechanism as 2,3,7,8-TCDD because of their molecular size and their ability to adopt a planar conformation.

Dioxins and dioxin-like PCBs are generally not detected in the different matrices as single compounds but as complex mixtures of the different congeners. To express the toxicity of individual congeners, the concept of toxic equivalency factors (TEFs) has been introduced[11]. TEFs are based on the consideration that dioxins and dioxin-like PCBs are structurally similar compounds that have the same structural mechanism of action (activation of the aryl hydrocarbon receptor AhR) and produce similar toxic effects: it is the binding between dioxins and the receptor that is the key step in the subsequent triggering of toxic effects. The TEFs are calculated by comparing the binding affinity of the different organochlorine compounds to the AhR receptor with that of 2,3,7,8-TCDD, taking the affinity of this molecule as the unit reference value. To express the total concentration of dioxins in the different matrices, the concept of toxic equivalent (TEQ) was introduced, which is obtained by summing the products of the TEF values of the different congeners and their respective concentrations (C_i) expressed in the unit of measurement of the matrix in which they are found, i.e: $TEQ= \sum (TEF_i * C_i)$.

Another important aspect of the impact of PCBs on the environment and human health is their ubiquitous distribution. Persistence in the environment is related to the long half-life of the compounds, which vary from months to years, and to the ability of the congeners to be adsorbed in soils, a property that increases with the degree of chlorination. From soils, contaminants can migrate into other environmental matrices and then they can be transferred through the food chain. The main source of human exposure, apart from environmental pollution, is the consumption of contaminated food. PCBs bioaccumulate in the food chain: they are rapidly absorbed by the gastrointestinal tract of living organisms and due to their lipophilic nature, accumulate in fatty tissues. PCBs have been observed to cross the placenta and be excreted in milk, thus accumulating in the foetus and infant.

Polycyclic Aromatic Hydrocarbons (PAHs) are another class of organic compounds that have been studied extensively because of their potential effects on the environment and human health.

PAHs belong to the family of hydrocarbons (compounds containing only carbon and hydrogen atoms); the term "polycyclic aromatics" refers to the presence of two or more aromatic rings fused together. Within this class, the Environmental Protection Agency (EPA) and the World Health Organisation (WHO) have identified 16 compounds defined as "priority pollutants", i.e. molecules that have shown significant toxic effects and whose presence in the environment and in food must be strictly regulated[12]. The main chemical-physical characteristics of PAHs are low solubility in water (hydrophobic compounds), high lipophilicity, low volatility and low vapour pressure. These properties vary according to the number of rings in the compound and hence the molecular weight. PAHs can be formed during the slow ripening of organic matter (petrogenic origin) and during the incomplete combustion or pyrolysis of organic matter (pyrogenic origin). Emission sources of PAHs can be divided into natural and anthropogenic. Sources of natural origin are mainly related to: volcanic eruptions, fires, while anthropogenic sources are related to: combustion of fossil products, combustion of organic substances, incineration of municipal solid waste, exhaust from internal combustion engines, power plants. Due to these numerous and widespread sources, PAHs are ubiquitous and can be found in all environmental compartments (air, water, soil, sediment, biota) and consequently enter the food chain.

In unprocessed foods, the presence of PAHs is mainly due to environmental contamination: deposition from atmospheric particulate matter (e.g. on cereals, fruit and vegetables), uptake from contaminated soil (e.g. for vegetables), uptake from contaminated river and sea water (e.g. for fish and shellfish). Common sources in processed or cooked foods are heat treatments (in particular: grilling, roasting and baking and frying) and manufacturing processes. The latter include in particular drying processes using combustion fumes, e.g. in the case of vegetable oils, and smoking processes. The effects of PAHs on human health depend mainly on the duration of exposure, the concentration, the toxicity of the PAHs and the route of exposure; they can therefore be grouped into short-term (or acute) and long-term (or chronic) effects. Given the important relevance of PAHs to both the environment and food, the presence of these contaminants in various matrices is

regulated by the European Commission. EC Regulation No. 915/2023 sets concentration limits in food for the sum of the four substances benzo(a)pyrene, chrysene, benzo(a)anthracene and benzo(b)fluoranthene[13].

3.2. PLASTICIZERS AND BISPHENOLS

The definition of a plasticizer adopted by IUPAC in 1951 is as follows "a plasticizer is a substance or material incorporated into a material (usually plastic or elastomer) to increase its flexibility, workability or expandability. A plasticizer can reduce the softening viscosity, lower the second order transition temperature or reduce the modulus of elasticity of the product"[14]. This is due to the fact that the plasticizer molecules insert themselves between the polymer chains, reducing their intramolecular attractive forces and allowing the polymer chains to displace each other: their presence thus transforms a hard and rigid polymer into a soft and flexible material. There are several possible classifications of plasticizers based on their prevalence and practical use, such as phthalates (phthalic acid esters), adipates (adipic acid esters), citrates (citric acid derivatives) and trimellitates (extraction resistant). Of these, the class of phthalates is by far the most widely used.

Phthalates are a family of organic chemicals synthesised by the double esterification of 1,2-benzenedicarboxylic acid (phthalic acid) with linear or branched alcohols and used in various industrial applications. Low molecular weight phthalates, e.g. diethyl phthalate (DEP) and dibutyl phthalate (DBP), have been used since the 1930s in personal care and hygiene products such as perfumes, shampoos, soaps, lotions, cosmetics, fabric softeners, as plasticizers for cellulose acetate and in the manufacture of paints, varnishes, lubricating oils, adhesives, inks, insecticides and coatings. High molecular weight phthalates such as di(2-ethylhexyl) phthalate (DEHP), di-isononyl phthalate (DINP) and di-n-octyl phthalate (DnOP) are mainly used as plasticizers in the production of flexible vinyl, which in turn is used in products such as floor and wall coverings, toys, food contact applications and medical devices. Phthalates, which also include di-isodecyl phthalate (DIDP), di- methyl phthalate (DMP) and butyl benzyl phthalate (BBP), are used as intermolecular

lubricants, giving plastics durability, flexibility, malleability and elasticity by allowing polymer molecules to flow together.

Worldwide, up to 8 million tonnes of phthalates are produced each year, including more than 2 million tonnes of DEHP. In Europe, around one million tonnes of phthalates are produced annually, of which 900,000 are used to plasticise PVC (polyvinyl chloride)[15].

Since phthalates are not covalently bound to the polymer matrix, they tend to migrate or dissociate from it, especially when in contact with lipophilic substances and under mechanical or thermal stress. They tend to adsorb to soil particles and sediments; in general, phthalates persist in the atmosphere for hours and in soil for months, while in sediments they may persist for years. Phthalates can bioaccumulate in invertebrates, fish and plants, while they are efficiently metabolised and excreted in complex animals.

Exposure to phthalates can occur by direct contact, as in the case of migration from a product containing them and from medical devices, or by transfer of these compounds from one product to another, as in the case of food packaging and environmental contamination. The main route of exposure is through ingestion, and this mainly concerns phthalates. For the population, the main source of exposure through ingestion is contaminated food, either during the production process or during packaging and storage.

The intake of phthalates through food consumption depends on several factors such as the initial contamination level of the ingredients used in production, specific production technologies, fat content, packaging material used, contact time, contact temperature. Phthalates can contaminate raw materials in the field, where they can reach, given their moderate resistance to degradation, through atmospheric deposition from funnel emissions and incineration of plastic products during disposal. After use, industrial and household liquid products containing phthalates, particularly DBP and DEHP, are discharged into the sewage system, which, after specific treatment, carries the phthalates into rivers and the aquatic environment. Contamination of the aquatic environment is inevitably reflected in contamination of fish and crops. Phthalates in soil and agricultural soils are available to

grazing livestock and can therefore pass into animal tissues and secretions, such as milk. Contamination of food with phthalates can also occur during production, handling, transport, packaging and domestic preparation. During the production process, food can be contaminated with phthalates from fumes from industrial vinyl flooring, PVC piping and other equipment. For example, DEHP can be transferred from equipment to milk during the various cheese-making processes. Contamination can also be caused by the components of packaging materials, such as printing inks used in flexible packaging, adhesives used for paper and plastics, seals for bottle and jar closures, in coatings and in foil-laminated paper. Five phthalates (DBP, BBP, DEHP, DINP, and DIDP) are authorised for use in food contact plastics as plasticizers or technical support agents, subject to the restrictions described in the latest consolidated version of the Plastics Regulation (EU) 10/2011[16]. With regard to phthalates authorised for use in plastic food contact materials, the European Food Safety Authority has updated the Tolerable Daily Intake (TDI) values in 2019. In practice, the TDI is an estimate of the amount of a substance that can be ingested daily over a lifetime without posing a significant health risk. The experts set a new safe level of 50 µg/kg body weight for four of the five phthalates (DBP, BBP, DEHP and DINP). For DIDP, EFSA set a separate TDI of 150 µg/kg bw per day based on its effects on the liver[17]. It is important to note that there are no Maximum Residue Limits (MRLs) for these compounds, i.e. the highest levels of phthalates that are legally tolerated in food have not been set by any regulation.

Other compounds used in the manufacture of plastics, resins and other polymeric materials are bisphenols. Bisphenols are a class of chemical compounds consisting of two phenols linked by an alkyl group to which various functional groups are added or substituted, depending on the specific compound. The main bisphenol is 2,2-bis(4-hydroxyphenyl) propane, known as bisphenol A (BPA), synthesised in 1891, whose main property was to act as a plasticizer once converted to polymeric form. Since then, this compound has been widely used in the production of polycarbonates, epoxy resins, thermal paper and polyvinyl chloride.

In recent years, several studies have also been carried out on structural analogues of BPA (such as bisphenol A diglycidyl ether -BADGE- and its derivatives, bisphenol S, bisphenol AF, bisphenol F, bisphenol B, etc.). These compounds are structural analogues in that they share the basic bisphenol structure on which various functional groups are substituted, or, as in the case of bisphenol A diglycidyl ether (BADGE) and its derivatives, they are basic monomers of epoxy resins (after reaction of bisphenol A (BPA) with epichlorohydrin).

Due to their properties of resistance to high temperatures, corrosion by acids and oils and transparency, polycarbonates are widely used in tableware and food and drink containers, while epoxy resins coat the inside of metal cans and drinking water tanks. Bisphenols are also used in the manufacture of a wide range of non-food products such as tax receipts, toys, contact lenses, some electronic components, flame retardants, medical components, paints and printing inks. Their widespread use in consumer products has led to an increasing focus on their ecotoxicological role in humans and other organisms, particularly as endocrine disruptors. The mass production and widespread use of BPA has resulted in significant release and widespread distribution of this substance in the natural environment; it has been measured at relevant concentrations in soils in the United States, Europe and Korea, in surface waters and river sediments worldwide, with peaks in Taiwan and China[18].

Biological and toxicological studies have confirmed that this compound can have very adverse effects on both the environment and exposed organisms, as has already been demonstrated for human health. Indeed, numerous studies have demonstrated the presence of BPA in blood, urine, adipose tissue, placenta and amniotic fluid, linking its ubiquity to a range of disorders, including reproductive and fertility problems, cancer and cardiovascular disease, childhood obesity and type 2 diabetes. At the European level, it has been observed that the drive to replace BPA with alternatives has also led to an increase in the release of other bisphenols and bisphenol derivatives into the environment. As there is no real awareness of the potential effects associated with these compounds, it is clear that there is a need for appropriate regulation across a broad spectrum and covering the

different sources of exposure and release. To give an example, ECHA published a statement in 2020 stating that 187,000 tonnes of bisphenol S based thermal paper will be placed on the EU market in 2019, but that the widespread use of this substance is of concern due to suspected effects on the human reproductive and endocrine systems[19]. Of course, this is not an issue limited to Europe; in fact, there are numerous scientific publications internationally highlighting the presence of environmental contamination by bisphenols. Although the presence of BPA is described in more detail, there is also significant evidence of other analogues such as bisphenol S, bisphenol F and bisphenol AF[20]. In fact, these analogues are the main substitutes for BPA in the manufacture of polycarbonate plastics and epoxy resins. The close chemical similarity to BPA also leads to the classification of bisphenol analogues as endocrine disrupters. Despite this, studies and research into the effects of these bisphenols are still very limited, as is the supporting legislation. In this context, it should be noted that BPA is authorised for use in plastic food contact materials in accordance with European Commission Regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food and subsequent revisions and is subject to a specific migration limit (SML) of 0.05 mg kg-1[16]. The SML is the maximum permitted amount of a substance that can migrate from the packaging into the food. This limit ensures that the food contact material does not pose a risk to human health. It is an essential tool to ensure the health protection of all consumers, including the most sensitive populations. In January 2015, EFSA reduced the tolerable daily intake (TDI) from 50 to 4 µg/kg bw per day. At that time, EFSA's experts only set a temporary TDI as uncertainties remained and highlighted the need to address gaps and conflicting opinions found in the scientific data. In 2017 and 2019, the experts carried out extensive preparatory work, including the publication and subsequent testing of a hazard assessment protocol. The review resulted in a TDI of 0.2 ng/kg bw per day (April 2023), replacing the previous provisional level of 4 µg/kg bw. The new TDI has therefore been reduced by 20 000[21].

On 9 February 2024, the European Commission launched a public consultation on a draft new regulation that will ban the use of bisphenol A together with other bisphenols in food contact materials and articles and repeal Regulation (EU) 2013/2018[22].

To prevent BPA from being replaced by equally hazardous substances, the new regulation extends its caution to all other bisphenols used in food contact materials, which will have to be risk assessed and authorised before use to ensure that they do not pose a risk to human health. There are also no MRLs for bisphenols in food and this regulatory gap needs to be filled. To this aim, the European Chemicals Agency (ECHA) and Member States evaluated a group of 148 bisphenols and recommended that more than 30 of them be restricted due to their potential endocrine or reproductive effects[23].

To speed up the identification of chemicals requiring regulatory action, the authorities decided to deal with them in groups of structurally related substances rather than as individual substances. A group restriction was identified as the best way to manage the risks of 34 bisphenols[23]. This number may change as more information is generated for these and other bisphenols. Three bisphenols (bisphenol A, bisphenol B and bisphenol P) have already been identified as substances of very high concern. Harmonised identification or classification and labelling is proposed for other bisphenols for which sufficient hazard information is already available. However, for many members of the group, more data is needed before potential endocrine disrupting and reproductive toxicity properties can be confirmed.

For example, the German authorities are already preparing a proposal to restrict the use of bisphenol A and other bisphenols with endocrine disrupting properties in the environment. Once it is clearer which bisphenols will be covered by the German proposal, ECHA and the European Commission will assess the need for further regulatory action on bisphenols[23].

3.3. TOXIC AND POTENTIALLY TOXIC ELEMENTS

Toxic and potentially toxic elements are non-degradable compounds in both organic and inorganic forms. They are classified as essential (e.g. Copper, Zinc, Chrome and Selenium), probably essential (eg Vanadium, Cobalt and Nickel), potentially toxic and toxic (e.g. Aluminium, Arsenic, Lead, Cadmium and Mercury)[24]. Non-essential elements can be tolerated by the body up to a certain concentration, above which they become toxic. Elements such as arsenic, cadmium, lead and mercury are toxic at low concentrations, cannot be broken down or destroyed and are subject to the phenomenon of bioaccumulation. The toxicity of a chemical element is related to its chemical form (speciation) and bioavailability. Chemical speciation is the form in which the element occurs in the environment (free ion or complexed structure). The determination of the chemical form in which the element occurs is particularly important for assessing the bioavailable fraction that accumulates in organisms and is transferred through trophic chains. Organisms, in the process of exchanging matter and energy with their environment, may be involved in enrichment effects of potentially toxic elements present in the biosphere, either naturally or as a result of anthropogenic contamination. Bioaccumulation is the process that then determines the increasing accumulation of these elements along the food chain.

Toxic and potentially toxic elements are normally found in nature: in soil, water and the atmosphere. They can also be found in food as a result of contamination during processing or storage, or as residues from human agricultural and industrial activities. Humans can therefore be exposed to these toxic elements through the environment or by ingesting contaminated water or food, although the dietary route appears to be the most relevant to date. Let us take a few examples: chromium, used in the production of metal alloys, paints, paper, cement and rubber, tends to accumulate in the aquatic environment[25]. It can be found in fish and aquaculture products, but also in brewer's yeast, meat, cheese and whole grains. Long-term exposure can cause damage to the kidneys (mainly through urinary excretion of the metal), liver, circulatory system and nervous system. Cadmium originates from zinc processing and is widely recycled (e.g. batteries, plating, alloys, electronic waste,

pigments). Concentrations of cadmium can also be found as an impurity in many products, including fertilisers, from which it passes into agricultural products for human consumption or into animal feed. Food, especially seaweed, fish and seafood, and chocolate are the main sources of cadmium exposure for the population. EFSA's Panel on Contaminants in the Food Chain (CONTAM) has reduced the TWI (Tolerable Weekly Intake) for cadmium to 2.5 micrograms per kilogram of body weight (µg/kg bw)[26]. In the body, cadmium accumulates mainly in the kidneys and liver and has a half-life of 10 to 30 years; it is also a carcinogen (mainly in the lungs, but also in the endometrium, bladder and breast), causes bone damage with osteoporosis and immunological deficits.

Lead is an environmental contaminant that occurs both naturally and as a result of human activities such as mining. Measures have been in place in Europe since the 1970s to regulate lead levels in petrol, paint, food cans and pipes, but concern is still high. Although lead is present in the environment mainly in inorganic form, human exposure occurs mainly through food and water, and to some extent also through air, dust and soil. The EFSA Panel has identified a number of foods that may contribute most to dietary exposure to lead, including cereals, vegetables, drinking water. In 2024, EC Regulation 915/2023[13], also based on an EFSA opinion, set new limit values for lead to reduce the exposure of infants and children up to 3 years of age as well as women of childbearing age and to revise some limit values set for certain plant species. An important change concerns honey, for which there was no limit. The new entries for lead are cereal-based foods and other foods for infants and young children <0.050mg/kg; foods specifically intended for infants marketed in powder form <0.020mg/kg, in liquid form <0. 010 mg/kg; beverages intended for infants and young children, labelled and sold as such, marketed in liquid form or to be reconstituted <0.020 mg/kg; leafy vegetables and fresh herbs, mushrooms and seaweed <0.10 mg/kg; cranberries, currants, elderberries and strawberries <0.20 mg/kg; honey <0.10 mg/kg. Duration and level of Pb exposure cause various human damages, the most common toxic effects being kidney, haemoglobin synthesis, gastrointestinal tract, reproductive system, joints and bones (where it has a half-life of 20-30 years) and nervous system[27]. Arsenic is a naturally occurring metalloid often associated with rocks and

minerals. However, it can also contaminate water resources and enter the food chain. The most common sources of arsenic exposure are contaminated drinking water, food grown in contaminated soil and food from animals exposed to arsenic. The adverse effects of arsenic on the human body are well documented. Chronic exposure can lead to a range of health problems, including nervous system damage, cardiovascular disease, diabetes and, in extreme cases, cancer. Their presence in food is therefore of great concern and requires immediate action by regulators and the food industry. After collecting data on the presence of inorganic arsenic in food, EFSA assessed chronic dietary exposure to inorganic arsenic in its Scientific Report 2021 and concluded that the main contributors to dietary exposure to inorganic arsenic are rice, rice products, cereals and cereal products not containing rice, and drinking water. EFSA 2024[28] also concluded that some foods targeted at the young population (e.g. cereal-based foods for infants and young children, rusks and biscuits for infants, infant formulae, follow-on formulae, toddler formulae, baby foods and fruit juices) contribute significantly to the dietary exposure of this population to inorganic arsenic. It was therefore considered appropriate to set new maximum levels for the products that contribute most to arsenic exposure. The products concerned by the revision of the maximum levels were cereals and cereal products, infant formulae, follow-on formulae, and young children and infant formulae (marketed in powder and liquid form), reconstituted concentrated fruit juices and fruit nectars[28].

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Chapter 4

AIM AND RESEARCH LINES

The aim of this thesis was to provide an overview of food safety in different types of agri-food chains. In particular, food of animal origin (including milk and dairy products, meat, fish products and honey) were thoroughly assessed for their quality and safety with the final aim to ensure consumer health. Significant space was devoted also to the analytical side of these studies. Indeed, the extraction technique known as "QuEChERS" (Quick, Easy, Cheap, Effective, Rugged and Safe) was in depth considered for the extraction of organic and inorganic contaminants. This sample preparation techniques is simple, fast and inexpensive and is ideal for extraction, purification and multi-residue analysis in a wide range of food matrices. In addition, much effort was put into optimising and validating new green analytical methods to achieve increasingly sustainable and at the same time efficient methods. A second objective of this work was to demonstrate how chemometric tools can be of great help in the assessment of food traceability and authenticity, and how certain experimental variables correlate with the food origin.

During the three years of the PhD programme, several lines of research were started and followed, including the BIOTRAK project 'Innovation and technology transfer of agro-industrial wastes and by-products for animal feed with a view to the circular economy and sustainability of the livestock supply chain', approved and funded under the POFESR measure 1.1.5. The project was contextualised in the field of animal nutrition and quality of animal production, since it focused on the development of an innovative system of traceability of the supply chain by formulating feed for dairy cows integrated with olive oil derivatives. This project, hence, assessed the quality and safety of milk, cheese and oil waste to ensure safe products for the consumer. BIOTRAK strongly supported the evergreen concept of circular economy, that promotes the application of technological innovations to reduce waste and pollution, with the aim of recovering and reusing Sicilian olive oil waste.

Within the dairy research line, another project followed during the PhD period was the CHEESHAL project 'Technological Product and Process Innovations for the Development of the Halal Dairy Chain'. The CHEESHAL project aimed to produce innovative dairy products that meet the demands of increasingly aware consumers. In fact, the overall objective of the project was to develop and define an innovative supply chain process for the Halal certification of traditional dairy products. Two scientific research units were involved: the University of Catania, with the role of testing the suitability of using aqueous kiwi extract as a vegetable coagulant in the cheese making process, and the University of Messina, with the role of assessing the nutritional quality of milk and cheese and validating the zootechnical system of animal management. Within the framework of the intergovernmental European Cooperation Programme in Science and Technology (COST), created to reduce the fragmentation of research in the European Research Area (ERA) and to support research, innovation and networking activities, the four-year project programme 'BeSafeBeeHoney: Beekeeping products valorisation and biomonitoring for the safety of bees and honey' has been the most exciting and challenging line of research. The project is still ongoing, involves partners with different capacities, such as universities, research and development centres and SMEs, from up to 27 European and non-European countries and, thanks to its multi-stakeholder approach, brings together different competencies in chemistry, biology, ecology, veterinary science, nutrition, economics and policy to provide innovative results and insights into the most important and controversial aspects of beekeeping. The main topics addressed by BeSafeBeeHoney are nutritional and medicinal properties of honey and other beekeeping products; beekeeping products as indicators of abiotic stressors and anthropogenic pollutants in the environment; prevalent diseases and biotic stressors that threaten honeybee colonies; honeybees as pollinators in agriculture and the consequences of bee decline in agricultural ecosystems; policy research and market analysis related to beekeeping activities. During the three-year PhD, there was the opportunity to actively participate in the project team by delving into the contamination aspects of honey.

Chapter 5

RESEARCH LINES RELATED TO DAIRY PRODUCTS

Dairy products are considered in the global economy as a major market due to their high consumption. World milk production has increased by about 20% in the last decade, from 694 million tonnes in 2008 to 843 million tonnes in 2021.[1] Cow's milk is the most consumed and accounts for about 48% of total milk production. Dairy products are exposed to a variety of contaminating hazards, ranging from biological to chemical. Chemical contamination of milk and dairy products can result from: application of agrochemicals, use of veterinary drugs, contaminated feed and fodder, use of chemicals during production, processing and packaging. Several studies have shown that more than 90% of human exposure to contaminants appears to occur through food intake.[2] Dairy products seem to be good indicators of the presence of contaminants in the food chain. Phthalates, the world's most widely used plasticizers, and bisphenols (BP), used in the production of polycarbonate plastics and epoxy resins, are the focus of the scientific community's attention as previously discussed in Chapter 3. Due to their lipophilic nature, phthalates are mainly found in high-fat dairy products, can accumulate in feedstuffs and ruminant tissues, in fatty muscles, and pass from the animal's digestive tract to milk and, consequently, to humans. Many studies have been conducted on the migration of phthalates from PVC pipes to milking machines used on farms. Bisphenols can enter the dairy chain through animal feed, production machinery, environmental contamination and packaging materials. Bisphenols can be accumulated in the adipose tissue of the animal, secreted in milk fat and stored in fatty dairy products.[3] Other contaminants such as pesticides, PCBs and PAHs can be transferred through the food chain, and transfer through milk and dairy products in particular is known to be high. These contaminants are highly lipophilic and resistant to degradation and can be biomagnified through the food chain (see chapter 3). In addition, their highly toxic nature can cause adverse effects on both human health and the environment.

Due to the nutritional importance of dairy products, knowledge of their composition in macro and micro elements is important from both a nutritional and safety point of view. Cow's cheese is considered to be an excellent source of mineral elements, including macro-elements such as Ca, K, Mg, Na and essential micro-elements such as Zn, Fe, Cu and Se. The mineral content of dairy products depends on the genetic characteristics of the dairy cows, the lactation status and the diet of the animal. Toxic and potentially toxic elements (Cd, As, Pb, Al and Hg) can also come from various sources, such as contamination during the production or preparation of cheese, contact with processing equipment, accidental contamination during packaging or storage. According to other studies, the determination of potentially toxic elements in dairy products can also be a tool to assess the environmental pollution.[4]

Particular attention is paid in Italy to the contamination of the dairy chain as the dairy industry is the main food industry. Among Italian dairy products, semi-hard pasta filata cheeses are widespread in Southern Italy but are also highly appreciated in the rest of the peninsula. Provola pasta filata cheese produced in Ragusa (Sicily) is particularly appreciated by consumers for its sensorial characteristics such as taste and consistency, and it is a very common ingredient in many gastronomical preparations.Several studies conducted in the ruminant livestock sector have demonstrated the crucial role of feeding strategies in influencing the chemical and nutritional profile of food of animal origin.[5] In particular, the use of agro-industrial by-products as feed supplements, while respecting animal welfare, can improve the nutritional quality of animal products.

Furthermore, the need to solve the problem of waste disposal and to reduce the production costs of animal feed has made the use of agro-industrial by-products the best strategy. An important issue is the reuse of waste biomass from olive oil production as a suitable strategy to overcome environmental and economic problems.

Olive cake, the residual solid phase obtained from olive, has attracted the attention of the scientific community due to its high concentration of macro- and micronutrients and offers the possibility of being used as a supplement in the diets of farm animals. The use of unconventional feeds could be

an economical and sustainable way to improve the quality of dairy products and reduce the environmental impact of their difficult disposal.[6] In this context, a Provola cheese with innovative and sustainable characteristics, while maintaining a high nutritional profile, could be welcomed by the modern consumer.

As part of the BIOTRAK project, four papers have been produced and published on the quality and safety of dairy products with a vie w to the circular economy, sustainability and valorisation of production in the agri-food chain. In the following sections, each of these papers will be discussed in more detail:

- Liotta, L., **Litrenta, F**., Lo Turco, V., Potortì, A. G., Lopreiato, V., Nava, V., Bionda, A., & Di Bella, G. (2022). EVALUATION OF CHEMICAL CONTAMINANTS IN CONVENTIONAL AND UNCONVENTIONAL RAGUSANA PROVOLA CHEESE published in the journal *Foods 11*(23), 3817 (https://doi.org/10.3390/foods11233817)[7]
- Potortì, A. G., Lopreiato, V., Nava, V., **Litrenta, F**., Lo Turco, V., Santini, A., Liotta, L., & Di Bella, G. (2024). THE USE OF OLIVE CAKE IN THE DIET OF DAIRY COWS IMPROVES THE MINERAL ELEMENTS OF PROVOLA CHEESE published in the journal *Food Chemistry*, *436*, 137713 (https://doi.org/10.1016/j.foodchem.2023.137713)[8]
- Attard, G., Bionda, A., **Litrenta, F**., Lopreiato, V., Di Bella, G., Potortì, A. G., Lo Turco, V., & Liotta, L. (2024). USING OLIVE CAKE AS A SUSTAINABLE INGREDIENT IN DIETS OF LACTATING DAIRY COWS: EFFECTS ON NUTRITIONAL CHARACTERISTICS OF CHEESE published in the journal *Sustainability 16*(8), 3306. (https://doi.org/10.3390/su16083306)[9]
- Calabrese, F. M., Russo, N., Celano, G., Pino, A., Lopreiato, V., **Litrenta, F.**, Di Bella, G., Liotta, L., De Angelis, M., Caggia, C., & Randazzo, C. L. (2023). EFFECT OF OLIVE BY-PRODUCTS FEED SUPPLEMENTATION ON PHYSICOCHEMICAL AND MICROBIOLOGICAL PROFILE OF PROVOLA CHEESE published in the journal *Frontiers in Microbiology 14*, 1112328. (https://doi.org/10.3389/fmicb.2023.1112328)[10]

In the last decade, there has been a growing demand for artisanal cheeses, produced according to traditional methods closely linked to the territory and with unique characteristics that make them worthy of protection and distinguish them from similar products produced on an industrial scale. In recent years, various factors, such as the high price of calf rennet, religious concerns (e.g. Islam and Judaism), diet (vegetarianism) or the ban on the use of genetically modified rennet, have encouraged the search for alternative sources of milk coagulation.[11]

The alternative coagulants should have biochemical properties similar to those of calf rennet, such as high milk coagulant power, high specificity towards k-casein, proteolytic activity at pH and cheesemaking temperature, and sufficient thermolability to ensure whey products without residual coagulant activity.[12]

Alternatives of tradition animal rennet included the use of recombinant bovine chymosin, and microbial coagulants used in the dairy industry for cheesemaking. In fact, the Food Drug Administration (FDA) has authorized the use of microbial coagulants in cheese production.[13] According to the Dairy Research Institute, microbial rennet is advantageous as it is halal, kosher and vegetarian certified. Microbial rennet uses proteolytic enzymes produced by certain microorganisms as a coagulant; the most used are *Criphonectria parasitica*, *Rhizomucor miehei* and *Rhizomucor pusillus*. The protease from *Rhizomucor miehei* is one of the best substitutes for calf rennet due to its specificity in cleaving the peptide bonds of k-casein, precisely because it has many similar properties to calf rennet.

Furthermore, an alternative to animal-derived coagulant is plant-derived coagulant, whose enzymes can be extracted from a variety of plants and whose coagulation activity is comparable to that of animal-derived coagulants. Using simple purification procedures, plant proteases can be isolated from a variety of readily available plants such as kiwi fruit (*Actinidia deliciosa*), nettle leaves (*Urtica dioica*), ginger rhizome (*Zingiber officinale*).[14] Among plant derived enzymes, actinidin, is a protease from kiwi, which has shown great potential for use as a milk coagulant in cheese production.

As part of the CHEESHAL Project, the quality and safety of fresh Sicilian cheeses produced with a vegetable coagulant based on kiwi extract, traditional calf rennet and Halal-certified microbial rennet were evaluated.

- **Litrenta, F.**, Liotta, L., Potortì, A. G., Lo Turco, V., Randazzo, C.L., La Cava, R., Nava, V., Rando, R., Di Bella, G., (2024). QUALITY OF SICILIAN FRESH CHEESE MADE USING A VEGETABLE COAGULANT FROM KIWI FRUIT COMPARED TO CONVENTIONAL COAGULANTS submitted to the journal *Food Chemistry*.

Also in the Sicilian dairy sector, the safety of milk from cows from different areas of Sicily has been assessed in terms of potentially toxic elements and natural toxins. The following papers have been produced:

- Messina, L., Licata, P., Bruno, F., **Litrenta, F**., Costa, G. L., Ferrantelli, V., Peycheva, K., Panayotova, V., Fazio, F., Bruschetta, G., Tabbì, M., & Nava, V. (2024). OCCURRENCE AND HEALTH RISK ASSESSMENT OF MINERAL COMPOSITION AND AFLATOXIN M1 IN COW MILK SAMPLES FROM DIFFERENT AREAS OF SICILY, ITALY published in the journal *Journal of Trace Elements in Medicine and Biology*, 127478. (https://doi.org/10.1016/j.jtemb.2024.127478)[15]

5.1 EVALUATION OF CHEMICAL CONTAMINANTS IN "RAGUSANA PROVOLA" CHEESES

The aim of this study was to assess the food safety of Provola Ragusana cheese considering the different types of products obtained from Friesian dairy cows fed a conventional diet (CTR group) and an unconventional diet (BIO group) enriched with olive oil cake (OC). In order to assess safety, residual levels of plasticizers, bisphenols, pesticides, PCBs and PAHs were determined. This study was conducted in accordance with the QS (Sicilian Quality) standard approved by the Sicilian Region, which foresees the use of pitted olive cake in animal feed as a tool for the sustainability of the agri-food chain.

5.1.1 Samples Collection

Provolas samples were collected from March to July 2021 at a commercial cheese-making located in Ragusa (Sicily, Italy). Provola cheese was produced from milk of Friesian cows subjected to two different feeding systems: a conventional diet (CTR) and an unconventional diet (BIO) similar in composition; only difference was pitted olive cake (OC) that was added in BIO diet at 8% level on Dray Matter (DM). The olive cake (used in the Biotrak diet) were produced in a Sicilian oil mill (Adrano, CT, Italy). The cow's milk obtained from both groups was cheesed separately, with the same production process, in a commercial cheesemaking located in Ragusa (Sicily, Italy), for the typical provola cheese production. Monthly, 400 litres of milk from each group were cheesed into 80 provolas according to the production diagram shown in the figure 1. Four representative samples of provolas (obtained from five provolas randomly selected from 20 provolas) were analyzed for each group each mount for a total of 40 analyses. In addition, the dried and pitted olive cake used as a supplement in the BIO diet was stored at the beginning of the trial and then sampled and analyzed monthly.

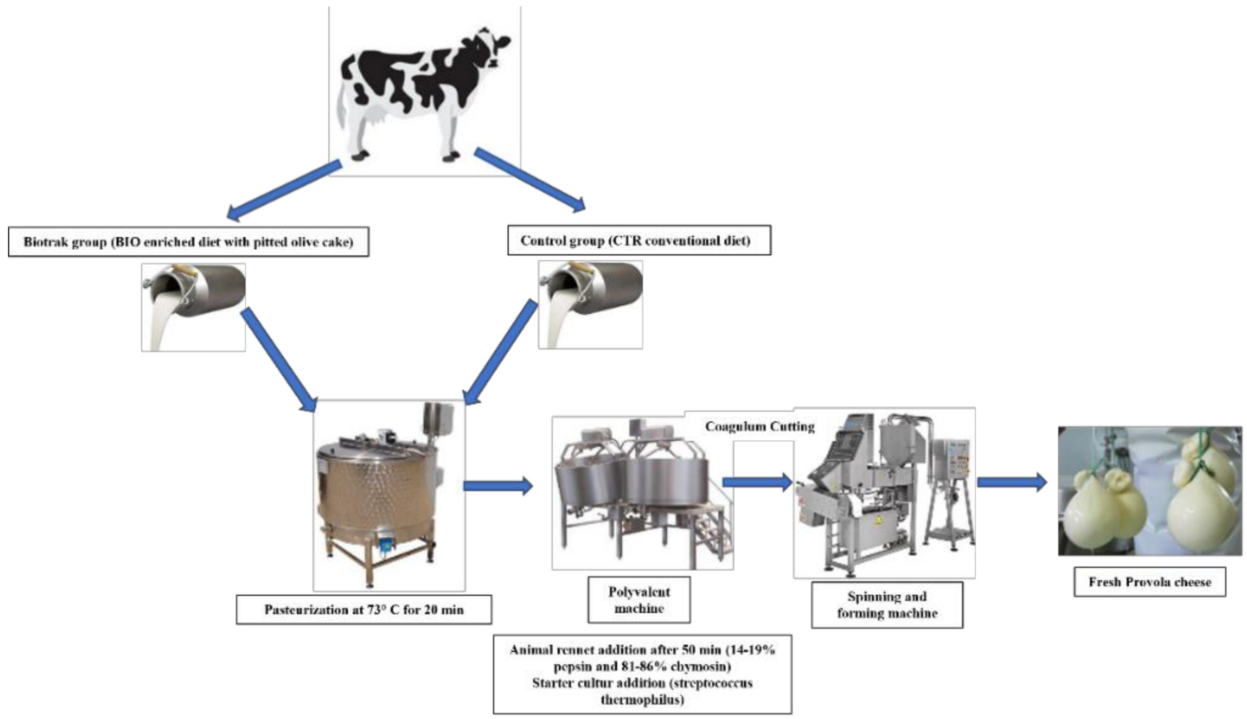

Figure 1. Production process diagram of provola samples.

5.1.2. Instrumentation and analytical conditions

The simultaneous analysis of 109 pesticides, 18 PCBs, 13 PAHs belonging to different classes and of 10 phthalate acid esters (PAEs) and 8 non-phthalate plasticizers (NPPs) were performed using a Shimadzu TQ8030 HRGC-MS/MS (Shimadzu Italia, Milan, Italy), equipped with a Shimadzu AOC-20s autosampler and a Supelco SLB-5MS capillary column (5% polydiphenylsiloxane, 95% polydimethylsiloxane) 30 m × 0.25 mm i.d., 0.25 µm film thickness, operating in electronic ionization (EI) mode. For pesticides, PCBs, PAHs the instrumental conditions were: He carrier gas at flow rate 0. 5 mL/min; Ar collision gas; column temperature: 60°C (1 min), 15°C/min up to 150°C, 10°C/min up to 270°C, 2°C/min up to the final temperature of 300°C (2 min); injector temperature: 250°C; ion source temperature (EI, 70 eV): 230°C; transfer line temperature: 290°C; injection volume was 1 µL, in splitless mode (1 min) and finally with a split ratio of 1:10.

All quantitative and qualitative data were acquired in multiple reaction monitoring (MRM) mode. All details are reported in Tables 1 and 2. For PAEs and NPPs the instrumental conditions were: He carrier gas at flow rate 0.68 mL/min; column temperature was initially varied from 60°C to 190°C, then 8°C/min up to 240°C (5 min), 8°C/min up to the final temperature of 315°C (5 min); injector temperature: 250°C; ion source temperature (EI, 70 eV): 200°C; injection volume was 1 µL, in split-less mode (1 min), and finally with a split ratio of 1:15. The assay was performed in single-ion monitoring (SIM) mode. SIM was done using three characteristic mass fragments: the first being the target ion (T) and the last two the qualifying ions (Q1 and Q2) for each target analyte. All details are reported in Table 3.

Table 1. GC-MS/MS acquisition parameters for the 140 organic pollutants under analysis.

Chemical class	Compound	Reaction 1	CE 1	Reaction 2	CE 2
Carbamates (Cs)					
	(±)-Indoxacarb	218 → 203	10	218 → 134	20
	Bendiocarb	166 → 151	10	166 → 109	20
	Carbaryl	144 → 115	20	115 → 89	20
	Carbofuran	164 → 149	15	164 → 103	20
	Carbophenothion	157 → 121	20	157 → 77	20
	Diethofencarb	267 → 225	10	267 → 168	20
	Ethiofencarb	168 → 107	10	168 → 77	25
	Furathiocarb	194 → 105	20	194 → 165	15
	Phenoxycarb	116 → 88	15	186 → 109	15
	Pirimicarb	238 → 166	10	206 → 166	15
Carbamates (Cs)/Acaricides (As)					
	Mecarbam	296 → 196	10	296 → 168	10
Fungicides (Fs)					
	Azoxystrobin	344 → 329	20	344 → 183	20
	Boscalid	342 → 140	10	342 → 112	25
	Bupirimate	208 → 165	15	108 → 140	15
	Captafol	151 → 79	20	151 → 122	10
	Captan	107 → 79	10	107 → 77	20
	Cyproconazole isomer II	222 → 125	20	224 → 127	20
	Diclobutrazol	270 → 159	10	270 → 137	25
	Fenarimol	251 → 139	20	251 → 111	25
	Fenhexamid	177 → 78	20	177 → 113	20
	Fluodioxonil	248 → 127	20	248 → 154	25
	Flusilazole	233 → 165	20	233 → 152	20
	Imazalil	215 → 173	15	215 → 145	25
	Kresoxim methyl	206 → 131	10	206 → 116	10
	Metalaxyl-M	160 → 130	20	160 → 144	20
	Mepronil	269 → 119	10	210 → 181	20
	Penconazole	248 → 157	20	248 → 192	20
	Prochloraz	180 → 138	15	180 → 69	20
	Procymidone	283 → 96	10	285 → 96	15
	Pyrimethanil	198 → 118	30	199 → 198	25
	Quintozen	237 → 143	20	237 → 119	20
	Tebuconazole	250 → 125	15	125 → 89	25
	Tolchlophos methyl	265 → 250	20	265 → 93	24
	Triadimefon	208 → 181	10	208 → 127	15

Chemical class	Compound	Reaction 1	CE 1	Reaction 2	CE 2
	Trifloxystrobin	190 → 130	15	190 → 102	25
	Vinclozolin	212 → 177	15	212 → 145	20
Herbicides (Hs)					
	Amandryn	227 → 170	10	227 → 185	10
	Atrazine	200 → 122	15	215 → 200	10
	Diflufenican	266 → 183	25	246 → 218	25
	Linuron	160 → 133	15	160 → 125	15
	Methabenzthiazuron	164 → 136	15	127 → 109	20
	Oxyfluorfen	300 → 223	20	252 → 170	25
	Propazine	214 → 172	15	214 → 94	20
	Propyzamide	173 → 145	15	173 → 109	25
	Simazine	201 → 173	7	201 → 186	8
	Terbuthilazine	214 → 104	15	214 → 132	10
	Trifluralin	264 → 160	15	264 → 206	10
Insect growth regulators (IGRs)					
	Buprofezin	175 → 132	15	175 → 117	20
	Cyromazine	151 → 109	15	165 → 123	20
	Pyriproxyfen	136 → 78	20	136 → 96	20
Organochlorine pesticides (OCPs)					
	2,4’-DDD	235 → 165	20	237 → 165	20
	2,4’-DDE	246 → 176	20	318 → 248	20
	2,4’-DDT	235 → 165	20	237 → 165	20
	4,4’-DDD	235 → 165	20	237 → 165	20
	4,4’-DDE	246 → 176	30	318 → 248	30
	4,4’-DDT	235 → 165	20	237 → 165	20
	Alachlor	188 → 160	15	161 → 146	15
	Aldrin	263 → 193	20	293 → 258	20
	cis-Chlordane	373 → 266	20	373 → 264	20
	Dicofol	250 → 139	20	250 → 215	10
	Dieldrin	263 → 193	20	263 → 228	20
	Endosulfan sulfate	272 → 237	15	274 → 239	15
	Endosulfan α	241 → 206	25	241 → 170	25
	Endosulfan β	195 → 160	10	195 → 125	20
	Endrin	263 → 193	20	281 → 245	15
	Methoxychlor	227 → 169	20	227 → 141	25
	trans-Chlordane	373 → 266	20	373 → 264	20
	α-HCH	181 → 145	10	219 → 183	10
	β-HCH	181 → 145	15	219 → 183	10
	γ-HCH	181 → 145	15	219 → 183	10
Organophosphorous pesticides (OPPs)					
	Acephate	136 → 94	10	136 → 119	8
	Andhion	231 → 175	15	231 → 129	20
	Azinphos ethyl	160 → 132	5	160 → 77	10
	Chlorpyriphos	197 → 169	15	197 → 169	15
	Chlorpyriphos methyl	286 → 93	25	286 → 271	20
	cis-Chlorfenvinphos	267 → 159	20	269 → 161	20
	Coumaphos	226 → 163	20	226 → 135	25
	Diazinon	137 → 84	15	179 → 137	20
	Dimethoate	125 → 79	20	125 → 79	8
	Fenamiphos	303 → 154	15	303 → 195	10
	Fenchlorphos	285 → 270	20	285 → 240	20
	Fenitrothion	125 → 79	15	277 → 125	18
	Fenthion	278 → 109	20	278 → 125	22
	Fenthion Sulfone	310 → 105	20	310 → 109	30
	Fenthion Sulfoxide	278 → 109	15	278 → 169	25
	Malathion	173 → 99	15	173 → 117	15
	Methidathion	145 → 85	10	145 → 58	20
	Omethoate	156 → 110	10	156 → 79	30
	Parathion methyl	263 → 109	15	263 → 246	6
	Phenthoate	274 → 125	15	274 → 121	15
	Phosalone	182 → 111	20	182 → 75	30
	Phosmet	160 → 77	25	160 → 133	15
	Phoxim	109 → 81	15	109 → 91	15

Chemical class	Compound	Reaction 1	CE 1	Reaction 2	CE 2
	Quinalphos	146 → 118	15	146 → 91	30
	trans-Chlorfenvinphos	267 → 159	20	269 → 161	20
	Triphenyl phosphate	325 → 169	20	325 → 77	25
Organophosphorous pesticides (OPPs)/Acaricides (As)					
	Carbophenothion	157 → 121	20	157 → 77	20
	Pirimiphos-methyl	290 → 125	15	290 → 151	15
Polychlorobiphenyls (PCBs)					
	PCB28	256 → 186	15	258 → 186	15
	PCB52	290 → 220	15	292 → 222	15
	PCB77	290 → 220	20	292 → 222	20
	PCB81	290 → 220	20	292 → 222	20
	PCB101	324 → 254	20	326 → 256	20
	PCB105	324 → 254	20	326 → 256	20
	PCB114	324 → 254	20	326 → 256	20
	PCB118	324 → 254	20	326 → 256	20
	PCB123	324 → 254	20	326 → 256	20
	PCB126	324 → 254	20	326 → 256	20
	PCB138	360 → 290	25	362 → 292	25
	PCB153	360 → 290	25	362 → 292	25
	PCB156	360 → 290	30	362 → 292	30
	PCB157	360 → 290	30	362 → 292	30
	PCB167	360 → 290	30	362 → 292	30
	PCB169	360 → 290	30	362 → 292	30
	PCB180	394 → 324	20	396 → 326	20
	PCB189	394 → 324	25	396 → 326	25
Polycyclic aromatic hydrocarbons (PAHs)					
	Acenaphthylene	152 → 126	30	152 → 102	30
	Anthracene	178 → 152	25	176 → 150	25
	Benzo[a]anthracene	228 → 226	30	228 → 202	20
	Benzo[a]pyrene	252 → 250	35	252 → 226	20
	Benzo[b]fluoranthene	252 → 250	35	126 → 113	10
	Benzo[g,h,i]perylene	276 → 274	45	276 → 272	50
	Benzo[k]fluoranthene	252 → 250	35	126 → 113	10
	Chrysene	228 → 226	30	228 → 202	20
	Dibenzo[a,h]anthracene	278 → 276	30	278 → 252	20
	Fluorene	166 → 165	15	165 → 164	20
	Indeno[1,2,3-cd]pyrene	276 → 274	30	137 → 136	15
	Phenanthrene	178 → 152	25	176 → 150	25
	Pyrene	202 → 200	20	202 → 152	30
Pyrethroid insecticides (PYRs)					
	cis-Fluvalinate	250 → 55	15	252 → 55	20
	cis-Permethrin	183 → 153	15	183 → 168	15
	Cypermethrin isomer I	181 → 152	20	163 → 91	15
	Cypermethrin isomer II	181 → 152	20	163 → 91	15
	Cypermethrin isomer III	181 → 152	20	163 → 91	15
	Deltamethrin	181 → 152	20	253 → 93	15
	trans-Fluvalinate	250 → 55	15	252 → 55	20
	trans-Permethrin	183 → 153	20	183 → 168	20
	Λ-Cyhalothrin	181 → 152	25	197 → 141	10
Synergists (SYNs)					
	Piperonyl butoxide	176 → 131	15	176 → 103	20

Table 2. Linearity, LOD, LOQ, for the 140 organic pollutants under analysis.

Compound	R^2	LOD (ng/g)	LOQ (ng/g)
(±)-Indoxacarb	0.995	0.14	0.51
2,4’-DDD	1.000	0.02	0.04
2,4’-DDE	0.996	0.08	0.21
2,4’-DDT	0.996	0.05	0.11
4,4’-DDD	0.998	0.05	0.15
4,4’-DDE	0.994	0.04	0.12
4,4’-DDT	0.997	0.08	0.23
Acenaphthylene	0.980	0.10	0.29
Acephate	0.990	0.15	0.56
Alachlor	0.998	0.04	0.14
Aldrin	0.992	1.14	4.21
Ametryn	0.989	0.06	0.2
Anthracene	0.987	0.13	0.42
Atrazine	0.994	0.93	3.61
Azinphos ethyl	1.000	0.03	0.09
Azoxystrobin	0.986	1.32	4.37
Bendiocarb	0.998	0.06	0.17
Benzo[a]anthracene	0.955	0.12	0.38
Benzo[a]pyrene	0.985	0.32	1.05
Benzo[b]fluoranthene	0.922	2.54	8.38
Benzo[ghi]perylene	0.972	0.42	1.31
Benzo[k]fluoranthene	0.980	2.55	8.36
Boscalid	0.989	0.26	0.86
Bupirimate	0.990	1.38	4.23
Buprofezin	0.995	2.32	8.22
Captafol	0.992	0.07	0.23
Captan	0.992	0.41	1.22
Carbaryl	0.982	0.82	2.92
Carbofuran	0.996	0.13	0.48
Carbophenthion	0.986	0.25	0.82
Chlorpyriphos	1.000	0.12	0.36
Chlorpyriphos methyl	0.999	0.15	0.47
Chrysene	0.972	0.35	1.17
cis-Chlordane	0.994	0.11	0.34
cis-Chlorfenvinphos	0.992	0.31	0.99
cis-Fluvalinate	0.998	2.45	8.24
cis-Permethrin	0.994	2.49	8.18
Coumaphos	0.996	0.12	0.4
Cypermethrin isomer I	0.999	5.09	14.5
Cypermethrin isomer II	0.984	5.51	19.3
Cypermethrin isomer III	0.992	4.85	15.8
Cyproconazole isomer II	0.978	0.32	1.29
Cyromazine	0.952	0.54	1.62
Deltamethrin	0.999	0.11	0.33
Diethofencarb	0.998	0.05	0.17
Diazinon	1.000	0.12	0.38
Dibenz[a,h]anthracene	0.961	5.11	17.08
Diclobutrazol	0.989	0.18	0.65
Dicofol	0.999	0.06	0.18
Dieldrin	0.990	0.16	0.54
Diflufenican	0.996	0.09	0.31
Dimethoate	0.962	0.52	1.62
Endosulfan sulfate	0.996	0.21	0.65
Endosulfan α	0.998	0.09	0.29
Endosulfan β	0.996	0.16	0.55
Endrin	0.998	2.33	8.23
Ethiofencarb	0.985	0.26	0.95
Ethion	0.986	0.82	2.85
Fenamiphos	0.992	0.19	0.62
Fenarimol	0.998	0.12	0.36
Fenchlorphos	0.996	0.12	0.53
Fenhexamid	0.998	0.09	0.31
Fenitrothion	0.998	0.15	0.46
Fenthion	1.000	0.07	0.23

Compound	R^2	LOD (ng/g)	LOQ (ng/g)
Fenthion Sulfone	0.972	0.09	0.29
Fenthion Sulfoxide	0.978	0.12	0.38
Fluodioxonil	0.980	0.14	0.42
Fluorene	0.985	0.04	0.14
Flusilazole	0.986	0.28	0.94
Furathiocarb	0.998	0.12	0.42
Imazalil	0.987	0.35	1.13
Indenopyrene	0.969	0.36	1.15
Kresoxim methyl	0.999	0.29	0.91
Linuron	0.996	1.68	6.04
Malathion	0.984	0.76	2.64
Metalaxyl-M	0.999	0.08	0.27
Methabenzthiazuron	0.984	0.29	0.92
Methidathion	0.992	0.09	0.28
Methoxychlor	0.999	0.11	0.35
Mecarbam	0.986	0.09	0.29
Mepronil	0.998	0.07	0.21
Omethoate	0.990	0.07	0.23
Oxyfluorfen	0.998	0.19	0.58
Parathion methyl	0.970	0.69	2.55
PCB28	0.995	0.04	0.13
PCB52	0.992	0.09	0.28
PCB77	1.000	0.07	0.20
PCB81	0.994	0.12	0.35
PCB101	0.999	0.06	0.16
PCB105	1.000	0.11	0.30
PCB114	0.997	0.04	0.10
PCB118	1.000	0.04	0.10
PCB123	0.998	0.10	0.29
PCB126	0.999	0.07	0.18
PCB138	1.000	0.11	0.29
PCB153	0.997	0.13	0.30
PCB156	1.000	0.11	0.37
PCB157	0.998	0.14	0.46
PCB167	0.999	0.12	0.38
PCB169	0.999	0.09	0.30
PCB180	1.000	0.08	0.22
PCB189	1.000	0.08	0.24
Penconazole	0.996	0.06	0.22
Phenanthrene	0.989	0.12	0.35
Phenoxycarb	0.996	0.14	0.49
Phenthoate	0.999	0.09	0.32
Phosalone	0.998	0.08	0.24
Phosmet	0.982	0.34	1.15
Phoxim	0.982	0.13	0.46
Piperonyl butoxide	1.000	0.24	0.92
Pirimicarb	1.000	0.06	0.19
Pirimiphos-methyl	0.978	0.74	2.75
Prochloraz	0.996	0.33	1.13
Procymidone	0.996	0.14	0.34
Propazine	0.992	0.15	0.52
Propyzamide	0.998	0.04	0.14
Pyrene	0.990	0.11	0.24
Pyrimethanil	0.990	0.13	0.38
Pyriproxyfen	0.994	0.12	0.41
Quinalphos	0.996	0.09	0.28
Quintozen	0.990	0.28	0.86
Simazine	1.000	0.1	0.31
Tebuconazole	0.994	0.17	0.48
Terbuthilazine	0.990	0.1	0.34
Tolchlophos methyl	1.000	0.11	0.32
trans-Chlordane	0.996	0.12	0.42
trans-Chlorfenvinphos	0.997	0.09	0.38
trans-Fluvalinate	0.999	2.42	8.02
trans-Permandhrin	0.996	2.38	8.21
Triadimefon	0.990	0.39	1.18
Trifloxystrobin	0.994	0.52	1.76

Compound	R^2	LOD (ng/g)	LOQ (ng/g)
Trifloxystrobin	0.992	0.08	0.26
Trifluralin	1.000	0.1	0.3
Triphenyl phosphate	0.999	0.05	0.14
Vinclozolin	0.999	0.1	0.33
α-HCH	0.994	0.1	0.32
β-HCH	0.986	0.09	0.29
γ-HCH	0.988	0.11	0.37
Λ-Cyhalothrin	1.000	0.06	0.18

Table 3. List of the investigated plasticizers. tr: Retention Time; T: target ion; Q1 e Q2: qualifying ions; linearity (**r^2**), LOD (mg/kg), LOQ (mg/kg).

Compound	Abbreviation	t_r(min)	T, Q1, Q2 (m/z)	r^2	LOD	LOQ
dimethyl phthalate	DMP	13.7	163, 92,164	0.9954	0.007	0.023
diethyl phthalate	DEP	15.8	149, 177, 176	0.9923	0.005	0.017
dipropyl phthalate	DPrP	18.8	149, 150, 209	0.9939	0.007	0.020
dibutyl phthalate	DBP	23.3	149, 150, 223	0.9948	0.007	0.023
diisobutyl phthalate	DiBP	20.9	149, 150, 223	0.9921	0.007	0.023
butyl benzyl phthalate	BBP	30.2	149, 91, 206	0.9883	0.037	0.121
diphenyl phthalate	DPhP	34.4	225, 226, 104	0.9945	0.015	0.051
dicyclohexyl phthalate	DcHexP	33.8	149, 167, 150	0.9985	0.027	0.087
diheptyl phthalate	DHepP	31.9	149, 99, 265	0.9954	0.177	0.553
di(2-ethylhexyl) phthalate	DEHP	34.1	149, 167, 279	0.9988	0.007	0.020
dimethyl adipate	DMA	10.1	114, 101, 111	0.9965	0.010	0.030
diethyl adipate	DEA	12.5	111, 157, 128	0.9943	0.013	0.037
benzyl benzoate	BB	19.0	105, 91, 212	0.9899	0.012	0.033
dibutyl adipate	DBA	18.6	129, 185, 111	0. 9853	0.023	0.068
diisobutyl adipate	DiBA	17.1	129, 185, 111	0.9955	0.008	0.027
di(2-ethylhexyl) adipate	DEHA	30.9	129, 112, 147	0.9865	0.013	0.037
di(2-ethylhexyl) terephthalate	DEHT	37.5	149, 112, 261	0.9883	0.070	0.233
di(2-ethylhexyl) sebacate	DEHS	38.2	185, 149, 112	0.9933	0.018	0.053

The determination of 9 bisphenols (BPs) were performing using HPLC system (Shimatzu, Kyoto, Japan), consisting of an LC-20ADXR binary pump, a SIL-20AXR autosampler, and temperature-controlled column operator. The detector was a LCMS-8040 triple quadrupole mass spectrometer with Electrospray ionisation (ESI) source. LabSolutions software was used for data control and analysis. Chromatographic separation was performed on an Agilent Zorbax SB-C18 column (5microm 4.6 x 250 mm). The flow rate was 0.7 mL/min. Mobile phases A and B were ultrapure water for HPLC-MS and acetonitrile, respectively. The following linear gradient was used: 0 min, 20%B; 7 min, 40%B; 25 min, 90%B; 35min, 20%B. The injection volume was 20 µL and the column temperature was set at 40 °C.

The MS was operated in negative ESI mode under the following specific conditions: nebulizer gas flow 3.0 L/min, nebulizer gas pressure 770 KPa, drying gas flow 15.0 L/min, DL temperature 250 ºC, CID gas 230 KPa. The dwell time was set to 500 ms.

All quantitative and qualitative data in this study were acquired in multiple reaction monitoring mode (MRM). All details are reported in Table 4.

Table 4. List of the investigated bisphenols with Retention Time and Monitored ions (m/z), linearity, LOD (µg/kg), LOQ (µg/kg).

Compound	Monitored ions (*m/z*)	r^2	LOD	LOQ
4,4′-Sulfonyldiphenol (BPS)	107.9, 92.0, 156.0	0.9993	0.30	1.0
4,4′-Methylenediphenol (BPF)	93.1, 105.1	0.9911	0.45	1.5
1,1-Bis(4-idrossifenil) etan (BPE)	198.0, 194.9, 176.9	0.9931	0.30	1.0
4,4'-(propan-2,2-diil) difenol (BPA)	212.1, 133.0, 211.1	0.9907	0.45	1.5
4- [2- (4-idrossifenil) butan-2-il] fenol (BPB)	212.0, 211.0	0.9944	0.30	1.0
2,2-Bis(4-idrossifenil) esafluoropropan (BPAF)	265.0, 177.0, 69.0	0.9995	0.30	1.0
1,1-Bis(4-idrossifenil) -1-fenil-etan (BPAP)	274.1, 273.1, 211.0	0.9954	0.45	1.5
1,1-Bis(4-idrossifenil)-cicloesan (BPZ)	145.0, 173.1, 222.9	0.9984	0.45	1.5
1,4-Bis(2-(4-idrossifenil) -2-propil) benzene (BPP)	330.1, 133.1, 314.9	0.9992	0.45	1.5

Underlined ions were considered for quantitative analysis.

5.1.3. Extraction of contaminants from cheese samples

The extraction of the different classes of contaminants was conducted using different QuEChERS methods according to the matrix type. The QuEChERS method is based on dispersive solid phase extraction (d-SPE), QuEChERS being short for 'Quick, Easy, Cheap, Effective, Rugged and Safe'. Briefly, the procedure followed consisted of a first step (extraction) in which the homogenized sample was extracted with acetonitrile (ACN), followed by the addition of salts such as anhydrous magnesium sulphate ($MgSO_4$), sodium chloride (NaCl) and sodium acetate (NaOAc). In a second step (clean-up), an aliquot of the upper layer was cleaned with primary and secondary amine (PSA), $MgSO_4$ and C_{18}. Tables 5 and 6 show the details of analytical methods.

Table 5. Analytical methods for PAEs, NPPs and BPs.

Matrix	Analytes	Extraction: Solvent and salts	Extraction: Clean-up	Instrument	Reference
Provola cheese	10 PAEs 8 NPPs	5 g of homogenized sample with 80.0 µL of the IS (20 µg/mL) was extracted with 10.0 mL of ACN 3 g NaCl was added and centrifuged for 5 min.	2.0 mL of the top layer of ACN was transferred into QuEChERS d-SPE (300 mg $MgSO_4$, 50 mg PSA, 50 mg C18) and shaken vigorously for 5 min, then centrifuged for 5 min 1.0 mL of the upper layer was dried with N_2 flow at 40°C, the residual was dissolved in 1.0 mL n-hexane.	GC-MS/MS	16
Provola cheese	9 BPs	5 g of homogenized sample spiked with IS* was extracted with 5.0 mL of ACN 2 g NaCl and 1 g $MgSO_4$ was added, shaken for 5 min and centrifuged for 5 min.	2.0 mL of the top layer of ACN was transferred into QuEChERS d-SPE (0.25 mg $MgSO_4$, 0.1 mg PSA, 0.1 mg C18), vortexed for 30s, and centrifuged for 5 min 1.0 mL of the upper layer was filtered through a PTFE Millipore filter (0.22 µm).	HPLC-MS/MS	17
Olive cake	10 PAEs 8 NPPs	3 g of dried olive cake with 80.0 µL of the IS (20 µg/mL) was extracted with 7ml of H_2O and 10 ml of ACN 4g $MgSO_4$ and 1g NaCl were added and vortexed for 1 min; the extract was centrifuged for 10 min.	5.0 mL of the top layer of ACN was transferred into QuEChERS d-SPE (750 mg $MgSO_4$, 250 mg PSA and 250 mg C18), shaken vigorously for 5 min, and centrifuged. 1.0 mL of supernatant was recovered, dry-carried and reconstituted with 1 ml of n-hexane.	GC-MS/MS	18
Olive cake	9 BPs	3 g of homogenized sample spiked with IS* was extracted with 5.0 mL of ACN 2 g NaCl and 1 g $MgSO_4$ was added, shaken for 5 min and centrifuged for 5 min.	2.0 mL of the top layer of ACN was transferred into QuEChERS d-SPE (0.25 mg $MgSO_4$, 0.1 mg PSA, 0.1 mg C18), vortexed for 30s, and centrifuged for 5 min 1.0 mL of the upper layer was filtered through a PTFE Millipore filter (0.22 µm).	HPLC-MS/MS	17

PAE, phthalate acid esters; NPP, non-phthalate plasticizers; BP, bisphenols; IS: dibutylphthalate-d4 (DBP-d4) and bis(2-ethylhexyl) phthalate-d4 (DEHP-d4) in nonane; IS*: $_{13}C^{12}$-BPA; HPLC-MS/MS, high performance liquid chromatography- tandem mass spectrometry; GC-MS/MS, Gas chromatography-tandem mass spectrometry.

Table 6. Analytical methods for Pesticides, PCBs and PAHs.

Matrix	Analytes	Extraction		Instrumental	Reference
		Solvent and salts	Clean-up		
Provola cheese	106 Pesticides 18 PCBs 13 PAHs	15 g of homogenized sample with bromophos-methyl (50 ng/mL) was extracted with 20 mL ACN for 5 min. 6 g $MgSO_4$ and 1.5 g NaOAc were added and vortexed for 1 min; the extract was centrifuged for 15 min.	5 mL of the top layer of ACN was transferred into QuEChERS d-SPE (1200 mg $MgSO_4$, 400 mg PSA, 400 mg C18) and shaken vigorously for 5 min, then centrifuged for 5 min 1 mL of supernatant was recovered, brought to dryness and reconstituted with 1ml of n-hexane.	GC-MS/MS	19
Olive cake	106 Pesticides 18 PCBs 13 PAHs	3 g of dried olive cake with bromophos-methyl (50 ng/mL) was extracted with 7 ml of H_2O and 10 ml of ACN. 4 g $MgSO_4$ and 1g NaCl were added and vortexed for 1 min; the extract was centrifuged for 10 min.	5 mL of the top layer of ACN was transferred into QuEChERS d-SPE (750mg $MgSO_4$, 250mg PSA and 250mg C18), shaken vigorously for 5 min, and centrifuged. 1 mL of supernatant was recovered, brought to dryness and reconstituted with 1ml of n-hexane.	GC-MS/MS	20

PCB, polychlorinated biphenyls; PAH, polycyclic aromatic hydrocarbons; GC-MS/MS, Gas chromatography-tandem mass spectrometry.

5.1.4. Statistical Analysis

The statistical analyses were carried out using JMP® 16 software (SAS Institute Inc., Cary, NC 1989-2021). For each parameter, descriptive statistics was generated and reported as mean ± standard deviation (SD). A two-way analysis of variance (ANOVA) with interaction was used applied to all the measured parameters and the Tukey-Kramer post-hoc test was used to identify the different levels when the model factors resulted significative; the included factors are reported below:

$$Yijk = m + Di + Mj + (DM)ij + eijk$$

Where m is the mean, Di is the diet (CTR vs BIO), Mj is the month in which the cheese was produced (from March 2021 to July 2021), (DM)ij is the interaction between the diet and the month, and eijk is the random residual. A principal component analysis (PCA) was performed using all the measured contaminants.

5.1.5. Results

The concentrations of bisphenols and plasticizers detected in Provolas samples, together with the significance of each factor for each parameter analysed are shown in Table 7. Bisphenols BPF, BPE, BPAP, BPZ, BPP, BPB, BPA and plasticizers BBP, DPhP, DeHexP, DHepP, DEA, BB, DBA, and DiBA were not detected in any samples. In Provola samples, the concentrations of BPAF and BPS varied from 2.06 to 2.84 μg/Kg and from 1.69 to 2.52 μg/Kg, respectively. Significant differences are observed between BIO and CTR for the months of March and June. In July only, significant differences are observed for BIO Provolas. While a significant increase is observed for CTR from March to May and from May onward, however, the differences are not significant. Significant seasonal variability is observed for both BIO and CTR in the phthalates detected. Significantly lower phthalate values were registered in April and June. No significant differences were observed

between BIO and CTR in March and July. Significant differences by sampling period were always shown for DMA and DEHA in Provolas, with significantly lower values in April and May. Furthermore, no significant differences were shown for DMA and DEHA among BIO and CTR in months. DEHT was found only in the Provolas with values in the range of 51.43 μg/Kg to 124.08 μg/Kg. In July, DEHT was significantly higher for both BIO and CTR and the concentrations were significantly different from each other.

Table 7. Bisphenols and plasticizers (µg/Kg) in Provola samples.

		BPAF	BPS	DMP	DEP	DPrP	DiBP	DBP	DEHP	DMA	DEHA	DEHT
CTR												
	March	2.06 ± 0.07^{h}	1.69 ± 0.05^{c}	4.26 ± 0.02^{d}	6.07 ± 0.05^{b}	3.22 ± 0.07^{ab}	16.08 ± 0.03^{c}	6.10 ± 0.01^{f}	21.05 ± 0.04^{a}	4.60 ± 0.07^{h}	98.26 ± 0.03^{b}	51.45 ± 0.13^{g}
	April	2.24 ± 0.02^{g}	2.20 ± 0.07^{ab}	4.04 ± 0.02^{h}	5.38 ± 0.02^{d}	2.67 ± 0.02^{f}	15.08 ± 0.01^{d}	5.76 ± 0.02^{g}	19.04 ± 0.02^{e}	5.17 ± 0.02^{d}	50.29 ± 0.05^{g}	60.07 ± 0.03^{c}
	May	2.84 ± 0.02^{a}	2.19 ± 0.61^{ab}	4.20 ± 0.02^{e}	5.23 ± 0.01^{de}	3.31 ± 0.01^{a}	16.02 ± 0.03^{c}	6.22 ± 0.02^{f}	20.21 ± 0.02^{b}	4.89 ± 0.01^{f}	46.50 ± 0.99^{h}	52.30 ± 0.03^{f}
	June	2.72 ± 0.12^{abc}	2.46 ± 0.04^{ab}	4.33 ± 0.02^{c}	7.02 ± 0.02^{a}	3.01 ± 0.02^{cd}	12.19 ± 0.02^{e}	6.53 ± 0.03^{c}	20.30 ± 0.04^{b}	7.03 ± 0.04^{b}	90.28 ± 0.02^{d}	54.10 ± 0.01^{d}
	July	2.80 ± 0.02^{ab}	2.52 ± 0.08^{a}	5.02 ± 0.01^{b}	7.11 ± 0.03^{a}	2.85 ± 0.04^{e}	17.41 ± 0.2^{a}	9.05 ± 0.01^{a}	14.20 ± 0.02^{g}	4.75 ± 0.02^{g}	95.06 ± 0.04^{c}	124.08 ± 0.07^{a}
BIO												
	March	2.32 ± 0.01^{fg}	2.03 ± 0.03^{bc}	4.26 ± 0.01^{d}	6.10 ± 0.01^{b}	3.16 ± 0.16^{b}	16.10 ± 0.04^{c}	6.23 ± 0.02^{e}	21.10 ± 0.07^{a}	4.58 ± 0.03^{h}	99.06 ± 0.05^{a}	51.43 ± 0.10^{g}
	April	2.54 ± 0.07^{de}	2.05 ± 0.04^{abc}	4.17 ± 0.01^{ef}	5.72 ± 0.21^{c}	2.60 ± 0.06^{f}	15.12 ± 0.03^{d}	5.82 ± 0.09^{g}	19.16 ± 0.06^{e}	5.25 ± 0.03^{c}	51.21 ± 0.04^{f}	59.96 ± 0.06^{c}
	May	2.59 ± 0.03^{cd}	2.00 ± 0.01^{bc}	4.11 ± 0.02^{g}	5.17 ± 0.02^{e}	3.17 ± 0.01^{b}	16.07 ± 0.06^{c}	6.16 ± 0.01^{ef}	20.05 ± 0.10^{c}	4.98 ± 0.02^{e}	45.19 ± 0.03^{i}	53.00 ± 0.02^{e}
	June	2.41 ± 0.03^{ef}	2.17 ± 0.05^{abc}	4.13 ± 0.03^{fg}	7.03 ± 0.02^{a}	3.06 ± 0.02^{c}	12.09 ± 0.05^{f}	6.41 ± 0.01^{d}	19.86 ± 0.05^{d}	7.12 ± 0.02^{a}	89.79 ± 0.16^{e}	54.08 ± 0.02^{d}
	July	2.66 ± 0.02^{bcd}	2.44 ± 0.02^{ab}	5.11 ± 0.01^{a}	7.06 ± 0.01^{a}	2.93 ± 0.02^{de}	16.61 ± 0.01^{b}	8.96 ± 0.04^{b}	15.19 ± 0.07^{f}	4.30 ± 0.02^{i}	95.10 ± 0.02^{c}	119.70 ± 0.07^{b}
p-value												
	Diet	0.2093	0.2666	**0.0371 I**	**0.0231 II**	**0.0191 I**	**< 0.0001 I**	0.1379	**< 0.0001 II**	**0.0006 I**	0.87	**< 0.0001 I**
	Month	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
	Diet x Month	**< 0.0001**	**0.0366**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**

Different letters represent significantly different mean values ($p < 0.05$). Next to the significant p-values for the diet is a II if the values are greater for the biotrak (BIO) diet and I if they are greater in the conventional diet (CTR).

The concentrations of pesticides and PAHs detected in Provolas together with the significance of each factor for each parameter analysed are shown in Table 8. PCBs values in all Provola samples were below the limit of quantification (LOQ). In Provolas were detected anthracene and fluorene with concentrations in the ranges of 0.39-0.45 μg/Kg and 0.26-0.31 μg/Kg, respectively. No significant difference was observed for CTR in months, while for BIO higher values were observed in April and lower values in June. Among the pesticides sought, only eight residues were determined (Carbaryl, t-Fluvalinate, Pyriproxyfen, Dieldrin, Phosalone, Clorpirifos, Tebuconazole, and Fenchlorfos). Carbaryl and t-Fluvalinate had the highest concentrations. No differences were observed between the CTR and BIO samples.

Table 8. PAHs and pesticides content (µg /kg) in Provola samples.

		Antracene	Fluorene	Carbaryl	t-Fluvalinate	Pyriproxyfen	Dieldrin	Phosalone	Clorpirifos	Tebuconazole	Fenchlorfos
CTR											
	March	0.43±0.02	0.30±0.01^{ab}	10.32±0.11^{ab}	5.19±0.02^{bcde}	3.43±0.03^{e}	0.52±0.01^{a}	0.34±0.04^{a}	0.31±0.01^{a}	0.12±0.01^{abc}	0.02±0.01^{d}
	April	0.42±0.02	0.29±0.01^{ab}	10.40±0.01^{ab}	5.29±0.08^{abc}	3.95±0.10^{d}	0.43±0.07^{b}	0.36±0.10^{a}	0.29±0.07^{a}	0.10±0.01^{bc}	0.02±0.01^{d}
	May	0.44±0.06	0.28±0.02^{abc}	10.35±0.06^{ab}	5.40±0.10^{a}	3.75±0.32^{de}	0.47±0.04^{ab}	0.41±0.08^{a}	0.30±0.02^{a}	0.13±0.01^{ab}	0.03±0.01^{cd}
	June	0.42±0.03	0.29±0.01^{abc}	10.24±0.05^{ab}	5.29±0.09^{abcd}	4.06±0.06^{cd}	0.46±0.05^{ab}	0.34±0.05^{a}	0.22±0.02^{cd}	0.12±0.01^{abc}	0.04±0.01^{cd}
	July	0.41±0.01	0.29±0.01^{abc}	10.28±0.03^{ab}	5.15±0.07^{de}	4.14±0.05^{bcd}	0.50±0.02^{ab}	0.34±0.06^{a}	0.20±0.02^{d}	0.09±0.02^{c}	0.05±0.01^{abc}
BIO											
	March	0.41±0.01	0.30±0.01^{ab}	10.66±0.46^{a}	5.33±0.02^{ab}	4.67±0.20^{a}	0.27±0.01^{c}	0.42±0.01^{a}	0.23±0.03^{c}	0.12±0.01^{abc}	0.07±0.01^{a}
	April	0.45±0.04	0.31±0.02^{a}	10.29±0.01^{ab}	5.14±0.04^{e}	4.57±0.14^{ab}	0.29±0.02^{c}	0.40±0.01^{a}	0.26±0.01^{bc}	0.13±0.02^{a}	0.04±0.02^{bc}
	May	0.43±0.01	0.28±0.01^{bc}	10.28±0.05^{ab}	5.10±0.05^{e}	4.40±0.40^{abc}	0.52±0.02^{a}	0.33±0.02^{a}	0.27±0.01^{b}	0.11±0.01^{abc}	0.06±0.01^{ab}
	June	0.39±0.01	0.26±0.01^{c}	9.90±0.48^{b}	5.07±0.02^{e}	4.49±0.04^{abc}	0.51±0.01^{a}	0.33±0.01^{a}	0.21±0.01^{cd}	0.10±0.01^{c}	0.07±0.01^{a}
	July	0.40±0.01	0.29±0.02^{ab}	10.30±0.01^{ab}	5.14±0.05^{de}	4.59±0.07^{a}	0.48±0.04^{ab}	0.31±0.01^{a}	0.20±0.01^{d}	0.10±0.01^{abc}	0.07±0.01^{a}
p-value											
	Diet	0.6252	0.5969	0.6823	**< 0.0001 I**	**< 0.0001 II**	**< 0.0001 I**	0.8715	**0.001 I**	1	**< 0.0001 II**
	Month	0.0605	**0.0001**	**0.0107**	**0.0033**	**0.0064**	**< 0.0001**	0.0647	**< 0.0001**	**0.013**	**< 0.0001**
	Diet x Month	0.2138	**0.0175**	**0.0499**	**< 0.0001**	**0.0008**	**< 0.0001**	**0.0322**	0.0571	**0.0006**	**0.0459**

Different letters represent significantly different mean values (p < 0.05). Next to the significant p-values for the diet is a II if the values are greater for the biotrak (BIO) diet and I if they are greater in the conventional diet (CTR).

Monthly, from the stored olive cake a sample was taken and subjected to analysis. The results were not significantly different over the months. The obtained average concentrations for each contaminant found in olive cake are shown in Table 9.

BPA, BPZ, BPB and BPS in olive cake samples were determined. Except for BPS, which content is ubiquitous in all samples, the other bisphenols were not detected in Provolas. In olive cake, BPB was the most abundant, with a concentration of 20.99±0.04 µg/Kg, followed by BPA 14.50±0.36 µg/Kg, and BPZ 13.12±0.05 µg/Kg. All plasticizers found in Provola were found in olive cake, except DEP, DiBP, and DPrP.

Pesticide residues determined in the olive cake were different from those in Provolas, except for carbaryl and tebuconazole residues, that were also determined in the Provolas. In olive cake, of all the PAHs analyzed, only anthracene and fluorene residues were found, as well as in Provolas.

Table 9. Bisphenols, plasticizers, PAHs and pesticides average content (µg /kg) in olive cake(n=5).

		Value			**Value**
Bisphenols			Pesticides		
	BPA	14.50±0.36		Carbaryl	0.50±0.04
	BPZ	13.12±0.05		Tebuconazole	0.33±0.02
	BPB	20.99±0.04		Carbophenothion	3.12±0.04
	BPS	3.27±0.15		Azinphos-ethyl	0.75±0.03
Plasticizers				Dicofol	0.23±0.02
	DMP	9.20±0.01		Fenarimol	0.62±0.02
	DBP	11.12±0.03		Furathiocarb	5.43±0.14
	DEHP	12.91±0.42		Acenaphthylene	0.49±0.02
	DMA	7.64±0.05		Phosmet	0.67±0.02
	DEHA	45.52±0.10		Omethoate	20.84±0.31
	DEHT	15.38±0.03		Endosulfan	0.95±0.07
PAHs				Quinalphos	0.93±0.03
	Anthracene	0.66±0.02			
	Fluorene	0.92±0.02			

5.1.6. Results of PCA analysis

According to Kaiser Criterion, only those PCs with eigenvalues greater than unity were retained. The six extracted principal component (eigenvalues 6.7212, 3.4867, 2.3888, 1.8118, 1.5805 and 1.4958, respectively) explains up to 83.260% of the total variance (32.006%, 16.603%, 11.375%, 8.628%, 7.526% and 7.123%, respectively). The first component shows the highest positive correlations with DBP (0.36), DEHT (0.35), DMP (0.34) and DEP (0.30), while negative correlations can be observed for DEHP (-0.34) and Chlorpyrifos (-0.29); DiBP and to a lesser extent fluorene and carbaryl have positive correlation with the second component (0.47, 0.33 and 0.32, respectively) while DMA had negative correlation (-0.48). In the PCA, samples tended to group according to the month factor (Figure 2). In particular, the first principal component, which explained 32% of the overall variability, clearly distinguished the Provolas produced in June and July. In particular, the July samples are characterized by higher values of DBP, DEHT, dimethyl phthalate (DMP), for both BIO and CTR. Whereas the June samples are characterized by significantly higher values of DMA and lower val-ues of DiBP. As expected, the diets were not clearly distinguished; however, BIO samples were plotted at PC1 value more positive than the CTR samples of the same month in most of the cases. The greatest difference between the two diets was seen in June and May; in May, we could also observe a greater variability with respect to the other months.

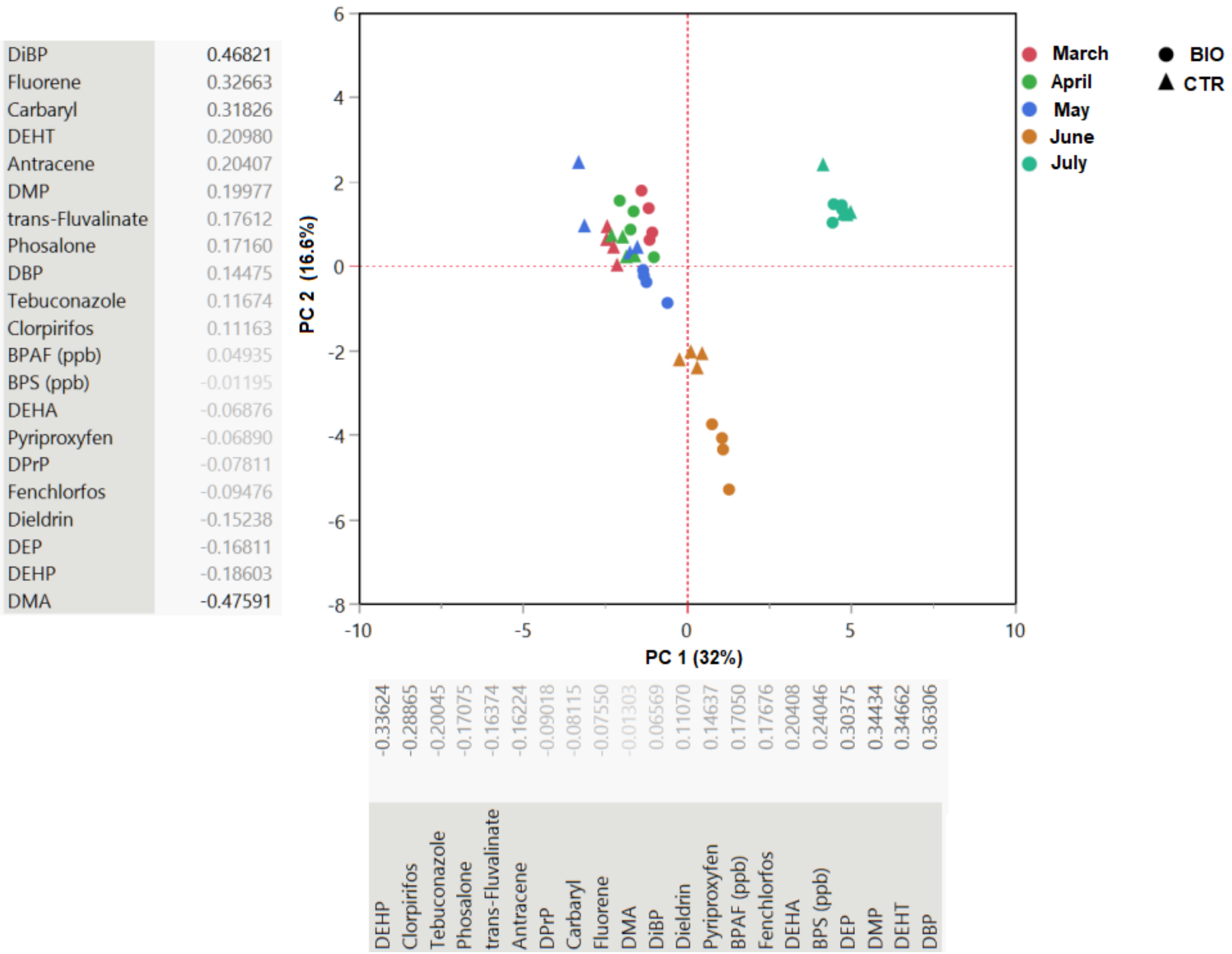

Figure 2. Scores plot for the first two principal components. Each month is represented by a different color; BIO and CTR samples are represented by circles and triangles, respectively.

5.1.7. Discussion of results

Information on contamination levels of BPAF and BPS analogues in dairy products is still limited. Furthermore, with the exception of BPS, all bisphenols found in olive cake are not present in Provola, suggesting that the contribution of bisphenols in Provola is not dependent on dietary supplementation of olive cake in the animal's diet. Bisphenols present in OC were probably metabolized and excreted by the animal through urine in accordance with Sonker et. al[21].

The levels of ubiquitous contamination of Provola cheese suggest that BPAF and BPS could result from contact with the plastic material in the machinery during the production process, and heat treatments during milk processing could have promoted the leaching of BPs into the cheese[22,23]. Furthermore, another likely source could be environmental contamination during the farming phase[24].

PAEs and NPPs in Provolas and olive cake were determined. In Provola samples from April and June lower phthalate values were found, probably due to seasonal variations. These results are in accordance with a previous study[25] in which PAEs levels appeared to vary seasonally. The same trend was observed for DMA and DEHA. In accordance with previous studies, there are several sources of possible migration of phthalates and adipates during the entire process; it is known, for example, that, heat accelerates the migration of phthalates[17,18], thus facilitated by pasteurization, or through contact with PVC tubes or plastic seals in the machinery used during processing.

Higher values of DEHT were found in Provolas, in July for both BIO and CTR, which is probably due to the high seasonal temperature[17]. Furthermore, some studies show that DEHT, due to its fewer hazardous properties, is used as a substitute for DEHP in the machinery used in dairy production. This justifies the presence of DEHT in Provola as process contamination. The results showed that BIO samples had similar or lower levels for almost all the contaminants.

5.1.8. Dietary exposure to plasticizers and bisphenols through Provola cheese

The presence of bisphenols and plasticizer such as DEP, DBP, DEHP and DEHA in the analyzed samples necessitates the assessment of their dietary exposure. The present study is the first to investigate human exposure to bisphenols and plasticizers through the consumption of Provola cheese. Based on the results obtained from the Provola samples, the estimated dietary intake (EDI) of these compounds was calculated using the following equation[26]:

$$EDI = C \cdot D/BW$$

where C (µg/kg) is the average measured concentration of the analytes in the provola samples. D (Kg/day) is the daily consumption dose (0.045 kg/day) recommended by Food and Agriculture Organization of the United Nations (FAOSTAT) and BW (kg) is the average adult body weight (60 kg). For the health risk assessment, the hazard quotient (HQ) for each plasticizer detected was calculated using the following equation:

$$HQ = EDI/TDI$$

HQ values of less than 1 are considered safe while values higher than 1 indicate that there is a possibility of inducing adverse health effects in the consumer[27].

EFSA in 2005 established a TDI of 0.01 mg/kg bw/day and 0.05 mg/kg bw/day for DBP and DEHP, respectively. The TDI for DEP was set in 2003 by the World Health Organization (WHO), which proposed a TDI of 5.00 mg/kg bw/ day. The Scientific Committee on Food has confirmed for DEHA a TDI of 0.30 mg/kg bw/day in 2000. In 2019, EFSA set a group-TDI of 50 µg/kg bw/day for DBP, BBP, DEHP, and DiNP.

Due to the unavailability of TDI values for bisphenols analogues, it was not possible to calculate the hazard quotient for analogues.

The main results of EDI and HQ calculation are reported in table 10. In this study, the average EDI in the CTR samples was $1.9 \times 10^{-03} \pm 2.7 \times 10^{-04}$ µg/kgbw/day and $1.7 \times 10^{-03} \pm 2.4 \times 10^{-04}$ µg/kg

bw/day for BPAF and BPS, respectively. While in BIO samples it was 1.9 x 10^{-03} ± 1.0 x 10^{-04} µg/kg bw/day and 1.6 x 10^{-03} ± 1.4 x 10^{-04} µg/kgbw/day for BPAF and BPS, respectively. These results indicate that the estimated dietary intake is similar in the two diets.

For DEP, DBP, DEHA and DEHP both EDI and HQ can be calculated. HQ values are well below 1 for all determined plasticizers, suggesting that there is not hazard for consumer linked with these contaminants in analyzed Provola samples.

Table 10. Estimated Daily Intake (mean ± standard deviation, µg/kgbw/day), Hazard Index and Tolerable Daily Intake (µg/kgbw/day) for plasticizers and bisphenols from Provola samples.

	EDI		HQ		TDI
	CTR	**BIO**	**CTR**	**BIO**	
Plasticizers					
DMA	$4.0 \times 10^{-03} \pm 7.5 \times 10^{-04}$	$3.9 \times 10^{-03} \pm 8.3 \times 10^{-04}$	-	-	n.a.
DMP	$3.3 \times 10^{-03} \pm 2.9 \times 10^{-04}$	$3.3 \times 10^{-03} \pm 3.2 \times 10^{-04}$	-	-	n.a.
DEP	$4.6 \times 10^{-03} \pm 6.6 \times 10^{-04}$	$4.7 \times 10^{-03} \pm 6.2 \times 10^{-04}$	$9.2 \times 10^{-07} \pm 1.3 \times 10^{-07}$	$9.3 \times 10^{-07} \pm 1.2 \times 10^{-07}$	5000
DPrP	$2.3 \times 10^{-03} \pm 2.0 \times 10^{-04}$	$2.2 \times 10^{-03} \pm 1.8 \times 10^{-04}$	-	-	n.a.
DiBP	$1.2 \times 10^{-02} \pm 1.5 \times 10^{-03}$	$1.1 \times 10^{-02} \pm 1.4 \times 10^{-03}$	-	-	n.a.
DBP	$5.0 \times 10^{-03} \pm 9.9 \times 10^{-04}$	$5.0 \times 10^{-03} \pm 9.5 \times 10^{-04}$	$5.0 \times 10^{-04} \pm 9.9 \times 10^{-05}$	$5.0 \times 10^{-04} \pm 9.5 \times 10^{-05}$	10
DEHA	$5.7 \times 10^{-02} \pm 1.9 \times 10^{-02}$	$5.7 \times 10^{-02} \pm 1.9 \times 10^{-02}$	$1.9 \times 10^{-04} \pm 6.4 \times 10^{-05}$	$1.9 \times 10^{-04} \pm 6.4 \times 10^{-05}$	300
DEHP	$1.4 \times 10^{-02} \pm 2.1 \times 10^{-03}$	$1.4 \times 10^{-02} \pm 1.7 \times 10^{-03}$	$2.8 \times 10^{-04} \pm 4.1 \times 10^{-05}$	$2.9 \times 10^{-04} \pm 3.4 \times 10^{-05}$	50
DEHT	$5.1 \times 10^{-02} \pm 2.3 \times 10^{-02}$	$5.0 \times 10^{-02} \pm 2.1 \times 10^{-02}$	-	-	n.a.
∑DBP and DEHP	$1.9 \times 10^{-02} \pm 1.8 \times 10^{-03}$	$1.9 \times 10^{-02} \pm 1.5 \times 10^{-03}$	$3.8 \times 10^{-04} \pm 3.7 \times 10^{-05}$	$3.8 \times 10^{-04} \pm 3.0 \times 10^{-05}$	50
Bisphenols					
BPAF	$1.9 \times 10^{-03} \pm 2.7 \times 10^{-04}$	$1.9 \times 10^{-03} \pm 1.0 \times 10^{-04}$	-	-	n.a.
BPS	$1.7 \times 10^{-03} \pm 2.4 \times 10^{-04}$	$1.6 \times 10^{-03} \pm 1.4 \times 10^{-04}$	-	-	n.a.

n.a., not available.

5.1.9. Conclusions

In this study, the presence of plasticizers, bisphenols, pesticides, PCBs and PAHs was evaluated in Provola from dairy cows fed a conventional diet and an unconventional diet enriched with dried olive cake. In order to assess whether the addition of olive oil cake could influence the contamination process, olive pomace was also analyzed. The results show that for most parameters, the trend of the two diets had minimal differences, indicating that the contamination does not depend on the olive pomace supplemented in the BIO diet. The results show that OC supplementation in the animals' diets does not affect the contamination levels of Provola cheese. Therefore, in this case, the source of contamination is not attributable to the feeding of dairy cows, but probably depends on the pro-duction process or ubiquitous environmental contamination. It can be concluded that BIO Provola is as safe for the consumer as CTR Provola and therefore represents a good strategy for sustainable animal husbandry.

5.2. OLIVE CAKE IN DAIRY COW DIET IMPROVES THE MINERAL ELEMENTS IN PROVOLA CHEESES

The aim of the present study was to investigate the concentrations of mineral elements in Provola cheese obtained from dairy cows fed two different diets: a conventional diet and a diet supplemented with dried and pitted olive cakes (see sample collection section *5.1.1*), in order to evaluate the effects of supplementation on the concentrations of mineral components. The results obtained were subjected to statistical analysis in order to try to discriminate between the two different types of cheese obtained. In addition, the intake of macro-, micro- and potentially toxic elements from cheese consumption was evaluated. The values obtained for macro and trace elements were compared with the Recommended Dietary Allowance (RDA) or Adequate Intake (AI), and for potentially toxic elements with the Tolerable Daily Intake (TDI) or Tolerable Weekly Intake (TWI) to assess human exposure to these elements.

5.2.1. Elemental analysis and validation methods

A closed vessel microwave digestion system (ETHOS 1, Milestone, Bergamo, Italy) was used for the acid digestion process of provola cheese samples. Briefly, 0.5 g of sample was weighed in PTFE vessels, fortified with 1 mL of Re used as internal standard (0.5 mg/L), and 7 mL of HNO_3 and 1 mL of H_2O_2 were added to perform acid digestion of the sample. The digestion procedure included a first step of 15 min at a temperature from 0 to 180°C, and a second step of 15 min at a constant temperature of 180°C, with a constant micro-wave power of 1000 W. The samples were successively diluted to a volume of 25 mL with ultrapure water and filtered using 0.45 µm PTFE filters. The blank solution (HNO_3 and H_2O_2, 7:1 v/v) underwent the same pretreatment. Skimmed Milk Powder (ERM-BD150) was used as certified matrix (containing the following mineral elements: Ca, K, Mg, Na, Cd, Cu, Fe, Hg, Mn, Pb, Se, Zn). When the element was not certified in the reference material, the matrix was spiked with a known amount of analyte.

Twenty elements (As, B, Ba, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Se, Sr, Ti, V and Zn) were determined by a single quadrupole inductively coupled plasma mass spectrometer (ICP-MS, iCAP-Q, Thermo Scientific, Waltham, USA). The iCAP-Q ICP-MS was powered by a solid-state generator at 27 MHz radiofrequency, equipped with a PFA cyclonic spray chamber and an autosampler (ASX520, Cetac Technologies Inc, Omaha, NE, USA) coupled to an integrated sample introduction system.

The collision cell operating in He KED (Kinetic Energy Discrimination) mode was used to reduce/remove spectral (polyatomic and isobaric) and non-spectral interferences. In addition, KED-attenuated polyatomic interferences were corrected using elemental interference equations included in the instrument software, while isobaric interferences were checked by measuring multiple isotopes for each analyte and corrected using the most abundant isotope for the element of interest. The isotopes monitored in all samples were: 11B, 23Na, 24Mg, 39K, 44Ca, 48Ti, 51V, 52Cr, 55Mn, 56Fe, 60Ni, 63Cu, 66Zn, 75As, 80Se, 88Sr, 98Mo,114Cd, 138Ba and 208Pb. For non-spectral interferences, a stock standard solution of 45Sc, 73Ge and 115In was used as an on-line internal standard (at a level of 1.5 mg/L) to reduce/eliminate non-spectral interferences and to correct for instrumental drift and signal suppression or enhancement due to matrix effect.

All samples were analysed using the following operating conditions: RF power 1550 W; plasma gas (Ar) 14 L/min; auxiliary gas (Ar) 0.8 L/min; carrier gas (Ar) 1.1 L/min; collision gas (He) 4.7 mL/min; spray chamber temperature was 2.7˚C; sample depth and sample introduction were 5 mm and 0.93 mL/min, respectively. Integration times were 0.5 s/point for V, Fe, Se and As, 0.01 s/point for Mg, Na, K and Ca; and 0.1 s/point for the other elements. Three points for each mass acquisition and three replicates were used for peak integration. Thermo Scientific Qtegra™ Intelligent Scientific Data System software was used for instrument control and data acquisition. Quantitative analysis was based on a seven-point calibration curve.

All samples were analysed in triplicate, together with blanks and analytical standard. Hg was determined by Direct Mercury Analyser (DMA-80, Milestone S.r.l., Italy) using thermal decomposition-atomic absorption spectrophotometry (TDA-AAS) according to EPA method 7473. The analytical procedure was as follows: ~100 mg of each sample was first dried at 200 °C for 3 min and then thermally decomposed at 650 °C for 2 min. The absorbance at the typical wavelength of mercury (253.7 nm) was then measured to determine the Hg content. Quantitative analysis was based on a seven-point calibration curve. All samples were analysed in triplicate.

Linearity, LOD, LOQ, accuracy and precision were validated according to Eurachem criteria for each analyte investigated. The validation data are given in Table 11. For all elements, linearity was checked by constructing calibration curves using seven multi-standard solutions at concentrations in the range 0.5-50.0 µg/L. The calibration curve for Hg was in the range 1-100 µg/L.

Each solution was injected six times ($n = 6$). All calibration curves were linear in the concentration range considered and the R^2 values ranged from 0.9980 (for Ca) to 0.9999 (for Cd). LOD and LOQ were calculated as 3.3 σ/b and 10 σ/b, respectively, where σ is the standard deviation of the analytical blank ($n = 6$) and b is the slope of the calibration curve. The LODs and LOQs ranged from 1.0 - 1156.0 µg/L and 3.0 - 3815.0 µg/L, respectively. For mercury, LOD was 1.0 µg/L and LOQ was 3.3 µg/L.

Table 11. Analytical validation of ICP-MS and DMA-80 analysis performed in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy (n=6) and precision (n=6).

				Skimmed Milk Powder - ERM-BD150				
Analyte	**R^2**	**LOD (µg/L)**	**LOQ (µg/L)**	**Experimental value (mg/Kg)**	**Expected value (mg/Kg)**	**Recovery (%)**	**Precision (RSD%)**	
							Intraday	**Interday**
Macro-elements								
Ca	0.9980	1156.0	3814.8	12466	13900	89.68	1.1	1.3
Na	0.9984	1105.0	3646.5	3751	4180	89.74	1.2	1.4
K	0.9983	1131.0	3732.3	15634	17000	91.97	1.0	1.3
Mg	0.9990	55.0	181.5	1201	1260	95.32	1.0	1.1
Micro-elements								
Zn	0.9995	49.0	161.7	42.9	44.8	95.76	1.3	1.5
Fe	0.9994	18.0	59.4	4.4	4.6	95.65	0.3	0.4
Mn	0.9997	3.0	9.9	0.281	0.289	97.23	1.1	1.5
Cu	0.9993	5.0	16.5	1.04	1.08	96.30	0.5	0.8
Se	0.9994	3.0	9.9	0.185	0.188	98.40	0.3	0.8
Cr*	0.9995	10.0	33.0	1.96	2.00	98.00	0.4	0.6
Ti*	0.9994	32.0	105.6	1.94	2.00	97.00	0.6	0.7
V*	0.9994	1.0	3.3	1.97	2.00	98.50	0.9	1.0
Ba*	0.9993	11.0	36.3	1.93	2.00	96.50	0.7	1.0
B*	0.9993	3.0	9.9	1.94	2.00	97.00	0.9	1.1
Mo*	0.9998	3.0	9.9	1.97	2.00	98.50	0.3	0.5
Sr*	0.9991	34.0	112.2	1.95	2.00	97.50	0.7	0.9
Potentially toxic								
Pb	0.9998	1.0	3.3	0.0195	0.0193	101.04	0.6	1.0
Cd	0.9999	1.0	3.3	0.0116	0.0114	101.75	1.3	1.5
Hg	0.9997	1.0	3.3	0.057	0.060	95.00	0.6	0.8
Ni*	0.9995	3.0	9.9	1.98	2.00	99.00	0.3	0.3
As*	0.9997	1.0	3.3	1.99	2.00	99.50	1.1	1.3

* Recovery and precision tested on the matrix spiked with standard solutions.

Skimmed milk powder - ERM-BD150, analysed in six replicates, was used for accuracy. The difference between the experimental value and the reference value was reported as percentage recovery. However, surrogate recoveries were used to estimate the accuracy of elements do not present in the reference material. The lowest and highest mean recoveries were observed for Ca (89.68%) and Cd (101.75%). The repeatability in terms of precision was evaluated by analysing the certified matrix and the spiked sample on the same day. The intermediate precision was evaluated over a longer period (1 week). The precision and intermediate precision, expressed as relative standard deviation (RSD%), were less than 1.3% and 1.7%, respectively.

5.2.2. Statistical analysis

All statistical calculations were performed using SPSS 13.0 software for Windows (SPSS Inc., Chicago, IL, USA). Statistical methods were conducted on three data sets. For the first, the initial

multivariate matrix was made up of 40 cases (Provola samples analysed) and 18 variables (concentrations of elements determined in the samples analysed). The data were divided into two groups according to the diet (Control and Biotrak).

The second and third data sets each consisted of a matrix of 20 cases (only provola samples from conventional diets and only provola samples from unconventional diets) and the same 18 variables as in the first data set; the cases were divided into five groups according to the time of sampling (from March to July). In all datasets, Li, Be, Al, Co, As, Pb, Bi and Hg were not included because they were always below the limit of quantification (LOQ) (the only exception was Al, which was below the LOQ in 60% of the samples); when concentrations were below the LOQ in only a few samples (Cr), they were replaced by half the limit of detection (LOD/2).

Firstly, the significance of the differences between samples from different diets was assessed using the non-parametric Mann-Whitney U test. In a second step, a one-way ANOVA with Tukey Honestly Significant Difference (HSD) post hoc multiple comparison test was performed to check the differences in element levels during the sampling period within samples of the same diet.

Subsequently, the first data sets were normalized to obtain independence of the scale factors of the different variables. After verifying the adequacy of the initial data using the Kaiser-Meyer-Olkin (KMO) test and Bartlett's test, the data were subjected to Principal Component Analysis (PCA) to try to differentiate between samples from different diets. Finally, Canonical Discriminant Analysis (CDA) was performed to classify the different provola samples.

5.2.3. Results and discussions

The average concentrations of macro and micro elements in the provola cheese samples are shown in Table 12. The comparison of elements between the Provola samples from different diets, carried out by the Mann-Whitney U test with a significant p level below 0.05, showed that no differences were detected between the two groups for Ba, Cd, B and V (Table 12); all the Biotrak provolas had

significantly higher values for all the other elements, except for Sr and Cr, which were higher in the control provolas.

Table 12. Categorization of Provola samples depending on the diet and the results of the Mann-Whitney test. The concentrations of each mineral elements are expressed in mg/kg.

	Ca	Na	K	Mg	Zn	Ti	Sr	Fe	Ni
Control									
Mean	7673.80	2857.90	349.70	178.90	15.39	6.960	3.260	1.50	0.74
Std.dev.	185.00	284.10	22.80	15.40	0.52	2.400	0.650	0.65	0.07
Biotrak									
Mean	8922.60	4999.60	521.50	209.8	17.21	8.820	2.690	1.75	0.86
Std.dev.	166.60	228.70	17.60	12.8	0.40	2.580	0.220	0.32	0.08
Mann_Whitney U	400.000	400.000	400.000	381.500	400.000	304.000	89.500	294.500	338.000
Wilcoxon W	610.000	610.000	610.000	591.500	610.000	514.000	299.500	504.500	548.000
Z	5.412	4.410	5.411	4.910	5.412	2.813	-2.989	2.557	3.733
p-value	**0.000 (II)**	**0.000 (II)**	**0.000 (II)**	**0.000 (II)**	**0.000 (II)**	**0.004 (II)**	**0.002 (I)**	**0.009 (II)**	**0.000 (II)**
	Ba	**Cr**	**Mn**	**Cu**	**Se**	**Cd**	**Mo**	**B**	**V**
Control									
Mean	0.547	0.219	0.162	0.154	0.104	0.104	0.088	0.067	0.0080
Std.dev.	0.047	0.099	0.038	0.025	0.018	0.024	0.016	0.036	0.003
Biotrak									
Mean	0.560	0.103	0.216	0.179	0.210	0.109	0.104	0.076	0.009
Std.dev.	0.025	0.037	0.038	0.023	0.080	0.012	0.010	0.030	0.003
Mann_Whitney U	262.000	40.000	338.000	306.000	348.000	190.000	315.500	239.500	262.500
Wilcoxon W	472.000	250.000	548.000	516.000	558.000	400.000	525.500	449.500	472.500
Z	1.678	-4.345	3.734	2.869	4.007	-0.271	3.126	1.069	1.708
p-value	0.096	**0.000 (I)**	**0.000 (II)**	**0.004 (II)**	**0.000 (II)**	0.799	**0.001 (II)**	0.289	0.091

Bold values are significant at $p<0.05$. Next to the significant p-values is a I if the values are greater for conventional diet (Control), and II if they are greater for unconventional diet (Biotrak).

Among the macro-elements, the highest mean concentration in all the analysed Provolas was observed for Ca, with 7673.8 mg/kg (control) and 8922.6 mg/kg (Biotrak), followed by Na (2857.9 mg/kg and 4999.6 mg/kg), K (349.70 mg/Kg and 521.5 mg/kg) and Mg (178.9 mg/kg and 209.8 mg/kg). According to other studies, Ca is the most abundant mineral element in dairy products. The higher concentrations of Ca, Na, K and Mg in the Biotrak group than in the control group may be related to the olive cake added to the Biotrak diet. In fact, the concentrations of macro-elements

in olive cake were quite high, as shown in Table 13. The most abundant element was Ca (2674.0 mg/kg), followed by K (1293.8 mg/kg), Na (85.790 mg/kg) and Mg (81.82 mg/kg).

The microelement content is ordered in the following trend Zn > Ti > Sr > Fe > Ba > Cr > Mn > Cu > Se > Mo >B > V, as shown in Table 12. No significant differences were observed in the two groups (Control and Biotrak) for Ba, B and V. For the Biotrak group, significantly higher values (p-value < 0.05) were observed for Zn (17.21 mg/kg), Fe (1.75 mg/kg), Mn (0.216 mg/kg), Cu (0.179 mg/kg), Se (0.210 mg/kg), while Sr (3.260 mg/kg); Cr (0.219 mg/kg) was significantly higher in the control group. The presence of essential elements is an important parameter and has acquired considerable importance as an index of the production process, environmental pollution and the quality of animal feed, which can affect the characteristics of the cheese. Zn plays an important role in many physiological processes. However, high concentrations of Zn cause nausea, vomiting, anaemia and cholesterol problems in adults.[28] The amount of Zn found in Provolas cheese was lower than that found in other studies.[29,30] Dairy products, especially cow's milk cheeses, are considered to be very poor sources of Fe and the concentrations of Fe reported in the literature are variable. In this study, Provola Biotrak resulted in significantly higher Fe contractions (p<0.05) compared to Provola Control. This is probably due to the consistent amount of Fe in the olive cake as shown in Table 13.

Table 13. Elements contents expressed in mg/kg (mean ± standard deviation) in olive cake.

	Ca	**Na**	**K**	**Mg**	**Zn**	**Ti**	**Sr**	**Fe**	**Ni**
Mean	2674.0	85.79	1293.8	81.82	1.44	2.57	3.70	17.8	0.20
Std.dev.	202.4	5.92	226.6	0.93	0.37	0.84	0.64	5.9	0.02

	Ba	**Cr**	**Mn**	**Cu**	**Mo**	**B**	**V**	**Se**
Mean	0.21	0.29	1.14	1.77	0.03	3.20	0.13	0.17
Std.dev.	0.04	0.02	0.18	0.09	0.01	0.29	0.08	0.06

The microelement content in the olive cake is ordered in the following trend Fe > B > Cu > Zn > Mn > Cr > Ba > Se > V > Mo (Table 13). The supplementation of olive cake in the biotrak diet had, in some cases, a low element transfer effect in the cheeses obtained. This effect is most pronounced for K and less so for B, Cr, Cu, Fe, Mn and V. Several studies have explained this by the fact that most of the ingested K (but this could also apply to the other elements) is excreted in the urine and not in the milk.[31]

Selenium is one of the most interesting essential elements to pay attention to, as it performs different biological functions. The Se content in the olive cake was 0.170 mg/kg as shown in Table 13. In Biotrak Provolas the Se content was in the range of 0.112 mg/kg to 0.281 mg/kg, about twice the Se content in Provolas Control. Studies have shown that dairy products contain variable levels of Se, which is directly related to the presence of this element in the feed.[32,33]

One approach to providing Se is to fortify foods. Selenium contributes to the antioxidant capacity of living cells and protects cell membranes from free radical attack.[34]

A diet low in Se can cause liver necrosis, cardiomyopathy, cardiac ischaemia, joint and muscle diseases, reduced fertility and various forms of cancer (prostate, lung, digestive tract, skin).

Furthermore, according to some studies, adequate selenium supplementation is associated with an increase in the number of T lymphocytes, which strengthens the immune system against the influenza virus and the recent coronavirus (COVID-19).[35]

Therefore, the concentrations of Se found in Provola Biotrak are interesting from the point of view of a greater contribution of this element in the human diet. A product richer in most of the essential elements (Na, K, Mg, Zn, Fe, Mn, Cu and Se) was obtained by supplementing the cows' diet with dried and pitted olive cake. Elements such as Cd, Pb, Ni, As and Hg, are of real importance because of their correlation with environmental pollution.[36] No quantifiable levels of Pb, As and Hg were found in any of the samples analysed. The concentrations of potentially toxic elements are given in Table 12. The mean concentrations of Cd in Control and Biotrak Provolas were 0.104 mg/kg and 0.109 mg/kg, respectively, while the mean concentration of Ni was 0.74 mg/kg in Control Provolas

and 0.86 mg/kg in Biotrak Provolas. For Cd, no significant difference was observed between the two groups (p-value 0.799). For Ni, significantly higher values were observed in the Biotrak group (p-value < 0.000).

The amount of Ni found in olive cake was 0.20 mg/kg, while the Cd content was below the LOQ. Ni and Cd in dairy products can originate from different sources, from feed to food, from atmospheric deposition or during processing, via milking machines.[37] Ni is an essential element in small doses, but it can be dangerous when the maximum tolerable levels are exceeded and can cause adverse effects on human health. Studies show that excessive doses cause various effects in humans, including heart failure, cancer, allergic skin reactions and dermatitis.[28]

The use of dried and pitted olive cake as a supplement in the diet of cows resulted in a product that was safe in terms of toxic element content, as their content in Biotrak and Control provolas was comparable.

After the evaluation between the two diets, an assessment of the seasonal variability was made for both groups. Significant differences in elemental concentrations between Provola samples from different months were estimated using the Kruskal-Wallis test with significant p levels below 0.05 within samples from the same diet. The results, presented in Tables 14 and 15, showed that significant differences were observed in the samples of both groups for all variables, except for Ca, Na and V.

On the other hand, for Ca higher concentrations were observed in the analysed cheeses in winter than in summer.[38] Within the Control group, the April and May samples showed significantly higher levels of Sr, Mo and Fe.

The May samples differed from the April samples by a higher content of Cr and Zn. The June and July samples were characterised by significantly lower levels of B, Ti and Sr. Finally, the March samples differed significantly by higher levels of Ni and lower levels of Zn. Within the Biotrak group, the June and July samples showed significantly lower levels of Cr, B and Ti. The April and May samples were characterised by significantly higher levels of K and Se.

Seasonal variation in mineral content may be due to a combined effect of climate change and changes in the metabolic status of dairy cows as lactation progresses. In fact, as observed by some authors, a general variation in mineral content in summer cow's milk is probably due to a decrease in osmotic pressure in the mammary gland in hot climates.[39,40]

Table 14. Elements contents (mean ± standard deviation in mg/kg) and results of Kruskal-Wallis test for Control Provolas during the sampling period.

	Ca	Na	K	Mg	Zn	Ti	Sr	Fe	Ni
March	7793.7 ± 145.70	2868.7 ± 361.4	311.20^{A} ± 9.20	173.5^{AB} ± 11.60	14.60^{A} ± 0.03	8.200^{B} ± 0.110	3.070^{B} ± 0.020	1.04^{A} ± 0.02	0.84^{C} ± 0.01
April	7466.2 ± 194.40	2624.7 ± 413.5	349.20^{AB} ± 11.40	157.5^{A} ± 13.70	15.23^{B} ± 0.03	9.020^{B} ± 0.030	3.910^{C} ± 0.010	1.47^{B} ± 0.01	0.63^{A} ± 0.01
May	7746.0 ± 177.40	3042.1 ± 137.8	366.90^{B} ± 17.40	184.3^{AB} ± 10.50	16.17^{D} ± 0.02	9.330^{B} ± 0.150	4.120^{C} ± 0.010	2.73^{C} ± 0.02	0.76^{B} ± 0.01
June	7605.9 ± 149.70	2793.6 ± 158.5	356.10^{AB} ± 3.90	193.8^{B} ± 4.80	15.66^{C} ± 0.05	4.060^{A} ± 0.040	2.640^{A} ± 0.010	1.12^{A} ± 0.010	0.75^{B} ± 0.02
July	7757.3 ± 70.1	2960.6 ± 167.1	365.10^{B} ± 2.80	185.2^{AB} ± 3.50	15.27^{B} ± 0.04	4.220^{A} ± 0.140	2.590^{A} ± 0.010	1.13^{A} ± 0.010	0.72^{AB} ± 0.01
F stat.	6.737	4.743	14.157	13.138	17.795	17.729	18.286	18.189	17.329
p-value	0.150	0.315	**0.007**	**0.011**	**0.001**	**0.001**	**0.001**	**0.001**	**0.002**

	Ba	Cr	Mn	Cu	Se	Cd	Mo	B	V
March	0.533^{A} ± 0.011	0.132^{A} ± 0.0060	0.168^{AB} ± 0.009	0.130^{A} ± 0.005	0.086^{A} ± 0.008	0.115^{B} ± 0.008	0.0760^{A} ± 0.005	0.092^{BC} ± 0.007	0.0070 ± 0.002
April	0.565^{AB} ± 0.047	0.099^{A} ± 0.010	0.146^{AB} ± 0.009	0.187^{B} ± 0.006	0.119^{B} ± 0.005	0.0590^{A} ± 0.006	0.102^{B} ± 0.009	0.080^{B} ± 0.013	0.0090 ± 0.003
May	0.620^{B} ± 0.011	0.363^{B} ± 0.030	0.231^{B} ± 0.009	0.159^{AB} ± 0.006	0.117^{B} ± 0.006	0.122^{B} ± 0.007	0.107^{B} ± 0.007	0.099^{C} ± 0.007	0.012 ± 0.004
June	0.509^{A} ± 0.004	0.273^{AB} ± 0.014	0.133^{A} ± 0.009	0.124^{A} ± 0.008	0.081^{A} ± 0.007	0.116^{B} ± 0.004	0.0720^{A} ± 0.004	0.027^{A} ± 0.004	0.0070 ± 0.003
July	0.509^{A} ± 0.006	0.231^{AB} ± 0.003	0.133^{A} ± 0.003	0.172^{B} ± 0.007	0.116^{B} ± 0.005	0.107^{B} ± 0.005	0.0830^{A} ± 0.004	0.026^{A} ± 0.005	0.0060 ± 0.001
F stat.	16.230	18.286	16.534	16.966	14.085	14.225	16.437	16.433	8.983
p-value	**0.003**	**0.001**	**0.002**	**0.002**	**0.007**	**0.007**	**0.002**	**0.002**	0.062

n.d., not determinable (< LOQ). Bold values are significant at $p<0.05$. Different letters represent significantly different mean values.

Table 15. Elements contents (mean ± standard deviation in mg/kg) and results of Kruskal-Wallis test for Biotrak provolas during the sampling period.

	Ca	Na	K	Mg	Zn	Ti	Sr	Fe	Ni
March	8867.5 ± 209.3	5001.1 ± 316.8	514.90^{A} ±15.10	191.9^{A} ±10.8	16.56^{A} ±0.04	10.28^{B} ±0.23	2.730^{B} ±0.03	1.29^{A} ±0.02	0.75^{A} ±0.01
April	9024.0 ± 124.2	4824.1 ±238.7	540.60^{B} ±15.10	216.3^{AB} ±12.0	17.10^{AB} ±0.05	10.37^{B} ±0.04	3.080^{C} ±0.02	2.03^{B} ±0.01	0.83^{AB} ±0.08
May	8859.6 ±195.2	5144.8 ±67.3	533.80^{B} ±17.80	215.6^{AB} ±7.0	17.50^{B} ±0.02	11.54^{B} ±0.34	2.470^{A} ±0.01	1.46^{AB} ±0.02	0.95^{B} ±0.01
June	8965.4 ±211.8	4934.9 ±266.9	507.00^{A} ±3.40	204.8^{AB} ±2.6	17.18^{AB} ±0.19	5.400^{A} ±0.04	2.550^{AB} ±0.01	1.92^{AB} ±0.01	0.84^{AB} ±0.01
July	8896.7 ±84.8	5093.1 ±108.6	511.20^{A} ±3.40	220.3^{B} ±3.4	17.68^{B} ±0.05	5.990^{A} ±0.02	2.610^{AB} ±0.01	2.04^{B} ±0.01	0.94^{B} ±0.01
F statistic	3.001	4.736	11.836	13.729	17.430	17.474	18.286	17.674	16.001
p-value	0.558	0.316	**0.019**	**0.008**	**0.002**	**0.002**	**0.001**	**0.001**	**0.003**

	Ba	Cr	Mn	Cu	Se	Cd	Mo	B	V
March	0.449^{B} ±0.26	0.124^{B} ±0.0050	0.194^{AB} ±0.017	0.146^{A} ±0.007	0.112^{A} ±0.007	0.104^{AB} ±0.007	0.0910^{A} ±0.008	0.081^{B} ±0.004	0.0090 ± 0.003
April	0.539^{A} ±0.017	0.131^{B} ±0.006	0.160^{A} ±0.012	0.198^{B} ±0.007	0.281^{C} ±0.022	0.0930^{A} ±0.007	0.109^{AB} ±0.008	0.099^{C} ±0.005	0.012 ±0.004
May	0.580^{B} ±0.012	0.053^{AB} ±0.008	0.261^{C} ±0.008	0.196^{B} ±0.008	0.281^{C} ±0.022	0.119^{B} ±0.009	0.0990^{AB} ±0.005	0.091^{BC} ±0.006	0.010 ±0.002
June	0.532^{A} ±0.009	n.d.^{A}	0.220^{B} ±0.005	0.161^{AB} ±0.006	0.120^{A} ±0.003	0.108^{AB} ±0.005	0.107^{AB} ±0.004	0.048^{A} ±0.011	0.0070 ±0.003
July	0.569^{AB} ±0.005	n.d.^{A}	0.246^{BC} ±0.005	0.194^{B} ±0.006	0.256^{B} ±0.010	0.120^{B} ±0.003	0.114^{B} ±0.004	0.041^{A} ±0.003	0.0090 ±0.001
F statistic	14.093	18.116	18.071	14.876	15.607	14.740	14.060	17.415	4.339
p-value	**0.007**	**0.001**	**0.001**	**0.005**	**0.004**	**0.005**	**0.007**	**0.002**	0.362

n.d., not determinable (< LOQ). Bold values are significant at p<0.05. Different letters represent significantly different mean values.

5.2.4. Principal Component Analysis and Canonical Discriminant Analysis.

PCA was applied to the normalised data using as variables the concentrations of the elements found to be significantly different in the diet analysis (Ca, Na, K, Mg, Zn, Ti, Sr, Fe, Ni, Cr, Mn, Cu, Se and Mo). The suitability of the data for factor analysis was verified.

The Kaiser-Meyer-Olkin sampling adequacy measure gave a value of 0.718 and Bartlett's sphericity test showed an approximate chi-square value of 785.042 (at a p-level of less than 0.0001), so the correlation matrix was factored and suitable for PCA. Analysis of the correlation matrix shows that the highest positive correlations were observed for K-Na (0.932), Na-Ca (0.932), Zn-K (0.927) and K-Ca (0.923), whereas the highest negative correlations were observed for Zn-Cr (-0.619), Na-Cr (-0.607), Ca-Cr (-0.607) and Sr-Mg (-0.596).

According to the Kaiser-Guttman criterion, three principal components with eigenvalues greater than one (7.817, 2.517 and 1.054) were extracted, which together explain 81.341% of the total variance (55.837%, 17.977% and 7.527%, respectively). Since there were no variables with low saturation in each factor and the communality was always higher than 0.603, the extracted components were able to reproduce all the variables well.

The first component has the highest positive correlation with Zn, K and Na, while negative correlations can be observed for Sr and Cr; Sr, and to a lesser extent Fe, has a positive correlation with the second component; the dominant variables in the third component were Ti and Cr with a positive correlation.

Figure 3 shows the 2D scatterplots on the plane defined by PC1 and PC2 (part A) and by PC1 and PC3 (part B) for the 40 Provola samples. Two groups are clearly distinguishable: the Biotrak provolas are separated from the Control provolas on the first component, which explains 55.837% of the total variance; the Biotrak samples are placed at positive values of PC1 and are characterised by higher values of all variables except Sr and Cr, instead the Control samples are placed at negative values of PC1 and have the highest concentrations of Sr and Cr, while all the other concentrations were lower than the Biotrak samples.

At PC2, a trend towards within-group separation was observed for the control group, and at PC3 for the Biotrak group; this trend towards within-group separation could be attributed to seasonal physiological changes in animal metabolism and changes in climatic conditions.

As can be seen in Figure 3A, the April samples (circled in green) had positive PC2 values, and the other samples had negative PC2 values: this is related to the highest content of Sr. Figure 3B shows that the June and July samples (circled in yellow) had negative PC3 values while the other samples had positive PC3 values.

This is due to the lower Ti content and the Cr value below the LOQ. Intra-group segregation also occurred in the control group. Figure 3A shows the April and May samples (circled in orange) with positive PC2 values due to the highest values of Sr, Ti and Mo. The May samples (circled in red) with positive PC3 values due to the highest values of Cr.

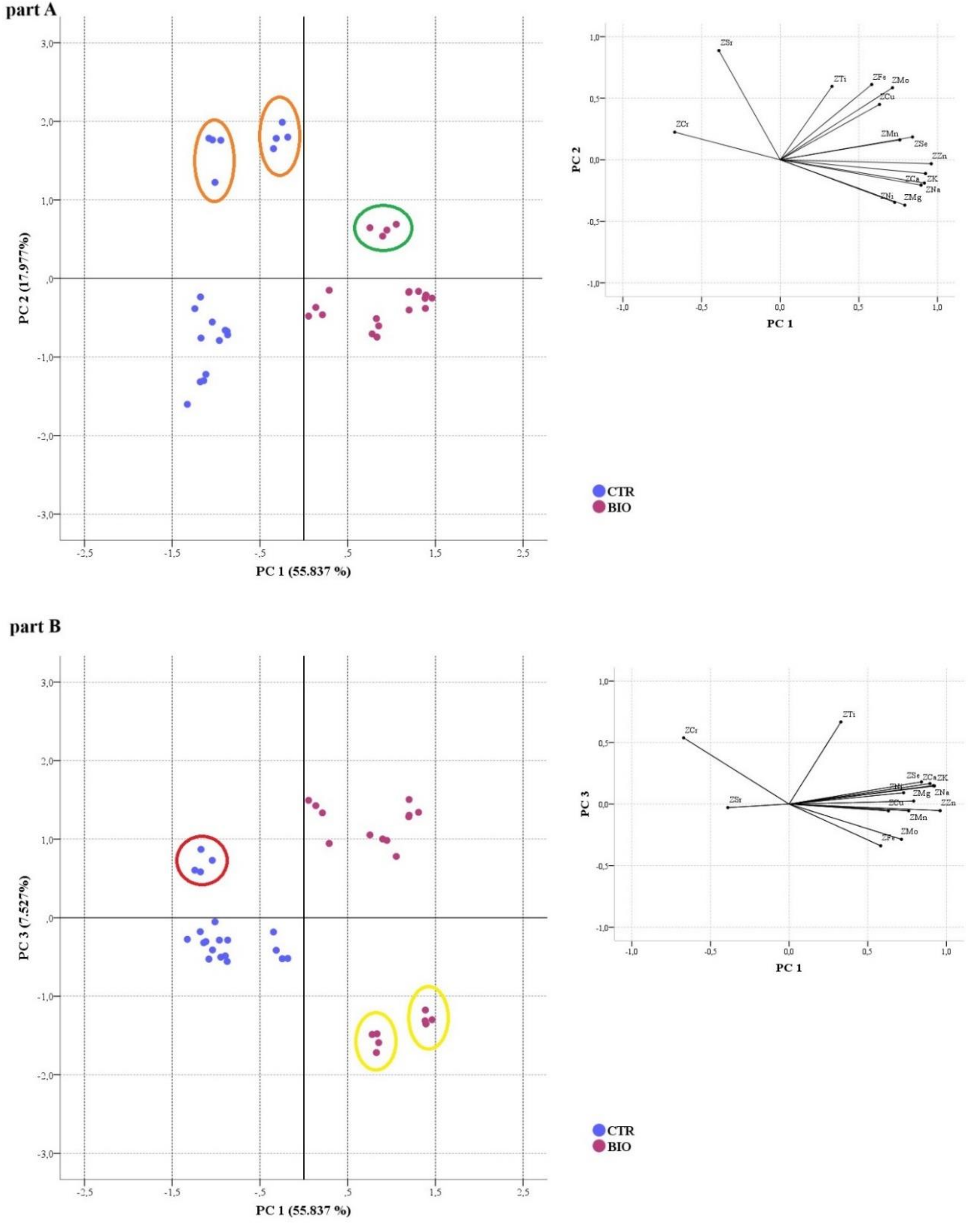

Figure 3. Score plots for 40 provola cheese samples categorized by Control and Biotrak. Plane defined by PC1 and PC2 (part A) and by PC1 and PC3 (part B); circled in green Biotrak samples of April, circled in yellow Biotrak samples of June and July, circled in orange Control samples of April and May and circled in red Control samples of May.

Canonical Discriminant Analysis (CDA) was then performed on the initial classification of samples from the biotrak and control groups. The canonical discriminant function obtained showed high discriminatory power, with a high correlation value (0.999) and a low Wilks' lambda value (0.002), as shown in Table 16.

Table 16. Canonical discriminant function parameters.

Eigenvalue	% of variance	Cumulative %	Canonical correlation	Wilks' Lambda
439.402	1.000	1.000	0.999	0.002

The values of the non-standardised and standardised coefficients are shown in Table 17. The non-standardised coefficients were used as coefficients of the canonical variables to calculate the canonical variable values for each case, while the standardised coefficients showed that the variables that discriminated best were Cr (-3.883), Ti (6.188), Mn (-3.620), Fe (-3.264), Zn (2.100) and Mo (3.175). The data set was analysed using the canonical discriminant function. Each case was assigned to the group in which it achieved the highest probability of group membership. The classification results (Table 18) show that 100% of the total samples were correctly classified.

Table 17. Matrix of components and variable commonalities.

Mineral elements	Unstandardized coefficients	Standardized coefficients
Cr	-2.990	**-3.883**
B	-1.411	-0.782
Na	6.972	0.552
Mg	11.143	0.860
K	36.798	1.951
Ti	17.446	**6.187**
V	-1.399	-0.468
Mn	-17.833	**-3.620**
Fe	-11.141	**-3.264**
Ni	0.019	0.001
Cu	-8.140	-1.235
Zn	71.898	**2.100**
Se	-4.157	-1.384
Sr	-2.939	-0.444
Mo	22.564	**3.175**
Cd	7.771	1.681
Ba	-0.503	-0.033
Ca	58.290	1.265

Bold values indicate the most discriminative variables.

Table 18. Classification matrix obtained using the canonical discriminant function.

		Planned group membership		
		Control	Biotrak	Total
Original	Count	20	0	20
		0	20	20
	%	100.0	0	100.0
		0	100.0	100.0
Cross-validation	Count	20	0	20
		0	20	20
	%	100.0	0	100.0
		0	100.0	100.0

5.2.5. Uptake of elements by Provola samples

Regulation (EU) No 1169/2011 of the European Parliament sets the Recommended Dietary Allowance (RDA) for adults at 800 mg/day for Ca, 2000 mg/day for K, 375 mg/day for Mg, 14 mg/day for Fe, 10 mg/day for Zn, 1 mg/day for Cu, 2 mg/day for Mn, 55 µg/day for Se, 50 µg/day for Mo and 40 µg/day for Cr. No RDA is available for Na, but the Adequate Intake (AI) is available and is set at 2000 mg/day. The RDAs and AIs for all elements are shown in Table 19.

Table 19. Percentage of RDA and AI for non-toxic elements, and of TWI and TDI for potentially toxic elements from analyzed Provola samples.

Non-toxic elements										
	Ca	**K**	**Mg**	**Fe**	**Zn**	**Mn**	**Cu**	**Se**	**Mo**	**Cr**
RDA (mg/day)	800	2000	375	14	10	2	1	0.055	0.050	0.040
% RDA Control group	43.17	0.79	2.15	0.48	6.93	0.36	0.69	8.51	7.92	24.64
% RDA Biotrak group	50.19	1.17	2.52	0.56	7.74	0.49	0.81	17.18	9.36	11.59
	Na									
AI (mg/day)	2000									
% AI Control group	6.43									
% AI Biotrak group	15.00									
Potentially toxic elements										
	Cd	**Ni**								
TWI (µg/kg$_{bw}$ /week)	2.5									
% TWI Control group	18.7									
% TWI Biotrak group	19.6									
TDI (µg/kg$_{bw}$ /day)		13								
% TDI Control group		3.65								
% TDI Biotrak group		4.25								

The EFSA Panel on Contaminants in the Food Chain recommended a tolerable weekly intake (TWI) of 2.5 μg/kg body weight for Cd to provide adequate protection for all consumers. For Ni established a tolerable daily intake (TDI) of 13 μg/kg body weight.

In the analysed samples, the estimated contribution of the metal was calculated on the basis of the recommended daily intake for cheese of 45 g/day by the Food and Agriculture Organization of the United Nations.[41]

The results showed that the amount of Provola provided the following percentages of the European RDA for the control and Biotrak groups, respectively Ca 43.17% and 50.19%, K 0.79% and 1.17%, Mg 2.15% and 2.52%, Fe 0.48% and 0.56%, Zn 6.93% and 7.74%, Mn 0.36% and 0.49%, Cu 0.69% and 0.81%, Se 8.51% and 17.18%, Mo 7.97% and 9.36%, Cr 24.64% and 11.59%.

The adequate intake percentage for Na was 6. 43% for the control group and 15. 00% for the Biotrak group.

The highest RDA was found for Ca, in agreement with Crupi et al.[42] The intakes of Ca, Na, K, Fe, Zn, Mn, Cu, Se and Mo were found to be consistently higher in Biotrak Provolas, especially for Se, indicating the enrichment of essential minerals for humans by the consumption of Biotrak Provolas.

The percentage of TWI for Cd and the percentage of TDI for Ni were calculated taking into account the recommended daily intake for cheese, the average concentrations of Cd and Ni in the two groups (Control and Biotrak) and the average weight of a 70 kg adult.

The %TWI and %TDI for Cd and Ni, respectively, were similar in the two groups (Cd: Control 18.7% and Biotrak 19.6%; Ni: Control 3.65% and Biotrak 4.25%).

Exposure to the toxic elements Cd and Ni is not of concern compared to TWI and TDI. In fact, both the weekly and daily intakes of these toxic elements from the consumption of provola are below the reference intakes for both groups. It can be concluded that the consumption of these provolas does not have a negative effect on the health of the consumer.

5.2.6. Conclusions

The results of the present research show that provola cheese from the Biotrak group has a higher value of essential elements for almost all the elements studied, proving the higher quality of the product obtained from the unconventional diet. The Se content in provola Biotrak was particularly interesting, as it was found to be about twice as high as in provola Control, covering on average 17.18% of the RDA. Furthermore, Pb, As and Hg were found at non-quantified levels in both Biotrak and Control provolas, whereas Cd and Ni were found at low levels, ensuring that the samples analysed are safe for the consumer. Thus, the use of olive cake in animal feed is a good strategy in the circular economy, accelerating the implementation of "Transforming our world: the 2030 Agenda for Sustainable Development" (https://sdgs.un.org/2030agenda) as well as the European Commission's Circular Economy Action Plan (https://environment.ec.europa.eu/strategy/circular-economy-action-plan_en).

5.3. USING OLIVE CAKE AS A SUSTAINABLE INGREDIENT IN DIETS OF LACTATING DAIRY COWS: EFFECTS ON NUTRITIONAL CHARACTERISTICS OF CHEESE

In previous chapters it has been emphasized that food safety is closely linked to quality. The aim of this study was to investigate the chemical composition, fatty acid profile and polyphenolic content of Provola cheese produced from milk obtained from cows fed with olive cake (see sample collection section *5.1.1).* The study was conducted with a herd of four hundred and sixty healthy, multiparous Holstein Friesian dairy cows. The animals were separated into two groups (CTR and TEST) of two hundred and thirty cows each. The animals were randomly assigned so that each treatment group was homogeneous in terms of body condition score (3 ± 0.5), calving interval (90–120 d) and milk performance (27 ± 3 kg/d). The two groups were managed according to local traditional practices. The cows in both groups were housed in a free-stall barn and kept on "deep litter" straw bedding. The barn was equipped with an automatic cooling system with fans and sprinklers that were activated during hot periods. Animals were given access to pasture for at least 6 h during the day (08:00 to 14:00 CEST). Both groups were given isoenergetic and isoproteic diets as a total mixed ration of 20 kg dry matter (DM)/head twice daily at 08:00 and 14:00 CEST. Diets were based on grass hay (crude protein: 110.9 g/kg dry matter, ether extract: 25.0 g/kg dry matter and neutral detergent fibre: 521.9 g/kg dry matter) supplemented with a concentrate pellet. The control group (CTR) received a concentrate without OC inclusion, while the experimental group (TEST) received a specially formulated concentrate containing 8% on a DM basis of olive cake (OC).

After a 3-week adaptation period, the trial started on lactation day 45 (day 0; April) and lasted 45 days (June). All cows were milked twice daily in the milking parlour at 04:30 and 16:30 CEST. The milk from the two groups was collected and kept separate during transport to the dairy plant in refrigerated tanks (4 ± 2 °C). From each 400-litre batch, 80 Provola cheeses were produced per group.

5.3.1. Nutritional Analyses

All provola and OC samples were analysed for moisture, protein, total fat, fatty acids and total polyphenols. The moisture content in OC and Provola was determined following the procedure described by official method AOAC 934.01, whereas protein content was determined according to Kjeldahl method (AOAC method 992.15).[43] Moisture and protein analysis for both OC and Provola cheese were done in triplicates.

The AOAC method 920.39 was used for the determination of total fat in OC.[43] An aliquot (20.0 g) of dried olive cake was extracted with n-hexane for 6 h using a Soxhlet apparatus. The extract was evaporated, and the dry extraction yield was determined gravimetrically.

The total lipids of Provola were extracted according to the Folch method, with modifications. Approximately 5 g of samples were weighed into 50 ml tubes. Folch's solution (chloroform/methanol 2:1 v/v), 37% concentrated HCl (to pH 1.00) and 0.73% NaCl were added and the mixture vortexed for 1 min and then centrifuged. The bottom layer was collected in a previously weighed flask and dried. Yield was determined gravimetrically.

Fatty acid methyl esters (FAME) were prepared by transmethylation of OC and Provola lipid extracts according to the ISO 5509 2000[44] method as follows: 1 mL of a 9:1 v/v methanol/sulphuric acid mixture was added to 1 mL of each extract and the mixture was placed in an oven at 110 °C for 1 hour. The supernatant was collected and diluted with n-hexane (1:2 v/v).

The fatty acid methyl ester (FAME) organic layer was injected into a gas chromatograph (GC) equipped with a split/splitless injector and flame ionization detector (FID) (Dani Master GC1000, Dani Instrument, Milan, Italy). A Supelco Omegawax 250 was used with the appropriate operating protocol: column temperature from 50°C (hold time 2 min) to 240°C (hold time 15 min) at 3°C/min. Helium was held constant at a linear velocity of 30 cm/s. Injector and detector temperatures were both set at 240°C.

The injection volume was 1 µL with a split ratio of 1:50. Clarity Chromatography Software v4.0.2 (DataApex, Prague, Czech Republic) was used to process the output data. All samples were

analyzed in quadruplicate with analytical blanks. By direct comparison with the retention times of the compounds present in the reference standard mixture, FAMEs were identified.

The Folin-Ciocalteu method, with some modifications, was used for the spectrophotometric determination of total polyphenols.[45]

Approx. 1 g of the homogenized samples were added to 2.5 ml of 95% ethanol and left at 0°C for 48 hours, 1 ml of the upper layer was transferred to a test tube and mixed with 95% ethanol and ultrapure water; Folin-Ciocalteu reagent (50%) and Na_2CO_3 (5%) were added. Using 95% ethanol as an analytical blank, the absorbance was read at 760 nm on a UV-visible spectrophotometer. A calibration curve was constructed using appropriate dilutions of a gallic acid standard solution (95% in ethanol). Determinations were carried out in triplicate together with the blank solutions.

5.3.2. Lipid Quality Indices of Provola

The saturated fatty acids were classified as pro-atherogenic because they promote the adhesion of lipids to cells of the immune system, while the unsaturated fatty acids were classified as anti-atherogenic because they inhibit plaque aggregation while reducing the levels of esterified fatty acids, cholesterol and phospholipids, thus preventing the occurrence of coronary heart disease.

The results of the fatty acid composition were used to calculate the atherogenicity index (AI) and the thrombogenicity index (TI) following equations:

$$AI = \frac{C12{:}0 + 4 * C14{:}0 + C16{:}0}{\sum MUFA + \sum n6 + \sum n3}$$

$$TI = \frac{C14{:}0 + C16{:}0 + C18{:}0}{0.5 * \sum MUFA + 0.5 * \sum n6 + 3 * \sum n3 + (\sum n3 \,/\, \sum n6)}$$

where C12:0, C14:0, C16:0 and C18:0 is lauric, myristic, palmitic and stearic acids respectively; MUFA, n6 and n3 are the sum of monounsaturated, polyunsaturated n6 and polyunsaturated n3 fatty acids.

5.3.3. Results and Discussion

The analytical analyses of the olive cake used in the treatment diet, shown in Table 20. The average protein content was similar to that reported by Molina-Alcaide et al.[46], while the total lipid and polyphenol contents were also in agreement with those reported by Dal Bosco et al.[47]

The fatty acid profiles were in agreement with those described by Vargas-Bello-Pérez et al.[48], with high amounts of C18:1 n-9 (66.63%), followed by C16:0 (16.14%) and C18:2 (10.66%). Other fatty acids present in significant amounts were C16:0 and C18:2, while C18:0 and C18:3 was marginal.

Table 20. The chemical, fatty acids composition, and polyphenols content of the dried and pitted olive cake integrated into the concentrate of the treatment experimental group (TEST). Values are reported as mean ± standard deviation.

	Olive cake
Chemical composition (g/kg dry matter)	
Moisture	36.47 ± 0.57
Ashes	34.13 ± 0.81
Crude fat	180.80 ± 0.26
Energy (ME) (Kcal/Kg)	3535.1
Crude protein (total N×6.25)	61.00 ± 1.73
Neutral detergent fibrer	410.33 ± 0.20
Acid detergent fibre	320.53 ± 0.34
Total polyphenols	10.18 ± 1.59
Fatty acids (g/100 g fat)	
C14:0	0.02 ± 0.01
C16:0	16.14 ± 0.17
C16:1	0.64 ± 0.07
C17:0	0.32 ± 0.07
C17:1	0.32 ± 0.02
C18:0	3.80 ± 0.16
C18:1n-9	66.63 ± 0.19
C18:2	10.66 ± 0.16
C18:3	0.57 ± 0.05
C20:0	0.53 ± 0.05
C20:1	0.33 ± 0.03
C22:0	0.03 ± 0.01
C24:0	0.01 ± 0.01

The composition, including polyphenol content and FA profile, of Provola cheeses from both treatment groups was analyzed, and evaluated for the effects of diet, month/season, and for diet×season interaction.

The main parameters of interest resulting from this model are presented in Table 21. Cheeses from the milk of animals fed the treatment diet have a higher protein concentration and less moisture content than those produced from the control group.

Table 21. Results of the model for the main parameters of interest. The least-square mean values of the test and control (CTR) groups and the standard errors of the mean (SEM) are reported, as well as the p-value for the three analyzed factors.

	TEST	CTR	SEM	Diet	Season	Diet × Season
Moisture (%)	42.02	43.62	0.34	0.0025	<0.0001	<0.0001
Proteins (%)	25.02	23.49	0.31	0.0019	0.7570	0.0004
Total Lipids (%)	20.94	20.91	0.41	0.9598	0.1259	<0.0001
Total polyphenols (mg/kg)	142.42	107.19	2.01	<0.0001	<0.0001	<0.0001
Σ FA	95.34	95.02	0.18	0.2206	0.0102	0.4615
Σ MUFA	23.17	22.71	0.16	0.0460	0.0073	<0.0001
Σ PUFA	3.88	3.66	0.05	0.0028	<0.0001	0.9269
Σ SFA	68.29	68.66	0.22	0.2517	0.0012	0.0006
Σ n-3	0.72	0.65	0.02	0.0034	<0.0001	0.0489
Σ n-6	3.03	2.72	0.04	<0.0001	<0.0001	0.0181
Σ n-6 / Σ n-3	4.26	4.20	0.09	0.6950	0.5710	0.5962
Undefined FA	4.66	4.98	0.18	0.2206	0.0102	0.4615
Atherogenic Index	2.98	3.17	0.02	<0.0001	0.0535	<0.0001
Thrombogenic Index	3.53	3.70	0.03	0.0004	0.0303	<0.0001
C12:0	3.60	3.62	0.04	0.7454	<0.0001	0.6527
C14:0	11.40	11.54	0.06	0.1115	<0.0001	0.8423
C16:0	31.01	32.84	0.09	<0.0001	0.0006	0.0000
C18:0	12.17	10.71	0.30	0.0016	0.4862	0.8813
C18:1 n-9	20.10	19.39	0.18	0.0087	0.5608	<0.0001
C18:2 n-6 cis	2.74	2.44	0.03	<0.0001	<0.0001	0.0053
C18:2 n-6 trans	0.10	0.10	0.01	0.6852	<0.0001	0.0108
C18:3 n-3	0.50	0.40	0.01	<0.0001	<0.0001	<0.0001
C20:4 n-6	0.02	0.01	0.002	0.0759	0.0154	0.4670
C20:5 n-3	0.04	0.04	0.003	0.2410	<0.0001	0.1347

Trends in higher milk protein from animals fed diets supplemented with olive cake have already been reported.[49,50] This could be an effect of 1) a metabolic/physiological process by which cows fed diets with olive cake inclusion produce more milk protein, and/or 2) the quality of protein in the milk from animals fed diets with olive cake inclusion has a tendency for higher coagulation within the cheese curd thereby increasing the concentration of protein in the final product. Russo et al.,[51] reported that destoned olive cake supplementation was able to modulate the fecal microbiota by increasing the Firmicutes phylum, which is associated with increased dietary nutrient availability, and decreasing the energetically less beneficial Bacteroidetes.

A significant effect of the regime diet on the biosynthesis of carbohydrates, fatty acids, lipids and amino acids was revealed by predicting metabolic pathways. Neither of the two diets had a significant effect on the total fatty acids present in the Provola cheese.

However, the FA profile was significantly affected, an observation that is consistent with the findings of several other authors that investigated milk or cheese produced by dairy ruminants fed with olive products. Some of the observed changes in the FA profile of the test cheeses can be considered beneficial from a consumer's health point of view. Total MUFAs and PUFAs were higher in cheese made from the milk of cows fed the treatment diet. Similarly, the same cheese had significantly higher levels of the total amount of n-6 and n-3 FAs, however, the ratio of n-6:n-3 remained constant at about 4:1. Since human and porcine organs are morphologically and physiologically similar, Duan et al.[52] used porcine as a model to investigate optimal dietary proportions of n-6:n-3 PUFAs and concluded that, overall, n-6:n-3 PUFAs have different effects on lipid metabolism and inflammation and that optimal proportions fall within a range of 1:1 to 5:1. Human consumption of PUFAs is associated with reducing LDL cholesterol, and thus decreasing the risk of coronary diseases.

The SFAs that are of relevance with regards to the already well-known overall beneficial effects of food and that registered as significantly affected by the diet in this study were C16:0 and C18:0. Cheese made from milk from cows on the treatment diet registered lower C16:0 and higher C18:0

when compared to cheese from the control group. In addition, the cheese from the treatment group had lower concentrations of palmitic and myristic acids, both of which have been shown to increase LDL cholesterol, and higher concentrations of stearic acid (C18:0), which did not appear to be associated with adverse health effects.

The MUFAs, n-3 and n-6, and C16:0 and C18:0 all contribute towards the calculations of the atherogenic and thrombogenic indexes of food. In this study, the incorporation of olive cake in the diets of dairy cows had an overall lowering effect on both the atherogenic and thrombogenic indexes, making the cheese from milk of cows fed OC supplement a healthier option. The C18:2 cis and C18:3 n-3 acids both registered significantly higher in the test cheeses.

These essential FAs are also precursors of other FAs with beneficial properties: linoleic acid is essential for the synthesis of arachidonic acid (C20:4, n-6), the precursor of prostaglandins and prostacyclins (involved in the reproductive function) or thromboxanes (playing a role in the haemostasis function). Conjugated linoleic fatty acid (CLA), which is made up of a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds, has been reported to have a wide range of beneficial effects, including anticarcinogenic, antiatherogenic and antiobesity activities. The α-linolenic acid is the precursor of eicosapentaenoic acid which in turn is a precursor of several compounds essential for the heart, retina, brain, and immune system functions. It is possible that this increase in conjugated linoleic fatty acid content is the result of a decreased biohydrogenation rate of oleic and linoleic intermediate by *Butyrivibrio genus* and *B. proteoclasticus.*[53] These changes in the cheeses' FA profile are in partial agreement with those reported by Castellani et al.[54] These authors observed an increase in MUFAs and a decrease in SFAs of milk and cheese whereas no effect of OC on PUFAs was found. They claim that dietary inclusion of olive cake reduced palmitic acid and atherogenic and thrombogenic indices while increasing oleic, vaccenic, stearic, and CLA in both milk and cheese.

In agreement with Vasta et al.,[55] the polyphenols recorded throughout the study period in the cheese from the test group were significantly higher than those detected in the cheese from the control

group. With a 32.9% increase in polyphenols, the cheese from the TEST group has greater functional nutrients and properties than the cheese from the CTR group. The consumption of these bioactive compounds is considered to be beneficial to human health as they enhance the natural defense system by decreasing the formation of free radicals and harmful oxidative events in metabolism.[56] While the changes in the FA profile and polyphenol content can have positive health implications on the consumer, the same observation can influence consumer acceptance due to a potentially altered taste. Thus, the cheeses are currently being evaluated on their organoleptic properties and related results will be published.

The present trial extended over the spring and summer seasons in Ragusa (Sicily). Having a typical Mediterranean climate during which the thermal comfort zone is frequently challenged and at times surpassed during the summer months, animals are prone to experience heat stress due to the combined effect of elevated environmental temperatures coupled with high relative humidity. Heat stress affects the physiological, metabolic, endocrine and molecular mechanisms of the animal, leading to reduced productivity. Two key factors generally trigger these mechanisms: 1) a reduction in feed intake and 2) an increase in water intake.

Such a situation tends to negatively affect milk production and also its composition.[57] Indeed, milk production of dairy cows subjected to chronic heat stress is closely related to dry matter and water intake.[58] Such environmental conditions have a significant influence on the organic and inorganic composition of milk. They also affect cheese yield and quality Bernabucci et al.[59] report a slightly lower casein concentration in milk during the summer when compared to winter which may account for the lower cheese production generally observed during the summer period, while according to Bouraoui et al.[60] cheese made from milk produced in summer has a reduced fat and protein content when compared to that manufactured in spring.

The data obtained in this study indicate that C12:0, C14:0, C18:2 n-6 cis, C18:2 n-6 trans, C18:3 n-3, C20:4 n-6, C20:5 n-3, undefined FAs, Σ n-3, Σ n-6, and Σ PUFA were recorded to be significantly

higher during the cooler spring period, whereas C16:0, C18:3 n-6, Σ FA, Σ MUFA and Σ SFA registered significantly higher in summer.

The effects of heat stress on milk fat content are still not fully understood, and controversial results have been reported in the literature. Bernabucci et al.[59] show a substantial and significant decrease in milk fat during summer as compared to spring. A study by Summer et al.,[61] also observed a decrease in milk fat content during the summer period compared to the autumn. In the case of Cowley et al.,[62] they found no significant differences between cows under normal conditions or under heat stress. The summer period also registered cheese have significantly higher moisture (%), thrombogenic index, and total polyphenols (mg/kg).

Contrary to the conventional school of thought that dictates a lowering of protein content during the summer period, in this study changes in protein did not register as significant.

The statistical model showed that the diet×season interaction had a significant effect on a number of parameters of importance, namely: moisture (%), proteins (%), total lipids (%), total polyphenols (mg/kg), Σ MUFA, Σ SFA, Σ n-3, Σ n-6, AI and TI, C16:0, C18:2 n-6 cis, C18:2 n-6 trans, and C18:3 n-3. The interaction plots for these parameters are reported in Figures 4, 5, 6.

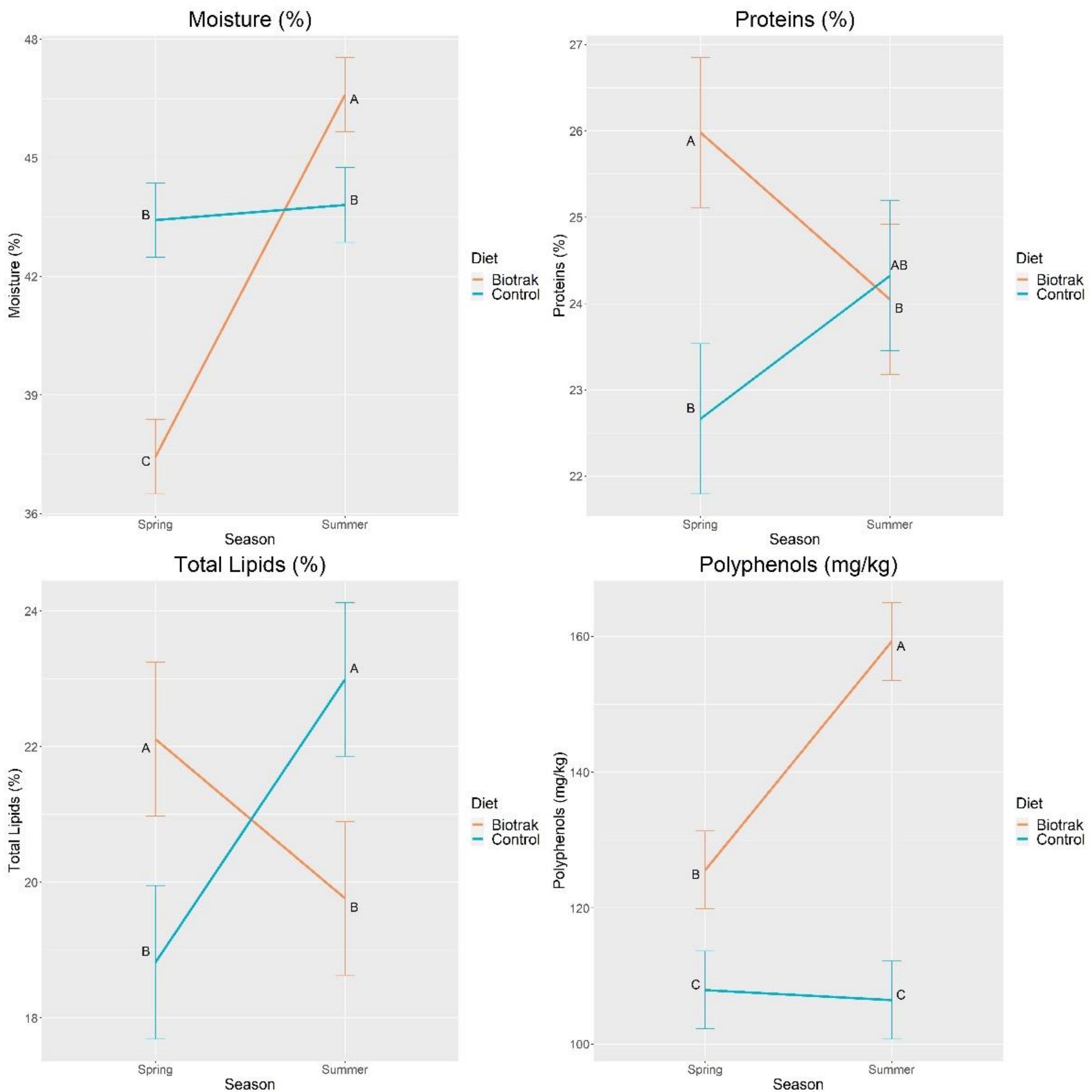

Figure 4. Diagrams of the interaction diet×season for moisture (%), protein (%), total lipids (%), and total polyphenols (mg/kg). Different letters indicate significantly different values according to Tukey-Kramer test.

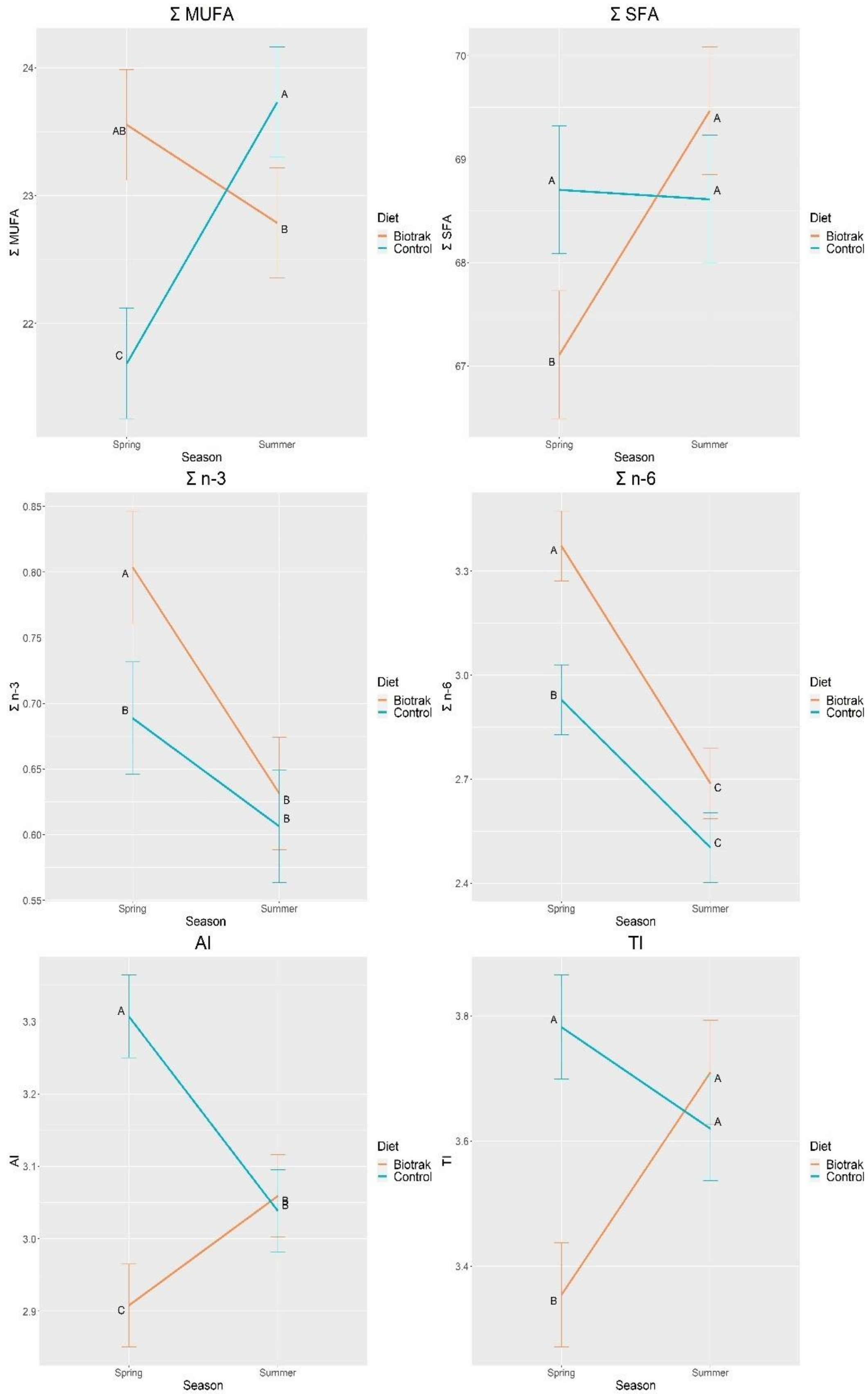

Figure 5. Diagrams of the interaction diet×season for Σ MUFA, Σ SFA, Σ n-3, Σ n-6, and atherogenic and thrombogenic indices (AI and TI). Different letters indicate significantly different values according to Tukey-Kramer test.

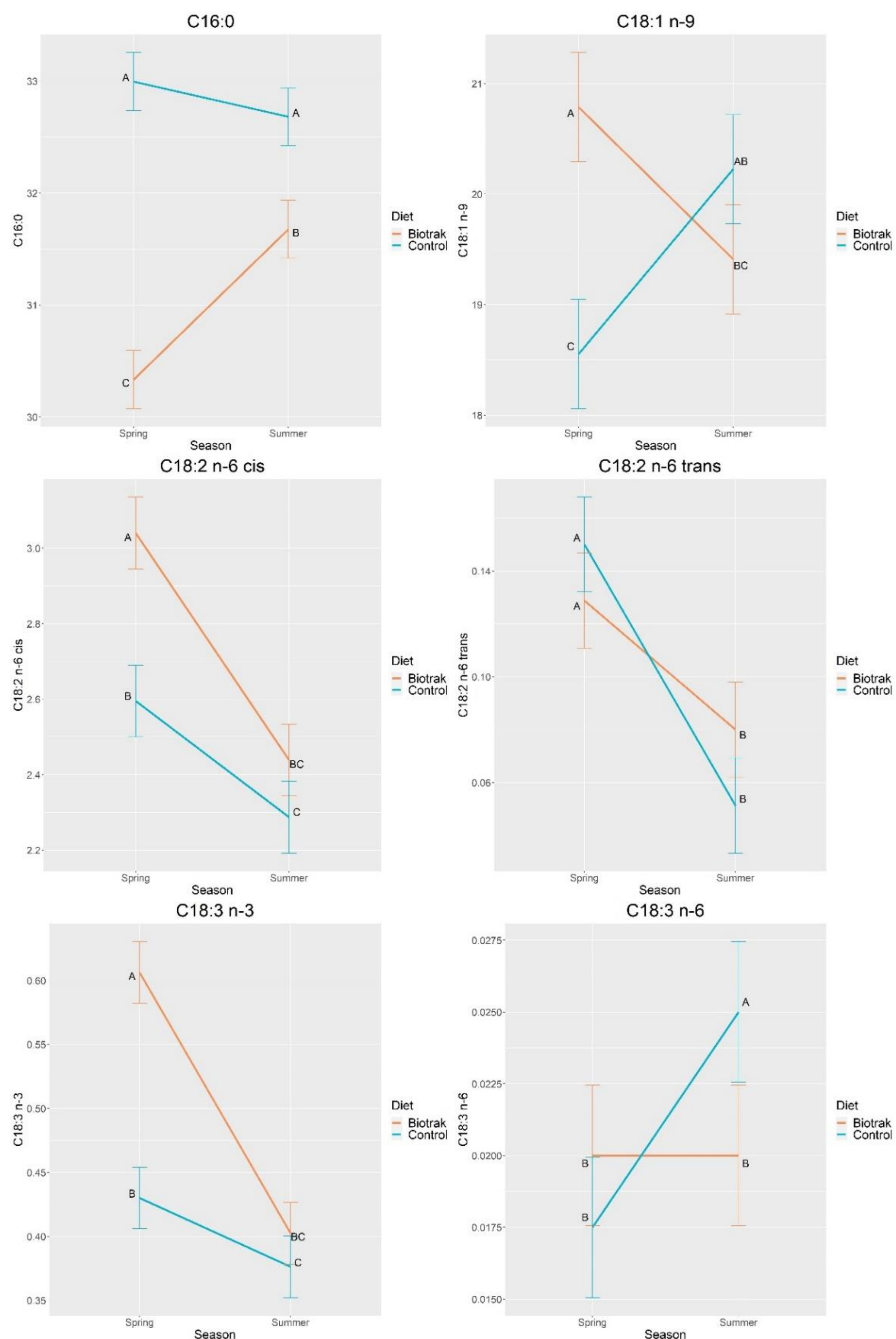

Figure 6. Diagrams of the interaction diet×season for C16:0, C18:2 n-6 cis, C18:2 n-6 trans, and C18:3 n-3. Different letters indicate significantly different values according to Tukey-Kramer test.

The treatment diet registered the following overall highest values for spring: proteins, Σ n-3, Σ n-6, C18:2 n-6 cis, C18:3 n-3, C18:3 n-6, while registered the lowest value for C16:0, atherogenic and thrombogenic indexes, moisture and Σ SFA. With regards to the summer period the treatment diet registered the overall highest for moisture and total polyphenols and the overall lowest for Σ n-3.

The control diet registered the following overall highest values for spring: C16:0, C18:2 n-6 trans, atherogenic and thrombogenic indexes, and Σ n-6, while registered the lowest value for proteins, total lipids, polyphenols, and Σ MUFA.

With regards to the summer period the treatment diet registered the overall highest total lipids, moisture, and Σ MUFA and the overall lowest for C18:2 n-6 trans, C18:3 n-3, polyphenols, Σ n-3 and Σ n-6.

Literature on the interactions of diets incorporating olive cake with season on dairy cows is scarce. The results presented in Table 22 show an element of inversion between diet type and season.

Table 22. Least square mean and standard error of the mean (SEM) of the main parameters of interest together with the respective p-value for diet×season interaction effect.

	TEST		**CTR**		**SEM**	**p-values**
	Spring	**Summer**	**Spring**	**Summer**		
Moisture (%)	37.44	46.60	43.43	43.81	0.481	<0.0001
Protein (%)	25.98	24.05	22.66	24.32	0.444	0.0004
Total Lipids (%)	22.11	19.76	18.82	22.99	0.578	<0.0001
Total Polyphenols (mg/kg)	125.61	159.24	107.94	106.45	2.922	<0.0001
C16:0	30.33	31.68	33.00	32.68	0.132	<0.0001
C18:1 n-9	20.79	19.77	18.43	19.91	0.252	<0.0001
C18:2 n-6 cis	3.04	2.44	2.60	2.29	0.049	0.0053
C18:2 n-6 trans	0.129	0.080	0.150	0.051	0.009	0.0108
C18:3 n-3	0.606	0.403	0.430	0.376	0.012	<0.0001
Σ MUFA	23.55	22.79	21.69	23.73	0.220	<0.0001
Σ n-3	0.80	0.63	0.69	0.61	0.022	0.0489
Σ n-6	3.37	2.69	2.93	2.50	0.052	0.0181
Σ SFA	67.11	69.47	68.70	68.61	0.315	0.0006
Atherogenic Index	2.91	3.06	3.31	3.04	0.029	<0.0001
Thrombogenic Index	3.35	3.71	3.78	3.62	0.042	<0.0001

Nonetheless, there tends to be a strong indication that the cheese with the highest nutritional benefit was manufactured from milk produced by the test diet in spring, while the one with the least nutritional benefit is the one from the control group from milk produced in spring. The data further indicates that irrespective of season, the cheeses with the highest nutritional benefit are those manufactured with milk from cows fed the treatment diet with the incorporation of olive cake.

5.3.4. Conclusions

Increased awareness of the need to reduce environmental impact and make better use of available resources has led researchers to focus on the possibility of identifying by-products of the agro-food chain as potential sources of active compounds with potential health benefits. The diversion of these identified by-products from the waste stream makes a significant contribution to environmental sustainability. As such, their use would benefit both human health and the environment by reducing the need for disposal. In the context of the circular economy, the olive oil processing cycle could serve as an example of the reuse of by-products from agri-food waste. The results of this study indicate that the incorporation of olive cake in the diet of lactating dairy cows has a significant effect on reducing the atherogenic and thrombogenic indexes, while also contributing to increasing the total polyphenols in the cheese product. These innovative and functional features are likely to be welcomed and appreciated by consumers, both for their health benefits and for the incorporation and reuse of biomass waste, which also contributes to the well-being of the environment and, ultimately, of all citizens. This study has again highlighted that the qualitative composition of cheeses is significantly influenced by the season. Therefore, cheeses, especially those with a quality guarantee mark, must clearly indicate whether they are summer or winter cheeses, in order to facilitate consumer information and ultimately choice. This will be in line with the requirement to include nutrition and health information on the label in accordance with Regulation (EC) No 1924/2006.[63] This would be the final objective, i.e. to provide direct information to the final consumer.

5.4. EFFECT OF OLIVE BY-PRODUCTS FEED SUPPLEMENTATION ON MICROBIOLOGICAL PROFILE OF PROVOLA CHEESE

The aim of this work was to evaluate the effects of dietary supplementation on microbiological profiles of olive cake and Provola cheese. The two groups were called Control (Ctr) and Experimental (Exp) and were previously described in section 5.1.1.

5.4.1. Microbiological analysis and DNA isolation

The microbiological analysis of the control and experimental 1-day cheese samples, collected monthly from March to July, was performed according to Commission Regulation (EC) No 2073/2005 of 15 November 2005.[64] In detail, both core and surface sections (25 g) were taken from each cheese sample and processed as described above. Microbiological counts were performed using the following media and agar conditions: Kanamycin Aesculin Azide (KAA) Agar, incubated aerobically at 37°C for 24-48 h, for enterococcal counts; Mannitol Salt Agar (MSA), incubated at 32°C for 48 h, for staphylococcal counts; M17, supplemented with 5 g/L lactose, incubated at 30°C for 24-48 h, for lactococci; de Man Rogosa and Sharpe agar (MRS), adjusted to pH 5.4, incubated at 32°C for 72 h under microaerophilic conditions, for lactobacilli; and Sabouraud Dextrose Agar (SDA), supplemented with chloramphenicol (0.05 g/L) incubated at 25°C for 3–5 days, for yeasts and molds count. VRBA and PCA were used as previously described for Enterobacteria, faecal coliforms and total mesophilic aerobic bacteria count. Listeria monocytogenes (ISO (International Organisation for Standardisation) (ISO 11290-1:2017) was enumerated as previously reported. Furthermore, according to the International Organisation for Standardisation (ISO), a two-step enrichment method was used for the detection of Salmonella spp. (ISO 6579-1:2017). Control and experimental cheeses were subjected to total bacterial DNA isolation using the DNeasy® mericon® Food kit (Qiagen, Germany) following the manufacturer's instructions.

The DNA concentration was determined with the Qubit 4.0 fluorometer (Invitrogen, Carlsbad, CA, USA) before being stored at -20°C until use.

The results of the microbiological analysis were calculated as mean values of three determinations and standard deviations and were expressed as log cfu/g.

DNA isolated from the cheese samples was subjected to meta-taxonomic analysis of the 16S rRNA gene. Specifically, the V3 region of the 16S rRNA gene sequence was amplified using the Probio-Uni e/Probio-Rev primer pair (Milani et al., 2013) and subjected to MiSeq (Illumina) by GenProbio srl (Parma, Italy). Bioinformatic analysis was performed as reported by Vaccalluzzo et al.[65] The raw 16S rRNA data from the study were deposited at the NCBI Sequence Read Archive (SRA1) under accession code PRJNA 903126.

5.4.2. Statistical analysis

With the aim of searching for statistically significant changes in alpha and beta diversity estimates and starting from Bray-Curtis, Jaccard, Weighted UniFrac, Unweighted UniFrac distance matrices, QIIME II nested plugins were used. The PERMANOVA test was computed in same software environment. Taxa abundance in the two compared groups was inspected by mean of a compositionality-aware test, i.e., the Analysis of Composition of Microbiomes test (ANCOM), but also with the White's non-parametric test corrected for multiple tests. In the case of White's test, results have been shown in terms of boxplot.

5.4.3. Results

Results of microbiological analysis carried out on both control and experimental cheese samples are shown in Table 23. Overall, *Salmonella spp*., *L. monocytogenes, E. coli*, fecal coliforms, and molds were never detected in the analyzed samples, except for the control cheese collected in July, where coliforms reached the value of 2.00 log cfu/g.

Lactic acid bacteria (LAB) achieved an average value of about 6.84 log cfu/g in control samples and of 7.19 log cfu/g in experimental cheeses.

In detail, control samples reached the highest values (7.99 log cfu/g) in April and the lowest (6.04 log cfu/g) in June, while the experimental ones achieved the highest (7.96 log cfu/g) in April and the lowest (6.55 log cfu/g) in July (Table 23).

The lactococci group showed the same average value (about 8.00 log cfu/g) in both samples, while marked fluctuations were revealed during sampling period in the counts of enterococci, Enterobacteriaceae and coagulase-positive staphylococci groups (Table 23), with the lowest average value always found in the experimental samples.

Significant differences ($p \leq 0.05$) among control and experimental samples were revealed for total mesophilic bacteria in April and May, while the yeast count showed similar values in both samples, reaching average value of 5.17 log cfu/g (Table 23).

Table 23. Microbial counts of cheese samples.

Microbial groups	March		April		May		June		July		Mean	
	Ctr	Exp	Ctr	Exp	Ctr	Exp	Ctr	Exp	Ctr	Exp	Ctr	Exp
Enterococcus spp.	<1	<1	3.00±0.06^{a}	2.43±0.18^{b}	<1	<1	2.96±0.17^{a}	<1^{b}	4.69±0.25^{a}	2.93±0.04^{b}	2.13±2.05^{a}	1.07±1.48^{b}
Enterobacteriaceae	2.61±0.48^{b}	3.35±0.49^{a}	5.08±0.13^{a}	3.66±0.12^{b}	2.04±0.06	2.13±0.18	<1b	2.66±0.08^{a}	2.70±0.13^{a}	<1^{b}	2.49±1.81	2.36±1.45
Coagulase-positive staphylococci	<1^{b}	2.39±0.75^{a}	2.31±0.01^{a}	<1^{b}	<1	<1	2.84±0.01^{b}	4.08±0.13^{a}	3.16±0.97^{a}	<1^{b}	1.66±1.55	1.29±1.87
Total mesophilic bacteria	2.81±0.10	3.33±0.47	5.93±0.03^{a}	5.06±0.41^{b}	6.62±0.16a	5.74±0.12^{b}	4.84±0.04^{b}	8.03±0.08^{a}	6.59±1.57	7.20±0.01	5.35±1.61	5.87±1.84
Yeasts	4.43±0.06	4.39±0.56	6.67±0.21	6.24±0.09	3.51±0.05	3.20±0.28	4.80±0.03	5.07±0.15	6.61±0.38	6.78±0.02	5.20±1.41	5.14±1.43
Lactococcus spp.	6.92±0.18	7.33±0.02	8.77±0.10	8.86±0.12	6.96±0.05b	7.70±0.24^{a}	8.79±0.01^{b}	8.68±0.16^{a}	8.73±0.25^{a}	7.47±0.06^{b}	8.03±0.79	8.01±0.63
Lactic Acid Bacteria	6.95±0.07	7.16±0.37	7.99±0.02	7.96±0.02	6.76±0.07b	7.47±0.09^{a}	6.04±0.06^{b}	6.80±0.07^{a}	6.44±0.01	6.55±0.16	6.84±0.65^{b}	7.19±0.50^{a}

Data are presented as mean log cfu/g ± standard deviation, based on 3 replicates. Different superscript letters within the same line indicate significant differences at $p < 0.05$ between control and experimental samples for each sampling month.

5.4.4. Read statistics and taxonomic annotation

High-throughput sequencing output determined the relative abundance of bacterial species in Provola cheese samples esteemed based on denoised and taxonomically assigned reads. A total of 496,430 reads from 10 cheese samples were retained after denoising steps. The mostly abundant genus was *Streptococcus*, which accounts for the great majority of relative abundance (99%) and the contribution of other detected taxa was very low. Noteworthy, among those genera that contributed with abundance percentages lower than 1%, *Lactobacillus*, *Lactococcus*, and *Acinetobacter* were found in all samples and can thus be considered as "core" taxa, whereas unclassified genera belonging to the family of *Moraxellaceae* and *Chromohalobacter* were not evenly distributed. Specifically, the *Chromohalobacter* genus was only detected in the experimental sample gathered in June.

The great majority of taxa resulted not statistically significant in the performed Welch's statistical test. The only one statistically significant difference found at the phylum level was between the control and treated groups sampled during the summer seasons. No other significant taxa were found when other taxonomic levels were inspected.

5.4.5. Discussion

Overall, microbiological data revealed no significant effects of experimental diet on cultivable microbiota. We hypothesize that in addition to the shared milking, cheese-making, the starter cultures contributed to concealing the effect of diet strategy. However, the slight mean decrease of Enterococci and coagulase-positive staphylococci groups in experimental cheeses could be attributed to polyphenols content of olive cake, which can exert an inhibitory effect on microorganisms.[66] In addition, Milutinović et al.[67] demonstrated the inhibitory effect of polyphenols against several pathogens, including *E. faecalis*. These findings were almost confirmed by the16S taxa assignment, where really few evidence emerged.

Only one statistically significant taxon, i.e., Proteobacteria, was found to be decreased in experimental sample during the summer season ($p<0.05$). Although this phylum abundance is not supported by statistical significance at lower taxonomic levels, it could reasonably involve the *Chromohalobacter* and *Moraxellaceae* taxa, embed as minor contributors in cheeses, most largely present in both the equipment and environments from the production area.

Within the *Moraxellaceae* family, the presence of *psychrotrophic* bacteria genus *Acinetobacter*, detected as a core taxon, by 16S rRNA analysis, could be linked to proteases milk spoilage components in un-ripened cheese. The alpha and the beta diversities, estimated based on various computed distances, did not show neither a divergent behavior of sample collapsed by fed groups, nor a separation by month/season.

5.4.6 Conclusion

In this study, the feeding of dairy cows with an olive oil cake supplement resulted in several advantages, such as a better product quality. In addition, an improvement in the microbiological quality of the experimental cheeses was obtained, which was related to the reduction of certain microbial groups associated with spoilage and did not affect the main taxa. In conclusion, this work sheds light on the use of the by-product of olive oil cake in agricultural practices as a management aimed at obtaining a more sustainable and safer dairy food product with a lower environmental impact.

5.5. QUALITY OF SICILIAN FRESH CHEESE MADE USING A VEGETABLE COAGULANT FROM KIWI FRUIT COMPARED TO CONVENTIONAL COAGULANTS

In the Sicilian dairy sector in recent years have focused on the development of innovative processes and products as an alternative to traditional ones. The combination of factors such as animal breed, climate, variety and plant composition of natural pastures, and all the cultural and historical aspects that characterize a specific geographic area, leads to the production of a wide variety of typical cheeses, linked to their territory of origin, with the possibility of obtaining distinctive quality marks. In particular, the Sicilian fresh cheeses are widely produced and appreciated throughout the country. With this in mind, the aim of this work was to assess the quality (in terms of nutritional properties and sensory profiles) of fresh Sicilian cheeses produced with a mixture of cow's/sheep's milk and a vegetable coagulant derived from kiwi extract (cK), as well as with traditional calf rennet (cT) and Halal-certified microbial rennet (cH).

5.5.1. Preliminary study on a laboratory scale

This study is part of the CHEESHAL project, which aims to design, test and integrate innovative production processes to diversify and increase cheese production by opening up new cheese products made with vegetable rennet. As part of this project, Nicosia et al.[68] carried out a preliminary study to develop a rapid and economical preparation of aqueous kiwi extract for use in laboratory-scale cheese production. Specifically, they prepared the aqueous extract from kiwi fruit, determined the presence of *actinidin* in fruit tissues at different levels of ripeness, evaluated the effect of temperature and pH on milk clotting activity and proteolytic activity, and conducted a laboratory-scale cheese-making trial using different amounts of aqueous extract from kiwi pulp.

The data reported by Nicosia et al.[68] showed the presence of the enzyme in both the skin and pulp of the fruit; the aqueous extract obtained from the pulp showed hydrolytic activity towards κ-casein. A temperature of 40°C combined with a pH of 5.5 gave the best performance in terms of coagulation and proteolytic activity of the kiwi pulp extract. The data showed a higher hydrolytic activity of the

enzyme preparation obtained from ripe kiwi than from unripe kiwi, suggesting the use of ripe kiwi pulp extract in laboratory scale cheese production. The data also showed that 3% (v/v) ripe kiwi pulp extract resulted in a curd yield of 20.27%, comparable to the yields obtained using chymosin (20.93%) and microbial coagulant (20.06%).

Given these excellent conditions, the present study analysed cheese samples produced according to the laboratory-scale cheesemaking protocol proposed by Nicosia et al.[68] in order to assess the quality of the products obtained on an industrial scale.

5.5.2. Sampled cheeses and coagulants

Sicilian fresh cheese samples were collected in May 2022 from three different batches in a commercial dairy in Randazzo, Sicily. They consisted of nine cheeses (3 x each batch) made with vegetable coagulant from kiwi (cK), nine cheeses made with traditional calf rennet (cT) and nine cheeses made with Halal-certified microbial rennet (cH).

The cheeses were made from mixed milk (50% Pezzata Rossa cow's milk and 50% Pinzirita sheep's milk). The cheeses were tasted after 7 days of ripening. The cheeses weighed 0.5 kg each and were cylindrical in shape. After removing 0.5 cm of rind, portion of 200 g was sampled and finely ground for chemical composition and the remaining cheese for sensory analysis.

The commercial factory that supplied the cheese also provided the following information about the coagulants. The vegetable coagulant used in cheesemaking was a kiwifruit aqueous extract developed by Nicosia et al.[68]

Briefly, it was obtained by the following procedure: the fruits were washed, peeled, and weighted. The aqueous extract of kiwifruit pulp was obtained by pressure using a manual stainless-steel press. The obtained juice was filtered twice through two layers of sterile gauze to separate all the seeds and the coarse content from aqueous extract.

Traditional calf rennet was a dark brown paste with a composition of 75% chymosin and 25% pepsin. The coagulating power of the rennet was 115 IMCU. The calf rennet was derived from lamb

stomachs and was free from microbial coagulants, pig pepsin and fermentation chymosin. It is not derived from genetically modified organisms, in accordance with EC Regulation 1830/2003 and the traceability and labelling of genetically modified foods.[69]

The microbial rennet (Halal-certified) was obtained from the controlled fermentation of *Rhizomucor miehei*, one of the best substitutes for calf rennet due to its specificity in cleaving k-casein peptide bonds. The microbial rennet was a brownish granulate, had a coagulation strength of 2400 IMCU and contained preservatives including up to 1% sodium benzoate and sodium chloride. The rennet did not contain genetically modified recombinant DNA enzymes in accordance with Regulation (EC) cited above. The rennet was Halal certified. Figure 7 shows the three types of cheeses obtained.

Figure 7. Cheeses produced with three different types of coagulant.

5.5.3. Chemical Analysis

Cheese samples were analyzed for pH, moisture, total proteins, total lipids, fatty acids profile, sterols, total polyphenols, individual polyphenols, and mineral elements. All details of the analyses are shown in Table 24. All samples were analyzed in triplicate. Details of HPLC-MS/MS method validation are given in Table 25.

Table 24. Determination of pH, moisture, total proteins, total lipids, fatty acid (FA), sterols, total polyphenols and minerals in Sicilian fresh cheese samples.

Analysis	Methods	Technique or instrumentation	References
pH	pH was measured with a portable digital pH meter, in 100 ml water solution containing 10 g cheese.	Digital pH meter (model 3510, Jenway, UK)	70
Moisture	The water content of the cheese was determined by thermogravimetry using a thermobalance.	Sartorius MA35 thermobalance (Milan, Italy)	71
Total proteins	Crude protein was determined by Kjeldahl method. Briefly, the samples were digested with sulphuric acid (98%), copper (II) selenite dihydrate and potassium sulphate using a SpeedDigester K-439 equipped with a scrubber for gases and then analyzed using a KjelMaster System K-375.	SpeedDigester K-439 (Büchi, Switzerland); KjelMaster System K-375 (Büchi, Switzerland)	72
Total lipids and FA	Total lipid extraction was performed by Folch methods. Briefly, 10 g of cheese were homogenized with 2:1 chloroform:methanol (v/v) mixture, and then a 0.73% NaCl water solution was added. The lipid fraction was taken and subjected to hot esterification with 9:1 methanol:sulfuric acid (v/v) mixture in an oven for one hour at 110 °C. The supernatant was recovered and diluted to 1:2 with hexane and analyzed by GC-FID.	GC-FID (Dani Master GC, Dani Instrument, Milan, Italy)	73
Sterols profile	Extraction was performed according to the method described in EU Regulation 1348/2013. The total lipids were saponified with an ethanol-potassium hydroxide solution after the addition of an internal standard (5α-cholesterol). The unsaponifiable matter was extracted with diethyl ether. The sterol fraction was separated by chromatography on a silica gel plate. This fraction was analyzed by gas chromatography after derivatization by BSTFA: TMCS 99:1 to obtain the corresponding trimethylsilyl ethers.	GC-FID (Dani Master GC, Dani Instrument, Milan, Italy)	74
Total polyphenols	Approximately 5 g of the homogenized sample was added to 10 mL of methanol/water (80/20, v/v). The two phases were separated by centrifugation. The supernatant was transferred to a flask, evaporated and dissolved in 5 mL of acetonitrile. The acetonitrile solution was finally evaporated to dryness by rotation. The mixture was then redissolved in 5 mL of methanol. The Folin-Ciocalteu method was used to determine the total polyphenols content spectrophotometrically. A 1 mL aliquot of each extract was mixed with 5 mL of H_2O and 1 mL of Folin-Ciocalteu phenol reagent (1N). A total of 10 mL of saturated Na_2CO_3 solution (7.5 %) and 5 mL of H_2O were then added and allowed to stand for 90 minutes in the dark. Absorbances were read using a UV-visible spectrophotometer at 760 nm with methanol as analytical blank. Appropriate dilutions of a gallic acid standard solution were used to construct a calibration curve. Determinations were carried out in triplicate together with the blank solutions.	UV-visible spectrophotometer (UV-2401PC Shimadzu)	75
Polyphenols profile	Individual polyphenols (gallic acid, caffeic acid, coumaric acid, cinnamic acid, quercetin, and catechin) were determined by HPLC-MS/MS analysis. Briefly, 20 µL of each sample extract was analysed using an HPLC-MS/MS system and a Zorbax SB-C18 (5microm 4.6 × 250 mm) reverse phase column. Gradient elution was solvent A (0.1% formic acid in water) and solvent B (methanol) at a flow rate of 1 mL/ min. The initial mobile phase composition was 0–3 min, 5–20% B; 3–8 min, 20–20% B; 8–12 min, 20–30% B; 12–13 min, 30–40% B; 13–15 min, 40–40% B; 15–17 min, 40–50% B; 17–19 min, 50–80% B; 19–22 min, 80–5% B. All solutions prepared were filtered through 0.45 µm filters.	HPLC system (Shimatzu, Kyoto, Japan) and MS-8040 triple quadrupole mass spectrometer with Electrospray ionisation (ESI) source.	76
Mineral elements	0.5 g of sample was mineralized with HNO_3 and H_2O_2 using a microwave digestion system with a temperature of 0-200 °C in 10 min (step 1) and 200 °C maintained for 10 min (step 2). The digested samples were then diluted with internal standard in ultrapure water and analyzed by a single quadrupole inductively coupled plasma mass spectrometer (ICP-MS). An external calibration procedure combined with internal standard normalization was used for quantification.	Microwave digestion system (Ethos 1, Milestone, Bergamo, Italy); ICP-MS iCAP Q (Thermo Scientific, Waltham, MA, USA)	77

Table 25. List of the investigated polyphenols with monitored ions (m/z), linearity, LOD, LOQ.

Compound	$[M-H]^-$ (m/z)	Products (m/z)	R^2	LOD (mg/kg)	LOQ (mg/kg)
Gallic acid	168.9	79, 125	0.9986	1.0	3.3
Caffeic acid	178.9	135, 117	0.9936	1.0	3.3
Coumaric acid	163.0	119, 93	0.9995	0.5	1.7
Cinnamic acid	149.0	131,103	0.9931	0.5	1.7
Quercetin	300.9	150.7, 96	0.9990	1.0	3.3
Catechin	288.9	245, 205	0.9987	0.5	1.7

*Underlined ions were considered for quantitative analysis.

5.5.4. Sensory analysis

Descriptive sensory analysis was conducted according to ISO 13299:2016 in order to evaluate the effect of the coagulant types on the sensory profile of cheeses. The panel consisted of 11 members (6 women and 5 men, aged between 27 and 60 years). The number of panellists was relatively limited to ensure a comparable level of experience to ensure accuracy, sensitivity, and repeatability of the judgement, as well as to highlight any particular shortcomings or strengths of the products. The panellists were selected from among technicians, researchers, and professors at the University of Messina with a solid experience in the food sector, including sensory analysis, although they were not specifically trained in cheese evaluation.

Considering the three types of cheeses analysed, the attributes considered were colour intensity, odour intensity, salty, bitter, hardness, and chewiness. Cheeses were presented as 20 g cubes and assessed in individual booths with a room temperature set at 21 ± 2 °C, neutral coloured wall and furniture, and under standard lighting conditions.

Triplicate sensory tests were carried out. Panellists were asked to drink plain water at the beginning of the sensory evaluation and between the samples to try to make the palate conditions for each sample for each sample. The intensity of each attribute was measured on a linear, unstructured scale from 0 (absence of the parameter) to 8 (maximum intensity of the parameter). At the end of sensory evaluation, panellists were asked to rank the overall cheese acceptability.

5.5.5. Statistical analysis

SPSS 13.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. One starting multivariate matrix was constituted by 27 cases (samples under analysis) and 73 variables (chemical composition, sterols, total and single polyphenols, fatty acids and mineral elements determined in analyzed samples). The data were subdivided into three groups according to the coagulant used (K is vegetable rennet by kiwifruit extract; H is microbial rennet Halal-certified; and T is traditional calf rennet). Initially, the one-way ANOVA with Kruskal-Wallis post hoc multiple comparison test was used for study the significance of the differences among cheese samples from different rennet.

The data set was normalized and subjected to the Principal Component Analysis (PCA). Previously, Kaiser-Meyer-Olkin test (KMO test) and Bartlett's test, were carried out to verify the adequacy of the initial data.

5.5.6. Results of chemical analysis

The chemical parameters of the three cheese groups, including pH, moisture, total lipids, protein, sterols, total and single polyphenols, were statistically different among the groups ($p<0.05$) and are shown in Table 26. The cK group cheeses had significantly lower values for pH (5.09), total lipids (27.56 g/100g), protein (22.01 g/100g), cholesterol (84.45 mg/100g) and significantly higher values for moisture (46.51 g/100g) and polyphenols (441.16 mg/kg). On the other hand, the cH and cT cheese groups had significantly lower values for moisture (37.49 g/100g and 38.34 g/100g) and polyphenols (155.04 mg/kg and 136.33 mg/kg) and significantly higher values for pH (5.79 and 5.95), total lipids (30.66 g/100g and 31.70 g/100g), protein (26.84 g/100g and 25.15 g/100g) and cholesterol (98.69 mg/100g and 99.25 mg/100g).

The individual polyphenols were selected on the basis of the literature and the standards available to us, which is why the number is rather small, but still provides a basis for further investigation. The results obtained in this study with regard to the polyphenols are interesting; the cK group

contained coumaric acid and cinnamic acid, which were not present in the cheeses of the cH and cT groups; furthermore, only gallic acid was not significantly different among the three groups, whereas caffeic acid, quercetin and catechin were significantly higher in the cK group. The results are in line with the findings of Serra et al.[75]

The chemical composition varied significantly among the groups; certainly, the type of coagulant used in the cheesemaking process influenced these parameters. However, possible variations could also be due to processing techniques, incubation and coagulation times and rind temperature. In fact, the pH may vary depending on the strength of the coagulants used, as suggested by Tabet et. al.[78] The higher moisture content in the cK group could be due to difficulties in draining the whey due to clogging of the drainage filter by fine particles. Another reason could be the longer coagulation time for plant proteases, resulting in a higher moisture content in the final product.[79] The higher syneresis of the curd obtained with microbial rennet Halal-certified and traditional calf rennet is responsible for the lower moisture content of these cheeses compared to those obtained with vegetable rennet with kiwi extract (Table 26). This is consequently related to the higher content of lipids and proteins of the cH and cT groups due to a "concentration" effect.

Table 26. Chemical composition, sterols, total and individual polyphenols content, SEM (Standard Error of Mean) and p-value in Sicilian fresh cheese according to the coagulant used.

	C_K	C_H	C_T	SEM	p-value
pH	5.09 ^{A}	5.79 ^{B}	5.95 ^{B}	0.018	**0.002**
Moisture (g/100g)	46.51 ^{B}	37.49 ^{A}	38.34 ^{A}	0.019	**0.000**
Total lipids (g/100g)	27.56 ^{A}	30.66^{B}	31.70 ^{B}	0.012	**0.000**
Proteins (g/100g)	22.01 ^{A}	26.84 ^{B}	25.15 ^{B}	0.017	**0.000**
Total polyphenols (mg/kg)	441.16 ^{B}	155.04 ^{A}	136.33^{A}	0.103	**0.000**
Sterols (mg/100g of cheese)					
Cholesterol	84.45 ^{A}	98.69 ^{B}	99.25 ^{B}	0.015	**0.000**
Stigmasterol	0.19	n.e.	n.e.	-	-
Campesterol	0.14	n.e.	n.e.	-	-
β-sitosterol	0.49	n.e.	n.e.	-	-
Individual polyphenols (mg/kg)					
Gallic acid	45.37	45.33	45.23	0.084	0.815
Caffeic acid	50.77^{B}	15.33^{A}	14.21^{A}	0.018	**0.000**
Coumaric acid	36.51	n.e.	n.e.	-	-
Cinnamic acid	22.74	n.e.	n.e.	-	-
Quercetin	14.72^{B}	7.62^{A}	7.55^{A}	0.026	**0.000**
Catechin	15.67^{B}	5.22^{A}	5.53^{A}	0.031	**0.000**

Letters A and B indicate homogeneous groups at α=0.05: sample types which do not differ from each other are designated by same letter. Bold values are significant at p<0.05. n.e.: not estimable.

The use of kiwi rennet resulted in a significant increase in total polyphenols (Table 26). The kiwi extract produced cheeses that were 2.7 and 3.4 times richer in polyphenols than cheeses from the cH and cT groups, respectively. The increased concentration of total polyphenols in cheeses made with vegetable coagulants is widely confirmed in the literature.[75]

This result is encouraging as it demonstrates the possibility of enriching cheese with a very important nutraceutical component.

Cheeses from the cK group contained not only a lower cholesterol content (closely related to their composition), but also some phytosterols such as stigmasterol, campesterol and β-sitosterol.

This result was also obtained by Serra et al.[75] in buffalo and sheep's milk cheeses made with the addition of kiwi juice as a coagulant.

The results show that the use of kiwi extract results in the transfer of small but not negligible amounts of these compounds, whose cholesterol-lowering properties are well known. However, the enrichment is not such as to suggest a possible direct nutraceutical effect on humans.

The FA profile of the cheeses is typical of products made from mixed cow and sheep milk. The FA composition of the analyzed cheeses expressed as % of total FA is shown in Table 27.

Table 27. Fatty acid composition expressed as % of total FA, SEM (standard error of mean) and p-value of different cheese types.

	C_K	C_H	C_T	SEM	p-value
C4:0	1.91	1.71	1.60	0.021	0.552
C6:0	2.68	2.35	2.39	0.311	0.060
C8:0	2.79	2.71	2.51	0.214	0.059
C10:0	7.87	7.76	7.28	0.126	0.091
C11:0	0.09	0.09	0.07	0.023	0.585
C12:0	4.43	4.31	4.28	0.053	0.145
C13:0	0.11	0.14	0.13	0.026	0.461
C14:0	10.88	11.83	11.06	0.027	0.467
C15:0 *iso**	0.31	0.34	0.33	0.024	0.126
C15:0 *anteiso**	0.53	0.60	0.54	0.034	0.279
C15:0	1.23	1.22	1.27	0.025	0.461
C16:0	26.15	26.41	26.71	0.022	0.168
C17:0	0.72	0.81	0.82	0.022	0.558
C18:0	9.33	9.78	9.85	0.025	0.557
C20:0	0.30	0.27	0.31	0.028	0.452
C21:0	0.09	0.11	0.10	0.023	0.555
C22:0	0.16	0.18	0.21	0.025	0.454
C23:0	0.10	0.09	0.09	0.022	0.356
C24:0	0.09	0.11	0.11	0.024	0.386
C14:1	0.42	0.43	0.41	0.020	0.549
C15:1	0.01	0.01	0.01	0.020	1.000
C16:1	1.19	1.11	1.10	0.038	0.164
C16:1 *trans**	0.20	0.20	0.23	0.029	0.284
C16:1 n-5	0.17	0.16	0.13	0.026	0.195
C17:1	0.32	0.30	0.30	0.020	0.130
C18:1 *cis*9	16.79	16.84	16.87	0.031	0.289
C18:1 *trans*9	0.24	0.31	0.31	0.025	0.163
C18:1 *cis*11*	2.62	2.56	2.11	0.029	0.125
C18:1 *trans*11*	0.49	0.57	0.51	0.024	0.557
C20:1 n-9	0.04	0.03	0.05	0.037	0.368
C22:1 n-9	0.03	0.03	0.02	0.047	0.110
C24:1 n-9	0.02	0.04	0.02	0.058	0.174
C16:3 n-4*	0.50	0.41	0.49	0.020	0.471
C18:2 *cis*9 *cis*12	1.63	1.33	1.61	0.119	0.063
C18:2 *trans*9 *trans*12	0.70	0.71	0.67	0.057	0.158
C18:3 *cis*6 *cis*9 *cis*12	0.05	0.03	0.04	0.076	0.099
C18:3 *cis*9 *cis*12 *cis*15	1.26	1.30	1.28	0.035	0.513
C20:2 n-6	0.01	0.01	0.03	0.064	0.161
C20:3 n-6	0.03	0.02	0.04	0.085	0.197
C20:3 n-3	0.01	0.02	0.02	0.062	0.202
C20:4 n-6	0.11	0.07	0.11	0.049	0.167
C20:5 n-3	0.09	0.07	0.11	0.033	0.478
C22:2	0.02	0.03	0.02	0.159	0.080
C22:6 n-3	0.05	0.02	0.05	0.078	0.181
SFA	69.81	70.83	69.67	0.121	0.067
MUFA	22.54	22.59	22.07	0.022	0.563
PUFA	4.48	4.02	4.47	0.030	0.474
n3	1.42	1.42	1.46	0.025	0.458
n6	2.54	2.16	2.49	0.024	0.571
n6/n3	1.79	1.52	1.71	0.035	0.361

* Individual fatty acids not included in Mix 37 FAMEs.

The average content of saturated fatty acids (SFA) was 70%, while the average content of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) was 22% and 4%, respectively. Among SFA, palmitic acid (C16:0) was most representative in all three groups, followed by myristic acid (C14:0) and stearic acid (C18:0); among MUFA, oleic acid (C18:1 cis9) followed by cis- and trans-vaccenic acid (C18:1 cis11 and C18:1 trans11).

Among PUFA, linoleic and α-linolenic acids (C18:2 cis9 cis12 and C18:3 cis9 cis12 cis15) were the most representative in all three groups. Fatty acids were not found to be significantly different in the three groups ($p>0.05$). Thus, the type of coagulant appears not to be a significant factor in the variation of fatty acids. However, the slight difference in FA composition could be related to the fact that calf rennet and halal-certified cheeses contained less water and, therefore, more dry matter and more lipids for the reasons mentioned above. In other words, as expected, the use of kiwi pulp does not lead to any significant variation in FA content. Even α-linolenic acid (C18:2 cis9 cis12), the most abundant fatty acid in kiwi, does not differ between cheeses produced with the different rennet types.

The profile of mineral elements is particularly interesting. The obtained concentrations of the mineral elements found in the cheeses of the three groups cK, cH and cT are listed in Table 28.

Table 28. Concentration of mineral elements (mg/kg), SEM (standard error of mean) and p-value of different cheese.

	C_K	C_H	C_T	SEM	p-value
Ca	7152.47	7162.89	7212.79	0.000	0.401
Na	4150.03^{A}	5550.29^{B}	5320.37^{B}	0.025	**0.000**
K	1188.91^{B}	737.87^{A}	709.41A	0.047	**0.000**
Mg	219.00	207.53	187.12	0.016	0.107
Fe	1.84	1.78	1.26	0.020	0.540
Zn	20.25	18.92	19.32	0.007	0.318
Cu	0.20	0.17	0.17	0.020	0.751

Letters A and B indicate homogeneous groups at $\alpha=0.05$: sample types which do not differ from each other are designated by same letter. Bold values are significant at $p<0.05$.

The results showed that the most representative macro-element was Ca with a concentration ranging from 7152.47 mg/kg for cheeses in the cK group to 7162.89 mg/kg for cheeses in the cH group. Among the macro-elements found, only Na and K were statistically different ($p < 0.05$). The Na content was significantly higher in cheeses made with microbial rennet Halal-certified and traditional calf rennet. In contrast, the K content was significantly higher in cheeses made with vegetable rennet. The higher Na content is probably due to the Na already present in the two types of rennet, cH, and cT, in the form of sodium chloride and sodium benzoate. These two salts are usually added as preservatives. The higher K content is probably due to the already high K content in the kiwi pulp. Literature data confirms that kiwi is one of the fruits with the lowest sodium content and the highest potassium content.[80]

5.5.7. Results of sensory analysis

The sensory analysis of the cheeses is illustrated by the spider graphs in Figure 8. The mean values of most attributes such as hardness and chewiness were lower ($p<0.05$) in the cheeses made with the vegetable coagulant from kiwifruit than in the cheeses made with traditional calf rennet and microbial rennet Halal certified. The only attribute with an opposite trend was the bitterness, which was significantly higher in cK compared to cH and cT.

Also, Ojha et al.81 as well as Fguiri et al.,82 Mahdian Dehkordi et al.,83 had found slight bitter taste in cheeses made with kiwi-based vegetable coagulant. This could be attributed to secondary proteolysis that promotes the formation of peptides with low molecular weight responsible for the defect such as bitter as reported by Nicosia et al.68 In addition, the cK cheese had a significantly lower fat content, and it is known that cheeses with low fat content have a lower ability to mask bitterness, as suggested by Fguiri et al.82

Finally, no significant differences were found between the characteristics of cH and cT. According to the acceptability test, acceptability votes of 68%, 73%, and 74% were obtained for cK, cH and cT cheeses, respectively; thus, confirming the overall palatability of cheeses made by kiwi extract.

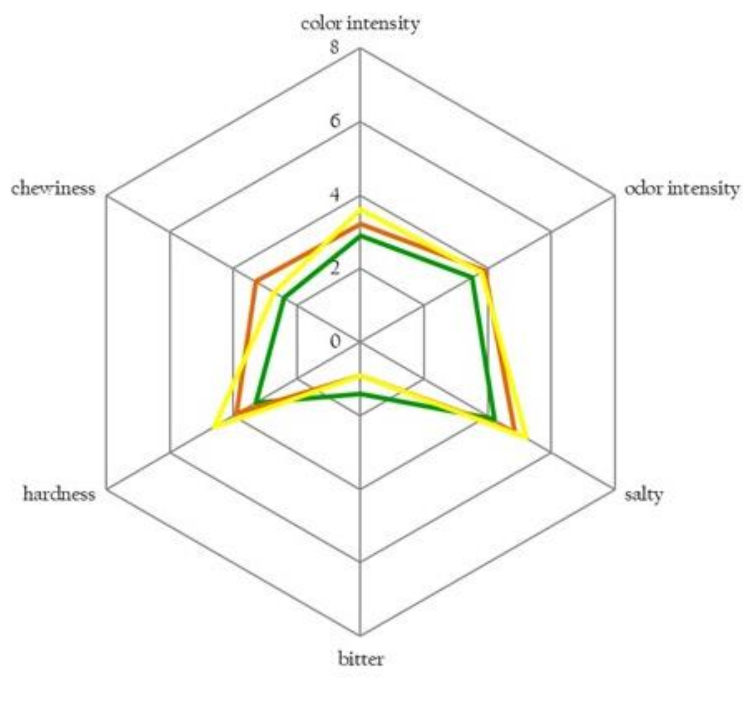

Figure 8. Spider graphs of sensory attributes for cheese under analysis.

5.5.8. Results of PCA

The results of the multiple comparisons showed significant differences with a p-level below 0.05 for many of the variables analyzed, as can be seen in Tables 26 and 28. Only variable significantly different were normalized and used in PCA.

In correlation matrix the highest positive correlations were observed for K and β-sitosterol (0.996), K and stigmasterol (0.993), K and campesterol (0.990), stigmasterol and polyphenols (0.989), and campesterol and polyphenols (0.989); instead, the highest negative correlations were observed for K and Na (-0.990), Na and β-sitosterol (-0.989), Na and stigmasterol (-0.984), and Na and polyphenols (-0.974). Three principal components with eigenvalues of 10.157, 1.562 and 1.246 were extracted according to the Kaiser-Guttman criterion.

The extracted components together explain 81.029% of the total variance (63.484%, 9.761% and 7.785% respectively). The commonality is always higher than 0.788, except for pH, which has a value of 0.652; no lower saturation values were observed for any of the factors, so the extracted components reproduce the original variables well.

The 2D scatter plots (Figure 9) showed that two groups of samples were well separated in the plane defined by PC1 and PC2. The PC2 axis separated the cheese samples with kiwi rennet extract from the others.

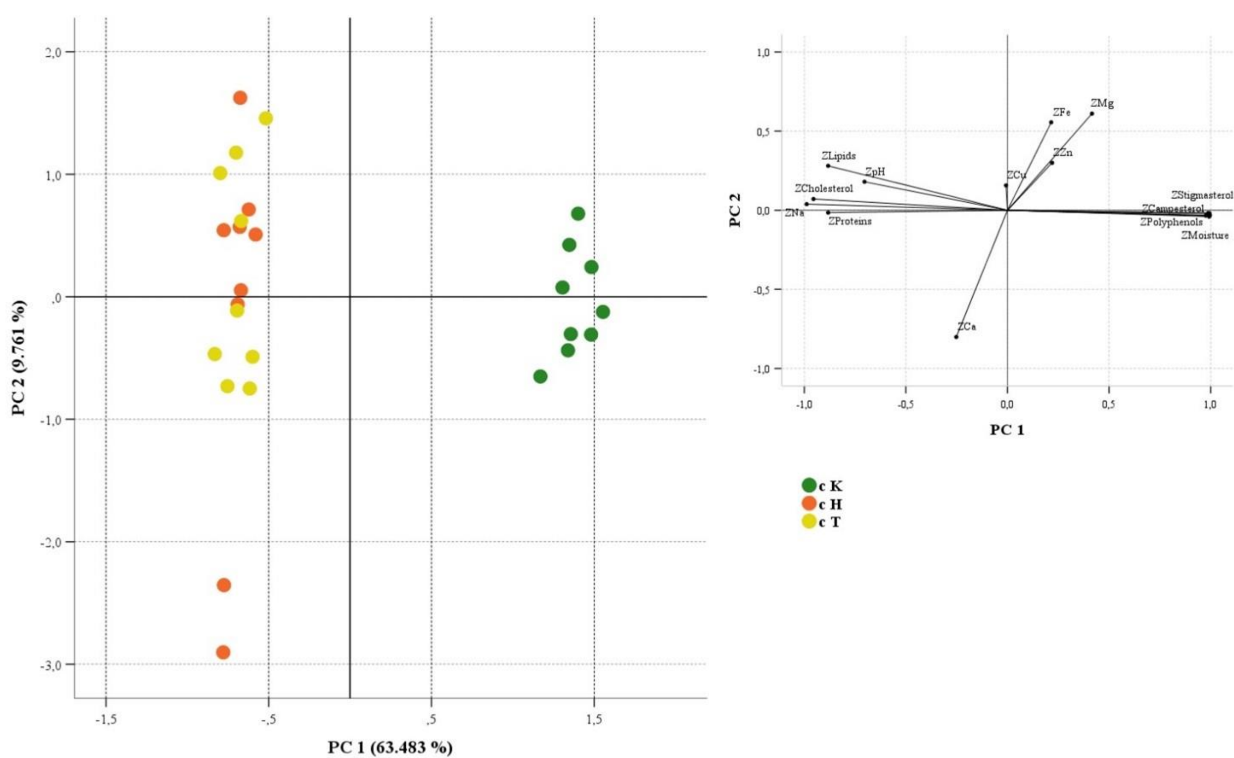

Figure 9. 2D Scatterplots for the cheese samples categorized by coagulant. Insert: loading plot for PC1 and PC2.

A strong positive correlation was observed with stigmasterol, campesterol and polyphenols and a strong negative correlation with protein. In contrast, there was an overlap of negative PC1 values in cheese samples with Halal-certified microbial rennet and conventional calf rennet.

These two groups of samples were characterized by a positive correlation with lipids, cholesterol and Na and a strong negative correlation with Ca. According to the ANOVA results, almost all the

parameters analyzed (pH, moisture, total lipids, proteins, sterols, total and single polyphenols, and mineral elements), each related to the type of rennet used, contributed significantly to the differentiation of the cK cheese samples from the other two types.

5.5.9. Conclusion

This study evaluated the effect of vegetable coagulant with kiwi extract, as well as microbial coagulant Halal-certified and traditional calf rennet in the production of a Sicilian fresh cheese. The data obtained showed that most of the chemical parameters analyzed were affected by the nature of coagulant used.

Cheeses produced with vegetable coagulant showed the following positive characteristics: lower total lipid, cholesterol and Na contents, higher polyphenol and K contents, non-negligible phytosterol content. The use of a coagulant obtained from kiwi pulp therefore showed interesting potential, particularly in terms of the transfer of healthy compounds (polyphenols and phytosterols), and also improved the nutritional properties of the cheeses obtained. Coumaric acid and cinnamic acid were not found in the cheeses of the cH and cT groups.

Therefore, they can be used as good indicators to evaluate the benefits of using kiwi in cheese making. It is possible to conclude that kiwi extract could be an excellent alternative to other coagulant in the clotting of the milk, combining the qualitative and nutritional aspects of a traditional product with the technological aspects of an innovative product. From a sensory point of view, the vegetable coagulant with kiwi extract provided a very slight bitter taste to the CK cheese, which 68% of the panelists found pleasant overall. This aspect is very important, since the acceptance of cheese by the consumer is based on the high quality of the product and specific sensory attributes. Combining the quality of the product with acceptable sensory attributes therefore provides the basis for increasing the chances of these cheeses succeeding in the market. This will make it possible to meet the growing demands of consumers and respond to the needs of an evolving society.

5.6. OCCURRENCE AND HEALTH RISK ASSESSMENT OF MINERAL COMPOSITION AND AFLATOXIN M1 IN COW MILK SAMPLES FROM DIFFERENT AREAS OF SICILY, ITALY

The aim of the present study was to evaluate the mineral elements and aflatoxins M1 content in cow milk samples from different areas of Sicily. Moreover, the elements contribution derived by milk ingestion considering the potential health risk for consumers were evaluated and to compare the determined concentrations with the Recommended Dietary Allowance (RDA) and Adequate Intake (AI) values for essential trace elements.

Exposure to toxic elements were evaluated to compare toxic with tolerable weekly intake (TWI), tolerable daily intake (TDI), or benchmark dose lower confidence limit (BMDL01).

5.6.1. Samples collection

In this study, a 180 raw cow milk samples were collected from various areas from Sicily (figure 10), a region of Southern Italy. Samples were received because of the mandatory controls of the Regional Microbiological, Chemical and Physical Risk Control Plan on Food and Beverages in the Experimental Zooprophylactic Institute of Palermo, Sicily (IZSSi) "Chemical and Food Technology Laboratories and Residue Laboratory" in the three-year period 2020–2022 from the Sicilian areas of Palermo, Catania, and Messina.

In detail, 20 samples were chosen per province per year, numbered in progressive order from 1 to 20 followed by the signature of the area of origin of the milk and year.

Figure 10. Geographical origins of the milk samples.

5.6.2 Sample preparation for aflatoxin M1 by HPLC-FLD

About 50 g of milk at room temperature was placed in a 50 mL Falcon centrifuge tube and 25 µl of the reference solution (100 µg/kg) was added to the blank. The sample was thus mixed with Vortex for 1 min, and then centrifuged at 4000 rpm for 15 min. Finally, the upper lipid phase was removed by Pasteur pipette and the lower aqueous phase used for subsequent purification followed by the actual analysis.

Before the analysis, the parameters were checked, and the calibration curve constructed. The data collection and processing were managed by the SILAB computer system. The chromatographic conditions were described in Table 29.

Table 29. Chromatographic conditions for M1 aflatoxin determination.

Chromatographic Conditions	Settings
Column	Agilent Zorbax C18 5µm, length 150x4,6 mm
Loop	100µl
Flow rate	0.9 ml/min
Column temperature	30 ºC
Stop time	12 min
Eluent	Acetonitrile, water (25:75; v/v)
Fluorescence detector	
Mode of isocratic elution	

5.6.3 Method validation

The validation of the ICP-MS and DMA-80 methods was performed in accordance with the Eurachem criteria.[84] As shown in Table 30, the methods were validated in terms of linearity (R^2), sensitivity (LODs and LOQs), and accuracy (% of recovery). To evaluate the linearity, 5 standard solutions were injected, each for six times (n = 6). This was useful to build calibration curves for each analyte. Good linearity was observed in each concentration range, with R^2 values always equal or greater than 0.9990. The limits of detection (LODs) and quantification (LOQs) were determined as 3.3 s/S and 10 s/S, respectively ("s" is the standard deviation of the response of 10 blanks; "S" is the slope of the calibration curve). The limits of detection (LODs) were from 0.001 to 1.420 µg/kg. The limits of quantification (LOQs) were from 0.003 to 4.477 µg/kg. Aflatoxin M1 measurement ranged from 0.005 to 0.16 µg/kg and the limit of detection (LOQ) was of 0.009 µg/kg and the limit of quantification (LOD) was of 0.007 µg/kg.

Accuracy was assessed evaluating six determinations over skimmed milk powder certified reference material (ERM-BD150) and it was reported as the percent recovery between the value found with the calibration curve and the true value showed in the certified reference materials. When the element was not certified in the reference material, the matrix was spiked with the known amount of analyte and was analyzed following the procedures discussed before. The analytical characteristic (linearity, sensitivity, accuracy and precision) can be considered appropriate for the aim of the analysis.

Table 30. Analytical characteristics of method.

Element	LOD (µg/kg)	LOQ (µg/kg)	R^2	Recovery (%)
Na	1.420	4.477	0.9990	85.29
Ca	1.277	4.214	0.990	88.17
Mg	0.037	0.122	0.9994	95.00
Al	0.081	0.267	0.9995	95.50
K	0.221	0.729	0.9990	88.86
Cr	0.001	0.003	0.9996	98.50
Mn	0.001	0.003	0.9998	98.62
Fe	0.014	0.046	0.9997	94.57
Ni	0.018	0.054	0.9997	98.00
Cu	0.015	0.050	0.9993	95.37
Zn	0.037	0.122	0.9992	97.50
As	0.010	0.033	0.9998	101.10
Se	0.032	0.106	0.9992	95.21
Cd	0.010	0.033	0.9999	103.74
Pb	0.010	0.033	0.9999	99.74
Hg	1.000	3.300	0.9998	98.75

5.6.4. Mineral composition

Table 31 shows the concentration of the sixteen elements found in cow milk samples. The concentrations of mineral elements in milk can be due to various factors, such as diet, breed, season, environmental conditions. Furthermore, this study made it possible not only to establish the safety of this milk, but also to use these milk samples as indicators of environmental pollution and to evaluate the possible toxic risk for the animal. The different classes of elements were subdivided in three categories: macro-elements (Ca, K, Mg, Na), essential trace elements (Cr, Cu, Fe, Mn, Se, Zn), and toxic and potentially toxic elements (Al, As, Cd, Hg, Ni, Pb).

Table 31. Element contents (mean (mg/L) ± standard deviation) in cows' milk from Palermo, Catania, and Messina (Sicily) and production years (2020, 2021, 2022).

Element (mg/L)	Milk Palermo 2020 (n=20)	Milk Palermo 2021 (n=20)	Milk Palermo 2022 (n=20)	Milk Catania 2020 (n=20)	Milk Catania 2021 (n=20)	Milk Catania 2022 (n=20)	Milk Messina 2020 (n=20)	Milk Messina 2021 (n=20)	Milk Messina 2022 (n=20)
Na	354.28±3.55	387.11±4.76	402.32±5.11	329.17±3.01	334.29±2.96	361.95±4.12	405.44±2.55	423.19±2.97	456.20±3.08
Ca	1175.11±8.67	1301.48±8.81	1365.29±8.72	1056.22±9.32	1148.25±8.49	1236.91±8.11	1265.48±7.34	1369.99±7.51	1423.12±7.23
Mg	105.29±2.87	112.77±2.66	125.93±3.12	156.39±4.01	160.12±3.67	165.73±3.88	133.80±2.13	145.61±2.48	159.14±2.07
K	1523.48±10.11	1552.08±9.34	1588.12±9.25	1232.98±8.90	1262.14±10.11	1291.87±9.43	1802.48±8.87	1831.29±8.55	1845.83±9.30
Al	0.68±0.10	0.73±0.08	0.71±0.08	0.53±0.05	0.55±0.04	0.50±0.06	0.39±0.03	0.36±0.04	0.35±0.03
Cr	0.36±0.06	0.40±0.05	0.39±0.05	0.51±0.07	0.49±0.05	0.54±0.08	0.27±0.04	0.33±0.03	0.34±0.03
Mn	0.02±0.00	0.05±0.01	0.06±0.01	0.04±0.01	0.04±0.01	0.05±0.01	0.03±0.00	0.04±0.00	0.06±0.01
Fe	0.39±0.08	0.41±0.07	0.44±0.07	0.80±0.11	0.83±0.10	0.93±0.13	0.48±0.08	0.54±0.10	0.65±0.08
Ni	0.04±0.01	0.04±0.01	0.04±0.00	0.04±0.00	0.05±0.01	0.05±0.01	0.01±0.00	0.02±0.00	0.02±0.00
Cu	0.10±0.02	0.21±0.05	0.24±0.04	0.21±0.03	0.35±0.06	0.38±0.06	0.33±0.05	0.35±0.04	0.35±0.05
Zn	2.51±0.21	2.66±0.15	2.80±0.23	7.79±0.54	7.95±0.51	8.01±0.55	3.85±0.34	3.93±0.29	4.07±0.35
As	0.010±0.002	0.008±0.002	0.010±0.003	0.015±0.004	0.017±0.006	0.013±0.004	0.011±0.003	0.013±0.005	0.014±0.004
Se	0.02±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.05±0.02	0.02±0.01	0.04±0.01	0.04±0.01
Cd	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Pb	0.010±0.003	0.005±0.001	0.008±0.002	0.005±0.001	0.006±0.001	0.009±0.002	0.004±0.001	0.004±0.001	0.004±0.001
Hg	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Regarding macro-elements, the K concentration found was the highest (range: 1232.98±8.90–1845.83±9.30 mg/L), followed by Ca (range: 1056.22±9.32–1423.12±7.23 mg/L), Na (range: 329.17 ±3.01–456.20±3.08 mg/L) and Mg (range: 105.29±2.87–165.73 ±3.88 mg/L). Potassium concentrations in our samples were in according to values found in cow milk from Mediterranean Areas (mean K concentration: 1461 mg/L) by Fantuz et al.[85] and in cow milk from Jersey (mean K concentration: 1503.8 mg/L) by Proskura et al.[86], but with higher values (mean K concentration: 828.05±0.82 mg/L) than reported by Elbagermi et al.[87]

Calcium concentrations were in accordance to data reported (mean: 1153 mg/L) by Fantuz et al.[85], but higher than Proskura et al. (mean: 587.57±0.87 mg/L and 643.1 mg/L, respectively). Sodium levels found in our milk samples were in line with the sodium range concentration (range: 40–58 mg/100 g) showed by Zamberlin et al.[88] Moreover, sodium levels were agreed with those reported (mean: 391.7 mg/L) by Fantuz et al.[85] and (mean: 349.2 mg/L) by Proskura et al.[86], but lower than those reported (mean: 512.38±0.30 mg/L) by Elbagermi et al.[87] Finally, milk samples showed a magnesium content lower than reported (mean: 214 mg/L) by Birghila et al.[89] in fresh cow milk, but agree with the results of Malbe et al.[90] that reported a mean Mg content of 113 mg/L.

The levels of essential trace elements were found to be different than data reported in the literature. Among the essential elements, zinc was the most abundant, with concentrations ranging from 2.51±0.21 mg/L to 8.01±0.55 mg/L, followed by iron, with contents ranging from 0.39 ±0.08 mg/L to 0.93±0.13 mg/L, and chromium (range: 0.27 ±0.04–0.54±0.08 mg/L). In the present study, the highest zinc content was found in milk samples from cows reared in the Catania area (7.79±0.54–8.01 ±0.55 mg/L). These values were higher than those reported in the literature: 3.28 mg/L for Slaˇcanac et al.; 2.21±1.58–2.86 ±1.93 mg/L for Monteverde et al.[91]; 3435 μg/L for Fantuz et al.[85]; 4.36±1.27 μg/g for Chen et al.[92]

However, these results showed in literature were in line to zinc levels determined in our milk samples from Messina and Palermo areas. The samples from the Catania area showed the highest contribution of iron, with values ranging from 0.80 ±0.11–0.93±0.13 mg/L. In general, the iron content determined in the following study was significantly lower than that reported by Monteverde et al.[91] for milk samples from cows reared in areas of Sicily close to industrial areas (range: 14.54±4.36–16.78±2.92 mg/L). However, our results from Messina and Palermo were in accordance with the iron levels obtained by Newton et al.[93] in milk cows (mean: 223.9 μg/L), whereas those from Catania were a little higher. Regarding the chromium content, the concentration ranged from 0.27±0.04–0.54 ±0.08 mg/L, with the milk from Catania that had the most abundant level of this element. The data obtained were higher than those reported by Chen et al.[92] with a chromium concentration of 15.0±9.2 ng/g in cow milk from China. In other study conducted by Di Bella et al.[94] on milk cow from Sicilia, the chromium contents were always below the detection limit (<0.070 mg/kg). Above all chromium (VI) is absorbed more easily both by inhalation and orally. Ingestion of high doses of chromium (VI) compounds causes acute, potentially fatal, effects on the respiratory, cardiovascular, gastrointestinal, hepatic, renal, and neurological systems. Furthermore, due to the corrosive nature of some chromium (VI) compounds, dermal exposure can lead to skin ulcers while chronic exposure to chromium (VI) compounds can also cause allergic responses (e.g. asthma and allergic dermatitis) in sensitized individuals. A possible cause of the high levels of chromium found in the tested samples could be due to the addition of Cr-containing additives to cow diets. Considering that the feed available to dairy cows generally has a low concentration of chromium and that several studies have observed an improvement in dry matter intake and milk production after feeding these feeds with a high Cr content, this choice is becoming increasingly common. In addition, chromium can contaminate animal feed from soil and water near industrial sites and landfills. In fact, the highest concentration of Chromium was found especially in milk samples from Catania where could be possible a contamination due to the presence of small industries in the areas close to the farms. The WHO sets the maximum limit for chromium contamination in milk at 0.1

mg/kg. Among the other essential trace elements, manganese showed similar levels in all the samples analyzed. The concentration ranged from 0.02±0.00 mg/L to 0.06±0.01 mg/L. However, these levels were lower than those reported by Singh et al.[95], who obtained Mn content in cow's milk samples between 0.036± 0.008 and 0.06±0.01 mg/100 g, but according to those (0.0037± 0.0001 mg/100 g) obtained by Fantuz et al.[85] (mean: 25.8 μg/L). Low concentrations of Selenium were found in all milk samples analyzed. Its content ranged from 0.02±0.01–0.05 ±0.02 mg/L. These levels were similar to those reported in literature.

Copper content was similar in milk samples from cows reared in Messina (0.33±0.05–0.35±0.05 mg/L) and Catania (0.21±0.03–0.38±0.06 mg/L), while milk samples from Palermo showed slightly lower Cu levels (0.10±0.02–0.24±0.04 mg/L). These results were higher than those (mean: 56.0 μg/L) reported by Fantuz et al.[85], and by Monteverde et al.[91] (0.07±0.06–0.08±0.06 mg/L), but according to those shown (mean: 0.178±0.030 mg/L) in the study by Elbagermi et al.[87]

5.6.5. Toxic and potentially toxic elements

Human activities cause the accumulation of toxic and potentially toxic elements. The industries, the traffic cause the contamination of the environment and, consequently, of the fodder consumed by the animals. According to European Regulation 915/2023[96] in milk is reported a maximum residual level (MRL) of 0.02 mg/L only for lead and all samples analyzed in this study were within this limit. In fact, the Pb concentration range in our samples was from 0.004±0.001 mg/L to 0.010±0.003 mg/L and its continuous surveillance is required to maintain the safety of the products and to safeguard the consumers. Aluminum was the most abundant potentially toxic element in milk. The samples from Palermo showed the highest content of this element with concentrations ranging from 0.68±0.10 mg/L to 0.73±0.08 mg/L, followed by those from Catania (0.50±0.06–0.55±0.04 mg/L) and Messina (0.35±0.03–0.39±0.03 mg/L). Our results were comparable to those (0.493±0.196 μg/g) of Chen et al. but significantly higher than those (mean: 63.7 μg/L) of Newton et al.[93]

In all samples analyzed the cadmium and mercury content was always below the limit of quantification (LOQ). Finally, arsenic was present at low levels, with almost similar concentrations in milk samples from the different Sicilian areas (range: 0.008±0.002–0.017±0.006 mg/L). These results were in line with other studies. In 2014, some authors published a monitoring study on heavy metals in milk from Calabria, a little industrialized territory as Sicily. The concentrations of Pb and Cd found in the samples were lower than those shown in the literature.[97,98] However, in Calabria, unlike the present study, all samples tested were positive for Pb levels even if the concentrations detected did not present a toxicological risk for the health of the consumer. Another study conducted in Sicily reported the content of heavy metals (cadmium, mercury and lead) and showed the highest concentration of these elements in the industrialized areas located along the northern coast. In fact, high levels of cadmium have often been associated with the presence of textile and mechanical metal industries, power plants and waste incinerators (present in the industrial area of Termini Imerese and Milazzo).[99]

5.6.6. Aflatoxin M1 content

Aflatoxin M1 concentrations were lower than the limit of detection (< 0,009 mcg/kg) in all milk samples from the areas considered in this study. The establishments where the samples were taken are small to medium size industry, mainly located in rural areas, outside the urban center. Furthermore, good practices regarding the management of animals by farmers and the storage of feed in places with controlled temperatures and humidity can influence the concentration of these contaminants. In fact, a study conducted by Cammilleri et al.[100], showed a different positivity to Aflatoxins M1 in sheep and goat's milk based on the season since, in the summer period, the animals feed mainly on fresh grazing grass while in the winter period the diet is richer in feed which is often stored in unsuitable places, favoring the development of AFM1.

5.6.7. Elements uptake by cows' milk

Table 32 shows the evaluation of the element contribution derived from the consumption of milk from cows raised in the three areas of Sicily (Palermo, Catania, Messina). The daily exposure (mg/d) was estimated by multiplying the average concentrations (mg/L) in the samples by the quantity consumed (mL/ milk/d). Calculations were based on the daily consumption of a 250 mL of milk. For essential trace elements, the values obtained were compared with the Recommended dietary Allowance (RDA) and Adequate Intake (AI); instead, the exposure to toxic and potentially toxic elements, calculated considering an adult of 70 kg, was compared with the tolerable daily intake (TDI), the tolerable weekly intake (TWI) or the lower confidence limit of the reference dose (BMDL01).

Table 32. Elements uptake (%) for milk consumption.

Element (%)	Milk Palermo 2020	Milk Palermo 2021	Milk Palermo 2022	Milk Catania 2020	Milk Catania 2021	Milk Catania 2022	Milk Messina 2020	Milk Messina 2021	Milk Messina 2022
Na	5.9	6.5	6.7	5.5	5.6	6.0	6.8	7.1	7.6
Ca	36.7	40.7	42.7	33.0	35.9	38.7	39.6	42.8	44.5
Mg	7.0	7.5	8.4	10.4	10.7	11.1	8.9	9.7	10.6
K	19.0	19.4	19.9	15.4	15.8	16.2	22.5	23.0	23.1
Al	1.7	1.8	1.8	1.3	1.4	1.3	1.0	1.0	0.9
Cr	225.0	250.0	243.8	318.8	306.3	337.5	168.8	206.3	212.5
Mn	0.3	0.6	0.8	0.5	0.5	0.6	0.4	0.5	0.8
Fe	0.7	0.7	0.8	1.4	1.5	1.7	0.9	1.0	1.2
Ni	0.7	0.7	0.7	0.7	0.8	0.8	0.2	0.3	0.3
Cu	2.5	5.3	6.0	5.3	8.8	9.5	8.3	8.8	8.8
Zn	6.3	6.7	7.0	19.5	19.9	20.0	9.6	9.8	10.2
As	11.9	9.5	11.9	17.9	20.2	15.5	13.1	15.5	16.7
Se	9.1	13.6	13.6	13.6	13.6	22.7	9.1	18.2	18.2
Cd	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Pb	7.1	3.6	5.7	3.6	4.3	6.4	2.9	2.9	2.9
Hg	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a

According to the opinions of EFSA and the European Commission, the reference RDAs for Ca, Cr, Cu, Fe, K, Na, Mg, Mn, Se, and Zn were 800, 0.040, 1, 14, 2000, 1500, 375, 2, 0.055, and 10 mg/day, respectively. For the toxic and potentially toxic elements, we considered the reference TDI and BMDL01 for As 0.3 μg/kgb.w./day, Hg 4 μg/kgb.w./day, Ni 22 μg/kgb.w./day, Pb 0.5

µg/kgb.w./day,the reference TWI for Cd 2.5 µg/kgb. w./week and Al 1 mg/kgb.w./week. This study has shown that milk is a good source of macronutrients.

Calcium reported the highest RDAs, ranging from 33.0 % to 44.5 %, with milk from cows reared in Catania having the lowest percentages, followed by potassium (between 15.4 % and 23.1 %), magnesium (between 7.0 % and 11.1 %) and sodium (between 5.5 % and 7.6 %). The essential trace elements, chromium uptake in the milk samples was very high, ranging from 168.8 % to 337.5 %. These results suggest that we should be particular attention to chromium concentrations because there is no legislation establishing a maximum level of this element in milk. We know that chromium (III) is not a carcinogen and that it is an essential human nutrient the deficiency of which can have adverse effects on human health. However, considering that the ICP-MS analysis carried out determined the total Cr content, there could also be the possible presence of Cr (VI), classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). For this reason, continuous monitoring of the Cr concentration in the milk samples analyzed is of paramount importance for the health of consumer. For the other essential trace elements (Cu, Fe, Mn, Se, Zn), percentages were 20 % or less. For the most of toxic and potentially toxic elements, the results are not worrying. However, special attention should be paid to uptake of arsenic and lead, which reached 20.2 % and 7.1 % respectively in all milk samples analysed.

5.6.8. Conclusions

The monitoring conducted over this three-year period has provided reassuring data regarding the chemical risk from heavy metals and M1 aflatoxins in cow milk from different areas under study. Despite this, the guard should not be lowered; on the contrary, it is essential to continue monitoring these contaminants widely recognized as toxic to animals and humans. Consumer needs have changed compared to past years, as they intend to purchase a product with a high biological value, but which is also safe for health. To this end, monitoring contaminants in products of animal origin provides both data on the food safety of the product itself and data on the health of the environment

in which we live. In general, none of the milk samples showed lead levels above the maximum limit set by European Regulation 915/2023. However, to make it easier to determine how safe or unsafe a milk sample is, it would be necessary to establish maximum levels for all mineral elements present in milk. In addition, the absorption rates of each element were calculated based on a daily milk consumption of 250 mL. Our results showed that the milk samples analyzed reported a low toxicological risk, except for chromium absorption, which was above 100 % in all samples. However, to determine the risk posed by this element it would be appropriate to carry out an analysis to distinguish between the two different forms of chromium (Cr (III) and Cr (VI)). In this context, further studies will be carried out to assess and to monitor the concentration of this element and to establish safety for the consumer. Moreover, the low levels of mineral elements found in milk samples analyzed suggest no significant contamination currently based on law limits. In conclusion, continuous research and possible prevention in the formation and spread of aflatoxin, arsenic, cadmium, lead and mercury in cow milk is of crucial importance with regards to food safety and milk quality. Furthermore, the concentrations of M1 aflatoxins found in cow's milk samples were below the limit of detection and this indicates that the areas subjected to the investigations are not at toxicological risk for consumer. In fact, cow milk is considered a basic food for the Mediterranean diet and it is consumed mainly by children. Finally, close collaboration between producing plants and institutions is necessary to effectively address the challenges related to the safety of cow milk in order to protect and safeguard the health of consumer.

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Chapter 6

RESEARCH LINES RELATED TO HONEY

Honey is a natural, complex, and plant-based sugar product produced by honeybees (*Apis mellifera L.*) and extensively consumed in the world not just as a direct food but also as a natural flavouring and sweetener agent.[1] Honey mainly consists of fructose and glucose but also includes fructo-oligosaccharides and numerous enzymes, vitamins, minerals, phenolic acids, and flavonoids that provide it with healthy and therapeutic properties. The quality of honey relies both on the botanical source of pollen, nectar, and honeydew and on the production context, which includes the quality of the soil, water, and air and the presence of chemical pollutants as well.[2] Environmental contaminants from agricultural, industrial, and urban areas are ingested by bees when they collect nectar, pollen, and honeydew from flowers and drink water, or the contaminants accumulate in their bodies via contact with contaminated surfaces. As a result, environmental pollutants are transferred within hives and accumulated in the honey, which can be considered a potential indicator for environmental contamination. However, to have the most benefit, honey must be free from any type of contaminant. Monitoring the chemical safety of honey is essential not only to ensure product quality and consumer health protection, but also to preserve the environment, landscapes and biodiversity. The surrounding environment with its urban, industrial and agricultural activities is not the only source of contamination. Plastic additives such as plasticizers (PAEs and NPPs) and bisphenols (BPs) may also be present in honey, and their presence can be explained not only by the chemical load of the raw material itself, given the ubiquitous presence of these contaminants, but also by the processing that involves direct contact of honey with plastic materials.[3] In order to protect the authenticity of honey from adulteration, precise physico-chemical quality standards have been established by the Codex Alimentarius, which have been further adapted by the European Community. However, the international regulatory framework for the chemical safety of these bee products is still fragmented. In fact, the international Codex standard for honey only refers to

contaminants such as pesticides and heavy metals and suggests that the corresponding maximum residue limits (MRLs) and maximum levels (MLs) should be in line with the standards set by the Codex Alimentarius Commission for contaminants in food.[4] In this context, the EU took the initiative to regulate bee products in September 2018 by approving the "technical guidelines for determining the extent of pesticide residues in honey and setting maximum residue levels in honey". Similar issues exist when considering process contaminants. Specific migration limits (SMLs) have been applied by the EU to contact materials containing plastic additives that have the potential to leach into food. As discussed in Chapter 3, SMLs were set by Commission Regulations No 10/2011 for certain PAEs and No 213/2018 and No 1907/2006 for bisphenol A and S and, in the absence of legal limits for process contaminants in food, are often used, albeit inappropriately, to give an idea of the level of contamination in food. Since the EU market is a major importer of honey and has a strong influence on the agricultural practices of exporting countries, beekeepers around the world tend to use the above-mentioned EU legislation as a reference for honey quality control. During the three years of the PhD, many efforts were made to search for organic and inorganic contaminants in honeys from different countries; two scientific articles were published evaluating the contamination of Moroccan and Algerian honeys, which are discussed in detail below:

- Massous, A., Ouchbani, T., Lo Turco, V., **Litrenta, F.**, Nava, V., Albergamo, A., & Di Bella, G. (2023). MONITORING MOROCCAN HONEYS: PHYSICOCHEMICAL PROPERTIES AND CONTAMINATION PATTERN published in the journal *Foods*, *12*(5), 969 *Foods* (https://doi.org/10.3390/foods12050969)[5]
- Derrar, S., Lo Turco, V., Albergamo, A., Sgrò, B., Ayad, M. A., **Litrenta, F.,** Said, M. S., Potortì, A. G., Aggad, H., Rando, R., & Di Bella, G. (2024). STUDY OF PHYSICOCHEMICAL QUALITY AND ORGANIC CONTAMINATION IN ALGERIAN HONEY published in the journal *Foods*, *13*(9), 1413. (https://doi.org/10.3390/foods13091413)[6]

Over the past decade, interest in the development of new analytical methods preparation has resulted from an increased need to reduce the environmental impact. The greening of sample preparation and analysis methods has involved techniques that require reduced amounts of sample, as well as lower amounts of solvents and reagents, with positive implications on the reduction of waste production. At the same time, the decrease in total extract amounts has been accompanied by much more sensitive, energy-efficient and user-friendly analytical instruments.

Research in the determination of bisphenol residues in honey is very difficult due to the extremely low concentrations of bisphenols in food (μg/kg-ng/kg), the complex composition of some matrices and the lack of standardised analytical methods. The most widely used method for the determination of bisphenols is solid phase extraction (SPE) and analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS). However, the use of this procedure is associated with higher sample amounts, large solvent volumes (high costs, environmental pollution) and long analysis times.

The evaluation of analytical methodologies in the context of green chemistry is very difficult due to the diversity of analytes and analytical methods, the complexity of sample matrices and the analytical criteria that have to be considered. This is why we must emphasise that, in some cases, it is impossible to meet all green chemistry criteria in analytical methodologies and therefore efforts to improve the method are necessary. In 2013, the 12 principles of green analytical chemistry (GAC) have been established and recently revised to focus more directly on the sustainable preparation of the analytical sample. As a result, the 10 principles of green sample preparation (GSP) have been implemented.[7]

Tobiszewsky et al.[8] pointed out that the least environmentally friendly step in the analytical process is the preparation of the sample. A very interesting approach to assess the ecology of an analytical method, called the Eco-Scale, has been proposed by Van-Aken et al.,[9] based on assigning penalty points to the parameters of an analytical process that do not correspond to the ’ideal green’ analysis (score of 100 in the Eco-Scale). The higher the score, the greener and cheaper the analytical process.

This concept can be adapted to evaluate green analytical methods. During my PhD, I had the opportunity to develop and validate a new approach, based on a method incorporating aspects of green analytical chemistry, and derived from a method previously developed for conventional analysis and then applied to real samples, in particular honey samples:

- Potortì, A. G., **Litrenta, F**., Sgrò, B., Di Bella, G., Albergamo, A., Mansour, H. B., Beltifa, A., Benameur, Q., & Lo Turco, V. (2023). A GREEN SAMPLE PREPARATION METHOD FOR THE DETERMINATION OF BISPHENOLS IN HONEYS published in the journal *Green Analytical Chemistry*, *5*, 100059. (https://doi.org/10.1016/j.greeac.2023.100059)[10]

6.1. MONITORING MOROCCAN HONEYS: PHYSICOCHEMICAL PROPERTIES AND CONTAMINATION PATTERN

Due to its important floristic, faunal and landscape diversity, Morocco is endowed with an important and unique beekeeping potential, resulting one of the most valuable territories for honey production in the Mediterranean area. Here, beekeeping is a well-rooted tradition and one of the most profitable businesses, thanks to the conspicuous production not only of honey, but also of pollen, propolis, beeswax, and royal jelly. A market overview showed that the Moroccan honey production increased from 4.7 tons to almost 8 tons between 2010 and 2020, with a turnover of around 101 million[11]. Moreover, a vibrant modernization of the sector has started in the last decade, thanks to the leverage effect of the Green Morocco Plan (GMP), the National Initiative for Human Development (NIHD) and, not least, the Moroccan Ministry of Agriculture, setting a referential catalog for high-quality terroir products, including honey, with the final aim to label them under Geographical Indications, Designations of Origin or Agricultural Labels, thus, promoting their consumption[12].

The Béni Mellal-Khénifra region, placed between the High and Middle Atlas Mountain ranges and the Tadla Plain, is well known for its rich and varied botanical diversity allowing for significant honey production[13]. Particularly, the region boasts the monofloral Euphorbia honey of Tadla-Azilal, labeled with Protected Geographical Origin (PGI) and produced from Euphorbia resinifera, an endemic Moroccan species mainly distributed in Azilal and Béni Mellal areas[14].

A literature overview suggests that, over the past decade, major efforts have been devoted to the characterization of physicochemical, melissopalynological, antioxidant and microbiological traits of Moroccan honey[12,13,14], while very few and fragmentary data are available on contaminants[15,16]. However, the chemical safety of such a product showing a considerable ability to accumulate xenobiotics from the surrounding environment should not be overlooked, also in view of the patchy regulatory framework, as well as the ongoing modernization facing Moroccan beekeeping and its labelled products.

The aim of the study was to investigate the physico-chemical characteristics and contamination patterns of unifloral honeys from different areas of the Béni Mellal-Khénifra region, including the PGI Euphorbia honey from Tadla-Azilal. Data on the main quality indicators (moisture, sugars, pH, conductivity and acidity) and a range of organic chemicals, including regulated (pesticides, PAE, NPP and BP), banned/restricted (OCP, PCB and PAP) and inorganic pollutants (potentially toxic elements) were used to statistically assess the relationship between honey quality and safety and current production scenarios in the Moroccan region. In addition, the dietary exposure to contaminants from the consumption of these honeys was assessed.

6.1.1. Study Area

The Béni Mellal-Khénifra region is a new Moroccan region created according to the new administrative subdivision of 2015; it represents 4% of Morocco's national territory and covers 28.088 km^2, of which more than 65% is mountainous. The region is located in central Morocco, between the High and Middle Atlas and the Tadla Plain, and comprises five provinces: Azilal, Béni

Mellal, Fquih Ben Salah, Khénifra and Khouribga (Figure 1). Thanks to diversified climates and landscapes, this region has a rich natural heritage and high biodiversity, with a significant potential for agricultural development. Indeed, the Béni Mellal-Khénifra region has an urbanization rate (49%) lower than the national average (60.36%), and more than half of the population lives in rural areas (51%), being strongly committed to the agricultural sector, which not only constitutes the major economic activity of the region, but it is also in the process of modernization as required by the GMP[17,18]. However, intense agricultural practices have been seen as a relevant factor for environmental pollution. In this respect, recent studies have reported in diverse areas cases of agricultural soils and groundwater/wastewater intended for irrigation being contaminated, especially in terms of heavy metals and pesticides.[19,20,21]

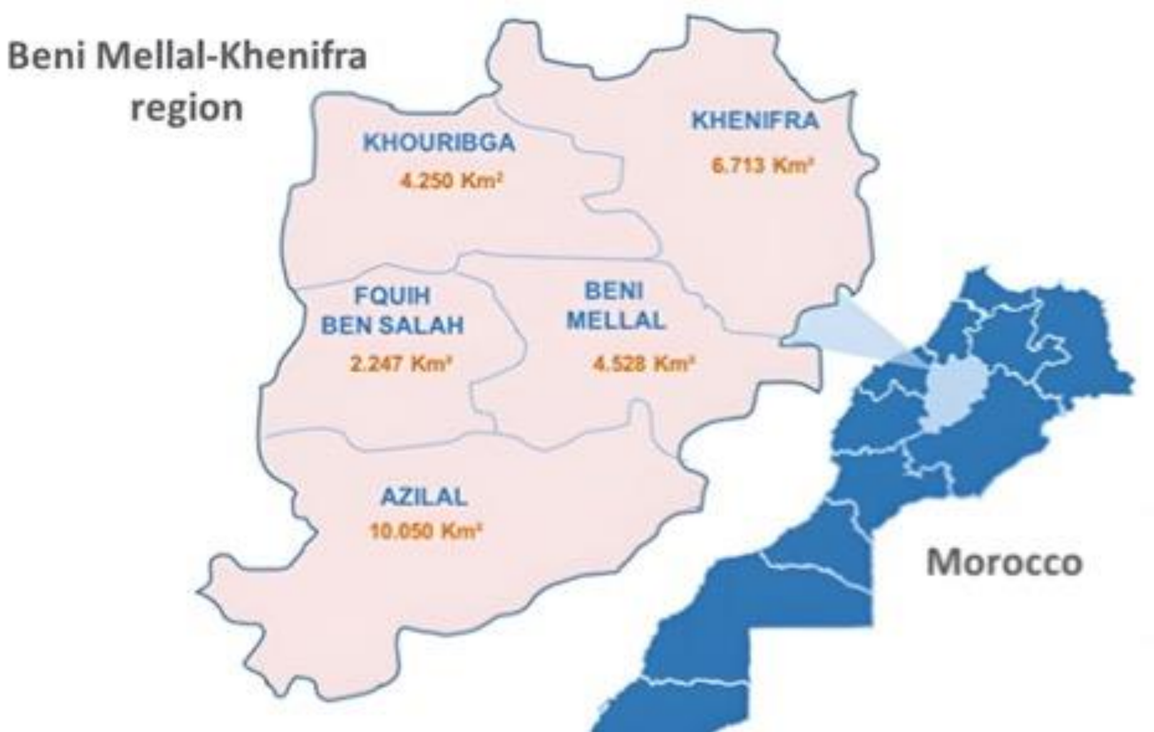

Figure 1. Geographical map of the Moroccan region Béni Mellal-Khénifra and its provinces

6.1.2. Honey Samples

The study was conducted on 12 honey samples produced in 2021 by beekeepers located in different provinces from the Béni Mellal-Khénifra region of Morocco. They included n = 3 honey samples from *Ziziphus lotus* (i.e., jujube honey) produced in Khénifra, n = 3 honeys from *Citrus sinensis* (i.e., sweet orange honey) collected in the Béni-Mellal province, n = 3 PGI honeys from *Euphorbia resinifera* (i.e., Euphorbia honey) produced in the Tadla-Azilal area, and n = 3 honeys from *Globularia alypum* obtained from the Fquih Ben Salah province. Honeys derived from pooling a given type of honey from different hives of a given area and they were collected in glass jars of ~125 g and stored at room temperature in a dark place until analysis.

6.1.3. Physicochemical Parameters

Moisture (%) and total soluble solids (TSS) represented by soluble sugars content and expressed as °Brix, were obtained from the tables of correspondence between a given water content/°Brix and the refractive index calculated for each sample at 20 °C. If the index was not determined at a temperature of 20 °C, the correction temperature was considered, and the result was reduced to a temperature of 20 °C. Free, combined, and total acidity were determined by the titrimetric method proposed by Bogdanov and colleagues[22]. Briefly, the titration of the honey sample (10 g diluted with 75 mL of distilled water) was carried out with 0.05 N NaOH to pH 8.5 (free acidity). Then, a 10 mL volume of NaOH was added and titrated again with 0.05 N HCl to pH 8.3 (combined acidity). Total acidity was calculated obtained by the sum of free and combined acidities. The pH and electrical conductivity for every honey sample were determined by a pH/conductivity meter. Approximately 10 g honey was dissolved in 75 mL distilled water and the pH and electrical conductivity were measured. For electrical conductivity, the quantity of honey to be weighed was calculated using the following equation:

$$M = 20 \times \frac{100}{100 - A}$$

M: mass of honey (g); 20: is the theoretical nominal mass of honey; A: water content in %. The ashes were obtained by drying 5 g of every honey sample at 600 °C until constant weight, according to the AOAC protocol[23]. For the determination of minerals (K, Mg, Na, and Ca) and essential trace elements (Mn, Fe, Cu, Zn, Se, Cr, Co and Ni), see Section *5.2.1. Elemental analysis and validation methods* in Chapter 5.

6.1.4. Extraction of residues from honey samples

For the extraction of pesticides, PCBs and PAHs, 10 g of honey was weighed into a tube with 10 mL of water and 10 mL of acetonitrile, and vortexed for 5 min.

Then, Q-sep QuEChERS kit (4 g MgSO4 and 1 g NaCl) and clean-up (750 mg MgSO4, 250 mg of PSA and 125 mg C18) was added and centrifuged for 5 min (5000 rpm). At the end, 5 mL of the organic phase was collected, reduced to 1 mL in a rotary evaporator at 30 °C and reduced to a volume of 0.5 mL volume under a stream of nitrogen. Before instrumental analysis, a known amount of every IS was added to every sample. The multiresidue screening was performed by a Shimadzu GCMS-TQ8030 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Separation conditions, and mass spectrometry (MS) details can be found in *5.1.2. Instrumentation and analytical conditions* in Chapter 5. Compound identification occurred by comparison of their retention times and mass spectra with those of corresponding commercial standards. The quantitative procedure was carried out in multiple reaction monitoring (MRM) mode, exploiting the IS normalization. The MRM transitions, as well as the main figures of merit of analytical validation are reported in Table 1 and 2 (chapter 5). Every honey sample was monitored in triplicate, along with analytical blanks.

The extraction of plasticizers from the various honey samples was performed according to a method already reported in Liotta et. al.[24], with some modifications. Briefly, 5 g of honey was weighed into a tube and extracted with 10 mL of acetonitrile. Then Q-sep QuEChERS was added and centrifuged for 5 min (5000 rpm). Approximately 2 mL of the organic phase were collected, evaporated to 1 mL

in a rotary evaporator at 30 °C and finally reduced to a volume of 0.5 mL volume under nitrogen stream. Before instrumental analysis, a known amount of DBP-d4 and DEHP-d4 was added to every sample. The multiresidue screening was carried out by a gas chromatography system (GC-2010, Shimadzu, Japan) equipped with an autosampler (HT300A, HTA, Italy) and coupled to a single quadrupole mass spectrometer (QP-2010 Plus, Shimadzu, Japan). Identification of PAEs and NPPs occurred by comparison of their retention times and mass spectra with those of corresponding commercial standards, while the quantitative assay was performed in SIM mode, considering the base peak ion out of three characteristic mass fragments for each target analyte (Table 3, chapter 5) and using the IS normalization. Measurements were conducted in triplicate for every sample, alternated with analytical blanks.

For the extraction of the nine bisphenols, the method already proposed by Liotta et al.[24] with some modification, was applied. Briefly, 5 g of honey was placed in centrifuge tubes with 10 mL of ultrapure water and 10 mL of acetonitrile, and vortexed for 5 min. Then, 4 g of MgSO4 and 1.5 g of NaCl were added. The obtained mixture was vortexed for 5 min, and centrifuged at 4000 rpm for 10 min. Then, 5 mL of supernatant was added to the QuEChERS d-SPE cleaning tube, vortexed and placed in centrifuge at 4000 rpm for 10 min. Hence, 1 mL of supernatant was recovered and filtered by 0.22 µm nylon filter and analyzed by HPLC-MS/MS.

Analysis was performed on an LC apparatus (Prominence UFLC XR system, Shimadzu, Kyoto, Japan) consisting of a controller (CBM-20 A), binary pumps (LC-20AD-XR), degasser (DGU-20A3R), column oven (CTO-20AC), and autosampler (SIL-20 A XR). An electrospray ionization (ESI) source interfaced the LC system to a triple quadrupole mass spectrometer (MS) (LCMS-8040, Shimadzu, Kyoto, Japan). Data were acquired in MRM mode and the resulting ion transitions were used for the identification and quantification (internal standard method) of BPs. MRM transitions and the main figures of merit of analytical validation for every target analyte are reported in Table 4, chapter 5. Every honey sample was monitored in triplicate along with analytical blanks.

Honey samples were mineralised for the determination of mineral elements. Mineralization of samples was carried out following the method proposed by Di Bella and coworkers[25]. About 0.5 g of each honey sample was weighed, and 1 mL of IS at 0.5 mg/L was added. The samples were digested with 7 mL of HNO3 (65%, v/v) and 1 mL of H2O2 (30%, v/v) in a microwave ETHOS 1 digestion system (Milestone, Bergamo, Italy) using the following instrumental parameters: 15 min at 1000 W up to 200 °C, 15 min at 1000 W at 200 °C.

The digested samples were conveniently diluted with ultrapure water and their analysis was carried out by means of a single quadrupole inductively coupled plasma-mass spectrometer (ICP-MS, iCAP-Q, Thermo Scientific, Waltham, MA, USA) according to the operating conditions already reported in section *5.2.1. Elemental analysis and validation methods,* chapter 5.

All samples were processed in triplicate along with analytical blanks. The analytical validation of the ICP-MS method is reported in Table 11 in chapter 5.

6.1.5. Statistical Analysis

Statistical analysis was carried out using the SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL, USA). Initially the non-parametric Kruskal–Walli's test was applied on log-transformed data to assess differences between honey samples, with a statistical significance at $p < 0.05$. Subsequently, a Principal Component Analysis (PCA) was conducted on a starting data matrix where the cases (12) were the analyzed honey samples and the variables (54) were the values of physicochemical parameters, as well as pesticides, PCBs, PAHs, plasticizers, BPs residues and element concentrations that were higher than their respective LOQs. When concentrations were below the limit of quantification (LOQ), these were replaced with half the limit of detection (LOD/2). Then, the data set was normalized to achieve independence of the different variables scale factors and a PCA was performed to evaluate the differentiation of honey samples in relation to the different production context and/or floral origin according to the investigated variables.

6.1.6. Assessment of the Dietary Exposure to Contaminants

To evaluate the health risks of organic and inorganic contaminants derived from the intake of Moroccan honey, the relative estimated daily intakes (EDI) were calculated by multiplying the mean contaminant concentration found in every sample (mg/Kg or µg/Kg) by the amount of honey consumed daily (g/day) and dividing the obtained result by the consumer's body weight (Kg bw). Hazard quotient (HQ), which is the ratio between a given EDI and the corresponding oral reference dose (RfD) proposed by the U.S. Environmental Protection Agency (US EPA,) was also employed to assess the plausibility of risk. An HQ (dimensionless) >1 entails a high non-carcinogenic risk.

6.1.7. Results and Discussion

The values of moisture, TSS, free, combined, and total acidity, and pH of Moroccan honeys are shown in Table 1. The moisture of honey is strictly related with the harvest time and practices performed by beekeepers, and, not least, the level of honey maturity reached in the hive.[26] This parameter influences honey flavor, color, density, and viscosity, and determines its stability and granulation during storage. In the honeys investigated, moisture values were always below the maximum limit (20%) set by the Codex Alimentarius and EU standards. This may be due not only to the correct time of extraction by Moroccan beekeepers, but also to the current use of modern hives with better moisture control. Specifically, moisture values ranged between 14.93–16.57%, thus indicating a good degree of maturity of all products.

Despite the small variability, the upper and lower moisture values, represented respectively by the jujube honey from Khénifra and the sweet orange honey produced in Béni Mellal province, were significantly different ($p < 0.05$), which may suggest a variation in such parameters in relation to the climatic conditions.[27] For TSS, minimum/maximum values of 82.67 °Brix/85.83 °Brix were found respectively in sweet orange and *G. alypum* honeys, fall within the acceptability range (78.8 and 85 °Brix). Indeed, TSS values are known to decrease with the increasing concentration of starch,

molasses, glucose, and distilled water. As a result, this parameter is inversely related to the moisture content and useful in the detection of adulteration events.[28]

Honey acidity and pH are correlated with each other, being dependent on the level of organic acids and enzymatic activity in honey. As a result, their variation in honey samples could be attributed to the floral origin rather than the environmental context. These physicochemical parameters are generally intended as a marker of honey freshness since the higher the acidity and the lower the pH, the better the environment that inhibits microorganism growth. In the present study, all products showed acidity values below the EU standards 50 meq/kg, thus suggesting the absence of undesirable fermentation and/or bacterial spoilage. Specifically, free, and total acidity values ranged from 15.41 meq/kg and 16.39 meq/kg in sweet orange honeys (Khénifra) to 39.28 meq/kg and 39.88 meq/kg in G. alypum honeys (Fquih Ben Salah), thus yielding significantly different results in these types of honey ($p < 0.05$). All samples analyzed showed an acidic character. Although non significantly different ($p > 0.05$), pH values were in accordance with the acidity values and varied probably due to the different floral origin. In fact, the lowest pH was observed in G. alypum honey (3.98), while the highest values were found in sweet orange and jujube honey (4.24).

Table 1. Physicochemical traits of monofloral honeys from the Béni Mellal-Khénifra region. Values are expressed as mean ± standard deviation of n = 3 samples. Statistics from Kruskal–Walli's test are also reported.

	Jujube Honey (Khénifra)	***Sweet Orange Honey (Béni Mellal)***	***Euphorbia Honey (PGI, Azilal)***	***G. alypum Honey (Fquih Ben Salah)***	**F Statistic**	**p-Value**
Moisture (%)	$14.93^{a} \pm 0.15$	$16.57^{b} \pm 0.21$	$15.47^{ab} \pm 0.25$	$16.40^{b} \pm 0.26$	9.667	**0.022**
TSS (°Brix)	$85.00^{ab} \pm 1.00$	$82.67^{a} \pm 0.58$	$84.27^{ab} \pm 0.64$	$85.83^{b} \pm 1.26$	8.048	**0.045**
Free acidity (meq/kg)	$25.46^{b} \pm 0.15$	$15.41^{a} \pm 0.07$	$27.49^{b} \pm 0.34$	$39.28^{c} \pm 0.85$	10.395	**0.016**
Combined acidity (meq/kg)	$0.99^{b} \pm 0.01$	$0.98^{b} \pm 0.01$	$1.50^{c} \pm 0.02$	$0.60^{a} \pm 0.10$	9.721	**0.021**
Total acidity (meq/kg)	$26.45^{b} \pm 0.16$	$16.39^{a} \pm 0.08$	$28.98^{b} \pm 0.33$	$39.88^{c} \pm 0.83$	10.385	**0.016**
pH	4.24 ± 0.08	4.24 ± 0.06	4.10 ± 0.10	3.98 ± 0.06	7.307	0.063

a–c indicates homogeneous sample groups at $\alpha = 0.05$ and honeys which do not differ from each other are designated by the same letter. Bold p-values showed significantly different results at $p < 0.05$ between different honeys.

The electrical conductivity, ash, and mineral profile (K, Ca, Na, and Mg) and essential trace elements (Mn, Fe, Zn, Cu, Se, Cr, Co, and Ni) of investigated Moroccan honey are reported in Table 2. The electrical conductivity of the honey is closely related to the concentration of minerals and organic acids and its assessment is useful in the discrimination between blossom and honeydew honeys. In fact, such a parameter tends to be higher in honeydew honeys and it varies in relation to the same honeydew content. The Codex Alimentarius and EU legislation require blossom honeys to have conductivity values not higher than 800 µS/cm.[29] As a result, honeydew honeys generally show higher values than 800 µS/cm.

Additionally, in monofloral honeys, this parameter shows great variability according to the floral origin.[30] In Moroccan honeys, conductivity varied from 157.00 µS/cm to 633.67 µS/cm, respectively in sweet orange and *G. alypum* honey, which consequently gave significantly different results ($p < 0.05$). On the other hand, jujube and Euphorbia honeys showed intermediate and non-significantly different conductivities (respectively, 381.33 µS/cm and 362.67 µS/cm, $p > 0.05$). All values were below the maximum limit (800 µS/cm) set by the Codex Alimentarius and EU standards for such parameter. Differently from conductivity, there is no specific legislation on maximum level of ash, minerals, and trace elements content in honey, which, consequently, are not yet considered as a quality parameter by either the Codex Alimentarius or the EU. However, they are very important quality markers of honey, reflecting both the floral source of honey as well as its environmental context of production.[31]

Ashes followed the same trend of electrical conductivity in investigated honeys, ranging from 0.34 g/Kg in sweet orange honey from Béni Mellal to 1.17 g/Kg in jujube honey from Khénifra ($p < 0.05$). In terms of concentrations, similar considerations could also be made for the element profile. Considering minerals, K was the most abundant mineral in all honeys analyzed, followed by Ca, Na and Mg. The predominance of K over the other minerals was already highlighted in other honeys Moroccan and not being a peculiar characteristic of such a bee product.[32]

G. alypum honey from the Fquih Ben Salah province had the highest concentration of K (849.73 mg/Kg), while the lowest value of K was found in the sweet orange honey from Béni Mellal (102.80 mg/Kg). The highest Ca content was found in the PGI *Euphorbia* honey from Azilal (125.62 mg/Kg), and the lowest in sweet orange honey from Béni Mellal (81.70 mg/Kg). In jujube and *G. alypum* honeys, Na was the third mineral element with a concentration of 76.84 mg/Kg and 89.99 mg/Kg, respectively; while in sweet orange and PGI Euphorbia honeys, Mg was the third most abundant mineral, with a concentration of 65.46 mg/kg and 69.54 mg/Kg, respectively. The mineral content of honeys under study agreed with the range of values reported for jujube and sweet orange honeys from the Béni Mellal-Khénifra region[33], as well as for the PGI Euphorbia honey.

For essential trace elements, the most significant contributions to the element profile came from Fe, Zn and Mn. Specifically, the highest concentration of Fe was found in G. alypum honey with a concentration of 16.51 mg/Kg, while the sweet orange honey had the lowest amount (6.89 mg/Kg). On the other hand, the PGI Euphorbia honey showed the most abundant concentrations of Mn and Zn (4.00 mg/Kg and 6.98 mg/Kg, respectively). Other essential trace elements (i.e., Cu, Se, Cr, Co, and Ni) were revealed at concentrations ≤1 mg/Kg.

Differently from major elements, no efforts have been devoted to the screening of trace elements in honey from the Béni Mellal-Kenifra region. However, Bettar et al.[34] and Moujanni et al.[30] recently revealed lower Fe and Mn contents for the PGI Euphorbia honey (respectively, 4.37–5.5 mg/Kg and < 1 mg/kg.

Overall, it could be argued that the elemental profiles of different honeys are greatly affected by the floral source. Indeed, elements are primarily introduced from the soil into the nectar via the root system of the plant. Additionally, bees are in contact with the surrounding environment during foraging and further amounts of inorganic elements can be accidentally transferred from soil, water and soil to the hive. As a result, the elemental profile of honey is a bio-accumulative picture of the geographical context as well as of the activity near the apiary site.

Table 2. Conductivity, ash and profile of minerals and essential trace elements of monofloral honeys from the Béni Mellal-Khénifra region. Values are expressed as mean ± standard deviation of n = 3 samples. Statistics from the Kruskal–Wallis test are also reported.

	Jujube Honey **(Khénifra)**	***Sweet Orange Honey*** **(Béni Mellal)**	***Euphorbia Honey*** **(PGI, Azilal)**	***G. alypum Honey*** **(Fquih Ben Salah)**	**F Statistic**	**p-Value**
Conductivity (µS/cm)	$381.33^{b} \pm 18.23$	$157.00^{a} \pm 1.00$	$362.67^{b} \pm 8.62$	$633.67^{c} \pm 8.02$	9.974	**0.019**
Ash (g/Kg)	$1.17^{b} \pm 0.18$	$0.34^{a} \pm 0.05$	$0.97^{b} \pm 0.08$	$1.51^{c} \pm 0.10$	9.112	**0.025**
K (mg/Kg)	$753.25^{c} \pm 72.86$	$102.80^{a} \pm 2.71$	$695.87^{b} \pm 38.61$	$849.73^{c} \pm 1.83$	9.667	**0.022**
Ca (mg/Kg)	$98.18^{a} \pm 0.89$	$81.70^{a} \pm 0.80$	$125.62^{b} \pm 1.00$	$110.75^{b} \pm 1.68$	10.385	**0.016**
Na (mg/Kg)	$76.84^{bc} \pm 0.32$	$35.21^{a} \pm 0.67$	$60.41^{b} \pm 1.06$	$89.99^{c} \pm 2.14$	10.385	**0.016**
Mg (mg/Kg)	$57.87^{a} \pm 2.42$	$65.46^{ab} \pm 0.94$	$69.54^{ab} \pm 0.91$	$85.24^{b} \pm 1.67$	10.385	**0.016**
Mn (mg/Kg)	$1.35^{a} \pm 0.11$	$0.57^{a} \pm 0.03$	$4.00^{b} \pm 0.11$	$2.24^{ab} \pm 0.12$	10.385	**0.016**
Fe (mg/Kg)	$9.50^{a} \pm 0.63$	$6.89^{a} \pm 0.10$	$14.34^{b} \pm 0.29$	$16.51^{b} \pm 0.24$	10.385	**0.016**
Zn (mg/Kg)	$2.81^{ab} \pm 0.06$	$1.41^{a} \pm 0.03$	$6.98^{c} \pm 0.51$	$3.60^{b} \pm 0.18$	10.385	**0.016**
Cu (mg/Kg)	$0.27^{a} \pm 0.09$	$0.27^{a} \pm 0.06$	$0.86^{b} \pm 0.06$	$1.59^{b} \pm 0.18$	9.539	**0.025**
Se (mg/Kg)	$0.25^{c} \pm 0.03$	$0.09^{a} \pm 0.01$	$0.16^{b} \pm 0.01$	$0.17^{b} \pm 0.01$	9.462	**0.024**
Cr (mg/Kg)	$0.09^{a} \pm 0.01$	$0.06^{a} \pm 0.01$	$0.11^{a} \pm 0.01$	$0.94^{b} \pm 0.05$	10.385	**0.016**
Co (mg/Kg)	$0.04^{ab} \pm 0.01$	$0.01^{a} \pm 0.01$	$0.24^{b} \pm 0.11$	$0.05^{ab} \pm 0.02$	9.585	**0.022**
Ni (mg/Kg)	$0.54^{b} \pm 0.11$	$0.17^{a} \pm 0.04$	$0.17^{a} \pm 0.01$	$0.21^{a} \pm 0.03$	8.231	**0.041**

a–c indicates homogeneous sample groups at $\alpha = 0.05$ and honeys which do not differ from each other are designated by the same letter. Bold p-values showed significantly different results at $p < 0.05$ between different honeys.

No honey samples were shown to be free of pesticides. However, among the n = 108 pesticides investigated, no OCPs were revealed, and only n = 11 pesticides were found at levels higher than the respective LODs, mostly belonging to the organophosphate class (OPs) and its metabolites (Table 3).

The jujube honey from the Khénifra province was among the samples with the highest number of quantifiable pesticides (n = 8), detected moreover at the highest levels. Such honey stood out for the highest level of carbaryl (1060.90 µg/Kg, $p < 0.05$), acephate (1251.19 µg/Kg, $p < 0.05$) and cyromazine (2060.99 µg/Kg, $p < 0.05$). Additionally, it was the only honey to show quantifiable residues of quinalphos (5.92 µg/Kg) and fenthion sulfoxide (16.53 µg/Kg). Intermediate and similar levels of contamination were found in the sweet orange honey produced in Béni Mellal and the PGI Euphorbia honey collected in Azilal, in which the most abundant residues were carbaryl (146.30 µg/Kg and 277.41 µg/Kg, $p > 0.05$) and cyromazine (223.72 µg/Kg and 113.60 µg/Kg, $p > 0.05$). Finally, the G. alyphum honey from the Fquih Ben Salah province had the lowest number of quantifiable pesticides (n = 5). In such honey, the most abundant residues were confirmed to be carbaryl, acephate and cyromazine.

However, they were found at very low levels when compared with the other honey samples ($p < 0.05$). According to the Regulation (EC) No. 396/2005 and subsequent amendments, 75% of investigated samples (all samples from jujube, sweet orange, and the PGI Euphorbia honeys) widely exceeded the MRL of 0.05 mg/kg for carbaryl and cyromazine, as well as the MRL of 0.02 mg/Kg for acephate, while 50% of the samples (all samples from jujube and sweet orange honey) exceeded the MRLs of 0.01 mg/kg for dimethoate and diazinon. All samples of jujube honey greatly exceeded the MRLs of 0.01 mg/kg, 0.05 mg/kg, and 0.01 mg/kg respectively for alachlor, carbofuran and fenthion sulfoxide. As mentioned in the introduction section, very few efforts have been devoted to the assessment of the chemical safety of Moroccan honeys.

A recent study conducted on the Euphorbia honey reported the identification and quantification of the 202 pesticides, including the ones detected in this study. However, contrasting results were

obtained, since the detected residues were always within the set MRLs, thus indicating a good quality of the PGI product. The pesticide fingerprint of different honeys clearly reflects the different agronomic practices of the different provinces of such Moroccan region and, more specifically, a more pronounced and prolonged use of OPs in the Khénifra province. The persistence of these pesticides on plants and soil can create shifts in the entire food chain. In fact, regardless of the type of honey, worker bees may transfer such contaminants from the pollen and nectar of plants to the hive, thus being inevitably incorporated into the different hive products. Of the n = 18 PCBs under analysis, n = 2 compounds were found. Specifically, PCB118 was detected in all honey samples and quantified only in jujube honey from Khénifra province (0.71 µg/Kg, $p < 0.05$) and PCB180 was revealed in all types of honey and quantified in 75% of samples (0.42–0.73 µg/Kg, $p < 0.05$), apart from G. alypum honey from Fquih Ben Salah province (Table 3). Of the n = 13 PAHs investigated, n = 6 congeners were present in all samples, but quantified in 50% of them (i.e., jujube and sweet orange honeys). In particular, the jujube honey from Khénifra was the most contaminated product, with n = 5 PAHs detected at a level >LOQ. Between these, chrysene (2.10 µg/Kg), anthracene (1.54 µg/Kg) and fluorene (1.14 µg/Kg) were the most abundant toxicants. To follow, n = 4 PAHs were quantified in the sweet orange honey from Béni Mellal, with the most abundant compounds represented by benzo[a]anthracene (1.71 µg/Kg) and chrysene (1.62 µg/Kg) (Table 3). As previously mentioned, there are still no regulatory limits for PCBs and PAHs in honey and no toxicological consideration can be made in reference to the Reg. (EC) No. 1881/2006, since it fixes the ML of just one PAH, namely the benzo[a]pyrene, and establishes a ML for the sum of PCBs, taking into account the share of fat in food. However, the monitoring of PCBs and PAHs in Moroccan honey is very scarce. To the best knowledge of the authors, only Chakir et al.[35] investigated diverse honey samples from different South, Center–South and East Moroccan regions and from many floral origins, including C. sinensis and E. resiniphera. The study reported that a small share of samples was contaminated with PCBs, with concentration levels between 0.06 and 5.1 µg/kg. Furthermore, PAHs were present in all investigated samples with levels in the same range

or slightly higher than those observed in this study (0.26–7.58 μg/kg). However, congeners such as dibenzo(a,h)anthracene and acenaphthylene were revealed at the highest levels. A literature review pointed out that honey from the Mediterranean area produced during the last decade was poorly monitored with respect to pesticides, PCBs, and PAHs. In this respect, few recent works on Italian honey generally showed higher standards of chemical safety. In fact, organophosphorus pesticides were detected in the order of ng/g and not exceeding the relative MRLs; in addition, PAHs, such as acenaphthylene, fluorene, phenanthrene and pyrene, were found in the range >LOD-7.70 ng/g PCBs were absent in all honeys investigated.

Table 3. Residues of pesticides, PCBs, and PAHs detected in several honeys from the Béni Mellal- Khénifra region. Data are expressed as mean ± standard deviation of n = 3 samples analyzed per honey. Statistics from the Kruskal–Wallis test are also reported.

Analyte (μg/kg)	***Jujube Honey* (Khénifra)**	***Sweet Orange Honey* (Béni Mellal)**	***Euphorbia Honey* (PGI, Azilal)**	***G. alypum Honey* (Fquih Ben Salah)**	**F statistic**	**p-Value**
Carbaryl	1060.90^{c} ± 71.34	146.30^{b} ± 7.24	277.41^{b} ± 23.24	16.62^{a} ± 0.70	10.385	**0.016**
Dimethoate	72.01^{c} ± 4.92	14.31^{b} ± 1.28	< LOQ	< LOQ	10.649	**0.014**
Carbofuran	77.30^{c} ± 1.90	5.30^{b} ± 1.00	< LOQ	< LOQ	10.649	**0.014**
Diazinon	< LOQ	25.50^{c} ± 0.81	27.31^{c} ± 1.56	2.41^{b} ± 0.35	10.116	**0.018**
Alachlor	22.66^{c} ± 1.21	9.05^{b} ± 0.24	8.75^{b} ± 0.44	1.37^{a} ± 0.17	9.667	**0.022**
Metalaxyl-M	< LOQ	11.43^{b} ± 1.19	28.35^{c} ± 1.10	< LOQ	10.649	**0.014**
Quinalphos	5.92^{b} ± 0.30	< LOQ	< LOQ	< LOQ	10.735	**0.013**
Fenthion Sulfoxide	16.53^{b} ± 1.05	< LOQ	< LOQ	< LOQ	10.735	**0.013**
Fenthion Sulfone	< LOQ	5.26^{b} ± 0.74	6.11^{b} ± 3.25	< LOQ	9.598	**0.022**
Acephate	1251.19^{b} ± 147.67	11.46^{a} ± 0.69	25.49^{a} ± 1.61	11.45^{a} ± 1.25	9.359	**0.025**
Cyromazine	2060.99^{c} ± 75.05	223.72^{b} ± 12.71	113.60^{b} ± 3.86	21.72^{a} ± 1.48	10.385	**0.016**
PCB 118	0.71^{b} ± 0.01	< LOQ	< LOQ	< LOQ	10.800	**0.013**
PCB 180	< LOQ	0.43^{b} ± 0.03	0.72^{c} ± 0.07	0.42^{b} ± 0.04	9.565	**0.023**
Acenaphthylene	0.66^{b} ± 0.05	< LOQ	< LOQ	< LOQ	10.735	**0.013**
Fluorene	1.14^{b} ± 0.10	0.86^{b} ± 0.05	< LOQ	< LOQ	10.649	**0.014**
Phenanthrene	0.65^{b} ± 0.04	< LOQ	< LOQ	< LOQ	10.735	**0.013**
Anthracene	1.54^{b} ± 0.11	< LOQ	< LOQ	< LOQ	10.735	**0.013**
Benzo[a]anthracene	< LOQ	1.71^{b} ± 0.09	< LOQ	< LOQ	10.735	**0.013**
Chrysene	2.10^{b} ± 0.06	1.62^{b} ± 0.06	< LOQ	< LOQ	10.649	**0.014**

a–c indicate homogeneous sample groups at $\alpha = 0.05$ and honeys which do not differ from each other are designated by the same letter. Bold p-values showed significantly different results at $p < 0.05$ between different honeys.

Plasticizer and bisphenol residues revealed in the several honeys from the Béni Mellal-Khénifra region are shown in Table 4. Five PAEs (i.e., DEHP, DEP, DPrP, DiBP, and DBP) and five NPPs (i.e., DEA, DiBA, DBA, DEHA and DEHT) were determined at a concentration >LOQ in 100% samples. Among PAEs, DEP was the most abundant congener (0.94–3.17 mg/Kg $p < 0.05$) in the various honey, except for the G. alypum honey (0.94 mg/Kg, $p < 0.05$), followed by DBP (0.49–1.05 mg/Kg, $p < 0.05$) and DiBP (0.45–0.79 mg/Kg, $p < 0.05$) in all honey samples. Among the NPPs, DBA was the most abundant compound (8.62–12.42 mg/Kg, $p < 0.05$) in the Moroccan honeys, except for the G. alypum honey (0.50 mg/Kg, $p < 0.05$), followed by DEA, with a concentration ranging between 1.30–5.65 mg/kg ($p < 0.05$).

Furthermore, three BPs were also determined, namely BPA, BPB and BPAF. BPA was detected in 100% samples but quantified only in sweet orange and PGI Euphorbia honey samples (respectively, 7.74 µg/kg and 8.07 µg/kg, $p > 0.05$); BPAF was determined in all samples except the G. Alypum honey (1.48–158 µg/kg, $p > 0.05$); and BPB was the most abundant BP in all Moroccan honeys (4.16–8.75, $p < 0.05$). Among the determined plasticizers, Reg. (EU) No. 10/2011 has established SMLs from food contact material for DBP (0.3 mg/Kg), DEHP (1.5 mg/Kg), as well as DEP, DiBP and DEHT (60 mg/Kg)[36]. On this basis, all Moroccan honeys had an excessive amount of DBP, this PAE being at a concentration level 1.5–3 times higher than the relative SML. On the other hand, the regulatory SML for BPA migration from food contact material is 600 ng/g[37].

Table 4 shows that none of the honey samples contained BPA at concentrations higher than the SML. Similarly to pesticides, PAHs and PCBs in the jujube honey from Khénifra and the sweet orange honey from Béni-Mellal demonstrated the highest levels of plasticizers and BPs, while the G. alypum honey from the Fquih Ben Salah province was generally the least contaminated product. However, in such honey, one PAE, i.e., DEHP, and one NPP, i.e., DEHT, were found at the highest levels (1.06 mg/kg and 1.14 mg/Kg, $p < 0.05$). To the best of our knowledge, there is no literature regarding plasticizers and BPs in Moroccan honey.

However, recent efforts can be observed in the determination of such chemicals in honey from the Mediterranean basin. In this respect, Lo Turco and coworkers[38] determined much lower levels of PAEs (e.g., DEP, 0.006 mg/Kg; DiBP, 0.042 mg/Kg; DBP, 0.039 mg/Kg; and DEHP, 0.191 mg/Kg) in Sicilian and Calabrian honeys than those determined in Moroccan honeys, and BPA was lower than analytical LOQ in all samples.

More recently, Notardonato and colleagues[39] confirmed the higher purity of Italian honey, pointing out lower frequencies of PAE determination in honey samples, with only the DEHP found at concentrations (0.005–0.960) similar to those of the Moroccan honeys under study. Indeed, other PAEs, such as DEP (0.020–0.371 mg/Kg), DiBP (0.028–0.299 mg/Kg), DBP (0.019–0.550 mg/Kg) yielded much lower levels. However, Italian honeys revealed higher levels of BPA (18.8–996.8 µg/kg). Since plasticizers and BPs could be easily released from the plastic components of honey production equipment (e.g., honey extractor and uncorkers), it may be assumed that the Moroccan honey could be contaminated by such compounds during production steps, as already observed not only in honey but also in a variety of processed food.[40,41]

Additionally, prolonged periods of storage with given conditions in terms of temperature, humidity, and light, may affect not only the peculiar physicochemical properties of honey, but also cause gradual polymer degradation, and, consequently, the migration of plastic additives from the plastic packaging into the honey. However, due to the ubiquitous presence of plasticizers and BPs in the environment, the contamination of honey from the nectar source should always be considered.[42]

Table 4. Plasticizers (PAEs and NPPs) and BPs detected in several honeys from the Béni Mellal- Khénifra region. Data are expressed as mean ± standard deviation of n = 3 samples analyzed per honey. Statistics from the Kruskal–Wallis test are also reported.

Analyte (mg/kg)	*Jujube Honey* (Khénifra)	*Sweet Orange Honey* (Béni Mellal)	*Euphorbia Honey* (PGI, Azilal)	*G. alypum Honey* (Fquih Ben Salah)	F statistic	p-Value
			Plasticizers (mg/kg)			
DEHP	$0.59^{ab} \pm 0.03$	$0.36^{a} \pm 0.02$	$0.35^{a} \pm 0.03$	$1.06^{b} \pm 0.03$	9.359	**0.025**
DEP	$2.95^{b} \pm 0.10$	$3.17^{b} \pm 0.05$	$2.42^{b} \pm 0.28$	$0.94^{a} \pm 0.06$	10.385	**0.016**
DPrP	$0.61^{b} \pm 0.03$	$0.65^{b} \pm 0.02$	$0.57^{b} \pm 0.01$	$0.42^{a} \pm 0.01$	9.974	**0.019**
DiBP	$0.77^{b} \pm 0.04$	$0.79^{b} \pm 0.03$	$0.60^{ab} \pm 0.07$	$0.45^{a} \pm 0.02$	9.359	**0.025**
DBP	$0.89^{b} \pm 0.06$	$1.05^{b} \pm 0.05$	$0.84^{b} \pm 0.04$	$0.49^{a} \pm 0.04$	9.667	**0.022**
DEA	$5.65^{b} \pm 0.48$	$5.30^{b} \pm 0.07$	$1.35^{a} \pm 0.09$	$1.30^{a} \pm 0.16$	8.436	**0.038**
DiBA	$0.85^{b} \pm 0.05$	$0.82^{b} \pm 0.03$	$0.76^{b} \pm 0.01$	$0.53^{a} \pm 0.08$	9.462	**0.024**
DBA	$12.42^{c} \pm 0.31$	$12.23^{c} \pm 0.40$	$8.62^{b} \pm 0.65$	$0.50^{a} \pm 0.04$	9.667	**0.022**
DEHA	0.39 ± 0.06	0.36 ± 0.08	0.36 ± 0.05	0.63 ± 0.05	9.368	0.095
DEHT	0.59 ± 0.03	0.52 ± 0.07	0.58 ± 0.09	1.14 ± 0.10	6.897	0.075
			Bisphenols (μg/kg)			
BPA	< LOQ	$7.74^{b} \pm 0.69$	$8.07^{b} \pm 0.08$	< LOQ	9.598	**0.022**
BPB	$8.75^{b} \pm 1.15$	$5.72^{ab} \pm 0.61$	$4.62^{a} \pm 0.31$	$4.16^{a} \pm 0.10$	10.385	**0.016**
BPAF	1.48 ± 0.17	1.55 ± 0.24	1.58 ± 0.17	< LOQ	6.668	0.083

a–c indicates homogeneous sample groups at α = 0.05 and honeys which do not differ from each other are designated by the same letter. Bold p-values showed significantly different results at p< 0.05 between different honeys.

The profile of potentially toxic trace elements of Moroccan honeys is reported in Table 5. Among investigated elements, Al was the most abundant metal and, differently from the trend of other contaminants, the highest and lowest levels of such metal were found respectively in the PGI Euphorbia honey from Azilal (5.56 mg/Kg, $p < 0.05$) and in the jujube honey produced in Khénifra (1.69 mg/Kg, $p < 0.05$). Aluminum may be related to the soil acidification caused by elevated industrial emissions and poor mining practices occurring in such Moroccan regions, and it may be increasingly bioavailable to organisms through plant roots. Inevitably, the metal can then spread up the food chain through pollen, and nectar collection.[43]

To follow, equal amounts of Pb and As (0.06–0.16 mg/Kg, $p > 0.05$) were determined in all samples, while Cd was lower than LOQ (0.003 µg/Kg) in any case. Regulation (EU) No. 2015/1005, amending Regulation (EC) No. 1881/2006 for the ML of Pb in certain foods, has introduced a ML of 0.1 mg/Kg in honey. Accordingly, 3 out of the 4 types of Moroccan honey, namely the sweet orange, the PGI Euphorbia and the G. alypum honeys, were characterized by a Pb content higher than the regulatory limit. Anthropogenic activities, including industrial applications and agricultural chemicals, are responsible for the heavy metal contamination of surroundings. As with other contaminants, bees and bee products including honey are exposed to these contaminants via polluted pollen, water and air. The role of honeybees as "filters" of heavy metals and their protective function against honey contamination have been commonly accepted.[44,45]

In contrast, honey is still considered as a typical indicator of heavy metal pollution related not only to anthropogenic activities (e.g., agriculture, industry etc.) but also to the entire production process, as poor beekeeping practices may be also source of heavy metal residues in honey. In the last decade, the issue of potentially toxic trace elements in honey from Mediterranean countries has been approached and, as expected, great data variability was pointed out. For example, carob honey from different Moroccan areas and the PGI Euphorbia honey did not display heavy metals such as Cd and Pb, probably because of the high instrumental LOQ values. Honey from several Libyan locations had very high levels of Pb (2.42–10.98 mg/Kg), Cd (0.125–0.150 mg/Kg), but low As contents (0.006– 0.018 mg/Kg).[46]

Despite honeys from different Italian regions generally showing Cd and Al at higher levels than Moroccan honeys (respectively, 0.003–0.02 mg/Kg and 0.52– 26 mg/Kg), Pb was always under the regulatory limit (0.05–0.06 mg/Kg) and As at lower levels than those detected in this study (0.01 mg/Kg). On the other hand, different varieties of Spain honeys were marked by lower levels of As (0.004–0.010 mg/Kg) and Pb (0.011–0.041 mg/Kg).[47]

Table 5. Potentially toxic elements of several honeys from the Béni Mellal-Khénifra region. Data are expressed as mean ± standard deviation of n = 3 samples analyzed per honey. Statistics from the Kruskal–Wallis's test are also reported.

Analyte (mg/kg)	*Jujube Honey* (Khénifra)	*Sweet Orange Honey* (Béni Mellal)	*Euphorbia Honey* (PGI, Azilal)	*G. alypum Honey* (Fquih Ben Salah)	F statistic	p-Value
Al	$1.69^{a} \pm 0.15$	$2.73^{ab} \pm 0.19$	$5.56^{b} \pm 0.12$	$2.67^{ab} \pm 0.12$	9.359	**0.025**
Pb	0.06 ± 0.31	0.16 ± 0.01	0.15 ± 0.01	0.12 ± 0.01	3.873	0.276
Cd	< LOQ	< LOQ	< LOQ	< LOQ	-	-
As	0.06 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	9.430	0.249

a–b indicates homogeneous sample groups at α = 0.05 and honeys which do not differ from each other are designated by the same letter. Bold p-values showed significantly different results at $p < 0.05$ between different honeys.

6.1.8. PCA Analysis

In the present study, PCA provided information on the most significant variables describing the whole data set, enabling data reduction at the same time with a minimum loss of original information. Four principal components (PCs) with eigenvalues exceeding one (325.627, 18.195, 7.575 and 1.257) were extracted according to the Kaiser Criterion, and they explained up to 97.51% of total variance (i.e., 47.457%, 33.695%, 14.029% and 2.327%, respectively). Figure 2 illustrates the bidimensional score and loading plots.

Defined by the first two PCs accounting for more than 81% of the variability of the system, the score plot (Figure 2a) showed four distinguished clusters of honey samples. Such clusters correspond to the four types of Moroccan honey investigated, which differed from each other not only in their botanical origin (i.e., jujube, sweet orange, Euphorbia and G. alypum honeys) but also for the production area (i.e., provinces of Khénifra, Béni Mellal, Azilal and Fquih Ben Salah).

Due to such a sample arrangement in the study, it is somewhat troublesome to define whether the clustering of honeys occurred according the botanical or geographic origin. However, based on the array of variables investigated, the obtained data and the provided considerations, it may be argued that both factors noticeably contributed to such sample differentiation. Indeed, the loading plot (Figure 2b) shows that, in accordance with the results of the Kruskal–Wallis test, almost all

investigated parameters (i.e., physicochemical indicators, minerals, organic and inorganic contaminants), each related to the floral and/or geographical origin, contributed significantly to the differentiation of honey samples, except for pH, DEHT, DEHA, BPAF, Pb and As. By overlapping the loading and score plots, it becomes clear that variables such as pesticides, PAHs, plasticizers and BPs weighed more on the jujube honey from Khénifra and the sweet orange honey from Béni Mellal which, in fact, were the honey samples most affected by organic contamination. The PGI Euphorbia honey was in general less contaminated than the above honeys, but it was still characterized by the highest content of pesticides, such as diazinon and metalaxyl-M, and potentially toxic metals, such as Al. On the other hand, the *G. alypum* honey was the least contaminated Moroccan product and, moreover, it was marked by the most convenient physicochemical traits (i.e., TSS, acidity and conductivity), as well as precious contents of minerals and essential trace elements.

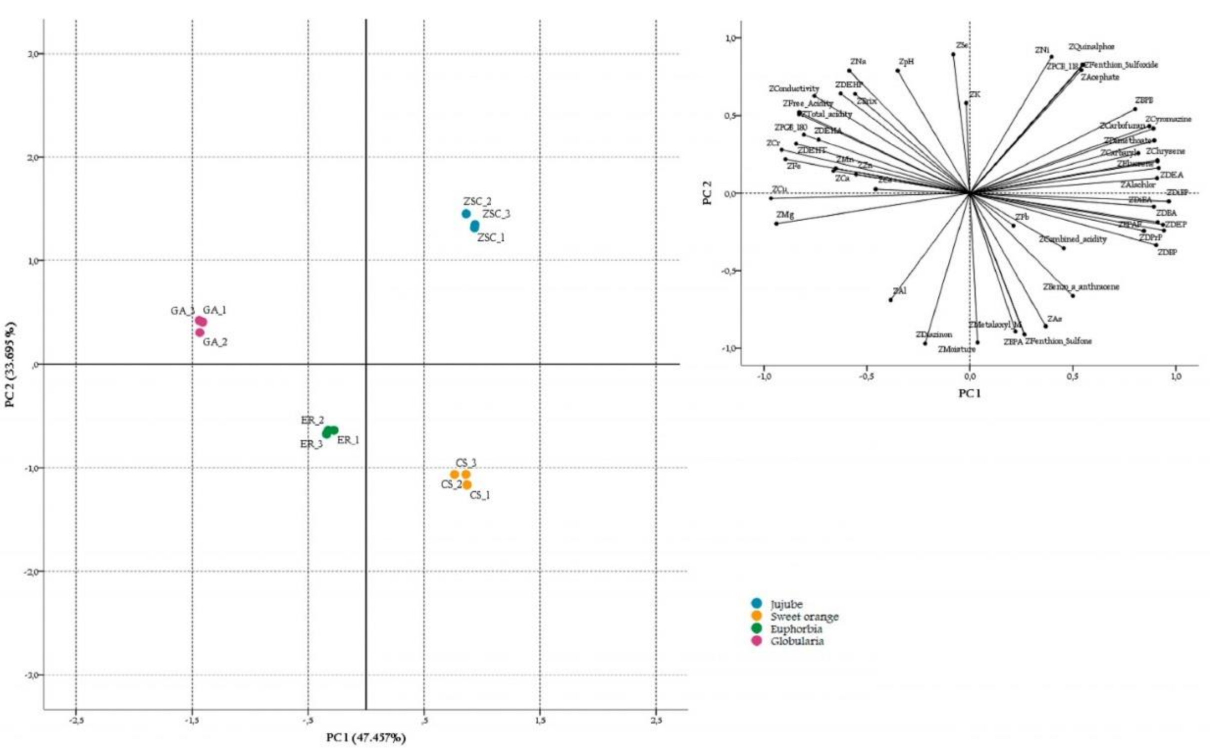

Figure 2. PCA score (left) and loading (right) plots of PC1 and PC2 showing the differentiation of honey samples in the component space based on the array of variables investigated.

6.1.9. Dietary Exposure to Contaminants

The quality of Moroccan honeys and the potential health risk to consumers were assessed by calculating the EDI and the non-carcinogenic risk (HQ) of organic and inorganic contaminants (Table 6). EDIs and HQs were calculated by considering the amount of honey consumed daily in the diet by an adult consumer (70 Kg) from Europe (1.8 g/day) and North Africa (0.3 g/day), according to FAO[48], as well as guideline values recommended by international organizations. As shown in Table 6, the EDIs calculated were well below the intake levels of relative pollutants recommended by international regulatory bodies, thus indicating that Moroccan honey can be safely consumed through the provided dietary amounts.

For the non-carcinogenic risk assessment, HQ did not exceed the threshold value of 1 for each contaminant potentially ingested by adults through honey in both European and North- African diets, thus, indicating that non-carcinogenic health effects derived from the consumption of these Moroccan honeys were not significant.

Table 6. EDIs (mg/Kgbw/day or *µg/Kgbw/day) and HQs calculated for investigated Moroccan honeys daily assumed by adult consumers both from Europe and North Africa.

	Jujube honey (Khénifra)				**Sweet orange honey (Béni Mellal)**				**Euphorbia honey (PGI, Azilal)**				***G. alypum* honey (Fquih Ben Salah)**			
	Europe		**North Africa**		**Europe**		**North Africa**		**Europe**		**North Africa**		**Europe**		**North Africa**	
	EDI	HQ	EDI	HQ	EDI	HQ	EDI	HQ	EDI	HQ	EDI	HQ	EDI	HQ	EDI	HQ
									Plasticizers and BPs							
DEHP	$1.52x10^{-05}$	<1	$2.53x10^{-06}$	<1	$9.25x10^{-06}$	<1	$1.54x10^{-06}$	<1	$9.00x10^{-06}$	<1	$1.50x10^{-06}$	<1	$2.73x10^{-05}$	<1	$4.54x10^{-06}$	<1
DEP	$7.59x10^{-05}$	<1	$1.26x10^{-05}$	<1	$7.97x10^{-05}$	<1	$1.32x10^{-05}$	<1	$6.22x10^{-05}$	<1	$1.04x10^{-05}$	<1	$2.42x10^{-05}$	<1	$4.03x10^{-06}$	<1
DBP	$2.29x10^{-05}$	<1	$3.81x10^{-06}$	<1	$0.27x10^{-05}$	<1	$0.45x10^{-05}$	<1	$0.21x10^{-04}$	<1	$3.60x10^{-06}$	<1	$1.26x10^{-05}$	<1	$2.10x10^{-06}$	<1
DEHA	$1.00x10^{-05}$	<1	$1.67x10^{-06}$	<1	$9.25x10^{-06}$	<1	$1.54x10^{-06}$	<1	$9.25x10^{-06}$	<1	$1.54x10^{-06}$	<1	$1.62x10^{-05}$	<1	$2.70x10^{-06}$	<1
BPA*	-	-	-	-	$0.20x10^{-03}$	<1	$3.31x10^{-05}$	<1	$0.21x10^{-03}$	<1	$3.46x10^{-05}$	<1	-	-	-	-
									Potentially toxic elements							
Al	$3.04x10^{-04}$	<1	$5.07x10^{-05}$	<1	$4.91x10^{-04}$	<1	$8.19x10^{-05}$	<1	$1.00x10^{-03}$	<1	$1.67x10^{-04}$	<1	$4.81x10^{-04}$	<1	$8,01x10^{-05}$	<1
Pb	$1.54x10^{-06}$	<1	$2.57x10^{-07}$	<1	$4.11x10^{-06}$	<1	$6.86x10^{-07}$	<1	$3.86x10^{-06}$	<1	$6.43x10^{-07}$	<1	$3.09x10^{-06}$	<1	$5.14x10^{-07}$	<1
As	$1.54x10^{-03}$	<1	$2.57x10^{-04}$	<1	$2.83x10^{-03}$	<1	$4.71x10^{-04}$	<1	$2.31x10^{-03}$	<1	$3.86x10^{-04}$	<1	$1.54x10^{-03}$	<1	$2.57x10^{-04}$	<1

6.1.10. Conclusions

For the first time, a comprehensive characterization of the physicochemical traits and contaminants of four monofloral honeys from different provinces of the Moroccan region Béni Mellal-Khénifra was carried out, thus corroborating the scarce literature on Moroccan honey. According to the physicochemical parameters, all honeys under analysis were in line with those EU standards established for assuring the authenticity of such bee products. However, a critical contamination pattern was outlined, with several toxicants often exceeding the EU regulatory limits available for honey. Specifically, the jujube honey from Khénifra and the sweet orange honey from the Béni Mellal province were the most contaminated products, as opposed to the *G. alypum* honey from the Fquih Ben Salah province, which was shown to be the least contaminated one. In this arrangement, the PGI Euphorbia honey from the Azilal area had an intermediate contamination degree. However, the dietary exposure assessment highlighted that small amounts of all honeys can be safely introduced both in European and North African diets on a daily basis. Hopefully, findings from this study should not only encourage the enforcement and harmonization of the international regulatory framework on the chemical safety of honey, which is still suffering from evident flaws and shortcomings, but also serve as a “wake- up call” for Moroccan governmental bodies to strengthen monitoring activity in beekeeping and to find suitable solutions for accomplishing more sustainable agricultural practices and modernize the beekeeping sector.

6.2. STUDY OF PHYSICOCHEMICAL QUALITY AND ORGANIC CONTAMINATION IN ALGERIAN HONEY

Algeria is considered a traditional consumer of honey, but national production does not achieve self-sufficiency because of the lack of national legislation and the rural organization of this ancient practice.[49] The northern region of Algeria, characterised by a Mediterranean climate and great diversity of flora, lends itself to beekeeping, while the high steppe plateau and the large Saharan plateau in the south of Algeria are less suitable for beekeeping. In 2021, the national honey production was estimated to be 5165 t, with a yield of 4 to 8 kg per hive, which is very low considering the potential offered by Algeria and the 150,000 tons of honey per year imported from other countries.[50] In the absence of national legislation, there are no criteria to check the safety and the quality of Algerian honey. Considering this scenario, the aim of the present study was to investigate the physicochemical parameters of honey (i.e., moisture, total soluble solids, pH, electrical conductivity, and acidity) and the presence of organic chemical residues in samples from different areas of Algeria. The aim was to monitor the quality and safety of honey from different geographical areas and to assess dietary exposure to contaminants by exploiting the Algerian guidelines.

6.2.1. Honey Sample Collection

A total of 54 honey samples were collected during 2022 and 2023 by beekeepers located in three different provinces of Algeria (i.e., Tiaret, Laghouat, and Tindouf), as detailed in Table 7. Samples with the same geographical and botanical origins were grouped together.

Laghouat and Tiaret are two of the most famous Algerian regions for their production of honey. Laghouat is an ancient oasis in the southern foothills of the Saharan Atlas, characterized by the presence of flora very similar to that present in Mediterranean regions. Tiaret is situated in the western steppe region of Algeria, and, in the north, there are dense forest areas that contain many different species of plants.

Tindouf, located in the natural region of the Sahara Desert, is characterised by the low diversity and abundance of plant species due to the extreme environmental conditions. Figure 3 shows the geographical map of the sampling sites considered for the study.

The honey samples obtained from these areas of Algeria were collected in glass containers of approximately 150 g and stored in a dark place at ambient temperature until analysis.

Table 7. Honey samples and their botanical and geographical origins.

Code	N. of Samples	Botanical Origin	Geographical Origin
M_T	6	*Multifloral*	Tiaret
E_T	3	*Echinops*	Tiaret
ES_T	3	*Eruca sativa*	Tiaret
ZL_T	3	*Ziziphus lotus*	Tiaret
BM_T	3	*Bunium mauritanicum*	Tiaret
TE_L	3	*Tamarix* and *Euphorbia orientalis*	Laghouat
EO_L	6	*Euphorbia orientalis*	Laghouat
EG_L	3	*Eucaliptus globulus*	Laghouat
M_L	3	Multifloral	Laghouat
Z_L	6	*Ziziphus lotus*	Laghouat
E_D	3	*Echinops*	Tindouf
ES_D	6	*Eruca sativa*	Tindouf
EO_D	3	*Euphorbia orientalis*	Tindouf
PH_D	3	*Peganum harmala*	Tindouf
Total	54		

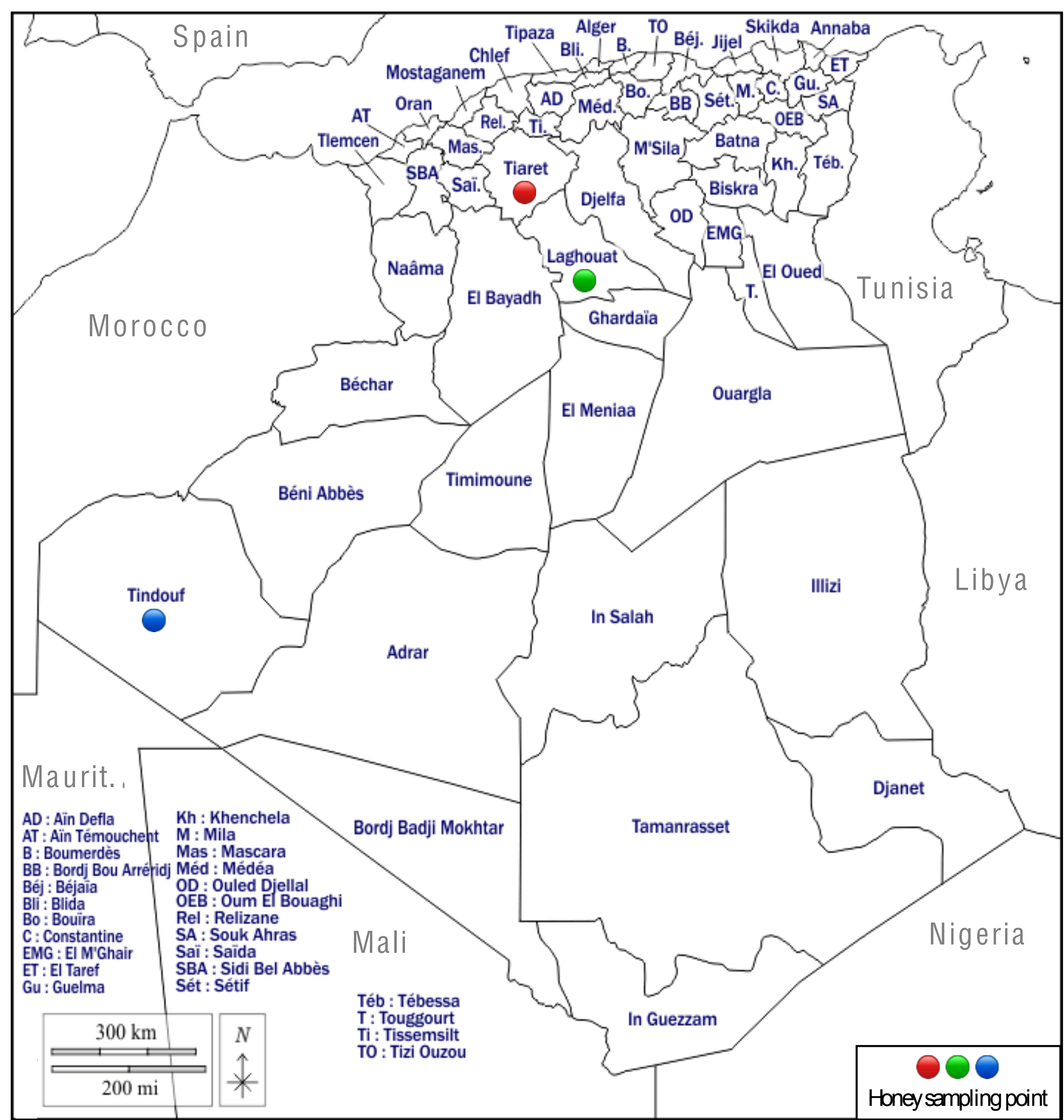

Figure 3. Geographical origin of honey samples considered for the study.

6.2.2. Chemical analysis

The physiochemical parameters of each honey sample were determined according to the official methods described in section *7.1.3*. The method adopted for the extraction of pesticides, PCBs and PAHs from honey samples was previously discussed in the section *7.1.4.*

The multiresidue analysis was carried out using a Shimadzu GCMS-TQ8030 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The GC-MS conditions can be found in Section *5.1.2.*

Instrumentation and analytical conditions in Chapter 5. The identification of pesticides, PAHs, and PCBs was carried out by comparing their mass spectra and retention times to those of corresponding commercially available standards. The Multiple Reaction Monitoring (MRM) mode was used for the quantification of analytes, exploiting the IS normalisation.

The MRM transitions, as well as the analytical method validation, are reported in Table 1 and 2 (chapter 5). The LabSolutions software 4.01 (Shimadzu) was used for data acquisition and quantification. Each honey sample was analysed three times, along with analytical blanks, to ensure the accuracy and reliability of the measurements.

The extraction method of plasticizer residues from honey samples as reported in the section *7.1.4* was adopted in the present work. PAEs and NPPs were identified by comparing their mass spectra and retention times to those of commercially available standards. Quantitative analysis was carried out in Single-Ion Monitoring (SIM) mode, taking into account the base peak ion among three characteristic mass fragments for each target analyte, reported in Table 2 (chapter 5), and employing internal standard (IS) normalisation. The LabSolutions software 4.01 (Shimadzu) was used for data acquisition and quantification. Each sample was analysed in triplicate with analytical blanks, to ensure the accuracy and reliability of the measurements. Glass equipment was used to avoid plasticizer contamination.

The extraction of bisphenols was performed using the Micro-QuEChERS procedure, developed and validated by Potortì et al. (the procedure in detail will be discussed in the next section 7.3)

6.2.3. Statistical Analysis

Experimental data are presented as the means ± standard deviation of three replicate measurements for each sample. The statistical analysis was performed using the SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for each independent variable to show statistically significant differences. When a significant F was determined (Fcalculated > Fcritical), Tukey's honestly significant difference (HSD) test was

performed for all pairwise comparisons of means. For each variable examined, statistical significance was accepted at the level of $p < 0.01$. To identify the differences in organic contaminants in honey from different regions of Algeria, the dataset was standardised to achieve uniform significance for all variables and the principal component analysis (PCA) was performed, including only the contaminants quantified in at least 60% of the samples analysed.

6.2.4. Assessment of the Dietary Exposure to Contaminants

In order to assess the health risks associated with the organic contaminants present in Algerian honey, the relative Estimated Daily Intakes (EDIs) were first determined. EDIs were calculated by multiplying the mean concentration of contaminants quantified in each sample (expressed in mg/kg or μg/kg) by the daily consumption of honey (in grams) and dividing the resulting value by the body weight of the consumers (in kilograms). Additionally, the chronic non-carcinogenic risk derived from dietary exposure to the chemical was calculated in terms of Hazard Quotient (HQ). HQ is obtained by dividing a specific EDI by the corresponding Acceptable Daily Intake (ADI) or Tolerable Daily Intake (TDI). When $HQ < 1$, there are no health risks to the exposed population.

6.2.5. Results and Discussions

Table 8 shows the values of moisture; TSS; conductivity; pH; and the free, combined, and total acidity of honey from different regions of Algeria.

Moisture is a crucial parameter in controlling the quality of honey because high moisture content can cause its undesirable fermentation.[51] This parameter depends on environmental conditions (i.e., pedoclimatic conditions and soil characteristics), activities conducted by beekeepers, harvest time, and honey maturity level. The moisture influences honey taste, colour, flavour, viscosity, density, crystallisation, and fermentation during storage. In the honey analysed, moisture values were in the range of 12.32–16.55% ($p < 0.01$), lower than the maximum limit set by the Codex Alimentarius and EU regulation (20%). This indicates that all honey samples investigated in this study have reached a good level of maturity.

The TSS of honey reflects the sugar compounds present in honey, and it is inversely correlated to the moisture. In the honey analysed, the maximum value was 84.91 °Brix in *E. globulus* honey from Laghouat while the minimum was 81.28 °Brix in multifloral honey samples from Tiaret ($p < 0.01$). High values of TSS contribute to osmotic stress for selected microorganisms.

Table 8. Physicochemical parameters (moisture, TSS, conductivity, pH, and acidity) of Algerian honey. Values are expressed as means ± standard deviation of three replicates for each sample with the same botanical and geographical origin.

	Moisture (%)	**TTS (°Brix)**	**Conductivity (µS/cm)**	**pH**	**Free Acidity (meq/kg)**	**Combined Acidity (meq/kg)**	**Total Acidity (meq/kg)**
M_T	14.51 ± 1.59 $^{a–d}$	81.25 ± 1.30	490.65 ± 24.47 ^{a,b}	4.26 ± 0.07 ^{a,b}	46.67 ± 0.99 ^{a,e,f}	0.86 ± 0.03 ^{a}	47.54 ± 0.97 ^{a,d}
E_T	14.39 ± 0.13 $^{a–d}$	84.65 ± 1.32	468.55 ± 7.56 ^{a,b}	4.37 ± 0.09 ^{a,b}	29.54 ± 0.64 ^{b,c}	0.87 ± 0.02 ^{a}	30.31 ± 0.64 ^{b,e,c}
ES_T	14.15 ± 0.16 $^{a–d}$	82.36 ± 1.58	326.94 ± 6.77 ^{a,c}	4.30 ± 0.02 ^{a,b}	41.84 ± 0.97 ^{a,d,e}	0.86 ± 0.02 ^{a}	42.71 ± 0.96 ^{a,c,d}
ZL_T	13.66 ± 0.20 $^{a–c}$	84.09 ± 1.30	525.79 ± 7.74 ^{a,b}	4.65 ± 0.07 ^{b,d}	26.37 ± 0.73 ^{b,c}	0.88 ± 0.03 ^{a}	27.25 ± 0.74 ^{b,e}
BM_T	16.55 ± 0.13 ^{d}	82.22 ± 1.36	439.15 ± 5.55 $^{a–c}$	4.40 ± 0.06 ^{a,b}	34.12 ± 0.75 ^{b,d}	4.18 ± 0.04 ^{b}	38.30 ± 0.71 ^{a,b}
TE_L	16.15 ± 0.08 ^{a,b,d}	81.89 ± 1.43	565.43 ± 8.10 ^{b,e}	4.41 ± 0.04 ^{a,b}	38.09 ± 0.64 ^{a,b,d}	0.84 ± 0.02 ^{a}	38.93 ± 0.63 ^{a,b}
EO_L	14.12 ± 0.76 $^{a–c}$	83.65 ± 1.29	544.96 ± 187.42 ^{b}	4.50 ± 0.09 ^{b,f}	29.80 ± 7.23 ^{d}	2.12 ± 0.07 ^{c}	31.92 ± 7.16 ^{b,g}
EG_L	12.51 ± 0.20 ^{c}	84.91 ± 1.61	471.05 ± 6.33 ^{a,b}	4.39 ± 0.10 $^{a–c}$	42.11 ± 0.46 ^{a,d,e}	5.23 ± 0.03 ^{d}	47.34 ± 0.43 ^{a,d,f}
M_L	14.03 ± 0.17 $^{b–d}$	83.07 ± 1.47	428.21 ± 5.79 $^{a–c}$	4.50 ± 0.11 ^{a,b}	31.27 ± 0.50 ^{b,d}	4.89 ± 0.03 ^{d}	36.16 ± 0.47 ^{b,c,f,g}
Z_L	13.16 ± 0.54 ^{c}	84.53 ± 1.24	471.75 ± 44.76 ^{a,b}	4.86 ± 0.07 ^{b}	20.10 ± 2.26 ^{c}	2.85 ± 0.33 ^{e}	22.95 ± 2.57 ^{e}
E_D	13.93 ± 0.12 ^{a,c}	83.19 ± 1.16	330.28 ± 5.03 ^{a,c}	3.97 ± 0.05 ^{a,c,e}	42.33 ± 0.60 ^{d,e}	2.57 ± 0.04 ^{e}	44.90 ± 0.60 ^{a,d,f}
ES_D	15.34 ± 0.98 ^{a,b,d}	82.08 ± 1.91	241.48 ± 7.04 ^{c}	3.61 ± 0.43 ^{e}	30.73 ± 5.99 ^{b}	0.86 ± 0.02 ^{a}	31.58 ± 5.98 ^{b,g}
EO_D	13.79 ±0.20 ^{a,c}	82.57 ± 1.02	337.07 ± 6.78 ^{a,c,e}	3.93 ± 0.04 ^{a,e}	52.96 ± 0.58 ^{e}	0.86 ± 0.01 ^{a}	53.82 ± 0.57 ^{d}
PH_D	12.32 ± 0.09 ^{c}	81.97 ± 1.34	376.50 ± 9.07 $^{a–c}$	4.08 ± 0.07 $^{a,d–f}$	37.34 ± 0.52 ^{b,d,f}	1.74 ± 0.04 ^{f}	39.08 ± 0.55 ^{a,g}
***p*-Value**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**

a–g Different superscript letters within the same column denote significantly different values for a specific parameter ($p < 0.01$ by post hoc Tukey's HSD test); same superscript letters denote not significantly different values for a specific parameter ($p > 0.01$ by post hoc Tukey's HSD test). Bold p-values indicate significantly different results at $p < 0.01$ between different types of honey.

The electrical conductivity of honey depends mainly on mineral content but also on organic acids in honey and it is influenced by the geographic location and botanical source. This parameter is useful in discerning between nectar and honeydew honey because it is generally higher in honeydew honey. In Algerian honey, the conductivity varied from 241.48 µS/cm to 565.43 µS/cm ($p < 0.01$), respectively, in *E. sativa* honey from Tindouf and Tamarix and E. orientalis honey from Laghouat. The Codex Alimentarius and EU regulation fixed the electrical conductivity lower than 800 µS/cm for nectar honey, so this parameter demonstrates that all the samples analysed were honey obtained from nectar. An acidic character was observed in all analysed samples. A normal pH value for honey falls within the range of 3.2 to 4.5. pH values of Algerian honey ranged between 3.61 in E. sativa

honey from Tindouf and 4.86 in *Z. lotus* honey from Laghouat ($p < 0.01$). Both analysed types of *Z. lotus* honey are characterized by a pH value out of the suggested range.

Honey acidity is correlated to pH, and vice versa. Good honey is characterised by high acidity and low pH, parameters that inhibit microorganism growth.[52] Acidity is determined via the content of organic acids (mainly gluconic acid, derived from the enzymatic reaction of glucose oxidase with glucose in the presence of water) and, therefore, via the enzymatic activity, and it is related to the freshness of the honey. The acids are in a fluctuating equilibrium between their free and combined forms represented by lactones. For this reason, the value of the total acidity of honey is given through the sum of free and combined acidity. The maximum level of free acidity is set at 50 meq/kg from the EU and Codex Alimentarius. Most of the samples analysed in this study showed a level of free acidity below the limit fixed, specifically in the range between 20.10 and 46.67 meq/kg ($p < 0.01$) in *Z. lotus* from Laghouat and multifloral honey from Tiaret, respectively. The same samples showed the lowest and the highest values of total acidity, respectively (22.95 meq/kg in ZL and 47.54 meq/kg in MT). The maximum level of combined acidity is found in the E. globulus honey from Laghouat. Only the *E. orientalis* honey from Tindouf showed a level of free acidity higher than the EU limits (52.96 meq/kg). The two analysed types of Z. lotus honey, with the higher pH value, also have lower levels of free and total acidity than all the samples.

For all parameters studied, statistically significant differences were found due to the great variability of the botanical and geographical origins of the analysed honey. Only for TTS did Tukey's HSD test show that there is no significant difference between the means of any pair despite a p-value lower than 0.01.

In general, moisture, TTS, conductivity and pH of honey samples evaluated in this study were in line with the results reported in recent studies concerning Algerian honey from different regions. However, the values of acidity were higher than the data reported in the literature.[53,54] These data show that Algerian honey displays medium quality parameters.

Table 9. Residues of pesticides, PCBs, and PAHs detected in several honey varieties from Algeria. Data are expressed as means ± standard deviation of three replicates for each sample with the same botanical and geographical origin.

Analyte	Tiaret					Laghouat					Tindouf				*p*-Value
(µg/kg)	**M_T**	**E_T**	**ES_T**	**ZL_T**	**BM_T**	**TE_L**	**EO_L**	**EG_L**	**M_L**	**Z_L**	**E_D**	**ES_D**	**EO_D**	**PH_D**	
Bendiocarb	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.20 ± 0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
Carbaryl	0.94± 0.42 ^{a}	7.61 ± 0.61 ^{b,e}	<LOQ	1.39 ± 0.17 ^{a,d}	1.18 ± 0.13 ^{a,d}	0.62 ± 0.06^{a,d}	1.08 ± 1.14 ^{a}	9.46 ± 0.87 ^{b}	0.67 ±0.06 ^{a,d}	1.49 ± 0.92 ^{a,d}	15.81±1.48^{c}	3.91±4.25^{a,e}	6.20 ± 0.68 ^{b,d,e}	4.51 ± 0.47^{a,b}	**<0.01**
Furathiocarb	<LOQ	<LOQ	<LOQ	2.15 ± 0.27	<LOQ	<LOQ	2.35 ± 2.57	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**0.89**
Metalaxyl-M	0.42 ± 0.10 ^{a,b,e}	0.31 ± 0.04 ^{b}	0.32 ± 0.02 ^{a,b}	0.63 ± 0.07 ^{c,e}	1.10 ± 0.09 ^{d,f}	0.78 ± 0.08 ^{c}	1.26 ± 0.13 ^{d}	0.84 ± 0.05 ^{c,f}	0.75 ± 0.08 ^{c}	0.46 ± 0.08 ^{a,b,e}	0.30±0.03^{a,b}	0.34±0.03^{a,b}	0.79 ± 0.08 ^{c}	0.27 ± 0.02^{a,b}	**<0.01**
Quintozen	<LOQ	0.35 ± 0.04 ^{a}	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	4.86 ± 0.57 ^{b}	0.37 ±0.03 ^{a}	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**<0.01**
Methabenzthiazuron	<LOQ	<LOQ	<LOQ	0.27 ± 0.02	<LOQ	<LOQ	0.35 ± 0.36	0.82 ±0.08	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**0.06**
Propazine	1.18 ± 1.28	<LOQ	<LOQ	0.46 ± 0.06	1.93 ± 0.13	0.47 ± 0.04	1.28 ± 0.15	<LOQ	0.42 ± 0.04	<LOQ	<LOQ	0.30 ±0.29	<LOQ	<LOQ	**<0.01**
Propyzamide	<LOQ	0.12 ± 0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
Simazide	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.69 ±0.05	<LOQ	<LOQ	0.31 ± 0.21	<LOQ	<LOQ	**0.07**
Cyromazine	103.60±94.12^{a,c,d}	40.28 ± 4.37 ^{a,b,d}	16.16 ± 1.54 ^{a,b}	50.63±4.48$^{a–c}$	163.58±16.20^{c}	55.90±5.48^{a,b,c}	123.08±9.66^{c,d,f}	10.32±1.33^{a,b}	58.38±4.39^{a,b,c}	12.77±14.02^{b,e}	0.30±0.04^{b,e}	43.21±16.24^{a,b,e,f}	6.48±0.68^{a,e}	9.94±0.92^{a,e}	**<0.01**
Pyriproxyfen	<LOQ	<LOQ	<LOQ	3.82 ±0.36	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
Alachlor	<LOQ	<LOQ	<LOQ	0.15 ±0.03 ^{a,c}	0.54 ±0.05 $^{a–c}$	0.14 ± 0.02 ^{a}	0.58 ± 0.25 ^{c}	0.36 ± 0.03$^{a–c}$	0.76 ± 0.06 ^{b}	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**<0.01**
Methidathion	<LOQ	0.22 ± 0.03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
Omethoate	<LOQ	<LOQ	<LOQ	<LOQ	4.82±0.44^{a}	<LOQ	11.55±12.64^{a,b}	13.52±1.12^{a,b}	<LOQ	<LOQ	14.56 ± 1.34 ^{a,b}	2.87 ± 3.10 ^{a}	4.24 ± 0.38 ^{a}	27.54 ± 2.44 ^{b}	**<0.01**
Carbophenothion	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.43 ± 0.48	0.95 ± 0.09	<LOQ	<LOQ	<LOQ	**0.11**
cis-Permethrin	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.48 ±0.03	0.29 ±0.29	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.44 ± 0.04	<LOQ	**0.44**
Acenaphthylene	0.25 ± 0.25 ^{a}	<LOQ	<LOQ	<LOQ	<LOQ	0.20 ± 0.05 ^{a}	0.36 ± 0.36 ^{a,b}	<LOQ	<LOQ	<LOQ	<LOQ	0.82 ± 0.08 ^{b}	0.22 ± 0.02 ^{a,b}	<LOQ	**<0.01**
Anthracene	<LOQ	1.23 ± 0.19 ^{a,c}	<LOQ	0.38 ± 0.02 ^{b,d}	0.36 ± 0.03 ^{b,d}	<LOQ	0.23 ± 0.22 ^{b}	1.55 ± 0.17 ^{c}	0.53 ± 0.07 ^{b}	<LOQ	0.91 ± 0.09 ^{a,b}	0.28 ± 0.28 ^{b}	0.46 ± 0.04 ^{b,d}	0.48 ± 0.07 ^{b,d}	**<0.01**
Benzo[a]ntracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.24 ± 0.23	<LOQ	<LOQ	1.60 ± 1.40	<LOQ	<LOQ	<LOQ	<LOQ	**0.04**
Chrysene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.11 ± 0.09	<LOQ	<LOQ	7.39 ± 8.02	<LOQ	<LOQ	<LOQ	<LOQ	**0.04**
Fluorene	1.33 ± 1.45 ^{a}	<LOQ	<LOQ	0.20 ± 0.01 ^{a}	1.44 ± 0.09 ^{a}	1.81 ± 0.14 ^{a}	0.35 ± 0.37 ^{a}	5.73 ± 0.93 ^{b}	1.56 ± 0.10 ^{a}	<LOQ	0.28 ± 0.04 ^{a}	0.17 ±0.16 ^{a}	0.30 ± 0.03 ^{a}	0.26 ± 0.03 ^{a}	**<0.01**
Phenanthrene	<LOQ	1.16 ± 0.14 ^{a}	<LOQ	0.22 ± 0.02 ^{b}	0.25 ± 0.03 ^{b}	<LOQ	0.30 ± 0.28 ^{b}	2.33 ± 0.39 ^{c}	0.40 ± 0.07 ^{b}	<LOQ	0.43 ±0.04 ^{b}	0.24 ± 0.24 ^{b}	0.29 ± 0.03 ^{b}	0.19 ± 0.02 ^{b}	**<0.01**
PCB 77	<LOQ	0.48 ± 0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
PCB 126	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.11 ± 0.06	0.18 ± 0.02	<LOQ	**0.09**
PCB 138	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.59 ± 0.16	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
PCB 153	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.28 ± 0.05 ^{a}	4.67 ± 0.41 ^{b}	0.23 ± 0.03 ^{a}	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**<0.01**
PCB 180	0.28 ± 0.05 ^{a,b}	0.37 ± 0.03 ^{a}	0.17 ± 0.02 ^{a,b}	<LOQ	0.25 ± 0.03 ^{a,b}	0.14 ± 0.01 ^{a,b}	0.36 ± 0.04 ^{a}	0.27 ± 0.02 ^{a,b}	0.22 ± 0.03 ^{a,b}	0.17 ± 0.04 ^{b}	<LOQ	0.13 ± 0.13 ^{b}	<LOQ	<LOQ	**<0.01**
PCB 189	0.29 ± 0.06 ^{a,b}	0.43 ± 0.04 ^{b}	0.17 ± 0.02 ^{a,c}	<LOQ	0.16 ± 0.01 ^{a,c}	0.12 ± 0.02 ^{c}	0.18 ± 0.02 ^{a,c}	0.16 ± 0.02 ^{a,c}	0.14 ± 0.01 ^{a,c}	0.14 ± 0.11 ^{c}	<LOQ	<LOQ	<LOQ	<LOQ	**<0.01**

a–f Different superscript letters within the same line denote significantly different values for a specific parameter ($p < 0.01$ by post hoc Tukey's HSD test); same superscript letters denote not significantly different values for a specific parameter ($p > 0.01$ by post hoc Tukey's HSD test). Bold p-values indicate significantly different results at $p < 0.01$ between different honey samples.

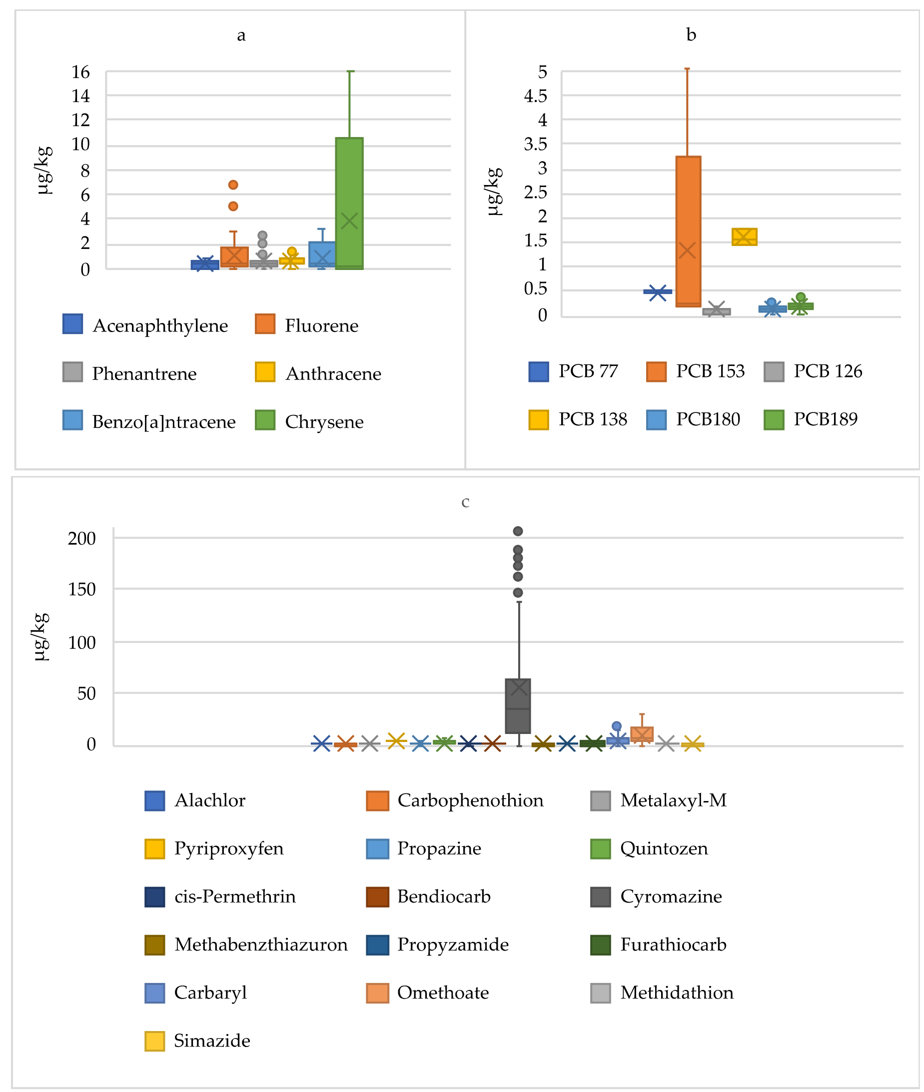

Figure 4. Box-plots of PAH (a), PCB (b), and pesticide (c) concentrations found in Algerian honey.

Pesticides, PCBs, and PHAs residues revealed in the several honeys from Algeria are shown in Table 9 and Figure 4. Pesticide residues were detected in all honey samples. Among the pesticides investigated, 16 pesticides were detected: exactly 4 herbicides, 3 carbamates, 2 fungicides, 2 insect growth regulators, 2 OPPs, 2 pyrethroid insecticides, and 1 OCP. *E. orientalis* honey from Laghouat (EOL) shows the highest number of quantifiable pesticides (n = 9) while the lowest number of pesticides (n = 2) was detected in E. sativa honey from Tiaret (EST). Cyromazine and metalaxyl-M were detected in all samples, followed by carbaryl, detected in 13 samples.

Cyromazine was the pesticide detected in the highest concentration in all samples (in the range from 163.58 µg/kg in *B. mauritanicum* honey from Tiaret to 6.48 µg/kg in *E. orientalis* honey from Tindouf) except for Echinops honey from Tindouf, which has the highest concentration of carbaryl (0.30 vs. 15.81 µg/kg).

Omethoate and propazine showed concentrations above the LOQ in 50% of the samples analysed. The highest concentration of omethoate was detected in P. harmala honey from Tindouf (27.54 µg/kg), while the highest concentration of propazine was found in B. mauritanicum honey from Tiaret (1.93 µg/kg). Alachlor was detected in two samples from Tiaret and four samples from Laghouat at concentrations lower than 1 ppb. Bendiocarb, propyzamide, pyriproxyfen, and methidathion were found in only one sample at different concentrations from 0.12 to 3.82 µg/kg. Most of the pesticides found in Algerian honey are not authorised for use in the European Union, except for metalaxyl-M, propyzamide, and pyriproxyfen, according to Regulation (EU) No. 540/2011.[55]

In accordance with the European Community Regulation No. 396/2005 and subsequent revisions, the concentration of pesticides was below the maximum residual limits (MRLs), except for cyromazine, which greatly exceeds its MRL of 50 µg/kg in 43% of the investigated samples (MT, ZLT, BMT, TEL, EOL, and ML). Among the 13 PAHs analysed, 6 compounds were detected. Anthracene, fluorene, and phenanthrene were found in most of the samples.

The highest concentration of these compounds was found in E. globulus honey from Laghouat (1.55, 5.73 and 2.33 µg/kg of anthracene, fluorene and phenanthrene, respectively). Similar to pesticides, E. orientalis honey from Laghouat exhibited the highest level of contamination among the products,

with n = 9 PAHs detected at a level > LOQ. E. sativa honey from Tiaret is the only sample with the concentration of all PAHs under analysis lower than the corresponding LOQ.

Among the 18 PCBs analysed, 6 compounds were detected. PCB 180 and PCB 189 were found in most of the samples at very low concentrations (0.12–0.43 μg/kg, $p < 0.01$). Similar to PAHs, E. globulus honey from Laghouat exhibited the highest level of contamination among the products, with n = 4 PCBs detected at a level >LOQ. Among these, PCB 153 (4.67 μg/kg) and PCB 138 (1.59 μg/kg) were the most abundant. In two types of honey from Tindouf (ED and PHD), there were no PCB residues. The Regulation (EU) No 915/2023 established the limit for benzo[a]pyrene; the sum of benzo[a]pyrene, benz(a)anthracene, benzo(b)fluoranthene, and chrysene; and the sum of dioxins and dioxin-like PCBs in various foods, excluding honey.[56]

Therefore, it is not possible to make toxicological considerations and evaluate the safety of honey samples in relation to these substances.

In the literature, there is no study concerning the organic contamination of Algerian honey. Moreover, the organic contamination of Algerian foods different from honey is little investigated. This was the first study to assess organic contamination in honey samples from Algeria.

The pesticide profile observed in honey samples from Algeria reflects the diversity of agricultural practices. Cyromazine is an insect growth regulator still used in Algeria and, despite no literature data on cyromazine in food being available, the high concentration found in the honey from this study may imply its large use in agriculture.

The presence of this insecticide in the environment could be unhealthy for bees, resulting in poor honey production. Also, metalaxyl-M, a fungicide found in all samples, is the active ingredient used in several plant protection products on the Algerian market.[57] However, although carbaryl is one of

the 23 substances banned from the Algerian market, residues of this pesticide were found in almost all samples. Referring to the literature data, traces of metalaxyl were found in apples, grapes, nectarines, plums, pears, peaches, and tomatoes from Algeria.

The widespread adoption and extensive use of pesticides in Algeria are considered essential for controlling pests, diseases, and weeds; minimising or preventing yield losses; and upholding a high level of productivity. The extended persistence of pesticides on plants and soil can indeed lead to issues across the entire food chain. In fact, bees may inadvertently carry these contaminants from plant pollen and nectar back to the hive. As a result, these substances have the potential to be assimilated into various hive products.

Concerning PCB and PAH contamination, there are no studies related to Algerian foods. The available studies concern only the contamination of soil and water. Among these, Halfadji et al. indicated that in northwest Algeria, the main sources of PAHs derive from pyrogenic activities and petrogenic contributions, such as coal and wood combustion, fossil fuel and waste incineration, and industrial processes. Additionally, the main origins of PCBs are attributed to commercial PCB mixtures used for industrial applications, including oil-filled insulators and dielectric fluids in transformers and capacitors. In fact, the same study found the presence of PCBs and PAHs in agricultural areas because of their proximity to industrial sites and urban areas.

Recent studies on the monitoring of pesticides, PCBs, and PAHs in honey produced in the Mediterranean area have generally shown differences in the type of contamination with respect to honey from Algeria. No traces of cyromazine were identified in honey from European countries in contrast to honey from Morocco.

On the contrary, the presence of OCPs and their toxic metabolites was detected in honey from industrialised areas and intensive apple orchards from Italy. The presence of different pesticides in honey, therefore, depends mainly on the agricultural practices used in different countries, hence the importance of honey as an indicator of environmental pollution. PCB and PAH residues were found in honey from Italy and Turkey, confirming that these contaminants are ubiquitous.[58]

Table 10. Residues of plasticizers (PAEs and NPPs) detected in several honey samples from Algeria. Data are expressed as means ± standard deviation of three replicates for each sample with the same botanical and geographical origins.

Analyte (mg/kg)	Tiaret					Laghouat					Tindouf				*p*-Value
	M_T	**E_T**	**ES_T**	**ZL_T**	**BM_T**	**TE_L**	**EO_L**	**EG_L**	**M_L**	**Z_L**	**E_D**	**ES_D**	**EO_D**	**PH_D**	
DEP	0.014 ± 0.014	<LOQ	0.038 ± 0.012	0.023 ± 0.006	0.034 ± 0.013	0.026 ± 0.009	0.013 ± 0.013	0.021 ± 0.002	<LOQ	1.656 ± 1.808	<LOQ	<LOQ	<LOQ	<LOQ	**0.02**
DPrp	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.016 ± 0.016	<LOQ	<LOQ	-
DBP	0.048 ± 0.006 ^{a}	0.073 ± 0.005 ^{b,c}	0.097 ± 0.007 ^{b}	0.038 ± 0.007 ^{a,c}	0.042 ± 0.006 ^{a,c}	0.037 ± 0.006 ^{a,c}	0.037 ± 0.005 ^{a}	0.041 ± 0.006 ^{a,c}	<LOQ	0.041 ± 0.006 ^{a}	0.048 ± 0.005 ^{a,c}	0.055 ± 0.014 ^{b,c}	0.037 ± 0.003 ^{a,c}	0.044 ± 0.004 ^{a,c}	**<0.01**
DiBP	0.036 ± 0.005 ^{a}	0.070 ± 0.009 ^{a,c}	0.042 ± 0.006 ^{a,d}	0.040 ± 0.006 ^{a,d}	0.050 ± 0.007 ^{a,d}	0.039 ± 0.006 ^{a,d}	0.052 ± 0.010 ^{a,d}	0.063 ± 0.006 ^{c,d}	0.040 ± 0.008 ^{a,d}	0.058 ± 0.023 ^{a,d}	0.266 ± 0.032 ^{b}	0.175 ± 0.141 $^{b–d}$	0.194 ± 0.021 $^{b–d}$	0.232 ± 0.027 ^{b,c}	**<0.01**
BBP	<LOQ	0.041 ± 0.006	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.115 ±0.019	0.020 ± 0.020	<LOQ	<LOQ	<LOQ	<LOQ	**<0.01**
DPhP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.070 ± 0.012	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
DEHP	0.050 ± 0.009 ^{a}	0.118 ± 0.012 ^{b}	0.045 ± 0.007 ^{a}	0.051 ± 0.004 ^{a}	0.053 ± 0.007 ^{a}	0.058 ± 0.004 ^{a}	0.048 ± 0.008 ^{a}	0.073 ± 0.007 ^{a}	0.049 ± 0.009 ^{a}	0.065 ± 0.024 ^{a}	0.070 ± 0.005 ^{a}	0.058 ± 0.013 ^{a}	0.068 ± 0.004 ^{a}	0.073 ± 0.008 ^{a}	**<0.01**
DEA	0.100 ± 0.108 ^{a,b}	0.175 ± 0.025 ^{b}	<LOQ	0.027 ± 0.005 ^{a,b}	0.047 ± 0.009 ^{a,b}	<LOQ	0.068 ± 0.037 ^{a,b}	0.033 ± 0.006 ^{a,b}	0.046 ± 0.008 ^{a,b}	0.013 ± 0.013 ^{a}	<LOQ	0.020 ± 0.021 ^{a}	<LOQ	<LOQ	**<0.01**
DEHT	0.042 ± 0.012 ^{a}	0.128 ± 0.019 ^{a,b}	0.038 ± 0.009 ^{a,b}	0.048 ± 0.009 ^{a,b}	0.139 ± 0.015 ^{b}	0.103 ± 0.021 ^{a,b}	0.053 ± 0.018 ^{a,b}	0.144 ± 0.017 ^{a,b}	0.055 ± 0.012 ^{a,b}	0.102 ± 0.077 ^{a,b}	0.108 ± 0.008 ^{a,b}	0.076 ± 0.033 ^{a,b}	0.094 ± 0.013 ^{a,b}	0.089 ± 0.014 ^{a,b}	**<0.01**

a–d Different superscript letters within the same line denote significantly different values for a specific parameter ($p < 0.01$ by post hoc Tukey's HSD test); same superscript letters denote not significantly different values for a specific parameter ($p > 0.01$ by post hoc Tukey's HSD test). Bold p-values indicate significantly different results at $p < 0.01$ between different honeys.

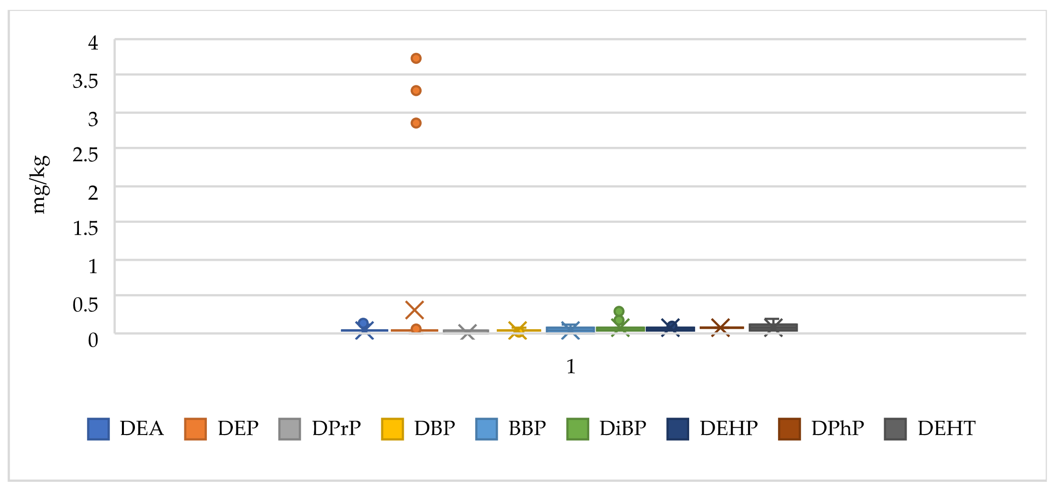

Figure 5. Box-plot illustrating the concentrations of plasticizer residues in Algerian honey.

Seven PAEs (i.e., DEP, DPrp, DBP, DiBP, BBP, DPhP, and DEHP) and two NPPs (i.e., DEA and DEHT) were detected in honey samples as shown in Table 10 and Figure 5.

DiBP, DBP, DEHP, and DEHT were determined at a concentration >LOQ in all the samples. The honey samples from Tindouf were characterised by a higher concentration of DiBP (in the range of 0.175–0.266 mg/kg) than the honey from the other two regions (in the range of 0.039–0.070 mg/kg), with statistically significative differences ($p < 0.01$). DEP was the plasticizer found at the highest concentrations in the Z. lotus honey from Laghouat (1.656 mg/kg) but the concentrations of this plasticizer were not statistically different ($p = 0.02$). The Z. lotus honey from Laghouat was also the sample with the highest number of quantifiable plasticizers ($n = 7$).

To the best of the authors' knowledge, there is no literature concerning plasticizers in Algerian honey. Since plasticizers can leach from plastic equipment used in honey production processes (such as honey extractors and uncorkers), there is a possibility of honey contamination during the production steps. However, contamination during honey storage can be excluded because the honey was stored in glass jars.

Nevertheless, it can be considered that plasticizers are ubiquitous in the environment, so the contamination of the nectar cannot be excluded. In this regard, DEHP is the most frequently identified plasticizer in honey samples.[38,39]

Regarding bisphenols, the concentration of BPA and all its analogues was below the LOQ in all the samples analysed. The only study in the literature on bisphenols in honey from Algeria and Tunisia showed the presence of BPA, BPAP, BPF, BPS, and BPZ residues in Algerian honey at very low concentrations.[10]

6.2.6. Principal Components Analysis

In this study, a PCA was exploited to differentiate honey samples by geographical origin based on contamination data. The variables considered in the PCA included only carbaryl, metalaxyl-M, cyromazine, fluorene, PCB 180, PCB 189, DBP, DiBP, DEHP, and DEHT, as these contaminants were found in at least 60% of the samples. All other contaminants were excluded from the PCA, since the inclusion of those contaminants present in <60% of samples would not have allowed the PCA.

Excluding contaminants present in only a few samples, the KMO (Kaiser–Meyer–Olkin) value (0.581) indicates that the data structure is suitable for PCA, and the Bartlett's test of sphericity indicates that the correlations between the variables are not all equal to zero.

Based on the Kaiser Criterion, three principal components (PCs) with eigenvalues greater than 1.0 were extracted. The three components showed a variance of 36.482%, 23.539%, and 13.112%, respectively, for a total of 73.132%.

Figure 6 displays the bidimensional score and loading plots of the first two principal components (PC1 and PC2), which in total explain 60.02% of the variability within the system. To achieve greater system variability, Figure 5 also shows the plots obtained by considering PC1 and PC3.

By overlaying the loading and score plots of Figure 6, variables such as metalaxyl-M, cyromazine, fluorene, PCB 180, and PCB 189 appeared to have a greater influence on the contamination of honey from Tiaret and Laghouat, which indeed exhibited the highest concentrations of these organic contaminants. Moreover, it is clear that honey from Tindouf differed from the others mainly because of the high level of DiBP.

Regarding Figure 7, the loading and score plots show that carbaryl and plasticizers weighed more on Tindouf honey samples, as also demonstrated through the higher carbaryl concentrations in these samples.

Consequently, as we can see from the score plots in Figures 6 and 7, the honey samples from Tindouf tend to cluster separately from the honey from the other two areas, namely Laghouat and Tiaret. However, a separation/differentiation between honeys from these two areas was not possible.

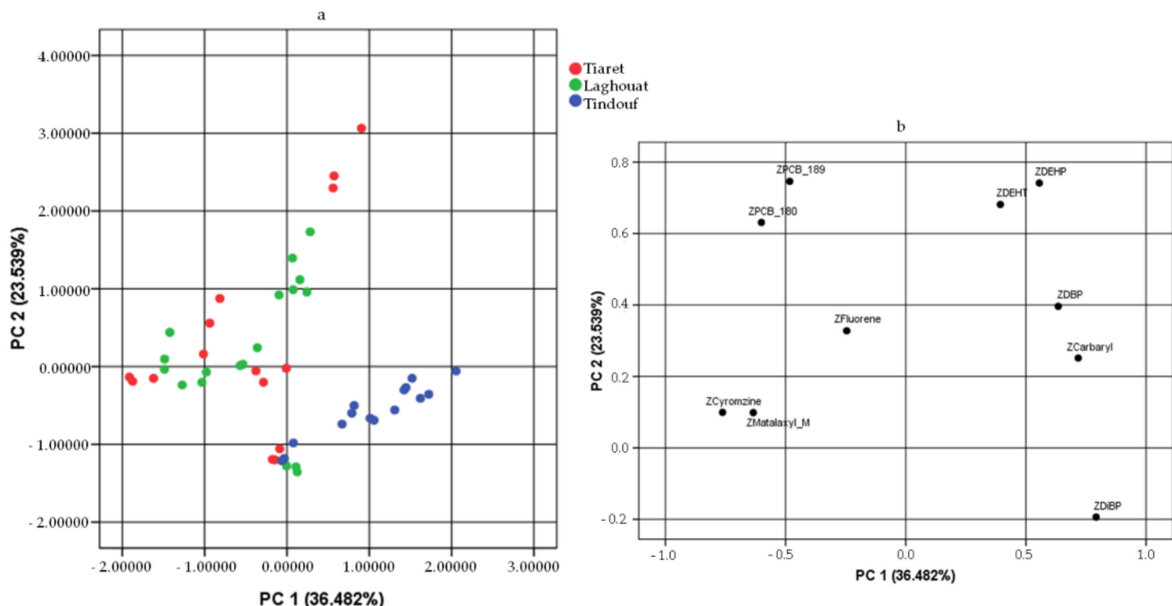

Figure 6. Score plot (a) and loading plot (b) of PC1 and PC2, explaining honey samples differentiated by geographical origin. For the analysis, only the data on 10 contaminants detected in at least 60% of the samples were considered.

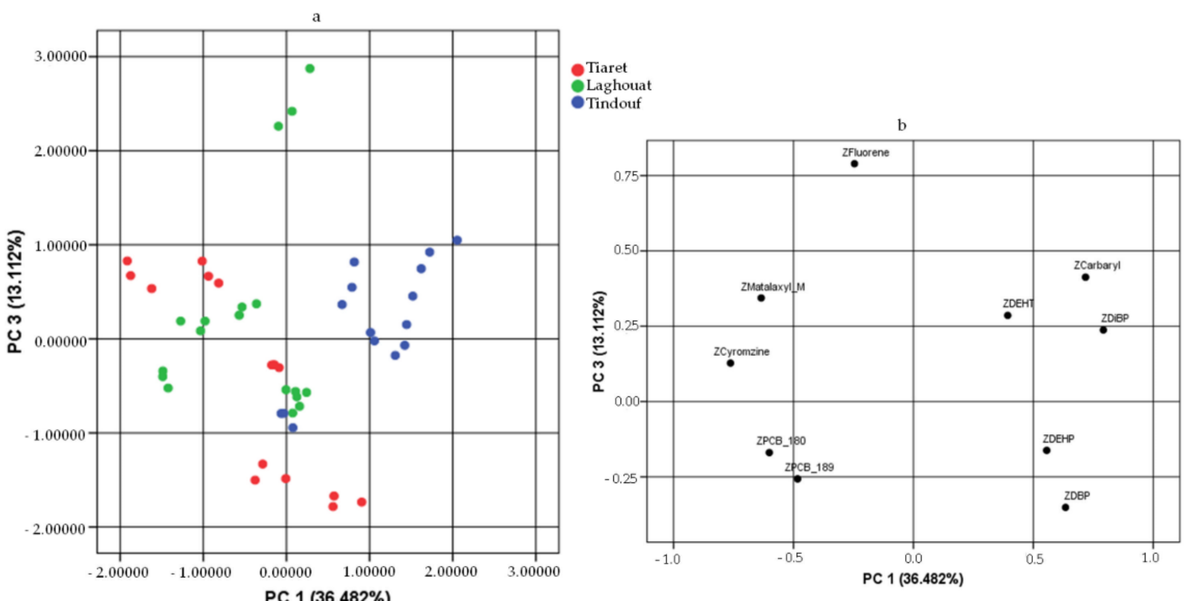

Figure 7. Score plot (a) and loading plot (b) of PC1 and PC3, explaining honey samples differentiated by geographical origin. For the analysis, only the data on 10 contaminants detected in at least 60% of the samples were considered.

6.2.7. Dietary Exposure to Contaminants

In order to assess the quality of Algerian honey and the potential health risks to consumers, the estimated daily intake (EDI) and non-carcinogenic risk (HQ) of pesticides and plasticizers were calculated, as shown in Table 11. Based on the obtained results, EDIs were calculated by considering the amount of honey consumed daily by a typical-sized adult (70 kg) from Algeria (0.33 g/day) and Europe (1.59 g/day), according to FAO. For the health risk assessment, the HQ for each contaminant detected was less than 1, indicating that the honey is safe for the consumers when ingested at the Algerian and European dietary levels. In fact, the calculated EDIs were well below the ADI for pesticides and the TDI for plasticizers, set by international regulatory bodies. This indicates that no adverse health effects result from the consumption of Algerian honey.

Table 11. Maximum and minimum values of EDIs (µg/kgbw/day or mg/kgbw/day) and HQs calculated for Algerian honey consumed daily by typical-sized (70 kg) adult consumers both from Algeria and Europe.

	Algeria				**Europe**			
	EDI_{min}	**HQ**	**EDI_{max}**	**HQ**	**EDI_{min}**	**HQ**	**EDI_{max}**	**HQ**
				Pesticides				
Bendiocarb *	9.43×10^{-7}	<1	9.43×10^{-7}	<1	4.54×10^{-6}	<1	4.54×10^{-6}	<1
Carbaryl *	2.92×10^{-6}	<1	7.45×10^{-5}	<1	1.41×10^{-5}	<1	3.59×10^{-4}	<1
Furathiocarb *	1.01×10^{-5}	<1	1.10×10^{-5}	<1	4.88×10^{-5}	<1	5.32×10^{-5}	<1
Metalaxyl-M *	1.27×10^{-6}	<1	5.94×10^{-6}	<1	6.13×10^{-6}	<1	2.86×10^{-5}	<1
Quintozen *	1.65×10^{-6}	<1	2.29×10^{-5}	<1	7.95×10^{-6}	<1	1.10×10^{-4}	<1
Methabenzthiazuron *	1.60×10^{-6}	<1	3.87×10^{-6}	<1	6.13×10^{-6}	<1	1.86×10^{-5}	<1
Propazine *	1.32×10^{-6}	<1	9.10×10^{-6}	<1	6.36×10^{-6}	<1	4.38×10^{-5}	<1
Propyzamide *	5.66×10^{-7}	<1	5.66×10^{-7}	<1	2.73×10^{-6}	<1	2.73×10^{-6}	<1
Simazide *	1.37×10^{-6}	<1	3.25×10^{-6}	<1	6.59×10^{-6}	<1	1.57×10^{-5}	<1
Cyromazine *	1.41×10^{-6}	<1	7.71×10^{-4}	<1	6.81×10^{-6}	<1	3.72×10^{-3}	<1
Pyriproxyfen *	1.80×10^{-5}	<1	1.80×10^{-5}	<1	8.68×10^{-5}	<1	8.68×10^{-5}	<1
Alachlor *	6.60×10^{-7}	<1	3.58×10^{-6}	<1	3.18×10^{-6}	<1	1.73×10^{-5}	<1
Methidathion *	1.04×10^{-6}	<1	1.04×10^{-6}	<1	5.00×10^{-6}	<1	5.00×10^{-6}	<1
Omethoate *	1.34×10^{-5}	<1	1.30×10^{-4}	<1	6.45×10^{-5}	<1	6.26×10^{-4}	<1
Carbophenothion *	2.03×10^{-6}	<1	4.48×10^{-6}	<1	9.77×10^{-6}	<1	2.16×10^{-5}	<1
cis-Permethrin *	1.27×10^{-6}	<1	2.26×10^{-6}	<1	6.13×10^{-6}	<1	1.09×10^{-5}	<1
				Plasticizers				
DEA **	5.66×10^{-8}	-	8.25×10^{-7}	-	2.73×10^{-7}	-	3.98×10^{-6}	-
DEP **	5.66×10^{-8}	<1	7.81×10^{-3}	<1	2.73×10^{-7}	<1	3.76×10^{-2}	<1
DPrp **	7.07×10^{-8}	-	7.07×10^{-8}	-	3.41×10^{-7}	-	3.41×10^{-7}	-
DiBP **	1.70×10^{-7}	-	1.25×10^{-6}	-	8.18×10^{-7}	-	6.04×10^{-6}	-
DBP **	1.74×10^{-7}	<1	4.57×10^{-7}	<1	8.40×10^{-7}	<1	2.20×10^{-6}	<1
BBP **	8.96×10^{-8}	-	5.42×10^{-7}	-	4.32×10^{-7}	-	2.61×10^{-6}	-
DEHP **	2.12×10^{-7}	<1	5.56×10^{-7}	<1	1.02×10^{-6}	<1	2.68×10^{-6}	<1
DPhP **	3.30×10^{-7}	-	3.30×10^{-7}	-	1.59×10^{-6}	-	1.59×10^{-6}	-
DEHT **	1.79×10^{-7}	-	6.79×10^{-7}	-	8.63×10^{-7}	-	3.27×10^{-6}	-

* µg/kg_{bw}/day. ** mg/ kg_{bw}/day.

6.2.8. Conclusions

The presence of pesticides, PCBs, PAHs, and plasticizers in food is a global problem. The impact of these substances on bees and their products is significant. Firstly, pesticide residues have a detrimental effect on bees, causing a decline in their population and reducing their ecological services. Secondly, through the contamination of the food chain, negative effects such as endocrine, carcinogenic, reproductive, and neurological effects can affect human life. Increased effort in monitoring and greater public intervention are, therefore, needed to reduce the use of pesticides, PCBs, PAHs, and plasticizers and, hence, minimise the exposure of the whole planet to these substances, including human beings. In this regard, the characterization of Algerian honey, which includes not only physicochemical parameters but also organic contaminants, was performed for the first time, adding to the limited existing literature on Algerian honey. In terms of physicochemical parameters, the honey analysed complied with the parameters set by the European Union EU to guarantee the authenticity of these bee products, with the exception of one sample (Euphorbia orientalis honey from Tindouf) with slightly high acidity levels. In addition, the level of contamination did not appear to be critical because the concentration of contaminants was very low and under the EU regulatory limits available for honey. The only exception was found in cyromazine, whose concentration exceeded the EU limit in most samples from Tiaret and Laghouat. In terms of the number of toxicants detected, the Euphorbia orientalis honey from Laghouat was the most contaminated samples while the Eruca sativa honey from Tiaret was the least contaminated. According to the dietary exposure assessment, a small amount of Algerian honey can be safely consumed daily in both European and Algerian diets.

In conclusion, it is hoped that the Algerian authorities will monitor beekeeping activities, find appropriate measures to reduce organic pollution, and harmonise and apply the international regulatory framework concerning the chemical safety of honey, in order to obtain honey of ever higher quality.

6.3. A GREEN SAMPLE PREPARATION METHOD FOR THE DETERMINATION OF BISPHENOLS IN HONEYS

The aim of this research was the determination of BPs in honey using a method based on Micro-QuEChERS technique and ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) derived by previous method (section 6.1) based on standard QuEChERS and HPLC-MS/MS. The two methods were compared using the Analytical Eco-Scale as a tool to verify that the method proposed in this study was more sustainable than the previous method. The method proposed, amongst other things, can analyse simultaneously a wide range of bisphenols. In detail, an accurate, sensitive, rapid, inexpensive, and environmentally friendly method for the extraction, separation, and detection of 9 bisphenols was applied to 15 samples of Algerian honey and 10 samples of Tunisian honey of different floral origin.

6.3.1. Sample collection

A total of 25 honey samples were collected in 2020 from different beekeepers in North Africa. Fifteen honey samples came from eight districts of Algeria, while ten honey samples from five district of Tunisia (Table 12). Algerian Honey samples was labelled as “A” (A1−A15), and Tunisian Honey samples was labelled as “T” (T1−A10). The samples of Algerian honeys were all multifloral, whereas Tunisian honey samples had different floral origin: multifloral, eucalyptus, prickly pear, red-flowered eucalyptus, lemon, thyme, almond, rosemary, and jujube as reported in Table 1. All samples were stored in a dark, cool, and dry place and in glass containers until analysis.

Table 12. Floral and geographic origin of Algerian and Tunisian honey samples.

Samples	Floral origin	Geographic origin
Algerian honey		
1A	Multifloral	Algiers
2A	Multifloral	Algiers
3A	Multifloral	Laghouat
4A	Multifloral	Ghardaïa
5A	Multifloral	Algiers
6A	Multifloral	Algiers
7A	Multifloral	El Bavadh
8A	Multifloral	M'sila
9A	Multifloral	Bouïra
10A	Multifloral	Tlemcen
11A	Multifloral	Tlemcen
12A	Multifloral	Tlemcen
13A	Multifloral	Tlemcen
14A	Multifloral	Naâma
15A	Multifloral	Tlemcen
Tunisian honeys		
1T	Multifloral	Sidi Bouzid
2T	Eucalyptus	Sidi Bouzid
3T	Prickly pear	Sidi Bouzid
4T	Red-flowered eucalyptus	Sidi Bouzid
5T	Lemon	Nabeul
6T	Thyme	Béja
7T	Multifloral	Béja
8T	Almond	Sfax
9T	Rosemary	Kairouan
10T	Jujube	Béja

6.3.2. Preparation of samples

Bisphenol analogues in honey samples were extracted according to the Micro-QuEChERS procedure developed from a previous study.[5] In order to optimize a more eco- friendly method, a sealed pack of Q-sep QuEChERS was used for ten extractions, so that the 750 mg amount of Q-sep QuEChERS extraction kit was used for a single sample; the amount of solvent was also reduced proportionally. In addition, in the clean-up step, the amount of sorbent was reduced according to the amount of sample used. Thus, the details of the method were as follows: an aliquot of 1.5 g of honey was mixed in a glass tube with 3 mL of ultrapure water, 100 µL of $^{13}C_{12}$BPA at 0.8 mg/L, shaken vigorously and left in the dark for 24 h before the extraction procedure, which was carried out with the specified Q-sep QuEChERS described above and 3 mL of ACN.

The mixture was stirred and centrifuged at 4000 rpm for 5 min. The top layer was transferred to the Clean-up Kit (25 mg octadecyl sorbent (C18)), stirred for 1 min and centrifuged at 4000 rpm for 5 min. Then 1 mL of ACN extract was filtered through a PVDF syringe filter (0.22 µm) and injected

into the UHPLC-MS/MS system. To avoid bisphenol contamination, glass equipment was used, and all samples and reagents were stored in the dark in vials and glass bottles.

6.3.3. Determination of BPs

A UHPLC Shimadzu (Shimadzu, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer MS-8040 (Shimadzu, Tokyo, Japan), with an electrospray ionization source negative (ESI−), was employed for the analysis of nine bisphenol analogues in honey samples. The UHPLC-ESI (-)-MS/MS system was equipped with a binary pump (LC20ADXR), autosampler (SIL-20AXR), temperature-controlled column operator and DGU-20A5R degasser. Chromatographic separation was performed on a reversed-phase analytical column Phenomenex C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size) (Phenomenex, CA, USA). The mobile phases were ultrapure water (A) and acetonitrile (B); linear gradient elution was used: 0 min, 20% B; 2 min, 40% B; 6 min, 90% B; 8 min, 20% B, totalling 8 min of the chromatographic run. The flow rate was 0.4 mL/min, and the injection volume was 2 μL.

The MS was operated in multiple reaction monitoring (MRM) mode. The MS conditions were DL temperature 250 ºC, CID gas 230 KPa, nitrogen gas nebulizer at 3 L/min, nebulizer gas pressure 770 KPa; argon gas (99.999%) was used as collision gas. The optimization of the MS/MS conditions was carried out initially by direct injection of individual standard at 1 mg/L. Then the ESI negative mode was used and [M-H]− precursor ions were selected. Finally, the two most intense transitions were selected for the identification and quantification of all target bisphenols after checking the fragmentation of each precursor ions and collision energy, and the collision energy were optimised for each transition in the range 10 – 40 eV. For each compound, one precursor and two daughter ions (one quantifier and one qualifier) were monitored, giving a total of four identification points. The quantifier ion was assigned as the most abundant m/z ion of the two daughters. The two transitions and related CE values as well as the ratio based on the intensity of quantifier to qualifier ions are showed in Table 13.

LabSolutions software was used for data acquisition and quantification. Acetonitrile and water solvents were tested for BPs. No bisphenol residues were found in solvents. The inclusion of acidity modifiers in the mobile phase was not necessary as it was found that the peak shape of the analytes was better when only acetonitrile was used as the organic phase.

Table 13. Chemical structure, retention time, MS/MS condition for nine targeted bisphenols.

Compounds	Structure	Rt (min)	Quantification transition (T1)	CE1 (eV)	Confirmation transition (T2)	CE2 (eV)	Intesity ion ratio (%)
BPS		2.57	249.2 → 107.9	15	249.27 → 156.0	12	2.4
BPF		3.50	199.2 → 93.1	13	199.23 → 105.1	14	1.8
BPE		3.82	213.3 → 198.0	38	213.26 →194.9	40	3.7
BPA		4.05	227.3 → 212.1	17	227.29 → 133.0	18	4.1
BPB		4.58	241.3 → 212.0	20	241.31 → 211.0	21	2.5
BPAF		4.73	335.3 → 265.0	35	335.30 → 177.0	33	3.7
BPAP		4.78	289.4 → 274.1	10	289.36 → 273.1	10	3.2
BPZ		4.92	267.3 → 145.0	17	267.30 → 173.1	18	4.1
BPP		5.01	345.5 → 330.1	33	345.46 → 133.1	34	3.8

6.3.4. Method validation

Validation procedures were performed for the developed UHPLCMS/MS method to ensure the quality of the analytical results. The developed method was validated for linearity, limits of detection (LODs) and limits of quantification (LOQs), recovery, precision, and matrix effect (Table 14). The linearity was assayed by analysing five mixtures of standard solutions with increasing concentrations (1 μg/L, 5 μg/L, 10 μg/L, 30 μg/L, 50 μg/L) prepared in neat solvent and matrix extract. Three replicate analyses were performed at each concentration and each experimental value corresponds to the average of these measurements. The standard response curves, obtained for each analyte plotting target ion peak areas versus concentrations. LODs and LOQs were calculated for each compound based on the standard deviation (SD) of the lowest detectable concentration. They correspond to 3 and 10 times the standard deviation of the blank, respectively, as: LOD = mean + (3 × SD); LOQ = mean + (10 × SD). For calibration curves derived from standard solution in neat solvent good linearity was observed with correlation coefficient (R2) from 0.9907 to 0.9995. The LOD and the LOQ for all analytes investigated were found in the range from 0.30 to 0.45 μg/L and from 1.0 to 1.5 μg/L, respectively. For calibration curves derived from matrix extract R^2 were between 0.9890 and 0.9981. The LOD and LOQ varied between 0.27 and 0.47, and between 0.8 and 1.6, respectively. R^2, LOD and LOQ value determined in two cases (neat solvent and matrix extract) were statistically evaluated by Mann-Whitney U test: no significant differences were detected, so the value in neat solvent were used for quantification analysis. From these data, comparing the LOQ value with specific migration limits, it seems clear that the method was sufficiently sensitive to detect in honey samples the levels of bisphenols under analysis. In order to evaluate the matrix effect, a comparison between standard curves derived from solutions in neat solvent and in matrix extract was performed. As reported in Table 14 the value of *t-calculated* for the slope comparison test were lower than critical value of 2.447 for a confidence level of 95% and a degree of freedom corresponding to the two compared slope.

Therefore, it is acceptable to assume that the matrix effect is negligible. Recovery and precision tests were carried out by fortifying the honey sample (1A, in which all target bisphenols were below their respective LOQs). Three different concentration levels (5, 10, 50 μg/L) for all bisphenols under analysis were prepared. The same extraction procedures were applied to spiked sample after 24 h and UHPLC-MS/MS conditions described above were used for the recovery studies. Recovery ranged from 85.9 (for BPA, level I) to 104.4 (for BPAP level III). Precision expressed in% RSD was always better than 7.8%. Thus, the method provided satisfactory recovery and precision for all BPs.

Table 14. Linearity (R2), limits of detection (LOD) and limits of quantification (LOQ), recovery, precision and matrix effect for analyte prepared in matrix extract (M) and in neat solvent(S).

Compounds	R^2_S / R^2_M	LOD_S/LOD_M (µg/kg)	LOQ_S/LOQ_M (µg/kg)	Recovery (%)	Precision (RSD %)	Matrix effect t_{cal}
BPS	0.9993/0.9905	0.30/0.27	1.0/0.8	95.9[a]	2.5[a]	0.673
				96.6[b]	2.8[b]	
				98.2[c]	1.9[c]	
BPF	0.9911/0.9984	0.45/0.35	1.5/1.2	89.1[a]	6.7[a]	0.842
				92.3[b]	7.8[b]	
				93.0[c]	5.6[c]	
BPE	0.9931/0.9890	0.30/0.35	1.0/1.2	90.6[a]	6.2[a]	0.064
				93.1[b]	5.9[b]	
				96.4[c]	6.5[c]	
BPA	0.9907/0.9956	0.45/0.41	1.5/1.3	85.9[a]	4.2[a]	1.013
				90.0[b]	3.5[b]	
				93.4[c]	5.6[c]	
BPB	0.9944/0.9912	0.30/0.28	1.0/0.9	90.8[a]	5.6[a]	0.777
				94.0[b]	4.2[b]	
				96.7[c]	5.5[c]	
BPAF	0.9995/0.9899	0.30/0.27	1.0/0.8	99.2[a]	3.8[a]	0.090
				101.5[b]	4.3[b]	
				103.1[c]	3.1[c]	
BPAP	0.9954/0.9927	0.45/0.47	1.5/1.6	98.8[a]	3.4[a]	0.739
				101.6[b]	4.9[b]	
				104.4[c]	3.0[c]	
BPZ	0.9984/0.9981	0.45/0.43	1.5/1.4	96.7[a]	3.7[a]	1.109
				97.1[b]	4.3[b]	
				98.5[c]	3.0[c]	
BPP	0.9992/0.9976	0.45/0.39	1.5/1.3	94.6[a]	6.4[a]	0.587
				95.2[b]	5.1[b]	
				97.0[c]	7.0[c]	

[a]level I 5 µg/kg, [b]level II 10 µg/kg, [c]level III 50 µg/kg

6.3.5. Assessment the greenness of analytical procedures

The tool used to assess the sustainability of analytical methods is the Analytical Eco-Scale developed in 2012. The concept of the Analytical Eco-Scale is that the ideal green analysis has a value of 100. Penalty points are assigned for each of the parameters of the analytical procedure that deviate from the ideal green analysis (reagent use, risk, energy and waste). The sum of the penalty points is then subtracted from 100 to obtain the numerical value of the Analytical Eco-Scale.

Table 15 shows the calculations of the Analytical Eco-Scale score for the analytical method proposed in this paper and for the method described in the section 6.1.

Penalty points were assigned using the following metric: one penalty point for each hazard pictogram corresponding to a chemical; if the substance was described with the term "Hazard", the number of pictograms was multiplied by 2, while the score did not change if the chemical was described with the term "Warning". The hazard score was multiplied by 1 if the amount of reagent or solvent was less than 10 mL or g, by 2 if the amount was between 10 and 100 mL or g. The emission of vapours to air during the analytical process resulted in 3 penalty points for both methods, while the generation of waste was given 3 penalty points for amounts between 1 and 10 mL or g and a score of 5 for larger amounts of waste. In addition, the non-treatment of waste resulted in 3 additional points. The method from this study achieved a score of 79 (100–21 PPs) on the Analytical Eco-Scale, while the method presented in the section 6.1 achieved a score of 71 (100–29 PPs). A score above 75 is considered ’excellent green analysis’; a score between 50 and 75 is considered ’acceptable green analysis’; a score below 50 is considered ’inadequate green analysis’. The result obtained by using this metric allows us to state that the method proposed in this study can be considered as an excellent eco-friendly alternative from the point of view of sample preparation, instrumental analysis and waste generation, thus fulfilling certain green chemistry criteria in analytical methods.

Table 15. Methods analytical Eco-Scale score.

Method	Analytical Eco-Scale score calculations	
UHPLC-MS/MS	Reagents and solvents	Penalty points (PPs)
	Acetonitrile (3 mL)	4
	Ultrapure water (3 mL)	0
	Magnesium sulphate anhydrous and sodium chloride (750 mg)	0
	Octadecyl sorbent (C18) (25mg)	0
	Isotopically labelled standards $^{13}C_{12}$-BPA (100 µL)	8
	Instruments	
	UHPLC-MS/MS	0
	Occupational hazard	
	Vapours are released	3
	Waste	
	3.2 mL	3
	No treatment	3
	Total PPs	
	Score	21
HPLC-MS/MS	Reagents and solvents	
	Acetonitrile (10 mL)	8
	Ultrapure water (10 mL)	0
	Magnesium sulphate anhydrous and sodium chloride (5 g)	0
	Octadecyl sorbent (C18) (125mg)	0
	Isotopically labelled standards $^{13}C_{12}$-BPA (100 µL)	8
	Instruments	
	HPLC-MS/MS	2
	Occupational hazard	
	Vapours are released	3
	Waste	
	24.0 mL	5
	No treatment	3
	Total PPs	
	Score	29

6.3.6. BPs occurrence in honey samples

Bisphenol concentrations in honey samples are given in Table 16 as µg/L based on the honey density in the analysed samples. Amongst the nine researched bisphenols, four (BPAF, BPE, BPP, BPB) were below their LOQ in all samples. In Algerian honeys were found bisphenols in 53% of samples: BPS, BPF, BPA, BPAP, and BPZ were detected. BPS was detected in three honey samples (20%) with concentrations in the ranges from 2.27 ± 0.06 µg/L to 8.56 ± 0.08 µg/L, while BPA was detected in four honey samples (27%) with concentrations in the ranges from 4.64 ± 0.07 µg/L to 10.17 ± 0.06 µg/L. The most abundant bisphenol was BPF and was found only in honey sample 14A, with a concentration of 34.20 ± 0.13 µg/L. BPAP and BPZ were detected only in the honey sample 7A with a concentration of 7.56 ± 0.06 µg/L and 4.54 ± 0.03 µg/L, respectively. In Tunisian honeys were found bisphenols in 30% of samples: BPA, BPAP and BPZ were detected. BPA were found in multiflora honey samples 1T and 7T with a concentration of 16.50 ± 0.18 µg/L and 9.00 ± 0.27 µg/L, respectively. BPAP and BPZ were found in samples 2T and 7T with a concentration of 4.95 ± 0.04 µg/L and 4.15 ± 0.06 µg/L, respectively. Similar data was obtained from Inoue et al.,[59] who detected BPA in 16% honey sam ples from European and non-European countries. The recoveries of BPA from honey, in the study cited, were of 93.9–116.4%, similar to those obtained in this study. Residues of BPA, BPAF, BPE, BPF, BPS and BPZ was found in honey sample from European and non-European countries by Česen et al.,[60] but the concentrations were higher than those reported in our research. Furthermore, compared to the study cited, in the present study, no residues of BPE were found in the analysed samples. Higher concentrations of BPA were found in 43% of honey samples analysed by Notardonato et al.,[39] which used a method with lower recovery values than those reported in this study. Rosa Peñalver et al.[61] had detected residues of BPA in multifloral honey stored in polyethylene terephthalate package with concentrations much higher and much longer analysis times than those found in this study. Comparing this study with other studies using standard procedures, our LODs and LOQs are fully acceptable.[5,39] In our case, the reduced consumption of reagents and solvents is advantageous, since it has effectively led to a reduction in waste production.

The minimisation of sample, chemical and material amounts along with analytical instrumentation such as UHPLC are amongst the 10 principles of Green Sample Preparations (GPS).

Table 16. Content of bisphenol analogues (μg/L) (mean ± standard deviation) in Algerian and Tunisian honey samples

Samples	BPS	BPF	BPA	BPAP	BPZ
Algerian honey					
1A	n.d.	n.d.	n.d.	n.d.	n.d.
2A	n.d.	n.d.	n.d.	n.d.	n.d.
3A	n.d.	n.d.	6.57 ± 0.13	n.d.	n.d.
4A	2.96 ± 0.03	n.d.	7.62 ± 0.08	n.d.	n.d.
5A	n.d.	n.d.	n.d.	n.d.	n.d.
6A	2.27 ± 0.06	n.d.	n.d.	n.d.	n.d.
7A	n.d.	n.d.	n.d.	7.56 ± 0.06	4.54 ± 0.03
8A	n.d.	n.d.	n.d.	n.d.	n.d.
9A	8.56 ± 0.08	n.d.	n.d.	n.d.	n.d.
10A	n.d.	n.d.	4.64 ± 0.07	n.d.	n.d.
11A	n.d.	n.d.	n.d.	n.d.	n.d.
12A	n.d.	n.d.	10.17 ± 0.06	n.d.	n.d.
13A	n.d.	n.d.	n.d.	n.d.	n.d.
14A	n.d.	34.20 ± 0.13	n.d.	n.d.	n.d.
15A	n.d.	n.d.	n.d.	n.d.	n.d.
Tunisian honeys					
1T	n.d.	n.d.	16.50 ± 0.18	n.d.	n.d.
2T	n.d.	n.d.	n.d.	4.95 ± 0.04	n.d.
3T	n.d.	n.d.	n.d.	n.d.	n.d.
4T	n.d.	n.d.	n.d.	n.d.	n.d.
5T	n.d.	n.d.	n.d.	n.d.	n.d.
6T	n.d.	n.d.	n.d.	n.d.	n.d.
7T	n.d.	n.d.	9.00 ± 0.27	n.d.	4.15 ± 0.06
8T	n.d.	n.d.	n.d.	n.d.	n.d.
9T	n.d.	n.d.	n.d.	n.d.	n.d.
10T	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected (≤ LOQ)

6.3.7. Conclusions

This study evaluated the use of a complete analytical procedure based on the Micro-QuEChERS technique and ultra-high performance liquid chromatography coupled to tandem mass spectrometry for the determination of BPA, BPAP, BPAF, BPB, BPE, BPZ, BPP, BPF and BPS in honey samples. Bisphenol residues were extracted from the matrix using a simple and rapid method that uses little extraction solvent, reagent and sample. A volume of only 3 mL of ACN and 750 mg of Q-sep QuEChERS proved suitable for the complete and simultaneous extraction of all BPS. In addition, the use of small volumes of organic solvents in UHPLC-MS/MS meets many green chemistry

requirements. Furthermore, a metric tool such as the Analytical Eco-Scale was successfully applied to assess the ecology of the method. The proposed method achieves a score of more than 75%, making it an excellent alternative to non-metric methods. The results also showed that contamination by residues of bisphenol analogues was minimal and therefore the analysed honeys do not represent a health risk to consumers.

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Chapter 7

RESEARCH LINES RELATED TO SEAFOOD PRODUCTS

Plasticizers, such as phthalic acid esters (PAEs) and non-phthalic plasticizers (NPPs), are among the most commonly found additives in plastic residues collected from various environmental compartments.[1] The anthropogenic release of chemical pollutants is potentially one of the greatest environmental threats to mankind. Without proper treatment, these pollutants and their transformation products end up in the aquatic environment where they degrade water quality. In recent decades, plastic pollution has become a major source of marine pollution.[2]

Indeed, plasticizers are ubiquitous and continue to be used as the material of choice. Various studies have highlighted PAE pollution in air, water, soil, sediment, biota and humans. The environmental and human health effects of plasticizers are mainly related to interference with the hormonal and endocrine systems of living organisms, as described above in the section 3.2., chapter 3.

As a result, six EAPs have been listed as priority pollutants by the United States Environmental Protection Agency (US EPA) and the European Union (EU), and PAE concentrations have been regulated for water consumption.[3,4,5,6]

The use of PAEs is now subject to stricter controls and some PAEs have been banned or their reduction recommended in many plasticised materials[4]. With economic development and the expansion of urbanisation, industrial and domestic wastewater discharges, together with the flow dynamics of seawater, have become the main sources of plasticizers. The lack of sophisticated wastewater treatment technologies and systematic discharge plans, the lack of environmental awareness among the population, and the discharge of domestic wastewater into urban marine waters lead to the rapid accumulation of plasticizers and in aquatic organisms, and consequently become harmful to human health.

Data on environmental biomonitoring of plasticizers are relatively numerous, with a focus on freshwater ecosystems.[7,8,9,10,11,12,13]

In Tunisia, although several studies have monitored the presence of PAEs and NPPs in food, cosmetics and pharmaceuticals, few studies have evaluated the fate of these contaminants in marine compartments.[14,15] Therefore, understanding the distribution and accumulation of plasticizers in marine matrices is necessary for the protection and management of the aquatic environment.

As part of this research line, a paper was prepared and published investigating the presence of PAEs and NNPs in different marine compartments of the Mahdia coast in Tunisia, such as sediments, seagrass and mussels:

- Souaf, B., Methneni, N., Beltifa, A., Lo Turco, V., Danioux, A., **Litrenta, F**., Sedrati, M., Mansour, H.B., & Di Bella, G. (2023). OCCURRENCE AND SEASONAL VARIATION OF PLASTICIZERS IN SEDIMENTS AND BIOTA FROM THE COAST OF MAHDIA, TUNISIA published in the journal *Environmental Science and Pollution Research*, *30*, 48532-48545. (https://doi.org/10.1007/s11356-023-25687-1)[16]

Over the last half century, industrial manufacturers have turned to the production of NPPs to overcome the regulatory and safety issues associated with PAEs. From the outset, NPPs, which mainly include terephthalates, sebacates, benzoates and adipates, appeared to be a good alternative to PAEs due to lower leaching rates and the absence of restrictions on their use in PVC products.[15] Although NPPs do not have the characteristics of persistent chemicals, they can still be present in the environment due to their continuous production and release, leading to continuous environmental and human exposure.[15]

In aquatic environments, organisms at all levels of the food chain have been reported to absorb and bioaccumulate NPPs. For example, DEHT (the most common of the NNPs) is the structural isomer of DEHP and is characterised by similar hydrophobicity and solubility in water[17] indicated that DEHP has negative effects on osmotic regulation and energy metabolism.

Mincarelli et al.[18] showed that the combination of stressors, such as heat stress, can affect the timing and success of the reproductive season in invertebrates such as the sentinel *M. galloprovincialis*.

Recently, Andreyeva et al.[19] showed that DEHP accumulates mainly in the hepatopancreas and induces immunosuppressive effects in bivalve haemocytes. However, data on the bioaccumulation and toxicity of NPPs in *M. galloprovincialis* are practically lacking.

Therefore, the research line followed during the PhD was to provide information on the bioaccumulation and toxic effects of the most common NPP, i.e. DEHT, on *M. galloprovincialis*, a bio-indicator of plasticizer pollution:

- Porretti, M., Impellitteri, F., Caferro, A., Albergamo, A., **Litrenta, F**., Filice, M., Imbrogno, S., Di Bella, G., & Faggio, C. (2023). ASSESSMENT OF THE EFFECTS OF NON-PHTHALATE PLASTICIZER DEHT ON THE BIVALVE MOLLUSCS *MYTILUS GALLOPROVINCIALIS* published in the journal *Chemosphere*, *336*, 139273. (https://doi.org/10.1016/j.chemosphere.2023.139273)[20]

Another line of research in the field of seafood products that was conducted during the PhD course was the study of cooking methods for the species *Engraulis encrasicolus*.

The *Engraulis encrasicolus*, known as the anchovy, plays an extremely important role in the human diet due to its high content of protein, minerals, vitamins and polyunsaturated fatty acids, of which seafood is the only significant food source.

In terms of food safety, this line of research aimed to show that certain cooking methods, which are necessary to make food safe, also play a key role in preserving nutrients.

Foods such as meat and fish become edible and more digestible when cooked, but heat treatment can lead to undesirable changes such as the formation of toxic and undesirable substances and the loss of nutrients.[21]

For example, cooking processes are critical steps in the preservation of essential fatty acids such as EPA and DHA from fish, as EPA and DHA are highly sensitive to oxidation. The result of this research has been the publication of a scientific paper:

- Di Bella, G., **Litrenta, F.**, Pino, S., Tropea, A., Potortì, A. G., Nava, V., & Lo Turco, V. (2022). VARIATIONS IN FATTY ACID COMPOSITION OF MEDITERRANEAN ANCHOVIES (ENGRAULIS ENCRASICOLUS) AFTER DIFFERENT COOKING METHODS published in the journal *European Food Research and Technology*, *248*, 2285-2290. (https://doi.org/10.1007/s00217-022-04043-6)[22]

7.1. OCCURRENCE AND SEASONAL VARIATION OF PLASTICIZERS IN SEDIMENTS AND BIOTA FROM THE COAST OF MAHDIA, TUNISIA

The objective of this paper aimed to improve knowledge about the accumulation profiles and seasonal variation of several plasticizers (diethyl phthalate (DEP), DiBP, DBP, DEHP, and DEHT) in three different marine samples: sediments, seagrass plants (*Posidonia oceanica* L. Delile), and mussels (*Mytilus galloprovincialis*) in the Rejiche seacoast (Government of Mahdia, Tunisia). This region is a probable pollutant-release area, being an industrial and urban zone as well as possessing a sewage treatment plant nearby its coast sea. *P. oceanica* is an aquatic plant, being an interesting bioindicator of the coastal sea environmental quality. The *M. galloprovincialis* is the most common species found in the Rejiche coast and are frequently consumed by the local population. In addition, mussels have been commonly used as sentinel organisms for assessing environmental pollution in coastal and estuarine ecosystems[23] due to their extensive distribution, abundance, easy sampling, low mobility, their filtration of large volumes of water for nutrition, and economic and ecological interests.[24]

The findings of this scientific report can be used to provide important data for developing sediment, mussel, and seagrass plant quality criteria of PAEs and NPPs in Tunisia.

7.1.1. Study area and sample collection

Rejiche is a municipality in the governorate of Mahdia (Tunisia) lying on the coast of Tunisia and facing the Mediterranean Sea. This coastal site is characterised by high marine biodiversity. Nevertheless, the environmental quality of this area is impacted by the huge volumes of wastewater discharged by a nearby sewage treatment plant and by industrial activities including medical, tourism, and aquaculture (Figure 1). Sampling was conducted during the wet (January–February-March) and dry season (June-July–August) in 2020. Surface sediment samples were gathered at a depth of 4 m using Niskin bottles with a Van Veen bucket with three different spots at the sampling point and immediately transferred to glass bottles. The collected sediments were freeze dried and sieved with screen mesh (250 µm), and then, the meshed samples were stored at−20 °C until further processing. Seagrass samples were collected at a depth of 4 m, and wild mussels were caught by local fishermen from the bottom of the sea at a depth ≈10 m. Then, they were immediately transported to the laboratory where they were stored at−20 °C until analysis. A total number of 54 of sediment samples for two seasons and 252 for seagrass samples and equally for mussels were collected for plasticizers analysis. All samples were performed in three replicates.

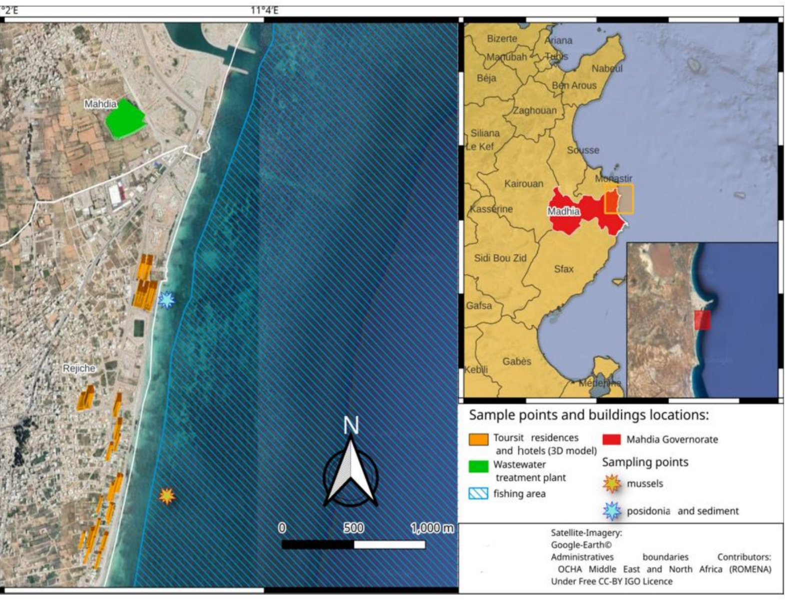

Figure 1. Map illustrating the geographical location of Rejiche in Mahdia governorate (Tunisia) and the sampling sites of sediment, seagrass (*P. oceanica*), and mussels (*M. galloprovincialis*)

7.1.2. Sample preparation

Sediments were freeze-dried for 48 h (Martin Christ Alpha 1–2/LD Plus, Germany), homogenised, and sieved (2 mm). Then, 5 g of samples was spiked with 0.001 mg of DBP-d4 and 0.001 mg of DEHP-d4. Then, a centrifuge glass tube was prepared, containing the spiked sample with anhydrous sodium sulphate and 30 mL of n-hexane:acetone solution (1:1, v/v). The obtained mix was ultrasonically extracted for 10 min and then was centrifuged at 3000 rpm during 10 min (Awel MF 20-R centrifuge, Awel SAS, France) to separate the organic supernatant from the bottom layer. The obtained supernatant was further extracted three times, according to the same procedure[25]. The final extract was completely dried using rotavapor (BUCHI Labortechnik AG, Switzerland) and re-suspended in 1 mL of hexane. The subsequent solid-phase extraction (SPE) was executed by a glass column (30 cm × 10 mm) packed with 5 g of Florisil (previously activated at 140 °C for 16 h) and 1 g of anhydrous sodium sulphate, which catches water molecules and prevents its passage to the extract. Eluate was processed with 60 mL of a diethyl ether:n-hexane solution (1:1, v/v) and evaporated to dryness[25].

Mussel (*M. galloprovincialis*) samples were freeze-dried for 72 h and then grinded into consistent powder. Afterwards, samples were spiked as described above and subjected to a matrix solid-phase dispersion (MSPD). A mix was prepared, consisting of 0.1 g of sample, 0.2 g of anhydrous sodium sulphate, and 0.4 g of solid sorbent (Florisil), which were homogenised, all together, using mortar and pestle. The obtained mix was transferred into a glass Pasteur pipette, pre-flled with 0.1 g of Florisil, and provided with a small amount of glass wool at the bottom. Targeted plasticizers were eluted with n-hexane:acetone (1:1, v/v) and dehydrated using rotavapor. For seagrass (*P. oceanica*) analysis, all samples were dried then subjected to homogenisation and are weighted to around 0.05 g each. Then, they were spiked by Iss at a concentration level of 5 mg/L and were, subsequently, extracted by n-hexane, 5 mL. All samples were transferred to a 15 mL centrifuge tube prior packaged with 900 mg magnesium sulphate ($MgSO_4$) and 150 mg PSA for disperse SPE and then was centrifuged at 4500 rpm for 10 min.

The obtained extracts were fltered through sodium sulphate (Na_2SO_4) and concentrated to about 0.5 mL by evaporation. Each sample set extraction has a corresponding relative blank and contains only solvents and reagents. Blanks are used for quality assurance.

7.1.3. GC–MS analysis

Analysis was determined by a gas chromatography system (GC-2010, Shimadzu, Japan) equipped with an autosampler (HT300A, HTA, Italy) and combined to a single quadrupole mass spectrometer (QP-2010 Plus, Shimadzu, Japan). Chromatographic separations were carried out on a SPB5MS capillary column (30 m×0.25 mm i.d.×0.25 μm film thickness, Supelco, USA). The oven temperature program was as follows: from 60 to 190 °C at 8 °C/min (5 min hold), from 190 to 240 °C at 8 °C/min (5 min hold), and from 240 to 315 °C at 8 °C/min. The injection port was at 260 °C and was provided with a narrow inlet liner (0.75 mm ID, Agilent Technologies). Sample injection occurred in splitless mode, with sampling time of 60 s, then split ratio 1:15. Injection volume was 1 μL. Carrier gas (He, 210.0 kPa, pressure control mode) was operated at a linear velocity of 30 cm/s. As for the MS setup, the temperature of the EI source was set at 200 °C, ionisation energy and emission current were 70 eV and 250 μA, respectively, while interface temperature and electron multiplier voltage were, respectively, equal to 300 °C and 1.0 kV. Data acquisition was executed both in full scan (mass range: 40–400 m/z) and selected ion monitoring (SIM) by monitoring three characteristic mass fragments for every analyte. Data acquisition and processing occurred by GC–MS solution software. Identifcation of plasticizers were performed by comparison of their retention times and mass spectra with those of corresponding commercial standards. The quantitative analysis was realised in SIM mode, taking into account the relative base peak ions and exploiting the internal standard normalisation. In the table 3 section *5.1.2*, one target ion (T) and the two qualitative ions (Q1 and Q2) used for each analyte are shown.

7.1.4. Statistical analysis

Descriptive data analysis, including mean and median minimum and maximum concentrations, was carried out. Spearman correlation was applied to assess the relationship between phthalate levels in sediment, seagrass, and mussel samples, using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). A statistical comparison of every plasticizer level among different marine samples and during different months was carried out by one-way ANOVA, followed by Tukey's honestly significant difference (HSD) post hoc test in order to investigate seasonal variations. Statistical significance was defined as $p<0.05$.

7.1.5. Occurrence of PAEs and NPP in sediments and biota

Four PAE congeners (DEP, DiBP, DBP, and DEHP) and one NPP (DEHT) were analysed in sediment, seagrass and mussel samples and the obtained results have been presented in Figure 2 and Table 1 PAEs and DEHT were detected in all marine samples with obvious quantity differences. Figure 2 shows a significantly greater Σ4PAE content in sediments compared to the concentrations detected in the seagrass and the mussels.

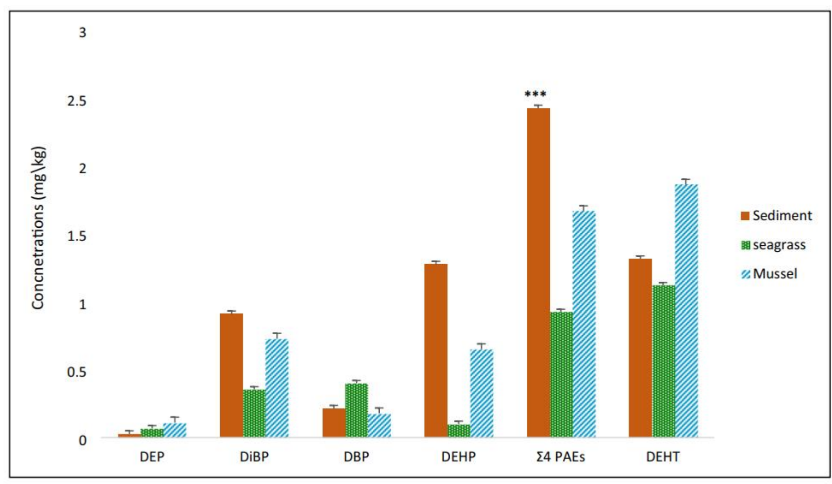

Figure 2. Concentration of single PAE congener, total phthalates esters (Σ4PAEs), and DEHT in sediment, seagrass, and mussel samples (DEP, diethyl phthalate; DiBP, diisobutyl phthalate; DBP, dibutyl phthalate; DEHP, di(2-ethylhexyl) phthalate; PAE, phthalates acid esters; DEHT, di-2-ethylhexyl terephthalate; ***significant at 0.001 level).

The concentrations of Σ4PAEs in the analysed sediment samples ranged between 0.839 and 3.88 mg/ kg, dry weight (dw), with a median and mean of 4.732 and 2.423 mg/kg, respectively. Among the four analysed PAEs, DEHP exhibited the highest concentration (mean: 1.275 mg/kg, median: 2.208 mg/kg, dw), followed by DiBP (mean: 0.909 mg/kg, median: 1.707 mg/kg). DiBP and DEHP showed the highest detection rate 98% and 97.1%, respectively, in all investigated sediment samples.

Table 1. Concentrations (range, mean, and median) of Σ4PAEs+DEHT content (expressed in mg/kg) and their detection rate in the single marine matrix from Rejiche coastal sea (Mahdia governorate, Tunisia)

	Compound	Range	Mean	Median	Detection rate (%)
Sediment (mg/kg, dw)	DEP	0.016–0.047	0.027	0.022	90.0
	DiBP	0.379–1.734	0.909	1.707	98.0
	DBP	0.130–0.326	0.214	0.413	96.0
	DEHP	0.314–1.773	1.274	2.590	97.1
	DEHT	0.674–2.000	1.181	2.008	95.0
	Σ4PAEs	0.839–3.880	2.423	4.732	
	Total	1.513–5.880	3.670	6.250	
Seagrass (mg/kg, dw)	DEP	0.037–0.098	0.068	0.209	100
	DiBP	0.050–1.216	0.355	0.077	100
	DBP	0.027–0.054	0.068	0.039	100
	DEHP	0.082–0.150	0.101	0.090	100
	DEHT	0.315–2.541	1.121	1.000	100
	Σ4PAEs	0.196–1.518	0.592	0.414	
	Total	0.511–4.059	1.713	1.414	
Mussel (mg/kg, dw)	DEP	0.071–0.169	0.110	0.097	100
	DiBP	0.363–1.961	0.727	0.473	100
	DBP	0.119–0.219	0.178	0.176	100
	DEHP	0.454–1.223	0.650	0.521	100
	DEHT	0.578–5.682	1.860	0.997	100
	Σ4PAEs	1.007–3.572	1.665	1.267	
	Total	1.585–9.254	3.525	2.264	

DEP diethyl phthalate; DiBP: diisobutyl phthalate; DBP: dibutyl phthalate; DEHP: di(2-ethylhexyl) phthalate; DEHT: di-2-ethylhexyl terephthalate; PAE: phthalate acid esters; dw: dry weight

The results of the Pearson correlation analysis of the PAEs and DEHT concentration data in the analysed marine samples are shown in Table 2. Positive correlations have been recorded between the Σ4PAE among the three studied marine samples. The greatest correlation was observed in seagrass samples between DiBP and DBP levels ($r = 0.83$, $p < 0.05$). The lowest correlation was

found in mussels between DBP and DEP levels (r=0.03). In seagrass samples, DiBP and DBP displayed positive correlations. While in sediment samples, only DiBP exhibits positive correlations with other congeners. Overall, DEHT was correlated with Σ4PAE with the exception of DBP.

Table 2. Spearman correlation analysis of total phthalates esters (Σ4PAEs) +DEHT in sediment, seagrass, and mussel samples.

	Compounds	DEP	DiBP	DBP	DEHP	DEHT
Sediment	DEP	1				
	DiBP	0.6	1			
	DBP	– 0.14	0.37	1		
	DEHP	– 0.37	0.26	– 0.03	1	
	DEHT	0.3	0.3	– 0.26	0.6	1
Seagrass	DEP	1				
	DiBP	– 0.6	1			
	DBP	– 0.43	0.83*	1		
	DEHP	0.12	0.41	0.75	1	
	DEHT	0.37	0.09	0.08	0.49	1
Mussel	DEP	1				
	DiBP	0.54	1			
	DBP	0.03	– 0.38	1		
	DEHP	– 0.09	0.09	0.41	1	
	DEHT	0.6	0.6	– 0.41	0.37	1

DEP diethyl phthalate, DiBP diisobutyl phthalate, DBP dibutyl phthalate, DEHP di(2-ethylhexyl) phthalate; DEHT di-2-ethylhexyl terephthalate, PAE phthalate acid esters *Significant at 0.05 level

7.1.6. Seasonal variation

The seasonal variation of PAEs and DEHT in every marine sample is shown in Figure 3. For the analysed sediment samples, although all four PAEs single and DEHT average concentrations in the wet season increased compared with those in the dry season, this seasonal variation was not significant. On the other hand, for the seagrass *P. oceanica*, DEP and DEHT concentrations were significantly higher in the wet season (0.089 mg/kg, 1.608 mg/kg, respectively, $p < 0.05$). The other PAE average concentrations were not significantly different among dry and wet seasons ($p>0.05$). Considering the *M. galloprovincialis*, only DEHT showed a significant content increase during the wet season (0.961 mg/kg). The seasonal distribution of the PAEs in mussel samples was not significant ($p>0.05$).

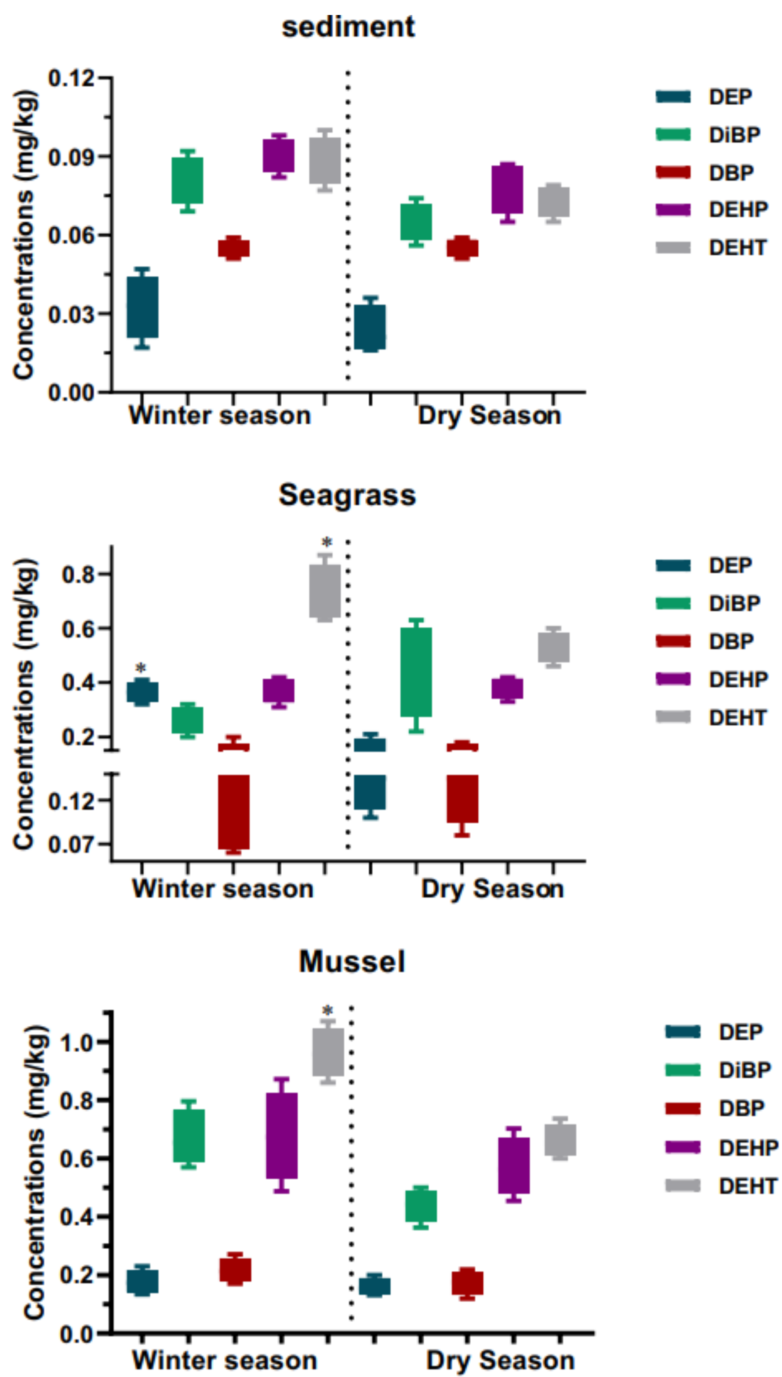

Figure 3. Seasonal variation of single PAE (phthalates esters) congeners, and DEHT in sediment, seagrass, and mussel samples collected from Rejiche coastal sea (Mahdia governorate, Tunisia) during wet and dry seasons (DEP, diethyl phthalate; DiBP, diisobutyl phthalate; DBP, dibutyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DEHT, di-2-ethylhexyl terephthalate; *significant at 0.05 level.

7.1.7. Discussion of results

In Tunisia, the coastline of Mahdia is one of the most important aquatic ecosystems occupying a very strategic geographical territory with approximately 75 km of coastline, presenting a preponderant fishing, heritage, and agricultural resources. However, the coast of Mahdia is subject to several sources of urban and industrial pollution. Once discharged into the environment, numerous micropollutants can cause environmental disturbances and threaten the maintenance of aquatic ecosystems and public health, such as phthalates and their alternatives. As a matter of fact, PAEs are the plasticizers most employed globally. This has created a serious concern given the rising levels of plastic pollution and PAEs presence in the marine environment; therefore, a reliable monitoring is essential to study and compare their dispersion pattern. In our study, (i) we monitored the traces of four PAE congeners (namely, DEP, DBP, DiBP, and DEHP) and one NNP, namely DEHT, in sediments, *P. oceanica* and *M. galloprovincialis* collected from Rejiche coast in Mahdia and (ii) we compared their seasonal distributions. The analytical screening by GC–MS revealed that PAEs and DEHT were detected in all marine samples, suggesting their ubiquitous distribution in the marine compartments of the Rejiche coast. DEHP and DiBP are the PAE compounds that showed the maximum level in sediments. From the obtained results, it can be stated that, despite the strictly regulated use, DEHP and DiBP remain still the most abundant PAEs detected in the environment.[9,10,26,27,28]

Van Wezel et al.[29] set a guideline for environmental risk limits and established a limit of 0.7 mg/kg for DEHP in marine sediments. Based on the scientific literature of the last 15 years, the collected results in this study were in accordance with the previous studies reporting dominance of DEHP and DiBP in sediments in Tunisia[15], Germany[30], China[26,27], South Africa[31], and France-Belgium[32]. However, the concentrations of PAEs in sediment samples from Rejiche coast were greater than other marine regions previously studied in the Korean bays (0.82× 10−3–0.46 mg/kg dw)[33], the Asan Lake of Korea (0.52× 10−3–0.38 mg/kg dw)[13], and the coastal areas of the False Creek Harbor (Vancouver, Canada, USA) (0.004–2.9 mg/ kg dw)[25], with exception of Kaohsiung Harbor

(Taiwan), and Dutch North sea (Netherlands) which showed higher DBP (0.29 mg/kg) and DEHP levels (0.17–3.39 mg/kg), respectively[8].

High concentrations of DEHP and DiBP in sediments may indicate their lower degradation rates and stronger sorption capacities. Moreover, the abundance of DEHP and DiBP in sediments could be attributed to the routine discharge of industrial effluents into the coastline of Mahdia. Further hypothesis could be attributed to the physicochemical properties of sediments, such as the total organic carbon (TOC) and the particle size, which plays an important factor in PAEs dissemination.[10,15]

Concerning the contamination by phthalate alternatives, only one recent work monitored DEHT in sediments from Korean semi-enclosed bays and coast showed considerably lower DEHT content (semi-enclosed bays: 0.0043 mg/kg, coast: 0.0097 mg/kg, dw) with respect to the coastal sediments from this work.[13,33]

Posidonia oceanica samples were studied for plasticizers, and DiBP was established as the most abundant compound among PAEs. With respect to previous literature, only one study was conducted in Mahdia Coasts (in 2018–2019) reported lower concentrations of DiBP (0.355 mg/kg > 0.101 mg/kg, dw), however, DBP and DEHP were higher at levels of 0.389 mg/kg, 0.729 mg/ kg, respectively[15]. These results suggest that seagrass plants, such as *P. oceanica*, could act as potential "adsorbents" of emerging chemicals in the marine ecosystem. Indeed, it has shown its significantly in adsorbing organic and inorganic pollutants[34,35]. Similarly to the seagrass plant, DiBP was found to be the highest PAE compound detected in *M. galloprovincialis*, whereas with comparison to previous studies, the collected results for DEP, DBP, and DEHP revealed lower concentrations in mussels than those detected in Spanish aquaculture (3.20–6 mg/kg, 6–32 mg/kg, 2–12 mg/kg, respectively)[36]. Furthermore, Spanish research on wild raw *M. galloprovincialis* demonstrated higher DBP and DEHP contents (0.68 mg/kg, 2.65 mg/kg, respectively) with the exception of DEP which displayed less content level (0.05 mg/kg)[37].

An English survey examining the ability of mussel bioaccumulation showed greater concentrations of DiBP and DEHP (4.4 mg/ kg, 4.1 mg/kg, respectively)[38]. The relatively high lipid content in marine organisms, fish, and mussels may boost the uptake of such pollutants and facilitate their bioaccumulation. Variability in PAE concentrations observed in mussel species may be due to variation in lipid content that can lead to differences in body burden of contaminants.

In fact, mussels appeared to be excellent toxicants bio-monitoring markers due to their tolerance to variations in the water environment, as well as their accumulation capability of various contaminants, especially plasticizers with low polarity index.[39,40,41]

Hence, the high bioaccumulation potential of the PAE congeners indicates that these compounds may reach high concentrations in aquatic organisms. To the knowledge of the authors, there are no comparative studies on alternative phthalate DEHT from marine biota that can be inferred from previous papers.

PAEs and DEHT were detected in all marine samples. Although these chemicals were present in all matrices, their concentrations in the individual matrix were quite different. For example, DiBP and DEHP compounds were among the compounds frequently detected at high levels in sediment and mussel samples, whereas this was not the case in seagrass samples. This fnding is probably due to a quite complex bioaccumulation regime involving different uptake pathways[42] and high small-scale variance of exposure. In general, given that the seagrass *P. oceanica* represents a lower trophic level as a primary producer species compared with consumer species (like mussels), it would be expected for it to bioaccumulate less of these contaminants in its tissues. Furthermore, the application patterns, emission events, and industrial, agricultural, or urban runoff during rain events could influence the presence of a contaminant in the aquatic environment[43]. A Pearson correlation analysis was performed to evaluate the strength of relationships between concentrations of single PAEs and NPPs. PAE congeners were positively correlated in the three marine samples; this correlation may be explained by the continuous and simultaneous consumption of both plasticizers and their

alternatives[13,33]. DEHT and DEHP shared a high positive correlation in sediment, seagrass, and mussel samples (r=0.6, 0.49, and 0.3, respectively). This finding may support the hypothesis of the growing usage of DEHT coherently with DEHP with the evidence that NPPs are increasingly replacing conventional PAEs in the industrial sector and to their similar usage profile and geochemical behaviour in the marine ecosystem[13], determining the strength and direction of the monotonic relationship between both compounds.

Considering the seasonal variations, DEP, DiBP, DBP, DEHP, and DEHT displayed a comparable temporal distribution in sediments. Although no significant diffrences have been recorded, PAEs were slightly higher in wet season. The decrease of PAE levels in the dry season could be partly due to high photolytic activity, microbial degradation, and oxidation. In addition, the seasonal variations of such compounds could be explained by weather-related factors, such as rainfall, stormwater flooding, and atmospheric fluctuations. In fact, the concentrated precipitation may cause large amounts of land-sourced pollutants from multiple sources to be carried by surface runoff into the seacoast[8]. Some papers have also stated that atmospheric deposition and rainfall runoff are the main factors affecting changes in the concentration of pollutants in marine sediments[10,13]. PAE congeners were also detected during dry season (from June to August). This could be related to tourism activities particularly because DEHP level increased during summer. On the other hand, the extent of industrial and urban activities as well as sewage treatment stations, generate preferentially inputs of plasticizers in the coastal seawater. Furthermore, the governorate of Mahdia benefits from a wealthy fishing harbour and intense maritime traffic that are predominant during summertime. Findings in *P. Oceanica* and *M. galloprovincialis* showed comparable seasonal PEA profiles with the exception of DEP which exhibited significantly higher levels in the wet season in seagrass samples, while DEHT was recorded to be significantly higher during wet season in both matrices. Although the seasonal variation of PAEs and their alternatives in marine biota has not been yet investigated with greater focus, it may be stated that the seasonal characteristics of rainfall and behaviour of runoffs and streams may play a leading role in the transmission and accumulation of

anthropogenic pollutants in the aquatic environment, affecting the exposure risk of marine organisms[44]. Taking all results into consideration, we could speculate that sediments do not only act as plasticizers final sink and reservoir, but they may contribute to their routes as an intermediate in plasticizers transport from environmental media to biological organisms in an aquatic ecosystem[13,15]. In fact, PAEs in sediments may have low to moderate potential risks of aquatic organisms[44]. In summary, the discharge of industrial and domestic wastewater, rainfalls, and surface runoffs may transport a large amount of PAEs in the drainage basin of the Rejiche seacoast causing pollutant accumulation in sediments, which may pose a risk of harm to aquatic organisms.

7.1.8. Conclusion

A comprehensive investigation of the occurrence, source, and seasonal trends of four PAE congeners (DEP, DBP, DiBP, and DEHP) and one NPP (DEHT) in sediment, seagrass, and mussel samples from Rejiche seacoast in Mahdia governorate (Tunisia) was performed in this study. Among the investigated PAEs, DEHP and DiBP were the most abundant and frequently detected congeners in every marine compartment. However, the coast of Rejiche was more polluted by DEHT than Σ4PAEs being coherent, in terms of routes, with conventional phthalates. ΣPAEs with DEHT screening in sediments were alike in mussels, suggesting that these contaminants could bioaccumulate through the food chain, whereas *P. oceanica* showed lower loads of these pollutants, probably due to its low capacity to uptake and bioaccumulate plasticizers from sediment. The PAE concentrations in this present study were at a medium level, compared to previous research. Our results suggest that the detected plasticizers in the studied area might be related to the discharge of domestic sewage as well as touristic and industrial inputs. The seasonal changes might affect their temporal distribution probably due to anthropogenic activities and weather-related factors.

7.2. ASSESSMENT OF THE EFFECTS OF NON-PHTHALATE PLASTICIZER DEHT ON THE BIVALVE MOLLUSCS MYTILUS GALLOPROVINCIALIS

The aim of this study was to provide a detailed framework on the bioaccumulation and toxic effects of DEHT on *M. galloprovincialis* by performing a controlled laboratory experiment using a multidisciplinary approach. Specifically, adult mussels were exposed to environmentally relevant doses and the bioaccumulation of DEHT was first assessed by gas chromatography coupled to mass spectrometry (GC-MS). Subsequently, alterations in lipid and protein metabolism were assessed by screening total protein and lipid in whole organisms and fatty acid (FA) composition by gas chromatography coupled to flame ionisation detector (GC-FID). The overall stress response to DEHT exposure was assessed by determining the cell viability of the haemolymph and digestive gland of mussels using appropriate staining assays. Antioxidant and lipid peroxidation peroxidation biomarkers and protein oxidative damage were also in the gills and digestive gland of the experimental mussels (Figure 4).

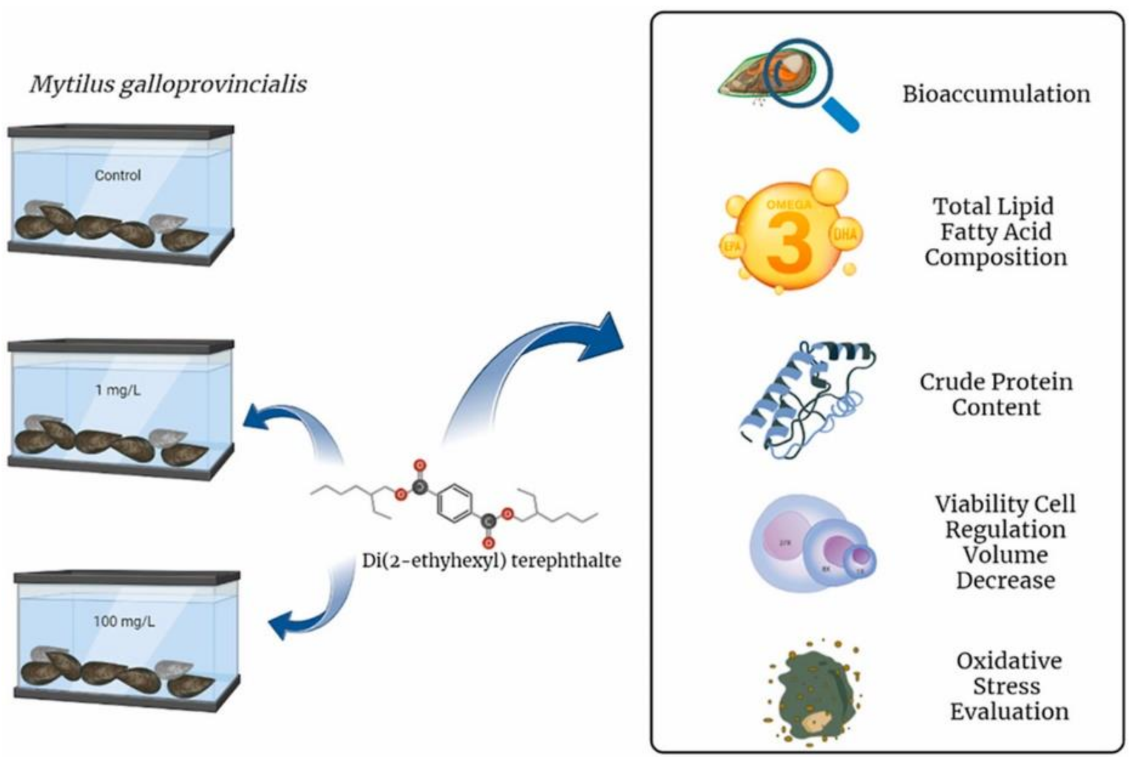

Figure 4. Experimental design.

7.2.1. Experimental design

The study was conducted during the months of November and December 2022 and the specimens *Mytilus galloprovincialis,* (Lamarck, 1819) was selected to investigate the toxicity of the plasticizer DEHT. A total of n = 150 adult mussels with a mean length of 4.6 ± 0.38 cm and a mean weight of 8.2 ± 2.1 g, were collected at the shellfish farm FARAU SRL Frutti di Mare located at "Lago di Faro", a coastal lagoon in Messina (Italy, 38◦15′ 39.95″ N 15◦37′ 01.9″ E) which is renowned for the shellfish culture, in accordance with the guidelines of Directive (2010)/63/EU on animal testing.
The environmental background DEHT contamination of the site was considered low, since despite the anthropic pressure mainly related to the presence of several farming activities, the area is part of the "Laguna di Capo Peloro" natural reserve and it is continuously monitored. Collected mussels were transported to the laboratory in aerated natural seawater and acclimated for one week in artificial saltwater (Premium REEF-Salt, Tropical Marine Centre, Chorleywood, UK) at constant temperature (18.2 ± 0.12 ◦C) and aeration. Following the acclimatisation period, they were randomly subdivided into six continuously aerated glass tanks (20l) and two experimental groups, and a control group were created in duplicates (n = 25 specimens per tank). The experimental groups were separately exposed to two different concentrations of DEHT (purity ≥96%, Sigma-Aldrich, Steinheim, Germany), namely DEHT1 (1 mg/l, environmental concentration, EC) and DEHT100 (100 mg/l, 100xEC). On the other hand, the control groups did not receive any exposure treatment. The exposure time lasted 14 days. During the study period, mussels were constantly kept under natural light condition and were not fed; while the saltwater was renewed three times per week (at day 2, 4, 6, 8, 10, and 12), with the same concentrations of DEHT being added immediately afterwards. No mortality was observed during the entire exposure period. The DEHT concentration of 1 mg/l was chosen based on the DEHT levels recently detected in seawaters from the Mediterranean area (0.78 mg/l[15]; 0.68–1.21 mg/l[14]), where the concentration 100 mg/l, which

corresponded to 100 times the environmental concentration, was also considered in relation to previous work[45].

A 14-day exposure was chosen considering that DEHT is not a highly persistent and accumulative plasticizer and on the basis of previous study[46].

7.2.2. Sample collection

For the chemical analysis n = 16 organisms were randomly selected from each group, and their whole body was dissected, pooled, freeze-dried, grounded into a fine powder, and stored at 80 ◦C until the bioaccumulation study was performed, as well total lipid, protein and FA composition were assessed.

Additionally, n = 8 water samples (50 mL) from each experimental/control group were collected in glass bottles before and after each water exchange and stored at + 4 ◦C until further analysis. For the biological analysis, n = 4 organisms were randomly selected from each experimental group and pooled for assessing cell viability and the ability to regulate volume decrease. In this case, haemolymph was collected from the anterior adductor muscle of mussels from each experimental/control group, using a glass syringe with a 23-gauge needle, according to Bolognesi and Fenech[47]. After haemolymph collection, each mussel pool was sacrificed on an ice flake and digestive glands (DGs) were dissected and collected. For antioxidant biomarkers and oxidative protein damage, n = 6 mussels were randomly selected, and their gills and DGs were collected and stored at 80 ◦C until use.

During the exposure study and the subsequent experimental procedures, every effort was made to significantly reduce the potential background DEHT contamination of solvents, reagents, and laboratory materials. All samples were processed using glassware and stainless-steel instruments, that were previously washed with acetone, rinsed with hexane, and dried at 120 ◦C for at least 4 h. Sodium sulphate was heated for 4 h at 140 ◦C and, after cooling, kept in a tightly sealed glass vial. Organic solvents were tested for background levels after concentration and GC–MS analysis. Only

the batches of solvents marked by negligible levels of contamination were used throughout the analytical procedure.

7.2.3. Determination of DEHT in mussel and water samples

The determination of DEHT content in experimental and control mussels, as well as in saltwater, was carried out according to the method proposed by Jebara et al[15].

In brief, 0.1 g of every powdered sample was spiked with a known amount of deuterated standard (DEHT-d4 in nonane, 100 µg/mL, Cambridge Isotope Laboratories Inc., Andover, MA, USA) and mixed with 0.2 g of anhydrous sodium sulphate (Sigma-Aldrich) and 0.4 g of solid sorbent magnesium silicate (Florisil®, 60–100 mesh, coarse powder, Fluka, Sigma-Aldrich). The obtained mixture was added on the top of a glass Pasteur pipette which was previously prepared with 0.1 g of Florisil and glass wool, and subsequently sealed at the top with additional glass wool. The extract was eluted with a solution of *n*-hexane: acetone (1:1 *v/v;* >99.9% grade, Sigma-Aldrich) and evaporated to dryness by a rotating evaporator. DEHT in saltwater samples was determined using a C18 cartdrige (Supelclean C18, 3 mL, 500 mg sorbent, Supelco), previously conditioned with 5 mL ethyl acetate (>99.9% grade, Sigma-Aldrich) and 5 mL methanol (>99.9% grade, sigma-Aldrich). After passing the samples, the cartridge was washed with 4 mL deionised water (Sigma-Aldrich), followed by 6 mL of a methanol: water solution (35%, v/v). DEHT was eluted with 5 mL ethyl acetate and evaporated to dryness by a rotating evaporator.

All dried extracts were re-suspended in n-hexane and analysed by a gas chromatography system (GC-2010, Shimadzu, Japan) equipped with an autosampler and coupled to a single quadrupole mass spectrometer (QP-2010 Plus, Shimadzu, Japan). Chromatographic separations occurred on a SLB-5MS capillary column (30 m × 0.25 mm i. d. × 0.25 µm film thickness, Supelco, USA). The oven temperature program was from 60 ◦C to 190 ◦C at 8 ◦C/min (5 min hold), from 190 ◦C to 240 ◦C at 8 ◦C/min (5 min hold), and from 240 ◦C to 315 ◦C 8 ◦C/min. The injection port was at 260 ◦C and was provided with a narrow inlet liner (0.75 mm ID, Agilent Technologies). Sample injection

took a place in splitless mode, with sampling time of 60 s, then split ratio 1:15. Injection volume was 1 µL. Carrier gas (He, 210.0 Kpa, pressure control mode) was used at a linear velocity of 30 cm/s. The MS setup was EI source temperature; 200 ◦C; ionization energy and emission current, 70 eV and 250 µA; interface temperature and electron multiplier voltage, 300 ◦C and 1.0 kV.

Acquisition was performed both in full scan (mass range 40–400 *m*/*z*) and selected ion monitoring (SIM) by monitoring three characteristic mass fragments of the analyte. Data acquisition and processing were performed by GC-MS solution software. Identification of DEHT occurred by comparison of its retention time and mass spectrum with that of the corresponding commercial standard (DEHT, purity >99%, Aldrich Chemical, Chicago, IL, USA). The quantitative procedure was carried out in SIM mode, considering the relative base peak ion and exploiting the internal standard normalization. Triplicate measurements were conducted for every sample, alternated with analytical blanks (solvent) and, in the case of mussel pools, results were expressed on a converted fresh weight (fw) basis. During the entire study period, trace contents of DEHT (1.07 µg/kg) were detected in these blanks, and they were constantly subtracted from the analysis of sample extracts.

7.2.4. Chemical composition

For the lipid extraction from pooled mussels, the method recommended by the Organization for Economic Cooperation and Development test guidelines was followed. Briefly, every powdered sample (~4 g) was homogenized with 40 mL of a mixture chloroform: methanol (1:2, v/v, HPLC grade, Carlo Erba), and then placed in an ultrasonic bath for 30 min. The mixture was added with 10 mL of NaCl 0.73%, shaken and centrifugated at 6300×*g* for 10 min (Awel Industries, France, MF 20-R). The lower chloroform phase was transferred to a flask, while the upper phase was extracted again according to the same procedure. Then, the two extraction phases were pooled, filtered with anhydrous ammonium sulphate, and dried. After determining the extraction yield (%) gravimetrically on a fw basis, the lipid extract was recovered by 1 mL n-hexane, added with 1 mL of sodium methoxylate and heated at 100 ◦C for 15 min. After cooling down the mixture, 1 mL of

boron trifluoride/methanol (14%) was added, and again heated under the same conditions. Approximately 1 mL of *n*-hexane and 4 mL of a saturated sodium chloride solution were added to the cooled mixture. The organic layer with fatty acid methyl esters (FAMEs) was collected and analysed by a gas chromatograph (GC) equipped with a split/splitless injector and a flame ionization detector (FID) (Dani Master GC1000, Dani Instrument, Milan, Italy). A Supelco SLB-IL100 capillary column, 60 m × 0.25 mm ID, 0.20 µm film thickness (Supelco, Sigma Aldrich, USA) was employed.

The following operating conditions were used: oven temperature program 60 °C for 2 min rate 13 ◦C/min, 150 °C at rate 2 °C /min, and 240 °C for 10 min, injector and detector temperatures were respectively 220 °C and 250 °C; helium at a linear velocity of 30 cm/s (constant). The injection volume was 1 µL, with a split 75.0 mL/min. Data acquisition and management was performed using Clarity Chromatography Software v4.0.2. All samples were analysed in triplicate along with analytical blanks. FAMEs of nutritional interest were identified by direct comparison with the reference retention times of compounds present in the commercial standard mixture (FAMEs reference standards C4–C24, Supelco (Bellefonte, PA, USA). For every sample, individual FAME percentages were calculated with respect to the total chromatogram area in triplicate analysis. Crude protein was determined from every powdered sample (~1 g) consisting of pooled mussels, by using the Kjeldahl method.

7.2.5. Cell viability assays

Haemolymph and DGs from pooled mussels were considered to evaluate the effects of DEHT on cell viability. The tests were conducted using two different colorimetric assays, namely the Trypan Blue (TB, Sigma-Aldrich) exclusion method and Neutral Red (NR, Sigma-Aldrich) retention assay. The first method mentioned entails staining the cells with Trypan blue, which has the ability to enter only non-viable cells with compromised cell membranes. This is because, under normal circumstances, viable cells would prevent the uptake of Trypan blue dye.

The percentage of unstained cells represents the percentage of viable cells in the cell suspension. This assessment helps determine the extent of damage caused to the cells. Additionally, the stability of the lysosomal membrane was assessed using the neutral red retention assay (NR). According to Torre et al.[48], this assay relies on living cells' ability to absorb and bind the neutral red dye in their lysosomes, after incubating the cells for 15 min.

The extent of dye retention provides insights into the stability of the lysosomal membrane. To calculate the percentage of cell viability, the following formula was used:

$$\text{Cells viability (\%)} = \frac{(\text{n° of viable cells})}{(\text{total number of cells})} \text{ x } 100$$

DG cells were isolated according to the method of Pagano and coworkers[49]. The DGs was cut in an isosmotic solution in a calcium, magnesium free solution (CMSF), then transferred in a glass tube with 0.01% collagenase (CLS IV, 175 U/mg, Sigma-Aldrich) and placed in a thermostatic bath for 60 min at 18 °C. The cellular suspension was filtered through 200 µm and 50 µm filters, resuspended with a physiological solution and then centrifuged for 10 min at 4 °C, at 400×g (Awel Industries, France, MF 20-R).

The supernatant was removed, and cells were resuspended twice with a physiological solution. The samples were transferred again into the thermostatic bath (18 °C) for another hour. The drop of cells sample was put on a slide and observed under a light microscope (Carl Zeiss Axioskop 20, Wetzlar, Germany) connected to a digital camera (Canon 550D).

The sample was gently washed with an isotonic solution and three images were taken in sequence. Subsequently, the sample was gently washed with a hypotonic solution (800 mOsm), for the first 10 min, the images were taken every 1 min and then every 5 min during the last 20 min. Cells area from exposed mussels was compared with the control group with the software ImageJ (NIH, Bethesda, MD, USA). Images were taken of 15 cells from each experimental group.

7.2.6. *Determination of antioxidant and lipid peroxidation biomarkers and oxidatively modified proteins*

Gills and digestive gland (n = 6 for each experimental and control condition) for each experimental and control condition) were homogenized in cold 100 mM Tris/HCl buffer (pH 7.2), containing a mixture of protease inhibitors to assess superoxide dismutase (SOD) activity, lipid peroxidation (LPO) and oxidatively modified proteins (OMP). SOD activity was determined by using the method described by (Marklund and Marklund, 1974), modified according to (Filice et al., 2023). The inhibitory effect of SOD on the auto-oxidation of pyrogallol at pH 8.20 was assayed spectrophotometrically at 420 nm and 25 °C.

The reaction was run in 50 mM Tris-HCl, 1 mM EDTA, 0.2 mM pyrogallol and monitored every 30 s for 5 min. One unit of SOD activity was defined as the amount of the enzyme that inhibits 50% of pyrogallol auto-oxidation. Results were expressed in U/mg protein. LPO was determined by measuring the concentration of 2-thiobarbituric acid-reacting substances (TBARS), as reported by Filice et al.[50]

A reaction mixture containing sample homogenate (10% w/v), 2-thiobarbituric acid (TBA, 0.8%), and trichloroacetic acid (TCA; 20%) was kept in a water bath at 100 °C for 10 min and then centrifuged at 5000×g for 10 min. TBARS levels were determined in the supernatant by assessing malondialdehyde (MDA) concentration at 540 nm (MDA extinction coefficient: 156,000 M−1 cm−1); TBARS values were reported as nmoles MDA/mg protein. OMP was evaluated by measuring the carbonyl groups content according to the 2,4-dinitrophenylhydrazine (DNPH) method, described by Levine et al.[51]

Aliquots of homogenates were incubated for 1 h at room temperature with 10 mM DNPH in 2 M HCl; proteins were then precipitated with 2 volume of TCA and centrifuged at 5000×g for 10 min (Sigma Laborzentrifugen, Germany, 1–15). Pellet was washed thrice with ethanol-ethyl acetate (1:1; v/v) to remove DNPH excess and then dissolved in 6 M guanidine in 2 N HCl. The concentration of carbonyl groups was measured spectrophotometrically at 370 nm (aldehydic derivates) and at

430 nm (ketonic derivates) using the extinction coefficient of 22,000 M^{-1} cm^{-1}. Results were expressed as nmol/mg protein.

7.2.7. Statistical analysis

Experimental data from the determination of the DEHT content, the total lipid content, the FA composition, the total protein content, the cell viability and the RVD were expressed as mean ± standard deviation (SD) of triplicate analysis. The statistical analysis was conducted by using the software Graphpad Prism, version 5 (Graphpad Software Ldt., USA, 2003). After running a Shapiro–Wilk test to verify the normal distribution of experimental data, the one way-ANOVA was applied for every independent variable to produce an F-statistic, i.e., the ratio of the variance calculated among the means to the variance within the samples. The one way-ANOVA was followed by a Tukey's honestly significant difference (HSD) post-hoc test for multiple comparative analyses between experimental and control groups. Statistical significance was always accepted at $p < 0.05$. Oxidative stress data were expressed as means ± standard error (SE) of individual experiments performed in duplicate; significance was assessed by one way-ANOVA followed by Tukey's HSD post-hoc multiple comparative test. Differences were considered significant at $p < 0.05$.

7.2.8. Results

Levels of DEHT detected by GC-MS in water and mussel samples are reported in Table 3.
Water from the control tank was considered DEHT-free, as DEHT was below the instrumental limit of detection (<LOD, where LOD = 0.04 μg/kg). In both exposure treatments, the detected DEHT concentrations were >92% of the nominal concentration throughout the exposure period, and thus significantly different from each other (DEHT1:0.95 mg/l and DEHT100: 96.36 mg/l, $p < 0.05$). Mussels maintained in DEHT-free saltwater exhibited no bioaccumulation of such NPP. Mussels coming from the DEHT1 treatment bioaccumulated an average of 123.69 mg/kg of this plasticizer, whereas DEHT100 mussels bioaccumulated DEHT to an average concentration of 595.13 mg/kg, which was significantly different from each other ($p < 0.05$).

Table 3. Concentration of DEHT revealed by GC-MS in experimental waters (mg/l) and control and treated pools (i.e., DEHT1 and DEHT100) of *M. galloprovincialis* (mg/Kg, fw). Data are presented as mean ± SD of triplicate analysis. LOD = instrumental Limit of Detection of DEHT (0.04 µg/kg).

Samples	Test groups		
	Control	DEHT1	DEHT100
Water	<LOD	0.95 ± 0.02a	96.36 ± 11.54b
Mussels pool	<LOD	123.69 ± 23.52a	595.13 ± 41.99b

a–b: different superscript letters in the same row indicate significantly different values ($p < 0.05$ by post hoc Tukey's HSD test).

Total lipid and the FA profile obtained from control and test mussel pools are reported in 4. Total lipids were equal to 5.52% fw in the control group. On the other hand, DEHT exposure caused a strong lipid increase, since DEHT1 and DEHT100 groups showed respective lipid contents of 8.75% and 8.41% fw. Statistical analysis showed a significant difference ($p < 0.05$) for both DEHT1 and DEH100 groups compared to the control condition (Table 4). Regardless of the control or experimental condition, the FA composition of *M. galloprovincialis* was characterized by a high content of polyunsaturated FAs (PUFA, 36.71–39.88%, $p < 0.05$), followed by an intermediate level saturated FAs (SFAs, 27.28–32.19%, $p < 0.05$) and a lower amount of monounsaturated FAs (MUFAs, 16.11–17.39%, $p > 0.05$).

The most abundant fatty acids revealed in the soft tissues of mussels, were palmitic acid (C16:0, 19.33–22.90%, $p < 0.05$), stearic acid (C18:0, 4.67–6.01%, $p < 0.05$), palmitoleic acid (C16:1 ω-7, 3.36-5.84.47-6.41, $p < 0.05$), eicosapentaenoic acid (C20:5 ω-3, 12.86–14.59%, $p < 0.05$) and docosahexaenoic acid (C22:6 ω −3, 14.92–15.91%, $p > 0.05$) (Table 3). DEHT exposure affected the lipid metabolism of *M. galloprovincialis* with respect to the control condition.

Conversely, non-significant changes were observed between DEHT1 and DEHT100 mussels, which were basically similar in both lipid content and FA composition.

Overall, an increase in total lipid from 5.52% in control mussels to 8.75–8.41% ($p < 0.05$) in exposed organisms was reported. Also, a general decrease in SFAs, particularly reflected in the variation of palmitic acid from 22.90% (control) to 20.73–19.33% (DEHT exposure, $p < 0.05$), and

a general increase in PUFAs, especially shown in the variation of ω3 FAs from 30.82% (control) to 33.96–33.38% (DEHT exposure, $p < 0.05$), were highlighted. Regarding the latter, most of PUFAs were characterized by an upward trend after DEHT exposure (e.g., the stearidonic acid (C18:4 ω-3) increased from 1.24% to 1.59–1.71%, $p < 0.05$), except for arachidonic acid (C20:4n-6), which slightly decreased from 4.35% to 4.04–4.01% ($p > 0.05$). Interestingly, the plasticizer DEHT did not induce significant changes in MUFAs.

Table 4. Total lipid (%, fw) and FA profile (%of total Fas, fw) of control and treated pools (i.e., DEHT1 and DEHT100) of M. galloprovincialis. Data are presented as mean ± SD of triplicate analysis.

Analyte (%)	**Test groups**		
	Control	DEHT1	DEHT100
Total lipid	5.52 ± 0.53^{a}	8.75 ± 0.36^{b}	8.41 ± 0.94^{b}
C14:0	2.07 ± 1.36^{a}	2.63 ± 0.22^{a}	2.35 ± 0.19a
C16:0	22.90 ± 0.35^{a}	20.73 ± 0.76^{b}	19.33 ± 0.42^{b}
C17:0	1.21 ± 0.02^{a}	$0.98 \pm 0.04^{a,b}$	0.93 ± 0.08^{b}
C18:0	6.01 ± 0.52^{a}	$4.81 \pm 0.29^{a,b}$	4.67 ± 0.39^{b}
SFA	32.19 ± 0.84^{a}	29.15 ± 0.91^{b}	27.28 ± 0.97^{b}
C16:1 ω-7	4.47 ± 0.11^{a}	5.84 ± 0.25^{b}	6.41 ± 0.19^{c}
C17:1	1.12 ± 0.26^{a}	1.25 ± 0.08^{a}	1.05 ± 0.05^{a}
C18:1 ω-9	5.81 ± 0.24^{a}	3.36 ± 0.15^{b}	3.53 ± 0.25^{b}
C18:1 ω-7	2.23 ± 0.41^{a}	2.30 ± 0.07^{a}	2.20 ± 0.11^{a}
C20:1 ω-11	1.51 ± 0.51^{a}	1.69 ± 0.14^{a}	1.54 ± 0.10^{a}
C20:1 ω-7	0.97 ± 0.06^{a}	1.11 ± 0.08^{a}	1.03 ± 0.05^{a}
C20:1 ω-9	1.87 ± 0.18^{a}	1.84 ± 0.17^{a}	1.60 ± 0.13^{a}
MUFA	16.11 ± 0.54^{a}	17.39 ± 0.14^{b}	17.36 ± 0.01^{b}
C18:2 ω-6	0.92 ± 0.07^{a}	1.01 ± 0.01^{a}	0.96 ± 0.05^{a}
C18:3 ω-6	0.06 ± 0.01^{a}	0.11 ± 0.01^{a}	0.10 ± 0.01^{a}
C18:3 ω-3	0.48 ± 0.04^{a}	0.41 ± 0.04^{a}	0.44 ± 0.04^{a}
C18:3 ω-4	0.18 ± 0.01^{a}	0.27 ± 0.02^{b}	0.29 ± 0.01b
C18:4 ω-3	1.24 ± 0.12^{a}	1.59 ± 0.10^{b}	1.71 ± 0.17^{b}
C20:2 ω-6	0.26 ± 0.03^{a}	0.36 ± 0.04^{a}	0.38 ± 0.03^{a}
C20:3 ω-6	0.18 ± 0.01^{a}	0.14 ± 0.01^{b}	0.31 ± 0.03^{c}
C20:4 ω-6	4.35 ± 0.39^{a}	4.04 ± 0.41^{a}	4.01 ± 0.20^{a}
C20:5 ω-3	12.86 ± 0.47^{a}	14.59 ± 0.67^{b}	14.08 ± 0.15^{b}
C22:5 ω-3	1.26 ± 0.05^{a}	1.35 ± 0.08^{a}	1.40 ± 0.10^{a}
C22:6 ω-3	14.92 ± 0.99^{a}	15.91 ± 0.82^{a}	15.66 ± 0.20^{a}
PUFA	36.71 ± 0.20^{a}	39.88 ± 0.39^{b}	39.34 ± 0.74^{b}
Σω3	30.82 ± 0.32^{a}	33.96 ± 0.14^{b}	33.38 ± 0.47^{b}
Σω6	5.71 ± 0.29^{a}	5.59 ± 0.37^{a}	5.81 ± 0.24^{a}
ω6/ω3	0.18 ± 0.01^{a}	0.16 ± 0.01^{a}	0.17 ± 0.01^{a}

a–c: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter ($p > 0.05$ b y post hoc Tukey's HSD test.

Total protein from control and test mussel pools are described in Table 5. The mean protein content of *M. galloprovincialis* in the control group was equal to 15.95% and decreased after DEHT exposure, as DEHT1 and DEHT100 mussels had respectively mean protein levels of 14.76% and

11.46%. Statistical analysis showed a significant decrease ($p < 0.05$) for the DEHT100 group compared to the control group and for the DEHT1 group compared to the DEHT100 group.

Table 5. Total protein (%, fw) of control and treated pools (i.e., DEHT1 and DEHT100) of *M. galloprovincialis*. Data are presented as mean ± SD of triplicate analysis.

	Test groups		
	Control	DEHT1	DEHT100
Total protein (%)	15.95 ± 1.03^{a}	14.76 ± 0.29^{a}	11.46 ± 0.08^{b}

a–b: different superscript letters in the same row indicate significantly different values for a given parameter (*p* < 0.05 b y post hoc Tukey's HSD test).

After exposure of *M. galloprovincialis* to 1 mg/l and 100 mg/l of DEHT, haemolymph cells showed a high stability of the lysosomal membrane and high viability (≥93%) (Table 6, and 7), thus resulting non significantly different ($p > 0.05$) from the control group (≥95%). Similar considerations could be made for DG cells coming from control mussels and organisms exposed to 1 mg/l of the plasticizer (≥97%, $p > 0.05$). DG cells from DEHT1 mussels showed a significantly lower viability respect to the control group after staining with NR (i.e., 98.5% vs. 97.1%, $p < 0.05$), and there is also significance between the two exposure groups DEHT1 and DEHT100 staining with NR (i.e., 97.1% vs. 97.9%, $p < 0.05$).

Table 6. Effects of DEHT1 and DEHT100 exposure on the viability of haemolymph and digestive gland (DG) cells of *Mytilus galloprovincialis* assessed by the Trypan Blue exclusion method. Data are expressed as % of viable cells (mean ± SD of ten duplicates analysis).

Trypan Blue exclusion method (%)	Test groups		
	Control	DEHT1	DEHT100
Haemocytes	95.4 ± 2.1^{a}	93.6 ± 4.9^{a}	93.6 ± 4.9^{a}
DG Cells	97.2 ± 0.5^{a}	98.0 ± 0.3^{a}	97.5 ± 0.4^{a}

a–c: different superscript letters in the same row indicate significantly different values for a given parameter (p < 0.05 b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter (p > 0.05 b y post hoc Tukey's HSD test).

Table 7. Effects of DEHT1 and DEHT100 exposure on the viability of haemolymph and digestive gland (DG) cells of Mytilus galloprovincialis assessed by the Neutral red retention assay. Data are expressed as % of viable cells (mean ± SD of ten duplicates analysis).

Neutral red retention assay (%)	Test groups		
	Control	DEHT1	DEHT100
Haemocytes	96.9 ± 1.6^{a}	97.0 ± 2.9^{a}	96.9 ± 2.9^{a}
DG Cells	98.5 ± 1.5^{a}	97.1 ± 1.8^{b}	97.9 ± 1.4^{a,c}

a–c: different superscript letters in the same row indicate significantly different values for a given parameter (p < 0.05 b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter (p > 0.05 b y post hoc Tukey's HSD test).

Under physiological conditions, DG cells *M. galloprovincialis* can regulate their volume in the presence of hypotonic solution, by swelling and gradually returning to their original volume over time. In Figure 5, DG cells in the control group slowly reached their maximum volume after 10 min of exposure to the hypotonic solution.

Conversely, DG cells from exposure groups (DEHT1 and DEHT100) showed a lower degree of swelling and did not return to their initial volume. Specifically, the final volume of DEHT1 cells was even higher than their initial volume, and the volume of DEHT100 cells was even lower than control cells.

Overall, statistical analysis showed that DEHT1 and DEHT100 mussels has a significantly lower ability to regulate their DG cell volume with respect to the control condition ($p < 0.05$). Moreover, this ability appeared to be significantly more impaired with increasing exposure levels.

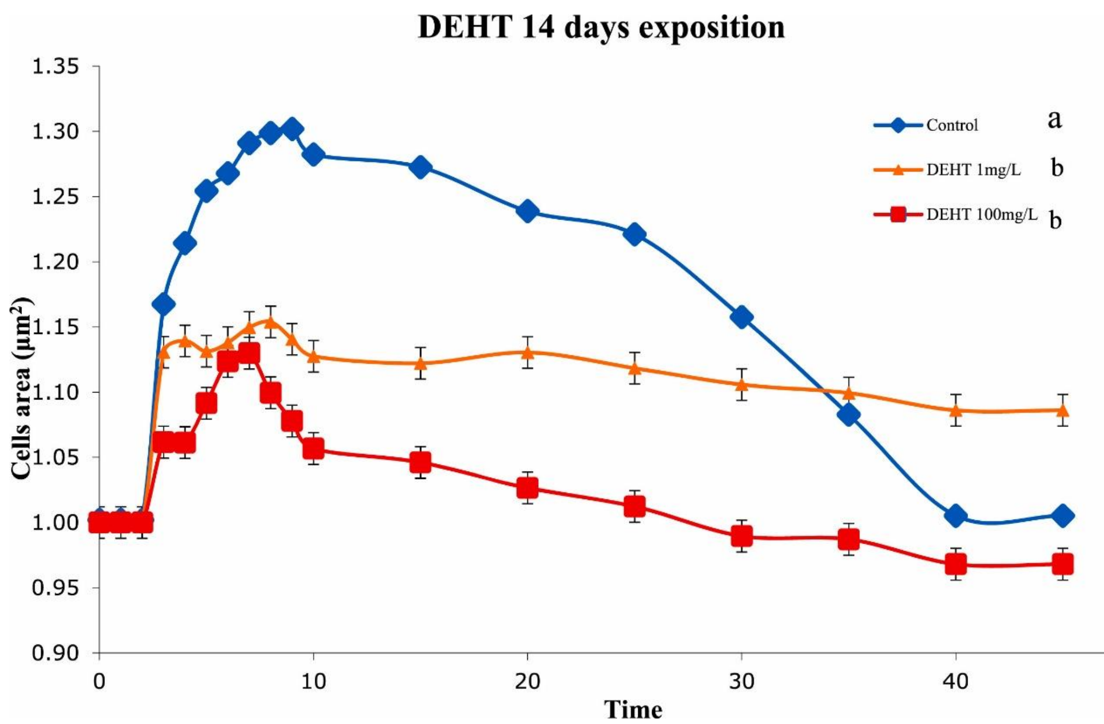

Figure 5. Effects of DEHT1 and DEHT100 exposure on the regulatory volume decrease of DG cells of *Mytilus galloprovincialis*. Data are expressed as mean cells area ±SD of triplicate measurements. A–b: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter ($p > 0.05$ b y post hoc Tukey's HSD test).

The activity of antioxidant enzymes, as well as the levels of oxidative products, are typically used as indexes of an altered oxidative homeostasis, thus reflecting the general health status of an organism[50]. Accordingly, the activity of SOD and the levels of LPO and OMP in gills and DG of *M. galloprovincialis* exposed to the plasticizer were measured and reported in Figure 6, and 7. In both gills and DG, SOD activity showed a slight, not significant ($p > 0.05$), concentration-dependent increase in DEHT-exposed animals (gills: ctrl = 31.27 U/mg prot, DEHT1 = 33.67 U/mg prot, DEHT100 = 38.61U/mg prot; DG: ctrl = 14.05U/mg prot, DEHT1 = 16.71U/mg prot, DEHT100 = 16.05 U/mg prot). No differences were observed in the levels of TBARS, and aldehydic and ketonic derivatives, in the three experimental conditions.

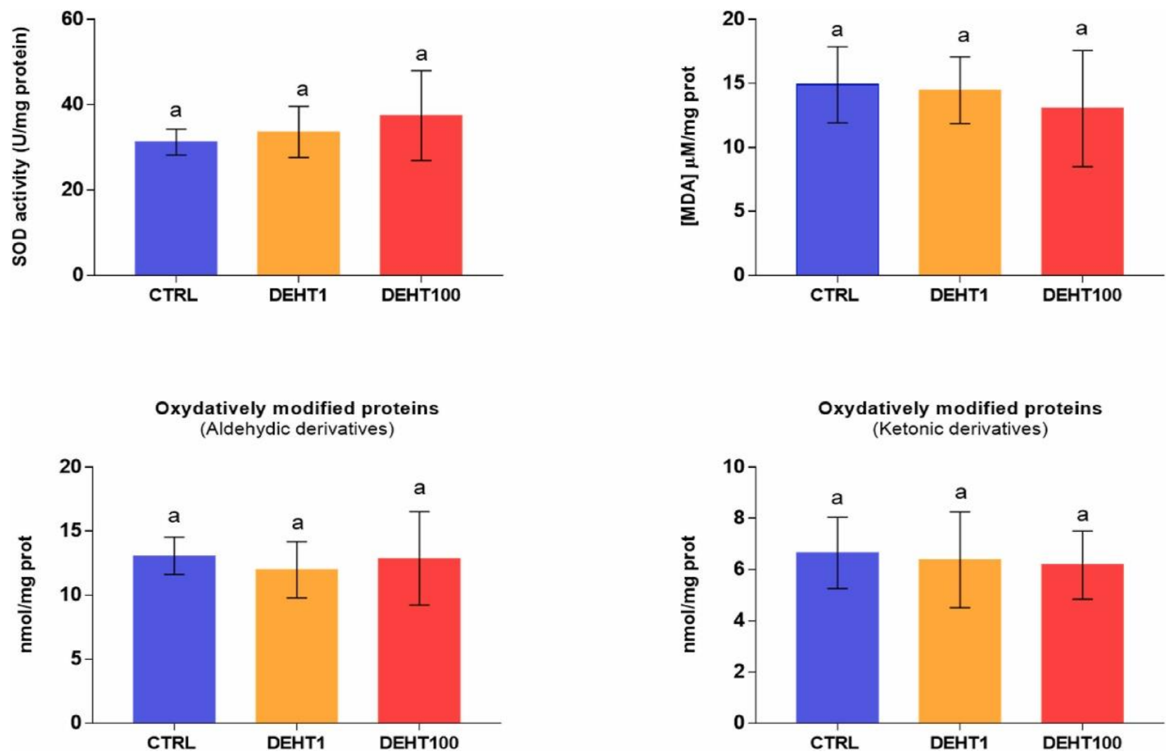

Figure 6. Effects of DEHT1 and DEHT100 exposure on the SOD activity, TBARS, and OMP of the gills of *M. galloprovincialis*. Data are expressed as mean ± S.E. of absolute values of individual experiments performed in duplicate.

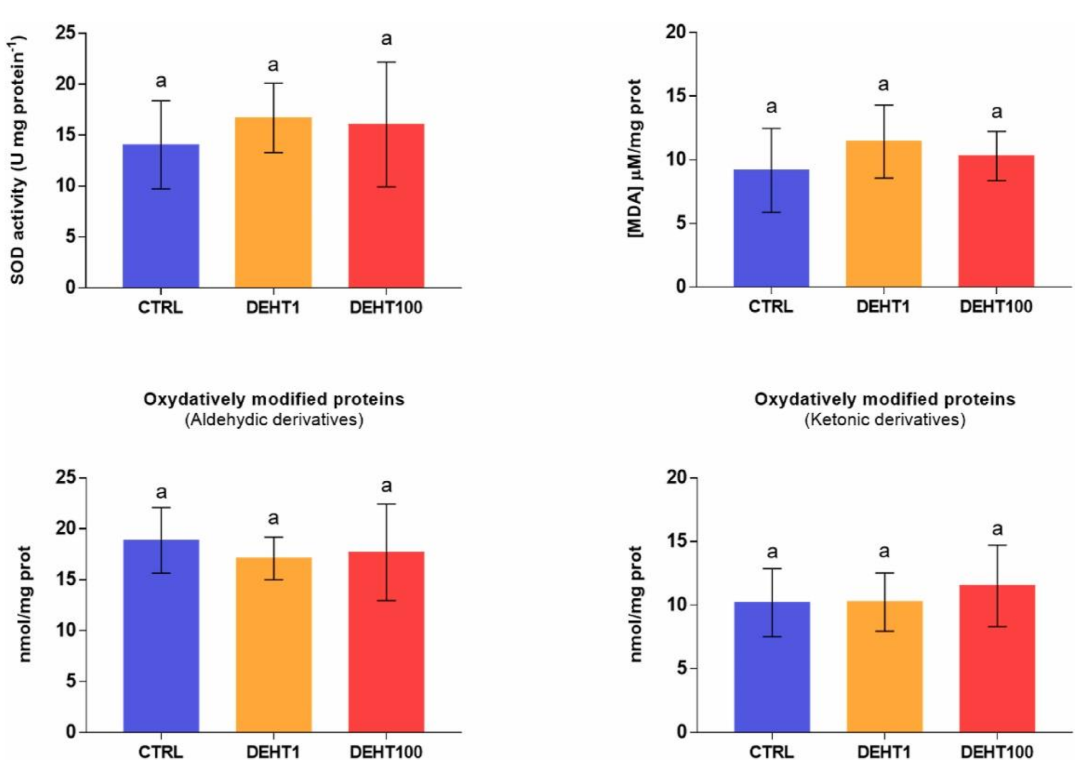

Figure 7. Effects of DEHT1 and DEHT100 exposure on the SOD activity, TBARS, and OMP in the DG of *M. galloprovincialis*. Data are expressed as mean ± S.E. of absolute values of individual experiments performed in duplicate.

7.2.9. Discussion

Due to the high production volumes and numerous applications, plastics of various sizes have been detected in a wide range of aquatic environments and have already been shown to bioaccumulate through the food chain, thus, causing adverse toxic effects. In this scenario, additives, such as PAEs and NPPs, represent emerging pollutants that are intrinsically linked to plastic pollution, but have not yet been adequately addressed for the range of sublethal effects they can induce in aquatic organisms, including the sentinel *M. galloprovincialis.*

In this study, we demonstrated for the first time that the NPP DEHT can bioaccumulate in *M. galloprovincialis* and consequently induce alterations in lipid and protein metabolism and certain physiological processes, without affecting oxidative stress processes. Although defined as a not-highly persistent and accumulative plasticizer, the first evidence of DEHT bioaccumulation in aquatic organisms dates back to a study by Liu et al.[52], which found very low levels of DEHT in the tissues of Chinese water snake and common carp from a pond polluted with e-waste (3.2 μg/kg and 12 μg/kg respectively).[52]

More recently, DEHT was revealed at higher concentrations in Mediterranean coastal waters (range 0.25–0.93 mg/g, and range 0.68–1.21 mg/l) and sediments (range:0.67–2.86 mg/kg dw), as well as in biota. Indeed, the sea grass *Posidonia oceanica* (0.32–11.1 mg/kg dw)[15], the gilthead sea bream (14.9–37.7 mg/kg, dw), the shark *Hexanchus griseus* (liver: 2.40 mg/kg dw, Salvo et al., 2020) and *M. galloprovincialis* (0.58–5.68 mg/kg dw) reasonably bioaccumulated such NPP at higher concentrations than abiotic matrices. Clearly, these previous field studies highlighted much lower DEHT levels than those found in mussels exposed to 1 mg/l and 100 mg/l of DEHT under laboratory conditions (respectively, 123.69 mg/kg and 595.13 mg/kg on a fw basis), but they still give an idea of the ubiquitous presence of this plasticizer also in the Mediterranean area, and the urgent need to explore its toxic effects on aquatic life at environmental realistic concentrations.

Macronutrients, such as lipids and proteins, are involved in many vital functions of aquatic organisms. They have already proved to be useful trophic markers for tracing predator-prey relationships and defining the food webs of the marine environment[53], since some of them can only be obtained from peculiar food resources (e.g., essential nutrients such as essential FAs). However, they were claimed to be also good biomarkers of ecosystem health and indicators of stress in response to xenobiotic exposure. On this basis, results from this study confirmed that DEHT exposure caused evident alterations in lipid of *M. galloprovincialis*. Although lipids vary depending on the diet and the surrounding environmental conditions from which the mussel comes, specimens from the control group showed total lipid and FA profile quite comparable to those displayed in previous works on mussels from the same collection site, especially with respect to the major SFAs, MUFAs and PUFAs. However, after laboratory exposure to both experimental DEHT concentrations, mussels were characterized by an increase in total lipids and their FA profile showed peculiar variations depending on the FA class (i.e., decreased SFAs, increased PUFAs, and unchanged MUFAs). The interpretation of these findings is a challenging task, since, to the best knowledge of the authors, there has been no previous attempt to investigate lipid and FA profile changes after exposure of aquatic organisms to DEHT.

However, similarly to this study, Henderson and Sargent[54] pointed out that the rainbow trout fed with a DEHP-based diet (2%) showed a reduction in the content of palmitic acid in liver, muscle and adipose, as well as an increase in the eicosapentaenoic and docosahexaenoic acids and a slight decrease in arachidonic acid in the same tissues. Despite the decrease observed in the content of palmitic acid, the crustacean body showed an increase in the percentage level of palmitoleic acid, eicosapentaenoic and docosahexaenoic acids.[55]

Overall, it may be speculated that, similarly to PAEs, also NPPs may impact the organism's lipidome by altering the expression of genes involved in lipid metabolism (e.g., desaturase and elongase genes) and stress response.[56,57]

For example, bivalves are capable of the de novo synthesis of certain FAs, namely non-methylene-interrupted fatty acids (NMIFAs), such as the dihomo-γ-linolenic acid (C20:3 ω-6) which is synthesized from γ-linolenic acid (C18:3 ω-6) by activating the elongase activity. On this basis, the increase in the dihomo-γ-linolenic acid reported in mussels exposed to 100 mg/l of DEHT could be due to a potential impairment of the elongation pathway induced by the NPP plasticizer. However, according to our results, organisms exposed to an environmental concentration of DEHT (1 mg/l) showed levels of such FA comparable to the control mussels, probably due to the well-known phenomenon of hormesis.[58]

DEHT exposure induced significant change also in the protein metabolism of *M. galloprovincialis*. Mussels under physiological conditions (control group) showed a much lower protein content than that reported in literature for other Mytilus species. For example, M. edulis from China had total protein varying from 42.23% to 45.62%, although on a dw basis. No other studies were found on the variation of protein content in *M. galloprovincialis*; the study reported by Chi et al.[59] represents a point of comparison that is distant from ours, both in terms of species and origin of the study. However, total protein tended to progressively decrease from the control group to DEHT100 group, with intermediate levels found in DEHT1 mussels.

Similarly, to lipid, the comparison of our data with previous literature is somewhat troublesome, since, to the best knowledge of the authors, there are no previous studies focusing on protein changes after exposure of aquatic organisms to DEHT.

However, similarly to this study, *Daphnia magna* exposed to a concentration of DEHP ranging from 0 to 811 µg/l show a variable decrease in protein content, ranging from 241 µg/daphnia to 128 µg/daphnia. To better understand the health status of the model organism, cell viability tests are performed on haemolymph and digestive gland cells[60].

The uptake of NR by bivalve hemocytes occurs through mechanisms such as pinocytosis or passive diffusion across cell membranes. This phenomenon is often used to assess the effect of stressors on the stability of lysosomal membranes in bivalve cells. Indeed, lysosomes are of great importance,

and extensive research has been devoted to investigating their role as a target organelle for monitoring the aquatic environment in which these animals live. Effective regulation of this process is likely to contribute to the ability of certain organisms to tolerate stressful and polluted environments. A cell is considered viable if lysosomal stability and viability exceed 80%. Viability tests conducted on mussels exposed to DEHT showed that there was a slight decrease in viability, although this was not significant. Only digestive gland cells showed high significance ($p \leq 0.01$) in the DEHT100 group compared to the control.

The study by Choi and co-workers[61] examined the exposure of these organisms to polyethylene terephthalate microfibres and showed a dose-dependent increase in the presence of apoptotic and necrotic haemocytes in exposed mussels compared to controls. This is an indication that the presence of plasticizers influences haemocytes, as in our case, since they are the first cells involved in cellular immune responses. Furthermore, exposure to microplastics >0–80 μm and to polystyrene microplastics showed that there is a significant reduction in lysosomal membrane stability, as in our case, evidenced by NR staining, ranging from 99% in the control to 97%, with a significance of $p \leq 0.01$.

The trypan blue dye exclusion test is used to determine the number of viable cells in a cell suspension. It relies on the fact that living cells have intact cell membranes that prevent certain dyes, like trypan blue, from entering, while dead cells do not. However, in our case, the trypan blue method data showed no significant changes in cell viability among the tested groups, with an average viability of over 90%. This finding is not uncommon, as another study investigating the impact of microplastics (polystyrene, 35–50 μm) on our experimental model also found no significant changes in cell viability after trypan blue staining[60].

It is worth noting that although the integrity of the cell membrane may remain intact, the cell's overall viability, including its ability to grow or function, could still be compromised.

The DG of *M. galloprovincialis* plays a critical role in the absorption and processing of essential nutrients needed for the animal's survival.

The hepatopancreas will therefore be the organ most exposed to xenobiotics and it is consequently susceptible to damage. Exposure to terephthalate reduces the ability of these cells to regulate their own cell volume when exposed to a hypotonic solution. Even at the environmental concentration of DEHT, the cells could not reach the maximum volume achieved by the control group, nor could they return to their original volume, remaining with an area greater than 1 (exactly 1.09 μm2), significance $p < 0.05$. Even the cells exposed to the 100-fold higher concentration showed an irregular pattern and their volume was significantly reduced at the end (0.97 μm^2) compared to the control group ($p < 0.01$). These tests assess the ability of cells to regulate their volume in response to a hypotonic solution, which is important for determining cell functionality and health.

Similar tests have been conducted on other contaminants in the aquatic environment, such as pesticides, fungicides, heavy metals, additives and pharmaceuticals, to confirm how these substances can affect cell functionality.

Marine bivalves dispose of an efficient antioxidant defence system which allows them to face oxidative stress when exposed to pollutants. In *M. galloprovincialis*, the influence of plastics pollutants on antioxidant enzyme activity, as well as on the expression levels of oxidative biomarkers is, to date, poorly investigated.

The few available studies suggest effects which strictly depend on the specific substance used, along with the intensity and duration of the treatment. Data mainly refer to changes in the activity of antioxidant enzymes, such as Glutathione S-transferases (GST) and Catalase (CAT), while information on lipid peroxidation and protein carbonylation are lacking or even absent. In cells of the digestive gland from *M. galloprovincialis*, exposure to either bisphenol A (BPA), or perfluoro octane (PFOS), induces a different modulation of GST and CAT[62].

Similarly, exposure to sub-lethal concentration of plastic leachates revealed the capacity of various polymers to differently affect the activity of antioxidant enzymes, as well as the levels of lipid peroxidation in gills and DG of *M. galloprovincialis*.

A recent paper by Andreyeva and coworkers[19] reports that in the DG of M. galloprovincialis the exposure to DEHP (4.0 mg/l) did not influence SOD activity following 24 h exposure period but induced a 1.8-fold increase after 48 h of treatment. In our experimental conditions, the exposure to either 1 mg/l or 100 mg/l of DEHT did not affect the activity of the SOD enzyme in gills and DG of *M. galloprovincialis*, as well as the levels of oxidative products.

This allows us to hypothesise that at the concentrations and the exposure time of DEHT used in this study, *M. galloprovincialis* does not suffer from oxidative stress. The possibility that the basal levels of antioxidant enzymes are enough to avoid a DEHT-induced oxidative status needs to be further investigated.

7.2.10. Conclusion

In conclusion, this study highlights the negative effects of DEHT exposure on the sentinel species *Mytilus galloprovincialis*, including changes in physiological and biochemical parameters, as well as alterations in nutritional parameters such as fatty acid profiles, total lipids, and total proteins. The presence of terephthalate in Mediterranean waters and biota is increasing, which is concerning as it can accumulate in organisms higher up in the food chain, such as humans.

This emphasizes the urgent need to reduce plastic pollution in our aquatic ecosystems and to better understand its impact on marine life and ultimately on human health. As plasticizers can alter the nutritional value of seafood, which is an important source of nutrients for humans, this kind of pollution can have significant implications for human health.

Further studies will undoubtedly be necessary, including on other species, to better understand the toxic effects of this compound, not only on its own, but also in combination with other chemical compounds, because unfortunately there is no single plasticizer in the water, but a variety that can act in synergy or in opposition to each other.

7.3. VARIATIONS IN FATTY ACID COMPOSITION OF MEDITERRANEAN ANCHOVIES (ENGRAULIS ENCRASICOLUS) AFTER DIFERENT COOKING METHODS

In this research anchovies (*Engraulis encrasicolus*) were analysed for a comparative studies of fatty acid content after the application of five different cooking treatments (boiling, marinating, frying, roasting, and boiling). In particular, the aim was evaluated what cooking method among those commonly used for fish cooking, is overall healthier on the base of the preserved n-3 long-chain PUFA amounts.

7.3.1. Samples

In this study, a total of three kilograms of fresh anchovies were purchased in May 2021 from a local fish market located in Sciacca, Sicily, Italy. The anchovy samples had an average weight of 8.50±0.90 g, an average length of 11.05±0.40 cm. From the carefully eviscerated samples, six pools of 300 g of edible portion were formed. Each pool was divided into three samples of 100 g each for a total of 18 samples. One portion was analysed as a raw sample; the others were subjected to cooking. The samples were processed using the different cooking techniques described in next section. Three replicates were performed for both the raw sample and each cooking treatment.

Different cooking treatments were selected as common anchovies cooking procedures used by consumers. These were steaming, boiling, marinating, frying, and roasting. For the steaming treatment: the anchovies were cooked in a steamer for 5 min. For boiling treatment: the anchovies were immersed in 500 mL of cold water and slowly brought to 95 °C for 3–4 min. For marinating treatment: the anchovies were entirely immersed in lemon juice (pH value of 2. 3) for about 30 min. For frying treatment: the anchovies were immersed in extra virgin olive oil in a pan for 4 min at 180 °C. For roasting treatment: the anchovies were cooked on electric grill for 10 min at 200 °C. Cooking temperatures were monitored with a digital probe. After cooking, the samples were allowed to cool to room temperature, and then the fillets were collected and intended to the subsequent analyses.

7.3.2. Fatty acids analysis

Total lipid extraction was performed by Folch method[63]. Samples (10 g) were weighed and homogenized with 2:1 chloroform: methanol (v/v) mixture, and then a 0.73% NaCl water solution was added. Samples were vortexed and centrifuged at 1000 rpm for 15 min at 4 °C using a refrigerated centrifuge. The lipid fraction was taken and subjected to hot esterification with 9:1 methanol: sulfuric acid (v/v) mixture in an oven for one hour at 110 °C. The supernatant was recovered and diluted to 1:2 with hexane. A gas chromatograph (GC) equipped with a split/splitless injector and a fame ionization detector (FID) (Dani Master GC, Dani Instrument, Milan, Italy) was used to analyse the methylated lipid samples containing the fatty acid methyl esters (FAMEs) using the method suggested by Tropea et al.[64]

A Supelco SLB-IL100 capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm, Supelco, Sigma Aldrich, USA) was used. The following operating conditions were used: column oven temperature of 180 °C to 270 °C (holding time 2 min) at 3 °C/min; injector and detector temperatures were set to 220 °C and 240 °C, respectively; helium was at a linear velocity of 30 cm/s (constant). The makeup gases were N_2, H_2, and air at a constant linear velocity of 25.0 mL/min, 40 mL/min, and 280 mL/min, respectively. The injection volume was 1 µL, with a split ratio of 1:50. Data acquisition and management were performed using Clarity Chromatography v4.0.2 software. All samples were analysed in triplicate along with analytical blanks. FAMEs of nutritional interest were identified by direct comparison with the reference retention times of the compounds present in the standard Supelco 37-component FAME mix. The individual percentage of FAMEs was calculated relative to the total chromatogram area.

7.3.3. Statistical analysis

All statistical calculations were made by SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL, USA). The studies of significant differences were carried out by Kruskal–Wallis tests.

Principal Component Analysis (PCA) was carried out to try to discriminate among uncooked and cooked samples using the parameters that best described the variability of the data.

7.3.4. Results and discussions

Fatty acid distribution Table 1 shows the fatty acid composition of the uncooked and cooked anchovy analysed samples. Only fatty acid content above 0.1% is reported. The predominant fatty acids were palmitic acid (C16:0), DHA and EPA in raw anchovy. These results agree with those obtained by Zotos et al.[65] and in partial with those obtained by Turhan et al.[66]

The same trend was observed in steamed, boiled, marinated, and roasted anchovy, whereas the predominant fatty acids were oleic (C18:1n-9), palmitic and linoleic (C18:2n-6) acids in fried anchovy. Frying causes an extensive absorption of fat; thus the fatty acids composition of fried fish tends to that of the fat used. The C18:1n-9 and C18:2n-6 fatty acids contents in fried samples increased from 9.33±0.53% to 54.34±0.45%, and from 1.88±0.31% to 9.03±0.43%, respectively. This is probably due to the fact that olive oil itself has a signifcant content of this fatty acid. Compared to uncooked sample, a significant decrease in polyunsaturated fatty acids and, specifically, in EPA and DHA content was observed in fried samples. EPA decreased at 1.71±0.1% (85% of the initial value), and DHA decreased at 7.56±0.24% (66% of the initial value).

In the other cooking techniques, the percentage concentration of these two fatty acids remains constant. This apparent decrease, however, does not affect the nutritional quality of the fried product, as explained later. For statistical analysis one starting multivariate matrix was built using as variables the 38 parameters listed in Table 8 and as cases the 18 analysed samples.

The data were subdivided into six groups according to the cooking method. The variables were normalized and then a Factor Analysis by Principal Components extraction was performed to fnd a potential discrimination among samples. Using the Kaiser Criterion, four principal components with eigenvalues exceeding one (26.296, 6.460, 2.643, and 1.320) were extracted; those explained the 69.199%, 17.000%, 6.954%, and 3.473% of total variance, respectively. The 248igni component

shows the highest positive correlation with C14:0 (0.995), C20:5n-3 (0.993), C22:6n-3 (0.991), and ∑n-3 (0.990), while negative correlations can be observed for MUFA (− 0.997), C18:1n-9 (− 0.996) and n-6: n-3 ratios (− 0.985). The dominant variables in the second component are C20:3n-6 (0.894) and C20.0 (− 0.891). In Fig. 1, the 2D Scatterplot for the 18 samples under analysis is showed.

As can been seen, groups of fried and roasted anchovies are clearly distinguished, whereas the groups of uncooked, steamed, and boiled anchovies overlap each other. Marinated anchovies are also very close to the latter but distinguishable. The fried samples showed negative PC1, and they were characterized by higher C18:1n-9, C18:2n-6, MUFA, ∑n-6, and n-6: n-3 ratios.

Samples of roasted anchovies showed negative PC2; so, these samples show the highest C20:0 and the lowest C20:3n-6 concentrations. Samples of uncooked, steamed, and boiled anchovies had positive PC1 and PC2; as a result, these samples show the highest C18:3n-3 and C18:4n-3 contents. Marinated samples had the same PC1 scores of uncooked, steamed, and boiled samples, but had little negative PC2 scores; so, their fatty acids profile is different principally for the highest C24:0 content.

Table 8. Fatty acid composition (% of total FA) in cooked and uncooked Engraulis encrasicholus

	Raw	Steamed	Boiled	Marinated	Roasted	Fried
C12:0	0.18 ± 0.03 (B)	0.19 ± 0.04 (B)	0.12 ± 0.06 (AB)	0.12 ± 0.03 (AB)	0.17 ± 0.02 (B)	0.01 ± 0.00 (A)
C14:0	7.45 ± 0.42 (B)	7.47 ± 0.28 (B)	6.97 ± 0.10 (B)	8.24 ± 0.08 (B)	7.59 ± 0.51 (B)	0.72 ± 0.07 (A)
C15:0	1.15 ± 0.05 (B)	1.43 ± 0.35 (B)	0.63 ± 0.05 (AB)	1.09 ± 0.05 (B)	0.53 ± 0.07 (A)	0.17 ± 0.07 (A)
C16:0	22.80 ± 0.61 (A)	22.62 ± 0.55 (A)	22.33 ± 0.33 (A)	26.18 ± 0.32 (B)	24.12 ± 0.39 (B)	18.24 ± 0.35 (A)
C17:0	0.77 ± 0.10 (B)	0.66 ± 0.06 (B)	0.32 ± 0.05 (A)	0.68 ± 0.04 (B)	0.37 ± 0.09 (AB)	0.21 ± 0.02 (A)
C18:0	3.56 ± 0.25 (AB)	3.28 ± 0.31 (AB)	4.54 ± 0.10 (B)	4.20 ± 0.06 (B)	4.42 ± 0.33 (B)	2.42 ± 0.30 (A)
C20:0	0.14 ± 0.03 (A)	0.34 ± 0.08 (AB)	0.16 ± 0.02 (A)	0.34 ± 0.04 (AB)	1.50 ± 0.29 (B)	0.34 ± 0.08 (AB)
C21:0	0.22 ± 0.03 (B)	0.21 ± 0.02 (B)	0.11 ± 0.03 (AB)	0.13 ± 0.02 (AB)	0.01 ± 0.00 (A)	0.01 ± 0.00 (A)
C22:0	0.19 ± 0.03	0.19 ± 0.03	0.16 ± 0.03	0.16 ± 0.04	0.14 ± 0.02	0.19 ± 0.03
C23:0	0.14 ± 0.02 (B)	0.13 ± 0.02 (B)	0.01 ± 0.01 (A)	0.14 ± 0.04 (B)	0.01 ± 0.00 (A)	0.01 ± 0.00 (A)
C24:0	0.84 ± 0.07 (A)	0.84 ± 0.05 (A)	0.78 ± 0.10 (A)	2.08 ± 0.16 (B)	1.67 ± 0.31 (B)	0.36 ± 0.06 (A)
∑ SFA	37.50 ± 1.04 (AB)	37.42 ± 0.31 (AB)	36.19 ± 0.21 (A)	43.42 ± 0.25 (B)	40.58 ± 0.21 (B)	22.74 ± 0.46 (A)
C14:1	0.31 ± 0.05 (B)	0.26 ± 0.06 (A)	0.27 ± 0.06 (AB)	0.24 ± 0.04 (AB)	1.67 ± 0.09 (B)	0.02 ± 0.00 (A)
C15:1	0.19 ± 0.04 (B)	0.14 ± 0.02 (B)	0.16 ± 0.02 (B)	0.18 ± 0.05 (B)	0.27 ± 0.06 (B)	0.01 ± 0.00 (A)
C16:1	7.00 ± 0.16 (B)	6.83 ± 0.10 (B)	7.56 ± 0.31 (B)	6.71 ± 0.14 (B)	5.76 ± 0.24 (A)	2.45 ± 0.07 (A)
C17:1	0.22 ± 0.02 (B)	0.23 ± 0.03 (B)	0.26 ± 0.07 (B)	0.25 ± 0.03 (B)	0.05 ± 0.00 (A)	0.01 ± 0.00 (A)
C18:1n9	9.33 ± 0.53 (B)	8.91 ± 0.66 (B)	9.30 ± 0.16 (B)	9.18 ± 0.17 (B)	9.57 ± 0.28 (B)	54.34 ± 0.45 (A)
C20:1n9	0.68 ± 0.14 (A)	0.82 ± 0.08 (A)	0.78 ± 0.10 (A)	1.25 ± 0.08 (B)	1.39 ± 0.16 (B)	0.48 ± 0.07 (A)
C22:1n9	0.48 ± 0.13 (A)	0.52 ± 0.11 (A)	0.57 ± 0.09 (AB)	0.64 ± 0.04 (B)	1.18 ± 0.25 (B)	0.01 ± 0.00 (A)
C24:1n9	0.07 ± 0.02 (A)	0.12 ± 0.03 (AB)	0.15 ± 0.03 (B)	0.18 ± 0.01 (B)	0.01 ± 0.00 (A)	0.01 ± 0.00 (A)
∑ MUFA	18.28 ± 0.48 (B)	17.84 ± 0.55 (B)	19.04 ± 0.50 (B)	18.64 ± 0.25 (B)	19.90 ± 0.69 (A)	57.33 ± 0.44 (A)
C18:2n6	1.88 ± 0.31 (B)	2.17 ± 0.21 (B)	1.91 ± 0.21 (B)	1.41 ± 0.33 (B)	1.54 ± 0.30 (B)	9.03 ± 0.43 (A)
C18:3n6	0.32 ± 0.05 (B)	0.32 ± 0.03 (B)	0.24 ± 0.03 (AB)	0.26 ± 0.03 (AB)	0.17 ± 0.04 (A)	0.08 ± 0.01 (A)
C18:3n3	1.75 ± 0.12 (B)	1.57 ± 0.15 (B)	1.80 ± 0.07 (B)	0.58 ± 0.05 (A)	0.69 ± 0.08 (AB)	0.72 ± 0.11 (AB)
C18:4n3	3.16 ± 0.35 (B)	3.41 ± 0.34 (B)	2.75 ± 0.16 (B)	1.19 ± 0.06 (AB)	0.14 ± 0.03 (A)	0.21 ± 0.02 (A)
C20:2n6	1.63 ± 0.19 (B)	1.60 ± 0.13 (B)	1.52 ± 0.10 (B)	0.25 ± 0.04 (A)	1.33 ± 0.06 (AB)	0.06 ± 0.02 (A)
C20:3n6	0.15 ± 0.03 (B)	0.11 ± 0.02 (B)	0.09 ± 0.01 (B)	0.13 ± 0.03 (B)	0.01 ± 0.00 (A)	0.06 ± 0.02 (AB)
C20:3n3	0.12 ± 0.04 (AB)	0.14 ± 0.02 (B)	0.13 ± 0.02 (B)	0.05 ± 0.00 (A)	0.31 ± 0.16 (B)	0.01 ± 0.01 (A)
C20:4n6	0.88 ± 0.06 (AB)	0.88 ± 0.05 (A)	0.84 ± 0.11 (A)	0.91 ± 0.04 (B)	1.41 ± 0.38 (B)	0.20 ± 0.01 (A)
C20:5n3	11.39 ± 0.56 (B)	11.30 ± 0.25 (B)	11.57 ± 0.48 (B)	11.36 ± 0.11 (B)	11.73 ± 0.76 (B)	1.71 ± 0.10 (A)
C22:2	0.85 ± 0.03 (B)	0.89 ± 0.03 (B)	0.74 ± 0.03 (AB)	0.73 ± 0.05 (A)	0.73 ± 0.09 (A)	0.29 ± 0.06 (A)
C22:6n3	22.09 ± 0.35 (B)	22.34 ± 0.20 (B)	23.18 ± 0.27 (B)	21.08 ± 0.24 (B)	21.45 ± 0.72 (B)	7.56 ± 0.24 (A)
∑ PUFA	44.22 ± 1.52 (B)	44.74 ± 0.62 (B)	44.77 ± 0.59 (B)	37.94 ± 0.12 (A)	39.52 ± 0.67 (AB)	19.93 ± 0.89 (A)
∑ n3	38.51 ± 1.23 (B)	38.77 ± 0.25 (B)	39.43 ± 0.66 (B)	34.26 ± 0.35 (B)	34.32 ± 0.59 (B)	10.21 ± 0.42 (A)
∑ n6	4.86 ± 0.28 (B)	5.08 ± 0.38 (B)	4.60 ± 0.10 (B)	2.95 ± 0.30 (B)	4.47 ± 0.17 (B)	9.43 ± 0.46 (A)
n6/n3	0.13 ± 0.00 (B)	0.13 ± 0.01 (B)	0.12 ± 0.00 (B)	0.09 ± 0.01 (B)	0.13 ± 0.00 (B)	0.92 ± 0.02 (A)
AI	0.86 ± 0.05 (B)	0.85 ± 0.01 (B)	0.80 ± 0.01 (B)	1.06 ± 0.01 (B)	0.93 ± 0.03 (B)	0.27 ± 0.01 (A)
TI	0.25 ± 0.01 (B)	0.25 ± 0.01 (B)	0.24 ± 0.00 (B)	0.31 ± 0.00 (A)	0.29 ± 0.00 (AB)	0.33 ± 0.02 (A)

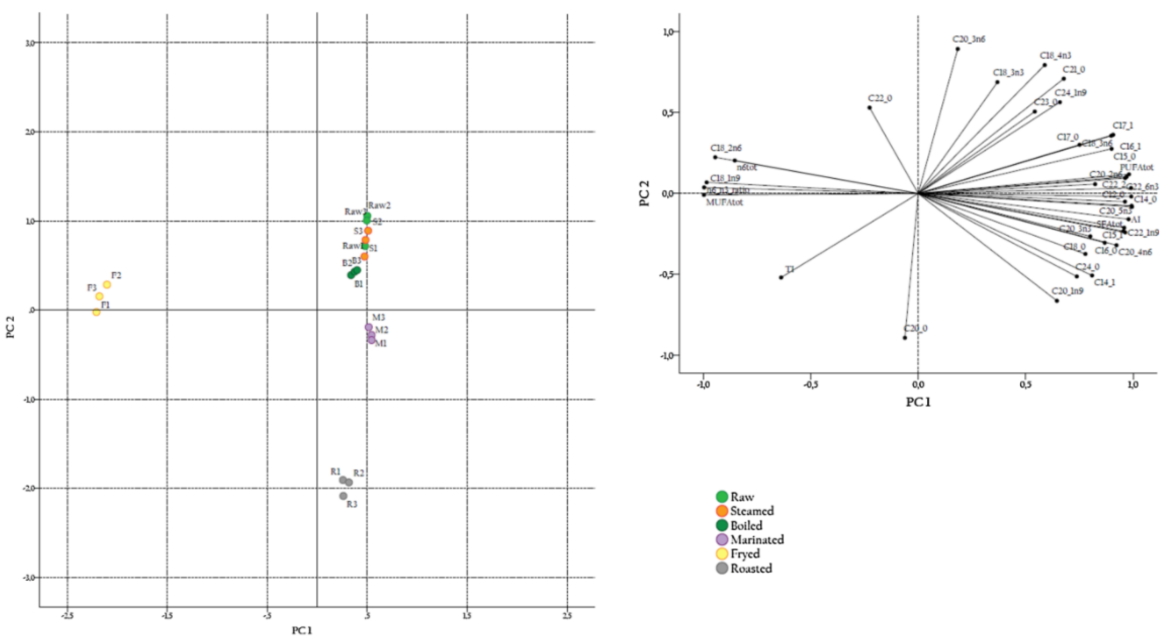

Figure 8. 2D Scatterplots for the 18 anchovy samples categorized by 251ignifica cooking methods. Insert: loading plot for PC1 and PC2

7.3.5. Lipid quality indices and fatty acid intake

A comparison between the atherogenicity index (AI) and thrombogenicity index (TI) values of uncooked and cooked anchovy is shown in Table 1. The AI and TI values in the uncooked sample were 0.86±0.05 and 0.25±0.01, respectively. It is assumed that low values of AI and TI (<1) are beneficial to human health. In cooked samples, all AI and TI indices were below 1, except for AI in marinated samples where it was slightly exceeding the value of 1 (1.06± 0.01). The obtained results indicate that the change in fatty acid profile of anchovy due to frying in olive oil however do not significantly modify the thrombogenicity index that remained at satisfactory level, and decreased the value of atherogenicity index, highlighting that frying, if done with olive oil, does not produce a product low in nutritional quality. Considering the total lipid content and the quantity of the fatty acid that are important for their nutritional value (C18:2n-6, C18:3n-3, ∑n-6, ∑n-3, EPA + DHA) in each of the six groups, fatty acids intakes were calculated (Table 9). It is recommended to eat fsh at least twice a week. Each serving of fish should be about 85 g. This consumption of anchovies

assures a good intake of ∑n-3 and of EPA + DHA for all cooking methods considered. The same consumption of fried anchovies also provides a good intake of C18:2n-6 and ∑n-6. These results indicate that olive oil does not lead to a decrease in the quality of fried anchovies related to fatty acids profle.

Table 9. Lipid Yield and percentage contribute to recommended value for nutritional fatty acid in cooked and uncooked *Engraulis encrasicholus*.

	Raw	Steamed	Boiled	Marinated	Roasted	Fried
Lipid content (g/100 g)	10.50 (A)	10.00 (A)	10.00 (A)	9.10 (A)	9.30 (A)	35.0 (B)
C18:2n-6	0.48 (A)	0.53 (A)	0.46 (A)	0.31 (A)	0.35 (A)	7.67 (B)
C18:3n-3	0.41 (A)	0.39 (A)	0.29 (A)	0.28 (A)	0.20 (A)	0.35 (A)
∑ n-3	61.38 (A)	58.85 (A)	59.84 (A)	47.32 (A)	48.45 (A)	54.24 (A)
∑ n-6	1.94 (A)	1.93 (A)	1.75 (A)	1.02 (A)	1.58 (A)	12.52 (B)
EPA + DHA	341.50 (A)	326.85 (A)	337.60 (A)	286.77 (A)	299.82 (A)	315.29 (A)

7.3.6. Conclusions

The fatty acid profile of uncooked a cooked Mediterranean anchovy (*Engraulis encrasicolus*) is influenced by cooking methods. Uncooked, steamed and boiled anchovies had the highest C18:3n-3 and C18:4n-3 contents; marinated and roasted anchovies had the highest C24:0 and C20:0 content, respectively; the fried samples showed the higher C18:1n-9, C18:2n-6, MUFA, ∑n-6, and n-6: n-3 ratios values, and the lower EPA and DHA concentrations. All samples appeared to be a good dietary source of beneficial fatty acids. In particular, also samples fried with olive oil maintained a high-level nutritional quality.

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Chapter 8

MEAT RESEARCH LINE

Food of animal origin can have value if it is associated with the geographical origin, breed or production system from which it is derived, especially if it emphasises animal welfare and environmental sustainability. Indeed, consumers are generally willing to pay a higher price for products with a clearly defined geographical origin, such as Protected Designation of Origin (PDO) brands[1]. Production systems specific to the Mediterranean area include the use of local breeds that are extensively grazed and slaughtered at an advanced age to obtain high quality matured products. One example is pigs, whose performance, carcass composition and quality of meat and meat products depend on many factors, such as genotype and rearing conditions, in both intensive (indoor) and outdoor systems[2]. Particularly in the outdoor system, vegetation typicality and seasonality can confer unique characteristics.

The “Nero dei Nebrodi” pig is an autochthonous breed from Sicily, characterised by its small size and dark, bristly coat, with a straight frontal-nasal profile and small ears pointing obliquely upwards with the tips carried horizontally forward. These animals are also characterised by a high degree of rusticity and are mainly reared outdoors in the Nebrodi area, a hilly and mountainous zone that reaches an altitude of 1.847 m. This breed has been the subject of both genomic[3,4] and growth performance evaluations, as well as meat and fat quality in indoor and outdoor rearing[5]. After the demographic decline suffered by the “Nero dei Nebrodi” pig up to 15 years ago, conservation and development programmes were developed to prevent the extinction of this native breed, which currently has a population of about 900 sows (450 sows in 2010)[6]. The animals are mainly bred in Sicily, in 127 farms out of a total of 128, where these pigs are reared in the open air for traditional and social reasons rather than for strictly commercial purposes.

In particular, 104 farms are located in the Nebrodi area, where 78.38% (685 sows) of the 874 sows reared in Sicily are concentrated. A few years ago, the Sicilian Regional Ministry of Agriculture set up a promotion committee to apply to the Ministry of Agricultural, Food and Forestry Policies (MASAF) for recognition of the Protected Designation of Origin (PDO) for fresh meat and its processed products. The granting of a geographical indication mark certifies a name registered with the European Community to designate a typical product of high quality whose area of origin and the traditions still practiced in its production make it so distinctive that it must be protected against counterfeiting[1]. Indeed, food traceability studies are currently focusing on the correlation between the geographical origin of food and its chemical composition through chemometric tools[7].

However, although the presence of the 'Nero dei Nebrodi' pig is historically documented there is little information on how the rearing area may contribute to the quality of the meat and, as far as we know, no study has evaluated the composition of stable isotopes and mineral elements in 'Nero dei Nebrodi' meat as a traceability tool.

As part of this line of research, the following scientific paper has been produced in collaboration with the Research and Innovation Centre of the Edmund Mach Foundation, which carries out scientific research, develops technologies and promotes innovation in the fields of agriculture, bioeconomy, ecology, biodiversity, environment and food:

- **Litrenta, F.**, Cavallo, C., Perini, M., Pianezze, S., Lo Turco, V., Di Bella, G., Liotta, L. (2024). ASSIGNING THE GEOGRAPHICAL ORIGIN OF “NERO DEI NEBRODI” PIG MEAT AND ANIMAL REARING SYSTEM USING CHEMICAL AND ISOTOPIC FINGERPRINTS submitted in the journal *Journal of Food Composition and Analysis.*

8.1. ASSIGNING THE GEOGRAPHICAL ORIGIN OF “NERO DEI NEBRODI” PIG MEAT AND ANIMAL REARING SYSTEM USING CHEMICAL AND ISOTOPIC FINGERPRINTS

In this study, the chemical-nutritional parameters, stable isotope composition, fatty acid and sterol profile and mineral elements were correlated with the geographical origin of 'Nero dei Nebrodi' *longissimus dorsi* meat from pigs reared in two different geographical areas of north-eastern Sicily. The aim was to establish a correlation between 'Nero dei Nebrodi' meat and its geographical origin, with a view to using these correlations for traceability and protecting consumers against fraud and commercial disputes.

8.1.1. Animals, study area and sampling

This study was carried out on samples of commercial meat purchased directly from the farm shop. Therefore, the study did not involve animal experimentation or the introduction of farming practices other than those normally used, and it was not necessary to seek the assessment and approval of an ethics committee. The study was conducted in two geographical areas of northern Sicily in the province of Messina: Mirto Areale, inside the Nebrodi National Park and Valle del Mela Areale, outside the Nebrodi National Park. A total of 20 female pigs aged 12 months were randomly divided into two homogeneous groups, namely: the 'Nebrodi group' (NG) and the 'External Nebrodi group' (ENG). The animals came from the same commercial farm but from different litters and were weaned 90 days after birth. The NG group remained on the farm of origin, in Mirto Areale, while the ENG group was transferred to a farm outside the Nebrodi area. As can be seen in Figure 1, Mirto Areale lies in the heart of the Nebrodi National Park; the Nebrodi Mountains are part of the Sicilian Apennines, facing the Tyrrhenian Sea to the north, while their southern limit is marked by Mount Etna. The main elements that characterise the natural landscape of Nebrodi are the dissymmetry of the two faces, the rich vegetation, and the wetlands. The Valle del Mela Areale, outside the Nebrodi Natural Park, is located on the western slopes of the Peloritani Mountains and offers a rich variety of landscapes.

The study was conducted during the finishing phase, from June 2022 to January 2023. All pigs were housed under the same conditions in a traditional outdoor system in the two geographical areas mentioned above; the diet was mainly based on natural pasture with some supplementation of cereals (mainly barley and never corn) and broad beans during periods of low pasture availability. All pigs had *ad libitum* access to water throughout the study. Approximately 24 hours after slaughter (20 ± 2 months of age; 110 ± 10 kg), a meat sample corresponding to the *longissimus dorsi* (LD) between the 10th and 14th ribs was taken from the left half-carcass of each animal. All samples were vacuum-packed and shipped to the laboratory at a controlled temperature of 4-5°C within 2 hours of collection. Each muscle sample weighed approximately 300 g. Once at the laboratory, samples were trimmed of fat, minced, homogenised and analysed in triplicate.

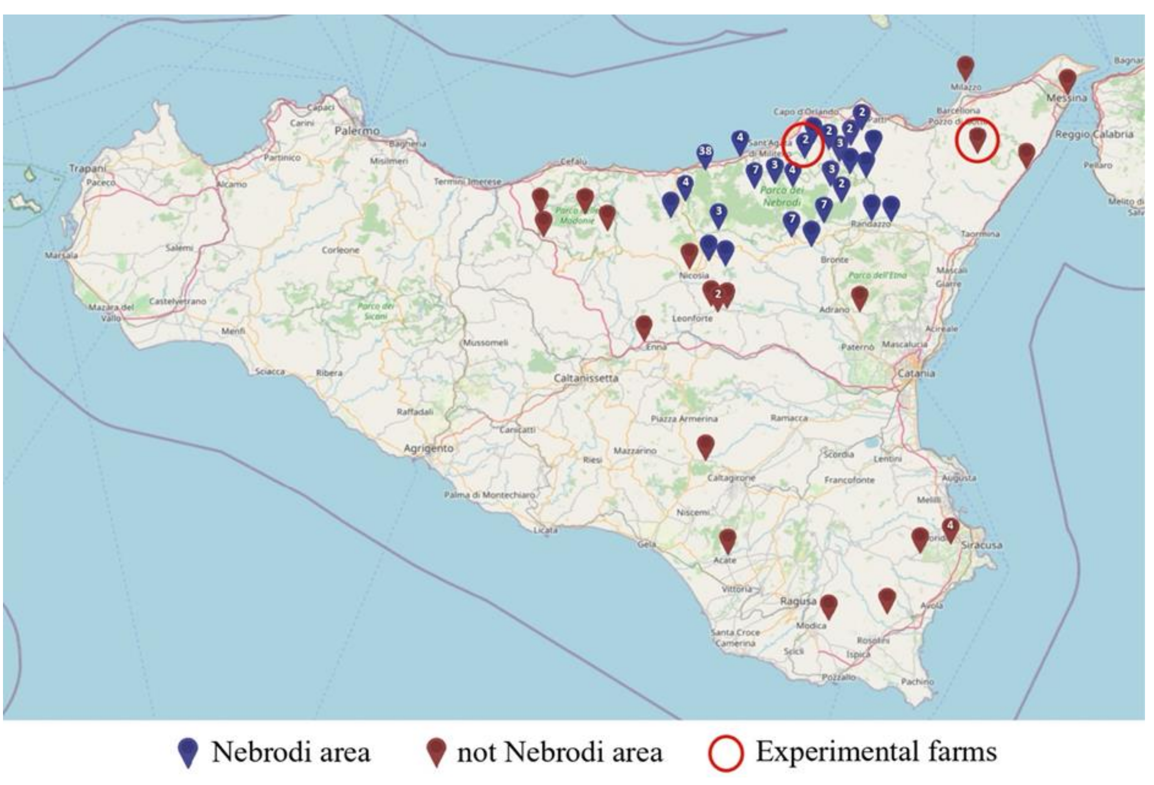

Figure 1, Map of Sicily illustrating the geographical location of all “Nero dei Nebrodi” pig farms and two farms considered in this study.

8.1.2. Chemical composition analysis

Each LD sample was homogenized individually after removal of the fat cover. Moisture and protein were determined in triplicate for each sample[8]. Intramuscular fat (IMF) was instead determined by extraction with chloroform/methanol (2:1 v/v) according to the rapid solvent extraction method described by Folch et al[9].

8.1.3. Stable isotope analysis

The samples were frozen or cooled during transport to the respective isotope laboratories. The meat was cut, minced, and lyophilized with a 5PASCAL (Trezzano sul Naviglio MI, Italy) freeze dryer model LIO5P DIGITAL. Afterwards, the samples were defatted with a soxhlet apparatus using petroleum ether as reported by Perini et al.[10]

The fat-free residue (or dry mass) was collected and saved for the C, N, S, O and H analyses.

All samples were weighed into silver and tin capsules for OH− and CNS−isotope measurements, respectively. The 2H/1H and 18O/16O ratios were measured using an isotope ratio mass spectrometer (IRMS) (Finnigan DELTA XP, Thermo Scientific, Bremen, Germany) coupled with a pyroliser (Finningan DELTA TC/EA, high-temperature conversion elemental analyser, Thermo Scientific). Before analysis, the silver capsules containing the samples or standards were kept for >24 h in a vacuum desiccator with silica. The 13C/12C, 15N/14N and 34S/32S were measured using an isotope ratio mass spectrometer (IsoPrime, Isoprime Limited, Langenselbold, Germany) after total combustion in an elemental analyser (VARIO CUBE, Isoprime Limited).

The measured isotope ratios are reported in the delta (δ) notation corresponding to the relative deviations of the molar ratio (R) of the heavy elements (i.e., 13C, 2H, or 18O) to light elements (i.e., 12C, 1H, or 16O) isotopes in the samples from those in international standards V−PDB (Vienna−Pee Dee Belemnite) for δ13C, V−SMOW (Vienna−Standard Mean Ocean Water) for δ18O and δ2H, V−CDT (Vienna−Canyon Diablo Troilite) for δ34S, and Air (atmospheric N2) for δ15N, as shown in the following equation:

$$\delta^h E_{sample} = \left[\frac{R(^hE/^lE)_{sample}}{R(^hE/^lE)_{standard}}\right] - 1$$

The delta values are here multiplied by 1000 and expressed in the more common unit "per mil" (‰) rather than, as required by the International System of Units (SI), in milliurey units (mUr).[11]

The measured values (i.e., 13C, 2H, and 18O values based on working gas cylinders of CO2, H2, and CO, respectively) were normalized to the VPDB-LSVEC and VSMOW-SLAP scales using three- or four-point calibration with international reference materials (RMs). The standards that have been used in the isotopic analyses were international reference materials or in-house working standards that have been calibrated against them. In particular, the international standards that have been used are: for 13C/12C, fuel oil NBS−22 (δ13C) = −30.03 ± 0.05‰), sucrose IAEA−CH−6 (δ13C) = −10.45 ± 0.04‰) (IAEA−International Atomic Energy Agency, Vienna, Austria), and L−glutamic acid USGS 40 (δ13C) = −26.39 ± 0.04‰) (U.S. Geological Survey, Reston, VA, USA); for 15N/14N, L−glutamic acid USGS 40 (δ15N) = −4.52 ± 0.06‰) (U.S. Geological Survey, Reston, VA, USA), ammonium sulfate salts IAEA−N−1 (δ15N) = +0.43 ± 0.07‰) and IAEA−N−2 (δ15N) = +20.41 ± 0.12‰) and potassium nitrate IAEA−NO3 (δ15N) = +4.7 ± 0.2‰); for 34S/32S, USGS 42 (δ34S) = +7.84 ± 0.25‰), USGS 43 (δ34S) = +10.46 ± 0.22‰), Barium sulphate IAEA−SO−5 (δ34S) = +0.5 ± 0.2‰) and NBS 127 (δ34S) = +20.3 ± 0.4‰); for 2H/1H and 18O/16O fuel oil NBS−22 (δ2H = −119.6 ± 0.6‰) and Keratins CBS (Caribou Hoof Standard δ2H = −157 ± 2‰ and δ18O = +2.4‰± 0.1‰) and KHS (Kudu Horn Standard δ2H = −35 ± 1‰ and δ18O =+21.2 ± 0.2 ‰) from U.S. Geological Survey.

Each reference material was measured in duplicate at the start and end of each daily group of analyses of samples (each sample was also analyzed in duplicate).

A control sample was also included in the analyses of each group of samples to check the efficiency of the measure. The maximum standard deviations of repeatability accepted were 0.3‰ for δ13C and δ15N, of 0.4‰ for δ34S, 0.5‰ for δ18O and of 3‰ for δ2H.

8.1.4. Fatty acid and sterols content

The lipid fraction was collected and subjected to hot esterification with a 9:1 methanol: sulphuric acid (v/v) mixture in an oven at 110°C for one hour. The supernatant was collected and diluted 1:2 with hexane and analysed on a gas chromatograph (GC) equipped with a split/splitless injector and a flame ionisation detector (FID) (Dani Master GC, Dani Instrument, Milan, Italy) according to the method proposed in the section 7.3.

Aliquots of the lipid extracts were saponified with a solution of ethanol and potassium hydroxide after the addition of the internal standard (5α-cholesterol). The unsaponifiable fraction was extracted with ethyl ether. The sterol fraction was separated by chromatography on silica gel. This fraction was analysed by gas chromatography after derivatisation with derivatising reagent BSTFA: TMCS (99:1) to obtain the corresponding trimethylsilyl ethers. The extraction was carried out according to the EU Regulation 1348/2013[12].

8.1.5. Analyses of mineral elements

Mineral elements screening (Ca, Na, Mg, Mn, Fe, Zn, Be, Co, Cr, Cu, Mo, Ni, Sb, Sn, Pb, Cd and As) was performed using optimised method described in detail in section 5. The analysis was performed with a quadrupole ICP-MS iCAP-Q (Thermo Scientific, Waltham, MA, USA). Briefly, muscle aliquots of 0.5 g each were digested with 7 mL HNO_3 and 2 mL H_2O_2 using the Ethos 1 microwave digestion system (Milestone, Bergamo, Italy). All samples were analysed in triplicate together with analytical blanks and data acquisition was performed using Qtegra™ Intelligent Scientific Data Solution (Thermo Scientific™). Each analyte was quantified by constructing a six-point calibration curve (R2 between 0.9984 (Na) and 0.9999 (Cd)). Triplicate measurements together with analytical blanks were performed for each sample. The ICP-MS procedure was analytically validated in terms of linearity, limit of detection (LOD) and limit of quantification (LOQ), accuracy, intra- and inter-assay variability, as reported in detail in section 5.

Quality control of the analysis was achieved by measurement of the blank and the certified reference material 'ERM®- BB184 Bovine Muscle' (Joint Research Centre Institute for Reference Materials and Measurements, Belgium), prepared under the same conditions as the samples. The certified reference material contained the following mineral elements: Ca, Mg, Na, As, Cd, Cu, Fe, Mn, Zn. The matrix was fortified with a known amount of these analytes if the element was not certified in the reference material, as in the case of Be, Co, Cr, Mo, Ni, Sb, Sn and Pb.

8.1.6. Statistical analysis

Statistics were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). There were 20 cases (samples analysed) and 80 variables (all parameters analysed) in an initial multivariate matrix. The data were divided into two groups according to the area of origin (NG inside the park and ENG outside the park). First, the significance of the differences between the groups was assessed using the non-parametric Mann-Whitney U test.

Then, the data set was normalized in order to obtain the independence of the scale factors of the different variables. Only those variables that were found to be significantly different ($p < 0.05$ and $p < 0.01$ depending on the variable) between the two groups were used. After verifying the adequacy of the initial data using the Kaiser-Meyer-Olkin (KMO) test and Bartlett's test, a factor analysis with principal component extraction (PCA) was carried out to try to differentiate the samples according to area of origin.

8.1.7. Results and discussions

The chemical composition and sterol profile of the samples analysed are shown in Table 1. Moisture and protein contents were significantly different between groups ($p < 0.05$). Moisture was significantly higher in the ENG samples than in the NG samples. Both values are in line with those found in other studies, e.g. Ren et al.,[13] found moisture values between 69.65% and 72.00% in Queshan black pigs and Yorkshire pigs, respectively, while Kim et al.[14] found average values of

70% in native Korean black pigs. The protein content was significantly higher in NG samples with a mean value of 23.08%. The lipid content was on average lower in NG than in ENG samples, but the differences were not statistically different ($p > 0.05$). However, the results obtained are in line with those of other authors.[13,14,15]

The sterol profile is also shown in Table 1 and is very interesting; in addition to cholesterol, brassicasterol, campesterol, campestanol, Δ-5,24-stigmastanol, Δ-7-stigmastanol, Δ-7-avenasterol were found in all samples analysed. The cholesterol content of the two groups was not statistically different, with a mean value of 56.96 mg/100 g in the NG samples and 63.55 mg/100 g in the ENG samples. In contrast, brassicasterol, campesterol, campestanol and Δ-5,24-stigmastanol were significantly higher in the NG samples ($p<0.05$).

Phytosterols play an important role in immune regulation and exert positive effects on disease resistance and anti-inflammatory activity and may benefit animal health and growth.

Son et al.,[16] evaluated the effect of plant sterol supplementation on growth performance and biochemical indicators in pigs and found that phytosterols effectively improved pig growth performance and nutrient digestibility, as well as their blood lipid status. Furthermore, another study conducted by researchers Naji et al.,[17] evaluated the effects of dietary phytosterols on growth and gene expression in broiler muscle and concluded that the administration of phytosterols was a good dietary programme for adequate morphological development of pectoral muscle.

The mountain flora of Nebrodi is particularly rich in regional endemisms[18] such as the wild brassica (*Brassica montana* and *Brassica rupestris*), the Nebrodi broom (Genista aristata), the pug thistle (Carlina nebrodensis), the Boccone hellebore (Helleborus bocconei) and the Boccone oak (Quercus gussonei), which are a source of valuable biomolecules for the animals bred in the area. The results of our study could be related to the rich wild vegetation in the Nebrodi area, which has a positive influence on meat quality.

Table 1. Chemical composition and sterol profile in muscle samples from the two geographical areas of origin: NG "Nebrodi group", ENG "External Nebrodi group".

Item	NG	ENG	SEM[1]	*p-value*[2]
Moisture %	66.94	70.46	0.01	**0.027**
Proteins %	23.08	20.88	0.01	**0.001**
Lipids %	4.30	6.08	0.09	0.269
Sterols profile (mg/100g)				
Cholesterol	56.96	63.55	0.03	0.215
Brassicasterol	0.54	0.07	0.26	**0.000**
Campesterol	0.42	0.17	0.12	**0.000**
Campestanol	0.09	0.05	0.10	**0.009**
Δ-5,24-Stigmastanol	0.11	0.07	0.11	**0.009**
Δ-7-Stigmastanol	0.06	0.05	0.12	0.964
Δ-7-Avenasterol	0.04	0.05	0.11	0.685

[1]SEM (Standard Error of Mean)
[2]Bold values are significant at $p<0.05$

Table 2 shows the results relating to the analysis of the stable isotopes ratios of carbon, nitrogen, sulphur, oxygen, and hydrogen of pig samples raised in both NG and ENG areas. The defatted meat δ13C and δ15N resulted statistically different ($p<0.01$) when comparing NG and ENG animals, revealing a difference in the composition of their diet.

Table 2. Isotopic ratios of defatted meat samples (‰) divided into two homogeneous groups: the 'Nebrodi group' (NG) and the 'External Nebrodi group' (ENG).

Item	$\delta^{13}C$ (‰, vs V-PDB)	$\delta^{15}N$ (‰, vs AIR)	$\delta^{34}S$ (‰, vs V-CDT)	$\delta^{2}H$ (‰, vs V-SMOW)	$\delta^{18}O$ (‰, vs V-SMOW)
NG					
Mean	-22.8	6.3	-0.1	-86	14.3
SD	0.4	0.3	0.5	2	0.4
ENG					
Mean	-23.9	7.5	-0.3	-78	15.6
SD	0.2	0.7	0.4	2	0.6

Carbon isotopic composition is mainly correlated with plant photosynthetic cycles, according to which they can be classified into three distinct groups: C3, C4 and CAM plants.[19,20]

C3 plants, which represent about 85% of the planet's plant species, use the Calvin cycle to fix carbon via the enzyme Rubisco. These plants have δ13C oscillating between -33‰ and -23‰. On the other hand, both C4 and CAM plants evolved alternative photosynthetic pathways to meet the challenges of hot and arid environments by reducing water loss through stomatal evapotranspiration. C4 plants fix CO2 through an enzyme other than Rubisco, producing oxaloacetic acid, from which they take their name, and have δ13C ranging between -14‰ and -12‰. Finally, CAM plants have δ13C values intermediate between C3 and C4 and adopt a water-saving mechanism known as Crassulaceae acid metabolism (CAM), which temporarily separates CO2 fixation from sugar synthesis[21].

In the analysed samples the reported values (average -22.8‰ for NG and -23.9‰ for ENG) are compatible with a diet mainly based on C3 plants, the representative vegetation of the sampling area. In the two breeding sites, the animals had the same composition supplements based on germinated barley (C3) with no C4 ingredient (such as corn) included. A possible explanation for the higher δ13C of the NG group could be represented by the exclusive presence of some C4 plants in the Nebrodi area vegetation, as reported by Tuttolomondo et al..[18]

These include the Amaranthus genus (e.g. cruentus, hybridus, hypocondriacus, retroflexus), ingested by grazing animals along with other plants. The presence of C4 species in the Nebrodi area exclusively can result in the discrimination between their meat and other competitor products, based on the δ13C.

The δ15N and δ34S of defatted meat are correlated with the same parameters of fodder plants consumed by animals. Also, it has long been known that the δ15N of animal tissues is higher than the δ15N of the diet they consume (trophic level effect)[22]. Plant and soil δ15N values are generally correlated, being the former more negative than the latter. The difference between the parameters (δ15Nplant-δ15Nsoil) increases with decreasing mean annual temperature (MAT)[23]. While no

fractionation processes occur to sulphur between the soil and the plant roots, resulting in no differences between their δ34S, leaves and grains (for instance, wheat ones) are enriched up to 2‰ compared to soil sulphate. Different factors influence soil nitrogen and sulphur isotopic composition. In the present study, the effect of fertilization (chemical or organic), which is reported to strongly affect both δ15N and δ34S can be excluded, since we are dealing with pastures where it is not applied.[24] As plants absorb nitrogen from the soil through the roots, their δ15N will reflect the value of the soil (average values ranging between -2‰ and +10‰). Leguminous plants opt for an alternative way to absorb nitrogen, picking it up directly from the air through the leaves. Therefore, the δ15N of these plants will reflect the air value (around -7‰). Different concentrations of leguminous plants were probably present in NG and ENG diets, providing a possible explanation for the difference between the nitrogen values ($p<0.05$) of their meat (Table 2). Among Nebrodi area exclusive endemic plants are the legumes Genista aristata C. Presl and Trifolium bivonae Guss. (Fam. Leguminosae).[25]

The plants δ34S are mainly influenced by the geology of the soil on which the plant grew (for example, the presence of sulfates or sulfides in the soil and the type of underlying local bedrock), as well as the proximity to the sea (sea-spray effect), since seawater sulphates reaching the coast have δ34S values of 21–22‰ approximately.[26] Volcanic, igneous rocks have a δ34S value close to 0‰, while values diverging from 0‰ indicate rocks containing sulphur of sedimentary, biological or (bio) chemical origin (Tcherkez and Tea 2013). The δ34S signal is transferred from plants to animals and it is not altered in food chains.[27]

The reported correlation between δ34S value of biomass and primary sulphur sources is only valid for bulk materials and not for individual compounds or tissues. The δ34S values measured in the defatted meat of the sample group ENG and NG are not significantly different ($p>0.05$). This probably reflects a certain homogeneity in the geological composition of the soil (Calabride Units, pre-Paleozoic and Paleozoic crystalline basement nappes sutured by a syntectonic terrigenous deposit) as already demonstrated by Lentini et al..[28]

The distance from the sea (about 6 Km) is also the same for the two sampling sites, excluding a different influence of the sea-spray effect. The hydrogen and oxygen isotopic composition of animal defatted meat is related to the feed and water the animal ingeste.[27] The hydrogen isotopic composition of intracellular water (resulting in the defatted meat one) depends on extracellular water hydrogen (81%) and intracellular metabolic hydrogen (19%).[29]

The δ2H of the defatted meat fraction is mainly influenced by the δ2H of the plants the animals have been consuming, while Perini et al.,[10] demonstrated that only approximately 30% of it depends on the isotopic composition of the drinking water. The δ18O isotope ratio mainly depends on the water source the animals have been drinking, be it groundwater (tap water), surface water or feed water (for example, water in pasture grass). The relatively high local temperature and humidity increase the soil/plant evapotranspiration, which leads to 2H and 18O enrichment in plant tissues (i.e., higher 2H and 18O values) and consequently in animal products with respect to the primary source, represented by the rainfall. Rainfall figures as the ultimate factor of influence for plants and drinking water δ2H and δ18O.

According to the literature, the main variability factors determining rainfall δ2H and δ18O are the temperature effect, also correlated to the altitude (the colder the air temperature, the lower the isotopic composition of precipitation) and the continental effect (the greater the distance from the coast, the more depleted the precipitation), resulting in "heavier" precipitation on the coast with respect to the inland. While the δ2H and δ18O of rain and of water resulting from the evapotranspiration of plants are essentially the same, the water contained in fresh food (e.g. grass) is significantly enriched in its isotopic composition compared to the groundwater the plant absorb[29].

In the absence of direct measurement of δ2H and δ18O on rainwater in the sampling sites, the Water Isotope database was used. On the site http://wateriso.utah.edu monthly weighted average precipitation data are available for sites around the world. The GPS coordinates and the altitude in meters above sea level of the two pig breeding locations were entered into the portal so as to obtain the expected δ2H and δ18O values of the rainwater in those sampling sites. The NG site of Mirto

(ME) presents rainwater average values of -6.3‰ for δ18O and -38‰ for δ2H, while the ENG site of Valle del Mela of -5.6‰ for δ18O and -34 ‰ for δ2H. These rainfall δ18O and δ2H values were significantly lower ($p<0.05$) for the Nebrodi park (mean +14.3‰ and -86‰ respectively) compared to the external sampling site ENG (mean +15.6‰ and -78‰ respectively), which seems to justify the results obtained for meat δ18O and δ2H. The correlation between the isotopic composition of the rain and the proteinic animal fraction is therefore confirmed and it allows to discriminate the samples coming from the two studied areas.

To the best of our knowledge, no works in the literature reported isotopic values of pigs raised in Sicily, probably due to the fact that this is not a typical intensively farmed animal of this region (as it happens in other Italian regions, such as Friuli). In a recent study on cattle raised on open air, values similar to those found in our study are reported, with averages for the δ2H isotopic ratio (-87 ± 3.5‰).[30] Meat quality is closely related to the fatty acid composition of IMF. Table 3 shows the fatty acid profile of the samples analysed and as can be seen, most of the fatty acids are statistically different in the two groups. Regarding the fatty acid classes, SFA, MUFA, PUFA, n3, n6 and n6/n3 were statistically different in the two groups. Specifically, SFA and MUFA were higher in the NG samples (55.65% vs 42.37%) compared to the ENG samples where PUFA, n3, n6 and n6/n3 were higher (8.15% vs. 14.10%, 0.89% vs. 1.02%, 6.05% vs. 12.96%, 6.91% vs. 12.85%, respectively). Among the SFAs, the most abundant fatty acid in both groups was C16:0, followed by C18:0, but the differences between the two groups in these fatty acids were not significant ($p > 0.05$). The fatty acids C12:0, C15:0, C17:0, C22:0, C24:0 was significantly lower in the NG. The LD muscle lipids of the NG samples were characterised by the highest content of total monounsaturated fatty acids, of which C18:1 n-9 fatty acid represented 41.49%. This higher percentage of oleic acid could be related to both the higher content of this fatty acid in the diet and the higher Δ9-desaturase activity in the adipose tissue of pigs.[31] The higher MUFA content in the NG group was due to the higher content of the following fatty acids: C16:1, C18:1, C20:1 and C22:1 of the n-7 and n-9 series, which were significantly higher in the NG samples ($p < 0.05$). This result was particularly interesting as

there is a correlation between the above fatty acids and the rearing environment in the NG group. As shown by several studies, the Nebrodi area has a high level of plant biodiversity and is considered one of the most important and interesting naturalistic areas in the Mediterranean[18]. As mentioned above, Tuttolomondo et al.,[18] in a study on the widespread use of wild plant species for medicinal purposes in the Nebrodi Regional Park, highlighted the presence of many species' endemic to this area. It is the *Brassicaceae* that caught our attention, as another study by Barthet et al.[32] identified the n-7 and n-9 fatty acids C16:1, C18:1, C20:1 and C22:1 in 12 Brassica species. In our study, as shown in Table 3, these n-7 and n-9 fatty acids were always significantly higher in the Nebrodi group. This is probably due to the fact that the animals in this area feed on the very rich and widespread wild vegetation. In contrast, the ENG samples had significantly higher PUFA contents (14.10%). The most common PUFAs were C18:2 n-6, C20:2 n-6, C18:3 n-3, C20:3 n-6. Nevrkla et al.,[33] state that a higher proportion of fatty acids in pig meat, mainly PUFAs, increases the palatability of the meat. In our study, therefore, it could be deduced that meat from pigs of the Nebrodi group (which have a significantly higher PUFA content, see Table 3) is more appreciated by consumers. ICP-MS represents one of the most advanced and accurate analytical methods used for multi-element determinations. The mineral element content of animal meat is influenced by the diet, reflecting the composition of the soil, pasture and drinking water and correlating the results with the geographical origin, as highlighted by several authors[34,35]. The mineral content of the muscle samples, expressed in mg/kg fresh meat, is shown in Table 4. Seventeen mineral elements were sought. Concentrations of Mg, Na, Ca, Fe and Zn were found in all muscle samples. Among the macro-elements, Mg and Ca were significantly higher in the NG samples than in the ENG samples ($p<0.05$). The most representative element was Mg, with a mean concentration of 2940.79 mg/kg in the NG samples and 2724.97 mg/kg in the ENG samples. Data from the literature show that Mg sources have a beneficial effect on some aspects of pig production[36]

Table 3. Fatty acids composition expressed as % of total FA in muscle samples. [1]SEM (Standard Error of Mean); [2]Bold values are significant at p<0.05

	Fatty acids	NG	ENG	SEM[1]	p-value[2]
SFA	C6:0	0.01	0.01	0.07	0.146
	C8:0	0.03	0.01	0.17	**0.002**
	C10:0	0.12	0.13	0.06	0.689
	C12:0	0.09	0.25	0.14	**0.000**
	C14:0	1.48	1.23	0.03	**0.005**
	C15:0	0.05	0.13	0.12	**0.000**
	C16:0	23.57	23.88	0.01	0.755
	C17:0	0.18	0.36	0.09	**0.000**
	C18:0	9.65	10.91	0.02	0.057
	C20:0	0.15	0.22	0.07	0.081
	C22:0	0.03	0.11	0.16	**0.000**
	C23:0	0.04	0.04	0.13	0.123
	C24:0	0.03	0.10	0.15	**0.001**
MUFA	C14:1	0.04	0.01	0.18	**0.011**
	C16:1 n-9	0.24	0.14	0.07	**0.000**
	C16:1 n-7*	3.99	2.16	0.11	**0.001**
	C17:1	0.27	0.17	0.09	**0.008**
	C18:1 n-9	41.49	34.95	0.02	**0.000**
	C18:1 n-7*	4.05	1.95	0.09	**0.000**
	C20:1 n-7*	3.35	1.61	0.12	**0.003**
	C20:1 n-9	1.83	0.29	0.32	**0.000**
	C22:1 n-7*	0.10	0.03	0.16	**0.000**
	C22:1 n-9	0.06	0.01	0.22	**0.000**
	C24:1 n-9	0.04	0.13	0.18	**0.001**
PUFA	C16:2 n-4*	1.14	0.01	0.57	**0.000**
	C18:2 n-6	4.87	10.28	0.09	**0.000**
	C18:3 n-6	0.02	0.07	0.17	**0.000**
	C18:3 n-3	0.20	0.27	0.05	**0.005**
	C18:4 n-3	0.06	0.15	0.13	**0.000**
	C20:2 n-6	0.17	0.74	0.18	**0.000**
	C20:3 n-6	0.14	0.24	0.08	**0.000**
	C20:3 n-3	0.05	0.06	0.08	0.087
	C20:5 n-3	0.03	0.06	0.09	**0.002**
	C22:2	0.11	0.06	0.16	**0.003**
	C22:5 n-3	0.52	0.31	0.10	0.652
	C22:6 n-3	0.03	0.16	0.07	**0.001**
	SFA	35.46	37.42	0.01	**0.011**
	MUFA	55.65	42.37	0.03	**0.000**
	PUFA	8.15	14.10	0.26	**0.001**
	n3	0.89	1.02	0.06	**0.000**
	n6	6.05	12.96	0.04	**0.037**
	n6/n3	6.91	12.85	0.09	**0.000**

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High concentrations of Mg have a positive effect on animal behaviour, reduce their sensitivity to stress and improve pork quality.[37] The concentrations of Mg found in this study are higher than those found by other authors.[38,39,40] Regarding the two geographical areas considered in this study, it is hypothesised that the higher concentration of Mg in pigs in the NG group is related to the higher concentration of this element in this area, as confirmed by the study conducted by Raab et al.[41].

Na was also significantly different between the groups, but the ENG samples had higher concentrations of this element (2680.46 mg/kg vs 2066.54 mg/kg). As shown in Table 4, Fe and Zn contents were found in all samples analysed but were not significantly different between the two groups ($p>0.05$).

Among the potentially toxic elements, Pb concentrations with an average value of 1.07 mg/kg were found in the ENG samples. In contrast, NG samples showed Pb concentrations <LOQ. Commission Regulation (EU) 2023/915 (2023) on maximum levels of certain contaminants in foodstuffs stipulates that the maximum level of Pb in pig meat must not exceed 0.1 mg/kg of fresh product. In all samples analysed from the ENG group, Pb levels were above the established limit. This is probably due to the various anthropogenic activities in the Valle del Mela area, such as the refining industry, oil-fired power plants, waste incineration and road traffic, which release fine atmospheric particles that can contaminate soils. This probability is supported by several studies carried out on the area's soil[42], water[43], and animal[44].

Table 4. Mineral contents expressed as mean and standard error of the mean (SEM) and results of Mann-Whitney test for n = 10 muscle samples.

Mineral elements (mg/kg)	**NG**	**ENG**	**SEM**	***p-value***
Mg	2940.79	2724.97	0.02	**0.019**
Na	2066.54	2680.46	0.04	**0.000**
Ca	56.66	44.90	0.04	**0.008**
Fe	20.66	22.94	0.03	0.216
Zn	20.37	23.38	0.03	0.085
Pb	-	1.07	0.48	**0.000**

8.1.8. Differentiation of samples from different geographical areas

The significance of the differences in all the parameters analysed between the NG and ENG samples was estimated using the Mann-Whitney U test with a significance level below 0.05. The comparison of all parameters between the samples from different geographical areas, presented in Tables 1, 2, 3 and 4, showed that the NG samples had significantly higher values for proteins, 13C, C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7, C20: 1 n-9, C22:1 n-7, C22:1 n-9, C16:2 n-4, MUFA, brassicasterol, campesterol, campestanol, Δ-5,24-stigmastanol, Mg and Ca; while the samples ENG had significantly higher values of moisture, 15N, 2H, 18O, C18:2 n-6, C18:3 n-3, C20:2 n-6, SFA, PUFA, n3, n6, n6/n3, Na, Fe, Zn and Pb. The suitability of the data for factor analysis was checked. The Kaiser-Meyer-Olkin measure of sampling adequacy gave a value of 0.735 (greater than 0.500). Bartlett's sphericity test gave an approximate chi-square value of 153.869. Therefore, the correlation matrix was factored and suitable for PCA analysis. Seven principal components were extracted, which together explain 91.296 % of the total variance (66.532%, 7.480%, 4.427%, 4.134%, 3.662%, 2.816% and 2.246% respectively). There were no variables with low saturation in each factor, and the commonality was always greater than 0.723, which allowed the extracted components to reproduce satisfactory results for all variables. Correlation matrix analysis showed that the highest positive correlations were observed for n6/n3-C18:2 n-6 (0.995), Pb-C22:0 (0.974), C18:2n-6 - C22:0 (0.968), MUFA-C18:1n-9 (0.964), Pb-C17:0 (0.920), C13(protein)-MUFA (0.913), and C13(protein)-C16:2n4 (0.902), while the highest negative correlations were observed for Pb-MUFA (-0.992), MUFA-C22:0 (-0.981), Pb-C22:1 n9 (-0.977), brassicasterol-Pb (-0.968), and Pb-C18:1 n9 (-0.958), C13(protein)-C20:2n6 (-0.928) and C13(protein)-C18:2n6 (-0.900).

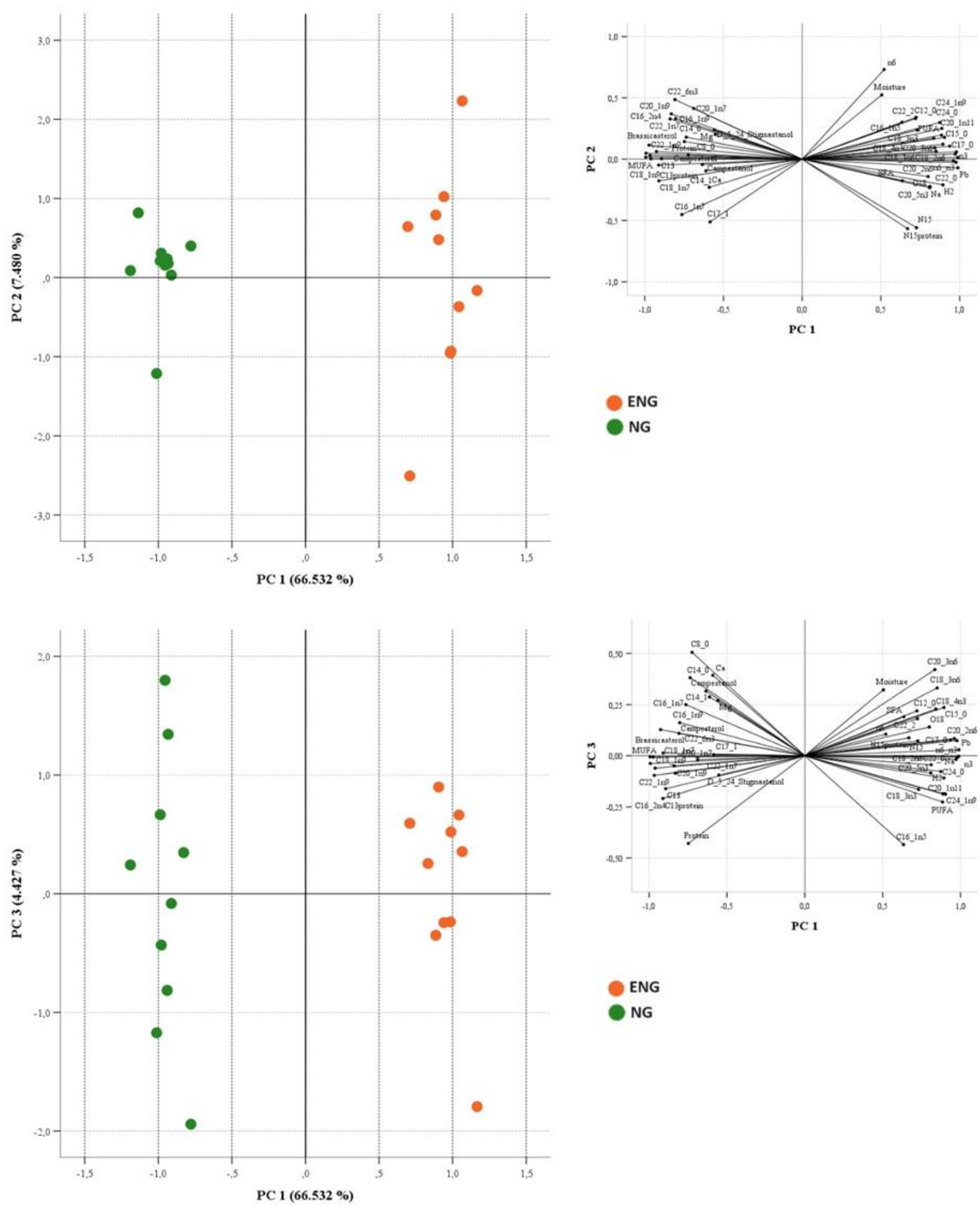

Figure 2. 2D scatter plots for fresh muscle samples classified by area of origin. Inset: loading diagram for PC1-PC2 and PC1-PC3.

8.1.9. Conclusion

In order to authenticate 'Nero dei Nebrodi' pig meat from two different geographical areas of north-eastern Sicily, a complete characterisation was carried out combining chemical-nutritional parameters with stable isotope and mineral element analysis. The rich vegetation endemic to the Nebrodi area was found to influence chemical-nutritional parameters such as the presence of

brassicasterol, campesterol and n-7 and n-9 fatty acids, which were significantly higher in the NG samples. In addition, the δ13C, δ15N, δ2H and δ18O of defatted meat were statistically different between animals coming from the two sampling areas. Statistical isotopic differences are mainly based on the different composition of diet (δ13C, δ15N) and drinking water (δ2H, δ18O). The isotopic results show the potential of these parameters for product traceability. Furthermore, the high levels of Pb found in the samples from the ENG group, probably due to the different anthropic activities present in the "Valle del Mela" area, confirm the possibility of differentiating the two rearing areas. In conclusion, this study could be used as a reliable tool for the authorities to authenticate the 'Nero dei Nebrodi' breed and use these correlations for traceability and consumer protection against fraud and commercial disputes.

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Chapter 9

OTHER RESEARCH LINES

Other lines of research followed during the three years of the PhD course are reported in this chapter and led to the writing and publication of the following scientific articles:

- Amar, Y. M. B., Potortì, A. G., Albergamo, A., **Litrenta, F**., Rando, R., Mouad, L. B., Mouad, L.B., Brigui, J., Chouaibi, N., & Di Bella, G. (2024). STUDY OF THE LIPID FRACTION OF MOROCCAN AND ITALIAN CAROBS (*CERATONIA SILIQUA* L.) published in the journal *European Journal of Lipid Science and Technology*, 2400036. (https://doi.org/10.1002/ejlt.202400036)[1]
- Lo Turco, V., **Litrenta, F**., Nava, V., Albergamo, A., Rando, R., Bartolomeo, G., Potortì, A.G., & Di Bella, G. (2023). EFFECT OF FILTRATION PROCESS ON OXIDATIVE STABILITY AND MINOR COMPOUNDS OF THE COLD-PRESSED HEMPSEED OIL DURING STORAGE published in the journal *Antioxidants*, *12*, 1231. (https://doi.org/10.3390/antiox12061231)[2]
- Nava, V., Albergamo, A., Bartolomeo, G., Rando, R., Litrenta, F., Lo Vecchio, G., Giorgianni, M. C., & Cicero, N. (2022). MONITORING CANNABINOIDS AND THE SAFETY OF THE TRACE ELEMENT PROFILE OF LIGHT *CANNABIS SATIVA* L. FROM DIFFERENT VARIETIES AND GEOGRAPHICAL ORIGIN published in the journal *Toxics*, *10*, 758. (https://doi.org/10.3390/toxics10120758)[3]
- Di Bella, G., Porretti, M., Cafarelli, M., **Litrenta, F.**, Potortì, A. G., Turco, V. L., Albergamo, A., Xhilari, M., & Faggio, C. (2023). SCREENING OF PHTHALATE AND NON-PHTHALATE PLASTICIZERS AND BISPHENOLS IN SICILIAN WOMEN'S BLOOD published in the journal *Environmental toxicology and pharmacology*, *100*, 104166. (https://doi.org/10.1016/j.etap.2023.104166)[4]

9.1. STUDY OF THE LIPID FRACTION OF MOROCCAN AND ITALIAN CAROBS (*CERATONIA SILIQUA* L.)

The aim of the present study was to investigate the lipid fraction of the Mediterranean carob in relation to the part of the fruit (i.e., pulp and seeds) and the geographical origin (i.e., Morocco and Italy). Besides the assessment of total fat and its fatty acid (FA) composition, minor lipophilic bioactives (e.g., phytosterols, squalene, and tocopherols) were evaluated. Hopefully, the study will contribute to

deepen the chemical composition of Mediterranean carob with the final aim to contribute toward a broader and more informed market of carob-based products.

9.1.1. Samples

The present study was conducted during September‑July 2023 and considered the raw pulp and seeds of carob (*C. siliqua* L.) pods collected from domesticated trees within agro-forestry systems in different Moroccan and Italian areas. In Morocco, carobs from the following areas were studied: provinces of Fahs-Anjra, Al Hoceima Al Hoceima, and Tangier-Asilah (region of Tanger-Tetouan-Al Hoceima), province of Rehamna (region of MarrakechMarrakech-Safi), and province of Agadir-Ida-Ou Tanane (region of Souss-Massa). In Italy, carobs from the provinces of Messina and Siracusa (region of Sicily) and the provinces of Cosenza and Reggio Calabria (region of Calabria) were investigated (Figure 1).

To ensure a number of samples representative of each selected area, n = 100 ripe (black) and defect-free fruits were collected from five different localities in every province, for a total of n = 900 carob pods considered in the study (i.e., 20 pods × 5 localities of a given province × 9 provinces).

Hence, for every locality of a given province, pods (n = 20) were separately pooled and freeze-dried (Alpha 1-2 LD Plus, Martin Christ). Dried pods were manually kibbled, and the seeds were manually separated from the pulp, so that pooled seeds and pulps were grounded and then sifted to produce respective fine powders. Pulp and seed samples, respectively, showed a moisture content of 13.88%

and 10.11%. All samples were labelled with the sampling location and the province they belonged to and stored at +4◦C in polyethylene bags until further analysis.

Figure1. Map of the sampling sites considered in the study. The Moroccan and Italian provinces under study are indicated, respectively, with green and purple points.

9.1.2. Production areas

The production potential of *C. siliqua* is difficult to evaluate, and the official data provided from year to year are often conflicting, probably because carobs occur as scattered trees within orchards or different agroforestry systems; several causes, such as the abandonment of the plantations, the presence of damaged specimens, and the inaccessibility of the site, make it difficult to make official estimates. However, the Statistical Database of Food and Agriculture Organization Corporate (FAOSTAT) reported that, in 2022, the species was harvested throughout the world for more than 15000 ha, with a production of more than 56000 t per year. Most of the commercial production (99%) remains concentrated in the Mediterranean basin, with Turkey (25106 t) being the leading producer, followed by Morocco (22059.05 t), Spain, Portugal, and Italy.[5]

Morocco is a carob-producing country with a worldwide reputation. In the present study, carobs were collected from three Moroccan regions—already well known to produce pods—which are distributed along a latitudinal gradient from North to South and characterized by many types of vegetation and habitats spread from subhumid to semiarid climates.[6] Carob production in Italy has always been mainly concentrated on the island of Sicily, followed by regions of the mainland, such as Calabria and Puglia.[7] According to the Italian National Institute of Statistics (ISTAT), Italy produced more than 355000 q of carobs in 2022, of which 5000 q were harvested in the south of Italy (mainly regions of Calabria and Puglia) and more than 350000 q in Sicily.[8]

9.1.3. Chemical analysis

The total fat of carob pulp and seeds was evaluated according to the AOAC 920.39 reference extraction method.[9] Sample aliquots (10.0 g) were extracted with *n*-heptane for 6 h, using a Soxhlet apparatus. Finally, lipid extracts were evaporated, and the extraction yield was gravimetrically assessed.

The transmethylation of FAs occurred by dissolving the lipid extract (0.1 g) in 1 mL of *n*-heptane and shaking the mixture with 0.2 mL of a potassium hydroxide solution in methanol. Then, the *n*-heptane layer was analysed by a gas chromatograph system provided with a split/splitless injector and a flame ionization detector (GC-FID, Dani Master GC1000, Dani Instrument).

The chromatographic analysis was carried out with a capillary column (Zebron ZB-WAX, 30 m × 0.25 mm ID × 0.25 μm, Phenomenex) and according to the following conditions: oven temperature from 130 to 200°C at a rate of 3°C min^{-1} (20 min hold); injector and detector temperature set, respectively, at 220 and 240°C; He at a linear velocity of 30 cm s^{-1} (constant). The injection occurred with a sample volume of 1 μL and exploiting a split ratio equal to 1:75. FAME identification was achieved by comparing the retention times of sample analytes and commercial standards, whereas their quantification occurred by considering the area of every FAME peak in relation to the total

area of chromatogram, and by expressing results in relation to the weight of extracted fat. Every sample was analysed on triplicate along with proper analytical blanks.

The protocol reported by EU Regulation no. 1348/2013 was considered for the study of the sterol composition.[10] Every lipid extract (0.1 g) was added with the internal standard α-cholestanol (2.33 mg mL^{-1}), saponified by a potassium hydroxide solution in ethanol, and extracted with ethyl ether to obtain the unsaponifiable matter. Then, thin-layer chromatography separated sterols from the unsaponifiable fraction. Briefly, every ethyl ether solution was loaded on glass plates (20 × 20 cm^2) coated with a basic silica gel previously heated at 110°C for 90 min. Elution was performed in 45 min with 100 mL of a solution of *n*-hexane: ethyl ether (65:35, v/v) in a glass developing chamber (27.0 × 26.5 × 7.0 cm^3). Hence, every plate was sprayed with 2,7-dichlorofluorescein in ethanol (0.2%, w/v) to read diverse bands under an UV source at 366 nm. The sterol band was scraped off from the gel and extracted with 10 mL of hot ethyl acetate.

The obtained residue was dried under vacuum and subsequently derivatized by 0.1 mL of BSTFA-TMCS (99:1, v/v) for 30 min at room temperature. Trimethylsilyl ether derivatives were then analyzed by a GC-FID provided with a capillary column (SPB-1, 15 m × 0.20 mm ID × 0.20 µm, Supelco). The oven temperature was from 240°C (5 min hold) to 290°C (5min hold) at a rate of 2°C min^{-1}. Injector and detector temperatures were set, respectively, at 280 and 290°C, and the He gas was at the linear velocity of 30 cm s^{-1} (constant). Every sample was injected by exploiting a volume of 1 µL and a split ratio of 1:50. The identification of phytosterols occurred by comparing the retention times of commercial standards, whereas their quantification by suitable external calibration plots was normalized by the internal standard. Triplicate analyses were conducted for every sample, along with analytical blanks.

Tocopherol isomers, namely, α-, γ-, and δ-, were assessed according to the procedure already reported in literature.[11] Every lipid sample (0.1 g) was added with 1mL of *n*-hexane and mixed with 1 mL of potassium hydroxide solution in methanol. The solution was stirred at 20°C for 10 min and then centrifuged at 4000 × *g* and +4°C, for 10 min. The *n*-hexane was filtered and analyzed a by

high-performance liquid chromatography system coupled to a fluorescence detector (HPLC-FD, Prominence HPLC System with RF-20A Detector, Shimadzu). For the chromatographic run, a LiChrosorb Si60 column (250 mm × 4.6 mm ID, 5 µm particle size) was placed in the column oven at 40°C. The analysis was performed with a mobile phase of *n*-hexane/ethyl acetate (90:10, v/v), under isocratic conditions, and with a flow rate of 0.8 mL min^{-1}. A sample volume of 20 µL was injected. Instrument control and data processing occurred thanks to the Lab-Solutions software ver. 5.10.153 (Shimadzu). Single tocopherols were identified with the help of reference compounds and by setting the detector with excitation and emission wavelengths, respectively, of 295 and 330 nm. On the other hand, the quantitative analysis was based on an external calibration procedure. Triplicate determinations were conducted for every sample, along with suitable analytical blanks.

For the determination of squalene, each lipid sample (0.1 g) was first fortified with the internal standard tetradecane (1.23 mg mL^{-1}) and then subjected to a solid-phase extraction, conducted with a Discovery DSC-Si silica cartridge (Supelco, 6 mL, 1 g) and n-hexane as eluent.

The subsequent analysis was carried out by a GC system (GC-2010, Shimadzu) coupled to a single-quadrupole mass spectrometer (QP-2010 Plus, Shimadzu). The analysis occurred by a capillary column (SPB-5 MS, 30 m × 0.25 mm ID × 0.25 µm, Supelco). The temperature program of the GC oven was from 80°C (1 min hold) to 140°C at a rate of 20°C min^{-1}, and finally to 290°C (2 min hold) at a rate of 5°C min^{-1}. Injection occurred at a temperature of 250°C, a sample volume of 1 µL, and a split ratio of 1:10.

The MS setup was: temperature of interface and EI source 290 and 230°C, respectively; ionization energy and emission current, respectively, at 70 eV and 250 µA. Squalene was identified in full scan (mass range: 40‑400 *m*/*z*) and quantified in selected ion monitoring (SIM) mode by considering characteristic mass fragments (i.e., 121, 137, 161, and 175 *m*/*z*), and exploiting the internal standard method.

Triplicate analyses of every sample were carried out along with analytical blanks.

9.1.4. Statistical analysis

The SPSS 13.0 software package was used for the statistical analysis. A descriptive analysis, involving the expression of experimental data as the mean±standard deviation of n =5 pools of pulps (or seeds) per province, was carried out.

To check whether the independent sample groups (carob pulps or seeds) were the same or different on the variables of interest, the one-way analysis of variance (ANOVA) was applied, and the significance of p-value was set at 0.001.

Then, to explore the effect of geographical origin on the chemical composition of Mediterranean carob, two data matrices corresponding to the two types of carob samples (i.e., the first matrix with all pulp samples and the second matrix with all seed samples) were built up so that the cases were the data from Moroccan and Italian provinces, and the variables were single FAs, sterols, tocopherols, and squalene.

Hence, every dataset was normalized to obtain equal importance for all variables, and a factor analysis with principal component (PC) extraction was performed to evaluate the differentiation of investigated samples based on the different geographical origins.

9.1.5. Results and discussion

The percent lipid content of carob pulp and seeds with different geographical origins is reported as the mean on a dw basis in Table 1.

On average, lipids from pulp and seed were 1.71% and 2.46% respectively, thus indicating that, like many other vegetable species, carob seeds are higher in fat than the relative fruit. Significant variability among the different provinces ($p < 0.001$) was highlighted.

For example, the carob from the province of Al Hoceima had the lowest lipids both in the pulp (1.18%) and seeds (1.71%); on the other hand, the most abundant lipid fractions were observed in fruits from Reggio Calabria (pulp: 2.46% and seeds: 3.19%) and Rehamna (pulp: 2.04% and seeds: 3.83%) provinces.

Along with its nutritional significance, fat defines the consistency, plasticity, and melting of a food all characteristics that must be considered in the formulation of a carob-based product. Both carob pulp and seeds are already well known for their low-fat content.[13] Hence, carob may be regarded not only as a healthy food source but also as an ideal ingredient to formulate low-fat products. The FA composition of carob pulp and seeds from the Mediterranean area is reported in Tables 2 and 3. Based on the one-way ANOVA, carob pulps had FAs that significantly varied among the investigated Moroccan and Italian provinces ($p < 0.001$), except for lignoceric acid (C24:0, $p > 0.001$). For carob seeds, 6 out of 17 FAs did not vary significantly. They were mainly saturated FAs such as stearic (C14:0, $p > 0.001$), behenic (C22:0 $p > 0.05$), lignoceric ($p > 0.001$), and montanic (C28:0, $p > 0.01$) acids. In both parts of the pod, the predominant FAs were palmitic (C16:0), oleic (C18:1$n - 9$), and linoleic acid (C18:2$n - 6$). However, although palmitic and oleic acids were higher in pulp (respectively, 20.21%‐26.50% and 37.63%‐44.49%) than seeds (respectively, 13.93%‐17.26% and 31.92%‐36.66%), linoleic acid was much more abundant in seeds than pulp (38.19%‐43.45% vs. 8.32%‐15.46%).

As a result, carob pulp was marked by consistent levels of SFA (29.60%‐41.56%) and MUFA (42.01%‐46.61%) and a reduced content of PUFA (11.55%‐23.30%), whereas seeds had a greater amount of PUFA (38.88%‐45.14%), a discrete content of MUFA (33.97%‐38.49%), and lower SFA (18.61%‐21.53%). According to these data, the carob pod shares a similar FA composition with pequi (*Caryocar brasiliense*), palm (*Elaeis guineensis*), and shea (*Vitellaria paradoxa*) fruits, characterized by high levels of palmitic and oleic acids.[13] On the other hand, the seeds with linoleic and oleic acids as major components show a FA profile comparable to that of sunflower (*Helianthus annuus*), sesame (*Sesamum indicum*), pumpkin (*Cucurbitamaxima*), cumin (*Cuminum cyminum*), and apple (*Malus communis*) seeds.[13]

Table 1. Total lipids (g/100 g of carob product, dw) of pulp and seeds of carobs from different sites in the Mediterranean basin. Data are expressed as mean±standard deviation of n=5 pools of pulps (or seeds) per province, where every pool was analyzed in triplicate. Bold p-values are statistically significant according to the one way-ANOVA (p<0.001).

Province	Fahs-Anjra	Al Hoceïma	Tangier-Assilah	Rehamna	Agadir-Ida -Ou Tanane	Messina	Siracusa	Reggio Calabria	Cosenza	p-value
Pulp	1.62±0.20	1.18±0.29	1.50±0.29	2.04±0.30	1.42±0.22	1.53±0.13	1.63±0.29	2.46±0.17	1.85±0.21	**<0.001**
Seeds	1.88±0.30	1.71±0.18	2.22±0.44	3.83±0.22	2.63±0.19	1.67±0.20	2.63±0.24	3.19±0.38	2.11±0.23	**<0.001**

Table 2. FA composition (g/100 g of lipid extract) of carob pulp coming from different Moroccan and Italian provinces. Data are expressed as mean±standard deviation of n=5 pools of pulp per province, where every pool was analyzed in triplicate. Bold p-values are statistically significant according to the one way-ANOVA (p<0.001). SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids.

	Fahs-Anjra	Al Hoceïma	Tangier-Assilah	Rehamna	Agadir-Ida-Ou Tanane	Messina	Siracusa	Reggio Calabria	Cosenza	p-value
C14:0	0.73±0.05	0.55±0.08	0.37±0.04	0.62±0.07	0.51±0.04	0.81±0.07	0.44±0.07	0.41±0.03	0.86±0.04	**<0.001**
C16:0	22.79±0.60	20.21±1.37	23.80±0.34	22.39±0.43	24.07±0.36	24.49±0.41	24.91±0.42	24.91±0.53	26.50±0.77	**<0.001**
C18:0	4.30±0.28	4.26±0.19	3.58±0.33	4.06±0.41	4.01±0.54	7.88±0.45	5.19±0.62	5.13±0.60	6.02±0.69	**<0.001**
C20:0	0.48±0.07	0.20±0.12	0.59±0.14	0.73±0.14	0.69±0.07	1.13±0.24	0.55±0.13	0.40±0.16	0.74±0.15	**<0.001**
C22:0	0.39±0.12	0.67±0.12	0.44±0.16	0.46±0.10	0.63±0.13	0.22±0.06	0.30±0.08	0.55±0.15	0.41±0.10	**<0.001**
C24:0	0.53±0.13	0.72±0.11	0.72±0.12	0.53±0.20	0.45±0.09	0.76±0.14	0.46±0.13	0.68±0.17	0.59±0.15	**>0.001**
C26:0	3.12±0.66	2.52±0.58	2.45±0.80	3.47±0.51	3.61±0.71	5.51±0.68	5.07±1.48	3.34±0.95	5.36±0.97	**<0.001**
C28:0	0.46±0.12	0.46±0.14	0.24±0.08	0.25±0.13	0.37±0.10	0.77±0.11	0.58±0.16	0.48±0.17	0.60±0.14	**<0.001**
SFA	**32.80±0.66**	**29.60±1.77**	**32.20±1.20**	**32.51±1.11**	**34.33±0.89**	**41.56±1.34**	**37.50±1.49**	**35.90±1.51**	**41.09±1.99**	**<0.001**
C16:1n-9	0.93±0.10	1.15±0.33	0.96±0.30	1.28±0.54	1.10±0.40	0.40±0.16	0.66±0.17	0.74±0.15	0.75±0.23	**<0.001**
C16:1n-7	1.47±0.40	4.47±0.75	3.52±0.55	2.31±0.34	1.44±0.33	1.12±0.15	1.51±0.34	1.36±0.34	0.90±0.26	**<0.001**
C18:1n-9	39.69±0.80	38.90±0.88	37.63±1.60	38.70±1.23	38.26±0.97	44.49±0.53	43.19±1.45	42.19±0.58	42.19±0.55	**<0.001**
C18:1n-7	1.23±0.35	1.19±0.21	1.35±0.34	1.15±0.36	1.21±0.17	0.59±0.13	0.30±0.09	0.53±0.20	0.24±0.06	**<0.001**
MUFA	**43.32±0.80**	**45.72±1.78**	**43.46±1.37**	**43.44±1.16**	**42.01±0.78**	**46.61±0.62**	**45.67±1.31**	**44.82±0.89**	**44.07±0.86**	**<0.001**
C18:2n-6	14.66±0.97	15.46±0.60	15.19±0.36	13.84±0.33	13.47±0.73	8.32±0.51	12.39±0.58	13.34±0.55	10.90±1.24	**<0.001**
C18:3n-6	0.90±0.16	0.54±0.25	0.41±0.20	0.50±0.12	0.85±0.23	0.28±0.11	0.16±0.12	0.25±0.08	0.18±0.11	**<0.001**
C18:3n-3	4.19±0.77	4.40±0.56	4.24±0.47	4.93±0.93	5.10±0.68	1.31±0.29	2.74±0.57	2.86±0.63	1.50±0.31	**<0.001**
C18:4n-3	1.52±0.36	1.40±0.22	1.63±0.83	1.86±0.34	2.36±0.79	0.86±0.24	0.73±0.07	1.05±0.43	0.96±0.38	**<0.001**
C18:4n-1	0.86±0.14	1.50±0.19	1.26±0.33	1.63±0.48	1.59±0.26	0.78±0.36	0.47±0.19	0.26±0.11	0.53±0.23	**<0.001**
PUFA	**22.13±1.96**	**23.30±1.23**	**22.74±1.49**	**22.75±0.98**	**23.37±1.85**	**11.55±0.65**	**16.50±0.91**	**17.77±0.19**	**14.07±0.63**	**<0.001**

Table 3. FA composition (g/100g of lipid extract) of carob seeds coming from different Moroccan and Italian provinces. Data are expressed as mean±standard deviation of n=5 pools of seeds per province, where every pool was analyzed in triplicate. Bold p-values are statistically significant according to the one way-ANOVA (p<0.001). SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids.

	Fahs-Anjra	**Al Hoceïma**	**Tangier-Assilah**	**Rehamna**	**Agadir-Ida-Ou Tanane**	**Messina**	**Siracusa**	**Reggio Calabria**	**Cosenza**	**p-value**
C14:0	0.05±0.04	0.13±0.07	0.15±0.06	0.16±0.08	0.19±0.07	0.10±0.04	0.25±0.09	0.18±0.08	0.13±0.07	>0.001
C16:0	15.12±0.30	14.51±0.82	13.93±0.34	15.33±0.59	14.57±0.58	15.85±0.42	17.26±0.82	17.17±0.58	16.22±0.74	**<0.001**
C18:0	2.76±0.37	3.67±0.52	3.24±0.25	3.30±0.43	2.87±0.23	3.79±0.26	3.78±0.65	4.06±0.45	3.77±0.30	**<0.001**
C20:0	0.36±0.07	0.31±0.16	0.30±0.09	0.35±0.16	0.29±0.11	0.47±0.14	0.46±0.07	0.43±0.09	0.52±0.13	>0.05
C22:0	0.13±0.06	0.18±0.05	0.24±0.10	0.20±0.05	0.23±0.07	0.16±0.04	0.27±0.14	0.14±0.05	0.24±0.09	>0.05
C24:0	0.33±0.04	0.45±0.05	0.26±0.05	0.35±0.06	0.33±0.09	0.29±0.06	0.25±0.08	0.26±0.12	0.27±0.07	>0.01
C26:0	0.23±0.06	0.33±0.04	0.32±0.04	0.26±0.04	0.24±0.09	0.64±0.13	0.51±0.12	0.48±0.10	0.50±0.11	**<0.001**
C28:0	0.17±0.05	0.18±0.07	0.17±0.04	0.09±0.05	0.19±0.09	0.22±0.09	0.28±0.04	0.22±0.08	0.25±0.10	>0.01
SFA	**19.16±0.74**	**19.79±0.38**	**18.61±0.19**	**20.05±1.15**	**18.92±0.80**	**21.53±0.53**	**23.06±1.65**	**22.95±0.29**	**22.02±0.90**	**<0.001**
C16:1n-9	0.31±0.04	0.29±0.03	0.21±0.07	0.32±0.04	0.22±0.06	0.10±0.06	0.19±0.08	0.08±0.04	0.17±0.12	**<0.001**
C16:1n-7	0.65±0.09	0.66±0.17	0.63±0.10	0.56±0.08	0.57±0.14	0.43±0.10	0.34±0.08	0.34±0.09	0.39±0.09	**<0.001**
C18:1n-9	31.92±1.01	33.78±1.08	34.17±1.11	33.94±0.94	33.70±1.02	36.66±0.72	35.26±1.02	36.18±0.73	36.47±1.24	**<0.001**
C18:1n-7	2.03±0.49	2.64±0.70	2.55±0.97	1.97±0.19	1.85±0.38	1.48±0.17	1.10±0.23	1.40±0.33	1.07±0.15	**<0.001**
MUFA	**33.97±0.74**	**38.18±0.73**	**38.35±1.00**	**36.31±0.62**	**36.75±0.33**	**38.67±0.86**	**37.07±0.16**	**37.76±0.42**	**38.49±1.49**	**<0.001**
C18:2n-6	43.45±1.08	40.84±1.51	41.19±1.39	40.63±0.98	41.73±1.33	39.07±0.60	38.57±0.88	38.27±1.06	38.19±0.65	**<0.001**
C18:3n-6	0.06±0.04	0.05±0.02	0.06±0.07	0.03±0.03	0.02±0.03	0.04±0.02	0.05±0.03	0.03±0.04	0.04±0.03	>0.05
C18:3n-3	1.29±0.52	1.37±0.20	1.11±0.37	1.12±0.23	1.33±0.22	0.72±0.25	0.57±0.18	0.65±0.19	0.48±0.14	**<0.001**
C18:4n-3	0.26±0.10	0.19±0.09	0.22±0.08	0.21±0.11	0.23±0.07	0.07±0.02	0.06±0.02	0.09±0.02	0.07±0.03	**<0.001**
C18:4n-1	0.08±0.04	0.04±0.03	0.04±0.04	0.05±0.05	0.07±0.04	0.05±0.02	0.04±0.04	0.06±0.02	0.09±0.05	>0.05
PUFA	**45.14±0.64**	**42.49±1.47**	**42.63±1.51**	**42.04±0.76**	**43.38±1.30**	**39.94±0.61**	**39.27±0.90**	**39.10±1.21**	**38.88±0.67**	**<0.001**

Interestingly, very long-chain saturated FAs, such as cerotic (C26:0) and montanic acids, were detected in both carob pulp and seeds with a content <1%, except for cerotic acid in pulp, which oscillated between 2.45% and 5.51%. These FAs have only recently been revealed in the FA composition of carob pods, and cerotic acid, in particular, was previously detected in other underutilized Nigerian legumes, such as *Brachystegia eurycoma* and *Mucuna flagellipes* (*Fabaceae*).[14]

Indices of the FA composition, such as the mean SFA/UFA ratio and the mean oleic/linoleic acid (O/L) ratio, slightly dropped going from the pulp (respectively, 0.55 and 3.25) to the seeds (respectively, 0.26 and 0.87). The higher the SFA/UFA and O/L values, the greater the resistance to oxidative stress and, hence, lipid oxidation.

Additionally, the O/L chemistry may directly correlate with the development of off flavors over time and ultimately be an important measure of product quality, as flavor is a primary driver of consumer acceptance.[15]

Hence, carob pulps have a higher oxidative stability than seeds, and, consequently, they better tend not to develop off-flavors and to be preserved over time.

Considering the study areas, Al Hoceima and Tangier-Assilah, respectively, had pulp and seed samples with the lowest levels of palmitic acid (20.21% and 13.93%). Moreover, the areas of Tangier-

Assilah and Fahs-Anjra, respectively, showed pulp and seeds with the least abundant oleic acid (37.63% and 31.92%), whereas the provinces of Messina and Cosenza, respectively, reported pulp and seed samples with scarce amounts of linoleic acid (8.32% and 38.19%). On the other hand, pulp and seed samples with the highest concentrations of palmitic acid (26.50% and 17.26%) were sampled, respectively, in the provinces of Cosenza and Siracusa. The area of Messina was characterized by carob pulp and seeds with the most abundant oleic acid (44.49% and 36.66%), whereas the highest contents of linoleic acid were recorded in the pulp and seeds, respectively, from the areas of Al Hoceima and Fahs-Anjra (15.46% and 43.45%).

Literature agrees that palmitic, oleic, and linoleic acids are typically the most abundant FAs of *C. siliqua* pods. However, scarce and inconsistent results on their content were reported. Yatim et al. studied four carob ecotypes from three Moroccan areas, namely, the Saiss plain, the High Atlas, and the Middle Atlas. They pointed out that the pulp had decreased levels of palmitic acid (13.97%‑16.58%) and comparable levels of linoleic acid (10.42%‑15.41%) with respect to the Moroccan and Italian carobs from this study.[16] However, the content of oleic acid (35.83%‑39.06%) was comparable to that detected in Moroccan pulps but lower than that found in Italian samples.

On the other hand, seeds showed a slightly higher concentration of palmitic acid (15.8%‑20.3%), lower levels of oleic acid (8.4%‑11.0%), and amuchmore abundant amount of linoleic acid (52.1%‑61.6%) thanMoroccan and Italian carobs.[16]

During the last decade, the FA composition of seeds of Turkish carobs was also explored. At first, Matthaus and Ozcan reported that seeds from wild and cultivated carobs had palmitic acid equal to 10.3% and 12%, oleic acid amounting to 30.4% and 26.5%, and linoleic acid as high as 49.1% and 51%, respectively.[17] More recently, Fidan et al. pointed out that seeds from commercial carobs were characterized by 16.6% of palmitic acid, 45.0% of oleic acid, and 32.4% of linoleic acid.[18]

The sterol %composition of carob pulp and seeds with different geographical origin is reported, respectively, in Tables 4 and 5. According to the one-way ANOVA, all sterols significantly varied in the pulp among the investigated areas ($p < 0.001$), except for brassicasterol ($p > 0.05$) and clerosterol ($p > 0.001$). In seeds, a higher number of sterols, such as cholesterol ($p > 0.05$), brassicasterol ($p > 0.05$), clerosterol ($p > 0.01$), Δ-5-avenasterol ($p > 0.05$), and Δ-7-stigmastenol ($p > 0.05$), did not significantly vary. In carob pulp, *β*-sitosterol (57.49%‑72.47%, equivalent to 2013.83‑6045.28 mg kg^{-1} of lipid extract), Δ-5-avenasterol (10.26%‑21.27%, equivalent to 817.33‑1695.79 mg kg^{-1} of lipid extract), and stigmasterol (3.97%‑8.11%, equivalent to 139.41‑284.08 mg kg^{-1} of lipid extract) accounted for more than 70% of total sterols. In seeds, *β*-sitosterol alone represented more than 70% of total sterols (70.76%‑75.87%, equivalent to 12616.47‑

13527.58 mg kg^{-1} of lipid extract), followed by stigmasterol (10.09%‑15.67%, 2721.55‑4226.63 mg kg^{-1} of lipid extract), and Δ-7-avenasterol (2.10%‑7.53%, equivalent to 390.75‑1401.11 mg kg^{-1} of lipid extract). The abundance of these sterols varied not only in relation to the part of the fruit considered, as seeds had much higher sterols than pod, but also to the geographical area of origin.

Table 4. Sterol composition (g/100g of lipid extract) of carob pulp from different Mediterranean areas. Data are expressed as mean±standard deviation of n=5 pools of pulp per province, where every pool was analyzed in triplicate. Bold p-values are statistically significant according to the one way-ANOVA (p<0.001).

	Fahs-Anjra	**Al Hoceïma**	**Tangier-Assilah**	**Rehamna**	**Agadir-Ida-Ou Tanane**	**Messina**	**Siracusa**	**Reggio Calabria**	**Cosenza**	**p-value**
Cholesterol	0.59±0.03	0.55±0.03	0.60±0.06	0.62±0.04	0.64±0.05	0.38±0.04	0.40±0.03	0.41±0.04	0.46±0.04	**<0.001**
Brassicasterol	0.15±0.05	0.11±0.04	0.13±0.04	0.18±0.04	0.15±0.04	0.11±0.03	0.16±0.03	0.12±0.03	0.14±0.06	>0.05
24-methylene-cholesterol	0.61±0.15	0.72±0.13	0.65±0.14	0.56±0.11	0.73±0.08	1.20±0.10	1.08±0.16	1.13±0.11	0.93±0.29	**<0.001**
Campesterol	1.58±0.17	1.43±0.09	1.49±0.14	1.77±0.12	1.67±0.16	2.29±0.44	3.01±0.30	2.21±0.23	2.65±0.27	**<0.001**
Campestanol	0.27±0.05	0.42±0.15	0.24±0.06	0.30±0.07	0.33±0.07	0.17±0.04	0.21±0.05	0.15±0.03	0.18±0.03	**<0.001**
Stigmasterol	4.55±0.24	5.10±0.91	5.34±0.75	3.97±0.47	4.30±0.40	6.39±0.58	5.90±0.44	8.11±0.59	7.62±0.40	**<0.001**
Δ-7-campesterol	0.76±0.09	0.68±0.07	0.73±0.07	0.56±0.11	0.84±0.07	0.66±0.10	0.89±0.08	0.71±0.12	0.83±0.07	**<0.001**
Clerosterol	1.39±0.13	1.28±0.10	1.50±0.13	1.43±0.31	1.56±0.16	1.85±0.18	1.92±0.65	1.76±0.10	1.67±0.26	>0.01
β-Sitosterol	65.78±1.99	71.54±1.00	72.47±2.47	71.42±3.71	70.77±3.16	57.49±2.27	63.72±3.07	58.79±4.32	61.30±2.19	**<0.001**
Δ-5-avenasterol	19.34±2.11	13.71±2.39	11.97±2.12	10.26±1.39	12.55±1.93	21.27±3.33	15.74±2.01	18.44±2.74	18.92±1.12	**<0.001**
Δ-5,24-stigmastadienol	1.24±0.15	1.27±0.08	1.67±0.24	1.50±0.22	1.37±0.28	1.69±0.33	1.18±0.07	1.11±0.17	1.41±0.12	**<0.001**
Δ-7-stigmastenol	1.66±0.24	1.75±0.24	0.96±0.35	1.57±0.33	1.35±0.28	1.64±0.28	1.48±0.25	1.27±0.18	1.80±0.23	**<0.001**
Δ-7-avenasterol	2.84±0.49	1.77±0.26	2.82±0.46	5.36±0.53	4.36±1.48	4.88±1.03	2.73±0.64	5.55±1.49	2.29±0.80	**<0.001**

Table 5. Sterol composition (g/100g of lipid extract) of carob seeds coming from different Mediterranean areas. Data are expressed as mean±standard deviation of n=5 pools of seeds per province, where every pool was analyzed in triplicate. Bold p-values are statistically significant according to the one way-ANOVA ($p<0.001$).

	Fahs-Anjra	Al Hoceïma	Tangier-Assilah	Rehamna	Agadir-Ida-Ou Tanane	Messina	Siracusa	Reggio Calabria	Cosenza	p-value
Cholesterol	0.41±0.06	0.45±0.07	0.32±0.08	0.35±0.05	0.36±0.08	0.40±0.15	0.45±0.09	0.43±0.07	0.37±0.11	>0.05
Brassicasterol	0.10±0.02	0.16±0.05	0.18±0.06	0.08±0.04	0.13±0.05	0.12±0.04	0.11±0.02	0.13±0.04	0.10±0.05	>0.05
24-methylene-cholesterol	0.48±0.07	0.34±0.10	0.37±0.07	0.40±0.09	0.25±0.07	0.62±0.10	0.55±0.09	0.67±0.11	0.73±0.11	**<0.001**
Campesterol	4.34±0.79	5.60±1.27	5.08±1.46	4.18±0.70	4.91±0.97	2.82±0.24	2.95±0.90	3.90±0.69	3.24±0.54	**<0.001**
Campestanol	0.23±0.10	0.27±0.08	0.36±0.08	0.31±0.09	0.25±0.07	0.40±0.16	0.44±0.15	0.52±0.17	0.59±0.12	**<0.001**
Stigmasterol	11.93±1.27	11.49±1.41	10.09±1.45	10.35±1.32	12.12±1.66	15.67±1.11	14.20±1.77	13.97±1.09	13.26±1.58	**<0.001**
Δ-7-campesterol	0.26±0.07	0.33±0.11	0.22±0.06	0.18±0.08	0.28±0.11	0.49±0.12	0.37±0.06	0.42±0.09	0.39±0.15	**<0.001**
Clerosterol	0.91±0.11	1.39±0.52	0.94±0.40	1.12±0.59	1.66±0.81	0.80±0.12	0.75±0.06	0.73±0.13	0.87±0.08	>0.01
β-Sitosterol	75.79±2.10	72.04±1.84	72.13±2.33	74.31±2.44	70.76±1.87	73.92±2.65	75.40±1.48	72.03±1.62	75.87±1.78	**<0.001**
Δ-5-avenasterol	0.92±0.13	0.87±0.30	0.97±0.28	1.21±0.43	1.17±0.37	1.36±0.35	0.93±0.24	1.10±0.23	1.20±0.32	>0.05
Δ-5,24-stigmastadienol	0.95±0.15	0.90±0.17	0.86±0.13	0.83±0.31	0.94±0.19	0.51±0.17	0.60±0.21	0.62±0.14	0.68±0.14	**<0.001**
Δ-7-stigmastenol	1.42±0.19	1.31±0.33	1.24±0.31	1.11±0.21	1.08±0.27	1.27±0.47	1.41±0.11	1.00±0.22	1.17±0.27	>0.05
Δ-7-avenasterol	2.98±0.57	5.39±0.87	7.53±1.45	5.47±1.17	6.50±1.73	2.10±0.57	2.63±0.22	4.07±0.55	2.26±0.40	**<0.001**

The Italian provinces of Messina and Agadir-Ida-Ou Tanane showed the least abundant amounts of *β*-sitosterol, respectively, in carob pulp and seeds (57.49% and 70.76%); the provinces of Rehamna and Tangier-Assilah were, respectively, characterized by pulp and seed samples with the lowest content of stigmasterol (3.97% and 10.09%); however,Δ-5-avenasterol and Δ-7-avenasterol were at the lowest levels, respectively, in pulp samples from Rehamna (10.26%) and seeds from Messina (2.10%).

On the other hand, *β*-sitosterol was most abundant in pulp samples from Tangier-Assilah (72.47%) and in seeds from Cosenza (75.87%); however, Reggio Calabria and Messina provinces reported the highest levels of stigmasterol, respectively, in pulp and seeds (8.11% and 15.67%). Pulp samples from Messina and seed samples from Tangier- Assilah were, respectively, marked by the highest concentrations of Δ-5-avenasterol (21.27%) and Δ-7-avenasterol (7.53%).

According to the present study, carob has been demonstrated to be a precious source of phytosterols, although their profile can considerably vary depending on the province of origin. Although the absorption of phytosterols in the human organism is lower than that of cholesterol, they can still reduce serum LDL-cholesterol and cardiovascular risk. Moreover, *β*-sitosterol showed to decrease in vitro in the viability of cancer cells.

In literature, the sterol composition of carob is roughly studied, and *β*-sitosterol and stigmasterol are the most frequently reported compounds. Yatim et al. focused on the assessment of sterols in pulp and seeds of four carob ecotypes from three Moroccan areas.[16] Contrary to our study's evidence, *γ*-sitosterol was the most abundant sterol in the pulp of all four carob samples (58.99–178.78 mg kg^{-1} of lipid extract). To follow, *β*-sitosterol occurred in three out of four samples (9.25–41.63 mg kg^{-1} of lipid extract), and stigmasterol was detected in only one sample (8.95–9.54 mg kg^{-1} of lipid extract). On the other hand, seeds from all Moroccan ecotypes were characterized by *β*-sitosterol (440–1410 mg kg^{-1} of lipid extract), *δ*-sitosterol (620–790 mg kg^{-1} of lipid extract), stigmasterol (170–260 mg kg^{-1} of lipid extract), and campesterol (70–150 mg kg^{-1} of lipid extract).[16]

Except for stigmasterol present in seeds at amounts comparable to those found in the seeds from our study, the other sterols were detected at much lower levels than those reported in both pulp and seeds of Moroccan and Italian carobs.

However, Turkish seeds demonstrated to have a sterol profile more similar to that obtained in this study, on a %basis. In fact, Matthaus and Ozcan pointed out that seeds from cultivated and wild carobs displayed β-sitosterol equal to 78.62% and 72.04%, respectively, and stigmasterol amounting to 0.58% and 11.43%. More recently, Faidan et al. highlighted that seeds from commercial carobs had 74.2% of β-sitosterol and 12.8% of stigmasterol.[18]

The content of α-, γ-, and δ-tocopherols of carob pulp and seeds coming from different Moroccan and Italian provinces is shown in Tables 6 and 7, respectively, and they are expressed in terms of mg/100 g of lipid extract. The one-way ANOVA highlighted that tocopherol significantly varied among the investigated sites of origin ($p < 0.001$), considering both pulp and seed samples.

In particular, γ-tocopherol was the most abundant tocopherol in pulp (10.37–22.78 mg/100 g) and seeds (4.95–22.86 mg/100 g), followed by α-tocopherol (pulp: 4.31–8.77 mg/100 g; seeds: 2.86–7.59 mg/100 g) and δ-tocopherol (pulp: 1.41–5.23 mg/100 g; seeds: 0.88–2.62 mg/100 g). Differently from other lipid components, the tocopherol profile was comparable in both parts of carob fruit.

Concerning the site of origin, the carob pulp had the lowest levels of α-, γ-, and δ-tocopherols in samples from the province of Reggio Calabria (respectively, 4.31 mg/100 g, 10.37 mg/100 g, and 1.41 mg/100 g); however, the highest contents were found in samples from Al Hoceima (respectively, 8.77 mg/100 g, 22.78 mg/100 g, and 5.23 mg/100 g).

Table 6. Tocopherols (mg/100 g of lipid extract) of carob pulp coming from different Moroccan and Italian provinces. Data are expressed as mean ± standard deviation of n=5 pools of pulp per province, where every pool was analyzed in triplicate. Bold p-values are statistically significant according to the one way-ANOVA (p<0.001).

	Fahs-Anjra	**Al Hoceïma**	**Tangier-Assilah**	**Rehamna**	**Agadir-Ida-Ou Tanane**	**Messina**	**Siracusa**	**Reggio Calabria**	**Cosenza**	**p-value**
α-tocopherol	6.30±1.46	8.77±1.47	7.28±1.52	6.64±1.39	6.98±1.48	4.53±1.20	5.50±1.23	4.31±1.55	5.23±2.21	**<0.001**
γ-tocopherol	17.61±2.74	22.78±1.93	21.18±2.72	19.37±2.42	16.13±2.53	10.88±2.49	13.13±1.98	10.37±2.47	14.89±1.55	**<0.001**
δ-tocopherol	2.07±0.47	5.23±1.48	2.29±1.33	1.93±1.01	1.73±0.65	2.70±0.92	2.55±0.78	1.41±0.93	1.63±0.63	**<0.001**

Table 7. Tocopherols (mg/100 g of lipid extract) of carob seeds from different Moroccan and Italian provinces. Data are expressed as mean±standard deviation of n=5 pools of seeds per province, where every pool was analyzed in triplicate. Bold p-values are statistically significant according to the one way-ANOVA (p<0.001).

	Fahs-Anjra	**Al Hoceïma**	**Tangier-Assilah**	**Rehamna**	**Agadir-Ida-Ou Tanane**	**Messina**	**Siracusa**	**Reggio Calabria**	**Cosenza**	**p-value**
α-tocopherol	6.86±1.77	5.44±1.58	7.59±1.56	6.63±2.70	5.50±2.25	4.86±2.25	2.86±1.19	3.85±0.93	3.61±1.65	**<0.001**
γ-tocopherol	22.86±2.78	17.19±1.90	15.70±1.61	13.52±1.50	15.31±1.50	12.08±1.18	9.32±1.56	4.95±1.58	8.47±1.51	**<0.001**
δ-tocopherol	2.18±0.81	2.09±0.89	1.57±0.63	2.62±0.98	2.27±0.50	0.88±0.30	1.02±0.69	1.20±0.56	2.24±0.82	**<0.001**

Carob seeds showed the lowest amounts of tocopherols in the Italian provinces of Siracusa (*α*-tocopherol: 2.86 mg/100 g), Reggio Calabria (*γ*-tocopherol: 4.95 mg/100 g), and Messina (*δ*-tocopherol: 0.88 mg/100 g); conversely, tocopherols were most abundant in the Moroccan provinces of Tangier-Assilah (*α*-tocopherol: 7.59 mg/100 g), Fahs-Anjra (*γ*-tocopherol: 22.86 mg/100 g), and Rehamna (*δ*-tocopherol: 2.62mg/100 g).

Yatim et al. evaluated tocopherols in pulp and seeds of four carob ecotypes from three Moroccan areas. In the pulp, apart from the determination of two *α*-tocopheroids, namely, *α*-tocospiro A and *α*-tocospiro B, vitamin E was detected only in two of the four ecotypes and ranged between 0.325 and 2.677 mg/100 g. On the other hand, *γ*-tocopherol (3‑16 mg/100 g) and *α*-tocopherol (2‑7 mg/100 g) characterized the seed samples of all Moroccan ecotypes.[16]

In Turkey, commercial carob seeds showed the presence of *α*-, *β*-, *γ*-, and *δ*-tocopherols, and, similarly to our study, *γ*-tocopherol (53.1%) and *α*-tocopherol (41.1%) were the most abundant isomers.[18]

Matthaus and Ozcan highlighted that wild carob seed oil had higher levels of tocopherols than the oil obtained from cultivated carobs.[17]

In fact, oil from wild carob seeds revealed 114.3 mg/100 g of *γ*-tocopherol, 70.4 mg/100 g of *α*-tocopherol, and 10.7 mg/100 g of *δ*-tocopherol, whereas cultivated carob seeds showed 101.2 mg/100 g of *γ*-tocopherol, 69.1mg/100 g of *α*-tocopherol, and 8.7 mg/100 g of *δ*-tocopherol. Overall, wild and cultivated carob seeds had much higher and not comparable levels of tocopherols than those revealed in our study.

Literature also shows evidence of tocopherols in Tunisian carob seeds. In this respect, Ben Ayache et al. reported that the total tocopherol content of carob seeds was 3 mg/100 g, including *γ*-tocopherol, *α*-tocopherol, and *δ*-tocopherol at lower amounts than those recorded for seeds from different Moroccan and Italian areas (respectively, 2.02 mg/100 g, 0.09 mg/100 g, and 0.12mg/100 g).[19]

Overall, although tocopherols may vary due to the production context, carob pulp and seeds are a good source of tocopherols, which may contribute to the antioxidant potential of the fruit as such or as an ingredient of many foods product. The levels of squalene in pulp and seed samples belonging to several Moroccan and Italian areas are reported in Table 8, and they are expressed in terms of mg/kg of lipid extract.

Table 8. Squalene (mg/kg of lipid extract) of carob pulp and seeds from different Moroccan and Italian provinces. Data are expressed as mean±standard deviation of n=5 pools of pulp (or seeds) per province, where every pool was analyzed in triplicate. Bold p-values are statistically significant according to the one way-ANOVA (p<0.001).

	Fahs-Anjra	**Al Hoceïma**	**Tangier-Assilah**	**Rehamna**	**Agadir-Ida-Ou Tanane**	**Messina**	**Siracusa**	**Reggio Calabria**	**Cosenza**	**p-value**
Pulp	3.86±0.38	4.68±0.80	5.67±0.75	3.03±0.40	2.06±0.26	0.38±0.10	1.12±0.16	0.53±0.10	1.69±0.16	**<0.001**
Seeds	43.48±4.38	37.56±1.81	40.87±4.24	30.39±2.79	27.51±3.85	2.92±0.74	4.59±0.68	3.20±0.51	2.93±0.47	**<0.001**

Based on the one-way ANOVA test, the content of squalene of carob pulp and seeds significantly differed among the selected provinces ($p < 0.001$). Carob seed samples accumulated squalene at a higher content than pulp samples (0.38‑5.67 vs. 2.93‑43.48 mg kg^{-1}). Additionally, Moroccan provinces housed carobs with higher squalene levels than Italian provinces, both considering pulp (2.06‑5.67 vs. 0.38‑1.69 mg kg^{-1}) and seeds (27.51‑43.48 vs. 2.92‑4.59 mg kg^{-1}).

Squalene is a sesquiterpene of great biological relevance, present in the unsaponifiable fraction of plant oils and shark liver oil. Indeed, it provides certain health benefits to the consumer health, namely, the reduction of cholesterol and triglyceride serum levels and protection from a variety of cancers. To the best of our knowledge, squalene has never been determined in carob, and, consequently, a comparison with literature data is not possible. However, the levels of such terpene in carob seeds are comparable to those revealed in a variety of vegetable oils, such as avocado (42.93 mg kg^{-1}), macadamia (22.90 mg kg^{-1}), and coconut (20.37 mg kg^{-1}) oils, as well as seed oils, including rapeseed (20.8‑64.6 mg kg^{-1}), soybean (16.3‑45.6 mg kg^{-1}), and camellia (15.3‑42.5mg kg^{-1}).[20]

As already discussed, the one-way ANOVA showed significant differences ($p < 0.001$) between carob pulps (or seeds) with different geographical origins for almost all experimental variables.

Therefore, any variable was excluded for the subsequent statistical elaboration. In the PC analysis (PCA) of pulp, the Kaiser criterion was satisfied, as eight PCs had eigenvalues >1.0 for a total variance of 81.02%. The first two PCs showed the higher values of variance (respectively, 43.74% and 9.33%), and consequently, they were selected for developing the relative score and loading plots (Figure 2).

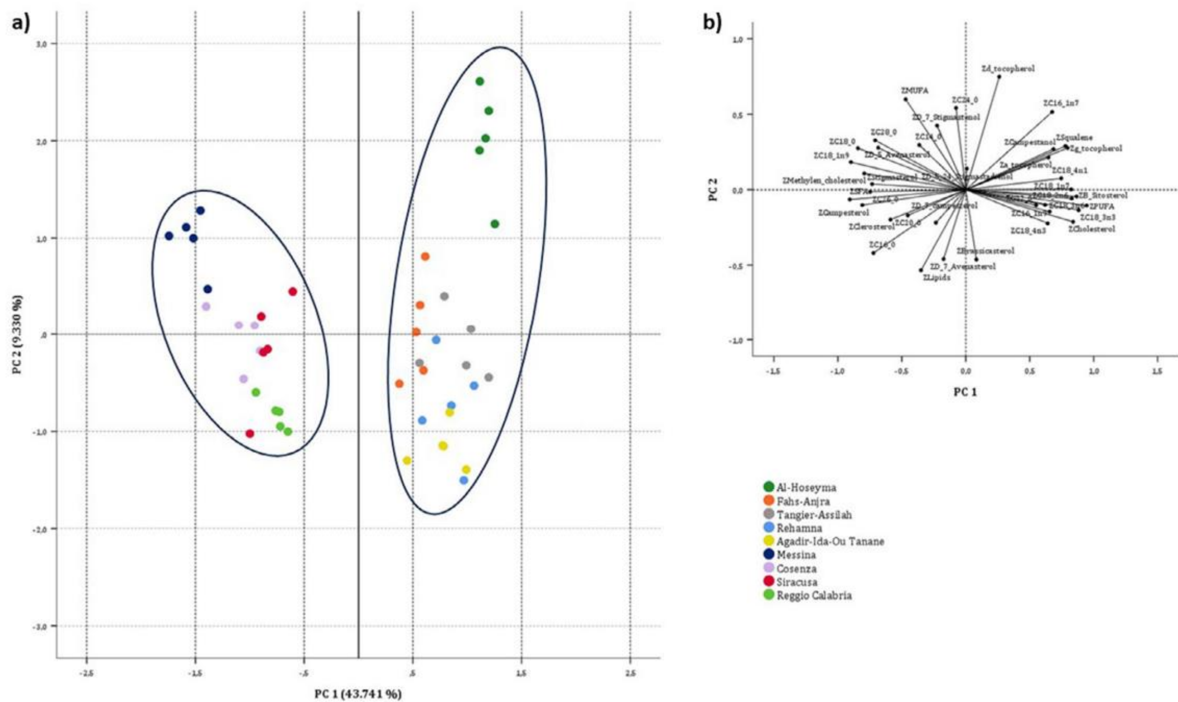


Figure 2. 2D principal component analysis (PCA) score (a) and loading (b) plots of the first two pcs obtained from all experimental variables investigated in carob pulps. drawn ellipses suggest the natural grouping of samples according to the geographical origin.

A significant separation between Moroccan and Italian carob pulps was highlighted due to different geopedoclimatic contexts that affect the lipid fraction of the fruit pulp. Specifically, all Moroccan samples were placed in the right area of the score plot characterized by the highest PC1 and PC2 scores. Indeed, the clustering of such samples was influenced by strongly positive correlations of PC1 with PUFA (0.946), α-linolenic acid (0.882), sitosterol (0.841), cholesterol (0.839), and linoleic acid (0.829). Other relevant positive correlations were with γ-tocopherol (0.797) and squalene (0.779). Interestingly, carob pulps from the Al Hoceima province well differentiated from the other Moroccan samples thanks to higher levels of δ-tocopherol and palmitoleic acid, which, consequently, showed positive correlations with the PC2 axis (respectively, 0.748 and 0.516).

On the other hand, all Italian samples were plotted on the left side of the score plot, being marked by the most negative PC1 scores. Indeed, these samples clustered in correspondence of variables such as SFA, oleic and stearic acids, which displayed the strongest negative correlations with PC1

(respectively, −0.907, −0.899, and −0.844) and, to follow, with campesterol (−0.809), stigmasterol (−0.733), and Δ-5-avenasterol (−0.685). As a result, Italian carob pulps were characterized by the highest content of such FAs and sterols. Moreover, it should be noted that variables marked by positive associations with PC2 (i.e., MUFA: 0.599, stearic acid: 0.275, and oleic acid: 0.183) determined the differentiation of carob pulps from Messina area from the other Italian samples. Indeed, the carob pulp from this area had the highest amounts of MUFA, stearic acid, and oleic acids.

With respect to the PCA of seeds, 11 PCs had eigenvalues >1.0 for a total variance of 82.08%. Having shown the highest values of variance (respectively, 37.27% and 7.49%), the first two PCs were used for constructing the relative score and loading plots (Figure 3). Although these two PCs retained less than 45% of the total variance, a sample discrimination based on the geographical origin is still possible, thus pointing out that the region of growth affects the lipids of carob seeds. By overlapping the score and loading plots, it is noticeable how all Moroccan samples clustered around variables characterized by the most positive PC2 scores, such as squalene (0.950), PUFA (0.894), linoleic acid (0.837), and γ-tocopherol (0.818).

Sterols such as Δ-5-24-stigmastadienol (0.649) and Δ-7-avenasterol (0.628) were also positively associated with PC1. Among Moroccan samples, carob seeds from the Al Hoceima province markedly separated from samples of the other provinces thanks to higher levels of γ-tocopherol and linoleic acid, which, consequently, showed positive correlations with the PC2 axis (respectively, 0.419 and 0.284). Conversely, seeds from the different Italian provinces were plotted on the left half of the score plot, which was characterized by the most negative PC1 scores for SFA (−0.884), cerotic (−0.829), palmitic (−0.797), and oleic (−0.793) acids. Other negative correlations were displayed with 24-methylene-cholesterol (−0.761) and campestanol (−0.675).

As a result, the clustering of such samples against the Moroccan ones was attributable to their higher content of SFA, including cerotic and palmitic acids, oleic acid, and sterols, such as 24-methylene-cholesterol and campestanol.

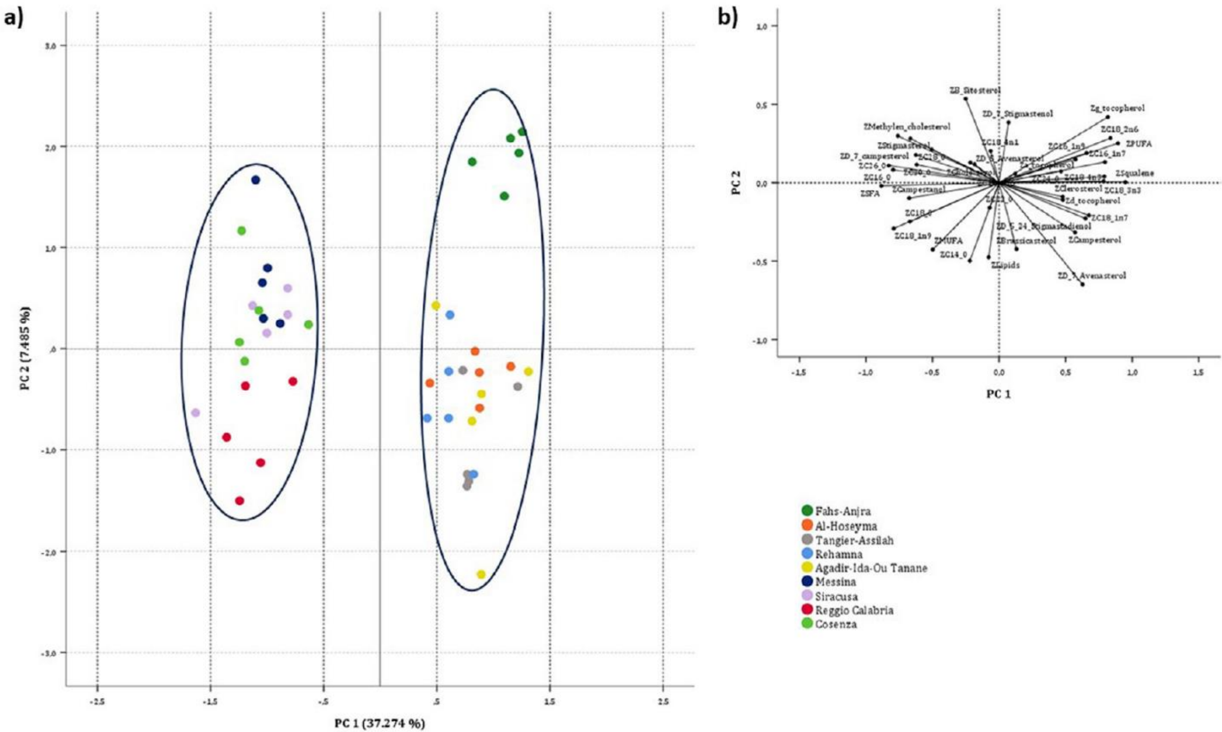


Figure 3. 2D principal component analysis (PCA) score (a) and loading (b) plots of the first two PCs obtained from all experimental variables investigated in carob seeds. Drawn ellipses suggest the natural grouping of samples according to the geographical origin.

9.1.6. Conclusions

Being characterized by a favorable FA composition and beneficial amounts of minor lipophilic compounds with bioactive properties, the Mediterranean carob is marked by a lipid fraction with a relevant nutritional value and promising technological properties. Although lipids of carob vary in relation to the fruit part, pulp and seeds can both be regarded as sustainable value-added products. Additionally, a proper statistical analysis pointed out that the lipid profile of carob may be affected by the geo-pedoclimatic context in which the plant grows, as demonstrated by evident compositional differences in the seeds and pulp of Moroccan and Sicilian carobs. Consequently, both the fruit part and the geographical origin of carob are factors that should be taken into account when defining dietary requirements and designing functional foods.

9.2. EFFECT OF FILTRATION PROCESS ON OXIDATIVE STABILITY AND MINOR COMPOUNDS OF THE COLD-PRESSED HEMPSEED OIL DURING STORAGE

The aim of this study was to comprehensively evaluate the effectiveness of filtration on the oxidative stability and minor compounds of the cold-pressed hempseed oil during a 12-week storage in transparent glass bottles. To this purpose, the hydrolytic and oxidative status, fatty acid (FA) composition, tocopherols, pigments, phenols, squalene, and inorganic elements were monitored in experimental filtered and non-filtered oils over the study period, statistically elaborated, and discussed to evaluate the effectiveness of the filtration process on the cold-pressed hemp oil as well as its convenience for future commercial applications.

9.2.1. Production of Non-Filtered and Filtered Cold-Pressed Hempseed Oils

Virgin cold-pressed hempseed oils were provided in 2022 by Sativa Molise (Palata, Italy), and they were obtained from seeds of *Cannabis sativa* L. subsp. Sativa cv. Finola. The seeds were mechanically separated from inflorescences (~95% pure seed and ~5% dockage), dried at 25±2°C up to a humidity of ~8–10%, packaged in polyethylene bags, and stored in a dry room (21±3°C, 20–30% RH) for a maximum of two weeks. Hempseeds were cold-pressed in an expeller press with a 1.8 kW electric motor and a capacity of 5–7 kg/h. The press was first heated to 80°C, then the heater was turned off and the material to be pressed was released. At that time, the temperature of the press head was 40°C, while the temperature of the outflowing oil was ~50°C, as an effect of pressure and friction. The speed of the screw press was operated at a constant rotating speed of 25 rpm. The obtained oil was then decanted into a storage tank for 24 h. To produce filtered oil samples, the cold-pressed oil was filtered within 24 h of pressing through a filter press equipped with cellulose acetate membranes (thickness: 0.81 mm) and operating at a pressure of 6 bar.

9.2.2. Samples

Non-filtered and filtered hempseed oils (respectively, NF-HO and F-HO) were separately blotted in triplicate by employing transparent glass bottles of 100 mL each with screw caps (~3% headspace),

for a total of n = 12 NF-HO bottles and n = 12 F-HO bottles. Under these experimental conditions, the oil oxidation may be attributable to autoxidation—occurring just with atmospheric oxygen ($^3O_2$)—and photooxidation—related to the presence of light, sensitizers, and $^3O_2$.

Changes in the oxidative state and minor compounds of various oil samples were monitored during 12 weeks of storage by keeping all bottles in a controlled environment at room temperature (22 ± 1.2 °C), under a 12/12 h light/dark regime, and rotating them every 10 days[21].

Hence, three bottles from every treatment were considered at the beginning of the experimental trial (T0) and every 4 weeks (T4, T8, and 12), and the oil from each bottle was analyzed in triplicate.

9.2.3. Chemical analysis

For the determination of free acidity and peroxide value (PV), the procedures already reported by Costa and colleagues [33] were followed. For the acidity, 90 mL of a solution of ethyl alcohol/diethyl ether (1:2, v/v) was mixed with a few drops of 1% phenolphthalein and subsequently neutralized with 0.1 N KOH. The mixture was then added to 5 g of the oil sample and titrated with 0.1 N KOH until the colour changed. The acidity was calculated according to the following equation and expressed as a % of oleic acid:

$$Oleic\ acid\ (\%) = \frac{V\ x\ N\ x\ MWOA}{Ws\ x\ 10}$$

where V is the volume of titrant (mL of KOH), N is the normality of KOH (0.1), MWOA is the molecular weight of oleic acid (282 g/mol), and Ws is the weight of the oil sample (g).

For the determination of PV, 25 mL of a solution of glacial acetic acid/chloroform (3:2, v/v) was mixed with 500 µL of a saturated KI solution. After vigorously shaking, the solution was allowed to stand in the dark for ~5 min. Then, 75 mL of distilled water and starch indicator were added to the mixture, and a titration with 0.01 N Na_2S2O_3 was conducted until the color changed.

PV was defined as milliequivalents of reactive oxygen content per 1 kg of oil sample (mEq/O_2/kg), and it was derived by following the equation:

$$\text{PV (mEq/O2/Kg)} = \frac{V\,x\,N\,x\,1000}{Ws}$$

where V is the volume of titrant (mL of $Na_2S_2O_3$), N is the normality of $Na_2S_2O_3$ (0.01), and Ws is the weight of the oil sample (g).

Finally, the spectrophotometric exam was conducted by measuring specific UV absorbances at 232 and 270 nm and expressing them as extinction coefficients K232 and K270.

For the elucidation of the FA profile of hemp oil samples, the protocol already employed by Sdiri and colleagues was considered[22]. Approximately 0.1 g of hemp oil was mixed with 2 mL of n-heptane and 0.2 mL of methanolic KOH solution for 30 s at room temperature and decanted. Then, the upper layer containing FAMEs was injected into a gas chromatograph (GC) (Dani Master GC1000) equipped with a split/splitless injector and a flame ionization detector (FID) (Dani Instrument, Milan, Italy). For the chromatographic separation, a SLB-IL100 capillary column (60 m × 0.25 mm ID, 0.20 µm film thickness, Supelco, Sigma Aldrich, Burlington, MA, USA) was employed with the following operating conditions: column oven temperature from 165°C to 210°C at 2°C/min (10 min hold); injector and detector temperatures of 250°C; He gas at a linear velocity of 30 cm/s (constant); injection volume of 1 µL, with a split ratio of 1:100. Data acquisition and handling were performed using Clarity Chromatography Software v4.0.2. FAMEs of nutritional interest were identified by direct comparison with the retention times of reference compounds and expressed as the relative percent area of the total chromatogram.

For the determination of α-, γ-, and δ-tocopherols, ~200 mg of oil sample was diluted in 1.8 mL of n-hexane, filtered through a 0.20 µm PTFE syringe filter, and analyzed by a high-performance liquid chromatography system coupled to fluorescence detector (HPLC-FD, Shimadzu, Milan, Italy) according to the conditions already reported by Albergamo and coworkers[23].

Specifically, the chromatographic separation was carried out by a LiChrosorb® Si60 column (250 mm × 4.6 mm I.D., 5 µm particle size, Merck, Darmstadt, Germany), and by exploiting a mobile phase consisting of n-hexane/ethyl acetate (90:10 v/v) under isocratic conditions. HPLC-FD

analyses were performed at 40 °C with an injection volume of 20 µL and a flow rate of 0.8 mL/min. Data processing occurred by the LabSolutions software, ver. 5.10.153 (Shimadzu). The identification of tocopherols was carried out by a direct comparison with the retention time of relative commercial standards at respective excitation and emission wavelengths of 295 nm and 330 nm. The quantitative analysis was performed by constructing appropriate external calibration curves for every investigated tocopherol.

For the determination of chlorophyll (Chl) a, chlorophyll (Chl) b, and total carotene, the protocol of Blasi and colleagues[24] was considered. Briefly, 1 g of every oil sample was mixed with 50 mL of diethyl ether, vortexed, and sonicated for 1 min. The absorbance of solutions was measured by an UV spectrophotometer (UV-2401 PC, Shimadzu, Milan, Italy). Chl a and Chl b showed the maximum absorbances at 663 nm (A663) and 640 nm (A640), respectively, while total carotene content was determined at 470 nm (A470). The concentration (µg/mL) of these pigments was calculated according to the formulas:

$$\text{Chl a} = 9.93 \times \text{A663} - 0.78 \times \text{A640}$$

$$\text{Chl b} = 17.60 \times \text{A640} - 2.81 \times \text{A663}$$

$$\text{Chl a} + \text{b} = 7.12 \times \text{A663} - 16.80 \times \text{A640}$$

$$\text{Total carotene } = \frac{(1000\ x\ A470 - 0.52\ x\ Chl\ a - 7.25\ x\ Chl\ b)}{226}$$

For the extraction of polyphenols, approximately 6 g of oil samples were mixed with 6 mL of a methanol/water solution (80:20, v/v), stirred for 2 min, and kept at room temperature until phase separation.

Then, the colorimetric assay was conducted according to what had already been reported by Aghraz and coworkers[25]. Specifically, 0.2 mL of the upper part of the mixture was collected, and 1.8 mL of distilled water was added along with 8 mL of Na_2CO_3 (20%) and 10 mL of Folin–Ciocalteu reagent. The mixture was kept in the dark for 30 min and read at 700 nm with an UV-visible spectrophotometer (UV-2401 PC, Shimadzu, Milan, Italy). The quantification procedure occurred

through an external calibration curve of gallic acid, and the total phenol content was calculated as milligrams of gallic acid equivalent in 1 L of hemp oil (mg GAE/kg).

Squalene was extracted from every oil sample by means of a solid phase extraction (SPE) exploiting Supelco Discovery DSC-Si Silica cartridges and n-hexane and analyzed by a gas chromatography system (GC-2010, Shimadzu, Tokyo, Japan) coupled to a single quadrupole mass spectrometer (QP-2010 Plus, Shimadzu, Japan). Chromatographic separations occurred on a SPB-5 MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Supelco, Bellefonte, PA, USA). The oven temperature program was from 80 °C (1 min hold) to 140 °C at 20 °C/min, and finally to 290 °C (2 min hold) at 5 °C/min. The injection port temperature was set at 250 °C, and the injection volume was 1 μL with a split ratio of 1:10. The MS conditions were: EI source temperature 230 °C; ionization energy and emission current 70 eV and 250 μA, respectively; interface temperature 290 °C. The identification occurred in full scan (mass range: 40–400 m/z) by comparing both retention time and mass spectrum with those of commercial standards, while quantification was performed in selected ion monitoring (SIM) by monitoring four characteristic mass fragments (121, 137, 161, and 175 m/z). Hence, the amount of the compound was derived by considering the relative base peak ion and exploiting the internal standard normalization with the internal standard tetradecane.

9.2.4. Element Analysis

Around 0.5 g of each oil sample were mineralized with 8 mL of HNO_3 and 2 mL of H_2O_2 by a microwave digestion system (Ethos 1, Milestone, Bergamo, Italy) with a temperature of 0–200 °C in 10 min (step 1) and 200 °C held for 10 min (step 2) and a power of 1000 W. Digested samples were cooled down at room temperature and properly diluted with the internal standard Re in ultrapure water. Elemental analyses were carried out by a quadrupole ICP-MS iCAP Q (Thermo Scientific, Waltham, MA, USA), equipped with an ASX-520 autosampler (Cetac Technologies Inc., Omaha, NE, USA). For quantification purposes, an external calibration procedure combined with an internal standard normalization was exploited.

Instrumental control and data acquisition were performed by Thermo Scientific's Qtegra™ Intelligent Scientific Data System software (Thermo Fisher Scientific, Bremen, Germany).

9.2.5. Statistical Analysis

The data were statistically analyzed by R Studio v. 3.6.1 (Boston, MA, USA) for Windows. A descriptive analysis, including the mean and standard deviation, was conducted for all the experimental data obtained from this study. After running a Shapiro–Wilk test to verify the normal distribution of experimental data, every parameter was statistically elaborated in all oil samples by (i) the one-way ANOVA followed by a post hoc Tukey's HSD to study the effect of storage and highlight significant differences in NF-HO samples (or F-HO samples) during T0-T12, and (ii) a two-tailed Student's t-test for unpaired data to evaluate the effectiveness of filtration and point out significant differences between NF-HO and F-HO samples. Statistical significance was accepted at $p \leq 0.05$.

9.2.6. Hydrolytic and Oxidative Status of Cold-Pressed Hempseed Oils

The hydrolytic and oxidative status of cold-pressed hempseed oil was explored in NF-HO and F-HO samples (Figure 4) and compared with the available quality parameters fixed for edible fats and oils not covered by individual standards by the Codex Alimentarius for free acidity (2% of oleic acid or 4 mg KOH/g of oil) and PV (15 mEqO2/kg of oil).

9.2.7. Results

Free fatty acids are more susceptible to autooxidation than esterified fatty acids, and they also stimulate the hydrolysis of phenolics, thus contributing to the deterioration of the shelf life of the edible oil. At the beginning of the experimental trial, fresh NF-HO and F-HO samples were characterized by similar and acceptable acidities (respectively 0.87% and 0.90%). By exploiting the highest formation rate during the first 4 weeks of storage (NF-HO: +54%; F-HO: +49%), free fatty acids increased, but still within the Codex limits, in both types of oil (NF-HO: 0.87–1.87%, p <

0.05; F-HO: 0.90–1.77%, $p < 0.05$), in agreement with the recent literature, which, however, has also reported fresh oils with a very high acidity.[26,27,28]

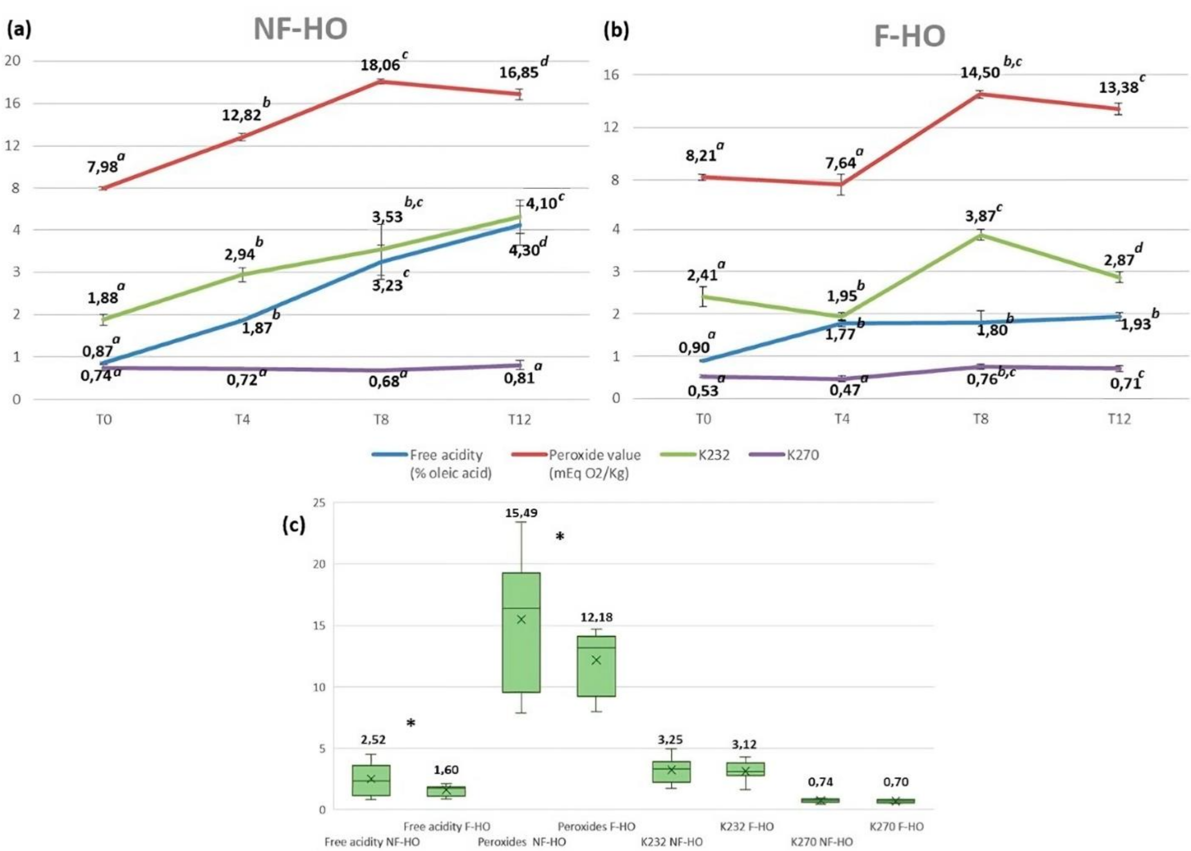


Figure 4. Evolution of free acidity, peroxide value (PV), K232, and K270 of non-filtered (NF-HO, (a)) and filtered (F-HO, (b)) cold-pressed hempseed oil during 12 weeks of storage in transparent glass bottles. Data are expressed as the mean ± sd of n = 3 oil bottles, each analyzed in triplicate. a–d: different superscript letters in the same line indicate significantly different values for a given parameter ($p < 0.05$ by post hoc Tukey's HSD test); the same superscript letters in the same line indicate not significantly different values ($p > 0.05$ by post hoc Tukey's HSD test). Figure (c) illustrates the free acidity, PV, K232, and K270 of total NF-HO and F-HO samples. Data are expressed as the mean ± sd of n = 12 oil bottles, each analyzed in triplicate. In each box, "×" indicates the average value. In the comparison between NF-HO and F-HO samples, "*" indicates significantly different values ($p < 0.05$ by Student's t-test) for a given parameter; conversely, the absence of "*" indicates non-significantly different values ($p > 0.05$ by Student's t-test).

With the advancement of storage (T4-T12), lower formation rates were recorded, and only NF-HO reported acidities higher than the Codex guide value, the free acidity of NF-HO and F-HO samples being respectively from 1.87% to 4.30% ($p < 0.05$) and from 1.77% to 1.93% ($p < 0.05$) (Figure 4

a, b). Overall, the NF-HO showed a worse hydrolytic quality than the F-HO (mean acidities: 2.52% and 1.60%, $p < 0.05$), thus suggesting an ameliorative effect of filtration on the oil acidity (Figure 4c).

Notoriously, proper seed storage conditions (in terms of humidity, light and temperature) as well as good seed quality (in terms of moisture level and percent dockage) are essential requirements to produce an oil with a favorable hydrolytic status. However, according to the results of this study, the filtration also had a positive effect on the acidity of the cold-pressed hempseed oil during storage, probably because less water was dispersed in the filtered oil samples. This would be consistent with the recent work of Tura and colleagues[27], in which stable and compliant acidities were recorded in cold-pressed hempseed oils subjected in a laboratory to cotton gauze filtration and centrifugation before storage in amber glass bottles for 90 days.

Additionally, Fregapane et al.[29] reported that filtration reduced the rate of hydrolysis of the triacylglycerol matrix with positive effects on the oxidative stability of the virgin olive oil. However, on the other hand, Frega and colleagues[30] suggested that filtration increased the oil's susceptibility to oxidative degradation by removing suspended solid materials. From their point of view, suspended solids and free fatty acids may react to form a precipitated residue that is not capable of an oxidative reaction.

PV is indicative of the total peroxidic-bonded oxygen present in an oil and, consequently, of its oxidative status, especially at the early stages of storage. As a result, lipid peroxyl radicals and hydroperoxides are typically defined as primary oxidation products. This parameter can vary in relation to the oxygen partial pressure in the headspace, the type of oxygen (i.e., $^1O_2$ is much more reactive with lipids than $^3O_2$), temperature (i.e., the solubility of oxygen in oil generally increases as the temperature increases), light (i.e., light promotes production of $^1O_2$ in the presence of sensitizer pigments and $^3O_2$), and minor oil components (i.e., metals, free fatty acids, mono- and diacylglycerols, and phospholipids generally accelerate oil oxidation). Fresh NF-HO and F-HO samples had similar and acceptable PVs (respectively 7.98 $mEqO_2/kg$ and 8.21 $mEqO_2/kg$).

However, similarly to the acidity, they showed an increase within 4 weeks of storage in both sets of samples (NF–HO: 7.98–14.39 mEqO_2/kg, $p < 0.05$, and F–HO: 8.21–12.64 mEqO_2/kg, $p < 0.05$), while remaining lower than the Codex value.

Values from fresh hempseed oils can be hardly compared with literature since peroxides quickly react to generate other radical forms, thus showing a considerable fluctuation even in the same oil.[26,27,28]

Interestingly, during the first 4 weeks of storage, the highest rate of peroxide formation was observed both in NF-HO (+45%) and F-HO (+35%) samples. This can be interpreted as a trigger effect generated by the initial and variable level of $^3O_2$ dissolved in the oil during the production and bottling phases[31].

With the advancement of storage (T4-T12), however, the PV of NF-HO samples became higher than the Codex guidance value. Indeed, PV of NF-HO and F-HO samples varied respectively from 12.82 mEqO_2/kg to 16.85 mEqO_2/kg ($p < 0.05$) and from 7.64 mEqO_2/kg to 13.38 mEq O_2/kg ($p > 0.05$) (Figure 4 a, b). Overall, the effectiveness of filtration on oil peroxides is also supported by the fact that F-HO showed a better oxidative status than NF-HO (mean PVs: 12.18 mEqO_2/kg and 15.49 mEqO_2/kg, $p < 0.05$) (Figure 4c).

Specific extinction coefficients (K) at the UV wavelengths of 232 nm and 270 nm are helpful in studying the progress of the autooxidation of vegetable oils. In fact, when hydroperoxides are formed, double bond shifting and isomerization occur, producing primary oxidation products such as conjugated dienes, which exhibit intense absorption at 232–234 nm, and subsequently conjugated trienes, which typically adsorb at 268–270 nm. At this point, the decomposition of conjugated systems into secondary oxidation products (i.e., aldehydes, ketones, alcohols, and short-chain hydrocarbons) is commonly observed.

According to our results, the extinction K232 rose from 1.88 to 4.10 ($p < 0.05$) in NF-HO and from 2.41 to 2.87 ($p < 0.05$) in F-HO. Similarly, K270 of NF-HO and F-HO samples increased, respectively, in the ranges 0.74–0.81 ($p < 0.05$) and 0.53–0.71 ($p < 0.05$). In line with previous

studies on hempseed oil, both NF-HO and F-HO were characterized by K232 and directly correlated with the evolution of PV [27]. Moreover, similarly to PV, K232 and K270 showed the highest increase during the first 4 weeks of storage, both in NF-HO (+43% and +36%) and F-HO (+32% and +30%) samples. Subsequently, a steady state of oxidation was observed during storage. In fact, K232 and K270 were not significantly different both in NF-HO and F-HO during T4-T8 ($p > 0.05$), probably due to the establishment of an equilibrium between the formation of diene systems and their decomposition into secondary products, such as hexanal, 2-decenal, or 2-heptenal, potentially responsible for the off-flavor of the oxidized oils. From the discussed data, a weak effect of filtration on the formation of dienes and trienes in the hempseed oil during storage may be argued.

This is also suggested by the fact that NF-HO had slightly higher conjugated dienes (mean values: K232 = 3.25 vs. 3.12, $p > 0.05$) and trienes (mean values: K270 = 0.74 vs. 0.70, $p > 0.05$) than F-HO (Figure 1c).

A possible explanation of the different oxidative behaviour of hempseed oils is that filtration may reduce all those minor oil components that may cause the initial increase in PV and conjugated systems by various mechanisms, such as autooxidation (i.e., free fatty acids), increase of the rate of oil oxidation (i.e., transition metals), and increase of the diffusion rate of oxygen from the headspace into the oil (i.e., free fatty acids, mono- and diacylglycerols, and phospholipids).[32] Consequently, a greater presence of these compounds in NF-HO samples would be indicative of lower stability against oxidative degradation. Additionally, NF-HO would be more affected by the light from the use of transparent bottles than F-HO, since light may trigger $^1O_2$ oxidation pathways and, consequently, further increase the level of primary oxidation products.

Table 9 reports the profile of the most nutritionally relevant FAs present in NF-HO and F-HO samples stored for 12 weeks in transparent glass bottles.

Table 9. Evolution of the FA composition (%) in non-filtered (NF-HO) and filtered (F-HO) cold-pressed hempseed oil during 12 weeks of storage in transparent glass bottles. Data are expressed as the mean ± sd of n = 3 oil bottles, each analyzed in triplicate.

	NF-HO				F-HO			
	T0	T4	T8	T12	T0	T4	T8	T12
C16:0	7.20 ± 0.45^{a}	6.30 ± 0.44^{a,b}	6.82 ± 0.33^{a,b}	5.87 ± 0.31^{b}	6.77 ± 0.23^{a}	5.62 ± 0.53^{b}	5.57 ± 0.32^{b}	5.53 ± 0.19^{b}
C18:0	3.24 ± 0.29^{a}	2.94 ± 0.08^{a}	2.18 ± 0.34^{a}	2.40 ± 0.67^{a}	2.95 ± 0.08^{a}	2.58 ± 0.61^{a}	2.18 ± 0.12^{a}	2.27 ± 0.08^{a}
SFA	10.44 ± 0.74^{a}	9.24 ± 0.39^{a,b}	9.00 ± 0.03^{b,c}	8.27 ± 0.48^{b,c}	9.72 ± 0.24^{a}	8.20 ± 1.14^{a,b}	7.75 ± 0.38^{b}	7.80 ± 0.12^{b}
C18:1 n-9	10.42 ± 1.08^{a}	9.41 ± 1.03^{a}	9.04 ± 0.23^{a}	9.06 ± 0.67^{a}	9.18 ± 0.33^{a}	8.75 ± 0.21^{a,b}	8.80 ± 0.33^{a,b}	8.42 ± 0.19^{b}
C18:1 n-7	0.93 ± 0.13^{a}	1.13 ± 0.44^{a}	0.96 ± 0.22^{a}	0.87 ± 0.08^{a}	1.00 ± 0.12^{a}	0.88 ± 0.08^{a}	0.81 ± 0.03^{a}	0.87 ± 0.09^{a}
MUFA	11.35 ± 1.21^{a}	10.54 ± 1.46^{a}	10.01 ± 0.15^{a}	9.93 ± 0.74^{a}	10.18 ± 0.42^{a}	9.63 ± 0.13^{a,b}	9.61 ± 0.35^{a,b}	9.29 ± 0.21^{b}
C18:2 n-6	55.56 ± 0.61^{a}	55.18 ± 1.62^{a,b}	52.74 ± 0.90^{b,c}	52.36 ± 0.87^{c}	56.27 ± 0.40^{a}	56.13 ± 1.25^{a}	54.61 ± 0.64^{a}	54.29 ± 0.49^{a}
C18:3 n-6	5.34 ± 0.95^{a}	5.47 ± 0.46^{a}	5.86 ± 0.37^{a}	5.40 ± 0.83^{a}	5.18 ± 0.36^{a}	4.89 ± 0.14^{a}	4.71 ± 0.17^{a}	4.94 ± 0.06^{a}
C18:3 n-3	18.85 ± 0.75^{a}	17.92 ± 0.34^{a}	18.37 ± 0.76^{a}	18.70 ± 0.35^{a}	19.18 ± 0.40^{a}	18.63 ± 0.68^{a}	18.80 ± 0.33^{a}	19.07 ± 0.19^{a}
C18:4 n-3	0.99 ± 0.12^{a}	0.90 ± 0.21^{a}	1.00 ± 0.23^{a}	1.25 ± 0.38^{a}	1.02 ± 0.13^{a}	0.99 ± 0.24^{a}	1.33 ± 0.34^{a,b}	1.76 ± 0.11^{b}
PUFA	80.74 ± 2.20^{a}	79.47 ± 2.30^{a}	77.97 ± 1.38^{a}	77.71 ± 1.44^{a}	81.87 ± 0.41^{a}	80.64 ± 0.78^{a,b}	79.44 ± 0.23^{b}	80.06 ± 0.72^{b}
PUFA/SFA	7.77 ± 0.75^{a}	8.62 ± 0.59^{a,b}	8.67 ± 0.13^{a,b}	9.41 ± 0.37^{b}	8.43 ± 0.18^{a}	9.98 ± 1.54^{a}	10.27 ± 0.55^{a}	10.27 ± 0.09^{a}
n-6/n-3	3.07 ± 0.10^{a}	3.22 ± 0.03^{a,b}	3.03 ± 0.12^{a}	2.90 ± 0.13^{a,c}	3.05 ± 0.05^{a}	3.11 ± 0.13^{a,b}	2.95 ± 0.13^{a}	2.84 ± 0.02^{a,c}

a–c: Different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ by post hoc Tukey's HSD test); the same superscript letters in the same line indicate not significantly different values ($p > 0.05$ by post hoc Tukey's HSD test).

Minimal differences were recorded in single FAs of NF-HO and F-HO samples at T0. However, major classes of SFA and MUFA were more concentrated in NF-HO than F-HO (SFA: 10.44% and 9.72%, MUFA: 11.35% and 10.18%); conversely, PUFA were higher in F-HO than NF-HO (81.87% and 80.74%).

During storage of NF-HO samples, no significant variations were detected between the initial (T0) and final (T4) percent content of most single FAs. In fact, only the palmitic and linoleic acids decreased significantly over 12 weeks of storage (i.e., 7.20–5.87% and 55.56–52.36%, $p < 0.05$) and, in parallel, total SFA and PUFA reduced significantly from 10.44% to 8.27% ($p < 0.05$) and from 80.74% to 77.71% ($p < 0.05$). Accordingly, the n-6/n-3 ratio was lowered from 3.07 to 2.07. Conversely, in F-HO samples, while most FAs decreased, the linoleic acid remained stable (56.27–54.29%, $p > 0.05$) and the stearidonic acid increased (1.02–1.76, $p < 0.05$) during the study period. Accordingly, SFA, MUFA, PUFA, and n-6/n-3 significantly decreased in the respective ranges of 9.72–7.80% ($p < 0.05$), 10.18–9.29% ($p < 0.05$), 81.87–80.06% ($p < 0.05$), and 3.05–2.84 ($p < 0.05$). As shown in Figure 5, the filtration process impacted the FA composition. In fact, SFA and MUFA were on average more abundant in NF-HO than F-HO (respectively, 9.24% and 8.37%, $p < 0.05$;

10.45% and 9.67%, $p < 0.05$). Conversely, PUFA and PUFA/SFA were lower in NF-HO and higher in F-HO (respectively, 78.97% and 80.50%, $p < 0.05$; 8.61% vs. 9.64%, $p < 0.05$).

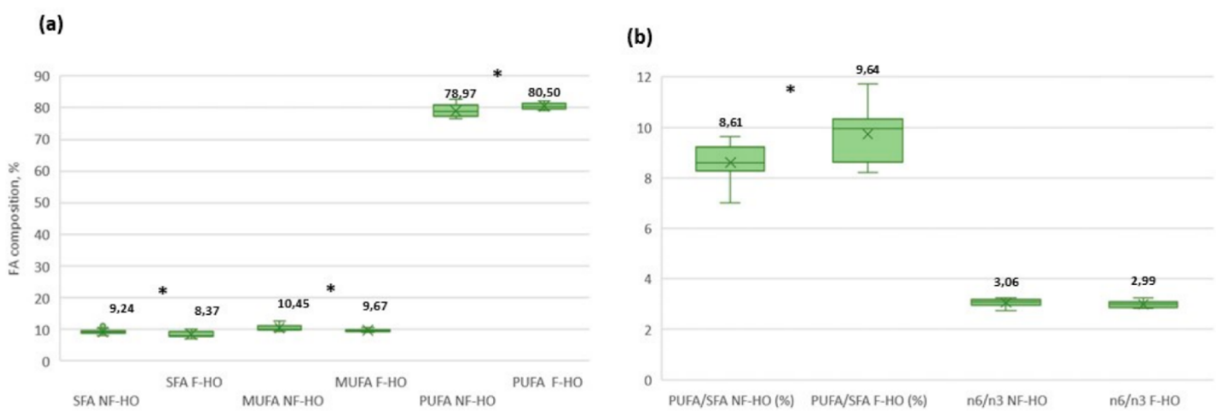


Figure 5. Main figures of the FA composition from total non-filtered (NF-HO) and filtered (F-HO) cold-pressed hempseed oil samples (a, b). Data are expressed as the mean ± sd of $n = 12$ oil bottles, each analyzed in triplicate. In every box, "×" indicates the average value, whereas the outlier points display the outlier data lying above the upper whisker. In the comparison between NF-HO and F-HO samples, "*" indicates significantly different values ($p < 0.05$ by Student's *t*-test) for a given parameter; conversely, the absence of "*" indicates non-significantly different values ($p > 0.05$ by Student's *t*-test).

Overall, the FA composition of the cold-pressed hempseed oil, revealed in both NF-HO and F-HO samples, was within the ranges reported by recent literature, which already highlighted high levels of linoleic (50–80%) and α-linolenic (15–25%) acids in the hempseed oil[33,34], as well as a peculiar amount of stearidonic acid (0.5–1.5%). [34] In addition, the oil from this study belonged to the Finola hemp variety, which has already been shown to contain up to 2% stearidonic acid and up to 4% γ-linolenic acid.[35]

Vegetable oils with a high unsaturation degree are characterized by a greater formation rate and amount of primary oxidation compounds accumulated over time.

The rates of autooxidation and $^1O_2$ oxidation depend on the rate of the lipid alkyl radical formation, which in turn depends mainly on the type of FA. In this respect, unsaturated FAs, such as oleic, linoleic, and linolenic acids, are particularly prone to oxidation and consequently more easily degraded in oil.

This would explain the reduction of MUFA and PUFA observed in the hempseed oils during 12 weeks of storage and, in general, the lower oxidative stability of high PUFA oils. The factor "light" must also be considered during storage since it is well known that the oxidation of unsaturated FAs is accelerated by exposure to light, especially when pigments, such as chlorophylls, are present in the oil. The influence of light on the FA composition of hempseed oil has not been investigated in any study.

However, Rastrelli and colleagues[36] found out that the sum of linoleic and α-linolenic acids decreased more significantly in the clear glass oil bottles than in the dark ones during 1 year of storage. Therefore, under our experimental conditions, both the oxygen dissolved in the oil and the light may contribute to the decreasing stability of unsaturated FAs in the hempseed oil.

Considering the filtration process, no significant differences in single FAs were revealed between F-HO and NF-HO, thus confirming that oil treatments do not cause drastic changes in the FA profile of a vegetable oil. However, Golimowsky and colleagues[37] recently applied a low-temperature bleaching to the cold-pressed hempseed oils with positive effects on the major classes of FAs rather than single FAs, as the oils displayed a reduced SFA proportion and a consequent growth in the proportion of PUFA and SFA/PUFA ratio. Accordingly, results from this study pointed out that filtration may better preserve the MUFA and PUFA of the cold-pressed hempseed oil during storage.

Tocopherols are the most important antioxidants present in vegetable oils since they reduce the extent of oil autooxidation by competing with unsaturated FAs for alkoxyl and peroxyl radicals in synergistic action with polyphenols. In the present study, the content of α-, γ-, and δ-tocopherol in NF-HO and F-HO samples stored for 12 weeks in transparent glass bottles is illustrated in Figure 6. As expected, fresh NF-HO and F-HO displayed very different contents of α- (42.03 mg/kg and 8.44 mg/kg), γ- (1059.56 mg/kg and 172.94 mg/kg), and δ- (33.37 mg/kg and 6.44 mg/kg) tocopherols. Additionally, a further reduction in all isomers occurred in all oils with increasing storage time, especially at the initial stage (T0-T4).

Specifically, γ-tocopherol decreased in NF-HO from 1059.56 mg/kg to 549.16 mg/kg ($p < 0.05$) over the study period, with a 24% reduction observed during T0-T4, while in F-HO it showed a reduction from 172.94 mg/kg to 110.14 mg/kg ($p < 0.05$), with a 12% loss observed during the first 4 weeks of storage. Alpha-tocopherol varied in NF-HO from 42.03 mg/kg to 14.64 mg/kg ($p < 0.05$) during 12 weeks of storage, lowering by 28% during T0-T4. The same isomer was reduced in F-HO from 8.44 mg/kg to 4.25 mg/kg ($p < 0.05$), with an 18% decrease during T0-T4. Delta-tocopherol was lowered in the range 33.37–24.31 mg/kg ($p < 0.05$) and 6.44–3.76 mg/kg ($p > 0.05$), respectively, in NF-HO and F-HO samples stored over T0-T12, with respective reduction rates of 19% and 9% during the first 4 weeks of storage (Figure 6 a, b).

Differently from other isomers, α-tocopherol showed higher degradation rates in NF-HO along with statistically significant quantitative changes ($p < 0.05$) observed at every storage step. Conversely, it had a lower consumption rate in F-HO samples, together with relatively stable contents during T4-T12 (Figure 6 a, b). Overall, the filtration procedure demonstrated that it markedly affected the content of tocopherols in oil. In fact, NF-HO samples showed higher mean tocopherol contents than F-HO (γ-tocopherol: 790.00 mg/kg vs. 139.61 mg/kg, $p < 0.05$; α-tocopherol: 27.82 mg/kg vs. 6.21 mg/kg, $p < 0.05$; δ-tocopherol: 27.90 mg/kg vs. 5.47 mg/kg, $p < 0.05$) (Figure 6c). Additionally, from the data discussed above, it is evident that filtration affected the degradation rate of tocopherols, since it was generally more pronounced in NF-HO than F-HO samples. Cold-pressed hempseed oils from this study shared the highest content of γ-tocopherol followed by α- and δ-isomers, thus resulting in line with the previous literature[26,27]. However, while the freshly produced NF-HO samples were marked by tocopherol levels consistent with most recent studies dealing with non-treated hempseed oils (Table 2), the F-HO samples were characterized by much lower amounts of such antioxidants. However, differently from this study, Tura and colleagues[28] did not find significant differences between filtered and non-filtered commercial hempseed oils.

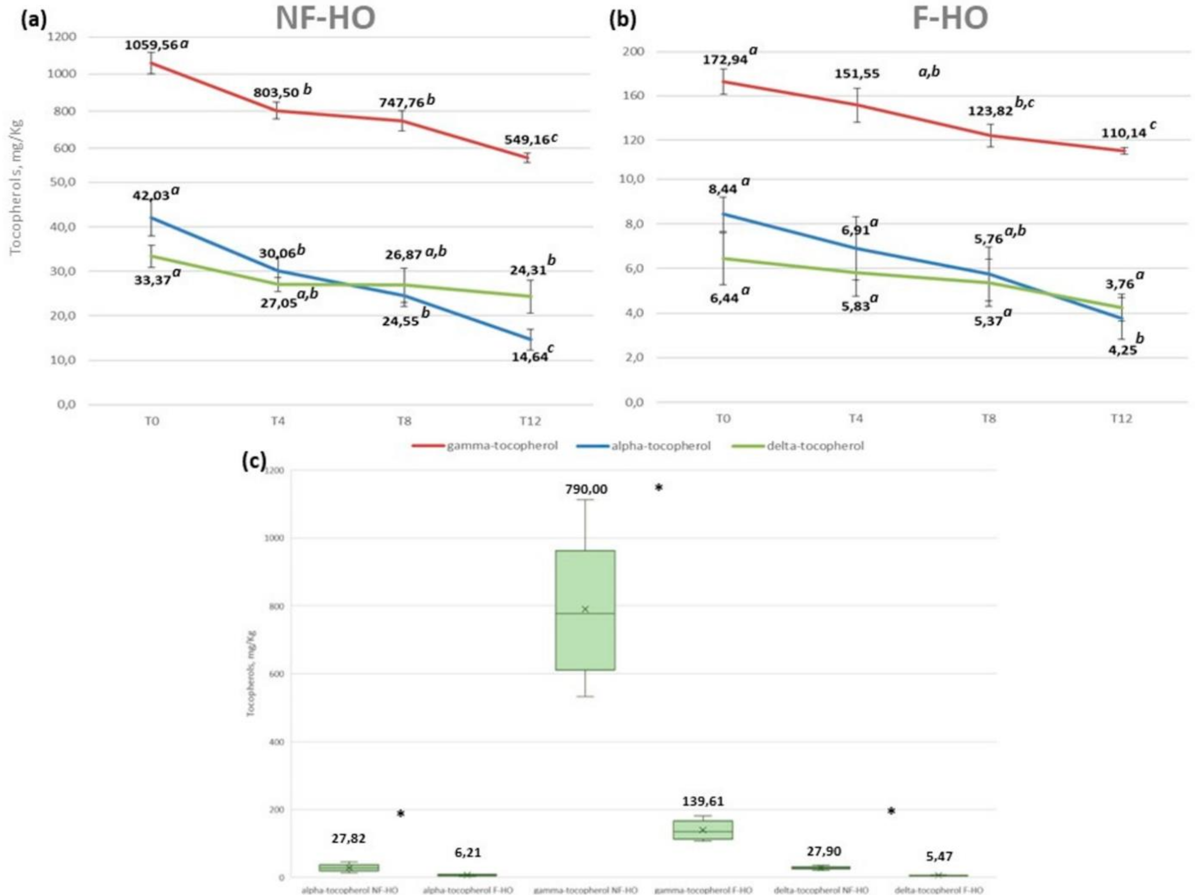


Figure 6. Evolution of the content of α- γ- and δ-tocopherol in non-filtered (NF–HO, (a)) and filtered (F–HO, (b)) cold-pressed hempseed oil during 12 weeks of storage in transparent glass bottles. Data are expressed as the mean ± sd of n = 3 oil bottles, each analyzed in triplicate. a–c: different superscript letters in the same line indicate significantly different values for a given parameter ($p < 0.05$ by post hoc Tukey's HSD test); the same superscript letters in the same line indicate not significantly different values ($p > 0.05$ by post hoc Tukey's HSD test). Figure (c) illustrates the α- γ- and δ-tocopherol of total NF-HO and F-HO samples. Data are expressed as the mean ± sd of n = 12 oil bottles, each analyzed in triplicate. In each box, "×" indicates the average value. In the comparison of every analyte between NF-HO and F-HO samples, "*" indicates significantly different values ($p < 0.05$ by Student's t-test).

Figure 7 illustrates the trend of total chlorophylls (intended as the sum of chlorophylls a + b) and total carotenes recorded in NF-HO and F-HO samples over 12 weeks of storage in transparent glass bottles.

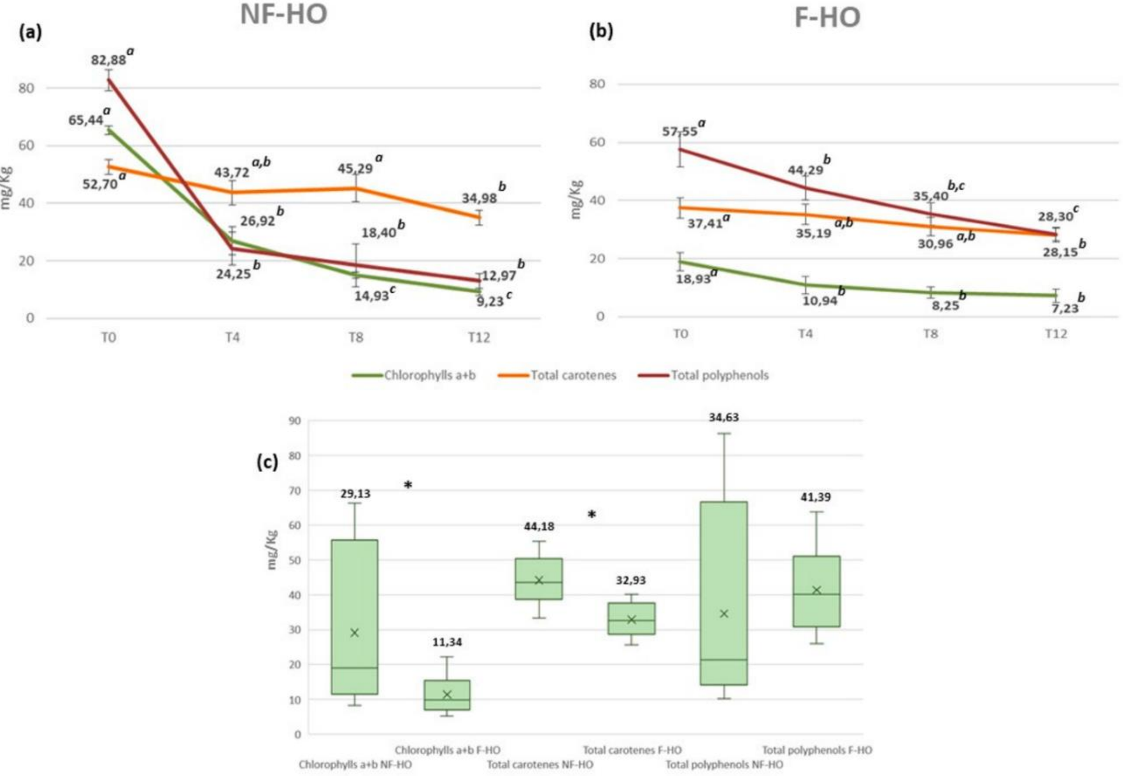


Figure 7. Evolution of the content of chlorophylls a + b, total carotenes, and total polyphenols in non-filtered (NF–HO, (**a**)) and filtered (F–HO, (**b**)) cold-pressed hempseed oil during 12 weeks of storage in transparent glass bottles. Data are expressed as the mean ± sd of $n = 3$ oil bottles, each analyzed in triplicate. $^{a–c}$: different superscript letters in the same line indicate significantly different values for a given parameter ($p < 0.05$ by post hoc Tukey's HSD test); the same superscript letters in the same line indicate not significantly different values ($p > 0.05$ by post hoc Tukey's HSD test). Figure (**c**) illustrates chlorophylls a + b, total carotenes, and total polyphenols of all NF-HO and F-HO samples. Data are expressed as mean ± sd of $n = 12$ oil bottles, each analyzed in triplicate. In each box, "×" indicates the average value. In the comparison of every analyte between NF-HO and F-HO samples, "*" indicates significantly different values ($p < 0.05$ by Student's *t*-test); conversely, the absence of "*" indicates non-significantly different values ($p > 0.05$ by Student's *t*-test).

The filtration induced a consistent pigment decrease in fresh F-HO compared to the NF-HO counterpart (chlorophylls: 65.44 mg/kg and 18.93 mg/kg; carotenes: 52.70 mg/kg and 37.41 mg/kg). A further reduction was observed with increasing storage weeks. Specifically, total chlorophylls and carotenoids reduced from 65.44 mg/kg to 9.23 mg/kg ($p < 0.05$) and from 52.70 mg/kg to 34.98 mg/kg ($p < 0.05$) in NF-HO samples, with the greatest reduction rates at T0-T4 (respectively, −59% and −17%). A downward trend was also observed in the F-HO samples, as chlorophylls a + b and carotenoids decreased in the ranges of 18.93–7.23 mg/kg ($p < 0.05$) and 37.41–28.15 mg/kg ($p < 0.05$), with the highest deterioration rates equal to −42% and −6% observed after 4 weeks of storage. For both types of pigment, high degradation rates were recorded in NF-HO samples along with statistically significant quantitative changes ($p < 0.05$) during T4-T12. Conversely, F-HO samples displayed lower consumption rates together with relatively stable pigment contents after 4 weeks of storage (Figure 7 a, b). Additionally, although a proper color assessment could not be made, fresh NF-HO samples were characterized by a deep and cloudy green color that turned brown at the end of storage, whereas fresh F-HO samples had a more stable color that varied from a brilliant yellow to a slightly darker hue after 12 weeks of storage. As expected, filtration had a great impact on such pigments, as all NF-HO and F-HO samples showed a mean chlorophyll level amounting to 29.13 mg/kg and 11.34 mg/kg ($p < 0.05$) and a mean carotene content equal to 44.18 mg/kg and 32.93 mg/kg ($p < 0.05$) (Figure 7 c).

Total chlorophylls tend to degrade to pheophytins and pheophorbides, with greater rates observed during the initial stages of storage and upon exposure to light. Chlorophylls and derivatives are primarily involved in the photooxidation process since they can act as sensitizers in the presence of light and $^3O_2$ to produce $^1O_2$, thus causing a faster deterioration of the oxidative stability, color change, and an inevitable reduction in the nutritional and economic value of the oil[38]. On the other hand, carotenoids slow down photooxidation by light filtering, $^1O_2$ quenching, sensitizer inactivation, and free radical scavenging.

Among carotenoids, β-carotene could be protected from degradation by α-tocopherol, to prevent both the autooxidation and the photooxidation of an oil with a synergistic effect. In the presence of chlorophyll, carotenoids counteract the progress of photooxidation by physical or chemical $^1O_2$ quenching. However, while the physical quenching does not alter their structure, the chemical quenching leads to their degradation and conversion into epoxide or carbonyl derivatives. As a result, an overall reduction in carotenoids in the oil, as well as its discoloration, can be expected to occur during storage and in the presence of light[39].

Results from this study also pointed out that filtration lowered the pigment content of the fresh hempseed oil and, at the same time, reduce its consumption during storage. Indeed, greater pigment deterioration rates were recorded in NF-HO samples than in F-HO samples over the study period. This may be explained by the higher content of chlorophyll in non-filtered oils, which, reacting with the atmospheric oxygen ($^3O_2$) dissolved in the oil, triggers the process of photooxidation to a greater extent, especially at the initial stages of storage.

As a result, carotenoids were also consumed at higher rates in non-filtered oils to counteract the oxidation process. Therefore, oil filtration has become highly desirable not only to limit the photooxidation process by chlorophylls and derivatives but also to slow down the consumption of antioxidant carotenoids over time.

Similarly to pigments, fresh NF-HO showed a higher level of total phenols than fresh F-HO samples, and an overall reduction in such secondary metabolites was observed in both series of hempseed oil over storage. Specifically, total polyphenols decreased in the range 82.88–12.97 mg/kg ($p < 0.05$) and 57.55–28.30 mg/kg ($p < 0.05$) respectively in NFHO and F-HO samples (Figure 7a, b), with higher oxidation rates recorded in NF-HO than F-HO samples, especially in the early storage (T0-T4: −71% and −23%, respectively). However, both arrays of oil samples shared a non-statistically significant reduction in total polyphenols at every storage step analyzed during T4-T12 ($p > 0.05$, Figure 7a, b). Interestingly, F-HO was characterized by a non-statistically significant higher total phenol content than NF-HO (respectively, 41.39 mg/kg and 34.63 mg/kg, $p > 0.05$) (Figure 7c).

The activity of polyphenols is notably supported by the presence of tocopherols in the oil. In fact, a clear synergism between polyphenols, acting both as metal chelators and radical scavengers, and tocopherols, acting as radical scavengers, has already been displayed in previous studies dealing with cold-pressed oils with high PUFA levels. However, the antioxidant activity of phenolics is strictly related to the compound family and content, which in turn depend on genotype, geographical origin, cultivation practices of the hemp, and extraction and storage conditions of the oil.

While considering that total polyphenols by the Folin–Ciocalteu method might be biased by several interfering non-phenolic compounds, such as amino acids, ascorbic acid, reducing sugars, and transition metals. The total polyphenol content of fresh oils from our study would be more similar to that reported by Tura and colleagues[27].

Indeed, these studies revealed that the oxidation of phenolic compounds was linearly related to the formation of primary oxidation products over storage, probably because they were increasingly involved in the scavenging of hydroperoxide radicals. The influence of light on the degradation rate of polyphenols was studied in olive oil stored in dark and transparent glass bottles, and non-significant changes were observed in the content of such secondary metabolites over time. Therefore, it can be concluded that, under our experimental conditions, the oxygen dissolved in the oil and the content of primary oxidation products would most affect the stability of polyphenols[40].

The filtration process had a clear effect on the cold-pressed hempseed oil. From the obtained results, it is evident that filtration can lower the water-soluble phenolic content in the fresh hempseed oil and at the same time reduce its deterioration rate during storage. This was probably because filtration improved the oxidative stability of the hempseed oil, with relevant implications for reduced consumption and better stability of the polyphenol content during storage. On the other hand, the lower oxidative stability of non-filtered oils resulted in a faster consumption of these antioxidants.

The literature showed contradictory results on the influence of filtration and storage on the polyphenol content of vegetable oils. Some studies confirmed the progressive decrease in polyphenols during the storage of virgin olive oils. However, higher deterioration rates were

recorded in filtered oils than in non-filtered oils, which consequently showed higher total phenol contents. Conversely, other research highlighted minimal or no differences in total phenols between filtered and non-filtered olive oils during storage.

Figure 8 reports the content of squalene in NF-HO and F-HO samples over the considered storage period. As expected, fresh NF-HO showed a higher amount of squalene than fresh F-HO (557.01 mg/kg and 152.22 mg/kg).

However, squalene decreased over storage in both NF-HO and F-HO within the respective ranges of 557.01–280.53 mg/kg ($p < 0.05$) and 152.22–92.38 mg/kg ($p < 0.05$), again with more pronounced reduction rates observed in NF-HO than F-HO samples, especially during the first 4 weeks of storage (respectively, −23% and −21%) (Figure 8 a, b). On average, NF-HO was characterized by a higher amount of squalene than F-HO (respectively, 401.74 mg/kg and 117.71 mg/kg, $p < 0.05$) (Figure 8b).

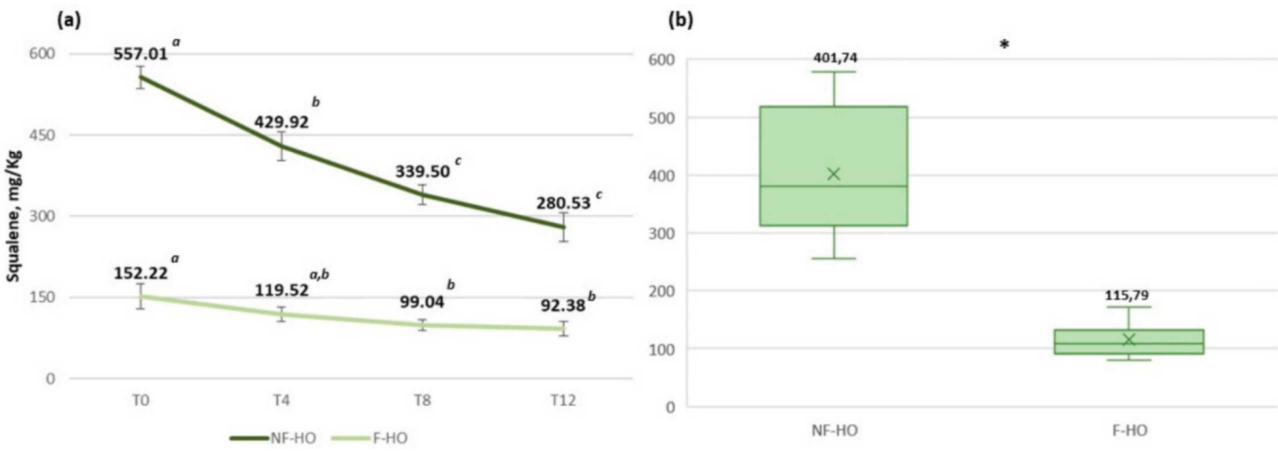


Figure 8. Evolution of the content of squalene in non-filtered (NF-HO) and filtered (F-HO) cold pressed hempseed oil during 12 weeks of storage in transparent glass bottles (**a**). Data are expressed as the mean ± s.d. of n = 3 oil bottles, each analyzed in triplicate. a–c: different superscript letters in the same line indicate significantly different values for a given parameter ($p < 0.05$ by post hoc Tukey's HSD test); the same superscript letters in the same line indicate not significantly different values ($p > 0.05$ by post hoc Tukey's HSD test). Figure (**b**) illustrates the mean content of squalene in all NF-HO and F-HO samples. Data are expressed as the mean ± s.d. of n = 12 oil bottles, each analyzed in triplicate. In each box, "_" indicates the average value. In the comparison of squalene between NF-HO and F-HO samples, "*" indicates significantly different values ($p < 0.05$ by Student's t-test).

The influence of filtration and storage on the content of squalene has not yet been explored in minor cold-pressed oils. However, the consumption of this antioxidant typically occurs during the storage of virgin olive oil, and the diffuse lighting of the oil did not seem to play a major role in the degradation process. As a result, the consumption of this compound observed in the hempseed oils during storage would be attributable to the scavenging of peroxyl radicals and the consequent conversion into quite stable degradation products that are not involved in further propagation reactions[41]. As proof, the olive oil refined and subsequently enriched with squalene showed lower acidity, PV, and absorbances at 237 nm and 270 nm with respect to the crude olive oil during storage, thus confirming the role of such antioxidants in the delay of autooxidation.[42] The profile of inorganic elements in NF-HO and F-HO samples recorded during storage is reported in Table 10. Freshly produced NF-HO and F-HO samples were characterized by similar element profiles.

Specifically, Na was found at the highest content (NF-HO: 238.95 mg/kg and F-HO: 254.14 mg/kg), followed by other major elements, such as K (NF-HO: 46.40 mg/kg and F-HO: 45.39 mg/kg), and Mg (NF-HO: 13.99 mg/kg and F-HO: 12.92 mg/kg).

Among essential trace elements, the most abundant elements were Fe (NF-HO: 4.11 mg/kg and F-HO: 5.28 mg/kg) and Zn (NF-HO: 7.86 mg/kg and F-HO: 7.95 mg/kg). Considering the potentially toxic trace metals, Al was the most concentrated metal (NF-HO: 5.74 mg/kg and F-HO: 4.58 mg/kg), followed by a much lower amount of Pb (NF-HO: 0.26 mg/kg and F-HO: 0.41 mg/kg). Additionally, both fresh NF-HO and F-HO samples were marked by levels of Pb and As lower than the limit fixed by the Codex Alimentarius for such heavy metals (0.1 mg/kg).

For greater convenience, only the data from T0 and T12 were reported in Table 10, as both NF-HO and F-HO samples showed overlapping and non-statistically different element contents ($p > 0.05$) from the start to the end of the storage period. Not only the storage in clear glass bottles but also the filtration process did not alter these minor components in the cold-pressed hempseed oil since, as reported in Figure 9, all NF-HO and F-HO samples showed non-significantly different mean concentrations of each investigated analyte ($p > 0.05$).

Table 10. Element profile (mg/kg) of non-filtered (NF-HO) and filtered (F-HO) cold-pressed hempseed oils before the experimental trial (T0) and after 12 weeks of storage in transparent glass bottles (T12). Data are expressed as the mean ± sd of n = 3 oil bottles, each analyzed in triplicate. Limit of Detection (LOD) of Se: 0.002 mg/kg.

	NF-HO		**F-HO**	
	T0	**T12**	**T0**	**T12**
Na	238.95 ± 17.54[a]	233.95 ± 21.91[a]	254.14 ± 6.71[a]	246.83 ± 13.30[a]
Mg	13.99 ± 3.22[a]	12.45 ± 2.76[a]	12.92 ± 3.71[a]	11.22 ± 1.11[a]
K	46.40 ± 4.67[a]	48.47 ± 5.25[a]	45.39 ± 6.31[a]	47.80 ± 10.28[a]
Fe	4.11 ± 2.36[a]	3.17 ± 0.89[a]	5.28 ± 2.54[a]	4.84 ± 1.42[a]
Cu	0.028 ± 0.026[a]	0.071 ± 0.035[a]	0.047 ± 0.025[a]	0.070 ± 0.019[a]
Mn	1.17 ± 0.20[a]	1.25 ± 0.19[a]	1.12 ± 0.23[a]	1.43 ± 0.58[a]
Zn	7.86 ± 3.28[a]	6.49 ± 1.46[a]	7.95 ± 2.42[a]	5.83 ± 1.50[a]
Se	<LOD	<LOD	<LOD	<LOD
Ni	0.023 ± 0.009[a]	0.014 ± 0.004[a]	0.016 ± 0.004[a]	0.020 ± 0.009[a]
Cr	0.008 ± 0.003[a]	0.006 ± 0.002[a]	0.006 ± 0.004[a]	0.007 ± 0.003[a]
Al	5.74 ± 2.15[a]	5.74 ± 1.28[a]	4.58 ± 2.60[a]	4.94 ± 2.88[a]
Cd	0.08 ± 0.004[a]	0.011 ± 0.005[a]	0.015 ± 0.004[a]	0.012 ± 0.006[a]
Pb	0.26 ± 0.08[a]	0.33 ± 0.07[a]	0.41 ± 0.24[a]	0.38 ± 0.14[a]
As	0.08 ± 0.004[a]	0.009 ± 0.001[a]	0.009 ± 0.003[a]	0.009 ± 0.007[a]

The same superscript letter “a” in the same line indicates not significantly different values (p > 0.05 by post hoc Tukey’s HSD test).

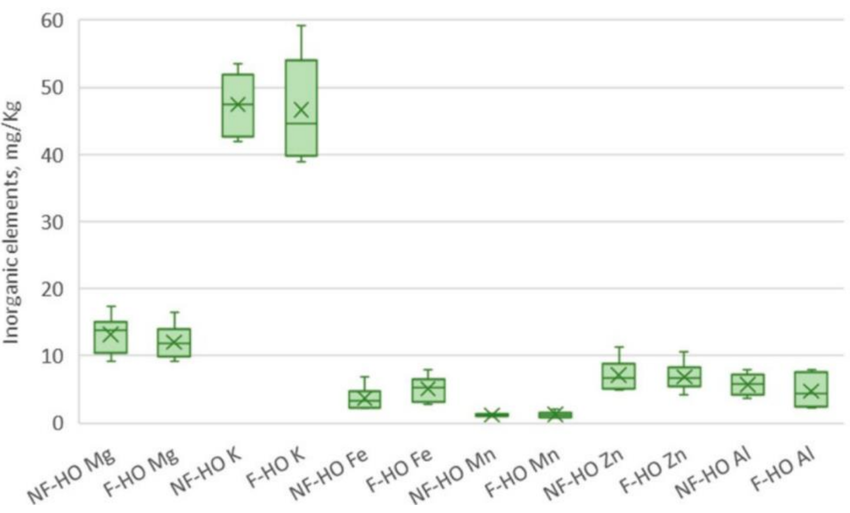


Figure 9. Mean content (mg/kg) of the most abundant elements present in total non-filtered (NF-HO) and filtered (F-HO) cold-pressed hempseed oil samples. Data are expressed as the mean ± sd of n = 12 oil bottles, each analyzed in triplicate. For each element, “×” indicates the average value. In the comparison of each element between NF-HO and F-HO samples, the absence of asterisk indicates non-significantly different values (p > 0.05, by Student’s t-test).

The effect of storage and filtration processes on the inorganic elements of the cold-pressed hempseed oil during storage has not been addressed elsewhere, nor is there any comparative literature on other vegetable oils. The stable and comparable element profile obtained during the storage of both NF-HO and F-HO may be related (i) to the absence of inorganic matter decomposition during the storage of the oils and, not least, (ii) to the non-responsiveness of these minor compounds to the filtration process. However, this compositional aspect should be worthy of more in-depth investigation, as the oxidative stability of edible oils is influenced not only by storage conditions, energy inputs, oxygen type, and triacylglycerol matrix but also by a variety of minor compounds, including metals. In fact, metals catalyze the initiation step in the autooxidation, and, not least, they produce $^1O_2$ and hydroxy radicals, respectively, from $^3O_2$ and hydrogen peroxides, which inevitably accelerate the overall oil oxidation. Among elements, transition elements such as Fe and Cu are known to accelerate these processes[32]. Additionally, Fe also causes the decomposition of phenolics, thus further deteriorating the oil's oxidative stability [43].

9.2.8. Conclusions

The present study showed that the filtration treatment could improve the oxidative stability and extend the shelf life of the hempseed oil bottled in transparent glass, resulting in an effective and sustainable alternative to other severe refining procedures in view of maintaining the good nutritional standards typical of cold-pressed oils. Benefits from the hempseed oil filtration include, certainly, an improvement in the hydrolytic and oxidative status, as well as a better preservation of MUFA and PUFA content within 12 weeks of storage. By removing a large amount of chlorophyll, filtration can not only produce lighter and brighter-colored oils but also promote the use of clear glass bottles for packaging, which has a positive impact on consumer acceptance. As expected, fresh filtered oils were characterized by reduced carotenoids, tocopherols, polyphenols, and squalene. However, findings from this study highlighted the "protective effect" of filtration on such

antioxidants, resulting in lower degradation rates and better preservation of these minor compounds than crude oils over storage.

9.3. MONITORING CANNABINOIDS AND THE SAFETY OF THE TRACE ELEMENT PROFILE OF LIGHT CANNABIS SATIVA L. FROM DIFFERENT VARIETIES AND GEOGRAPHICAL ORIGIN

The term "cannabis" refers to the plant native to Central Asia which belongs to the genus of *Cannabaceae* of the *Moraceae* family. Its taxonomy has always been quite complex and troublesome. However, the genus is monospecific (*Cannabis sativa* L.), and includes two subspecies (*Cannabis sativa* L. subsp. sativa and *Cannabis sativa* L. subsp. indica (Lam.)) which are, in turn, characterized by several varieties, conferring to the genus a relevant genetic variability. Cannabis varieties show a variable degree of adaptation to different environmental conditions, versatility and suitability for processing, also in relation to the geo pedoclimatic conditions of growth and agronomic requirements[44]. *C. sativa* L. is a herbaceous plant with an annual cycle, and it is a dioecious species with female or male flowers borne either individually or in conspicuous inflorescences. However, only the female inflorescences can produce more than 100 cannabinoids, the most important ones being Δ9- tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN)[45].

THC is well known to induce psychoactive effects by directly acting on the cannabinoid receptors mostly present in the brain areas devoted to temporal and sensory perception, pleasure, thinking, memory and coordination; while CBD and CBN are molecules with no psychotropic effects and different therapeutic properties, such as pain-relieving and relaxing effects, as well as antibacterial, antioxidant and anti-inflammatory activities[45]. Additionally, CBD may contrast some negative effects of THC. The THC and CBD content, and the derived THC/CBD ratio, notoriously allow it to establish the type of cannabis, or its "chemotype"[45]. On this basis, three categories can be

outlined: (i) the cannabis with chemotype >1 (chemotype I), THC between 0.3–20% and CBD <0.5%, which is typically exploited for the preparation of well-known drugs, such as hashish and marijuana; (ii) the cannabis with chemotype = 1 (chemotype II), THC between 0.3–2% and CBD >0.5%, which can be employed for the treatment of various pathological conditions, such as chronic pain, multiple sclerosis or spinal cord injury, as well as anxiety or depressive disorders [46]; and (iii) the cannabis with chemotype <1 (chemotype III), THC <0.3% and CBD, typically varying between 2–3%, and potentially reaching up to 40%, is commonly defined as "industrial" or "light" cannabis or, more simply, hemp. According to the Council Directive 2002/53/EC, the cultivation of hemp is allowed by using seeds only from certain certified cannabis varieties, which are included in the EU catalog updated from year to year[47]. EU Regulations no. 1307/2013 and 1155/2017 and the Italian Law n. 242/2016 encourage the cultivation of such cannabis to produce food, cosmetics and semi-finished products (i.e., fiber, powder, oil or fuel), as well as material aimed at bioengineering, green building or phyto-remediation of contaminated sites[48]. Lately, such products have been also defined by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) as "Low-THC cannabis products" [48]. Many studies have already defined hemp as an excellent phytoremediation agent, since it can adsorb from soil and accumulate potentially toxic elements, such as lead (Pb), nickel (Ni), cadmium (Cd), arsenic (As), mercury (Hg) and chromium (Cr), thanks to the type of root it possesses. Hemp has already been tested for its heavy metal tolerance and accumulation in semi-natural conditions, and for cleaning up environments with emulated Chernobyl conditions based on radio cesium levels (^{137}Cs). The latter experiment, specifically, led to the detection of radioactivity in all plant tissues, as well as retting water, fiber and seed oil that could potentially end up in the hands of consumers. However, there are several factors that may influence the extent of adsorption and accumulation of metals by *C. sativa* L. These include soil pH, its organic matter content, redox potential, clay content, cation exchange capacity, nutrient balance, humidity and temperature. While hemp has proved to be valuable for the soil remediation of contaminated areas, its tendency to adsorb toxic elements from soil may pose a serious threat to consumers.

This is especially true for the extracts from the hemp inflorescence and fruit, which are considered "Novel Food" due to the relevant levels of CBD and other non-psychoactive cannabinoids, according to the Regulation (EU) n. 2283/2015. Nonetheless, the law calls also for the continuous evaluation of the safety of any product able to be placed on the EU market as novel food or food ingredient. For this reason, the levels of metals present in hemp products should be constantly monitored, and suitable regulatory standards should be developed to keep them under control. On this basis, an array of inflorescences produced in four Italian areas and belonging to different varieties of *C. sativa L*. (chemotype III) were investigated to evaluate the compliance with the Law n. 242/2016, by monitoring the contents of main cannabinoids, and their safety, by the screening of trace elements. The experimental dataset was statistically explored to evaluate the power of cannabinoids and trace elements on the discrimination of investigated inflorescences based on botanical variety and/or geographical origin.

9.3.1. Samples

In this study, 24 types of dried inflorescences from *Cannabis sativa* L. (chemotype III) were provided during April–June 2022 by seven agricultural cooperatives distributed throughout Italy and dedicated to the outdoor cultivation of industrial hemp and the consequent processing of the inflorescences. For the sampling, six different varieties of *C. sativa* L. (i.e., Finola, Antal, Futura 75, Tiborzallasi, Kompolti and Carmagnola) grown in four Italian regions (i.e., Sicily, Lazio, Apulia and Lombardy) were collected in triplicate, for a total of 72 samples. Further details on the inflorescences, their variety and geographical origin are shown in Table 11. All inflorescence samples were packed in polyethylene bags and transported to the laboratory, where they were removed from their twigs, the ground, and passed through a 150-mesh sieve to obtain fine powders. All samples were stored at room temperature until further analysis.

Table 11. List of the inflorescence samples collected for the present study.

Sample code	Species	Variety	Geographical origin	No. samples
Finola_SR	*C.sativa L*	Finola	Siracusa (Sicily)	3
Antal_SR		Antal		3
Futura75_SR		Futura75		3
Tiborszallasi_SR		Tiborszallasi		3
Kompolti_SR		Kompolti		3
Carmagnola_SR		Carmagnola		3
Finola_RM		Finola	Rome (Lazio)	3
Antal_RM		Antal		3
Futura75_RM		Futura75		3
Tiborszallasi_RM		Tiborszallasi		3
Kompolti_RM		Kompolti		3
Carmagnola_RM		Carmagnola		3
Finola_BA		Finola	Bari (Apulia)	3
Antal_BA		Antal		3
Futura75_BA		Futura75		3
Tiborszallasi_BA		Tiborszallasi		3
Kompolti_BA		Kompolti		3
Carmagnola_BA		Carmagnola		3
Finola_LO		Finola	Lodi (Lombardy)	3
Antal_LO		Antal		3
Futura75_LO		Futura75		3
Tiborszallasi_LO		Tiborszallasi		3
Kompolti_LO		Kompolti		3
Carmagnola_LO		Carmagnola		3
Total samples				**72**

9.3.2. Cannabinoid Analysis

Around 0.2 g of sample was weighed, mixed with 2 mL of petroleum ether, and sonicated in an ultrasonic bath (FALC instruments ultrasonic, Treviglio, Italy) for 15 min at room temperature. Subsequently, the sample was filtered and evaporated by means of a rotary evaporator (Buchi B-491, Büchi, Flawil, Switzerland). Hence, the dried extract was resuspended in chloroform, added with 0.5 mL of the internal standard 4-androstene-3,17-dione (final concentration, 3mg/mL) and injected into a gas chromatograph (GC) equipped with a split/splitless injector and a flame ionization detector (FID) (Dani Master GC1000, Dani Instrument, Milan, Italy). A SPB-1 column (15 m × 0.2 mm i.d., 0.2 μm film thickness, Supelco, Sigma Aldrich, St. Louis, MO, USA) was used.

For the analysis, the following operating conditions were employed: column oven temperature from 200°C (hold time 5 min) to 280 °C (hold time 17 min) at 4 °C/min; injector and detector temperatures, 280 °C. Carrier and makeup gases were He: 30 cm/s, N_2: 25 mL/min; H_2: 40 mL/min, air: 280 mL/min. The injection volume was 1 μL, with a split ratio of 1:100 and a split flow of 50

mL/min. Data handling and acquisition were performed by means of the Clarity Chromatography Software v. 4.0.2 (Dani Instrument, Milan, Italy). Cannabinoids such as THC, CBD and CBN were identified by direct comparison with the retention times of reference compounds and quantified by appropriate external calibration curves with an internal standard normalization. All samples were run in triplicate.

9.3.3. Mineral elements analysis

Element analysis involved a sample pretreatment based on a process of acid digestion conducted in a closed-vessel microwave digestion system (ETHOS 1, Milestone, Bergamo, Italy). Approximately 0.3 g of each sample was accurately weighed in PTFE vessels, added with 1 mL of internal standard Re at a concentration of 0.5 mg/L, and mixed with 7 mL of HNO_3 and 1 mL of H_2O_2. The operating conditions of the digestion program were: 20 min at a temperature ranging from 0 to 180°C (step 1), 15 min at a constant temperature of 180°C (step 2) with a constant power microwave of 1100 W. Subsequently, samples were cooled at room temperature, diluted to a volume of 25 mL with ultrapure water and filtered using 0.45 μm PTFE filters. Both the blank solution (HNO_3 and H_2O_2, 7:1 v/v) and the certified reference material (white clover- BCR402, Institute for Reference Materials and Measurements, European Commission, Joint Research Centre, Belgium) were prepared under the same conditions as the samples. The certified reference material (white clover-BCR402) contained the following mineral elements: Cr, Fe, Co, Ni, Zn, As, Se, Mo.

The screening of trace essential (Fe, Mn, Cu, Mo, Zn, Se, Co, Cr, Ni) and potentially toxic elements (As, Cd, Pb, Hg, Al) was carried out by the single quadrupole inductively coupled plasma-mass spectrometer (ICP-MS, iCAP-Q, Thermo Scientific, Waltham, USA) powered by a 27 MHz radiofrequency solid-state generator, and equipped with a PFA cyclonic spray chamber with a port accepting a 4 mm i.d. and 6 mm o.d. nebulizer, nickel sampler and skimmer cones of 1.1 mm and 0.5 mm. The instrument was also provided with an autosampler (ASX520, Cetac Technologies Inc., Omaha, NE, USA) coupled to an integrated sample introduction system.

Before analysis, the ICP-MS method was optimized to reduce spectral (polyatomic and isobaric) and non-spectral interferences potentially affecting the multianalyte determination. For each investigated analyte, isobaric interferences were monitored by means of its several isotopes and corrected according to the most abundant isotope. As a result, 27Al, 52Cr, 55Mn, 56Fe, 59Co, 60Ni, 63Cu, 66Zn, 75As, 80Se, 98Mo, 114Cd and 208Pb were the isotopes monitored in all samples. On the other side, non-spectral interferences were reduced by means of online internal standards with a mass number close to that of the analytes to minimize instrumental drifts and matrix effect. Hence, 45Sc was chosen to monitor the signal of 52Cr, 55Mn, 56Fe and 59Co; while 73Ge for 60Ni, 63Cu, 66Zn, 75As, 80Se and 98Mo; 115In for 111Cd; and 209Bi for 208Pb.

Inflorescence samples were then analyzed according to the following conditions: RF power, 1550 W; plasma gas (Ar) flow rate, 14 L/min; auxiliary gas (Ar), flow rate, 0.8 L/min; carrier gas (Ar) flow rate, 1.1 L/min; collision gas (He) flow rate, 4.7 mL/min; spray chamber temperature, 2.7°C; sample depth and sample introduction flow rate, respectively 5 mm 0.93 mL/min. Integration times were 0.5 s/point for V, Fe, Se and As, and 0.1 s/point for the other elements. To integrate the peaks, 3 points for each mass and 3 replicate acquisitions were taken. Thermo Scientific Qtegra™ Intelligent Scientific Data System software (Thermo Scientific, Waltham, MA, USA) was employed for instrumental control and data acquisition. An external calibration procedure, based on the construction of seven-point calibration plots, with an internal standard normalization was adopted for quantitative purposes. All samples were analyzed in triplicate, along with analytical blanks.

Hemp samples were also assessed for the Hg content by a Direct Mercury Analyser (DMA-80, Milestone S.r.l., Italy), based on the thermal decomposition amalgamation-atomic absorption spectrophotometry (TDA-AAS). DMA-80 was used instead of ICP-MS for several reasons. Firstly, it is a more versatile analytical instrumentation than ICP-MS, as it allows direct sample analysis, without the need for pre-treatment.

In addition, operators do not encounter mercury exposure. Another important reason is that mercury analysis by ICP-MS requires the use of additional instrumental devices to clean the entire

instrumentation, as mercury tends to accumulate in different parts and cause saturation phenomena in the instrument. This problem is overcome, however, in the DMA-80 where mercury vapours are trapped thanks to a gold amalgamator inside the instrumentation.

Specifically, according to the EPA method 7473 (SW-846) [49]. Briefly, ~100 mg of every sample was initially dried at 200°C for 3 min and subsequently thermally decomposed at 650°C for 2 min. The Hg content was then determined by measuring absorbance at 253.7 nm according to an external calibration, exploiting a seven-point calibration curve.

The linearity of the signal concentration for every analyte was determined based on the linear least-square regression method. To this purpose, multi-standard solutions were used to construct seven-point calibration curves in the range 0.5–50.0 μg/L. However, the concentration range of the Hg curve was 5–100 μg/L. The obtained R^2 values ranged from 0.9992 (for Cu) to 0.9999 (for Cd). The following experimental formulas were used to calculate LOD and LOQ: 3.3 σ/b and 10 σ/b, respectively, where σ is the standard deviation of the analytical blank (n = 6) and b is the slope of the relative calibration curve. LODs ranged, respectively, from 0.001 μg/kg and 0.003 μg/kg (Co, As and Cd) to 0.067 μg/kg and 0.221 μg/kg (for Al) (Table 12).

Table 12. Analytical validation of ICP-MS and TDA-AAS methods of analysis performed in terms of linearity, LOD, LOQ, accuracy (n=6) and precision (n=6).

				BCR-402 (white clover)				
Analyte	**R^2**	**LOD (mg/Kg)**	**LOQ (mg/Kg)**	**Experimental value (mg/Kg)**	**Expected value (mg/Kg)**	**Recovery (%)**	**Precision (RSD%)**	
							Intraday	**Interday**
Al*	0.9995	0.067	0.221	1.96	2.00	98.00±0.50	1.1	1.3
Cr	0.9996	0.002	0.007	5.11	5.19	98.46±0.39	0.3	0.5
Mn*	0.9998	0.003	0.010	1.98	2.00	99.00±0.50	1.0	1.4
Fe	0.9995	0.024	0.079	250.1	244.00	102.50±0.45	0.2	0.5
Co	0.9998	0.001	0.003	0.172	0.178	96.63±1.12	0.4	0.7
Ni	0.9996	0.002	0.007	7.99	8.25	96.85±0.56	0.3	0.2
Cu*	0.9992	0.017	0.056	1.92	2.00	96.00±1.00	0.5	0.9
Zn	0.9994	0.061	0.201	24.8	25.2	98.41±0.79	1.2	1.5
As	0.9998	0.001	0.003	0.094	0.093	101.08±1.08	0.9	1.2
Se	0.9993	0.002	0.007	6.65	6.70	99.25±0.15	0.3	0.6
Cd*	0.9999	0.001	0.003	2.00	2.00	100.00±0.50	1.3	1.7
Pb*	0.9997	0.002	0.007	1.98	2.00	99.00±0.50	0.7	0.8
Mo	0.9997	0.002	0.007	6.78	6.93	97.84±0.44	0.3	0.4
Hg*	0.9998	0.002	0.007	1.96	2.00	98.00±1.00	0.6	1.0

*Analyte does not present in the certified matrix.

For the accuracy assessment, the BCR-402 (white clover) was analyzed in six replicates, and the difference between the mean experimental value and the reference value was reported as mean percent recovery. However, the accuracy of elements non-present in the reference material was estimated by the surrogate recovery.

In a separate experiment, a sample of inflorescences was spiked with a known number of elements such as Al, Mn and Cu and analyzed in replicate alongside the same unspiked sample. The mean difference between these two values corresponded to the recovered part of the spiked analyte. The lowest and the highest average recovery were observed, respectively, for Cu (96.00%) and Fe (102.50%). Repeatability was evaluated in terms of precision, by considering the analyses of the certified matrix and the spiked sample performed in the same day, and intermediate precision, by considering a longer period of time (1 week). For the evaluation of precision, six replicates were carried out. Precision and intermediate precision, expressed as relative standard deviation (RSD%), were, respectively, below 1.3% and 1.7% (Table 12).

9.3.4. Statistical Analysis

The statistical data elaboration was performed by R studio version 3.6.1 (R Studio: Integrated Development for R., Boston, MA, USA) for Windows. A descriptive analysis, including mean, median and standard deviation, of inorganic elements and cannabinoids overall inflorescence samples was conducted and shown in Tables 13 and 14. After running a Shapiro–Wilk test to verify the normal distribution of experimental data, the one way-ANOVA was applied for every independent variable to produce an F-statistic, i.e., the ratio of the variance calculated among the means to the variance within the samples. Statistical significance was accepted when $F_{calculated} > F_{critical}$ ($F_{critical} = 1.75$ with $\alpha < 0.001$).

Then, an exploratory PCA was performed to explore sample discrimination in relation to the botanical variety and/or geographical origin of inflorescences.

Table 13. Percentage content of major cannabinoids (i.e., THC, CBD and CBN) in the different samples of inflorescences from *C. sativa* L. Results are expressed as mean ± standard deviation (mg/Kg, dw) of n=3 samples, where every sample was analyzed three times.

Sample	THC %	CBD %	CBN %
Finola_SR	0.28±0.03	5.55±0.99	0.07±0.00
Antal_SR	0.19±0.04	4.86±0.10	0.08±0.02
Futura75_SR	0.33±0.03	7.65±0.09	0.12±0.05
Tiborszallasi_SR	0.37±0.06	7.76±0.09	0.11±0.00
Kompolti_SR	0.34±0.06	7.76±0.13	0.07±0.00
Carmagnola_SR	0.08±0.03	5.30±1.06	0.04±0.00
Finola_RM	0.42±0.05	1.05±0.05	0.14±0.03
Antal_RM	0.37±0.12	1.24±0.08	0.22±0.00
Futura75_RM	0.33±0.06	2.12±0.12	0.11±0.01
Tiborszallasi_RM	0.36±0.06	3.26±1.21	0.20±0.06
Kompolti_RM	0.22±0.03	1.90±0.06	0.26±0.08
Carmagnola_RM	0.15±0.03	2.52±0.19	0.27±0.07
Finola_BA	0.25±0.04	5.74±0.05	0.12±0.01
Antal_BA	0.25±0.06	7.62±0.09	0.26±0.01
Futura75_BA	0.11±0.05	6.35±0.62	0.16±0.01
Tiborszallasi_BA	0.39±0.06	6.03±0.94	0.30±0.05
Kompolti_BA	0.13±0.04	8.78±2.03	0.05±0.01
Carmagnola_BA	0.20±0.07	5.45±0.97	0.14±0.01
Finola_LO	0.18±0.05	2.92±0.06	0.03±0.01
Antal_LO	0.27±0.04	2.35±0.49	0.20±0.02
Futura75_LO	0.30±0.05	1.72±0.06	0.24±0.05
Tiborszallasi_LO	0.10±0.05	3.03±0.11	0.20±0.06
Kompolti_LO	0.13±0.04	1.74±0.06	0.07±0.00
Carmagnola_LO	0.15±0.05	2.57±0.07	0.07±0.00
Mean	0.24	4.39	0.14
Median	0.25	4.06	0.13
Standard deviation	0.10	2.44	0.08
Fcalculated	11.35	45.30	18.01

Table 14. Profile of inorganic elements of the different samples of inflorescences from C. sativa L. Results are expressed as mean ± standard deviation (mg/Kg, dw) of n=3 samples,where every sample was analyzed three times.

Sample	As	Cd	Co	Cr	Cu	Fe	Mn	Mo	Ni	Pb	Se	Zn	Al	Hg
Finola_SR	0.01±0.00	0.06±0.03	0.01±0.00	0.03±0.01	2.61±0.11	47.56±2.08	49.96±0.15	0.50±0.05	0.15±0.02	0.04±0.02	0.17±0.02	110.67±9.42	4.59±0.11	<LOD
Antal_SR	0.01±0.01	0.02±0.01	<LOD	0.05±0.02	2.73±0.11	49.08±0.14	39.60±2.08	0.76±0.10	0.12±0.04	0.08±0.03	0.12±0.04	111.24±3.64	5.01±0.07	<LOD
Futura75_SR	0.01±0.01	0.04±0.02	<LOD	0.11±0.02	0.67±0.13	38.56±2.36	72.21±0.19	0.48±0.03	0.10±0.04	0.06±0.03	0.15±0.04	103.81±0.96	1.88±0.07	<LOD
Tiborszallasi_SR	0.01±0.00	0.02±0.01	0.01±0.00	0.04±0.01	0.89±0.07	40.81±3.93	41.16±1.24	0.54±0.09	0.05±0.02	0.02±0.01	0.11±0.03	150.43±8.07	8.78±0.24	<LOD
Kompolti_SR	0.01±0.01	0.02±0.02	<LOD	0.06±0.02	3.06±0.11	44.03±2.74	36.18±0.87	0.66±0.08	0.11±0.04	0.05±0.02	0.14±0.02	97.36±1.41	6.81±0.19	<LOD
Carmagnola_SR	0.01±0.01	0.04±0.02	0.01±0.01	<LOD	0.56±0.12	32.73±2.90	30.23±1.27	0.54±0.09	0.12±0.06	0.07±0.02	0.11±0.04	90.54±2.06	1.22±0.20	<LOD
Finola_RM	<LOD	<LOD	<LOD	0.01±0.01	0.33±0.08	4.49±0.47	3.59±0.32	0.06±0.02	0.02±0.01	0.01±0.00	0.97±0.07	45.81±0.57	4.50±0.33	0.01±0.00
Antal_RM	<LOD	<LOD	<LOD	<LOD	0.22±0.11	2.99±0.16	2.15±0.15	0.05±0.02	0.02±0.01	0.02±0.01	0.93±0.09	43.87±0.80	2.06±0.17	<LOD
Futura75_RM	<LOD	<LOD	0.01±0.00	<LOD	0.05±0.02	1.87±0.30	18.44±0.34	0.03±0.01	0.01±0.00	<LOD	1.07±0.03	44.22±0.45	1.27±0.17	<LOD
Tiborszallasi_RM	<LOD	<LOD	<LOD	0.01±0.00	0.09±0.03	3.57±0.39	2.04±0.16	0.03±0.02	0.01±0.00	0.01±0.01	1.08±0.06	52.39±0.83	4.13±0.16	<LOD
Kompolti_RM	<LOD	<LOD	<LOD	<LOD	0.35±0.06	2.44±0.22	2.58±0.31	0.02±0.01	0.03±0.02	<LOD	1.07±0.06	54.43±0.46	2.00±0.08	0.01±0.00
Carmagnola_RM	<LOD	<LOD	<LOD	0.01±0.00	0.10±0.05	1.36±0.31	1.67±0.27	0.04±0.01	0.01±0.00	0.01±0.00	0.88±0.11	39.94±1.51	1.15±0.17	<LOD
Finola_BA	<LOD	<LOD	0.46±0.10	0.02±0.01	2.27±0.22	5.38±0.71	16.18±3.28	0.12±0.07	0.07±0.03	0.79±0.12	0.13±0.03	84.42±1.95	23.86±0.29	0.02±0.02
Antal_BA	<LOD	<LOD	0.35±0.07	0.03±0.01	3.09±0.10	4.17±0.32	17.67±1.24	0.13±0.06	0.11±0.04	0.44±0.08	0.15±0.05	80.54±2.35	25.04±2.87	0.03±0.02
Futura75_BA	<LOD	<LOD	0.39±0.08	0.01±0.01	1.28±0.20	6.44±0.27	16.18±1.61	0.01±0.00	0.02±0.01	0.41±0.11	0.11±0.04	67.60±1.56	87.29±2.40	<LOD
Tiborszallasi_BA	<LOD	<LOD	0.93±0.09	<LOD	2.82±0.18	8.86±1.90	20.52±1.03	0.13±0.03	0.19±0.11	0.61±0.15	0.11±0.06	72.93±1.90	134.07±7.15	0.04±0.02
Kompolti_BA	<LOD	<LOD	0.75±0.10	0.02±0.01	2.31±0.17	7.88±0.21	19.57±0.35	0.03±0.02	0.15±0.05	0.35±0.09	0.15±0.02	85.40±1.79	122.60±3.02	<LOD
Carmagnola_BA	<LOD	<LOD	0.33±0.05	<LOD	1.24±0.11	3.54±0.22	12.04±1.14	0.01±0.01	0.01±0.01	0.31±0.11	0.10±0.05	60.66±2.33	102.08±2.77	0.02±0.01
Finola_LO	<LOD	0.01±0.00	0.01±0.00	0.47±0.07	20.89±3.22	21.13±0.45	15.98±0.13	0.21±0.09	2.11±0.05	0.03±0.01	0.03±0.02	18.07±2.44	15.44±0.21	<LOD
Antal_LO	0.01±0.01	<LOD	0.01±0.01	0.44±0.10	22.80±3.64	22.57±0.12	17.34±0.32	0.18±0.05	1.88±0.16	0.03±0.01	0.01±0.01	12.80±1.78	21.72±0.26	<LOD
Futura75_LO	<LOD	0.01±0.00	0.02±0.01	0.33±0.04	19.62±5.10	9.28±0.42	35.47±2.60	0.01±0.01	1.92±0.16	0.01±0.01	0.01±0.00	12.43±3.23	4.72±0.27	<LOD
Tiborszallasi_LO	0.01±0.00	<LOD	0.01±0.01	0.36±0.06	20.97±3.38	23.39±0.25	17.91±0.30	0.10±0.05	1.09±0.03	0.03±0.01	0.05±0.03	13.47±1.63	38.33±3.55	<LOD
Kompolti_LO	<LOD	<LOD	<LOD	0.39±0.07	21.43±3.86	14.63±0.25	6.67±0.20	0.13±0.05	1.55±0.32	0.02±0.02	0.09±0.07	6.30±2.27	14.17±0.18	<LOD
Carmagnola_LO	<LOD	<LOD	<LOD	0.30±0.05	18.55±3.68	1.64±1.41	13.21±0.49	0.01±0.00	1.07±0.06	<LOD	0.01±0.00	8.54±2.17	1.26±0.28	<LOD
Mean	**0.004**	**0.009**	**0.13**	**0.11**	**6.21**	**16.60**	**21.19**	**0.20**	**0.45**	**0.14**	**0.32**	**61.16**	**25.99**	**0.005**
Median	**0.000**	**0.000**	**0.01**	**0.03**	**2.29**	**8.37**	**17.50**	**0.11**	**0.11**	**0.03**	**0.12**	**57.54**	**5.91**	**0.000**
Standard deviation	**0.006**	**0.017**	**0.26**	**0.16**	**8.64**	**16.58**	**17.36**	**0.24**	**0.71**	**0.22**	**0.40**	**38.80**	**39.72**	**0.011**
Fcalculated	**14.99**	**9.96**	**120.72**	**66.16**	**59.92**	**421.38**	**650.83**	**66.53**	**68.87**	**44.91**	**211.54**	**461.89**	**1252.51**	**14.59**

*Fcritical =1.75 at α<0.001

9.3.5. Results and discussion

Figure 10 and Table 13 report the variable percentage contents of the main cannabinoids (i.e., THC, CBD and CBN), determined in hemp samples by GC-FID, with results expressed on a dry weight basis. Based on the one-way ANOVA, the F calculated of each cannabinoid is also shown in comparison with the set F critical. Although the F values of THC, CBD and CBN were generally much lower than those identified for the elements, they were still higher than the F critical value (11.35–45.30 vs. 1.75), as they significantly varied between investigated samples.

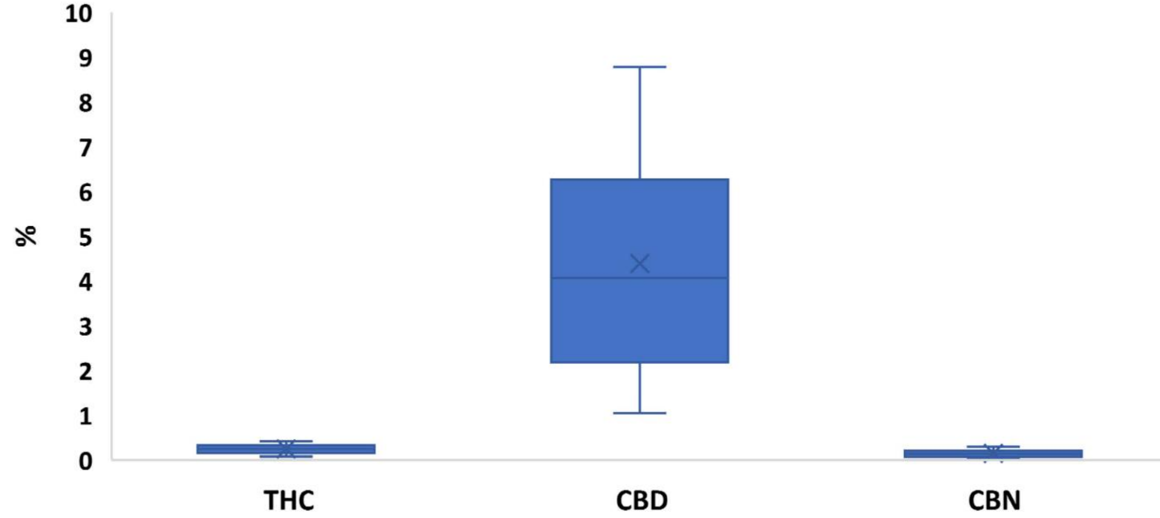


Figure 10. Box plot reporting the variable percentage of cannabinoids (Δ9- tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN)) in investigated hemps. The "×" indicates the average value of each cannabinoid.

All hemp samples complied with the Council Directive 2002/53/EC on the common catalogue of varieties of agricultural plant species and Italian Law 242/2016 on the provisions for the promotion of hemp cultivation and its agro-industrial supply chain, due to a percentage content of the active ingredient THC <0.6%. The THC ranged from 0.08% to 0.42%, with mean and median levels, respectively, equal to 0.24% and 0.25%. The consistency between mean and median THC content highlighted a little variation of such cannabinoid over hemp samples. Coherently, THC showed the lowest F value (11.35).

When determining the THC content of Cannabis products by GC-FID, the effect of THCA decarboxylation to THC due to the high instrumental temperature of the injector should be considered. In our case, however, a minimal and negligible effect of decarboxylation may be assumed, as the THC obtained in all hemps still did not exceed the legal 0.6%.

With reference to the geographical origin, products from Latium and Lombardy showed in general the highest and lowest THC levels (0.10–0.30% and 0.15–0.42%, respectively). Concerning the variety, Tiborzallasi hemps and Carmagnola inflorescences were characterized by the most and least abundant levels of the psychoactive molecule (0.10–0.37% and 0.08–0.20%, respectively) (Table 13).

In light hemp, the lower levels of THC are typically juxtaposed with the higher levels of CBD, a cannabinoid with anti-seizure activity, also, it has anti-inflammatory, antitumor, analgesic and antipsychotic activity[50].

In the present study, CBD ranged from 1.05% to 8.78%, with mean and median contents of 4.39% and 4.06%. Overall, a consistent variation of such cannabinoids over Italian hemps was pointed out, as confirmed also by the higher F value of 45.30. Apulian and Latium hemps showed the most and least abundant CBD content (5.44–8.78% and 1.05–3.26).

On the other hand, Kompolti and Finola varieties reported in general the highest and lowest CBD levels (1.74–8.78% and 2.92–5.74%) (Table 13). The cannabinoid CBN is known for its sedative, anti-inflammatory and anti-microbial effects[50]. Such a molecule has also been experimentally demonstrated to stimulate the appetite as an efficient and non-toxic alternative to THC[51].

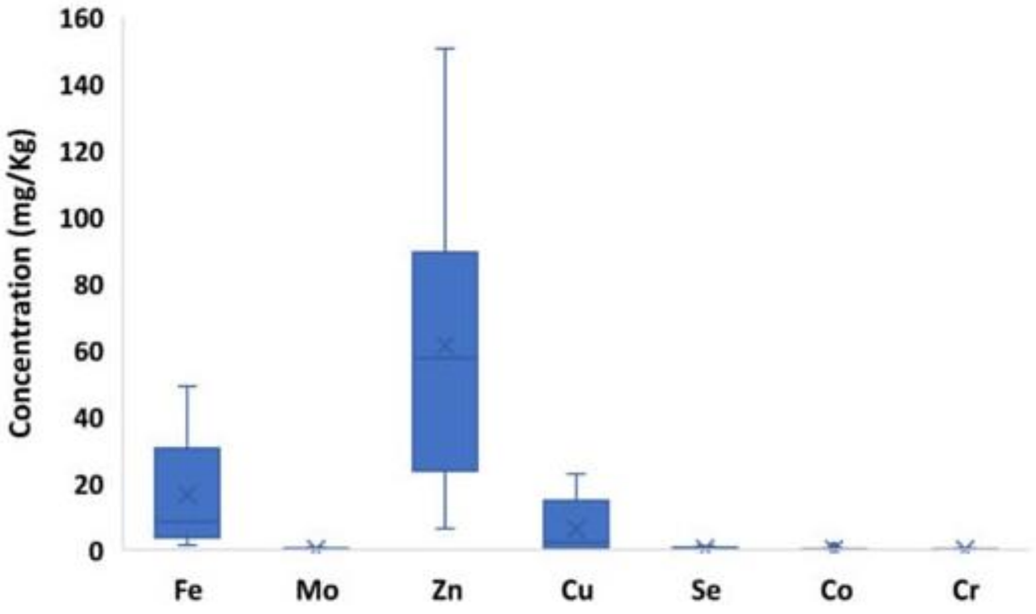
According to Table 13, CBN varied between 0.03–0.30% with mean and median values, respectively of 0.14% and 0.13%. The F value of 18.01 indicated a lower variation of such cannabinoid over-investigated hemps than CBD.

In relation to the geographical origin, products from Sicily and Latium were the least and most concentrated in CBN (respectively, 0.04–0.12% and 0.11–0.27%). On the other hand, according to

the variety, Finola and Tiborzallasi inflorescences showed the highest and lowest content of such cannabinoid (respectively, 0.03–0.14% and 0.11–0.30%).

Although various classifications of trace elements have been proposed, elements with nutritional significance from this study will be classified into essential elements, probably essential elements, and potentially toxic elements according to WHO.

Table 14 and Figure 11, Figure 12 and Figure 13 show the variability of the trace element profile over the hemp samples revealed by validated ICP-MS and TDA-AAS methods, with results expressed on a dry weight basis. Based on the one-way ANOVA, the Fcalculated of each element



is also shown in comparison with the set Fcritical.

Figure 11. Box plot of essential trace elements (Fe, Mo, Zn, Cu, Se, Co, Cr) concentration in hemps. The “×” indicates the average value of each element.

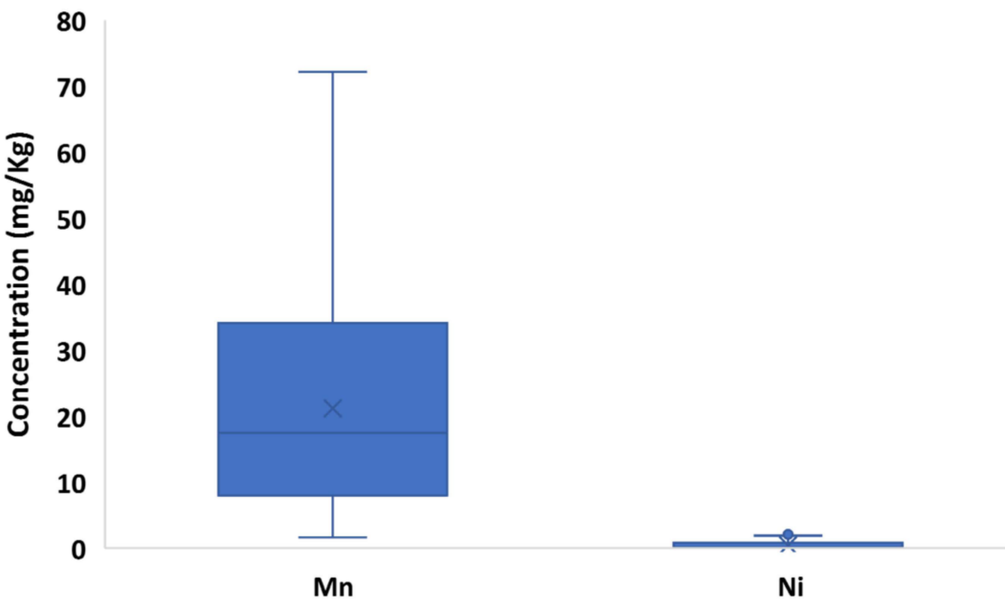


Figure 12. Box plot of probably essential trace elements (Mn, Ni) concentration in hemps. The "×" indicates the average value of each element, whereas the outlier points display the outlier data lying either below the lower whisker line or above the upper whisker line.

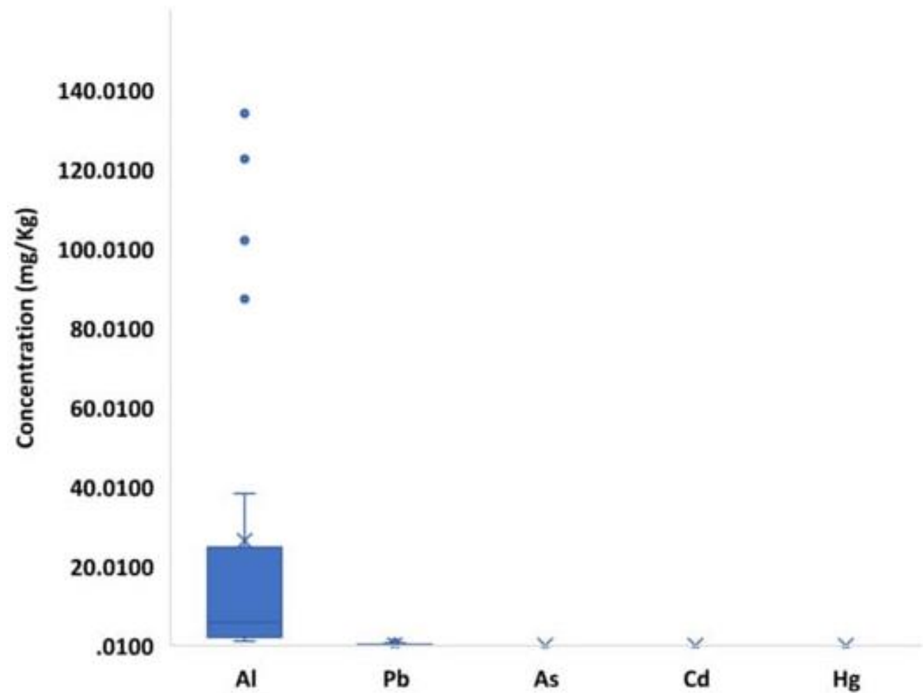


Figure 13. Box plot of potentially toxic element (Al, Pb, As, Cd, Hg) concentration in hemps. The "×" indicates the average value of each element, whereas the outlier points display the outlier data lying either below the lower whisker line or above the upper whisker line.

The mean/median content of essential trace elements in the hemp samples followed the decreasing order: Zn (61.16/57.54 mg/Kg) > Fe (16.60/8.37 mg/Kg) > Cu (6.21/2.28 mg/Kg) > Se (0.32/0.12 mg/Kg) > Mo (0.20/0.11 mg/Kg) > Co (0.13/0.01 mg/Kg) > Cr (0.11/0.03 mg/Kg). However, the great dissimilarity between the mean and median values of every metal reflects a consistent value dispersion over the investigated sample range. Overall, the highest F values were highlighted for Zn (461.89) Fe (421.38), Se (211.54) and Co (120.72) thus, indicating that these essential elements were responsible for the highest variation within the samples (Table 14). Considering the producing areas, Sicilian hemps showed the highest levels of Fe (32.73–49.08 mg/Kg), Zn (90.54–150.43 mg/Kg) and Mo (0.48–0.76 mg/Kg), while inflorescences from Lazio had the lowest levels of Fe (1.36–4.49 mg/Kg) and Mo (0.02–0.06 mg/Kg). The lowest levels of Zn were revealed in samples from Lombardy (6.30–18.07 mg/Kg).

However, hemps from Lombardy were characterized by the greatest Cr and Cu contents (18.55–22.80 mg/Kg and 0.30–0.47 mg/Kg); whereas samples from Apulia and Lazio showed the most abundant levels, respectively of Co and Se (0.33–0.93 mg/Kg and 0.88–1.08 mg/Kg). Conversely, samples from Lazio were characterized by the lowest concentrations of Cr (<LOD–0.01 mg/Kg), Cu (0.05–0.35 mg/Kg) and Co (<LOD–0.01 mg/Kg) and hemps from Lombardy with the smallest levels of Se (0.01–0.09 mg/Kg). The variability of each trace essential element between different varieties was less pronounced than that between different production areas, due to more similar concentration values from one variety to another. The Antal variety showed slightly higher levels of Cr (0.00–0.44 mg/Kg), Mo (0.05–0.76 mg/Kg) and Cu (0.22–22.80 mg/Kg) than other varieties. Kompolti hemp was marked by increased levels of Se (0.09–1.07 mg/Kg) and Fe (2.44–44.03 mg/Kg); whereas Finola inflorescences had greater levels of Zn (18-07-110.67 mg/Kg) than other varieties (Table S2). In the investigated hemps, the mean/median content of probably essential trace elements showed the decreasing order: Mn (21.19/17.50 mg/Kg) > Ni (0.45/0.11 mg/Kg).

Once again, the difference between mean and median values for both analytes are indicative of a significant value dispersion over the considered samples. Interestingly, the F value calculated for Mn (650.83) was among the highest F calculated values of the study (Table 14).

With respect to the provenience, Sicilian inflorescences showed the highest Mn contents (30.23–72.21 mg/Kg) followed by hemps produced in Apulia (12.04–20.52 mg/Kg) and Lombardy (6.67–35.47 mg/Kg). On the other hand, hemps from Lombardy showed the highest Ni levels (1.07–2.11 mg/Kg) followed by Sicilian samples (0.05–0.15 mg/Kg) (Table 14). Considering the variety factor, Futura 75 and Carmagnola hemps highlighted the highest and lowest levels of Mn (respectively, 16.18–72.21 mg/Kg and 1.67–30.23 mg/Kg); whereas Finola samples showed just slightly higher Ni contents (18-07-110.67 mg/Kg) than other varieties (Table 14).

The mean/median concentrations of potentially toxic trace elements were revealed in the investigated samples according to the order: Al (25.99/5.91 mg/Kg) > Pb (0.14/0.03 mg/Kg) > Cd (0.009/0.000 mg/Kg) > Hg (0.005/0.000 mg/Kg) > As (0.004/0.000 mg/Kg).

The consistent gap between the mean and median content of Al and Pb points out a relevant variation of such metals over hemp samples. Interestingly, Al revealed the highest F value (1252.51) of the study by one-way ANOVA. However, the gap narrows for the mean/median concentrations of Cd, As and Hg, thus, suggesting a lower variability of hemps in relation to these heavy metals. Coherently, these elements showed the lowest F values (9.96–14.99).

With reference to the geographical origin, Al and Pb were greatly accumulated in Apulian hemps (Al: 23.86–134.07 mg/Kg and Pb: 0.31–0.79 mg/Kg) and, to follow, in samples from Sicily (Pb 0.02–0.08 mg/Kg) and Lombardy (Pb: 1.26–38.33 mg/Kg). Among other heavy metals, Cd and Hg were most abundant, respectively, in Sicilian (0.02–0.06 mg/Kg) and Apulian (0.02–0.04 mg/Kg) inflorescences. Most of the potentially toxic trace elements showed comparable contents in the different hemp varieties, except for Al, which resulted at higher levels in the Tiborzallasi (4.13–134.07 mg/Kg) and Kompolti (2.00–112.6 mg/Kg) varieties (Table 14).

Recent efforts have been devoted to the assessment of inorganic elements in relation to different geographical producing areas and/or hemp varieties[46]. Among essential and probably essential trace metals, Amendola and colleagues[52], as well as Zafeiraki and coworkers[46] and Douvris et al.[53], highlighted Zn, Fe Cu and Ni as the most abundant elements. However, they were generally found at much higher and non-comparable concentrations with respect to the contents revealed in the present study. Interestingly, investigated hemps showed higher levels of Se than those shown in literature (0.10–0.15 mg/Kg)[46]. Ultimately, the profile of inorganic elements of light hemp highly varies depending on several factors related to its cultivation (i.e., variety of the plant, geopedoclimatic context, agronomic practices, soil properties and not least, the proximity to industrial plants or pollution sources) and its subsequent processing (i.e., drying methods and storage conditions). For example, elements such as Cd, Cr, Cu, Ni, Pb and Zn are highly influenced by the pH value of the soil. Precisely, they become more bioavailable under acidic soil conditions.

However, if, on one hand, the accumulation of some inorganic elements may be advantageous (i.e., essential trace elements), on the other hand, it is not, because, as already described in the introduction section, hemp remarkably accumulates also potentially toxic elements, which may cause hazardous effects to the consumer. In fact, heavy metals fall into the US Federal Drug Administration Class 1 category substances, i.e., "human toxicants that have limited or no use in the manufacture of pharmaceuticals". Elements such as As, Cd and Ni are classified by the International Agency for Research on Cancer as carcinogenic to humans (Group 1), and their inhalation may also contribute to cardiovascular disease and smoking-related lung diseases.

The EU has not yet established a regulatory framework concerning the monitoring of hazardous toxicants in cannabis products, including inorganic elements. Nonetheless, data from this study can be compared with the limits set by the WHO framework recommending the continuous monitoring of raw herbal materials intended for medicinal use in terms of potentially toxic metals, other than pesticide residues[54,55], as well as with the thresholds fixed by the US Pharmacopeia (USP) based on a typical consumption of 10 g/day of cannabis products for medical purposes[55].

With reference to the WHO framework, it was found that the experimental concentrations of Cr and heavy metals were lower than fixed limits in all hemps. Differently from WHO, the USP regulates a larger number of essential (i.e., Mo, Cu, Co and Cr) and probably essential metals (i.e., Ni), as well as potentially toxic elements (i.e., Pb, As, Cd and Hg), generally established for the latter lower thresholds than WHO limits. As shown in Table 15, all Italian hemps may be safely ingested due to sub-threshold contents of Mo, Cu, Co, Cr, As, Cd and Hg. However, among investigated samples, two Apulian inflorescences from Finola and Tiborzallasi varieties exceeded the safety ingestion limits for Pb.

Table 15. Number of Italian hemps exceeding the World Health Organization (WHO) and US Pharmacopeia (USP) limits in terms of inorganic elements. WHO limits refer to the maximum content of elements allowable in raw herbal materials intended for medicinal use. USP sets the threshold of elements for both oral intake and inhalation of cannabis products, considering the consumption of 10 g/day.

Element	WHO 2007		Oral concentration		Inhalation concentration	
	Limit (mg/Kg)	No. samples	Limit (mg/Kg)	No. samples	Limit (mg/Kg)	No. samples
Mo	-	-	300	0	1	0
Cu	-	-	300	0	3	8
Co	-	-	5	0	0.3	6
Cr	2	0	1100	0	0.3	6
Ni	-	-	20	0	0.5	6
Pb	10	0	0.5	2	0.5	2
As	5	0	1.5	0	0.2	0
Cd	0.3	0	0.5	0	0.2	0
Hg	0.2	0	3	0	0.1	0

A different situation was observed for the consumption of Italian hemps via inhalation. In fact, all Italian hemps may be safely inhaled due to sub-limit levels of Mo, As, Cd and Hg. Concerning the other regulated elements, all hemp varieties from Lombardy showed levels of Cu, Cr and Ni above the fixed limits, while all investigated varieties of Apulian cannabis were above the threshold set for Co. Additionally, Apulian products from the varieties Finola and Tiborzallasi accumulated levels of Pb hazardous to inhalation. Overall, the discussed results pointed out that Apulia and Lombardy represented the most contaminated areas in terms of potentially toxic elements and that hemp varieties such as Finola and Tiborzallasi were more susceptible to the accumulation of toxic heavy

metals. Cases of metal toxicity from cannabis consumption are described in literature. Busse and coworkers[56] highlighted that regular consumer of cannabis in Leipzig reported typical symptoms of Pb intoxication (i.e., abdominal cramps, nausea, anemia, fatigue and in most cases neurologic symptoms) due to marijuana adulterated with Pb during processing.

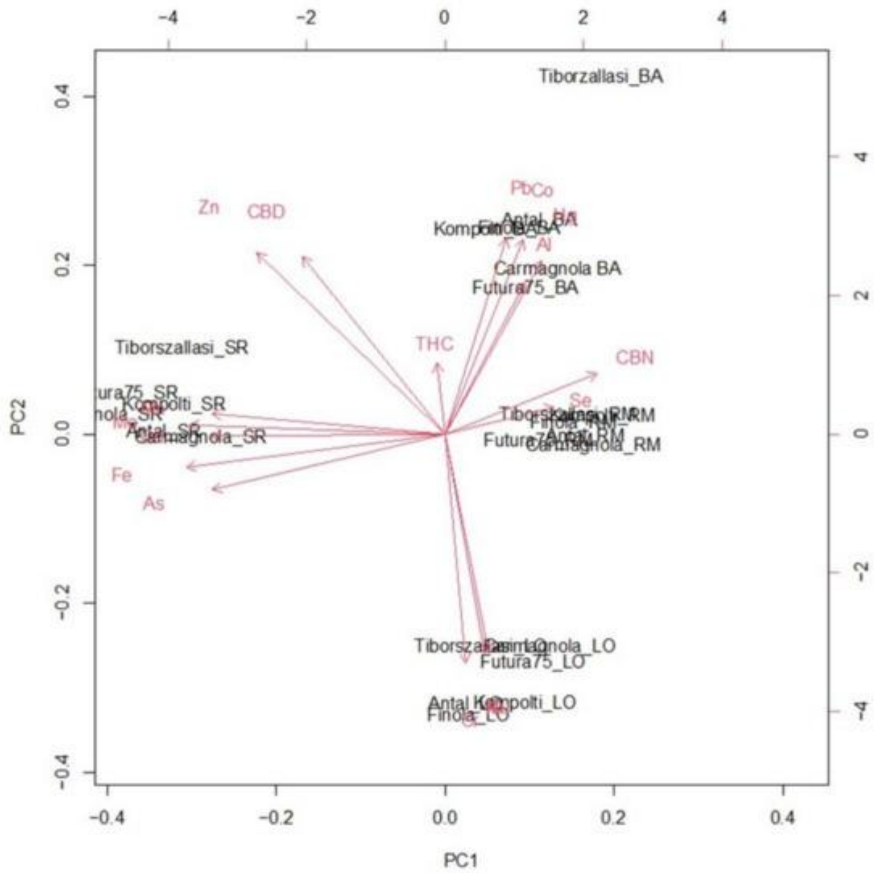
Combemale and colleagues[57] asserted a case of arteritis associated to the consumption of cannabis with very high levels of As. Overall, the combustion and deep inhalation of cannabis products may expose the consumers to very high levels of metals and make them more susceptible to toxic metals, thus, creating a variety of adverse health effects.[58,59]

9.3.6. PCA of Mineral Elements and Cannabinoids

A PCA was carried out by considering a data matrix with 17 columns (i.e., Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Pb, Hg, THC, CBD and CBN) and 24 rows (i.e., number of representative hemp samples) in order to visually reduce data dimensionality and identify those combinations of variables most responsible for sample variability, namely the principal components (PCs).

Most sample variability was described by the first two PCs, namely PC1 and PC2, which accounted, respectively, for 33.22% and 28.39% of the total variance. The biplot resulting from the first two PCs is reported in Figure 14.

Figure 14. Two-dimensional principal component analysis (PCA) biplot of the first two principal components (PCs) obtained from the variables (i.e., inorganic elements and cannabinoids) investigated in the Italian hemp samples.



Most of analyzed samples were separated on PC2 according to the geographical origin rather than botanical variety. Only hemp varieties from Sicily are plotted on PC1 (Figure 14). Specifically, all Apulian hemps were marked by the highest mean levels of Al, Pb and Hg and the lowest concentrations of Ni, Cu and Cr. Conversely, samples from Latium were characterized by a positive correlation with Se and CBN, and a negative correlation with Fe, Mo, Mn, Cd and As. However, these elements are plotted in correspondence to Sicilian hemps, thus, being crucial for their discrimination from other hemps. On the other hand, inflorescences produced in Lombardy were distinguished by a strong positive correlation with Cu, Cr and Ni (Figure 14).

Accordingly, the score plots (Figure 15) showed that investigated inflorescences sharply clustered according to the production area rather than variety, thus confirming that most variables responsible for the discrimination (i.e., inorganic elements) varied considerably with reference to the geopedoclimatic context in which *C. sativa* grew.

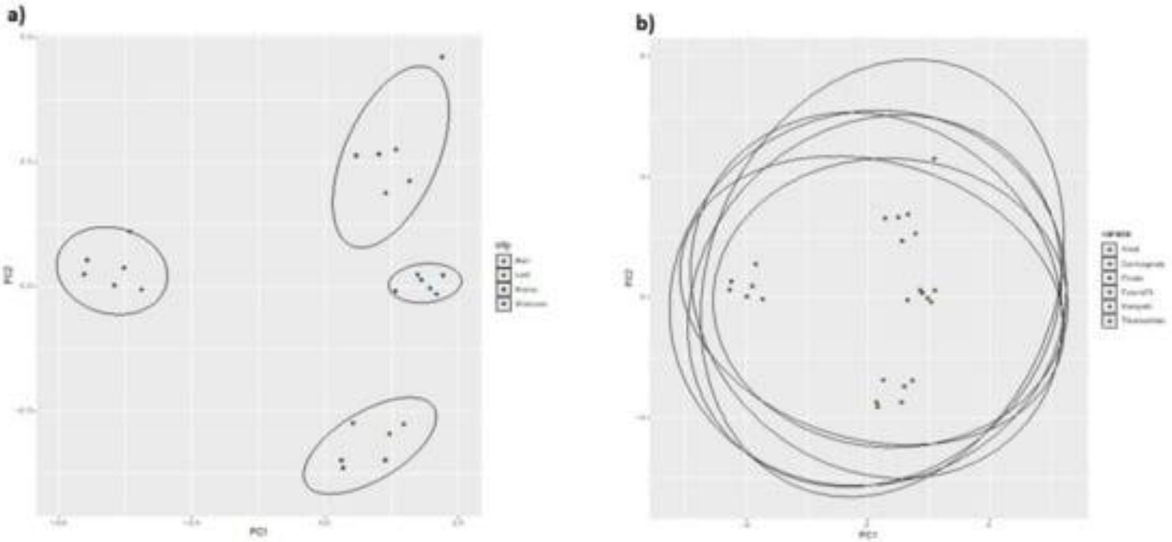


Figure 15. Score plots of the investigated Italian hemps. Drawn ellipses suggest the clustering of samples according to the geographical origin (**a**) botanical variety (**b**).

9.3.7. Conclusions

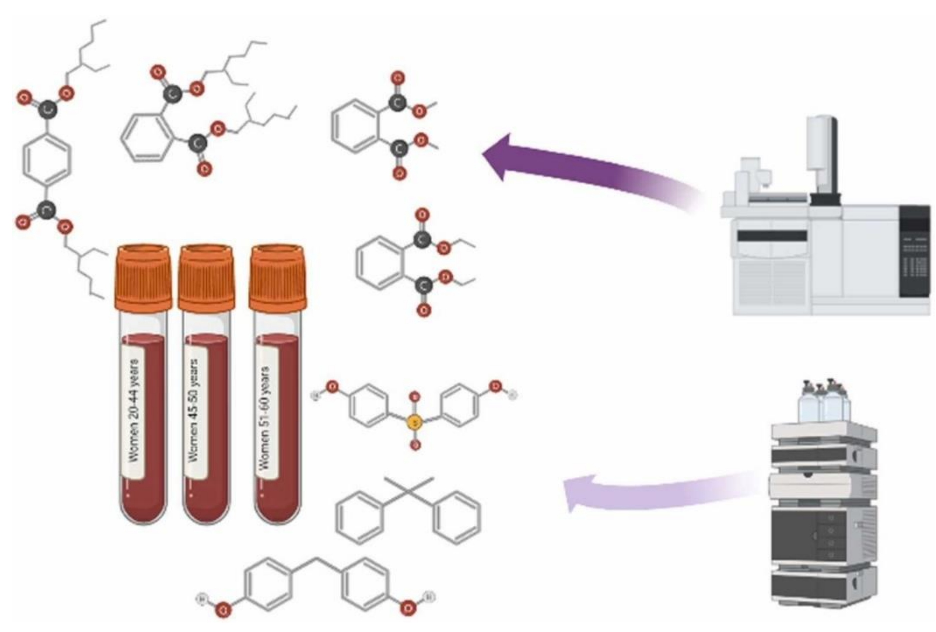
Since the introduction of Italian Law 242/2016, the trade of hemp products has literally exploded in small retail shops in many Italian cities. In particular, light cannabis inflorescences are increasingly sold for recreational use, thanks to a lower content of the psychotropic THC, and higher levels of CBD with therapeutic effects. In the present study, 72 samples of hemp produced in four Italian regions and belonging to six botanical varieties were screened for trace elements and main cannabinoids. Overall, all samples complied with the Council Directive 2002/53/EC on the common catalogue of varieties of agricultural plant species and, the Italian Law 242/2016 on the provisions for the promotion of hemp cultivation and its agro-industrial supply chain, due to a THC content $<0.6\%$. However, the trace element profiles of hemp samples highlighted the urgency to regulate the content of probably essential and potentially toxic elements in hemp products both at the EU and national level, as well as the necessity to constantly monitor such elements to ensure consumers'

safety and health, since several Italian hemps exceeded the current safety limits of Co, Cu, Cr, Ni and Pb set by the available regulatory frameworks (i.e., USP).

Aside from the assessment of the compliance of Italian hemp with available regulations, experimental data were also statistically interpreted and a PCA pointed out that most investigated variables (i.e., inorganic elements) allowed to discriminate hemp products based on the geographical origin rather than botanical variety, thus, reaching a first milestone toward the development of an effective traceability system for the Italian light cannabis.

9.4. SCREENING OF PHTHALATE AND NON-PHTHALATE PLASTICIZERS AND BISPHENOLS IN SICILIAN WOMEN'S BLOOD

The aim of the study was to study PAEs, NPPs and BPs in blood from healthy females aged between 20 and 60 years and living in Sicily (Italy). Also, a statistical interpretation, based on univariate and multivariate tools, was performed to find a potential relationship between the blood content of such compounds and the variable "age". Hopefully, this study will provide insights on the issue of plasticizers in female blood, and it will lay also the groundwork for a better understanding of the early or late onset of those diseases linked to the presence of these compounds in biological matrices at given age groups.



9.4.1. Samples

During the period May 2021–October 2021, many female volunteers were asked to complete a questionnaire regarding their gender, age, residence, and general health status with particular attention to the presence of infertility issues. As a result, n = 75 healthy females living in the province of Messina (Sicily, Italy) and aged between 20 and 60 years were selected for the present study.

For each volunteer, informed consent was obtained, and 5 mL of whole venous blood was collected in sterilized glass tubes imbibed with 30 μL of 0.5 M ethylenediamonotetraacetic acid (EDTA) at pH 8.0 and stored at − 20 °C until analysis. Then, blood samples were subdivided into 3 cohorts according to women's age, the first group being between 20 and 44 years old (reproductive women, n = 25), the second between 45 and 50 years old (pre-menopausal women, n = 25), and finally 51–60 years old (menopausal women, n = 25) (Table 16).

Table 16. Information about the female participants in the study.

Age group	Age range	Age expressed as mean ± sd	Provenance
Reproductive women	20-44	32.67 ± 6.34	
Pre-menopausal women	45-50	47.25 ± 1.87	Sicily (province of Messina)
Menopausal women	51-60	56.83 ± 2.96	

To minimize contamination, in addition to the glass tubes properly capped with aluminium foil, glass syringes were employed throughout the sampling procedure to avoid a potential contamination by the most common PAEs or BPs present in medical equipment.

9.4.2. Plasticizers analysis

The extraction of PAEs and NPPs was performed according to the method of Haishima et al.[60] Briefly, 120 μL of every blood sample was transferred into screw-capped glass tubes, which were filled with distilled water up to the level of 1 mL. Sodium chloride (10 mg) was added to every sample and incubated for 30 min at room temperature. Then, hexane (2 mL) was added, and the

obtained mixture was stirred for 20 min at room temperature, and the organic phase was collected and evaporated to dryness.

Before GC-MS analysis, all dried extracts were re-suspended in 500 μL of n-hexane and spiked with 500 μL of each internal standard. For analytical validation purposes, the described procedures were also applied to additional and representative blank samples which were previously analysed by the GC-MS protocol reported ahead and revealed no plasticizer contamination.

Plasticizers were determined in all samples by a gas chromatography system; chromatographic separations occurred on a SPB-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Supelco, USA). Full details of the analytical method can be found in the section *5.1.2 'Instrumentation and analytical conditions*' of Chapter 5.

9.4.3. Bisphenols analysis

The BPs extraction was carried out according to a protocol already reported by Li et al.[61]

To every blood sample, 6 mL of acetonitrile were added and stirred at 250 rpm for 30 min, followed by an ultrasonic treatment for 40 min. Then, the mixture was centrifugated at 4000 rpm for 10 min, and the supernatant was transferred to a clean tube. The residue was extracted again by repeating the procedure. All extracts were evaporated under a nitrogen stream and diluted with 5 mL of water containing 0.5 mM ammonium acetate. A further purification was conducted by passing the extract through an Envi-C18 cartridge (Sigma-Aldrich, Oakville, ON, Canada), which was previously conditioned with 5 mL of methanol and 5 mL of water (containing 2.5 mM ammonium acetate). After the sample loading, the cartridge was rinsed with 5 mL of 10 % methanol in water, and target analytes were eluted with 5 mL of methanol containing 0.1 % ammonia. The eluent was evaporated under a gentle nitrogen stream, and the final volume was adjusted to 50 μL with methanol/water (50:50) before instrumental injection. BP residues were determined in every extract by an HPLC system; MRM transitions and analytical validation for every target analyte can be found in the section *5.1.2 'Instrumentation and analytical conditions*' of Chapter 5.

9.4.4. Statistical analysis

Statistical analysis was carried out using the SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL). Initially, the non-parametric Kruskal Wallis's test was applied on log-transformed data to assess differences between blood samples, with a statistical significance at $p < 0.05$. Subsequently, a Principal Component Analysis (PCA) was conducted on a starting data matrix where the cases (75) were the analyzed blood samples, and the variables (9) were the plasticizers and BPs residues reliably determined in blood samples. To this purpose, the data set was normalized to achieve independence of the different variables scale factors, outliers were removed and a PCA was performed to (i) evaluate the differentiation of blood samples in relation to the women's age, and (ii) reduce data dimensionality, while identifying those combinations of variables, which provide the largest contribution to sample variability, commonly known as principal components (PCs). Accounting for a major portion of the variance in the data set, and having the largest eigenvalues, the first PCs are typically used for sample differentiation.

9.4.5. Results

Table 17 show the concentrations of plasticizers determined in every blood sample divided into 3 groups according to the age of the subjects. The results of GC-MS/MS analysis demonstrated the presence of PAEs and NPPs in every sample. Specifically, of the plasticizers analysed, 5 PAEs and 2 NNPs were detected in all samples, while the other compounds were below the relative limit of detection (LOD).

Table 17. Profile of PAEs (mg/L) and NPPs (mg/L) and total content of plasticizers (ΣPAEs+NPPs, mg/L) and phthalates (ΣPAEs, mg/L) revealed in Sicilian females' blood aged 20–44 years (reproductive females), 45–50 years (premenopausal females), and 51–60 years (menopausal females). Results are expressed as mean concentration and standard deviation of *n* = 25 subjects selected for each group. Statistics from Kruskal-Wallis's test is also reported.

Blood samples		**PAEs**					**NPPs**		**ΣPAEs+NPPs**	**ΣPAEs**
		DMP	**DEP**	**DiBP**	**DEHP**	**DBP**	**DEHT**	**DEHA**		
Reproductive females	mean ± sd	0.40 ±0.02[a]	0.27 ±0.03	0.62 ±0.09[a]	0.64 ±0.08[a]	0.40 ±0.06	0.57 ±0.12[a]	0.41 ±0.03	3.34 ± 0.38	2.36 ±0.15[a]
	% positive samples	84	79	100	100	57	100	100	88.6	84
Premenopausal females	mean ± sd	0.39 ±0.00[a]	0.30 ±0.06	0.75 ±0.12[b]	0.65 ±0.05[a]	0.42 ±0.05	0.38 ±0.15[b]	0.40 ±0.07	3.29 ± 0.18	2.52 ±0.19[b]
	% positive samples	86	71	100	100	48	100	100	86.4	81
Menopausal females	mean ± sd	0.35 ±0.02[b]	0.29 ±0.05	0.59 ±0.10[a]	0.62 ±0.10[b]	0.40 ±0.07	0.89 ±0.29[c]	0.39 ±0.06	3.56 ± 0.62	2.28 ±0.11[c]
	% positive samples	83	67	100	100	44	83	100	82.4	78.8
F statistics		31.89	0.24	19.05	7.12	0.34	23.60	1.51	0.16	17.25
p-value		**< 0.001**	0.89	**< 0.001**	0.03	0.84	**< 0.001**	0.47	0.93	**< 0.001**

a–c indicates homogeneous sample groups at α = 0.05 and blood samples which do not differ from each other are designated by same letter. Bold p-values showed significant differences at p< 0.05 between different sample groups.

PAEs were determined in the three sample groups according to the concentration order: DEHP ≈ DiBP > DBP > DMP > DEP. DEHP and DiBP were the only PAEs detected in 100 % samples and resulted significantly different between the investigated sample groups ($p < 0.05$). In fact, they were least abundant in menopausal females (respectively, 0.59 mg/L and 0.62 mg/L) and had greater contents in premenopausal and (respectively, 0.75 mg/L and 0.65 mg/L) and reproductive (respectively, 0.62 mg/L and 0.64 mg/L) females.

DBP, DMP and DEP shared the same trend of detection frequency, as they were less detected in menopausal women (respectively, 83 %, 67 % and 44 %) than premenopausal (respectively, 86 %, 71 % and 48 %) and reproductive (respectively, 84 %, 79 % and 57 %) females. However, DMP ranged between 0.35 and 0.40 mg/L ($p < 0.001$), with the same concentration trend described above, while DBP and DEP were not statistically significant between investigated samples (respectively 0.40–0.42 mg/L, $p > 0.05$ and 0.27–0.30 mg/L, $p > 0.05$).

Considering NPPs, DEHT and DEHA were reliably quantified respectively in 94.3 % and 100 % samples. Interestingly, DEHT was least detected in menopausal females (83 %). However, in these blood samples such NPP was at the highest level (0.89 mg/L, $p < 0.001$), thus, resulting less abundant in reproductive and premenopausal women (0.57 mg/L and 0.38 mg/L, $p < 0.001$). Conversely, DEHA was non statistically different between the examined groups (0.39–0.41 mg/L, $p > 0.05$).

On this basis, the total mean content of plasticizers, intended as sum of PAEs and NPPs, ranged from 3.29 mg/L (in 86.4 % premenopausal females) to 3.56 mg/L (in 82.4 % menopausal females), being non significantly different between the age groups ($p > 0.05$), while the total content of PAEs spanned from 2.28 mg/L (in 78.8 % of menopausal females) to 2.52 mg/L (in 81 % of premenopausal females), being significantly different in relation to age ($p < 0.001$).

Table 18 shows the BPs determined in Sicilian females' blood. The HPLC-MS/MS analysis revealed the presence of 2 of the 9 BPs investigated in every blood sample. Indeed, only BPA and BPS were successfully determined in most blood samples, the other BPs (i.e., BPF, BPE, BPB, BPAF, BPAP,

BPZ and BPP) being lower than relative LODs. Interestingly, both BPA, BPS and ΣBPs were detected at higher frequencies in premenopausal and reproductive women's blood than in samples from menopausal females characterized by lower frequencies.

Table 18. Profile of BP residues (µg/L) and total content of BPs (ΣBPs, µg/L) revealed in Sicilian females' blood aged 20–44 years (reproductive females), 45–50 years (premenopausal females), and 51–60 years (menopausal females). Results are expressed as mean concentration (µg/L) and standard deviation of n = 25 subjects selected for each group. Statistics from Kruskal-Wallis's test is also reported.

Blood Samples		BPA	BPS	∑BPs
Reproductive Females	mean ± sd	2.19 ± 0.20^{a}	1.04 ± 0.08^{a}	3.23 ± 0.23^{a}
	% positive samples	100	98	99
Premenopausal Females	mean ± sd	1.86 ± 0.10^{b}	0.80 ± 0.06^{b}	2.66 ± 0.11^{b}
	% positive samples	87	82	84.5
Menopausal Females	mean ± sd	1.56 ± 0.07^{c}	0.58 ± 0.07^{c}	2.14 ± 0.09^{c}
	% positive samples	93	85	89
F Statistics		58.69	63.09	63.14
P-Value		**< 0.001**	**< 0.001**	**< 0.001**

a–c indicates homogeneous sample groups at α = 0.05 and blood samples which do not differ from each other are designated by same letter. Bold p-values showed significant differences at p< 0.05 between different sample groups.

Similar conclusions can be done also for the concentrations of BPs, since they were at the lowest and highest levels respectively in menopausal and reproductive females. Specifically, BPA was detected in 93 % samples (100 % of positive reproductive females and 87–93 % of positive pre- and menopausal females) and was in the range 1.56–2.19 µg/L ($p < 0.001$), while BPS had lower detection frequencies (98 % of positive reproductive females and 82–85 % of positive pre- and menopausal females) and varied between 0.58 and 1.04 µg/L ($p < 0.001$). Accordingly, ΣBPs significantly decreased from reproductive to menopausal women (i.e., 3.23–2.14 µg/L, $p < 0.001$).

9.4.6. Principal component analysis

The PCA analysis was performed by using only those variables resulted to significantly different between the sample groups and the normalized data set. The suitability of the data set was pre-checked. The Kaiser–Meyer–Olkin measure revealed a value of 0.745, greater than 0.600, and the

Bartlett's test of sphericity showed a Chi-squared value equal to 716.326 (at p level below 0.001), thus, supporting the suitability of the correlation matrix. According to the Kaiser Criterion, only those principal components (PCs) with eigenvalues greater than unity were retained.

Accordingly, three PCs with respective eigenvalues of 3.45, 1.73, and 1.16, were extracted and they explained up to 79.21 % of the total variance (43.065 %, 21.645 % and 14.50 %, respectively). The three PCs and the relative communalities (h2), namely the total amount of variance a variable share with all other variables, are reported in Table 19. Variables with low saturation in each component were not identified and all communalities were ≥ 0.620, therefore the extracted PCs were able to satisfactorily reproduce all original variables. Specifically, PC1 showed the highest positive correlation with BPA, BPS and ΣBPs phthalates, while a negative correlation could be observed for DMP. PAEs, such as DiBP, to a lesser extent DEHP, and ΣPAEs, had positive correlations with PC2, while the dominant variable in PC3 was DEHT.

Table 19. Extracted PCs and relative variables communalities (h^2).

Compound	PC1	PC2	PC3	h2
DMP	**-0.663**	-0.160	-0.410	0.633
DiBP	-0.342	**0.736**	-0.431	0.845
DEHP	-0.340	**0.568**	0.427	0.620
DEHT	-0.228	0.202	**0.769**	0.684
∑PAEs	-0.436	**0.771**	-0.130	0.802
BPA	**0.902**	0.240	-0.001	0.872
BPS	**0.905**	0.279	-0.117	0.911
∑BPs	**0.947**	0.266	-0.051	0.971

Bold values indicate the dominant variables in each PC.

As shown in the bidimensional score plots of figure 16, groups of reproductive and menopausal females are clearly separated into two distinct clusters. However, the cluster of perimenopausal women covered the central part of the plot, while partially overlapping with that of reproductive females. The role of PC1 in separating the different female groups is quite evident and the female blood is ranked according to a decreasing score order, namely samples from fertile, perimenopausal and menopausal females. In such order, BPs exhibited the best discrimination power.

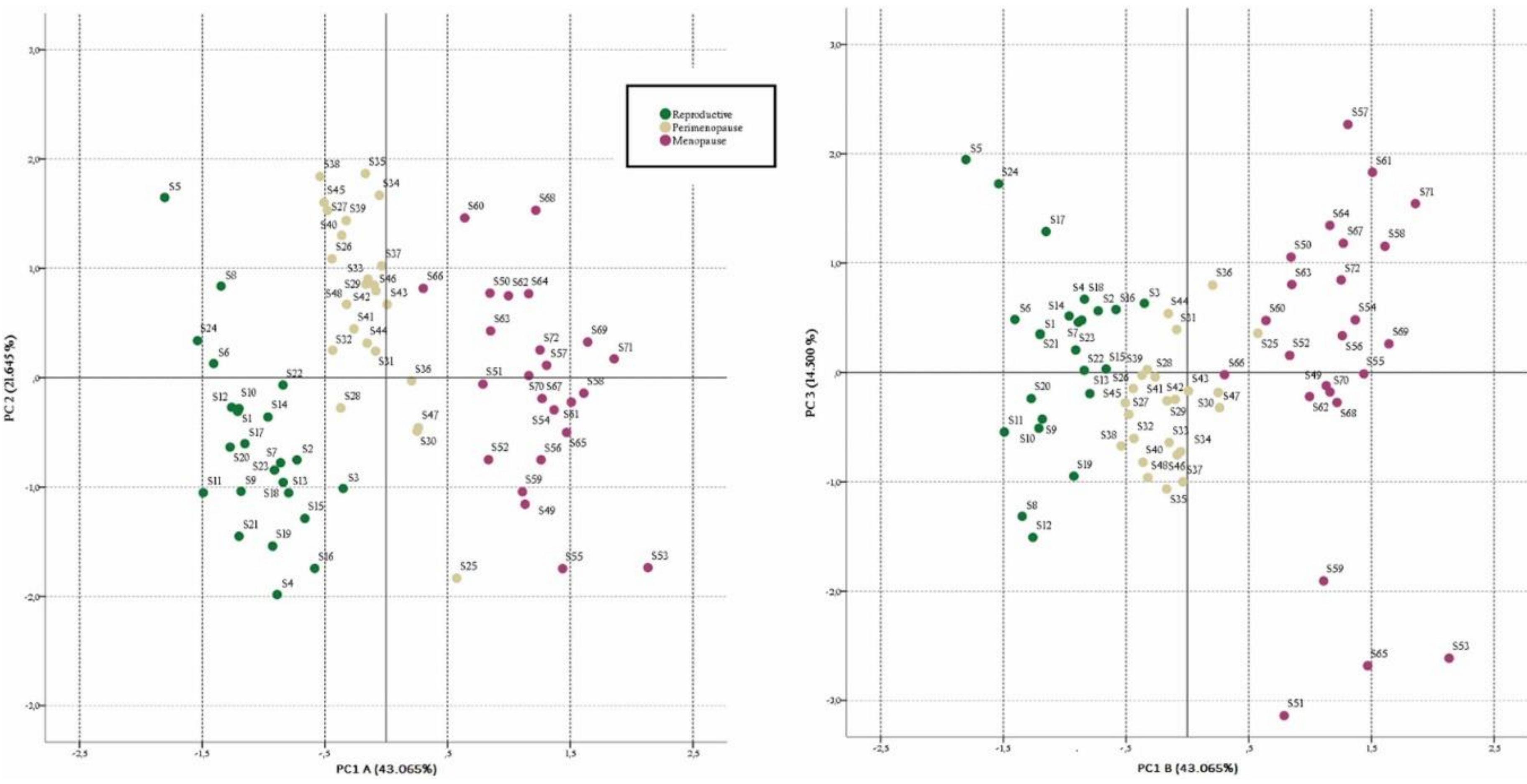


Figure 16. Bidimensional score plots obtained by plotting the three main PCs, namely PC1viz. PC2 (A) and PC1viz. PC3 (B). The definition of three clusters, corresponding to the blood samples from the three investigated female groups, is most evident in plot (A), explaining a greater cumulative variance.

9.4.7. Discussion

The research of microplastics (MPs) in the human body is rapidly evolving, with more studies expected to emerge in the near future. Despite available studies are still limited to a few world regions, MPs have been already detected in a variety of human tissues and biological matrices, including blood[62,63]. In blood, moreover, MPs are inevitably throughout body organs via the circulation system.[63] MPs contain several chemicals that are added during the manufacturing process, including plasticizers with known endocrine disrupting activity, such as PAEs, NPPs and BPs. Such intrinsic chemicals were reported to have a constant movement along the concentration gradient, as plastics kept being fragmented into MPs, and they may be translocated when MPs come into contact with body surfaces, organs or tissues, thus, interfering with endogenous hormones, even at low concentrations.[64] In the last two decades, the monitoring of plasticizers in human blood was carried out[65,66,67,68,69,70,71], also in references to vulnerable female groups from Italian population[68,71]. In accordance with previous literature and with the ubiquitous nature of these compounds, the present study confirmed the widespread exposure to diverse PAE and BP residues also in Sicilian women's blood with different ages, while NPPs, such as DEHT and DEHA, were detected in this study for the first time. Regardless of the age group, the most abundant residues found in female blood were DiBP, DEHP, DEHT and BPA. Between them, DEHP is still the most common plasticizer employed in plastic products despite the European Union has restricted its use in many commercial products (i.e., toys and childcare products) in the past twenty years, DEHT has been emerging as one of the main replacement plasticizers in PVC materials[72], and BPA is the main plasticizer employed in the manufacturing of most common PC products. Moreover, their abundancy in biological matrices may be explained by a relatively high molecular weight, lower water solubility and, consequently, higher accumulation ability of such compounds in body tissues[73]. Based on the obtained data, higher concentration ranges of PAEs were alarmingly displayed in this study with respect to previous literature[66,70], thus, correlating with the drastic increase of plastic production volumes observed in the

last decades. Apart from the reliable determination of PAEs, NPPs and BPs, this study proposed for the first time a valid statistical approach able to display how certain compounds varied significantly based on the females' age. In fact, premenopausal and reproductive females shared greater contents of PAEs, such as DEHP and DiBP, and BPA and BPS than older menopausal women. Based on the evidence that human blood contains information about short-term exposure to xenobiotics, and that plasticizers have a relatively short half-life as they are rapidly metabolized in human body[74,75], the greater contents of DEHP and DiBP in reproductive and premenopausal woman correlates well with younger age groups, who are prone to use at higher quantities a variety of PVC and PC products containing such common plasticizers, such as toys, cosmetics, baby care and body care products, food wraps, bottles, food containers etc. Additionally, although the content of plasticizers, such as DEP and DBP, showed no discrimination power in relation to the females' age, they were still characterized by different detection frequencies between the investigated age groups, which may underline a more frequent use of PVC products by younger than older females.

9.4.8. Conclusions

Results from this study indicate that the environmental abundance of plastics with their MPs, constantly exposes also the modest portion of female participants in this study to the wide spectrum of plasticizers, and that a correlation between the women's age and the profile of PAEs, NPPs and BPs outlined in blood, can be reasonably defined. However, the lack of information regarding dietary habits, education, lifestyle, employment etc. of participating subjects, prevented us from discovering the exposure routes in the study population, as well as correlating the obtained data with clear environmental sources of plasticizers. As a result, the sources of non-occupational/occupational exposure to such xenobiotics should be in-depth explored.Undoubtedly, there is a need for a radical change in approaching the study of contaminants in human health, as more emphasis should be first placed on monitoring programs, population-based data, and epidemiological studies to derive reliable

relationships between human exposure and health outcomes. By doing so, better predictive models of human response to toxicants may be established.

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