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Development of a non-target screening methodology using liquid chromatography coupled to high-resolution mass spectrometry for the detection of olive oil adulteration

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Ανάπτυξη μεθοδολογίας μη στοχευμένης σάρωσης με υγροχρωματογραφία συζευγμένη με φασματομετρία μάζας υψηλής διακριτικής ικανότητας για την ανίχνευση της νοθείας του ελαιόλαδου

ΤΖΑΒΕΛΛΑΣ ΗΛΙΑΣ

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ABSTRACT

Extra virgin olive oil (EVOO) is known to be the core of the Mediterranean diet, mainly because of its high nutritional value and also for its unique taste. Olive oil superiority, which has been validated by the EU establishment responsible for verifying the scientific substantiation of the health claims on olive oil polyphenols (EU 432/2012), has laid the foundation in the field for in-depth studies on olive oil authenticity and uniqueness. On many occasions, due to its high economic value compared to other food products, olive oil is considered at high risk of some kind of adulteration. According to FDA ranking, fraud concerning the substitution of olive oil with vegetable oils comes first as compared to other food. Having said that, in order to highlight the product's special qualities, attention must also be paid to the assurance of geographical origin and variety.

In the present study, for the purpose of achieving detection of geographical origin and varietal adulteration in EVOO samples, integrated non-target screening workflows based on LC-QToF-MS analysis have been developed and optimized. As such, two different studies on adulteration were designed and carried out. Samples of different EVOO varieties and geographical origin were analysed, including samples of adulterated olive oil. The adulterated samples were constructed in the lab, in different adulteration ratios varied from 10 to 50%. Potential authenticity markers of origin and variety for EVOO discrimination were introduced and notable differentiations in their profile were recorded. Evaluation and data analysis in non-target HRMS screening workflows were carried out. The advanced processing tools in combination with unsupervised chemometric techniques that were used were of great assistance.

Subject Area: Analytical Chemistry, Food Authenticity

Keywords: Olive oil, Adulteration, LC -QToF-MS, Chemometrics

ΠΕΡΙΛΗΨΗ

Το εξαιρετικά παρθένο ελαιόλαδο (EVOO) είναι γνωστό ότι αποτελεί τον πυρήνα της μεσογειακής διατροφής, κυρίως λόγω της υψηλής θρεπτικής του αξίας αλλά και λόγω της μοναδικής του γεύσης. Η ανωτερότητα του ελαιόλαδου, η οποία έχει επικυρωθεί βάσει του κανονισμού ΕΕ 432/2012 περί ισχυρισμών υγείας για τις πολυφαινόλες ελαιόλαδου, έθεσε τα θεμέλια στον τομέα για μελέτες σε βάθος σχετικά με την αυθεντικότητα και μοναδικότητα του ελαιόλαδου. Σε πολλές περιπτώσεις, λόγω της υψηλής οικονομικής του αξίας σε σύγκριση με άλλα προϊόντα διατροφής, το ελαιόλαδο θεωρείται ότι διατρέχει υψηλό κίνδυνο νοθείας. Σύμφωνα με την κατάταξη του FDA, η απάτη σχετικά με την αντικατάσταση του ελαιόλαδου με φυτικά έλαια έρχεται πρώτη σε σύγκριση με άλλα τρόφιμα. Ως εκ τούτου, προκειμένου να αναδειχθούν οι ιδιαίτερες ιδιότητες του προϊόντος, πρέπει επίσης να δοθεί προσοχή στη διασφάλιση της γεωγραφικής προέλευσης και της ποικιλίας.

Στην παρούσα μελέτη, με σκοπό την επίτευξη ανίχνευσης της νοθείας με βάση τη γεωγραφική προέλευση και την ποικιλία σε δείγματα EVOO, έχουν αναπτυχθεί και βελτιστοποιηθεί ενσωματωμένες ροές εργασιών μη-στοχευμένης σάρωσης με βάση την ανάλυση LC-QToF-MS. Ως εκ τούτου, σχεδιάστηκαν και πραγματοποιήθηκαν δύο διαφορετικές μελέτες σχετικά με τη νοθεία. Αναλύθηκαν δείγματα EVOOs διαφορετικών ποικιλιών και γεωγραφικής προέλευσης, συμπεριλαμβανομένων δειγμάτων νοθευμένου ελαιόλαδου. Τα νοθευμένα δείγματα κατασκευάστηκαν στο εργαστήριο και το εύρος της αναλογίας νοθείας κυμαινόταν από 10 έως 50%. Παρουσιάστηκαν πιθανοί δείκτες γνησιότητας προέλευσης και ποικιλίας για τη διάκριση των EVOOs και καταγράφηκαν αξιοσημείωτες διαφοροποιήσεις στο προφίλ τους. Πραγματοποιήθηκε αξιολόγηση και ανάλυση με τεχνικές μη στοχευμένης-επεξεργασίας δεδομένων HRMS. Τα προηγμένα εργαλεία επεξεργασίας σε συνδυασμό και με μη επιτηρούμενες χημειομετρικές τεχνικές που χρησιμοποιήθηκαν αποδείχθηκαν πολύ χρήσιμα.

Θεματική Περιοχή: Αναλυτική Χημεία, Αυθεντικότητα Τροφίμων
Λέξεις Κλειδιά: Ελαιόλαδο, Νοθεία, LC -QToF-MS, Χημειομετρία

''To my brother''

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CHAPTER 1
INTRODUCTION

1.1 Olive Oil

It is widely known that olive oil is one of the most significant food products in the Mediterranean countries. The olive tree counts among the oldest and most important oil-producing crops, after palm oil. Edible plant oils are a commonly used cooking ingredient around the world, while due to their high unsaturated fat content, are often considered to be an important component of a healthy diet. Olive oil in particular, is plant oil extracted from the pressings of olives and is an important fat source in many Mediterranean countries and throughout the world. Extra virgin olive oil (EVOO) is the highest quality olive oil and its health benefits have been studied extensively. When consumed as part of a traditional Mediterranean diet, EVOO has shown to be beneficial in preventing heart- and obesity-related health issues. The health benefits of EVOO are largely attributed to high levels of monounsaturated fatty acids as well as anti-oxidant and anti-inflammatory properties of minor phenolic compounds found in the oil. Olive oil and especially Extra Virgin Olive Oil (EVOO) utilization in dietary patterns world-wide is increasing due to its particular flavor, aroma, nutritional and health beneficial effects [1, 2].

1.1.1 Virgin (or natural) olive oil

The initial division is based on extraction methods used and oil is classified as “virgin” olive oil when it is extracted from the fruit by mechanical means, in a way which will not alter the oil characteristics. This process excludes oils obtained using solvents or re-esterification processes and any mixture with oils of any other kind or source. Modern methods of extraction of virgin oil involve washing, grinding or crushing the fruit, mixing or malaxing the paste, and then centrifuging to separate the oil from the pomace. This category, virgin olive oil, can be further divided, based on oil quality, into the following three groups [3]:

1. Extra virgin olive oil, must meet chemical and organoleptic limits. In particular, the free fatty acids (measured as oleic acid) must be less than, or equal to 0.8% w/w. In addition, the sensory requirements of this grade require that the oil has zero “defects” and positive attributes of fruitiness in excess of zero, based on sensory assessment.

2. Virgin olive oil must have less than, or equal to 2.0% w/w free fatty acids. The sensory requirements are that the oil be between 0 to 3.5 defects, as described by the International Olive Council (IOC), and greater than zero for fruitiness. Ordinary virgin olive oil is a category used by IOC but not all countries use this grade. It describes virgin olive oil with a free fatty acid level of not more than 3.3% w/w.

3. Lampante virgin olive oil, also referred as “crude” in some standards, refers to any virgin olive oil exceeding the free fatty acid limits discussed above or has a peroxide value (a measure of vegetable oil rancidity) in excess of 20 millequivalents (mEq) peroxide oxygen per kg/oil per the IOC standard, while California’s standard lowers this value to 15 [3]. This oil is considered unfit for human consumption in this state and requires refining or is otherwise used for industrial purposes. All grades of edible virgin olive oil should have a peroxide value of less than, or equal to 20 mEq peroxide oxygen per kg/oil. For lampante oil, there is no limit for peroxide value.

1.1.2 Refined olive oil

Refined olive oil, sometimes called ‘Pure Olive Oil’, is olive oil suitable for consumption after it goes through a food grade refining process. Any process used for refining must not alter the glyceridic structure of the oil. Due to refining, the free fatty acid content is low and for this grade it must be no more than 0.3% by weight [4].

Olive oil composed of refined olive oil and virgin olive oil, is a blend of refined olive oil and virgin olive oil (other than lampante oil). In this case the free fatty acids must be not more than 1.0% by weight.

Crude olive pomace oil is the oil extracted, using solvents such as hexane, from the solid waste, after producing virgin olive oil. The oil requires refining if used for human consumption. Otherwise it is used for industrial purposes.

• Refined olive pomace oil is obtained by refining crude olive pomace oil in a way that does not change the glyceridic structure of the oil. The free fatty acid concentration must not exceed 0.3% by weight.

• Olive pomace oil composed of refined olive pomace oil and virgin olive oils. As the name implies, this grade of oil is a blend of virgin olive oil and refined olive pomace oil. The free fatty acid content should not exceed 1.0% by weight.

1.1.3 Olive oil chemical classes

However, despite the large number of studies available in the literature regarding EVOO constituents, its thorough characterization still remains challenging due to the highly complex nature and the variation of factors affecting its composition. The importance accorded to olive oil is mainly due to the presence of its high content in monounsaturated fatty acids, specifically oleic acid (60– 80%) and to its richness in some minor components. The latter include squalene, pigments, tocopherols and phenolic compounds, which are involved in the preservation of the chemical quality of oil [4]. Among its several minor constituents, phenolic compounds are attracting considerable attention because they contribute to the virgin olive oil (VOO) stability against auto-oxidation [3, 4]. Moreover, polyphenols strongly affect sensory properties of VOO such as the typical bitter and pungent taste and they are also known for their beneficial properties in health. Characteristic classes of polar compounds abundant in EVOO are phenolic acids, phenylalcohols,

secoiridoids, flavonoids and lignans. From the phenylalcohol group, the most characteristic ones are hydroxytyrosol and tyrosol while secoiridoids constitute the major component of the unsaponifiable fraction of EVOO. Specifically, oleocanthal, oleacein and their structurally related molecules oleuropein and ligstroside aglycons together with elenolic acid and its derivatives such as methyl- and ethyl- esters of elenolic acid as well as the dialdehydic form of decarboxymethyl elenolic acid (EDA) are the most prominent secoiridoids of EVOO [3-5]. The amount of polyphenols in extra-virgin olive oil (EVOO) varies depending on several factors such as geographical zone, agro-climatic conditions, degree of fruit ripeness and oil-extraction process [3, 4]. Additionally, the phenolic fraction of olive oil can greatly vary among cultivars.

Olive oil phenolic fraction can be classified into different chemical classes to follow, according to their structural arrangement. **Table 1** summarizes the chemical classes while an indicative compound from its group is also quoted.

Tocopherols

Tocopherols are fat-soluble compounds consisting of a single benzene ring with a hydrocarbon tail attached. Several isoforms of tocopherols exist, all known as vitamin E, which is known to prevent lipid oxidation. Extra virgin olive oil is high in tocopherols with 23 g of olive oil providing approximately 25% of the RDI for vitamin E [6].

Sterols

Sterols, also known as steroid alcohols, are a sub-class of steroids, and are found in low to moderate levels in O. europaea. Many phytosterols are found in the olive, which are known to reduce the absorption of cholesterol in mammals. The sterol profile of olive oil can be used to determine the original cultivar and can elucidate oil adulteration with other vegetable oils [7].

Fatty Acids

The composition of olive oil is chiefly mixed triacylglycerols as well as free fatty acids, mono- and diacylglycerols, which together constitute 98-99% of the oil weight. For each commercial category of olive oil, the International Olive Council (IOC) sets compositional limits for the level of fatty acids. Monounsaturated fatty acids comprise the bulk of the fatty acids, with oleic acid being the most predominant type (55-83%), whilst polyunsaturated and saturated fatty acid represent 4-10% and 8-14% respectively. The consumption of monounsaturated fatty acids has been shown to decrease low-density lipoprotein levels [7, 8].

Hydrocarbons

Part of the unsaponifiable fraction of olive oil, hydrocarbons may be indicator of oil quality and origin. Squalene, a triterpenoid, comprises 80-90% of the hydrocarbon fraction in olive oil, and is widely used as an emollient in cosmetic preparations [9].

Phenols

The polar phenolic compounds identified within Olea europaea demonstrate a diverse subset of chemical structures found in varying concentrations with the leaf, fruit, and oil. Phenols are defined by their aromatic ring structure with one or more hydroxyl groups. These compounds contribute to the stability, antioxidant capacity, and bitterness of olive oil. A range of agronomic, genetic and environmental factors govern the phenolic composition of the olive [10-13]. The bioactive properties of these phenolic compounds continue to be explored.

Coumarins

Present in many plant species, coumarins are contained within the leaves of the olive. This subclass includes esculetin and scopoletin

Flavonoids

A class of plant metabolites defined by their 15-carbon skeleton. Considerable in vitro work has elucidated their antioxidant and anti-inflammatory activities. Apigenin, luteolin and their derivatives are the most abundant in olives and olive products.

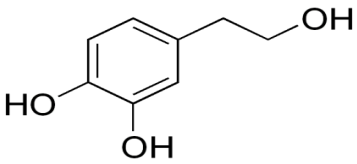
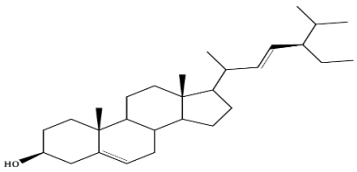
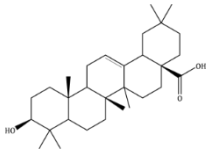
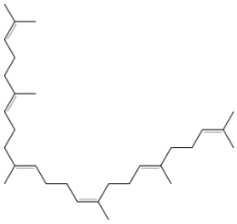
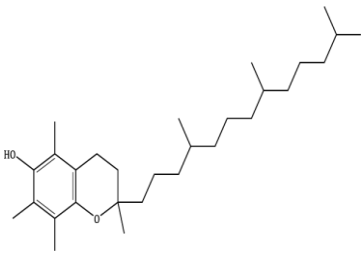
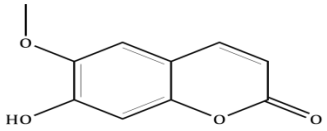
Secoiridoids

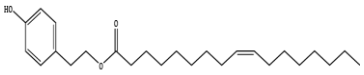
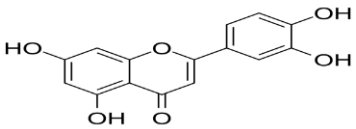
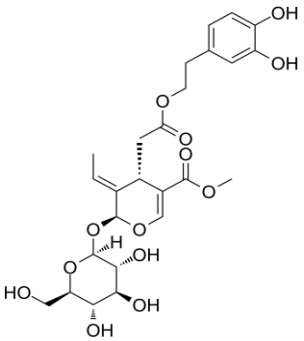
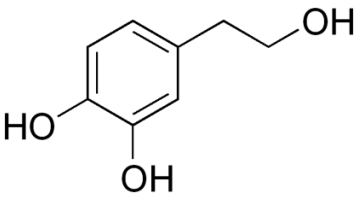
Secoiridoids are characterised by the presence of elenolic acid in its glucosidic or aglyconic forms. These compounds occur mainly in the Oleaceae species and are abundant in the leaves and fruit of O. europaea. The most common secoiridoids are oleuropein, demethyloleuropein, ligstroside and their aglycones.

Simple Phenols

An aromatic ring with either one hydroxymethyl and/or one to two hydroxyl groups. This group includes the phenylethanoids tyrosol and hydroxytyrosol, known for their antioxidant activity profiles.

Table 1. Structures of bioactive compounds from different chemical classes.

Chemical Class	Indicative Compound	Structure
Phenols	Hydroxytyrosol	
Sterols	Stigmasterol	
Triterpenic acids	Oleanolic acid	
Hydrocarbons	Squalene	
Tocopherols	α-tocopherol	
Fatty acids	Oleic acid	
Coumarins	Scopoletin	

Phenolic Fatty Acid Esters	1-oleyltyrosol	
Flavonoids	Luteolin	
Secoiridoids	Oleuropein	
Simple Phenols	Hydroxytyrosol	

1.2 Phenolic molecules in virgin olive oil

Oleuropein belongs to a specific group of coumarin-like compounds, the secoiridoids, which are abundant in Oleaceae. Secoiridoids are compounds that are usually glycosidically bound and produced from the secondary metabolism of terpenes. The secoiridoids, found only in plants belonging to the family of Olearaceae that includes Olea europaea L., are characterised by the presence of elenolic acid in its glucosidic or aglyconic form, in their molecular structure. In particular, they are formed from a phenyl ethyl alcohol (hydroxytyrosol and tyrosol), elenolic acid and, eventually, a glucosidic residue. Oleuropein is an ester of hydroxytyrosol (3,4-DHPEA) and the

elenolic acid (EA) glucoside (oleosidic skeleton common to the secoiridoid glucosides of Oleaceae). Secoiridoids of VOO in aglyconic forms arise from glycosides in olive fruits by hydrolysis of endogenous β-glucosidases during crushing and malaxation. These newly formed substances, having amphiphilic characteristics, are partitioned between the oily layer and the vegetation water, and are more concentrated in the latter fraction because of their polar functional groups. During storage of VOO hydrolytic mechanisms that lead to release of simple phenols, such as hydroxytyrosol and tyrosol, from complex phenols as secoiridoids may be involved.

The phenolic compounds are so interesting because of:

– The levels and chemical structure of antioxidant phenols in different plant foods, aromatic plants and various plant materials.

– The probable role of plant phenols in the prevention of various diseases associated with oxidative stress such as cardiovascular and neurodegenerative diseases and cancer.

– The ability of plant phenols to modulate the activity of enzymes, a biological action not yet understood.

– The ability of certain classes of plant phenols such as flavonoids (also called polyphenols) to bind to proteins. Flavonol–protein binding, such as binding to cellular receptors and transporters, involves mechanisms which are not related to their direct activity as antioxidants.

– The stabilization of edible oils, protection from formation of off-flavors and stabilization of flavours.

– The preparation of food supplements.

Focusing on phenolic compounds of virgin olive oil and bearing in mind the reasons for being so important, attention must be paid to the fact that this class of compounds has not been completely characterized due to the complexity of their chemical nature and the complexity of the matrix in which they are found. Health aspects linked to phenols in VOO is an integral ingredient of the Mediterranean diet and accumulating evidence suggests that

it may have health benefits which include reduction of risk factors of coronary heart disease, prevention of several types of cancers, and modification of immune and inflammatory responses. VOO can be considered as an example of a functional food, with a variety of components that may contribute to its overall therapeutic characteristics.

VOO contains at least 30 phenolic compounds. The major phenolic compounds are oleuropein derivatives, based on hydroxytyrosol which are strong antioxidants and radical scavengers. Phytochemical compounds such as oleuropein and oleuropein aglycon have been intensively studied for their promising results with respect to their effects on human health and their potential medicinal properties. It has been found that diets containing olive oil phenols may increase in vivo resistance of LDLs to oxidation; the effectiveness of oleuropein has been explained in part through its ability to act as an antioxidant and in part through a hypocholesterolaemic effect. In an investigation it was found that when a diet rich in oleuropein was fed to rabbits, the ability of LDL to resist to oxidation increased, thanks to its antioxidant capacity; moreover, a significant reduction of the plasmatic levels of total, free and ester-derivatives of cholesterol was found.

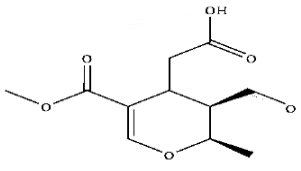
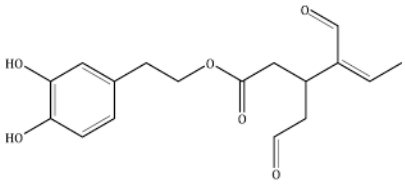
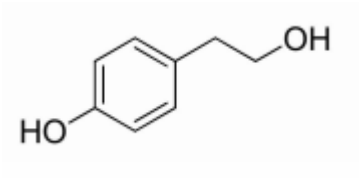
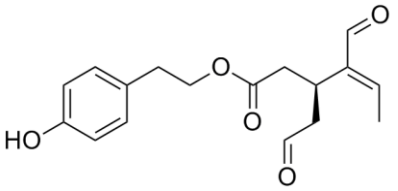
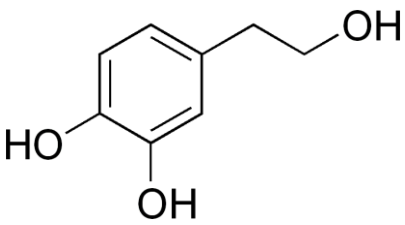
In the last few years, VOOPs have received growing attention because of chemoprevention activity against some human cancers. Several processes are essential for cancer development: DNA damage, sustained proliferation and insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, tissue invasion, metastatization and inflammation. Some VOOPs affect many if not all these processes thus interfering with carcinogenesis both in vitro and in vivo. This chemopreventive activity of VOOPs is the result of specific gene regulation effects some of which are now identified. Oxidative DNA damage plays a central role in both the stages of cancer initiation and promotion/progression. The protection effect of different VOOPs against the H2O2-induced DNA damage has been investigated in HL60 human lymphoblasts and in peripheral blood mononuclear cells (PBMC). HT (3,4-DHPEA) significantly reduced the extent of DNA damage at concentrations as low as 1 mM, as evaluated by single cell gel

electrophoresis (SCGE or Comet assay). Other compounds structurally related to HT showed the same effect, but with a different degree of potency. In particular, tyrosol, showed less efficacy than HT (3,4-DHPEA). These VOOPs also prevented the oxidative DNA damage induced in human lymphocytes by the co-culture with PMA stimulated monocyte-macrophages, an ex vivo model that mimics the pathophysiology of oxidative stress of an inflammatory lesion. In this experimental system, tyrosol was more effective than HT in preventing oxidative DNA damage.

Individual EVOO-derived complex phenolic compounds such as hydroxytyrosol and oleuropein aglycon efficiently inhibited proliferation and induced apoptotic cell death in human-derived breast cancer cell lines bearing high levels of the tyrosine kinase receptor HER2 (erbB-2), an oncoprotein which is found overexpressed in ~15-30% of human breast carcinomas. Moreover, it was established that isolated individual complex phenolic compounds and phenolic fractions mainly containing a sole phenolic component were not equivalent in their abilities to inhibit HER2-driven cell growth and to down-regulate the activity and expression of the HER2 protein itself. It is necessary to consider that because the biological effects of phenolic compounds, including breast cancer cytotoxic actions, are varied and compound specific, combinatorial effects (i.e., addition, antagonism or synergism) can occur in EVOO naturally exhibiting enriched or low levels of specific phenolic compounds. Although these experimental studies support the hypothesis of EVOO-derived complex phenols as breast cancer inhibiting compounds, forthcoming studies assessing the in vivo accessibility of EVOO phenolics to tumor tissues should be performed before suggesting that anticancer activity of EVOO-derived complex phenols should be expected from their direct local effects on the breast cancer tissues. Unfortunately, the knowledge available on the metabolic fate of EVOO derived complex phenols is still scarce. While absorption and bioavailability studies have revealed that tyrosol and hydroxyltyrosol can be retrieved in plasma and urine after olive oil consumption, there is an urgent need of data regarding the plasma/urine concentration of the free forms of various secoiridoid aglycones. Indeed, it is

reasonable to suggest that limited bioavailability of EVOO-derived complex polyphenols and their conversion into less-active metabolites (e.g., glucuronidated or sulfated forms) could significantly affect their antibreast cancer potential in vivo. Conversely, it has been suggested that the unabsorbed fraction of EVOO-derived lignans such as pinoresinol can be used by intestinal flora to produce the mammalian lignans enterodiol and enterolactone, which have been shown to reduce invasion in breast cancer cell lines. Although enrichment with the lignans fraction closely related to lower breast cancer cytotoxic activities as assessed by MTT-based cell viability assays in vitro, caution must be applied when trying to extrapolate in vitro results into clinical practice because dietary lignans have been repeatedly related with reduction of breast cancer risk. Moreover, methylation by catechol-O-methyltransferase (COMT), which has been described in vitro and in animal studies regarding the polyphenol (-)-epigallocatechin-3-gallate [EGCG], is a potential effect that could significantly alter the potent cytotoxic effects of secoiridoid aglycones in vitro against breast carcinomas in vivo.

Table 2. Bioactive compounds

Indicative Compound		**Structure**	**Bioactivity**
Elenolic Acid	Ester of hydroxytyrosol (3,4- DHPEA)		Antimicrobial and Antiviral Effects. Strong antioxidants and radical scavenger. chemoprevention activity
Oleuropein-aglycone di-aldehyde	3,4-DHPEA-EDA		Lipid-Regulating and Antiobesity Effects
Tyrosol	2-(4-hydroxyphenyl)-ethanol		Lipid-Regulating neuroprotective effectpreventing oxidative DNA damage.
Oleocanthal	p-HPEA-EDA		Mimic the pharmacological effects of ibuprofen modulator of inflammation and analgesia
hydroxytyrosol	hydroxytyrosol		Antiatherogenic and Cardioprotective Effect Neuroprotective Effects Antidiabetic, Lipid-Regulating and Antiobesity Effects

1.2.1 Olive oil and health benefits

Olive oil has a significant number of health benefits in health. Among others, EVOO has anti-oxidant activity due to the presence of polyphenols and vitamins A–E and K, and may reduce risk factors of coronary heart disease, prevent several types of cancers and modify immune and inflammatory responses. EVOO contains high levels of monounsaturated fatty acids (MUFAs) (considered a healthy dietary fat) and a higher MUFA/saturated FAs (SFAs) ratio. In the long term, these properties contribute to the protective effects by lowering “bad” cholesterol and raising “good” cholesterol. Indeed, the consumption of olive oil, as the predominant fat intake, provides high oleic acid content and polyphenols, which have atherogenic, antioxidant and anti-inflammatory effects and reduce the cholesterol/high density lipoprotein (HDL) ratio and the concentration of the oxidized low-density lipoprotein (LDL) [14]. Several studies demonstrated that diets with MUFA-rich EVOO can reduce the risk of obesity in childhood. Moreover, due to the high level of FAs and fat-soluble vitamins, EVOO is a source of high-density energy and is sometimes recommended in case of premature birth, a condition that requires a large amount of calories in a small quantity of food. The Mediterranean diet (MD) principal protective compounds against diabetes are contained in fibers and vegetable fats; in particular, this protection is guaranteed by EVOO intake (rich in MUFAs) used in cooking, spreading, dressing and frying foodstuffs. The presence of oleic, linoleic, and linolenic acids contribute to the development and growth of babies’ bones and brain. Indeed, EVOO and some other components of the MD (i.e., walnuts and moderate quantities of wine), or foods with antioxidant properties or rich in polyphenols, are independently associated with better cognitive function and high plasma levels of ω-3 FAs. This evidence suggests that the association between MD adherence and cognitive functions may be mediated by vascular factors, but also by non-vascular biological mechanisms, such as oxidative stress, inflammation and metabolic disorders, supporting the importance of the MD in

health, lifestyle and aging. Moreover, EVOO and other vegetable fats, containing polyunsaturated FAs (PUFAs), are reported as inversely correlated to the upper digestive, stomach and urinary tract cancer development risk.

1.3 EVOOs Adulteration

Virgin olive oil is considered a price premium product compared to other vegetable oils. It is very prone to adulteration leading to economic losses, disloyal competition among producers, and break in consumer trust. Recent crises in the food sector, such as with dioxin in poultry, bovine spongiform encephalopathy (BSE), and the controversy about genetically modified organisms (GMO) have highlighted the need for more strict food quality control, which should include determination of the origin of a product and the raw materials used in it. That is why a well-documented traceability system has become a requirement for quality control in the food chain.

1.3.1 Market dynamics

While olive oil production has gradually increased from 2,206 million tons in the 1992/93 season to a projected 3,314 million tons in 2017/18, there is substantial fluctuation in the annual production. Supply shortages are usually due to poor harvests, particularly in Spain. In 2012, olive trees in Spain were exposed to an unexpected frost in the spring, combined with a severe drought later in the year, leading to a drop in olive oil production from 1,615 million tons in the 2011/12 season to 618 million tons in 2012/13.4 The consequences were an impressive increase in olive oil prices, reported to be over 60% in the months from July – September 2012. A similar impact was observed after a drought hit the Andalusia region of Spain in 2014/15. In the same season, olive trees in Puglia, the main olive producing area in Italy, were affected by blight, again leading to a shortage in olive oil supply and a spike in prices. The blight is caused by an infection with the bacterium Xylella fastidiosa, which colonizes the olive tree’s water-conducting tissues, reducing water flow to leaves and branches, which eventually dry out and die. Unscrupulous suppliers may “compensate” for supply shortages and price increases by diluting olive oil for packaged food with other vegetable oils, e.g.,

canola/colza/rapeseed (Brassica napussubsp. napus, Brassicaceae), sunflower (Helianthus annuus, Asteraceae), or hazelnut (Corylus avellana, Betulaceae) oil, or with extracts from olive pomace. Price pressure on higher quality olive oils (virgin or extra virgin oils) means in turn that undeclared lower grade olive oils may be added in order to be competitive in the market.

IOC pricing provides average monthly prices at various sites in Europe. For refined olive pomace oil, wholesale prices in Spain ranged from US $1.68-2.62/kg in 2018. Refined olive oil fetched US $2.77-4.24/kg, while EVOO was sold at US $3.04-4.42/kg.25 EVOO prices in the United States fluctuated between approximately US $2.80–5.80/kg from 2004-2014, with prices highest in summer 2006, and lowest in summer 2012. Apparently, this price drop was caused by the increased substitution of EVOO with lower grade olive oils in the USA, allowing suppliers to sell their merchandise at a lower cost.26 Wholesale pricing for some other common vegetable oils is available from the US Department of Agriculture,27 which lists the following costs (in US $/kg) for 2018: Canola oil: 0.81-0.85; corn (oil: 0.58-0.68; cottonseed oil: 0.63-0.72; palm oil: 0.60- 0.75; palm kernel oil: 1.23-1.40; peanut oil: 1.37-1.50; soybean oil: 0.60-0.68; sunflower oil: 1.16-1.22. Retail pricing for all grades of olive oil in the US multi-outlet channel varied between US $2.46-93.88/L* in April 2019. For the most commonly sold volume (500 mL), the costs varied between US $6.83-83.03/bottle, with an average price of US $15.64 (± US $8.83). The majority of these 500 mL products were offered at US $9-15/bottle. High-priced products represent gourmet oils which are produced on a small scale.)The ability to deliver safe and authentic food is of high priority for manufacturers. However, often due to a complex network of suppliers, the potential of unintentional contamination of food exists considerably. The deliberate contamination driven by economic gain has also become harder to detect and track. As a result, the vulnerability of products and the risks inherent to food fraud is of considerable concern. An example is fake or diluted extra virgin olive oil (EVOO). Olive oil production is of paramount economic importance in selected Mediterranean regions,

particularly in Spain, Italy, and Greece, although the production of olive oil is also deeply rooted in countries as Syria, Tunisia, or Morocco [15].

1.4 EU Legislation

Food safety and food quality have always been issues of great importance; efforts to address them have been made in recent years. In light of this, 4 Food Fraud operational criteria have been established by the European Union. These are namely: violation of EU Food Law, intention, economic gain and deception of customers [5]. In an effort to limit and control food fraud, it has been EU's priority to enhance collaboration and trust among different services at a European level, namely: food experts (inspectors), Police/Customs (with investigative powers) and Justice within the EU countries.

EU networks were coordinating and exchanging information with Police/Customs (Europol/ European Anti-Fraud Office (OLAF) and Eurojust but not with Food Fraud experts since before the "horse meat" crisis. This was until July 2013, when the EU Food Fraud Network (FFN) was created.

In 2006, in order to limit Food Fraud, the EU financed the program, ''Metabolomics for Plant, Health and OutReach (META-PHOR)'' as a way to establish an international consortium of multi-disciplinary experts to develop common strategies and standards in food research. META-PHOR's aim is *''to generate knowledge on these metabolites in our food which determine key characteristics such as nutritional value, quality and health by developing the advanced tools required for their detection'' [16].*

Figure 1: The reorganised network of EU against Food Fraud based on mutual trust [5].

Following the horse meat crisis of 2013, EU has strived to enhance its control system in its entirety, in order to detect and counter frauds traced in the food chain. EU's initiatives in that respect were:

- Creation of an EU FFN, with representatives from the European Commission and all EU countries including Switzerland, Norway and Iceland, to achieve a more efficient cross-border administrative assistance and cooperation
- Development of the Administrative Assistance and Cooperation System (AAC), a dedicated IT tool, which would allow the rapid exchange of information of potential cross-border fraud cases, among network members. The system is operating since November 2015
- Specialised training (in the framework of Better Training for Safer Food initiative) for food inspectors, police, customs officers and judicial authorities of the EU countries, on new investigation and control techniques concerning food fraud (including eCommerce). Each year five trainings are being held
- Coordinated Control Plans at EU level
- The new Official Controls Regulation (OCR)

- Coordinated Control Plans at EU level
- The new Official Controls Regulation (OCR)

Figure 2:Current initiatives and dedicated activities of EU against Food Fraud [5]

To sum up, some basic strategies have been published for the protection against food adulteration, namely:

- safety assessment on ingredients and additives
- determination of ingredients and additives suitability for use in food, always in line with federal regulations
- sanitary processing, packaging, storage, transportation, handling assurance
- implementation of ongoing, periodic or voluntary inspections
- development and implementation of a food safety/HACCP plan

Regarding the EU, the aforementioned principles are covered in Regulation (EC) No 178/2002 of the European Parliament and the Council of 28 January

2002, where the general principles and requirements of food law were laid down, as well as the procedures in matters of food safety. The Regulation also established the European Food Safety Authority [17].

In the USA and Canada, these principles are detailed in the Food and Drugs Administration (FDA) (RSC, 1985, Chapter F-27) [18].

CHAPTER 2
DETECTION OF POSSIBLE ADULTERATION OF OLIVE OILS - LITERATURE REVIEW

2.1 Introduction

Olive oil has been recognized as an emblematic food of Mediterranean diet both for its distinctive taste and its high nutritional value. EVOO in particular due to its beneficial properties derived from its consumption, is often subjected to some kind of fraud, with partial substitution with oils of lower quality.

Different methodologies have been applied in order to declare product's special qualification and reassure its labelling compliance. Mass spectrometry techniques are the most prevailed to address this issue, taking into account not only a specific list of compounds (target screening) but also broadening the range of screening in masses yet to be identified but crucial in authenticity studies (non-target screening).

2.2 Sample Treatment

In their majority these methods are based on Liquid-Liquid Extraction (LLE). The solvent mixture used is the appropriate one, whereas in many cases as a clean-up technique of Solid Phase Extraction (SPE) is also being used. In LLE, it is essential that the optimum extraction solvent is selected, since it has to be able to efficiently extract target analytes but at the same time to also minimize the extraction of other matrix constituents so that undesirable matrix effects are avoided. It has been reported that in olive oil authenticity studies, different extraction solvents in different volume ratios have been used. For example, a simple dilution of the olive oil sample, with satisfying results, is used, so that triacylglycerols (TAGs) and the total chromatographic fingerprint

are determined [19-22]. In order to extract polyphenols and/or other polar compounds, the main extraction solvents, in different rations are methanol (MeOH) and water (H_2O), with MeOH: H_2O 80/20 v/v being the prevailing solvent ratio [23-31]. In most cases, the procedure of extraction is repeated 3 times [32-34] and in some rare cases, to ensure better analyte extraction from the matrix it is repeated 4 times [35]. Lastly, in some sample preparation procedures, a step of evaporation - reconstitution is also brought into the workflow. In such cased, the reconstitution solvent of the final, injection-ready, sample is MeOH [30, 36] or MeOH: H_2O 50/50 v/v [37-40].

2.3 Analytical Techniques- Liquid Chromatography coupled to Mass Spectrometry (LC-MS)

The food industry has employed a combination of MS and chromatography as a “golden standard” for the quantification and semi-quantitative screening of particular compounds in food. Both LC and MS, as opposed to GC-MS techniques, have proven to be great instruments for sensitive and selective determination of higher polar or ionic contaminants in trace levels in food [41, 42] including veterinary medicines [43, 44], pesticides [45, 46] and toxins [44, 47].

2.3.1 Reversed Phase Ultra High-Performance Liquid Chromatography (RP-UHPLC)

Progress in the area of chromatography allows more rapid and highly efficient LC separations [48, 49] and favors the analysis of ionic or polar compounds [50-52]. More specifically, UHPLC is a promising technique that uses particles of small-diameter in the stationary phase and short columns. This is how fast and high- resolution separation that increases LC-MS sensitivity and minimizes matrix interference is achieved [50]. UHPLC is usually carried out in reversed-phase (RP) mode using C18 columns. The mobile phase consists of an aqueous and an organic solvent.

2.3.2 Mass spectrometry (MS)

The developed analytical methods in many studies [44, 47, 53] comprise of liquid chromatography in combination with tandem mass spectrometry, using low resolution mass analyzers, usually triple quadrupole (QqQ), for a more reliable qualitative and quantitative determination [54]. LC-MS includes a variety of ionization techniques that can be used for the determination of chemical compounds in food; however, the most common among them remains electrospray ionization (ESI) [55]. It should also be mentioned that in light of ESI's overwhelming popularity atmospheric pressure chemical ionization (APCI) [56] seems to have been left out. This could be a result of the increasing number of wider range analytes, or it could be a reflection of the progress made in source and probe design of ESI that cannot be matched by APCI [57]. The excellent performance of LC-HRMS enables full spectral information, with the added bonus of high mass-resolving power which increases selectivity and capability for accurate mass measurement and aids identification with the additional advantage of retrospective analysis [58-60]. In theory, possession of full-spectral accurate-mass data makes the identification of an unlimited number of analytes present in a sample possible, because the acquisitions have been made as 'all ions all the time' [61]. It could be said that LC-HRMS is one of the most commonly used techniques in analytical chemistry [62], since it combines the simultaneous determination of a broad number of compounds in one injection with a corresponding reduction of time and cost even when reference standards are not available. One of the most commonly used HRMS analyzers is Time-of-flight (TOF). It is widely used because of its desirable specification compared to other instruments (see table 1) and it can be easily combined with ultra high-performance liquid chromatography (UHPLC). Mass accuracy is usually lower than 2 ppm and mass resolution ranges from 20,000 up to 80,000 FWHM.

Table 3. Common parameters used to compare performance of mass spectrometers used for LC-MS [63]

Mass analyser type	Resolving Power ($\times 10^3$)	Mass accuracy (ppm)	Upper limit of m/z range ($\times 10^3$)	Acquisition speed (Hz)	Linear dynamic range	Price
Q	3-5	Low	2-3	2-10	10^5-10^6	Low
IT	4-20	Low	4-6	2-10	10^4-10^5	Moderate
ToF	10-60	1-5	10-20	10-100	10^4-10^5	Moderate
Orbitrap	100-240	1-3	4	1-5	5×10^3	High

2.3.3 Tandem mass spectrometry (MS/MS)

MS/MS involves three distinct steps of selection-fragmentation-detection: precursor ions are selected, then fragmented; usually by collision-induced dissociation (CID), and the m/z ratio of the product ions formed is measured for the purpose of their detection. MS/MS involves two approaches: tandem in space and tandem in time. Tandem-in-space instruments are not of typical design; separate independent mass analyzers are located on different parts of the instrument. Hybrid mass spectrometers combine analyzers of different types. Hybrid configurations increase the analyzer's potential for screening purposes and offer structural information by ensuring accurate-mass product ion spectra following MS/MS experiments, i.e. Quadrupole-Time-of-flight (QqToF) (Figure 6), [54]. Some other examples of tandem mass spectrometers include, but are not limited to, triple/tandem quadrupole (QqQ), and Orbitrap hybrid instruments.

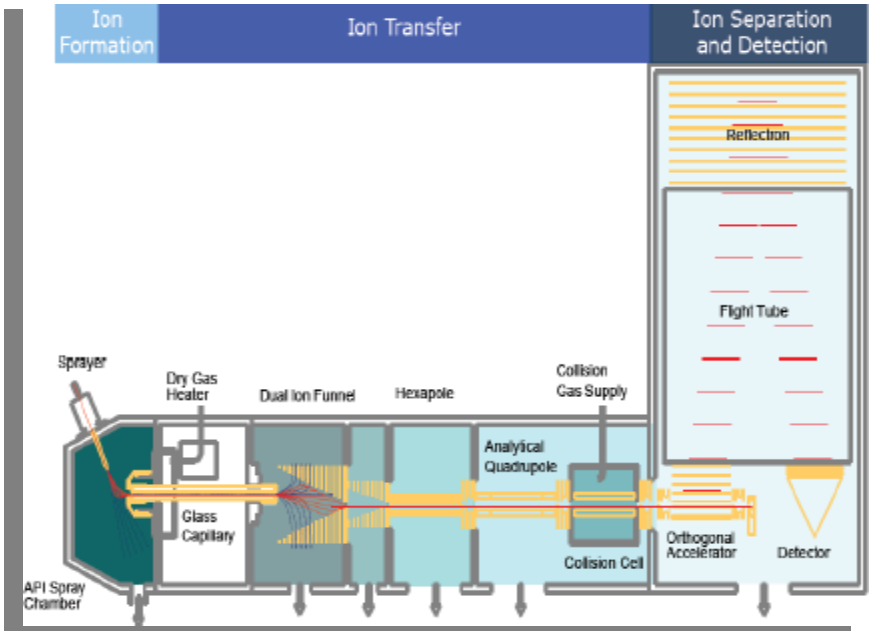


Figure 6: Course of ions in the QTOF sections (maXis Impact, Bruker)

Tandem-in-time instruments are typically described as ion-trapping mass spectrometers, comprising of Fourier transform ion cyclotron resonance (FTICR) instruments, linear ion traps (LIT) and 3-D quadrupole ion traps (QIT). During the experiment, the various stages of MS take place at difference times but within the same physical trapping volume [57].

2.3.3.1 Data Dependent Acquisition (DDA)

The DDA method involves first a full scan defined as the survey scan and “on-the-fly” processed data for the purpose of determining candidates of interest based on predefined selection criteria, such as intensity threshold or suspect inclusion list. MS/MS analysis is triggered and MS/MS scans (data-dependent) are performed [54, 60] provided that the selection criteria are met.

2.3.3.2 Data Independent Acquisition (DIA)

With DIA, the need to pre-select the precursor ion is not necessary. With one injection full scan spectra can be obtained at different collision energies. In a single run, using one high and one low collision energy scan, DIA produces accurate mass data of parent compounds and fragment ions at the same time. Application of low energy (LE) in the collision cell yields no 32 fragmentation. Information on the parent ion (the (de)-protonated molecule) and sometimes the adduct ions and the in-source fragments is obtained through a full-scan spectrum. Through the application of high energy (HE) in the collision cell, fragmentation is carried out and a spectrum as per MS/MS experiments is obtained. According to the QTOF manufacturer, the above approach is known as all-ions MS/MS, MSE or bbCID [44].

2.4 Workflow

After the LC-HRMS analysis the workflow processing contain three different approaches. Target, suspect and non-target screening of raw data as shown in **Figure 10**.

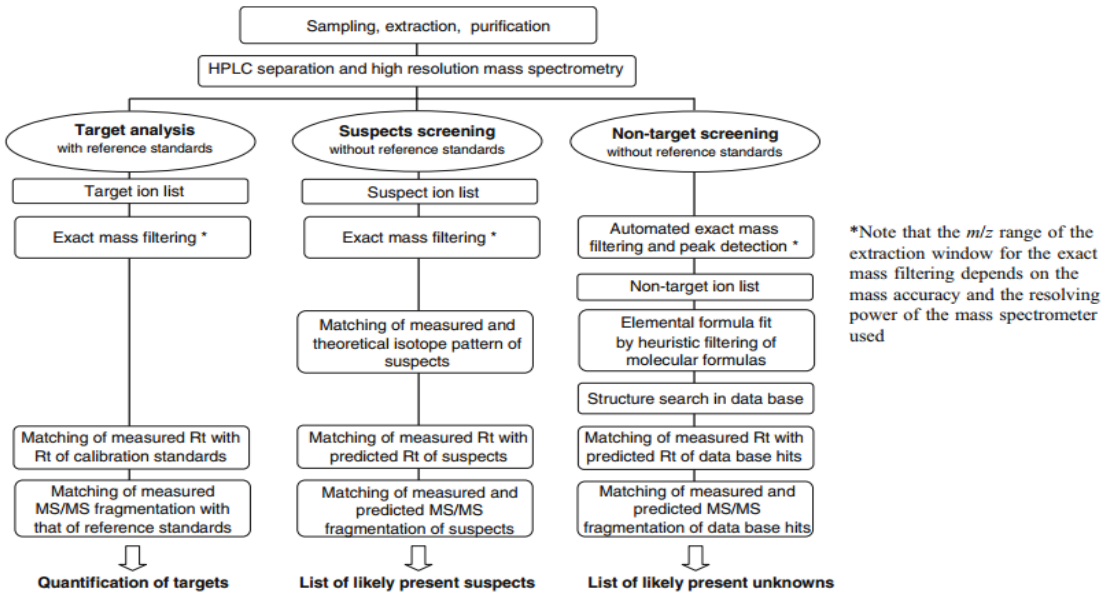


Figure 10:Systematic workflow for target, suspect and non-target screening by LC-HRMS/MS [60]

2.4.1 Target analysis

The method of target analysis is used for the determination of analytes already known and the identification process is performed by using standard solutions. A reference standard is needed in order to match and compare the MS/MS fragmentation and the experimental time as well for purposes of quantification [63]. In studies about olive oil authentication, a target list is generated based on the literature, comprised of significant classes of compounds in olive oil already identified and reference standards are available. As far as target analysis is concerned, data independent acquisition (DIA) is most suitable and it has to do with simultaneously acquiring accurate mass data in low and high collision energy. When applying low energy in the collision cell, there is no fragmentations and the acquired information is a full scan MS spectrum. Fragmentation of all ions takes place at high collision energy and MS/MS spectra is obtained. With data independent acquisition, in a single acquisition without the need of pre-selection of analytes, both molecular and fragment ions are acquired. In target screening the parameters that are evaluated are peak area thresholds and ion intensity, isotopic fit and mass accuracy thresholds. The reference standard's MS/MS data and the retention time should match with those of the sample. Target HRMS methodologies that are used to investigate EVOOs/VOOs authenticity are at a disadvantage since they do not study the entire fingerprint but instead only a few compounds.

2.4.2 Suspect Screening

LC-HRMS with suspect screening depends on isotope information and accurate mass of the precursor ion. Data depended acquisition (DDA) is often used for suspect screening. In order to ensure a reliable identification, both high resolving power and high mass spectral accuracy are required to increase selectivity against the matrix background and to assign correct molecular formulas to the unknown peaks. For the purpose of screening compounds, expected to exist in the samples, the exact mass of their

pseudomolecular ions in negative ($[M–H]^-$) or positive ($[M+H]^+$) ESI is used. The chromatogram is used to extract the exact mass of each suspect compound which is then evaluated. Following that, the procedural blank is subtracted and peak thresholds and ion intensity are set. Further information such as isotopic fit, mass accuracy, MS/MS spectra interpretation and peak score are crucial [64]. In order to confirm the suspected analyte, it is imperative to either compare MS/MS information with spectral libraries, provided they are available, like MassBank [65], FOODB [66], or Metlin [67] or to use *in silico* fragmentation tools. Target protocols have been used in combination with suspect screening protocols for the determination and quantification of some important phenolic compounds, to investigate EVOOs adulteration [68] and for the determination of biomarkers that distinguish between organic and conventional varieties [27].

2.4.3 Non-target screening

Non-target screening involves identifying compounds for which no a priori information is available. Target and suspect screening precede non-target screening [69]. Similar to suspect, non-target screening uses data depended analysis. Peak picking is the first step for data processing. Comparing the samples with control or blank samples is important in order to exclude irrelevant peaks. Removal of noise peaks, mass recalibration and componentization of isotopes and adducts, usually carried out automatically, is the second step. The molecular formula is assigned to accurate mass by using heuristic filters, i.e. the seven golden rules of Kind and Fiehn [70]. Candidate structures can be derived from the exploration of online databases such as ChemSpider [71] or PubChem [72]. Hence, the search of databases can be limited by the information on the parent compound (molecular formula, substructures) and it is likely that possible candidates are proposed. In order to rank the candidate structures, information from MS/MS spectra has to be examined by comparing in silico mass spectral fragmentation with fragmentation pattern [73] and then with reference spectrum libraries [65]. To

a point, tentative candidates' retention time can be predicted by validated computational models which are based on QSRR [74, 75]. Additionally, in order to search known unknown masses via their *in silico* fragmentation MS/MS and experimental MS/MS so as to accelerate the identification process a relevant database is necessary. Kalogiouri's et al. [28] work, where a database of 1608 natural compounds found in olive oil matrices was compiled, achieved this. Bearing in mind that in the majority of cases there is no available reference standard to confirm the existence of the possible candidates, for communication reasons, the use of identification levels was proposed [76]. Based on this, level 1 is linked to confirmed structures with reference standards, level 2 corresponds to plausible structures (level 2a: evidence by spectra matching from literature or library; level 2b: diagnostic evidence where no other structure fits the experimental MS/MS information), level 3 to tentative candidate(s), level 4 to unequivocal molecular formulas and level 5 to exact mass(es) of interest. Following this identification workflow, three olive oil authenticity works have been published, providing markers which are responsible for the production type [27] or the organoleptic profile [29]. Non-target methodologies in combination with suitable chemometric tools might be able to upgrade traditional target analysis and create new prospects for novel applications [77-79]. As non-target HRMS methodologies can lead to the generation and detection of a great number of markers (m/z), identifying is a challenging task. The combination with chemometric tools could result to significant decrease of the number of the detected features and to introduce the most meaningful m/z to the investigation of crucial EVOOs/VOOs authenticity issues [80].

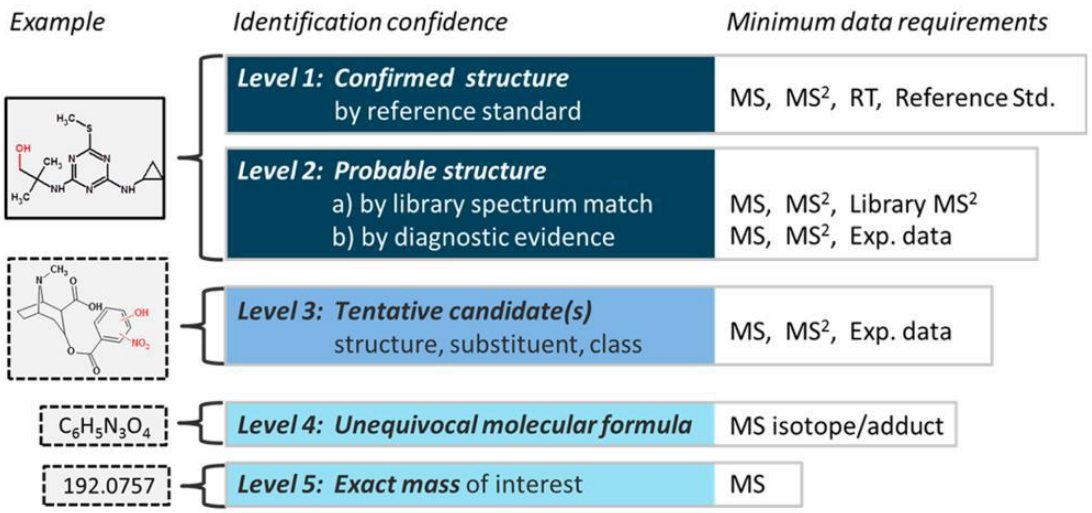


Figure 3: Identification confidence levels in HRMS [76]

2.5 Chemometrics

The inter-disciplinary field of chemometrics is defined as the science of studying a chemical process/system using mathematics or multivariate statistics [81]. Through the application of chemometrics the optimal condition for operating the multi-factorial experiment can be derived since its scope is not limited only to studies that involve interpreting instrumental data. As environmental or food samples often contain too many markers that are difficult to identify, chemometrics are used to solve these complex problems referring to pattern recognition of the samples' chemical profile. In HRMS data analysis, the primary objective is to examine the specific biomarkers that can clarify the categories to which the samples belong. In this respect, the entire samples' chemical profile could be focused on these m/z values that have a peak area or intensity that varies significantly among categories. In order to achieve this, the unsupervised or supervised classification method could be applied, based on prior knowledge of the categories of sample sets. To sum up, the basis and specific application of chemometric methods together with HRMS screening strategies, especially in the field of foodomics, will be discussed.

2.6 Research for possible adulteration in EVOOs – Analytical methods performed

Extra virgin olive oil, or EVOO, seems to significantly differ in quality and nutrients, subject to the variety of the olive oil and/or geographical origin due to its greater chemical specificity. Compared to other oils, EVOOs are quite susceptible to adulteration thus producing lower quality olive oils in cases of mixing-adulteration. It is, therefore, imperative that indicators based on the chemical identity of olive oils are found so as to identify and separate them [19, 27, 82, 83]. The typical classes of polar compounds in EVOO are phenolic acids, phenylalcohols, secoiridoids, flavonoids and lignans. From the phenylalcohol group, the most characteristic ones are hydroxytyrosol and tyrosol, while secoiridoids are the main component of the EVOO unsaponifiable fraction. In particular, oleocanthal, oleacein and the structurally related molecules of oleuropein and ligstroside, as well as the anhydrous derivatives of these forms are the best known secoiridoids found in EVOOs. Certain polyphenols, such as hydroxytyrosol and its derivatives, have been proven to protect blood lipids from oxidative stress above a certain concentration, as stated in the Commission Regulation (EU) No 432/2012 [84]. This showcases the importance of identifying the phenolic compounds of olive oil as well as their quantification [1, 85]. In the Bibliography, a variety of different techniques have been used to better determine phenolic compounds at very low concentrations.

The techniques are described in more detail in Table 1 below, where they are separated based on the:

- variety of olive oil analyzed
- markers proposed for the identification of the respective adulteration
- detection limits (LOD), where these are indicated
- technique-instruments selected
- sample pre-treatment, where required

Table 3. Literature review

Samples	Markers	LOD (mg/ml)	Method of Analysis	Sample Pre-Treatment	Bibliography
126 EVOO samples from six different Mediterranean regions	• hydroxy elenolic acid • decarboxymethyl • hydroxytyrosol • hydroxy decarboxymethyl • luteolin • elenolic acid (isomer a) • oleuropein aglycone (isomer a) • desoxy elenolic acid • oleuropein aglycone (isomer a) • oleuropein aglycone (isomer c) • ligstroside aglycone (isomer b) • acetoxypinoresinol • ligstroside aglycone (isomer b) • ligstroside aglycone (isomer a) • oleuropein aglycone (isomer b)	-	LC-MS negative **Intensity Solo C18 column** (2.1 × 100 mm, 1.8 µm) (Bruker Daltonik), protected with an AQUITY UPLC BEH C18 VanGuard precolumn (2.1 × 5 mm, 1.7 µm) (Waters, Manchester, UK). **Phase** A: water and phase B: ACN, both of them acidified with 0.5% of AcH. **The mobile phase gradient**: 0 to 2 min, 5%–30% B; 2 to 7 min, 30%–50% B; 7 to 8 min, 50%–90% B; 8 to 8.2 min, 90%–95% B, 8.2 to 10 min, 95%–99.9% B (kept for 3.9 min), and 13.9 to 14 min, 99.9%–5% B. **Run time**:14 min **Flow:** 0.4 mL/min; it was just increased at 0.6 mL/min (and kept at this value) from 10 to 14 min to speed up the elution of the most apolar components. After that, it was set again to the initial value. **Injection volume**: 2 µL.	The LLE protocol used for preparing the EVOO extracts was the result of a modification carried out to simplify the procedure and to facilitate the supernatant collection after the centrifugation step. Solvents: EtOH/H2O (60:40, v/v) mixture, which presents higher density than the oily phase, was used in the first place, followed by two consecutive extraction steps with the less dense EtOH/H2O (80:20, v/v) mixture. In that way, four 6 mL portions of extractant solvent were replaced by three 10 mL portions of the EtOH/H2O mixtures, which led to equivalent extraction rates.	[86]

			MS detection conditions: • positive and negative ionization modes • nebulizer pressure (2 bar) • drying gas flow (8 L/min) • drying temperature (200 ºC). **QTOF analyzer**/LC system: • 100 Vpp for both 1 and 2 RF funnels, • 50 Vpp for hexapole RF, • 5 eV foquadrupole ion energy, • 6 eV for collision energy • 1000 Vpp for collision RF • Full scan spectra was recorded (from 30 to 1000 m/z)		
	• hexyl cinnamaldehyde • Apigenin • ligstroside aglycone (isomer a) • diosmetin • 4-octylbenzoic acid • Luteolin		**LC-MS positive**		[86]
	• hydroxy decarboxymethyl elenolic acid • decarboxymethyl oleuropein aglycone • desoxy elenolic acid		**LC-MS combined**		[78, 86]

	• elenolic acid (isomer I) • oleuropein aglycone (isomer I) decarboxymethyl ligstroside aglycone • □-sitostero • Cycloartenol • Δ5-avenasterol • Methylencycloartanol • palmitoleic acid • tyrosol • Hydroxytyrosol • ligstroside aglycone (isomer III) • ligstroside aglycone (isomer II) • glyceryl linoleate		**GC-MS** • Column: BR-5 (30 m × 0.25 mm i.d., 0.25 µm) (Bruker Daltonik). • Flow: 1 mL/min. 1 µl of the silylated extract was injected at a split ratio of 1:20 • Injector temperature of 250 ºC. • The transfer line was kept at 290 ºC. • APCI interface were: 2000 V for capillary voltage, 2000 nA for corona discharge, 3.5 bar for nebulizer pressure, 2.5 L/min for dry gas flow and dry temperature of 280 ºC. • 200 Vpp for funnel 1 RF, 100 Vpp for funnel 2 RF, 50 Vpp for hexapole RF, 4 eV for the quadrupole ion energy, 8 eV for the collision energy, 300 Vpp for the collision RF, transfer time of 100 µs and pre pulse storage of 2 µs. • Full scan spectra was recorded (from 30 to 1000 m/z) at a scan rate of 3 Hz.		[86]

78 samples of extra virgin olive oil collected from ten different countries: Argentina(5),Chile(6), France (1), Greece (1), Italy (1), Morocco (4), Peru (32), Portugal (1), Spain (26), and Syria	Gallic acid Hydroxytyrosol Tyrosol Vanillic acid Caffeic acid Syringic acid Gibberellic acid p-Coumaric acid Ferulic acid Oleuropein Resveratrol Luteolin • Apigenin	0,08 1,25 0,33 0,04 0,09 0,01 0,01 0,05 0,01 0,05 0,04 0,02	**LC-QTOF-MS** HPLC system • Agilent Series 1200, • Column: a reversed-phase C18 analytical column of 100 mm×4.6 mm and particle size of 1.8 µm (Agilent Zorbax XDB-C18) • Injection volume: 10 µL • Mobile phases A and B were water with 0.1 % formic acid and acetonitrile.Lineat gradient: (10 % B) constant for 5 min, followed by a linear gradient to 100 % B at 25 min. Then, 100 % B remained constant during 5 min. • Flow rate: 0.5 mL min−1. the time-of-flight mass spectrometer Agilent TOF 6220 (equipped with an electrospray interface operating in the negative ionization mode. Conditions: • capillary voltage 2,500 V • drying gas 9 L min−1 • gas temperature 325 °C • nebulizer pressure 40 psig, and mass spectra were recorded across the range of m/z 50–1,000.	SPE procedure involves a preconditioning step of the cartridges with 10 mL of MeOH and 10 mL of n-hexane at a flow rate of 2 mL min−1. 3 g olive oil was mixed with 3 mL of nhexane and was loaded into the cartridge at a flow rate of 3 mL min−1. The nonpolar fraction was removed by passing 15 mL of nhexane through the cartridge. The retained phenols were eluted with 10 mL of MeOH at 1 mL min−1 and collected in a 15-mL centrifuge tube. This eluate was then evaporated until near dryness by a gentle nitrogen stream using a TurboVap LV from Zymark, with a water bath temperature of 35°C and a N2 pressure of 10 psi. The samples were then taken up with 1 mL of MeOH and 2 mL of Milli-Q water. The extract filtered through a 0.45-µm PTFE filter	[13, 82]

phenolic fraction of 156 VOO samples extracted from olives samples collected from 7 different north Moroccan regions	Quinic acid Hydroxytyrosol Tyrosol p-Coumaric acid Pinoresinol Ferulic acid Cafeic acid Oleuropein Luteolin Apigenin	0,008 0,025 0,008 0,012 0,013 0,009 0,047 0,010 0,010 0,04	**LC-ESI-IT MS** Agilent 1260-LC system (Agilent Technologies, Waldbronn, Germany) • Column: Zorbax C18 (4.6 x 150 mm, 1.8 µm particle size). • Room temperature. • Flow rate:0.8 mL/min. • Mobile phases: water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) • Solvent gradient conditions: 0 to 10 min, 5 % B; 10 to 12 min, 30% B; 12 to 17 min, 38% B; 17 to 20 min, 50% B; 20 to 23 min, 95% B. • Injection volume: 10 µL. • DAD (240 and 280 nm), and • mass spectrometry detector. The mass spectrometer • the mass range from m/z 50 to 800 with a spectra rate of 1 Hz. • The capillary was set at +4000V, the End Plate offset at -500V, • Nebulizer Gas at 2 Bar • Dry Gas at 9 L/min at 250°C. • External mass spectrometer calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA)	Isolation of phenolic compounds from olive oil samples. 4.0 ± 0.001 g of olive oil were weighed in a test tube with a screw cap and 0.05 mL of internal standard solution was added. The solvent was evaporated using nitrogen, and phenolic compounds were extracted three times, adding every time 2 mL of n-hexane and 4 mL of methanol/water (60/40); the extract was centrifuged at 3500 rpm for 6 minutes. The supernatants were collected and the resulting solution was evaporated to dryness using a rotary evaporator under reduced pressure and a temperature of 30°C. Finally, the residue was redissolved in 2 mL of methanol and filtered through a 0.45 µL membranes (Millipore) filter.	[87]

26 samples [7] 52 EVOOs [8]	Cyanidin Luteolin Tyrosol Ferulate Sesamin Matairesinol Resveratrol		**UHPLC-ESI/QTOF** • A 1290 liquid chromatography system, Electrospray JetStream ionization source, coupled to a G6550 mass spectrometer detector (Agilent Technologies, Santa Clara, CA, USA). mass spectrometer • positive ionization mode 50-1000 m/z range. • Column: Agilent Zorbax eclipse plus C18 analytical column (50 x 2.1 mm, 1.8 µm). • Phase: water-methanol gradient elution (from 10% to 90% organic in 34 minutes). • Injection volume: 3 µL. • Source conditions: nitrogen was used both as sheath gas (10 L/min and 350 ° C) • as drying gas (8 L / min and 330 ° C), nebulizer pressure was 60 psig, nozzle voltage was 300 V and capillary voltage was 3.5 KV	Liquid-liquid extraction of phenolics and sterols Phenolic compounds and sterols were extracted in triplicate from each sample as follows: an aliquot 3 g of oil was weighted into conical centrifuge tube, added with 3 mL of 80 % methanol solution (v/v). The mixtures were vortexed vigorously and then centrifuged at 6,000 x g for 10 min at 4 °C. The methanol fractions were collected, whilst the residues were rejected. The resulting supernatants were filtered through 0.22 µm cellulose syringe filters and stored in amber vials at -20°C	[27, 88]

CHAPTER 3
SCOPE

Following the literature review, it is noted that due to the high nutritional value of olive oil there have been various studies many of which focus on finding adulteration indicators. The aim of this research is to develop a non-targeted scanning methodology for finding characteristic olive oil adulteration markers, based on the overall metabolomic footprint of each sample. The study will focus on finding indicators among different varieties and geographical origins. Specifically, the olive oils that will be used as samples come from three different varieties (Koroneiki, Adramytiani and Kolovi) from three different geographical areas of Greece (Lesvos, Crete and Peloponnese).

CHAPTER 4
MATERIALS AND METHODS

4.1 Chemicals and Materials

All standard chemicals and reagents used were of high-purity grade (>95%). In detail, luteolin and hydroxytyrosol were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), catechin, ferulic acid, epicatechin, quercetin, pinoresinol, syringic acid, salicylic acid, myricetin, and eriodictyol from Sigma-Aldrich (Stenheim, Germany), while caffeic acid, vanillin, apigenin and naringenin were purchased from Alfa Aesar (Karlsruche, Germany). Ligstroside aglycone, oleacein, oleocanthal, oleocanthalic acid, oleomissional and oleuropein aglycone were acquired from Prof. P. Magiatis laboratory (Laboratory of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens, Greece). The standards had been isolated from olive oil extracts and confirmed through NMR study in terms of structure and purity.

Methanol (MeOH) (LC-MS grade) was purchased from Merck (Darmstadt, Germany). Sodium hydroxide monohydrate for trace analysis ≥99.9995%, ammonium acetate and formic acid 99% were purchased from Fluka (Buchs, Switzerland) when ammonium formate from Fisher Chemical (Geel, Belgium). A Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA) was used for distilled water. Lastly, regenerated cellulose syringe filters (RC filters, pore size 0.2 μm, diameter 15mm) were provided by Phenomenex (Torrance, CA, USA) and stock standard solutions of individual compounds (1000 mg L-1) were prepared in MeOH and then stored in dark glass bottles at -20 °C.

4.2 Sampling, Sample Preparation and Storage

A total of 145 samples of extra virgin olive oil cultivated in Greece, differentiated in terms of variety and geographical origin were selected.

Samples were stored in amber glass bottles at 4 ^{o}C until analysis. Before LC-QTOF/MS analysis, EVOOs were set at room temperature and subjected to liquid-liquid extraction (LLE) with MeOH:H_2O (80:20%, v/v) as extraction solvent, previously described by Kalogiouri et al. [27]. Briefly, 0.5g of each sample was weighed and 0.5 mL of methanol–water (80:20, v/v) was added to 2-mL Eppendorf tubes. The mixture was vortexed for 2 min and centrifuged for 5 min at 13,400 rpm. The upper phase was collected and filtered through RC syringe filters. Finally, a two-fold dilution was performed in the received extracts, with MeOH:H_2O (80:20%, v/v) in order to avoid saturation in LC-analysis. Procedural blank was additionally prepared to detect potential contamination.

Regarding adulteration, two different experimental case studies were applied; one part concerned the study of EVOOs adulteration in terms of variety, where 29 samples from three different varieties (Kolovi, Koroneiki, Adramytiani) were analyzed, whereas the second part involved EVOOs adulteration among 34 samples of different geographical region of Greece (Crete, Lesvos, Peloponnese), as shown in **Table 4.** To simulate adulteration, fully-characterized EVOOs admixtures with EVOOs of different variety and geographical origin were constructed at low, medium and high adulteration level (10, 20, 30, 40 and 50% respectively).

Table 4. EVOOs Sampling

VARIETY	KORONEIKI	KOLOVI	ADRAMYTIANI
NUMBER OF SAMPLES	9	10	10

ORIGIN	LESVOS	CRETE	PELOPONNESE
NUMBER OF SAMPLES	8	13	13

4.3 Instrumentation

An Ultra-High Performance Liquid Chromatography (UHPLC) system (UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany) coupled to a Quadrupole- Time of Flight Mass Spectrometer (QToF-MS) (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used for the analysis of the samples. The UHPLC apparatus consists of a solvent rack degasser, an auto-sampler, a column and a binary pump with solvent selection valve (HPG-3400). The QTOF-MS apparatus consists of an Electrospray Ionization (ESI) source operating in positive and negative mode.

Figure 4: UHPLC-QToF-MS, Maxis Impact, Bruker Daltonics

In our analysis, a reversed-phase chromatographic run was performed in negative and positive ESI modes. An Acclaim RSLC 120 C18 column (2.1 × 100 mm, 2.2 µm) (Dionex Bonded Silica Products, Thermo Scientific, Dreieich, Germany), preceded by an ACQUITY UPLC BEH C18 1.7 µm guard column of the same packaging material (VanGuard Pre-Column, Waters, Dublin, Ireland), and thermostated at 30 ^{o}C, was used.

During negative ionization the mobile phases comprised of water/methanol (90:10 v/v, solvent A) and methanol (solvent B), whereas in positive ionization the mobile phases comprised of water/methanol 90/10 (solvent A) and methanol (solvent B) both contained 5mM ammonium formate and 0.01%

formic acid. For both the negative and positive ionization modes, a gradient elution program was applied, starting for 1 min with 1% B (flow rate of 0.2 mL min-1), at 2 min it was increased to 39% and then in another 11 mins to 99.9% (flow rate of 0.4mL min-1).

Solvent (B) was kept constant for 2min (flow rate of 0.48 mL min-1) and for 3 min initial chromatographic conditions were restored by re-equilibrating the column and the injection volume was set up to 5 μL. The UPLC-QTOF-MS system, operating in positive and negative mode, was furnished with an electro spray ionization interface (ESI). The ionization parameters consisted of a nebulizer gas pressure of 2 bar (N2), a capillary voltage of 3500 V, an end-plate offset of 500 V, drying gas flow at 8 L min-1 and a dry temperature of 200 ^{o}C.

4.4 Data treatment

The software used for starting the data evaluation in the targeted approach applied was TASQ Client 2.1 and DataAnalysis 5.1 (Bruker Daltonics). The MetaboScape® 4.0 software (Bruker Daltonics), which is compatible with LC-QTOF data (T-ReX 3D) was used in order to perform non-targeted data treatment. For the pre-processing of data, the algorithm T-Rex was used along with mass calibration, bucketing, peak picking, time alignment and procedural blank removal. Mass calibration, by using sodium formate as reference solution (this method was also used during in external calibration which came before the instrumental analysis) was automatically for all samples. The samples were categorized based on variety and origin prior to feature extraction and the result was effective bucketing of the data into manageable parts for the statistical analysis that will follow. Furthermore peak picking parameters were optimized to ensure reliable data extraction. This was accomplished by setting intensity threshold at 2000 counts, adjusting minimum peak length at 5 spectra for data acquired from LC-QTOF analysis (DDA mode). Lastly, procedural blank removal was performed in the software environment and in order to filter positive from false peaks and to eliminate potential contamination, the default value was set at 3. The only features

taken into account for feature annotation and statistical analysis were those that satisfied the three times above the intensity noted in blank sample criteria. The data were also assessed through multivariate statistical analysis which included pairwise PLS and partial least squares discriminant analysis (PLS-DA). PLS, which was performed via MetaboScape statistical tool, was used in order to evaluate possible clustering of EVOOs subject to their varietal background. PLS-DA was performed using an in-house program called ChemoTrAMS10 in the R environment (RStudio, Version 1.1.463, Boston, MA, USA), trying to discriminate EVOOs in terms of geographical origin and variety.

CHAPTER 5
RESULTS AND DISCUSSION

As mentioned under 2.4.3, the approach followed was non-target screening through the Metaboscape 4.0. Next, pairwise PLS was conducted in each of the two different case studies using the following pairs each time. In the first case study involving EVOOs from different Greek regions the pairs that underwent PLS were Crete-Peloponnese, Crete-Lesvos, and Peloponnese-Lesvos. The same procedure was also followed for olive oils of different varieties (Koroneiki, Kolovi, Adramytiani).

5.1 Variety discrimination

In the first case study involving samples of different varieties, peak picking was performed using parameters as described under 4.4 in each one of the different experiments (negative – positive ionization). In order to achieve the optimal discrimination through peak-picking step, only m/z that were detected in 80% percentage of samples of each group were allowed, while m/z that were detected individually were filtered out of the process. Following peak-picking procedure in each ionization data set (2 different bucket lists were initially created for positive and negative runs), the two data sets were merged into a single data set. Afterwards, pairwise PLS was conducted for Koroneiki-Kolovi, followed by Kolovi-Adramytiani and Koroneiki-Adramytiani resulting in 3 different groups of the most significant m/z with respect to the separation of the varieties at hand. m/z markers with importance greater than 1.50 were kept from each group and a unified m/z list was compiled (**Table 5**).

Table 5. Variety Markers

A/A	m/z	Retention Time (min)	Primary Ion detected	m/z (Fragment Ions)	Propable elemental Composition	Mass Error (mDa)	Marker	Tentative identification	Level of identification
1	177.0562	6.99	[M-H]-	117.0365; 145.0318	C10H10O3	1.8	ADR	-	3
2	207.1380	9.21	[M+H]+	147.1164; 171.1163; 119.0854	C13H18O2	0.1	ADR	1-(4-amylphenoxy)ethane-1,2-diol	2b
3	223.0601	6.2	[M+H]+	167.0336; 149.0229; 181.0491	C11H10O3	0.0	ADR	2-allylperoxycarbonylbenzoic acid	2b
4	239.1637	10.54	[M+H]+	164.0704; 147.1165; 179.0622	C14H22O3	0.1	ADR	-	3
5	255.0865	6.21	[M+H]+	181.0492; 167.0335; 149.0228	C12H14O6	0.1	ADR	2-(4-formyl-2,6-dimethoxy-phenoxy)propionic acid	2b
6	309.2059	10.78	[M+H]+	291.1948	C18H28O4	0.3	ADR	6-[2-[(E)-3,4-dihydroxyhex-1-enyl]cyclohexa-2,4-dien-1-yl]hexanoic acid	2b
7	343.0944	6.7	[M+H]+	121.0647; 137.0594	C22H14O4	2.4	ADR	-	3
8	351.1934	12.35	[M+H]+	195.1194; 263.1049	C23H26O3	0.2	ADR	4-[1-(1,1,4,4,7-pentamethylisochroman-6-yl)vinyl]benzoic acid	2b
9	372.3114	13.87	[M+NH4]+	263.2371; 245.2268; 337.2739	C21H38O4	0.3	ADR	-	3
10	405.131	6.62	[M+H]+	300.104; 151.075; 137.060	C24H20O6	2.8	ADR	3-[[(2-formylphenoxy)-(2-formylphenyl)methoxy]methyl]-4-methoxy-benzaldehyde	2b
11	471.3469	12.5	[M+H]+	201.1638; 227.2001	C30H46O4	1.0	ADR	Unknown compound	4
12	569.439	12.49	[M+H]+	227.1750; 326.2440; 340.2601	C33H60O7	2.9	ADR	methyl 2,28-diacetoxy-24-oxo-octacosanoate	2b
13	391.2246	13.42	[M+H]+	149.0228; 195.1220	C26H30O3	2.7	ADR	(4-cyclohexylbenzoyl) 4-cyclohexylbenzoate	2b

14	359.2913	13.34	[M+H]+	285.279; 201.148; 123.117;	C21H42O4	0.3	ADR,KOR	-	3
15	239.0916	7.74	[M+H]+	165.0545; 137.0599	C12H14O5	0.3	KOL	-	3
16	257.1023	4.98	[M+H]+	165.0544; 151.0386; 123.0437	C12H16O6	0.3	KOL	1-[2-hydroxy-3-[(2-hydroxy-1-methylol-ethyl)peroxymethyl]phenyl]ethanone	2b
17	361.1313	7.78	[M-H]-	291.0875; 101.0244	C19H22O7	0.7	KOL	Ligstroside aglycone	1
18	305.1389	6.53	[M+H]+	121.0651; 122.0687; 165.0549	C17H20O5	0.7	KOL	-	3
19	363.1442	8.19	[M+H]+	121.0650; 122.0682; 225.0757	C19H22O7	-0.8	KOL	5-[5-(2-ethoxyphenoxy)pentoxy]-4-keto-pyran-2-carboxylic acid (Ligstroside aglycone derivative)	2b
20	411.1651	8.41	[M+H]+	121.0650; 137.0598; 165.0546	C20H26O9	0.2	KOL	Unknown Compound	4
21	449.1211	5.98	[M+K]+	225.1959; 417.0941; 389.0.980	C20H26O9	0.2	KOL	7-hydroxy-6-[3-methyl-4-[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-methylol-tetrahydropyran-2-yl]oxy-butyl]coumarin	2b
22	727.295	5.72	[M+Na]+	375.141; 343.115;	C36H48O14	-0.1	KOL	-	3
23	351.1438	5.72	[M-H]-	95.0494; 183.0670; 139.0777	C18H24O7	1.3	KOL, KOR	3-[2-[(E)-3,8-diketo-8-methoxy-oct-1-enyl]-3-hydroxy-5-keto-cyclopenten-1-yl]butyric acid	2b
24	377.1242	8.41	[M-H]-	275.057; 147.046; 95.051	C19H22O8	0.2	KOR,KOL	Oleuropein aglycone	1
25	138.0194	5.61	[M-H]-	108.0219;	C6H5N03	1.5	KOR	Unknow Compound	4
26	169.0855	5.69	[M+H]+	151.0751; 123.0804	C9H12O3	0.3	ADR	6-isobutyl-4-hydroxy-2-pyrone	2b
27	811.3170	6.76	[M+Na]+	417.1525; 385.1267; 357.1318	C40H52O16	1.0	KOL	Oleomissional derivative - isomer	2b
28	391.1509	7.05	[M+H]+	137.0600	C20H19N5O4	0.1	KOL	2-(1-(6-Hydroxyquinoxalin-2-yl)piperazine-4-carboxamido)benzoic acid	2b
29	319.2246	12.38	[M+H]+	317.2095; 301.2132	C20H30O3	0.2	ADR	Unknown Compound	4

30	335.1984	12.38	[M+H]+	247.1301; 256.2636	C23H26O2	2.6	ADR	Benzoic acid, 4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)ethenyl)-, (E)-	2b
31	583.455	12.79	[M+NH4]+	227.1755; 340.2596; 453.3436	C29H59NO9	0.3	ADR	Unknown Compound	4
32	511.3193	13.15	[M+H]+	229.1444	C35H42O3	2.9	ADR	Unknown Compound	4
33	545.3244	13.44	[M+H]+	413.2426; 215.1261; 283.2625	C35H44O5	3.5	KOR	-	3
34	367.2247	13.61	[M+H]+	309.2390; 365.2676; 347.2575	C24H30O3	2.6	ADR	Unknown Compound	4
35	393.2406	13.87	[M+H]+	149.023; 195.1225; 133.0856	C26H32O3	2.1	ADR	Unknown Compound	4
36	635.5258	13.91	[M-H]-	281.251	C39H72O6	2.9	ADR	Unknown Compound	4

The table provides information for the retention time, ions detected, its fragments, the possible formula, mass accuracy (expressed in mDa), the variety to which each m/z is assigned as marker and its possible name as well as the identification level. To produce these results the MetaboScape 4.0 was used as well as the tools and possibilities of the program. Using the Smart Formula program we derived the possible formula of each m/z utilizing precursor ion information reflected on its MS spectrum. Then, with Compound Crawler we obtained possible names and structures as research took place for the specific molecular formula in libraries from the PubChem library. Then, through the MetFrag in silico fragmentation took place that identified the MS/MS spectrum of the pre-selected m/z with the spectra derived from all possible compounds. Via the MetFrag the final result was derived in the annotation with a score of 1.0/1.0, referring that all fragments proposed are found in MS/MS spectrum. Finally, target and suspect EVOOs databases were also used, resulting in the highest confidence level (that of 1) of identification procedure.

Preliminary discrimination study was performed among mono-varietal EVOOs (**Figure 5**), where clear discrimination was observed.

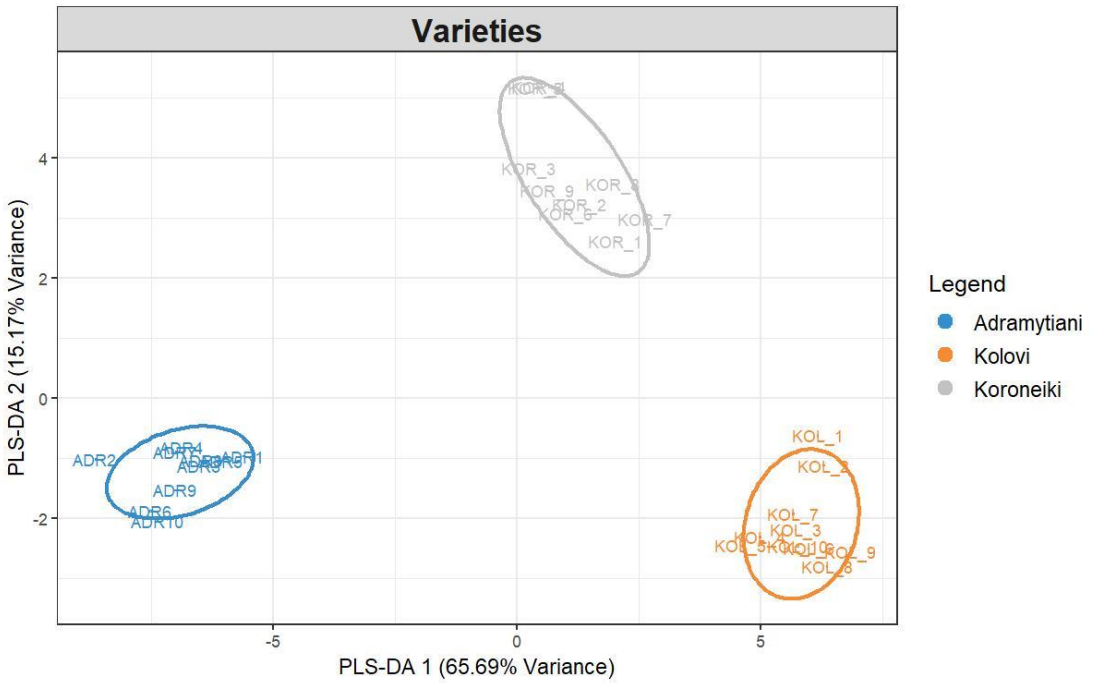


Figure 5: PLS-DA Variety Discrimination

5.1.1 Building prediction models

All m/z retrieved from pairwise PLS, as previously reported and further identified, were included in PLS-DA prediction models. More specifically, we performed PLS-DA with all the m/z markers found in order to distinguish single-variety EVOOs from adulterated. **Figures 6, 9, 12** illustrate PLS-DA prediction models built that enable sufficient discrimination even in low adulteration levels (e.g. 10%). Finally, in order to evaluate model's prediction ability, a batch of adulterated samples in all different adulteration levels, from 50 to 10% was used as a test-set.

The first two components (PC1 and PC2) of the model interpreted the majority of the results (higher than 50%), (**Figures 7, 10, 13)**.

Figures 8, 11, 14 show first two components contribution to the model, as well as the most important markers for the discrimination achieved.

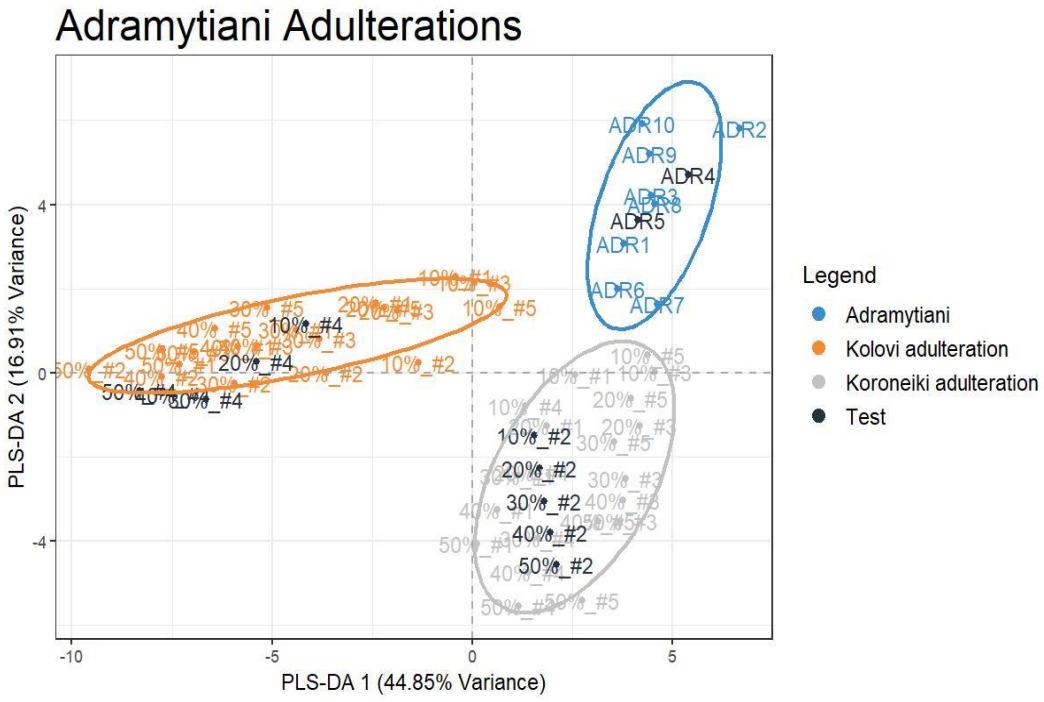


Figure 6: PLS-DA Adramytiani

For Adramytiani mono-varietal EVOOs, as shown in **Figure 6**, adequate discrimination is being achieved as far as Kolovi and Koroneiki varieties used as adulterants. The model predicts successfully adulteration till 10% in both adulterated samples.

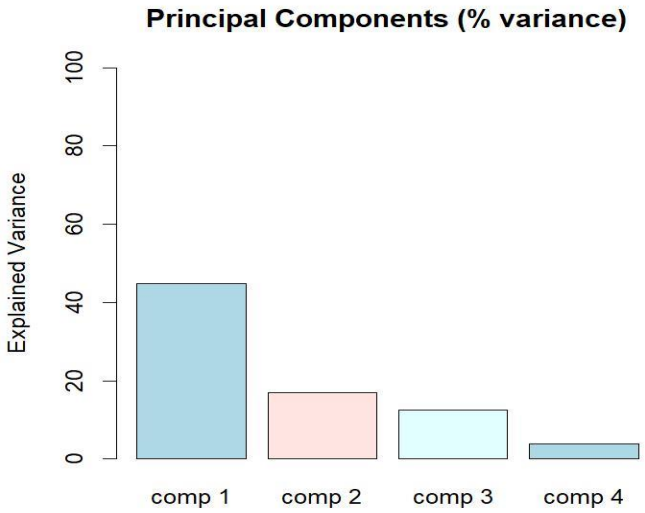


Figure 7: Principal components of Adramytiani case study (% variance)

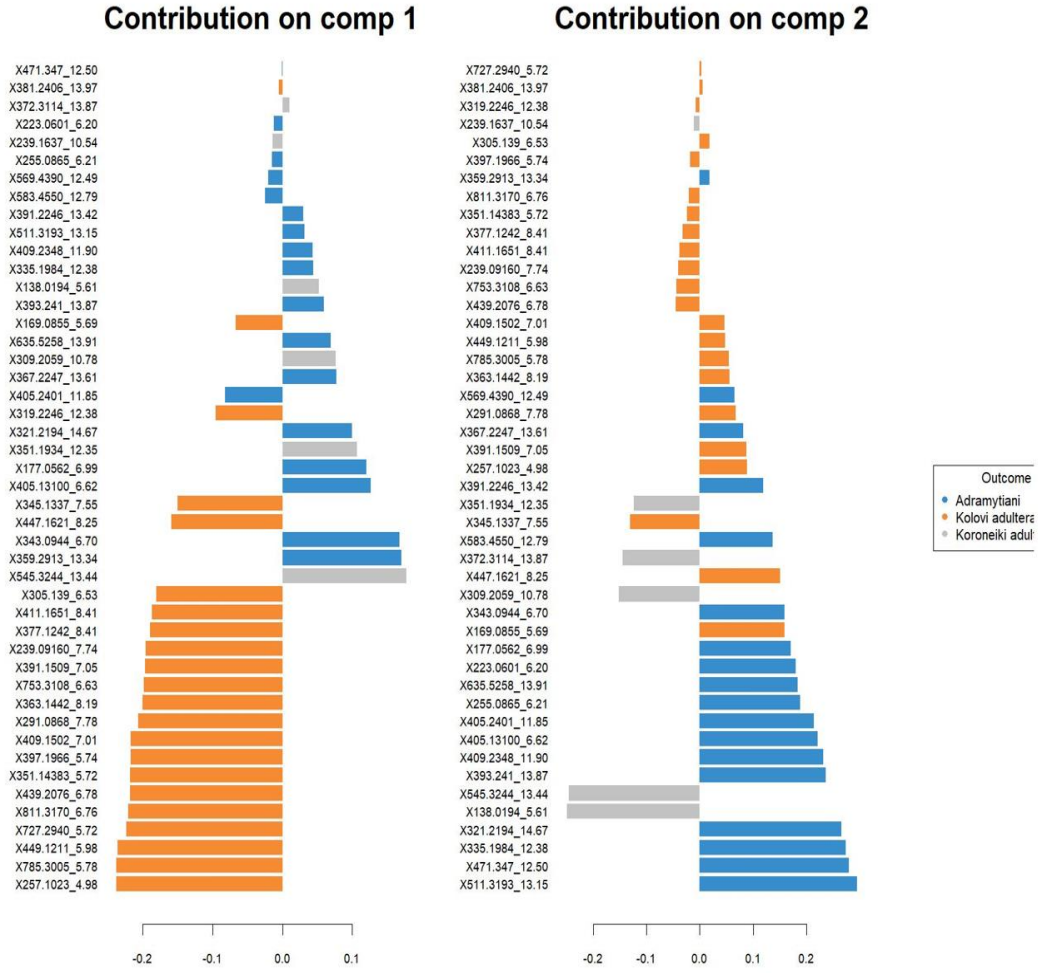


Figure 8: VIP score of the first two PCs of Adramytiani adulteration study

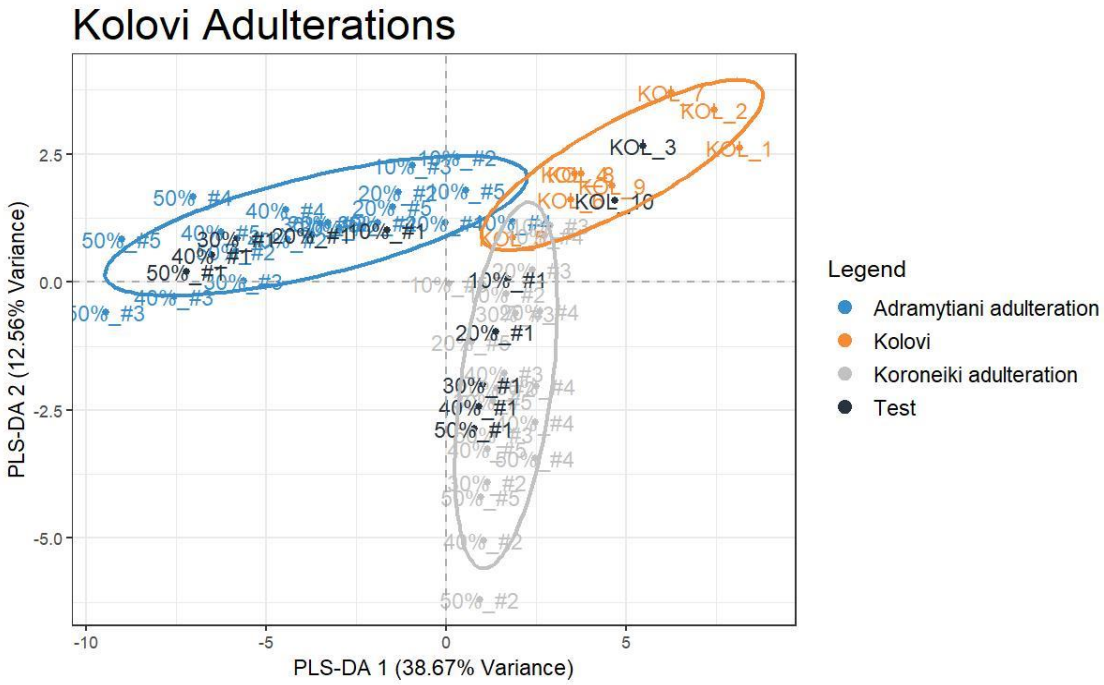


Figure 9: PLS-DA Kolovi

For Kolovi mono-varietal EVOOs, as shown in **Figure 9**, adequate discrimination is being achieved as far as Adramytiani and Koroneiki varieties used as adulterants.

However, in Koroneiki adulteration case study, while the test set data are accurately classified into adulterated ones, although some data used in the training set (at 10% adulteration level) are coincided those of pure Kolovi.

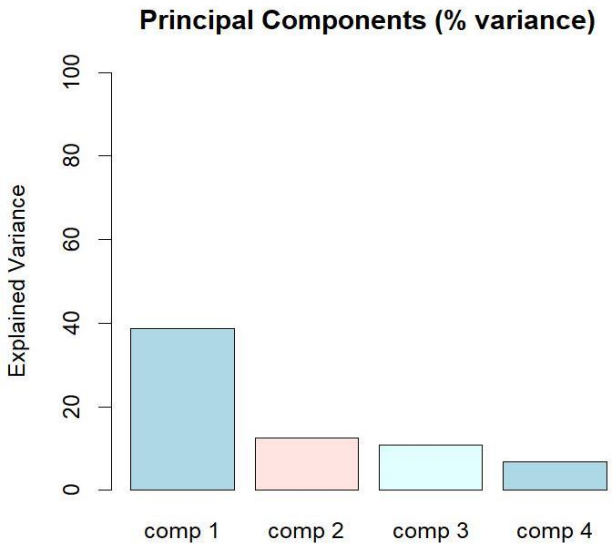


Figure 10: Principal components of Kolovi case study (% variance)

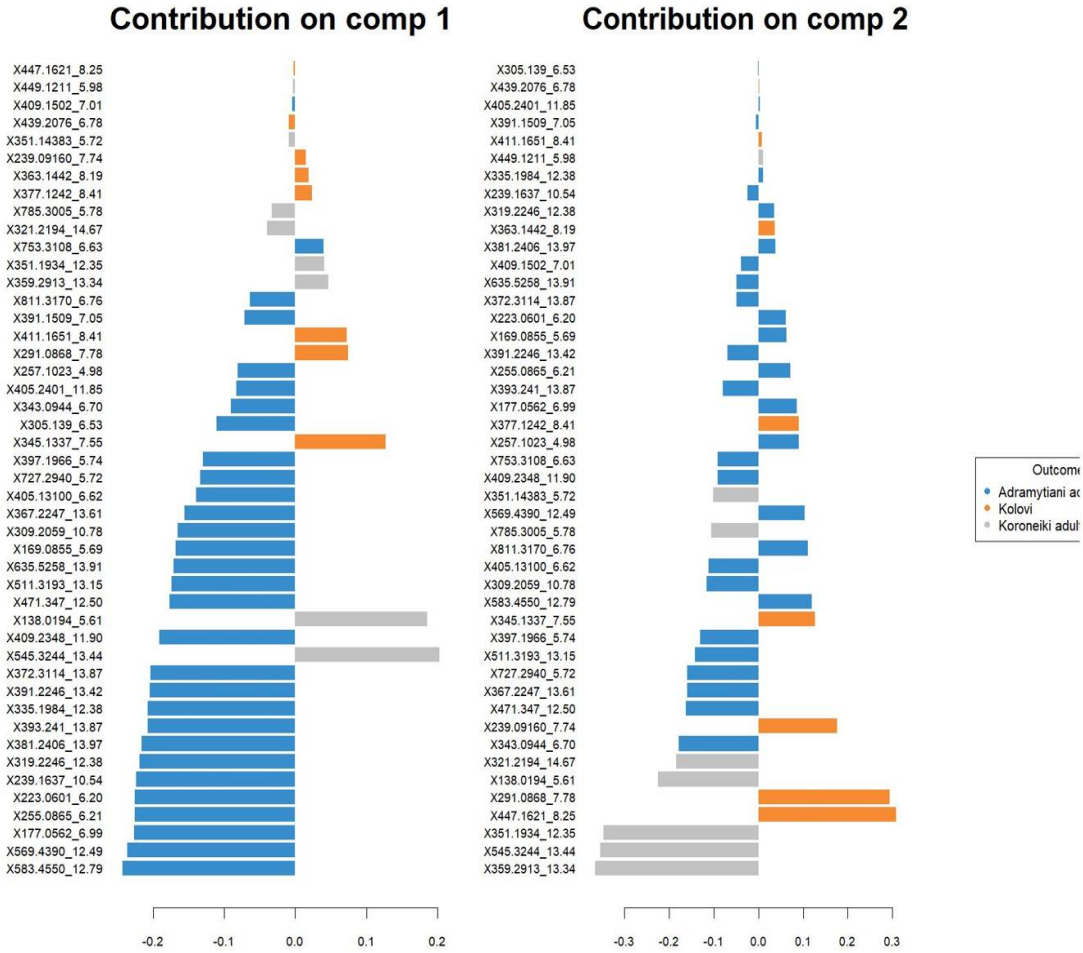


Figure 11: VIP score of the first two PCs of Kolovi adulteration study

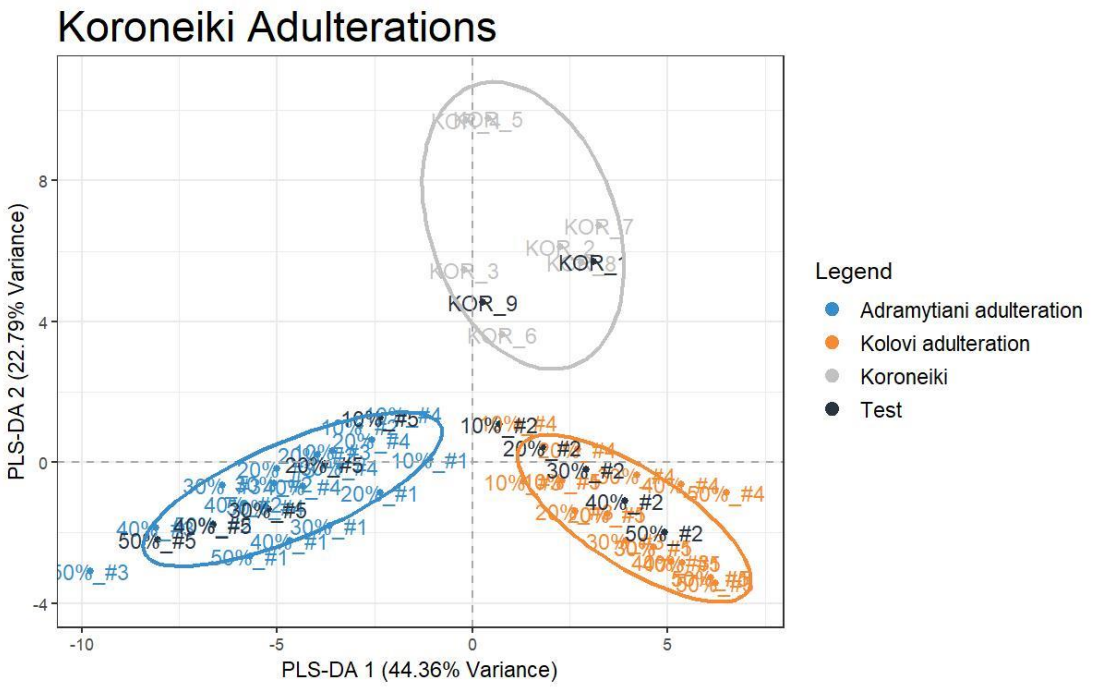


Figure 12: PLS-DA Kolovi

For Koroneiki mono-varietal EVOOs, as shown in **Figure 12**, adequate discrimination is being achieved as far as Kolovi and Adramytiani varieties used as adulterants. The model predicts successfully adulteration till 10% in both adulterated samples.

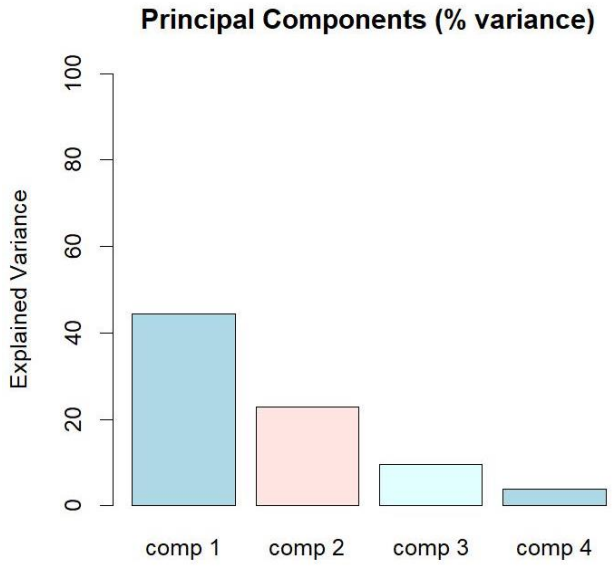


Figure 13: Principal components of Koroneiki case study (% variance)

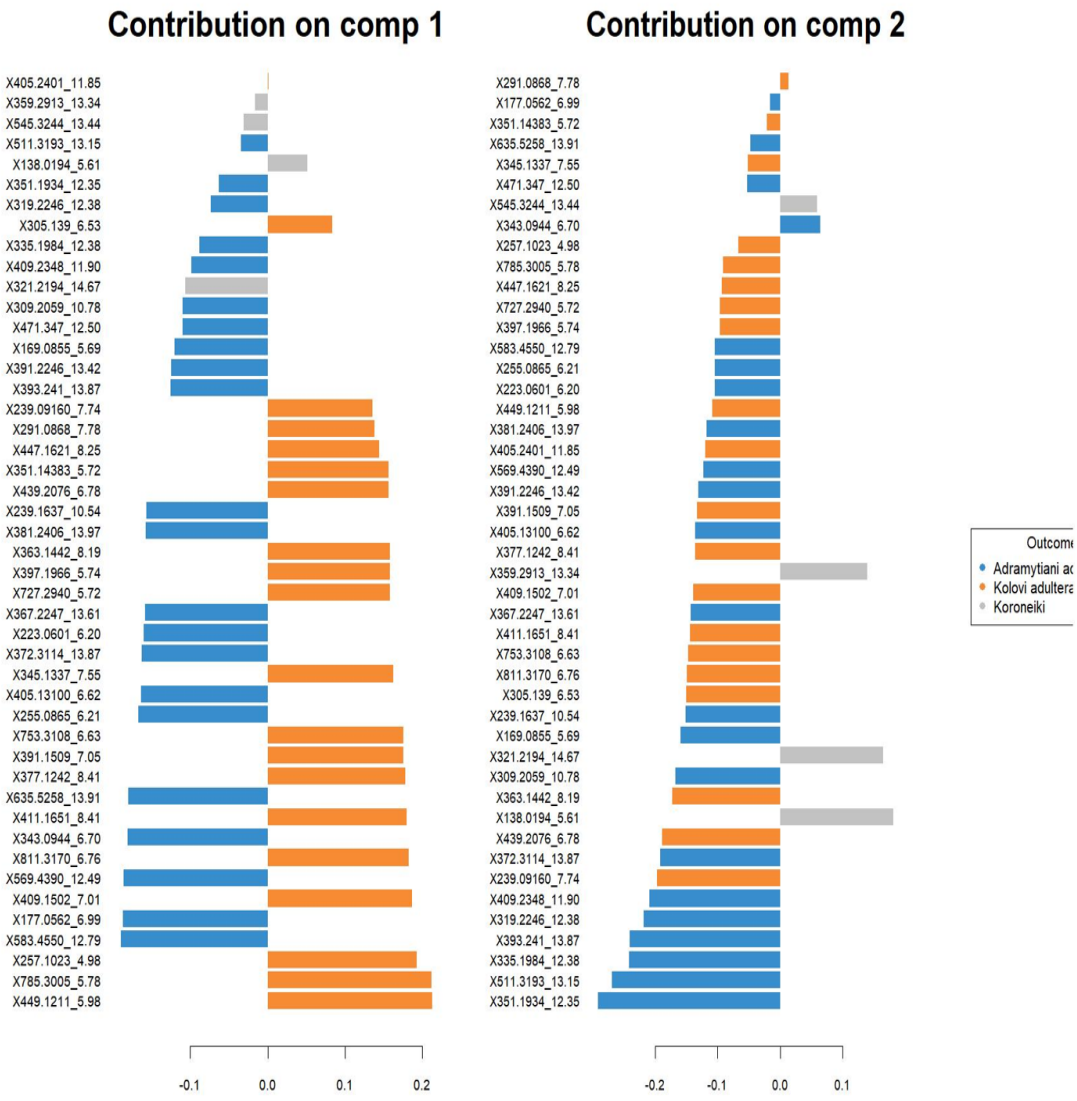


Figure 14: VIP score of the first two PCs of Koroneiki adulteration study

5.1.2 Adulterations experiments – Trend analysis

The trend across different adulteration levels of the most significant VIP marker of each variety was examined. Especially for Adramytiani, the markers with the highest VIP score (**Figure 8**) are 139.02671 m/z for Koroneiki adulteration and 256.0950 m/z for Kolovi adulteration. Adulteration levels of 10 to 50% were examined regarding linearity and trend across ratios, whereas correlation coefficient (R^2) was calculated. The markers with the highest VIP score for Kolovi and Koroneiki, based on **Figures 11, 14** were similarly studied.

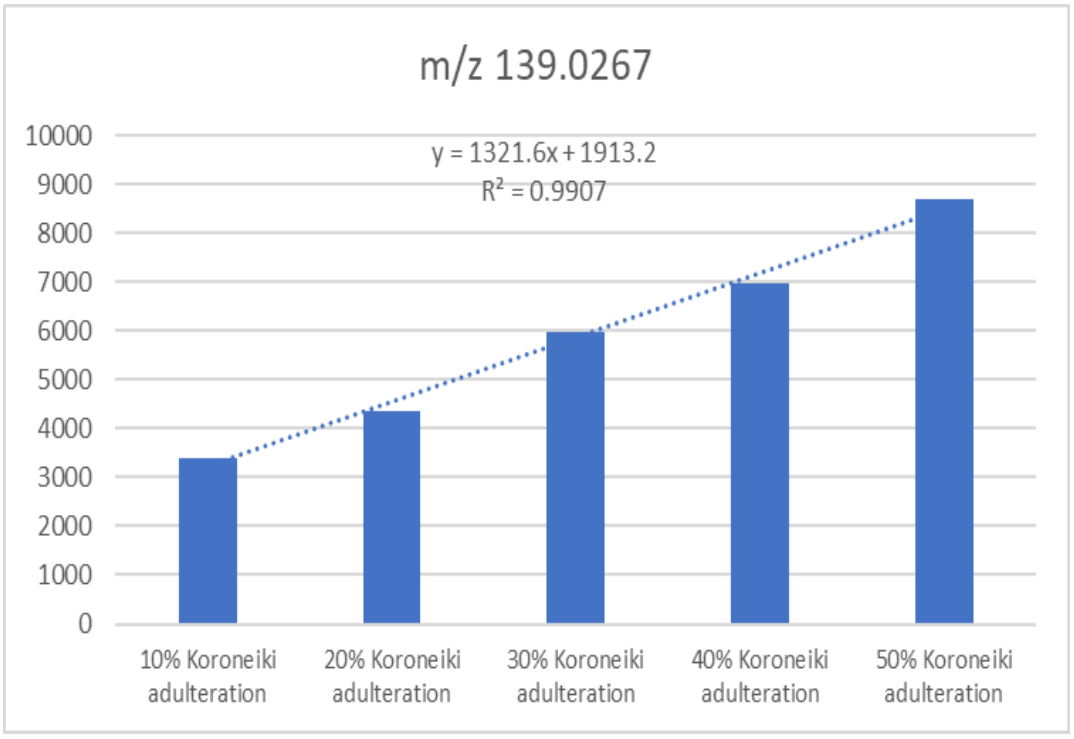


Figure 15: Adramytiani marker, m/z 139.0267

As shown in **Figure 15**, the marker with m/z 139.02671 follows a linear trend, (R^2 = 0.9907)

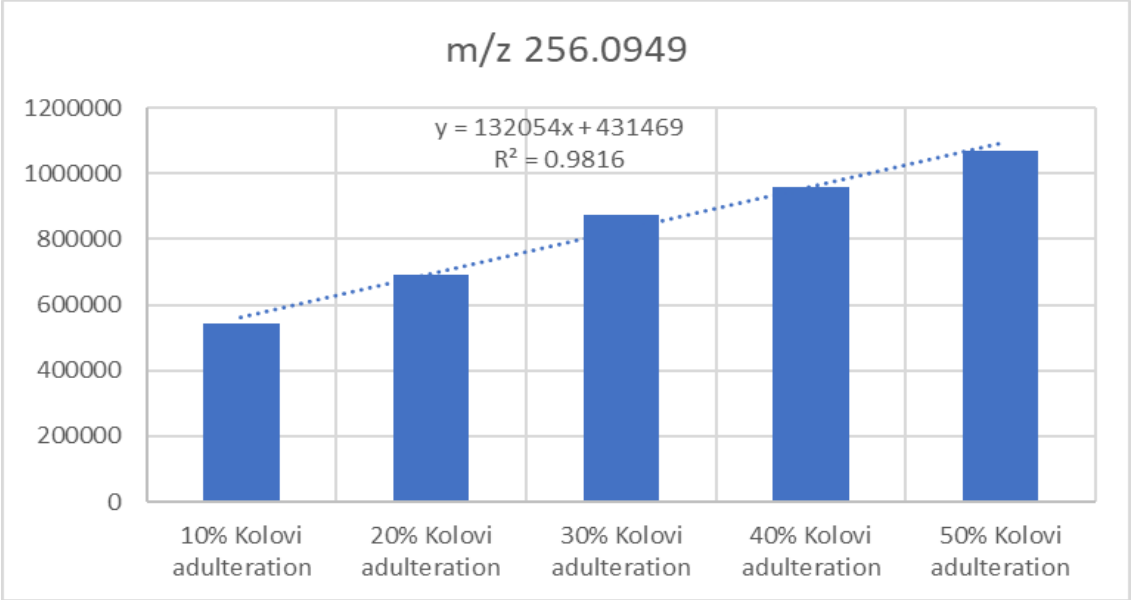


Figure 16: Adramytiani marker, m/z 256.0949

As shown in **Figure 16,** the marker with m/z 256.0949 follows a linear trend, (R^2 = 0.98)

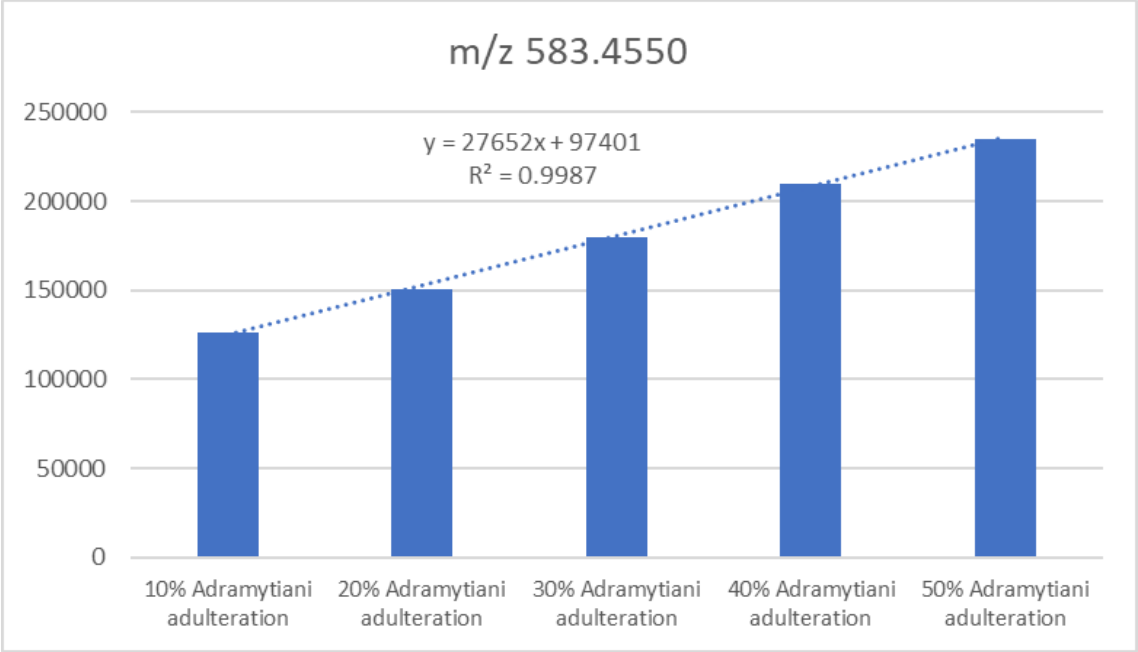


Figure 17: Kolovi marker, m/z 583.4550

As shown in **Figure 17**, the marker with m/z 583.4550 follows a linear trend, (R^2 = 0.998)

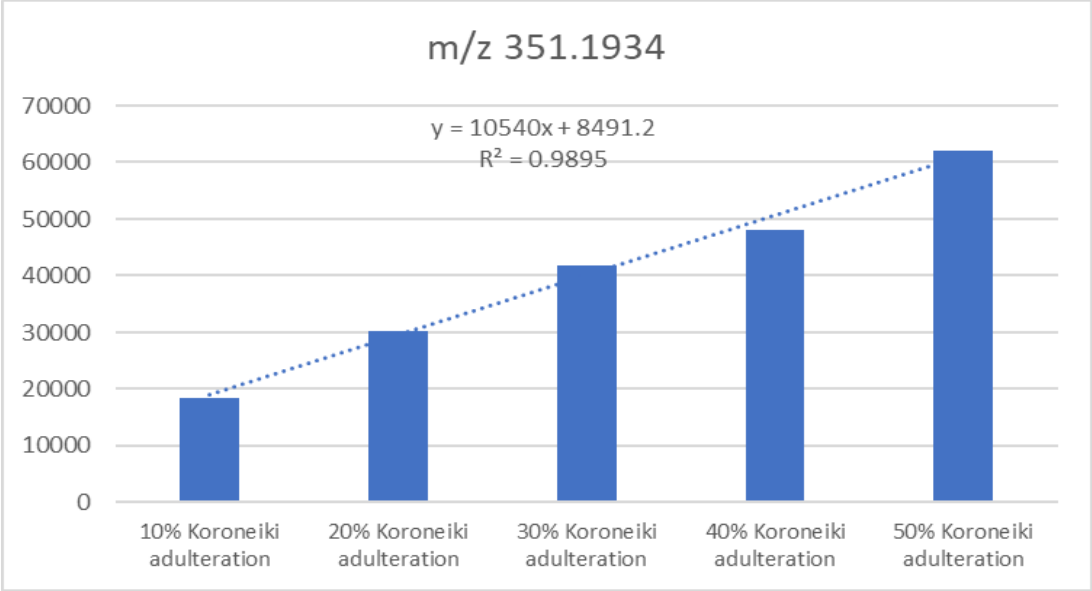


Figure 18: Kolovi marker, m/z 351.1934

As shown in **Figure 18**, the marker with m/z 351.1934 follows a linear trend, (R^2 = 0.99)

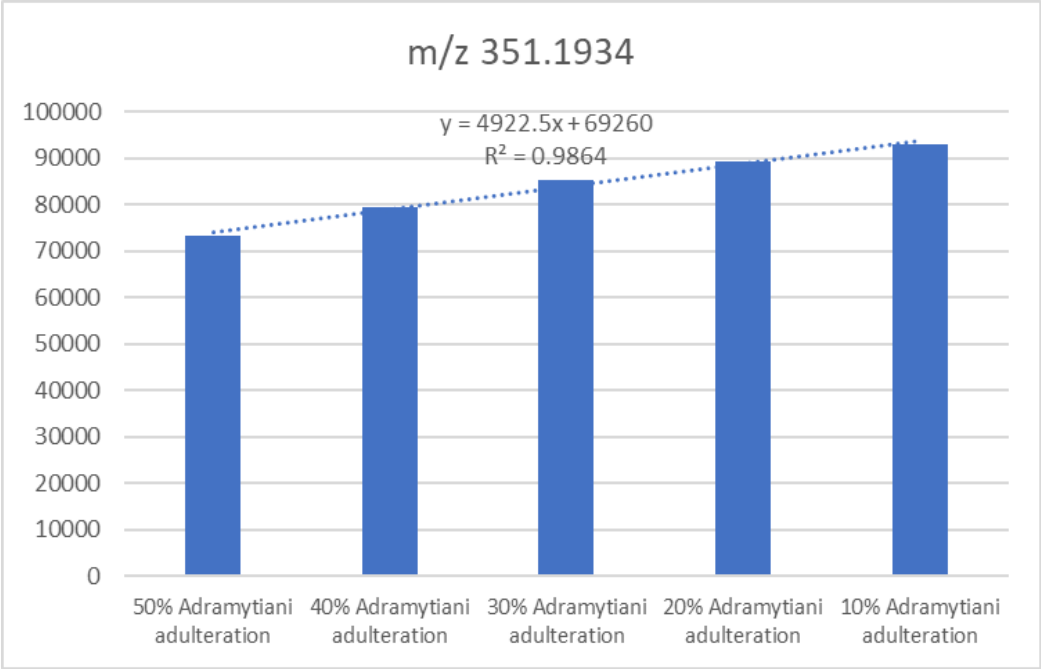


Figure 19: Koroneiki marker, m/z 351.1934

As shown in **Figure 19**, the marker with m/z 351.1934 follows a linear trend, (R^2 = 0.99).

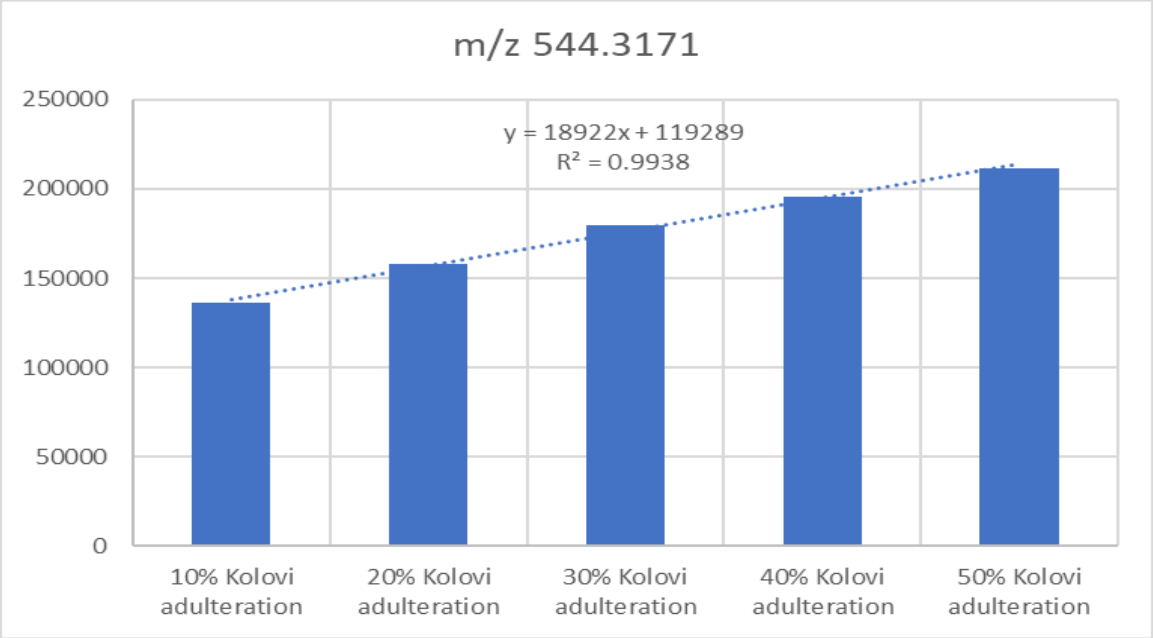


Figure 20: Koroneiki marker, m/z 544.31713

As shown in **Figure 20**, the marker with m/z 544.3171 follows a linear trend, (R^2 = 0.994).

5.2 Origin discrimination

The second case study involving samples of different region of Greece (Crete, Lesvos, Peloponnese). Peak-picking step was performed using parameters as

described under 4.4 in each one of the different experiments (negative – positive ionization) using a filter that produced the m/z that are traced in a percentage greater that 80% of the samples, as previously described. Pairwise PLS was conducted for Crete-Peloponnese, followed by Lesvos-Crete and Peloponnese-Lesvos, resulting in 3 different groups of the most significant m/z with respect to the separation of the origin at hand. m/z markers with importance greater than 1.50 were kept from each group and a unified m/z list was compiled. As shown in **Table 6** identification of these m/z was performed when possible.

Table 6. Origin Markers

A/A	m/z	Retention Time (min)	Primary Ion detected	m/z (Fragment Ions)	Propable elemental Composition	Mass Error (mDa)	Marker	Tentative identification	Level of identification
1	121.0648	9.04	[M+H]+	103.0561; 93.0506	C8H8O	1.8	CRE	Acetophenone	2b
2	193.0488	5.02	[M+H]+	151.039; 123.045;	C10H704	0.9	CRE	2-(3-ketopropanoyl)benzoic acid	2b
3	293.1021	6.54	[M+H]+	205.090; 165.058; 137.063;	C15H14O6	0.4	CRE	(2S)-4-[[(5S)-7-allyl-2-methoxy-3-bicyclo[3.2.0]hepta-1,3,6-trienyl]oxy]-2-hydroxy-4-keto-butyric acid	2b
4	309.1736	12.09	[M-H]-	183.0137; 96.9609	C11H25N4O6	-0.3	CRE	Unknown Compound	3
5	377.1642	8.17	[M+H]+	121.0664	C13H28O12	1.2	CRE	Unknown Compound	3
6	389.1360	9.03	[M+K]+	167.0701; 149.0242; 121.0648	C12H24N2O12	0.4	CRE	Unknown Compound	3
7	157.1236	8.97	[M-H]-	-	C9H17O2	1.1	CRE/LES	2,6-Dimethylheptanoate	2b
8	173.1157	4.47	[M+H]+	153.092; 111.082	C9H15O3	0.3	CRE/LES	1-ethyl-5-hydroperoxy-4-methyl-cyclohex-3-en-1-ol	2b
9	205.1596	12.28	[M-H]-	189.1351	C14H21O	0.2	CRE/LES	Unknown Compound	3
10	353.2090	12.86	[M+H]+	233.151; 147.114;	C19H25N6O	1.1	CRE/LES	Unknown Compound	3
11	433.1260	9.22	[M+H]+	189.019; 165.015; 121.066	C25H19O7	1.3	CRE/LES	piperonylic acid [4-[(E)-3-(2,5-dimethoxyphenyl)-3-keto-prop-1-	2b

								enyl]phenyl] ester	
12	137.0590	8.46	[M+H]+	119.051	C8H7O2	0.7	CRE/PEL	1-hydroperoxy-4-vinyl-benzene	2b
13	285.0402	7.38	[M-H]-	133.0309; 151.0056	C15H10O6	1.1	CRE/PEL	Luteolin	1
14	335.1494	5.78	[M-H_2O+H]+	137.0604	C9H15N14O	1.8	CRE/PEL	Unknown Compound	4
15	337.2355	13.83	[M-H]-	255.235	C20H34O4	1.2	CRE/PEL	Unknown Compound	4
16	365.2666	14.55	[M-H]-	283.2651	C22H38O4	0.3	CRE/PEL	Unknown Compound	3
17	377.1604	9.22	[M+H]+	121.0649; 165.0543	C20H24O7	1.2	CRE/PEL	Isoolivil	2b
18	393.1545	8.46	[M-H_2O+H]+	137.0592; 121.0645; 165.0543	C20H24O8	0.8	CRE/PEL	2-Hydroxy-1,2-bis(3,4,5-trimethoxyphenyl)ethanone	2b
19	428.1920	6	[M+NH_4]+	137.0592; 165.0538; 225.0753	C20H29NO9	0.5	CRE/PEL	Unknown Compound	3
20	449.1209	7.98	[M+K]+	415.1375; 387.1408	C25H20O8	1.7	CRE/PEL	3-(4-Methoxyphenoxy)-4-oxo-4H-chromen-7-yl 2,6-dimethoxybenzoate	2b
21	585.4857	13.98	[M-H]-	281.2505	C38H66O4	0.5	CRE/PEL	Unknown Compound	3
22	698.4167	14.62	[M-H]-	508.2824; 283.2673; 232.1346	C31H57N9O9	0.3	CRE/PEL	Unknown Compound	4
23	758.4378	14.62	[M-H]-	508.2837; 509.2860; 283.2649	C32H65N5O15	0.9	CRE/PEL	Unknown Compound	4
24	138.0195	5.63	[M-H]-	108.0204	C6H5NO3	0.4	LES	Nitrophenol	2b

25	243.1958	10.76	[M+H]+	153.1260; 149.1305	C14H26O3	0.6	LES	L-Menthyl (R,S)-3-hydroxybutyrate	2b
26	250.1802	7.95	[M+NH4]+	135.1159; 233.1531; 175.1102	C15H23NO2	0.6	LES	Alprenolol	2b
27	279.2323	12.39	[M-H_2O+H]+	-	C18H30O2	0.3	LES	Linolenic Acid	2b
28	295.2227	11.37	[M-H]-	195.1389	C18H30O3	0.6	LES	2-{2-[4-(1,1,3,3-Tetramethylbutyl)phenoxy]ethoxy}ethanol	2b
29	615.4593	12.39	[M+H]+	319.2245; 317.2090; 320.2279	C38H64O6	2.8	LES	Unknown Compound	4
30	427.1154	6.35	[M+K]+	137.0583; 165.0528	C33H14O	2.7	LES/PEL	Unknown Compound	4
31	197.1165	5.41	[M+H]+	179.1052; 133.1001; 161.0950	C11H16O3	0.7	PEL/CRE	Isobutyl 2-furanpropionate	2b

The **Table 6** provides information for the retention time, ions detected, its fragments, the possible formula, mass accuracy (expressed in mDa), the variety to which each m/z is assigned as marker and its possible name as well as the identification level.

Preliminary discrimination study was performed among EVOOs of single geographical origin (**Figure 5**), where clear discrimination was observed.

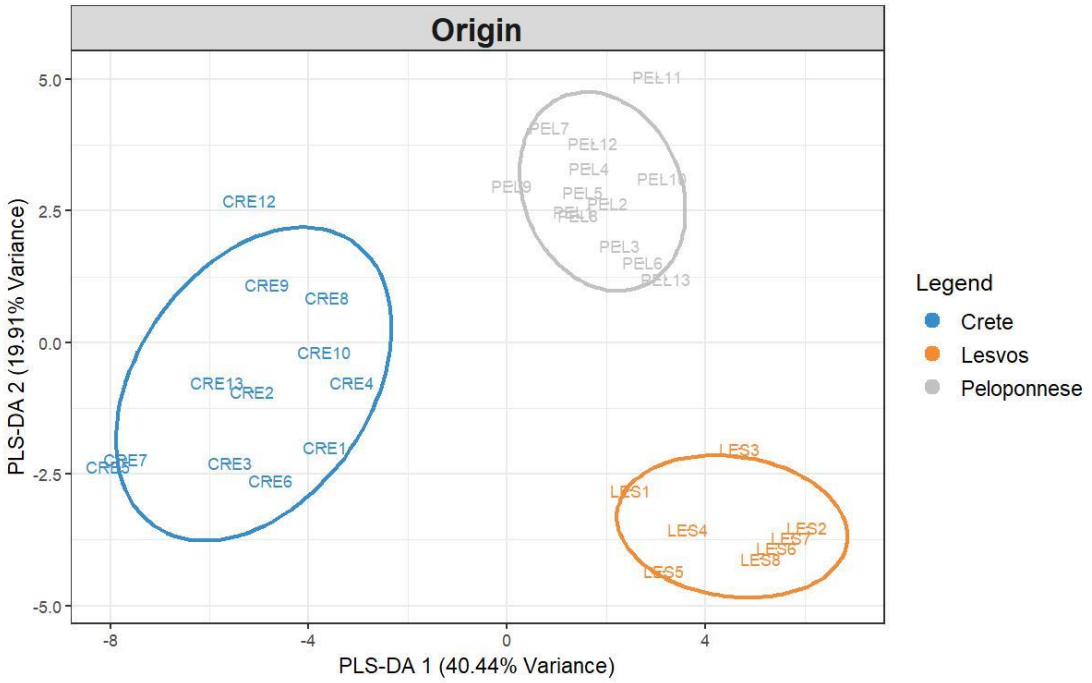


Figure 21: PLS-DA Origin Discrimination

5.2.1 Building prediction models

All m/z retrieved from pairwise PLS, as previously reported and further identified, were included in PLS-DA prediction models. More specifically, we performed PLS-DA with all the m/z markers found in order to distinguish EVOOs of each region from adulterated. **Figures 22, 25, 28** illustrate PLS-DA prediction models built that enable sufficient discrimination even in low adulteration levels (e.g. 10%). Finally, in order to evaluate model's prediction ability, a batch of adulterated samples in all different adulteration levels, from 50 to 10% was used as a test-set.

The first two components (PC1 and PC2) of the model interpreted the majority of the results (higher than 50%), (**Figures 23, 26, 29)**. **Figures 24, 27, 30** show first two components contribution to the model, as well as the most important markers for the discrimination achieved.

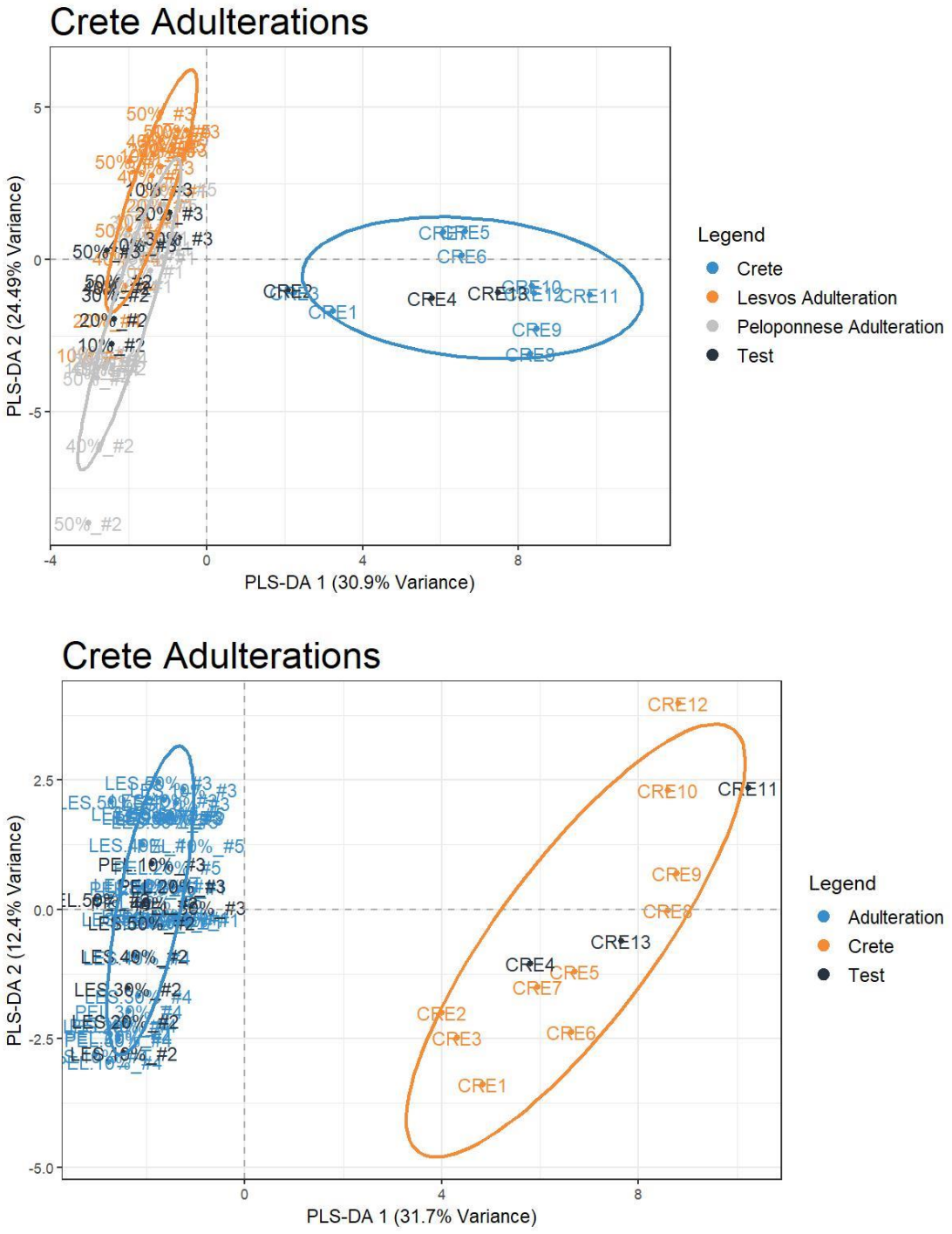


Figure 22: PLS_DA Crete

For Crete mono-varietal EVOOs, as shown in **Figure 22**, adequate discrimination is being achieved as far as samples from Lesvos and Peloponnese used as adulterants. The model predicts successfully the adulteration from both region but cannot predict the origin of adulteration.

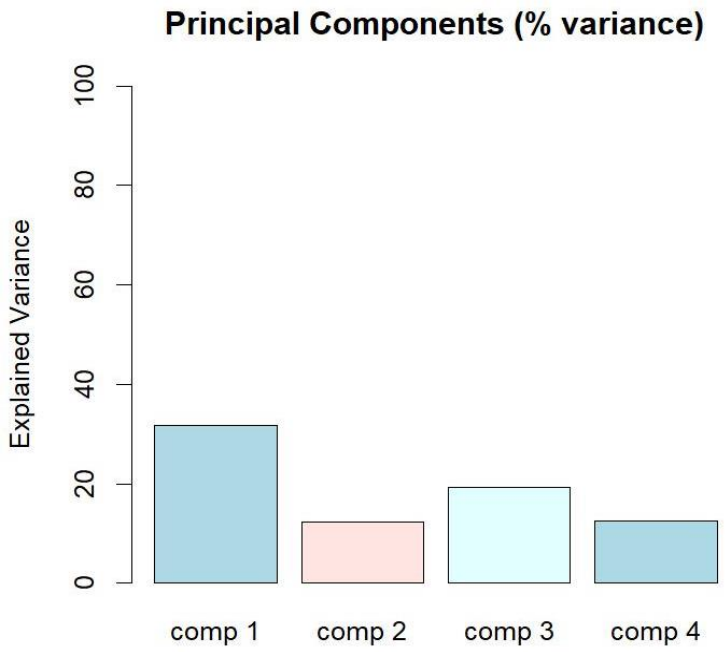


Figure 23: Principal components of Crete case study (% variance)

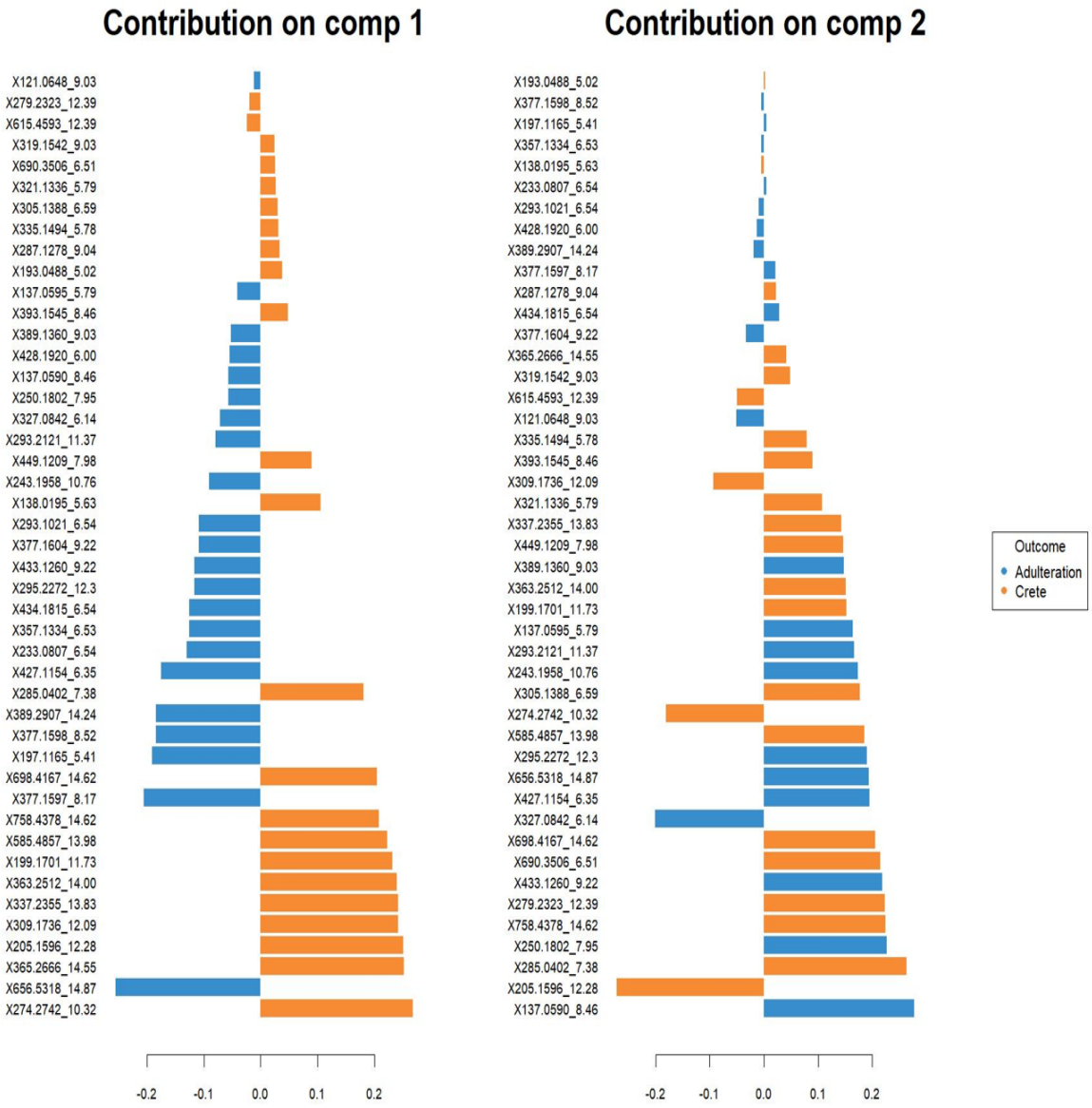


Figure 24: VIP score of the first two PCs of Crete adulteration study

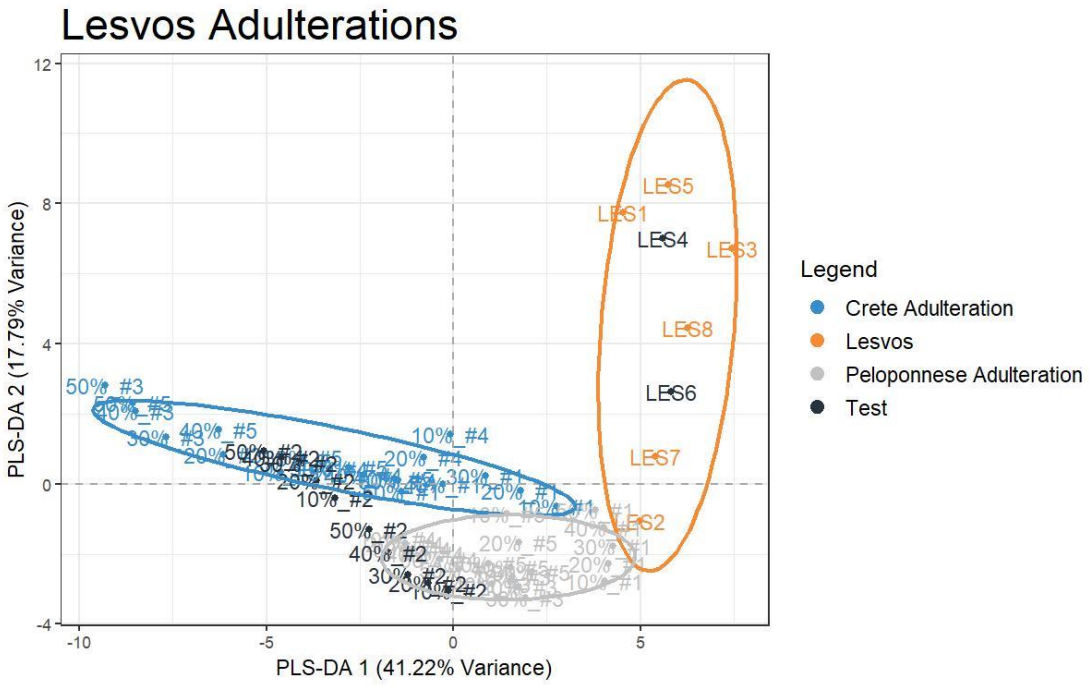


Figure 25: PLS_DA Lesvos

For Crete monovarietal EVOOs, as shown in **Figures 25**, adequate discrimination is being achieved as far as samples from Crete and Peloponnese used as adulterants. The model predicts successfully adulteration till 10% in both adulterated samples.

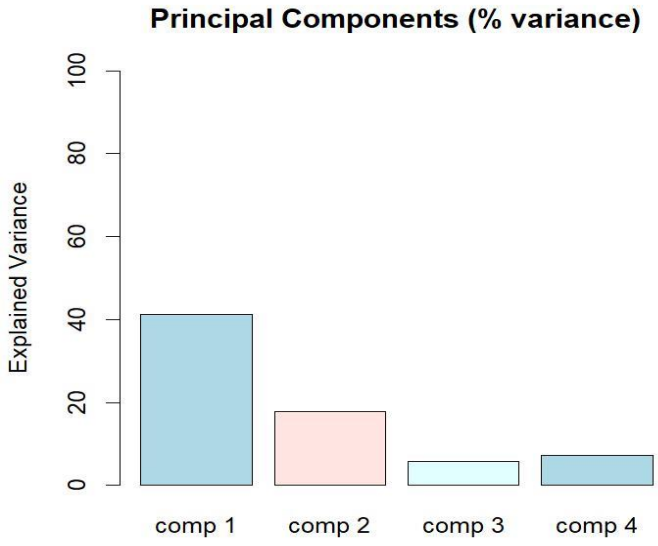


Figure 26: Principal Components of Lesvos case study (%variance)

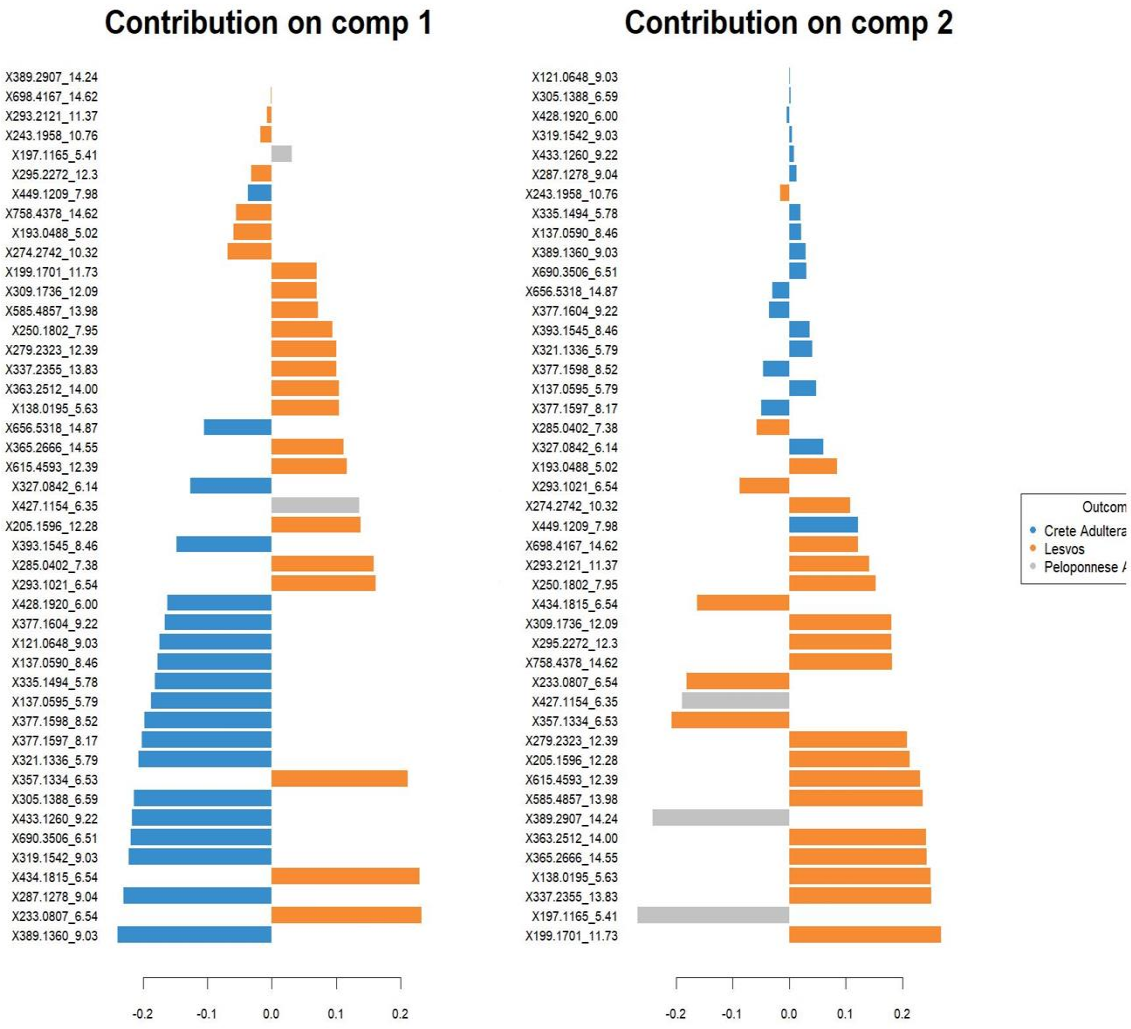


Figure 27: VIP score of the first two PCs of Lesvos adulteration study

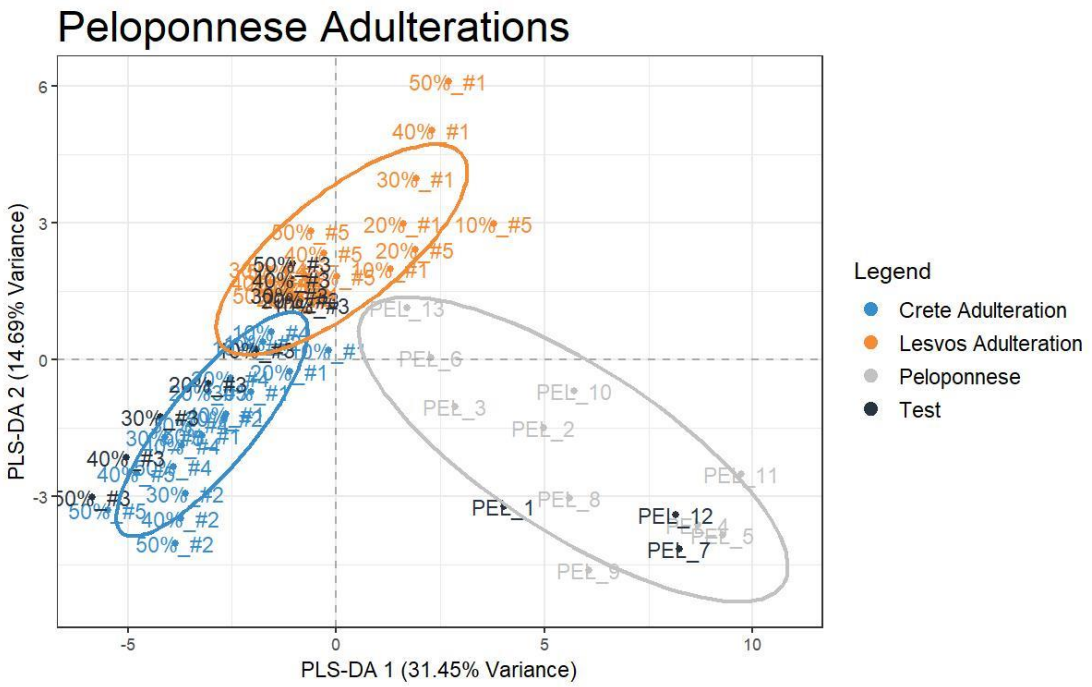


Figure 28: PLS_DA Peloponnese

For Crete monovarietal EVOOs, as shown in **Figures 28**, adequate discrimination is being achieved as far as samples from Crete and Lesvos used as adulterants. The model predicts successfully adulteration till 10% in both adulterated samples but it cannot predict the origin of adulteration in range lower than 20%, in Crete adulteration case study.

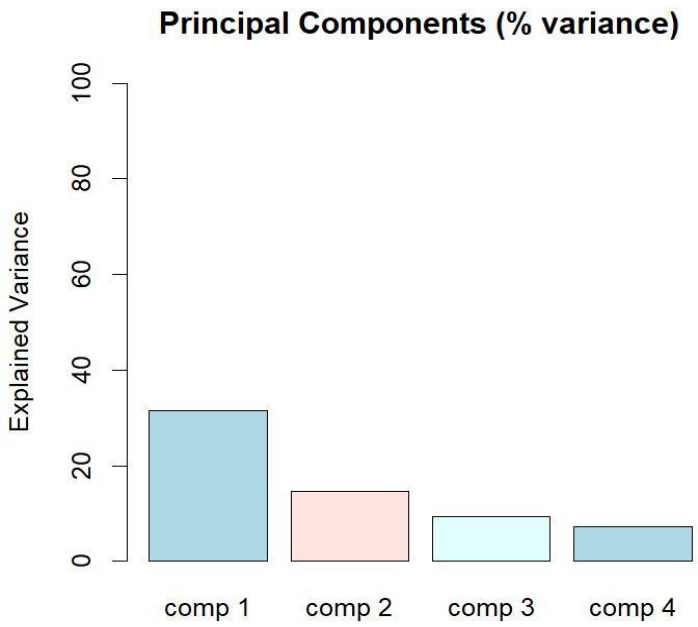


Figure 29: Principal Components of Peloponnese case study (%variance)

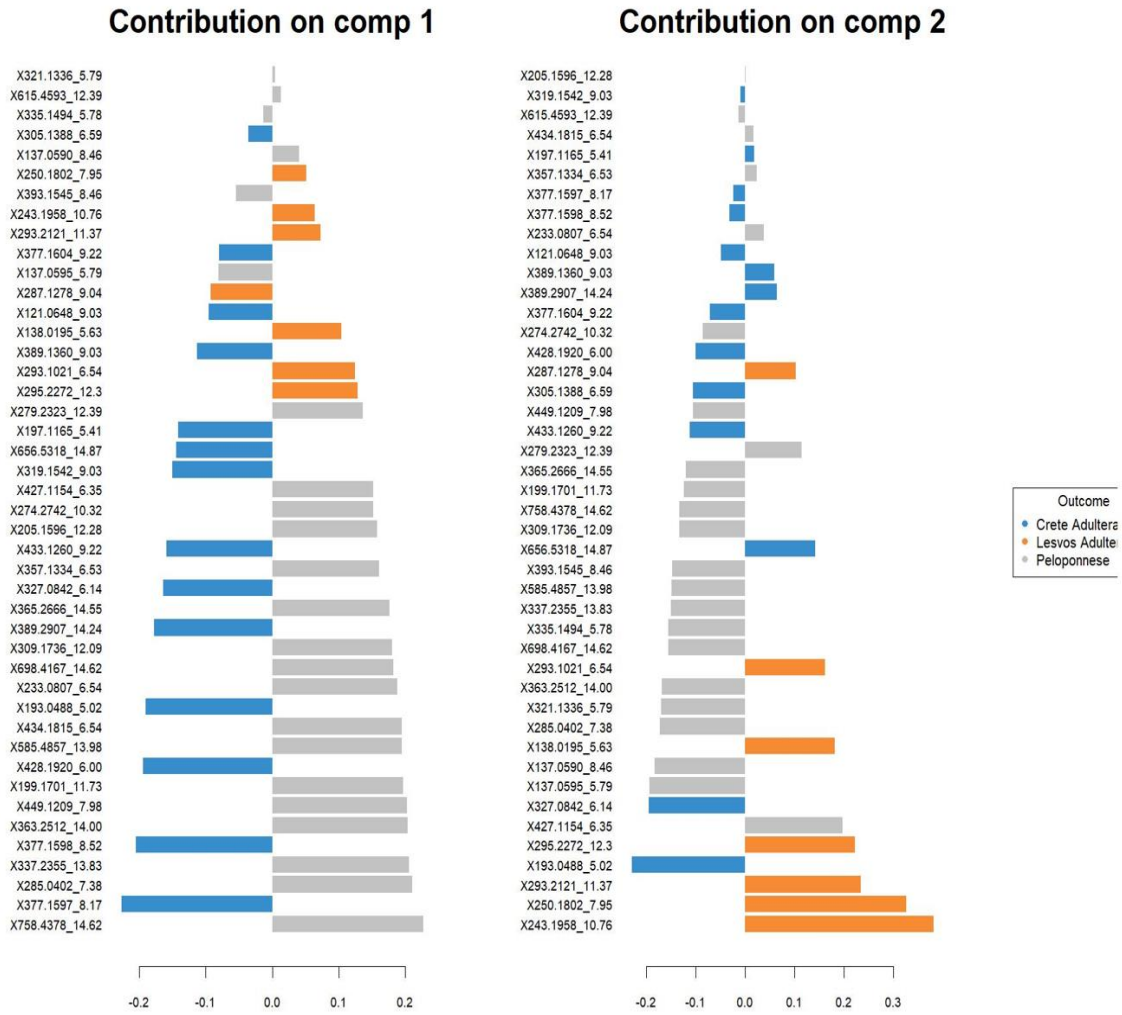


Figure 30: VIP score of the first two PCs of Peloponnese adulteration study

5.2.2 Adulteration experiments – Trend analysis

The trend across different adulteration levels of the most significant VIP marker of each variety was examined. Especially for Crete, the markers with the highest VIP score (**Figure 24**) are 295.2272 m/z for Lesvos adulteration and 427.1154 m/z for Peloponnese adulteration. Adulteration levels of 10 to 50% were examined regarding linearity and trend across ratios, whereas correlation coefficient (R^2) was calculated. The markers with the highest VIP score for Lesvos and Peloponnese, based on **Figures 27,30** were similarly studied.

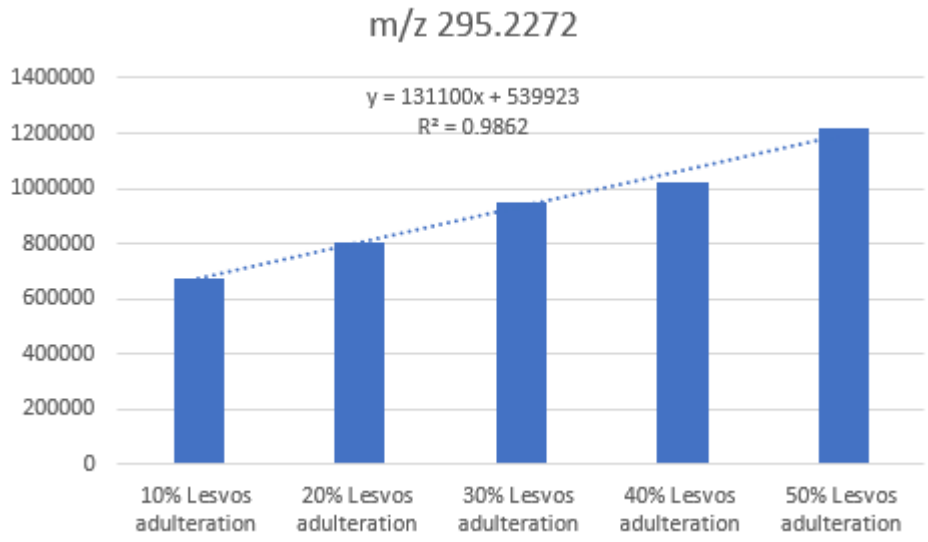


Figure 31: Crete marker, m/z 295.2272

As shown in **Figure 31**, the marker with m/z 295.2272 follows a linear trend, (R^2 = 0.99).

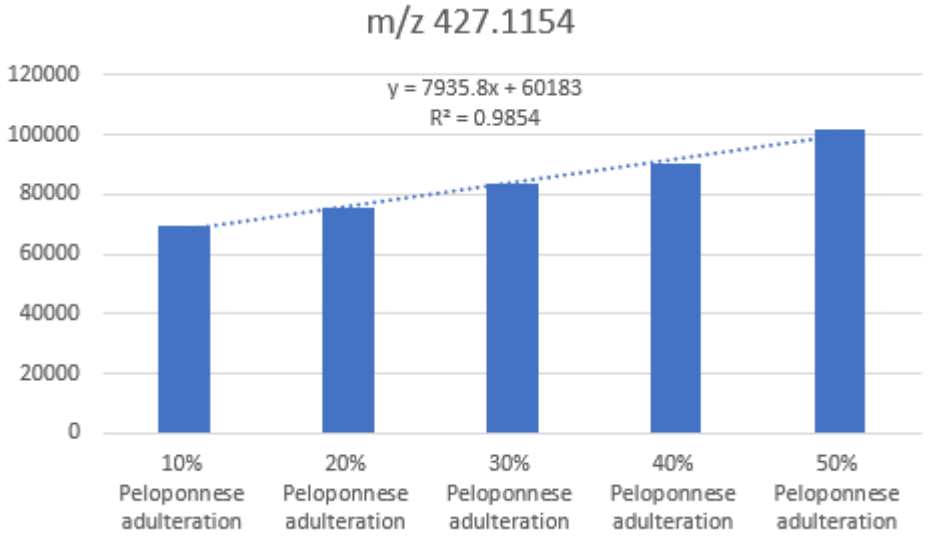


Figure 32: Crete marker, m/z 427.1154

As shown in **Figure 32**, the marker with m/z 427.1154 follows a linear trend, (R^2 = 0.98).

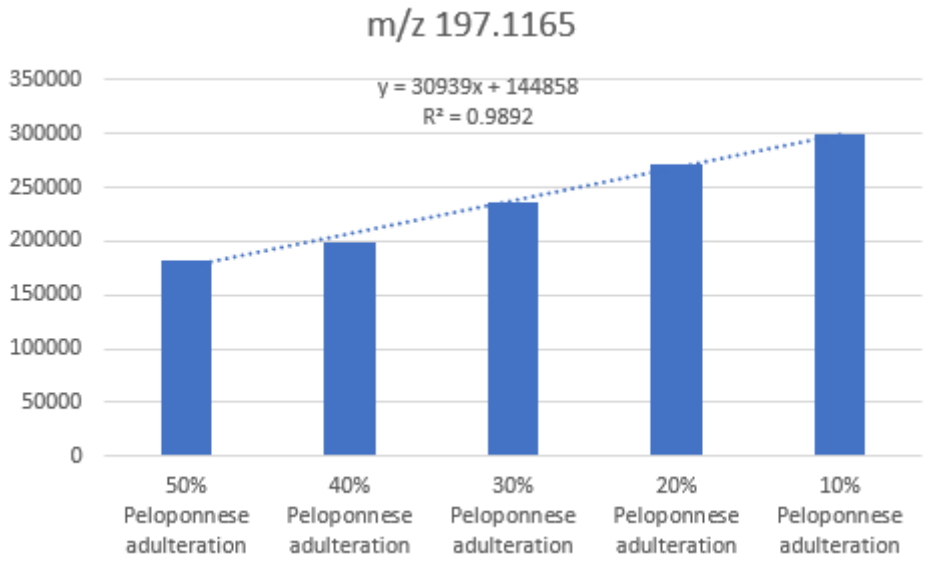


Figure 33: Lesvos marker, m/z 197.1165

As shown in **Figure 33**, the marker with m/z 197.1165 follows a linear trend, (R^2 = 0.99).

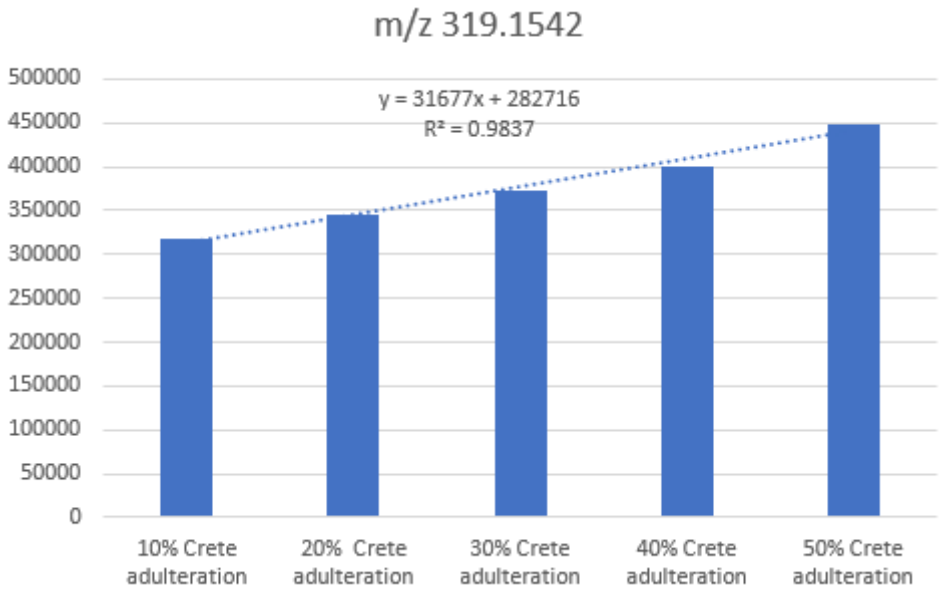


Figure 34: Lesvos marker, m/z 319.1542

As shown in **Figure 33**, the marker with m/z 319.1542 follows a linear trend, (R^2 = 0.98).

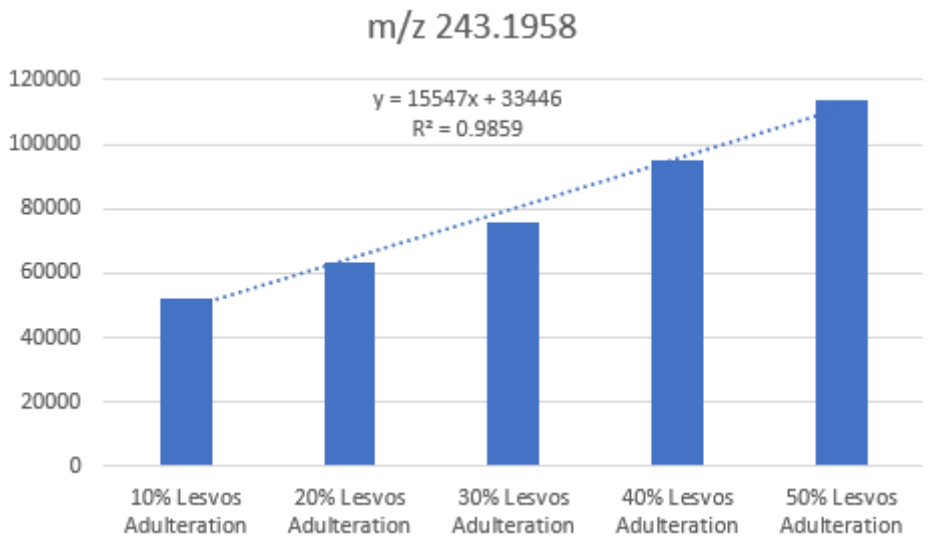


Figure 35: Peloponnese marker, m/z 243.1958

As shown in **Figure 35**, the marker with m/z 243.1958 follows a linear trend, (R^2 = 0.98).

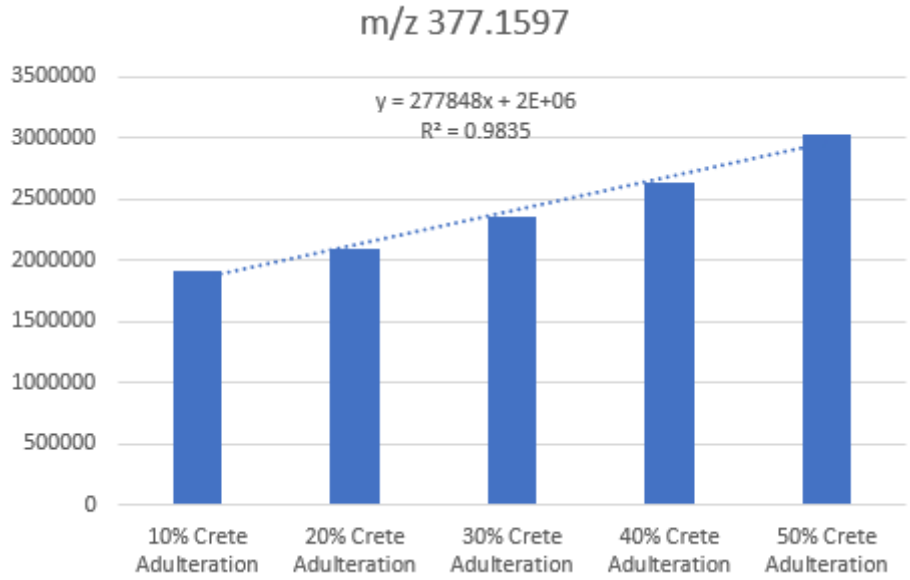


Figure 36: Peloponnese marker, m/z 377.1597

As shown in **Figure 36**, the marker with m/z 377.1597 follows a linear trend, (R^2 = 0.98).

CHAPTER 6
CONCLUSIONS

Monovarietal olive oil samples were analyzed and processed through non-target screening in order to evaluate important markers that discriminate varieties. Taking a step forward, adulterated samples were conducted in different ratios from 10 to 50%. Reliable prediction models were built, based on PLS-DA resulting in the highlighting and further identification of crucial authenticity markers.

ABBREVIATIONS – ACRONYMS

APCI	Atmospheric pressure chemical ionization
bbCID	broad-band Collision Induced Dissociation
DAD	Diode Array Detector
DDA	Data Depended Acquisition
DIA	Data Independent Acquisition
EC	European Council
EIC	Extracted Ion Chromatogram
EU	European Union
EMA	Economically Motivated Adulteration
ESI	Electrospray Ionization
EU	European Union
FDA	Food and Drugs Administration
FFN	Food Fraud Network
FLD	Fluorescence Detector
HACC	Hazard Analysis and Critical Control Points
HE	High Energy
HRMS	High-Resolution Mass Spectrometry
IS	Internal Standard
LC-HRMS	Liquid Chromatography – High-Resolution Mass Spectrometry
LC-MS	Liquid Chromatography – Mass Spectrometry
LE	Low Energy
LOD	Limit of Detection
ME	Matrix Effect
META-PHOR	Metabolomics for Plant, Health and OutReach
MLOD	Method limit of Detection
MLOQ	Method limit of Quantification
MS/MS	Tandem mass spectrometry
MVA	Multi-Variate Analysis
OCR	Official Control Regulation

OLAF	European Anti-Fraud Office
PC	Principal Component
QC	Quality Chart
QqQ	Triple quadrupole
QTOF	Quadrupole-Time-of-flight
RC	Regenerated Cellulose
RP	Reversed-Phase
RT	Retention time
SD	Standard Deviation
TAGS	Triacylglycerols
TOF	Time-of-flight
UHPLC	Ultra High Performance Liquid Chromatography
US	United States
USC	United States Code

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