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Innovative Mass Spectrometry Techniques for Food Quality and Authenticity

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INNOVATIVE MASS SPECTROMETRY TECHNIQUES FOR FOOD QUALITY AND AUTHENTICIY

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QUASIORA

QUAlità SIcurezza ORigine degli Alimenti

Nothing in life is to be feared,

it is only to be understood.

Now is the time to understand more,

so that we may fear less.

Marie Skłodowska-Curie

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CHAPTER 1.

Food quality: Nutraceuticals, Safety and Authentication

1.1 Introduction to food quality

Food quality represents one of the most critical issues of the present day. It is pretty common among consumers to consider, in advance, products like fresh fruits and vegetables as free of any risk to human health. Recent illnesses connected with the consumption of fruits, vegetables, juices and milk, however, demonstrated that making this assumption is not always proper. (Jha, 2010) For that reason, ensuring the quality of food products is an increasing focus for producers and governments, even if the goal is not easy to reach because of the change in what represents food security and nutritional quality worldwide. (Vincente et al., 2014; Caswell, 1998)

The DIN ISO 9000 defines quality as "the totality of the features and characteristics of a product that bear on its ability to satisfy stated or implied needs". So, it can mean different things within the distribution chain. Good examples are sensory characteristics, which can be perceived directly by the human senses. They depend on the connection between human sensory perception and other brain functions such as memory, culture, values, and emotions. Regarding fruits and vegetables, this category includes size, shape, texture, colour, and flavour. There are also hidden attributes, like nutritional and safety, that can be ensured using more sophisticated techniques. On the other hand, it should also consider the ideological and the religious factors. Indeed, the ultimate objective of the production and distribution of food is to satisfy the needs and expectations of consumers whose behaviour and desires change constantly as society does. (Jha, 2010; Caswell, 1998; Muller & Steinhart, 2006; Perri, 2006)

A model to ensure food quality should look at a series of parameters that can be distinguished into two groups depending on whether the product is seen as food or an object of trade. The product as food, in turn, contains two subcategories: product requirements and psychological requirements. The first one includes commodity requirements established by law, voluntary regulations, customary practices, and safety, sensory and nutritional requirements. The combination of nutritional and sensory requirements leads to biological quality, the foundation of food quality. These four requirements, however, are not enough to satisfy consumer expectations. For that reason, the subcategory of psychological requirements is important and incorporates requirements concerning the production context, such as the product's

origin or use of organic agriculture, and ethical requirements, which are the values conditioning consumers' behaviour.

Apart from this, there is the side of the product as an object of trade. In this case, extremely significant are certification, authenticity and traceability. (Perri, 2006)

1.1.1 Nutritional parameters

A correct diet is composed of distinct groups of food collectively providing the nutrients to satisfy the human body's needs for optimum health. That includes cereals and cereal products, fruits and vegetables, roots and tuber, milk with other dairy products, meats, fish and eggs. (Geissler & Powers, 2017) Comparing animal and vegetable products, the second ones have a much wider variability in nutritional composition and represent a convenient alternative to conventional animal-based products. (Nolden & Forde, 2023) For that reason, but also for the growing interest in facing heart disease, stroke, cancer and diabetes, which are the main causes of mortality in the world, it is worth focussing attention on how fruits and vegetables affect human health. (Vincente et al, 2014)

The nutrient composition of vegetables is quite complex to estimate due to all the genetic and environmental factors that affect the levels of plant metabolites. Amongst them, not only natural factors such as light, humidity, temperature, and type of soil but also transportation and storage conditions may be relevant. All the physical and chemical changes during these stages could lead to the loss of beneficial components. (Hounsome et al., 2008)

Besides water, constituting up to 95% of the mass and representing the most abundant single component in fruit and vegetables (Vincente et al., 2014), the nutrient compounds are the product of primary and secondary plant metabolism. Primary metabolites are produced using nearly the same biochemical pathways: carbohydrates, amino acids, lipids and organic acids (Hounsome et al., 2008). In contrast, secondary metabolites such as flavonoids, carotenoids, sterols, phenolic acids and alkaloids are obtained from the secondary metabolism of plants and have attracted the attention of the scientific world for their potential therapeutic effect on human health. (Hounsome et al., 2008; Fernandes de Arujo et al., 2021)

- **Carbohydrates.** The amount of carbohydrates in fruits and vegetables varies from as little as 2% (melons, cucumbers) to 50-80% of dry weight. Consequently, they represent the most abundant constituents after water and occur in different forms. Primarily, carbohydrates are present in all vegetables as sugar monosaccharides. Examples are glucose, fructose, and arabinose, water-soluble compounds responsible for the sweet taste of fruits and vegetables. Then there are disaccharides (sucrose, maltose), sugar alcohols (sorbitol, mannitol), oligosaccharides (raffinose, stachyose) and polysaccharides (starch, cellulose, hemicellulose, pectin). (Vincente et al., 2014; Hounsome et al., 2008; Lee, 2018)

  Considering their nutritional role, carbohydrates are distinguished as available and unavailable. Examples of available carbohydrates are sucrose and digestible starch; in fact, they are hydrolyzed to monosaccharides by enzymes of the human gastrointestinal system. Available carbohydrates represent the most accessible source of energy in the human body. On the other hand, unavailable carbohydrates are not hydrolyzed and can be fermented in the large intestine by microorganisms. (Hounsome et al., 2008) Some non-digestible compounds, such as cellulose, pectins and resistant starch, are dietary fiber. A diet low in fiber may increase the risk of colon cancer, cardiovascular diseases, and obesity. (Vincente et al., 2014; Hounsome et al., 2008)

- **Amino acids**. In fruits and vegetables, proteins constitute less than 1% of the fresh weight but should be considered because dietary protein is the main source of structural material for the human body: amino acids. In fact, enzymes break down most proteins to form amino acids, which are absorbed in the small intestine. Humans can biosynthesize some amino acids such as alanine, asparagine, glutamic acid, aspartic acid, cysteine, glycine, proline and tyrosine. At the same time, nine specific amino acids are called essential, related to the fact that they can only be obtained from diet. These essential amino acids, which are tryptophan, valine, lysine, methionine, phenylalanine, leucine, histidine, isoleucine, and arginine, are contained in vegetables, but

sometimes in an incredibly low amount and cannot satisfy the human need. Potatoes and asparagus have elevated arginine levels, while histidine can be found in broccoli, cauliflower and phenylalanine in carrots, spinach, and tomatoes. (Hounsome et al., 2008; Lee, 2018)

- **Lipids**. The total fat concentration in fruits and vegetables varies a lot, but it can be considered that the majority has a content that is less than 1%: 0.2% for grapes, 0.1% for bananas and 0.06% for apples. There are some exceptions. For example, avocado and olive have a fat content within 35-70% of dry mass. Lipids are mainly found as triglycerides, esters of glycerol and three fatty acids. (Vincente et al., 2014) Fatty acids comprise a hydrophilic group attached to a long hydrocarbon chain, providing energy and structural material for the human body. (Hounsome et al., 2008)

  There are different classification methods to distinguish between fatty acids. One of the simplest is considering the number of double bonds in the hydrocarbon chain. That way, there are saturated fatty acids that do not have double bonds. The most abundant is palmitic acid, which can be found in almost all types of vegetable oil. Then there are the monounsaturated fatty acids with only one double bound and the polyunsaturated fatty acids with two or more double bonds. (Shahidi & Senanayake, 2008; Moghadasian & Shahidi, 2017) The last category contains four independent families: ω-3, ω-6, ω-7 and ω-9 where ω-3 and ω-6 are the essential fatty acids. Fatty acids are necessary for some functions in the human body. They are used to build lipids and hormones to regulate blood pressure, blood clotting and inflammatory response. The human body can synthesize most fatty acids. Exceptions are linoleic acid and α-linolenic acid, which must be introduced through diet and are quite common in plant oils. (Vincente et al., 2014; Das, 2006)

- **Organic acids**. The most abundant organic acids in fruits and vegetables are aliphatic, such as citric acid, malic acid and tartaric acid. (Vincente et al., 2014) Some of these acids can accumulate in considerable amounts, so fruits and vegetables are normally acidic. Citric acid is the principal acid in citrus fruits,

strawberries, pineapples, pomegranates, and pears, while malic acid characterizes apples, plums, cherries, apricots and bananas. Therefore, the range of acidity is quite extensive. The content is particularly low in some vegetables, while in some fruits, such as lemon, grape, and loganberry, it can reach extremely elevated levels. Lemon, for example, contains over 3% of citric acid (Lee, 2018)

Organic acids also play a significant role as flavour enhancers and natural antimicrobial agents. (Hounsome et al., 2008)

1.1.2 Safety contaminants

Assuring safety in food cannot be achieved at 100% due to the impossibility of testing every single product for every imaginable contaminant, adulterant, toxin, or food pathogen. (Bochers et al., 2010) Governments all over the world are doing the best possible to improve the safety of food. The agri-food sector must respect increasingly rigorous and stringent quality control procedures. Despite this, illnesses connected to foods remain a significant health issue for developed and developing countries. Food can be contaminated at any point, for example, during harvesting or processing, storage, distribution, transportation, and even during preparation. (Bochers et al., 2010; Jha, 2010; Kamboj et al., 2020)

For example, urban activities can be responsible for pesticides in fruits and vegetables or heavy metals such as cadmium, mercury and arsenic in water, air and soil. The contamination can also occur during transportation due to exhausts of petrol and diesel or cross-contamination in the vehicle. Another step of possible contamination is cleaning one during food processing. It is essential to eliminate microorganisms, but great attention must be paid to the chemicals used as cleaners or disinfectants. Not all the products can be used because they could leave unsafe residues. One pretty underrated aspect is the food preparation. Frying is a cooking process that can generate various toxic compounds in the food.

Likewise, food packaging provides protection and ensures a better shelf life of the product, but the direct contact between the food and the packaging results in the migration of harmful compounds. Indeed, there is strict legislation in Europe for the materials that can be safely used for food packaging. (Kamboj et al., 2020)

The main concern is microbiological contamination. Over 2300 types of *Salmonella exist,* and the presence of a chemical contaminant whose source is much more difficult to trace. (Jha, 2010; Kamboj et al., 2020) These contaminants are generally called hazards, where a hazard is "a biological, chemical or physical agent in food with the potential to cause an adverse health effect". (Kamboj et al., 2020) (**Figure 1.1.1**) All the main groups of chemical contaminants found in food share three characteristics. First, they are not intentionally added to food. This allows us to distinguish between contaminants and other chemicals in food, like vitamins and additives. Then, the contamination can happen in one or more stages of food production, and the disease usually manifests itself if enough of the contaminant is ingested. Common chemical contaminants are naturally occurring toxicants, pesticides, veterinary drugs and persistent environmental chemicals. (Watson, 2001)

- **Mycotoxins**. Mycotoxins are secondary metabolites secreted by fungi growing on organic substrates that cause fatal or side effects when ingested by humans. (Adeyeye, 2020) The ingestion can occur for direct consumption of contaminated food or indirectly through ingesting food from animal sources such as eggs, meat and milk. Some conditions that promote the formation of these toxins are moisture, temperature, and water vapour in the air. The formation of mycotoxins may start in the preharvest phase of the crop and then accumulate during harvest, transportation, and storage. (Shahazad, 2021)

	Type of hazard		
	Biological	**Chemical**	**Physical**
Considerations	Organisms that can cause harm through infection or intoxication	Chemicals that can cause harm through toxic effects, either immediate or long-term	Items that can cause harm through direct injury or choking
Examples	Pathogenic bacteria, e.g. *Escherichia coli*, *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *C. botulinum* (non-proteolytic), *C. perfringens*, *Salmonella* spp, *Shigella* spp, *Staphylococcus aureas*, *Vibrio parahaemoliticus*; Viruses, Protozoan parasites, e.g. *Cryptosporidium parvum*, *Giardia intestinalis*,	Mycotoxins, e.g. aflatoxins, patulin, vomitoxin, fumonisin; pesticides, allergenic materials, heavy metals, PCBs dioxins, cleaning chemicals	Glass, metal, stones, wood, plastic, pests, intrinsic natural materials, e.g. bone, nut shell

Figure 1.1.1. Type of hazard (Kamboj et al., 2020).

Even if mycotoxins are present in tiny amounts, they significantly impact human health. They can cause alteration of genome expression, kidney diseases, they can affect the central nervous system, and disturb the intestine, and some of them are known to be carcinogens. (Adeyeye, 2020; Shahazad, 2021)

The most common mycotoxins are aflatoxins, which are carcinogenic secondary metabolites of fungal origin and are linked to diseases such as liver cancer and chronic gastritis. (Adeyeye, 2020) In combination with hepatitis B, aflatoxins are responsible for thousands of deaths in non-industrialized tropical countries. Another important mycotoxin is ochratoxin A, which has been reported to be related to many urinary tract cancers and kidney damage. (Adeyeye, 2016)

- **Pesticides.** The use of pesticides in agriculture has increased in the last decades in correlation with population growth and rapid urbanization.

(Narenderan et al., 2020) They improve the yield of crops such as corn, potatoes, and vegetables, but the residues of pesticides can contaminate soils and water, enter the food chain, and be ingested by humans. (Carvalho, 2006) Exposure to pesticides, or their metabolites and breakdown products, leads to potentially dangerous effects on human health. Indeed, they can cause depression and neurological deficits, but also diabetes, respiratory diseases and, in extreme cases, they can cause cancer, fetal death, spontaneous abortion, and genetic diseases. (Narenderan et al., 2020)

For this reason, many governments are trying to reduce the use of dangerous pesticides, but there is a fundamental difference in the behaviour of developed and developing countries. Developed countries, including the European Union, USA, and Canada, pass increasingly stringent laws on the use of agrochemicals. The aim is to protect consumers by reinforcing lower concentration limits for residues in food and water. For example, in the EU, the limit concentration of pesticides in drinking water is set at 0.1 µg/L. For developing countries, the situation is not the same. They need to increase agriculture production and protect crops, and the use of pesticides is an effortless way to achieve it. Nowadays, new pesticides are less persistent in the environment than classic ones, but they are more expensive and cannot be afforded by developing countries that keep cheaper alternatives, such as DDT and HCH. (Carvalho, 2006) Pesticides are divided into four families: organochlorines (OCs), organophosphates (OPs), carbamates and pyrethroids. (Narenderan et al, 2020)

The OCs pesticides, such as DDT, have high toxicity and bioaccumulation. They are also carcinogenic and environmentally persistent, with an expected life of 10-30 years. So, they are banned for agricultural purposes in Europe, America and many Asian countries, and organophosphorus compounds and carbamates have largely replaced them. (Watson, 2001; Narenderan et al., 2020) These two groups are mainly used due to their price, low-persistent characteristics in environmental conditions and the capability to kill many pests. They also have toxic aspects but are lower if compared to OCs. The pesticides are classified as extremely hazardous, highly hazardous,

moderately hazardous, and slightly hazardous by the World Health Organization. (Narenderan et al., 2020)

- **Veterinary drugs**. The presence of veterinary drugs in food has been recognized later than pesticide residues, but several of them are likely to cause adverse effects in humans. The main types of veterinary drugs are antimicrobial agents used to treat and prevent diseases caused by bacteria and fungi. (Watson, 2001; Baynes et al., 2016) Above the regulatory concentration, their residues in food products could result in allergic reactions, disruption of normal intestinal human flora, cancer and development of antimicrobial resistance, making it more difficult to treat human infections.

  Veterinary drugs include beta-agonists, used to increase feed efficiency. Many adverse health effects in humans have been reported, such as muscle tremors, cardiac palpitations, nervousness, headache, muscular pain, nausea, vomiting and fever. (Baynes et al., 2016)

- **Persistent environmental chemicals**. Among all the environmentally persistent chemicals, the most studied are polychlorinated biphenyls (PCBs) and dioxins. They can enter food products for direct contamination due to an industrial accident or migration from packaging. (Watson, 2001) PCBs can cause many adverse effects on human health regarding the immune, reproductive, nervous and endocrine systems. The International Agency for Research on Cancer (IARC) classify PCBs as group 1 human carcinogens. They were banned from commercial production in the 70s. However, a vast quantity of PCBs is still contaminating the environment. (Saktrakulka et al., 2020)

  Dioxins are ubiquitous environmental contaminants due to their persistence. The principal sources of dioxins include incinerators and vehicle exhausts, manufacture and use of organic chemicals and metallurgical processors. The contamination of food may occur through atmospheric deposition and the spreading of food on farmland. (Watson, 2001)

1.2 Nutraceuticals: beyond diet and before drug

In recent years, human diet habits have changed due to the constant increase of diseases connected to improper nutrition and consumption of junk food. Among them, there are obesity, heart disease, cancers, osteoporosis and arthritis. Therefore, people seek alternative beneficial products, making nutraceuticals particularly appealing. (Das et al., 2012) The term nutraceutical was coined in 1989 by Stephen Defelice from the Union of Nutrition and Pharmaceuticals. He defined "a nutraceutical as a substance that is a food or a part of food and provides medical or health benefits, including the prevention and treatment of disease." This definition concerns isolated nutrients, herbal products, and dietary supplements. It is important to highlight that a functional food generally provides the human body with the correct amounts of vitamins, fats, proteins, carbohydrates and all the compounds necessary for healthy survival. It becomes a nutraceutical only when the functional food helps prevent or treat diseases. (Das et al., 2012; Pandey et al., 2010)

Despite the growing interest, there is no regulatory definition. In the European Union, nutraceuticals are considered the same as food supplements. In the recent regulation, there is special attention to novel food, but nutraceutical is still not officially recognized or mentioned. According to this regulation, the European Food Safety Authority (EFSA) does not distinguish between food supplements and nutraceuticals. (Santini et al., 2017)

The benefits that nutraceuticals can give include the increase of health value of the diet, the possibility of extending life expectancy and avoiding some medical conditions. Moreover, nutraceuticals can be perceived as more natural than traditional medicine and less likely to produce side effects (Pandey et al., 2010).

There exist diverse ways to classify nutraceuticals; some of the most common are based on food sources, mechanism of action and chemical nature. According to food sources, nutraceuticals are classified as:

- Probiotics.
- Prebiotics.
- Polyunsaturated fatty acids.
- Antioxidant vitamins.
- Bioactive compounds: polyphenols, terpenoids and alkaloids.

Considering a broader classification, nutraceuticals can be distinguished into two groups:

- ❖ Potential nutraceuticals
- ❖ Established nutraceuticals.

A potential nutraceutical becomes established after clinical data on its health effects are obtained (Das et al., 2012), but it is quite disappointing that most nutraceutical products are in the potential category (Pandey et al., 2010).

1.2.1 Probiotics and Prebiotics

Nutraceuticals can also include live microorganisms, such as in the case of probiotics. Probiotics have been defined by the World Health Organization (WHO) as "live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host" (Damian et al., 2022; Aureli et al., 2011). Prebiotics, as shown in **Figure 1.2.1**, are food ingredients that, at low levels, promote the growth of helpful bacteria with a good impact on health. For example, lactobacillus and Bifidobacterium use prebiotic fibre as substrate (Jain et al., 2022).

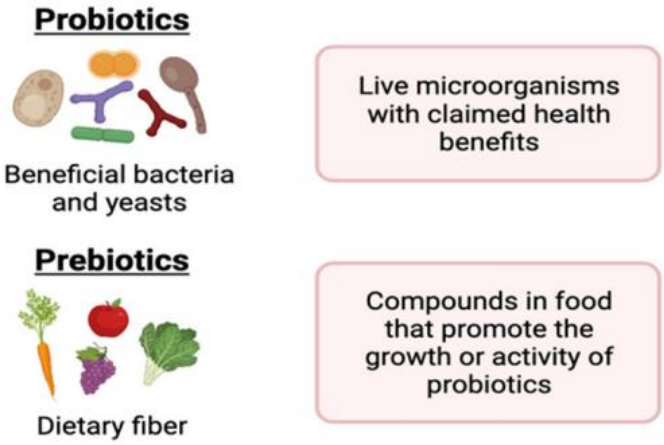

Figure 1.2.1. Probiotics and Prebiotics definition (Damian et al., 2022).

Probiotics generally include some bacteria categories, such as lactobacilli, gram-positive cocci and bifidobacterial, and are available in various forms, such as liquid, gel, or powder (Ds et al., 2012). The assumption of probiotics has been shown to help

the immune system against pathogens, suppress autoimmune responses, shorten the duration of infectious diarrhoea, increase gastrointestinal tolerance to antibiotics, improve treatment of women with bacterial vaginosis and decrease the incidence of dental caries (Jain et al., 2022).

The health benefits of prebiotics, on the other hand, incorporate improved lactose tolerance, anti-tumour properties, neutralization of toxins and stimulation of the intestinal immune system, reduction of constipation and reduction of blood lipids and blood cholesterol levels (Das et al., 2012).

The food industry presented many functional foods obtained by adding probiotic bacteria as ingredients in the formulation and as additives. Apart from dairy-based functional food that contains probiotics, there are also plant-based and fruit-based products containing probiotics. Good examples are fruit juices and juice drinks; adding prebiotics produces bioactive compounds that increase the antioxidant capacities through the fermentation of sucrose and sugars in the fruit (Damin et al., 2022).

1.2.2 Polyunsaturated fatty acids.

The polyunsaturated fatty acids contain more than one double bond in their hydrocarbon chain and are also called essential fatty acids because they are crucial for the body's function and must be introduced externally through the diet (Das et al., 2012).

The primary families are the ω-3, which results from α-linolenic acid (ALA, 18:3) and the ω-6, which comes from linoleic acid (LA, 18:2) (Das, 2006). Such fatty acids make up the cellular membranes and help maintain homeostasis, providing the correct functionality for proteins. Generally, polyunsaturated fatty acids are considered beneficial for human health, but the ω-3 and ω-6 families can have opposite effects on human metabolism. A diet rich in ω-6 relates to inflammatory processes, obstruction of blood vessels and platelet aggregation. On the one hand, acute inflammatory responses can protect the body from infections and wounds, but on the other hand, excessive stimulation of the inflammatory system could produce a favourable environment for the growth of tumours. Indeed, a persistent inflammatory condition is linked to the development of tumours and metastases.

Equally, chronic inflammation led to atherosclerosis with the outbreak of cardiovascular diseases (Saini et al., 2018).

The relation between the consumption of ω-3 and ω-6 has changed over time. Nowadays, industrialized societies are characterized by a higher consumption of saturated fatty acids, ω-6 and trans fatty acids, with a decreased intake of ω-3. The fatty acids now represent between 28 and 42% of the total energy for European populations, while, in the past, it was only 20-30%. The increased consumption of vegetable oil rich in linoleic acid has led to a higher assumption of ω-6 against the ω-3 one. An optimal diet requires a ratio of 1-4:1 between ω-6 and ω-3 intake. However, nowadays, this ratio has increased up to 10:1 or 20:1. Concurrently, there has been an increase in the incidence of diseases involving inflammatory processes, such as cardiovascular problems, diabetes, obesity, rheumatoid arthritis, and cancer (Patterson et al., 2012).

The high intake of ω-6 increases the plasma concentration of its metabolic products that contribute to the formation of thrombus and atheroma in blood vessels, to the development of inflammatory diseases and allergies and excessive cell proliferation. The ω-3, instead, counteract these deleterious effects, determining the production of compounds with vasodilatory activity and strong anti-inflammatory activity (Yashodhara et al., 2009).

Because they present such conflicting effects, to understand the beneficial effects on the human organism ω-3 and ω-6, it is necessary to enter more into the details of these two families of compounds.

- **The ω-3 family**. The first evidence of the beneficial effect of a diet rich in ω-3 occurred with the observation of an extremely low incidence of inflammatory and autoimmune diseases, such as psoriasis, asthma and diabetes, in the Inuit population. The Inuit, as well as Japanese, have in their diet a high consumption of ω-3 from fish and a low incidence of myocardial infarction and autoimmune and inflammatory diseases. For that reason, today, many studies have highlighted the fundamental role played by ω-3 in preventing and treating coronary diseases, hypertension, arthritis, and cancer (Simopoulos, 2022).

The main ω-3 fatty acids are α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The body can use all three of these ω-3 to perform various functions. EPA is mainly found in fish and fish oil, while DHA is in fish and seaweed oil. ALA, instead, is found in seeds, vegetable oils and green vegetables. ALA can also be converted to EPA and DHA in the body, as shown in **Figure 1.2.2**. EPA play a vital role in prenatal development and the cardiac and circulatory system development. Despite this, DHA is the main ω-3 fatty acid considered when dealing with human health during gestation and infant nutrition (Shahidi & Senanayake, 2008). The American Heart Association suggests that the intake of EPA and DHA in a range of 500-1800 mg/day can reduce the risk of death due to cardiovascular diseases, while The Food and Drug Administration affirms that the intake of EPA + DHA is safe up to 3000 mg per day. Switching to excessive consumption has negative sides on the immune system and can inhibit blood clotting.

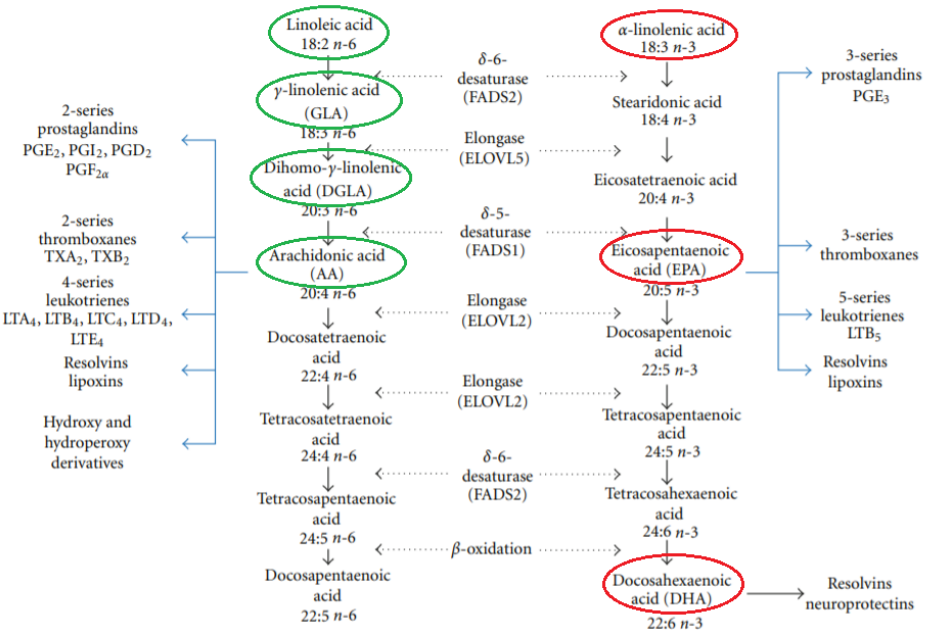

Figure 1.2.2. Metabolic processes of ALA and LA (Patterson et al., 2012).

Regarding ALA, however, the U.S. Institute of Medicine has established that the adequate amount to be taken is equal to 1.1 g per day for adults (Moghadasina, 2017).

The eicosanoids comprise different families, such as the prostaglandins (PGEs), the thromboxane (TXs) and the leukotrienes (LTs). They can be synthesized not only from α-linolenic acid but also from linoleic acid (ω-6). In this case, they are called bad eicosanoids, even if the distinction is unclear, and they are also necessary for the body.

The assumption of EPA and DHA from fish and fish oil has been demonstrated to promote the diminution of prostaglandin PGE_2, of thromboxane A_2, a powerful vasoconstrictor and platelet aggregator, and of leukotriene B_4, and inflammation inductor. On the contrary, they also lead to a higher concentration of thromboxane A_3, a weak platelet aggregator and vasoconstrictor, and leukotriene B_5, a weak inflammation stimulant. Therefore, ω-3 modulates the prostaglandin metabolism and reduces the concentration of triglycerides, and in considerable amounts, it also reduces cholesterol, leading to anti-inflammatory properties.

Between all the beneficial effects of ω-3 on the heart, it is possible to find the reduction of arrhythmia and heart rate. Patients with myocardium heart attack treated with fatty acids ω-3 showed a reduced risk of developing ventricular arrhythmia (minus 38%) and death (minus 28%). Finally, a diet rich in ω-3 delays the progression of coronary atherosclerosis reverses the coronary arteries' stenosis and prevents restenosis following coronary angioplasty. Another beneficial effect is that in patients with type 2 diabetes mellitus, they may reduce insulin resistance (Patterson et al., 2012; Yashodhara et al., 2009; Simopoulos, 2022).

- **The ω-6 family.** The principal ω-6 fatty acids are the linoleic acid (LA), the γ-linolenic (GLA) that can be directly introduced by diet or obtained from linoleic acid, dihomo- γ-linolenic (DGLA) and arachidonic acid (AA). The linoleic acid can be very easily found in different foods such as vegetable oils, meat, eggs and milk (Al-Khudairy et al., 2015), the GLA in milk and hempseed oil, the DGLA in human milk, kidneys and liver, while the AA in human and cow milk, meat and some seeds. It has been estimated that the intake of arachidonic acid daily is around 100-200 mg, more than enough for the

normal production of prostaglandins, which is around 1mg per day (Das, 2006).

It has already been demonstrated that inflammation is the base of numerous diseases, like cardiovascular and atherosclerosis, but the contribution of ω-6 fatty acids to the occurrence of these diseases is controversial. Some studies assert that substituting saturated fatty acids with ω-6 fatty acids relates to a decrease in pathologies of the cardiovascular system. In contrast, others affirm that high levels of ω-6 in diet have a pro-inflammatory effect and worsen the occurrence of such diseases (Al-Khudairy et al., 2015).

It is important to highlight that inflammation is a natural and necessary process of the human body and that it is used as a defence mechanism against pathogenic organisms and as a response to lesions, but if it becomes pathological, it can lead to the onset of diseases. This occurs when regulatory processes lead to excessive inflammation that can damage tissues. However, the interaction of ω-3 and ω-6 with the inflammatory processes is not yet fully understood (Inees & Clder, 2018), so to maintain good health and avoid chronic illnesses, the ω-3 and ω-6 intake should be balanced (Saini et al., 2018). The American Heart Association recommends an intake of about 500 mg of ω-3 and 15 mg per day of linoleic acid (Al-Khudairy et al., 2015).

1.2.3 Antioxidant vitamins.

Vitamins have extraordinarily strong antioxidant properties, so they can be used as nutraceuticals and play a significant role in maintaining human health (Jain et al., 2022). Vitamins can act singly and synergistically to prevent oxidative reactions that can lead to several degenerative diseases like cancer, cardiovascular diseases, cataracts, and many others (Das et al., 2012). The vitamins known today are usually classified as fat-soluble, more specifically vitamin A (retinol), vitamin D, E and K, and water-soluble, such as vitamin C and B complex (**Figure 1.2.3**) (Vincente et al., 2014).

Figure 1.2.3. Food distribution of vitamins.

Vitamin A involves vision, cell division and differentiation, bone development and reproduction. It is common to observe vitamin A deficiency in populations where the diet is based almost on a single starch-based crop that does not contain enough pro-vitamin A carotenoids. It is estimated that vitamin A deficiency affects almost 1/3 of children under the age of five, and most of them are blind or die (Vincente et al., 2014).

Vitamin C has two main forms: ascorbic acid and dehydroascorbic acid (**Figure 1.2.4**). It is a common metabolite in many plants and animals, but humans cannot synthesize it due to a series of gene mutations responsible for catalyzing the last enzymatic step in ascorbate synthesis. However, the right amount of vitamin C can be introduced by diet. Regarding plasma concentrations, values above 28 µmol/L are considered adequate, while values between 11 and 28 µmol/L indicate slight deficiency and values below 11 µmol/L specify deficiency. The lack of vitamin C can cause scurvy, leading to blood vessel fragility, connective tissue damage, tiredness and death. Even in industrialized countries, it is possible to easily find slight deficiency with an incidence of 15% of the general population.

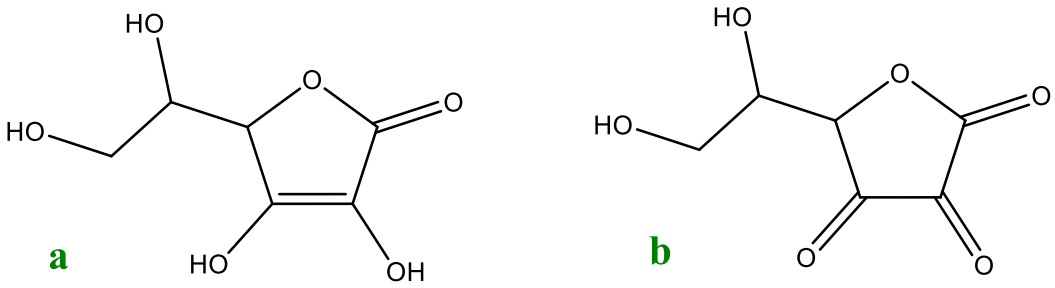

Figure 1.2.4. Chemical structure of ascorbic acid (a) and dehydroascorbic acid (b).

The recommended dietary allowance (RDA) for vitamin C in the United States and Canada is set to 90mg/day for adult men and 75 mg/day for adult women, but these values change worldwide. For example, in the United Kingdom, the RDA is 40 mg/day. In France and Belgium, it is 110 mg/day; in Germany, it is 110 mg/day for men and 95 mg/day for women (Granger & Eck, 2018; Grosso et al., 2013).

Vitamin C is a potent reducing agent that protects cells against oxidative damage. This antioxidant property is correlated to its ability to reduce potentially damaging ROS by forming relatively stable ascorbate free radicals. The excessive production of ROS led to diseases such as cancer, diabetes, cardiovascular problems, atherosclerosis, and ageing. Moreover, ascorbic acid prevents DNA mutation and can be considered a potential anti-cancer mechanism, and vitamin C may also function as a cancer cell killer (Grosso et al., 2013).

The main sources of dietary vitamin C are fruits, vegetables, and juices. In the United States, fruits and vegetables represent 90% of the vitamin C food supply. Reliable sources are kiwifruit, strawberry, citrus fruit, spinach and broccoli. The amount of ascorbic acid can vary within species due to genetic and environmental factors, such as exposure to sunlight. More sunlight received during growth increases the concentration of ascorbic acid.

Vitamin D is usually present as ergocalciferol cholecalciferol. It helps the absorption and use of calcium and phosphate, but it occurs in trace amounts in fruits and vegetables. On the contrary, vitamin D is found in higher amounts in fortified dairy products, eggs, liver and salmon. (Vincente et al., 2014). The lack of vitamin D is the most widespread in developed nations, but in this case, the deficiency does not necessarily determine an explicit disease. For example, it is involved in protection

against age-related bone illnesses such as osteoporosis and a lower risk for breast cancer and cardiovascular diseases (Heaney, 2008).

Vitamin K is especially important for blood coagulation; it helps to prevent vascular calcifications, and it helps to prevent fractures due to osteopenia and osteoporosis. Moreover, vitamin K inhibits the growth of human cancers such as hepatoma. Its deficiency is very uncommon because it is abundant in lettuce, spinach, cauliflower and cabbage, and it can also be produced by gut microflora (Vincente et al., 2014; DiNicolantonio et al., 2015).

Vitamin E includes eight compounds, namely α-, β-, γ-, δ-tocopherol and α-, β-, γ-, δ-tocotrienol where the terms α, β, γ and δ refer to the number and position of methyl groups on the chromanol ring (**Figure 1.2.5**).

Each form has vitamin E activity, but α-tocopherol is the most active and the most usual form of vitamin E in human tissues, followed by γ-tocopherol, while tocotrienols are usually not detected in tissues. It is a cytoprotective factor that helps prevent inflammatory and degenerative processes in the liver during exposure to xenobiotics, environmental pollutants, and dietary factors. The α-tocopherol was also reported to prevent cholesterol-induced atherosclerosis lesions and associated cardiovascular complications.

Dietary intake of vitamin E is established in many countries in relation to its antioxidant activity (protection against lipid peroxidation). The RDA vary between 3 and 15 mg/day depending on the country and the age of the person. For example, in the United States, the RDA is 15 mg/day of α-tocopherol for both adult men and women. The European Safety Authority (EFSA) established an adequate intake of 13 mg/day for men, 11 mg/day for women and 5-13 mg/day for children depending on age. However, vitamin E intake is generally low worldwide in both developing and industrialized countries (Galli et al., 2017). The main dietary sources of vitamin E are oily seeds, olives, nuts, avocados, and almonds. Broccoli and leafy vegetables are the better sources of vitamin E among fruits and vegetables, even if they contain tocopherols less than fats and oils (Vincente et al., 2014).

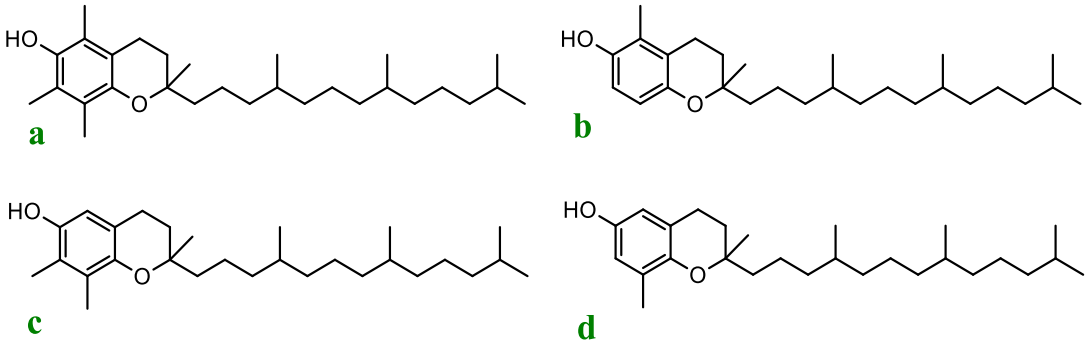

Figure 1.2.5. Chemical structure of α-tocopherol (a), β-tocopherol (b), γ-tocopherol (d) and δ-tocopherol (d).

The vitamin B complex contains B1 (thiamine), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B9 (folate/folic acid), biotin, choline and B12 (cyanocobalamin). Thiamine plays a key role in the metabolism of carbohydrates. Its recommended daily intake ranges between 1-2 mg for adults, and legumes are especially rich in thiamine.

Regarding vitamin B2, the RDA is around 1-2 mg/day. It is usually found in good amounts in green vegetables such as beans, peppers and spinach, while starchy vegetables and fruits are relatively poor sources of riboflavin.

Niacin, which represents vitamin B3, has a recommended daily intake of 10-25 mg. It is a precursor of NADH, so it is essential for living organisms. It can be introduced from the diet. For example, almonds, gooseberries and avocadoes are useful sources, but they can also be synthesized in the body starting from tryptophan. The problem with niacin is that it can be stored in the organism for a period longer than 10-14 days, so in case of improper intake, the deficiency symptoms rapidly develop.

Pantothenic acid, or vitamin B5, occurs widely in fresh fruits and vegetables like beans, peas, broccoli, mushrooms, and potatoes. When its intake is insufficient, the deficiency leads to tiredness, headaches, sleep disorders and tingling of hands (Vincente et al., 2014).

Vitamin B9 comprises folic acid and folates, where folic acid rarely occurs in nature. Its synthetic form is added to food to obtain fortified food and dietary supplements. Folate in foods mainly occurs in the polyglutamate form that needs to be hydrolyzed to the mono-glutamate form before absorption. Its deficiency generates the typical symptom of megaloblastic anaemia, leading to a volume increase of red blood cells.

Moreover, a low folate status is related to an increased risk of chronic illnesses such as cardiovascular diseases, cancer (colorectal, breast and prostate cancer) and cognitive dysfunctions. For that reason, there is a recommended daily intake of folates in various countries. The RDA is set to 240 μg/day for people aged 18. In the case of pregnant women, the RDA has an addition of 240 μg/day for a total of 480 μg/day, while for lactating women, there is an additional 100 μg/day for a total of 340 μg/day. The maximum tolerable intake level of folic acid has been estimated as 1000 μg/day to avoid adverse effects. The problem does not concern a normal diet but dietary supplements. A high folic acid intake could mask pernicious anaemia, resulting in neurological disorders (Ebara, 2017).

1.2.4. Bioactive compounds: polyphenols, terpenoids and alkaloids.

Polyphenols are a wide range of bioactive compounds that are secondary metabolites produced by fruits and vegetables. Over 8000 different classes of polyphenols are known, with a general structure characterized by one or more aromatic rings with variable degrees of hydroxylation. They can be free or conjugated with sugars, acids, and other biomolecules soluble or insoluble in water. In general, polyphenols engage in the defence of plants. They function as deterrents for many insects and other animals and as antimicrobials. They also protect plants against UV radiation and oxidative stress. Moreover, polyphenols contribute to the colour and smell of plants (Vincente et al., 2014; Hounsome et al., 2008; Das et al., 2012; Fernandes de Arujo, 2021). Polyphenols introduced by diet can benefit human health because they possess antioxidant, anti-inflammatory, antimicrobial and cardioprotective activities, and they also have a role in preventing neurodegenerative diseases and diabetes mellitus. They are mainly known for their antioxidant activity, which is more effective than vitamins E and C due to their chemical structure (Das et al., 2012). Polyphenols are grouped into phenolic acids, flavonoids, lignans, stilbenes, tannins, coumarins and lignin (Vincente et al., 2014).

- **Phenolic acids**. Phenolic acids are naturally found in fruits and vegetables. They can be divided into two classes: the ones deriving from hydroxybenzoic acid and the ones deriving from hydroxycinnamic acid, whose structures are highlighted in **Figure 1.2.6**.

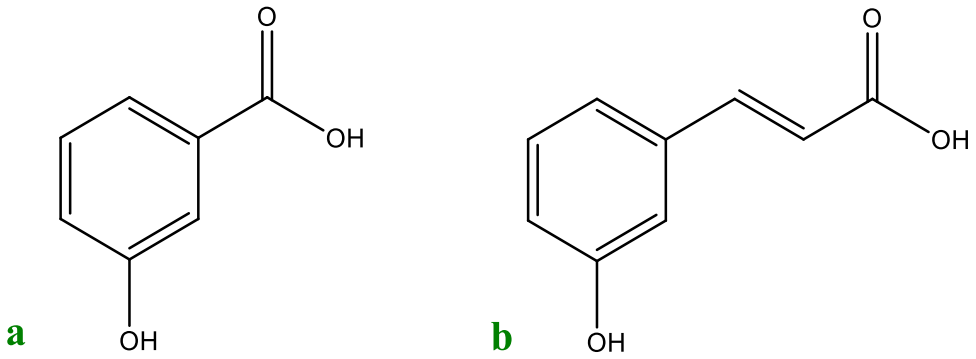

Figure 1.2.6. Chemical structure of hydroxybenzoic acid (a) and hydroxycinnamic acid (b).

The compounds derived from hydroxybenzoic acid are characterized by one carboxylic group, and the most common are ρ-hydroxybenzoic acid, gallic acid, protocatechuic acid and vanillic acid. The compounds derived from hydroxycinnamic acid, instead, are characterized by a two-carbon skeleton, and the main are ρ-hydroxycinnamic acid, ρ-coumaric acid, caffeic acid and ferulic acid (Fernandes de Arujo et al., 2021). These acids are popular in fruits and vegetables but are rarely present as free and mainly as acid derivatives. For example, caffeic acid is esterified with quinic acid, forming chlorogenic acid, the most popular phenolic acid in apples and pears (Lee, 2018). Gallic and caffeic acid are found in lettuce, vanillic and cinnamic acid in onions, parsley, and spinach, and coumaric acid in tomatoes, carrots, and garlic (Hounsome et al., 2008). The potential of phenolic acids on human health is as wide as their structural diversity. They can act as antidepressant, antihypertensive, anti-inflammatory, neuroprotective, antihyperglycemic, anticancer and anti-diarrheal (Fernandes de Arujo et al., 2021). For example, it has been reported that benzoic, vanillic and caffeic acids have antimicrobial antifungal action, while chlorogenic, ferulic and coumaric acids have strong antioxidant activity due to the inhibition of lipid oxidation (Hounsome et al., 2008).

Moreover, it has been demonstrated that about 100 mg/Kg/day for 8 weeks of chlorogenic acid is effective against metabolic syndrome. It determines an

increase in gut microbiota diversity. The intake of 60 mg/Kg/ day of gallic acid for 7 days by intragastric injection reduces the expression of pro-inflammatory biomarkers. In addition, gallic acid helps reduce weight gain (Fernandes de Arujo et al., 2021).

- **Flavonoids**. Flavonoids are the most common phenolic compounds found in fruits and vegetables. Two aromatic rings characterize their structure, usually indicated as A and B, attached to a tetrahydropyran ring called C. This pyran ring may vary and allows to distinguish within six groups, whose basic structure is shown in **Figure 1.2.7** and are flavonols, flavanones, flavanols, flavones, anthocyanins and isoflavones. Most flavonoids are naturally associated with sugar in conjugated form and may be monoglycosidic or diglycosidic.

  They are related to the colour and taste of flavonoids. For example, anthocyanidins, chalcone and flavones are pigments that determine the colour of vegetables. The composition of flavonoids in plants depends on a series of factors, including the genetic variety of the species, the part of the plant, the condition of growth and the degree of maturation (Hounsome et al., 2008; Fernandes de Arujo et al., 2021; Tapas et al., 2008). Flavonoids introduced by diet benefit human health; indeed, they possess antiviral, anti-inflammatory, antihistamine, and antioxidant properties. They are also involved in synthesising enzymes and vitamins, and they play a significant role in limiting the oxidation of lipids. Damage to lipids and proteins has been linked to cancer, atherosclerosis, ischemic injury, and neurodegenerative diseases such as Parkinson's and Alzheimer's. Flavonoids protect low-density lipoprotein cholesterol from being oxidized. In this way, they prevent the formation of atherosclerotic plaques in the arterial wall (Hounsome et al., 2008).

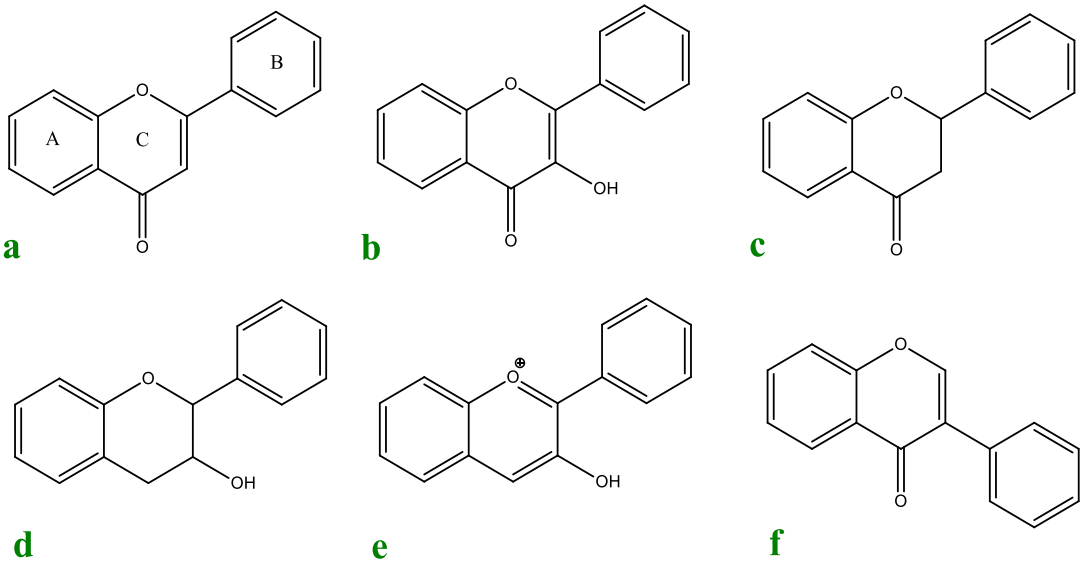

Figure 1.2.7. Basic structure of flavones (a), flavonols (b), flavanones (c), flavanols (d), anthocyanidins (e) and isoflavons (d).

Common flavones are rutin, luteolin and apigenin, while the most abundant flavonols are quercetin and kaempferol. The quercetin can be found in considerable amounts in onions and the peel of blueberries. Flavones are present in citrus but mostly in the peel. It is always possible to find a great number of flavanone glycosides, such as hesperidin, in citrus.

Anthocyanidins are pigments that give some fruits the characteristic colour red or purple. They are widely present in fruits and vegetables and are considered ordinary antioxidants in the human diet. The most common are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. They are usually glycosylated with glucose, galactose, rhamnose and arabinose. For instance, in fruits, the monoglycoside forms represent ca. 70-100% of the total (Vincente et al., 2014).

Apart from polyphenols, another important class of bioactive compounds is represented by terpenoids. They include numerous compounds characterized by the repetitive fusion of branched 5-carbon isoprene units. They can be found in plants, where they play distinct roles. They are components of membranes (sterols) or photosynthetic pigments such as carotenoids, electron carriers and hormones. The

main terpenoids introduced by diet are carotenoids, tocopherols and tocotrienols, quinones and sterols (Hounsome et al., 2008).

- **Carotenoids**. They are pigments synthesized by plants, but not animals; the major human diet sources are fruits and vegetables. More than 600 carotenoids exist in nature, but only around 40 are present in a typical human diet, and of this 40, only 20 were identified in human blood and tissues. β-carotene, α-carotene, lycopene, lutein and cryptoxanthin represent almost 90% of carotenoids in the diet and human body. The main sources of β-carotene and α-carotene are yellow-orange vegetables and fruits, while dark green vegetables provide lutein and tomatoes lycopene. Lack of carotenoids in the human diet can lead to xerophthalmia (night blindness) and premature death. On the contrary, a diet rich in carotenoids has beneficial effects on human health, reducing the risk of some forms of cancer, coronary diseases, and cataracts. For example, lycopene plays a significant role in preventing prostate, breast, lung, gastrointestinal and ovarian cancer and prevents the emergence of cardiovascular diseases. Carotenoids are biological antioxidants that contribute to disorders such as osteoporosis and hypertension (Hounsome et al., 2008; Rao & Rao, 2007).

- **Sterols**. Sterols play a key role in plants; they are structural components but have a regulatory function. They may exist as free sterols, as esters with fatty acids or as sterol glycosides. Due to this diversity of types of sterols, in plants, they play a wide range of functions. For example, some sterols are precursors of plant hormones, the brassinosteroids that participate in plant fertility and growth, cell division and embryonic development (Hounsome et al., 2008). The main sterols that can be found in plants are β-sitosterol (65%), campesterol (32%) and stigmasterol (3%). When introduced by diet, plant sterols are non-nutritive compounds, and their absorption is low if compared to cholesterol, which can vary from 30% to 60%. The absorption of campesterol is 9.4-14.8%, while the absorption of β-sitosterol and stigmasterol are lower, respectively, 3.1-4.5% and around 4%. The higher absorption of cholesterol relies on the presence of a double bond, which

increases the absorption rate, while in phytosterols, the longer chain length decreases its absorption. Due to their similar structure, plant sterols decrease cholesterol absorption from the diet and decrease the synthesis of endogenous cholesterol in the body.

Between the beneficial effects of sterols on human health, there is evidence of the inhibitory action of phytosterols on lung, stomach, and ovarian cancer. They also influence the immune system; some studies show their anti-inflammatory capacity (Jain & Bathla, 2015).

In the end, there are the alkaloids, a group of compounds that contain nitrogen, mainly derived from amino acids. Usually, alkaloids are classified based on the common molecular precursor. In this way, there are coniine and nicotine that derive from pyridine, and there are atropine and cocaine, which derive from tropane. Morphine and codeine have isoquinone as a common precursor. Caffeine is obtained starting from purine, and solanine derives from steroids. Most alkaloids are toxic, but they still play necessary roles in plant protection against herbivores, microorganisms, and damage by UV light. For example, alkaloids like barberine, palmatine and mahanine have antimicrobial and cytotoxic activities.

Some vegetable alkaloids are toxic to humans. Tomatoes and potatoes, when exposed to light, contain solanine and chaconine. Solanine can cause neurological and gastrointestinal symptoms, including depression of the activity of the central nervous system. However, consuming these vegetables is problematic only if they are in excessive proportion.

Some plant glucoalkaloids, such as tomatine and chaconine, are called saponins. Saponins can be found in peas, beans, tomatoes, spinach, onions, and potatoes. Dietary saponins determine a reduction of blood cholesterol, inhibit the growth of cancer cells, and stimulate the immune system. Also, some saponins can be toxic to humans, such as sapotoxin. They irritate membranes of the respiratory and digestive tract (Hounsome et al., 2008).

1.3 Determining food traceability and authenticity.

During the last few years, consumers have shown increasing concern and attention about the quality and safety of the food they buy. Therefore, they request more transparency from food companies and more information about the food they eat, not only from a nutritional point of view but also regarding the origin, safety, traceability, and authenticity of food. The companies, therefore, should be able to certify the content and the origin of their products to protect consumers against adulteration and fraud. There is a growing incentive in the adulteration of food resulting from the growing desire of consumers to pay more money for products with organoleptic characteristics, and due to factors which create opportunities for food fraud such as international trade, long and complex food supply chain and e-commerce. Food adulteration usually has no risk to human health. An example is an incorrect geographical origin on labels, while in other cases, the food may contain health hazards due to toxic and allergenic substances. Different techniques of food fraud have been discovered; good examples are substitution, dilution, mislabelling and concealment. In any case, the food adulteration remains an unfair market competition.

For all this reason, it is necessary to have tools to reassure consumers that traceability and authenticity are fundamental. Traceability is the process of ensuring the food source at any point in the production chain, while authenticity is evaluating food and assessing whether it complies with the labelled information.

In addition, the governments are introducing stricter legislation to achieve control on the food from the field to the market. For example, the European Union established two levels of recognition of food products. This is the Protected Designation of Origin, known as PDO, where the food' main characteristics depend on the territory of origin, a series of strict production rules, and the Geographical Indication (PGI) that can be attributed to a food that has specific characteristics and quality due to a specific area of production (Amaral, 2021; Fanelli et al., 2021).

To ensure food authentication, several analytical techniques can be applied. The majority are based on targeted methods. In this case, some characteristic compounds or adulterants are selected, and the attention is focused on them. These methods have some limits and are not so valuable when there is no information about the

possible adulterants present or when unconventional adulterants are added. In this case, non-targeted methods are more efficient because they are not based on the analysis of a few selected analytes. The aim is to study a global fingerprint that should be as comprehensive as possible. Moreover, this approach generally requires a simpler sample preparation than the targeted methods, which usually require complex and expensive extraction processes. For what concerns the instrumental techniques between them, it is possible to find chromatography, spectrometry, and spectroscopy with the support of chemometric approaches (Amaral, 2021).

1.3.1 Analytical methods for food traceability and authenticity.

Many methods exist to ensure food traceability and authenticity of food deriving from animals and plants. For animal-based food, the principal frauds concern the substitution of an ingredient and the geographical origin of the animal, while for plant-based food, the fraudulent practices are much more. There is incorrect information about the geographical origin of the product reported on the label, the adulteration and contamination of products, the use of distinct species of different varieties compared with those declared on the label, and levels of additives higher than the one permitted. In the first case, the principal techniques applied are based on vibrational spectroscopy and DNA typing of animal species. In the second case, the approaches are more variable (Fanelli et al., 2021).

The most commonly used techniques include liquid and gas chromatography, isotope ratio and elemental analysis, spectroscopic techniques, DNA-based techniques, and sensor techniques. Liquid and gas chromatography are the most widespread in chemical analyses. They usually require long and expensive sample purification steps, isotope ratio, elemental analysis and DNA-based methods. Looking for more rapid, non-destructive, and non-invasive approaches, is it possible to find vibrational spectroscopy, hyperspectral fluorescence, and nuclear magnetic resonance, but sometimes they display lower sensitivity. Some of the techniques commonly used are summarised in **Table 1.1**.

Detection technique	Food matrix	References
HPLC-MS	Wine	Versari et al., 2014
	Apple juice	Kamiloglu, 2019
	Orange juice	Kamiloglu, 2019
GC-MS	Extra-virgin olive oil	Melucci et al., 2016
	Orange juice	Bocharova et al., 2017
	Tea	Kamiloglu, 2019
IRMS	Wine	Dinca et al., 2016
	Tea	Lagad et al., 2013
NIRS	Extra-virgin olive oil	Sinelli, et al., 2010
	Lime juice	Shafiee & Minaei, 2018

Table 1.1. Summary of techniques used for determining the authenticity and traceability of food.

In food analysis, liquid chromatography (LC) is widely applied to analyze, separate, and quantify chemical components in different matrices. It is also used to assert traceability and authenticity by detecting adulterants and pollutants and the chromatographic profile of primary and secondary metabolites such as flavonoids. Liquid chromatography can be coupled with different detectors, UV or fluorescence, but when coupled with mass spectrometry, it has even more power for the traceability aim of agro products. Gas chromatography (GC) is usually applied to analyze volatile and semi-volatile compounds, aromatic molecules, and contaminants like pesticides. Depending on the nature of the compounds, the GC can be coupled with different detectors, such as flame ionization and electron capture, but mass spectrometry, also in this case, remains the most potent detector in food research. The isotope ratio mass spectrometry (IRMS) detects precise and exact deviations in the natural isotopic abundance of light stable isotope, so it is widely used to evaluate the authenticity and geographical origin of agro products. In the end, also the spectroscopic techniques provide useful chemical information regarding the sample. Between them, the main techniques applied for determining the adulteration and authenticity of food products are the near-infrared (NIR), mid-infrared (MIR), Raman Spectroscopy and hyperspectral imaging (Wadood et al., 2020).

1.3.2 Chemometric techniques for food traceability and authenticity

In some cases, the application of the analytical techniques can generate a large amount of data that needs to be interpreted. Advanced multivariate chemometric tools are the perfect instrument to extract as much information as possible from the data (Gonzalez-Dominiguez & Fernandez-Recamales, 2022). Information can be structured at two levels: qualitative and quantitative. The qualitative information usually shows groupings of samples that can be more or less clearly defined. The quantitative information allows the detection of trends and ordered paths. These multivariate tools are usually divided into unsupervised and supervised families, as shown in **Figure 1.3.1.** The unsupervised methods have no prior information about the classes of the samples. They function as an exploration of the data and reveal patterns between the samples. Among the unsupervised techniques, there is the principal component analysis (PCA) and the cluster analysis (CA) (Oliveri et al., 2021). The PCA is a data visualization method, and it is beneficial to reduce the dimension of vast data sets. It compresses the information into a lower number of components named principal components (PCs). Each PC is a new variable for the sample obtained as a linear combination of the original variables. In this way, the first PCs contain as much information as possible and are used to draw plots. Examining these plots allows us to recognize patterns that can be groupings or trends among the samples.

The CA is devoted explicitly to identifying the presence of clusters. The procedure usually uses the Euclidean distance, the squared Euclidean distance, the Manhattan distance, and the Pearson correlation coefficient. The clustering methods are categorized into hierarchical, optimizing, density-seeking and clumping techniques. The most common is the hierarchical cluster analysis (HCA), which yields dendrograms (Gonzalez-Dominiguez & Fernandez-Recamales, 2022; Oliveri et al., 2021).

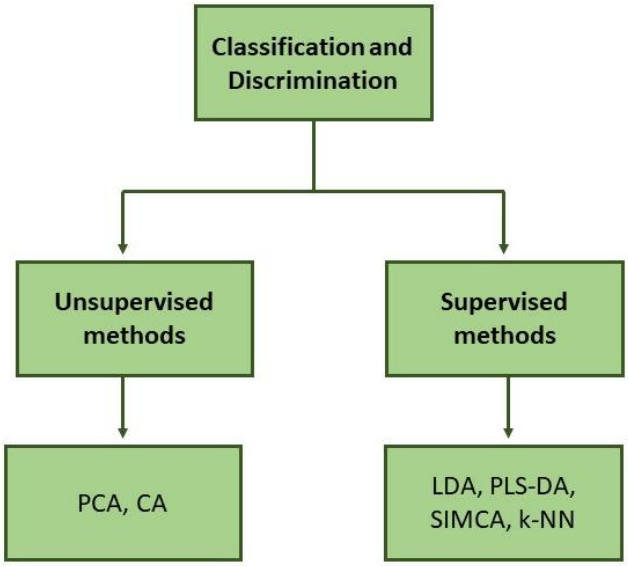

Figure 1.3.1. Classification of the chemometric techniques usually used in food analyses.

These unsupervised methods build descriptive models. On the contrary, the supervised methods require prior information about the presence of patterns within data, and then this is used to build models that can be applied to new data for prediction purposes. The supervised classification methods can be divided into two families: discriminant classifiers that require at least two classes and class-modelling methods, which are also suitable for one-class classification. The first discriminant method is linear discriminant analysis (LDA). It is a very robust method that provides a linear delimiter but has limitations. LDA requires more rows than columns, which means more samples than variables. In some applications, this problem can be overcome by applying LDA after performing a data compression method such as PCA. Another discriminant method is k nearest neighbours (k-NN), which is non-linear and free of statistical hypothesis. It classifies a new sample to a given class based on its distance from all the samples in each class.

There is also partial least square discriminant analysis (PLS-DA) and soft independent modelling of class analogy (SIMCA) (Oliveri et al., 2021).

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CHAPTER 2.

Mass Spectrometry as a tool to ensure food quality

2.1 Introduction to mass spectrometry

In the last years, food quality has attracted more and more public concern, making it one of the fundamental pillars of the European Union. For such reason, a crucial task of modern analytical chemistry is the possibility of obtaining accurate and reliable analytical results. Mass spectrometry has been considered one of the most suitable techniques and has increased within the sector due to its high sensitivity, selectivity, and throughput. Moreover, the combination of mass spectrometry with separation techniques such as liquid and gas chromatography has been widely applied in food analysis because they are easy to automate and possess high-throughput capability (Picò 2015; Wang et al., 2013). Mass spectrometry was introduced in 1913 by Sir Joseph John Thomson. It provided structural information on the analytes by measuring the mass-to-charge ratio (*m/z*) of the charged molecules and tandem mass spectrometry (MS/MS) fragments. On the market, it is possible to find both high and low-resolution MS instruments, and they all contain three main elements: the ionization source, the mass analyzer and the detector (Awad et al., 2015).

Before the 1980s, the primary ionization source applied was electron impact ionization (EI), where electrons interact with atoms or molecules in the gas phase to produce ions. Still, it only allows the analysis of small molecules (Picò 2015). Therefore, other ionization techniques were developed, such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption ionization (MALDI), and desorption electrospray ionization (DESI) (Figure 2.1.1) (Awad et al., 2015).

- **Electrospray ionization** (**ESI**) is based on a high voltage applied to a liquid supplied through a metallic capillary. The voltage, which can be positive or negative according to the nature of the analytes, produces charged droplets at the end of the electrospray tips that pass through a curtain of heated inert gas or a heated capillary to eliminate the solvent. Then, they pass down a pressure and a potential gradient toward the analyzer.
- **Matrix-assisted laser desorption ionization** (**MALDI**), instead, is a soft ionization technique based on the application of a short laser pulse of nitrogen, around 237 nm, to ionize molecules incorporated into a matrix

crystal. The crystals are formed by spotting a mixture of the analytes and the matrix solution on a plate, and then they are dried by air or under a vacuum (Awad et al., 2015; Pico, 2015).

- **Atmospheric pressure chemical ionization** (**APCI**) establishes that ionization occurs at atmospheric pressure. In this case, the spray droplets pass through a vaporization chamber to eliminate the solvent and then through a corona discharge electrode to generate the ionization. Then, the formed ions are sent into the vacuum system through differentially pumped regions (Awad et al., 2015; Fang et al., 2020).

The choice of the ionization source can impact the MS analysis; indeed, it has been shown that by varying the ionization, the lower limit of quantification and the linear range can be altered (Awad et al., 2015).

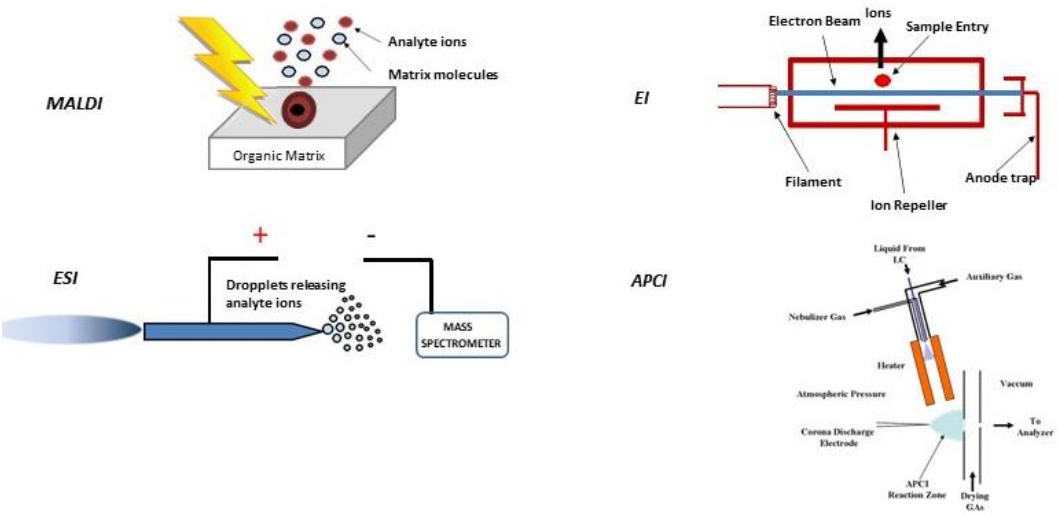

Figure 2.1.1. Scheme of the most common ionization sources (Pico, 2015).

Successively, during the 1990s, the attention moved from the ionization sources to the mass analyzers, which are responsible for the resolutions of the ions according to their mass-to-charge ratio (*m/z*). Different types of mass analyzers in commerce can be classified into four major groups: quadrupole-base, ion traps, time-of-flight (TOF), and Fourier transform analyzers. Moreover, there also exist combinations or hybrids of these analyzers. Choosing one mass analyzer over another depends on a series of

factors, including the desired *m/z* range, the analyte's mass, the analyser's necessary resolving power and the limit of detection required (Haag, 2016; Pico, 2015).

- **Quadrupole**. It is composed of four cylindrical or hyperbolic rods in parallel with each other. Opposite rods are connected electrically to a radio frequency potential (RF) and a direct current potential (DC). The RF and DC potential combination makes the ions oscillate when they pass through the quadrupole. For such reason, quadrupoles work basically as filters. Only the ions with a specific *m/z* can have a stable trajectory depending on the DC and RF values. All the others will collide into the rods and be excluded (March 1997). The quadrupole is one of the most popular mass analyzers due to its low cost and compact shape. In addition, it is reliable for long periods of time and does not need repeated calibrations. The main disadvantages are related to both the limited mass ranges and the poor resolution, so it is not suitable for analyzing complex mixtures of compounds with similar masses.

  When three quadrupoles are organized in linear form, it is known as a triple quadrupole (QqQ). **Figure 2.1.2** shows the scheme of both a single and a triple quadrupole. Generally, mass spectrometers that have two or more mass analyzers consecutively are known as tandem mass spectrometers, and the triple quadrupole is one of the best examples. In this case, the first analyzer is called Q1 and can either scan across a range of *m/z* values or filter ions with a specific *m/z*. Then, the ions pass in a second quadrupole, known as Q2, that works differently from the previous one. Q2 is a collision cell used to fragment the ions selected from Q1. The ions are fragmented by colliding with chemical inert gas at low pressure. The process is known as *CID*. In the end, the product ions pass in the Q3, which can be scanned to obtain a mass spectrum, or the Q3 can be fixed to monitor a particular ion. The combination of the Q1 and Q3 work modalities determines the type of scan performed, as shown in **Figure 2.1.3** (Cooks, 1995; Yost and Enke, 1978).

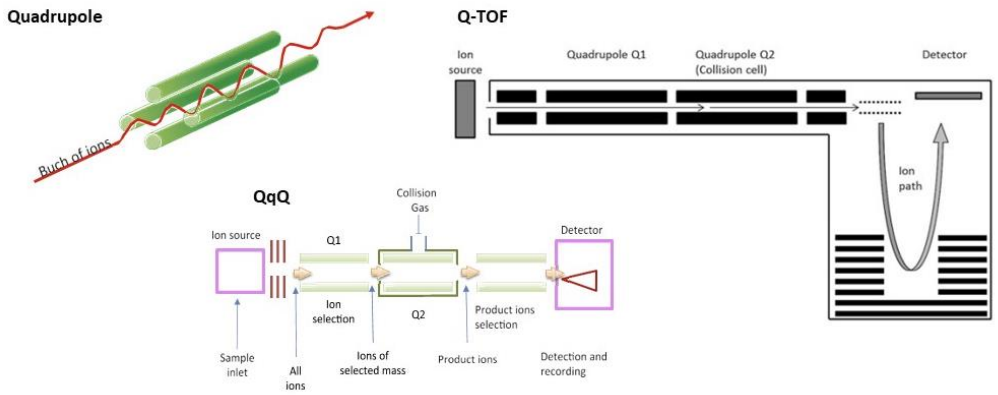

Figure 2.1.2. Scheme of quadrupole, triple quadrupole and Q-TOF (Haag, 2016).

The *product ion scan* mode provides structural information about the analytes, while the *precursor ion scan* allows determining the m/z of precursor ions with identical product ion. The *neutral loss scan* mode is helpful to track ions before and after the loss of a neutral group. The *selected reaction monitoring*, also known as *multiple reaction monitoring* (MRM), is very popular to quantify compounds in mixtures because it allows the monitoring of specific fragment ions originated from a compound of known mass (Haag, 2016).

Another tandem (hybrid) configuration, which involves the use of quadrupoles, is the Q-TOF setup. In this case, to allow tandem mass spectrometry experiments, the TOF is placed in an orthogonal configuration after the quadrupole, as shown in **Figure 2.1.2**. This configuration is necessary to inject the filtered ions, coming from the quadrupole, as a packet into the TOF analyzer. The main advantage of Q-TOF, compared with triple quadrupole, is the higher accuracy, resolution and scan speed (Chernushevich et al., 2001; Haag, 2016).

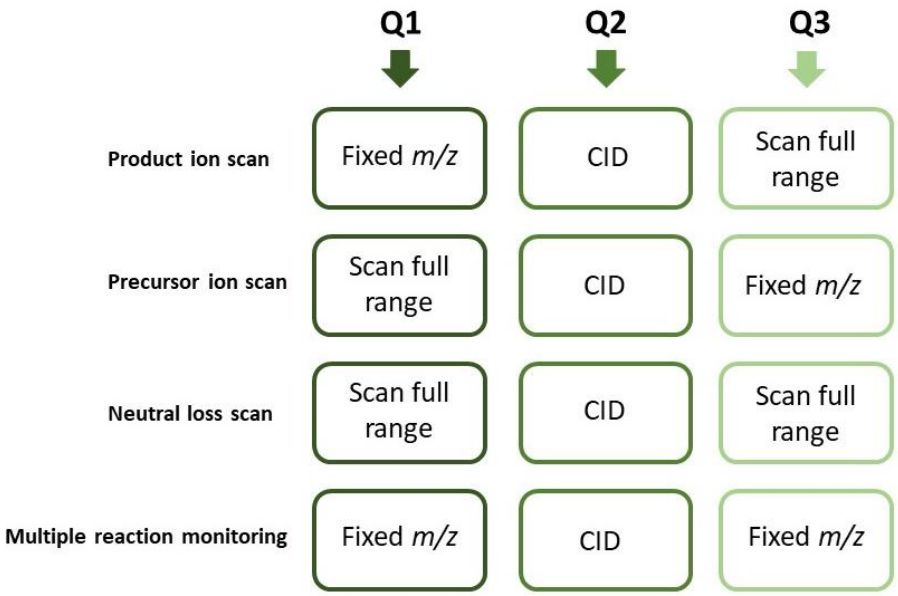

Figure 2.1.3. Table of different scan modes of triple quadrupole instruments (Haag, 2016).

- **Ion trap**. The ion trap mass analyzer is an adaptation of a normal quadrupole. The most famous one until twenty-first century was the ion trap known as the Paul Trap or 3D ion trap, but nowadays, the linear ion trap has become more popular. The 3D one consists of a hyperbolic ring between two hyperbolic electrode plates facing each other (**Figure 2.1.4**). The ions are trapped between the electrodes by applying an oscillating radio frequency and a direct current. That occurs because the forces are in all three dimensions, instead of two, as in the case of quadrupole, so the stable motion of ions allows to trap them. Varying the RF potential, is it possible to selectively eject ions based on their *m/z*. The linear trap is the equivalent of a quadrupole, but a potential field is applied to each end of the quadrupole so that the ions are trapped.

  The high sensitivity is amongst the main advantages of ion traps, determined by the ability to accumulate ions over time. Moreover, they are very small and affordable. They can also perform multiple-stage mass spectrometry (MS^n) and are very useful in studying the kinetics and equilibrium of ion/molecule reactions because it is possible to vary the reaction time. In contrast, one of the main disadvantages is the low-resolution power; particularly, the 3D models have only single unit mass resolution (Wong & Cooks; Haag, 2016).

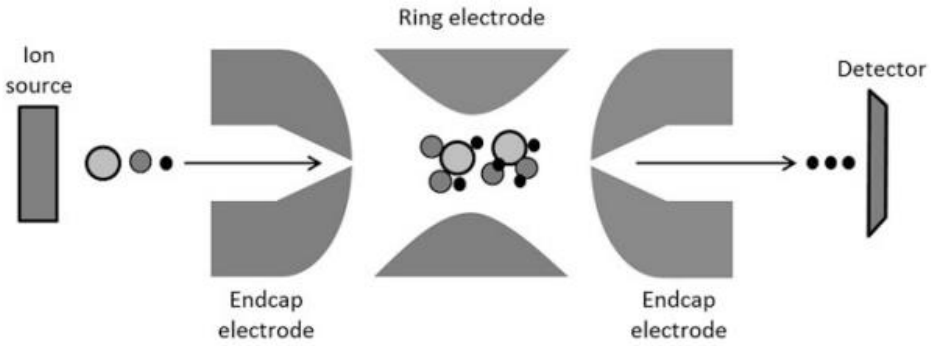

Figure 2.1.4. Scheme of a 3D ion trap mass analyzer (Haag, 2016) .

- **Time of flight**. The scheme of a time-of-flight mass analyzer comprises a flight tube and an acceleration grid that accelerates the ions from the ionization source to the MS detector. If two ions with different *m/z* are accelerated with the same kinetic energy and then allowed to drift in a field-free region of the flight tube, they will arrive at the detector at different times. The main problem, in this case, is the resolution because ions with the same mass do not arrive at the detector at the same time due to a difference in the initial kinetic energy. To solve this problem, modern TOF employ a reflectron that permits correcting these slight differences in the initial kinetic energy of the ions. The reflectron reflects the ions back in the direction of the ions source before being detected. The scheme of both linear TOF and reflectron TOF is shown in **Figure 2.1.5**. TOF analyzers have the widest mass range; they can separate ions from a few Daltons to over 100kDa and are perfect to analyze biomolecules with high mass, such as proteins (Cotter, 1999; Mamyrin, 2001; Haag, 2016).

  As in the case of quadrupole, two time-of-flight mass analyzers can be aligned to obtain a TOF/TOF with a CID fragmentation in between, as shown in **Figure 2.1.6**. In particular, the ions are, firstly, separated according to their *m/z* in the flight tube on the base of their velocity; then, the ions with specific *m/z* are selected, and all the others are left out. This selection happens using a gate, a timed-ion-selector, filters ions according to the time they arrive. The filtered ions are later de-accelerated and directed to a collision cell for CID. In the end, the product ions obtained are re-accelerated into the second flight

tube and analyzed. In this way, it is possible to perform tandem mass spectrometry experiments on biological compounds such as peptides and oligonucleotides (Haag, 2016).

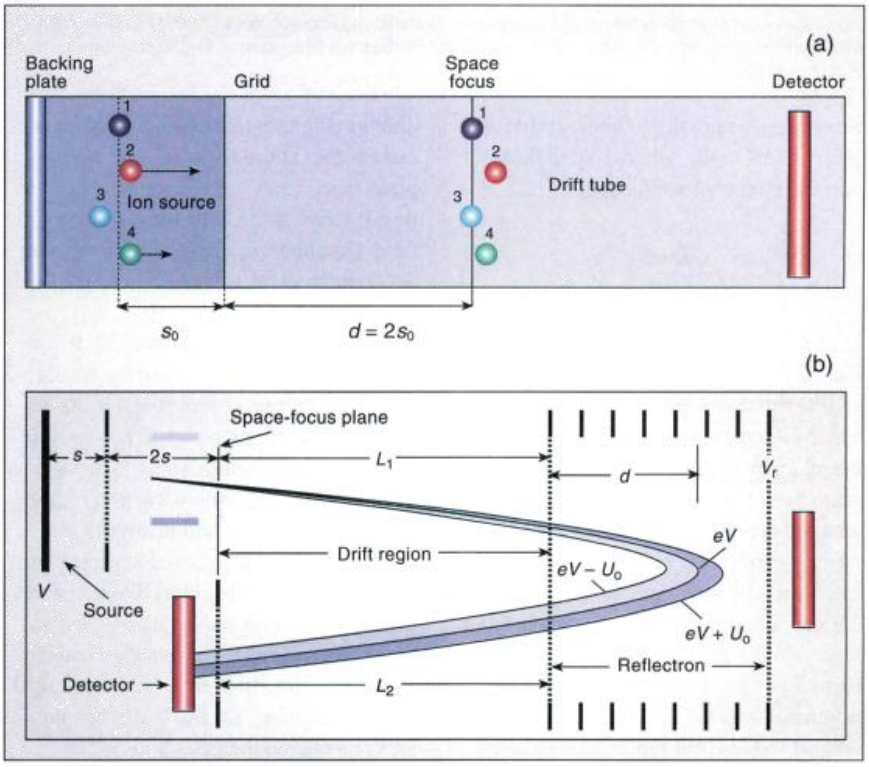

Figure 2.1.5. Scheme of a linear TOF mass spectrometer (a) and a reflectron mass spectrometer (b) (Cotter, 1999).

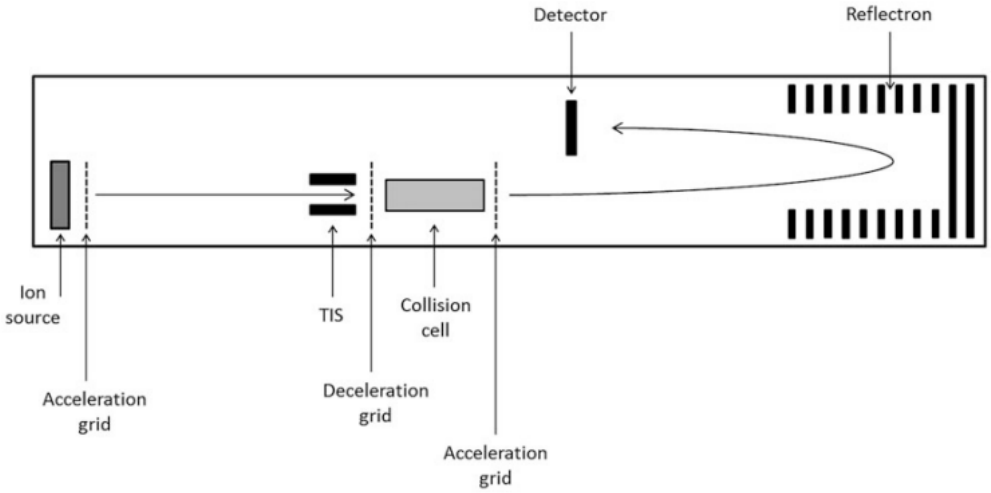

Figure 2.1.6. Scheme of a TOF/TOF mass spectrometer (Haag, 2016).

- **Orbitrap**. The orbitrap analyzer can be considered a modification of a Kingdom trap with specially shaped inner and outer electrodes and a

quadrupole ion trap. There is a substantial difference with the latter. Indeed, in the orbitrap, the ions are trapped using a static electric field, while the quadrupole ion trap applies an oscillating electric field (Hu et al., 2005). The orbitrap is composed of an inner spindle electrode inside two hollow outer concave electrodes facing each other (**Figure 2.1.7**). The two outer electrodes are separated with a thin ring of dielectric material. Applying a voltage between the inner and outer electrodes creates an electric field between them. Thanks to that field, the ions rotate with a spiral path around the inner electrode when they enter the orbitrap. Due to the conical shape of the electrodes, the ions start to oscillate back and forth, and that oscillation is detected by the outer electrodes that work, also as receiver plates. The orbitrap, moreover, uses a Fourier transformation, and the signal produced by the oscillation of the ions is transformed from the time domain to a frequency domain. The main advantages of the orbitrap mass analyzer include the high resolution and accuracy power. Still, they are slower if compared with TOF, and so, less ideal for performing experiments where fast acquisition is more important than very high resolution or accuracy (Haag, 2016).

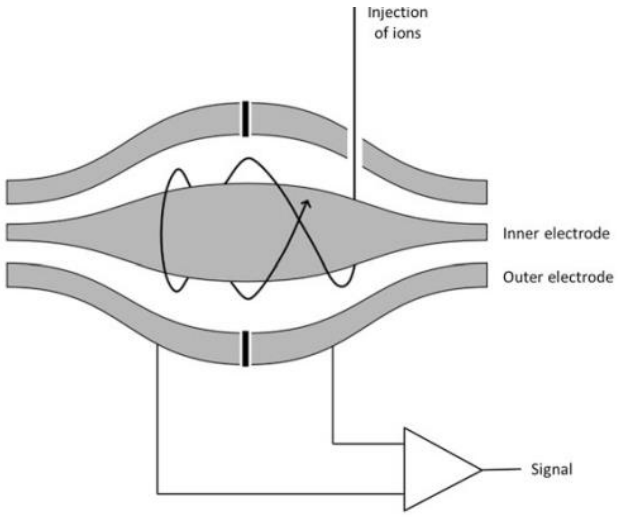

Figure 2.1.7. Scheme of Orbitrap mass analyser (Haag, 2016).

2.2 Classical mass spectrometry techniques in food analysis

Mass spectrometry is considered an important tool in food analysis for quality control due to its advances in ionization techniques and mass analyzers that have improved

the range of applicability and sensitivity. A crucial point for food applications was the impact of new ionization techniques such as electrospray and matrix-assisted laser desorption, but also the use of quadrupole and time of flight mass analyzers and the possibility to perform tandem mass spectrometry analyses (Careri et al., 2002). Moreover, the coupling of MS with separation techniques has shown good convenience for food analysis because they are among the most sensitive and selective analytical methodologies. These are two fundamental parameters when dealing with food-based matrices that are very complex. The hyphenated methodologies mostly applied are GC-MS and HPLC-MS, with GC-MS resulting as the most widely used for food analysis with more or less 200-300 papers for years. On the other hand, HPLC-MS is less used, but it is important to highlight that it is the one that is growing the most among the analytical methodologies in food analysis. Indeed, the papers based on the application of GC-MS doubled in the last years, while those based on HPLC-MS increased almost 10 times in the same period (Di Stefano et al., 2012).

2.2.1 Liquid Chromatography- Mass Spectrometry

HPLC-MS is a general technique that can be used to characterize different classes of molecules. When dealing with foodstuff, it is important to know that there is a large variety of molecules with different structures and biological and nutritional properties. For example, there are compounds that are always present at minor or trace levels but are very important from a nutritional point of view. In other cases, a component can be very abundant in a food and found in trace in another. Moreover, the variability of components in foodstuffs covers a wide range of polarity. Some are apolar, while others are strongly polar. For such reasons, it is necessary to apply specialized methodologies, there is no analytical technique that covers all the aspects of food analysis.

Regarding HPLC-MS, a fundamental point is the choice of the ionization technique. Electrospray ionization (ESI) is excellent to study polar, ionized or ionizable molecules in positive or negative ion mode. At the same time, it is not very useful for compounds with low polarity; the best choice, in this case, is atmospheric pressure chemical- or photoionization (APCI and APPI) (Di Stefano et al., 2012).

Other important parameters include the column inner diameter and the particle size; by reducing them, it is possible to increase sensitivity, peak resolution, and efficiency. Also, the analysis times can be shortened. The reduction of particle size at 1.7 and 1.8 µm introduced ultra-high performance liquid chromatography (UHPLC). There are some problems related to liquid chromatography that neither the UHPLC has been able to overcome completely, such as peak coelution and compounds that elute with the solvent front. This separation problem can be solved using two-dimensional liquid chromatography (LC x LC). The peak capacity with an analysis of a few hours grows from a few hundred in 1D-LC to 3000 in 2D-LC (Stoll and Carr, 2017; Picò, 2015).

The HPLC-MS methodology has been applied to ensure food quality and safety on a wide range of compounds, including macronutrients like lipids, carbohydrates, and proteins, but also minor nutraceutical compounds such as vitamins and flavonoids and possible dangerous compounds for human health, just like mycotoxins and pesticides. Some of the applications are gathered in **Table 2.2.1;** for each one, it is reported the target compounds, the matrix and the LC-MS methodology highlighting the ionization source and the mass analyzer used.

Another important aspect to highlight is that food quality and safety control are pronominally reached through target analysis. This implies the use of analytical standards to quantify natural compounds present in food or contaminants (Picò, 2015). This approach has been largely applied to determine the exact content of various compounds found in foodstuffs. Good examples concern the quantification of flavanones in citrus juices and other beverages (Di Donna et al., 2013) and the content determination of hydroxytyrosol and tyrosol in olive oil (Mazzotti et al., 2012). Alongside this, the introduction of the “omics” techniques, such as proteomics and metabolomics, was based on obtaining spectra with a large range of masse by a non-targeted approach.

Compounds analysed	Matrix	HPLC-MS technique	Reference
Food Components			
Phenolic compounds	Ginger	HPLC-QqQ-MS/MS	(Tohma et al., 2017)
Phenolic compounds	Hazelnut	HPLC-ESI-IonTrap-MS^n	(Jakopic et al., 2011)
Phenolic compounds	Pomegranate	UHPLC-HESI-Q-Orbitrap-MS	(Di Stefano et al., 2018)
Polyphenols	Fruits and Vegetables	HPLC-ESI-QTOF/MS	(Gu et al., 2019)
Polyphenols	Blueberry and strawberry	HPLC-ESI-QqQ-MS/MS	(Mustafa et al., 2022)
Flavonoids	Citrus juices	HPLC-ESI-qMS	(Dugo et al., 2005)
Flavanones	Citrus juices and beverages	UHPLC-ESI-QqQ-MS/MS	(Di Donna et al., 2013)
Anthocyanins	Grape Juice	HPLC-ESI-Qtrap-MS HPLC-ESI-Qtrap-MS/MS	(Oh et al., 2008)
Tyrosol and Hydroxytyrosol	Olive oil	HPLC-ESI-QqQ-MS/MS	(Mazzotti et al., 2012)
Folates	Soybean	HPLC-ESI-QqQ-MS/MS	(Agyenim-Boateng et al., 2022)
Catechins	Tea	UHPLC-ESI-QqQ-MS/MS	(Spacil, et al., 2010)
Food Contaminants			
Mycotoxin	Food supplements	HPLC-ESI-QqQ-MS/MS	(Di Mavungu et al., 2009)
Mycotoxin	Baby foods	HPLC-ESI-Qtrap-MS/MS	(Rubert et al., 2012)
Pesticides	Baby foods	HPLC-ESI-QqQ-MS/MS	(Radford et al., 2014)
Pesticides	Apple, lemon, lettuce and wheat grain	HPLC-ESI-QqQ-MS/MS	(Gonzalez-Cubelo et al., 2014)
Pesticides	Lemon, maize, oat, green bean and red cabbage	UHPLC-HESI-Q-Orbitrap-MS	(Feng et al., 2020)
Bisphenol A and alkylphenols	Cereals	HPLC-ESI-QqQ-MS/MS	(Niu et al., 2012)

Table 2.2.1. Application of LC-MS for Food Quality and Safety.

In this case, the quantification is performed only in the second stage. After that, the identification of the compounds of interest is achieved. The non-target analysis can be divided into nontarget metabolomics and nontarget fingerprint. A fingerprint is the characteristic spectrum of a food, so that it can be related to its properties and to its authenticity in the same ways as a human fingerprint is specific for a single person and identifies them unequivocally (Picò, 2015).

Obtaining a fingerprint involves applying an overall analytical methodology that permits to ensure the identity of a food product. In this way, it is possible to identify any deviation from it, and consequently, it may reveal adulteration, contamination, etc. A fingerprint can be obtained with three different strategies: applying the methodology directly on the original material or on a solution of the whole material, performing before separation and applying the method only on a fraction selected, or applying a chemical reaction on the original material or the isolated fraction (Cuadros-Rodriguez et al., 2021).

2.2.2 Gas Chromatography-Mass Spectrometry

GC-MS has been widely used in the determination of food composition and contaminants. The combination of the separative power of gas chromatography with mass spectrometry creates a unique tool for the reliable characterization of a complex mixture. Moreover, in comparison with LC, it is faster and allows better separation efficiency. The major problem with this technique is that it is very suitable for volatile and semi-volatile compounds and very thermally stable analytes but not for polar compounds. To resolve the problem, it is possible to perform derivatization reactions to convert the polar group of the target molecules into less polar moieties. The only disadvantage is that most of the time, the derivatization process is time-consuming and requires intensive and tedious labour. Another vantage, instead, is the possibility of using the EI-MS spectrum library to identify the unknown components of a complex food matrix.

Regarding food analysis, the most traditional way to perform GC-MS is with a capillary column that can have variable dimensions and stationary phases, an EI ionization source and a single quadrupole mass spectrometer. Sometimes, it is necessary to

extract one or more specific compounds over a complex matrix, and the GC-MS methodology can be combined with extraction techniques such as solid-phase microextraction (SPME). Apart from a single quadrupole, depending on the specific analytical aim, GC can be coupled with other mass analyzers, like a triple quadrupole or a time-of-flight, to achieve high resolution and accuracy. One problem of GC is that it suffers from long times of analysis. To speed up analysis, fast gas chromatography has been proposed using short columns and higher flow rates.

As for liquid chromatography, exist the two-dimensional gas-chromatography (GC x GC) that consists of two columns connected one after the other so that the analytes emerging from the first column enter the second and are analyzed sequentially (Picò, 2015; Wang et al., 2013; Hussain & Maqbool, 2014).

GC-MS has been applied to detect a great number of natural compounds and contaminants in food. Some examples are summarized in **Table 2.2.2**.

Compounds analysed	Matrix	GC-MS technique	Reference
VOCs	Wines	GCxGC-TOF-MS	(Vestner et al., 2011)
Furans, lactones, volatile phenols, acetals	Wines	GCxGC-TOF-MS	(Perestrelo et al., 2011)
Organic pollutants	Drinking water, grape, fish, cucumber	GC-TOF-MS	(Ibanez et al., 2012)
PCBs	Meat and Sea Food	GC-QqQ-MS/MS	(Huo et al., 2012)
Pesticides	Wheat, rapeseed, cumin and tea	GC-Q-Orbitrap	(Belarbi et al., 2021)
Pesticides	Rice and wheat flour	GC-QqQ-MS/MS	(Grande-Martinez et al., 2016)
Pesticides	Orange, nectarine and spinach	GC-APCI-Q-TOF-MS	(Potoles et al., 2010)
Pyrethroids	Apple Juice	GC-qMS	(Wong et al., 2010)

Table 2.2.2. Application of GC-MS for Food Quality and Safety

2.3 Ambient mass spectrometry for food quality

The ionization techniques, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), applied to food analysis, have worked very well to separate the target analytes from matrix solutions at atmospheric pressure and to transfer the free ions into a vacuum environment to perform mass spectrometry analysis. However, a problem related to these ionization sources is the necessity of complex and expensive sample preparation that is also time-consuming (Black et al., 2016). Trying to resolve these problems leads to the introduction of ambient ionization mass spectrometry techniques. The main merit of these ionization sources is that the samples are exposed to the ionization medium under ambient conditions, and it is not necessary to perform a previous step of extraction and separation. In this way, food analysis is extraordinarily easy and efficient (Wang et al., 2013).

Fenn envisaged paper spray mass spectrometry (PS-MS) describing the possibility of employing cellulose-based materials as ionization substrate. However, the first PS-MS study was published in 2010. For this reason, desorption electrospray ionization (DESI) is yet recognized as the first ambient ionization technique (Black et al., 2016).

To classify an ionization source as ambient, it needs to satisfy some requirements:

- The ability to perform ionization in the open air to interrogate objects of unusual shape or size.
- The ability to perform direct surface analysis avoids time-consuming sample preparation steps that are traditionally required in the analysis of solid samples through mass spectrometry.
- The versatility to be interchangeable in any mass spectrometer equipped with an atmospheric pressure interface.
- The capacity to generate ions without significant in-source fragmentation simplifies mass spectra interpretation when analyzing complex samples (Nollet and Munjanja, 2019).

After DESI, the next ambient ionization source introduced was DART or direct analysis in real-time and ASAP or atmospheric pressure solid analysis probe, both in 2005 (Black et al., 2016). In the following years, a plethora of ambient techniques were developed.

For what concern food analysis, AMS techniques provide some advantageous characteristics. Good examples include the possibility of performing *in-situ* analysis, the very small amount of sample and organic solvents required, the capacity to obtain fast and high-throughput analysis, the minimal or absent sample preparation needed and the relatively low matrix effects. Amongst all the ambient mass spectrometry methodologies developed, the most applied in food studies are DESI and DART, probably because they are the first techniques introduced and the devices are more available in commerce. Apart from them, other AMS techniques often used are: Desorption atmospheric pressure chemical ionization (DAPCI), Desorption atmospheric pressure photoionization (DAPPI), Easy ambient sonic-spray ionization (EASI), Extractive electrospray (EESI), Laser ablation electrospray ionization (LAESI), Leaf spray, Paper Spray (PS), Low temperature plasma (LTP), Matrix-assisted laser desorption electrospray ionization (MALDESI), Nano-desorption electrospray ionization (Nano-DESI) and Rapid evaporative ionization mass spectrometry (REIMS). Moreover, the simplicity of AMS allows their easy modification to adapt them to the sample and the analytical problem. Therefore, new approaches are being constantly introduced to perform target analysis of organic contaminants in food, to detect food fraud and to determine food authenticity (Nollet and Munjanja, 2019).

All these AMS methodologies combine different desorption methods with different ionization techniques, making their classification very challenging. A possible classification is based on the ionization mechanism: spray or jet ionization, electric discharge ionization, and gas-, heat- or laser-assisted desorption/ionization. Others are based, instead, on the intrinsic extraction/desorption/ionization mechanism involved. In this case, there are methodologies based on direct ionization where the analytes, in a liquid phase, are directly ionized by an electric field without any pre-treatment. Another group is based on the mechanism of desorption/ionization. In this case, some charged and reactive species generated from the ambient source are directed to the surface of the sample to allow the desorption and the following ionization. The last category regards the two-step ionization, where the analytes are desorbed applying a laser or through thermal desorption or desorption/ablation and then the ionization happens as a second and independent step (Huang et al., 2011).

In the end, another classification method is the so-called "ionization trees", where, like in a family tree, each ambient ionization technique is correlated to the classical methodology it comes from. The tree with the main methodologies is shown in **Figure 2.3.1**, where the mainstream techniques are highlighted in brown, while some variations are highlighted in red and green.

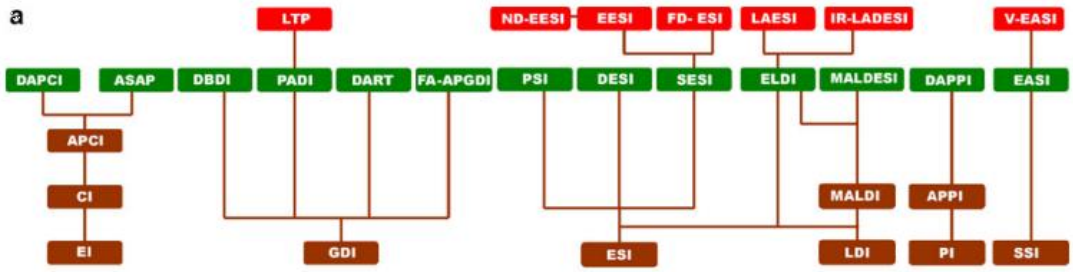

Figure 2.3.1. The ionization tree of ambient mass spectrometry techniques.

2.3.1 APCI-related techniques

Atmospheric Pressure Chemical Ionization (APCI) was introduced over 30 years ago. It is based on a support gas, usually N_2, that is ionized by a stream of accelerated electrons. A series of reactions, which depend on the gas composition, generate the reagent ions. The most used APCI source is the corona discharge (CD). In a CD, there is a high voltage (3-5 kV) applied to a needle electrode. When the aerosol, obtained from the meeting of the sample with the heated inert gas, arrives at the needle electrode, the ionization occurs (Andrade et al., 2008).

DAPCI (Desorption Atmospheric Pressure Chemical Ionization) is the ambient MS variant of APCI. The main difference is that only the inert gas goes through the needle electrode, and then the charged molecules of gas interact with the sample, which is located outside the instrumentation, allowing its ionization. As shown in **Figure 2.3.2,** the DAPCI source consists of a cylindric electrode located near the exit of a capillary that is inserted in a T-joint that allows the use of a sheath gas. A high voltage (3-6kV) is applied to the electrode to ionize the gas molecules that are later sent to the sample surface to ionize the target analytes. This typology of the source should be arranged in a position that creates an incident angle (30-45°) and a desorption angle (15-30°) between the source, the sample and the inlet of the mass analyzer. This technique permits the analysis of both volatile and non-volatile species, and it is very

useful for the determination of small and low-polar compounds (Pitman & La Course, 2020).

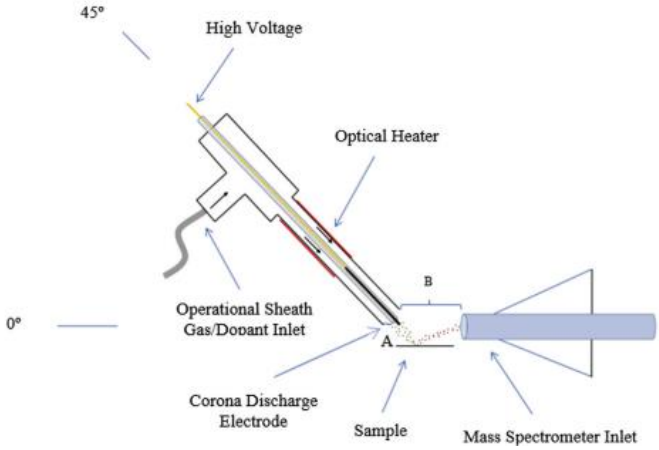

Figure 2.3.2. Scheme of DAPCI source (Pitman & La Course, 2020).

Another AMS obtained from APCI is **ASAP** or Atmospheric Pressure Solid Analysis Probe. The solid sample is positioned in a probe that is a fusion temperature capillary built with borosilicate and inserted in the typical APCI chamber (**Figure 2.3.3**). The mechanism is based on a corona discharge effect where a solvent creates a spray that is used to desorb the analytes from the probe (Alberici et al., 2010). This source is good to analyze both polar and non-polar compounds.

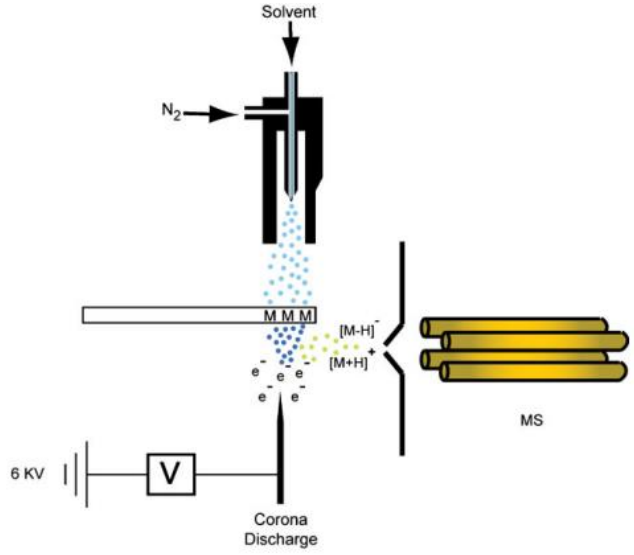

Figure 2.3.3. Scheme of ASAP source (Alberici et al., 2010).

2.3.2 GDI related techniques

The GDI or Glow Discharge Ionization is an ion source used for mass spectrometry for more than 80 years. It is based on the formation of a plasma, which generates atoms, ions and electrons when the correct voltage is applied between two electrodes and the inert gas breaks down. The generated ions allow us to maintain the plasma but also to use it as an ionization source for mass spectrometry. For at least 40 years, indeed, it has been the preferred technique for the analysis of trace compounds in metals and semiconductors. Only recently, GDI has been applied for the analyses of liquids and gases.

Direct Analysis in Real Time (**DART)** is a variant of GDI, introduced for the first time in 2005 by Cody and Laramèe. Even though the DART is one of the most complex ambient mass spectrometry techniques, it has been widely applied for the analysis of liquid, solid and gaseous samples because it gives good results regardless of the molecule polarity. As shown in the scheme in **Figure 2.3.4**, the DART is composed of a series of chambers which pass an inert gas, usually He or N_2, at a speed of 1-3 L/min. The glow discharge is created when a potential of 1-5 kV is applied, producing ions, electrons and neutral species in an excited state. Then, the gas stream is adjusted at a temperature between 250 and 500 °C, and ions of opposite polarity are removed to prevent signal loss. When the DART gas makes contact with the sample, with an angle of 0°, the ionization occurs.

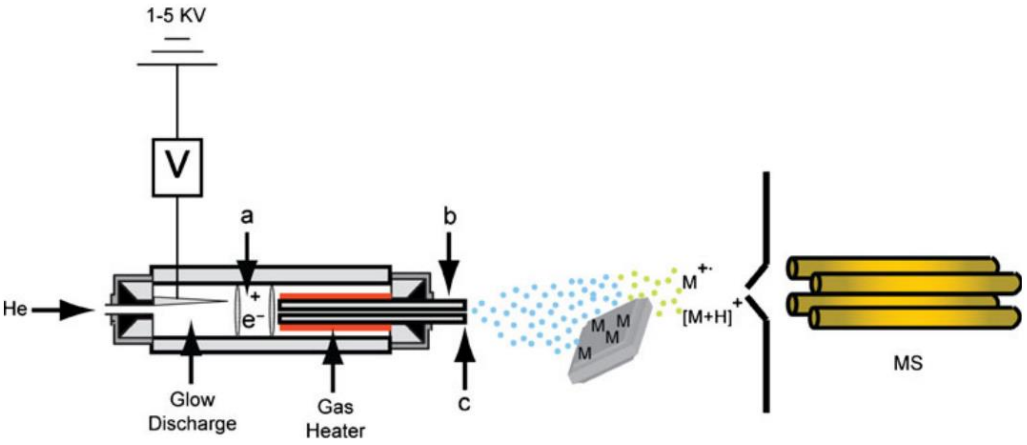

Figure 2.3.4. Scheme of DART source (Alberici et al., 2010).

The ionization mechanism is the Penning ionization because the ions are removed from the gas stream after the electrical discharge. For such reason, the sample is

exposed to a stream that contains hot but neutral gas atoms and molecules. What happens is that nitrogen or helium, N* and He* excited species are generated in the GD, which transfer energy to the sample analytes, forming $M^{+\cdot}$ and an electron e^- (Alberici et al., 2010).

Other related AMS techniques based on atmospheric pressure discharges, although using AC voltages, are Plasma-Assisted Desorption/Ionization (PADI), Dielectric Barrier Discharge Ionization (DBDI) and Low-Temperature Plasma (LTP). The main features of PADI and DBDI are the direct exposure of the sample to the plasma and the use of radiofrequency to avoid instabilities at the electrodes instead of a direct current. In **PADI**, the radiofrequency is applied to a stainless-steel wire, usually between 200 and 500V; this determines the creation of a plasma at a temperature close to that of the surrounding ambient. That cold plasma also allows the analysis of thermally sensitive samples (Alberici et al., 2010; Andrade et al., 2006).

The **DBDI** uses a Dielectric Barrier Discharge (DBD) to create the plasma. A stainless-steel needle works as the discharge electrode, while the opposite electrode is a copper sheet, and a piece of glass located in the copper sheet works as the discharge barrier. The piece of glass also works as a sample substrate. The distance between the needle and the sample can be selected. When an inert gas passes through the needle, the applied voltage determines the creation of the plasma, which desorbs and ionizes the sample on the glass support (Alberici et al., 2010; Na et al., 2007).

The **LTP**, instead, is a variation of PADI, even if it shares some features with DBDI because LTP applies a similar mechanism of desorption/ionization. The LTP probe is a glass tube in which there is a stainless-steel electrode. Outside, there is a second electrode; more specifically, it consists of a copper wire that covers the glass tube. When a potential between 2.5 and 5 kV is applied at a frequency of 2-5 kHz, the dielectric barrier discharge is created (Alberici et al., 2010; Harper et al., 2008). **Figure 2.3.5** shows the structure of the LTP probe. The created plasma reaches a maximum temperature of 30°C, so it is not dangerous for most surfaces and human skin. It produces active species like electrons at high energy and radical ions that determine the sample ionization. Another important characteristic is that the high-voltage electrode is isolated electrically from the discharge region, so there is no possibility of electric shock for the sample.

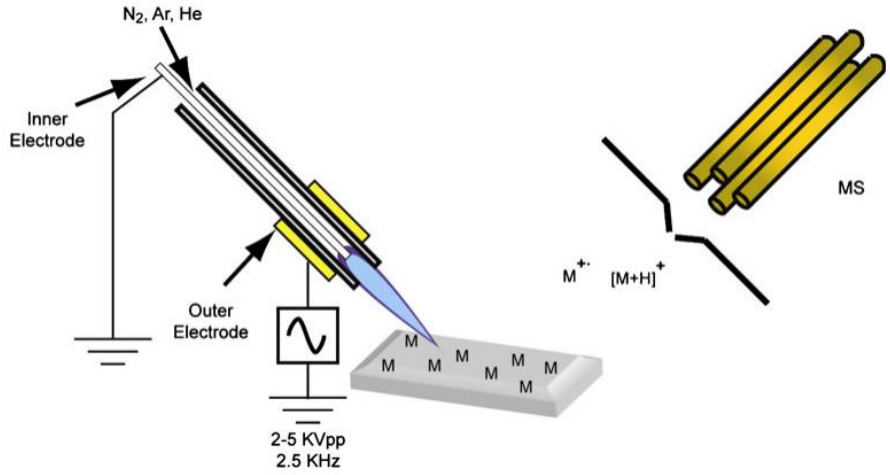

Figure 2.3.5. Scheme of LTP source (Alberici et al., 2010).

2.3.3 APPI related techniques

Atmospheric Pressure Photoionization (**APPI)** is a relatively novel soft ionization technique. APPI is based on the photoionization phenomenon, introduced over 30 years ago as a detection method for gas chromatography and later for liquid chromatography. There are 3 necessary steps to allow determination by means of photoionization: eluent vaporization, the generation of photo-ions, the interaction of a photon emitted from a UV source and the analytes, and the detection that can be achieved by mass spectrometry. To generate ions that are reactive only to the analytes and not to the solvent, the photon should have an ionization energy that is higher than the ionization energy of the analytes and lower than the ionization energy of the solvent. Moreover, a doping agent can be employed to increase the number of ions formed (Raffaelli & Saba, 2003).

Based on the APPI, Kostiainen and Kotiaho created the first ambient mass spectrometry technique with photoionization, Desorption Atmospheric Pressure Photoionization, **DAPPI** (Alberici et al., 2010). In this ion source, a jet of heated vapour and photons emitted from a krypton discharge lamp are cotemporally directed to the sample. The combination of the two allows the desorption and the ionization of the analytes from the surface of the sample. Later, the obtained ions are collected into the mass spectrometer (Alberici et al., 2010; Raffaelli & Saba, 2003).

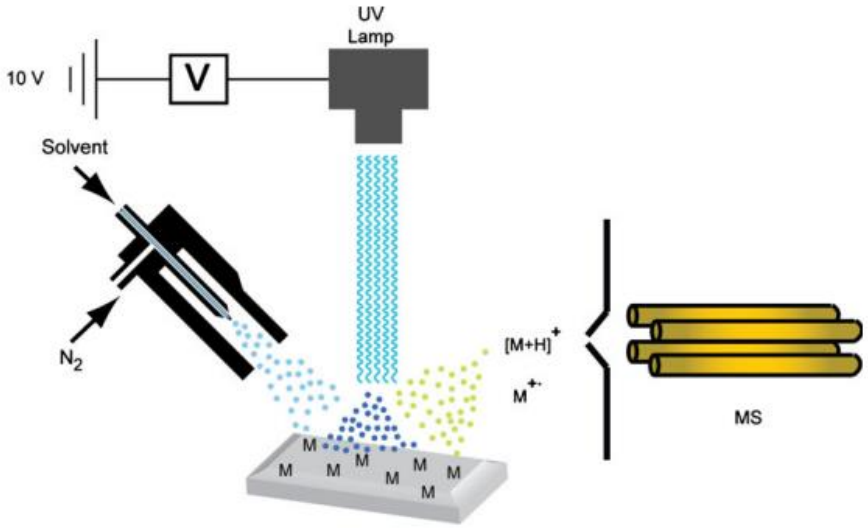

Figure 2.3.6. Scheme of DAPPI source (Alberici et al., 2010).

In order to work correctly and to obtain the best results, it is very important how each part of the ionization source is arranged. As shown in **Figure 2.3.6**, the vapour jet and the sample are arranged in an axial position with respect to the inlet of the mass analyzer, while the photoionization lamp is positioned at 10mm above the sample spot. One of the best features of using a nebulizer chip is that it is very easy to modify the temperature. The heating and cooling times are fast enough and allow the implementation of different temperatures to different samples on the same sample plate, reducing the analysis time (Haapala et al., 2007).

2.3.4 SSI related techniques

In 1994, Hirabayashi and his collaborators introduced Sonic Spray Ionization or **SSI**. This ion source presented a new way of producing ions; it is possible to produce ions without the application of current, radiation or heat. The charged droplets are obtained simply by spying an acidified solution of the analytes in methanol at sonic speed.

A reduced flow of charged droplets provides an efficient desorption and ionization of samples in their natural ambient conditions. That introduced the Easy Ambient Sonic Spray Ionization, **EASI**. Explicitly, it is an ambient ionization technique based on SSI. The scheme of the ion source is shown in **Figure 2.3.7**.

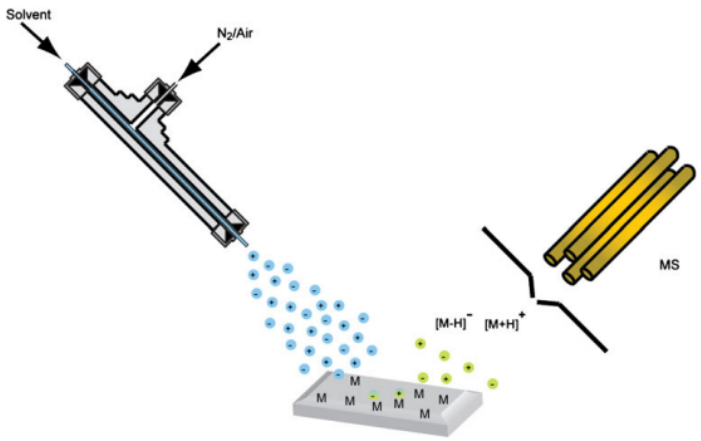

Figure 2.3.7. Scheme of EASI source (Alberici et al., 2010).

The acid solution of water and methanol, with the help of the gas, permits the formation of the charged droplets by sonic spray. The charged droplets arrive on the sample surface, allowing the desorption and ionization of the analytes that are then directed towards the inlet of the mass spectrometer. The main advantages of EASI are:

- its extreme simplicity, only compressed air and nitrogen are necessary.
- its ability to produce, at the same time, both negative and positive charged droplets, so there is no need to change potentials from EASI (+) to EASI (-).
- the low charge concentration on the droplets seems to reduce the noise related to the solvent; this promotes the ionization of the analytes and improves the signal-to-noise ratio.
- the extreme softness of the ionization process.
- the absence of thermal degradation and interferences that, instead, occur in ESI and ESI-based techniques.

The high-speed spray obtained from EASI can deeply penetrate the matrix when dealing with solid samples, providing homogeneous sampling and long-lasting signals. The main limitation concerns the very high speed of the stream, which can easily blow away the samples. This is not a problem when dealing with solid samples, crystallized samples, rough surfaces and viscous oils (Alberici et al., 2010).

The EASI-MS has been successfully applied for the analysis of different analytes in equally different matrices; between them, there is also the analysis of food to ensure security (Teunissen et al., 2017).

The introduction of the Venturi effect simplifies the mechanism of EASI and permits the development of Venturi Easy Spray Ionization or **V-EASI**. This technique uses the forces of a sonic stream of N_2 or air to cause the combined result of a self-pumping solution through the Venturi effect and the ionization via sonic spray (Alberici et al., 2010). The Venturi effect happens when a high-speed stream goes through a very narrow tube; this determines a pressure reduction and a self-pumping effect. This high-speed stream, apart from creating the Venturi effect, determines the SSI and thus, the V-EASI is obtained. It is a very easy technique which allows the analysis of both solid and liquid samples without the necessity of an electric pump, **Figure 2.3.8** (Santos et al., 2011).

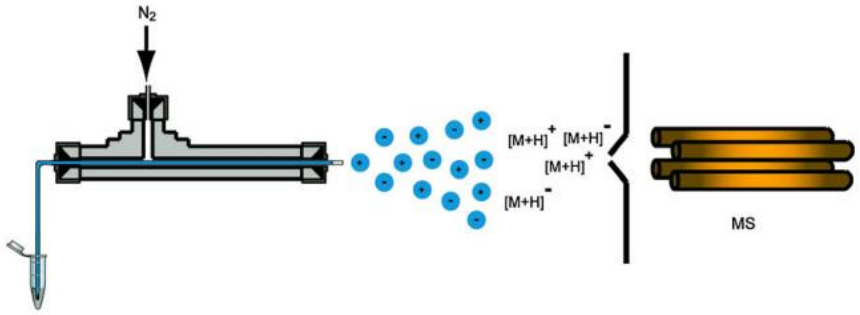

Figure 2.3.8. Scheme of EASI source (Alberici et al., 2010).

As EASI, V-EASI works at ambient temperature and does not require potential or heat or radiations; for such reason, it has no interferences. Its higher simplicity, although, makes it more suitable for real-time analysis (Alberici et al., 2010).

2.3.5 ESI related techniques

The mechanism of the ESI source is similar to the one of APCI. A potential of ±5kV is applied to a capillary in which flows a solution. This capillary is contained in a tube filled with Argon, which works as a nebulizer gas. The action of the gas with the electric field determines the creation of an aerosol with charged droplets. The droplets then start to evaporate until the charge passes on the analytes.

On the basis of ESI, Desorption electrospray ionization, or simply **DESI**, was developed and now it is one of the most used ambient mass spectrometry techniques. It can be applied directly to the target molecules in their original matrix, or a support can be employed like glass, metal or plastic (Alberici et al., 2010). The DESI possess high sensibility; it gives an instant result, and it can be applied for both the study of organic molecules with little dimensions and proteins and other molecules of biological origin. This technique combines the characteristics of ESI with that of DI or Direct Desorption/Ionization. It is based on an electrospray emitter that generates solvent ions and charged droplets that are then directed to the sample, as shown in **Figure 2.3.9**. A potential is applied to the spray solution, and the nebulization helps the desolvation, so the DESI is really like ESI. For what concern DI, the correlation with DESI is at a phenomenological level. In all the DI methods, there is the impact of photos or exited atoms or energetic ions with the sample in a condensed phase, but it happens under a vacuum while the DESI is at ambient conditions. This is the main characteristic that discriminates DESI from the other DI methods (Takats et al., 2005).

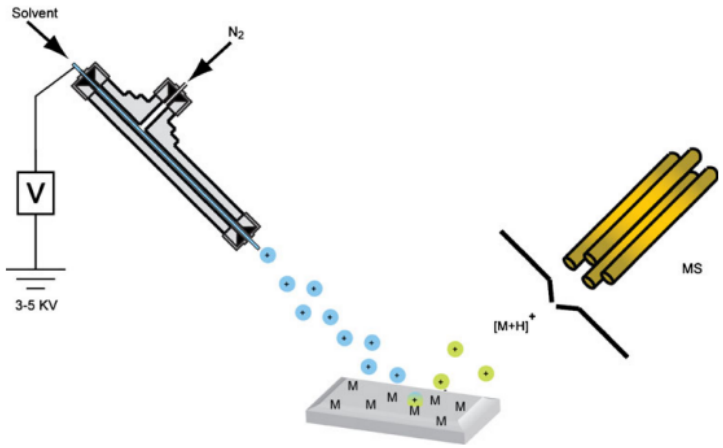

Figure 2.3.9. Scheme of DESI source (Alberici et al., 2010).

A negative aspect of DESI concerns the sprite that occurs when the stream of gas and droplets arrives on the sample surface. To eliminate this problem, the **nano-DESI** has been developed. As shown in **Figure 2.3.10**, in this case, the analytes are desorbed from the surface at the point where two capillaries, filled with liquid, touch the surface. In this way, the sample is analyzed by a charged solvent bridge created between the two capillaries. The first one guides the charged solvent droplets to the

sample surface, while the other one generates a nanospray that contains the analyzed and directs it to the mass spectrometer. The potential difference between the first capillary and the inlet of the mass analyzer is 3-5 kV, while the distance is around 0.5mm (Roach et al., 2010).

Another ionization source based upon ESI is **SESI** or Secondary Electrospray Ionization. The mechanism of SESI is similar to the one of APCI, but in this case, the ions are produced by an electrospray process and allow a better ionization efficiency if compared with ESI (Tam & Hill, 2004).

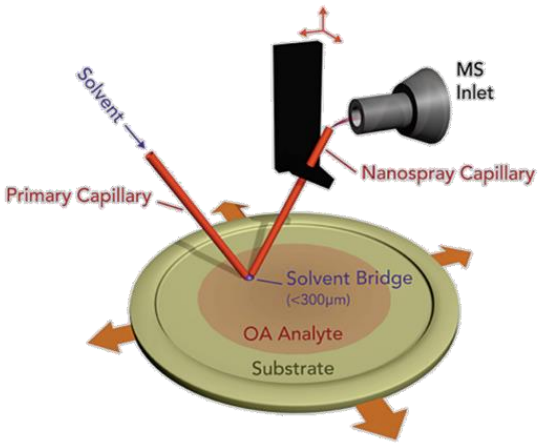

Figure 2.3.10. Scheme of nano-DESI source (Roach et al., 2010).

In 2006, Cooks and his collaborators introduced a variation of SESI named Extractive Electrospray Ionization or **EESI**. It was created to solve the limitations of DESI with volatile analytes that are complicated to manage because they quickly evaporate from the surface (Alberici et al., 2010). As it is possible to see in the scheme in **Figure 2.3.11**, in this case, there is no need to put the sample on a substrate. A sample flow containing the analytes in the gas phase is analyzed by directing it into the ESI spray. The collision of the aerosol particles with the solvent droplets allows the extraction of the soluble components from the aerosol. When the solvent evaporates from the charged droplets, the analyte ions are ejected through a Coulomb explosion mechanism and enter the mass spectrometer (Gallimore & Kalberer, 2013).

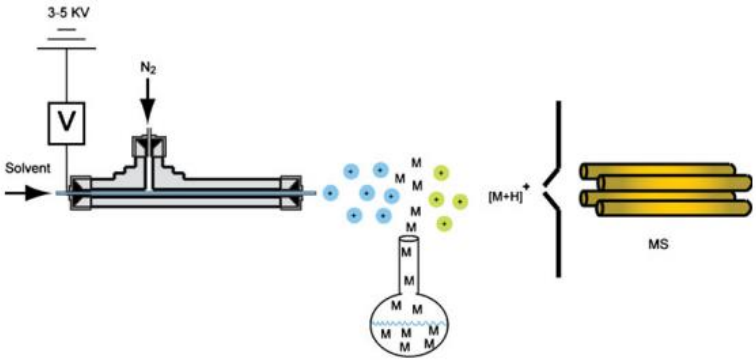

Figure 2.3.11. Scheme of EESI source (Alberici et al., 2010).

One of the fundamental parameters for the correct operation of EESI is the nature of the solvents used to generate the ESI spray and sample aerosol. Depending on their nature, it is possible to observe an increase or decrease of the analyte ion signal (Law et al., 2010). Another important ionization source based on ESI is **PSI** or Paper Spray Ionization, detailed in *Paragraph 2.4*.

2.3.6 LDI-related techniques

The main LDI technique is Matrix-Assisted Laser Desorption Ionization or **MALDI**, but it is still a methodology based on the application of vacuum conditions. The ambient mass spectrometry techniques, in this case, derive from the combination of LDI and ESI, therefore from the combination of the desorption power of applying a laser and the ability of the charged droplets, generated with ESI, to generate ions at ambient conditions (Alberici et al., 2010).

Among them, there is **ELDI** or Electrospray Assisted Laser Desorption Ionization. The ionization process of ELDI occurs in two separate steps. In the first step, the analytes are deposited on a surface and are desorbed through a laser that operates at 337nm, generating gaseous species. In the second step, the desorbed particles are ionized, blending with the charged solvent droplets produced by ESI (Peng et al., 2010). The scheme of ELDI is reported in **Figure 2.3.12**. The distance between the electrospray capillary and the plate where the sample is loaded should be less than 3mm to obtain an efficient mix of analytes and the ESI plume. Moreover, the sample should touch the initial stages of the plume to achieve a high ionization efficiency (Shiea et al., 2005).

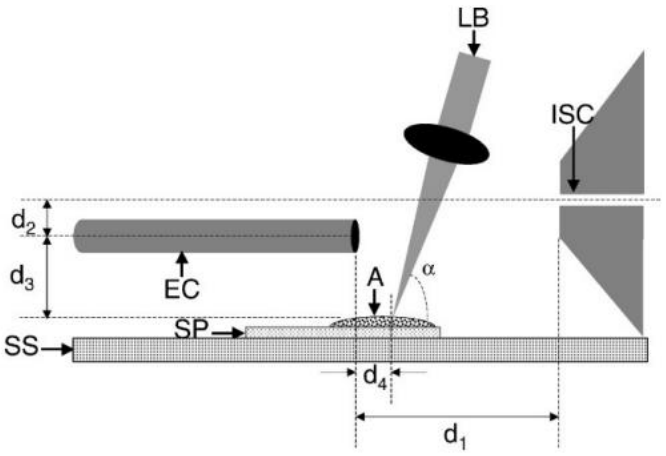

Figure 2.3.12. Scheme of ELDI source (Shiea et al., 2005).

Two variations of ELDI are the Laser Ablation Electrospray Ionization, **LAESI,** and the Infrared-Laser Assisted Desorption Electrospray Ionization, **IR-LADESI**. Both techniques do not require the application of a matrix and differ from ELDI in the use of two types of radiation, UV and IR (Alberici et al., 2010).

La LESI applies a laser in the mid-infrared to excite the O-H vibrations in the sample water molecules and to generate a plume of neutral ions that are then ionized through the charged droplets produced by nano-electrospray. The charged ions obtained are directed to the mass spectrometer (Etalo et al., 2018).

The IR-LADESI, instead, differs from LAESI for the incident angle of the IR laser on the surface of the sample and for the low-energy pulsation that allows not only the desorption but also the ablation of the analytes from the surface. The angle is 45° with respect to the support where the sample is located (Alberici et al., 2010; Rezenom et al., 2008).

Another methodology that is a variation of MALDI is **MALDESI** or Matrix Assisted Laser Desorption Electrospray Ionization. As in the case of ELDI, the ESI is performed on a plume of neutral and ionized molecules, but, in this case, the plume is generated by MALDI applying a supporting matrix. For this reason, MALDESI combines MALDI and ESI. The arrangement of MALDESI is identical to the one of ELDI; the unique difference is the use of a supporting matrix, **Figure 2.3.13**.

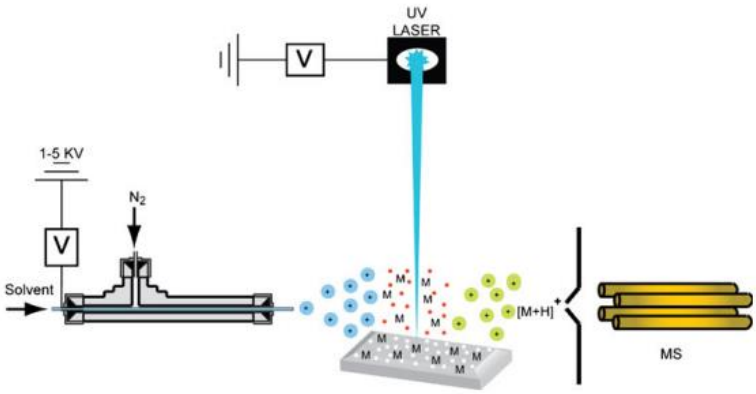

Figure 2.3.13. Scheme of MALDESI source (Alberici et al., 2010).

2.4 Paper spray mass spectrometry

The development of paper spray, and in general of all the ambient ionization sources, finds its roots in the need to introduce liquid samples into the high vacuum environment in which the mass spectrometer works. The emergence of PSI as an ionization source derives, initially, from its ability to detect analytes from samples of dried blood (DBS) on paper. This ability to analyze complex matrices in the presence of the matrix itself, without the need for any kind of sample preparation, is in great demand in the analytical field and is, therefore, the motivation behind the development of this and other ambient techniques. The advantage of using paper spray for DBS analysis, compared to other techniques, is attributed to its ability to retain matrix interferents thanks to the paper porous structure (McBride et al., 2019). Moreover, before the development of PSI by Professor Cooks and his collaborators, it was demonstrated that the paper could be used to generate electrospray. In fact, the authors themselves placed a sample on a piece of paper and, applying a high voltage to the wet paper, were able to generate ions and analyze them using a mass spectrometer.

The paper is widely available and cheap and showed great potential in the development of cheap diagnostic methods. Good examples of paper-based tests are pregnancy tests and tests for blood glucose. Thus, the use of paper and paper spray is the right solution for a fast quantitative analysis at a very low cost (Liu et al., 2010).

2.4.1. Mechanism of paper spray ionization source

The arrangement of Paper Spray ionization is very simple. It consists of a triangular-shaped piece of paper (usually 10 mm long and 5 mm wide) held by a copper clamp with the tip of the paper pointing towards the inlet of the mass spectrometer at a distance of 3 or more millimetres from it, as shown in **Figure 2.4.1**.

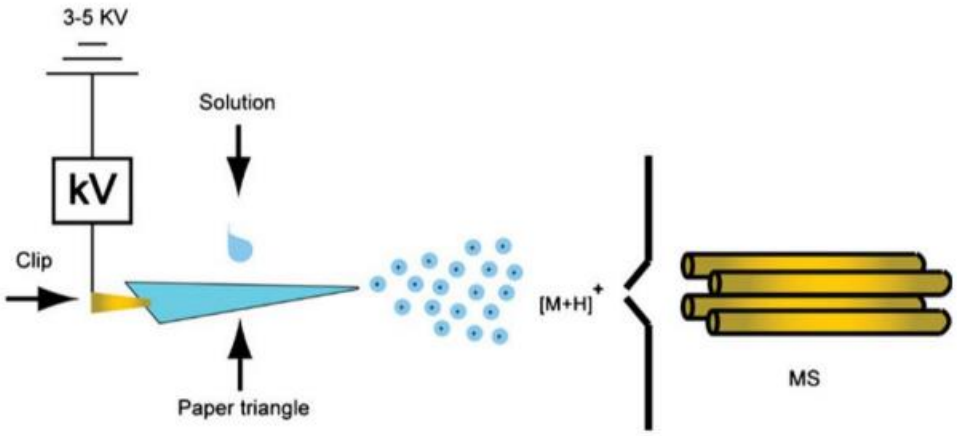

Figure 2.4.1. Scheme of paper spray ionization source (Alberici et al., 2010).

The sample is loaded into the paper support, and, adding around 30 µl of solvent and applying a voltage between 3 and 5 kV, the ionization is performed. Indeed, paper, generally made from cellulose, is a porous hydrophilic material that can hold a certain amount of liquid. The wet paper conducts the current, and the structure of cellulose allows the transport of liquids, including dissolved analytes. The voltage applied between the paper and the inlet of the mass spectrometer generates an electric field, which produces a charge that tends to accumulate at the tip of the paper triangle. When the solvent with the analytes reaches the tip, the ionization occurs with a mechanism like electrospray. In fact, the Coulomb force breaks the interactions between the molecules of the liquid, allowing the formation of charged droplets, which then undergo a desolvation process to generate the analyte ions. Paper Spray Ionisation does not require a supporting gas and works well even if the capillary of the mass spectrometer inlet is at room temperature (McBride et al., 2019; Liu et al., 2010; Zhang et al., 2014).

The greatest utility of an analytical method is obtained when a deep knowledge of how it works is possessed. Over the years, many efforts have been made to improve the knowledge of PSI mechanisms, such as Epsy and his collaborators did, investigating the spray formation by using a FASTCAM 1024 PCI with fibre-optic backlighting. The obtained image is shown in **Figure 2.4.2**.

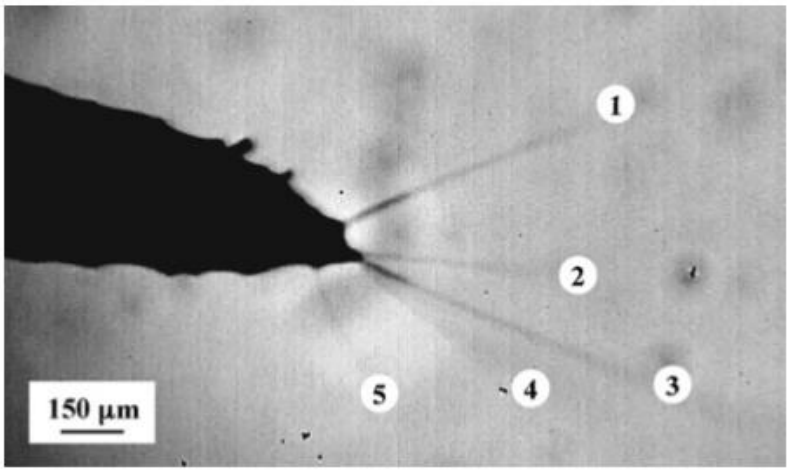

Figure 2.4.2. Jets generated by PSI (Espy et al., 2014).

It is possible to notice the presence of many jets, of which five are clearly identifiable, and three show the typical Taylor cone structure. The primary jet is the number 3 as it is the densest, and it is located right at the tip of the triangle. Number 1 and number 5 are secondary because they are at a distance of 60-80 µm from the tip, while numbers 2 and 4 are unclear; they could be off-axis casts as they also appear blurred. The high number of jets formed is probably the result of the high electric field. In fact, from 2 to 5 jets can be observed depending on the composition of the solvent used, the solvent flow and the voltage applied (Espy et al., 2014).

The PSI has often been compared with Nanospray Ionisation because they have some characteristics in common in terms of the volume of solvent that is used and the droplet size of the spray (McBride et al., 2019). In PSI, the spray has a higher voltage than in nano-ESI, >2.8 kV versus >0.8 kV. The signal in the PSI increases with increasing applied voltage, whereas in the nano-ESI the signal increases in the 0.5-2.0 kV voltage range, and then there is a gradual decrease in the range 2.0-4.5 kV (Zhang et al., 2014).

In addition to the comparison with nano-ESI, to highlight the high simplicity of PSI, it is useful to compare it with all the other ambient mass spectrometric techniques, highlighting which conditions are required for the correct operation of each ionization source, **Table 2.4.1**.

	Voltage	Carrier gas	Heating	Lamp or laser	Supporting matrix
DAPCI	☑	☑	☑		
ASAP	☑	☑	☑		
DART	☑	☑	☑		
LTP	☑	☑			
DAPPI	☑	☑		☑	
EASI		☑			
V-EASI		☑			
DESI	☑	☑			
EESI	☑	☑			
ELDI	☑	☑		☑	
MALDESI	☑	☑		☑	☑
PSI	☑				

Table 2.4.1. Comparison of ambient ionisation techniques.

The simplicity of the PSI methodology is very evident. Only with EASI and V-EASI it is important to do some clarifications. Both require only the use of carrier gas; in fact, they are listed among the simplest ionization methodologies, and they are the only ones where it is not necessary to apply a voltage to generate ionization. Despite this, the EASI requires a pump to push the solvent, as the latter must generate a vaporization at the speed of sound so that when it impacts the sample, it is able to produce desorption and ionization. In the V-EASI, on the other hand, there is no need for a pump because the Venturi effect is used to self-pump the solution containing the analytes. It, therefore, remains an extremely simple technique and is suitable for

miniaturization. However, it is not as simple as PSI, where a paper triangle is wet with the solvent directly by hand without the need for special support.

2.4.2. Fundamentals parameters of PSI.

There are several considerations that need to be made when setting up a PS-MS analysis. The performance of the method depends not only on the efficiency in transferring the compounds of interest but also on the efficiency of the spray that is created (McBride et al., 2019; Zhang et al., 2014). Therefore, the performance of the PSI is the result of the choice of the paper substrate, the amount of sample loaded, the position of the paper with respect to the inlet of the mass spectrometer and the geometry of the paper. Among these, the type of paper support plays a crucial role (Fernandez & Garcia-Reyes, 2017).

- *Types of paper*. As it is well known, paper is composed mainly of cellulose and semi-cellulose with different hydroxyl groups on the surface. When used for separative purposes, it allows the differentiation of compounds on the basis of polarity. Because of this characteristic, paper is widely used as a chromatographic support. The paper is also very important in paper spray ionization because the elution and ionization efficiency of the compounds of interest depends on the physical structure and chemical properties of the paper substrate (Schulz, 1991). Already in the work where paper spray was presented, six different types of paper, commercially available, were tested. Of these, the chromatographic paper produced the MS spectrum with the best quality, while the worst performance was obtained with paper with glass fibres (Liu et al., 2010).

  The main problem with paper made from cellulose and semi-cellulose is that when you want to analyze compounds of polar nature, strong hydrogen bridge bonds and Van der Waals interactions are created with the paper support, affecting the elution and analytical sensitivity of the target molecules. This leads to a dramatic reduction in PSI performance. To this end, significant progress has been made in the modification of paper substrates using various approaches, such as atomic layer deposition, dip-coating, spray deposition and electrostatic

spraying methods. Many types of both hydrophilic and hydrophobic paper substrates have been manufactured. Among the most important, there is a *paper substrate coated with silica*. The silica is easily applied by vacuum filtration. The modified paper has the advantage of a homogeneous surface and a well-defined thickness, providing good repeatability for PS-MS analyses. There also exist *paper substrates modified with nanoparticles of noble metals and those coated with metal oxides*. The latter are also obtained by vacuum filtration, and in various studies in which they have been employed, they showed high performances. Another type is the paper substrates *coated with MOFs* or metal-organic frameworks. They are created to decrease the interactions formed between the paper and the target analytes. Another is the *polymer-coated type*. In order to improve selectivity and hydrophobicity, paper has been modified with various types of polymers. Also important are *graphene-coated substrates*, which have received considerable attention due to their characteristic properties, such as electron mobility and thermal conductivity (Han et al., 2018).

- *Angle of the paper tip.* This parameter is one of the key ones in the generation of the spray. The spray formation has been studied at different angles, taking into consideration different paper shapes. It was observed that the initial voltage needed rises as the angle of the paper substrate increases. The voltage is 3.0 kV for points with angles of 30° and 60°, while it increases to 4 kV for angles of 90° and 120°. When the voltage exceeds 6.0 kV, no spray formation is observed for angles of 150°. Conversely, the spray current gradually decreases as the angle increases (Zhang et al., 2014). In **Figure 2.4.3**, there are shown the plumes obtained by using different shaped paper substrates; they differ in the size of the angle of the paper tip. It shows that acute angles generate stronger sprays at lower voltages. Therefore, paper triangles are usually used in PS-MS with an angle between 30-45° (Klampfl & Himmelsbach, 2015).

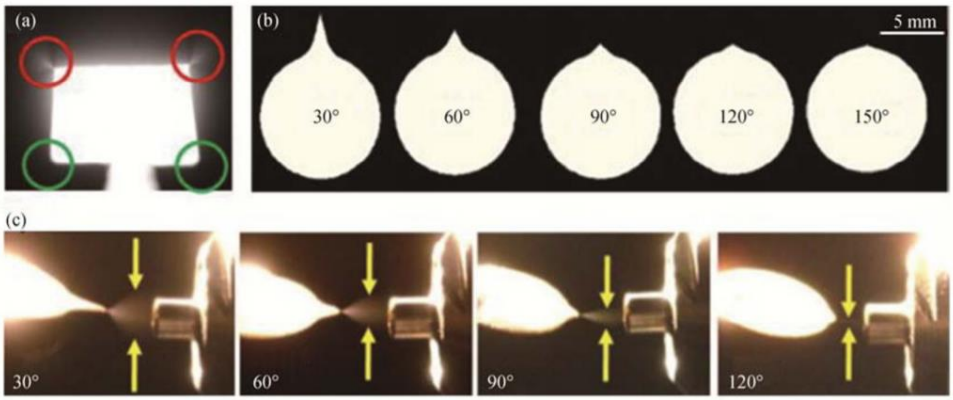

Figure 2.4.3. Spray formation with paper substrates of different angles (Zhang et al., 2014).

- *Solvent*. In paper spraying, the solvent used determines the efficiency of elution of the analytes from the surface of the paper substrate and the efficiency of the spray formation at the tip of the paper. What plays a key role is the polarity and volatility of the solvent. It is possible to increase the sensitivity of the analysis by adding low polar or nonpolar solvents to the polar one. This increases the efficiency of desolvation during electrospray. In addition, it is possible to lower the LOD (limit of detection) by adding a certain percentage of a solvent with a low boiling point, such as dichloromethane (Zhang et al., 2014). The most widely used solvent is methanol; it is a volatile solvent with a low surface tension and can facilitate the ionization process. The main problem with pure methanol is its rapid evaporation, which results in a limited spray generation time. For this reason, it is often used in a mixture with water or by using media to delay evaporation, such as the application of a Teflon tube (Cao & Huang, 2018).

2.4.3. Paper spray ionization variants.

There exist variants of paper spray ionization which are not based on the use of a paper substrate. In this case, the ionization is carried out directly on materials of other nature. A practical summary of the most used materials is shown in **Figure 2.4.4**.

The **Leaf Spray** was born as a development of the paper spray. If a leaf has a sharp, pointed shape, it can be used directly in its entirety, or a triangular-shaped piece can be cut.

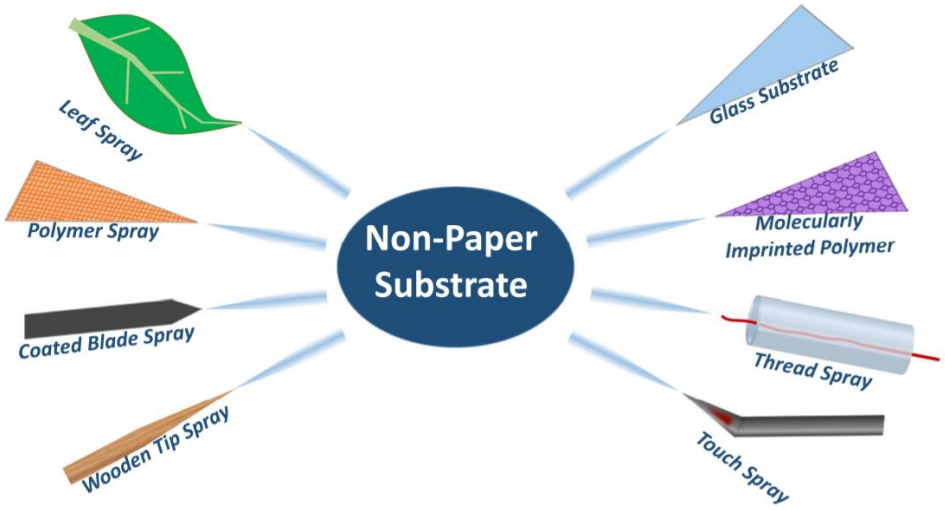

Figure 2.4.4. Outline of the main non-paper-based substrates (Etalo et al., 2018).

The use of this technique is not restrictive only towards the leaves, but any part of the plant can be used, such as flowers, roots and seeds. The optimum dimensions are a thickness of 3 mm, a base length of 5 mm and a height of 10 mm. The addition of solvent drops to generate the spray, in this case, is not always necessary. For a pulpy fruit, its natural lymph is sufficient to create the spray and obtain high-quality mass spectra. A scheme of leaf spray arrangement is shown in **Figure 2.4.5**.

This methodology has been tested and proven for the analysis of amino acids, carbohydrates, fatty acids and lipids. Very important is the application in the field of agriculture; without the need for pre-treatment, such as grinding, it is possible to perform fast analyses capable of detecting the presence of pesticide residues, allergens and any other molecules essential for quality control (Etalo et al., 2018).

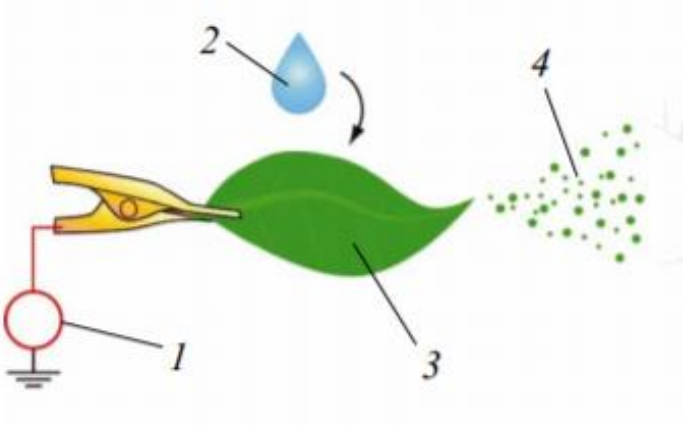

Figure 2.4.5. Scheme of leaf spray ionization (Lebedev, 2015).

The **Wooden Tip Spray** was first introduced in 2011 with the use of toothpicks. The sample preparation in this method is simple. The sample solutions can be applied to the tip of the wooden support using a pipette; this allows the creation of a coating at the tip. The hydrophilic and porous nature of wood allows the sample to adhere to the surface without the need to make modifications to the surface itself. The very narrow stick shape of the wooden substrate generates less diffusion of the solution compared with Paper Spray, where the paper substrate is flat.

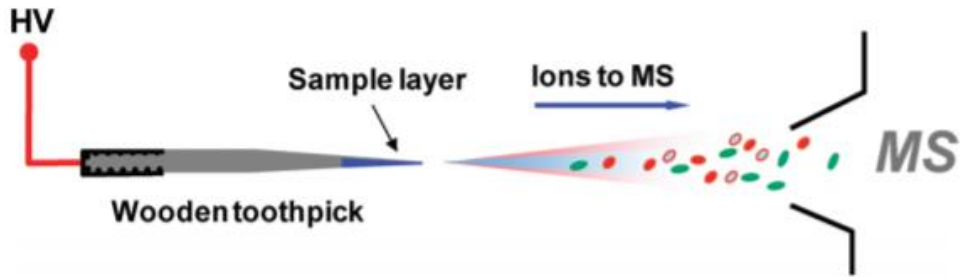

Figure 2.4.6. Scheme of Wooden tip spray ionization (Hu et al., 2011).

This reduces the evaporation area and the tendency of the solutions to dry out, allowing the acquisition of a longer-lasting signal. The structure of the Wooden Tip Spray is shown in **Figure 2.4.6** and is very simple. This technique can be used for the analysis of various compounds, such as peptides, proteins and samples that are less analyzable with classical ESI, such as unfiltered slurries, powders and, finally, has high potential in the forensic field (Hu et al., 2011).

In **Polymer Spray**, the substrate consists of a polymer such as polyester and polyethylene. These are much stronger media than paper, where its fibrous nature lacks structural integrity and makes it unsuitable for support reuse (Song et al., 2018). Substrates of a polymeric nature do not have this problem and, cut into triangles, behave like Paper Spray. The absence of hydroxyl groups, however, leads to better responses from the deposited analytes, which increases the sensitivity. Finally, the use of conducting polymers, such as polymethyl methacrylate, eliminates the difficulty of extracting the analytes from the pores (Frey et al., 2019).

The **Coated Blade Spray** (CBS) is a technology based on solid phase microextraction (SPME), which was developed to perform a fast extraction of analytes from complex matrices followed by direct ionization under ambient mass spectrometry conditions. CBS is an ideal compromise between sample preparation and direct coupling with mass spectrometry. It is, essentially, a sheet of stainless steel cut in the shape of a sword and coated with C18-polyacrylonitrile (C18-PAN). The SPME coating extracts the analytes from the sample without removing the matrix. In this way, only the analytes of interest and the technique allow to eliminate artefacts that can lead to errors in over- or under-estimation of the analytes. Ions are formed in the same way that Paper Spray works: an electric field is applied, and a small volume of solvent is used to generate desorption and, subsequently, ionization (**Figure 2.4.7**).

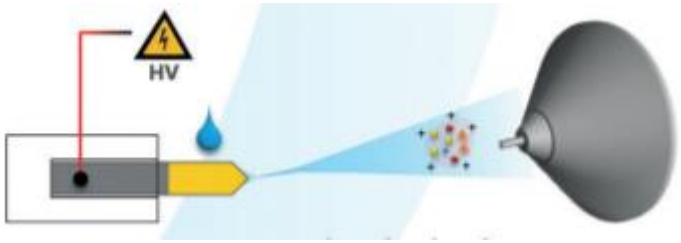

Figure 2.4.7. Scheme of Coated Blade Spray (Gomez-Rios & Pawliszyn, 2014).

The main difference with PSI is in the fact that CBS is built of a conducting material instead of a porous, non-conducting substrate such as paper, so a static electric field is generated at the tip of the CBS. The result of the presence of such a field is a more efficient and reproducible ion formation (Gomez-Rios & Pawliszyn, 2014).

The **Thread Spray** uses a simple thread to perform the ionization. This methodology has been developed and demonstrated for the analysis of various capsaicinoids within peppers without destroying the sample. The efficiency of the technique changes depending on the chemical and physical characteristics of the wire used. The set-up consists of a wire, approximately 35 mm long, inserted into a glass capillary. The sample is previously deposited on the wire, and this is placed in front of the mass spectrometer, as shown in **Figure 2.4.8**. The application of an electric field to the wet wire allows the generation of charged liquid droplets containing the analytes, which then enter the mass spectrometer (Jackson et al., 2018).

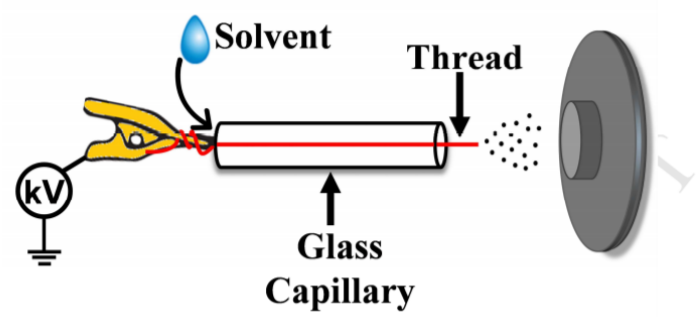

Figure 2.4.8. Scheme of Thread Spray (Jackson et al., 2018).

Finally, **Touch Spray** uses a probe, for example, a needle, on which the sample and solvent are placed. Then, like all other techniques based on paper spray, through the application of a voltage, the transfer and ionization of the analytes is obtained. Then, the analytes are directed towards the mass spectrometer (**Figure 2.4.9**).

This methodology has the capability of adsorbing on the probe materials such as solid powders, trace solids, solutions and heterogeneous matrices such as tissues (Kerian et al., 2014). A particular variation of this method, used in various works, is the **Swab Touch Spray,** in which the probe is a swab (Bain et al., 2018).

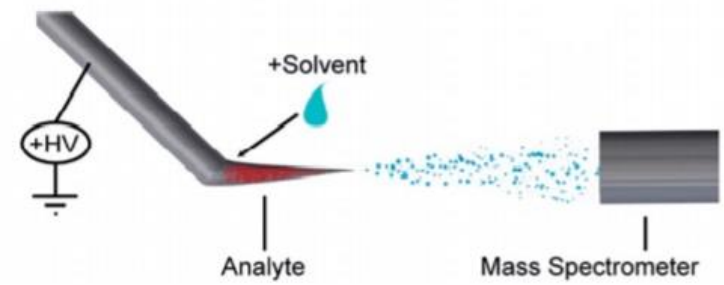

Figure 2.4.9. Scheme of Touch Spray (Kerian et al., 2014).

2.4.4. Application of Paper spray mass spectrometry for food quality and authenticity.

Like all the other ambient ionization techniques, paper spray provides a series of advantageous characteristics. It is helpful to remember the very small sample and organic solvents required, the capacity to obtain fast and high-throughput analysis, the minimal or absent sample preparation needed and the relatively low matrix effects.

In particular, PS-MS is one of the simplest ambient mass spectrometry techniques, and it is straightforward to modify and adapt to reach a specific goal. This led to the introduction of new derived methodologies, such as leaf spray and wooden tip spray. Moreover, the paper support can be appropriately modified to promote the selective ionization of the analytes from the sample, improving the selectivity and sensitivity of the mass spectrometry analysis. All these characteristics made PS-MS attractive and have been applied for qualitative and quantitative analyses in many fields (Teodoro et al., 2017). Starting from the application in the clinical field using matrices like plasma, urine, saliva and tissues (McBride et al., 2019; Manicke et al., 2016) for the determination of metabolic substances or illicit drugs and narcotics (Epsy et al., 2014; Manicke et al., 2011), to the environmental one (Zhu et al. 2016; McBride et al., 2019) The PS-MS methodology has been used, also for the characterization of dangerous substances in cosmetics, to check the authenticity of documents, to monitor chemical reactions and to discriminate microorganisms (Klampfl & Himmelsbach, 2015).

In the end, there is the field of food quality and authenticity, the main applications for the determination of food components and food contaminants are shown in **Table 2.4.2**, while the main applications of paper spray mass spectrometry, coupled with chemometric tools for food authenticity and traceability are reported in **Table 2.4.3**.

Compounds analysed	Matrix	Reference
Food components		
Anti inflammatory dialdehydes	Olive oil	Mazzotti et al., 2013
Vitamin E	Extra virgin olive oil	Bartella et al., 2019a
Hydroxytirosol and Tyrosol	Extra virgin olive oil	Bartella et al., 2020
Aldehydes	Vegetables, fruits, and meat products	Lin et al., 2022
Benzoic acid and vitamin C	Beverages	Yu et al., 2018
Lutein	Fresh and commercial fruits and vegetables	Alsaggaf, 2022
Theobromine, theophylline and caffeine	Cocoa products	Bartella et al., 2019b
Resveratrol	Red wine	Di Donna et al., 2017
Food contaminants		
Sudan azo dyes	Chili powder	Taverna et al., 2013
Antimicrobial residues	Beef, chicken meat, milk, and egg	Su et al. 2018
4-methylimidazole	Caramel and beverages	Li et al., 2013
Pesticides	Oranges, tomatoes, grapes	Evard et al., 2015

Table 2.4.2. Main PS-MS applications for the determination of food components and food contaminants.

Approach	Matrix	Chemometric tools	Reference
Fingerprint approach	Scottish whiskies	PCA PLS-DA	Teodoro et al., 2017
Fingerprint approach	Coffee	PCA HCA	Garrett et al., 2013
Fingerprint approach	Beers	PCA PLS-DA	Pereira et al., 2016
Fingerprint approach	Arabica and Robusta coffees	PCA PCA-LDA	Pumba et al., 2023
Metabolic profile	Tomatoes	PCA	Santos et al., 2022

Table 2.4.3. Main PS-MS applications for the determination of food authenticity and traceability.

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CHAPTER 3.

Paper Spray Mass Spectrometry for food quality

Part I.

High-throughput determination of flavanone-O-glycosides in citrus beverages by paper spray tandem mass spectrometry

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High-throughput determination of flavanone-*O*-glycosides in citrus beverages by paper spray tandem mass spectrometry

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ABSTRACT

A fast and accurate methodology for the quantification of the most abundant flavanone glycosides in citrus beverages has been developed. The approach relies on the use of paper spray mass spectrometry, which allows to record data in few minutes and without sample pre-treatment. The experiments have been carried out in Multiple Reaction Monitoring scan mode, in order to obtain the best specificity and sensitivity. The analytical parameters were all satisfactory. The results coming from the analysis of real samples were compared to the data obtained by the commonly used chromatographic method, proving the robustness of the proposed approach.

1. Introduction

It is now well recognised worldwide that to maintain a good and healthy life it is important to change some habits, such as the consumption of junk food or an extremely sedentary lifestyle. Moreover, there is a strong connection between the increase of fruits and vegetables in the diet with the risk reduction of some diseases like the cardiovascular ones and cancer. In particular, the attention is now focused on polyphenols, secondary metabolites present in food derived from plant (Khan et al., 2014). A significant class of polyphenols is represented by flavonoids, they have strong antioxidant activity (Aviram & Fuhrman, 2022; Barreca et al., 2017; Le Marchand, 2022) and are associated with reduced risk for certain chronic diseases, with prevention of cardiovascular disorders and some typologies of cancers. Also, flavonoids have antiviral, antimicrobial and anti-inflammatory activities. The dietary intake is the main source of flavonoids for humans, depending on dietary and also cultural habits, the average intake is between 70 and 170 mg/day. They are usually found in fruits, vegetables, and cereals, even if the food preparation and processing of fresh fruits and vegetables may decrease flavonoids content by 50%. *Citrus* fruits and *Citrus* juices represent the major dietary source for phenolic compounds and, in particular, flavonoids (Gattuso et al., 2007) In addition, *Citrus* plant, belonging to the Rutacease family, are cultivated worldwide and their fruits and juices such as oranges, mandarins, grapefruits and chinotto, are very common for almost all human population (Barreca et al., 2017). Fresh fruits and their juices mostly contain flavones, and flavanones, this last class accounts for approximately the 95% of the total content of flavonoids in citrus fruits. These flavanones are not equally distributed in the fruit, they are more present in the albedo than in the pulp. For that reason, when preparing juices, the albedo is discarded and so, in juices the actual level of flavanones is lower (Testai and Calderone, 2017). The flavanones can contribute to fruit and juice in many ways, they can influence the taste and the nutritional value. For example, hesperidin can cause cloudiness in lemon and orange juice, or naringin influences the bitterness of grapefruits and bergamot juices. Flavanones are mainly present in *Citrus* fruits as their glycosyl derivatives. The equivalent forms without the sugar moieties, occur less frequently in juices due to their lipophilic nature and their low solubility in water. The most common sugar moieties include D-glucose and L-rhamnose. The glucosides are

usually O-glycosides, with the sugar moiety bound to the aglycone hydroxyl group at C-7. In *Citrus* juices the flavanone-O-glycosides are divided in two group of compounds, and both are L-rhamnosyl-D-glucosyl derivatives: rutinose which have a α-1,6 interglycosidic linkage, and neohesperidose, with a α-1,2 interglycosidic bond (**Figure 3.1.1**) (Khan et al., 2014).

The abundance of these flavanone-O-glycosides change between the *Citrus* species. Considering oranges, they are divided in sweet oranges and sour oranges. The sweet oranges are all members of the specie *Citrus sinensis*, which has four subclasses: common, novel, Valencia and blood. The dominant flavanone glycosides in sweet oranges are hesperidin and narirutin, while in sour oranges the two predominant flavanone glycosides are neohesperidin and naringin. The sugar rutinose of hesperidin and narirutin, determines a neutral taste, while the neohesperidose of neohesperidin and naringin imparts a tangy or bitter taste (Peterson et al., 2006).

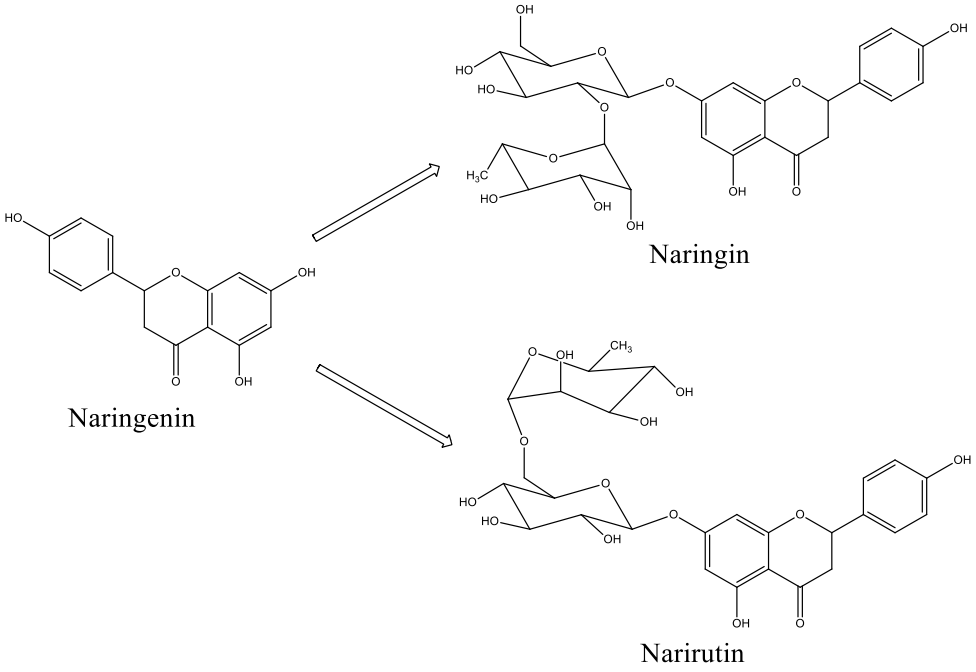
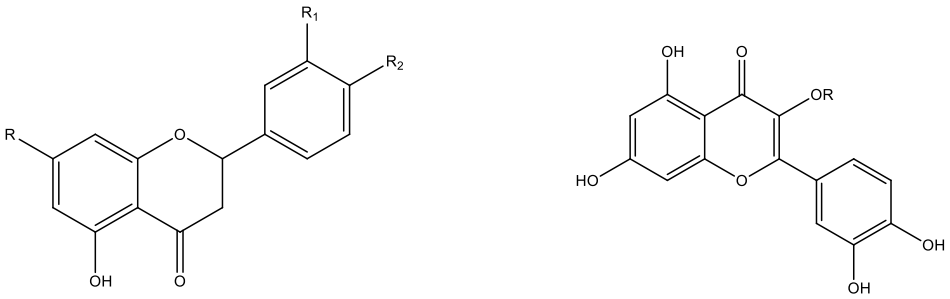


Figure 3.1.1. Chemical structure of flavanone aglycone Naringenin, and its O-glycosides derivatives: Naringin (7-O-neohesperidoside) and Narirutin (7-O-rutinoside).

For what concern hesperidin in sweet oranges, it is the main component followed by narirutin, the two have a content thar range from 35 to 147 mg/100g (Chanet et al., 2012). The general specie for tangerine is *Citrus reticulata*, between the others there are *Citrus clementina* and *Citrus deliciosa*. They have the same flavanone composition

as sweet orange, but in less amount. The flavanone hesperidin is also the predominant compound in lime and lemon juice, followed by eriocitrin. Naringin, neohesperidin, neoeriocitrina and eriocitrin are the most abundant flavonones in chinotto and sour orange (Barreca et al., 2017 and Peterson et al., 2006). The content of naringin, neoeriocitrin and neohesperidin in bergamot juice is on the order of hundreds of ppm, but there is also other compound with good antioxidant activity and present in considerable amount. These flavonoids carry a 3-hydroxy-3-methylglutaric acid moiety and are brutieridin, melitidin and peripolin, the HMG conjugates of, respectively, neohesperidin, naringin and neoeriocitrin. The HMG moiety makes these molecules more soluble in water and, consequently, easier to be absorbed after oral ingestion (Bartella et al., 2022; Di Donna et al., 2009) Commonly the quantitative analysis of the flavanone glycosides in *Citrus* juices is performed using liquid chromatography coupled with mass spectrometry or ultraviolet for detection. (Belajova & Suhaj, 2004; Di Donna et al., 2013; Dugo et al., 2005; Russo et al., 2011) Mass spectrometry is a very powerful technique that allows to obtain specific and sensitive results. Moreover, it has rapidly evolved in the last decades and now the attention is focused on a new class of methodologies, the ambient mass spectrometry techniques. In this case the sample is directly analysed in its proper natural state and that reduces or completely eliminates the need of long and laborious sample pre-treatments. (Cooks e al, 2006) Among them, paper spray mass spectrometry is one of the simplest techniques. In this case the sample is loaded on a triangular piece of paper placed in front of the mass spectrometer inlet and applying a high voltage through a metallic clip, the compound ionization is performed. This methodology has already been employed in different matrices (Bartella, 2020; Chiang et al., 2018; Frey et al., 2020) and, in particular, on foodstuff (Bartella et al., 2019a; Bartella et al., 2019b; Chen et al., 2017; Li et al., 2013). For what concern flavonoids, paper spray mass spectrometry has been applied for their quantification in ginkgo plant (Zhou, 2019).



Eriocitrin	R = O-rutinose, R_1 = OH, R_2 = OH
Hesperidin	R = O-rutinose, R_1 = OH, R_2 = OCH_3
Naringin	R = O-neohesperidose, R_1 = H, R_2 = OH
Narirutin	R = O-rutinose, R_1 = H, R_2 = OH
Neoeriocitrin	R = O-neohesperidose, R_1 = OH, R_2 = OH
Neohesperidin	R = O-neohesperidose, R_1 = OH, R_2 = OCH_3
Neoponcirin	R = O-rutinose, R_1 = H, R_2 = OCH_3
Poncirin	R = O-neohesperidose, R_1 = H, R_2 = OCH_3
Brutieridin	R = O-(6"-HMG)-neohesperidose, R_1 = OH, R_2 = OCH_3
Melitidin	R = O-(6"-HMG)-neohesperidose, R_1 = H, R_2 = OH

Quercetin 3-glucoside R = glucose

Figure 3.1.2. Chemical structures of flavanones and internal standard.

For that reason, a new methodology for the rapid quantification of flavanone-O-glucosides in *Citrus* beverage through paper spray mass spectrometry has been developed. The compounds analysed are shown in **Figure 3.1.2** with a multiple reaction monitoring (MRM) approach, using quercetin-3-O-glucoside as intern standard.

1. Experimental

2.1 Chemicals

Solvents (HPLC grade) were commercially available (Sigma-Aldrich, St. Louis, MO). Flavanones-O-glycosides standards quercetin-3-O-β-Dglucoside, used as internal standard, were purchased from Extrasynthese (Genay Cedex, France). The pure standards brutieridin and melitidin were obtained as reported elsewhere (Mon et al., 2020).

2.2 Sample preparation

Nine Citrus beverages were used to test the developed methodology. All the samples, two orange soda beverages, two lemon soda beverages, a chinotto soda beverage, one grapefruit juice, one tangerine juice, one bergamot juice and a cola soft drink, used as blank matrix, were purchased in a local store. The beverages were stored at 4 °C. Before the PS-MS/MS analysis, the samples were added with the appropriate

amount of internal standard solution at a concentration of 200 mg/L, centrifuged at 12000 rpm and properly diluted with water. In particular, tangerine and lemon drinks were diluted two times; bergamot and grapefruit, 10 and 20 times, respectively; the orange beverages were analysed both undiluted and diluted 5 times; lastly, the chinotto drink was analysed as it is. The same samples without the internal standard were submitted to HPLC-UV analysis.

2.3 Paper spray mass spectrometry

The MS analyses were carried out with a TSQ Quantum Vantage (Thermo Fisher Scientific, San José, CA) triple-stage quadrupole mass spectrometer equipped with a homemade paper spray ionization source. Qualitative Whatman filter paper n◦ 1 (pore size 11 µm, thickness 180 µm) was used for the experiments. The sample spotting volume was 15 µl, and once dried, the paper triangle was wetted of the same volume of methanol to allow the desorption of the ions. The paper spray ionization was performed in negative ion mode. The working conditions were the following: voltage 5.0 kV, applied directly to the paper triangle; vaporizer and capillary temperatures 280 and 290 ◦C, respectively. The collision gas was argon used at a pressure in the collision cell (Q2) of 1.5 mTorr, and the mass resolution at the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da at full width at half-maximum. The scan time was set at 0.4 s while the number of micro scans at 2. The collision energy (CE) was optimized individually per compound, ranging from 22 to 35 eV; S-lens values was set at 130 V for each analyte. The assay was performed by using the multiple reaction monitoring (MRM) scan mode, following specific gas phase transitions from the deprotonated precursor ion $[M- H]^-$ (**Table 3.1.1**). The average of the ion current of each monitored transition (quan transition), over the total acquisition time, was used for the quantitative analyses.

Compound	Transition	CE (eV)	S-Lens (V)
Naringin/Narirutin	*m/z* 579 → *m/z* 271 (quan)	30	130
	m/z 579 → *m/z* 459 (qual)	35	
Neoeriocitrin	*m/z* 595 → *m/z* 459 (quan)	35	130
	m/z 595 → *m/z* 287 (qual)	30	
Eriocitrin	*m/z* 595 → *m/z* 287 (quan)	35	130
	m/z 595 → *m/z* 459 (qual)	40	
Neohesperidin/Hesperidin	*m/z* 609 → *m/z* 301 (quan)	28	130
	m/z 609 → *m/z* 489 (qual)	35	
Poncirin/Neoponcirin	*m/z* 593 → *m/z* 285 (quan)	30	130
	m/z 593 → *m/z* 473 (qual)	35	
Melitidin	*m/z* 723 → *m/z* 579 (quan)	25	130
	m/z 723 → *m/z* 271 (qual)	28	
Brutieridin	*m/z* 753 → *m/z* 609 (quan)	25	130
	m/z 753 → *m/z* 301 (qual)	28	
Quercetin-3-*O*-glucoside	*m/z* 463 → *m/z* 301 (quan)	22	130
	m/z 463 → *m/z* 300 (qual)	22	

Table 3.1.1. Selected gas-phase transitions and optimized instrumental parameters.

2.4 HPLC-UV analysis

The HPLC-UV analyses were performed using a FractionLynx system from Waters (Milford, MA) working in analytical mode, equipped with a 2535 quaternary pump and a 2989 UV/visible detector. The analytical column used for the chromatographic separation was a C18 reversed-phase column, Luna (250 × 4.6 mm, 5 µm, Phenomenex). The injection volume was 20 µl. The elution was carried out with 0.1% formic acid in water (solvent A) and methanol (solvent B) under gradient conditions. The gradient steps were the following: 80% A in isocratic for 7 min, from 80 to 40% A (7–40 min), 40% A isocratic for 5 min, from 40 to 20% A (45–50 min), 20% A in isocratic for 7 min, from 20 to 80% A (57–62 min) and then an isocratic flow (8 min) to equilibrate the system before starting the new analysis. The total run time was 70 min, while the flow rate was set at 1 mL/min and the UV detector was set at 280 nm. The concentration of flavanone glycosides was evaluated using an external

calibration curve gained by standard solutions of their standards at a concentration ranged from 10 to 400 µg/mL (Giuffre et al., 2019).

2.5 Limit of detection and limit of quantification (LOD and LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry (McNaught and Wilkinson, 1997), as follows:

$S_{LOD} = S_{RB} + 3\sigma_{RB}$

$S_{LOQ} = S_{RB} + 10\sigma_{RB}$

where S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the ratio of the signals given by the transitions of the analyte and of the internal standard from the blank sample (cola soda drink diluted two times), and σ_{RB} is its standard deviation. The concentrations were calculated by the standard curve.

2. Results and discussion

Between the polyphenols usually found in food derived from plant, there the flavonoids, compounds with strong antioxidant, anti-inflammatory, antiviral and anti-microbial activity. In *Citrus* fruits and juices, the most abundant are the flavanone-O-glycosides, that are L-rhamnosyl-D-glucosyl derivatives of flavanones. The aim of this work was the development of an innovative methodology based on paper spray mass spectrometry for the rapid analysis and quantification of flavanone-O-glycosides in commercial citrus juices.

The analyses were carried out in negative ionization mode, because of the presence of several acidic hydrogens on the aglycon skeleton which increase the ionization efficiency in negative conditions, and also to minimize possible interference effects due to the presence of cations and sugars. The quantification was performed under multiple reaction monitoring (MRM) scan mode in order to ensure a higher specificity. The MRM methodology was determined observing the fragmentation behaviour of each single compound under tandem mass spectrometry experiments.

As shown in **Figure 3.1.3**, the MS/MS spectra of flavanone-O-glycosides (B, C) in negative ionization mode are characterized by few products ions. The base pick is the deprotonated aglycone which is obtained from the loss of the disaccharide portion. The same thing happens for the internal standard quercetin-3-O-glucoside (A), in the MS/MS the base pick derives from the loss of the glucose moiety. To perform the quantification analysis, for almost each analytes, the gas-phase transition elected are those leading to the deprotonated aglycone from the parent ion $[M-H]^-$. Exceptions are neoeriocitrin, brutieridin and melitidin. For what concern neoeriocitrin, the gas-phase transition chosen is originated by the breaking of the C ring. This behaviour is probably due to the simultaneous presence of neohesperidose sugar and catechol on the aglycon moiety, which probably force a particular folding of the sugar unit. For the HMG conjugated melitidin and brutieridin, the monitored transitions originate from the loss of the HMG moiety from the deprotonated precursor ions (**Figure 3.2.2** E, F). To perform PS-MS/MS experiments, a drop of the diluted sample with the addition of the internal standard is placed onto the paper and the ionization of the analytes is obtained applying a high voltage and adding 10 µl of methanol every 30 sec, with a total acquisition time of 2 minutes.

In citrus juices there are both favanone-7-O-neohesperidosides and flavanone-7-O-rutinosides, which are structural isomers and isobars, and so they cannot be distinguished using PS-MS/MS experiments due to the absence of a chromatographic steps and because the gas-phase transitions are the same for both precursors. Nevertheless, the purpose is still achieved, in fact the flavanone glycosides are quantified as the sum of the two structural isomers.

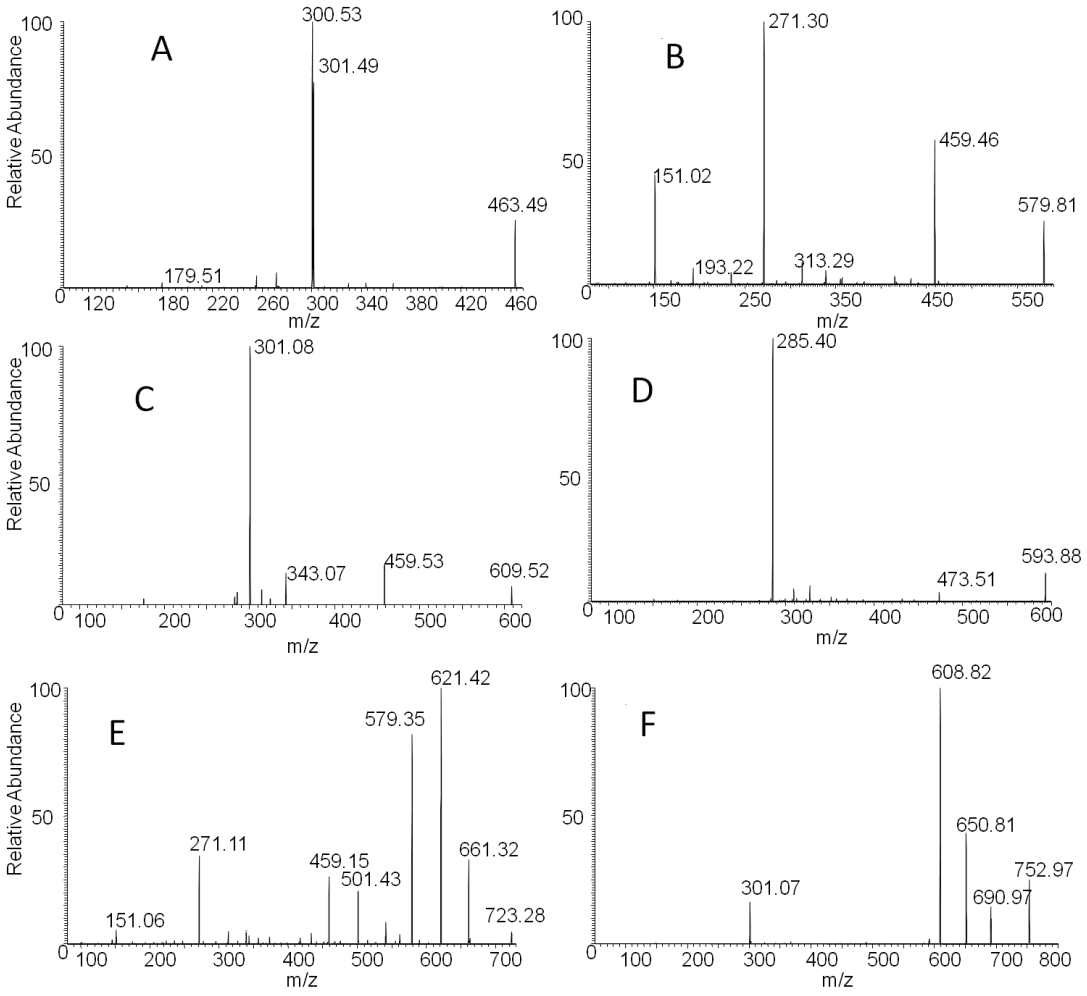


Figure 3.1.3. MS/MS spectra of: quercetin-3-O-glucoside (IS, A), naringin (B), neohesperidin (C), poncirin (D), melitidin (E), brutieridin (F).

The quantitative analysis was based on the internal standard calibration, using quercetin-3-O-glucoside as standard, a flavanol mono-glycosides not found in citrus juice. The neohesperidosides isomers were used as reference compounds for all quantitative experiments. For each compound, the calibration curve was obtained by analyzing five standard solutions in the range from 2 to 20 mg/L, with the internal standard at a fixed concentration of 5 mg/L. Each standard solution was submitted to PS-MRM analysis in triplicate, as described in the experimental section. The method showed a good linearity in the selected range of calibration, providing correlation coefficient values higher than 0.98 for each flavanone glycoside investigated. The limit of detection (LOD) and quantification (LOQ) were determined by analyzing a cola

soda beverage used as blank, fortified with the internal standard at a concentration of 5 mg/L.

In **Table 3.1.2** there are the values of LOQ and LOD calculated in solution after dilution. For all compounds, the LOQ values were below the lowest calibration level (2 mg/L), which means that the PS-MS/MS approach provides good sensitivity for their determination in citrus-based beverages. LOD values ranged from 0.21 to 0.88 mg/L.

Then the methodology was applied to fortified blank samples for the evaluation of the analytical parameters' accuracy reproducibility and repeatability. The accuracy was estimated analysing two spiked samples prepared at representative concentrations of the calibration curve edges, 2.5 mg/L and 18 mg/L. The accuracy values ranged from 95% to 115% for all compounds. The methodology aim was to quantify the main flavanone-O-glycosides as the sum of the two structural isomers, using only the neohesperidoside isomer as calibration standard. For that reason, it was important to evaluate the accuracy for the quantification of rutinosides employing this strategy. So, two blank samples fortified with flavanone rutinosides (eriocitrin, narirutin, hesperidin and neoponcirin) were prepared and quantified using the calibration curves build with neohesperidosides standard solutions. The obtained accuracy values ranged from 90% to 120% for all compounds, except for eriocitrin. It is possible to explain this disagreement observing the MS/MS spectrum of the two isomers, in which the product ions are obtained at the same collision energy. It is possible to notice a difference relative abundance.

The MS/MS spectrum of neoeriocitrin is characterized by the base peak at m/z 459, probably due to a breakage of the C ring, while the main product ion from precursor eriocitrin corresponds to the peak at m/z 287, relative to the formation of the deprotonated aglycon (**Figure 3.1.4**).

	Spiked (S1) 2.5 mg/L	RSD%	Accuracy	Spiked (S2) 18 mg/L	RSD%	Accuracy	LOD mg/L	LOQ mg/L	RSD%* (S2)
Naringin	2.39 ± 0.22	9.32	95.62	17.15 ±0.97	5.65	95.26	0.70	1.60	12.4
Poncirin	2.50 ± 0.33	13.18	100.05	17.57 ± 1.99	11.30	97.61	0.72	1.76	13.6
Neohesperidin	2.49 ± 0.16	6.32	99.60	17.67 ± 2.69	15.21	98.15	0.21	0.47	14.8
Neoeriocitrin	2.52 ± 0.34	13.66	100.95	18.59 ± 2.40	12.89	103.26	0.42	1.11	10.8
Melitidin	2.61 ±0.35	13.54	104.36	20.49 ± 1.74	8.49	113.84	0.88	1.69	11.2
Brutieridin	2.69 ± 0.28	10.37	107.77	20.52 ± 2.47	12.04	113.98	0.88	1.04	12.5
Eriocitrin	2.51 ± 0.29	11.49	100.34	17.11 ± 2.48	14.51	95.04	0.79	1.90	10.6

*The reproducibility was determined by analyzing the one spiked sample 3 times over a period of 1 week.

Table 3.1.2. Analytical parameters of accuracy, LOQ, LOD, and reproducibility*

Juice/Soda	Naringin/ Narirutin	RSD%	Poncirin/ Neoponcirin	RSD%	Neoeriocitrin / Eriocitrin	RSD%	Neohesperidin/ Hesperidin	RSD%	Melitidin	RSD%	Brutieridin	RSD%
Chinotto	3.01 ± 0.22	7.16					4.15 ± 0.58	13.89				
Orange 1	17.59 ± 1.55	8.83	2.94 ± 0.13	4.37			49.13 ± 6.15	12.52				
Orange 2	21.65 ± 0.59	2.71	4.73 ± 0.14	2.99			45.27 ± 3.96	8.75				
Tangerine	9.04 ± 0.90	9.92					14.47 ± 2.27	14.68				
Bergamot	129.11 ± 6.64	5.14			96.39 ± 4.44	4.61	94.75 ± 7.91	8.35	19.42 ± 2.96	15.24	41.37 ± 6.39	15.44
Lemon 1					27.62 ± 2.15	7.79	35.48 ± 5.56	15.66				
Lemon 2					14.36 ± 1.18	8.24	6.81 ± 0.95	13.95				
Grapefruite	282.06 ± 27.07	9.60										

Table 3.1.3. Amount of flavanones-*O*-glycosides found in analyzed samples

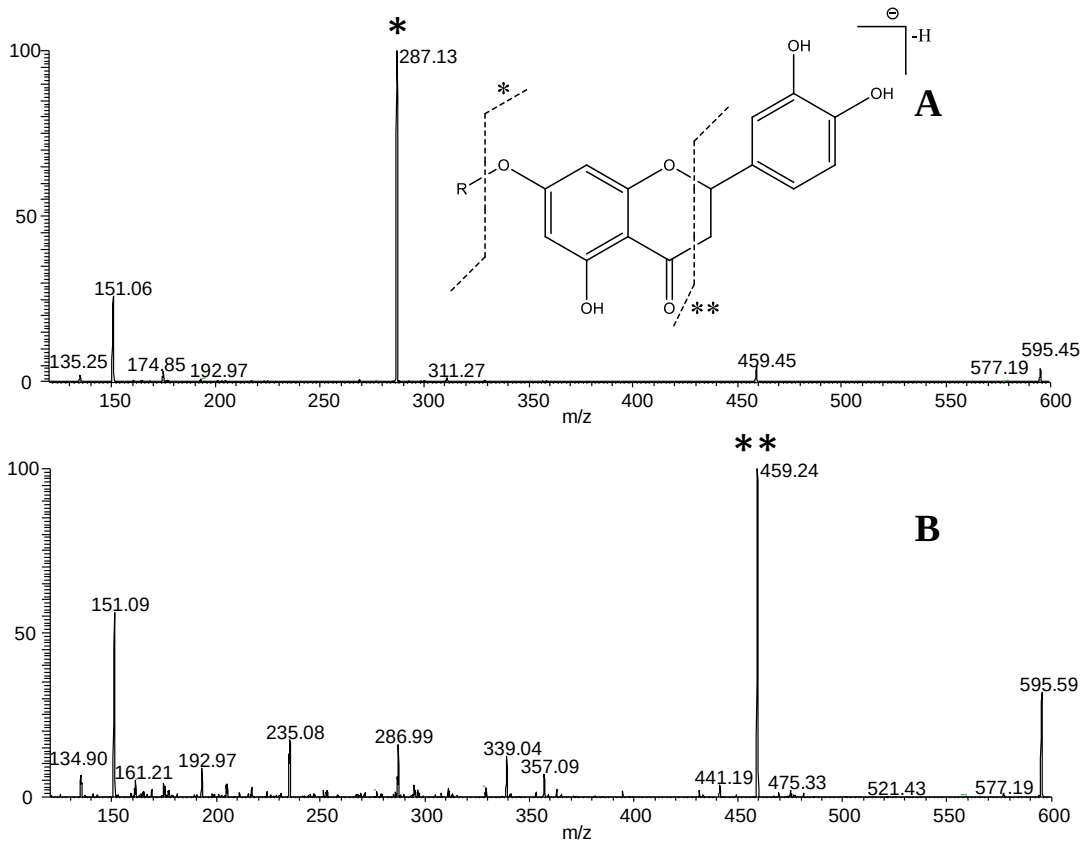


Figure 3.1.4. ESI (-) MS/MS of eriocitrin (A) and neoeriocitrin (B) acquired with a collision energy of 25 eV

Only for this compound, it was necessary to use eriocitrin as calibration standard, by monitoring the transition relative to the main fragmentation: from the deprotonated molecule ($[M-H]^-$) to the product ion at m/z 287.

The method reproducibility (RSD%) was checked on different days from independent analysis of the spiked sample and was always lower than 15%. The RSD% was also below 15% and was calculated by performing three instrumental analyses proving a goof repeatability (**Table 3.1.2**). The recovery test is not reported, because no extraction process was carried out, as the samples were centrifuged and appropriately diluted.

After the evaluation of accuracy, the proposed approach was tested on nine real samples: juices and citrus-based soda beverages. The sample preparation was very rapid and easy, in fact the sample is centrifuged for 3 minute and diluted 5 or 10 tomes with water, after adding the appropriate amount of internal standard. **Table 3.1.3** shows the amounts of flavonoids found in the investigated samples.

At last, to prove the reliability and the robustness of the developed PS-MS/MS methodology, the same samples were submitted to the more traditional but time-consuming HPLC-UV analysis. The values obtained are comparable with the data provided by PS-MRM determination, supporting the quality of the proposed methodology (**Table 3.1.4**).

Juice/Soda	Naringin/Narirutin		Poncirin/Neoponcirin		Neoeriocitrin/Eriocitrin		Neohesperidin/hesperidin		Melitidin		Brutieridin	
	PS-MS	LC-UV	PS-MS	LC-UV	PS-MS	LC-UV	PS-MS	LC-UV	PS-MS	LC-UV	PS-MS	LC-UV
Chinotto	3.01	5.16	-	-	-	-	4.15	5.30	-	-	-	-
Orange 1	17.59	20.94	2.94	3.21	-	-	49.13	54.66	-	-	-	-
Orange 2	21.65	18.62	4.73	4.07	-	-	45.27	41.58	-	-	-	-
Tangerine	9.04	8.41	-	-	-	-	14.47	12.94	-	-	-	-
Bergamot	129.11	120.98	-	-	96.39	92.14	94.75	88.35	19.42	18.72	41.37	38.10
Lemon 1	-	-	-	-	27.62	30.35	35.48	33.28	-	-	-	-
Lemon 2	-	-	-	-	14.36	10.97	6.81	6.87	-	-	-	-
Grapefruit	282.06	279.09	-	-	-	-	-	-	-	-	-	-

Table 3.1.4. Comparison of Flavanone glycosides amount (mg/L) found in the analyzed samples by PS-MS and HPLC-UV analysis.

4 Conclusions

A rapid methodology for the quantitative determination of the main flavanone glycosides has been developed. The assay is based on the paper spray ionization coupled with tandem mass spectrometry. The methodology is very fast and specific thanks to the employment of MRM scan mode. The consistency of this novel approach is highlighted by the good analytical parameter values and by the comparison with the classic HPLC-UV analysis. This novel approach could be used for the flavonoid determination in other vegetable matrices.

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Part II. Paper spray mass spectrometry for determination of flavanone-O-glycosides isomers in citrus juices

Abstract

A new methodology for the determination of flavanone-O-glucosides isomers in citrus juices has been developed. The methods overcome the main limitation faced by paper spray mass spectrometry that relies in the presence, in citrus juices, of both favanone-7-O-neohesperidoses and flavanone-7-O-rutinosides, compounds that are structural isomers and isobar. The method quality has been established through analytical parameters and comparison with the classic HPLC-MS/MS analysis.

1. Introduction

In the last years, the constant growth of the global population has led to an increasing demand for food and, therefore, the necessity to ensure food quality has attracted more and more public concern. Food quality concerns food authentication and adulteration, including ingredients such as lipids, proteins, carbohydrates, vitamins, and minor compounds, like the antioxidant ones, that possess nutraceutical benefits for human health. Moreover, food quality and authenticity have received wide attention from governments that have increased the number of legislations in this field (Lu et al., 2018; Wang et al., 2013). Consequently, the whole field of food science has experienced great growth. Due to its advantages of high sensitivity and selectivity, mass spectrometry is one of the most suitable techniques for the analysis of food and beverages (Wang et al., 2013). Traditionally, mass spectrometry is coupled with liquid or gas chromatography (LC-GC/MS). They allow to detect specific compounds in food samples, but most of the time, they are accompanied by time-consuming and tedious sample pre-treatment, such as extraction, enrichment, or separation. For this reason, the mentioned typical methodologies may not be suitable for the direct analysis of real food sample in contemporary food science (Lu et al., 2018). Alternatively, Ambient mass spectrometry (AMS), a subdiscipline of direct MS techniques introduced for the first time in 2004 by Cooks, attracts attention because it can be performed in the open air, so in the natural ambient of samples. Moreover,

most of the time, they require minimal or absent sample pre-treatments (Klampfl et al., 2018).

However, the specificity of mass spectrometry could be in question when more than one compound shows the same precursor ions and similar chromatographic properties, such as in the case of isomer analysis (Crotti et al., 2023). The interest in mass spectrometry for isomer characterization is constantly increasing. A cohesive example is the enantiomers. This type of isomers, most of the time, have identical physical and chemical properties, but they show different biological activities in chiral environments, such as in biological systems. This difference can be expressed in potency, antagonism or even toxic effects. Various methods have been applied to separate enantiomers and are categorized into classical and modern methods. The classical ones include the use of enzymatic degradation of one of the enantiomers or preferential crystallization of the enantiomers. Modern methods concern chromatographic and electrophoretic separation techniques (Awad & El-Anned, 2013).

Apart from this, some attempts have been made to resolve isomers only using mass spectrometry. One example is based on the reaction of an isomer with a mixture of isotopically labelled/unlabelled enantiomers to form complexes with mass differences. This is a very simple approach but cannot be used to perform quantitative analysis of isomers (Crotti et al., 2023; Sawada et al., 1998). After the introduction of ion mobility instruments, isomer resolution became possible due to the influence of molecular shape on drift times, but this technique requires an expensive instrumental system (Lapthorn et al., 2013). On the other hand, there are the MS/MS methods that are widely applied due to their high selectivity and dynamic range but show poor specificity in isomer distinction. For such reason, some attempts were made in order to resolve isomers by MS/MS. Generally, it is possible to use diagnostic fragments, but it is necessary that the molecules with the same mass have sufficient differences in the fragmentation routes. When dealing with isomers, although, they usually show the same product ions, limiting the application of this method in a mixture. In such cases, there exist alternative approaches like the use of MS^3 or the use of higher fragmentation energy in order to establish secondary fragmentation pathways and to obtain more structural information.

Another important approach is based on the assumption that a mixture of compounds with the same fragmentation pattern will generate MS/MS spectra that are a combination of fragments from each component summed in proportions to their relative concentration (Crotti et al., 2023). In this way, it is possible to calculate the concentration of the isomers in unknown samples using an algorithm such as the linear deconvolution analysis (LEDA) (Menicatti et al., 2020).

In the present work, a similar method, based on the combined MS/MS spectrum obtained for a couple of isomers, has been developed to perform the direct quantification of flavanone-O-glycosides isomers in citrus juices by paper spray mass spectrometry.

Paper spray mass spectrometry is one of the simplest ambient mass spectrometry techniques. The sample is loaded on a triangular piece of paper placed in front of the mass spectrometer inlet, and when a high voltage is applied through a metallic clip, the compound ionization is performed. The technique has already been used for quantification purposes on foodstuff (Bartella et al., 2019a; Bartella et al., 2019b; Chen et al., 2017; Li et al., 2013) and, in particular, on citrus juices where both favanone-7-O-neohesperidoses and flavanone-7-O-rutinosides are present. These compounds are structural isomers and isobars and have already been quantified as the sum of the two structural isomers through PS-MS/MS experiments (Mazzotti et al., 2021).

2. Experimental

2.1 Chemicals

Solvents (HPLC grade) were commercially available (Sigma-Aldrich, St. Louis, MO). Flavanones-O-glycosides standards were purchased from Extrasynthese (Genay Cedex, France).

2.2 Sample preparation

The samples used to develop the methodology were five citrus beverages, in particular an orange juice, an orange (40%) – tangerine (40%) juice, a bergamot juice, a tangerine (70%) – bergamot (30%) juice, a grapefruit juice, and a coca cola soft

drink, used as blank matrix. All the samples were purchased in local stores and were stored at 4 °C. Before the PS-MS/MS analysis, each sample was centrifugated at 1200 rpm for three minutes and then diluted 10 times. The same sample preparation was performed before the HPLC-MS/MS analysis, but in this case, the caffeic acid was added as an internal standard at a concentration of 100 µg/L, and different dilutions were performed. In particular, the orange juice and the grapefruit were diluted 100 times, the orange-tangerine juices and the bergamot juice were diluted 1000 times, and the lemon juice and the tangerine-bergamot juice were diluted 500 times.

2.3 PS-MS analysis

The MS analyses were carried out with a TSQ Quantum Vantage (Thermo Fisher Scientific, San José, CA) triple-stage quadrupole mass spectrometer equipped with a homemade paper spray ionization source. Qualitative Whatman filter paper n°1 (pore size 11 µm, thickness 180 µm) was used to perform the experiments. The sample spotting volume was 15 µl, and once dried, the paper triangle was wetted with the same volume of methanol to allow the desorption of the ions. The working conditions were the following: voltage 5.0 kV, vaporizer and capillary temperatures were respectively 280°C and 290 °C. The collision gas applied was argon at a pressure in the collision cell (Q2) of 1.5 mTorr, and the mass resolution at the first (Q1) and third (Q3) quadrupoles was set a 0.7 Da at full width at half-maximum. The scan time was set a 0.4 s and the number of micro scans at 2. The collision energy (CE) was optimized per compound and set at 25 eV for all three isomer couples, while the S-lens values were set at 160 V for each analyte. The analyses were performed by using the multiple reaction monitoring (MRM) scan mode, following specific gas phase transitions from the deprotonated precursor ion $[M-H]^-$ as shown in **Table 3.2.1**.

2.4 HPLC-MS/MS analysis

The HPLC-MS/MS analyses were performed using a Thermo Scientific UHPLC instrument coupled to a TSQ Quantum Vantage (Thermo Fisher Scientific, San José, CA) triple-stage quadrupole mass spectrometer. The chromatographic separation was achieved using a C_{18} reversed-phase analytical column (2.1 x 100 mm, 3 µm). The sample injection volume was 5 µl, while the flow rate was set at 0.3 mL/min, using as

elution solvents H_2O, 0.1% HCOOH (solvent A) and Methanol (solvent C) under gradient conditions. The gradients steps were the following: 95% A in isocratic for 1.5 min, from 95 to 70% A (1.5 – 3.5 min), 70% A isocratic for 1 min, from 70% to 60 % A (4.5 – 8 min), 60% A isocratic for 1 min, from 60% to 40% A (9 – 11 min), 40% A isocratic for 1 min, from 40% to 30% A (12 – 14 min), 30% A isocratic for 1 min, from 30% to 20% A (15 – 16 min), 20% A isocratic for 2 min, from 20% to 95% A (18 – 20 min), and then an isocratic flow for 6 min to equilibrate the system before starting a new analysis. The total run time was 26 min.

The electrospray ionization MS/MS analysis was performed on a triple quadrupole mass analyser with an ESI source operating in negative ion mode. The instrument conditions were the following: spray voltage, 4 kV, vaporizer and capillary temperature, 270 °C, and sheath and auxiliary gas at 40. For the MS/MS experiment, the parameters are the same as reported in section 2.3 for the PS-MS analyses. The concentration of flavone glucosides was evaluated using an external calibration curve at a concentration ranging from 25 to 400 µg/L with an internal standard of 100 µg/L.

Compound	Transition	CE (eV)	S-Lens (V)
Naringin/Narirutin	*m/z* 579 → *m/z* 151	25	160
	m/z 579 → *m/z* 271	25	
	m/z 579 → *m/z* 459	25	
Neoeriocitrin/Eriocitrin	*m/z* 595 → *m/z* 287	25	160
	m/z 595 → *m/z* 459	25	
Neohesperidin/Hesperidin	*m/z* 609 → *m/z* 301	25	160
	m/z 609 → *m/z* 325	25	
	m/z 609 → *m/z* 489	25	160

Table 3.2.1. Selected gas-phase transitions and optimized instrumental parameters.

2.4 Matrix effect evaluation

The matrix effect was evaluated using the following equation:

$$ME\ (\%) = \frac{B}{A} \times 100$$

where A is the peak area of the analytes obtained from the standard solution, and B is the peak area of the analytes obtained from the blank matrix spiked with the target compounds (Matuszewski et al., 2003).

2.5 Data analysis

The results obtained from PS-MS/MS and HPLC-MS/MS analyses were subjected to statistical analysis using Microsoft Excel's Data Analysis according to Student's *t-test* ($\alpha = 0.05$). The test was performed to verify the percentage quantification power of the paper spray calibration curves for a couple of flavanone-O-glucoside isomers and to verify the effective comparability between the data given by the two different instrumental analyses.

3. Results and discussion

The main limitation of the methodology for the quantification of flavanone-O-glycosides by paper spray mass spectrometry relies on the presence, in citrus juices, of both favanone-7-O-neohesperidoses and flavanone-7-O-rutinosides. In particular, neoeriocitrin and eriocitrin, naringin and narirutin, and neohesperidin and hesperidin. These compounds are structural isomers and isobars, and due to the absence of a chromatographic step and because the gas-phase transitions are the same for both precursor ions, they can only be quantified as the sum of the two structural isomers in PS-MS/MS experiments. As reported by Mazzotti et al., 2021, when evaluating the quantification accuracy for rutinosides using neohesperidosides calibration curves, it came to attention a difference in the relative abundance of product ions in the MS/MS spectrum of neoeriocitrin and eriocitrin.

This work aims to take advantage of this difference in the isomers MS/MS spectrum and develop a new methodology that allows percentage determination of flavanone-O-glucosides isomers directly with paper spray mass spectrometry. Considering the couple neoeriocitrin and eriocitrin, the difference in the MS/MS spectrum is very clear. As shown in **Figure 3.2.1**, the base peak completely changes between the two isomers. For neoeriocitrin, the base peak corresponds to *m/z* 459, which probably results from a breakage of the C ring, while for eriocitrin, the base peak is the *m/z* 287 corresponds to the formation of the deprotonated aglycon.

In the other couples of isomers, the difference is not as pronounced as in the last case. The base peak remains the same for the two forms, *m/z* 271 for the couple narigin - narirutin and *m/z* 301 for the couple hesperidin – neohesperidin. What changes is the relative abundance of the other peaks. In the case of naringin and

narirutin, the abundance of the peak at *m/z* 459 greatly decreases in the rutinoside isomer. For neohesperidin and hesperidin, the peak at *m/z* 325 increases its abundance for hesperidin, while the *m/z* at 489 completely disappears.

The percentage quantitative methodology was based on the intensity change of the previously highlighted gas-phase transitions. Considering naringin and narirutin, the calibration curve was built using the ratio between the intensity of the phase-gas transition *m/z* 579 → *m/z* 459 over the sum of the total intensity of all transitions, including the previous one. The same approach was applied for neoeriocitrin and eriocitrin, considering as the main gas-phase transition the *m/z* 595 → *m/z* 459. In the case of neohesperidin and hesperidin, the transition *m/z* 609 → *m/z* 489 was discarded due to its complete disappearance for hesperidin, and the calibration curve was build considering the ratio between the intensity of the transition *m/z* 609 → *m/z* 325 over the sum of the intensity of the previous transition plus the transition *m/z* 609 → *m/z* 301. For each couple of isomers, the calibration curve was built analysing six standard solutions, 100 - 0%, 80 - 20%, 60 – 40%, 40 – 60%, 20 – 80% and 0 – 100% with a total concentration of 10 µg/L, where the first percentage refers to the neohesperidoside isomer.

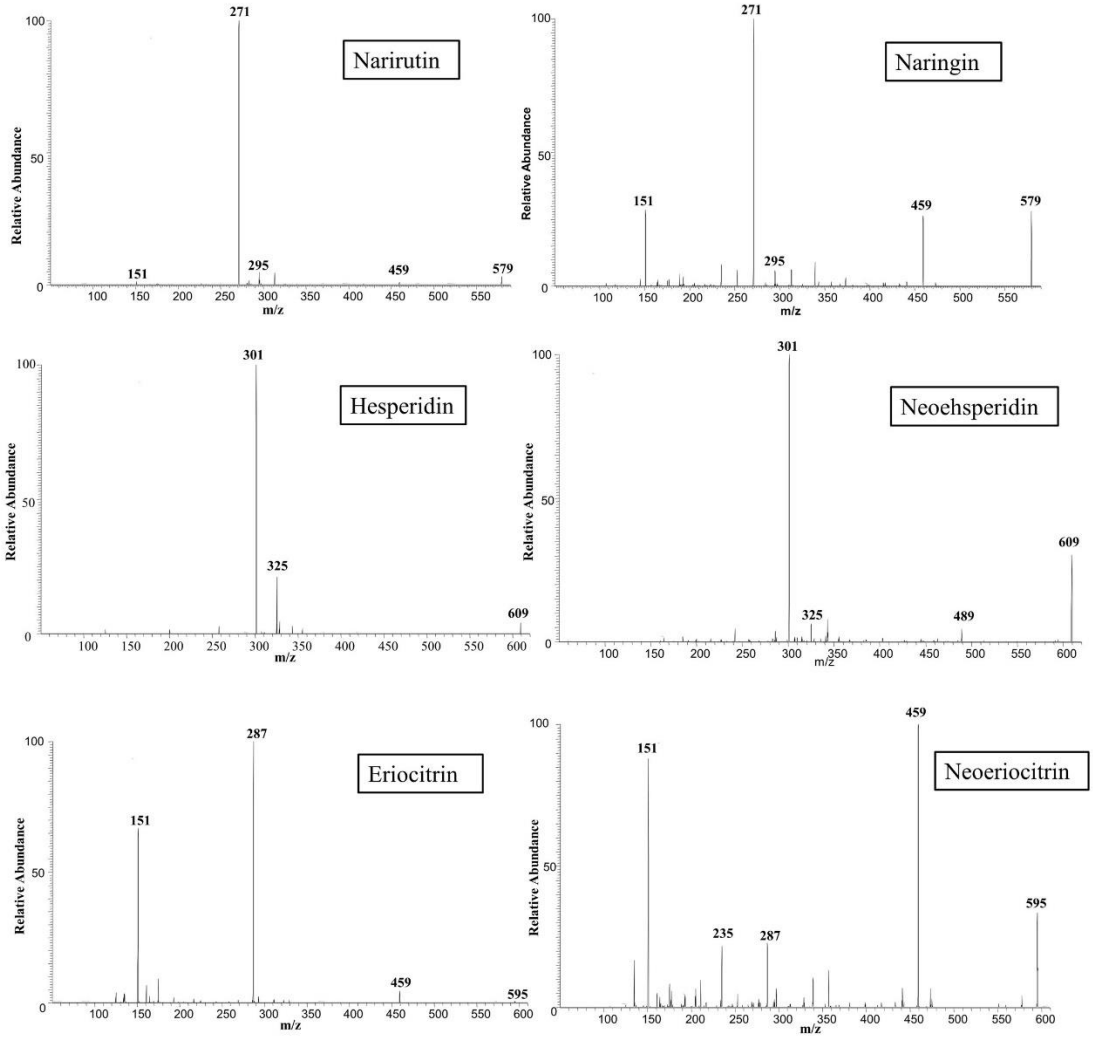

Figure 3.2.1. PS (-) MS/MS of analysed isomers acquired with a collision energy of 25 eV.

In the first attempt, the standard solutions for the calibration curve were made in water, and each solution was submitted to PS-MS/MS MRM analysis in triplicate. The method showed good linearity with correlation coefficient values higher than 0.98 for each couple of isomers. The accuracy of the calibration curves was determined analysing some spiked black samples obtained by adding the right amounts of standards to a diluted cola soda beverage used as blank. It was evaluated using a total concentration of 10 µg/L at 80% of neohesperidosides and 20% of rutinosides. The cola soda beverage was prepared at four different dilutions: 20 times (S1), 10 times (S2), 5 times (S3) and 2 times (S4). **Table 3.2.2** shows the accuracy values for the examined solutions.

The accuracy values for the couple neoeriocitrin/eriocitrin do not change with the increasing presence of the matrix; it ranged from 96% to 101%. Otherwise, for neohesperidin and hesperidin, the accuracy decreases at 85% when the soda cola beverage is diluted only two times, but it is still acceptable. The main problem is for naringin and narirutin, where the accuracy drastically falls when the matrix gets less diluted, reaching 68% when diluted 5 times.

There is an evident matrix effect for these compounds that was evaluated using the spiked sample diluted 10 times and the equation in *Section 2.4*. The calculate values were 81% for the couple naringin/narirutin, 102% for neoeriocitrin/eriocitrin and 100% for neohesperidin/hesperidin, confirming the presence of a matrix effect for naringin and narirutin.

For this reason, in the second attempt, the standard solutions for the calibration curve were prepared using a blank matrix a cola soda beverage diluted 10 times spiked with the right amount of the compounds. The method reported, also in this case, a good linearity with correlation coefficient values higher than 0.99 for each couple of isomers (**Figure 3.2.2**).

The methodology was then applied to fortified blank samples to evaluate accuracy and reproducibility. Because the curves are already built using cola soda beverage as the blank matrix, a different matrix was used. Specifically, a bergamot juice was passed through a C_{18} cartridge to remove the compounds of interest, and the non-adsorbed part was used as a blank matrix. The accuracy was estimated analysing six spiked blank samples prepared at the edges of the percentage calibration curves. In particular, 100 – 0%, 90 – 10%, 10 – 90%, 0 – 100% and also two intermediate points, 95 – 5% and 5 – 95%, the first percentage refers to the concentration of the neohesperidosides isomers.

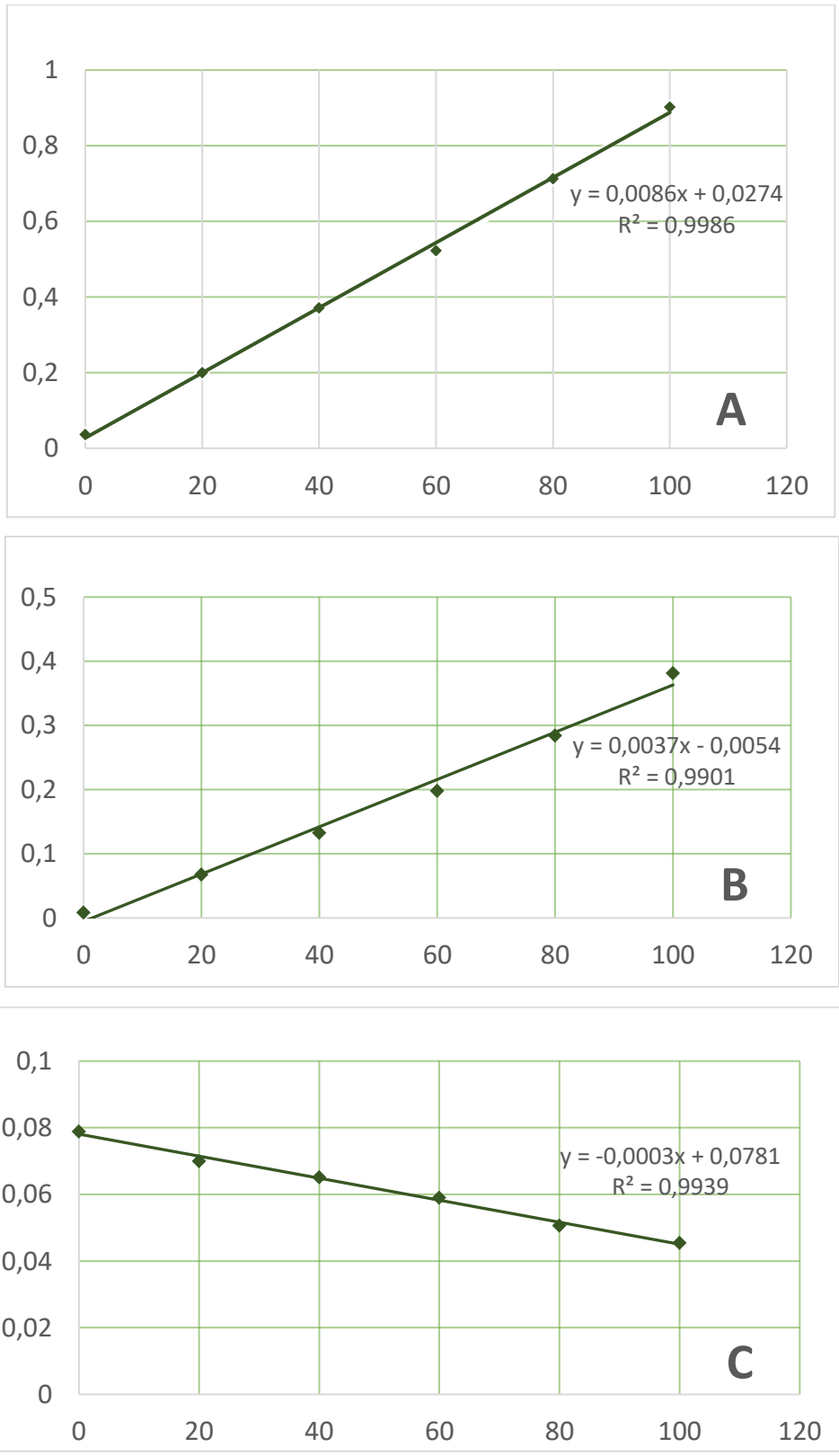

Figure 3.2.2. Calibration curves of (A) neoeriocitrin/eriocitrin, (B) naringin/narirutine and (C) neohesperidin/hesperidin.

	Naringin/ Narirutin			Neoeriocitrin/ Eriocitrin			Neohesperidin/ Esperidin		
Total concentration of 10 µg/L	Concentration %	RSD%	Accuracy	Concentration %	RSD%	Accuracy	Concentration %	RSD%	Accuracy
Spiked (S1) 20 times	77.34 ± 1.27	1.7	97	78.59 ± 0.91	0.1	98	73.85 ± 7.7	10.4	92
Spiked (S2) 10 times	67.75 ± 1.44	2.1	85	80.54 ± 0.64	0.8	101	73.90 ± 1.91	2.6	92
Spiked (S3) 5 times	54.40 ± 1.52	2.8	68	78.75 ± 1.38	1.8	98	72.79 ± 3.24	4.5	91
Spiked (S4) 2 times	-	-	-	77.11 ± 0.62	0.8	96	67.94 ± 7.32	10.7	85

Table 3.2.2. Analytical parameter of accuracy for calibration curves in water.

	Naringin/ Narirutin			Neoeriocitrin/ Eriocitrin			Neohesperidin/ Hesperidin		
Total concentration of 10 µg/L	Concentration %	RSD%	Accuracy	Concentration %	RSD%	Accuracy	Concentration %	RSD%	Accuracy
Spiked 100 – 0%	104.88 ± 3.22	3.1	104	101.56 ± 0.40	0.4	102	110.57 ± 9.59	8.7	111
Spiked 90 – 10%	90.78 ± 2.87	3.2	101	91.777 ± 0.18	0.2	102	89.77 ± 1.29	1.4	100
Spiked 0 – 100 %	4.8 ± 0.35	2.8	96	1.67 ± 0.16	9.8	98	-15.98 ± 3.62	-22.7	116
Spiked (S4) 10 – 90%	13.93 ± 0.31	2.24	96	11.85 ± 0.81	6.8	98	12.99 ± 2.29	17.7	97
Spiked 95 – 5 %	97.47 ± 2.65	3.10	102	95.21 ± 1.94	2.0	100	89.28 ± 4.87	5.45	94
Spiked 5 – 95 %	8.78 ± 0.74	8.42	96	6.18 ± 0.49	7.9	99	22.60 ± 6.19	27.4	82

Table 3.2.3. Evaluation of the analytical parameter of accuracy.

For what concern the couples neoeriocitrin/eriocitrin and naringin/narirutin, the accuracy values ranged for all the spiked samples from 96% to 104%, as shown in **Table 3.2.3**, while slightly different is the situation for neohesperidin/hesesperidin. First, they show overall higher values of RSD% that can be related to the lower slope of the calibration curve if compared with the other two couples of isomers. Then, the accuracy falls to 82% for the spiked sample at 5 – 95%. Considering these two factors, some t-tests (α = 0.05) were performed to highlight if there is real difference between the point 100 – 0%, 95 – 5%, 90 – 10%, 10 – 90%, 5 – 95%, and 0 – 100 % for all the compounds. The results presented p value ≤ 0.05 for neoeriocitrin/eriocitrin, indicating that there is a significant difference between the points. For the couples neohesperidin/hesperidin and naringin/narirutin, the t-tests gave p value ≥ 0.05 when the points differ only by 5%, indicating that there is no significant difference between them. Therefore, the methodology does not allow to discern between samples that differ for less than 10% in the content of neohesperidin and hesperidin, and naringin and narirutin.

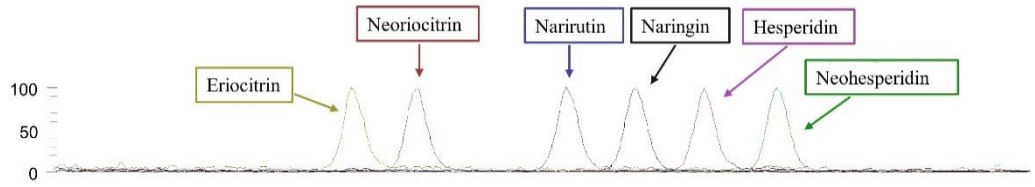

Figure 3.2.2. HPLC-MS/MS chromatogram of favanone-7-O-neohesperidoses and flavanone-7-O-rutinosides standard solutions at a concentration of 100 mg/L.

After the evaluation of the accuracy, the methodology was applied to five real citrus juices as illustrated in *Section 2.2,* and the results are shown in **Table 3.2.4**, including the results obtained with the reference HPLC-MS/MS analysis **(Figure 3.2.2**). To test the robustness and reliability of the developed method, a *t-test* (α = 0) was performed between the PS-MS/MS and the HPLC-MS/MS data for the tangerine-bergamot juice that contains all the six flavanone-O-glucosides analysed. The test gave p value ≥ 0.05 for all the analytes, indicating the absence of significant difference between the data and, therefore, the quality of the developed methodology.

	Naringin		Narirutin		Neoeriocitrin		Eriocitrin		Neohesperidin		Hesperidin	
Juice	PS-MS/MS	HPLC-MS/MS	PS-MS/MS	HPLC-MS/MS	PS-MS/MS	HPLC-MS/MS	PS-MS/MS	HPLC-MS/MS	PS-MS/MS	HPLC-MS/MS	PS-MS/MS	HPLC-MS/MS
Orange			94.05 ± 0.45	100							102.53 ± 1.79	100
Orange - tangerine			95.39 ± 2.38	100							94.10 ± 4.30	100
Bergamot	103.14± 2.13	100			97.65 ± 0.46	100			97.24 ± 1.65	100		
Tangerine - bergamot	75.21 ± 3.67	76.06 ± 0.32	24,06 ± 3.04	23.94 ± 0.32	85.49 ± 2.89	84.47 ± 0.24	14.23 ± 2.67	15.78 ± 0.23	17.78 ± 6.94	13.07 ± 0.39	82.23 ± 6.94	86.93 ± 0.40
Grapefruit	123 ± 4.32	100										

Table 3.2.4. Percentage amount of flavanone-O-glucosides (mg/L) found in the analysed sample by PS-MS/MS and HPLC-MS/MS

4. Conclusion

A new and very fast methodology for the percentage determination of flavanone-O-glucosides has been developed. The approach is based on paper spray mass spectrometry and overcomes its limitations related to the absence of a chromatographic step when facing the presence of structural isomers. The method quality has been established through analytical parameters and comparison with the classic HPLC-MS/MS analysis.

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Part III.

Paper spray tandem mass spectrometry for assessing oleic, linoleic and linolenic acid content in edible vegetable oils.

separations

MDPI

Article

Paper Spray Tandem Mass Spectrometry for Assessing Oleic, Linoleic and Linolenic Acid Content in Edible Vegetable Oils

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Abstract: Oleic, linoleic and linolenic acids exert several beneficial effects on human health, some of which are also certified by recent European and U.S. regulations. The goal of the presented work was to develop an innovative methodology to evaluate their content in edible vegetable oils, in order to increase the value of oils from a nutraceutical perspective. The protocol is based on the use of paper spray ionization coupled with tandem mass spectrometry experiments, which allowed the recording of data very quickly and with high specificity. All investigated compounds gained a good linear relation (r^2 higher than 0.98). Accuracy values are near 100% for all concentration levels examined, and the repeatability and reproducibility data result lower than 15%, highlighting the consistence of the methodology. The developed approach was successfully applied for the analysis of different real samples, and its robustness was confirmed by comparing the results obtained with those coming from the classical and official methodology.

Keywords: fatty acid methyl esters; vegetable oils; paper spray ionization; tandem mass spectrometry

1. Introduction

Vegetable oils represent an important resource for human diet not only as daily energy intake but also for the presence of nutraceuticals compounds that have beneficial effects on health. They are composed in large amount by triacylglycerols (TAGs), molecules obtained from the esterification of a molecule of glycerol and three fatty acids. Then, there are also diacylglycerols and monoacylglycerols, phospholipids, free sterols, tocopherols, tocotrienols, triterpene alcohols, hydrocarbons, and fat-soluble vitamin in very small amounts. (Guidoni et al., 2019, Dubois et al., 2007, Orsonova et al., 2015) A fatty acid is a hydrocarbon chain that can be saturated or not, with a methyl group at one end, and a carboxylic function at the other. A saturated fatty acid, therefore, possess a structure with a fully saturated hydrocarbon chain, while an unsaturated one possesses one or more double bond along the chain, and they are classified as monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). (Dubois et al., 2007). The chain length and the degree of unsaturation may have great influence on the chemical and biological properties of these compounds. (Guidoni et al., 2019). The polyunsaturated fatty acids are also called essential fatty acids because they are crucial for the body's function and need to be introduced externally through the diet (Das et al., 2012). The main families are the ω-3 that results from α-linolenic acid (ALA, 18:3) and the ω-6 that comes from linoleic acid (LA, 18:2) (Das, 2006). Such fatty acids make up the cellular membranes and help maintain the homeostasis providing a correct functionality for proteins. Generally, the polyunsaturated fatty acids are considered beneficial for human health, actually the ω-3 and ω-6 families can have opposite effects on the human metabolism. A diet rich in ω-6 relates to inflammatory processes, obstruction of blood vessels and platelets aggregation. On one hand, acute inflammatory responses can protect the body from infections and wounds, but on the other hand, an excessive stimulation of the inflammatory system could produce a favourable environment for the growth of tumours. Indeed, a persistent inflammatory condition is linked to the development of tumours and metastases. Equally, a chronic inflammation led to atherosclerosis with the outbreak of cardiovascular diseases (Saini et al., 2018).

The relation between the consumption of ω-3 and ω-6 has changed during time. Nowadays, the industrialized societies are characterized by a higher consumption of saturated fatty acids, ω-6 and trans fatty acids, with a related decreased intake of ω-3. The fatty acids now represent the 28-42% of the total energy for European populations, while, in the past it was approximately only the 20-30%. The increase in the consumption of vegetable oil rich in linoleic acid has led to a higher assumption of ω-6 against the ω-3 one. An optimal diet requires a ratio of 1-4:1 between ω-6 and ω-3 intake, but nowadays this ratio has increased up to 10:1 or 20:1. Concurrently, there has been an increase in the incidence of diseases involving inflammatory processes, such as cardiovascular problems, diabetes, obesity, rheumatoid arthritis, and cancer (Patterson et al., 2012).

The high intake of ω-6 increases the plasma concentration of its metabolic products that contribute to the formation of thrombus and atheroma in blood vessels, to the development of inflammatory diseases and allergies and excessive cell proliferation. The ω-3, instead, counteract these deleterious effects, determining the production of compounds which have vasodilatory activity and have strong anti-inflammatory activity (Yashodhara et al., 2009).

In general, several studies have reported that the appropriate intake of PUFAs may prevent cardiovascular and inflammatory diseases. (Kris-Etherton & Su, 1997, Abeywardena & Head, 2001; Shapiro, 2003).

In addition, for both fatty acids there exits specific health claims emanated from the European Food Safety Authority (EFSA) whose purpose is the evaluation of the scientific base of the claim's affirmations. For that reason, the EFSA can determine if such claims can be applied on an industrial and commercial level, because they play an important role for what concern the consumer orientation toward a particular type of product instead of another one. Indeed, the consumer is very. susceptible to all the information concerning health and, therefore, the food industries are really interested in using all the possible health claims in their advertising campaign (Picchieri et al., 2020).

There exist different kind of health claims: *Function Health Claims, Risk Reduction Claims and Claims referring to children's development*. The first ones concern the role that specific nutrients play in the development and preservation of human body. The

second ones specify which nutrients are capable of determine a risk reduction for some diseases. In the end, the last ones are related to the optimal growth and development of children.

For oils and, in particular, olive oil, there four health claims proposed by EFSA and certified by the regulation CE 432/2012. Three out of this four are *Function Health Claims*:

- The polyphenols in olive oil contribute to the protection of the hematic lipids from oxidative stress.
- ***Source of vitamin E***. The vitamin E contributes to the protection of cells from oxidative stress.
- ***Rich in unsaturated fatty acids***. The substitution in the diet of saturated fatty acids with unsaturated ones contributes to maintain the normal levels of cholesterol in blood.

The fourth and last claim, instead, is of the type *Risk Reduction Claims*:

- ***Rich in unsaturated fatty acids***. It has been demonstrated that the substitution in the diet of saturated fatty acids with unsaturated fatty acids, decrease the cholesterol in blood. Cholesterol is a high factor related to the development of coronary diseases.

Among these claims the only one that is specific only for olive oil is the first one, the remaining can be applied also to other foods and, in particular, to other types of oil.

Indeed, the claim that says *Rich in unsaturated fatty acids* concern the content of oleic, linoleic and linolenic acid.

In particular, for the linoleic acid, the related claim can be found in the European Regulation n°432/2012, and it says that *the linoleic acid contributes to maintain the normal levels of cholesterol in blood. This health claim ca be employed only for a food than contains at least 1.5g of linoleic acid (AL) per 100g and per 100 Kcal. The consumer should be informed that the beneficial effect can be obtained with the assumption of 10g of AL per day*.

For what concern the linolenic acid, the claim is present in the European Regulation n°432/2021 and it says *that the ALA or α-linolenic acid contributes to the maintenance of the normal levels of cholesterol in blood. This claim can be employed only for a food*

that is source of α-linolenic acid, as specified in the claim Source of omega-3 fatty acids that can be found in the CE n°1924/2006. The consumer should be informed that the beneficial effect is reached with the assumption of 2d of α-linolenic acid per day.

The information regarding the *Source of omega-3 fatty acids* says that *a food is a source of omega-3 fatty acids if it contains at least 0.3g of α-linolenic acid per 100g and per 100 kcal or at least 40mg of the sum between eicosapentaenoic acid and docosahexaenoic acid per 100g and 100kcal.*

In the end, there is the case of oleic acid that doesn't possess a specific claim in the European regulation. Indeed, the CE regulation 432/2012 refers to oleic acid affirming that *the substitution in the diet of saturated fatty acids with unsaturated ones contributes to maintain the normal levels of cholesterol in blood. The oleic acid is an unsaturated fatty acid. This claim can be used only for a food with a high content of unsaturated fatty acids as specify in the claim Rich in unsaturated fatty acids that can be found in the CE regulation n°1924/2006*.

A food can be considered rich in unsaturated fatty acids only if at least the 70% of fatty acids present in the food are unsaturated and that they determine more than 20% of the total energetic value of the product.

As can be seen, this claim does not refer exclusively to oleic acid, but instead to all the unsaturated. The situation is slightly different outside Europe. For example, in the United States, the FDA or Food and Drug Administration considered a claim specific for oleic acid. It asserts that "Daily consumption of edible oil with at least 10 grams of oleic acid per serving (one tablespoon) reduces the risk of coronary heart disease. To achieve this benefit, oleic acid containing oils with at least 10 grams of oleic acid per serving should replace a similar amount of saturated fat and not increase the total number of calories you eat in a day. One serving of [x] oil provides [x] grams of oleic acid (which is [x] grams of monounsaturated fatty acid)".

Although there is evidence that the intake of oleic acids coming from olive oil and sunflower oil reduces the risk of coronary diseases, the FDA affirmed that the claim doesn't have the necessary characteristics to become an authorized FDA claim. For that reason, the FDA suggest other claims that can be used in substitution: "Supportive but not conclusive scientific evidence suggests that daily consumption of about 1½ tablespoons (20 grams) of oils containing high levels of oleic acid, when

used to replace for fats and oils higher in saturated fat, may reduce the risk of coronary heart disease. To achieve this possible benefit, oleic acid-containing oils should not increase the total number of calories you eat in a day. One serving of [x] oil provides [x] grams of oleic acid (which is [x] grams of monounsaturated fatty acid).” “Supportive but not conclusive scientific evidence suggests that daily consumption of about 1½ tablespoons (20 grams) of oils containing high levels of oleic acid, may reduce the risk of coronary heart disease. To achieve this possible benefit, oleic acid containing oils should replace fats and oils higher in saturated fat and not increase 29 the total number of calories you eat in a day. One serving of [x] oil provides [x] grams of oleic acid (which is [x] grams of monounsaturated fatty acid)” In short, the claim affirms that the intake of 1½ spoon, around 20g, of oil with high levels of oleic acid, has the power to reduce the incidence of coronary diseases. However, the scientific base is not yet definitive.

To develop a new method for the rapid analysis of these three fatty acids it is important to have an idea of which type of oils contain them the most and also which are the common analytical methods used for their quantification.

In **Figure 3.3.1** there is a diagram showing the fatty acids composition of some of the most used oils. Indeed, each oil has a particular distribution of fatty acids that depends on the original plant. For that reason, each oil has a different effect on human health, it can be beneficial but also dangerous and causes serious illnesses.

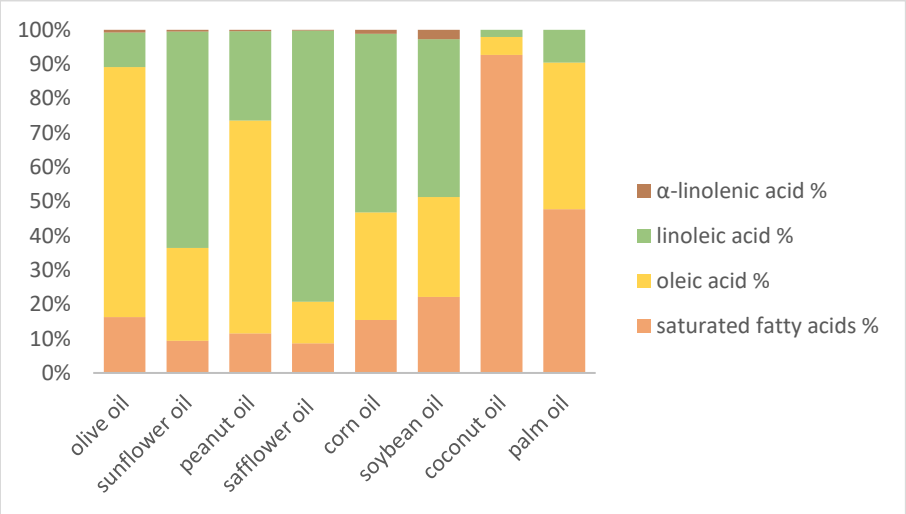

Figure 3.3.1. Summary of the fatty acids composition in different oils.

For example, the coconut and palm oil are characterized for the majority of saturated fatty acids. Olive oil, instead, has over the 70% of oleic acid, while sunflower,

safflower and corn oil possess the highest amount of linoleic acid, respectively around 63%, 77% and 52%. In the end, the oils with the major content of α-linolenic acid are corn and soybean oil, it is about 1% for corn oil and 3% for soybean oil. (Orsonova et al., 2015, Alves et al., 2019, Dubois et al., 2007, Yang et al., 2012).

The method usually used for the analysis of fatty acids, as methyl esters, is based on gas chromatography with a flame ionization detector (GC-FID). Before performing the analysis, the sample is pre-treated with a transesterification reaction using KOH in methanol. (Shantha, 1992; Seppanen-Laakso et al., 2002; Elagbar et al., 2016)

Apart from GC-FID, other techniques applied for the analysis of fatty acids are GC-MS and GC with vacuum ultraviolet as detectors. (He et al, 2018, Fan et al, 2016) and also UHPLC-MS (Wabaidur, et al, 2016).

All these methods apply chromatography, so the current study aimed to find a new methodology for fast and accurate quantification of the total oleic, linoleic, and linolenic acid in vegetable oils typically consumed in our daily diet, using paper spray mass spectrometry (PS-MS). Paper spray mass spectrometry is one of the simplest ambient mass spectrometry techniques. The sample is loaded on a triangular piece of paper placed in front of the mass spectrometer inlet, and applying a high voltage (4-5 kV) through a metallic clip, the compound ionization is performed. This methodology has already been employed for the quantification of bioactive compounds in different matrices (Bartella, 2020; Chiang et al., 2018; Frey et al., 2020) and on foodstuff (Bartella et al., 2019a; Bartella et al., 2019b; Chen et al., 2017, Li et al., 2013). In this case multiple reaction monitoring (MRM) is used as a scanning mode to quantify the fatty acids, with the aid of erucic acid methyl ester as an internal standard. The sample preparation is based on a transesterification reaction which provides the methyl esters (**Figure 3.3.2**), directly analyzed via PS-MSMS. The entire experiment is very fast; in fact, after the transesterification reaction, the internal standard is added to the mixture, properly diluted, and directly submitted to the PS-MSMS analysis, whose scanning time is two minutes. Furthermore, to demonstrate the robustness of the methodology, the values obtained from the analysis of the investigated edible oils have been compared with those obtained by the application of the official EU method.

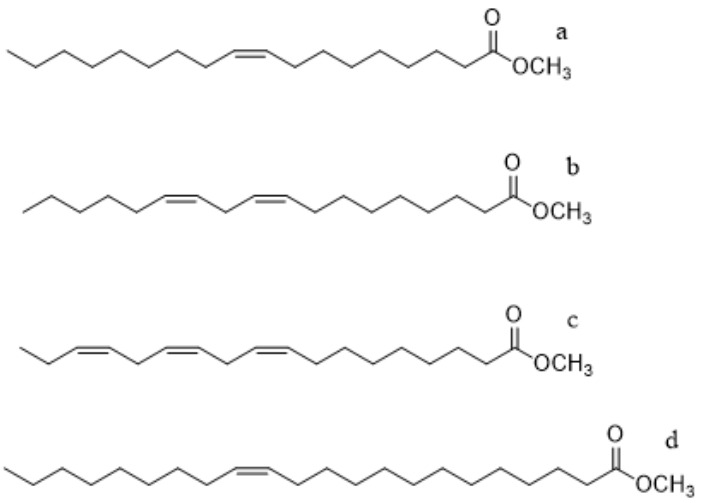

Figure 3.3.2. Chemical structured of oleic acid methyl ester (**a**), linoleic acid methyl ester (**b**), linolenic acid methyl ester (**c**) and internal standard (**d**).

2. Experimental

2.1 Chemicals

All solvents used were HPLC grade and commercially available from Sigma-Aldrich (St. Louis, MO, USA). Pure compounds, methyl oleate (methyl cis-9-octadecenoate), methyl linoleate (methyl cis,cis-9,12-octadecadienoate), methyl linolenate (methyl cis,cis,cis-9,12,15- octadecatrienoate), methyl erucate (methyl cis-13-docosenoate), triolein (oleic acid triglyceride) and trilinolein (linoleic acid triglyceride) were also purchased from Sigma-Aldrich.

2.2 Sample preparation

Each sample was submitted to a transesterification reaction following the official procedure of the European Union 2015/1833, Annex X. Essentially, 50mg of each oil were weighed and then were added 50 µl of potassium hydroxide 2M and 2 ml of *n*-hexane. The solution was shaken for ca. 30 seconds and allowed to stratify. To perform the PS-MS/MS analysis, the supernatant was properly diluted with *n*-hexane and the internal standard was added. The olive oil samples were diluted 1:100 for the quantification of methyl oleate, while for methyl linoleate and linolenate, they were diluted respectively 1:200 and 1:10. The seeds oil samples were diluted 1:1000 for methyl oleate and linoleate and 1:100 for the quantification of methyl linolenate.

2.3 Paper Spray Mass Spectrometry

PS-MS determinations were carried out in positive ion mode by using a TSQ Quantum Vantage (Thermo Fisher Scientific, San José, CA, USA) triple-stage quadrupole mass spectrometer coupled with an in-house implemented paper spray source. The paper spray source is composed of a triangular piece of paper (qualitative Whatman filter paper n ◦ 1) on which 15 µL of sample are loaded and put in front of the triple quadrupole inlet using a metallic clip. After letting it dry for about a minute, a high voltage was applied to the triangle through the clump, and 15 µL of methanol was added every 30 s to permit the spray desorption. The total scan time was 2 min. The MS working conditions were set as follows: applied voltage +5000 V, vaporizer temperature 280 °C and capillary temperature 290 °C. The gas used for CID experiments was argon, with a pressure in the collision cell (Q2) of 1.5 mTorr. Mass resolution at the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da at full width at half-maximum. The scan time was set at 0.4 s while the number of micro scans was set at 2. The collision energy (CE) was optimized for each compound and ranged from 12 to 20 eV; S-lens values was set at 120 V for all investigated compounds. The quantitative determination was conducted under multiple reaction monitoring (MRM) conditions, using the ion current generated by two gas phase transitions from the protonated compounds $[M+H]^+$, the first one for the quantitative assay, and second for confirmation (**Table 3.3.1**).

Compound	Transition	CE (eV)	S-Lens (eV)
Oleic acid methyl ester	*m/z* 297 → *m/z* 265 (quan)	12	120
	m/z 297 → *m/z* 247 (qual)	15	120
Linoleic acid methyl ester	*m/z* 295 → *m/z* 263 (quan)	12	120
	m/z 295 → *m/z* 245 (qual)	15	120
Linolenic acid methyl ester	*m/z* 293 → *m/z* 261 (quan)	15	120
	m/z 293 → *m/z* 243 (qual)	18	120
Erucic acid methyl ester (IS)	*m/z* 353 → *m/z* 321 (quan)	18	120
	m/z 353 → *m/z* 303 (qual)	20	120

Table 3.3.1. Selected gas-phase transitions and optimized instrumental parameters.

2.4 GC-FID

The gas chromatographic analyses were carried out following the EU official method 2015/1833, Annex X using a GC-FID instrument from Varian (Palo Alto, CA, USA) The

detector temperature was set at 225 ◦C and the flame of detector was kept at 250 ◦C with 30 mL/min H_2 and 300 mL/min air. The chromatography was performed with a 100 m × 0.25 mm DB-23 capillary column with a film thickness of 0.25 µm from Agilent Technologies. Sample injection volume was 1 µL and the oven temperature started from 100 to 240 °C with a speed rate of 3 °C/min and 5 min of plateau. The total time of analysis was 43 min.

3. Results and discussion

The first approach for the development of the current method concerned the transesterification of some oils samples in order to determine the best analysis conditions and to verify that the reaction is quantitative. Through direct injection in ESI-MS, the positive ionization mode was selected because has a better efficiency for the fatty acid methyl esters investigated. The best spray solvent was methanol which provided good signals for *m/z* 297, *m/z* 295 and *m/z* 293 that are, respectively, the protonated fatty acid methyl ester $[M+H]^+$ ions of oleic, linoleic and linoleic acids. Then the conditions for the quantitative analysis were optimized, in particular the multiple reaction monitoring was used as scan mode following specific transition for the analytes. These specific transitions were determined observing the typical fragmentation pattern of methyl esters in tandem mass spectrometry. In **Figure 3.3.3** are shown the fragmentation profile for the methyl ester of oleic, linoleic, linolenic and erucic acid. As all fatty acid methyl esters, the MS/MS spectra are characterized by few diagnostic fragments. The base pick corresponds to the acyl ion that originates from the formal loss of a methanol neutral molecule from the protonated molecule $[M+H]^+$. The second most intense product ion is produced by the formal loss of one molecule of water from the latter, aided by the transfer of a proton in alpha position with respect to the carbonyl oxygen.

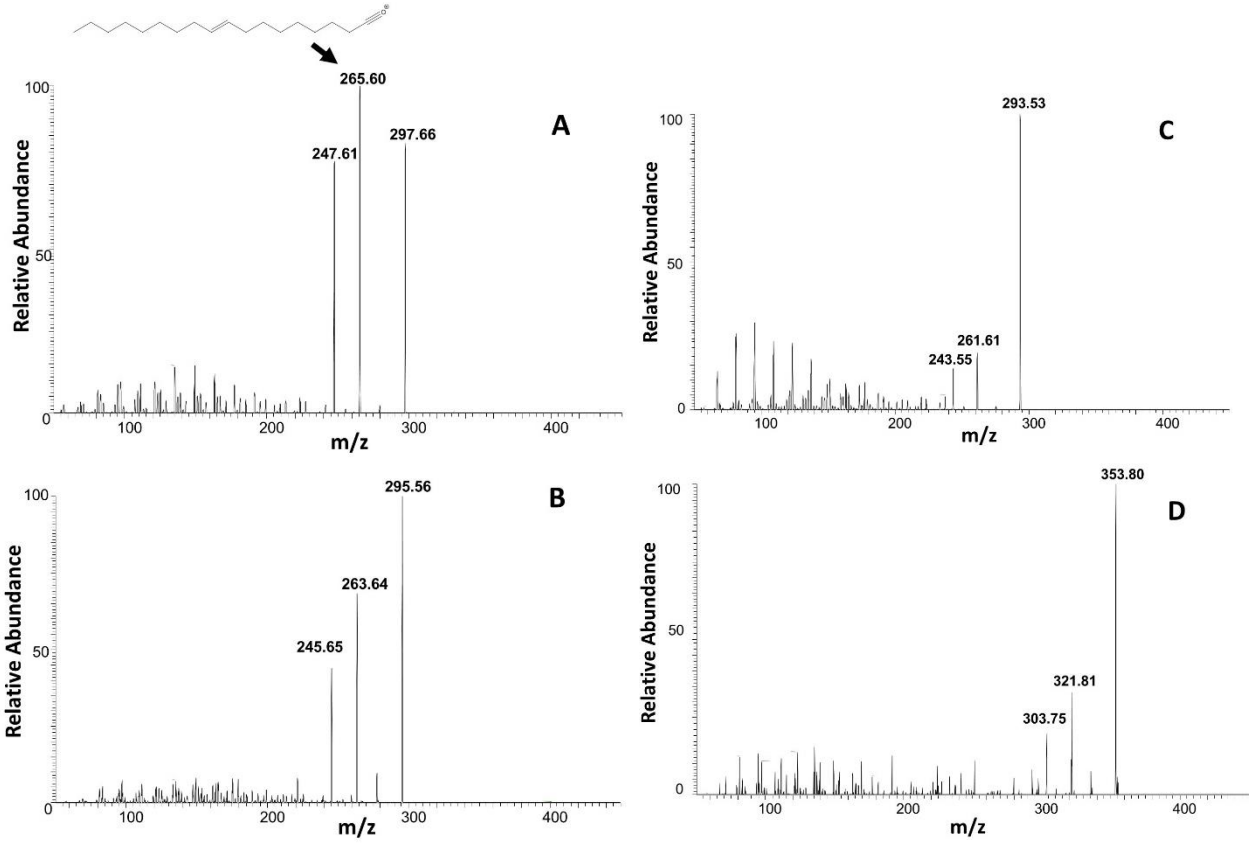

Figure 3.3.3. ESI (+)-MS/MS spectra of $[M+H]^+$ ions of oleic acid methyl ester (A), linoleic acid methyl ester (B), linolenic acid methyl ester (C), and erucic acid methyl ester-IS (D).

The methyl ester of erucic acid was used as internal standard to perform the quantification of the total fatty acids content. Indeed, it possess a chemical structure very similar to the investigated compounds and it is not found in the vegetable oils considered for this study. The selected standard, moreover, shows the same fragmentation behaviour of the other analytes. The most abundant product ion is generated by the loss of a methanol molecule from the $[M+H]^+$ parent ion and correspond to the *m/z* 321, while the consequent formal loss of water gave the signal at *m/z* 303. **Table 3.3.1** summarizes the selected gas-phase transitions, the loss of methanol was used for the quantitative analysis, while the subsequent dehydration was used to confirm the signal. The collision energy for each transition was optimized to achieve the highest signal.

The calibration curves were built in a range from 2.5mg/L to 20mg/L with the internal standard at a concentration of 10mg/L. Each standard solution was analysed in

triplicate, and the calibration curves showed good linearity with a correlation coefficient (r^2) higher than 0.98 for all analytes. (**Figure 3.3.4**)

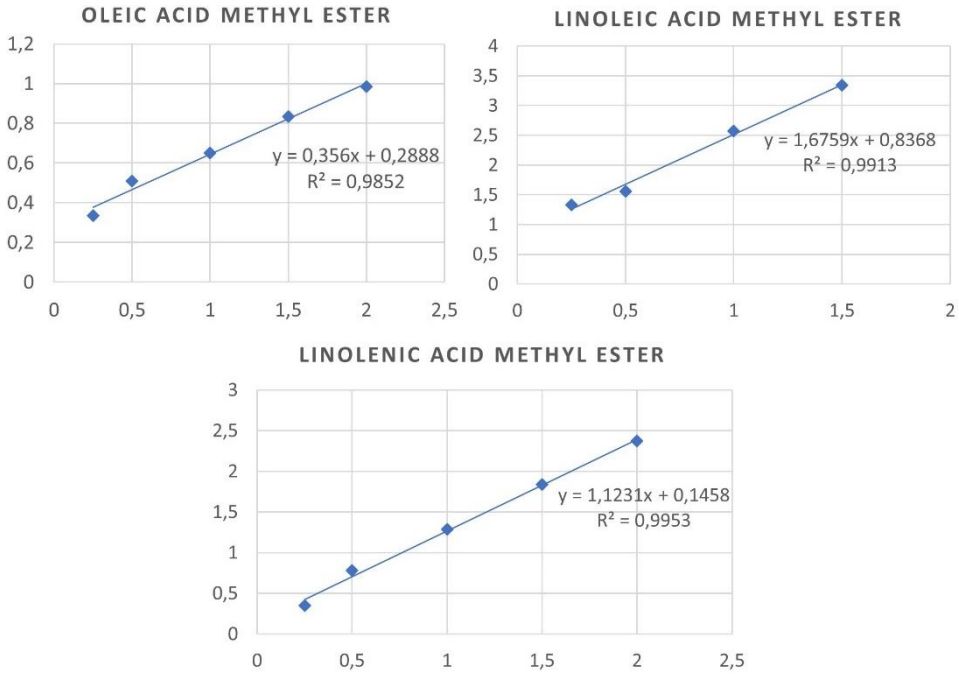

Figure 3.3.4. Calibration curves of oleic acid methyl ester, linoleic acid methyl ester and linolenic acid methyl ester.

The calibration reproducibility (RSD%) was evaluated by preparing two standard solutions at concentrations corresponding to the linear dynamic range edges (2.5 and 20 mg/L) and analysed three times over a period of a week. For both concentrations' levels, the percentage relative standard deviation (RSD%) was less than 15%, highlighting a good reproducibility for what concerns the instrumental response. Due to the lack, the accuracy of the whole procedure was disclosed by submitting two mixtures of triolein and trilinolein to transesterification process and then analyzed. The mixtures were prepared at the following ratios: S1 80/20 and S2 20/80 (triolein/trilinolein), in order to mimic an oil with a high oleic and linoleic acid content, respectively. After transesterification, both samples were diluted 1:1000 and 1:100 using hexane and submitted to PS-MS/MS analysis. In all cases, the accuracy values were around 100%. The same samples were also employed for evaluating the repeatability and reproducibility of the methodology, both expressed as RSD%. The first one was assessed by performing instrumental analyses for each sample in

triplicate, while the reproducibility was calculated by analysing the samples three times at one-week intervals. An RSD% below 15% was obtained for each experiment. **Table 3.3.2** shows the value of analytical parameters discussed above.

	Sample		Calculated Amount (*w/w* %)	Accuracy (%)	Repeatability (RSD %)	Reproducibility (RSD %)
S1 (*w/w* %)	Oleic acid	80%	82 ± 7	102	8.5	9.2
	Linoleic acid	20%	18 ± 2	90	11.1	12.0
S2 (*w/w* %)	Oleic acid	20%	21 ± 2	105	9.5	10.2
	Linoleic acid	80%	77 ± 6	96	7.7	8.4

Table 3.3.2. Accuracy, repeatability, and reproducibility values.

After evaluating the calibration linearity, accuracy and reproducibility, the developed protocol was applied to real samples of vegetables oils purchased from a local store. In particular, three extra virgin olive oils, three corn oils, two soybean oils and one sunflower oil were submitted to PS-MS/MS analysis after transesterification reaction. **Table 3.3.3** shows the total fatty acid content (w/w %) found in the samples tested, which were also analysed in parallel by the classical GC method to corroborate the data obtained by PS-MS.

	Methyl Oleate (*w/w* %)		Methyl Linoleate (*w/w* %)		Methyl Linolenate (*w/w* %)	
Sample	PS-MS	GC-FID	PS-MS	GC-FID	PS-MS	GC-FID
Olive oil 1	60 ± 8	69	9 ± 1	8	0.8 ± 0.1	1.0
Olive oil 2	55 ± 9	68	6 ± 1	6	0.6 ± 0.2	0.7
Olive oil 3	63 ± 9	70	7 ± 1	5	0.45 ± 0.05	0.8
Corn oil 1	28 ± 4	31	60 ± 11	53	0.8 ± 0.2	1.2
Corn oil 2	24.5 ± 4.0	32	47 ± 6	52	1.6 ± 0.2	1.6
Corn oil 3	25 ± 5	31	46 ± 3	51	1.5 ± 0.3	1.3
Sunflower oil 1	29 ± 4	25	57 ± 6	63	1.1 ± 0.1	0.8
Soybean oil 1	20 ± 4	24	55 ± 8	53	3.7 ± 0.5	5.3
Soybean oil 2	23.5 ± 4.5	23	51 ± 11	51	6.5 ± 0.5	6.1

Table 3.3.2. Methyl esters amount (w/w%) found in the investigated oil samples by PS-MS/MS and GC-FID analysis.

The results obtained by PSMS were compared to GC data using a standard t-test (α = 0.05). For all vegetable oils under investigation, a p value ≥ 0.05 was obtained. This emphasizes there are no significant differences between the data and shows the reliability of the proposed method. This cross-validation emphasises also that the PS-MS approach is not affected by sample preparation and/or by matrix composition.

It is necessary to highlight some considerations regarding the relation of the fatty acids composition of the analysed oil samples and the health claims regulations reported in the introduction. The amount of linoleic acid in the sample of corn, soybean and sunflowers oils satisfies the EU regulation 432/2012 for what regard the claims on the "maintenance of normal blood cholesterol levels". For what concern nutraceutical linolenic acid, it appears from the data, that all the samples may be considered as sources of ω-3 fatty acids, while no olive oil may be considered as high-grade oleic acid content under the statements of the FDA regulations.

4. Conclusion

Paper spray mass spectrometry was first employed to determine the total content of important fatty acids in vegetable oils, such as oleic, linoleic and linolenic acids. The comparability between the results gained by classical GC-FID and PS-MS analysis demonstrates that the developed protocol may be applied for very rapid screening of these compounds in oils and similar matrices as an alternative to the determination employing chromatographic separation. The relevance of the presented methodology lies in the potential of its use to address recent EU and US regulations on the health claims for these nutraceutical compounds, enabling important health indications to be directly reported on the food labels and enhancing the products' value.

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Part IV

Rapid analytical method for simultaneous determination of curcuminoids and gingerols in food containing Turmeric and Ginger.

1. Introduction

In recent years, great attention has been paid to diet and lifestyle changes to improve health and try to reduce the incidence of some of the principal causes of death in the world. Indeed, it has been demonstrated that essential benefits come with a healthier diet that prioritises consuming plant materials, such as fruit, vegetables, grain, and oils (Poswal et al., 2019; Khan et al., 2014). Different and non-traditional dietary sources can be employed to reach this final goal. For example, the Government of the Netherlands introduced some national dietary guidelines where it is recommended to drink three cups of green or black tea per day due to their potential to reduce the incidence of stroke and high blood pressure (Poswal et al., 2019). Alongside green and black tea, or tea in general, obtained from leaves of *Camellia sinensis,* there is another class of water infusions known as herbal teas or tisanes. These are prepared from the infusion and decoction of plants that are *non-Camellia sinensis* (Ruchika, 2022).

For this reason, they do not contain caffeine, and usually, they are a mixture of several ingredients, like dried leaves, grasses, seeds, nuts, roots, fruits, and flowers. Moreover, herbal teas can consist of only one main ingredient or a blend of herbal ingredients. In the market, there exist herbal teas with different therapeutic and medicinal purposes. Between them, it is possible to find herbal teas that can relax the body and mind or help with stomach and digestive problems. Still, they also help strengthen the immune system or provide antioxidants to the body (Ruvikumar, 2014).

Historically, people have always used herbs for culinary purposes, for example, to enhance the flavour of foods, but also for providing health benefits due to the presence of active substances, such as phenolic acids and flavonoids. In the Chinese,

Indian and various indigenous medical system, the therapeutic use of herbal teas is deeply rooted. Among the most popular herbal teas, chamomile exhibits calming, anti-inflammatory and anti-mutagenic properties, and peppermint provides stress relief and relaxing effects on the gastrointestinal tract (Kinki, 2021; Poswal et al., 2019; Ruvikumar, 2014).

In general, herbal teas are known for their antioxidant, anti-diabetic, anticancer, anti-mutagenic, anti-microbial, anti-atherogenic and chemo-preventive properties. Furthermore, it has come to attention from different studies that natural antioxidants present in traditional herbs, like flavonoids, phenolic compounds, and tannins, are more potential antioxidants than normal dietary antioxidants (Ruchika, 2022). For such reasons, the World Health Organization has highlighted in its 2014-2023 strategy the importance of Traditional Medicine, with the aim of providing access to effective and affordable alternatives to medicine that are also coherent with people's cultural practices. So, the importance of ensuring efficacy, safety and quality through new methods has grown very fast. For example, there is growing concern about the adulteration of herbs with dyes and bulking agents (Poswal et al., 2019).

In this work, the attention was focused on herbal teas that contain turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*), two herbaceous rhizomatous plants, members of the *Zingiberaceae* family that are cultivated in tropical and subtropical regions around the world. In India and Asian countries, it has been used for medical purposes for many centuries, but it is also extensively used as a colouring and flavouring agent. (Amalraj et al., 2017; Hewlings and Kalman, 2017). Regarding its composition, turmeric is a root composed predominately of water (80-90%), then carbohydrates account for around 13%, proteins for 2% and lipids for less than 1%. The rest of the minor compounds represent the 10% turmeric powder. The most abundant compound in this category is curcumin, a yellow pigment that can compose between 62 and 90 mg/g of commercial powders. Then there are the demethoxy curcumin (9-23 mg/g) and the bisdemethoxycurcumin (0.3-14 mg/g). The World Health Organization affirmed that the acceptable daily intake of total curcuminoids as a food additive should range from 0 to 3 mg/Kg. These three molecules, whose structures can be found in **Figure 3.4.1**, are the main biologically active compounds of turmeric and have potential pharmacological effects. (Munekata et al., 2021; Shah et al.,

2022). Among them, curcuminoids possess antioxidant and anti-inflammatory activities (Nahar and Seeram, 2015; Meng et al., 2021), but they are anticancer agents, in particular, in cases of gastrointestinal, breast, and lung cancer. Curcuminoids have also been shown to reduce triglycerides and cholesterols levels and to have antimicrobial activity, including antibacterial, antiviral, antifungal, and antimalarial. (Shah et al., 2022).

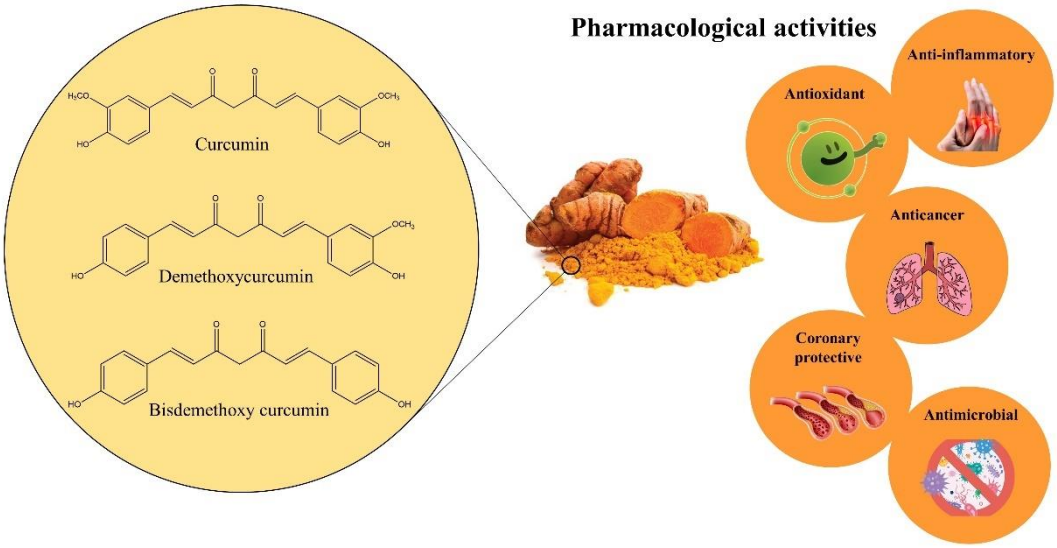

Figure 3.4.1. Chemical structure of Turmeric biologically active compounds and their pharmacological properties.

Despite its health benefits, one of the major problems with curcumin is that it is poorly soluble in water and has low bioavailability due to poor absorption, rapid metabolism, and rapid elimination. However, it is possible to block the metabolic pathway of curcumin through agents like piperine, which can increase the bioavailability of curcumin by 2000%. (Hewlings and Kalman, 2017).

Ginger has been used as traditional herbal medicine since ancient times in China, India or Mexico. It has also been used as an important cooking spice and was one of the first oriental spices to be cultivated in Europe. It was introduced by the Romans, who got it from Arab traders. (Ma et al., 2021, Shahrajabian et al., 2019). Ginger is composed mainly of water, protein, carbohydrates, fibre, sugar and lipids. Then, there are minor compounds whose profile depends on ginger growth cycles, regions of growth, temperature, and humidity. Moreover, new compounds could be

generated or synthesized during ginger storage periods. These compounds are classified as volatile oils, gingerols, and diarylheptanoids. Gingerols are the main pungent compounds in fresh ginger, while shogaols are responsible for the pungency in dry ginger. Indeed, gingerols are phenols with an alkyl chain that could have different lengths, and the most abundant is 6-ginerol, followed by 8-gingerol and 10-gingerol (**Figure 3.4.2**). They are unstable during heating or drying, and the OH group at the C-5 position is eliminated. This elimination leads to the formation of a double bond between the C-4 and the C-5 position, and the shogaols are generated. (Ma et al., 2021).

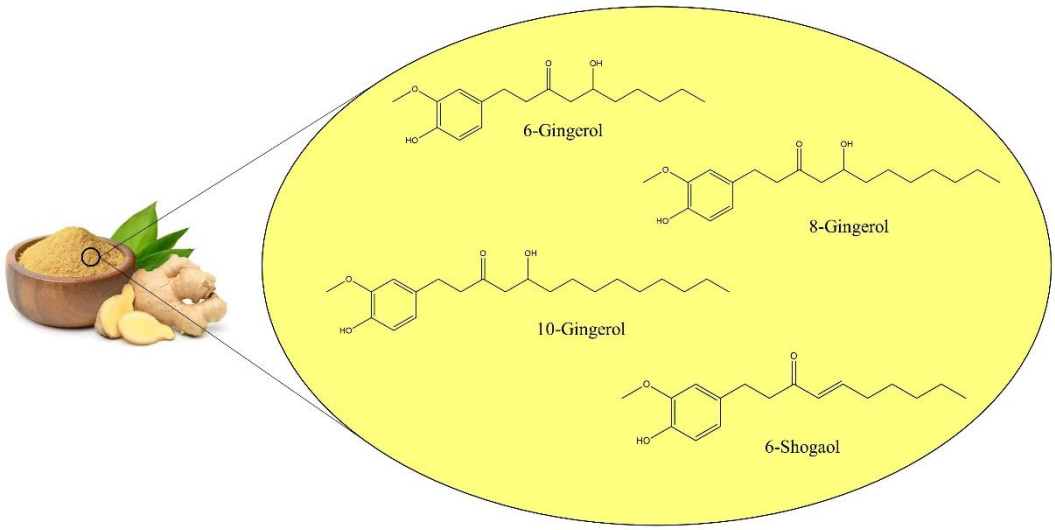

Figure 3.4.2. Chemical structure of Ginger biologically active compounds.

Gingerols and shogaols might have many therapeutic effects on human health. It is used to treat several gastrointestinal diseases, such as peptic and duodenal ulcers. Ginger protects gastric mucosa against several ulcerogenic agents. (Jabir Al-Awwadi, 2017). Furthermore, related gingerol compounds have been shown to possess antimicrobial, antifungal and anti-infective properties. (Ekwenye et al., 2005) Ginger has antioxidant and anti-inflammatory properties. For example, 6-gingerol possesses strong antioxidant activity both in vivo and in vitro. Since ancient times, inflammatory disorders such as rheumatic conditions have been treated with ginger or ginger-derived formulation. Then, gingerol can also be used as a cholesterol-lowering and blood pressure-lowering agent. (Gunathilake and Rupasinghe, 2015).

Commonly, the quantitative analysis of curcuminoids and gingerols is performed using an HPLC method with ultraviolet (Malasoni et al., 2013; Jayaprakasha et al., 2002; Lee et al., 2007) or mass spectrometry as detection. (Kroon et al., 2023). Considering the great attention that, nowadays, is paid to biologically active compounds such as curcuminoids and gingerol, it is very useful to develop new and faster methods to perform the quantitative analysis of the latter compounds and, therefore, assert the quality of the products containing them. In this field, mass spectrometry is a very powerful technique that allows us to obtain specific and sensitive results, and it has rapidly evolved in the last decades. There is an all-new class of methodologies, the ambient mass spectrometry techniques, where the sample is directly analysed in its proper natural state, and that reduces or eliminates the need for long and laborious sample pre-treatments. (Cooks et al., 2006) Among them, paper spray mass spectrometry is one of the simplest techniques. In this case, the sample is loaded on a triangular piece of paper placed in front of the mass spectrometer inlet, where the compound ionization is performed by applying a high voltage through a metallic clip. This methodology has already been employed in different matrices (Bartella, 2020; Chiang et al., 2018; Frey et al., 2020) and, in particular, on foodstuff (Bartella et al., 2019a; Bartella et al., 2019b; Chen et al., 2017; Li et al., 2013).

2. Experimental

2.1 Chemicals

All solvents used were HPLC grade and commercially available from Sigma-Aldrich (St. Louis, MO, USA). Pure compounds, curcumin, demethoxycurcumin, bisdemethoxycurcumin, labelled curcumin d-6, 6-gingerol, 8-gingerol and 10-gingerol were also purchased from Sigma-Aldrich.

2.2 *Sample preparation*

All eight real samples, two Turmeric and Ginger herbal teas (*HT1 and HT3*), one Turmeric and Dandelion herbal tea (*HT2*), one Turmeric, Ginger, and Tropical Fruits herbal tea (*HT6*), two Ginger and Lemon herbal tea (*HT4 and HT5*), one Turmeric powder and one Ginger powder were purchased at a local store. Each herbal tea was

submitted to a simple extraction procedure. Briefly, around 5mg of the sample were weighed, then 1mL of methanol was added, and the solution was extracted for 30 min with ultrasounds. Before performing the analysis in PS-MS/MS and HPLC-UV, the obtained extracts were centrifuged for 3 min at 1200 rpm and filtered using a 0.45µm filter. For the PS-MS/MS experiments, each sample was diluted, and the correct amount of internal standard was added. The samples HT1, HT2 and HT3 were diluted 1:10 for the quantification of curcumin and 6-gingerol, while they were diluted 1:2 for demethoxycurcumin, bisdemethoxycurcumin, 8-gingerol and 10-gingerol. The samples HT4 and HT5 were diluted 1:5 for the quantification of 6-gingerol and not diluted for 8-gingerol and 10-gingerol. The HT6 was used without dilution for the quantification of all analytes, while the turmeric and ginger powders were diluted. Respectively, 1:50 and 1:10 for the quantification of curcumin and 6-gingerol, and 1:10 and 1:2 for demethoxycurcumin, bisdemethoxycurcumin, 8-gingerol and 10-gingerol.

2.3 *HPLC-UV analysis*

The HPLC-UV analyses were performed using an HPLC 1100 system (Agilent Technologies, Waldbronn, Germany) working in analytical mode, equipped with a quaternary pump and a UV/visible detector. The analytical column used for the chromatographic separation was a C18 reversed-phase column, Luna (250 × 4.6 mm, 5 µm, Phenomenex). The injection volume was 25 µl.

The elution was carried out for curcuminoids with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) under gradient conditions. The gradient steps were the following: from 60% to 50% A (0–20 min), from 50% to 20% A (20–22 min), 20% A in isocratic for 5 min, from 20 to 60% A (27–30 min) and then an isocratic flow (5 min) to equilibrate the system before starting the new analysis. The total run time was 35 min, while the flow rate was set at 1,5 mL/min, the column was set at 45°C, and the UV detector was set at 420 nm. The concentration of curcuminoids was evaluated using an external calibration curve gained by standard solutions of their standards at a concentration ranging from 1.5 to 15 mg/L.

The elution of gingerols was carried out with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) under gradient conditions. The gradient steps were the

following: 55% A in isocratic for 5 minutes, then from 55% to 50% A (5–13 min), from 50% to 35% A (13–20 min), from 35% to 10% A (20-45 min), from 10% to 55% A (45-50 min), and then an isocratic flow (5 min) to equilibrate the system before starting the new analysis. The total run time was 55 min, the flow rate was 1 mL/min, the column was 45°C, and the UV detector was 282 nm (Lee et al., 2007). The concentration of gingerols was evaluated using an external calibration curve constructed by standard solutions ranging from 2.5 to 40 mg/L.

2.4 PS-MS/MS analysis

The MS analyses were conducted with a TSQ Quantum Vantage (Thermo Fisher Scientific, San Jose, CA) triple-stage quadrupole mass spectrometer equipped with a homemade paper spray ionization source. Qualitative Whatman filter paper n◦ 1 (pore size 11 µm, thickness 180 µm) was used for the experiments. The sample spotting volume was 15 µl, and once dried, the paper triangle was wetted with the same volume of methanol to allow the desorption of the ions. The paper spray ionization was performed in negative ion mode. The working conditions were: voltage 5.0 kV, applied directly to the paper triangle; vaporizer and capillary temperatures: 280 and 290 °C, respectively. The collision gas was argon used at a pressure in the collision cell (Q2) of 1.5 mTorr, and the mass resolution at the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da at full width at half-maximum. The scan time was set at 0.4 s, and the number of micro scans was 2. The collision energy (CE) was optimized individually per compound, ranging from 15 to 21 eV; S-lens values were set at 75V for each analyte. The assay was performed by using the multiple reaction monitoring (MRM) scan mode, following specific gas phase transitions from the deprotonated precursor ion $[M- H]^-$ (**Table 3.4.1**). The average ion current of each monitored transition over the total acquisition time was used for the quantitative analyses.

Compounds	Transition	CE (eV)	S-Lens (V)
Curcumin	m/z 367 → m/z 149	22	75
Demethoxycurcumin	m/z 337 → m/z 119	18	75
Bisdemethoxycurcumin	m/z 307 → m/z 119	15	75
6-Gingerol	m/z 293 → m/z 99	19	75
8-Gingerol	m/z 321 → m/z 127	20	75
10-Gingerol	m/z 349 → m/z 155	20	75
Curcumin-d6 (IS)	m/z 373 → m/z 152	22	75

Table 3.4.1. Selected gas-phase transitions and optimized instrumental parameters.

2.5 Limit of detection and limit of quantification (LOD and LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry (McNaught and Wilkinson, 1997), as follows:

$S_{LOD} = S_{RB} + 3\sigma_{RB}$

$S_{LOQ} = S_{RB} + 10\sigma_{RB}$

where S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the ratio of the signals given by the transitions of the analyte and of the internal standard from the blank sample, an extract of a mixture of tea and chamomile, and σ_{RB} is its standard deviation. The concentrations were calculated by the standard curve.

3. Results and discussion

Herbal teas are well known for their antioxidant, anti-diabetic, anticancer, anti-mutagenic, anti-microbial, anti-atherogenic, and chemo-preventive properties. In this work, the attention was focused on herbal teas that contain turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*) and their biologically active compounds. For turmeric, the compounds considered were curcumin, demethoxycurcumin, and bisdemethoxycurcumin, while for ginger, they were 6-gingerol, 8-gingerol, and 10-gingerol. Mass spectrometry allows us to obtain good results with good accuracy and

very few minutes of analysis, particularly when Ambient Mass Ionization techniques are involved. For this reason, this work aims to develop a new method based on ambient mass spectrometry for the quantification of curcuminoids and gingerols in Turmeric and Ginger food products, as these nutraceutical molecules are considered important quality markers. Paper Spray was selected as an ion source because it is one of the easiest to apply for direct analysis of different molecules in complex mixtures. The sample is located on a triangular piece of paper, and a high voltage is applied. Through the employing of a few microliters of solvent, the ionization is performed.

The developed method was tested on products containing the two spices generally used in the diet, such as herbal teas, cooking powders, and dietary supplements. A quite simple procedure, proposed by Lee and co-workers (Lee et al., 2007) was applied to extract the analytes of interest. The mass spectra were acquired in negative ionization mode due to the phenolic-based structure of curcuminoids and gingerols, which increases the ionization efficiency with the negative ionization mode. The quantitative determination, instead, was carried out through multiple reaction monitoring (MRM) scan mode to improve the specificity and sensitivity. Each analyte gas-phase fragmentation for MRM experiments was selected according to the output of collision-induced dissociation (CID) experiments performed on the deprotonated parent ions and are reported in **Table 3.4.1**.

In the case of curcuminoids, the MS/MS spectra show the main product ions derived from a β-hydrogen shift to the double bond of the diketone form of the precursor ions, leading to the loss of a neutral moiety with styrene-like structure (Jiang et al., 2006) (**Figure 3.4.3** A, B, C, D), while for the gingerols family, the most abundant product ion is generated by the cleavage of the C_4-C_5 bond with the presumed neutral loss of 4-(4-hydroxy-3-methoxyphenyl) butan-2-one (Lee et al., 2007) (**Figure 3.4.3** E, F, G).

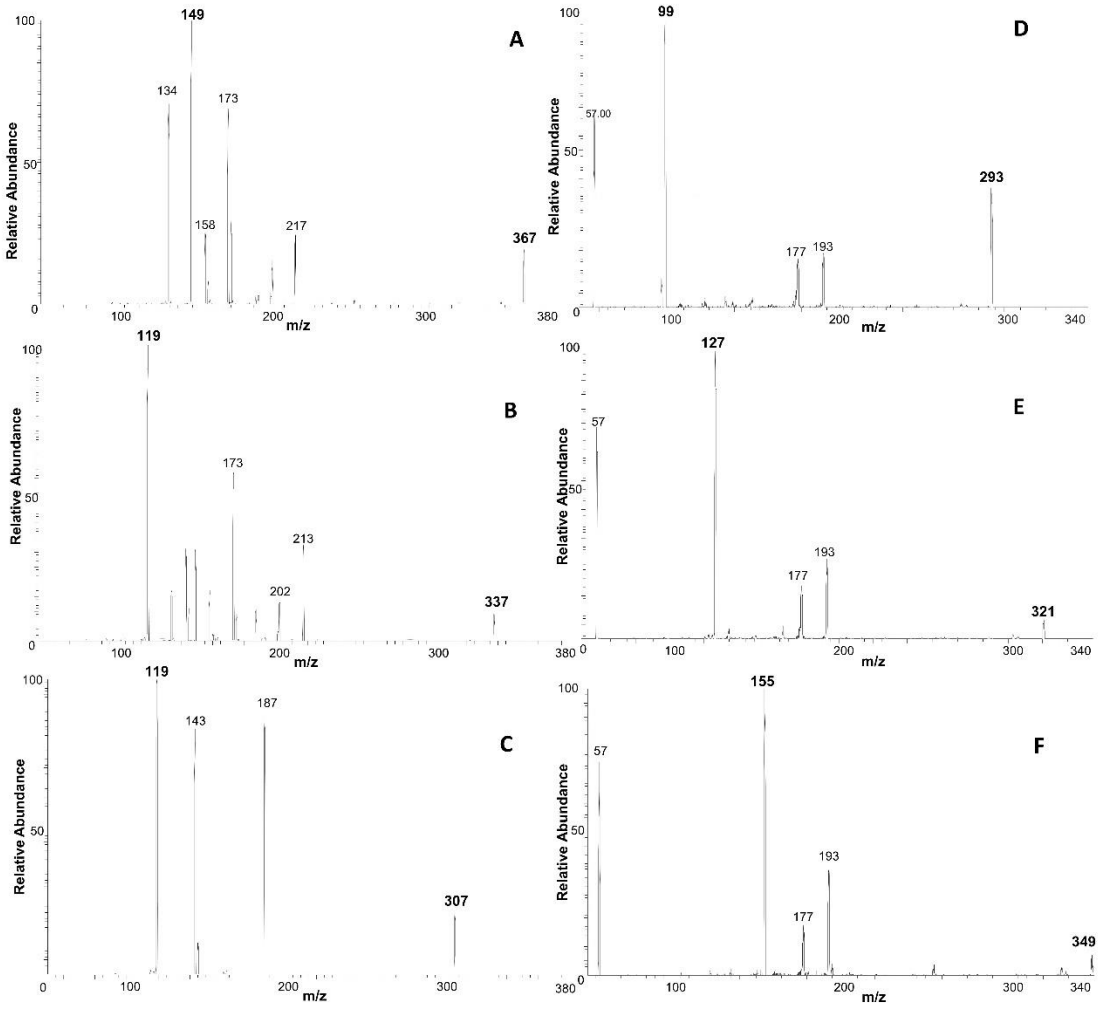

Figure 3.4.3. MS/MS spectra of Curcumin (A), Demetoxycurcumin (B), Bisdemetoxycurcumin (C), 6-gingerol (D), 8-gingerol I and 10-gingerol (F).

When performing a quantitative determination, it is advisable to employ an internal standard, particularly when the detector is a mass spectrometer, because it improves the accuracy and reproducibility of the measurements. In this work, the labelled d_6-curcumin was employed as the internal standard for all the analytes under investigation. The MS analyses, based on PS-MS, were very fast (an acquisition time of two minutes). The crude extract, after an appropriate dilution, was directly loaded onto the paper triangle, and the analyte ions were desorbed, dropping a few µl of methanol onto the paper every 30 s during the total run time. For each analyte, the calibration curve was built by analyzing in triplicate five standard solutions whose analyte concentration ranged from 1 µg/mL to 10 µg/mL, keeping the concentration of the internal standard fixed at 5 µg/mL. As shown in **Figure 3.4.4**, the instrumental

response was extremely linear, within the concentration range, obtaining correlation coefficient values (r^2) exceeding 0.98 for all the analytes under investigation.

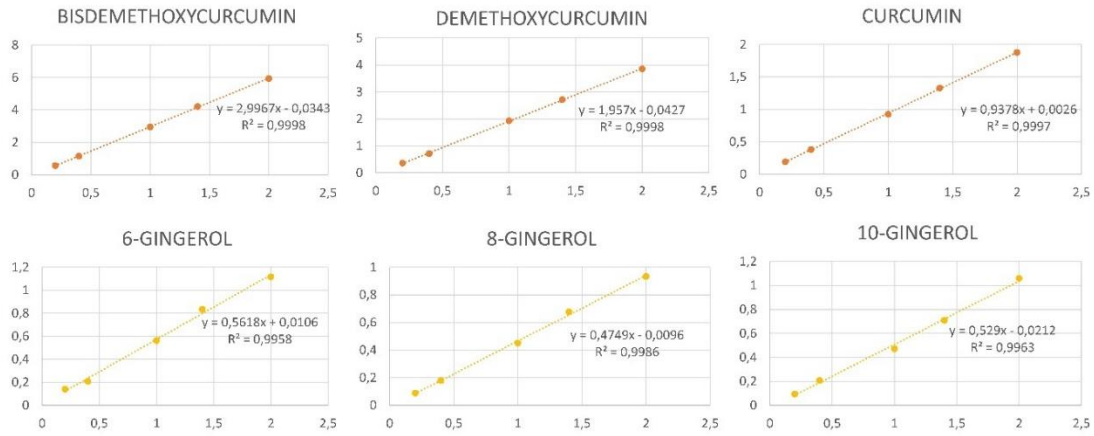

Figure 3.4.4. PS-(-)-MS/MS calibration curves for curcuminoids and gingerols.

LOD and LOQ were calculated using a blank matrix (chamomile tea) in the presence of the internal standard. LOQ and LOD were also determined in solution for all curcuminoids and gingerols analysed. The obtained values are reported in **Table 3.4.2**. LOQ values were below the lowest level of the calibration range, highlighting that the developed PS-MS/MS method provides a satisfactory sensitivity for the quantification of all analytes in the investigated matrices. LODs ranged from 0.10 to 0.62 mg/L.

Other parameters investigated to evaluate the proposed methodology were accuracy and precision (repeatability and reproducibility). In this case, two blank samples were fortified with a known concentration of analytes, and then submitted to the analytical procedure. As for LOQ and LOD, the black matrix consisted of a chamomile-based tea spiked with the analytes at two different concentration levels near the calibration edges. The first spiked sample, S1, was added with a low amount of each curcuminoid and gingerols to achieve, in the final extract, a concentration of 1.5 mg/L. In contrast, the second one, S2, was fortified to obtain a final concentration of 9 mg/L for each analyte. The obtained accuracy values are shown in **Table 3.4.2**. For curcuminoids, the values ranged from 96% to 117%, while for gingerols, they ranged from 100% to 104%. The same samples were used to evaluate the precision, expressed as the

	Spiked (S1) 1.5 mg/L	RSD%	Accuracy	Spiked (S2) 9 mg/L	RSD%	Accuracy	LOD mg/L	LOQ mg/L	RSD%* (herbal tea 1)
Curcumin	1.44 ± 0.02	1.5	96	8.57 ± 0.28	3.2	95	0.38	0.62	10.9
Demethoxycurcumin	1.71 ± 0.12	6.9	114	9.07 ± 1.36	14.9	101	0.62	0.87	12.1
Bisdemethoxycurcumin	1.75 ± 0.26	14.7	117	10.08 ± 1.14	11.3	112	0.34	0.68	14.8
6-Gingerol	1.50 + 0.17	11.2	100,3	8.69 ± 1.01	11.7	97	0.22	0.32	12.3
8-Gingerol	1.56 ± 0.22	14.2	104	8.92 ± 10.83	9.3	99	0.17	0.22	10.9
10-Gingerol	1ù.50 ± 0.19	12.4	100	8.82 ± 1.28	14.6	98	0.10	0.21	9.3

Table 3.4.2. Analytical parameters of accuracy, LOQs, LODs, repeatability and reproducibility
The reproducibility was determined by analyzing one sample (herbal tea 1) 3 times over a period of 1 week.

Samples	Curcumin (mg/g)	RSD%	Demethoxy -curcumin (mg/g)	RSD%	Bisdemethoxy -curcumin (mg/g)	RSD%	6-Gingerol (mg/g)	RSD%	8-Gingerol (mg/g)	RSD%	10-Gingerol (mg/g)	RSD%
Herbal tea 1	5.48 ± 0.13	2.4	1.68 ± 0.12	7.2	1.43 ± 0.10	7.2	4.04 ± 0.11	2.7	0.84 ± 0.09	10.5	1.18 ± 0.07	5.9
Herbal tea 2	6.03 ± 0.16	2.6	2.20 ± 0.09	3.9	2.31 ± 0.20	8.8	-	-	-	-	-	-
Herbal tea 3	4.79 ± 0.20	4.3	1.75 ± 0.14	8.3	1.31± 0.16	12.3	4.19 ± 0.36	8.5	0.77 ± 0.05	6.3	1.05 ± 0.08	7.5
Herbal tea 4	-	-					3.61 ± 0.29	8.1	0.72 ± 0.03	3.8	0.97 ± 0.02	2.3
Herbal tea 5	-	-	-	-	-	-	4.25 ± 0.33	7.7	1.48 ± 0.18	12.1	1.27 ± 0.18	14.5
Herbal tea 6	1.62 ± 0.10	6.4	1.07 ± 0.15	14.1	1.55 ± 0.12	7.9	1.64 ± 0.10	6.1	0.69 ± 0.11	15.3	0.45 ± 0.06	13.4
Turmeric powder	24.60 ± 2.91	11.8	7.04 ± 0.41	5.8	5.41 ± 0.56	10.3	-	-	-	-	--	-
Ginger powder	-	-	-	-	-	-	11.53 ± 1.69	14.6	2.19 ± 0.19	8.5	2.69 ± 0.21	7.4
Supplement dietary 1	27.52 ± 1.19	4.3	7.82 ± 1.18	15.1	5.82 ± 0.85	14.6	7.04 ± 0.61	8.7	1.11± 0.12	10.8	2.09 ± 0.11	5.3
Supplement dietary 2	7.62 ± 1.05	13.8	2.16 ± 0.25	11.6	1.61 ± 0.15	9.3	-	-	-	-	-	-
	Curcumin (mg/100ml)	RSD%	Demethoxy -curcumin (mg/100ml)	RSD%	Bisdemethoxy- curcumin (mg/100ml)	RSD%	6-Gingerol (mg/100ml)	RSD%	8-Gingerol (mg/100ml)	RSD%	10-Gingerol (mg/100ml)	RSD%
Herbal tea 1 in hot water	-	-	-	-	-	-	2.11 ± 0.21	10.0	-	-	-	-
Herbal tea 3 in hot water	-	-	-	-	-	-	2.16 ± 0.28	13.0	-	-	-	-

Table 3.4.3. Amount of analytes in mg/g found in analyzed real samples period of 1 week.

percentage of relative standard deviation and calculated by analyzing each fortified sample in triplicate.

The RSD values were below 15% in all cases, indicating good repeatability. The reproducibility, instead, was assessed by the analysis of one real sample (herbal tea 1) three times over a week, and the obtained values were also below 15%.

After the evaluation of the method, it was applied for the analysis of some real samples purchased from local stores: two cooking powders, two dietary supplements and five herbal teas. The results obtained for the quantification of curcuminoids and gingerols in these samples are reported in **Table 3.4.3**.

The extraction and analysis procedures were straightforward. They consisted of weighing 5 mg of the sample and extracting it in 1 mL of methanol using an ultrasonic bath. Then, the raw extract was loaded directly onto the triangular-shaped paper support to perform PS-MS/MS analyses. The recovery of the analytes, according to this procedure, is quantitative, as reported in the literature (Lee et al., 2007). In the end, to verify the validity of the proposed method, the same samples were analyzed by the classical HPLC-UV method. The amounts found are like those obtained by PS-MS analysis (**Table 3.4.4**). The results obtained by PS-MS were compared with the values determined by the validated HPLC method using a standard t-test ($\alpha = 0.05$); for all the tested samples, a p value ≥ 0.05 was obtained, demonstrating the non-existence of significant variation among the data, and supporting the robustness of the proposed methodology.

The last test consisted of preparing two herbal teases in hot water for 5 minutes to check the amount of gingerols and curcuminoids. The results shown in Table 3.3.4 highlighted that in herbal teas, only 6-gingerol is present in remarkable amounts. About 70 per cent of the quantity in the raw sample is transferred into the water. Probably, this trend is explained by the low lipophile nature of 6-gingerol compared to the other molecules considered. Thus, to reach the correct intake, the daily dose of gingerols and curcuminoids, it is preferable to take supplements rather than herbal teas.

Samples	Curcumin		Demethoxycurcumin		Bisdemethoxycurcumin		6-Gingerol		8-Gingerol		10-Gingerol	
	PS-MS	LC-UV	PS-MS	LC-UV	PS-MS	LC-UV	PS-MS	LC-UV	PS-MS	LC-UV	PS-MS	LC-UV
Herbal tea 1	5.48	6.10	1.68	2.13	5.46	6.10	4.04	4.03	0.84	0.78	1.18	1.27
Herbal tea 2	6.03	7.35	2.20	2.70	2.31	2.58	-	-	-	-	-	-
Herbal tea 3	4.79	5.17	1.75	1.88	1.31	1.24	4.19	4.14	0.77	0.76	1.05	1.23
Herbal tea 4	-	-	-	-	-	-	3.61	3.60	0.72	0.62	0.97	1.04
Herbal tea 5	-	-	-	-	-	-	4.25	4.57	1.48	0.85	1.27	1.92
Herbal tea 6	1.62	1.74	1.07	0.98	1.55	1.42	1.64	1.71	0.69	0.54	0.45	0.55
Turmeric powder	24.60	23.50	7.04	7.35	5.41	5.36	-	-	-	-	-	-
Ginger powder	-	-	-	-	-	-	11.53	10.98	2.19	1.82	2.69	1.45
Dietary supplement 1	27.52	28.14	7.82	8.61	5.82	6.15	7.04	7.41	1.11	1.23	2.09	2.01
Dietary supplement 2	7.62	8.05	2.16	2.31	1.61	1.82	-	-	-	-	-	-

Table 3.4.4. Comparison of analytes amount (mg/L) found in the analyzed samples by PS-MS and HPLC-UV analysis.

4. Conclusions

The presented study aims to propose a rapid and specific analytical approach for the quantification of curcuminoids and gingerols in food products generally consumed in our diet based on Turmeric and Ginger. The protocol relies on the use of paper spray ionization coupled with tandem mass spectrometry. The reliability of our protocol is demonstrated by the satisfactory values of the analytical parameters and by the comparison with the classic HPLC-UV determination. The methodology could also be used for the determination of similar molecules in other natural matrices, with the aim of monitoring their quality.

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CHAPTER 4.

Paper Spray Mass Spectrometry to ensure food authenticity and traceability.

Part I.

Paper spray mass spectrometry profiling of olive oil unsaponifiable fraction for commercial categories classification.

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Paper spray mass spectrometry profiling of olive oil unsaponifiable fraction for commercial categories classification

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Chemometrics

ABSTRACT

A new method for a fast molecular profiling of olive oil unsaponifiable fraction has been developed. This approach, based on paper spray mass spectrometry, allows obtaining MS data with only a few minutes of analysis and without significant solvent and disposable consumption. Tandem mass spectrometry and high-resolution mass spectrometry experiments have been performed to identify the main ions detected. The MS data coming from the analyses of sixty-three samples of three different olive oil categories: extra virgin olive oil (EVOO), virgin olive oil (VOO), and pomace olive oil (POO), have been used to test the discriminative potential. Both unsupervised (PCA and HCA) and supervised (kNN and LDA) chemometric procedures have been applied with good results in prediction. The same approach was tested using direct infusion mass spectrometry data to confirm the ability of paper spray fingerprinting to classify different olive oils correctly.

1. Introduction

Extra virgin olive oil is one of the most famous foods of the Mediterranean Diet, well known and profoundly studied because of its beneficial effects on the human health. Its nutraceutical features are mainly related to the high content of monounsaturated fatty acids and minor components such as phenolic compounds, vitamins, and phytosterols (Finotti et al., 2001; Leon et al., 2011; Nocella et al., 2018; Zarrouk et al., 2010). Olive oil is composed of two fractions, the saponifiable fraction, which represents 98-99% of the total weight, and the remaining unsaponifiable portion (1-2%), which consists of a complex mixture of hydrocarbons, tocopherols, pigments, waxes, aromatic and aliphatic alcohols, phytosterols and triterpene acids. This smaller fraction is the most relevant from a health perspective, exhibiting different biological activities against several diseases (Yang et al., 2018; Lukic et al., 2021), and it is also crucial for quality assessment and frauds detection (Boskou et al., 2006; Kycyk et al., 2016). In fact, the content of specific minor components such as phenolic compounds, sterols and triterpene diols is one of the parameters required to verify the nutraceutical properties and the authenticity of olive oils (EEC No 2568/91; Reg. (EU) No 432/12). Despite the existence of strictly European regulations highlighting the criteria for olive oil quality grade and commercial categories (Reg. (EU) 29/2012; Reg. (EU) 1308/2013), the extra virgin olive oil is still a leading target for fraudulent practises, which have become increasingly sophisticated. In fact, among the most common adulterations are the marketing of VOO as EVOO, or EVOO with the addition of small amounts of seed oil and pomace oil (POO) (Casadei et al., 2021; Tsimidou et al., 2016; Jabeur et al., 2017). In many instances, simple chemical and/or organoleptic analyses are not sufficient to detect this type of adulterations (Casadei et al., 2021; Circi et al., 2017).

Because of the extensive olive oil culture and its innumerable health benefits, substantial efforts have been devoted to its molecular characterization, to develop new methodologies for quantifying nutraceutical markers, and for the determination of possible adulterations, geographical and botanic origin (Benito-Cambra et al., 2020; Pacetti et al., 2019; Yuan et al., 2017).

In this regard, great attention has been dedicated to the development of new methods for olive oil classification, by applying chemometric tests as useful tool. These approaches have been already employed mainly after classical and time-consuming chromatographic separation methods coupled with ultraviolet detector and mass spectrometry (GC-MS and HPLC-MS), using both targeted and untargeted approaches (Zarrouk et al., 2010; Agozzino et al., 2010; Garcia-Nicolas et al., 2020; Gerhardt et al., 2019; Kalogiouri et al., 2020; Kritikou et al., 2021; Lerma-Garica et al., 2011; Nagy et al., 2005; Quintanilla-Casas et al., 2022), focusing also on the fingerprint of the sterols fraction (Bagur-Gonzalez et al., 2015). Therefore, it would be important to have methods to establish the possible presence of adulterants in olive oil by specific molecular fingerprints obtained using fast instrumental analysis. Ambient Mass Spectrometry (AMS) is surely the most useful tool in this respect (Bartella et al., 2020; Birse et al., 2022; Pumbua et al., 2023), because it combines the specificity of mass spectrometry with very brief analysis times due to minimal or no sample treatment and no separation steps. Paper Spray mass spectrometry (PS-MS) is probably one of the most straightforward and cost-effective AMS approaches, which permits to obtain specific molecular profiles within a few minutes of analysis (Bartella et al., 2022; Lara-Ortega et al., 2018). In a typical PS-MS experiment, the sample is loaded on a triangular piece of paper, and after applying solvent and high voltage (3-5 kV), analytes ionization is promoted mainly by an electrospray-like mechanism (Liu et al., 2010). PS-MS has been applied in many analytical fields (Manicke et al., 2016; McBride et al., 2019), and for foods and beverages quality control (Bartella et al., 2019a; Bartella et al., 2023; Mazzotti et al., 2021; Taverna et al., 2016; Zhang et al., 2012; Zhang et al., 2014). Furthermore, this approach has already been successfully used to determine nutraceutical compounds in EVOO samples, such as vitamin E (Bartella et al., 2019b), and phenolic compounds (Bartella et al., 2020). Considering the versatility, low-cost, and speed of the PS-MS, together with the interest in monitoring the authenticity and quality of a product such as olive oil using its unsaponifiable fraction, herein, we present a study in which the PS-MS approach has been applied for the first time to direct analysis of olive oil unsaponifiable fraction. The PS-MS performance, in terms of molecular detection

capability, has been confirmed by high-resolution MS experiments. A fingerprinting MS approach coupled with chemometrics has been used for the development of a classification method by using three groups of commercial olive oil that are usually target for fraudulent practises: extra virgin olive oil (EVOO), virgin olive oil (VOO), and pomace olive oil (POO). The same procedure has been performed with high-resolution mass spectrometry (APCI-TOF), which has allowed to evaluate the goodness of the results coming from PS-MS analysis. In addition, to classify the oils according to their untargeted content, various statistical tests were applied, including principal component analysis (PCA-LDA), cluster analysis of original variables, and K-NN.

2. Experimental

2.1 Chemicals

Methanol (MeOH, <99%), Chloroform (<99%), Isopropanol (<99%), hexane (<99%) and diethyl ether (<99%) were supplied by Merck-Sigma Group (Darmstadt, Germany). Standards of β-Amyrin (≥98,5%), campesterol (~65%), β-Sitosterol (95%) and fucosterol (93%) were purchased by Sigma Aldrich (Saint Louis, MO, US).

2.2 Sample Preparation

Sixty-three olive oils belonging to three different categories: Extra virgin Olive Oil (EVOO) (*n* = 21), Virgin Olive Oil (VOO) (*n* = 21) and Pomace Olive Oil (POO) (*n* = 21) were used as trials of the method development. All the sample were provided by Laboratorio Tello (Jaèn, Spain) and regularly tested to verify their quality grade and commercial category. The extra virgin Olive oil come from different regions and cultivar of Spain (19) and Morocco (2). For the Spanish samples, 4 belong to Cornicabra, 4 to Arbequina, 1 to Manzanilla, 3 to Picual, 3 to Hojiblanca, 1 to Arroniz, 2 comes from Extremadura with no information about the cultivar and the last one is a coupage.

To perform multivariate analysis, for each category, oil samples were split randomly into two data sets, a training set (15 samples for each group) and a test set (6 samples for each group). All the samples were submitted to saponification reaction according

to the EU official method (Regulation EEC 2568/91).. The unsaponifiable fractions were dissolved in 2 ml of chloroform and directly used for PS-MS analysis with the ion trap. The same solutions were diluted with isopropanol (1: 200 and 1: 2500) to perform the HPLC-APCI-TOFMS and direct infusion APCI-TOFMS analysis for identification and comparison purposes.

2.3 Paper spray mass spectrometry

The MS analyses were done with a Thermo LTQ linear ion trap mass spectrometer (Thermo Scientific, San José, CA, USA) with an in-built paper spray ionization source. Whatman 42 filter paper, with a triangular shape, was used for the experiments. The sample volume was 10 µL, and once dried, a high voltage was applied to the paper through the clamp, and later it was wetted with the same amount of methanol to allow the transfer of the analytes to the gas phase. The paper spray ionization was performed in positive ion mode, and the working conditions were the following: voltage (kV),5.0. Capillary voltage (V), 35. Capillary temperature (°C), 275, and tube lens (V), 100. The number of micro scans per spectrum was set at 3. Mass spectra were recorded in the mass range from 300 to 700 amu. Collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) experiments were carried out to identify compounds in the samples using a collision energy of 15 eV. Acquired data were processed using Xcalibur 3.0 (Thermo Scientific) software.

2.4 High-resolution mass spectrometry

2.4.1 HPLC-APCI-TOFMS analysis

The HRMS measurements were performed using an LC-MS system (Agilent 1290/Agilent 6220 TOF). The analytical column used for the chromatographic separation was a C_{18} reversed-phase column, Zorbax Eclipse plus (150 x 4.6 mm) (Agilent, Santa Clara, California, USA). The injection volume was 20 µL. The elution was accomplished with methanol (solvent A) and isopropanol (solvent B) under gradient conditions. The gradient steps were the following: from 0 to 50 % of B in 2 min, from 50 to 90% of B (2-15 min), from 90 to 100 % B (15-18 min), 100 % B isocratic for 2 min, from 100 to 50 % of B (20-23 min) and then a 2 min flow to equilibrate the

system at the initial condition before starting the new analysis. The total run time was 25 min, while the flow rate was 0.400 mL/min. The full-scan accurate mass spectra were obtained using a time-of-flight mass spectrometer (Agilent 6220 accurate mass TOF, Agilent) coupled with an APCI source working in positive ionization mode. The instrumental parameters used are gas temperature (°C), 250. Vaporizer, 300. Gas flow (l/min), 5.0. Nebulizer (psi), 30, and fragmentor voltage (V), 250. Mass spectra were recorded across the mass range from *m/z* 300 to 500. The full-scan data were recorded with Agilent Mass Hunter Acquisition software (version B.04.00) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.04.00).

2.4.2 Flow-injection APCI-TOFMS analysis

For flow-injection experiments, the APCI parameters were the same described in the previous section. The injection volume was 20 µl while the mobile phase used to transport the injected sample was 50:50 methanol and isopropanol with a flow rate of 0.300 mL/min. In this case, the spectra were acquired in the mass range from to *m/z* 300-500, in order to reduce the number of variables considering that PS-MS experiments highlighted that no interesting masses are present above *m/z* 500.

The direct infusion analyses were performed with an APCI source in positive ionization mode coupled to a time-of-flight mass spectrometer (Agilent 6220 accurate mass TOF, Agilent). The instrumental parameters are gas temperature (°C), 250. Vaporizer, 300. Gas flow (l/min), 5.0. Nebulizer (psi), 30 and Fragmentor voltage (V), 250. Mass spectra were recorded across the mass range from *m/z* 200 to 500. The full-scan data were recorded with Agilent Mass Hunter Acquisition software (version B.04.00) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.04.00).

2.5 Statistical analysis

A chemometric study was performed using both unsupervised and supervised approaches. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were employed as the unsupervised method to visualize the objects in a

multidimensional space and understand patterns and groupings among a data set by means of a tree of clusters. Two supervised classification techniques were employed, which rendered a delimiter between classes and permit the assignment of each new sample to the class with the highest probability. The two approaches used were K Nearest Neighbours (K-NN) and Linear Discriminant Analysis (LDA). K-NN is a straightforward distance-based technique and was performed both on the original variable and on PCA scores. LDA, instead, is a probabilistic technique only relevant if applied to the PCA scores for the case studied. (Oliveri et al., 2021). In this study, PCA, K-NN, and LDA were performed using the multivariate data analysis software CAT (R. Leardi, C. Melzi, G. Polotti, CAT (Chemometric Agile Tool), freely downloadable from http://gruppochemiometria.it/index.php/software), while HCA was performed using SPSS software (SPSS for Windows 26.0, SPSS Inc., USA).

3. Results and discussion

The unsaponifiable fraction of olive oil contains several bioactive compounds, ranging from hydrocarbons, aliphatic alcohols to different sterol derivates. Therefore, the MS fingerprinting of this fraction may provide insights into the origin and quality of the product. One of the objectives of the present work was to develop an innovative methodology for direct analysis of olive oil unsaponifiable fraction relying on the use of paper spray mass spectrometry. Since this fraction is more stable than for instance phenolic compounds or volatiles, the proposed approach could be more rugged to enable olive oil classification. The MS data obtained were submitted to chemometric analysis to verify their capability in olive oil categories classification.

3.1 Optimization of PS-MS performance

In order to optimize the paper spray mass spectrometry performance, several experimental conditions were tested. Both positive and negative ionization modes were employed, and different spray solvents were evaluated to increase the molecular detection capability. To screen the whole extract, the first experiments were carried out over a wide *m/z* mass range, from *m/z* 100 to 1000. The best ionization outcome was obtained by spotting just 10 µl of the unsaponifiable extract

onto the paper, using positive polarity and methanol as spray solvent at the beginning of the MS acquisition.

In the mass spectra, a remarkable difference was observed between the onset of the MS acquisition, when the paper is wet with methanol, and the last seconds, when the triangle is dry (**Figure 4.1.1**). In fact, only when the paper has dried the ions related to the non-polar compounds, specific to unsaponifiable fractions, are detected. The ionization of these compounds is due to a corona discharge effect between the tip of the triangle and the mass spectrometer inlet (Lara-Ortega et al., 2018).

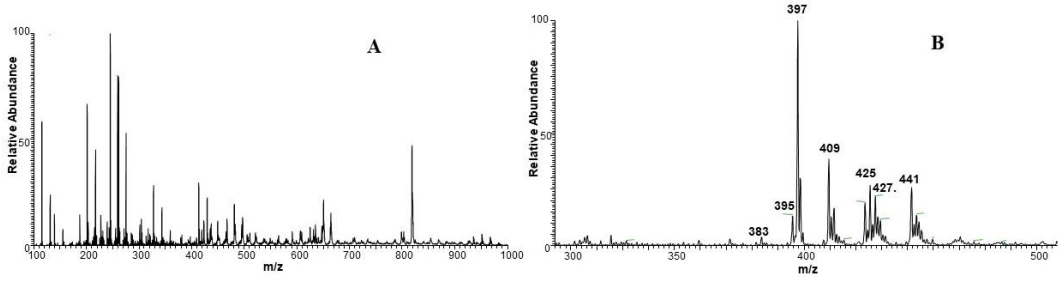

Figure 4.1.1. PS (+) MS spectra of wet paper (A) and zoomed dry paper (B) of the unsaponifiable fraction of olive oil.

The ions detected in PS-MS spectra were related to sterol compounds as $[M - H_2O + H]^+$ ions. In fact, sterols easily ionize in positive mode due to the presence of a hydroxyl group, which after protonation, is eliminated as a neutral loss of water. Considering the non-polar characteristics of the compounds found in the investigated fraction, also hexane was tested as a spray solvent, which also allowed the detection of squalene (*m/z* of 411). The use of hexane causes, on the other hand, a decrease in the sterols ion intensities; for this reason, methanol was selected as the spray solvent. Several paper spray tandem mass spectrometry experiments were performed to identify the main ions present in the full scan spectra: *m/z* 383, *m/z* 395, *m/z* 397, *m/z* 409, *m/z* 423, *m/z* 425, m/z 441, and *m/z* 443. The first four ions show the typical fragmentation pattern of sterols, while the MS/MS spectra of the ions at *m/z* 423, 425, 441, and 443 are characterized by very few product ions. It was possible to confirm that $[M - H_2O + H]^+$ ions at *m/z* 383 and *m/z* 397 are relative to campesterol and the β -Sitosterol, respectively. In fact, for both ions, some characteristic

fragments were detected, the product ion at *m/z* 243, which derives from the breaking of the D ring, and the fragment ions at *m/z* 161 and 147 from the breaking of the C ring in two different positions (**Figure 4.1.2**).

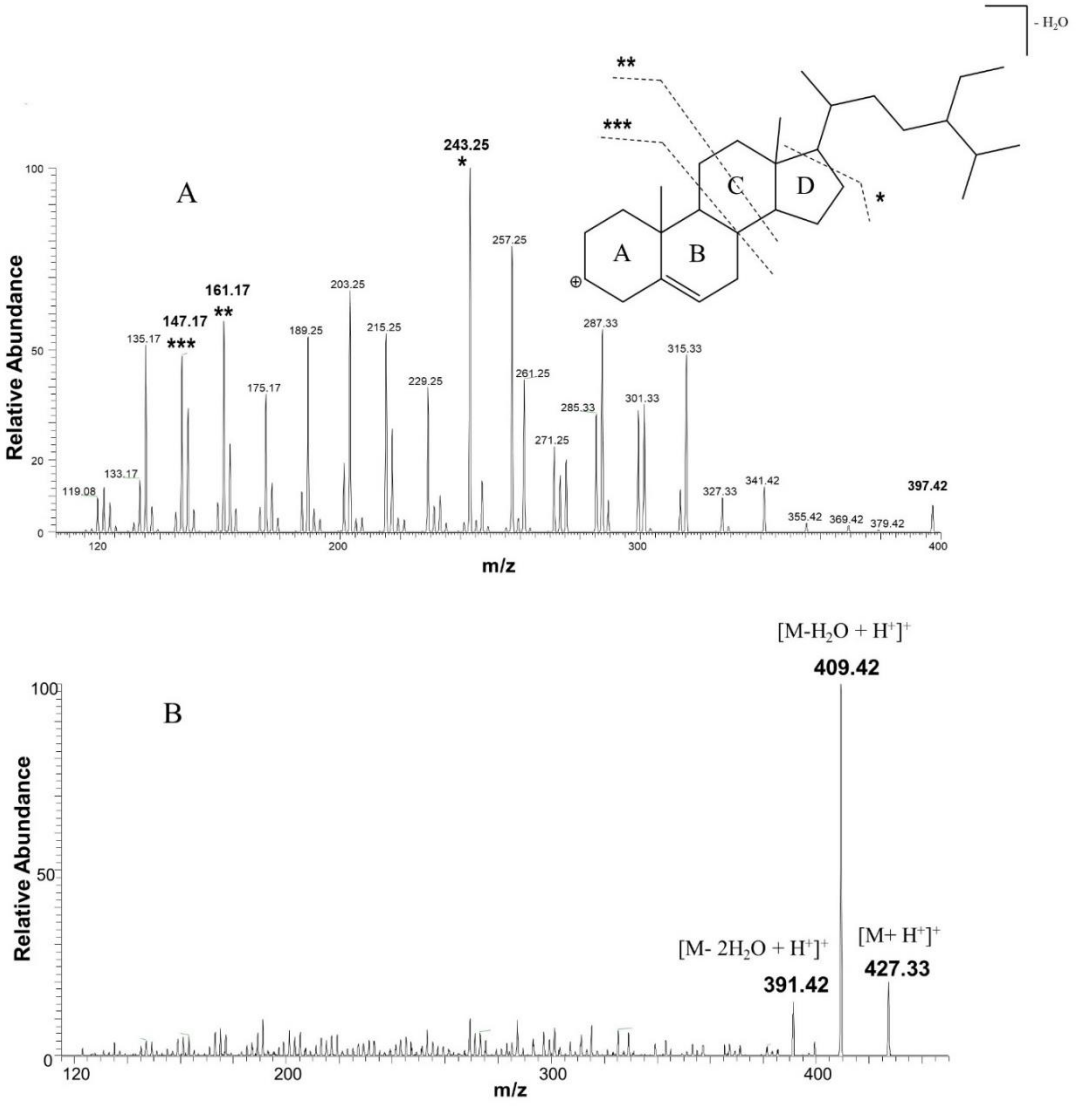

Figure 4.1.2. PS (+) MS/MS spectra of m/z 397 (A) and m/z 441 (B).

The ion at *m/z* 395 could be related to fucosterol, stigmasterol and 5-avenasterol, three isobar sterols found in olive oil. The molecular ion at *m/z* 409 is attributable to β-amyrin, cycloartenol, and lupeol. The ion at *m/z* 443 could be referred to as protonated erythrodiol and uvaol ($[M+H]^+$) together with the $[M - H_2O + H]^+$ ion at *m/z* 425, referred to as the same molecules. The same behaviour was observed in the case of the ions at *m/z* 441 and *m/z* 423.

Due to the absence of chromatography, it is only possible to presume the presence of all these compounds as a sum with paper spray experiments. In order to confirm sterols identification, high-resolution mass spectrometry analyses were performed using the HPLC-APCI-TOFMS system together with available standards.

Table 4.1.1 shows the tentative chemical assignment for the main ions detected in the olive oil unsaponifiable fraction. Campesterol (*m/z* 383), fucosterol (*m/z* 395), β-sitosterol (*m/z* 397), and β-amyrin (*m/z* 409) were unambiguously identified not only by accurate mass measurements but also by retention time comparison with standards. The other primary ions detected are related to their isomers.

After PS-MS approach optimization, 63 different olive oil samples were submitted for analysis to obtain an MS fingerprinting in the mass range from *m/z* 300 to 700.

	Standards MIX	Sample					
m/z	**RT (min)**	**Compound**	**RT (min)**	**Observed mass**	**Predicted formula**	**Calculated exact mass**	**Error (ppm)**
383	8,882	campesterol	8,837	383,3679	$C_{28}H_{47}$	383,3672	1,75
395	8,634	fucosterol	8,595	395,3685	$C_{29}H_{47}$	395,3672	3,22
395	-	stigmasterol/Δ-5-avansterol	7,784	395,3674	$C_{29}H_{47}$	395,3672	0,44
397	9,202	β-sitosterol	9,190	397,3842	$C_{29}H_{49}$	397,3829	3,33
409	9,367	β-amyrin	9,306	409,3834	$C_{30}H_{49}$	409,3829	1,28
409	-	cycloartenol/lupeol	8,316	409,3833	$C_{30}H_{49}$	409,3829	1,03
409	-	cycloartenol/lupeol	7,193	409,3835	$C_{30}H_{49}$	409,3829	1,52
423 **441**	-	unknown	9,144 9,144	423,3996 441,4099	$C_{31}H_{51}$ $C_{31}H_{53}O$	423,3985 441,4091	2,06 1,83

Table 4.1.1. Compounds detected by LC/high resolution MS experiments.

3.2 Unsupervised and supervised chemometric approaches for discriminative purposes.

The MS fingerprint of olive oil unsaponifiable fraction, obtained as previously described, has been used to discriminate between oil samples belonging to different commercial categories, EVOO, VOO and POO. Several multivariate analyses were performed using sixty-three samples. Each sample was submitted to PS-MS analysis five times, providing an MS spectrum of 400 total variables (*m/z* values). Before performing the statistical analysis, it was necessary to perform a pre-processing of

the data: the abundance of each detected ion was normalized to the sum of the intensities of all ions, and the normalized m/z signals were filtered according to the instrumental repeatability value (RSD% < 20%). Finally, a data set consisting of 400 variables for 45 samples has been built for qualitative pattern recognition analysis (PCA and HCA).

Figure 4.1.3 shows the score plot (A) and the loading plot (B) for PS-MS data in the space of the first PCs (PC1 vs PC2 vs PC3). The first three principal components explain 80,4% of the total variance, and it gets very close to 100% by the tenth PC. Good discrimination among the three different olive oil categories can be observed. PC1 highlights the separation between POO samples and EVOO and VOO samples. The first ones are placed at positive values of PC1, while the others are mainly at negative values along PC1, even if, in the centre, there is a slight overlapping of a sample belonging to all three categories. The difference between EVOO and VOO comes from PC3; in this case, EVOO samples are placed at positive values of PC3 and VOO samples at negative ones. From the loadings plot, it is possible to note that few ions influence the PCs and allow the formation of group trending. For PC1, which accounts for 52,6% of the total variance, *m/z* 397 (β-sitosterol) together with *m/z* 443 and 425, tentatively $[M+H]^+$ and $[M-H_2O+H]^+$ of erythrodiol and uvaol, determine the discrimination between EVOO-VOO and POO samples. Indeed, pomace olive oils present a considerable higher content of erythrodiol and uvaol than virgin olive oil (Lukic et al., 2021; Jabeur et al., 2017). The separation between EVOO and VOO, instead, occurs on PC3 (13,4 % of variance), where *m/z* 423 and *m/z* 441 have the biggest loading values followed by β-sitosterol. These compounds haven't been identified, and moreover the EEC 2568/91 reports the same parameters for the sterol composition of EVOO and VOO. The group trending, in this case, could be an outcome of the total specific fingerprint of the sterol fraction for the two different commercial categories highlighting its potential power in allowing a discrimination. While *m/z* 443 and 425, tentatively $[M+H]^+$ and $[M-H_2O+H]^+$ of erythrodiol and uvaol, have their major influence on PC2 (14,3% of variance) but do not play any role in the discrimination process.

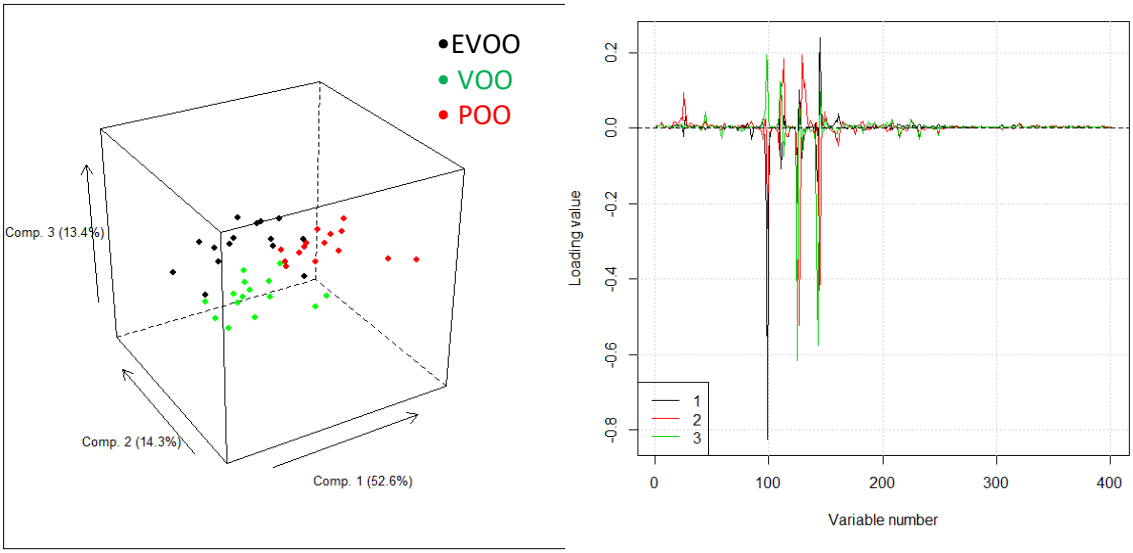

Figure 4.1.3. PCA scores plot (A) and loading plot (B) of the PS(+)-MS spectra (45 samples, 400 variables) (80,4% of the total variance).

In addition to Principal Component Analysis, Hierarchical Cluster Analysis was carried out. **Figure 4.1.4** shows the dendrogram obtained performing the analysis on the first 20 PCs coming from PCA. Based on their PS-MS fingerprint, the 45 olive oil samples could be divided into four classes. Cluster 1 comprises 86% of VOO samples and the remaining 14% of EVOO samples, while Cluster 2 has contributions from all three categories; it is 21% of POO, 57% of EVOO, and 21% of VOO. Cluster 3 is very small, composed of 100% of EVOO samples, whereas Cluster 4 consists of only POO samples (100%). Cluster 2 composition agrees with the PCA outcome, where overlapping of some samples was observed. The results of the two unsupervised techniques (PCA and HCA) permit us to affirm that PS-MS fingerprints can be used to discern between the different olive oil categories investigated. The following step was the development of a classification method involving additional multivariate techniques. Linear Discriminant Analysis (LDA) and k-Nearest Neighbour (k-NN) were the two classification methods selected and compared. The two algorithms were tested and evaluated using a training set and external samples set (test set), considering the first 20 PCs gained after PCA analysis.

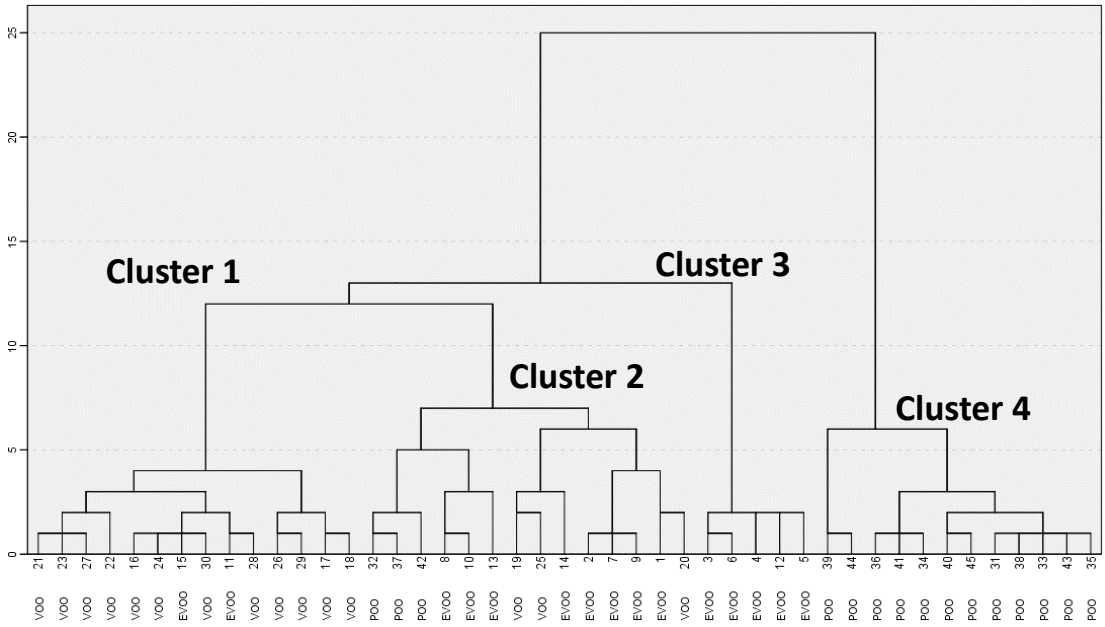

Figure 4.1.4. Hierarchical Cluster Analysis (HCA) based on PS(+)-MS fingerprints of EVOO, VOO and POO samples.

The models' validation was achieved by repeated five-fold cross-validation on training set matrices. The k-NN and PCA-LDA correctly classified the samples into their categories with a total correct prediction of 91.1 % and 93.3%, respectively. For k-NN applied to the external samples (test set 18 samples), the overall precision was the following: 83% for EVOO, 80% for VOO and 86% for POO; and accuracy results were: 89% for EVOO, 83% for VOO and 94% for POO. The mismatch samples were one EVOO and two VOO samples; in particular, the extra virgin olive oil (T6) was classified as the minor category VOO, while one of the VOO samples (T7) was identified as EVOO and the other one (T8) as pomace olive oil. On the other hand, PCA-LDA provides for precision, 83% for EVOO, 83% for VOO, 100% for POO and accuracy, 89% for EVOO, 89% for VOO and 100% for POO. In this case, two test set samples were mismatched, an EVOO sample classified as VOO (T4) and a VOO one (T7) classified as EVOO. In general, the best classification was obtained by the PCA-LDA model. More information about the classification can be found in **Table 4.1.2**.

No	True label	Predicted label		Classification	
		K-NN	PCA-LDA	K-NN	PCA-LDA
T1	EVOO	EVOO	EVOO	Match	Match
T2	EVOO	EVOO	EVOO	Match	Match
T3	EVOO	EVOO	EVOO	Match	Match
T4	EVOO	EVOO	VOO	Match	Mismatch
T5	EVOO	EVOO	EVOO	Match	Match
T6	EVOO	VOO	EVOO	Mismatch	Match
T7	VOO	EVOO	EVOO	Mismatch	Mismatch
T8	VOO	POO	VOO	Mismatch	Match
T9	VOO	VOO	VOO	Match	Match
T10	VOO	VOO	VOO	Match	Match
T11	VOO	VOO	VOO	Match	Match
T12	VOO	VOO	VOO	Match	Match
T13	POO	POO	POO	Match	Match
T14	POO	POO	POO	Match	Match
T15	POO	POO	POO	Match	Match
T16	POO	POO	POO	Match	Match
T17	POO	POO	POO	Match	Match
T18	POO	POO	POO	Match	Match

Table 4.1.2. Samples classification for k-NN and PCA-LDA on paper-spray mass spectrometry analysis.

3.3 Confirmation with direct infusion APCI-TOFMS analysis.

As confirmation, the same multivariate procedure was applied using MS data obtained from direct infusion APCI-TOF analysis. The reason lies in the need to understand whether the prediction percentages from the PS-MS method are good. The same 63 investigated samples were diluted and submitted to APCI-TOF analysis through direct infusion injection. For each sample, three different MS acquisitions were carried out. The outcomes of PCA and HCA analyses are shown in **Figure 4.1.5** and **Figure 4.1.6**. The first 3 components explain 61.2% of the total variance, but the first 2 (PC1 37,3% and PC2 12,3%) permit good discrimination between the three

categories. PC1 allows to separate POO, EVOO, and VOO samples; the first ones are situated at positive values of PC1, while the other 2 categories are at negative values.

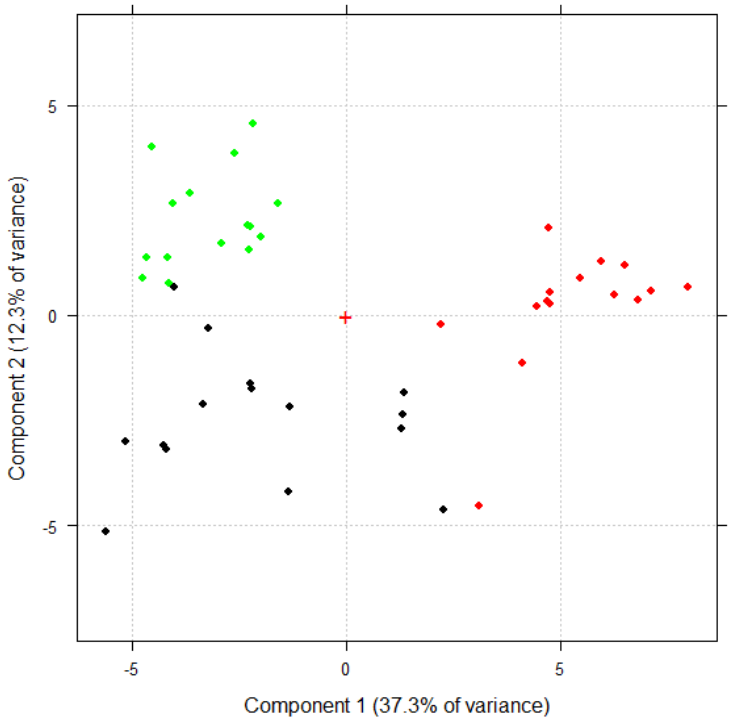

Figure 4.1.5. PCA scores plot of the APCI-TOF spectra (49,6% of the total variance)

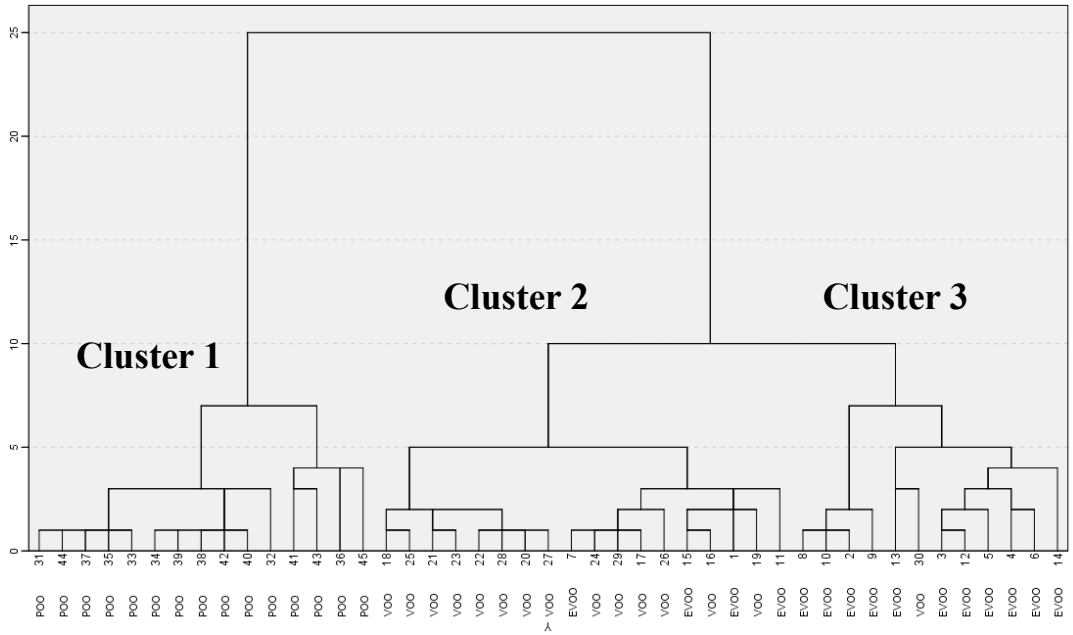

Figure 4.1.6. Hierarchical Cluster Analysis (HCA) based on APCI-TOF experiments of EVOO, VOO and POO samples.

This is consistent with the PCA analysis on PS-MS data. The separation between EVOO and VOO happens on PC2. VOO samples are located at positive values and EVOO at negative ones. Regarding HCA analysis, the 45 olive oil samples could be divided into 3 classes. Cluster 1 is composed of 100 % of POO samples, while Cluster 2 is composed of 78% of VOO samples, and the remaining 22% are EVOO. Ultimately, Cluster 3 is almost characterized by EVOO samples (92% EVOO and 8% VOO). Then, as in the PS-MS method, k-NN and PCA-LDA were applied. After 5-fold cross-validation (training set 45 samples), the correct prediction was 93,3% for both supervised statistical approaches. When performing k-NN and PCA-LDA on the external samples (test set 18 samples), the overall precision and accuracy results show, also in this case, slightly better results for PCA-LDA. Accuracy: 100% for EVOO, 94% for VOO and 95% for POO. Only 1 sample (T16) is mismatched; indeed, it is a pomace olive oil, and it is classified as VOO. More information is provided in **Table 4.1.3** and **Table 4.1.4**.

	Precision (%)		**Accuracy (%)**	
	K-NN	**PCA-LDA**	**K-NN**	**PCA-LDA**
EVOO	100	100	94	100
VOO	75	86	89	94
POO	100	100	95	95

Table 4.1.3. Precision and Accuracy values for APCI-TOFMS analysis

No	True label	Predicted label		Classification	
		K-NN	PCA-LDA	K-NN	PCA-LDA
T1	EVOO	EVOO	EVOO	Match	Match
T2	EVOO	EVOO	EVOO	Match	Match
T3	EVOO	EVOO	EVOO	Match	Match
T4	EVOO	EVOO	EVOO	Match	Match
T5	EVOO	EVOO	EVOO	Match	Match
T6	EVOO	VOO	EVOO	Mismatch	Match
T7	VOO	VOO	VOO	Match	Match
T8	VOO	VOO	VOO	Match	Match
T9	VOO	VOO	VOO	Match	Match
T10	VOO	VOO	VOO	Match	Match
T11	VOO	VOO	VOO	Match	Match
T12	VOO	VOO	VOO	Match	Match
T13	POO	POO	POO	Match	Match
T14	POO	POO	POO	Match	Match
T15	POO	POO	POO	Match	Match
T16	POO	VOO	VOO	Mismatch	Mismatch
T17	POO	POO	POO	Match	Match
T18	POO	POO	POO	Match	Match

Table 4.1.4. Samples classification for k-NN and PCA-LDA on APCI-TOFMS analysis

The precision and accuracy values resulting from the application of both techniques are very similar and near 100%, confirming the reliability of the proposed streamlined PS-MS method for olive oil discriminative purposes (**Table 4.1.5**).

Model	Method	Overall Precision (%)		Overall Accuracy (%)	
Three-class model: EVOO, VOO, POO		k-NN	PCA-LDA	k-NN	PCA-LDA
	PS-MS	83	89	89	93
	APCI-TOF	92	95	93	97

Table 4.1.5. Comparison of the results of the different classification methods for both PS(+)-MS and Direct infusion APCI-TOF data.

4. Conclusions

The importance of the study lies in combining the advantages of mass spectrometry with little or absent sample preparation requirements. In this work, PS-MS was successfully applied for the first time for streamlined discrimination of different olive oil commercial categories. Indeed, two different techniques, two distinct ionization methods, and mass spectrometry resolutions were compared with the same good outcome. Different unsupervised (PCA, HCA) and supervised (k-NN, LDA) chemometric procedures have been applied successfully. The information extracted from the paper spray MS fingerprinting allowed the discrimination between three categories of olive oil (EVOO, VOO, and POO) with a high percentage of prediction. The performance of PCA-LDA was better than the no linear k-NN, but only by 4% for overall accuracy. PS-MS is successfully used for a discriminative purpose. Indeed, it permitted to obtain, using PCA-LDA, a 90% in precision and 95% in accuracy, for the classification of EVOO, VOO, and POO samples. Despite being an ambient mass spectrometry technique, the quality of the method stands out when compared with the APCI-HRMS results. Moreover, the comparison with classical high-resolution mass spectrometry analysis allowed us to confirm the goodness of the developed methodology.

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Part II.

Paper Spray Mass Spectrometry for quality assessment of wine.

Abstract

The present research explores the potential of a fast approach based on Paper Spray Mass Spectrometry (PS-MS) supported by an untargeted data analysis strategy to enhance the information provided by molecular profiling of wine. A total of 114 samples of several commercial wines from different geographical origins were analysed using this innovative workflow. Multiple-level information from the molecular profile of the wines was obtained, allowing the characterisation of a southern Italian brand of wine, "Terre Grecaniche", compared to several other types of commercial wine. Furthermore, the same dataset also provided interesting information regarding the wine categories within the same brand.

1. Introduction

Wine is globally renowned as one of the most popular and widely consumed alcoholic beverages. It is produced through the fermentation of fresh grapes and possesses a rich and enduring tradition, particularly in Mediterranean nations. The United States, France, Italy, Germany, and China stand as the primary consumers of wine, collectively consuming an average of 120 million hectolitres each year. The enjoyment of savouring wine and the deep-rooted cultural traditions in certain countries are among the driving factors that entice consumers to choose it over other alcoholic beverages (Gutierrez-Escobar et al., 2021).

Italy, in particular, holds a prominent position on the global stage in viticulture and oenology. It ranks fourth worldwide with 705,000 hectares of vineyards, secures the second spot in grape production with 8.6 million tons, takes the lead in wine production with 54.8 million hectolitres, and reports wine consumption of 22.4

million hectolitres, as indicated by the International Organisation of Vine and Wine (OIV) Focus for 2018 (Arapitas et al., 2020).

Assessing the quality of wine involves a plethora of factors rooted in the chemical constituents of the beverage. These parameters include color, flavour, sweetness, and dryness, all of which significantly contribute to the overall quality. In general, wine is composed of several key components, such as alcohol, sugars, acids, tannins, minerals, proteins, and various other compounds like organic acids, volatile compounds, and phenolic compounds (Garrido and Borges, 2013).

Organic acids are primary metabolites found in grapes, and analyzing them in wines is imperative for quality control and tracking the changes in acidity throughout the various stages of winemaking. Significant alterations in wine can be identified through shifts in the acid content, making this analysis an essential aspect of the winemaking process. The presence of acetic acid, for example, can serve as an indicator of microbiological changes or the utilization of low-quality raw materials in the preparation of these products (Monteiro Coelho et al., 2018; Silva et al., 2015; Lima et al., 2014; Robles et al., 2019). Moreover, the level of phenolic maturity in grapes at harvest is a significant factor that greatly influences the quality of red wine. Many sensory characteristics of wine, such as astringency and bitterness, are closely linked to the composition of anthocyanins and proanthocyanins. This composition is heavily impacted by the level of ripeness achieved (Kontoudakis et al., 2011).

Bearing in mind the high marketing value that wine possesses within the Mediterranean countries, its adulteration by fraudulent practices remains a timely concern. There is a need for appropriate analytical methods to gain deeper insights into the chemical composition and its changes associated with adulteration (Versari et al., 2014).

Since factors such as origin, grape variety, winemaking technique and ageing process are often associated with the final price of the products, there is a growing interest in establishing links between wine physicochemical properties and their oenological attributes. Traditional assessment of sensory parameters are frequently conducted by a panel of expert tasters. However, this approach may become impractical when dealing with a large number of samples. Therefore, more streamlined strategies

based on analytical methods may be necessary to efficiently evaluate and compare wines (Serrano-Lourido et al., 2012). The most well-established analytical methods involve profiling trace elements, phenolic compounds and volatiles. These analyses are usually conducted using a variety of chromatographic and spectroscopic techniques (Versari et al., 2014). Among them, HPLC-MS and GC-MS have been widely employed to determine the composition and identify the difference between wines, not only with a targeted approach but also with an untargeted one (Arapitsas et al., 2020; Flamini et al., 2013). Indeed, for wine discrimination, the integration of instrumental analyses with chemometrics approaches has proven to be effective in categorizing different wine products based on their grape varieties, geographical origins, and specific aspects of the winemaking process. Multivariate statistical techniques are a potent tool for examining and classifying grapes and wines using large datasets containing chemical or sensory information (González & Peña-Méndez, 2000).

However, the utilization of classical methods can be time-consuming, mainly due to the sample pre-treatment and the time needed for the chromatographic separation. It would be preferable for the analytical methods employed not to require complicated sample pre-treatment to keep the time for both chemical analysis and chemometrics as brief as possible (De Beer et al., 2004; Villagra et al., 2012).

For this reason, Ambient Mass Spectrometry represents a valid option due to the very rapid determination because of the direct ionization of analytes in their native environment. Among the AMS techniques, Paper Spray Mass Spectrometry (PS-MS) is one of the most cost-effective and straightforward ionization sources due to its simple setup comprising a metallic clip and a paper substrate. In a PS-MS experiment, a relatively modest sample volume is placed onto the paper in front of the mass spectrometer inlet, and the ionization is prompted by applying a high voltage and a spray solvent.

The PS-MS approach has already been employed in red wine analyses. Good examples include the quantification of resveratrol (Di Donna et al., 2017) and the determination of multiple pesticides (Guo et al., 2019). Moreover, the capability of PS-MS of analysed wine for quality purposes has been compared with other ambient

mass spectrometry techniques. It has provided the most informative and discriminative MS profile with more compounds and higher signal intensity (Bartella et al., 2022). For this reason, the present study has opted for this approach as the method to attempt the differentiation of Terre Grecaniche wines from other commercial varieties, employing an untargeted fingerprinting approach.

2. Experimental

2.1 Chemicals and reagents

All solvents used, methanol, ethanol and formic acid, were analytical grade and commercially available (Sigma-Aldrich, St. Louis, MO). Ultrapure water was obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA).

2.2 Wine samples

Commercial wines (N = 57) were purchased from a local supermarket (Rende, Italy). Southern Italian territory brand wines were kindly provided by Terre Grecaniche - Italy (N = 57). A detailed list of samples is reported in **Table 4.2.1**. All wine samples were stored in amber glass bottles at 4°C and submitted to MS analysis as raw samples.

WINE NAME	YEAR OF PRODUCION	ORIGIN	NUMBER OF BOTTLES
Terre Grecaniche			
ARANGHIA	2015	Palizzi (RC)	8
ARANGHIA BIO	2017	Palizzi (RC)	7
ARANGHIA BIO	2018	Palizzi (RC)	7
ARANGHIA BARRIQUE	2014	Palizzi (RC)	7
HERAKLION	2015	Palizzi (RC)	6
HERAKLION	2016	Palizzi (RC)	7
HERAKLION BIO	2017	Palizzi (RC)	8
CATOI BIO	2018	Palizzi (RC)	7
Commercial wines			
CL 2019	2019	Cirò Marina (KR)	6
DL 2019	2019	Lamezia Terme (CZ)	8
AC 2019	2019	Reggio Calabria	6
CH 2019	2019	Toscana	8
LR 2020	2020	Sicilia	8
DUE 2018	2018	Puglia	7
DUE 2019	2019	Puglia	7
NT 2019	2019	Puglia	7

Table 4.2.1. List of samples belonging to Terre Grecaniche and Commercial wines.

2.3 PS-MS analysis

MS fingerprints of investigated wines were obtained using a triple quadrupole mass spectrometer TSQ Quantum Vantage (Thermo Fisher Scientific, San José, CA) equipped with an open source in-house implemented for paper spray MS applications. This source is composed of a small triangle of paper to support the sample, held in front of the mass spectrometer inlet through a metal clip used to apply a high voltage to the paper. Paper spray ionization occurred according to the following steps: 15 µL of each sample were deposited onto a paper triangle held in front of the mass spectrometer inlet and left to dry. A high voltage was applied, and 15 µL of methanol, employed as spray solvent, was added to allow the ions spray desorption every 15 s for a total acquisition time of 2 min. MS measurements were performed in full-scan mode within the mass range of 100 - 900 amu using negative ionization polarity. PS-MS working conditions were the following: needle voltage – 5 kV; capillary temperature 290 °C. For MS/MS investigations, several experiments were performed at different collision energies (CE), from 25 to 35 eV, using argon as collision gas at a pressure within the collision cell (Q2) of 1.5 mTorr. The mass resolution at the first (Q1) and third (Q3) quadrupole was set at 0.7 amu at full width at half-maximum (FWHM). Scan time was set at 0.5 sec.

2.4 LC-HRMS analysis

Wine samples were also submitted to high-resolution MS and tandem MS experiments using a Vanquish HPLC system coupled with an Exploris 120 high-resolution mass spectrometer (Thermo Fisher Scientific, San José, CA, USA). The chromatographic separation was carried out employing an Accucore C18 reversed-phase column (100 × 2.1 mm, 2.6 µm, Thermo Scientific, Bremen, Germany) maintained at 30°C. The elution was performed under gradient conditions using 0.1% formic acid in water (solvent A) and methanol (solvent B). The gradient was built as follows: 5% B for 1 min, from 5% to 30% B in two min, 30% B for 2.5 min, from 30% to 50% B in 3 min, 50% B for 4 min, from 50% to 60% B in 1.5 min, 60% B for 1.5 min, from 60% to 70% B in 1.5 min, 70% B for 1.5 min, from 70% to 95% in 1 min, remain at 95% B for 5 min, from 5% to 5% B in 0.5 min, finally an isocratic flow (5 min) to

equilibrate the system. The injection volume was 2 µl. The run time was 30 min, and the flow rate was set at 0.3 ml/min. All MS measurements were performed both in negative and positive ionization mode with the following working conditions: spray voltage was 3.5 kV and 3.3 kV in positive and negative polarity, respectively; ion transfer tube and vaporizer temperature were both 290°C; sheath gas and auxiliary gas flow rate were 40 and 10 units, and S-lens value was 70% of the maximum value. Full-scan spectra were recorded in the mass range 100-1000 m/z with a resolution of 120,000, and the molecular formulae were obtained by Excalibur software (Thermo Fisher Scientific, San José, CA, USA).

2.5 Data acquisition and statistical analysis

Three PS-(-)-MS fingerprints were acquired for each wine sample. The total ion current coming from PS-MS analysis was averaged over the total scan time (2 min), and the abundance of each detected ion, significantly different from the blank sample, was normalised to the sum of all ion abundances in the investigated mass range. The normalised m/z signals were filtered based on instrumental repeatability value (RSD% < 20%) to eliminate insignificant masses and reduce the number of variables from 800 to 623. After performing a first explorative PCA using CAT software (R. Leardi, C. Melzi, G. Polotti, CAT (Chemometric Agile Tool), freely downloadable from http://gruppochemi ometria.it/index.php/software), the proprietary feature selection algorithm in ChromCompare+ software (SepSolve Analytical, Peterborough, UK) was used to perform data reduction. This algorithm uses a multivariate method to assess the covariance between features. This allowed the top 25 most significant features of the known sample classes to be found. Furthermore, a Hierarchical Cluster analysis was performed with the hclust function in package stat. using MetaboAnalyst 5.0 (Pang, Chong et al. 2021). The heatmap was column normalized, and each feature underwent a square root transformation to make feature intensity more comparable.

3. Results and discussion

3.1 Molecular fingerprinting of wines by PS-MS

The primary objective of this study was to establish a quick and effective method for distinguishing between various wine brands, ultimately enhancing the qualitative value of these products by ensuring their authenticity. The methodology, in particular, was optimized using a set of red wine samples belonging to a Calabrian winery, "Terre Grecaniche".

The project was based on testing Paper Spray Mass Spectrometry to produce specific wine fingerprints with the widest molecular coverage in non-targeted mode. The further point was to evaluate the capability of the data set produced by PS-MS analysis coupled with chemometric methods to obtain a statistical model for wine differentiation and classification. Paper spray MS was selected among different ambient ionization sources based on the results obtained from our previous work, which highlighted the ability of this Ambient MS source to produce the best molecular profiles in terms of molecular detection and signal intensity (Bartella et al., 2022). Moreover, Paper Spray is one of the simplest and least expansive Ambient MS sources, composed of just a small paper triangle placed in a metallic clip with the tip in front of the mass spectrometer inlet. The experimental conditions were selected based on our previous published paper; the analyses were performed using the raw samples without pretreatment or matrix dilution. Using negative ionisation mode, themolecular fingerprints were acquired under full-scan MS conditions in the mass range between 100 and 900 uma. The negative polarity was chosen since it showed a better ionization efficiency over the whole mass range analyzed due to the acidic nature of the more abundant compounds in the wine matrix.

114 red wines were subjected to PS-MS analysis: 57 samples belong to Terre Grecaniche winery, while the other 57 samples are commercial wines from different Italian regions.

A typical PS-MS profile of a red wine from Terre Grecaniche is shown in **Figure 4.2.1**. From the spectrum, it is possible to observe that the most intense ions are present in the mass range between 100 and 300 amu, and are related to deprotonated molecules $[M-H]^-$ of wine-characteristic organic acids, such as succinic acid (m/z 117),

malic acid (m/z 149), tartaric acid (m/z 149), isopropylmalic acid (m/z 175), citric acid (m/z 191), galacturonic acid (m/z 193) and gluconic acid (m/z 195).

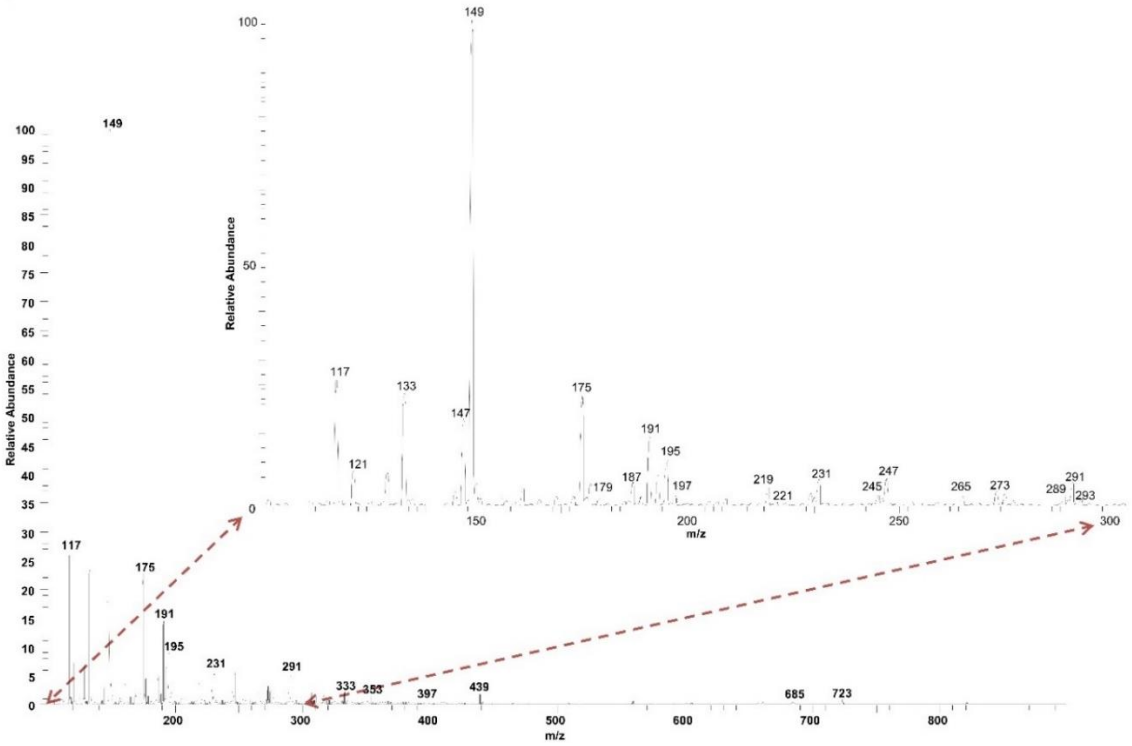

Figure 4.2.1. PS-(-)-MS spectrum of a red wine from Terre Grecaniche winery.

3.2 Application of PS-MS for quality assessment of wine

The MS fingerprints of the red wines, generated using the paper spray experiments as previously described, were subjected to unsupervised chemometric analysis to determine if they exhibited any distinctive qualitative patterns. Following the data pre-processing steps, an initial dataset was created, comprising 623 variables for 114 samples, which was then analyzed using Principal Component Analysis (PCA). The resulting PCA score plot revealed a clear and distinct separation into two clusters. One cluster corresponded to the commercial wines used in the study, while the other cluster represented the wines produced by the Terre Grecaniche winery. Given that the fingerprint approach involved a high number of variables, efforts were made to reduce this dimensionality and extract more meaningful information from the PCA results. In particular, focus was placed on the first 25 variables responsible for the discrimination between these two classes. These variables have been selected applying an algorithm and they are, in order: m/z 155, m/z 156, m/z 179, m/z 220,

m/z 221, m/z 223, m/z 427, m/z 577, m/z 578, m/z 366, m/z 545, m/z 574, m/z 369, m/z 206, m/z 111, m/z 193, m/z 385, m/z 573, m/z 516, m/z 191, m/z 224, m/z 383, m/z 202, m/z 501, m/z 446. Among these variables, there are mass values that may correspond to typical compounds found in red wine, as previously discussed. It is worth noting that adjacent mass values could potentially relate to the same compound, a consequence of the limitations in the nominal mass output process of the low-resolution mass spectrometer.

The output of PCA analysis performed using these 25 variables is reported in **Figure 4.2.2**. It shows the score plot (A) in two dimensions and (B) in three dimensions in the space of the first PCs (PC1 vs PC2 vs PC3) for the 25 variables selected.

The first three principal components explain 77,3% of the total variance, and it gets very close to 100% by the eighth PC. The discrimination between the two groups of samples is very clear, and it occurs mainly on PC1, which accounts for 48,1% of the total variance. Indeed, the samples belonging to the group Terre Grecaniche are located at positive values of PC1, while all the other commercial wines have negative values of PC1.

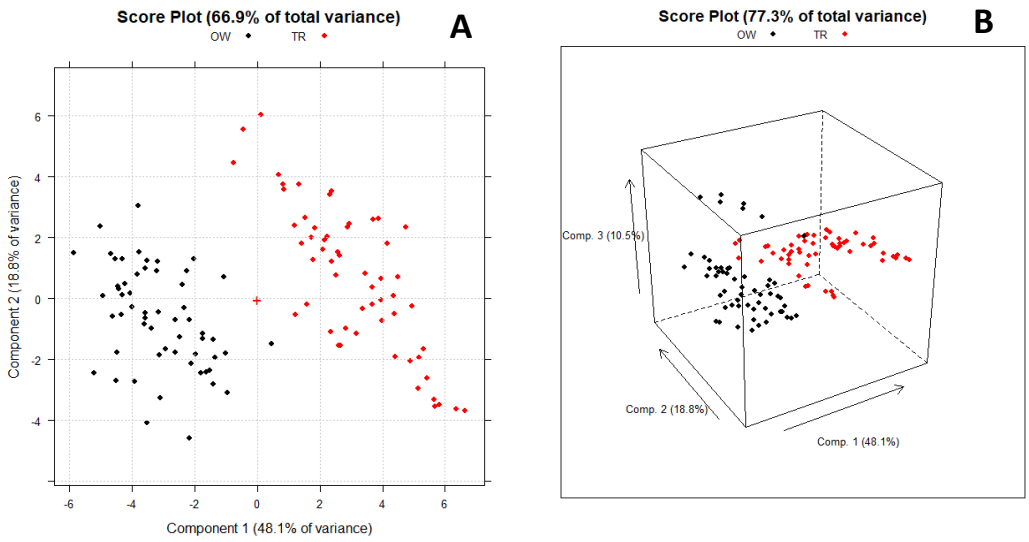

Figure 4.2.2. PCA score plot (A) in two dimensions and (B) in three dimensions of the PS(-)-MS spectra (114 samples, 25 variables)(77,3% of the total variance).

Looking at the loading plots in **Figure 4.2.3**, it is possible to recognise the m/z values determining the discrimination. Since the differentiation of the two groups occurs on PC1, all the variables have an influence on that PC and allow the formation of group

trending. Only m/z 202 has a loading very close to zero on PC1. Its primary influence is on PC2 but does not play any role for this discrimination purposes.

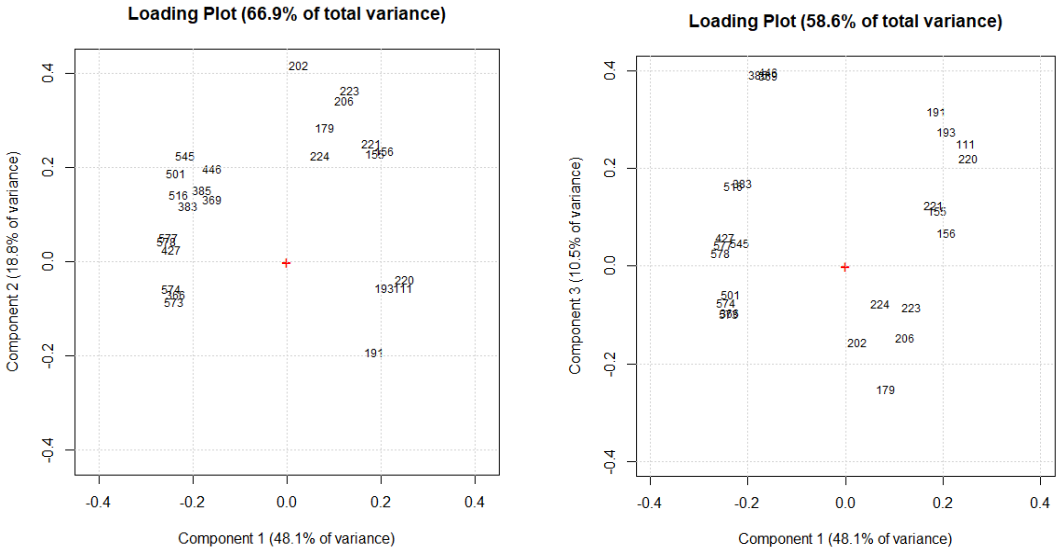

Figure 4.2.3. Loading plots of PS-(-)-MS of the 25 variables in the space of PC1, PC2 and PC3.

The masses characterizing the most the samples belonging to Terre Grecaniche are common organic acids such as m/z 191 which could be $[M-H]^-$ of citric acid, m/z 193 that can be either $[M-H]^-$ of galacturonic acid and $[M-H]^-$ of glucuronic acid, m/z 111 which tentative identification could be $[M-H]^-$ of furoic acid and m/z 221 or $[M-H]^-$ of ethyl glucuronide, a metabolite product of ethanol, which has been found as naturally occurring in wine (Muller and Iwersen-Bergmann, 2018). On the other hand, the wine samples belonging to the group of other wines are predominantly characterized by higher values of m/z. Between them it is possible to find m/z 577, maybe $[M-H]^-$ of Procyanidin B1, B2, B3 and B4, m/z 427 which could correspond to $[M-H]^-$ of gallic acid-O-exoside and m/z 545 that could be related to $[M-H]^-$ of 4'-O-Methylellagic acid 3-(2'',3''-diO-acetyl)-α-L-rhamnoside, a derivative of gallic acid.

Moreover, looking at the 3D score plot in **Figure 4.2.2.**, it is possible to notice how, also between the same class, there is the formation of smaller independent clusters. This can be better visualized in **Figure 4.2.4**, where each typology of wine is identified with an exclusive colour label.

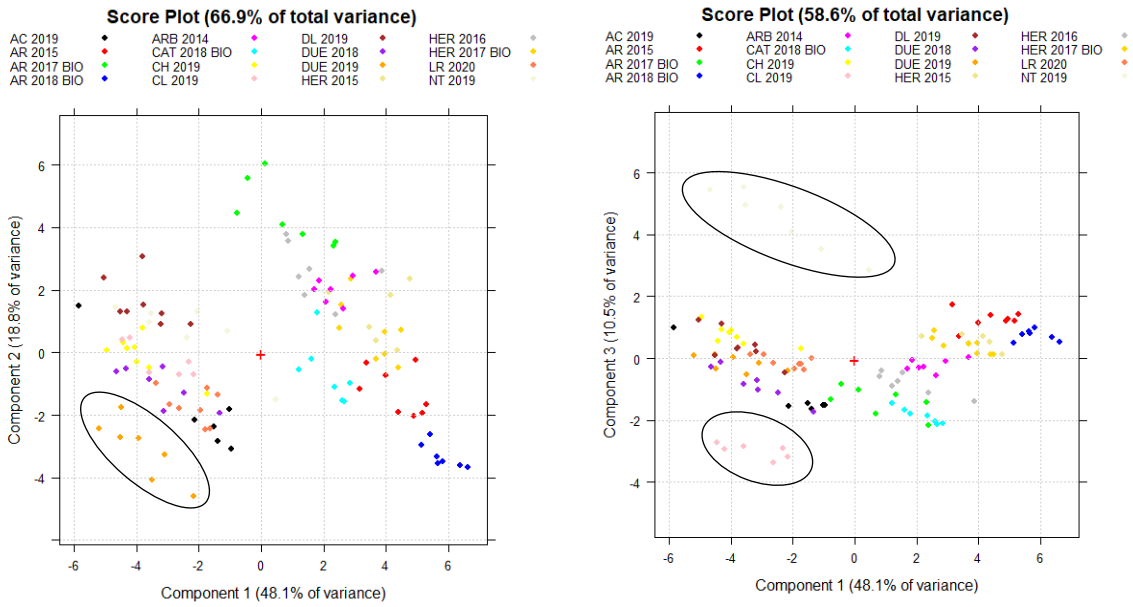

Figure 4.2.4. PCA score plot of the PS(-)-MS spectra highlighted for each kind of red wine (114 samples, 25 variables)(77,3% of the total variance).

Many factors get involved when dealing with the authenticity and traceability of wines. These factors include variations in the geographical regions where the grapes are cultivated, the diversity in grape varieties employed, and the various stages of production and storage. All of these elements can significantly impact the quality of wine and are reflected in the results of the PCA analysis. Considering the only commercial wine category, PC2 (18,8%) and PC3 (10,5%) allow the discrimination of some samples, like the wine DUE 2019 labelled with orange, but also CL 2019 (pink) and NT 2019 (grey). It is an expected result, considering that these wines come from different Italian regions. Notably, even among the wines of Terre Grecaniche, it is possible to visualize some cluster formation. To emphasise this result, a PCA analysis was conducted only on this category of wine and the result is reported in **Figure 4.2.5**. The first three principal components collectively explain 79.7% of the overall variance. One of the most noticeable outcomes is the significant differentiation between Catoi wine and all other samples. This differentiation primarily occurs along PC2, where Catoi wines are positioned at negative PC2 values, while all other wines cluster around zero or exhibit positive PC2 values. This pronounced distinction is attributed to the production methods employed for the wine and underscores the effectiveness of the PS-(-)-MS fingerprint in facilitating such discrimination.

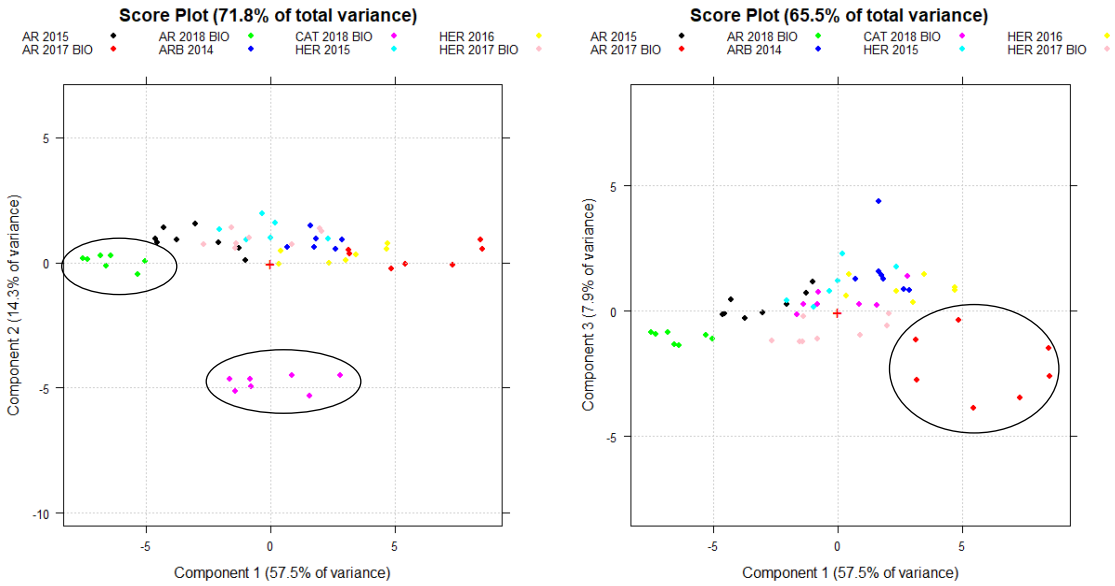

Figure 4.2.5. PCA score plot of the PS(-)-MS spectra for the Terre Grecaniche wine category (79,7% of the total variance).

Indeed, both Catoi and Aranghia wines are crafted from a selection of grape varieties, including Nerello Mascarese, Calabrese, Alicante, and Syrah. However, they differ in terms of their production processes. Another noteworthy factor is the influence of the production year, which is evident in the distribution of Aranghia wines from 2014, 2015, 2017, and 2018 along PC1. Specifically, Aranghia 2018 (depicted in green) and Aranghia 2015 (in black) occupy positions with negative PC1 values, while Aranghia 2014 (in blue) and Aranghia 2017 (in red) are situated with positive PC1 values. In contrast, no significant year-to-year variation is observed in the Heraklion samples despite their utilization of the same grape varieties, except Syrah.

A Hierarchical Clustering Analysis (HCA) was performed using the top 25 most discriminatory features, applying the similarity measure based on Pearson's correlation and the complete linkage where the clustering uses the farthest pair of observations between the two groups. The data are visualised by means of heat-map along with the dendogram (**Figure 4.2.6**). The coloured cells on the map represent the relative intensity of each feature after PQN normalisation and Square root transformation.

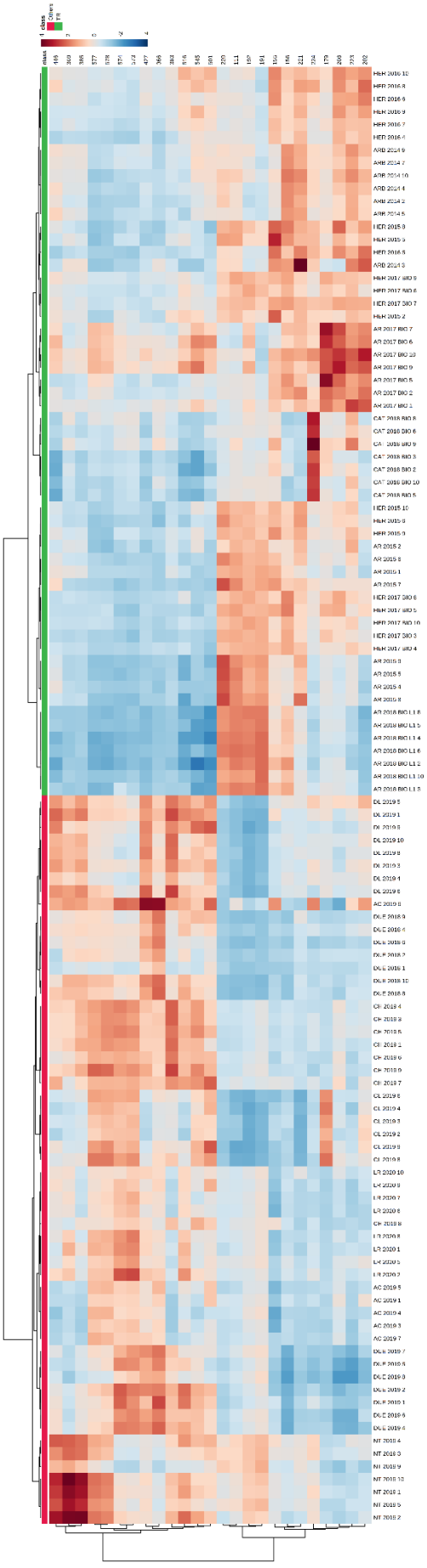

Figure 4.2.6. Hierarchical Clustering Analysis based on Pearson's correlation.

The HCA resulted in two main clusters of features according to the two categories considered. The first one, highlighted with green in **Figure 4.2.6**, correspond to the Terre Grecaniche wine category. This cluster is more characterized by some of the 25 most discriminative variables, such as m/z 111, m/z 179, m/z 191, m/z 193, m/z 221 and m/z 223 whose tentative identification has been reported in *Section 3.3*. Instead, the samples labeled with red belong to the other wines category and the m/z 427, m/z 545 and m/z 577 are higher in this cluster. These results are completely congruent with the ones obtained from principal component analysis.

3.3 Tentative identification of the 25 most influential variables.

Following the chemometric analyses, which facilitated the discrimination between wines from Terre Grecaniche and others, an attempt was made to identify compounds based on the 25 most influential variables. Some experiments were conducted using HPLC-Orbitrap-MS, and **Table 4.2.2** presents the provisional chemical assignment of certain ions responsible for the observed patterns in the PCA analysis. Please note that this identification is preliminary due to the unavailability of standards. It relied on accurate mass measurements and existing literature. Notably, common organic acids such as caffeic acid and citric acid were recognized. Among the higher mass ions, only m/z 545, tentatively representing $[M-H]^-$ of 4'-O-Methylellagic acid 3-(2'',3''-diO-acetyl)-α-L-rhamnoside, and m/z 577, which may correspond to $[M-H]^-$ of one or more of the Procyanidin B1, B2, B3, and B4 compounds, were provisionally identified. For all other masses that remain unidentified, additional experiments, including MS/MS analyses, are required to both confirm and establish their precise identities.

m/z	Compound	Observed mass	Predicted formula	Calculated exact mass	Error (ppm)
111	Furanoic acid	111,0089	$C_5H_3O_3$	111,00767	11,49
179	Caffeic acid	179,0350	$C_9H_7O_4$	179,03389	6,31
191	Citric acid	191,0198	$C_6H_7O_7$	191,01863	6,23
193	Galacturonic acid and Glucoronic acid	193,0355	$C_6H_9O_7$	193,03428	6,28
221	Ethyl glucuronide	221, 0667	$C_8H_{13}O_7$	221,06558	5,19
223	Sinapic acid	223,0612	$C_{11}H_{11}O_5$	223,06010	5,09
427	Gallic acid-O-exoside	427,0342	$C_{13}H_{15}O_{16}$	427,03546	-3,00
545	4'-O-Methylellagic acid 3-(2'',3''-diO-acetyl)-α-L-rhamnoside	545, 9044	$C_{25}H_{21}O_{14}$	545,09258	3,31
577	Procyanidin B1, B2, B3 and B4	577,1355	$C_{30}H_{25}O_{12}$	577,13405	2,51

Table 4.2.2. Tentative identification of some of the most influent variables performed using HPLC-Orbitrap-MS.

4. Conclusions

In this work, a PS-MS fingerprint approach, in combination with chemometrics analysis, has been employed to differentiate between some products of Terre Grecaniche winery and other commercial wines. The PCA results showed the formation of two independent clusters relative to the two categories, and interesting discrimination patterns were observed within the single class of Terre Grecaniche. That highlighted the workflow power of discriminating between wines with different origins, different grape cultivars and different ages, but also different winemaking procedures.

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High-throughput determination of flavanone-*O*-glycosides in citrus beverages by paper spray tandem mass spectrometry

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ABSTRACT

A fast and accurate methodology for the quantification of the most abundant flavanone glycosides in citrus beverages has been developed. The approach relies on the use of paper spray mass spectrometry, which allows to record data in few minutes and without sample pre-treatment. The experiments have been carried out in Multiple Reaction Monitoring scan mode, in order to obtain the best specificity and sensitivity. The analytical parameters were all satisfactory. The results coming from the analysis of real samples were compared to the data obtained by the commonly used chromatographic method, proving the robustness of the proposed approach.

1. Introduction

Citrus plants are widespread in many areas of the world and their fruits are well known for the positive effects exerted on the human health. Their daily intake has been associated to a reduction of many types of diseases, such as cardiovascular related diseases and cancer (Gupta et al., 2015). The juice of citrus fruits, either as is or in small percentages, is employed mainly to produce commercial beverages and soda drinks. The beneficial effects of these fruits can be associated to their chemical composition, which is characterized not only by nutritional compounds, such as vitamins and minerals, but also by many phytochemicals, including flavonoids, an important class of polyhydroxylated aromatic compounds, which are known to exert a strong antioxidant activity and to possess a wide range of biological and health properties (Hertog et al., 1993; Kim et al., 1998; Nijveldt et al., 2001; Hollman, 2001; Aviram & Fuhrman, 2002; Le Marchand, 2002). Generally, in fruits and vegetables, these molecules occur as flavonoid glycosides; in Citrus fruits, the site of glycosylation is located at position 7 of the flavanone unit. The sugar portion is represented by a disaccharide, commonly rutinose (rhamnosyl-(α1 → 6)-glucose) and neohesperidose (rhamnosyl-(α1 → 2)-glucose). Among these, the most representative flavanone-7-*O*-glycosides are: eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, neoponcirin and poncirin (Merken & Beecher, 2000; Rouseff et al., 1987). Furthermore, also "statin-like" flavanones such as melitidin and brutieridin, which may have possible hypocholesterolaemic activity are found in particular Citrus (Di Donna et al., 2011; Fiorillo et al., 2018) (Fig. 1). Due to their health-related properties, these molecules can be considered food quality markers, and, in this context, the development of rapid, accurate and sensitive approaches for their determination assumes great significance. Commonly, the assay of flavonoids is performed by using liquid chromatography coupled with ultraviolet and/or mass spectrometric detection (Belajová & Suhaj, 2004; Gattuso et al., 2007; Di Donna et al., 2013). To date, mass spectrometry has become the most useful technique to obtain specific and sensitive results, in terms of identification, structural characterization and quantitative determination of a wide range of compounds (Bartella et al., 2017, 2018; Beneduci et al., 2019; Di Donna et al., 2018). Moreover, mass spectrometry has rapidly evolved during the past decades, especially with the development of a new class of methodologies, namely ambient ionization techniques. The most important feature of the latter is the rapidity of the MS determination; ambient ionization techniques allow direct analyses of many molecules in different samples and in their native state, with minimal or no sample pretreatment. Among these MS techniques, the most employed are DESI – desorption electrospray ionization, and DART – direct analysis in real time, together with other complementary methods such as PS – paper spray ionization (Bartella et al., 2019a, 2019b; Manicke et al., 2011; Takats et al., 2004, 2005; Taverna et al., 2016;

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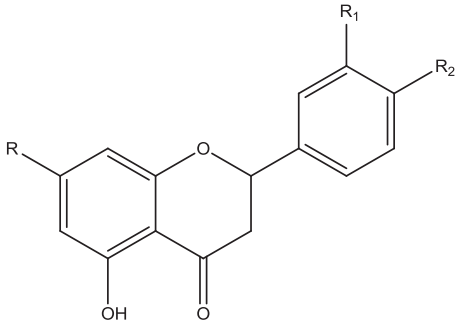

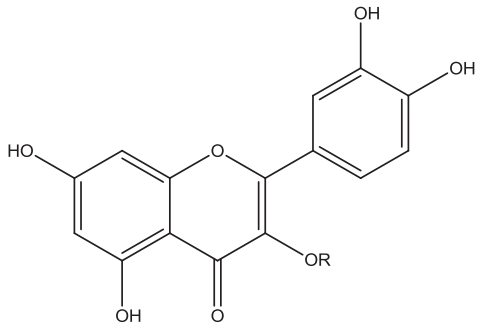

Eriocitrin	R = O-rutinose, R_1 = OH, R_2 = OH
Hesperidin	R = O-rutinose, R_1 = OH, R_2 = OCH_3
Naringin	R = O-neohesperidose, R_1 = H, R_2 = OH
Narirutin	R = O-rutinose, R_1 = H, R_2 = OH
Neoeriocitrin	R = O-neohesperidose, R_1 = OH, R_2 = OH
Neohesperidin	R = O-neohesperidose, R_1 = OH, R_2 = OCH_3
Neoponcirin	R = O-rutinose, R_1 = H, R_2 = OCH_3
Poncirin	R = O-neohesperidose, R_1 = H, R_2 = OCH_3
Brutieridin	R = O-(6"-HMG)-neohesperidose, R_1 = OH, R_2 = OCH_3
Melitidin	R = O-(6"-HMG)-neohesperidose, R_1 = H, R_2 = OH

Quercetin 3-*O*-glucoside R = glucose

Fig. 1. Chemical structures of flavanones and internal standard.

Weston et al., 2005; Wiseman et al., 2008). Paper spray is surely one of the most rapid and cheap ambient ionization tools, especially for its simple set-up. PS-MS ion source consists in a small paper triangle (used to support the sample and positioned in front of the mass spectrometer inlet) to which a high voltage is applied through a metallic clip (Bartella et al., 2020a, 2020b; Wang et al., 2011). Paper spray ionization, as well as other ambient MS techniques, has already been employed in the analysis of beverages: for molecular fingerprinting and quantification of bioactive compounds (Teodoro et al., 2017; Tosato et al., 2018; Silva et al., 2020; Yu et al., 2018), and also to assay particular flavonoids in plant extracts (Zhou et al., 2019; Bagatela et al., 2015). Moreover, there are many papers which add the advantage of high-resolution mass spectrometry to verify the quality and safety of food (Rubert, Zachariasova and Hajslova, 2015). Herein we present an innovative, very fast and time/solvent-saving methodology to assay the most abundant and characteristic flavanone-7-*O*-glycosides in citrus beverages (juices and soda drinks). This approach makes it possible to analyze these quality markers directly in the sample or after a simple dilution, by means of paper spray mass spectrometry and multiple reaction monitoring (MRM) scan mode, using quercetin-3-O-glucoside as internal standard (Fig. 1). The novelty of our method relies on the simplicity of the overall procedure, due to the absence of tedious sample preparation and chromatography, and on the specificity given by tandem mass spectrometry. The whole process takes few minutes: after the addition of the appropriate amount of internal standard, the sample is centrifuged for 3 min and, if necessary, diluted with water. The samples are submitted to PS-MS/MS experiment whose acquisition time is just 2 min; a drop of the diluted beverage containing the analytes and the internal standard is placed onto the paper and the ions are generated by applying a high voltage and adding from time to time (every 30 *sec*) few μL of methanol.

Table 1
Selected gas-phase transitions and optimized instrumental parameters.

Compound	Transition	CE (eV)	S-Lens (V)
Naringin/Narirutin	*m/z* 579 → *m/z* 271 (quan)	30	130
	m/z 579 → *m/z* 459 (qual)	35	
Neoeriocitrin	*m/z* 595 → *m/z* 459 (quan)	35	130
	m/z 595 → *m/z* 287 (qual)	30	
Eriocitrin	*m/z* 595 → *m/z* 287 (quan)	35	130
	m/z 595 → *m/z* 459 (qual)	40	
Neohesperidin/ Hesperidin	*m/z* 609 → *m/z* 301 (quan)	28	130
	m/z 609 → *m/z* 489 (qual)	35	
Poncirin/Neoponcirin	*m/z* 593 → *m/z* 285(quan)	30	130
	m/z 593 → *m/z* 473 (qual)	35	
Melitidin	*m/z* 723 → *m/z* 579 (quan)	25	130
	m/z 723 → *m/z* 271 (qual)	28	
Brutieridin	*m/z* 753 → *m/z* 609 (quan)	25	130
	m/z 753 → *m/z* 301(qual)	28	
Quercetin-3-*O*-glucoside	*m/z* 463 → *m/z* 301(quan)	22	130
	m/z 463 → *m/z* 300 (qual)	22	

2. Experimental

2.1. Chemicals

Solvents (HPLC grade) were commercially available (Sigma-Aldrich, St. Louis, MO). Flavanones-O-glycosides standards quercetin-3-*O*-β-D-glucoside, used as internal standard, were purchased from Extrasynthese (Genay Cedex, France). The pure standards brutieridin and melitidin were obtained as reported elsewhere (Mon et al., 2020).

2.2. Sample preparation

Nine Citrus beverages were used as trials for the developed methodology. All the samples, two orange soda beverages, two lemon soda beverages, a chinotto soda beverage, one grapefruit juice, one tangerine juice, one bergamot juice and a cola soft drink, used as blank matrix, were purchased in a local store. The beverages were stored at 4 °C. Before the PS-MS/MS analysis, the samples were added with the appropriate amount of internal standard solution at a concentration of 200 mg/L, centrifuged at 12000 rpm and properly diluted with water. In particular, tangerine and lemon drinks were diluted two times; bergamot and grapefruit, 10 and 20 times, respectively; the orange beverages were analyzed both undiluted and diluted 5 times; lastly, the chinotto drink was analyzed as is. The same samples without the internal standard were submitted to HPLC-UV analysis.

2.3. Mass spectrometry

The MS analyses were carried out with a TSQ Quantum Vantage (Thermo Fischer Scientific, San José, CA) triple-stage quadrupole mass spectrometer equipped with a homemade paper spray ionization source. Qualitative Whatman filter paper n° 1 (pore size 11 μm, thickness 180 μm) was used for the experiments. The sample spotting volume was 15 μl, and once dried, the paper triangle was wetted of the same volume of methanol to allow the desorption of the ions. The paper spray ionization was performed in negative ion mode. The working conditions were the following: voltage 5.0 kV, applied directly to the paper triangle; vaporizer and capillary temperatures 280 and 290 °C, respectively. The collision gas was argon used at a pressure in the collision cell (Q2) of 1.5 mTorr, and the mass resolution at the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da at full width at half-maximum. The scan time was set at 0.4 s while the number of micro scans at 2. The collision energy (CE) was optimized individually per compound, ranging from 22 to 35 eV; S-lens values was set at 130 V for each analyte. The assay was performed by using the multiple reaction monitoring (MRM) scan mode, following specific gas phase transitions from the deprotonated precursor ion $[M-H]^-$ (Table 1). The average of the ion current of each monitored transition (quan transition), over the total acquisition time, was used for the quantitative analyses.

2.4. HPLC-UV analysis

The HPLC-UV analyses were performed using a FractionLynx system from Waters (Milford, MA) working in analytical mode, equipped with a 2535 quaternary pump and a 2989 UV/visible detector. The analytical column used for the chromatographic separation was a C_{18} reversed-phase column, Luna (250 × 4.6 mm, 5 μm, Phenomenex). The injection volume was 20 μl. The elution was carried out with 0.1% formic acid in water (solvent A) and methanol (solvent B) under gradient conditions. The gradient steps were the following: 80% A in isocratic for 7 min, from 80 to 40% A (7–40 min), 40% A isocratic for 5 min, from 40 to 20% A (45–50 min), 20% A in isocratic for 7 min, from 20 to 80% A (57–62 min) and then an isocratic flow (8 min) to equilibrate the system before starting the new analysis. The total run time was 70 min, while the flow rate was set at 1 mL/min and the UV detector was set at 280 nm. The concentration of flavanone glycosides was evaluated using an external calibration curve gained by standard solutions of their standards at a concentration ranged from 10 to 400 μg/mL. (Giuffrè et al., 2019)

2.5. Limit of detection and limit of quantification (LOD and LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry (McNaught and Wilkinson, 1997), as follows:

$$S_{LOD} = S_{RB} + 3\sigma_{RB}$$
$$S_{LOQ} = S_{RB} + 10\sigma_{RB}$$

where S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the ratio of the signals given by the transitions of the analyte and of the internal standard from the blank sample (cola soda drink diluted two times), and σ_{RB} is its standard deviation. The concentrations were calculated by the standard curve.

2.6. Data analysis

The results obtained from PS-MS/MS and HPLC-UV analyses were subjected to statistical analysis using Microsoft Excel's Data Analysis according to Student's *t*-test ($\alpha = 0.05$). The test was performed for all analytes, in order to verify the effective comparability between the data given by the two different instrumental analysis.

3. Results and discussion

Flavanone glycosides are typical phenols found in large amount in citrus juices; they can be considered a value-added for this food due to their nutraceutical properties. This work aims to develop an innovative and time saving methodology for their quantification in order to highlight and monitor the quality of the citrus-based beverages, by employing paper spray ionization coupled with tandem mass spectrometry. The latter has been implemented in our laboratory, and already used for the assay of other bioactive compounds in different food products. The analyses were carried out in negative ionization mode, because of the presence of several acidic hydrogens on the aglycon skeleton which increase the ionization efficiency in negative conditions, and also to minimize possible interference effects due to the presence of cations and sugars; while the quantitative determination was performed under multiple reaction monitoring (MRM) scan mode in order to ensure the highest specificity. The MRM method has been accomplished by the observation of the fragmentation behavior of compounds in tandem mass spectrometry experiments. The MS/MS spectra in negative ion mode of flavanone glycosides are characterized by few product ions; among these, the base peak is represented by the deprotonated aglycone, which is generated by the loss of the disaccharide portion. The same fragmentation pattern can be seen for the internal standard MS/MS spectrum, quercetin-3-O-glucoside, which, in this case, loses the glucose moiety. For the most of analytes, the monitored gas-phase transitions are those leading to deprotonated aglycone from the parent ion ($[M-H]^-$); the only exception is represented by neoeriocitrin, brutieridin and melitidin: the chosen MRM transition for neoeriocitrin is originated by the breaking of the C ring (Table 1). This special behavior is probably due to the simultaneous presence of neohesperidose sugar and catechol on the aglycon moiety, which probably force a particular folding of the sugar unit; this effect is not observed in the rutinose analog eriocitrin. For the HMG conjugates flavonoids, melitidin and brutieridin, the monitored transitions originate by the loss of HMG moiety from the deprotonated precursor ions (Table 1).

It is worth to remember that citrus juices contain both flavanone-7-*O*-neohesperidosides and flavanone-7-*O*-rutinosides, which are structural isomers and isobars, and for that they cannot be distinguished using a PS-MS/MS experiment; this is due mainly to the absence of chromatography and because the gas-phase transitions are the same for both precursors. Nevertheless, the technique still allows to achieve the purpose by assaying the flavanone glycosides as the sum of the two structural isomers. On the other hands, this can be seen as an advantage since the nutraceutical properties of these molecules are not related to the disaccharide moiety, but to the chemical structure of aglycone portion.

The quantitative assay was based on the internal standard calibration, using quercetin-3-*O*-glucoside as standard, a flavanol monoglycosides not found in citrus juice. Neohesperidoside isomers were used as reference compounds for all quantitative experiments; for each compound, the calibration curve was obtained using unweighted least squares, by analysing five standard solutions in the range from 2 to 20 mg/L, containing the internal standard at a fixed concentration of 5 mg/L. Each standard solution was submitted to PS-MRM analysis in triplicate, as described in the experimental section. The method response showed a good linearity in the selected range of calibration, providing correlation coefficient values higher than 0.98 for each flavanone glycoside investigated (Fig. S1). In order to assess the reproducibility of the calibration curves, two flavonoids standard solutions were prepared at a concentration corresponding to the linear dynamic range borders (2 and 20 mg/L) and analyzed 3 times over a period of one week. In all cases, the analyses provided an instrumental response in agreement to that of calibration curves levels, with the RSD% values below 15%. The limit of detection (LOD) and quantification (LOQ) were determined by

Table 2
Analytical parameters of accuracy, LOQ, LOD, and reproducibility*

	Spiked (S1) 2.5 mg/L	RSD%	Accuracy	Spiked (S2) 18 mg/L	RSD%	Accuracy	LOD mg/L	LOQ mg/L	RSD%* (S2)
Naringin	2.39 ± 0.22	9.3	96	17.15 ± 0.97	5.7	95	0.70	1.60	12.4
Poncirin	2.50 ± 0.33	13.2	100	17.0 ± 2.0	11.3	98	0.72	1.76	13.6
Neohesperidin	2.49 ± 0.16	6.3	100	17.7 ± 2.7	15.2	98	0.21	0.47	14.8
Neoeriocitrin	2.52 ± 0.34	13.7	101	18.6 ± 2.4	12.9	103	0.42	1.11	10.8
Melitidin	2.61 ± 0.35	13.5	104	20.5 ± 1.7	8.5	114	0.88	1.69	11.2
Brutieridin	2.69 ± 0.28	10.4	108	20.5 ± 2.5	12.0	114	0.88	1.04	12.5
Eriocitrin	2.51 ± 0.29	11.5	100	17.1 ± 2.5	14.5	95	0.79	1.90	10.6

*The reproducibility was determined by analyzing the one spiked sample 3 times over a period of 1 week.

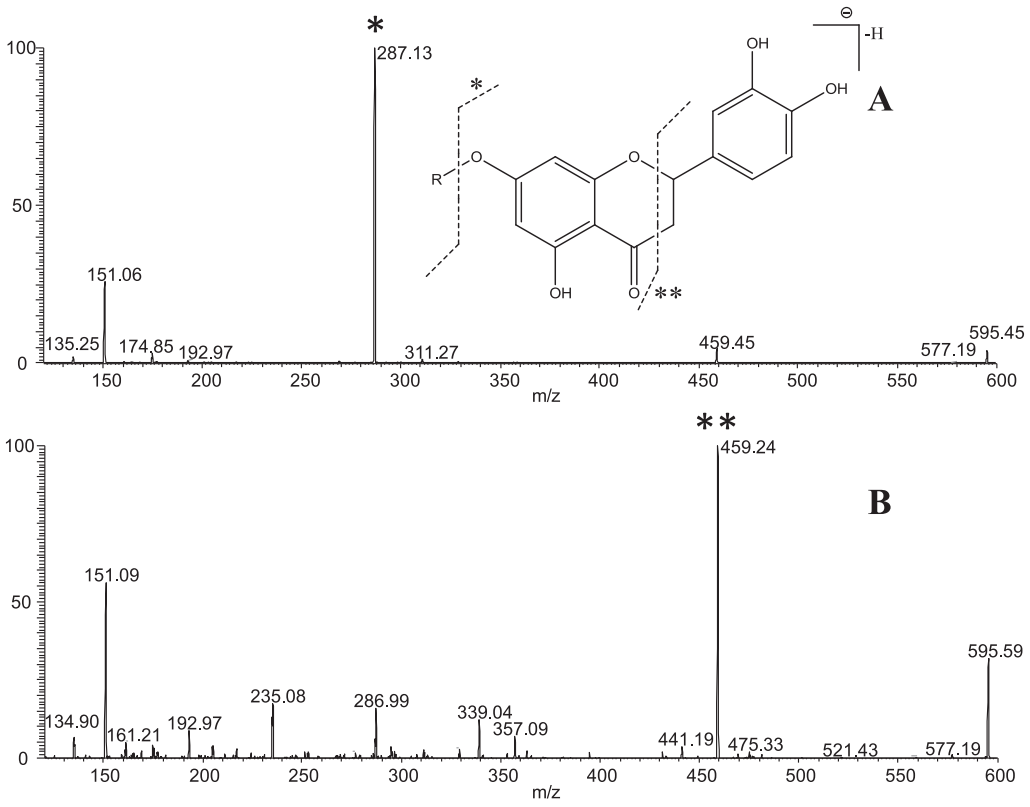

Fig. 2. ESI (-) MS/MS of eriocitrin (A) and neoeriocitrin (B) acquired with a collision energy of 25 eV.

analysing a cola soda beverage used as blank (see Experimental), fortified with the internal standard at a concentration of 5 mg/L. Table 2 shows the values of LOQ and LOD calculated in the blank matrix. For all compounds, the LOQ values were below the lowest calibration level (2 mg/L), which means that the PS-MS/MS approach provides good sensitivity for their determination in citrus-based beverages. LOD values ranged from 0.21 to 0.88 mg/L.

The described methodology was applied to fortified blank samples for the evaluation of the analytical parameters: accuracy, reproducibility and repeatability. The accuracy of the developed approach was estimated analyzing two spiked blank samples (cola soda drink missing of flavonoids) prepared at representative concentrations of the calibration curve edges. In particular, sample S1 was fortified with a low amount of the investigated flavonoids to obtain, after dilution, a final concentration of 2.5 mg/L, whereas the other sample, S2, was spiked to reach a concentration of 18 mg/L. Table 2 shows the accuracy values for the two examined solutions which ranged from 95% to 114%, for all compounds (Table 2).

As already discussed, the developed methodology intends to quantify the main flavanone-O-glycosides as the sum of two structural isomers, using only the neohesperidoside isomer as calibration standard. It was important to evaluate the accuracy for the quantification of rutinosides employing this strategy. In this regard, two blank samples fortified with flavanone rutinosides (eriocitrin, narirutin, hesperidin and neoponcirin) were prepared and quantified by using the calibration curves built with neohesperidosides standard solutions. The obtained accuracy values ranged from 90% to 120% for all compounds, except for the eriocitrin. This disagreement can be explained by observing the MS/MS spectrum of the two isomers, in which the product ions obtained at the same collision energy, show different relative abundance. The MS/MS spectrum of neoeriocitrin is characterized by the base peak at *m/z* 459, probably due to a breakage of C the ring (Cuyckens and Claeys, 2002), while the main product ion from the precursor eriocitrin corresponds to the peak at *m/z* 287, relative to the formation of the deprotonated aglycon (Fig. 2). Only for this compound, it was necessary to use eriocictrin as calibration standard, by monitoring the transition relative to the main fragmentation: from the deprotonated molecule ($[M-H]^-$) to the product ion at *m/z* 287.

The method reproducibility (RSD%), always lower than 15%, was checked on different days from independent analysis of the spiked sample prepared with a concentration of 18 mg/L. The RSD%, calculated by performing three instrumental analyses was also below 15%,

Table 3
Flavanone glycosides amount (mg/L) found in the analyzed samples by PS-MS/MS and HPLC-UV analysis.

	Naringin/Narirutin		*Poncirin/Neoponcirin*		*Neoeriocitrin/ Eriocitrin*		*Neohesperidin/ Hesperidin*		*Melitidin*		*Brutieridin*	
Juice/ Soda	**PS-MS/MS**	**LC-UV**	**PS-MS/ MS**	**LC-UV**	**PS-MS/ MS**	**LC-UV**	**PS-MS/ MS**	**LC-UV**	**PS-MS/ MS**	**LC-UV**	**PS-MS/ MS**	**LC-UV**
Chinotto	3.01 ± 0.22	5.2 ± 0.4					4.15 ± 0.58	5.3 ± 0.6				
Orange 1	17.6 ± 1.6	21.0 ± 3.0	2.94 ± 0.13	3.2 ± 0.3			49.1 ± 6.2	54.7 ± 2.6				
Orange 2	21.7 ± 0.6	19.0 ± 2.0	4.73 ± 0.14	4.1 ± 0.5			45.0 ± 4.0	41.6 ± 2.6				
Tangerine	9.0 ± 0.9	8.4 ± 1.1					14.5 ± 2.3	13.0 ± 0.8				
Bergamot	129.0 ± 7.0	121.0 ± 8.0			96.4 ± 4.4	92.0 ± 6.0	94.8 ± 7.9	88.4 ± 4.2	19.0 ± 3.0	18.7 ± 2.1	41.4 ± 6.4	38.0 ± 2.0
Lemon 1					27.6 ± 2.2	30.4 ± 2.1	35.5 ± 5.7	33.0 ± 3.0				
Lemon 2					14.4 ± 1.2	11.0 ± 2.0	6.81 ± 0.95	6.9 ± 0.5				
Grapefruit	282.0 ± 27.0	279.0 ± 14.0										

providing a good repeatability (Table 2). The recovery test is not reported, because no extraction process was carried out, as the samples were centrifuged and appropriately diluted.

After the evaluation of accuracy, the proposed approach was tested to nine real samples: juices and citrus-based soda beverages. Table 3 shows the amounts of flavonoids found in the investigated samples.

At last, to prove the reliability and the robustness of the developed PS-MS/MS methodology, the same samples were submitted to the more traditional but time-consuming HPLC-UV analysis. The comparability between PS-MRM and HPLC-UV results were assessed by *t*-test ($\alpha = 0.05$). For almost all investigated flavonoids, a p value ≥ 0.05 was obtained, highlighting the absence of significant differences between the data and supporting the quality of the proposed methodology (Table 3). The only exception concerns the amount of naringin in chinotto drink, which seems to be overestimated by HPLC-UV analysis. This is probably due to co-elution of compound which underlines the poor selectivity of HPLC-UV analysis.

4. Conclusions

A rapid methodology for the quantitative determination of the main flavanone glycosides has been developed. The assay is based on the paper spray ionization coupled with tandem mass spectrometry. The methodology is very fast and specific thanks to the employment of MRM scan mode. The consistence of this novel approach is highlighted by the good analytical parameter values and by the comparison with the classic HPLC-UV analysis. This novel approach could be used for the flavonoid determination also in other vegetable matrices.

CRediT authorship contribution statement

Fabio Mazzotti: Conceptualization, Visualization, Validation, Investigation, Writing - review & editing. **Lucia Bartella:** Conceptualization, Methodology, Visualization, Validation, Supervision, Writing - original draft, Writing - review & editing. **Ines Rosita Talarico:** Formal analysis, Investigation. **Anna Napoli:** Investigation. **Leonardo Di Donna:** Conceptualization, Supervision, Resources, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130060.

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separations

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Article

Paper Spray Tandem Mass Spectrometry for Assessing Oleic, Linoleic and Linolenic Acid Content in Edible Vegetable Oils

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Abstract: Oleic, linoleic and linolenic acids exert several beneficial effects on human health, some of which are also certified by recent European and U.S. regulations. The goal of the presented work was to develop an innovative methodology to evaluate their content in edible vegetable oils, in order to increase the value of oils from a nutraceutical perspective. The protocol is based on the use of paper spray ionization coupled with tandem mass spectrometry experiments, which allowed the recording of data very quickly and with high specificity. All investigated compounds gained a good linear relation (r^2 higher than 0.98). Accuracy values are near 100% for all concentration levels examined, and the repeatability and reproducibility data result lower than 15%, highlighting the consistence of the methodology. The developed approach was successfully applied for the analysis of different real samples, and its robustness was confirmed by comparing the results obtained with those coming from the classical and official methodology.

Keywords: fatty acid methyl esters; vegetable oils; paper spray ionization; tandem mass spectrometry

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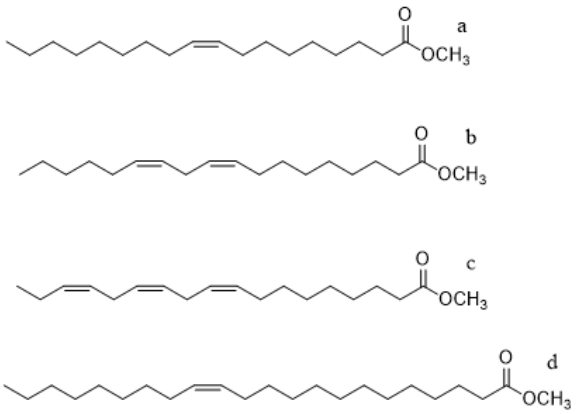
1. Introduction

Vegetable oils are one of the main fat constituents of the human diet, and represent an important daily energy source. These fat food matrices are composed, for the most part, by triacylglycerols (TAGs), which consist of two or three fatty acid chains esterified to glycerol molecules [1,2]. Fatty acid composition both affects the chemical and physical characteristics of oils and their health properties. For example, high levels of unsaturated fatty acids are positively associated with a reduction of cardiovascular disease by influencing the concentration of blood lipids (HDL, LDL, and triglycerides); in this regard, unsaturated fatty acids such as oleic, linoleic, and linolenic are the most noteworthy [3–5]. Oleic acid (C18:1), a monounsaturated (MUFA) ω-9 fatty acid, is the main component of many vegetable oils, especially olive oil, where it represents 70–80% of the composition. The beneficial effect of olive oil intake is partly related to its high oleic acid content, which has also shown to have anti-inflammatory, breast cancer protective and immune system function-enhancing effects [6–8]. Linoleic acid (C18:2) and α-linolenic acid (C18:3) are among the most important polyunsaturated fatty acids (PUFAs). They belong to the omega-6 and omega-3 fatty acids family, respectively, and are found mainly in soybean, sunflower, corn, and olive oils [9,10]. Several studies have reported that appropriate PUFAs intake may prevent cardiovascular and inflammatory diseases [11–13]. In addition, for both fatty acids, the recent Regulation 432/2012 of European Union allows the following health claim: "linoleic acid (LA)/α-linolenic (ALA) acid contributes to the maintenance of normal blood cholesterol levels". For LA, the sentence may be used only for a food containing at least 1.5 g of linoleic acid per 100 g and per 100 kcal; while for ALA, the nutraceutical declaration may be used for food which is a source of ALA (0.3 g of ALA per 100 g and per 100 kcal) as referred to in the claim regarding the source of ω-3 fatty acids listed in

the Annex to Regulation (EC) No 1924/2006 [14]. In the case of oleic acid, the European regulation provides no specific health claim, but the U.S. Food and Drug Administration (FDA) has established that there is evidence to support a claim stating that "consumption of $1^{1/2}$ tablespoons, about 20 g of oil containing high levels of oleic acid is able to reduce the incidence of coronary heart disease" [15]. Oleic, linoleic, and linolenic acids are, therefore, quite relevant for their beneficial effects and, in this context, the use of fast, accurate and reliable methodologies is essential for their quantitative determination; also, in order to address the above-mentioned regulations. Commonly, fatty acids in vegetable oils are quantified as methyl esters after transesterification reaction, while the instrumental analysis is performed by gas chromatography (GC) coupled with flame ionization detection (FID), as described by the EU official method (regulation 2015/1833, Annex X) [16], or mass spectrometry (MS) [16–20]. To date, mass spectrometry is certainly the most widely used instrument for both structural investigations and quantitative determinations, due to its high specificity and sensitivity [21–24].

The current study aimed to find a new methodology for a fast and accurate quantification of the total oleic, linoleic, and linolenic acid content in vegetable oils typically consumed in our daily diet, such as extra virgin olive oil, sunflower, soybean, and corn oil. For this purpose, we employed paper spray mass spectrometry (PS-MS), a methodology belonging to the ambient ionization techniques which has quickly become popular, due to its operative easiness [25–27]. The paper spray source is composed of a metallic alligator clip which holds a paper triangle in front of the mass spectrometer inlet. To perform this type of experiment, a small volume of sample solution is deposited on the thin paper triangle. The paper spray ion generation is gathered by applying a high voltage (4–5 kV) to the metal clip and, at the same time, by dropping few microliters of an extraction/spray solvent to the paper; in this way, charged droplets are accumulated on the paper tip and a Taylor cone is generated, providing ions through an electrospray-like process [28,29]. This technique has already been used for quantitative determination of other bioactive compounds in foods and beverages and also for molecular fingerprinting used for quality control purposes [30–37]. Multiple reaction monitoring (MRM) is used as a scanning mode to quantify the fatty acids, with the aid of erucic acid methyl ester as an internal standard. The sample preparation is based on a transesterification reaction which provides the methyl esters (Scheme 1), directly analyzed via PS-MSMS. The innovation of the proposed procedure stands on the simplicity and specificity of analysis, underlined by the absence of any chromatographic step and by the use of the tandem mass spectrometry. The entire experiment is very timesaving; in fact, after the transesterification reaction, the internal standard is added to the mixture, properly diluted, and directly submitted to the PS-MSMS analysis, whose scanning time is two minutes. Furthermore, in order to demonstrate the robustness of the presented methodology, the values obtained from the analysis of the investigated edible oils have been matched with those obtained by the application of the official EU method.



Scheme 1. Chemical structures of oleic acid methyl ester (**a**), linoleic acid methyl ester (**b**), linolenic acid methyl ester (**c**) and internal standard (**d**).

2. Materials and Methods

2.1. Chemicals and Reagents

All solvents used were HPLC grade and commercially available from Sigma-Aldrich (St. Louis, MO, USA). Pure compounds, methyl oleate (methyl *cis*-9-octadecenoate), methyl linoleate (methyl *cis*,*cis*-9,12-octadecadienoate), methyl linolenate (methyl *cis*,*cis*,*cis*-9,12,15-octadecatrienoate), methyl erucate (methyl *cis*-13-docosenoate), triolein (oleic acid triglyceride) and trilinolein (linoleic acid triglyceride) were also purchased from Sigma-Aldrich.

2.2. Standard Solutions

Standard solutions of fatty acid methyl esters were achieved at a concentration of 3000 mg/L by solubilizing the standard compounds in *n*-hexane. The calibration curves were obtained by analyzing five standard solutions of methyl oleate, methyl linoleate and methyl linolenate at increasing concentrations, in the range from 2.5 to 20 mg/L, and internal standard (methyl ester of erucic acid) at fixed concentration of 10 mg/L.

2.3. Sample Preparation

Each oil sample was submitted to transesterification reaction following the EU official procedure 2015/1833, Annex X [16], with minor amendments. Briefly, 50 mg of oil were weighted, added of 50 µL of 2 M potassium hydroxide methanolic solution and 2 mL of *n*-hexane. The resulting solution was vigorously shaken for 30 s and allowed to stratify until the supernatant was clear. Before the PS-MS/MS analysis, the supernatant was diluted by using *n*-hexane and the appropriate amount of internal standard was added. Olive oil samples supernatant was diluted 1:1000 for the quantification of methyl oleate; for methyl linoleate and linolenate, it was diluted 1:200 and 1:10, respectively. For seeds oil samples, the supernatant was diluted 1:1000 for the assay of methyl oleate and linoleate, and 1:100 for the quantification of methyl linolenate.

2.4. Paper Spray Mass Spectrometry

PS-MS determinations were carried out in positive polarity by using a TSQ Quantum Vantage (Thermo Fischer Scientific, San José, CA, USA) triple-stage quadrupole mass spectrometer coupled with an in-house implemented paper spray source. The latter is composed of a small triangular shaped piece of paper (qualitative Whatman filter paper n° 1) which supports the sample and held in front of the triple quadrupole inlet using a metallic clip. Up to 15 µL of sample solution were deposited onto the paper and left to dry for 1 min. After this time, a high voltage, set using the power supply enclosed in the mass spectrometer for the electrospray source, was applied to the triangle through the clump, and 15 µL of methanol was added every 30 s to permit the spray desorption. The total scan time was 2 min. The MS working conditions were set as follows: applied voltage +5000 V, vaporizer temperature 280 °C and capillary temperature 290 °C. The gas used for CID experiments was argon, with a pressure in the collision cell (Q2) of 1.5 mTorr. Mass resolution at the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da at full width at half-maximum. The scan time was set at 0.4 s while the number of micro scans was set at 2. The collision energy (CE) was optimized for each compound and ranged from 12 to 20 eV; S-lens values was set at 120 V for all investigated compounds. The quantitative determination was conducted under multiple reaction monitoring (MRM) conditions, using the ion current generated by two gas phase transitions from the protonated compounds $[M+H]^+$, the first one for the quantitative assay, and second for confirmation (Table 1). For each quantitative transition, the ion current was averaged over the total scanning time. Instrument control was carried out by means of Xcalibur 2.1 software.

2.5. GC-FID

The gas chromatographic analyses were carried out following the EU official method 2015/1833, Annex X [16] by using a GC-FID instrument from Varian (Palo Alto, CA, USA) The detector temperature was set at 225 °C and the flame of detector was kept at 250 °C

with 30 mL/min H_2 and 300 mL/min air. The chromatography was performed with a 100 m × 0.25 mm DB-23 capillary column with a film thickness of 0.25 µm from Agilent Technologies. Sample injection volume was 1 µL and the oven temperature started from 100 to 240 °C with a speed rate of 3 °C/min and 5 min of plateau. The total time of analysis was 43 min.

Table 1. Selected MRM transitions, instrumental parameters and calibration curve equations.

Compound	Transition	CE (eV)	S-Lens (eV)	Linearity
Oleic acid	m/z 297 → m/z 265 (quan)	12	120	y = 0.356x + 0.2888
methyl ester	m/z 297 → m/z 247	15	120	R^2 = 0.9852
Linoleic acid	m/z 295 → m/z 263 (quan)	12	120	y = 1.6759x + 0.8368
methyl ester	m/z 295 → m/z 245	15	120	R^2 = 0.9913
Linolenic acid	m/z 293 → m/z 261 (quan)	15	120	y = 1.1231x + 0.1458
methyl ester	m/z 293 → m/z 243	18	120	R^2 = 0.9953
Erucic acid	m/z 353 → m/z 321 (quan)	18	120	
methyl ester (IS)	m/z 353 → m/z 303	20	120	

3. Results

The vegetable oils under investigation were submitted to transesterification to convert the triacylglycerols to fatty acid methyl esters. The procedure was very simple and required just few minutes. The reaction products were initially analyzed by direct injection ESI (+)-MS analysis to verify that the reaction conversion was quantitative. The positive mode was chosen for the paper spray MS experiments because a better ionization efficiency for the fatty acid methyl esters investigated was observed. After several trials involving different extraction spray solvents, we decided to use methanol for the ionization step which provided a good signal at m/z 297, m/z 295 and m/z 293 for the protonated fatty acid methyl esters $[M + H]^+$ of oleic, linoleic and linolenic acids, respectively. The quantitative determinations were performed using multiple reaction monitoring (MRM) scan mode by following specific transitions for the analytes. The latter were selected by observing the fragmentation behaviour of methyl esters in tandem mass spectrometry (MS/MS) experiments. For all fatty acid methyl esters, the MS/MS spectra were characterized by few diagnostic fragments; the base peak is relative to the formation of the acyl ion, generated by the formal loss of a methanol neutral molecule from the protonated molecule $[M + H]^+$, while the second most intense product ion is produced by a formal loss of one molecule of water from the latter, aided by the transfer of a proton in alpha position with respect to the carbonyl oxygen (Figure 1).

An internal calibration method was used to quantify the total fatty acids content, employing the methyl ester of erucic acid (methyl *cis*-13-docosenoate) as internal standard. The selected standard is a monounsaturated fatty acid methyl ester with a chemical structure similar to the investigated compounds and not present in the vegetable oils under study. Methyl *cis*-13-docosenoate exhibits the same fragmentation behaviour of the other analytes: the product ions provided by the loss of methanol molecule from the $[M + H]^+$ parent ion and the subsequent formal loss of water gave signals at m/z 321 and m/z 303, respectively. Table 1 summarizes the gas-phase reaction generated from the protonated species $[M + H]^+$ and monitored during the assay of the oils. In particular, the loss of the neutral methanol was used for quantitative analysis, while the subsequent dehydration was used to confirm the signal. The collision energy for each transition was optimized to achieve the highest signal.

The calibrations curves, built by analyzing in triplicate each of the five standard solutions at increasing concentrations of analytes, show a good linearity in the chosen range, with a correlation coefficient (r^2) higher than 0.98 for all analytes (Table 1). The range of concentrations of the fatty acid methyl esters in the standard solutions was selected from 2.5 to 20 mg/L, while the internal standard was maintained at 10 mg/L. The

calibration reproducibility (RSD%) was evaluated by preparing two standard solutions at concentrations corresponding to the linear dynamic range edges (2.5 and 20 mg/L) and analyzed three times over a period of a week. For both concentration levels, the percentage relative standard deviation (RSD%) was less than 15%, highlighting a good reproducibility for what concerns the instrumental response. Due to the lack of a blank matrix, the accuracy of the whole procedure was disclosed by submitting two mixtures of triolein and trilinolein to transesterification process and then analyzed. The mixtures were prepared at the following ratios: S1 80/20 and S2 20/80 (triolein/trilinolein), in order to mimic an oil with a high oleic and linoleic acid content, respectively. After transesterification, both samples were diluted 1:1000 and 1:100 using hexane and submitted to PS-MSMS analysis. In all cases, the accuracy values were around 100%. The same samples were also employed for evaluating the repeatability and reproducibility of the methodology, both expressed as RSD%. The first one was assessed by performing instrumental analyses for each sample in triplicate, while the reproducibility was calculated by analyzing the samples three times at one-week intervals. An RSD% below 15% was obtained for each experiment. Table 2 shows the value of analytical parameters discussed above.

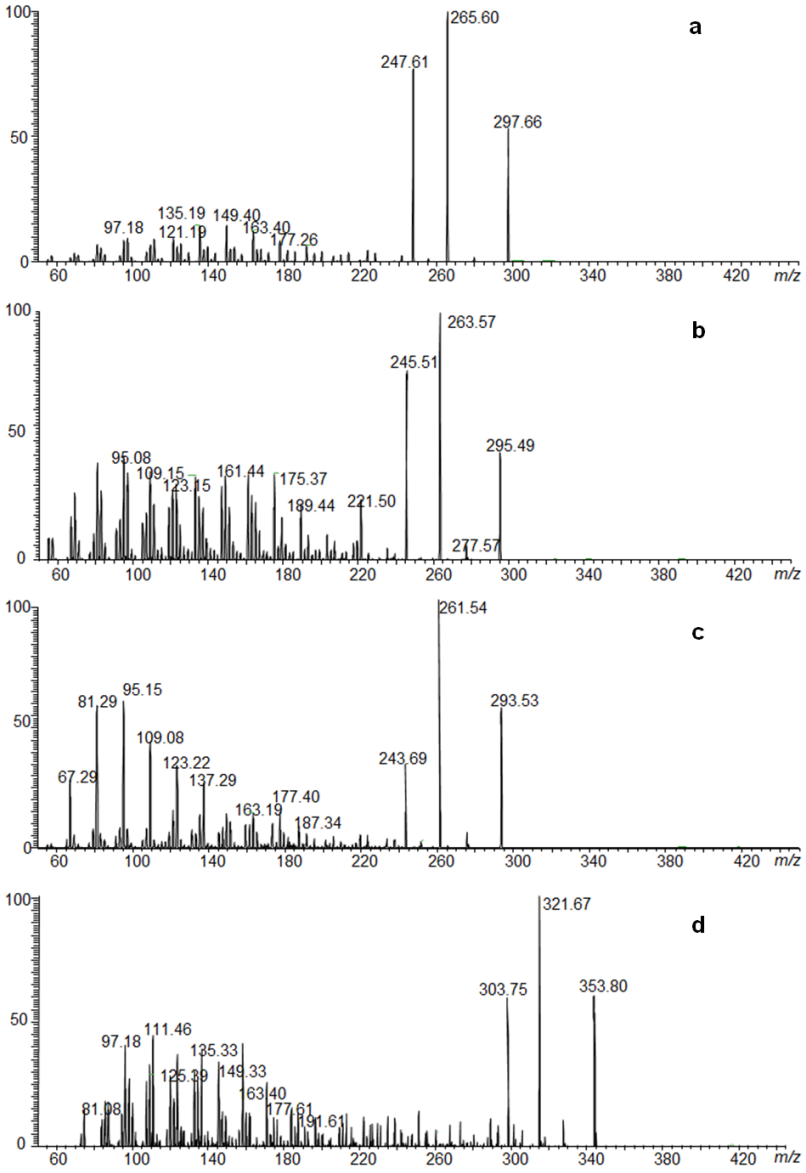


Figure 1. ESI(+)-MS/MS spectra of $[M+H]^+$ ions of oleic acid methyl ester (**a**), linoleic acid methyl ester (**b**), linolenic acid methyl ester (**c**), erucic acid methyl ester—IS (**d**).

Table 2. Accuracy, repeatability, and reproducibility values.

Sample			Calculated Amount (*w*/*w* %)	Accuracy (%)	Repeatability (RSD %)	Reproducibility (RSD %)
S1 (*w*/*w* %)	**Oleic acid**	**80%**	82 ± 7	102	8.5	9.2
	Linoleic acid	**20%**	18 ± 2	90	11.1	12.0
S2 (*w*/*w* %)	**Oleic acid**	**20%**	21 ± 2	105	9.5	10.2
	Linoleic acid	**80%**	77 ± 6	96	7.7	8.4

After evaluating the calibration linearity, accuracy and reproducibility, the developed protocol was applied to real samples of vegetables oils purchased from a local store. In particular, three extra virgin olive oils, three corn oils, two soybean oils and one sunflower oil were submitted to PS-MS/MS analysis after transesterification reaction. Table 3 shows the total fatty acid content (*w*/*w* %) found in the samples tested, which were also analysed in parallel by the classical GC method to corroborate the data obtained by PS-MS.

Table 3. Methyl esters amount (*w*/*w*%) found in the investigated oil samples by PS-MS/MS and GC-FID analysis.

	Methyl Oleate (*w*/*w* %)		Methyl Linoleate (*w*/*w* %)		Methyl Linolenate (*w*/*w* %)	
Sample	**PS-MS**	**GC-FID**	**PS-MS**	**GC-FID**	**PS-MS**	**GC-FID**
Olive oil 1	60 ± 8	69	9 ± 1	8	0.8 ± 0.1	1.0
Olive oil 2	55 ± 9	68	6 ± 1	6	0.6 ± 0.2	0.7
Olive oil 3	63 ± 9	70	7 ± 1	5	0.45 ± 0.05	0.8
Corn oil 1	28 ± 4	31	60 ± 11	53	0.8 ± 0.2	1.2
Corn oil 2	24.5 ± 4.0	32	47 ± 6	52	1.6 ± 0.2	1.6
Corn oil 3	25 ± 5	31	46 ± 3	51	1.5 ± 0.3	1.3
Sunflower oil 1	29 ± 4	25	57 ± 6	63	1.1 ± 0.1	0.8
Soybean oil 1	20 ± 4	24	55 ± 8	53	3.7 ± 0.5	5.3
Soybean oil 2	23.5 ± 4.5	23	51 ± 11	51	6.5 ± 0.5	6.1

4. Discussion

The assay of three fatty acids, important from a nutraceutical point of view, were performed in the present work. Numerous studies have highlighted the health properties of oleic, linoleic and linolenic acids; for the last two, a claim is also included in EU Regulation 432/2012, while for oleic acid, the FDA has also expressed an indication about its beneficial effects. The main purpose of our study was to develop a methodology that could have been a suitable alternative to the official method used, which involves gas chromatographic separation coupled to FID detection. The disadvantages of the latter rely on the long analysis times; furthermore, it is based on a relative quantitative determination calculated on the areas of the chromatographic peaks without the use of calibration curves. The proposed protocol takes advantages of the specificity of tandem mass spectrometry MRM scanning mode, but the most important advancement concerns the use of the paper spray ionization source which provides high-throughput determinations. The application of the paper-spray-based method allows accurate results within minutes, through a direct sample ionization, without the need for chromatographic separation steps and with minimal solvent consumption. Furthermore, the use of internal standard improves the accuracy and precision of analyses. The results obtained by PSMS were compared to GC data using a standard *t*-test (α = 0.05). For all vegetable oils under investigation, a p value $\geq$ 0.05 was obtained. This emphasizes there are no significant differences between the data and shows the reliability of the proposed method (see Table 3). This cross-validation emphasises also that the PS-MS approach is not affected by sample preparation and/or by matrix composition. Regarding the analytical parameters, accuracy values near 100% and

precision (repeatability and reproducibility) always below 15%, highlighted the reliability of methodology. Recovery is quantitative for all compounds, since no extraction procedure is involved. Some considerations may be provided around the fatty acid composition of the oil samples analyzed and its relation to the health claim regulation reported in the introduction. The amount of linoleic acid in the samples of corn, soybean and sunflowers oil satisfies the EU regulation 432/2012 for what regards the claims on the "maintenance of normal blood cholesterol levels"; for what concerns the nutraceutical linolenic acid, it appears from the data, that all the samples may be considered as source of ω-3 fatty acids, while no olive oil may be considered as high-grade oleic acid content, under the statements of the FDA regulation [15].

5. Conclusions

Paper spray mass spectrometry was first employed for determining the total content of important fatty acids in vegetable oils, such as oleic, linoleic and linolenic acids. The comparability between the results gained by classical GC-FID and PS-MS analysis demonstrates that developed protocol may be applied for very rapid screening of these compounds in oils and similar matrices, as an alternative to the determination employing chromatographic separation. The relevance of the presented methodology lies in the potential of its use to address recent EU and US regulations on the health claims for these nutraceutical compounds, enabling important health indications to be directly reported on the food labels, enhancing the products' value.

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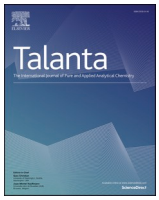

Paper spray mass spectrometry profiling of olive oil unsaponifiable fraction for commercial categories classification

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ABSTRACT

A new method for a fast molecular profiling of olive oil unsaponifiable fraction has been developed. This approach, based on paper spray mass spectrometry, allows obtaining MS data with only a few minutes of analysis and without significant solvent and disposable consumption. Tandem mass spectrometry and high-resolution mass spectrometry experiments have been performed to identify the main ions detected. The MS data coming from the analyses of sixty-three samples of three different olive oil categories: extra virgin olive oil (EVOO), virgin olive oil (VOO), and pomace olive oil (POO), have been used to test the discriminative potential. Both unsupervised (PCA and HCA) and supervised (kNN and LDA) chemometric procedures have been applied with good results in prediction. The same approach was tested using direct infusion mass spectrometry data to confirm the ability of paper spray fingerprinting to classify different olive oils correctly.

1. Introduction

Extra virgin olive oil is one of the most famous foods of the Mediterranean Diet, well known and profoundly studied because of its beneficial effects on the human health. Its nutraceutical features are mainly related to the high content of monounsaturated fatty acids and minor components such as phenolic compounds, vitamins, and phytosterols [1–4]. Olive oil is composed of two fractions, the saponifiable fraction, which represents 98–99% of the total weight, and the remaining unsaponifiable portion (1–2%), which consists of a complex mixture of hydrocarbons, tocopherols, pigments, waxes, aromatic and aliphatic alcohols, phytosterols and triterpene acids. This smaller fraction is the most relevant from a health perspective, exhibiting different biological activities against several diseases [5,6], and it is also crucial for quality assessment and frauds detection [7,8]. In fact, the content of specific minor components such as phenolic compounds, sterols and triterpene diols is one of the parameters required to verify the nutraceutical properties and the authenticity of olive oils (EEC No 2568/91; Reg. (EU) No 432/12). Despite the existence of strictly European regulations highlighting the criteria for olive oil quality grade and commercial categories (Reg. (EU) 29/2012; Reg. (EU) 1308/2013), the extra virgin olive oil is still a leading target for fraudulent practises, which have become increasingly sophisticated. In fact, among the most common adulterations are the marketing of VOO as EVOO, or EVOO with the addition of small amounts of seed oil and pomace oil (POO) [9–11]. In many instances, simple chemical and/or organoleptic analyses are not sufficient to detect this type of adulterations [9,12].

Because of the extensive olive oil culture and its innumerable health benefits, substantial efforts have been devoted to its molecular characterization, to develop new methods for quantifying nutraceutical markers, and for the determination of possible adulterations, geographical and botanic origin [13–15].

In this regard, great attention has been dedicated to the development of new methods for olive oil classification, by applying chemometric tests as useful tool. These approaches have been already employed mainly after classical and time-consuming chromatographic separation

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methods coupled with ultraviolet detector and mass spectrometry (GC-MS and HPLC-MS), using both targeted and untargeted approaches [4,16–23] focusing also on the fingerprint of the sterols fraction [24]. Therefore, it would be important to have methods to establish the possible presence of adulterants in olive oil by specific molecular fingerprints obtained using fast instrumental analysis.

Ambient Mass Spectrometry (AMS) is surely the most useful tool in this respect [25–27], because it combines the specificity of mass spectrometry with very brief analysis times due to minimal or no sample treatment and no separation steps. Paper Spray mass spectrometry (PS-MS) is probably one of the most straightforward and cost-effective AMS approaches, which permits to obtain specific molecular profiles within a few minutes of analysis [28,29]. In a typical PS-MS experiment, the sample is loaded on a triangular piece of paper, and after applying solvent and high voltage (3–5 kV), analytes ionization is promoted mainly by an electrospray-like mechanism [30]. PS-MS has been applied in many analytical fields [31,32], and for quality and authenticity control of foods and beverages quality control [33–40]. Furthermore, this approach has already been successfully used to determine nutraceutical compounds in EVOO samples, such as vitamin E [39], and phenolic compounds [25]. Considering the versatility, low-cost, and speed of the PS-MS, together with the interest in monitoring the authenticity and quality of a product such as olive oil using its unsaponifiable fraction, herein, we present a study in which the PS-MS approach has been applied for the first time to direct analysis of olive oil unsaponifiable fraction. The PS-MS performance, in terms of molecular detection capability, has been confirmed by high-resolution MS experiments. A fingerprinting MS approach coupled with chemometrics has been used for the development of a classification method by using three groups of commercial olive oil that are usually target for fraudulent practises: extra virgin olive oil (EVOO), virgin olive oil (VOO), and pomace olive oil (POO). The same procedure has been performed with high-resolution mass spectrometry (APCI-TOF), which has allowed to evaluate the goodness of the results coming from PS-MS analysis. In addition, to classify the oils according to their untargeted content, various statistical tests were applied, including principal component analysis (PCA-LDA), cluster analysis of original variables, and K-NN.

2. Experimental

2.1. Chemicals

Methanol (MeOH, <99%), Chloroform (<99%), Isopropanol (<99%), hexane (<99%) and diethyl ether (<99%) were supplied by Merck-Sigma Group (Darmstadt, Germany). Standards of β-Amyrin (≥98,5%), campesterol (~65%), β-Sitosterol (95%) and fucosterol (93%) were purchased by Sigma Aldrich (Saint Louis, MO, US).

2.2. Sample preparation

Sixty-three olive oils belonging to three different categories: Extra Virgin Olive Oil (EVOO) ($n = 21$), Virgin Olive Oil (VOO) ($n = 21$) and Pomace Olive Oil (POO) ($n = 21$) were used as trials of the method development. All the sample were provided by Laboratorio Tello (Jaén, Spain) and regularly tested to verify their quality grade and commercial category. The EVOO come from different regions and cultivar of Spain (19) and Morocco (2). For the Spanish samples, 4 belong to Cornicabra, 4 to Arbequina, 1 to Manzanilla, 3 to Picual, 3 to Hojiblanca, 1 to Arroniz, 2 of them, came from Extremadura with no information about the cultivar and the last one is a *coupage*.

To perform multivariate analysis, for each category, oil samples were split randomly into two data sets, a training set (15 samples for each group) and a test set (6 samples for each group). All the samples were submitted to saponification reaction according to the EU official method (Regulation EEC 2568/91). The unsaponifiable fractions were dissolved in 2 mL of chloroform and directly used for PS-MS analysis with the ion trap. The same solutions were diluted with isopropanol (1: 200 and 1: 2500) to perform the HPLC-APCI-TOFMS and direct infusion APCI-TOFMS analysis for identification and comparison purposes.

2.3. Paper spray mass spectrometry

The MS analyses were done with a Thermo LTQ linear ion trap mass spectrometer (Thermo Scientific, San José, CA, USA) with an in-built paper spray ionization source. Whatman 42 filter paper, with a triangular shape, was used for the experiments. The sample volume was 10 μL, and once dried, a high voltage was applied to the paper through the clamp, and later it was wetted with the same amount of methanol to allow the transfer of the analytes to the gas phase. The paper spray ionization was performed in positive ion mode, and the working conditions were the following: voltage (kV),5.0. Capillary voltage (V), 35. Capillary temperature (°C), 275, and tube lens (V), 100. The number of micro scans per spectrum was set at 3. Mass spectra were recorded in the mass range from 300 to 700 amu. Collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) experiments, with N_2 as inert gas, were carried out to identify compounds in the samples using a collision energy of 15 eV. Acquired data were processed using Xcalibur 3.0 (Thermo Scientific) software.

2.4. High-resolution mass spectrometry

2.4.1. HPLC-APCI-TOFMS analysis

The HRMS measurements were performed using an LC-MS system (Agilent 1290/Agilent 6220 TOF). The analytical column used for the chromatographic separation was a C_{18} reversed-phase column, Zorbax Eclipse plus (150 × 4.6 mm, 3.5 μm, fully porous) (Agilent, Santa Clara, California, USA). The injection volume was 20 μL. The elution was accomplished with methanol (solvent A) and isopropanol (solvent B) under gradient conditions. The gradient steps were the following: from 0 to 50% of B in 2 min, from 50 to 90% of B (2–15 min), from 90 to 100% B (15–18 min), 100% B isocratic for 2 min, from 100 to 50% of B (20–23 min) and then a 2 min flow to equilibrate the system at the initial condition before starting the new analysis. The total run time was 25 min, while the flow rate was 0.400 mL/min. A time-of-flight-MS was coupled to the HPLC system using an APCI source, operating in positive ion mode to obtain the full-scan accurate mass spectra. The instrumental parameters used are gas temperature (°C), 250. Vaporizer, 300. Gas flow (l/min), 5.0. Nebulizer (psi), 30, and fragmentor voltage (V), 250. Mass spectra were recorded across the mass range from m/z 300 to 500. The full-scan data were recorded with Agilent Mass Hunter Acquisition software (version B.04.00) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.04.00).

2.4.2. Flow-injection APCI-TOFMS analysis

For flow-injection experiments, the APCI parameters were the same described in the previous section. The injection volume was 20 μL while the mobile phase used to transport the injected sample was 50:50 methanol and isopropanol with a flow rate of 0.300 mL/min. In this case, the spectra were acquired in the mass range from to m/z 300–500, in order to reduce the number of variables considering that PS-MS experiments highlighted that no interesting masses are present above m/z 500.

The direct infusion analyses were performed with an APCI source in positive ionization mode coupled to a time-of-flight mass spectrometer (Agilent 6220 accurate mass TOF, Agilent). The instrumental parameters are gas temperature (°C), 250. Vaporizer, 300. Gas flow (l/min), 5.0. Nebulizer (psi), 30 and Fragmentor voltage (V), 250. Mass spectra were recorded across the mass range from m/z 200 to 500. The full-scan data were recorded with Agilent Mass Hunter Acquisition software (version B.04.00) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.04.00).

2.5. Statistical analysis

A chemometric study was performed using both unsupervised and supervised approaches. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were employed as the unsupervised method to visualize the objects in a multidimensional space and understand patterns and groupings among a data set by means of a tree of clusters. Two supervised classification techniques were employed, which rendered a delimiter between classes and permit the assignment of each new sample to the class with the highest probability. The two approaches used were K Nearest Neighbours (K-NN) and Linear Discriminant Analysis (LDA). K-NN is a straightforward distance-based technique and was performed both on the original variable and on PCA scores. LDA, instead, is a probabilistic technique only relevant if applied to the PCA scores for the case studied. (Oliveri et al., 2021). In this study, PCA, K-NN, and LDA were performed using the multivariate data analysis software CAT (R. Leardi, C. Melzi, G. Polotti, CAT (Chemometric Agile Tool), freely downloadable from http://gruppochemiometria.it/index.php/software), while HCA was performed using SPSS software (SPSS for Windows 26.0, SPSS Inc., USA).

3. Results and discussion

The unsaponifiable fraction of olive oil contains several bioactive compounds, ranging from hydrocarbons, aliphatic alcohols to different sterol derivates. Therefore, the MS fingerprinting of this fraction may provide insights into the origin and quality of the product. One of the objectives of the present work was to develop an innovative method for direct analysis of olive oil unsaponifiable fraction relying on the use of paper spray mass spectrometry. Since this fraction is more stable than for instance phenolic compounds or volatiles, the proposed approach could be more rugged to enable olive oil classification. The MS data obtained were submitted to chemometric analysis to verify their capability in olive oil categories classification.

3.1. Optimization of PS-MS performance

In order to optimize the paper spray mass spectrometry performance, several experimental conditions were tested. Both positive and negative ionization modes were employed, and different spray solvents were evaluated to increase the molecular detection capability. To screen the whole extract, the first experiments were carried out over a wide *m/z* mass range, from *m/z* 100 to 1000. The best ionization outcome was obtained by spotting just 10 μL of the unsaponifiable extract onto the paper, using positive ion mode and methanol as spray solvent at the beginning of the MS acquisition.

In the mass spectra, a remarkable difference was observed between the onset of the MS acquisition, when the paper is wet with methanol, and the last seconds, when the triangle is dry (Fig. 1). In fact, only when the paper has dried the ions related to the non-polar compounds, specific to unsaponifiable fractions, are detected. The ionization of these compounds is due to a corona discharge effect between the tip of the triangle and the mass spectrometer inlet [29].

The ions detected in PS-MS spectra were related to sterol compounds as $[M + H–H_2O]^+$ ions. In fact, sterols easily ionize in positive mode due to the presence of a hydroxyl group, which after protonation, is eliminated as a neutral loss of water. Considering the non-polar characteristics of the compounds found in the investigated fraction, also hexane was tested as a spray solvent, which also allowed the detection of squalene (*m/z* of 411). The use of hexane causes, on the other hand, a decrease in the sterols ion intensities; for this reason, methanol was selected as the spray solvent.

Several paper spray tandem mass spectrometry experiments were performed to identify the main ions present in the full scan spectra: *m/z* 383, *m/z* 395, *m/z* 397, *m/z* 409, *m/z* 423, *m/z* 425, *m/z* 441, and *m/z* 443. The first four ions show the typical fragmentation pattern of sterols, while the MS/MS spectra of the ions at *m/z* 423, 425, 441, and 443 are characterized by very few product ions. It was possible to confirm that $[M + H–H_2O]^+$ ions at *m/z* 383 and *m/z* 397 are relative to campesterol and the β -Sitosterol, respectively. In fact, for both ions, some characteristic fragments were detected, the product ion at *m/z* 243, which derives from the breaking of the D ring, and the fragment ions at *m/z* 161 and 147 from the breaking of the C ring in two different positions (Fig. 2).

The ion at *m/z* 395 could be related to fucosterol, stigmasterol and 5-avenasterol, three isobar sterols found in olive oil. The molecular ion at *m/z* 409 is attributable to β-amyrin, cycloartenol, and lupeol. The ion at *m/z* 443 could be referred to as protonated erythrodiol and uvaol ($[M+H]^+$) together with the $[M + H–H_2O]^+$ ion at *m/z* 425, referred to as the same molecules. The same behaviour was observed in the case of the ions at *m/z* 441 and *m/z* 423.

Due to the absence of chromatography, it is only possible to presume the presence of all these compounds as a sum with paper spray experiments. In order to confirm sterols identification, high-resolution mass spectrometry analyses were performed using the HPLC-APCI-TOFMS system together with available standards.

Table 1 shows the tentative chemical assignment for the main ions detected in the olive oil unsaponifiable fraction. Campesterol (*m/z* 383), fucosterol (*m/z* 395), β-sitosterol (*m/z* 397), and β-amyrin (*m/z* 409) were unambiguously identified not only by accurate mass measurements but also by retention time comparison with standards. The other primary ions detected are related to their isomers.

After PS-MS approach optimization, 63 different olive oil samples were submitted for analysis to obtain an MS fingerprinting in the mass range from *m/z* 300 to 700.

3.2. Unsupervised and supervised chemometric approaches for discriminative purposes

The MS fingerprint of olive oil unsaponifiable fraction, obtained as

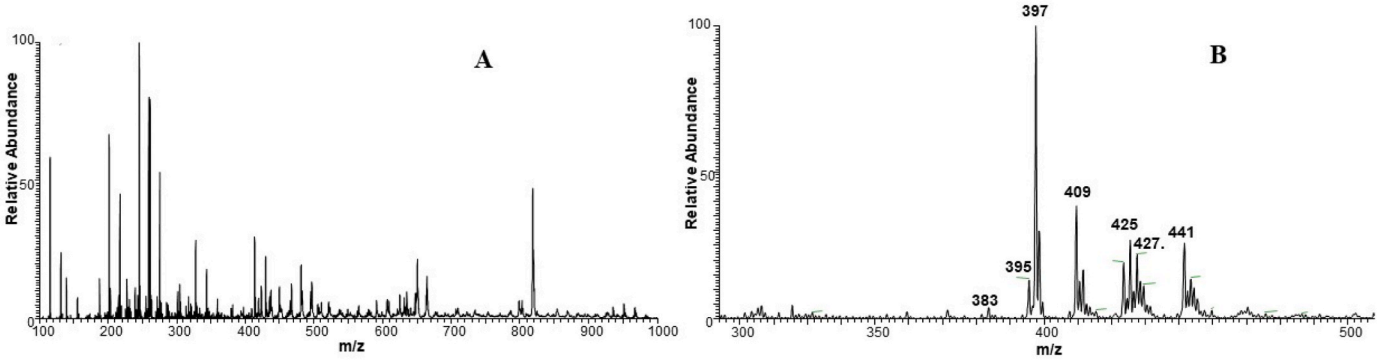

Fig. 1. PS (+) MS spectra of wet paper (A) and zoomed dry paper (B) of the unsaponifiable fraction of olive oil.

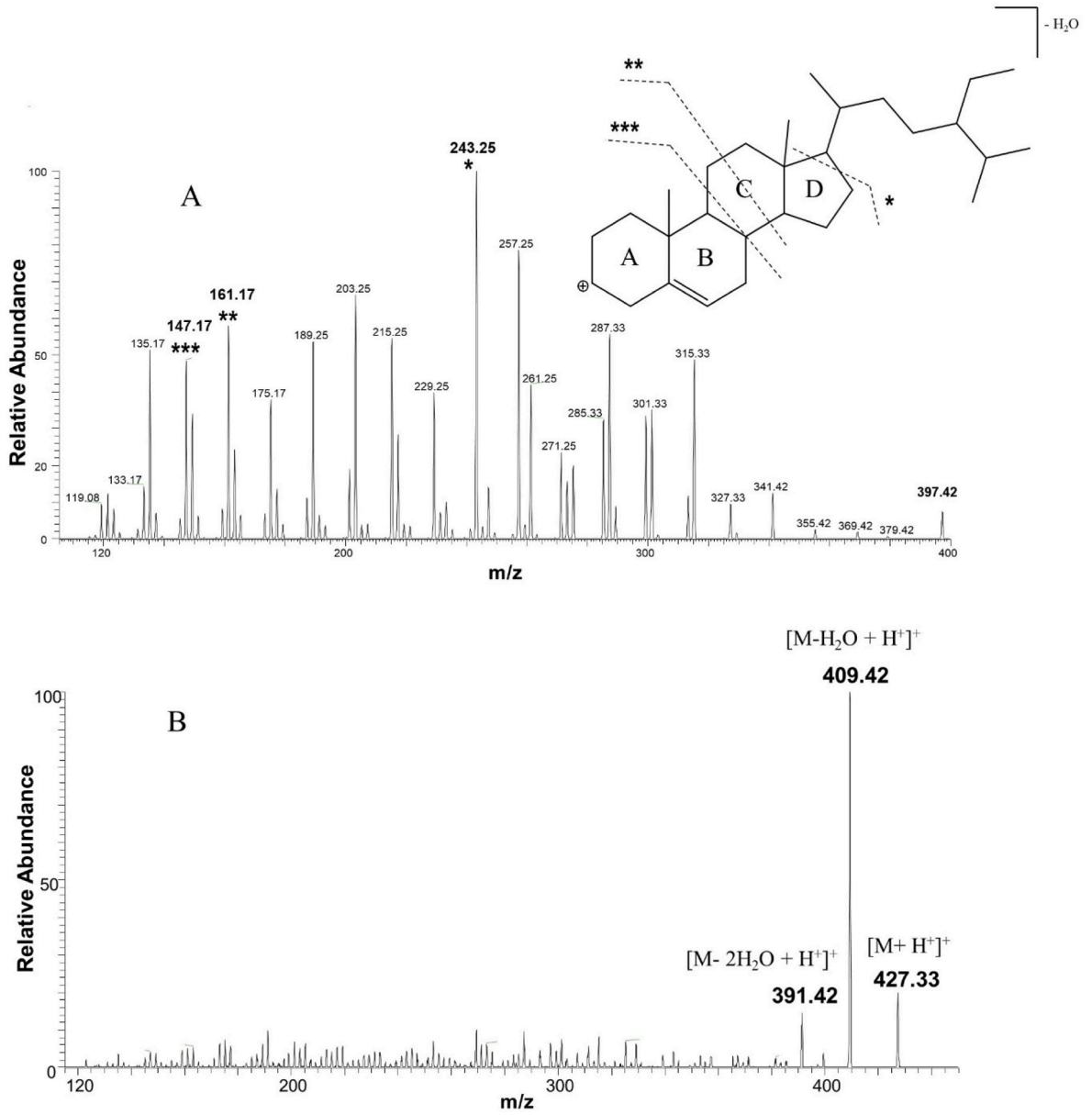

Fig. 2. PS (+) MS/MS spectra of *m/z* 397 (A) and *m/z* 441 (B).

Table 1
Compounds detected by LC/high resolution MS experiments.

	Standards MIX	Sample					
m/z	*RT (min)*	*Compound*	*RT (min)*	*Observed mass*	*Predicted formula*	*Calculated exact mass*	*Error (ppm)*
383	8.882	campesterol	8.837	383.3679	$C_{28}H_{47}$	383.3672	1.75
395	8.634	fucosterol	8.595	395.3685	$C_{29}H_{47}$	395.3672	3.22
395	–	stigmasterol/Δ-5-avansterol	7.784	395.3674	$C_{29}H_{47}$	395.3672	0.44
397	9.202	β-sitosterol	9.190	397.3842	$C_{29}H_{49}$	397.3829	3.33
409	9.367	β-amyrin	9.306	409.3834	$C_{30}H_{49}$	409.3829	1.28
409	–	cycloartenol/lupeol	8.316	409.3833	$C_{30}H_{49}$	409.3829	1.03
409	–	cycloartenol/lupeol	7.193	409.3835	$C_{30}H_{49}$	409.3829	1.52
423	–	unknown	9.144	423.3996	$C_{31}H_{51}$	423.3985	2.06
441			9.144	441.4099	$C_{31}H_{53}O$	441.4091	1.83

previously described, has been used to discriminate between oil samples belonging to different commercial categories, EVOO, VOO and POO. Several multivariate analyses were performed using sixty-three samples. Each sample was submitted to PS-MS analysis five times, providing an MS spectrum of 400 total variables (*m/z* values). Before performing the statistical analysis, it was necessary to perform a pre-processing of the data: the abundance of each detected ion was normalized to the sum of the intensities of all ions, and the normalized *m/z* signals were filtered according to the instrumental repeatability value (RSD% $<$ 20%). Finally, a data set consisting of 400 variables for 45 samples has been

built for qualitative pattern recognition analysis (PCA and HCA).

Fig. 3 shows the score plot (A) and the loading plot (B) for PS-MS data in the space of the first PCs (PC1 vs PC2 vs PC3). The first three principal components explain 80.4% of the total variance, and it gets very close to 100% by the tenth PC. Good discrimination among the three different olive oil categories can be observed. PC1 highlights the separation between POO samples and EVOO and VOO samples. The first ones are placed at positive values of PC1, while the others are mainly at negative values along PC1, even if, in the centre, there is a slight overlapping of a sample belonging to all three categories. The difference between EVOO and VOO comes from PC3; in this case, EVOO samples are placed at positive values of PC3 and VOO samples at negative ones. From the loadings plot, it is possible to note that few ions influence the PCs and allow the formation of group trending. For PC1, which accounts for 52.6% of the total variance, *m/z* 397 (β-sitosterol) together with *m/z* 443 and 425, tentatively $[M+H]^+$ and $[M+H–H_2O]^+$ of erythrodiol and uvaol, determine the discrimination between EVOO-VOO and POO samples. Indeed, pomace olive oils present a considerable higher content of erythrodiol and uvaol than virgin olive oil [6,11]. The separation between EVOO and VOO, instead, occurs on PC3 (13.4% of variance), where *m/z* 423 and *m/z* 441 have the biggest loading values followed by β-sitosterol. These compounds haven't been identified, and moreover the EEC 2568/91 reports the same parameters for the sterol composition of EVOO and VOO. The group trending, in this case, could be an outcome of the total specific fingerprint of the sterol fraction for the two different commercial categories highlighting its potential power in allowing a discrimination. While *m/z* 443 and 425, tentatively $[M+H]^+$ and $[M+H–H_2O]^+$ of erythrodiol and uvaol, have their major influence on PC2 (14.3% of variance) but do not play any role in the discrimination process.

In addition to Principal Component Analysis, Hierarchical Cluster Analysis was carried out. Fig. 4 shows the dendrogram obtained performing the analysis on the first 20 PCs coming from PCA. Based on their PS-MS fingerprint, the 45 olive oil samples could be divided into four classes. Cluster 1 comprises 86% of VOO samples and the remaining 14% of EVOO samples, while Cluster 2 has contributions from all three categories; it is 21% of POO, 57% of EVOO, and 21% of VOO. Cluster 3 is very small, composed of 100% of EVOO samples, whereas Cluster 4 consists of only POO samples (100%). Cluster 2 composition agrees with the PCA outcome, where overlapping of some samples was observed. The results of the two unsupervised techniques (PCA and HCA) permit us to affirm that PS-MS fingerprints can be used to discern between the different olive oil categories investigated. The following step was the development of a classification method involving additional multivariate techniques. Linear Discriminant Analysis (LDA) and k-Nearest Neighbour (k-NN) were the two classification methods selected and compared. The two algorithms were tested and evaluated using a training set and external samples set (test set), considering the first 20 PCs gained after PCA analysis.

The models' validation was achieved by repeated five-fold cross-validation on training set matrices. The k-NN and PCA-LDA correctly classified the samples into their categories with a total correct prediction of 91.1% and 93.3%, respectively. For k-NN applied to the external samples (test set 18 samples), the overall precision was the following: 83% for EVOO, 80% for VOO and 86% for POO; and accuracy results were: 89% for EVOO, 83% for VOO and 94% for POO. The mismatch samples were one EVOO and two VOO samples; in particular, the extra virgin olive oil (T6) was classified as the minor category VOO, while one of the VOO samples (T7) was identified as EVOO and the other one (T8) as pomace olive oil. On the other hand, PCA-LDA provides for precision, 83% for EVOO, 83% for VOO, 100% for POO and accuracy, 89% for EVOO, 89% for VOO and 100% for POO. In this case, two test set samples were mismatched, an EVOO sample classified as VOO (T4) and a VOO one (T7) classified as EVOO. In general, the best classification was obtained by the PCA-LDA model. More information about the classification can be found in Table S1.

3.3. Confirmation with direct infusion APCI-TOFMS analysis

As confirmation, the same multivariate procedure was applied using MS data obtained from direct infusion APCI-TOF analysis. The reason lies in the need to understand whether the prediction percentages from the PS-MS method are good. The same 63 investigated samples were diluted and submitted to APCI-TOF analysis through direct infusion injection. For each sample, three different MS acquisitions were carried out. The outcomes of PCA and HCA analyses are shown in Fig. S1 and Fig. S2. The first 3 components explain 61.2% of the total variance, but the first 2 (PC1 37.3% and PC2 12.3%) permit good discrimination between the three categories. PC1 allows to separate POO, EVOO, and VOO samples; the first ones are situated at positive values of PC1, while the other 2 categories are at negative values. This is consistent with the PCA analysis on PS-MS data. The separation between EVOO and VOO happens on PC2. VOO samples are located at positive values and EVOO at negative ones. Regarding HCA analysis, the 45 olive oil samples could

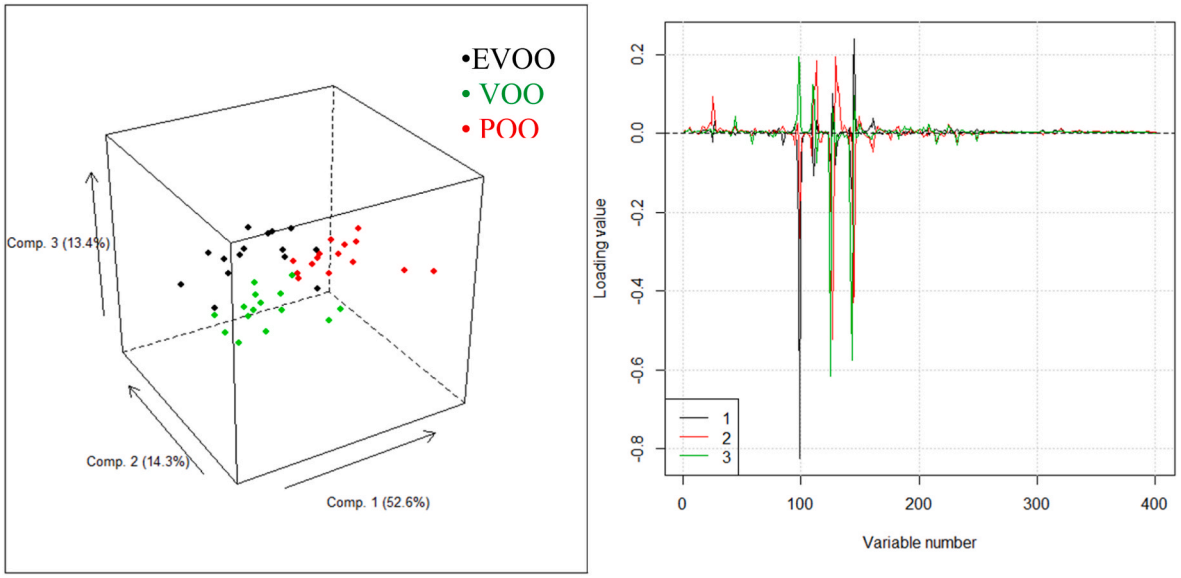

Fig. 3. PCA scores plot (A) and loading plot (B) of the PS(+)-MS spectra (45 samples, 400 variables) (80,4% of the total variance).

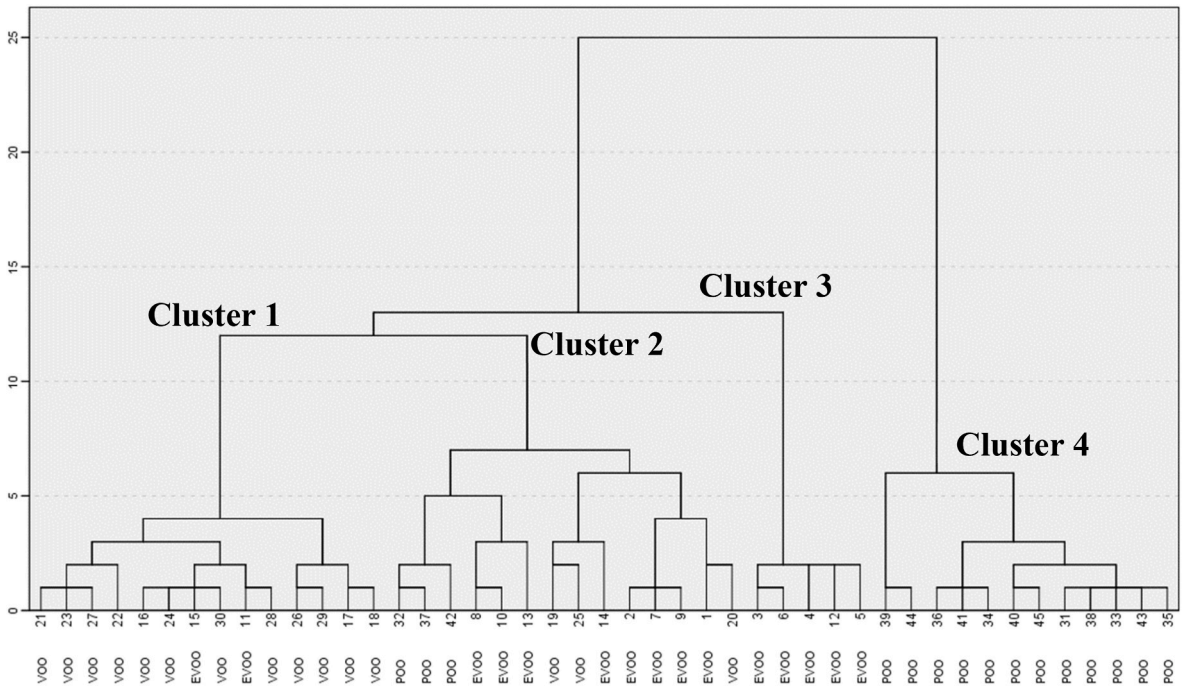

Fig. 4. Hierarchical Cluster Analysis (HCA) based on PS(+)-MS fingerprints of EVOO, VOO and POO samples.

be divided into 3 classes. Cluster 1 is composed of 100% of POO samples, while Cluster 2 is composed of 78% of VOO samples, and the remaining 22% are EVOO. Ultimately, Cluster 3 is almost characterized by EVOO samples (92% EVOO and 8% VOO). Then, as in the PS-MS method, k-NN and PCA-LDA were applied. After 5-fold cross-validation (training set 45 samples), the correct prediction was 93,3% for both supervised statistical approaches. When performing k-NN and PCA-LDA on the external samples (test set 18 samples), the overall precision and accuracy results show, also in this case, slightly better results for PCA-LDA. Accuracy: 100% for EVOO, 94% for VOO and 95% for POO. Only 1 sample (T16) is mismatched; indeed, it is a pomace olive oil, and it is classified as VOO. More information is provided in Tables S2 and S3.

Table 2 highlights the precision (%) and accuracy (%) results obtained from the application of both MS techniques. For PS-MS, the values are lower than those achieved from APCI-MS, and this could lead to misclassification errors (false negatives and/or false positives). Despite that, both MS techniques, the results are very similar and near 100%, confirming the reliability of the proposed streamlined PS-MS method for olive oil discriminative purposes.

4. Conclusions

The importance of the study lies in combining the advantages of mass spectrometry with little or absent sample preparation requirements. In this work, PS-MS was successfully applied for the first time for streamlined discrimination of different olive oil commercial categories. Indeed, two different techniques, two distinct ionization methods, and mass spectrometry resolutions were compared with the same good outcome. Different unsupervised (PCA, HCA) and supervised (k-NN, LDA) chemometric procedures have been applied successfully. The information extracted from the paper spray MS fingerprinting allowed the discrimination between three categories of olive oil (EVOO, VOO, and POO) with a high percentage of prediction. The performance of PCA-LDA was better than the no linear k-NN, but only by 4% for overall accuracy. PS-MS is successfully used for a discriminative purpose. Indeed, it permitted to obtain, using PCA-LDA, a 90% in precision and 95% in accuracy, for the classification of EVOO, VOO, and POO samples. The developed method, based on an Ambient MS technique, due to the open-air ionization is certainly affected by lower MS signal stability when compared to MS techniques using classical ionization sources. This is evident in the comparison of classification results (accuracy and precision values) for the investigated samples. Nevertheless, the quality of the method stands out when compared with the APCI-HRMS results. Moreover, the comparison with classical high-resolution mass spectrometry analysis allowed us to confirm the goodness of the developed method.

Table 2
Comparison of the results of the different classification methods for both PS (+)-MS and Direct infusion APCI-TOF data.

Model	Method	Overall Precision (%)		Overall Accuracy (%)	
Three-class model: EVOO, VOO, POO		**k-NN**	**PCA-LDA**	**k-NN**	**PCA-LDA**
	PS-MS	**83**	**89**	**89**	**93**
	APCI-TOF	**92**	**95**	**93**	**97**

Credi author statement

Ines R Talarico: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing, **Lucia Bartella**: Conceptualization, Investigation, Methodology, Writing – review & editing and Funding acquisition, **Priscilla Rocio-Bautista**: Conceptualization, Investigation, Methodology, Writing – review & editing, **Leonardo Di Donna**: Conceptualization, Writing – review & editing and Funding acquisition, **Antonio Molina-Díaz** Conceptualization, Writing – review & editing, & Funding acquisition, **Juan F García-Reyes** Conceptualization, Writing – review & editing Project administration & Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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