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Advanced Non-Targeted Methodologies applied to Food Authenticity

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Advanced Non-Targeted Methodologies applied to Food Authenticity

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A thesis submitted for the degree of

Doctor of Phylosophy

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“C'est en faisant n'importe quoi qu'on devient n'importe qui”

Rémi Gaillard

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"*Choose a job you love and you will never have to work a day in your life*". This is undoubtedly the most precious teaching I will carry with me for the rest of my life. Within the last 3 years, I have never felt in a research lab, nor in normal workplace. From the very beginning, the Department of Food and Drug has been my new home, and my colleagues my new family. As someone says, family is not an important thing. It's everything

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Preface

Most of the regulations issued in the food sector over the past decades have been mainly centred on safety aspects (e.g. chemical and biological). Consequently, today's surveillance programmes are essentially based on reactive strategies which start monitoring a specific health hazard after a public incident take place. Such reactive approach has proven to be appropriate in the short-run; yet, it has shown ineffective in protecting consumers over the medium-long period. In fact, food poisoning/adulteration outbreaks still remain a global issue and, as demonstrate by recent fraud scandals (e.g. Spanish Toxic Oil Syndrome in 1981, "Chinese milk" in 2008), fraudster does not always have the expertise to assess whether a manipulation can pose toxicological or hygienic risks to the consumers. Therefore, even though the ultimate goal of food fraud is normally to gain extra profit, it may well result in a public health harm. In addition, the episodic nature and alarming regularity of these food-related incidents unavoidably lead the consumer to wonder when the next food integrity outbreak will occur. As a result, competent authorities are losing credibility, companies are losing money and consumers are losing trust in the food system and competent authorities.

While several proactive strategies have been proposed and tested at the management level, the present dissertation focuses on the monitoring stage, where reliable analytical methods accounting for the disguising nature of food adulteration, are increasingly demanded. The specific aim of the thesis was to investigate the potential of non-targeted analytical approaches to solve food authenticity questions, as well as to describe the scientific and analytical constraints to be faced within their development and subsequent implementation in routine analysis. In particular, the manuscript is structured as follow:

- **Introduction** (**Section 1**). After a broad introduction of current food authenticity issues, related root-causes and potential consequences (**Section 1.1**), the inadequacy of common (safety-based) monitoring programmes for tackling food adulteration was illustrated (**Section 1.2**). Afterwards, more emphasis was placed, in **Section 1.3**, on the emerging non-targeted fingerprinting methods for the rapid screening of non-compliance, along with the main challenges encountered in the development of food authentication methods in general (**Section 1.4**);
- **Experimental studies** (**Section 2**). Three scientific studies focused on the development of suitable and fit-for-purpose non-targeted food fingerprinting methods are reported in this section. Diverse detection principles, i.e. vibrational spectroscopy (**Chapter 1**), electronic noses (**Chapter 2**), ambient mass spectrometry (**Chapter 3**), have been tested for the authentication of honey, extra-virgin olive oil and dried oregano, respectively;
- **Discussion (Section 3)**. Since the above-mentioned scientific work are all provided with its respective "Discussion", general considerations about the analytical techniques employed during the PhD programme have been made in **Section 3.1**. Afterwards, advantages of non-targeted

fingerprinting methods over traditional analytical protocols have been reviewed in **Section 3.2**, while their intrinsic drawbacks and limitations are summarized in **Section 3.3**. Particular attention is given to the critical issues to be addressed during the method development and validation. Since multivariate statistics constitutes an integral part of the non-targeted analytical workflow, considerations concerning the today's use (and misuse) of chemometrics in the data treatment and evaluation are summarized in **Section 3.4**.

- **Conclusions** (**Section 4**). Lastly, future perspectives of non-targeted fingerprinting approaches in official food control are illustrated from the author's point of view, along with the urgent needs and gaps to be bridged in order to shift from "academic exercises" to relevant implementations in routine analysis.
- **Annexes I-IV** provide additional details about the employed analytical platforms and the mainly used chemometrics techniques, respectively.

The thesis' introduction and discussion have been adapted from a critical review entitled "Untargeted Fingerprinting Approaches for Food Authenticity: Where Do We Stand?", which is in preparation with the aim of being submitted on Trends in Food Science & Technology. Besides, the three chapters were either published or submitted on peer-reviewed scientific journals (see respective chapters).

For purpose of presentation, the present dissertation has been focused on the application of non-targeted approaches to food authenticity issues. Nevertheless, the author has been involved in several different research topics during his PhD programme - from the development of a liquid chromatography tandem mass spectrometry method for the simultaneous accurate quantification of regulated and emerging mycotoxins, to the assessment of inter-platform and inter-laboratory reproducibility of Travelling Wave Ion Mobility-derived Collision Cross Section measurements and prediction, to the study of plant metabolic response to mycotoxins exposure (Deoxynivalenol, T2 and HT2 toxin) exposure through the application of high resolution mass spectrometry-based metabolomics strategies. For additional details see section "Author".

1. Introduction

Quality is a concept; it is not a physical entity or instance [1]. Finding a comprehensive description of food quality may not be easy and a number of definitions, highlighting different facets, have been proposed [2]. The standard ISO 9001–1:1994 defines the quality as the "Totality of characteristics of an entity that bears on its ability to satisfy stated and implied needs". In the accompanying notes it also states: "The term 'quality' is not used as a single term", but "a qualifying adjective should be used" [3]. Moreover, the valued attributes in a food product may differ according to the viewpoint of different stakeholders (i.e. regulator bodies, food manufacturers, consumers) [4]. Aside from debates about the most appropriate definition, what can be agreed is that several qualities exist, each related to different attributes that influence a product's value to the consumer (or stakeholder).

Being strictly related to consumer's satisfaction, thus somewhat subjective, the quality perception has dramatically evolved in the last 100 years along with economic and cultural changes of the modern society. Research progresses have played an important role in this regard as they have provided scientific evidences of the health-promoting effects induced by the regular consumption of certain food commodities. Besides, the information availability at people's fingertips has increased their awareness towards food's nutritional properties - boosting the interest, for instance, in the so-called "functional foods" [5]. Consequently, also the choices and preferences of today's consumers have significantly changed in industrialized countries; they are nowadays driven by lifestyle, health, economic and, in the modern multicultural society, also religious concerns. As a results, an ever broader range of high- and consistent-quality food products is demanded for competitive prices, regardless of seasonality or local availability [6,7]. In the early 1990s, a large Western supermarket carried an assortment of 10,000 articles on average; nowadays it offers more than 30,000 items. Such agri-food system globalization and trade barriers drop have mutated the food production and distribution chains beyond recognition [6]. To meet the demand, food industries and retailers are pushed to source their products all over the world and even fresh products shipped from halfway around the globe can be offered at fairly low prices [7]. However, with food travelling over larger distances, food safety- and quality-related concerns are often raised [8].

In response, national and international governments are issuing new legislation and regulations that place more emphasis on safety/quality monitoring, food products traceability, as well as environment protection themes [6]. On a global scale, the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) published the Codex Alimentarius in 1963, which consists of a collection of standards, guidelines, codes of practice, and advisory texts on food safety and

quality [9]. For instance, Codex standards issue from specific raw and processed materials characteristics (e.g. hygiene, pesticides and contaminants residues, labelling, etc.), to sampling procedure and analytical protocols to be used in surveillance. It represents the benchmarks for policy making against which national food measures and regulations are evaluated [6]. The publication of the Codex Alimentarius has been followed by an enormous increase in food standards and legislation at national and international levels. In this development, there has been a shift from the former end-of-line product inspection to a new "from-farm-to-fork" approach where the links in the food chain assume responsibility for safety through control of their processes [6]. Within the EU, food and feed businesses carry the full responsibility for the safety of the products that they import, produce, process, place on the market, or sell [10]. This has prompted food operators to introduce internal monitoring programmes. Moreover, concerning the traceability, companies are obliged to keep registration of raw materials supplies and customer deliveries on a transaction basis.

In light of the above, food products can be considered safer than ever before; yet, paradoxically, the safety and quality perception of consumers has decreased significantly in the past decades. Each year worldwide, 600 million foodborne diseases cases and 420 000 deaths caused by unsafe food are estimated by the WHO [11]. At the EU level, a joint Europol-Interpol operation (OPSON) in 2017 reported that 9 800 tonnes, 26.4 million litres and 13 million items of counterfeited and potentially harmful food and drink products were seized across 21 European Union member states, with an estimated value of EUR 230 million [12]; recall announcements can be easily found almost weekly in any newspaper; as highlighted by the Rapid Alert System for Food and Feed (RASFF) and Administrative Assistance and Cooperation System (AAC) 2018 annual report, an increased number of 'alert' and 'non-compliance' have been notified by the EU Member States respect to the previous two years (**Figure 1**) [13].

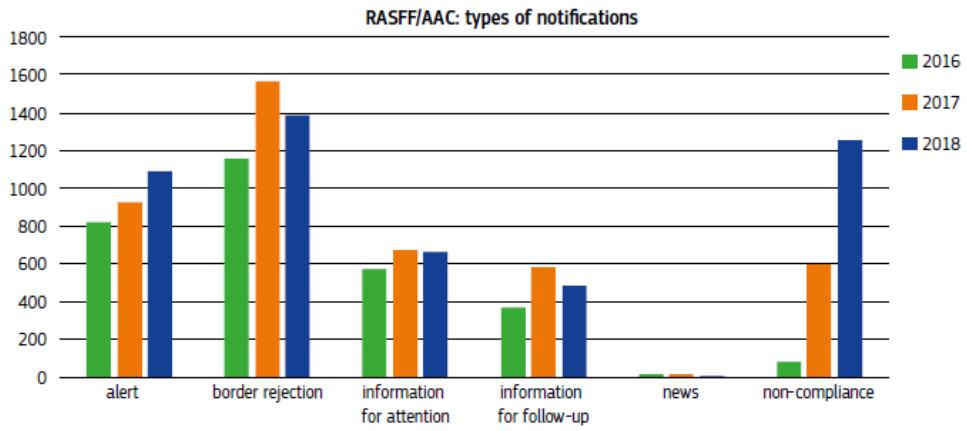

Figure 1. Number of notifications in RASFF and AAC over the period from 2016 to 2018. An 'alert' is sent when a food, feed or food contact material presenting a serious risk is on the market and a rapid action is or might be required. 'Non-compliance' refers to fraudulent activities along the agri-food chain as well as any other nonconformity notified by the EU Member States. For more detail refers to [13,14]. Adapted from [13].

These figures could be said to be directly related to the globalization process described above, as well as the higher vulnerability of modern and faster distribution systems [7]. Nevertheless, it must be pointed out that the increasing reactivity and collaboration between Member States has enabled the implementation of solid control programmes within the food supply networks [13]. Furthermore, knowledge and technology advances in food analysis have led to the continuous development of more effective and reliable testing methods, which may have increased the detection of product non-conformities [15]. On the other hand, the growing demand for highly-processed food products, ever more competitive prices and the extremely complex supply chains have certainly provided more chances for (unintentional or intentional) food contamination and adulteration [8,16].

1.1 Food Authenticity

A food product is authentic when "it is what it says it is" [17]. Although overlapping, and sometimes confusing, terminology is frequently used nowadays, "food fraud" is the expression widely employed in the relevant literature and has been defined by the Global Food Safety Initiative (GFSI) as a "collective term encompassing the deliberate and intentional substitution, addition, tampering or misrepresentation of food, food ingredients or food packaging, labelling, product information or false/misleading statements made about a product for economic gain that could impact consumer health" [18]. Motivation and aims may be different, ranging from personal revenge to ideological objectives, even though economic purposes are certainly the main drivers. In order to place more emphasis on this aspect, the Food and Drug Administration (FDA) introduced the term "economically motivated adulteration" (EMA) - defined as "the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing its apparent value or reducing its cost of production". Apart from the dictionary terminology (comprehensively reviewed in [17] and [19]), what can be agreed is that food fraud includes any illegal, intentional and profit-making deception using food. Besides, "food authentication" has been defined as the process that verifies the consistency between the product characteristics and the reported label description (e.g. origin, production method, processing technologies, etc.) [17,20]; it can be broadly seen as the set of countermeasures put in place by regulators, producers, and even consumers to uncover potential fraud practices [21]. Despite one might think that the authentication process only concerns the final food products to protect the end-consumer, it rather regards the whole supply chain. In fact, food brand manufacturers are generally in the unique position of being victim of fraud activities, as well as largely liable for adulteration incidents [17]. This leads to more stakeholders being actively involved in the fight against food fraud and

makes the development and implementation of effective countermeasures important not only from the legal standpoint, but also from the commercial perspective [20].

The phenomenon of food adulteration is by no means a contemporary problem and it is probably as old as the food processing and production systems themselves [7,22]. Since the times of ancient Rome, common adulteration practices were mixing of spoilt with fresh food, substitution of inferior local products for expensive goods from abroad, or the fraudulent introduction of colouring/masking additives [23]. In the "modern" scientific era, the first to address this issue was the German analytical chemist Frederick Accum in 1820, with a treatise on adulteration of food and culinary poisons (reporting the use of red lead, Pb_3O_4, to give a vibrant colour to cayenne pepper) [24]. More recently, food adulteration/contamination scandals have helped refocus attention on the topic, thanks to the international impact gained due to the resultant media attention [22,25]. The "Chinese milk" and "Horsegate" scandals in 2008 and 2013, respectively, are just part of a long list of events that have laid bare the vulnerability of the modern food global network. Few of the major incidents in recent history include: Spanish Toxic Oil Syndrome in 1981, polyethylene glycol (anti-freeze) and raw methanol adulteration of wines in 1985, carcinogenic red Sudan-I dye found in chilli powder and tomato-based products in 2003 and the so-called 'Irish pork crisis' in 2008 [22,26]. Some of these occasions fortunately did not have lethal consequences; by contrast, the melamine event and other incidents, such as the Spanish Toxic Oil Syndrome, did lead to the loss of life [26]. Indeed, even though the purpose of food fraud is normally to gain extra profit, it can result in public health harm as fraudster does not always have the expertise to assess whether a manipulation poses any toxicological or hygienic risks to the consumer [16]. For these reasons, food fraud has emerged as a serious quality and safety risk that ranks as one of the biggest concerns for both the food industry and government regulators [19]. The episodic nature of the above-mentioned incidents and their alarming regularity unavoidably leads to wonder when another largescale food safety/integrity event will occur [7]. This has not only economic implications, but the increased magnitude and spread of these outbreaks is undermining the consumers' trust in the food system and competent authorities [15,27].

According to van Ruth et al., the fraud vulnerability of a certain food commodity depends on several factors, which can be condensed into three key elements: opportunities, motivations, and control measures (**Figure 2**) [16].

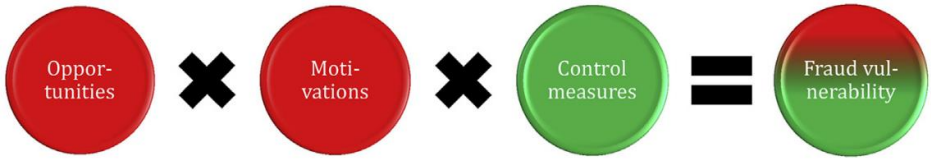

Figure 2. Schematic representation of the food fraud vulnerability concept. Adapted from [16].

The opportunities point out why offenders are able to commit fraud. For instance, fraud opportunities increase when potential malefactors have physical access to the location in which the fraud can be committed (i.e. product storage, processing lines, etc.). Complex supply chain networks enhance fraud vulnerability, since they generally exhibit lower transparency. Motivations - typically economic - detail why offenders are bent on committing the fraud. Food adulteration normally involves the substitution of a higher value product with less expensive or lower quality alternative(s); therefore, products exhibiting a high price/Kg, as well as claiming valuable attributes (e.g. production systems, origin and processing labels, etc.), are expected to add temptation to fraudsters. Other incentives are gaps existing between physical product availability and market demand or prices shift due to regional or global supply shortages. Accordingly, fraud vulnerability can be seen as the result of the interaction between motivated offenders, and the opportunities presented by victims [28]. Such vulnerability can be minimized by the control measures in place. Intuitively, the availability of a detection method for certain illicit practices, as well as the effectiveness of fraud monitoring systems, certainly reduce the susceptibility of the commodity [16].

While staple foods, such as grains, bread, wine and milk, were the typical candidates for adulterations in antiquity, fraudulent practices were soon extended to more "luxurious" food commodities imported from overseas. For example, coffee, which was an extremely expensive commodity in the Middle Ages, was adulterated with chicory, roasted wheat, or burnt sugar; tea leaves were often mixed with leaves from other plant species; sugar would be blended with sand. Nowadays, there are several ways in which food, and even feed, commodities can be counterfeited. Typical authenticity issues are adulteration and counterfeiting (e.g. dilution of beverages with water, replacement of one ingredient by a cheaper one, etc.), sophistication processes (e.g. undeclared use of ingredients and/or additives), incorrect and false botanical/geographical origin or production process (e.g. organic vs non-organic) declarations [29,30]. Moore and co-workers developed a database of food ingredient fraud and EMA from publicly available articles in scholarly journals [31]. Therein, olive oil, milk, honey, and spices shown to be the most common targets for adulteration. Accordingly, the 2018 annual report of the EU Food Fraud Network highlighted the same product categories being among the most vulnerable within the EU (**Figure 3**).

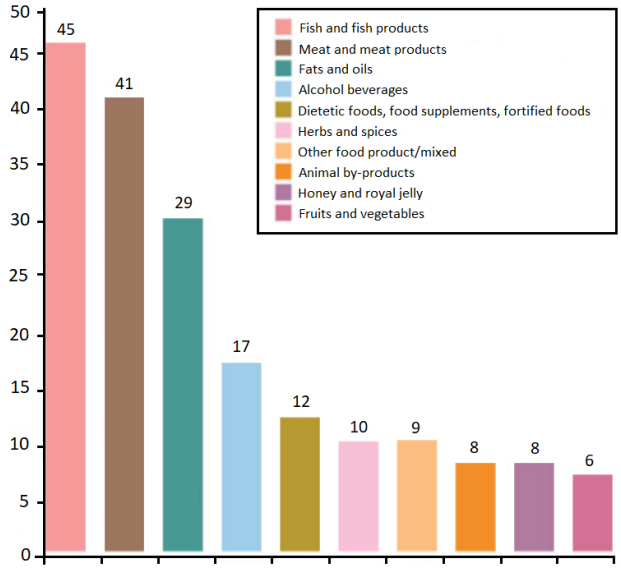

Figure 3. Top 10 categories in the AAC in 2018 according to the number of requests for administrative assistance concerning fraud suspicions from the EU Member States. Adapted from [14].

Three of these highly-vulnerable food commodities were investigated in the present thesis, namely honey, olive oil and dried herbs. The experimental studies reported in **Section 3**, focuses on potential analytical methods for the detection of specific fraud actions. Nevertheless, vulnerability issues related to the supply chains of these commodities, have been thoroughly described in literature [32–37] and the reader is referred to these manuscripts for more details.

1.2 From Targeted to Non-targeted Approaches

Most of the newly introduced regulations regarding food products have been mainly centred on safety aspects, with the relative protection plans involving "prevention", "intervention" and "response", with a process cycle back to "prevention" [31].

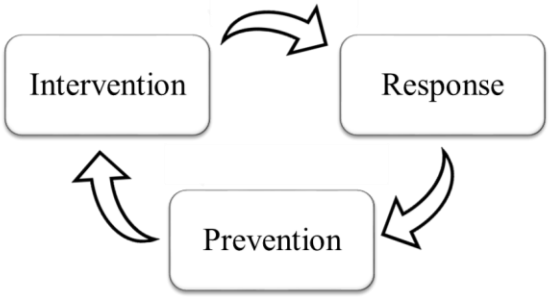

Figure 4. Food protection plan progression. Adapted from [31].

As a matter of fact, such control systems are essentially based on reactive strategies since they start monitoring a specific contaminant after a public health incident occur ("Intervention" step) [19]. Although reactive approaches have proven to be effective in the short-run, they are limited in their scope over the medium to long-term period and, as proof of such inadequacy, food poisoning/adulteration outbreaks still remain a global issue [8,16]. When it comes to this discussion, a landmark is represented by the China's international milk crisis in 2008. It involved melamine, a nitrogen-rich organic compound widely used in plastic manufacture, which was added to food products (including milk) in order to increase their apparent protein content [22]. The sequence of events started in May 2007 when a number of cats and dogs died in the USA after ingesting melamine-contaminated pet food imported from China [38]. The following December, sick babies with discoloured urine were reported in China with the first child fatality occurring on 1 May 2008. By the end of the year, there were an estimated 300 000 victims in China, including 860 infants hospitalised and six children death due to kidney damage. The contamination widened and spread to several other countries, where several other food products, such as egg powder, biscuits, confectionary and baking powder, were tested positive for melamine. During and following the outbreak, lot of research efforts have focused on the development of analytical protocols for the detection of melamine in a wide range of products, which are nowadays included in routine control programmes [39,40]. Melamine and other structurally related compounds testing is now one of the "must-do's" when exporting feed materials to the USA and EU from third-party countries. The example of melamine clearly points out the intrinsic limitations of current food safety ploys. Melamine was not a substance on the control system radar and was not detected in infant formula and powdered milk by routine analysis. The "early warning" was the public health incident and the detection was a response to human illness and death. Moreover, it underlines that also a food adulteration actions may have also severe safety consequences.

Due to the situation, the food industry, agencies and academics have started working together to develop and implement holistic, all-encompassing countermeasures [19]. As opposed to reactive approaches, a proactive management aims to prevent the nonconforming food items reaching the point of sale, in order

to reduce both the risk and impact of food-related accidents [41]. Examples of proactive strategies implemented at a management level for the identification of emerging safety issues are early warning system, chain vulnerability assessment and predictive model based on retrospective analysis of occurrence trends [42,43]. A parallel can be drawn at the control and monitoring stage, where it is important that equipment and infrastructure keep abreast of new scientific developments in order to continuously improve the efficiency of food control activities. As will be discussed in the following sections, the disguising and highly dynamic nature of food fraud is among the main challenges to be faced and reliable analytical methods must take these features into account. Traditionally, authenticity assessment of food has been based on the (targeted) analysis of specific marker compounds, indicative for a certain product's property. The subsequent comparison of the measured value with an established threshold enables the assessment of the product's compliance, or not, with a required standard. Such conventional food authentication approaches are based on stable isotope analysis, molecular biology, mass spectrometry-based marker quantification, etc. [44–48]. Being focused on definite compounds and/or food components whose analytical "behaviour" is known beforehand, selective sample preparations can be optimized in order to enhance the analyte responses and the overall method efficiency [49]. On the other hand, targeted methods for authenticity purposes also present serious, and unbridgeable in some cases, drawbacks to be taken into consideration. First, classical analytical protocols are generally time- and labour-consuming, requiring highly trained personnel; therefore, they strive to meet the demands of modern and fast-paced food global networks [22,25]. In contrast, the food industry's stakeholder (e.g. producers, retailers, regulators, etc.) are expressly seeking more high-throughput and user-friendly screening methods for the rapid detection of food fraud. These methods should ideally rely on portable, ruggedized, and potentially handheld or remotely controlled equipment, so that they can be taken to or positioned on/at/in-line at points of vulnerability. It is also preferable that these approaches would require a minimum amount of training for new users [7]. Second, by the very nature of the adulteration, only the criminal knows how the product has been manipulated. Especially as regard to the addition/substitution of food ingredients, current protection systems are not designed to look for the undefinable number of potential adulterants which can, and have been used [4,31]. Indeed, in order to circumvent routine controls, fraudsters often end up using unconventional and in some cases non-food ingredients which are unlikely to be detected by traditional routine controls [25]. Finally, food integrity assessment cannot always be simply restricted to the identification of a single or a few marker compounds [50]. Typical food authentication questions (e.g. geographical/biological origin, organic vs non-organic, etc.) usually require complex and sophisticated laboratory studies and finding a reliable marker(s) related to the problem under investigation may not be an easy task because of the intrinsic biological and technical sources of variation [51]. For these reasons, common safety ploys have shown to be ineffective for tackling food adulteration [16]. Besides, the need of

more “holistic” analytical methods, which bring into focus the whole food matrix instead of one single analyte, has boosted the development of the so-called non-targeted testing strategies.

As opposed to targeted methods, non-targeted strategies for detecting food adulteration “model” the properties of the authentic material rather than the features of the known adulterants. Instead of trying to find some chemical which may - or may not - be present, the focus is on detecting something that somehow differs from a defined reference standard by merging interdisciplinary expertise, including analytical chemistry, statistics and food science [52]. From the analytical standpoint, the underlying idea is to capture as many compounds, or features, as technically possible in order to gain comprehensive insights of the tested sample [51]. This concept is based on the so-called *metabolomics* [53] and has been widely adopted in the scientific literature. However, it must be mentioned that non-targeted methodologies have been developed by scholars working in a multitude of disciplines, and each seems to have established his own terminology [52]. In this regard, the United States Pharmacopeia (U.S. Pharmacopeia) has recently published a guidance document intended to standardize some of the key terms related to the development and validation of non-targeted analytical methods specifically intended for the detection of EMA-related adulterants in food [54]. Therein, a non-targeted method is defined as an “analytical measurement coupled with statistical tools that recognize deviations from the signal associated with the nominal material” [54]. Given this broad definition of non-targeted analytical methods, different approaches can be distinguished based on the adopted detection and data evaluation principles. In particular, as reviewed by Esslinger et al. [51]:

Food profiling refers to the analysis of a specific group of metabolic products, or a class of compounds, optionally followed by multivariate data analysis. Such strategy is based on a prior knowledge of the biological system and, thus, should be rather considered as a targeted analysis. Profiling approaches have been widely applied for food authentication of various food matrices focusing on different analyte classes, such as trace elements, fatty acids, phenolic and volatile compounds [55–58].

Foodomics is the term firstly introduced by Cifuentes et al. [59,60] to define the global discipline that studies the food- and nutrition-related domains through the application of advanced *omics* technologies (i.e. “genomics”, “proteomics”, “metabolomics”) in combination with bioinformatics approaches. Foodomics has been applied in food safety control (e.g. evaluation of microbial toxins, allergens, pesticides), food quality (organoleptic properties and nutritional value), food authenticity and traceability [60–64].

Fingerprinting approaches are typically based on a high-throughput screening of samples after, if necessary, a (simple) sample preparation. The most widely used methods are based on spectroscopic techniques, such as nuclear magnetic resonance (NMR), NIR or FT-IR spectroscopy. The spectral profile yielded by these technologies contain information about potentially all the molecular absorbers present in

the matrix. Being characteristic of the specific analysed item, the resultant spectrum can be seen as a chemical "fingerprint" of the sample.

It must be mentioned that these terminologies are frequently used interchangeably in literature. For instance, the term "fingerprint" is often used when a specific class of compounds [65–67], as well as multi-elemental and isotopic ratio profiles [68–71], are considered for the discrimination between sample groups using multivariate statistics. Based on the above, the term "profiling" should rather be employed due to the *a priori* knowledge of the analytes under investigation. Souard et al., even used all the terms "metabolomics", "fingerprint", "untargeted" and "profiling" in their manuscript's title [72]. Notwithstanding, such nomenclatures cannot be defined as "wrong" since a universally accepted definitions is still lacking (the U.S. Pharmacopeia guidance refers to generic "non-targeted analysis" [54]).

1.3 Non-targeted Fingerprinting

Despite some substantial differences between the above-mentioned non-targeted approaches, to some extent, they all follow the common workflow reported in **Figure 5**.

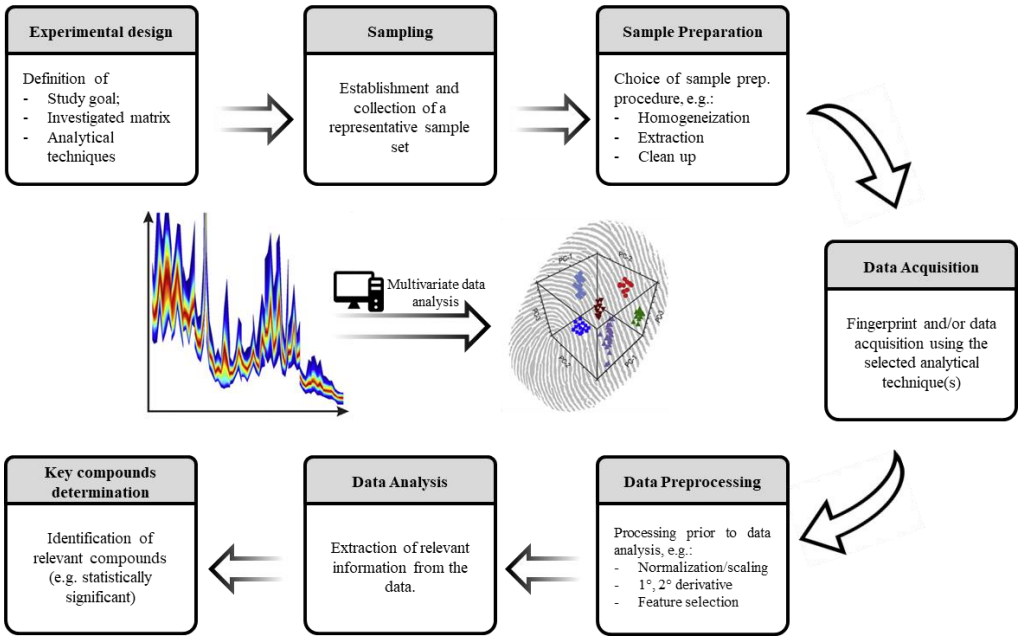

Figure 5. Workflow of non-targeted approaches for food analysis. Adapted from [51].

Briefly, the process of developing a non-targeted method begins with the definition of the "Applicability Statement". This is a general statement about the purpose and scope of the method, addressing the key aspects of the specific situation and circumstances (e.g. threat to be covered, sample material and its characteristics, etc.), specifying the chosen analytical technology and adopted data processing/analysis strategy, defining the minimum performance requirements and expected achievements [54]. In addition, the types and concentration range of the adulterants have to be defined in this stage by means of a thorough risk assessment. The following steps of the workflow are the sample collection, which normally represents one of the most demanding task in food authentication studies (see **Section 1.4**), preparation and analysis (according to the applicability statement). At this point, the acquired data undergoes to pre-processing and multivariate analysis, aimed at extracting the relevant information and/or make the decision on the sample integrity. Optionally, statistically-relevant compounds responsible for the sample classification/discrimination can be determined and structurally elucidated. For more details, the reader is directed to the several published review that thoroughly describe all these steps [49,51,73–76].

Even though *foodomics* and food profiling approaches have been increasingly applied in food safety and quality control, the focus of the present thesis was on non-targeted fingerprinting methods (**Chapter 1**, **2**

and **3)** and larger attention is given to such approach in the present section. In this regard, also the U.S. Pharmacopeia guidance, despite citing generic "non-targeted analysis", refers rather explicitly to the development of non-targeted fingerprinting methods, which couple a rapid analytical measurement with a one-class classification model for the detection of non-compliance [54]. The fundamental purpose is to assign an unknown sample as "Typical" (authentic) or "Atypical" (not authentic) by comparing the recorded fingerprint with a reference data library [54]. Therefore, the statistical analysis (chemometrics) aim at answering the following question: "Is the tested sample Typical or Atypical compared to a reference set of Typical samples?". A "Typical" outcome imply that, within the known performance of the method and the applied statistical conditions, the tested sample exhibits similar properties to the reference set. Besides, an "Atypical" outcome suggests that the unknown sample is not consistent with the reference set, potentially revealing misdescription or adulterations of the product [49,54].

According to the USP guidance, two sets of samples are required for a proper method development: the so-called "reference" and "test set" (**Figure 6**).

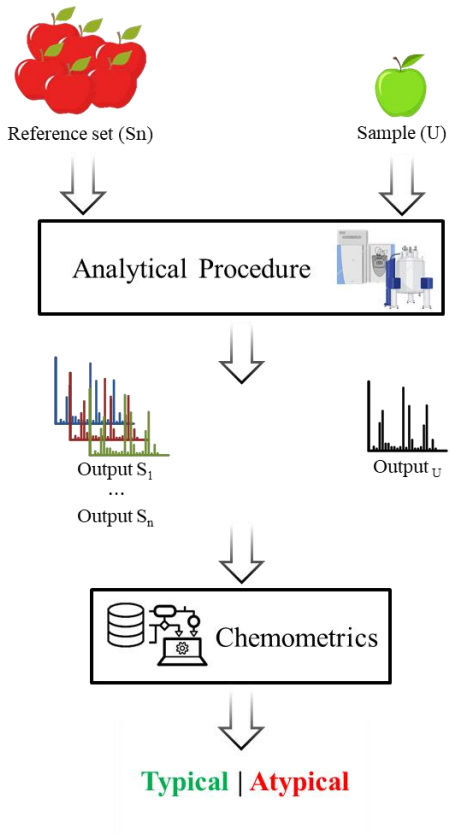

Figure 6. Schematic representation of a non-targeted fingerprinting workflow for the detection of food authentication.

The reference set consists of a fully representative population of authentic samples covering all the relevant sources of variations, as well as data acquisition conditions (e.g. instruments, operators, etc.), associated with the research question. Besides, the test set is an approximately equal mix of Typical (which must not be used also in the reference set), and "Atypical" samples; as the name suggests, it is used as "blind" sample set to challenge the developed statistical model and assess its fitness-for-purpose. Ideally, when the addition of a specific adulterant is being investigated, at least three replicates should be used at varying levels of concentration - one at levels around the risk threshold (specified in the Applicability Statement), the others around half and somewhat exceeding twice that level. Furthermore, it must be ensured that deliberate spiking does not compromise the sample matrix unduly. The intention is to make the sample as ″genuinely adulterated″ as possible [54]. Further details and recommendations can be found in [49,54]. If the method performance shows promise, it has to be further validated using a so-called validation set, which is similar to the test set (both include an approximately equal number of typical and atypical samples) but must be independent from any other samples used in the method development. Methods that cannot meet the requirements of the applicability statement require its modifications in the analytical procedure or threshold/boundary [52]. Method validation arguably represents the most challenging part of the entire development process, which must guarantee the reliability and robustness of the analytical protocol. Unfortunately, there are no clear-cut reference procedures for the validation of non-targeted fingerprinting methods and most if left at the discretion of the analyst. As a general rule, all the possible experimental factors, such as instrument drifts, environmental influences, different operators/laboratories, changes in food matrix composition during the storage, etc. have to be comprehensively taken into consideration through appropriate sample collection and data analysis [49]. As will be discussed in **Section 3.3**, such lack of standardization represents one of the main bottlenecks of non-targeted fingerprinting methods.

Unlike *foodomics* (or metabolomics in general), food fingerprinting does not deal with the identification of significant metabolites, but on the recognition of patterns [49]. Data are evaluated at a multivariate level and model performance are customarily expressed as sensitivity and specificity rate for the chosen decision thresholds. Sensitivity rate is the number of correct Atypical predictions from the method divided by the total number of Atypical samples. Besides, specificity rate is the number of correct Typical predictions from the method divided by the total number of Typical samples:

$$Sensitivity = \frac{Correct\ Atypicals}{Total\ Atypicals}$$

$$Specificity = \frac{Correct\ Typicals}{Total\ Typicals}$$

Other terminologies often used when evaluating the outcomes of one-class classifier are “True Positive” for the samples belonging to the class of interest correctly recognised as compliant; “False Negative” if they are erroneously rejected. Correspondingly, samples not belonging to the class of interest are labelled as “False Positive” if they are wrongly assigned to the class, and “True Negative” if they are correctly refused [77]. More details can be found in [77].

Even though the present dissertation has been focused on qualitative questions related to food authenticity, it must be mentioned that fingerprinting-based methods are also largely applied for the quantitative determination of key components of food and food ingredients. For instance, the rapid determination of protein content by near-infrared spectrometry and multivariate calibration [78] can be considered as a non-targeted fingerprinting approach [49]. The generic workflow followed for these studies, although similar to what reported in **Section 1.3**, will not be covered in the present thesis. For an overview of the quantitative fingerprinting approaches and the related data analysis methodologies, the reader is referred to [79].

1.4 Challenges in Food Authenticity

As mentioned in **Section 1.2**, reliable analytical procedures accounting for the disguising nature of food adulteration are highly acclaimed by the industry, agencies and regulatory bodies. Besides, analytical and food chemists are demanded to develop effective methods to detect fraudulent practices and a significant amount of literature has been produced in the last two decades. While more technical aspects of modern non-targeted fingerprinting methods will be discussed in **Section 3.2** and **3.3**, some of the main challenges typically faced within a food authentication study in general are summarized below.

Fraudsters vs control organism. Normally, fraudsters are expected to be aware of current authenticity monitoring programs and to conceive unconventional solutions to avoid detection. This represents one of the biggest issues in food authenticity which requires a continuous effort to improve the analytical power to uncover sophisticated products adulterations [25,30]. It can be seen as a competition between vigilance organisms, pursuing analytical methods for detecting newly-identified adulterations, and malefactors seeking new ways to circumvent routine protocols [7]. The challenge for quality assurance laboratories is to think one step ahead of fraudsters; however, in this “endless cycle”, they are always one step behind [52]. The required knowledge about the specific analytes to be screened poses a serious weakness for traditional targeted analysis and, as explained in **Section 1.2**, represents the main reason

behind the growing interest towards non-targeted analytical methods [25]. The latter possess significant practical benefits, but due diligence is required in their development, validation, and implementation (see **Section 3**).

Choosing the right approach. When a newly-uncovered fraud practice is faced, finding the most suitable approach to guarantee its reliable detection is among the first questions and often represents one of the greatest challenges to the analyst. From the analytical standpoint, by choosing one or technique, the focus is placed on certain food components/substances which can be best used to reveal specific fraud practice. For instance, DNA-based techniques are largely used for species/variety determination, stable isotope ratio ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$) are deemed suitable to confirm the production method (e.g. conventional vs organic) and geographic origin, metabolomics and proteomics are employed to identify a low-molecular weight analyte or a peptide sequence indicative of the adulteration/substitution presence, etc. Clearly, the choice is strictly related to the problem under investigation and a comprehensive discussion of all the possible solution cannot be provided herein. Nevertheless, the reader is directed to the several excellent reviews published in literature [22,29,80].

Although the present thesis focuses on non-targeted food testing strategies for the early detection of food adulteration, it is by no means against targeted analytical approaches in general. As will be discussed in **Section 3.3**, targeted approaches have explicit advantages over non-targeted methods. In fact, a widespread strategy is to use non-targeted analytical approaches to perform preliminary investigations and finding chemical substances, or DNA/RNA sequences, which can be used as potential adulteration marker(s); after a proper validation of these markers, the appropriate (targeted) analytical protocol for their quantification can be developed [62,81]. In this regard, several successful applications of such two-stage strategy can be found in literature [48,62,82–84]. Nevertheless, it must be mentioned that discovering reliable markers for the food/ingredient, adulterant in question, processing and/or production origin, is not always an easy task. In fact, all the environmental/biological factors affecting its natural variation, which must be limited, have to be accurately characterized [80]. Honey represents an illustrative example. If in the one hand two decades of studies investigating potential honey's floral and geographic authenticity markers have been reviewed by Kaškonienė et al.[85], in the other hand, there is a range factors (e.g. beekeeping technique, harvest and storage environmental conditions, etc.) affecting its chemical composition that can lead to strong seasonal fluctuations of these compounds. Moreover, their stability over long-period storage and/or processing must be ensured [36,86].

Sample collection. A more practical challenge encountered in the development of either targeted or non-targeted methods concerns the sample collection. In general, the number and type of reference samples

and the sampling procedure strictly depend on the problem in question and the chosen analytical approach. As an example, DNA-based strategies (e.g. determination of species/varieties) may require only a single sample for reliable detection since DNA sequences are almost constant against exogenous factors. In contrast, the sample's chemical composition and metabolome can be affected by the environmental fluctuations (e.g. weather conditions, soil, pesticides) and all the relevant sources of variability associated with the research question should be covered within the sampling process. To this end, large sample sets are normally needed and, after the actual method development stage, reference products must still be regularly sourced and analysed to monitor such influences over the long-term period (see **Section 3.3**), particularly in these times of increasing climate change [74]. Furthermore, the collection of complementary metadata (e.g. GPS coordinates, use of fertilizers/pesticides, etc.) is mandatory since it can help to consider the various influencing factors and allow a meaningful interpretation of the results [74,87]. However, authentic reference materials are seldom readily available and obtaining both representative typical and atypical samples may be a very demanding task [52,80]. For instance, reference products must be sourced directly from foreign countries when dealing with geographical origin questions [80]. In addition, normal market conditions should be reproduced using standardized sampling procedures in order to create reference databases applicable to real-world situations [87]. In this regard, the sampling period is another important factor since variations in the chemical composition of several natural products (e.g. wine, olive oil, honey, etc.) have been reported also within the same harvesting season [30]. Due to these difficulties, many scientific studies published in literature have been based on not-suitably authenticated samples [25].

References

[1] H.A. Bremner, Toward practical definitions of quality for food science, Crit. Rev. Food Sci. Nutr. 40 (2000) 83–90. https://doi.org/10.1080/10408690091189284.

[2] J.R. Botta, Evaluation of Seafood Freshness Quality, 1st ed., Wiley, 1995.

[3] ISO - ISO 9001:1994 - Quality systems — Model for quality assurance in design, development, production, installation and servicing, (n.d.). https://www.iso.org/standard/16534.html (accessed May 9, 2020).

[4] J. Spink, The Challenge of Intellectual Property Enforcement for Agriculture Technology Transfers, Additives, Raw Materials, and Finished Goods against Product Fraud and Counterfeiters, J. Intellect. Prop. Rights. 16 (2011) 183–193.

[5] C. Peri, The universe of food quality, Food Qual. Prefer. 17 (2006) 3–8. https://doi.org/10.1016/j.foodqual.2005.03.002.

[6] J. Trienekens, P. Zuurbier, Quality and safety standards in the food industry, developments and challenges, Int. J. Prod. Econ. 113 (2008) 107–122. https://doi.org/10.1016/j.ijpe.2007.02.050.

[7] D.I. Ellis, H. Muhamadali, S.A. Haughey, C.T. Elliott, R. Goodacre, Point-and-shoot: Rapid quantitative detection methods for on-site food fraud analysis-moving out of the laboratory and into the food supply chain, Anal. Methods. 7 (2015) 9401–9414. https://doi.org/10.1039/c5ay02048d.

[8] R. Nayak, P. Waterson, Global food safety as a complex adaptive system: Key concepts and future prospects, Trends Food Sci. Technol. 91 (2019) 409–425. https://doi.org/10.1016/j.tifs.2019.07.040.

[9] E. Kirchsteiger-Meier, T. Baumgartner, eds., Global Food Legislation: an Overview., Wiley, 2014. https://doi.org/https://doi.org/10.1002/9783527680795.

[10] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food saf, Off. J. L31 (2002) 1–24.

[11] Estimating the burden of foodborne diseases, (n.d.). https://www.who.int/activities/estimating-the-burden-of-foodborne-diseases (accessed May 9, 2020).

[12] EUR 230 million worth of fake food and beverages seized in global OPSON operation targeting food fraud | Europol, (n.d.). https://www.europol.europa.eu/newsroom/news/eur-230-million-worth-of-fake-food-and-beverages-seized-in-global-opson-operation-targeting-food-fraud (accessed September 14, 2020).

[13] The Rapid Alert System for Food and Feed 2018 Annual Report, Luxembourg, 2019. https://ec.europa.eu/food/sites/food/files/safety/docs/rasff_annual_report_2018.pdf (accessed May 9, 2020).

[14] The EU Food Fraud Network and the System for Administrative Assistance - Food Fraud, Luxembour, 2019. https://ec.europa.eu/food/sites/food/files/safety/docs/ff_ffn_annual-report_2018.pdf (accessed May 9, 2020).

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

[16] S.M. van Ruth, W. Huisman, P.A. Luning, Food fraud vulnerability and its key factors, Trends Food Sci. Technol. 67 (2017) 70–75. https://doi.org/10.1016/j.tifs.2017.06.017.

[17] K. Robson, M. Dean, S. Haughey, C. Elliott, A comprehensive review of food fraud terminologies and food fraud mitigation guides, Food Control. 120 (2021) 107516. https://doi.org/10.1016/j.foodcont.2020.107516.

[18] GFSI Position Paper on Mitigating the Public Health Risk of Food Fraud - The Consumer Goods Forum, (n.d.). https://www.theconsumergoodsforum.com/press_releases/gfsi-position-paper-on-mitigating-the-public-health-risk-of-food-fraud/ (accessed October 12, 2020).

[19] J.W. Spink, Food Fraud Prevention, Springer New York, 2019. https://doi.org/10.1007/978-1-4939-9621-6.

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

[21] S. Charlebois, A. Schwab, R. Henn, C.W. Huck, Food fraud: An exploratory study for measuring consumer perception towards mislabeled food products and influence on self-authentication intentions, Trends Food Sci. Technol. 50 (2016) 211–218. https://doi.org/10.1016/j.tifs.2016.02.003.

[22] D.I. Ellis, V.L. Brewster, W.B. Dunn, J.W. Allwood, A.P. Golovanov, R. Goodacre, Fingerprinting food: Current technologies for the detection of food adulteration and contamination, Chem. Soc. Rev. 41 (2012) 5706–5727. https://doi.org/10.1039/c2cs35138b.

[23] J.F. Bush, “By Hercules! The More Common the Wine, the More Wholesome!” Science and the Adulteration of Food and Other Natural Products in Ancient Rome on JSTOR, Food Drug Law J. 52 (2002) 573–602. https://www.jstor.org/stable/26660496 (accessed September 15, 2020).

[24] Friedrich Accum, A treatise on adulterations of food, and culinary poisons, Longman, Hurst, Rees, Orme, and Brown, London, 1820.

[25] T.F. McGrath, S.A. Haughey, J. Patterson, C. Fauhl-Hassek, J. Donarski, M. Alewijn, S. van Ruth, C.T. Elliott, What are the scientific challenges in moving from targeted to non-targeted methods for food fraud testing and how can they be addressed? – Spectroscopy case study, Trends Food Sci. Technol. 76 (2018) 38–55. https://doi.org/10.1016/j.tifs.2018.04.001.

[26] D.I. Ellis, R. Goodacre, Detecting food authenticity and integrity, Anal. Methods. 8 (2016) 3281–3283. https://doi.org/10.1039/c6ay90015a.

[27] J. Barnett, F. Begen, S. Howes, A. Regan, A. McConnon, A. Marcu, S. Rowntree, W. Verbeke, Consumers' confidence, reflections and response strategies following the horsemeat incident, Food Control. 59 (2016) 721–730. https://doi.org/10.1016/j.foodcont.2015.06.021.

[28] S.M. van Ruth, P.A. Luning, I.C.J. Silvis, Y. Yang, W. Huisman, Differences in fraud vulnerability in various food supply chains and their tiers, Food Control. 84 (2018) 375–381. https://doi.org/10.1016/j.foodcont.2017.08.020.

[29] S. Lohumi, S. Lee, H. Lee, B.K. Cho, A review of vibrational spectroscopic techniques for the detection of food authenticity and adulteration, Trends Food Sci. Technol. 46 (2015) 85–98. https://doi.org/10.1016/j.tifs.2015.08.003.

[30] S. Medina, J.A. Pereira, P. Silva, R. Perestrelo, J.S. Câmara, Food fingerprints – A valuable tool to monitor food authenticity and safety, Food Chem. 278 (2019) 144–162. https://doi.org/10.1016/j.foodchem.2018.11.046.

[31] J.C. Moore, J. Spink, M. Lipp, Development and Application of a Database of Food Ingredient Fraud and Economically Motivated Adulteration from 1980 to 2010, J. Food Sci. 77 (2012) R118–R126. https://doi.org/10.1111/j.1750-3841.2012.02657.x.

[32] P. Galvin-King, S.A. Haughey, C.T. Elliott, Herb and spice fraud; the drivers, challenges and detection, Food Control. 88 (2018) 85–97. https://doi.org/10.1016/j.foodcont.2017.12.031.

[33] I.C.J. Silvis, S.M. van Ruth, H.J. van der Fels-Klerx, P.A. Luning, Assessment of food fraud vulnerability in the spices chain: An explorative study, Food Control. 81 (2017) 80–87. https://doi.org/10.1016/j.foodcont.2017.05.019.

[34] A. Bajoub, A. Bendini, A. Fernández-Gutiérrez, A. Carrasco-Pancorbo, Olive oil authentication: A comparative analysis of regulatory frameworks with especial emphasis on quality and authenticity indices, and recent analytical techniques developed for their assessment. A review, Crit. Rev. Food Sci. Nutr. 58 (2018) 832–857. https://doi.org/10.1080/10408398.2016.1225666.

[35] L. Conte, A. Bendini, E. Valli, P. Lucci, S. Moret, A. Maquet, F. Lacoste, P. Brereton, D.L. García-González, W. Moreda, T. Gallina Toschi, Olive oil quality and authenticity: A review of current EU legislation, standards, relevant methods of analyses, their drawbacks and recommendations for the future, Trends Food Sci. Technol. (2019). https://doi.org/10.1016/j.tifs.2019.02.025.

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

[37] A.J. Siddiqui, S.G. Musharraf, M.I. Choudhary, A. ur Rahman, Application of analytical methods in authentication and adulteration of honey, Food Chem. 217 (2017) 687–698. https://doi.org/10.1016/j.foodchem.2016.09.001.

[38] R.L.M. Dobson, S. Motlagh, M. Quijano, R.T. Cambron, T.R. Baker, A.M. Pullen, B.T. Regg, A.S. Bigalow-Kern, T. Vennard, A. Fix, R. Reimschuessel, G. Overmann, Y. Shan, G.P. Daston, Identification and Characterization of Toxicity of Contaminants in Pet Food Leading to an Outbreak of Renal Toxicity in Cats and Dogs, Toxicol. Sci. 106 (2008) 251–262. https://doi.org/10.1093/toxsci/kfn160.

[39] S.A. Tittlemier, Methods for the analysis of melamine and related compounds in foods: A review, Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 27 (2010) 129–145. https://doi.org/10.1080/19440040903289720.

[40] G. Venkatasami, J.R. Sowa, A rapid, acetonitrile-free, HPLC method for determination of melamine in infant formula, Anal. Chim. Acta. 665 (2010) 227–230. https://doi.org/10.1016/j.aca.2010.03.037.

[41] Z. Xiaobo, H. Xiaowei, M. Povey, Non-invasive sensing for food reassurance, Analyst. 141 (2016) 1587–1610. https://doi.org/10.1039/c5an02152a.

[42] J.M. Park, J.M. Kim, J.W. Hong, S. Ghim, Introduction of highly effective proactive food safety management programs into food distribution channels: For safe food labeling and safe advertisements, J. Food Saf. 40 (2020). https://doi.org/10.1111/jfs.12751.

[43] H.J.P. Marvin, G.A. Kleter, L.J. Frewer, S. Cope, M.T.A. Wentholt, G. Rowe, A working procedure for identifying emerging food safety issues at an early stage: Implications for European and international risk management practices, Food Control. 20 (2009) 345–356. https://doi.org/10.1016/j.foodcont.2008.07.024.

[44] A. Caligiani, M. Nocetti, V. Lolli, A. Marseglia, G. Palla, Development of a Quantitative GC-MS Method for the Detection of Cyclopropane Fatty Acids in Cheese as New Molecular Markers for Parmigiano Reggiano Authentication, J. Agric. Food Chem. 64 (2016) 4158–4164. https://doi.org/10.1021/acs.jafc.6b00913.

[45] F. Camin, R. Wehrens, D. Bertoldi, L. Bontempo, L. Ziller, M. Perini, G. Nicolini, M. Nocetti, R. Larcher, H, C, N and S stable isotopes and mineral profiles to objectively guarantee the authenticity of grated hard cheeses, Anal. Chim. Acta. 711 (2012) 54–59. https://doi.org/10.1016/j.aca.2011.10.047.

[46] M.I.S. Veríssimo, J.A.F. Gamelas, D. V. Evtuguin, M.T.S.R. Gomes, Determination of 5-hydroxymethylfurfural in honey, using headspace-solid-phase microextraction coupled with a

polyoxometalate-coated piezoelectric quartz crystal, Food Chem. 220 (2017) 420–426. https://doi.org/10.1016/j.foodchem.2016.09.204.

[47] R. Khaksar, T. Carlson, D.W. Schaffner, M. Ghorashi, D. Best, S. Jandhyala, J. Traverso, S. Amini, Unmasking seafood mislabeling in U.S. markets: DNA barcoding as a unique technology for food authentication and quality control, Food Control. 56 (2015) 71–76. https://doi.org/10.1016/j.foodcont.2015.03.007.

[48] B. Prandi, M. Varani, A. Faccini, F. Lambertini, M. Suman, A. Leporati, T. Tedeschi, S. Sforza, Species specific marker peptides for meat authenticity assessment: A multispecies quantitative approach applied to Bolognese sauce, Food Control. 97 (2019) 15–24. https://doi.org/10.1016/j.foodcont.2018.10.016.

[49] J. Riedl, S. Esslinger, C. Fauhl-Hassek, Review of validation and reporting of non-targeted fingerprinting approaches for food authentication, Anal. Chim. Acta. 885 (2015) 17–32. https://doi.org/10.1016/j.aca.2015.06.003.

[50] A.P. Sobolev, F. Thomas, J. Donarski, C. Ingallina, S. Circi, F. Cesare Marincola, D. Capitani, L. Mannina, Use of NMR applications to tackle future food fraud issues, Trends Food Sci. Technol. 91 (2019) 347–353. https://doi.org/10.1016/j.tifs.2019.07.035.

[51] S. Esslinger, J. Riedl, C. Fauhl-Hassek, Potential and limitations of non-targeted fingerprinting for authentication of food in official control, Food Res. Int. 60 (2014) 189–204. https://doi.org/10.1016/j.foodres.2013.10.015.

[52] B. Gao, S.E. Holroyd, J.C. Moore, K. Laurvick, S.M. Gendel, Z. Xie, Opportunities and Challenges Using Non-targeted Methods for Food Fraud Detection, J. Agric. Food Chem. 67 (2019) 8425–8430. https://doi.org/10.1021/acs.jafc.9b03085.

[53] S.G. Oliver, M.K. Winson, D.B. Kell, F. Baganz, Systematic functional analysis of the yeast genome, Trends Biotechnol. 16 (1998) 373–378. https://doi.org/10.1016/S0167-7799(98)01214-1.

[54] Appendix XVIII: USP Guidance on Developing and Validating Non-Targeted Methods for Adulteration Detection, (2018). http://members.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/Food_Auth/2019USPC-Appendix XVIII_Guidance_on_Developing_and_Validating_Non-Targeted_Methods_for_Adulteration_Detection-FCC_Forum_December_2018.pdf. (accessed September 17, 2020).

[55] L.M. Crawford, J.L. Janovick, N. Carrasquilla-Garcia, E. Hatzakis, S.C. Wang, Comparison of DNA analysis, targeted metabolite profiling, and non-targeted NMR fingerprinting for differentiating cultivars of processed olives, Food Control. 114 (2020) 107264. https://doi.org/10.1016/j.foodcont.2020.107264.

[56] M.A. Farag, N. Hegazi, E. Dokhalahy, A.R. Khattab, Chemometrics based GC-MS aroma profiling for revealing freshness, origin and roasting indices in saffron spice and its adulteration, Food Chem. 331 (2020) 127358. https://doi.org/10.1016/j.foodchem.2020.127358.

[57] A.C.M. Junior, C. Maione, R.M. Barbosa, M. Gallimberti, A.C.C. Paulelli, F.R. Segura, V.C.O. Souza, B.L. Batista, F. Barbosa, Elemental fingerprint profiling with multivariate data analysis to classify organic chocolate samples, J. Chemom. 32 (2018) e3036. https://doi.org/10.1002/cem.3036.

[58] S. Van Ruth, M. Alewijn, K. Rogers, E. Newton-Smith, N. Tena, M. Bollen, A. Koot, Authentication of organic and conventional eggs by carotenoid profiling, Food Chem. 126 (2011) 1299–1305. https://doi.org/10.1016/j.foodchem.2010.11.081.

[59] A. Cifuentes, Food analysis and foodomics, J. Chromatogr. A. 1216 (2009) 7109. https://doi.org/10.1016/j.chroma.2009.09.018.

[60] M. Herrero, C. Simó, V. García-Cañas, E. Ibáñez, A. Cifuentes, Foodomics: MS-based strategies in modern food science and nutrition, Mass Spectrom. Rev. 31 (2012) 49–69. https://doi.org/10.1002/mas.20335.

[61] J.P. Antignac, F. Courant, G. Pinel, E. Bichon, F. Monteau, C. Elliott, B. Le Bizec, Mass spectrometry-based metabolomics applied to the chemical safety of food, TrAC - Trends Anal. Chem. 30 (2011) 292–301. https://doi.org/10.1016/j.trac.2010.11.003.

[62] A. Erban, I. Fehrle, F. Martinez-Seidel, F. Brigante, A.L. Más, V. Baroni, D. Wunderlin, J. Kopka, Discovery of food identity markers by metabolomics and machine learning technology, Sci. Rep. 9 (2019) 1–19. https://doi.org/10.1038/s41598-019-46113-y.

[63] H. Davies, A role for “omics” technologies in food safety assessment, Food Control. 21 (2010) 1601–1610. https://doi.org/10.1016/j.foodcont.2009.03.002.

[64] S. Li, Y. Tian, P. Jiang, Y. Lin, X. Liu, H. Yang, Recent advances in the application of metabolomics for food safety control and food quality analyses, Crit. Rev. Food Sci. Nutr. (2020). https://doi.org/10.1080/10408398.2020.1761287.

[65] L. Lucini, G. Rocchetti, D. Kane, M. Trevisan, Phenolic fingerprint allows discriminating processed tomato products and tracing different processing sites, Food Control. 73 (2017) 696–703. https://doi.org/10.1016/j.foodcont.2016.09.020.

[66] M. Ben Mohamed, G. Rocchetti, D. Montesano, S. Ben Ali, F. Guasmi, N. Grati-Kamoun, L. Lucini, Discrimination of Tunisian and Italian extra-virgin olive oils according to their phenolic and sterolic fingerprints, Food Res. Int. 106 (2018) 920–927. https://doi.org/10.1016/j.foodres.2018.02.010.

[67] Y. Li, B. Jiang, Y. Lou, Q. Shi, R. Zhuang, Z. Zhan, Molecular characterization of edible vegetable oils via free fatty acid and triacylglycerol fingerprints by electrospray ionization Fourier transform ion

cyclotron resonance mass spectrometry, Int. J. Food Sci. Technol. 55 (2020) 165–174. https://doi.org/10.1111/ijfs.14258.

[68] J. Griboff, M. V. Baroni, M. Horacek, D.A. Wunderlin, M. V. Monferran, Multielemental + isotopic fingerprint enables linking soil, water, forage and milk composition, assessing the geographical origin of Argentinean milk, Food Chem. 283 (2019) 549–558. https://doi.org/10.1016/j.foodchem.2019.01.067.

[69] C. Brodie, O. Kracht, A. Hilkert, Tracing the Geographical Origin of Roasted and Green Coffee Using Isotope Fingerprints, J. AOAC Int. 102 (2019) 653–654. https://doi.org/10.5740/jaoacint.18-0314.

[70] H. Liu, Q. Zhao, X. Guo, C. Tang, X. Yu, T. Zhan, Y. Qin, J. Zhang, Application of isotopic and elemental fingerprints in identifying the geographical origin of goat milk in China, Food Chem. 277 (2019) 448–454. https://doi.org/10.1016/j.foodchem.2018.10.144.

[71] A.C.M. Junior, C. Maione, R.M. Barbosa, M. Gallimberti, A.C.C. Paulelli, F.R. Segura, V.C.O. Souza, B.L. Batista, F. Barbosa, Elemental fingerprint profiling with multivariate data analysis to classify organic chocolate samples, J. Chemom. 32 (2018) e3036. https://doi.org/10.1002/cem.3036.

[72] F. Souard, C. Delporte, P. Stoffelen, E.A. Thévenot, N. Noret, B. Dauvergne, J.M. Kauffmann, P. Van Antwerpen, C. Stévigny, Metabolomics fingerprint of coffee species determined by untargeted-profiling study using LC-HRMS, Food Chem. 245 (2018) 603–612. https://doi.org/10.1016/j.foodchem.2017.10.022.

[73] P. Oliveri, R. Simonetti, Chemometrics for Food Authenticity Applications, in: Adv. Food Authent. Test., Elsevier Inc., 2016: pp. 702–728. https://doi.org/10.1016/b978-0-08-100220-9.00025-4.

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

[75] B. Shao, H. Li, J. Shen, Y. Wu, Nontargeted Detection Methods for Food Safety and Integrity, Annu. Rev. Food Sci. Technol. 10 (2019) 429–455. https://doi.org/10.1146/annurev-food-032818-121233.

[76] J. Donarski, F. Camin, C. Fauhl-Hassek, R. Posey, M. Sudnik, Sampling guidelines for building and curating food authenticity databases, Trends Food Sci. Technol. 90 (2019) 187–193. https://doi.org/10.1016/j.tifs.2019.02.019.

[77] P. Oliveri, Class-modelling in food analytical chemistry: Development, sampling, optimisation and validation issues – A tutorial, Anal. Chim. Acta. 982 (2017) 9–19. https://doi.org/10.1016/j.aca.2017.05.013.

[78] C. Lin, X. Chen, L. Jian, C. Shi, X. Jin, G. Zhang, Determination of grain protein content by near-infrared spectrometry and multivariate calibration in barley, Food Chem. 162 (2014) 10–15. https://doi.org/10.1016/j.foodchem.2014.04.056.

[79] J.J. Roberts, D. Cozzolino, An Overview on the Application of Chemometrics in Food Science and Technology—An Approach to Quantitative Data Analysis, Food Anal. Methods. 9 (2016) 3258–3267. https://doi.org/10.1007/s12161-016-0574-7.

[80] S. Primrose, M. Woolfe, S. Rollinson, Food forensics: Methods for determining the authenticity of foodstuffs, Trends Food Sci. Technol. 21 (2010) 582–590. https://doi.org/10.1016/j.tifs.2010.09.006.

[81] D. Granato, P. Putnik, D.B. Kovačević, J.S. Santos, V. Calado, R.S. Rocha, A.G. Da Cruz, B. Jarvis, O.Y. Rodionova, A. Pomerantsev, Trends in Chemometrics: Food Authentication, Microbiology, and Effects of Processing, Compr. Rev. Food Sci. Food Saf. 17 (2018) 663–677. https://doi.org/10.1111/1541-4337.12341.

[82] D. Cavanna, S. Zanardi, C. Dall'Asta, M. Suman, Ion mobility spectrometry coupled to gas chromatography: A rapid tool to assess eggs freshness, Food Chem. 271 (2019) 691–696. https://doi.org/10.1016/j.foodchem.2018.07.204.

[83] L. Righetti, J. Rubert, G. Galaverna, K. Hurkova, C. Dall'Asta, J. Hajslova, M. Stranska-Zachariasova, A novel approach based on untargeted lipidomics reveals differences in the lipid pattern among durum and common wheat, Food Chem. 240 (2018) 775–783. https://doi.org/10.1016/j.foodchem.2017.08.020.

[84] B. Senizza, G. Rocchetti, S. Ghisoni, M. Busconi, M. De Los Mozos Pascual, J.A. Fernandez, L. Lucini, M. Trevisan, Identification of phenolic markers for saffron authenticity and origin: An untargeted metabolomics approach, Food Res. Int. 126 (2019) 108584. https://doi.org/10.1016/j.foodres.2019.108584.

[85] V. Kaškonienė, P.R. Venskutonis, Floral Markers in Honey of Various Botanical and Geographic Origins: A Review, Compr. Rev. Food Sci. Food Saf. 9 (2010) 620–634. https://doi.org/10.1111/j.1541-4337.2010.00130.x.

[86] C. Pita-Calvo, M. Vázquez, Honeydew Honeys: A Review on the Characterization and Authentication of Botanical and Geographical Origins, J. Agric. Food Chem. 66 (2018) 2523–2537. https://doi.org/10.1021/acs.jafc.7b05807.

[87] M. Creydt, M. Fischer, Omics approaches for food authentication, Electrophoresis. 39 (2018) 1569–1581. https://doi.org/10.1002/elps.201800004.

2. Experimental studies

In general, food analysis has often been the beneficiary of technical advances initially developed for other fields (e.g. petroleum, pharmaceutical, chemical manufacturing, environmental, etc.). Through technology adaptation, many analytical approaches well-established in other sectors, were applied by food chemists and analysts. Examples are chiral separations needed in the pharmaceutical industry for enantiomeric isomers identification adapted to natural flavors, or capillary electrophoresis introduced for biochemical separations applied to oligosaccharides and protein analysis [1]. This has been also the case of food authentication as, in the last years, a growing range of technologies has been developed and/or adapted to verify legal requirements related to the declared ingredients, processing and origin [2]. The various techniques nowadays available for the routine testing of both raw materials and finished food products integrity include mass spectrometry [3–5] and vibrational spectroscopy [6] in their various formats, stable isotope ratio analysis [2,7], immunochemical methods [8,9] and e-sensing technologies [10,11].

Concerning the investigated food commodities, authenticity issues of honey, olive oil and dried herbs were examined in the following chapters. As highlighted by the database of food ingredient fraud developed by Moore and co-workers [12], these goods are among the most vulnerable food commodities, accounting for 31% of the total scholarly reports.

References

[1] R.J. Mcgorrin, One Hundred Years of Progress in Food Analysis, J. Agric. Food Chem. 57 (2009) 8076–8088. https://doi.org/10.1021/jf900189s.

[2] S. Primrose, M. Woolfe, S. Rollinson, Food forensics: Methods for determining the authenticity of foodstuffs, Trends Food Sci. Technol. 21 (2010) 582–590. https://doi.org/10.1016/j.tifs.2010.09.006.

[3] H. Davies, A role for "omics" technologies in food safety assessment, Food Control. 21 (2010) 1601–1610. https://doi.org/10.1016/j.foodcont.2009.03.002.

[4] C. Black, O.P. Chevallier, C.T. Elliott, The current and potential applications of Ambient Mass Spectrometry in detecting food fraud, TrAC - Trends Anal. Chem. 82 (2016) 268–278. https://doi.org/10.1016/j.trac.2016.06.005.

[5] M. Herrero, C. Simó, V. García-Cañas, E. Ibáñez, A. Cifuentes, Foodomics: MS-based strategies in modern food science and nutrition, Mass Spectrom. Rev. 31 (2012) 49–69. https://doi.org/10.1002/mas.20335.

[6] S. Lohumi, S. Lee, H. Lee, B.K. Cho, A review of vibrational spectroscopic techniques for the detection of food authenticity and adulteration, Trends Food Sci. Technol. 46 (2015) 85–98. https://doi.org/10.1016/j.tifs.2015.08.003.

[7] F. Camin, L. Bontempo, M. Perini, E. Piasentier, Stable Isotope Ratio Analysis for Assessing the Authenticity of Food of Animal Origin, Compr. Rev. Food Sci. Food Saf. 15 (2016) 868–877. https://doi.org/10.1111/1541-4337.12219.

[8] L. Asensio, I. González, T. García, R. Martín, Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA), Food Control. 19 (2008) 1–8. https://doi.org/10.1016/j.foodcont.2007.02.010.

[9] A. Vlachos, I.S. Arvanitoyannis, P. Tserkezou, An Updated Review of Meat Authenticity Methods and Applications, Crit. Rev. Food Sci. Nutr. 56 (2016) 1061–1096. https://doi.org/10.1080/10408398.2012.691573.

[10] M. Peris, L. Escuder-Gilabert, A 21st century technique for food control: Electronic noses, Anal. Chim. Acta. 638 (2009) 1–15. https://doi.org/10.1016/j.aca.2009.02.009.

[11] M. Śliwińska, P. Wiśniewska, T. Dymerski, J. Namieśnik, W. Wardencki, Food analysis using artificial senses, J. Agric. Food Chem. 62 (2014) 1423–1448. https://doi.org/10.1021/jf403215y.

[12] J.C. Moore, J. Spink, M. Lipp, Development and Application of a Database of Food Ingredient Fraud and Economically Motivated Adulteration from 1980 to 2010, J. Food Sci. 77 (2012) R118–R126. https://doi.org/10.1111/j.1750-3841.2012.02657.x.

Chapter 1: Vibrational Spectroscopy

Vibrational spectroscopy in conjunction with chemometrics have been massively used as fast and non-destructive solutions for the analysis of quality-related parameters and authentication of a wide variety of agro-food samples. As a major advantage, these techniques are comparatively low-cost and require limited training for new users. A more detailed description of theoretical principles of near-infrared (NIR), mid-infrared (MIR) and Raman spectroscopy, as well as their main application in food analysis is provided in **Annex I**.

The following manuscript, published on Foods (https://www.mdpi.com/journal/foods) within the special issue "Novel Analytical Methods in Food Analysis", presents a capabilities comparison between three spectroscopic techniques as fast screening platforms for honey authentication purposes, using the provenance discrimination of Argentinian honeys as case study.

Vibrational Spectroscopy Coupled to a Multivariate Analysis Tiered Approach for Argentinean Honey Provenance Confirmation

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Abstract: In the present work, the provenance discrimination of Argentinian honeys was used as case study to compare the capabilities of three spectroscopic techniques as fast screening platforms for honey authentication purposes. Multifloral honeys were collected among three main honey-producing regions of Argentina over four harvesting seasons. Each sample was fingerprinted by FT-MIR, NIR and FT-Raman spectroscopy. The spectroscopic platforms were compared on the basis of the classification performance achieved under a supervised chemometric approach. Furthermore, low- mid- and high-level data fusion were attempted in order to enhance the classification results. Finally, the best-performing solution underwent to SIMCA modelling with the purpose of reproducing a food authentication scenario. All the developed classification models underwent to a "year-by-year" validation strategy, enabling a sound assessment of their long-term robustness and excluding any issue of model overfitting. Excellent classification scores were achieved by all the technologies and nearly perfect classification was provided by FT-MIR. All the data fusion strategies provided satisfying outcomes, with the mid- and high-level approaches outperforming the low-level data fusion. However, no significant advantage over the FT-MIR alone was obtained. SIMCA modelling of FT-MIR data produced highly sensitive and specific models and an overall prediction ability improvement was achieved when more harvesting seasons were used for the model calibration (86.7% sensitivity and 91.1% specificity). The results obtained in the present work suggested the major potential of FT-MIR for fingerprinting-based honey authentication and demonstrated that accuracy levels that may be commercially useful can be reached. On the other hand, the combination of multiple vibrational spectroscopic fingerprints represents a choice that should be carefully evaluated from a cost/benefit standpoint within the industrial context.

Keywords: honey; vibrational spectroscopy; geographical origin; chemometrics; data fusion

1. Introduction

According to the European Union Council Directive 2001/110/EC [1] and FAO/WHO Codex Alimentarius [2], honey is defined as the natural substance produced by *Apis mellifera* bees from plant nectar or excretions of plant-sucking insects. As a relative expensive food commodity, honey is known to be highly vulnerable to adulteration with the main concern historically being its dilution with cheaper sugars and/or syrups. Nowadays, the premium price usually commanded by mono-floral and mono-geographic products encourages other fraud practices such as false origin labelling or misdescription [3].

Reliable analytical methods for the honey authenticity assessment are highly claimed and lot of research has been undertaken in this field. Botanical origin is traditionally confirmed by melissopalynology, a microscopy-based qualitative and quantitative characterization of pollen [4]. This technique has been tested also for geographical discrimination purposes, but its application suffers from methodological shortages and limitations [3,5]. Consequently, novel alternative approaches have been proposed, including those based on mass spectrometry, vibrational spectroscopy and molecular biology [6–8]. The targeted quantification of specific compounds indicative for certain properties and/or origin would represent the most straightforward approach for food authentication; the comparison of the measured parameter with a control limit would empower the direct assessment of the product compliance and might also be used for forensic purposes [9]. However, finding reliable authenticity markers for honey's botanical/geographical origin proved to be a hard task due to the number of factors affecting its chemical composition (e.g., beekeeping technique, harvest and storage environmental conditions, etc.). In addition, the analytical output may strongly depend on the adopted sample preparation procedure, hindering the data comparison and interpretation [10].

Over the past few years, new food testing strategies based on the so-called fingerprinting approaches have been introduced. The intrinsic aim of food fingerprinting is the non-targeted detection of as many features as technically possible, by means of high-throughput techniques, to gain a comprehensive insight into the sample composition. The recorded output consists of multidimensional datasets which, beside relevant information, may also contain unintended systematic and random variation. For this reason, mathematical and statistical tools (multivariate analysis/chemometrics) constitute an integral part of the fingerprinting workflow for the extraction of meaningful information from the raw data [9]. A review of the main fingerprinting technologies has been published by Ellis et al., with particular interest toward vibrational spectroscopy techniques, namely Raman, near- and mid-infrared spectroscopy [11]. These platforms offer non-destructive and cost-effective solutions to get quick spectral information about the tested material; the easy-of-use and potential on/in-line implementation represent further advantages over traditional methods that contributed to their spread in virtually all branches of agricultural and food industries [12].

In the honey authenticity field, the potential of vibrational spectroscopy coupled to multivariate data analysis to confirm the product's claimed provenance [6,13–17] and/or botanical origin [18–20] has been widely investigated. Most of the published works are represented by truthful feasibility studies that demonstrated the capability of the employed technologies to capture differences between the analysed honey samples. To this end, discriminant analysis (DA) techniques have been used to develop supervised classification models that would correctly assign each sample to its belonging class. However, in real-world authentication contexts, no information is normally available about the alternative classes to which the tested item may belong. Indeed, the goal is typically to establish whether the analysed sample is compliant or not with a defined reference standard. For these reasons, DA methods have been defined inappropriate for solving food authenticity problems by several authors [21–23]. In contrast, one-class classifier (OCC) approaches should be preferred. Furthermore, the sample collection in the above-mentioned studies was most often limited to 1–2 years, thus hardly representative of the potential seasonal variability. This has certainly posed some limitations for a solid validation of the achieved classification results. As a matter of fact, the adaption of existing models to new harvests is a problem scarcely addressed in pilot studies, usually due to the limited samples and/or resources availability. Nevertheless, it represents an essential challenge to be faced for a relevant implementation of non-targeted fingerprinting approaches in routine analysis [24].

The present work deals with the geographical origin discrimination of Argentinian honeys. Multifloral honeys were collected from three main honey-producing Argentinian provinces (i.e., Buenos Aires, Catamarca, Misiones) and the sampling was repeated over four harvesting seasons, from 2014 to 2017. Each sample was fingerprinted by near-infrared (NIR), Fourier-transform mid-infrared (FT-MIR) and Raman (FT-Raman) spectroscopy. The main intention was not the development of a multivariate model able to correctly classify the analysed samples according to their provenance. Rather, the aim was to use this survey as a case study to compare the capabilities of the employed spectroscopic techniques as fast screening platforms for honey authentication purposes. In order to further improve the results obtained by the individual techniques, different data fusion strategies were attempted. Finally, the best-performing solution (i.e., either individual or fused data) was further modelled using an OCC approach with the purpose of reproducing a food authentication scenario and establish whether commercially useful accuracy levels can be reached. All the developed classification models underwent to a "year-by-year" validation strategy that enabled a sound assessment of their long-term robustness and excluded any issue of model overfitting.

2. Materials and Methods

2.1. Sample Collection

Authentic and traceable multifloral honey samples were collected from three main honey-producing provinces of Argentina: Buenos Aires (BA), Catamarca (Cat) and Misiones (Mis) (Figure S1), within the framework of the Argentinean National Projects PICT 3264/2014 and PICT 0774/2017, following the instructions depicted on the Projects' analytical plan, and used for the scope of the present study. The samples (about 1 Kg of raw honey each) were provided directly by beekeepers and/or honey producer cooperatives along with farming information: harvest date and conditions, declared botanical origin, field or hive address and GPS coordinates, agricultural system, treatments, etc. The honeys were harvested between April and August and the sampling was repeated over four harvesting seasons (i.e., 2014, 2015, 2016 and 2017). Collected information on honey samples are to be considered part of the above-mentioned projects, and may be available upon request according to the data protection policy.

From here on, the sample batches (i.e., honeys from each harvest) are referred to as HN2014, HN2015, HN2016, HN2017, respectively. The total number of samples was n = 502 and an overview of the sample set is given in Table S1. After collection, the honeys were stored in screw-capped glass containers, in the dark and at 4 °C, until analysis.

2.2. Instrumental Analysis

All the collected samples were fingerprinted by means of FT-MIR, FT-Raman and NIR. After the collection, each sample batch (i.e., harvest) was scanned over a 14-day period. Prior to the analysis, the honeys were incubated at 40 °C and manually stirred in order to dissolve any crystalline residue material. Quality control materials were scanned throughout the whole analysis in order to monitor potential batch-to-batch instrumental drift.

FT-MIR spectra were recorded in attenuated total reflection (ATR) mode, on a Vertex 70 FT-IR spectrometer (Bruker, Billerica, MA, USA), equipped with a Globar source, a DLaTGS detector and a Golden Gate ATR cell (Specac Ltd., Orpington, UK). Analyses were carried out in triplicate, placing the honey samples directly on the ATR crystal. All the spectra were computed at 4 cm^{-1} resolution, across the spectral range 4000–600 cm^{-1} and averaging a total of 64 scans. Data export was performed by Opus 7.2 software (Bruker).

FT-Raman spectra were collected on a Vertex 70 equipped with the RAM II add-on module (Bruker), a laser source emitting at 1064 nm and a $Ge^{(418\text{-}T/R)}$ detector cooled by liquid N_2. The laser power was set to 0.8 W. Honey samples were placed in a glass tube and analyzed in duplicate, across the spectral range

3600–0 cm^{-1}, at a nominal resolution of 4 cm^{-1}. Each spectrum was obtained by averaging 128 scans and exported with Opus 7.2 software (Bruker).

NIR spectroscopic analysis was performed on an XDS Vis/NIR spectrometer (FOSS Analytical, Hilleroed, Denmark) equipped with a tungsten halogen lamp and a dual detector Si (400–1100 nm) and PbS (1100–2500 nm). The spectra were recorded in transflectance mode, directly depositing the honey on the golden reflector. The analysis ran in duplicate and a total of 16 scans were averaged for each spectrum, at a nominal resolution of 2 nm, across the spectral range 400–2500 nm. Signal acquisition and export were performed by ISIscan software (FOSS Analytical).

2.3. Statistical Data Analysis

All the chemometric computations were carried out using Matlab v2019b (The Mathworks, Inc., Natick, MA, USA) and the PLS Toolbox (Eigenvector Research, Inc., Manson, WA, USA).

2.3.1. Data Preprocessing

Prior to any exploratory or classification analysis, spectral preprocessing was applied to reduce the impact of unwanted sources of variability on the overall signal, thus highlighting the chemical information contained in the spectra. Different algorithms for spectral pretreatment, namely 1st and 2nd order derivative according to the Savitzky–Golay method (S-G), multiplicative scatter correction (MSC) and standard normal variate (SNV), were tested both on their own and in combination. The SNV and MSC are both designed to remove from reflectance spectra part of the variability that may be caused by scattering effects. In many cases, these two spectral pretreatment produced very similar results, so that they are widely regarded as exchangeable [25]. S-G derivative filter emphasizes band width, position, and separation while simultaneously reducing baseline and background effects [26].

2.3.2. Unsupervised Pattern Recognition

After the preprocessing, principal component analysis (PCA) was performed as exploratory data analysis for the detection of evident outlying samples and/or potential data structures in a reduced-dimension space. The underlying concept of the PCA is to decrease the dimensionality of a dataset containing a large number of interrelated variables, while retaining as much as possible of the initial data variation. The original descriptors are “compressed”, through linear combination, into a new set of uncorrelated variables (i.e., principal components, PCs), which point in the directions of maximal variance. The so-called scores and loadings constitute the main output of the PCA. The scores represent the newly computed latent variables onto which the objects are projected, therefore they can be interpreted in exactly

the same way as any other variable. On the other hand, the loadings are the weights given to the original variables during the computation of the PCs; thus, they determine what a PC represent. Both scores and loadings can be graphically plotted as line or scatter plots [27].

2.3.3. Supervised Pattern Recognition and Validation Strategy

The employed spectroscopic techniques were compared on the basis of the classification performance achieved under a supervised chemometric approach, by using partial least squares discriminant analysis (PLS-DA) as classification algorithm. PLS-DA is arguably the most widely used DA technique, particularly suitable for dealing with data matrices characterized by a large number of highly correlated variables, such as spectroscopic data. PLS-DA can be regarded as a linear two-class classifier, although extension to more than two groups is also possible. The method aims to find a linear decision function(s) that divides the multidimensional variable space into as many regions as the number of classes. The objects are then projected onto lines orthogonal to this function and their distance along this discriminator is considered as discriminant score [28].

Binary PLS-DA models were generated on each data block, considering two geographical regions at once (i.e., BA-Mis, BA-Cat, Cat-Mis). At first, the models were built including all the harvesting seasons and optimized through "leave-one-out" cross-validation. Afterwards, the so-called receiver operating characteristic (ROC) curves were derived. ROC curves are widely used in many application fields as they allow a straightforward comparison of binary classifier systems. In the multivariate case, the curves are built varying the criterion threshold at which the classification is performed. Model's sensitivity (i.e., fraction of compliant objects correctly accepted) and specificity (i.e., fraction of alien objects correctly rejected) are computed at each step and graphically represented in a two-axis Cartesian plot, in which 1-specificity is usually reported on the x-axis against the sensitivity on the y-axis. Experimental outcomes are connected by a line that constitutes the ROC curve. The area under the curve (AUC) is often used as summary measure of the general discrimination quality of the model. Intuitively, the larger the AUC, the higher the model classification ability. The ideal situation would be with both sensitivity and specificity equal to 1, which corresponds to a curve passing through the top-left corner of the graph and an $AUC = 1$; in contrast, a curve lying on the diagonal bisector (corresponding to an $AUC = 0.5$) suggests no discrimination [23].

Since ROC curves were built upon a cross-validation procedure, which may be prone to overfitting, the results reliability was ensured by the following validation strategy. At first, models were trained on the HN2014 and the provenance of HN2015 was predicted. Afterwards, the training set was augmented with the HN2015 samples and the models, upon re-optimization, were applied for the prediction of HN2016 provenance. As final step, HN2014, HN2015 and HN2016 were included in the training set and the HN2017

samples were classified. In this manner, the whole process involved three external validation steps independent of each other; thus, it can be considered much more reliable than a cross-validation approaches [29]. The validation scheme is summarized in Figure S2.

2.3.4. Data Fusion

Since each honey was fingerprinted by three spectroscopic techniques, three different data matrices for the same sample set were obtained. The process of integrating multiple data blocks into a single global model is called data fusion (DF) and can lead to improvements of the classification accuracy respect to the individual data sources. Essentially, three DF strategies have been proposed in literature according to the degree of information merged: low, mid- and high-level data fusion (LL-, ML- and HL-DF, respectively). In LL-DF, data from all sources are simply concatenated column-wise into a single array. The merged matrix is then processed by the desired chemometric technique. ML-DF operates in a similar way, but relevant features are previously extracted from each data sources, separately. These features can be original descriptors identified as relevant or, more commonly, latent variables (e.g., PCA scores). The so-extracted variables are then concatenated prior to the multivariate data analysis. Lastly, in the HL-DF, separate models are built on the individual data blocks and the fusion occurs at the decision level, i.e., the individual predictions are integrated into a single final response. A more detailed description of DF methodologies employed in food and beverage authentication can be found in [30].

In the present study, LL-, ML- and HL-DF were attempted for the HN2017 prediction (i.e., last step of the year-by-year validation) with the aim of improving the performance of the single techniques. Briefly:

LL-DF: FT-MIR, FT-Raman and NIR data blocks consisted of 1349, 3009 and 751 variables, respectively. Each dataset was preprocessed according to its optimal spectral pretreatment prior to the concatenation. As a result, each sample was described by 5109 predictors. Autoscaling was applied to the fused matrix before further modelling;

ML-DF: PCA was separately performed on the training set of each data block. HN2017 objects were projected onto the PCs space so that both training and test sets were described by the same (latent) variables. Thereafter, PCA scores obtained from the individual blocks were merged and used for subsequent modelling;

HL-DF: The provenance of HN2017 samples was separately predicted carrying out PLS-DA on the individual data blocks as described in Section 2.3.3. Therefore, three column vectors containing the predicted classes were obtained and merged into a single array. The final decision on the class membership was made upon majority vote criterion.

2.3.5. Soft Independent Modelling of Class Analogy

Soft independent modelling of class analogy (SIMCA) was the first class-modelling method introduced in the literature. It is a non-probabilistic distance-based modelling which relies on the assumption that the main systematic variability of the class of interest can be captured by a PCA model of appropriate dimensionality. The results of the PCA decomposition of the target category are used to define the so-called SIMCA inner space. At this point, the membership of the tested objects is decided on the basis of some statistical criterion for outlier detection. A comprehensive tutorial of SIMCA, and OCC methods in general, is provided in [23].

In the present study, being the most represented within the sample set, BA was set as target class whereas Catamarca and Misiones honeys were used as alien objects to challenge the model. The "degree of outlyingness" with respect to the target category was computed as combination of the Mahalanobis distance to the center of the inner space (T^2) and the orthogonal distance (Q). For multivariate models whose assignation rule is based on the combined T^2-Q distances, the classification outcome can be graphically represented in a Cartesian plot reporting the T^2 and Q of the tested objects on the x- and y-axis, respectively. Roughly, the further from the origin (down-left corner) the sample is, the higher is its degree of outlyingness.

The same validation strategy described in Section 2.3.3 was adopted to ensure the reliability of the obtained classification results.

3. Results

3.1. Data Exploration

Prior to any chemometric manipulation, the recorded raw spectra of all honey samples were plotted and visually inspected (Figure S3). While very consistent FT-MIR and NIR spectra were obtained, FT-Raman spectra exhibited evident baseline drift, likely due to fluorescence phenomena. Therefore, the optimal combination of spectral filters and/or mathematical preprocessing was found to be SNV + S-G derivative (1st order derivative, 2nd order polynomial, 9 points window) + Mean centering for FT-MIR and NIR spectra, whereas a baseline correction step (manually-selected points, 3rd order polynomial, 5 regions) prior to SNV + Mean centering was included in the FT-Raman data preprocessing workflow.

As explained in Section 2.1, each sample batch (i.e., harvest) was scanned within 14 days after the collection. However, the analysis of the whole sample set was performed over a 4-years period. Therefore, the spectra recorded from the quality control materials were both visually examined and inspected through PCA in order to reveal any batch-to-batch instrumental drifts. No substantial spectral differences and/or

separation in the scores plot were observed further to the application of SNV as data pretreatment (data not shown).

Once the data consistency had been ensured, PCA was carried out on the preprocessed honey spectra. The first three PCs accounted for more than 87% of the total variance in all the datasets. Regardless of the used platform, the PC1 vs. PC2 scores plot highlighted a noteworthy separation between BA and Mis honeys, whereas Cat samples were more scattered (Figure 1). Visual examination of higher order PCs did not reveal any greater degree of separation. Here too, no apparent clustering related to the harvesting year was noticed.

As denoted by the PC1 and PC2 loadings (Figure S4), the variables that shown the highest relevance in the PCs definition all corresponded to chemically meaningful spectral intervals. Specifically, most of the dispersion among the samples is explained by the wavelength range 1500–600 cm^{-1} for FT-MIR, 3000–2900 and 1500–0 cm^{-1} for FT-Raman, 480–600 and 1850–2500 nm for NIR.

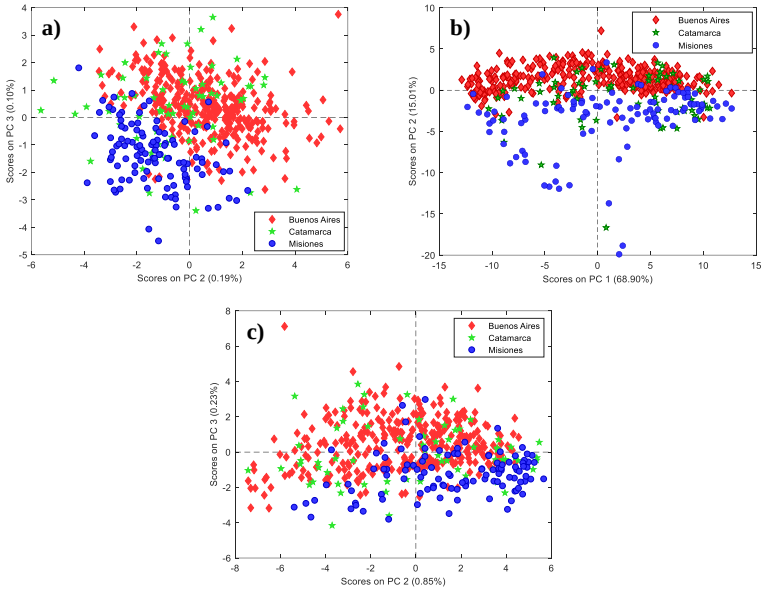

Figure 1. Scatter plot of PC1 vs PC2 scores obtained from FT-MIR (**a**), FT-Raman (**b**) and NIR (**c**) data. Objects are marked according to the provenance region (Red diamond: BA; Green star: Cat; Blue circle: Mis).

According to previous reports, carbohydrate moieties are chiefly responsible for absorptions in these ranges of the honey spectra [14,15,31]. Noisy and/or uninformative spectral regions, i.e., CO_2 band and flat regions, were excluded from the subsequent data treatment. As a result, the considered wavelength ranges were, respectively, 3800–2400 cm^{-1} and 1990–600 cm^{-1} for FT-MIR, 3600–2500 cm^{-1} and 1800–0 cm^{-1} for FT-Raman; 400–700 nm and 1300–2500 nm for NIR (Figure S3).

Band assignment was not the main goal of the study as the general tendency in fingerprinting methods is to use the entire spectra in the multivariate data analysis [32]. Nevertheless, description of the main

peaks/bands responsible for the sample discrimination might be helpful for future research. Therefore, illustration of the statistically-significant spectral signals and of the three datasets has been reported in Supplementary Materials (Figure S5). Furthermore, assignment of the relevant peaks/bands was carried out based on the literature [6,13,20,31,33–37].

3.2. Techniques Comparison under a Supervised Chemometric Approach

Classification outcomes provided by the individual spectroscopic techniques, as well as the fused datasets, are summarized in this section. ROC curves were constructed as described in Section 2.3.3 and graphically reported in Figure 2.

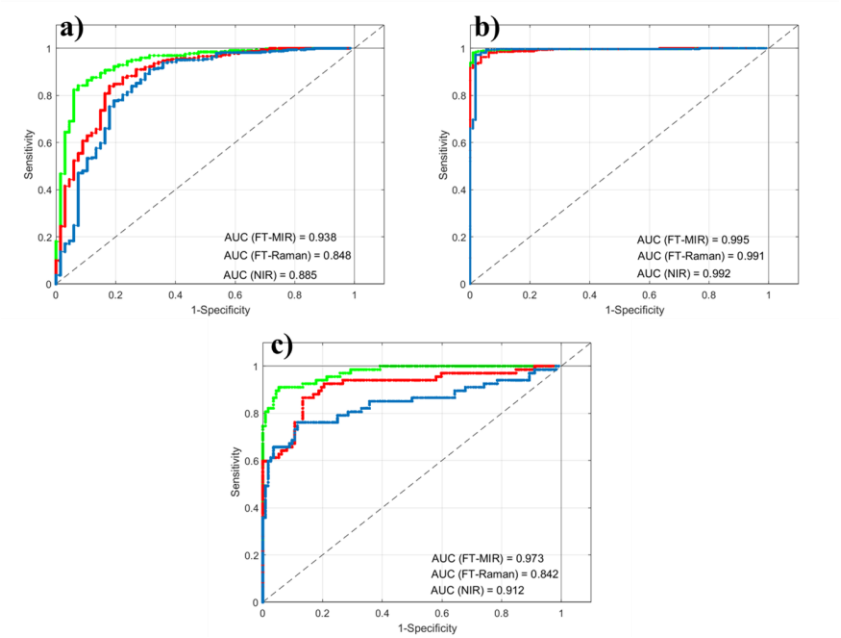

Figure 2. ROC curves related to the binary classification models (**a**) BA vs. Cat; (**b**) BA vs. Mis; (**c**) Cat vs. Mis.

As expected from the unsupervised pattern recognition, better results were reached in the discrimination of Mis honeys (i.e., BA-Mis and Cat-Mis models). In particular, the BA-Mis model produced nearly perfect classification, with AUC always above 0.99 regardless the spectroscopic technique. In contrast, the BA-Cat model provided slightly lower AUC, ranging from 0.88 (NIR) to 0.93 (FT-MIR), perhaps due to unbalanced number of samples available. Concerning the inter-platforms comparison, FT-MIR provided yielded the largest AUC in all the binary models, while the lowest score was always obtained by FT-Raman spectroscopy.

The results of the validation procedure are summarized as correct classification rates (i.e., ratio between correctly classified and total tested objects, CCRs) in Table 1. For purposes of presentation, only the scores

provided by FT-MIR data were reported, while FT-Raman and NIR data are available in Supplementary materials (Tables S2 and S3).

Table 1. PLS-DA prediction results expressed as correct classification rates (FT-MIR data).

Predicted Harvest	Correct Classification Rate (%)		
	BA vs. Cat	BA vs. Mis	Cat vs. Mis
2015	84.6	88.4	92.3
2016	91.8	100.0	92.5
2017	91.9	100.0	95.5

The model validation confirmed what was highlighted by the ROC curves. The best performance was offered by FT-MIR and, here too, the best classification was reached for Mis honeys, whatever the spectroscopic technique. Interestingly, in the case of FT-MIR, an overall improvement of the models' prediction ability was achieved as more harvesting seasons were included in the training set, with all the binary models reaching CCRs $> 90\%$ in the prediction of HN2017 (i.e., last step of the validation scheme). It must be pointed out that small differences (e.g., $0.1-0.2\%$) between the results have to be assessed with caution since these classification outcomes cannot be tested for statistical significance. Nevertheless, the overall trends have been clearly evidenced.

To further enhance the obtained results, the DF strategies described in Section 2.3.4. were attempted and the CCRs achieved in the HN2017 prediction summarized in Table 2.

Table 2. PLS-DA classification results of HN2017, expressed as correct classification rates, according to the different DF strategies.

Predicted Harvest	Correct Classification Rate (%)		
	BA vs. Cat	BA vs. Mis	Cat vs. Mis
LL-DF	85.4	91.7	80.0
ML-DF	87.0	98.6	80.0
HL-DF	93.5	98.6	95.5

All the DF methods provided satisfying classification performance, with HL-DF showing the highest scores, followed by ML-DF and LL-DF. The HL-DF reached comparable results respect to the FT-MIR (Table 1), with slightly better scores in the BA-Cat model and lower CCRs achieved in the BA-Mis honeys discrimination. A further attempt was made by combining the data blocks from two platforms only (i.e., FT-MIR+FT-Raman, FT-MIR+NIR and FT-Raman+NIR). However, no significant classification improvement was achieved (data not shown).

3.3. SIMCA Modelling

FT-MIR dataset underwent to SIMCA modelling as, in the light of the above results, it proved to be the most promising option for a hypothetical fingerprinting method for honey authentication. BA was set as target category to be modelled; thus, Cat and Mis samples represented the alien objects to be rejected by the model. Five PCs were considered sufficient for proper modelling as they accounted for $> 95\%$ of the original data variance. The confidence level was set to $\alpha = 0.05$ and the classification rule was based on the so-called T^2-Q augmented distances. The same year-by-year validation was adopted.
SIMCA results are reported as sensitivity, specificity and overall CCRs in Table 3.

Table 3. SIMCA modelling results of class BA (FT-MIR data) according to the different harvesting seasons, expressed as sensitivity, specificity and overall correct classification rates.

Predicted Harvest	Sensitivity (%)	Specificity (%)	CCR (%)
2015	61.0	89.7	69.6
2016	90.6	75.0	85.8
2017	86.7	91.1	88.8

Highly sensitive and specific models were produced, confirming what expected from the excellent classification previously obtained. In accordance with the PLS-DA results (Table 1), the inclusion of 2015 and 2016 harvest in the model training led to an overall enhancement of the model performance. Remarkably, within the prediction of HN2017, 39 out of 45 BA samples were correctly recognized as belonging to the target class (86.7% sensitivity), while 15 out of 17 Cat and 26 on 28 Mis honeys were rightly rejected by the model (91.1% specificity). T^2 and Q distances of the predicted HN2017 samples are graphically represented in Figure 3.

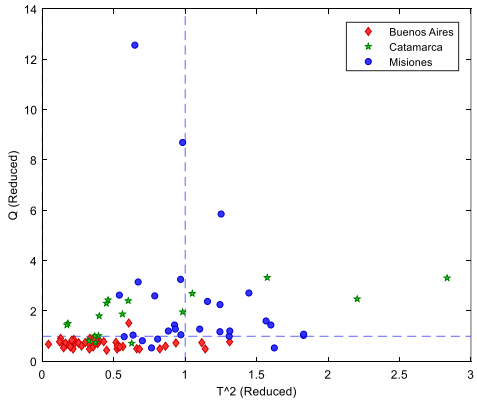

Figure 3. SIMCA modelling on FT-MIR data. Projection of HN2017 objects onto the T^2 (reduced) vs. Q (reduced) model space of class BA.

4. Discussion

Since the analysis of all the collected honeys was carried out over a 4-years period, ensuring the absence of instrumental drift among the analysis batches was the first concern. The routine maintenance of the equipment, typically performed once a year, includes the substitution of overused components (e.g., source) and re-alignment of the interferometer, which may easily result in signal intensity (i.e., absorbance) shifts. Such technical variations, if not properly handled, may give rise to fingerprint deviations that prevent the use of the classification model for its ultimate purpose: the prediction of new harvests [24]. As an example, Woodcock et al. observed a clear separation between honey samples analysed in two consecutive years. However, the authors were not able to definitely attribute such trend to different sample's characteristics, rather than the use of a non-standardized instrument [13].

Both the unsupervised and supervised chemometric approaches evidenced the presence of actual differences between the honeys having diverse provenance. Such differences are unlikely due to random variation or overfitting issues. In fact, it is worth stressing that the employed validation strategy allowed any developed model to be challenged with independent external test sets. With the main factor under investigation being the geographical origin, it is reasonable to ascribe the samples separation to the distinct environmental features of the three Argentinian regions. Variations of soil and weather conditions likely result in different melliferous floras foraged by the bees, which is known to have the greatest influence on the honey's chemical composition [38]. Buenos Aires province is located within the ecoregion *Pampeana*, where the temperate climate and abundant rainfall encourage extensive crop cultivation. Misiones province is characterized by its typical flora, known as "Missionary Forest", favored by the subtropical weather of the ecoregion *Selva Paranaense*. The peculiar characteristics of this ecoregion might underlie differences in the honeys' physiochemical properties, which would explain the better results achieved in the classification of Mis samples. While Buenos Aires and Misiones regions show fairly uniform climate conditions, five different ecoregions are recognized in Catamarca (i.e., *Yungas*, *Chaco Seco*, *Monte de Sierras and Bolsones*, *Puna* and *Altos Andes*) and therefore a number of microclimates can be encountered, from the subtropical rains in the east, to the arid highland in the west [39]. Therefore, the larger overlap of Cat samples over the other classes might be due to this climate, and thus botanical, heterogeneity.

All the employed spectroscopic techniques provided more than satisfying performance, confirming the high potential of vibrational spectroscopy as rapid screening tool for honey authentication. Although lot of research has been done in the application of vibrational spectroscopy for honey testing, cross-platform comparisons have been scarcely documented. Tahir and co-workers observed equivalent performance of FT-MIR and FT-Raman spectroscopy for the prediction of phenolic compounds content and the antioxidant activity in honey [40]. Ballabio et al. recently evaluated five different technologies, including FT-MIR, NIR and FT-Raman spectroscopy, for the botanical origin identification of honeys [41]. The authors reported

better classification provided by NIR, respect to FT-MIR and FT-Raman spectroscopy. Nevertheless, the same authors pointed out that such outcomes have to be assessed with caution due to the small size of the sample set. Within the present work, FT-MIR shown to be the best option for honey fingerprinting, providing always the largest AUC within the ROC curves, as well as superior CCRs (>90%) through the validation process. The reason probably lies in the better sensitivity and higher S/N normally provided by FT-MIR instruments respect to NIR and FT-Raman, since fundamental absorptions are being measured in the MIR region [42].

As pointed out in Section 3.2, LL-DF provided the poorest results among the attempted DF strategies. This is consistent with the literature, where LL-DF approach either did not produce substantial classification/prediction improvement over the single techniques or was outperformed by higher-level DF [40,41,43]. The explanation can be found in the high collinearity of vibrational spectroscopy data. In fact, LL-DF introduces, along with useful information, a large number of redundant and irrelevant variables. Such noise is, for example, reduced in the ML-DF by the features extraction prior to the concatenation. Concerning the ML-DF and HL-DF, despite the noteworthy results, no significant classification enhancement was reached respect to the FT-MIR only. On the basis of the present outcomes, the combination of vibrational spectroscopic data cannot be regarded as worthwhile as no evident advantage has been provided over the individual techniques. The authors attributed the ineffectiveness of DF to the lack of information orthogonality between the combined data sources, which is crucial for the successful application of DF [30].

When evaluated under conditions “closer” to a real authentication scenario, FT-MIR still yielded remarkable classification scores. The lower CCRs achieved by SIMCA respect to PLS-DA are not surprising as DA algorithms use information about the modelled classes to maximize the group differences, whereas OOC methods “do not know anything about existence of alternative classes or samples”. In fact, despite the widespread opinion that “PLS-DA may go further than SIMCA”, performance comparisons of these two algorithms are not even consistent as they employ diverse amounts of modelling information [21]. As mentioned in Section 1, DA algorithms are not suited for one-class problems where only one target category is modeled against a heterogeneous group of off-specification products [23]. For this reason, the authors believe that the SIMCA results (Section 3.3) are more representative of the potential performance of a routine screening method based on FT-MIR fingerprinting. The classification achieved in the HN2017 prediction can be considered excellent for a rapid screening platform and demonstrated that, under a proper characterization of the class of interest, FT-MIR spectroscopy can be a powerful tool for honey authenticity purposes.

In the authors’ opinion, the results herein obtained can be sensibly extended to problems of honey’s floral origin. In fact, botanical/varietal and geographical origin of food products are often treated as separate

issues in food authenticity studies; however, they are highly correlated and hard to be considered individually, especially in the case of natural products such as honey. For example, distinct geographic areas do not only provide different climatic conditions affecting the accumulation of phytochemicals in pollen and nectar, but also normally offer diverse melliferous flora foraged by the bees. All these factors and relationships cannot be ignored in the development of methods for honey's origin confirmation.

Despite the remarkable outcomes, in must be pointed out that the development of a comprehensive model able to identify the geographic origin of an unknown sample is unrealistic; it would require an exhaustive sampling of world honeys over several harvest years. Furthermore, honeys from different localities may not have unique spectral signatures due to similarities in vegetation. Thus, it is unlike to reach similar performance at a world-level. We believe that a fundamental knowledge of the limits and capabilities of the chosen methods is essential for their correct utilization and interpretation. Screening platforms based on spectroscopic fingerprints find the best applicability at a company-level, where the "boundaries" of the application can be clearly defined. Typical examples are internal quality assurance or the management of incoming raw materials from suppliers with established relationships. In these contexts, the target classes can be appropriately outlined and sampled in a representative way.

5. Conclusions

Honey authenticity remains a challenging issue to deal with as reliable and manageable methods for its floral and geographical origin confirmation are still lacking. Several feasibility studies have been reported in literature to demonstrate the capabilities of vibrational spectroscopy for the discrimination of honey's botanical and/or geographical origin.

A key feature of the present work was the realistic and rather large variability included in the sample set. All the collected honeys were multifloral, thus covering differences in nectar sources. Besides, seasonal climate fluctuations were also considered by repeating the sampling over four consecutive harvesting seasons. This extra variation is of great benefit for the robustness of any developed model and crucial to demonstrate its capabilities under real-world conditions.

Excellent classification scores were achieved by all the technologies and the adopted validation strategy allowed to exclude any issue related to model overfitting. The nearly perfect classification results provided by FT-MIR suggested its major potential for honey fingerprinting. DF strategies yielded satisfying outcomes, however, no significant improvement in discrimination power was achieved respect to FT-MIR. Therefore, within an industrial context, a multi-platforms spectroscopic fingerprint is a choice that should be carefully evaluated from a cost/benefit standpoint. In fact, it must be considered that a multiple sample

fingerprinting would represent an increased expense in terms of equipment and expertise, making the food control process more time and labour-demanding.

SIMCA modelling was successfully applied on the FT-MIR dataset and demonstrated that the use of large and representative training sets can definitely improve the model robustness over analytical and biological factors. The year-by-year validation not only ensured the results reliability, but also well reproduced a hypothetical quality control context where, reasonably, spectral libraries are gradually enlarged with newly recorded spectra. In the author's opinion, such results can be considered a reliable performance estimation of a potential FT-MIR-based fingerprinting method.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/10/1450/s1, Figure S1: Geographical map of the three Argentinian provinces included in the study, Figure S2: Scheme of the adopted validation strategy, Figure S3: Recorded raw spectra of FT-MIR, FT-Raman and NIR, Figure S4: Line plots of PC1 and PC2 loadings obtained from FT-MIR, FT-Raman and NIR data, Figure S5: Line plots of the VIP scores extracted from the PLS2-DA model generated on FT-MIR, FT-Raman and NIR data, Table S1: Sample set overview, Table S2: PLS-DA prediction results expressed as correct classification rates (FT-Raman data), Table S3: PLS-DA prediction results expressed as correct classification rates (NIR data). Experimental data are openly available as supplementary information.

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References

1. The Council of the European Commission. *Council Directive 2001/110/CE Concerning Honey*; 2001. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32001L0110&from=EN (accessed on 12th October 2020).

2. Codex Alimentarius Commission. Codex Alimentarius Commission Standards. *Codex Stan 12-1981* **2001**, 1–8. Available online: http://www.fao.org/input/download/standards/310/cxs_012e.pdf (accessed on 12th October 2020)

3. Soares, S.; Amaral, J.S.; Oliveira, M.B.P.P.; Mafra, I. A Comprehensive Review on the Main Honey Authentication Issues: Production and Origin. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*.

4. Geana, E.I.; Ciucure, C.T. Establishing Authenticity of Honey via Comprehensive Romanian Honey Analysis. *Food Chem.* **2020**, *306*, 125595.

5. Von Der Ohe, W.; Persano Oddo, L.; Piana, M.L.; Morlot, M.; Martin, P. Harmonized Methods of Melissopalynology. *Apidologie* **2004**, *35* (Suppl. 1), S18–S25.

6. Latorre, C.H.; Crecente, R.M.P.; Martín, S.G.; García, J.B. A Fast Chemometric Procedure Based on NIR Data for Authentication of Honey with Protected Geographical Indication. *Food Chem.* **2013**, *141*, 3559–3565.

7. Dong, H.; Xiao, K.; Xian, Y. Isotope Ratio Mass Spectrometry Coupled to Element Analyzer and Liquid Chromatography to Identify Commercial Honeys of Various Botanical Types. *Food Anal. Methods* **2017**, *10*, 2755–2763.

8. Soares, S.; Grazina, L.; Mafra, I.; Costa, J.; Pinto, M.A.; Duc, H.P.; Oliveira, M.B.P.P.; Amaral, J.S. Novel Diagnostic Tools for Asian (Apis Cerana) and European (Apis Mellifera) Honey Authentication. *Food Res. Int.* **2018**, *105*, 686–693.

9. Esslinger, S.; Riedl, J.; Fauhl-Hassek, C. Potential and Limitations of Non-Targeted Fingerprinting for Authentication of Food in Official Control. *Food Res. Int.* **2014**, *60*, 189–204.

10. Pita-Calvo, C.; Vázquez, M. Honeydew Honeys: A Review on the Characterization and Authentication of Botanical and Geographical Origins. *J. Agric. Food Chem.* **2018**, *66*, 2523–2537.

11. Ellis, D.I.; Brewster, V.L.; Dunn, W.B.; Allwood, J.W.; Golovanov, A.P.; Goodacre, R. Fingerprinting Food: Current Technologies for the Detection of Food Adulteration and Contamination. *Chem. Soc. Rev.* **2012**, *41*, 5706–5727.

12. McGrath, T.F.; Haughey, S.A.; Patterson, J.; Fauhl-Hassek, C.; Donarski, J.; Alewijn, M.; van Ruth, S.; Elliott, C.T. What Are the Scientific Challenges in Moving from Targeted to Non-Targeted Methods for Food Fraud Testing and How Can They Be Addressed?—Spectroscopy Case Study. *Trends Food Sci. Technol.* **2018**, *76*, 38–55.

13. Woodcock, T.; Downey, G.; Kelly, J.D.; O'Donnell, C. Geographical Classification of Honey Samples by Near-Infrared Spectroscopy: A Feasibility Study. *J. Agric. Food Chem.* **2007**, *55*, 9128–9134.

14. Woodcock, T.; Downey, G.; O'Donnell, C.P. Near Infrared Spectral Fingerprinting for Confirmation of Claimed PDO Provenance of Honey. *Food Chem.* **2009**, *114*, 742–746.

15. Hennessy, S.; Downey, G.; O'Donnell, C. Multivariate Analysis of Attenuated Total Reflection—Fourier Transform Infrared Spectroscopic Data to Confirm the Origin of Honeys. *Appl. Spectrosc.* **2008**, *62*, 1115–1123.

16. Hennessy, S.; Downey, G.; O'Donnell, C.P. Attempted Confirmation of the Provenance of Corsican PDO Honey Using FT-IR Spectroscopy and Multivariate Data Analysis. *J. Agric. Food Chem.* **2010**, *58*, 9401–9406.

17. Guelpa, A.; Marini, F.; du Plessis, A.; Slabbert, R.; Manley, M. Verification of Authenticity and Fraud Detection in South African Honey Using NIR Spectroscopy. *Food Control.* **2017**, *73*, 1388–1396.

18. Ruoff, K.; Luginbühl, W.; Künzli, R.; Iglesias, M.; Bogdanov, S.; Bosset, J.O.; Von Der Ohe, K.; Von Der Ohe, W.; Amadò, R. Authentication of the Botanical and Geographical Origin of Honey by Mid-Infrared Spectroscopy. *J. Agric. Food Chem.* **2006**, *54*, 6873–6880.

19. Giordano, A.; Retamal, M.; Fuentes, E.; Ascar, L.; Velásquez, P.; Rodríguez, K.; Montenegro, G. Rapid Scanning of the Origin and Antioxidant Potential of Chilean Native Honey Through Infrared Spectroscopy and Chemometrics. *Food Anal. Methods* **2019**, *12*, 1511–1519.

20. Bisutti, V.; Merlanti, R.; Serva, L.; Lucatello, L.; Mirisola, M.; Balzan, S.; Tenti, S.; Fontana, F.; Trevisan, G.; Montanucci, L.; et al. Multivariate and Machine Learning Approaches for Honey Botanical Origin Authentication Using near Infrared Spectroscopy. *J. Near Infrared Spectrosc.* **2019**, *27*, 65–74.

21. Rodionova, O.Y.; Titova, A.V.; Pomerantsev, A.L. Discriminant Analysis Is an Inappropriate Method of Authentication. *TrAC—Trends Anal. Chem.* **2016**, *78*, 17–22.

22. Granato, D.; Putnik, P.; Kovačević, D.B.; Santos, J.S.; Calado, V.; Rocha, R.S.; Cruz, A.G.; Jarvis, B.; Rodionova, O.Y.; Pomerantsev, A. Trends in Chemometrics: Food Authentication, Microbiology, and Effects of Processing. *Compr. Rev. Food Sci. Food Saf.* **2018**, *17*, 663–677.

23. Oliveri, P. Class-Modelling in Food Analytical Chemistry: Development, Sampling, Optimisation and Validation Issues—A Tutorial. *Anal. Chim. Acta* **2017**, *982*, 9–19.

24. Riedl, J.; Esslinger, S.; Fauhl-Hassek, C. Review of Validation and Reporting of Non-Targeted Fingerprinting Approaches for Food Authentication. *Anal. Chim. Acta* **2015**, *885*, 17–32.

25. Fearn, T.; Riccioli, C.; Garrido-Varo, A.; Guerrero-Ginel, J.E. On the Geometry of SNV and MSC. *Chemom. Intell. Lab. Syst.* **2009**, *96*, 22–26.

26. Zimmermann, B.; Kohler, A. Optimizing Savitzky-Golay Parameters for Improving Spectral Resolution and Quantification in Infrared Spectroscopy. *Appl. Spectrosc.* **2013**, *67*, 892–902.

27. Bro, R.; Smilde, A.K. Principal Component Analysis. *Anal. Methods* **2014**, 6, 2812–2831.

28. Brereton, R.G.; Lloyd, G.R. Partial Least Squares Discriminant Analysis: Taking the Magic Away. *J. Chemom.* **2014**, *28*, 213–225.

29. Esbensen, K.H.; Geladi, P. Principles of Proper Validation: Use and Abuse of Re-Sampling for Validation. *J. Chemom.* **2010**, *24*, 168–187.

30. Borràs, E.; Ferré, J.; Boqué, R.; Mestres, M.; Aceña, L.; Busto, O. Data Fusion Methodologies for Food and Beverage Authentication and Quality Assessment—A Review. *Anal. Chim. Acta* **2015**, *891*, 1–14.

31. Fernández Pierna, J.A.; Abbas, O.; Dardenne, P.; Baeten, V. Discrimination of Corsican Honey by FT-Raman Spectroscopy and Chemometrics. *Biotechnol. Agron. Soc. Environ.* **2011**, *15*, 75–84.

32. Downey, G. Vibrational Spectroscopy in Studies of Food Origin. In *New Analytical Approaches for Verifying the Origin of Food*; Elsevier Ltd.: Amsterdam, The Netherlands, 2013; pp. 94–116.

33. Subari, N.; Mohamad Saleh, J.; Shakaff, A.Y.M.; Zakaria, A. A Hybrid Sensing Approach for Pure and Adulterated Honey Classification. *Sensors* **2012**, *12*, 14022–14040.

34. Gallardo-Velázquez, T.; Osorio-Revilla, G.; Zuñiga-de Loa, M.; Rivera-Espinoza, Y. Application of FTIR-HATR Spectroscopy and Multivariate Analysis to the Quantification of Adulterants in Mexican Honeys. *Food Res. Int.* **2009**, *42*, 313–318.

35. Tewari, J.C.; Irudayaraj, J.M.K. Floral Classification of Honey Using Mid-Infrared Spectroscopy and Surface Acoustic Wave Based z-Nose Sensor. *J. Agric. Food Chem.* **2005**, *53*, 6955–6966.

36. Gok, S.; Severcan, M.; Goormaghtigh, E.; Kandemir, I.; Severcan, F. Differentiation of Anatolian Honey Samples from Different Botanical Origins by ATR-FTIR Spectroscopy Using Multivariate Analysis. *Food Chem.* **2015**, *170*, 234–240.

37. Corbella, E.; Cozzolino, D. The Use of Visible and near Infrared Spectroscopy to Classify the Floral Origin of Honey Samples Produced in Uruguay. *J. Near Infrared Spectrosc.* **2005**, *13*, 63–68.

38. Kaškonienė, V.; Venskutonis, P.R. Floral Markers in Honey of Various Botanical and Geographic Origins: A Review. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 620–634.

39. Arana, M.D.; Martìnez, G.A.; Oggero, A.J.; Natale, E.S.; Morrone, J.J. Map and Shapefile of the Biogeographic Provinces of Argentina. *Zootaxa* **2017**, *4341*, 420.

40. Tahir, H.E.; Xiaobo, Z.; Zhihua, L.; Jiyong, S.; Zhai, X.; Wang, S.; Mariod, A.A. Rapid Prediction of Phenolic Compounds and Antioxidant Activity of Sudanese Honey Using Raman and Fourier Transform Infrared (FT-IR) Spectroscopy. *Food Chem.* **2017**, *226*, 202–211.

41. Ballabio, D.; Robotti, E.; Grisoni, F.; Quasso, F.; Bobba, M.; Vercelli, S.; Gosetti, F.; Calabrese, G.; Sangiorgi, E.; Orlandi, M.; et al. Chemical Profiling and Multivariate Data Fusion Methods for the Identification of the Botanical Origin of Honey. *Food Chem.* **2018**, *266*, 79–89.

42. Abbas, O.; Dardenne, P.; Baeten, V. Near-Infrared, Mid-Infrared, and Raman Spectroscopy. In *Chemical Analysis of Food: Techniques and Applications*; Elsevier Inc.: Amsterdam, The Netherlands, 2012; pp. 59–89.

43. Huang, F.; Song, H.; Guo, L.; Guang, P.; Yang, X.; Li, L.; Zhao, H.; Yang, M. Detection of Adulteration in Chinese Honey Using NIR and ATR-FTIR Spectral Data Fusion. *Spectrochim. Acta—Part A Mol. Biomol. Spectrosc.* **2020**, *235*, 118297.

Supplementary information for:

Vibrational Spectroscopy Coupled to a Multivariate Analysis Tiered Approach for Argentinean Honey Provenance Confirmation

Figure S1. Geographical map of the three Argentinian provinces included in the study.

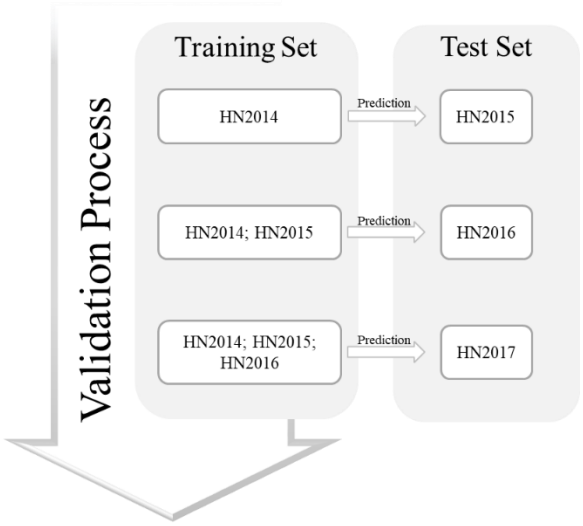

Figure S2. Scheme of the adopted validation strategy.

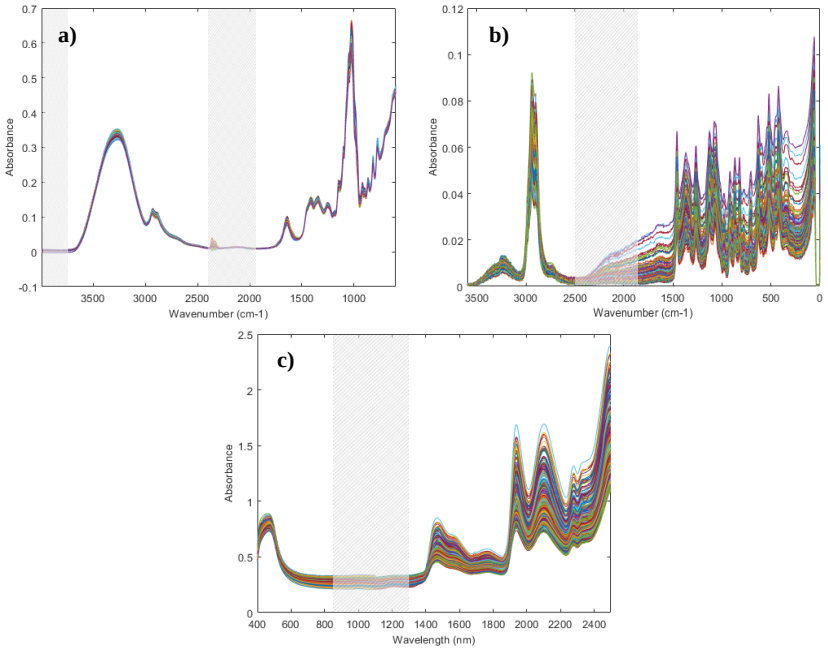

Figure S3. Recorded raw spectra of FT-MIR (**a**), FT-Raman (**b**) and NIR (**c**). Grey bands show spectral regions excluded from the data analysis.

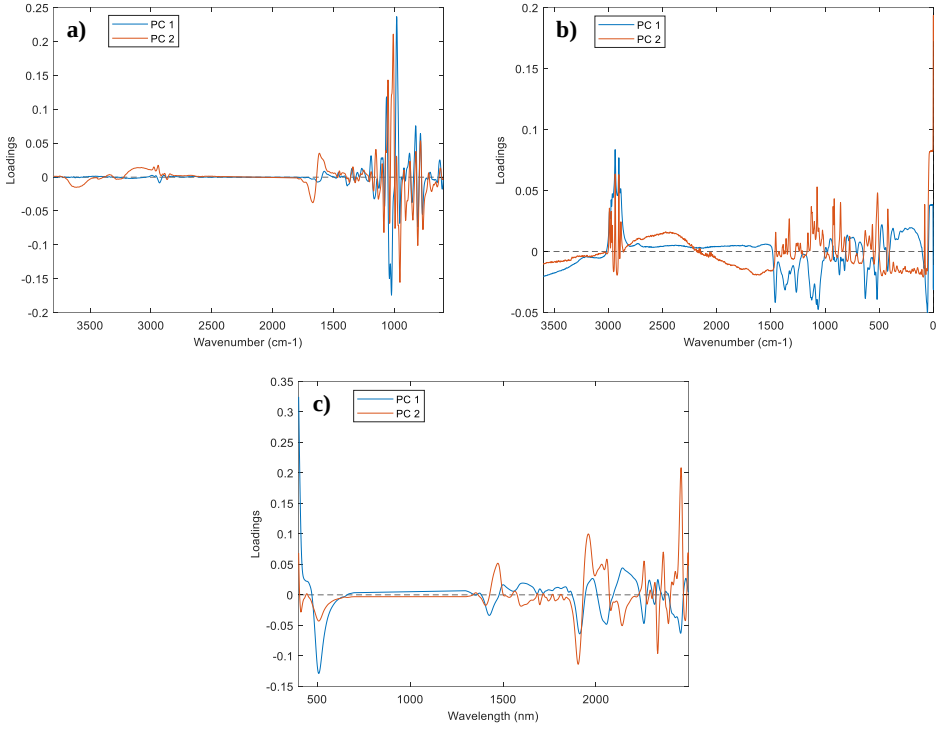

Figure S4. Line plots of PC1 and PC2 loadings obtained from FT-MIR (a), FT-Raman (b) and NIR (c) data.

Band assignment

Investigation of the main peaks/bands relevant for the samples segregation was carried out with the aid of Variable Importance in Projection (VIP) extracted from a PLS model. VIP scores summarize the influence that the original descriptors have had on the PLS model generation. In addition, these scores are scaled in a way that variables exhibiting VIP values greater than 1 can be considered statistically-relevant in a given model. In order to obtain such importance estimates, PLS2-DA models were generated considering all the geographical regions at once (i.e. BA vs Cat vs Mis) on the data coming from the different instrumental techniques. A 5-fold cross validation (venetian blinds) was adopted. Illustration of the VIP scores for the three classification models are reported in **S. Fig 5**.

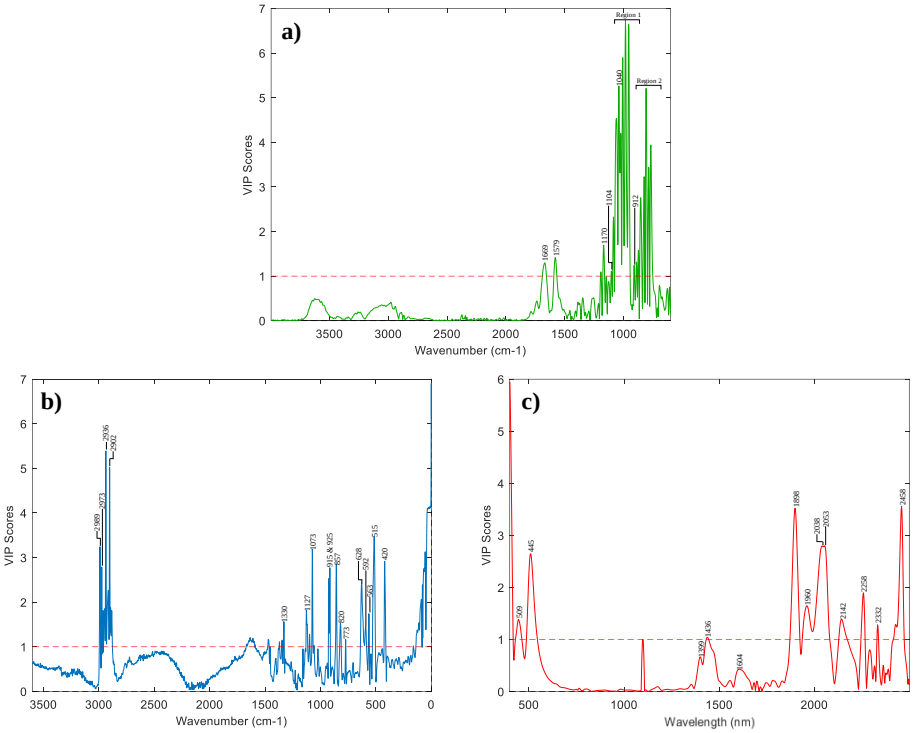

Figure S5. Line plots of the VIP scores extracted from the PLS2-DA (i.e. BA vs Cat vs Mis) model generated on FT-MIR (a), FT-Raman (b) and NIR (c) data. The red dashed line represents the threshold value for significant contribution (VIP=1). Wavenumber/wavelength corresponding to the maximum band absorbance are highlighted.

Concerning the FT-MIR data, as can be seen, Region 1 contains the highest VIP scores, with peaks at 956, 983 and 1006, 1024, 1040, 1061, 1086 and 1104 cm^{-1}. According to the literature, Region 1 (1120–940 cm^{-1}) corresponds to the C–O and C–C stretching of the three major carbohydrates of honey: fructose, glucose and sucrose [17]. Indeed, the peak at 1040 cm^{-1} has been assigned to the C–O stretch in the C–OH group in the carbohydrate structure [18,19]. Furthermore, the small peak at 1104 cm^{-1} corresponds to the C–O stretching band of the C–O–C linkage (the C–O–C is present in sucrose as a glycoside bond) [18]. High VIP values can be also observed in Region 2 (920–750 cm^{-1}) with peaks at 767, 784, 806, 824, 854

872, 889 and 912 cm^{-1}. This is the anomeric region, characteristic of the saccharide configuration [17,18]. In particular, the peak at 912 cm^{-1} is due to the C–H bending of the carbohydrate. No statistically relevant bands were highlighted in the 1540–1175 cm^{-1} range, which has been assigned by Gok and co-workers to the O–H stretching/bending, C=O stretching of ketones and the C–O & C–H stretching of carbohydrates [20].

With regard to FT-Raman data, the two peaks at 2936 and 2902 cm^{-1} are related to the asymmetric stretching of CH_2 and C-H stretching vibrations, respectively. In addition, several peaks characteristic of different chemical groups can be observed in the fingerprint region (200-1500 cm^{-1}). Most of these signals are consistent with the literature and thorough band assignment can be found in our previous work [21].

Finally, VIP extracted from NIR data highlighted two statistically-relevant bands, 1400-1490 nm and 1870-1930 nm, both corresponding to O–H, C–H, and C–H_2 deformations [22]. Besides, the spectral range between 2000 and 2100 nm corresponds to the C–H combinations [22,23], the minor peak at 2332 nm has been related to the C–H bonds [24] and the 2430-2490 nm band is characteristic of the O–H, N–H, and C–H deformation [22]. Furthermore, signals at 1470 and 1960 nm have been associated with the O-H stretch (first and second overtone, respectively) [25].

Table S1. Sample set overview.

Year	Buenos Aires	Catamarca	Misiones
2014	78	25	31
2015	93	11	28
2016	107	15	25
2017	45	17	28

Table S2. PLS-DA prediction results expressed as correct classification rates (FT-Raman data).

Predicted harvest	Correct classification rate (%)		
	BA vs Cat	BA. vs Mis	Cat vs Mis
2015	80.7	95.8	79.4
2016	81.9	96.2	80.0
2017	82.3	97.2	84.4

Table 3. PLS-DA prediction results expressed as correct classification rates (NIR data).

Predicted harvest	**Correct classification rate (%)**		
	BA vs Cat	BA. vs Mis	Cat vs Mis
2015	70.1	96.6	76.5
2016	80.3	87.1	65.0
2017	88.7	94.5	82.2

References

1. Subari, N.; Mohamad Saleh, J.; Md Shakaff, A.; Zakaria, A. A Hybrid Sensing Approach for Pure and Adulterated Honey Classification. Sensors 2012, 12 (10), 14022–14040. https://doi.org/10.3390/s121014022.

2. Gallardo-Velázquez, T.; Osorio-Revilla, G.; Loa, M. Z. de; Rivera-Espinoza, Y. Application of FTIR-HATR Spectroscopy and Multivariate Analysis to the Quantification of Adulterants in Mexican Honeys. Food Res. Int. 2009, 42 (3), 313–318. https://doi.org/10.1016/j.foodres.2008.11.010.

3. Tewari, J. C.; And, †; Irudayaraj, J. M. K. Floral Classification of Honey Using Mid-Infrared Spectroscopy and Surface Acoustic Wave Based z-Nose Sensor. 2005. https://doi.org/10.1021/jf050139z.

4. Gok, S.; Severcan, M.; Goormaghtigh, E.; Kandemir, I.; Severcan, F. Differentiation of Anatolian Honey Samples from Different Botanical Origins by ATR-FTIR Spectroscopy Using Multivariate Analysis. Food Chem. 2015, 170, 234–240. https://doi.org/10.1016/j.foodchem.2014.08.040.

5. Fernández Pierna, J. A.; Abbas, O.; Dardenne, P.; Baeten, V. Discrimination of Corsican Honey by FT-Raman Spectroscopy and Chemometrics. Biotechnol. Agron. Soc. Environ. 2011, 15 (1), 75–84.

6. Bisutti, V.; Merlanti, R.; Serva, L.; Lucatello, L.; Mirisola, M.; Balzan, S.; Tenti, S.; Fontana, F.; Trevisan, G.; Montanucci, L.; Contiero, B.; Segato, S.; Capolongo, F. Multivariate and Machine Learning Approaches for Honey Botanical Origin Authentication Using near Infrared Spectroscopy. J. Near Infrared Spectrosc. 2019, 27 (1), 65–74. https://doi.org/10.1177/0967033518824765.

7. Woodcock, T.; Downey, G.; Kelly, J. D.; O'Donnell, C. Geographical Classification of Honey Samples by Near-Infrared Spectroscopy: A Feasibility Study. J. Agric. Food Chem. 2007, 55 (22), 9128–9134. https://doi.org/10.1021/jf072010q.

8. Latorre, C. H.; Crecente, R. M. P.; Martín, S. G.; García, J. B. A Fast Chemometric Procedure Based on NIR Data for Authentication of Honey with Protected Geographical Indication. Food Chem. 2013, 141 (4), 3559–3565. https://doi.org/10.1016/j.foodchem.2013.06.022.

9. Corbella, E.; Cozzolino, D. The Use of Visible and near Infrared Spectroscopy to Classify the Floral Origin of Honey Samples Produced in Uruguay. J. Near Infrared Spectrosc. 2005, 13 (2), 63–68. https://doi.org/10.1255/jnirs.458.

Chapter 2: Alternative Rapid Techniques

Within the present PhD thesis, part of the work has regarded the application of rapid techniques for the sample's headspace analysis. Gas chromatography (GC) coupled to mass spectrometry represents the "golden standard" for the analysis of volatile organic compounds (VOCs). However, the overall high cost, analysis time and expertise demanded have prompted the development of alternative approaches for the analysis unresolved mixture of VOCs. Among them, chemical sensors, such as electronic noses coupled to multivariate data analysis has shown promising capabilities for food quality and process control and nowadays represent an active and growing field of research.

The next manuscript, published on Microchemical Journal (https://www.journals.elsevier.com/microchemical-journal), shows the first application of two emerging headspace-based techniques, namely gas-chromatography ion mobility spectrometry (GC-IMS) and flash gas-chromatography electronic nose (FGC-Enose), for the detection of EVOO adulteration with soft-refined olive oils using a non-targeted fingerprinting approaches. Further information about the employed analytical techniques is reported in **Annex II**.

GC-IMS and FGC-Enose fingerprint as screening tools for revealing Extra Virgin Olive Oil blending with soft-refined olive oils: a feasibility study

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Abstract: The undeclared blending of EVOO with soft-refined oils is one of the main issue in the olive oil sector. Despite the efforts, reliable markers related to soft-refinement processes have not been found yet. In the present work, two rapid headspace-based techniques, namely gas-chromatography ion mobility spectrometry and flash gas chromatography electronic nose, were proposed and tested as rapid screening tools for the detection of this fraud practice. Since real counterfeited samples are not commercially available, soft refined and deacidified olive oils were recreated at a laboratory scale and mixed with EVOO at different percentages. Commercial EVOOs sampled over three harvesting seasons (2015/2016, 2016/2017 and 2017/2018), along with the in-house prepared blends, were analysed by means of the above-mentioned techniques. SIMCA was chosen as classification algorithm to discern the illicit mix from the authentic EVOOs. Both the analytical techniques exhibited notable robustness and stability over the time in terms of intra- and inter-day reproducibility. Concerning the samples discrimination, the final outcome was found to be greatly affected by the inclusion (or exclusion) of the EVOO15/16 group in the model training. When EVOO from more recent harvests (i.e. EVOO16/17, EVOO17/18) were used to calibrate the model, a practically 100% specificity was achieved by both the techniques and even the lowest-percentage adulterated samples (i.e. 10%) were recognized to be non-authentic EVOOs. On the other hand, poorer classification was achieved including the EVOO15/16 in the model training. The present work demonstrated that focusing on the volatile fraction might be the right strategy to overcome the lack of clear and specific process-related marker formed upon soft-refinement processes. At the same time, it highlighted how the EVOO chemical (in)stability would be a crucial aspect to be considered in the development of fingerprinting methods.

Keywords: extra-virgin olive oils, soft-deodorization, deacidification, fingerprint, GC-IMS, FGC-Enose**.**

1. Introducton

Olive oil is part of the Mediterranean culture and, due to its unique nutritional and organoleptic properties, is arguably the most appreciated among edible oils [1]. Due to the coexistence of international (International Olive Council, Codex standards), European (European Economic Community) and new national legislation in the emerging producing countries (e.g. USA, Australia, New Zealand), the olive oil sector is one of the most regulated markets worldwide [2,3]. Despite the slight differences between these regulations, olive oils are basically grouped into three quality grades, namely "extra-virgin olive oil" (EVOO), "virgin olive oil" (VOO) and "lampante olive oil" (LOO), on the basis of specific chemical and organoleptic criteria [2].

Referring to the European Regulation No. 2568/91 and subsequent amendments, VOO must be obtained using solely mechanical or other physical means, under conditions which do not alter the product's integrity. In contrast, LOO category includes those oils which do not fulfil the minimum VOO quality criteria and, therefore, are not intended for direct human consumption [4]. Finally, EVOO represents the top-quality grade due to its superior sensory attributes and the claimed health-promoting effects [5]. Its limited production, higher price and growing consumers' demand represent explicit fraud drivers that make EVOO particularly susceptible to adulteration [2]. In fact, while in 2018 "fats and oils" was the third-most notified category within the EU Food Fraud Network, in 2019 it ranked first with the "olive oil" being the most reported product in the system [6].

Undeclared blending of EVOO with lower-quality and cheaper oils is probably the main fraud issue in the olive oil sector. In particular, the addition of VOO or LOO whose organoleptic and physiochemical defects have been removed by refinement procedures, is a frequent adulteration practice which has shown to be exceptionally challenging to be addressed [7]. Trans-fatty acids level and stigmastadienes profile have been long considered as suited markers for the detection of unpermitted thermal treatment and the regulation (EEC) 2568/1991 set maximum limits for these compounds [8]. Moreover, several methods based on the determination of further compounds (i.e. chlorophyllic derivatives, conjugated linoleic acid, dimers, and 1,3-diacylglycerol isomers) have been proposed to verify whether VOO had been thermally treated [9]. However, developments in the field of fat and oil technology have led to refinement (neutralization and/or deodorization) processes carried out under milder conditions in order to avoid the formation of such degradation products at detectable levels [8].

The term ''soft-deodorization" refers to a deodorization process carried out at lower temperature respect to the conventional process (i.e. 100°C instead of 180-200°C). It basically consists of a vacuum steam distillation using nitrogen to strip out all the volatiles compounds responsible for the undesirable odours [7]. Deacidification is designed to remove the free fatty acids (FFA), which speed up oxidation processes and are involved in the development of rancid flavour. It is normally achieved through the addition of alkali

(e.g. sodium hydroxide) to the oil; this results in the precipitation of the FFA as an insoluble soap dreg, subsequently removed by centrifugation and/or filtration [10].

Since the use of lower temperatures does not seem to produce substantial compositional and structural modification in the bulk, the resulting soft-refined olive oils (SROO) are best used to blend EVOO and create illicit mixtures no longer detectable by current methods [3]. For instance, isomerization of fatty acids and formation of steradienes start at about 170 °C and 150 °C, respectively. Therefore, they are not expected to arise in SROO [8]. Attempting to overcome this problem, Pérez-Camino and co-workers suggested fatty acid ethyl and methyl esters (FAEEs, FAMEs) as indirect markers of soft-deodorization, when the process is applied on oils defected for fermentative reasons [9]. Indeed, the FAEEs content is strictly related to fermentative processes occurring in damaged or over-ripe drupes and these compounds are not removed by mild refinements. On the other hand, not all low-quality oils necessarily show a high content of FAEEs (e.g. rancid oils) [3].

A recent work by our research group exploited the potential of liquid chromatography coupled to high-resolution mass spectrometry for the annotation of twelve chemical compounds identified as potential markers of the undeclared EVOO-SROO blending. In addition, seven of these twelve compounds were further confirmed within an inter-laboratory study [11].

Relying on targeted quantification of specific compounds, all the above-mentioned analytical approaches would empower a straightforward assessment of the product compliance through the comparison with a defined control limit [12]. Nevertheless, considerable disadvantages have to be taken into account. Firstly, they are based on time-consuming and complex procedures that require intensive sample preparations before analysis [13]. In addition, the normal occurrence of the chosen compound(s), along with the method uncertainty, have to be thoroughly investigated in order to ensure results reliability [8,12]. Moreover, it is known that defrauders often possess appropriate knowledge to adjust their products in the way that they would meet the official criteria. Therefore, counting only on analytical parameters listed by legal regulations may increase the risk that the manipulation remains undetected [8]. For all these reasons, more “unconventional”, rapid and environmental friendly methods have received special attention by the scientific community [13]. As recently reviewed by Valli et al., novel application of optical techniques (e.g. UV-Vis, NIR, MIR, Raman and fluorescence spectroscopy), electronic chemical sensors (e.g. electronic noses and tongues), as well as the measurement of electrical characteristics (e.g. electrical impedance spectroscopy, amperometry, cyclic voltammetry) have shown large potential and promising capabilities of these technologies within the quality and authenticity monitoring of olive oils [13].

In the present work, two emerging headspace (HS) based techniques, namely gas-chromatography ion mobility spectrometry (GC-IMS) and flash gas-chromatography electronic nose (FGC-Enose) were tested for the detection of EVOO blending with SROO. As discussed above, the main changes due to soft refining

are not expected to occur in the bulk fraction. Therefore, targeting the volatile profile might be a more fruitful strategy. The working principle of IMS is the separation of ionic species according to their relative ionic mobility. To this purpose, the analytes are introduced into the ionization chamber of the IM spectrometer and subsequently accelerated by a uniform weak electric field through a linear drift tube subjected to a counter flowing drift gas. Since complex matrices being analysed often result in ion-ion interactions and/or overlapping peaks, the separation efficiency can be enhanced by coupling the IM spectrometer with a prior GC separation [14]. This technology was originally developed for the rapid detection of explosives and chemical warfare agents. However, in the last years, it has found novel applications in food quality and authenticity control [15]. Concerning the FGC-Enose employed in the present study, it consisted of a gas-chromatographic system equipped with two parallel short columns, each coupled to a flame ionization detector (FID). Although its detection system differs from the conventional e-sensing devices based on arrays of broadly tuned (non-specific) sensors, such equipment is often referred as e-nose [16]. In fact, it is often used following the same working principle of e-noses analysis: generate a characteristic sample fingerprint which can be evaluated on a pattern level by means of multivariate data analysis (MDA) tools [17].

Both GC-IMS and FGC-Enose have been tested, among others, in the olive oil quality and authenticity field. Substantial work has been done by Garrido-Delgado et al. in the application of GC-IMS for the monitoring of different quality aspects of olive oil [18–22], from the shelf-life assessment to the detection of EVOO blending with other vegetable oils [23]. Concerning the FGC-Enose, most of the studies carried out thus far have been mainly focused on quality and authenticity assessment of alcoholic beverages [24–26], while applications in the vegetable oils field have been less reported [27–29].

Further to the remarkable results reached in the olive oil authenticity, the goal of the study was to assess and compare the capabilities of these platforms as untargeted screening tools for the detection of illicit EVOO-SROO blending. To this end, SROOs were in-house reproduced under controlled conditions and mixed with authentic EVOOs at different percentages, thus reproducing a potential counterfeiting action. Afterwards, each sample was analysed by means of the two above-mentioned HS-based techniques. The following MDA aimed at developing classification models able to distinguish between authentic and adulterated EVOOs. To the best of our knowledge, the present work shows the first application of GC-IMS and FGC-Enose for the detection of EVOOs adulteration with SROOs.

2. Materials and Methods

2.1. Chemicals

For the soft-refining process, Sodium Hydroxyde and Sodium Sulphate were purchased from VWR International, Ltd (Poole, United Kingdom), while water was purified using a Milli-Q system (Millipore, Bedford, MA).

2.2. Samples set

A total of 43 commercial EVOOs, produced in different Italian regions and over three harvesting seasons 2015/2016 ($n = 18$), 2016/2017 ($n = 8$) and 2017/2018 ($n = 17$), were collected and included in the study. Henceforth, the EVOO groups will be referred as EVOO15/16, EVOO16/17 and EVOO17/18, respectively. All the samples were stored in the dark and at 4 °C until the analysis. Soft-deodorization and deacidification were carried out on commercial VOOs and LOOs. Both the refining processes were replicated on different oils in order to check the procedure repeatability. At the end, 3 soft-deodorized (DEO) and 2 deacidified (DEA) oils were obtained. Furthermore, two aliquots of the DEA oils underwent also to the soft-deodorization process, gaining two olive oil samples both deacidified and soft-deodorized (DEA+DEO).

In order to create counterfeited samples potentially compliant with the legislation, the official EVOO physico-chemical quality parameters [30] were analysed in these refined oils. Based on the obtained results, 21 illegal mixtures were prepared at different percentage (from 10 to 60%), by blending the so-obtained SROOs with EVOOs randomly chosen from the sample set. A detailed samples list is provided in the Supplementary material (Table S1). Moreover, 2 commercial EVOOs suspected to be frauded with SROOs, were included in the sample set.

2.3. Soft Deodorization and deacidification

A soft-refining process was in-house reproduced and applied to non-EVOO samples (i.e. virgin and lampante olive oil). The soft-deodorization process was carried out partially modifying the protocol proposed by Aparicio-Ruiz et al. [7]. The olive oil sample (400 mL) was introduced into a triple-neck round-bottom flask containing a magnetic stir bar. Two necks were used to connect, respectively, the vacuum tubing and a dropping funnel containing Milli-Q grade water. A thermometer was introduced in the left neck in order to constantly check the system temperature and, finally, the apparatus was placed on a magnetic stirring hotplate (VELP Scientifica, Italy) operating at 300 rpm. In order to start the distillation, 0.2 mL of water were added when the oil temperature reached an initial temperature of 60 °C. Thereafter, the temperature was raised to 100 °C and maintained for 60 minutes. After evaporation of the initial amount of water, further 0.2 mL were added to carry on the distillation process. The time/temperature parameters

were chosen according to the results achieved by Aparicio-Ruiz et al. [7]. At the end of the procedure, the sample was cooled down and stored in the dark at 4 °C until the analyses.

For the deacidification, 600 mL of defective oil were weighted into a beaker, and the latter placed on a magnetic stirring plate (VELP Scientifica, Italy) operating at room temperature. The neutralization was achieved adding NaOH and Na_2SO_4 directly to the oil. The volume of NaOH to add was computed according to the following formula while the amount of Na_2SO_4 was twice the so-calculated value.

$$NaOH\ (mL) = \left[\frac{(A-B)\cdot C\cdot D}{(\frac{E}{100})}\right]\cdot\left[\frac{(\frac{100}{F})}{G}\right]$$

Where:

- A and B are, respectively, the starting and intended value of FFA% of the oil (expressed as percentage of oleic acid equivalent). In the present work $B = 0.4\%$;
- C is the sample weight (g);
- D and E are, respectively, the NaOH and oleic acid molecular weight ($D = 40\ g/mol$; $E = 282\ g/mol$);
- F and G are, respectively, the concentration and the density of the NaOH solution concentration ($F = 50\%$, $G = 1.52$ g/mL);

After 5 minutes stirring, the sample was centrifuged (5000 rpm) and the supernatant (neutralized oil) collected and stored at 4 °C before the analyses. An aliquot of the so-obtained deacidified oil underwent to the soft-deodorization process described above.

2.4. Instrumental Analysis

The GC-IMS analyses were performed on an IMS commercial instrument (FlavourSpec® - G.A.S., Dortmund, Germany), equipped with a 2.5 mL Hamilton syringe, an automatic headspace sampling unit and a gas chromatograph. For the analysis, 0.2 g of olive oil was placed into a 20 mL HS vial and incubated at 60 °C for 10 minutes. Afterwards, 500 µL of sample's HS were sampled and injected into the GC–IMS equipment, with a speed rate of 30 mL min^{-1} and a syringe temperature of 80 °C. The GC was equipped with a SE-54-CB-1 capillary column (5%phenyl-1%vinyl-94% methylpolysiloxane), 15 m × 0.53 mm × 1 µm film thickness. The separation was achieved in isothermal conditions (40 °C), using N_2 as carrier gas with a flow ramp starting with 2 mL min^{-1} for 5 min, increasing up to 70 mL min^{-1} in 20 min and finally maintaining 70 mL min^{-1} for 5 min. The total GC runtime was 30 min. Subsequently, the eluting compounds were guided into the IMS ionization chamber for the ion mobility separation and detection (instrumental details in Supplementary Table 2).

FGC-Enose analysis were performed on the Heracles II commercial system (Alpha MOS, Toulouse, France). The instrument was equipped with two parallel short GC columns: a non-polar column (MXT5: 5% diphenyl, 95% methylpolysiloxane, 10 m x 0.180 mm x 0.4 µm film thickness) and a slightly polar column (MXT1701: 14% cyanopropylphenyl, 86% methylpolysiloxane, 10 m x 0.180 mm x 0.4 µm film thickness). For the analysis, 0.2 g of olive oil was sealed into a 20 mL HS vial and incubated at 60 °C for 20 min in a shaker-incubator. Thereafter, 5 mL HS were sampled and injected into the FGC-Enose equipment using H_2 as carrier gas. In order to remove the air and moisture excess, as well as to concentrate the analytes, the sampled volume was adsorbed on a CARBOWAX trap (40 °C for 65 s) prior to the chromatographic separation. For the compounds desorption, the trap temperature was increased up to 240 °C in 30 s and the volume was driven into the chromatographic unit. The total chromatographic separation time was 110 s, employing the following chromatographic temperature program: starting temperature of 40 °C (kept for 2 s), increased up to 270 °C (at 3 °C s^{-1}) and held for 21 s. Each column was equipped with a FID operating at 270 °C, therefore, two GC traces were recorded during the analysis.

All the GC-IMS and FGC-Enose analysis took place over a 7-months period (from October 2017 to May 2018). Each sample was analysed in duplicate for the GC-IMS and in triplicate for the FGC-Enose. The replicates' average was used for the following data processing.

2.5. Data treatment and statistics

2.5.1. GC-IMS and FGC-Enose data

The GC-IMS output consists of a 3D array, graphically represented as a topographic plot. Due to the twofold separation, the analytes occur as 2D-signals (spots) characterized by a chromatographic RT (y-axis), an ion mobility drift time (x-axis) and the intensity values represented by a colour scale. LAV software was used to visualize the data (i.e. topographic plots), pinpoint the relevant spots and manually box the areas to carry the integration on, taking the peak height as intensity value [31]. GC-IMS spectra alignment respect to the reactant ion peak (RIP) position was automatically performed by the software.

As explained in the previous section, two GC chromatograms are obtained from each FGC-Enose run. As proposed by Melucci et al., the obtained GC traces were aligned and arranged consecutively. All the detected peaks above an established intensity threshold were selected and automatically integrated by the Alphasoft software [29].

2.5.2. Multivariate data analysis

In order to compare the performance of the employed devices, GC-IMS and FGC-Enose datasets underwent to chemometric analysis, separately. Data visualization and exportation was carried out by

means of the commercial software LAV 2.2.1 (G.A.S., Dortmund, Germany) and Alphasoft V14 (Alphamos, Tolouse, France), respectively for GC-IMS and FGC-Enose instruments. The obtained datasets were imported in Matlab R2019b (The Mathworks Inc., Natick, MA, USA, 2007) and processed using PLS Toolbox 8.7 (Eigenvector Research, Inc., Manson, WA, USA).

Prior to any exploratory or classification analysis, different data pretreatments (i.e. mean centering, autoscaling, Pareto scaling, Log10 transformation) were tested, both on their own and in combination, in order to highlight the relevant variation contained in the data. Thereafter, principal component analysis (PCA) was used for a preliminary data exploration in a reduced dimension space.

Supervised pattern recognition aimed at developing multivariate models able to correctly discern between EVOOs and the adulterated mixtures. Many food authentication and quality control scenarios involve a single target class, whereas off-specification products represent a heterogeneous group hard to be appropriately outlined. In such situations, one-class classifiers approaches should be adopted [32,33]. Soft Independent Modelling of Class Analogies (SIMCA) is arguably the most used class-modeling technique in chemometrics. It relies on the assumption that the main systematic variability of the modeled class can be represented by a PCA model. Based on such PCA decomposition, an acceptance area (i.e. the so-called SIMCA inner space) around the class of interest is defined and the tested objects are recognized as "compliant" whether they fall within such boundaries. The "degree of outlyingness" with respect to the target category is usually computed as combination of the Mahalanobis distance to the centre of the inner space (T^2) and the orthogonal distance (Q). The latter are a lack-of-fit statistic that measure the magnitude of the variation remaining in each sample after projection. Thus, they can be considered as a representation of how well each object conforms to the model. On the other hand, the Hotelling's T^2 measure how far each sample is from the centroid of the target class (i.e. within-model variation). A comprehensive tutorial of SIMCA, and one-class classifiers methods in general, is provided in [33].

Class modelling performance is generally reported in terms of sensitivity and specificity. Sensitivity is also called true positive ratio and consists of the fraction of samples actually belonging to the target class and correctly recognised as compliant. Specificity, or true negative ratio, is the fraction of alien objects correctly rejected by the model [33].

3. Results and Discussion

In food authenticity studies, the sample collection often represents the most demanding part of the workflow. A large number of authentic and adulterated samples are needed for the development and a reliable validation of any analytical method. In particular, real counterfeited samples are (obviously) not commercially available. In order to overcome this shortcoming, many researchers attempt to recreate

adulterated products at a laboratory scale [34]. This was the case of the present study, where a soft-refining process was in-house reproduced and applied to non-EVOO samples in order to obtain SROOs as described in Section 2.3.

GC-IMS is a rapid and easy-to-use technology designed for in-line quality control testing. For this purpose, the commercial LAV software was designed as a user-friendly interface to perform the data processing; the spots considered to be relevant can be manually boxed and easily exported as dataset. Accordingly, 52 markers were selected and included in the dataset (73 objects x 52 variables) for the following MDA. A detailed marker list is provided in Supplementary Material (Supplementary Table 3). Being affected by the operator's subjectivity in the marker selection, such an approach might present some weakness in terms of reliability. For this reason, a more systematic method exploiting the entire 3D spectrum as sample's fingerprint and was attempted [20]. Briefly, a prior data preprocessing (i.e. baseline correction, Savitzky-Golay smoothing and peak alignment using the spline interpolation algorithm) was performed on the GC-IMS data. Afterwards, the 3D-dataset was unfolded and the IMS spectra of each samples arranged consecutively in a row, thus obtaining a final matrix of 73 objects x 1698780 variables [35]. Comparable and consistent results were provided by both the strategies. However, it must be highlighted that the latter exploit a great volume of data ($\approx 10^6 - 10^7$ data points) for modelling [18]. Consequently, besides potential overfitting issues due to the massive number of variables, it is more demanding in terms of computing power, time and operator's expertise, therefore less convenient for industrial implementation. For these reasons and for purpose of presentation, only the outcome of the first approach are shown.

Concerning the FGC-Enose data, the two chromatograms obtained for each sample were merged into a single comprehensive trace. The aim was to reduce the risk of discarding useful information by selection of relevant RT ranges. On the other hand, it must be taken into account that the same analyte could generate peaks in both chromatograms, leading to redundant information in the dataset [29]. At the end, 74 chromatographic peaks were found to be significant (73 objects x 74 variables).

As expected, strong differences were found between GC-IMS topographic plots of EVOOs and SROOs, with all the volatile organic compounds (VOCs) eluting between 1.89 and 14.1 minutes, and drift time ranging from 7.8 to 15.35 ms. Likewise, FGC-Enose chromatograms of EVOOs showed a larger number of peaks (higher in intensity) respect to the SROOs traces (Fig. 1). This is not surprising as almost all the VOC's are supposed to be stripped out during the deodorization process.

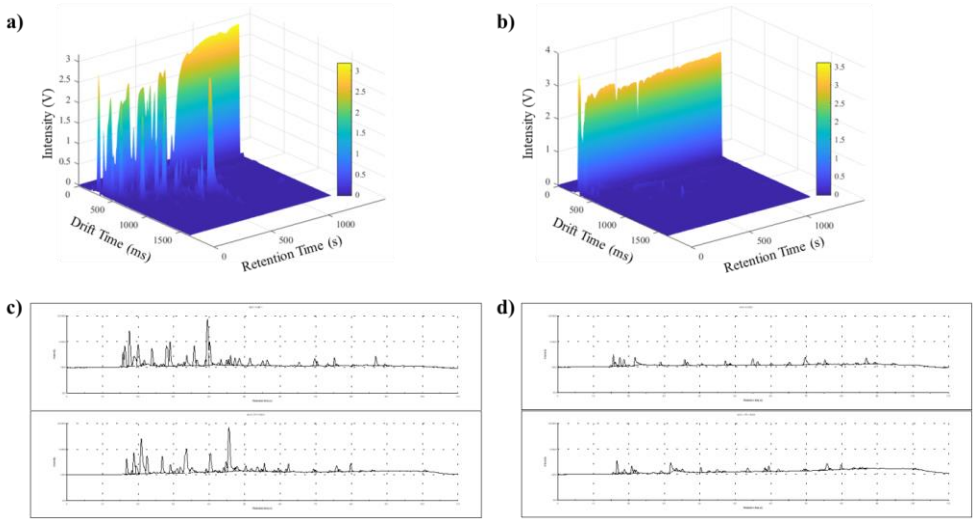

Figure 1. GC-IMS 3D topographic plot of EVOO (a) and soft-deodorized oil (b); FGC-Enose chromatograms of EVOO (c) and soft-deodorized oil (d).

As noteworthy aspect of the present work, all the analyses were carried out over a 7-months period, thereby covering potential instrumental fluctuations, a critical aspect to be faced for fingerprinting method development. In manufacturing quality control contexts, samples collection and analysis are normally performed within long-term monitoring programmes. In fingerprint-based methods, technical variations (i.e. environmental condition, instrumental drift, etc.) not properly handled may produce measurement deviations that would ultimately lead to unreliable predictions. Therefore, the experimental design should account also for these sources of variability in order to avoid overestimation of the method performance [12].

In this regard, the GC-IMS measurement intra-day and inter-day repeatability were assessed, by analysing several replicates of the same reference sample within the same day and over the whole analysis period. The most intense spot was taken as reference and the recorded intensity values used to calculate the intra-day and inter-day repeatability as relative standard deviation (RSD) of the measurements [22]. The same computation was performed on the FGC-Enose data, taking the highest peak as reference. The resulting intra-day and inter-day repeatability for GC-IMS and FGC-Enose are summarized in Table 1. As can be seen, both the equipment showed remarkable stability over the time, which is a great advantage for any fingerprinting method.

Table 1. Intra-day and inter-day reproducibility of the GC-IMS and FGC-Enose equipment, expressed as RSD of the highest peak measured in the reference sample.

Technique	Intra-day (%)	Inter-day (%)
GC-IMS	1.3	4.7
FGC-Enose	2.2	3.4

3.1. Unsupervised Pattern Recognition

PCA was carried out as exploratory analysis on the whole sample set. In both cases the first four PCs accounted for more than 60% of the total variation (64.97% and 66.34% for GC-IMS and FGC-Enose, respectively). As can be observed in the PCA scores plot (Figure 2), the SROO replicates (i.e. three replications of DEO, two of DEA and DEA+DEO) are fairly close to each other, which denotes that the refinement protocol was rather reproducible and properly performed. Furthermore, plotting PC1 versus PC2 revealed a rather evident trend among the EVOO groups. While a clear clustering between EVOO16/17, EVOO17/18 and the illicit mixtures can be appreciated, the latter are largely overlapped by the EVOO15/16. Hence, it seems that the EVOO samples get closer to the SROO blends admixtures according to the harvesting year. Such a trend suggests, albeit in mere qualitative terms, that during the storage/ageing the EVOO's volatile fraction might undergoes to changes similar to those produced by the soft-refinement in a shorter time-scale.

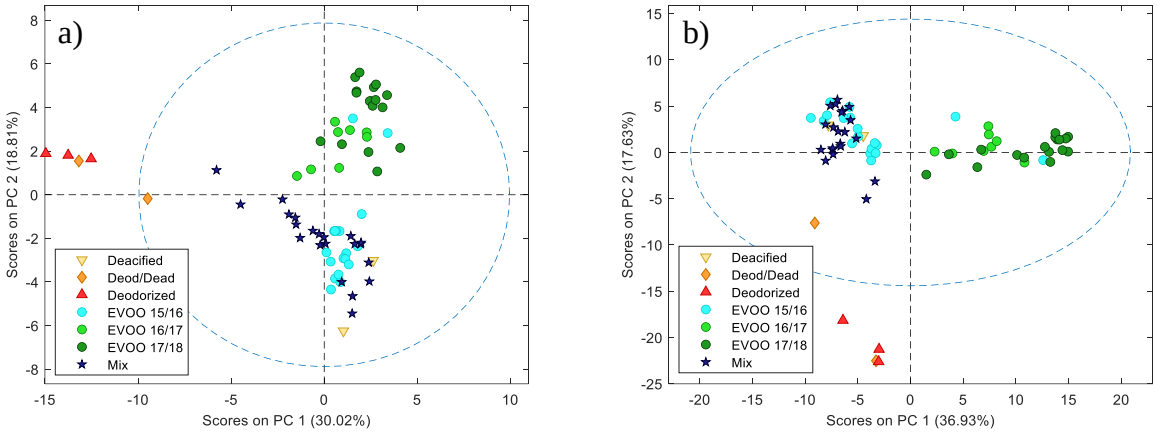

Figure 2. Scatter plots of PC1 vs PC2 scores of GC-IMS (a) and FGC E-nose (b) data.

3.2. SIMCA Modelling

Thus far, several works have demonstrated the feasibility of GC-IMS and FGC-Enose as valuable screening tools for olive oil authentication [20,22,29]. To this end, chemometrics has often focused on the classification of samples belonging to different oil categories by means of discriminant analysis techniques. As mentioned in Section 2.5.2, authentication problems should be addressed using class modelling approaches [33]. Accordingly, SIMCA was chosen as classification algorithm.

As discussed in the previous section, a rather clear trend among the EVOO groups was highlighted by the PCA and further investigations are needed to draw any conclusion. Nevertheless, the EVOO stability in ordinary shelf-life conditions (i.e. room temperature and exposed to light) rarely exceeds one year. In fact, the physico-chemical quality descriptors risk to be no longer compliant with the legislation [36,37]. For these reasons, only the EVOO16/17 and EVOO17/18 samples were modelled as target class, whereas EVOO15/16 were excluded, at a first instance, from the modelling. The in-house prepared mixtures, along with five EVOO samples (i.e. two EVOO16/17 and three EVOO17/18), were used as external test set to challenge the model. The SIMCA model complexity was determined through a cross-validation procedure, retaining the number of PCs that provided the minimum RMSECV (Root Mean Squared Error Cross Validation). Six PCs were found to be the optimal dimensionality for both the GC-IMS and FGC-Enose datasets, explaining the 80.9% and 98.8% of the data variance, respectively. The confidence level was set to α=0.05 and the classification rule was based on the augmented T^2-Q distances. The best data preprocessing for GC-IMS and FGC-Enose data was found to be autoscaling and mean centering, respectively.

Notably, both the developed models were able to correctly recognize the SROO blends as non-authentic products, even at the lowest adulteration percentage (i.e. 10%) (Table 2). Furthermore, only one EVOO sample was wrongly recognized as alien object. It must be pointed out that the limited number of samples available prevented an exhaustive model training and validation; therefore, these results have to be regarded as preliminary outcomes as only few objects could be included in the test set. Nevertheless, they confirmed the high potential of the two employed techniques and demonstrated that targeting the sample's HS may be a feasible strategy for the detection of undeclared SROO blending.

Table 2. Sensitivity/specificity obtained by SIMCA modelling of the GC-IMS and FGC-Enose data. The results are referred to the external test set (i.e. illicit mixtures and five EVOO samples).

Technique	Preprocessing	PCs	Sensitivity (%)	Specificity (%)
GC-IMS	Autoscaling	6	80	100
FGC-Enose	Mean Center	6	80	100

As mentioned in **Section 2.5.2**, recent implementations of SIMCA use the combined T^2-Q model distances to assign the observation to one of the available classes. To this purpose, a distribution of the residuals Q (i.e. non-modeled variation) is generated for each sample and summarized in the "distance-to-model" parameter (also called DModX) [38]. The investigation of such residual distribution may highlight the features that largely differ from their PCA model representation, thus likely responsible of the sample rejection. As an example, Q residual distribution of three EVOOs versus DEA and DEO oils, respectively, is reported in Figure 3 (GC-IMS data). As can be observed, deacidification and deodorization seem to affect the VOCs profile in a different direction. DEA samples (Figure 3a) shown a significantly higher response of two features (i.e. spot 26 and spot 38) with respect to the target class. This is probably due to their increase in formation upon deacidification. On the other hand, several compounds in DEO oils are decreased in concentration compared to EVOOs (Figure 3b). This can likely be ascribed to the VOC's stripping during the soft-deodorization process. Since the main aim of this study was the development of a fingerprinting method for the rapid screening of oil samples within an industrial plant, marker identification was not considered as a major goal. However, in order to develop a target analytical method to be used for suspect sample confirmation, chemical annotation of the statistically significant markers should be carefully performed.

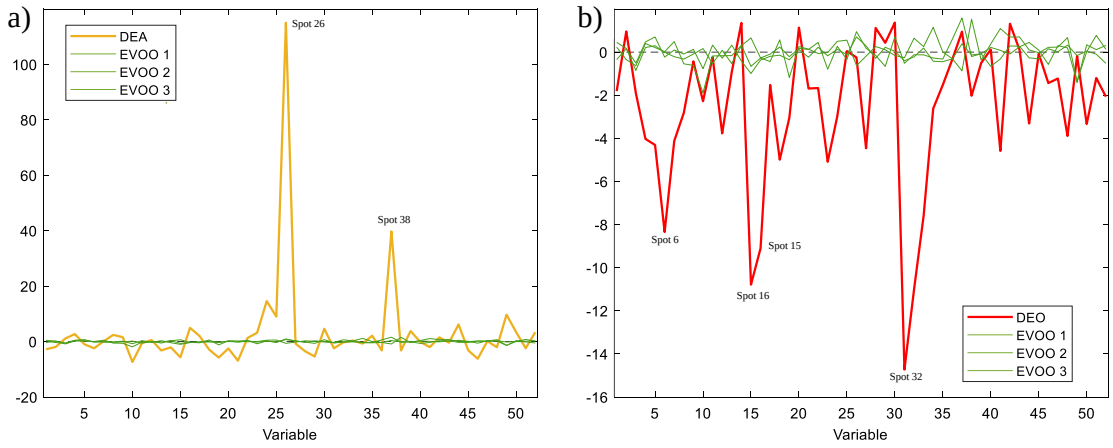

Figure 3. Distribution of Q residuals of DEA (a) and DEO (b) respect to the model representation of the target class (GC-IMS data). Q residuals distributions of three EVOOs are also reported for purpose of comparison.

At this point, EVOO15/16 were included in the modelling to assess the method's reliability towards oils of uncertain characteristics. At first, EVOO15/16 samples were classified by means of the aforementioned SIMCA model. As a result, all the objects were rejected and recognized as non-compliant with respect to the target class. A graphical representation of the classification outcome is shown in Figure 4 (magnifications of the critical zones) and in Figure S1 (original plots) by plotting the T^2 vs Q statistics of the tested objects. Such misclassification might be due to major changes in the volatile profile occurred

during the storage, leading to a HS fingerprint that largely differs from more recent EVOOs. On the other hand, influence of the olive cultivars and/or process conditions cannot be excluded. This is a further aspect to be included in future studies intended to a thorough validation of fingerprinting methods, in order to ensure the model's robustness towards these factors potentially affecting the oils' chemical-sensory characteristics.

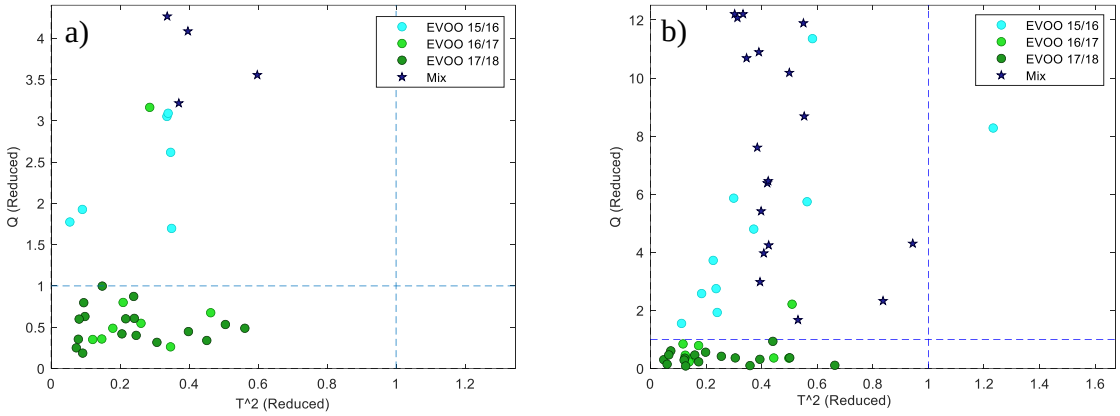

Figure 4. Projection of the samples onto the T^2 red vs Q red model space of the target class (EVOO16/17 and EVOO17/18). GC-IMS and FGC E-nose data reported in the left (a) and right (b) figure, respectively. The dashed lines indicate the confidence limit corresponding to α=0.05. Objects lying below the dashed lines (bottom-left quarter) are accepted by the model, while those falling above are rejected. The figures are magnifications of the region close to the decision boundaries. The original plots are reported in supplementary materials

As a further attempt, the EVOO15/16 group was included in the model training. Thus, the SIMCA model was calibrated over all the available EVOO samples (i.e. EVOO15/16, EVOO16/17, EVOO17/18) and challenged with the same external test set described above (i.e. illicit mixtures and five EVOO samples). As a results, only 15 and 8 blends were correctly rejected from the model built on GC-IMS and the FGC-Enose, respectively (Table 3).

Table 3. Sensitivity/specificity obtained by SIMCA modelling of the GC-IMS and FGC-Enose data including EVOO15/16 in the model calibration. The results are referred to the external test set (i.e. illicit mixtures and five EVOO samples).

Technique	Preprocessing	PCs	Sensitivity (%)	Specificity (%)
GC-IMS	Autoscaling	8	80	60
FGC-Enose	Mean Center	8	80	34.7

Therefore, within the present study, the inclusion/exclusion of the EVOO15/16 group in the model training greatly affected the classification. As a matter of fact, EVOO is known to be a non-stable food matrix prone to chemical changes in ordinary shelf-life conditions. Such modifications, concerning both

the bulk and the volatile fraction, may play a significant role in fingerprinting measures. Despite the reduced number of samples, it seems that the oil ageing and storage conditions represent crucial aspects to be taken into account for the development of untargeted fingerprinting methods.

4. Conclusions

In the present work, the authors dealt with one of the main issues of the EVOO sector: the undeclared blending with SROOs. As discussed in Section 1, several targeted analytical approaches have been proposed thus far. However, they suffer from serious drawbacks and limitations in terms of reliability.

Within the present study, GC-IMS and FGC-Enose were tested as rapid screening platforms for the detection of this common fraud practice. These two techniques offer rapid, high throughput and non-destructive solutions for quality and authenticity testing, highly claimed by modern food industries. The required sample preparation was minimal, providing global information about VOCs profile in a short time, and both the equipment exhibited notable robustness and stability over the time.

Despite being a feasibility study, the present results confirmed the high potential of the two employed techniques and demonstrated that focusing on the EVOO volatile fraction might be the right strategy to overcome the lack of clear and specific process-related markers formed upon soft-refinement processes. At the same time, it highlighted crucial aspects to be taken into account in future works intended to a thorough untargeted method development and validation.

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.

References

[1] J.L. Barjol, Introduction, in: Handb. Olive Oil Anal. Prop., Springer US, 2013: pp. 1–17. https://doi.org/10.1007/978-1-4614-7777-8_1.

[2] A. Bajoub, A. Bendini, A. Fernández-Gutiérrez, A. Carrasco-Pancorbo, Olive oil authentication: A comparative analysis of regulatory frameworks with special emphasis on quality and authenticity indices, and recent analytical techniques developed for their assessment. A review, Crit. Rev. Food Sci. Nutr. 58 (2018) 832–857. https://doi.org/10.1080/10408398.2016.1225666.

[3] L. Conte, A. Bendini, E. Valli, P. Lucci, S. Moret, A. Maquet, F. Lacoste, P. Brereton, D.L. García-González, W. Moreda, T. Gallina Toschi, Olive oil quality and authenticity: A review of current EU

legislation, standards, relevant methods of analyses, their drawbacks and recommendations for the future, Trends Food Sci. Technol. (2019). https://doi.org/10.1016/j.tifs.2019.02.025.

[4] European Commission Regulation 183/93 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis, Official Journal of the European Communities, 1993, January 29, L 22, 58-68.

[5] M. Meenu, Q. Cai, B. Xu, A critical review on analytical techniques to detect adulteration of extra virgin olive oil, Trends Food Sci. Technol. 91 (2019) 391–408. https://doi.org/10.1016/j.tifs.2019.07.045.

[6] European Commission, The EU food fraud network and the Administrative Assistance and Cooperation System Health and Food Safety, Retrieved from https://ec.europa.eu/food/safety/food-fraud/ffn_en

[7] R. Aparicio-Ruiz, I. Romero, D.L. García-González, C. Oliver-Pozo, R. Aparicio, Soft-deodorization of virgin olive oil: Study of the changes of quality and chemical composition, Food Chem. 220 (2017) 42–50. https://doi.org/10.1016/j.foodchem.2016.09.176.

[8] C. Gertz, B. Matthäus, I. Willenberg, Detection of Soft-Deodorized Olive Oil and Refined Vegetable Oils in Virgin Olive Oil Using Near Infrared Spectroscopy and Traditional Analytical Parameters, Eur. J. Lipid Sci. Technol. 122 (2020) 1900355. https://doi.org/10.1002/ejlt.201900355.

[9] D. Carmen Pérez-Camino, A. Cert, A. Romero-Segura, R. Cert-Trujillo, W. Moreda, Alkyl Esters of Fatty Acids a Useful Tool to Detect Soft Deodorized Olive Oils, J. Agric. Food Chem. 56 (2008) 6740–6744. https://doi.org/10.1021/jf801131b.

[10] C. Vaisali, S. Charanyaa, P.D. Belur, I. Regupathi, Refining of edible oils: a critical appraisal of current and potential technologies, Int. J. Food Sci. Technol. 50 (2015) 13–23. https://doi.org/10.1111/ijfs.12657.

[11] D. Cavanna, K. Hurkova, Z. Džuman, A. Serani, M. Serani, C. Dall'Asta, M. Tomaniova, J. Hajslova, M. Suman, A Non-Targeted High-Resolution Mass Spectrometry study for extra virgin olive oil adulteration with soft refined oils: preliminary findings from two different laboratories, ACS Omega 2020, in press.

[12] S. Esslinger, J. Riedl, C. Fauhl-Hassek, Potential and limitations of non-targeted fingerprinting for authentication of food in official control, Food Res. Int. 60 (2014) 189–204. https://doi.org/10.1016/j.foodres.2013.10.015.

[13] E. Valli, A. Bendini, A. Berardinelli, L. Ragni, B. Riccò, M. Grossi, T. Gallina Toschi, Rapid and innovative instrumental approaches for quality and authenticity of olive oils, Eur. J. Lipid Sci. Technol. 118 (2016) 1601–1619. https://doi.org/10.1002/ejlt.201600065.

[14] A.B. Kanu, H.H. Hill, Ion mobility spectrometry detection for gas chromatography, J. Chromatogr. A. 1177 (2008) 12–27. https://doi.org/10.1016/j.chroma.2007.10.110.

[15] Z. Karpas, Applications of ion mobility spectrometry (IMS) in the field of foodomics, Food Res. Int. 54 (2013) 1146–1151. https://doi.org/10.1016/j.foodres.2012.11.029.

[16] F. Röck, N. Barsan, U. Weimar, Electronic Nose: Current Status and Future Trends, (2008). https://doi.org/10.1021/cr068121q.

[17] M. Peris, L. Escuder-Gilabert, A 21st century technique for food control: Electronic noses, Anal. Chim. Acta. 638 (2009) 1–15. https://doi.org/10.1016/j.aca.2009.02.009.

[18] N. Arroyo-Manzanares, A. Martín-Gómez, N. Jurado-Campos, R. Garrido-Delgado, C. Arce, L. Arce, Target vs spectral fingerprint data analysis of Iberian ham samples for avoiding labelling fraud using headspace – gas chromatography–ion mobility spectrometry, Food Chem. 246 (2018) 65–73. https://doi.org/10.1016/j.foodchem.2017.11.008.

[19] R. Garrido-Delgado, M.M. Dobao-Prieto, L. Arce, J. Aguilar, J.L. Cumplido, M. Valcárcel, Ion mobility spectrometry versus classical physico-chemical analysis for assessing the shelf life of extra virgin olive oil according to container type and storage conditions, J. Agric. Food Chem. 63 (2015) 2179–2188. https://doi.org/10.1021/jf505415f.

[20] R. Garrido-Delgado, L. Arce, M. Valcárcel, Multi-capillary column-ion mobility spectrometry: A potential screening system to differentiate virgin olive oils, Anal. Bioanal. Chem. 402 (2012) 489–498. https://doi.org/10.1007/s00216-011-5328-1.

[21] R. Garrido-Delgado, M.D.M. Dobao-Prieto, L. Arce, M. Valcárcel, Determination of volatile compounds by GC-IMS to assign the quality of virgin olive oil, Food Chem. 187 (2015) 572–579. https://doi.org/10.1016/j.foodchem.2015.04.082.

[22] R. Garrido-Delgado, F. Mercader-Trejo, S. Sielemann, W. de Bruyn, L. Arce, M. Valcárcel, Direct classification of olive oils by using two types of ion mobility spectrometers, Anal. Chim. Acta. 696 (2011) 108–115. https://doi.org/10.1016/j.aca.2011.03.007.

[23] R. Garrido-Delgado, M. Eugenia Muñoz-Pérez, L. Arce, Detection of adulteration in extra virgin olive oils by using UV-IMS and chemometric analysis, Food Control. 85 (2018) 292–299. https://doi.org/10.1016/j.foodcont.2017.10.012.

[24] M. Śliwińska, P. Wiśniewska, T. Dymerski, W. Wardencki, J. Namieśnik, Application of Electronic Nose Based on Fast GC for Authenticity Assessment of Polish Homemade Liqueurs Called Nalewka, Food Anal. Methods. 9 (2016) 2670–2681. https://doi.org/10.1007/s12161-016-0448-z.

[25] P. Wiśniewska, M. Śliwińska, T. Dymerski, W. Wardencki, J. Namieśnik, Differentiation Between Spirits According to Their Botanical Origin, Food Anal. Methods. 9 (2016) 1029–1035. https://doi.org/10.1007/s12161-015-0280-x.

[26] Y. Yang, Y. Zhao, S. Zhang, Y. Ni, J. Zhan, Qualitative analysis of age and brand of unblended brandy by electronic nose, in: IFIP Adv. Inf. Commun. Technol., Springer, Berlin, Heidelberg, 2012: pp. 619–628. https://doi.org/10.1007/978-3-642-27278-3_64.

[27] L. Xu, X. Li, J. Huang, P. Gao, Q. Jin, X. Wang, Rapid Measuring Flavor Quality Changes of Frying Rapeseed Oils using a Flash Gas Chromatography Electronic Nose, Eur. J. Lipid Sci. Technol. 121 (2019). https://doi.org/10.1002/ejlt.201800260.

[28] Y. Zhang, G. Wu, C. Chang, Y. Lv, W. Lai, H. Zhang, X. Wang, Q. Jin, Determination of Origin of Commercial Flavored Rapeseed Oil by the Pattern of Volatile Compounds Obtained via GC–MS and Flash GC Electronic Nose, Eur. J. Lipid Sci. Technol. 122 (2020). https://doi.org/10.1002/ejlt.201900332.

[29] D. Melucci, A. Bendini, F. Tesini, S. Barbieri, A. Zappi, S. Vichi, L. Conte, T. Gallina Toschi, Rapid direct analysis to discriminate geographic origin of extra virgin olive oils by flash gas chromatography electronic nose and chemometrics, Food Chem. 204 (2016) 263–273. https://doi.org/10.1016/j.foodchem.2016.02.131.

[30] European Commission Regulation 2016/2095 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis, Official Journal of the European Communities, 2016, September 26, L 326, 1–6.

[31] D. Cavanna, S. Zanardi, C. Dall'Asta, M. Suman, Ion mobility spectrometry coupled to gas chromatography: A rapid tool to assess eggs freshness, Food Chem. 271 (2019) 691–696. https://doi.org/10.1016/j.foodchem.2018.07.204.

[32] O.Y. Rodionova, A. V. Titova, A.L. Pomerantsev, Discriminant analysis is an inappropriate method of authentication, TrAC - Trends Anal. Chem. 78 (2016) 17–22. https://doi.org/10.1016/j.trac.2016.01.010.

[33] P. Oliveri, Class-modelling in food analytical chemistry: Development, sampling, optimisation and validation issues – A tutorial, Anal. Chim. Acta. 982 (2017) 9–19. https://doi.org/10.1016/j.aca.2017.05.013.

[34] D. Cavanna, L. Righetti, C. Elliott, M. Suman, The scientific challenges in moving from targeted to non-targeted mass spectrometric methods for food fraud analysis: A proposed validation workflow to bring about a harmonized approach, Trends Food Sci. Technol. 80 (2018) 223–241. https://doi.org/10.1016/j.tifs.2018.08.007.

[35] N. Gerhardt, M. Birkenmeier, D. Sanders, S. Rohn, P. Weller, Resolution-optimized headspace gas chromatography-ion mobility spectrometry (HS-GC-IMS) for non-targeted olive oil profiling, Anal. Bioanal. Chem. 409 (2017) 3933–3942. https://doi.org/10.1007/s00216-017-0338-2.

[36] F. Gutie, Ä. Rrez, J.L. Ferna, Ä. Ndez, Determinant Parameters and Components in the Storage of Virgin Olive Oil. Prediction of Storage Time beyond Which the Oil Is No Longer of "Extra" Quality, (2002). https://doi.org/10.1021/jf0102158.

[37] M.G. Di Serio, L. Giansante, G. Di Loreto, L. Di Giacinto, Shelf life of extra-virgin olive oils: First efforts toward a prediction model, J. Food Process. Preserv. 42 (2018) e13663. https://doi.org/10.1111/jfpp.13663.

[38] M. Bylesjö, M. Rantalainen, O. Cloarec, J.K. Nicholson, E. Holmes, J. Trygg, OPLS discriminant analysis: Combining the strengths of PLS-DA and SIMCA classification, J. Chemom. 20 (2006) 341–351. https://doi.org/10.1002/cem.1006.

[39] B. Gao, S.E. Holroyd, J.C. Moore, K. Laurvick, S.M. Gendel, Z. Xie, Opportunities and Challenges Using Non-targeted Methods for Food Fraud Detection, J. Agric. Food Chem. 67 (2019) 8425–8430. https://doi.org/10.1021/acs.jafc.9b03085.

Supplementary information for:

GC-IMS and FGC-Enose fingerprint as screening tools for revealing Extra Virgin Olive Oil blending with soft-refined olive oils: a feasibility study

Table S1. Samples set overview.

Category	**Harvest Year**	***n***
EVOO	2015/2016	18
EVOO	2016/2017	8
EVOO	2017/2018	17
Deodorized	/	3
Deacidified	/	2
Deodorized + Deacidified	/	2
Mix	/	21
Suspect frauded	/	2

Table S2: IMS instrumental parameters.

Drift tube length	**9.8 cm**
Drift gas flow rate	150 mL min^{-1}
Drift time	30 ms
Drift Voltage	5 kV
Drift tube temperature	45 °C
Ionization mode	Positive

Table S3*:* GC-IMS global area set integration parameters.

Spot	**RT min (min)**	**RT max (min)**	**DT min (ms)**	**DT max (ms)**
Peak1	1.89	2.38	7.80	9.58
Peak2	2.32	2.69	8.78	9.99
Peak3	2.43	3.37	7.96	8.97
Peak4	2.59	2.91	8.54	9.31
Peak5	3.19	3.70	10.22	11.18

Peak6	3.24	3.76	8.41	9.32
Peak7	3.50	3.88	9.05	9.82
Peak8	3.52	4.75	7.98	9.06
Peak9	4.18	4.58	9.02	10.16
Peak10	3.98	4.44	10.37	12.35
Peak11	3.98	4.24	9.43	10.12
Peak12	5.04	5.46	9.69	12.14
Peak13	4.75	5.07	10.06	10.72
Peak14	4.75	5.10	10.27	11.09
Peak15	4.98	5.76	8.38	9.67
Peak16	5.96	6.36	9.10	10.77
Peak17	5.37	6.10	7.82	9.18
Peak18	5.94	6.21	11.29	12.84
Peak19	3.86	4.44	9.68	11.10
Peak20	6.08	6.18	10.38	11.46
Peak21	6.05	6.21	11.02	11.63
Peak22	6.24	6.44	10.36	11.53
Peak23	6.15	6.50	8.50	9.47
Peak24	6.37	6.58	8.85	10.40
Peak25	6.33	6.52	11.34	12.30
Peak26	6.37	6.54	12.23	12.79
Peak27	6.99	7.29	9.68	10.85
Peak28	6.97	7.14	11.58	12.18
Peak29	6.98	7.18	12.07	12.42
Peak30	6.98	7.38	12.20	12.81
Peak31	7.57	7.79	9.80	11.02
Peak32	7.92	8.42	8.45	10.40
Peak33	8.00	8.60	10.86	13.50
Peak34	8.58	9.07	9.77	11.34
Peak35	12.58	14.00	10.13	11.39
Peak36	8.28	8.73	9.46	10.63
Peak37	2.45	3.06	10.54	11.13
Peak38	7.38	7.95	12.90	13.38
Peak39	12.86	13.66	14.11	14.78

Peak40	6.57	6.80	9.63	10.40
Peak41	6.75	6.97	8.98	9.77
Peak42	6.58	6.85	10.42	11.64
Peak43	6.59	6.87	11.17	12.46
Peak44	6.60	6.85	12.66	13.49
Peak45	10.84	11.76	9.17	10.74
Peak46	2.90	3.20	9.53	10.30
Peak47	2.53	2.81	9.33	10.77
Peak48	7.54	7.99	11.29	12.36
Peak49	8.50	8.78	13.59	15.35
Peak50	8.64	9.00	12.38	14.20
Peak51	7.11	7.49	9.38	9.98
Peak52	3.48	3.80	10.45	11.52

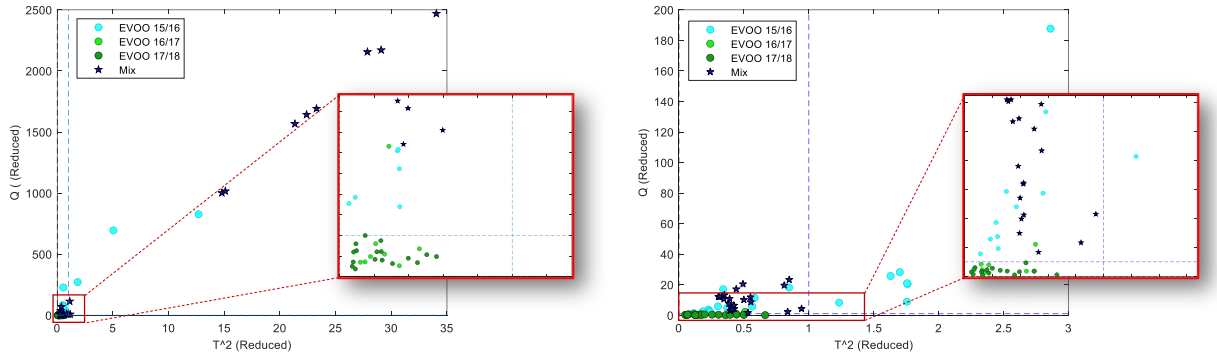

Figure S1. Projection of the samples onto the T2 red vs Q red model space of the target class (EVOO16/17 and EVOO17/18). GC-IMS and FGC E-nose data reported in the left and right figure, respectively. The dashed lines indicate the confidence limit corresponding to α=0.05. Magnifications of the region close to the decision boundaries are reported in the inset.

Chapter 3: Ambient Mass Spectrometry

Ambient mass spectrometry (AMS) is a relatively new field of analytical chemistry which has shown the potential to overcome these major issues related to conventional MS-based analytical methods [4]. In fact, AMS allows the analysis of samples under open-air conditions, enabling direct, rapid, real-time, and high-throughput analyses with little or no sample preparation.

The following study evaluated the fitness-for-purpose of two AMS techniques, i.e. Direct Analysis in Real Time (DART) and Atmospheric Solid Analysis Probe (ASAP) Mass Spectrometry (MS), for the high-throughput authenticity screening of commercial dried herb products through a fingerprinting approach. A thorough description of the employed AMS techniques is reported in **Annex III**. The manuscript has been accepted for publication in Talanta (https://www.journals.elsevier.com/talanta**)**.

Critical evaluation of ambient mass spectrometry coupled with chemometrics for the early detection of adulteration scenarios in *Origanum vulgare* L.

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Abstract: Nowadays, most of the screening methods in food manufacturing are based on spectroscopic techniques. Ambient Mass Spectrometry is a relatively new field of analytical chemistry which has proven to offer similar speed and ease-of-use when compared to other fingerprinting techniques, alongside the advantages of good selectivity, sensitivity and chemical information. Numerous applications have been explored in food authenticity, based either on the target detection of adulteration markers or, less frequently, on the development of multivariate classification models. The aim of the present work was to evaluate and compare the capabilities of Direct Analysis in Real Time (DART) and Atmospheric Solid Analysis Probe (ASAP) Mass Spectrometry (MS) for the high-throughput authenticity screening of commercial herbs and spices products. The gross addition of bulking material to dried Mediterranean oregano was taken as case study. First, a pilot sample set, constituted by authentic dried oregano, olive leaves (a frequently reported adulterant) and mixtures thereof at different levels (i.e. 10, 20, 30 and 50% w/w) was used. Each sample was fingerprinted by both ambient-MS techniques. After appropriate pre-processing, the whole mass spectra were used for the subsequent multivariate data analysis. Soft Independent Modelling of Class Analogy was adopted as classification algorithm and the model was challenged with both new authentic oregano and *in-house* prepared blends. To the best of our knowledge, this is the first report of DART-MS and ASAP-MS used in full scan mode and coupled to chemometric modelling as rapid fingerprinting approach for food authentication. Although both the techniques provided satisfactory results, ASAP-MS clearly showed greater potential, leading to reproducible, diagnostic feature-rich mass spectra. For this reason, ASAP-MS was further tested under a more convoluted scenario, where the training and validation sets were enlarged with additional authentic oregano samples and a wider range of adulterant species, respectively. Overall good results were achieved, with 93% model predictive accuracy, and screening detection capability estimated between $5-20\%$ (w/w) addition, depending on the adulterant considered with the only exception of majorana. Investigation of Q residuals could highlight the statistically-relevant chemical markers which could be tentatively annotated by coupling the ASAP probe with a high resolution mass analyser. The results from the validation study confirmed the great potential of ASAP-MS in combination with chemometrics as fast MS-based screening solution and demonstrated its feasibility for classification model building.

Keywords: DART, ASAP, Ambient Mass Spectrometry, Oregano, Authenticity, Chemometrics.

1. Introduction

Food adulteration is by no means a contemporary problem and it is probably as old as the food processing and production systems themselves [28]. Nowadays, the Grocery Manufacturers Association estimates that fraud may cost the global food industry $10-15 billion per year, affecting approximately 10% of all commercially sold food products [29]. Recent food adulteration/contamination scandals have helped refocus attention on this topic thanks to the gained media attention and consequent international impact. Nevertheless, vulnerabilities have been revealed in current surveillance systems, primarily designed to protect the consumer from non-compliant practises relating to biological and chemical food safety. In fact, fraudsters are expected to put all the efforts in concealing their illegal activities and, especially as regard the addition/substitution of food ingredients, conventional (targeted) analytical methods are not designed to screen for the undefinable number of potential adulterants which can, and have been used [14,30]. For these reasons, new testing strategies accounting for the disguising nature of food adulteration are highly claimed [30]. Moreover, the food industry and regulatory bodies are explicitly demanding high-throughput and user-friendly screening methods that can be readily implemented at/on-line, along the dynamic food supply chains [31].

In this context, the so-called "food fingerprinting" represents an emerging approach that has caught the attention of researchers and industrial users in the past decade [28,32]. In general terms, food fingerprinting aims at the non-targeted detection of as many features as technically possible, followed by intense chemometric analysis and comparison of the recorded fingerprint with an authentic sample set. The analytical workflow for the detection of potential food misdescription/adulterations by means of different non-targeted strategies has been thoroughly described in [33]. At the time of writing, most of the proposed fingerprinting methods are based on vibrational spectroscopic techniques as they offer non-destructive and cost-effective solutions to obtain quick spectral information about the tested material [15]; notwithstanding, ambient mass spectrometry (AMS) is a relatively new field of analytical chemistry which has shown the potential to overcome the major limitations related to traditional MS-based protocols [4]. In fact, AMS platforms are designed for "the ionization of unprocessed or minimally modified samples in their native environment", enabling rapid and cost-effective analysis of complex samples with little or no sample preparation [34]. Such characteristics meet the demands of the fast-paced global networks and, consequently, several studies have focused on the implementation of emerging AMS techniques in food quality and safety analysis [35].

As reviewed by Black et al., numerous applications have been explored in the food authenticity field, based either on the target detection of adulteration markers (e.g. melamine in milk and dairy products, Sudan dyes in spices, etc.) or, less frequently, on the development of multivariate classification models [4]. Yet, despite the demonstrated potential, AMS is still mostly used in specialized laboratories and/or for

research purposes, whereas translation into everyday life practice has so far not been realized. Indeed, AMS also presents some important technical hurdles, in terms of hardware design and data evaluation, that need to be overcome for a widescale adoption in routine analysis. For example, weak analyte desorption/ionization and insufficient mass transport usually lead to significantly lower sensitivity compared to conventional methods, thus constraining the analysis of trace compounds. In addition, non-controlled ion suppression effects and minor changes in ambient conditions could substantially alter the recorded spectral profiles; as a result, fluctuations of the absolute signal intensities are frequently observed under repeatability and, at a higher extent, reproducibility conditions [36]. This clearly hampers the construction of predictive models and the exploitation of non-targeted approaches.

In the present work, two AMS techniques, namely Direct Analysis in Real Time (DART) and Atmospheric Solid Analysis Probe (ASAP) Mass Spectrometry (MS), were evaluated to address a well-known fraud issue of the herbs and spices sector. DART is a plasma-based ionization technique firstly introduced by Cody et al. in 2005 and proposed as versatile ion source for the analysis of materials under open-air conditions [37]. In the same year, McEwen and co-workers developed the ASAP probe slightly modifying a commercial ESI/APCI ion source to allow rapid analysis of volatile and semi-volatile liquid or solid materials [38]. Although operating under not-identical working principles, DART and ASAP are both solvent-free techniques, relying upon APCI-like ionization fundamentals observed in flowing solvent-based systems. Therefore, in terms of molecular coverage, these platforms are broadly recognized as amenable to polar and non-polar, low molecular weight analytes [39]. The reader is directed to the excellent reviews published in literature for more details and technical explanations about these ambient ionization sources [39–41].

With regard to the considered adulteration scenario, herbs and spices represent attractive categories for potential offenders as they exhibit explicit fraud drivers such as high value per kilogram product, and gaps between physical product availability and market demand [42,43]. Historically, the main concern has been the use of unapproved colorants (i.e. dyes) to enhance the product's aesthetic [44]. Nevertheless, the bulk addition of extraneous material is another common practice [43,45]. In particular, the present study dealt with the undeclared blending of dried oregano with cheaper and/or worthless non-aromatic plants. Mediterranean oregano substitution with plant leaves having similar silvery grey colour (e.g. sumac, cistus, myrtle, olive leaves) has been repeatedly reported in literature, along with detection methods based on different principles (**see Section 3.4**) [46–50].

The aim of the present work was to assess and compare the feasibility of the employed AMS techniques for the detection of gross addition of bulking materials to dried herbs. Specifically, the previously described adulteration of Mediterranean oregano was taken as case study and a non-targeted fingerprinting approach was adopted. At first, DART-MS and ASAP-MS performance were evaluated and compared over a

relatively small sample set, constituted by authentic dried oregano, olive leaves (a frequently reported adulterant) and *in-house* prepared blends. All the samples were fingerprinted by both the techniques and the recorded spectra underwent to multivariate data analysis with the purpose of developing a classification model able to discern between genuine and tampered products. In the second stage of the work, the best-performing platform was further tested under a more convoluted scenario. The sample set was enlarged with more authentic oregano in order to add extra biological variability within the training set and allow a more faithful assessment of the model reliability. Furthermore, a wider range of adulterants was collected and new illicit admixtures were prepared. Following the same analytical protocol, the so-obtained blends were then used to challenge the model and determine whether the non-compliance could be detected regardless the adulterant type.

2. Materials and Methods

2.1. Experimental design and sample collection

As mentioned in the previous section, the present study was divided into two stages. The first part of the work aimed at evaluating the feasibility of the application, as well as comparing the performance of the two AMS platforms. To this end, a pilot sample set constituted by authentic dried oregano, olive leaves and their admixtures was used. Dried oregano samples (*Origanum vulgare subs. heracleoticum*, $n = 32$) harvested in the 2018 and 2019 seasons, were directly provided by Italian producers actively involved in the project (see **Acknowledgements** section). Being supplied as whole sprigs, the products' authenticity was ensured. Indeed, oregano leaves were manually detached from the branches, thereby excluding any intentional/unintentional addition of foreign plants. Afterwards, certified ISO sieves (Endecotts, London, UK) were used to standardize the particle size to that usually found in the retail market ($2mm < \emptyset < 1\ mm$). Fresh olive leaves (*Olea europaea*, $n = 20$) were hand-picked from plants belonging to different cultivars ($n = 9$) and dried under both controlled (oven, 40 °C, 24 hours, ≈ 5 m^3/h) and non-controlled conditions (sun drying). After drying, the samples were ground with a laboratory mill (MultiDrive basic, IKA, Stauffen, Germany) and sifted to the same particle size as for oregano in order to fully reproduce a potential counterfeiting action. At this point, illicit admixtures were created by blending the so-treated olive leaves with oregano samples randomly chosen from the sample set. Five mixes at four different levels (10, 20, 30 and 50% w/w) were prepared.

For the second stage of the study the sample set was enlarged with the purpose of reproducing circumstances closer to a real-world scenario, where many different adulterants can be used to effect the substitution. New authentic oregano were included in the sample set (final number, $n = 75$) and used for

both model training and testing. On the other side, a wider range of adulterants was sourced from commercial producers or collected from growing plants of known species, i.e. olive leaves (*Olea europaea*), cistus (*Cistus incanus* and *Cistus cyprius*), rhododendron (*Rhododendron indicum*), thyme (*Thymus serpyllum*), two marjoram (*Origanum majorana*), and used to prepare new blends. In this case, illicit admixtures for each adulterant were prepared at five different levels (5, 10, 20, 30 and 50% w/w). A schematic illustration of the two-stages experimental design is reported in **S. Figure 1**.

All the samples were stored at room temperature and in presence of light until analysis with the aim of reproducing normal storage conditions of commercial dried oregano. A detailed samples list is provided in the Supplementary material (**Table S1**).

2.2. Chemicals and reagents

All solvents (LC-MS grade, or higher) were purchased from Honeywell international Inc. (Morris Plains, NJ, USA). Formic acid was supplied by Sigma–Aldrich (St. Luis, MO, USA).

2.3. Sample preparation

Samples were extracted by adding 10 mL of CH_3OH + 0.1% of formic acid to 0.2 g of finely ground plant material directly into 15mL falcon tubes, mixed in an automatic shaker (VWR, Radnor, PA) for 5 minutes at 1500 rpm. After a mild centrifugation (4 min at 1000 rpm), the supernatant was collected and further diluted with the extraction solvent (1:3 and 1:5 dilution for the DART-MS and ASAP-MS analysis, respectively) in order to reduce ionization suppression phenomena, as well as potential carry-over into the ion sources.

Pooled quality control samples (QC) were prepared by mixing an equal volume of all the extracts and analysed across the entire sequence in order to reveal any issue related with instrumental drift.

2.4. Instrumental Analysis

Both DART (IonSense Inc., Saugus, MA) and ASAP (Waters, Wilmslow, UK) ion sources were coupled to the Waters QDa system (Waters, Wilmslow, UK), a single-quadrupole mass spectrometer detector.

DART-MS analysis were performed via the QuickStrip (QS) module (IonSense, Inc., Saugus, MA, **S. Figure 2**), a consumable 12-position sample card with stainless steel meshes used as loading surface. The liquid extracts were spotted (9 μL) onto the QS using manual micropipettes (Gilson Inc., Middleton, WI). Prior to the analysis, the solvent was allowed to dry for ≈ 60 s. Ten (technical) samples' replicates were analysed (one QS card was used for each sample). Blank extraction solvent was placed in the last slot of each QS in order to acquire a spectrum of the background for each sample. Analysis were performed using

the ion source in a horizontal alignment, with the QS carried through the ionization region by a motorized linear rail (IonSense) at the speed of 0.8 mm/s. The DART ion source was operated using the following conditions: positive ionization mode; gas temperature 350 °C; gas flow 2.5 L/min**;** cone voltage 10 V; source temperature 120 °C. For operation, helium was used as plasma gas, whereas nitrogen gas was employed in the standby mode. The data were recorded in full scan, continuum mode over the range 100-650 *m/z*, with a sampling frequency of 5 Hz. The quadrupole mass analyser was tuned at unit mass resolution. For operation, the DART source was controlled by the web-based software SVP-Source controller (IonSense), while the MassLynx v. 4.2 (Waters, Wilmslow, UK) software was used for data acquisition.

For ASAP-MS analysis, a prototype ASAP source incorporating a horizontal sampling geometry and "Z" configuration of the ion guides was employed to enhance de-clustering and neutrals clean-up, thus increasing ions transmission efficiency. Practically, the pre-existing QDa source design (normally equipped with an ESI source) was modified by the inclusion of a corona discharge pin and the removal of the spray capillary and nebulizer housing from the ESI inlet, as described by McCullough and co-workers [51]. Sealed glass capillaries (1.9 mm diameter) were used as sampling surface, spotting the liquid extracts (10 µL) onto the capillary tip and, as for the DART-MS, allowing the solvent to dry for ≈ 60 s. Prior to the sample loading, each capillary was introduced into the ASAP source and exposed to the hot N_2 stream for ≈ 60-80 s in order to remove potential contaminants from the capillary surface ("bake-out" step), thus significantly reducing the spectral background. Three (technical) replicates were analysed for each sample. Optimal setup for ASAP-MS analysis was as follows: positive ionization mode, gas flow 3.0 L/min; gas temperature 450°; corona voltage 3.0 kV (constant voltage); cone voltage 15 V source temperature 150 °C. All the analysis were carried out using N_2 as desorption gas, although ambient air could also be employed by the QDa [51]. ASAP-MS spectra were collected in continuum mode over the range 100-1000 *m/z*, with a sampling frequency of 2 Hz.

Experimental parameters used for DART- and ASAP-MS are summarized in **Table S2**.

2.5. Statistical Data Analysis

Data pre-processing and chemometrics analysis was performed in R (v. 4.0.2) using internal statistical functions and external packages (mdatools for multivariate data analysis and ggplot2 for plots creation).

The general tendency in fingerprinting approaches is to process the entire spectral profiles in the multivariate data analysis. Therefore, raw spectra were visually inspected by means of the MassLynx software and then exported as a data matrix having as many rows as the number of samples and as many columns as the number of *m/z* (binning resolution of 0.06 *m/z*). Once exported in the form of absolute signal intensities, raw spectra underwent to a data pre-treatment step in order to reduce the impact of systematic

and/or random sources of variability, thus highlighting the chemical information therein contained. In particular, spectral normalization according to the total ion current (TIC) and smoothing with the Savitzky–Golay method (2^{nd} order polynomial, 3 points window) were applied. Being a common practice in processing AMS data, the benefits of the background spectrum subtraction from the sample traces were evaluated on both DART-MS and ASAP-MS data. Mass spectra of the technical replicates were averaged prior to modelling and mean-centering was used for data scaling.

Principal component analysis (PCA) was performed as exploratory data analysis to assist the identification of evident outlying samples and/or potential data structures in a reduced-dimensions space. Thereafter, Soft Independent Modelling of Class Analogy (SIMCA) was used as classification algorithm for the detection of non-authentic samples. SIMCA is a non-probabilistic distance-based modelling method relying on the key assumption that the main systematic variability of the target class can be captured by a PCA model of opportune dimensionality. Proposed by Wold et al. in 1976, it was the first class-modelling method introduced in the literature and further details about available algorithms and theoretical principles can be found in [52–54]. As will be shown in **Section 3.3**, investigation of the residuals distributions computed for each tested object can be used to pinpoint the statistically-relevant features likely responsible for the sample classification. Further explanation about residuals is provided in **Supplementary Materials**.

3. Results and Discussions

3.1. Data exploration and experimental considerations

Being a first suitability evaluation of the employed AMS techniques as non-targeted fingerprinting approaches, it was decided to carry out the analysis on liquid extracts in order to minimize reproducibility issues derived from inconsistent sample's properties or irreproducible sample introduction. In fact, concerns regarding the sample representativeness normally arise when heterogeneous materials are being analysed in their native state [36]. Notwithstanding, the possibility of performing the measurement on solid sample with no prior preparation would definitely improve the method efficiency and throughput, as well as being more suited for a potential on/at-line implementation. For these reasons, further studies to assess the feasibility of such approach are ongoing. Different solvents, namely methanol, ethanol, acetonitrile and their mixtures with water (organic/water 75:25, v/v) were tested and compared for the extraction. Preliminary experiments revealed that the presence of solvent traces on the sampling surface greatly affected the final spectral profile. This is in accordance with the ionization mechanisms described in literature for the two techniques. Concerning DART, although the primary ionizing species are metastable

helium atoms (He^*), ionization of analyte molecules is mostly effected by secondary ions produced within a cascade of gas-phase reactions with nitrogen (via Penning ionization) and water [40]:

$$He^* + N_2 \rightarrow He + N_2^{+\bullet} + e^-$$
$$N_2^{+\bullet} + N_2 + N_{2\ (3rd\ body)} \rightarrow N_4^{+\bullet} + N_{2\ (3rd\ body)}$$
$$N_4^{+\bullet} + H_2O \rightarrow 2N_2 + H_2O^{+\bullet}$$
$$H_2O^{+\bullet} + H_2O \rightarrow H_3O^+ + OH^\bullet$$

Besides, ASAP follows a similar pathway, with the N_2 being ionized directly by the corona discharge [55]:

$$N_2 + e^- \rightarrow N_2^{+\bullet} + 2e^-$$
$$N_2^{+\bullet} + 2N_2 \rightarrow N_4^{+\bullet} + N_2$$
$$N_4^{+\bullet} + H_2O \rightarrow 2N_2 + H_2O^{+\bullet}$$
$$H_2O^{+\bullet} + H_2O \rightarrow H_3O^+ + OH^\bullet$$

In both cases, a dry atmosphere leads to the formation of molecular ions $M^{+\bullet}$ via charge transfer from radical nitrogen or metastable He^*. However, especially in the case of ASAP, residual moisture from either the source or the sample may support the formation of protonated ion species $[M+H]^+$. Since the alteration of spectral profiles might represent a serious problem for building predictive models, the complete solvent evaporation was waited prior to the analysis in order to avoid mixed-mode ionization events and to obtain more reproducible mass spectra. As a consequence of this, the use of aqueous extraction mixtures was ruled out due to their significantly longer time for drying. Among the organic solvents, the highest-quality and informative mass spectra, in terms of both signal intensities and number of diagnostic features, were provided by CH_3OH + 0.1% of formic acid. Comparable spectra were obtained by using CH_3CN as extraction solvent, however, methanol was preferred due its cost-effectiveness and availability.

Concerning the other experimental conditions and parameters reported in **Section 2.3**, gas flow and temperature proved to be among the most impactful settings, hence they were finely optimized. High temperature (> 350 °C) guaranteed the efficient analytes desorption and ionization over the whole mass range. On the other hand, overheated gas flows may cause degradation of thermally labile compounds and lead to an intensity signal drop for susceptible components [56].

Because of the absence of a prior chromatographic separation, the analysis of more concentrated extracts not necessarily led to overall higher spectral signals and the sample dilution factor turned out to be a critical parameter to be defined. In fact, most of the AMS techniques suffers from a reduced ionization and transmission efficiency – it is estimated that only $< 1\%$ of the generated ions is transferred into the mass spectrometer [57]. Strong charge-competition and suppression phenomena arise when complex matrices are being analysed, resulting in either major sample constituents or compounds more prone to

desorption/ionization dominating the mass spectrum, although sometimes representing statistically irrelevant features [36,58,59]. Moreover, the analysis of diluted extracts reduces the risk of carry-over and/or contamination, especially in the case of the ASAP (enclosed) source. Given the above, a further 1:3 and 1:5 dilution of the supernatant with the extraction solvent (reaching an overall dilution factor of 1:150 and 1:250) was found to be best compromise between the number of detected features and their signals intensity for DART-MS and ASAP-MS, respectively (**Section 2.3**).

TIC traces obtained from DART-MS and ASAP-MS analysis are reported in **Figure 1**. As can be seen, DART-MS analysis performed on the QS module results in twelve distinct peaks in the TIC trace, one for each loading position. Whereas ASAP-MS traces exhibit a single peak that starts with the sample introduction into the source and decreases as the analytes are gradually desorbed from the glass capillary surface. For DART-MS, each spectrum was taken by considering the whole peak width at 10% of the baseline. Concerning the ASAP-MS, upon examination of the consecutive mass spectra, it was found that the mass spectrum profiles are not constant over all scan points of the TIC peak, as both absolute intensities and ion ratios can vary over the run (**S. Figure 4**). Lower-mass features tend to decrease with the analysis time, while high *m/z* signals are much more intense in the last region of the peak. This is not surprising as the analytes characterized by a higher volatility are expected to be desorbed earlier and dominate the mass spectra in the first instance, whilst less volatile compounds tend to appear afterwards. For the sake of consistency and for reaching higher replicate-to-replicate reproducibility, sample spectra were taken averaging the scans at 70% of the TIC peak height (**Figure 1**). It is worth mentioning that higher gas flow rates were preferred as they provided sharper TIC peaks and higher run-by-run reproducibility.

DART-MS and ASAP-MS full-scan spectra obtained from the same oregano sample are displayed in **Figure 2**. Strong spectral differences between the two techniques appear. In the DART-MS spectrum, the majority of the relevant signals arise between 115 and 375 *m/z*. The acquisition was indeed limited to the 100-650 *m/z* range as no meaningful peaks were observed at higher *m/z*. In contrast, ASAP-MS produced richer spectra characterized by numerous features across the entire mass range, (spectra recorded over the 100-1000 *m/z*). Another substantial difference lies in the signal intensities. In fact, signals $>$ 10 times higher were provided by ASAP-MS compared to DART-MS. The reason can be attributed to the enclosed source of the ASAP-MS equipment, which likely led to a more efficient analyte desorption/ionization, while reducing the random fluctuations caused by the environment (e.g. possible sources of fluctuations affecting DART ionization could be air conditioners, rapidly moving personnel, housekeeping products etc.) [36,60]. An overlay of the previous traces with olive leaves' spectra are provided in **S. Figure 5**. Visible differences in the detected spectral features can be observed all over the spectra, even though some overlapping peaks were present.

Occurrence of contaminant's spectral features is expected when working with AMS techniques, especially in open-air configurations such as DART. For this reason, the background spectra were examined (**S. Figure 6**). A substantial background was found in DART-MS spectra, with interfering features arising over the entire *m/z* range with significantly high signals (even higher than those in the oregano spectrum). Further experiments revealed that interfering features most likely come from indoor air contaminants, rather than solvent residues (data not shown). In contrast, a "noisy" background was observed in the ASAP-MS, with the interfering signals being almost two orders of magnitude less intense than the sample features. This represented a main concern for the DART-MS data processing as several interfering ions (e.g.149.1, 135.9, 331.0 *m/z*, etc.) were still detectable in the sample spectra. Although the best solution would be the selective removal (or reduction) of such unwanted features before the MS detection, their presence can hardly be avoided [61]. Therefore, to overcome this problem, a common practice consists of subtracting the background from the sample's spectra, albeit it must be assumed constant over the analysis time [60]. As a qualitative assessment of the impact that this can have on the data reproducibility, PCA was carried out considering 10 technical replicates of 10 oregano samples, with and without applying the background-subtraction prior to the mathematical pre-processing (TIC normalization + Savitzky–Golay smoothing). The resulting scores plots are reported in **S. Figure 7**. While data points resulted to be more spread by applying mathematical pre-processing only, the further background-subtraction led to a tighter grouping of the samples' replicates. For this reason, the latter was included in the data pre-processing workflow prior to modelling. On the other side, background-subtraction did not produce any evident benefit in the case of ASAP-MS (data not shown) and, therefore, was not applied. In this regard, it must be considered that the interfering signals are expected to be substantially decreased by the presence of a complex matrix as the bulk will compete for the ionization. In fact, none of the background features was observed in the sample spectra.

3.2. DART-MS and ASAP-MS evaluation and comparison

PCA was carried out on the pre-processed spectra in order to explore the data structure in a reduced dimension space. The first three principal components (PCs) accounted for more than 95% of the total variance in both datasets. PC1 vs PC2 scores plot (**Figure 3**) revealed a clear separation between pure oregano and olive leaves samples, as expected from the large differences in the raw spectra. The tight clustering of the pooled QCs in the plot center denotes a good stability of both systems along the analysis sequence. Furthermore, two clusters among the oregano samples can be clearly recognized in the ASAP-MS data. Marking the objects according to the different oregano producers, such separation was found to be related to the suppliers of origin (**S. Figure 8**). Further investigations were carried out by means of

liquid-chromatography coupled to high-resolution mass spectrometry and the results will be presented elsewhere (manuscript in preparation).

The present work addresses a typical authentication question, where the goal is to establish whether the tested sample is compliant or not with the reference class (i.e. authentic oregano). As pointed out by several authors [53,62] and the recently published U.S. Pharmacopeia "Guidance on Developing and Validating Non-Targeted Methods for Adulteration Detection" [63], one-class classifier approaches should be used as classification algorithm in these circumstances. Therefore, both the datasets underwent to SIMCA modelling to assess whether a multivariate classification model able to discern between authentic and counterfeited products can be developed. As explained in **Section 2.1**, five mixtures at four adulteration levels were prepared using five randomly chosen oregano samples. The SIMCA model was calibrated over the remaining oregano samples ($n = 27$). The illicit admixtures ($n = 20$), along with the oregano samples used for their preparation ($n = 5$), constituted the (independent) test set for the model performance evaluation. Optimal model dimensionality was defined upon cross-validation procedure (leave-one-out). Six and three PCs were considered sufficient for proper modelling of the DART-MS and ASAP-MS dataset, respectively (**Table 1**), as they provided the minimum RMSECV (Root Mean Squared Error Cross Validation) and accounted for more than 95% of the original data variance. The confidence level was set to $\alpha = 0.05$ and the classification rule was based on the combined T^2-Q [53]. SIMCA modelling of DART-MS data produced some misclassification. In particular, three out of five oregano samples were wrongly rejected by the model, while four mixtures (i.e. three 10% and one 20%) were incorrectly recognized as authentic products. In contrast, a full classification (with less retained PCs) was reached on ASAP-MS data and the smaller number of retained PCs ensure that such perfect outcomes is not due to an overfitted model. Modelling results are summarized in **Table 1** in terms of sensitivity and specificity. The first is defined as the fraction of reference samples correctly assigned to the target class (true positive assignation). Conversely, specificity refers to the proportion of alien objects correctly rejected by the model (true negative assignation) [53].

DART-MS provided results that can be considered satisfying for a rapid screening platform. However, despite being a pilot study with a limited number of samples available, a greater potential of ASAP-MS was clearly evidenced. The reasons can be attributed to the fundamental differences between the two ionization sources. Being less susceptible to non-controlled environment conditions, the enclosed ASAP source enabled the acquisition of higher quality and reliable MS data, a mandatory condition for building predictive classification models.

3.3. Model Validation

According to the results shown in the previous section, ASAP-MS proved to be the best performing technique within the present study. For this reason, it was decided to carry out the second stage of the work on this platform, aiming at a sound capabilities assessment of the proposed method in a real-world scenario. To this purpose, the sample training set was enlarged with new authentic objects (i.e. oregano); furthermore, a wider range of different adulterants ($n = 8$) was used (**Section 2.1**). In this case, illicit admixtures were prepared at five different levels (5, 10, 20, 30 and 50% w/w); therefore, a total 40 blends were used in the model validation stage. Moreover, thanks to the larger number of samples available, the calibration set was split and more oregano samples ($n = 15$) were included in the validation set to assess the model sensitivity (ability to correctly recognize authentic objects as belonging to the target class). It is worth stressing that the present work represents a proof-of-concept study with the goal of developing a classification model able to effectively recognize genuine from counterfeited products, regardless the used adulterant, can be built. The plant materials herein used to reproduce the fraud action were chosen according to both previous literature reports and their availability. Thus, they do not necessarily represent adulterants actually used at the present time.

The same modelling approach described in the previous section was followed. Five PCs were retained as optimal model dimensionality (minimum RMSECV), which accounted for 78.5% of the training data variance (confidence level was set to $\alpha = 0.05$ and the classification was based on the T^2-Q augmented distances). Overall, the lowest detectable level was found to be between 5 and 20% for all mixes, except for the marjoram mixes. Specifically, *Cistus cyprus*, thyme and one olive leaves mixtures were identified at 20% levels, while the other olive leaves blend was detected at 10% level (as in the first stage of the work, **Section 3.2**). Furthermore, *Cistus incanus* and rhododendron admixtures were recognized as non-compliant even at the lowest percentage (5% w/w). In the authors' opinion, such results can be considered commercially-useful as herbs adulteration is expected to occur at gross levels (over 70% w/w substitution was reported by Wielogorska and co-workers [48], otherwise it would hardly be convenient for fraudsters from a risk/benefit standpoint. On the other hand, both the marjoram admixtures were assigned as non-authentic only at the highest adulteration level (50%). Marjoram belongs to the same botanical family (Lamiaceae) and genus (*Origanum*) of the Mediterranean oregano; it was purposely included in the study in order to investigate the performance of a potential screening method when dealing with chemotaxonomically similar species. It is indeed known that species of the same family added for dilution purposes are more difficult to detect, as phytochemical traits may be extremely similar. Even the use of unequivocal chemical markers (or their ratios) may be hampered by natural variability [64,65].

To perform the assignation of a new tested sample, SIMCA compute the class-belonging probability (π) according to its T^2-Q deviation from a critical limit defined according to the T^2-Q theoretical distributions

(estimated from the calibration data). Basically, the closer the object's T^2-Q to the threshold value, the higher its π [66]. A π values below the pre-defined threshold determines the object rejection by the model. Class-belonging probabilities of the illicit admixes are reported in **Figure 4**. As can be seen, higher-level adulteration admixtures resulted in lower π, as they lie farther from the class acceptance area. Since the confidence level was set to $\alpha = 0.05$, the probability threshold for acceptance/rejection is $\pi = 0.5$ [67]. Concerning the authentic oregano, 14 out of 15 samples were correctly accepted by the model (sensitivity = 93.4%), thus denoting a method's low risk of false-negative detection.

Since the present work evaluated DART and ASAP coupled to a nominal mass analyser as non-targeted fingerprinting methods, the whole mass spectra were used as sample profiles for the subsequent multivariate data analysis. Although markers identification was not the main goal of the study, it is possible to determine the statistically-relevant features upon investigation of the residuals distributions that SIMCA computes for each tested sample (see **Supplementary Materials**). For instance, residual profiles of thyme blends (from 10 to 50%) over the 340-385 *m/z* range have been reported in **Figure 5**. As can be seen, for some features, the corresponding residuals increase along with the adulteration percentage, while remaining close to zero in the case of oregano. Since increasingly differing from the model representation of the training data (i.e. authentic oregano), these variables are definitely related to the adulterant, thus responsible for the sample rejection. In case one is interested in annotating such chemical markers, DART or ASAP ion sources can be coupled with a high resolution mass analyser – the compound's elemental formula and, where possible, potential structure from fragments analysis can be inferred. In this context, the possibility to fit both DART and ASAP sources to higher-resolution mass spectrometers represents an appealing solution, not only for marker identification purposes.

With respect to the present case, coupling ambient ionization sources with such equipment would certainly lead to a larger number of detected features, rising the amount of information available for modelling. Nevertheless, this solution would likely present also higher purchase- and running-costs; being often the main drivers in industry frameworks, such drawbacks represent important aspects to be carefully evaluated.

As reviewed by Riedl et al., validation of non-targeted fingerprinting approaches represents a thorny challenge to be faced and the results' reliability/generalisability heavily relies on the representativeness of the sample set (both training and validation set) with respect to the true picture [68]. The SIMCA classification scores shown above have been achieved on a rather heterogeneous sample set (reference oregano samples were gathered from different producers, provenance and harvesting years). For this reason, the authors believe that such results can be considered reasonably representative of the potential performance of a routine screening based on ASAP-MS fingerprinting. Moreover, the methods' capabilities could be further enhanced once implemented at an industrial-level, where the application "boundaries" can

be clearly defined and the target class sampled in a fully representative way (e.g. internal quality control). In this regard, as a follow-up of this feasibility study, an on-site testing of the ASAP-MS equipment is being carried out within an industrial plant (see **Acknowledgements** section). The aim is to assess the future potential for non-lab operation and point-of-control testing, as well as to evaluate the possibility of data/model transfer between multiple devices.

3.5. Comparison of ASAP-MS with today's available approaches

A few authors already dealt with the bulk addition of extraneous plant material to dried Mediterranean oregano and proposed various detection methods based on different technologies. Thus, it is worth wondering how the ASAP-MS can be positioned within the current scenario of available analytical approaches.

As mentioned in **Section 1**, vibrational spectroscopy, so far, represents the "golden standard" for fingerprinting analysis, particularly praised for its quickness, easy-of-use, and low running-costs. Being the same qualities somehow offered by the AMS techniques herein proposed, a performance comparison between these analytical platforms would certainly be very interesting. Previous scholarly reports about the use of infrared spectroscopy for the authentication of Mediterranean oregano are available in literature. Rodionova & Pomerantsev recently employed Near-Infrared (NIR) spectroscopy in conjunction with chemometrics to discern genuine oregano from several other foreign plant materials (e.g. hazelnut, olive leaves, nettle, celandine) [69]. Although such study was more centred on the popular misuse of discriminant analysis (DA) for authentication scenario, rather than the NIR's analytical capabilities, the authors found harder to recognize adulterants botanically closer to oregano, such as thyme and marjoram (in accordance with **Section 3.3**). If on the one hand such work strongly supports our results, on the other hand, a straightforward comparison between NIR and ASAP-MS cannot be made. In fact, unlike the present work, no *in-house* prepared admixtures were analysed; therefore, an approximate limit of detection (LOD) could not be estimated. The same goes for Black et al., who applied Fourier-transform Infrared (FT-IR) but did not test the developed method on counterfeited mixtures [47]. In addition, the authors only employed DA techniques to discriminate pure oregano from the adulterants. As well explained by Rodionova and co-workers, this approach implies a prior knowledge of the adulterant to be sought – a requirement that is hardly fulfilled in real-world authentication scenarios, where no information is normally available about the sample being tested [62].

In food authentication, DNA-based techniques are largely used for species/variety discrimination since DNA sequences can be used as suitable molecular markers [2]. Marieschi and co-workers identified both Randomly Amplified Polymorphic DNA (RAPD) and Sequence Characterized Amplified Region (SCAR) suitable for a reliable detection of numerous adulterants (e.g. *Cistus incanus, Rhus coriaria, Olea europae,*

etc.) in Mediterranean oregano, down to 1% presence [64,70,71]. Yet, SCAR-PCR analysis requires tailored primers to be previously identified for each adulterant. Therefore, such approach suffers from the same limitation mentioned for targeted methods: the fraud is likely to remain undetected if a different plant material is used. More suited is the RAPD-PCR method [70], since no prior information about the sequence to be amplified is needed. Nonetheless, as pointed out by the same authors in the following study [64], poor reliability and replicability are the major drawbacks of RAPD primers. In addition, it must be considered that DNA-based techniques rely on extensive sample preparations (e.g. overnight DNA extraction) and analytical protocols that have to be carried out by highly-prepared personnel; thus, they are not best suited for primary routine screening of a large number of batches. On the other hand, genomic techniques allow the detection of extremely low amounts of adulterant and can guarantee unequivocal results without the tedious establishment of reference libraries within the method development stage (DNA sequences are almost constant against exogenous factors [72]). Such superior sensitivity and reliability make them extremely valuable platforms for confirmatory (targeted) analysis, also suitable for forensic investigations.

In comparison, the ASAP-MS equipment is amenable for basic-laboratory environments and to be handled by non-specialized users upon minimum training, thus it can meet the demands from industrial contexts. The authentication method herein proposed involves a quick and straightforward sample preparation, along with very short analysis time (< 3 minutes), enabling the immediate identification of suspect samples. Within the investigated scenario, the screening detection capability was estimated to be ranging between 5 and 20% (w/w) addition, depending on the used adulterant. In fact, more difficulties were encountered in detecting the addition of chemotaxonomically similar species. AMS may definitely constitute an appealing alternative for rapid food testing since extra information about the detected non-compliance (i.e. *m/z*, fragments analysis, etc.) can be gathered. At the same time, compared to well-established analytical platforms such as NIR or FT-IR, the use of AMS is still in its infancy. As said in **Section 3.3**, further research is ongoing to verify the robustness and long-term stability of multivariate classification models built on AMS data, a crucial aspect for a relevant implementation in routine analysis [68].

4. Conclusions

In the last 15 years, innovations in the field of AMS have pushed the technology far beyond its point of conception, and numerous applications have been explored in food authenticity, showing great potential in some circumstances. The present work evaluated DART-MS and ASAP-MS as fast fingerprinting methods for the detection of gross addition of bulking agents to herbs and spices. To this end, the adulteration of Mediterranean oregano with cheaper and/or worthless non-aromatic plants was taken as case study. To the

best of our knowledge, this is the first report of DART-MS and ASAP-MS used in full scan mode and coupled to chemometric modelling as rapid food fingerprinting approaches.

The first preliminary evaluation of DART-MS and ASAP-MS performance clearly highlighted a major potential of ASAP-MS. Therefore, the latter was further tested to assess whether the non-compliance could be detected regardless the adulterant type. The screening detection capability was estimated to be ranging between 5 and 20% (w/w) addition, depending on the adulterant considered. However, a lower detection capability (50%) was found when majorana is used as adulterant. Taking into consideration a possible analytical blindness in detecting the addition of chemotaxonomically similar species must be taken into account during method development for herbs adulteration.

Despite the compounds playing a key role in the sample classifications could not be elucidated in the present study due to the low mass resolution of the employed equipment, **Section 3.3** described how to pinpoint the statistically-relevant features upon investigation of the Q residuals. Coupling the two AMS sources with a high-resolution mass analyser would allow the tentative identification of the chemical markers.

It is the author's opinion that the obtained results showed the ASAP coupled to a single quadrupole mass analyser to be *fit-for-purpose* as a rapid screening for gross fraud detection and demonstrated its feasibility for building multivariate classification models. The achieved detection capabilities (5 – 20% for all adulterant herbs, with the only exception of majorana) can be considered a reliable performance estimation of a potential screening method based on ASAP-MS fingerprinting. Besides, the approach herein proposed can be sensibly extended to other food commodities subjected to gross addition of bulking material, such as spices.

CRediT authorship contribution statement

Tito Damiani: Conceptualization, Methodology, Investigation, Software, Formal Analysis, Visualization, Validation, Writing - Original Draft, Writing - Review & Editing. **Nicola Dreolin:** Methodology, Investigation, Validation, Writing - Review & Editing. **Sara Stead:** Supervision, Resources, Writing - Review & Editing. **Chiara Dall'Asta:** Supervision, Project administration, Resources, 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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References

[1] D.I. Ellis, V.L. Brewster, W.B. Dunn, J.W. Allwood, A.P. Golovanov, R. Goodacre, Fingerprinting food: Current technologies for the detection of food adulteration and contamination, Chem. Soc. Rev. 41 (2012) 5706–5727. https://doi.org/10.1039/c2cs35138b.

[2] L. Manning, Food fraud: policy and food chain, Curr. Opin. Food Sci. 10 (2016) 16–21. https://doi.org/10.1016/j.cofs.2016.07.001.

[3] J.C. Moore, J. Spink, M. Lipp, Development and Application of a Database of Food Ingredient Fraud and Economically Motivated Adulteration from 1980 to 2010, J. Food Sci. 77 (2012) R118–R126. https://doi.org/10.1111/j.1750-3841.2012.02657.x.

[4] S.M. van Ruth, W. Huisman, P.A. Luning, Food fraud vulnerability and its key factors, Trends Food Sci. Technol. 67 (2017) 70–75. https://doi.org/10.1016/j.tifs.2017.06.017.

[5] D.I. Ellis, H. Muhamadali, S.A. Haughey, C.T. Elliott, R. Goodacre, Point-and-shoot: Rapid quantitative detection methods for on-site food fraud analysis-moving out of the laboratory and into the food supply chain, Anal. Methods. 7 (2015) 9401–9414. https://doi.org/10.1039/c5ay02048d.

[6] S. Medina, J.A. Pereira, P. Silva, R. Perestrelo, J.S. Câmara, Food fingerprints – A valuable tool to monitor food authenticity and safety, Food Chem. 278 (2019) 144–162. https://doi.org/10.1016/j.foodchem.2018.11.046.

[7] S. Esslinger, J. Riedl, C. Fauhl-Hassek, Potential and limitations of non-targeted fingerprinting for authentication of food in official control, Food Res. Int. 60 (2014) 189–204. https://doi.org/10.1016/j.foodres.2013.10.015.

[8] T.F. McGrath, S.A. Haughey, J. Patterson, C. Fauhl-Hassek, J. Donarski, M. Alewijn, S. van Ruth, C.T. Elliott, What are the scientific challenges in moving from targeted to non-targeted methods for food

fraud testing and how can they be addressed? – Spectroscopy case study, Trends Food Sci. Technol. 76 (2018) 38–55. https://doi.org/10.1016/j.tifs.2018.04.001.

[9] C. Black, O.P. Chevallier, C.T. Elliott, The current and potential applications of Ambient Mass Spectrometry in detecting food fraud, TrAC - Trends Anal. Chem. 82 (2016) 268–278. https://doi.org/10.1016/j.trac.2016.06.005.

[10] C.L. Feider, A. Krieger, R.J. Dehoog, L.S. Eberlin, Ambient Ionization Mass Spectrometry: Recent Developments and Applications, (2019). https://doi.org/10.1021/acs.analchem.9b00807.

[11] H. Lu, H. Zhang, K. Chingin, J. Xiong, X. Fang, H. Chen, Ambient mass spectrometry for food science and industry, TrAC - Trends Anal. Chem. 107 (2018) 99–115. https://doi.org/10.1016/j.trac.2018.07.017.

[12] T.H. Kuo, E.P. Dutkiewicz, J. Pei, C.C. Hsu, Ambient Ionization Mass Spectrometry Today and Tomorrow: Embracing Challenges and Opportunities, Anal. Chem. 92 (2020) 2353–2363. https://doi.org/10.1021/acs.analchem.9b05454.

[13] R.B. Cody, J.A. Laramée, H.D. Durst, Versatile new ion source for the analysis of materials in open air under ambient conditions, Anal. Chem. 77 (2005) 2297–2302. https://doi.org/10.1021/ac050162j.

[14] C.N. McEwen, R.G. McKay, B.S. Larsen, Analysis of solids, liquids, and biological tissues using solids probe introduction at atmospheric pressure on commercial LC/MS instruments, Anal. Chem. 77 (2005) 7826–7831. https://doi.org/10.1021/ac051470k.

[15] D.J. Weston, Ambient ionization mass spectrometry: Current understanding of mechanistic theory; Analytical performance and application areas, Analyst. 135 (2010) 661–668. https://doi.org/10.1039/b925579f.

[16] J.H. Gross, Direct analysis in real time-a critical review on DART-MS, Anal. Bioanal. Chem. 406 (2014) 63–80. https://doi.org/10.1007/s00216-013-7316-0.

[17] R.J. Fussell, D. Chan, M. Sharman, An assessment of atmospheric-pressure solids-analysis probes for the detection of chemicals in food, TrAC - Trends Anal. Chem. 29 (2010) 1326–1335. https://doi.org/10.1016/j.trac.2010.08.004.

[18] I.C.J. Silvis, S.M. van Ruth, H.J. van der Fels-Klerx, P.A. Luning, Assessment of food fraud vulnerability in the spices chain: An explorative study, Food Control. 81 (2017) 80–87. https://doi.org/10.1016/j.foodcont.2017.05.019.

[19] P. Galvin-King, S.A. Haughey, C.T. Elliott, Herb and spice fraud; the drivers, challenges and detection, Food Control. 88 (2018) 85–97. https://doi.org/10.1016/j.foodcont.2017.12.031.

[20] S.A. Haughey, P. Galvin-King, Y.C. Ho, S.E.J. Bell, C.T. Elliott, The feasibility of using near infrared and Raman spectroscopic techniques to detect fraudulent adulteration of chili powders with Sudan dye, Food Control. 48 (2015) 75–83. https://doi.org/10.1016/j.foodcont.2014.03.047.

[21] A.S. Wilde, S.A. Haughey, P. Galvin-King, C.T. Elliott, The feasibility of applying NIR and FT-IR fingerprinting to detect adulteration in black pepper, Food Control. 100 (2019) 1–7. https://doi.org/10.1016/j.foodcont.2018.12.039.

[22] L. Drabova, G. Alvarez-Rivera, M. Suchanova, D. Schusterova, J. Pulkrabova, M. Tomaniova, V. Kocourek, O. Chevallier, C. Elliott, J. Hajslova, Food fraud in oregano: Pesticide residues as adulteration markers, Food Chem. 276 (2019) 726–734. https://doi.org/10.1016/j.foodchem.2018.09.143.

[23] C. Black, S.A. Haughey, O.P. Chevallier, P. Galvin-King, C.T. Elliott, A comprehensive strategy to detect the fraudulent adulteration of herbs: The oregano approach, Food Chem. 210 (2016) 551–557. https://doi.org/10.1016/j.foodchem.2016.05.004.

[24] E. Wielogorska, O. Chevallier, C. Black, P. Galvin-King, M. Delêtre, C.T. Kelleher, S.A. Haughey, C.T. Elliott, Development of a comprehensive analytical platform for the detection and quantitation of food fraud using a biomarker approach. The oregano adulteration case study, Food Chem. 239 (2018) 32–39. https://doi.org/10.1016/j.foodchem.2017.06.083.

[25] M. Marieschi, A. Torelli, F. Poli, A. Bianchi, R. Bruni, Quality control of commercial Mediterranean oregano: Development of SCAR markers for the detection of the adulterants Cistus incanus L., Rubus caesius L. and Rhus coriaria L., Food Control. 21 (2010) 998–1003. https://doi.org/10.1016/j.foodcont.2009.12.018.

[26] M. Marieschi, A. Torelli, F. Poli, G. Sacchetti, R. Bruni, RAPD-Based Method for the Quality Control of Mediterranean Oregano and Its Contribution to Pharmacognostic Techniques, J. Agric. Food Chem. 57 (2009) 1835–1840. https://doi.org/10.1021/jf8032649.

[27] B.J. McCullough, K. Patel, R. Francis, P. Cain, D. Douce, K. Whyatt, S. Bajic, N. Lumley, C. Hopley, Atmospheric Solids Analysis Probe Coupled to a Portable Mass Spectrometer for Rapid Identification of Bulk Drug Seizures, J. Am. Soc. Mass Spectrom. 31 (2020) 386–393. https://doi.org/10.1021/jasms.9b00020.

[28] S. Wold, Pattern recognition by means of disjoint principal components models, Pattern Recognit. 8 (1976) 127–139. https://doi.org/10.1016/0031-3203(76)90014-5.

[29] P. Oliveri, Class-modelling in food analytical chemistry: Development, sampling, optimisation and validation issues – A tutorial, Anal. Chim. Acta. 982 (2017) 9–19. https://doi.org/10.1016/j.aca.2017.05.013.

[30] M. Bevilacqua, R. Bucci, A.D. Magrì, A.L. Magrì, R. Nescatelli, F. Marini, Classification and Class-Modelling, in: Data Handl. Sci. Technol., Elsevier Ltd, 2013: pp. 171–233. https://doi.org/10.1016/B978-0-444-59528-7.00005-3.

[31] A.D. Ray, J. Hammond, H. Major, Molecular Ions and Protonated Molecules Observed in the Atmospheric Solids Analysis Probe Analysis of Steroids, Eur. J. Mass Spectrom. 16 (2010) 169–174. https://doi.org/10.1255/ejms.1069.

[32] J. Hajslova, T. Cajka, L. Vaclavik, Challenging applications offered by direct analysis in real time (DART) in food-quality and safety analysis, TrAC - Trends Anal. Chem. 30 (2011) 204–218. https://doi.org/10.1016/j.trac.2010.11.001.

[33] L. Meier, C. Berchtold, S. Schmid, R. Zenobi, Extractive electrospray ionization mass spectrometry. Enhanced sensitivity using an ion funnel, Anal. Chem. 84 (2012) 2076–2080. https://doi.org/10.1021/ac203022x.

[34] Z. Li, Y.W. Zhang, Y.D. Zhang, Y. Bai, H.W. Liu, Rapid analysis of four Sudan dyes using direct analysis in real time-mass spectrometry, Anal. Methods. 7 (2015) 86–90. https://doi.org/10.1039/c4ay02409e.

[35] L. Vaclavik, J. Rosmus, B. Popping, J. Hajslova, Rapid determination of melamine and cyanuric acid in milk powder using direct analysis in real time-time-of-flight mass spectrometry, J. Chromatogr. A. 1217 (2010) 4204–4211. https://doi.org/10.1016/j.chroma.2010.03.014.

[36] S.R. Kumbhani, L.M. Wingen, V. Perraud, B.J. Finlayson-Pitts, A cautionary note on the effects of laboratory air contaminants on ambient ionization mass spectrometry measurements, Rapid Commun. Mass Spectrom. 31 (2017) 1659–1668. https://doi.org/10.1002/rcm.7951.

[37] X. Guo, A.P. Bruins, T.R. Covey, Characterization of typical chemical background interferences in atmospheric pressure ionization liquid chromatography-mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 3145–3150. https://doi.org/10.1002/rcm.2715.

[38] O.Y. Rodionova, A. V. Titova, A.L. Pomerantsev, Discriminant analysis is an inappropriate method of authentication, TrAC - Trends Anal. Chem. 78 (2016) 17–22. https://doi.org/10.1016/j.trac.2016.01.010.

[39] Appendix XVIII: USP Guidance on Developing and Validating Non-Targeted Methods for Adulteration Detection, (2018). http://members.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/Food_Auth/2019USPC-Appendix XVIII_Guidance_on_Developing_and_Validating_Non-Targeted_Methods_for_Adulteration_Detection-FCC_Forum_December_2018.pdf. (accessed September 17, 2020).

[40] M. Marieschi, A. Torelli, A. Bianchi, R. Bruni, Detecting Satureja montana L. and Origanum majorana L. by means of SCAR-PCR in commercial samples of Mediterranean oregano, Food Control. 22 (2011) 542–548. https://doi.org/10.1016/j.foodcont.2010.10.001.

[41] A.C. Figueiredo, J.G. Barroso, L.G. Pedro, J.J.C. Scheffer, Factors affecting secondary metabolite production in plants: Volatile components and essential oils, Flavour Fragr. J. 23 (2008) 213–226. https://doi.org/10.1002/ffj.1875.

[42] A.L. Pomerantsev, O.Y. Rodionova, Concept and role of extreme objects in PCA/SIMCA, J. Chemom. 28 (2014) 429–438. https://doi.org/10.1002/cem.2506.

[43] S. Kucheryavskiy, mdatools – R package for chemometrics, Chemom. Intell. Lab. Syst. 198 (2020). https://doi.org/10.1016/j.chemolab.2020.103937.

[44] J. Riedl, S. Esslinger, C. Fauhl-Hassek, Review of validation and reporting of non-targeted fingerprinting approaches for food authentication, Anal. Chim. Acta. 885 (2015) 17–32. https://doi.org/10.1016/j.aca.2015.06.003.

[45] O.Y. Rodionova, A.L. Pomerantsev, Chemometric tools for food fraud detection: The role of target class in non-targeted analysis, Food Chem. 317 (2020) 126448. https://doi.org/10.1016/j.foodchem.2020.126448.

[46] S. Primrose, M. Woolfe, S. Rollinson, Food forensics: Methods for determining the authenticity of foodstuffs, Trends Food Sci. Technol. 21 (2010) 582–590. https://doi.org/10.1016/j.tifs.2010.09.006.

[47] M. Marieschi, A. Torelli, F. Poli, G. Sacchetti, R. Bruni, RAPD-Based method for the quality control of mediterranean oregano and its contribution to pharmacognostic techniques, J. Agric. Food Chem. 57 (2009) 1835–1840. https://doi.org/10.1021/jf8032649.

[48] M. Marieschi, A. Torelli, A. Bianchi, R. Bruni, Development of a SCAR marker for the identification of Olea europaea L.: A newly detected adulterant in commercial Mediterranean oregano, Food Chem. 126 (2011) 705–709. https://doi.org/10.1016/j.foodchem.2010.11.030.

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

Figures

Figure 1. Typical TIC traces obtained from DART-MS (top) and ASAP-MS (bottom) analysis. DART-MS analysis performed on the QS module results in twelve distinct peaks (one for each sample position). Ten sample replicates were loaded on the corresponding position, while the first slot was left empty and blank extraction solvent was spotted onto the last grid. ASAP-MS TIC trace exhibits a single broad peak starting with the capillary introduction into the ion source and gradually decreasing over the time. Red arrows show how the average spectra were taken in both cases. Maximum recorded TIC intensity is reported in the top-right corner of each trace.

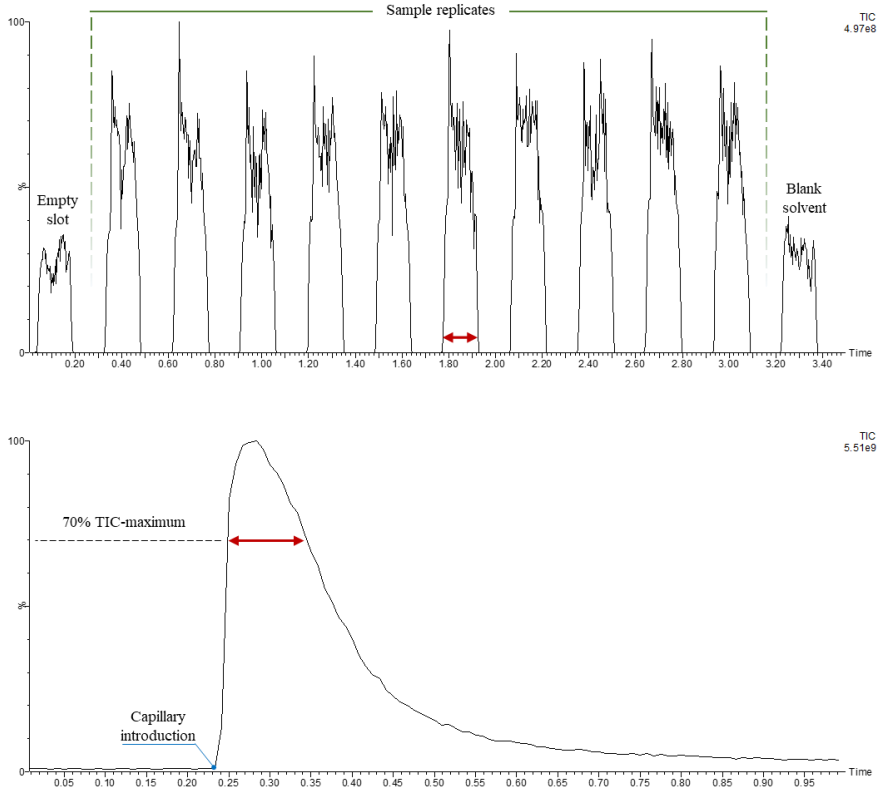


Figure 2. DART-MS (top) and ASAP-MS (bottom) raw spectra recorded from the same oregano sample. Base peak intensity is reported in the top-right corner of each spectrum.

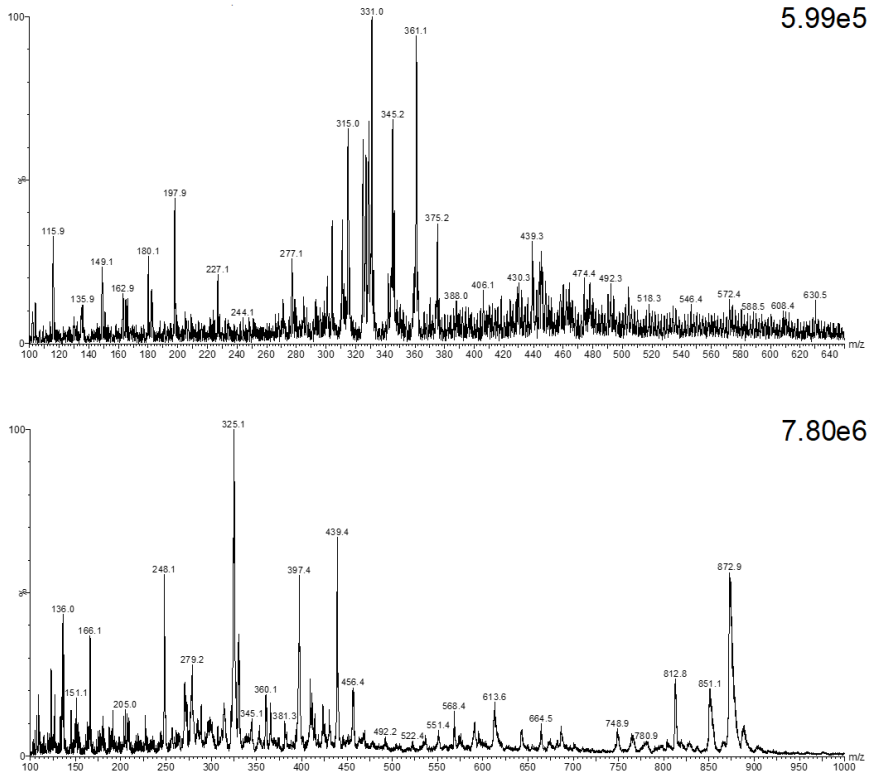


Figure 3. Scatter plot of PC1 vs PC2 scores obtained from DART-MS (left) and ASAP-MS (right) data. Confidence ellipses (95%) are reported as dashed blue lines.

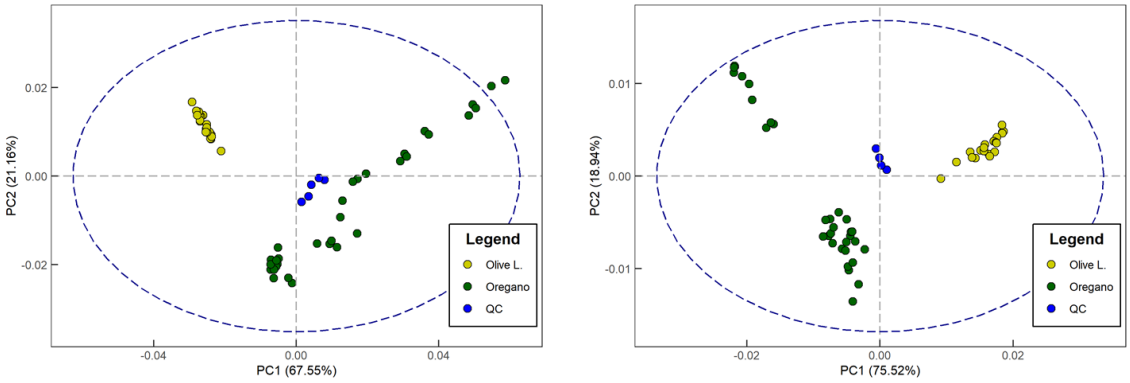


Figure 4. Bar chart reporting the SIMCA class-belonging probabilities (π) computed for the illicit mixtures. Probability threshold ($\pi = 0.5$) corresponding to 95%-tolerance interval is shown as red dashed line. Objects associated to a $\pi < 0.5$ are recognized as alien and rejected by the model, whereas when $\pi > 1$ the probability is set to 1 [67]. Adulteration level is labelled on the top of each bar.

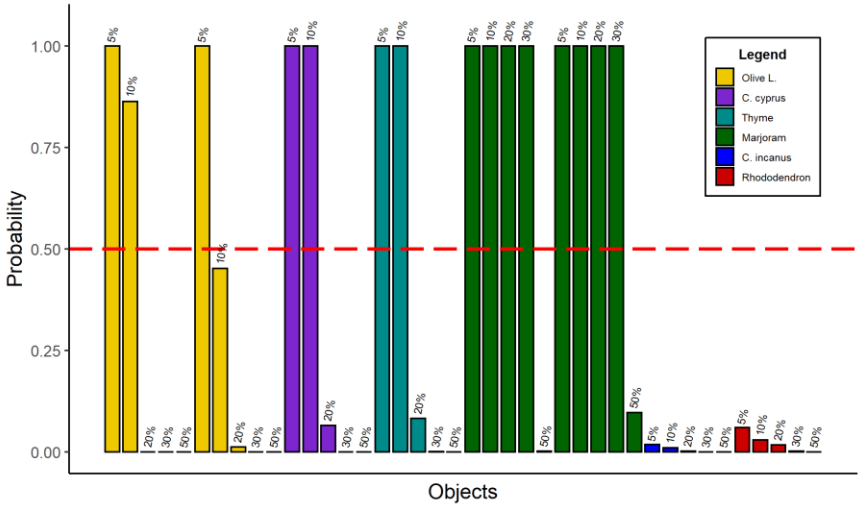
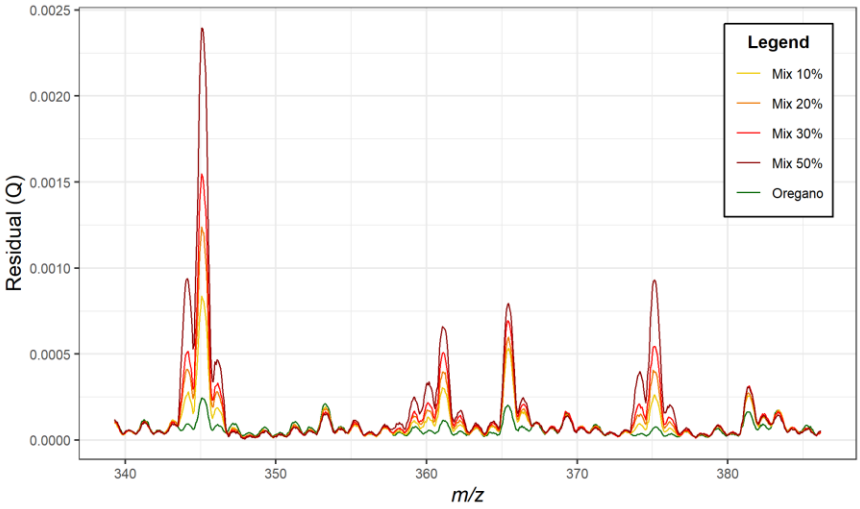


Figure 5. Residuals (e_{unk}), calculated as the difference between the measurements (x_{unk}) and its back-projection ($\hat{x}_{unk}$), with respect to the principal component representation of thyme blends (from 10 to 50%) over the range 340-385 *m/z*. Residuals of one oregano sample are reported as reference.



Tables

Table 1. Results of SIMCA modelling of authentic results and applied to illicit mixtures. Results are expressed as sensitivity and specificity.

Technique	PCs	Sensitivity (%)	Specificity (%)
DART-MS	6	40	80
ASAP-MS	3	100	100

Supplementary information for:

Critical evaluation of ambient mass spectrometry coupled with chemometrics for the early detection of adulteration scenarios in dried herbs

Table S1. Samples set overview.

Sample	Genus	Species	Provenance	Harvest
Oreg-1	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-2	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-3	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-4	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-5	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-6	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-7	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-8	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-9	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-10	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-11	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-12	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-13	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-14	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-15	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-16	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-17	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-18	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-19	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-20	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-21	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-22	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-23	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-24	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019

Oreg-25	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-26	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-27	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-28	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-29	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-30	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-31	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-32	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-33	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-34	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-35	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-36	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-37	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-38	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-39	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-40	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-41	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-42	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-43	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-44	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-45	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-46	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2018
Oreg-47	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-48	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-49	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-50	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-51	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-52	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-53	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-54	*Origanum*	*vulgare* ssp. *heracleoticum*	Italy	2019
Oreg-55	*Origanum*	*vulgare*	Greece	2017
Oreg-56	*Origanum*	*vulgare*	Greece	2017

Oreg-57	*Origanum*	*vulgare*	Greece	2017
Oreg-58	*Origanum*	*vulgare*	Greece	2017
Oreg-59	*Origanum*	*vulgare*	Greece	2017
Oreg-60	*Origanum*	*vulgare*	Greece	2017
Oreg-61	*Origanum*	*vulgare*	Greece	2017
Oreg-62	*Origanum*	*vulgare*	Greece	2017
Oreg-63	*Origanum*	*vulgare*	Greece	2017
Oreg-64	*Origanum*	*vulgare*	Greece	2017
Oreg-65	*Origanum*	*vulgare*	Greece	2017
Oreg-66	*Origanum*	*vulgare*	Greece	2017
Oreg-67	*Origanum*	*vulgare*	Greece	2017
Oreg-68	*Origanum*	*vulgare*	Greece	2018
Oreg-69	*Origanum*	*vulgare*	Greece	2018
Oreg-70	*Origanum*	*vulgare*	Greece	2018
Oreg-71	*Origanum*	*vulgare*	Greece	2018
Oreg-72	*Origanum*	*vulgare*	Greece	2018
Oreg-73	*Origanum*	*vulgare*	Greece	2018
Oreg-74	*Origanum*	*vulgare*	Greece	2018
Oreg-75	*Origanum*	*vulgare*	Greece	2018
Oliv-1	*Olea*	*europaea* v. Rama Pendula	Italy	2019
Oliv-2	*Olea*	*europaea* v. *Rama Pendula*	Italy	2019
Oliv-3	*Olea*	*europaea* v. *Rama Pendula*	Italy	2019
Oliv-4	*Olea*	*europaea* v. *Leccino*	Italy	2019
Oliv-5	*Olea*	*europaea* v. *Leccino*	Italy	2019
Oliv-6	*Olea*	*europaea* v. *Leccino*	Italy	2019
Oliv-7	*Olea*	*europaea* v. *Leccino*	Italy	2019
Oliv-8	*Olea*	*europaea v. Leccino*	Italy	2019
Oliv-9	*Olea*	*europaea* v. *Ascolana*	Italy	2019
Oliv-10	*Olea*	*europaea* v. *Ascolana*	Italy	2019
Oliv-11	*Olea*	*europaea* v. *Ascolana*	Italy	2019
Oliv-12	*Olea*	*europaea* v. *Ascolana*	Italy	2019
Oliv-13	*Olea*	*europaea* v. *Leucocarpa*	Italy	2019

Oliv-14	*Olea*	*europaea* v. *Leucocarpa*	Italy	2019
Oliv-15	*Olea*	*europaea* v. *Pendolino*	Italy	2019
Oliv-16	*Olea*	*europaea* v. *Pendolino*	Italy	2019
Oliv-17	*Olea*	*europaea* v. *Maurino*	Italy	2019
Oliv-18	*Olea*	*europaea* v. *Carboncella*	Italy	2019
Oliv-19	*Olea*	*europaea* v. *Sargano*	Italy	2019
Oliv-20	*Olea*	*europaea* v. *Nocellara Etnea*	Italy	2019
Cistus-1	*Cistus*	*incanus*	Germany	2019
Cistus-2	*Cistus*	*cyprus*	UK	2019
Rhodo-1	*Rhododendron*	*indicum*	UK	2019
Thym-1	*Thymus*	*serpyllum*	Italy	2019
Marjo-1	*Origanum*	*majorana*	Italy	2019
Marjo-2	*Origanum*	*majorana*	Italy	2019

Table S2. Optimized experimental parameters used for DART-MS and ASAP-MS analysis.

Experimental parameter	**DART-MS**	**ASAP-MS**
Sampling support	Quick Strip module	Glass capillary
Sample volume (µL)	9	10
Rail speed (mm/s)	0.8	NA
Cone voltage (V)	10	15
Corona discharge (kV)	NA	3
Gas temperature (°C)	350	450
Gas flow (L/min)	2.5	3
Mass range (*m/z*)	100-650	100-1000
Polarity	DART+	ASAP+
Sampling frequency (Hz)	5	2

Figure S1. Schematic illustration of the two-stage experimental design.

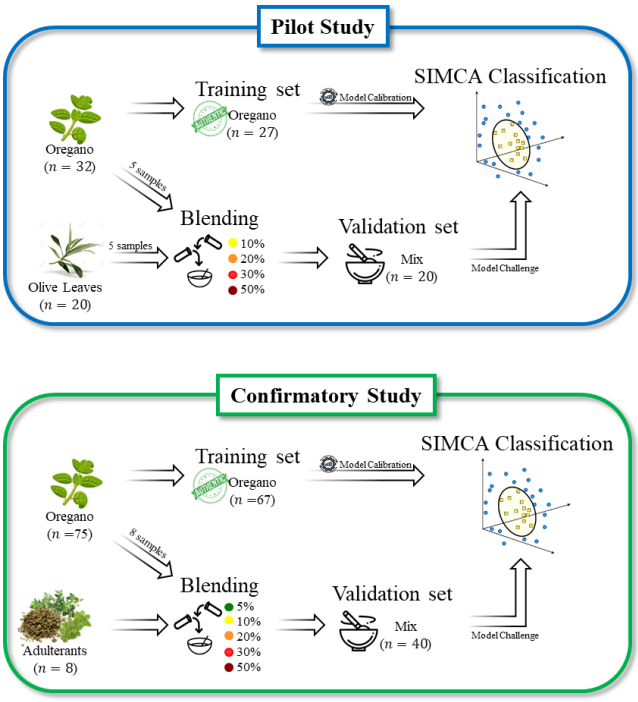
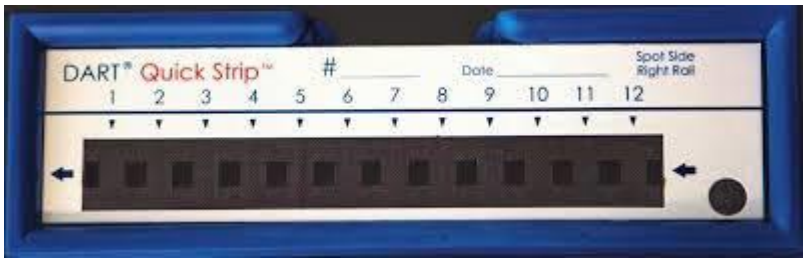


Figure S2. "QuickStrip" module. Liquid or solid samples can be loaded onto the stainless steel meshes (12 positions) by both manual micropipettes and robotic systems. For analysis, the QuickStrip is loaded on an automatic linear rail, running through the ionization region (between the DART source and the mass spectrometer inlet) at controlled speed. Being a consumable card, no cross-contamination occurs.



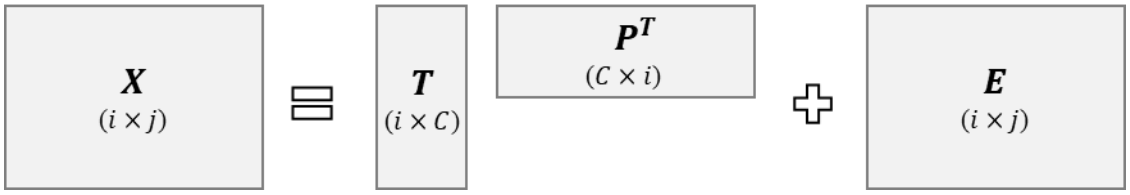
Investigation of Residuals distribution

As explained by Bro & Smilde, a meaningful evaluation of the model's summarizing capability can be obtained by assessing how representative is the prior PCA decomposition in terms of replacing the original data matrix $\boldsymbol{X}$ [73]. This can be done by exploiting the relationship between $\boldsymbol{X}$ and the model's components:

$$\boldsymbol{X} = \boldsymbol{T}\boldsymbol{P}^T + \boldsymbol{E}$$

where $\boldsymbol{X}$ is the experimental data from the training samples, $\boldsymbol{T}$ and $\boldsymbol{P}$ are the scores and loadings matrices, respectively, and $\boldsymbol{E}$ is the array collecting the residuals (representing the unmodelled variation in $\boldsymbol{X}$) [54]. The product matrix $\boldsymbol{T}\boldsymbol{P}^T$ is often indicated as $\widehat{\boldsymbol{X}}$, since it serves as a model representation of $\boldsymbol{X}$. A schematic illustration of the PCA model components is provided below:

Figure S3. Illustration of a *C*-component PCA model generated from a data matrix $\boldsymbol{X}$ having i objects and j variables.



S. Figure 3 further highlights that $\boldsymbol{E}$ and $\boldsymbol{X}$ have the same structure (i.e. dimensions). Therefore, any useful visualization of the original data would also be suited for inspecting the residuals - $\boldsymbol{E}$ and $\boldsymbol{X}$ also have the same magnitude. For instance, residuals calculated on spectral data would literally correspond to the residual spectra, thus potentially providing tangible chemical information about the unexplained spectral variation.

Within SIMCA, $\boldsymbol{E}$ is normally used to compute the sum of squared residuals (Q-statistics) - which summarizes the distances between each object and the model (hyper)plane – employed to verify the compliance of test samples with the class model [53]. In the present case, the residual distribution of the newly-tested objects has been used to pinpoint the relevant (original) variables responsible of the sample classification. Specifically, when the unknown sample is being analysed, the row vector of measurements x_{unk} is obtained and then projected onto the inner-space PCs:

$$t_{unk} = x_{unk}\boldsymbol{P}$$

This provides the object's scores coordinates t_{unk} within the PCs space. At this point, the scores t_{unk} are back-projected onto the original variable space:

$$\hat{x}_{unk} = t_{unk}\boldsymbol{P}^T$$

The so-obtained $\hat{x}_{unk}$ vector is the model representation of the tested sample. The difference between $\hat{\boldsymbol{x}}_n$ and the experimental measurements x_{unk} corresponds to the residual vector:

$$e_{unk} = x_{unk} - \hat{x}_{unk}$$

In the case of mass spectrometry data, e_{unk} appears as a mass spectrum with "peaks" arising in correspondence of the features that largely differed from the model representation (see **Figure 5**). It must be noticed that residuals change according to the number of retained PCs [73]; therefore, their investigation should be carried out once the model dimensionality has been defined. More details can be found in [54,66].

Figure S4. Consecutive spectra (5 scan point each) obtained from the same ASAP-MS TIC trace of dried oregano. Magnification of two mass ranges are reported: 250-450 *m/z* (top) and 750-950 *m/z* (bottom). The first (red), central (green) and last (blue) portion of the TIC peak are highlighted in different colours. Spectra are shown with linked vertical axes.

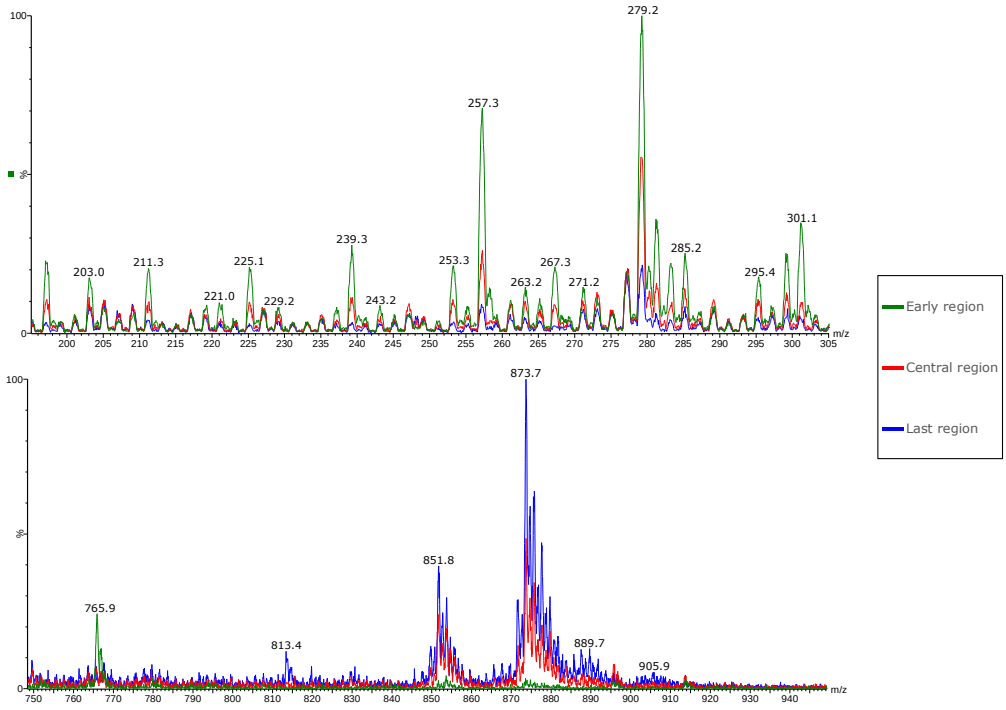


Figure S5. Overlay of oregano and olive leaves raw spectra recorded with DART-MS (top) and ASAP-MS (bottom) raw spectra recorded from the same oregano sample.

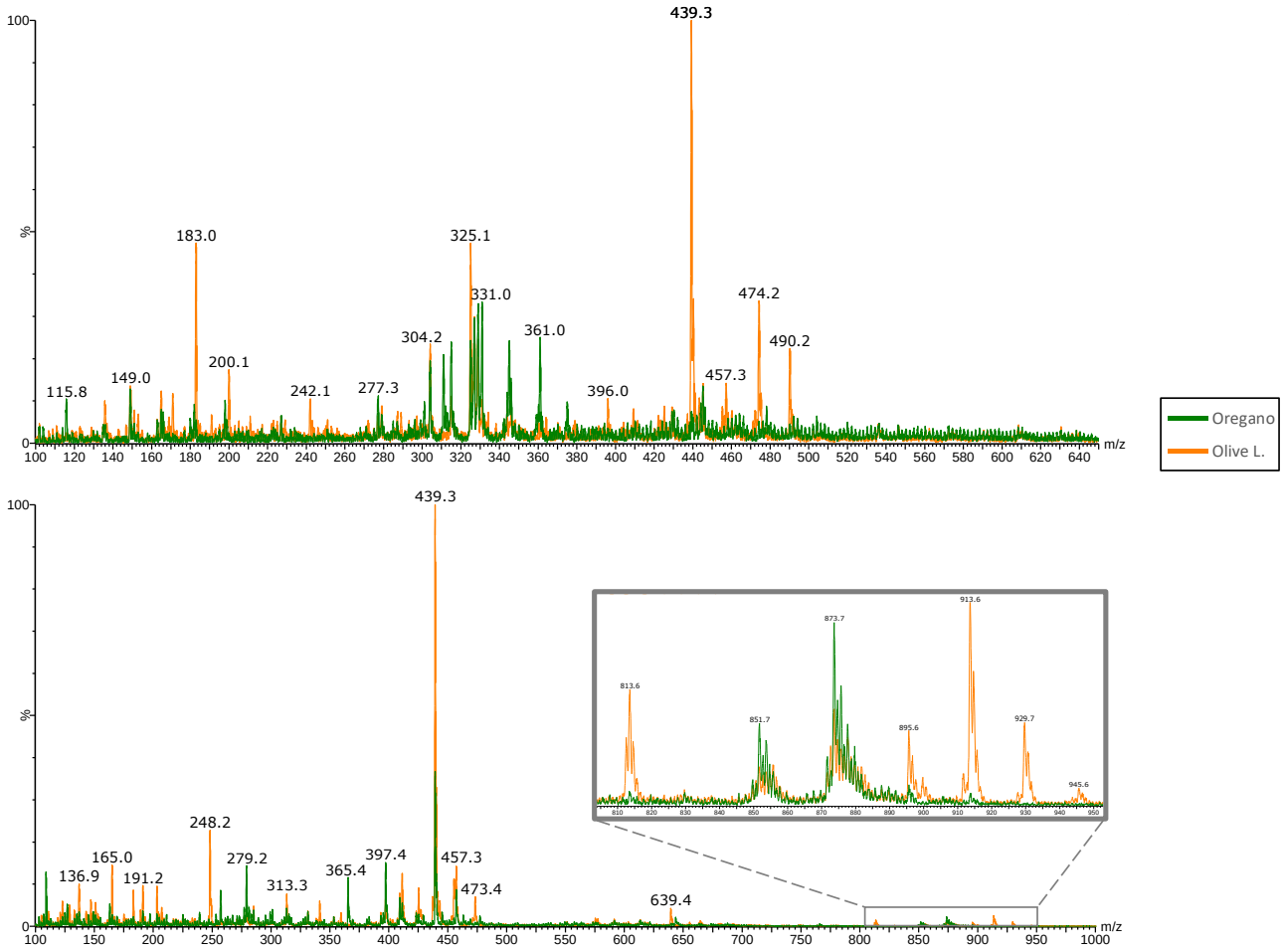


Figure S6. Background's raw spectra recorded in DART-MS (top) and ASAP-MS (bottom) analysis. DART-MS background was recorded on the last QS card position spotted with blank extraction solvent Concerning the ASAP-MS, the displayed spectrum refers to the end of the "bake-off" process, prior to loading the sample onto the glass capillary surface. Base peak intensity is reported in the top-right corner of each spectrum.

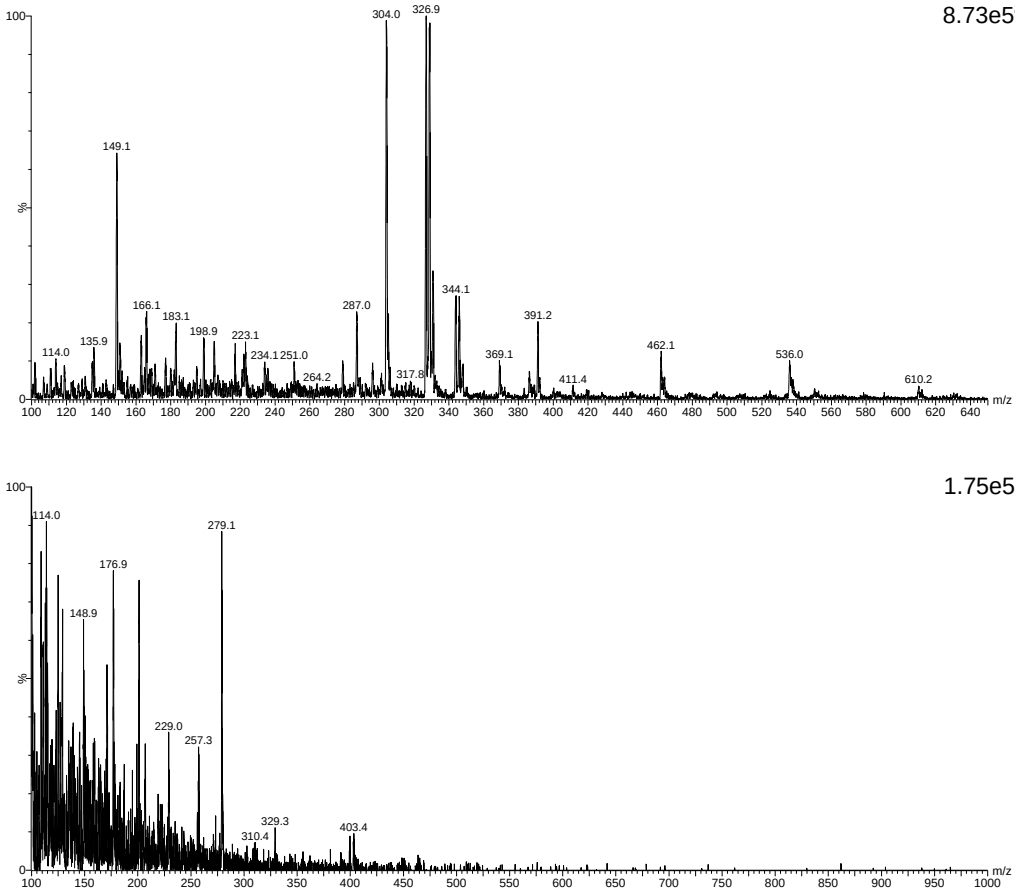


Figure S7. Scatter plot of PC1 vs PC2 scores obtained from 10 technical replicates of 10 analysed oregano samples by applying mathematical preprocessing only (left) or mathematical preprocessing and background subtraction (right). Each sample is marked with a different colour. Confidence ellipses (95%) are reported as dashed blue lines.

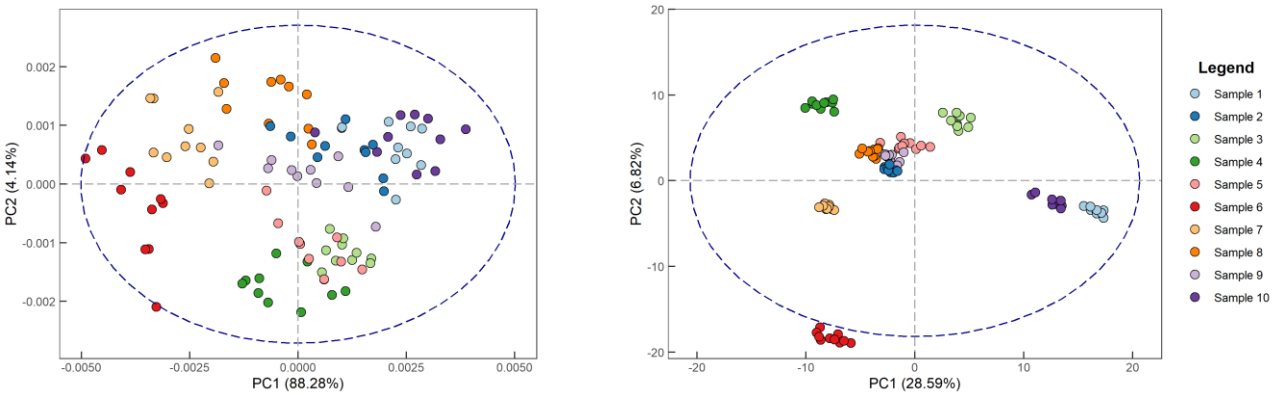
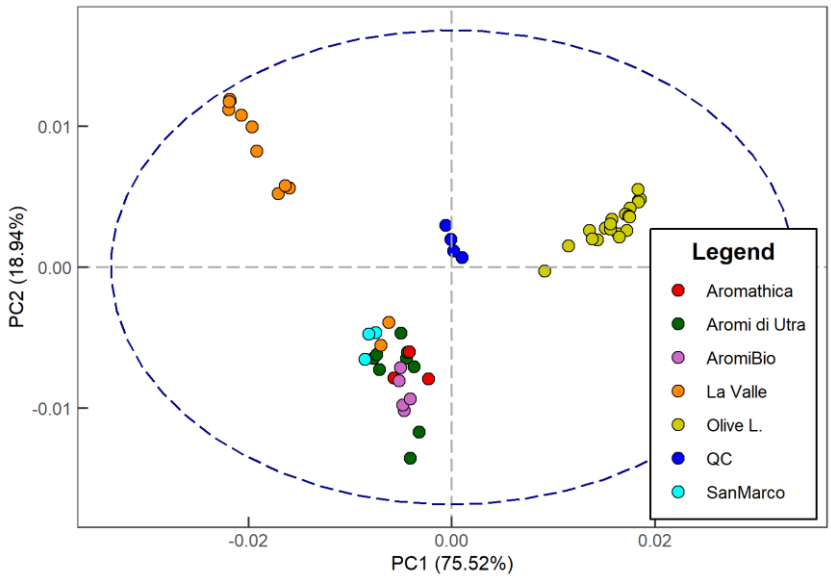


Figure S8. Scatter plot of PC1 vs PC2 scores obtained from ASAP-MS data. Objects are marked according to the different oregano producers. Confidence ellipses (95%) are reported as dashed blue lines.



References

[1] R. Bro, A.K. Smilde, Principal component analysis, Anal. Methods. 6 (2014) 2812–2831. https://doi.org/10.1039/c3ay41907j.

[2] M. Bevilacqua, R. Bucci, A.D. Magrì, A.L. Magrì, R. Nescatelli, F. Marini, Classification and Class-Modelling, in: Data Handl. Sci. Technol., Elsevier Ltd, 2013: pp. 171–233. https://doi.org/10.1016/B978-0-444-59528-7.00005-3.

[3] P. Oliveri, Class-modelling in food analytical chemistry: Development, sampling, optimisation and validation issues – A tutorial, Anal. Chim. Acta. 982 (2017) 9–19. https://doi.org/10.1016/j.aca.2017.05.013.

[4] A.L. Pomerantsev, O.Y. Rodionova, Concept and role of extreme objects in PCA/SIMCA, J. Chemom. 28 (2014) 429–438. https://doi.org/10.1002/cem.2506.

3. Discussion

In the following sections, critical considerations about the employed analytical techniques are made. Afterwards, the main advantages and intrinsic drawbacks of non-targeted fingerprinting, with respect to traditional analytical protocols, have been summarized and discussed (**Section 3.2** and **3.3**, respectively). In addition, considerations are made about the chemometrics involved in the data treatment/evaluation and its potential misuse.

3.1 Instrumental techniques evaluation

At present, an ever-growing number of instrumental platforms are available and increasingly employed in non-targeted fingerprinting methods. Clearly, all the detection methods nowadays available cannot be covered herein; indeed, they would very easily fill several volumes. Therefore, the present section has been focused on the analytical techniques employed during the PhD programme (see **Section 2**) is provided in the present section.

Although relying on totally different detection principles, the employed platforms all proved to be suitable for the problem under investigation. Overall, spectroscopic possesses a number of major advantages; indeed, the majority of food fingerprinting protocols are nowadays based on spectroscopic techniques such as NMR, NIR or FT-IR spectroscopy. Normally, the little or no sample preparation required hold the analysis time down to few seconds, providing a quick and non-destructive solution at low running costs. In addition, once a method has been developed, the equipment (NIR spectroscopy in particular) can be moved out of the laboratory for *in situ* or online analysis and run by new users upon limited training, making the entire process more labour- and cost-saving [1]. In the food processing industry, this is particularly valuable for continuous monitoring at all stages of the process, from production and collection of raw ingredients, to the quality control of finished products. In terms of portability, more recently handheld portable FT–IR and Raman instruments are also becoming more widely available, though not yet as mature as NIR [2].

Similar characteristics are offered by e-sensing technologies (e-nose and e-tongue), which are becoming more and more popular in food control [3]. In addition to the advantages already mentioned for vibrational spectroscopic techniques, e-noses and e-tongues are particularly suited for merging the responses from

multiple devices through data fusion strategies in order to enhance the method's capabilities [4]. Last but not least, a further interesting feature for the food industry is the potential correlation with data from human sensory for the rapid and objective evaluation of quality descriptors and organoleptic attributes [5–7]. It is worth mentioning that, thanks to the collaboration between Barilla G.R. F.lli SpA and the University of Parma, both the equipment employed in **Chapter 2** have been already implemented in the industry plant for the routine quality screening of incoming raw materials [8,9].

While spectroscopic (including NMR) and e-sensing technologies nowadays represents the "golden standard" for food fingerprinting thanks to the above-mentioned attributes [3,10], MS has seen a huge growth in the last 15-20 years concerning ionization techniques, faster and higher-resolution detectors. Yet, the major limitations of MS-based analytical techniques have historically been their destructive nature, the higher purchasing- and running-costs and the need of skilled personnel. Nevertheless, as discussed in **Chapter 3**, AMS has shown the potential to overcome these major issues [11] and exhibit attributes similar to the above-mentioned techniques. As proof of this, in their literature review on "food fingerprints", Medina and co-workers found that more than 75% of the cited publications involved MS technology [12]. Such large and perpetual interest toward MS lies in the reduced effort for sample preparation compared to hyphenated techniques (e.g. GC-MS, LC-MS); besides, AMS can potentially provide additional information (i.e. *m/z*, fragments analysis, etc.) about the detected non-compliance respect to the spectroscopic and e-sensing techniques. However, the use of AMS is still in its infancy compared to the others well-established platforms and, despite the demonstrated potential, AMS is still mostly used in specialized laboratories and translation into a larger scale has so far not been realized [13]. Indeed, such emerging platforms must prove to be successful in validation trials among multiple laboratories. In this regard, as a follow-up of the feasibility study reported in **Chapter 3**, an on-site testing of the ASAP-MS equipment is being performed in collaboration with the food company Barilla G.R. F.lli SpA. The aim is to assess the instrument capabilities and robustness within an industrial plant and to evaluate the possibility of data/model transfer between multiple devices.

3.2 Advantages of fingerprinting approaches

The number of published studies focusing on fingerprinting approaches for food authentication purposes has grown exponentially in the past few years. Overall, the attention of researchers and industrial users has been caught in two cases:

i) Official or universally accepted analytical methods were available for the problem under investigation, yet relying on time- and labour-consuming protocols or expensive equipment. The most illustrative example is arguably the determination of major components' content by means of NIR spectroscopy coupled to multivariate regression methods. Laborious and demanding conventional methods (e.g. Kjeldahl method for protein content estimation) have been replaced by fast, cost-effective and high-throughput NIR scanning providing acceptable, if not comparable, analytical accuracy [14]. Another meaningful example is the melissopalynological analysis for the discrimination of unifloral honey botanical origin. Melissopalynology represents the most used method for honey's origin confirmation, even though it suffers from several shortages and limitations, such as the extensive analysis time, the need of highly skilled personnel and the great seasonal variation of pollen content [15,16]. Consequently, novel alternative approaches have been proposed, as mentioned in **Chapter 1**;

ii) Complex research questions for which no analytical method was available. Most of the cases involve challenging scientific topics (e.g. geographical and/or botanical origin issues) that could hardly be addressed with a single measurement/parameter by using classical targeted approaches. Hence, "holistic" non-targeted food fingerprinting approaches have been, and are being tested.

Multivariate statistics. The first advantage of non-targeted fingerprinting over classical methods is related to the multivariate statistics involved in the data treatment. Traditional univariate approaches attempt to relate a measured variable x to the investigated sample's property y, either quantitative (e.g. analyte concentration) or qualitative (e.g. belonging class), through a function (model) in the form of a $y = f(x)$. The exact same concept is pursued by modern fingerprinting methods, with the only difference being that x represents the recorded fingerprint. Therefore, a large set of measured variables x_i is simultaneously considered and associated to the outcome variable y. This not only offers a higher chance to find a x_i that strongly correlates with y but, is also more effective respect to the univariate vision where any covariation between variables is explicitly neglected, leading to important features being potentially ignored [17]. The multivariate approach is particularly suitable for complex analytical tasks, such as labelling claim confirmation, which usually require information (both physical and chemical) on a wide range of food constituents [18]. In this regard, chemometrics can be also applied with the purpose of "features selection" (e.g. iPLS-DA), aiming at identifying few statistically-relevant variables and discarding the signals regions

that are not-related to the response variation. Among the main benefits, variable selection allows a more straightforward data visualization and interpretation, while potentially providing improved prediction performance and model robustness [19].

Investigation of multiple objectives. A further peculiar advantage over conventional analytical approaches, where a single measurement is performed to answer one question, is the possibility to investigate multiple objectives with the same method [20]. As said, ensuring the authenticity of food products from an official control perspective, may require several properties to be considered. Such augmented complexity can be addressed by decision trees composed of multiple statistical models rather than an individual multivariate model [21]. Examples are the simultaneous quantification of food components and trace compounds [22,23], as well as the determination of quality parameters [24,25] which can eventually be used for authenticity purposes [26]. It must be noted that all the above-mentioned methods answer to quantitative question. The development of fingerprinting protocol for multiple classification is undoubtedly possible, but would require appropriate and comprehensive sampling, model training, and validation processes covering all the investigated questions to obtain reliable results.

Multiplatform Sample Characterization. An emerging trend in several branches of (bio)analytical chemistry consists in the combination of outputs from multiple instrumental sources [4,27]. Fusion of data from complementary platforms can provide a more comprehensive sample characterization, yielding better inferences than the individual techniques, i.e. lower uncertainty in the analyte concentration prediction and improved prediction ability of multivariate models. For this reason, multiplatform analysis of food samples with subsequent data fusion is gaining growing attention in the food authenticity field, also promoted by the ever increasing availability of rapid and non-destructive analytical techniques in industrial processes and laboratories [4].

In general terms, the common workflow adopted by most of the authors consists of the following steps. First, the instrumental platforms to be used for the analysis are selected. Obviously, the instrumental responses must be related to the investigated food properties (e.g. sensory descriptors, organoleptic attributes, geographical/botanical differences, etc.). Furthermore, the chosen analytical techniques have to be evaluated in order to find possible redundancy and/or complementarity. In fact, the fused data source must provide orthogonal information about the sample. Otherwise, the risk is to introduce, along with useful information, a large number of redundant variables (noise), thus hampering the multivariate modelling (**Chapter 1**). After the measurement and data generation, the fusion level (low-, mid- or high-level) is decided according to the amount and form of the generated data. In this respect, new ideas for data preprocessing, variable selection and model validation are often required to enhance the synergy between

the merged data sources and highlight the chemical information therein contained. Overall, several data fusion strategies have proven capable to enhance the global model classification/prediction ability and/or decrease the uncertainty of each individual result. Nevertheless, the chemical knowledge about the samples composition and the problem at hand is fundamental for its successful application [4,28].

"Machine Learning" algorithms. Being a subset of the Machine Learning (ML) domain, most of statistical methods used in chemometrics are shared between these fields (e.g. classification and regression algorithms). ML algorithms are designed to build mathematical models that learn from the provided training data in order to perform tasks without being explicitly programmed to do so [29]. Within the food fingerprinting workflow, this is accomplished in the model training stage, where the algorithm attempts to find a meaningful relationship between the training data and the outcome variable. In addition, ML algorithms are typically intended to "improve automatically through experience", which essentially means that the confidence in the final outcome is increased upon enlargement of the training dataset. In general, such data-driven approach may allow the end-user to make decisions even without a full understanding of what underlies the obtained result. The possibility offered by fingerprinting methods to be used as "black box" is highly attractive for the industry as the analysis could be run by non-specialized personnel. This is among the fundamental reasons behind the success of vibrational spectroscopy in this field [10] and, in this respect, emerging technologies and new equipment are indeed demanded to be ever more user-friendly and require minimum training for new users (**Chapter 2** and **3**).

Potential data sharing. Connected with the purpose of applying non-targeted food authentication in routine analysis and food surveillance, the potential data exchange between different laboratories may lead to the establishment of joint comprehensive databases to be used for the development of overarching multivariate models. This would exponentially increase the effectiveness and applicability range of modern non-targeted fingerprinting methods. Spectra can be shared easily in networks, instead of samples and analysts interested in applying newly-developed authenticity based on non-targeted fingerprint could use historical data to build the statistical model, rather than starting the research from the beginning.

The fundamental problem arises when the samples to be predicted are measured on a different instrument or under diverse environmental factors from those used to build the model; the changes in spectral variations between the two conditions may make the model invalid for prediction in the new system. In the case of quantitative applications, to circumvent the need of a full recalibration each time a new instrument is used, standardization and preprocessing methods have been proposed to enable multivariate calibration models to be effectively transferred between two systems, thus eliminating the need for a full recalibration [30].

Examples of qualitative applications, though less frequently, can also be found in literature [31]. Nevertheless, data exchange between instruments of different vendors is not always readily achievable. Evaluation of spectra produced from different equipment with one central model would require a rigorous standardization of the analytical workflow, which would ensure the generation and exchange of consistent and comparable measurement data. In addition, databases often have limited availability and access due to intellectual property issues. These are hurdles that have to be addressed for the development of comprehensive multivariate models relying on joint databases and further emphasizes the need for harmonized analytical protocols and universally accepted validation schemes, as already mentioned in **Section 1.4** [21].

"Black box" approach. Despite fingerprinting approaches are particularly praised for their quickness and easy-of-use, the method development and monitoring are generally demanding for the company in terms of time and money. For this reason, the past few years have seen a growing tendency of certain instrument manufacturers to offer, along with the equipment, also "ready-to-use" multivariate models to accomplish specific tasks. In this manner, a push-button solution is provided to the end user who only needs to perform the acquisition, while the tedious works of sample collection, analysis and model development/validation are left to the manufacturer. Moreover, the latter often takes care also of periodical models' update or re-building. Essentially, such fingerprinting methods can be used as "black-boxes" in industrial environments, and this is particularly appealing for the food companies as they might not possess sufficient expertise to carry out the whole method development and no specialized personnel is needed to run the analysis.

Successful instances of this policy are the MilkoScan™ and Infratec™ from FOSS Analytical (Hilleroed, Denmark) [32,33], well-established analytical solutions for the simultaneous determination of numerous constituents and quality-related parameters of milk and cereals, respectively. Further examples are the JuiceScreener™, WineScreener™ and Honey-Profiling™ provided by Bruker BioSpin GmbH (Rheinstetten, Germany) [34–36]. The latter are fully automated NMR-based targeted/untargeted screening that are not only designed to perform quantitative estimation of several macro-components and trace compounds in the respective food matrices; they also offer a (qualitative) assessment of the product's authenticity using an untargeted fingerprinting approach. Multivariate models are built on large spectral databases recorded from reference samples over consecutive years and routinely updated. For example, grape variety, vintage and appellation (e.g. DPO) can be confirmed for wine, while geographical/botanical origin of honey can be verified. In all these cases, reference libraries are routinely enlarged with data recorded from the users and the models updated accordingly. In order to make this possible, standardized

procedures must be followed by the analysts and data processing strategies for data and model exchange/transfer has been successfully implemented by the manufacturers.

Bruker has provided the first commercial solutions of non-targeted food fingerprinting applied to classification problems, proving to be a pioneer company in this respect. However, in the author's opinion, the development of comprehensive fingerprinting-based models for geographical and botanical identity is extremely arduous, not to say unrealistic, since all the sources of variability can hardly be covered within the model calibration. In fact, the development of a classification model for the geographic origin discrimination applicable at a "global-level", would require an exhaustive sampling to be carried out on a world scale and repeated over several harvest years, regardless the used analytical techniques. Furthermore, it cannot be guaranteed that two samples coming from different localities will have unique spectral signatures. In fact, it must be stressed that such methods are *in-house* validated by the company itself and, not surprisingly, no approval has been obtained by accredited bodies. As reported in the company website, the "Wine-Profiling 4.0" method for authenticity and integrity testing is still under ISO17025 accreditation [34].

3.3 Limitations of fingerprinting approaches

Along with the above-mentioned advantages, fingerprinting approaches also presents not-trivial intrinsic drawbacks. The latter must be taken into account within the development of a non-targeted method along with the common challenges in setting up a food authentication study (listed in **Section 1.4**). Regrettably, these major challenges often reduce the published works to truthful feasibility studies that demonstrate the capability of the employed methodology, yet do not find practical application in industry environment.

Sample preparation and detection principle. While in classical targeted methods the instrumental output is normally converted in a concrete quantity (e.g. µg/L, mg/Kg), non-targeted fingerprinting data are evaluated on a pattern level; therefore, as mentioned in the previous section, changing the employed sample preparation procedure and/or instrumental settings would utterly compromise the use of the initial model. For this reason, the analytical protocol must be necessarily optimized during the development stage and cannot be changed in the future to ensure the consistency of newly-acquired data. In addition, in conventional targeted methods, both sample preparation and instrumental parameters are typically tailored to the investigate analyte(s) in order to maximize the signal response. In contrast, in non-targeted analysis the sample preparation should be kept as non-specific as

possible, so that the widest spectrum of matrix components can be captured and loss of potential information is avoided [21]. The optimal procedure must be carefully selected as any step may strongly impact on the final outcome [20]. For instance, the choice of the solvent composition represents a critical question when a solid-liquid extraction step is required; it is well-known that no extraction method is able to exhaustively cover the whole metabolites range of a biological sample (e.g. phytochemicals) [37]. Alternatively, either multiple or multi-phase extractions procedures have been proposed to produce different extract fractions of the same sample that will be analyzed separately [38,39]. As a drawback, it certainly lengthens the total analysis time, making the process more time- and labor-demanding.

Besides, enzymatic and/or oxidative processes may occur during the storage and transportation of reference materials; they can heavily influence the resulting data profiles, thereby representing a big concern for a proper sample characterization. As an example, DNA, elemental and isotopic profiles are relatively stable [40], whereas solvent-derived artefacts may occur in metabolomics and proteomics research, even during a short storage of sample extracts [41,42]. Freeze-drying can be useful for long-term storage of biological samples; enzymatic reactions are forced back and, in the case of NMR fingerprinting, the negative impact of water signals on the measurements is reduced [43]. At the same time, this introduces additional sample handling steps that require particular attention (e.g. thawing) [44] and do not reproduce ordinary market conditions [40]. For these reasons, in the past few years the interest of food industries and researchers has been caught by analytical techniques which involve minimal, or no sample preparation. In this regard, vibrational spectroscopy it is a well-established platform which offer the possibility to carry out non-destructive analysis of a wide variety of agro-food samples (**Chapter 1**). In addition, ambient mass spectrometry [11] and e-sensing [3] constitute further emerging "sample-preparation free" approaches which have shown to be highly attractive for non-targeted analysis.

Clearly, the optimal sample preparation protocol strictly depends on the chosen detection principle (e.g. DNA-based, spectroscopic, mass spectrometry, etc.). In addition, also the equipment-specific features (e.g. sensitivity, resolution, etc.) must be taken into account as they impact on the amount of recorded information. Overall, high-resolution methods may be preferred since the informative depth of the method increases as the information content rises. However, they are usually expensive, require a high level of maintenance and trained personnel - which is not what the food industry is demanding (**Section 1.2**). For these reasons, low-resolution equipment is used when possible [43].

"Mandatory" chemometrics. If in one hand the multivariate statistics involved in fingerprinting approaches represent a clear advantage over classical (univariate) methods (**Section 3.2**), on the other side the wealth of information contained in the large and complex datasets is impossible to access without chemometrics. Here too, vibrational spectroscopy provides a representative example. Raw spectra of

biological materials (such as food) are virtually impossible to interpret with the unaided eye (see **Annex I**) and a range of mathematical preprocessing methods have been developed to reduce the impact of sample inhomogeneity, thereby highlighting the chemical information herein contained (**Figure 7**) [45,46].

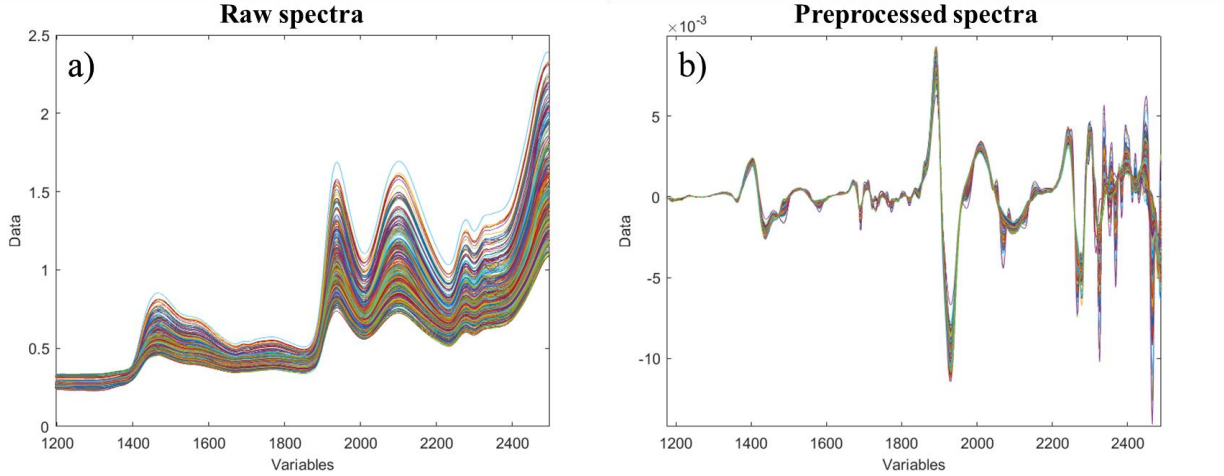


Figure 1. Raw (left) and preprocessed (right) NIR spectra of honey (data sourced from Chapter 1). Upon application of mathematical preprocessing, i.e. standard normal variate + Savitzky-Golay derivative (2nd order derivative, 3rd order polynomial, 9 points window), chemical information and differences between the samples are highlighted.

Another typical problem of high-dimensional dataset is their visualization. Obviously, they cannot be presented in tabular form and their inspection plotting few features at a time in a two- or three-dimensional Cartesian plot is impracticable as hundreds, or even thousands, of variables are measured. Chemometrics tools (e.g. principal component analysis, factor analysis, etc.) for dimensionality reduction can be used to "compress" the data in a reduced-dimension space while retaining their (original) meaningful properties. This allows an easier and effective data displaying to investigate the presence of evident outlying samples and/or potential underlying data structures (**Figure 8**).

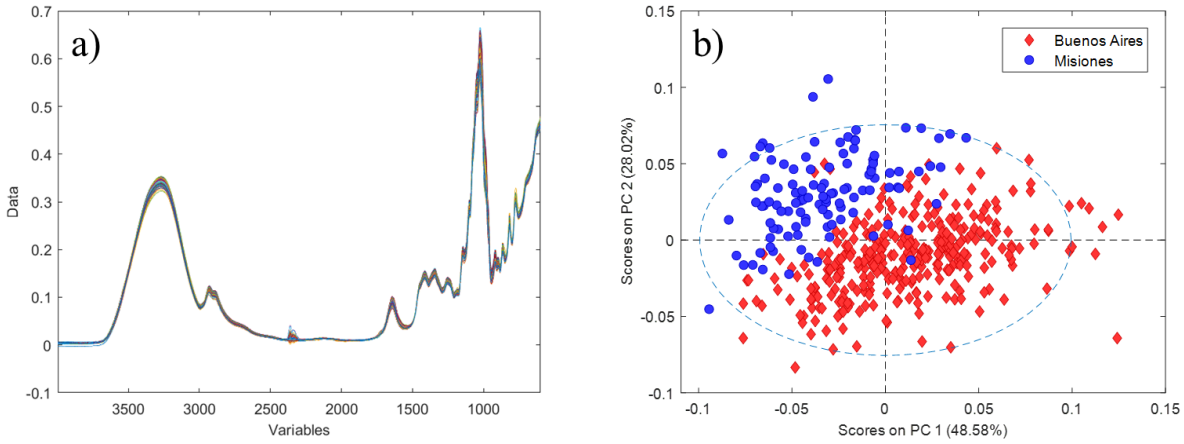


Figure 2. Raw MIR spectra ($\boldsymbol{n = 435}$) are reported on the left figure, while the PC1 vs PC2 scores plot is reported on the right-figure (data sourced from Chapter 1). It can be seen as all the raw spectra look identical upon visual inspection. Nevertheless, PCA highlighted spectral differences existing between the samples, revealing a clear separation between two samples group.

The essential need of chemometrics tools for handling fingerprinting data imply, at least, their basic understanding by the user. As discussed in **Section 3.2**, such inconvenience has been partially addressed by the ready-to-use solutions offered by instrument manufacturers. However, the lack of fundamental knowledge and the use of these methods as "push-button" tools is clearly risky as it may lead to erroneous decisions unconsciously made by the user

Method validation. Although fingerprinting approaches are intended to be labour- and cost-saving, the initial stage of both method development and validation generally requires a significant expenditure of time and money. In addition, deep chemometrics expertise beyond the above-mentioned data investigation/visualization is needed for a proper capabilities assessment of the developed models [47]. Within the method development stage, lot of effort have to made for the collection and analysis of a meaningful sample set covering all the possible source of variability [48]. In fact, the reliability of the modelling results that will be obtained afterwards, heavily relies on the representativeness of the training samples, respect to the true picture. In fact, validation of fingerprinting-based protocols likely represents their most important downside respect to conventional approaches nowadays.

Although considered integral part of the method development, broadly speaking, method validation can be seen as the process that confirms whether the analytical procedure is able to provide performance capabilities consistent with what required by the application [49]. In general, targeted results are evaluated compound-by-compound using univariate statistics to carry out hypothesis testing in order to assess whether the measured value is violating the established limit, taking into account the measurement uncertainty. As

a result, analytical determinations standing "beyond reasonable doubt" can be yielded, enabling its possible forensic utilization [21]. In contrast, non-targeted fingerprinting results cannot be evaluated according to such classical parameters, particularly with respect to classification issues, due to the multivariate nature of fingerprinting data themselves. This entails a series of drawbacks to be faced [20]. For instance, while confidence intervals for single univariate distributions can be readily described by an upper and a lower bound, their definition in the multivariate case requires different approaches (e.g. confidence regions) and strong theoretical assumption [50]; therefore, hypothesis tests cannot be performed in the same straightforward way.

Another important shortcoming is related to the so-called "curse of dimensionality" – a series of phenomena and statistical challenges arising when dealing high-dimensional data [51]. In simple words, when the dimensionality increases, the volume space increases so fast that the available data become sparse. This sparsity is problematic for any method that requires statistical significance. In fact, in order to obtain a statistically-reliable result, the amount of needed sample size grows exponentially along with the dimensionality, quickly becoming unmanageable (**Figure 9**).

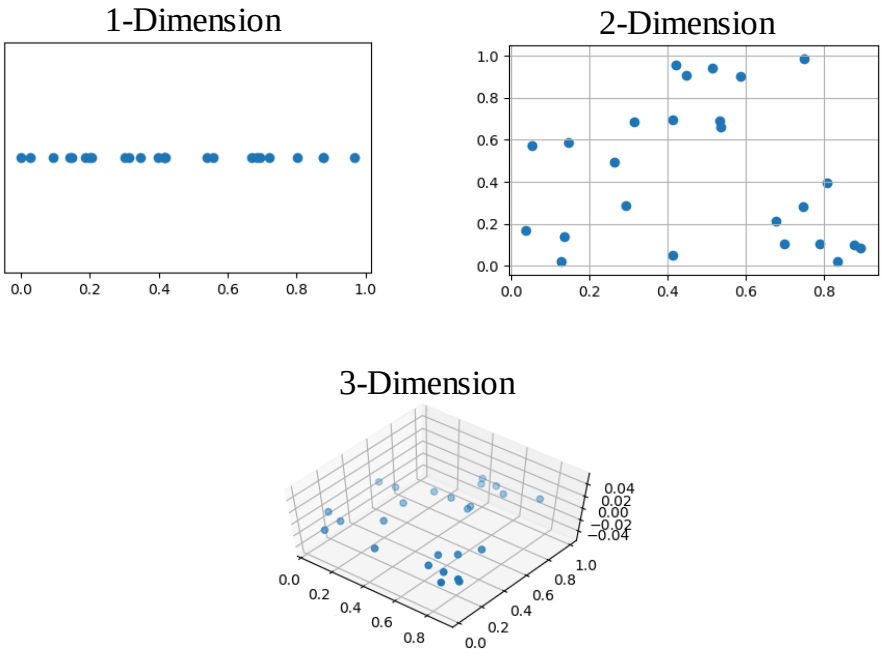


Figure 3. Schematic representation of the "curse of dimensionality". The same number of data points quickly become sparse as the dimensionality increases.

One solution to handle data multidimensionality is to use ML algorithms [51]. However, several critical aspects must be taken into account when using ML on complex data. First, fingerprinting data typically comprise far more variables than observations; this may lead to a significant risk of model overfitting and,

therefore, result misinterpretation and/or overestimation [21]. In addition, ML algorithms require data to be properly pre-treated (e.g. normalization, scaling) to adjust the variance magnitude of each measured variable and deal with instrumental/experimental "data artefacts", often characteristic of the different analytical techniques, such as baseline shifts in spectroscopy and peak shifts in LC-MS or NMR spectra. In this respect, the adopted data preprocessing may strongly influence the final outcome and it is still almost not-possible to predict with certainty the optimal preprocessing to use (or avoid) [52].

A further crucial concern for the implementation of multivariate statistical models in routine analysis is their long-term stability and robustness. All methods are built on historical data; data are generated by instruments; instruments are subject to wear, as well as non-controlled variations (e.g. environmental changes in laboratory, different operators, etc.). In addition, routine maintenance of the equipment typically includes the substitution of worn components or adjustments that easily results in substantial signal shifts [20] (**Chapter 1**). Such technical variations may produce important signal deviations that, if not covered by the original model, may prevent the prediction of new unknown objects. Moreover, especially in the case of natural products, fingerprint variations may arise also from matrix-intrinsic properties (e.g. seasonal/environmental shifts, sample's composition stability and homogeneity, matrix effects induced from process-related variations, etc.) [53]. Therefore, multivariate models must be continually monitored for performance and, potentially, remedial action of model adjustment or re-building could be required [18].

For all these reasons, an adequate model validation strategy in the case of untargeted approaches cannot be limited to the analytical aspects (i.e. sample preparation, instrumental analysis, etc.), but must also cover the subsequent steps of data analysis to ensure the relevance of modelling results. It is indeed not surprising that the adaption of existing models to new analysis batches (e.g. new harvest years) is a problem scarcely addressed in pilot food authentication studies [21].

Lack of Standardization. In the food authenticity field, it must be considered that a detected non-conformity is likely to cause subsequent legal disputes. For this reason, validated and legally-incontestable control measures are increasingly demanded by food industries and regulatory bodies. However, with respect to the emerging non-targeted approaches, so far, lots of effort has been directed towards the analytical development, whereas the data evaluation and method validation/standardization are still in their infancy [21].

Although official standards (e.g. ISO or CEN) do not exist for validation measures of analytical methods, the term "validation" has been well defined for classical targeted analysis along with many guidelines and performance indicators (e.g. selectivity, limit of detection/quantification, accuracy, measurement uncertainty), either from current practice or defined in specific areas [54,55]. In contrast, such entrenched validation parameters cannot be readily adapted to non-targeted multivariate approaches, particularly with

respect to classification issues. As a result, equal guidelines for non-targeted analysis are far to be established, which have certainly limited their extensive implementation in routine analysis thus far [21]. As a result, alternative and case-by-case approaches are being used by researchers to validate the analytical methodology, necessary for a faithful evaluation and comparison of results' quality [21,49].

This latter issue has been the focus of recent European research projects (e.g. QSAFFE and Food Integrity) dealing with the harmonization of authenticity testing. Thanks to the effort made in recent years [21,56–59], a great step forward has been represented by the recently-published U.S. Pharmacopeia guidance on the development and validating of non-targeted methods for adulteration detection [53]. As said in **Section 1.3**, they are explicitly directed to the development of non-targeted fingerprinting approaches, involving a rapid analytical measurement coupled with a classification method for the detection of non-compliant samples [53]. Nevertheless, they do not go further than generic recommendations such as "select an appropriate analytical approach" or "consider all the relevant sources of variation within the sampling", etc. For obvious reasons, they cannot cover neither all the possible methodologies, nor the fields of application.

Thus far, the lack of universally accepted standards arguably represents the main limitation of non-targeted fingerprinting methods, which prevent the unbiased results evaluation/interpretation and, thus, their forensic utilization [21]. Moreover, harmonized analytical protocols would also allow data comparability and exchange, dramatically expanding the potential and capabilities of such approaches [20].

As mentioned in the previous section, harmonized analytical procedures and data processing strategies are needed for data standardization and to build joint databases. In this regard, another problem holding back the spread of non-targeted fingerprinting methods is the fact that model developed on one instrument cannot easily transferred to another device (e.g. of a different brand). Clearly, re-measuring every sample and constructing a new model every time a new instrument is purchased is not a practical solution and, due to the substantial cost and time needed for carrying out robust calibrations. For this reason, model transfer has become very desirable [31]. In the case of vibrational spectroscopic-based methods, several chemometric approaches have been developed to allow data and model transferability [30,60]. Nevertheless, the same approaches are highly needed also for other emerging analytical techniques.

3.4 Use and misuse of Chemometrics

Nowadays, food scientists and technologists are dealing with massive amounts of data produced by sophisticated instrumental techniques and modern approaches to food analysis [61]. Fingerprinting measures are no exception to this as they normally produce high-dimensional datasets, which need to be somehow "simplified" to allow the extraction of relevant information and minimize spurious sources of variation (systematic and/or noisy) that might be present [52,62]. Therefore, the fundamental question is how to get the best out of these complex and unwieldy sets of numbers [63]. Chemometrics is the discipline that allow to tackle the wide range of challenges posed by such degree of complexity [63,64] and, in fact, constitutes an integral part of the of the non-targeted analytical workflow [2,61]. In its arsenal, there are methods to deal with all steps of analytical procedures, starting from the design of an experiment, through extraction of information, to the final decision making [65].

After its origin in the 1960s, the use of chemometrics has increased steeply in the last 20 years thanks to the enhanced processing capacity of affordable computers. The profession of chemometrician has spread accordingly and many research groups have included a data analysist in the team. At first, chemometrician were often computer-oriented scientists becoming active in data analysis and modelling. However, as more people heard the word "chemometrics", more wanted to use it. Nevertheless, the modern fast-paced and competitive society, often does not give the time to learn about the computational and statistical basis of chemometrics methods; scientist are demanded to use the methods "immediately". The spreading of ready-to-use toolboxes with user-friendly interfaces (SIMCA, PLS Toolbox, Unscrambler, Pirouette), have led to an enormous growth in the number of people ordinarily dealing with chemometrics in their work [66,67]. Yet, most application scientists view data analysis as the last step to be carried out within a study, with the aim of producing some nice-looking plots to be used in a paper. In addition, commercial software companies advertise their all-purpose packages where everything can be solved by paying for a licence and attending a training course of few afternoons. As a result, many of the today's chemometrics practitioners (called "Friday-afternoon chemometrician" by Brereton in its "Short history of chemometrics" [67]) do not possess an adequate knowledge of basic statistics and mathematical modelling. It is estimated that around 2% of people encountering chemometrics in their research can be considered real experts [67]. However, data mining is an extremely skilled task requiring years of practice and this gap between users and experts may represents a serious problem since application and interpretation of multivariate data is brimmed with pitfalls for non-statisticians [47,67]. The first consequence of this today's use of chemometrics is that sophisticated techniques are often applied by uninitiated scientists who do not have a fundamental knowledge of the capabilities and the limits of the chosen method. As a result, literally thousands of incorrect data treatments and/or inconsistent conclusions have been, and are being published [47]. On the

other hand, many novel efficient methods are not applied in practice just because they are not implemented in the commercially-available software [47].

After these considerations about the modern use of chemometrics, the present section aims at highlighting some of the common misunderstandings and mistakes related to the use of chemometrics in food authentication studies, from raw data preprocessing to model interpretation and validation, that can be easily encountered in scientific paper published in literature. Many others still frequently occur and further details can be found in the several excellent reviews [1,47,65–70].

Sample size. Even though is not a step of the data analysis workflow, the sample collection – thus, its size - may strongly affect the multivariate model performance. In fact, the use of large and representative sample sets can definitely improve the model robustness over analytical and biological factors (see **Chapter 1**). In most of the cases, the more samples are used during the model training, the more accurate predictions will be obtained. However, authentic reference materials may not always be readily accessible. To overcome the limited samples availability for model construction and validation, authors often resort to cross-validation and/or resampling procedures in order to get some model diagnostic indicators to be included in their manuscript and please the referees and editors. To make matters worse, such practice is often accepted uncritically by the literature. A reasonable number of samples is vital to draw any statistically-relevant conclusions and increase the confidence in the final outcome. In fact, by small sample sizes, validated misclassification rates can be very sensitive to the choice of segmentation. Hence, it can be difficult to assess whether an obtained rate of misclassification is substantially biased relative to random results. If you toss a coin ten times, and obtain 8 heads and 2 tails, does that imply the coin is biased? [68,69] In this regard, McGrath et al. recommended a minimum of 200 samples for each class using spectroscopic methods [1]. However, while it can be agreed that a substantial portion of literature may not use large enough sample sets to develop and validate a robust non-targeted method, the minimum sample number cannot be defined *a priori*, but must be evaluated on a case by case basis. It depends upon various factors, such as the applicability statement, problem under investigation, strategy for data evaluation, sample groups, etc. For instance, in multi-class problems (e.g. botanical/geographical origin) all the considered categories must be meaningfully defined and sampled in a fully representative way [48].

Data preprocessing. The majority of chemometrics methods are general, which means that they can be applied to any type of analytical experiment and to any type of instrumental output. However, there are problems associated with specific sort of signals which require special treatment [52]. For this reason, after the instrumental acquisition, the first step of the data treatment is normally represented by the data preprocessing (or data preparation) in which raw data is transformed to ‘‘cleaned’’ data. Unwanted variation

(e.g. instrumental and experimental artefacts) are removed, so that pre-processed data is better suited to the data analysis goals. On the other side, if not properly performed, pre-processing can also introduce unwanted variation in the data. For these reasons, within the fingerprinting workflow, the data preparation is a particularly critical step that may strongly affect the final outcome [71].

Depending on the analysis' goal and the different properties of the data, many different pre-processing methods have been developed. The choice of the optimal pre-processing method, or combination of methods, requires very careful thought as their effect are not transparent when dealing with large data sets [52]. Lot of excellent scientific material has been published on this topic [45,52,71–74]. However, here too, there are no clear-cut guidelines when to use or to avoid certain pre-processing methods; thus, it is still largely impossible to robustly predict whether a certain data pre-treatment brings the analysis goal closer [52]. Only with the knowledge of the system studied and the principles of the measurements performed can well-suited methods be best chosen [65].

Choose the appropriate classification technique. Getting meaningful results requires not only meaningful data but also a reasonable understanding of the theoretical principles behind the chosen analysis [69]. A common mistake regards the choice of a chemometrics algorithm to be used in the non-targeted analytical workflow. Discriminant analysis (DA) techniques perfectly suit tasks of discrimination between multiple sample groups and pinpointing of the statistically-relevant features, e.g. differentiation of samples originated from Region 1 and Region 2. However, confirming that a specific sample truly originated from Region 1 is a different matter. There, the general properties of the samples from Region 1 have to be characterized, independently of other classes and region. Indeed, in real-world authentication contexts, no information is normally available about the alternative classes to which the tested item may belong. For this reasons, although successfully applied in many 'omics' applications, several authors have defined discriminant analysis methods inappropriate for solving food authenticity problems [48,75,76]. In the latter, the goal is typically to establish whether the analysed sample is compliant, or not, with a defined reference standard and class-modelling approaches (e.g. UNEQ, SIMCA, OCPLS, etc.) should be preferred.

Analysts often confuse authentication task with discrimination problems and it is common practice to investigate the potential of a new application by applying DA techniques to correctly assign each sample to its belonging category [77–87]. As a result, the literature has been "filled" with proof-of-concept studies that demonstrates the feasibility of the employed approach, but do not reproduce real-world authentication conditions. For instance, in the review of Riedl et al., 42 food authentication studies were presented [21]. In total, 56 chemometric methods were applied; 46 of which were various discrimination methods, 5 one-class classifier techniques and 5 unsupervised Principal Component Analysis.

In addition to this, several published studies have been devoted to the comparison of classification algorithms (such as DA and class-modelling techniques) according to the achieved success rates - e.g. one method exhibits 90% success rate and the other 93%, so the latter is viewed as better). In this regard, there is a wide-spread opinion that PLS-DA (discriminant analysis) "may go further" than SIMCA (class modelling) as it better separates the various classes. Yet, it is not consistent to compare methods that employ various amounts of modelling information [76]. Moreover, the model classification/discrimination performance dramatically depends on a number of aspects, such as the nature of the investigated data, adopted validation strategy, actual independence of the independent test set, pre-treatment and pre-processing methods used, etc. [67].

Objective assessment of the Model Performance. A problem that often arises when multivariate statistical models are developed upon small sample sets is the potential overestimation of the algorithm's capabilities due to model "overfitting" issues. Overfitting phenomena essentially occur either when irrelevant predictors and/or components are included in the model or when modelling approaches more flexible than necessary are applied. If the algorithm is too complex or flexible (e.g. not properly regularized), it can end up "learning" from the noise instead of the actual signals; thus it fits unusually well the training dataset, yet it will poorly fit with new datasets [88]. It must be stressed that chemometrics algorithms are designed to associate the training data with the outcome variable and this will always be accomplished. What has to be evaluated is the relevance of such relationship, which strongly depends on several factors, such as the size of the sample set, the optimum number of variables/components selected, validation strategy, etc. For instance, if an excessive number of variables is retained, too much redundancy in the independent variables is modelled. On the other hand, the use of few components will lead to an "underfitted" models - not able to capture the variability in the dataset [61].

The best way to ensure the absence of overfitting issues is through the implementation of integrated validation strategies, which guarantee reliable results and the model robustness for application. As strongly recommended by Riedl et al., a "system challenge approach" should be implemented as validation scheme, where instrumental, spatial and temporal variation in non-targeted fingerprints are considered to ensure the model long-term stability and the absence of overfitting issues [21]. Even so, the same authors have identified the improper (or even lack of) stringent validation strategies as the major default of the current food authenticity literature. In fact, although statistical model validation is ordinarily performed within most of the studies, external validation and system challenge using independent test sets are rarely applied [21].

References

[1] T.F. McGrath, S.A. Haughey, J. Patterson, C. Fauhl-Hassek, J. Donarski, M. Alewijn, S. van Ruth, C.T. Elliott, What are the scientific challenges in moving from targeted to non-targeted methods for food fraud testing and how can they be addressed? – Spectroscopy case study, Trends Food Sci. Technol. 76 (2018) 38–55. https://doi.org/10.1016/j.tifs.2018.04.001.

[2] D.I. Ellis, V.L. Brewster, W.B. Dunn, J.W. Allwood, A.P. Golovanov, R. Goodacre, Fingerprinting food: Current technologies for the detection of food adulteration and contamination, Chem. Soc. Rev. 41 (2012) 5706–5727. https://doi.org/10.1039/c2cs35138b.

[3] M. Peris, L. Escuder-Gilabert, A 21st century technique for food control: Electronic noses, Anal. Chim. Acta. 638 (2009) 1–15. https://doi.org/10.1016/j.aca.2009.02.009.

[4] E. Borràs, J. Ferré, R. Boqué, M. Mestres, L. Aceña, O. Busto, Data fusion methodologies for food and beverage authentication and quality assessment - A review, Anal. Chim. Acta. 891 (2015) 1–14. https://doi.org/10.1016/j.aca.2015.04.042.

[5] J. Newman, T. Egan, N. Harbourne, D. O'Riordan, J.C. Jacquier, M. O'Sullivan, Correlation of sensory bitterness in dairy protein hydrolysates: Comparison of prediction models built using sensory, chromatographic and electronic tongue data, Talanta. 126 (2014) 46–53. https://doi.org/10.1016/j.talanta.2014.03.036.

[6] S. Shakoorjavan, S. Akbari, M.H. Kish, M. Akbari, Correlation of sensory analysis with a virtual sensor array data for odour diagnosis of fragrant fabrics, Meas. J. Int. Meas. Confed. 90 (2016) 396–403. https://doi.org/10.1016/j.measurement.2016.04.074.

[7] H. Tian, F. Li, L. Qin, H. Yu, X. Ma, Quality Evaluation of Beef Seasonings Using Gas Chromatography-Mass Spectrometry and Electronic Nose: Correlation with Sensory Attributes and Classification According to Grade Level, Food Anal. Methods. 8 (2015) 1522–1534. https://doi.org/10.1007/s12161-014-0031-4.

[8] D. Cavanna, S. Zanardi, C. Dall'Asta, M. Suman, Ion mobility spectrometry coupled to gas chromatography: A rapid tool to assess eggs freshness, Food Chem. 271 (2019) 691–696. https://doi.org/10.1016/j.foodchem.2018.07.204.

[9] T. Damiani, D. Cavanna, A. Serani, C. Dall'Asta, M. Suman, GC-IMS and FGC-Enose fingerprint as screening tools for revealing extra virgin olive oil blending with soft-refined olive oils: A feasibility study, Microchem. J. 159 (2020) 105374. https://doi.org/10.1016/j.microc.2020.105374.

[10] M. Lin, B.A. Rasco, A.G. Cavinato, M. Al-Holy, Infrared (IR) Spectroscopy-Near-Infrared Spectroscopy and Mid-Infrared Spectroscopy, in: Infrared Spectrosc. Food Qual. Anal. Control, Elsevier Inc., 2009: pp. 119–143. https://doi.org/10.1016/B978-0-12-374136-3.00006-7.

[11] C. Black, O.P. Chevallier, C.T. Elliott, The current and potential applications of Ambient Mass Spectrometry in detecting food fraud, TrAC - Trends Anal. Chem. 82 (2016) 268–278. https://doi.org/10.1016/j.trac.2016.06.005.

[12] S. Medina, J.A. Pereira, P. Silva, R. Perestrelo, J.S. Câmara, Food fingerprints – A valuable tool to monitor food authenticity and safety, Food Chem. 278 (2019) 144–162. https://doi.org/10.1016/j.foodchem.2018.11.046.

[13] T.H. Kuo, E.P. Dutkiewicz, J. Pei, C.C. Hsu, Ambient Ionization Mass Spectrometry Today and Tomorrow: Embracing Challenges and Opportunities, Anal. Chem. 92 (2020) 2353–2363. https://doi.org/10.1021/acs.analchem.9b05454.

[14] C. Lin, X. Chen, L. Jian, C. Shi, X. Jin, G. Zhang, Determination of grain protein content by near-infrared spectrometry and multivariate calibration in barley, Food Chem. 162 (2014) 10–15. https://doi.org/10.1016/j.foodchem.2014.04.056.

[15] W. Von Der Ohe, L. Persano Oddo, M.L. Piana, M. Morlot, P. Martin, Harmonized methods of melissopalynology, Apidologie. 35 (2004) S18–S25. https://doi.org/10.1051/apido:2004050.

[16] S. Soares, J.S. Amaral, M.B.P.P. Oliveira, I. Mafra, A Comprehensive Review on the Main Honey Authentication Issues: Production and Origin, Compr. Rev. Food Sci. Food Saf. 16 (2017) 1072–1100. https://doi.org/10.1111/1541-4337.12278.

[17] R. Bro, A.K. Smilde, Principal component analysis, Anal. Methods. 6 (2014) 2812–2831. https://doi.org/10.1039/c3ay41907j.

[18] G. Downey, Vibrational spectroscopy in studies of food origin, in: New Anal. Approaches Verif. Orig. Food, Elsevier Ltd, 2013: pp. 94–116. https://doi.org/10.1533/9780857097590.2.94.

[19] J.A. Fernández Pierna, O. Abbas, V. Baeten, P. Dardenne, A Backward Variable Selection method for PLS regression (BVSPLS), Anal. Chim. Acta. 642 (2009) 89–93. https://doi.org/10.1016/j.aca.2008.12.002.

[20] S. Esslinger, J. Riedl, C. Fauhl-Hassek, Potential and limitations of non-targeted fingerprinting for authentication of food in official control, Food Res. Int. 60 (2014) 189–204. https://doi.org/10.1016/j.foodres.2013.10.015.

[21] J. Riedl, S. Esslinger, C. Fauhl-Hassek, Review of validation and reporting of non-targeted fingerprinting approaches for food authentication, Anal. Chim. Acta. 885 (2015) 17–32. https://doi.org/10.1016/j.aca.2015.06.003.

[22] T. De Nardo, C. Shiroma-Kian, Y. Halim, D. Francis, L.E. Rodriguez-Saona, Rapid and simultaneous determination of lycopene and β-carotene contents in tomato juice by infrared spectroscopy, J. Agric. Food Chem. 57 (2009) 1105–1112. https://doi.org/10.1021/jf802920z.

[23] C. Li, E. Li, M. Ma, X. Liu, J. You, Y. Wu, W. Jiang, Z. Liu, Y. Qin, Simultaneous determination of six alkaloids in tobacco and tobacco products by direct analysis of real-time triple quadrupole mass spectrometry with a modified pretreatment method, J. Sep. Sci. 43 (2020) 1603–1613. https://doi.org/10.1002/jssc.201900946.

[24] M.Y. Talpur, H. Kara, S.T.H. Sherazi, H.F. Ayyildiz, M. Topkafa, F.N. Arslan, S. Naz, F. Durmaz, Sirajuddin, Application of multivariate chemometric techniques for simultaneous determination of five parameters of cottonseed oil by single bounce attenuated total reflectance Fourier transform infrared spectroscopy, Talanta. 129 (2014) 473–480. https://doi.org/10.1016/j.talanta.2014.04.002.

[25] W. Dong, Y. Ni, S. Kokot, A near-infrared reflectance spectroscopy method for direct analysis of several chemical components and properties of fruit, for example, Chinese hawthorn, J. Agric. Food Chem. 61 (2013) 540–546. https://doi.org/10.1021/jf305272s.

[26] M. Ottavian, P. Facco, L. Fasolato, E. Novelli, M. Mirisola, M. Perini, M. Barolo, Use of near-infrared spectroscopy for fast fraud detection in seafood: Application to the authentication of wild European sea bass (Dicentrarchus labrax), J. Agric. Food Chem. 60 (2012) 639–648. https://doi.org/10.1021/jf203385e.

[27] G. Tsiliki, S. Kossida, Fusion methodologies for biomedical data, J. Proteomics. 74 (2011) 2774–2785. https://doi.org/10.1016/j.jprot.2011.07.001.

[28] A. Biancolillo, R. Boqué, M. Cocchi, F. Marini, Data Fusion Strategies in Food Analysis, in: Data Handl. Sci. Technol., Elsevier Ltd, 2019: pp. 271–310. https://doi.org/10.1016/B978-0-444-63984-4.00010-7.

[29] J.R. Koza, F.H. Bennett, D. Andre, M.A. Keane, Automated Design of Both the Topology and Sizing of Analog Electrical Circuits Using Genetic Programming, in: J.S. Gero, F. Sudweeks (Eds.), Artif. Intell. Des. '96, Springer, Dordrecht, 1996: pp. 151–170. https://doi.org/10.1007/978-94-009-0279-4_9.

[30] R.N. Feudale, N.A. Woody, H. Tan, A.J. Myles, S.D. Brown, J. Ferré, Transfer of multivariate calibration models: A review, Chemom. Intell. Lab. Syst. 64 (2002) 181–192. https://doi.org/10.1016/S0169-7439(02)00085-0.

[31] C. McVey, T.F. McGrath, S.A. Haughey, C.T. Elliott, A rapid food chain approach for authenticity screening: The development, validation and transferability of a chemometric model using two handheld near infrared spectroscopy (NIRS) devices, Talanta. 222 (2021) 121533. https://doi.org/10.1016/j.talanta.2020.121533.

[32] MilkoScanTM FT3 – Foss Analytics. https://www.fossanalytics.com/en/products/milkoscan-ft3 (accessed August 22, 2020).

[33] Infratec NOVA – Foss Analytics. https://www.fossanalytics.com/en/products/infratec-nova (accessed August 22, 2020).

[34] Wine Profiling - Bruker. https://www.bruker.com/products/mr/nmr-food-screening/wine-profiling.html (accessed August 18, 2020).

[35] Juice Profiling - Bruker. https://www.bruker.com/products/mr/nmr-food-screening/juice-profiling.html (accessed August 22, 2020).

[36] Honey Profiling - Bruker. https://www.bruker.com/products/mr/nmr-food-screening/honey-profiling-module-of-the-nmr-foodscreener.html (accessed August 22, 2020).

[37] S. Bijttebier, A. Van der Auwera, K. Foubert, S. Voorspoels, L. Pieters, S. Apers, Bridging the gap between comprehensive extraction protocols in plant metabolomics studies and method validation, Anal. Chim. Acta. 935 (2016) 136–150. https://doi.org/10.1016/j.aca.2016.06.047.

[38] T. Frank, B. Scholz, S. Peter, K.H. Engel, Metabolite profiling of barley: Influence of the malting process, Food Chem. 124 (2011) 948–957. https://doi.org/10.1016/j.foodchem.2010.07.034.

[39] M.A. Salem, J. Jüppner, K. Bajdzienko, P. Giavalisco, Protocol: A fast, comprehensive and reproducible one-step extraction method for the rapid preparation of polar and semi-polar metabolites, lipids, proteins, starch and cell wall polymers from a single sample, Plant Methods. 12 (2016) 45. https://doi.org/10.1186/s13007-016-0146-2.

[40] M. Creydt, M. Fischer, Omics approaches for food authentication, Electrophoresis. 39 (2018) 1569–1581. https://doi.org/10.1002/elps.201800004.

[41] F. Maltese, F. Van Der Kooy, R. Verpoorte, Solvent derived artifacts in natural products chemistry, Nat. Prod. Commun. 4 (2009) 447–454. https://doi.org/10.1177/1934578x0900400326.

[42] C. Sauerschnig, M. Doppler, C. Bueschl, R. Schuhmacher, Methanol generates numerous artifacts during sample extraction and storage of extracts in metabolomics research, Metabolites. 8 (2018). https://doi.org/10.3390/metabo8010001.

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

[44] H.K. Kim, R. Verpoorte, Sample preparation for plant metabolomics, Phytochem. Anal. 21 (2010) 4–13. https://doi.org/10.1002/pca.1188.

[45] T. Fearn, C. Riccioli, A. Garrido-Varo, J.E. Guerrero-Ginel, On the geometry of SNV and MSC, Chemom. Intell. Lab. Syst. 96 (2009) 22–26. https://doi.org/10.1016/j.chemolab.2008.11.006.

[46] A. Savitzky, M.J.E. Golay, Smoothing and Differentiation of Data by Simplified Least Squares Procedures, Anal. Chem. 36 (1964) 1627–1639. https://doi.org/10.1021/ac60214a047.

[47] E. Pretsch, C.L. Wilkins, Use and abuse of chemometrics, TrAC - Trends Anal. Chem. 25 (2006) 1045. https://doi.org/10.1016/j.trac.2006.10.011.

[48] P. Oliveri, Class-modelling in food analytical chemistry: Development, sampling, optimisation and validation issues – A tutorial, Anal. Chim. Acta. 982 (2017) 9–19. https://doi.org/10.1016/j.aca.2017.05.013.

[49] S. Naz, M. Vallejo, A. García, C. Barbas, Method validation strategies involved in non-targeted metabolomics, J. Chromatogr. A. 1353 (2014) 99–105. https://doi.org/10.1016/j.chroma.2014.04.071.

[50] J. Korpela, E. Oikarinen, K. Puolamäki, A. Ukkonen, Multivariate Confidence Intervals, Proc. 17th SIAM Int. Conf. Data Mining, SDM 2017. (2017) 696–704. http://arxiv.org/abs/1701.05763 (accessed August 22, 2020).

[51] S. Adachi, Rigid geometry solves "curse of dimensionality" effects in clustering methods: An application to omics data, PLoS One. 12 (2017) e0179180. https://doi.org/10.1371/journal.pone.0179180.

[52] J. Engel, J. Gerretzen, E. Szymańska, J.J. Jansen, G. Downey, L. Blanchet, L.M.C. Buydens, Breaking with trends in pre-processing?, TrAC - Trends Anal. Chem. 50 (2013) 96–106. https://doi.org/10.1016/j.trac.2013.04.015.

[53] Appendix XVIII: USP Guidance on Developing and Validating Non-Targeted Methods for Adulteration Detection, (2018). http://members.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/Food_Auth/2019USPC-Appendix XVIII_Guidance_on_Developing_and_Validating_Non-Targeted_Methods_for_Adulteration_Detection-FCC_Forum_December_2018.pdf. (accessed September 17, 2020).

[54] T. Pihlström, A. Valverde, S. Reynolds, A.R. Fernández-Alba, H. Mol, S. Chemist, M. Jezussek Bavarian Health, D. Stajnbaher, G. Valenciana, S. Paula Medina, M. Erecius Poulsen, O. Malato, C. Ferrer Amate, Method Validation and Quality Control Procedures for Pesticide Residues Anaysis in Food and Feed, 2019. https://www.eurl-pesticides.eu/docs/public/tmplt_article.asp?CntID=727 (accessed May 9, 2020).

[55] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Off. J. L221/8 (2002).

[56] L.W. Sumner, A. Amberg, D. Barrett, M.H. Beale, R. Beger, C.A. Daykin, T.W.M. Fan, O. Fiehn, R. Goodacre, J.L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A.N. Lane, J.C. Lindon, P. Marriott, A.W. Nicholls, M.D. Reily, J.J. Thaden, M.R. Viant, Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI), Metabolomics. 3 (2007) 211–221. https://doi.org/10.1007/s11306-007-0082-2.

[57] O. Fiehn, L.W. Sumner, S.Y. Rhee, J. Ward, J. Dickerson, B.M. Lange, G. Lane, U. Roessner, R. Last, B. Nikolau, Minimum reporting standards for plant biology context information in metabolomic studies, Metabolomics. 3 (2007) 195–201. https://doi.org/10.1007/s11306-007-0068-0.

[58] C.F. Taylor, D. Field, S.A. Sansone, J. Aerts, R. Apweiler, M. Ashburner, C.A. Ball, P.A. Binz, M. Bogue, T. Booth, A. Brazma, R.R. Brinkman, A. Michael Clark, E.W. Deutsch, O. Fiehn, J. Fostel, P. Ghazal, F. Gibson, T. Gray, G. Grimes, J.M. Hancock, N.W. Hardy, H. Hermjakob, R.K. Julian, M. Kane, C. Kettner, C. Kinsinger, E. Kolker, M. Kuiper, N. Le Novère, J. Leebens-Mack, S.E. Lewis, P. Lord, A.M. Mallon, N. Marthandan, H. Masuya, R. McNally, A. Mehrle, N. Morrison, S. Orchard, J. Quackenbush, J.M. Reecy, D.G. Robertson, P. Rocca-Serra, H. Rodriguez, H. Rosenfelder, J. Santoyo-Lopez, R.H. Scheuermann, D. Schober, B. Smith, J. Snape, C.J. Stoeckert, K. Tipton, P. Sterk, A. Untergasser, J. Vandesompele, S. Wiemann, Promoting coherent minimum reporting guidelines for biological and biomedical investigations: The MIBBI project, Nat. Biotechnol. 26 (2008) 889–896. https://doi.org/10.1038/nbt.1411.

[59] H. Ćwiek-Kupczyńska, T. Altmann, D. Arend, E. Arnaud, D. Chen, G. Cornut, F. Fiorani, W. Frohmberg, A. Junker, C. Klukas, M. Lange, C. Mazurek, A. Nafissi, P. Neveu, J. Oeveren, C. Pommier, H. Poorter, P. Rocca-Serra, S.A. Sansone, U. Scholz, M. Schriek, Ü. Seren, B. Usadel, S. Weise, P. Kersey, P. Krajewski, Measures for interoperability of phenotypic data: Minimum information requirements and formatting, Plant Methods. 12 (2016) 44. https://doi.org/10.1186/s13007-016-0144-4.

[60] T. Fearn, Standardisation and Calibration Transfer for near Infrared Instruments: A Review, J. Near Infrared Spectrosc. 9 (2001) 229–244. https://doi.org/10.1255/jnirs.309.

[61] J.J. Roberts, D. Cozzolino, An Overview on the Application of Chemometrics in Food Science and Technology—An Approach to Quantitative Data Analysis, Food Anal. Methods. 9 (2016) 3258–3267. https://doi.org/10.1007/s12161-016-0574-7.

[62] M.M.W.B. Hendriks, F.A. va. Eeuwijk, R.H. Jellema, J.A. Westerhuis, T.H. Reijmers, H.C.J. Hoefsloot, A.K. Smilde, Data-processing strategies for metabolomics studies, TrAC - Trends Anal. Chem. 30 (2011) 1685–1698. https://doi.org/10.1016/j.trac.2011.04.019.

[63] E.K. Kemsley, M. Defernez, F. Marini, Multivariate statistics: Considerations and confidences in food authenticity problems, Food Control. 105 (2019) 102–112. https://doi.org/10.1016/j.foodcont.2019.05.021.

[64] D. Granato, P. Putnik, D.B. Kovačević, J.S. Santos, V. Calado, R.S. Rocha, A.G. Da Cruz, B. Jarvis, O.Y. Rodionova, A. Pomerantsev, Trends in Chemometrics: Food Authentication, Microbiology, and Effects of Processing, Compr. Rev. Food Sci. Food Saf. 17 (2018) 663–677. https://doi.org/10.1111/1541-4337.12341.

[65] M. Daszykowski, B. Walczak, Use and abuse of chemometrics in chromatography, TrAC - Trends Anal. Chem. 25 (2006) 1081–1096. https://doi.org/10.1016/j.trac.2006.09.001.

[66] C.A. Nunes, V.O. Alvarenga, A. de Souza Sant'Ana, J.S. Santos, D. Granato, The use of statistical software in food science and technology: Advantages, limitations and misuses, Food Res. Int. 75 (2015) 270–280. https://doi.org/10.1016/j.foodres.2015.06.011.

[67] R.G. Brereton, A short history of chemometrics: a personal view, J. Chemom. 28 (2014) 749–760. https://doi.org/10.1002/cem.2633.

[68] R.G. Brereton, Consequences of sample size, variable selection, and model validation and optimisation, for predicting classification ability from analytical data, TrAC - Trends Anal. Chem. 25 (2006) 1103–1111. https://doi.org/10.1016/j.trac.2006.10.005.

[69] K. Kjeldahl, R. Bro, Some common misunderstandings in chemometrics, J. Chemom. 24 (2010) 558–564. https://doi.org/10.1002/cem.1346.

[70] D. Cavanna, L. Righetti, C. Elliott, M. Suman, The scientific challenges in moving from targeted to non-targeted mass spectrometric methods for food fraud analysis: A proposed validation workflow to bring about a harmonized approach, Trends Food Sci. Technol. 80 (2018) 223–241. https://doi.org/10.1016/j.tifs.2018.08.007.

[71] R.A. van den Berg, H.C.J. Hoefsloot, J.A. Westerhuis, A.K. Smilde, M.J. van der Werf, Centering, scaling, and transformations: Improving the biological information content of metabolomics data, BMC Genomics. 7 (2006) 142. https://doi.org/10.1186/1471-2164-7-142.

[72] T. De Meyer, D. Sinnaeve, B. Van Gasse, E.R. Rietzschel, M.L. De Buyzere, M.R. Langlois, S. Bekaert, J.C. Martins, W. Van Criekinge, Evaluation of standard and advanced preprocessing methods for the univariate analysis of blood serum 1H-NMR spectra, Anal. Bioanal. Chem. 398 (2010) 1781–1790. https://doi.org/10.1007/s00216-010-4085-x.

[73] X. Wei, X. Shi, S. Kim, L. Zhang, J.S. Patrick, J. Binkley, C. McClain, X. Zhang, Data preprocessing method for liquid chromatography-mass spectrometry based metabolomics, Anal. Chem. 84 (2012) 7963–7971. https://doi.org/10.1021/ac3016856.

[74] B. Horn, S. Esslinger, M. Pfister, C. Fauhl-Hassek, J. Riedl, Non-targeted detection of paprika adulteration using mid-infrared spectroscopy and one-class classification – Is it data preprocessing that makes the performance?, Food Chem. 257 (2018) 112–119. https://doi.org/10.1016/j.foodchem.2018.03.007.

[75] O.Y. Rodionova, A. V. Titova, A.L. Pomerantsev, Discriminant analysis is an inappropriate method of authentication, TrAC - Trends Anal. Chem. 78 (2016) 17–22. https://doi.org/10.1016/j.trac.2016.01.010.

[76] D. Granato, P. Putnik, D.B. Kovačević, J.S. Santos, V. Calado, R.S. Rocha, A.G. Da Cruz, B. Jarvis, O.Y. Rodionova, A. Pomerantsev, Trends in Chemometrics: Food Authentication, Microbiology, and Effects of Processing, Compr. Rev. Food Sci. Food Saf. 17 (2018) 663–677. https://doi.org/10.1111/1541-4337.12341.

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

[78] S. Hennessy, G. Downey, C. O'Donnell, Multivariate Analysis of Attenuated Total Reflection—Fourier Transform Infrared Spectroscopic Data to Confirm the Origin of Honeys, Appl. Spectrosc. 62 (2008) 1115–1123. https://doi.org/10.1366/000370208786049033.

[79] A.S. Wilde, S.A. Haughey, P. Galvin-King, C.T. Elliott, The feasibility of applying NIR and FT-IR fingerprinting to detect adulteration in black pepper, Food Control. 100 (2019) 1–7. https://doi.org/10.1016/j.foodcont.2018.12.039.

[80] S. Hennessy, G. Downey, C.P. O donnell, Attempted confirmation of the provenance of corsican PDO honey using FT-IR spectroscopy and multivariate data analysis, J. Agric. Food Chem. 58 (2010) 9401–9406. https://doi.org/10.1021/jf101500n.

[81] T. Woodcock, G. Downey, C.P. O'Donnell, Near infrared spectral fingerprinting for confirmation of claimed PDO provenance of honey, Food Chem. 114 (2009) 742–746. https://doi.org/10.1016/j.foodchem.2008.10.034.

[82] T. Woodcock, G. Downey, J.D. Kelly, C. O'Donnell, Geographical classification of honey samples by near-infrared spectroscopy: A feasibility study, J. Agric. Food Chem. 55 (2007) 9128–9134. https://doi.org/10.1021/jf072010q.

[83] E. Wielogorska, O. Chevallier, C. Black, P. Galvin-King, M. Delêtre, C.T. Kelleher, S.A. Haughey, C.T. Elliott, Development of a comprehensive analytical platform for the detection and quantitation of food fraud using a biomarker approach. The oregano adulteration case study, Food Chem. 239 (2018) 32–39. https://doi.org/10.1016/j.foodchem.2017.06.083.

[84] C. Black, S.A. Haughey, O.P. Chevallier, P. Galvin-King, C.T. Elliott, A comprehensive strategy to detect the fraudulent adulteration of herbs: The oregano approach, Food Chem. 210 (2016) 551–557. https://doi.org/10.1016/j.foodchem.2016.05.004.

[85] S.A. Haughey, P. Galvin-King, Y.C. Ho, S.E.J. Bell, C.T. Elliott, The feasibility of using near infrared and Raman spectroscopic techniques to detect fraudulent adulteration of chili powders with Sudan dye, Food Control. 48 (2015) 75–83. https://doi.org/10.1016/j.foodcont.2014.03.047.

[86] E. Achten, D. Schütz, M. Fischer, C. Fauhl-Hassek, J. Riedl, B. Horn, Classification of Grain Maize (Zea mays L.) from Different Geographical Origins with FTIR Spectroscopy—a Suitable Analytical Tool for Feed Authentication?, Food Anal. Methods. 12 (2019) 2172–2184. https://doi.org/10.1007/s12161-019-01558-9.

[87] D. Cavanna, C. Loffi, C. Dall'Asta, M. Suman, A non-targeted high-resolution mass spectrometry approach for the assessment of the geographical origin of durum wheat, Food Chem. 317 (2020) 126366. https://doi.org/10.1016/j.foodchem.2020.126366.

[88] D.M. Hawkins, The Problem of Overfitting, J. Chem. Inf. Comput. Sci. 44 (2004) 1–12. https://doi.org/10.1021/ci0342472.

4. Conclusions and outlook

The inadequacy of current safety ploys for ensuring food integrity and authenticity has been the starting point of the present thesis. The transition from conventional reactive to modern proactive approaches have been thoroughly discussed from the analytical perspective, with the main focus being the development and validation of non-targeted fingerprinting methods. In this regard, three scientific studies have been carried out during this PhD programme and presented in **Section 2**. This has allowed the author to gain hands-on experience, as well as a proper understanding of both advantages and limitations of such testing approaches. This last section summarizes, according to the author's personal view, the current state and future perspectives of this food analysis field, highlighting the urgent needs for bridging the major gaps with respect to traditional food testing methods.

The promise of non-targeted methods for food fraud detection is great and research efforts in this area have increased massively in recent years. Yet, the proportion of non-targeted methods adopted in routine applications is still comparatively low [1]. Nowadays, many companies already possess the infrastructure to develop and use these methods more broadly; however, they may still have important questions about how to implement these novel approaches. Confusion in terminology and lack of consistent information about the procedures for proper method development and validation are certainly among the main reasons for the somewhat slow adoption of these techniques by food industries and regulatory agencies [2]. For instance, despite ranking as one of the biggest concerns for the entire food sector [3], even the definition of "food fraud" is not univocal; in their review, Robson et al. revealed twelve different definitions of "food fraud" in peer-reviewed literature, while further fifteen definitions have been found in standards and publications from government bodies and stakeholders [4]. Although the majority of definitions agree that food fraud is an intentional deception for economic gain using food, such lack of systematic terminology makes comprehensive literature searches harder for users and researchers not-familiar with the field [5]. Besides, from a more pragmatic standpoint, standardized analytical protocols would promote data comparability and exchange between laboratories. This would not only assist the development of reliable and fit-for-purpose analytical methods, but the availability of joint comprehensive databases and transferable models (ideally between different devices) would definitely raise their applicability from the company-level to a global scale. For these reasons, harmonization of both terminology and methodologies (ultimately resulting in legislation) constitutes the major priorities for the scientific community and the starting point for boosting the implementation of non-targeted strategies in routine analysis. As mentioned in **Section 3.2**, the recently-published U.S. Pharmacopeia guidance has represented a great step forward in

this respect. However, in the author's opinion, if the intention is to produce legally uncontestable outputs, more application- and/or method-specific guidelines would be of greater help. Methodological steps, such as adequate system challenges, must be developed to identify systematic variations and calibration procedures to ensure data consistency and allow the adaption of existing models to samples with new properties (e.g. harvest year, storage, etc.). Nevertheless, this is mostly dependent on the problem under investigation and the employed analytical technique(s). For instance, exogenous factors that can potentially cause fingerprint deviations over the long term are certainly different for vibrational spectroscopy equipment, respect to ambient mass spectrometry platforms; or, as said in **Section 1.4**, the relevant external influences (e.g. weather conditions) to be considered within the method development will strictly depend, among other factors, on the employed detection principle (e.g. metabolic fingerprinting or DNA sequencing).

From the academic perspective, even though validation constitute the last step of the workflow, it should be among the first concerns before starting the entire process of method development. Questions like "Are the instrumental drift and environmental/seasonal variations (in case of natural products) manageable over the long period? Could this method be transferred out of this laboratory? Does a new user need to re-performer the calibration from the beginning?" should be wondered in the very early stages of the work. Otherwise, the risk is to keep on filling the scientific literature with feasibility studies which, however, will never find practical application in industry environment due to the lack of a proper validation.

At the current stage, it is hard to believe that non-targeted approaches could be used, in the short term, as stand-alone platforms for the unequivocal detection of food fraud. Nevertheless, despite all the drawbacks mentioned within the present thesis, fingerprinting methods continue to be developed for food and other applications because of the significant advantages that a successful model would offers over other candidate analytical methods. While traditional thinking on food fraud detection focuses on whether a known adulterant is present or absent. For non-targeted methods, finding food fraud is a probability mindset and there still remains important space to be employed as rapid screening tools with suspect samples subjected to some form of targeted/confirmatory analysis [2]. As stated in the U.S. Pharmacopeia guidance, "a single 'Atypical' result does not generally provide a sufficient degree of evidence to deem a material as adulterated, but rather should be a trigger for additional analyses to verify the nature of the material [6]. Moreover, even though in many cases a thorough method validation cannot be properly fulfilled (due to the several reasons discussed above), such non-targeted screening could act as a deterrent, discouraging potential fraudster to carry out illicit practices when dealing with the company in question [7]. In fact,the implementation of additional countermeasures at a company-level, even if for internal control only, could motivate other supply chain's stakeholders (e.g. manufacturers, suppliers, distributors) to contribute at maintaining the security of the food chain.

Before closing, a last broad consideration concerning the fight against food fraud is worth to be done. Many articles published on this topic (arguably the majority of those reported in the **References** section), as well as the present thesis itself, essentially adopt solution-based approaches, i.e. a backward process where applications are searched for available technologies. This tendency is particularly emphasized in the food testing sector, where technical advances initially developed for other fields have been adapted afterwards. However, the most effective weapon to combat and mitigate food fraud is through its prevention and, as thoroughly explained by Spink in his book, prevention is a "problem-based" research which starts with the a deep understanding of the problem and its root causes [3]. Research and efforts against food fraud cannot be limited to the mere development of new testing solutions. In contrast, the latter must be part of an integrated systems of proactive management strategies (see **Section 1.2**) and reliable detection methods.

A more comprehensive research approach including other disciplines such as social science, criminology and business decision-making, should be adopted. Particular attention should be placed on the root causes of the problem through a prior vulnerability assessment of certain food commodities and supply chains that create the fraud opportunity [3,8–11]. Subsequently, effective testing methods can be best developed and implemented.

References

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

[2] K. Xie, S. Holroyd, K. Laurvick, S. Gendel, Tackling Food Fraud - Checking What is Not There, (2019). https://www.usp.org/sites/default/files/usp/document/about/newsroom/2019-2-6-twfi-non-targeted-methods-guidance-kenny-xie-final.pdf (accessed October 10, 2020).

[3] J.W. Spink, Food Fraud Prevention, Springer New York, 2019. https://doi.org/10.1007/978-1-4939-9621-6.

[4] K. Robson, M. Dean, S. Haughey, C. Elliott, A comprehensive review of food fraud terminologies and food fraud mitigation guides, Food Control. (2020) 107516. https://doi.org/10.1016/j.foodcont.2020.107516.

[5] J.C. Moore, J. Spink, M. Lipp, Development and Application of a Database of Food Ingredient Fraud and Economically Motivated Adulteration from 1980 to 2010, J. Food Sci. 77 (2012) R118–R126. https://doi.org/10.1111/j.1750-3841.2012.02657.x.

[6] Appendix XVIII: USP Guidance on Developing and Validating Non-Targeted Methods for Adulteration Detection, (2018). http://members.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/Food_Auth/2019USPC-Appendix XVIII_Guidance_on_Developing_and_Validating_Non-Targeted_Methods_for_Adulteration_Detection-FCC_Forum_December_2018.pdf. (accessed September 17, 2020).

[7] T.F. McGrath, S.A. Haughey, J. Patterson, C. Fauhl-Hassek, J. Donarski, M. Alewijn, S. van Ruth, C.T. Elliott, What are the scientific challenges in moving from targeted to non-targeted methods for food fraud testing and how can they be addressed? – Spectroscopy case study, Trends Food Sci. Technol. 76 (2018) 38–55. https://doi.org/10.1016/j.tifs.2018.04.001.

[8] S.M. van Ruth, W. Huisman, P.A. Luning, Food fraud vulnerability and its key factors, Trends Food Sci. Technol. 67 (2017) 70–75. https://doi.org/10.1016/j.tifs.2017.06.017.

[9] I.C.J. Silvis, S.M. van Ruth, H.J. van der Fels-Klerx, P.A. Luning, Assessment of food fraud vulnerability in the spices chain: An explorative study, Food Control. 81 (2017) 80–87. https://doi.org/10.1016/j.foodcont.2017.05.019.

[10] J. Spink, D.L. Ortega, C. Chen, F. Wu, Food fraud prevention shifts the food risk focus to vulnerability, Trends Food Sci. Technol. 62 (2017) 215–220. https://doi.org/10.1016/j.tifs.2017.02.012.

[11] S.M. van Ruth, P.A. Luning, I.C.J. Silvis, Y. Yang, W. Huisman, Differences in fraud vulnerability in various food supply chains and their tiers, Food Control. 84 (2018) 375–381. https://doi.org/10.1016/j.foodcont.2017.08.020.

ANNEX I – Vibrational Spectroscopy

In general terms, "spectroscopy" can be defined as the study of the interaction between matter and electromagnetic radiation. In its turn, electromagnetic radiation is characterized by wavelength, wavenumber and frequency. The wavelength (λ) is the distance over which the wave's shape repeats and is typically expressed in cm or nm; the wavenumber, or spatial frequency, ($\tilde{\upsilon}$) is defined as the number of wavelengths per unit distance (cm^{-1}); the frequency (υ) represents the number of vibration per unit time Hz [1]. The amount of energy carried by an electromagnetic radiation is directly proportional to the photon's electromagnetic frequency:

$$E = h\tilde{\upsilon} = \frac{hc}{\lambda}$$

Where h is the Planck constant ($6.6256 \cdot 10^{-34}\ J \cdot s$) and c is the speed of light in vacuum ($2.998 \cdot 10^{10}\ cm/s$). Therefore, the higher the photon's frequency, the higher its energy. Equivalently, the longer the photon's wavelength, the lower its energy.

The entire distribution of electromagnetic radiation, according to frequency or wavelength, is called electromagnetic spectrum; it covers electromagnetic waves with frequencies ranging from below $1\ Hz$ to above $1025\ Hz$, corresponding to wavelengths from thousands of kilometers down to a fraction of the size of an atomic nucleus. This frequency range is conventionally divided into separate bands; starting from the lower frequencies: radio waves, microwaves, infrared, visible light, ultraviolet, X-rays, and gamma rays (**Figure A1**).

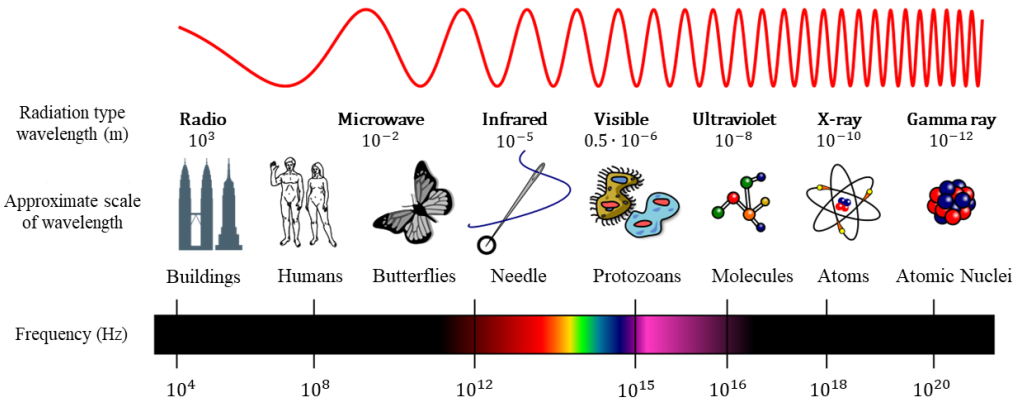


Figure A1. Diagram of the electromagnetic spectrum across the range of frequencies and wavelengths.

Each wavelength bands has different characteristics - especially in terms of interaction with matter - and most of them are somehow used by spectroscopic techniques to study such interactions and obtain relevant information about the sample [2].

Since NIR, FT-MIR and FT-Raman spectroscopy have been employed during the PhD programme, they will be covered in more detail in the following section. As suggested by the name, these techniques use the infrared radiation ($780\ nm < \lambda < 1\ mm$) which, upon interaction with a molecule, might cause changes in both its vibrational and rotational (ro-vibrational) states. Indeed, nuclei are in constant motion and the majority of the population occupies the lowest-energy level ($v = 0$). However, an absorption may occur when the frequency of the incident radiation matches the energy gap between two ro-vibrational states, thus leading to a state transition. The frequencies (or wavelengths) causing such absorption are characteristic of the bond type and, in addition, they are affected by the specific environment in which the molecule is located. Therefore, measurement of the radiation absorption, over an appropriate wavelength (or frequency) range, can potentially provide specific and meaningful information about the analysed sample. "Vibrational spectroscopy" is the term used to describe studies of the interaction between electromagnetic radiation and the vibrational states of atomic nuclei within their respective molecules.

Vibrational energy, like other molecular energies, is quantized and only certain vibrational alterations are permitted by quantum theory. Therefore, energy levels that can be occupied by the molecules are defined by integer numbers ($v = 0, 1, 2, ...$). Vibrational transition from the ground state ($v = 0$) to the first energy level ($v: 0 \rightarrow 1$) is the most common; yet, transitions to higher vibrational states (i.e. $v: 0 \rightarrow 2$, $v: 0 \rightarrow 3$) are also possible and called "first" and "second overtone", respectively. Higher-order overtones may also arise although the absorption intensity rapidly decreases as the overtone level rises [1]. For further details about the theory of vibrational spectroscopy the reader is directed to [1].

Broadly speaking, the spectroscopic analysis basically consists of passing a beam of incident light through the sample and scanning the transmitted, or reflected, radiation (see below). As a result, a spectrum of the light absorbance (or transmittance) is produced as function of the incident light frequency (or wavelength). Examination of such spectrum reveals how much energy was absorbed at each frequency.

When a sample is irradiated with light, part of the radiation can be reflected, transmitted, and absorbed. According to energy conservation law, the sum of all these fractions is 1.0 and their proportions depend on the light wavelength and sample properties (composition and thickness among others). The fraction of absorbed light is called "absorbance" (A) and it cannot be directly measured. However, A can be derived from the light fraction that passes through the sample and reaches the detector, indeed referred as "transmittance". Transmittance (T) is defined as the ratio of radiation passing a sample per unit area (P) divided by the initial radiation power (P_0), expressed as percentage:

$$T = \frac{P}{P_0} \cdot 100$$

Absorbance and transmittance are related by the following equation:

$$A = log\left(\frac{1}{T}\right) = log\left(\frac{P_0}{P}\right)$$

$Log(1/T)$ is called apparent absorbance because the effects from light dispersion in the sample are not taken in account.

Concerning the reflected radiation, it consists of two main components: specular and diffuse reflectance. The specular fraction has the same reflection angle as the incident light (**Figure A2**) and achieves its maximum intensity when the irradiation is perpendicular to a smooth sample surface. It lacks relevant information due to its minimum interaction with the sample. On the other hand, the diffuse component refers to the part of the incident beam which is scattered within the sample and reflected after a certain degree of interaction. It can be correlated to absorbance through the following equation:

$$A = -log(R_{rel}) = \log\left(\frac{1}{R_{rel}}\right)$$

Where, relative reflectance (R_{rel}) is defined as the ratio of the sample measured reflectance (R_{sample}) over the measurement from a highly-reflective material (R_{std}), with reflectance approximately 100%, such as Teflon or Spectralon:

$$R_{rel} = \frac{R_{sample}}{R_{std}}$$

According to the types of interaction occurring between the sample and the incident radiation discussed above, two main working modes can be recognized among vibrational spectroscopic systems: transmission or diffuse reflection. The primary difference between these two operating modes lies in the arrangement of the detector and the light source respect to the sample position. Transmittance instruments are configured with sample compartment between the source and the detector, located at opposite sides. Differently, diffuse reflectance detectors use integrating sphere or ellipsoidal mirrors to collect the diffused radiation [3].

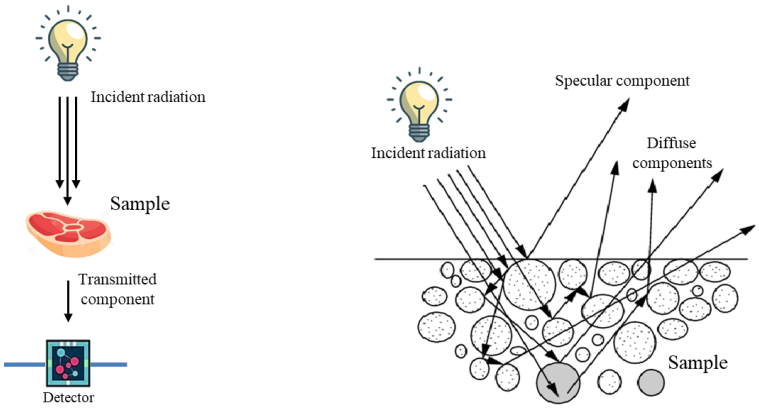


Figure A2. Instrument configuration for the acquisition of transmittance (left) and reflectance (right).

Transmitted radiation is expected to carry more sample information because of the longer optical path, and thus larger interactions, experienced by the light. Besides, the sample path length is a critical factor and must be kept constant for transmittance measurements which, indeed, cannot be performed on large-volume or thick solids. Furthermore, above $2500\ nm$ (MIR region) absorption phenomena become stronger, limiting the applicability of transmittance mode. On the other hand, diffuse reflection mode allows working with thicker and denser samples without inducing as much heating as transmission [4]. For this reason, reflectance mode has been widely used in commercial spectrometers due to the simple arrangement and flexibility in sample presentation. Nevertheless, especially at higher wavelengths, reflectance is highly dependent on physical characteristics of the sample such as density or packing, particle size, and material absorption. Any sample changes will be source of additional variability and noise, strongly affecting the measure reproducibility [5].

Near-Infrared Spectroscopy

After its discovery by Herschel in 1800, NIR was ignored for about 150 years as, compared to MIR, it was considered lacking of relevant chemical information. Indeed, NIR spectroscopy involves radiation in the wavelength range $780-2500\ nm$ (12 820 to 4000 cm^{-1}) which is the most energetic infrared region. This has direct consequences on the resulting spectra, which are characterized by overlapping absorptions, mainly related to overtones and vibrational combinations (i.e. simultaneous transitions involving two or more different vibrational modes) of C-H, N-H and O-H chemical bonds. In fact, while frequencies of fundamentals transitions are in the MIR spectral region, overtones fall in the NIR region and as their frequencies normally correspond to about two or three times the fundamental one [1]. As a consequence,

the entire NIR spectra contains up to four overtones from the absorptions of methyl C-H, aromatic C-H, methylene C-H, methoxy C-H, carbonyl associated C-H, N-H from primary and secondary amides, N-H from amides (primary, secondary, and tertiary), N-H of amine salts, O-H (alcohols and water), S-H, and C=O groups [6]. Such redundancy of information results in broader and overlapping bands, hard to be interpreted and/or assigned to a specific chemical compound in a straightforward manner as for MIR spectra. Moreover, recorded spectra often contain information about unimportant sample properties and spurious source of variability. For instance, powdered samples may provide strong interferences from scattered light which is affected by particle size, packing density, etc.

Most of these problems were solved by enhanced computing power provided by the modern computer technologies. A range of pre-processing mathematical treatments (e.g. derivative filter, multiplicative scatter correction, standard normal variate transformation, etc.) have been developed to significantly reduce the interferences from sample inhomogeneity and highlight relevant information (**Section 3.3**). Multivariate regression methods were applied to correlate NIR spectral features with reference values, paving the way for the determination of food components through prior calibration [7]. In this regard, the major breakthrough for NIR spectroscopy was in the early 1960s when Norris and co-workers were able to determine moisture content from whole seeds using NIR bands with a multivariate calibration approach [8]. In the 1970s and 1980s, there was an explosion of agricultural applications for NIR developed through government and university research programs, with the first official NIR method adopted by the Canadian Grain Commission for protein testing in wheat [9]. In the 1982, the American Association of Cereal Chemists (AACC) accepted NIR reflectance methods for measuring protein in wheat, and the list was later expanded with further methods (i.e. grain protein content, fat determination in soybeans, hardness determination of wheat, etc.) [10].

Nowadays, NIR has found application in several agricultural and food fields as it offers a straightforward, rapid, and cost-effective alternative over the traditional reference methods for physicochemical characterization of food matrices. Historically, the quantitation of predominant constituents has been the major focus [11–14]. However, qualitative analysis such as varietal discrimination of plant products, detection of potential adulteration and production process control (e.g. fermentation) have also been performed spectroscopically [15–17]. Furthermore, NIR spectroscopy has proven to be particularly feasible for in- and on-line implementation in manufacturing contexts. For these applications, the essential prerequisite represents the suitability of the spectrometer construction for process integration. Depending on the application conditions, the device generally needs to withstand harsh process environment, such as temperature fluctuations, vibrations, and humidity. Moreover, collecting reliable spectra from intact and moving samples under industrial conditions is not an easy task. Being able to meet

all these requirements, NIR spectroscopy is widely accepted as one of the most promising on/in-line process control techniques upon food processing [18].

Mid-Infrared Spectroscopy

As discussed in the previous section, after the discovery of the infrared radiation, the development of IR-based analytical methods was mainly focused on the MIR spectral region. The first commercial instruments appeared in the 1940s, intended for the rubber and petroleum fields and requiring, however, highly trained personnel for routine operation. Prior to the 1980s, conventional MIR spectrometers were dispersive instruments with spectral acquisition limited by scan-speed shortcomings. An important technological advance was represented by the Fourier transform infrared spectrometers (FT-IR) advent [19]. Some of the advantages of FTIR include speed of analysis, higher S/N and sensitivity, cost, and precision. Nowadays, most of the MIR instruments are based on the FT-IR technology, using interferometers instead of monochromators. As a result, the term "MIR spectroscopy" and "FT-IR spectroscopy" are often used interchangeably [9].

The MIR region extends from 2500 to 25 000 nm (4000 to 400 cm^{-1}) and radiation absorption in this region is associated with fundamental molecular stretching and bending transitions [19]. Stretching vibrations consists in bond length changes with the atoms staying on the same axis. Instead, bending vibrations include atoms movement that change bond angles. Overall, bending alterations need less energy than vibration transitions, thus, the corresponding absorption bands can be found at lower frequencies in the MIR spectrum [20]. Within the FT-MIR spectral range, four regions may be generally considered; the X-H stretch region ($4000-2500\ cm^{-1}$, including hydroxyl or amino groups), triple bond region ($2500-2000\ cm^{-1}$), the double bond region ($2000-1500\ cm^{-1}$, including acyl chain, carbonyl and alkene groups) and the "fingerprint" region ($1500-600\ cm^{-1}$, mainly showing bending vibrations and some stretching vibrations of the acyl chain and functionalized groups). The latter owes its name to the higher specificity provided by the large number of occurring bands. Indeed, two structure-related compounds might exhibit similar profiles at $\lambda > 1500\ cm^{-1}$. However, their spectra will rarely overlap at $\lambda < 1500\ cm^{-1}$ [1]. Absorbance bands in the first three regions are generally stable and can be identified rather easily. This is not the case for the fingerprint region, where most of the bending and skeletal vibrations are subject to significant wavenumber shifts arising from relatively small electronic or steric effects. Consequently, no attempt is generally made to identify specific peaks in this region but rather the absorbance of the complete region is used as a fingerprint of the material under study. For this reason,

similarly to NIR, many FT-MIR-based applications require the use of chemometrics tools to deconvolute and correlate the recorded spectrum to the investigate the sample's properties [19].

To date, FT-MIR is a well-established analytical platform that has found practical applications in virtually all branches of agricultural and food industries [9]. It shares the same advantages mentioned for NIR over the more labor- and time-consuming traditional methods. However, when compared to NIR, a major specificity, due to the narrower and well-defined bands, is often claimed as a substantial advantage of the FT-MIR. Moreover, overall better sensitivity and higher S/N are provided by modern FT-MIR instruments since fundamental absorptions (which are the strongest) are being measured. On the other hand, despite appearances, the higher energy of NIR radiation and the implication of combination vibrations paradoxically enable NIR spectroscopy to provide more complex structural information [1]. Moreover, water is a strong absorber in certain MIR spectrum regions and might mask contributions from other species [19]. Concerning the in/online implementation, FT-MIR instrument and materials (e.g. fiber optics, transmission cell, etc.) are generally more expensive and less flexible for this purpose. In addition, the minor penetration depth (usually few μm) of the used radiation, limits its application, for example, in the analysis of food throughout intact packaging [9,21].

Raman Spectroscopy

The principle involved in Raman spectroscopy is somewhat different from NIR or MIR. This spectroscopy technique is substantially based on the "Raman scattering" phenomenon, which was firstly reported in 1928 by Raman and Krishnan [22]. Basically, Raman spectroscopy is performed by pointing a monochromatic laser beam at the sample and recording the intensity and wavelength of the scattered light. As a result of the interaction between the incident beam and the molecule, the scattered photons may exhibit the same (Rayleigh or elastic scattering) or different frequency (Raman or inelastic scattering) than the incident light [23]. The change in photon's wavelength observed in Raman scattering is due to an energy transfer (either from the molecule to the photon or vice versa) that causes excitation, or relaxation, of vibrational modes of a molecule [24]. In extremely simple terms, if the molecule gains energy as overall result of the interaction, scattered photons will show longer wavelengths; otherwise, when the molecule release energy, the scattered photons will be characterized by shorter wavelengths. These two mechanisms are named anti-Stokes Raman and Stokes Raman scattering [25] (**Figure A3**).

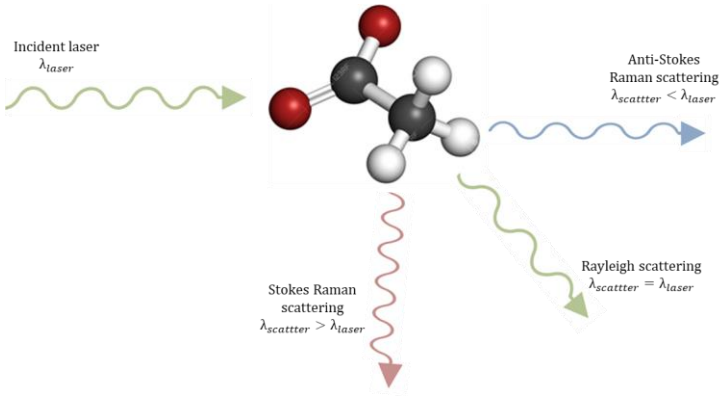


Figure A3. Schematic representation of the possible types of scattering occurring when light interacts with a molecule.

More detailed description of the theory and mechanism behind Raman spectroscopy, as well as current instrumentation and trends can be found in literature [25–28].

Raman and Krishnan described the Raman scattering phenomenon as "feeble compared with the ordinary scattering". Indeed, a very small portion ($10^{-6} - 10^{-8}$) of the light undergoes to inelastic scattering, proportionally to the magnitude of the change in molecular polarizability [19,24]. However, since there has been interaction between the sample and the incident light, the scattered radiation contains information about the vibrational states of the sample's molecules. The occurring frequency shifts are recorded by the spectrometer and presented as spectrum. Raman spectra are plots of scattered intensity as a function of the energy difference between the incident and Raman-scattered photons (**Figure A4**). This frequency difference is termed "Raman shift" and is expressed as cm^{-1}.

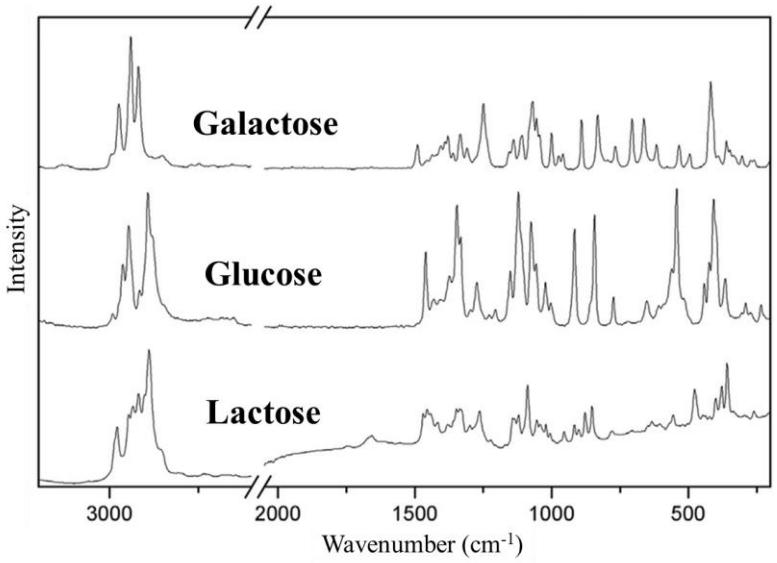


Figure A4. Raman spectra of crystalline structures: galactose, glucose and lactose. Reproduced from [29].

Raman peaks are spectrally narrow and, in the case of single-compound analysis, they can be associated with the vibration of chemical bonds (or single functional group) in the molecule [23]. In particular, the wavelength shift corresponds to the frequency of the fundamental MIR absorbance band of the molecule's bond involved in the excitation/relaxation [1]. While IR absorbance are strongest for vibrations involving a large change in dipole moment, Raman bands are strongest for large alterations in polarizability. This makes the information from IR and Raman spectroscopy rather complementary [19].

Because of the inherently small intensity of the Raman signal, the sensitivity limits of available detectors, and the intensity of the excitation sources, the use of Raman spectroscopy was restricted for many years [24]. In addition, a number of further constraints severely limited its use on biological samples. Examples are the fluorescence interference which may completely obscure the Raman scattering signal, photodecomposition due to the employed high-powered IR lasers and the difficulty of attaining high-resolution spectra with the classic dispersive Raman spectrometer [21]. However, major instrumental advances have rekindled the interest in this technique and greatly extended the range of useful application in many diverse disciplines, including agricultural products and food analysis [25,30]. The emergence of Fourier-transform (FT) Raman spectrometers equipped with a Nd:YAG laser (NIR monochromatic light excitation) allowed to reduce the fluorescent background impact [30]. Further developments in the technology and instrumentation enabled higher accuracy of wavelength calibration and significantly lessen analysis time, thus reducing potential thermal degradation of the sample [19,30].

Similar to NIR and MIR, when complex multicomponent systems are studied, the large number of functional groups and their diverse microenvironments usually result in overlapping broad spectral bands. Therefore, Raman spectra are often used as sample fingerprint and relevant information is extracted through chemometrics tools [30]. Remarkable advantages of Raman spectroscopy over NIR and FT-MIR can be mentioned such as the high-level (and often complementary) spectral information, very low band absorption from water, fast scan speed and ruggedness due to the absence of any moving part other than the shutter in front of the detector [19]. Furthermore, glass has a very weak Raman spectrum, making the technique suitable for the analysis of sample directly into the bottle [21]. As a drawback, Raman instrumentation is often expensive and less suitable for on/in-line implementation respect to NIR equipment [31].

References

[1] O. Abbas, P. Dardenne, V. Baeten, Near-Infrared, Mid-Infrared, and Raman Spectroscopy, in: Chem. Anal. Food Tech. Appl., Elsevier Inc., 2012: pp. 59–89. https://doi.org/10.1016/B978-0-12-384862-8.00003-0.

[2] D.A. Skoog, D.M. West, J.F. Holler, S.R. Crouch, Fundamentals of Analytical Chemistry, 9th ed., Brooks Cole, 2004.

[3] Blitz, Diffuse Reflectance Spectroscopy, in: F.M. Mirabella (Ed.), Mod. Tech. Appl. Mol. Spectrosc., Wiley, 1998.

[4] L.E. Agelet, C.R. Hurburgh, A tutorial on near infrared spectroscopy and its calibration, Crit. Rev. Anal. Chem. 40 (2010) 246–260. https://doi.org/10.1080/10408347.2010.515468.

[5] O. Berntsson, L.G. Danielsson, S. Folestad, Estimation of effective sample size when analysing powders with diffuse reflectance near-infrared spectrometry, Anal. Chim. Acta. 364 (1998) 243–251. https://doi.org/10.1016/S0003-2670(98)00196-2.

[6] J. Workman, L. Weyer, Practical Guide to Interpretive Near-Infrared Spectroscopy, CRC Press, Inc., NW Boca Raton (FL), 2007.

[7] B.G. Osborne, T. Fearn, Near Infrared Spectroscopy in Food Analysis, John Wiley & Sons Inc., New York, 1986.

[8] K.H. Norris, J.R. Hart, Direct Spectrophotometric Determination of Moisture Content of Grain and Seeds, J. Near Infrared Spectrosc. 4 (1996) 23–30. https://doi.org/10.1255/jnirs.940.

[9] M. Lin, B.A. Rasco, A.G. Cavinato, M. Al-Holy, Infrared (IR) Spectroscopy-Near-Infrared Spectroscopy and Mid-Infrared Spectroscopy, in: Infrared Spectrosc. Food Qual. Anal. Control, Elsevier Inc., 2009: pp. 119–143. https://doi.org/10.1016/B978-0-12-374136-3.00006-7.

[10] Cereals & Grains Association, Approved Methods of the American Association, 11th ed., St. Paul (MN), 1999. http://methods.aaccnet.org/about.aspx.

[11] M.M. Cascant, C. Breil, A.S. Fabiano-Tixier, F. Chemat, S. Garrigues, M. de la Guardia, Determination of fatty acids and lipid classes in salmon oil by near infrared spectroscopy, Food Chem. 239 (2018) 865–871. https://doi.org/10.1016/j.foodchem.2017.06.158.

[12] C. Lin, X. Chen, L. Jian, C. Shi, X. Jin, G. Zhang, Determination of grain protein content by near-infrared spectrometry and multivariate calibration in barley, Food Chem. 162 (2014) 10–15. https://doi.org/10.1016/j.foodchem.2014.04.056.

[13] C. Rébufa, I. Pany, I. Bombarda, NIR spectroscopy for the quality control of Moringa oleifera (Lam.) leaf powders: Prediction of minerals, protein and moisture contents, Food Chem. 261 (2018) 311–321. https://doi.org/10.1016/j.foodchem.2018.04.066.

[14] J.C. Hashimoto, J.C. Lima, R.M.S. Celeghini, A.B. Nogueira, P. Efraim, R.J. Poppi, J.A.L. Pallone, Quality Control of Commercial Cocoa Beans (Theobroma cacao L.) by Near-infrared Spectroscopy, Food Anal. Methods. 11 (2018) 1510–1517. https://doi.org/10.1007/s12161-017-1137-2.

[15] J. Nogales-Bueno, L. Feliz, B. Baca-Bocanegra, J.M. Hernández-Hierro, F.J. Heredia, J.M. Barroso, A.E. Rato, Comparative study on the use of three different near infrared spectroscopy recording methodologies for varietal discrimination of walnuts, Talanta. 206 (2020) 120189. https://doi.org/10.1016/j.talanta.2019.120189.

[16] P. Vermeulen, M. Suman, J.A. Fernández Pierna, V. Baeten, Discrimination between durum and common wheat kernels using near infrared hyperspectral imaging, J. Cereal Sci. 84 (2018) 74–82. https://doi.org/10.1016/j.jcs.2018.10.001.

[17] S. Barbon, A.P. Ayub Da Costa Barbon, R. Gomes Mantovani, D. Fernandes Barbin, Machine Learning Applied to Near-Infrared Spectra for Chicken Meat Classification, (2018). https://doi.org/10.1155/2018/8949741.

[18] H. Huang, H. Yu, H. Xu, Y. Ying, Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: A review, J. Food Eng. 87 (2008) 303–313. https://doi.org/10.1016/j.jfoodeng.2007.12.022.

[19] G. Downey, Vibrational spectroscopy in studies of food origin, in: New Anal. Approaches Verif. Orig. Food, Elsevier Ltd, 2013: pp. 94–116. https://doi.org/10.1533/9780857097590.2.94.

[20] N. Colthup, L.H. Daly, S.E. Wiberley, Introduction to Infrared and Raman Spectroscopy, 3rd ed., Academic Press, 1990.

[21] D.L. García-González, V. Baeten, J.A. Fernández Pierna, N. Tena, Infrared, raman, and fluorescence spectroscopies: Methodologies and applications, in: Handb. Olive Oil Anal. Prop., Springer US, 2013: pp. 335–393. https://doi.org/10.1007/978-1-4614-7777-8_10.

[22] C. V. Raman, K.S. Krishnan, Polarisation of scattered light-quanta [7], Nature. 122 (1928) 169. https://doi.org/10.1038/122169a0.

[23] Z. Movasaghi, S. Rehman, I.U. Rehman, Raman spectroscopy of biological tissues, Appl. Spectrosc. Rev. 42 (2007) 493–541. https://doi.org/10.1080/05704920701551530.

[24] C.L. Haynes, A.D. McFarland, R.P. Van Duyne, Surface-enhanced: Raman spectroscopy, Anal. Chem. 77 (2005). https://doi.org/10.1021/ac053456d.

[25] D. Yang, Y. Ying, Applications of raman spectroscopy in agricultural products and food analysis: A review, Appl. Spectrosc. Rev. 46 (2011) 539–560. https://doi.org/10.1080/05704928.2011.593216.

[26] P.J. Larkin, Infrared and Raman Spectroscopy - Principles and Spectral Interpretation, Elsevier, 2018. https://doi.org/10.1016/C2015-0-00806-1.

[27] R.S. Das, Y.K. Agrawal, Raman spectroscopy: Recent advancements, techniques and applications, Vib. Spectrosc. 57 (2011) 163–176. https://doi.org/10.1016/j.vibspec.2011.08.003.

[28] P. Worsfold, A. Townshend, C. Poole, M. Miró, eds., Encyclopedia of Analytical Science - 3rd Edition, Elsevier, 2019.

[29] J.K.F. Torres, R. Stephani, G.M. Tavares, A.F. de Carvalho, R.G.B. Costa, C.E.R. de Almeida, M.R. Almeida, L.F.C. de Oliveira, P. Schuck, Í.T. Perrone, Technological aspects of lactose-hydrolyzed milk powder, Food Res. Int. 101 (2017) 45–53. https://doi.org/10.1016/j.foodres.2017.08.043.

[30] E.C.Y. Li-Chan, The applications of Raman spectroscopy in food science, Trends Food Sci. Technol. 7 (1996) 361–370. https://doi.org/10.1016/S0924-2244(96)10037-6.

[31] J. Grabska, K.B. Beć, C.G. Kirchler, Y. Ozaki, C.W. Huck, Distinct Difference in Sensitivity of NIR vs. IR Bands of Melamine to Inter-Molecular Interactions with Impact on Analytical Spectroscopy Explained by Anharmonic Quantum Mechanical Study, Molecules. 24 (2019) 1402. https://doi.org/10.3390/molecules24071402.

Annex II - Alternative Rapid Techniques

Profile of volatile compounds could serve as a valuable tool for several purposes in food analysis, from quality and process control, to origin and adulteration identification. Gas chromatography (GC) coupled to mass spectrometry represents the "golden standard" for the analysis of volatile compounds. However, the overall high cost, analysis time and expertise demanded by traditional analytical methods have prompted the development of alternative approaches for the analysis unresolved mixture of volatiles compounds. Among them, chemical sensors, such as electronic noses coupled to multivariate data analysis has shown promising capabilities for food quality and process control and nowadays represent an active and growing field of research [1].

Electronics noses (e-nose) were introduced as "an attempt to mimic the principles of smelling that gives another view on the whole scene of volatiles compared to its biological inspiration" [2]. As an example, one of the initial hopes was to instrumentally assess attribute descriptors such as fruity, grassy, earthy, malty, etc. However, despite 25 years of research, this is still not possible due to fundamental differences between the human nose and a technical device. The e-nose is more and less at the same time. It offers the capability to detect some important non-odorant gases; for instance, water vapour or carbon monoxide are normally detectable by e-noses, whereas humans have no receptors for these gaseous compounds [2]. On the other hand, it is not suitable for substances of daily importance in mammalian life such as the scent of other animals, foodstuff, or spoilage. Nevertheless, e-noses have found large application in quality control and processes as they offer a non-destructive, rapid and inexpensive alternative to traditional analytical methods. The basic training required for new users and the minimal running cost are further major stimulants for continuous ongoing developments in this field [3].

Classical e-noses are designed to detect and discriminate complex odours using a sensor array. The latter consists of broadly tuned (non-specific) sensors that are treated with a variety of odour-sensitive chemical or biological materials. Upon interaction with an odour stimulus, the sensor array generates a characteristic fingerprint. Patterns or fingerprints from known odours are used to construct a database and train pattern recognition algorithms so that new unknown odours can subsequently be classified and identified. Therefore, (most of) the e-nose instruments do not give information on the sample composition. Rather, they recognize whether a digital fingerprint is "similar", or not, with a reference standard. Calibration of e-noses can be done with chemicals or, in the case of complex samples, equipment responses are often calibrated onto reference materials [4].

In recent years, the classical sensor types used for e-noses have been enhanced and complemented by other technologies introduced in this field. Nevertheless, in general terms, e-nose instruments are essentially

constituted by three elements: (i) sample handling system, (ii) detection system, and (iii) data processing system [4].

Sample handling is always a critical step in analytical chemistry as it may strongly affect the final outcome. In the case of e-noses, several sampling techniques have been proposed to improve the analytical reproducibility. Among others, static headspace procedures represent the most common and easy-to-use techniques which focuses on the volatile compounds present in the sample's headspace (HS). Normally, the sample is placed in a hermetically sealed vial until equilibrium between the matrix and the gaseous phase has been reached. Thereafter, an aliquot of the HS is drawn and injected in the detection system. To this end, autosampler apparatus are often used to overcome the poor repeatability of manual injection. Static HS sampling is the most straightforward and simplest technique which, however, suffers of low sensitivity because no pre-concentration of the analytes is performed. In this regard, solid-phase micro-extraction (SPME) is a user-friendly method routinely used for the analytes pre-concentration. Basically, a silica fiber covered with a thin adsorbent layer is exposed in the sample's HS so that the analytes remain trapped onto the fiber surface. Sample temperature, equilibration time, vial size and sample quantity are the main parameters that have to be optimized. It must be pointed out that the pre-concentration introduce a supplementary step in the method, increasing the analysis time and, moreover, analytical artefacts (i.e. memory effects, bleeding or irreversible adsorption) may arise [4].

The classical e-nose use an array of gas sensors as detection system. A chemical sensor is a device capable of converting a chemical quantity into an electrical signal that is related to the concentration of specific particles. The types of sensors used in e-noses must respond to molecules in the gas phase, typically volatile organic compounds (VOCs). Piezoelectric, electrochemical, optical and thermal sensors, as well as biosensors, are the main used types of sensors and new ones are being steadily developed. A deep discussion of this aspects is not the aim of the present thesis and more detailed description of sampling techniques and sensor types can be found in [4]. Gas sensors represent the most common detection system, however, their functioning is affected by several problems, such as sensor poisoning, the strong influence of moisture, profile masking by major sample's constituents (e.g. ethanol) and the non-linearity of signals. To overcome some of these issues, new technologies such as MS and ion mobility spectrometry (IMS) have recently entered this field. Even though such instrumentations operate upon different detection principles, they are often referred to as e-noses [2]. MS-based e-noses uses mass spectrometry analysers (e.g. single quadrupole spectrometer) as detector without prior chromatographic separation. Volatile compounds are introduced into the ionization chamber and, after electronic or chemical ionization, the resulting ions (and potential fragment ions) are detected according to their *m/z*. Each measured *m/z* of the mass spectrum acts as a "sensor" and its abundance is equivalent to the sensor signal. Therefore, a variable and readily modifiable number of sensors can be used in MS-based e-noses. Moreover, additional chemical information about the

sample can be obtained. For instance, the chemical structure of relevant compounds can be inferred from the ion-fragmentation patterns. Despite its higher technical complexity, MS-based e-noses are, in general, not better suited for odour detection respect to classical e-noses but has advantages for defined tasks (e.g. peptide detection) [2]. The major constraint of all the mass spectrometers is that they operate under vacuum conditions, and therefore, this introduces cost and limitations respect to the classical e-noses [4]. IMS also represents an emerging approach as e-noses' detection principle. This technique is based on the ionization of the analytes and subsequent drift through an electric field. In this case, volatile compounds are separated according to their different ionic mobility, instead of their *m/z*. The recorded signal is integrated over defined time intervals and treated as a virtual sensor array response. Since IMS operates at atmospheric pressure, it normally requires smaller, lighter weight and easier use analytical units, as well as lower power requirements, respect to MS [4].

Chromatographic techniques provide a further way to add a separation dimension to classical e-noses. Intuitively, since e-noses focus on volatile compounds, GC is the natural choice. While MS and IMS separate the analytes along the *m/z* and ionic mobility dimension, respectively, GC-based e-noses resolve complex mixture according to compounds' retention in the GC column. Since e-noses are intended to be simple and rapid in use, fast or ultrafast GC have been widely proposed and different parameters (e.g. carrier gas flow rate, ramp temperature, column length, etc.) have to be adapted to reduce the runtime [2].

Within the present PhD thesis, part of the work has been done on headspace-gas chromatography-ion mobility (HS-GC-IMS) and flash GC-enose (FGC-Enose). Therefore a summary description of these equipment is provided below, whereas more details about e-nose platforms can be found in literature [2,4,5].

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As mentioned above, IMS represent an emerging approach in the field of e-noses. Essentially, the working principle of IMS is the separation of ionic compounds according to their mobility through a gas-filled drift region. To this purpose, compounds somehow extracted from the samples are introduced into the ionization chamber of the IM spectrometer by a carrier gas (usually N_2). A schematic representation of a classic ion mobility spectrometer is reported in **Figure A7**.

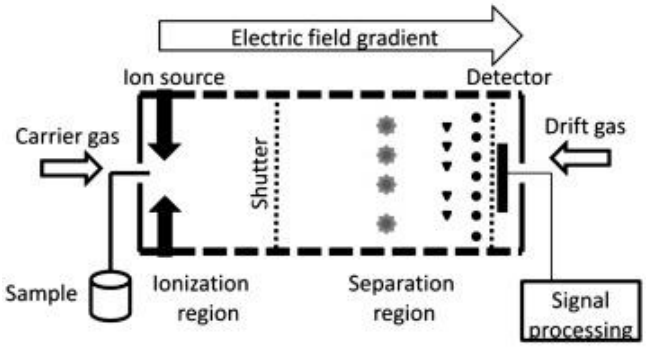

Figure A7. Schematic of a classic ion mobility spectrometer. Ions characterized by different mobility values will reach the detector plate at different times. The plot of ion current as a function of the drift time yields the mobility spectrum (bottom). Reproduced from [6].

Ionization sources based on different mechanism are available. As an example, tritium radioactive source is based on the emission of primary electrons, which collide with N_2 molecules. This triggers a series of reactions, leading to the generation of water clusters $[(H_2O)_n+H^+]$ as reactant ions. The reactant ion peak (RIP) in the spectrum represents the total available ions generated in the source (see **Figure A8**). The analyte molecules reaching the ionization chamber interact with the so-formed reactant ions and, if the analyte' proton affinity is higher than that of water, protonated monomers (or proton bound dimers depending on the experimental conditions) are yielded. Ionization selectivity can be modified by doping the drift gas with various additive such as NH_3 vapour, acetone, chlorinated solvents or others. Substances with electron-capturing capabilities, like halogenated compounds, can be detected as negative ions by potential inversion. Another often used alternative for compounds with sufficiently low ionization potential is the UV photoionization.

Regardless the ionization mechanism, the newly formed ions are then accelerated through a linear drift tube where, exposed to a uniform weak electric field and a counter flowing drift gas, they separate according to their physiochemical properties. Small, lightweight, compact ions travel faster and will reach the detector at the end of the drift tube before larger and heavier ions. Depending on the ion impact, a current is generated and measured over the drift time. The analytical output, the so-called mobility spectrum, is a plot of the so-

measured ion current intensity against the drift time (**Figure A8)** [7]. Further details about the fundamental principles behind IM measurements can be found in several excellent reviews [8–11].

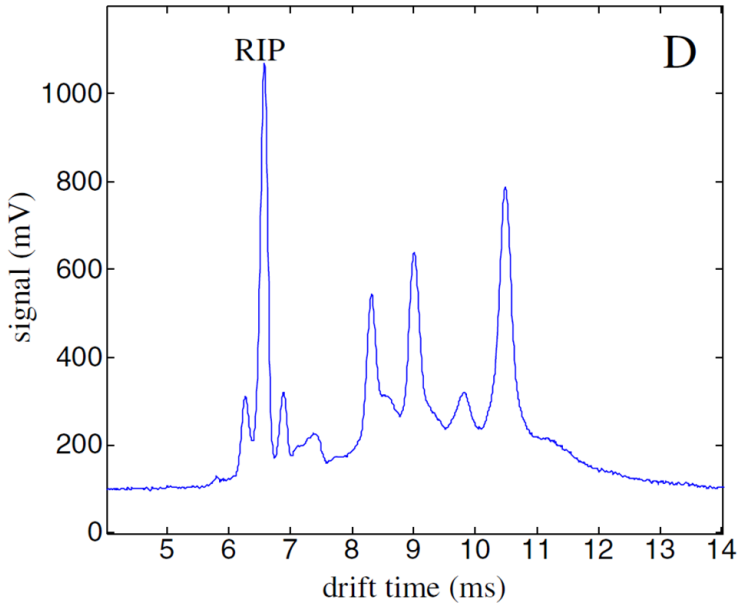

Figure A8. Mobility spectrum obtained from a virgin olive. The reactant ion peak (RIP) has been highlighted in the figure. Reproduced from [12].

IMS was originally developed for the detection of explosives and chemical warfare agents. However, in the last years, it has found novel applications in the field of foodomics, such as muscle food freshness and spoilage, process control in the beer industry, detection of illicit and toxic chemicals in food products, wine quality, etc. [6]. However, it must be pointed out that too complex matrices being analysed often result in ion-ion interaction and/or overlapping peaks. Since the drift tube length cannot be extended at will, a possibility to enhance the separation efficiency is to interface the IM spectrometer with a GC separation. In the first place, the analytes are separated on the basis of their retention in the GC column. In addition, co-eluting compounds can be differentiated by their drift time in the IM cell as a second dimension of separation [13]. Although this certainly increases the analysis time, further sample's chemical information may be highlighted as a 2D-map of the aroma compounds is obtained (see **Chapter 3**). Indeed, while chromatography operates on a timescale of minutes, ion mobility separation is carried out within milliseconds and several spectra at a given retention time can be processed. Therefore, such additional separation increases the peak capacity while enhancing the analytical sensitivity by reducing ion suppression due to ionization competition phenomena [14].

The obtained 2D data can be treated following essentially two chemometric strategies (see **Chapter 3**). A first approach uses the information contained in the whole spectral fingerprint. All data are taken into

consideration, even those signals which may not be visually appreciated. The second strategy consisted of a prior selection of individual markers throughout the spectra to carry out the chemometric treatment. These targets are often selected based on prior knowledge of the matrix under investigation or according to literature records [15].

GC-IMS has proven to be an effective separation technique because of its comparatively simple system setup, benchtop size, robustness, and price. Thanks to the headspace sampling, time-consuming sample-preparations are usually not required and almost untreated samples are analysed. Furthermore, the possibility to operate under atmospheric pressure conditions represent a significant advantage as smaller analytical units and lower power supplies are required. Several studies focused on the application of IMS and GC-IMS for olive oil [12,16–19], Iberian ham [15] and other food commodities [20,21] have been published in literature, proving the great potential of this technology for food quality and authenticity testing.

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The analysis of volatiles and semi-volatiles in food samples has been generally carried out by conventional GC-based protocols. The goal of any GC method is the total separation of the main sample components in the minimum time. Obviously, it would be preferable to deliver entirely resolved analytes to the detector system. Conventional GC capillary columns are around $25-30\ m\ x\ 0.25-0.32\ mm$ i.d. (internal diameter) column. They normally ensure satisfactory separations (often over-resolved) on simple-to-medium complex samples. The main drawback is represented by the cost in analytical time (for most food applications this is around 20-30 minutes). This becomes a substantial disadvantage for laboratories with a high daily sample throughput and/or where there is a need for quick and reliable results [22].

Assuming that the most selective conventional GC column for a specific application is being used, chromatographic parameters (e.g. gas flow rate and/or the temperature program ramp) can be adjusted until the lowest degree of acceptable analyte separation is attained. Shortening the used GC columns may not be the most convenient choice. For this reason, an ever-increasing interest has been shown over the past years for high-speed GC approaches. Several classification of rapid GC techniques have been proposed by different authors. GC analysis are normally defined, according to the total runtime, as fast (3-12 min), very fast (1-3 min) and ultrafast (< 1 min) [22]. A recently published review provides an overview of the state-of-the-art of fast GC-MS methodologies [23].

In terms of detection, mass detector would obviously be a highly desirable option. However, reliable, rugged, low-costing and rapid acquisition rate flame ionization detectors (FIDs) represent the most widely used solution for fast GC applications [22]. Most of fast GC systems are traditionally equipped with one chromatographic column. However, devices with different designs are also manufactured. One of the examples is the Heracles II (Alpha MOS, France).

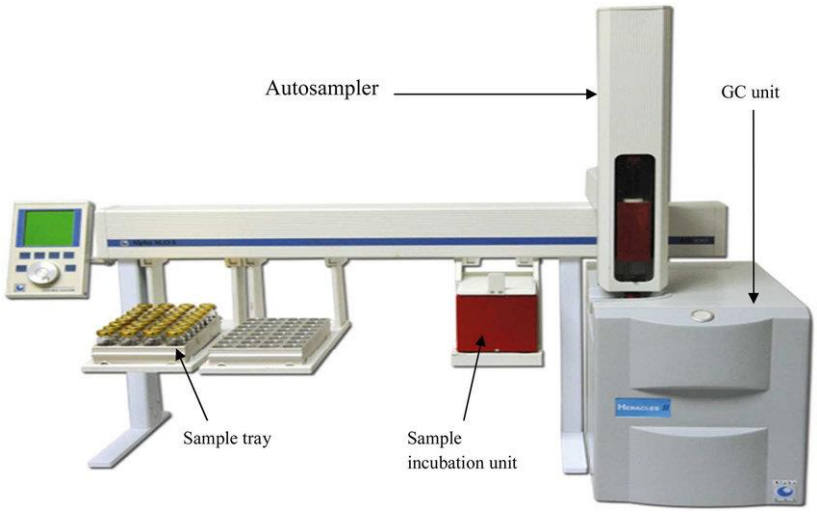

Figure A9. Heracles II instrument quipped with an automated HS sampler unit. Reproduced from [24].

It is a flexible and user-friendly "all-in-one instrument", often referred as e-nose, intended for rapid volatile sample fingerprint within industrial conformity and/or quality control contexts. The Hercales II features two independent chromatographic columns of different polarities mounted in parallel and coupled to two FID detectors. As a result, 2 chromatograms are obtained simultaneously, thus providing broader information about the sample composition.

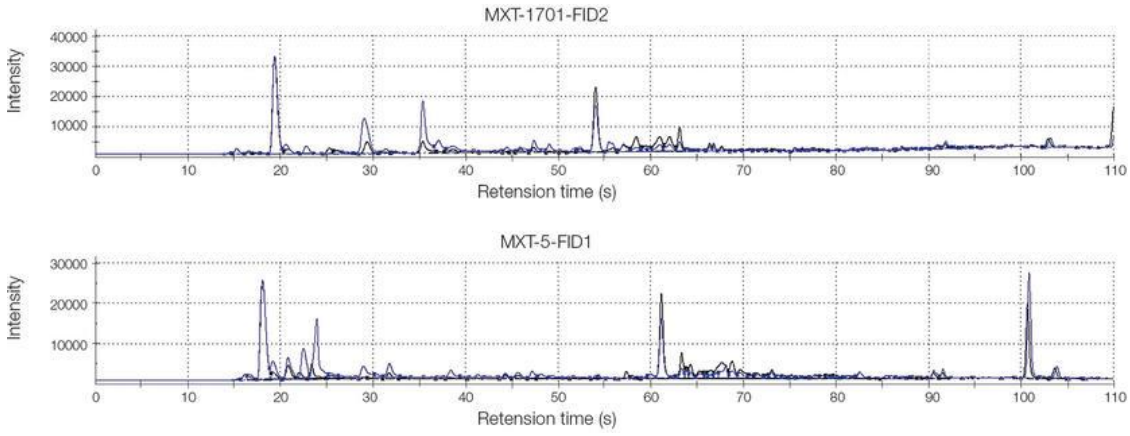

Figure A10. Examples of simultaneously-recorded chromatograms obtained from equipped with the MXT-5 and MXT-1701 columns.

The automated “sample preparation” and sampling systems enable faster and reproducible analysis, while the integrated solid adsorbent trap thermoregulated by Peltier cooler (0-260°C) achieves an efficient pre-concentration of light volatiles enhancing the system sensitivity [25].

Most of the work carried out thus far have been mainly focused on authenticity an quality assessment of spirits and alcoholic beverages [25–27]. Nevertheless, application of FGC e-nose for the origin and quality control of rapeseed oil [28,29] and geographic origin discrimination of extra virgin olive oil [30] have been also reported.

References

[1] E.A. Baldwin, J. Bai, A. Plotto, S. Dea, Electronic Noses and Tongues: Applications for the Food and Pharmaceutical Industries, Sensors. 11 (2011) 4744–4766. https://doi.org/10.3390/s110504744.

[2] F. Röck, N. Barsan, U. Weimar, Electronic Nose: Current Status and Future Trends, (2008). https://doi.org/10.1021/cr068121q.

[3] Z. Xiaobo, H. Xiaowei, M. Povey, Non-invasive sensing for food reassurance, Analyst. 141 (2016) 1587–1610. https://doi.org/10.1039/c5an02152a.

[4] M. Peris, L. Escuder-Gilabert, A 21st century technique for food control: Electronic noses, Anal. Chim. Acta. 638 (2009) 1–15. https://doi.org/10.1016/j.aca.2009.02.009.

[5] M. Śliwińska, P. Wiśniewska, T. Dymerski, J. Namieśnik, W. Wardencki, Food analysis using artificial senses, J. Agric. Food Chem. 62 (2014) 1423–1448. https://doi.org/10.1021/jf403215y.

[6] Z. Karpas, Applications of ion mobility spectrometry (IMS) in the field of foodomics, Food Res. Int. 54 (2013) 1146–1151. https://doi.org/10.1016/j.foodres.2012.11.029.

[7] A.B. Kanu, H.H. Hill, Ion mobility spectrometry detection for gas chromatography, J. Chromatogr. A. 1177 (2008) 12–27. https://doi.org/10.1016/j.chroma.2007.10.110.

[8] R. Cumeras, E. Figueras, C.E. Davis, J.I. Baumbach, I. Gràcia, Review on Ion Mobility Spectrometry. Part 1: Current instrumentation, Analyst. 140 (2015) 1376–1390. https://doi.org/10.1039/c4an01100g.

[9] R. Cumeras, E. Figueras, C.E. Davis, J.I. Baumbach, I. Gràcia, Review on Ion Mobility Spectrometry. Part 2: Hyphenated methods and effects of experimental parameters, Analyst. 140 (2015) 1391–1410. https://doi.org/10.1039/c4an01101e.

[10] J. Puton, J. Namieśnik, Ion mobility spectrometry: Current status and application for chemical warfare agents detection, TrAC - Trends Anal. Chem. 85 (2016) 10–20. https://doi.org/10.1016/j.trac.2016.06.002.

[11] H.E. Revercomb, E.A. Mason, Theory of Plasma Chromatography/Gaseous Electrophoresis. A Review, Anal. Chem. 47 (1975) 970–983. https://doi.org/10.1021/ac60357a043.

[12] R. Garrido-Delgado, L. Arce, M. Valcárcel, Multi-capillary column-ion mobility spectrometry: A potential screening system to differentiate virgin olive oils, Anal. Bioanal. Chem. 402 (2012) 489–498. https://doi.org/10.1007/s00216-011-5328-1.

[13] M. del M. Contreras, N. Arroyo-Manzanares, C. Arce, L. Arce, HS-GC-IMS and chemometric data treatment for food authenticity assessment: Olive oil mapping and classification through two different devices as an example, Food Control. 98 (2019) 82–93. https://doi.org/10.1016/j.foodcont.2018.11.001.

[14] J.C. May, J.A. McLean, Ion mobility-mass spectrometry: Time-dispersive instrumentation, Anal. Chem. 87 (2015) 1422–1436. https://doi.org/10.1021/ac504720m.

[15] N. Arroyo-Manzanares, A. Martín-Gómez, N. Jurado-Campos, R. Garrido-Delgado, C. Arce, L. Arce, Target vs spectral fingerprint data analysis of Iberian ham samples for avoiding labelling fraud using headspace – gas chromatography–ion mobility spectrometry, Food Chem. 246 (2018) 65–73. https://doi.org/10.1016/j.foodchem.2017.11.008.

[16] R. Garrido-Delgado, M. Eugenia Muñoz-Pérez, L. Arce, Detection of adulteration in extra virgin olive oils by using UV-IMS and chemometric analysis, Food Control. 85 (2018) 292–299. https://doi.org/10.1016/j.foodcont.2017.10.012.

[17] R. Garrido-Delgado, F. Mercader-Trejo, S. Sielemann, W. de Bruyn, L. Arce, M. Valcárcel, Direct classification of olive oils by using two types of ion mobility spectrometers, Anal. Chim. Acta. 696 (2011) 108–115. https://doi.org/10.1016/j.aca.2011.03.007.

[18] R. Garrido-Delgado, M.M. Dobao-Prieto, L. Arce, J. Aguilar, J.L. Cumplido, M. Valcárcel, Ion mobility spectrometry versus classical physico-chemical analysis for assessing the shelf life of extra virgin olive oil according to container type and storage conditions, J. Agric. Food Chem. 63 (2015) 2179–2188. https://doi.org/10.1021/jf505415f.

[19] R. Garrido-Delgado, M.D.M. Dobao-Prieto, L. Arce, M. Valcárcel, Determination of volatile compounds by GC-IMS to assign the quality of virgin olive oil, Food Chem. 187 (2015) 572–579. https://doi.org/10.1016/j.foodchem.2015.04.082.

[20] D. Cavanna, S. Zanardi, C. Dall'Asta, M. Suman, Ion mobility spectrometry coupled to gas chromatography: A rapid tool to assess eggs freshness, Food Chem. 271 (2019) 691–696. https://doi.org/10.1016/j.foodchem.2018.07.204.

[21] N. Gerhardt, M. Birkenmeier, S. Schwolow, S. Rohn, P. Weller, Volatile-Compound Fingerprinting by Headspace-Gas-Chromatography Ion-Mobility Spectrometry (HS-GC-IMS) as a Benchtop Alternative to 1H NMR Profiling for Assessment of the Authenticity of Honey, Anal. Chem. 90 (2018) 1777–1785. https://doi.org/10.1021/acs.analchem.7b03748.

[22] P. Donato, P.Q. Tranchida, P. Dugo, G. Dugo, L. Mondello, Rapid analysis of food products by means of high speed gas chromatograpy, J. Sep. Sci. 30 (2007) 508–526. https://doi.org/10.1002/jssc.200600407.

[23] M. Zoccali, P.Q. Tranchida, L. Mondello, Fast gas chromatography-mass spectrometry: A review of the last decade, TrAC - Trends Anal. Chem. 118 (2019) 444–452. https://doi.org/10.1016/j.trac.2019.06.006.

[24] S.M. Yimenu, J.Y. Kim, B.S. Kim, Prediction of egg freshness during storage using electronic nose, Poult. Sci. 96 (2017) 3733–3746. https://doi.org/10.3382/ps/pex193.

[25] M. Śliwińska, P. Wiśniewska, T. Dymerski, W. Wardencki, J. Namieśnik, Application of Electronic Nose Based on Fast GC for Authenticity Assessment of Polish Homemade Liqueurs Called Nalewka, Food Anal. Methods. 9 (2016) 2670–2681. https://doi.org/10.1007/s12161-016-0448-z.

[26] P. Wiśniewska, M. Śliwińska, T. Dymerski, W. Wardencki, J. Namieśnik, Differentiation Between Spirits According to Their Botanical Origin, Food Anal. Methods. 9 (2016) 1029–1035. https://doi.org/10.1007/s12161-015-0280-x.

[27] Y. Yang, Y. Zhao, S. Zhang, Y. Ni, J. Zhan, Qualitative analysis of age and brand of unblended brandy by electronic nose, in: IFIP Adv. Inf. Commun. Technol., Springer, Berlin, Heidelberg, 2012: pp. 619–628. https://doi.org/10.1007/978-3-642-27278-3_64.

[28] L. Xu, X. Li, J. Huang, P. Gao, Q. Jin, X. Wang, Rapid Measuring Flavor Quality Changes of Frying Rapeseed Oils using a Flash Gas Chromatography Electronic Nose, Eur. J. Lipid Sci. Technol. 121 (2019). https://doi.org/10.1002/ejlt.201800260.

[29] Y. Zhang, G. Wu, C. Chang, Y. Lv, W. Lai, H. Zhang, X. Wang, Q. Jin, Determination of Origin of Commercial Flavored Rapeseed Oil by the Pattern of Volatile Compounds Obtained via GC–MS and Flash GC Electronic Nose, Eur. J. Lipid Sci. Technol. 122 (2020). https://doi.org/10.1002/ejlt.201900332.

[30] D. Melucci, A. Bendini, F. Tesini, S. Barbieri, A. Zappi, S. Vichi, L. Conte, T. Gallina Toschi, Rapid direct analysis to discriminate geographic origin of extra virgin olive oils by flash gas chromatography electronic nose and chemometrics, Food Chem. 204 (2016) 263–273. https://doi.org/10.1016/j.foodchem.2016.02.131.

Annex III - Ambient Mass Spectrometry

Most of the analytical methods, especially those based on mass spectrometry measurement, nowadays employed in the food testing require long and complex sample preparation and/or assay times. Ambient mass spectrometry (AMS) is a relatively new field of analytical chemistry which has the potential to overcome these issues whilst giving results that are comparable with other conventional techniques [1]. In fact, AMS allows the analysis of samples under open-air conditions, enabling direct, rapid, real-time, and high-throughput analyses with little or no sample preparation. Furthermore, AMS techniques represent an effective solution for the surface compositions investigation of various biological solids thanks to the non-invasive sampling [2].

The first appearance of AMS dates back to 1998 in the patent of John Fenn. Among the electrospray ionization methods, a direct ionization method employing cellulose based materials (paper spray mass spectrometry) was described [3]. However, the first published work concerned desorption electrospray ionisation (DESI) was published in 2005 by Takats et al. [4]. For this reason, DESI is widely regarded as the first ambient ionisation technique to be created [1]. In 2005, two more ambient mass spectrometry techniques were introduced: direct analysis in real time (DART) by Cody et al. and atmospheric pressure solid analysis probe (ASAP) McEwen et al. [5,6]. Nowadays, there are over thirty different techniques available often combining separate desorption and ionization processes in a single method offer unprecedented flexibility in sample analysis in the open environment [7]. Thanks to this wide selection of potential desorption and ionization methods (i.e. laser-desorption, thermal desorption, upon impact by ions or charged droplets), both highly polar/non-volatile and non-polar/volatile analytes can generally be ionized and detected. In most of the AMS systems the analytes ionization occurs predominantly through electrospray (ESI) and atmospheric pressure chemical ionization (APCI) processes; therefore, the resulting mass spectra are very similar to that by conventional ESI or APCI techniques [2].

Excellent comprehensive reviews about the currently available ambient ionization mass spectrometry techniques can be found in [7,8]. Herein, DART and ASAP will be covered more in detail as they were employed in the present thesis work (**Chapter 3**). These AMS platforms are both solvent-free techniques using an electrical discharge to effect the analytes ionization during the sample desorption. They are broadly recognized as relying upon the same APCI fundamental principle observed in flowing solvent-based systems and, therefore, amenable to polar and non-polar, low molecular weight analytes [7].

Direct Analysis in Real Time

The DART source comprises an enclosed primary ionization device and a reaction zone extending from its exit, through the open atmosphere, to the inlet orifice of the mass spectrometer. The cutaway view of the first commercial version of the DART ionization block is shown in **Figure A5**.

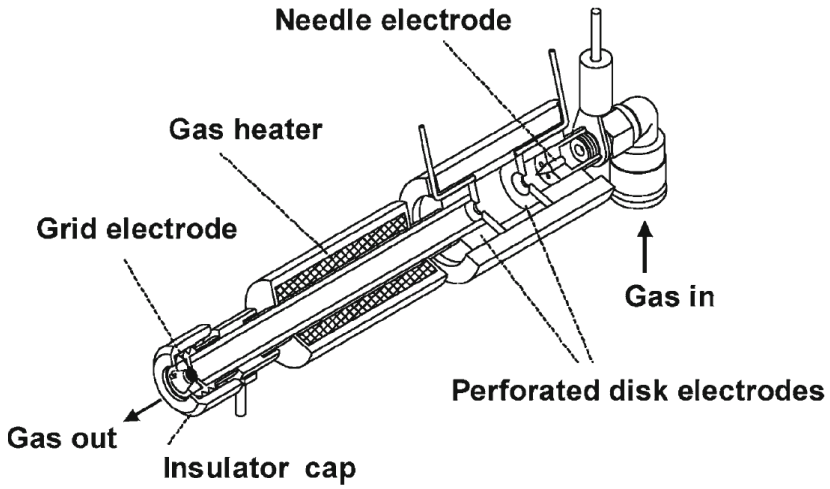

Figure A5. Cutaway view of the DART ionization source. Reproduced from [5].

In DART source, the gas (He or N_2) is guided through an axially segmented tube at a flow rate of approximately 3.5 $L\ min^{-1}$. In the first compartment, a corona discharge between a needle electrode and a first perforated disk electrode produces ions, electrons and excited atoms. The corona discharge operates at $2\ mA$ discharge current and a gas temperature of $50-60\ °C$. Next, an ancillary heating element variably heats the gas stream ($50\ °C\ to\ 550\ °C$) while cations, anions and electrons are removed by a perforated and a grid electrodes. Therefore, the gas stream exiting the ionization source into the atmospheric reaction zone contains only electronic or vibronic excited neutral species. The sample is placed in the $5-25\ mm$ gap between the gas exit orifice and the atmospheric pressure ionization interface (ionization zone) of the mass spectrometer. Both desorption and ionization processes occur simultaneously; the desorption is carried out by the hot gas hitting the sample surface, whereas the ionization goes through an APCI-like process involving ion–molecule reactions with excited gas molecules [9].

Helium is the most effective and almost exclusively used gas because its (2^3S) excited electronic state has an energy ($19.8\ eV$) clearly above the ionization energy of any potentially relevant molecule. Although the primary ionizing species are metastable helium atoms (He^*), ionization of analyte molecules is mostly effected by secondary ions created from the surrounding air. Indeed, upon exiting into the atmosphere, He^* can initiate a cascade of gas-phase reactions with nitrogen, oxygen, water and basically with any other

gaseous species in the laboratory atmosphere (including the analyte molecules M) [10]. In the case of positive ionization:

$$He^* + N_2 \rightarrow He + N_2^{+\cdot} + e^-$$
$$N_2^{+\cdot} + N_2 + N_{2\,(3rd\ body)} \rightarrow N_4^{+\cdot} + N_{2\,(3rd\ body)}$$
$$N_4^{+\cdot} + H_2O \rightarrow 2N_2 + H_2O^{+\cdot}$$
$$He^* + M \rightarrow He + M^{+\cdot} + e^-$$

Direct charge transfer from $N_4^{+\cdot}$, $O_2^{+\cdot}$, and NO^+ to analyte molecules may also occur, yielding molecular ions $M^{+\cdot}$. Moreover, the primary $H_2O^{+\cdot}$ may effectively undergo reactions leading to formation of protonated water clusters:

$$H_2O^{+\cdot} + H_2O \rightarrow H_3O^+ + OH^{\cdot}$$
$$H_3O^+ + nH_2O \rightarrow [(H_2O)_n + H]^+$$

These water cluster ions may then act as reagent ions for analyte ion generation by an APCI mechanism:

$$M + [(H_2O)_n + H]^+ \rightarrow [M + H]^+ + nH_2O$$

Therefore, DART ionization may lead to both even-electron and odd-electron species. The prevailing process of analyte ion formation for a given sample strongly depends on such analyte properties. More detailed discussion about the ionization processes in positive and negative polarity can be found in [10]. In rare cases, nitrogen has also been used for DART ionization [11] for improved cost efficiency, but for most analytes nitrogen is not able to effect analyte ion formation [12].

Since solution-phase processes are excluded, DART requires some sort of volatility of the analyte. This is effected by the gas heater element which provide the energy for evaporation or thermal desorption processes. Having analyte molecules present in the gas phase is a prerequisite and, indeed, the overall efficiency is defined by the ability of a compound to evaporate without thermal degradation rather than by the DART ionization processes [10]. This may impose limits, as observed in the analysis of nucleotides [13], or may potentially be an advantage when small molecules are to be detected in a high-mass matrix [12].

DART has become an established technique for rapid mass spectral analysis of numerous types of sample. It is suitable for the analysis of bulk materials, either solid or liquid, as may be required in quality control or to quickly investigate the identity of a compound from chemical synthesis [10]. From the very beginning, DART has been employed for the analysis of plant materials, pesticide monitoring on vegetables, forensic and safety applications (e.g. detection of explosives, warfare agents, illicit drugs, etc.) [5,14]. In food analysis, DART has found application in the fast detection of contaminants such as dyes

[15,16], veterinary and pesticides residues [17–20], mycotoxins [21–24] and alkaloids [25,26] in a wide range of food and feed matrices. In these contexts, DART clearly showed many benefits such as lack of sample preparation, quick assay running times and fairly comparative qualitative results respect to conventional techniques. However, the latter are still preferred for quantitation analysis, mainly for reasons of sensitivity and measurement reproducibility [27].

DART sensitivity is primarily governed by the following factors: (1) the efficiency of desorption and (2) ionization of the analyte in the hot and excited helium gas flow and (3) the effective collection and transmission of the generated ions to the mass spectrometer. In most of the ASM techniques, the overall ionization efficiency is often reduced by strong competition and suppression effects arising as "everything" happen in a shorter timescale [15,27]. Therefore, the compounds more prone to be desorbed and ionize will likely dominate the mass spectrum. Concerning the ion transmission, it is much lower in DART respect to vacuum systems; it is estimated that less than 1% of the ions cloud generated with ambient ion sources could be transferred into the mass spectrometer due to gas-phase collisions, Columbic repulsion and ambient gas flow [28–30]. Lot of research and efforts has been done to address these shortcomings. For example, Häbe & Morlock optimized the DART source configuration geometry in order to improve the analyte desorption and ions transmission [31], while Sekimoto et al. added a corona discharge device to the DART apparatus to enhance the ionization yield [9]. Furthermore, a prior sample separation and/or pretreatment aiming at removing the matrix components and concentrate the relevant analytes can effectively improve the method performance [17,32,33]; on the other hand, it would sacrifice the quickness and throughput of DART analysis [34].

A further issue in DART quantitative analysis is represented by large signal fluctuations between repeated measures, which may lead to quantitation inaccuracy and low measurement reproducibility. This probably attributes to that in situ ionization is influenced by surroundings with an unstably moving air environment and interfered with abundant co-existed ingredients in complex matrices [34]. Several works reported measurement reproducibility around 10-15% in quantitative analysis using matrix matched calibration [15,23,24,27]. In order to compensate for signal fluctuations, the authors introduced stable isotope-labeled analogue of the analytes as internal standards, considerably improving the methods reproducibility [34]. In this regard, it is worth to mention that Vaclavik et al. found d_6-melamine not to be suitable as isotopic-labelled internal standard for melamine quantification in milk powder products, as instant deuterium/hydrogen exchange occurred during DART ionization [27].

Finally, the natural drawback of all the AMS techniques is the lack of additional separation to the *m/z* dimension, which increases the chances of unknown ion interferences. In this regard, attempts to couple DART source to ion mobility devices have been made with the purpose of adding a separation dimension without increasing analysis time [35,36].

In the food authenticity field, lot of research has been carried out by the research group of Jana Hajslova, Tomas Cajka and Lukas Vaclavik. Some of the above-mentioned studies focused on the quantification of undeclared addition of Sudan dyes (I–IV) in chili powder and melamine to milk powder and milk-based products [27], which are well-known fraud activities. In contrast to such targeted approaches, the capabilities of DART-MS coupled to subsequent multivariate data analysis for authentication purposes were also investigated. Vaclavik et al. were able to detect down to 6% adulteration of extra virgin with the cheaper hazelnut oil through DART-MS profiling and linear discriminant analysis [37]. Cajka et al. attempted to use DART–MS for the retrospective control of chickens feed fraud [38]. The authors tried to distinguish poultry fed with and without chicken bone meal through the analysis of polar and non-polar extracts of chicken muscles. Hrbek et al. investigated the possibility to discriminate between organic and conventional cow's milk, goat's milk and sheep's milk as well as the detection of undeclared addition of plant oils in milk-based foods [39].

Furthermore, DART-HRMS was proposed for the authentication of spices commodities. Avula et al. employed DART-HRMS and subsequent PCA data exploration for the discrimination between "true" *Cinnamon verum* and the cheaper *Cinnamon cassia* [40], while Pavlovich et al. tried to distinguish different spices according to their commercial brand [41]. More recently, DART-HRMS has found applications also in the assessment of salmon freshness and authenticity [42,43].

Atmospheric Pressure Solids Analysis Probe

Similarly to DART, the atmospheric pressure solids analysis probe (ASAP) also addresses the routine analysis of single compounds or simple mixtures [10]. ASAP has been proposed as a method for rapidly analysing volatile or semi-volatile liquid or solid materials using only slightly modified commercial ESI or APCI ion sources. In fact, ASAP can be implemented on any ESI/APCI ion source by simple introduction of the material of interest into a stream of heated gas (usually N_2), that effects vaporization of the analyte from the tube's surface. Ionization is then fulfilled by the corona discharge of an APCI source [10].

In its first appearance, ASAP was implemented on a Waters Micromass Qtof I mass spectrometer (Waters Corp., Beverly, MA), slightly modifying the original Z-Spray ion source (**Figure A7**). Basically, a 5-mm hole was cut in the glass sleeve surrounding the ionization region of the Z-Spray source in order to allow the direct introduction of a borosilicate capillary melting point (mp) tube. The normal ESI/APCI probe was used to deliver a hot $(350-500\,°C)$ N_2 desolvation and nebulizing gas stream, without any solvent flow. The mp glass tube was dipped into the sample solution and introduced through the hole, thus

exposing the capillary tip to the N_2 stream. Mp tubes have been chosen for the application as they are free of volatile components and inexpensive. Ionization occurs by a corona discharge at atmospheric pressure using the same voltages and discharge apparatus of the APCI source [6].

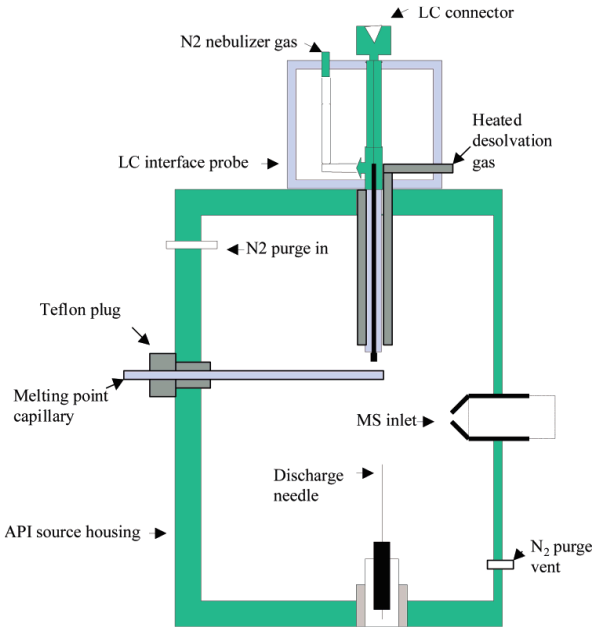

Figure A6. Cross-sectional drawing of an atmospheric pressure LC/MS ion source modified for ASAP analysis. Reproduced from [6].

In terms of ionization, for obvious reasons, principles and processes of the ASAP are closely related to APCI. The N_2 is ionized by the corona discharge:

$$N_2 + e^- \rightarrow N_2^{+\cdot} + 2e^-$$

$$N_2^{+\cdot} + N_2 + N_{2\ (3rd\ body)} \rightarrow N_4^{+\cdot} + N_{2\ (3rd\ body)}$$

In a dry source environment, the $N_2^{+\cdot}$ and $N_4^{+\cdot}$ act as reagent ions with charge transfer being the most likely pathway for ionisation. Differently, in the presence of water vapour, the formation of protonated water clusters is favoured:

$$N_4^{+\cdot} + H_2O \rightarrow 2N_2 + H_2O^{+\cdot}$$

$$H_2O^{+\cdot} + H_2O \rightarrow H_3O^+ + OH^{\cdot}$$

$$H_3O^+ + H_2O + N_2 \rightarrow [(H_2O)_2 + H]^+ + N_2$$

In ASAP there is no water being continually pumped into the source as in the case of LC-APCI/MS, thus explaining its similarity to the DART processes; however, some residual water vapour from either the source or the sample may be present. Therefore, the ionization path highly depends on the environment within the source block. A dry atmosphere will lead to the formation of analyte molecular ions via charge

transfer from radical nitrogen. Moist surroundings will support the constitution of protonated/deprotonated ion species. Moreover, mixed ionization mechanisms may also occur as observed by Ray et al. in steroids [44].

Thus far, ASAP has been mainly used in the field of forensic [45,46], drugs [47–49] and chemical materials analysis [50–54], while has been far less used in in food testing compared to DART-MS. In the first work on ASAP, McEwen et al. were able to readily observe a range of different analytes such as drugs and natural compounds from fresh biological samples [6,55]. Fussell et al. provided a proof-of-concept for the fast detection of (e.g. dyes, pesticides, etc.) in different food commodities. However, the authors stated that further experiments are required to assess possible applications of ASAP in residue analysis [56]. More recently, ASAP was employed for the semi-quantification of flavonoids for pollen characterization [57] and for the direct analysis of fatty acids on seed surface [58].

Being an AMS technique, ASAP-MS overall shows advantages and shortcomings similar to those mentioned for DART-MS. Easy-of use and reduced analysis time and costs, as well as ionization competition and suppression effects are features common to most of the AMS technologies. On the other hand, the enclosed source is expected to reduce the instability and interferences of an open-air ionization like DART, thus yielding better measurement reproducibility. As an advantage over DART-MS which requires a dedicated source, all ASAP needs is modification of a commercial APCI source by addition of a small port in the housing around the spray region to mount and safely insert the glass tube [10]. Likely for this reason, ASAP has already been coupled with ion mobility (IM) as post-ionization separation in several studies [59–64]. As mentioned for DART, the hyphenation with IM-MS add an extra dimension of separation, increasing selectivity.

References

[1] C. Black, O.P. Chevallier, C.T. Elliott, The current and potential applications of Ambient Mass Spectrometry in detecting food fraud, TrAC - Trends Anal. Chem. 82 (2016) 268–278. https://doi.org/10.1016/j.trac.2016.06.005.

[2] M.Z. Huang, S.C. Cheng, Y.T. Cho, J. Shiea, Ambient ionization mass spectrometry: A tutorial, Anal. Chim. Acta. 702 (2011) 1–15. https://doi.org/10.1016/j.aca.2011.06.017.

[3] J.B. Fenn, Method and apparatus for electrospray ionization, US6297499B1, filed July 3, 1998, and issued October 2, 2001.

[4] Z. Takáts, J.M. Wiseman, R.G. Cooks, Ambient mass spectrometry using desorption electrospray ionization (DESI): instrumentation, mechanisms and applications in forensics, chemistry, and biology, J. Mass Spectrom. 40 (2005) 1261–1275. https://doi.org/10.1002/jms.922.

[5] R.B. Cody, J.A. Laramée, H.D. Durst, Versatile new ion source for the analysis of materials in open air under ambient conditions, Anal. Chem. 77 (2005) 2297–2302. https://doi.org/10.1021/ac050162j.

[6] C.N. McEwen, R.G. McKay, B.S. Larsen, Analysis of solids, liquids, and biological tissues using solids probe introduction at atmospheric pressure on commercial LC/MS instruments, Anal. Chem. 77 (2005) 7826–7831. https://doi.org/10.1021/ac051470k.

[7] D.J. Weston, Ambient ionization mass spectrometry: Current understanding of mechanistic theory; Analytical performance and application areas, Analyst. 135 (2010) 661–668. https://doi.org/10.1039/b925579f.

[8] C.C. Mulligan, N. Talaty, R.G. Cooks, Desorption electrospray ionization with a portable mass spectrometer: In situ analysis of ambient surfaces, Chem. Commun. (2006) 1709–1711. https://doi.org/10.1039/b517357d.

[9] K. Sekimoto, M. Sakakura, T. Kawamukai, H. Hike, T. Shiota, F. Usui, Y. Bando, M. Takayama, Improvement in ionization efficiency of direct analysis in real time-mass spectrometry (DART-MS) by corona discharge, Analyst. 141 (2016) 4879–4892. https://doi.org/10.1039/c6an00779a.

[10] J.H. Gross, Direct analysis in real time-a critical review on DART-MS, Anal. Bioanal. Chem. 406 (2014) 63–80. https://doi.org/10.1007/s00216-013-7316-0.

[11] J.T. Shelley, J.S. Wiley, G.C.Y. Chan, G.D. Schilling, S.J. Ray, G.M. Hieftje, Characterization of Direct-Current Atmospheric-Pressure Discharges Useful for Ambient Desorption/Ionization Mass Spectrometry, J. Am. Soc. Mass Spectrom. 20 (2009) 837–844. https://doi.org/10.1016/j.jasms.2008.12.020.

[12] K. Jorabchi, K. Hanold, J. Syage, Ambient analysis by thermal desorption atmospheric pressure photoionization, Anal. Bioanal. Chem. 405 (2013) 7011–7018. https://doi.org/10.1007/s00216-012-6536-z.

[13] M. Curtis, M.A. Minier, P. Chitranshi, O.D. Sparkman, P.R. Jones, L. Xue, Direct analysis in real time (DART) mass spectrometry of nucleotides and nucleosides: Elucidation of a novel fragment [C5H5O] + and its in-source adducts, J. Am. Soc. Mass Spectrom. 21 (2010) 1371–1381. https://doi.org/10.1016/j.jasms.2010.03.046.

[14] F.M. Fernández, R.B. Cody, M.D. Green, C.Y. Hampton, R. McGready, S. Sengaloundeth, N.J. White, P.N. Newton, Characterization of Solid Counterfeit Drug Samples by Desorption Electrospray Ionization and Direct-analysis-in-real-time Coupled to Time-of-flight Mass Spectrometry, ChemMedChem. 1 (2006) 702–705. https://doi.org/10.1002/cmdc.200600041.

[15] Z. Li, Y.W. Zhang, Y.D. Zhang, Y. Bai, H.W. Liu, Rapid analysis of four Sudan dyes using direct analysis in real time-mass spectrometry, Anal. Methods. 7 (2015) 86–90. https://doi.org/10.1039/c4ay02409e.

[16] R. Wen, D. Zeng, Z. Yang, L. Jiang, M. Ma, B. Chen, T.A. Van Beek, Rapid Analysis of Illegal Cationic Dyes in Foods and Surface Waters Using High Temperature Direct Analysis in Real Time High-Resolution Mass Spectrometry, J. Agric. Food Chem. 66 (2018) 7542–7549. https://doi.org/10.1021/acs.jafc.8b02388.

[17] G. Germán, A. Gómezgómez-Ríos, E. Gionfriddo, J. Poole, J. Pawliszyn, Ultrafast Screening and Quantitation of Pesticides in Food and Environmental Matrices by Solid-Phase Microextraction–Transmission Mode (SPME-TM) and Direct Analysis in Real Time (DART), (2017). https://doi.org/10.1021/acs.analchem.7b01553.

[18] T. Guo, P. Fang, J. Jiang, F. Zhang, W. Yong, J. Liu, Y. Dong, Rapid screening and quantification of residual pesticides and illegal adulterants in red wine by direct analysis in real time mass spectrometry, J. Chromatogr. A. 1471 (2016) 27–33. https://doi.org/10.1016/j.chroma.2016.09.073.

[19] F.J. Lara, D. Chan, M. Dickinson, A.S. Lloyd, S.J. Adams, Evaluation of direct analysis in real time for the determination of highly polar pesticides in lettuce and celery using modified Quick Polar Pesticides Extraction method, J. Chromatogr. A. 1496 (2017) 37–44. https://doi.org/10.1016/j.chroma.2017.03.020.

[20] X.Q. Li, H.M. Li, S. Xu, Y. Gao, Q.H. Zhang, Y. Zhang, M.Y. Feng, Rapid quantification of trace chloramphenicol in honey under ambient conditions using direct analysis via real-time QTRAP mass spectrometry, Food Chem. 276 (2019) 50–56. https://doi.org/10.1016/j.foodchem.2018.09.130.

[21] M. Busman, C.M. Maragos, Determination of T-2 and HT-2 toxins from maize by direct analysis in real time mass spectrometry, World Mycotoxin J. 8 (2015) 489–497. https://doi.org/10.3920/WMJ2014.1854.

[22] M. Busman, J. Liu, H. Zhong, J.R. Bobell, C.M. Maragos, Determination of the aflatoxin AFB1 from corn by direct analysis in real time-mass spectrometry (DART-MS), Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 31 (2014) 932–939. https://doi.org/10.1080/19440049.2014.900572.

[23] M. Busman, J.R. Bobell, C.M. Maragos, Determination of the aflatoxin M1 (AFM1) from milk by direct analysis in real time - mass spectrometry (DART-MS), Food Control. 47 (2015) 592–598. https://doi.org/10.1016/j.foodcont.2014.08.003.

[24] L. Vaclavik, M. Zachariasova, V. Hrbek, J. Hajslova, Analysis of multiple mycotoxins in cereals under ambient conditions using direct analysis in real time (DART) ionization coupled to high resolution mass spectrometry, Talanta. 82 (2010) 1950–1957. https://doi.org/10.1016/j.talanta.2010.08.029.

[25] C. Li, E. Li, M. Ma, X. Liu, J. You, Y. Wu, W. Jiang, Z. Liu, Y. Qin, Simultaneous determination of six alkaloids in tobacco and tobacco products by direct analysis of real-time triple quadrupole mass spectrometry with a modified pretreatment method, J. Sep. Sci. 43 (2020) 1603–1613. https://doi.org/10.1002/jssc.201900946.

[26] A.D. Lesiak, K.L. Fowble, R.A. Musah, A Rapid, High-Throughput Validated Method for the Quantification of Atropine in Datura stramonium Seeds Using Direct Analysis in Real Time-High Resolution Mass Spectrometry (DART-HRMS), in: Methods Mol. Biol., Humana Press Inc., 2018: pp. 207–215. https://doi.org/10.1007/978-1-4939-8579-1_18.

[27] L. Vaclavik, J. Rosmus, B. Popping, J. Hajslova, Rapid determination of melamine and cyanuric acid in milk powder using direct analysis in real time-time-of-flight mass spectrometry, J. Chromatogr. A. 1217 (2010) 4204–4211. https://doi.org/10.1016/j.chroma.2010.03.014.

[28] Q. Yu, Z. Diao, K. Ni, X. Qian, F. Tang, X. Wang, Design and study of an atmospheric pressure ion funnel by computer simulations, Rapid Commun. Mass Spectrom. 29 (2015) 1055–1061. https://doi.org/10.1002/rcm.7195.

[29] L. Meier, C. Berchtold, S. Schmid, R. Zenobi, Extractive electrospray ionization mass spectrometry. Enhanced sensitivity using an ion funnel, Anal. Chem. 84 (2012) 2076–2080. https://doi.org/10.1021/ac203022x.

[30] Z. Li, J. Zhang, Y. Zhang, Y. Bai, H. Liu, New Strategy for Further Improving the Detection Sensitivity of Direct Analysis in Real Time-Mass Spectrometry, J. Anal. Test. 1 (2017) 1–6. https://doi.org/10.1007/s41664-017-0005-9.

[31] T.T. Häbe, G.E. Morlock, Improved desorption/ionization and ion transmission in surface scanning by direct analysis in real time mass spectrometry, Rapid Commun. Mass Spectrom. 30 (2016) 321–332. https://doi.org/10.1002/rcm.7434.

[32] O. Kiguchi, K. Oka, M. Tamada, T. Kobayashi, J. Onodera, Thin-layer chromatography/direct analysis in real time time-of-flight mass spectrometry and isotope dilution to analyze organophosphorus insecticides in fatty foods, J. Chromatogr. A. 1370 (2014) 246–254. https://doi.org/10.1016/j.chroma.2014.10.037.

[33] M.F. Mirabelli, J.C. Wolf, R. Zenobi, Direct Coupling of Solid-Phase Microextraction with Mass Spectrometry: Sub-pg/g Sensitivity Achieved Using a Dielectric Barrier Discharge Ionization Source, Anal. Chem. 88 (2016) 7252–7258. https://doi.org/10.1021/acs.analchem.6b01507.

[34] N. Zhang, M. Lu, X. Duan, C.C. Liu, H. Wang, In situ calibration of Direct Analysis in Real Time-mass spectrometry for direct quantification: Urine excretion rate index creatinine as an example, Talanta. 201 (2019) 134–142. https://doi.org/10.1016/j.talanta.2019.03.092.

[35] J.D. Keelor, P. Dwivedi, F.M. Fernández, An effective approach for coupling direct analysis in real time with atmospheric pressure drift tube ion mobility spectrometry, J. Am. Soc. Mass Spectrom. 25 (2014) 1538–1548. https://doi.org/10.1007/s13361-014-0926-8.

[36] I. Ayodeji, T. Vazquez, R. Bailey, T. Evans-Nguyen, Rapid pre-filtering of amphetamine and derivatives by direct analysis in real time (DART)-differential mobility spectrometry (DMS), Anal. Methods. 9 (2017) 5044–5051. https://doi.org/10.1039/c7ay00892a.

[37] L. Vaclavik, T. Cajka, V. Hrbek, J. Hajslova, Ambient mass spectrometry employing direct analysis in real time (DART) ion source for olive oil quality and authenticity assessment, Anal. Chim. Acta. 645 (2009) 56–63. https://doi.org/10.1016/j.aca.2009.04.043.

[38] T. Cajka, H. Danhelova, M. Zachariasova, K. Riddellova, J. Hajslova, Application of direct analysis in real time ionization-mass spectrometry (DART-MS) in chicken meat metabolomics aiming at the retrospective control of feed fraud, Metabolomics. 9 (2013) 545–557. https://doi.org/10.1007/s11306-013-0495-z.

[39] V. Hrbek, L. Vaclavik, O. Elich, J. Hajslova, Authentication of milk and milk-based foods by direct analysis in real time ionization-high resolution mass spectrometry (DART-HRMS) technique: A critical assessment, Food Control. 36 (2014) 138–145. https://doi.org/10.1016/j.foodcont.2013.08.003.

[40] B. Avula, T.J. Smillie, Y.H. Wang, J. Zweigenbaum, I.A. Khan, Authentication of true cinnamon (Cinnamon verum) utilising direct analysis in real time (DART)-QToF-MS, Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 32 (2015) 1–8. https://doi.org/10.1080/19440049.2014.981763.

[41] M.J. Pavlovich, E.E. Dunn, A.B. Hall, Chemometric brand differentiation of commercial spices using direct analysis in real time mass spectrometry, Rapid Commun. Mass Spectrom. 30 (2016) 1123–1130. https://doi.org/10.1002/rcm.7536.

[42] G.M. Fiorino, I. Losito, E. De Angelis, M. Arlorio, A.F. Logrieco, L. Monaci, Assessing fish authenticity by direct analysis in real time-high resolution mass spectrometry and multivariate analysis:

discrimination between wild-type and farmed salmon, Food Res. Int. 116 (2019) 1258–1265. https://doi.org/10.1016/j.foodres.2018.10.013.

[43] G.M. Fiorino, I. Losito, E. De Angelis, A.F. Logrieco, L. Monaci, Direct analysis in real time coupled to high resolution mass spectrometry as a rapid tool to assess salmon (Salmo salar) freshness, J. Mass Spectrom. 53 (2018) 781–791. https://doi.org/10.1002/jms.4260.

[44] A.D. Ray, J. Hammond, H. Major, Molecular Ions and Protonated Molecules Observed in the Atmospheric Solids Analysis Probe Analysis of Steroids, Eur. J. Mass Spectrom. 16 (2010) 169–174. https://doi.org/10.1255/ejms.1069.

[45] E. Jagerdeo, J.A. Clark, J.N. Leibowitz, L.J. Reda, Rapid analysis of forensic samples using an atmospheric solid analysis probe interfaced to a linear ion trap mass spectrometer, Rapid Commun. Mass Spectrom. 29 (2015) 205–212. https://doi.org/10.1002/rcm.7097.

[46] D. Carrizo, I. Nerín, C. Domeño, P. Alfaro, C. Nerín, Direct screening of tobacco indicators in urine and saliva by Atmospheric Pressure Solid Analysis Probe coupled to quadrupole-time of flight mass spectrometry (ASAP-MS-Q-TOF-), J. Pharm. Biomed. Anal. 124 (2016) 149–156. https://doi.org/10.1016/j.jpba.2016.02.033.

[47] C. Petucci, J. Diffendal, Atmospheric solids analysis probe: a rapid ionization technique for small molecule drugs, J. Mass Spectrom. 43 (2008) 1565–1568. https://doi.org/10.1002/jms.1424.

[48] M. Doué, G. Dervilly-Pinel, A. Gicquiau, K. Pouponneau, F. Monteau, B. Le Bizec, High throughput identification and quantification of anabolic steroid esters by atmospheric solids analysis probe mass spectrometry for efficient screening of drug preparations, Anal. Chem. 86 (2014) 5649–5655. https://doi.org/10.1021/ac501072g.

[49] B.J. McCullough, K. Patel, R. Francis, P. Cain, D. Douce, K. Whyatt, S. Bajic, N. Lumley, C. Hopley, Atmospheric Solids Analysis Probe Coupled to a Portable Mass Spectrometer for Rapid Identification of Bulk Drug Seizures, J. Am. Soc. Mass Spectrom. 31 (2020) 386–393. https://doi.org/10.1021/jasms.9b00020.

[50] S. Trimpin, K. Wijerathne, C.N. McEwen, Rapid methods of polymer and polymer additives identification: Multi-sample solvent-free MALDI, pyrolysis at atmospheric pressure, and atmospheric solids analysis probe mass spectrometry, Anal. Chim. Acta. 654 (2009) 20–25. https://doi.org/10.1016/j.aca.2009.06.050.

[51] M.J.P. Smith, N.R. Cameron, J.A. Mosely, Evaluating Atmospheric pressure Solids Analysis Probe (ASAP) mass spectrometry for the analysis of low molecular weight synthetic polymers, Analyst. 137 (2012) 4524–4530. https://doi.org/10.1039/c2an35556f.

[52] Z. Du, Y. Zhang, A. Li, S. Lv, Rapid identification of polymer additives by atmospheric solid analysis probe with quadrupole time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 28 (2014) 2035–2042. https://doi.org/10.1002/rcm.6998.

[53] L. V. Tose, M. Murgu, B.G. Vaz, W. Romão, Application of Atmospheric Solids Analysis Probe Mass Spectrometry (ASAP-MS) in Petroleomics: Analysis of Condensed Aromatics Standards, Crude Oil, and Paraffinic Fraction, J. Am. Soc. Mass Spectrom. 28 (2017) 2401–2407. https://doi.org/10.1007/s13361-017-1764-2.

[54] M. Driffield, E. Bradley, L. Castle, A. Lloyd, M. Parmar, D. Speck, D. Roberts, S. Stead, Use of atmospheric pressure solids analysis probe time-of-flight mass spectrometry to screen for plasticisers in gaskets used in contact with foods, Rapid Commun. Mass Spectrom. 29 (2015) 1603–1610. https://doi.org/10.1002/rcm.7255.

[55] C. McEwen, S. Gutteridge, Analysis of the Inhibition of the Ergosterol Pathway in Fungi Using the Atmospheric Solids Analysis Probe (ASAP) Method, J. Am. Soc. Mass Spectrom. 18 (2007) 1274–1278. https://doi.org/10.1016/j.jasms.2007.03.032.

[56] R.J. Fussell, D. Chan, M. Sharman, An assessment of atmospheric-pressure solids-analysis probes for the detection of chemicals in food, TrAC - Trends Anal. Chem. 29 (2010) 1326–1335. https://doi.org/10.1016/j.trac.2010.08.004.

[57] X. Xiao, L.L. Miller, K.J. Parchert, D. Hayes, J.M. Hochrein, Atmospheric solids analysis probe mass spectrometry for the rapid identification of pollens and semi-quantification of flavonoid fingerprints, Rapid Commun. Mass Spectrom. 30 (2016) 1639–1646. https://doi.org/10.1002/rcm.7601.

[58] M. Cechová, I. Hradilová, P. Smýkal, P. Barták, P. Bednář, Utilization of atmospheric solids analysis probe mass spectrometry for analysis of fatty acids on seed surface, Anal. Bioanal. Chem. 411 (2019) 1169–1180. https://doi.org/10.1007/s00216-018-1551-3.

[59] C. Barrère, F. Maire, C. Afonso, P. Giusti, Atmospheric solid analysis probe-ion mobility mass spectrometry of polypropylene, Anal. Chem. 84 (2012) 9349–9354. https://doi.org/10.1021/ac302109q.

[60] H. Pan, G. Lundin, Rapid Detection and Identification of Impurities in Ten 2-Naphthalenamines Using an Atmospheric Pressure Solids Analysis Probe in Conjunction with Ion Mobility Mass Spectrometry, Eur. J. Mass Spectrom. 17 (2011) 217–225. https://doi.org/10.1255/ejms.1125.

[61] X. Xiao, L.L. Miller, R. Bernstein, J.M. Hochrein, Thermal degradation of β-carotene studied by ion mobility atmospheric solid analysis probe mass spectrometry: full product pattern and selective ionization enhancement, J. Mass Spectrom. 51 (2016) 309–318. https://doi.org/10.1002/jms.3755.

[62] E. Cossoul, M. Hubert-Roux, M. Sebban, F. Churlaud, H. Oulyadi, C. Afonso, Evaluation of atmospheric solid analysis probe ionization coupled to ion mobility mass spectrometry for characterization

of poly(ether ether ketone) polymers, Anal. Chim. Acta. 856 (2015) 46–53. https://doi.org/10.1016/j.aca.2014.12.013.

[63] C. Barrère, M. Hubert-Roux, C. Afonso, A. Racaud, Rapid analysis of lubricants by atmospheric solid analysis probe-ion mobility mass spectrometry, J. Mass Spectrom. 49 (2014) 709–715. https://doi.org/10.1002/jms.3404.

[64] J. Vieillard, M. Hubert-Roux, F. Brisset, C. Soulignac, F. Fioresi, N. Mofaddel, S. Morin-Grognet, C. Afonso, F. Le Derf, Atmospheric Solid Analysis Probe-Ion Mobility Mass Spectrometry: An Original Approach to Characterize Grafting on Cyclic Olefin Copolymer Surfaces, Langmuir. 31 (2015) 13138–13144. https://doi.org/10.1021/acs.langmuir.5b03494.

Annex IV - Chemometrics

Nowadays, modern high-throughput instrumental techniques produce massive amounts of data with little effort in sample preparation and analysis; in this context, the risk for food scientists is to be inundated by non-informative data if appropriate data analysis strategies are not adopted [1,2]. Normally, the best way to extract relevant and useful information from high-dimensional data is to process them by multivariate statistics and pattern recognition techniques which, unlike univariate methods, takes into account the inter-correlations between the measured variables [3]. The term "chemometrics", coined in 1972 by Svante Wold, denotes the chemical discipline that use mathematical, statistical and other methods employing formal logic to: i) design or select optimal measurement procedures and experiments; ii) provide maximum relevant chemical information by analysing chemical data [4]. Some of the formal definitions of chemometrics would make no distinction from statistics in analytical chemistry. However, what somehow distinguishes chemometrics is primarily the use of computationally intense approaches, most of which are multivariate [5].

Multivariate statistical methods were first proposed theoretically in the early 20th century [6–8]; yet, in most of the cases, the computational demands were so high that chemometrics only began to be used in practice fifty years later, with the scientific computing becoming generally accessible. Therefore, although chemometrics' origin can be traced back to the 1960s [5], the use of mathematical and statistical methods in food science and technology has steeply increased only in the last 20 years (**Figure A11**).

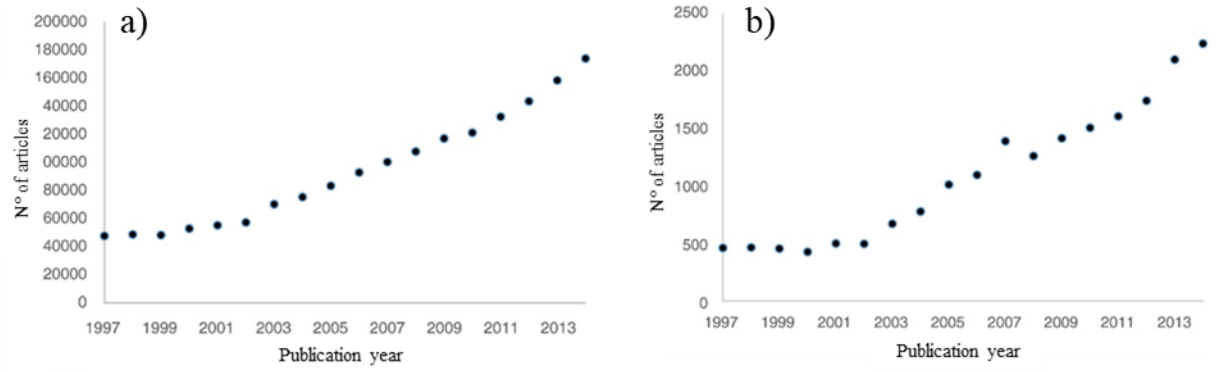

Figure A11. Number of published articles in ScienceDirect from 1997 to 2014 containing the terms "statistical analysis" (a) or "chemometrics" (b) in the title. Reproduced from [9].

Such trend has certainly been boosted by the enhanced processing capacity of affordable computers, along with the spread of user-friendly software and computational packages, leading to an increased number of people ordinarily using chemometrics in their work [9].

Historically, the majority of chemometric applications in food science have regarded the quantitative association of spectral data (e.g. NIR, FT-MIR) with one (or more) compositional parameter and/or chemical compound's concentration through a function or model $y = f(x)$, where x represents the recorded spectrum while y is the reference value (often measured by a conventional methods) [1]. On the other hand, application of chemometrics to qualitative problems, such as claims/label confirmation or the compliance with reference standards, has been growing in the past few years [10]. In these cases, pattern recognition techniques are used for both exploratory and classification purposes. It must be noted that the employed mathematical algorithms are often the very same, but y now represents a category/condition to which the tested sample belongs [11].

The following sections are intended to prove a minimum background of the main chemometric techniques used in the food authenticity field and, in particular, in the present PhD thesis.

Data Preprocessing

In most of the cases, chemometrics algorithms require data to be properly pre-treated to improve the quality of digital signals by reducing the impact of both random and systematic noise. Random noise is generally caused by random interferences phenomena (e.g. electric interference or surrounding environmental fluctuations). Besides, systematic bias may be due to difference in variance magnitude between the measured variables and/or instrumental/experimental "data artefacts", normally adjusted by scaling the data and promoting their "normality". Clearly, data pre-treatment must have minimal impact on the quality of information contained in the dataset [12].

Usually, each analytical method requires its own typical pre-processing steps and methodologies. Typical examples are chromatographic peak alignment in LC-MS data for compensating minimal changes in the chromatographic system during the analysis sequence or, as shown in **Section 3.2**, SNV processing in FT-IR data to emphasize differences between spectra and reduce light scattering effect [13,14]. Therefore, the choice of the best approach clearly depends on the problem under investigation and the nature of experimental data. For obvious reasons, all the possible data preprocessing solutions cannot be covered herein, Nevertheless, the reader is directed towards the several excellent reviews published in literature [13,15–19].

Even if all artefacts are removed from the data, all the measured variables often do not contain information important for the scientific question at hand. Moreover, important variables can be masked by the variability of many unimportant variables, specifically – but not exclusively – when variables are expressed on different scales [20]. Normally, the deviation from an intercept and/or offset often represents the interesting variation in the data. Mean-centering subtract each variable of its mean value computed row-wise across the data table; thus, it helps focusing on the "fluctuating" part of the data. In fact, all the measured values are converted to fluctuations around zero, instead of around the mean.

$$z_{ij} = x_{ij} - m_j$$

Where x_{ij} is the value of the j^{th} variable measured on the i^{th} element, and and m_j is the mean of the j^{th} variable. This is particularly useful in latent variable projections-based algorithms accounting for the maximum variation in the data (e.g. PCA). Otherwise, any type of biological variation in the data will be masked [21].

Scaling is used to equalize the potential of each variable to contribute to the eventual model. The type of scaling should be applied partly depends on the model itself. Here too, algorithms pointing at the largest data variance are sensible to variables with high variance, which are likely to dominate the model. In this respect, a commonly-used scaling method the so-called "unit-variance" scaling, or autoscaling, where all the measured values of variable are divided by their standard deviation after a prior mean centering:

$$z_{ij} = \frac{x_{ij} - m_j}{d_j}$$

Where d_j is the standard deviation of the j^{th} variable. The aim of autoscaling is to give equal weights to all of the features which will equally contribute to the multivariate model. In turn, it must be taken into consideration that also unimportant and/or noisy variables (often, characterized by a low standard deviation) become equally important; thus, information about the contribution of each variable to the total variance can be ''hidden''. For this reason, other scaling methods (e.g. Pareto scaling, range scaling, etc.) have been proposed in order to equalize the contribution of different variables by assigning different weights to each variable [20].

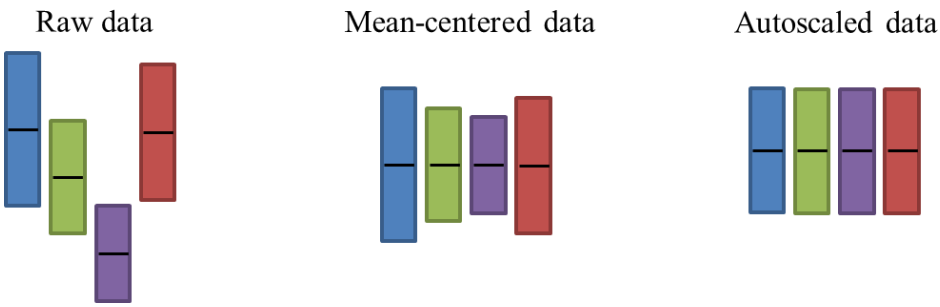

Figure A12. Schematic illustration of the effect of mean centering and autoscaling on. Four variables are depicted as rectangles of different colours. The rectangle's "height" represents the standard deviation ($\boldsymbol{st.dev.}$) of the measured values, while the $\boldsymbol{mean}$ is portrayed by a short horizontal line inside each bar. In raw data, each variable has its own $\boldsymbol{mean}$ and $\boldsymbol{st.dev.}$; after mean centering, all the variables have $\boldsymbol{mean = 0}$ and their own $\boldsymbol{st.dev.}$; after autoscaling, all the variables have zero $\boldsymbol{mean = 0}$ and $\boldsymbol{st.dev. = 1}$.

Transformations are nonlinear conversions of the data, which are generally applied to correct for heteroscedasticity, to convert multiplicative into additive relations, and to make skewed distributions (more) symmetric. For instance, NMR and MS data are generally subject to heteroscedastic noise (i.e. noise increases at higher signal intensity) and the distributions of the features can be non-Gaussian. Unlike centering and scaling, which operate column-wise, transformations work element-by-element. As an example, Log transformation replaces each value x_{ij} in the data matrix with a $\log(x_{ij})$. Thanks to its easy-of-use, it is one of the most popular method used to address skewed data in a range of research areas. It must be noted that, since the log transformation tend to reduce high values in the dataset relatively more than low, it has also a "pseudo-scaling" effect [21].

It must be stressed that the adopted data preprocessing processing can make or break subsequent data analysis; yet, it is still almost not-possible to predict with certainty the optimal preprocessing to use (or avoid) [20] and, in many cases, pre-processing strategies are selected based on past experience and/or using a ''trial-and-error'' approach [20].

Exploratory Data Analysis

Exploratory analysis is often the first step in data analysis workflows (even when the final goal is predictive modeling) carried out by means of different statistical techniques with the main of finding patterns, uncovering hidden correlations, detecting outliers, testing assumptions, etc.

Projection method are necessary in situations where the number of measured variables largely exceeds the number of analysed samples ("underdetermined" experiment), therefore, graphical inspection of the data in the original multi-dimensional space may result difficult or even unfeasible. Indeed, as the name suggests, these methods operate by projecting the data on a reduced-dimension space, allowing a more convenient data exploration/visualization by means of plots and figures. Such "data compression" allows a more convenient data exploration/visualization by means of plots and figures; besides, it is often used reduces the size and complexity of the original data prior to the actual modelling and/or to carry out a selection of the most relevant features [22].

The most common multivariate projection method is the Principal Component Analysis.

Principal Component Analysis

Principal Component Analysis (PCA) is arguably the most widely used method for data compression [23]. The central idea is to reduce the dimensionality of a dataset (especially when consisting of a large number of interrelated variables) while retaining as much as possible of the original data variation. This is basically done by "condensing" the original descriptors into a new set of uncorrelated variables, i.e. the principal components (PCs).

In order to provide an easy-to-understand geometric interpretation of PCA, let us consider a X matrix with 3 variables and N objects, i.e. a 3-dimensional space, using measurements $[x_1, x_2, x_3]$ (**Figure A13a**).

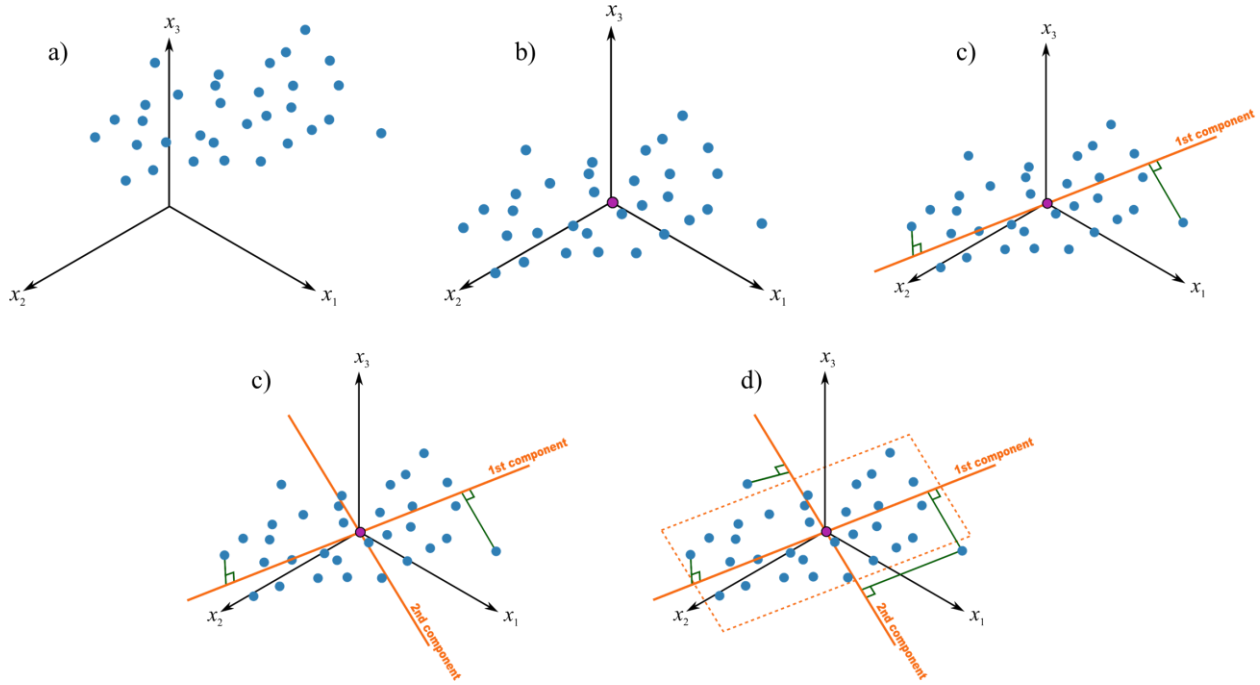

Figure A13. Geometric illustration of the PCA algorithm steps.

The first step in PCA is to move the data to the centre of the coordinate system by a prior mean-centering of the data (**Figure A13b**). If necessary (e.g. variables measured are in different units or having different magnitude), data can also be scaled to unit-variance. At this point, the best-fit line is drawn through the cloud of points (**Figure A13c**) so that it will best explain all the observations with the minimum residual error (it is a common saying that "it points toward the direction of maximum variance of the data"). Such best-fit line is the first PC and it is built as a sort of weighted average (i.e. linear combination) of the original variables:

$$t = p_1 \cdot x_1 + p_2 \cdot x_2 + \cdots + p_k \cdot x_k$$

where t is the so-called score vector (see below), $[x_1, \dots, x_k]$ are the k variables within the data matrix X and $[p_1, \dots, p_k]$ constitutes the so-called loadings vector. Loadings are the weights given to the variables $[x_1, \dots, x_k]$ in the generation of the PC. Therefore, they basically define what a particular PC represents in terms of original variables. Loadings are needed to interpret the scores and explain their meaning within the model [23]. Scores are the coordinates of the original data points' projection onto the PCs. In fact, when the direction of the best-fit line is found, each object is perpendicularly projected onto this line and its distance from the origin corresponds to the "score". Therefore, each observation gets its own score value on every PC. Scores can also be sees as (latent) variables; thus they can be interpreted in exactly the same way as any other variable. Concerning their visualization, they are most often plotted as a line plots (samples vs scores PC_x) or scatter plot (scores PC_x vs scores PC_y). The former highlights which samples has positive, or negative, scores for each PC. Samples having positive (or negative) scores on PC_x will exhibit relatively lower values of the original variables characterized by positive (or negative) loadings. $PC_x \; vs \; PC_y$ scatter plots are used to find clusters and patterns in the data. Close samples will be similar in terms of what the PCs represent (defined by the loadings). If the scores of the two visualized components are on the same scale, the distances between samples in the corresponding scatter plot will reflect distances in the original data.

A second component can be defined so that it starts at the origin and is perpendicular to PC_1 (**Figure A13c**). It should be noted that such axis could point in any direction as long as it remains perpendicular to the PC_1. However, it is rotated around until the direction of (residual) greatest variance in the data is found. The PC_1 and PC_2 jointly define a plane (**Figure A13d**). This plane is the *latent variable model* with two components. With one component the latent variable model is just a line, with two components the model is a plane, and with 3 or more components the model is defined by a hyperplane.

The non-linear iterative partial least squares (NIPALS) algorithm is one of the available methods to compute the principal components. Other algorithms have been proposed, such as the Eigenvalue

decomposition and the Singular Value Decomposition; however, NIPALS algorithm is used by most of the computer packages as it has two main advantages: it handles missing data and calculates the components sequentially. As said, each component is extracted sequentially and always oriented towards the direction of greatest variance. The NIPALS-PCA iterations are graphically represented in **Figure A14**:

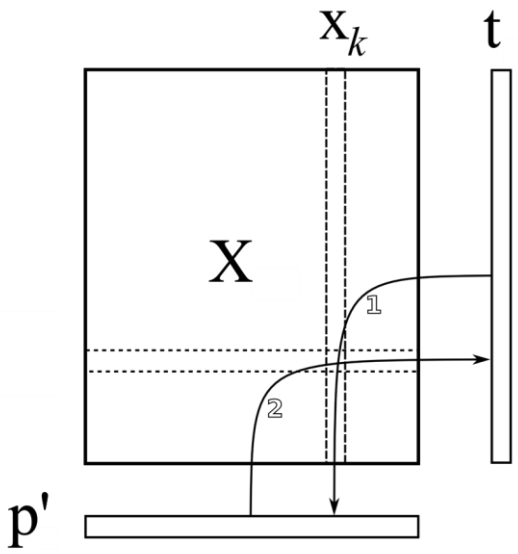

Figure A14. Schematic representation of the PCA-NIPALS algorithm. Adapted from [24].

Given a pre-processed - usually centered and scaled – X matrix, briefly:

1) The t column vector is arbitrarily created using random numbers or use a column from the X matrix. Anything can be used as long as it is not a column of zeros;
2) Each column of X is regressed (one at a time) onto the vector t (arrow in the figure) and the corresponding regressions coefficients are stored in p:

$$p_k = \frac{t^T X_k}{t^T t}$$

Where X_k is the k^{th} column of X and p_k is the k^{th} value of the p vector. Note that an ordinary least squares regression ($y = \beta x$) is performed, where the x-variable is the t column and the y-variable is the regressed column from X;

3) The loading vector p is normalized to unit length:

$$p^T = \frac{p}{\sqrt{p^T p}} \cdot p^T$$

4) Each column of X is regressed (one at a time) onto the normalized vector p and the corresponding regressions coefficients are stored in the t column. Here too, the x-variable is the p vector and the y-variable is the regressed column from X;

5) Steps 2, 3 and 4 are iterated until convergence of t vector (i.e. changes between current and previous values are within a predefined tolerance, e.g. 10^{-5}). Most data sets require no more than 200 iterations before achieving convergence;
6) On convergence, the score and loading vectors t and p are stored as column in the T and P matrices, respectively;
7) Finally, the X matrix is deflated ($\hat{X}$) and the residuals matrices E is calculated:

$$E = X - tp^T = X - \hat{X}$$

After deflation the algorithm is repeated for the next component. For the first PC, X is just the pre-processed raw data. Then, the second component is actually calculated on the residuals E, i.e. fraction of data variation not captured by the PCA model. Therefore, each subsequent component sees only the variation remaining after the deflation of the previous. This explains why each PC is orthogonal to the others: there is no possibility that two PCs explain the same variability.

From the mathematical perspective, one of the fundamental results of PCA is the decomposition of the data matrix X as:

$$X = TP^T + E$$

where P and T are the loadings and scores matrices, respectively (**Figure A15**). The (algebraic) multiplication of T and P^T gives the so-called model representation of the original data $\hat{X}$, then used to calculate the residuals E. As can be seen in **Figure A15**, the residuals matrix has the same structure of the original data; therefore, any useful data visualization will also be suited for the residuals investigation. For instance, for spectral data the residuals would literally correspond to the residual spectra - magnitude of the residuals will be the same of the modelled data. Residuals investigation can provide important chemical information about what spectral variation has not been explained by the developed model. In addition, residuals can be plotted as histograms or scatter plots (e.g. normal probability plots) in order to assess whether they follow a normal distribution.

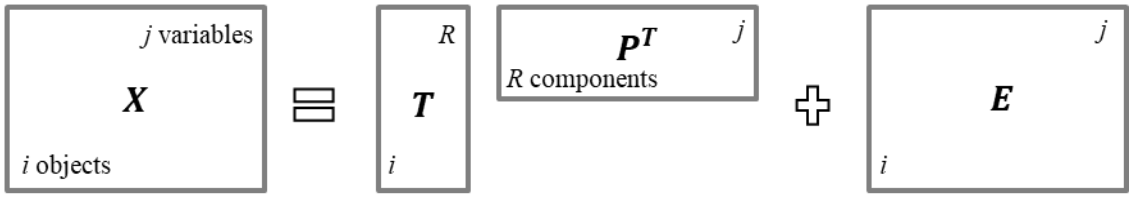

Figure A15. Illustration of a PCA model structure. Adapted from [23].

Thorough explanation and mathematical derivation of PCA can be found in several excellent reviews published in literature [23,25,26].

Classification Analysis

Broadly speaking, the term "classifying" means to assign a sample to one, or more, categories - collection of objects sharing similar characteristics - based on a set of measurements used to describe or characterize the object itself. From the chemometric standpoint, all these problems fall into the domain of pattern recognition or, more specifically, classification [11].

Classification methods can be grouped classified in different ways, yet the fundamental distinction is generally made between methods focused on discriminating among different categories and those rather directed towards modelling classes. In the first case, one speaks of "discriminant classification", while in the latter, of "class modelling". In general terms, the classification rules resulting from discriminant techniques are built focusing on the differences between samples coming from different classes and result in hypersurfaces, dividing all the variable space into as many non-overlapping regions as the number of available categories so that a sample is always univocally assigned (**Figure A15**). On the other hand, class-modelling methods are aim at capturing the similarities among samples from the same category rather than the differences among the classes. This is reflected by the fact that every category is modelled individually, irrespectively of the others. Geometrically, this corresponds to identifying a volume in the multidimensional space enclosing the class, so that if a sample falls within that volume it is accepted by the particular category, while if it falls outside, it is rejected by that class model. In addition, regions where a sample is accepted by more than one category, or by none, can occur (**Figure A15**).

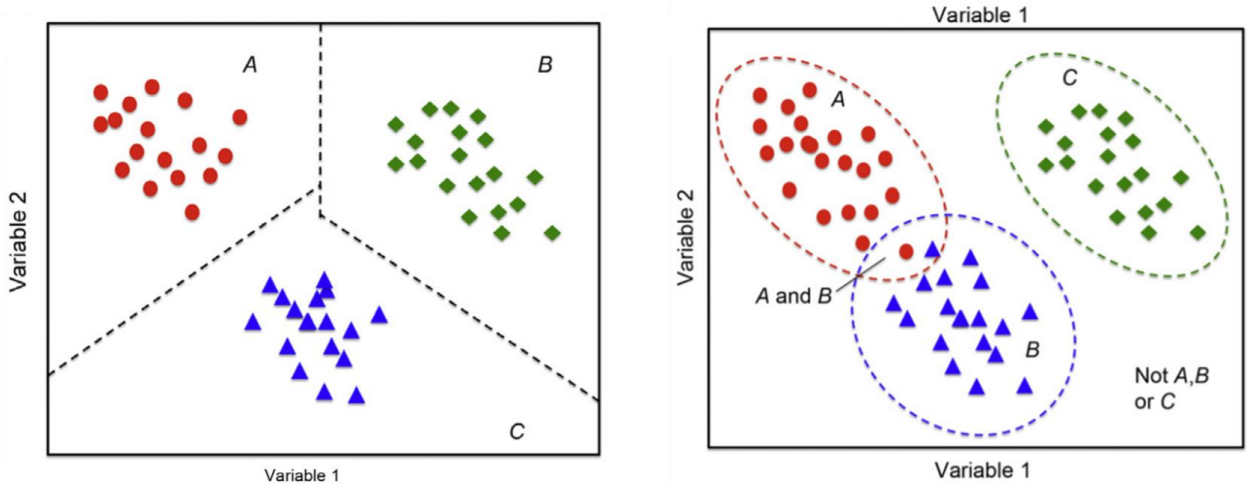

Figure A16. Illustration of the differences between discriminant (left) and modelling (right) approaches in the case of a problem involving three classes in a two-dimensional space. Reproduced from [11].

Partial Least Squares Discriminant Analysis (PLS-DA) and Soft Independent Modelling for Class Analogies (SIMCA) are among the mainly used chemometric techniques for discriminant analysis and class modelling, respectively, and will be briefly explained in the next sections.

Partial Least Squares - Discriminant Analysis

The purpose of discriminant analysis is to build statistical models which can best differentiate the categories under investigation. From the geometrical perspective, this can be visualized as defining boundaries in the multivariate space which separate and delineate the different classes. From a predictive standpoint, this concept translates to the outcome that each sample is attributed to one, and only one, of the classes represented [22].

PLS-DA is probably the most used algorithm for discrimination and, at the same time, one of the most misunderstood and misused methods in chemometrics [27]. It can be loosely seen as a modified PCA since both perform a dimension reduction in order to overcome the problem of highly-collinear data matrices; yet, while PCA relies on the sample (total) variance/covariance matrix, PLS is driven by the between-group sum-of-squares and cross-product matrix [28]. For the same reasons, PCA may provide decent results in terms of classification accuracy when the major variation in fingerprints represents the between-group separation; however, it is far from optimal when the within-group variation dominates over between-group variance [29]. Historically, PLS algorithm was introduced for prediction purposes (e.g. analyte concentrations) by regressing the data matrix X against one, or more, continuous variables [30]. The extension of PLS to classification problems was based on the following simple idea: instead of regressing against a continuous variable, the calibration is made over (discrete) numeric labels assigned to the samples according to their belonging classes. Therefore, in both cases (i.e. regression or classification) there is a Y block matrix summarizing the so-called outcome variables, either continuous or discrete, and the problem is reduced to a regression between the scores for the original data X and the Y block. For instance, in a two-classes problem (often referred as PLS1), the Y block usually consists of a vector containing $+1$ and -1, or 0 and $+1$. The extension of PLS to more than two groups (i.e. PLS2) is possible, but requires a number of difficult decisions and hardly-verified assumptions, which most users are unaware of [27].

Several different algorithms have been proposed for PLS-DA (Andersson listed 9 methods in its paper [31] while a number of other approaches are available in literature); the first and most used algorithm was developed by Wold (also called NIPALS-PLS) and it is based on the properties of the NIPALS algorithm [32]. NIPALS-based PLS-DA algorithm proceeds in exactly the same way for PCA, except for the fact that

PLS iterates through both X and Y blocks, simultaneously. In PCA terms, X and Y blocks can be written as follow:

$$X = TP^T + E$$
$$Y = TQ^T + F$$

Where, T and P are the PLS scores and loadings matrices, respectively; E and F are the residuals matrices. It must be noted that the same notation is often used interchangeably in literature for PCA and PLS; however, they do not have the same properties. For instance, unlike PCA, here TP^T is not the best-fit least squares model for X.

The NIPALS-PLS iterations are graphically represented in **Figure A16**:

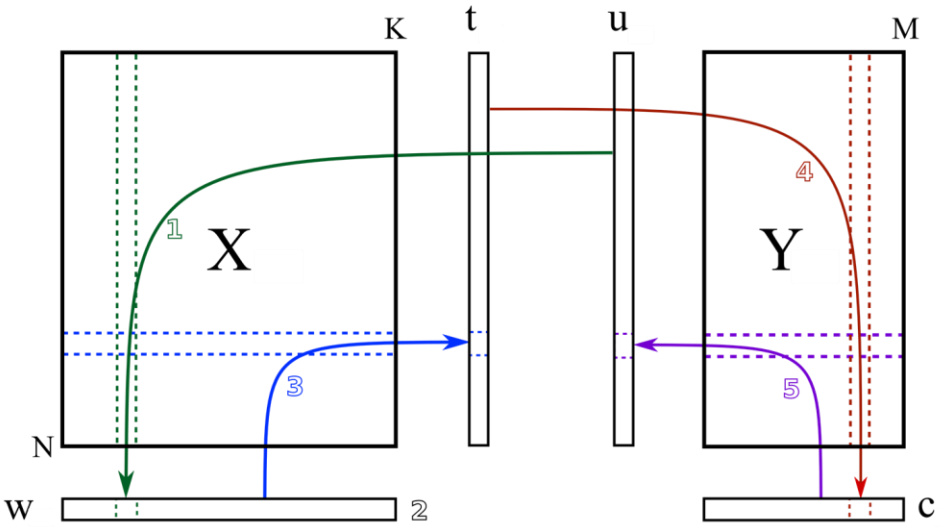

Figure A17. Schematic representation of the PLS-NIPALS algorithm. Adapted from [24].

Given the data matrix X and the outcome variables block Y, briefly:

1) A column is selected from Y as initial estimate of u. In one-dimensional Y case, then whole y vector is taken;
2) Each column of X is regressed onto the vector u (arrow 1) and the corresponding regressions coefficients are stored in w:

$$w = \frac{1}{u^T u} \cdot X^T u$$

Columns in X which are strongly correlated with u will have large weights in w (positive or negative values according to the correlation). Unrelated columns will have weights close to zero;

3) The vector w is normalized to unit length:

$$w = \frac{w}{\sqrt{w^T w}}$$

4) Each row of X is regressed onto the weight vector w (arrow 3) and the corresponding regressions coefficients are stored in t:

$$t = \frac{1}{w^T w} \cdot Xw$$

Rows in X that have a similar pattern to that described in w will have large values in t. Observations that are totally different to w will have near-zero score values.

5) Each column in Y are regressed onto the so-obtained score vector t (arrow 4) and the corresponding regression coefficients are stored in c:

$$c = \frac{1}{t^T t} \cdot Y^T t$$

In case of multi-dimensional Y, these coefficients indicate which Y-variables are modelled by the respective PLS component;

6) Each rows in Y is regressed onto this new weight vector c (arrow 5). A number I of regressions is performed (in one go) and the corresponding regression coefficients are stored in a new u vector:

$$u = \frac{1}{c^T c} \cdot Yc$$

Observations in Y that are strongly related to c will have large positive or negative slope coefficients in u;

7) The algorithm is iterated until convergence of u vector (i.e. changes between current and previous values are within a predefined tolerance, e.g. 10^{-5}). On convergence, the vectors w, t, c and u are store and jointly define the first component. When there is only one *y*-variable, Y loadings are set equal to 1 and the NIPALS-PLS algorithm converges after one iteration [33].

8) X and Y are deflated in $\hat{X}$ and $\hat{Y}$ and residuals matrices E and F are calculated (as seen for PCA). Deflation removes the explained variability from X and Y.

While the purpose of PCA is to describe X as better as possible (i.e. minimize $\|E\|$), PLS aims to best describe Y (i.e. minimize $\|\mathrm{F}\|$) simultaneously accounting for an acceptable variance in the X and Y blocks [27]. In simple words, the deviation from the PCA factors is needed to improve the correlation at the cost of some decrease in the explained variance. For further details, the reader is directed to [11,27,28,30,34].

A major advantage of PLS-DA over other techniques (e.g. LDA, QDA, etc.) is the possibility to investigate relationships between samples using scores, and between variables using loadings and weights. For instance, the significance of variables is often obtained in terms of statistical indicators or via graphical inspection of loadings and weights [34]. On the other hand, scores, loadings and weights may slightly differ according to the used algorithm, although the final predictions will be identical. In fact, another common PLS-DA algorithm was developed by Martens [35] and, as thoroughly explained by Brereton and Lloyd, it

has complementary and contrasting properties respect to the NIPALS method [34]. For instance, a common misconception is to plot the scores obtained from the NIPALS algorithm as orthogonal axes, introducing distortions in the representation. For this reason, any PLS-related plot should provide information about the method used to obtain it [34].

Soft Independent Modelling for Class Analogies

Class-modelling techniques operate with a different approach respect to discriminant algorithms. Instead of maximizing class differences, they focus on modelling the peculiar characteristics of the individual categories, hence the name [36]. Basically, they characterize the normal variation of the target category and, to assign a new unknown sample, check whether its registered signal falls within the identified normal range of variation. In fact, class-modelling methods are very similar, in some cases coincident, to outlier detection methods.

SIMCA was the first class-modelling method introduced in the literature. Its key assumption is that, the main variability within the target class can be captured by a PCA model of opportune dimensionality. Given a data matrix X, as seen above, a C-component PCA model summarizes the data as follow:

$$X = TP^T + E$$

The results of the PCA decomposition are used to define the class model according to an outlier detection criterion. In the original implementation of SIMCA, the information about the residuals was used to evaluate the samples acceptance. Such information is encoded in the form of a standard deviation:

$$s_0 = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{v} e_{ij}^2}{(v-C)(n-C-1)}}$$

Where v and n are the number of variables and objects, respectively, of the training set, C is the number of PCs of the PCA model, and e_{ij}^2 is the ith row and jth column element of the residual matrix E. The s_0 represents an indication of the typical data variation within the modelled class and can be considered as a measure of the distance between the training samples set and its model representation. This concept of "distance to the model" constitutes the basis to check the degree of outlyingness of unknown samples and, consequently, whether it is accepted or not by the category.

Given an unknown sample characterized by the row vector of measurements x_{unk}, it is first projected onto the PCs space:

$$t_{unk} = x_{unk} \cdot P$$

Where the t_{unk} vector contains the scores of the unknown sample onto the C principal components. Afterwards, the model representation of x_{unk} is obtained by back-projecting the scores onto the original variable space:

$$\hat{x}_{unk} = t_{unk} \cdot P^T$$

At this point, the residuals of the sample respect to its PCs representation in the target class can be computed as shown in the previous sections:

$$e_{unk} = x_{unk} - \hat{x}_{unk}$$

The vector of residuals e_{unk} is used to define the distance of the unknown sample to the model which, in the original implementation of SIMCA, is expressed under the form of a standard deviation:

$$s_{unk} = \sqrt{\frac{e_{unk} e_{unk}^T}{(v - C)}} = \sqrt{\frac{\sum_{j=1}^{v} e_{unk,j}^2}{(v - C)(n - C - 1)}}$$

Where $e_{unk,j}$ is the jth element of the residual vector e_{unk}. In this framework, an F test is carried out to compare s_{unk} with s_0 and check whether the sample distance to the model is comparable, or too large, to respect to the normal variability of the target class. In particular, the 95th percentile of the F distribution ($v - C$ and $n_A - C - 1$ degrees of freedom for the numerator and the denominator, respectively) is used to define a threshold values for the distance:

$$s_{crit} = \sqrt{F_{(0.95, v-C, n-C-1)}\, s_0}$$

Accordingly, if the distance to the class model s_{unk} is lower than the threshold distance s_{crit}, then the unknown sample accepted by the class, otherwise it is rejected. If more than one class are modelled, the same procedure is repeated for each category and it may also happen that the sample is accepted by more than one, or by none, of the available classes [11]. Other SIMCA implementations have been proposed in the literature introducing the score distance in the PC space as additional parameter to calculate s_{unk}. Further details can be found in [11].

Classification performance of class-modelling method are usually expressed as sensitivity and specificity. As explained in **Section 1.3**, sensitivity is the fraction (often expressed as percentage) of samples truly belonging to the target category which are correctly accepted by the class model; besides, specificity is the percentage of alien objects which are correctly rejected by the class model.

References

[1] J.J. Roberts, D. Cozzolino, An Overview on the Application of Chemometrics in Food Science and Technology—An Approach to Quantitative Data Analysis, Food Anal. Methods. 9 (2016) 3258–3267. https://doi.org/10.1007/s12161-016-0574-7.

[2] J. Riedl, S. Esslinger, C. Fauhl-Hassek, Review of validation and reporting of non-targeted fingerprinting approaches for food authentication, Anal. Chim. Acta. 885 (2015) 17–32. https://doi.org/10.1016/j.aca.2015.06.003.

[3] P. Oliveri, R. Simonetti, Chemometrics for Food Authenticity Applications, in: Adv. Food Authent. Test., Elsevier Inc., 2016: pp. 702–728. https://doi.org/10.1016/b978-0-08-100220-9.00025-4.

[4] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. de Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimetrics, 1st ed., Elsevier Science, 1997.

[5] R.G. Brereton, A short history of chemometrics: a personal view, J. Chemom. 28 (2014) 749–760. https://doi.org/10.1002/cem.2633.

[6] K. Pearson, On lines and planes of closest fit to systems of points in space, Informa UK Limited, 1901. https://doi.org/10.1080/14786440109462720.

[7] R.A. Fisher, Statistical Methods for Research Workers, in: Springer, New York, NY, 1992: pp. 66–70. https://doi.org/10.1007/978-1-4612-4380-9_6.

[8] H. Hotelling, Analysis of a complex of statistical variables into principal components, 1933. https://doi.org/10.1037/h0071325.

[9] C.A. Nunes, V.O. Alvarenga, A. de Souza Sant'Ana, J.S. Santos, D. Granato, The use of statistical software in food science and technology: Advantages, limitations and misuses, Food Res. Int. 75 (2015) 270–280. https://doi.org/10.1016/j.foodres.2015.06.011.

[10] G. Downey, Vibrational spectroscopy in studies of food origin, in: New Anal. Approaches Verif. Orig. Food, Elsevier Ltd, 2013: pp. 94–116. https://doi.org/10.1533/9780857097590.2.94.

[11] M. Bevilacqua, R. Bucci, A.D. Magrì, A.L. Magrì, R. Nescatelli, F. Marini, Classification and Class-Modelling, in: Data Handl. Sci. Technol., Elsevier Ltd, 2013: pp. 171–233. https://doi.org/10.1016/B978-0-444-59528-7.00005-3.

[12] D. Granato, P. Putnik, D.B. Kovačević, J.S. Santos, V. Calado, R.S. Rocha, A.G. Da Cruz, B. Jarvis, O.Y. Rodionova, A. Pomerantsev, Trends in Chemometrics: Food Authentication, Microbiology, and Effects of Processing, Compr. Rev. Food Sci. Food Saf. 17 (2018) 663–677. https://doi.org/10.1111/1541-4337.12341.

[13] S. Castillo, P. Gopalacharyulu, L. Yetukuri, M. Orešič, Algorithms and tools for the preprocessing of LC-MS metabolomics data, Chemom. Intell. Lab. Syst. 108 (2011) 23–32. https://doi.org/10.1016/j.chemolab.2011.03.010.

[14] T. Fearn, C. Riccioli, A. Garrido-Varo, J.E. Guerrero-Ginel, On the geometry of SNV and MSC, Chemom. Intell. Lab. Syst. 96 (2009) 22–26. https://doi.org/10.1016/j.chemolab.2008.11.006.

[15] P. Mishra, A. Biancolillo, J.M. Roger, F. Marini, D.N. Rutledge, New data preprocessing trends based on ensemble of multiple preprocessing techniques, TrAC Trends Anal. Chem. 132 (2020) 116045. https://doi.org/10.1016/j.trac.2020.116045.

[16] L.C. Lee, C.Y. Liong, A.A. Jemain, A contemporary review on Data Preprocessing (DP) practice strategy in ATR-FTIR spectrum, Chemom. Intell. Lab. Syst. 163 (2017) 64–75. https://doi.org/10.1016/j.chemolab.2017.02.008.

[17] H. Tian, B. Li, G. Shui, Untargeted LC–MS Data Preprocessing in Metabolomics, J. Anal. Test. 1 (2017) 187–192. https://doi.org/10.1007/s41664-017-0030-8.

[18] B. Reiz, A. Kertész-Farkas, S. Pongor, M.P. Myers, Data Preprocessing and Filtering in Mass Spectrometry Based Proteomics, Curr. Bioinform. 7 (2012). https://doi.org/10.2174/157489312800604363.

[19] J. Sun, Statistical analysis of NIR data: Data pretreatment, J. Chemom. 11 (1997) 525–532. https://doi.org/10.1002/(SICI)1099-128X(199711/12)11:6<525::AID-CEM489>3.0.CO;2-G.

[20] J. Engel, J. Gerretzen, E. Szymańska, J.J. Jansen, G. Downey, L. Blanchet, L.M.C. Buydens, Breaking with trends in pre-processing?, TrAC - Trends Anal. Chem. 50 (2013) 96–106. https://doi.org/10.1016/j.trac.2013.04.015.

[21] R.A. van den Berg, H.C.J. Hoefsloot, J.A. Westerhuis, A.K. Smilde, M.J. van der Werf, Centering, scaling, and transformations: Improving the biological information content of metabolomics data, BMC Genomics. 7 (2006) 142. https://doi.org/10.1186/1471-2164-7-142.

[22] E.K. Kemsley, M. Defernez, F. Marini, Multivariate statistics: Considerations and confidences in food authenticity problems, Food Control. 105 (2019) 102–112. https://doi.org/10.1016/j.foodcont.2019.05.021.

[23] R. Bro, A.K. Smilde, Principal component analysis, Anal. Methods. 6 (2014) 2812–2831. https://doi.org/10.1039/c3ay41907j.

[24] K. Dunn, Latent Variable Modelling, in: Process Improv. Using Data, 2020: pp. 315–391. https://learnche.org/pid/PID.pdf?73d667 (accessed October 20, 2020).

[25] S. Wold, K. Esbensen, P. Geladi, Principal component analysis, Chemom. Intell. Lab. Syst. 2 (1987) 37–52. https://doi.org/10.1016/0169-7439(87)80084-9.

[26] J.E. Jackson, A Use's Guide to Principal Components, John Wiley & Sons, Inc., Hoboken, NJ, USA, 1991. https://doi.org/10.1002/0471725331.

[27] R.G. Brereton, G.R. Lloyd, Partial least squares discriminant analysis: Taking the magic away, J. Chemom. 28 (2014) 213–225. https://doi.org/10.1002/cem.2609.

[28] M. Barker, W. Rayens, Partial least squares for discrimination, J. Chemom. 17 (2003) 166–173. https://doi.org/10.1002/cem.785.

[29] T. Rajalahti, R. Arneberg, A.C. Kroksveen, M. Berle, K.M. Myhr, O.M. Kvalheim, Discriminating variable test and selectivity ratio plot: Quantitative tools for interpretation and variable (biomarker) selection in complex spectral or chromatographic profiles, Anal. Chem. 81 (2009) 2581–2590. https://doi.org/10.1021/ac802514y.

[30] P. Geladi, B.R. Kowalski, Partial least-squares regression: a tutorial, Anal. Chim. Acta. 185 (1986) 1–17. https://doi.org/10.1016/0003-2670(86)80028-9.

[31] M. Andersson, A comparison of nine PLS1 algorithms, J. Chemom. 23 (2009) 518–529. https://doi.org/10.1002/cem.1248.

[32] H. Wold, Nonlinear estimation by iterative least square procedures, in: David IFN (Ed.), Res. Pap. Stat. Festschrift J. Neyman, Wiley, New York, 1966.

[33] N.L. Afanador, T.N. Tran, L.M.C. Buydens, Use of the bootstrap and permutation methods for a more robust variable importance in the projection metric for partial least squares regression, Anal. Chim. Acta. 768 (2013) 49–56. https://doi.org/10.1016/j.aca.2013.01.004.

[34] R.G. Brereton, G.R. Lloyd, Partial least squares discriminant analysis for chemometrics and metabolomics: How scores, loadings, and weights differ according to two common algorithms, J. Chemom. 32 (2018) e3028. https://doi.org/10.1002/cem.3028.

[35] H. Martens, T. Næs, Multivariate Calibration, in: B.R. Kowalski (Ed.), Chemom. - Math. Stat. Chem., Springer, Dordrecht, 1992. https://doi.org/10.1007/978-94-017-1026-8_5.

[36] Appendix XVIII: USP Guidance on Developing and Validating Non-Targeted Methods for Adulteration Detection, (2018). http://members.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/Food_Auth/2019USPC-Appendix XVIII_Guidance_on_Developing_and_Validating_Non-Targeted_Methods_for_Adulteration_Detection-FCC_Forum_December_2018.pdf. (accessed September 17, 2020).

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Studies

Tito Damiani got the Master degree in Food Science (University of Parma, Italy) in July 2017, thesis titled "Fumonisins: Analytical and Processing Issues". His master degree's thesis focused on the analytical and processing issues related to Fumonisins analysis. Subsequently, November 2017, Tito Damiani started his PhD in Food Science (University of Parma, Italy), centred on "Advanced Untargeted Methodologies applied to Food Analysis", under the supervision of Prof. Chiara Dall'Asta. The PhD research work has been associated with a strong collaboration with the Department of Food Science (University of Parma, Italy) and national and international partners, including R&D Unit at Barilla G. e R. Fratelli – s.p.a. (Italy), Department of Valorisation of Agricultural Products at Wallon Research Centre (CRA-W, Belgium), Scientific Operation Unit at Waters Corporation (UK).

During his 1st year, Tito Damiani has been selected for the "Young Scientist mobility programme" within the Food Integrity EU project. He spent 4 months at the CRA-W in Gembloux (Belgium) as visiting student working on vibrational spectroscopy (i.e. FT-MIR, FT-Raman, NIR) coupled to chemometrics for rapid screening analysis of food. In September 2019 he moved to the European Headquarter of Waters Corporation in Wilmslow (UK). There, he spent over 9 months focusing on the application of ambient mass spectrometry and liquid chromatography coupled to high-resolution mass spectrometry as rapid screening and confirmatory platforms for food authenticity. Overall, his doctoral research aimed at assessing quality and authenticity of food using advanced non-targeted analytical approaches coupled to intense chemometrics analysis. Some of the developed methods shown to be fit-for purpose and are now either implemented or being tested within the industry plant of Barilla G.R. F.lli SpA.

In addition, he has worked and contributed to several other projects dealing with the development of a LC-MS/MS method for the simultaneous quantification of relevant mycotoxins, plant metabolomics studies, assessment of the inter-platform reproducibility ion mobility-derived collision cross section, as well as their prediction using machine learning algorithms (see below).

Scientific activities

Review
"Untargeted Fingerprinting Approaches for Food Authentication: Where Do We Stand?"
Tito Damiani, Vincent Baeten, Juan Antonio Fernández Pierna, Sara Stead, Chiara Dall'Asta;
Trends in Food Science & Technology, (*In preparation*);

Original papers
"Analytical issues related to fumonisins: a matter of sample comminution?"
Tito Damiani, Laura Righetti, Michele Suman, Gianni Galaverna, Chiara Dall'Asta
Food Control, 95 (2019), 1-5, https://doi.org/10.1016/j.foodcont.2018.07.029;

"Exploiting the potential of micropropagated durum wheat organs as modified mycotoxin biofactories: The case of deoxynivalenol"
Laura Righetti, Enrico Rolli, Tito Damiani, Michele Suman, Renato Bruni, Chiara Dall'Asta
Phytochemistry, 170 (2020), e112194, https://doi.org/10.1016/j.phytochem.2019.112194;

"GC-IMS and FGC-Enose fingerprint as screening tools for revealing extra virgin olive oil blending with soft-refined olive oils: A feasibility study"
Tito Damiani, Daniele Cavanna, Andrea Serani, Chiara Dall'Asta, Michele Suman
Microchemical Journal, 159 (2020), e105374, https://doi.org/10.1016/j.microc.2020.105374;

Contribution to "Travelling Wave Ion Mobility-derived Collision Cross Section for mycotoxins: Interplatform and interlaboratory reproducibility"
Laura Righetti, Nicola Dreolin, Alberto Celma, Mike McCullagh, Gitte Barknowitz, Juan V. Sancho, Chiara Dall'Asta
Journal of Agricultural and Food Chemistry, 68 (2020), 10937–10943,
https://doi.org/10.1021/acs.jafc.0c04498;

"Vibrational Spectroscopy Coupled to a Multivariate Analysis Tiered Approach for Argentinean Honey Provenance Confirmation"
Tito Damiani, Rosa Maria Alonso-Salces, Ines Aaubone, Vincent Baeten, Quentin Arnould, Chiara Dall'Asta, Sandra Fuselli, Juan Antonio Fernández Pierna
Foods, 9 (2020), e1450, https://doi.org/10.3390/foods9101450;

"Critical evaluation of ambient mass spectrometry coupled with chemometrics for the early detection of adulteration scenarios in *Origanum vulgare* L."
Tito Damiani, Nicola Dreolin, Sara Stead, Chiara Dall'Asta
Talanta, (*Accepted for publication*);

"Quantification of regulated mycotoxins by LC-MS/MS: Addressing the challenges related to the analytical workflow"
Nicola Dreolin, Tito Damiani, Sara Stead, Chiara Dall'Asta
Food Control, (*In preparation*);

"Prediction of Collision Cross Section values using machine learning algorithms: Application to non-intentionally added substance identification in food contact materials"
Xuechao Song, Nicola Dreolin, Tito Damiani, Cristina Nerín
Food Chemistry, (*In preparation*).

Communications
"Authenticity of Honey: is DART-MS an effective tool?"
Tito Damiani, Nicola Dreolin, Sara Stead, Emiliano De Dominicis, Elisa Gritti, Chiara Dall'Asta
6th MS Food Day, Camerino, Italy, 25-27 September, 2019.

Poster Presentation
"Fumonisins in maize (Zea mays L.): effect of particle size on the analytical performance"
Tito Damiani, Marco Spaggiari, Laura Righetti, Chiara Dall'Asta
8th International Symposyum on Recent Advance in Food Analysis, Prague, Czech Republic, 7-10th November, 2017;

"Exploiting the potential of HRMS for deciphering mycotoxin modification in micropropagated wheat: the case of deoxynivalenol"
Tito Damiani, Laura Righetti, Enrico Rolli, Michele Suman, Renato Bruni, Chiara Dall'Asta
XXII International Mass Spectrometry Conference, Florence, Italy, 26-31st August, 2019;

"Near infrared microscopy and hyperspectral imaging for spices adulteration: a feasibility study"
Tito Damiani, Marco Spaggiari, Laura Righetti, Chiara Dall'Asta
Food Integrity, Nantes, France, 14-15th November, 2018;

"Authenticity of Honey: is Direct-MS an effective screening tool?"
Tito Damiani, Marco Spaggiari, Laura Righetti, Chiara Dall'Asta
9th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, 5-8th November, 2019;

"Atmospheric Solid Analysis Probe mass spectrometry for food compliance screening: oregano authenticity case study"
Tito Damiani, Nicola Dreolin, Sara Stead, Chiara Dall'Asta
68th ASMS Conference on Mass Spectrometry, Houston, Texas, 1-12th June, 2020;

Participation to Phd School and workshop

Workshop: "Vibrational spectroscopy and chemometrics for monitoring of food and feed products and contaminants´ detection", Prague, Czech Republic, 7th November, 2017;

"Foundamentals of Toxicology", Parma, Italy, 6-8th March, 2018;

"11th Vibrational spectroscopy and Chemometrics annual training", Gembloux, Belgium, 12-16th March 2018;

"Genetic taste & Food Preferences", Parma, Italy, 3rd May, 2018;

"Joint seminar Parma-Wageningen", Parma, Italy, 18th October, 2018;

1st REIMS and Ambient Mass Spectrometry User Meeting, Wilmslow, UK, 18th October 2019;

Workshop: "Vibrational Spectroscopy and Chemometrics: the laboratory moves to the sample", Czech Republic, 5th November, 2019;

IMaSS webinar on Data Analysis, online meeting, 16-20th April, 2020;

Study of the aromatic fraction of food: methods for its determination and analysis, 24-28th April, 2020;

1st GNPS User Meeting, online meeting, 26 August, 2020;

Intensive School for Advanced Graduate Studies - Machine Learning, Pavia, Italy, 7-17th September, 2020.

Teaching and didactic activities

- Master's degree course in "*Xenobiotics in Foods*" (Lecturer - Prof. Chiara Dall'Asta) for the academic years 2016/2017 and 2017/2018: 36 hours of laboratory experience;
- Bachelor's degree course in "*Food Chemistry"* (Lecturer - Prof. Martina Cirlini) for the academic years 2017/2018: 16 hours of laboratory experience;
- Lecture dedicated to the Master's degree course in *Food Science and Technology* students held at the University of Parma, Department of Food and Drug on 12th April 2019: "Introduction to Chemometrics";
- Tutoring of master's degree in Food Science and Technology students during their traineeship and thesis project period. Thesis are reported below:
  - "Factors affecting fumonisins accumulation in maize: climate, hybrid and geographical origin";
  - "Application of the strontium isotope analysis methodology ($^{87}Sr/^{86}Sr$) for the territorial and qualitative characterization of wine products";
  - "Investigation of the multi-mycotoxin profile in food and biological samples: two elaborate surveys";
  - "Effect of enriching raw material with ω-3 fatty acids and functional ingredients on the nutritional properties and the quality of dry cured 'Culatta' ";
  - "Occurrence of glyphosate in cereals and study of its distribution into milling fractions by IC-HESI-HRMS";

Other activities

Active collaboration with "*Food Hub*" magazine, writing article "Frodi nel settore alimentare" for divulgation purposes (https://www.foodhubmagazine.com/2019/05/16/frodi-nel-settore-alimentare/);

Participation at the joint seminar "*Ph.D. students from Parma and Wageningen*" held at the University of Parma, Centro Santa Elisabetta, Italy on 18 October 2018.